Available online at www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

Der Pharma Chemica, 2017, 9(19):34-39
(http://www.derpharmachemica.com/archive.html)

Studies on Biodegradability of Copolymer from PET-LA Copolymerization in Presence of Hexavalent Chromium

Prachi Agarwal[1*], Ajit Joshi[2], Himanshu Sharma[3]

[1]Department of Biochemistry, Mewar University, Gangrar, Chittorgarh, Rajasthan, India
[2]Department of Chemistry, Mewar University, Gangrar, Chittorgarh, Rajasthan, India
[3]Department of Applied Chemistry, MIET, Meerut, U.P, India

ABSTRACT

Heavy metals present in high concentrations in aquatic systems are posing serious problems to aquatic life. Among the heavy metals, chromium has been used in many industries and hence its removal from waste waters is significant. Biodegradation is an economic and ecofriendly method employed in the removal of heavy metals. Use of microbes like bacteria, algae, yeasts and fungi as degrading agent for heavy metal removal has received interest because of high surface to volume ratio, availability, rapid kinetics of adsorption and desorption and low cost. In present paper, the microbial degradation of polymeric material was carried out by incubating the polymeric films with microbes like Pseudomonas fluorescens, Phanerochaete chrysosporium, Trametes versicolor, Streptomyces species, Aspergillus niger, Nocardia sp, Bacillus subtilis etc., in presence of hexavalent chromium ions (Cr^{6+}). Degradation impact by microbes on the crystalline structure was seen in Scanning Electron Microscopy (SEM) micrographs. A record of loss in weight and tensile strength were also made. Esterase was found to be involved in the biodegradation. Polymeric films that are incubated with P. fluorescens in presence of Cr^{6+} ions, biodegradation was faster as compared to other microbial species. Further SEM analysis confirmed the presence of abundant colonies of branched mycelium throughout the infected transparency sheet was clearly visible.

Keywords: Heavy metals, Copolymer, Polymeric films, Degradation

INTRODUCTION

Rapid industrialization and uncontrolled extraction of natural resources results in generation of large amounts of toxic chemicals which pollute the soil and water. Decontamination of polluted sites requires state-of art and cost effective technologies to convert them to safe for human habitations [1]. Heavy metal pollution is a most important problem, as it leads to toxicity, risk to human survival and disruption of ecological balance [2]. The occurrence of toxic heavy metals in the environment result in geo-accumulation, bioaccumulation and biomagnification processes which have intense ecological and public health implications [3].

As chromium is non-biodegradable and toxic in nature, removal of Cr from the industrial effluents is mandatory before disposing them in water bodies. Bacteria detoxify chromium mainly by reducing Cr (VI) to Cr (III), through Cr (V) and Cr (IV) intermediates [4] and it is a potentially useful process in the remediation of Cr (VI)-affected environments. Reduction of Cr (VI) to Cr (III) can be performed by a wide range of bacteria including, *Pseudomonas aeruginosa* [5], *Pseudomonas synxantha* [6], *Pseudomonas putida* [7], *Pseudomonas ambigua* [8], *Pseudomonas fluorescens* [9], *Pseudomonas dechromaticans* and *Pseudomonas chromatophila* [10].

Hexavalent chromium is toxic to bacteria present in contaminated soil or waste water. The bacterial strains growing in toxic conditions are assumed to be tolerant/resistant to chromium [11]. *Pseudomonas* sp., was the first hexavalent chromium resistant strain isolated from waste water [12]. Resistance is the ability of the microorganism to survive toxic effects of metal exposure by means of detoxification mechanisms produced in direct response to the metal concerned. Tolerance is the ability of the microorganism to survive metal toxicity by means of intrinsic properties and or environmental modification of toxicity [13]. The reduction of Cr (VI) can be identified by using diphenyl carbazide and read at 560 nm. Bacterial strains isolated from electroplating industry showed higher reduction rate [14].

In an attempt to conserve energy and minimize waste, research towards producing biodegradable polymers using starch is in effect. It has been proved [15] that microbes like *Phanerochaete chrysosporium, Trametes versicolor, Streptomyces species, Aspergillus niger, Pleurotus ostentus, Geophyllum trabeum, Thermonospora* and *Actinomadura* have the ability to degrade polymers. Later it was attempted to prepare biodegradable co-polymer or bioplastics using a *Pseudomonas* strain [16]. Active researches in the area of manufacturing biodegradable plastics that will decompose in natural aerobic (composting) and anaerobic (landfill) environments are also in effect [17]. Under regulated conditions

biodegradable plastics could potentially degrade to the point where microorganisms can metabolize them.

