ARTICLE

Biodegradation of low-density polyethylene by bacteria isolated from serpentinization-driven alkaline spring

Denisse Yans Z. Dela Torre, Lee A. Delos Santos, Mari Louise C. Reyes and Ronan Q. Baculi*

Department of Biology, College of Science, University of the Philippines Baguio, Baguio City, Philippines

Low-density polyethylene (LDPE), a commonly-used packaging material that is resistant to degradation under natural conditions, contributes to environmental pollution. Recently, considerable attention has been focused on microorganisms capable of degrading polyethylene-based plastic waste. In this study, bacterial strains with unique capabilities to use low-density polyethylene as sole carbon source were isolated from a hyperalkaline spring (pH 11) through enrichment culture procedures. The process of biodegradation was observed for 90 days in a synthetic medium containing LDPE films. The bacterial isolates, phylogenetically-affiliated with *Bacillus krulwichiae*, *Bacillus pseudofirmus*, *Prolinoborus fasciculus,* and *Bacillus* sp., were able to reduce the weight of the residual polymer up to 9.9%, 8.3%, 5.1%, and 6.3%, respectively. The viability of the isolates was correlated with an increased protein density of the biomass. SEM analysis revealed that the strains exhibited strong adhering capabilities as indicated by morphological changes that occurred on the surface of LDPE films incubated with bacterial isolates. Fourier transform infrared spectra showed changes in keto carbonyl index, ester carbonyl bond index, internal double bond index, and vinyl bond index supporting the depolymerization activity of the isolates. Reduction in percent crystallinity of the films incubated with isolates was also observed. This study confirmed the ability of the selected microorganisms to utilize LDPE as carbon source, and to degrade the films without thermal and oxidative pre-treatments.

*Corresponding author
Email Address: rqbaculi@up.edu.ph
Date received: January 13, 2018
Date revised: March 21, 2018
Date accepted: April 14, 2018

KEYWORDS

alkaline spring, biodegradation, Fourier transform infrared spectroscopy, low- density polyethylene

INTRODUCTION

Plastics are long-chain synthetic polymer that are used in various fields as replacement for paper and other cellulose-based products for packaging due to their bio-inert and excellent moisture barrier properties (Andrady 2011). The annual global demand for plastics has consistently increased over the years and presently stands at about 245 million tons. Unfortunately, the accumulation of plastic wastes in the environment causes pollution, reduces soil fertility, minimizes water percolating capacity of plants, threatens animal life by plastic ingestion, and releases harmful chemicals that lead to health problems (Pramila and Ramesh 2015). These negative effects are clearly apparent as plastics are resistant to the natural process of degradation

(Kumar et al. 2007). One of the most common type of synthetic polymers found in solid waste is low-density polyethylene (LDPE).

LDPE is one of the most inert plastic materials, a characteristic that is largely contributed by its high molecular weight, three-dimensional structure and hydrophobicity as proven by several long-term degradation studies (Hadad et al. 2005). In another study (Potts, 1978), no sign of biodegradation was observed in polyethylene sheet incubated in moist soil for 12 years. Further, polyethylene films incubated in soil for 32 years showed only partial degradation (Otake et al. 1995). Several biodegradation studies, however, reported the potential of photo- and thermal-based pre-treatments in enhancing biodegradation rate of polyethylene. In a long-term study on the biodegradation conducted in soil, the UV-irradiated ^{14}C-labelled polyethylene samples, released < 0.5% carbon dioxide by weight after 10 years. Consistent with its inert nature, the non-irradiated^{14}C-labelled polyethylene sheet incubated in soil for the same length of time evolved < 0.2 % carbon by weight (Albertsson and Karlsson 1990). It has been proposed that UV exposure and thermal treatment generate macro radical in the amorphous region of the polyethylene film and that through a series of reactions, these free radicals will then be converted to carbonyl groups that can be utilized by microorganisms in the degradation process (Raut et al. 2015).

Biodegradation entails the utilization of polymers by microorganisms as sources of carbon and energy for their growth (Albertsson et al. 1997). Microbial degradation involves the use of extracellular and intracellular depolymerases by organisms to break down organic substances. Exoenzymes from microorganisms introduce a chain cleavage of the polymer producing oligomers and monomers that can be absorbed and utilized for microbial metabolism. Most microbes that demonstrated the capacity to degrade polyethylene were identified as bacterial species belonging to genera *Pseudomonas*, *Streptococcus*, *Staphylococcus*, *Micrococcus*, and fungi species belonging to genera *Aspergillus* and *Trichoderma* (Swift1997).

Although the the abovementioned microorganisms were found to thrive in highly favorable environments, several studies have reported that extremophiles inhabiting extremely harsh conditions can also degrade polyethylene. Hadad et al. (2005) and Duddu et al. (2015) have recently reported the degradation of polyethylene by the thermophilic bacteria *Brevibacillus borstelensis* and *Streptomyces coelicoflavus*. These reports showed a metabolically diverse nature of extremophiles and incited a compelling need to identify other organisms with potential for degradation. Microorganisms isolated from alkaline environments have been extensively investigated for various biotechnological applications but their potential for synthetic polymer degradation remains to be explored. These microorganisms are present in naturally-occurring alkaline environments, such as ocean water, soda lakes and deserts, underground alkaline water, and alkaline springs that are distributed worldwide (Horikoshi 2011). In the Philippines, ophiolites have been identified as potential sources of hyperalkaline waters driven by a geological process called serpentinization. This process involves the weathering of mineral-rich mafic and ultramafic rocks upon exposure to carbon dioxide-charged waters producing serpentinite, hydroxide, and carbonates along the process (Tiago et al. 2004). During dissolution of minerals in rocks, protons are used and the dominant hydroxide ions drive up the pH of the aqueous system, generating an extremely alkaline pH in these serpentinizing systems. Poon Bato Spring, a natural alkaline spring in Zambales ophiolite complex of the Philippines, is a typical Ca^{2+} - OH^- type water sourced in actively serpentinizing host rocks (Cardace et al. 2015).

In the present study, the potential of LDPE-degrading bacteria isolated from high pH spring in Zambales, Philippines was determined *in vitro* by measurement of bacterial biomass on the film, monitoring of change in weight of the LDPE samples, variation in physical properties of the LDPE film, and changes in the infrared spectra of the film after biotic exposure.

MATERIALS AND METHODS

Substrate

Low-density polyethylene (LDPE) in the form of garbage bags were bought from a local supermarket. The substrate was confirmed as LDPE after comparison with a spectral library having 93% match to low-density polyethylene.

Sample collection

Water samples were collected from rock crevices of the Poon Bato spring in Botolan, Zambales, Philippines (15˚18'52.34" N 120˚03'52.85" E). The samples were transferred aseptically into sterile bottles for analysis. Temperature, pH, salinity, and dissolved oxygen were measured using a water quality multisensory equipment during sample collection. Water samples were analyzed at the water testing laboratory of the Department of Science and Technology (DOST)-Cordillera Administrative Region (CAR).

Pretreatment and preparation of LDPE powder

LDPE bags were cut into small pieces of size 1.5 cm x 1.5 cm and boiled with xylene for 15 minutes. After boiling, the pieces were crushed with a blender at 3,000 rpm for 10minutes. The resulting LDPE powder was washed with 70 % ethanol, dried overnight in a hotoven at 60^0C and stored at room temperature (Das and Kumar 2014).

Enrichment and isolation of LDPE-degrading bacteria

Enrichment cultures were prepared by adding 10 mL of water sample to 90 mL of synthetic medium (SM) containing (g/L of distilled water): NH_4NO_3, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 1.0; $CaCl_2 \cdot 2H_2O$, 0.1; KCl, 0.15; yeast extract (Difco), 0.1; and 1.0 mg for each of the following micronutrients: $FeSO_4 \cdot 6H_2O$; $ZnSO4 \cdot 7H_2O$ and $MnSO_4$ (Balasubramanianet al. 2010) . The solution was supplemented with 300 mg of LDPE powder while the pH of the medium was adjusted to pH 11 by the addition of Na_2CO_3. The Na_2CO_3 was autoclaved separately from the other components of the medium and was mixed after cooling to 50°C. The sample solutions were incubated at 37^0C in a shaker incubator for 5 days.

