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Research Article

Acceleration of the Degradation of Low-density Polyethylene using Endolichenic Fungal Spore Suspension

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Abstract

The involvement of fungal spores in the biodegradation of recalcitrant substances has garnered significant attention in recent years. The present study evaluated the potential of an endolichenic fungal spore suspension of *Phanerochaete chrysosporium* to enhance the biodegradation of low-density polyethylene (LDPE). A spore suspension containing 2×10^6 spores mL^{-1} was developed and optimized for application to LDPE, with 1.20 mL per 2.40 g LDPE and pH 7.0 identified as the minimum effective volume and optimum pH, respectively. Under optimized *in vitro* conditions, incubation for 105 days resulted in 19.44% weight loss, 29.37% reduction in tensile strength, and 1.60% water absorption of LDPE. Changes in Fourier Transform Infrared (FTIR) spectra, including the disappearance of the CH_3 deformation band at 1375 cm^{-1} and formation of oxygen-containing functional groups, together with Scanning Electron Microscopy (SEM)-observed cracks, cavities and holes, confirmed polymer degradation. Gas Chromatography-Mass Spectrometry (GC-MS) analysis detected low-molecular-weight alkanes, alkenes, carboxylic acids, esters, ethers and alcohols among the degradation products. In addition, 574.1 mg of gaseous carbon evolved from spore suspension-treated LDPE compared with 28.2 mg in the control. Laboratory-scale soil application further demonstrated enhanced degradation following spore application, with 20.91 ± 0.20% weight loss and 1.57 ± 0.09% water absorption, compared with 13.90 ± 0.15% and 0.60 ± 0.07%, respectively, without spore inoculation. The isolation of *P. chrysosporium* from treated soil after 105 days confirmed successful germination and persistence of the introduced fungus alongside native soil microflora. Overall, the findings demonstrate that *P. chrysosporium* spore suspension can substantially enhance LDPE biodegradation under both *in vitro* and soil conditions and has potential as a promising biological approach for polyethylene waste management.

Keywords: biodegradation, low-density polyethylene, *Phanerochaete chrysosporium*, spore suspension, enhancer.

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1. Introduction

Polyethylene is a naturally durable polymer, and once it enters the environment, it persists for many years. The accumulation of polyethylene waste poses a significant ecological threat. Various strategies, including microbiological approaches, have been initiated to address and reduce the environmental impact of polyethylene accumulation.

Fungi are a biotechnologically valuable group of organisms. Because fungi occupy a vast array of habitats and must compete for survival, they have evolved various survival strategies and distinctive traits (Hyde *et al.*, 2019). These unique characteristics make fungi highly promising for use in biotechnology and industry. The xenobiotic degradation capacity of most fungi is due to extra-cellular, non-specific enzymes. Fungi are extensively utilized in bioremediation because of their resilience and the wide variety of enzymes they produce (Mahalakshmi and Andrew 2012). These non-specific enzymes enable several advantages which are not found in other bioremediation systems.

Endolichenic fungi (ELF) are fungi that occur asymptomatically within lichen thalli and are considered a relatively underexplored source of metabolically diverse microorganisms. Their association with lichenized environments may require adaptation to nutritionally limited and physiologically stressful conditions, potentially favouring the production of extracellular enzymes and metabolites involved in the utilization or transformation of complex substrates (Santhirasegaram *et al*., 2020). In the present study, *Phanerochaete chrysosporium* was selected from a previously established endolichenic fungal culture collection because this isolate had shown promising LDPE degradation and possessed a rich depolymerizing enzymatic profile in earlier investigations (Perera *et al*., 2022). *Phanerochaete chrysosporium* (anamorph, *Sporotrichum pulverulentum*), is a white-rot basidiomycete capable of producing lignolytic enzymes associated with the transformation of chemically recalcitrant substrates. They typically produce key lignin-degrading enzymes, including lignin peroxidase, manganese peroxidase, and laccase. (Kumar and Chandra 2020; Perera *et al*., 2021, 2022). Degradation of high-molecular-weight polyethylene sheets by P. chrysosporium has been reported, and previous studies have also documented the involvement of the ligninolytic enzyme system of *P. chrysosporium* in the transformation of polyethylene (Wei *et al*., 2017). Its previously reported ability to degrade polyethylene therefore provided the primary biological basis for its selection in the present study.

In addition to its polyethylene biodegradation capability, there is ample evidence of P. chrysosporium's involvement in fungal biotechnology and industrial microbiology (Sedighi *et al*., 2009; Ghasemzadeh *et al*., 2018). For these purposes, viable fungal cells and spore suspensions are basically involved, and in any biodegradation study, it is crucial to prepare viable inoculum to initiate biological degradation.

The laboratory-scale application of strategies to address polyethylene waste, particularly in soil, offers numerous advantages. It allows for the feasibility assessment of approaches in a controlled yet representative environment, providing insights into practicality and effectiveness before full-scale implementation. By evaluating the environmental impact at a laboratory-scale soil treatment, it is possible to understand how a strategy interacts with soil ecosystems, thus aiding in refining the approach based on real-world conditions (Pearson *et al.*, 2020). Targeting soil as a source for this application is justified for several reasons.

Polyethylene waste is often found in these environments due to improper disposal and runoff, making them key areas for direct treatment. Additionally, addressing polyethylene pollution at the source in the soil helps to minimize its ecological threat to the vital ecosystems (Kumar *et al.,* 2021). In summary, laboratory-scale soil applications provide a strategic and comprehensive solution to the pressing environmental problem of polyethylene accumulation.

Soil sampling is a fundamental step in understanding the environment's initial conditions when assessing the impact of microbiological strategies on polyethylene waste (Puglisi *et al*., 2019). The use of a spore suspension in the context of polyethylene degradation involves inoculating sampled soil with microbial spores to enhance the polymer's natural degradation by introducing microorganisms specifically adapted for this purpose. The importance of testing the survivability of introduced spores cannot be overstated. This testing is crucial for gauging the resilience and adaptability of selected microorganisms in the target environment, ensuring their longevity and sustained polyethylene degradation. Additionally, assessing survivability helps to identify any potential adverse effects on the native soil microbiota, ensuring a harmonious coexistence between introduced spores and the existing microbial community (Ali *et al*., 2023). In summary, the combination of soil sampling, spore suspension, and survivability testing provides a comprehensive approach to understanding and implementing effective microbiological strategies to mitigate polyethylene accumulation in the environment.

