Comparative in situ biodegradation studies of polyhydroxybutyrate film composites

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Abstract Application of polyhydroxybutyrate (PHB) to plastic industry has expanded over the last decades due to its attracting features over petro-based plastic, and therefore, its waste accumulation in nature is inevitable. In the present study, a total of four bacterial strains, viz., MK3, PN12, PW1, and Lna3, were formulated into a consortium and subsequently used as biological tool for degradation of biopolymers. The consortium was tested through $\lambda_{\max}$ shifts under in vitro conditions for utilization of PHB as sole carbon source. Talc-based bioformulations of consortium were used for the degradation of PHB film composites under in situ conditions. After 9 months of incubation, the recovered samples were monitored through Fourier transform infrared (FT-IR) spectroscopy and scanning electron microscopy (SEM), respectively. Analytical data, viz., changes in $\lambda_{\max}$ shifts (212–219 nm), FT-IR spectra, and SEM micrographs, revealed the biodegradation potential of developed consortium against PHB film composites, i.e., higher degradation of copolymer films was found over blend films. The used consortium had enhanced the rate of

natural degradation and can be further used as a natural tool to maintain and restore global environmental safety.

Keywords Consortium · Polyhydroxybutyrate (PHB) · Talc-based bioformulations · In vitro and in situ conditions · Fourier transform infrared spectroscopy · Scanning electron microscopy

Introduction

In the past decades, an enormous work has been conducted to develop biodegradable polymers. In this context, polyhydroxybutyrate (PHB), a member of class polyhydroxyalkanoates (PHAs) (Santos et al. 2015), has emerged as one of the most promising biodegradable plastic materials substitute for petro-based plastics (Sinha and Rathore 2015). These are of great interest because of properties, viz., bio-derived, biodegradable, biocompatible, recyclable, and having low processing time (Yoshie et al. 2002; Bugnicourt et al. 2014) and characteristic features, viz., crystallinity, melting point, strength, density, and modulus comparable to those of polyolefin commodities like polypropylene (PP) (Jedra 2014). Therefore, these remarkable attributes offers their wide range of utility in various fields, viz., medical (Aburas 2016), food packaging (Darani and Bucci 2015), agricultural, household, marine (Hawas et al. 2016), etc.

Nonetheless, the physico-chemical properties and quality of PHB have been improved by heat treatment, copolymerization (Barham et al. 2006), blending (Zhang and Thomas 2011), addition of plasticizers (Savenkova et al. 2000), chemical modification (Kai and Loh 2014), and formation of self-assembled micelles (Loh et al. 2009). The global bioplastic production was approximately 890,000 metric tons in 2012 and it is estimated to grow at

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an annual growth rate of 25% through 2017 reaching more than 2.5 million metric tons (Smithers Rapra 2012). Consequently, the increasing consumption of bioplastic is also inevitable. Although the waste and slow rates for complete degradation, i.e., 2–5 years were observed in a field test of biodegradable plastics in the soil which is a serious socio-environmental concern (Suyama et al. 1998).

In recent years, as the output of PHB polymer is increasing, studies to evaluate their degradation in natural environments acquiring alarming significance. The previous reports revealed that PHB blends and its copolymer can be degraded in various natural environments such as soil, compost, and natural water (Suyama et al. 1998; Volova et al. 2015). PHB polymer can be broken down by enzymes known as PHB depolymerases (Orts et al. 2008). These enzymes have been studied in both Gram-positive and Gram-negative bacteria (Schöber et al. 2000), moulds (Oda et al. 1997), and fungi (Garcia-Hidalgo et al. 2013). In addition, there are reports supporting the evidence of existence of aerobic and anaerobic PHB-degrading bacteria in all terrestrial and aquatic ecosystems (Shah et al. 2007).

However, the impact of thickness of PHB blend and its copolymer film on the biodegradability characteristics has not been addressed and documented. Nevertheless, PHB biodegradation using known bacterial consortia has been rarely studied (Volova et al. 2010). Thus, the present study was aimed to evaluate the in situ biodegradation of different PHB film composites (blend and copolymer) using known bacterial consortium. To this end, the potential of respective consortium was checked under in vitro studies using PHB homopolymer pellets prior to in situ biodegradation trial. Therefore, this investigation will give a better insight of its chemical and physical properties on biodegradation under natural conditions and also provide a moderate way to resolve the waste management issue naturally.

