BIODEGRADATION OF HIGH DENSITY POLYETHYLENE AND POLYURETHANE FILMS BY FUNGI

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, **BIODEGRADATION OF HIGH DENSITY POLYETHYLENE AND POLYURETHANE FILM BY FUNGI** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July 2002 to July 2007 under the supervision of Dr. Ramasare Prasad, Associate Professor, Dr. Ben. M. J. Pereira, Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

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This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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ABSTRACT

Total 12 fungal isolates were isolated from buried polyethylene film samples collected from riverbed and dumpsites. The growth characteristics and colony morphology of all the isolates were studied using standard methods. To select the potential plastic degrading microorganism, all the twelve fungal isolates were screened for cell-surface hydrophobicity, percentage adherence to hydrocarbon, extracellular esterase enzyme production, biofilm formation and survival of fungal isolates on polyethylene surface and in planktonic phase. Out of 12 fungal isolates, strain F-8, F-9 and F-12 were found to be highly hydrophobic with high percentage of adherence and hydrolytic enzyme secretion compared to the other isolates, which enabled it to form a dense biofilm on the polyethylene surface with a large number of viable spores along with hyphae. Based on this data, these three isolates were further selected for biodegradation studies and identified. These isolates were deposited at Identification services, Indian Type Culture Collection (ITCC), Indian Agricultural Research Institute (IARI), New Delhi for identification. Isolate number F-8, F-9 and F-12 were identified as *Aspergillus flavus* (ITCC No. 6051), *Aspergillus fumigatus* (ITCC No. 6050) and *Aspergillus niger* (ITCC No. 6052) respectively.

All the degradation studies were carried out in pure shake flask system for 30 days. To facilitate the degradation activity, pretreatment of polyethylene film was done by giving thermal treatment at 70°C for a period of 10 days in hot air oven followed by chemical treatment in freshly made disinfectant to check microbial contamination. Degradation of the polyethylene film was studied for physical or chemical changes using

weight loss measurement, reduction in tensile strength, surface changes using Scanning Electron Microscopy (SEM), changes in functional groups using Fourier Transform Infra red Spectroscopy (FTIR), morphological changes such as melting (T_m) and onset Temperature (T_o) using Differential Scanning Calorimetery (DSC), structural changes like changes in crystallinity ($\%C_{XRD}$) using X-Ray diffraction (XRD), degradation products analysis by Gas Chromatography-Mass Spectrometry (GC-MS).

A. flavus (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) showed 4.41, 3.45 and 1.16 % reduction in weight and 61.33, 60 and 58.77% reduction in tensile strength of the polyethylene film after incubation with the respective isolate for 30 days. These data suggest that these isolates could be potential polyethylene (HDPE) degrader. Degradation was further investigated by scanning electron microscopy (SEM). All the three isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) showed good colonization on the polyethylene surface, though a little variation was observed in the extent of colonization. SEM studies showed clear degradation features such as shearing, tearing, hyphae penetration, formation of cavities and holes on the polyethylene film surfaces.

FTIR spectrum of heat treated polyethylene film showed a typical carbonyl peak at 1715 cm^{-1}. Incubation of heat-treated polyethylene film with all the three isolates showed a significant reduction in carbonyl content. The reduction in carbonyl content was also estimated in terms of carbonyl index (CI). The changes in onset temperature (T_o) and melting temperature (T_m) was also evaluated to determine the morphological changes induced by biological treatment. The significant changes were observed in T_m and T_o after treatment with fungal isolates compared to control. Samples incubated with

these three isolates showed a significant reduction in $\%C_{XRD}$. Culture broth was analyzed for the presence of degradation products of HDPE by GC-MS and identification of compounds was done by comparison with NBS database and major products identified were toluene, 1,2-Benzene Dicarboxylic acid, Diisooctyl ester, Propanoic acid, Phenol, 4,6-Di(1,1-Dimethylethyl)-2-Methyl, Methyl carbamate, Phenol,2,6-Bis(1,1-Dimethylethyl)-4-Methyl-,Methylcarbamate, 2,6-Di-T-Butyl-4-Methylphenol acetate (ester), 1,3,5-cycloheptatriene.

Polyurethane degradation ability of these isolates was also studied under similar conditions using polyurethane as carbon source. In contrast to polyethylene (HDPE), polyurethane (PU) is reported to be relatively susceptible to microbial attack. All the three isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) showed great potential for polyurethane degradation as revealed from weight loss, SEM, FTIR and DSC studies. The degradation of polyurethane was faster as compared to polyethylene as expected due to its susceptibility for microbial attack. The degradation of polyurethane by *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) reported in the present study is the first direct report of degradation by these fungal isolates.

Hydrolytic enzymes particularly Esterase, Lignin Peroxidase (LiP) and Manganese Peroxidase (MnP) producing abilities of *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) were measured in 30 days old minimal media culture broths inoculated by these isolates containing polyethylene or polyurethane as carbon source. High esterase activity (136U/50ml), (152U/50ml) and (130 U/50ml) respectively were observed in the culture broth of *A. flavus* (ITCC No.

6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) containing HDPE as carbon source. SDS-PAGE and in gel assay showed the secreted molecular weight in the range of 45-48 kDa. A significant MnP (8-22U/50ml) and LiP (6-9U/50ml) activities were also observed in the culture broths of these isolates, though it was much lower compared to esterase activity. The presence of significant activities of esterase, LiP and MnP suggest their possible role in plastic degradation. The biodegradation of high density polyethylene (HDPE) and polyurethane (PU) by *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) in the present study is the first direct report.

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ABBREVIATIONS USED

%	Percentage
$\%C_{XRD}$	Percentage of crystallinity
µg	Microgram
ASTM	American Society for Testing Materials
CFU	Colony Forming Unit
CI	Carbonyl Index
cm	centimetre
DBI	Double Bond Index
DSC	Differential Scanning Calorimetry
EPS	Extracellular Polysaccharide
FDA	Fluorescein diacetate
Fig.	Figure
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography-Mass Spectrometry
HDPE	High Density Polyethylene
LDPE	Low Density Polyethylene
LiP	Lignin Peroxidase
mg	Milligram
ml	Millilitre
mM	millimole
MnP	Manganese Peroxidase
MSM	Mineral Salt Medium
nd	Not detected
°C	°Centigrade
PAGE	Poly-acrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PDA	Potato Dextrose Agar
pNP	p-nitrophenol
pNPA	p-nitrophenyl acetate
PU	Polyurethane
rpm	Revolutions per Minute

S.D	Standard Deviation
S.E	Standard Error
SDS	Sodium dodecyl sulfate
sec	Second
SEM	Scanning Electron Microscopy
T_m	Melting Temperature
T_o	Onset melting Temperature
UV	Ultra violet
v/v	Volume/Volume
w/v	Weight/Volume
XRD	X-Ray Diffraction
µ	micron
h	Hour
min	Minute
g	Gram
l	Litre
M	Molarity
N	Normality
γ	Gamma

CHAPTER 1

INTRODUCTION

The term plastic is generally used to describe polymers, long chain molecules that have been made from the reaction of smaller molecules called monomers. Plastics are of two major types: thermo (e.g HDPE, LDPE) and thermoset (Polyurethane, PET) plastics. Polyethylene (PE) is the most widely used plastic throughout the world and high density Polyethylene (HDPE) is the most widely used type of polyethylene.

Plastics have many industrial and commercial applications and have become the integral part of our lives. They can be stretched to wide variety of strength and shapes. They are cheap, light, chemically stable and durable that makes them popular to be used as wrappers and packaging materials. They have molecular weight ranging from 50,000 to 1,000,000 Da. Because of high durability, they accumulate in the environment at a rate of 25 million tons per year (Orhan and Buyukgungor, 2000).

The drastic rise in the use of plastic materials has not been accompanied by a corresponding development of procedures for the safe disposal or degradation of these materials. As a consequence, plastic waste accumulating in the environment pose an ever increasing ecological threat to terrestrial and marine wildlife (Gilan *et al.*, 2004). Their extreme resistance to microbial attack is considered to be a disadvantage from the view point of environmental problem and solid waste management (Kenji *et al.*, 2001). Excessive molecular size seems to be mainly responsible for the resistance of these plastics to biodegradation and their persistence in soil for long time (Atlas, 1993). Several hundred thousand tons of plastics are discarded into marine environment every year and accumulate in oceanic regions. Many of the plastic products entering marine environment degrade very slowly. The accumulating debris poses increasingly significant threat to marine mammals, seabirds, turtles, crustaceans and fishes.

Polyethylene waste, unlike other wastes from other plastics is in the form of bags and films which are light in weight but voluminous. They cause problems ranging from littering leading to choking of drains, streams and water clogging, animal deaths due to consumption of the bags and emission due to roadside burning of plastic waste. Littering is very common problem in India. The proper management and disposal of plastic waste has been a great problem.

There are various methods of plastic waste disposal like incineration, landfills, mechanical and chemical recycling. Landfill has been a very popular method of disposing plastic waste. But the problems encountered in this case are the availability of space, slow degradation of plastics, leading to leacheates causing ground and surface water contamination. The Incineration of plastic waste is a widely used method in waste management and has been one of the option dealing with non-biodegradable plastics, but other than being expensive, it is also dangerous and is connected with environmental problems e.g the formation of dioxines and chlorinated compounds from polyvinyl chloride is reported (Kaminsky, 1992). Harmful chemicals like hydrogen chloride and hydrogen cyanide are released during incineration (Atlas, 1993; Johnston, 1990). Burning of polyethylene releases formaldehyde and acetaldehyde which are suspect carcinogens. Recycling of plastics is highly hazardous. Plastics generate many toxic effluents and emissions during its production and recycling. Recycled plastic will contain more harmful substances than virgin material because if toxic substances are in source material then they will be magnified in recycled products. Considering the impacts of conventional methods of plastic waste management, there is an urgent need for safe and eco friendly way of plastic waste management.

There could be two possible ways to overcome this problem: one is to develop synthetic polymers (biodegradable plastics) susceptible to biodegradation and the other is to develop the latent ability of naturally occuring microorganisms to degrade these non-biodegradable plastics. The need for safe, eco friendly atmosphere has led to a paradigm shift towards the use of biodegradable materials. Past two decades have shown a growing interest in the development of biodegradable plastics and its degradation by microorganisms. However, they create new challenges on waste management with respect to policies and laws, waste management technologies, economic concerns, poor physical properties compared with synthetic plastics, safety standards and shortage to meet the present day demand. Because of their availability in large quantities at low cost and favourable functional characteristics such as good tensile and shear strength, synthetic plastics are still being produced and used in large quantities. Thus, there is emergent need for the development of eco friendly way to manage the commonly used synthetic plastic and biological methods have gained a lot of attention.

According to one recent survey, out of the total amount of plastic used, 92% is thermo plastics, which are relatively resistant to microbial degradation due to their structure and only 8% is thermoset plastics, which are found to be relatively susceptible to biodegradation. Since the major contributors are these synthetic thermo plastics, the environmental pollution caused by these is a serious concern. Safe disposal of these kind of plastics have gained attention during the past few years.

The biodegradation of plastics is desirable to be estimated in natural environment, where the waste plastics are exposed as such. Biodegradation is a process in which naturally occurring microorganisms such as bacteria or fungi act on the material and

consume it (Orhan *et al.*, 2004). Major hurdle in the biodegradation of polyethylene is its high surface hydrophobicity which interferes with the microbial adhesion to the surface. Due to its relatively resistant nature to microbial attack, the biodegradation of plastic has been found to be a very slow and complex process under natural conditions. However it has been reported that pretreatment of plastics such as UV photo oxidation (Gilan *et al.*, 2004; Hadad *et al.*, 2005), thermal oxidation (Albertsson *et al.*, 1998; Volke-Sepulveda *et al.*, 2002) or chemical oxidation (Brown *et al.*, 1974) prior to exposure to microorganisms enhanced the biodegradation by changing the physical properties of polyethylene particularly by forming carbonyl bond, making hydrophobic surface relatively hydrophilic and thus, increasing their susceptibility to microbial attack. As a result of which the long carbon chains are broken to shorter segments and their molecular weight is reduced. Microorganisms can then assimilate the polyolefins monomeric and oligomeric breakdown products previously derived from photo and chemical degradations (Bonhomme, 2003; Yamada *et al.*, 2001; Zuchowska *et al.*, 1998). Biodegradation resulting from the utilization of polyethylene as a nutrient (i.e. a carbon source) may be more efficient if the degrading microorganism forms a biofilm on the polyethylene surface.

In the present study, emphasis has been on the second alternative and an attempt has been made to isolate the potential fungal isolates with ability to degrade the commonly used high density polyethylene (HDPE) plastic bags and polyurethane (PU). The major objectives of the work and the approach used for studying the biodegradation of plastics are as follows:

1. Isolation of fungal isolates from buried polyethylene samples collected from dumpsites.
2. Screening, morphological study and identification of potent plastic degrading fungal isolates.
3. Study the degradation of high density polyethylene (HDPE) and polyurethane (PU) film by selected potential fungal isolates.
4. Hydrolytic enzyme producing ability of fungal isolates and their possible role in biodegradation of plastics.

Details of investigation on the above aspect of biodegradation of plastics have been presented in this thesis.

CHAPTER 2

LITERATURE REVIEW

Plastics: Overview

With the improvement in technologies, more and more synthetic products are coming our way since the past few decades. Out of these, plastics have been the most successful in achieving a long-term existence. The use of plastics is growing due to the new and numerous applications that are frequently emerging. Plastics have now become an important part of modern life and are used in different sectors of applications like packaging, building materials, consumer products and much more. Plastics are man-made long chain polymeric molecules. Worldwide production of plastics is more than 78 million tons per year. Demand for plastics in India was about 4.3 million tons in the year 2001-02 and increased to about 8 million tons in the year 2006-07. Currently, however, the per capita consumption of plastics in India is only about 3 kg compared to 30-40 kg in the developed countries. The present market in India is of about Rs 25,000 crore (Biodegradable plastics, 2003).

Plastics are unique in chemical composition, physical forms, mechanical properties, and applications. High versatility of the carbon to carbon and carbon to non-carbon (C-C, C-R and C-H) bonds and substituent groups, the possible configurations, stereochemistry and orientation provide basis for variations in chemical structures and stereochemistry (Odian, 1991). Very small variations in chemical structure may result in large differences in terms of biodegradability. Because of this structural versatility, they are widely used in product packaging, insulation, structural components, protective coatings, medical implants, drug delivery carriers, slow release capsules, electronic insulation, telecommunication, aviation and space industries, sporting and recreational equipments, building consolidants etc (Lemaire *et al.*, 1992; Pitt, 1992).

Synthetic polymers designated as plastics have become technologically significant since the 1940s and since then they have come to replace glass, wood, masonry and other constructional materials, and even metals in many industrial, domestic, commercial and environmental applications (Cain, 1992). The widespread applications are not only due to their favourable mechanical and thermal properties but mainly due to stability and durability of plastics. Synthetic plastics have been extensively used due to their structural stability and properties such as chemical stability, light weight, hydrophobicity, versatility, and resistance to chemical and biological deterioration (Volke-Sepulveda *et al.*, 1999). Petrochemical-based plastics such as polyolefins, polyesters, polyamides etc. have been increasingly used as packaging materials because of their availability in large quantities at low cost and favourable functional characteristics such as good tensile and shear strength, good barrier properties to O_2 and heat sealability, ideal insulating properties. They have a very low water vapour transmission rate and most importantly they are non-biodegradable (Tharanathan and Saroj, 2001).

2.1 Types of plastics and their applications

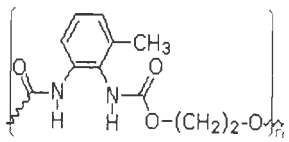
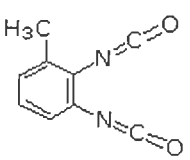
Plastics are synthetic polymers and two main processes are used for the manufacture of these synthetic polymers (Alauddin, 1995). The first involves the breaking of double bond in the original olefin by additional polymerization leading to the formation of new carbon-carbon bonds, to form carbon chain polymers. The second process is based on the elimination of water (or condensation) between a carboxylic acid and an alcohol or amine to form polyester or polyamide.

Plastics are divided into two major groups: thermoplastics and thermoset plastics (Alauddin, 1995). Thermoplastics are the products of the first kind of reaction mentioned above. They can be repeatedly softened and hardened by heating and cooling. In these types of plastics, the atoms and molecules are joined end to end into a series of long chains solely made up of carbon, which are independent of others (Allen *et al.*, 1999). This type of structure makes thermoplastics resistant to degradation or hydrolytic cleavage of chemical bonds because of which thermoplastics are considered non-biodegradable plastics.

Thermoset plastics are synthesized by second type of reaction mentioned above. They are solidified after being melted by heating. The process of changing from the liquid state to solid state is irreversible (Alauddin, 1995). They have cross-linked structure. Main chain of thermoset plastics is composed of heteroatoms, making them potentially susceptible to be degraded by the hydrolytic cleavage of chemical bonds such as ester bonds or amide bonds (Zheng *et al.*, 2005).

Thermoplastics are widely used in packaging and fabrication of bottles and films. They include linear, low-density polyethylene (LLDPE), high density polyethylene (HDPE), polyvinyl chloride (PVC), low density polyethylene (LDPE), polypropylene (PP), polystyrene (PS) and other resins. The major types of thermoset plastics include polyesters like polyethylene terephthalate (PET) and polyurethane (PU) (Avella *et al.*, 2001). The various types of plastics and their possible applications are listed in Table 1.

Table 1. Major types of plastics and their applications.

Name(s)	Formula	Monomer	Properties	Uses
Low density **Polyethylene** (LDPE)	$-(CH_2-CH_2)_n-$	ethylene $CH_2=CH_2$	soft, waxy solid	film wrap, plastic bags
High density **Polyethylene** (HDPE)	$-(CH_2-CH_2)_n-$	ethylene $CH_2=CH_2$	rigid, translucent solid	electrical insulation bottles, toys
Polypropylene (PP) different grades	$-[CH_2-CH(CH_3)]_n-$	propylene $CH_2=CHCH_3$	atactic: soft, elastic solid isotactic: hard, strong solid	similar to LDPE carpet, upholstery
Poly(vinyl chloride) (PVC)	$-(CH_2-CHCl)_n-$	vinyl chloride $CH_2=CHCl$	strong rigid solid	pipes, siding, flooring
Poly(vinylidene chloride)	$-(CH_2-CCl_2)_n-$	vinylidene chloride $CH_2=CCl_2$	dense, high-melting solid	seat covers, films
Polystyrene (PS)	$-[CH_2-CH(C_6H_5)]_n-$	styrene $CH_2=CHC_6H_5$	hard, rigid, clear solid soluble in organic solvents	toys, cabinets packaging (foamed)
Polyacrylonitrile (PAN, Orlon, Acrilan)	$-(CH_2-CHCN)_n-$	acrylonitrile $CH_2=CHCN$	high-melting solid soluble in organic solvents	rugs, blankets clothing
Polytetrafluoroethylene (PTFE, Teflon)	$-(CF_2-CF_2)_n-$	tetrafluoroethylene $CF_2=CF_2$	resistant, smooth solid	non-stick surfaces, electrical insulation
Poly(methyl methacrylate) (PMMA)	$-[CH_2-C(CH_3)CO_2CH_3]_n-$	methyl methacrylate $CH_2=C(CH_3)CO_2CH_3$	hard, transparent solid	lighting covers, signs skylights
Poly(vinyl acetate) (PVAc)	$-(CH_2-CHOCOCH_3)_n-$	vinyl acetate $CH_2=CHOCOCH_3$	soft, sticky solid	latex paints, adhesives
cis-Polyisoprene natural rubber	$-[CH_2-CH=C(CH_3)-CH_2]_n-$	isoprene $CH_2=CH-C(CH_3)=CH_2$	soft, sticky solid	requires vulcanization for practical use
Polychloroprene (cis + trans) (Neoprene)	$-[CH_2-CH=CCl-CH_2]_n-$	chloroprene $CH_2=CH-CCl=CH_2$	tough, rubbery solid	synthetic rubber oil resistant
Polyurethane (PU)		$HOCH_2CH_2OH$ 	both soft and hard, solid	Coating, insulation, paints, packing

According to a recent survey by American Plastics Association, out of the total plastics used in North American countries (Mexico, USA, Canada), 92% of plastics employed are thermoplastics while only 8% of the total plastics are comprised of thermoset plastics (Zheng *et al.*, 2005). The percentage distribution of various plastic resins and an overview of current consumption level of various polyethylene polymers in world are given in Table 2 and 3.

Table 2. Percentage distribution of various plastic resins (Zheng *et al.*, 2005).

Type of plastic resin	Percentage distribution
Polypropylene(PP)	18.4%
Polyvinyl chloride (PVC)	15.8%
Polystyrene (PS)	6.7%
High density Polyethylene (HDPE)	17.4%
Low density polyethylene (LDPE)	8.2%
Linear low density polyethylene (LLDPE)	12.1%
Other thermoplastics	12.5%
Thermoplastics total	92.0%
Thermoset and other plastics	8.0%
Total	100%

Out of 154 million tons of various polymers used, 54 million tons is from the polyethylene family, which is the largest share. More than 60% of 54 million tons polyethylene polymer are consumed in making films to produce various flexible packages (Ghosh, 2004). Since their introduction in 1977, plastics grocery bags have

become a part of daily life in developed countries (Williamson, 2003). The demand is ever increasing and according to an estimate, the plastic waste generation will grow by 15% per year for the next decade (Chau *et al.*, 1999).

Table 3. Consumption of polyethylene polymers in world (Ghosh, 2004).

Consumption of Polyethylene polymers in World	
Polymer	**Consumption, M. ton, year 2000**
LDPE	17,000,000
HDPE	23,000,000
LLDPE	14,000,000
Total PE	54,000,000
Total Polymer	154,000,000

A very visible portion of municipal and industrial waste consists of polyethylene (PE) films utilized on a massive scale as wrapping material and commonly used consumer shopping bags, a typical example for the end-consumer being shopping bags. The high rate of accumulation of these waste are of major concern because of their adverse environmental impact. Beside, though thermoset plastics constitute only 8% of the total amount of plastics, their susceptibility to biodegradation also raises attention (Zheng *et al.*, 2005).

2.2 Plastics: A threat to environment

Widespread use and high durability has led to the accumulation of plastic waste material at a rate of about 25 million tons per year in the environment (Orhan and Buyukgungor, 2000). According to American statistics, 160 tons of solid wastes are annually thrown into the environment, of which 6% to 7% are plastic waste, representing

about 30% of the total volume. Now a days, a wide variety of petroleum based synthetic polymers are produced worldwide to the extent of approximately 140 million tons per year and remarkable amount of these polymers are introduced in to ecosystem as industrial waste products (Shimao, 2001). As a result, plastic litter has become omnipresent in our environment (Kounty *et al.*, 2006a). As the new uses for these materials are being developed, applied in the practices and made available to more people, the quantity of plastic debris entering the marine environment has undergone a corresponding increase. Since they are also buoyant, an increasing load of plastic debris is being dispersed over long distances and when they finally settle in sediments, they may persist for centuries (Goldberg, 1997; Hansen, 1990; Ryan, 1987).

