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SUSCEPTIBILITY OF LOW DENSITY POLYETHYLENE FILMS TO POND WATER MICROFLORA

TABASSUM MUMTAZ[1], HUSNA PARVIN NUR[2] AND M. R. KHAN[*]

Laboratory of Microbiology, Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh

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Abstract

Low density polyethylene (LDPE) films were kept in pond water at laboratory room temperature for more than two years. Biofilm that developed on the material was examined for microbial type and erosion characteristics. Samples which showed considerable deformities in surface morphology were selected for subsequent cultivation and isolation of dominant microorganisms. Organisms well adapted to grow on LDPE films in nutrient deficient environment were used to study their ability *in vitro* to degrade LDPE as per guidelines of ASTM, ISO authorities. In liquid culture, the value of total water soluble organic carbon (TOC) increased threefold after 14 days of incubation but dropped below initial level within next two weeks. Fifteen - 58 % coverage by *Penicillium* sp. only after 65 days and 44 - 81 surface growth of *Humicola* sp. on LDPE film showed their affinity towards polyethylene. In clear zone assay, no halo zones around the colonies were observed but all bacterial isolates showed enhanced growth in the presence of polyethylene (100 mg/l).

Introduction

LDPE films have been reported to undergo both abiotic (Photodegradation and oxidative degradation) and biotic (Biodegradation and bio-oxidation) degradation (Spore and Bethea 1972, Albertsson *et al*. 1987, 1990, Andrady 1990, David *et al.* 1992). Biodegradation of amended polyethylene *e.g.* LDPE/cellulose blends, starch-PE, plastic-PE etc. have also been investigated (Foust *et al.* 1997, Lee *et al.* 1991, El-Shafei *et al.* 1998, Billingham *et al.* 2002).

Degradation of commercially available conventional LDPE films under soil conditions has been reported (Mumtaz 2005). This paper deals with isolation of microorganisms from polyethylene film immersed in pond water and the susceptibility of weathered and emulsified polyethylene strips to isolated microorganisms.

Material and Methods

LDPE films were cut into strips and placed in 2L conical flasks containing pond water. Flasks were kept on laboratory bench at room temperature for two years. Routine examination was made throughout the period.

Isolation of microorganisms: Polyethylene strips which exhibited considerable deterioration under microscope were selected for isolation of associated dominant microorganisms. For isolation, two types of culture media were formulated *viz.* SPYM (Soil extract 250 ml, potato extract 250 ml, yeast extract 2.0 g, malt extract 5.0 g, phosphate buffer (pH 6.5) solution 200 ml, trace-salt solution 1 ml, distilled water 300 ml, final pH 7.0) and OASP (Oat meal 5.0 g, L-aspargine 0.5 g, soil extract 250 ml, potato extract 250 ml, distilled water 50 ml, pH 7.0). Freshly prepared agar plates were inoculated with sample pieces. Growth which appeared after three - five days, were isolated and purified by repeated streaking on nutrient agar plates.

Isolated microorganisms were charged with LDPE films for testing their ability to degrade the polymer.

*Corresponding author. [1]Microbiology & Industrial Irradiation Division, IFRB, AERE, Savar. [2]Fibre & Polymer Division, Dhaka Laboratories, BCSIR, Dhaka-1215, Bangladesh.

Liquid culture test: Biofilm developed on polyethylene film was charged with fresh polyethylene flakes. Degradation was quantified by the estimation of water soluble total organic carbon (TOC) and pH of the culture media.

Chu's media ($MgSO_4.7H_2O$ 0.25 g; Na_2SiO_3 0.025 g, $NaCO_3$ 0.020 g; K_2HPO_4 0.010 g; ferric citrate 0.003 g, citric acid 0.003 g per litre with pH 7.2) was employed for the cultivation of microbes for 48 hrs prior to inoculation. Five hundred ml flasks plugged with cotton were labelled as M, M + C, M + PE, M + PE + C where M stands for media, C for culture and PE for polyethylene. M + C served as cell control (No film) whereas, M + PE served as film control (No inoculation). 10 % v/v of culture was inoculated in flasks and 0.1% w/v of polyethylene flakes were used as substrate. Final volume in each flask was 200 ml.

