

SCREENING OF PLASTIC DEGRADING MICROBES FROM VARIOUS DUMPED SOIL SAMPLES

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Abstract –

Plastic and polythene waste accumulating in the environment are posing an ever increasing ecological threat. Biodegradable plastics are environment friendly; they have an expanding range of potential application and are driven by the growing use of plastics in packaging. Low Density Polyethylene (LDPE) is one of the polymers that is up till date nearly impossible to be degraded safely. However, considering their abundance in the environment and their specificity in attacking plastics, biodegradation of plastics by microorganisms and enzymes seems to be the most effective process. This study introspected the comparative extent of plastic biodegradation by employing bacterial species and fungal species which were isolated from dumped soil samples from pallikaranai and harbour at Chennai. The screening of plastic degrading microbes was done by using opaque method separately for bacteria and fungi. Three bacterial species and three fungal species which formed most opaque were used for further studies. The bacterial species was identified as *Bacillus sp.*, *Pseudomonas sp.* and *Streptococcus sp.* and fungal species were identified as *Aspergillus sp.* and two *Fusarium sp.* by biochemical test. Then the identified bacterial species were inoculated in the Nutrient broth and the fungal species were inoculated into Potato Dextrose Broth. Their effectiveness on the degradation of commercial polythene carry bags of LDPE was studied over a period of 30 days in shaker culture under laboratory conditions by weight determination method and it was found that *Bacillus sp.* isolated from petroleum soil degrades the plastic up to 23% and the *Fusarium sp.* degrades the plastic up to 44%. Thus it may takes 120 days complete degradation of plastics by using bacteria and its takes approximately 75 days while using fungi for complete degradation of plastic.

Key words: Plastic, Low Density Polyethylene, Biodegradation

1. INTRODUCTION

Plastics are polymers that consist of monomers linked together by chemical bonds [20]. A general estimate of worldwide plastic waste generation is annually about 57 million tons [21]. The polymers include polyethylene, polypropylene, polystyrene, polyurethane, nylon etc., Polyethylene is a thermoplastic polymer produced by combining monomers of ethylene. Plastics have become an indispensable ingredient of human life [19]. Their enormous use is a matter of great environmental and economic concern, which has motivated the researchers and the technologists to induce different degrees of degradations in the plastic [1]. Plastic is the general term for a wide range of synthetic or semi synthetic polymerized products. The polythene is the most typically found non degradable solid waste that has been recently recognized as a major threat to marine life [6]. They are composed of organic condensation or addition polymers and may contain other substances to improve performance or economics. Discarded plastics, besides being highly visible are a rapidly increasing percentage of solid waste in landfills, resistant to biodegradation leading to pollution, harmful to the natural environment [11]. The term biodegradable plastics normally refer to an attack by microorganism on non water soluble polymer based materials [15]. Plastics are resistant to microbial attack, because their short time of presence in nature evolution could not design new enzyme structures capable of degrading synthetic polymers [12]. The term is often used in relation to ecology, waste management, environmental remediation and to plastic materials, due to their long life span [13]. Plastics can be classified by the chemical process that is used in their synthesis [18]. Pure plastics generally have low toxicity due to their insolubility in water and relative chemical inertness. Many microorganisms accumulate PHA as intracellular energy and storage of carbon inclusions when the carbon is in excess to the other nutrients such as nitrogen, sulphur, phosphorus and oxygen [10]. The finished plastic is non toxic, the monomers that is used in the manufacture of the parent polymers may be toxic [9]. Biodegradation is the process by which organic substances are broken down by living organisms.

2. MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

Soil samples were collected from two different regions in and around Chennai. One sample from garbage soil at pallikaranai and other sample from petroleum soil at harbour.

2.2 PREPARATION OF LDPE POWDER (LOW DENSITY POLY ETHYLENE)

LDPE sheets were cut into bits and immersed in 20 ml of xylene. It was boiled for 15mins as xylene dissolves the LDPE film and the residue was crushed while it was warm by using band gloves. The LDPE powder so obtained was washed with ethanol to remove residual xylene and allowed to evaporate to remove ethanol. The powder was dried in hot air oven at 60°C over night.

