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Molecular identification of cellulase-producing thermophilic fungi isolated from Sungai Pinang hot spring, Riau Province, Indonesia

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Abstract. *Saryono, Novianty R, Suraya N, Piska F, Devi S, Pratiwi NW, Ardhi A. 2022. Molecular identification of cellulase-producing thermophilic fungi isolated from Sungai Pinang hot spring, Riau Province, Indonesia. Biodiversitas 23: 1457-1465.* Thermostable cellulolytic enzymes have become a subject of interest in industrial processes due to their ability to degrade cellulosic polysaccharides at elevated temperatures produced by microorganisms such as fungi and bacteria. In the present study, cellulase-producing thermophilic fungi were isolated and identified from Sungai Pinang hot spring, Riau Province, Indonesia. Morphological identification was carried out by macroscopic and microscopic observations. The ability of the thermophilic fungi to produce cellulase was determined using the clear zone test on carboxymethyl cellulose medium with a congo red staining. Isolate with the highest activity was identified molecularly using universal primers ITS1F and ITS4R. The results showed 19 isolates were morphologically identified as *Aspergillus* sp. and *Penicillium* sp. Based on qualitative testing, 3 of the19 isolates showed cellulase activity. The isolate performing the most considerable cellulase was LBKURCC293, which was identified as *Aspergillus fumigatus* with a cellulase activity of 2.6 x 10^{-2} IU/mL and a specific cellulase activity 8.0 x 10^{-3} IU/mg protein after 96 days of incubation.

Keywords: *Aspergillus fumigatus*, cellulase, hot spring, identification, thermophilic fungi

INTRODUCTION

Cellulose is a carbohydrate consisting of a linear polymer chain of repeating units of cellobiose joined by β-1,4 linkages. Cellulose chains interact through hydrogen bonds and form rigid and insoluble crystal structures (Yamada et al. 2013; Vázquez-Montoya et al. 2019). Cellulose can be hydrolyzed by cellulases that can be produced by bacteria and fungi (Zumrotiningrum et al. 2004; Islam and Roy 2018). This enzyme plays an essential role in industry and biotechnology, namely food, medicine, cosmetics, detergents, textile, pulp, paper, waste treatment, and pharmaceutical industries (Soares et al. 2012; Utami et al. 2019). Degradation of cellulose to glucose usually involves a mixture of several enzymes, including three specific enzymes, namely endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (Saroj et al. 2018; Rezaei et al. 2008).

Some industrial processes require high temperatures. Therefore, there is an increasing demand for thermophilic and thermostable producers of cellulolytic enzymes, particularly those resistant to product inhibition and highly stable (Arora et al. 2014; Mallerman et al. 2015). Research on thermophilic microorganisms conducted such as by Ardhi et al. (2020), has successfully isolated and identified thermophilic bacteria in several hot springs in West Sumatra, Indonesia. Fachrial et al. (2019) have also reported the isolation and molecular identification of thermophilic bacteria in North Sumatra, Indonesia.

Because of their ability to produce stable and active thermotolerant extracellular enzymes at high pH and temperature, thermophilic fungi are widely used for enzyme production for industrial application. Fungi were chosen because they can produce more enzymes than other microorganisms such as bacteria, which due to more practical extraction and purification processes (Vázquez-Montoya et al. 2019). Research on the production of cellulase enzymes from fungi has attracted many researchers. Saroj et al. (2018) isolated fifteen thermophilic fungi from soil with optimal activity from crude thermostable cellulase obtained at 60°C, whereas FPase, β-glucosidase, and xylanase were at 50°C. Meanwhile, Gautam et al. (2011) isolated two cellulase-producing fungi, *Aspergillus niger* and *Trichoderma* sp., from municipal solid waste with the optimum temperature of 40 and 45°C, and pH 6.7 and 6.5, respectively.

Thermophilic fungi can survive at high temperatures, in which hot spring is one of their habitats. Indonesia is rich

in hot springs with potential as a habitat for thermophilic fungi. The thermophilic fungi isolated from extreme environment of hot springs in Sungai Pinang, Riau, Indonesia would have industrial prospect to be developed especially in the field of biotechnology. For this reason, this research was conducted to isolate, characterize and identified thermophilic fungi from Sungai Pinang hot spring in Riau as well as to test their cellulase activity.

