Enhanced in vitro biodegradation of low-density polyethylene using alkaliphilic bacterial consortium supplemented with iron oxide nanoparticles

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Low-density polyethylene (LDPE) has been used extensively leading to its overaccumulation in the environment. Recent studies have shifted their focus on devising techniques that could accelerate the biodegradation process such as the formulation of microbial consortia and the use of nanoparticles. In this study LDPE biodegradation was carried out by using a bacterial consortium composed of the two most efficient LDPE-degrading alkaliphilic bacterial strains isolated from hyperalkaline spring (pH 11) in Zambales, Philippines, through enrichment techniques. Phenotypic and molecular analyses revealed that the isolates were phylogenetically affiliated to *Bacillus pseudofirmus* and *Bacillus agaradhaerens*. The supplementation of iron oxide nanoparticles (IONPs) significantly increased the bacterial growth, along with shortened lag phase and longer stationary phase. The bacterial consortium, in the presence and absence of IONPs, was able to reduce the weight of the residual polymer up to 18.3 ± 0.3% and 13.7 ± 0.5%, respectively, after 60 days of incubation. Bacterial adhesion to hydrocarbon test demonstrated higher hydrophobicity of the consortium with IONPs. This result was corroborated by an increased protein content of the cells adhered to the films. End-product analysis by Fourier transform infrared and scanning electron microscopy revealed chemical bond shifting and pronounced disruption of surface texture in the presence of IONPs, respectively, thereby confirming the biodegradation process. The combination of the two isolates supplemented with IONPs exhibited maximum degradation of LDPE as revealed by various analyses. Bacteria-nanoparticle interactions are significant, and the formulation of alkaliphilic bacterial consortium grown in the presence of nanoparticles accelerates the rate of LDPE film degradation.

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Email Address: rqbaculi@up.edu.ph
Date received: February 15, 2019
Date revised: June 23, 2019
Date accepted: June 25, 2019

KEYWORDS

biodegradation, bacterial consortium, iron oxide nanoparticles, low-density polyethylene

INTRODUCTION

Plastic is an extensively used synthetic polymer due to its low cost, durability, and thermal properties. One prevalent type of plastic is polyethylene, a semicrystalline polymer built from ethylene subunits. Low-density polyethylene (LDPE) is a specific type characterized by a density of less than 940 kg/m and a high degree of short and long chain branching (Lepoutre 2006). This inert material has been used extensively leading to its overconsumption and accumulation in the environment.

Consequently polymer degradation has been used to alleviate the effects of the excessive accumulation of polyethylene. Common approaches in polyethylene degradation include thermal

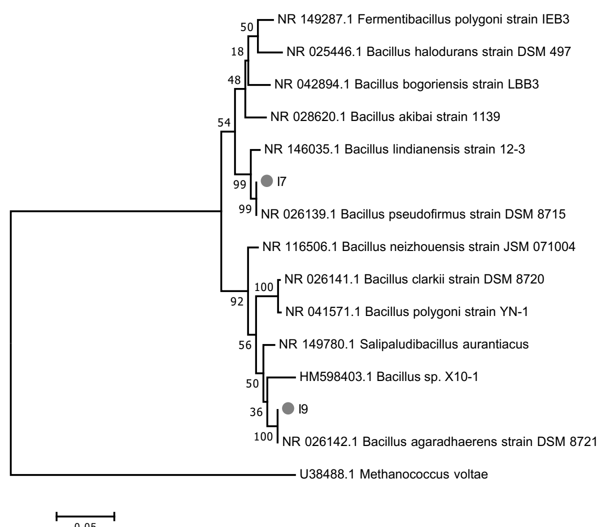

Figure 1: Phylogenetic tree based on 16S rRNA gene sequences highlighting the phylogenetic position of the two isolates relative to other alkaliphilic strains. *Methanococcus voltae* was used as an outgroup. Sequences were aligned by using ClustalW, and phylogenetic inferences were obtained by using the Maximum Likelihood method within the Mega 7 software. Bootstrap values are expressed by percentage of 1000 replicates with a Kimura 2 parameter test and shown at the branching points. The branches of the tree are indicated by the National Centre for Biotechnology Information gene accession numbers followed by the genus and species name of the type strains.

degradation, photo-induced degradation, and chemical degradation (Yousif and Haddad 2013). However, extensive use of such methods is known to be costly and detrimental to the environment (Ramakrishnan 2012). Hence there is a need to reduce such pollutants by means of a cost-effective biological approach such as microbial degradation. Biodegradation involves the use of microorganisms that produce enzymes to break down different materials such as plastics into nontoxic compounds (Sharma and Reddy 2004). Previous studies revealed that several bacteria have the ability to degrade polyethylene. Plastic-degrading bacteria isolated from mangrove and landfill soils were able to utilize polythene and LDPE, respectively, as their carbon and energy source (Kathiresan 2003; Esmaeili et al. 2013). The isolate of *Serratia marcescens* was also discovered to degrade polyethylene film up to 8% (Azeko 2016). However, focus is being directed toward the isolation of microorganisms from extreme environments such as those living in alkaline environments. Extreme microorganisms such as those isolated from alkaline environments have been extensively investigated for various biotechnological applications, but their potential for synthetic polymer degradation remains to be explored. With extremophiles' remarkable mechanisms of adaptation and novel metabolic pathways allowing them to display unique physiological capabilities, this metabolically diverse group could potentially serve as attractive sources of catalysts for biodegradation processes. Recently several *Bacillus* strains with capabilities to use LDPE were isolated from a hyperalkaline spring in the Philippines and reported a maximum weight loss of 9.9% after 90 days of incubation using the individual isolates (Dela Torre et al. 2018).

Despite the promising results in using different microorganisms in biodegradation, the rates of degradation of synthetic plastics are still relatively slow (Devi et al.2016). Thus the use of microbial consortia is being considered as it may reduce the time required for the degradation process through the positive relationship of the strains (Sarkar et al. 2011). The effectiveness of a consortium formed by using four strains isolated from polluted soil was superior to the degrading ability of the individual strains (Gallego et al. 2007). The degradation of hydrocarbons by a consortium with six bacterial strains isolated from contaminated soil was more efficient and effective than the degrading ability of a consortium with a lesser number of strains (Ghazali et al. 2004). Apart from the utilization of microbial consortia, nanoparticles are also being explored for their potential in enhancing the rate of biodegradation. Nanoparticles, with a size range of about 1–100nm, are said to have a greater surface area per weight than larger particles, allowing them to be more reactive to other molecules (Taylor et al. 2013). These particles could enhance biodegradation by increasing the growth rate of microorganisms, thereby amplifying the production of hydrolytic enzymes (Bhatia et al. 2013). Biodegrading bacteria doped with Fullerene-60 nanoparticles exhibited a significant increase in biodegradation efficiency (Sah et al. 2010). Similarly supermagnetic IONPs can enhance the degradation of LDPE by accelerating the growth of LDPE-biodegrading bacterial consortia (Kapri et al. 2010). As an alternative approach, enzymatic biodegradation has also been utilized as a pollutant biodegradation strategy because enzyme-catalyzed reactions are not constrained by nutrient requirements for microbial growth. Nanoparticles can be packaged with enzymes without compromising their catalytic activities and extending their stability under natural environmental conditions (Wang et al. 2015).

The ability of alkaliphilic bacterial consortia supplemented with nanoparticles in the biodegradation of LDPE is currently less explored. In our study the potential of an alkaliphilic bacterial consortium supplemented with IONPs in accelerating the rate of LDPE biodegradation was determined by measuring the bacterial biomass on the film, monitoring the change in weight of the LDPE samples, the variation in physical properties of the LDPE film, and the changes in the infrared spectra of the film after exposure to the bacterial isolates in vitro.

MATERIALS AND METHODS

Sampling site and sample collection

Water samples were collected from Poon Bato hyperalkaline spring in Botolan, Zambales, Philippines (15° 17' 22.5600" N, 120° 1' 28.2000" E). The samples were aseptically transferred into sterile containers for transport and analysis. Physicochemical characteristics including water temperature, pH, conductivity, and dissolved oxygen were recorded during the sampling. Analysis was also conducted to determine the concentration of elements such as calcium, magnesium, iron, manganese, and chemicals such as sulfate, chloride, and nitrate.

Enrichment and isolation of LDPE-degrading bacteria

Enrichment cultures were prepared by adding 10 mL water sample to 90 mL synthetic media (SM) which contained g/L of distilled water: NH_4NO_3, 1.0; $MgSO_4 \cdot 7H_2O$, 2.0; K_2HPO_4, 1.0; $CaCl_2 \cdot 2H_2O$, 0.1; KCl, 0.15; yeast extract, 0.1; and 1.0 mg of each of the following trace elements: $FeSO_4 \cdot 6H_2O$; $ZnSO_4 \cdot 7H_2O$, and $MnSO_4$ (Balasubramanian 2010). About 300 mg LDPE powder was supplemented to the sample before adjusting the solution to pH 11 by using Na_2CO_3, which was separately autoclaved and cooled to 50° C. The sample solutions were incubated in a shaker incubator at 37ºC for five days.