The current study leads to the development of a spore suspension using ELF and its application as an enhancer for low-density polyethylene (LDPE) biodegradation. The specific novelty of the present study is the development of *P. chrysosporium* spores as a standardized inoculum and evaluation of their ability to enhance LDPE biodegradation in both *in vitro* conditions and as a laboratory-scale assessment in soil environment. This approach enabled assessment of polymer degradation through complementary physicochemical and microscopic parameters and, importantly, evaluation of the survival of the introduced fungus within a native soil microbial community.

2. Materials and methods

2.1 ELF culture collection and identification

From the previously isolated ELF culture collection (of the Department of Chemistry, University of Kelaniya, Sri Lanka), *Phanerochaete chrysosporium* (MT507810) was selected for the present study based on its promising results on degradation of LDPE and rich depolymerizing enzymatic profile (Perera *et al*., 2022). The identity of the ELF isolate was authenticated using morphological characterization and ITS gene sequencing.

2.2 Preparation of the ELF spore suspension

The development of spore suspension followed a previously established method (Ghasemzadeh *et al.,* 2018). Actively growing cultures of the ELF isolate were obtained on Potato Dextrose Agar (PDA, Oxoid Ltd., Basingstoke, UK). The plates were flushed with 5.0 mL sterile distilled water. The suspension was subsequently filtered, and the resulting spore suspension was centrifuged at 3,000 rpm for 5 minutes, and the supernatant was discarded. The pellets were resuspended in 5.0 mL of sterile distilled water–agar (0.500 g L^{-1}) supplemented with inorganic salts, including $(NH_4)_2SO_4$ (1.000 g L^{-1}), K_2HPO_4 (1.000 g L^{-1}),

NaCl (1.000 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.500 g L^{-1}), KH_2PO_4 (0.200 g L^{-1}), $FeSO_4 \cdot 7H_2O$ (0.010 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.002 g L^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.001 g L^{-1}), $MnSO_4 \cdot H_2O$ (0.001 g L^{-1}), and $CuSO_4 \cdot 5H_2O$ (0.001 g L^{-1}). All chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The final pH was adjusted to 7.0 ± 0.2, and the preparation was maintained at 28 ± 2 °C.

A diluted spore suspension was prepared to achieve an average absorbance of 0.5 at 650 nm in a 1-cm path-length cuvette (Sedighi *et al*., 2009; Ghasemzadeh *et al*., 2018). The number of spores per millilitre was quantified using the Hemocytometer counter (Paul Marienfeld GmbH & Co. KG, Germany) under the optical microscope (Olympus Cx21 Microscope CX21FS1, Olympus Corporation, Japan). Until further use, spore suspension was stored in the refrigerator (4 °C) in dark conditions. A method described in previous studies was applied to ensure the viability of the fungal spores in the suspension (Pearson *et al*., 2020). Briefly, viability was assessed by serial dilution and by periodically plating the spore suspension on PDA (Oxoid Ltd.) containing Streptomycin (10 µg mL-1) (initially, after 1 month, 2 months, and 3 months). The plates were incubated at room temperature (28 ± 2 °C) for 3-5 days.

2.3 Optimizing the conditions for the in vitro application

The application of fungal spore suspension on the LDPE substrate was performed with few modifications to a method described previously (Ghasemzadeh *et al*., 2018). To determine the minimum effective inoculum volume, 2.40 g of surface-sterilized LDPE strips (10 cm × 3 cm) were placed in 250 mL Erlenmeyer flasks containing 0.60, 1.20, 1.80, 2.40 and 3.00 mL of the standardized spore suspension. The flasks were agitated at 150 rpm and maintained at 28 ± 2 °C, with degradation assessed at 21, 42, 63, 84 and 105 days. Three replicates were used for each treatment, while control LDPE strips were treated under identical conditions without fungal spore inoculation.

Following identification of the minimum effective volume, the effect of pH was evaluated by preparing the spore suspension at pH 4.5, 7.0 and 10.0, while maintaining the other experimental conditions constant. The minimum effective volume identified in the preceding experiment was used for each pH treatment.

Temperature and agitation speed were maintained at 28 ± 2 °C and 150 rpm, respectively, throughout the optimization experiments and were not independently optimized. These conditions were selected to maintain consistent fungal growth and application conditions based on the established methodology used for preparation and application of the spore suspension.

2.4 Biodegradation of LDPE under the optimized conditions for the in vitro application

LDPE strips were subjected to in vitro biodegradation studies under optimized conditions. The degradation of polymer properties was studied by varying the incubation period in three-week gaps of 42, 63, 84 days and 105 days. All determinations were performed in three replicates. Control LDPE strips were treated with the same conditions, without fungal spore inoculation.

2.5 Confirmation of the degradation of LDPE for the in vitro application

Confirmation of the degradation was carried out based on the tests described previously (Perera *et al*., 2022). Briefly, analyses on percent weight loss, reduction of tensile properties,

increment in degree of water absorption, changes in the peaks of Attenuated Total Reflection - Fourier Transform Infrared (ATR-FTIR) spectra, structural changes as revealed by Scanning Electron Microscopy (SEM) images and Gas Chromatography-Mass Spectrometry (GC-MS) product analyses. In addition to the above tests, the amount of gaseous carbon evolved by the spore suspension-treated LDPE strips was determined using the respirometer method, as described in ISO 17556;2019(E).

2.6 Sampling of soil for the laboratory-scale soil application of the spore suspension

Soil sampling was carried out using a method described in a previous study (Ameh and Kawo 2017). Soil samples were collected from 3 locations in an urban waste dumping site at Karadiyana Mihisaru Viyamana Resource Management Center, Karadiyana, Sri Lanka. Loose, unconsolidated topsoil (7 cm depth) was used to attain a composite sample. The sample was carried to the laboratory in a sterile container inside an ice pack. The soil sample was passed through a 4 mm sterile sieve.