MATERIAL AND METHODS

Isolation and screening of cellulase-producing ability

The hot water sample was collected from Sungai Pinang hot spring, Riau Province (Figure 1), using a random sampling technique. The sample was inoculated using the pour plate method, in which a total of 1 mL of homogeneous sample was put in the center of a sterile petri dish and followed with the pouring of Potato Dextrose Agar (PDA) medium. After the agar was solid, the plate was inverted and incubated according to the actual temperature (45 and 50°C) for five days. Purification was carried out until a pure colony was obtained. The fungal isolates' ability to degrade cellulose was observed through the clear zone formation on carboxymethyl cellulose (CMC) medium with 0.1% congo red staining. The formation of a clear zone indicated that the isolate could degrade cellulose (Mohammad et al. 2017), which involved specific cellulose enzyme complexes, namely endo-1,4-β-D-glucanase, Exo-1,4-β-D-glucanase, and β-D-glucanase (Ikram-ul-Haq et al. 2005)

Morphological characterization

Macroscopic characterization was conducted by observing the growth of the fungal colonies on PDA, which included the shape, the edge, and the color of the colony. Mycelium growth, and the characteristics of the conidia were observed using an Olympus CX23LEDRFS1 microscope coupled with Optilab Viewer 3.0. The morphological characteristics observed were based on the characteristics described by Samson et al. (2010).

Molecular identification

Molecular identification was carried out based on sequences of Internal Transcribed Spacer (ITS) regions, for the isolates that showed highest ability to hydrolyze cellulose. Cellulase enzyme activity was determined using CMC as a substrate. The isolate was grown on PDA using the spread plate method. The genomic DNA of the fungal mycelia was extracted using the Wizard Genomic DNA Purification Kit (Promega). PCR amplification of the ITS region was carried out using universal primers ITS1F (5 'TCC GTA GGT GGA CCT GCG G' 3) and ITS4R (5 'TCC TCG GCT TAT TGA GC' 3), in a total volume of 50 µL containing 2 µL of genomic DNA is, 10 µL of each primer (2 pmol), 5 µL dNTP mix (2 mM), 10 µL buffer + 5 µL $MgCl_2$, 7.75 µL H_2O, and 0.25 µL Go Taq DNA Polymerase (Promega).

