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IDENTIFICATION OF POTENTIAL FUNGAL DEGRADERS OF LOW-DENSITY POLYETHYLENE (LDPE)

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ABSTRACT

Low Density Polyethylene (LDPE) is one of the most important constituents of waste generated, since the time taken for its natural degradation is long. Therefore, this study mainly focused on identifying efficient fungal degraders of LDPE. Partially degraded polyethylene was collected from different places in Kaduwela area, Sri Lanka to isolate the abundant fungi in degrading polyethylene. For biodegradation studies, these fungal isolates were inoculated on streptomycin incorporated potato dextrose agar (PDA) medium which comprised 20-micron LDPE film. After 90 days of incubation, gravimetric analysis, light microscopy, Fourier- Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) were conducted to determine the potent of these fungal isolates in degrading LDPE. These fungal isolates were identified by sequencing internal transcribed spacer region (ITS). Multiple alignments of partial sequences were performed by CLUSTAL W and the phylogeny was analyzed using MEGA 6.0 software. According to the one-way Analysis of Variance, the mean values of weight loss of control and Aspergillus niger isolate PS3. (P=0.000), control and Fusarium sp. isolate PS3 (P=0.031) and control and Penicillium sp. isolate PS2 (P=0.010) were significantly different. Scanning electron microscope images and light microscopic observations showed the presence of fungal colonization and surface erosion, cracks, folding and firm fungal attachment. The FTIR spectroscopy images of Fusarium sp. isolate PS3 treated LDPE films showed the early stage of degradation by initiating bonds such as carboxylic bonds and aldehyde bonds. The fungal isolates were identified as Fusarium sp. isolate PS3, Penicillium sp isolate PS2 and Aspergillus niger isolate PS3 by analyzing the ITS region sequencing. Therefore, it can be concluded that the fungal species, Fusarium sp., Aspergillus sp. and Penicillium sp. are capable of colonizing on LDPE films and has the potential to be developed into an inoculum for expedited LDPE degradation.

Keywords: Biodegradation, Low Density Polyethylene, *Penicillium*, *Fusarium*, *Aspergillus*

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1.0 INTRODUCTION

The attention in environmental problems is growing and there are rising demands to develop ecofriendly material which do not harm the environment expressively. Plastic is a broad name given to different recalcitrant polymers with high molecular weight, which cannot be degraded by biological processes easily [1]. Among these plastic derivatives, the usage of polyethylene is growing day by day with the population growth of the world [2]. Degradation of polythene is one of the big challenges faced by people who are involved in the waste management of the globe since the polyethylene is 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 do 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 increase the durability [3]. However, considering their abundance in the environment and their specificity in attacking plastics, biodegradation of plastics by microorganisms and enzymes seems to be an effective and environmental friendly process [4].

Low-density polyethylene (LDPE) can be degraded in various methods as follows: chemical degradation, photo-degradation and biological degradation [5]. The microbial species associated with the degradation of polymers are bacteria (*Pseudomonas, Streptococcus, Staphylococcus, Micrococcus*) and fungi *(Aspergillus niger, Aspergillus glaucus, Aureobasidium sp. Fusarium sp. Trichoderma* sp.) [6]. Microbial enzymes are known to enhance the rate of biodegradation of LDPE very effectively without causing any harm to the environment. The extracellular enzymes are considered to be too large to penetrate deeply into the polymer material and as a result, they act only on the polymer surface and the biodegradation of plastics is usually a surface erosion process. It is found that UV irradiation (photo-oxidation), thermal and chemical oxidation of polyethylene prior to its exposure to a biotic environment enhances biodegradation [7].

Several countries in the world have deployed microorganisms for composting processes. However there are only few applications of biodegradation of polyethylene [8]. Most important biological factors are bacteria and fungi. Bacteria includes species *of Bacillus, Rhodococcus, Pseudomonas, Klebsiella, Streptomyces, Micromonospora* and *Mycobacterium*. Some bacteria can get accumulated on the polymer up to 90 % of their dry mass [9]. Fungi that are reported to be involved in the biodegradation process are nearly *Sporotrichum, Talaromyces, Phanerochaete, Ganoderma, Thermoascus, Thielavia, Paecilomyces, Thermomyces, Geotrichum, Cladosporium, Phlebia, Trametes, Candida, Penicillium, Chaetomium and Aerobasidium* [6]. The carbon-carbon bonds in polyethylene require a huge amount of energy to breakdown the polymer structure. So carbon-carbon bonds cannot be readily degraded in the environment by microorganisms [10]. Therefore, it takes a long time for natural degradation under natural environmental condition. However, there are some microorganisms which are able to breakdown carbon-carbon bonds in LDPE in low rates as above mentioned. Genetically engineered microorganisms with high rates of degradation will be a solution to address this limitation. Thus, identification of potential LDPE degrading microorganisms is vital for further development of environmental biodegradation of low-density polyethylene.

