3.1 Experimental

Present work is concerned with the degradation of waste as well as recycled polyethylene films of different grades by using native microbes (i.e. biodegradation). For the biodegradation study, isolated as well as lab isolate microbes have been used. Microorganisms have been isolated from the soil samples collected from different plastic/garbage waste dumpsites. Various parameters have been optimized for the effective biodegradation. Details of the chemicals and materials used and procedures employed are given in the following sections.

3.2 Materials

3.2.1 Collection of waste and recycled polyethylene

Low density polyethylene (LDPE), high density polyethylene (HDPE) and recycled polyethylene films of different grades used in this work were collected from the local market, Lanka, Varanasi, Uttar Pradesh, India. Thickness of the films were measured by using "Electronic outside micrometer, Schut".

3.2.2 Collection of soil samples from plastic waste dumpsites

Soil samples were collected from different locations of plastic waste dumpsites situated at Bhagwanpur, Lohtubeer and Ramana in Varanasi, UP, India. The particular sites have been chosen since they are being used for dumping plastics since long, hence increasing the chances of finding organisms that can degrade plastics. Soil samples were brought to the laboratory and stored at 4°C until used.

3.3. Chemicals and reagents

Chemicals and reagents used in different experiments are presented in table 3.1.

Table3.1 Chemicals and reagents used in experiments

S.N.	Chemicals/materials	Grade	Source
1	Potato Dextrose Agar	Analytical grade	Himedia
2	Potato Dextrose Broth	Analytical grade	Himedia
3	Nutrient Broth	Analytical grade	Titan Biotech
4	Nutrient Agar	Analytical grade	Titan Biotech
5	Tween-80	Analytical grade	Sigma
6	Bleaching powder	Analytical grade	Himedia
7	Ethanol	Analytical grade	Sigma
8	Sodium dodecyl sulfate	Analytical grade	Sigma
9	Diethyl ether	Analytical grade	Sigma
10	Gluteraldehyde	Analytical grade	Sigma
11	K_2HPO_4	Analytical grade	Sigma
12	HCl	Analytical grade	Sigma
13	NaOH	Analytical grade	Sigma
14	Guaiacol	Analytical grade	CDH
15	Sodium acetate	Analytical grade	Titan Biotech
16	Glacial acetic acid	Analytical grade	CDH

3.4 Isolation, screening and identification of polyethylene degrading microbes

3.4.1 Media preparation

For the preparation of Nutrient Agar (NA) media 500 ml of distilled water was taken in a 1L conical flask. 14 g of nutrient agar powder and 7.5 g 100 ml^{-1} of agar powder were mixed well in the distilled water and the volume was further maintained to 1000 ml. This solution was heated for 5 minutes in order to get a homogenate solution. The media was autoclaved at 121º C, 15psi, for 30 min.

For the preparation of Potato Dextrose Agar (PDA) media, 500 ml of distilled water was taken in a 1L conical flask. 20 gm of Potato Dextrose Agar (PDA) powder and 7.5 gm 100 ml^{-1} of agar powder were mixed well in the distilled water and the volume was further maintained to 1000ml. The media solution was heated for a few minutes and autoclaved at 121º C, 15psi, for 30 min.

3.4.2 Isolation and purification of microbial culture

In the present study, serial dilution technique was used to isolate the microorganisms from the mixed culture in sample. The soil sample was collected from plastic dumping sites near Ramana, Lohtubeer and Bhagwanpur, Varanasi. Soil was dug few meters in depth and was collected in a container. Isolation of the bacterial and fungal colonies was done by using the serial dilution method and the spread plate technique.

