Research Article

Biodegradation of Low Density Polyethylene by *Aspergillus sps* and *Pseudomonas sps* Isolated from Plastic Dumped Site – A SEM Analysis

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ABSTRACT

This study was the plastic degrading bacteria and fungi were isolated from plastic dumped soil. The bacteria was isolated and identified based on the cultural, morphological and biochemical characteristics and confirmed as Pseudomonas species using Bergey's Manual of Systematic Bacteriology. Fungal strains Aspergillus niger, Aspergillus flavus and Aspergillus oryzae were selected for polythene degradation under laboratory condition. Their effectiveness on the degradation of commercial polythene carry bags of low density polyethylene was studied over a period of 20 to 60 days. Further, SEM analysis and FTIR confirmed the degradation revealing the presence of porosity and fragility of the fungal degraded polythene surface. These microbial isolates were responsible for the decreasing weight of LDPE films by adhering on this inert surface and also utilizing it as the only carbon and energy source which was evident by increase in the fungal growth. The biodegradation of plastic bags in beneficial an it is cost effective and efficient for the solid waste management.

Keywords: Polythene, Degradation, Aspergillus sp, Pseudomonas sp, Carbon source.

INTRODUCTION

Plastics are polymers derived from petrochemicals which are further synthetically made from monomers by some chemical processes to produce these long chain polymers. Plastics are light weight, low cost, highly durable and are of high strength. In our daily life, the plastics are available in various forms such as nylon, polycarbonate, polyethylene terephthalate, polyvinylidene chloride, Urea formaldehyde, polyamides, polyethylene, polypropylene, polystyrene, polytetraflouroethylene, polyurethane and polyvinyl chloride[1]. The annual production of plastics has doubled over the past 15 years to 245 million tonnes. Production of plastic has increased from 204 million tonnes in 2002 to 310 million tonnes in 2013, representing a 46.6 % increased in 2015. During the past three decades, plastic materials are widely used in transportation, food, clothing, shelter construction, medical, recreation industries, fishing nets, packaging, food industry and agricultural field[2].

Under the natural condition degradable or non-degradable organic materials are considered as the major environmental problem (*e.g.*, plastics). The accumulation of these plastic wastes created serious threat to environment and wild life. The environmental concerns include air, water and soil pollution. The dispersal of urban and industrial wastes contaminates the soil. The soil contaminations are mainly made by human activities[3]. Environmental pollution is caused by synthetic polymers, such as wastes of plastic and water-soluble synthetic polymers in waste water.

Biodegradable Polymers

Over the last ten years, there has been a shift away from investigation of the degradability of traditional plastics, with more and more emphasis placed on the development of novel biodegradable polymers. Many biodegradable polymers currently exist, both natural and synthetic; however, the two major barriers to their incorporation in current plastic-based applications are increased production costs and inferior material properties, e.g., decreased durability[4]. Production costs can be minimised through the continued development of manufacturing protocols and increasing efficiency, but substantial research is still required to produce biodegradable polymers with comparable physical properties to conventional plastics. Regardless, some progress has been made in the field of biodegradable plastics, and a number of strategies have emerged for their development.

Factors Affecting the Biodegradability of Plastics

The properties of plastics are associated with their biodegradability. Both the chemical and physical properties of plastics influence the mechanism of biodegradation. The surface conditions (surface area, hydrophilic, and hydrophobic properties), the first order structures (chemical structure, molecular weight and molecular weight distribution) and the high order structures (glass transition temperature, melting temperature, modulus of elasticity, crystallinity and crystal structure) of polymers play important roles in the biodegradation processes.

Mechanism of Polythene Biodegradation

The degradation of polythene begins with the attachment of microbes to its surface. Various bacteria (*Streptomyces viridosporus*, *Streptomyces badius* and *Streptomyces setonii*) and wood degrading fungi produced some extracellular enzymes which lead of degradation of polythene. In wood degrading fungi, the extracellular enzymatic complex (lignin lytic system) contains peroxidases, lactase and oxidases which lead to the production of extracellular hydrogen peroxide[5]. Depending upon the type of the organism or strain and culture condition, the characteristics of this enzyme system varies[6]. For degradation of lignin, three enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and phenol oxidase containing copper also known as lactase[7].