In the present work, attention was focused on the active role of microbes present in the environment in biodegrading a strongly bonded polymer was found to be degrading. The sheet was brittle at places and had white coloured microbes growing on it along with a foul smell too. This transparency sheet was polyester; hence possibility of secretion of esterase by the microbe for biodegradation has also been looked into. Packaging manufacturers use polyethylene terephthalate plastic mainly because of its strength, thermo-stability and transparency [18]. We have attempted in studying the physical and chemical impact of degradation.

EXPERIMENTAL SECTION

Materials

The ± DL lactic acid (AR) and ethylene glycol were from Merck, terephthalic acid was from Sigma-Aldrich Chemical Co. while stannous chloride was from Qualigens. All reagents were used as received

Synthesis of PET-LA by copolymerization

Ethylene glycol (70.5 ml) is reacted with terephthalic acid (40 g) at 240-260°C and 300-500 kPa, to yield bis(hydroxyethyl) terephthalate (BHET) [19,20]. Now, 35 g of BHET react with lactic acid (9 ml) in the presence of stannous chloride (0.8 weight%) along with p-toluene sulphonic acid [21]. This reaction mixture was again taken in a 500 ml round bottom flask which was to be immersed in an oil bath at a temperature of 180-210°C kept for 6-8 h. As the process continues, water and glycol was removed from the reaction and the remaining mixture was dissolved in chloroform which was later precipitated in methanol filtered and dried at 90°C [22] has been shown in Figure 1. The films were cut into pieces of 1 cm^2 and sterilized at UV light for 10 min. Each film was then aseptically transferred and individually placed into sterile medium.

Characterization of copolymer

Copolymer was synthesized by condensation reaction and characterized for FTIR and NMR.

Fourier transform infra-red (FTIR) spectroscopy

The FTIR spectra of synthesized copolymers were recorded using Perkin Elmer model spectrum BX Series FTIR. The spectra were recorded by using KBr Pellets. A total scan 16 scan per sample at resolution of 4 cm^{-1} were obtained over mid-IR region of 4000-400 cm^{-1}.

Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy

Chemical and sequential structures of co-polyester were determined by 300 MHz ^{1}H-NMR analysis on a Bruker DPX300 spectrometer using chloroform-d as a solvent and Tetramethylsilane (TMS) reference with a repetition delay of 2 sec.

Microorganisms

Isolation of fungi

In order to isolate fungi from the degraded polymeric films, different fungal growth media were used like seaboards agar, Potato Dextrose Agar (PDA) and a complex medium consisting of malt extract, glucose yeast extract and peptone agar. 20 ml of respective media was poured into test tubes and slants were prepared after sterilizing it at 121°C for 30 min.

Isolation of bacteria and actinomycetes

The media tried were Actinomycetes Isolation Agar (AIA), Glycerol Asparagine Agar Base (GAAB), Alternate Thioglycolate Broth (ATGB), Bushnell and Hass Broth (BHB), Luria Bertani Tributyrin media (LBT). 20 ml of respective media was poured into test tubes and slants were prepared after sterilizing it at 121°C for 30 min.

Metal solution preparation

The stock solution of Cr (VI) metal was prepared by dissolving 1000 mg of Potassium Dichromate ($K_2Cr_2O_7$) in 10 ml of double distilled water. Working concentrations of chromium solutions with different initial concentrations were made from the stock solution using metal free distilled water. Films were inoculated onto the test medium with a concentration of 1 mg ml^{-1} of Cr^{6+}. The pH of the medium was maintained at 5.2 ± 0.2. Cultures were incubated at 10, 25 and 35 ± 1°C in dark and light (3000 Lux) conditions. Growth was recorded at regular intervals.

