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 [Premraj and Doble, 2005]. Polymer materials are solid, non-metallic compounds of high molecular weights. They are comprised of repeating macromolecules, and have varying characteristics depending upon their composition. Each macromolecule that comprises a polymeric material is known as a mer unit. A single mer is called a monomer, while repeating mer units are known as polymers [Kolybaba *et al*., 2003].

Polymers are of two types: synthetic and natural. 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. These characteristics and others (such as rigidity, permeability and transparency) can be controlled by changing the polymer synthesis process, molecular weight and/or by the use of specific additives [Kyrikou and Briassoulis, 2007].

The attributes like light weight yet strong, least energy consumption and minimum emission of pollutants in the air and water during production, inert characteristics, excellent water resistance and barrier properties, excellent insulation and dielectric characteristics, ease of fabrication into variety of shapes and structure – have all made polymers not only a material of choice for an array of applications, use of polymers has become essential in every sphere of our modern life. The long life of polymer products has added to the convenience [Indian Centre for Plastics in the Environment, 2010]. This has resulted in single use of polymer products for mass consumption, and consequently a large volume of such products as carrybags, packaging materials, water bottles, dairy containers etc. are being thrown into the garbage [Maiti and Jana, 2005].

Synthetic plastics accumulate at a rate of 25 million tons per year in the terrestrial and marine coastal environment [Sudhakar *et al*., 2008]. Discarded, non-degradable polymers show undesirable environmental problems. These polymers create a threat to diverse animal populations. They have a direct impact on marine ecosystems and are believed to be responsible for the death of a very large number of birds by ingestion or strangulation [Scott and Wiles, 2001]. It is estimated that one million tons of polymers are dumped in the sea annually. Entanglement can readily occur in materials with holes or plastic bags. Non-biodegradable polymers also have the capacity to act as disease foci because they persist in the environment for a very long period of time enabling organisms to accumulate [Jayasekara *et al*., 2005].

Thus polymers become a red herring to the environmentalists, and by their pressure governments are forced to enact laws which are not conducive to the growth and development of polymer industry. Some of the over-enthusiastic groups call for a ban on use of the mass consumed plastic items [Maiti and Jana, 2005]. Since 1990 the polymers industry, as individual companies and through organizations such as APC (*American Polymers Council*), has invested more than $1 billion to support increased recycling and educate communities. Despite the continuing growth of recycling, source reduction and energy recovery, some proportion of the waste will always require disposal [Kyrikou and Briassoulis, 2007].

Polymers can and do degrade by many routes. The polymers can be fragmented through physical forces. Fragmentation often plays an important role in the early stages of degradation and can be brought about by physical forces of mechanical nature. Chemical changes within the polymer can occur and may begin with abiotic degradation [Kyrikou and Briassoulis, 2007]. Degradation brought by chemical reactions generally involves chain scission-fragmentation of the polymer chains. Surface erosion can be the result of chain scission resulting from chemical hydrolysis. At some point, some specific polymers may be attacked effectively by microorganisms-the onset of biodegradation. All of these pathways are potential routes for polymer degradation as shown in fig. 1.1 [Arutchelvi *et al*., 2008].

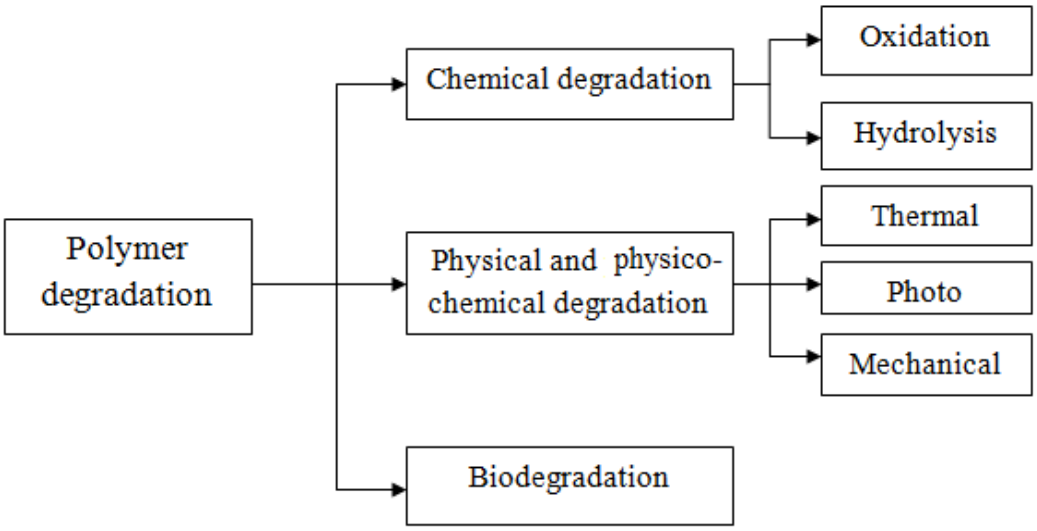

Fig. 1.1 Overview of degradation of polymers

Many synthetic polymers are produced and utilized because they are resistant to chemical and physical degradation. On the other hand, polymers resistant to degradation present disposal problems when their usefulness ceases. The degradation of used polymers is not a simple process, when referring to environmental degradation. One of the best ways of disposal is biodegradation [Kyrikou and Briassoulis, 2007].

Biodegradation or biotic degradation is chemical degradation of materials (e.g. polymers) brought about by the action of naturally occurring microorganisms such as bacteria, fungi and algae [Kyrikou and Briassoulis, 2007]. Biodegradation can be aerobic or anaerobic.

Aerobic biodegradation [Bastioli, 2005]:

$$C_{POLYMER} + O_2 \longrightarrow CO_2 + H_2O + C_{RESIDUE} + C_{BIOMASS}$$

Anaerobic biodegradation [Bastioli, 2005]:

$$C_{POLYMER} \longrightarrow CO_2 + CH_4 + H_2O + C_{RESIDUE} + C_{BIOMASS}$$

Biodegradation is generally considered as consisting of both enzyme-catalyzed hydrolysis and non-enzymatic hydrolysis [Wackett and Hershberger, 2001]. Enzymatic degradation can be carried out either by extracellular enzymes present in the microorganism's environment or by intracellular enzymes. Both result in chain scission whereby the polymer chains are cleaved into smaller segments. The enzymes may be either endoenzymes, which cleave internal linkages within the chain or exoenzymes, which cleave terminal monomer units sequentially [Kyrikou and Briassoulis, 2007]. Endoenzymes cleave the internal chain linkages randomly which results in a rapid decrease in molecular weight. Under some conditions microorganisms contribute to degradation of polymers through ingestion, mastication and excretion.

The kinetics of polymer degradation depend on whether the environment is dry air, humid air, soil, a landfill, a composting environment, sewage, freshwater or a marine environment. Each environment has its own characteristic concentration profile of important factors: oxygen, water, and other chemicals, day-light and degrading microorganisms [Albertsson *et al.,* 1987].

According to the nature of the environment, there may be a relatively more efficient or less efficient mechanism by which degradation can occur. In one environment, a very efficient degradation mechanism may be available, whereas in another environment, the same mechanism might not be available at all for lack of appropriate conditions. Also, according to the nature of the environment, there may be a larger or a smaller concentration of chemicals that react with the polymer during the degradation process [Kyrikou and Briassoulis, 2007].

There is a strong synergism between biodegradation and environmental factors, and biodegradation can, in practice, never be entirely separated from the purely physical and chemical; mainly auto-oxidative progressive ageing, always present as an unavoidable slow but cumulative background effect. Biodegradation is seldom due to a single cause, but a combined effect, including heat, UV light, stress and water. The presence of water is necessity for biodegradation [Albertsson *et al*., 1987].

Biodegradability of the polymer is essentially determined by the following important physical and chemical characteristics: (1) Availability of functional groups that increases hydrophobictiy, (2) Size, molecular weight and density of the polymer, (3) Amount of crystalline and amorphous regions, (4) Structural complexity, (5) Presence of easily breakable bonds such as ester or amide bonds, (6) Molecular composition (blend) and (7) Nature and physical form of the polymer such as whether it is in the form of films, pellets, powder or fibers [Arutchelvi *et al*., 2008].

As the polymer usage is unavoidable, ways have to be found to (1) Enhance the biodegradability of the polymers by blending them with biodegradable natural polymers; (2) Mixing with prooxidants so that they are easily degraded and (3) Isolate and improve microorganisms that can efficiently degrade these polymers [Arutchelvi *et al*., 2008]. Since most of the polymers are resistant to degradation, research over the past couple of decades has focused on developing biodegradable polymers, which are degraded and catabolized ultimately to CO_2 and water by bacteria, fungi under natural environment (they should not generate any harmful substances) [Premraj and Doble, 2005].

Biodegradable polymers may be naturally occurring or may be synthesized by chemical means [Jakubowicz, 2003]. These polymers can be classified into three major categories: (1) polyesters produced by microorganisms, (2) natural polysaccharides and other biopolymers like starch and (3) synthetic polymers like aliphatic polymers (eg., poly L-lactide and polybutylene succinate, etc. are commercially produced) [Premraj and Doble, 2005].

The development of environmentally degradable polymers (EDPs) was initiated among several other attempts in the early 1980s to address an emerging global plastic waste problem, following decades of fast development and explosive growth of plastic utilization [Scott, 2000]. Environmentally degradable polymeric materials and plastics (EDPs) comprise new kinds of plastic items, which are designed to exhibit a significant degradation resulting in environmentally compatible end products, namely CO_2, water, and cell biomass within an acceptable time frame [Krzan *et al*., 2006].

Chapter-2

LITERATURE REVIEW

With the advances in technology and the increase in the global population, polymers have found wide applications in every aspect of life and industries [Tokiwa *et al*., 2009]. Polymer accumulates in the environment at the rate of 25 million tons per year throughout the world. Burning of the polymer waste and burying of the polymers releases harmful toxic material which is a major pollutant in the environment. About 3% of polymer material is recycled while remaining remains as litter or landfiller [Kumari *et al*., 2009]. It is therefore essential that the risks involved be eliminated or at least reduced to an acceptable level [Poliakoff *et al*., 2002].

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 ease of processing to various products. The range of their applications includes food packaging, textiles, lab equipment, and automotive components [Arutchelvi *et al*., 2008]. PP has a methyl group instead of one of the hydrogen present in PE, on every other carbon, which gives rise to the existence of three stereo-isomeric forms namely, atactic, isotactic, and syndiotactic [Baker and Mead, 2002]. This stereoregular polymer was synthesized by Ziegler and Natta with propylene as the monomer.

