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Characterization and virulence of two indigenous entomopathogenic fungal isolates from decayed oil palm empty fruit bunches against *Spodoptera litura* (Lepidoptera: Noctuidae)

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Abstract. *Sahid A, Kusumaningtyas P. 2023. Characterization and virulence of two indigenous entomopathogenic fungal isolates from decayed oil palm empty fruit bunches against* Spodoptera litura *(Lepidoptera: Noctuidae). Biodiversitas 24: 1192-1199.* The objective of this study was to characterize (morpho-molecular characterization and lignocellulolytic activity) and evaluate the virulence of two indigenous EPF isolates from decayed oil palm empty fruit bunched (OPEFB) against the common cutworm *Spodoptera litura* Fabr. (Lepidoptera: Noctuidae) under laboratory conditions. The fungi were isolated from OPEFB samples collected from oil palm plantations in Indonesia, and were identified as *Fusarium keratoplasticum* (isolate TKKS-1) and *Metarhizium anisopliae* (isolate TKKS-2). Both fungal isolates were tested for their pathogenicity against the 3rd instar larvae of *Spodoptera litura* Fabr. at the concentration of 1×10^6 conidia/mL. Both fungal isolates showed 100% mortality rate after 7 days of application. Virulence of the EPF isolates against the 3rd instar of *S. litura* larvae was estimated based on the LC_{50} values calculated using probit regression analysis. The LC_{50} values of *M. anisopliae* and *F. keratoplasticum* isolates were 1.41×10^5 and 1.02×10^6 conidia/mL, respectively. The LT_{50} at the concentration of 1×10^6 conidia/mL was 3.82 days for *F. keratoplasticum* and 3.52 days for *M. anisopliae*. *M. anisopliae* showed cellulolytic activity, while *F. keratoplasticum* exhibited ligninolytic activity. This finding suggests that two EPFs isolated from OPEFBs were highly virulent against *S. litura* larvae and potential decomposers for lignocellulose. These characteristics indicated significant biocontrol and decomposing properties of lignocellulose waste.

Keywords: Decomposer, *Fusarium keratoplasticum*, lignocellulose, *Metarhizium anisopliae*, pathogenicity

INTRODUCTION

Indonesia is a major producer of various tropical agricultural products which contribute to its national economy, including oil palm, rubber, cocoa, paddy, and coffee. However, pests are a threat to crop production in tropical agriculture (Pathak and Khan 1994). The infestation of insect pests has caused a considerable 18-20% loss in worldwide annual crop production, with an estimated value of more than US$470 billion. Moreover, the loss is more severe in the developing tropics of Asia and Africa (Sharma et al. 2017). Insect defoliators (leaf-eating insects) are the most common insect pests that impact crop production (Speight 2016). At high infestation levels, defoliators cause massive defoliation to crop plants. The prolonged defoliation causes significant loss as the small roots and twigs are dropped and the plants eventually dies (Wagner et al. 2008). In many cases, defoliator outbreaks occur spontaneously making it difficult to observe the damage. Lepidopterous defoliators (psychid, slug caterpillar, nettle caterpillar, and bagworm) attack on oil palm tree and reduce yield loss by up to 50% (Kalidas 2012). Most farmers in developing countries rely heavily on broad-spectrum synthetic insecticides to control insect defoliators in crop plantations. Improper use of insecticides causes environmental pollution that threatens local ecosystems and endangers the health of surrounding communities (Aktar et al. 2009). Synthetic insecticides not only kill pest insects, but also other beneficial insects, such as parasitoids, predators, flower-pollinating insects, honey bees, and neutral insects. To reduce the use of synthetic insecticides as pest control, microbial control agents, such as viruses, bacteria, and fungi can make good alternatives (Chattopadhyay et al. 2017; Samada et al. 2020). Among these microbial control agents, entomopathogenic fungi are one of the most important biocontrol agents for the control of insect pests for having a wide host range.

