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Original article

Isolation and identification of lontar-degrading fungus in Bali island, Indonesia

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Abstract

Lontar is a palm-leaf or lontar-based manuscript containing a Balinese script passed down from generation to generation. Lontar is derived from cellulose-rich palm leaf, which can serve as a substrate for the growth of fungi producing cellulase enzymes. Nevertheless, the activity of these fungi could potentially damage the lontar itself. The objective of this study is to identify the fungi that contaminated the Balinese lontar. The fungus was isolated from several Lontar storage areas in Balinese society using swabs. In addition, the fungus was cultivated on PDA media prior to isolation and identification. Seven lontar-degrading fungus isolates, including *Penicillium restricum, Aspergillus fumigatus, Mucor racemosus, Candida krusei, A. niger, Fusarium sp*., and *Rhodotorula mucilaginosa*, have been identified as the primary causes of damage to the Balinese lontar. Of these, *P. restricum*, *A. fumigatus*, *M. racemosus*, and *A. niger* were frequently found fungi at all sampling sites.

Keywords: lontar-degrading fungus, Balinese Lontar, isolation, identification

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Introduction

Lontar is a palm-leaf or lontar-based manuscript containing a Balinese script passed down from generation to generation. Classical or ancient manuscripts written on lontar leaves are typically worth preserving and religious (Geriani, 2010; Sedana *et al*., 2013). In ancient times, Balinese people often used palm leaves to make lontar before the invention of paper, which are used to record their writings. A substantial proportion of knowledge was written on the lontar, including architecture in the form of procedures for building houses in Bali, known as asta Kosala kosali, a law in the form of customary regulations (*awig-awig*) that Balinese people must obey and respect, and astrology as a guide for farming by Balinese people (Sancana, 2014).

The palm tree is majorly grown in South and Southeast Asia (Palmweb, 2017). This species of palm tree is a member of the Palmae and Arecaceae families. The Latin name for this plant species is *Borassus flabellifer* Linn (Hi, 2016; Sukamaluddin et al., 2016). It was also stated that this plant is known as ental or palm leaf in Bali. In ancient times, manuscripts, letters, and royal documents were written on palm leaves.

Bali, as part of Indonesia, has humid environmental conditions, making lontar extremely susceptible to damage. In addition to insects, the presence of microorganisms such as fungi causes harm to the Lontar. According to data submitted by the Bali Province Bali Language Extension Coordinator in 2016, of the 8.370 lontar units observed in all regencies and cities in Bali, 5.804 (69.3 %) are in good condition but require conservation, while 2.562 (30.1 %) are in poor state. The damage will worsen over time, thus conservation efforts are highly required, otherwise, the source of Balinese knowledge will be destroyed (Lestari, 2016). Palm leaves, which are used to make Lontar, are mostly composed of cellulose components, which are excellent substrates for fungal growth. Cellulose is a carbohydrate constituent of plant cell walls that is easily damaged by the cellulase enzyme (Yosmar et al., 2015). According to Sancana (2014), Lontar manuscripts are cultural objects that are susceptible to environmental influences. In general, the Balinese people still store lontars in a simple manner. Because the storage area is still in an open state, moisture can easily enter and mold growth is very likely. Such conditions can cause the lontar to degrade quickly. Previous research found that several fungi genera, including *Aspergillus, Penicillium, and Fusarium*, can harm the Balinese Lontar (Sancana, 2014).

In light of these observations, it is crucial to identify the types of fungi that are contaminating and degrading the Balinese Lontar in order to save and preserve it as the ancestral heritage of the Balinese people.

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Methods

Place and time of the research

This research was conducted from May 2020 to August 2020 at the Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, Udayana University, Bali, Indonesia.

Materials and instrumentation

Lontar from Bali and awar-awar leaves (Ficus septica) were used as the primary materials in this study. Maceration procedure was exploited using methanol PA solvent (Pro Analysis). The sample was isolated using water and sterile cotton. Potato Dextrose Agar (PDA) was used to culture the isolated fungi. Chloramphenicol (Indofarma) was used as antibacterial in PDA. Lactophenol Blue was used for fungi identification (Merck). The instruments used in this study, including cooler box (Marina), glass jar, separating funnel (Pyrex), blender (Miyako), knife, scissors, Petri dish (Pyrex), support rod, analytical balance (AND EK-300i), ErlenMayer (Pyrex), test tube (Pyrex), vortex (Poway), cock borer (Usbeck Germany), autoclave (Yazumi Autoclave Gas, SG-41280), beaker (Schott), stir bar, volumetric flask (Iwaki CTE 33), dropper (Pyrex), glass object (Sail Brand), cover glass (Sail Brand), Epi microscope (Nikon/Upright Microscope Eclips Ni-U), micropipette (Dumo and Parsons), blue and yellow tip (One Med), wire ose (Usbeck), filter paper number 1 (Whatman), counter (Novus), caliper (Sigma), aluminum foil (Klin Pak), Biological Safety Cabinet (Biosafety BH 2000), incubator (Memmert).