Industrially applicable PE was first synthesized in 1933 by Eric Fawcett and Reginald Gibson at ICI chemicals [Trossarelli and Brunella, 2003]. PE is totally linear and available with varying range of densities from 0.91-0.97 g/cm^3. Low density PE has branching at random places leading to low packing of the polymer chains, whereas the high density PE is more linear with minimal branching leading to high packing density [Baker and Mead, 2002].

In recent years, considerable attention has been focused on biodegradability of polymeric materials. Such materials need to be resistant to degradation both prior to and during use and should be capable of being degraded, if discarded after use, without causing any environmental problems [Upreti and Srivastava, 2003]. Two possible approaches to reduce the 'vices of polymeric materials' are (a) to develop biodegradable commodity plastic [Ratajska and Boryniec, 1999], and (b) to identify potential microorganisms and develop protocol to effectively biodegrade the polymeric materials [Kawai, 1995].

The first biodegradable synthetic polymer is '*polyglycollic acid*' which was invented in 1954. Due to poor thermal and hydrolytic stabilities this polymer is not used as a regular plastics material. This polyglycollic acid is mostly used as a biodegradable suture material in surgery [Maiti and Jana, 2005]. The word 'bio-plastic' is used confusingly. In our understanding, however, bio-plastics consist of either biodegradable plastics (*i.e.,* plastics produced from fossil materials) or bio-based plastics (*i.e.,* plastics synthesized from biomass or renewable resources). The inter-relationship between biodegradable plastics and bio-based plastics is shown in fig. 2.1 [Tokiwa and Calabia, 2008].

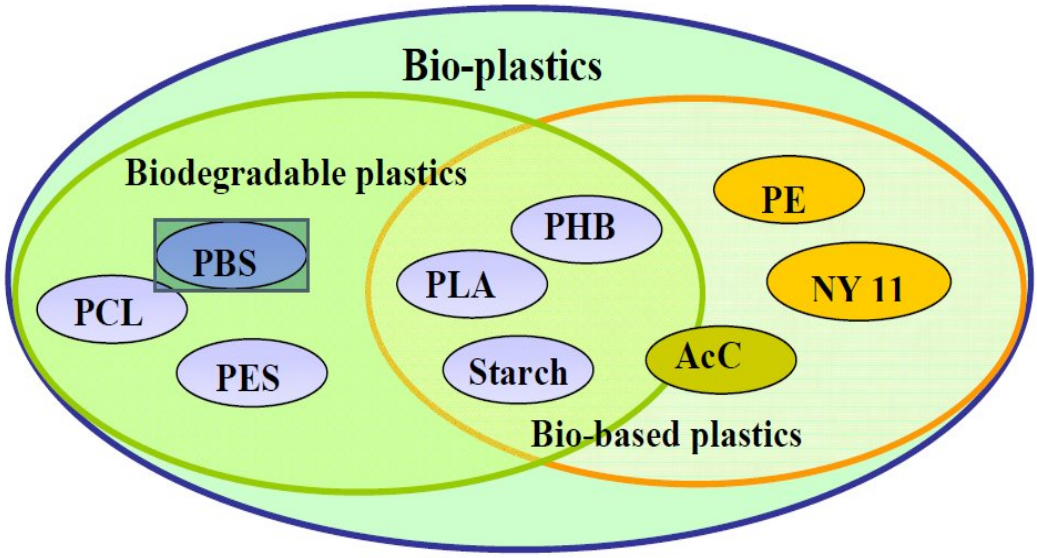

Fig. 2.1 Bioplastics comprised of degradable plastics and bio-based plastics

Polycaprolactone (PCL), and poly(butylene succinate) (PBS) are petroleum based, but they can be degraded by microorganisms. On the other hand, poly(hydroxybutyrate) (PHB), poly(lactide) (PLA) and starch blends are produced from biomass or renewable resources, and are thus biodegradable. Despite the fact that polyethylene (PE) and Nylon 11 (NY11) can be produced from biomass or renewable resources, they are non-biodegradable. Acetyl cellulose (AcC) is either biodegradable or non-biodegradable, depending on the degree of acetylation. AcC's with a low acetylation can be degraded, while those with high substitution ratios are non-biodegradable [Tokiwa *et al*., 2009].

The microorganism reported for biodegradation include fungi (*Aspergillus niger*, *A. flavus*, *A. oryzae*, *Chaetomium globusum*, *Penicillium funiculosum*, *Pullularia pullulans*), bacteria (*Pseudomonas aeruginosa*, *Bacillus cereus*, *Coryneformes bacterium*, *Bacillus* sp., *Mycobacterium*, *Nocardia*, *Corynebacterium*, *Candida* and *Pseudomonas*) and Actinomycetales (*Streptomycetaceae*) [Sudhakar *et al*., 2008].

The first report demonstrating bacterial degradation of the oxidized polyethylene in pure culture was described by Lee *et al*. [1991]. The known lignin-degrading bacteria *Streptomyces viridosporus* T7A, *S. badius* 252, and *S. setonii* 75Vi2 and fungus *Phanerochaete chryosporium* were used. They described the pure culture system for evaluating the biodegradability of degradable plastic films containing pro-oxidant and 6% starch. Biodegradability was evaluated by weight loss, tensile strength loss, changes in percent elongation, and changes in polyethylene molecular weight distribution. This is the first pure culture study to demonstrate that lignin-degrading microorganisms can actually degrade the oxidized polyethylene component of degradable plastics (indicated by molecular weight reductions).

El-Shafei *et al.* [1998] investigated the ability of fungi and *Streptomyces* species to attack degradable plastics (disposable polyethylene bags containing 6% starch) in pure shake-flask culture studies. Eight different *Streptomyces* strains were isolated and two fungi *Mucor rouxii* NRRL 1835 and *Aspergillus flavus* were used. Ten-day heat treated (70°C) polyethylene films were chemically disinfected and incubated at 30°C, 125 rpm in 0.6%

yeast extract medium (pH 7.5) for *Streptomyces* spp. and for the fungi in 3% yeast extract medium (pH 5.5) for 1, 2 and 4 weeks along with an uninoculated control for each treatment. Active enzymes caused changes in the film's mechanical properties and weight.

Biodegradation depends upon polymer characteristics, organism type and nature of pretreatment [Shah *et al*., 2008]. The pretreatment of polyethylene is very significant for its biodegradation. Physical rupturing of the polyethylene and chemical washing by ethanol might have added value to its degradability. To support this, Volke-Sepulveda *et al*. [2002] showed that addition of ethanol to fungus cultures containing polyethylene improved the degradation rate of the polymer. Despite the fact that the isolated bacteria were native to the site of polyethylene disposal and might show some degradability in natural conditions, yet they also exhibited biodegradation in laboratory conditions on synthetic media. This gives some clue that these bacteria can be used in both natural and artificial conditions for the purpose of degradation of polymers [Nanda and Sahu, 2010].

Remarkable work had been done by using *Rhodococcus ruber*. Gilan (Orr) *et al*. [2004] isolated a strain of *Rhodococcus ruber* C208 that utilized polyethylene as a sole carbon and energy source. This strain was found to be highly hydrophobic, which enabled it to form dense biofilm on the polyethylene surface and improved its biodegradability capacity. Sivan *et al*. [2006] continued the work and investigated the kinetics of the biodegradation activity of the biofilm formed on polyethylene by C208. They monitored the early formation of the three-dimensional structures and followed the growth, differentiation and survival of the biofilm over an extended period of time. In earlier two works, *Rhodococcus ruber* C208 had been isolated that degrade polyethylene films. Mor and Sivan [2008] then monitored the kinetics of biofilm formation by C208 on polystyrene, determining the physiological activity of the biofilm and analyzing its capacity to degrade polystyrene.

Kumari *et al*. [2009] isolated, screened and identified bacteria degrading polyethylene (PE) and also optimized the conditions of polyethylene degradation by using and

comparing two methods viz; % weight loss and CO_2 evolution. Thirty two bacterial isolates were obtained from soil by soil burial method followed by enrichment culture technique in film culturing (FC) media. Bacterial isolates differing in morphology were selected, purified and maintained at 4°C. Thirty % of these isolates were found to be Gram negative and 50% showed positive starch hydrolysis test and were screened for their ability to degrade low density polyethylene (untreated, UV and heat strips) in film culturing media and percent weight loss of polyethylene after 4^{th} week was determined. Among various isolates, highest degradation was by Is 3, Is 22 and Is 31 to the range of 25- 27%, of UV treated polyethylene strips. High temperature (40°C) was found to enhance degradation rate of polyethylene more effectively by 24-28% compared to low temperature at 30°C (18-21%). Degradation of treated polyethylene strips (UV, heat steam) was up to 4% by compost treatment as studied by using CO_2 evolution, an estimation tool to analyze % degradation.

Nanda *et al*. [2010] evaluated the biodegradability of natural and synthetic polyethylene (disposable plastic bags containing 6% starch) by three different *Pseudomonas* spp. isolated from variant locations (household garbage and vegetable waste; textile effluents drainage site; soil dumped with sewage sludge) in laboratory conditions by a pure culture shake-flask incubation method. They concluded that natural polyethylene gave a rapid biodegradation. *Pseudomonas* spp. from sewage sludge dump was found to degrade polyethylene efficiently; in contrast, *Pseudomonas* spp. from the household garbage dump gave the lowest biodegradability.

Chatterjee *et al*. [2010] studied the biodegradation of heat treated commercial polyethylene (extruded low-density polyethylene commonly available in the market as 20 µm thick carrier bags) by *Staphylococcus epidermis* BP/SU1. The growth of BP/SU1 is supported by the presence of shredded LDPE as its only carbon source in inorganic salt minimal nutrient medium. SEM analysis of the films showed pores of almost bacterial dimensions. LDPE supplemented liquid mineral culture medium showed the presence of extracellular enzymes with appropriate molecular weight of 55 kDa and 35 kDa. This is

the first evidence of enzyme-mediated biodegradation of commercial grade petrochemical-based polyethylene after heat treatment alone.

Temperature plays an important role in controlling the nature and efficiency of microbial hydrocarbon degradation, which is of major significance for in situ bioremediation [Leahy & Colwell 1990]. Degradation of long chain alkanes by mesophiles at temperatures between 25 and 28°C has been extensively studied in contrast with biodegradation pathways in thermophiles that are not yet well characterized [Mishra *et al*., 2001; Sepic *et al*., 1996]. It is recognized that enzymes from thermophiles are more resistant to physical and chemical denaturation, while another advantage of using thermophiles in bioremediation processes would be faster growth rates [Meintanis *et al*., 2006].

Relative studies suggest that thermophilic hydrocarbon degraders of *Bacillus*, *Thermus*, *Thermococcus* and *Thermotoga* species occurring in natural high-temperature or sulfur-rich environments are of special significance as they could be efficiently used for bioremediation of oil-polluted desert soil, sediments in semi-arid climates with long hot summers and in composting processes [Feitkenhauer *et al*., 2003; Shimura *et al*., 1999].

