ISOLATION OF MICROORGANISMS CAUSING BIODEGRADATION OF PLASTICS

Thesis submitted in partial fulfillment of the requirement for the award of degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

By

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CERTIFICATE

This is certified that the thesis entitled **"Isolation of microorganisms causing biodegradation of plastics",** is an authentic record of my own work carried out as requirements for the award of degree of M.Sc. (Biotechnology) at Thapar University, Patiala, under the guidance of Dr. Pramod K. Bajpai (Distinguished Professor, ChED) and Dr. Haripada Bhunia (Assistant Professor, ChED) during January to June 2011.

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ABSTRACT

The present study describes the isolation of microorganisms from compost with the ability to degrade BOPP film. Several bacterial strains and actinomycetes strains were isolated by three techniques: i. by compost burial method; ii. by incubating BOPP film in synthetic media; and iii. isolation from air. Bacterial strains attached on the BOPP film, after compost burial for 45 days, were found to be Gram positive, rod shaped bacteria and identified as *Bacillus* sp. Actinomycetes strains isolated were showing Gram positive, rod shaped character. All the isolated strains (bacteria and actinomycetes) were screened for their ability to degrade BOPP films in synthetic media. As the growth of microbes proportionally increased in synthetic media so it was predicted that the microbes were solely dependent on BOPP films for its carbon source. Biodegradation of polymer films was evaluated by percent weight loss of polymer after degradation and Fourier transform infrared spectroscopy. Percent weight loss of polymer after degradation was found to be higher in compost burial method (8.2%) than culturing in synthetic media (2.01%). Fourier transform infrared spectroscopy confirmed the degradation of the polymer.

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LIST OF ABBREVIATIONS

ATR-FTIR	Attenuated total reflectance- Fourier transform infrared spectroscopy
APP	Atactic polypropylene
B.O.D	Biological oxygen demand
BOPP	Bi-axially oriented polypropylene
DMTA	Dynamic Mechanical Thermal Analysis
DSC	Differential scanning calorimetry
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography-mass spectrometry
GPC	Gel permeation chromatography
HDPE	High density polyethylene
IPP	Isotactic polypropylene
IR	Infrared spectroscopy
kT	kilo tones
LDPE	Low density polyethylene
LLDPE	Linear, low density polyethylene
NA	Nutrient agar
NB	Nutrient broth
NMR	Nuclear magnetic resonance spectroscopy
PCL	Polycaprolactone
PE	Polyethylene
PET	Polyethylene terepthalate
PLA	poly(lactide)
PP	Polypropylene
PS	Polystyrene
PU/PUR	Polyurethane
PVC	Polyvinyl chloride
SEM	Scanning electron microscopy
SM	Synthetic media
TGA	Thermo gravimetric analysis

CHAPTER-1

INTRODUCTION

Approximately 140 million tons of synthetic polymers are produced worldwide every year. Since polymers are extremely stable, their degradation cycles in the biosphere are limited. In Western Europe alone it is estimated that 7.4% of municipal solid waste are plastic, which are classified as 65% polyethylene/polypropylene, 15% polystyrene, 10% PVC, 5% polyethylene terephthalate and remaining others. Environmental pollution by synthetic polymers, such as waste plastics and water-soluble synthetic polymers in waste-water has been recognized as a major problem. In view of this, energetic, chemical and biological polymer-degrading techniques have been studied extensively during the last three decades. The energetic agencies can be either thermal or radiant. The radiant energy may be high-energy radiation like gamma rays, ion beams, and electrons or even low energy radiation like ultra-violet (UV) rays. Chemical degradation is caused using certain chemicals like acids and alkalis, etc. Usage of certain microorganisms and enzymes to degrade polymers are classified as the biodegradation method of polymers (Premraj and Doble, 2005).

The word plastic comes from the Greek word “plastikos”, which means ‘able to be molded into different shapes’ (Joel, 1995). They are natural and synthetic macromolecules composed of smaller units called monomers that are bonded together. Examples of natural polymers include proteins, polysaccharides and nucleic acids (Chandra and Rustgi, 1998). Synthetic polymers have been developed for durability and resistance to all forms of degradation (Chiellini *et al*, 2003). These characteristics and others, such as rigidity, permeability and transparency can be controlled by changing the polymer synthesis, molecular weight and/or by the use of specific additives. The resulting plastics’ versatility allows them to be used in a very wide range of applications, including agriculture.

Plastics are divided into two groups: thermoplastics and thermoset plastics (Alauddin *et al.*, 1995). In thermoplastics, the atoms and molecules are joined end-to-end into a series of long, sole carbon chains. These long carbon chains are independent of the others

(Allen *et al.*, 1999). This kind of structure in which the backbone is solely built of carbon atoms makes thermoplastics resistant to degradation or hydrolytic cleavage of chemical bonds. Consequently, thermoplastics are considered non-biodegradable plastics. Distinguished from the linear structure of thermoplastics, thermoset plastics have a highly cross-linked structure (Alauddin *et al.*, Scott, 1999). Since the main chain of thermoset plastics is made of heteroatoms, it is possible that they are potentially susceptible to be degraded by the hydrolytic cleavage of chemical bonds such as ester bonds or amide bonds (Muller *et al.*, 2001).

Thermoplastics are widely used in packaging and fabrication of bottles and films (Table 1.1). The major types of thermoplastic material include linear, low density polyethylene (LLDPE), high density polyethylene (HDPE), polyvinyl chloride (PVC), low density polyethylene (LDPE), polypropylene (PP), polystyrene (PS) and other resins. Thermoset plastics include a) polyester, one of which is polyethylene terephthalate (PET); and b) polyurethane (PUR) (Avella *et al.*, 2001).

TABLE 1.1 Main plastics and their applications

Plastics	Applications
Low density polyethylene (LDPE), linear low density polyethylene (LLDPE), polyvinylchloride (PVC).	Films and Packaging
Polyethylene terephthalate (PET), PVC, high density polyethylene (HDPE).	Bottles, tubes, pipes, insulation molding
Polystyrene (PS), polypropylene (PP), PVC.	Tanks, jugs, containers
LDPE, LLDPE	Bags
Polyurethane (PUR).	Coating, insulation, paints, packing

Polypropylene (PP) and polyethylene (PE), expressed as C_nH_{2n}, are the most widely used linear hydrocarbon polymers. The versatility of these polymers arises from the fact that they are made from cheap petrochemical feed stocks through efficient catalytic

polymerization process and their ease of processing to various products. The range of their applications include; food packaging, textiles, lab equipments and automotive components. PP has a methyl group instead of one of the hydrogens present in PE, on every other carbon, which gives rise to the existence of three steroisomeric forms namely, atactic, isotactic, and syndiotactic. This stereo-regular polymer was first synthesised by Zieglar and Natta with propylene as the monomer. Mettalocene catalysts can also be used for its synthesis (Arutchelvi *et al*, 2008). Commercial polypropylene (PP) is predominantly isotactic. Isotactic polypropylene (IPP) is a stiff, highly crystalline polymer that has the lowest density of the major plastics and possesses a very high strength-to weight ratio. It has a crystalline melting point of 165–175°C. IPP has good tensile strength, modulus, hardness, stiffness, and so forth and is mainly used as a molding material. Because of its hardness, it is blended with elastomers and used in many applications. Atactic polypropylene (APP) is predominantly amorphous, semi tacky, and intermediate between a wax and a rubber. It swells extensively in aliphatic and aromatic hydrocarbons at room temperature. APP is used in conjunction with bitumen in coating compounds for roofing materials and sealing strips, to which it confers improved aging properties, and in road construction, in which it improves the stability of asphalt surfaces.

During the past 3-decades, plastic materials have been increasingly used in food, clothing, shelter, transportation, construction, medical and recreation industries. Plastics are advantageous as they are strong, light-weight, inexpensive, easily process able, and durable. However, they are disadvantageous as they are resistant to biodegradation, leading to pollution, harmful to the natural environment. Because of their durability and visibility in liter, plastics (polymers) have attracted more public and media attention than any other component of the solid waste stream. The plastic waste stream emerges from domestic, industrial and municipal refuse (Jayasekara *et al*, 2005).

The increasing problems posed by waste management of packaging materials have stimulated interest in biodegradable materials. Indeed, biodegradation is an efficient and rapid way for eliminating some plastic wastes under composting and land filling conditions (Massardier - Nageotte *et al.*, 2006).

The term “biodegradable plastics” normally refers to an attack by microorganisms on non-water soluble polymer-based materials (plastics). This implies that the biodegradation of plastics is usually a heterogeneous process. Because of a lack of water-solubility and the size of the polymer molecules, microorganisms are unable to transport the polymeric material directly into the cells where most biochemical processes take place; rather, they must first excrete extracellular enzymes which depolymerize the polymers outside the cells (Figure 1.1). As a consequence, if the molar mass of the polymers can be sufficiently reduced to generate water-soluble intermediates, these can be transported into the microorganisms and fed into the appropriate metabolic pathway(s). As a result, the end-products of these metabolic processes include water, carbon dioxide and methane (in the case of anaerobic degradation), together with a new biomass. The extracellular enzymes are too large to penetrate deeply into the polymer material, and so act only on the polymer surface; consequently, the biodegradation of plastics is usually a surface erosion process (Muller, 2005).

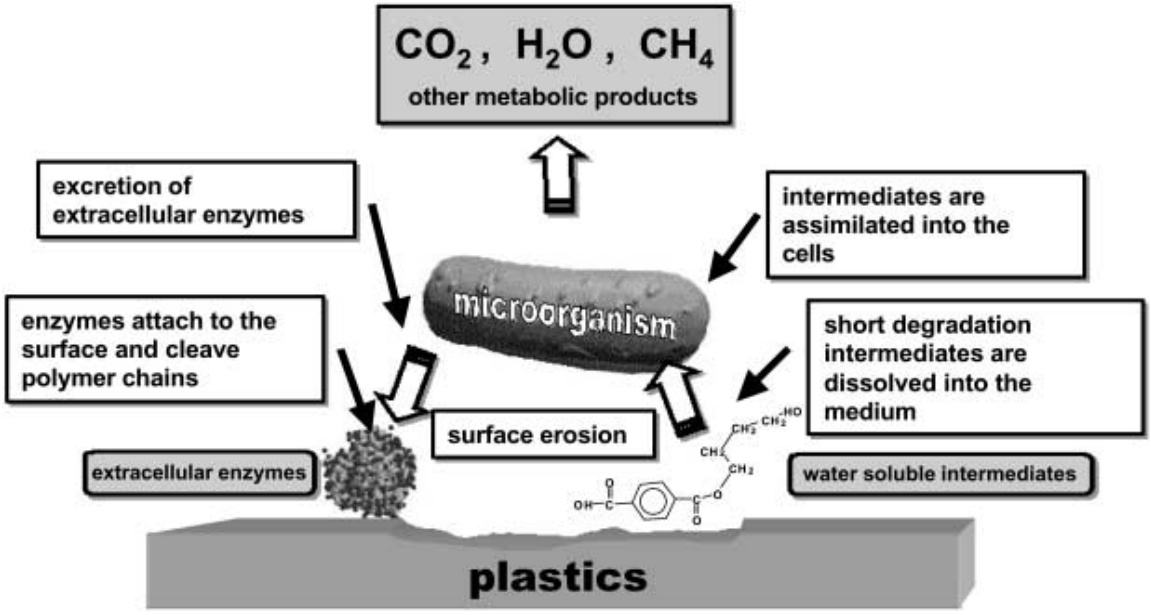


Fig 1.1 General mechanism of plastics biodegradation (Muller, 2005)

Although the enzyme-catalyzed chain length reduction of polymers is in many cases the primary process of biodegradation, non-biotic chemical and physical processes can also act on the polymer, either in parallel or as a first stage solely on the polymer. These non-

biotic effects include chemical hydrolysis, thermal polymer degradation, and oxidation or scission of the polymer chains by irradiation (photo degradation). For some materials, these effects are used directly to induce the biodegradation process [e.g., poly (lactic acid); pro-oxidant modified polyethylene], but they must also to be taken into account when biodegradation is caused predominantly by extracellular enzymes. Because of the co-existence of biotic and non-biotic processes, the entire mechanism of polymer degradation could - in many cases - also be referred to as environmental degradation (Muller, 2005).