The plastic waste accumulation in ocean has become a serious problem and cause threat to marine life (Laist, 1987; Pruter, 1987; Thiel *et al.*, 2003; Thompson *et al.*, 2004; Wong *et al.*, 1974). The types of plastic debris most dangerous to marine life are fishing nets and net fragments, plastic strapping bands, plastic bags, synthetic rope and line, small plastic objects, such as plastic cups, which degrade into small floating fragments and raw plastic pellets. Plastic items have now become major component of man-made debris in the world's oceans Plastic debris is ingested by sea animals. This ingested debris may block the digestive track and provide a source of toxic chemicals. Mortality due to entanglement in marine debris contributes significantly to declining trend of population on islands (Laist, 1987). Most distressing are the facts that over a billion seabirds and mammals are dying annually from ingestion of plastics (Baker, 2002).

Due to slow decomposition and long persistence in the environment, plastic bags are inhibiting the breakdown of biodegradable materials in and around it (Stevens, 2001).

Their persistence in environment is adding to the growing water and surface waste litter problems, which has raised concerns about plastics (Kawai, 1995). Surface waste disposal leads to choking of drains leading to floods. By clogging sewer pipes, plastic grocery bags also create stagnant water that serves as the ideal habitat for mosquitoes and other parasites, which have the potential to spread a large number of diseases.

Toxic emissions produced during the extraction of materials for the production of plastic grocery bags, their manufacturing, and roadside burning of plastic waste causes adverse effect on environment. According to a report, manufacturing of two plastic bags produces 1.1 kg of atmospheric pollution, which contributes to acid rain and smog. Acid rain is recognized as a serious threat to natural and human-made environments (Environmental Literacy Council, 2005; Institute for Lifecycle Environmental Assessment, 1990; National Plastic Bags Working, Group 2002). Impacts on human health are perhaps the most serious of the effects associated with plastic grocery bags, ranging from health problems associated with emissions to death.

2.3 Various options for management of plastic waste and related issues

Increased use of synthetic packaging material films has led to serious ecological problems due to their total non-biodegradability. Lack of degradability and increasing depletion of landfill sites as well as growing water and land problems have led to concern about plastics (Kawai, 1995). Keeping in mind the hazardous impacts of plastic waste on terrestrial and aquatic life, there is an emergent need to consider this problem as a major issue, which need to be addressed. Emphasis now is on developing methods for effective management of plastic waste. Table 4 summarizes the different management options of plastic waste and issues related with them.

Table 4. Pros and Cons of major plastic waste management technologies (Ren, 2003)

Technology	Pros	Cons
Recycling (Mechanical and Chemical)	• Reduce amount of wastes for disposal • Save resources and energy in virgin production • Extend product's lifetime • Conserve resources	• High cost of separation • Not everything economically recyclable • Recycling consumes energy • Emit pollutants • Recycled product inferior in quality, thus only lower grade application, limited market
Incineration	• Reduce waste substantially by volume/ weight • Generate energy • Need small space • Reduce burden of landfill	• High capital and operational costs • Emission of hazardous substances (Dioxins etc) • More stringent in operation and control
Landfilling	• Final and indispensable disposal of wastes, residues from recycling, incineration etc. • Relatively easy to build and operate	• Suitable sites become scare worldwide • Cost is increasing significantly due to higher environmental and sanitary requirements. • Leachate and gas emission problems • Ground and surface water contamination
Composting	• Reduce load of landfill by digesting the organics • End product useful for soil amendment • Need less energy than recycling, incineration	• Economics still unfavorable • Risk of odor and pest problem • No reliable market for end product (compost)

Landfill has been a very popular method of disposing plastic waste and it does provide solution to this problem to some extent but until there is a brake on the consumption levels, we cannot successfully use them. Problems encountered during the landfilling of plastic waste are the availability of space, slow degradation of plastics, leading to leacheates. Incineration is another option for plastic waste management but a number of harmful chemicals and gases like hydrogen chloride and hydrogen cyanide are released during the Incineration of plastics (Atlas, 1993).

Because of their persistence in the environment, the increased cost of solid waste disposal (owing to the reductions in available landfill space) as well as the potential hazards from waste incineration (such as dioxin emission from PVC incineration), plastics have become more a waste deposit problem (Zeid, 2001).

Recycling is obviously a better choice at a higher cost but most countries cannot afford to recycle all its polymer wastes. Moreover, all polymers are not recyclable since their properties after recycling are poor compared to their original ones and they are of less economic value. Recycling of plastics is hazardous. Plastics generate many toxic effluents and emissions during their recycling. Recycled plastics will contain more harmful substances than virgin material (Fletcher, 1993).

2.4 Biodegradation: an environment friendly approach to plastic waste management

Currently, the annual worldwide use of plastic materials is gradually increasing and plastic disposal by conventional physicochemical techniques (incineration, recycling, and landfill disposal) involves technical and economical problems and they further add to environmental pollution. For this reason, the importance of both developing a new and improving an existing technology for the eco-friendly management of waste plastic

materials is greatly emphasized (Kim and Rhee, 2003). An alternative to dispose synthetic plastics is biodegradation, a biological process where certain microorganisms degrade them to obtain energy for their growth (Cain, 1992). Biodegradability of plastics has been proposed as a solution for the plastic waste problem (Palmisano and Pettigrew, 1992). Two possible strategies have been used in this regard:

1. To develop biodegradable polymers which could be easily degraded by microbes.
2. To exploit the inherent ability of microorganisms to degrade presently used so called non-biodegradable synthetic plastics particularly polyethylene.

Polymers are potential substrates for heterotrophic microorganisms including bacteria and fungi. Microorganisms are involved in the deterioration and degradation of both synthetic and natural polymers (Gu *et al.*, 2000), but very little is known about the biodegradation of synthetic polymeric materials. The reason is probable due to the recent development and manufacture of this class of materials and the relatively low rate of degradation in natural environments

The degradation of plastics in nature is a very slow process that involves environmental factors such as temperature, humidity, pH and solar energy followed by the action of wild microorganisms. Polymer biodegradation is initiated by extracellular enzymes that break polymeric chains, releasing oligomers and monomers that can be transported into the cell (Starnecker and Menner, 1996). Many fungi possess highly unspecific oxidative enzymes (oxygenases) that are able to oxidize several substrates that could also attack polymeric substrates by cometabolic processes. The generalized pathway for polymer biodegradation is shown in Figure 1.

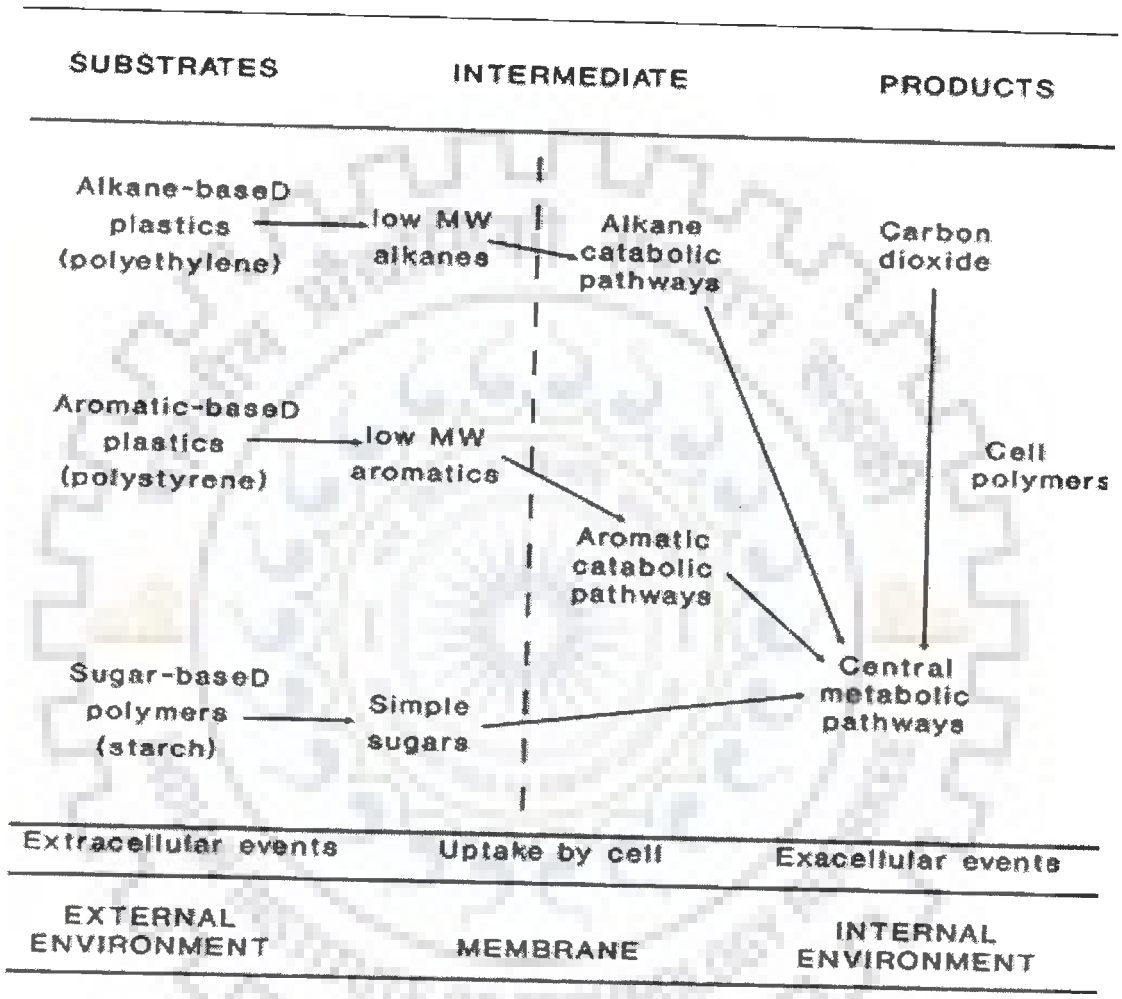


Figure 1. General pathway for polymer biodegradation (Chandra and Rustgi, 1998).

2.4.1 Development of Biodegradable polymers as a suitable alternative

Continuous awareness by all towards environmental pollution by plastic litter and the need for safe, eco friendly atmosphere, has led to a paradigm shift towards the use of biodegradable materials. The past two decades have witnessed a growing public and scientific concern regarding the use of biodegradable materials as an alternative to conventional plastics offering a solution for the existing grave problem of plastic waste (Bichler *et al.*, 1993). The worldwide consumption of biodegradable polymers has increased from 14 million kg in 1996 to an estimated 68 million kg in 2001 (Gross and Kalra, 2002). Biodegradable plastics opened the way for new considerations of waste management strategies since these materials are designed to degrade under environmental conditions or in municipal and industrial biological waste treatment facilities.

Biodegradable plastics are designed to be completely biodegradable. The polymer matrix is derived from natural sources (such as starch or microbially grown polymers), and the fiber reinforcements are produced from common crops such as flax or hemp. Microorganisms are able to consume these materials entirely, eventually leaving carbon dioxide and water as by products (Kolybaba *et al.*, 2003). The American Society for Testing Materials (ASTM) and the International Standard Organization (ISO) define biodegradable plastics as those that undergo a significant change in chemical structure under specific environmental conditions.

Over the last few years, much emphasis have been given to develop biodegradable plastics and various types of biodegradable plastics have indeed been successfully developed to meet the specific demands in various fields and industries (Amass *et al.*, 1998; Aminabhavi *et al.*, 1990; Muller *et al.*, 2001; Ohtake *et al.*, 1994;

Sasikala and Ramana, 1996; Tharanathan, 2003). There are large number of reports regarding biodegradation of degradable plastics by microorganisms (Brandl and Puchner, 1992; Gamal and Seliger, 2004; Muller, 2006; Reddy *et al.*, 2003; Witt *et al.*, 1995). The types of biodegradable plastics and their suitable applications have been described below:

2.4.1.1 Types of biodegradable polymers

Biodegradable plastics can be classified into three groups: starch-based biodegradable plastics, chemically synthesized biodegradable plastics, microorganism based biodegradable plastics and their classification is shown in Table 5.

Table 5: Classification of biodegradable polymers

Type	Product name	Constituents
Starch-based plastics		
	MATER-BI	Starch (60%) / PVA alloy
	NOVON	Starch (90-95%) + additive
	AMIPOL	Starch (100%)
Chemical synthesis based		
	Polylactic acid	Polylactic acid
	Plockcelton	
	BIONOLLE	Polycaprolactone
		Aliphatic polyester
Microorganisms based		
	BIOPOL	Copolymer of polyhydroxybutyrate and valeric acid (PBH/V)

2.4.1.1.1 Starch-based biodegradable plastics

In this type, starch is added as filler and cross-linking agent to produce a blend of starch and plastic (e.g starch polyethylene). By varying the synthetic blend component and its miscibility with starch, the morphology and properties can be regulated. Soil microorganisms degrade the starch easily, thus breaking down the polymer matrix. This result in significant reduction of degradation time, but these types of plastics are only partially degradable. The fragments left after the removal of starch is recalcitrant and remain in the environment for a long time. Italian company Novamont produced the first commercial product with brand name Mater-Bi that consists of approximately 60% starch. The main applications are for a variety of packaging and personal-care uses. The US Company Warner-Lambert has also developed a starch-based plastic, Novon (Glover, 1993; Gross and Kalra, 2002; Khanna and Srivastava, 2005). There are several studies on biodegradation of these starch based biodegradable plastics (Kim and Rhee, 2003; Nakamura *et al.*, 2005).

2.4.1.1.2 Chemically synthesized biodegradable plastics

Polyglycollic acid, polylactic acid, poly (ε- caprolactone), polyvinyl alcohol, poly (ethylene oxide) fall into this category. These are susceptible to enzymatic or microbial attack. They do not match all the properties of plastics, therefore they are not commercially used as a substitute for plastics (Khanna and Srivastava, 2005). There are several reports showing the biodegradation of this class of biodegradable plastics (Lesinky *et al.*, 2005; Muller *et al.*, 2001).

2.4.1.1.3 Microorganisms based biodegradable plastics

Microorganism based plastics are the only 100% biodegradable polymers. They are the polyesters of various hydroxyalkanoates, which are synthesized by numerous microorganisms as energy reserve materials when an essential nutrient such as nitrogen or phosphorus is available only in limiting concentrations in the presence of excess carbon source. They possess properties similar to various synthetic thermoplastics and hence can be used in their place. They are completely degraded to water and CO_2 under aerobic conditions and to methane under anaerobic conditions by microbes (Khanna and Srivastava, 2005). Biopol, a truly biodegradable plastic product is a linear polyester copolymer of poly (hydroxy butyrate) and poly (hydroxyl valerate) and produced by the fermentation of sugars by *Alcaligenses eutrophus.* bacteria is an example of microorganism based plastics (Bastiolli, 1998; Glover, 1993). There are several studies showing the biodegradation of these types of biodegradable plastics by microorganisms (Brandl and Puchner, 1992; Colak and Guner, 2004)

2.4.1.2 Drawbacks and limitations of biodegradable polymers

Though the demand for biodegradable plastics is increasing and they have been developed as a solution for the waste problem, acceptance of biodegradable polymers is likely to depend on factors like customer response to costs, the achievement of total biodegradability, waste management with respect to policies and laws, waste management technologies. For the design of new and improved materials and the evaluation of the degradation behaviour under other environmental conditions, work has to be done to elucidate the degradation mechanism of these interesting groups of biodegradable polymers (Muller *et al.*, 2001). Economic concerns must be addressed

objectively as biopolymer materials are developed, because the future of each product is dependent on its cost competitiveness, and society's ability to pay for it. Many governments are introducing initiatives designed to encourage research and development of biologically based polymer (Kolybaba *et al.*, 2003).

2.4.2 Biodegradation of synthetic plastics (thermo and thermoset plastics)

Widespread use of synthetic plastics has led to their accumulation in the environment at such a rate that the environmental problem caused by them has now become an issue of major concern. Though biodegradable polymers have been suggested as an alternative to the problem of plastic waste management, still there are certain issues that need to be taken into account. New forms of plastics must retain all of the physical properties needed by the consumer and must fulfill the safety standards both when it is being used and after it has been discarded. Until then, polymers that are not biodegradable will continued to be used. According to a recent survey, biodegradable plastics forms only a part of 8% plastics used and rest 92% of the total plastics used are synthetic non-biodegradable polymers (Zheng *et al.*, 2005). The environmental impact caused by these plastics is a major concern. Biodegradation of these inert synthetic plastic materials has been a major concern. Synthetic plastics (polyethylene) are found to be relatively resistant to microbial attack because of which they keep on accumulating in the ecosystem and takes hundreds of year to degrade.

Polymer biodegradability depends on molecular weight, crystallinity and physical forms (Gu *et al.*, 2000). The extent of polymer degradation in an ecosystem is affected by material processing, the inherent characteristics of the substrate to be degraded, and

various microbiological and environmental factors. A series of parameters can influence the rate of biodegradation and the life time of a plastic material in nature, including the type of environment, the presence of a microbial population and its microbial activity, the availability of water, the temperature, the section thickness of the plastic material, its surface texture, its porosity and the presence of second components in the plastic, such as fillers or coloring agents (Brandl *et al.*, 1995; Brandl and Puchner, 1992,). These factors are all interdependent and contribute synergistically to inert synthetic plastic degradation. Generally an increase in molecular weight results in decline of polymer degradability by microorganisms. High molecular weight results in sharp decrease in solubility making them unfavourable for microbial attack because microbes require the substrate be assimilated through the cellular membrane and further degraded by cellular enzymes. However concurrent abiological and biological processes may facilitate the degradation of polymers (Albertsson and Karlsson, 1990; Gu, 2003, 2007; Gu and Gu, 2005). At least two categories of enzymes are actively involved in biological degradation of polymers: extracellular and intracellular depolymerases (Gu *et al.*, 2000). During degradation, exoenzymes from microorganisms break down complex polymers yielding short chains or smaller chains e.g. oligomers, dimers and monomers, that are smaller enough to pass the semi permeable outer membrane of microbial cell, and then to be utilized as carbon and energy sources (Gu, 2003).

2.4.2.1 Role of biofilm formation in polyethylene degradation

Deterioration of a polymeric material is caused by adhering microorganisms that colonize their surface and form biofilm (Flemming, 1998; Seneviratne *et al.*, 2006). Microbial adhesion is the first in the series of events that occurs in the colonization of

solid substratum (Brown, 1946; Christensen *et al.*, 1995; Costerton *et al.*, 1995; Flemming, 1998; Webb *et al.*, 1999) which leads to subsequent formation of a biofilm. Adhesion to the materials such as plastics or glass is caused by non specific interactions between cell surface and substratum (Webb *et al.*, 2000). As far as commercial plastics are concerned, it is the plasticizers and fillers used in the formulations which render them susceptible to attack. This attack usually manifests itself in the form of a surface biofilm that causes little adverse effect to the physical or chemical integrity of the material (Morton and Surman, 1994).

Polyethylene is highly hydrophobic in nature, which interferes with colonization and biofilm formation (Gilan *et al.*, 2004; Sivan *et al.*, 2006). Biodegradation resulting from utilization of polyethylene as a nutrient source (i.e carbon source) may be more efficient if the degrading microorganism forms a biofilm on polyethylene film (Hadad *et al.*, 2005). Since polyethylene is not soluble in aqueous solution, biofilm producing microorganisms may be more efficient in biodegradation of synthetic polymers. There are several reports where biofilm formation on polyethylene surface by various bacteria and fungi is attributed to its degradation, which are described in more detail in later section (Gilan *et al.*, 2004; Sivan *et al.*, 2006).

2.4.2.2 Biodegradation of thermoset plastics (Polyurethane)

Polyurethanes (PUs) are a versatile class of man-made polymers and has been found to be susceptible to biodegradation by naturally occurring microorganisms irrespective of its xenobiotic origin. Polyurethanes (PUs) were first investigated and produced by Dr. Otto Bayer in 1937. PU is a polymer in which repeating unit contains a urethane moiety. Urethanes are derivatives of carbamic acids that exist in the form of

their esters (Dombrow, 1957). Variations in R group and substitutions of the amide hydrogen produce multiple urethanes. Although all PUs contain repeating urethane groups, other moieties such as urea, ester, ether and aromatic may be included (Saunders and Frisch, 1964). Variation in the number of substitutions and the spacing between and within the branch chains produce PUs ranging from linear to branched and flexible to rigid. PU are synthetic polymer widely used as a raw material for various industries. PUs can be found in products such as furniture, coatings, adhesives, constructional materials, fibers, paddings, paints, elastomers and synthetic skins (Howard, 2002a,b). PUs are replacing the old polymers for various reasons like increased tensile strength and melting point. Their resistance to degradation by water, oils, and solvents make them excellent for the replacement of thermoplastics (Saunders and Frisch, 1964).

All the PU based materials are susceptible to microbial attack. However, there is found to be variation in their biodegradability. The variations in the degradation patterns of different samples of PUs were attributed to many properties of PUs such as molecular orientation, crystallinity, cross linking and chemical groups present in the molecular chains which determine the accessibility to degrading- enzyme system (Pathirana and Seal, 1983). PUs with long repeating units and hydrolytic groups is less likely to pack into high crystalline regions as normal polyurethanes and these polymers were more accessible to biodegradation. The microbial degradation process can roughly be divided into the degradation of urethane bonds and the degradation of polyol segments, which are the major constituents of PU and its degradability is greatly influenced by the chemical structure of the polyol segments (polyester or polyether type) (Howard, 2002a).

Due to its susceptibility to microbial attack, biodegradation of PUs has been one of the thrust area during the last few decades. There are several reports describing biodegradation of PUs by various fungi (Barratt *et al.*, 2003; Crabbe *et al.*, 1994; Darby and Kaplan, 1968; Kaplan *et al.*, 1968; Ossefort and Testroet, 1966) and bacteria (Akutsu *et al.*, 1998; Allen *et al.*, 1999; Blake and Howard, 1998; Howard, 2002b; Kay *et al.*, 1991, 1993; Nakajima-Kambe *et al.*, 1997). There are review articles describing various aspects of PU degradation (Howard, 2002a). It has been observed that polyester type PUs is more susceptible to microbial attack than other forms (Kanavel *et al.*, 1966; Labrow *et al.*, 1996).

Boubendir (1993) isolated two fungi *Chaetomium globosum* and *Aspergillus terrus* with esterase and urethane hydrolase activities which causes the degradation of PU. Crabbe et al. (1994) isolated four species of fungi *Curvularia senegalensis*, *Fusarium solani*, *Aureobasidium pullulans* and *Cladosporium* sp. with an ability to utilize a colloidal polyester PU (Impranil DLN ^{TM}) as sole carbon and energy source. Attempt has also been made to assess the potential of *Aspergillus foetidus* for polyurethane degradation (Upreti and Srivastava, 2003).

