Environ. Eng. Res. 2021; 26(3): 200167
https://doi.org/10.4491/eer.2020.167

pISSN 1226-1025
eISSN 2005-968X

Research

Identification and characterization of low density polyethylene-degrading bacteria isolated from soils of waste disposal sites

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ABSTRACT

The current study focused on an environment friendly method for degradation of Low Density Polyethylene (LDPE) using bacteria. A total of 36 bacterial strains were isolated from waste disposal sites in which six strains showed potential biodegradation activities. In this study, we reported 2 new strains i.e. *Bacillus siamensis* and *Bacillus wiedmannii* for LDPE degradation. The percent weight loss of LDPE films for isolates was; *B. siamensis* (8.46 ± 0.3%), *B. cereus* (6.33 ± 0.2%), *B. wiedmannii* (5.39 ± 0.3%), *B. subtilis* (3.75 ± 0.1%), *P. aeruginosa* (1.15 ± 0.1%) and *A. iwoffii* (0.76 ± 0.1%) after 90 d of incubation. The LDPE films showed slight surface disruption as observed in Field Emission Scanning Electron Microscopy (FE-SEM) and Fourier Transform Infrared Spectroscopy (FTIR) showed formation of typical carbonyl peaks which were markedly reduced after incubation as measured by carbonyl index. The X-Ray Diffraction (XRD) analysis presented an increase in percent crystallinity and there was no apparent change in total carbon percentage. Different genes responsible for degradation of LDPE like Laccase (167 bp), Alk1 (330 bp) and Alk2 (185 bp) were identified in bacterial isolates and further sequenced. The low degradation values in this study indicate that LDPE degradation is a slow, continuous and time dependent process.

Keywords: *Bacillus siamensis*, *Bacillus wiedmannii*, Biodegradation, Low Density Polyethylene

1. Introduction

Polyethylene (PE) is man-made polymers used in many aspects of human life. It is widely used due to its light weight, inexpensive, strong and durable nature. The worldwide production rate of synthetic polymers is about 140 million tons and its utility is increasing at the rate of 12% per annum [1]. Approximately 500 billion to 1 trillion Low Density Polyethylene (LDPE) bags are consumed annually around the globe, this enormous production and utilization of LDPE leads to their accumulation in the environment. Since PE is characteristically resistant to natural degradation, due their strong C-H and C-C bond, their disposal evokes a big ecological issue and is one of the leading sources of pollution [2]. Thus the rapid biodegradation of plastics has been a subject of interest as the disposal strategies are critical and need attention as it makes up to 40% of total municipal waste [3]. Moreover, oceans are getting contaminated with plastic wastes and marine animals by chance, ingest the plastics leading to their death [4].

Different methods are used for the degradation of LDPE including physical, chemical and biological methods. The physical and chemical methods are expensive, producing toxic wastes which in turn pollute the environment. On the other hand, biodegradation by microorganisms have become the focus of interest for eco-friendly disposal of plastics. Biodegradation is the result of utilization of a polymer as a carbon source by the microorganisms. These microorganisms produce extracellular enzymes which lead to chain cleavage of polymer into small monomers and oligomers. These are taken up by microbial cells and are metabolized into water and carbon dioxide [5, 6].

Several microorganisms have been reported to degrade LDPE, however, its high molecular weight, hydrophobic nature and lack of functional groups, recognized by microbial enzymes makes it adverse towards degradation [7]. Different treatments like Ultra Violet (UV), thermal and chemical, leads to the oxidation of the

Received March 31, 2020 **Accepted** June 24, 2020

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polymer surface and decreases the hydrophobicity of the surface favoring the microbial degradation [8]. The biodegradation assay was performed with variable range of cultural conditions. Different studies reported an in-vitro biodegradation of LDPE films in liquid culture system for 40-120 d of incubation at 30-37℃ and 120-180 rpm [2, 8-12].

Microorganisms such as *Brevibacillus* spp, *Aneurinibacillus* spp [13], *Bacillus cereus*, *Brevibacillus borstelensis* [14], *Pseudomonas aeruginosa* [15] and *Enterobacter* spp [16] have been reported in recent studies to degrade LDPE. Other studies showed *Acinetobacter baumannii*, *Micrococcus luteus*, *Bacillus* spp and *Staphylococcus* spp [17], *Bacillus vallismortis*, *Pseudomonas protegens*, *Stenotrophomonas* spp and *Paenibacillus* spp [18] to be involved in biodegradation. Various studies have been done to investigate the efficacy of genus *Bacillus* and are most frequently identified among LDPE biodegrading genera. Which includes *B. cereus*, *B. thuringiensis*, *B. subtilis*, *B. pseudomycoides*, *B. mycoides*, *B. toyonesis*, *B. sphericus* and *B. amyloliquefaciens* [7, 9, 10, 14, 19-21].