Environmental factors not only influence the polymer to be degraded, they also have a crucial influence on the microbial population and on the activity of the different microorganisms themselves. Parameters such as humidity, temperature, pH, salinity, the presence or absence of oxygen and the supply of different nutrients have important effects on the microbial degradation of polymers, and so these conditions must be considered when the biodegradability of plastics is tested (Muller, 2005).

The physical and chemical structures of the polymers are the basic properties that affect the degradation and the biodegradation. The biodegradability of polymers depends primarily not only on their molecular structure but also on: the length of the polymer chain (it seems that the shorter the chain, the easier the degradation), the crystallinity of the polymer (crystalline parts are more difficult to degrade than amorphous parts) and the more complex the formula, the less degradable because several microorganisms are required to attack the different functions of the polymer (as an example polymers containing rings seem to be more difficult to degrade) (Massardier - Nageotte *et al*, 2006).

Other parameters that influence the biodegradability of polymers are: conditions and properties of the test systems such as volume and shape of the vessels, open or closed bottles, temperature, way of mixing or shaking and oxygen supply and test duration. If the polymer is water soluble, it is easier to degrade. Indeed, water is favourable to the development of microorganisms (Massardier - Nageotte *et al.*, 2006).

The successful production and marketing of biodegradable plastics will help alleviate the problem of environmental pollution. In the past 10 years, several biodegradable plastics have been introduced into the market. However, none of them is efficiently biodegradable in landfills. For this reason, none of the products has gained widespread use (Anonymous l999). Hence, there is an urgent need to develop efficient microorganisms and their products to solve this global issue.

CHAPTER 2

LITERATURE REVIEW

The consumption of plastics in the year 2006 in India was around 4545 kT, and out of this polypropylene (PP) accounted for 1200 kT. The consumption of thermoplastics was 40 million tons in European countries (Plastemart.com website, 2004). These figures give the scenario of the alarming rate at which plastic will be dumped in the environment after its useful life. Natural weathering, which includes solar radiation, UV rays and ambient temperature, affect the properties of the polymer to some extent, but generally these changes take place at a slow rate (Suits and Hsuan, 2003). Biodegradation is the result of the utilization of the polymer as a carbon source by the microorganisms. This process is facilitated if the microorganism initially forms a biofilm over the polymer surface (Hadad *et al*., 2005). In the case of polymers such as PP, a continuous chain of repetitive methylene units makes it resistant to degradation. The hydrophobic nature of PP hinders the attachment of microorganism on its surface. Studies reported on biodegradation of PP are given in Table 2.1 (Arutchelvi *et al*., 2008).

Table 2.1 Various literature reports on biodegradation of polypropylene and its blends.

Title of the paper	Polymer	Organism	Conditions	Analytical techniques	Observation	Reference
Isotactic PP biodegradation by microbial community	Isotactic PP	Microaerophilic microbial community	Mineral medium containing Na lactate & glucose	IR, NMR, GC-MS	Organism & mycelia with known adaptability & metabolic flexibility can degrade isotactic PP	Cacciari *et al.*, 1993
UV-Irradiated biodegradability of ethylene-propylene copolymers	Ethylene-propylene copolymer	Fungal species	Composting & culture environment	FTIR, SEM, VISCOSITY	Viscosity decrease & increase in carbonyl/ hydroxyl region	Pandey & Singh, 2001

					in FTIR	
Biodegradation of γ-sterilized biomedical polyolefins blends	Isotactic PP	Fungal species	Composting & culture environment	FTIR, SEM, VISCOSITY	Viscosity decrease & increase in chain scission	Sameh *et al*., 2006
Calorimetric & thermo gravimetric studies of UV-irradiated PP/ starch based materials aged in soil	PP/ starch	Soil	Soil burial tests	DSC and TGA	Biodegradation not affects the thermal stability, photo oxidation decrease the thermal stability of the mixture	Morancho *et al*., 2006
Effect of short wavelength UV- irradiation on ageing of PP/ cellulose compositions	PP/ cellulose	Soil	Composted in garden soil	ATIR-FTIR, TENSILE & SEM	Significant mechanical and surface changes found	Kaczmarek *et al*., 2005
Mechanical behavior of biodegradable polyolefins	(HDPE)/ PP	Soil	Soil burial tests	DMM, Viscoelastic & DSC	A significant change in mechanical behavior observed	Jakubowicz , 2003
Structure & properties of degradable polyolefin-starch blends	Polyolefin-starch	*Phanerochaete chrysoporium*	Liquid fungus culture & soil burial test	Tensile DMTA, GPC, intrinsic, FTIR & optical microscopy	Increased susceptibility to biodegradation	Zuchowska *et al*., 1998
Enzymatic degradation of plastics containing PCL.	PCL/ PP	*Rhizopus arrhizus* lipase	Enzymatic condition	SEM & Spectrometric	Blends of PCL and LDPE or PP retained high biodegradability of PCL	Iwamoto & Tokiwa, 1994

Thermal degradation of PP/ starch based materials with enhanced biodegradation	PP/ starch based materials	Soil	Soil burial tests	TGA, FTIR	Biodegradability observed more in starch based material rather than PP matters	Ramis *et al*., 2004
Characterization by thermal analysis of HDPE/PP blends with enhanced biodegradation	Blends of HDPE/ PP with different biodegradable additives	Soil	Soil burial tests	TG, DSC and dynamic-mechanical spectroscopy	Additive more affected by degradation than the polymeric matrix. Changes both in the crystalline morphology and activation energies of relaxation processes happens at different time & depends on the additives used	Contat-Rodrigo *et al*., 2002

As evident from the table, the work carried out on this area is scarce. Apart from fungal species (*Aspergillus niger*), microbial communities such as the species of *Pseudomonas* and *Vibrio* have been reported to biodegrade PP (Cacciari *et al.*, 1993). A decrease in viscosity and formation of new groups namely carbonyl and hydroxyl were observed during the degradation process (Sameh *et al*., 2006, Iwamoto & Tokiwa, 1994). Except for one report (Cacciari *et al.,* 1993), all the studies deal with degradation of pretreated PP. The pretreatment techniques reported include UV- irradiation (Huang *et al*., 2005; Kaczmarek *et al.*, 2005 and Sameh *et al*., 2006), γ- sterilization (Iwamoto & Tokiwa, 1994) and thermal treatment (Abd El-Rehim *et al*., 2004). These pretreatments either decrease the hydrophobicity of the polymer thereby making it more compatible with the organism or introduces groups such as C=O (carbonyl) or –OH (hydroxyl), which are

more prone to degradation. It is reported that UV- treated PP sample is more susceptible to degradation than LDPE (Sameh *et al*., 2006). Biodegradation of polypropylene/starch or polypropylene/cellulose blends has been reported using soil organisms. It is observed that the organisms easily degrade starch or cellulose leaving behind the polymer (Albertson *et al.,* 1995, Shah *et al.,* 2008, Ramis *et al.,* 2004). These carbohydrates or fillers increase the adhesion of the organisms to the surface of the polymer. Polycaprolactone (PCL) blended PP has also been reported to degrade in the presence of lipase (Weiland *et al*., 1995). PCL is an ester and since lipase is well known to degrade ester linkages, degradation of this polymer is facile. Lipase cannot affect the carbon-carbon present in PP. There are no reports available on the effect of tacticity on the nature and rates of biodegradation as well as on the use of marine organisms to achieve biodegradation. Isotactic polypropylene exposed to bacterial consortia for 175 days had 40% methylene chloride extractable compounds, and this extract was identified to be a mixture of hydrocarbons (between $C_{10}H_{22}$ and $C_{31}H_{64}$) (Cacciari *et al*., 1993). Thirty to sixty percent growth of *A. niger* was observed on gamma irradiated PP films at the end of 6 weeks, which indicated that the fungus was able to utilise this polymer as its sole carbon source (Alariqi *et al*., 2006).

Though polyolefins are quiet resistant to acids and bases it was found to be oxidized by concentrated sulphuric acid (Neu, 1996). Sulphuric, chromic and nitric acids are reported to oxidize PP at high concentrations. The strong oxidizing agent like Fenton's reagent which can generate OH radical can also be used to oxidize a polymer. This treatment is widely used in the bioremediation of dyes and in waste water treatment plants.

Unlike polypropylene, more research articles are published on studies relating to biodegradation of polyethylene. Fungi that include *A. niger*, *Penicillium funiculosum*, *Fusarium redolens* and *A. vesicolor*, and soil microorganisms (mixed culture as well as *Rhodococcus rhodochorus*, *Cladosporium cladosporoides*) have been reported to degrade neat PE (Albertson *et al.,* 1995, Shah *et al.,* 2008, Ramis *et al.,* 2004). DSC or FTIR and other mechanical and physical techniques such as weight loss, changes in tensile strength have been the commonly used analytical techniques to monitor the nature of

biodegradation. Thermal, UV, photo and corona treated PE has been found to degrade faster than the untreated polymer. Biodegradation of starch blended and modified PE with protein hydrolysate has also been studied (El-Shafei *et al.*, 1998).

Biodegradation resulting from the utilization of polyethylene as a nutrient (that is, a carbon source) may be more efficient if the degrading micro-organism forms a biofilm on the polyethylene surface. However, the hydrophobicity of the polyethylene interferes with the formation of a microbial biofilm. Attempts to facilitate colonization of polyethylene by adding nonionic surfactants to the culture medium promoted the biodegradation of polyethylene (Albertsson *et al*. 1993; Ehara *et al*. 2000). Albertsson and Karlsson (1990) found that polyethylene subjected to 26 days of artificial UV irradiation before being buried in soil evolved less than 0.5% carbon (as CO_2) by weight after 10 years. Without prior irradiation, less than 0.2% carbon dioxide was produced. Earlier reports have shown that no signs of deterioration could be observed in polyethylene sheet incubated in moist soil for 12 years (Potts and Jelinek, 1978), and only partial degradation was observed in a polyethylene film buried in soil for 32 years (Otake *et al*., 1995). Nevertheless, several studies have demonstrated partial biodegradation of polyethylene after UV irradiation (Cornell *et al*., 1984), thermal treatment (Huang *et al*., 1990; Kwpp and Jewell, 1992) or oxidation with nitric acid (Mochizuki *et al*., 1999). It is reported that there is a synergistic effect between photo-oxidation and biodegradation of polyethylene (Albertsson *et al*., 1987). An increase in the biodegradation of polyethylene was observed with increase in the time of exposure to UV (Hadad *et al*., 2005).