Although there are relatively few reports on degradation of PU by bacteria compared to fungi, however both gram-positive and gram negative bacteria have been reported as PU degraders (Kay *et al.*, 1991; Nakajima-Kambe *et al.*, 1995). In a large-scale test of bacterial activity against PUs, *Corynebacterium* sp. and *Pseudomonas aeruginosa* have been reported to degrade PU in basal media and there was significant reduction in tensile strength and elongation after three days of incubation (Kay *et al.*,

1991). Infrared spectrophotometer analysis revealed the ester segment of the polymer to be main site of attack. In another study, isolates from PU military aircraft paint *Acinetobacter calcoaceticus*, *Pseudomonas cepacia* and *Arthrobacter globiformis* were capable of utilizing PU as sole carbon and energy source (Halim EI-Sayed *et al.*, 1996). Nakajima-Kambe et al. (1995) isolated *Comamonas acidovorans* TB-35 strain from soil with ability to degrade polyester PU. Blake and Howard (1998) reported ability of *Bacillus* sp. and *Pseudomonas* sp. to degrade and utilize polyester PU (Impranil DLN ™) as sole carbon and energy source.

Attempts have been made to understand the possible mechanism of PU biodegradation. It has been suggested that microbial degradation of PU is due to hydrolytic enzymes such as ureases, proteases and esterases (Allen *et al.*, 1999; Black and Howard, 1998; Evans and Levisohn, 1968; Flilip, 1978; Griffin, 1980; Hole, 1972; Nakajima-Kambe *et al.*, 1997). Study from both fungi and bacteria indicated that PU degradation was due to hydrolysis of ester bond and the involvement of esterase enzyme has been proposed to be a major mechanism of PU degradation (Boubendir, 1993; Howard and Blake, 1999; Nakajima-Kambe *et al.*, 1995; Wales and Sagar, 1988). So far, only two types of PUase enzymes have been isolated and characterized: a cell associated, membrane bound PU-esterase (Akustu *et al.*, 1998) and soluble, extracellular PU-esterases (Allen *et al.*, 1999; Ruiz *et al.*, 1999; Vega *et al.*, 1999). Purification and characterization of two PU esterase enzymes has been done from *Pseudomonas fluorescens* (Howard and Blake, 1999).

2.4.2.3 Biodegradation of thermoplastics (low density polyethylene and high density polyethylene)

Thermoset plastics constitute 92% of total amount of plastic used. Out of which, Low-density polyethylene (LDPE) and high-density polyethylene (HDPE) forms a major part. They are considered to be inert polymers and are resistant to microbial attack, therefore, their long persistence in the environment is a major concern. In lieu of this, several groups have studied the biodegradation of polyethylene during the last two decades. It has been reported that many microorganisms can utilize paraffins as a carbon source (Fuhs, 1961). Therefore, the idea that such microbes could also catabolize polyethylene has been of interest and provide the sound basis to explain the possibility of polyethylene utilizing microbes. Polyethylene and paraffins were first compared in degradation experiments by Jen-Hou and Schwartz (1961), who counted the number of bacteria growing on these alkenes as a measure of polyethylene utilization (Jen-Hou and Schwartz, 1961). Study showed that such microbes could grow on a low molecular weight polyethylene, but not on high-molecular-weight polyethylenes. It was reported that linear paraffin molecules below ca 500 molecular weight were utilized by several microorganisms (Haines and Alexander, 1974; Potts *et al.*, 1972, 1973). It has been suggested that in order to help the mechanism through which the microorganisms can assimilate the carbon contained in the polyethylene, the polymer must be first transformed to more oxidized compounds of low molecular weight. When polyolefins e.g polyethylene is subjected to biodegradation by microorganisms, slow changes takes place (Albertsson and Banhidi, 1980; Albertsson *et al.*, 1978; Albertsson and Ranby, 1979). Scott concluded in 1975 that an attack on polyethylene by microorganism is a secondary

process. The literature on the biodegradation of synthetic polymers is sharply divided between those suggesting that microbiological attack can only occur if polymers could be degraded to extremely short chain lengths and those suggesting that synthetic polymers can also be metabolized at relatively high molecular weights (Gu, 2003).

Degradation of polyethylene in nature is a very slow and complex process where both abiotic and biotic factors are found to play a major role in the biodegradation of these types of plastics by microorganisms. It is known that oxidation of polyethylene molecules by means of physicochemical treatments prior to their exposure to microorganisms facilitates the action of microorganisms (Albertsson *et al.*, 1987; Palmisano and Pettigrew, 1992). UV photooxidation (Cornell *et al.*, 1984), thermal oxidation (Albertsson *et al.*, 1998; Khabbaz *et al.*, 1999; Volke-Sepulveda *et al.*, 2002) or chemical oxidation with nitric acid (Brown *et al.*, 1974) of the polyethylene prior to its exposure to biotic environment have been reported to enhance biodegradation. It has been reported that these treatments enhance biodegradation by altering certain properties of polyethylene like increasing surface area for colonizing or by reducing molecular weight (Palmisano and Pettigrew, 1992), reducing the polymeric chain size (Albertsson *et al.*, 1987) and by forming oxidized groups (carbonyl, carboxyl and hydroxyl) (Gilan *et al.*, 2004), modifying the properties (crystallinity level, morphological changes) of the original polymer (Byungtae *et al.*, 1991), by generation of free radicals able to oxidize the polymeric molecules, resulting in the rupture of chains (Palmisano and Pettigrew, 1992). The thermal treatment has been used to make polyethylene more susceptible to biodegradation, at temperatures higher than the melting point. The thermal treatment decreases the fusion heat and increases the carbonyl content (Volke-Sepulveda *et al.*,

2002; Weiland and David, 1994). Presence of photodegradative enhancers, antioxidants, hydro peroxides and carbonyl compounds produced during manufacture increase their potential susceptibility to degradation (Albertsson *et al.*, 1987). Growth of microorganisms on plastic polymers can be related to their capability to cause changes in the polymer molecular weight and on some of their measurable physical and chemical properties (Cain, 1992; Premraj and Doble, 2005).

High surface hydrophobicity of polyethylene avoids the adherence of microorganisms to the surface, thus making it difficult for microbes to utilize them as carbon or energy source. Several attempts have been made to overcome this obstacle of polyethylene surface hydrophobicity. Addition of a surfactant to the culture media containing polyethylene was shown to enhance the rate of biodegradation (Albertsson and Karlsson, 1993; Karlsson *et al.*, 1988; Liyoshi *et al.*, 1998). The surfactant apparently increased the hydrophilicity of the polyethylene surface and thus facilitated the adhesion of bacteria to the polymer. In another study, the effect of mineral oil on biodegradation was studied (Gilan *et al.*, 2004). The mineral oil found to increased both biofilm formation and the subsequent biodegradation of polyethylene presumably by increasing the hydrophobic interactions between the bacterial biofilm and the polymer surface. Studies were conducted to show physical evidences of colonization of polyethylene by both fungi and bacteria (Clutario and Cuevas, 2001; Sivan *et al.*, 2006). In a similar attempt, several researchers have mentioned that the active colonizers of polymer are able to adhere to their substrates because of their ability to produce exocellular polymers composed primarily of nonionic and anionic polysaccharides. Such adhesion to surfaces of substrates is found be decisive step in microbially induced corrosion (Kaeppeli and

Fiechter, 1976; Milstein *et al.*, 1992; Sietsma and Wessels, 1981; Whitekettle, 1991). It was hypothesized that after adhesion, solubilizing agents are produced and secreted by many microorganisms capable of utilizing water-immiscible compounds (Gutnick and Minas, 1987; Reddy *et al.*, 1982).

2.4.2.3.1 Degradation study of low density polyethylene (LDPE)

Being a major contributor to plastic waste problem, much emphasis has been given in past two decades to study the biodegradation of LDPE. There are several reports on biodegradation of LDPE under compost (Chiellini *et al.*, 2003; Chiellini, 2004; Dave *et al.*, 1997; Greizerstein *et al.*, 1993; Hueck, 1974; Jones *et al.*, 1974; Jakubowicz, 2003; Vallini *et al.*, 1994; Yabannavar and Bartha, 1994) or pure shake flask culture conditions (Anthony *et al.*, 1992; Byungtae *et al.*, 1991; EI-Shafei *et al.*, 1998). Most of the studies have been carried out on thermally treated LDPE containing additives like starch (Goheen and Wool, 1991; Otey and Westhoff, 1982) or prooxidant (Byungtae *et al.*, 1991; Weiland *et al.*, 1995). Byungtae et al. (1991) reported the lignin-degrading fungi *Phanerochaete chrysosporium* and *Streptomyces badius* 252, *S. Setonii* 75Vi2 and *S. Viridosporus* T7A to degrade low-density polyethylene containing starch and pro-oxidant in pure shake flask culture. It was also been reported that degradation was due to secretion of extracellular enzymes(s), capable of attacking and modifying the polyethylene (Anthony *et al.*, 1992, 1993; Byungtae *et al.*, 1991). The ability of fungi *Mucor rouxii* and *Streptomyces* strains to attack disposable polyethylene bags containing 6% starch was studied in pure shake flask culture and study confirmed the involvement of extracellular enzyme in degradation (Ei-Shaefai *et al.*, 1998). Biological treatment with *Phanerochaete chrysosporium* in a blend of LDPE and sugar cane bagasse for 32 days

modified the crystalline morphology (Manzur *et al.*, 1997). Jolanta et al. (2003) studied the biodegradation of low-density polyethylene (LDPE) film modified with Bionolle® by *Aspergillus niger, Penicillium funiculosum* and mixed fungi inoculum and showed that filamentous fungi can biodegrade polyethylene. Biodegradation of thermally oxidized LDPE with additive was studied under different conditions using fungal consortia (*A.niger, Penicillium funiculosum, Pascilomyces variotii* and *Gliocladium virens*) on soild agar and in liquid medium using *Streptomyces badius, S.setonii* and *S.viridosporus* (Weiland *et al.*, 1995).

In addition, biodegradation of LDPE has also been reported by some bacteria. Colonization, biofilm formation and degradation of LDPE containing UV-photosensitizer by a strain of *Rhodococcus ruber* has been reported (Gilan *et al.*, 2004; Sivan *et al.*, 2006). Thermophilic bacterium *Brevibaccillus borstelensis* strain 707 has been reported to utilize LDPE as sole carbon source (Hadad *et al.*, 2005). Biodegradation of low density polyethylene has also been reported by marine bacteria *Brevundimonas vesicularis, Bacillus sphericus, Bacillus cereus* and *Vibrio* furnissii under sea water condition (Trishul *et al.*, 2005). In a recent study, the biodegradation of various polyolefins by marine bacterial isolates *Pseudomonas* sp. and anaerobic, heterotrophic and iron reducing bacteria has been reported (Sudhakar *et al.*, 2007)

In comparison to LDPE containing additives (starch and prooxidant), only a few studies have been carried out on LDPE without additives. Biodegradation of thermally treated LDPE was studied using a consortium of four filamentous fungal strains *Aspergillus niger, Gliocladium virens, Penicillium pinophilum* and *Phanerochaete chrysosporium* in shake flask culture conditions (Manzur *et al.*, 2004). Biodegradation of

thermooxidised LDPE by two filamentous fungi *Aspergillus niger* and *Penicillium pinophilum* under axenic cultures conditions have been reported (Volke-Sepulveda *et al.*, 1999, 2002).

2.4.2.3.2 Degradation study of high density polyethylene (HDPE)

Because of inertness and resistance to microbial attack, the degradation of HDPE is a very slow process (Volke-Sepulveda *et al.*, 2002). Attempts have been made to enhance the biodegradability by incorporating additives like starch or prooxidant in HDPE. It has been reported that inertness and resistance to microbial attack was reduced by incorporating starch and prooxidant (Albertsson *et al.*, 1987; Albertsson and Karlsson, 1988). Several reports are showing the biodegradation of HDPE with additives. However only a few studies on biodegradation of HDPE without additives have been carried out. Inertness and resistance to microbial attack was reduced by incorporating starch and prooxidants. Albertsson and Banhidi (1980) examined the biodegradation of high-density (linear) polyethylene (HDPE) film (Mw 93000) for 2 years and found that the short chain oligomeric fraction contained in HDPE film is the main degraded component. A polyethylene sheet kept in contact with moist soil for 12 years showed no biodegradation (Potts, 1978). Partial degradation was observed in polyethylene films buried in soil for 32 years (Otake *et al.*, 1995). Biodegradation of polyethylene have been carried out on natural soil (Kathiresan, 2003) and compost (Orhan *et al.*, 2004) as biotic environment

There are a number of studies where the biodegradation of HDPE were carried out in axenic bacterial or fungal cultures amended with polyethylene under shake flask conditions (Dave *et al.*, 1997; Liyoshi *et al.*, 1998; Yamada *et al.*, 2001). Use of a single strain rather than a mixture makes it easier to manipulate the culture conditions. In

addition, the accessibility of the film to microbial attack appears to increase with direct contact with cultures under shake flask conditions. *Pseudomonas aeroginosa* grown in carbon-free media with HDPE (with 3% additives) as the sole carbon source showed a positive bacterial growth and a weight loss of 2.2% for degraded polyethylene samples (Agamuthu, 2005). Biodegradability of high density polyethylene film (HDPE) and low density polyethylene film (LDPE) both containing a balance of antioxidants and prooxidants were studied with defined microbial strains particularly with *Rhodococcus rhodochorus* and *Nocardia asteroids* in mineral medium (Kounty *et al.*, 2006b). In a recent study, biodegradation of polyolefins (HDPE, LDPE, PP) containing antioxidant and stabilizer was studied in sea water (Sudhakar *et al.*, 2007). A weight loss of 0.5-0.8% was observed for HDPE after six-month study.

Inert synthetic polyethylene without additive is the major part of the plastic waste. The biodegradation of polyethylene without additives have been of major concern. In the recent few years, emphasis has been given to study the degradation of polyethylene without additives. In contrast to earlier studies with polyethylene containing starch as carbon source to help microorganisms grow, biodegradation of polyethylene without additives was investigated. A strain of the fungus *Penicillium simplicissimum* YK with ability to biodegrade high density polyethylene (HDPE) was isolated (Yamada *et al.*, 2001). The result showed that with fungal activity, polyethylene with starting molecular weight in the range of 4000 to 28,000 was degraded to units with a low molecular weight of 500 after 3 months of cultivation, which indicated the biodegradation of polyethylene. Biodegradation of high-molecular weight polyethylene membrane by lignin-degrading fungi IZU154 *Phanerochaete chryosporium* and *Trametes veriscolor* was investigated

under various nutritional conditions and enzymes related to its degradation were also investigated (Liyoshi *et al.*, 1998). The results suggest that polyethylene degradation is related to lignolytic activity of lignin degrading fungi and Manganese peroxidase (MnP) is the key enzyme in polyethylene biodegradation by lignin-degrading fungi (Liyoshi *et al.*, 1998). In another study, *Aspergillus niger* a fungus was used in the degradation of commercially available thermoplastic polyethylene films and it was found that the external substrate (sucrose) in the growth medium influenced the biodegradation process of polyethylene and adapted microorganisms were able to metabolize a small portion of polyethylene (Raghavan and Torma, 2004). Effect of γ-sterilization on biodegradation of high density polyethylene has been studied under compost and fungal culture (Alariqi *et al.*, 2006). Significant changes in carbonyl content and surface erosion were observed in samples with high doses of γ-sterilization suggesting that γ-sterilization petreatment facilitate the biodegradation of neat polymer matrix in biotic conditions significantly (Alariqi *et al.*, 2006).

Thus, it is clear that compared to large number of reports regarding biodegradation of biodegradable plastics and polyethylene with additives such as starch and prooxidant, there are only few reports on biodegradation of polyethylene without additives. During the recent few years, attention has been given to study the possibility of biodegradation of polyethylene without additives. It has been hypothesized that if right kind of microorganisms are selected, then the so called non biodegradable polyethylene can be degraded. In the present work, an attempt has been made to study the biodegradation of high density polyethylene (HDPE) without additives by fungi under axenic culture conditions.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and polymers used

All chemicals used were of analytical grades and purchased from Sigma (USA), Merck (Germany) and Hi Media (India).

The test materials used for present study were high density polyethylene (HDPE) and polyurethane (PU). HDPE of 0.95 g/cm^3 density and 20 μ thickness was kindly supplied by Carry n Packers, Surat, India and was used as such without any modification. The polyurethane (PU) used for the present study was of the type polyester polyurethane and was supplied as sheets by Instapak^R, USA. Its composition was not known since the product was patented.

3.2 Chemical structure of HDPE and PU

The chemical structures of HDPE and PU, polymers chosen for the degradation studies is shown in Fig.2.

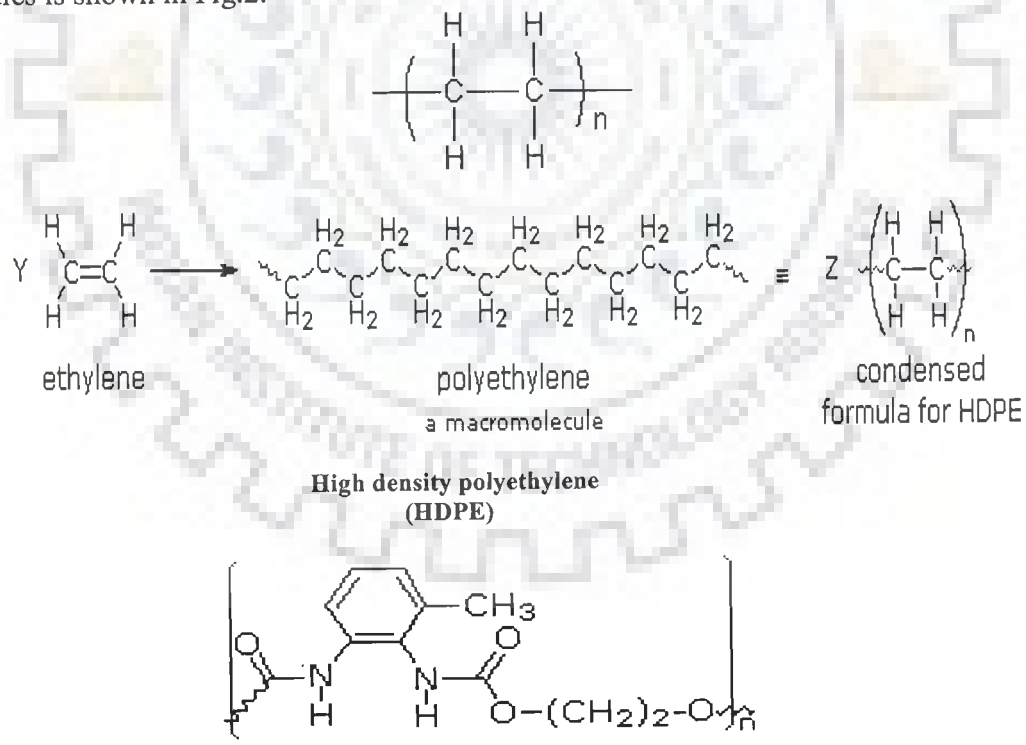


Fig. 2 Chemical structures of HDPE and PU

3.3 Pretreatment (Physicochemical treatments) of high density polyethylene (HDPE) prior to biodegradation

3.3.1 Thermal oxidation of polyethylene

Since the polyethylene films are relatively resistant to microbial attack in normal condition. To accelerate the degradation activity, sheets of polyethylene films were placed in a 70°C preheated air oven for a maximum of 10 days. For constant heating, the orientation of sheets were changed every 4 days. Prior to transfer to liquid media, the polyethylene films were cut into strips of size 4 x 1 inch (10 x 2.5 cm each) and ensured that they are free of edge nicks and creases (Fig. 3).

3.3.2 Sterilization by chemical disinfection

The disinfection of the preheated polyethylene films was carried by soaking and stirring the strips for 30-60 min at room temperature, into a fresh solution of disinfectant (7 ml tween-80, 10 ml bleach and 983 ml of sterile water). The films were then removed aseptically using sterile forceps in laminar flow-hood and placed into a covered beaker filled with sterile water and stirred for 60 min at room temperature. The films were then aseptically transferred into 70% (v/v) ethanol solution and left for 30 min. Each film was then placed into a pre weighed sterile petri dish. Petri dish containing films were placed in an oven at 50°C to dry overnight. They were equilibrated to room temperature and weighed (Fig. 3).

3.4 Pretreatment of Polyurethane (PU)

Polyurethane sheet was cut into films of size 4 x 3 cm and 1mm thickness. Films were sterilized with the help of absolute alcohol and kept in desiccator. Films were weighed before inoculation.

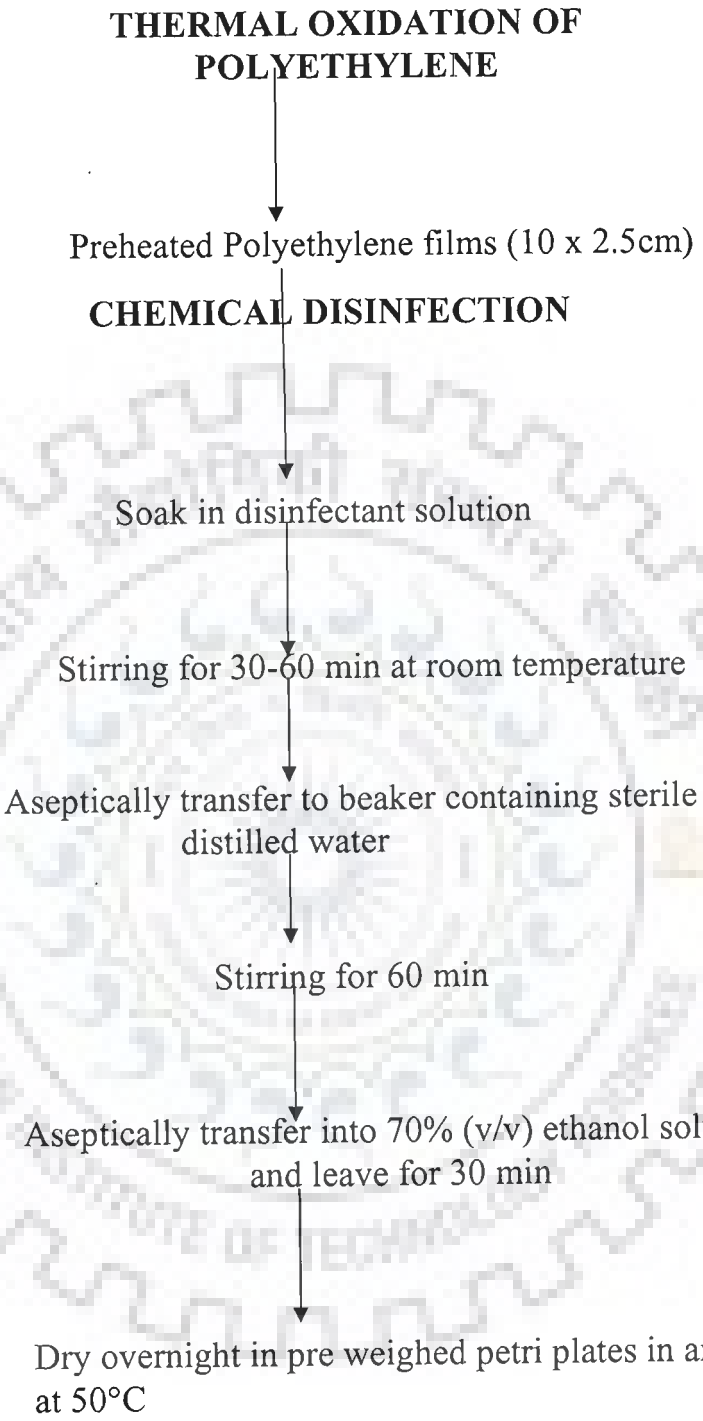


Fig 3. Flow chart of the procedure for physicochemical treatment of high density polyethylene.