3.4.3 Serial dilution

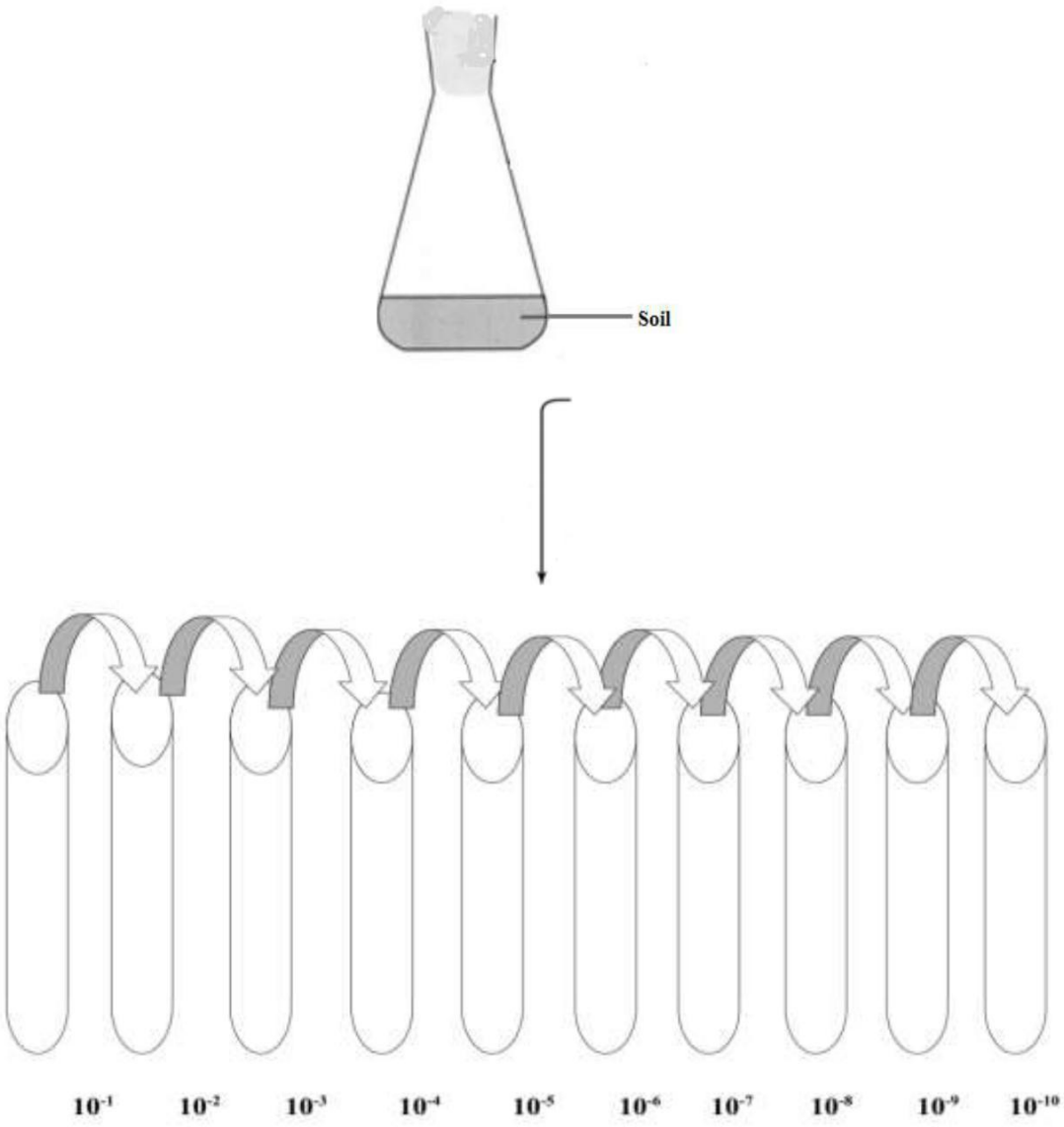


Fig.3.1 Serial dilution

(A). 1g of soil sample was mixed with 99ml distilled water and was stirred for 23 h and was serially diluted.

Ten sterilized test tubes were taken with 9 ml of autoclaved distilled water in each. 1 ml of sample was taken from stock solution and added in the first test tube and the whole solution was mixed thoroughly. From the first test tube, one ml sample was added to the next test tube. Subsequently, the same process was repeated till the last test tube.

Micropipette was used for measuring 1 ml solution. The dilution was performed under sterilized conditions in the laminar air flow.

(B). 20 µL of diluted samples were taken from the test tubes. The diluted samples were properly spread on different plates (NA and PDA) with a sterilized spreader and the polythene strips of 3×3 cm were cut and placed on different plates.

(C). The plates of selective media (NA and PDA) were transferred in the incubators maintained at 25-40±1^{o}C for 12 h for bacterial and at 30-50±1°C for 24-48 h for fungal strains and waited for 12 h for the observation of bacterial and 24-48 h for the observation of fungal strains. After the incubation the growth of microorganism were examined on the polythene strips. (Fig.3.2)

(D). Morphologically distinct colonies that appeared on the plates were further purified. The purified isolates were routinely sub-cultured at an interval of 30 days and preserved at 4°C in the refrigerator on NA and PDA slants and at –20°C as glycerol stocks in the deep freeze for short & long-term storage, respectively.