In Integrated Pest Management (IPM), entomopathogenic fungi (EPF) become an integral part of the mycoinsecticide in horticulture, forestry, and plantation crops (Gul et al. 2015). This fungus infects the insects through cuticles or orally (Mannino et al. 2019). The EPFs have hyphae to penetrate host organisms (insects) and spores to effectively transmit from one host to the next, and many species produce toxic compounds (mycotoxin). In some insect orders, the nymph and larval stages are more susceptible to fungal infections than the adult stage (Maina et al. 2017). The mass production of EPF is affordable and it requires easy and efficient storage at wide range of humidity and temperature. The complex modes of action of

EPF make it difficult for pests to develop resistance. This approach is considered environmentally friendly and safer to use in IPM programs in the field and production of organic agricultural products (Khan et al. 2012). Efforts have been done to explore and isolate the potential entomopathogenic fungi from various agricultural ecosystems (Safitri et al. 2018; Herlinda et al. 2020; Shahriari et al. 2021).

EPFs are heterogeneous organisms that play multiple ecological roles as endophytes of plants, parasites of insects, or saprophytes in soil (McGuire and Northfield 2020). The vast majority of fungi are saprophytic feeding on dead organic material. Saprophytes fungi contribute significantly in the decomposition of organic matter and nutrition recycling, particularly carbon and nitrogen. For this reason, EPF must be isolated from empty palm fruit bolls (OPEFB), which are left to decay in oil palm plantations as mulch. OPEFB is a complex lignocellulosic material consisting of polymer organic matter, including 57.8% cellulose, 21.2% hemicellulose, and 22.8% lignin (Shariff et al. 2014). Several species of fungi have been reported to produce a series of extracellular lignocellulolytic enzymes (Sahadevan et al. 2016) that can decompose lignocellulosic materials, such as *Trichoderma* sp. (Sapareng et al. 2017; Siddiquee et al. 2017), *Lichtheimina ramose* and *Neurospora crassa* (Tahir et al. 2019). The ability of fungi to produce a wide range of extracellular enzymes allows them to efficiently degrade the recalcitrant lignocellulose matrix that other microorganisms are unable to decompose (Sahadevan et al. 2016). The presence of hemicellulose both in the lignocellulose structure composing the OPEFB and in the chitin composing the insect cuticle provides the assumption that fungi which have the ability to break down the complex solid material containing lignocellulose are also able to break down the insect cuticle composed of chitin. Therefore, the aim of the present study was to evaluate the virulence of two EPFs isolates against a leaf-eating caterpillar pest *Spodoptera litura* (Lepidoptera: Noctuidae), and their potential in degrading lignin and cellulose. The identification of EPFs that are able to degrade OPEFB can provide the basic information to the development of a novel biological control strategy in the management of oil palm plantations. EPFs in oil palm plantations can play dual roles as a biological control agent in suppressing the insect pest and as a decomposer in degrading OPEFB waste.

MATERIALS AND METHODS

Fungal isolates

EPF isolates (TKKS-1 and TKKS-2) were isolated from decayed OPEFB samples taken from an oil palm plantation in Semuntai district, East Kalimantan Province, Indonesia (01°40'36.9"S, 116°07'44.5"E) using Tenebrio molitor bait method. Isolates were grown on potato dextrose agar (PDA) medium added with 1% yeast extract and 0.2 g/L tetracyclines at 26 ± 1 °C with 80-85% relative humidity for 7-14 days. Fungi were sub-cultured to obtain pure culture and kept on a PDA slant.

Morpho-molecular identification of EPF isolates

For the morphological identification, a small portion of the fungal colonies on PDA were transferred onto glass slides and observed under a light microscope for their hyphal color and structure, shape, and size of conidia and conidiophores. Fungal isolates were identified using the identification key proposed by Barnett and Hunter (1972). Morphological identification of the isolated EPFs was further confirmed by molecular identification. In molecular characterization, the mycelia of fungal isolates were pelleted by centrifuging at 10,000 rpm for 10 min for extraction using the PowerSoil DNA Isolation Kit (Catalog No.12888-100 MOBIO Laboratories, Carlsbad, California). The extracted DNA was used as a template for PCR amplification. The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified by PCR with specific primers for ITS1 (5′- TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′- TCCTCCGCTTATTGATATGC-3′) as described by White et al. (1990). DNA amplification was carried out with a total volume of 25 µL under PCR cycle conditions: 1 denaturation cycle at 95°C for 3 minutes, followed by 34 denaturation cycles at 95°C for 1 minute, annealing at 52°C for 30 seconds, and elongation at 72°C for 1 minute, with a final extension stage of 72°C for 10 minutes. PCR products were visualized in 1% agarose gels. The amplified PCR products were sent to the First Base (Malaysia) for purification and sequencing using the same primers. The sequence data were compared to the GeneBank database using the BLAST software available on the National Center for Biotechnology Information (NCBI) website.