Samples taken from the enrichment culture were serially diluted and subsequently plated onto SM agar. All plates were incubated at 37^0C for 5 days. Distinct colonies were picked and purified by streak plating method using the same medium. The cultures were examined for their cultural characteristics, Gram reaction, and were subjected to biochemical tests such as catalase and oxidase tests. Pure cultures were maintained in 40% glycerol stock solution and stored at -70^0C biofreezer.

Sequencing and phylogenetic analysis of 16S rRNA genes of the isolates

Amplification of 16S rRNA gene of the isolates was performed by Macrogen Inc., Seoul, Korea using the bacterial primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGGTTACCTTGTTACGACTT) (Lane 1991). Sequencing was carried out by using Big Dye terminator cycle sequencing kit v.3.1 (Applied BioSystems, USA). ChromasPro software

Table 1: Physicochemical characteristics of water samples obtained from Poon Bato Spring

Variable	Measurement
pH	11
Temperature (˚C)	28
Conductivity (µS/cm)	781
Dissolved Oxygen (mg/L)	3.6
Calcium (mg/L)	50.3
Chloride (mg/L)	20.3
Sulfate (mg/L)	4.16
Magnesium (mg/L)	1.31
Iron (mg/L)	0.12
Nitrate (mg/L)	<LoD*
Manganese (mg/L)	ND**

*LoD — Limit of Detection (0.43 mg/L for nitrate)
**ND — Not Detected

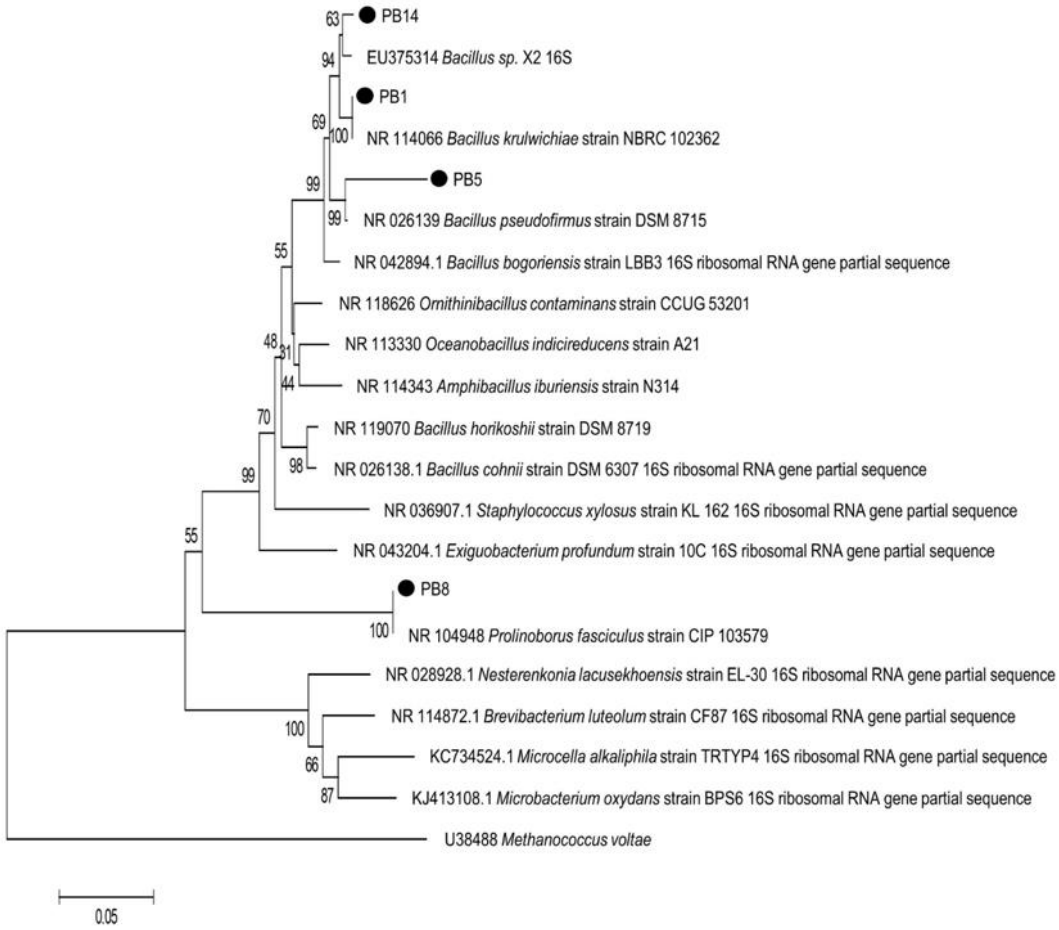

Figure 1: Phylogenetic tree showing the relationship among 16S rRNA gene sequences of the bacterial isolates with reference sequences obtained through BLAST analysis. The sequences alignment was performed using Clustal W program and the tree was constructed using Neighborhood-Joining with Kimura 2 parameter distances in MEGA 6 software. Bootstrap values (1000) replicates are shown at the nodes. Sequences designated as PB were obtained from the present study. *Methanococcus voltae* was used as outgroup.

(*http://www.technelysium.com.au/)* was used to manually evaluate the sequences and to remove low quality regions usually at the start and end of the fragment. DNA sequences were analyzed using the BLAST tool at the National Centre for Biotechnology Information (NCBI) server (*http://blast.ncbi.nlm.nih.gov*). The sequences were submitted for multiple alignments with reference sequences from the GenBank database using Clustal W. Phylogenetic trees were constructed with the Maximum Likelihood algorithm of MEGA 6 software (Tamura et al. 2011) with evolutionary distances calculated according to Kimura's two-parameter correction method. The phylogenetic trees were evaluated through bootstrap analysis from 1000 bootstrap replicates. All sequences generated have been deposited in the GenBank database under accession numbers MF407325-MF407328.

Polyethylene film biodegradation assay

Each isolate collected was tested for LDPE-degrading efficiency. Flasks containing 300 mL of SM broth were added with pre-weighed LDPE strips (1.5 cm x 1.5 cm size) that had been dried overnight at 60°C, weighed, disinfected for 30 minutes in 70% ethanol, air-dried for 15 minutes in laminar airflow chamber, and treated with mineral oil (0.05%) to allow bacterial adhesion (Das and Kumar 2014). One mL of a 24-hour bacterial culture maintained at a turbidity equivalent to 0.5 McFarland standards was added to the suspension. Flasks containing non-inoculated SM medium supplemented with

Table 2: Phenotypic characteristics of bacterial isolates from Poon Bato Spring

Isolate	Cultural characteristics (color, colony form, margin, surface, elevation	Cell morphology	Gram Reaction	Catalase Test	Oxidase Test
PB1	Colorless, circular, entire, smooth, raised	Rod	+	+	+
PB5	Yellow, circular, curled, smooth, convex	Rod	+	+	+
PB8	Colorless, circular , entire, smooth, raised	Rod	_	+	+
PB14	Colorless, circular, entire, smooth, flat	Rod	+	+	+