2.7 Biodegradation of LDPE in the laboratory-scale soil application of the spore suspension

Soil burial studies for LDPE strips were carried out with few modifications to a previously described method (La Mantia *et al.,* 2020). The experiment was carried out as two set-ups, each in three replicates. Soil samples from the waste dump yard were added into the sterile containers (500.00 g, approx. 1000 cm3 of soil per container). A weighed amount (2.40 g) of sterile LDPE strips were placed in each container (of the two set-ups) and buried in the soil, such that the soil covered the LDPE strips from all sides (to a depth of 1-cm). Set-up 1 remained without spore inoculation, while set-up 2 was added with the spore suspension. A container with sterile LDPE strips and sterile soil, without fungal spore suspension, was used as the control. The degradation of polymer properties was studied after 105 days. Confirmation assays were carried out as described under 2.5, for the LDPE strips, taken out from each container, in soil environment.

2.8 Isolation and identification of abundant bacteria and fungi

Initial suspensions and decimal dilutions were prepared for microbial isolation, with few modifications to a previously described method (Ameh and Kawo 2017). Soil was used as the test samples, separately sampled from the top and bottom of set-up 2 containers after the 105-day incubation period. A soil sample (1.00 g) was added to sterile water (9.0 mL), followed by homogenization to obtain 10^{-1} dilution. Further tenfold serial dilutions were made for the colony counting. The microbial colonies were isolated with few modifications to a previously described methods (Ameh and Kawo 2017). For bacterial isolation, isolation streaks were drawn on Nutrient Agar (NA) supplemented with Cycloheximide (100 µg $mL^{-}1$). Plates were incubated at 37 ± 2 °C for 24 hours. The bacterial colonies were stored on NA slants and kept at 4 °C, for further characterization and identification. For fungal isolation, hyphal tips from morphologically distinct colonies were placed on PDA plates and subjected to several subculturing to obtain pure cultures. Plates were incubated at room temperature (28 ± 2 °C) for 3-5 days. The isolated fungal colonies were stored on PDA slants and kept at 4 °C, for further characterization and identification.

During the identification of bacterial isolates, set of biochemical tests were carried out on pure culture of each isolate following microscopic examinations. Results were interpreted with the identification schemes (Bergey, 1994). DNA-based bacterial species confirmation

was achieved by comparing the sequence of the 16S rRNA gene using universal primers 27F and 1492R. The fungi were identified based on their colony characteristics, morphology of hyphae and spores. Microscopic analysis was done using the Lacto Phenol Cotton Blue staining method (McClenny, 2005). In fungal species identification, the nuclear rDNA ITS region was amplified using fungal-specific ITS1 forward and ITS4 reverse universal primers (Perera *et al*., 2022).

The molecular identification of bacteria and fungi involved DNA extraction, amplification, and sequencing techniques. The PCR-amplified products were sequenced using Sanger dideoxy sequencing at Genetech Institute in Colombo, Sri Lanka. The sequences were manually refined using BioEdit Sequence Alignment Editor (Version 7.2.5) and compared with GenBank sequences through BLAST to determine homology. The DNA sequences of the identified bacterial and fungal species were submitted to the NCBI database, and accession numbers were acquired.

2.9 Statistical analysis

The quantitative data obtained from the laboratory-scale soil application, namely percent weight loss, degree of water absorption and percent reduction in tensile strength, were arranged according to a completely randomized design. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was performed to determine significant differences among the experimental setups. Three replicates were used for each setup. Statistical analyses were performed using SPSS (IBM, Armonk, NY), and differences were considered statistically significant at $P < 0.05$.

3. Results and Discussion

Most of the processes currently used to reduce accumulated polyethylene waste are laborious, expensive, time-consuming, and not environmentally friendly. Under such circumstances, microbial-mediated degradation seems to be an important approach in decimating polyethylene waste. As the study is focused on the development of a spore suspension from potential ELF, which can be used in answering the burning issue of polyethylene accumulation, prior the spore suspension development, the identity of *P. chrysosporium* was further authenticated using morphological and DNA barcoding techniques. During the morphological studies, aerial hyphae appeared as hyaline, thin-walled, smooth, septate, and branched, and these branches often developed as conidiophores (Supplementary Fig. S1). DNA-based species identity was achieved by comparing the sequence of ITS rDNA region with the established (vouchered /published) sequences with the highest percent similarity (100%) of the GenBank accessions and was confirmed as *P. chrysosporium* (MT507810).

In the preparation of the spore suspension, spores were harvested from seven days old lawn cultures of PDA. This resulted in a mixed suspension of spores and mycelia and, it was filtered and centrifuged to remove mycelial fragments. Pellets were resuspended in sterile distilled water and sterile agar, with inorganic salts added. Agar provided stability to the suspension, preventing spore sedimentation and enabling precise quantification (Caligiore-Gei and Valdez 2015). Spore germination typically requires environmental nutrients to initiate spore activation. Low molecular weight nutrients and inorganic salts can activate spore germination (Chitarra, 2003). In the current study, the addition of carbon sources was avoided during the formulation of spore suspension, because the carbon sources would act as

competitors with LDPE, in the application of this spore suspension for biodegradation purposes.

From the initial spore suspension, diluted suspension was prepared, to achieve an average absorbance of 0.5 at 650 nm. As suggested in the previous studies, prior to the applications on *P. chrysosporium* spore suspensions, dilution up to this level was recommended (Sedighi *et al*., 2009; Ghasemzadeh *et al*., 2018). The number of spores per milliliter of the diluted spore suspension was quantified using the Hemocytometer counter. It was found that there were 2 x 10^6 spores per milliliter of diluted suspension. These results were consistent with the previous results (Ghasemzadeh *et al.,* 2018), in the preparation of *P. chrysosporium* spore suspensions. In the microscopic view (Fig.1), spores in the suspension appeared as hyaline, densely granular contents with thickened walls, and were variable in sizes and shapes (ovoid or elliptical).

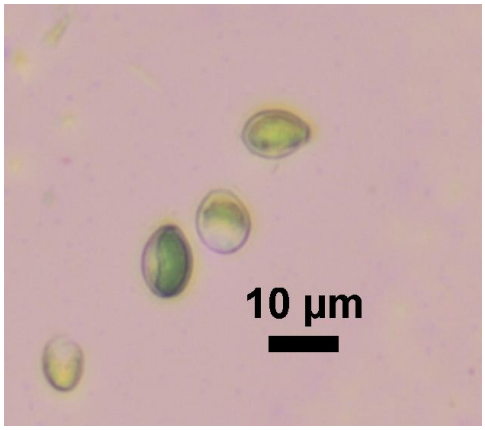


Fig.1. *Phanerochaete chrysosporium* spore suspension under the optical microscope (at magnification ×1000)

Until further use, spore suspension was stored at refrigeration temperature (4°C), at dark conditions (after wrapping in Al foils), to minimize the germination of the spores, while in the suspension. Temperature is known to play a critical role in the germination of most fungal spores. In a previous study on the effect of temperature on fungal conidial spore germination at 5 °C, overall results showed no conidial germination of Botryosphaeriaceous fungi (Úrbez-Torres *et al.*, 2010). The viability of the spores in the refrigerated suspension was ensured by plating on PDA periodically in 1-month time gaps, for 3 months.