Gilan *et al*. (2004) isolated a strain of *Rhodococcus ruber* that was found to colonize and degrade polyethylene. The ability of this bacterium to form a biofilm on polyethylene was attributed to the hydrophobicity of its cell surface. Addition of a small amount of mineral oil to the culture medium increased both biofilm formation and the subsequent biodegradation of the polyethylene, presumably by increasing the hydrophobic interactions between the bacterial biofilm and the polymer (Gilan *et al*., 2004). In another study, Hadad *et al*. (2005) isolated a thermophilic bacterial strain, *Bravibacillus borstelensis*, which was found more effective in degrading branched low-density

polyethylene than the *Rhodococcus* strain. Watanabe *et al*. (2009) isolated and identified three types of low-density polyethylene (LDPE) degrading microbes *Bacillus circulans*, *Bacillus brevies*, and *Bacillus sphaericus*, by soil burial method. Different fungal strains for example, *Mucor rouxii* NRRL 1835 and *Aspergillus flavus* (El-Shafei *et al*., 1998), *Penicillium simplicissimum* YK (Yamada-Onodera *et al*., 2001), and *Phanerochaete chrysosporium* (Iiyoshi *et al*., 1998), have been reported as degrading PE.

Microorganisms capable of degrading other polymers like polystyrene (PS), polycaprolactone (PCL), polyurethane (PU), polyamides (nylons), poly(lactide) (PLA) etc. have also been reported. Polystyrenes have been found to be degraded by fungal cultures like *Trichoderma* sp. and *P. pullulans*; when carbohydrate molecules like glucose, sucrose and lactose were linked to polystyrene-maleic anhydride (Galgali *et al.,* 2004). It was shown in this study that structural features can be incorporated to non-biodegradable polymer to induce biodegradability. *Streptomyces halstedii, Bacillus megaterium, Sphingobacterium spiritivorum, Bacillus cerus* capable of degrading styrene, were isolated from the bed of an experimental biofilter purifying exhaust gases from a cable factory's coil-wire varnishing division (Przybulewska *et al.*, 2006).

Crabbe *et al*. (1994) isolated four fungal species, *Curvularia senegalensis, Fusarium solani, Aureobasidium pullulans* and *Cladosporium* sp., from soil and found to degrade ester-based polyurethane. Nylon is produced in large quantities as fibers and plastics all over the world but they are non-biodegradable due to its strong inter-molecular cohesive forces caused by hydrogen bonds between molecular chains. A thermophillic bacterium, *Geobacillus thermocatenulatus,* capable of degrading nylon 12 and nylon 66, was isolated from soil by enrichment culture technique at 60°C (Tomita *et al.*, 2003). Most of the PLA-degrading microorganisms phlogenetically belong to the family of *Pseudonocardiaceae* and related genera such as *Amycolatopsis*, *Lentzea, Kibdelosporangium, Streptoalloteichus* and *Saccharothrix* (Tokiwa and Calabia, 2006).

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Compost

The mature compost (municipal solid waste) was obtained from a compost plant, New Delhi Municipal Council, Okhla, New Delhi, India. The compost inoculum was free from larger inert materials (glass, stones, metals, etc.) as much as possible. These items are removed manually as much as possible to produce a homogenous compost inoculum. The compost had the following basic properties: total solids (%TS) 81%; volatile solids at 550°C (%VS) 18%; pH 7.2; C/N ratio 15.3. It was used for isolation of polymer degrading microorganisms.

3.1.2 BOPP films (Bi-axially oriented polypropylene films)

BOPP films (Transparent Plain one side corona treated suitable for Printing and Lamination shown in figure 3.1) of thickness 18µm were obtained from Max Specialty Products which was having following features.

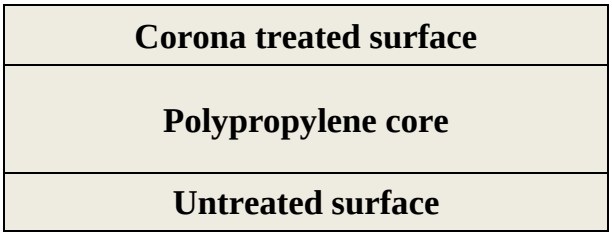


Figure 3.1 Structure of BOPP

i) Features of BOPP films

- Outstanding clarity and gloss.
- Good stiffness and mechanical properties.
- Good printability and suitable for lamination with other substrates.
- Good barrier to moisture.

3.1.3 Media for cultivation and degradation experiments

Nutrient broth (Table 3.1) and nutrient agar (Table 3.2) were obtained from HIMEDIA Laboratories Ltd. Minimal synthetic media (SM), devoid of any carbon source was used for degradation experiments (Table 3.3). Media sterilization was performed by autoclaving at 121ºC and 15 lbs pressure for 15 minutes.

Table 3.1 Composition of Nutrient broth

Chemicals	**Quantity (g/l)**
Peptic digest of animal tissue	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50

Table 3.2 Composition of Nutrient agar

Chemicals	**Quantity (g/l)**
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Agar	15.00

Table 3.3 Composition of synthetic media

Chemicals	Quantity
NH_4NO_3	1.0 g/l
$MgSO_4.7H_20$	0.2 g/l
K_2HPO_4	1.0 g/l
$CaCl_2.2H_2O$	0.1 g/l
KCl	0.15 g/l
Yeast extract	0. 1 g/l
$FeSO_4.6H_2O$	1.0 mg/l
$ZnSO_4.7H_2O$	1.0 mg/l
$MnSO_4$	1.0 mg/l

3.2 Methodology

3.2.1 Isolation of polymer degrading microorganisms

The aim was to isolate the microorganisms which can degrade the synthetic polymer. The polymer used was BOPP. The polymer degrading microorganisms were isolated from compost and air.

3.2.1.1 Composting

BOPP films were cut into 3cm×3cm size, weighed, disinfected in 70% ethanol for 30 minutes and air-dried for 15 minutes in laminar air flow chamber. Three such films were buried in 600g of compost taken in a beaker. It was kept in B.O.D. incubator (Model NSW-152 of Narang Scientific Works Pvt. Ltd., India) at 37ºC for 45 days. Moisture conditions and aerobic conditions were maintained manually as much as possible for controlled composting.

i) Isolation of polymer degrading microbes

After 45 days, BOPP films were taken from compost, remove the loosely attached material, rinsed with sterilized distilled water and placed on nutrient agar plates, at 37ºC for few days to observe the microbial growth.

During rinsing with sterilized water, some polymer degrading microbes are also washed off. So, to procure them, rinsed water was also spreaded on NA plates.

3.2.1.2 Biodegradation in synthetic media with polymer as a carbon source

Biodegradation tests were performed with samples of BOPP films (3×3 cm) that had been dried overnight at 60°C, weighed, disinfested (30 min in 70% ethanol), air-dried for 15 minutes in Laminar air flow chamber and added to flasks, each containing 100 ml of SM (three BOPP films). Flasks were inoculated with 1 ml of compost suspension. Then cultures were incubated on a rotary shaker (New Brunswick Scientific, USA, Excella E-24 model) at 37ºC and 130 rpm for 2 months. Each test consisted of three flasks (replicates).

Synthetic medium, devoid of any carbon source, was used as general medium for isolation of strains capable of growing on polymer as the sole carbon source. BOPP films added in SM were the only source of carbon and energy required for growth of microorganisms.

After 2 months, BOPP films were taken from media, rinsed with sterilized distilled water and placed on nutrient agar plates, at 37ºC for few days to observe the microbial growth.

3.2.1.3 Biodegradation in air using synthetic media

BOPP films were added in SM taken in petriplates and were kept in the open in different surroundings in order to collect polymer degrading microorganisms from air. BOPP film added in SM was the only source of carbon and energy required for growth of microorganisms for 2 months.

After 2 months, BOPP films were taken from media, rinsed with sterilized distilled water and placed on nutrient agar plates, at 37ºC for few days to observe the microbial growth.

3.2.2 Identification of Bacterial isolates

The identification of Bacterial isolates with the ability to degrade BOPP films was performed on the basis of macroscopic and microscopic examination. The bacterial isolates were identified macroscopically by examining colony morphology; surface pigment, shape, size, margin, surface on nutrient agar plates and microscopic examination including; Gram's staining, to study the staining behavior, shape and cell arrangement; and spore staining.

i) Gram staining

Gram staining is used to determine Gram status to classify bacteria broadly. It is based on the composition of their cell wall. Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall), which stains purple while Gram-negative bacteria have a thinner layer (10% of cell wall), which stains pink. Gram staining uses crystal violet to stain cell walls, iodine (as a mordant), and safranin (counter stain) to mark all bacteria. Gram status is important in medicine; the presence or absence of a cell wall change the bacterium's susceptibility to some antibiotics.

3.2.3 Determination of dry weight of residual polymer

A simple and quick way to measure the biodegradation of polymers is by determining the weight loss. Microorganisms 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 since biodegradation usually is initiated at the surface of the polymer.

To facilitate accurate measurement of the weight of the residual polyethylene, the bacterial biofilm was washed off the polymer surface with a 2% (v/v) aqueous sodium

dodecyl sulphate solution for 4 h and then with distilled water. The washed polymer film was placed on a filter paper and dried overnight at 60^0C before weighing.

3.2.4 Growth kinetics of different microbes in Nutrient broth

Growth pattern of all the 9 strains isolated from compost was seen in Nutrient broth. Growth of microbes at different time intervals was tested by taking growth O.D in U.V-VISIBLE spectrophotometer (Model Lambda 35-Perkin Elmer, USA) at 600 nm. If growth O.D does not come between the range 0.1-0.9 then diluted the sample with blank media.

3.2.5 Growth kinetics of different microbes in synthetic media with polymer as a carbon source

Growth pattern of all the 9 strains isolated from compost was also seen in synthetic media. In these experiments, 100 ml of synthetic media was autoclaved, BOPP films (3cm×3cm) were added and 5 ml of 24 hour old microbial culture in nutrient broth, was added as inoculum. These flasks were kept in rotary shaker at 130 rpm; 37°C. Growth of microbes at different time intervals was tested by taking growth O.D in U.V-VISIBLE spectrophotometer at 600 nm.

3.2.6 Fourier Transform Infrared (FTIR) and Attenuated Total Reflectance (ATR) spectroscopy

FTIR analysis is a useful tool to determine the formation of new or disappearance of functional groups. So degradation products, chemical moieties incorporated into the polymer molecules such as branches, co-monomers, unsaturation and presence of additives such as antioxidants can be determined by this technique. Fourier transform-attenuated total reflectance (FT-ATR) infrared spectroscopic studies were carried out on film samples using a Thermo SCIENTIFIC FT-IR spectrophotometer (Model Nicolet iS10, software OMNIC) in the horizontal ATR mode, using a zinc-selenide crystal. A total of 3 scans were taken.

