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To enhance polythene degradation property of bacteria by strain improvement

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Abstract
Plastic are defined as the polymers which are man-made long chain polymer units. There are various methods of polythene degrading but ecofriendly acceptable method is by using microbes. The present study deal with isolation, characterization, screening and identification and enhance degrading activity of bacteria by strain improvement. The different bacteria isolated from different soil (Dumping, Petrol, Village, Village Dump) out of them some bacteria show capacity to use polythene as carbon source and degrade them. The capacity was checked by zone of clearance and growing them on minimal media contain polythene powder as carbon source then the capacity was increased by chemical mutation treatment then observed that the bacteria show more efficiency toward polythene degradation.

Keywords: polythene degradation, EMS, minimal media, biochemical tests

Introduction

Plastic are defined as the polymers which are man-made long chain polymer units. Synthetic plastic are those which are synthesized artificially. Any synthetic fiber is a chain of small unit is a chemical substance. Many such small units joined and form large single unit called a polymer. Plastics are polymers that consist of monomers linked together by chemical bonds [1]. A general estimate of worldwide plastic waste generation is annually about 57 million tons [2]. 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 [3].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 [4]. 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 [5]. 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 [6]. The term biodegradable plastics normally refer to an attack by microorganism on non-water Soluble polymer based materials [7]. 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 [8]. The term is often used in relation to ecology, waste management, Environmental remediation and to plastic materials, due to their longlife span [9]. Plastics can be classified by the chemical process that is used in their synthesis [10]. 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 [11]. The finished plastic is nontoxic, the monomers that is used in the manufacture of the parent polymers may be toxic [12]. Biodegradation is the Process by which organic substances are broken down by living organisms.

Materials and Methods

Soil Sample Collection: Soil samples were collected from different areas of Garbage soil sample (dump area), village dump soil, and local petrol pump area soil from Solapur. Soil was collected from depth of 3-5cm.

Plastic samples: Polyethylene bags were collected from local market. The polythene sample collected is 20mic and 40mic and cut into small squares and used.

Isolation of microorganisms: Serial dilution method was first used for isolation of microorganisms.1gm of all the five samples were suspended separately in 9ml sterile saline and serially diluted. 10^{-5} and 10^{-6} dilutions were plated on nutrient agar and incubated at 37ºC for 24-48 hr to isolate different bacterial strains. The colonies with different colony morphology were sub cultured onto nutrient agar plates.

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 ºC for 24 hrs.

Screening of polythene degrading microorganism

This was carried out by zone of clearance method where the 0.5 concentrations of PEG were used in minimal media containing salts of ammonium and potassium and the zone of clearance around the colonies were observed by staining with Coomassie blue this indicate its capacity to utilize polythene as C-source and degrade polythene. Polyethylene 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. 15 ml sterilized medium was poured before cooling in each plate. The isolated organisms were inoculated on polymer containing agar plates and then incubated at 25-30°C for 2-4 weeks. The organisms, producing zone of clearance around their colonies were selected for further analysis.

Identification test for microorganisms

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 mordent (Gram's iodine), and then decolorized by ethyl alcohol. Some of the bacteria remain their stain colour in certain conditions, while some are decolorized. 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 decolourization. The colour of Gram-positive bacteria remains purple while the Gram-negative bacteria were stained to appear red.

Biochemical Test

Catalase test: The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Catalase expedites the breakdown of hydrogen peroxide (H2O2) 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.

Indole test: The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole. Inoculate the tube of tryptone broth with a small amount of a pure culture and incubate at 37°C for 24 to 48 hrs. 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

Methyl red test: This test is to detect the ability of an organism to produce and maintain stable acid end product from glucose fermentation. Methyl Red (MR) is a pH indicator, which remains red in color at a pH of 4.4 or less. The sterile tubes were taken and the broth was poured [13]. 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.

Starch Hydrolysis test: Inoculate the plates of starch agar with the assigned bacteria's and incubated at 37ºC 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.

