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Isolation and screening of cellulolytic fungi from *Salacca zalacca* leaf litter

SITI LUSI ARUM SARI♥, RATNA SETYANINGSIH, NOVITA FITRIATUL AINI WIBOWO
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57 126, Central Java, Indonesia. Tel./Fax. +62-271-663375, ♥email: arumprasojo@gmail.com

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Abstract. *Sari SLA, Setyaningsih R, Wibowo NFA. 2017. Isolation and screening of cellulolytic fungi from Salacca zalacca leaf litter. Biodiversitas 18*: *1282-1288.* Cellulases are the main enzymes in a bioconversion of lignocellulosic biomass. Fungal cellulases have been proven to be better candidates for this process than other microbial cellulases since they secrete free cellulase complex including endoglucanases, cellobiohydrolases, and β-glucosidase. This study was conducted to isolate and screen of cellulolytic fungi from *Salacca* leaf litter. There were about 12 fungal isolates which were obtained in this research and 8 isolates showed cellulolytic activity. Based on morphological characters, these cellulolytic fungi were identified as belonging to 4 genera, i.e. *Penicillium*, *Aspergillus*, *Paecilomyces*, and *Thielaviopsi*s. Based on clear zone formation, isolate SLL03, SLL06 and SLL10 showed highest cellulolytic activity. Whereas, based on 18S rRNA gene sequence, these isolates were identified as *Aspergillums flavus* (SLL03), *Penicillium* sp. (SLL06) *Thielaviopsis ethacetica* (SLL10). Therefore, these isolates would be good candidates for cellulase producer.

Keywords: Cellulase, cellulolytic, fungi, leaf litter, *Salacca*

INTRODUCTION

Cellulases are a group of enzymes which play an important role in the hydrolysis process of β-1,4-glycosidic linkage in cellulose. The complex of cellulolytic enzymes consist of endo-cleaving (endoglucanases), exo-cleaving (cellobiohydrolases) and β-glucosidases. A complete hydrolysis of crystalline cellulose involves synergistic actions of these cellulolytic enzymes (Lynd et al. 2002; Dashtban et al. 2009; Sari et al. 2016).

Cellulases are the third most significant commercial enzyme in the world market. Cellulases, solely or in a mixture with other enzymes, are involved in several industries including biofuel, food, feed, beverages, paper, textile, pharmaceutical, agricultural etc (Kuhad et al. 2011). Cellulase is one of the most important enzymes that can be used in the pulp and paper processes. The application of cellulolytic enzymes in the bioleaching process is environment-friendly in nature and can improve the quality of pulp and paper (Jerusik 2010). The addition of cellulase to the silage production process can improve the quality of silage fermentation as it can increase both the fiber degradation and the content of water-soluble carbohydrates (WSC) which are substrates for lactic acid bacteria (Li et al. 2014).

Recently, researches on cellulolytic enzymes have been done intensively due to their important role as the lignocellulosic material source in the process of bioethanol production (Okeke et al. 2015). Cellulase plays important role in the process of fermentable sugars production from cellulose, which is the primary polysaccharides in lignocellulosics. Cellulase production is the key phase of the enzymatic cellulose hydrolysis process. Since the cost of enzyme production reaches 50% of the total cost of cellulose hydrolysis process, the current research is mainly focused on the suppression of production cost of enzymes (Howard et al. 2003). Isolation and characterization of cellulolytic microbes provide a good starting point for the discovery of such beneficial enzymes. Therefore, much research is aimed to obtain new microorganisms producing cellulase with higher specific activities (Rathnan et al. 2012).

Cellulase is produced by large number microorganisms, including fungi and bacteria. Fungi are eukaryotic organism which are the main producers of extracellular cellulase. Cellulolytic fungi include many species such as genus *Trichoderma* (Atanasova et al. 2010) *Aspergillus*, *Eurotium*, *Penicillium, Rhodotorula* (Herculano et al. 2011) and *Neurospora* (Hildebrand et al. 2015). Most of the cellulolytic fungi belong to Ascomycota and Basidiomycota phylums. Filamentous fungi are used in many industrial processes for the production of enzymes and metabolites. Some of advantages employing fungi for enzyme production are low-cost material with high productivity, faster production, and amenable modified enzymes. Furthermore, the extracellular enzymes which is normally secreted outside cells can be easily recoverable from the culture media (Vishwanatha et al. 2010; de Souza et al. 2015).

