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BIOREMEDIATON OF PLASTIC FROM POLYETHYLENE DEGRADING MICRO-ORGANISM PRESENT IN THE SOIL ENVIRONMENT USING THEIR ENZYME ACTIVITY.

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Abstract

Synthetic plastics are fundamental in our modern way of life and their accumulation is therefore one of the great concern for the environment and human health.(Petro)polymers derived from petroleum such as polyethylene (PE), polyethylene terephthalate (PET), polyurethane (PU),Polystyrene (PS), polypropylene (PP) and polyvinyl chloride (PVC) are extremely resistant to natural pathways of biodegradation. To degrade such plastics which are harmful for natural environment is the aim of this research. Some micro-organisms capable of doing this Petro polymers degrading under in vitro conditions have been isolated and characterized which was found to be belonging to the group of endospore forming bacillus and mucor fungal species. In this experimental research study the enzymes expressed by these microbes have been extracted and processed as a part of degradation procedures. The procedure is quite lengthy takes upto 60 days or more depending on the isolated organism. Several similar 15-20 research papers were referred from online journal for studying the methods and outcome. The rate of biodegradation of the polymer depends on several factors, including chemical structures, molecular weights and degrees of crystallinity they are polymers of large molecules with both regular crystals (crystalline region) and irregular groups(amorphous region), where the latter provides flexibility to the polymers. PET-based plastics have a high degree of crystallinity, the main reasons for their decreased microbial degradation. The enzymatic degradation occurs in two phases: adsorption of the enzymes to the polymer surface, followed by hydrolysis of the bonds using PETase or other such enzymes. Here the traditional broth medium was used in degrading method. The sources of plastic degradation enzymes can be found in micro-organisms from different environments such as soil, riverside, beaches etc. There are multiple case studies of india and other Asian countries where the water bodies are contaminated by plastic waste, few fertile lands where plastic dumps are present on ground soil.To find a solution for eliminating this harmful plastic waste from environment which is dangerous for animals, humans and other living organsim in future this research is carried out. Microbial and enzymatic degradation of petro plastics waste is a promising strategy for depolymerization of petro plastics waste into polymeric monomers for recycling or for converting waste plastics into enhance bioproducts, such as biodegradable polymers. Bioplastic as an application was prepared and demonstrated using organic sources. It provides as an aid towards harmful plastic present in the environment as it biodegradable in nature.

KEYWORDS: Polyethylene (PE), Polyethylene terephthalate (PET), Polyurethane (PU), Polystyrene (PS), Polypropylene (PP) and Polyvinyl chloride (PVC), Biodegradation, Petro polymers, Depolymerization, Bioplastics.

Introduction: Plastic are utilized for a variety of purposes in our day-to-day lives, plastics can be considered building materials (Gnanavel et al.). ,2012). On the other hand, because they are stable, they accumulate in the environment and cause environmental pollution (Hemashenpagam et al.). ,2013;). In the greater part of the nations this plastic contamination are caused because of ill-advised reusing and squander the executives frameworks (Jayasiri et al, 2013). According to Gu et al. (2000), biodegradation is an emerging trend in the field of degradation because it involves microorganisms like bacteria and fungi that can degrade polythene. Enzymatic reactions break down polymers into monomers and oligomers during microbial cell metabolism, which is the process by which plastic is degraded by microbes. High-impact digestion prompts the creation of carbon dioxideand water (Starnecker and Menner,1996) and running against the norm anaerobic digestion creation of carbon dioxide, water and methane as the final results (Gu et al. ,2000). According to Shimao (2000), approximately 140 million tons of synthetic polymers are produced annually, and the world's consumption of polyethylene is rising at a rate of 12% annually. They take a thousand years to degrade effectively. There is a huge accumulation of polythene in the environment, so disposing of it poses a significant ecological challenge. A few potential strategies are there for this object are biodegradation and biorecycling (Yang et al. ,2005). For the treatment of plastic waste, enzymatic degradation is currently the most common method. Plastic is a complex polymer that has degraded for a long time. Because plastic is a polymer with a long structure that repeats, cutting the chains to form a molecule with short chains takes quite a bit of time. Plastic is a product that is frequently used by the community for everything from packaging for household goods to office equipment and public facilities. According to, 220 million Indonesians generated approximately 1,35 million tons of waste in 2003, while the government's waste management capacity was between 20% and 30%. The world's population has increased, and so has the use of plastic materials. Plastic is getting more requests because it offers more benefits than other materials. Raw plastic materials are typically lighter, more efficient as an insulator, and less expensive to produce. Plastic consumption rises at the risk of going to waste and could pollute the land. Plastic's characteristics, which naturally accumulate in waste ground and pile up in the ground, are difficult to break down. The ground's biological activity will be affected. a government-mandated proscession that is restricted only by the accumulation in the landfill area Biodegradable plastic made from microorganisms, which is cheap and good for the environment, is one of the most talked-about solutions. Biodegradation is the process by which microorganisms break down natural polymers like lignin and cellulose as well as synthetic polymers like polyethylene and polistrin. Polymer is broken down by every kind of microorganism. According to, the primary component of the biosphere that contributes to the breakdown of organic compounds is made up of microorganisms like fungi and bacteria. Endoenzymes and eksoenzymes in microorganisms that can break down a substrate into its simpler component Microorganisms can make use of the components as a source of carbon and energy. Biofilms form on the surface of a polymer when microorganisms degrade it. The examination was led to determination microscopic organisms from the Last Removal Spot of trash which can be utilized for corrupts the polymer of plastic. The purpose of this study was to select an indigenous bacterial strain that was isolated from the Tamangapa landfill in Makassar to degrade plastic.