Materials and methods

Starting materials

Polyhydroxybutyrate (PHB) homopolymer pellets blend and copolymer films were procured from Fraunhofer Institute, IPK, Berlin (Sigma-Aldrich, Germany). These polymer composites are commercially available under the name Biomer®P304, ECOMANN20000 (265 and 100 µm thickness) and ECOMANN20010 (100 µm thickness), respectively. The homopolymer PHB pellets (Biomer®P304) were converted into powdered form through boiling with chloroform, followed by evaporating the solvent under ambient conditions. Later, the powdered PHB was washed with 70% ethanol, dried at 50 ± 1 °C (dry oven) for 1 h, and then

used as primary carbon source during in vitro studies (Raghuwanshi et al. 2016). For in situ biodegradation studies, PHB films of dimensions 4×4 cm² were cut using blade (Zafar et al. 2013) and further both powder and films were thoroughly surface sterilized with 70% ethanol for 10 min and dried in vacuum at room temperature. Furthermore, the incorporation of indigenous bacterial consortium was carried out with the help of talc (HiMedia Laboratories Pvt. Ltd., Mumbai, India) based bioformulations. Moreover, nutrient broth and Minimal broth Davis w/o dextrose (HiMedia Laboratories Pvt. Ltd., Mumbai, India) which is deprived of carbon source were used as medium for bacterial growth and in vitro utilization studies, respectively.

Bacterial consortium

Four bacterial cultures were retrieved from the departmental culture collection of microbiology, CBSH, G. B. Pant University of Agriculture and Technology, Pantnagar, India (Supplementary Table 1). These cultures, viz., *Microbacterium* sp. Strain MK3 (DQ318884), *Bacterium* Te68R Strain PN12 (DQ423487), *Pseudomonas putida* Strain PW1 (EU741798), and *Enterobacter* sp. Strain Lna3 (DQ205431), were selected on the basis of their pre-identified potential to act upon LDPE (Soni et al. 2009; Kapri et al. 2010) and epoxy and its blends (Negi et al. 2009; Raghuwanshi et al. 2015), respectively. The cultures were revived by inoculating into 5.0 mL nutrient broth test tubes at optimum pH (7 ± 0.2) and temperature (35 ± 1 °C). Furthermore, aliquots of 500 µL overnight culture were used to inoculate into 10 mL nutrient broth and incubated for 4 h at ambient growth conditions to obtain mid-log phase active culture. The calculated amount (CFU mL⁻¹) of each mid-log phase bacterial strain was mixed to form bacterial consortium (Goel et al. 2011). The compatibility test of each strain was conducted prior to the preparation of consortium as described earlier (Raghuwanshi et al. 2015; Goel et al. 2011).

Determination of optimum tolerance level of PHB

The optimum concentration of PHB tolerated by the consortium was determined by inoculating the active consortium into 5 mL minimal broth. And then, powdered PHB was added at increasing concentration from 0 to 6 mg mL⁻¹ and incubated overnight at optimum growth temperature (35 ± 1 °C) with continuous shaking (120 rpm). The absorbance was recorded for all the treatments at 600 nm wavelength with spectrophotometer after filtration (using Whatman filter paper of 2.5 µm particle retention size) of non-biodegraded compound. The experiment was performed in triplicates and the values were expressed as their means.

In vitro PHB-utilization studies

Sterilized powdered PHB was added to 250 mL Erlenmeyer flasks containing 100 mL autoclaved Minimal broth (pH 7.0 ± 0.2). Thereafter, 150 μL of active consortium was mixed and taken as a positive control. Minimal broth with PHB (optimum concentration) was served as a negative control for its respective treatment. In addition, the flask containing Minimal broth with consortium and powdered PHB (optimum concentration) served as treatment. Flasks were incubated at optimum growth temperature ($35 \pm 1^\circ\text{C}$) with continuous shaking (120 rpm) for a period of 7 days. The absorbance (OD_{600}) and the shift in λ_{max} of treatments and their respective controls were determined after regular intervals of 24 h during incubation period.