3.5 Isolation, selection and identification of potential plastic degrading fungi

3.5.1 Collection of sample for isolation

Polyethylene films buried under soil in riverbed of Solani River, Roorkee (Uttarakhand) and local municipal waste dumpsites were collected and used for the isolation of polyethylene degrading fungi.

3.5.2 Media for isolation, cultivation and maintenance of fungal isolates

3.5.2.1 Media composition

Potato dextrose agar (PDA) media supplied by Himedia was used for growth and maintenance of fungal isolates and its composition is listed in Table 6. Media showing the growth or discolouration was discarded. Media sterilization was done by autoclaving at 121°C and 15 lb pressure for 20 min. The pH values of the media were adjusted with 0.1 M sodium hydroxide or hydrochloric acid to the desired value.

Table 6. Composition of Potato dextrose agar (PDA) (Himedia-India)

Constituents	Amount (g/l)
Potato (peeled)	200.0
Dextrose	20.0
Agar	15.0

Distilled water was added to make up to 1 liter volume, pH 5.6 ± 0.2

3.5.2.2 Isolation of plastic degrading fungi and growth conditions

Polyethylene sample was washed gently using sterile water to remove soil debris and it was then cut into small pieces. One gram of the sample infested with microorganism was transferred into the conical flask containing 99 ml of sterile water. It was shaken vigorously for 30 min for its equal distribution. Supernatant was then serially

diluted. A dilution series up to 10^{-6} was made and aliquots of 0.1 ml from each dilution were spread on potato dextrose agar (PDA) plates and then incubated at 28 ± 2°C. Growth on the petri plates was observed after 48 h of incubation. The distinct isolated colonies were picked and sub cultured to get pure culture. Pure cultures were then transferred on PDA plates and were incubated at 28 ± 2°C for 5 days (Fig. 4).

3.5.2.3 Maintenance and preservation of fungal strains

The isolated strains were maintained on potato dextrose agar slants, stored at 4°C and were sub-cultured on PDA slants for routine use after one month interval. All stock cultures were preserved at -80°C as glycerol stocks in air tight vials. Cultures were also maintained in lyophilized state.

3.5.3 Selection of potential plastic degrading strains

3.5.3.1 Evaluation of fungal cell surface hydrophobicity

Fungal cell-surface hydrophobicity was estimated by Microbial adhesion to hydrocarbons (MATH) assay (Rosenberg *et al.*, 1980), which is based on affinity of fungal cells for hydrocarbon hexadecane. The more hydrophobic a cell is, greater affinity for hydrocarbon, resulting in transfer of cells from aqueous to organic phase and a consequent reduction in the turbidity of the culture. Fungal cells at a concentration of 10^7 cells/ml were suspended in 10 mM potassium phosphate buffer (pH 7.0). Next, 200 µl of hexadecane was added to 3 ml of cell suspension and vortexed for 20s. The solution was then allowed to stand for 10 min and phases allowed to cleanly separate. The absorbance of the aqueous suspension before (A_0) and after (A_t) mixing was recorded at 600 nm. The percentage of cells adhering to organic phase was calculated with the following formula:

$$\text{Percentage adhering (\%)} = (A_0 - A_t) / A_0 \times 100.$$

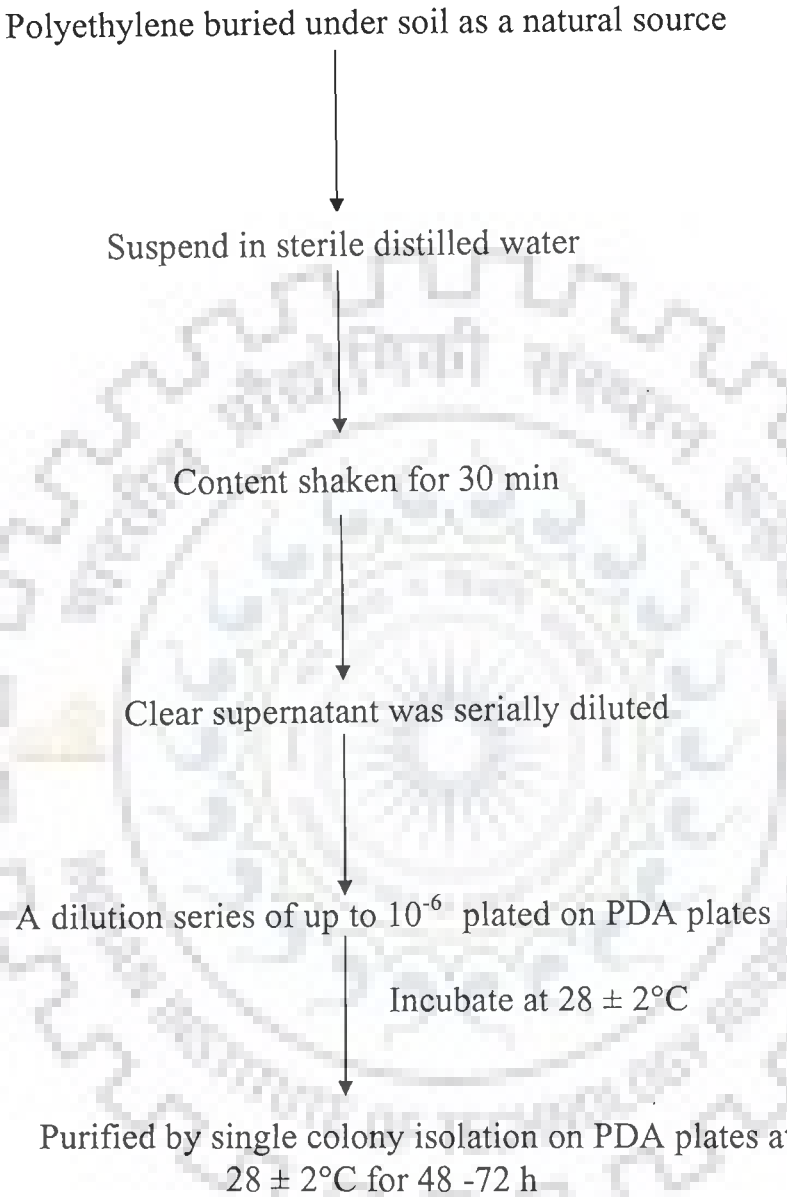


Fig. 4. Flow chart for the procedure used for isolation of plastic degrading microorganisms

3.5.3.2 Esterase producing ability

Culture broths were further analyzed for the presence of esterase enzyme that are found to have role in biodegradation of polymers. Esterase activity in culture broths of different fungal isolates grown in presence of HDPE or PU in minimal media was analyzed using zone formation assay on esterase substrate plates (Singh *et al.*, 2006). The cell free culture broth was used as a source of esterase enzyme. Chromogenic substrate plates were prepared by using phenol red (0.01%) along with 1% lipidic substrate [tributyrin (himedia)], 10 mM $CaCl_2$ and 2% agar. The pH was adjusted to 7.3-7.4 by using 0.1 N NaOH. A small disc of filter paper was impregnated with culture broth from inoculated culture and placed on top of chromogenic substrate plates. The plates were incubated at 37°C for 15-20 min. Disc impregnated with control broth served as control.

3.5.3.3 Screening for ability to utilize polyethylene or polyurethane as carbon source

Ability of the fungal isolate to utilize polyethylene or polyurethane as carbon source was estimated in terms of their growth in minimal media containing polyethylene or polyurethane. Determination of dry weight of fungal biomass was done after 7, 14, 21 and 30 days of incubation. For the determination of dry weight of fungal biomass, the culture broth was filtered through Whatman No. 4 filter paper. The filtered mycelia were washed thoroughly with distilled water. In case of HDPE, fungal hyphae adhering to the surface of films was scrapped off using flat edges of scalpel. Scrapped cells and one obtained after filtration were pooled and were dried at 50°C and weighed.

3.5.4 Morphological study and identification of fungal isolates

Morphological study of fungal isolates was done using Light microscopy and Scanning Electron Microscopy (SEM).

3.5.4.1 Colony morphology

PDA grown colony morphology features of various fungal isolates were studied based on appearance of colony, colour of spores, mycelia and media colouration.

3.5.4.2 Light Microscopy and Scanning Electron Microscopy (SEM)

For light microscopy, samples were stained with lactophenol cotton blue and observed under compound microscope (Axioscope, Zeiss, Germany) supplemented with a camera).

The detailed morphological study of fungal strains were carried out using Scanning Electron Microscopy (SEM) Pieces of fungal matte were taken and primary fixation was done overnight in 4% glutaraldehyde in 0.05 M Phosphate buffer saline (PBS), pH 7.3. After primary fixation, samples were rinsed with 0.05 M PBS (3 times, 10 min each). Samples were then subsequently dehydrated in a series of ethanol washes (70%, 80%, 90% for 5 min each and 100% 3 changes, 5 min each). The dehydrated samples were dried, mounted and sputter coated with gold and then observed under scanning electron microscope (SEM LEO 435 VF, LEO electron microscopy, England). Electron micrographs were obtained at desired magnifications. Composition of PBS buffer is given in Table 7.

Table 7. Composition of PBS Buffer

Constituents	Amount (g/l)
NaCl	8
KCl	0.2
Na_2HPO_4	1.44
KH_2PO_4	0.24

Adjust pH to 7.3.

3.5.4.3 Identification of potent plastic degrading fungal isolates

The isolated strains were deposited at identification services, Indian Type Culture collection (ITCC), Indian Agricultural Research Institute (IARI), New Delhi for identification according to standard methods.

3.6 Study on colonization and biofilm formation on polyethylene surface

3.6.1 Media composition

Colonization, biofilm formation and degradation were studied using minimal media containing 5.03g l^{-1} Na_2HPO_4, 1.98g l^{-1} KH_2PO_4, 0.2 g l^{-1} $MgSO_4.7H_2O$, 0.2g l^{-1} NaCl, 0.05g l^{-1} $CaCl_2.2H_2O$, 0.01% malt extract and 1 ml of trace element solution in distilled water Table 8. pH was adjusted to 5.5 and was autoclaved for 15 min at 121°C.

Table 8. Composition of minimal media for colonization, biofilm formation and biodegradation study of polyethylene

Constituents	Amount (g/l)
Na_2HPO_4	5.03
KH_2PO_4	1.98
$MgSO_4.7H_2O$	0.2
NaCl	0.2
$CaCl_2.2H_2O$	0.05
malt extract	0.01 %

3.6.2 Culture conditions for colonization and biofilm formation

Screening of isolated fungal strains for their ability of colonization and biofilm formation on polyethylene surface was carried out in minimal media mentioned above. High density polyethylene films of size 10 x 2.5 cm were pre weighed, chemically disinfected and aseptically transferred to 100 ml sterilized culture medium before inoculation and incubated for 24 h with shaking to ensure asepsis.

For the preparation of inoculum, fungal strains were transferred to PDA petri plates and incubated at 28 ± 2°C for 5 days. Spores were harvested by scraping the growth from the agar surface with a sterile wire loop. The fungal growth was vigorously shaken in a test tube with sterile distilled water and was poured through sterile glass wool to separate spores from mycelia. Spores were further isolated by a series of filtration through sterile Whatman filter paper of smaller pore size. This was continued until investigation under microscope indicated that only spores remained. This spore suspension was washed three times with sterile distilled water to remove nutrients. Spores were counted using Haemocytometer (counting chamber) and resuspended in the appropriate volume of sterile water to result in a concentration of 10^7 spores/ml.

Culture media were then inoculated with respective fungal isolates having a spore concentration of $2x10^5$ spores/ml and were incubated with shaking at 120 rpm for 30 days at 28 ± 2°C. For control, heat treated polyethylene films were incubated in culture media under similar conditions without inoculation.

3.6.3 Estimation of colony forming units (CFU)

Fungal population density was estimated using procedure from Webb et al. (2000). Polyethylene films were removed from culture medium after 7, 14, 21 and 30

days of incubation. Polyethylene films were washed in sterile distilled water and were then transferred to Petri plate containing 5 ml of sterile distilled water and scrapped three times on both sides using flat edges of a sterile scalpel blade. Polyethylene film, scalpel and water were then transferred to tubes and shaken for 1 min. A dilution series up to 10^{-5} was prepared from each tube and aliquots of 0.1 ml from each dilution series were spread on Potato dextrose agar (PDA) plates in triplicate. Viable counts were performed after 3-7 days of incubation at 28 ± 2°C. The number of colonies appearing on plates were counted, averaged and multiplied by the dilution factor to find out CFU.

3.6.4 Estimation of fungal biofilm viability

The activity and viability of fungal biofilm on polyethylene surface was determined by Fluorescein diacetate (3', 6'-diacetylfluorescein) FDA hydrolysis (Lopes *et al.*, 2002). The cytotoxic test was improved by combining fluorescein diacetate (FDA) and ethidium bromide (EB) that showed a strong contrast between living and dead cells (Lopes *et al.*, 2002).

For viability testing, 1 g of each sample was suspended in 1 ml of distilled water. Thereafter 0.1 ml of a 1:1 mixture of a fluorescein diacetate (FDA) solution (2 µg/ml in PBS buffer, pH 7.4) and ethidium bromide (50 µg/ml in PBS buffer, pH 7.4) was added to 0.1 ml of the suspension. This mixture was incubated at 25°C for 30 min. Samples were viewed under fluorescent microscope (Zeiss, Germany).

3.6.5 Determination of Extracellular polysaccharide (EPS) and protein in biofilm

EPS analysis was performed as per the method described by Sivan *et al.*, (2006) with slight modification. Samples of polyethylene films (10 x 2.5 cm) that had been

disinfested were added to flasks each containing 100 ml of minimal medium. The flasks were inoculated as described above. Each test was performed in triplicate. At regular time intervals, films were removed from each flask, gently rinsed with distilled water and incubated in flasks containing 50 ml of 1.5% (v/v) aqueous SDS solution for 5 h. The polyethylene films were removed and the suspension containing the detached biofilm was centrifuged for 10 min at 8000 rpm. The resulting supernatant was dialyzed overnight in dialysis tubes (6,000–8,000 kDa cut-off). The samples were then tested for presence of polysaccharides and proteins.

Extracellular polysaccharides were determined by Phenol-sulfuric acid method (Dubois *et al.*, 1956) and protein concentration was determined using Bradford method (Bradford, 1976).

3.6.6 Scanning Electron Microscopy for biofilm analysis

Polyethylene films containing biofilm were removed from culture medium at selected time intervals (14, 30, 60 days) and washed in distilled water. Biofilms were then placed in a fixative (4% glutaraldehyde in 0.05 M phosphate buffer) overnight at 4°C. The samples were rinsed in 0.05 M PBS (3 times, 10 min each) and subsequently dehydrated in a series of ethanol washes (70%, 80%, 90% for 5 min each and 100% 3 x 5 min each). They were finally dried under vacuum. Samples were then mounted, sputter coated with gold and examined under SEM LEO 435 VF (LEO electron microscopy, England).

3.7 Degradation study of polyethylene by fungal isolates

3.7.1 Media composition and growth conditions for degradation

The composition of media used for biodegradation study of polyethylene is shown in Table 8.

The composition of mineral salt medium (MSM) used for polyurethane was as follows (gm/l) (Table 9): $NaNO_3$, 2.0; KH_2PO_4, 0.7; K_2HPO_4, 0.3; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.1. The pH was adjusted to 6.0-6.5.

The degradation tests were carried out in 250 ml flask with a working volume of 100 ml as pure shake flask culture. For polyethylene, culture media was then inoculated with respective fungal isolates having a spore concentration of $2x10^5$ spores/ml and were incubated with shaking at 120 rpm for 30 days at 28 ± 2°C. For control heat treated and unheated sterile polyethylene films were incubated in culture media under similar conditions without inoculation.

For polyurethane, Inoculum concentration of 4.8×10^6 spores/ml was adjusted with the help of a haemocytometer. Test films were used in triplicates and were suspended aseptically in flasks containing 100 ml of mineral salt medium. Flasks were inoculated with 3 ml per 100 ml of test inoculum concentration. Controls were also kept. All the exposures were kept at 28 ± 2°C in an incubator for 30 days with shaking at 120 rpm.

Table 9. Media composition for biodegradation study of polyurethane

Constituents	Amount (g/l)
$NaNO_3$	2.0
KH_2PO_4	0.7
K_2HPO_4	0.3
KCl	0.5
$MgSO_4.7H_2O$	0.5
$FeSO_4.7H_2O$,	0.1

3.7.2 Film harvest

After 30 days of incubation, polyethylene and polyurethane films were harvested. They were rinsed with sterile water, then washed in 70% ethanol to remove cells mass from the residual films as much as possible and then dried in pre weighed petri plates at 45°C overnight.

3.7.3 Evaluation of degradation using analytical methods

3.7.3.1 Determination of starch content of polyethylene film

The starch content of polyethylene was determined according to the phenol-sulfuric acid procedure of Fratzke et al. (1991). A 60 mg of polyethylene sample was dissolved in 4 ml of decalin and heated at 150°C for 40 min. After brief cooling, 5 ml of 1M HCl was added to solubilize, hydrolyze and extract the starch at 100°C for 30 min. After heating, the decalin was removed with Pasteur pipette, 1 to 2 ml of the aqueous phase was transferred to another tube and the phenol-sulfuric analysis was performed. The sample aliquot was added to the distilled water (total volume 1 ml) plus 1 ml of 6% phenol and 5 ml of concentrated H_2SO_4. After 30 to 60 min, the A_{490} values of phenol sulfuric acid samples relative to that of distilled water were read. A standard curve was prepared with native corn starch.

3.7.3.2 Weight loss measurement

After drying at 45°C overnight, the polyethylene and polyurethane films were equilibrated to room temperature. The post-incubation weight was compared to the weight prior to incubation. The weight of the films in the control was also measured for comparison. The percent weight reduction was computed with the formula: percentage

weight reduction (%) = $(W_1-W_2/W_1) \times 100$, where W_1 is the pre-incubation weight and W_2 is the post incubation weight of the polyethylene or polyurethane films.

3.7.3.3 Tensile strength determination

Polyethylene degradation was measured by changes in tensile strength (the stress measured at the fracture of the specimen). Tensile strength was determined on Instron model 1128 Universal (Instron Corporation, Canton, MA) Tensile tester at room temperature and 500 mm per min with a 5 cm gap. All samples were equilibrated to 50% relative humidity for at least 40 h before analysis (ASTMI 882-83, Standard Test Method for Tensile Properties of thin Plastic Sheet). Reduction in tensile strength of fungus treated polyethylene film as compared to those of untreated film was calculated.

3.7.3.4 Scanning Electron Microscopy (SEM)

Pieces of polyethylene and polyurethane films were washed in distilled water. They were fixed overnight at 4°C in 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) and washed three times (10 min each) in phosphate buffer. Samples were dehydrated through 70, 80, 90 and 100% ethanol (5 min in each stage) and three changes in 100% ethanol at room temperature. Samples were then dried under vacuum. They were mounted on stubs and coated with gold in a sputter coater and examined under SEM LEO 435 VF (LEO electron microscopy, England) and photographed (Lopez and Valientet, 1993).

3.7.3.5 Differential Scanning Calorimetry (DSC)

DSC studies were conducted using Perkin Elmer (Pyris Diamond) thermal analyzer under nitrogen atmosphere. The instrument was calibrated using Indium. Samples of 5-10 mg were sealed in aluminium pans and subjected to heating-cooling

cycles. Previous thermal effects were minimized by initially heating the samples until melting. The samples were then cooled to room temperature at a constant heating rate of 5°C/min to favour crystallization. Subsequently, the second heating was recorded at 10°C/min in the temperature range of 25-170°C/min resulting in endothermic curves (Chandra and Rustgi, 1997). Melting (T_m) and onset melting (T_o) temperatures were calculated from thermograms obtained with DSC (Manzur *et al.*, 2004).

For polyurethane, the reference sample was alumina. Nitrogen was used as purge gas at the pressure of 100ml/min. The temperature range used was 0-500°C.

3.7.3.6 Fourier Transform Infrared Spectroscopy (FT-IR)

The chemical changes of polyethylene and polyurethane films during incubation with different fungal isolates were evaluated by changes in the film infra red spectra measured on a Thermo Nicolet Corporation, Madison (NEXUS FT-IR spectrometer). Films were affixed directly to standard infrared sample plates. A spectrum of untreated sample was recorded as control. Relative intensities of the carbonyl band at 1715 cm^{-1} (carbonyl index) and double bond band at 1615 cm^{-1} (Double bond index) to that of the methylene band at 1465 cm^{-1} and ether bond (Ether index) at 1105 cm^{-1} to 1413 cm^{-1} were evaluated.

3.7.3.7 X-ray Diffraction (XRD)

Changes in the final crystallinity ($\%C_{XRD}$) of polyethylene film were estimated using the X-ray diffraction technique (XRD). The XRD patterns were recorded with X-Ray diffractometer (Bruker AXS D8 Advance) fitted with a scintillation counter, a pulse-height analyzer, and a graphite crystal monochromator placed in the scattered beam. CuKα radiation (λ=1.5418Å) was used and the scattered radiation was registered in the

angular interval (2θ) from 12–28°. $\%C_{XRD}$ of HDPE samples was calculated by using XRD data , with the following relation:

$$\%C_{XRD} = \frac{A_c^{110} + A_c^{200}}{A_a + A_c^{110} + A_c^{200}}$$

Where A_a and A_c^{hkl} are the areas under the amorphous halo and the *hkl* reflections, respectively.

3.7.3.8 Gas chromatography-Mass Spectrometry (GC-MS)

Degradation products were separated and identified by means of a Perkin Elmer gas chromatograph equipped with HP-1 column coupled with a mass spectrometer. The oven temperature was held for 1 min at 50°C, then increased at 5°C/min to 310°C and held for 10 min. The carrier gas was helium. The injector temperature was 250°C and the interface was maintained at 300°C. Compounds were identified by comparison with NBS database. Culture broth was examined for degradation products. It was acidified with concentrated HCl to pH 2.0, extracted with 2x100 ml diethyl ether, the ether extracts were bulked, dried over anhydrous Na_2SO_4, filtered and 1.0 µl samples were injected onto gas chromatograph.