2.3 ISOLATION OF MICROORGANISM

Soil sample was collected from the municipal solid waste landfill area, Pallikaranai, Chennai. 1g of soil sample was serially diluted from 10^{-1} to 10^{-9} and inoculated in sterilized Synthetic Medium (SM). SM contains the following constituents in 1000ml distilled water (K_2HPO_4 , 1g; KH_2PO_4 , 0.2g; NaCl, 1g; $CaCl_2 \cdot 2H_2O$, 0.002g; $(NH_4)_2SO_4$, 1g; $MgSO_4 \cdot 7H_2O$, 0.5g; $CuSO_4 \cdot 5H_2O$, 0.001g; $ZnSO_4 \cdot 7H_2O$, 0.001g; $MnSO_4 \cdot H_2O$, 0.001g and $FeSO_4 \cdot 7H_2O$, 0.01g). 100mg of LDPE powder was added were incubated at room temperature for 1 week. Soil samples from each place has been taken in a zip cover and brought to laboratory from which 1g of soil was weighed and mixed with sterile water [17]. The collected samples were serially diluted up to 10^{-9} dilution using sterile water as a blank and the diluted samples 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} and 10^{-9} of garbage and petroleum soil up to 20 μ l were plated into the sterile nutrient agar using L-rod by spread plate method. The nutrient agar was incubated at 37°C for 24 hrs.

2.4 CULTURING OF MICROORGANISMS

To study the characteristics of single species. That particular species must be separated from all other species by pure cultures. This pure culture was obtained by streak plate technique.

2.4.1 SPREAD PLATE TECHNIQUE

Nutrient agar was prepared and sterilized and poured in the sterilized Petri plate and allowed to solidify. The sample was serially diluted from 1 to 10. From each dilution 0.1 ml of sample was transferred into the Petri plate with the help of sterile micropipette [4]. The 0.1 ml of sample present on the surface of agar was spread evenly by using L-rod plates were incubated at 37°C for 24 hrs to get the isolated colonies.

2.4.2 STREAK PLATE TECHNIQUE

The microbial mixture was transferred into the edge of the agar plate with the help of the inoculation loop and then streaked out over the surface. This inoculation thin out the bacteria and they are separated from each other. The plates were incubated at 37 °C for 24 hrs.

2.5 SCREENING OF POLYETHYLENE DEGRADING MICROORGANISMS

OPACITY METHOD: Agar separately amended with substrates like 1% starch, 1% gelatin, 1% tween-80 served as the suitable medium for the action of enzymes present in culture extracts of bacteria and fungi. Agar amended with each substrate was separately sterilized at 15 lbs for 15mins. About 5-20ml of sterilized substrate was poured into sterile Petri plate [14]. The plates were surface dried over night and the wells were cut aseptically to load the culture filtrates of isolated bacteria and fungi. The plates loaded with culture filtrates were incubated at 37°C for 3-4 hrs. After incubation, opacity was observed around the well surface which indicated the positive result for the respective substrates. Further quantitative assay was performed.

2.6 IDENTIFICATION TEST FOR MICROORGANISMS

2.6.1 PRELIMINARY TEST

2.6.1.1 SIMPLE STAINING

A simple stain consists of a solution of single dye. Basic solution such as acetylene blue, Grams safranin or Grams crystal violet is useful for staining most bacteria. These stains will readily give up a hydroxide ion or accept a hydrogen ion which leaves the stain positively charged. Since the surface of the most bacterial cells is negatively charged, these positively charged stains adhere readily to the cell surface [7]. Prepare and heat fixes a smear of the organism to be studied. Cover the smear with the staining solution. If crystal violet or safranin is used, allow one min for staining. Then use of methylene blue requires 3-5 mins [2]. Carefully wash off the dye with tap water and blot the slide dry with blotting paper.

2.6.1.2 GRAMS STAINING

The Grams staining method is one of the widely used differential staining methods in bacteriology. The bacteria was stained by basic primary stain, followed by the addition of a mordant (Gram's iodine), and then decolorized by ethyl alcohol. Some of the bacteria remain their stain colour in certain conditions, while some are decolorized [16]. Therefore the bacteria can be classified into two groups, the former is Grams positive bacteria and the latter is Grams negative bacteria. For clear observations a final step of counter staining with safranin or basic fushin is applied after the decolorization. The colour of Gram positive bacteria remains purple while the Gram negative bacteria were stained to appear red. The Gram positive bacteria and the Gram negative bacteria turn up differently on their chemical,

