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Website: www.mutagens.co.in
E-mail: submit@mutagens.co.in
researchsubmission@hotmail.com

Research Paper

ISOLATION AND IDENTIFICATION OF LOW DENSITY POLYETHYLENE (LDPE) DEGRADING BACTERIAL STRAINS FROM POLYTHENE POLLUTED SITES AROUND GWALIOR CITY (M.P.)

Jyoti Singh, Dr. K.C.Gupta[1] and Archana shrivastava

Cancer college and research institute(CHRI) , Gwalior [1, 3]
[1]Department of Microbiology, Government V.R.G. P.G. College, Gwalior, India.

Abstract
Plastic and polythene waste, including low and high density polyethylene, is accumulating continuously in the environment. It is posing an ever increasing ecological threat. The low density polyethylene is comparatively more vital environmental pollutant and its biodegradation was the focus of the present of study. This study reveals the biodegradation with the help of bacterial strains isolated from polythene polluted soil around Gwalior city,(M.P) India. Eighteen bacterial isolates were isolated from polythene polluted soil. Isolation was done by enrichment in mineral salt medium with LDPE as sole carbon and energy source. The screening of isolates was based on their ability to utilize LDPE as a primary carbon source. During in vitro degradation, three isolate P.E.5, P.E.7 and P.E.10 showed the highest degradation . The increase in Bacterial growth and weight loss of LDPE in the medium, were recorded at regular time intervals. The degradation ability was analyzed by clear zone technique, weight reduction of LDPE and sturm test. These bacterial isolates were identified as ***P****seudomonas putida* (P.E.7) *Pseudomonas aeruginosa* (P.E.10) and *Bacillus amyloliquefaciens* (P.E.5) ,on the basis of biochemical characteristics and 16S rRNA sequence analysis.
Key words: LDPE, Biodegradation, Degradation ability, *Pseudomonas sp., Bacillus amyloliquefaciens*.

INTRODUCTION

The polyethylene is one of the major sources of environmental pollution, which is a polymer made of long chain monomers of ethylene with different densities. The worldwide utility of polyethylene is expanding at a rate of 12% per annum and approximately 140 million tones of synthetic polymers are produced each year at international level (Shimao, 2001) With such huge amount of polyethylene getting accumulated in the environment, their disposal evokes a big ecological issue. Because of its very slow natural degradation, thus accumulates in environment in huge amount. It posses the main environmental pollution problems. The biodegradation is a promising method of solving this environmental issue among other physical and chemical degradation method. Our study illustrated the biodegradation of LDPE with the help of certain bacterial sps. isolated from polythene polluted sites.

Microorganisms utilizing this organic complex polymer as carbon and biologically transforming to simpler one. The microorganisms secrete several LDPE degrading enzymes in different quantities, which expressed its degradation efficiency of the microorganism. (Bhardwaj *et al*,

2012). In several studies, bacteria were considered favorable for the degradation of LDPE due to their higher ability to form hydrophobic enzyme proteins, which helped the bacterial sps. to form biofilm and helps in attachment to the polymer surface (Seneviratne, *et al.,* 2006 and Kershaw M.L. ,1998). Kim and Rhee, 2003) also recorded several bacterial sps. As biodegrading agents but the faster growth of fungal biomass was observed when compared to the bacterial sps. (Shah A.A. 2008). (Frazer A.C. 1994) concluded that the extra cellular enzymes were responsible for such degradation process. He also recorded that these microbes attached to the inert surface of polyethylene with the help of enzymes secreted by them and grow on film by utilizing the LDPE and the polymers are depolymerized and are degraded by the process of mineralization into the carbon dioxide (CO_2), water (H_2O) or methane (CH_4). The aim of this study was to isolate the soil bacteria which were native to the site of polyethylene disposal sites and showing degradability in natural conditions. The LDPE degradation efficiency of isolates in laboratory condition was determined.

MATERIALS AND METHODS

Collection of fresh samples

Low density polyethylene (LDPE) sheets were obtained from Gwalior Plastic Industry (Gwalior).

