

## Isolation and Screening of Actinomycetes for Biodegradation of Low Density Polyethylene from Mangrove Sediment

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### ABSTRACT

Polyethylene is widely used as a packing material for different purposes of human life. Accumulation of polyethylene in the environment may cause ecological threat. To prevent the accumulation of polyethylene in the environment, a total of seven actinomycetes were isolated from Koringa Mangrove sediments, near Kakinada, Andhra Pradesh. These isolates were able to grow on mineral salts medium containing polyethylene as a sole source of carbon. Of these, one isolate M4 showed prominent result with redox probe TTC (2,3,5-triphenyltetrazolium chloride) as a viability indicator, which forms red coloured insoluble TPF (triphenylformazan) on mineral salts medium containing emulsified polyethylene within five to seven days, and was selected for detailed analysis. A significant reduction in weight of the polyethylene films were observed after four weeks of incubation with selected isolate in the mineral salts medium containing PE films as a carbon source. The viability and metabolic activity of isolate M4 growing on the polyethylene surface was confirmed using a TTC reduction test. The microbial degradation of LDPE was also analyzed by the change in pH of the culture media and microscopic analysis. Optimization of pH and temperature range for polyethylene degradation by this isolate was studied. The isolate was also able to grow on other polymers such as polyvinyl acetate, polycaprolactone, polyethylene oxide and polyethylene glycol. It could be concluded that the PE degrading actinomycete isolate selected in this study showed diverse and varying capacities to degrade polyethylene and other polymers and can be exploited for cleaning up polyethylene contaminated sites.

**Keywords:** Low density polyethylene, microscopic analysis, mineral salts media, 2,3,5-triphenyl tetrazolium chloride

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### INTRODUCTION

The use of synthetic plastic especially low density polyethylene has changed the nature of waste in last 3-4 decades [1]. Over this period, it has replaced natural material in various aspects of human life and is non-degradable causing serious environmental and human health problems. These plastic materials are used widely because of their availability, durability and light weight. The UV irradiation (photo-oxidation) [2], thermal and chemical oxidation of PE prior to its exposure to a microbial environment enhances biodegradation [3]. These pre-treatments increase surface hydrophilicity of the polymer by forming polarizable groups such as carbonyl, hydroxyl groups that can be easily utilized by microorganism

[4-6]. Among these plastics, low density polyethylene (LDPE) is used mainly as carrier bags, in agricultural and other industrial applications constitutes the major portion of waste problem. LDPE is chemically inert and microbial attack resistance as it is hydrophobic and absence of carbonyl and hydroxyl groups.

The generation of biodegradable polyethylene requires modifying the properties that are responsible for the polyethylene resistance to degradation. A standard test to determine the biodegradation of plastic materials when exposed to soil was developed by the ASTM D 5988 [7]. Moreover, plastics pollute beaches and oceans and kill marine fauna.

These issues raise concerns regarding the use of plastic materials. Since, the plastics have become an integral part of modern life; it is not possible to discontinue the use of these waste materials. Therefore, there is an emergency requirement of biotechnological/microbiological approaches to solve these environmental challenges related to plastics. Disintegration of LDPE and subsequent degradation of polymers by using microorganisms is the main strategy [8].

Furthermore, actinomycetes also play an important role in polyester degradation [9]. Most of studies at high temperature were focused on bacteria for polyethylene succinate (PES)-degradation. A thermophilic bacterium, for example, is able to degrade PES at 50 °C [10]. Pranamuda et al. [11] had isolated a PLA-degradable actinomycete, *Amycolatopsis* strain HT-32, and Jarerat and Tokiwa [12] had isolated the *Saccharothrix wayanrdensis* and *Kibdelosporangium aridum*. Sanchez et al. [13] had found the PCL-degrading fungi, *Aspergillus* sp. However, the studies related to PES degradation by actinomycetes at high temperature are still rare in the literature. The use of actinomycetes for biodegradation of polymers such as polyethylene succinate, polyhydroxybutyrate, polycaprolactone at different temperatures, have gained notable importance because of the inefficiency of the physical and chemical treatments for disposal of these plastic materials in the environment [14]. Biodegradability of plastic waste under natural conditions is required in the management of these plastic waste materials [15]. Therefore, the present study focused mainly on the isolation and screening of actinomycetes for LDPE degradation.