2.0 MATERIALS AND METHODOLOGY

2.1 Chemicals and polyethylene

All reagents used in this study were of high purity and analytical grade. The LDPE films of 20 µm thickness (Polystar Machinery (Pvt.) and India) were used in this study.

2.2 Sample collection

The LDPE films used in this study were collected from Kaduwela and Homagama polythene dumping sites in Sri Lanka. For the biodegradation studies, purchased LDPE films were cut into small squares (2cm x 2cm). The cut squares sterilized with 70 % ethanol and placed on square per plate.

2.3 Isolation of abundant fungal isolates

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

2.4 Monitoring of LDPE degradation

A 3.5cm x 3.5cm square 20-micron LDPE (washed with 90 % alcohol) was placed on streptomycin treated 25 % PDA plate. Three abundant fungal species (0.1 mL of 1x103spores/mL) were inoculated on PDA plate and sterilized polythene under aseptic condition. All the fifty replicates were placed invert position for 90 days' incubation period at 37 °C.

2.5 Calculation of LDPE degradation

Dry weight of residual LDPE was measured. Then the treated LDPE films were recovered from the degradation medium and they were washed with 2 % (v/v) sodium dodecyl sulfate (SDS) solution and further rinsed with distilled water [11]. The washed LDPE film was dried overnight at 40 °C before weighing and the percentage of weight loss was determined [12].

2.6 Microscopic analysis

Electron microscopy

After incubation, the cover slip was carefully placed on a glass slide containing a drop of water with Lacto Phenol Cotton Blue indicator. The prepared slide was observed under the high power (10x40) of the light microscope (model, company, country) and photographs were taken.

Scanning electron microscopy

The treated samples after 90 days of incubation with fungal isolates named as UY1, UB1 and UY2 were subjected to SEM analysis after washing with 2 % (v/v) aqueous SDS followed by distilled water for few minutes and wiped with 70 % ethanol to remove the adhered cells [11]. The samples were analyzed by using high resolution scanning electron microscope (ZEISS, Germany).

FTIR (Fourier- Transform Infrared Spectroscopy) analysis

The changes in the polymer bond of LDPE film were determined using FTIR spectrophotometer (Company and Country).

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

Grown mycelia (1.0g) were collected by scraping the mycelia and put into 300µl extraction buffer [200mMTris- HCl (pH 8.3), 20mM NaCl, 25mM EDTA (pH 8.3) and 1 % SDS] containing eppondorf tube and samples were incubated under room temperature for 72 hours. Mycelia were crushed using sterilized blunt pipette tip. All the DNA samples were centrifuged at 12000 rpm for 5 minutes. A 150 µl of sodium acetate was added. After that, the samples were centrifuged at 10000 rpm for 5 minutes. The supernant was pipetted out into new tube containing 500µl ice cold isopropanol. The samples were centrifuged in 4 °C at 12000 rpm for 1 minute. Then the supernant was discarded and pellet was air dried for 30- 45 minutes. Afterwards the pellet was dissolved in 50 µl of TE buffer (10mM 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 sent to Macrogen, Korea for sequencing. A BLAST (Basic Local Alignment Search Tools) was used to search for closest match sequences in the GenBank database. Multiple sequence alignments of partial sequences were performed by CLUSTALW and the phylogeny was created using MEGA 6.0 software by using the sequences of other identified fungal degraders. An un-rooted tree was built using the neighbor-joining method [13].

