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BIO-DEGRADATION OF POLYHYDROXYALKANOATES (PHA) FILMS IN SOIL AND LAKE ENVIRONMENT

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ABSTRACT

The use of petroleum-based synthetic plastics has led to a deleterious solid waste management especially in the form of marine debris and presents a major growing global pollution problem. In response to these issues, the application of biobased and biodegradable polymers as an alternative to synthetic plastics has been proposed. Polyhydroxyalkanoates (PHA) is a biodegradable microbial polymer. In this study, the biodegradation of this PHA both in soil and lake environment was evaluated. The percentage of degradation of the PHAs with various monomers such as poly (3-hydroxybutrate) [P(3HB)] and its copolymers poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB] in soil and lake was carried out. Besides, the modifications of these biopolymers into salt-leached films were also tested. Based on the results obtained, P(3HB-*co*-4HB) films showed the highest rate of degradation for both lake and soil environment. Also the degradation of PHA, mainly caused by microbial activity, isolation and identification microorganisms capable of degrading PHA was also carried out. Based on the degradation index, *Pseudomonas* species and *Acidovorax* species were isolated.

Key words: PHA, P(3HB-*co*-4HB), degradation

INTRODUCTION

Synthetic plastic which is most commonly derived form petrochemicals, is extremely versatile material and is ideal for a wide range of consumer and industrial applications. Every year synthetic plastic is manufactured over 200 million tons (Atlee *et al.*, 2016). However, the excessive use of these petroleum-based plastic has led to deleterious solid waste management problems. The detrimental effect of plastic debris cause around 1 million deaths of marine habitatants anually (Debbarma *et al.*, 2017). The versality and impreviousness of synthetic plastic which is comparatively low in cost results in its accelerating rise in demand and production. As it is, over nine billion tons of plastic has been generated over the last six decades. Every year, 9 million tons of plastic is being dumped into our oceans causing a detrimental effect to our marine organisms and the ecosystem. These plastic pollutants which enters our oceans or ends up in landfills, take up about 500 years to decompose while leaching toxic chemicals into the ground.

The harmful effects of this petrochemical derived plastic has garrnered attention worlwide and prompted a global scientific drive to develop an alternative green, ecofriendly and biodegradable polyesters as plastic substitutes. Polyhydroxyalkanoate (PHA), biodegradable microbial plastic produced from natural substrate has gained significant attention and popularity over the years globally (Sudesh & Doi, 2005). These polymers are produced under limiting conditions of nitrogen, phosphate, oxygen or magnesium sources as an intracellular energy storage material accumulated as granules within the cytoplasm (Martinez-Toboon *et al*., 2018).

As the volume of PHA production increases and the span of application widens, it is relevant to study the mechanism of biodegradation under

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various natural conditions. In this study, the biodegradation of PHAs such as poly(3-hydroxybutrate)[P(3HB)], poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB] and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P3HB-*co*-3HV)] have been investigated. As the degradation of PHA is mainly caused by microbial activity, isolation and identification microorganisms capable of degrading PHA was also carried out.

MATERIALS AND METHODS

Fabrication of polymer films

Homopolymer P(3HB) natural origin (Sigma-Aldrich) powder was used as a polymer substrates for the media. The copolymer P(3HB-*co*-4HB) with molar fraction 14, 47 and 87mol% were obtained from Lab 318, School of Biological Science, Universiti Sains Malaysia (USM). PHA films were prepared by solvent casting and salt leaching techniques which were prepared according to the previously decribed method (Faezah *et al*., 2011).

Biodegradation of the polymer films

The biodegradation study of the polymer films was carried out at lakes located near the Restu, Saujana and Tekun hostels, USM and the soil degradation was carried out at the garden of Biological School, USM. The films were cut into small pieces 1.2 cm x 1.2 cm per piece. The thickness of the films was about 0.3 mm to 6 mm. The average weight of the scaffold was 0.0190 g. The films were placed into a 2 cm x 2 cm nylon pocket mesh with different color pockets, differentiating one scaffold from another. Immersion test method was used to study the rate of degradation in lake as previously described (Sridewi *et al*., 2006; Salim *et al.*, 2012). Soil burial method was used to study the rate of degradation in garden soil. A dimension of 1 x 0.3 m flower bed was made and divided into 5 portions. In each portion the samples were buried 10 cm below the soil. All the degradation samples were carried out in triplicates. The samples (solvent cast films) were later retrieved from the lakes and soil after 5 weeks whereas the salt-leached samples were retrieved after 2 weeks, rinsed with distilled water to remove soil particles. Then dried at room temperature (Salim *et al.*, 2012).