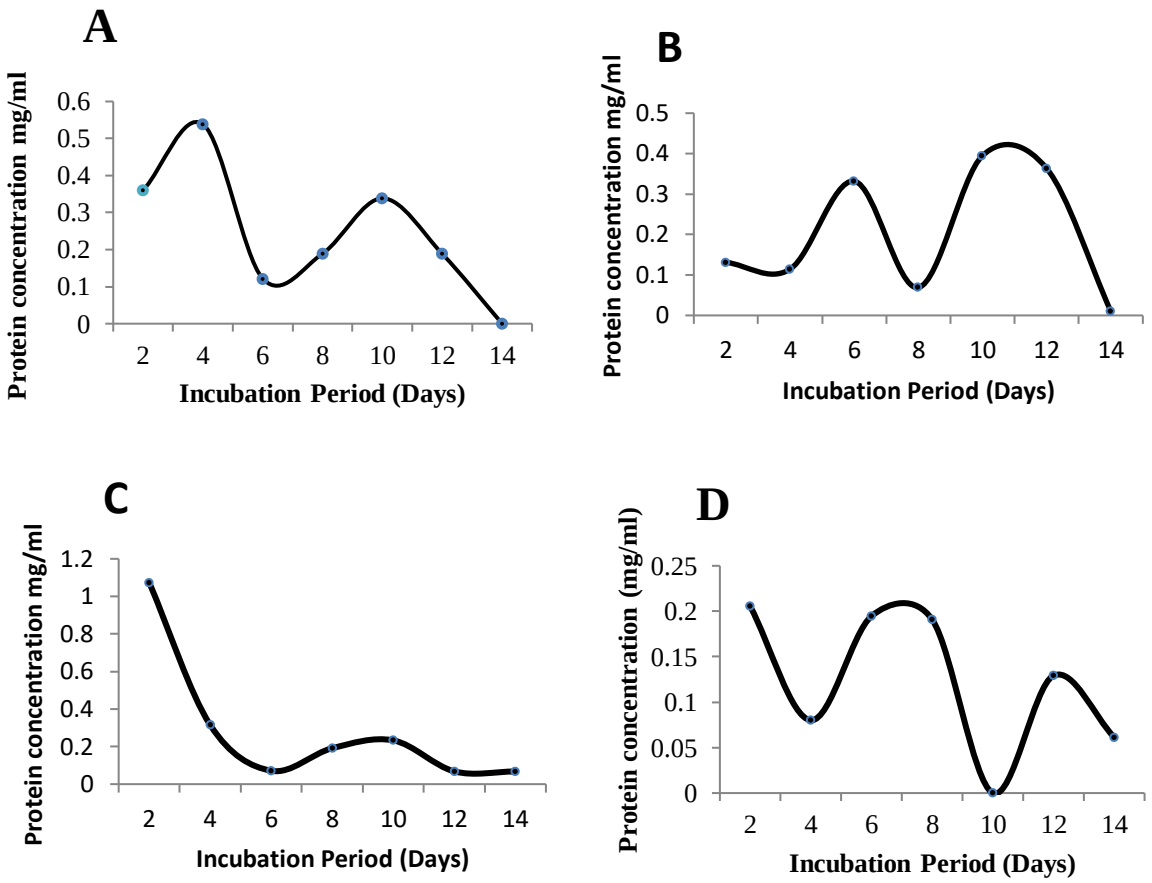

Figure 2: Protein content of attached bacterial isolates (A) *Bacillus krulwichiae* PB1 (B) *Bacillus pseudofirmus* PB5 (C) *Prolinoborus fasciculus* PB8 and (D) *Bacillus* sp. PB14 on the surface of low-density polyethylene film.

polyethylene served as the control. All flasks were incubated at 37°C for 90 days (Gajendiran et al. 2016).

Determination of dry weight of residual LDPE

To facilitate accurate measurement of the dry weight of residual LDPE, recovered LDPE films were washed with 2% (v/v) aqueous sodium dodecyl sulfate (SDS) solution for 4 hours and then with distilled water (Gilan et al.2004). The mineral oil added on the LDPE films was removed using chloroform and then SDS wash. The washed LDPE films were dried overnight on a filter paper at 60°C before weighing. The weight loss was calculated using the following equation: Percentage of weight loss = [(Initial weight - Final weight)/ Initial weight] x 100 (Konduri et al. 2010).

Quantitative estimation of bacterial population on LDPE films

Indirect estimation of the bacterial biomass was done by determining the protein concentration on the LDPE films utilizing the procedure of Hadad et al. (2005) with modifications. Each bacterial isolate was inoculated into 14 flasks each containing SM broth supplemented with 30 mg LDPE strips (1.5 cm x 1.5 cm size). The flasks were incubatedat 37°C from 1 to 14 days. The protein concentration was checked

Table 3: Bacterial isolates and their phylogenetic affiliations

Isolate	Nearest Phylogenetic Affiliation	Accession number of the nearest phylogenetic affiliation	% similarity	Phylogenetic group
PB1	*Bacillus krulwichiae*	NR_114066	97%	Firmicutes
PB5	*Bacillus pseudofirmus*	NR_026139	95%	Firmicutes
PB8	*Prolinoborus fasciculus*	NR_104948	99%	Proteobacteria
PB14	*Bacillus sp.*	EU375314	99%	Firmicutes

Table 4: Carbonyl index obtained from FTIR spectra of low density polyethylene (LDPE) incubated for 90 days with bacterial isolates vs non-inoculated polyethylene

	Carbonyl Index		
Isolate	**30 days**	**60 days**	**90 days**
Non-inoculated (Control)	0.009	0.011	0.006
PB1	0.009	0.005	0.008
PB5	0.003	0.004	0.005
PB8	0.014	0.009	0.007
PB14	0.012	0.015	0.007

every day by boiling the films with 5 mL of 0.5M of NaOH solution for 30 minutes. The solution was then centrifuged for 1 minute at 5000rpm. The supernatants were combined and subjected to UV-Vis spectrophotometer with the NaOH solution serving as blank solution. Finally, the concentration of proteins was computed using the equation described by Groves et al. (1968): Protein concentration (mg/mL) = A_{280} (1.55) – A_{260} (.76).

Fourier transform infrared (FTIR) spectroscopy analysis of polyethylene

After 90 days of incubation, the LDPE films were washed with 2% SDS followed by sterile distilled water and dried overnight at 50°C prior to submission for FTIR analysis. Changes in the structure of polyethylene samples incubated with the bacterial strains and non-inoculated control were analyzed by Fourier transform infrared (FTIR) spectroscopy (Shimadzu Prestige, Pike Technologies, USA). FTIR scan per sample was taken at spectrum ranging from 400 cm^{-1} to 4000 cm^{-1} at ambient room temperature. Characteristic peaks of the spectra were determined using Essential FTIR Software (Operant LLC, Madison, U.S.A). The relative absorbance intensities of the ester carbonyl bond (1740 cm^{-1}), the keto carbonyl bond (1715 cm^{-1}), the terminal double bond (vinyl) bond (1650 cm^{-1}) and the internal double bond (908 cm^{-1}) to that of the methylene bond (1465 cm^{-1}) were evaluated using the following equation (Albertsson et al. 1997): keto carbonyl bond index (KCBI) = I_{1715}/I_{1465}; ester carbonyl bond index (ECBI) = I_{1740}/ I_{1465}; vinyl bond index (VBI) = I_{1650}/I_{1465}; and internal double bond index (IDBI) = I_{908}/I_{1465}. The crystallinity percentage of the polymer was calculated using the following equation: % crystallinity = 100 – [{1 – (*Ia*/1.233*Ib*) / 1 + (*Ia*/*Ib*)} × 100]: where *Ia* is the absorbance at 1473 and *Ib* is absorbance at 1463. Carbonyl index was computed using the following equation: CI = absorption at 1740 cm^{-1} / absorption at 1460 cm^{-1} (Kyaw et al. 2012). Samples incubated with bacterial isolates were subjected to FTIR analysis after every 30 days of incubation.

Scanning electron microscopy of polyethylene

To examine the morphological changes on the surface of polyethylene incubated with bacterial isolates, polyethylene films were recovered from the SM media after 90 days of incubation. The samples were washed with 2% SDS and distilled water and finally flushed with 70% ethanol to remove surface-adhered organisms. The films were air-dried overnight, gold-coated, and visualized using scanning electron microscope (JEOL, Model JSM-6390LV).

Statistical analysis

Experimental results were expressed as the mean ± standard deviation of three independent replicates. Test of significance of the differences among mean values was determined by one-way analysis of variance (ANOVA) followed by Tukey's test at the 5% level of significance ($P < 0.05$) using IBM Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM Corporation, Armonk, NY, USA.

RESULTS AND DISCUSSION

Isolation and identification of LDPE-degrading bacteria

Bacterial isolates were obtained from Poon Bato Spring, a natural alkaline environment in the Zambales ophiolite complex of the Philippines. The measured pH was 11 and the temperature was 28˚C. Chemical analysis of the water samples revealed the presence of calcium, magnesium, sulfate, chloride, and iron. Calcium had the highest amount at 50.3 mg/L (Table 1). The high concentration of calcium detected in the site confirmed that the spring is typical Ca^{2+}-OH^- type water generated by active serpentinization process (Cardace et al. 2015). Initially, nine isolates were obtained by enrichment culture supplemented with LDPE as the sole carbon source. However, only four strains have significantly reduced the weight of the polymer after 90 days of incubation. The four isolates were found to be Gram-positive except PB8, catalase-positive, and oxidase-positive (Table 2). Analysis of the 16S rRNA gene sequences revealed that the isolates belong to two major groups of bacterial phyla, phylum Firmicutes and phylum Proteobacteria. Specifically, three of the isolates were affiliated with phylum Firmicutes, which contains low G+C Gram-positive bacteria, represented by the genus *Bacillus* while the remaining isolate was grouped under phylum Proteobacteria, a diverse group of Gram-negative bacteria, represented by genus *Prolinoborus*. All of the

sequences analyzed showed high similarities (95-99%) to 16S rRNA gene sequences of already characterized strains (Table 3). A phylogenetic analysis confirmed their similarity to the respective species (Fig. 1).