Thermophiles growing on medium chain and long chain alkanes constitute a great biotechnology perspective and the number of characterized thermophiles with biodegradation potential has been increased during the last years [Feitkenhauer *et al*., 2003; Hao *et al*., 2004]. Yet, little is known about the main alkane hydroxylase systems of the thermophilic degradation pathways. Homologues of the *Pseudomonas* genes that allow hydrocarbon degradation found in thermophilic bacteria can be related with high biodegradation rates exhibited at high temperatures [Shimura *et al*., 1999; Smits *et al*., 1999; Wang *et al*., 1996].

For above reason, Hadad *et al*. [2005] isolated *Brevibacillus borstelensis* strain 707 from soil, which was more effective in degrading branched low-density polyethylene than *Rhodococcus* strain, although the biofilm forming capacity of the former was not found to be as good as of the latter. Still *B. borstelensis* was able to show reduction gravimetric

and molecular weights by 11 and 30% respectively for UV irradiated polyethylene. *Brevibacillus borstelensis* also degraded polyethylene in the presence of mannitol. Biodegradation of u.v. photo-oxidized polyethylene increased with increasing irradiation time. FTIR analysis of photo-oxidized polyethylene revealed a reduction in carbonyl groups after incubation with the bacteria.

Itavaara *et al*. [2002] demonstrated the biodegradation of polylactide in both aerobic and anaerobic thermophilic conditions. In thermophilic conditions, anaerobic biodegradation of PLLA was even faster than biodegradation in aerobic conditions, probably due to lactic acid being a more favourable substrate for anaerobic than for aerobic microorganisms [Zellner *et al*., 1994]. The clear effect of temperature on the biodegradability of PLLA indicates that its polymer structure has to be hydrolyzed before microorganisms can utilize it as a nutrient source.

To prove the effectiveness of thermophillic bacteria, Nanda and Sahu [2010] compared the biodegradability of polyethylene by *Brevibacillus* to that by *Pseudomonas* and *Rhodococcus*. The initial and final dry weights of polyethylene before and after incubation in the culture medium were compared and the percentage of degradation was calculated. *Pseudomonas* was found most efficient in degrading polyethylene with its biodegradability of 40.5% followed by *Brevibacillus* with 37.5% and *Rhodococcus* with 33% biodegradability, respectively.

Kim and Yoon [2010] isolated thermophilic strains (*Geobacillus tepidamans*, *Brevibacillus brevis* and *Brevibacillus limnophilus*) for the first time using activated sludge retrieved from waste water treatment plant of a poly(vinyl alcohol) (PVA) producing factory for biodegradation of PVA at relatively high temperatures. Isolation of thermophilic bacteria is beneficial because waste water contaminated with PVA mostly originates from fabric dyeing factories and is discharged relatively hot, and thereby the treatment of the waste water is usually carried out at relatively high temperatures. PVA degradation activity of the isolated strains was assessed at first by the halo zone size formed around the colonies and finally by the modified Sturm test. These strains

degraded PVA at 55°C much faster than *A. cholinophagum* SB98, a mesophilic bacterium, which was reported to degrade PVA as a single strain [Lee and Kim, 2003]. *G. tepidamans* was thermally more stable than the symbiotic pair in that symbiotic pair lost the activity completely at 65°C, while *G. tepidamans* still formed clear halo at the same temperature.

Knowledge of the biodegradability of chemicals is one of the most important aspects of their environmental behavior because a biodegradable substance is expected to cause less ecological problems in the long term than a persistent one [Pagga, 1997]. As biodegradability is a basic prerequisite for chemicals in general a number of biodegradation tests have been developed and standardized in the last 35 years [Pagga *et al*., 2001].

Actually, several test methods aimed at defining the potential biodegradability of polymeric materials have been developed by International Standard Organization (ISO) and regional organizations such as American Society for Testing and Materials (ASTM), European Committee for Standardization (CEN) and German Institute for Standardization (DIN) [Yang *et al*., 2005]. The test methods should be standardized as far as possible but they may be different in various tests. One important factor which cannot be standardized is the inoculums, the source of microorganisms for the test, and their state of acclimatization and adaptation [Pagga 1997].

Most of the examples mentioned above deal with fungi and bacteria based degradation. Based on the literature one could conclude that pretreated polymers degrade more easily than the untreated polymers. Also, degradation is more facile with starch and cellulose blended polymers.

Cell surface hydrophobicity and addition of surfactants showed an important role in biofilm formation, which is prerequisite condition for biodegradation. Degradation leads to decrease in molecular weight, tensile strength and viscosity, formation of new functional groups such as carbonyl, hydroxyl, etc.

Most of the studies had been done on polyethylene but little is known about degradation of polypropylene. Moreover, mesophilic microorganisms are used in most of the researches. Thermophilic, although are more effective but used less. In my thesis, I have focused on the biodegradation of linear low density polyethylene (LLDPE) and polypropylene (PP) by using thermophilic bacteria. In the course of study, I investigated the kinetics of the biodegradation activity of the biofilm formed on LLDPE and PP.

Chapter-3

MATERIALS & METHODS

3.1 Materials

3.1.1 Polymers

Commercial grade of linear low density polyethylene (LLDPE) (trade name Halene-L, melt flow index (MFI) 1.0 gm/10 min with 2.16 kg standard die at 190°C, grade 71601S), was obtained from Haldia Petrochemical Ltd., Haldia, India. Bi-axially oriented polypropylene (BOPP) films (one side corona treated, thickness 20 μm, density 0.89-0.91 g/cc, melting point: 160-170°C) were obtained from Max Specialty Films, Max India Group, India.

3.1.2 Bacterial strain

Brevibacillus borstelensis (MTCC number 1952) was procured from Microbial Type Culture & Gene Bank, Institute of Microbial Technology, Chandigarh. This bacterium is a thermophilic, gram-positive, spore-forming rod with a growth optimum at 50°C.

3.1.3 Media for cultivation

Nutrient broth (NB) (g/l containing: 5g peptic digest of animal tissue, 5g sodium chloride, 1.5g beef extract and 1.5g yeast extract) & Nutrient agar (NA) (g/l containing 5g peptic digest of animal tissue, 5g sodium chloride, 1.5g beef extract, 1.5g yeast extract and 15g agar) obtained from HIMEDIA Laboratories Ltd., India, were used to maintain the bacterial culture. In case of nutrient broth, Liquid cultures (100 ml) were incubated in flasks (250 ml) on shaker incubator (New Brunswick Scientific, USA, Excella E-24) at 130 rpm, 50°C. Whereas in case of nutrient agar, medium was first poured in a Petri-dishes then the bacterium was inoculated by taking 1 ml inoculums.

3.1.4 Media for inoculation & degradation

Bacterial strain used, is capable of utilizing polymer films as the sole source of carbon and energy, were grown on minimal medium containing (g/l of distilled water): 1g NH_4NO_3, 0.2g $MgSO_4.7H_2O$, 1g K_2HPO_4, 0.1g $CaCl_2.2H_2O$, 0.15g KCl, and 0.1g yeast extract, and 1mg/l of each of the following micro-elements: $FeSO_4.6H_2O$, $ZnSO_4.7H_2O$ and $MnSO_4$. All the chemicals are obtained from HIMEDIA Laboratories Ltd., India.

3.2 Methodology

3.2.1 Aerobic biodegradation (ASTM D 5247)

According to ASTM D 5247, aerobic biodegradability of degradable polymers by specific microorganisms is determined. Here in this work, specific microorganism used is *Brevibacillus borstelensis* and polymers used are LLDPE & PP. Pre-weighed films of the polymers are taken in 250 ml Erlenmeyer flasks containing 100 ml minimal medium inoculated with the culture. The flasks were incubated in shaker incubator at 130 rpm, 50°C under aerobic conditions for 16, 30 and 60 days. ASTM is currently developing standard practices for exposing degradable plastics to "real systems" environments and reporting the resulting data. The specific microorganisms test method does not represent any real world waste management infrastructure but provides a standard test method to quantify biodegradability using well-defined microbial cultures commonly present in the environment.

3.2.1.1 Pretreatment of polymers

The polymer films were cut into small pieces (3cm × 3cm) and were allowed to dry overnight at 60°C, and disinfected (30 minutes in 70% ethanol), and then weighed. Ethanol was used to remove any organic matter adhering to the surface. Then the films are added to flasks, each containing 100 ml of mineral medium.

3.2.1.2 Culture conditions

The bacterium is provided in freeze-dried form in a sealed depressurized ampoule. The ampoule was marked near the middle of the cotton wool with a sharp file. The surface around the mark was disinfected with ethanol. A thick cotton wool was wrapped around the ampoule and it was then broken. The pointed top of the ampoule was removed gently (this step had to be performed very carefully as hasty opening will release bacterial spores into the atmosphere).

Nutrient broth of approximate 200 ml volume was sterilized by autoclaving at 121°C, 15 psig for 15 minutes (Equitron, India). Sterilized nutrient broth medium of 0.3 to 0.4 ml volume was added to the ampoule to make a cell suspension. The cell suspension was added to two Erlenmeyer flasks of 250 ml capacity each containing 100 ml nutrient broth. These flasks were incubated for 24 hours at 50°C and 130 rpm in the shaker incubator (New Brunswick Scientific, USA, Excella E-24).

3.2.1.3 Enrichment of *Brevibacillus borstelensis*

Enrichment of *Brevibacillus borstelensis* was done by using nutrient broth as a medium. Two 250 ml Erlenmeyer flasks each containing 100 ml of nutrient broth were autoclaved at 121°C, 15 psig for 15 minutes (Equitron, India). Out of two, one flask was inoculated by bacteria by taking 4 ml inoculums and other was kept blank. Then both the flasks were kept in shaker incubator at 50°C & 130 rpm.

3.2.1.4 Film culturing

Preweighed disinfected films were aseptically added to 100 ml sterilized culture media. Films in culture medium were incubated with shaking for 24 h before inoculation to ensure asepsis. Culture media were inoculated with 4 ml of culture from mid-exponential phase and were incubated with shaking at 130 rpm for 16, 30 and 60 days at 50°C. Three replicates were prepared for each different pretreated film.

3.3 Analytical/ Testing procedures

3.3.1 Growth pattern in NB

The flasks containing *Brevibacillus borstelensis* in NB media were kept in shaker incubator at 50°C & 130 rpm. Flasks were withdrawn at regular intervals and OD was checked at 620 nm in UV-Vis Spectrophotometer (Perkin Elmer, USA, Lambda 35 model). It gives the growth pattern of the *Brevibacillus borstelensis* i.e. lag, log, stationary and death phase of the culture under controlled conditions can be estimated by measuring the OD at regular intervals. OD directly gives the log of number of microorganisms.