Degradation of P(3HB), P(3HB-*co*-4HB) and P(3HB-*co*-3HV) of films

The percentage of degradation of the films was determined by using the following formula:

$$\% \text{ of weight loss} = \frac{\text{initial weight} - \text{final weight after degradation}}{\text{Initial weight of film}} \times 100$$

Isolation and identification of PHA-degrading bacteria

Debris from the samples was washed off using distilled water. The degrading films and different dilutions of the resulting suspension were inoculated onto P(3HB) agar plates (Salim *et al*., 2012). The different bacteria were later cultured onto new P(3HB) agar plate and clear zone around the colony was observed while the diameter of the halo zone was measured. The degradation index of the isolated bacteria was verified as follows:

$$\text{Degradation index} = \text{Halo zone diameter (mm)} / \text{bacterial colony diameter (mm)}$$

The PHA-degradading bacterial identification of 16S rRNA gene sequence was determined by direct sequencing of PCR-amplified 16S rDNA. Briefly, the genomic DNA extraction, PCR mediated amplification of the 16S rDNA and purification of the PCR product was carried out. The resulting sequence data from the strain was put into the alignment editor and aligned manually and compared with the representative 16S rRNA gene sequences of organisms (Amirul *et al.*, 2009).

Statistical analysis

The experimental values were represented as mean and standard deviation. All the data was analyzed using ANOVA and Tukey's test using SPSS 20.0 software. The significance level adopted was $p < 0.05$.

RESULTS AND DISCUSSION

The biodegradation of the solvent cast film

In this study, there are four types of polymers tested. These polymers are P(3HB), P(3HB-*co*-87% mol 4HB), P(3HB-*co*-47% mol 4HB) and P(3HB-*co*-14% mol 4HB). These were prepared using solvent casting and salt leaching techniques. In this experiment, these films were immersed in the lake or burried under the soil while the percentage of polymer degradation was recorded. Figure 1 shows the percentage of degradation of solvent cast films at the end of 5 weeks. The P(3HB-*co*-87% mol 4HB) showed the highest percentage of degradation in soil and lake at 98.9 ± 1.8% and 81.5 ± 2.9% respectively followed by P(3HB-*co*-47% mol 4HB) at 84.2 ± 2.9% and 56.7 ± 2.3% respectively. The P(3HB) scaffold showed the lowest percentage of degradation at 43.51 ± 4.8% and 43.53 ± 1.5% in lake and soil. However, P(3HB-*co*-14% mol 4HB) also showed low degradation in lake and soil at 44.8 ± 1.8% and 35.1 ± 2.6%. It can be deduced that the percentage of degradation of salt leached film is higher as compared to solvent-cast film. Based on