The rearing of *Spodoptera litura* larvae

The egg batches of *S. litura* were collected from long bean crops in Lempake village, Samarinda, East Kalimantan Province, Indonesia. Each of the egg batches was placed on wet cotton in a plastic container to be kept at 26 ± 1°C, 80-85% relative humidity, and 12:12 (L:D) h of photoperiod. Newly hatched larvae were transferred to a new container (30 × 50 × 30 cm) and fed with spinach leaves until the pupation. The newly formed pupae were collected and placed in a new plastic container until the moths came out. A 10% (v/v) honey solution in small cotton wool balls was provided as supplementary food for the moths. After the adult emerged, they were shifted to a rearing cage (30 cm in diameter and 50 cm in high) for mating and egg-laying. Adults were fed with 10% honey solution through a cotton layer hung on the top cage. The entire surface of the cage was covered with white gauze and an additional folded paper for the female laid egg. Egg masses were collected regularly and transferred into new containers to maintain the colony and for experimental uses. The 3rd instar larvae of *S. litura* were used for testing the pathogenicity of the EPF isolates in the experiments.

Bioassay of EPF isolates against *Spodoptera litura*

The two purified strains were cultured on a PDA medium and incubated at 26°C for fifteen days. Conidia were harvested by applying 10 mL of sterile sterilized 0.05% Tween 80 as a wetting agent to each Petri dish and gently scraping the surface of cultures with a sterile scraper. The conidial suspensions were filtered to remove rough parts of medium and mycelia and then transferred into a 15 mL test tube. Conidia concentration in each suspension was measured using an Improved Neubauer haemocytometer, while the pathogenicity assay of each isolate was evaluated by adjusting the conidia concentration to 1×10^6 conidia/mL. A complete randomized design was used to statistically evaluate pathogenicity, where two fungal isolates and a control treatment (three replicates each) were also maintained. The mortality rate of the third instar of *S. litura* larvae was corrected with that in control using the formula according to Abbott (1925) as follows:

$$P = \left[\frac{C-T}{C}\right] \times 100 \qquad (1)$$

Where, P = estimated percentage of larvae killed by the fungus, C = percentage of control larvae living, and T = percentage of treated larvae that lived after the treatment.

The virulence assay was conducted on the 3rd instar of *S. litura* larvae with four serial conidial concentrations of both fungal isolates: 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/mL. All treatments were conducted in a container (20 × 20 × 10 cm) containing 10 larvae. In each treatment, larvae were directly sprayed with the conidial suspension, while sterile 0.05% Tween 80 without fungal conidia was used in control treatment. All bioassay trials were performed in the laboratory at 26 ± 1°C and 80-85% relative humidity under a 12:12 (L:D) h of photoperiod. The number of dead larvae was observed daily until all larvae died or formed the pupa. The experiments were repeated three times to confirm the pathogenicity and virulence of two fungal isolates against *S. litura*.

Ligninolytic activity assay

The ability of fungal isolates in degrading lignin was tested using Bavendamm test, where fungal isolates were grown on PDA media supplemented with 0.1% w/v tannic acid and incubated at 26 ± 1°C in the dark for 8 days. The brown zone formed around colonies indicates the presence of phenol oxidase activity, showing that the fungal isolate has lignin-degrading properties.