According to Bhardwaj et al. (2012), biodegradation by microbial enzymes increases the rate of plastics' degradation without harming the environment. Polythenes build up in large quantities in the environment, causing problems for the environment. This paper attempts to isolate the microorganisms that degrade polythene because it is necessary to remove polythene from the atmosphere.

The decomposition of substances by microbial activity is known as biodegradation. This is a complicated procedure with multiple steps depolymerization (Microorganisms secrete enzymes and free radicals that can cleave polymer into oligomers, dimers, and monomers), assimilation (some molecules are recognized by receptors of microbial cells and can go across the plasma membrane), and mineralization (the release of simple molecules such as CO2, N2, CH4, H2O, and different salts from intracellular metabolites that are completely oxidized) are all examples of bio-deterioration.

Biodegradation can be defined as the decomposition of substances through microbial activ ity. This is a complex process which involves several steps bio-deterioration (the combined action of microbial communities and abiotic factors to fragment the materials into tiny frac tions), depolymerization (Microorganisms secrete enzymes and free radicals able to cleave polymer into oligomers, dimers and monomers, assimilation (some molecules are recognized by receptors of microbial cells and can go across the plasma membrane) and mineralization (simple molecules as

CO2, N2, CH4, H2O and different salts from intracellular metabolites that are completely oxidized are released) Christabel Ndahebwa Muhonja1 etal

Fig 1. Plastics accumulation on the sea shore.

MICROORGANISMS AND THEIR ENZYMES RESPONSIBLE FOR BIODEGRADATION OF PLASTICS

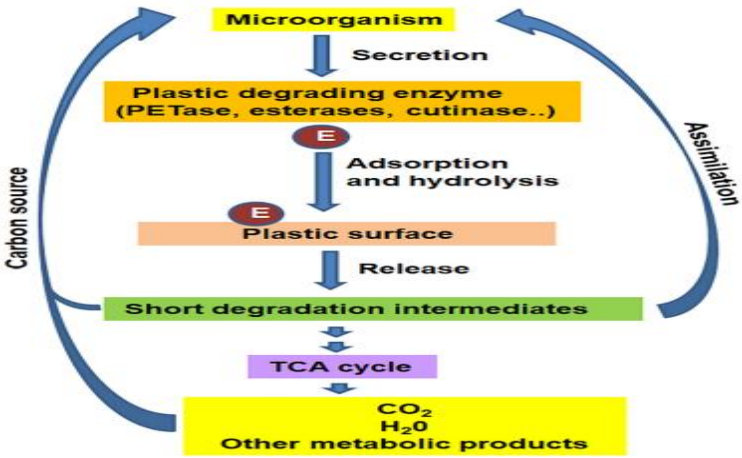


Fig no 2 Schematic representation of biodegradation

Several microorganisms like parasites and microscopic organisms are responsible for the debasement of engineered and normal plastics. Living space of polymer/plastic corrupting microorganisms fluctuates incredibly among soil,

manure, enacted slop and ocean water. As the microorganisms are available ubiquitously, they have special qualities of framing an association with material surfaces. The course of adherence of intricate microbial local area on plastic surfaces is known as "miniature fouling" or arrangement of biofilms and include the action of microorganisms and their extracellular polysaccharides. Biofilms are boundless in sea-going as well as earthly conditions and show high variety as far as existence. Microorganisms are responsible for debasement as they use hydrocarbons in the polymer spine as the essential carbon source. Plastic polymers having high sub-atomic weight are not appropriate for bacterial assault as it requires the phagocytosis of the substrate through plasma film and afterward it is corrupted by intracellular proteins. A few extracellular bacterial PHB and PHBV depolymerase have been accounted for to utilize PHB and other PHA particles. The subsequent enzymatically divided water dissolvable items are then

consumed by the microbial cells where they are additionally utilized. Carbon dioxide and water are the end products during oxygen consuming biodegradation while in anaerobic biocorruption, carbon dioxide, water and methane is created.