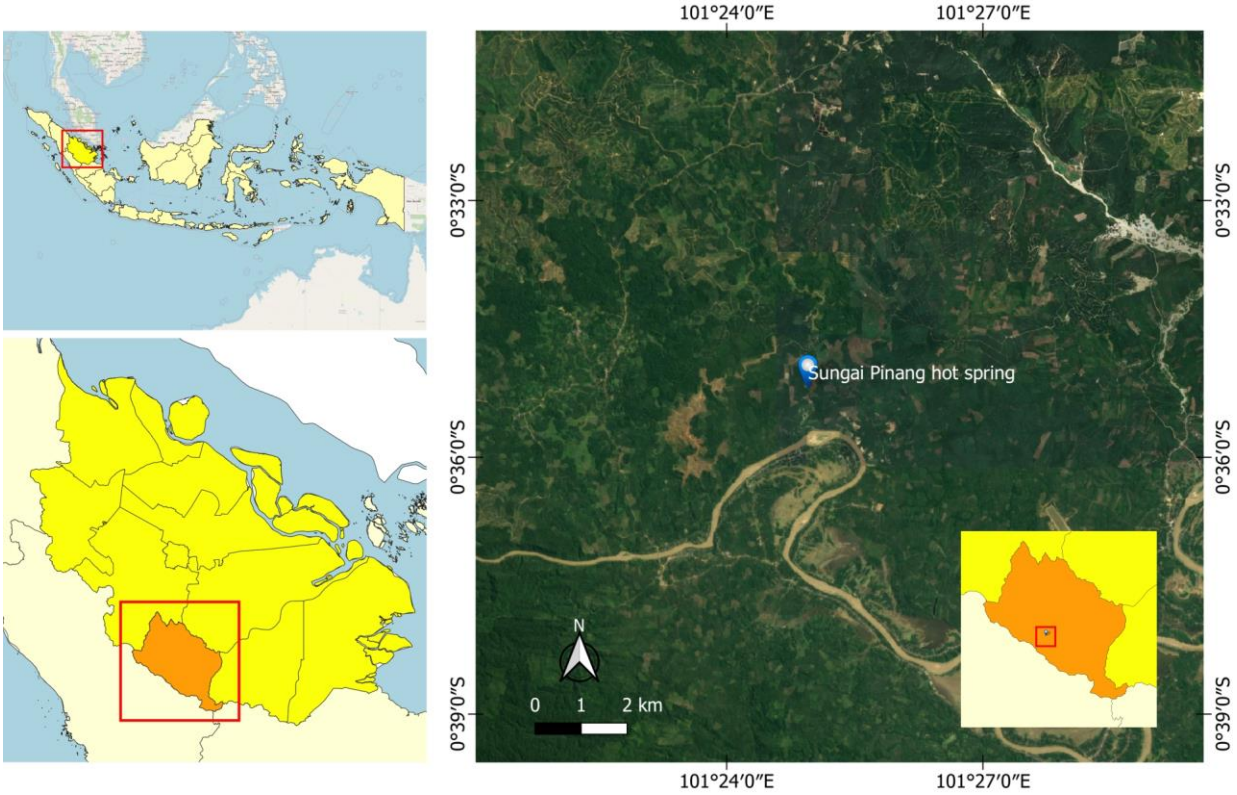


Figure 1. Location of Sungai Pinang hot spring, Riau, Indonesia

PCR cycles applied in this study were pre-denaturation performed at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. The amplified PCR products were visualized using 0.8% electrophoresis. The PCR product's molecular weight was calculated based on sample's migration distance compared to 1 kb DNA marker used. The PCR products were sequenced at the Eijkman Molecular Biology Research Center and determined their similarity with isolates found on GenBank accessed through BLAST (http://www.ncbi.nlm.nih.Gov/Blast.)

After sequencing, BLAST search was performed against Genbank database to find the similarity with the isolates in the database. Multiple sequences alignment was used to align the sequences for phylogenetic analysis based on Neighbour-Joining (N-J) method, utilizing MEGA6 software (Ardhi et al. 2020). The relatedness of the isolates was performed using Clustal X version 2.1 program.

Determination of cellulase activity and specific cellulase activity

The ability of the isolates to hydrolyze cellulase was determined using the methods by Mandels et al. (1976), followed with Nelson Somogyi method, which was expressed as μmol reducing sugar/mL minutes. Cellulase activity was performed using CMC as a substrate. A total of 1 mL of 2% CMC substrate was placed in a test tube, while the control tube was left empty. The substrate was incubated for 5 minutes in a water bath (40°C). The test and the control tubes were then added with 1 mL of enzyme solution and incubated for 30 minutes in a water bath. After that, the blank tube was filled with 0.05 M phosphate buffer solution pH 7 of 2 mL, then the test, control, and blank tubes were added with 1 mL of the Nelson-Somogyi reagent. The tubes were heated in a water bath (40°C) for 20 minutes and cooled to room temperature. Approximately, 1 mL of arsenomolybdate reagent was added to the tubes. After incubation for 5 minutes, 6 mL of distilled water was added into the blank tube, while in the test and control tubes, 7 mL of distilled water was added. All the tubes were homogenized, incubated for 30 minutes, and absorbance at a wavelength of 540 nm was measured using Thermo Scientific Genesys 10S UV-Vis spectrophotometer. The glucose solutions with the concentration range of 0-70 μg/mL were used to produce a glucose standard curve.

Enzyme activities were defined in International Units (IU), indicating the amount of enzyme that release 1 μmol reducing sugars/mL/minute. Meanwhile, specific cellulase activity is defined as the number of cellulase IU/mL divided by the protein concentration in mg/mL. Thus, it is quoted as IU/mg. The protein content of the enzymes produced was determined by the Lowry method (Lowry et al. 1951).