physical properties and their staining as well. Currently it is generally acknowledged that the Gram positive bacteria have unique complexes of nucleoprotein magnesium salt and polysaccharide. And their combinations between the complexes of the Gram positive bacteria and the complexes of the crystal violet and iodine (CV-1) within the inner and outer layer of the cell are strong. Therefore the Gram positive bacteria are not easy to be decolorized. In the contrast, the combinations between the complexes of the Gram negative bacteria and the complexes of crystal violet and iodine (CV-1) within the inner and outer layer of the cell are not so strong. It causes that the Gram negative bacteria cannot absorb the strain efficiently so that they can be decolorized easily [3]. Heat fixes the sample to the slide by carefully passing the slide with a drop or small piece of sample on it through a Bunsen burner. Add the primary stain (crystal violet) to the sample/slide and incubate for 1min. Rinse slide with a gentle stream of water for a maximum of 5sec to remove unbound crystal violet. Add Gram's iodine for 1 min this is a mordant or an agent that fixes the crystal violet to the bacterial cell wall. Gentle stream of water. Add the safranin to the slide and incubate for 1 min. Wash with a gentle stream of water for a maximum of 5sec. Then it was viewed under a microscope.

2.6.1.3 MOTILITY TEST

The simplest method to examine living micro organism and their motility is by hanging drop method. In this method the organism are observed in a drop that is suspended under a cover glass in a concave slide the hanging drop slides are usually observed in bright microscope. Cavity slide and cover slips were washed with distilled water, dried and wiped with alcohol. Vaseline was placed on the four edges of cover slip. A drop of culture was placed in the centre of the cover slip. The cavity slip was placed over the cover slip and contact was made between the cover slip and slide with the help of Vaseline. Curve was such taken that the drop should not touch the inner end of the cavity. The slide was turned upright and the edge of drop was observed under light microscope.

2.6.2 BIOCHEMICAL TEST

2.6.2.1 Catalase test

The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Catalase expedites the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen. This reaction is evident by the rapid formation of bubbles. The culture sample was taken and kept in the slide. The hydrogen peroxide solution was added drop by drop. The air bubbles were observed in the positive samples.

2.6.2.2 Indole test

The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole. It is used as part of the IMVpC (indole, MR-Vp Citrate) procedures, a battery of tests designed to distinguish among

members of the family Enterobacteriaceae. Inoculate the tube of tryptone broth with a small amount of a pure culture. Incubate at 37°C for 24 to 48 hrs. To test for indole production, add 5 drops of Kovac's reagent directly to the tube. A positive indole test is indicated by the formation of a pink to red color ("cherry red ring") in the reagent layer on top of the medium within sec of adding the reagent. If a culture is indole negative, the reagent layer will remain yellow or be slightly cloudy.

2.6.2.3 Methyl red test

This test is to detect the ability of an organism to produce and maintain stable acid end product from glucose fermentation. Some bacteria produce large amount of acids from glucose fermentation that they overcome the buffering action of the system. MR is a pH indicator, which remains red in color at a pH of 4.4 or less. MR broth media was prepared and sterilized. The sterile tubes were taken and the broth was poured [14]. Test organisms were inoculated and the tubes were kept into the incubator for 24 hrs. After 24 hrs the methyl red indicator was added to the tubes, and the color change is observed. Formation of red color indicates positive result and yellow color indicates negative result.

3.9.2.4 Starch Hydrolysis test

Inoculated the plates of starch agar with the assigned bacteria's and incubated at 37°C for 24-48 hrs. Dripped a small amount of Gram's iodine on the plate around the inoculated area and a small amount in an uninoculated area away from the Inoculum. A clear zone was observed around the Inoculum. Compared the inoculate area with the uninoculated area, and recorded the results.

2.6.2.5 Carbohydrate Utilization test

Transferred 0.1 ml of inoculum of each of the bacteria's into broths (glucose) and incubated the inoculated broths at 37°C for 24-48 hrs in a rotary shaker at 150 rpm. Results were observed for each broth and compared to the uninoculated controls.

2.6.2.6 Lactophenol cotton blue test

Place a drop of Lactophenol Blue Solution on a slide. Using an inoculating needle carefully spread the fungal culture into a thin preparation. Place a cover slip edge on the drop and slowly lower it. Avoid trapping air bubbles under the cover slip. Wait for about 5 mins. Observe under a microscope with low power for screening in low intensity.

2.7 Microbial growth studies

1 ml of three isolates was added to 250 ml of nutrient broth and standard flask culture experiments were carried out. These flasks were kept in shaker. 5ml of samples were

removed every day and microbial growth was monitored by calorimeter at 620 nm.