## **MATERIALS AND METHODS**

### **Low density polyethylene**

The low density polyethylene granules (35-65 mm) were collected from Sai polymers, Auto nagar, Visakhapatnam (India). The granules were dissolved in xylene (Fisher Scientific, 97% pure) by heating for 15 min, the residue was crushed while it was warm followed by filtration. The powder so obtained, 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 to remove residual solvent and obtain dry fine powder.

### **Collection of mangrove sediment**

The sediments were collected from Koringa mangrove environment (16°-30' to 17°-00' N latitudes and 82°-14' to 82°-23'E longitudes), near Kakinada, East Godavari district (Andhra Pradesh, India). The sediment samples were collected from 5-10 cm depth during the month of August 2014 by inserting sterile corer into the soil. The sample was transferred to a sterile polythene bag and transported immediately to the laboratory for further processing.

### **Sampling and isolation of actinomycetes**

From the sediment sample collected above, 1 g of sample was added in different test tubes containing 10 mL sterile distilled water and shaken well using vortex mixer. These test tubes were considered as stock samples for different sediment sample sites. From the stock samples, a volume of 1 mL was transferred aseptically and added to a test tube containing 9 mL of sterile distilled water and mixed well. From this test tube, 1 mL of aliquot was again transferred and mixed with another 9 mL of sterile distilled water to make 10<sup>-2</sup> dilution factor. Similarly, dilutions up to 10<sup>-8</sup> were made using serial dilution technique for all sediment samples. A volume of 1 mL of suspension from 10<sup>-7</sup> and 10<sup>-8</sup> serially diluted samples were taken and spread evenly with sterile glass rod over the surface of sterile starch casein agar plates aseptically using spread plating technique. Cycloheximide (50 µg/ mL) and Rifampicin (25 µg/ mL) were added in medium to inhibit fungal and bacterial contamination, respectively. The plates were incubated aerobically at 27 °C up to 7 days and observed intermittently during incubation [16]. After incubation, morphologically different actinomycete colonies on the plates were picked and further subcultured onto their respective isolation media. The actinomycete colonies were purified by streak plate method [17,18]. Once the pure colonies were obtained, each colony was further identified on the basis of its earthy like smell, colony morphology, colour of hyphae and the presence or absence of aerial and substrate mycelium. Then, selected and identified

colonies of actinomycetes were transferred from the plate to starch casein agar slant and incubated at 27 °C for their growth. After incubation, the slants containing pure isolated actinomycetes were stored at 4 °C and preserved as glycerol stocks at -20 °C for further studies.

#### **Screening of actinomycetes for polyethylene degradation**

These isolates obtained above were assayed for their ability to utilize polyethylene as the sole source of carbon and energy. They were grown in mineral salts medium containing (per liter): yeast extract, 0.1 g; FeSO<sub>4</sub>·7H<sub>2</sub>O, 10 mg; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 20 mg; NaCl, 0.1 g; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.5 mg; NaWO<sub>4</sub>·2H<sub>2</sub>O, 0.5 mg; MnSO<sub>4</sub>·H<sub>2</sub>O, 0.6 mg with a pH 7.2. Then, 20 µl of 1% TTC solution was added to 10 mL of medium as an indicator of viability [19]. Further, the screening actinomycetes were performed by comparing their growth ability in solid medium containing 0.1% LDPE powder as the sole source of carbon for the final screening step [2].

#### **Polyethylene film biodegradation assay**

The biodegradation tests were performed on samples of low-density polyethylene films (i.e., 1×1 cm pieces of polyethylene bags, that had been dried overnight at 60 °C, weighed, autoclaved at 105 °C for 1 h) and added to each flask (approximately 50.0 mg of polyethylene film per flask) containing 50 mL of basal medium. The flasks were inoculated with 1 mL of a mid-exponential phase culture, which are maintained in starch casein agar medium. The cell densities of the inoculums were adjusted to 1.5-1.6×10<sup>6</sup> colony forming units (CFU) per mL. The flasks containing non-inoculated basal medium supplemented with polyethylene film served as the control.