Cellulolytic activity assay

The fungal isolates were cultivated on basal medium supplemented with Carboxymethylcellulose (CMC) as suggested by Shahriarinoor et al. (2011) and were then incubated at 26 ± 1°C in the dark for 5 days. Fungal culture in the petri dishes was filled with 0.5% aqueous congo red for 30 min which was then de-stained using 1 M NaCl solution for 15-20 min. The yellow-opaque area formed around colonies against a red color for undegraded CMC indicates the presence of endoglucanase activity in CMC degradation.

Data analysis

Data were analyzed using one-way ANOVA, followed by Duncan's multiple range tests ($P<0.05$). Meanwhile, virulence data were examined by Probit Analysis to calculate the LC_{50} and LT_{50} of two EPF isolates against *S. litura* on the Statistical Package Social Sciences (SPSS) software version 22.

RESULTS AND DISCUSSION

EPF is regarded as a vital ecological factor that regulates insect pest populations in the field for having broad-spectrum activity, good persistence in soil, easy for mass-production, and no resistance on the insect host against entomopathogenic fungi. Results showed that two EPFs isolates were identified based on their morphological and molecular characteristics. The macroscopic characters of isolates were measured based on the colony color and shape, while the microscopic characters were confirmed by the phialides, conidiophores, and conidia at a magnification of 400×. The colony of isolate TKKS-1 on PDA appeared as yellowish white color (Figure 1A) with the presence of macro- and micro-conidia which was the most common morphological characteristics of genera *Fusarium* (Figure 1B). The macroconidia of isolate TKKS-1 were slightly curved apical cell with 2-septate with a length of 21.03 ± 2.64 µm and width of 5.39 ± 1.14 µm. The phialides of conidiophores were cylindrical, consisting of long monophialides with a size of 86.26 ± 16.36 µm long and 3.08 ± 0.54 µm wide. The ellipsoidal microconidia of isolate TKKS-1 were bounded with the tip of monophialides were 5.18 ± 1.11 µm long and 1.04 ± 0.31 µm wide. The white colony of isolate TKKS-2 changed into greenish and dark green or dark after 7 days of incubation (Figure 1C). Isolate TKKS-2 had only cylindrical shaped conidia measuring 6.76 ± 0.49 µm long and 2.39 ± 0.26 µm wide. The phialides of conidiophore were upright, layered, and branched with the size of 24.15 ± 6.85 µm long and 2.27 ± 0.17 µm wide (Figure 1D).

To confirm the morphological identification, the total DNA of two fungal isolates (TKKS-1 and TKKS-2) was amplified in the region of the rDNA-ITS gene by specific primers ITS1 and ITS4. The rDNA-ITS gene region was successfully amplified for both fungal isolates with the PCR product at around 550 bp (Figure 3). The sequence results of both fungal isolates were compared to the sequences of 18S rDNA accessed in GenBank using BLAST. Results showed that isolate TKKS-1 was 100% identical to *Fusarium keratoplasticum* with 100% query coverage and an E value of 0.0. Furthermore, isolate TKKS-2 showed 98.8% highly homology with *Metarhizium anisopliae* with 100% query coverage and E value of 0.0 (Figure 2). The presence of *Fusarium* and *Metarhizium* in decayed OPEFB samples might also correlate with better adaptability in acidic environment. These fungi are known as an organic acid producers in soil, such as oxalic acid by *Metarhizium* (St. Leger et al. 1987), and fusaric acid by *Fusarium* (Papizadeh et al. 2018).

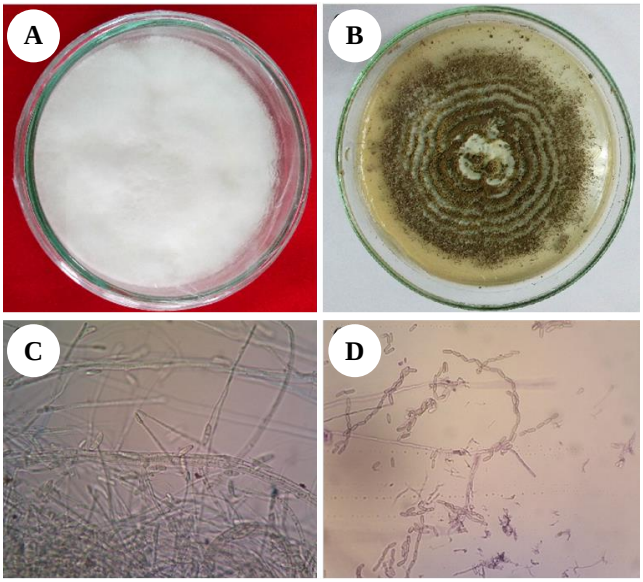

Figure 1. Colony of isolates after 7 days incubation: (A) TKKS-1, and (B) TKKS-2. Hyphae and conidia of isolates at 400× magnification: (C) TKKS-1, and (D) TKKS-2