Polyethylene is a polymer comprised of rehashing units of ethylene monomers. The utilization of this engineered polymer is developing at a pace of 12% each year and around 140 million tons of manufactured polymers are created overall every year.

Organisms cause cleavage of the polymer chain utilizing specific compounds and convert them into monomers and oligomers. The assorted metabolic capacity of organisms can be taken advantage of for bioremediation of plastic squanders that utilizes microbial strain created through choice, strain improvement and hereditary adjustments. Biodegradability of bioplastics has been broadly exposed in the public eye and the interest for bundling is quickly expanding among retailers and the food business at large scale.

BIOPLASTICS

Biomaterials are regular items that are combined and catabolised by various organic entities and that have found wide biotechnological applications. They can be assimilated by numerous species (biodegradable) and don't cause poisonous impacts in the host giving upon them a significant benefit with regard to other customary engineered items. Bioplastics are an extraordinary sort of biomaterial. They are polyesters, created by a scope of organisms, refined under various supplement and ecological circumstances. These polymers, which are generally lipid in nature, are gathered as stockpiling materials (as portable, nebulous, fluid granules), permitting microbial endurance under pressure conditions.

APPLICATION OF BIOPLASTIC

- Bioplastic Bags.
- Medical Equipments.
- Textile Materials.
- Cosmetic Packaging.
- Agro-Food Packaging.
- Stationery
- Childcare Products.

ADVANTAGES OF BIOPLASTIC

- Potentially a much lower carbon footprint.
- Lower energy costs in manufacture.
- Do not use scarce crude oil.
- Improved compostability from using biodegradable bioplastics.
- Improved acceptability to many households.

METHODOLOGY

- **SAMPLE COLLECTION**

  Soil sample was collected from two different areas where the plastics were accumulated i.e.

  - Creek soil (SAMPLE 1)
  - Garden soil (SAMPLE 2)

- **SERIAL DILUTION AND PROCESSING OF THE SAMPLES**

  - Each 1gram of the samples were inoculated in the sterile saline and vortexed for 1minute.
  - The vortexed 0.5ml of the samples was serial diluted in 4.5 saline separately in 2 sets of 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} dilutions
  - Last 3 dilutions were considered in order get pure isolates i.e 10^{-4} 10^{-5} 10^{-6}.
  - Spread plate technique was performed using 0.1ml of the aliquot from serially diluted samples.
  - Nutrient agar medium was used for plating out.

- **ISOLATION OF THE ORGANISMS**

  - After incubation at 37ºC for 24-48 hours bacterial colonies were isolated for further procedures.
  - After incubation at 37ºC for 5-7 days fungal colonies was isolated and further processed.
  - From Sample 1 and sample 2 total 7 isolates were obtained and futher carried out for the enrichment process.
  - The isolates were preserved on NA slants and SABOURAUDS slant

- **ENRICHMENT PHASE WITH PLASTIC FOR DEGRADATION**

  - For determination of plastic degradation 4x4cm polythene sheets were dry weighed and sterilized with ethanol which was then transferred in sterile petri plates and kept in hot air oven for drying.

  - **1st method-** These polyethylene sheets were then inoculated along with each isolates in 50ml of medium for each in nutrient broth and sabourauds broth respectively for a time period of 30-40 days.

  - **2nd method-**These 4x4cm sterile polythene sheets were then placed over the surface of nutrient agar and sabourauds agar plates containing each isolate which using pour plate method was inoculated .

- Futher it was incubated at 37°C FOR 30 days.
- For each method 1 control was maintained.

- **ANALYSIS OF DEGRADATION**
  - 1st method- On basis of difference between initial weight and final weight of the polyethylene sheets inoculated in broth.
  - 2nd method- On basis of structural changes on the surface of the polyethylene sheets layed over the agar.
  - This observation was done every 10-12 days after inoculation with the isolates upto 30days.
- **SELECTION OF THE ISOLATES**
  - Selection of 2 isolates was done on the basis of degradation of the polyethylene by the organisms after enrichment phase.
  - Isolates chosen after the degradation phase was as follows:
    a) **(CREEK) SAMPLE 1 – ISOLATE 1**
    b) **(GAREDEN) SAMPLE 2 – ISOLATE 7**
- **SCREENING OF THE ORGANISMS**
- Colony morphology
- Gram staining
- Lactophenol cotton blue stain
- Microscopic examination
- Biochemical tests

❖ **Colony Morphology:** The isolates were characterised on the basis of shape, size and colour. After gram stain slide was then observed under microscope to determine the morphology of selected isolate.

