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Research

Screening of Native Fungi For Biodegradation of High-Density Polyethylene (HDPE) Plastic in Mangroves Ecosystem

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ABSTRACT

Accumulation of high-density polyethylene (HDPE) plastic in the environment has become a global issue. A substantial amount of HDPE wastes ends up in the mangroves posing a significant menace to the ecology. Mitigation techniques using mycoremediation to treat the HDPE are gaining ground due to its sustainable approach. This study aims to screen and identify fungi isolated from a mangrove located in Kampung Kuala Lukut, Malaysia, that can degrade HDPE. From this study, eight fungi species were shown able to grow on HDPE as a sole carbon source in a Bushnell-Hass Broth culture. The identity of these isolates was confirmed using morphological observation and molecular identification using ITS1 and ITS4 primers. The ability of these eight fungi species to degrade plastic was assessed based on the percentage of weight loss of HDPE and the increment of fungal biomass. Results from this study show *Aspergillus niger* has the highest biomass increment, but *Leptobacillium sp.* shows the highest percentage of weight loss of HDPE. Thus, *Leptobacillium sp.* has the best potential to be developed as an efficient agent to degrade HDPE in an integrated plastic waste management system.

Key words: Environmental pollution, high-density polyethylene, plastic degradation, sustainable land management

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INTRODUCTION

According to Plastics Europe (2023) and the OECD (2022), global plastic material output ranges between 400 and 460 million tonnes in 2022, with only 9% of plastic waste recovered. This means that almost 350 million tonnes of plastic were wasted. This acceleration in plastic production can translate into a substantial amount of plastic waste entering the environment as pollutants. The amount of plastic waste is projected to exceed 12,0000 million tons by 2050 (Ekanayaka *et al*., 2022). Of all the plastics produced, polyethylene was the highest in terms of production (Danso *et al*., 2019), and is expected to exceed 121.4 million tons by 2026 (Tiseo, 2021). In particular, high-density polyethylene (HDPE) is widely used in the packaging industry due to its versatility, lightweight, affordable, durability, and ease of mass production (Begum *et al*., 2015). However, the consequence of utilizing HDPE is that this type of plastic takes a long time to degrade (Geyer *et al*., 2017), leading to its accumulation in the environment.

Presently, various technologies are employed to manage polyethylene plastic wastes (Zhang *et al*., 2020). According to Huang *et al*. (2022), the current methods of dealing with plastics are landfilling, recycling, pyrolysis, liquefaction, road construction and tar manufacture, and concrete production, with landfilling being the worst. However, the issue endures primarily due to the inherent limitations of the available

techniques. A particular example is disposing of plastic wastes using landfilling, an approach that is both cost-effective and convenient. However, landfilling can cause soil and water pollution through leachate and greenhouse gas emissions, adversely implicating the environment (Ferronato & Torretta, 2019). Such limitations call for an urgent need to seek a more efficient and sustainable alternative to deal with plastic, in particular HDPE waste. As a result, mycoremediation was proposed to reduce the environmental impact of PP pollution. Fungi are naturally decomposing organisms with long life cycles, a vast hyphal network, extensive biomass growth, and a broader metabolic competence (Kumar *et al*., 2021). Recent research demonstrated that fungi possess a wide range of enzymes that effectively interact with the chemical bonds in HDPE polymers, leading to polyethylene plastic degradation (Srikanth *et al*., 2022).

Mangroves particularly ended up as the repository of anthropogenic waste including huge amounts of plastic. Mangroves receive plastics from terrestrial, marine, and atmospheric sources, acting as a huge filter for the environment between land and sea (Wang *et al*., 2023). This poses a significant threat to the sensitive ecosystem of the mangrove which is a protected area (van Bijsterveldt *et al*., 2021). According to the Food and Agriculture Organisation, plastics on the mudflats of mangroves prevent the establishment of seeds and the growth of seedlings, resulting in detrimental consequences on the mangrove ecosystem's functions, economic activities, sustainable livelihoods, and the well-being of communities (FOA, 2002). However, mangrove environments also provide special conditions (high average & constant temperatures, high salinity, strong winds & anaerobic muddy soil) for the colonization of fungi that can create different types of enzymes for industrial purposes, recycling of plants and animals in the ecosystems, and the breakdown of pollutants (Devadatha *et al*., 2021).