#### **Weight loss measurements**

To facilitate accurate measurement of the residual polyethylene film weight, the microbial film colonizing the film surface was removed by washing the PE films with 2% (v/v) aqueous sodium dodecyl sulfate (SDS) solution. The flasks were then incubated for 4 h at 50 °C and further washed with warm distilled water [20]. The polyethylene films were collected on filter paper, rinsed with distilled water and then dried overnight at 60 °C before they were

finally weighed. The initial weight of the pre-incubated PE films was measured following the same procedure mentioned above.

#### **Viability and metabolic activity of the isolates**

The viability and metabolic activity of the surface-attached actinomycetes was measured with the redox probe 2,3,5-triphenyltetrazolium chloride (TTC), which facilitates the monitoring of actively respiring actinomycete isolates. The colorless TTC is readily reduced by the microbial electron transport system (ETS) to red-colored insoluble triphenylformazan (TPF). The respiration was monitored by measuring the concentration TPF [19]. Cell pellets were washed twice with 50 mM phosphate buffer at a pH of 7.6 and centrifuged at 5000 rpm for 10 min. Then, the pellets were re-suspended in 4.5 mL of buffer solution, and 0.5 mL of TTC (0.1 g/L) was added. The mixture was incubated at 37 °C for 15 min in a water bath. Then, 5 mL of 96% cold methanol was added to stop the reaction and to begin the extraction of TPF. The enzyme activity was measured at 480 nm in the spectrophotometer. Ice-cold methanol and a phosphate buffer solution at a 1:1 ratio served as a blank.

#### **Determination of pH change**

Study of pH change was used know about any metabolic activity of the actinomycete isolate in the supplemented medium, as metabolism shown by microbes may support the evidence of degradation. The pH of the medium inoculated with actinomycete was measured weekly during the study. Initial pH of the medium was ensured to be 7.2 ± 0.3. The medium uninoculated with microbe serves as negative control.

#### **Microscopic observation of the polyethylene films**

The untreated and treated films after four weeks of incubation with selected actinomycete isolates were subjected to microscopic analysis after washing thrice with 2 % (w/v) aqueous sodium dodecyl sulphate and followed by warm distilled water repeatedly through mild shaking for few minutes and additionally flushed with 70 % ethanol for the removal of cells, air dried overnight and then, the polyethylene

films were observed using trinocular microscope (America Inc., LABOMED USA CXR3 9122100).

### Optimization of polyethylene biodegradation

#### Effect of pH

The effect of pH on the ability of actinomycete isolate to utilize polyethylene as a sole source of carbon was determined by supplementing mineral salts medium with PE films (approximately 50 mg films per 50 mL medium) at different pH values (pH 3-10), to determine the pH value for greater polyethylene degradation, then the flasks were inoculated with selected isolate M<sub>4</sub> and kept on rotary shaker at 180 rpm at 27 °C for seven days. The weight loss of films was measured after incubation.

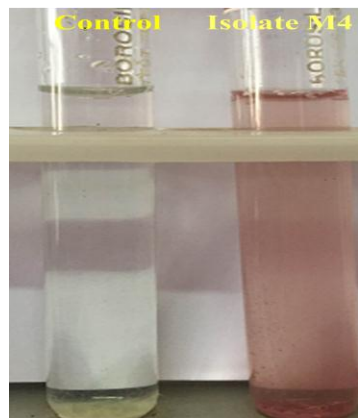
#### Effect of Temperature

To determine the effect of temperature on the ability of the isolate to utilize polyethylene as a carbon source, mineral salts medium supplemented with PE films (approximately 50 mg films per 50 mL medium) was inoculated and incubated in shaker incubator at 180 rpm at different temperatures (range between 10-50 °C) for seven days. The weight loss of PE films was measured after incubation.