The objective of the present study was To collect polyethylene dumped soil and polyethylene bags from waste disposed area of Vedharaniyam (TK), Tamil Nadu, India, to isolate and identify the bacteria and fungi from the soil using standard methods, to screen the bacteria and fungi for plastic degrading ability, to perform plastic degradation by simple plating method, to analyses the plastic degradation ability of fungi by Strum's Test, to study statistical analysis, to study the plastic degradation ability of fungi by FTIR and SEM analysis.

MATERIALS AND METHODS

Sample Collection

Soil samples and Low Density Polyethylene (LDPE) bags were collected from waste dumped area in Vedharaniyam (TK), Nagappattinam (DT), Tamil Nadu, India.

Preparation of LDPE powder

The LDPE films were cut into small pieces and dipped in xylene and heated, when the plastic dissolved, it was cooled to palm bearable heat and was crushed to fine particles. Later, it was kept to evaporate the xylene and it was washed with ethanol to remove xylene residues. Then it was dried in hot air oven at 50°C for overnight.

Isolation and identification of bacteria and fungi[8, 9]

Soil samples were collected and organisms were isolated on agar media by pour plate method and identified on the basis of morphological and biochemical characterization as per Bergey's Manual of Systematic Bacteriology [10].

The identification of fungi was performed on the basis of macroscopic and microscopic examination. The fungi were identified after staining with lacto phenol cotton blue. Single spore culture method was used for fungal identification. A portion of the growing edge of each colony was picked up with the help of a pair of needles and mounted on a clean slide with lacto phenol cotton blue.

Screening of polythene degrading bacteria and fungi

This was carried out by zone of clearance method where the 0.5 concentration of PEG were used in minimal media containing salts of ammonium and potassium and the zone of clearances around the colonies were observed by staining with coomassie blue. This indicates its capacity to utilize polythene as C-source and degrade polythene[11]. LDPE powder was added to synthetic medium at a concentration of 0.1% (w/v) and the culture was kept in shaker oven for one month at 28°C. The fungal colonies thus obtained, were isolated. These isolated sps were inoculated on LDPE powder containing Czapek dox agar plates and incubated at 28°C for 7 days. The development of clear zone clearance around the colonies was recorded. These organisms were selected for further analysis.

Colonization study of bacteria and fungi

The colonizing capacity of bacteria on LDPE films was studied by growing the bacteria in petriplates. LDPE sheets were cut into small pieces 2cm×2cm of similar weight, disinfected with 70% ethanol for 30 min and transferred to sterile distilled water for 20 min. Three LDPE sheets of same weight were placed in petriplates containing the nutrient agar medium and they were inoculated with bacteria taken from the nutrient agar and nutrient broth inoculated with plastics contaminated soil samples.

The colonizing capacity of the fungi on LDPE films was studied by growing the fungi in petriplates. Synthetic medium was aseptically poured into petriplates. LDPE sheets were cut into small pieces 2cm×2cm of similar weight, disinfected with 70% ethanol for 30 min and transferred to sterile distilled water for 20 min. Three LDPE sheets of similar weight were placed in petriplates containing the synthetic medium (without yeast extract). These sheets were inoculated with screened colonies of similar sized fungi using the cork borer. The petriplates were inoculated at 28°C temperature and results were determined after 1 to 4 weeks on the basis of increased weight of fungi (Fresh weight).

Petriplate methods[12]

Further the isolation of microorganism were carried out by spreading the dilution and the polythene strips of 3×3cm were cut and placed on the nutrient agar plates. After the incubation the growth of microorganism was seen on the polythene strips.

Degradation study of bacteria and fungi

The degradation study was analyzed in the LDPE (polythene) and the bacteria were applied in the LDPE (polythene) sheets. After 28 days of incubation, the following parameters were analyzed.