Preparation and mean-life determination of talc-based bioformulation

To prepare talc-based bioformulation, 200 mL of prepared active consortium was centrifuged at 5000 rpm for 10 min to remove the bacterial cells. Then, bacterial pellets were incorporated with 10 g talc under sterile conditions and mixed thoroughly and dried at room temperature ($28 \pm 1^\circ\text{C}$). The shelf-life of bacterial isolates in the bioformulation was deduced by serial dilution plating method on nutrient agar medium. For this purpose, 1.0 g of talc-based formulation was mixed in 1.0 mL of sterile distilled water and the suspension was further mixed with 9.0 mL of sterilized distilled water. The plates were incubated ($35 \pm 1^\circ\text{C}$) and the CFU mL^{-1} counts were checked initially after 2 and 4 days. Thereafter, the viability was determined after regular interval of 7 days for subsequent 21 days, followed by 15 day interval. The above pattern was followed to keep checking on rapidity of changes in viable counts during the storage. The plate counts were carried out in triplicate and the average of the three respective readings were taken.

Comparative in situ biodegradation studies

Experimental pit preparation and treatment design

The in situ net house experimentation was carried out into the experimental pits ($60 \times 30 \text{ cm}^2$), which were filled with top soil collected from crop research centre (CRC), Pantnagar, India. Briefly, the soil in pits was pulverized manually to facilitate uniform porosity and aeration and thereafter, $4 \times 4 \text{ cm}^2$ -sized PHB film coupons were subsequently buried inside the soil randomly

at varying depths. The pure film of PHB composite was served as a negative control. Whereas, the one buried in un-inoculated soil served as a positive control and the rest treated with active bioformulation are considered as treatments. This experiment was set up for 9 months under natural conditions. The parameters, i.e., moisture and aeration in the experimental pits were taken care by adding autoclaved distilled water and shoveling the soil at regular intervals of 5 days.

Recovery of degraded film samples from soil bed

The biodegraded samples were recovered after 6 and 9 months of incubation. Analyses of these samples were carried out to understand the regular pattern of changes in PHB film structure after treatments for longer duration. Henceforth, the recovered products were surface sterilized with 70% ethanol for 10 min and dried in a desiccator for 24 h under vacuum (Anwar et al. 2016).

Analysis of recovered film samples

Fourier transform infrared spectroscopy

The chemical changes in the residual polymer films obtained after the treatment were determined through fourier transform infrared spectrophotometer (Excalibur), SAIF, CDRI, Lucknow, different peaks relative to CH_2 deformation, viz., CH_2 bending (symmetrical), CH_2 bending (asymmetrical), CH_2 stretching (asymmetrical and symmetrical), CH_2 rocking (asymmetrical and symmetrical) and CH vibration mode, C–O–C bond stretching, C–C bond stretching, C–C bond stretching, CH_3 stretching (asymmetric), CH_3 bending (symmetric), C=O bond bending, and OH bending and stretching were compared and analyzed, while pure PHB film as a reference for different thickness variants. Bending, stretching, and rocking vibrations have been depicted by δ , ν , and ρ , with asymmetrical and symmetrical absorptions represented by subscripts “asym” and “sym”, respectively. The pure, untreated, and treated PHB composite samples were depicted by P, UN, and T, respectively. The spectra were recorded in potassium bromide (KBr) disc.

Scanning electron microscopy

The change in the morphology of the recovered polymer films was determined through the SEM studies. For this purpose, the samples were metalized with gold particles and analyzed by SEM (LEO 435 VP) at 15.00 kV EHT under three successive magnifications (1.00, 3.00 and 5.00 KX) performed at IIC, IIT, Roorkee, India.

Results and discussion

Determination of optimum tolerance level of PHB

Four bacterial isolates, viz., MK3, PN12, PW1, and Lna3, were formulated into consortium on the basis of biocompatibility of these strains with each other. The consortium was prepared by mixing 64, 160, 211, and 96 (CFU mL⁻¹) for all strains MK3, PN12, PW1, and Lna3, respectively (Supplementary Table 2). Moreover, the optimum tolerance level (OTL) of PHB powder that could allow the optimum growth of consortia was determined for further in vitro PHB-utilization studies. The varying growth of bacterial consortia with the increasing concentration of respective polymer was observed (Supplementary Table 3). Determinately, PHB concentration, i.e., 3 mg mL⁻¹ was found significant as OTL, where the growth of consortia was innocuous and found appropriate.