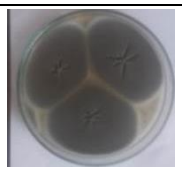
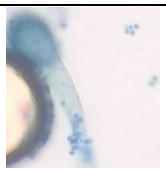
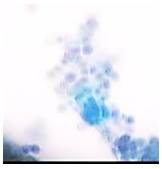
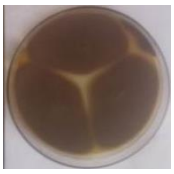
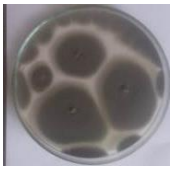
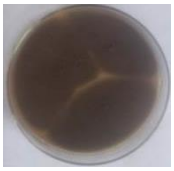
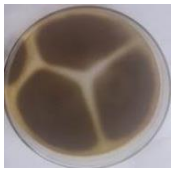
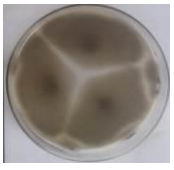
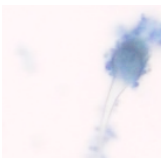
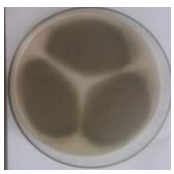
RESULTS AND DISCUSSION

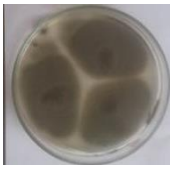
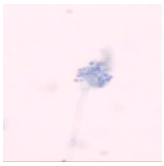
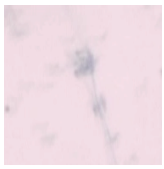
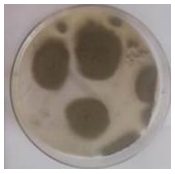
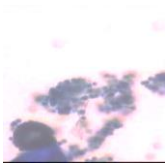
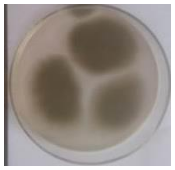
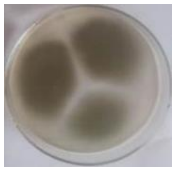
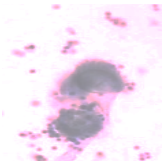
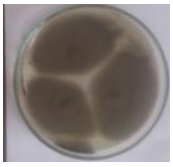
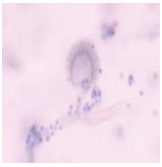
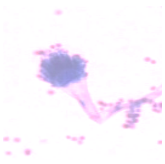
Isolation and screening of cellulase

A total of 19 thermophilic fungal isolates were isolated from the Sungai Pinang hot spring. Based on macroscopic and microscopic characteristics, the isolates were identified as *Aspergillus* sp. and *Penicillium* sp. Identification of the isolates and the cellulase activity screening are presented in Table 1.

Macroscopically, *Aspergillus* colony was initial white and turned to green with a smooth colony surface. The results were similar with a study by Gautam and Bhadauria (2012), who isolated a dark green colony with white edges identified as *A. fumigatus*. *A. fumigatus* can proliferate at 37°C and tolerate temperatures above 50°C (Bhabhra and Askew, 2005). Due to its capability to grow and survive at a wide range of temperatures, *A. fumigatus* is globally distributed and can be isolated from many environments. On starvation, *A. fumigatus* produces conidia (asexual spores) on specialized hyphal structures called conidiophores, which successively produces conidia on characteristic conidial heads. *A. fumigatus* colony is composed of septated multinucleated cells (van de Veerdonk et al. 2017). For *Penicillium*, initially the colony was white with dense hyphae, and changed to dark green interspersed with white mycelia. Microscopic features of *Aspergillus* was characterized by conidiophores, vesicles, and round shape conidia. Meanwhile, *Penicillium* was identified by the hyphae with septa and round-conidium presence. The formation of the clear zone indicated the ability of isolates to hydrolyze the cellulose substrate. The wider the diameter of the clear zone produced, the higher the enzyme activity. From 19 isolates successfully isolated from the hot spring, only three *Aspergillus* isolates could produce cellulase, which were LBKURCC286, LBKURCC288, and LBKURCC293 with the ratio of the colony and clear zone of 1:1.76, 1.72, and 1.99, respectively (Table 1) with isolate LBKURCC293 produced the largest clear zone.