Visual observation[13]

The evaluation of visible changes in plastics can be performed in almost all tests. Effects of degradation include roughening of the surface, formation of holes or

cracks, defragmentation, changes in color, or formation of bio-films on the surface. These changes do not improve the presence of a biodegradation process in terms of metabolism, but the parameters of visual changes can be used as a first indication of any microbial attack. To obtain information about the degradation mechanisms, more sophisticated observation can be obtained using microscope.

CO_2 evolution test- Modified Strum test [14]

Under aerobic condition, microbes use oxygen, carbon dioxide as one of the major metabolic end product. Consequently, the consumption of oxygen (respirometric test) or the information of carbon dioxide (Strum test) are good indicators for polymer degradation and are the most often used methods to measure biodegradation in laboratory tests. CO_2 evolved as a result of LDPE biodegradation when it was determined volumetrically by Strum test.

Volumetric analysis

The dissolved carbon dioxide present in the medium was also measured volumetrically using titration methods. Briefly, sample was taken in a conical flask and 0.05ml of 0.01N thiosulphate solution was added. After the addition of 2 drops methyl orange indicator, this solution was titrated against 0.02M sodium hydroxide solution. End point showed change in colour from orange red to yellow. Next phenolphthalein indicator was added and titration was continued till a pink colour developed. Volumes of the titrate used were noted and the amount of CO_2 evolved was calculated using the formula;

$$\text{Evolved } CO_2 = \frac{A \times B \times 50 \times 1000}{V}$$

Where,

A=ml of NAOH titrate

B= Normality of NAOH

V=ml of the samples

Measurement of biodegradation

Weight reduction

A simple way to measure the biodegradation of polymers is by determining the weight loss. To facilitate accurate measurement of the dry weight of the residual polyethylene, the fungal colonization was washed off the polymer surface by dipping the fungi-treated films with 2 %(v/v) aqueous sodium dodecylsulphate solution for 4 hrs and then washed with distilled water. The washed polymer film was placed on a filter paper and dried overnight at 60°C before weighting.

Change in p^H

The variation in the p^H level in the culture media possibly occurred due to the microbial activity that was measured at an interval of 60 days during the study.

SEM analysis[15]

The treated samples after 60 days of incubation with *Aspergillus sp* was subjected to SEM analysis after washing with 2 % (v/v) aqueous SDS and distilled water for few minutes and flushed with 70 % ethanol to remove the cells. The sample was pasted onto the SEM analysis stub using a carbon tube and the sample was coated with the gold for 40 s and analyzed under high-resolution scanning electron microscope.

The fungal attachment of surface films and different changes on surfaces such as micro-cracks pits on LDPE films by the growing fungi were visualized by SEM. Samples for SEM have to be prepared to withstand the vacuum condition and high energy of electrons, and have to be of a size that will fit on the specimen stage. Samples are generally mounted rigidly to a specimen holder or stub using a conductive adhesive. SEM is used extensively for defect analysis of semiconductor wafers, and manufacturers make instruments have champers that can examine any part of a 300 mm semiconductor wafer. Many instruments have champers that can tilt an object of that size to 45° and provide continuous 360° rotation.

FTIR analysis[16]

Fourier transform infrared (FTIR) measurements were carried out for identification of surface structural changes on polymer. Horizontal ATR – attenuated total reflectance. Allows measurement of aqueous solutions, elastic and viscous samples which are difficult to grind. Specular reflectance – allows measurement of thin films on metals. KBr Discs – allow suspension of powders or contaminants in IR transparent KBr so they may be analyzed. Gas cells for head space analysis. Solvent extractions of low level bulk organic compounds and surface contaminants. Solution cells – for measuring liquid sample in transmission mode.