Fungi are well known as decomposing agents of organic matters (Lynd et al. 2002). Lignocellulose-rich materials, such as leaf litter, compost soil, decayed wood and lignocellulosic waste are potential substrates for lignolytic fungi. Lignocellulose-rich materials should, therefore, be a good source for the discovery of lignolytic fungi producing novel cellulase. This study was conducted

to isolate and screen of cellulolytic fungi from *Salacca* leaf litters which are rich of lignocelluloses.

MATERIALS AND METHODS

Source of fungi

Fungi were isolated from *Salacca* leaf litter samples which were collected randomly in five points of *Salacca* plantation in Srumbung, Magelang, Central Jawa, Indonesia. Samples were collected in early October 2015.

Isolation and screening of cellulolytic fungi

Isolation of fungi was performed by serial dilution and spread plating method, in which 25 g of sample was suspended in 225 ml of sterile 0.85 % NaCl (10^{-1} dilution). From this dilution, 1 ml was taken and diluted serially from 10^{-2} to 10^{-5}. Diluted samples (10^{-2} to 10^{-5}) were plated on Potato Dextrose Agar (PDA) media added by 0.01 % (w/v) Chloramphenicol and incubated for 96 hours at 25 ^{o}C. For fungal purification, fragments of different colonies were transferred separately to PDA in petri dishes. After confirming the purity, fungal isolates were transferred to PDA slant and preserved at 4^{o}C.

Cellulolytic activity was screened based on clear zone formation on Carboxy methyl cellulose (CMC) media. The media comprised (per l): urea 0.3 g, $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2.0 g, $CaCl_2$ 0.3 g, $MgSO_4$ 0.3 g, yeast extract 0.25 g, peptone 0.75 g, CMC 10 g, and agar 17.7 g (Khokhar et al. 2012). Fungal isolates were inoculated at CMC media and incubated at 24 ^{o}C for 48 hours. To visualize the clear zone, the plates were flooded with an aqueous solution of Congo red 1% for 30 minutes and washed with 1 M NaCl (Theater and Wood 1982).

Morphological identification of cellulolytic fungi

Cellulolytic fungi were identified based on colony morphology on PDA and microscopic characters. Cellulolytic fungi were inoculated at PDA in petri dishes and incubated at 25 ^{o}C for 7 days.

Molecular identification

Fungal isolates which showed highest cellulolytic activities were identified based on 18S rDNA partial sequence. Genomic DNA extraction was done by DNeasy Plant Mini Kit (Qiagen). Fungal mycelium (100 g) was lyophilized in liquid Nitrogen and grinded to a fine powder using a mortar and pestle. The powder was transferred to sterile 1.5 ml microcentrifuge tube, which were then added with, 400 µl AP1 buffer and 4 µl RNase A (provided in the kit). The next step is followed the manufacturer's instruction of The DNeasy Plant Mini Kit Protocol.

The partial sequence of the 18S of the fungal rDNA was amplified using forward and reverse primers (nu-SSU-0817-5'-and nu-SSU-1536-3' (Borneman and Hartin 2000). DNA from fungi were amplified in 25 µl PCR mixtures containing the following final concentrations with total amounts: 18.8 µl of ddH_2O, 0.5 µL dNTP, 2.5 µL Taq buffer, 0.2 µL Taq polymerase, 1 µL reverse primer and 1 µL forward primer. All reagents were combined and heated at 94°C for 5 min. twenty-five cycles of PCR were then performed by using 95°C for 1 min, 55°C for 1 min, and 72°C for 1min. Purification and sequencing of the 18S rDNA were done in 1st Base Singapore. Similarities of the rDNA sequences to sequences in the GenBank database were determined by using BLAST (National Center for Biotechnology Information http: //www.ncbi.nlm.nih.gov/BLAST). Phylogenetic analysis was performed using the neighbor-joining method with MEGA 6.0. statistically evaluating the branching, bootstrap analysis was carried out with data resampled 1000 times (Sari et al. 2016)

RESULTS AND DISCUSSION

Isolation and screening of cellulytic fungi

This research successfully isolated 12 fungal isolates from *Salacca* leaf litters. The cellulolytic activities of these isolates were tested based on the clear zone formation on the minimal media containing CMC (Figure 1). The result of the screening showed that eight isolates had cellulolytic activities indicated by the clear zone formation around the colony (Table 1). The dimension of the clear zone ranged from 0.2527 cm^2 to 1.2158 cm^2. Three isolates which were most potential as cellulase producers were SLL03, SLL06, and SLL10 with the clear zone dimensions of 1.2158 cm^2, 0.8135 cm^2, and 1.1424 cm^2, respectively.

