IJASRM
International Journal of Advanced Scientific Research and Management, Volume 3 Issue 7, July 2018
www.ijasrm.com
ISSN 2455-6378

Screening and Optimization of Actinomycetes from compost soil for the biodegradation of LDPE

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Abstract

Plastics are polymers linked together by chemical bonds. It is a broad name given to different polymers with high molecular weight including polyethylene, polypropylene, polystyrene, polyurethane etc. With more and more plastics being employed in human lives and increasing pressure being placed on capacities available for plastic waste disposal, the need for biodegradable plastics and biodegradation of plastics wastes has assumed increasing importance in the last few years. This attempt has been carried out in the ease of screening out an efficient biodegrading organism and involving that organism in degrading low density polyethylene under natural circumstances. In the present study, *Actinomycetes* were selected as the process organisms. The *Actinomycetes* were isolated from compost soil samples, Pallikaranai. Totally ten isolates were obtained , out of which five isolates were screened to be efficient degrading strains by clear zone and BATH test. Further these strains were involved in degradation of low density polyethylene by using Soil extract as the medium with an intention of carrying out the degradation in a quite simple, cost effective and native.

Keywords: *Low density polyethylene, Soil extract, BATH test, Clear zone assay.*

1. Introduction:

The advancement in technology and increase in the global population have let to exponential use of plastics, they find wide applications in every aspect of life and industries. However, most conventional plastics such as polyethylene, polypropylene, polystyrene, poly-vinyl chloride etc are stable and resist degradation, leading to pollution and a serious threat to the ecosystem.

Low density polyethylene is a thermoplastic made from petroleum (Shah *et al.,* 2008). LDPE is the most widely used packaging material because of its versatile nature, excellent mechanical properties, barrier properties against water, light and water etc. Even though this polymer provides a lot of benefits to the human life, they also being agreat danger in affecting the living beings in many ways. With these huge amount of polyethylene getting accumulated in the environment, their disposal put forth a big ecological issue. It takes thousand years for their efficient degradation.

Disposal of polymers by degradation results in beneficial compounds will be a proper solution to overcome the problem of pollution. These degradation process can be achieved phytochemically, thermally and biologically (Goheen and Wool,1991).Out of all these, biological way of degradation will be a quite efficient , ease and cost effective under normal conditions without any prerequisites. Wide range of microbes like *Actinomycetes* were involved in this process (Gu, 2003).

In the present study, the *Actinomycetes* strains were isolated from municipal solid compost soil samples. Isolates were screened for its efficiency in degrading LDPE. The strains were further subjected to degradation studies under natural conditions by using a soil extract medium containing LDPE powder as a sole carbon source with a view to attempt the process of degradation in an

environmentally realistic condition which may help in safe disposal of plastics in future perspectives.

2. Materials and Methods:

Isolation of Actinomycetes:

2.1 Collection and processing of the sample:

Compost soil samples were collected in sterile plastic bags and they were transported to the laboratory for further processing. Soil extract media was used as a natural media without any added ingredients except LDPE as a sole source of carbon.

5gm of soil was diluted in 30 ml of sterile distilled water and was kept in the shaker under proper aeration and agitation at 30°C for two to three days.The broth was taken intermittently and was examined for *Actinomycetes* by wet mount and LPCB staining. Further the inoculum was taken from the broth and was spread plated onto soil extract agar medium. The plates were then incubated at 30°C for one week.

2.2 Identification and characterization of *Actinomycetes:*

The characterization of *Actinomycetes* culture was done using aerial mass colour, reverse side pigmentation, melanoid pigment, spore chain morphology and spore morphology.*Actinomycetes* were further identified based on standard biochemical methods (Shirling and Gottlieb, 1966; Pridham and Gottlieb, 1948).

