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Microbial Degradation of Plastic (LDPE) & domestic waste by induced mutations in *Pseudomanas putida*

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***Abstract* — The most common polymer in plastics is polyethylene (PE), which is made from ethylene monomers (CH2=CH2). In natural form it is not biodegradable. Low density polyethylene is a vital cause of environmental pollution. It occurs by choking sewer line through mishandling thus posing an everlasting ecological threat, the making of the genetically engineered microbes for bioremediation, the latter being a strategy to develop an accelerated evolution of pathways by DNA restructuring. To enhance the biodegradation of polyethylene, pretreatment strategies were followed. Three different pretreatment strategies were employed for the present study, three duration of *Pseudomanas putida* treatment to PE were analyzed on 7, 14, and 28th day. In the first, PE films were subjected for Bleach with Alkali treatment and in the second they were subjected to UV light (UV-C,>300nm wavelength). Third with the EMS induction of bacterial strains and assessed for polymer biodegradation by Biomass weight loss, estimation of total carbohydrates and total protein in the culture supernatant, followed by DNA isolation for Gel electrophoresis, and Mutated DNA Stability analysis by Capillary Gel electrophoresis were carried out.**

Index Terms— Microbial degradation of plastics, chemically treated polyethylene. Biomass, Sugar, Capillary Gel electrophoresis (key words)

I. INTRODUCTION

Biodegradation is necessary for water-soluble or water-immiscible polymers because they eventually enter streams which can neither be recycled nor incinerated. It is important to consider the microbial degradation of natural and synthetic polymers in order to understand what is necessary for biodegradation and the mechanisms involved. This requires understanding of the interactions between materials and microorganisms and the biochemical changes involved. Widespread studies on the biodegradation of plastics have been carried out in order to overcome the environmental problems associated with synthetic plastic waste.

Some studies have, demonstrated partial biodegradation of polyethylene after shorter periods of time.it has been suggested that the biodegradation of polyethylene is enhanced by oxidative pretreatment, which increases surface hydrophobicity by the formation of carbonyl groups that can be utilized by microorganisms. (1, 2, 3)
Some microorganisms are indeed capable of degrading the high molecular weight polymer (4) as was evident from a recent report on the biodegradation of thermooxidised polyethylene by *P.pinophilum* (5). A non ionic surfactant (Tween -80) to the culture medium *of Pseudomonas aeruginosa* .The surfactant apparently increased the hydrophobicity of the polyethylene surface and thus facilitated the adhesion of bacteria to the polymer (6).

Low density polyethylene is one of the major sources of environmental pollution. Polyethylene is a polymer made of long chains of ethylene monomers. The use of polyethylene growing worldwide at a rate of 12% per year and about 140 million tons of synthetic polymers are produced worldwide each year. With such a large amount of polyethylene gets accumulated in the environment, generating plastic waste ecological problems are needed thousands of years to efficiently degradation (7).

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). This study aims to isolate the bacteria from waste polyethylene plastics that can degrade polyethylene plastic.

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The selected *Pseudomanas putida* bacterial strains were assessed for polymer biodegradation by Biomass weight loss, estimation of total carbohydrates & total protein in the culture supernatant.

DNA isolation and stability studies carried out in capillary gel Electrophoresis.

II. MATERIALS & METHODS

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

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
- **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.

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 mins. 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

Induction of mutation by UV

Materials required:-

- UV germicidal light bulb (Sylvania G15T8; 254 nm wavelength) or Stratagene UV Cross linker
- 23^0c incubator
- *Pseudomonas putida* (MTCC NO: 2467**)**
- LB agar plate

Grow and mutagenize cells

1. Grow an overnight culture of the desired pseudomonas putida strain in 5 ml LB agar plate at 30^0c.
2. Determine the density of cell in the culture and record this number Adjust concentration to ~2×10^8 cells/ml if necessary. Transfer 1 ml of the culture to a sterile micro centrifuge tube.
3. Pellet cells in a micro centrifuge for 5 to 10 sec at maximum speed, room temperature. Discard supernatant and resuspend in 1 ml sterile water. Repeat wash. After the second wash, resuspend cells in 1ml of sterile water.

Plating:-

1. Make serial dilutions of the culture in sterile water so that each plate has 200 to300 viable cells.
2. Plate 0.1 and 0.2 ml of the diluted cells on separate sets of LB agar plate, using ten plates in each set. Incubate all plates for 3 to 4 days at room temperature.
3. Irradiate all but two plates from each set with UV light using a dosage of 300 ergs/mm^2 (there should be 40% to 70% survival). The nonirradiated plates will serve as controls to determine the degree of killing by the UV light.

