

Rhizopus Rotting on Agricultural Products in Jakarta

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ARTICLE INFO

Article history:

Received July 6, 2018

Received in revised form August 20, 2019

Accepted August 30, 2019

KEYWORDS:

Rhizopus rot,
ITS rDNA,
mold,
postharvest disease,
agriculture

ABSTRACT

Agricultural products may easily perish if they are not well taken care of during postharvest treatment. One of the main causes in damaged products is the biological contamination of pathogenic fungi, such as *Rhizopus* spp. that gives rise to *Rhizopus* rot symptoms. The purpose of this research was to isolate *Rhizopus* spp. from different agricultural products showing *Rhizopus* rot symptoms as well as to identify them. *Rhizopus* pure cultures were isolated on potato dextrose agar. Identification was done through molecular techniques using Phytopure™ DNA Extraction Kit and Maxwell® RSC Plant DNA Kit for DNA isolation, internal transcribed spacer (ITS4 and ITS5) as primers for amplification, and molecular evolutionary genetics analysis 7 (MEGA7) for the reconstruction of phylogenetic tree from sequence result. The phylogenetic tree using Maximum Likelihood statistics with 1,000 replications of bootstrap test showed five strains, namely AR9, AR10, AR11, AR13, and AR14, which belong to *R. delemar*, and the other seven remaining strains, AR1-AR7 belong to *R. stolonifer*. Identification was clarified with morphological and physiological data using *Rhizopus* growth at temperature control of 33 and 42°C as well as microscopic observation involving rhizoid, columella, measurement of sporangiospores, and sporangiophores.

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. Aside 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 1 α (EF-1 α), 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.

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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. Sporangiophores 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* CBS120 12T 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* CBS340 62T AB113010 and *R. sexualis* CBS336 39T 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

Table 1. Sample origin, strain code, location and date of isolation, and accession number acquired

Sample origin	Strain code	Location of isolation	Species	Accession number
Apple (Crab)	AR5	Jakarta	<i>R. stolonifer</i>	MF461024
Apple (Fuji)	AR11	Jakarta	<i>R. delemar</i>	MF445158
Banana (Cavendish)	AR4	Jakarta	<i>R. stolonifer</i>	MF461023
Banana (Latundan)	AR1	Jakarta	<i>R. stolonifer</i>	MF461020
Cherry tomato	AR14	Jakarta	<i>R. delemar</i>	MF445161
Grape	AR2	Jakarta	<i>R. stolonifer</i>	MF461021
Guava	AR6	Jakarta	<i>R. stolonifer</i>	MF461025
Peach	AR13	Jakarta	<i>R. delemar</i>	MF445160
Pear	AR7	Jakarta	<i>R. stolonifer</i>	MF461026
Strawberry	AR3	Jakarta	<i>R. stolonifer</i>	MF461022
Sweet potato	AR9	Jakarta	<i>R. delemar</i>	MF445156
Tomato	AR10	Jakarta	<i>R. delemar</i>	MF445157

