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Isolation and Identification of Thermophilic Cellulolytic Fungi

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A B S T R A C T

Indonesia has a substantial amount of cellulosic waste from the agricultural industry that can be further utilized by involving microorganisms capable of producing cellulase enzymes. These cellulase-producing microorganisms can be obtained through isolation and identification from samples sourced from compost of various leaves. The shift in Earth's temperature due to global warming demands a change in fermentation conditions to a thermophilic fermentation state. Therefore, this research aims to obtain thermophilic cellulolytic fungal isolates. In this study, the isolation process involved enrichment in CY broth media and isolation on PDA media with incubation at 45°C. The obtained isolates were tested for their cellulolytic ability on Cellulose Agar media at 45°C. Subsequently, isolates showing positive cellulolytic activity were identified to the species level. The initial isolation resulted in 13 isolates, and after a selection process, 4 thermophilic cellulolytic isolates were obtained. The identification results showed that these isolates were Rhizopus oligosporus (KDK2, KR1.b, and KR1tb) and Aspergillus terreus (KDK3).

Keywords: *Isolation, identification, thermophilic, cellulolytic.*

1. Introduction

Indonesia, as an agrarian country, has vast productive agricultural land, with 13,835,252 hectares of rice harvest area in 2013. The total harvest area for food crops such as rice, corn, soybeans, peanuts, mung beans, cassava, and sweet potatoes in Indonesia amounted to 20,136,282 hectares in 2013 (Anonymous$_1$, 2015). The extensive productive agricultural land impacts the processes in the agricultural industry, generating by-products that can be further utilized, such as cellulosic waste, husks, and straw (Hardjo et al., 1989). Common sources of cellulosic waste in Indonesia include straw and sugarcane fiber (bagasse).

The agricultural waste rich in cellulose has high value because it can be used as a renewable energy source, such as biofuel, or converted into various essential products like chemicals (organic acids and polylactic acid for biodegradable plastics) and enzymes. The abundant cellulosic waste from agriculture, industry, and forestry, along with the need for biodegradation and bioconversion of this waste into fermentable sugars, has increased the demand for more effective cellulase-producing microorganisms. Bioconversion holds a promising prospect for developing the utilization of cellulosic waste, using inexpensive raw materials to produce products beneficial to humans (Hardjo et al., 1989). Bioconversion involves microorganisms that can hydrolyze cellulose into desired products, including several bacteria and filamentous fungi (Zhang et. al., 2006).

In previous research, bioconversion was conducted by Aspergillus japonicus URM5620 using jatropha fruit waste with solid-state fermentation (SSF) to process jatropha oil waste, which has minimal toxin content, making it suitable for animal feed and fertilizer (Herculano, et al., 2011). Another study reported on other cellulase-producing fungal species, such as Rhizopus oligosporus, which can produce significant amounts of β-glucosidase during growth in solid-state systems and hydrolyze phenolic glucosides (Vattem DA, et al., 2002). These studies indicate a tendency to use fungi as bioconversion agents.

Microorganisms typically grow in current environmental conditions as mesophilic microorganisms, thriving at temperatures between 20-45°C (Prescott, 2008). This includes fungal species involved in bioconversion, which are mesophilic. However, recent environmental phenomena have affected microbiological processes. Global warming, causing unpredictable rises in environmental temperatures, has drawn global experts' attention. The increased temperatures affect fermentation or microbiological processes, necessitating anticipation to obtain microorganisms capable of physiological activity at higher temperatures than current environmental conditions. The availability of thermophilic microorganisms is crucial. Thermophilic microorganisms can perform physiological activities at temperature ranges of 45-65°C, higher than mesophilic microorganism activity temperatures (Prescott, 2008).

The thermophilic microorganisms suitable for their ability to hydrolyze cellulose and possessing thermophilic properties to be studied in this research are fungi. Known thermophilic fungi include ascomycetes from the orders Sordariales, Eurotiales, and Onygenales, or zygomycetes from the order Mucorales (Berka et al., 2011; Morgenstern et al., 2012). Thermophilic fungi are typically found in compost, sawdust, stored rice grains, and livestock manure (Johri et al., 1999). This aligns with the habitat of thermophilic fungi, which require high temperatures for growth. Compost, sawdust, stored rice grains, and livestock manure likely naturally contain thermophilic fungi.

Regarding the expected cellulolytic properties of the microorganisms studied, Maheswari et. al (2000) state that thermophilic fungi have varying abilities to degrade cellulose. Some microorganisms exhibit weak capabilities. Not all thermophilic fungi have the same cellulolytic ability at specific high temperatures, necessitating cellulase enzyme activity tests for each type for evaluation purposes. This research aims to obtain thermophilic cellulolytic fungal isolates, which are expected to be useful for processing agricultural waste and further research development.