The feasibility of the spore suspension for the application on LDPE was studied under *in vitro* conditions, using the minimum effective volume of spore suspension and under the optimum pH, for its proper activation. Inoculum volume and pH were selected as the primary variables because the former determines the amount of fungal inoculum available per unit mass of polymer, whereas pH can influence spore germination, fungal metabolic activity and the production/activity of extracellular depolymerizing enzymes. The objective was therefore to identify the minimum effective volume of the standardized spore suspension and a suitable pH condition for subsequent LDPE biodegradation experiments. Based on the results, 1.20 mL was obtained as the minimum effective volume of spore suspension to be applied for 2.40 g of LDPE strips to show detectable changes in the degradation of polymer properties under *in vitro* conditions. The formulation relevant to the pH 7.0 was selected as the optimum pH value.

The spore suspension used in these experiments was standardized to approximately 2×10^6 spores mL^{-1}, based on the established preparation procedure, and the concentration was verified using a hemocytometer. Spore concentration was therefore maintained constant rather than optimized independently, allowing the effects of application volume and pH to be evaluated without introducing an additional variable. It was evident that the degree of biodegradation increased with the incubation time. Therefore, the results were presented for 105 incubation days.

Percent weight loss and degree of water absorption of LDPE strips for a 105-day incubation period were recorded as 19.44% and 1.60%, respectively, while the percent reduction of tensile strength was 29.37%.

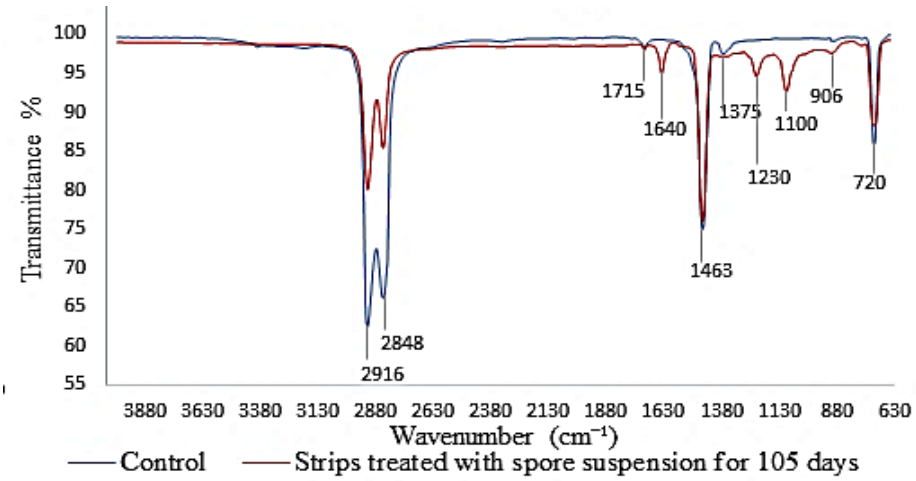


Fig.2. FTIR spectrum for control and spore suspension treated LDPE strips for the 105-day incubation period

FTIR spectroscopy was used to examine changes in the chemical structure of LDPE following the treatment with the *P. chrysosporium* spore suspension for 105 days (Fig. 2). The spectra showed qualitative changes in several characteristic absorption bands, including those at 2916, 2848, 1715, 1463 and 720 cm^{-1}. The bands at 2916 and 2848 cm^{-1} corresponded to asymmetric and symmetric C–H stretching of CH_2 groups, respectively, while the band at 1715 cm^{-1} was associated with carbonyl-containing groups (Torre *et al.,* 2018). The bands at 1463 and 720 cm^{-1} were attributed to CH_2 scissoring (Maalihan and Pajarito 2015) and C–H bending vibrations (Torre *et al.,* 2018), respectively.

A notable qualitative change was the disappearance of the absorption band at 1375 cm^{-1}, previously assigned to CH_3 symmetric deformation (Gulmine *et al.,* 2002), in the spore-suspension-treated LDPE spectrum. Simply, the disappearance of a band indicates the absence of the functional group after the reaction (Řeřicha *et al.,* 2005). It was reported that the formation of new peaks was due to the intermediate products of the biodegradation of polyethylene, such as carbonyl, ether, and carboxyl functional groups. New peaks at the

range 1000-1320 cm^{-1}, correlated with the –C-O stretch of alcohols, carboxylic acids, esters, and ethers (Vimala and Mathew 2016), were visible in the FTIR spectrum obtained for the spore suspension treated strips. In addition, a new peak correlated with vinyl C=C bonds (Torre *et al*., 2018) appeared around 1640 cm^{-1} in the spectrum. Similar patterns in formations of new carbonyl bonds were observed when the LDPE degrading efficiency of microbial isolates was analyzed in the previous studies (Chitarra, 2003; Khruengsai *et al*., 2021). These spectral changes indicate modification of the chemical environment of the polymer following incubation with the fungal spore suspension. These FTIR observations were further supported by GC-MS detection of alkanes, alkenes, carboxylic acids, esters, ethers and alcohols among compounds associated with the treated LDPE.

The replicate peak-area or peak-height measurements were obtained during the FTIR analyses, to monitor the quantitative changes in functional-group abundance. Accordingly, the increment of carbonyl index of spore suspension treated LDPE strips, relative to control was 5.13%.

The degradation of the LDPE strips treated with spore suspension was further confirmed using SEM analyses.