The experiment continued for six weeks and during each sampling approximately 15ml of culture broth was withdrawn from the flask for the analysis of TOC and pH. Glassware was washed with 3 M HNO_3 prior to use. The reaction mixture containing TOC was filtered through membrane filter (Sartorious, pore size 0.45 and 0.2 mm) and was estimated with a TOC 5000A Analyzer (Shimadzu Corp.).

Plate test with fungi: To test the ability of fungal strains to attack polyethylene films, strains were cultured on mineral base agar (MBA) medium described by ASTM G21-90 (1996). This carbon free synthetic medium was supplemented with 0.02 % yeast extract. pH was adjusted to 6.5.

White, opaque LDPE films submerged in pond water for two years were washed, air-dried and cut into 1 mm × 1 mm pieces. Specimens were sterilized with 5% aqueous solution of Na-hypochlorite for a few seconds in a pre-sterilized Petri plates (Pranamuda *et al.* 1997). Each piece weighing approximately 0.007 - 0.009 g was aseptically transferred to MBA plates. Plates were point inoculated with three - seven days' old fungal isolates. Fungal isolates were maintained on PDA slants (pH 5.5). Plates were kept at room temperature and were observed at regular intervals. Polyethylene pieces were transferred to fresh agar plates within 28 - 35 days. Surface area covered by each fungus was measured visually using transparent grid. The experiment was continued for seven weeks.

Quantitative assessment: Degradative ability of three fungal isolates was examined by the quantitative assessment of growth on polyethylene sample as per ASTM G21-90 (1996) specifications. A standard rating system by ASTM for the evaluation of fungal growth on polymeric materials, for biodegradation is 0 = no visible growth, l = less than 10 % growth, 2 = 10 - 30 % growth, 3 = 30 - 60 % growth, 4 = 60 - 100 % growth covering surface of polymer film.

Clear zone assay with bacteria: Five bacterial isolates were grown on PE-emulsified agar plates which were prepared as follows: White, fresh LDPE films were shredded by scissors. 0.05 g of PE strips were taken in a round bottom flask and were dissolved in 10 ml of xylene at 110ºC. The hot solution was transparent but gradually became cloudy when cooled to room temperature. The suspension was then poured in screw-capped test tube and sterilized in an autoclave at 121ºC for 20 minutes. MBA was prepared and adjusted to pH 7.2. Bromocresol purple (1.4 % alcoholic solution, 1 ml/l) was added to the media. Medium (500 ml) was taken in 1litre flask. A magnetic bar was kept in the flask. A few drops of Tween-80 was added to the flask prior to sterilization. After sterilization, polyethylene suspension was aseptically added to tempered agar medium and

was stirred with a magnetic stirrer for 15 - 20 min until polyethylene was homogenously dispersed into the liquid content. The resulting turbid medium was then dispensed into sterile Petri plates. Parallel sets of plates without PE were also prepared.

Well dried plates were inoculated with bacterial isolates and incubated at 37ºC for first four days and at 32ºC for rest of the days. Plates were observed at regular intervals.

Results and Discussion

Isolation of microorganisms: Microbial film developed on polyethylene samples consisted of blue-green algae, desmids, diatoms, some filamentous organisms, bacteria and spores. Chu's broth (Claus 1995) was selected for the cultivation of blue-green algae along with other microbial types. On the other hand, SPYM (ATCC 1992) and OASP plates were uaed for bacterial and fungal colonies. After purification, bacterial isolates were maintained on nutrient agar (NA) slants (pH 7.2) and fungal isolates on potato dextrose agar as well as on Czapek Dox agar (CAB 1968) slants having pH of 5.5.

