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Biodegradation of Low Density Polythene (LDPE) by Pseudomonas putida using induced mutations

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ABSTRACT: *Lack of degradability and the closing of landfill sites as well as growing water and land pollution problems have led to concern about plastics. With the excessive use of plastics and increasing pressure being placed on capacities available for plastic waste disposalIn the current research focused on the Biodegradation of Synthetic plastics (LDPE) using induced mutations in Bacteria Pseudomonas putida, bacterial growth curve were monitored and analyzed in Fourier transform infrared spectroscopy (FTIR) were followed, DNA isolation for Gel electrophoresis, and Mutated DNA Stability analysis by Capillary Gel electrophoresis were carried outSince the bacterial growth curve of Treated were showed near normal with well-maintained viability between 0.2 to 0.3 OD against positive control 0.18, when the OD read at 600nm. In the positive control (pseudomonas putida with plastic LDPE) DNA was found to be stable with a degradation capacity of 90.08 % as and when compared to Treated (pseudomonas putida -UV induced, with plastic LDPE) for 60 days DNA was found to be unstable with a degradation capacity of 78.01 % From the gel electrophoresis DNA run, the inference can be noted that, in the 450bp bands were stable except Positive control, at 200bp band of 30 days treatment followed by 900bp band in 60 days treated showed prominent band appearance, it could be due to UV induced mutation effect, and these genes could be certainly beneficial in LDPE degradation*

KEYWORDS: *Plastic (LDPE) degradation, mutation induced (UV), Pseudomonas putida, growth curve, FTIR, DNA run and Capillary Gel electrophoresis*

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I. INTRODUCTION

Petroleum plastics are the non-biodegradable synthetic polymers that accumulate at the rate of 25 million per year, contaminating the soil and water [1]. Low Density Polyethylene belongs to thermoplastics class [2] and is believed to have non-degradable nature due to hydrophobic backbone [3].

The synthetic plastics are thus, dumped into landfills or are incinerated. Incineration burns off the plastic waste completely, but at the same time causes heavy toxic fume generation [4, 5]. Recycling is a very environmentally-attractive solution, but a very small part of the plastics can be recycled where the remaining goes to the burial sites [6, 7].

Thus, there is a basic need for to developing affordable, cost effective, RRR –Eco-friendly degradation strategy

The composition of medium – chain length polyhydroxyalkanoates (PHA) accumulated by pseudomonads depends on the PHA syntheses, the carbon source, and the metabolic routes involved (1-3).In Pseudomonas putida two PHA synthase genes pha C1 and pha C2, which were separated by the PHA depolymerase encoding the phaZ gene, were identified and characterized.

Microorganisms can degrade plastic over 90 genera, from bacteria and fungi, among them; Bacillus megaterium, Pseudomonas sp., Azotobacter, Ralstonia eutropha, Halomonas sp., etc. [8].

Plastic degradation by microbes due to the activity of certain enzymes that cause cleavage of the polymer chains into monomers and oligomers. Plastic that has been enzymatically broken down further absorbed by the microbial cells to be metabolized. Aerobic metabolism produces carbon dioxide and water, instead of anaerobic metabolism produces carbon dioxide, water, and methane as end products [9].

Pseudomonas citronellolis is amongst the polyethylene degrading bacteria, [10,11] that belongs to P. aeruginosa group [12], it has shown upto 35% of degradation of poly- ethylene in sample taken from plastic waste dumping site [13].

The bacteria involve enzymes like monooxigenase, dioxigenase and dehydrogenase to carry out the degradation mechanism [14]. The enzymes involved cause microbial oxidation [1] by the capture of oxygen

from air as the initial step in biodegradation; further the UV irradiations cause photo-catalytic oxidation [15] and accelerate the biodegradation process in soil.

The varied bacterial species requires genomic identification to evade the phenotypic identification related problems [12]. Although genomic identification will help in genus identification but the strain still remains unknown for which a phylogenetic assessment is carried out using 16S rRNA sequencing. The 16S rRNA is a universal marker which is used in PCR assay for identification of bacterial species [16].

