



Research Paper

Identification of potential fungal degraders of low density polyethylene (LDPE)

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ABSTRACT

Municipal Solid Waste (MSW) is becoming a significant potential threat to the environment in Sri Lanka. Among the constituents of MSW, low density polyethylene (LDPE) is one of the most considerable portions of total waste generated, because it takes a long time to degrade naturally. Hence, this study mainly focused on identifying efficient fungal degraders of LDPE. To isolate the abundant fungi degrading polyethylene, partially degraded polyethylene were collected from different places in Kaduwela area, surface sterilized and placed on streptomycin incorporated PDA. Three fungi were isolated and purified. For biodegradation studies, these fungal isolates were inoculated on streptomycin incorporated ¼ strength potato dextrose agar medium which comprised 20 micron LDPE film and five replicates were used. After 90 days of incubation, gravimetric analysis, microscopic images, Fourier- Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) were conducted to determine whether there exists any potential in degrading LDPE by using these fungal isolates. These fungal isolates were identified using molecular identification tools. Multiple alignments of partial sequence were performed by CLUSTAL W and the phylogenetic was analyzed using MEGA 6.0 software. According to the One-way Analysis of Variance, the mean values for control and *Penicillium* sp. (P=0.000), control and *Fusarium* sp.KC-2010ba (P=0.031) and control and *Penicillium* sp.X9 (P=0.010) were significantly different. Scanning Electron Microscope (SEM) images and light microscopic observations revealed the presence of fungal colonization indicating surface erosion, cracks, folding and firm fungal attachment. The FTIR spectroscopy images of *Fusarium* sp. KC-2010ba and treated LDPE films showed the degradation initiating bonds such as carboxylic and aldehyde bonds. The fungal isolates were identified as *Fusarium* sp. KC-2010ba, *Penicillium* sp. and *Penicillium* sp. X9 by analyzing the ITS region sequencing. Therefore, it can be concluded that the fungal species *Fusarium* sp. and *Penicillium* sp. are capable of degrading and colonizing and has the potential to be developed into an inoculum for expedited LDPE degradation.

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INTRODUCTION

The attention in environmental problems is growing and there are snowballing demands to develop ecofriendly materials which do not burden the environment expressively (Shah et al., 2008). Plastic is a broad name given to different recalcitrant polymers with high molecular weight, which cannot be degraded by biological processes

easily. Among these plastic derivatives, the usage of polyethylene is growing daily with increasing population of the world. As per its degradation, it is one of the most challenges faced by people involved in waste management around the globe and this is as a result of the polyethylene being durable and the time taken for complete natural

degradation is still not known. It has a high degree of short and long chain branching, which means that it does not pack into the crystal structure as well. It has high intra molecular strength and is less tensile. This results in a lower tensile strength and increased durability. However, considering their abundance in the environment and their specificity in attacking plastics, biodegradation of plastics by micro-organisms and enzymes seems to be the most effective and environmental friendly process (Tokiwa et al., 2009).

Low-density polyethylene (LDPE) can be degraded using various methods such as chemical degradation, photo-degradation and biological degradation (da Luz et al., 2013). The microbial species associated with the degradation of polymers have been identified in several types of bacteria (*Pseudomonas*, *Streptococcus*, *Staphylococcus*, *Micrococcus*), fungus (*Aspergillus niger*, *Aspergillus glaucus*, *Fusarium* sp. and *Trichoderma*) are known to be associated with polythene (Swift, 1997). Microbial enzymes are known to enhance the rate of biodegradation of LDPE very effectively without causing harm to the environment. The extracellular enzymes are considered to be too large to penetrate deeply into the polymer material, and as a result act only on the polymer surface and the biodegradation of plastics is usually a surface erosion process. It is observed that UV irradiation (photo-oxidation) and thermal and chemical oxidation of polyethylene prior to its exposure to a biotic environment enhances biodegradation (Shah et al., 2008).