Identification of morphological character-based cellulolytic fungi

The cellulolytic fungi were identified based on the morphological characters. The identification result showed that the cellulolytic fungi were identified as belonging to 4 genera, i.e. *Penicillium, Aspergillus, Paecilomyces* and *Thielaviopsis* (Table 2).

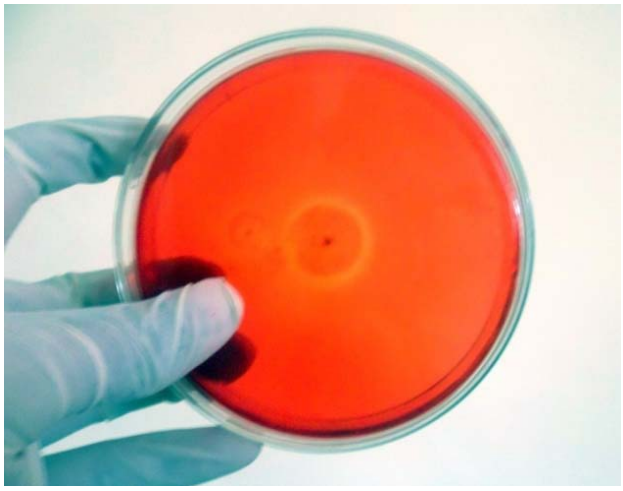

Figure 1. SLL03 on CMC medium after colouring with 1% Congo red. Clear zone surrounding a colony indicate cellulolytic activity

Table 1. Clear zone dimension showed cellulolytic activity of fungi isolated from *Salacca* leaf litter

Code of isolates	Clear zone dimension (cm^2)
SLL01	0
SLL02	0.2527
SLL03	**1.2158**
SLL04	0.3587
SLL05	0.4215
SLL06	**0.8135**
SLL07	0.4401
SLL08	0.5807
SLL09	0
SLL10	**1.1424**
SLL11	0
SLL12	0

Table 2. The identification result of cellulolytic fungi based on morphological characters

Code of isolates	Genera
SLL02	*Penicillium*
SLL03	*Aspergillus*
SLL04	*Paecilomyces*
SLL05	*Aspergillus*
SLL06	*Penicillium*
SLL07	*Thielaviopsis*
SLL08	*Penicillium*
SLL10	*Thielaviopsis*

Table 3. Identification result based on 18s rDNA partial sequences

Code of isolates	Description	Eva-luate	Query cover (%)	Identity (%)	Accession number
SLL03	*A. flavus*	0.0	95	99	LC127086.1
SLL06	*Penicillium* sp.	0.0	94	99	EU667999.1
SLL10	*T. ethacetica*	0.0	96	99	KR673899.1

Penicillium

Based on morphological characters, three isolates, i.e. SLL02, SLL06, and SLL08, were identified as belonging to the genera of *Penicillium.* Macroscopically, the colony of Isolates SLL02, SLL06 and SLL08 had different characters (Figure 2A-3F). Isolate SLL02 had a bluish color with a white color edge from the upper view and brownish from the reverse site. The surface of the colony looked flat with cotton-like hyphae growing neatly. Isolate SLL06 had a dark green colony with a yellowish edge from the upper view and yellow to orange from the reverse site. The colony had a velvet surface texture. Isolate SLL08 had a green color colony from the upper view and a blackish green color colony from the reverse site. The more solid color indicated that the isolate produced exudates to surrounding environment. The shape of the colony was round with flat edge, and the texture was cotton-like. Under a light microscope with a magnification of 400, the three isolates had almost the same characteristics, such as having septate hyphae and branched conidiophores supporting some bottle shaped phialides showed a brush-like appearance (Figure 2G-3I).