The phylum Firmicutes consists mostly of Gram-positive bacteria which are phenotypically- and physiologically-diverse, allowing them to inhabit a wide variety of environments including hypersaline habitats. Members of this phylum have been reported in bacterial diversity studies of alkaline environments such as the Lonar Lake in India (Joshi et al.2008; Kanekar et al. 2007), Cabeco de Vide aquifer in Portugal (Tiago et al. 2004), the Cedars in California (Morrill et al.2013) and the Manleluag hyperalkaline spring in the Philippines (Baculi et al. 2015) where the Firmicutes similarly made up the large portion of the total isolates in the aforementioned sites. The genus *Bacillus* is the most commonly found aerobic, eubacterial alkaliphiles both in soda lakes and in less selective environments (Horikoshi and Akiba 1982; Krulwich and Guffanti 1983). The high occurrence of *Bacillus* species in highly alkaline saline environments may be due to their nutritional versatility, stress-tolerant thick-walled endospores, and broad tolerance for environmental extremes (Satyanarayana et al. 2012). The *Bacillus krulwichiae* isolated in this study was closely related to an obligate alkaliphilic, halotolerant type strain bacterium isolated from alkaline Lonar Lake in India (Tambekaret al. 2014) and from soil in Japan (Yumuto et al. 2003). Another isolate obtained in this study was closely affiliated with *Bacillus pseudofimus* previously isolated from alkaline water and sediment samples in the study of Tambekar and Tambekar (2013) and Ma et al. (2012), respectively. Hence, it can be inferred that the alkaline pH of Poon Bato Spring can possibly support the growth of this bacterium.

The phylum Proteobacteria encompasses a major proportion of Gram-negative bacteria that are known to occur in a broad spectrum of habitats including marine, hypersaline, alkaline and acidic habitats due to their extreme metabolic diversity (Horikoshi et al.2011). Microbial diversity studies have previously reported the persistence of the members of the phylum in alkaline environments such as the Tablelands Ophiolite in Newfoundland, Canada (Brazelton et al. 2013), the ikaite columns and the surrounding Ikka Fjords in Greenland (Glaring et al. 2015), and the serpentinizing springs at The Cedars, California (Suzuki et al. 2014). Members of the genus *Prolinoborus* are distinct from other Gram-negative rods by using proline as the only source of carbon and nitrogen, lack of ability to use carbohydrate compounds, and nitrogenase activity (Brenner et al. 2005). The isolate PB8 exhibited high sequence similarity to the only species of this genus, *Prolinoborus fasciculus* (formerly *Aquaspirillum fasciculus*) which has been isolated from pond water and alkaline Lonar Lake in India (Strength et al. 1976; Tambekar et al.2014). The presence of an isolate in Poon Bato Spring that is closely related to *P. fasciculus* strain is a new discovery since it has not been reported in other serpentinization–hosted environment although it has been isolated from a soda lake in India (Tambekar et al. 2014).

Weight reduction analysis

Polyethylene degradation was initially monitored by dry weight reduction of polyethylene films incubated with the individual isolates for 90 days. The present findings show that four bacterial strains related to *Bacillus* and *Prolinoborus* were capable of utilizing LDPE as carbon source. Among the nine isolates tested, four were capable of LDPE degradation based on weight loss after 90 days of incubation with significant differences at $p<0.05$ when compared to the negative control. The percentage weight loss was calculated as 9.9± 0.05%, 8.3 ±0.1%, 5.1±0.2% and 6.3±0.2% for isolates identified as *Bacillus krulwichiae*, *Bacillus pseudofirmus*, *Prolinoborus fasciculus*, and *Bacillus sp.*, respectively. No weight loss was estimated in the polyethylene films incubated without any of the bacterial isolates.

Several studies have been undertaken in determining the role of bacteria in biodegradation. However, only a few studies have reported on the degrading capability of isolates from extreme environments. Studies conducted by Hadadet al. (2005) and Dudduet al. (2015) showed that the thermophilic bacteria *Brevibacillus borstelensis*and *Streptomyces coelicoflavus* were able to degrade LDPE with 11% and 30% weight loss in just 30 days and 120 days, respectively. Moreover, halophilic bacteria have been reported to have the capability to degrade oils (Hao and Lu 2009) and aromatic compounds (Nicholson and Fathepure 2004).

During the 90-day incubation period, the highest percentage of polyethylene biodegradation measured in terms of weight loss was 9.9±0.05% demonstrated by isolate PB1 which is related to *Bacillus krulwichiae*. The biodegradation level exhibited by this strain was higher than the previously reported 5% and 9% weight loss estimates of LDPE treated with *Pseudomonas sp.* and *Pseudomonas putida*, respectively (Tribedi and Sil 2012; Kyawet et al. 2012). In general, the biodegradation rate of LDPE demonstrated by the isolates was higher compared to the 3.5 to 8.4% weight loss values of polyethylene incubated for 10 years (Potts 1978; Albertsson and Karlsson 1990) and thermo-oxidized polyethylene incubated for 3-5 years with *Arthrobacter parrafineus* in the soil (Albertsson et al.1997).

The degradation of polymer is associated with several physical and chemical processes that lead to only small structural changes but significant damage to the quality of the material (Sudhakaret al. 2008). Microorganisms use their exo-enzymes to break down large and complex polymers into smaller chains of oligomers, dimers or monomers with the help of several enzymes such as dehydrogenases, dioxygenase and monooxygenase. These smaller molecules can then be absorbed by the semi-permeable membrane of microbes and be used as the carbon and energy source (Singh et al. 2016). The results obtained demonstrate the remarkable ability of *Bacillus* species to utilize LDPE as a carbon source. Several *Bacillus* species, which include *B. amyloliquefaciens*, *B. sphericus*, *B. cereus*, *B. pumilus*, *and B. subtilis* (Sudhakaret al. 2008), have been observed to utilize LDPE. However, *Bacillus krulwichiae* and *Bacillus pseudofirmus* isolated in this study have not been reported as showing the same capability. Similarly, the degradation of polyethylene by *Prolinoborus fasciculus* has yet to be cited in any biodegradation study inferring that such competency is a new discovery.

Quantitative estimation of bacterial population on LDPE films

Measurement of bacterial biomass on the polyethylene film was done through analysis of protein content of cells attached on the films. The results indicated a biphasic pattern of growth characterized by two exponential stages for most isolates (Fig. 2). Rapid colonization of the polyethylene occurred during the first two to six days of incubation with isolates PB1, PB5, and PB14. The initial increase in biomass can possibly be due to the utilization of mineral oil as a carbon source (Gilan et al. 2004).

Isolate PB8 was unable to utilize mineral oil as source of carbon. Mineral oil could enhance adherence of cells on the surface of polyethylene presumably by mediating the hydrophobic interactions between the cells on the bacterial biofilm, and the polymer (Balasubramanian et al. 2010; Gilan et al. 2004). The eventual decrease in bacterial biomass after the first exponential growth indicates a depletion of mineral oil as carbon source. Once the mineral oil has been consumed, a low-density population of cells capable of using LDPE as the carbon source

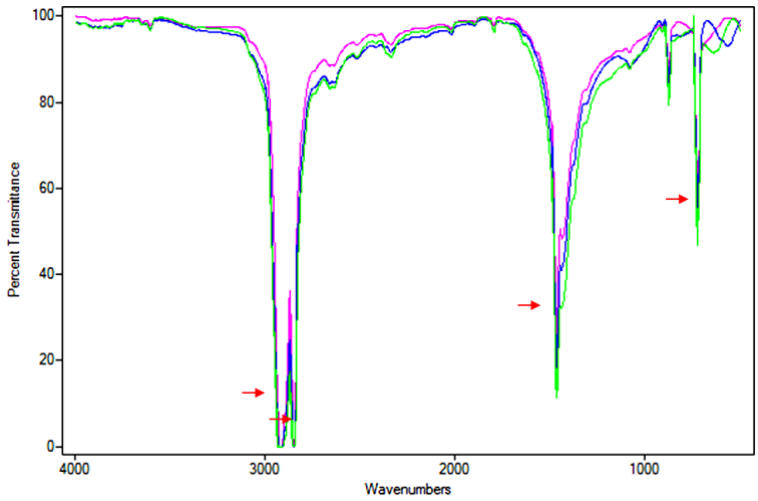

Figure 3: Fourier transform infrared spectrum of untreated LDPE after 90 days of incubation. The colors indicate the length of incubation period when the measurement was taken: green = 30 days of incubation, blue = 60 days of incubation, pink = 90 days of incubation). Red arrows indicate characteristic absorption bands assigned at different wave numbers: 719 cm^{-1} (C-H bend-mono), 1472 cm^{-1} (C=C stretch), and 2919 and 2850 cm^{-1} (both due to C-H stretch)