2.3 Optimization of growth parameters:

Optimization of the culture condition is a prime requirement to obtain the maximum yield from the desired microorganisms. For this purpose, suitable carbon source and physicochemical parameters were chosen based on criteria (Prit, 1975).

a) Carbon Source.
b) Temperature.
c) pH.

a) Influence of varying concentration of carbon source on growth of *Actinomycetes:*

The isolates were inoculated into soil extract broth with varying concentration of LDPE ranging from 0.5, 1 and 2%. The growth of the isolates were analyzed by turbidity method using spectrophotometer at regular intervals. The best opted concentration of carbon source was determined by its ability to bring about the maximum growth of *Actinomycetes*.

b) Influence of varying temperature on the growth of Actinomycetes:

In order to find out the optimum temperature required to obtain a maximum yield, the A*ctinomycetes* strains were inoculated into soil extract broth with 1% LDPE which is said to be the best opted concentration supporting the maximum growth of *Actinomycetes*, and incubated at varying temperature range of 30°C, 35°C and 40°C.The growth at every 30 min intervals was analysed by turbidity method using spectrophotometer to obtain a maximum growth kinetics.

c) Influence of varying pH on growth of *Actinomycetes*:

The isolates were inoculated in the soil extract broth and incubated at 30°C with varying pH range (Pridham and Gottlieb, 1948, Augusta *et al.,* 1993, Imam and Gould,1990).The growth kinetics was determined by turbidity method using spectrophotometer.

2.4 Screening for polyethylene degrading *Actinomycetes:*

Preparation of LDPE Powder:

Pure grade LDPE sheets were shredded into small bits and immersed in xylene .The sheets were then boiled in water bath until they dissolved completely to become a clear solution. It was cooled and smashed using a sterile rubber gloves.It was then flooded with ethanol to remove xylene residues. It was air dried in hot air oven to remove ethanol to obtain a pure form of powder.

a) Clear Zone Test:

LDPE powders were added to the soil extract medium with the concentration of 0.2%. Mixture was sonicated for 1 hour. The medium was sterilized at 121°C and a pressure of 15 lbs/inch^2 for 20 minutes.The medium was poured into the plates and was allowed for solidification. It was then inoculated with *Actinomycetes* isolates and the plates were incubated at 30°C for three to four weeks. The plates were then flooded with Coomassie brilliant blue solution and kept for ten minutes and drained off completely. The plates were flooded with 40% methanol and 10% of acetic acid for 20 minutes. The clear zones were visualized as a zone of clearance around the isolate against blue back ground (Augusta *et al.,* 1993).

b) Determination of hydrophobicity (BATH) Test:

To estimate the bacterial cell surface hydrophobicity that can be directly related to the ability to form an effective biofilm over any hydrophobic surfaces which was considered to be the initial and an important step in the process of degradation of LDPE. Hence Bacterial cell surface hydrophobicity (Bacterial adhesion to hydrocarbon) was determined by the BATH test using a standard protocol (Rosenberg *et al.,* 1980).

3. Results:

Three compost soil samples were collected, out of which 8 *Actinomycetes* were isolated.

Identification and characterization of *Actinomycetes:*

a) Aerial mass colour:

The colour of the substrate mycelium was determined by observing the plates after 7 to 10 days. It was done only after seeing the heavy spore mass surface on *Actinomycetes* agar plates. Table 1

Table 1: Aerial mass colour of the isolates

Isolates	Aerial mass colour
Isolate 1	Greyish black
Isolate 2	Greyish black
Isolate 3	White
Isolate 4	Greenish brown
Isolate 5	White
Isolate 6	Whitish grey
Isolate 7	Orange
Isolate 8	Orange

b) Reverse side and melanoid pigments:

The strains were divided into two groups according to their ability to produce pigments on the reverse side of the colony, namely distinctive (1) and not distinctive or none (0). Reverse side pigments and melanoid pigmentation was observed by the formation of greenish brown, brownish black or distinct brown pigment. Colour for not distinctive were pale yellow, olive or yellowish brown were marked as 0. Observation of reverse side pigments, melanoid pigments and not distinctive pigments produced by *Actinomycetes* on plates were recorded in Table 2.

