Low density polyethylene (LDPE) is used for making common shopping bags and plastic sheets and is a significant source of environmental pollution. The present study was aimed at testing the ability of bacterial strains identified as *Serratia* sp. KC1-MRL, *Bacillus licheniformis* KC2-MRL, *Bacillus* sp. KC3-MRL and *Stenotrophomonas* sp. KC4-MRL isolated from a limestone cave to degrade polyethylene. These strains were isolated from soil of Kashmir Smast, a limestone cave in Buner, Pakistan. These strains showed antibacterial activity against *Micrococcus luteus*, *Klebsiella* sp., *Pseudomonas* sp., and *Staphylococcus aureus*. The pieces of LDPE plastic were incubated along with bacterial strains for a period of one month and then analyzed. Degradation was observed in terms of growth of microorganisms used in consortia, chemical changes in the composition of LDPE by fourier-transform infrared spectroscopy, and changes in physical structure of LDPE by scanning electron microscopy. Maximum growth (10^7 to 10^5 CFU mL^-1) at 28 °C and subsequent change in chemical and physical properties of plastic were observed in the presence of calcium and glucose. The cave soil sample had a very high concentration of calcium. The microscopy showed adherence of bacteria with lots of mechanical damage and erosion on the surface of plastic films incubated with bacterial consortia. The spectroscopy showed breakdown and formation of many compounds, as evident by the appearance and disappearance of peaks in LDPE treated with bacterial consortia as compared to the untreated control. We conclude that antibiotic-producing cave bacteria were able to bring about physical and chemical changes in LDPE pieces and degradation of LDPE was enhanced in media augmented with calcium.

**INTRODUCTION**

Plastics are polymers of carbon, oxygen, and hydrogen and that are synthetically derived from petrochemicals and suitable for a wide range of usage. Since plastics are artificially manufactured, they are xenobiotic compounds and they resist degradation (Kawai, 2010).

Polyethylene is one of the most commonly used commercial plastics, found in various products ranging from simple plastic bags to artificial limbs (Orhan and Büyükgüngör, 2000; Shimao, 2001). Thermal and mechanical stability and their morphologies make polymeric substances one of the most popular commodity of the modern world (Rivard et al., 1995). Plastic waste is an environmental hazard. Plastic debris poses a direct threat to wildlife. The main dangers associated with plastic objects for most species are related to entanglement and ingestion. Juvenile animals, in particular, often become entangled in plastic debris, which can result in serious injury as the animal grows, not to mention restriction of movement, preventing animals from properly feeding and, in the case of mammals, breathing (Webb et al., 2012). Due to plastic’s resilience against degradation and its proliferation in industry, the issue of plastic pollution has evolved to become a threat to global ecology.

Management of plastic waste is an ever-increasing problem, and none of the current techniques of solid waste management completely alleviate all the concerns related to these recalcitrant polymers (Nkwachukwu et al., 2013). One way to deal with these polymers could be to alter the manufacturing process, and new formulations should be developed with special considerations on mechanism for their biodegradation. These alterations could include looking into factors that can aid in biodegradation like pH, temperature, chemical structure, polymeric morphology, presence or absence of certain additives, and most importantly, the type of organisms that can be involved (Gu and Gu, 2005).

Degradation of plastics is carried out by organisms that are chemoheterotrophs. Many studies have shown the presence of such bacteria in caves. Bacteria also have the ability to utilize hydrocarbons as a source of energy. Studies have shown that a variety of culturable chemoheterotrophs are present in microhabitats of caves and catacombs (De Leo et al., 2012). The microorganisms living under stressful or low nutrient habitats...
can develop the ability to use any available nutrient to survive. Studies on a bacterial strain belonging to the *Arthrobacter* genus from alpine ice showed biodegradation of phenol under various environmental conditions (Margesin et al., 2004). Bacteria present in caves are capable of carrying out a variety of biodegradative and biodeteriorative processes. Extensive studies have reported biodeteriorative effects of microorganisms in cave environment (Cuevza et al., 2012; Schabereiter-Gurtner et al., 2002).