Different group of enzymes have been reported to degrade LDPE including laccase from *Rhodococcus ruber*. As a result of polymer chain scission and oxidations, a reduction in the molecular weight and increase in the carbonyl groups were observed after two weeks of incubation [22]. Laccase, reported from *Trametes vasicolor* and *B. cereus* has been reported to reduce the molecular weight of LDPE [23, 24]. Another important enzyme, alkane hydroxylase from ALK-B family, has also been reported for degradation of LDPE [25, 26]. Alkane hydroxylase system from *P. aeruginosa* E7 showed 30% of degradation, a good indication of using them for biodegradation [15].

Landfills are the oldest and most common method of plastic waste disposal. Several studies has reported the PE degrading microorganisms from these waste disposal sites [14, 27]. This is the first study on isolation of local bacteria from waste disposal sites in Peshawar, Khyber Pakhtunkhwa (KP), Pakistan for LDPE degradation and we found two newly reported *Bacillus* strains, which can degrade LDPE efficiently. Moreover, we also confirmed that these isolates possess a Laccase and Alkane hydroxyase- producing gene which is the molecular explanation for the degradation of LDPE.

2. Material and Methods

2.1. Chemicals and Polyethylene Films

Additive free LDPE films of 230 micron thickness (ET311351/2, Goodfellow Cambridge Limited Huntingdon, England) were used in this study. Each film was cut into 2 cm x 2 cm pieces and was subjected to heat at 70°C for 10 d followed by irradiation for 20 d with equal exposure on either side under UV 365 nm lamp (SANKYO DENKI, 8 W Japan). After pretreatments, LDPE films were disinfected with universal disinfectant (10 mL bleach and 7 mL Tween 80 in 983 mL d H_2O) for 30-60 min. The films were transferred into sterile distilled water and stirred for 30 min followed by placing in 70% ethanol for 30 min. All the films were allowed to dry overnight at 60℃, prior to subjected in further experimental work [21, 28]. Mineral Salt Media (MSM) was prepared by using 0.5 K_2HPO_4, 0.04 KH_2PO_4, 0.1 NaCl, 0.002 $CaCl_2.2H_2O$, 0.2 $(NH_4)_2SO_4$, 0.02 $MgSO_4.7H_2O$, 0.001 $FeSO_4$ and 0.01 $MnSO_4$ in g/L of sterile double deionized distilled water followed by adding 0.1% Tween 80 and stirred well for 1 h [8]. All reagents used in this study were of analytical grade.

2.2. Sample Collection and Isolation of Low Density Polyethylene Degrading Bacteria

Soil samples were collected from waste disposal sites in Peshawar, KP, Pakistan. Since these sites have been used to dump plastic bags for a very long time (10-20 years) and hence have increased probability of potent LDPE degrading bacteria. LDPE bags were present at varying depth of approximately 5-20 cm. About 1 g of soil was aseptically scratched from each buried LDPE bag and was transported to laboratory in sterile Ziploc plastic bag within 24 h. At the time of sampling the soil temperature was 26-38℃ and pH was 8 ± 1. Isolation of bacteria was done by serial dilution and spread plate technique using Tryptic Soy Agar (TSA). Each plate was incubated at 37℃ until bacterial growth was appeared. All morphologically distinct colonies were sub-cultured to get pure isolates followed by preservation at -80℃ [5].

2.3. Inoculum Preparation

Bacterial isolates were initially cultured in sterile MSM (modified with 0.5% peptone) at 37℃ for 24-48 h in order to make them adapt to this environment. After subsequent sub-culturing in the same medium, the mid log phase was analyzed by determining its growth curve. Each bacterial culture was harvested at mid log phase and cells were collected by centrifugation at 10,000 rpm for 3-5 min [8]. The bacterial cells were then washed with sterile MSM to remove any residual media that will serve as a source of carbon contamination. This procedure was repeated three times and the washed cells in the pellet were resuspended in sterile MSM.

2.4. Screening and Preliminary Identification of Bacteria

The cell suspension containing washed cells (0.5 mL) were homogenously spread on sterile MSM agar plates and sterile LDPE film was placed in the center. The uninoculated MSM plate containing only LDPE film was taken as sterile control. Inoculated MSM plate without LDPE film was used as control to check whether the isolates could grow on MSM alone [8]. Plates were incubated at 37℃. Growth on LDPE films was preliminarily determined by observing the formation of a biofilm after 35-45 d. The growth of bacteria on the film showed its capability of degrading LDPE and was selected for further processing. The selected bacterial isolates were initially identified using Gram staining and then by Vitek 2 Identification System according to manufacturer's instructions (VITEK 2 Compact, Biomerieux, France).