Aspergillus

Isolate SLL03 and SLL05 were identified as belonging to the genera of Aspergillus. The two isolates had colonies with different characters (Figure 3A-4D). Isolate SLL03 had a round shape colony with moss green color on the upper surface and yellowish green color on the reverse site. The texture of the surface was like flour grains. Isolate SLL05 had a solid black color colony. The colony of this isolate appeared to be black dots, forming a fan-like pattern. The surface of the colony was like flour grains, forming concentric circles. Under a light microscope with a magnification of 400x, both Isolate had similarities, namely: having septate hyphae and conidiophores with bulging ends that form vesicles which support phialides (Figure 3E,4F).

Paecillomyces

Isolate SLL04 was identified into *Paecilomyces*. This isolate had yellowish green color colony with yellowish edges (Figure 4A). The surface of the colony had flour-like texture. The reverse side of the colony had more yellow and dense in color (Figure 4B). Under a light microscope with a magnification of 400, this isolate had septate hyphae and the series of septate hyphae appeared to be more bulging at one of its ends (Figure 4C). This isolate produced irregular or verticillate branching of conidiophores which was ended with long shape of philiades (Figure 4D), cylindrical or oval conidia which formed catenulate.

Thielaviopsis

Isolates SLL07 and SLL10 macroscopically had the same characters (Figure 7.A-7.D). The two isolates had blackish green color colonies and had white aerial hyphae, which stuck out the roof of the petri dish in the fifth day of incubation. Isolate SLL07 had a darker colony color than SLL10. The aerial hyphae of SLL07 spread whereas those of the SLL10 were dominantly in the middle part. The upper side of the colonies had a cotton-like texture. Meanwhile, the lower side of the colonies had a black color in the middle part with the white gray color edges and radial lines. Under a light microscope with a magnification of 400, Isolates SLL07 and SLL10 had septate hyphae, which produced oval or cylindrical conidia and chlamydospores which formed catenulate (Figure 5.E-F).

Molecular identification

Three isolates which showed the highest cellulolytic activities, i.e. SLL03, SLL06, and SLL10, were identified based on 18S rDNA partial sequences. The 18S rDNA amplicons of these isolates using nu-SSU-0817-5'-and nu-SSU-1536-3' primers are shown in Figure 6. Based on Blast result, the three isolates had a partial sequence similar to the database from the Gene Bank with the level of similarity of over 99% (Table 3).

Phylogenetic analysis

A general phylogenetic tree was generated with sequences obtained from the amplification of the 18S rDNA and some sequences from the GenBank database (Figure 7).