The maximum degraded LDPE films for 60 days was analyzed through Fourier transform infrared spectroscopy. Thus, it proved the efficacy of mutation induced (60min) Pseudomonas putida strain and incubated LDPE films for 60 days. The Stability analysis for induced mutation was carried out by genomic DNA isolation followed by Molecular weight determination with 2KB ladder.

II. MATERIAL & METHODS

II.1Materials: Low density polyethylene (LDPE) which is the major cause of environmental pollution was used for the study.

II.2 Microorganism collection

The bacteria Pseudomonas putida (MTCC NO: 2467) used in this study were procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh. Cultures were maintained on LB agar plate

II.3 Raw materials

Plastics is polyethylene (PE) as commercial plastic carry bags of LDPE were collected and cut into small strips and subjected for Chemical - alkali treatment.

II.4 Chemical - alkali treated polyethylene: polyethylene bags were cut into small strips & transferred to fresh solution containing 18ml tween, 10ml bleach, and 225ml of distilled water & stir it to 30-60mins. Bleach consists of 5gms of sodium chloride, 5gms of sodium hydroxide & 10 ml of glacial acetic acid. Strips were transferred to beaker with distilled water & stir it 2 one hour.

They were aseptically relocated to ethanol solution 70%v/v For 30 min. Finally, the polyethylene strips were transferred to petridish and inoculated at 45°-50°c overnight. Ethanol was used as disinfectant to polyethylene & removes any organic matter adhering to its surface

II.5 Induction of mutation by UV

Materials required:-

1. UV germicidal light bulb (Sylvania G15T8; 254 nm wavelength) or Stratagene UV Cross linker, induction with ideal 60 min exposure (for mutation) growth culture were selected
2. 370c incubator
3. Pseudomonas putida (MTCC NO: 2467)
4. LB agar plate

II.6 In vitro biodegradation assay

100 ml of growth medium in different flasks was inoculated with the individual obtained bacterial culture and then weighed LDPE sheet pieces were placed in each. LDPE with growth medium and bacterial strain with growth medium were taken as negative and positive controls, respectively. The flasks were then incubated for 24 hours at 37°C, 150 rpm. The OD at 600 nm was recorded after regular intervals of 24 hours till the bacterium reaches stationary phase [17, 18].

II.7 Recovery of degraded product and sample analysis

The LDPE sheets were recovered after incubation through filtration and were kept for evaporation. The product was then washed using ethanol by centrifugation to remove the bacterial biomass. The obtained product was kept for overnight drying and analysis of recovered LDPE samples was carried out by weight loss percent, SEM, FTIR and TGA [17, 10, and 3].

II.8 Gel electrophoresis:

Extraction and estimation of Genomic-DNA by gel electrophoresis- Amnion Bioscience KIT

II.9 Capillary Gel electrophoresis analysis:

Polyacrylamide gel-filled capillaries are usually employed, although new polymer formulations with greater stability to the applied electric field are likely to be introduced shortly. Agarose gels are unable to

withstand the heating produced by the high voltages used in capillary gel electrophoresis (CGE). The instrument CGE Pro 9600 – CGE Lauf-Nr 15315(Machine 3)

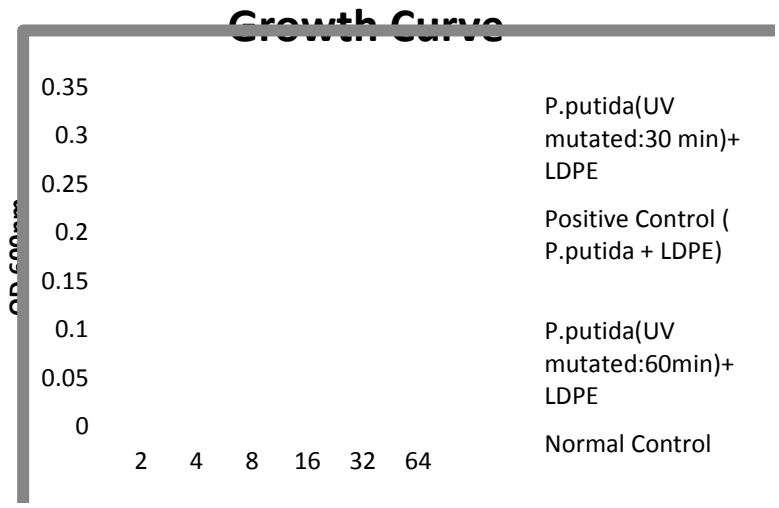
II.10 Capillary Gel electrophoresis was used: to analyse DNA fingerprinting is a useful tool for identifying the genotype of living organisms by determining their DNA sequence. For this technique, genomic DNA must be amplified by PCR. Capillary electrophoresis separates this amplified DNA with a one base pair resolution and creates specific peaks for each nucleotide to map the DNA sequence.