3.3.2 Growth kinetics in synthetic media (glucose as C-source)

The flasks containing *Brevibacillus borstelensis* in minimal media, taking glucose as a sole source of carbon, were kept in shaker incubator at 50°C & 130 rpm. Flasks were withdrawn at regular intervals and OD was checked at 620 nm in UV-Vis Spectrophotometer. This gives the curve representing how the culture behaves in different carbon sources. The composition of the media remains same as described earlier, except the carbon source.

3.3.3 Reducing sugar determination

Reducing sugar concentration was determined by dinitrosalicylic acid DNS using glucose as a standard. Sugars are assayed by using their reducing properties. One such compound is 3,5-dinitrosalicylic acid (DNS), which in alkaline solution gets reduced to 3-amino,5-nitro salicylic acid. The degree of color intensity formed due to the presence of reduced compound in the reaction mixture can be directly correlated with the amount of reducing sugar present in the media. The sugar must be estimated in cell free supernatant. 1 ml of media containing culture was centrifuged at 10,000 rpm, 4°C for 15 minutes in Eppendorf tubes.

The supernatant, then, was suitably diluted so that its sugar concentration falls between 0.1 to 1.0 mg/ml. O.D. of supernatant was measured at 540 nm. The standard curve was used to compute sugar concentration in media.

3.3.4 Evaluation of bacterial hydrophobicity

Bacterial cell-surface hydrophobicity was estimated by the microbial adhesion to hydrocarbon (MATH) test as described by Rosenberg et al. (1980), which is based on the affinity of bacterial cells for an organic hydrocarbon. The more hydrophobic the bacterial cells, the greater their affinity for the hydrocarbon, resulting in transfer of cells from the aqueous suspension to the organic phase and a consequent reduction in the turbidity of the culture.

For the MATH test, bacteria were cultured in NB media until mid-exponential phase, centrifuged at 10,000 rpm, 4°C for 15 min, washed, and suspended in phosphate urea magnesium (PUM) buffer containing (per liter): 22.2 g $K_2HPO_4.3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea and 0.02 g $MgSO_4.7H_2O$, pH 7.1. The washed cells were resuspended in PUM buffer to an optical density at 400 nm (OD_{400}) of 0.9-1.1. Cell suspension aliquots (2 ml) were then transferred to clean, round-bottom test tubes and 0.3 ml pet ether was added. After the mix was homogenized for 2 min, the hydrocarbon phase was allowed to separate completely and the aqueous phase was removed to determine the OD_{400}. Cell-free buffer served as the blank.

3.3.5 Screening of LLDPE and PP

LLDPE and PP films were taken with 100 ml of minimal media and 4 ml of inoculums in 250 ml Erlenmeyer flasks. The flasks were constantly monitored for first 16 days to confirm that the *Brevibacillus borstelensis* was able to form biofilm on polymer surfaces. 1.5 ml of media was taken every day from the flasks and measured the media at 620 nm to ensure the culture is alive in the media where polymer is the sole source of carbon.

3.3.6 Dry weight measurement

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 LLDPE and PP films, the bacterial biofilm was washed off the film surface with a 2% (v/v) aqueous sodium dodecyl sulphate solution for 4 hr and then with distilled water. The washed films was placed on a filter paper and dried overnight at 60°C before weighing.

3.3.7 Estimation of bacterial biomass colonizing the polymers

As the bacterial cells were strongly attached to the polymer surfaces, it was impossible to estimate the population density by standard techniques, such as direct cell counting or plating. Therefore, the population density of the biofilm on the polyethylene surface was estimated by determining the concentration of extractable protein. Colonized polymer samples taken from the bacterial liquid culture was washed briefly with water and then boiled for 30 min in 5 ml of 0.5 N NaOH. The suspension was centrifuged at 10,000 rpm, 4°C for 15 min, and the supernatant was kept aside and the pellet was subjected to the same procedure once again. The two supernatants were combined and the protein concentration was determined according to Folin-Lowry method as described by Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

3.3.8 Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared spectroscopy (FTIR) analysis is a useful tool to determine the formation of new and disappearance of functional groups. So degradation products, chemical moieties incorporated in the polymer molecules such as branched, co-

monomers, unsaturation and presence of additives such as antioxidants can be determined by this technique.

After incubation of polymer films in liquid medium for 16, 30 and 60 days, polymer films were analyzed by Fourier transform-attenuated total reflectance (FT-ATR) infrared spectroscopic using a ThermoSCIENTIFIC FT-IR spectrophotometer (Model Nicolet iS10) in the horizontal ATR mode, using a zinc-selenide crystal. A total of 2 scans were taken with a resolution of 4 cm^{-1}. It was used to detect the degradation on the basis of changes in the functional groups. A spectrum was taken at 400 to 4000 cm^{-1} wave numbers for each sample.

Chapter-4

RESULTS & DISCUSSION

4.1 Aerobic treatment

A wide variety of organic materials are easily degraded under aerobic conditions. In aerobic metabolism, O_2 is the terminal electron acceptor. When biodegradation follows this pattern, microbial populations quickly adapt and reach high densities. As a result, the rate of biodegradation quickly becomes limited by rate of supply of oxygen or some nutrient, not the inherent microbial capacity to degrade the polymer or other contaminant. Some organic compounds can also be degraded under anaerobic conditions. Under anaerobic conditions, oxygen is absent, nitrate (NO_3^-), sulphate (SO_4^{2-}), ferric iron (Fe^{3+}), manganese (Mn^{3+}, Mn^{4+}), and bicarbonate (HCO_3^-) can serve as terminal electron acceptors, if the microbes have the appropriate enzyme systems. The rate of degradation is usually limited by the inherent reaction rate of the active microorganisms; adaptation is slow, requiring months or years, and metabolic activity results in the formation of incompletely oxidized, simple organic substances, such as organic acids, and by-products such as methane or hydrogen gas.

So, aerobic treatment is much safer than anaerobic treatment. Microorganisms adapt easily and early in aerobic conditions; and the danger of release of harmful gases are less. Biodegradation results in complete oxidation of organic substances.

The present study deals with the biodegradation of linear low density polyethylene and polypropylene by using *Brevibacillus borstelensis* under aerobic conditions. The purpose to take polypropylene is that its usage is increasing day-by day but less biodegradation studied had been observed on it. This study also explains the degradation potential of *Brevibacillus borstelensis* by taking it in media having different carbon sources.

4.1.1 Growth pattern of *Brevibacillus borstelensis*

Growth pattern of *Brevibacillus borstelensis* should be discussed before doing further studies to evaluate the lag, log, stationary and death phases. 1 ml of inoculums was inoculated in 100 ml of nutrient broth taken in 250 ml Erlenmeyer flasks and kept in shaker incubator at 50°C, 130 rpm. Absorbance readings in terms of optical density (OD) were taken at fixed intervals at 620 nm (as shown in table 4.1 and fig. 4.1).

.**Table 4.1** Growth kinetics of *Brevibacillus borstelensis* in nutrient broth

Time (hours)	OD (at 620 nm)
0	0.006
1	0.009
2	0.01
3	0.015
5	0.041
6	0.274
7	0.497
9	0.72
12	0.978
13	1.009
14	1.052
15	1.087
16	1.075

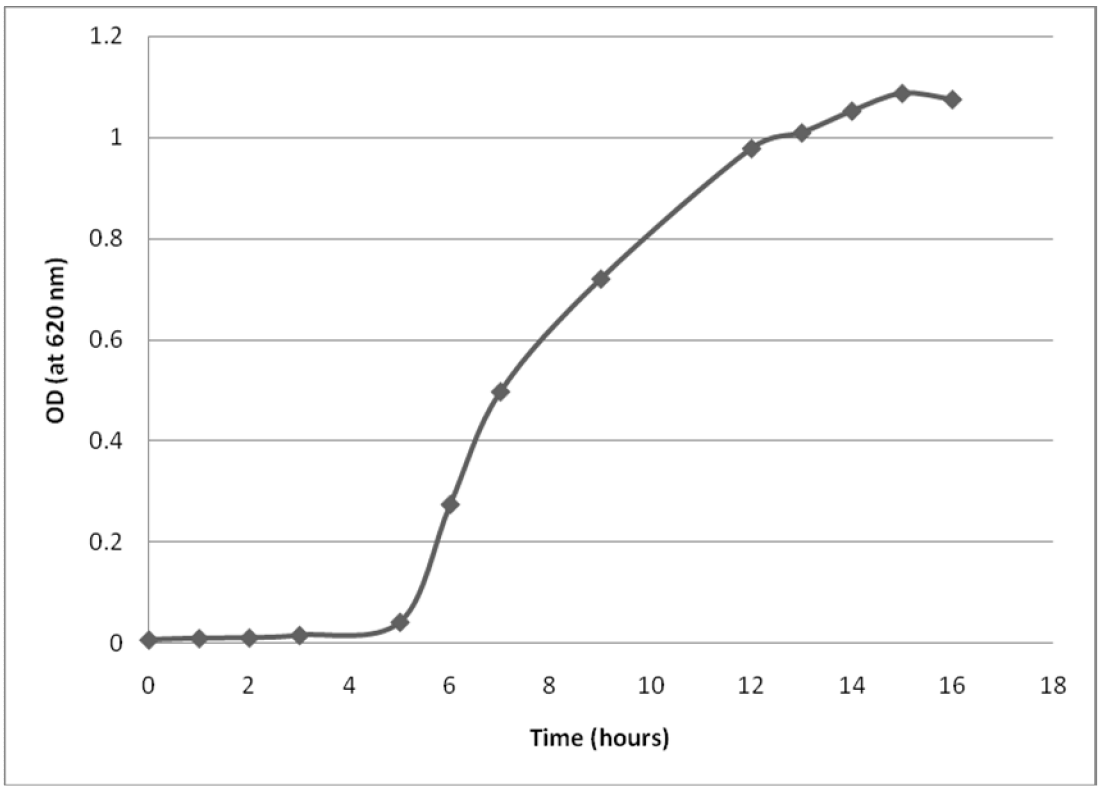

Fig. 4.1 Growth curve of *Brevibacillus borstelensis* in nutrient broth

By the fig. 4.1, we conclude that lag phase continues to 4-5 hours and then log or exponential phase goes from 5-13 hours then comes with stationary phase with little or no growth due to lack of nutrients. In this study, culture present in mid-exponential phase was taken for degradation purposes.

4.1.2 Growth kinetics in synthetic medium (glucose as a C-source)

Since the growth kinetics of *Brevibacillus borstelensis* in nutrient medium is understood, so now synthetic medium was taken to see its behavior in synthetic medium whose composition was same that of the medium used for polymer degradation, the only change is the carbon source i.e. here in this case glucose was taken instead of polymer to study its growth pattern in the media. Growth kinetics of *Brevibacillus borstelensis* in synthetic medium, glucose as a carbon source is shown in table 4.2 and fig. 4.2.