Preparation of LDPE Powder:

The process of Pramila and Ramesh (2011) was followed to prepare the LDPE powder from sheets. The LDPE sheets were cut into small pieces and immersed in xylene and boiled for 15 minutes. To remove the xylene from the solution LDPE solution was treated with ethyl alcohol. The xylene-ethyl alcohol was evaporated and thus obtained LDPE Powder was washed with ethanol to remove the residue of xylene and again it was allowed to evaporate. The powder was dried in hot air oven at 40-50°C for overnight.

Media used

SM contains the following constitutions in1000ml distilled water (K_2HPO_4, 1g;KH_2PO_4, 0.2 g; NaCl, 1g; $CaCl_2.2H_2O$, 0.002 g; $(NH_4)_2SO$, 1 g; $MgSO_4.7H2O$, 0.5 g;$CuSO_4.5H_2O$, 0.001 g; $ZnSO_4.7H_2O$,0.001g; $MnSO_4.H_2O$, 0.001g and $FeSO_4.7H_2O$, 0.01g.) (Pramila and Ramesh 2011).

Sample collection for Isolation.

Soil of different waste disposal sites, dumped with polythene bag and plastic waste, were collected from Gwalior city. The soil samples were collected from the depth of 5-10 cm in sterile container and it was air dried at room temperature. These soil samples were placed at 4°C for further studies.

Isolation of bacteria from soil associated with material (polyethylene bags and plastic bags).

Enrichment cultures were established to isolate the polymer degrading bacteria:
One gram of the soil was suspended in 10 ml of sterile water. It was vortexed for thorough mixing and then left undisturbed for 15 min. 5 ml of soil suspension was transferred into 250 ml Erlenmeyer flask containing 100 ml of the sterile minimal media and 0.1% polymer powder (PE) was added to it. After autoclaving the media the powder prepared previously was added. All Erlenmeyer flasks were incubated in an incubator shaker (Kuhner, Switzerland) at 120 rpm and at 37°C. After one week of growth, observed as OD_{600} in UV-Visible spectrophotometer (Model Lambda 35- Perkin Elmer, USA), 5 ml of the enrichment culture was transferred into 100 ml of freshly prepared minimal media with 0.1% polymer powder as the sole source of carbon and energy. The second and third transfers were performed successively under identical conditions. One hundred μl (0.1 ml) of the third enrichment culture of over night grown was plated on agar plates containing 0.1% polymer powder for isolation of bacteria. After 14 days of incubation at 37°C, individual colonies were picked and streaked on the Nutrient Agar plates for isolation and purification.

Screening of polyethylene degrading bacterial isolates by clear zone method

LDPE powder was added in mineral salt medium at a final concentration of 0.1% (w/v) respectively and the mixture was sonicated for 1hour at 120 rpm in shaker. After sonication, the medium was sterilized at 121°C and pressure for 15 lbs/inch^2 for 20 minutes. Sterilized medium was poured before cooling in each plate with medium containing LDPE powder . The isolated organisms were inoculated on polymer containing agar plates and then incubated at 37°C for bacteria for 2-4 weeks. The organisms, producing zone of clearance around their colonies were selected for further analysis.

Pretreatment of polyethylene samples

40 mm thick films of LDPE irradiated with 40w UV-B lamps of intensity between 285 & 360 nm with maxima at 315nm in air at room temp (30°C+1) on open racks positioned 8cm from the lamp (Roy et al .,2006). Uninterrupted exposer for 24 h per day was given and after 20 days the samples were recovered and then and were exposed for thermal oxidation in a hot air oven for 10 days at 70 ^{o} C (Nil et al.,2009).

Microbial degradation of pretreated and untreated LDPE films under laboratory condition by Liquid culture method.

Pretreated and untreated films of LDPE (10cm x 2.0 cm) were first washed with ethanol, rinsed in sterile distilled water and dried. .1 gm dried film of each LDPE was aseptically transferred into the conical flask containing 100 ml of sterile Mineral Salt Media and pH of the medium was adjusted to 7.0 . 3% (v/v) inoculum (24 h old bacterial culture) of screened polythene degrading bacteria, which was previously grown in the MSM broth medium was inoculated in the flask containing fresh medium.(Roy et al.,2008) and each pre-weighed films of LDPE in the flasks containing inoculum were maintained at 30°C with a rotary incubatory shaker (120 rpm) for.1month. Different 2 sets of controlled experiments were maintained i.e, the Untreated LDPE (UNLDPE), Pretreated LDPE(PRLDPE), in basal medium, which were devoid of bacterial culture.(Roy et al.,2008). All the experiments were run simultaneously in triplicate. Bacterial growth was checked by spectrophotometer at 600nm.