Several countries in the world have deployed micro-organisms for composting processes. However, there are only few applications of biodegradation of polyethylene (Bates, 2005). Most important biological factors are bacteria and fungi. Bacteria includes species of *Bacillus*, *rhodococcus*, *Pseudomonas*, *Klebsiella*, *Streptomyces*, *Micromonospora*, *Mycobacterium* and *Alcaligenes* etc. Some bacteria can get accumulated on the polymer up to 90% of their dry mass (Jayasekara et al., 2005). Fungi that are reported to be involved in the biodegradation process are *Sporotrichum*, *Talaromyces*, *Phanerochaete*, *Ganoderma*, *Thermoascus*, *Thielavia*, *Paecilomyces*, *Thermomyces*, *Geotrichum*, *Cladosporium*, *Phlebia*, *Trametes*, *Candida*, *Penicillium*, *Chaetomium* and *Aerobasidium* (Tuomela et al., 2000).

The carbon-carbon bonds in polyethylene require too much energy to breakdown the polymer structure. Hence, carbon-carbon bonds cannot be readily degraded in the environment by micro-organisms. If so, it takes long time for natural degradation under natural environmental condition, but there are some micro-organisms which can be able to breakdown carbon-carbon bonds in LDPE in low rate as earlier mentioned. If these micro-organisms will be modified by genetic engineering, definitely LDPE can be degraded naturally. Thus, identification of potential LDPE degrading micro-organisms is vital for further development of environmental biodegradation of LDPE.

MATERIALS AND METHODS

Chemicals and polyethylene

All reagents used in this study were of high purity and analytical grade. The LDPE films used in this study were purchased from China, which were 20- μ m-thick layers.

Sample collection

The LDPE films used in this study were collected from Kaduwela polythene dumping site, 1 m depth of soil layers in Homagama and Kaduwela area, in Sri Lanka which were 20 μ m-thick bags. For the biodegradation studies, LDPE films were cut into small squares and sterilized with 70% ethanol. Landfill soil and 1 m depth soil with polythene particles were collected for the isolation of LDPE-degrading micro-organisms.

Isolation of abundant fungal isolates

Triple sterilized polythene strips from selected sites were placed on the antibiotic treated PDA plates under aseptic condition and plates were incubated for 48 h at 37°C in an inverted position. Abundant fungal species were thereafter isolated and purified.

Monitoring of LDPE degradation

A 3.5 cm $\times$ 3.5 cm square 20 micron LDPE (washed with 90% alcohol) was placed on antibiotic treated 25% PDA plate. There are three abundant fungal species inoculated on PDA plates which consisted of polythene under aseptic condition. All the plates were placed at invert position for 90 days incubation period.

Calculation of LDPE degradation

Dry weight of residual LDPE was measured. Thereafter, the treated LDPE films were recovered from the degradation medium and washed with 2% (v/v) sodium dodecyl sulfate (SDS) solution and further rinsed with distilled water (Orr et al., 2004). The washed LDPE film was dried overnight at 40°C before weighing and the percentage of weight loss determined using the formula as described by Kyaw et al. (2012).

Microscopic analysis

Electron microscopy

After incubation, the cover slip was carefully dried and

placed on a glass slide containing a drop of water. The prepared slide was observed under the high power (10 × 40) of the light microscope and photographs were taken.

Scanning electron microscopy

The treated samples after 90 days of incubation with UY1, UB1 and UY2 were subjected to SEM analysis after washing with 2% (v/v) aqueous SDS and distilled water for few minutes and wiped with 70% ethanol to remove the adhered cells (Orr et al., 2004). The samples were analyzed by using High Resolution Scanning Electron Microscope.

FTIR (Fourier- Transform Infrared Spectroscopy) analysis

FTIR spectroscopy is a technique used to obtain an infrared spectrum of absorption of a solid, liquid or gas. FTIR analysis provides high resolution images about the bonds of a solid, liquid or gas. The changes in the polymer bond of *Fusarium* sp.KC-2010ba treated LDPE film were determined using FTIR spectrophotometer.