In vitro PHB-utilization and comparative growth profiling of bacterial consortium

The optimum concentration (3 mg mL⁻¹) was used under in vitro experimentation for 6 days during growth profiling of bacterial consortia. The presence of powdered PHB has not affected the growth pattern of the consortium as stationary phase was achieved within 3 days of incubation, similar in case of control (Supplementary Table 4). Whereas, in the presence of polymer, the consortium showed reduction in lag phase by 48 h, the earliest onsets of metabolically active log phase (Fig. 1). Furthermore, the bacterial biomass was found greatly significant in the treatment during incubation period than the controls. These findings clearly suggested that the presence of PHB accelerated the growth of consortia due to utilization of

PHB as sole carbon and energy source. In addition, a significant shift in $\lambda_{\max}$, i.e., from 212 to 219 nm in treatment was observed within 48 h of incubation and subsequently attained a value of 218, 217, 216, and 215 nm after a successive period of incubation, i.e., 3rd, 4th, 5th, and 6th day, respectively. This preliminary result affirmed that the consortium has utilized PHB powder as sole carbon source for their survival, as the growth medium was not containing any other carbon source. It has been shown and considered that the relative higher growth of bacterial consortium is achieved only when polymer is utilized as sole source of carbon and energy (Raghuwanshi et al. 2015; Anwar et al. 2016).

Viability and the average life of talc-based bioformulation

The formulation consisting of active entities must be preserved or maintained in viable condition to produce its biological effect (Arora et al. 2008; Ardakani et al. 2011; Shanmugam et al. 2011). The development of bioformulation furnishes pleasant handling and application in the fields. In this investigation, the talc-based bioformulation was found to maintain the viability of the consortium for a period of 70 days (Table 1) at ambient temperature. The observation suggested that the consortium was viable and stable in developed carrier-based bioformulation over the respective period of storage. With progression of storage, consortium showed that sustained viability and the counts dropped 4.93% after 70 days. Considering the vitality rate, consortium was selected for progressive PHB film composites biodegradation under natural conditions.

Comparative analysis of recovered PHB film samples

FT-IR spectra

The potential of consortium towards the biodegradation of PHB film composites was reflected through the shift in the wave numbers (cm⁻¹) in comparison to the untreated samples. The changes in the polymers incubated in the presence of consortium were monitored in the range of 4000–450 cm⁻¹. The PHB blend pure films (PHBB-P and PHBR-P of 100 and 265 μ m thickness, respectively) showed characteristic wave numbers (cm⁻¹) corresponding to 3848.51–3401.06 (ν OH), 2922.65 (ν_{asym} CH₂), 1723.81 (δ C=O), 1644.31 (δ OH), 1454.51 (δ_{asym} CH₂), 1384.09 (δ_{sym} CH₃), 1276.1 (γ CH), 1183.78 (δ C–C), 1096.58 (ν C–O–C), and 768.51 (ρ_{asym} CH₂), respectively, which proved the characteristic synthesis peaks of polyhydroxybutyrate and polylactic acid blend [Fig. 2A(a), B(a)] (Armentano et al. 2015).

Fig. 1 Growth profile of bacterial consortium grown in the presence of PHB as sole carbon source in liquid minimal medium

Table 1 Shelf life of bioformulation under ambient storage temperature

Consortium	Dilution factor	^a CFU mL ⁻¹ at subsequent time intervals (days)							
		2nd	4th	11th	18th	25th	40th	55th	70th
	10 ⁷	284 ± 2	283 ± 2	279 ± 2	280 ± 2	278 ± 2	275 ± 2	275 ± 2	273 ± 2
% Survival decrease		0	0.4	1.8	1.5	2.12	3.2	3.2	4.93

^a The data are average of triplicate experimental values

Fig. 2 Comparative FT-IR spectra of 100 μm -thick PHB blend films (A), 265 μm -thick PHB blend films (B), and 100 μm -thick PHB copolymer films (C): with pure films (a), untreated (b), and consortium treated films (c) over 9 months of incubation, respectively