3.8 Hydrolytic enzyme (Esterase, Lignin Peroxidase and Manganese Peroxidase) production

3.8.1 Esterase activity assay

The esterase activity was assayed by measuring the rate of hydrolysis of *p*-nitrophenyl ester as per methods of Kordel et al. (1991). Briefly, one volume of a 16.5 mM solution of *p*-nitrophenyl acetate (pNPA) in 2-propanol was mixed just before use with 9 volume of a 50 mM Tris-HCl buffer, pH 8.0 containing 0.4% (w/v) Triton X-100

and 0.1% gum arabic. Then 1.35 ml of this mixture was equilibrated at 37°C in a 1ml cuvette. The reaction was started by addition of 0.15 ml of the enzyme solution at an appropriate dilution in the 50 mM Tris-HCl buffer, pH 8.0. The variation of the optical density at 410 nm was monitored for 2-5 min against a blank without enzyme using a Varian Carry 100 Conc UV/VIS spectrophotometer (USA). The concentration of the liberated *p*-nitro phenol (pNP) was estimated using as extinction coefficient of 12.75 x 10^6 cm^2/mol. The value of molar extinction coefficient was determined from the absorbance of standard solution of pNP in the reaction mixture. One enzyme unit was the amount of enzyme liberating one µmol of pNP per min under above conditions.

3.8.2 Lignin Peroxidase (LiP) assay

Lignin peroxidase (LiP) activity was measured by monitoring the oxidation of veratryl alcohol to veratraldehyde at 310 nm (Arora and Gill, 1991). The standard reaction mixture consisted of 1ml of 125 mM sodium tartarate buffer (pH 3.0), 50 µl of 10 mM veratrylalcohol, 500 µl of 2 mM H_2O_2 solution and 500 µl of culture filtrate. The reaction was initiated by adding H_2O_2 and the change in the absorbance was monitored at 310 nm. One unit of enzyme activity was one µmole of veratraldehyde produced per min per ml of the culture filtrate. Specific enzyme activity was expressed as enzyme units per mg protein.

3.8.3 Manganese Peroxidase (MnP) assay

MnP activity was determined by a modified method as described by Wariishi et al. (1992). Each 1 ml (final volume) reaction mixture contained 50 mM sodium malonate (pH 4.5), 0.5 mM $MnCl_2$, 0.2 mM H_2O_2, and 5 to 50 µl of enzyme source. The reaction

was initiated at 25°C by adding H_2O_2 and the rate of Mn^{3+}-malonate complex formation was monitored by measuring the increase in absorbance at 270 nm (ε_{270}=11590 M cm^{-1}).

3.9 Partial characterization of esterase

3.9.1 SDS- Polyacrylamide gel electrophoresis

Culture broths were analyzed for the presence of esterase enzyme using SDS-PAGE, performed essentially as described by Laemmlli (1970) using 10% gels with a ratio of acrylamide to bis-acrylamide of 29.2:0.8. Electrophoresis was performed at a constant voltage of 100 V. Medium molecular weight molecular mass standards (GENEI, Bangalore, India) included the following: Phosphorylase b (97.4 KDa); Bovine serum albumin (66 KDa); Ovalbumin (43 KDa); Carbonic anhydrase (29 KDa); Lysozyme (14 KDa). Gels were stained with Coomassie brilliant blue and were observed.

3.9.2 Detection of esterase activity by Zymogram

Native polyacrylamide gel electrophoresis (PAGE) was performed using discontinuous gel system. Gels were cast with 5% stacking gel and 10% resolving gel. Proteins were allowed to stack at 100 V and to separate at 80 V. The gels were rinsed three times with distilled water and equilibrated in 50 mM Tris/HCl (pH 8.0) for 30 min at room temperature. The gels were overlaid with the molten chromogenic substrate (at 40°C), which was then allowed to solidify and incubated at 37°C. Depending on the amount of esterase, the activity was observed within 15 min as yellow band over a pink background. Chromogenic substrate was prepared using Phenol red (0.01%) along with 1% lipidic substrate (tributyrin, Himedia), 10 mM $CaCl_2$, and 2 % agar. The pH was adjusted to 7.3–7.4 by using 0.1 N NaOH.

3.10 Statistical analysis

All degradation studies were carried out in triplicate and experimental results represents the mean of three identical degradation studies. Standard deviation (SD) and standard error (SE) were calculated using following formula:

$$SD = \frac{\sqrt{\sum X^2}}{N}$$

$$SE = \frac{SD}{\sqrt{N}}$$

Instruments and equipments

Instrument	Company
Homogenizer	Remi motors, Mumbai, India
Gel electrophoresis	Hoefer Scientific Instruments, Sanfranciscs, USA
Laminar Air Flow	ACCO, India
Centrifuge	Hermle Labortechnik GmbH, Germany
Scanning Electron Microscopy (SEM) (Model LEO 435 VP)	Leo Electron Microscope Ltd., England
Sensitive balance	Samson, India
GC-MS	Perkin Elmer
µP pH meter (TMP-85)	Toshniwal Instruments Mfg. Pvt. Ltd. India
Autoclave	Vikrant Scientific Ltd., India
Incubator Universal (Memmert Type)	Hindustan Scientific Instruments company, India
Tensile Tester Instron model 1128 Universal	Instron Corporation, Canton, MA
Light microscope with camera	Zeiss, Germany
Orbital shaker incubator	Gallen Kamp, USA
Haemocytometer	Neubauer, Germany
Water bath	Branson 2200, USA
Gel documentation and analysis system	Alpha Infotech Corporation
Thermal Analyzer (DSC)	Perkin Eelmer (Pyris Diamond)
Ultrasonicator	Misonix, USA
NEXUS FT-IR spectrometer	Thermo Nicolet Corporation, Madison
X-Ray Diffractometer (Bruker AXS D8 Advance)	Bruker AXS Inc., Madison, WI USA
UV/VIS Spectrophotometer	Perkin Elmer

CHAPTER 4

RESULTS

A. ISOLATION, SELECTION AND IDENTIFICATION OF POTENTIAL PLASTIC DEGRADING FUNGAL ISOLATES

4.1 Isolation of fungal isolates

Total twelve fungal isolates were isolated from the buried polyethylene samples collected from the dumpsites. The growth characteristics and colony morphology of all the fungal isolates were studied on PDA plates. There was found to be much variation in shape, colony form, mycelia and spore colour. The growth characteristics and colony morphology of the isolates are summarized in Table 10.

4.2 Selection of plastic degrading fungal isolates

Since the major hurdle in polyethylene biodegradation is its high surface hydrophobicity and high molecular weight, it has been suggested and logical that microorganisms with high cell surface hydrophobicity and ability to utilize polyethylene as carbon and energy source could have potential for plastic degradation. Therefore, to select the potential polyethylene and polyurethane degrading fungi, all the twelve fungal isolates were screened for their cell surface hydrophobicity, ability to utilize HDPE and PU as carbon source and extracellular esterase production.

4.2.1 Cell surface hydrophobicity and percentage adherence to hydrocarbon

All the twelve fungal isolates obtained from the same source were screened for their cell surface hydrophobicity using MATH (Microbial adhesion to hydrocarbon) assay. MATH assay clearly showed the higher cell surface hydrophobicity of isolates F-8, F-9 and F-12 compared to other isolates obtained from same source (Fig. 5). The adhesion of fungal cells to hexadecane was high even at a very low concentration of the

hydrocarbon for these three isolates F-8, F-9 and F-12, resulting in a reduction in the turbidity of the suspension of about 10, 20 and 1.5 % respectively. Other isolates were found to be less hydrophobic. Percentage adherence to hydrocarbon data showed that adhesion of isolates F-8, F-9, and F-12 to hydrocarbon was higher compared to other isolates (Fig. 6).

4.2.2 Ability to utilize HDPE and PU as carbon and energy source

The ability of fungal isolates to utilize HDPE and PU as carbon source were studied by determining fungal growth and biomass in minimal media containing HDPE or PU as carbon source. When grown in minimal media in presence of HDPE or PU as carbon source, out of twelve isolates, five fungal isolates (F-1, F-4, F-8, F-9 and F-12) showed their ability to utilize polyurethane as carbon and energy source, while only isolates F-8, F-9 and F-12 showed their ability to utilize HDPE as carbon and energy source.

Growth of fungal isolate was estimated in terms of fungal biomass (dry weight in g/100 ml). A significant increase in fungal biomass of isolates F-8, F-9 and F-12 were observed with increase of incubation time. Biomass of values 6.81, 7.2, 5.19 g/100 ml of culture medium for polyethylene and 7.04, 9.02 and 6.13 g/100 ml of culture medium for polyurethane were obtained for isolates F-8, F-9 and F-12, respectively after 30 days of incubation (Fig. 7 A and B). Fungal isolates F-1 and F-4 were also found growing in minimal media containing polyurethane as carbon source. However, the yield of biomass was significantly less as compared to F-8, F-9 and F-12.

Table 10: Colony morphology of different fungal isolates

Organisms	Appearance		Color		
	Margin	**Colony form**	**Mycelium**	**Spore**	**Media**
Fungal isolate F1	Irregular	Irregular	White	Black	No pigment
Fungal isolate F2	Regular	Regular	Brown	Brown	No pigment
Fungal isolate F3	Regular	Regular	Light yellow	Brown	No pigment
Fungal isolate F4	Thread like	Round	Yellow	Black	No pigment
Fungal isolate F5	Thread like	Round	Cream	Cream	No pigment
Fungal isolate F6	Thread like	Round	Cream	Cream	No pigment
Fungal isolate F7	Round	Hairy	Light grey	Grey	No pigment
Fungal isolate F8	Round	Irregular	Dirty green	Dirty green	No pigment
Fungal isolate F9	Round (hairy)	Hairy	Brownish Yellow	Brownish	No pigment
Fungal isolate F10	Hairy (Thread Like)	Hairy	White	White	No pigment
Fungal isolate F11	Thread like	Round	Green	Green	No pigment
Fungal isolate F12	Thread like	Round	Yellow	Black	No pigment

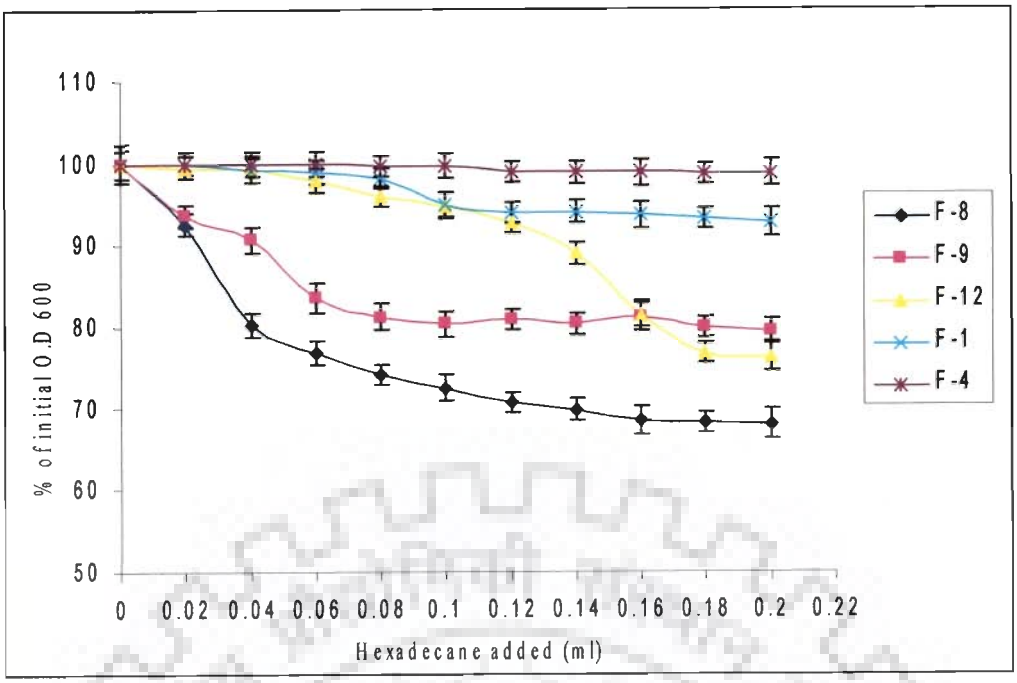


Figure 5. Cell-surface hydrophobicity of different fungal isolates. Each value represents mean ± SE of triplicates.

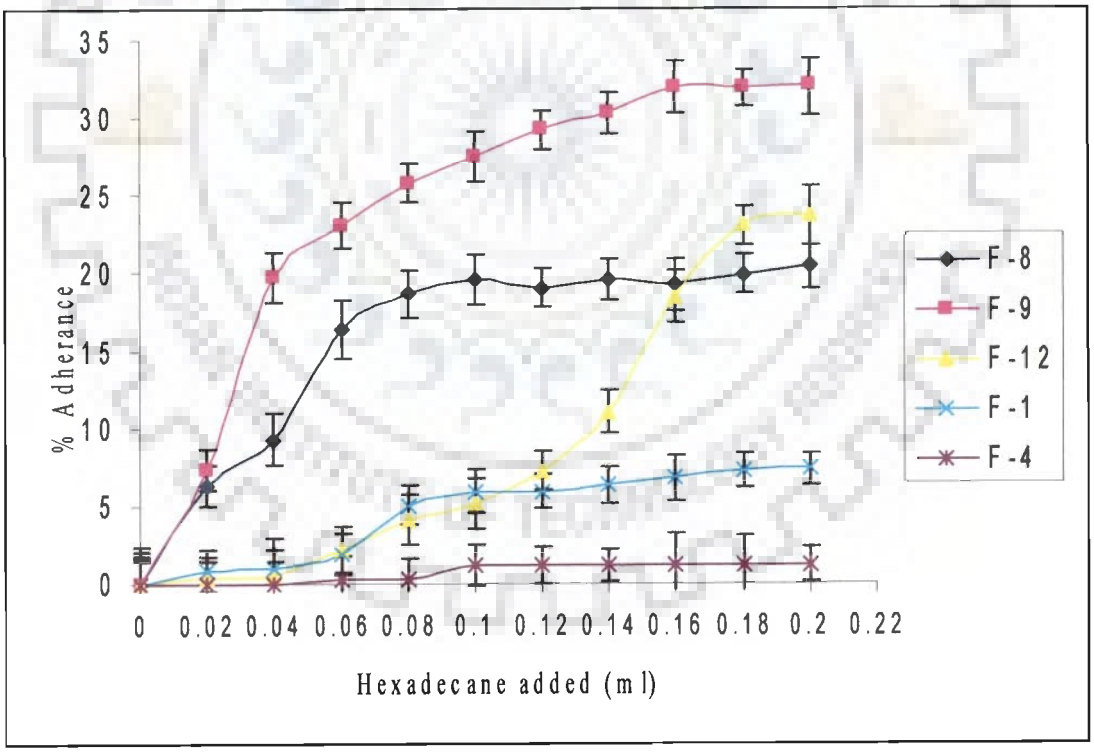


Figure 6. Percentage adherence of different fungal isolates to hydrocarbon . Each value represents mean ± SE of triplicates.

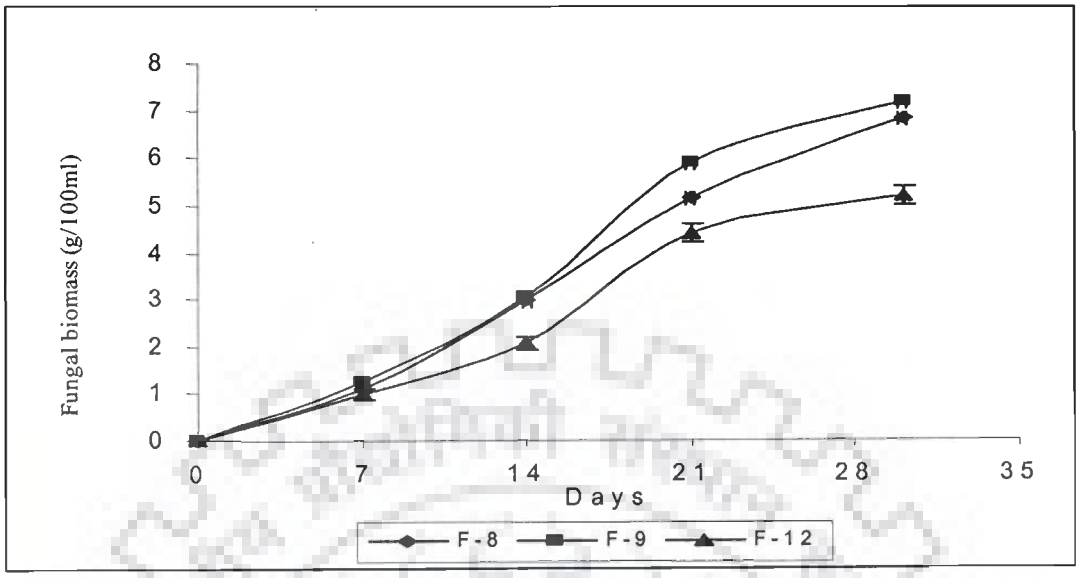


Figure 7 A. Screening of fungal isolates for their ability to utilize HDPE as carbon and energy source. The fungal isolates were grown in minimal media containing HDPE as carbon source. The biomass of fungal isolates showing significant growth in minimal media are shown. Each value represents mean ± SE of triplicates.

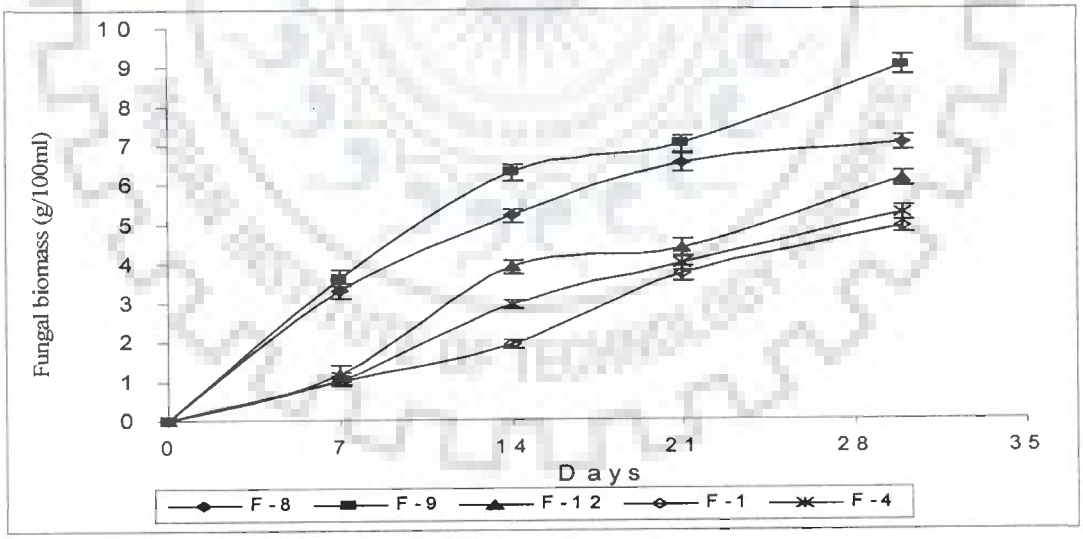


Figure 7 B. Screening of fungal isolates for their ability to utilize PU as carbon and energy source. The fungal isolates were grown in minimal media containing HDPE as carbon source. The biomass of fungal isolates showing significant growth in minimal media are shown. Each value represents mean ± SE of triplicates.

4.2.3 Esterase producing ability

Polymer degradation abilities of microbes have been reported to be associated with their hydrolytic enzyme system. Since the esterase enzymes are found to have a key role in biodegradation. Esterase activity in the culture broths of the different fungal isolates containing HDPE as carbon source was also estimated and values are shown in Fig. 8 with isolates F-8, F-9 and F-12 showing maximum esterase activity compared to other fungal isolates obtained from same source. Esterase activity of the culture broth of isolates F-8, F-9 and F-12 was also determined using zone formation assay using tributyrin as substrate. A change in colour from pink to yellow around the disc was observed in case of F-8, F-9 and F-12 culture broth impregnated disc while no change in colour was observed in control (Fig. 9A and B). There was found to be an increase in the diameter of the zone with increasing volumes of culture broth.

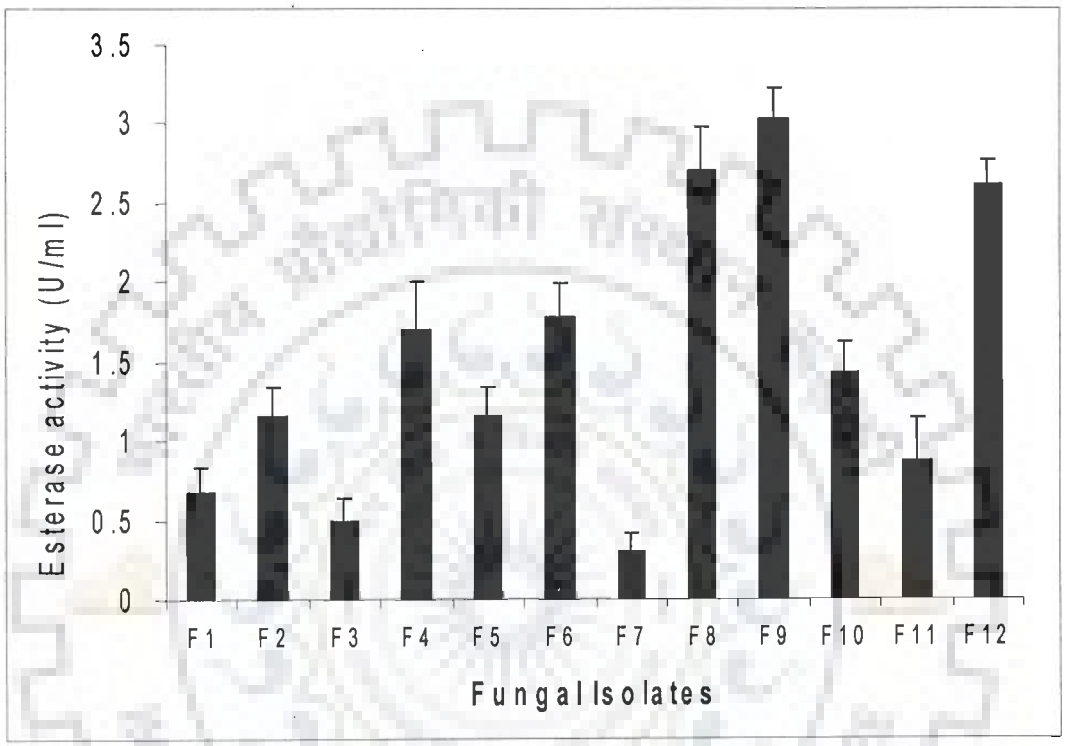


Figure 8. Esterase activity in the culture broths of the different fungal isolates. The fungal isolates were grown in minimal media using polyethylene films as carbon source for 30 days. The culture were centrifuged and esterase activity was determined using *p*-nitrophenyl acetate as substrate. Each value represents mean ± SE of triplicates.