2. Material and methods

2.1 Material and Tools

Leaf compost from the zinc roof parking lot of university and leaf and twig compost from dry bushes in Selomartani Village Garden. Dry straw from the Selokan rice fields and Selomartani Village rice fields. Media used includes CYA (Czapek Yeast Agar), PDA (Potato Dextrose Agar), MEA (Malt Extract Agar), CA (Cellulose Agar), and SS (Semi Solid) agar. Chemicals used for isolation and identification include 0.1% Tween 80, 20% lactic acid, and 100 ppm chloramphenicol. 250 ml erlenmeyer flask, 500 ml erlenmeyer flask, water bath shaker, scissors, spatula, petri dishes, 1 ml and 5 ml micropipettes, autoclave, oven, 30°C incubator, 25°C incubator, 50°C incubator, thermometer, spectrophotometer, 100 ml graduated cylinder, 250 ml graduated cylinder, 10 ml volumetric flask, test tubes, test tube rack, cotton plug, 100 ml bottles, analytical balance, and Laminar Air Flow.

2.2 Isolation of Aspergillus sp. and Rhizopus sp.

Isolation is performed by first preparing the sample, which involves collecting it from its original location. The sample is then reduced in size and placed into CY broth medium in a 250 ml Erlenmeyer flask, incubated at 45°C with shaking at 100 rpm. Once the fungi have grown, they are taken and inoculated onto PDA medium, each according to its morphological appearance. The next step is to isolate fungi that still have different morphological appearances from the initial isolate. Purification is then carried out. Once a pure isolate is obtained, a preliminary macroscopic test is conducted. This is followed by a qualitative cellulase test using the method of Kasana et al. (2008) and the calculation of the cellulolytic ratio according to Roza et al. (2013). The formula to determine the qualitative cellulolytic ability of fungal isolates can be calculated as follows:

The ratio (R) = Z/K (Eq. 1)

Z = diameter of the clear zone

K = diameter of the colony

This method concludes with the identification of the genus and species level for each isolate using the method of Samson et al. (1984).

2.3 Identification of Aspergillus sp. and Rhizopus sp.

The identification of the genera *Aspergillus* and *Rhizopus* conducted with several key steps, namely the rejuvenation of culture isolates, macroscopic observation, and microscopic observation. The working culture of the fungal isolate is taken with the pointed end of a sterile skewer, then aseptically dipped into semi-solid agar. Next, the fungal spores are collected with the sterile end of the skewer and aseptically inoculated onto Czapek Yeast Agar (CYA) media. The media is then incubated at 30°C for the genera *Rhizopus sp.* and at 25°C for the genera *Aspergillus sp*. Subsequently, macroscopic and microscopic identification of each isolate can be performed to determine the species of the isolate.

- Identification of the genus and species *Aspergillus sp.*

At this step, the identification is initiated with macroscopic observation. The colony characteristics was observed visually, including color, texture, and morphology. *Aspergillus terreus* typically forms colonies that are brown to reddish-brown with a dense, velvety texture. After obtaining matching characteristics, the observation is continued microscopically.

The grown colony was taken in a small portion and placed on a microscope slide. A stain such as lactophenol cotton blue was applied to highlight the structures. Examination is conducted under a microscope to identify hyphae, conidia, and vesicles. *Aspergillus terreus* has smooth conidiophores ending in round vesicles with brown, rough conidia. Next, the observations are compared with the morphological descriptions available in the literature, specifically the description by Samson (1984) for *Aspergillus terreus*. Matching of the characteristics such as vesicle size and shape, as well as conidia and hyphae features is conducted.

- Identification of the genus and species *Rhizopus sp.*

At this step, the identification is initiated with macroscopic observation. The colony characteristics was observed visually, including color, texture, and morphology. *Rhizopus oligosporus* typically forms colonies that are white to gray and have thick hyphae. After obtaining matching characteristics, the observation is continued microscopically.

The grown colony was taken in a small portion and placed on a microscope slide. A stain such as lactophenol cotton blue was applied to clarify the structures. Examination is conducted under a microscope to identify hyphae, sporangia, and spores. *Rhizopus oligosporus* has round sporangia with smooth spores and coenocytic (non-septate) hyphae. Next, the observations are compared with the morphological descriptions available in the literature,

specifically the description by Samson (1984) for *Rhizopus oligosporus.* Matching of the characteristics such as the size of the sporangia, the shape and size of the spores, and the characteristics of the hyphae is conducted.