Isolation and identification of lontar-degrading fungi

Griya Balun (DB); Griya Batu Kandik (DK); Griya Kediri (TK); Griya Penebel (TS); Griya Ketewel (GU1); and Griya Mas Ubud (GU2) are repositories of the variety of lontar-degrading fungi isolated from lontar. The surface of a damaged Lontar is swabbed with sterile cotton. Then, the cotton is placed in a test tube containing sterile water. On ten distinct lontars, swabs were performed. The sample is placed in a cooler box for laboratory analysis. The isolation of lontar-degrading fungi used serial dilution process. Briefly, the sample was diluted to a 10^{-2} (v/v) dilution (Nester et al., 2007). Then, 1 mL sample was transferred into 9 mL of sterile water (dilution 10^{-1}). The 10^{-1} dilution was homogenized using a vortex, and 1 mL was transferred to a second test tube containing 9 mL of sterile water (dilution 10^{-2}). A 10^{-1}-10^{-2} dilution was used to label Petridishes. In a Petridish filled with 20 µL of Chloramphenicol (500 mg dissolved in 5 mL of sterile water), 1 mL of each dilution series was added, followed by 15 mL of sterile PDA. The mixture was simultaneously shaken to achieve a uniform mushroom culture. The sample was incubated at 28°C for 96 h. 10^{-2} dilutions of fungal colonies were re-isolated to create pure cultures. After incubating pure cultures for five days, macroscopic and microscopic identification was performed. The fungus with the greatest distribution was chosen for further analysis.

The fungal isolates are identified based on macroscopic and microscopic observations. The fungal isolate was grown on PDA and cultivated for macroscopic observation. According to Gandjar (2000), several features of macroscopic observations include 1) the color and surface of the colony (granular, like flour, mountainous, slippery, presence or absence of exudate drops), 2) the presence or absence of radial lines from the center of the colony to the center of the colony, the direction of the colony's edge, and 3) the presence or absence of concentric circles. In addition, the microscopic characteristics were also observed. To conduct microscopic observations, a clean slide culture and then drop Lactophenol dye were prepared. A small amount of the pure isolate was taken with a needle, aseptically streaked on the dye, covered with a cover glass, and then observed under a microscope at 400x magnification.

The objective of microscopic observation is to examine the anatomical structure of fungi that is not visible to the naked eye. Observations included 1) hyphae with or without a septum, 2) hyphae with hyaline (colorless or blue when stained) or darkly pigmented hyphae (greenish or blackish brown, dark black, grayish black), 3) spiral-shaped hyphae, or nodules, or rhizoids, and 4) single cells (smooth or rough-walled, pigmented or not). Multicellular (smooth or rough walled, septate or not, number of compartments, only transverse septa, or longitudinal transverse, pigmented or not). The species identification guide utilizes Fungi and Food Spoilage, Second Edition (Pitt and Hocking, 1997) and Introduction to Food-Borne Fungi (Samson et al., 1981)

Results

Lontar is a heritage from the ancestors of the Balinese people that must be preserved. Fungal isolation from several stored lontars revealed the presence of seven isolates of fungi as one of the causes of lontar damage. Figure 1 depicts a variety of fungi isolates that were successfully cultured on PDA media for four days at room temperature. Both Griya Balun and Griya Sunantaya had six distinct types of fungi. In contrast, only three to five types of fungi were discovered in other regions, as shown in Table 1. In this study, seven fungal isolates were successfully isolated. Of these, *Aspergillus fumigatus, M. racemosus*, and *A. niger* were most frequently found in the six sampling sites. At the same time, *P. restricum* was discovered only in Griya Balun and Griya Kediri. *Fusarium* sp. and *Rhodoterula mucilaginous* were the rarest fungi discovered in Griya Sunantaya and Griya Balun.

Figure 1. The variety of fungi grown in PDA media at room temperature with an incubation period of 4 days.

The macroscopic and microscopic observation of the fungi isolated from lontar and collected from six different locations are presented as follows:

1. *Penicillium restricum*

The morphological characteristics of the fungi isolates, as determined by macroscopic observation and according to Pitt and Hocking's (1997) manual, were grayish-green, flat, and white, with a colony diameter of 18 mm. According to microscopic observation with 40x magnification, the fungi isolates included conidiophores, metula, phialides, and conidia (Fig. 2). Combining the macroscopic and microscopic characteristics from the

observations, it can be estimated that the fungus was *P. restricum*.