Despite much study, the knowledge is very limited about microbial life in diverse and extreme habitats like caves. There exists much potential for isolating and studying microbes in caves that have unique and unexplored characteristics of potential commercial applicability. These studies can also be helpful in investigating evolutionary relationships of microorganisms in cave environment. Most caves are characterized as having very low nutrient availability, constant low temperature, and high humidity. Caves can either be terrestrial or aquatic. Some may be rich in specific natural minerals or have exposure to nutrient sources, and therefore, different caves will have different types of microorganisms inhabiting various ecological niches (Zada et al., 2016). Fauna, environmental factors, temperature, humidity, organic matter, and other environmental factors influence activities such as nutrient cycling, and geomicrobiological activities including the formation or alteration of cave structures (Adetutu and Ball, 2014). Cave organisms have evolved some extraordinary abilities to survive and live in this inhospitable environment.

Polyethylene makes a significant contribution to solid waste in developed countries. Management of this waste can be carried out by chemical, physical, and biological methods. Various natural microflora of soil, including bacteria and fungi, are reported to degrade low-density polyethylene (LDPE) under various physical and chemical environments. This study determines the degradation capacity of four bacterial strains isolated from Kashmir Smast, Khyber Pakhtoon Khwa, Pakistan. The bacteria isolated were previously identified and tested positive for antibiotic production in the Microbiology Research Laboratory (MRL), Department of Microbiology, Quaid-e-Azam University, Islamabad. Since antibiotic production and LDPE degradation takes place under stressed environmental conditions, it was hypothesized that bacteria positive for the first character may also be positive for the other character. For this purpose, commercially available LDPE from a shopping bag was used. Bacterial isolates *Serratia* sp. KC1-MRL, *Bacillus licheniformis* KC2-MRL, *Bacillus* sp. KC3-MRL, and *Stenotrophomonas* sp. KCMRL isolated from the cave were used in consortium to carry out biodegradation of polyethylene. Nutrient agar medium was used for isolation of bacterial strains from cave soil. The strains were isolated using standard serial dilution methods and subsequent growth on Nutrient agar plates for 48h at 37 °C. All the isolated strains were screened for polyethylene degradation. For this purpose the strains were incubated for two weeks in 120 mL of mineral salt medium (g L−1): [KH2PO4, 2.0; K2HPO4, 7.0; MgSO4·7H2O, 0.1; ZnSO4·7H2O, 0.001; FeSO4·7H2O, 0.01; MnSO4·6H2O, 0.002; NH4NO3, 1.0; CuSO4·7H2O, 0.0001; pH 7.2] at 37 °C with pieces of polyethylene (1 by 1 cm) (Anwar et al., 2009). Polyethylene used was pretreated by exposing it to UV light for three minutes. At the end of two weeks of incubation, viable cells were counted as CFU mL−1 by serial dilution. Four bacterial strains were found active in terms of growth in the medium.

### Materials and Methods

#### Sampling Site and Sample Collection

Soil samples were collected from Kashmir Smast (cave), Nanser, Buner, Khyber Pakhtunkhwa (Fig. 1). The Kashmir Smast is a series of natural limestone caves probably of marine origin (Khan, 2013). These caves are located in the Babozai Mountains between Mardan and Buner in northern Pakistan. The only source of water was drip water. Two soil samples were collected from wall and ground surfaces of the cave in sterile Falcon tubes under aseptic conditions. Samples were collected from the dark end of the cave about 188 m from the entrance. This cave is located far away from human access, so human intervention is negligible (Zada et al., 2016). The samples were then brought to the laboratory in an icebox and stored at 4 °C for further processing. The pH and temperature of soil was recorded as 7.2 and 25 °C.

#### Soil Analysis by Atomic Absorption

For the quantitative analysis of elements in the soil sample, atomic-absorption spectrophotometry was performed with a AA240FS Fast Sequential Atomic Absorption Spectrophotometer. Soil digestion was performed to prepare samples for analysis. One gram each of soil from the cave floor and control soil from outside the cave were ground separately and were then mixed in 15 mL aqua regia, heated at 15 °C, and left overnight. Then 5 mL of HClO4 was added and again heated at 150 °C. The solution almost became dry before brown fumes were produced. Whatman filter paper (No. 42) was used for filtration, and the volume was made up to 50 mL using double-distilled water (Kelly et al., 2008).

