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Original Article

Sustainable disposal of polyethylene terephthalate (PET) waste degraded by fungi isolated from a Malaysian landfill

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ABSTRACT

The accumulation of polyethylene terephthalate (PET) has become a major environmental in Malaysia. Disposal of PET in the local landfill has resulted in additional problems, as plastic leachate is known to cause cancer and neurological impairment in humans. Mycoremediation using fungi with vast hyphal network and broader metabolic competence is seen as an eco-friendlier approach to tackle this issue. The objective of this research is to evaluate fungi remediation of PET *via* the detection of manganese peroxidase and laccase activity. Biodegradation of PET is shown *via* the enzymatic activity of laccase and manganese peroxidase measured at the wavelength of 450 nm and 610 nm, respectively. Of the 14 species of fungi isolated from the Jeram landfill, only 7 fungal species were able to biodegrade PET to obtain the carbon that supports fungal growth. Only one species, *P. janthinellum* was able to express relatively high MnP and laccase activity consistently, demonstrating the ability to biodegrade PET. This shows that *P. janthinellum* has the highest potential to be further developed as an agent to biodegrade PET in an integrated plastic waste management to restore soil pollution.

KEYWORDS: laccase, manganese peroxidase, soil restoration, sustainable land management.

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INTRODUCTION

Plastic pollution is currently seen as one of the most serious environmental issues worldwide. Among the many various plastics, polyethylene terephthalate (PET) is the most abundant in the environment. In 2020 alone, the global plastic usage reached 367 million tonnes, and out of that figure 27 million tonnes are PET packaging products [1]. PET is a strong, lightweight, durable [2], and flexible polyester [3] ubiquitously found in various terms used every day [4], from sports clothing to the packaging of mineral water, sauces, and cleaning products [2]. According to the British Plastic Federation (BPF), 70% of the world's soft drink containers is made of PET bottles [5].

Malaysia is no exception, in terms of plastic pollution, being ranked 28th in the world in 2021 (at 56 kg per capita annually) [6]. The exponential use and degradation resistance of synthetic PET plastics has resulted in their widespread accumulation which poses a serious hazard to the ecology [7]. During PET manufacturing, compounds added to enhance plastic properties are also found to be poisonous and dangerous to mammalian and marine life because such compounds can disrupt chemical signaling molecules in aquatic ecosystems [8]. PET has been shown to leach endocrine-disrupting chemicals (EDC) which interrupt hormone regulation resulting in the increasing probability of developing illnesses such as cancer

and neurological impairment in humans [9]. Neurological impairment is the main cause of behavioral changes and negatively affects the immune system of the fauna [10, 11]. Due to PET's potential toxicity, pollution caused by PET is often managed using conventional approaches including mechanical, thermal, and chemical-based treatments. These procedures are not only costly but also produce secondary pollutants which exacerbate the cleaning process. Thus, there is a need for a more cost-effective and environmentally beneficial technique to manage PET-based plastic. Enzymatic treatment or recycling is a key strategy for reducing PET pollution in a sustainable manner [12].

Fungi are decomposers of carbon polymers with long life cycles, a wide hyphal network, extensive biomass expansion, and a broad metabolic competence [13, 14]. Fungi secrete a wide range of extracellular enzymes [15], including manganese peroxidase and laccases [16]. Manganese peroxidase (E.C. 1.11.1.13. Mn^{2+}: H_2O_2 oxidoreductases) is a ligninolytic enzyme that has been reported to breakdown lignin and organic contaminants [17]. Laccases (EC 1.10.3.2, benzenediol: oxygen oxidoreductase), on the other hand metabolizes phenolic compounds with oxygen as the only byproduct [18]. The short chain of aliphatic molecules, coupled with an aromatic ring, causes the polymer to become a stiff molecule [19]. Thus, fungi have the potential to breakdown PET plastic since it is structurally composed of aliphatic carbon chain and benzene or phenolic ring. Hence, this study evaluates the potential of fungi in remediating PET by detecting the activities of manganese peroxidase and laccase.