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

Pre-treatment of polythene: The polyethylene bags were cut into the small strips and transferred to a fresh solution having 7 ml Tween 80, 1 ml bleach, and 92 ml distilled water and stirring for 30 to 60 minutes. Then the strips were transferred into a beaker with distilled water and stirred for 1 hour. Further, they were aseptically placed in the ethanol solution 70% v/v for 30 min.

Degradation of Pre-Treated Polythene: Initially weighed strips of 3×3-cm size of 10 and 40 micron polythene were aseptically transferred to the conical flask containing 50 ml of nutrient broth medium and inoculated with bacteria (0.5ml). Control was maintained with plastic discs in the microbe-free medium. Different flasks will be kept in a shaker for 10 to 25 days. After the respective duration of shaking, the polythene strips were collected, washed thoroughly using distilled water, shade-dried and then weighed for final weight and percentage weight loss were calculated using below formula.

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

Treatment of bacteria with chemical mutagens

Selection of culture for mutation: The bacterial strains which show their capacity for degrade the polythene i.e., use polythene as carbon source are treated with mutagenic agent (EMS)

Process for treatment of bacterial normal strain: EMS solution of 0.1%, 0.2% Prepared and the bacterial colonies which shows polythene degrading capacity are treated for 15 sec. respectively the culture this treatment used are Dump, Petrol, village Dump and village.

Inoculation of treated strain: The degrading capacity strain are inoculated in the liquid (LB Broth) media and kept on rotary shaker for 15-25 days respectively.

Weighing of polythene after treatment: The Polythene containing flask and the media contain mutated strain of Dump, petrol, Village dump and village weighed respectively after particular day of incubation.

The mutated strain use carbon source as the polythene in more way as compared to normal strain, the initial weight of polythene before treatment are more than the bacterial strain after mutation.

Observation

The pretreated polythene was added in liquid minimal media by using mutant bacterial strain. Degradation of polythene (Pretreated) by using strain improved bacteria was concluded by weight loss method.

Table 1: Observation of Normal Strain

Culture / Strain	Polythene type (20mi)	Polythene type (40mi)	Initial weight	Final weight	Weight loss
Dump	10 Days	20 Days	0.056	0.048	14 %
Petrol	20 Days	25 Days	0.0275	0.0181	34 %
Village	10 Days	30 Days	0.0181	0.0125	30 %
Village Dump	10 Days	40 Days	0.0307	0.0280	8.7%

Table 2: Observation of Mutant strain

Culture / Strain	Polythene type (20mi)	Polythene type (40mi)	Initial weight	Final weight	Difference
Village	20 Days	-	0.0174	0.0143	70%
Village Dump 3	20 Days	30 Days	0.0221	0.0216	22.62%
Petrol	-	30 Days	0.0366	0.0181	50.54%
Dump	20 Days	-	0.0402	0.0386	39%
Village dump2	-	20 Days	0.0386	0.0356	8.2%