#### Screening and Isolation of LDPE-Degrading Bacteria

Previously identified strains of *Serratia* sp. KC1-MRL, *Bacillus licheniformis* KC2-MRL, *Bacillus* sp. KC3-MRL, and *Stenotrophomonas* sp. KCMRL isolated from the cave were used in consortium to carry out biodegradation of polyethylene. Nutrient agar medium was used for isolation of bacterial strains from cave soil. The strains were isolated using standard serial dilution methods and subsequent growth on Nutrient agar plates for 48h at 37 °C. All the isolated strains were screened for polyethylene degradation. For this purpose the strains were incubated for two weeks in 120 mL of mineral salt medium (g L−1): [KH2PO4, 2.0; K2HPO4, 7.0; MgSO4·7H2O, 0.1; ZnSO4·7H2O, 0.001; FeSO4·7H2O, 0.01; MnSO4·6H2O, 0.002; NH4NO3, 1.0; CuSO4·7H2O, 0.0001; pH 7.2] at 37 °C with pieces of polyethylene (1 by 1 cm) (Anwar et al., 2009). Polyethylene used was pretreated by exposing it to UV light for three minutes. At the end of two weeks of incubation, viable cells were counted as CFU mL−1 by serial dilution. Four bacterial strains were found active in terms of growth in the medium.
Bacterial colonies were further purified and enriched on nutrient agar plates.

**Preparation of Inoculum**

About 10 mL of nutrient broth was inoculated with two or three loops of the pure culture of isolated strains. Bacterial growth was evaluated at 25 °C, 37 °C and 40 °C. Maximum growth was observed at 37 °C (O.D. at 600 nm). Consortia were developed by taking inocula from each test tube into a separate flask with 100 ml of nutrient broth. Five percent of this prepared consortia was used as inoculum for further biodegradation experiments.

**Medium Preparation and Incubation**

Different metabolites were used in combinations to study their effects on biodegradation of polyethylene by the cave-bacteria consortia. Glucose, yeast extract, and calcium were used as co-metabolites. About 1% w/v of glucose and yeast extract were used, whereas the concentration of calcium in the medium was maintained at 0.03% to match the natural concentration of calcium of the environment where the soil was taken.

In total six combinations of these metabolites in mineral salt medium with polyethylene pieces and bacterial consortia were incubated at 150 rpm at pH 7.2 and temperature 37 °C.

*Figure 1. Location and map of Kashmir Smast (Cave), Nanseer Buner, Khyber Pakhtunkhwa, Pakistan. Source Zada et al. (2016).*

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for four weeks. A negative control was set by incubating polyethylene pieces in mineral salt medium with no bacterial inoculum.

**Biodegradation Analysis**

Biodegradation of polyethylene was analyzed by determining CFU mL\(^{-1}\), Fourier-transform infrared spectra, and scanning electron microscope images. CFU mL\(^{-1}\) was determined after every week, whereas FTIR and SEM analysis were performed after one month of incubation. The viable cell count was done for bacterial growth determination through serial dilution and calculating CFU mL\(^{-1}\). Test LDPE samples were compared with the untreated control samples. FTIR (Jasco FT/IR – 620) analysis was performed to check the degradation of LDPE pieces after being mixed with the growing bacterial consortia in liquid medium. This analysis detects any change in the functional groups. Spectrum was recorded at 500-4000 wave-numbers cm\(^{-1}\) for all the LDPE samples. Surface morphology of LDPE pieces was observed by SEM (JSM 5910 Joel, Japan) to look for any change in structure or surface of LDPE piece after treating with microbial consortia. After rinsing of the LDPE pieces with autoclaved distilled water, LDPE pieces were mounted on the copper stubs with gold paint. Gold coating was carried out under vacuum by evaporation to make the samples conducting.