The bands chosen for polyethylene analysis were in the regions 3000–2800, 1550–1400 and 750–650 cm^{-1}. For the spectral resolution study, the inter ferograms were acquired with 0.5 cm^{-1} and the spectra was recalculated for 0.5 1, 2, 4 and 8 cm^{-1} resolution using the following mathematical treatments: Bartlet (triangular), Hamming, Medium with apodization and Boxcar, without apodization.

Statistical analysis[17]

Statistical analysis was performed by calculating Mean ± Standard deviation.

The formula for calculating standard deviation:

$$\text{Mean } x = \varepsilon x / N$$

RESULTS AND DISCUSSION

Isolation and identification of bacteria and fungi

Bluish green colour colonies were observed on *Pseudomonas* agar medium. The isolated colonies were gram negative rod (Table-1). The colonies showed

positive results for Methyl Red, Citrate utilization, Catalase, Oxidase tests and negative results for Indole, Voges Proskauer and Urease tests (Table-1).

Screening of polythene degradation bacteria and fungi

The screened microorganisms were further tested for their ability for degrading of plastics in laboratory condition. The microorganisms were incubated in suitable broth culture under shaking condition for time period of 2 months having 1gm of polythene strips. After a time intervals 20 days, 40 days and 60 days the plastic strips were collected from the culture, washed thoroughly with distilled water followed by ethanol and air dried. The strips were then weighed to study their final weight. The three fungal species revealed partial degradation of plastic strips utilizing them as sole carbon source. From the data collected, weight loss polythene strip was calculated and is shown in (Table-2 and Table-3).

Table 1: Morphological and biochemical characterization of isolated bacteria

S.NO	Morphological characteristics	*Pseudomonas sp*
1	Gram staining	Gram Negative
	Biochemical characteristics	
2	Motility test	Motile
3	Indole test	-
4	Methyl red test	+
5	Voges Proskauer test	-
6	Citrate utilization test	+
7	Oxidase test	+
8	Urease test	-
9	Catalase test	+

"+"- indicates Positive test

"-'' -indicates Negative test

Table 2: Degradation of plastic by *Pseudomonas* sp in Nutrient agar

S.NO	Incubation period (days)	Initial weight (mg)	Final weight (mg)	Weight Loss (%)
1	7	50±0.02	38	24
2	14	50±0.04	35	12
3	21	50±0.03	40	26
4	28	50±0.04	32	20

Values are represented as Mean ± Standard deviation

Table 3: Degradation of plastic by *Pseudomonas* sp in Nutrient broth

S.NO	Incubation period (days)	Initial weight (mg)	Final weight (mg)	Weight loss (%)
1	7	50±0.06	40	20
2	14	50±0.09	38	24
3	21	50±0.32	37	26
4	28	50±0.11	35	30

Values are triplicate and expressed as mean ± standard deviation

Colonization study of bacteria and fungi

The bacterial degradation process was observed in 7 days of interval for 28 days. The plates showed a gradual decrease in the weight of the LDPE film from 7th, 14th, 21st and 28th days ranging from 0.962 ± 0.02g, 0.808 ± 0.01g, 0.596 ± 0.03g to 0.483 ± 2.96g respectively.

The fungal biodegradation ability of *A. niger*, *A. flavus* and *A. oryzae* were found to be 38.0%, 31.2% and 26.1% for 60 days.

CO_2 evolution test of bacteria and fungi

CO_2 evolved as result of LDPE biodegradation was determined volumetrically by Strum's test. In the volumetric analysis, dissolved carbon dioxide present in the medium was also taken in a conical flask and 0.05 ml of 0.1N Thiosulphate solution was added, and the titration proceeds up to pink colour formation. In the volumetric analysis, the isolate organisms produced 5.3g/ L evolved amount of CO_2 (Table-4).

The most common end products of polyethylene degradation were CO_2 or H_2O. Mineralization is the evolution of CO_2 during depolymerization. Thus the level

of CO_2 was calculated from the control and reaction chamber after 60 d study. Theoretical carbon dioxide for 3% LDPE was calculated to be 11 g and the percentage of mineralization level of LDPE through evolved carbon dioxide from reaction chambers was determined (Table-5). As degradation of LDPE films by *A.niger* and *A.oryzae*, were maximum thus they produced large amount of CO_2 compared to other strains.