❖ **Gram Staining Method:** Principle of gram staining is the ability of the bacterial cell wall to retain the crystal violet dye during solvent treatment. Gram-positive microorganisms have higher peptidoglycan content, wherein gram-negative organisms have higher lipid content.

- **Procedure** - A clean grease free slide was taken and a smear of the culture was made on it with a sterile loop. Smear was air dried and heat fixed.

  Then it was subjected to staining reagents:

  (a)Flood with crystal violet for 1min followed by washing with water

  (b) Again flooded with Gram's iodine for 1 min and followed by washing with alcohol.

  (c)The slide was counterstained with saffranin for 30 seconds followed by washing with water.

❖ **Lactophenol cotton blue** – Is a stain that is used to examine fungal culture.Stain contains phenol, which kill the organisms, lactic acid which preserves fungal structures, cotton blue that stains the chitin found in the fungal cell walls.

- **Procedure** :*a*)Apply lactophenol blue on clean slide.
  b)Use fungal growth scraped from an agar medium and further mixed with the lactophenol blue dye , the slide is cover-slipped and viewed under the microscope.

❖ **Biochemical:** Biochemical identification of the isolated strains was done by using bergey's manual biochemical methods.

1) **Catalase Test-** The catalase test was performed to detect the presence of catalase enzyme by inoculating a loopful of culture into tubes containing 3% of hydrogen peroxide solution. Positive result was indicated by formation of effervescence or appearance of bubbles due to the breaking down of hydrogen peroxide to O_2 and H_2O.

2) **Mannitol Test-** To determine whether the bacteria is capable of fermenting mannitol sugar or not. Whenever organism ferment mannitol sugar, the pH of the media becomes acidic due to the production of acids. The fermentation of the media from red to yellow show positive result.

3) **Test for MR(Methylene red)** -Some bacteria have the ability to utilize glucose and convert it to a stable acid like lactic acid, acetic acid or formic acid as the end product.
4) **VP(Voges proskauer)** -Glucose nonfermenter.

5) **Citrate Test** This test determines the ability of the bacteria to convert citrate into oxaloacetate. Citrate is the only carbon source available to the bacteria in this method. Positive results are seen if the bacteria grows and the media turns into bright blue colour.
6) **Motility test**
7) **Oxidase test.**

➢ **EXTRACTION OF THE CRUDE ENZYME**

- From the 2 isolates enzyme was extracted.
- The culture inoculated broth medium was used as the suspensions
- The suspensions were centrifuged at 10000rpm for 15-20mins.
- The supernatant was collected as the crude form of enzyme.

- **BIOPLASTIC PRODUCTION**

  ORGANIC SOURCE- Corn starch ,glycerine , vinegar, food color.