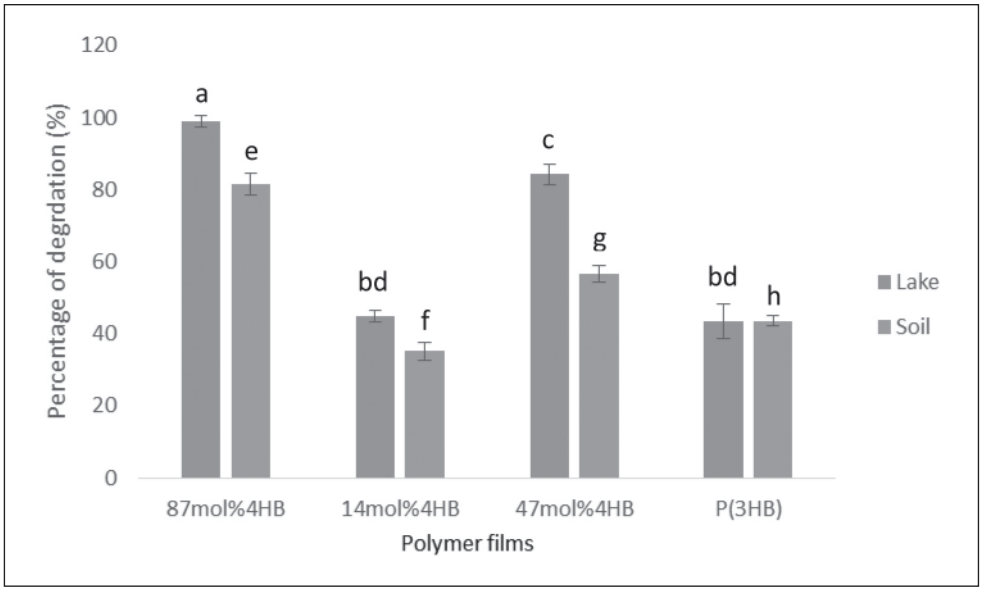


Fig. 1. Percentage of degradation of solvent-cast films in the lake and soil for a period 5 weeks. Degradation was calculated by observing weight changes of the replicates of three films. Means with different alphabets within the same column are significantly different at $p < 0.05$ level (Tukey test).

the results, salt leached films are able to achieve almost similar percentage of degradation as solvent cast film in only two weeks. Therefore, polymers that were prepared using salt leached film techniques can be degraded faster than solvent-cast film technique. Basically, degradation of PHA is determined by environmental conditions and properties of the PHA materials (Ong *et al.*, 2017). The rate of degradation of various PHAs in the soil and water could be affected by the surface porosity of these films (Faezah *et al*., 2011).

The biodegradation of salt leaching scaffold between lake and soil

Figure 2 showed the percentage of degradation of salt-leached films in both soil and lake for 2 weeks. In general, both conditions showed same pattern polymer degradation whereby highest percentage of degradation was P(3HB-*co*-87% mol 4HB) at 98.3%±2.9 and 67.0%±0.7 in lake and soil resepectively followed by the lowest percentage of degrdation of P(3HB) at 51.9 ± 3.2% and 32.1 ± 3.5% in lake and soil respectively. However the percentage of degradation in lake is higher as compared to soil. Table 1 and Table 2 showed physical changes of the solvent cast and salt-leached films in soil and lake. Similarly, the size of the polymer decreased into small fragments and the colour of the film changed to brownish for both the solvent cast and salt-leached films. Similar observation was reported in regards to the bio-degrdation of various P(3HB) and it's composites in soil environment (Altaee *et al*., 2016).

Isolation and screening PHA-degrading bacteria

Based on the degradation index we could assume the strength of the degradation enzyme produced among the bacterial colonies isolated. All the colonies showed the formation of clearzone (halozone) after 5 days of incubation. Table 3 showes the identification of PHA-degrading bacteria based on degradation index of the bacterial colonies. The secretion of extracellular depoly-merases by microorganisms possess the ability to hydrolyze PHA (Handrick *et al*., 2001) The identi-fication of the PHA-degrading bacteria was carried out using molecular analysis, 16s rRNA. Based on the results of 16s rRNA analysis, B1 and B6 were found to have similarities to *Pseudomonas* species. In fact, the first PHA-degrading microorganisms and enzymes were isolated from *Pseudomonas* strains (Numata *et al*., 2009). These microorganisms were found to excrete a number of extracellular PHA depolymerases which enables the degradation of environmental PHA and the decomposed compounds were utilized as nutrients. Meanwhile bacteria B4, B3 and B8 had been identified as *Massilia* species, *Thiomonas* species and *Acid-ovorax* species respectively. PHA degrading micro-organisms vary with respect to the type of PHA they can degrade. Most PHA degrading microorganisms have enzymes with substrate specificity for P(3HB). However, there are microorganisms that have enzymes with broader substrate specificity and are capable of utilizing wider range of PHA (Ong *et al*., 2017; Vigneswari *et al.*, 2015).