II.11 Bacterial Genomic DNA Extraction - was carried out using- Bacterial Genomic DNA Isolation Kit 05/16 (Catalog # K309-100; 100 isolations; Store at -20°C/RT)
Bacteria are also used in various industrial applications such as production of enzymes and biofuels. BioVision's bacterial genomic DNA isolation kit provides convenient and simple step-by-step method for isolating quality genomic DNA from gram-negative and gram-positive bacterial species.

This kit utilizes enzymatic reactions to release bacterial DNA from the cell. DNA release from the cell is coupled with adsorption of DNA onto a silica spin-column in the presence of high salt concentration, eliminating the use of toxic organic compounds or solvents. DNA purified by this kit is suitable for various downstream molecular biology applications such as PCR, cloning, DNA hybridization, and Southern Blotting.

III. RESULTS

Since the bacterial growth curve in treated group were showed near normal with well-maintained viability between 0.2 to 0.3 OD against positive control 0.18, when the OD read at 600nm



FTIR:

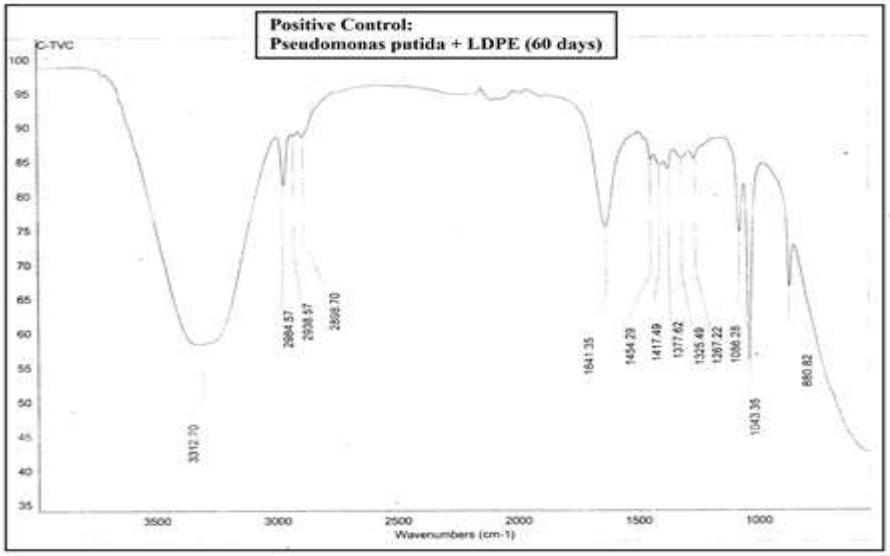
In the present study FTIR results in Positive Control: Pseudomonas Putida with LDPE showed that 1417.49 cm-1 to 1454.29 cm-1 O-H, 1641.53 cm-1 C=C, and 2988.57 cm-1 C-H, peaks were stretched in the treated group of Pseudomonas Putida (UV induced) with LDPE (60 days), i.e. 1420.55 cm-1 to 1457.35 cm-1 O-H, 1641.35 cm-1 C=C, and and 2981.50 cm-1 C-H, respectively