Weight loss

Fresh films of 1 cm^2 were weighed after sterilizing them. The isolated microbes were then inoculated onto the fresh (control) films in respective medium and Cr^{6+} ion solution. Change in weight of the film was recorded on day 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200. Weight loss of the films was calculated by the following expression:

$$\text{Weight loss} = \frac{\text{Weight of sample (before degradation} - \text{after degradation)}}{\text{Weight of sample before degradation}} \times 100$$

Scanning electron microscopy (SEM)

The SEM analysis was performed to analyze the surface texture of untreated and treated films against metals using JEOL JSM-840 scanning electron microscope. The film samples were dried and placed on a metallic support, aluminum standard stub. The samples were processed at 10-15 kV and 50-120 Pa using a Large-Field detector. After incubation with fungal cultures, the pieces of polymer were taken out from the culture and repeatedly rinsed with distilled water, fungal mycelium being removed carefully. The films were dried at 35°C and use for evaluation of biodegradation efficacy. Micrographs of the samples were taken at different magnifications to identify holes and other changes on the surface during the degradation process.

Esterase analyses

To confirm that the microbes were responsible for degradation of the film, the presence of the enzyme esterase was tested. Microbes which grew

on the respective media were further analysed to test for the presence of esterase. Briefly, bacteria were subcultured on Luria Bertani plates and allowed to grow at 30 and 35°C. After 72 h, when an appreciable amount of bacteria grew, they were subcultured on Luria Bertani media containing tributyrin and further grown at 30 and 35°C in the dark.

RESULTS AND DISCUSSION

Synthesis of copolymer

During the copolymerization process (Figure 1), the temperature was maintained at 180-200°C throughout the process. After completion of the copolymerization, both chloroform and methanol were added resulting in the formation of two layers with a white precipitate at the bottom part and in the upper part, all the non-copolymerized waste residues were obtained. After filtering and drying of the precipitate, a white powder was obtained which had a melting point of 170°C confirming the formation of copolymer and on melting it at the characteristic melting point, thin films were obtained and showed increased stability.

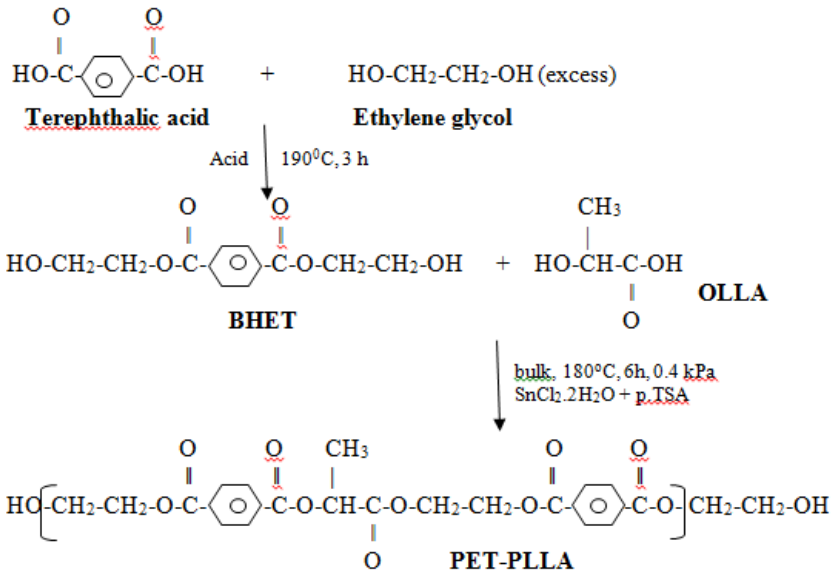

Figure 1: PET-LA copolymerization

Characterization of copolymer

FTIR spectroscopy

The FTIR studies were performed to study the extent of copolymerization of lactate monomers with BHET shown in Figure 2. Absorption peaks were observed at the wave numbers of 1716 and 1096 cm^{-1} representing the stretches of C=O and C-O bonds which confirms the presence of an ester group between BHET and Lactate monomers. The broad peak at 3447 cm^{-1} may be attributed to O–H str. of alcohol. The bands at 1507 cm^{-1} and 1575 cm^{-1} indicates C=C str. in aromatic hydrocarbon.