2.5. Molecular Identification of the Isolates

The genomic DNA was extracted from fresh culture. The cells were lysed using conventional L6 lysis buffer (5.25 M GuSCN, 50 mM Tris-HCl (pH 6.4), 20 mM EDTA, 1.3% Triton X-100, distilled water). Amplification was performed using universal primers 27F

(5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCT TGTTACGACTT-3'). A 50 μL mixture was prepared for polymerase chain reaction (PCR) containing; 2 μL of template DNA, 5 μL of i-Taq^{TM}10X buffer (Intron Biotechnology, Seoul, Korea), 4 μL of dNTP's, 2μL of each primer, 1 μL (2U) of iTaq^{TM} DNA polymerase and 34 μL of nuclease free water. The PCR was conducted at following conditions: initial denaturation at 95℃ for 5 min, followed by 35 cycles of 94℃ for 30 s, 55℃ for 30 s, 72℃ for 2 min and final extension at 72℃ for 7 min. The amplified product was analyzed and sequenced (Microgen Inc., Seoul, South Korea). The nucleotide sequences were analyzed and aligned using the CLUSTAL W program (BioEdit) and similarity search was conducted using BLAST of NCBI GenBank database. The phylogenetic tree was constructed using the Neighbor-joining method (MEGA version 7) after multiple sequence alignment with bootstrap value of 1000 replicates.

2.6. Biodegradation Studies

2.6.1. Percent weight loss determination

Sterile LDPE films (2 cm x 2 cm) were pre-weighted and aseptically transferred to conical flask containing 100 mL MSM, as a sole carbon source. 5 mL of washed suspension (approximately 10^8 cells per mL) [8] were added to MSM containing LDPE film. Negative control was maintained having plastic film in medium without inoculation for further reference and to compare reduction in weight. The flasks were incubated at 37℃ in shaking incubator for 90 d [2], at 100 rpm. For identifying any contamination in the medium, streak culture method was performed on TSA plates [21]. After incubation, the LDPE films were collected and washed thoroughly to remove cell debris with 2% Sodium Dodecyl Sulfate (SDS) solution for 4 h at 50℃ followed by rinsing with sterile double deionized distilled water and 70% ethanol. All the films were dried overnight at 60℃ before weighing and percent weight loss was calculated [7, 9].

2.7. PCR Amplification of LDPE Degrading Gene(s)

Total genomic DNA was extracted from bacterial isolates using Lysis buffer and the purified DNA was stored at -20℃ until used. DNA purity and concentration was determined using Nanodrop. Laccase and Alkane hydroxylase have been reported to be involved in enzymatic biodegradation of LDPE [14, 22, 26]. The genes for these enzymes were amplified using specific primers [29, 30], and conditions mentioned in Table S1 using the Program Temp Control system PC 707 (ASTEC, Fucota, Japan).

2.8. Analytical Procedures

2.8.1. Field Emission Scanning Electron Microscopy (FE-SEM) analysis

The LDPE films were analyzed to check for any changes in surface morphology (small holes, cracks and pits) after treating them with LDPE degrading bacteria. The air-dried samples were platinum coated (LICA EM ACE 600), and were then exposed to FE-SEM (SUPRA 55-VP, Carl Zeiss, Germany) [31].

2.8.2. Fourier Transform Infrared Spectroscopy (FTIR) analysis

Changes in the structure of plastic films following UV irradiation and subsequent incubation with bacteria were analyzed using FTIR spectrophotometer (Nicolet 6700, USA). Each sample was observed with a spectrum range from 4,000-650 cm^{-1}. Carbonyl Index (CI) was calculated in order to measure the degree of biodegradation. The CI was calculated based on the relative intensities of the carbonyl group at 1,712 cm^{-1} and CH_2 group at 1,462 cm^{-1} [32]. The equation (Eq. (1)) used to calculate CI is expressed as:

$$Carbonyl\ Index\ (CI) = \frac{Absorption\ at\ 1{,}712\ cm^{-1}\ (the\ maximum\ of\ carbonyl\ peak)}{Absorption\ at\ 1{,}462\ cm^{-1}\ (the\ maximum\ of\ carbonyl\ peak)} \quad (1)$$

2.8.3. X-Ray Diffraction (XRD) analysis

The XRD pattern of the LDPE films was analyzed using X-ray diffractometer (Rigaku, SmartLab, Japan) after biodegradation. The scattered radiation was recorded with the range of 10-40 degrees two theta on the polymer films under constant operating conditions at room temperature [31]. Before processing our XRD data for the peak analysis to calculate the percent crystallinity, we did convolution and subtracted the background using Lab 6 software to minimize the error due to noise. Percent crystallinity for each sample was calculate separately, by processing the three peaks around 19, 21 and 23.7 at 2 θ in the XRD data using originPro8.5 and the following equation (Eq. (2)).

$$\text{Percent crystallinity} = (A2 + A3)/(A1 + A2 + A3) * 100], \quad (2)$$

A1: Integrated area of the amorphous peak around 19 2 θ
A2 and A3: Integrated areas of the crystalline peaks.