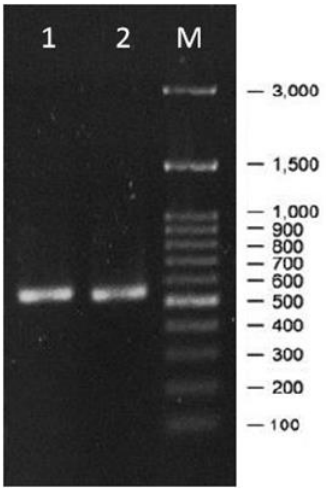

Figure 3. Agarose gel profile of the amplification product of the rDNA-ITS genes from two isolates. Lane 1= isolate TKKS-1, Lane 2: isolate TKKS-2, Lane M = marker of DNA Ladder 100-3,000 bp

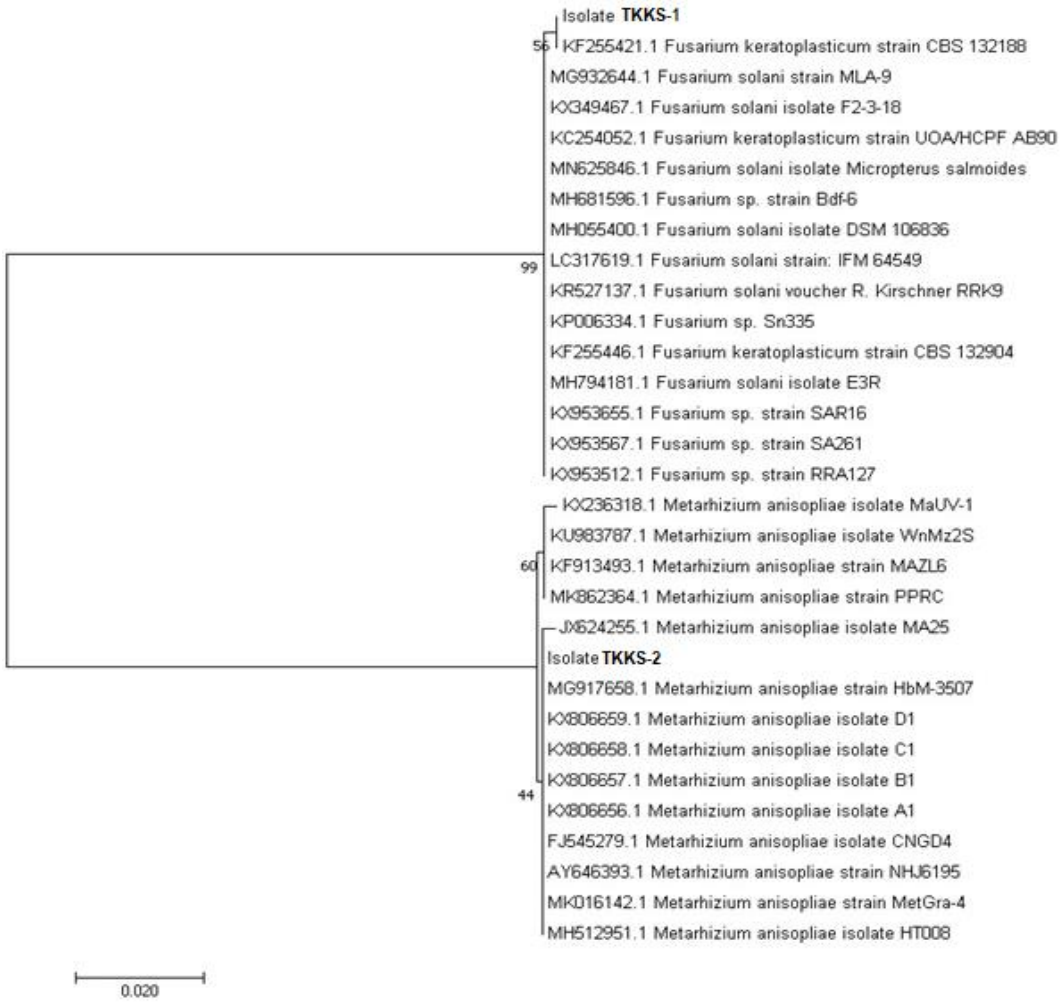

Figure 2. Phylogenetic tree based on the 5.8rDNA sequence of isolate TKKS-1 and isolate TKKS-2

Prior research showed that the pH at the end of the OPEFB decomposition process was rather acidic (Kusumaningtyas et al. 2022) because several decomposer fungi converted lignocellulose into organic acids (Mahgoub et al. 2022; Tran and Zhao 2022). Quesada-Moraga et al. (2007) also reported that the percentage of soil samples containing entomopathogenic fungi was progressively higher in the samples put in environment with increased humidity, acidity, and organic matter content.

The pathogenicity and virulence of the entomopathogenic *F. keratoplasticum* and *M. anisopliae* isolates were tested on the leaf-eating lepidopteran pest, *Spodoptera litura* larvae. This insect is a highly polyphagous migratory which causes significant economic loss and it is concerned as a serious pest in the Asia-Pacific region (Bragard et al. 2019). The present study revealed that both fungal isolates can cause 100% mortality on *S. litura* larvae at a concentration of 10^5 conidia/mL for 6 days after treatment (Figure 4). No larval mortality was observed on the control larvae and most of the control larvae changed into pupae on the 7th day. The dead larvae of *S. litura* swell and turn reddish brown. Hyphal growth and sporulation on dead *S. litura* larvae were observed on e 7th day for *Metarhizium* isolate and on the 11th day for *Fusarium* isolate. Overall, both EPF isolates were found highly pathogenic against the third instar of *S. litura* larvae at all conidial concentrations tested. The *S. litura* larvae infected by *Fusarium* species exhibited white mycelium that grew from their cuticles after 11th days (Figure 5A), while *S. litura* larvae attacked by *Metarhizium* species exhibited mycelia with greenish white to dark green or dark color in their cuticles after 7th day (Figure 5B).