Dry weight determination

LDPE sheets treated and untreated were recovered after 1 month of incubation. a bacterial biofilm was developed. It was washed the with 2 % (v/v) aqueous sodium dodecyl sulfate solution for 4 h (using shaker) followed by distilled water and finally with the 70 % ethanol to ensure maximum possible removal of cells and debris. The washed LDPE pieces were placed on a filter paper and dried overnight at room temperature before weighing.

Sturm test

100 ml capacity autoclavable plastic containers were used for the study. Separate setup was maintained with un-inoculated MSM supple-mented with LDPE powder. After the stipulated time [48 h] the KOH solution [1 M] that had trapped the CO2 liberated by the inoculant [after utilization of LDPE the sole carbon source] was gravimetrically quantified using barium chloride. The dissolved carbon dioxide present in the medium was also estimated using titration method. Briefly, sample (25 ml) was taken in a conical flask and 0.05 ml of 0.1 N Thiosulphate solution was added. After the addition of 2 drops of methyl orange indicator, this solution was titrated against 0.02 Sodium Hydroxide solution. End point was the change in color from orange red to yellow. Following this, two drops of phenolphthalein indicator were added and titration continued till a pink color developed. Volumes of the titrant used were noted and the amount of CO2 calculated using the formula.Separate quantification was performed for test as well as control.

$$\frac{A \times B \times 50 \times 1000}{V}$$

Where A= ml of NaOH titrant
B= normality of NaOH
V= ml of the sample. Separate quantification was performed for test as well as control.

Identification Polyethylene Degrading Microorganisms:

The three potential polyethylene degrading bacteria which showed the highest degradation were selected for further studies. The identification of bacteria was carried out on the basis of macroscopic and microscopic examination and biochemical test according to Bergey's manual of determinative bacteriology.For molecular identification, DNA was isolated from each isolate . The 16S rRNA was amplified through PCR using universal 16S1 (forward) and 16 S2 (reverse) primers. The PCR products were purified with PCR product purification kit, following the instructor's manual (fermantas). Sequencing was carried out using the same PCR primer on a 96 capillary model 3730xl system using the Big Dye Terminator kit from Applied Biosystems (Applied Biosystems, Foster city ,CA, USA). The sequences were edited with SeqED program (Applied Biosystems).

RESULT AND DISCUSSION

To isolate LDPE degrading bacteria the soil samples were added in MSM medium and isolated bi enrichment method. We isolated and screened 18 bacterial strains. Among them only three bacterial strains (P.E.5), (P.E.7) *and* (P.E.10).(fig 1) On the basis of morphological and biochemical characteristics the bacterial isolates P.E. 7 and P.E 10 , were identified as a member of *Pseudomonas and* P.E.5 was identified as a member of *Bacillus.* (table 1*)* This was further confirmed by assembled 16s rRNA sequences analysis and was identified as *Pseudomonas putida* (P.E.7) *Pseudomonas aeruginosa* (P.E.10) , and *Bacillus amyloliquefaciens* (P.E.5) respectively.

Pre-treatment initiated abiotic degradation.

The abiotic pre treatment involved the exposure of LDPE films to U.V radiations for total 20 days and thermal oxidation for 10 days. After this period , the polymer is chemically modified and is more susceptible to attack.(Bonhomme et al.,2003).The catalytic degradation in the presence of transition metal in polyethylene has been attributed to its ability to generate free radicals on the surface of polyethylene, which later react with oxygen to generate carbonyl groups.(Osawa et al., 1979; Osawa 1988).