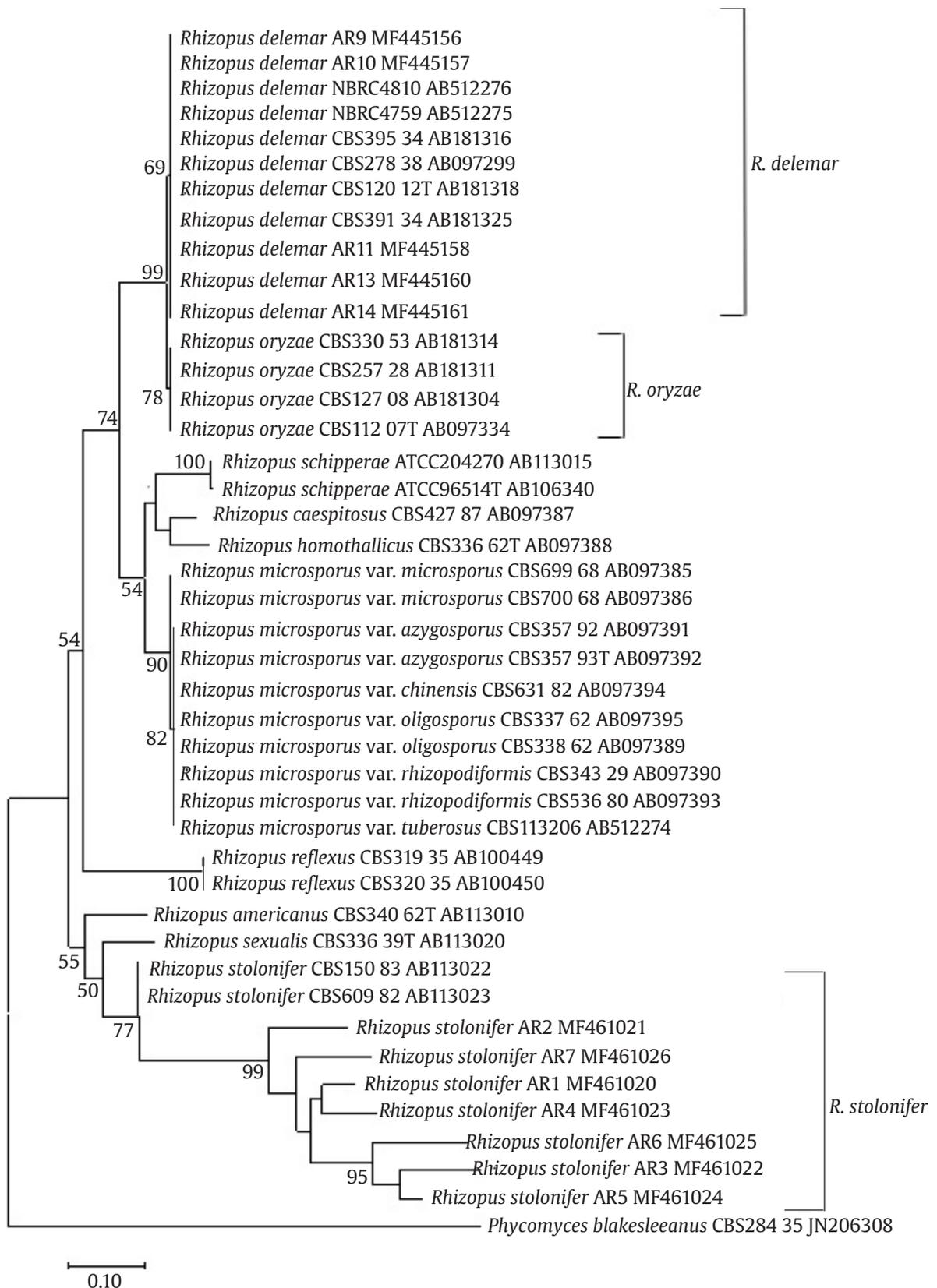


Figure 1. Phylogenetic tree of *Rhizopus* strains AR1-7, 9-11, 13-14 compared to the reference species using Maximum Likelihood statistics with 1,000 replications of bootstrap test