Three distinct types of fungal and five bacterial isolates were finally selected for the present study. Morphological, physiological and biochemical characteristics of bacterial isolates were studied.

Colour

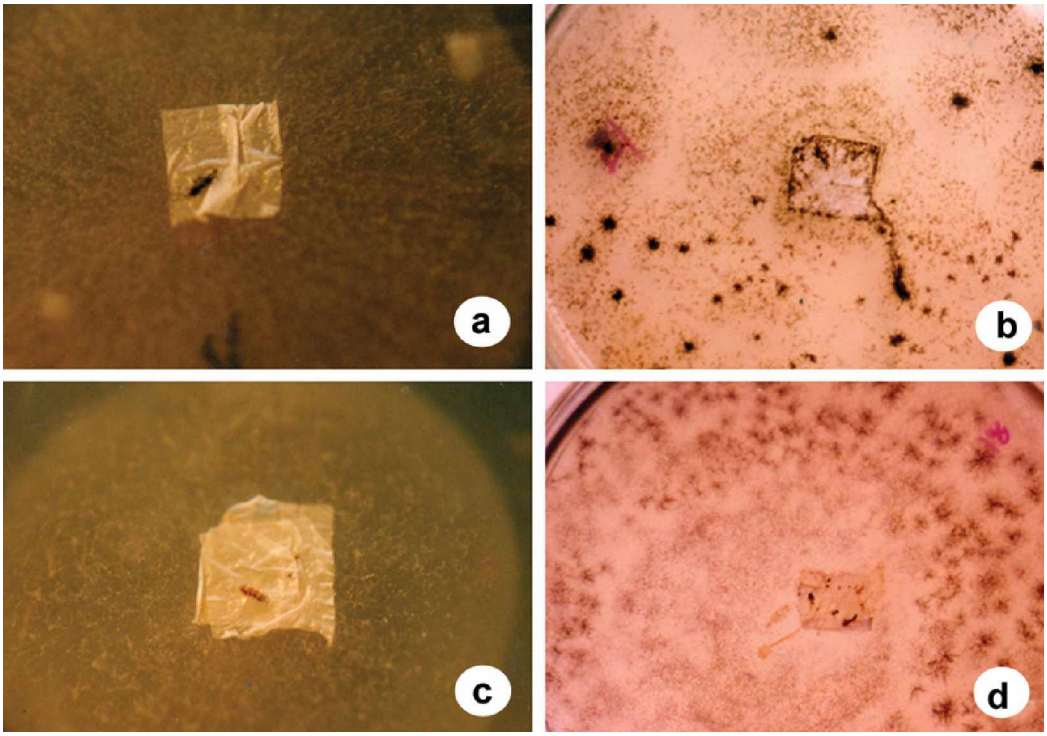

Fig. 1. Observed surface growth of S_{14}/F_1 (a, b) and S_{14}/F_2 (c, d) after two weeks and nine weeks of incubation in the plate test, respectively.

Identification: Preliminary examination of bacterial isolates in accordance with Bergey's

Manual of Systematic Bacteriology (Sneath *et al.* 1986) showed that spore forming strains belonged to the genus *Bacillus*. On the other hand, taxonomic identification of fungal strains up to genus included examination with optical microscope at different magnifications and comparing illustrations with that of Gilman (1957). S_{14}/F_1 belonged to the genus *Penicillium*, while S_{14}/F_2 was assigned to *Humicola* (Fig. 1). Taxonomic position of S_3/F_3 could not be ascertained.

Liquid culture test: The value of measured TOC (in ppm) during the experiment is shown (Table 1).

Table 1. Total organic carbon at different days under different conditions.