MATERIALS AND METHODS

Sampling and fungi identification

Plastic bags purchased from vendors were sent to the INBIOSIS, Universiti Kebangsaan Malaysia (UKM), Malaysia for FTIR analysis. Results of the FTIR analysis performed using wavelengths between 780 nm to 1 mm [20] confirmed that the plastic is made of polyethylene terephthalate (PET) with characteristic peaks [21] as shown in Figure 1. The PET plastic bags were manually shredded into around 0.5 cm and used in the subsequent experiment.

The topsoil samples (0 to 10 cm depth) were obtained from three locations within the Jeram Sanitary Landfill, Malaysia (geocoordinates: 3.1890889,101.3625547) using a stainless-steel shovel [22]. About 2 kg of topsoil was collected from each site. The soil samples were then diluted to 10^{-3} and 10^{-5} using sterilized water and mixed into the Rose Bengal agar (RBA) (OXOID, UK) in the ratio of 1:9 [23]. The RBA was then incubated at room temperature for 2 to 3 days. Single fungal isolate was picked and sub-cultured onto Potato Dextrose Agar (PDA) to obtain fresh and pure culture. The fungal culture was incubated in Potato Dextrose broth (PDB) for 3 to 4 days at room temperature, and the fungal biomass was used in the DNA extraction. The extracted DNA

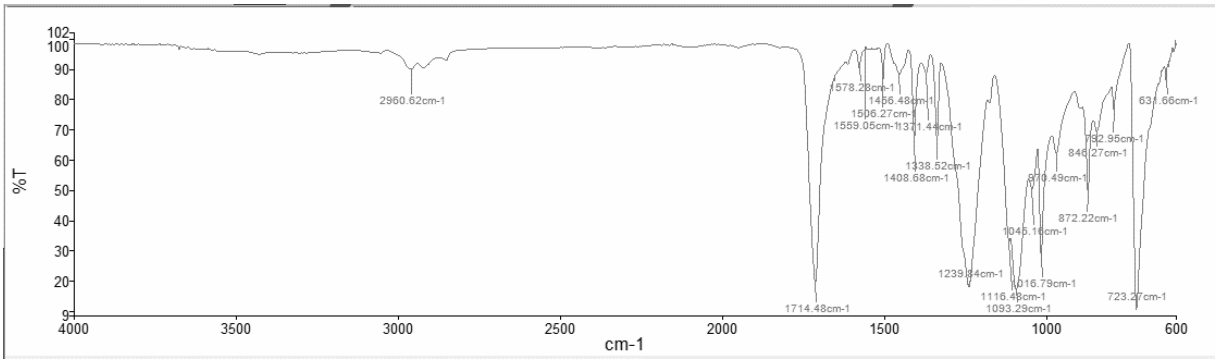

Figure 1. Identification of PET using FTIR analysis.

was then subjected to polymerase chain reaction (PCR) amplification using a pair of primers targeting the ITS1 (5'TCC GTA GGT GAA CCT TGC GG 3') and ITS4 (5'TCC TCC GCT TAT TGA TAT GC 3') region, following the Biotaq Polymerase protocol obtained from Bioline. The PCR product was then purified and sequenced by Genomics BioSci & Tech, Malaysia. The obtained DNA sequence was examined using the Basic Local Alignment Search Tool (BLAST).

Screening for PET metabolizing fungi species

A pure fungal colony was inoculated into a flask containing 50 mL of autoclaved Bushnell Haas Broth (BHB) (OXOID, UK) mixed with 0.04 g of shredded PET [24]. The BHB with PET without fungal inoculum served as a control. The flasks were shaken at 150 rpm for seven days at room temperature [25]. The experiment was carried out in triplicates. At the end of the incubation period, culture flasks containing fungi that can metabolise PET as their only carbon source can be observed to turn turbid.

Enzyme assay

Culture samples that turned turbid were used for enzyme assay. Cell-free supernatant was obtained by centrifuging the culture at 4000 rpm for 10 minutes at 4 °C [26]. The supernatant was then used as the source for laccase and manganese peroxidase enzyme assay.