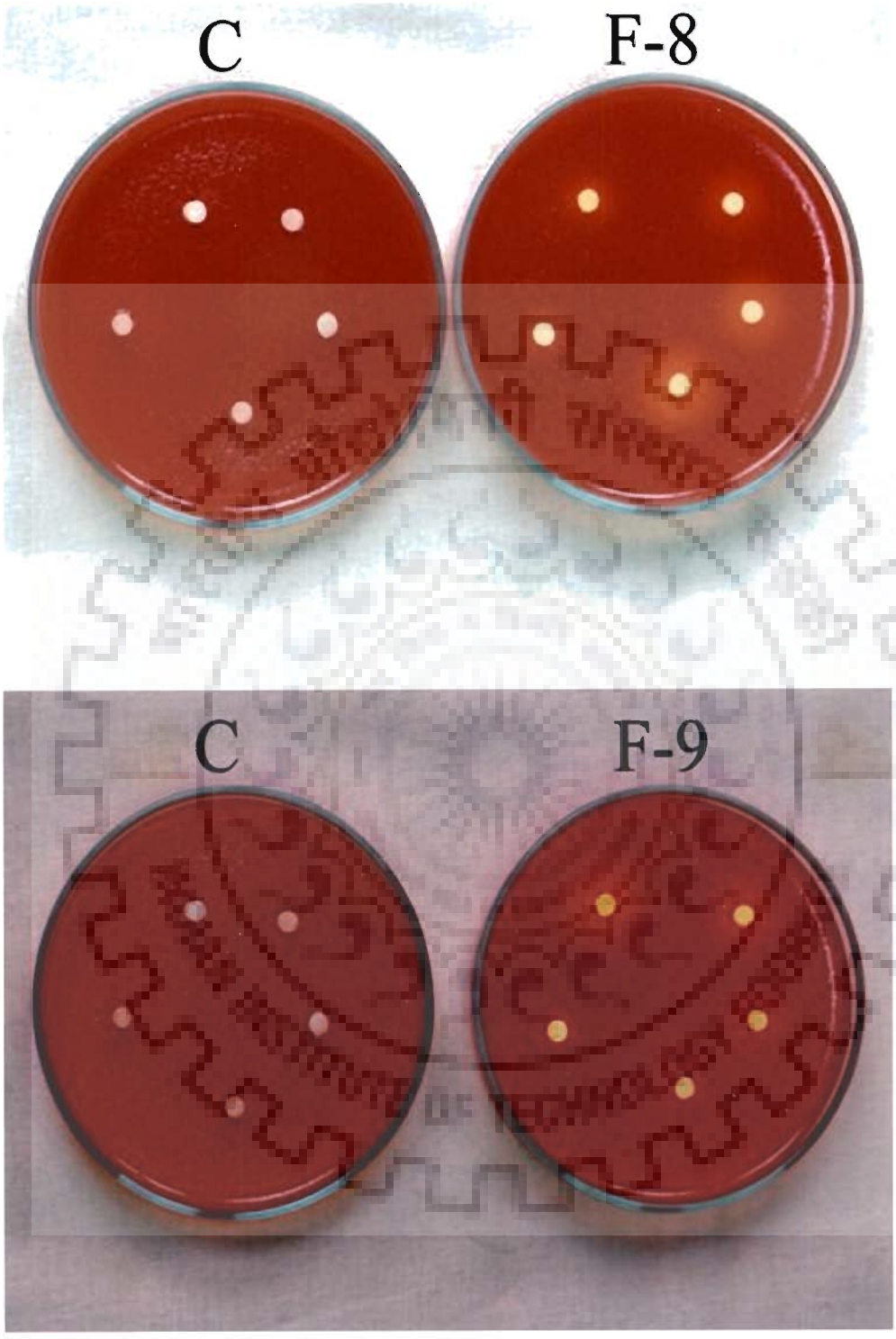


Figure 9A. Screening of different fungal isolates for their esterase secretion activity using zone formation assay. The clear zone could be seen in the background.

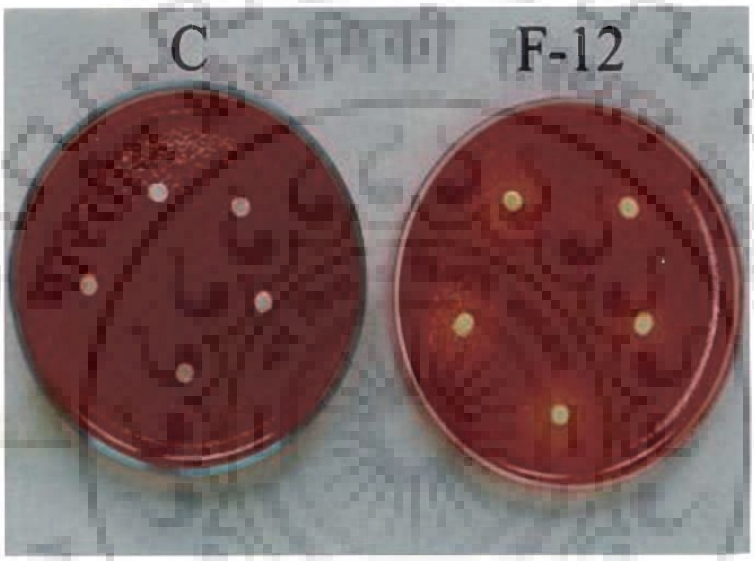


Figure 9 B. Screening of different fungal isolates for their esterase secretion ability using zone formation assay. The clear zone could be seen in the background.

4.3 Identification of the potential plastic degrading fungal isolates

Based on preliminary screening the fungal isolates F-8, F-9 and F-12 were found to be potential plastic degrading fungi as these were able to utilize HDPE and PU as carbon source. These three fungal isolates were further identified and used for detailed biodegradation studies.

4.3.1 Morphology study of fungal isolates

The morphology characteristics of spores and hyphae of these three isolates were studied using Light microscopy and Scanning electron microscopy (SEM). The characteristic features of F-8, F-9, and F-12 are shown in Fig. 10. Based on morphological features as observed from light microscopy (Fig. 10. A, B and C) and SEM (Fig. 10 D, E and F), the isolates were identified as *Aspergillus* sp. For further confirmation and identification, isolates were deposited at Identification services, Indian Type Culture Collection (ITCC), Indian Agricultural Research Institute (IARI), New Delhi.

4.3.2 Identification of fungal isolates

Based on the standard test and identification parameters, fungal isolates F-8, F-9 and F-12 were identified as *Aspergillus flavus* (ITCC N0. 6051), *Aspergillus fumigatus* (ITCC No. 6050) and *Aspergillus niger* (ITCC No. 6052) respectively by Identification/culture supply services, Indian Type Culture Collection (ITCC), Indian Agricultural Research Institute (IARI), New Delhi.

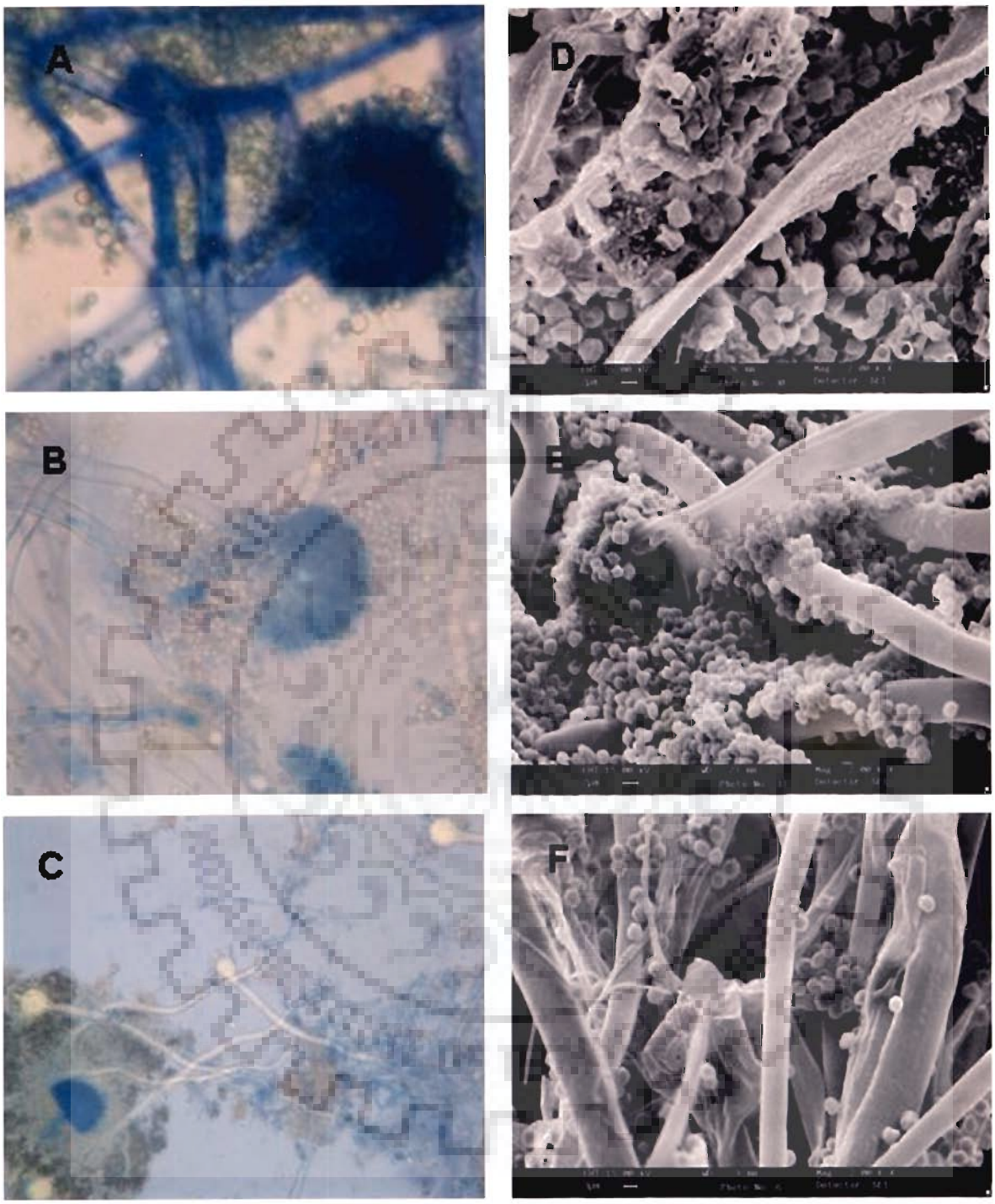


Figure 10. Morphology study of different fungal isolates using Light and Scanning Electron Microscopy (SEM). **A, B & C** are Lactophenol cotton blue stained light micrographs of F-8, F-9 and F-12, respectively (100 X magnification). **D, E & F** are scanning electron micrographs of the same (2KX magnification, bar-2µm). Spores and hyphae could be clearly seen.

B. COLONIZATION AND BIOFILM FORMATION ON HDPE SURFACE BY FUNGAL ISOLATES

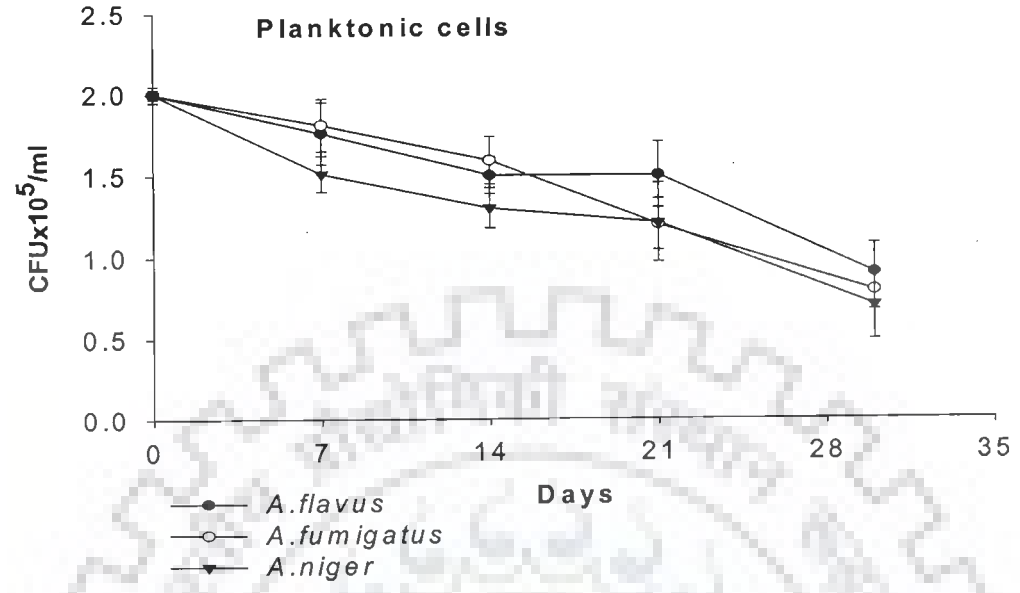
Adherence, colonization and subsequent biofilm formation by microorganism is a prerequisite for deterioration and degradation of the polymer to take place. Commercial synthetic plastics such as polyethylene are resistant to microbial degradation which is mainly due to non-adherence of microbes on plastic surface due to their high surface hydrophobicity. Hydrophobicity of polyethylene interferes with adherence, colonization and biofilm formation by the microbes. Since the polyethylene is not soluble in aqueous solutions, biofilm forming microbes may be more efficient in biodegradation. Colonization and biofilm formation ability of all the three isolates was studied.

4.4. Biofilm growth kinetic study

4.4.1 Estimation of colony forming units (CFU)

Biofilm development and growth kinetics was studied for *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) grown in minimal media containing polyethylene as carbon source. During the course of incubation, most of the fungal cells adhered to the polyethylene film. There was found a clear shift from planktonic growth to biofilm mode of growth. The cell density for biofilm and planktonic cells varied for each fungal isolate. Initially the planktonic cell number was higher but as the incubation progressed, the number of planktonic cells started decreasing while that of biofilm cells increased. A fully developed biofilm after 30 days of incubation has a cell density of 6.5×10^6, 7×10^6 and 5.2×10^6 cells/cm^2 while the planktonic cell density reduced to 0.9×10^5, 0.79×10^5 and 0.7×10^5 cells/ml for *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052), respectively (Fig. 11 A & B).

(A)



(B)

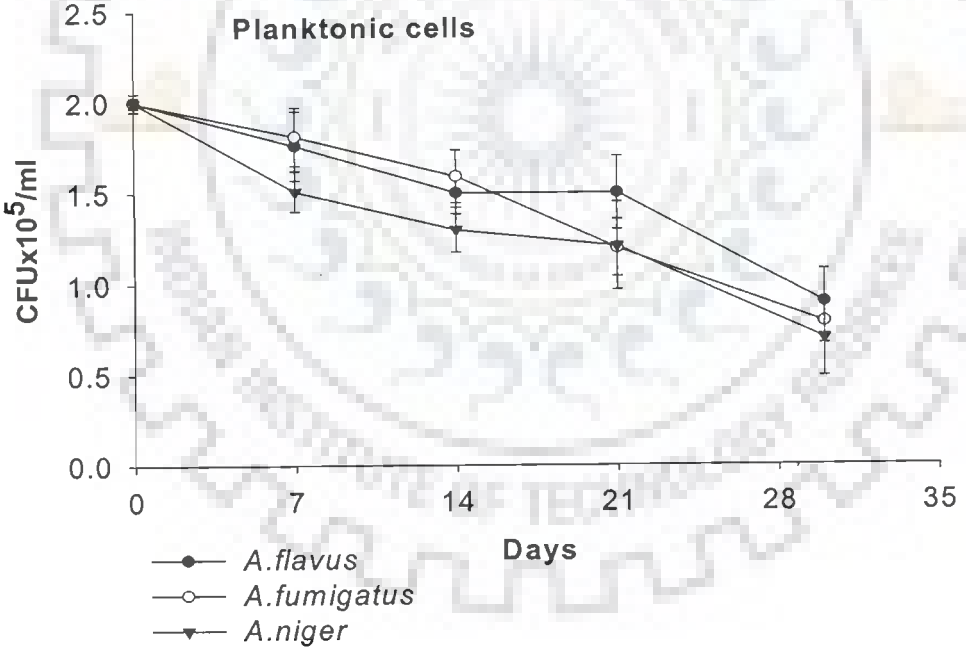


Figure 11. Kinetics of biofilm formation on polyethylene surface by *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052). **A**: Biofilm cells **B:** Planktonic cells. Each value represents mean ± SE of triplicates.

4.4.2 Fungal biofilm viability

Fungal cells started adhering HDPE surface after 7 days of incubation with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) in carbon limiting media (Fig. 12, 13 and 14). The number of planktonic cells were higher during the initial phase of incubation but gradually fungal cells started adhering to the HDPE surface and there was found a significant increase in the cells on polyethylene surface while decrease in planktonic cells with increase in the time of incubation. A biofilm with thick matte of viable cells along with few dead cells could be seen on HDPE film surface after 30 days of incubation. A large number of viable spores along with few hidden mycelia in background are visible. Since fungus was grown in minimal media, large numbers of spores compared to hyphae are likely due to initial adaptation. The biofilm survived up to 60 days with significantly high number of viable cells though the number of dead cells increased. The number of viable cells in planktonic and on HDPE surface varied with each fungal isolate.

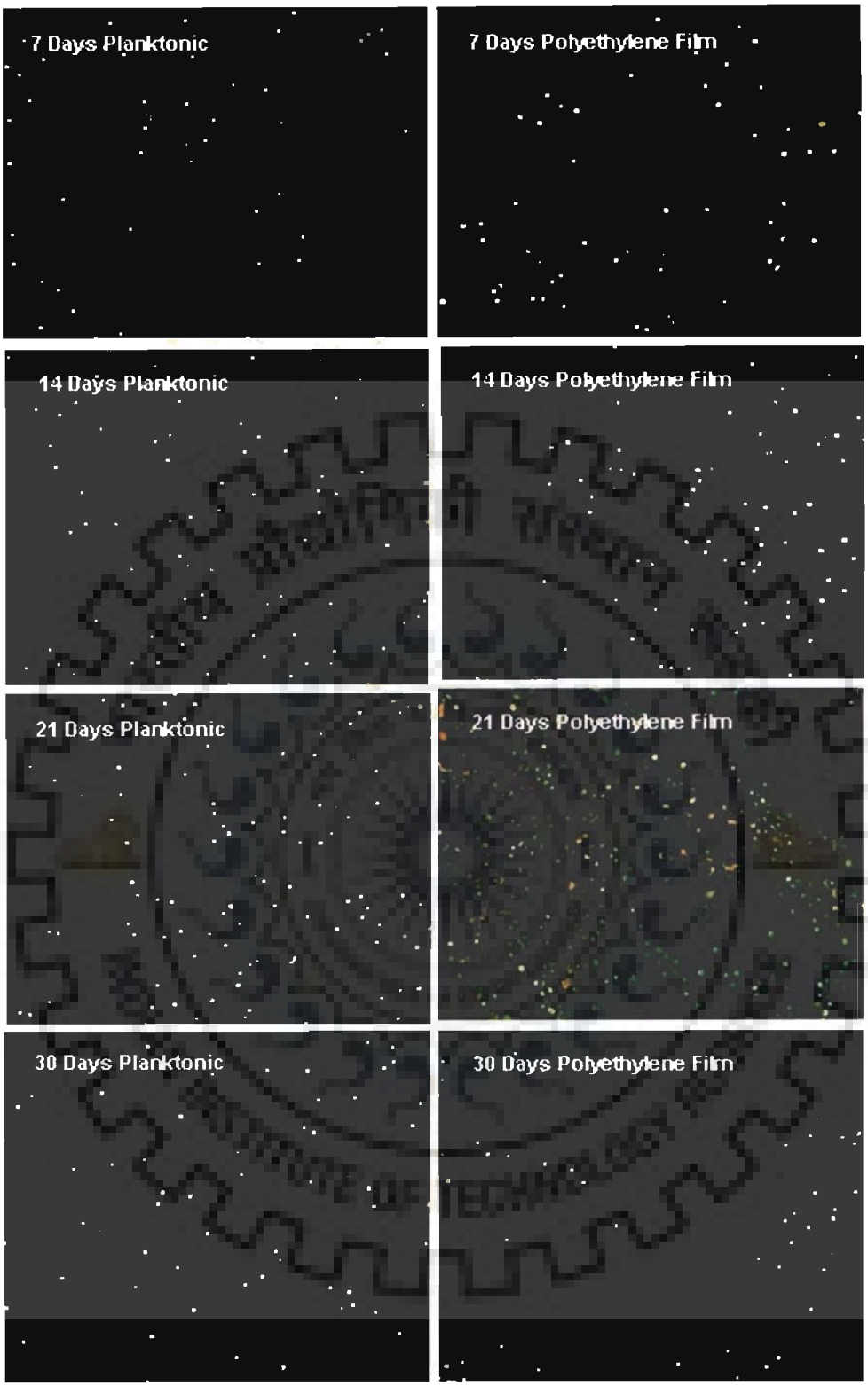


Figure 12. Photomicrographs of *A. flavus* (ITCC No. 6051) showing planktonic and biofilm population after 7, 14, 21 and 30 days in a liquid culture containing polyethylene film as carbon source. Planktonic and biofilm population were stained with Fluorescein Diacetate (FDA). Green cells are live and red cells are dead.

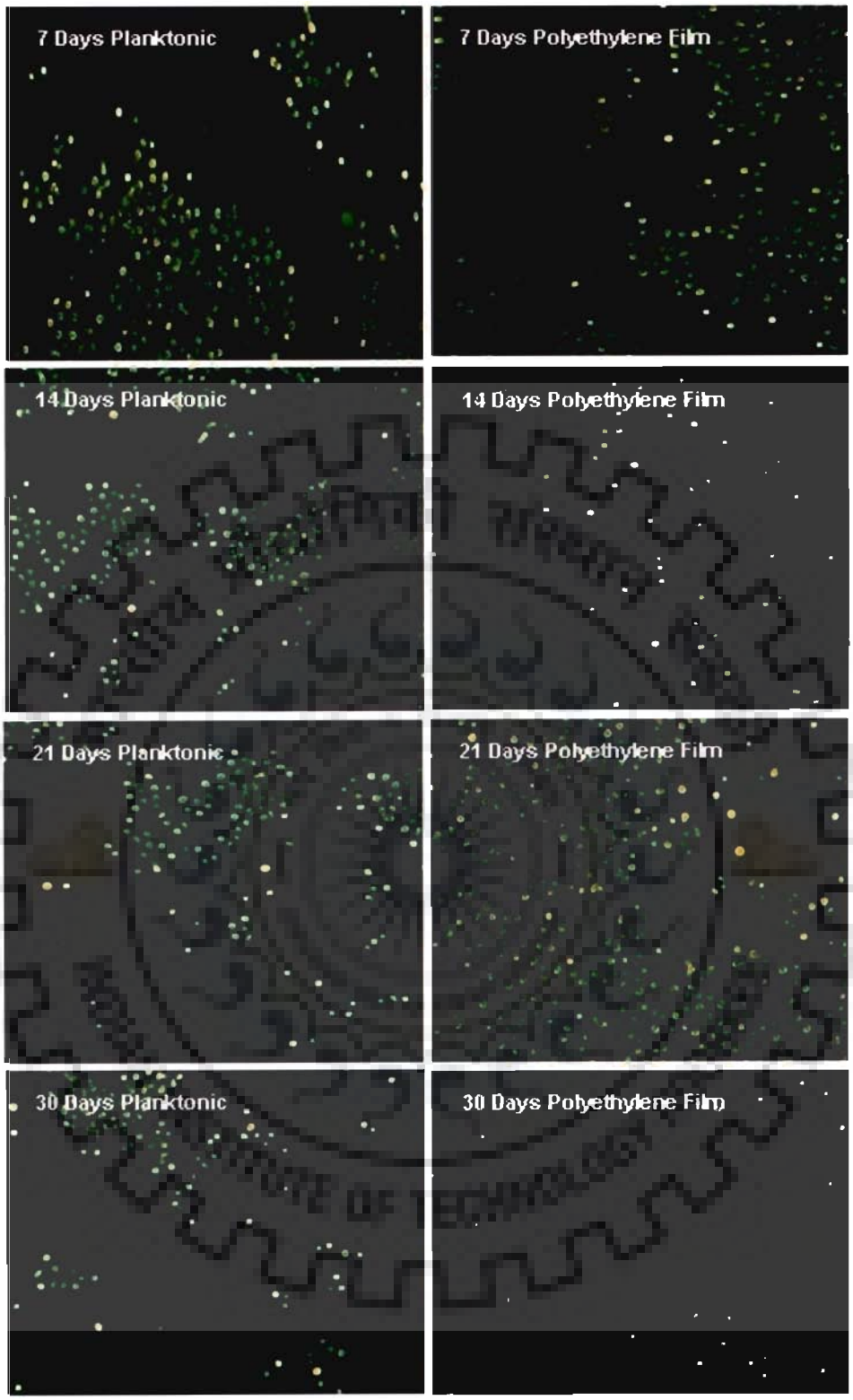


Figure 13. Photomicrographs of *A. fumigatus* (ITCC No. 6050) showing planktonic and biofilm population after 7, 14, 21 and 30 days in a liquid culture containing polyethylene film as carbon source. Planktonic and biofilm population were stained with Fluorescein Diacetate (FDA). Green cells are live and red cells are dead.