Days of observation	Water soluble TOC	
	M + C	M + C + PE
0	289.60	284.80
14	78.08	830.80
28	453.30	218.00

It was interesting to note that, in the film control (M + C), where the initial TOC value was 289.6 ppm, the microorganisms grew and utilized soluble carbon (yeast extract) of the medium up to 14 days, thereafter the TOC value increased after next two weeks and it could be due to the result of autolysis and release of organic carbon in the medium. On the other hand, in the experimental flask with medium plus cell plus polyethylene, the initial TOC value was close to the value of the control flask. After 14 days, the value rose up possibly due to the break down of carbon from substrate through microbial activity. After 28 days, the TOC value dropped down even below the initial value.

Results obtained could be explained by microbial assimilation during this period. This also indicated the ability of the organisms to survive in an environment where carbon was supplied from a recalcitrant polymer like polyethylene.

Plate test with fungi: Fungal plates were observed after 14 days. S_4/F_3 did not grow on MBA plates whereas *Penicillium* sp. and *Humicola* sp. showed extensive growth on culture media and over polyethylene pieces (Fig. 1).

Growth of *Humicola* sp. on polyethylene sample varied from 44 - 81% with surface growth observed after six - seven days of incubation. On the other hand, *Penicillium* sp. inoculated in triplicates showed 15 - 58 % coverage only after 65 days.

Clear zone assay with bacteria: Among five bacterial isolates, S_{14}/B_1, S_3/B_1 and C_1 showed considerable growth on PE-emulsified plates (designated as M + PE) only after four days. For all isolates, growth appeared to be enhanced in presence of 0.1 g/l raw polyethylene (Fig. 2). S_3/B_2 showed no growth on M + PE plates. S_8/B_1, on the other hand, showed faint growth after four days but considerable growth occurred after nine days. Bromocresol purple indicator was used to detect any change in the pH of the medium. The control plates were purple in colour, however, M + PE plates turned to sea-green after plating probably as a result of slight increase in acidity due to xylene.

The growth of bacterial isolates on carbon free media with polyethylene as a sole source of carbon reflects the utilization of polyethylene by the isolates.

In the present study, liquid culture test was employed to estimate TOC and pH during aerobic fermentation of polymer in an aqueous environment. To assess biodegradability of materials, a liquid environment is currently used despite the fact that the most polythenes are dumped in soil, compost or landfill (Calmon-Decriaud *et al.* 1998). On the other hand, Petri dish screen test and clear zone technique was followed in solid media to assess the resistance of plastics to microbial degradation.

Similar tests were also performed by several workers to evaluate the biodegradability of PCL, PTMS, PLA etc. (Tokiwa *et al.* 1976, Pranamuda *et al.* 1995, 1997). However, from their experiment, it appeared that, polymer degradation by hydrolysis progresses with the increase of water soluble TOC in a liquid culture. This increase also reflects exponential phase of microbial growth. After that, the value remains unaltered as the metabolic activity of microbial cells become slow. A drop of pH value was observed by the above workers after two weeks of incubation, but no such change was noticed in the present experiment.