2.8 Microbial Degradation of Plastics in Laboratory Condition

Preweighed plastic disc of size 2x2 is taken, washed with distilled water and then with acetone. Then each plastic strips were dried and their dry weight is measured using the weight balance. Sterilized culture medium is taken to inoculate the plastic disc with identified micro organisms. 50ml of Nutrient broth is taken in each flask for bacterial degradation and the preweighed plastic disc was inoculated into the flask. 50ml of Potato dextrose broth is taken each flask for fungal degradation and the identified fungal species were inoculated into it. The inoculated disc were taken on different days of the month and washed with distilled water and then with acetone [8]. Then the plastic were dried and weighed for final weight. The weight loss is measured using the formula

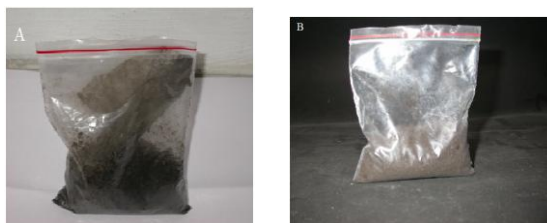
$$\% \text{ Weight loss} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

3. RESULTS AND DISCUSSIONS

The present study aimed to degrade the plastic strips using microbes isolated from various dumped soil samples. Many different bacterial and fungal isolates were obtained from the soil samples. But only three predominant bacterial colonies and fungal colonies were chosen by screening from opaque method and they were identified based on their Morphological and biochemical characteristics.

3.1 SAMPLE COLLECTION

The two soil samples were collected from pallikaranai and harbour at Chennai.



A. Garbage soil

B. Petroleum soil

Fig 1: Soil samples

3.2 ISOLATION OF MICRO ORGANISMS

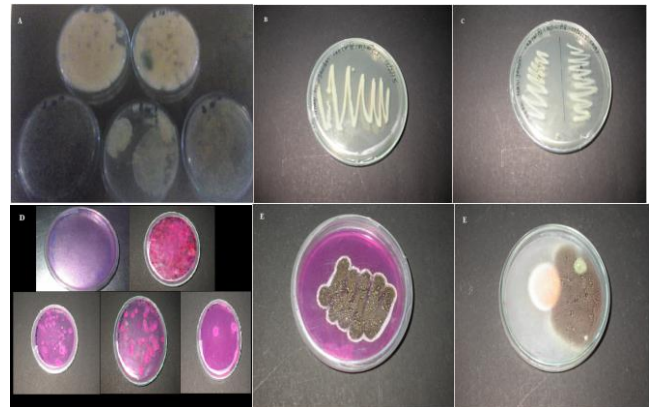


Fig 2: Isolation of microorganisms

A. Serial diluted bacterial culture; B. Isolate 1 C. Isolate 2&3

D. Serial diluted fungal culture E. Fungal Isolate 1, F. Fungal Isolate 2&3

The isolation of bacteria and fungi were done by serial diluting the samples. The culturing of bacteria was done by using Nutrient Agar medium and for culturing fungi Rose Bengal Agar medium was used.

3.3 SCREENING OF MICRO ORGANISMS

The screenings of potential plastic degrading micro organisms were done by using opaque method.

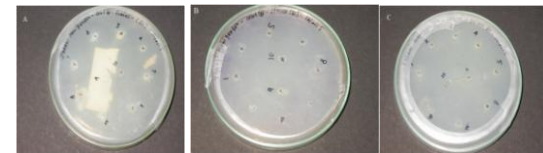


Fig: 3 Screened plastic degrading microbes by opaque method (A. By Gelatin B. By Starch C. By Tween 80)

Since Gelatin and Tween 80 is one of the derivative of the petroleum product, it was used for screening the potent microbes. In fig:3 (A) 1% of Gelatin was used as a substrate and it was amended with agar. In fig: 3 (C) 1% Tween 80 was used as substrate and poured onto agar plates. 10 wells were made by using the gel puncher and supernatant collected from the broth cultures were loaded to the wells and observed for opacity. Well number 9 and 10 were used as controls [5]. The microbes utilize the carbon source present in Gelatin and in Tween 80 and forms opacity in wells. The microbes which form most opaque were chosen for further studies. Starch is a carbon source material and it was used for screening the potent plastic degrading microbe. 1% starch was taken with agar and poured onto the agar plates. The wells were made using gel puncher and samples were loaded onto the wells and opacity was observed. The microbes which utilizes the starch as a sole carbon source and forms opacity. Three strains were isolated by their ability to forming opacity.