## RESULTS

### Isolation and screening of PE degrading actinomycetes

A total of seven actinomycete isolates were obtained on starch casein agar plates. They were identified based on morphological appearance, earthy like smell, powdery nature of colonies. The isolates were used for biodegradation of polyethylene under shaking conditions on rotary shaker. Out of these seven isolates, only one isolate M<sub>4</sub> had shown positive result with TTC as a viability indicator, and displayed intense pink colour (**Figure 1**), which indicates that the viability of the selected actinomycete isolate in the mineral salts medium containing polyethylene as sole source of carbon for its energy and the isolate M<sub>4</sub> had shown prominent growth on the solid medium containing polyethylene powder as sole source of carbon. This result agrees with the TTC reduction test, which was used to test the viability of isolate on mineral salts medium containing polyethylene as sole carbon source.



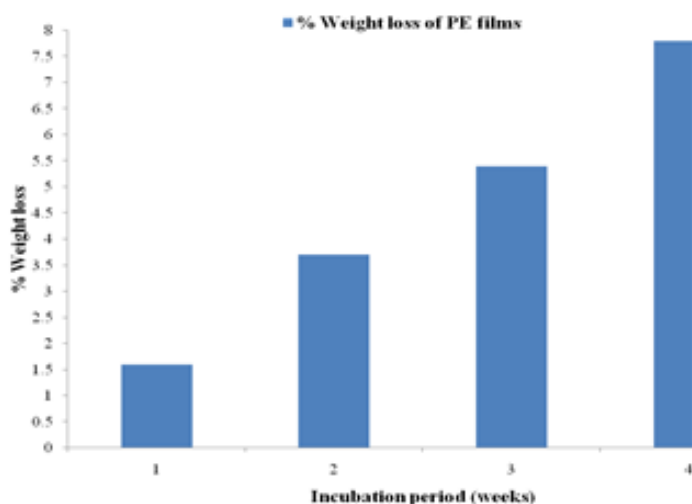
**Figure 1: Viability of actinomycete isolate M<sub>4</sub> in mineral salts medium containing TTC as redox indicator and polyethylene as a sole source of carbon**

### Determination of weight loss

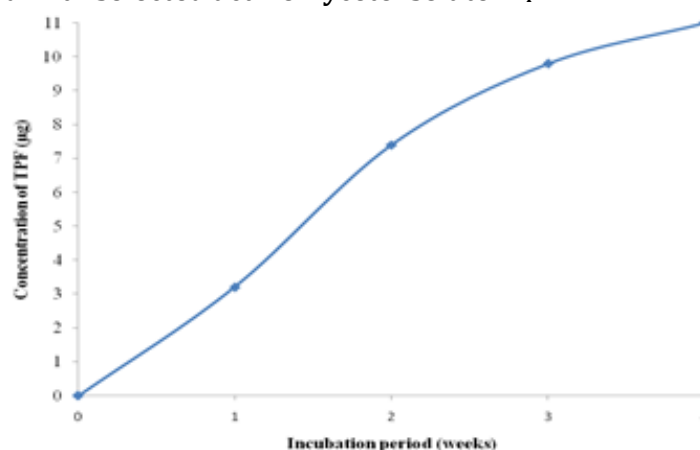
The actinomycete isolate M<sub>4</sub> screened above was allowed to degrade the polyethylene under shaking condition for 4 weeks to observe the percent weight loss of polyethylene films. Initially, after one week, the isolate M<sub>4</sub> had shown 1.6% weight loss of PE films, followed by increase in weight loss of the PE films up to 4<sup>th</sup> week (7.8%) of incubation with selected isolate M<sub>4</sub>. Increase in percentage weight loss of the PE films from 1<sup>st</sup> week to 4<sup>th</sup> week of exposure to selected actinomycete isolate M<sub>4</sub> was shown in (**Figure 2**). The weight loss of the polythene can be attributed to the breakdown of carbon backbone due to enzymatic activity by the selected isolate M<sub>4</sub>. The degradation was further confirmed based on viability of isolate M<sub>4</sub> in mineral salts medium containing polyethylene and microscopic examination of PE films.