CHAPTER 4

RESULTS AND DISCUSSION

The present study deals with the isolation of microorganisms from compost with the ability to degrade BOPP films. Several bacterial strains and actinomycetes strains attached on the BOPP film, after compost burial for 45 days; were isolated and characterized through macroscopic and microscopic studies. Biodegradation of polymer was measured by weight loss in the polymer and FTIR spectroscopy. Growth kinetics of all the isolated microorganisms was performed to study their growth pattern.

4.1 Macroscopic and Microscopic examination

A number of bacterial (*Bacillus sp.*) and actinomycetes strains were isolated from compost, synthetic media and air. They were characterized on the basis of macroscopic and microscopic tests.

4.1.1 From compost

Compost was collected from the environment that was rich in plastic wastes and was used as a source of isolating microorganisms having the ability to degrade BOPP films. 9 different strains were isolated from compost by compost burial method. Out of these 9 strains, 4 strains were obtained after water spreading and 5 strains were obtained by keeping polymer on NA. They were named as 1-4 and A-E respectively.

i. Actinomycetes strains

Out of these 9 strains, some of the strains were found to be actinomycetes like strain 1, strain 3, strain 4, strain A, strain B and strain C, when seen morphologically; and when seen under microscope, they were showing Gram positive, rod shaped character (as shown in figure 4.1). Their characteristics are as follows:

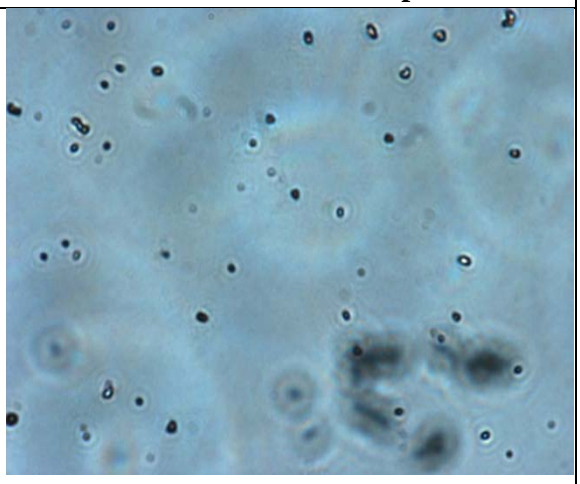
Strain 1 was yellow colored, round shaped, pinpoint sized colony, with shiny surface, spore forming, Gram positive, rod shaped. Strain 3 was yellow colored, round shaped,

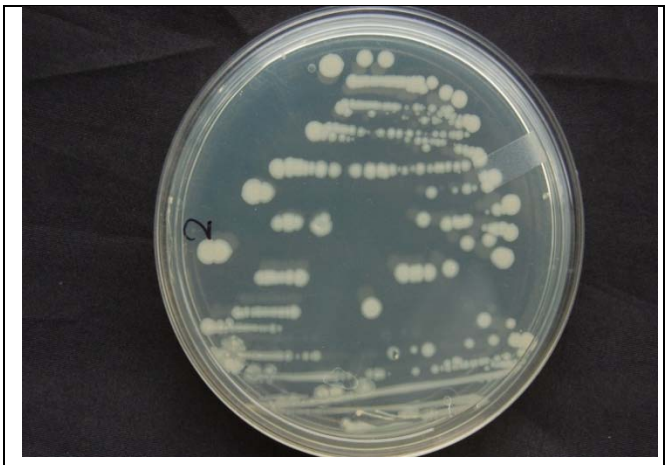
medium sized colony, with shiny surface, spore forming, Gram positive, rod shaped. Strain 4 was yellow colored, round shaped, small sized colony, with shiny surface, spore forming, Gram positive, rod shaped. Strain A was yellow colored, round shaped, small sized colony, with dull surface, spore forming, Gram positive, rod shaped. Strain B was yellow colored, round shaped, pinpoint sized, with dull surface, spore forming, Gram positive, rod shape bacteria. Strain C was yellow colored, round shaped, pinpoint sized, with dull surface, non-sporulating, Gram positive, rod shape bacteria.

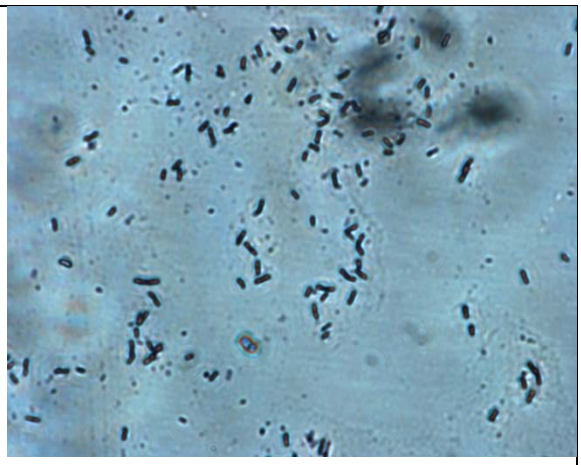
ii. Bacterial strains

The rest of the strains i.e. strain 2, strain D and strain E were found to be bacteria. They were identified as *Bacillus sp*. Their characteristics are as follows:

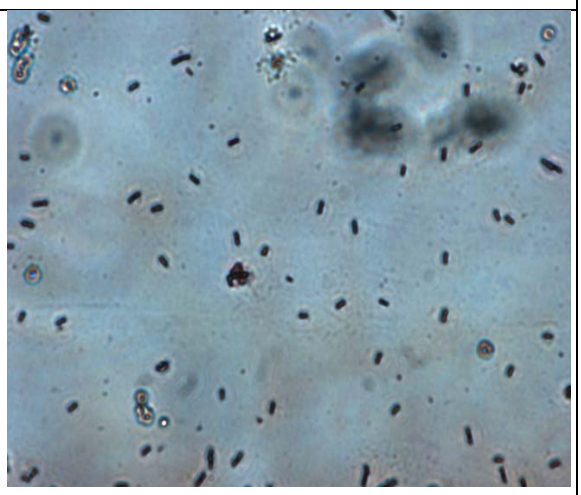
Strain 2 was yellow colored, round shaped, small colony, with dull surface, spore forming, Gram positive, rod shaped bacteria. Strain D was yellow colored, round shaped, medium sized, with dull surface, non-sporulating, Gram positive, rod shape bacteria. Strain E was yellow colored, round shaped, pinpoint sized, with dull surface, non-sporulating, Gram positive, rod shape bacteria.

Images of petriplates showing the isolated colonies	**Gram staining of the microorganisms isolated from the compost**
	
Strain 1	

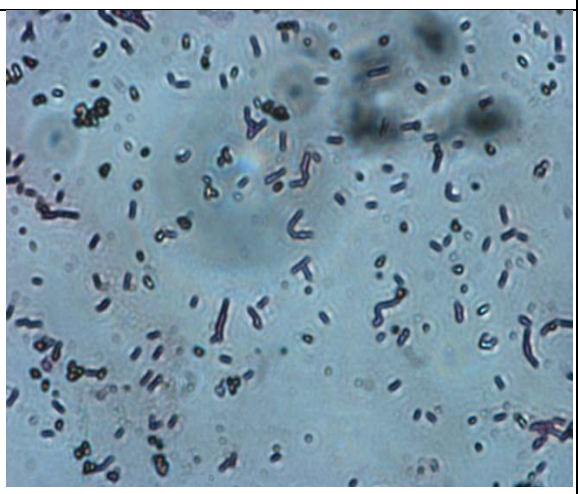




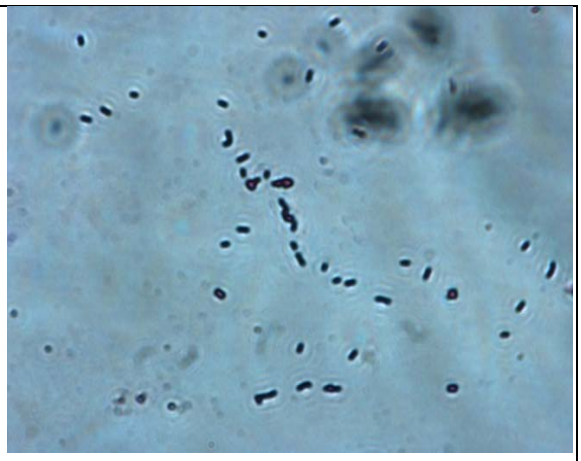
Strain 2



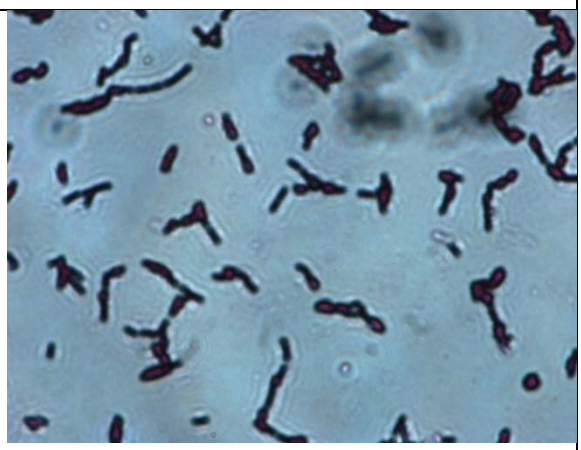
Strain 3



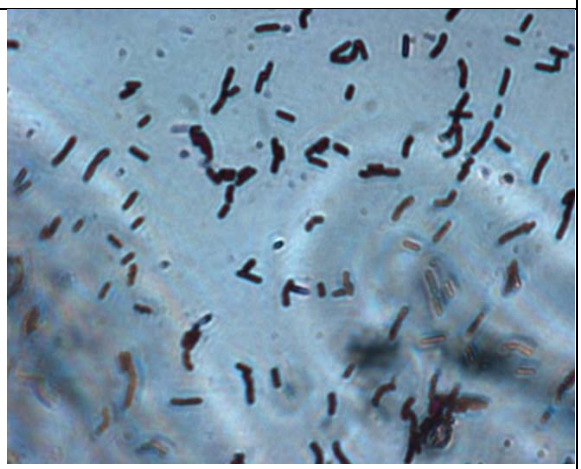
Strain 4



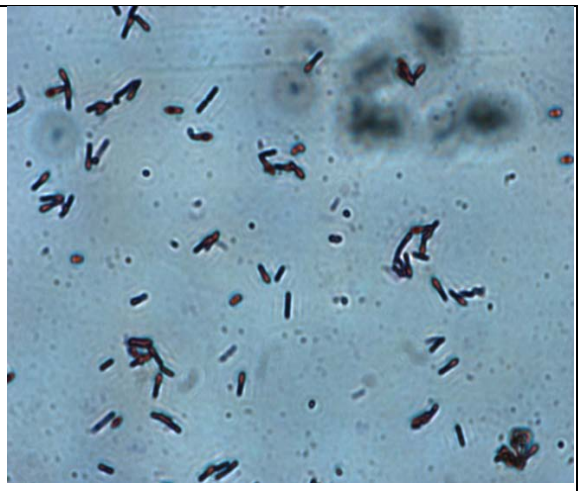
Strain A



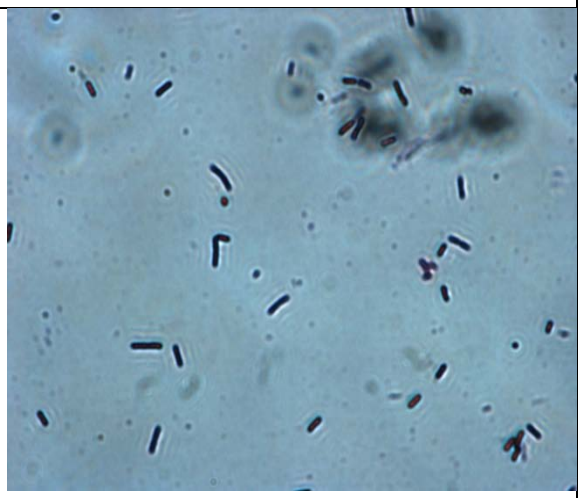
Strain B



Strain C



Strain D



Strain E

Fig 4.1 Photographs of the petriplates showing the isolated colonies and their Gram staining character

4.1.2 From synthetic media

After two months of incubation in SM at 37^0C and 130 rpm, polymer film was taken out, rinsed with sterilized water and placed on nutrient agar (as shown in Figure 4.2). Microbes grown were isolated and studied (as shown in Figure 4.3). Bacteria isolated from media were yellow colored, large colony with dull surface, non-sporulating, Gram positive rod shaped bacteria. Rods were arranged in chains (as shown in Figure 4.3).