It was also observed that *F. keratoplasticum* and *M. anisopliae* isolates showed higher mortality on *S. litura* larvae. Asi et al. (2013) reported the pathogenicity of *M. anisopliae* against the third instar of *S. litura* larvae was 43.0% at a similar concentration after 10 days of treatment. Anand and Tiwary (2009) reported the pathogenicity of *M. anisopliae* and *F. lateritium* on the larvae of *S. litura* were 88% and 89% mortality, respectively, at the higher concentration of 10^8conidia/mL. On the other side, *Isaria fumosorosea* killed the larvae of *S. litura* with a mortality rate ranging between 41.2-85.7% (Asi et al. 2013; Ullah et al. 2019), while *Beauveria bassiana* can kill the larvae of *S. litura* with the mortality of 33.3% (Ullah et al. 2019). These data indicated that the fungal isolates that the used fungal isolates showed high virulence and fast action against the leaf-eating lepidopteran pest *S. litura*. Prior researchers have also examined the pathogenicity of *Metarhizium* against several orders of insects other than Lepidoptera that included Coleoptera (Tuncer et al. 2019), Eriophyidae (Robles-Acosta et al. 2019), and Acrididae (Jiang et al. 2020). This denotes that *Metarhizium* isolate is a generalist entomopathogenic fungus as stated by Hu et al. (2014). On the contrary, *Fusarium* genus is known to be the most destructive plant pathogen (Sharma and Marques 2018). Only a few species of the *Fusarium* genus were identified as entomopathogenic fungi, including *F. oxysporum*, *F. solani*, *F. keratoplasticum* and *F. proliferatum* (Chehri 2017; Sharma and Marques 2018). The entomopathogenic effect of the genus *Fusarium* can be seen from the production of mycotoxins, such as beauvericin (Guo et al. 2018). Research on fusarium-insect pest is very limited because fusarium is generalized as opportunistic insect-pathogens (Sharma and Marques 2018). A large number of entomopathogenic *Fusarium* are facultative pathogens, especially for the lepidopteran and coleopteran orders that colonize their dead insect hosts as saprophytes (Teetor-Barsch and Robert 1983). Probit regression analysis of data regarding the percent corrected mortality of *S. litura* larvae revealed that *Metarhizium* isolate was more virulent than *Fusarium* isolate. The LT_{50} value as shown in Table 1, *Metarhizium* isolates killed the 3rd instar of *S. litura* larvae faster (3.52 days) compared to *Fusarium* isolate (3.82 days) at the concentration of 1×10^6 conidia/mL. Similarly, the median lethal concentration (LC_{50}) values for *Metarhizium* isolate were 1.41×10^5 conidia/mL on the 7th day and 1.02×10^6 conidia/mL on the 10th day for *Fusarium* isolate (Table 2).