Despite extensive research efforts, studies on local fungi in terms of identification, characterization, and metabolism related to HDPE degradation in Malaysia remain limited. From a financial perspective, mycoremediation is favored because it is cost-effective since the cultivation is relatively inexpensive. Furthermore, using fungi to remediate HDPE waste is environmentally friendly because no harmful by-products will be produced during the remediation process. Hence, the main objectives of this preliminary study to identify fungal strains capable of degrading HDPE from tropical mangrove areas were conducted and the effectiveness of the isolates in degrading HDPE.

MATERIALS AND METHODS

Fungal species isolation

A 3-5 cm depth of surface soil (litter in the soil was removed) was also collected into a plastic bag by using a plastic scoop (Yap *et al*., 2022) from three different locations of a mangrove forest located in Kampung Kuala Lukut, Malaysia (geo coordinates: 2.5580928, 101.8086683). The selection of the sampling sites was determined based on the presence of HDPE plastics and the diversity of fungal species in the mangroves (Kuswytasari *et al*., 2023). The soil samples were then diluted to 10^{-3} and 10^{-5} using sterilized water before mixing into the Rose Bengal agar (RBA) (OXOID, UK) in the 1:9 ratio. The RBA was then incubated at room temperature for 2 to 3 days (Samson *et al*., 2004). Single fungal isolate was subcultured onto Potato Dextrose Agar (PDA) to obtain a young and pure culture. Lastly, the pure culture was grown in the Potato Dextrose broth (PDB) to obtain biomass for DNA extraction.

Identification of fungal species

The isolated fungi were identified using the molecular approach. The fungal DNA was extracted using FavorPrep™ Plant Genomic DNA Extraction Mini Kit (Favorgen Biotech Corr, n.d.) The extracted DNA was then subjected to the polymerase chain reaction (PCR) amplification using a pair of primers targeting the ITS1 (5'TCC GTA GGT GAA CCT TGC GG 3') & 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 using the sequencing services provided by Apical Scientific Company, Malaysia. The DNA sequences obtained were then modified using the editing tool MEGA 11 software before being submitted to BLAST in the NCBI database to search for similar sequences.

The fungi species were also identified using the morphology approach. Each fungus grown on the PDA was analyzed visually in terms of colony formation patterns, colors, and textures and compared to the images and description from the reported study. Results from the morphology analysis were then matched against the results obtained through molecular identification for accuracy of the fungal species identification.

Screening for HDPE degrading fungal species

In triplicates, a single pure fungal isolate was inoculated into a flask containing autoclaved 50 mL of Bushnell Haas Broth (BHB) (OXOID, UK) mixed with 0.1 g of shredded HDPE in beads form which was obtained from the hardware shop. The flasks were then covered with parafilm and were incubated for 30 days at 25°C in a rotary shaker (120 rpm) (Ogunbayo *et al*., 2019). At the end of the incubation period, the fungal culture was observed with the naked eye for increased media turbidity. The BHB with HDPE in the absence of fungi culture was used as a control comparison (Ojha *et al*., 2017). A culture that showed turbidity indicated that the fungal isolate was able to metabolize HDPE as the single carbon source to support growth.

The remaining HDPE plastic residues in each medium were retrieved via filtration using Whatman filter paper No1. The HDPE plastic residues were soaked overnight in a 2% sodium dodecyl sulfate (SDS) solution to remove fungal biomass that was attached to the plastic surface and any residual BHB. Then, the plastics were thoroughly cleansed with 70% ethanol, rinsed thrice with distilled water, and the excess moisture was removed by placing the plastic into an oven at 60 °C (Gong *et al*., 2023). After drying, the final weight of HDPE plastic was measured using a digital weighing scale. The weight of the HDPE plastic in the control sample after 30 days was recorded as naturally degraded weight. The percentage of weight loss of HDPE plastic incubated with fungal isolate was calculated using the equation below:

$$\text{Weight loss \%} = \frac{\text{(Initial weight)-(Final weight+naturally degraded weight)}}{\text{Initial weight}} \times 100$$

Statistical analysis

The data of biomass of fungal isolates and the percentage weight loss of HDPE were analyzed using the software program Statistical Package for the Social Sciences (SPSS). The data were statistically analyzed using the One-way analysis of variance (ANOVA), with the Least Significant Difference (LSD) Post Hoc Test (Ong *et al*., 2019) performed at a 95% confidence level.