Table 1. Characterization and cellulase activity of thermophilic fungi isolated from Sungai Pinang hot spring, Riau, Indonesia

Temp. (°C)	Isolates (LBKURCC)	Characterization		Genus identification	Clear Zone ratio
		Macroscopic	Microscopic		
45	265	 - *dark green* - *granular colony* - *rough colony surface texture*	 - *septate hyphae* - *conidia round* - *round vesicles*	*Aspergillus*	0.00±0.00[c]
	266	 - *dark green* - *velvety texture*	 - *septate hyphae* - *clear and smooth conidiophores* - *round conidia*	*Penicillium*	0.00±0.00[c]
	267	 - *blackish green* - *granular colony* - *rough colony surface texture*	 - *septate hyphae* - *round conidia*	*Aspergillus*	0.00±0.00[c]
	268	 - *dark green* - *granular colony* - *rough colony surface texture*	 - *septate hyphae* - *semi-round vesicles* - *hyaline conidiophore*	*Aspergillus*	0.00±0.00[c]
	269	 - *blackish green* - *granular colony* - *rough colony surface texture*	 - *septate hyphae* - *semi-round vesicles*	*Aspergillus*	0.00±0.00[c]
	270	 - *green* - *granular colony* - *rough colony surface texture*	 - *septate hyphae* - *semi-round vesicles*	*Aspergillus*	0.00±0.00[c]
	271	 - *blackish green* - *granular colony* - *rough colony surface texture*	 - *septate hyphae* - *semi-round vesicles* - *hyaline conidiophore*	*Aspergillus*	0.00±0.00[c]
	272	 - *green* - *granular colony* - *rough colony surface texture*	 - *septate hyphae* - *round conidia* - *elliptical vesicles*	*Aspergillus*	0.00±0.00[c]
50	284	 - *dark yellowish-green* - *granular colony* - *rough colony surface texture*	 - *septate hyphae* - *round vesicles* - *hyaline conidiophore*	*Aspergillus*	0.00±0.00[c]
	285	 - *green with black color at the edge* - *granular colony* - *rough colony surface texture*	 - *septate hyphae* - *semi-round vesicles* - *hyaline conidiophore*	*Aspergillus*	0.00±0.00[c]

286		- *yellowish-green* - *granular colony* - *rough colony surface texture*		- *septate hyphae* - *round vesicles* - *hyaline conidiophore*	*Aspergillus*	1.76±0.22[b]
287		- *green* - *granular colony* - *rough colony surface texture*		- *conidium* - *round vesicles* - *hyaline conidiophore*	*Aspergillus*	0.00±0.00[c]
288		- *yellowish-green* - *granular colony* - *rough colony surface texture*		- *conidium* - *semi-round vesicles* - *hyaline conidiophore*	*Aspergillus*	1.72±0.15[b]
289		- *yellowish-green* - *granular colony* - *rough colony surface texture*		- *round conidia* - *hyaline conidiophore* - *semi-round vesicles*	*Aspergillus*	0.00±0.00[c]
290		- *yellowish-green* - *granular colony* - *rough colony surface texture*		- *round conidia* - *hyaline conidiophore* - *semi-round vesicles*	*Aspergillus*	0.00±0.00[c]
291		- *yellowish-green* - *granular colony* - *rough colony surface texture*		- *round conidia* - *hyaline conidiophore* - *semi-round vesicles*	*Aspergillus*	0.00±0.00[c]
292		- *green* - *granular colony* - *rough colony surface texture*		- *conidium* - *round vesicles* - *hyaline conidiophore*	*Aspergillus*	0.00±0.00[c]
293		- *green with white color at the edge* - *granular colony* - *rough colony surface texture*		- *round conidia* - *short conidiophore* - *semi-round vesicles*	*Aspergillus*	1.99±0.11[a]
294		- *green with black color at the edge* - *granular colony* - *rough colony surface texture*		- *septate hyphae* - *semi-round vesicles* - *hyaline conidiophore*	*Aspergillus*	0.00±0.00[c]