RESULTS AND DISCUSSIONS

- ✓ **ISOLATION OF THE ORGANISMS**

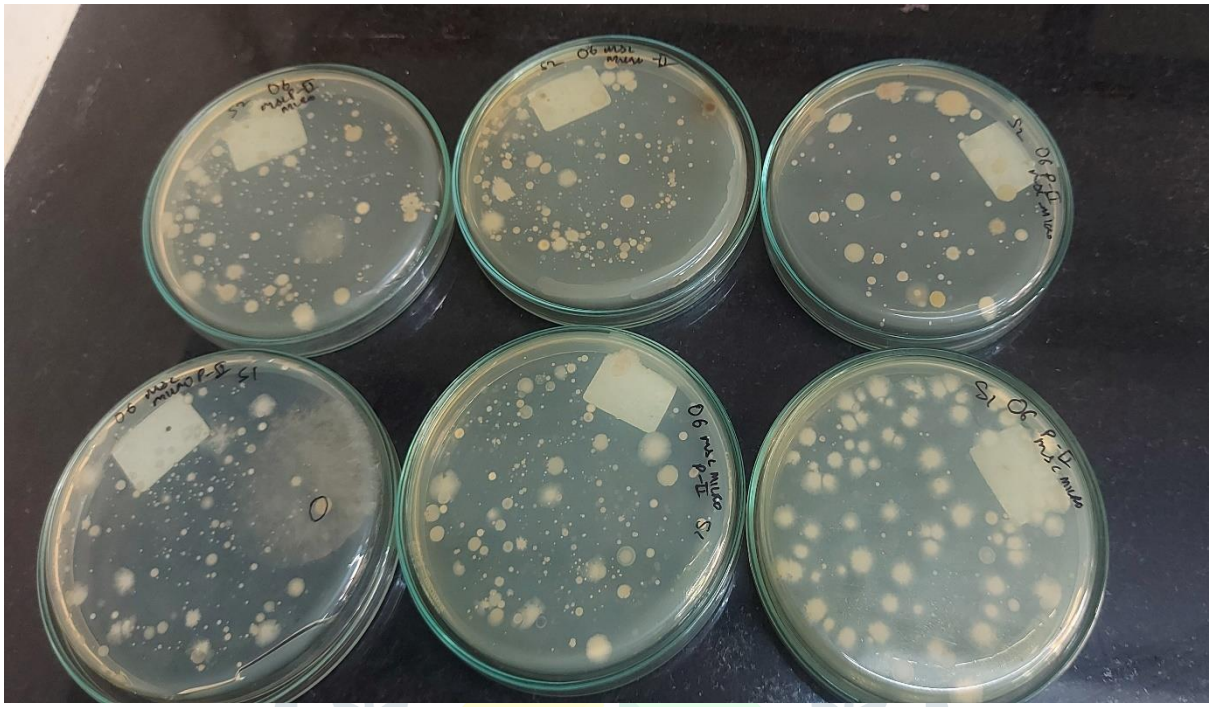


Fig no:2 Isolates obtained from the soil sample.

Fig no: 3 Seven different isolates on NA &SABOURAUD'S agar slants.

- ✓ After serial dilution of the 2 soil samples and plating out variety of colonies was found on the plates after 3-5 days of incubation at 37°C.
- ✓ Out of which 7 different colonies were picked and isolated separately.
- ✓ 4 bacterial and 3 fungal isolates were preserved on nutrient agar and sabourauds agar slants.

- ✓ **AFTER THE ENRICHMENT PHASE**

METHOD 1 & METHOD 2

Fig no: 4 Cultures inoculated with polyethylene sheet in the medium.

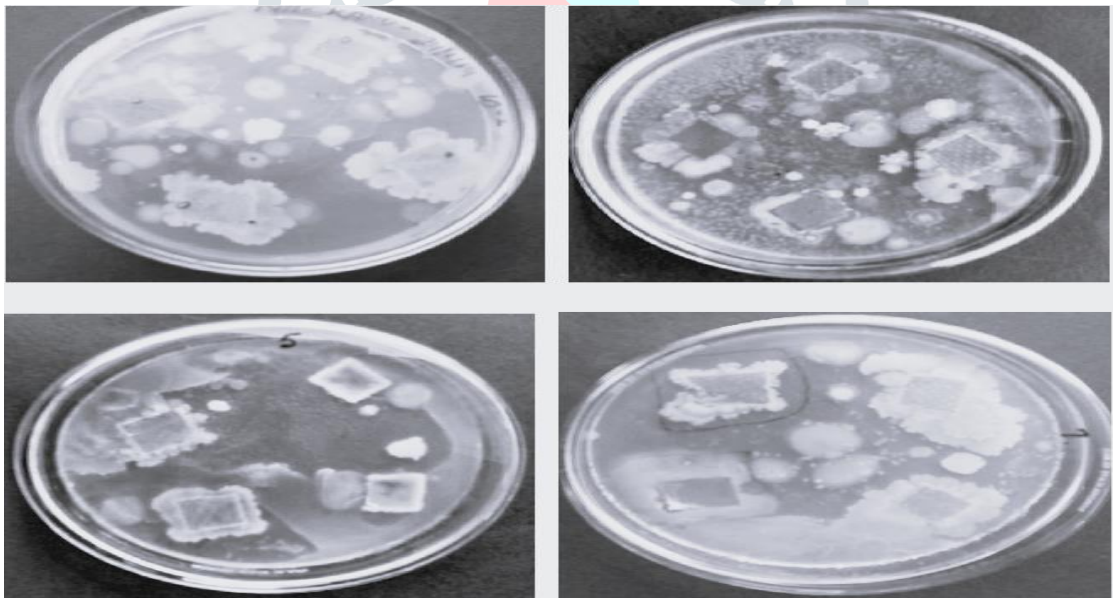


Fig no: 5 Cultures pour plated and polyethylene laid over it.