Bacterial growth on LDPE containing medium

Biotic exposure time to LDPE films results into growth of bacteria.(fig 2). Lioshi et al .,1998 stated that carbon limitation may facilitate the slow growth on polyethylene such as lignin. Among the polyethylene films, Pretreated LDPE enhances the growth of bacteria compared with the untreated LDPE sample. The culture flask containing LDPE inoculated with each bacterial strain separately, than Pretreated LDPE flask sample showed highest growth. after 30 days of incubation. At the same time the growth of organism was very poor in untreated LDPE sample containing medium. The pretreated LDPE uninoculated act as a control and all the flasks were monitored for contamination at regular time intervals by plating technique.(fig 2)

Vol. 4(8), 2015 pp. 3220-3228

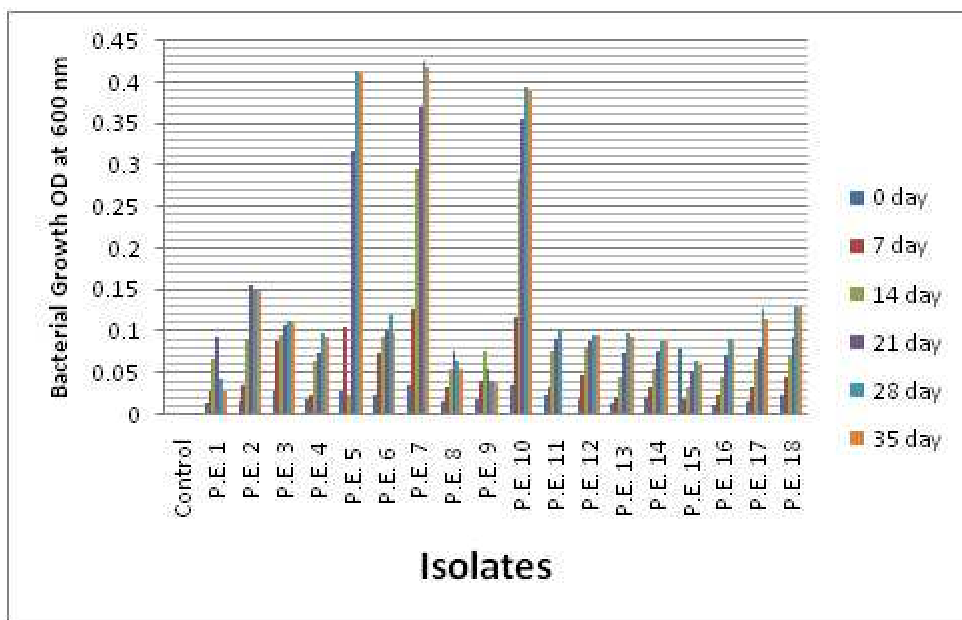

Fig 1: The growth of LDPE degrading bacteria in mineral salt medium containing 100 mg , at different time intervals (day), incubated at 30 ^{0}C, pH, and 120 rpm.

