Rhizopus Rotting on Agricultural Products in Jakarta

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1. Introduction

One prevalent cause of crop spoilage is biological contamination. A common group of microorganisms that contaminate agricultural products is Rhizopus spp., a pathogenic fungus that causes decay as well as disease of many fruits and vegetables. Medically, they were known to cause mucormycosis in man and animals. Whereas in agriculture and food, it plays an important role as fermentation and bioremediation agents yielding enzymes that could catalyze the formation of drugs. The beneficial impact of Rhizopus contributes the idea of collecting as many species for further useful applications in the future.

The taxonomy and molecular phylogeny of the genus Rhizopus have been intensively analyzed over several decades. The current classification of Rhizopus is based on revisions published by Schipper (1984), followed by Schipper and Stalpers (1984), who utilized morphological approaches and classified all the species of the genus into the stolonifer-group, R. oryzae, and microsporus-group. After this revision, classification based on morphology was further clarified with accurate phylogenetic relationships. A side from morphological features, Zheng et al. (2007) applied ribosomal RNA-encoding DNA (rDNA) with pyrG sequence analysis for phylogenetic data based on Liu et al. (2007) and re-classified the genus into 10 species and 7 varieties. This was further revised due to obscurities found in the rDNA ITS sequence of R. americanus, which had three distinct sequences similar to R. stolonifer, R. microsporus, and R. oryzae. Phylogenetic relationships were re-evaluated using actin gene (act1), translation elongation factor 1α (EF-1α), and rDNA ITS by Abe et al. (2010), who proposed R. niveus to be re-classified as R. delemar, R. americanus, and R. sexualis to be re-classified as R. stolonifer. Zheng et al. (2007) replaced R. arrhizus to be R. oryzae, which differed from Abe et al. (2010), who used the former name.

Morphological identification of Rhizopus as plant pathogen carried out in Indonesia was restricted to genus only. Standard method in every fungal taxonomy requires molecular phylogenetic analysis to reflect morphological features. The purpose of this research was to isolate Rhizopus spp. from varying agricultural products as well as to identify them into species level.
2. Materials and Methods

2.1. Materials
Materials used in this research were agricultural products carrying symptoms of *Rhizopus* rot, including apples (Crab and Fuji), bananas (Cavendish and Latundan), cherry tomato, grapes, guava, peach, pear, strawberry, sweet potato, and tomato obtained from several markets in Jakarta.

2.2. Methods
This research consisted of isolation of *Rhizopus*, molecular identification of *Rhizopus*, and clarification of *Rhizopus* species.

2.3. *Rhizopus* Isolation
*Rhizopus* rot from agricultural products was determined through microscopic clarification of rhizoid structure. Isolation was carried out using direct plating method in potato dextrose agar (PDA) plate containing 250 ppm chloramphenicol. The cultures were incubated at 28°C.

Suspension of spores from a culture aged 4 days old was conducted to acquire a single hyphae. The single hyphae was transferred to a fresh PDA medium. The single hyphae isolation was incubated at 28°C for two days. The single hyphae obtained was transferred to a fresh slanted PDA medium for culture collection.