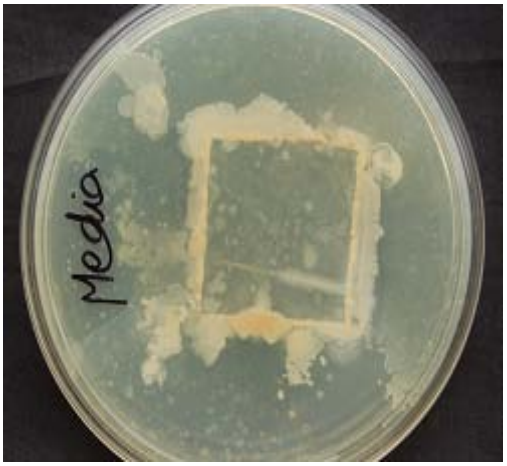


Figure 4.2 Photograph of the petriplate showing polymer kept in centre of NA plate

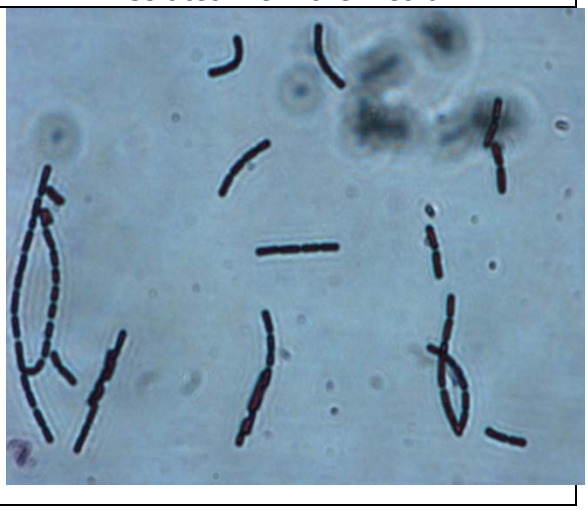
Photograph of petriplate showing the isolated colonies	**Gram staining of the microorganisms isolated from the media**
	

Figure 4.3 Photograph of the petriplate showing isolated colonies and their Gram staining character

4.1.3 From air

After 2 months, BOPP films were taken out from media kept in petridishes in the open, rinsed with sterilized distilled water and placed on NA plates (as shown in Figure 4.4), at 37ºC for few days to observe the microbial growth.

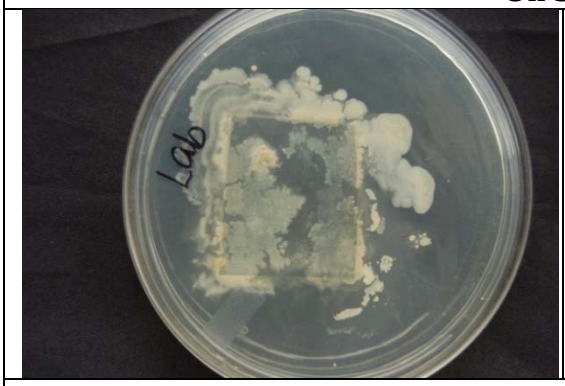
Photographs of petriplates showing polymer kept on NA plates	Photographs of petriplates showing the isolated colonies
	
On Stand	
	
In lab	
	
In Hostel	

Figure 4.4 Photographs of petriplates showing polymer kept on NA plate and isolated colonies

All the strains isolated from air were actinomycetes, when seen morphologically; and they were showing Gram positive, rod shaped character (Figure 4.5). They were non-sporulating, yellow in color with dull surface and round in shape. Some was having entire margins (from stand and lab) and other was having filamentous margins (from hostel).

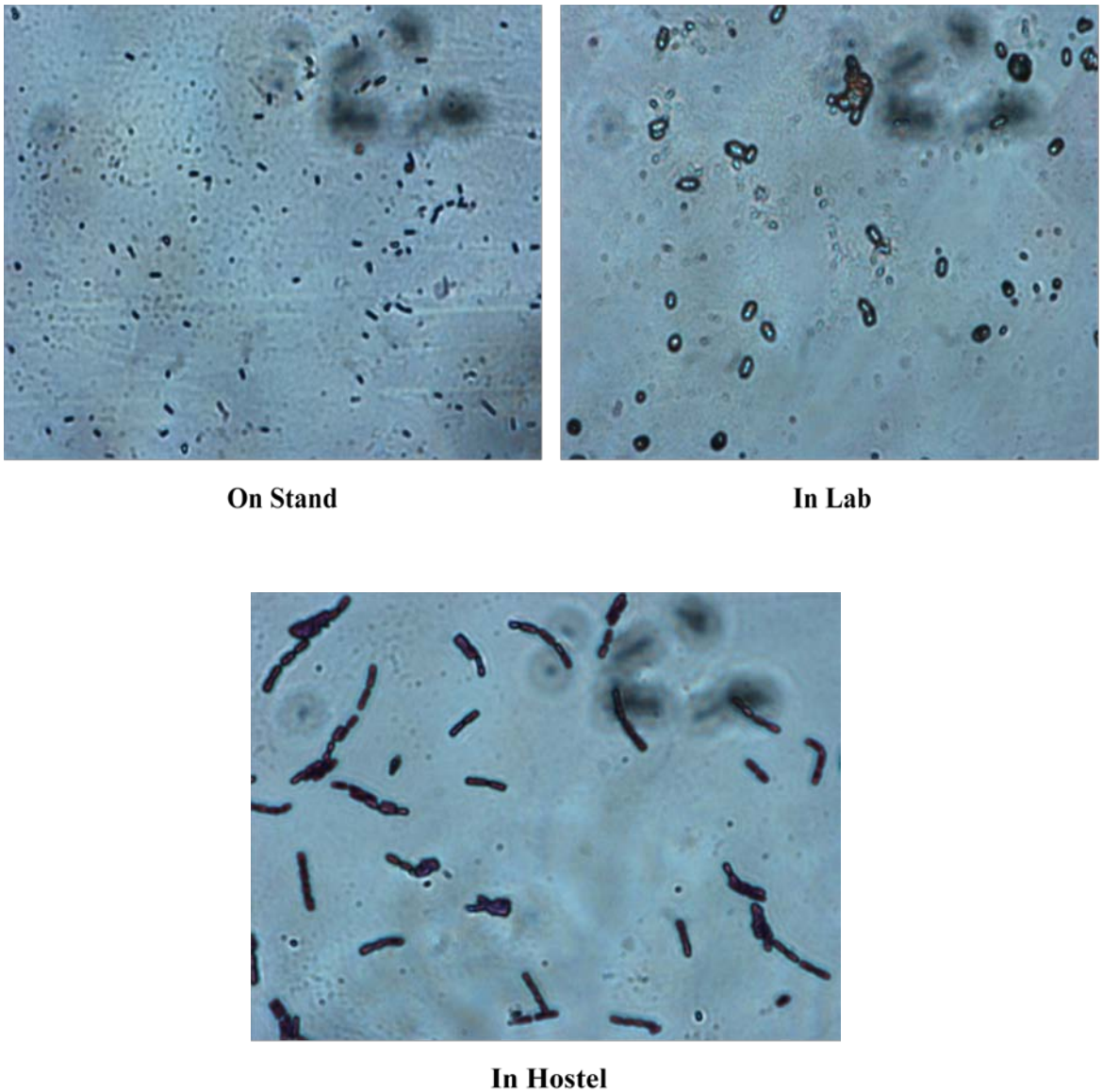


Figure 4.5 Gram staining of the microorganisms isolated from the air.

4.2 Summary of characteristics of polymer degrading microorganisms

The characteristics (Macroscopic and microscopic) of all the microorganisms observed are summarized here.

Table 4.1 Characteristics of polymer degrading microorganisms

Characteristics	**Strains**			
	1	**2**	**3**	**4**
Colony characteristics				
Shape	Round	Round	Round	Round
Size	pinpoint	Small	Medium	Small
Colour	Yellow	Yellow	Yellow	Yellow
Surface	Shiny	Dull	Shiny	Shiny
Margin	Entire	Entire	undulate	Entire
Morphology				
Straight rods	+	+	+	+
Cocci	-	-	-	-
Gram stain	+	+	+	+
Cell arrangement	Short rods, single	Short rods, single	Short rods, single	Short rods, single
Spore	+	+	+	+

Table 4.2 Characteristics of polymer degrading microorganisms

Characteristics	**Strains**				
	A	**B**	**C**	**D**	**E**
Colony characteristics					
Shape	Round	Round	Round	Round	Round
Size	Small	Pinpoint	pinpoint	Medium	pinpoint
Colour	Yellow	Yellow	Yellow	Yellow	Yellow
Surface	Dull	Dull	Dull	Dull	Dull
Margin	Entire	Entire	Entire	Entire	Entire
Morphology					
Straight rods	+	+	+	+	+
Cocci	-	-	-	-	-
Gram stain	+	+	+	+	+
Cell arrangement	Short rods	Curved rods	Curved rods	Medium sized rods	Medium sized rods
Spore	+	+	-	-	-

Table 4.3 Characteristics of polymer degrading microorganisms

Characteristics	**Media**	**Air**		
		Stand	**Lab**	**Hostel**
Colony characteristics				
Shape	Round	Round	Round	Round
Size	Large	Small	Medium	Medium
Colour	Yellow	Yellow	Yellow	Yellow
Surface	Dull	Dull	Dull	Dull
Margin	Entire	Entire	Entire	Filamentous
Morphology				
Straight rods	+	+	+	+
Cocci	-	-	-	-
Gram stain	+	+	+	+
Cell arrangement	Rods were arranged in chains	Short rods	Short rods	Short rods arranged in chains
Spore	-	-	-	-

4.3 Weight loss in polymer

Changes due to microbial degradation were assessed qualitatively by measuring weight loss of polymer. The weight loss of polymer after incubation may be purely because of microbial activity.

