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Comparative shelf life and efficacy of LDPE and PVC degrading bacterial consortia under bioformulation

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The present study elucidates the development of the talc-based formulation for long-term sustenance / storage of bacterial consortia, pertains to degrade polymer (LDPE and PVC). Four potential bacterial consortia were employed which consisted of *Microbacterium* sp., *Pseudomonas putida, P. aeruginosa, P. otitidis, Bacterium Te68R, Bacillus aerius, B. cereus and Acanthopleurobacter pedis*. The viability of consortia was ascertained by measuring the colony forming units per mL besides assuring the polymer biodegradation potential after formulation in talc by carrying out *in vitro* assays. The analysis was done by determination of λ-max shifts and scanning electron microscopy for changes in polymer composition and surface dissolution, respectively. It reveals that using the described formulations, all the consortia were able to successfully retain their characteristic biodegradation property even after 70 days of storage at ambient conditions. Furthermore, the SEM micrographs documented significant disruption of surface texture of polymer (LDPE and PVC) film by respective consortia recovered after storage. Thus, the talc-based formulation may be useful for the storage and handling of polymer-degrading consortia for large-scale applications to minimize the solid waste disposal.

Key words: biodegradation, bioformulation, talc, consortia, shelf life, LDPE, PVC, SEM

INTRODUCTION

The extensive use of polymeric materials (plastics) during past decade in all the sectors of life has created serious problems with plastic waste due to its accumulation in the environment (Magan et al., 2010). Further, thermoplastics are inert materials and resistant to biodegradation because of its high molecular weight, long carbon chain backbone, three-dimensional structure, hydrophobic nature (Hadad et al., 2005; Kawai, 2010) and lack of functional groups recognizable by existing microbial enzyme systems (Chiellini et al., 2003). However, several attempts were made earlier to investigate the microorganisms capable to utilize the thermoplastics (Onodera et al., 2001; Gilan et al., 2004; Booth et al., 2007; Shah et al., 2008; Gang et al., 2011). Further, the utilization of microbial consortia offers considerable advantages over the use of pure cultures in the degradation of recalcitrant compounds considering its multifunctional ability and can be more robust to environmental fluctuations (Gilbert et al., 2003; Roy et al., 2008).

The carrier based formulation of microbial cells has long been established for applications in various fields like agricultural (Trivedi and Pandey 2008; Kumar et al., 2010), industrial (Tanaka et al., 1993) and pharmaceutical (Kalyan et al., 2010) sectors. The aim of formulating viable cells in carriers is to facilitate the delivery and handling processes, and to ensure the adequate cell viability to maintain the efficacy of the cells (Bazilah et al., 2011). For bioremediation purposes, formulated microbial cells are often applied using wet (liquid) formulations i. e. by spraying inoculum suspensions on targeted sites, or using dry (solid) formulations like granules or dust (Sathiya moorthi et al., 2008). The selection for the type of formulation developed and used is dependent on the nature of the active cells and factors related to the site of application such as aquatic or terrestrial landscapes and temperature, etc (Tu and Randall, 2005). Most often, dry formulations are preferred over wet ones because they provide extended shelf life and are easier to store for long time and transport (Lumsden et al., 1995).

In this context, four efficient polymer-degrading bacterial consortia have been documented earlier. Among all, two consortia have documented to degrade synthetic po-

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lymers like low density polyethylene (LDPE) (Kapri et al., 2010a), epoxy and epoxy silicone blends (ESBs) (Negi et al., 2009). Moreover, the participating strains have also been used in combination with other bacteria to degrade high density polyethylene (HDPE) (Satlewal et al., 2008), non-poronized and poronized LDPE (Soni et al., 2009). These consortia have also been reported to degrade LDPE in the presence of superparamagnetic iron oxide nanoparticles (SPION), nanobarium titanate (NBT) and fullerene nanoparticles (Kapri et al., 2009; Sah et al., 2010; Kapri et al., 2010a, b). Further, another two consortia have been reported for the degradation and utilization of polyvinyl chloride (PVC) as carbon and energy source (Anwar, 2011). The viability of these four potential polymer-degrading consortia needed to be tested in carrier-based formulation (talc) after storage at room temperature (28 ± 1 °C). Thus, viability of inoculums in an appropriate formulation for a certain length of time is important for commercialization of the technology. For this purpose, the present study has been conducted with a view of developing microbial inoculants in carrier-based formulation for LDPE and PVC biodegradation. Further, their biodegradation efficiencies after 70 days of storage were tested.