2.8.4. Total carbon analysis

Total carbon was analyzed in LDPE film after incubation with the bacterial isolates. Uninoculated film was taken as control to compare percentage of total carbon reduced after biodegradation [33]. Approximately 10 mg of LDPE film was cut into small pieces and analyzed using elemental analyzer (Flash EA1112, Thermo electron corporation, USA).

3. Results and Discussion

3.1. Identification and Screening of Bacterial Isolates Capable of LDPE Biodegradation

Many distinct colonies were observed on TSA plates after incubation at 37℃ for 3-4 d. A total of thirty six morphologically distinct colonies were selected and sub-cultured for further screening. All bacterial isolates were screened for their degradation ability using MSM media and LDPE films. Clear growth was observed beneath the surface of LDPE films after 45 d of incubation at 37℃ (Fig. 1). Out of total thirty six isolates, only six showed biofilm formation revealing their capacity to utilize LDPE as a carbon source and were further characterized. The biofilm formation on LDPE films determines its biodegradation potential because biofilm causes the bacteria to efficiently utilize the insoluble polymer substrate [34]. No bacterial growth was seen on either control i.e., MSM with a LDPE films but without inoculation and MSM without a LDPE film but with inoculation (Fig. 1 (g), (h)).

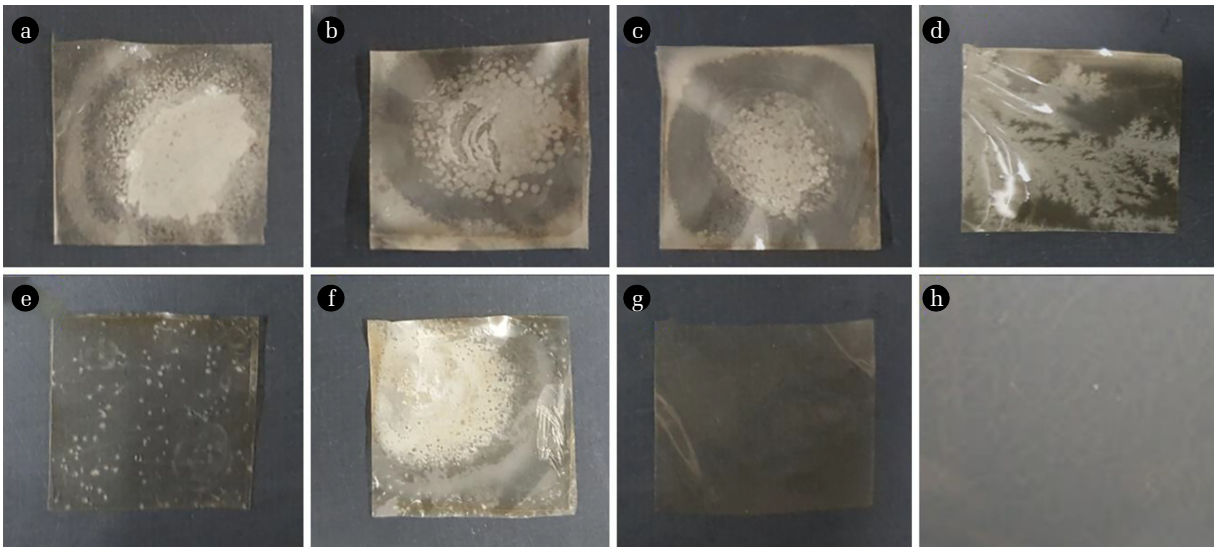

Fig. 1. Growth of bacterial biofilm on LDPE film's surface revealing their biodegradation ability. (a) *B. wiedmannii*, (b) *B. cereus*, (c) *B. siamensis*, (d) *A. iwoffii*, (e) *P. aeruginosa*, (f) *B. subtilis*, (g) *Sterile Control*, (h) MSM without LDPE film but with inoculation.