**Results**

**Soil Analysis**

Atomic absorption spectroscopy was performed to determine the concentration of elements in the cave soil sample (ZADA et al., 2016). Ca was 332.938 mg kg\(^{-1}\) as compared to 121.65 mg kg\(^{-1}\) in control soil. Mg was 1.2576 mg kg\(^{-1}\) in cave soil and 1.023 mg kg\(^{-1}\) in control soil. Ni 0.965 mg kg\(^{-1}\) in cave soil and 10.4 mg kg\(^{-1}\) in control soil, Cr 0.571 mg kg\(^{-1}\) in cave soil and 8.74 mg kg\(^{-1}\) in control soil, Co 0.266 mg kg\(^{-1}\) in cave soil and 0.810 mg kg\(^{-1}\) in control soil, Cu 1.824 mg kg\(^{-1}\) in cave soil and 4.7 mg kg\(^{-1}\) in control soil, Zn 12.7311 mg kg\(^{-1}\) in cave soil and 36.41 mg kg\(^{-1}\) in control soil, and Pb 1.31 mg kg\(^{-1}\) in cave soil and 8.14 mg kg\(^{-1}\) in control soil were much lower than those found in the control soil (Zada et al., 2016).

**Viable Cell Count**

The concentration of viable cells in CFU mL\(^{-1}\) was determined at time zero, before initial incubation, and then after every week for a period of one month (Table 1). Since polyethylene in the medium was the sole carbon source, CFU mL\(^{-1}\) is directly proportional to the ability of organisms to degrade polyethylene and use it as a carbon source. There was a consistent decrease in CFU mL\(^{-1}\) after three and four weeks of incubation. The soil from where the bacteria were isolated contained exceptionally high concentration of calcium. Considering this high amount of calcium in the native habitat
of the organism, extra calcium salt was added in the medium so that the organisms experience minimum deviation from their natural environment. Medium augmented with extra calcium showed increasing values of viable cell count in the first two weeks of incubation. Increase in the values of viable cell count was also observed in the first two weeks of incubation when the medium is augmented with glucose; these higher values of CFU mL⁻¹ in first two weeks were because the bacteria were provided with glucose that acted as growth activator. Additional amounts of calcium proved to be helpful for better growth of bacterial colonies.

**Fourier Transform Infrared Spectroscopy**

FTIR was carried out on LDPE films after incubation with bacterial consortia for four weeks. The peaks formed were compared with the control (Fig. 2).

LDPE in mineral salt medium containing bacterial consortia: Absorbance peaks formed at 2915 cm⁻¹ and 2848 cm⁻¹ suggest presence of C–H bonds. A peak of variable strength at 1472 cm⁻¹ and 1462 cm⁻¹ shows formation of C=C bonds. A strong peak at 1035 cm⁻¹ shows formation of stretch of C=O bonds. Absorbance peaks formed at 615 cm⁻¹, 718 cm⁻¹ and 730 cm⁻¹ show presence of =C–H bending bonds.

LDPE in MSM containing calcium carbonate and bacterial consortia: Maximum variety of peaks was observed in this film. Formation of peaks at 3251 cm⁻¹ and 3032 cm⁻¹ shows formation of O–H bonds. A peak at 2915 cm⁻¹ and 2848 cm⁻¹ represents formation of stretch of C–H bonds in the polyethylene. Peak formation at 2233 cm⁻¹, 2178 cm⁻¹, 2167 cm⁻¹, 2103 cm⁻¹, and 2013 cm⁻¹ shows that new Nitrile bonds of –C≡N are formed. Absorbance peaks at 1462 cm⁻¹ and 1472 cm⁻¹ show bending of –C-H-bonds. Peak at 1084 cm⁻¹ shows formation of stretch of C–O functional group. Absorbance peaks formed at 615 cm⁻¹, 718 cm⁻¹, and 730 cm⁻¹ shows presence of =C-H bending bonds.