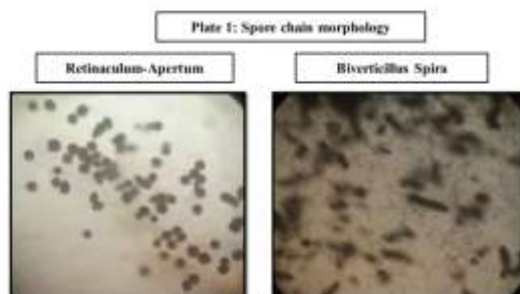
Table 2: Reverse side and melanoid pigments

Isolates	Melanoid pigments	Reverse side pigments	Soluble pigments
Isolate 1	0	0	1
Isolate 2	0	1	0
Isolate 3	0	0	1
Isolate 4	0	0	1
Isolate 5	0	0	1
Isolate 6	0	1	0
Isolate 7	1	1	0
Isolate 8	1	0	1

C) Spore chain morphology:

The slides were examined under the microscope. Spira was shown by maximum strains, only isolate 3 has shown the simple rectus spore chain and isolate 5 has shown Biverticillus-spira spore chain and two strains were shown Retinaculum-Apertum (RA).(Plate 1)

ISSN 2455-6378



Colony Morphology and characterization of Actinomycetes:

Five different *Actinomycetes* were identified and characterized by 16S rRNA sequencing as per the standard methods, which included *Streptomyces exfoliatus*, *Actinomyces slackii, Brevibacteriaceae bacterium, Nocardiopsis alba* and *Actinomyces marimammalium.*

a) *Streptomyces exfoliatus:*

Streptomyces are gram positive, spore-forming bacteria found in soil. They are characterized by their tough, leathery, frequently pigmented colonies and their filamentous growth. They are small, opaque, compact, frequently pigmented (brown, yellow, pink, etc.), often leathery, and appear dry and dull looking.

b) Actinomyces slackii:

They are gram-positive, non-motile, non-sporulating rods of 0.8 pm long by 0.56 pm wide. Catalase positive. Growth is stimulated by carbon dioxide in liquid media. Acid end products are acetic and lactic acids, with traces of succinic acid. The strains fail to hydrolyze esculin. Acid is produced from raffinose, salicin, and glucose. No acid is produced from L-arabinose, amygdalin, celobiose, inositol, D-mannitol, L-rhamnose, glycerol, sorbitol, melezitose, or D-xylose.

c) Brevibacteriaceae bacterium:

They are gram-positive, non-acid fast, non-spore-forming, aerobic, catalase positive, and oxidase-negative, non-motile.

d)Nocardiopsis alba:

It forms tan, peaked, circular, erose, rough, and opaque with aerial mycelia that develop over time. Cells are gram positive, acid fast positive, branched, and filament like.

e) *Actinomyces marimammalium:*

They are gram-positive, non-acid-fast and non-motile. Colonies are grey, entire, circular, convex and pin-point to 0±5 mm in diameter. Non-haemolytic. facultatively anaerobic and catalase-negative. Acid is produced from d-glucose, maltose and lactose. Acetoin is not produced. Nitrate is not reduced to nitrite. The end products of glucose metabolism are acetic, lactic and succinic acids.

Optimization of growth parameters.

The optimum temperature, pH & carbon source for the maximum growth of *Actinomycetes* were as follows: - 30°C, neutral pH and 0.5% of LDPE.

Screening for polyethylene degrading *Actinomycetes*

The characterized *Actinomycetes* were subjected to screening assay to select the better isolate to be involved in degradation.

1) Clear zone assay:

Initial screening of biodegradation of LDPE of *Actinomycetes* was done by clear zone assay - a semi quantitative methods was performed, it was observed that, out of 8 *Actinomycetes* strain 5 strains showed a maximum zone of clearance indicating their ability to depolymerise the polymer suggesting them to be the better isolates for the biodegradation of LDPE. (Plate 2)

Plate 2: Clear Zone Assay

2) Determination of hydrophobicity test (BATH):

BATH test was performed to determine the hydrophobicity; it was observed out of 8 strains, isolate number 4 and 6 showed maximum decrease in their level of hydrophobicity. (Table3)