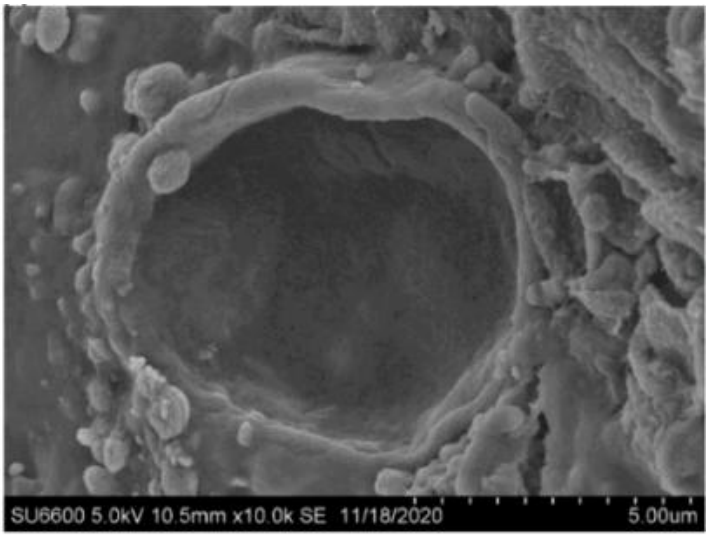


Fig.3.SEM image of a LDPE strip after 105-day incubation with spore suspension, under *in vitro* conditions

The SEM analysis revealed the degradation on the surfaces of the LDPE strips in the form of cracks, scions and holes (Fig.3). After the germination of spores, the fungi were colonized on the LDPE strips by utilizing LDPE as the sole carbon source, and the fungal hyphae have successfully coalesced with LDPE strips by growing over the surface. The degradation was enhanced by fungal biofilm formation, as reported in previous studies (Das and Kumar 2014; Khruengsai *et al*., 2021).

GC-MS product analyses and respirometer analyses on evolved gaseous carbon amount were further carried out for LDPE strips, with a 105-day incubation period, to confirm the boosted degradation process in spore suspension-treated LDPE strips. Based on the retention times and abundance of compounds obtained from the GC-MS scan method, the presence of the low molecular weight products was not detected in higher abundance in the extracts of the control strips (Supplementary Fig.S2a) while their presence was confirmed among the products extracted from the spore suspension treated strips, after 105 days of incubation.

Table 1. GC-MS library report for spore suspension treated LDPE strips under *in vitro* conditions, for 105-day incubation.

Retention Time	Area Percentage (%)	Library/ ID
13.871	3.39	Hexadecane
14.468	3.69	Z-8-Hexadecene
14.824	3.81	Heptadecane Nonadecane Hexadecane
15.719	6.72	Octadecane
16.316	3.95	1-Octadecene
16.569	2.56	Nonadecane
17.097	13.95	n-Hexadecanoic acid
17.395	3.65	Eicosane Heptadecane Hexacosane
17.981	3.23	n-Tetracosanol-1 Methyl triacontyl ether
18.659	3.94	Octadecanoic acid
19.508	2.51	2,3-Dimethylhexa-1,3-dien-5-ol 2-Hydroxyhexanedial
20.323	3.11	Tetracosane Eicosane Octadecane
21.391	3.50	Phthalic acid, bis(2-ethylhexyl) ester
29.383	39.76	Tris(2,4-di-tert-butylphenyl) phosphate

The presence of alkanes and several oxygenated compounds was confirmed in products extracted from strips treated with a spore suspension and incubated for 105 days under *in vitro* conditions. The degradation products extracted and identified by referring to previously saved data in the NIST14 and W9N11 libraries were primarily alkanes and alkenes (Table 1). Several carboxylic acids and esters were also present. Ethers such as Methyl triacontyl ether

and alcohols such as 2,3-Dimethylhexa-1,3-dien-5-ol, and n-Tetracosanol-1 were also present. The presence of aldehydes was also prominent. Formations of new peaks at the FTIR spectra, correlated with the functional groups such as carboxylic acids and esters were reconfirmed by these GC-MS analyses, on the intermediate products of LDPE degradation. In line with these results, after biodegradation, polyethylene produced oxidized products of low molecular weight (Albertsson *et al.*, 1987) and a wide variety of both volatile and semi-volatile compounds were eluted (Kyaw *et al*., 2012). The degradation products of LDPE included alkanes, aromatic compounds, oxygenated chemical compounds predominantly containing aldehydes/ ketones/ esters/ ether groups, long-chain fatty acids, and other unsaturated fatty acids (Kyaw *et al.,* 2012).

Respirometer analyses on evolved gaseous carbon amount in spore suspension-treated LDPE strips incubated under *in vitro* conditions, for 105 days, were indicated in Fig.4.

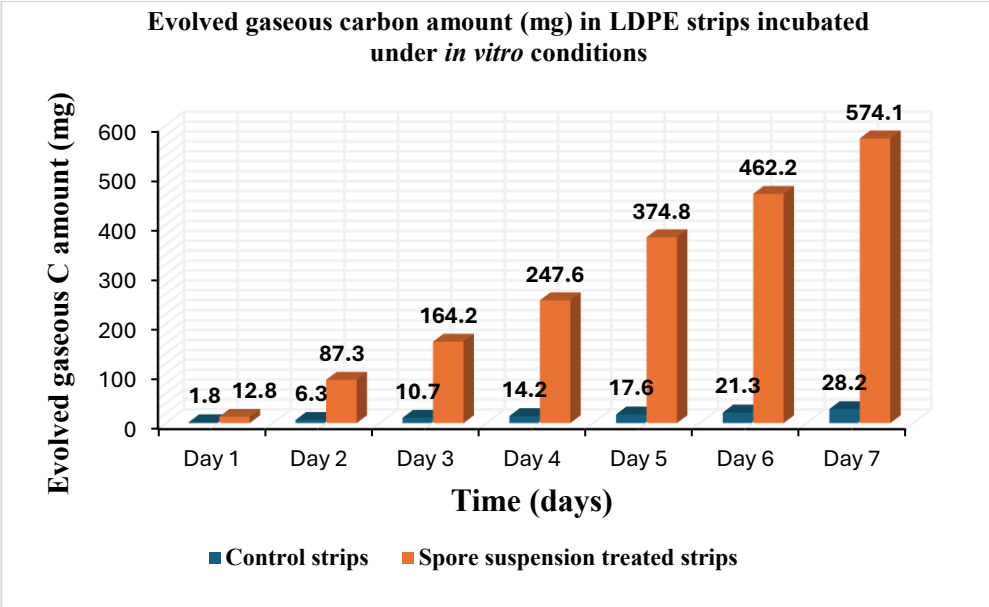


Fig.4. Evolved gaseous carbon amount (mg) in the control and spore suspension treated LDPE strips incubated under *in vitro* conditions for 105 days.