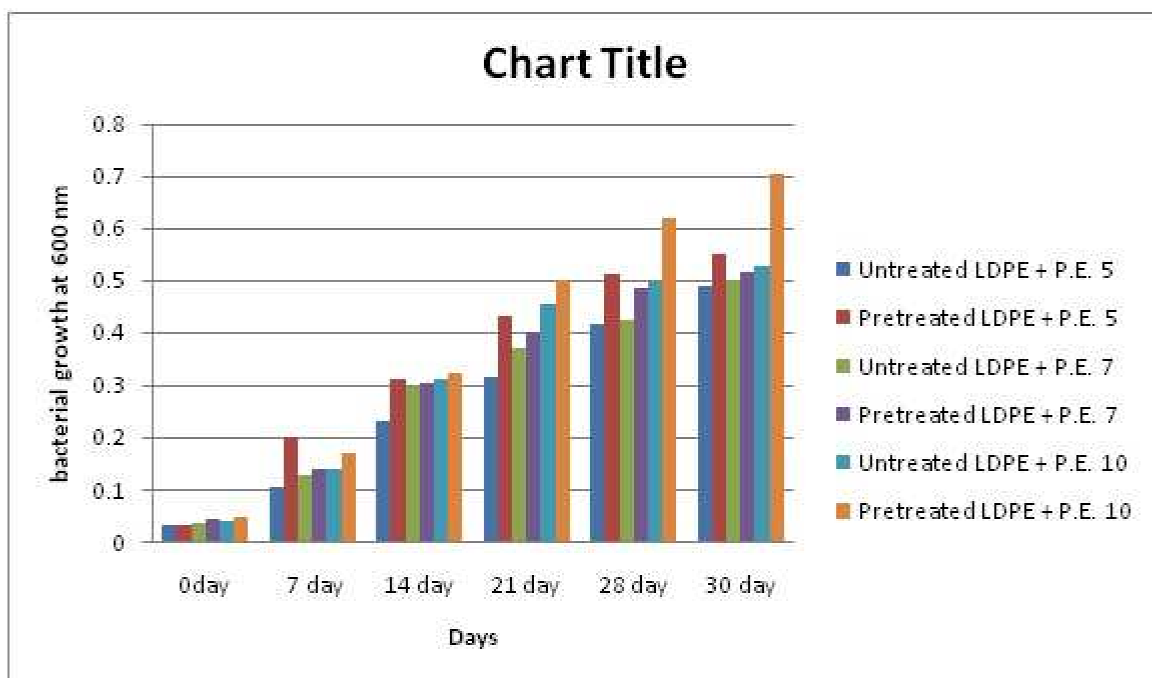

Fig 1: The growth of LDPE degrading bacteria in mineral salt medium containing 100 mg , at different time intervals (day), incubated at 30 ^{0}C, pH, and 120 rpm.

Table no 1: Identification of polyethylene (LDPE) degrading bacterial isolates .

Characteristics	Isolate P.E 5	Isolate P.E 7	Isolate P.E 10
Colony Characteristics			
Shape	Round	Round	Round
Size	Small	Large	Large
Colour	White	Green	Green
Surface	Dull, granular	Convex	Convex
Margin	Entire	Undulate	Undulate
Morphology			
Straight rod	+	+	+
Cocci	-	-	-
Gram stain	+	-	-
Cell arrangement	Short rod, Single	Short chain, Single	Short chain, Single
Spore	C	C	C
Motility	+	+	+
Enzyme production			
Amylase	+	-	-
Lipase	+	+	+
Gelatinase	+	-	+
Carbohydrate Fermentation			
Glucose	A/-	-	+
Fructose	A/-	-/-	-/-
Sucrose	A/-	-/-	-/-
Lactose	A/-	-/-	-/-
Mannitol	A/-	-/-	-/-
Urease	-	-	-
Casein	+	-	-
Nitrate reduction	+	+	+
Citrate	+	+	+
Oxidase	+	+	+
Catalase	-	+	+
Indole production	-	-	-
Methyl Red	-	-	-
Voges Proskauer	+	-	-
Citrate Utilization	-	+	+

Weight reduction

Changes due to microbial degradation were assessed qualitatively by measuring weight loss of polymer. Weight loss of polymer after incubation may be purely because of microbial activity. Films were weighed, with an accurate four-digit balance, before and after incubation in media. l Microorganisms that grow within the polymer lead to an increase in weight due to accumulation, whereas a loss of polymer integrity leads to weight loss. Weight loss is proportional to the surface area since biodegradation usually is initiated at the surface of the polymer. The reduction in weight was observed after the biodegradation of LDPE (Fig.3 and table 2).

Table 2: Result of degradation of polyethylene sample by bacteria after one month.

Strain no.	Initial wt(mg)	Final wt(mg)	Difference	Weight loss per month (in %)
Pseudomonas putida + untreated LDPE	100	85± 1.03	15	15%
.*Pseudomonas putida + Pretreated LDPE*	100	80± 1.00	18	20%
Pseudomonas aeruginosa + untreated LDPE	100	88± 1.12	12	12%
Pseudomonas aeruginosa + Pretreated LDPE	100	82± 1.01	18	18%
Bacillus amyloliquefaciens + untreated LDPE	100	90±1.21	10	10%
Bacillus amyloliquefaciens + Pretreated LDPE	100	88± 0.96	12	12%
Control	100	100	0	0%