Molecular identification

Molecular identification was carried out to ascertain the species of LBKURCC293 isolate. A phylogram of LBKURCC293 thermophilic fungi was obtained using the Neighbor-Joining Tree method with 10.000 bootstrap replicates. The bootstrap analysis is carried out to test the branches of a reliable phylogenetic tree and how well the phylogenetic tree model data sets are used. The higher the bootstrap repetition rate, the higher the accuracy of the phylogenetic tree obtained. Several studies also used 10.000 bootstrap replications in fungal and bacterial testing (Krimitzas et al. 2013; Anwar et al. 2016; Gordillo-Fuenzalida et al. 2019). The size of the LBKURCC293 isolate in the ITS region was 609 and based on the

phylogenetic analysis, the isolate was grouped with *A. fumigatus*. (Figures 2 and 3). BLAST search showed the isolate LBKURCC293 has 98% similarity with *A. fumigatus* with E-Value 0, which indicated a high level of significance.

In the present study, ITS was chosen as its region was probably the most widely sequenced region of DNA in fungi. ITS has two informative regions, namely ITS-1 and ITS1-2, located between the 18S ribosomal subunit of SSU and the 28S LSU and separated by the 5.8S ribosomal subunit (Figure 4). Moreover, universal primers are available, and the region has multicopy properties of rDNA repeats, making it relatively easy to amplify, even though from highly degraded DNA samples. ITS regions vary significantly among fungal species with different taxonomies or even isolates from the same species (Larena et al. 1999; Brasileiro et al. 2004).