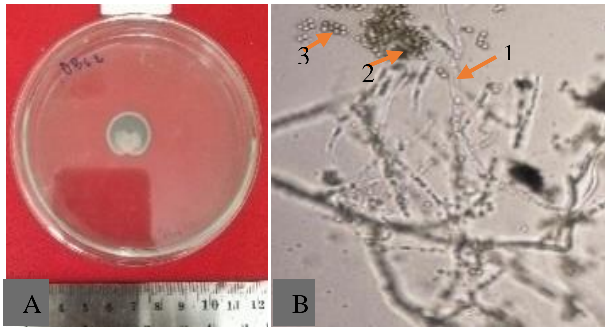

Figure 2. A. Colony of *P. restricum* on PDA media with an incubation period of 4 days. B. Microscopic (1. Conidiophores; 2. Metula; 3. Phialides and 3. Conidia)

2. *Aspergillus fumigatus*

According to the macroscopic observation and the Pitt and Hocking (1997) and Gandjar *et al*. (1999) manuals, the morphological characteristics of the fungi isolates were green, containing *aerial mycelia*, the flat edgesand white with a colony diameter of 50 mm. The incubation period of this fungi was seven days. In addition, based on microscopic characteristics, it has short conidiophores, vesicles, and round phialide conidia (Fig. 3). Based on these macroscopic and microscopic characteristics from the observations, it could be estimated that the fungus was *A. fumigatus*.

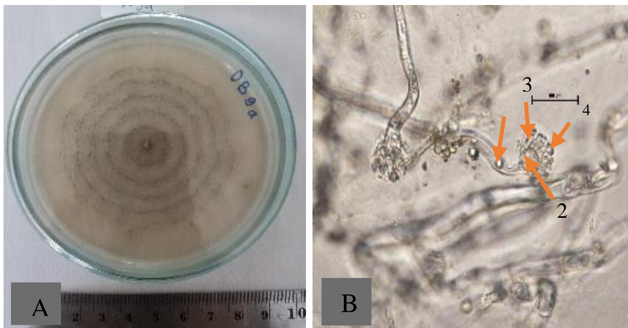

Figure 3. A: Colony of *A. fumigates* on PDA media within seven days of the incubation period B: Microscopic (1. Conidiophores; 2. Vesicles; 3. Phialides and 4. Conidia)

3. *Mucor racemosus*

According to macroscopic observation and the Pitt and Hocking (1997) manual, the morphological characteristics of the fungi isolates were grayish-yellow, and the hyphae grew rapidly until they filled the Petri dish.

As depicted in Figure 4, under 40x magnification, only chlamydospores were observed under the microscope. On the basis of the macroscopic and microscopic characteristics observed, it was possible to predict that the fungus was *M. racemosus.*

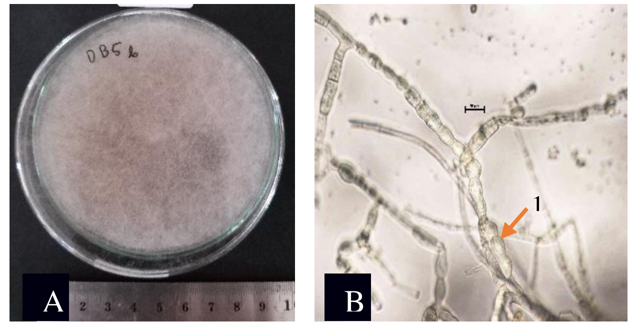

Figure 4. A: Colony of *Mucor racemosus* on PDA media with four days incubation period B : Microscopic *Mucor racemosus* (1. Chlamydiospores)

4. *Candida krusei*

Based on the macroscopic observation and Pitt and Hocking (1997), the morphological features of fungi isolates were white, uneven edges, and had a colony diameter of 12 mm after seven days of incubation. The microscopic features were found in oval-shaped cells with budding (Fig. 5). On the basis of these macroscopic and microscopic characteristics, it was possible to estimate that the fungus was *C. krusei*.