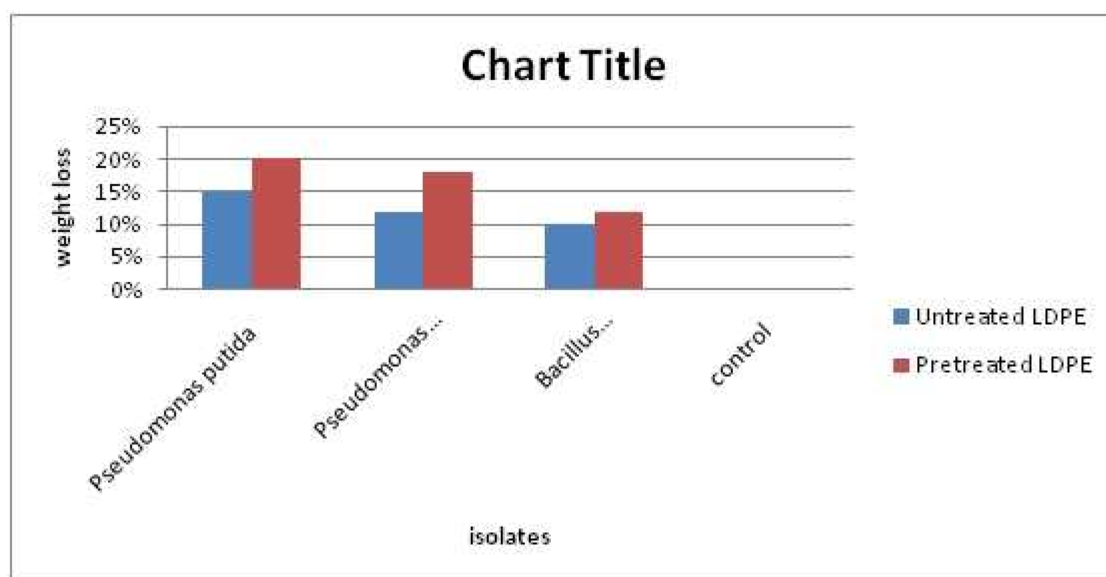

fig.3 Weight loss comparison (untreated = No U.V and Thermal treatment),Pretreated= U.V + Thermal treatment.

Sturm test

The carbon dioxide evolved as a result of polymer , was determined gravimetrically by sturm test. It was found that in case of test (without LDPE pieces) , the total amount of CO_2 produced by isolates (table 3). The result shows the potentiality of isolates and supports the fact of biodegradation and biomineralization of this hazardous polymer.

Table 3 (a) CO_2 Evolution level of bacterial isolates.

Isolates	Carbon dioxide evolution (g/L)(after 1 month)		
	Control (C) (Atmospheric)	Test (T) (Metabolic + Atmospheric)	T-C (Metabolic)
P.E. 10 + (UNLDPE)	6.90 ± 0.03	11.42 ± 0.09	4.52
P.E. 10 + (PRLDPE)	7.23 ±0.14	13.51 ± 0.15	6.28
P.E. 7 + (UNLDPE)	8.16 ±0.12	11.43 ± 0.08	3.27
P.E. 7+ (PRLDPE)	7.02 ±0.32	11.03 ± 0.012	4.01
P.E. 5 + (UNLDPE)	6.90 ±0.03	8.09 ± 0.011	1.19
P.E. 5 + (PRLDPE)	5.23 ±0.03	9.12 ± 0.010	3.89

In the present study the screening and identification of LDPE degrading bacteria from polythene polluted sites were focused. LDPE was degraded by the three potent bacterial strains after the exposure for period of 30 days. Among the bacterial species. *Pseudomonas putida* (22%), . *Pseudomonas aeruginosa* (18%) and *Bacillus amyloliquefaciens* (18%) showed degradation.

CONCLUSION

The problem of plastic pollution is now really a mess for mankind. There is no part of the world untouched from its impact. In the present era of globalization some stress must be given to plan safe disposal of products before making it commercial. Making science to the leap and forgetting the other side of coin lead to such conditions. In the present study, three isolated strains were found to be useful for the biodegradation which is first time reported with applicable evidences. This biodegradation approach is safe and eco-friendly. The results showed a promising hope to degrade LDPE faster rate than to be degraded naturally.

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