2.4. Molecular Identification of *Rhizopus*
*Rhizopus* genomic extraction was done with materials acquired from illustra Nucleon Phytopure™ Kit. Mycelia were collected from the previous culture collection and transferred to an Eppendorf tube with a 1.5 ml maximum capacity which already contained 500 μl milliQ. Centrifugation at 10,000 rpm for 10 min was done, followed by removal of supernatant. Pellet was pounded with a plastic pestle until the pellet formed a porridge-like substance. As much as 300 μl of reagent 1 was added, followed by resuspension. As much as 3 μl of RNAse (20 μg/ml) was added, resuspended, then incubated at 37°C for 30 min. As much as 200 μl of reagent 2 was added and shaken until homogeneous, then incubated at room temperature for 10 min and put in ice for 20 min. The suspension of mycelia was next added with 500 μl of cold Phenol: Chloroform: Isopropanol, then mixture was shaken for 10 min at room temperature and centrifuged at 10,000 rpm at 4°C for 10 min. The supernatant formed was transferred to a new Eppendorf tube, and then added with cold isopropanol as much as half the tube's volume while homogenizing the mixture slowly. Centrifugation was done at 13,000 rpm for 10 min to get the supernatant which was later removed. As much as 50 μl of cold ethanol 70% was added and centrifuged at 13,000 rpm for 10 min, then supernatant was removed and pellet was air dried for approximately 30 min. As much as 50 μl of NFW was added to the pellet, and then kept at -20°C. Preparation for ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3’) and ITS5 (5’-GGA AGT AAA AGT CGT AAC AAG G-3’) was done to amplify a gene at the site 18S-5.8S-28S using Gene Amp® PCR System 2400 with KAPA Taq Extra HotStart PCR kit. PCR reactions for each rDNA sample consisted of 1 μl of 100 ng DNA sample, 1.5 μl of 10 mm dNTP mix, 3.5 μl of 25 mm MgCl₂, 2.5 μl of 10 μm ITS4 and ITS5, 0.5 μl of 2.5 Uμl Taq polymerase enzyme, 10 μl of 5X KAPA Taq Extra Buffer, and 28.5 μl of NFW. PCR reaction cycle was operated 94°C, 2 min; 35 cycles at 94°C, 15 sec; 55°C, 30 min; 72°C, 1 min; 72°C, 5 min. PCR results were visualized at 1% agarose gel at 100 V for 45 min, and then followed by dyeing of ethidium bromide for 15 min. Gel was rinsed with aquades and observed using UV light (Hartanti *et al.* 2015).

Isolates unsuccessfully extracted and amplified with Phytopure™ DNA Extraction Kit were treated with a quick protocol from Promega Maxwell® RSC Plant DNA Kit with the Maxwell® RSC System (AS4500). As much as 20 mg sample was placed at the bottom of a ClickFit microtube with a 1.5 ml capacity. Liquid nitrogen was added to the sample to freeze the sample. Using a plastic pestle, the frozen sample was grinded against the tube wall. As much as 300 μl tail lysis buffer (TLA) was added to each tube, then followed by 10 μl RNAse. All tubes were vortexed briefly for 10 sec. and centrifuged at 13,000 rpm for 2 min. A deck tray and cartridges from the kit were set up. An amount of 300 μl of NFW was added to the first well of each Maxwell® RSC Plant DNA Kit reagent cartridge. The sample lysate, excluding any solid materials, was transferred from the extraction tube to the first well of the reagent cartridge. Samples were then programmed under Maxwell® RSC System into automated purification with 45 min. duration.

The results of PCR were sequenced by 1st Base, Malaysia. DNA sequence was edited using ChromasPro2. Edited sequence was aligned with DNA database reference in Gene Bank, including *Rhizopus* type species (www.ncbi.nih.gov/Genbank), using MEGA7 with outgroup *Phycomyces blakesleeanus*. Alignment result was used to construct the phylogenetic tree using MEGA7 with maximum likelihood (ML) method.
2.5. Clarification of *Rhizopus* Species

Morphological observation was made through Riddle's slide culture technique. Microscopic examination involved the measurement of sporangiophore's length and sporangiospore's diameter, as well as the sight of rhizoid and columella. Physiological observation utilized five PDA plates inoculated from each strain which were incubated at 33 and 42°C for two days.

3. Results

Twelve *Rhizopus* strains were successfully isolated from nine types of agricultural products (Table 1). Diversities of *Rhizopus* spp. in agricultural products were classified based on rDNA-ITS region tree generated from ML analysis which identified *R. delemar* (5 strains) and *R. stolonifer* (7 strains) with 1,000 bootstrap replications and Tamura-Nei model (Figure 1).

3.1. Maximum Temperature and Morphology of *Rhizopus*

Molecular data was clarified with physiological data. Strains AR1-AR7 showed no growth at 33°C, while strains AR9-AR11, AR13, and AR14 showed growth at 33°C, and no growth at 42°C (Table 2).

*Rhizopus delemar* strains were identified through microscope having sporangiophores measuring between 120.43-1088.5 μm with swellings commonly observed. Sporangiospores were seen usually in one cluster arising from rhizoid. Rhizoid was not well developed, not abundant, and appeared to resemble a finger. Its columella showed distinct apophyses. Sporangiospores were variable in shapes and sizes measuring between 2.31-10.23 μm (Figure 2). In macroscopic examination, all *R. delemar* strains showed colonies that were white initially, then becoming grey to black at the entire upper portion, showing a maturing process.