Method 1- DRY WEIGHT

ISOLATES	INITIAL WEIGHT	FINAL WEIGHT	DIFFERENCE
1	0.25	0.22	0.03
2	0.30	0.29	0.01
3	0.23	0.22	0.01
4	0.27	0.27	0
5	0.33	0.33	0
6	0.22	0.21	0.01
7	0.32	0.27	0.05

Table no: 1 Difference in Initial weight -final weight after 30 days of incubation.

- ✓ The difference in dry weight was obtained after 30 days of incubation at RT.
- ✓ The polyethylene degradation was found maximum by isolate 1 and isolate 7 which was 0.03 and 0.05 respectively.
- ✓ Thus these isolates i.e isolate 1 from sample 1 and isolate 7 from sample 2 were found to have ability to degrade the polyethylene over given period of time.

Method 2 - STRUCTURAL DEGRADATION

- ✓ The polyethylene sheet was laid over the culture medium of each isolate and it was found that the surface was fully or partially covered by the respective organisms.
- ✓ This shows that the structure of the plastic had changed due to the degradation taking place over it.
- ✓ Thus it was considered a positive result for the isolates to perform degradation process.

COLONY CHARACTERISTICS

Morphology	Isolate 1	Isolate 7
Color	Creamy white	Green
Shape	Circular	Irregular
Size	Small	Medium

Texture	Moist	Rough
Opacity	Opaque	Opaque
Elevation	Flat	Raised
Margin	Entire	Filamentous

Table no:2 Colony characteristics

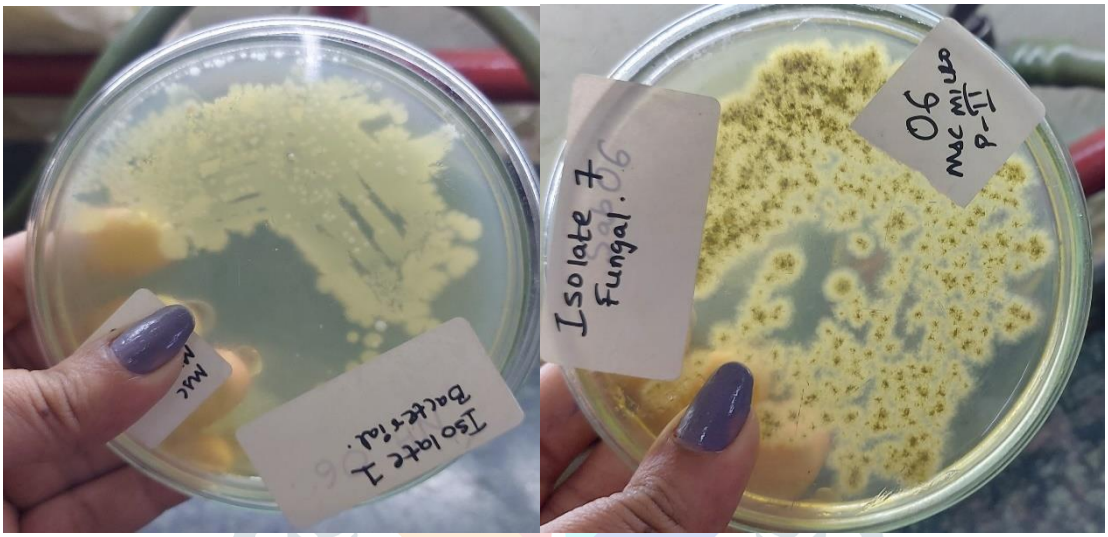


Fig no: 6 Polyethylene degrading organisms.

- **STAINING RESULTS**

SAMPLES	**Gram stain**	**Lactophenol cotton blue**
Isolate 1	Gram positive endospore forming bacillus spp.	_
Isolate 7	_	Filamentous fungi (mucor) spp.