Samples from the enrichment culture were serially diluted and plated onto SM agar. Bacterial isolates were then allowed to grow by incubating the plates at 37ºC for five days. Growing colonies were selected and streaked successively onto the same media for purification. Pure cultures were maintained by using a 40% glycerol stock solution.

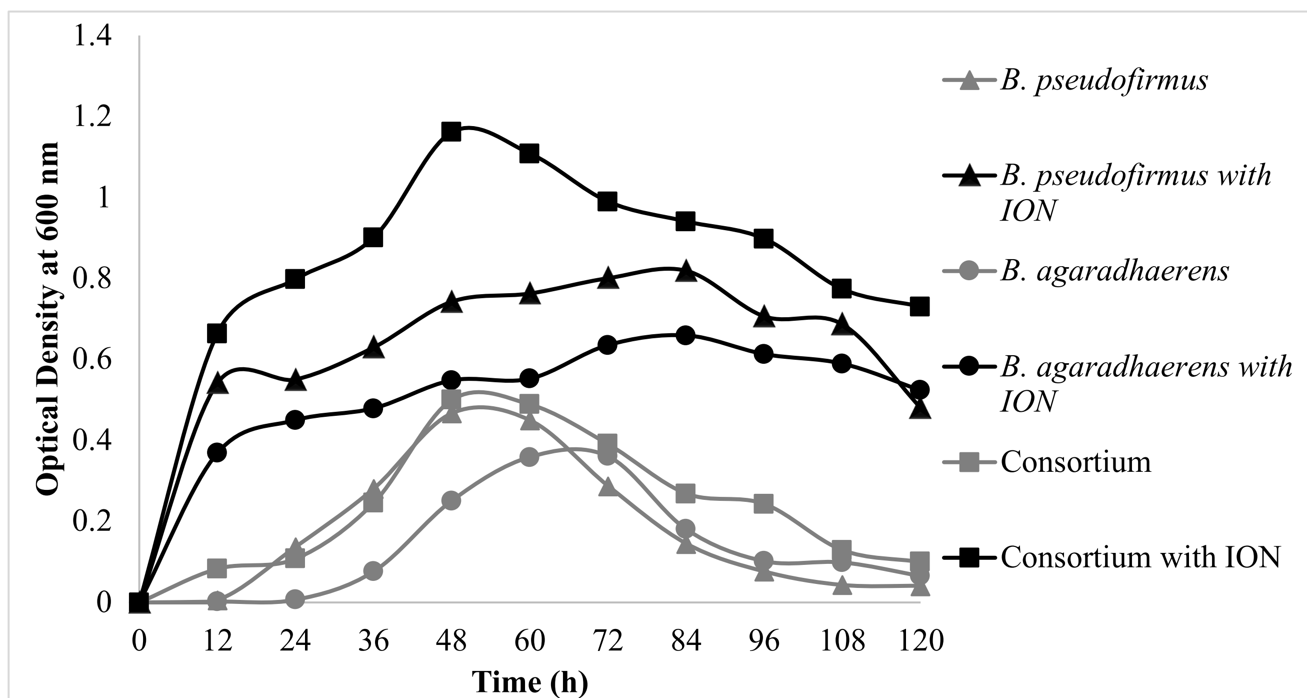

Figure 2: Comparative growth profiling of low-density polyethylene–degrading *B. pseudofirmus* I7, *B. agaradhaerens* I9, and consortium in the presence and absence of iron oxide nanoparticles (IONPs).

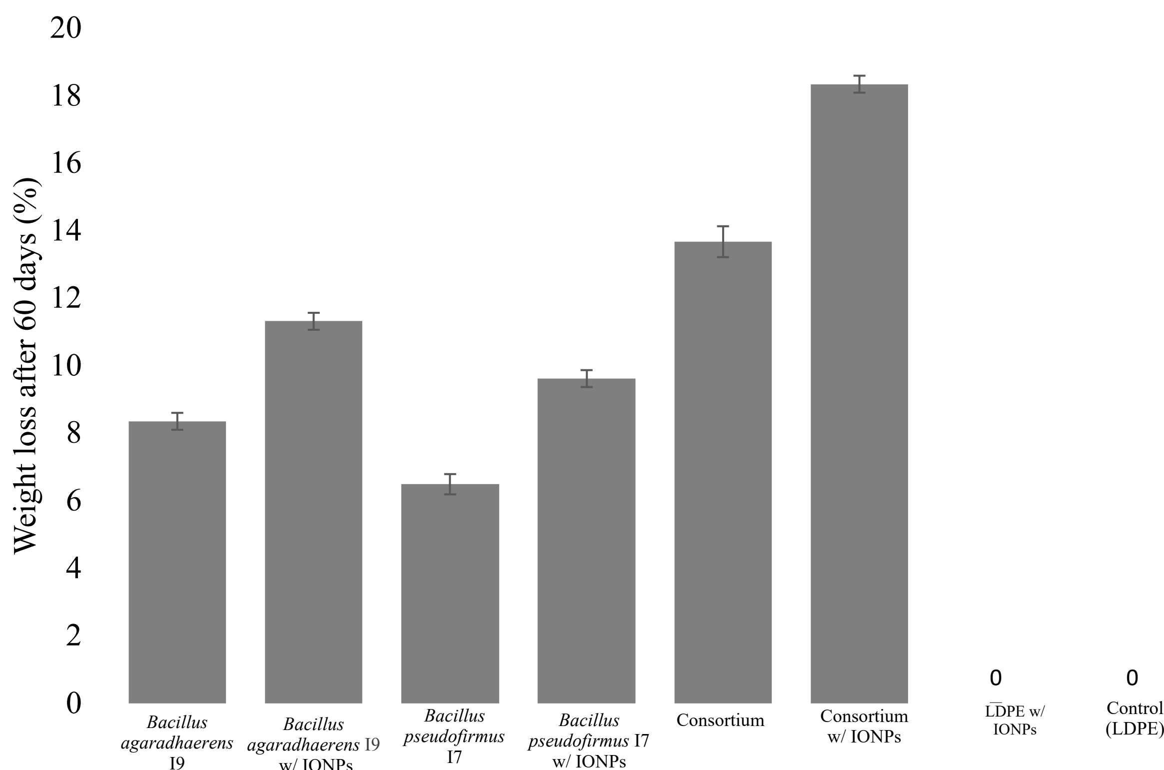

Figure 3: Weight loss percentage of low-density polyethylene (LDPE) films after 60-day incubation with bacterial isolates and consortium in the absence and presence of iron oxide nanoparticles (IONPs).

Phenotypic characterization of LDPE-degrading bacteria

The isolates were examined for their Gram reaction, endospore formation, and cultural characteristics, such as color, colony form, margin, surface, and elevation. Growth conditions such as temperature, NaCl, pH, and growth factor requirements were also determined. The determination of the NaCl requirement of the isolates was performed by using their respective solid media containing 1–15% NaCl concentrations. The growth of isolates on different pH (pH 7–12) was also assessed by using the same media adjusted by using 1 M Na_2CO_3. For the determination of optimum temperature for growth, plates were incubated at 4°C, 37°C, and 50°C for 24 hours. Selected biochemical tests (including hydrolysis of cellulose, starch, Tween 80, and gelatin), nitrate reduction, and activity of catalase, oxidase, protease, and keratinase enzymes were also performed.

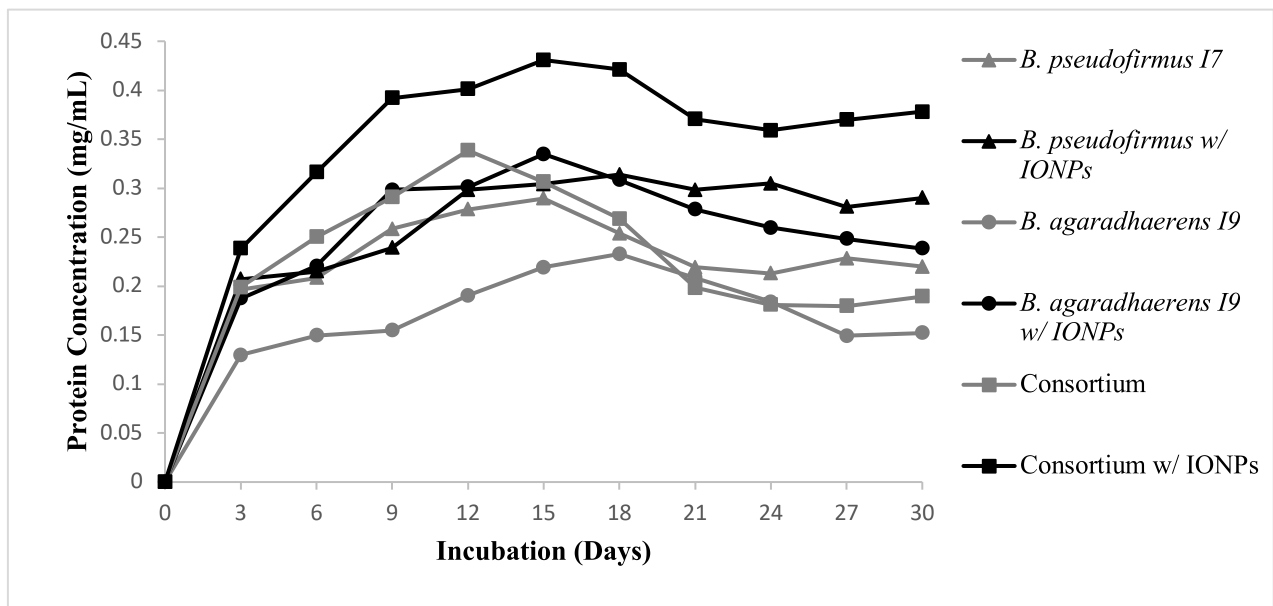

Figure 4: Protein content of surface-adhering bacterial isolates *B. pseudofirmus* I7, *B. agaradhaerens* I9, and their consortium on low-density polyethylene films in the presence and absence of iron oxide nanoparticles (IONPs).