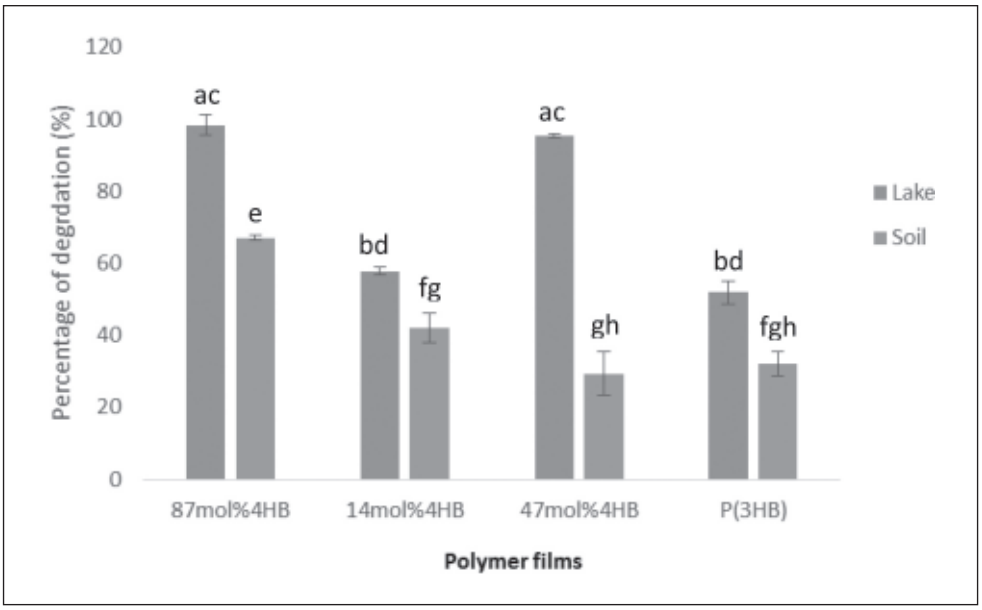


Fig. 2. Percentage of degradation of salt-leached films in the lake and soil for a period of 2 weeks. Degradation was calculated by observing weight changes of the replicates of three films. Means with different alphabets within the same column are significantly different at $P < 0.05$ level (Tukey test).

Table 1. Physical changes of the various PHA solvent-cast films in soil and lake for a period of 5 weeks

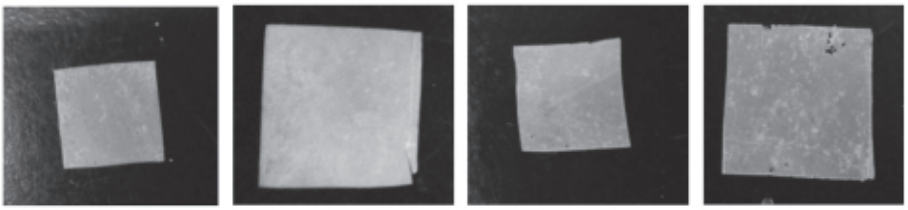
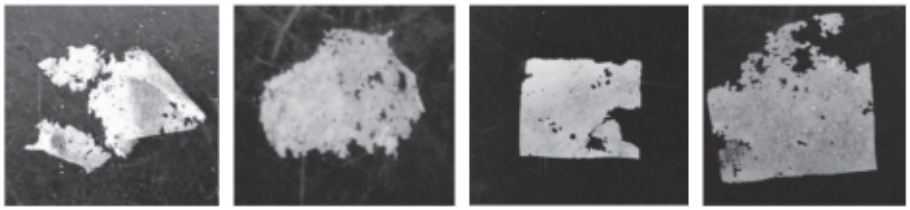
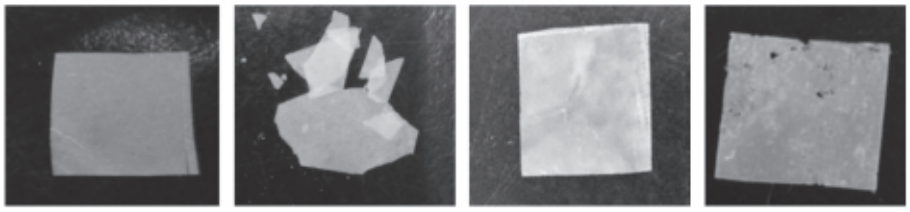
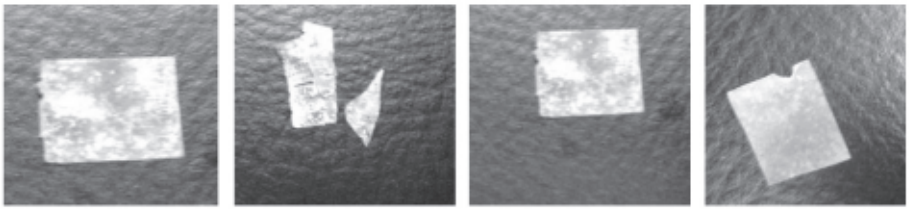
Types of polymer	Soil		Lake	
	Week 3	Week 5	Week 3	Week 5
P(3HB)				
P(3HB-*co*-87%4HB)				
P(3HB-*co*-47%4HB)				
P(3HB-*co*-14%4HB)				