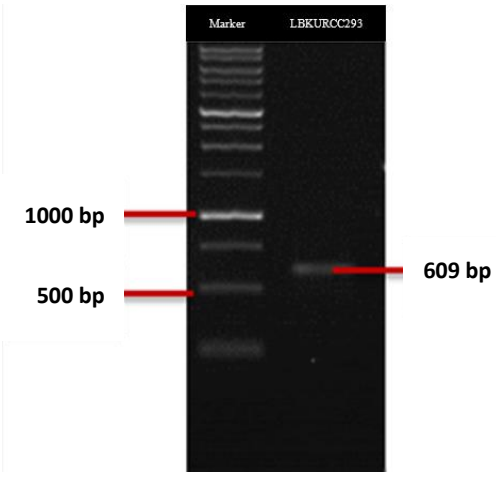


Figure 2. PCR product of LBKURCC293

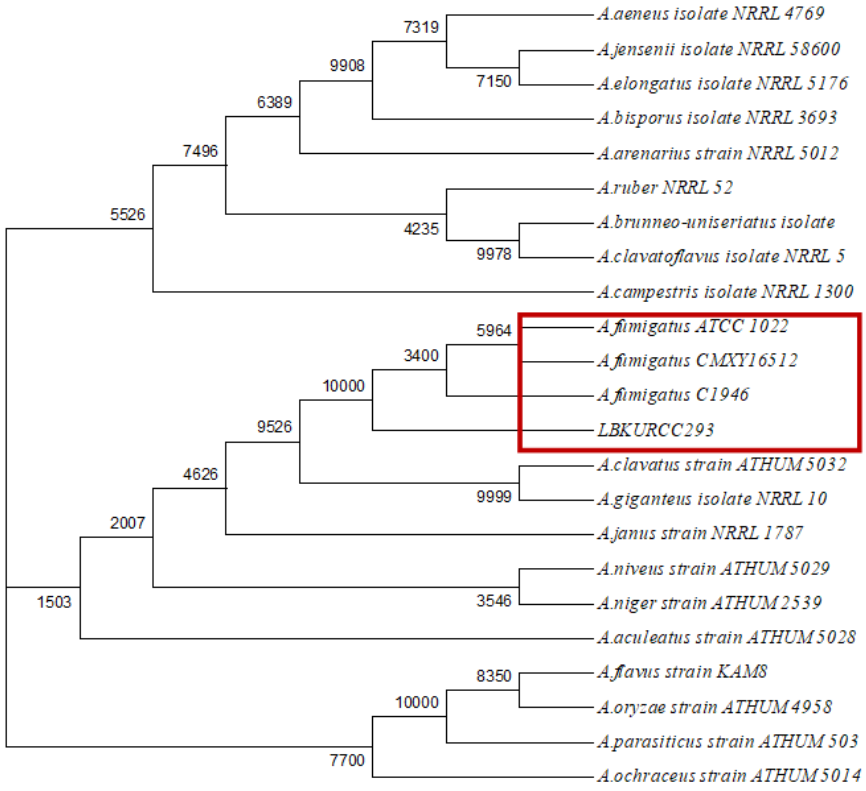


Figure 3. The phylogenetic analysis of LBKURCC293 isolate

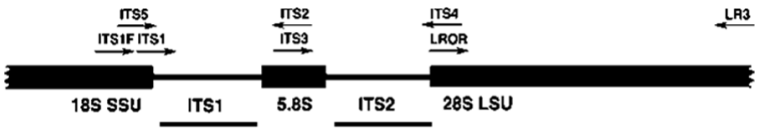


Figure 4. ITS regions for PCR amplification (Raja et al. 2017)

This molecular identification followed macroscopic and microscopic observation, which showed that the LBKURCC293 isolate was *Aspergillus* sp. *Aspergillus* is a genus widely utilized in biotechnology because it can ferment various substrates under extreme conditions that generally the group of bacteria and yeast cannot live (Krimitzas et al. 2013). Previous studies had successfully isolated cellulase-producing thermophilic *Aspergillus fumigatus* isolates that showed their ability to produce extracellular enzymes, such as lignocellulolytic and cellulolytic (Moretti et al. 2012; Saroj et al. 2018; Saroj et al. 2021). Even *Aspergillus* sp. and their consortium were also reported to be able to live in extreme environment of polluted soil and yielded long hydrocarbon-decomposing enzymes (Novianty et al. 2021). Saroj et al. (2022) reported that the cellulase from *A. fumigatus* JCM 10253 exhibited optimum activity at 50°C, which the purified cellulases showed significant tolerance against various metal ions, denaturing agents, and organic solvents. Another report found that the crude cellulase secreted by *A. fumigatus* Z5 was further applied to hydrolyze pretreated corn stover and the enzymatic hydrolysate was successfully used as a substrate for ethanol production by *Saccharomyces cerevisiae* (Liu et al. 2011). Srivastava et al. (2018) resumes that the main components of the microbial cellulase enzymatic system, namely endoglucanase, exoglucanase and β-glycosidase, effectively converted cellulosic substrates into fermentable sugars. On the other hand, Low et al. (2011) found that A. fumigatus was related to allergenicity due to its ability to produce major allergenic proteins that are more highly expressed at lower sporulation temperatures.

Determination of cellulase activity and specific cellulase activity

The results of optical density and cellulase activity of LBKURCC293 isolate from 168 hour-incubation are shown in Figure 5. The results showed that the optical density and cellulase activity peaked at 96 hours of incubation and subsequently decreased to 168 hours of incubation. Enzyme activity illustrated the ability of isolate to hydrolyze cellulose-containing substrates. In general, enzymes produced by thermophilic fungi were thermostable because they lived in extreme environments with temperatures between 45-80°C (Alrumman et al. 2018). The productivity of cellulase increased along with longer incubation time to reach the optimum point. However, after reaching the optimum point, enzyme activity decrease due to cell death, thus glucose produced is also decreased.