3.0 RESULTS AND DISCUSSION

3.1 Weight loss measurements

A simple and quick way to measure the biodegradation of polymers is the weight loss[14] Microorganisms utilize the polymer and this process leads to the polymer integrity degradation [15] 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. isolate PS3 showed 0.59 %, *Penicillium* sp. isolate PS2 showed 0.36 % and other Aspergillus *niger* isolate PS3 showed 0.35 % of weight loss after 90 days of incubation period whereas control did not show any weight loss. According to the one-way Analysis of Variance of fifty replicates and two trials, the mean values for control and Aspergillus *niger* isolate PS3 (P=0.000), control and *Fusarium* sp. isolate PS3 (P=0.031) and control and *Penicillium* sp. isolate PS2 (P=0.010) were significantly different[14] conducted the study on biodegradation of LDPE under laboratory conditions, the isolates are A. *niger*, A. *japonicas*, A. *terreus*, A. *flavus* and *Mucor* sp. from polluted soil sample, among these strains *Aspergillus japonicas* efficiently degraded 11.11 % of in 1 month of incubation time and A. *niger* degraded. 5.8 % in 1 month.

But in this study weight loss measurements were not sufficient parameter to evaluate the fungal degradation compared to previous studies. It could be mainly due to the difference in experimental

setup and inadequate fungal concentration during the incubation period. Because most of the early studies focus on the biodegradation of LDPE using broth cultures but in here solid plate culture technique was applied.

3.2 Microscopic analysis

After the initial degradation, crystalline spherolites appeared on the surface of LDPE films that could be observed by the light microscope (Figure 1). That can be explained by a preferential degradation of the amorphous polymer fraction, etching the slower- degrading crystalline parts out of the material [16].

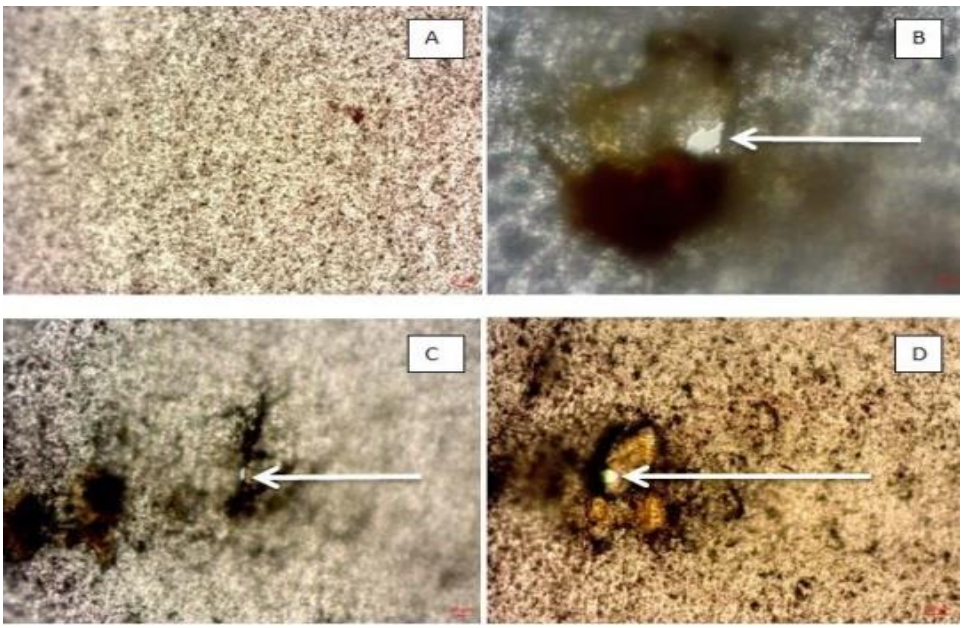


Figure 1: Light microscopic observation of LDPE films. (A) control; (B*) Aspergillus niger* isolate PS3; (C*) Penicillium* sp. isolate PS2; (D) *Fusarium* sp. isolate PS3 treated LDPE film; arrow keys show the surface fractions on fungal treated LDPE films (10x40).

SEM analysis was used to confirm that the surface of LDPE becomes physically weak after the biological treatment. The fungal colonization was observed on the surface of LDPE by SEM after 90 days of incubation (Figure 2). LDPE films exposed to *Fusarium* sp. isolate PS3, Aspergillus *niger* isolate PS3. and *Penicillium* sp. isolate PS2 showed surface erosion, cracks, folding and fungal colonization (Figure 2). This may be due to the extracellular metabolites and enzymes released by the fungus in response to the stress because ¼ strength PDA medium may not consist of enough nutrients for 90 days incubation [17].