Table 4.2 Growth kinetics in synthetic medium (glucose as a carbon source)

Time (hours)	OD (at 620 nm)
0	0.023
2	0.068
4	0.166
6	0.489
8	0.769
10	0.950
12	1.089
14	1.087
16	1.079
18	1.075

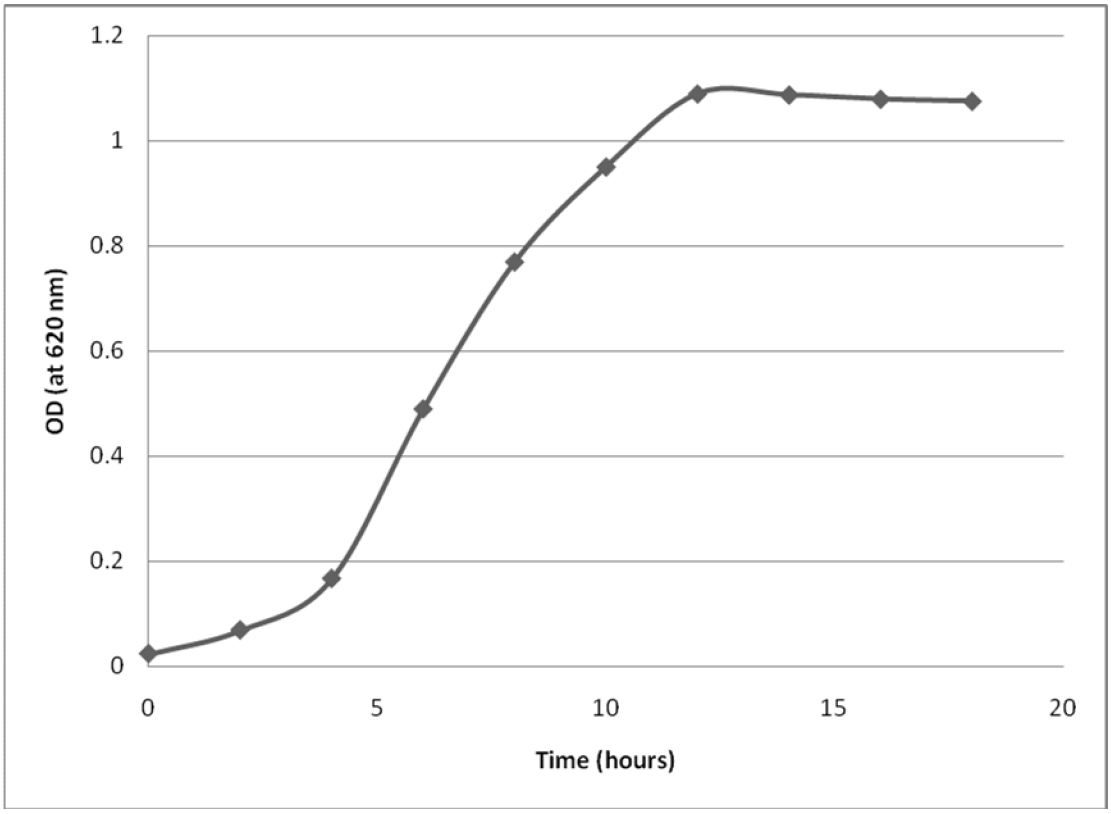

Fig. 4.2 Growth kinetics in synthetic medium (glucose as a carbon source)

From the fig. 4.2, we concluded that the growth curve was almost same as that of the nutrient medium. The culture was taken from mid-exponential phase in this case i.e. the culture was already active so takes less time to adapt in synthetic medium. Growth curve of culture represents the increase in number of microorganism with simultaneous decrease in glucose concentration.

4.1.3 Reducing sugar determination

With the increase of microbial growth in the synthetic medium containing glucose as a sole source of carbon, the concentration of glucose (carbon source) decreases. Readings were taken at fixed intervals at 540 nm for the determination of change of concentration of glucose. Table 4.3 and fig. 4.3 represent the data evaluated from it.

Table 4.3 Effect of incubation time on glucose concentration in terms of OD

Time (hours)	OD (at 540 nm)
0	1.089
2	1.079
4	0.986
6	0.821
8	0.681
10	0.549
12	0.485
14	0.412
16	0.409
18	0.399
20	0.389

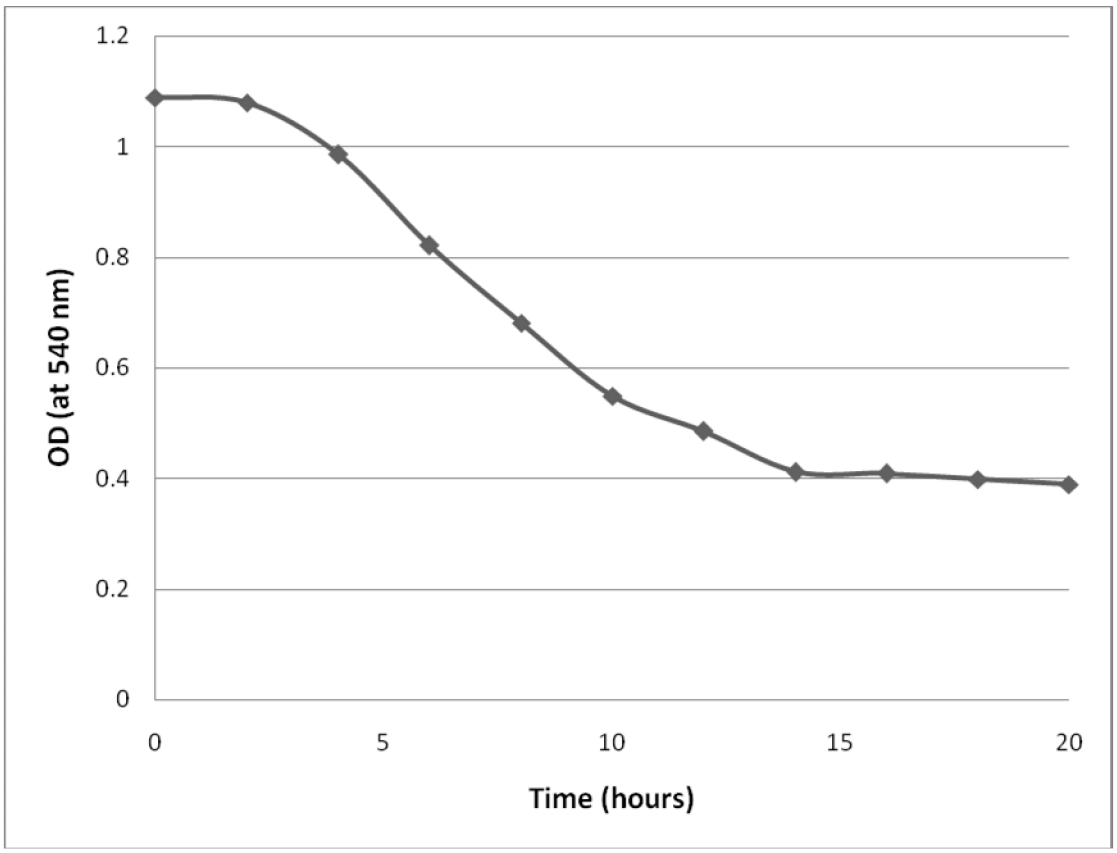

Fig. 4.3 Glucose concentration vs incubation time

OD is directly proportional to the concentration. The concentration of the unknown samples can be detected by the standard glucose curve which came from the OD measurement of the known concentration of glucose. The OD was compared with glucose standard curve to detect the concentration. The concentration that comes out is represented in table 4.4.

Table 4.4 Estimation of the glucose concentration

Time (hours)	OD (at 540 nm)	Conc.(mg/ml)
0	1.089	1.11
2	1.079	1.06
4	0.986	0.96
6	0.821	0.75
8	0.681	0.67
10	0.549	0.54
12	0.485	0.47
14	0.412	0.39
16	0.409	0.38
18	0.399	0.37
20	0.389	0.35

4.1.4 Estimating bacterial hydrophobicity

Cell surface properties are recognized as the key factors that influence bacterial adhesion to surfaces. Hydrophobic interactions define the strong attraction between hydrophobic molecules and surfaces in water [Kadam *et al*., 2009]. In biological systems hydrophobic interactions are the strongest long-range non-covalent interactions and are considered a determining factor in microbial adhesion to surfaces [Sanin *et al*., 2003]. The percentage of adhesion to hydrocarbons was calculated using the following expression:

Percentage of adhesion

$= 100 \times [OD_{400}$ (initial bacterial suspension)

$-OD_{400}$ (aqueous phase)] / OD_{400} (initial bacterial suspension)

Where,

OD_{400} (initial bacterial suspension) = 1.021

OD_{400} (aqueous phase) = 0.220

$$\text{Percentage of adhesion} = 100 \times [(1.021 - 0.220) / 1.021] = 78.45\%$$

Microorganisms can attach to the surface, if the polymer surface is hydrophilic. Since PP and PE have only CH_2 groups, the surfaces are hydrophobic. Initial physical or chemical degradation leads to the insertion of hydrophilic groups on the polymer surface making it more hydrophilic (insertion of hydrophilic groups also decreases the surface energy). Once the organism gets attached to the surface, it starts growing by using the polymer as the carbon source. The MATH assay clearly showed the higher hydrophobicity of *Brevibacillus borstelensis*. *Brevibacillus borstelensis* incubated in a liquid medium containing LLDPE and PP as the sole carbon source, colonized the films surfaces strongly and formed a thick biofilm. This finding was not surprising as formation of a biofilm on polymer films, which are hydrophobic, require the bacterial surface also to be hydrophobic, and the MATH assay demonstrated that the hydrophobicity of *Brevibacillus borstelensis* was very good.

4.1.5 Screening of LLDPE and PP

Table 4.5 and fig. 4.4 represent the growth behavior in two media containing same composition but different carbon source i.e. LLDPE and PP. OD readings were taken every day to watch the microbial activity. Screening is done to confirm the survival of *Brevibacillus borstelensis*. The results give the comparison of growth kinetics in two separate sources.