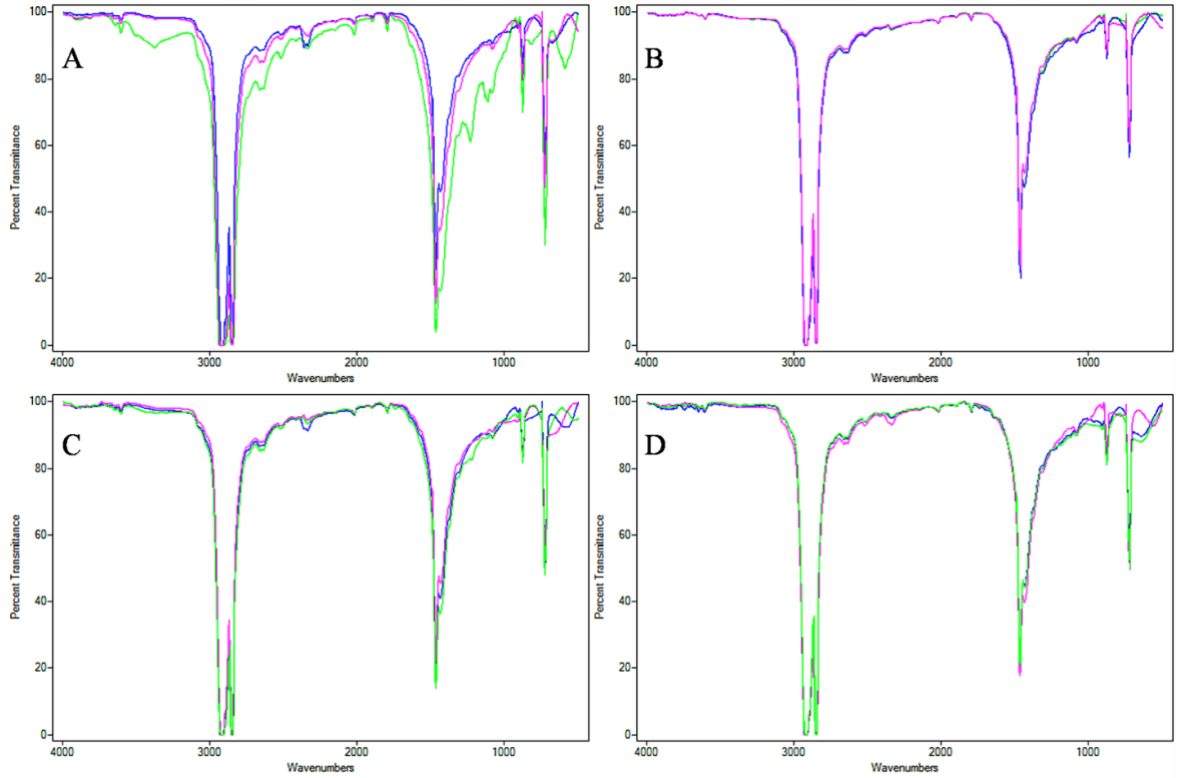

Figure 4: Fourier transform infrared spectra of LDPE after 90 days of incubation with bacterial isolates (A) *Bacillus krulwichiae* PB1, (B) *Bacillus pseudofirmus* PB5, (C) *Prolinoborus fasciculus* PB8, and (D) *Bacillus* sp. PB14. The colors indicate the length of incubation period when the measurement was taken: green = 30 days of incubation, blue = 60 days of incubation, pink = 90 days of incubation).

developed on the polyethylene. This observation confirms the results of Hadad et al. (2005) on biodegradation of polyethylene by the thermophilic bacterium, *Brevibacillus borstelensis.* In their study, bacterial colonization of the polyethylene measured as extractable protein by *B. bortelensis* initially increased followed by a gradual decrease leading to the absence of extractable protein until the 20th day of incubation. A slow and constantly proliferating biofilm eventually developed on the polyethylene film following the decline in the protein content of the fast-proliferating cells. Similarly, Gilan et al. (2004) reported the development of low-density biofilm of *Rhodococcus ruber* using LDPE film as carbon source after the utilization of mineral oil.

Fourier transform infrared (FTIR) spectroscopy analysis

Fourier transform infrared analysis enables the determination of degradation products, chemical moieties incorporated into the polymer such as branches, co-monomers, unsaturation, and presence of additives such as antioxidants (Sudhakar et al. 2008). Analyses were conducted to monitor the formation or disappearance of acids (1715 cm^{-1}), ketones (1740 cm^{-1}), and double bonds (1640 and 908 cm^{-1}) to explain the mechanisms of biodegradation process. The control spectra of the polyethylene film not treated with any of the isolates displayed distinctive bands that characterize a typical LDPE (Fig. 3). The characteristic absorption bands were assigned at 2920 cm^{-1} (CH_2 asymmetric stretching), 2850 cm^{-1} (CH_2 symmetric stretching),

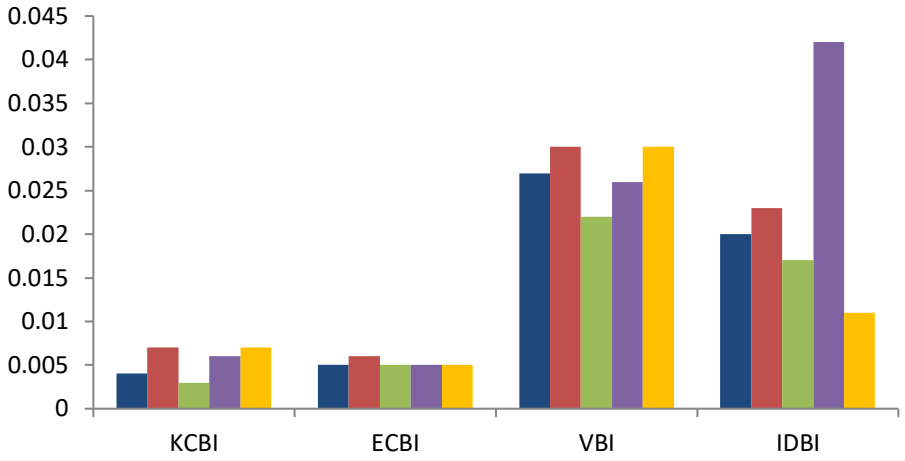

Figure 5: FTIR indices of LDPE films exposed to the isolates for 90 days. (KCBI-keto carbonyl index; ECBI-ester carbonyl index; VBI-vinyl bond index; IDBI-internal double bond index) [(Control ( );Bacillus krulwichiae PB1( );Bacillus pseudofirmus PB5( );Prolinoborus fasciculus PB8 ( ); Bacillus sp. PB14 ( )].