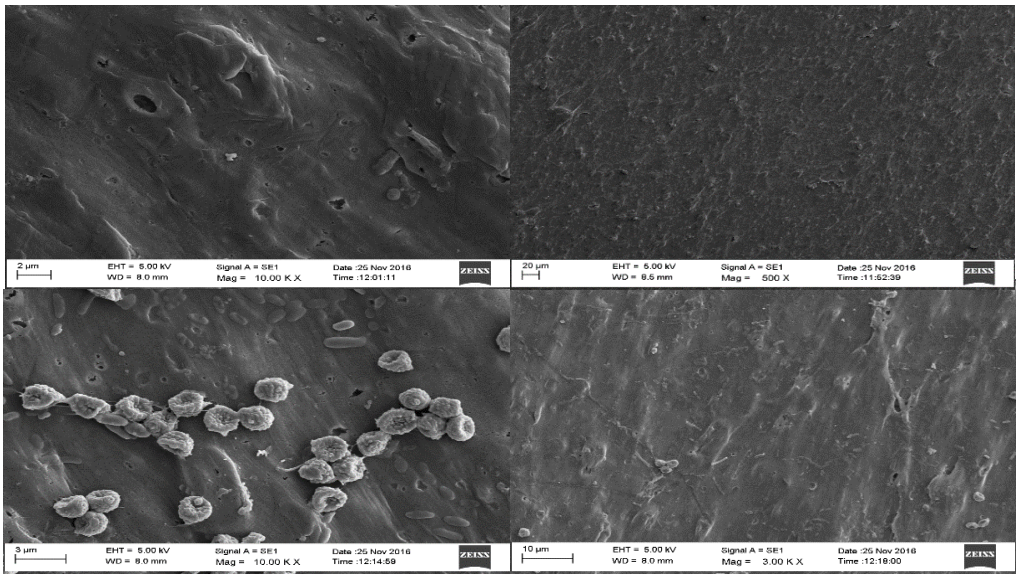
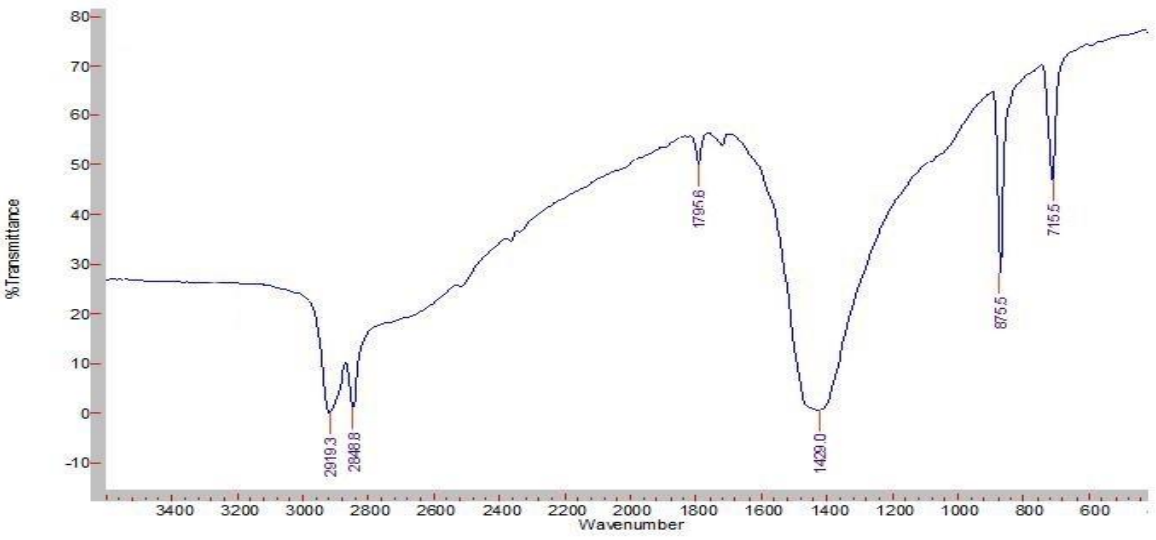


Figure 2: SEM images of LDPE films of *Penicillium* sp. isolate PS2 treated (upper left) control sample (upper right) and *Fusarium* sp. isolate PS3 treated (upper left and upper right) LDPE film (10.00 K x).