Induction of mutation by EMS

Materials required:- *Pseudomonas putida* (MTCC NO: 2467**)** Sterile water, LB agar plate, 0.1 M sodium phosphate buffer, pH 7.0, Ethyl methanesulfonate, 5% (w/v) sodium thiosulfate (autoclaved),13 × 100–mm culture tube, Vortex, 30^0c incubator with rotating platform

Grow and mutagenize cells

1. Grow an overnight culture of the desired yeast strain in 5 ml LB agar medium at 30^0c.
2. Determine the density of cell in the culture and record this number Adjust concentration to ~2×10^8 cells/ml if necessary. Transfer 1 ml of the culture to a sterile micro centrifuge tube.
3. Pellet cells in a micro centrifuge for 5 to 10 sec at maximum speed, room temperature. Discard supernatant and resuspend in 1 ml sterile water. Repeat wash. After the second wash, resuspend cells in 1.5 ml sterile 0.1 M sodium phosphate buffer pH 7.0.
4. Add 0.7 ml cell suspension to 1 ml buffer in a 13 × 100–mm culture tube. Save remaining cells on ice for a control.
5. Add 50 µl EMS to the cells and disperse by vortexing. Place on a rotating platform and incubate 1 hr at 30^0c. (EMS treatment should cause 40% of the cells to be killed).
6. Transfer 0.2 ml of the treated cell suspension to a culture tube containing 8ml sterile 5% sodium thiosulfate, which will stop the mutagenesis by inactivation of EMS. If cells are to be stored before plating, pellet in a tabletop centrifuge 5 min at 3000×g at 4^0c, resuspend in an equal volume of sterile water and store at 4^0c.
7.

Total Biomass:

About 1ml of the culture was transferred into 1.5ml micro centrifuge tube & pelleted down at 12000rpm at 4°c for 25 min. The pellet was dried overnight at 50°c & dry weight of the resulting biomass was calculated

Total proteins:

The total protein concentration in the supernatant was determined by the method reported by Lowry's method. Bovine serum albumin solutions were used as standards & observance was measured with a spectrophotometer at 595nm.

Total sugar:

The total sugars were analyzed by anthrone method. Glucose was used as the standard & the absorbance was measured at 495nm

Gel electrophoresis:

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

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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)

Capillary Gel electrophoresis was used to analyze 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.

III. RESULTS

Characterization of the isolates: based on growth these cultures were identified *as pseudomonas putida*

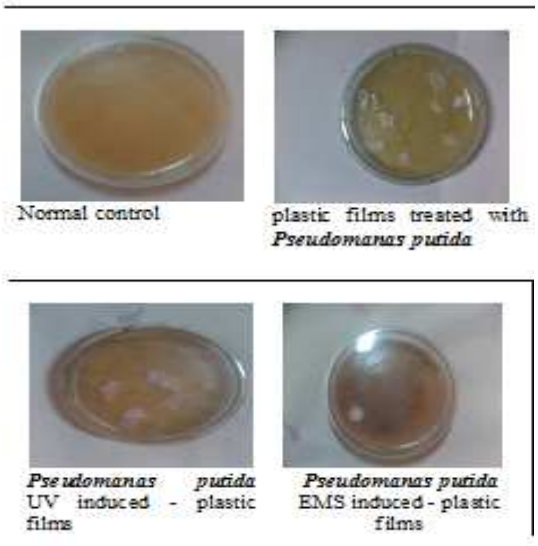
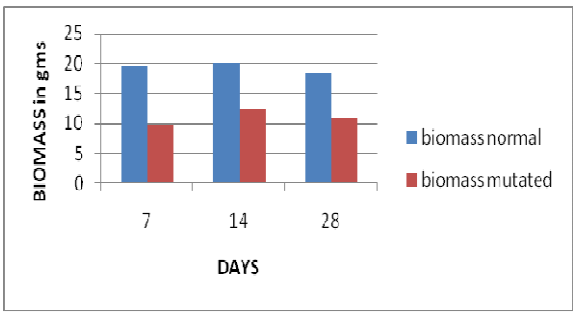
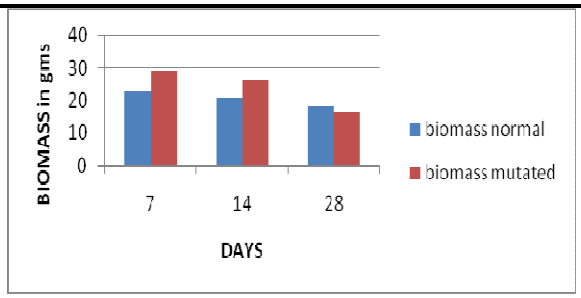