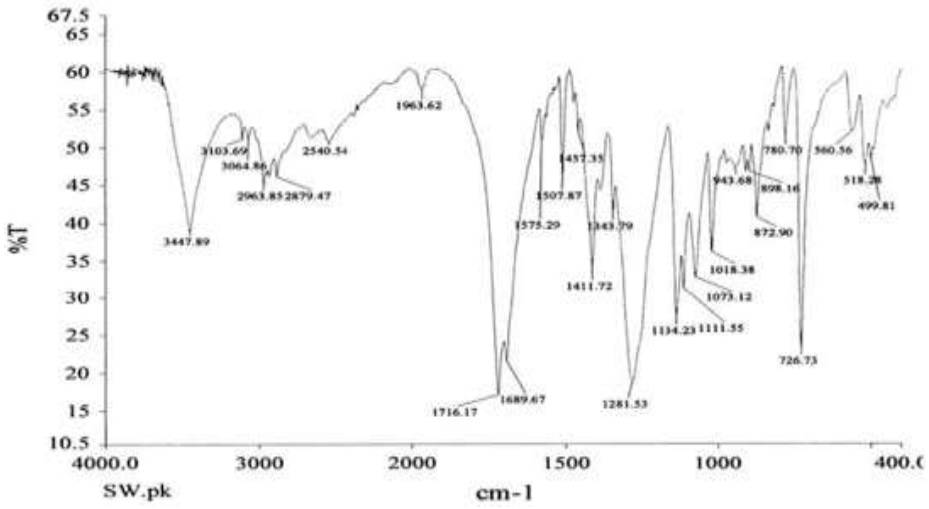

Figure 2: FTIR spectra of copolymer

^{1}H-NMR spectroscopy

The chemical shift and assignments of proton signals observed in NMR spectra are shown in Figure 3. The NMR spectra of copolymer show

signals associated with all possible E-centered triads and diads in 3.5-5.5 ppm region. This indicates that the copolymers are consisting of all similar possible connections to those synthesized from lactic acid and BHET [22].

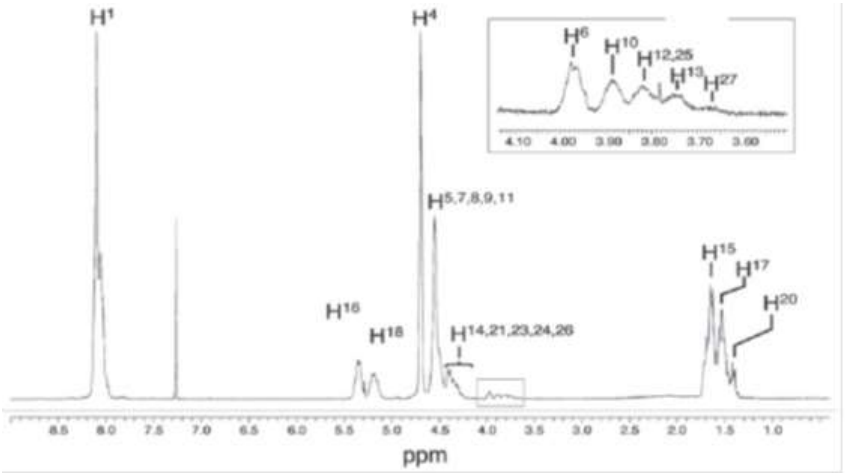

Figure 3: ^{1}H-NMR spectrum of co-polyester

Growth of fungi on different media

Fungus did not grow on any of the three fungal growth media, confirming that fungi are not involved in degradation.

Growth of bacteria on different media

Five different types of media (AIA, BHIA, GAAB, ATGB and BHB) are suitable for growth of both actinomycetes and bacteria. The most suitable temperature for the growth was 35°C. Microbes grew in both light and dark; however, the growth was better in the dark (Figure 4).

Figure 4: Growth of bacteria *(P. fluorescens)* from degrading copolymer on BHB media

All the above mentioned microbes that were isolated on different media were later grown on the control (uninfected) films to see which of them could degrade the polymer. The impact on physical property of films as well as chemical changes was also recorded.

Weight loss of degraded films in presence of Cr^{6+} ions (after 200 days)

The bacteria that were grown on various culture media had good source of nutrients, however it must be noted here that the microbes that grew on polymeric films only had the polyester as the source of carbon. Uninfected films were weighed and inoculated. As seen in Table 1, maximum weight loss of 9% on 200th day was recorded when the film was kept in contact with the microbe isolated from BHB medium and metal solution.