4.3.1 In compost

BOPP film was weighed, with an accurate four-digit balance, before and after burying in compost. The weight loss was found to be:
Weight of film before degradation: 16.1mg
Weight of film after degradation: 14.6mg
Weight loss (%): 8.2%

4.3.2. In synthetic media with polymer as a carbon source

Multiple samples were weighed before and after degradation and average value is reported here.

Table 4.4 Weight of polymer before and after biodegradation in media

S.No.	Weight before degradation (mg)	Weight after degradation (mg)	Weight loss (%)
1	16	15.3	4.375
2	15.1	15	0.66
3	15	14.8	1.33
4	15	14.8	1.33
5	16.5	16.3	1.25
6	16	15.5	3.125

On an average: 2.01%

These figures are represented in graphs (Figure 4.6 & 4.7)

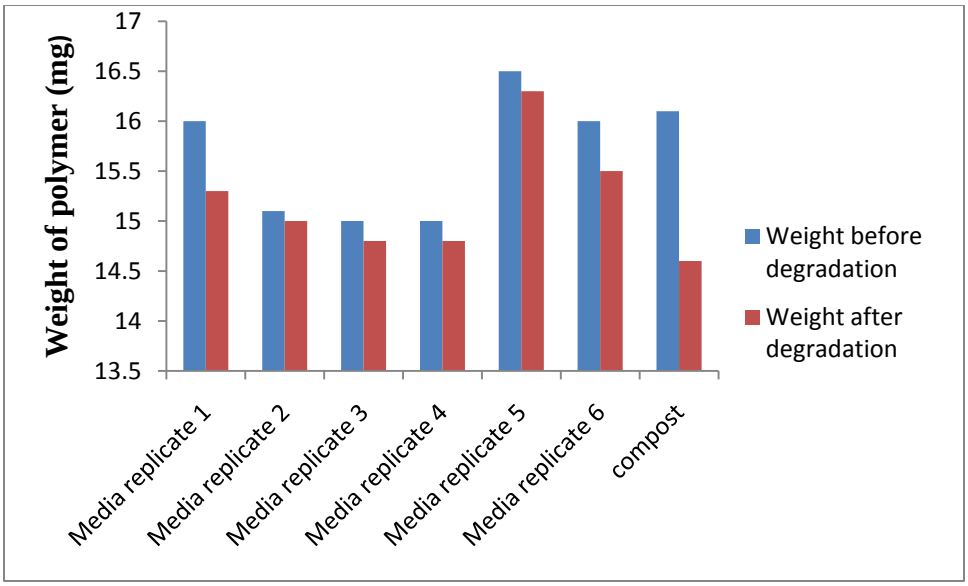


Fig 4.6 Weight of polymer before and after biodegradation

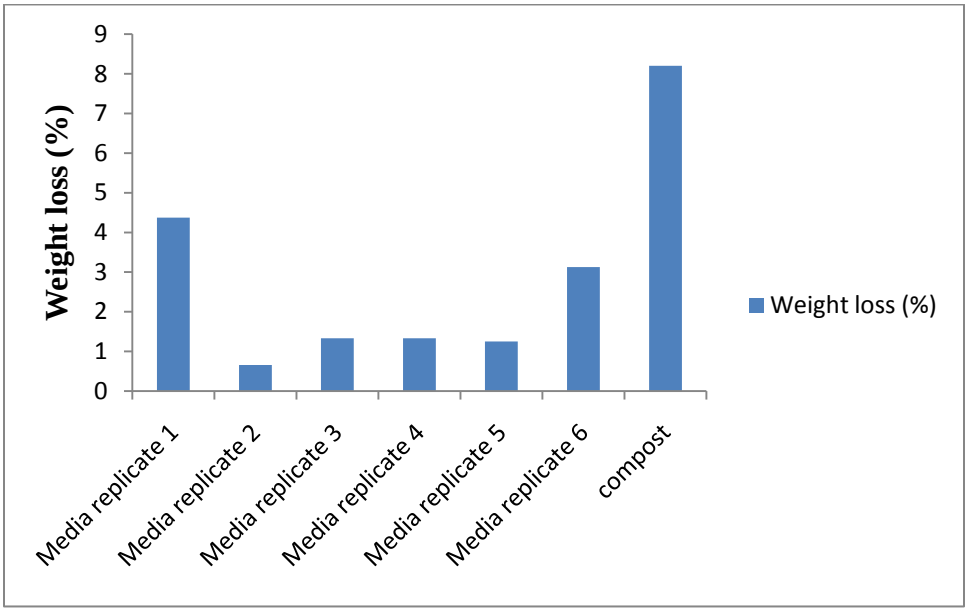


Fig 4.7 Percentage weight loss of plastic films after treatment

As there was a statistically significant decrease in polymer weight, therefore degradation was considered to have taken place.

4.4 Growth kinetics of isolated microorganisms in nutrient broth

The strains isolated from compost were selected, purified and their growth pattern was studied in nutrient broth as well as in synthetic media (as shown in figures 4.8-4.12). Growth phase of bacteria can be divided into – lag, log, stationary and death.

Table 4.5 Growth kinetics of isolated microorganisms in nutrient broth

Time (hours)	Optical Density				
	A	B	C	D	E
0	0.044	0.011	0.01	0.012	0.011
3	0.078	0.147	0.015	0.026	0.021
4	0.265	0.893	0.103	0.188	0.169
6	0.69	1.71	1.34	1.45	1.5
8	0.928	1.87	1.85	1.77	1.6
22	2.79	4.08	3.4	2.32	3.48
24	2.92	4.14	3.46	2.56	3.53
26	2.98	4.27	3.59	2.67	3.74
29	2.91	4.3	3.67	2.7	3.95
30	3.13	4.46	3.7	2.87	3.97

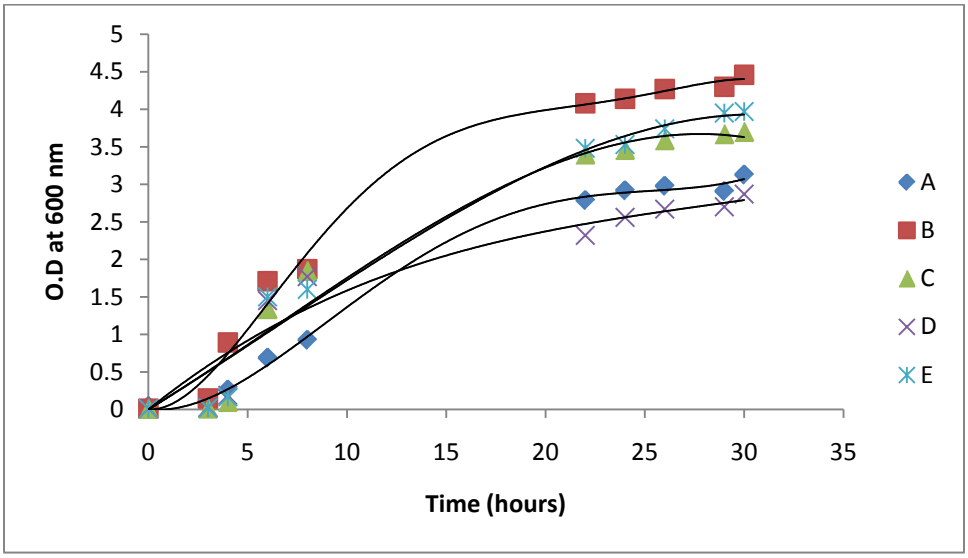


Figure 4.8 Growth curve of isolated microorganisms in nutrient broth

Table 4.6 Growth kinetics of isolated microorganisms in nutrient broth

Time (hours)	Optical Density			
	1	2	3	4
0	0.032	0.03	0.018	0.023
2	0.068	0.047	0.023	0.031
4	0.458	0.401	0.03	0.04
6	0.506	1.02	0.158	0.266
8	0.695	1.15	0.666	0.763
10	0.788	1.16	1.14	1.107
12	0.81	1.21	1.21	1.2
16	1.1	1.43	1.37	1.32
20	1.42	1.51	1.62	1.45
24	1.76	1.68	1.71	1.57
26	1.82	1.69	1.74	1.58

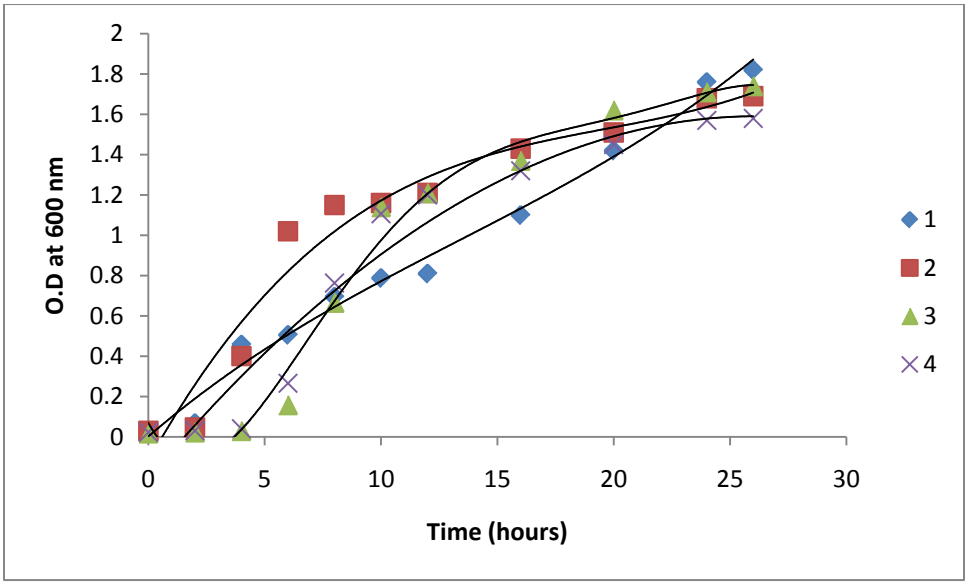


Figure 4.9 Growth curve of isolated microorganisms in nutrient broth

The growth pattern seen in fig 4.8-4.9 indicates that nutrient broth is favourable for the growth of microbes. This liquid culture served as inoculum source for further studies on

growth kinetics and biodegradation. The growth pattern obtained suggests that even after 27 hours the microorganism is growing at an exponential rate. The lag phase or the acclimatisation phase lasts for about 4 hours in nutrient broth. These results indicate that the bacteria used for the inoculum was in the log phase.

4.5 Biodegradation kinetics of isolated microorganisms in synthetic media

Studies on growth pattern of bacteria were carried out in 250 ml Erlenmeyer flasks containing 100 mL synthetic media and BOPP film as a carbon source. Inoculum used was 5 ml of liquid culture containing isolated strains in nutrient broth, incubated for 24 hours at 37°C. The growth of microbial culture was obtained by measuring the optical density (O.D.) of the sample at different times using UV-VIS spectrophotometer (Model Lambda 35-Perkin Elmer, USA).