Molecular taxonomic identification of selected fungal isolates using ITS region gene sequencing

The emerging fungal mycelia were inoculated in potato dextrose broth for 24 h days under 100 rpm at 30°C. Few of the grown mycelia (1.0 g) were selected and put into 300 µl extraction buffer [200 mM Tris- HCl (pH 8.3), 20 mM NaCl, 25 mM EDTA (pH 8.3) and 1% SDS] containing Eppendorf tube and samples were incubated under room temperature for 72 h. Mycelia were crushed using sterilized blunt pipette tip. All the DNA samples were centrifuged at 12000 rpm for 5 min. A 150 µl of sodium acetate was added. Thereafter, the samples were centrifuged at 10000 rpm for 5 min. The supernatant was pipetted out into new tube containing 500 µl ice cold isopropanol. The samples were centrifuged in 4°C at 12000 rpm for 1 min. Then the supernatant was discarded and the pellet air dried for 30 to 45 min. The pellet was dissolved in 50 µl of TE buffer (10 mM Tris-HCl, 1mM EDTA at pH 8.0).

PCR was conducted to amplify the internal transcribed spacer (ITS) region of the extracted DNA, using the primers ITS 1 and ITS 4 under the following conditions: 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 54°C for 1 min, 72°C for 2 min, and final extension at 72°C for 7 min. PCR amplicons were electrophorized in 1.2% agarose gel. The amplified PCR products were sequenced by Macrogen, Korea. A BLAST (Basic Local Alignment Search Tools) was used to search for closest match sequences in the GenBank database and selected fungal isolates identified. Multiple alignments of partial sequence were performed by

CLUSTALW and the phylogenesis was analyzed using MEGA 6.0 software by using the sequences of other fungal degraders. An un-rooted tree was built using the neighbor-joining method (Tamura et al., 2007).

RESULTS AND DISCUSSION

Biodegradation studies

After incubation of 90 days, the degrading capacity of the fungal isolates were analyzed using various parameters and the results were interpreted.

Weight loss measurements

A simple and quick way to measure the biodegradation of polymers is the weight loss. Micro-organisms utilized the polymer and this process led to the polymer integrity degradation. The reduction of weight loss of LDPE was observed after 90 days of incubation period because biodegradation is usually proportional to the weight loss of polymer. The fungal isolate *Fusarium* sp. KC-2010ba showed 0.59%, *Penicillium* sp. X9 showed 0.36% and other *Penicillium* sp. showed 0.35% of weight loss after 90 days of plate culture method incubation period whereas the control did not show any weight loss. According to the One-way Analysis of Variance, the mean values for control and *Penicillium* sp. (P=0.000), control and *Fusarium* sp.KC-2010ba (P=0.031) and control and *Penicillium* sp.x9 (P=0.010) were significantly different.

In this study, weight loss measurements were not sufficient parameters to evaluate the fungal degradation as compared to previous studies. It could be mainly due to the difference in experimental set-up and inadequate fungal concentration during the incubation period. Because most of the early studies focus on the biodegradation of LDPE using broth cultures but here solid plate culture technique was applied.

Microscopic analysis

After the initial degradation, crystalline spherulites appeared on the surface of LDPE films and was observed using the light microscope. This can be explained by a preferential degradation of the amorphous polymer fraction, etching the slower- degrading crystalline parts out of the material (Kikkawa et al., 2002) (Figure 1).

SEM analysis was used to confirm that the surface of LDPE becomes physically weak after the biological treatment (Figure 2). The fungal colonization was observed on the surface of LDPE by SEM after 90 days of incubation. LDPE films exposed to *Fusarium* sp. KC-2010ba, *Penicillium* sp. and *Penicillium* sp. X9 showed surface erosion, cracks,