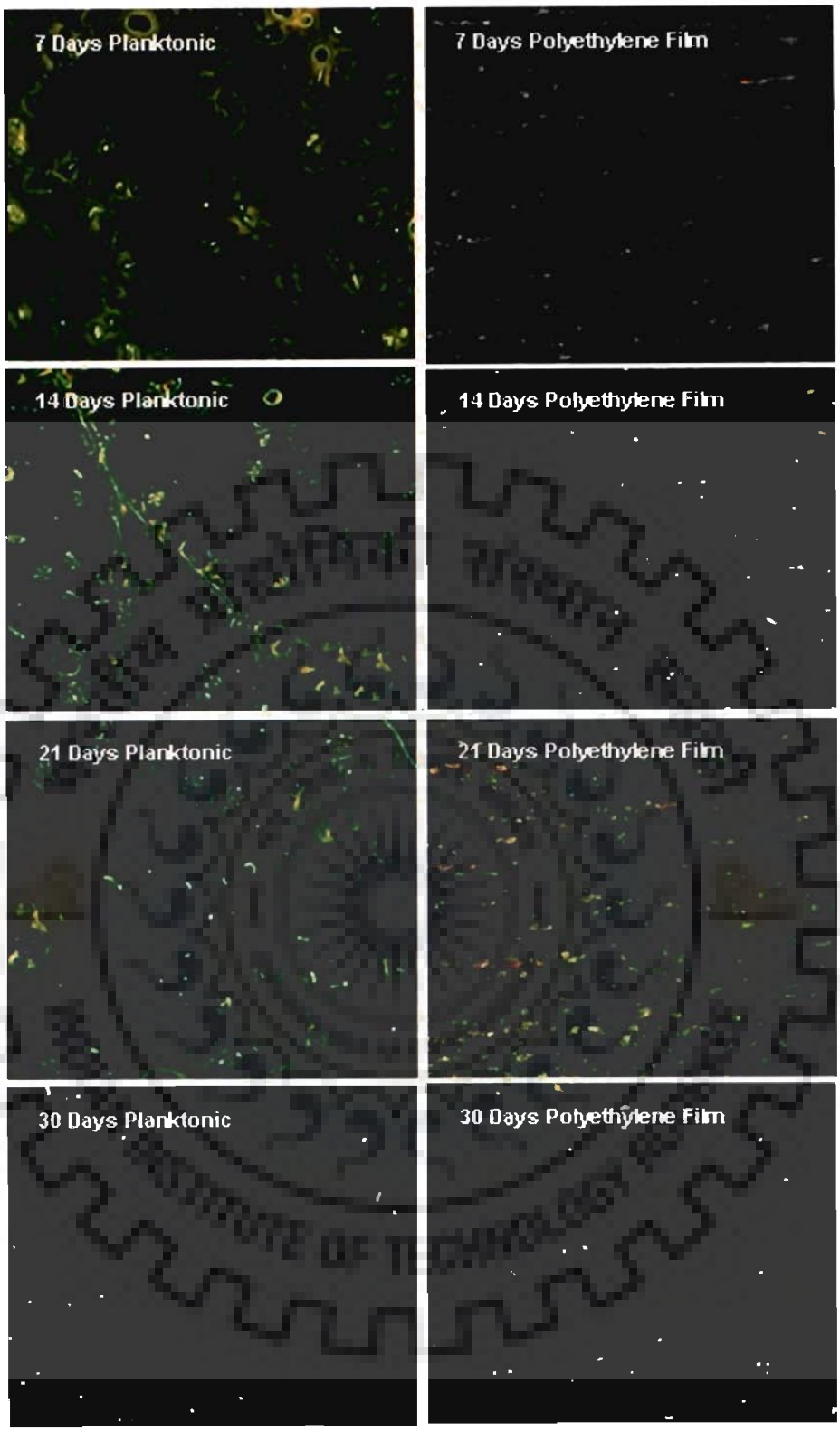
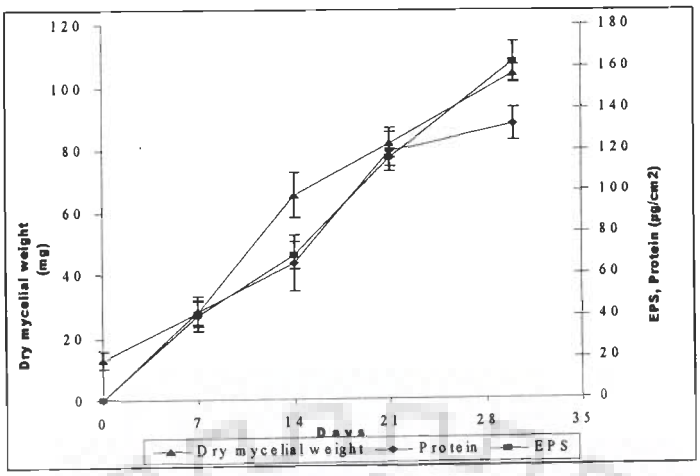


Figure 14. Photomicrographs of *A. niger* (ITCC No. 6052) showing planktonic and biofilm population after 7, 14, 21 and 30 days in a liquid culture containing polyethylene film as carbon source. Planktonic and biofilm population were stained with Fluorescein Diacetate (FDA). Green cells are live and red cells are dead.

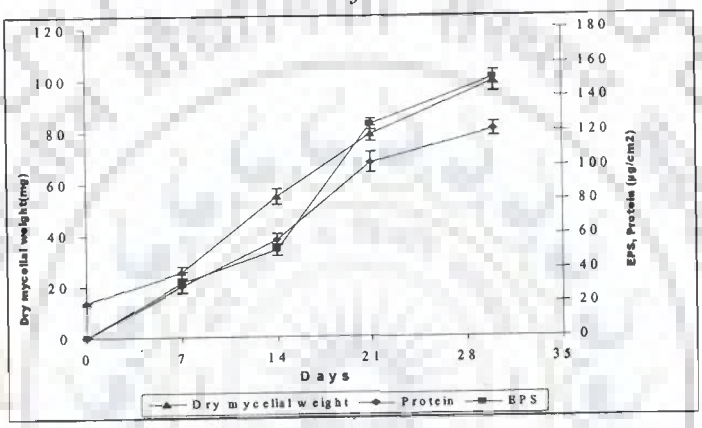
4.4.3 Determination of EPS, fungal biomass and protein content

Since extracellular polysaccharides have been found to be a major constituent of biofilm and play important role in its development, the EPS production was determined at different time intervals during 30 days of incubation with different fungal isolates. There was found to be increase in EPS content with values of 162, 151 and 153 $\mu g/cm^2$ that was in close agreement with the kinetics of biofilm development on polyethylene surface by *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) (Fig. 15).

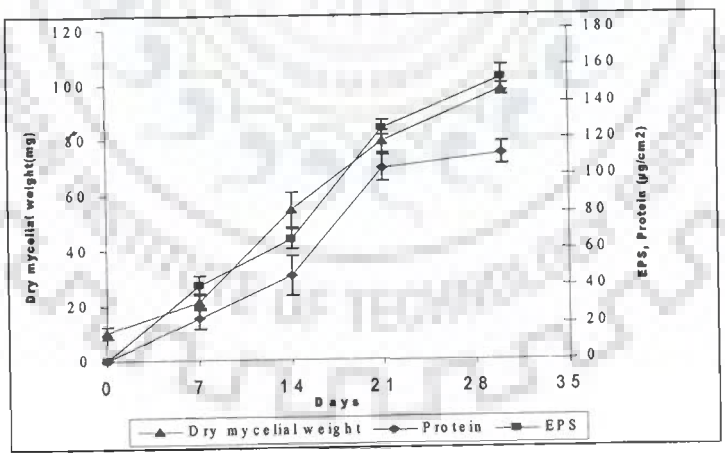
The growth of fungal cells on polyethylene and biofilm formation was further characterized in terms of fungal biomass density and protein content. As it is evident from biofilm kinetic data and photomicrographs, few number of fungal cells initially colonized the polyethylene surface resulting in little fungal biomass and protein content respectively. Once the fungal cells have colonized the polyethylene surface, they started proliferating on the surface resulting in increased fungal biomass resulting in increased protein content. There was found to be an increase in both dry mycelial weight and protein content of biofilm that reached maximum after 30 days of incubation for all the three isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) (Fig. 15).



A: *A. flavus*



B: *A. fumigatus*



C: *A. niger*

Figure 15 .Production of Extracellular polysaccharide (EPS), protein and dry mycelial weight in biofilm of **A**: *A. flavus* (ITCC No. 6051*)*, **B**: *A.fumigatus* (ITCC No. 6050*)*, **C**: *A.niger(*ITCC No. 6052).Biofilms were formed on 10x2.5 cm HDPE films. Each value represents mean ± SE of triplicates.

4.4.4 Longevity of biofilm

Since the process of polyethylene biodegradation is very slow, in order to degrade it is prerequisite for a microbe to form a biofilm that remain active and viable over a longer period. The viability of fungal biofilm formed on HDPE surface was checked during the course of incubation. A fully active and healthy viable biofilm was observed after 30 days of incubation with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052). Biofilm remained active up to 60 days though the number of live cells were reduced compared to 30 days (Fig. 16, 17 and 18). The number of live cells in biofilm varied with each fungal isolate with *A. niger* (ITCC No. 6052) having quite a higher number of dead cells compared to *A. flavus* (ITCC No. 6051) and *A. fumigatus* (ITCC No. 6050).

4.5 Scanning electron microscopy for biofilm analysis

The extent of colonization and biofilm formation and its development on polyethylene surface by different fungal isolates was studied using Scanning Electron Microscopy (SEM). SEM observation revealed the colonization of HDPE surface as early as 7 days by all the three isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052). However, the clear sign of colonization and initiation of biofilm formation by these isolates was seen after 14 days of incubation in carbon limiting media in the presence of polyethylene films. Further incubation of polyethylene film with fungal isolates showed increase in the size and density of cells forming a dense biofilm on the surface. Biofilm with fungal hyphae and spores forming a matte like structure on polyethylene surface was seen after a period of 30 days (Fig. 19, 20 and 21). The extent of colonization and biofilm formation varied with each fungal isolate. Biofilm surviving on the HDPE surface can be seen even after a period of 60 days.

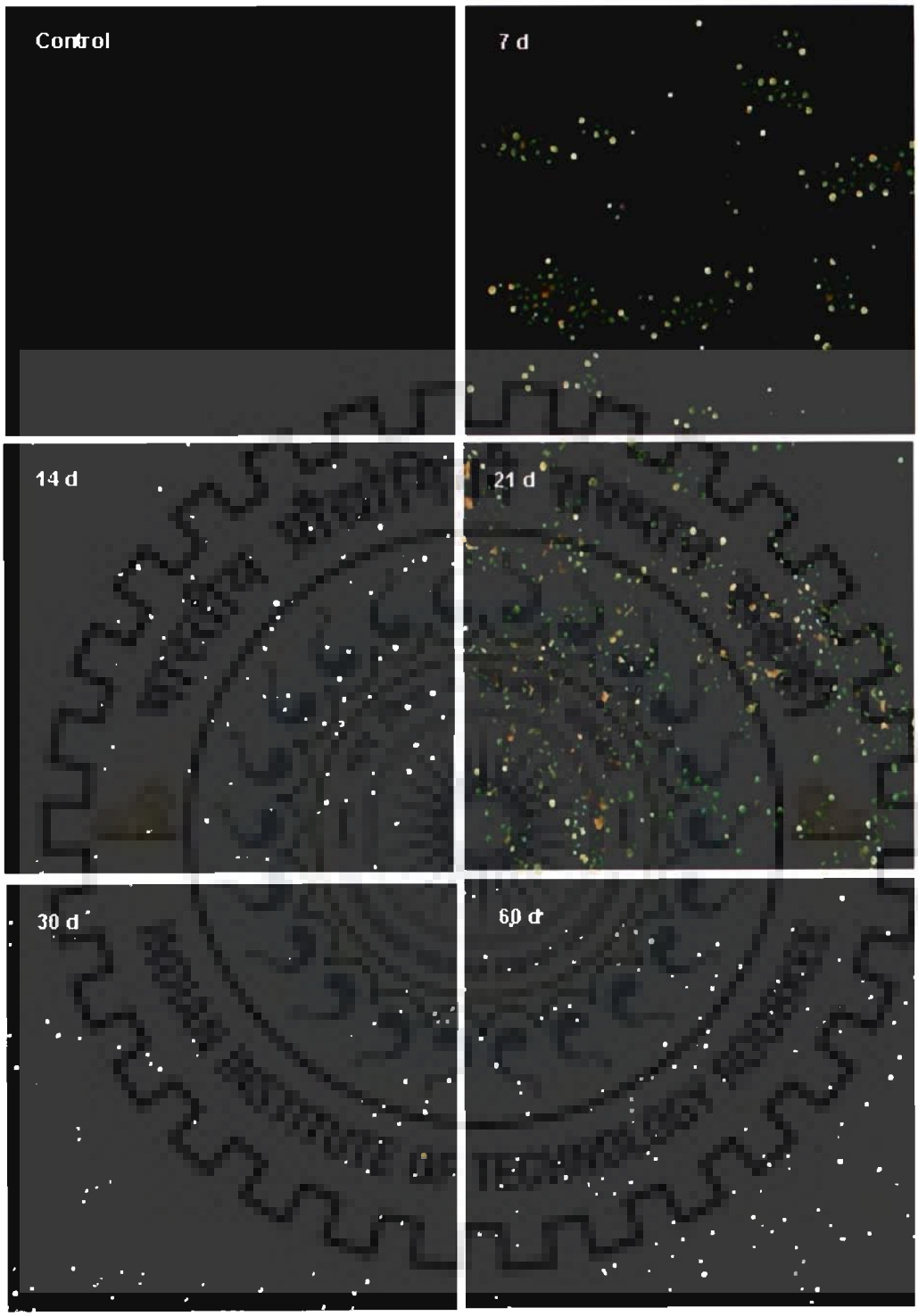


Figure 16. Survival of the biofilm of *A. flavus* (ITCC No. 6051). The biofilm was cultured in minimal liquid medium containing polyethylene film as carbon source. Cell viability was determined using FDA (Flourescein Diacetate) staining. Green cells are live and red cells are dead.

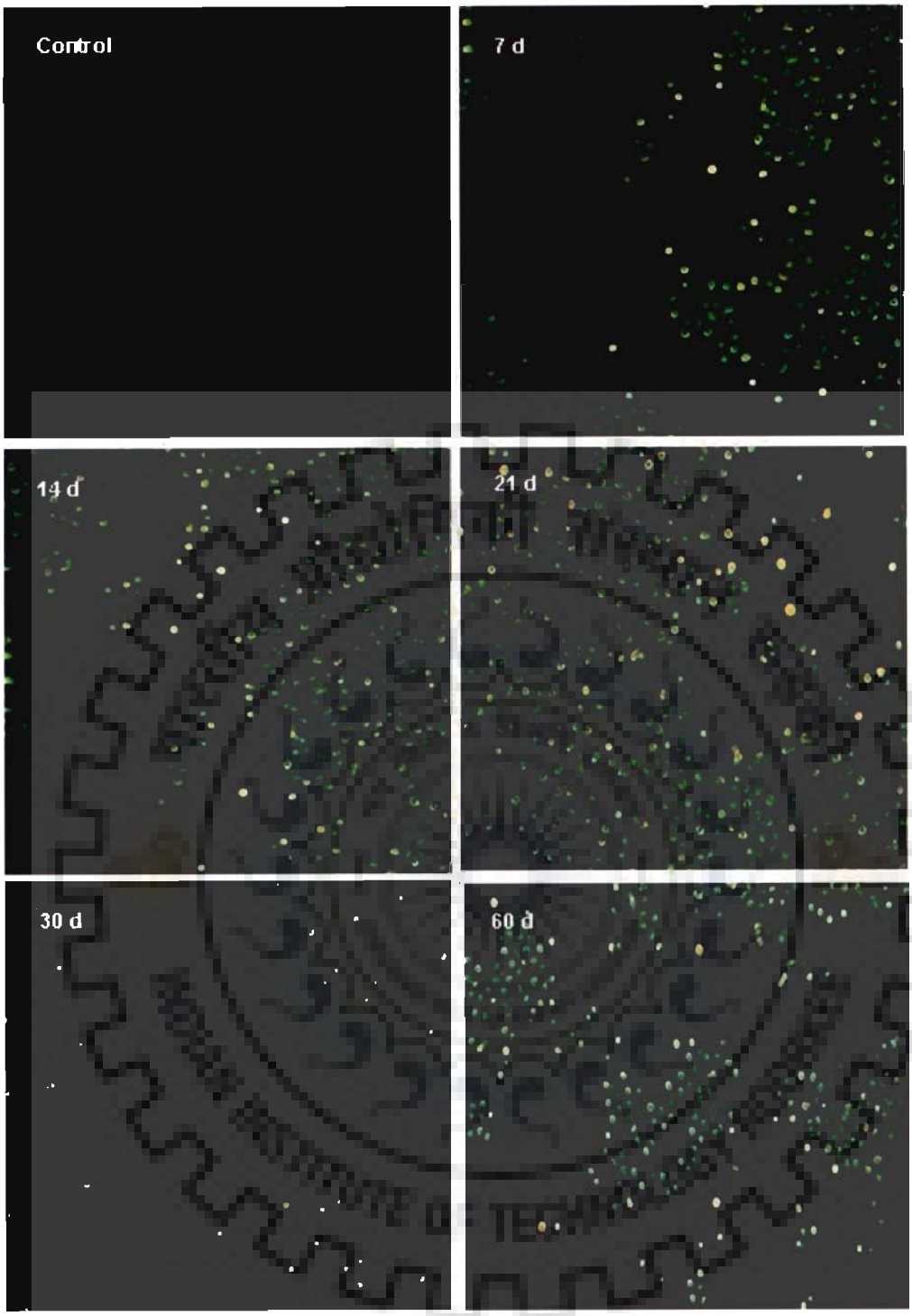


Figure 17. Survival of the biofilm of *A. fumigatus* (ITCC No. 6050). The Biofilm was cultured in minimal medium containing polyethylene film as carbon source. Cell viability was determined using FDA (Fluorescein Diacetate) staining. Green cells are live and red cells are dead.

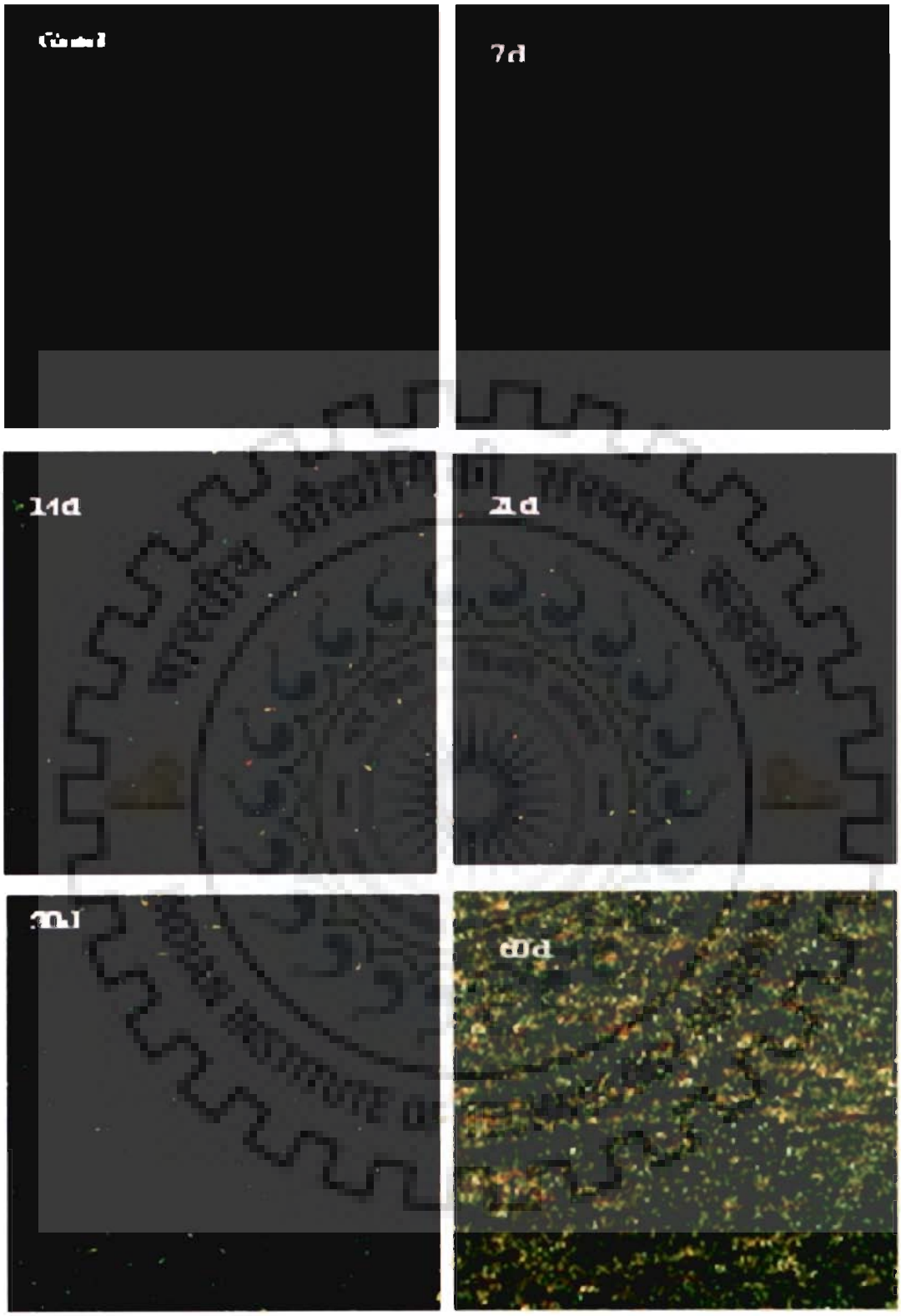


Figure 18. Survival of the biofilm of *A. niger* (ITCC No. 6052). The Biofilm was cultured in minimal medium containing polyethylene film as carbon source. Cell viability was determined using FDA (Fluorescein Diacetate) staining. Green cells are live and red cells are dead.