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

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

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

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-

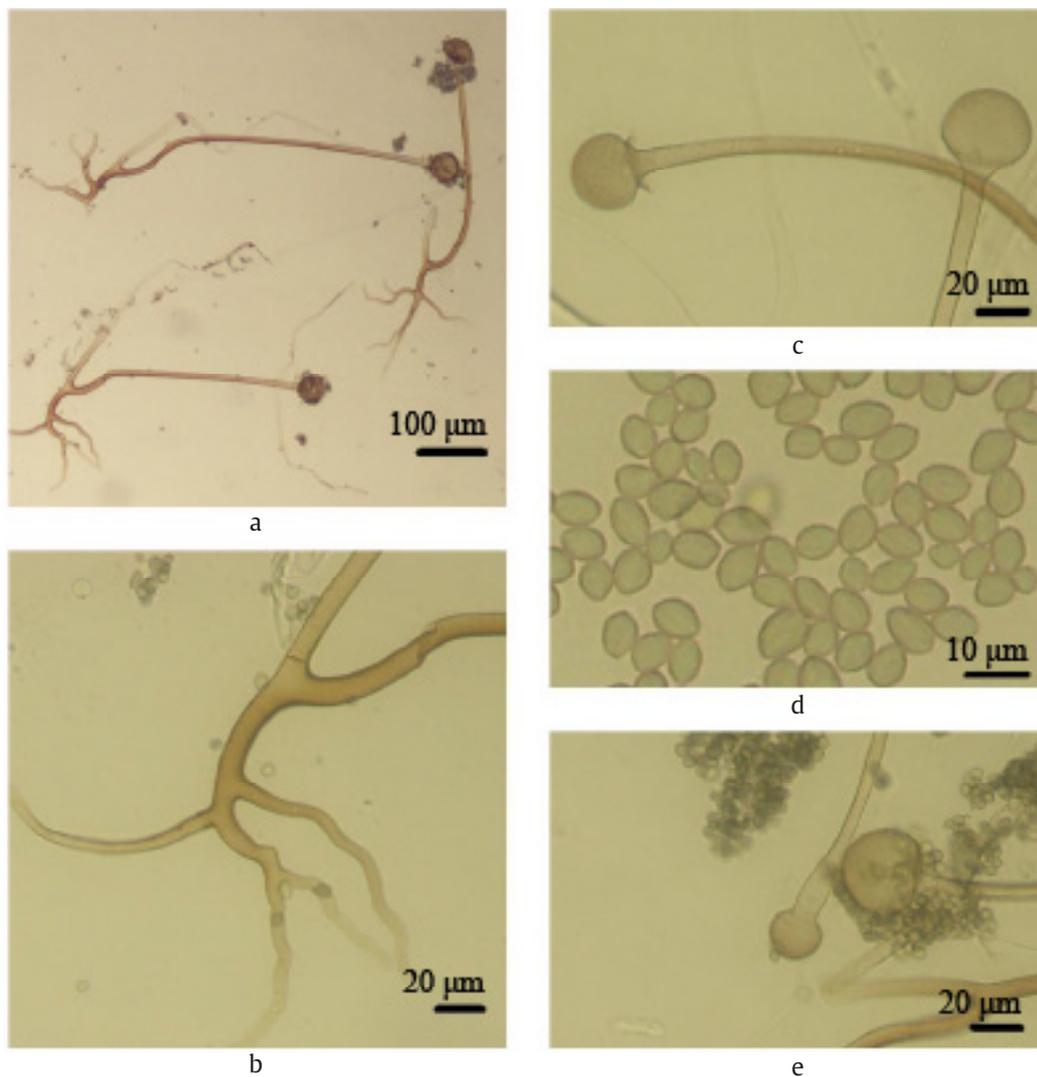


Figure 2. *Rhizopus delemar* strain collected from cherry tomato and peach: (a) sporangiophores in one cluster arising from rhizoid, (b) finger-like shaped rhizoid, (c) columella with distinct apophyses, (d) irregular shaped sporangiospores, (e) swelling on sporangiophore