MATERIALS AND METHODS

LDPE and PVC films

Commercially available branched LDPE (0.92 g cm^{-3}) and PVC films (3.0 ± 0.01 × 10^{-2} mm) were used in this study. LDPE film contained different additives in the form of Masterbatch (trade name), a mixture that contains cornstarch, linear low-density polyethylene (LLDPE), styrene-butadiene-styrene copolymer (SBS) and manganese stearate. The PVC film contains different additives in the form plasticizer.

Talc as carrier

Talc was purchased from HiMedia Lab Pvt. Ltd, Mumbai, India. It was composed of talcum steatite, talc fine powder and hydrous magnesium silicate.

Bacterial consortia

A total of twelve bacterial cultures were retrieved from the departmental culture collection of Microbiology, CBSH, G. B. Pant University of Agriculture and Technology, Pantnagar, India (Table 1). These were originally isolated from different plastic waste disposal sites and artificial soil beds (Satlewal et al., 2008; Negi et al., 2009; Kapri et al., 2009; Sah et al., 2010; Kapri et al., 2010 a, b). These cultures were selected based on their pre-identified potential to degrade a variety of polymers like LDPE (Satlewal et al., 2008; Soni et al., 2009; Kapri et al., 2009; Sah et al., 2010; Kapri et al., 2010 a, b), HDPE (Satlewal et al., 2008), epoxy, and epoxy silicone blends (Negi et al., 2009) and PVC (Anwar, 2011). These bacterial strains were characterized and developed into four different consortia in different combinations (Table 1). The cultures were revived by inoculating into 10 mL of nutrient broth (HiMedia, India) and maintained on nutrient agar (HiMedia, India) at optimum pH (7.0 ± 0.2) and temperature (37 °C). A single colony from each culture was inoculated into 20 mL flask containing 10 mL of nutrient broth (pH 7.0 ± 0.02) and active cultures were prepared by incubating the flask at 37 °C for 16 h with continuous shaking at 120 rpm. The calculated amount of colony forming unit per mL (CFU/mL) of each strain was mixed for the development of consortium as described by Goel et al., 2010.

Table 1. **Bacterial strains used in this study**

Consortium	Bacterial strains
H	*Microbacterium* sp. strain MK3 (DQ318884), *Pseudomonas putida* strain MK4 (DQ318885), *Bacterium* Te68R strain PN12 (DQ423487)
L	*Pseudomonas aeruginosa* strain PS1 (EU741797), *P. putida* strain PW1 (EU741798), *P. aeruginosa* strain C1 (EU753182)
CP-I	*Acanthopleurobacter pedis* strain SPA1 (GU598259), *Bacillus cereus* strain SPA2, (GU598260)
CP-II	*P. otitidis* strain SPT1, (GU598256), *Bacillus aerius* strain SPT2 (GU598257), *Acanthopleurobacter pedis* strain SPT3 (GU598258), *Bacillus cereus* strain SPK1 (GU598261)

Preparation of talc based formulation

Active consortium (200 mL) was divided into four parts, 50 mL each in centrifuge tubes and spun at 5000 rpm for 10 min to remove the cells. Later the supernatant was partially decanted and the tubes were vortexed for 15 min. Then, 2.5 g talc was weighed and added to each tube with pellets under sterile conditions. The tubes were vortexed again for homogenous mixing of talc with the bacterial suspension. With a sterile spatula the mixture is then emptied into glass petri-plates. The plates were kept at room temperature (28 ± 1 °C) aseptically for drying the mixture.

Shelf life of talc based formulation

The viability of bacterial isolates in the formulation was ascertained by serial dilution plating method. For this purpose, 50 mg of talc-based formulation was dissolved in 1 mL of sterile distilled water in an eppendorf tube. Later, the suspension was dissolved in 9 mL of sterilized distilled water. Likewise, dilution plating was done for consortium H, L, CP-I and CP-II in nutrient agar medium (Table 2). The plates were incubated at 37 ± 1 °C and viability was checked initially after 2 and 4 days. Thereafter, the CFU/mL counts were determined after regular interval of 7 days for subsequent 3 weeks followed by 15-day interval up to 70th day. The above pattern was followed keeping in view the rapidity of changes in viable counts during storage. The plate counts were carried out in triplicates and the final CFU/mL were the average of three readings.