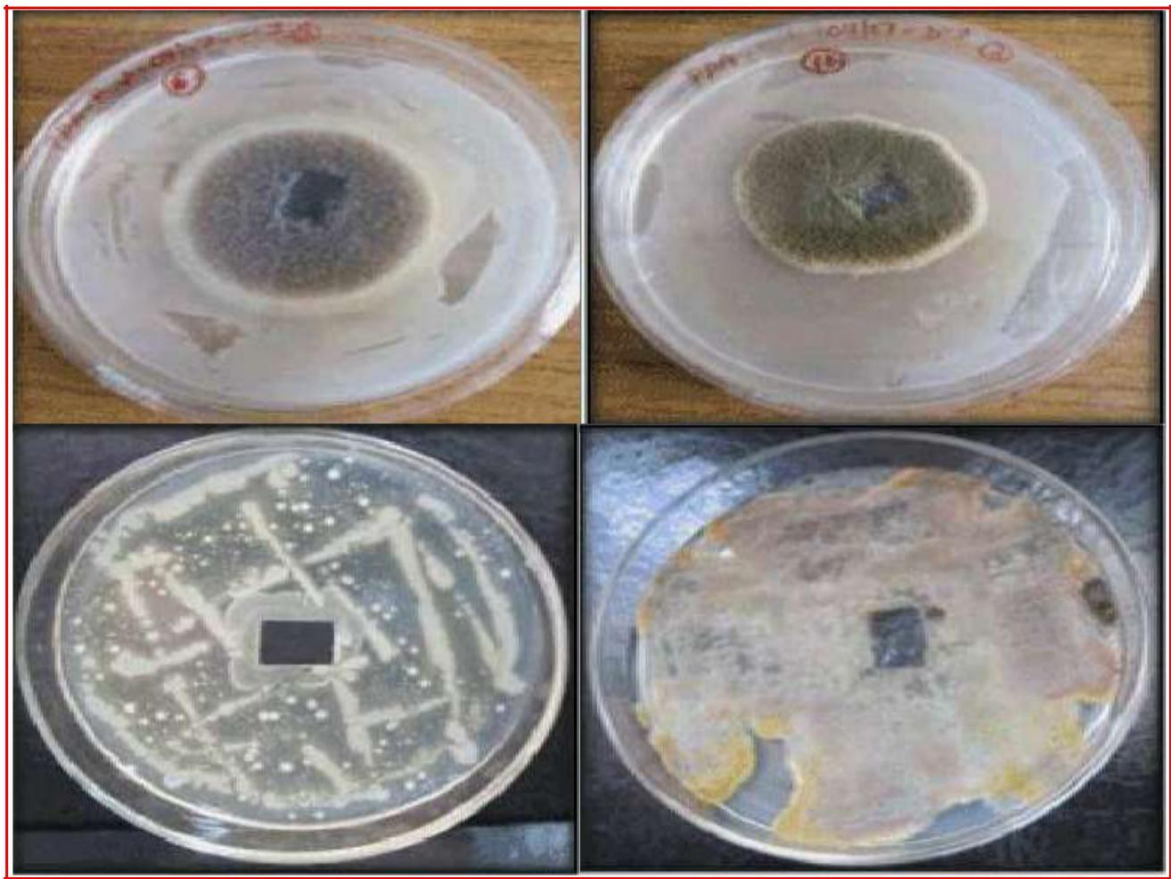


Fig.3.2 Showing different bacterial and fungal strains growing on the PE surface

3.4.4 Characterization of bacterial and fungal cultures

3.4.4.1 Morphological characterization

The bacterial and fungal cultures were evaluated for various characteristics such as the structure, shape, size, surface, margin, and texture of colony. The cell preparations stained through the Gram staining were examined under a microscope for various cellular characteristics such as shape, size, spore formation, and arrangement of cells.