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 such as fungal colonization, cracks and holes on LDPE degradation by *Aspergillus* sp. [18].

3.3 Spectroscopic analysis

The molecular structure analysis is one of the major parameters to identify and clarify the molecular changes during the LDPE degradation which is responsible for weight reduction. FTIR is sensitive to determine the molecular arrangement of interactions with macromolecules during the degradation process [19].



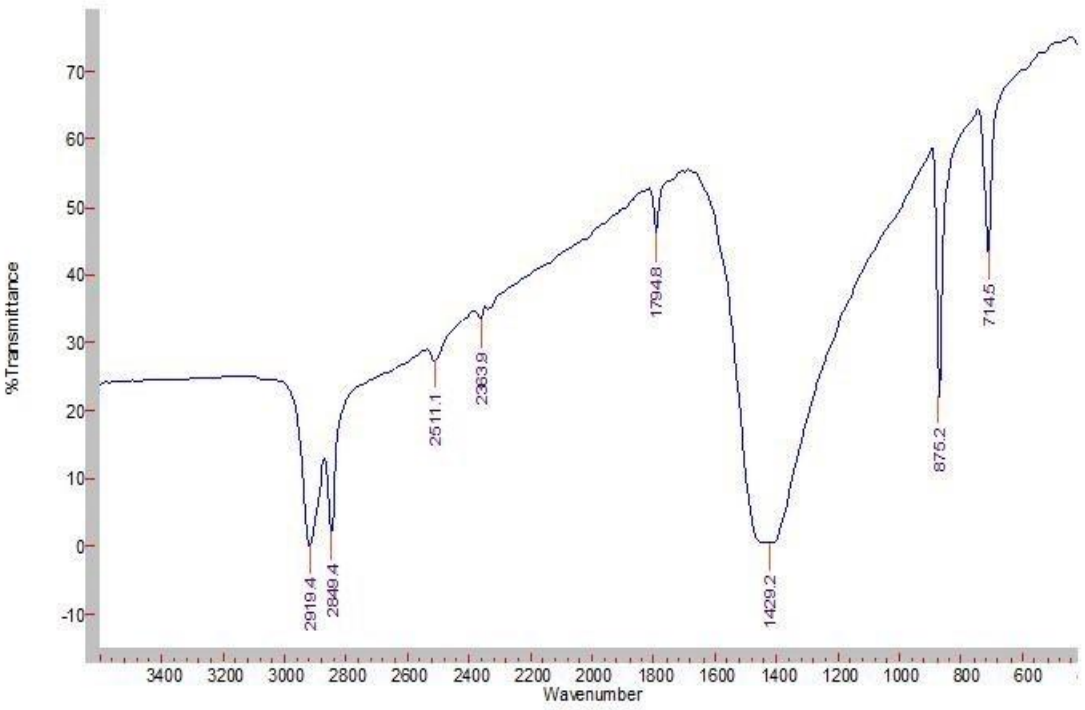


Figure 3: FTIR spectrum of degraded LDPE film after 90 days (A) without any inoculation; (B) with *Fusarium* sp. isolate PS3 inoculation.

FTIR analysis of the LDPE films of *Fusarium* sp. isolate PS3 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 875.2cm^{-1}, out of plane bending alkene group at 714.5cm^{-1} and carbonyl absorption bands in addition to an ammonium band at 2363.9 cm^{-1}. The band attributed to O-H carboxylic group and carbonyl absorption bands in addition to an ammonium bands were slightly present. However, the control sample showed only C-H stretching alkane groups at 2917.9 and 2848.9 cm^{-1} bending CH_3 bond at 1430.3 cm^{-1}, out of plane bending aromatic group at 875.5 cm^{-1} and out of plane bending alkene group at 716.3 cm^{-1}. [20] and [21] examined the same results in their studies and noticed the formation of new functional groups in their biodegradation studies by using *Bacillus amyloliquefaciens* strain and combination of different bacterial and fungal species. The differences in the peak value of functional groups also support the initiation of structural changes on LDPE. Hence, this FTIR analysis revealed that the *Fusarium* sp. isolate PS3 has a potential to initiate the degradation of LDPE film.