Table 2. **Enumeration of total viable count of respective consortia under carrier-based formulation**

Consortium	Dilution Factor	CFU/mL* at subsequent time intervals (days)							
		2nd	4th	11th	18th	25th	40th	55th	70th
H	10^6	279 ± 2	276 ± 2	271 ± 2	269 ± 2	267 ± 2	269 ± 2	269 ± 2	270 ± 2
L	10^7	174 ± 2	174 ± 2	130 ± 2	127 ± 2	116 ± 2	77 ± 2	54 ± 2	32 ± 2
CP-I	10^7	280 ± 2	266 ± 2	260 ± 2	256 ± 2	223 ± 2	146 ± 2	104 ± 2	74 ± 2
CP-II	10^6	223 ± 2	216 ± 2	210 ± 2	176 ± 2	171 ± 2	90 ± 2	76 ± 2	36 ± 2

* The data are average of triplicate experiment values

Functional characterization of carrier based formulation

Isolated viable colonies of bacteria constituting each consortium were picked up from the respective dilution plating of formulation on 70th day. The isolated colony of each bacterium was inoculated in a flask containing 10 mL nutrient broth and then incubated at 37 °C with continuous shaking (120 rpm) for overnight. Thereafter, active consortium was developed as described earlier and *in vitro* biodegradation assay was repeated in order to test and compare the biodegradation potential of the consortia before and after formulation in talc. For functional characterization of carrier based formulation, 100 mL minimal broth davis without dextrose (pH 7 ± 0.2) was taken in two 250 mL Erlenmeyer flasks for each consortium. The coupons of LDPE, size 2.54 × 2.54 cm^2 (1 $inch^2$) added to these flasks and them inoculated with 300 µl of active consortia L and H separately. Similarly, the stated treatment was conducted with PVC film using consortia CP-I and CP-II separately. The films were surface sterilized with 70% ethanol for 10 min prior to addition. The experiment was performed with respective positive (minimal broth + consortia) and negative (minimal broth + LDPE/PVC) controls. The flasks were incubated at 37 °C with continuous shaking (120 rpm). Samples were collected after regular intervals of 24 h and analyzed spectrophotometrically for OD at 600 nm and λ-max.

Scanning electron microscopy

Polymer (LDPE and PVC) film samples removed from the broth after 5 days of *in vitro* assay and surface sterilized with 70% ethanol for 10 min, before drying them in a desiccator for 24 h under vacuum. The samples were metallized with gold (3 discharges of 40 mA/50s in argon atm) in a high vacuum metallizator (Bal-Tec SCD 005) and analyzed by SEM (Leo, 435VF, UK) at 15.00 kV EHT and three successive magnifications (0.8, 1.5 and 3.0 KX).

RESULTS AND DISCUSSION

Shelf life of talc based formulation

The viability of all the consortia was tested in talc-based formulation for a storage period of 70 days. Each consortium differed markedly from each other with respect to CFU/mL counts after two days of storage. With the progression of storage, consortium H showed a sustained viability, whereby the counts dropped marginally from 279 × 10^6 to 270 × 10^6 after 70 days of storage. However, in case of consortium L, the cell viability decreased significantly after 11 days (from 174 × 10^7 to 130 × 10^7), and finally recording reduced counts of 32 × 10^7 after 70 days. Moreover, in case of consortium CP-I cell viability slightly decreased (from 280 × 10^7 to 223 × 10^7) up to 25th day, with remarkable decrease recorded after 40 days (146 × 10^7) and subsequently reduced to 74 × 10^7 CFU/mL. Consortium CP-II also followed a similar pattern of reduction in cell viability during storage period and reduced to 36 × 10^6 CFU/mL after 70 days of storage (Table 2). These observations suggested that the consortium H and CP-I were more stable and viable as compared to consortium L and CP-II in the developed formulations.

For commercialization, the viability of bioinoculants in a prescribed formulation for a certain period of storage is desirable (Smith, 1992; Bazilah et al., 2011). Similar studies of viability counts were also conducted on PGPR bioinoculants using sawdust as carrier (Arora et al., 2008). Talc based formulation has also been reported for PGPR strains for the storage and management of various plant pathogens (Shanmugam et al., 2011). In field of biodegradation, clay based formulation of *Pseudomonas* cells for degrading petrol has been reported earlier by Ting et al. (2010).