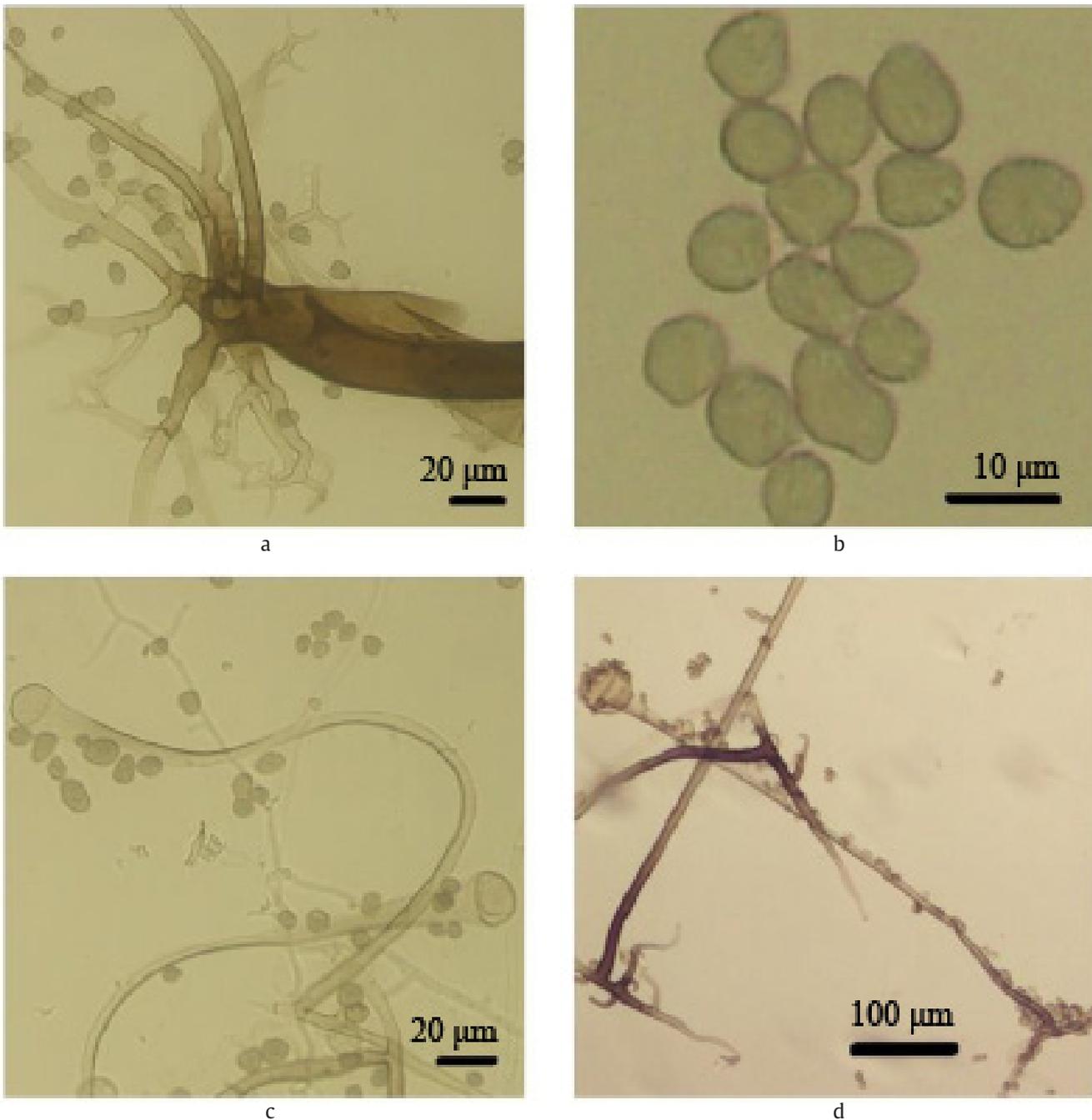


Figure 3. *Rhizopus stolonifer* strain collected from apple, banana, and pear: (a) profusely branched rhizoid, (b) irregular shaped sporangiospores, (c) columellae with distinct apophyses, (d) sporangiohores in single clusters arising from rhizoid

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).

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-1 α 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 occurred 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.

Acknowledgements

The authors would like to thank Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia for providing a fund to support this research.

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