Table no: 3 Results obtained after staining

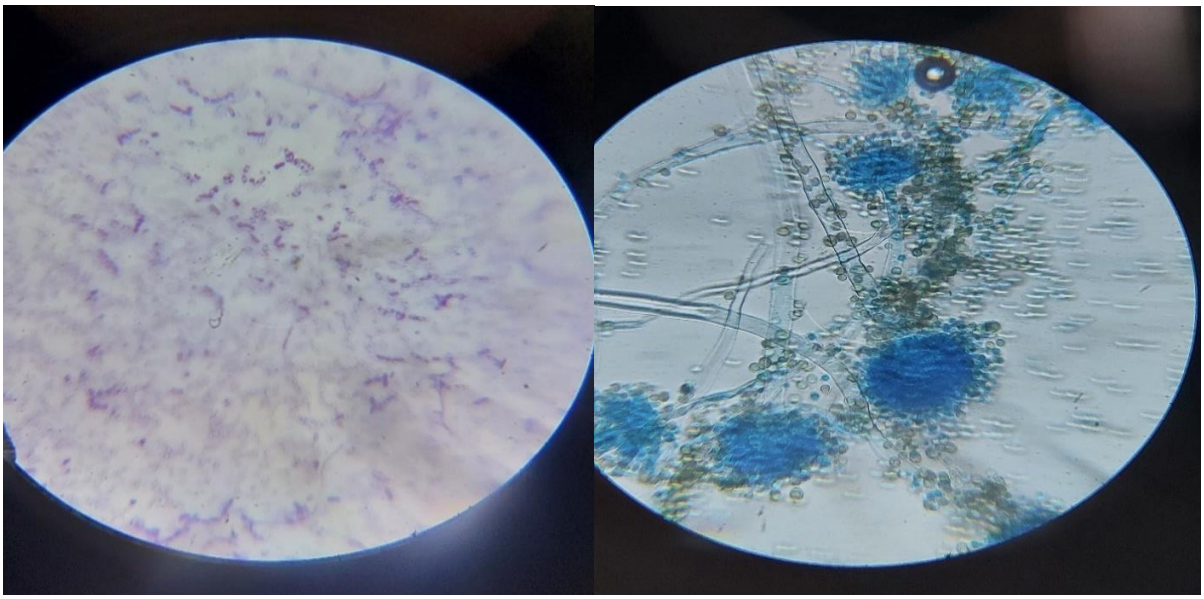


Fig no:7 Gram stain and lactophenol cotton blue stain under microscope

- **BIOCHEMICAL TESTS RESULTS FOR ISOLATE 1**

Test	Results
Catalase	+
Mannitol	-
MR	+
VP	-
Citrate	+
Oxidase	+
Motility	+
Organism	***BACILLUS CEREUS***

Table no:4 Results of biochemical test.

- **EXTRACTION OF CRUDE ENZYMES**

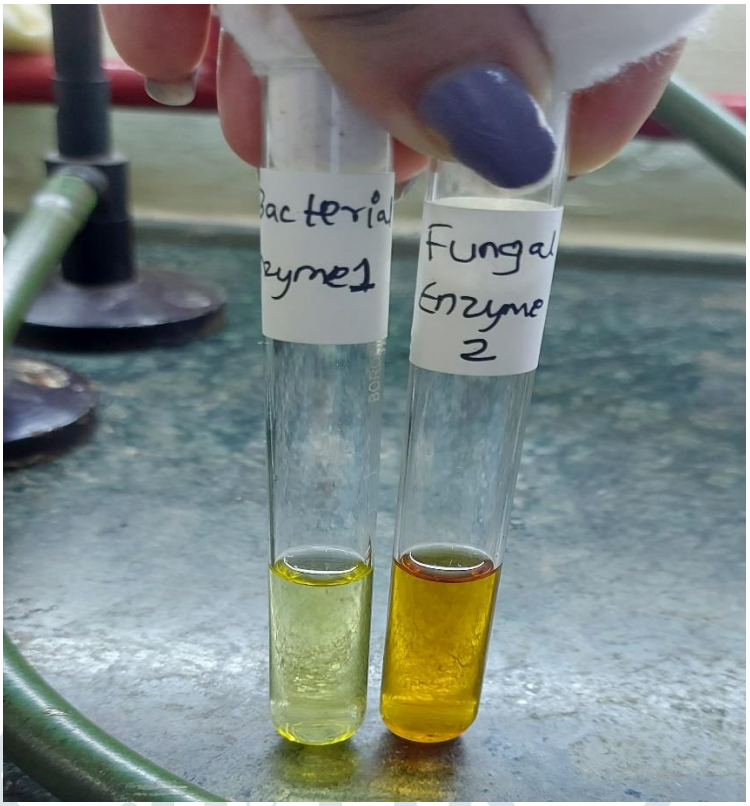
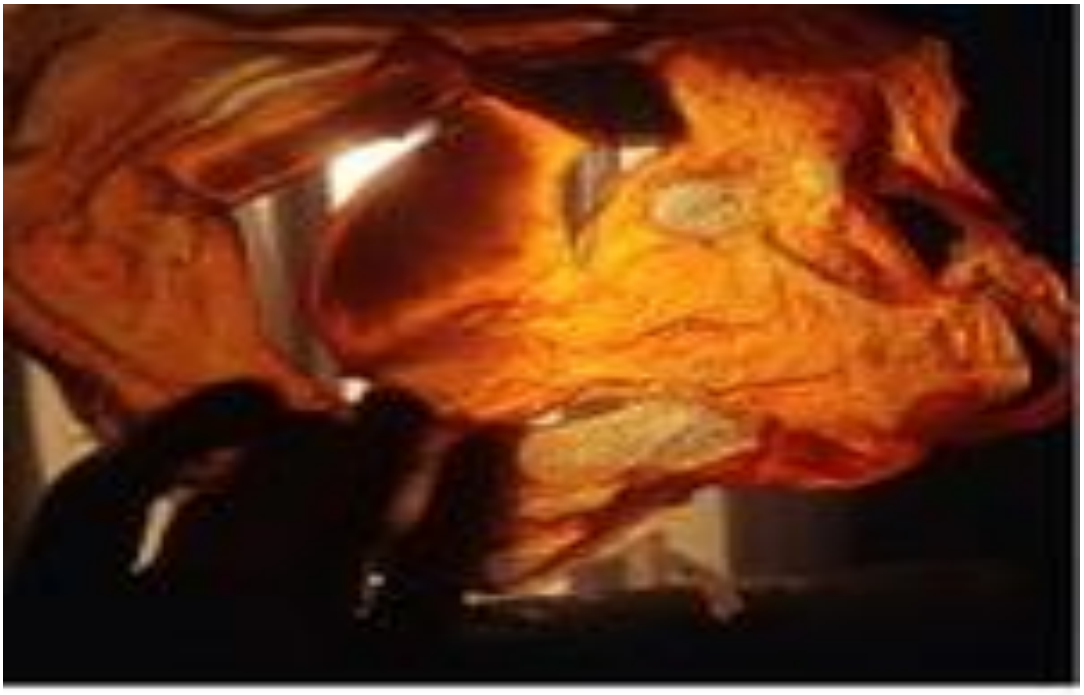