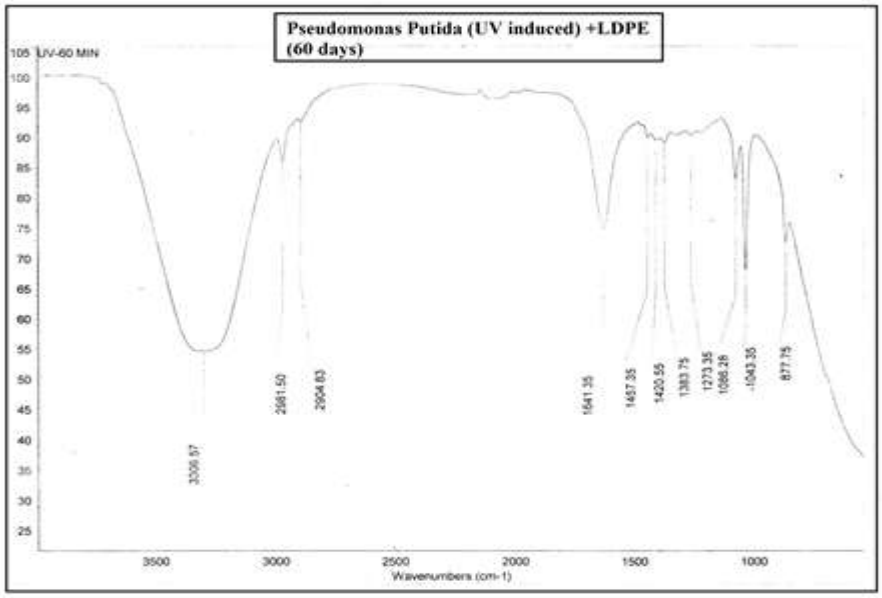
The biodegradation study has characterized A2 as LDPE degrading strain. Further, phylogenetic assessment has specified A2 strain as novel among the Pseudomonas citronellolis strains, and has been avowed as Pseudomonas citronellolis EMBS027 [20].

The LDPE degraded spectra of similar pattern was observed [13], where Bacillus and Arthrobacter species were implemented for plastic degradation and a study with Pseudomonas and Microbacterium species [21, 22].

The documented studies have illustrated the polymer degradation by the functional group analysis through FTIR. The addition, deletion and shifting of functional group peaks has been inferred as the major aspect representing the structural changes.

The additional peak at 1107.5 cm−1 and 1028.1 cm−1 were found respectively when LDPE was treated with consortium and was analyzed after in vitro study for10 days and in vivo study for 3 months, respectively. The frequency shifts had been observed due to hydrolysis that led to occurrence of carbonyl group as an additional peak [20, 21].





Capillary Gel electrophoresis analysis:

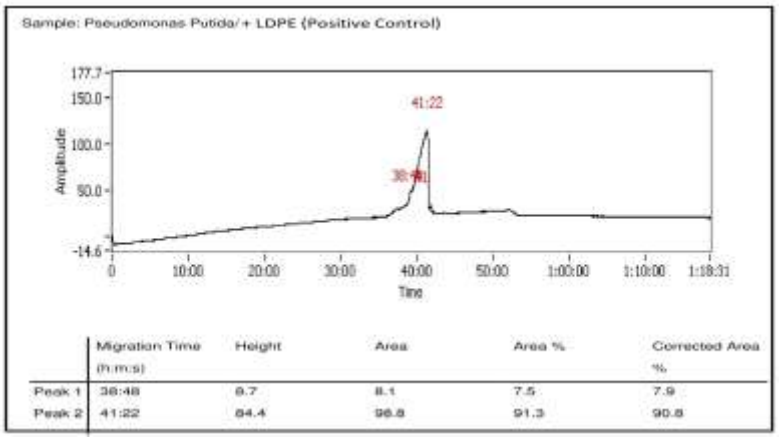
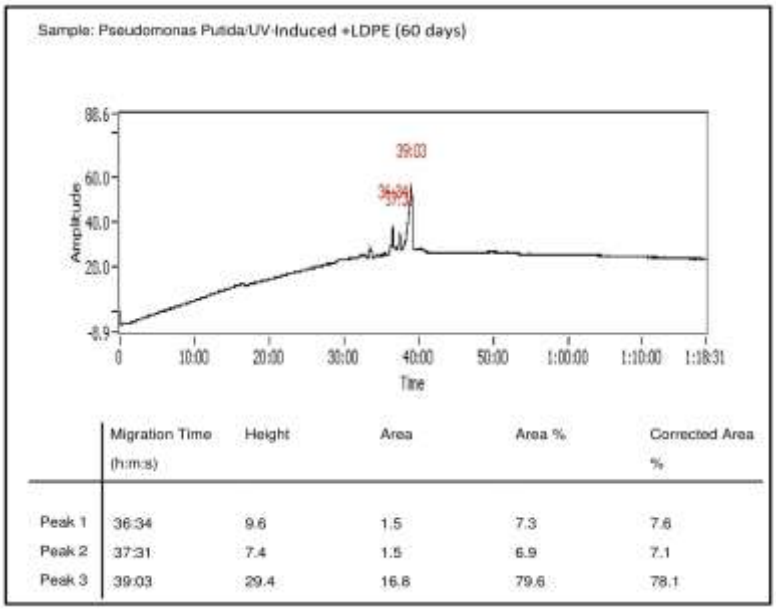