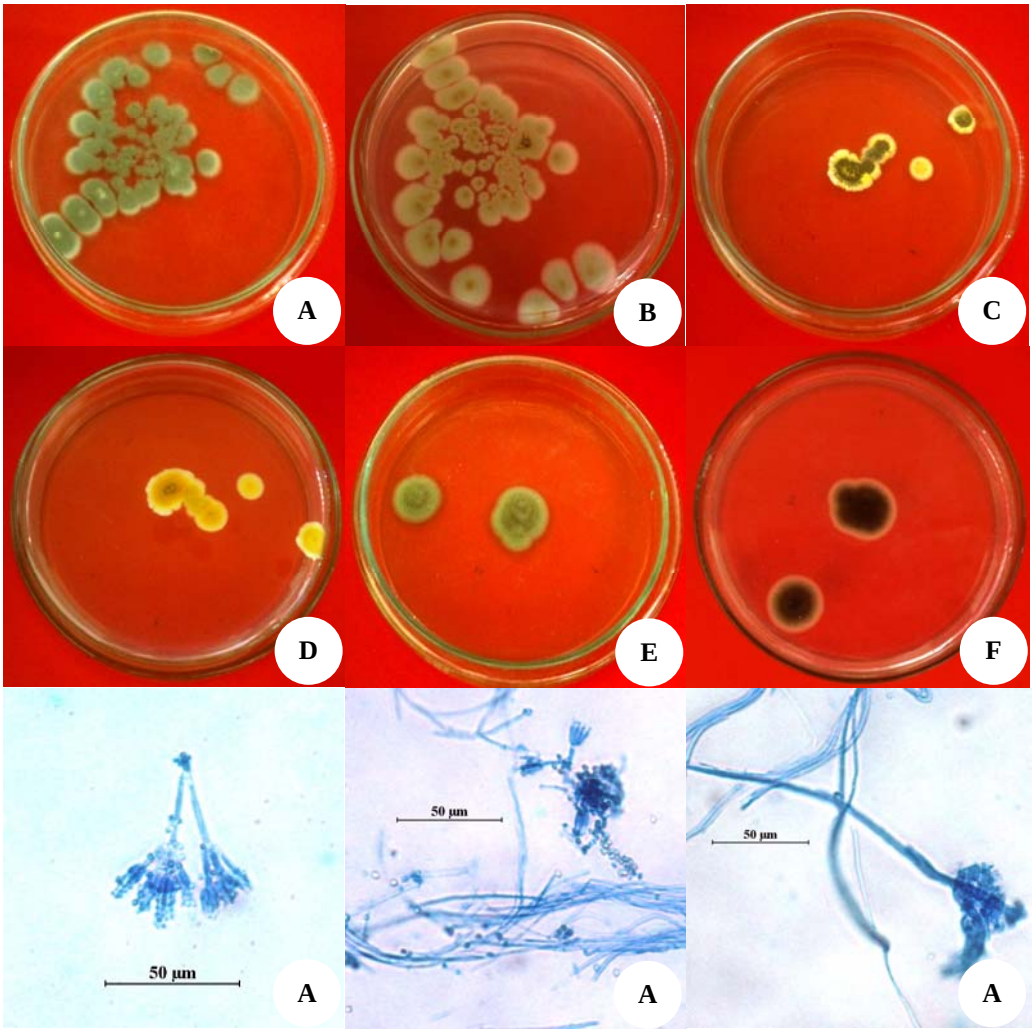

Figure 2. Colony of SLL02 captured from upper site (A) and reverse site (B), SLL06 captured from upper site (C) and reverse site (D), SLL08 captured from upper site (E) and reverse site (F) and microscopic of SLL02 (G), SLL06 (H) and SLL08 (I)

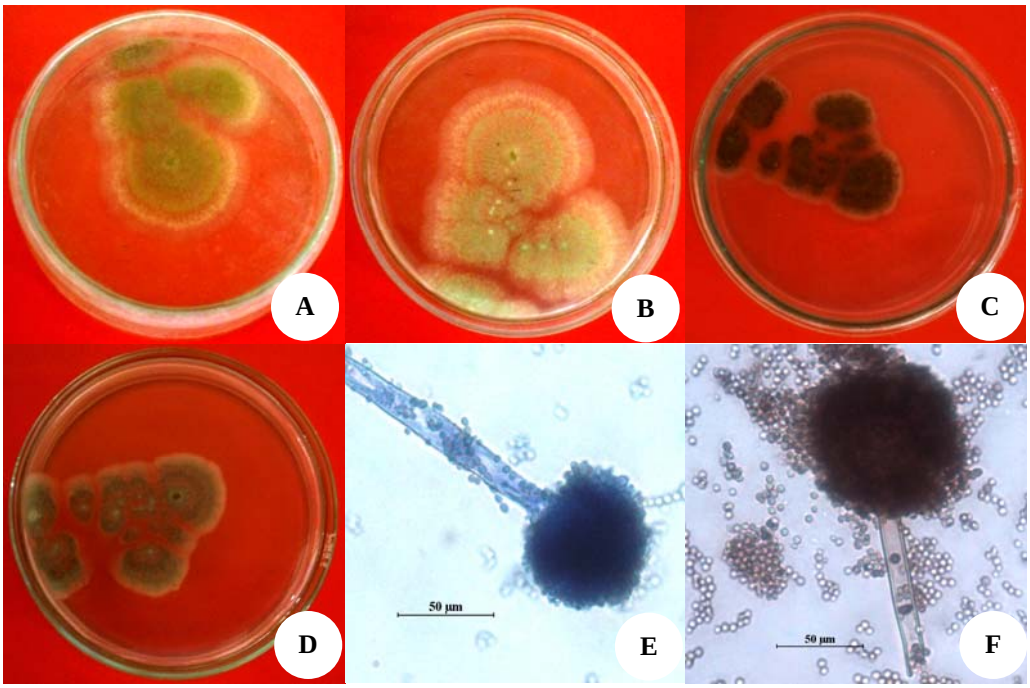

Figure 3. Colony of SLL03 captured from upper site (A) and reverse site (B), SLL05 captured from upper site (C) and reverse site (D), and microscopic of SLL03 (G) and SLL05 (I)