Table 4.5 Growth kinetics in synthetic media (LLDPE & PP as C-source)

Time (days)	**OD (at 620 nm)**	
	PP	**LLDPE**
0	0.036	0.035
1	0.28	0.297
2	0.336	0.368
3	0.322	0.256
4	0.426	0.436
5	0.465	0.476
6	0.483	0.531
7	0.515	0.627
8	0.55	0.689
9	0.575	0.725
10	0.61	0.76
11	0.65	0.805
12	0.69	0.841
13	0.725	0.861
14	0.745	0.889
15	0.765	0.912
16	0.775	0.923

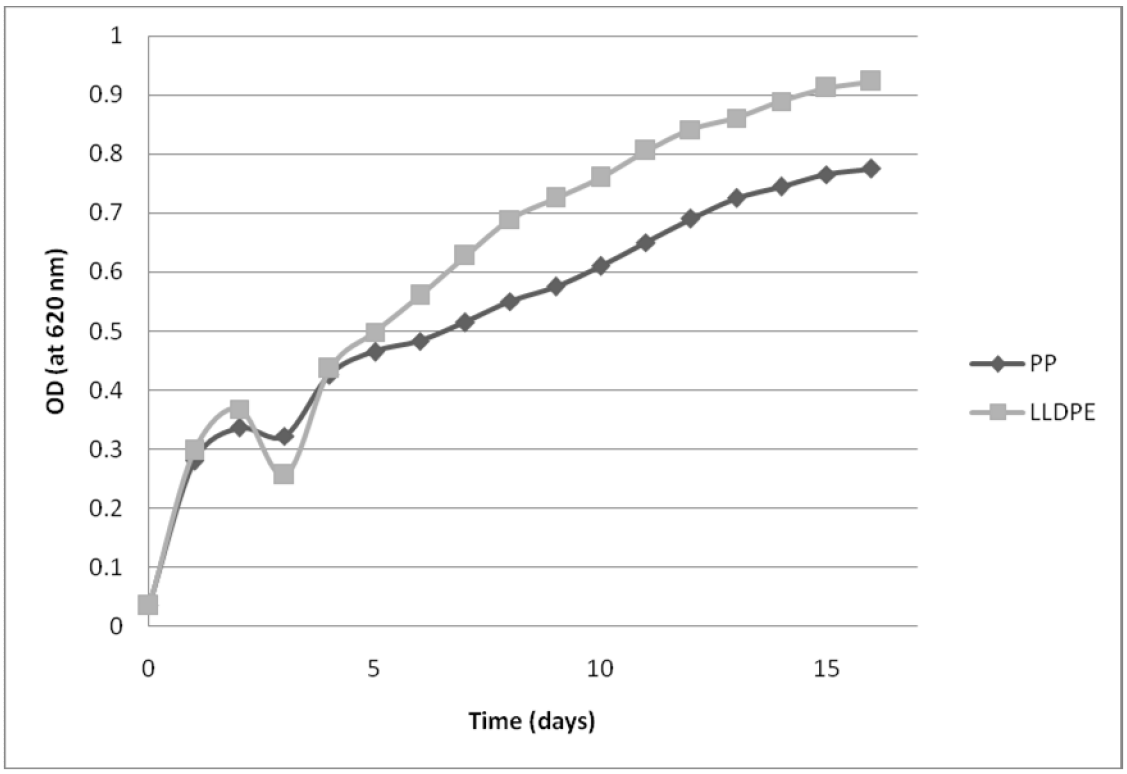

Fig. 4.4 Growth kinetics in synthetic media (LLDPE & PP as C-source)

It can be easily evaluated from fig.4.4 that growth of *Brevibacillus borstelensis* is higher in case of LLDPE as compared to PP. Initial increase was due to the presence of glucose in the media. Then there was sudden decrease in curve as culture took time to adapt in liquid media containing LLDPE and PP as a sole carbon source. Both the curves show the same pattern.

4.1.6 Dry weight measurement

Our standard biodegradation assay for LLDPE and PP lasts for *ca* 60 days. During this period, the bacteria utilize the carbonyl residues and reduce their concentration. Therefore, it was important to verify that the biodegradability of LLDPE and PP with a reduced level of carbonyls would not be diminished: in other words, would the bacteria consume a $[CH_2]_n$ backbone that was almost devoid of carbonyls? To answer this question, we tested the biodegradability of LLDPE and PP after 16, 30 and 60 days. After each incubation period, the polyethylene samples were washed, dried and weighed. The total reduction in weights of the LLDPE and PP samples is shown in table 4.6 and 4.7 respectively.

Table 4.6 Weight of LLDPE before and after incubation

No. of days	Weight before incubation (g)	Weight after incubation (g)	% weight loss
16	0.0659	0.0653	0.90
30	3.083	2.9046	5.70
60	3.346	3.006	10.16

Table 4.7 Weight of PP before and after incubation

No. of days	Weight before incubation (g)	Weight after incubation (g)	% weight loss
16	0.0298	0.0297	0.34
30	3.031	3.008	0.76
60	3.450	3.356	2.72

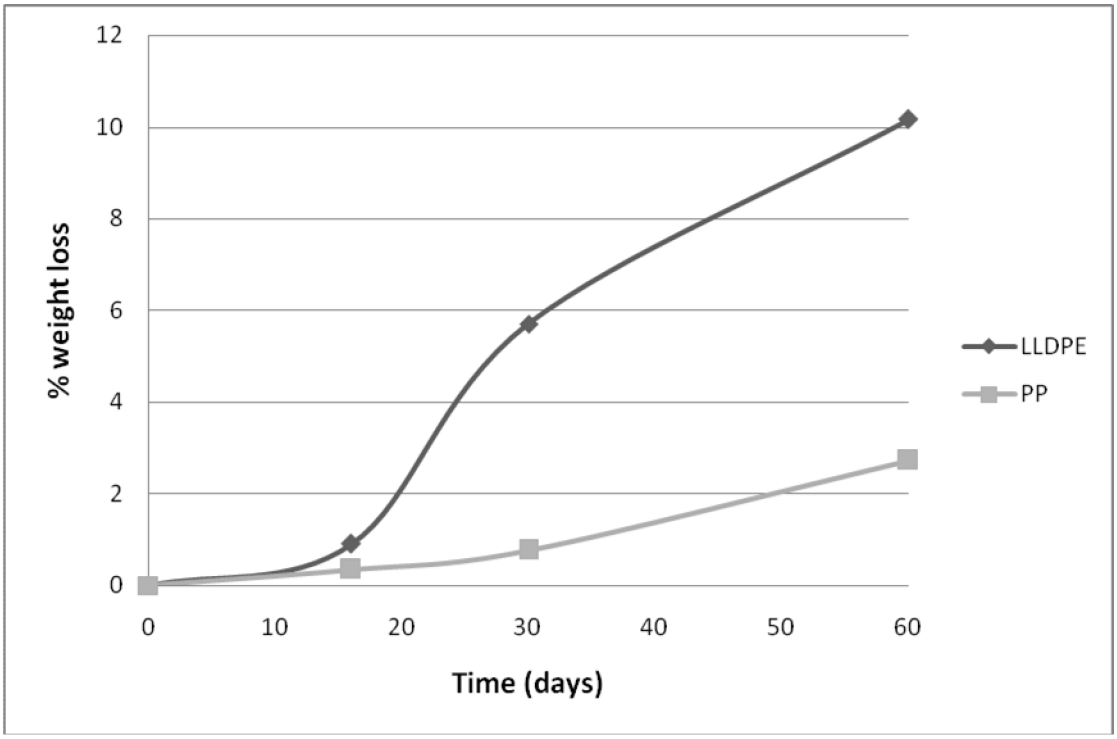

Fig. 4.5 Comparison of the % weight loss of both LLDPE and PP

Fig. 4.5 clearly shows the biodegradability in both the cases. LLDPE shows 10.16% loss in weight within 60 days whereas PP manages to degrade only 2.72%. One possible reason for this difference can be the structural difference of polymer. Even though PP is a polyolefin and prone to oxidative degradation similar to PE, the substitution of methyl in the place of hydrogen in the β-position makes it more resistant to microbial attack.

4.1.7 Protein estimation

Growth kinetics of biofilm formation on polymer surfaces was monitored by quantifying the total proteins extracted from the biofilm. The readings are taken after the interval of 16, 30 and 60 days at 660 nm. The OD was compared with the OD of the known sample i.e. bovine serum albumin (BSA) was taken as standard in this case. The resultant concentration is shown in table 4.8.

Table 4.8 Estimation of protein Concentration

Incubation time (days)	Protein concentration (µg/ml)	
	On LLDPE films	On PP films
0	0.06	0.08
16	4.8	3.4
30	2.1	1.7
60	1.9	1.4

The fig. 4.6 showed a biphasic pattern of biofilm formation on the polymer surfaces. The first phase was characterized by a steep increase in protein content during the first 16 days of incubation, reflecting an increase in the biofilm biomass. This phase was followed by a second period in which the protein content remained constant. Fig. 4.6 also represents the comparison of the concentration of protein extracted from LLDPE and PP films.