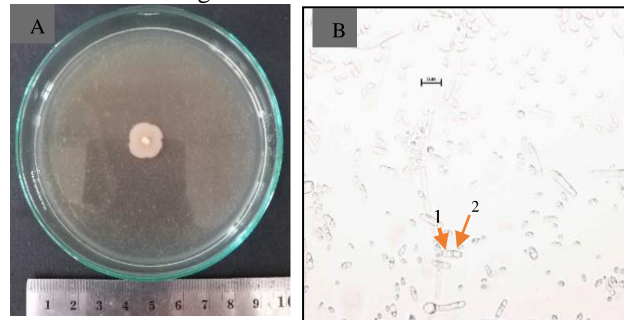

Figure 5. A: Colony of *Candida krusei* B: Microscopic (1.Vegetative cells ; 2.budding cell)

5. *Aspergillus niger*

On the basis of the macroscopic observations and the findings of Pitt and Hocking (1997) and Gandjar et al. (1999), the morphological characteristics of fungi isolates were blackish with white color and irregular edges, and the colony diameter was 40 mm after four days of incubation. Using a microscope with 40x magnification, the conidiophores, vesicles, and phialides conidia were observed to be spherical (Fig. 6). On the basis of these macroscopic and microscopic characteristics, the fungus was identified as *A. niger.*

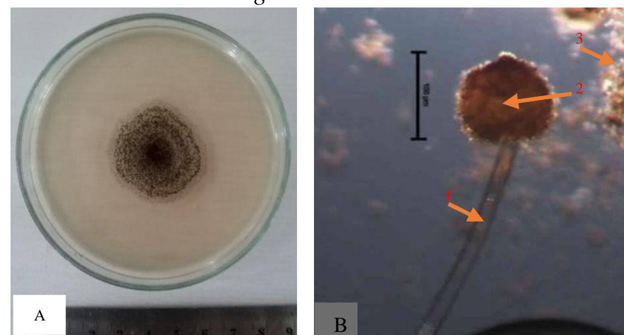

Figure 6. A: Colony of *A. niger* on PDA media with four days incubation period B: Microscopic (1. Conidiophores; 2. Vesicles; 3. Conidia)

6. ***Fusarium* sp.**

According to macroscopic observation and Pitt and Hocking (1997) and Gandjar et al. (1999), the morphological characteristics of fungal isolates were cotton-white, and as the incubation period increased, the bottom of the colony became brownish yellow, with white and uneven edges, and the colony diameter was 70 mm after 7 days of incubation. Based on the microscopic observations with a 40x magnification, macroconidia were coiled and partitioned on the inside, while microconidia were round, and chlamydospores were present on the hyphae (Fig. 7). Based on the macroscopic and microscopic characteristics, it could be estimated that the fungal isolates were *Fusarium* sp.

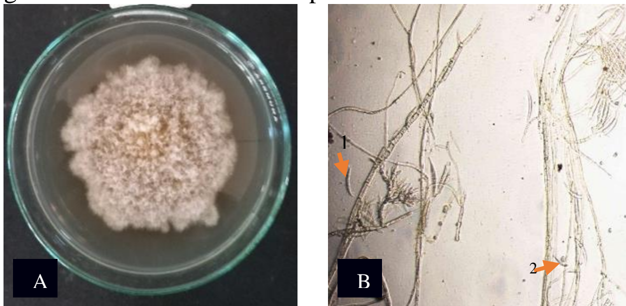

Figure 7. A: Colony of *Fusarium* sp. on PDA media with seven days incubation period B: Microscopic (1. Macroconidia; 2. Microconidia and chlamydospores)

7. ***Rhodoterula mucilaginosa***

Based on the macroscopic observation and the findings of Pitt and Hocking (1997), the morphological characteristics of the fungal isolate had a round shape with irregular edges and a mucoid, reddish-yellow surface. During the 4-day incubation period, the diameter of the colony was 20 mm. On microscopic examination using a microscope with a 40x magnification, the following results were obtained: the cells are elliptical, and there are shoots between them, as shown in Figure 8. Based on the macroscopic and microscopic characteristics, it could be estimated that the fungal isolate was *R. mucilaginosa*

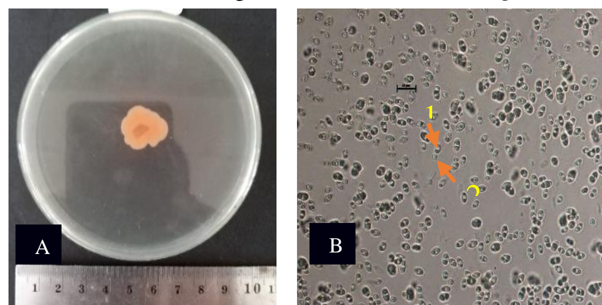

Figure 8. A: Colony of *Rhodotorula mucilaginosa* on PDA media with four days incubation period B: Microscopic (1. Vegetatif cells; 2. Budding cell