3. Result and Discussions

3.1 Isolation of Leaf Compost and Leaf-Twig Shrub Compost Samples

Isolation from samples of pine leaves (DC), leaves (D) from the zinc roof of the university parking lot, leaf compost (KD), wood compost (KK), twig compost (KR), dry twigs (RK), and dry leaf compost (KDK) from the shrub garden of Selomartani Village resulted in isolates shown in Table 4.1.

Table 3.1. Isolation from samples of pine leaves (DC), leaves (D) from the zinc roof of the university parking lot, leaf compost (KD), wood compost (KK), twig compost (KR), dry twigs (RK), and dry leaf compost (KDK) from the shrub garden

Source of sample	**Isolate code**	**Colony colour**	**Isolate characterictic**
Leaf of pine tree compost	DC_{1p} (Fungi in surface on CY Broth)	White fibrous cotton like and blackish fibrous cotton like	White fibrous cotton like DC_{1p} and blackish fibrous cotton like $DC_{1p\,2}$
	DC_{1t} (Fungi sink below surface of CY Broth)	White fibrous cotton like	White fibrous with black spotted
	DC_2 Fungi on second repetition	White soft fibrous cotton like	White soft fibrous cotton like
Leaf compost	D_3 (Fungi sink below surface of CY Broth)	White soft fibrous cotton like	White soft fibrous cotton like
	D_{3p} (Fungi in surface on CY Broth)	White fibrous cotton like	Dark green with white fibrous around
Leaf compost	KD_1	White rough fibrous	White fibrous with green spot in the center
Wood compost	-	-	-
Branch wood compost	KR_{1b}	White soft fibrous cotton like	White fibrous with black spotted
	$KR1_{b2}$	White rough fibrous	White fibrous with green spot in the center
	$KR1_{tb}$	White soft fibrous cotton like	White fibrous with brown-blackish spotted
Branch wood	RK_1	White soft fibrous cotton like	White soft fibrous cotton like
Dry leaf compost	KDK_2	White fibrous cotton like	White soft fibrous cotton like
	KDK_3	Green sticked on medium surface	Green sticked on medium surface with wrinkle under medium

Based on the Table 3.1., it is known that the isolates obtained from the isolation process consist of 13 thermophilic isolates. This refers to the ability of the isolates to grow during incubation at a temperature of 45°C for seven days. Then, the thirteen isolates proceeded for checking their cellulose degradation capability.

3.2 Screening or Selection of Sample Isolates

After obtaining fungal isolates that can grow at a temperature of 45°C, the next step is to screen their cellulolytic ability, which is the ability to degrade cellulose. This is done by growing the isolates on Cellulose Agar media and subsequently measuring the clear zone. The results of the characterization of thermophilic sample isolates and their cellulolytic abilities can be seen in Figure 3.1 and Figure 3.2.

KDK_3 KDK_2 $KR_{1b.2}$ KR_1tb $KR_{1.b}$ KD_1

Figure 3.1. Screening or selection of the isolates (from leaf and branch compost)

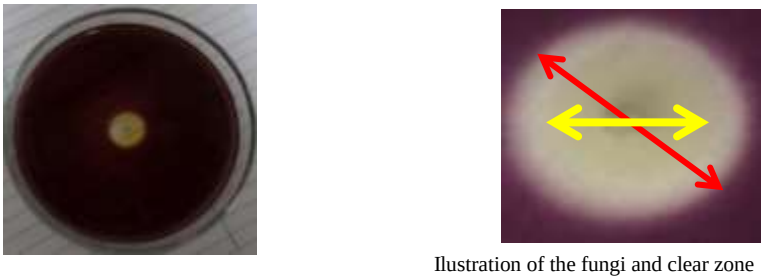
The result of screening from each isolated sample after flood by Gram's iodine (3-5 minutes)

DC_{1P} DC_{1T} DC_2 D_3 D_{3P}

Figure 3.2. Screening or selection of the isolates (from dry leaf)

The result of screening from each isolated sample after flood by Gram's iodine (3-5 minutes)

The results shown in the figures represent the grouping of selected outcomes from two sample groups: leaf-twig compost from the shrub habitat in Selomartani Village (Figure 3.1) and leaf compost from the zinc roof habitat of the university parking lot (Figure 3.2). These results display a clear zone indicating the differences between the growing fungal colonies and the clear zones produced by the colonies. The clear zones represent the media hydrolyzed by cellulase enzymes from each grown fungus. For the RK_1 sample, prediction tests were not conducted because the strain died during storage due to improper handling. Figure 3.1 visualizes the largest colonies and clear zones in the order $KR_{1tb} > KR_{1b} > KD_1 > KDK_2 > KR_{1b2} > KDK_3$. However, it is not this visualization that serves as the qualitative reference for the cellulolytic abilities of fungal isolates, but rather the calculation of the colony diameter compared to the diameter of the clear zone on the media.