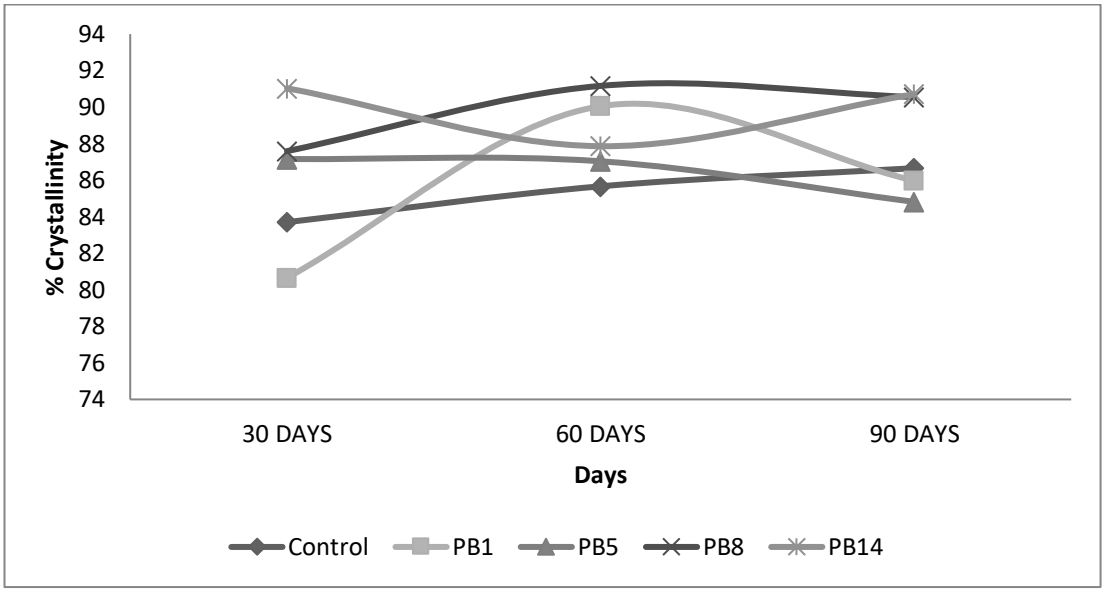

Figure 6: Kinetics of crystallinity changes of untreated LDPE films and films treated with bacterial isolates throughout the 90-day incubation period.

1473, 1462, and 1437 cm^{-1} (C=C bending deformation), and 727 and 723 cm^{-1} (C-H bend mono) (Gulmine 2002).

The intensity of peaks varied in different regions after 30, 60 and 90 days of incubation of the test samples with the bacterial isolates. Generally, there was a decrease in the intensity of peaks that corresponded to C=C bending deformation (1473, 1462, and 1437 cm^{-1}), and C-H bend mono (727 and 723 cm^{-1}) on the films incubated with the isolates (Fig. 4). Moreover, strong absorption peaks at 1462 cm^{-1} and 1437cm^{-1} became less prominent after microbial treatment. The intensity of these peaks was further reduced in the case of isolates PB1 and PB8.

Carbonyl residues at the typical carbonyl peak (1712 cm^{-1}) were reduced upon incubation with bacterial isolates PB1, PB8, and PB14 indicating carbonyl utilization (Fig. 4). The initial abiotic step in degradation of polyethylene involves polymer chain oxidation which forms carbonyl groups that subsequently forms carboxylic groups that eventually undergo β-oxidation. Complete degradation happens via citric acid cycle resulting in the formation of CO_2 and H_2O (Albertsson et al. 1997). The carbonyl index is the ratio between the absorbance peaks of carbonyl group to CH_2 at 1462-1463cm^{-1}. Initially, carbonyl index increased relative to the control for strains PB1, PB8, and PB14 after 30 days of incubation (Table 4). Prolonged exposure of the films to these isolates led to a decrease in carbonyl index presumably due to the utilization of low molecular weight oxidation products. Similar activity was reported in the polyethylene degradation studies by Gilan et al. (2004), Sudhakar et al. (2008), Hadad et al. (2005), and Volke-Sepulveda et al. (2001). On the other hand, the increase in carbonyl index was evident throughout the 90-day incubation of the polyethylene films with isolate PB5 which can be attributed to biological activities of the microorganisms that lead to the formation of ketone or aldehyde C=O groups (Esmaeili 2013) which was confirmed by peak formations. Moreover, this may also be due to microbial oxidation of short chains of alkanes forming carboxylic acids (Manzur et al.2004). This increase in carbonyl index as a result of biodegradation is similar to the results of Balasubramanian et al. (2010).

The KCBI, ECBI, and VBI of the polyethylene increased when incubated with most of the isolates (Fig. 5). Although KCBI and VBI were lower than the control when using isolate PB5, a consistent increase in KCBI and VBI values was observed with this isolate throughout the 90-day incubation time presumably due to the emergence of keto and ester carbonyl as the primary products of enzyme activity particularly involving oxidoreductase (Karlssonand Albertsson 1998). Furthermore,

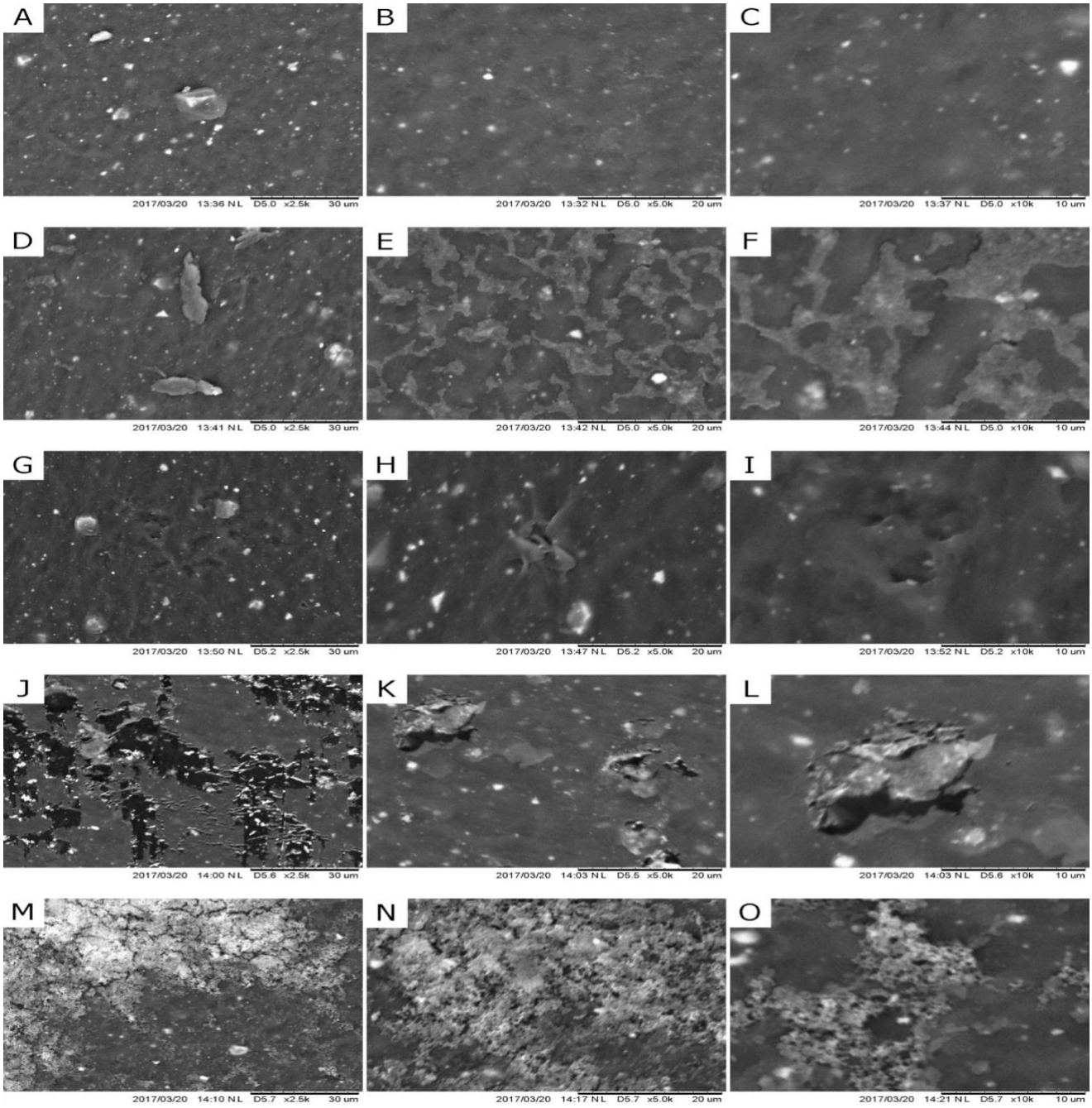

Figure 7: Scanning electron micrographs revealing the surface morphology of LDPE films after 90 days of incubation without bacterial isolate (A-C), and with bacterial isolates *Bacillus krulwichiae* PB1 (D-F), *Bacillus pseudofirmus* PB5 (G-I), *Prolinoborus fasciculus* PB8 (J-L), and *Bacillus* sp. PB14 (M-O) (2,500x, 5,000x and 10,000x).