Fig no 10: APPLICATION -PRODUCTION OF BIOPLASTIC FROM ORGANIC SOURCES.



Bioplastic from cornstarch ,glycerine and vinegar

➢ DISCUSSION

The study deals with the isolation , identification and degradative ability of plastic degrader microorganisms from soil environment. Different types of changes are observed by the isolates during structural and chemical analysis. Alternate area examined has been the biodegradation of plastic by the liquid culture method. It is clear that the polymers to some extent can be degraded in the appropriate environment in right concentration .Here, it was concluded that Sample 1 and sample 2 has high degradation activity on Polyethylene. It is also observed that the degradation of Polyethylene was seen within period of 30days by both the Bacillus cereus and Mucor spp. It also showed great difference in weight for Polyethylene sheet. Microbial degradation of a solid polymer like polyethylene requires the formation of biofilm which was seen due to the action of B.cereus and Mucor spp and has also been tested in this study.

➢ CONCLUSION

In this study bioremediation of plastic i.e polyethylene was carried out using soil micro-organism , wherein Isolation of polyethylene degrading organism was performed using 2 different methods liquid & solid method which further was tested for its characterisation and identification using staining and biochemical methods. Here it was observed that after inoculation of polyethylene sheets in the medium along with isolated culture at RT and 37^{O}C for the time period of 30 days the 2 isolates which was later found to be gram positive Bacillus cereus and Mucor spp respectively was able to degrade the plastic effectively to an extent by formation of biofilm over it. This shows the ability of naturally obtained microbial species which are present in soil environment that can degrade such harmful plastics. Thus the bioremediation of polyethylene was done and the enzyme was extracted and it was seen that the more fertile soil the more chances of bioremediation by the load of potential organism present in it. The production of bioplastic was

done using organic sources such as cornstarch , glycerine and vinegar. Hence It is an important aspect for environment safety that these plastic should be eliminated with the help of such bioremediation processes which has great potential to do so, also the soil fertility plays an important role and must be promoted as it carry a great number of different micro-organisms in it. Eco-friendly bioplastic must be encouraged for the better future of the earth's environment.

➢ FUTURE ASPECTS

- ✓ Enzyme can further be purified and proteins estimation can be done.
- ✓ Various other strains can also be isolated from natural environment and sequenced which further can be compared using bioinformatics tools for similarity between the degrading bacteria.
- ✓ Solutions can be prepared using such enzyme for disrupting or dissolving the plastic material easily.
- ✓ There is a very urgent need for safe and nontoxic alternatives to fossil fuel-based plastics, such as biodegradable plastics like polyhydroxyalkanoates (PHA), which can be more easily degraded by microorganisms.
- ✓ Biodegradable plastics can be produced from food waste, agricultural residue etc.

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