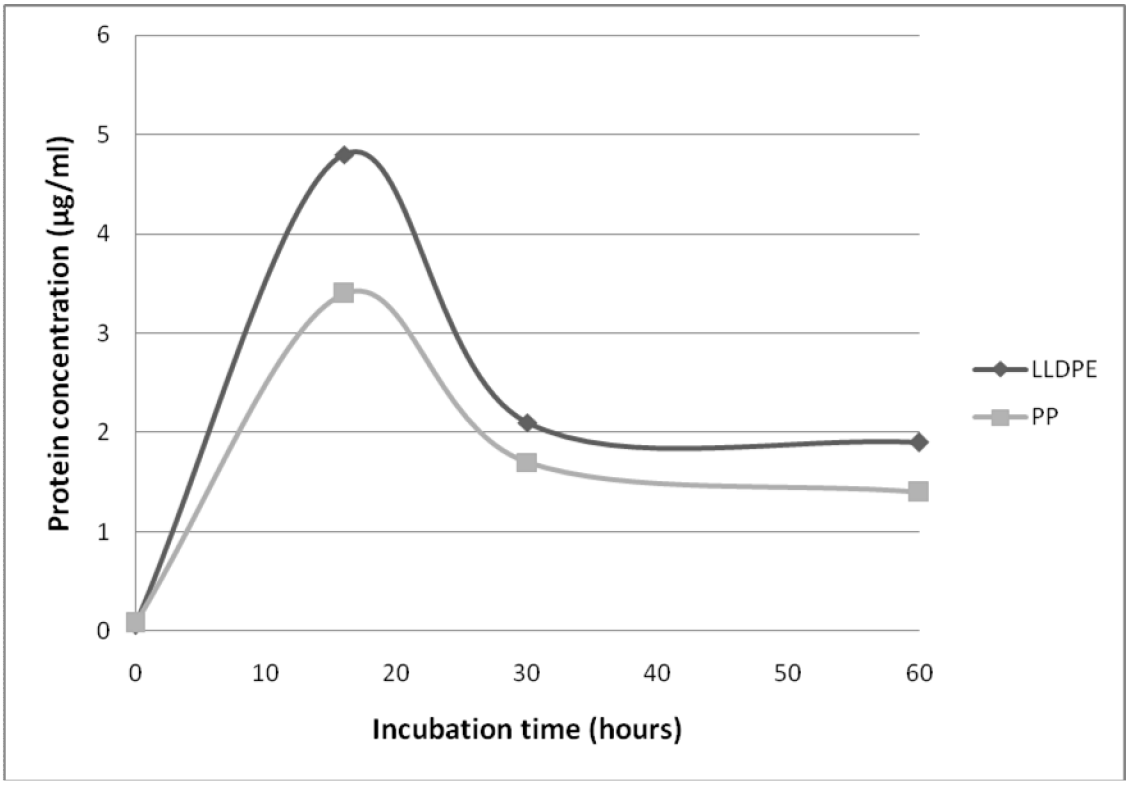

Fig. 4.6 Comparison of protein conc. in LLDPE and PP

4.1.8 Fourier transform infra-red (FTIR) spectroscopy

FTIR spectroscopy was performed to check the biodegradation of polymer films by the action of enzymes present in *Brevibacillus borstelensis*. FTIR analysis of polymer films showed decrease in peak from wavelength 2926.42 cm^{-1} to 2924.43 cm^{-1} (as shown in fig. 4.7-4.9). The decrease indicated the cleavage of C-H bonds. The appearance of some new peaks (shown by -C=C-) and increase in already existing peaks, at region of 1400-1600 cm^{-1}, indicating the formation of new intermediate products (as shown in fig. 4.7-4.13). Peaks present at 2865-2845 cm^{-1} and at 1485-1445 cm^{-1} are due to methylene C-H symmetric stretch and methylene C-H bend respectively. Peaks present in fig. 4.11-4.13 at 1470-1430 cm^{-1} and 1380-1370 cm^{-1} are due to methyl C-H asymmetric and symmetric bends respectively. Peak (shown in fig. 4.7) at 932.18 cm^{-1} is due to double bonds (H_2C=C-) which is absent in fig. 4.8-4.9, probably due to breakage of double bonds.