Table 3: BATH Test

Isolates	OD value before adding Hexadecane	OD value after adding Hexadecane	Percentage of microbes adhering to hydrocarbons
1	0.013	0.009	31%
2	0.013	0.011	15%
3	0.025	0.017	32%
4	0.044	0.022	50%
5	0.030	0.018	40%
6	0.023	0.012	48%
7	0.022	0.020	9%
8	0.012	0.012	0%

4. Discussion:

Polyethylene (PE) is one of the synthetic polymers of high hydrophobic level and high molecular weight. It is not easily degradable in its natural form. Therefore their use in the production of disposal or packaging materials causes dangerous environmental problems. Biodegradation requires certain modifications in their crystalline level, molecular weight and mechanical properties (Imam and Gould, 1990). This can be achieved by improving their hydrophilic level and reducing its polymer chain length to improve accessibility to microbial degradation (Orr *et al.,* 2004).

Biodegradation is defined as any physical or chemical change in a material caused by biological activity. Microorganisms like *Actinomycetes* are involved in the degradation process (Gu *et al.,* 2000). *Actinomycetes* are found in terrestrial habitat and widely distributed in a variety of other habitats lake bottoms, river muds, compost (Alexander, 1977). They are distributed microorganisms in most soils. The majority of *Actinomycetes* are free living saprophytic bacteria found widely distributed in soil water and colonising plants. *Actinomycetes* population has been identified as one of the major group of soil population (Kuster, 1968), which may vary with the soil type.

Actinomycetes possess many properties that make them good candidate for application in degradation of plastics in soils, contaminated with organic pollutants. They produce extracellular enzymes that degrade a wide range of complex organic compounds; in addition, the frequently occurring filamentous growth favours the colonization of soil particles (Ensign, 1968). Another interesting feature within this group of microorganisms, especially in the biodegradation of hydrophobic substance is their surfactant producing activity (Rapp *et al.,* 1979). Many *Actinomycetes* are known hydrocarbon degraders (Johnston and Cross, 1976), and also represents the dominant group among degraders (Vasile, 2000).In this present study, it was found that *Actinomycetes* were isolated in abundance accounting to be the soil microflora.

Augusta *et al.,*(1993) reported that the extracellular hydrolysing enzymes secreted by the target organism to hydrolyze the suspended polyethylene in the turbid agar medium into water soluble products thereby producing zones of clearance around the colony indicates that the organisms are at least able to depolymerise the polymer, which is the first step of degradation. This method is usually done to screen the organism that can degrade a certain polymer. In our study, out of 8 *Actinomycetes* strain 5 strains showed a maximum zone of clearance indicating their ability to depolymerise the polymer suggesting them to be the better isolates for the biodegradation of LDPE.This was found to be in consistent with the previous results.

Investigated role of microbes to attack the polyethylene in disposed polyethylene bags and suggested that *Actinomycetes* were found to be more efficient in degrading through biofilm formation which attributes to gradual decrease in hydrophobicity of its surface (Hadad *et al.,* 2005) Cell surface hydrophobicity plays a very important role in biofilm formation which in turn serve as a mechanism in reducing the hydrophobicity thereby enhancing the microbial adherence onto polyethylene film. This adherence ability is the initial and important step in the mechanism of degradation. Once it gets attached to the surface, it starts growing by using the polymer as the carbon source leading to further degradation (Kathiresan, 2003). Hence, this

microbial adherence paves a way for degradation so they both are directly proportional. This was found to be in agreement with our study which showed adherence of *Actinomycetes* onto LDPE sheets.

5. Conclusion:

LDPE can be biodegradable if the right microorganisms are isolated.It was proved that the hydrophobic LDPE film can act as a substratum for some group of organism which formed biofilm on the LDPE film. Moreover the isolates grew on minimal medium containing only LDPE as the carbon source even without any nitrogen source. Therefore, degrading the polyethylene by using the *Actinomycetes* that completely degrade the recalcitrant LDPE compound by providing right conditions in the right environment.

Acknowledgements:

We wish to thank the help and encouragement rendered by Ms. Summera Rafiq, HOD and Associate Professor and Dr. SK. Jasmine Shahina, Associate Professor, Department of Microbiology, JBAS College for Women towards this work.

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