The protein rose until 48 hours of incubation and remained stable during the incubation period (Figure 6). In comparison, the highest specific cellulase activity was produced at 96 hours. The highest cellulase activity in this study was at 96 hour-incubation (2.6 x 10^{-2} IU/mL). The result was considerably lower than reported by El-Hadi et al. (2014), which has maximum enzyme activity of 0.23 IU/mL with 96 hours incubation time. Another cellulolytic fungal strain, *Aspergillus fumigatus* NITDGPKA3 isolated from straw retting ground, produced cellulolytic activity at 6.53 IU/mL (Sarkar and Aikat, 2013). In a study by Saroj et al. (2018), thermophilic *Aspergillus fumigatus* JCM10253 that grew at 50°C, produced the maximum cellulase activity of 26.2 IU/mL at 144 hours. In their subsequent study, Saroj et al. (2021) optimized the cellulase production from the same fungal isolate and yielded 95.2 IU/mL of enzyme. Immanuel et al. (2006) produced cellulase from coir waste and sawdust using *A. fumigatus*, of which the highest cellulase (5.2 x 10^{-2} IU/mL) was obtained at pH 6, and temperature at 40°C, with maximum production was 0.290 U/mL.

The isolate LBKURCC293 in the present study demonstrated the ability to produce cellulase at high temperatures of 50°C with an incubation period of 96 hours. Meanwhile, the specific cellulase activity was equal to 8.0 x 10^{-3} IU/mg protein obtained after incubation for 96 hours. An increased protein generated by thermophilic fungi revealed the possibility of enzymes produced during incubation was not only cellulase, thus causing the activity of cellulase to be relatively small but had high protein levels. Crude cellulase has less specific activity than pure cellulase. Purification is needed to remove contaminants such as proteins or other molecules present in the mixture (Nema et al. 2016).

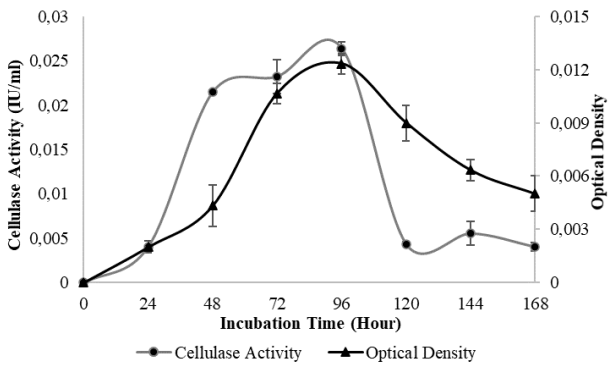


Figure 5. Cellulase activity and optical density of LBKURCC293 isolate

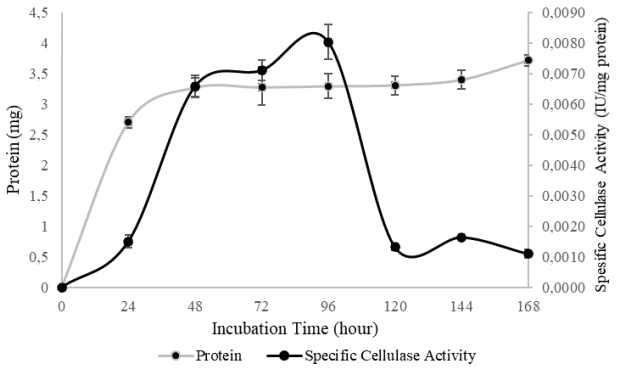


Figure 6. Protein content and specific cellulase activity of LBKURCC293 isolate

In conclusion, 19 isolates of thermophilic fungi were isolated from the Sungai Pinang hot spring, Riau Province, and morphologically identified as *Aspergillus* sp. and *Penicillium* sp. A total of 3 isolates from 19 isolates were able to produce cellulases. The isolate with the highest cellulase activity was LBKURCC293, which was molecularly identified as *Aspergillus fumigatus*, with the cellulase activity of 2.6 x 10^{-2} IU/mL and the cellulase specific activity of 8.0 x 10^{-3} IU/mg protein obtained at 96 hour-incubation time. The present findings demonstrated that the cellulase from the thermophilic isolate of *A. fumigatus* could become the potential candidate for cellulose hydrolysis in elevated temperatures, which is crucial and most preferred for industrial purposes. Future studies may be conducted to optimize the condition to produce the highest cellulase activity and continue with purification and characterization of the enzyme produced.

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