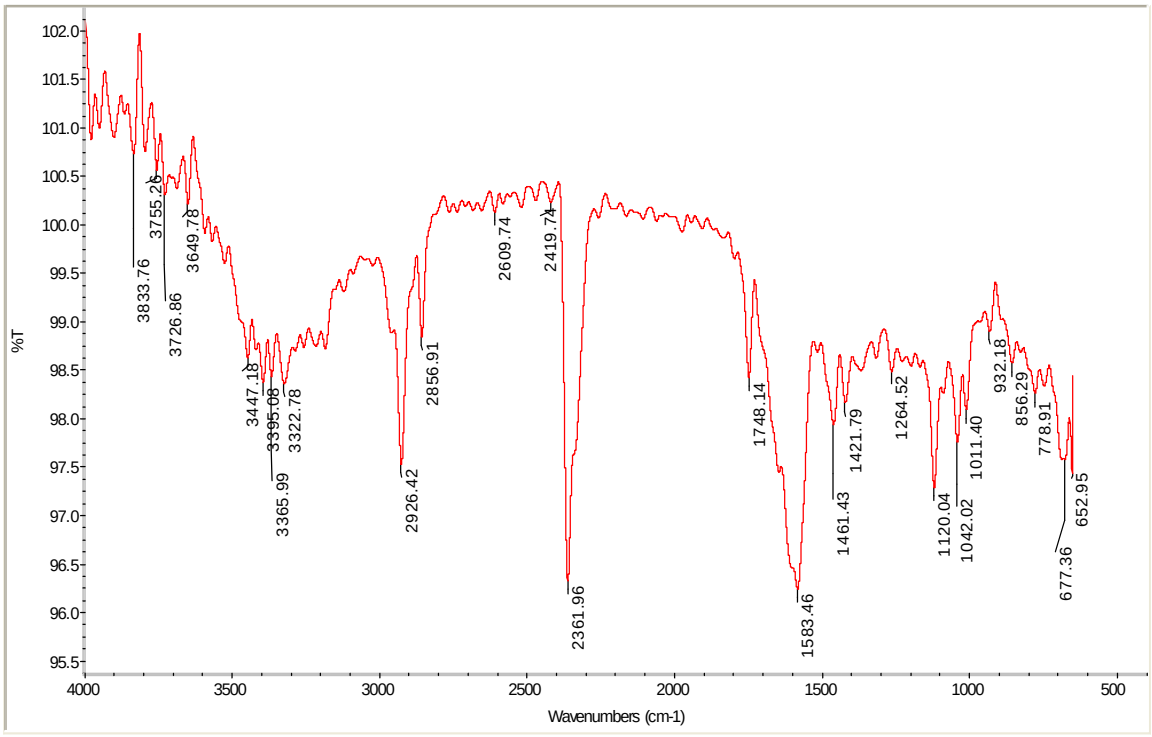

Fig. 4.7 FTIR spectra of untreated LLDPE

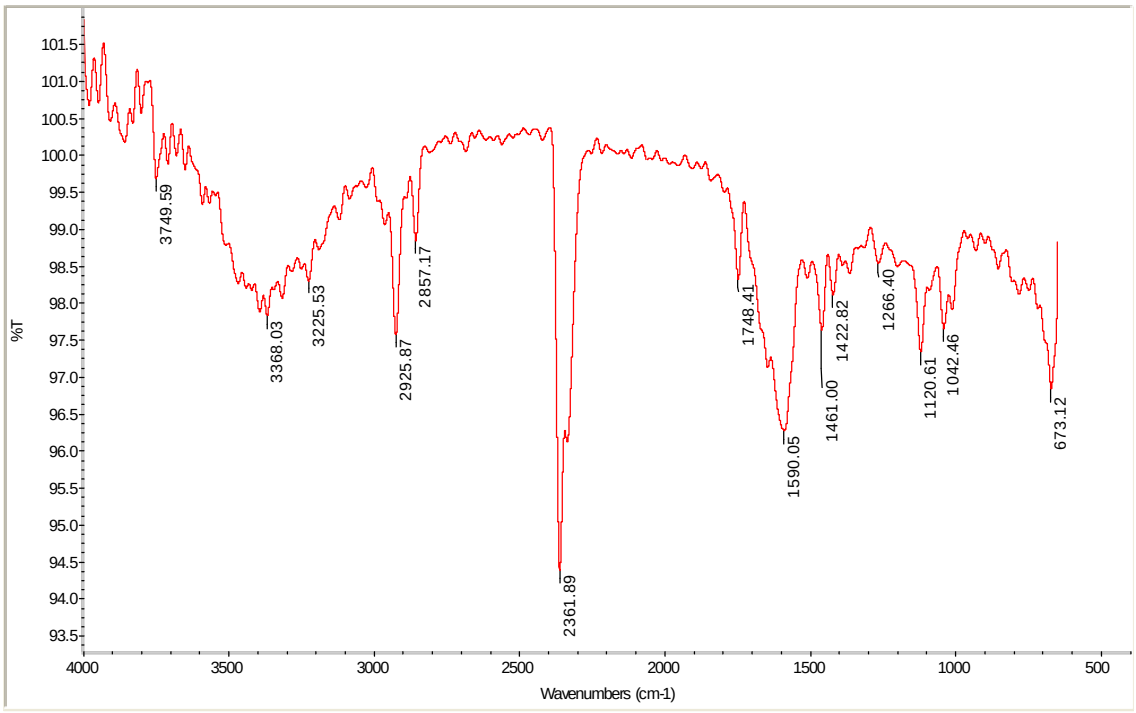

Fig. 4.8 FTIR spectra of LLDPE after 16 days of incubation

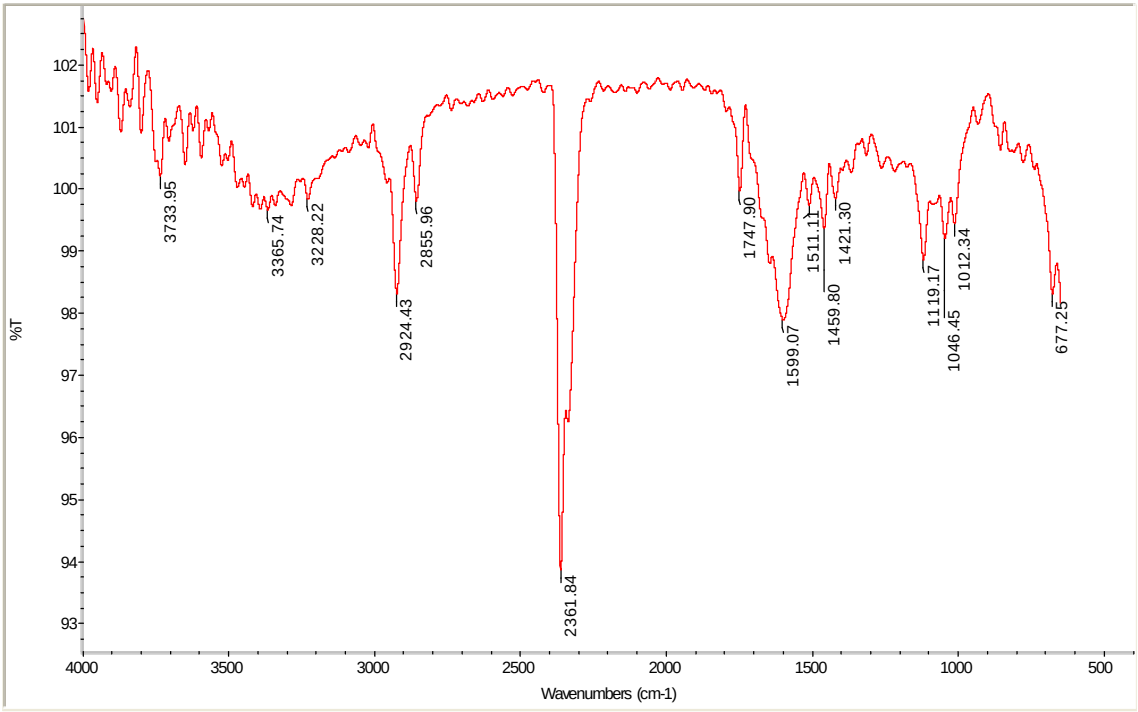

Fig. 4.9 FTIR spectra of LLDPE after 60 days of incubation

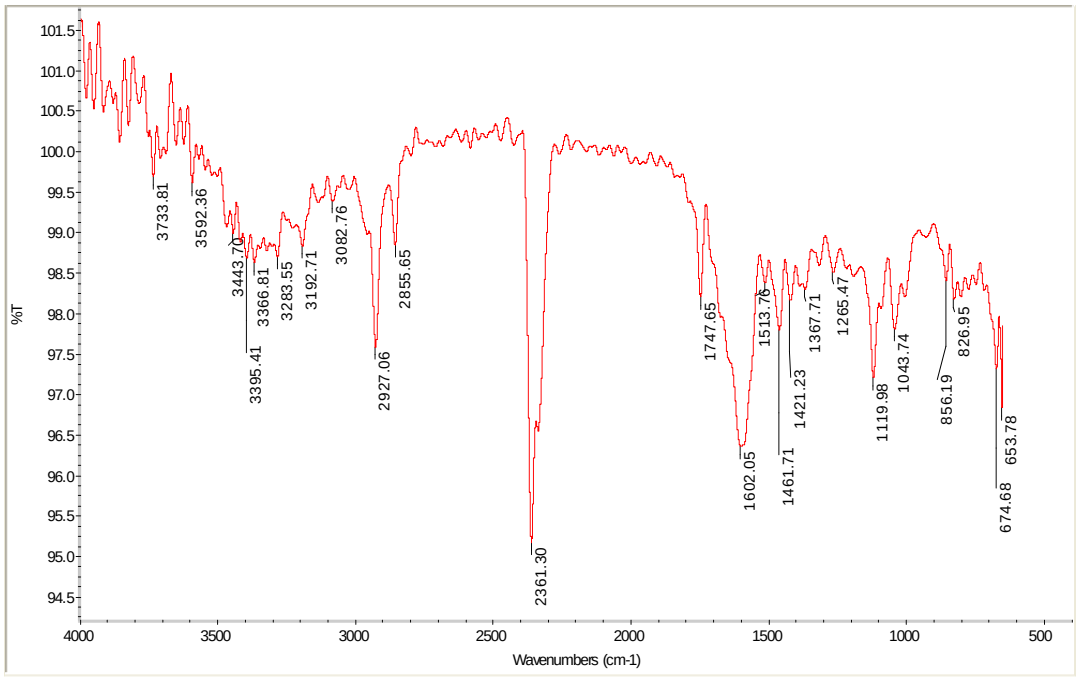

Fig. 4.10 FTIR spectra of untreated PP

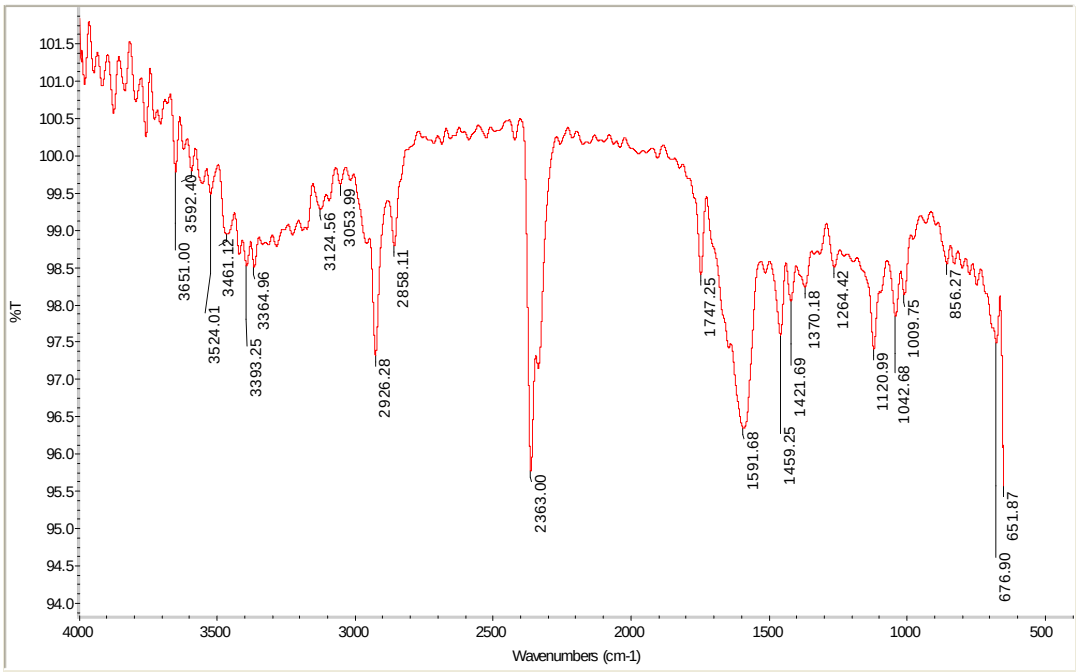

Fig. 4.11 FTIR spectra of PP after 16 days of incubation

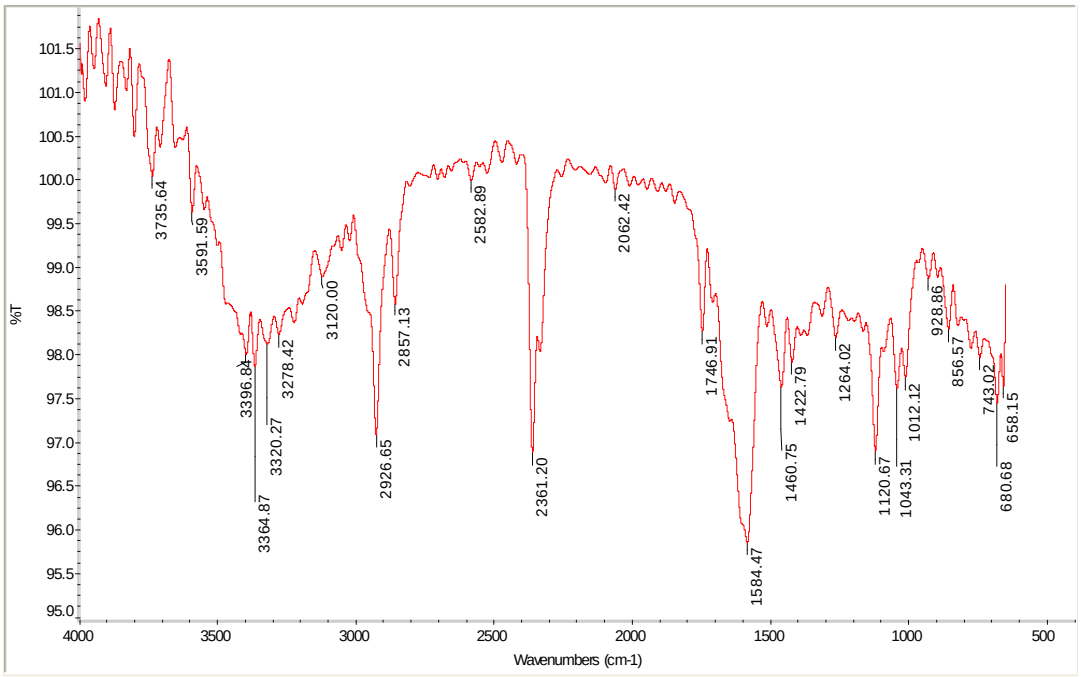

Fig. 4.12 FTIR spectra of PP after 30 days of incubation

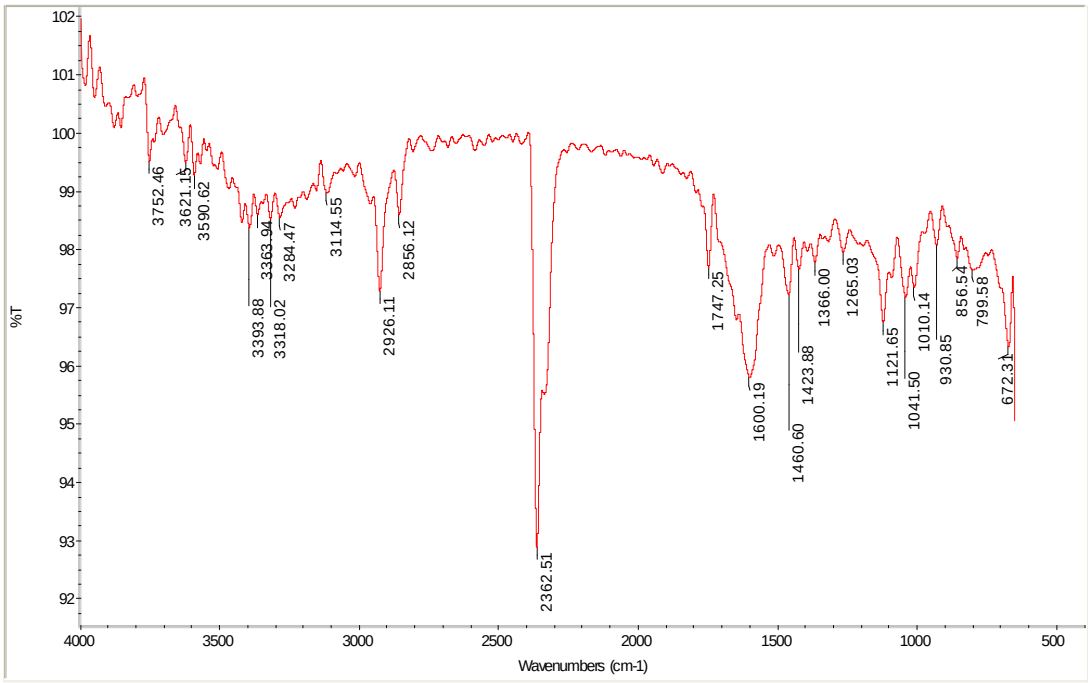

Fig. 4.13 FTIR spectra of PP after 60 days of incubation

Chapter-5

CONCLUSIONS & FUTURE PROSPECTS

5.1 Conclusions

One of the key advantages of a pure culture biodegradation assay is the ability to identify what portion of the degradation is due to chemical degradation and what can be attributed directly to biological degradation. This study demonstrates that polymers-considered to be inert-can be biodegraded if the right microbial strain is used. Enrichment culture methods were effective for enhancing the capabilities of a bacterium capable of utilizing LLDPE and PP as the sole carbon and energy sources. Maximal biodegradation was obtained in case of LLDPE. Although biodegradation of PP was less but culture was able to survive in PP media and also shows some degradation. It is clear from the study that *Brevibacillus borstelensis* was able to degrade the CH_2 backbone of polymers. Biodegradation leads to decrease in molecular weight and formation of new functional groups such as carbonyl, hydroxyl etc.

5.2 Future prospects

1. Modify the polymer for microbial utility by the (i) addition of natural polymers and/ or prooxidants to PP; (ii) modification of polymer by protein hydrolysates; and (iii) pretreatment of the polymer.
2. Modify the microbes to utilize the polymer by (i) modifying medium composition and thus enhancing the utilization of polymer; and (ii) genetically modify the microorganism to utilize the polymer.
3. Overexpress the enzyme, which is responsible for degradation and purify it and utilize for this purpose.

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