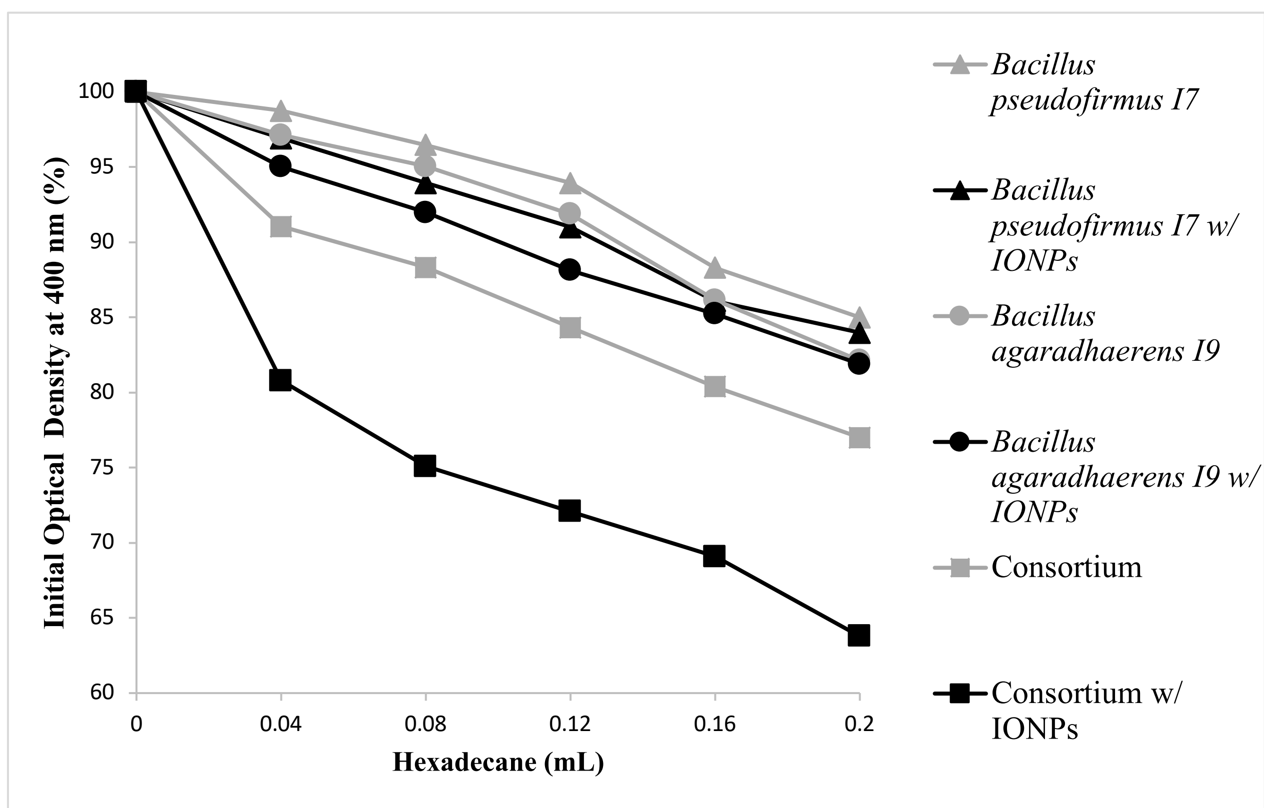

Figure 5: Hydrophobicity of the bacterial isolates and consortium in the absence and presence of iron oxide nanoparticles (IONPs) by BATH (bacterial adhesion to hydrocarbon) test. Cell suspensions were supplemented with increasing concentrations of hexadecane. The transfer of hydrophobic cells from the aqueous phase to the hexadecane is reflected as a decrease in the turbidity (optical density at 600 nm) of the bacterial suspension.

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

Pure bacterial isolates were sent for amplification and sequencing of 16S rRNA gene to Macrogen Inc., Seoul, South Korea. The amplification of 16S rRNA gene of the isolates was performed by 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 (*http://www.technelysium.com.au/)* was used to evaluate the sequences manually and to remove low-quality regions usually at the start and end of the fragment. DNA sequences were analyzed by using BLAST (basic local alignment search tool) at the NCBI (National Centre for Biotechnology Information) server (*http://blast.ncbi.nlm.nih.gov*). The sequences were submitted for multiple alignments with reference sequences from the GenBank database using Clustal W. A phylogenetic tree was constructed with the Maximum Likelihood algorithm of MEGA 7 software (Tamura et al. 2011) with evolutionary distances calculated according to Kimura's two-parameter correction method. The phylogenetic tree was evaluated through

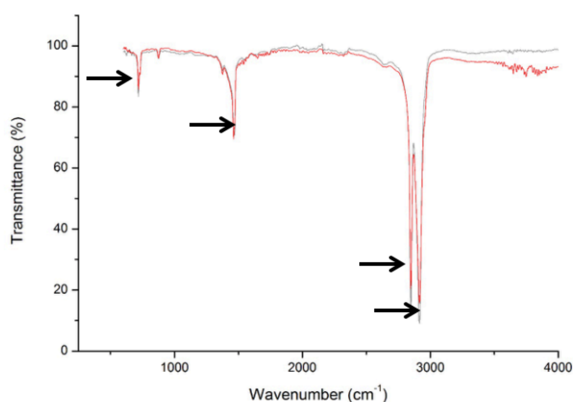

Figure 6: Fourier transform infrared spectrum of untreated low-density polyethylene after 30 and 60 days of incubation. The colors indicate the length of the incubation period when the measurement was taken: gray = 30 days of incubation, red = 60 days of incubation. Black arrows indicate characteristic 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).

bootstrap analysis from 1000 bootstrap replicates. All sequences generated in our study were submitted to GenBank under accession numbers MH517425-MH517426.

Formation of bacterial consortium for LDPE biodegradation

Two bacterial isolates that demonstrated the highest biodegradation ability based on weight loss of LDPE after 30 days were selected for the formulation of the consortium. The consortium was prepared in falcon tubes containing 35 mL of SM by adding 500 µL of each isolate that was maintained at a turbidity of 0.5 McFarland standards. The tubes were then incubated at 37 ºC for 10 hours with continuous shaking at 120 rpm (Satlewal et al. 2008).

Doping of iron oxide nanoparticles and profiling of bacterial growth rate

IONPs (Shanghai Xinglu Chemical Co., China) characterized to be 99.9% pure and 10–20 nm in size were homogenized through sonication using Soner 210H bath sonicator (Rocker Scientific Co., Ltd., China) at 50–60 Hz with 0.3 second repeating duty cycles for 2.5 minutes followed by UV sterilization (Biolab Scientific FH1000-X, Biolab Scientific Ltd., Canada). Nanoparticles were supplemented at a fixed concentration of 0.02% (w/v) in falcon tubes containing 35 mL SM inoculated with 1 mL isolate, which was maintained at a turbidity equivalent to 0.5 McFarland standards. The tubes were then incubated at 37ºC with continuous shaking at 150 rpm (Kapri et al. 2009).

The impact of IONPs in the growth rate of the isolates was monitored by obtaining OD_{600} using Hanon i8 UV-Vis spectrophotometer (Jinan Hanon Instruments Co., China) at regular intervals of 12 hours for 120 hours (Jones et al. 2013).

LDPE biodegradation assay

Various assays were performed in falcon tubes containing 35 mL SM broth (pH 11) and preweighed polyethylene strips (1.5x1.5 cm) to assess the biodegradation efficiency of the isolates and the consortium. The strips had been dried at 60°C for 24 hours, surface sterilized with 70% ethanol for 30 minutes, washed with distilled water for 20 minutes, dried in the incubator at 60°C, and treated with mineral oil (0.05%). Each assay was performed with separate tubes containing the following setups: (1) individual isolates with nanoparticles, (2) individual isolates without nanoparticles, (3) microbial consortium with nanoparticles, and (4) microbial consortium without nanoparticles. About 1 mL of 24-hour-old bacterial stock solution, maintained at a turbidity of 0.5 McFarland standards, was inoculated to each tube (Kyaw et al. 2012). By contrast, the control was prepared by adding LDPE strips to SM without bacteria. All tubes were incubated at 37°C for 60 days (Gajendiran et al. 2016).