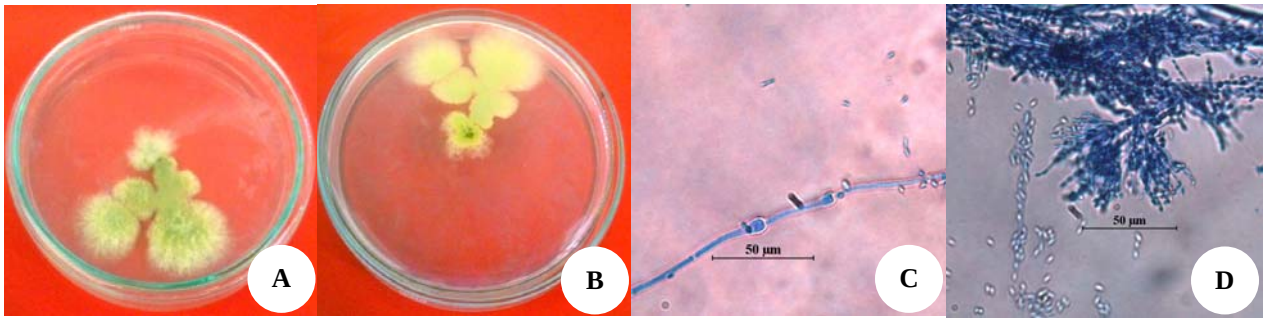

Figure 4. Isolate SLL04 captured from upper site (A) and reverse site (B). This isolate had septate hyphae that bulging at one of its ends (C) and irregular branching of conidiophores (D)

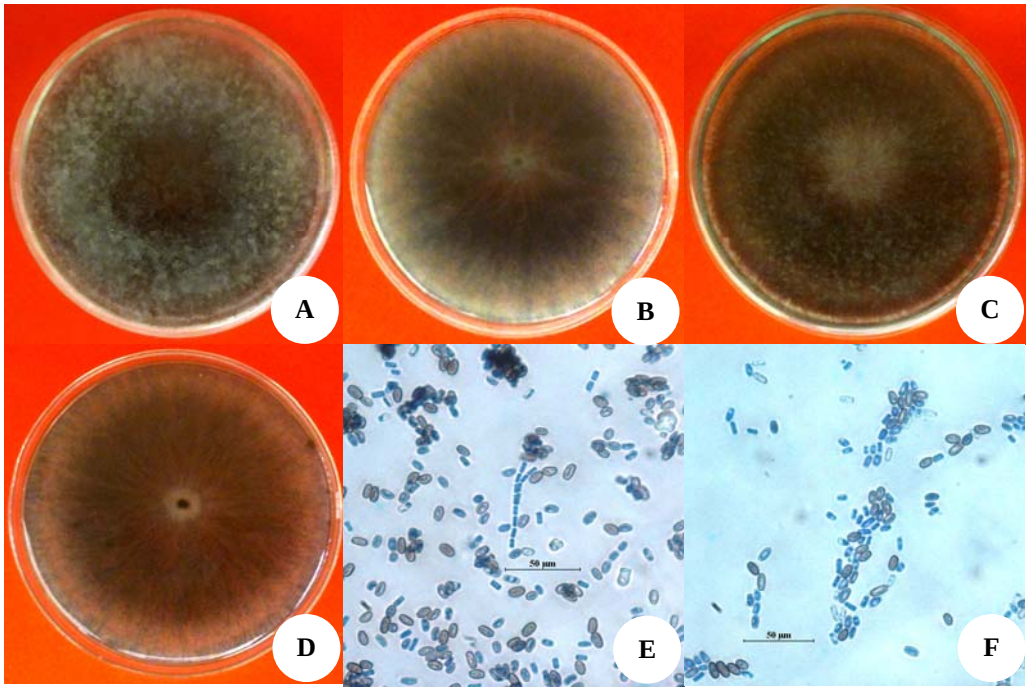

Figure 5. Colony of SLL07 were captured from upper site (A) and reverse site (B), SLL10 captured from upper site (C) and reverse site (D) and microscopic of SLL07 (E), SLL10 (F)