The PHB untreated blend (PHBB-UN) samples recovered after 9 months of incubation showed the wave numbers (cm^{-1}) at 3847.45–3400.58 (ν OH), 1643.56 (δ OH), 1384.84 (δ_{sym} CH₃), 1216.09 (γ CH), 1155.76 (δ C–C), 1068.79 (ν C–O–C), 770.19 (ρ_{asym} CH₂), and additional 668.91 (ν C–C–C) [Fig. 2A(b)]. In addition, the spectra of PHB treated blend (PHBB-T) samples showed wave number (cm^{-1}) corresponding to 3745.01–3401.42 (ν OH), 3019.63 (ν_{asym} CH₃), 2400.17 (ρ_{sym} CH₂), 1644.34 (δ OH), 1384.91 (δ_{sym} CH₃), 1215.42 (γ CH), 1155.83 (δ C–C), 1068.78 (ν C–O–C), and 669.12 (ν C–C–C) [Fig. 2A(c)]. Comparatively, complete degradation of δ OH and ρ_{asym} CH₂ and the introduction of additional absorptions (3019.63 and 2400.17 cm^{-1}) were clearly attributed to the action of consortium. The absorption of lowered γ CH was due to the potential of consortium.

Comparatively, PHB untreated blend (PHBR-UN) samples (265 μm) showed wave numbers (cm^{-1}) corresponding to 3430.65 (ν OH), 2939.25 (ν_{asym} CH₂), 3024.29 (ν_{asym} CH₃), 2402.94 (ρ_{sym} CH₂), 1750.26 (δ C=O), 1638.35 (δ OH), 1453.24 (δ_{asym} CH₂), 1381.99 (δ_{sym} CH₃), 1214.68 (γ CH), 1189.24 (δ C–C), 1093.85 (ν C–O–C), 761.70 (ρ_{asym} CH₂), and 669.60 (ν C–C–C), respectively [Fig. 2B(b)]. Whereas, the PHB treated blend (PHBR-T) samples showed absorptions corresponding to 2940.47 (ν_{asym} CH₂), 3025.32 (ν_{asym} CH₃), 2403.03 (ρ_{sym} CH₂), 1750.39 (δ C=O), 1638.88 (δ OH), 1453.19 (δ_{asym} CH₂),

1381.32 (δ_{sym} CH₃), 1213.86 (γ CH), 1188.78 (δ C–C), 1093.91 (ν C–O–C), 761.32 (ρ_{asym} CH₂), and 669.68 (ν C–C–C), respectively [Fig. 2B(c)]. Comparing between untreated and treated the lowering in δ_{sym} CH₃, γ CH and ν C–C–C and a bit increase in ν_{asym} CH₃ was due to the effect of consortium. Conclusively, a better degradation rate of PHBB was noticed over PHBR after the treatment of consortium. Degradability of the polymer was inversely proportional to the thickness of the polymer. Therefore, lesser the thickness of the polymer, more degradable was the polymer.

Moreover, Pure PHB copolymer (PHBC-P) film (100 μm) illustrated FT-IR absorptions (cm^{-1}) corresponding to 3439.45 (ν OH), 2987.80 (ν_{asym} CH₂), 3024.36 (ν_{asym} CH₃), 2404.87 (ρ_{sym} CH₂), 1735.57 (δ C=O), 1640.49 (δ OH), 1453.29 (δ_{asym} CH₂), 1381.78 (δ_{sym} CH₃), 1270.61 (γ CH), 1187.03 (δ C–C), 1094.91 (ν C–O–C), 758.66 (ρ_{asym} CH₂), and 668.96 (ν C–C–C), respectively, which shows the characteristic synthesis peaks of polyhydroxybutyrate and hydroxyvalerate copolymer [Fig. 2C(a)] (Lopez et al. 2012). The untreated copolymer (PHBC-UN) samples showed absorption spectral wave numbers (cm^{-1}) corresponding to 3438.78 (ν OH), 3024.98 (ν_{asym} CH₃), 2402.26 (ρ_{sym} CH₂), 1737.47 (δ C=O), 1639.18 (δ OH), 1453.33 (δ_{asym} CH₂), 1382.82 (δ_{sym} CH₃), 1263.97 (γ CH), 1187.93 (δ C–C), 1094.36 (ν C–O–C), 761.13 (ρ_{asym} CH₂), and 669.65 (ν C–C–C), respectively [Fig. 2C(b)].