Fig: 1. Colonies of *pseudomonas putida* showing Plastic degradation

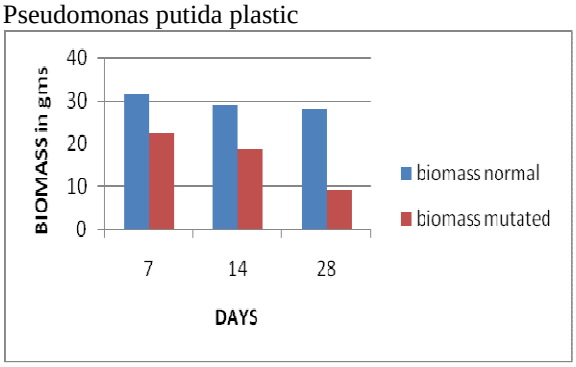
Total Biomass
Bacterial biomass is a direct measure of the growth of the culture in the medium. Chart shows the variation in biomass during 7, 14 & 28 days respectively



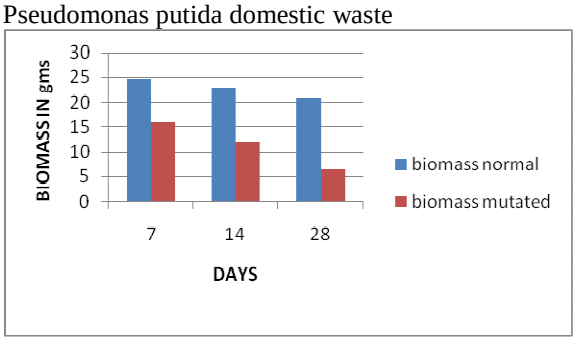
Pseudomonas putida control



Pseudomonas putida plastic

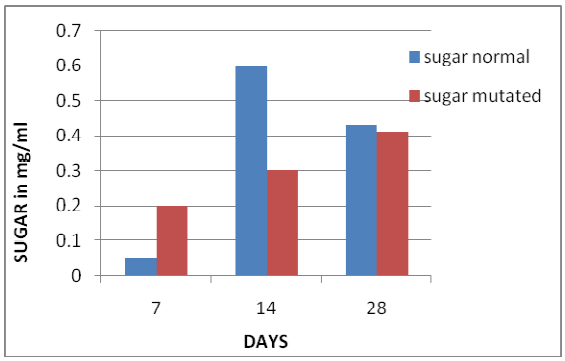


Pseudomonas putida domestic waste



Pseudomonas putida plastic + domestic waste

Total sugars in the obtained filtrate
The amount of total sugars produced by bacterial strain during 7, 14 & 28 days respectively



Sugar pseudomonas putida control

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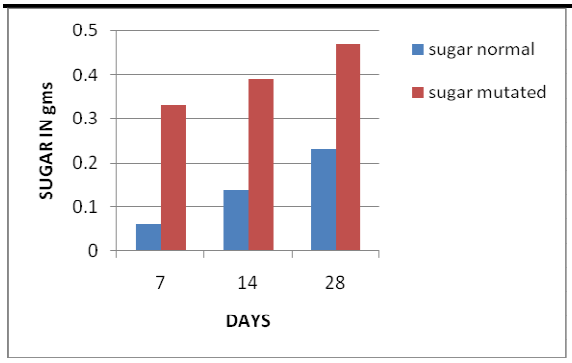
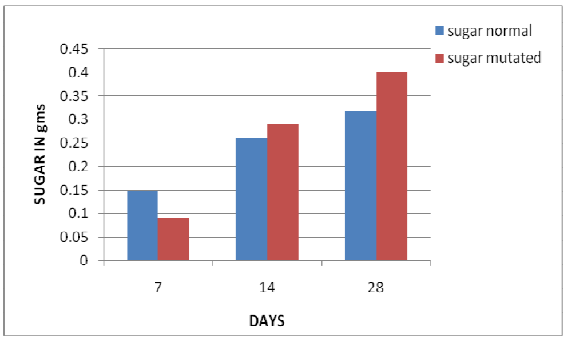
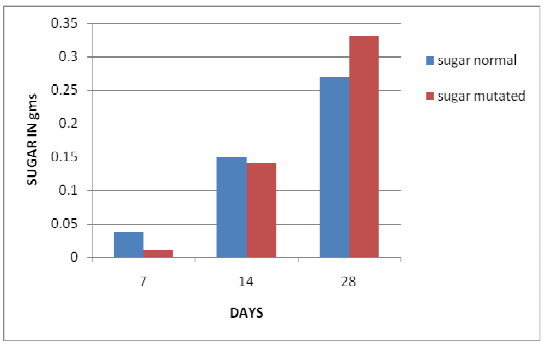