3.4 COLONY COLOUR

The obtained bacterial colonies were white and grayish white in colour. The fungal colonies were obtained as black, white and reddish colour.

3.5 Results for identification of microbes

3.5.1 Preliminary test

3.5.1.1 Simple staining

The isolate 1 shows spherical shape and the isolate 2&3 shows rod shaped were observed in three isolates through simple staining.

4.5.1.2 Grams staining

The isolate 1 showed gram positive rods and the isolate 2 showed gram negative rods and the isolate 3 showed gram positive rods respectively, through gram staining technique.

4.5.1.3 Motility test

The isolate 1 showed motile nature and isolate 2&3 showed non motile.

3.5.2 BIOCHEMICAL PROPERTIES OF THE OBTAINED ISOLATES

3.5.2.1 CATALASE TEST

All three isolates showed the negative results for this test by forming no bubbles.

3.5.2.2 INDOLE TEST

All three isolates showed positive results by colour changes from pink to red (Cherry red ring).

3.5.2.3 METHYL RED TEST

All three isolates showed negative results by no color changes to red.

3.5.2.4 STARCH HYDROLYSIS TEST

All three isolates forms clear zone around them after 48 hrs.

3.5.2.5 CARBOHYDRATE UTILIZATION TEST

All three isolates shows growth by utilizing the carbohydrate present in the medium.

3.5.2.6 LACTOPHENOL COTTON BLUE

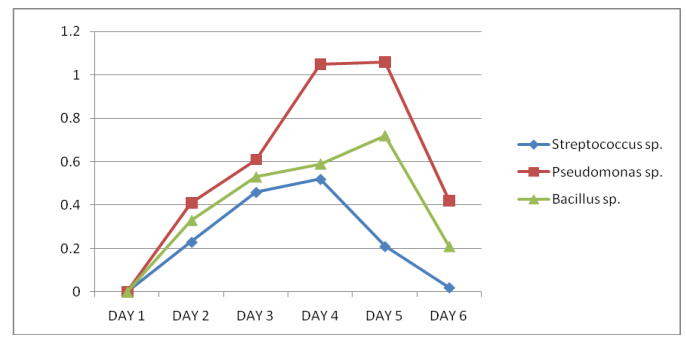
The identifying of fungal species is done by using Lacto phenol cotton blue test [2].

RESULTS OF BIOCHEMICAL TEST

The bacterial isolate 1 is identified as *streptococcus sp.* and isolate 2 is identified as *pseudomonas sp.* and the isolate 3 is identified as *bacillus sp.*

3.7 MICROBIAL GROWTH STUDIES

The growth of bacteria is measured on daily basis for 6 days at 620 nm and found that the stationery phase is reached on fourth day and the decline phase starts by fifth day.



Graph 1: Growth curve of isolated micro organisms

3.8 Microbial Degradation of Plastics in Laboratory Condition

Preweighed discs of 2x2 cm prepared from polythene bags were aseptically transferred to the conical flask containing 50 ml of culture broth medium, inoculated with different bacterial species and fungal isolates. Control was maintained with plastic discs in the microbe free medium. Different flasks were maintained for each treatment and left in a shaker. After one month of shaking, the plastic discs were collected, washed thoroughly using distilled water and then with acetone, shade dried and then weighed for final weight.

4. CONCLUSIONS

Plastic degrading microorganisms were isolated from two different soil samples and degradation of plastic strips by the isolated micro organisms was determined using weight loss method. The isolate which shows high opacity was selected and further used. The isolates obtained was subjected to standard biochemical test results showed the presence of *Streptococcus sp.*, *Pseudomonas sp.*, and *Bacillus sp.* for bacteria and *Aspergillus sp.* and *Fusarium sp.* were observed in fungi. The organisms identified was further inoculated into different culture media and their biodegradative ability was determined by loss of weight after a period of 30 days and observed that bacterial *sp.* degrades up to 23% and fungal *sp.* up to 44%. Thus fungi degrade more plastic than bacteria. Thus it may takes 120 days complete degradation of plastics by using bacteria and its takes approximately 75 days while using fungi for complete degradation of plastic. Further biochemical test can be done to identify the exact bacterial and fungal species. Further characterization of the obtained microbes can be done to increase the level of biodegradation of plastic.

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