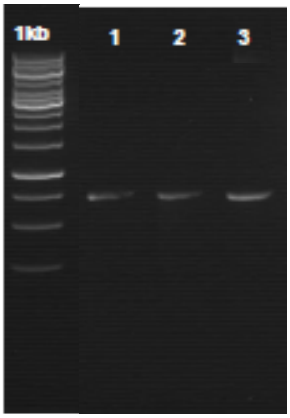

Figure 6. Fungal 18S rDNA PCR products using Primer using nu-SSU-0817-5'-and nu-SSU-1536-3'. The following DNA templates were used for PCR (by lane): 1, SLL03; 2, SLL06 and 3, SLL10.

Discussion

The results of this study showed that *Salacca* leaf litter was a potential source of cellulolytic fungi for cellulase production. There were about 12 fungal isolates which were obtained in this research and 8 isolates showed cellulolytic activity. *Salacca* leaf litter became a unique habitat for cellulolytic microbes because it contained abundant sources of lignocellulosic substrates as their important food substrates. This is in line with many studies mentioning that habitats containing lignocelluloses have a high variety of lignocellulolytic fungi. Okeke et al. (2015) have successfully isolated 32 isolates of cellulolytic fungi from soil-biomass mixture. Fungi are important agents of plant biomass-decomposition either as saprophytes or as parasites and play an important role in C cycle in the ecosystem. This decomposition process involves synergic actions of various enzymes such as oxidative, hydrolytic,

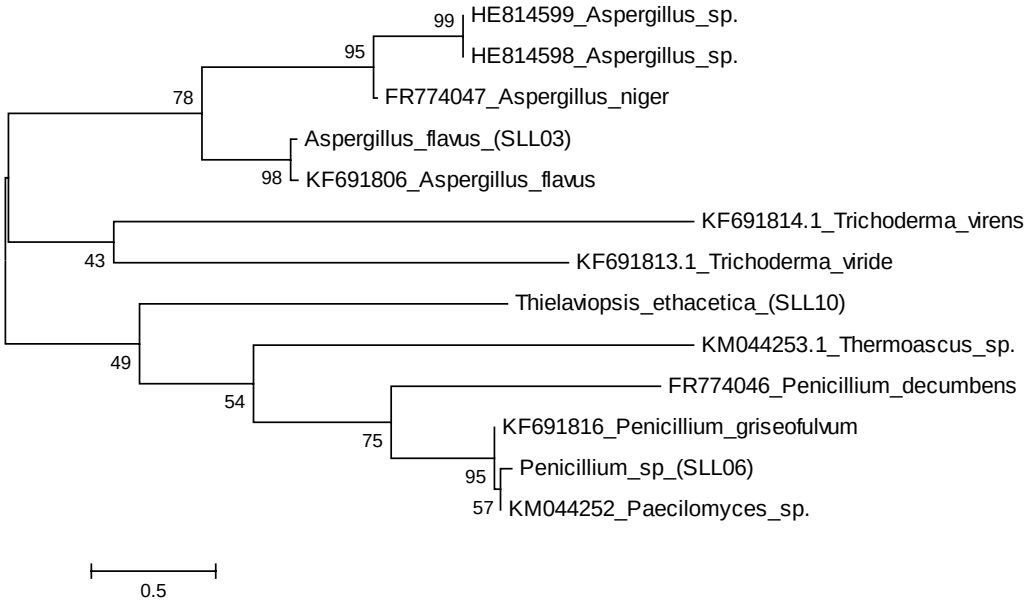

Figure 7. 18s rDNA partial sequences-based dendrogram showing phylogenetic relationships of cellulolytic fungi isolated from *Salacca* leaf litter (shown with SLL as a code of isolate) to members of the cellulolytic fungi from Gen Bank database. Bootstrap values (n=1000 replicates) of ≥43% are reported as percentages. The scale bar represents the number of changes per nucleotide position

and non-hydrolytic enzymes (Guerriero et al. 2015). Cellulolytic fungi produce cellulase which is one of the key enzymes in the plant biomass biodegradation process. This enzyme will hydrolyze cellulose, which the main component of plant's cell wall. Bacteria are known to produce cellulases, but only fungi can produce cellulose complexes which can completely degrade the lignocellulosic materials without pre-treatment. This indicates that the cellulolytic fungi have potential application in the bioprocess of lignocellulosic materials through Consolidated BioProcessing (Amore and Faraco 2012).