### Viability and metabolic activity of the isolate M<sub>4</sub>

The respiration of the surface-attached actinomycete on the polyethylene films was monitored. The formation of TPF increased sharply on the 4<sup>th</sup> day of incubation, followed by constant increase of TPF concentration up to 4<sup>th</sup> week of incubation (**Figure 3**). This observation indicates a regular increase in the respiration rate and suggests that the isolate viability and growth indicating that the isolate M<sub>4</sub> utilizes the polyethylene as a carbon source.



**Figure 2: Percent reduction in weight of PE films after four weeks of incubation when inoculated with selected actinomycete isolate M<sub>4</sub>.**



**Figure 3: The respiration of surface attached actinomycete isolate M<sub>4</sub> in mineral salts medium supplemented with polyethylene as a sole source of carbon. The respiration was measured by the reduction of TTC to TPF.**

### pH change

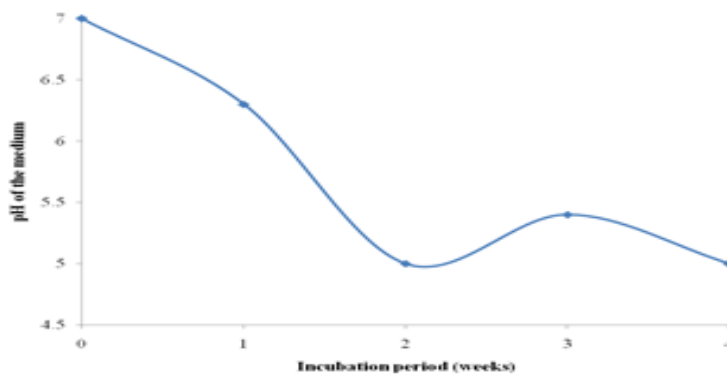
The pH is a key factor for the survival and activity of microorganisms and enzymes, respectively. The periodic measurements of the medium pH are depicted in (Figure 4). A decrease in pH of the medium was observed till the end of 3<sup>rd</sup> week of incubation, followed by slight increase in pH and decrease in pH after 4<sup>th</sup> week of incubation. The change in pH of the medium indicates the metabolic activity of the actinomycete isolate in the mineral salts medium containing polyethylene films as a sole source of carbon.

### Microscopic analysis of PE films

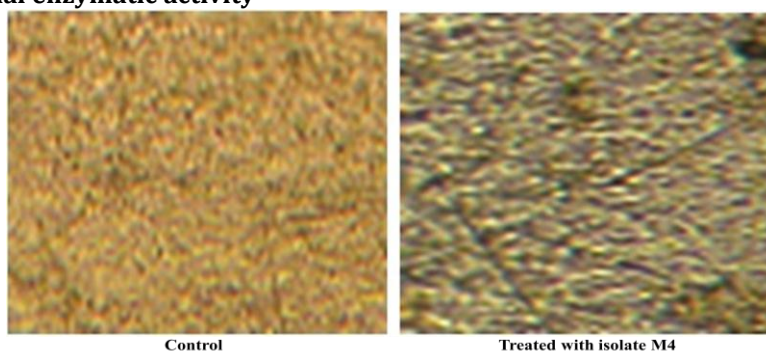
Microscopic examination of polyethylene films was performed to monitor the changes in the surface of the PE films. For this, films were incubated in liquid cultures as a

substrate for isolate to examine their surface erosion. The surfaces of PE films became rough and some localized degradation was observed on the films inoculated with isolate M<sub>4</sub> after four weeks of incubation (Figure 5). On the contrary, the surfaces were smooth on the films without inoculation of isolate M<sub>4</sub> (Figure 5). **Effect of pH and temperature**