Figure-2: Capillary Gel Electrophoresis -Genomic /DNA:

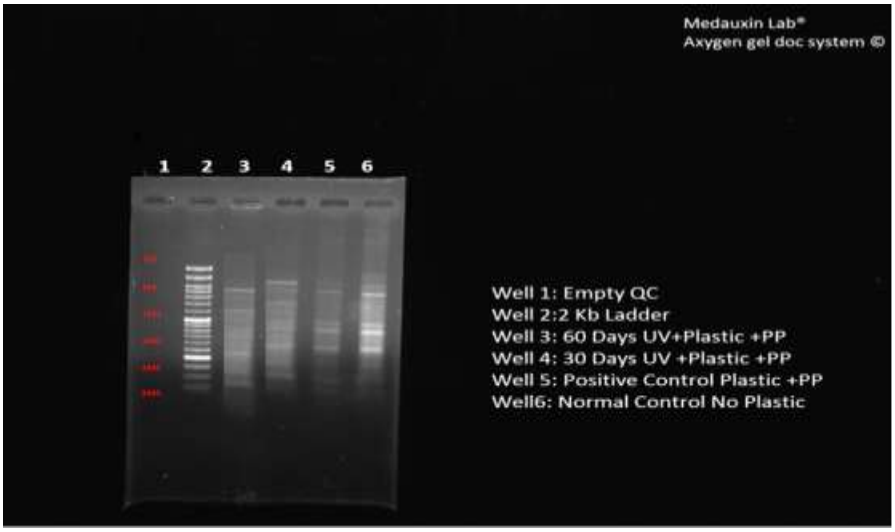
Pseudomonas Putida + LDPE (Plastic)-60 days



	Migration Time (h:m:s)	Height	Area	Area %	Corrected Area %
Peak 1	36:34	9.6	1.5	7.3	7.6
Peak 2	37:31	7.4	1.5	6.9	7.1
Peak 3	39:03	29.4	16.8	79.6	78.1

Figure-3: Capillary Gel Electrophoresis - Genomic /DNA:

Pseudomonas Putida (UV: induced + LDPE (Plastic) - 60 days)
Inferences: DNA of PP was found to be stable with a degradation capacity of 90.08 % with2 STR repeats. (Fig:
Inferences: DNA of PP was found to be unstable with a degradation capacity of 78.01 % with3 STR repeats . (Fig: 3).



DNA bands eluted in on 1% agarose gel- 100 to 2000bp, well 2: DNA marker ladder size: 2KB,
Well 3: P.putida (UV induced) and treated with LDPE -60 days
Well 4: P.putida (UV induced) and treated with LDPE -30 days
Well 5: Positive Control: P.putida treated with LDPE
Well 6: Normal Control: P.putida No LDPE

Note: Generally, good quality genomic DNA will have A260/280 of 1.7- 1.99 and exhibit one clear band of high molecular weight on 1% agarose gel