Sugar pseudomonas putida plastic



Sugar pseudomonas putida domestic waste



Sugar pseudomonas putida domestic waste + plastic

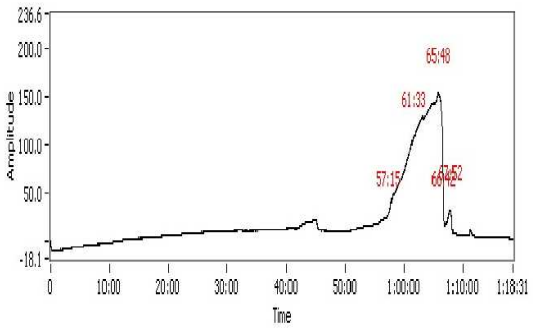


Figure-2: Capillary Gel Electrophoresis - Pro 9600 Sample: *Pseudomonas Putida*/EMS/Genomic /DNA/Plastic

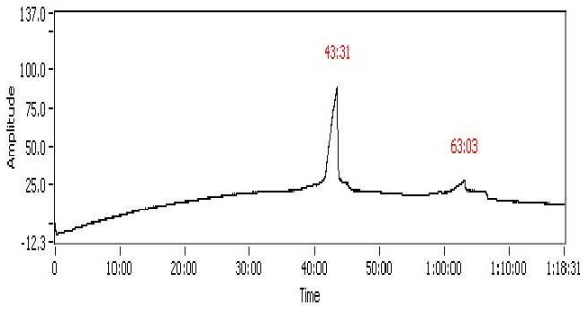


Figure-3: Capillary Gel Electrophoresis - Pro 9600 Sample: *Pseudomonas Putida*/EMS/Genomic /DNA/Plastic DW

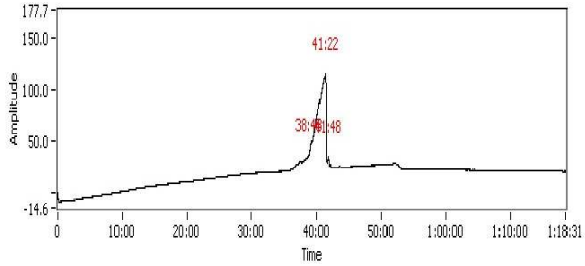


Figure-4: Capillary Gel Electrophoresis - Pro 9600 sample: *Pseudomonas Putida*/UV/Genomic /DNA/Plastic

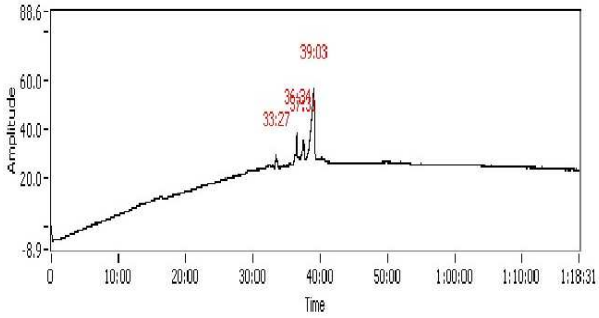


Figure-5: Capillary Gel Electrophoresis - Pro 9600 sample: Sample: *Pseudomonas Putida*/UV/Genomic/DNA/Plastic+ DW

Analysis of Capillary Gel electrophoresis:

Separations of oligonucleotides and DNA sequence products have been accomplished in polyacrylamide gels. For restriction fragments and larger oligos, gels with little or no crosslinker seem most effective due to the larger pore size of the gel. Separation of deoxyoligonucleotides such as poly (dA) 40-60 is readily accomplished in an 8% T gel with a buffer consisting of 100 mM Tris-borate, pH 8.3 with 2 mM EDTA and 7 M urea, in under 35 min with unit base resolution. Determining the purity of synthetic oligos is an important application of CGE.

***Pseudomonas Putida*/EMS/Genomic /DNA/Plastic**: DNA is Stable and the Elution happened at 67.6 Minutes, Heavy Molecular Weight - DNA. Lot of Pre and Post Elution of Genomic DNA, reason may be DNA Fragmentation. (Fig: 2).