Table 4.7 Biodegradation kinetics of isolated microorganisms in synthetic media

Time (days)	**Optical Density**			
	1	**2**	**3**	**4**
0	0.000	0.000	0.000	0.000
7	0.230	0.402	0.380	0.290
14	0.474	0.826	0.765	0.600
17	0.705	0.909	0.866	0.647
22	0.563	1.010	0.940	0.756

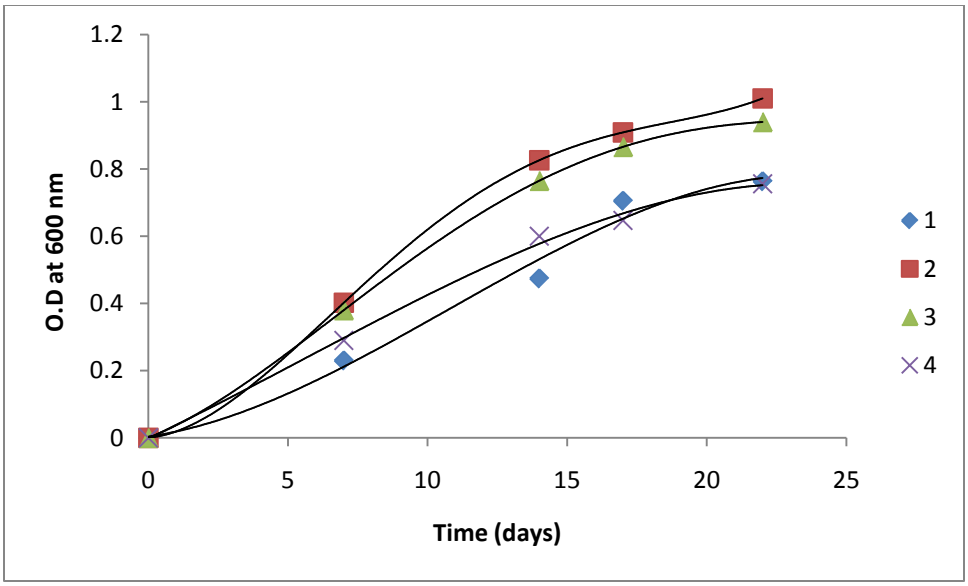


Figure 4.10 Growth curve of isolated microorganisms in synthetic media

Table 4.8 Biodegradation kinetics of isolated microorganisms in synthetic media

Time (days)	Optical Density				
	A	B	C	D	E
0	0.000	0.000	0.000	0.000	0.000
7	0.293	0.133	0.252	0.127	0.093
10	0.383	0.256	0.299	0.246	0.274
15	0.437	0.302	0.301	0.300	0.307
20	0.490	0.350	0.330	0.320	0.316

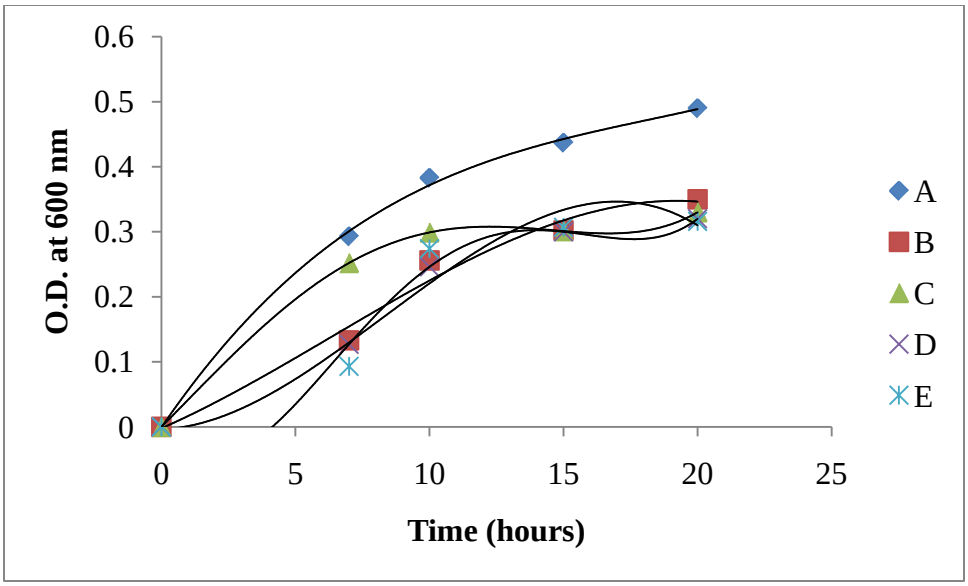


Figure 4.11 Growth curve of isolated microorganisms in synthetic media

Growth kinetics in synthetic medium indicates growth patterns of microorganisms as well as its survival potential in synthetic media. Growth pattern seen in figure 4.10 and 4.11 indicates that the synthetic medium is suitable for the growth of the microorganism. As growth proportionally increased in synthetic media, therefore degradation was considered to have taken place.

4.6 FTIR spectra of BOPP film before and after degradation in compost and in media

Figure 4.13 shows the FTIR spectra of BOPP film. Figure 4.14 and 4.15 shows the FTIR spectra of BOPP film after compost burial and incubation in SM respectively. The overlapping spectra in the expanded form are also provided for comparison, which clearly depict the changes observed (Figure 4.16-4.18).

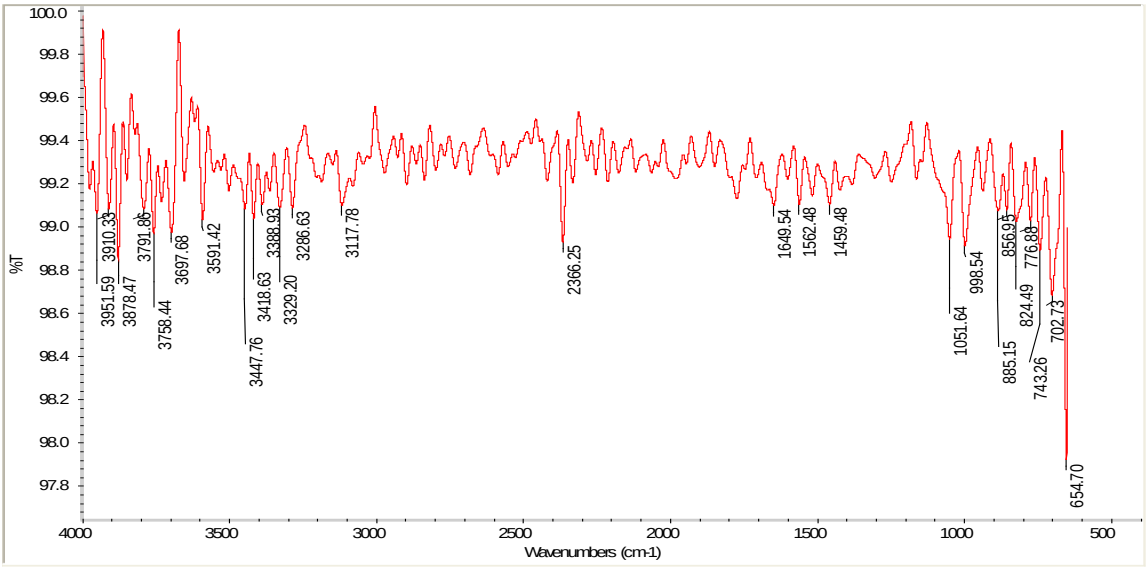


Figure 4.12 FTIR spectra of BOPP film before degradation

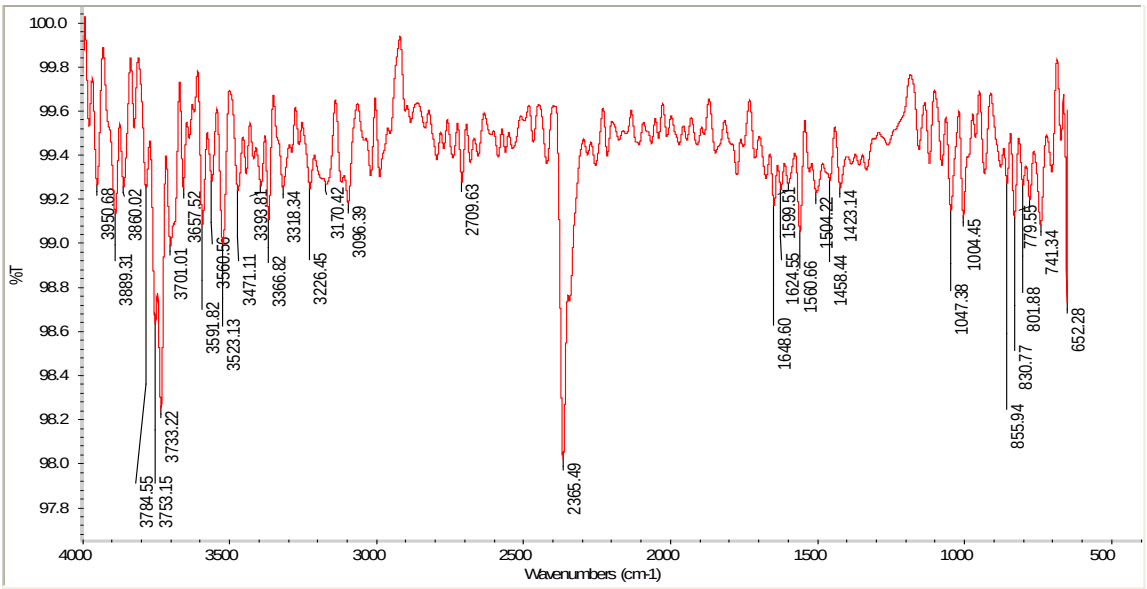


Figure 4.13 FTIR spectra of BOPP film after burial in compost for two months

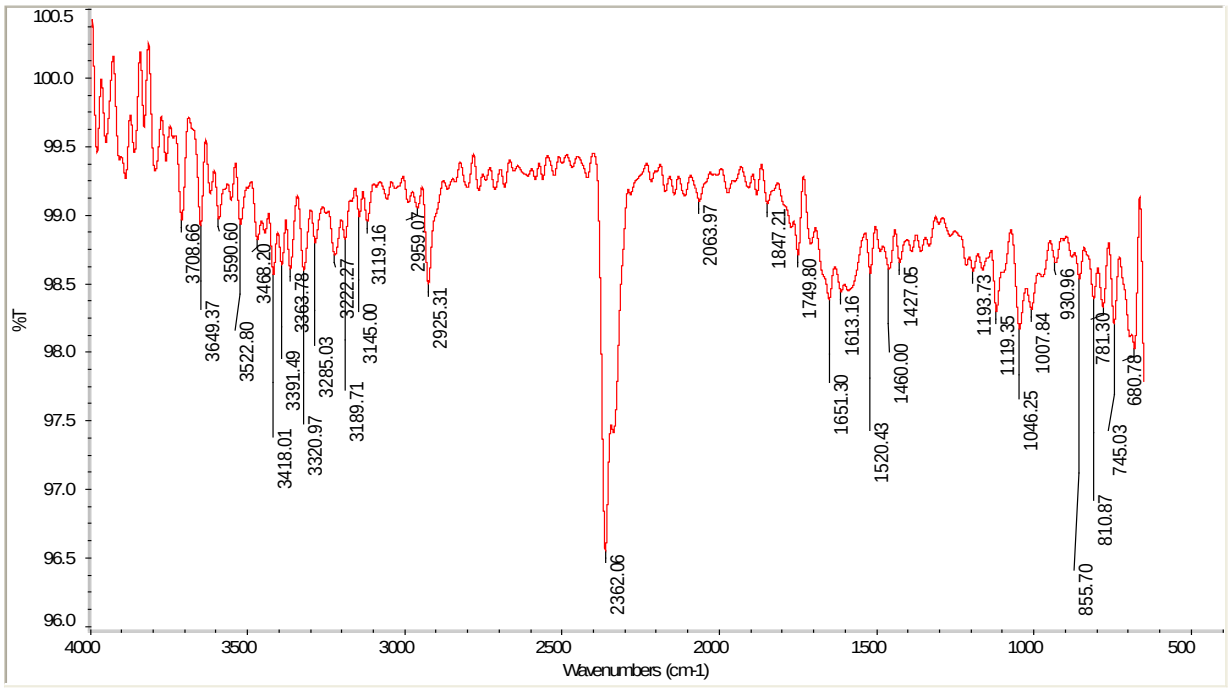


Figure 4.14 FTIR spectra of BOPP film after degradation in synthetic media for two months