Table 1: Weight loss (%) of degraded films kept in contact of microbe (isolated from BHB medium) and Cr^{6+} ions after 200 days

Days	Weight loss (%)				
	AIA	BHIA	GAAB	ATGB	BHB
20	-	-	-	-	0.262
40	-	-	-	-	0.259
80	-	0.128	0.325	-	0.252
120	-	0.128	0.319	0.23	0.247
160	0.247	0.127	0.317	0.23	0.241
200	0.238	0.126	0.312	0.22	0.238
Total	3.64	1.56	4	4.34	9.16

SEM of degraded films

The colony formation of the bacterial species inside copolymer was confirmed using scanning electron microscopic analysis. The SEM images of degraded film by *Pseudomonas fluorescens* in presence of Cr^{6+} ions are indicated in Figure 5. The bacterial colonies that formed inside the pores of degraded film can be clearly observed in Figure 5b, whereas empty pores are observed in Figure 5a.

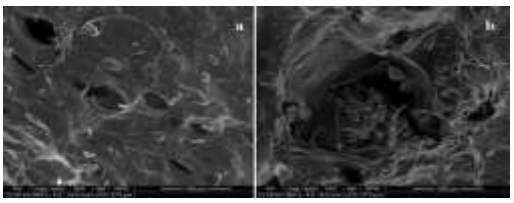

Figure 5: SEM micrographs of degraded film by *P. fluorescens* in presence of Cr^{6+} ions

Testing of degraded films (200 days)

Table 2 shows change in tensile strength and elongation of the films exposed to microbes isolated from different media and metal solution, on 200^{th} day. Microbes that grew in BHB medium were found to cause maximum loss in film weight and tensile strength also. These results prompted further analysis of the enzyme esterase present in microbes grown on BHB medium.

Table 2: Loss in tensile strength & elongation breakdown (%) of degraded films kept in contact of microbes and Cr^{6+} ions after 200 days

Media	Tensile strength (Kg/Sq.Mm)	Elongation breakdown (%)
Blank	93	6.23
Control	93	6.22
AIA	91	6.21
BHIA	92	6.22
GAAB	92	6.22
ATGB	91	6.22
BHB	76	6.20

Esterase analysis

Microbes that grew on BHB medium were further subcultured on Luria Bertani medium at 35°C in dark. Three types of microbial colonies appeared Figure 6a opaque white and circular, transparent white and circular and transparent colonies with irregular edges. A micrograph of the esterase producing microbe Figure 6b showed branching of *P. fluorescens* colony. Presence of esterase is confirmed by formation of a clear zone around a bacterial colony. On hydrolysis ester produces an acid and an alcohol apart from water, CO_2, CO and H_2. Analysis of acid content and esterase activity as an indicator for measuring the degradation of copolymer was performed.

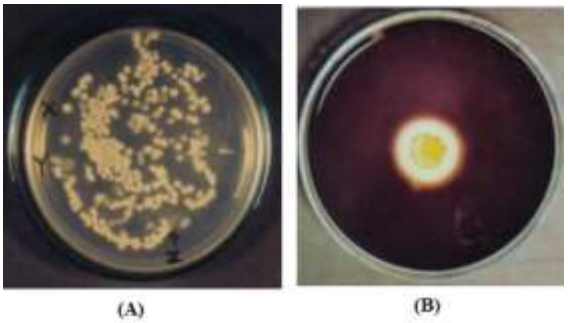

Figure 6: (A) Three types of microbial colonies appeared (x) opaque white and circular, (y) transparent white and circular and z) transparent colonies with irregular edges, (B) Presence of esterase is confirmed by formation of a clear zone around a bacterial colony

CONCLUSION

In the present work, no external source of microbes or microbial enzymes was used. The bacterial strain *P. fluorescens* was found to be most efficient for the degradation of polymer in presence of Cr^{6+} ions. It may provide a basis for the development of biodegradation strategies to remediate pollutants in the environment. Since, esterase was found to be associated with the biodegradation of polyethylene terephthalate it would be a good idea to develop a high esterase producing strain of *P. fluorescens* for the biodegradation of polyethylene terephthalate.

ACKNOWLEDGEMENTS

The corresponding author is thankful to DST, New Delhi for providing financial assistance for the research work.

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