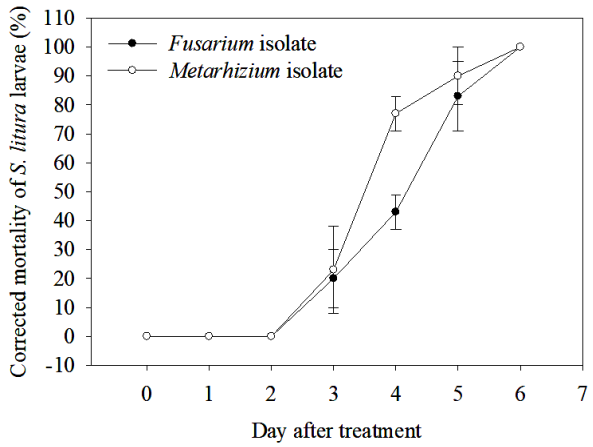

Figure 4. Mortality (%) rate of *S. litura* larvae treated with conidial suspension (1×10^6 conidia/mL) of *Fusarium keratoplasticum* and *Metarhizium anisopliae* isolate within 6 days after treatment

Figure 5. Symptoms of *S. litura* larvae infected by: A. *Fusarium keratoplasticum* isolate on 7th day, and B. *Metarhizium anisopliae* on 11th

Table 1. Median lethal time (LT_{50}) of indigeneous EPF isolates on the 3rd instar *S. litura* larvae under laboratory conditions

EPF Isolates	LT_{50} (day)	95% Confidence limits		X^2(df)	*P-value*
		Lower limits	Upper limits		
Fusarium	3.82	3.54	4.08	1.991 (4)	*P*>0.05
Metarhizium	3.52	3.26	3.76	1.794 (4)	*P*>0.05

Table 2. Median lethal concentration (LC_{50}) of indigeneous EPF isolates on the 3rd instar *S. litura* larvae under laboratory conditions

EPF Isolates	LC_{50} (conidia/mL)	95% Confidence limits		X^2(df)	*P-value*
		Lower limits	Upper limits		
Fusarium	1.02×10^5	2.69×10^5	$2{,}75 \times 10^6$	0.220 (2)	*P*>0.05
Metarhizium	1.41×10^5	1.41×10^5	1.23×10^7	1.201 (2)	*P*>0.05