The inference can be noted in the 450bp, bands were stable except Positive control, at 200bp band of 30 days treatment followed by 900bp band in 60 days treated showed prominent band appearance , it could be due to UV induce mutation effect , these genes can be certainly helpful in LDPE degradation

IV. DISCUSSION

Analysis of chemical composition of LDPE films by GC-MS revealed the presence of alkanes, aromatic hydrocarbon, chlorocarbon, saturated fatty acids as well as unsaturated fatty acid and other unknown compounds. The degradation of LDPE by microbes was facilitated by the formation of biofilm on the surface which enables them to breakdown the high molecular weight polymer into smaller fragments through enzymatic processes. The mechanism of degradation has been postulated to be under the presence of a series of enzymatic solubilisation; however the exact mechanism has not been fully understood.

These findings support the previous work on biodegra-dation of LDPE under natural environmental conditions although in vitro studies have not been fully investigated using individual bacterial strains [3].

Past research has isolated Pseudomonas putida from sludge in industrial waste and determined that it used o-chloronitrobenzene (o-CNB) as its only carbon, nitrogen, and energy source. Most importantly, the highest degradation of o-CNB (85%) by P. putida was found to be at 32°C and a pH of 8.0. Although o-chloronitrobenzene is not plastic, this research gives a general idea of ideal growing conditions for P. putida, and shows that it is capable of using one source as its only carbon, nitrogen, and energy source [23].

Microorganisms are unable to transport the polymeric material directly into the cell due to the lack of its solubility in water & its size. They excrete extra cellular enzymes which aid in the degradation of polymers outside the cells [24]. The superficial growth of hyphae on the polymer surface was a function of the oxidation levels of treated sample was observed [25] . Therefore pre-treated samples showed greater weight loss than untreated samples.

Since the continuous introduction of recalcitrant materials, microorganisms are challenged to develop new pathways by altering their own preexisting genetic components by mutation in single structural and/or regulatory gene or perhaps recruitment of single silent gene when they encounter the foreign compounds [26].

The microorganisms appeared on the surface of LDPE film causing some physical changes like cracks, formation of pits, cavities, erosions, sporangia and spore grown through however there were some areas where the film unchanged. This can be concluded that microbes utilized starch and created pores in the polyethylene film. SEM study has proven a useful tool to study colonization and the degradation of LDPE films by fungi and other soil microorganisms [27]. By microbial assimilation during this period, this also indicated the ability of the organisms is effective when it enriched with carbon sources for their viable growth

The obtained sequence of Pseudomonas citronellolis EMBS027 contains 1476 base pairs with Molecular Weight for single stranded and double stranded as 447604.00 Daltons and 898163.00 Daltons, respectively. GC content was 53.46% and AT content was 46.54%. In their present modelled structure gibbs free energy specifies the fold stability and also provide energy minimized structure, but can deviate in natural complexities of the system. The prediction is a proof of the stability of nucleotides in the novel Pseudomonas citronellolis EMBS027, though can be used for extracting useful information when implicated in future studies.

Biodegradation of polymer granules by the isolated organisms and it makes physical and structural changes over a period of time after microbial adhesion to the granules. To check the efficiency of biodegradation, weight method was performed under laboratory conditions for 2, 4 and 6 months. Experimental data revealed that Streptomyces sps have highest plastic degradation capacity and it degrades up to 46.7%, this degradation was followed by the Aspergillus niger (26.17%), bacterial species Pseudomonas sps (24.22%) and A. flavus (16.45%) for the period of 6 months [28]

The overall investigation can be concluded that Pseudomonas putida exhibited significant polythene degradation ability and in the near future, Pseudomonas putida with induced mutation and establishing and enrichment may be of its AlkB gene can be used to reduce the quantity of plastic waste, which is rapidly accumulating in the natural environment.

V. CONCLUSION

The Biodegradation of Synthetic plastics (LDPE) using induced mutations in Bacteria Pseudomonas putida, bacterial growth curve were monitored isolated DNA banding patterns, and Mutated DNA Stability were established in the Capillary Gel Electrophoresis, this research work indicates that the affordable, reliable, eco-friendly and adequate method of degradation using microbes could be ideal in near future.

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