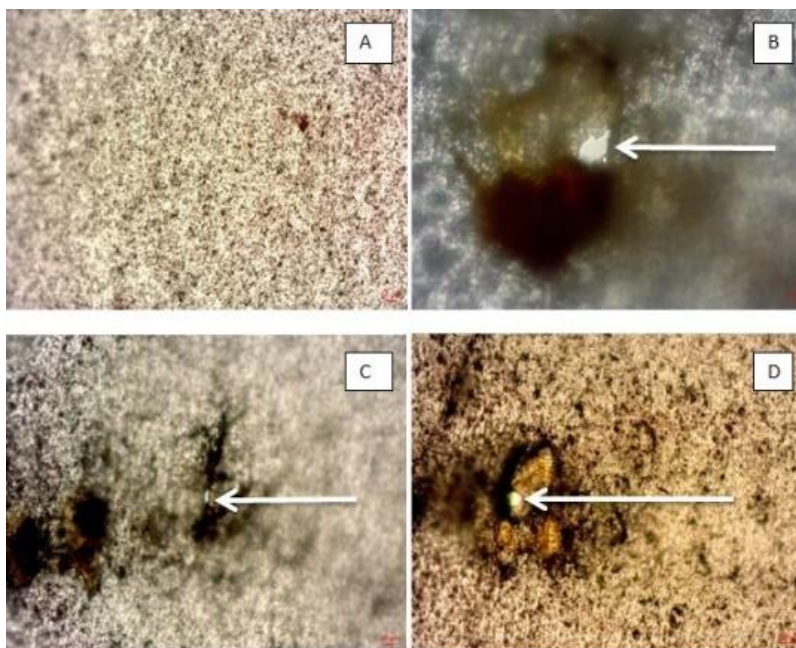


Figure 1: Light microscopic observation of LDPE films. (A) control (B) *Penicillium* sp. x9 (C) *Penicillium* sp. x9 (D) *Fusarium* sp.KC-2010ba treated LDPE film; arrow keys show the surface fractions on fungal treated LDPE films (10 × 40).

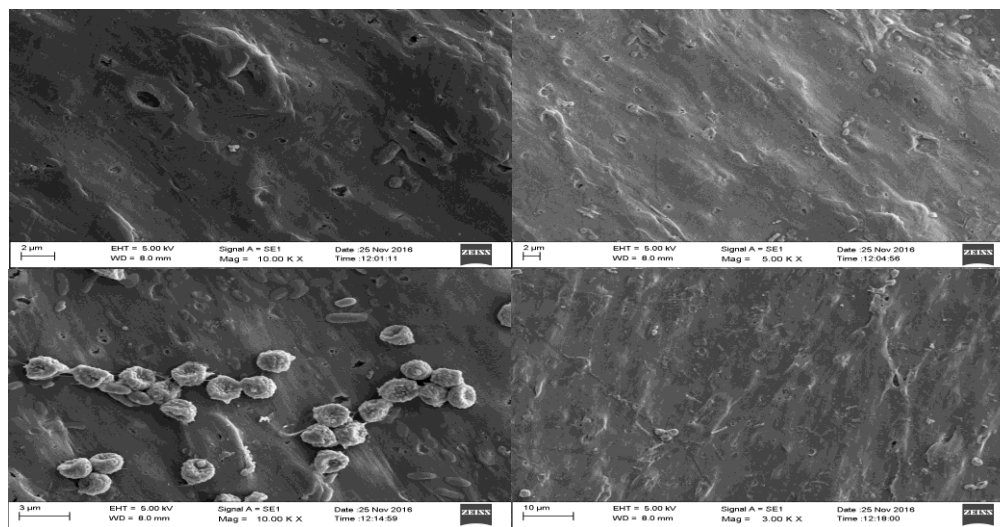


Figure 2: SEM images of LDPE films of *Penicillium* sp. x9 treated (upper left and upper right) and *Fusarium* sp.KC-2010ba treated (upper left and upper right) LDPE film.

folding and fungal colonization. This may be due to the extracellular metabolites and enzymes released by the fungus in response to the stress because $\frac{1}{4}$ strength PDA medium may not consist of enough nutrients for 90 days incubation.

Mass loss is primarily determined by the surface erosion of LDPE. As a cross reference to the earlier studies on the biodegradation of LDPE, many researchers have reported the same morphological changes on LDPE degradation by

Aspergillus sp. (Volke-Seplveda et al., 2002).