LDPE in MSM containing calcium carbonate, glucose, and bacterial consortia: Maximum variety of peaks was observed in this film. Formation of peaks at 3251 cm⁻¹ and 3032 cm⁻¹ shows formation of O–H bonds. A peak at 2915 cm⁻¹ and 2848 cm⁻¹ represents formation of stretch of C–H bonds in the polyethylene. Peak formation at 2233 cm⁻¹, 2178 cm⁻¹, 2167 cm⁻¹, 2103 cm⁻¹, and 2013 cm⁻¹ shows that new Nitrile bonds of –C≡N are formed. Absorbance peaks at 1462 cm⁻¹ and 1472 cm⁻¹ show bending of –C-H-bonds. Peak at 1084 cm⁻¹ shows formation of stretch of C–O functional group. Absorbance peaks formed at 615 cm⁻¹, 718 cm⁻¹, and 730 cm⁻¹ shows presence of =C-H bending bonds.

LDPE in MSM containing calcium carbonate, yeast extract, and bacterial consortia: In comparison with control, new peak was formed in this medium combination at 1033 cm⁻¹ that represents formation of C-O bond.

LDPE in MSM containing calcium carbonate, yeast extract and bacterial consortia: In comparison with control, anew peak was formed in this medium combination at 1033 cm⁻¹ that represents formation of C-O bond.

**Scanning Electron Microscopy**

SEM showed adherence of bacteria that caused mechanical damage and erosion on the surface of plastic films incubated with bacterial consortia as compared to the untreated control (Fig. 3). More changes in surface topology and attachment of cells, despite the washing, were observed on the LDPE piece incubated in the presence of glucose and calcium.
DISCUSSION

There is an increasing interest in investigating biodegradation of non-degradable plastics using efficient microorganisms (Bonhomme et al., 2003; Boonchan et al., 2000; Lee et al., 1991). In our present study, bacterial isolates were obtained from the soil of Kashmir Smast, which is a limestone cave in Khyber Paktoonkhuwa province, Pakistan. The four isolates were identified as Serratia sp. KC1-MRL, Bacillus licheniformis KC2-MRL, Bacillus sp. KC3-MRL, and Stenotrophomonas sp. KC4-MRL. These strains were used in consortia to check for the ability of these microbes to degrade polyethylene. Since both antibiotic production and polyethylene degradation occur under stressed environmental conditions, the hypothesis of the current study was that bacteria having the first property are more likely to give positive result for the other. Studies on Brevibacillus borstelensis 707 showed an increase in potential of polyethylene biodegradation when grown on nitrogen-limit stressed cultures (Hadad et al., 2005). It was also observed that not only nitrogen deprivation, based on the amount of KNO₃ in medium, but also carbon limitation in a mannitol-free medium alone enhanced degradation, which was also enhanced when used in combination. It was observed that mannitol-free medium supplemented with nitrogen source showed maximum biodegradation in 30 days of incubation. In the present study, it was found that bacterial consortia showed higher viable cells measured as CFU mL⁻¹ when the medium was supplemented with the nitrogen source.

Loss of tensile strength of plastic after incubation with Pseudomonas stutzeri suggests that the bacterium is capable of degrading the polymer (Sharma and Sharma, 2004). When bacteria are grown in different media compositions along with a polymer, maximum turbidity is observed on forty-fifth day (Ciferri, 1999). The increase in growth rate in glucose, as well as in minimal medium, suggests that the bacteria were completely depending upon polyethylene film for its source of carbon in the absence of glucose. CFU mL⁻¹ increased from 10¹ to 10³ from day 15 to day 30 (Table 1). These results suggest that the bacteria growing in Lascaux Cave, France are
capable of using plastics and other resins such as glue as their sole source of carbon. Higher values of CFU mL⁻¹ in the first two weeks were observed in all those media combinations in which 1% glucose w/v was added at the beginning. When the nutrients depleted, bacteria had polyethylene as the only carbon source. Addition of easily available substrate like glucose in MSM medium increases bacterial growth in initial stages of pesticide degradation (Cycoń et al., 2011). A similar effect on growth was observed when MSM media is augmented with yeast extract. The increased CFU mL⁻¹ was observed in first two weeks of the experiment and then the growth decreased, which indicates depletion of glucose in the medium.