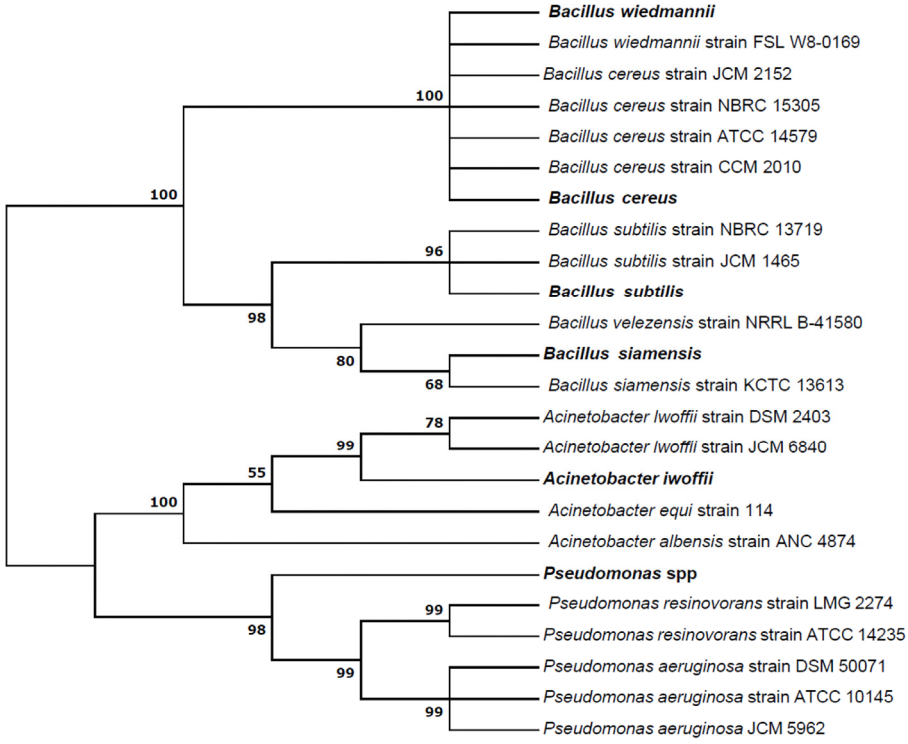

Fig. 2. Neighbor-joining phylogenetic tree of isolates based on 16SrDNA sequencing.

The bacteria isolated and identified in the current study were; *Bacillus wiedmannii* (NR152692.1, 100%), *Bacillus subtilis* (CP020102.1, 100%), *Bacillus siamensis* (KY643639.1, 99%), *Bacillus cereus* (KY628813.1, 99%), *Acinetobacter iwoffii* (NR113346.1, 99%), *Pseudomonas aeruginosa* (CP012001.1, 97%) and according to Vitek™ and 16SrDNA sequencing. The phylogenetic tree showing related species as per 16SrDNA sequencing is illustrated in Fig. 2.

3.2. Investigation of LDPE Degradation

The biodegradation of LDPE was monitored by weight loss estimation and was further confirmed by analytical procedures using FE-SEM, XRD, FTIR and total carbon analysis.

3.2.1. Percent weight loss determination

The identified bacterial isolates were tested for their degradation capacity that was measured in terms of percent weight loss (Table 1).

Table 1. Percent LDPE Degradation of the Bacterial Isolates After 90 Days of Incubation

S. No	Sample	Initial weight (mg)	Final weight (mg)	Weight loss (%)
1	*Bacillus siamensis*	100.0	91.54	8.46 ± 0.3
2	*Bacillus cereus*	179.35	168.0	6.33 ± 0.2
3	*Bacillus wiedmannii*	191.59	181.26	5.39 ± 0.3
4	*Bacillus subtilis*	187.69	180.66	3.75 ± 0.1
5	*Pseudomonas aeruginosa*	180.52	178.44	1.15 ± 0.1
6	*Acinetobacter iwoffii*	185.92	184.5	0.76 ± 0.1
7	*Negative Control*	159.24	159.0	0.15 ± 0.1

The highest percent weight loss was presented by *B. siamensis* (8.46 ± 0.3%) followed by *B. cereus* (6.33 ± 0.2%), *B. wiedmannii* (5.39 ± 0.3%) and *B. subtilis* (3.75 ± 0.1%). Among bacterial isolates *P. aeruginosa* (1.15 ± 0.1%) and *A. iwoffii* (0.76 ± 0.1%) were not further investigated in this study as their percent weight reduction was negligible.