Functional characterization of carrier based formulation

Growth profiling of used consortia was studied in the presence of respective polymers *viz.* LDPE and PVC before and after retrieval from the talc-based formulation (Fig. 1). In the case of LDPE biodegradation studies, the growth of consortium H was found to increase in the presence of LDPE as compared to the control under both conditions. Thus, the growth phases were not affected; the consortium reiterated the growth pattern in both cases (Fig. 1a). However in case of consortium L, the growth phases were insignificantly affected in the presence of LDPE and found to be sustained after 24 h of incubation (Fig. 1b). Further, λ-max of the biodegraded LDPE film samples was found to deviate from 209 nm in the control to average values of 203 and 215 nm by consortium H and L, respectively under both conditions (Fig. 1a, Fig. 1b).

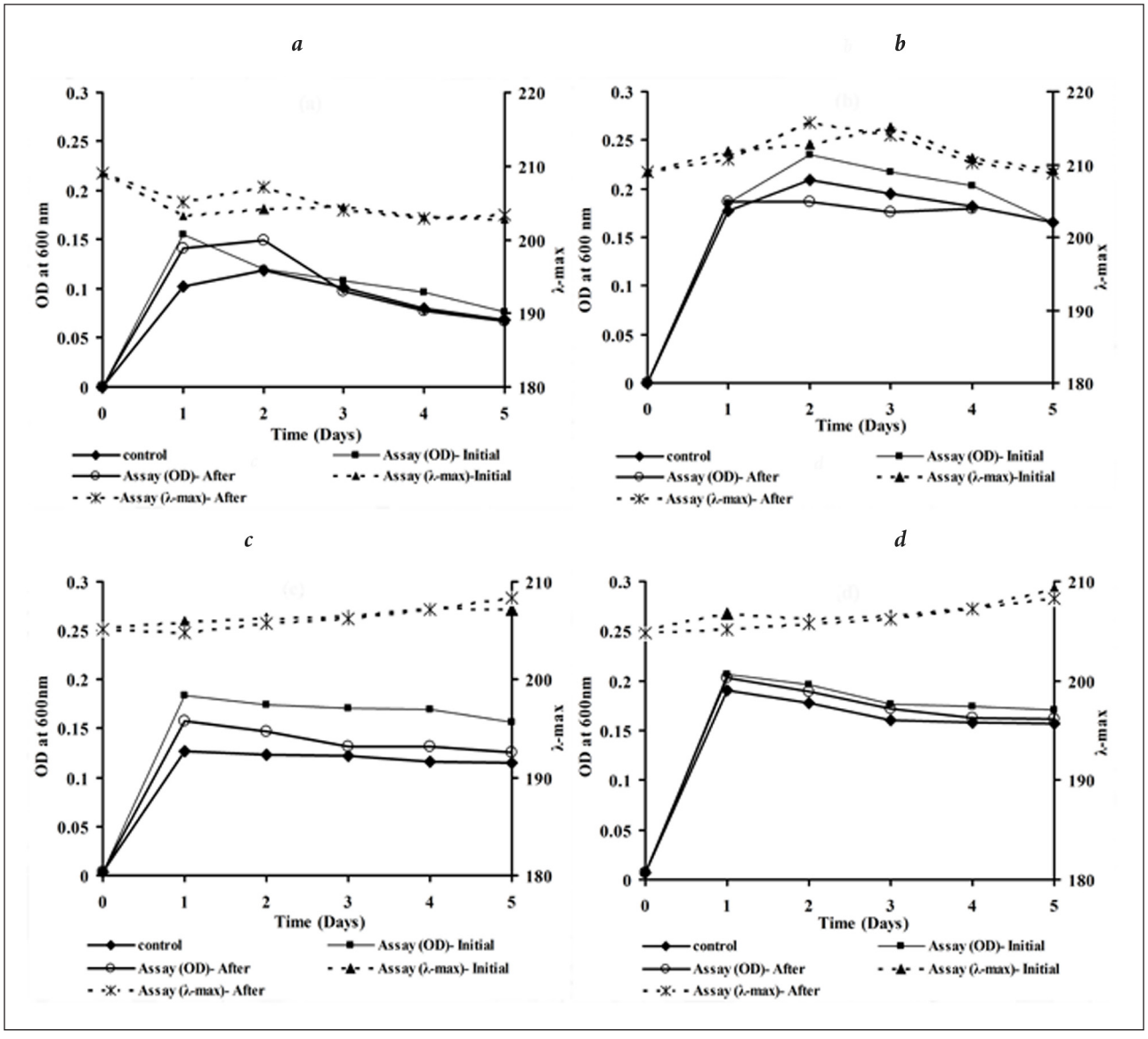

Fig. 1. Comparative *in vitro* biodegradation assay of LDPE film using consortium H (a), consortium L (b) and PVC film using consortium CP-I (c), consortium CP-II (d) before and after retrieval from talc based formulation