3.4 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 showed a maximum identity and a query cover were considered when identifying the unknown samples. Identification of the unknown fungal isolates and the accession numbers obtained from NCBI are presented in Table 1.

Table 1: Identity of the fungal isolates of LDPE degradation

Unknown sample code	Identification with BLAST	Accession Number
UY1*	*Aspergillus niger* isolate PS3	MN 235833
UB1	*Penicillium* sp. isolate PS 2	MN 148618
UY2	*Fusarium sp.* isolate PS3	MN 238763

Phylogenetic and molecular evolutionary analyses were conducted by MEGA 6.0 software with the neighbor-joining algorithm. The phylogenetic tree was constructed by using the sequences of other fungal degraders which were previously found (Figure 4).

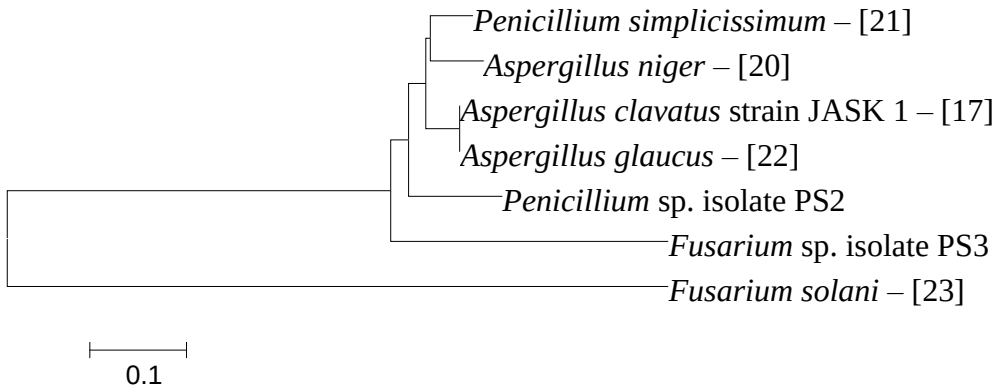


Figure 4: Phylogenetic relationship of *Fusarium* sp. isolate PS3 and *Penicillium* sp. isolate PS2 with existing polyethylene fungal degraders [19];[22]; [23];[24];[25].
Note: Bar represent the sequence divergence.

The previous studies conducted by [17] performed a comparative analysis between nine different Aspergillus sp. to identify the evolution relationship of Aspergillus *clavatus* strain JASK 1 with other isolated species. According to the results obtained from this study, Fusarium sp. isolate PS3 and *Penicillium* sp. isolate PS2 also clustered with other existing fungal degraders in evolution. (Esmaeili *et al*., (2013) reported that the soil microorganism such as *Aspergillus* sp., and *Lysinibacillus* sp were able to degrade LDPE efficiently and [26] showed that mangrove soil microorganisms including *Aspergillus* sp., *Fusarium* sp. and *Penicillium* sp. also degrade polythene. Hence, *Fusarium* sp. isolate PS3, *Aspergillus niger* isolate PS3 and *Penicillium* sp. isolate PS2 have a capability to degrade LDPE when the conditions are optimum for the degradation.

4.0 CONCLUSION

This article was conducted to identify the potential fungal degraders in Kaduwela area, Sri Lanka and assumed that the isolated fungal species have capability to degrade LDPE films within 90 days of incubation. The mean values for control and fungal treatments were significantly different. Scanning electron microscope images and light microscopic observations were shown the presence of fungal colonization indicating surface erosion, cracks, folding and firm fungal attachment. The images obtained from FTIR spectroscopy also showed that the initial degradation process has been started on the treated LDPE films. The fungal isolates were identified as *Fusarium* sp. isolate PS3, Aspergillus *niger* isolate PS2 and *Penicillium* sp. isolate PS2 by analyzing the ITS region sequencing. To the best of our knowledge that there were no supporting articles related to *Fusarium* sp. isolate PS3 and *Penicillium* sp. isolate PS2 on degradation of LDPE. Therefore, it can be concluded that the fungal species *Fusarium* sp., *Aspergillus* sp. and *Penicillium* sp. qualify as potential candidate to degrade LDPE.

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