Table 4: CO_2 evolution by bacterial degradation of LDPE

Bacteria isolates	Carbon dioxide evolution (g/L)		Mineralization level (%)
	Control	Test	
Pseudomonas sp	**12.54**	**5.36**	**2.48**

Table 5: CO_2 evolution by fungal degradation of LDPE

Fungal isolates	Carbon dioxide evolution (g/L)		Mineralization level (%)
	Control	Test	
Aspergillus niger	19.13	20.26	10.3
Aspergillus flavus	17.53	18.47	8.6
Aspergillus oryzae	18.17	19.38	13.02

SEM analysis

Scanning electron micrograph showed the attachment of fungi on LDPE surface and formation of various holes and irregularities whereas the control film was appeared with smooth surface having no any pits, cracks or any particles attached on its surface (Fig-1).

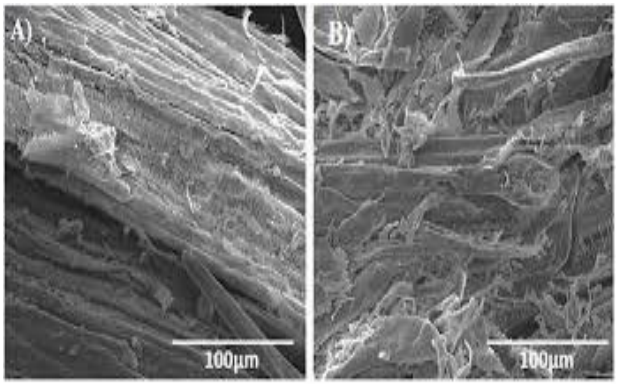

Figure 1: Scanning electron micrograph of untreated and treated sample of polymer sheet

A) Control untreated LDPE film B) LDPE treated with *Aspergillus*

FTIR analysis

The FTIR spectroscopy analysis exhibited several changes on the surface after the degradation of 60 d with different fungal isolates (Fig-2). The increase in 1079 cm^{-1} and 2418 cm^{-1} band due to formation of C=O and O- H stretch were observed. The peak at 2920 cm^{-1} was distorted after the treatment with fungal isolates. The films were more effected by *Aspergillus* species. Polymer degradation has been reflected in changes of bond scission, chemical transformation and formation of new functional groups.

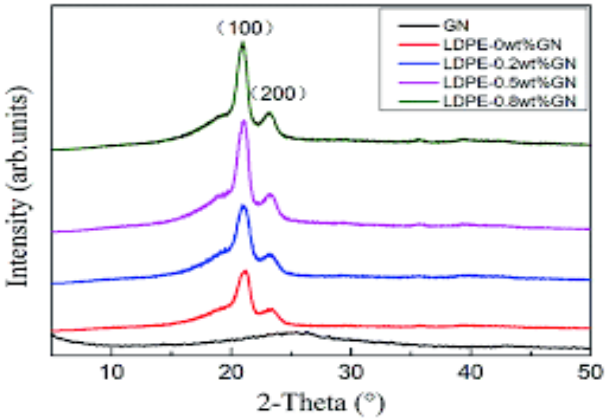

Figure 2: FTIR spectra of LDPE film after 60 days of incubation with fungal strains

CONCLUSION

The present research was conducted to analyze the ability of *Aspergillus sp*. And *Pseudomonas sp* isolated from plastic dumped site to degrade low density polyethylene. The impact of this research on the society is highly effective because it leads to the bioremediation of biodegradable plastic wastes using the two species, *Aspergillus sp* and *Pseudomonas sp*. The polluted environment containing plastic wastes can be cleaned easily without causing any harm to the environment. Moreover, the biodegradation of plastic bags in beneficial an it is cost effective and efficient for the solid waste management. Therefore, it is essential to degrade large amount of plastic bags which causes pollution at a higher rate to the environment.

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