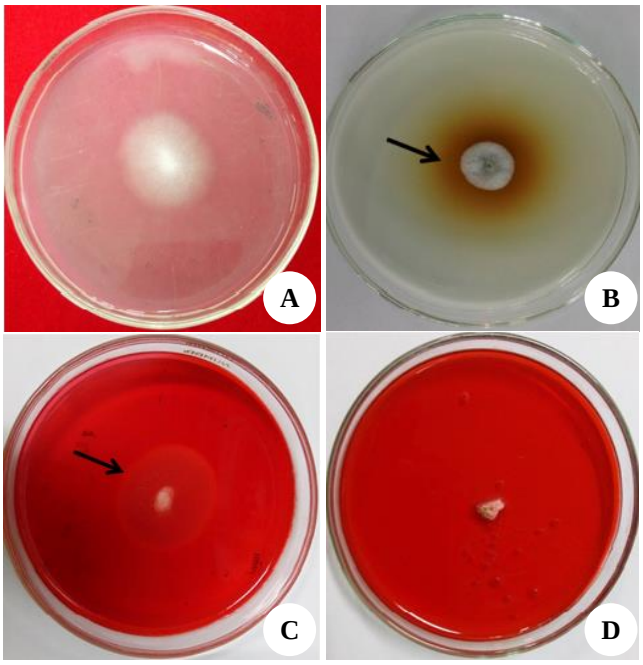

Figure 6. Ligninolytic and cellulolytic activity of *M. anisopliae* and *F. keratoplasticum* (A-D). Arrow showed the presence of zone formation around the colony

The lignocellulolytic activity of both fungal strains isolated from decayed OPEFB was examined to see their potential as decomposers in OPEFB degradation in the field. The result of Bavendamm test exhibited that ligninolytic activity of *M. anisopliae* was shown by the brown zone around the fungal colony (Figure 6B), while *F. keratoplasticum* did not produce any zone. The cellulolytic activity was indicated by the presence of a clear zone surrounding the colony on the CMC basal medium after coloring with 0.5% Congo red. Among both fungal strains isolated from decayed OPEFB, only *F. keratoplasticum* showed cellulolytic activity (Figure 6C), while *M. anisopliae* did not show cellulolytic activity. Therefore, both fungal strains isolated from decayed OPEFB have the potential in degrading lignocellulolytic materials when they were applied together as a consortium. *Metarhizium* and *Fusarium* belong to Sordariomycetes (Ascomycota division)- the most common fungal detected in decaying OPEFB that is assumed to contribute in OPEFB degradation (Tahir et al. 2019). The presence of *Fusarium* and *Metarhizium* in decayed OPEFB which is rich in organic material relates to the ability of these fungi to grow in the saprophagous phase (Tkaczuk et al. 2012). It can be assumed that entomopathogenic fungi inhabiting decayed OPEFB take the organic matter contained in OPEFB as their nutrient source. To prove this assumption, this study examined the ability of both fungal isolates in degrading lignin and cellulose based on the presence of polyphenol oxidase and endoglucanase enzymes. Our results revealed that *F. keratoplasticum* has cellulolytic activity and *M. anisopliae* has ligninolytic activity. This fact exhibited that *F. keratoplasticum* might be involved in the later stages of OPEFB degradation when more nutrients containing cellulose and hemicellulose are available. Whilst, *M. anisopliae* was found in OPEFB samples since the beginning of the decomposition process.

The attempt to isolate entomopathogenic fungi from the decayed OPEFB is intended to obtain potential indigenous entomopathogenic fungi for controlling insect pests in oil palm plantation and for the degradation of OPEFB that comprises the largest solid waste in oil palm production. The result of this study revealed that two fungal strains which might play multiple roles as natural enemies against insect pests attacking the oil palm tree and as the decomposers can enhance the composting process of OPEFB in the field. The conidia of *F. keratoplasticum* and *M. anisopliae* against *S. litura* larvae can be sprayed directly on the larvae pests, yet different doses of fungus need to be further examined for more comprehensive findings. The formulation of these effective strains can increase their efficacy, considering the formulation of other entomopathogenic fungi has increased their effectiveness and kept them viable for a longer period of time.

In summary, this study is the first report of lignocellulolytic activy of entomopathogenic *F. keratoplasticum* and *M. anisopliae*. The results of this preliminary research show promising contribution to the development of biological tools. Despite the need for more experiments, the isolates clearly show biotechnological potential to be employed as tools to control insect pests and

degrade the OPEFB in oil palm plantations at once. Future researchers should be encourage to investigate other culturing methods using different media to accelerate the mycelium growth and create abundant sporulation of the pathogen to develop an appropriate formulation to control insect pests.

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