Bacillus species were more efficient in degrading LDPE films in this study as compared to other isolates. Several studies in literature also identified the potential of *Bacillus* spp for PE degradation via percent weight loss, such as Harshvardhan and Jha 2013, reported 1.75% in 30 d [7] and Yang et al. [8] observed 10.7 ± 0.2% in 60 d of incubation. In the current study *B. cereus* showed 6.33% of weight loss of LDPE films (230micron), while previous study reported percent weight loss of 35.2% in 30 micron film after 120 d of incubation [35]. In present study, *B. subtilis* resulted in weight loss of 3.75 ± 0.1%, which is comparatively higher than 1.85% weight loss in 30 d of incubation [10] however another work reported 23.15% reduction in 60 d of treatment with *B. subtilis* [19]. The variation in percent weight loss in our study with comparison to literature might be attributed to the origin of bacterial isolates, film thickness and culture conditions such as incubation time. In this study, *B. siamensis* and *B. wiedmannii* resulted in the weight loss of 8.46 ± 0.3% and 5.39 ± 0.3%, respectively. To the best of our knowledge, we are reporting *B. siamensis* and *B. wiedmannii* for the first time in LDPE degradation which exhibits the ability of utilizing LDPE as a sole carbon source.

As Laccase and Alkane hydroxylase have been known to be involved in enzymatic biodegradation of LDPE, their genes(s) were amplified in all the selected bacterial isolates. Amplified segments were subjected to agarose gel electrophoresis. Laccase (167 bp) was amplified and bands appeared in all isolates indicating their presence in them (Fig. 3 (a)) Moreover, two set of primers were used for Alkane hydroxylase amplification. *B. subtilis and B. wiedmannii* were positive for Alk 1 (330 bp), while *B. cereus* and *B. siamensis* showed the presence of Alk2 (185 bp) (Fig. 3 (b)). Production of extracellular enzymes plays an important role in biodegradation of PE through depolymerization [36] and are then enzymatically degraded into intermediate products that can be utilized as a carbon source [14]. Extracellular laccases and Alkane hydroxylase are among the enzymes implicated in LDPE biodegradation [22, 26]. These enzymes have been reported in various bacteria, *Brevibacillus borstelensis*, *Pseudomonas putida*, *Brevibacillus parabrevis* and *Bacillus cereus*, for biodegradation of PE [14]. Alkane hydroxylase from *Pseudomonas* spp degraded PE by reducing its molecular weight indicating its capability in biodegradation [15, 26].

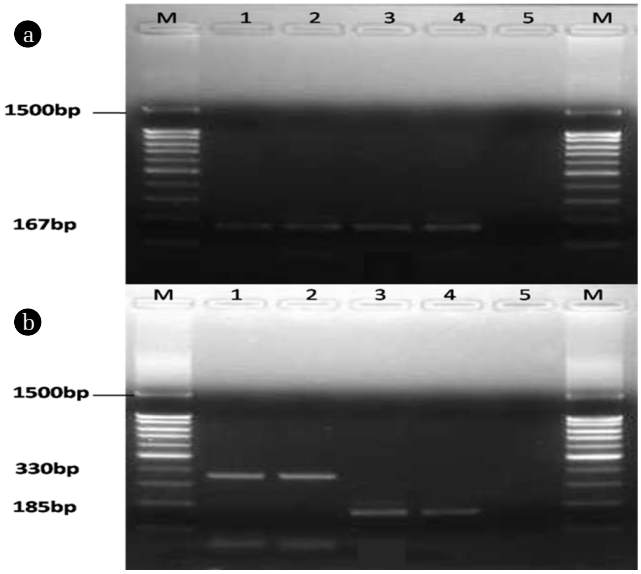

Fig. 3. (a) PCR product analysis of Laccase (167bp) from LDPE degrading bacteria, M: 100 bp Ladder; lane 1, *B. subtilis*; lane 2, *B. wiedmannii*; lane 3, *B. subtilis*; lane 4, *B. siamensis*; lane 5, Control and M, 100bp ladder. (b) PCR amplification of Alkane hydroxylase by Alk1 (330 bp) (lane 1-2) and Alk2 (185 bp) (lane 3-4); M, 100bp Ladder; lane 1, *B. subtilis*; lane2, *B. wiedmannii*; lane 3, *B. cereus*; lane 4, *B. siamensis*; lane 5, Control and M, 100 bp ladder.

3.2.2. FE-SEM analysis

Initial attack on LDPE films begins with surface colonization and penetration of bacterial isolates and these phenomena can be observed through FE-SEM. The treated LDPE films showed some physical changes (slight surface disruption) due to colonization of bacterial isolates, after 90 d of incubation. In contrast, the control retained a smooth surface under the same conditions (Fig. 4) These changes were not uniformly distributed on the entire surface as bacteria attached to only certain regions [37]. Similar observations have been reported in a previous study where formation of grooves were seen in the micrographs of LDPE films when treated with *Pseudomonas* sp AKS2 [38]. In one of the study Koutny et al. [39] reported that after 100 d of incubation with microorganisms, SEM analysis revealed no clear signs of bio-erosion on the surface of films. This study is quiet similar to our findings as our data suggest that bacterial action was only limited to the surface of LDPE film having slight surface disruption indicating biodegradation as a relatively slow and continuous process.