3.4.4.2 Molecular identification procedure of selected bacterial strains

DNA was isolated from the isolated bacterial cultures, and their quality was evaluated on 1.2% agarose gel. Fragments of 16S rDNA gene were amplified by PCR using 8F and 1492R primers from the above isolated DNA. The PCR amplicon was

purified and further processed for sequencing. Forward and Reverse DNA sequencing reactions of PCR amplicon were carried out with **704F** and **907R** primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of **1412 bp** 16S rDNA gene was generated from forward and reverse sequence data using the aligner software. The obtained 16S rDNA gene sequence was used to carry out BLAST alignment using search tool of NCBI gene bank database. Based on the maximum identity score, first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5.

3.4.4.3 Molecular identification procedure of selected fungal strains

DNA was isolated from the fungal cultures and their quality was evaluated on 1.2% agarose gel, a single band of high-molecular weight DNA has been observed. Isolated DNA was amplified with 18S rRNA Specific Primer (1Fand 4R) using Veriti® 99 well Thermal Cycler (Model No. 9902). A single discrete PCR amplicon band of 900 bp was observed. The PCR amplicon was enzymatically purified and further subjected to Sanger Sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with **1F** and **4R** primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of **905 bp** of 18S gene in SSU region was generated from forward and reverse sequence data using aligner software. The 18S gene in SSU region sequence was used to carry out BLAST alignment search tool of NCBI genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5.

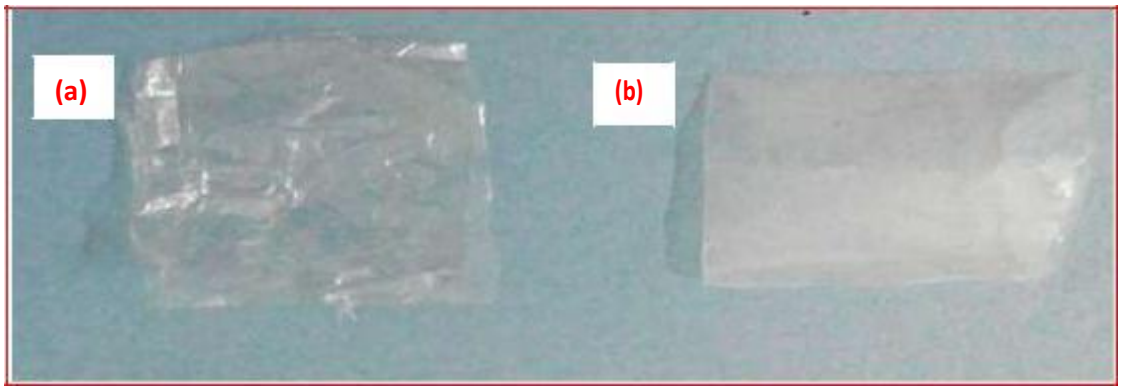
3.5 Biodegradation experiments

Table 3.2 presents list of microbes used in biodegradation study of polyethylene

Table 3.2 Various microorganisms used in the biodegradation study of HDPE and LDPE

S.N.	Microorganism	Accession No.	Isolation Condition/place of isolation	Degradation conditions
1	*Klebsiella pneumoniae* CH001	MF399051	Lohtubeer, Varanasi	Batch studies , shaking at 110rpm, temp.30ºC
2	*Brevibacterium leuteolum*	MF188996	Ramana, Varanasi	Batch studies , shaking 110 rpm, 37ºC
3	*Leifsonia shinshuensis* SA1	MF399050	Ramana, Varanasi	Batch studies shaking 120 temp. 35ºC
4	*Aspergillus fumigatus* fungus1	MF195023	Bhagwanpur, Varanasi	Batch studies shaking 140 rpm temp. 40ºC
5	*Aspergillus niger* fungus 2	MF195024	Lohtubeer, Varanasi	Batch studies, shaking at 135rpm temp. 40º C
6	*Aspergillus awamori* fungus 3	MF195025	Lohtubeer, Varanasi	Batch studies, shaking at 130rpm temp. 40º C
7	*Rhizopus oryzae* NS5	KT160362	Agricultural field, Banaras Hindu University (BHU), Varanasi	Batch studies, shaking at 120rpm temp. 40º C

Figure 3.3(a-b) and 3.4 show pictures of polyethylene films used in biodegradation assay