RESULTS AND DISCUSSION

A total of 11 fungal species were isolated from the mangrove soil samples of Kampung Kuala Kulut, Malaysia. Three fungal species were excluded from consideration due to their slow growth during the screening period. For the remaining eight isolates, seven were identified up to species level, including *Talaromyces rufus, Aspergillus niger, Purpureocillium lilacinum, Penicillium citrinum, Cladosporium pseudocladosporioides, Aspergillus flavus,* and *Aspergillus nidulans.* However, *Leptobacillium* sp. was identified only at the genus level.

HDPE plastic weight loss percentage

Figure 1 depicts the HDPE plastic weight loss percentage, which ranged from 0.25% to 0.86%. In the descending order of HDPE plastic weight loss percentage for each species were *Leptobacillium* sp., *P. lilacinum* and *P. citrinum*, *T. rufus*, *C. pseudocladasporioides*, *A. niger*, *A. flavus* and *A. nidulans.* However, the HDPE plastic weight loss percentage recorded showed no significant difference among all fungal species.

Notably, *Leptobacillium* sp. exhibited the most effective performance with a 0.85% weight loss in HDPE. Although it cannot be identified at the species level, the Sordariomycetes class was reported able to degrade HDPE in a study by Ekanayaka *et al*. (2022). On top of that, *P. lilacinum* and *P. citrinum* also exhibited relatively high HDPE weight loss (0.66%). Both belong to members of the *Penicillium* genus and were recognized for PE degradation (Ekanayaka., 2022). There is limited study on *P. lilacinum* in degrading HDPE, it is reported for the first time for causing damage to low-density PE (LDPE) surfaces, confirmed by Attenuated Total Reflectance Fourier-transform infrared spectroscopy (ATR-FTIR) analysis and Scanning Electron Microscope (SEM) images (Federica *et al*., 2021). The analysis showed a reduction in the relative amount of methyl groups in PE subjected to *P. lilacinum*, suggesting biodegradation at the expense of methyl terminal groups. HDPE and LDPE belong to PE and might share some common characteristics that are degradable by *P. lilacinum*. Similarly, there is also a limited study on *P. citrinum*-degraded HDPE. However, Khan *et al*., 2021 reported *P. citrinum* has rapid growth in LDPE, resulting in a 1.08% weight loss confirmed by FTIR, supporting the involvement of enzymes, and confirming LDPE depolymerization (Khan *et al*., 2021). Considering the similar polymer structures of LDPE and HDPE, it is conceivable that both *P. lilacinum* and *P. citrinum* can degrade HDPE. Their membership in the Sordariomycetes class and *Penicillium* genus further strengthens their potential as

candidates for further HDPE degradation research.