According to the respirometer test results, the amount of gaseous carbon released in spore suspension-treated LDPE strips incubated under *in vitro* conditions for 105 days was 574.1 mg. In control strips, the amount was 28.2 mg, and this could be due to the minor degradation of polymer-based products under the influence of one or more environmental factors, such as heat, light, and chemicals (Matusinovic and Wilkie 2014). Readings of this assay were recorded at 24-hour intervals throughout a period of 7 days, and during the whole period, there were striking increments in the amounts of gaseous carbon evolved in spore suspension-treated LDPE strips compared to controls.

However, enzyme activities were not determined in the present study; therefore, the direct contribution of any particular ligninolytic enzyme to the LDPE degradation cannot be established. The observed degradation is instead supported by the combined physicochemical

and structural evidence obtained in this study. The involvement of ligninolytic enzymes therefore remains a plausible hypothesis based on previous reports for *P. chrysosporium* and should be experimentally verified through targeted enzyme activity assays in future studies.
Afterwards, laboratory-scale soil application was carried out under two set-ups in three replicates in the soil environment. Set-up 1, was instrumental in determining the biodegradation effects of environmental soil microflora on LDPE in waste dump yard soil. Set-up 2, on the other hand, was effective in assessing the combined biodegradation effects of *P. chrysosporium* spore suspension and environmental soil microflora. The greater degradation observed in the spore-treated treatment indicates that application of the *P. chrysosporium* spore suspension enhanced LDPE degradation under the experimental conditions. Percent weight loss, degree of water absorption and percent reduction of tensile strength of LDPE strips after 105 days of incubation in each set-up were recorded in Table 2.

Table 2. Percent weight loss, degree of water absorption and percent reduction of tensile strength of LDPE strips buried in the soil environment.

Set-ups	LDPE strips buried in the soil environment		
	Percent weight loss (%)	Degree of water absorption (%)	Percent reduction in tensile strength (%)
Control	0^{c}	0^{c}	0^{a}
Set-up 1	13.90 ± 0.15^{b}	0.60 ± 0.07^{b}	7.28 ± 4.91^{a}
Set-up 2	20.91 ± 0.20^{a}	1.57 ± 0.09^{a}	15.65 ± 5.29^{a}

Mean of three replicates ± standard error.
Means that share a common superscript letter in each column are not significantly different ($P>0.05$) and means that do not share superscript letters are significantly different ($P<0.05$).

One-way ANOVA with Tukey's multiple comparison test indicated significantly highest percent weight loss and degree of water absorption in set-up 2, while the percent reductions of tensile strength values among set-ups were not significantly different.

FTIR spectroscopy was performed and the percentages of transmittance through LDPE strips were evaluated at signatures corresponding to the functional groups.

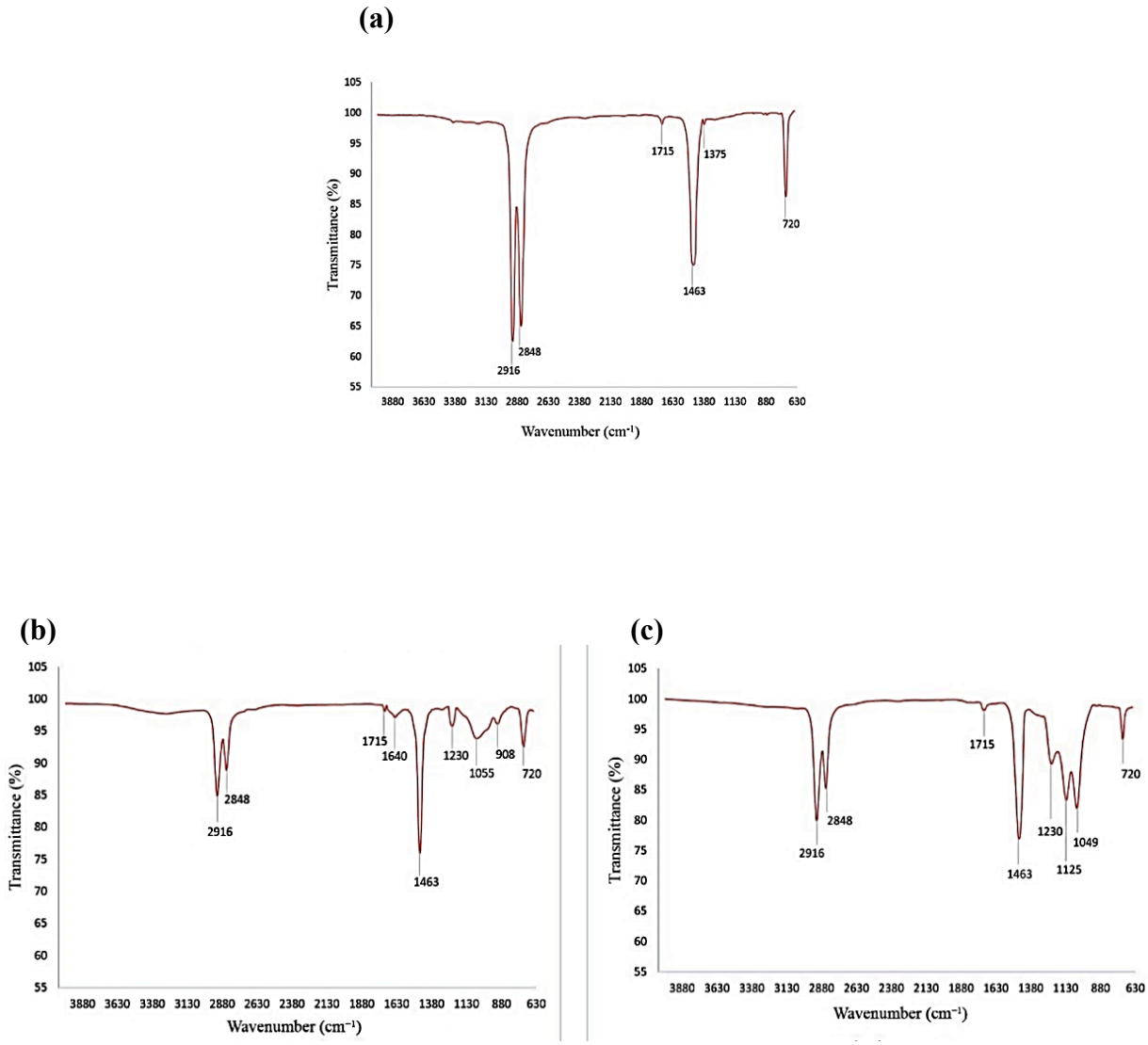


Fig.5. FTIR spectrum for LDPE strips buried in the soil environment (a) control (b) set-up 1 (c) set-up 2

In this study, as shown in Fig.5, notable changes visible in the FTIR of LDPE strips buried in set-up 1 and set-up 2 were formations of new peaks at 900-1200 cm^{-1} region due to the intermediate products of biodegradation of polyethylene, and fluctuations in percentage transmittance of existing peaks in control strips, indicating the evidence for the changes happened during the process of LDPE biodegradation. After the incubation period the carbonyl index values of LDPE strips in both set-up 1 and set-up 2 strips were found to be increased relative to the control strips. The highest percentage increase (10.07%) was observed in set-up 2, whereas it was only 6.05% in set-up 1.