IDBI increased when the polyethylene was incubated with isolates PB1 and PB8.

Low-density polyethylene is insoluble in nature due to their crystalline structure (Pierre and Chiellini 1986). In this study, there was an observed reduction in the crystallinity of the polyethylene after 90 days incubation with isolates PB5 and PB1 (Fig. 6). The decrease in crystallinity supports the conversion of crystalline structures of the LDPE films into an amorphous structure as a consequence of degradation. On the other hand, abiotic degradation results in the remaining polymer becoming more organized after release of the degradation products (Albertsson et al.1995) as indicated by the increase in percent crystallinity of the control. Furthermore, the observed increase in crystallinity in the film incubated with PB1 and PB8 after 60 days of incubation can be attributed to microbial attack on amorphous fractions rather than crystals because the latter are more resistant to enzymatic attack.The subsequent decrease in crystallinity at the end of the 90-day incubation for both strains was possibly caused by the microbial attack on smaller crystal structures which were formed during the initial attack on amorphous fractions (Manzur et al. 2004). The results obtained are similar to the findings of Manzur et al.(2004) on the biodegradation of physicochemically- treated LDPE by a consortium of filamentous fungi and of Volke-Sepúlveda et al.(2001) on biodegradation of LDPE.

Scanning electron microscopy (SEM) analysis

The changes in the surface morphology of the LDPE films were investigated by SEM after 90 days of incubation. The control sample, incubated without any of the bacterial isolates, had a smooth surface with no pits, cracks or any particles attached to the surface. In contrast, LDPE films treated with the bacterial isolates had surface alterations characterized by pits, cracks,

erosions, and roughness (Fig.7). The observed changes in surface morphology suggest an ability to colonize and probably adhere to the LDPE films by means of extracellular polymeric substances that are primarily polysaccharides (Raut et al. 2015). Although further investigation is needed to elucidate the underlying mechanism of LDPE degradation, it can be inferred that the process involves a series of enzymatic solubilization steps (Kyaw et al. 2012). The adherence of microorganisms through polymeric substances plays an important role in transporting depolymerizing enzymes (Raut et al.2015). The presence of pits and cavities may be due to the absence of a uniform distribution of short branches in the polymer matrix that suggests the penetration of bacteria into the LDPE matrix during degradation (Manzur et al. 2004). Surface erosion is the primary cause of mass loss from the surface of the film (Gajendiran et al. 2016). The occurrence of several non-uniformly scattered whitened areas and erosion zones indicates surface erosion mechanism involved in degradation which might be due to enzyme catalytic action (Bhatia et al. 2014). These findings are consistent with the biodegradation efficiency of bacterial isolates reported by Negi et al. (2009).

Generally, the isolates showed degradation efficiency based on the reduction in dry weight of the polymer, formation and disappearances of several functional groups as revealed by the changes in peaks of FTIR spectra, and morphological changes on the polymer as shown by SEM analysis.

CONCLUSIONS

The present study on *in*-vitro biodegradation clearly showed that bacteria isolated from a hyperalkaline spring can degrade low-density polyethylene, a polymer that is resistant to natural process of degradation. Bacterial isolates, phylogenetically related to *Bacillus krulwichiae, Bacillus pseudofirmus, Prolinoborus fasciculus,* and *Bacillus sp.*, were able to colonize and degrade LDPE films based on weight reduction, appearance and disappearance of various functional groups as revealed by FT-IR analysis, and structural changes on the film by SEM analysis. Moreover, the actively increasing metabolism of the isolates as revealed by protein quantification supported their ability to utilize LDPE as carbon source. The degradation capability of the bacterial strains could be due to the occurrence of certain enzymatic activities that lead to a chain cleavage and assimilation of the hydrocarbon backbone of the polymer. This study demonstrated the ability of the isolates to degrade polyethylene even in the absence of prior oxidation treatments. The results showed that selected microorganisms exhibited great potential for LDPE biodegradation, a discovery which can be used in reducing solid waste currently accumulating in natural environments. Determination of bacterial protein concentrations using samples incubated at longer periods combined with cell surface hydrophobicity analysis will lead to improvements in understanding the colonization and biofilm formation on the film. Microbial consortia can be formulated and evaluated for their efficacy in enhancing the rate of LDPE biodegradation.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Department of Biology, College of Science, University of the Philippines Baguio for providing laboratory facilities. Likewise, they would like to thank Dr. Elsie Jimenez for providing comments and for editing the article.

CONFLICTS OF INTEREST

The authors declare no potential conflicts of interests with respect to the research, authorship, and/or publication of this article.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Denisse dela Torre, Lee delos Santos and Louise Reyes contributed to the overall conceptualization and design of the study, acquisition, analysis and interpretation of data, writing and revision of the manuscript.

Baculi, Ronan: Contributed in the overall conceptualization and design of the study, writing and critical revision of the manuscript for intellectual content.

REFERENCES

Albertsson AC, Barenstedt C, Sigbritt K, Torbjorn L. Degradation product pattern and morphology changes as means to differentiate abiotically- and biotically-aged degradable polyethylene. Polymer 1995; 36:3075-3083.

Albertsson AC, Erlandsson B, Hakkarainen M, Karlsson S. Molecular weight changes and polymeric matrix changes correlated with the formation of degradation products in biodegraded polyethylene. J Environ Polym Degrad 1998; 6:187-195.

Albertsson AC, Karlsson S. The influence of biotic and abiotic environments on the degradation of polyethylene. Prog Polym Sci 1990; 15 ;177–192.

Albertsson AC, Andersson SO, Karlsson S. The mechanism of biodegradation of polyethylene. Polym Degrad Stab1997; 18:73–87.

Andrady AL. Microplastics in the marine environment. Marine Pollution Bulletin 2011; 62:1596–1605.

Baculi R, Lantican NB, delos Reyes III FL, Raymundo AK. Prokaryotic community analysis of a hyperalkaline spring in the Philippines using 16S rRNA gene clone library construction. Phil J Sci 2015:144 (1):1-12.

Balasubramanian V, Natarajan K, Hemambika B, Ramesh N, Sumathi CS, Kottaimuthu R, Rajesh Kanan V. High-density polyethylene (HDPE)-degrading potential bacteria from marine ecosystem of Gulf of Mannar, India. Lett Appl Microbiol 2010;51, 205-211.

Bhatia M, Girdhar A, Tiwari A, Nayarisseri A. Implications of a novel *Pseudomonas* species on low density polyethylene biodegradation: an *in vitro to in silico* approach. Springer Plus 2014; 3:497.

Brazelton WJ, Morrill PL, Szponar N, Schrenk MO. Bacterial communities associated with subsurface geochemical processes in continental serpentinite springs. Appl Environ Microbiol 2013; 79(13):3906–16.

Brenner DJ, Krieg NR, Staley JT, Garrity GM. *Bergey's Manual of Systematic Bacteriology*, 2nd ed., vol. 2, New York: Springer-Verlag, 2005.

Cardace D, Meyer-Dombard, D, Woycheese, K, Arcilla, C. Feasible metabolisms in high pH springs of the Philippines. Front Microbiol 2015; 6:10.

Das MP, Kumar S. An approach to low-density polyethylene biodegradation by *Bacillus amyloliquefaciens*. Biotech 2014;5:81-86.

Duddu M, Tripura K, Guntuku G, Divya D. Biodegradation of low-density polyethylene (LDPE) by a new biosurfactant-producing thermophilic *Streptomyces coelicoflavus* NBRC 15399^{T}.African J Biotech 2015; 14(4):327-340.

Esmaeili A, Pourbabaee AA, Alikhani HA, Shabani F, Esmaeili E. Biodegradation of low-density polyethylene (LDPE) by mixed culture of *Lysinibacillus xylanilyticus* and *Aspergillus niger* in soil. PLoS ONE 2013; 8(9): 71720.

Gajendiran A, Krishnamoorthy S, Abraham J. Microbial degradation of low-density polyethylene (LDPE) by *Aspergillus clavatus* strain JASK1 isolated from landfill soil. Biotech 2016; 6(1):52.

Gilan I, Hadar Y, Sivan A. Colonization, biofilm formation and biodegradation of polyethylene by a strain of *Rhodococcus ruber*. Appl Microbiol Biotechnol 2004; 65:97-104.