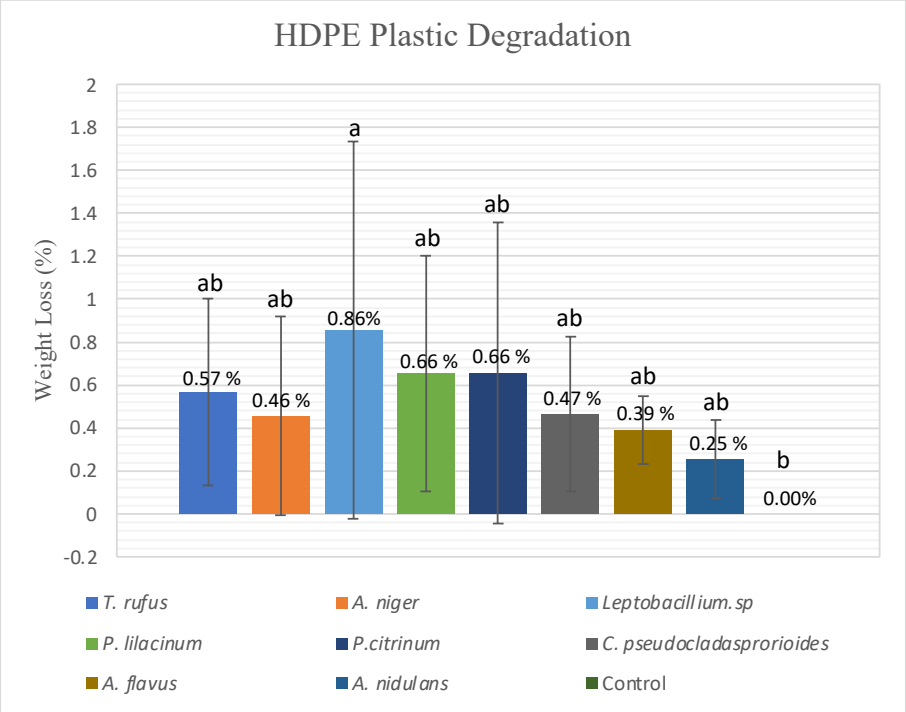

Fig. 1. HDPE plastic weight loss percentage of fungal species after 30 days of incubation.
Note: a, ab, b: different alphabet in each column shows the different significant means (Post Hoc Test, LSD, $P<0.05$).

Fungal biomass production

Figure 2 illustrates the biomass production on HDPE by each fungal species, ranging from 0.016 g to 0.058 g. Both *A. niger* and *P. citrinum* demonstrated a significant difference from *Leptobacillium* sp., *P. lilacinum, C. pseudocladasporioides, A. niger,* and *A. nidulans.* The descending order of biomass production on HDPE by each species was from *A. niger*, *P. citrinum*, *T. rufus, A. nidulans, A. flavus, P. lilacinum, C. pseudocladasporioides* and *Leptobacillium* sp. Despite numerous investigations into various HDPE-degrading fungi, there remains a limit of studies on fungal biomass production. In general, biomass production is low after 30 days of incubation in HDPE for all fungal species. The complexity of PE structure may contribute to the limited conversion of PE plastics into biomass by certain fungi. Yao *et al*. (2022) stated that the predominant composition of PE, primarily consisting of carbon and hydrogen with new functional groups, hinders its susceptibility to enzymatic attacks. This lack of functional groups, which are commonly found in more easily degradable organic compounds, causes PE to be chemically inert which means it provides fungi with fewer sites to interact with fungal enzymes to initiate degradation processes.

According to Figure 2, *A. niger* demonstrated the highest biomass production on HDPE with values of 0.058 g although this species showed the lowest percentage in HDPE weight loss. Nevertheless, a prior study suggested a correlation between the increase in fungal biomass over time and the weight loss percentage observed for LDPE caused by *A. niger*, attributed to the increased secretion of enzymes that degrade the LDPE compound (Altaee & Al-Dosarry, 2021). In contrast to *A. niger*, all other fungal species demonstrated results in biomass production on HDPE that contradicted their respective plastic weight loss percentage. Notably, *Leptobacillium* sp. displayed the lowest biomass yield of 0.016 g on HDPE although this species showed the highest percentage of HDPE weight loss. A study by Ene *et al*. (2014), highlighted the diverse metabolic flexibility developed by various fungal species in response to environmental and evolutionary stress, suggesting that differences in enzyme production rates and properties are influenced by distinct gene expressions within each species.

Another study focused on phospholipases produced by different colonies of *A. niger* (A1 & A2) and lipases from *A. niger* and *P. cyclopium*. The results showed distinct behaviors, with A1 and A2 producing 1.8 mg/mL and 8.5 mg/mL of phospholipase, respectively, under similar conditions (Mustranta *et al*., 1995). On top of that, these phospholipases exhibited different isoelectric point (pI) values of 4.3 and 6.0, respectively. Additionally, *A. niger* lipase and *P. cyclopium* lipase displayed diverse production rates (50 mg/g and 30 mg/g, respectively) and optimal pH levels (4.5 for *A. niger* lipase and 4.0 for *P. cyclopium*

lipase) (Mustranta *et al*., 1995). These variations in enzymatic production rates and properties provide insights into why certain fungal species yield contrasting results in terms of plastic degradation and biomass production.