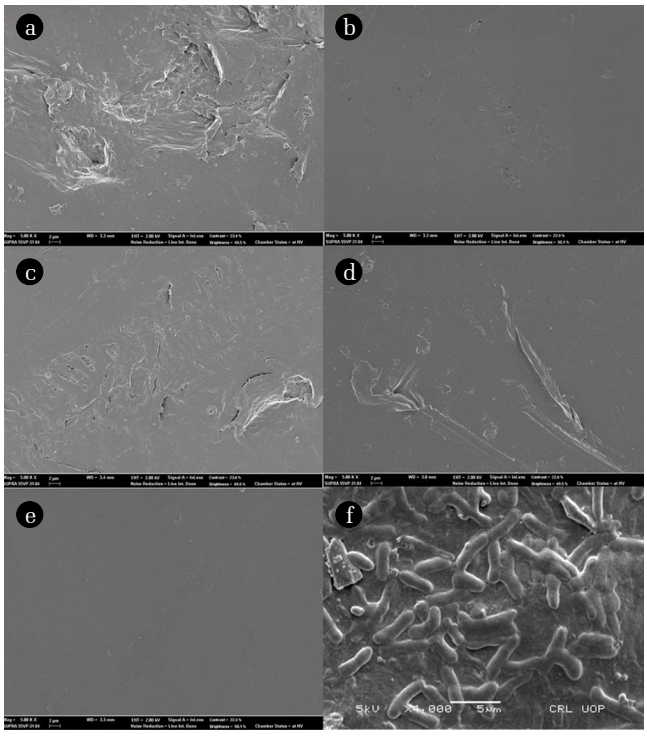

Fig. 4. FE-SEM micrograph of LDPE films after 90 days of incubation with bacteria showing slight surface disruption and small holes at 5.00 K X magnification. (a) *B. siamensis*, (b) *B. cereus*, (c) *B. wiedmannii*, (d) *B.subtilis*, (e) Control, (f) Depiction of bacterial attachment to LDPE film at 4,000 K X magnification.

3.2.3. FTIR analysis

The exposure of LDPE films to UV radiations causes an increase in the carbonyl group and provides attachment sites for the bacteria [40]. The FTIR analysis of LDPE films were peformed to moniter the changes in carbonyl groups or double bonds which is essential to elucidate the biodegradation process. The FTIR spectra of LDPE films showed formation of typical carbonyl peak at 1,712 cm^{-1} (except untreated control), which was markedly reduced after 90 d of incubation with bacteria. This formation and disappearance of carbonyl peaks is essential to explicate the mechanism of plastic biodegradation [41] and the same phenomenon has been observed in our study where there is formation of carbonyl peak at 1,712 cm^{-1} which is reduced after treating with bacteria for 90 d. The

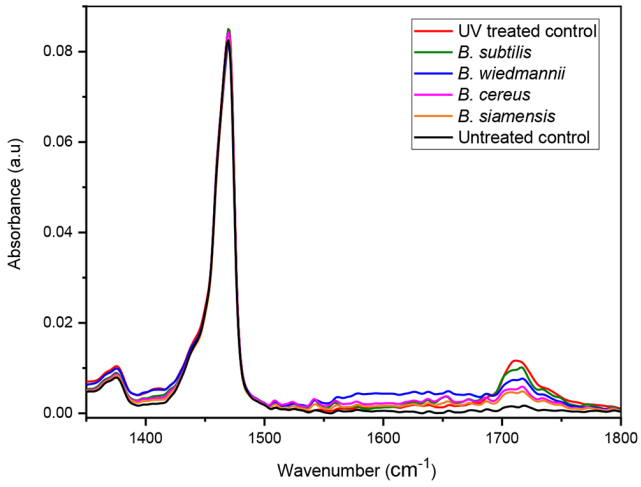

Fig. 5. FTIR spectra of LDPE films treated with bacteria; formation of carbonyl peak after pretreatment (UV treated control) and subsequent reduction in carbonyl peak with *B. subtilis, B. wiedmannii, B. cereus* and *B. siamensis*. No changes were observed in the untreated control.

same results have been reported by other researchers to monitor the formation or disappearance of functional groups to explain the mechanism of biodegradation process [7, 8], thereby supporting our results.

The formation and distinct reduction in the carbonyl residues (Fig. 5) was observed in all samples, suggesting microbial degradation. This reduction was also measured in terms of CI index as shown in Table 2. The maximum decrease in CI was observed for *B. siamensis* followed by *B. cereus*, *B. wiedmannii* and *B. subtilis*, showing *B. siamensis* has more potential for degradation. Several researchers also reported reduction in CI index values, suggesting microbial degradation, which may be due to the presence of oxidoreductases [21, 31].