Weight reduction analysis of LDPE

LDPE films were recovered from the SM broth and subsequently treated with chloroform, and washed with 2% sodium dodecyl sulfate (SDS) for 4 hours and distilled water for 10 minutes to remove mineral oil and biofilm residues on the surface. The washed LDPE was then placed on a filter paper and dried at 60 °C for 24 hours prior to weighing. The percentage weight loss of the LDPE was determined by using this formula (Kyaw et al. 2012): Weight loss (%) = [(Initial weight – Final weight)/Initial weight] x 100.

Evaluation of bacterial hydrophobicity

Bacterial cell surface hydrophobicity of the bacteria was determined by using the bacterial adhesion to hydrocarbon (BATH) test (Ambika 2014). Bacterial isolates were cultured in SM broth until the midexponential phase and were centrifuged at 10,000 rpm for 15 minutes. The pellets were subsequently washed with phosphate-urea-magnesium sulfate (PUM) buffer and resuspended in PUM buffer containing (gL^{-1}) K_2HPO_4, 17.0, KH_2PO_4, 7.26, Urea, 1.8, $MgSO_4.7H_2O$, 0.2 to an OD_{400} of 1.0–1.2. About 1.2 mL aliquot of the suspension was then transferred to a series of tubes with increasing volumes of hexadecane (0–0.2 mL). The tubes were shaken for 10 minutes and were allowed to stand for 2 minutes to facilitate phase separation. The aqueous phase was then pipetted from the tube and the OD_{400} using UV-Vis spectrophotometer. PUM buffer without cell culture served as the blank. The % hydrophobicity was evaluated by using the following formula: % Hydrophobicity = [(optical density of initial bacterial suspension – optical density of aqueous phase)/OD of initial bacterial suspension)] x 100.

Quantitative estimation of bacterial population on LDPE films

Bacterial biomass was estimated by determining the concentration of extractable protein in cells colonizing the surface of the LDPE sheets (Hadad et al. 2005). The bacterial isolates were inoculated into six sets of tubes, each set consisting of ten tubes with 35 mL SM broth supplemented with 30 mg of LDPE films (1.5 cm x 1.5 cm). The tubes were incubated at 37ºC, and the protein concentration was measured every 3 days for a duration of 30 days. Inoculated polyethylene sheets were removed from the culture, briefly washed with distilled water, and boiled in 5 mL of 0.5 M NaOH for 30 minutes. The suspension was then centrifuged for 1 min at 5000 rpm. All supernatants obtained from repeated centrifugation were combined and subjected to UV-Vis spectrophotometer with the NaOH solution serving as blank solution. The protein concentration was computed by using the following formula: $\text{mg ml}^{-1} = A_{280}(1.55) - A_{260}(0.76)$.

Fourier transform infrared spectroscopy analysis of polyethylene

The changes in the functional groups of the polymer incubated with the individual isolates and the bacterial consortium in the presence and absence of IONPs were analyzed through Fourier transform infrared (FTIR) spectroscopy (Shimadzu Prestige, Pike Technologies, USA). FTIR analysis of the samples was done on day 0, 30, and 60 of the incubation period. Prior to the analysis, LDPE films were washed with 2% SDS and distilled water followed by drying for 24 hours at 50ºC. Characteristic peaks of the spectra were determined by using Essential FTIR software (Operant LLC, Madison, U.S.A.). Relative absorbance

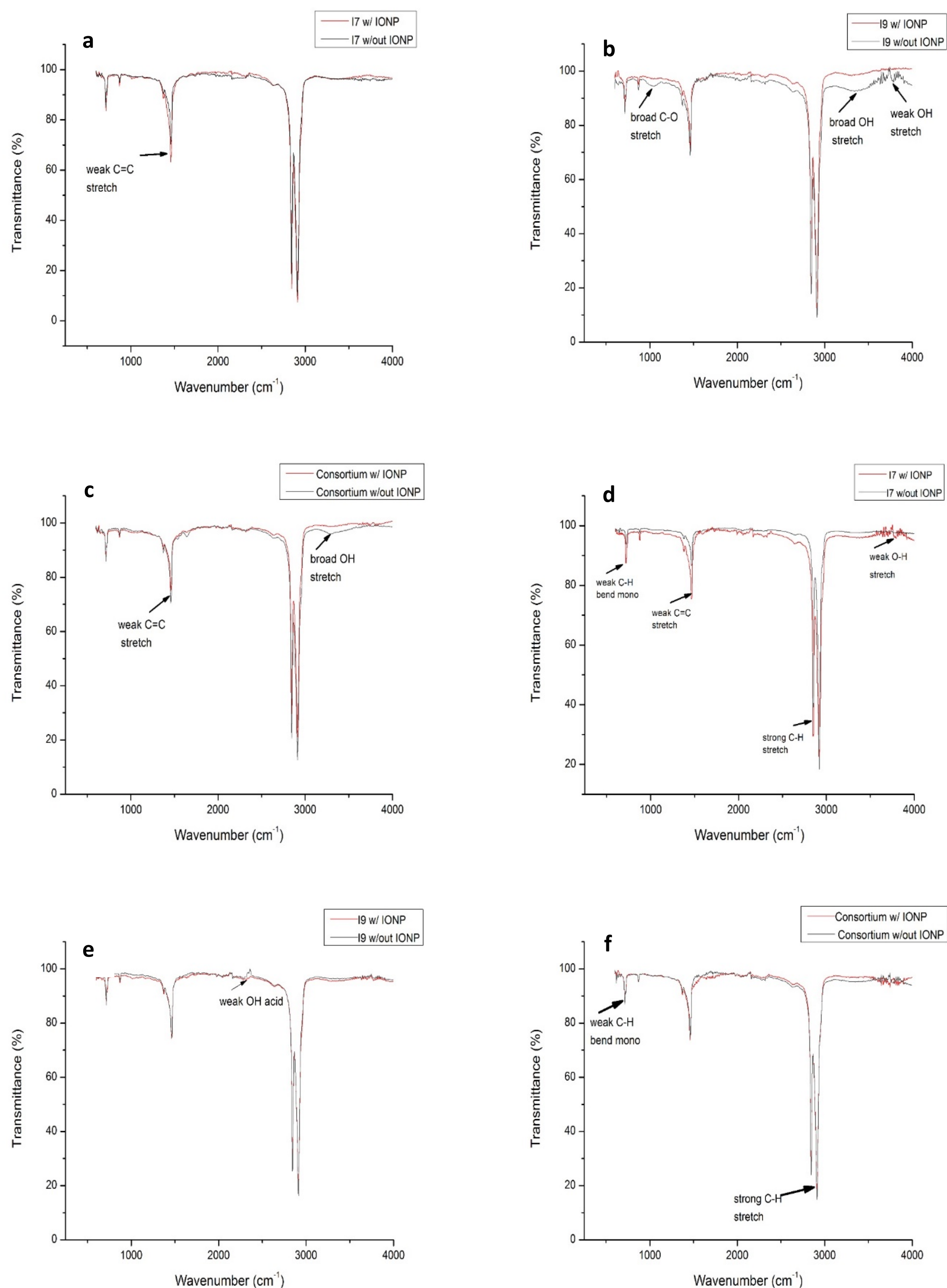

Figure 7: Fourier transform infrared spectra of low-density polyethylene after 30 days of incubation with bacterial isolates (a) *Bacillus pseudofirmus* I7, (b) *Bacillus agaradhaerens* I9, (c) consortium, and after 60 days of incubation with bacterial isolates (d) *Bacillus pseudofirmus* I7, (e) *Bacillus agaradhaerens* I9, and (e) consortium in the presence and absence of iron oxide nanoparticles (IONPs). The red color indicates incubation in the presence of IONPs, while the gray color indicates incubation in the absence of IONPS. The black arrows specify the formed peaks with their corresponding intensity and functional groups.

intensities of the ester carbonyl bond, keto carbonyl bond, terminal double bond (vinyl), and internal double bond were computed by using the following formulas (Albertson and Andersson 1987): Ester carbonyl bond index (ECBI) = I_{1740}/I_{1465}; Keto carbonyl bond index (KCBI) = I_{1715}/ I_{1465}; Vinyl Bond Index (VBI) = I_{1650}/I_{1465}; and Internal Double Bond Index (IDBI) = I_{908}/I_{1465}. Carbonyl index was calculated by using this formula (Kyaw et al. 2012): Carbonyl index (CI) = Absorption at 1740 cm^{-1}/Absorption at 1460 cm^{-1}.