Further, in the case of PVC degradation studies, the biomass of consortium CP-I was found to be higher in the presence of PVC as compared to the control under both conditions (Fig. 1c). However in case of consortium CP-II, this difference was insignificant under both conditions (Fig. 1d). Moreover, the consortia reiterated the growth pattern in both cases. The λ-max of the biodegraded PVC film samples was found to be deviated from 205 nm in the control to average values of 207 and 209 nm by consortium CP-I and II, respectively. Further, after talc-based formulation the λ-max of the biodegraded PVC film samples was found to be deviated from 205 nm in the control to average values of 208 nm for both consortia CP-I and II, (Fig. 1c, Fig. 1d). Similar shifts in the λ-max have been reported earlier in case of LDPE, HDPE biodegradation (Satlewal et al., 2008; Soni et al., 2009) and epoxy and ESB biodegradation (Negi et al., 2009). Thus, the observations suggest that all the consortia sustained their biodegradation efficiencies even after storage in talc-based formulation for 70 days.

Scanning electron microscopy

The SEM was performed to detect the change in morphology of the polymer film surface after treatment with the used consortia. The SEM micrographs of the undegraded LDPE (Fig. 2a) and PVC film (Fig. 3a) as control illustrated a smooth surface morphology. After 5 days of incubation, the LDPE film degraded by consortium H (Fig. 2c) and consortium L (Fig. 2e) recovered from the talc after 70 days of storage demonstrates occurrence of several non-uniformly

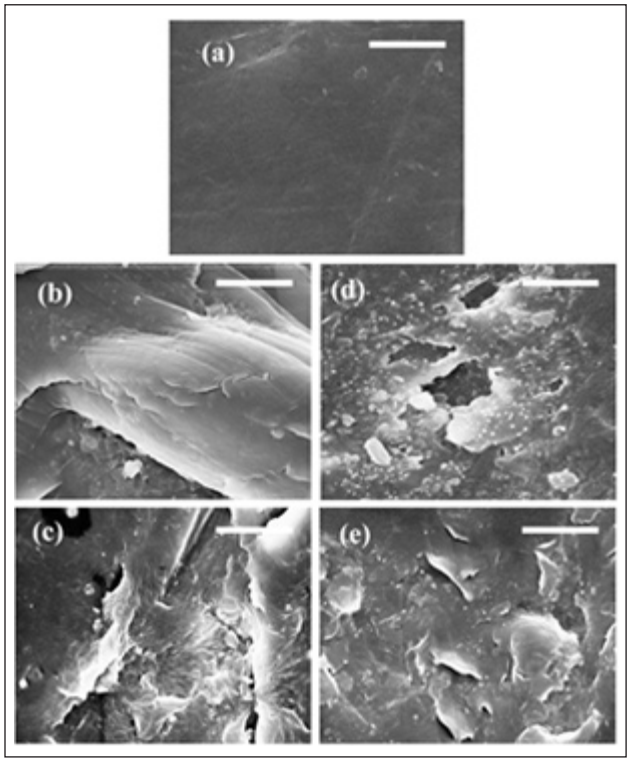

Fig. 2. Comparative SEM micrographs of LDPE film degraded by consortium H (b and c) and L (d and e) before and after formulation in talc, respectively, by taking untreated LDPE film as control (a). Scale bars = 10 µm; magnification = 3.00 KX