3.2.4. XRD analysis

The percent crystallinity of LDPE films was increased after 90 d of incubation with selected bacterial isolates (Table 2 and Fig. S1). These results could explain the phenomenon that once polymer is exposed to microorganism it mainly attacks the amorphous regions (which is less resistant to microbial attack than crystalline regions), causing an increase in the overall crystallinity. Later with

Table 2. The Carbonyl Index, Percent Crystallinity and Total Carbon Percentages of LDPE Films Before and After Treatment with Bacteria for 90 Days

Sample	Carbonyl Index (CI) CI = I1/I2a	Crystallinity (%) [(A2 + A3)/(A1 + A2 + A3) * 100]	Carbon (%)
B. siamensis	0.058489	35.35532592	85.5679
B. cereus	0.069845	31.46878474	85.6169
B. weidmanni	0.093827	31.15333231	85.4823
B. subtilis	0.119543	28.48387491	85.4114
UV Treated control	0.149755	27.92827197	85.3836
Untreated control	0.020487	-	-

a : I1 = Intensity at 1,712 cm^{-1} and I2 = Intensity at 1,462 cm^{-1}

the attack on smaller crystals located in the amorphous- crystalline phase or produced by initial consumption of amorphous phase, causes a decrease in crystallinity [42-44].

In a previous study an increase in the relative crystallinity of LDPE was observed in initial 3 months over treating with filamentous fungi [42]. Similarly Volke-Sepúlveda et al. [43] also reported an initial increase within 7-16 months of incubation by fungi followed by a decrease in crystallinity. In present study similar trend was observed by bacterial species in which the highest increase in percent cystallinity was shown by *B. siamensis* followed by *B. cereus, B. wiedmannii* and *B. subtilis*.

3.2.5. Total carbon analysis

In the current study there was no apparent change in total carbon (Table 2). This may be because of the short duration of incubation and thickness of the film as PE is hard to degrade and its degradation is a time dependent process. The negligible decrease in percent carbon content of 230 micron LDPE film was recorded, which is in contrast to a previously reported study in which author reported a decrease in carbon percentage from 93% to 91.7% and 91.6% to 90.1% for 20 and 40 micron PE film, respectively [33]. In another study different fungal strains resulted in significant decrease of total carbon of 15 micron thick PE sample [45]. The decrease in the carbon percentage may depend on the thickness of the film used.

4. Conclusions

In this study we isolated bacterial species from waste disposal sites of Peshawar, KP, Pakistan for LDPE degradation. We hereby conclude that the *Bacillus* species were capable of utilizing LDPE as a sole carbon source, as evidenced by various analytical tools; FE-SEM, FTIR, XRD and total carbon analysis. Among these species, *B. siamensis* and *B. wiedmannii* are reported for the first time that exhibit effective LDPE degradation, hence adding new information to the literature. Biodegradation was confirmed not only by the attachment of bacteria to the LDPE films and percent weight loss, but also by some physical and chemical changes in LDPE films like surface disruption, formation and reduction of carbonyl peaks, decrease in CI index and increase in percent crystallinity. There was negligible change in the total carbon content which attributes to the thickness of film used. The biodegradation by selected bacterial isolates was limited to surface of LDPE films and is relatively slow but continuous process. These isolates possesses LDPE degrading genes that encodes for enzymes Laccase and Alkane hydroxylase, which is the molecular explanation for the degradation of LDPE. In near future, different strategies are needed for optimum expression of these enzymes to increase and exploit their degradation potential.

Acknowledgment

This work was supported by Higher Education Commission (HEC) Pakistan, to be conducted in Lab of Infectious Diseases, Collage of Veterinary Medicine, Seoul National University. This study was partially supported by BK21 PLUS for Creative Veterinary Science Research and the Research Institute of Veterinary Science, Seoul National University, Republic of Korea. This article was presented at IWA 11th Micropol & Ecohazard Conference (2019) held on 20-24 October 2019 in Seoul, South Korea.

Author Contributions

L.M. (Ph.D. Student) conducted all the experiments. I.K. (Associate Professor, Ph.D) and B.A. (Professor) guided the author throughout the research. H.S.Y. (Professor) provided space and availability of the instruments in his laboratory with guidance throughout the research project. H.P (Ph.D. Student) and S.K. (Ph.D. Student) helped in the phylogenetic analysis of the isolates. S.A. (Assistant Professor) helped in conduct ing FTIR analysis through software.

Nomenclature

%	Percentage
°C	Temperature (Degree centigrade)
μ	Micro (Mu)
Θ	Theta
'	Prime

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