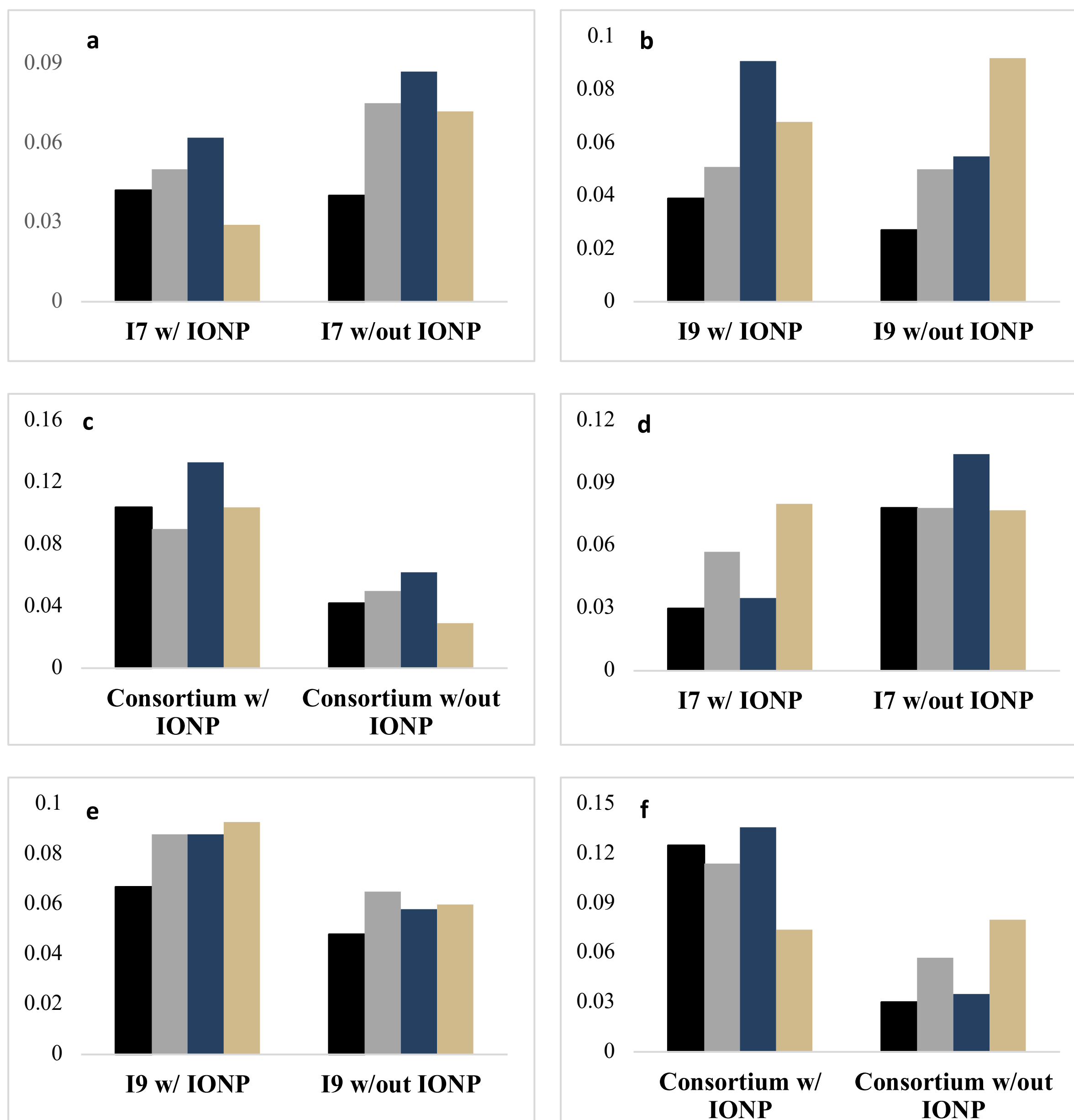

Figure 8: Fourier transform infrared indices of low-density polyethylene measured after 30 days of incubation with bacterial isolates (a) *Bacillus pseudofirmus* I7, (b) *Bacillus agaradhaerens* I9, (c) consortium, and after 60 days of incubation with bacterial isolates (d) *Bacillus pseudofirmus* I7, (e) *Bacillus agaradhaerens* I9, and (f) consortium in the presence and absence of iron oxide nanoparticles (IONPs). Colors indicate respective index: black = keto carbonyl bond index (KCBI), gray = ester carbonyl index (ECBI) , blue = vinyl bond index (VBI), and brown = internal double bond index (IDBI).

Scanning electron microscopy analysis of polyethylene
The surface morphology of the LDPE samples incubated for 60 days with the individual isolates and the bacterial consortium in the presence and absence of nanoparticles was investigated by using scanning electron microscopy (SEM) (Hitachi TM-3000-Accelerating voltage-5kV). The LDPE films were washed with 2% SDS and subsequently flushed with 70% ethanol. The films were then air-dried for 24 hours and coated with gold particles before imaging (Kyaw et al. 2012). Sample strips were mounted on the holder and scanned with 2000x, 5000x, and 8000x magnification.

Statistical analysis
All the experiments were carried out in triplicates (n = 3), and the results were expressed as mean values with standard deviation (Mean ± SD). One-way analysis of variance (ANOVA) was performed by using SPSS (IBM Statistical Package for the Social Sciences) version 20.0 to estimate the statistical significance at $P<0.05$.

RESULTS AND DISCUSSION

Isolation and identification of LDPE-degrading bacteria
Bacteria were isolated from Poon Bato spring, a natural alkaline environment in the Philippine Zambales ophiolite. The measured pH was 11, and the temperature was 28ºC. Chemical analysis of the water samples revealed the presence of dissolved oxygen, calcium, chloride, sulfate, magnesium, and iron (table 1). The high concentration of calcium detected in the site confirms that the spring is a typical Ca^{2+}-OH-type water driven by an active serpentinization process resulting in strongly alkaline circulating water. All continental sites of active serpentinization are characteristically high in ions such as

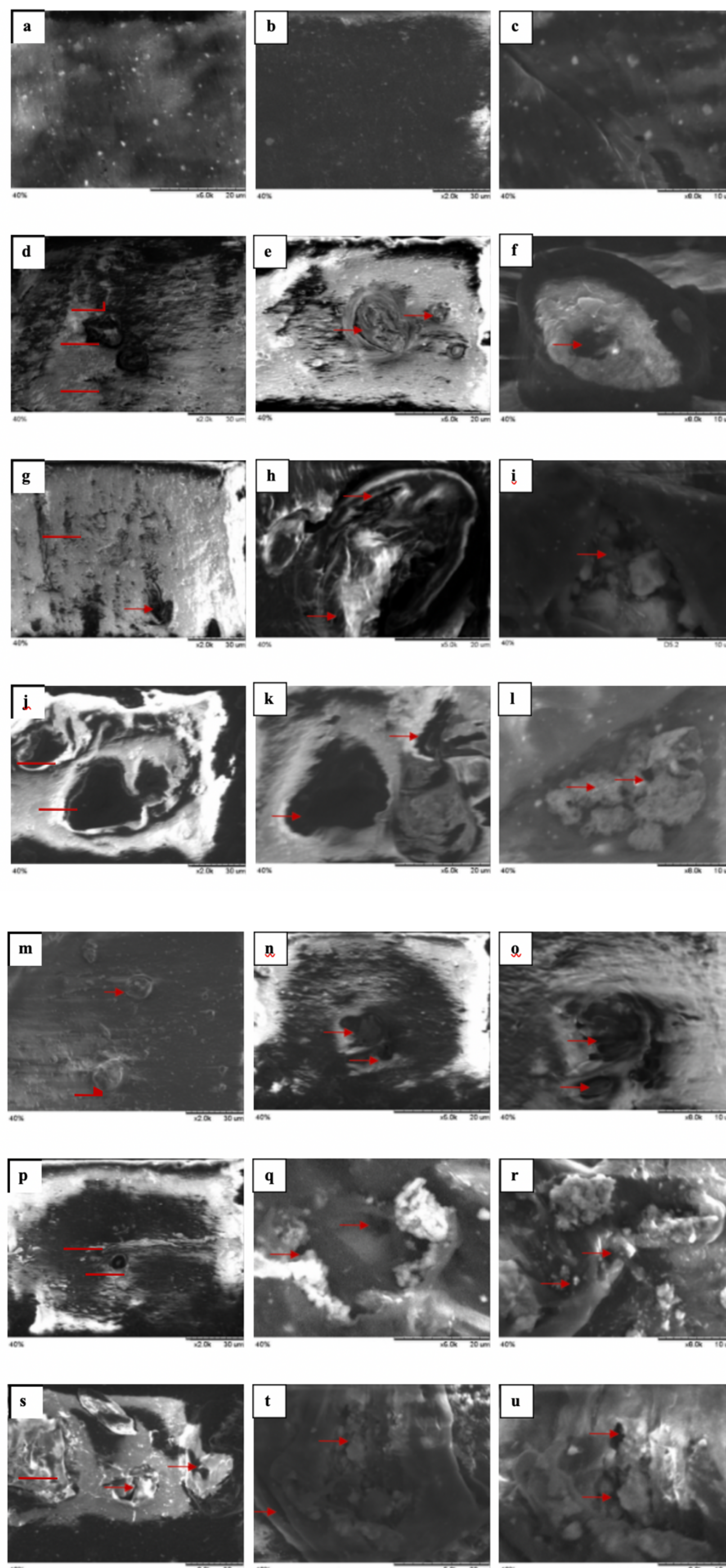

Figure 9: Scanning electron micrographs (SEMs) revealing the surface morphology of low-density polyethylene films after 60 days of incubation without bacterial isolate (a–c), and with *Bacillus pseudofirmus* I7 and iron oxide nanoparticle (IONP) (d–f), Bacillus pseudofirmus I7 without IONP (g–i), *Bacillus agaradhaerens* I9 with IONP (j–l), *Bacillus agaradhaerens* I9 without IONP (m–o), Consortia with IONP (p–r), and Consortia without IONP (s–u). The SEM images for every setup are arranged in order of increasing magnification (2000x, 5000x, and 8000x). The red arrows indicate the morphological changes in the samples characterized by surface dissolution, deepened cracks, and larger cavities with globular white areas.