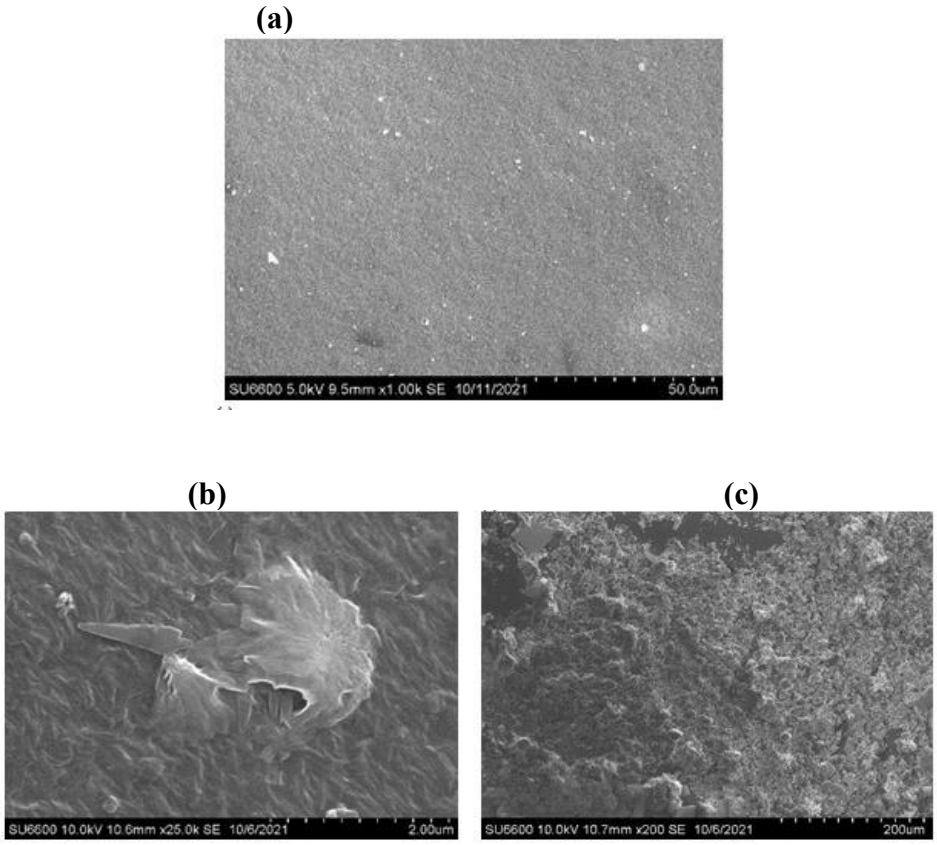


Fig.6. SEM image of LDPE strips buried in the soil environment (a) control (b) set-up 1 (c) set-up 2.

Various structural changes such as pits, cavities, roughening of surface and adsorption of LDPE onto the fungal hyphae were clearly visible in SEM images of both set-up 1 and set-up 2 strips (Fig.6). These changes were present only on a minor scale in the control strips.

The volatile organic compound analysis could improve the condition assessment and monitoring the degradation of LDPE films (Khruengsai *et al.,* 2021). GC-MS scan method was performed to confirm the presence of low molecular weight products, generated during the polyethylene biodegradation, of LDPE strips buried in waste dump yard soil. LDPE strips in control, where the strips were buried in the sterile soil without spore suspension, only showed a few minor peaks with comparatively low abundance values. In set-up 1 and set-up 2, the presence of alkanes, alkenes and several oxygenated compounds was confirmed.

Table 3. GC-MS library report for LDPE strips buried in set-up 1 in soil environment.

Retention Time	Area Percentage (%)	Library/ ID
12.912	1.25	3,3-Dimethylhexane
13.360	1.94	10-Methylnonadecane
14.003	1.39	Phthalic acid, 5-methylhex-2-yl ethyl ester
15.014	2.40	Eicosane 2-Methylhexacosane
15.416	3.28	Tetracosane
16.656	1.45	Hexadecane Cetane Docosane
17.161	11.95	n-Hexadecanoic acid
18.607	4.46	2-Methylhexacosane
20.261	17.79	9-Octadecenamide, (Z)-
20.720	2.88	Phenol,2,2'-methylenebis[6-(1,1-dimethylethyl) -4-methyl-
23.177	17.43	Squalene 4,9,13,17-Tetramethyl-4,8,12,16-octadecatetraenal
28.046	11.86	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)
30.583	21.23	Tris(2,4-di-tert-butylphenyl) phosphate

Table 4. GC-MS library report for LDPE strips buried in set-up 2 in soil environment.