Spectroscopic analysis

The molecular structure analysis is one of the major parameters used to identify and clarify the molecular changes during the LDPE degradation which is responsible for weight reduction. FTIR is sensitive in determining the

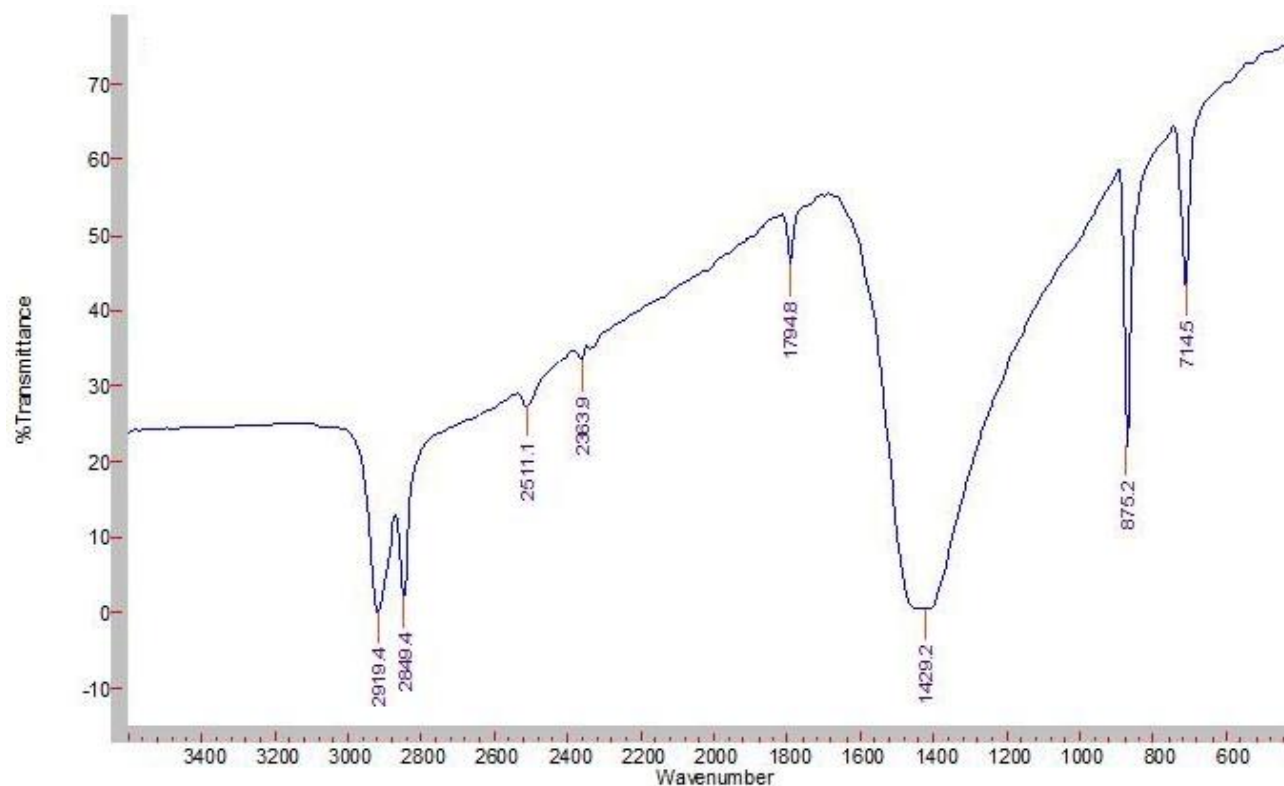


Figure 3: FTIR spectrum of degraded LDPE film after 90 days with *Fusarium* sp.KC-2010ba inoculation.

Table 1: Identity of the fungal isolates of LDPE degradation.

Unknown sample code	Identification with BLAST	Accession number
UY1	<i>Penicillium</i> sp.	-
UB1	<i>Penicillium</i> sp. x9	KJ958367.1
UY2	<i>Fusarium</i> sp. KC-2010ba strain	JQ364970.1

Note: Accession number is yet to be received.

molecular arrangement of interactions with macromolecules during the degradation process (Gajendiran et al., 2016) (Figure 3).