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

PARAMETER	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

chloride, sodium, calcium, and magnesium as a result of the weathering of mineral-rich ultramafic rocks upon exposure to CO_2-charged waters in these environments (Cardace et al. 2015). Similar geochemical characteristics were reported in the hyperalkaline groundwaters of Cabeco de Vide in Portugal (Tiago et al. 2004), Manleluag hyperalkaline spring in the Philippines (Baculi et al. 2015), and the Cedars springs in California (Morill et al. 2013).

Using enrichment media supplemented with LDPE as carbon source, 14 morphologically distinct isolates were obtained and further screened for LDPE degradation. Two bacterial isolates, I7 and I9, were selected on the basis of highest rate of weight reduction of the polymer after 30 days of incubation. Phenotypic characterization revealed that both isolates are rod-shape, endospore-forming, Gram-positive bacteria with the ability to hydrolyze various substrates. Both isolates were classified as mesophilic, obligate alkaliphiles based on their optimal growth at 37°C and pH 9 and 10. Phenotypic characteristics of the isolates are summarized in table 2. Analysis of the 16S rRNA gene sequences revealed that both isolates belong to the phylum Firmicutes, which mostly consists of low G + C Gram-positive bacteria. Specifically, isolates I7 and I9 showed a 99% similarity to the obligate alkaliphiles *Bacillus pseudofirmus* and *Bacillus agaradhaerens* strains, respectively. A phylogenetic analysis confirmed their similarity to the respective species (fig. 1).
Members of phylum Firmicutes are physiologically and metabolically diverse Gram-positive bacteria with a wide range of nutritional requirements, allowing them to inhabit various ecological niches including hypersaline habitats (Sen et al. 2015). Several bacterial diversity studies have reported the dominance of Firmicutes over other isolates in alkaline environments such as the Lonar Lake in India (Joshi et al. 2008), Cabeco de Vide aquifer in Portugal (Tiago et al. 2004), the Cedars in California (Morill et al. 2013), and Manleluag spring in the Philippines (Baculi et al. 2015). The genus Bacillus under this phylum is the most common aerobic or facultatively anaerobic, eubacterial alkaliphiles that can be found in soda lakes and in less selective environments (Horikoshi and Akiba 1982). Members of this genus form endospores that make them suitable for dispersal and tolerant to extreme conditions including high pH levels (Nicholson et al. 2000). Additionally, their nutritional versatility and ability to produce highly stable extracellular enzymes allow them to proliferate at a highly alkaline environment. Consequently, the presence of the isolated *Bacillus pseudofirmus* and *Bacillus agaradhaerens* in alkaline habitats such as serpentinization-driven springs has already been documented. The obligate alkaliphile *Bacillus pseudofirmus* has been isolated from an Ethiopian soda lake (Martins et al. 2000) and Lonar Lake (Tambekar and Tambekar 2012), while *Bacillus agaradhaerens* has been isolated from the Ethiopian and Egypt soda lakes (Martins et al. 2000; Ibrahim et al. 2011). Hence the alkaline pH and moderate temperature in Poon Bato spring may pose a favorable environment for the growth and proliferation of these species. The ability of *B. pseudofirmus* to degrade keratin wastes and LDPE has been previously reported (Kojima et al. 2006). To date, the ability of the isolate closely related to *B. agaradhaerens* strain to degrade LDPE is a new finding for our study.

Comparative growth profiling using iron oxide nanoparticles

The influence of IONPs on the growth of the isolates and the consortium was monitored through OD reading at 600 nm. IONPs were observed to increase the growth of the individual isolates and the consortium in a synthetic medium supplemented with LDPE (fig. 2). In the absence of IONPs, the individual isolates and the consortium displayed a lag phase of 12–24 hours and less than 12 hours, respectively, and a correspondingly sustained log phase until the 48th hour for both *Bacillus pseudofirmus* I7 and the consortium, while the sustained log phase until the 60th hour was observed for *Bacillus agaradhaeren*s I9. However, the growth of the individual isolates and the consortium in the presence of IONPs was characterized by an earlier onset of a log phase (before 12 hours) and a prolonged stationary phase. Moreover, the setups containing nanoparticles exhibited no drastic decline in the OD readings during the duration of growth profiling, thereby conforming to the prolonged stationary phase.

The demonstrated effect of IONPs in the dynamics of bacterial growth may be attributed to properties of the nanoparticles such as magnetism and electrostatic charge that can alter bacterial motion through signal transduction as well as the formation of cofactors produced in the medium in the presence of IONPs (Pal et al. 2007; Flores et al. 2004; Perez et al. 2003). Alterations in the growth of microorganisms characterized by a shortened lag phase and elongated log and stationary phases have been previously documented by using inorganic nanoparticles such as IONPs (Kapri et al. 2010), nanobarium nanoparticles (Kapri et al. 2009), cobalt ferrite nanoparticles (Flores et al. 2004), and nanometric silicon particles (Perez et al. 2003).

Weight reduction analysis of LDPE

Our study initially determined the rate of LDPE degradation by the weight loss method using the individual bacterial isolates and the consortium in the absence and presence of IONPs over a period of 60 days. The present findings show that *Bacillus pseudofirmus* I7 and *Bacillus agaradhaerens* strain I9 were capable of LDPE degradation based on weight loss with a significant difference in comparison to the negative control ($p< 0.05$). The isolates were tested individually for LDPE degradation and exhibited 6.49 ± 0.3% and 8.36 ± 0.3% weight loss respectively in the absence of IONPs, and 9.62 ± 0.3% and 11.32 ± 0.3%, correspondingly in the presence of IONPs. When the LDPE sample was incubated with the bacterial consortium consisting of the two strains, a degradation rate of 13.7 ± 0.5% was obtained. The observation is indicative of more efficient LDPE degradation by the formulated bacterial consortium than the degradation by its individual strains. Moreover, the bacterial consortium supplemented with IONPs exhibited a higher rate of degradation with a maximal weight loss of 18.3 ± 0.3%. No weight loss was observed in the control setup, which consists of an LDPE film without bacterial treatment. Similarly, no weight reduction was observed on LDPE films incubated with the IONPs alone, indicating that the nanoparticles cannot mediate biodegradation in the absence of bacterial isolates (fig. 3). To confirm significance at $P<0.05$, one-way ANOVA was performed resulting in a statistically significant difference between groups, indicating that the reduction of mass for each setup was due to the action of the isolates. A subsequent test involving multiple comparisons and Tukey post hoc test was also performed for each of the isolate and treatment (i.e., isolates

Table 2: Phenotypic characteristics of alkaliphilic bacterial strains from Poon Bato spring.

Characteristics	*B. pseudofirmus* I7	*B. agaradhaerens* I9	Characteristics	*B. pseudofirmus* I7	*B. agaradhaerens* I9
Cultural characteristics (color, colony form, margin, surface, elevation)	Yellow circular, undulate, contoured, raised	Creamy white, irregular, lobate, undulate, raised	Catalase	+	+
Cell shape	Rod	Rod	Oxidase	+	+
Gram reaction	+	+	Hydrolysis of cellulose	+	+
Temperature requirement (°C)	10-45	25-45			
($T_{(optimum)}$)	37	37	Hydrolysis of Tween 80	+	+
NaCl tolerance (%)	≤10	≤10			
($NaCl_{\ optimum}$)	6	3-5	Hydrolysis of skim milk	+	+
Growth factor requirement	+	+	Hydrolysis of gelatin	+	+
pH tolerance	8-11	8-11			
($pH_{\ optimum}$)	9	10	Hydrolysis of starch	+	+

Table 3: Carbonyl index obtained from Fourier transform infrared spectra of low-density polyethylene incubated for 60 days with bacterial isolates versus noninoculated polyethylene.

	Carbonyl Index	
Isolate	30 days	60 days
Control	0.091	0.076
B. pseudofirmus I7	0.084	0.077
B. agaradhaerens I9	0.056	0.077
Consortium	0.073	0.064
B. pseudofirmus I7 w/ IONPs	0.052	0.050
B. agaradhaerens I9 w/ IONPs	0.054	0.098
Consortium w/ IONPs	0.094	0.121

IONPs = iron oxide nanoparticles

with IONPs and isolates without IONPs). The results show that the mean differences are significant at 0.05 level, indicative of a faster rate of degradation in setups supplemented with IONPs.