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***Pseudomonas Putida*/EMS/Genomic /DNA/Plastic+DW:** Stable DNA 97.2 % Purity and Elution at 43.31, Mutation Stable. (Fig: 3).

***Pseudomonas Putida*/UV/Genomic /DNA/Plastic:** Stable DNA 90.8 % Purity and Elution at 41.22, Mutation Stable. (Fig: 4).

***Pseudomonas Putida*/UV/Genomic /DNA/Plastic+ DW**: Unstable Mutation Not Suggested to be Induced .Internal Folding. (Fig: 5).

IV. DISCUSSION

Although the bio-degradation and bio-deterioration of polyethylene has been demonstrated by several researchers, the enzymes involved and mechanisms associated with these phenomena are still unclear. Nevertheless, it is recognized that both enzymatic and abiotic factors (such UV light) can mediate the initial oxidation of polyethylene chains, and given the chemical similarity between polyethylene and olefins it has been suggested that the metabolic pathways for degradation of hydrocarbons can be used once the size of polyethylene molecules decrease to an acceptable range for enzyme action (typically from 10 to 50 carbons). The long-range structure and morphology of polyethylene have shown important roles, with amorphous regions being more prone to microbial attack than crystalline ones.

The Microbial Degradation of Plastic (LDPE) polyethylene & domestic waste mixture with plastic when were induced with *UV & EMS* in *Pseudomanas putida* successfully revealed the beneficial response in Biomass reduction for better yield against growth, sugar conversion along with proteins utilization consistently proven in both normal and mutated organism as the days succeeded may be by more than a month, soil mixture and domestic waste with plastic : polyethylene bags dumping can be eco-friendly manageable to degrade and utilize the biomass for agricultural cultivation of crops

May be Physico-chemically treated polyethylene films were found to be effectively degraded by the fungal isolates than untreated films. The hypothesis is that a physicochemical treatment of the polymer leads to its oxidation and subsequent breakdown assisting in the easy assimilation by the fungus and, hence, can be effectively used as a pretreatment strategy before subjecting the polymer to biodegradation (10). The oxidized polymer helps in adhesion of microorganisms (due to probable changes in the hydrophobicity of the polymer surface), which is a prerequisite for biodegradation (11). Similarly in the present study, a higher biomass was observed on the pretreated samples. Because carbohydrates in the medium constitute the main energy source for their growth and metabolism during the nonavailability of readily assimilating carbon source, microorganisms adhere to the polymeric surface during the formation of the biofilm, which is essential for bringing about degradation (12).

It is also based on research (13) these bacteria Pseudomonas sp. able to degrade the plastic by 8.16% and was able to degrade the polythene by 20.54% within one month incubation anaerobically. While this type of fungi Aspergillus Glaucus able to degrade the plastic by 7.26% and was able to degrade the polythene by 28.80% within one month incubation anaerobically, from the results of the degradation of polythene faster and easier than plastic degradation. Earlier publications interpreted the growth of microorganisms on polyolefins, e.g. polyethylene as being limited to the microbial action on the surface of an inert support without impact on the polymers (14). However, it was found that polyethylene is not only colonized but also biodegraded by various fungi mostly belonging to the genera Aspergillus, Fusarium or Penicillium (15). Polythene and plastics are two polymers with wide application, both are recalcitrant and thus remain inert to degradation and damage that leads to accumulation in the environment, and create serious environmental problems. Therefore, further research is needed to prevent environmental damage caused by plastic and polythene waste contamination (16).

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 (17).

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 (18). The superficial growth of hyphae on the polymer surface was a function of the oxidation levels of treated sample was observed (19). Therefore pretreated 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 either mutation(s) in single structural and/or regulatory gene or perhaps recruitment of single silent gene when they encounter the foreign compounds (20).

In *Pseudomonas Putida*, when **EMS** induced Genomic DNA isolated from **Plastic+DW** group showed: Stable DNA of 97.2 % Purity and Elution at 43.31, Mutation were Stable and beneficial. Same as *Pseudomonas Putida*, when **UV** induced Genomic DNA from **Plastic:** showed Stable DNA with 90.8 % Purity and Elution at 41.22, Mutation were Stable and beneficial.

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CONCLUSION

The Microbial Degradation of Plastic (LDPE) polyethylene & domestic waste mixture with plastic when were induced with *UV & EMS* in *Pseudomanas putida* successfully revealed the beneficial response better option for further utility in commercial or municipal dump yards at better strain improvement and longer duration could be an ideal organism for the sustainable technology

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