(A)

(B)

(C)

Fig. 3 Comparative FT-IR spectra of 100 μm -thick PHB blend films (A), 265 μm -thick PHB blend films (B), and 100 μm -thick PHB copolymer films (C): with pure films (a–c), untreated films (d–f and g–i) and consortium treated films (j–l and m–o) after 6 and 9 months of incubation under in situ conditions, respectively. Scale bars 10 μm , magnification 1.00, 3.00, and 5.00 KX, respectively

However, the absorption spectra of treated copolymer (PHBC-T) sample showed wave numbers (cm^{-1}) corresponding to 3682.36–3429.74 (ν OH), 3021.78 (ν_{asym} CH_3), 2401.4 (ρ_{sym} CH_2), 1632.94 (δ OH), 1417.93 (δ_{asym} CH_2), 1215.84 (γ CH), 1026.53 (ν C–O–C), 763.05 (ρ_{asym} CH_2), and 670.60 (ν C–C–C), respectively [Fig. 2C(c)]. Comparatively, PHBC-UN and PHBC-T samples showed the absence of ν_{asym} CH_2 , lowering in ρ_{sym} CH_2 as compared to PHBC-P film. This result could be due to the influence of environmental attributes.

Furthermore, the first region of investigation for degradation of PHB copolymer was found in the spectral range of 4000–1000 cm^{-1} . The effect of used consortium was found most significant and effective as depicted by the loss of an array of absorptions, i.e., δ CH_2 , δ C–C which are present in PHBC-P and PHBC-UN. The lowering in wave number, i.e., in case of ν CH_3 , ρ CH_2 , δ OH, δ_{asym} CH_2 , γ CH, and ν C–O–C was also observed.

A comparative account indicates that consortium has introduced the absorption corresponding to the formation of hydroxyl group (3682.36–3429.74 cm^{-1}) on the copolymer macromolecules with the complete degradation of carbonyl group (δ C=O) [Fig. 2C(c)] which clearly indicated that PHBC polymer chains get destroyed (Goel et al. 2011). In addition, higher degradation activity of consortium has been reflected in spectral range 1000–450 cm^{-1} . All these absorptions proved the higher potential of used consortium towards PHB copolymer degradation.

Conclusively, the FT-IR spectra have illustrated the degradation of both blend and copolymer PHB films of all used thicknesses upon in situ treatments with indigenous bacterial consortium. The comparative results have shown clear evidence that the bacterial consortium has potential to speed up the rate of natural degradation of all polymers. However, the consortium was more effective towards PHB copolymer (100 μm) followed by PHB blend (100 and 265 μm), respectively.

Scanning electron microscopy

The comparative study of the effect of bacterial consortium on the surface topographies of PHB films which were both blend (100 and 265 μm) and copolymer (100 μm) was analyzed after 6 and 9 months of incubation under natural conditions. The pure (P) and untreated (UN) biopolymer films were taken as controls against treated (T) samples for blend and copolymer, respectively. The pure PHB blend

and copolymer film images were smooth and homogenous [Fig. 3A, B, C (a–c)] (Arrieta et al. 2014). Upon incubation under un-inoculated soil bed, the films morphology was found to be changed and heterogeneous during incubation periods [Fig. 3A, B, C (d–f), (g–i)]. Moreover, the changes on the surface of untreated biopolymer were most probably due to the natural factors which were comparatively negligible with that of consortium treated films (Mousavioun et al. 2012). The SEM micrographs of treated biopolymer were visibly distinguishable from control and untreated films as the fractures and disintegrations on the surface morphology were more widened and prominent representing features like widened fissure, formation of wrinkled cavities, and distortion, etc. [Fig. 3A, B, C (j–l), (m–o)] (Zhao et al. 2003; Harmaen et al. 2016). However, among all recovered samples SEM micrographs of treated copolymer (100 μm), films were more intensively distorted and revealed that the extensive surface aberration was caused by the bacterial consortium which was most prominent in 9 month incubation of films comparing 6 month blend film composites and this substantiates the FT-IR results subsequently.

Conclusions

This investigation explored the comparative biodegradability analysis of PHB film composites with different thickness variants using indigenously developed bacterial consortium under natural conditions. The results suggested that the consortium can influence and accelerate the biodegradation process of PHB polymer which could be due to higher bacterial colonization on the polymer surface. Thus, these findings signify as instrumental for better understandings of the polymer biodegradation with respect to their physico-chemical properties towards the mitigation of this kind of polymer menace in the soil.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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