In general, microorganisms utilize hydrocarbons present in the polymer backbone, making them the key players for biodegradation (Skariyachan et al. 2014). The exoenzymes such as dehydrogenases, dioxygenase, and monooxygenase are used to facilitate depolymerization of complex high molecular weight polymers (Kumar and Maiti 2016). Several studies have assessed the biodegradability of polyethylene using nonextremophilic microorganisms by measuring the changes in the weight of the residual polymer after an incubation period. The rate of biodegradation of polyethylene after 10 years of exposure to microbial consortia of soil was very slow, accounting for a 3.5% to 8.4% weight loss of polyethylene (Albertsson and Karlsson 1990) and thermo-oxidized polyethylene incubated with *Arthrobacter parrafineus* in the soil for 3–5 years (Albertsson et al. 1997). The rate of biodegradation demonstrated by the bacterial strains used in our study was also 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; Kyaw et al. 2012). Moreover, LDPE sheets incubated with *Bacillus sphericus* and *Bacillus cereus* for a duration of one year resulted in a weight reduction of 10% and 3.5 %, respectively (Sudhakar et al. 2008). Although several studies have been made in determining the role of bacteria in biodegradation, only a few have reported biodegradation of plastics using extremophiles. Thermophilic bacteria *Brevibaccillus borstelensis* and *Streptomyces coelicoflavus* were able to reduce LDPE weight by 14% and 30% in 30 days, respectively (Hadad et al. 2005; Duddu et al. 2014). Although the isolate *B. pseudofirmus* has been reported to have a role in the biodegradation of hard keratinous wastes (Kojima et al. 2006), its role in the biodegradation of polyethylene has not yet been documented. The isolate *B. agaradhaerans* has not been cited in any polyethylene biodegradation study; we infer that such ability is a new discovery. In our study the formulated consortium exhibited a higher weight reduction compared to the previously reported consortia containing *Pseudomonas* spp., *Lysinibacillus* sp., and *Salinibacterium* sp. exhibiting a weight loss of 1.53% in 150 days and 2.3% in 180 days (Veethahavya et al. 2016; Syranidoul et al. 2017). The amplification and synergistic activity of different enzymes/cofactors utilized in the degradation process may account for the better degradation competence of the bacterial consortium (Soni et al. 2009).

The enhanced biodegradation in the presence of nanoparticles has been investigated in several studies. The use of supermagnetic IONPs has been reported to increase significantly the biodegradation efficiency of the consortium by increasing the duration of exponential and stationary phases (Kapri et al. 2010; Bhatia et al. 2013). The suggestive reason behind the accelerated effect of IONPs on bacterial growth dynamics is the promotion of nanoparticle-bacteria interaction mediated by the charges on the bacterial surface and the involvement of the magnetic property and electrostatic charge of IONPs (Kapri et al. 2010). Bacterial growth promotion and biofilm formation can be induced by exposure to IONPs, and this can be attributed to iron dissolution and the release of soluble Fe (III) (Borcherding et al. 2014). This is consistent with the report that the accelerated bacterial growth in the presence of IONPs is associated with the release of iron ions which could influence the upregulation of iron-dependent cellular metabolism (Current et al. 2017).

Quantitative estimation of bacterial population on LDPE films

In our study the measurement of extractable protein concentration of bacterial cells attached on the LDPE films was

Table 4: Peak changes in the characteristic spectra of low-density polyethylene in different setups after 30 and 60 days' incubation.

SETUP	WAVENUMBER (cm^{-1}) AND FUNCTIONAL GROUP									
	2920 (CH_2)	2850 (CH_2)	1473 (C=C)	1462 (C=C)	1437 (C=C)	727 (C-H)	723 (C-H)	1047 (C-O)	3348 (OH)	3792 (OH)
Control	19.97	16.49	70.09	60.95	76.71	90.37	90.66	97.37	77.57	88.46
B. pseudofirmus **I7 w/ IONP (30 days)**	11.65	15.26	72.16	63.29	79.03	89.82	90.18	96.58	96.28	97.30
B. pseudofirmus **I7 w/out IONP (30 days)**	14.86	18.59	75.84	70.43	85.01	91.03	91.36	97.08	96.33	96.54
B. agaradhaerens **I9 w/ IONP (30 days)**	17.96	21.33	78.23	70.99	78.23	91.01	91.32	97.19	98.63	100.53
B. agaradhaerens **I9 w/out IONP (30 days)**	12.34	18.38	75.89	68.88	82.35	89.80	89.55	94.45	92.58	96.00
Consortium w/ IONP (30 days)	25.93	31.66	80.99	74.95	86.53	91.31	91.53	96.42	98.89	100.02
Consortium w/out IONP (30 days)	16.84	22.48	78.35	70.40	84.49	90.99	91.26	96.87	96.43	98.97
B. pseudofirmus **I7 w/ IONP (60 days)**	24.32	30.12	80.70	75.43	86.29	92.65	92.22	97.38	96.03	95.79
B. pseudofirmus **I7 w/out IONP (60 days)**	28.36	40.47	89.93	86.72	93.46	96.31	96.03	98.47	98.71	97.75
B. agaradhaerens **I9 w/ IONP (60 days)**	21.06	27.65	80.24	86.47	86.47	91.41	91.39	96.49	95.59	95.77
B. agaradhaerens **I9 w/out IONP (60 days)**	20.54	26.32	79.49	74.71	86.30	92.59	92.34	97.68	96.30	96.44
Consortium w/ IONP (60 days)	20.77	27.27	79.69	73.72	85.99	91.48	91.31	96.49	95.59	95.77
Consortium w/out IONP (60 days)	20.77	27.27	79.69	73.72	85.99	91.48	91.31	97.68	96.30	96.44

done as an indirect measurement of the bacterial biomass and state of colonization on the films. The results showed that the bacteria in all setups were able to colonize the film on the third day of incubation, the highest of which is the setup containing the consortium supplemented with IONPs (fig. 4).

The formation of bacterial consortium is known to increase the growth rate of bacteria due to the positive interactions between the strains promoted by gene transfer and metabolic cross-feeding (Sarkar et al. 2011). IONPs are also known to influence bacterial growth positively by prolonging the exponential and stationary phases (Kapri et al. 2010). The combined effect of bacterial consortium and use of IONPs could have led to the drastic increase in the bacterial population density, thereby causing the higher extractable protein concentration. Moreover, the immediate increase in biomass could be attributed to the utilization of mineral oil serving as the initial carbon source of the bacteria. Mineral oil is known to play a functional role in allowing bacterial adherence to the surface of the polymer, presumably increasing the hydrophobic interactions between the bacterial cells and the polyethylene film (Balasubramanian et al. 2010; Gilan et al. 2004). The consequent decrease in bacterial biomass observed after 18 hours indicates the depletion of mineral oil as the carbon source. A slow and constantly proliferating biofilm capable of using LDPE as the carbon source eventually developed on the film following the decline in the protein content of the fast-proliferating cells (Gilan et al. 2004). The enhancing effect of mineral oil in bacterial colonization was also observed in another study (Hadad et al. 2005). In the presence of mineral oil the extractable protein content of the biofilm formed by *Brevibacillus borstelensis* significantly increased, with its peak recorded on the fourth day of incubation, as compared to the control. The same trend was observed in the protein content analysis of the biofilm of *Anthrobacter* sp. and *Pseudomonas* sp. in which a steep rise in the peak of the two strains was followed by a gradual decrease in the concentration of the extractable protein (Balasubramanian et al. 2010).

Overall the bacterial setups in our study were all able to colonize the LDPE surface, and this could therefore explain the sustained biodegradation of polyethylene through weight loss.

Evaluation of bacterial hydrophobicity

Hydrophobic interactions are vital in adhesion and proliferation of cells on highly hydrophobic polymer materials such as LDPE (Goldberg et al. 1990). The BATH test demonstrated the hydrophobicity of the individual strains in the presence and absence of IONPs; however, the setup containing the consortium supplemented with IONPs showed higher hydrophobicity than all other setups. The adhesion of the bacterial cells to the hydrocarbon in the setup containing the consortium treated with IONPs was evident even at the lowest concentration of hexadecane (0.04 mL) with a 20% reduction in the turbidity of the initial bacterial suspension (fig. 5). As for the individual isolates, *Bacillus agaradhaerens* I9 was observed to be more hydrophobic than *Bacillus pseudofirmus* I7. The higher hydrophobicity of *Bacillus agaradhaerens* is indicative of the increased colonization and degradation of LDPE as manifested by a higher weight loss reduction. The hydrophobicity of polyethylene interferes with the adhesion of bacteria to the surface; however, some bacterial strains exhibit increased surface hydrophobicity leading to a greater extent of colonization and degradation. These hydrophobic cells are known to adhere more strongly to hydrophobic surfaces leading to biofilm formation due to hydrophobic-hydrophobic interactions with the substrate (Heipieper et al. 2007; Kochkodan et al. 2008). As the polyethylene surface is hydrophobic in nature, a greater affinity to hydrocarbon indicates a higher biofilm formation on the polyethylene surface resulting in an increased diffusion rate and substrate utilization (Bacakova et al. 2011).