Discussion

Seven fungal isolates were successfully identified from the lontar of various stored locations, including Griya Balun and Griya Batu Kandik (Denpasar Regency); Griya Kediri and Griya Sunantaya (Tabanan Regency); Griya Ubud 1 and Griya Ubud 2 (Gianyar Regency). Six different types of fungi were discovered in Griya Balun and Griya Sunantaya. In contrast, only three to five types of fungi were discovered in other locations, as shown in Table 1. In this study, seven fungal isolates were successfully identified. Of these, *A. fumigatus, M. racemosus*, and *A. niger* were most frequently found at the six sampling sites. At the same time, *P. restricum* was only found in Griya Balun and Griya Kediri. *Fusarium* sp and *Rhodoterula mucilaginous* were the rarest types of fungi discovered in Griya Sunantaya and Griya Balun. It was hypothesized that the variety of fungi successfully isolated from these various locations depended on the method of lontar storage and environmental conditions. The most prevalent type of fungi was discovered in lontar stored in Griya Balun and Griya Sunantaya, whose storage conditions were in an open area, meaning that they were not stored in cupboards/glass shelves, making them susceptible to contamination by dust and water vapor. Griya Sunantaya, a mountainous region with an agricultural and plantation setting, contributed significantly to the discovery of *Fusarium* sp. in stored lontar. Sancana (2014) isolated this fungus from Balinese lontar and also reported its existence. In addition, *Penicillium* sp. and *Aspergillus* sp. have been isolated. *Fusarium* sp. was not conclusively identified as the primary destroyer of lontar, despite the fact that it is known to be pathogenic to plants. According to Sirait et al. (2018), one of the fungi that caused disease in Indonesian banana and grain crops was the *Fusarium* group.

A. flavus, A. niger, and A. fumigatus were successfully isolated from Balinese lontar at Griya Gede Siwa Manggis Manuaba, Nyanglan village, Klungkung regency, Bali (Setiani, 2018). Talantan et al. (2018) demonstrated that the Aspergillus group possessed the ability to produce cellulolytic enzymes capable of decomposing cellulose. The components of Balinese lontar are palm leaves or lontar leaves, which are majorly composed of cellulose. It is assumed that it could be harmed by the Aspergillus group, however, further research is required for confirmation. The discovery of a group of khamir in Balinese lontar, namely *C. krusei* and *Rhodoterula mucilaginosa*, was the most intriguing aspect of this study. According to Hachem et al. (2018), *C. krusei* could lead to candidemia in neutropenic patients. The discovery of this khamir in Balinese lontar in multiple locations (Griya Balun, Griya Kediri, and Griya Sunantaya) suggested that *C. krusei* was not the primary microorganism causing damage to Balinese lontar. *Rhodoterula mucilaginosa* is reportedly saprophytic in a variety of environments, including animals, humans, and a variety of foods and beverages. In addition, Deligios et al. (2015) found that khamir was an opportunistic pathogen that could affect immunocompetent and immunocompromised patients.

M. racemosus was a type of fungi that was discovered in nearly all sampling locations of the Balinese lontar. The growth of the colony on PDA media was grayish-yellow, and the hyphae expanded to the point where they passed through a Petridish. This is consistent with Pitt and Hocking's (1997) assertion that *M. racemosus* grows extremely rapidly on PDA media and forms a gray-yellow pigment. Al-Enazi et al. have also isolated this type of fungi from the soil of Al-Qassim, Saudi

Arabia (2017). The intracellular extract of *M. racemosus* was also found to inhibit the growth of *C. glabrata* and *C. norvegens*.

In conclusion, seven fungal isolates were identified from 6 different lontar storage sites in Bali province, including *Penicillium restricum*, *Aspergillus fumigatus, Mucor racemosus, Candida krusei, A. niger, Fusarium* sp., and *Rhodotorula mucilaginosa*. Of these, the most frequently found in all sampling sites were the *P. restricum*, *A. fumigatus, M. racemosus,* and *A. niger*.

Table 1. The fungi isolates were isolated and collected in lontar from several locations in Bali

No	Types of Fungi	The storage Lontar location					
		DB	DK	TK	TS	GU1	GU2
1	*Penicillium restricum*	√	-	√	-	-	-
2	*Aspergillus fumigatus*	√	√	√	√	√	√
3	*Mucor racemosus*	√	√	√	√	√	√
4	*Candida krusei*	√	-	√	√	-	-
5	*Aspergillus niger*	√	√	√	√	√	√
6	*Fusarium* sp.	-	-	-	√	-	-
7	*Rhodoterula mucilaginosa*	√	-	-	-	-	-

Note : √ : Present - : Not Present

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