The result of cellulolytic activity screening based on the clear zone dimension on the CMC media showed that three isolates, namely: SLL03, SLL06 and SLL10 had the highest cellulolytic activities. Morphologically, each of the three isolates belonged to *Penicillium, Aspergillus,* and *Thielaviopsis,* respectively. The identification using the sequence of 18s rDNA, showed that each of them was closely related to *Aspergillus flavus, Penicillium* sp. and *Thielaviopsis ethacetica.* Various researches reported that two of those three fungi i.e *Aspergillus* and *Penicillium* are being dominant and can potentially be used as extracellular cellulase producers in the fermentation industry process, but the research regarding with,*Thielaviopsis*, used as cellulase producers is still limited.

The result of phylogenetic analysis showed that *Aspergillus flavus* (SLL03) was in one group with *Aspergillus flavus* isolated by Ang et al. (2015) which had the ability to degrade untreated oil palm trunk (OPT) in a solid-state fermentation system (SSF). *Aspergillus flavus* is reported as a potential strain for cellulase production by Solid State Fermentation (SSF) using the lignocellulosic media (Sajith et al. 2014). Fungi of *Aspergillus*, which are reported as the cellulase producers among others are *A. nidulans*, *A. niger* and *A. oryzae* (Jabasingh 2011; Amore and Faraco 2012), *A. fumigatus* (Liu et al. 2011), and *A. ochraceus* (Asha et al. 2016). Fungi of *Aspergillus* has been known as the enzyme producers for the production of high extracellular protein, bearing good ability in the fermentation process, and the generation of various enzymes for wood-degradation. Fungi of *Aspergillus* can produce β-glucosidases, endoglucanases, and exoglucanases. Endoglucanases and β-glucosidases are dominant components of *Aspergillus* cellulase while exoglucanases are found in a smaller quantity (DeVries and Visser 2001).).

Penicillium is known to have a large potential as the cellulase producers for the lignocellulosic biomass saccharification process. *Penicillium* sp. (SLL06) is in a group with *Penicillium griceofulvum* which had the ability to degrade untreated oil palm trunk (OPT) in a solid-state fermentation system (SSF) (Ang et al. 2015). *Penicillium* sp.TG2 has cellulolytic activities which are comparable with those of *Trichoderma reesei* RUT-C30 which are strains used for commercial cellulase production (Jung et al. 2015). Okeke et al. (2015) report that *Penicillium janthinellum* is potential isolate to produce cellulase. The profile and enzymatic activities of this strain show that the generation of enzymes can be applied for the biomass conversion and biodegradation with an effective cost. Andersen et al. (2016) reported that *Penicillium chrysogenum* are good candidates for over-production of

enzymes in order to supply industrial enzyme blends or to boost the bioconversion of lignocellulose-rich biomass**.**

Thielaviopsis (Microascales: Ceratocystidaceae) is a group of fungi known as plant pathogens. *Thielaviopsis* can grow at lignocellulosic substrates, but the ability of the fungi to produce lignocellulose-degrading enzymes has not been much reported. Lucas et al. (2001) reported that *Chalara* (syn. *Thielaviopsis*) *paradoxa* showed endoglucanase activities during the late trophophase. Goluguri et al. (2012) reported that *Thielaviopsis basicola* can grow well in the rice straw media as the sole carbon source and to produce xylanases. This research showed that *T. ethacetica* can further be explored as a new cellulase producer.

This research succeeded in isolating the cellulolytic fungi from Salacca leaf litter. Salacca is a very important fruit product in Indonesia that has been cultivated throughout Indonesia. *Salacca zalacca* var Pondoh originaly from Sleman, Yogyakarta (Lestari et al., 2013). It become superior commodity that is widely cultivated in the northern slopes of Merapi mountain, including Sleman and Magelang areas. This plant produces abundant lignocellulosic biomass which has a low decomposition rate. Cellulolytic fungi that was isolated in this study has the potential aplication as a source of inoculum for biodegradation process of Salacca leaf litter so that it can be utilized to increase the rate of decomposition or lignocellulose conversion process for biotechnological application.

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