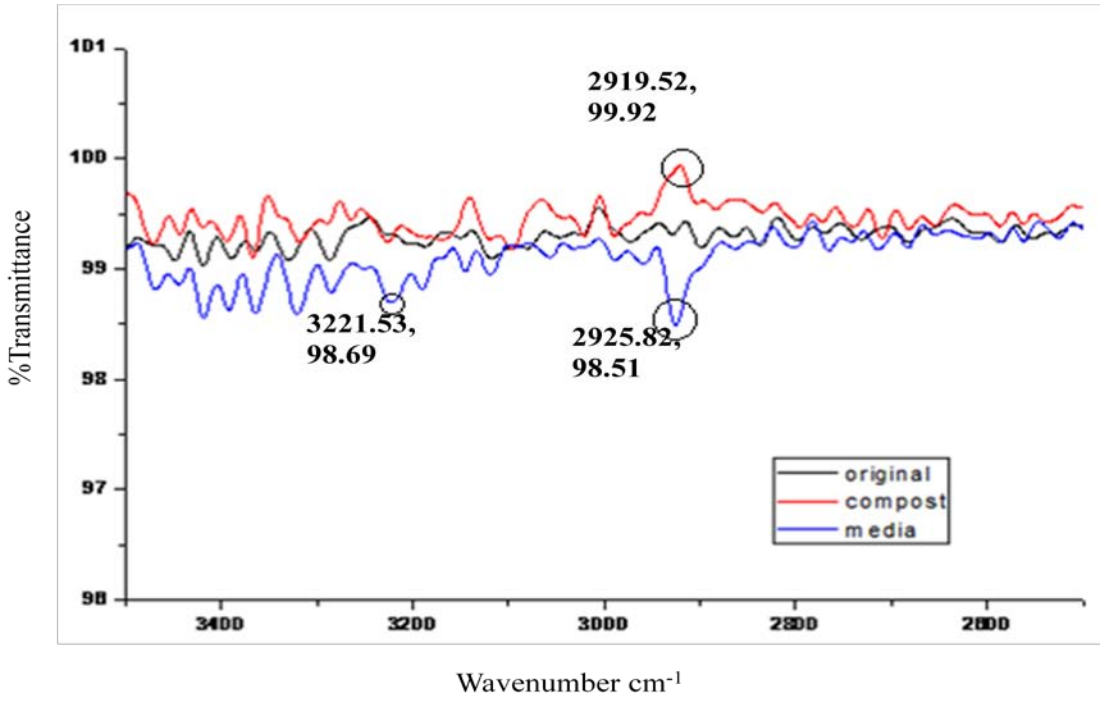


Figure 4.15 Overlapping FTIR spectra of all the three from 3500-2500 cm^{-1}

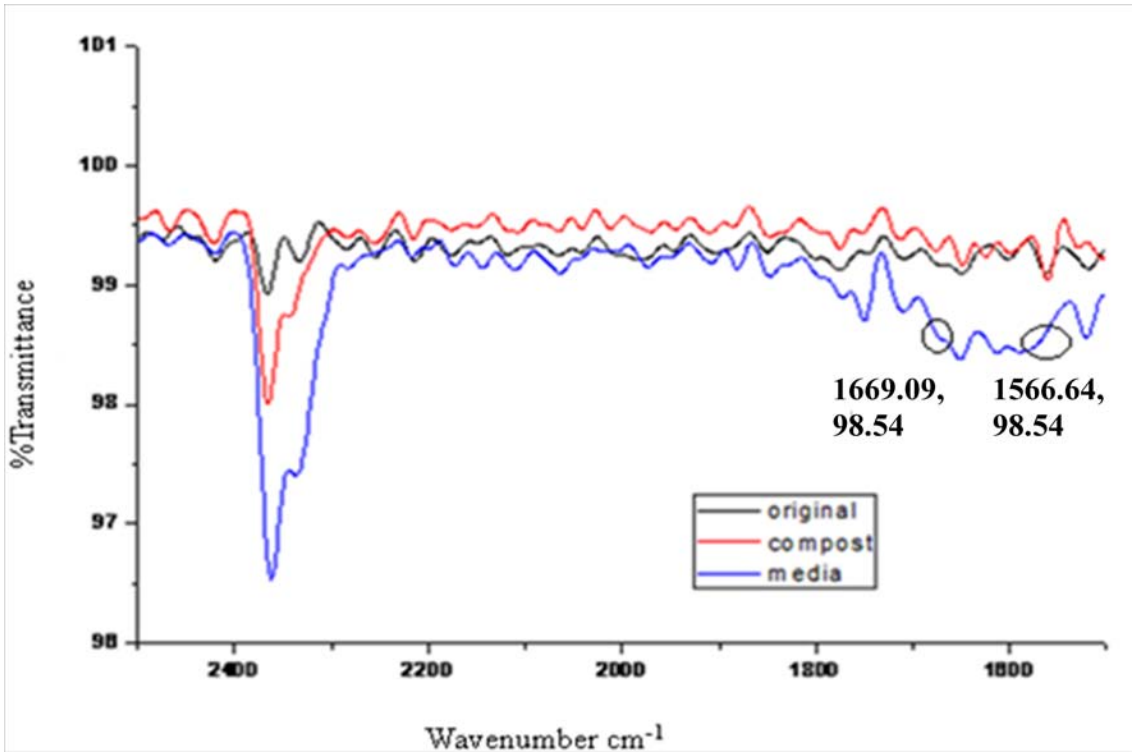


Figure 4.16 Overlapping FTIR spectra of all the three from 2500-1500 cm^{-1}

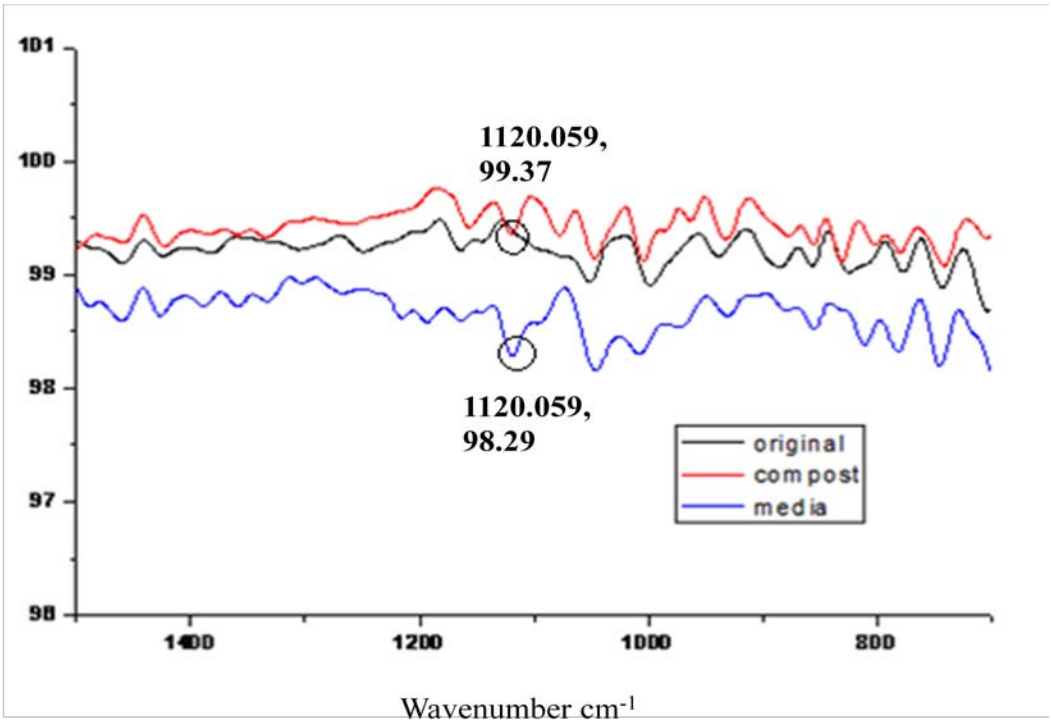


Figure 4.17 Overlapping FTIR spectra of all the three from 1500-700 cm^{-1}

From Figure 4.16-4.18, we find that

i) In case of BOPP film kept in synthetic media (blue line), the spectrum is showing peak at 3221.53cm^{-1} corresponding to O-H region and at 2925.82 cm^{-1} corresponding to methylene region. There is broadening in the region of 1669.09 to 1566.64 cm^{-1} corresponding to C-O (carboxylates) region and C=C (unsaturation). Peak can also be seen at 1120.059 cm^{-1} which corresponds to C-O bond of ether group.

ii) In case of BOPP film buried in compost (red line), the spectrum is showing peak at 2919.52 cm^{-1} corresponding to methylene region and at 1120.059 cm^{-1} which corresponds to C-O bond of ether group.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

A total of thirteen bacterial and actinomycetes strains capable of degrading BOPP have been isolated from compost and air. Most of them are actinomycetes and some are bacteria. The bacterial isolates are Gram positive and belong to genus *Bacilli.* Actinomycetes are showing Gram positive, rod shaped character. It can be concluded that soil contains the potential candidates for bioremediation of plastic wastes.

Weight loss of polymer kept in synthetic media for two months at 37°C varies from 0.66% to 4.38% (with an average value of 2.01%). The same value in compost is 8.2%. Weight loss in polymer confirms the degradation of polymer.

By growth kinetic study in synthetic media, it is concluded that isolated strains are solely dependent on BOPP films for its carbon source.

FTIR spectra also confirm the biodegradation of polymer as some chemical changes are seen in surface of polymer.

Future Recommendations

In the natural environment, different kinds of microorganisms play an important role in various steps involved in the degradation of synthetic polymers in general, and polyolefins in particular. Studying the synergism between those microorganisms will give insight for future efforts towards the biodegradation of these materials.

In addition to screening soil microorganisms, isolating microorganisms from marine, petroleum waste and polymer dump site could lead to new unexplored strains, with superior performance.

If one can characterize the genes responsible for the production of degrading enzymes and its regulation by using current genetic engineering tools, one can genetically modify the microorganisms and use them as a superbug for degrading the recalcitrant polyolefins.

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