Table 2. Physical changes of the various PHA salt-leached films in soil and lake for a period of 2 weeks

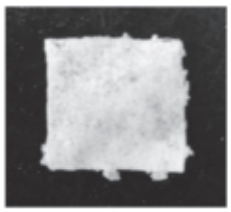
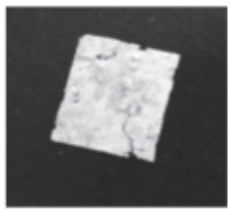
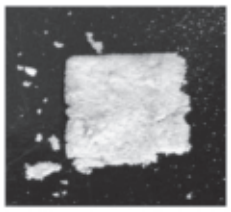
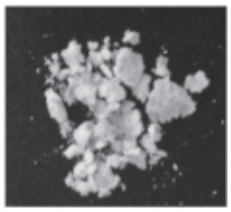
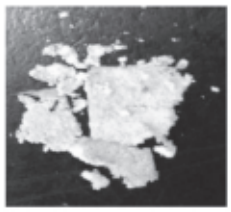
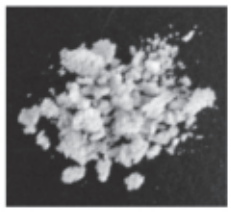
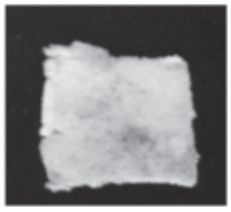
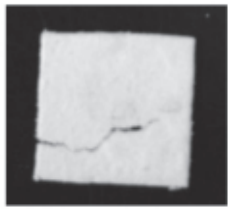
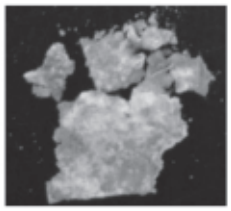
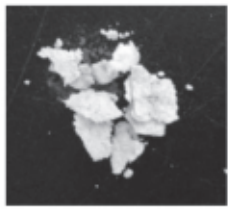
Type of polymer	Soil		Lake	
	Week 1	Week 2	Week 1	Week 2
P(3HB)				
P(3HB-*co*-87%4HB)				
P(3HB-*co*-14%4HB)				
P(3HB-*co*-47%4HB)				

Table 3. Bacterial identification based on 16s RNA and the degrdation index of the bacterial strain

Bacteria	Place	Degradation index	Bacterial identification
B1	Lake	6	*Pseudomonas* sp.
B3	Lake	6.5	*Thiomonas* sp.
B4	Lake	4.4	*Massilia* sp.
B6	Soil	6.9	*Pseudomonas* sp.
B8	Lake	7	*Acidovorax* sp.

CONCLUSION

The different types of PHA films and the different techniques of fabrication which were solvent cast film and salt-leached film used in this study degraded well in the both the soil and lake. It is also apparent that the salt-leached films degraded more rapidly than the solvent cast films due to differences in surface morphology. Although the degradation rate for P(3HB-*co*-14mol%4HB) was expected to be higher than that of P(3HB), in an uncontrolled environment. It was found that the percentage of degradation of these films were in general quite similar. Surface modification like salt-leaching of these PHA films may be useful for accelerating the percentage of degradation of PHA based materials.

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