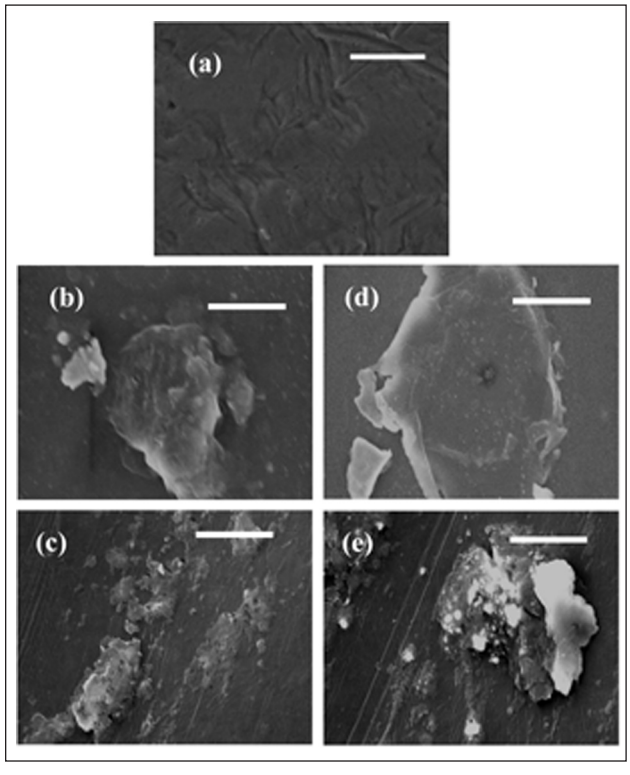

Fig. 3. Comparative SEM micrographs of PVC film degraded by consortium CP-I (b and c) and CP-II (d and e) before and after formulation in talc, respectively, by taking untreated PVC film as control (a). Scale bars = 10 µm; magnification = 3.00 KX

scattered whitened areas and erosion zones. Moreover, the worn-out areas with randomly distributed cracks and fissures reveal the disruption of surface texture of LDPE film. However, the extent of biodegradation is identical to the initial biodegradation efficiency documented by both consortia before talc formulation (Fig. 2b and Fig. 2d, respectively). Similar observations have also been reported by Kapri et al. (2010), where they have demonstrated similar results of SEM micrographs showing surface disintegration and disruption of the LDPE film samples in the presence of SPION and NBT. Further, the PVC film degraded using the respective consortia (CP-I and II) demonstrates the similar pattern of disruption in surface texture before (Fig. 3b, Fig. 3d) and after 70 days of storage in talc (Fig. 3c, Fig. 3e). Thus, the comparative biodegradation studies revealed that the biodegradation potential of all the consortia remained unaffected during and after the storage period in talc-based formulations.

Conclusively, the talc is managed to prolong the shelf life and sustain the efficacy of the bacteria suggesting the carrier is stable for the bacteria. Further, the study reveals that two LDPE degrading consortia (H and L) and two PVC degrading consortia (CP-I and II) have retained their characteristic biodegradation property of degrading LDPE and PVC, respectively, in the formulations. Moreover, consortia H and CP-I were found to be more viable in the formulations than consortia L and CP-II. Therefore, the present investigation may be a step towards field application and commercialization of talc as a carrier for long-term sustenance / storage of LDPE/PVC-degrading consortia, which may minimize solid waste disposal in the environment. However, further *in situ* trials are needed, and are already in progress in order to validate the material for large-scale application as an ultimate goal of this study.

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LDPE IR PVC POLIMERUS SKAIDANČIŲ BAKTERIJŲ MIŠINIŲ EFEKTYVUMAS IR JŲ GYVYBINGUMO TRUKMĖ BIOPREPARATUOSE

S a n t r a u k a

Tyrimo metu nustatytas talko užpildo pagrindu pagamintų polimerus – žemo tankio polietileną (LDPE) ir polivinilchloridą (PVC) – skaidančių bakterijų mišinio preparatų vartojimo ilgaamžiškumas. Tirti keturių bakterijų rūšių – *Microbacterium sp., Pseudomonas putida, P. aeruginosa, P. otitidis, Bacterium Te68R, Bacillus aerius, B. cereus ir Acanthopleurobacter pedis* – mišiniai. Įvertinus kolonijas sudarančių vienetų skaičių viename preparato mililitre, nustatytas bakterijų mišinio gyvybingumas bei išmatuotas talko pagrindu pagaminto biopreparato gebėjimas skaidyti polimerus *in vitro*. Polimero struktūra ištirta išmatavus maksimalios λ vertės pokyčius, o paviršiaus pokyčiai – apžiūrėjus nuskaitančiu mikroskopu (SEM). Tyrimu nustatyta, kad visi preparatuose esantys bakterijų mišiniai išsaugo reikiamas charakteristikas netgi po 70-ies eksploatavimo gamtinėmis sąlygomis parų. Be to, nuskaitančio mikroskopo nuotraukos rodo, kad, pasibaigus naudojimo laikui, polimerus (LDPE ir PVC) skaido preparatuose išgyvenusios bakterijos. Prieita prie išvados, kad talkas yra tinkamas komponentas, padedantis lengvai paskleisti preparatą ir palaikantis bakterijų mišinių gyvybingumą.

Raktažodžiai: biologinis skaidymas, bakterijų mišiniai, talkas, konsorciumai, galiojimo laikas, LDPE, PVC, SEM