The laccase enzyme activity was measured using the method described by Zhang *et al*. [27]. 1 mL of the supernatant was mixed with 1 mL of guaiacol and 3 mL of sodium acetate buffer (pH 6.7) and incubated for 10 minutes at room temperature. The reaction mixture was then transferred to a glass cuvette and measured at 450 nm wavelength on a spectrophotometer. The laccase activity was calculated using the formula below:

$$\text{Enzyme activity (U/L)} = \frac{A_{450} \times 10^6}{0.5mL\ (enzyme) \times 0.5\ \text{min} \times e\ (4460L,\ m^{-1}.\ cm^{-1})}$$

The manganese peroxidase (MnP) activity was measured using the method described in Silva *et al*. [28]. Two sets of reaction media were made for each sample by mixing 500 µL of supernatant, 50 µL of manganese sulfate (2.0 mM), 200 µL of bovine albumin (0.5% w/v), 50 µL of hydrogen peroxide (2.0 mM) in sodium succinate buffer (0.2 M, pH 4.5), 100 µL sodium lactate (0.25 M) and 100 µL of phenol red (0.01% w/v) in a test tube. One set of the reaction tube was subjected to boiling temperatures for 10 minutes to ensure there were no reactions within that sample. This media was used to blank the spectrophotometer at 610 nm. The other set was left at room temperature for 30 seconds to allow reaction to occur before terminating the reaction using 40 L sodium hydroxide (2.0 M). After that, 1 mL of the terminated reaction media was transferred to a cuvette and read at 610 nm using a UV spectrophotometer. The manganese peroxidase activity was calculated using the formula below:

$$\text{Enzyme activity (U/L)} = \frac{A_{610} \times 10^6}{0.5mL\ (enzyme) \times 0.5\ \text{min} \times e\ (4460L,\ m^{-1}.\ cm^{-1})}$$

RESULTS AND DISCUSSION

Soil samples collected from the Jeram Sanitary Landfill, Malaysia yielded a total of 14 fungi species, identified using universal molecular markers namely ITS1 and ITS4. All the 14 isolates were successfully identified up to specie level, namely *Cunninghamella bertholletiae*, *Cunninghamella polymorpha*, *Paecilomyces variotii*, *Trichoderma asperellum*, *Penicillium janthinellum*, *Penicilluim glaucoroseum*, *Penicillium simplicissimum*, *Penicillium cremeogriseum*, *Penicillium levitum*, *Talaromyces flavus*, *Talaromyces louisianensis*, *Aspergillus niger*, *Aspergillus tamarii* and *Trichosporon asahii.* Out of the 14 isolates, 7 fungal species were found to grow in BHB media using PET as the sole carbon source (Table 1). Out of the 7 fungi, 4 species were from the *Penicillium* genera, and 2 species were from the *Aspergillus* genera. These two fungi genera, members of the Eurotiomycetes and taxonomically placed under the Eurotiales, are commonly reported by existing literature to biodegrade plastic [29]. These 7 fungal species were analyzed for the detection of laccase and manganese peroxidase activities.

According to Figure 2, all fungal species showed the highest MnP activity in week 1 but it decreased

Table 1. Fungal species isolated from the landfill with demonstrated ability to grow in BHB media using PET as the sole carbon source.

Growth	No Growth
Penicillium janthinellum	*Cunninghamella bertholletiae*
Penicillium glaucoroseum	*Cunninghamella polymorpha*
Penicillium cremeogriseum	*Paecilomyces variotii*
Penicillium levitum	*Trichoderma asperellum*
Talaromyces flavus	*Penicillium simplicissimum*
Aspergillus niger	*Talaromyces louisianensis*
Aspergillus tamarii	*Trichosporon asahii*