Isolate with clear zone Ilustration of the fungi and clear zone

Figure 3.3. Illustration of Clear Zone Calculation on Cellulose Agar Media

The illustration of clear zone calculation which can help determine the cellulose degradation capability of the successfully isolated fungi. The cellulolytic ratio calculated using the (Eq. 1).

Table 3.2. The Iodine Test for Fungal Isolates from Two Sample Groups

Source of sample	Sample	Cellulolytic ratio
Leaf-twig compost from the shrub habitat of Selomartani Village	KR_{1tb}	R = 1.0278
	$KR_{1.b}$	R = 1.0303
	KDK_2	R = 1.0769
	KD_1	R = 1.3200
	$KR_{1b.2}$	R = 1.4500
	KDK_3	R = 1.4810

Leaf compost from the zinc roof parking lot habitat of FTP	D_3	R = 1.1300
	DC_{1T}	R = 1.2500
	DC_2	R = 1.2600
	D_{3P}	R = 1.2700
	DC_{1P}	R = 1.3200
	DC_{1P2}	R = 1.2500

From Table 3.2, it is known that the qualitative order of the cellulolytic ratio in leaf-twig compost samples from the shrub habitat of Selomartani Village is KDK3 > KR1b.2 > KD1 > KDK2 > KR1b > KR1.tb. For the order of the ratio in leaf compost samples from the zinc roof parking lot habitat of FTP, it is DC1P > D3P > DC2 > DC1T = DC1P2 > D3.

3.3. Identification of Genera and Species Levels

3.3.1. Rhizopus oligosporus

Table 3.3. Identification of Isolates Based on the Genus *Rhizopus*

Genera characteristic	Characteristics according to Samson et al., 1984	Characteristics according to the results (KR_{1tb}, KDK_2, KR_{1b})
Colony growth	Fast	Fast
Stolon	Present	Present
Rhizoid	Present	Present
Sporangiospore	Present	Present
Spora shape	Globose-ellips	Ellips (KR_{1b}), globose (KR_{1tb}, KDK_2).
Spora size	Generally large in size	Generally large in size
Spora color	White when young, turning blackish-brown as it matures.	Entirely cottony white when young, turning grayish-brown as it ages (KR1tb, KR1b), and dark brown to blackish as it further matures (KDK2).
Sporangium	Containing many spores	Containing many spores
Columella shape	Globose atau half-globose	Globose
Columella colour	Brown	Brown
Chlamidiospore	Present in several species	Present

Based on the presence of stolons, rhizoids, and sporangiophores (Table 3.3), which are key colony characteristics matching the genus *Rhizopus*, along with the matching characteristics of spores, sporangium, color of columella, and chlamydospores, colonies KR1tb, KDK2, and KR1b can be classified under the genus *Rhizopus*. Subsequently, the specific characteristics are matched with the species *Rhizopus oligosporus*.

Table 3.4. Identification of Isolates Based on Species *Rhizopus Oligosporus*

Species characteristic	Characteristics according to Samson et al., 1984	Characteristics according to the results (KR_{1tb})
Colony color	Brownish pale grey on Czapek Agar	Brownish pale grey on Czapek Agar
Colony size	1 mm high or more	3-5 mm
Sporangiophore	Present, compact in groups	Present, compact in groups
Sporangiophore size	Up to 1000 µm long and 10-18 µm	130 µm long and 12,5 µm diameter
Spora shape	Globose-elips	Globose
Spora size	7-10(24) µm	3,75 µm, 5 µm, 7,5 µm

Spora color	Becoming blackish brown with age	Becoming blackish brown with age
Sporangium and sporangium size	With many spores, diameter (50) 100-180 µm	With many spores, diameter (50) 100-180 µm
Columella shape	Globose or sub-globose with a funnel shape apophysis	Globose or sub-globose with a funnel shape apophysis
Chlamidiospore	Abundant, with no colour	Abundant, with no colour
Chlamidiospore size	7-30 µm atau 12-45 x 7-35 µm	10 x 22 µm
Suspected spesies		*Rhizopus oligosporus*

According to the matching characteristics of colony, sporangiophores, spores, sporangium, columella, and chlamidiospore (Tabel 3.4.) referring to the specific characteristics of the species R. oligosporus, it is determined that only colony KR1tb belongs to the species *Rhizopus oligosporus*.