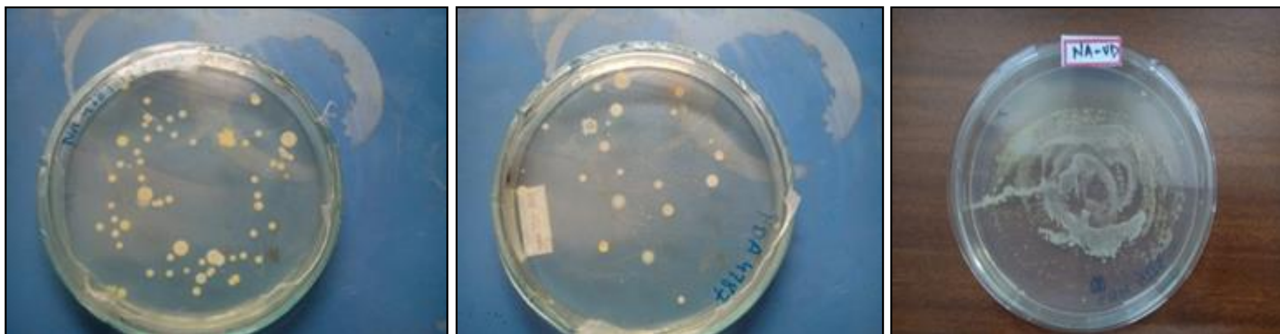

Fig 1: Isolation of microorganisms

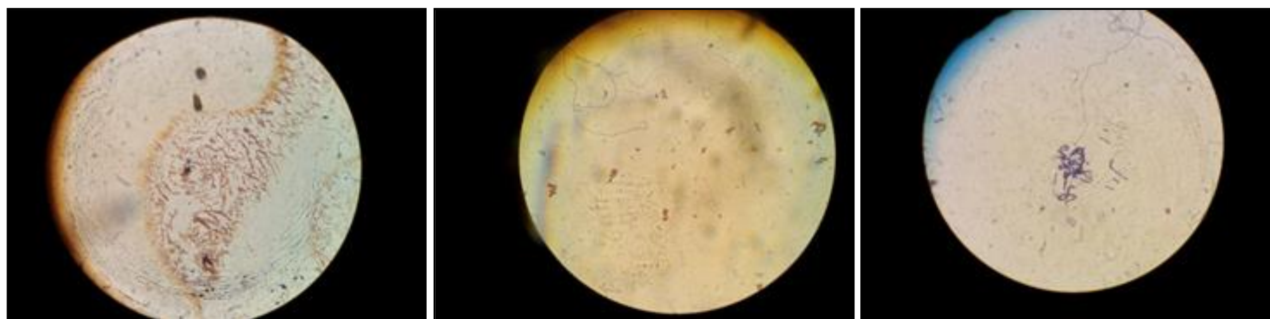

Fig 2: Gram staining (Gram -ve) Gram +ve

Fig 3: Starch hydrolysis test MR test (control & Test)

Fig 4: Catalase test

Fig 5: Glucose fermentation

Fig 6: Manitol fermentation

Fig 7: Oxidase test

Result

A total of 10 bacteria were recovered from different areas. Areas selected were petrol pump, hospital and local area. Four different samples were identified by microscopically and confirmed by various biochemical tests. Based on the colony morphology, gram staining, biochemical tests four different strains were identified. On this screening *Staphylococcus* spp, *Coryerbacterium kutesceri, Micrococcus, Enterobacter intermedius, Micrococcus varians* showed degradation activity. The isolated microorganisms from polyethylene dumped areas can be interacted with polythene and undergo changes. It is clear that synthetic polymers can be degraded to some extent by microbes. The biodegradation of plastics by isolated bacteria showed clear zone. It implies the initiation of biodegradation. Maximum degradation was found to be by *Microococcus* species and the minimum degradation was found to be by *Corynebacterium kutsceri* species. *Micrococcus* showed 34% degradation and *Corynebacterium kutsceri* showed 8.7% degradation by weight loss. These microbes confirmed its polyethylene degradation.

Screening and Identification of Polyethylene Degrading Microorganisms: The isolated colonies which showed growth on MSM agar plates were able to utilize polythene as the sole source of carbon Initial weight for dump, petrol, village and village dump area are 0.056gm,0.0275gm,0.0181gm,0.0307gm. The flasks were incubated in a shaker for 25-30 days. After incubation the polyethylene pieces were removed, washed with sterile distilled water, then sprayed with alcohol, air dried and weighed. The final weight of the polyethylene strips were noted as 0.048gm, 0.0181gm, 0.0125gm, 0.0280gm and percentage degradation was calculated as 14%, 34%, 30%, 8. 7%. The isolates with maximum weight loss- is 34% and minimum is 8.7%. After mutation by chemical treatment with EMS final weight of polythene films for village, village, dump3, petrol, dump and village dump 2 are 0.0143gm,1 0.0216gm,0.0181gm,0.0386gm,0.0356gm.

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