*Rhizopus stolonifer* strains were identified through microscope having well developed and abundant rhizoid repeatedly branching. Sporangiospores were irregular and measuring between 5.41-24.55 μm, while sporangiophores between 463.69-2683.87 μm. Its columella showed distinct apophyses (Figure 3). In macroscopic examination, all strains had their colonies white at the central portion and black at the outer edge.

4. Discussion

Twelve strains were identified until species level using molecular technique with supporting morphological and physiological data. According to phylogenetic analysis, strains AR9-11 and 13-14 were in concordance with type species *R. delemar* CBS12012T AB181318. The monophyletic clades of *R. delemar* and *R. oryzae* were further separated at 99% bootstrap value, which made both species be in different clades. Strains AR1-AR7 were confirmed as *R. stolonifer* within its subclades that weren't in the clade with the two types of species *R. americanus* CBS34062T AB113010 and *R. sexualis* CBS33639T AB113020. This occurrence is interesting to be studied further for taxonomic studies, using multigene approach in order to validate the identification of these strains.

The maximum temperatures of *R. delemar* and *R. stolonifer* were evaluated. *R. delemar* strains showed no growth at 42°C, but survived at 33°C. This is similar to Zheng et al. (2007), who stated that *R. arrhizus* var. *delemar* had a maximum temperature reaching 42°C. *R. arrhizus* var. *delemar* current name is *R. delemar*. *R. oryzae* had the same maximum temperature with *R. delemar*. *R. stolonifer* strains showed no growth at 33°C, which is also similar to Zheng et al. (2007), who stated that *R. stolonifer* had a maximum temperature not exceeding 33°C. The maximum temperature of every strain recorded adhered well with the molecular data.

Morphology results were compared to Zheng et al. (2007) as part of identifying strains AR1-AR14. All strains showed coherence with the reference. Colonies of *R. delemar* and *R. stolonifer* on PDA were found to be deep gray to nearly black. Development of rhizoids are well developed on *R. stolonifer* but less well developed on *R. delemar*. *Rhizopus delemar* had sporangiophores...
Figure 1. Phylogenetic tree of *Rhizopus* strains AR1-7, 9-11, 13-14 compared to the reference species using Maximum Likelihood statistics with 1,000 replications of bootstrap test.
measuring mostly within 1,000 μm, and reaching 1,700 μm or more in length; in this study 120.43-1,088.50 μm of sporangiophore’s length was acquired. R. stolonifer had sporangiophores measuring mostly within 2,500 μm, reaching 3,000 μm or more in length; in this study 463.69-2,683.87 μm of sporangiophore’s length was acquired. Swellings were supposedly seen mostly at the apex or the middle portion of the R. delemar sporangiophores; meanwhile, R. stolonifer had no swellings present. Both R. delemar and R. stolonifer are typically ovoid, ellipsoidal, or roundish conical shaped columellae. The diameter of sporangiospores on R. delemar is mostly 5-9 (±14.5) μm, and when irregular it reached 53 μm in length; in this study 2.31-

### Table 2. Physiological data of Rhizopus delemar and Rhizopus stolonifer conducted at temperature growth 33 and 42°C

| Strain code | 33°C | 42°C |
|-------------|------|------|
| R. stolonifer AR1 | - | - |
| R. stolonifer AR2 | - | - |
| R. stolonifer AR3 | - | - |
| R. stolonifer AR4 | - | - |
| R. stolonifer AR5 | - | - |
| R. stolonifer AR6 | - | - |
| R. stolonifer AR7 | - | - |
| R. delemar AR9 | + | - |
| R. delemar AR10 | + | - |
| R. delemar AR11 | + | - |
| R. delemar AR13 | + | - |
| R. delemar AR14 | + | - |

Annotation: (+) growth, (-) no growth

Figure 2. *Rhizopus delemar* strain collected from cherry tomato and peach: (a) sporangiophores in one cluster arising from rhizoid, (b) finger-like shaped rhizoid, (c) columella with distinct apophyses, (d) irregular shaped sporangiospores, (e) swelling on sporangiophore
42

Hartanti AT et al.

10.23 μm of sporangiospore’s diameter was acquired. Rhizopus stolonifer had sporangiospores with a diameter measuring mostly 5-12.5 (-19) μm; in this study 5.41-24.55 μm of sporangiospore’s diameter was acquired.