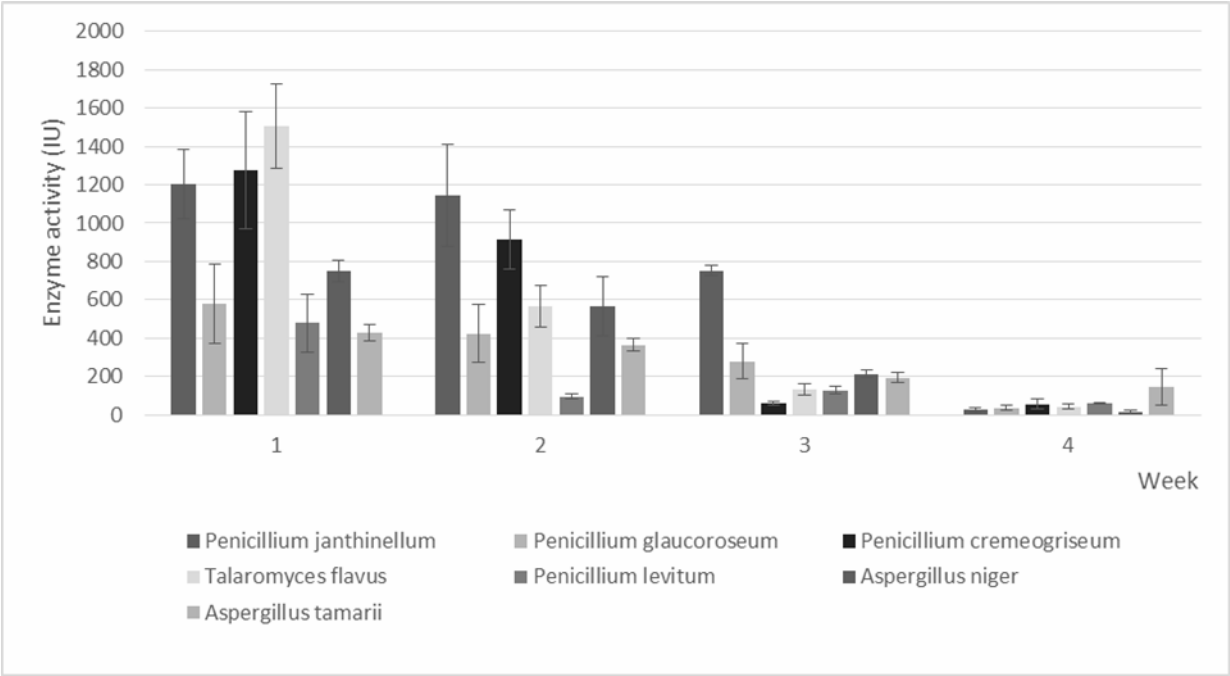

Figure 2. The manganese peroxidase activity (IU) of the fungal species metabolizing PET.

after week 2. Among all the species, *P. janthinellum* showed the best performance in terms of being consistent in expressing both MnP and laccase for a relatively longer period compared to the other fungi. From week 1 to week 3, *P. janthinellum* showed a consistent expression of MnP activity above 600 IU (Figure 2), whereas the expression of laccase activity of 10 IU and above was detected from week 1 to week 2 (Figure 3). This shows that *P. janthinellum* has the best potential to degrade PET. Although there is no known study on the ability of *P. janthinellum* to degrade PET, there are reports on *P. janthinellum* degrading other types of plastics. *P. janthinellum* has been shown to grow on polyurethane by degrading the plastic, as evidenced by the formation of a translucent zone around the fungal colony [30]. Since both polyurethane and polyethylene are members of the same polymer family and share similar structure where polyurethane is a synthetic resin that is made by polymerizing ethylene molecules [31], it is possible that *P. janthinellum*