Tensile strength 25 MPa **Tensile strength 30 MPa**

Fig.3.3 Images of waste LDPE (a) and HDPE (b) films

Tensile strength 19 MPa **Tensile strength 16 MPa** **Tensile strength 12 MPa**

(Grade I) **(Grade II)** **(Grade III)**

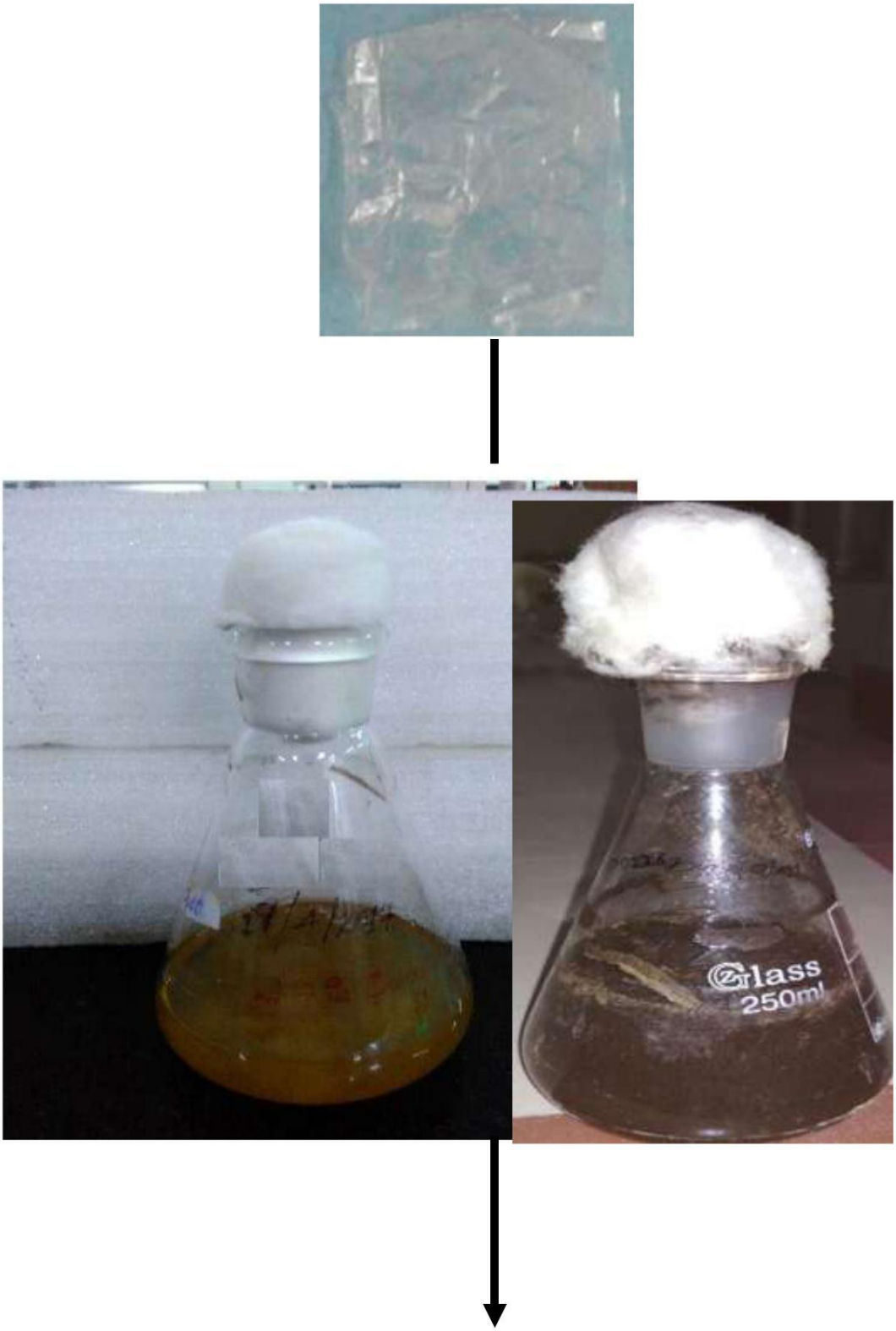
Fig.3.4 Recycled polyethylene films of different grades

3.5.1 Pretreatment of polyethylene films

To enhance the degradation of polyethylene films (Fig 3.3 and 3.4) the films were treated thermally by placing them in a preheated hot air oven at 70^{o}C for 10 days. Prior to transfer to liquid medium, strips were cut (10 × 4.5 cm), washed and disinfected with a solution containing 7 ml Tween-80, 10 ml bleach, and 983 ml sterile water. The films were then transferred aseptically into 70% (v/v) ethanol solution for 30 min to further sterilize them followed by drying overnight at 45 -50^{o}C and then were weighed.