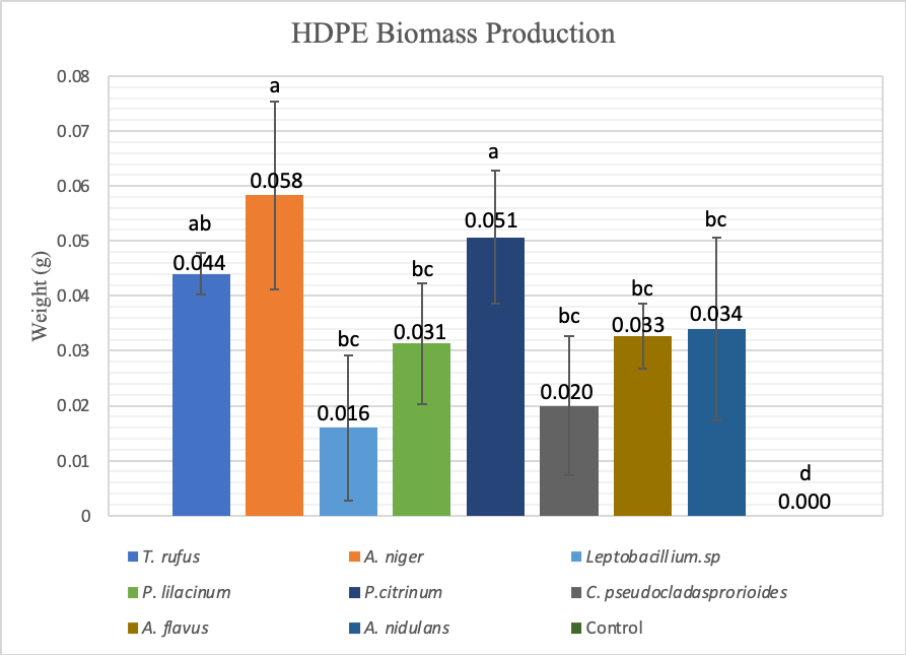

Fig. 2. Biomass production on HDPE by each fungal species after 30 days of incubation.
Note: a, ab, bc, d: different alphabet in each column shows the different significant means (Post Hoc Test, LSD, $P<0.05$).

Limitations of the study

There are limitations to this study including the incubation time, which could have been increased as studies in fungal biodegradation can last from 30 to 120 days (Ogunbayo *et al*., 2019; Raaman *et al*., 2012). This is due that some fungal species require a very long time to degrade plastic and demonstrate observable results (da Luz *et al*., 2019). Throughout this study, a notable observation was the limited well-established standard methods for the removal of HDPE attached biomass, where the complete removal of attached biomass was not assured which was reported that PE surfaces were confirmed to be penetrated by fungal biomass in SEM micrographs (Volke-Spulveda *et al*., 2022). This limitation consequently influenced the results in terms of plastic weight loss percentage and biomass production. This study also faced limitations such as numerous fungal species isolated from local mangroves have not been previously documented, hindering substantial comparisons or the establishment of standardized benchmarks. To improve the understanding of HDPE biodegradation mechanisms employed by these 8 fungal isolates, a more comprehensive study involving enzymatic activities and metabolite analysis is required.

CONCLUSION

This study comprises the isolation of 8 fungal species from a Malaysian mangrove location, demonstrating their potential to biodegrade HDPE and fungal biomass production. In this study, *Leptobacillium* sp. had the highest HDPE weight loss percentage (0.86%), while *A. niger* excelled in biomass production on HDPE (0.058 g). Despite both species performing well in different aspects, *Leptobacillium* sp. is more suitable to be an HDPE degrader compared to *A. niger*, in the context of PE degradation. Despite the unspecified species of *Leptobacillium* sp., in-depth studies could be conducted to elucidate its metabolic pathway and the mode of action of an enzyme related to PE degradation, along with the application of FTIR and SEM to assess its efficacy on PE degradation. Thus, making it a more promising candidate for advancing integrated PE plastic waste management and restoring PE-contaminated land.

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ETHICAL STATEMENT
Not applicable

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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