Glaring MA, Vester JK, Lylloff JE, Abu Al-Soud W, Sorensen SJ, Stougaard P. Microbial diversity in a permanently cold and alkaline environment in Greenland. PLoS ONE 2015; 10(4): e0124863.

Groves WE, Davis FC, Sells BH. Spectrophotometric determination of microgram quantities of protein without nucleic acid interference. Anal Biochem 1968; 22:195-210.

Gulmine JK, Janissek PR, Heise HM, Akcelrud L. Polyethylene characterization by FTIR. Polymer Testing 2002; 21:557-563.

Hadad D, Geresh S, Sivan A. Biodegradation of polyethylene by the thermophilic bacterium *Brevibacillus borstelensis*. J Appl Microbiol 2005; 98:1093- 1100.

Hao R, Lu A. Biodegradation of heavy oils by halophilic bacterium. Prog Nat Sci 2009; 19:997-1001.

Horikoshi K, Akiba T. Alkaliphilic microorganisms: A new microbial world. New York: Springer, 1982.

Horikoshi K, Antranikian G, Bull AT, Robb FT, Stetter KO. Extremophiles handbook, New York: Springer Science & Business Media 2011, 99-118.

Joshi AA, Kanekar PP, Kelkar AS, Shouche YS, Vani AA, Borgave SB, Sarnaik SS. Cultivable bacterial diversity of alkaline Lonar Lake, India Microb Ecol 2008; 55:163–172.

Kanekar, PP, Joshi AA, Kelkar AS, Borgave SB, Sarnaik, SS. Alkaline Lonar Lake, India – A treasure of alkaliphilic and halophilic bacteria. Proc Taal 2007; 12:1765-1774.

Karlsson S, Albertsson AC. Biodegradable polymers and environment interaction. Polym Eng Sci 1998; 38:1251-1253.

Konduri MKR, Anupam KS, Vivek JS, Kumar RDB, Narasu ML.Synergistic effect of chemical and photo treatment on the rate of biodegradation of high density polyethylene by indigenous fungal isolates. Int J Biotech Biochem 2010; 6:157-174.

Krulwich TA, Guffanti AA. Physiology of acidophilic and alkalophilic bacteria. Adv Microb Ecol 1983; 24:173-214.

Kumar S, Hatha AAM, Christi KS. Diversity and effectiveness of tropical mangrove soil microflora on the degradation of polythene carry bags. Int J Trop Biol 2007; 55:777–786.

Kyaw BM, Champakalakshmi R, Sakharkar MK, Lim CS, Sakharkar KR. Biodegradation of low-density polythene (LDPE) by *Pseudomonas* species. Indian J Microbiol 2012; 52(3): 411-419.

Lane DJ.16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, (Eds). Nucleic Acid Techniques in Bacterial Systematics. Chichester: John Wiley and Sons, 1991.

Ma C, Zhuang L, Zhou SG, Yang GQ, Yuan T, Xu RX. Alkaline extracellular reduction: Isolation and characterization of an alkaliphilic and halotolerant bacterium, *Bacillus pseudofirmus* MC02. J Appl Microbiol 2012; 112:883–891.

Manzur A, Limon-Gonzalez M, Favela-Torres E. Physicochemically treated LDPE by a consortium of filamentous fungi. J Appl Polym Sci 2004; 92:265-271.

Morrill PL, Kuenen JG, Johnson OJ, Suzuki S, Rietze A, Sessions AL, Nealson KH. Geochemistry and geobiology of a present-day serpentinization site in California: The Cedars. Geochim Cosmochim Ac 2013; 109:222-240.

Negi H, Kapri A, Zaidi M, Satlewal A, Goel R. Comparative *in vitro* biodegradation studies of epoxy and its silicone blend by selected microbial consortia. Int BiodeterBiodegrad2009; 63(5):553–558.

Nicholson C, Fathepure B. Biodegradation of benzene by halophilic and halotolerant bacteria under aerobic conditions. Appl Environ Microbiol 2004; 70(2):1222–1225.

Otake Y, KobayashiT, Ashabe H, Murakami N, Ono K. Biodegradation of low-density polyethylene, polystyrene, polyvinyl-chloride, and urea-formaldehyde resin buried under soil for over 32 years. J Appl Polym Sci 1995; 56:1789– 1796.

Pierre ST, Chiellini E. Biodegradability of synthetic polymers used for medical and pharmaceutical applications: Part 1 – Principles of hydrolysis mechanisms. J Bioact Compat Polym 1986; 1:467–497.

Potts JE. Biodegradation in aspects of degradation and stabilization of polymers. ed. Jelinek, H.H.G. New York: Elsevier 1978, 617-658.

Pramila R, Ramesh KV. Potential biodegradation of low-density polyethylene (LDPE) by *Acinetobacter baumannii*. African J Microbiol Res 2015; 7(3):24–28.

Raut S, Raut S, Sharma M, Srivastav C, AdhikariB, Sen S. *Curvularia lunata SG1* using particle swarm optimization strategy. Indian J Microbiol 2015; 55(3):258–268.

Satyanarayana T, Raghukumar C, Shivaji S. Extremophilic microbes: Diversity and perspectives. Current Science 2005; 89(1):78-90.

Satyanarayana T, Sharma A, Mehta D, Puri AK, Kumar V, Nisha M, Joshi S. Biotechnological applications of biocatalysts from the firmicutes *Bacillus* and *Geobacillus* species. In: Microorganisms in Sustainable Agriculture and Biotechnology. Netherlands: Springer, 2012; pp 343-379.

Singh G, Chandoha-Lee C, Zhang W, Renneckar S, Vikesland PJ, Pruden A. Biodegradation of nanocrystalline cellulose by two environmentally-relevant consortia. Water Res2016; 104: 137 -146.

Strength WJ, Isani B, Linn DM, Williams FD, Vandermolen GE, Laughon BE, Krieg NR. Isolation and characterization of *Aquaspirillum fasciculus* sp. nov., a rod-shaped, nitrogen-fixing bacterium having unusual flagella. Int J Syst Bacteriol 1976; 26:253-268.

Sudhakar M, Doble M, Sriyutha Murthy P, Venkatesan R. Marine-microbe-mediated biodegradation of low and high-density polyethylene. Int Biodeterior Biodegrad 2008; 61: 203-213.

Suzuki S, Kuenen JG, Schipper K, van der Velde S, Ishii Si, Wu A. Physiological and genomic features of highly alkaliphilic hydrogen-utilizing Betaproteobacteria from a continental serpentinizing site. Nat Commun 5 2014.

Swift G. Non-medical biodegradable polymers: Environmentally degradable polymers. In: Handbook of biodegradable polymers. Amsterdam: Hardwood Academic 1997; 473–511.

Tambekar S, Tambekar D. Compatibility and washperformance analysis of alkaline protease from *Bacillus pseudofirmus* with commercial detergents. Int J Pharm Chem Biol Sci2013; 3 (30):738-744.

Tambekar DH, Tiwari AA, Tambekar SD. Studies on production of antimicrobial substances from *Bacillus* species isolated from Lonar Lake. Ind J Appl Res2014; 4(8): 502-506.

Tambekar DH, Zanwar DP, Borkar PR. Detoxification of phenol by haloalkaliphilic bacteria isolated from Lonar Lake. Indian J Appl Res2014; 6:431-433.

Tamura K. MEGA6: Molecular evolutionary genetics analysis version. Mol Biol Evol 2013; 30(12):2725–2729.

Tiago I, Chung AP, Veríssimo A. Bacterial diversity in a nonsaline alkaline environment: heteroptrophic aerobic populations. Appl Environ Microbiol 2004; 73878-7387.

Tribedi P, Sil AK. Low-density polyethylene degradation by *Pseudomonas sp.* AKS2 biofilm. Environ Sci Pol Res 2012; 20:4146-4153.

Volke-Sepulveda T, Saucedo-Castaneda G, Gutierrez-Rojas M, Manzur A, Favela-Torres E. Thermally treated low-density polyethylene biodegradation by *Penicillium pinophilum* and *Aspergillus niger*. J Appl Polym Sci 2001; 83:305–314.

Yumoto I, Yamaga S, Sogabe Y, Nodasaka Y, Matsuyama H, Nakajima K, Suemori A. *Bacillus krulwichae* sp. nov., a halotolerant obligate alkaliphile that utilizes benzoate and hydroxybenzoate. Int J Syst Evol Microbiol 2003; 53:1531–1536.