3.5.2 Preparation of inoculums

100 ml NB and one loopful bacterial strains were incubated at 110 rpm and 37^{o}C for 24 h. Inoculum volume ranges from 1% (v/v) to 10% (v/v) were used for the biodegradation experiments and maximum biodegradation was found with 10% (v/v) actively growing inoculums having O.D. 0.8-1.0. Therefore, inoculum size of 10% (v/v) was used in all further experiments. 0.1ml was subsequently used as inoculum to study the effect of various factors/ conditions for biodegradation studies. In case of fungal strain 100 ml of Potato dextrose broth (PDB) and one loopful fungal strain were incubated at 120 rpm and 40^{o}C for 24 h. 10% (v/v) actively growing cultures having O.D. of 0.8-1.0 were used as inocula in all the further experiments. 0.1ml was subsequently used as inoculums to study the effect of various factors/ conditions for biodegradation studies. The biodegradation of polyethylene experiments were carried out in 250 ml Erlenmeyer flasks containing 100ml nutrient broth/potato dextrose broth supplemented with polyethylene (10 × 4.5 cm) films. The media were inoculated with respective bacterial or fungal culture by addition of inocula having uniform optical density (O.D. 0.8) (Fig 3.5).



Estimation of polyethylene biodegradation

Fig.3.5 Biodegradation assay

3.5.3 Optimization of various parameters affecting degradation of polyethylene (Batch mode under shaking condition)

The biodegradation experiments with bacterial strains were carried out in 250 ml Erlenmeyer flasks containing 100ml nutrient broth supplemented with PE films. The media were inoculated with respective bacterial strains by the addition of inocula having uniform cell density (O.D. 0.8).

For the biodegradation study with fungal strains 100 ml potato dextrose broth supplemented with PE films was taken in 250 ml Erlenmeyer flasks and media were inoculated with respective fungal strains by adding the inoculums with uniform cell density (O.D. 0.8). The process parameters optimized for the biodegradation of polyethylene films by bacterial and fungal strains were static/shaking condition, pH and temperature. To study the effect of pH it was adjusted using 0.01N NaOH and 0.01N HCl in the range of (3-10). To study the effect of temperature biodegradation study was carried out at different temperatures (25-50^{O}C) and to study the effect of static/shaking conditions experiments were performed at static condition as well as different shaking speeds ranging from 90 to 140rpm at 10 rpm interval with bacterial strains. In case of biodegradation studies by fungal strains range of these parameters were changed as follows: pH (3-10), temperature (25-50^{O}C) and shaking conditions (100-150rpm, 10 rpm interval). The uninoculated flasks kept under identical conditions were used as control. Samples (PE films) were withdrawn at regular interval (10 days/ 15 days) and got characterized by various techniques. In case of static conditions degradation was extremely slow and it was hardly possible to make even a rough estimation regarding the time necessary for the degradation to some substantial extent so further all the experiments were performed at shaking conditions.

3.5.4 Preparation of cell free extract and enzyme assays:

The isolated bacterial and fungal strains were incubated with and without polyethylene for 24 h. The resultant biomass was collected by centrifuging the respective broths at 8000 rpm for 15 minutes. The resultant cell pellets were sonicated for 1 minute using ultrasonication probe in 50mM sodium phosphate buffer (pH7.4) at 4^{o}C with 12 strokes of 30s. The sonicated cells were centrifuged and supernatant was used as the source of intracellular and extracellular enzymes. The enzyme activities were assayed spectrophotometrically for cell free extract with and without sonication at room temperature. One unit of enzyme activity is the amount of enzyme required to convert 1 µM of substrate per min. Laccase assay were performed as follow: 1 ml of the culture supernatant was added with one ml of 2mM guaiacol and 3ml 10mM sodium acetate buffer (pH 4.6). The reaction mixture was incubated at 30^{o}C for 15 mins. The color change was measured using spectrophotometer at 450 nm. One unit of laccase activity was defined as amount of enzyme required to hydrolyze guaiacol during incubation period. For the enzyme activity calculation of manganese peroxidase same procedure was used but for the reaction mixture 1 ml of H_2O_2 was added.