Retention Time	Area Percentage (%)	Library/ ID
12.912	2.73	Hexane, 3,3-dimethyl- Nonane, 5-methyl-5-propyl-
14.003	3.65	Phthalic acid, ethyl 2-methylbutyl ester Phthalic acid, 4,4-dimethylpent-2-yl propyl ester
15.025	3.55	Tetracosane 2-Methylhexacosane
15.438	4.37	Tetradecanoic acid Tridecanoic acid
16.655	1.38	Octacosane 2-Methyloctacosane
17.172	22.59	n-Hexadecanoic acid Pentadecanoic acid

18.607	10.73	Decanedioic acid, didecyl ester
20.157	1.19	Hexacosane Pentacosane
20.433	3.21	Pentacosane
21.501	3.95	Phthalic acid, di(2-propylpentyl) ester
30.606	39.13	7,8,17,18-Tetrahydro-35-methoxy-1,3,21,23-tetramethyl-16H,31H-5,9,15,19-dimethan

Previous researchers also have characterized the ability of several microbial strains to utilize LDPE as a sole carbon source and concluded that different microbes generate different hydrolysis products, during LDPE biodegradation (Montazer *et al*., 2019). In this study also, in set-up 1 and set-up 2, degradation was performed in the presence of different microbes and variations in hydrolysis products were observed. Notable changes visible in the peaks of FTIR spectra obtained for the two set-ups were reconfirmed by the GC-MS analyses, which revealed that the majority of volatile organic compounds (VOCs) present in all extracts were primarily hydrocarbons derived from the polymer chains in LDPE films. These compounds originated from oligomers formed during the polymerization of LDPE. Additionally, some VOCs containing oxygen were produced due to the oxidative degradation of LDPE during incubation (Khruengsai *et al.*, 2021). Some impurities, possibly from the soil environment, such as Tris(2,4-di-tert-butylphenyl) phosphate and 9-Octadecenamide, were also detected in the set-ups.

Based on the consequences of the analyses to confirm the biodegradation of LDPE, a comparative study was made between the two set-ups. Since a significantly higher degree of degradation was achieved in set-up 2, it was concluded that LDPE biodegradation by the environmental soil micro-flora was enhanced due to the application of the *P. chrysosporium* spore suspension. However, the experimental design did not include a heat-killed-spore treatment. Therefore, the present results cannot separate effects associated with viable fungal metabolism from potential abiotic effects or effects associated with non-viable fungal biomass. Future experiments incorporating heat-killed spores, together with appropriate abiotic controls, would allow the contribution of viable fungal activity to be more clearly established.

Afterwards, predominantly detected and morphologically distinct colonies from set-up 2 were isolated into pure cultures. In the present study, a total of 11 morphologically different microbial isolates were commonly and predominantly detected in set-up 2 containers, where LDPE strips were buried in the soil and treated with spore suspension. Isolates were subjected to microbial identification assays using morphological, biochemical approaches and eventually, species confirmation was achieved by comparing gene sequences. The identities of microbial isolates belonging to seven different species were revealed in the current study (three bacteria and four fungi as indicated in Fig.7 and Fig.8).

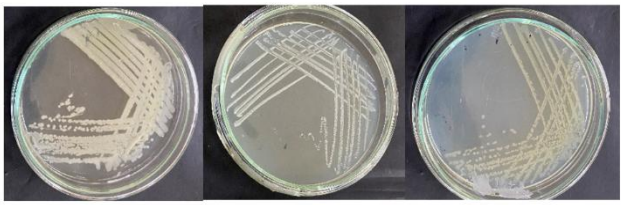


Fig.7. Isolated bacterial colonies grown in pure cultures on NA

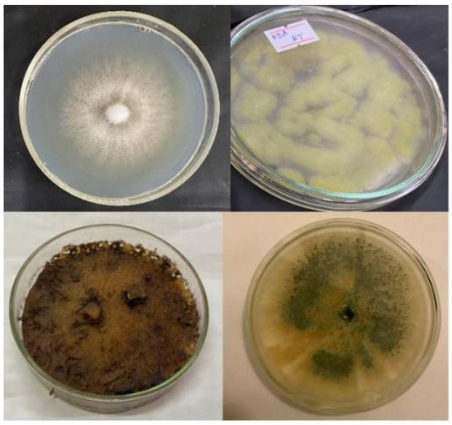


Fig. 8. Isolated fungal colonies grown in pure cultures on PDA.

Traditional microbial identification methods were based on observations of morphology, physiology, and biochemical characteristics. However, these methods often yielded inconsistent results due to variability in appearance and the influence of subjective judgments. The advent of DNA barcoding, which is more reliable and time-efficient, has significantly enhanced microbial identification and the study of microbial diversity (Sarjito *et al.*, 2009). In this study, bacterial species were confirmed by comparing the 16S rRNA gene sequences, while fungal species were confirmed by comparing the nuclear rDNA ITS region sequences with the most closely matching sequences in GenBank. Table 5 includes the GenBank accession numbers of the gene sequences submitted in this study.

Table 5. Bacterial and fungal species isolated and identified from set-up 2 soil.

Strain ID	**Bacteria / Fungi from NCBI BLAST search**	**% Homology**	**GenBank Accession Number**
KLN-N-002	*Phanerodontia chrysosporium* (MZ087900.1)	100.00%	OP422228.1
KLN-N-003	*Trichoderma harzianum* (MT341774.1)	99.82%	OP422229.1
KLN-N-006	*Rossellomorea marisflavi*	99.58%	OP420751.1

	(NR_025240.1)		
KLN-N-005	*Enterobacter cloacae* (NR_044978.1)	94.96%	OP493199.1
KLN-N-104	*Sphingomonas pseudosanguinis* (NR_042578.1)	99.59%	OP420752.1
KLN-N-105	*Mycoleptodiscus* sp. (MW775434.1)	100.00%	OP422230.1
KLN-N- 106	*Aspergillus clavatus* (MH858693.1)	99.47%	OP422231.1

The outcome of this section showed that diverse types of bacteria and fungi have been isolated from the normal flora of waste dump yard soil, and the presence of *P. chrysosporium* (KLN-N-002) among the isolates confirmed the proper germination and activation of spores in the ELF spore suspension, amidst of the natural circumstances. The study revealed that the abundance of bacteria and fungi was consistent with an environment characterized by high species richness and functional diversity. While some bacteria and fungi may have been overlooked, the research provided valuable insights into the culturable, aerobic bacteria and fungi that could coexist with *P. chrysosporium* in the soil environment.

4. Conclusion

This study demonstrated that spore suspension is effective for the biodegradation of LDPE, with significant results observed after a 105-day incubation period. Comparing LDPE strips in soil with and without spore suspension showed that the degradation by natural soil microflora was enhanced by the spore application. Additionally, the isolation of *P. chrysosporium* confirmed successful germination and activity of the spores amidst of the other soil micro-flora. The present study confirmed that the use of *P. chrysosporium* spores as inoculants can enhance LDPE degradation. Hence, it can be used to develop eco-friendly alternative treatment methods to degrade LDPE in the environment.

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Conflict of interest

No conflict of interest was declared.

Supplementary Files: Figure S1 and Figure S2.

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