In our study, fourier-transform infrared spectral analysis was carried out to check chemical degradation of polyethylene. Low- and high-density polyethylene are made of the elements carbon and hydrogen forming chains of repeating – CH₂ – units (Rajandas et al., 2012). In the process of biodegradation of LDPE, enzymes catalyze a specific series of biochemical reactions that lead to various kinds of chemical conversions, such as oxidation, reduction, hydrolysis, esterification, and molecular inner conversion (Harshvardhan and Jha, 2013). Keto and ester carbonyls have been reported as major products in the presence of oxidoreductase (Karlsson and Albertsson, 1998). Analysis of FTIR showed new peaks when LDPE is treated with bacterial consortia, indicating polymer breakdown and formation of new functional groups. The results of scanning electron microscopy have shown that all those media to which calcium was added showed strong biofilm development and hence increased biodegradation. It is also evident in several studies that bacteria release various surface-active substances extracellularly, which increase the bioavailability to the polymer. Studies on Pseudomonas sp. AKS2 showed that the strain was capable of degradation 5 ± 1 % of initial LDPE in 45 days (Tribedi and Sil, 2013). The degradation by Pseudomonas depends on its capability to colonize the surface of the polymer and degrade it. Addition of calcium increases biofilm development of Xylella fastidiosa under in vitro conditions (Cruz et al., 2012). The efficient increase in formation of biofilm was observed when at least 1.0 mM CaCl₂ was added in the medium. There was no effect of Ca on attachment when bacteria were treated with tetracycline, indicating that Ca has a regulatory role in colonization or attachment of the cells. In another study, Ehret and Böl (2013) showed that Ca ions crosslink alginites, which is the key constituent of the extracellular polymeric material produced by the mucoid P. aeruginosa strain to produce biofilms. Ciferri reported a list of bacteria responsible for degradation of paints (Ciferri, 1999).

In the present study, SEM showed discoloration, spots, erosion, and cracking on the surface of polyethylene film. Modification on the surface of polyethylene after bacterial treatment was also reported by (Matsunaga and Whitney, 2000). Formation of pits and erosion on the surface of LDPE when observed through electron microscopy when incubated with Fusarium sp. indicating adherence and degradation of LDPE (Hasan et al., 2007).

Bacteria capable of adhering to plastic surfaces, growing, and possibly degrading it by oxidation are commonly present in soil. Microorganisms that can adhere to the surface of pre-oxidized PE are also commonly present in soil. If the pre-oxidant technology is commonly employed in the PE manufacturing process, one can expect that these plastics will be able to degrade in waste disposal sites. In the current study, structural and surface changes in PE in the form of depression, pits, and erosions were visible in SEM. Physical erosion of the surface of polyethylene observed through SEM by fungi has been reported by Bonhomme et al. (2003). Polymer treated with microorganism loses its physical strength and disintegrates on applying mild pressure. Wide spread pits and holes in polycaprolactone surface are reported by Shaw et al. (2015) after ten days of incubation with thermophilic bacterium Ralstonia sp. strain MRL-TL. SEM has shown that biofilms develop on the surface of polyethylene with time by PE-degrading bacteria. It is known that formation of biofilm on the surface of plastics favors adhesion of bacteria on the surface and helps them survive under low nutrient conditions and to use polyethylene as their source of carbon (Linos et al., 2000).

**CONCLUSIONS**

Our study indicates that antibiotic producing bacteria in consortia isolated from a limestone cave could degrade the synthetic polymer polyethylene. Maximum biodegradation was observed when the medium was augmented with calcium salt, indicating higher degradation potential of bacterial consortia when in a medium close to the natural chemical composition of their native environment. Spectroscopy and microscopy results showed certain changes in low-density polyethylene test samples as compared to a control, indicating microbial breakdown of LDPE. Further research is needed to understand the mechanism of degradation of LDPE at a molecular level. All the bacterial strains were found to viable at the end of the experiment.

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