Rhizopus spp. can be divided into fumaric-malic acid producers, lactic acid producers, and producers of both. Although there wasn’t a clear correlation between organic producers and morphological classification, R. delemar and R. oryzae were distinct from each other by the acids they produced. Rhizopus delemar was reported to be lacking ldhA gene, which was responsible in the production of lactic acid; furthermore, R. delemar was classified to be a fumaric-malic acid producer. On the other hand, R. oryzae was capable of producing lactic acid, thus making R. oryzae different from R. delemar (Abe et al. 2007).

Figure 3. Rhizopus stolonifer strain collected from apple, banana, and pear: (a) profusely branched rhizoid, (b) irregular shaped sporangiospores, (c) columellae with distinct apophyses, (d) sporangiophores in single clusters arising from rhizoid

(a) profusely branched rhizoid (b) irregular shaped sporangiospores (c) columellae with distinct apophyses (d) sporangiophores in single clusters arising from rhizoid
Abe et al. (2010) reported that *R. americanus, R. sexualis, and R. stolonifer* shared morphological and physiological features. However, *R. stolonifer* is heterothallic, and both *R. americanus* and *R. sexualis* are homothallic, so these strains are classified as independent species in morphological taxonomy. A molecular phylogeny cluster occurring in phylogenetic trees based on rDNA-ITS, act1, and EF-1α consisted of *R. reflexus, R. stolonifer, R. sexualis, and R. americanus*. Abe et al. (2010) reclassified these species to be in the *R. stolonifer* group. However, high divergence and multiple types sequences in this cluster should be modified further for conclusive taxonomy.

*Rhizopus stolonifer* AR7, AR6, and AR2 is similar to *R. stolonifer* occurring on pear (Kwon and Lee 2006), guava (Ooka 1980) and grapes (Latorre et al. 2002). Although *R. delemar* AR10 and AR14 were found on cherry tomato and tomato, *R. stolonifer* has been reported to be the fruit pathogen as well (Kwon et al. 2001). *Rhizopus stolonifer* AR1, AR3, AR4, AR5 and *R. delemar* AR11, AR9 contradict reports of *R. oryzae* invading banana, apple, sweet potato, and strawberry (Kwon et al. 2011, 2012a, 2012b, 2014). Although *R. delemar* AR13 occured on peach, this product had the potential in experiencing *R. stolonifer* rot (Kwon and Lee 2006). Host nutrients and acidic pH are required for all *Rhizopus* species to germinate optimally, thus different agricultural products from different origins do not trigger species specific growth. There's no clear correlation between the host nutrients, in this case agricultural products, and *Rhizopus* species. However, environmental factor affects prominently in the infection of species specific pathogens, which might result in inconsistencies of *Rhizopus* spp. discoveries from different regions. The ecology and distribution of *Rhizopus* spp. on fruits or vegetables are inadequate in mild or tropical regions; therefore, it is hard to conclude whether some of the known fungi are limited to specific hosts and geography.

*Rhizopus* spp. are capable of producing significant amount of organic acids and enzymes that could be used in industries and medicinal uses. *Rhizopus delemar* and *R. stolonifer* produce fumaric acid used in plastic industry, and to a lesser extent, in the food industry (Roa Engel et al. 2008). *Rhizopus stolonifer* also produces lactic acid and is often used in dairy industry as preservatives or flavour enhancer (Soccol et al. 1994). *Rhizopus stolonifer* was reported to bring about hydroxylation in the synthesis of steroid used in treating hormonally imbalanced individuals, patients with auto-immune diseases, and as birth control pills (Nassiri-Koopaei and Faramarzi 2015). *Rhizopus delemar* was reported to produce extracellular lipase capable of acidolysis. The lipase acted strong on myristic, palmitic, palmitoleic, stearic, oleic, linoleic, and α-linolenic acids, hence, making it useful in oil companies or biodiesel production (Shimada et al. 1997). Protease produced by *R. stolonifer* is of greater importance due to its higher protease producing activity. Protease is essential in about 60% of total enzyme market and capable of digesting insoluble materials such as cellulose and protein (Kranthi et al. 2012). Naming and validating a specific *Rhizopus* species is an essential step to ensure the role and benefit of the actual sample acquired.

5. Conclusion

Twelve strains were successfully isolated and identified from nine different types of agricultural products. Five strains were identified as *R. delemar* and the other seven remaining strains were identified as *R. stolonifer*. These strains have the potential to yield useful organic acids and enzymes for many types of industries.

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