FTIR analysis of the LDPE films of *Fusarium* sp.KC-2010ba treated gave close view of C-H stretching alkane groups at 2919.4 and 2849.4 cm^{-1} , O-H carboxylic groups at 2511.1 cm^{-1} , C=O anhydride group at 1794.8 cm^{-1} , bending CH_3 bond at 1429.2 cm^{-1} , out of plane bending aromatic group at 875.2 cm^{-1} , out of plane bending alkene group at 714.5 cm^{-1} and carbonyl absorption bands in addition to an ammonium band at 2363.9 cm^{-1} (Figure 3). The band attributed to O-H carboxylic group and carbonyl absorption bands in addition to ammonium bands were slightly present. Hence, this FTIR analysis revealed that the *Fusarium* sp.KC-2010ba has a potential to initiate the degradation of LDPE film.

Molecular taxonomic identification of selected fungal isolates using ITS region gene sequencing

The fungal isolates used for LDPE degradation were identified by analyzing the ITS region sequences of each fungal isolate. The sequences which revealed a maximum identity and a query cover were considered when identifying the unknown samples. Table 1 shows the identification of the unknown fungal isolates and the accession numbers obtained from NCBI.

Phylogenetic and molecular evolutionary analyses were conducted by MEGA 6.0 software with the neighbor-joining algorithm. The phylogenetic tree was constructed using the sequences of other fungal degraders which were previously found (Figure 4). According to the results obtained from phylogenetic tree, *Penicillium* sp. X9 and *Fusarium* sp.

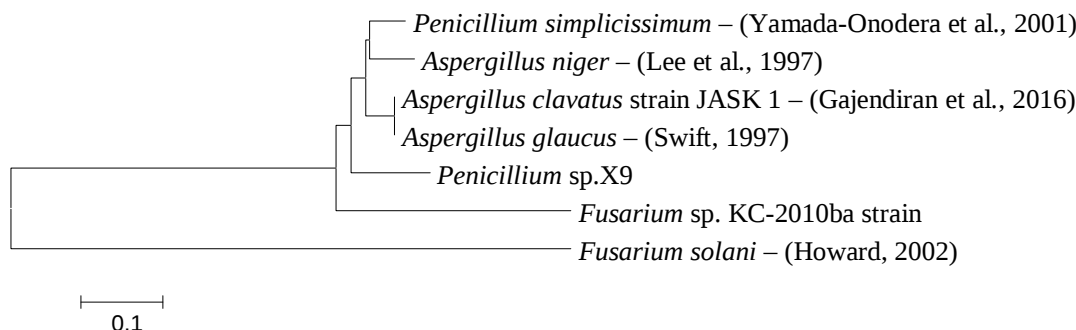


Figure 4: Phylogenetic relationship of *Fusarium* sp. KC-2010 ba strain and *Penicillium* sp. X9 with existing polyethylene fungal degraders (Gajendiran, Krishnamoorthy and Abraham, 2016) (Kim et al., 1997) (Yamada-Onodera et al., 2001) (Swift, 1997) (Howard, 2002).

KC-2010 ba strain have an evolutionary relationship with other fungal degraders.

Conclusion

After 90 days of incubation, the mean values for control and fungal treatments were significantly different. Scanning Electron Microscope (SEM) images and light microscopic observations revealed the presence of fungal colonization indicating surface erosion, cracks, folding and firm fungal attachment. The images obtained from FTIR spectroscopy showed that the initial degradation process has started. The fungal isolates were identified as *Fusarium* sp. KC-2010ba, *Penicillium* sp. and *Penicillium* sp. X9 by analyzing the ITS region sequencing. Therefore, it can be concluded that the fungal species *Fusarium* sp. and *Penicillium* sp. are capable of degrading the Low Density Polyethylene (LDPE) and has the potential to be developed into an inoculum for expedited LDPE degradation.

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