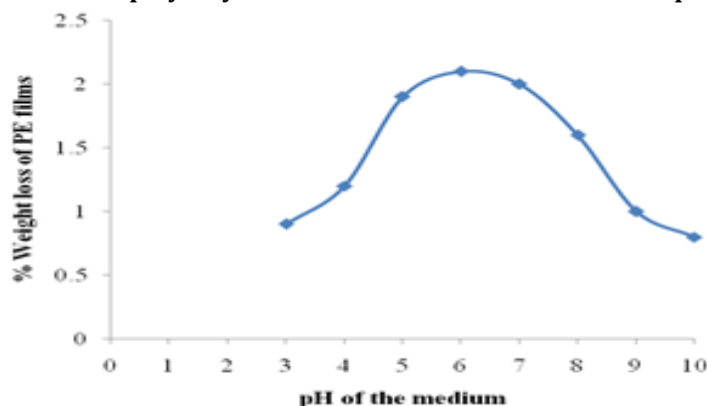
Mineral salt medium was prepared at different pH values in an attempt to determine the optimum pH required for greater weight loss of polyethylene. The results as depicted in (Figure 6) elucidated that greater degradation of PE films was occurred in the range from pH 5-7. As shown in (Figure 7), the temperature range for polyethylene degradation by the isolate M<sub>4</sub> was 35-45 °C.



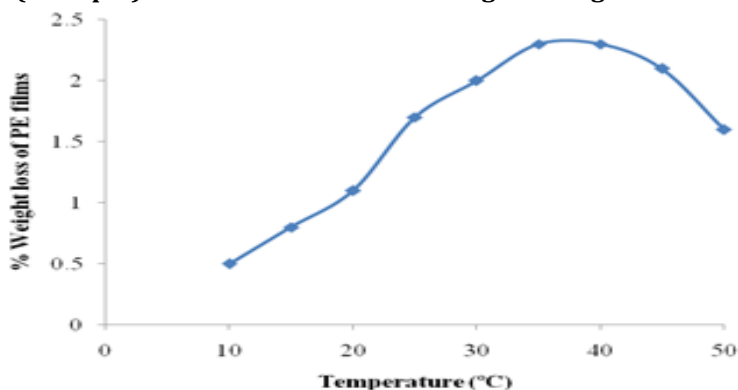
**Figure 4: Variation in pH of the medium during biodegradation of polyethylene due to microbial enzymatic activity**



**Figure 5: Surface structures of polyethylene films observed under microscope**



**Figure 6: Effect of pH of the medium on polyethylene degradation by selected isolate M<sub>4</sub> grown in mineral salt medium at different pH values containing PE films in shaking incubator (180 rpm) for one week and measuring the weight loss of PE films.**



**Figure 7: Effect of temperature on polyethylene degradation by selected isolate M<sub>4</sub> grown in mineral salt medium containing PE films for one week and measuring the weight loss of PE films**

### Degradation of other polymers

The pink colour formed not only on PE-emulsified broth but also on polycaprolactone (PCL), polyvinyl acetate (PVA), polyethylene glycol (PEG) and

polyethylene oxide (PEO) indicating that the actinomycete isolate M<sub>4</sub> is able to utilize these polymers for its growth and energy.

**Table 1: Viability and growth of the actinomycete isolate M<sub>4</sub> on other polymers using TTC as a viability indicator**

Polymer	Viability and growth
Polyethylene	++
Polycaprolactone	+
Polyvinyl acetate	++
Polyethylene glycol	+++
Polyethylene oxide	++

### DISCUSSION

Degradation of polyethylene by microorganisms had been known for several years and there is very few research information available on the degradation of polyethylene by microbes. This is the first report on low density polythene (LDPE) utilization as a carbon source under laboratory conditions by showing effective ability of the actinomycete isolate M<sub>4</sub> isolated from Koringa mangrove sediment sample, Kakinada (A.P.).