3.6 Analytical investigation

3.6.1 Microbial film harvesting

After incubation, PE films were removed from the conditioned media and washed with 2% (v/v) aqueous SDS and distilled water repeatedly through mild shaking for few minutes and finally flushed with 70 % (v/v) ethanol to remove cell mass from the residual films at the maximum and then dried at 45^{o}C -50^{o}C overnight and used for further analysis.

3.6.2 Measurement of change in weight of the polyethylene films

The weight loss was measured by measuring weight of film before incubation and after incubation with balance (Sartorius BSA224S-CW) and was calculated by the formula written below:

$$\text{Percentage of weight loss} = \frac{(\text{Initial weight} - \text{Final weight})}{\text{Initial weight}} \times 100$$

3.6.3 Measurement of change in mechanical strength:

The universal testing machine (Model INSTRON 4206) with a crosshead speed of 10 mm/min was used to estimate the mechanical strength of the treated and control PE strips at room temperature. Tensile strength of the incubated PE films was measured after regular time interval (days). Triplicates of samples were used for the measurement.

3.6.4 Measurement of Change in pH

Change in the pH of NB and PDB was measured before and after incubation using Elico LI614 pH Analyzer at regular time intervals for the biodegradation study of HDPE, LDPE and rLDPE.

3.6.5 Measurement of contact angle

PE films were washed with distilled water and the contact angle of PE films was measured at regular interval (days) at room temperature using a contact angle measuring unit (Digital drop method, Kruss, Contact Angle Analyzer Serial no: 30001712, Model no: DSA255). The wetting liquid used for this purpose was Millipore grade distilled water. Calculations were averaged from three measurements.

3.6.6 Microscopic analysis of polyethylene degradation

Microscopic analysis was performed to examine the surface morphology of film after degradation by the bacterial and fungal strains. PE films were recovered from the conditioned media and polyethylene films were fixed overnight by incubation at 4◦C in 4% glutaraldehyde in (pH 7.3). The samples were rinsed in 0.05 M phosphate buffer (three times, 10 min each) and were then subsequently dehydrated in a series of alcohol. Thereafter, the films were air-dried overnight, and the surface morphology of the microbe-treated and untreated films was examined by a scanning electron microscopy (SEM) (ZEISS, EVO18) and atomic force microscopy (AFM) (Oxford INSTRUMENT X-act NT-MDT). The polyethylene films were cut into small strips, coated with gold, and examined under SEM. In AFM analysis, all images were obtained with a scan speed of 1.0 Hz and a resolution of 512×512 pixels.

3.6.7 Fourier Transform Infrared (FTIR) Spectroscopy

The formation of new or disappearance of any functional groups from the samples of different incubation periods was measured between wave number 4000 and 500 cm^{-1} by using FTIR spectrophotometer (Perkin Elmer, version 10.03.05).

3.6.8 Gas chromatography mass spectroscopy (GC- MS) investigations

Compounds present in the blank and those formed after biodegradation of PE films were analysed by GC-MS. It is helpful in understanding the mechanism of PE biodegradation using GC-MS-QP-2010-Ultra (Shimadzu) mass spectrophotometer. Column oven temperature was $100^{O}C$. The temperature of program was set initially at $260^{O}C$ with 1 min hold time. The total analysis time was 45 min, injector and interface temperature was set at $270^{O}C$. The software used for data treatment was GC-MS real time analyzer. For this, the bacterial and fungal growth were removed by filtration after incubation and the filtrates were extracted with diethyl ether.

3.6.9 Kinetic study of microbial growth

For the kinetic study of microbial growth, weight of the bacterial pellets and fungal mycelia was taken at regular time intervals. For this bacterial culture and fungal mycelia were filtered after regular time period (days) of incubation and were vacuum dried for 24 h. Microbial weights for each flask were weighed by digital weighing balance and the microbial isolates with the highest weight were identified.

3.6.10 Statistical Analysis

Data were subjected to one-way ANOVA to observe the variation in weight loss, tensile strength, and media pH with incubation time. Post-hoc (Tukey) test ($P<0.05$) was performed to observe the significance of difference between the control and treated HDPE and LDPE films.Statistical analysis was carried out using the software SPSS-16.