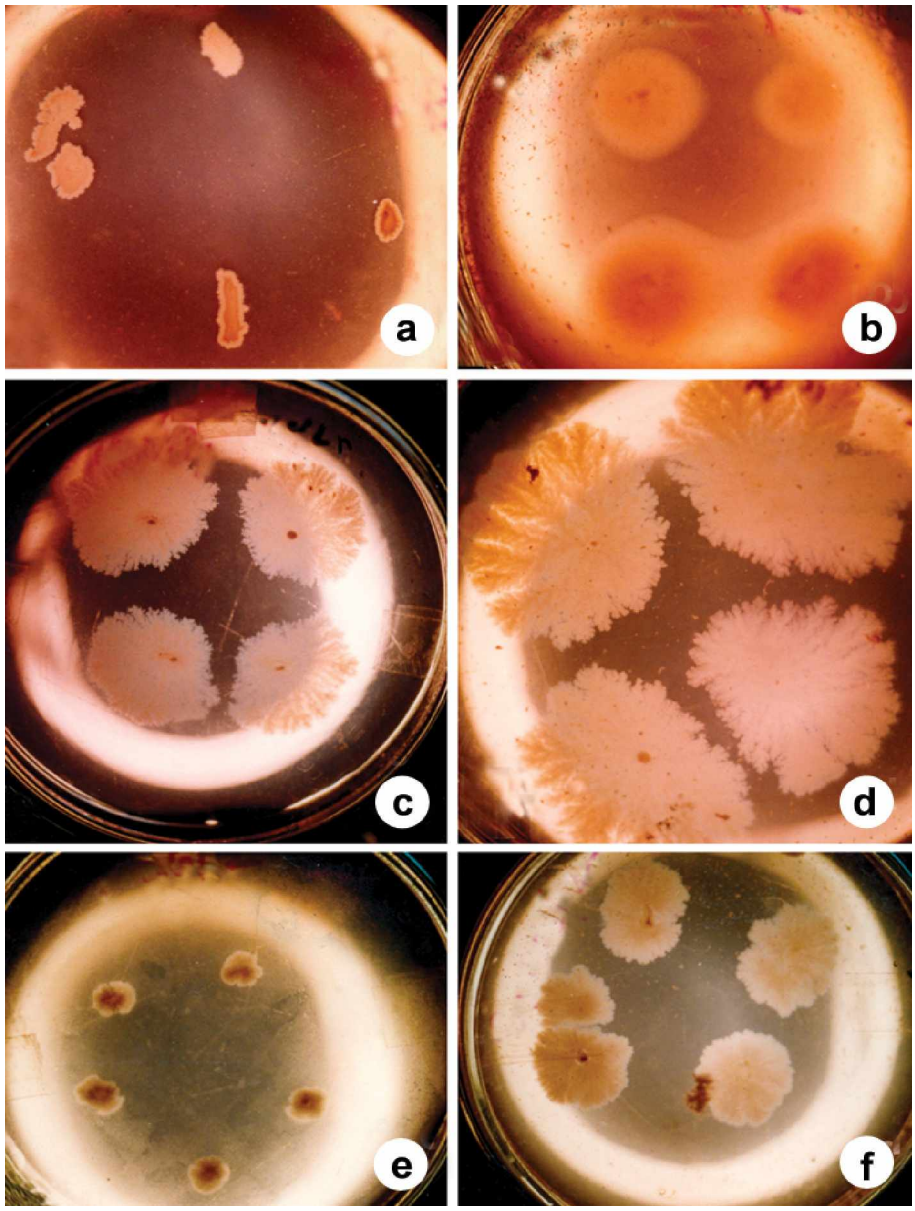

Fig. 3. Growth of bacterial isolates as observed after nine days. Growth of S_3/B_1 (a, b) S_{14}/B_1 (c, d) and C_1 (e, f) isolates in control (left) and PE-emulsified agar (right) plates.

In the plate test, weathered PE sample supported growth of fungi and therefore might be damaged by these fungal isolates.

Tansengco and Tokiwa 1998 confirmed degradation of polyethylene succinate (PES) by clear zone test. Clear zones were formed around the colonies of organisms on the turbid agar plates. Fields *et al.* (1974) stated that this happened when the polymer-degrading microorganisms excrete extracellular enzymes which diffuse through the agar and degrade the polymer to water soluble materials.

In the present experiment, no such clear zones were observed, even though biomass increased in presence of polyethylene. Absence of clear zones may be due to a number of reasons: (i) greater number of point inoculation per plate may cause complete consumption of polyethylene, therefore no clear areas were left, (ii) the concentration of the polymer in medium may be too low so that the media was not turbid enough to demonstrate clear areas around the colonies or, (iii) hydrolysis may not be the principal route for the degradation of polyethylene.

Recalcitrant polymers are unique in many ways and it is difficult to assess the initial degradation processes. The experimental results obtained in the present study demonstrated biodegradation of selected polyethylene material under laboratory conditions, although the extent of biodegradation was not quantified.

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