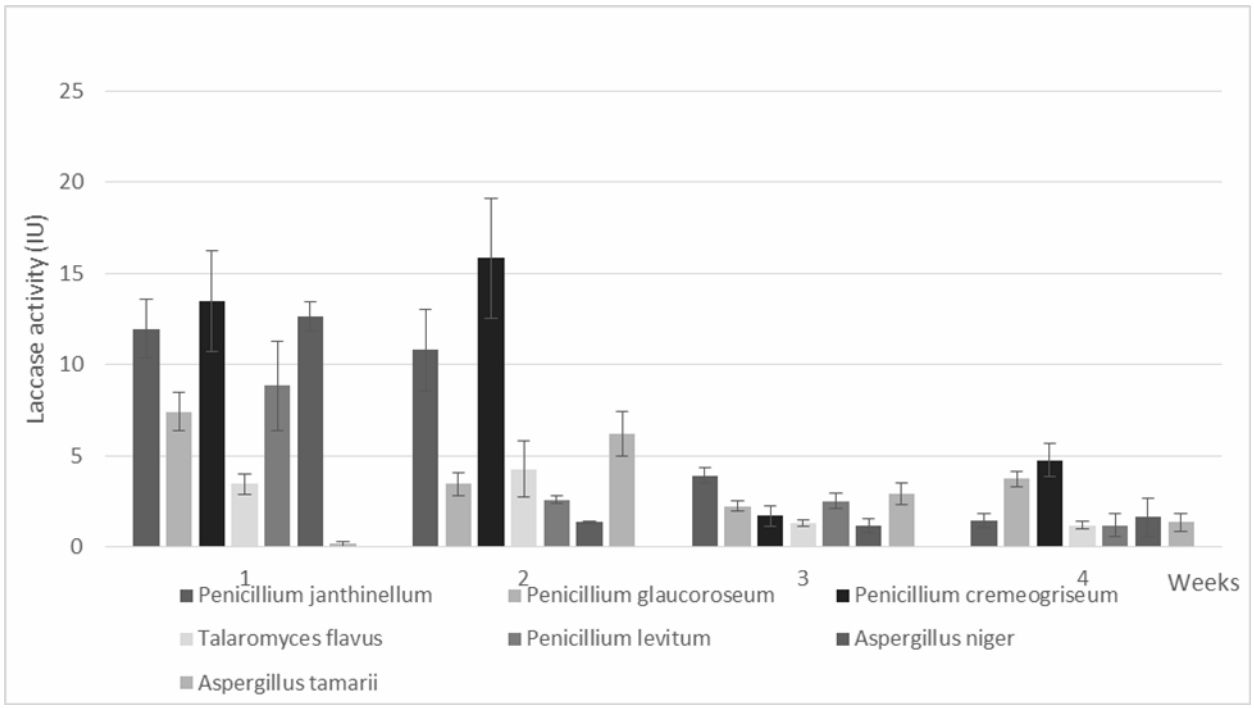

Figure 3. The laccase activity (IU) of the fungal species metabolizing PET.

also expresses the same MnP and laccase activity to degrade PET as well. Pathirana and Seal [32] noted that ester-type polyurethane-degrading enzymes such as esterases, proteases, lipases, and ureases have previously been found in fungi. Due to their comparable structures, polyurethane-degrading enzymes may be able to react with PET.

Figure 1 shows that both *P. cremeogriseum* and *T. flavus* expressed relatively high MnP activity (> 800 IU) in weeks 1 and 2, but low MnP activity (<200 IU) in weeks 3 and 4. Figure 2 shows that *P. cremeogriseum* reached the maximum laccase activity (>10 IU) on weeks 1 and 2, but the activity drops below 5 IU after that. In comparison to *P. janthinellum*, *P. cremeogriseum* has lower MnP and laccase activity. Information on *P. cremeogriseum* biodegrading PET is lacking, thus making this fungus a new potential candidate for further research. Similarly, there is lack of studies on PET biodegradation by *T. flavus*. However, there is a study that reported that *T. islandicus* biodegrades polyethylene bags [33], indicating that *T. flavus* is a potential candidate to biodegrade PET. Thus, both fungal species needed to be studied in future to gain a better understanding of what is the mechanism utilized to biodegrade PET.

Another fungal specie of interest is *P. levitum*, that expressed MnP activity exceeding 100 IU consistently from week 1 to week 4 (Figure 2). Despite *P. levitum* showing a relatively low laccase activity (<10 IU) on the starting week 1 that continued to fall (Figure 3), this fungal specie is one of the common species found in the soil [34]. *P. levitum* is native to most soil and has an advantage over other fungal species because the introduction of *P. levitum* into the soil to remediate PET contamination would not cause ecological disturbances in a fragile ecosystem. Since *P. levitum* remediating PET has not been reported, this fungal specie also deserved further investigation.

The current study has certain limitations because many fungal species isolated from the local landfills have never been reported before, making it difficult to make significant comparisons or set a guideline as a benchmark. A more in-depth research of enzyme activity and metabolite analyses need to be conducted to gain a better understanding of the mechanism of

biodegradation of PET by *P. janthinellum*, *P. cremeogriseum*, *T. flavus* and *P. levitum*.

CONCLUSIONS

In this study 7 fungal species were isolated from the landfill with the ability of biodegrading PET to support fungal growth. Of the 7 species, 6 species were from the Eurotiomycetes class, particularly the *Penicillium* genera. *P. janthinellum* was able to express relatively high MnP and laccase activities consistently. More extensive enzymatic studies and metabolite analysis can unravel the mechanism of PET degradation by this potential candidate, contributing towards the development of integrated PET plastic waste management and soil restoration.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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