The viability was found on mineral salts medium containing polyethylene as a sole source of carbon within 4-7 days of incubation (**Figure 1**) and growth development was also found rapid on solid medium containing polyethylene as a carbon source. In the present study, the color of the viable cells increased steadily during the first 7 days to 28 days of incubation (**Figure 3**). This indicates a regular increase in the cellular viability and growth of actinomycetes, whereas in bacteria the formation of TPF increased sharply on the first day of incubation, followed by a constant increase until the fourteenth day [22]. Because of its actively increasing metabolism after 4<sup>th</sup> week of incubation with selected actinomycete isolate M<sub>4</sub> supports the use of the isolate for biodegradation of low density polyethylene. It is the simplest method for investigating microbial degradation of aliphatic polymer, since the formation of pink colour in the mineral salts medium indicates solubilisation of the polymer by the enzyme secreted from the microbes [21, 11]. The pink colour was formed when strains

secrete enzymes in order to breakdown complex polymers into soluble materials. Dehydrogenases, a group of intracellular enzymes, are involved in microbial oxidoreductases mechanisms. These enzymes have been frequently used as an index of microbial activity in soil [19]. The activities of these enzymes are linked to respiratory and energy processes in the cell, which relates to the metabolic state of microorganisms. In order to determine dehydrogenase activity, tetrazolium salts such as TTC are used as artificial electron acceptors. TTC replaces oxygen as the final H<sup>+</sup>/ e<sup>-</sup> acceptor and is reduced by the aerobic cytochrome system to water-insoluble, red-colored formazans by microbial enzymatic activities [19, 23]. From this, it is clearly understand that the viability and growth of the isolate are occurring conventionally in the presence of LDPE as a sole carbon source.

A simple method to measure the biodegradation of polymers is by determining the weight loss. Microbes that grow within the polymer lead to an increase in weight due to accumulation, whereas a loss of polymer integrity leads to weight loss. Weight loss is proportional to the surface area of the polymer, since biodegradation usually is initiated at the surface of the polymer. The isolate M<sub>4</sub> showed significant weight reduction of PE films after 4<sup>th</sup> week of incubation (**Figure 2**). Kumar and Bhavanth [22] had also reported the weight loss was 1 ± 0.033%, 1.5 ± 0.038% and 1.75 ± .06% for *K. palustris* M16, *B. pumilus* M27 and *B. subtilis*

H1584, respectively after 30 days of incubation.

(Figure 4) shows the variation in pH of the medium during this biodegradation study by the selected isolate M<sub>4</sub>. Microorganisms secrete a wide variety of enzymes, which begin the breakdown of the polymers. Exoenzymes from the microbes first breakdown the complex polymers into monomers that are small enough to permeate through the cell walls to be utilized by microbes as carbon and energy sources by a process of depolymerization [24]. The similar results were also reported on *Bacillus amyloliquefaciens* [25]. The actinomycete isolate M<sub>4</sub> in the present study showed the production of some enzymes or metabolites with the indication of pH change supporting the metabolic activity of the isolate M<sub>4</sub> on the LDPE substrate and further the degradation of polymer.

The growth, viability, weight reduction, and pH change clearly supports the isolate M<sub>4</sub> responsible for polyethylene degradation, and further, changes in surface of PE films were elucidated by trinocular microscope. However, our study supports the occurrence of enzymatic activity on polyethylene as microscopic analysis of PE films showed localized degradation (Figure 5). Previous reports confirmed that the ability of actinomycete, *Streptomyces* to degrade PE. The *Streptomyces* shows the degradation of disposable polyethylene containing 6% starch [26] and *Microbispora* shows the disappearance of PES film within 6 days in liquid cultures at 50 °C [27].

#### CONCLUSION

Polyethylene, which has a wide range of applications in day to day human life, is accumulating in the environment. Its inert properties that resist deterioration and degradation are causing serious environmental problems. This *in vitro* biodegradation study suggests the suitability of the actinomycete isolate M<sub>4</sub> for the degradation of polyethylene. Based on the viability, growth results on the polyethylene surface, metabolic activity, microscopic analysis, we were able to determine that the isolate is responsible for degradation of polyethylene. Hence, further study on microbial enzymes from the isolate M<sub>4</sub> in degradation of the LDPE and plastic

will pave way for finding technology for degrading these environmentally hazardous plastic materials.

#### Conflict of interests

The author(s) have not declared any conflict of interest.

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