Fourier transform infrared spectroscopy analysis

FTIR analysis was used to monitor substantial changes in the chemical structure of LDPE after biodegradation involving the appearance or disappearance of functional groups. The LDPE film without any treatments displayed control spectra with distinctive bands that conform to the complex nature of LDPE

(fig. 6). The characteristic absorption bands were assigned at 2920 cm^{-1} (CH_2 asymmetric stretching), 2850 cm^{-1} (CH_2 symmetric stretching), 1473, 1462, and 1437 cm^{-1} (C = C bending deformation), and 727 and 723 cm^{-1} (C-H bend mono). In contrast, spectra of the films incubated with the isolate and consortium in the presence and in the absence of IONPs for 30 and 60 days revealed discrepancies in the intensities of bands. Generally, there was a decrease in the intensity of peaks at 1473, 1462, and 1437 cm^{-1} corresponding to C = C bending deformation and weaker absorption peaks at 727 and 723 cm^{-1} designated as C-H bend mono, due to the microbial action of the isolates (table 4 and fig. 7).

The biodegradation of polyethylene is initiated by abiotic processes such as oxidation exerting intramolecular modifications in the polymer chain. This process of oxidation results in molar mass reduction of the polymer and formation of various oxygenated groups dominated by carbonyl groups which are subsequently metabolized by microorganisms through beta-oxidation and citric acid cycle (Balasubramanian et al. 2011; Albertsson et al. 1987). Changes in these carbonyl groups were quantified by computing for the carbonyl index (CI), a ratio between the absorbance peaks of carbonyl group (1712 cm^{-1}) to CH_2 at 1460 cm^{-1}. Carbonyl index is the most commonly used indicator to measure the chemical oxidation of polyethylene reflecting the degradation of the mechanical properties of the polymer (Rouillon et al. 2016). In our study a decrease in the carbonyl index of polyethylene films was observed in setups containing *Bacillus pseudofirmus* I7, *Bacillus pseudofirmus* I7 with IONPs, and the one containing the consortium. Notably the consortium supplemented with IONPs gave the highest carbonyl index after 60 days of incubation (table 3). The decrease in carbonyl index observed in setups containing *Bacillus pseudofirmus* I7 and in the consortium can be attributed to the microbial consumption of low molecular weight oxidation products such as soluble carboxylic acids by the isolates (Albertsson 1995). A similar result was reported in biodegradation studies (Gilan et al. 2004; Sudhakar et al. 2008; Hadad et al. 2005; Esmaeili et al. 2013). Meanwhile, an increase in carbonyl index was evident after 60 days' incubation of the polyethylene films in setups containing *B. agaradhaerens* I9 and the consortium with IONPs. This increase in carbonyl index indicates a higher extent of oxidation and can be due to biological activities of the microorganisms leading to the formation of ketone or aldehyde C = O groups (Esmaeili 2013). This increase in carbonyl index as a result of biodegradation is similar to results reported elsewhere (Balasubramanian et al. 2010).

Oxidation reactions involving polyethylene are complex and result in the release of various products such as hydroxyl, carbonyl-containing functional groups (namely, ketones and esters), and vinyl groups (Yagoubi et al. 2015). In order to establish the extent of degradation of LDPE, changes in the functional groups of the polymer surface should be monitored, particularly the increase and decrease in keto carbonyl bond index (KCBI), ester carbonyl bond index (ECBI), vinyl bond index (VBI), and internal double bond index (IDBI). During oxidation of polyethylene, vinyl groups and different carbonyl-containing groups are increasingly produced; therefore, a close connection exists between the rate of degradation and the absorption occurring within these regions in the infrared spectrum of the polymer (Fodor et al. 1984). In our study there were an increase in ECBI in all setups and an increase in KCBI upon incubation with *B. agaradhaerens* I9 with IONPs and the consortium with IONPs after 60 days of incubation (fig. 8). The results indicate the degradation of the polyethylene films possibly mediated by the enzymatic action of the isolates. Notably the higher hydrophobicity observed for the isolate *B. agaradhaerens* I9 allows greater affinity to a polyethylene surface resulting in increased biofilm formation and substrate utilization. Thus, the observed increase in KCBI and ECBI could possibly be due to the formation of keto and ester carbonyl as major products of enzyme activity particularly oxidoreductase (Albertsson et al. 1998). The KCBI of the samples incubated with isolate *Bacillus pseudofirmus* I7 decreased after 60 days of incubation. The ketones produced by oxidation reactions can possibly undergo further chain scission via a Norrish type I process to generate carboxylic acid, thereby decreasing the amount of ketones (Yagoubi et al. 2015). By contrast, an increase in the IDBI observed for samples incubated with isolate *B. agaradhaerens* I9 and the consortium with IONPs is congruent with the high amount of carbonyl residues after 60 days of incubation in the aforesaid setups (fig. 8). Carbonyl groups in the polymer chain may be susceptible to attack by microorganisms, consequently exposing the unsaturated chains, hence resulting in the increase in IDBI (Albertson and Anderssen 1987). Furthermore, polymer chains ruptured upon biological treatment result in the formation of vinyl groups and a simultaneous increase in IDBI (Pathak et al. 2018). Interestingly the setup containing the consortium with IONPs demonstrated the highest KCBI, ECBI, and VBI values among the setups. This is consistent with having the highest amount of carbonyl groups generated after 60 days of incubation and indicative of a greater extent of degradation.

Overall the biodegradation efficiencies of all setups were confirmed by the formation and disappearances of several functional groups as revealed by the peak changes in their FTIR spectra. This was consequently affirmed by the quantification of these changes through FTIR indices. Note, however, that the incubation of LDPE generates changes in the concentration of the functional groups at the surface. These changes in the concentration can be due to the consumption or production of functional groups depending on the nature of the microorganism present (Pathak et al. 2018).

Scanning electron microscopy analysis of polyethylene

The morphological changes in LDPE films after 60 days of incubation under in vitro conditions were analyzed by using SEM. The LDPE films incubated without bacterial isolates exhibited smooth and homogenous surfaces (fig. 9: a–c). In contrast LDPE films incubated with the bacterial isolates and their consortium revealed the presence of surface alterations characterized by the formation of surface erosions, pits, cavities, and fissures (fig. 9: d–r). Furthermore, the LDPE films degraded by the consortium in the presence of nanoparticles exhibited greater surface dissolution, deepened cracks, and larger cavities with globular white areas (fig. 9: s–u). This was supported by the greater weight reduction of the films incubated in the presence of the consortium and IONPs.

The observed changes in the films indicate the physical degradation of the polymer through the action of the bacterial isolates. Pits and cavities on the surface may be due to the absence of a constant dissemination of short branches in the polymer matrix, in turn suggesting the association of the isolates within the films (Nowak et al. 2011). Consequently this association is important in the transportation of depolymerizing enzymes that cause the erosion of the polymer surface, thereby reducing its mass (Raut et al. 2015; Bhatia et al. 2014; Gajendiran et al. 2016). In addition, microbial growth on the polymer increases the pore size and promotes the formation of cracks on the film. These surface cracks eventually become sites of microbial activity. Lastly the random occurrence of surface alterations may be due to the nonuniform distribution of short polymer chains in the matrix as well as the initial attachment and localization of the consortia on the film (Kapri et al. 2010).

CONCLUSIONS

This in vitro biodegradation study of LDPE demonstrated the accelerated rate of biodegradation through the formation of alkaliphilic bacterial consortium grown in the presence of IONPs. The combination of isolates, phylogenetically affiliated with *Bacillus pseudofirmus* and *Bacillus agaradhaerens,* and supplemented with IONPs exhibited maximum degradation of LDPE as supported by its high weight reduction and increased hydrophobicity. The measurement of the extractable protein concentration then proved the viability of the isolates. An analysis of the degradation end products indicated the formation and disappearance of functional groups. SEM analysis further revealed pronounced morphological changes on the surface of the films. The ability of the consortium to degrade LDPE more efficiently may be due to the possible synergistic interactions of the isolates making up the consortium. By contrast, IONPs accelerate the process by enhancing the growth rate of the bacteria as demonstrated by the shortened log phase and prolonged stationary phase of the consortium. The formulation of a microbial consortium incorporated with IONPs exhibits a great potential for a more efficient degradation of LDPE; hence this may serve as a plausible technique for the reduction and management of solid wastes accumulating in the environment.

CONFLICT 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

Cada, Erika Joy: Contributed in the overall conception and design of the study, acquisition of data, analysis and interpretation of data, writing and revising the manuscript.
Muyot, Marion Lei: Contributed in the overall conception and design of the study, acquisition of data, analysis and interpretation of data, writing and revising the manuscript.
Sison, Joanna Maria: Contributed in the overall conception and design of the study, acquisition of data, analysis and interpretation of data, writing and revising the manuscript.
Baculi, R.Q.: Contributed in the overall conception and design of the study, writing and critical revision of the article for intellectual content.

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