3.3.2. Aspergillus terreus dan Aspergillus fumigatus

Table 3.5. Identification of Isolates Based on the Genus *Aspergillus*

Genera Characteristics	Characteristics (Samson *et.al.*, 1984)	Characteristics on Result (DC_{1P} , D_{3P}, DC_2 , DC_{1T} , $DC_{1P2,}$ $D_{3,}$ KDK_3 ,KD_1)
Colony color	White, yellow, brownish-yellow, blackish brown or green or with thick conidiophore	Green colony with thick conidiophore
Conidiophore	Aseptate, unbranched stipe	Aseptate, unbranched stipe
Phialide	Uniseriate , Biseriate	Uniseriate , Biseriate
Vesicle	Present	Present
Metulae	Present	Present
Conidia	Columnar or radiate, one celled, with or no ornament	Columnar

Based on the presence of vesicle and metulae, as well as the matching characteristics of colony color, conidiophore, phialide, and conidia (Table 3.5), which are key colony characteristics matching the genus *Aspergillus*, colonies DC_{1P} , D_{3P}, $DC_{2,}DC_{1T}$, $DC_{1P2,}$ $D_{3,}$ KDK_3 ,KD_1 can be classified under the genus *Aspergillus*. Subsequently, the specific characteristics are matched with the species *Aspergillus terreus* and *Aspergillus fumigatus*.

Table 3.6. Identification of Isolates Based on Species *Aspergillus terreus*

Species Characteristic	Characteristic (Samson *et.al.*, 1984)	Characteristic on Result (KDK_3)
Colony growth	On Czapek Agar, colony growth for seven day	On Czapek Agar, colony growth for seven day
Colony diameter	3,5 – 7 cm	5 cm
Conidia color	Brownish yellow and becoming darker at incubation	Brownish yellow and becoming darker at incubation
Conidia size	1,5 -2,5 µm	2 µm
Conidia shape	Globose or ellips	Globose
Conidiophore	Smooth walled	Smooth walled
Phialide size	Biseriate, 5-7 x 2-2,5 µm	Biseriate
Metulae size	5,5 x1,5 -2 µm	
Vesicle size	10 -20 µm, subglobose	11 µm , subglobose
Stipe		60 µm
Suspected species		*Aspergillus terreus*

According to the matching characteristics of colony; conidia color, size and shape; conidiophore; phialide, metulae, and vesicle size; as well as the presence of stipe (Tabel 3.5.), referring to the specific characteristics of the species *A. terreus*, it is determined that only colony KDK_3 belongs to the species *Aspergillus terreus*.

Table 3.7. Identification of Isolates Based on Species *Aspergillus fumigatus*

Species Characteristic	Characteristic (Samson *et.al.*, 1984)	Characteristic on Result (KD1, KR_{1b2}, DC_{1P} , D_{3P}, DC_2,DC_{1T} , $DC_{1P2,}$ D_3)
Colony growth	On Czapek Agar, colony growth for seven day	On Czapek Agar, colony growth for seven day
Colony diameter	3,5 – 7 cm	4 cm (KD1) and 5cm (D_3)
Conidia colour	Dense felt dark green	Dense felt dark green
Conidia size	2,5 – 3 µm	
Conidia shape	Conidia head : columnar, conidia : globose	Conidia head : columnar, conidia : globose
Conidiophore	Short, Smooth walled, green	Short, Smooth walled, green
Phialide size	Biseriate, 5-7 x 2-2,5 µm	Biseriate
Phialide colour	Green	Green
Vesicle size	10 -20 µm, subglobose	Subglobose
Suspected species		*Aspergillus fumigatus*

Based on the identification of species levels, the isolates KD_1, KR_{1b2}, D_3, DC_{1T}, DC_2, D_{3P}, DC_{1P}, and DC_{1P2} were identified as *Aspergillus fumigatus*. Since *Aspergillus fumigatus* is a pathogenic fungus, these isolates were not continued for further research. Therefore, only four isolates, namely KDK_3, KDK_2, KR_{1b}, and $KR_{1.tb}$, can be continued for testing their ability to produce cellulase enzyme activity.

4. Conclusion

The initial isolation resulted in 13 isolates, and after a selection process, 4 thermophilic cellulolytic isolates were obtained. The identification results showed that these isolates were Rhizopus oligosporus (KDK2, KR1.b, and KR1tb) and Aspergillus terreus (KDK3) could be continued for testing their ability to produce cellulase enzyme activity.

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