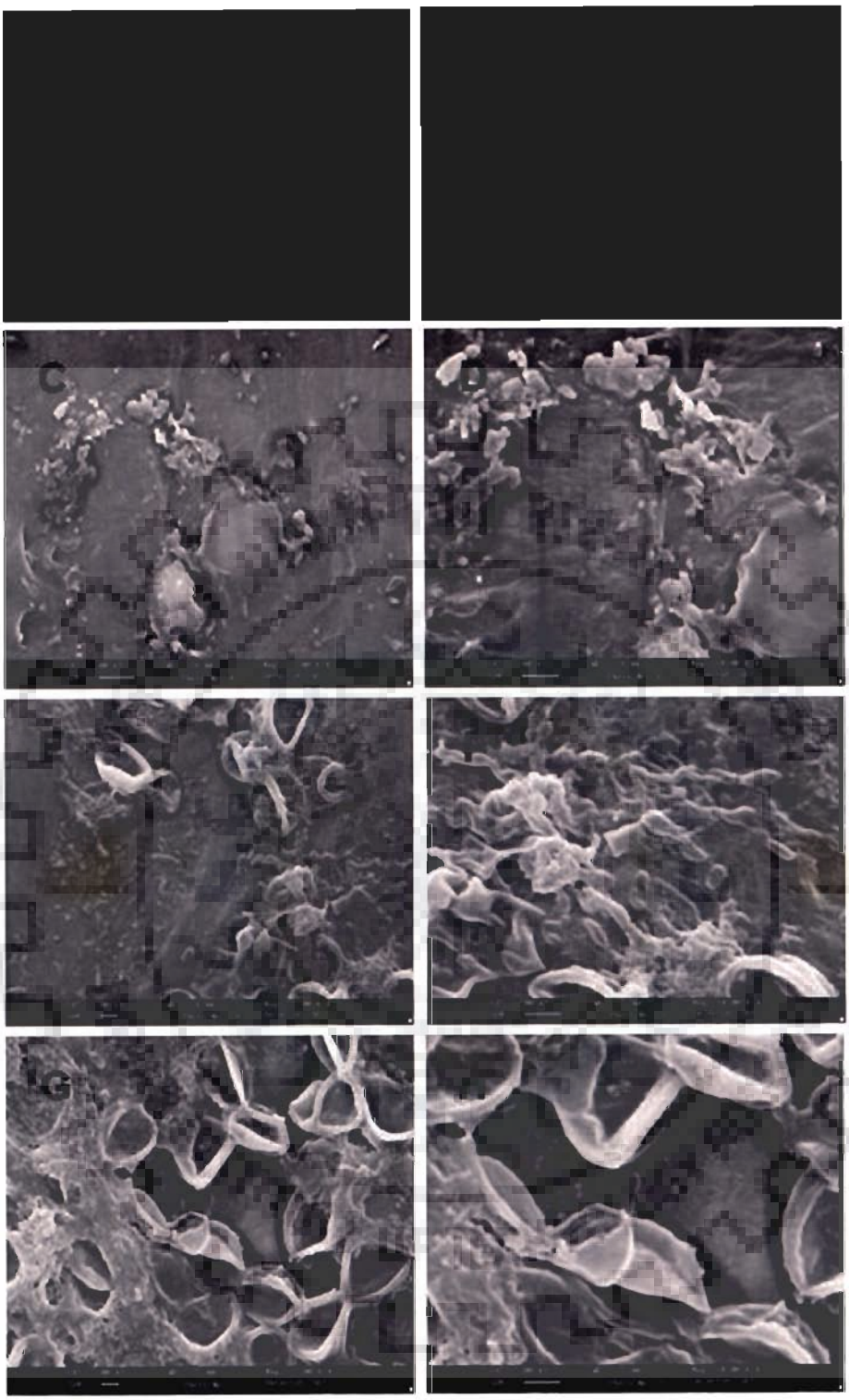


Figure 19. Scanning electron micrographs of colonization and biofilm formation by *A. flavus* (ITCC No. 6051) on HDPE surface. Fungal hyphae and spores can be clearly seen colonizing the polyethylene surface and formed a biofilm A: Control (2 KX, 1 μ bar); B: Control (5 KX, 1 μ bar);C: 14 days (5 KX, 1μ bar); D: 14 days (10 KX, 1μ bar); E: 30 days (5 KX, 1μ bar); F: 30 days (10 KX, 1μ bar); G: 60 days (5 KX, 1μ bar); H:60 days (10 KX, 1μ bar).

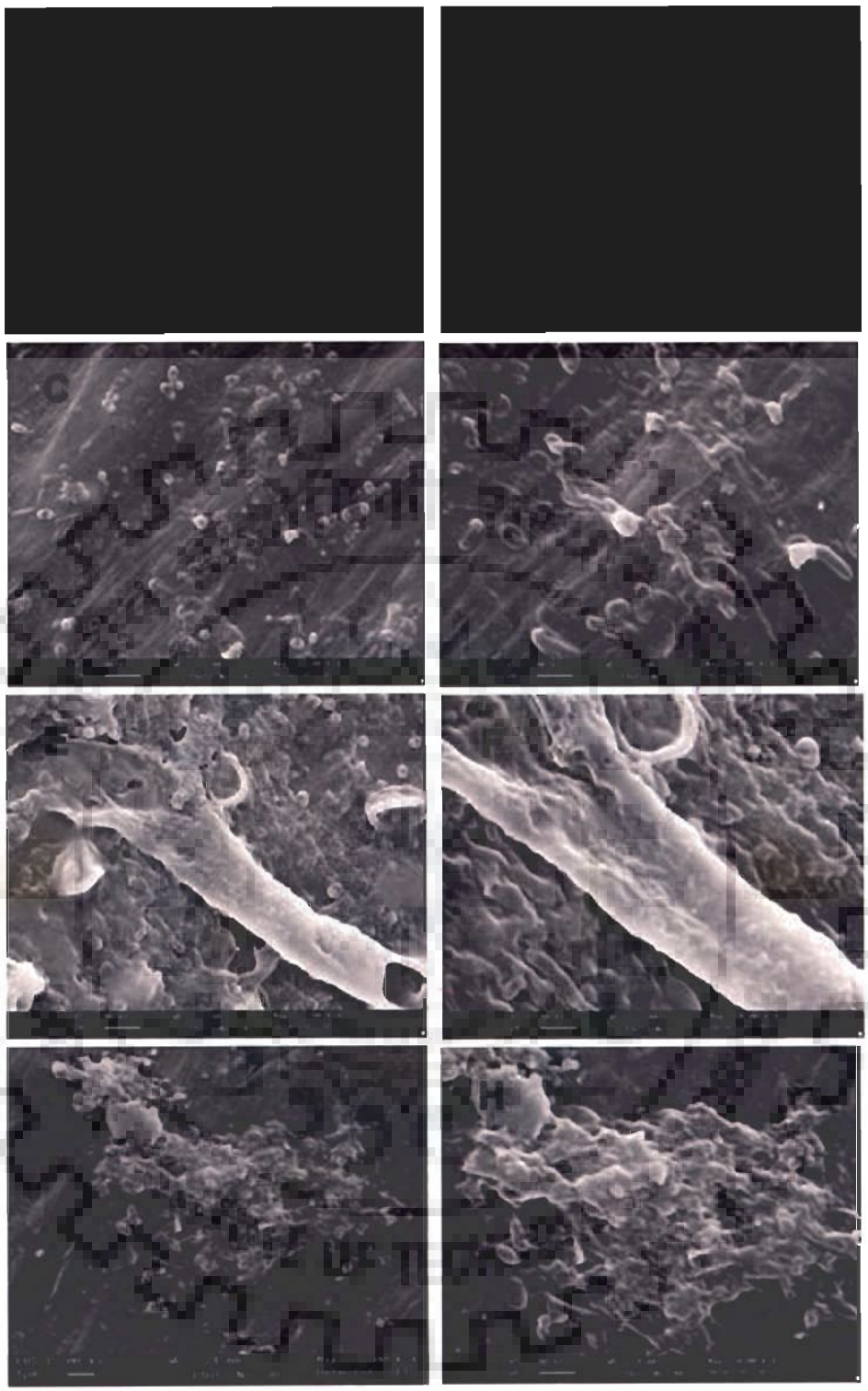


Figure 20. Scanning electron micrographs of colonization and biofilm formation by *A. fumigatus* (ITCC No. 6050) on HDPE surface. Fungal hyphae and spores can be clearly seen colonizing the polyethylene surface and formed a biofilm. **A**: Control (2 KX, 1 μ bar); **B**: Control (5 KX, 1 μ bar); **C**: 15 days (5 KX, 1 μ bar); **D**: 15 days (10 KX, 1 μ bar); **E**: 30 days (5KX, 1 μ bar); **F**: 30 days (10 KX, 1μ bar); **G:** 60 days (5 KX, 1μ bar); **H:** 60 days (10 KX, 1μ bar).

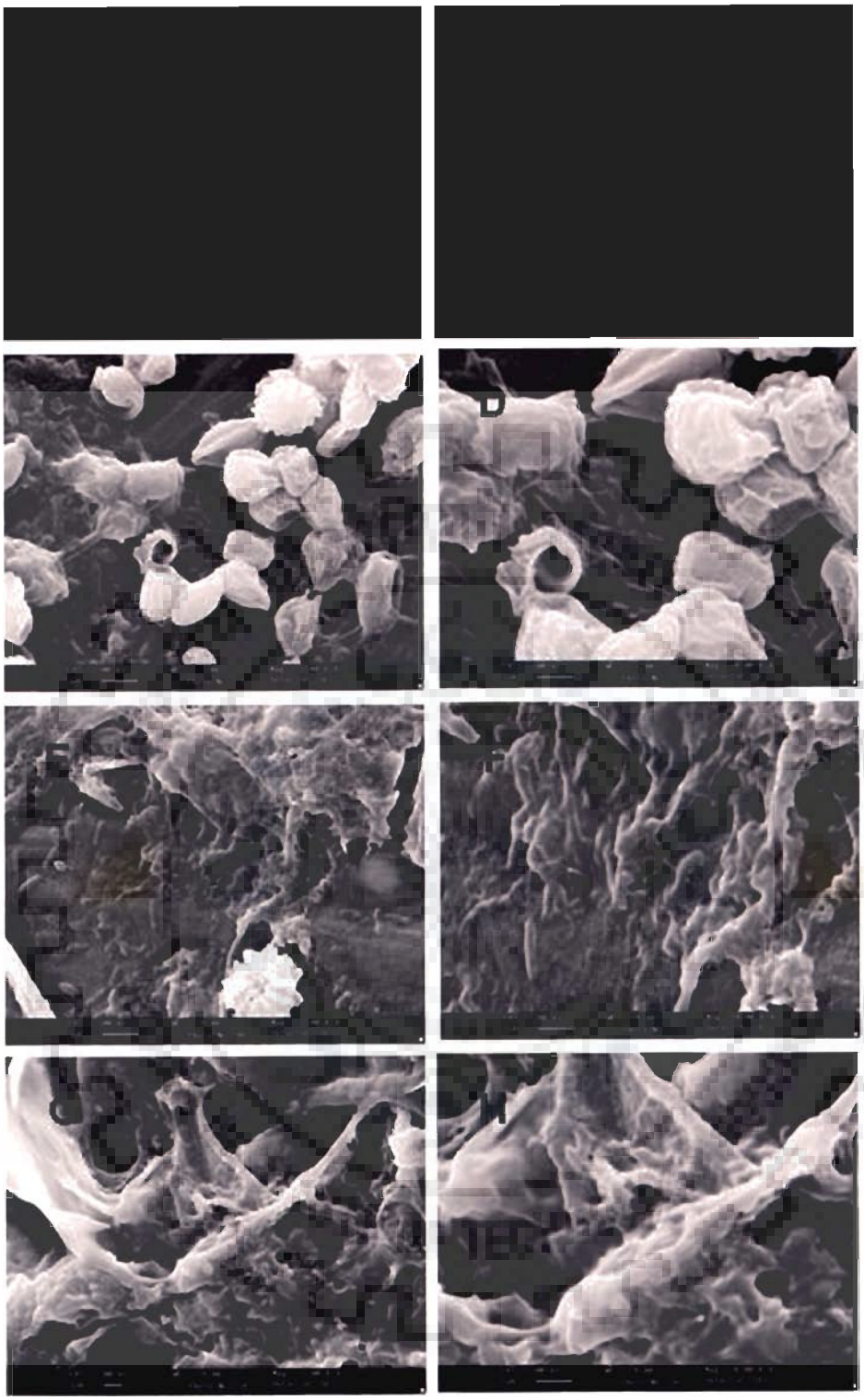


Figure 21. Scanning electron micrographs of colonization and biofilm formation by *A. niger* (ITCC No. 6052) on HDPE surface. Fungal hyphae and spores can be clearly seen colonizing the polyethylene surface and formed a biofilm A: Control (2 KX, 1 μ bar); B: Control (5 KX, 1 μ bar);C: 14 days (5 KX, 1μ bar); D: 14 days (10 KX, 1μ bar); E: 30 days (5 KX, 1μ bar); F: 30 days (10 KX, 1μ bar); G: 60 days (5 KX, 1μ bar); H:60 days (10 KX, 1μ bar).

C. DEGRADATION STUDY

4.6 Degradation study of high density polyethylene (HDPE)

It has been suggested that growth of microorganisms on plastic surface is related to their ability to cause changes in polymer molecular weight and on some of their measurable physical and chemical properties. To assess morphological and structural changes in polyethylene, some physical (weight loss and tensile strength measurement), physicochemical (thermal analysis, X-ray diffraction), microscopic (Scanning Electron Microscopy) and spectroscopic (Infra red) methods were used. Degradation of high density polyethylene by *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) in carbon limiting media after 30 days of incubation was studied using weight loss and tensile strength measurement, SEM, DSC, XRD, FTIR and GC-MS.

4.6.1 Polyethylene degradation rate

Polyethylene degradation was monitored by measuring the weight of the polyethylene film before and after incubation with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) in carbon limiting media (Fig. 22). After 30 days of incubation with *A. flavus, A. fumigatus* and *A. niger* in minimal media with polyethylene as carbon source, polyethylene lost 4.41, 3.45 and 1.16 % of its initial weight, respectively (Table 11).

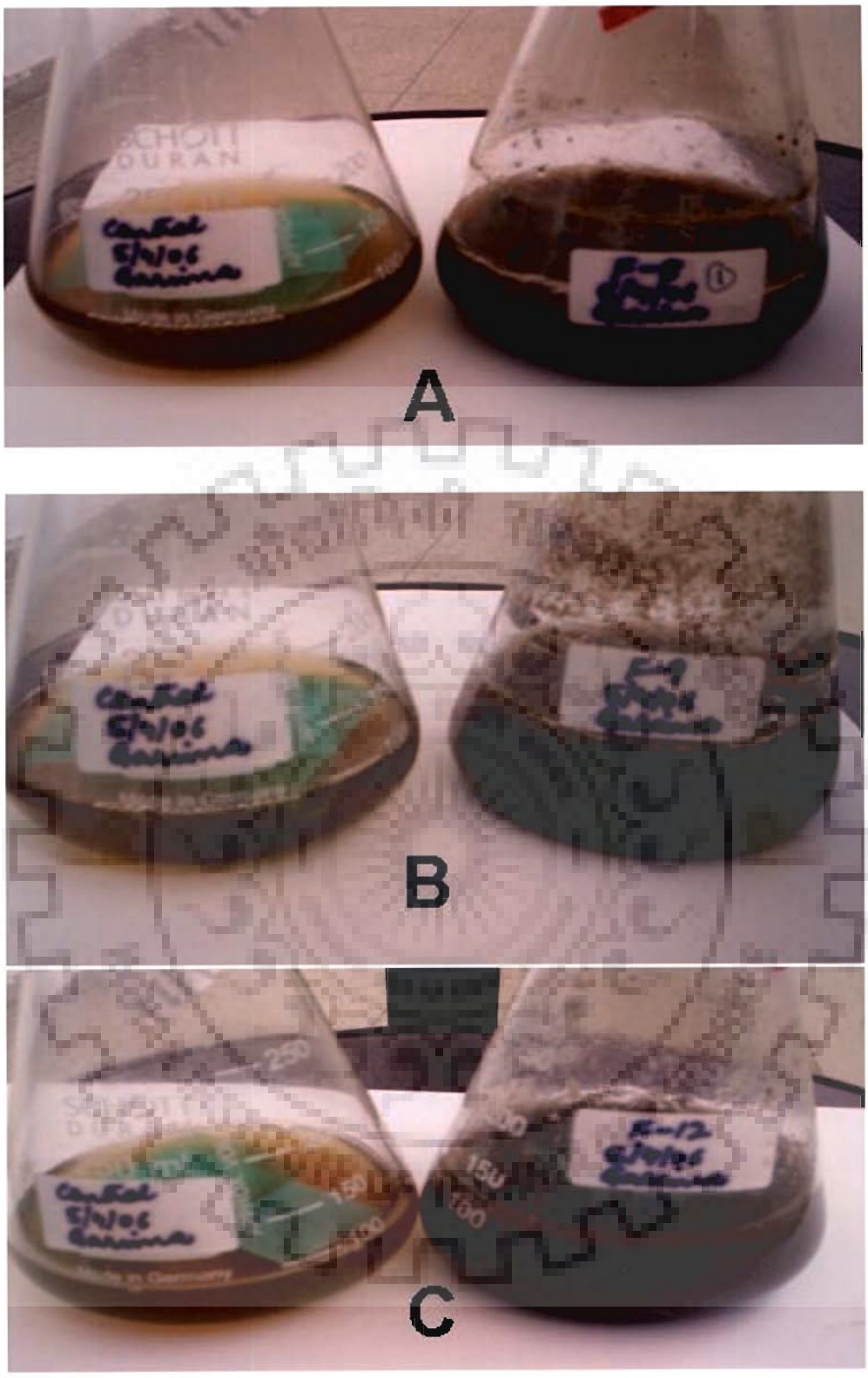


Figure 22. Pure shake flask culture set up for biodegradation studies of high density polyethylene (HDPE) film. Growth of different fungal isolates in minimal media containing polyethylene films as carbon source could be seen. **A**- *A. flavus* (ITCC No. 6051); **B**- *A. fumigatus* (ITCC no. 6050); **C**- *A. niger* (ITCC No. 6052).

Table 11. The change in the average weight of the HDPE film after 30 days of incubation with different fungal isolates compared to control

Sample	Initial weight of film (in grams)	Weight of film after 30 days of incubation (in grams)	Percentage reduction in weight
Control	0.0870±0.0001	0.0870±0.0001	0.00
A.flavus (ITCC No. 6051)	0.0838±0.0002	0.0801±0.0002	4.41
A.fumigatus (ITCC No. 6050)	0.087±0.0001	0.084±0.0001	3.45
A.niger (ITCC No. 6052)	0.086±0.0001	0.08504±0.0001	1.16

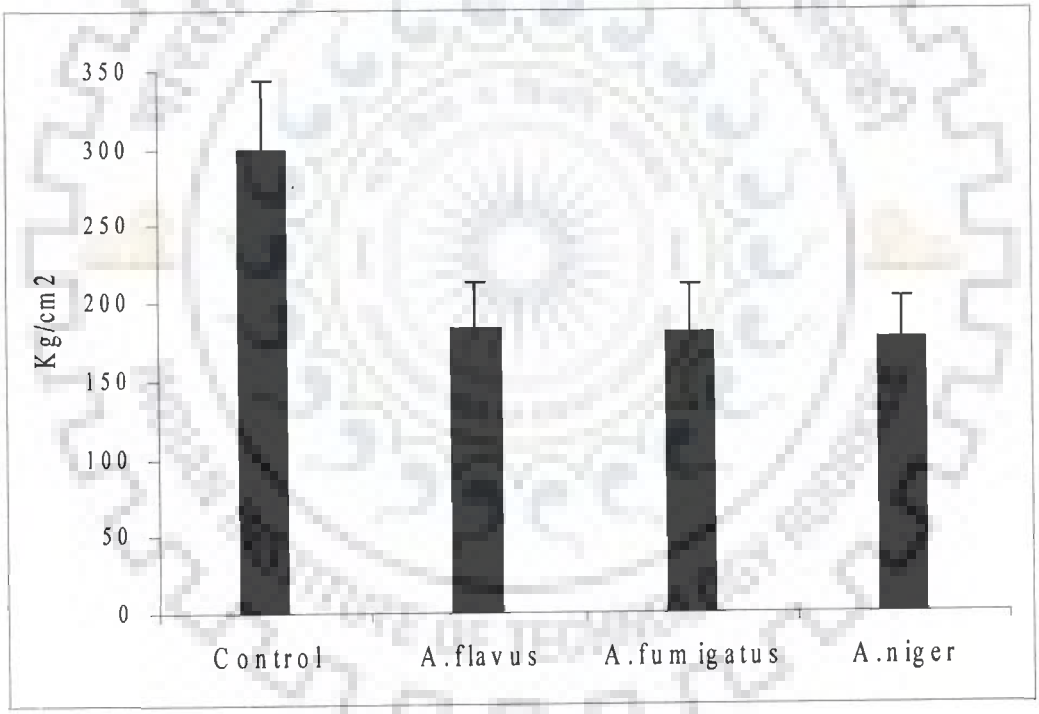


Figure 23. Reduction in tensile strength of polyethylene films after incubation with different fungal isolates. Fungal isolates were grown in minimal media containing polyethylene filmss as carbon source for 30 days. Film surface was washed and tensile strength were determined.

4.6.2 Tensile strength measurement

Degradation was assessed qualitatively by measuring the change in tensile strength after incubation with fungal isolates. The tensile strength of the control and polyethylene film inoculated with fungal isolates was determined. The change in the tensile strength is represented in Fig. 23. There was found to be 61.33, 60 and 58.77 % reduction in tensile strength of polyethylene films after incubation with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) respectively in comparison with the control.

4.6.3 Scanning Electron Microscopic (SEM) study

Biodegradation abilities of the isolates were also checked by monitoring the changes on the surfaces of the inoculated films in comparison to control. Since the initial attack generally begins with surface colonization, SEM gives a direct visualization of this kind of degradation. Changes were clearly seen on polyethylene film after 30 days of incubation with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052). Heavy colonization, hyphae penetration in the film and formation of cavities on the film surface after incubation with all three fungal isolates evidenced the biodegradation of polyethylene. The SEM micrographs of the control and inoculated films are shown (Fig. 24, 25 and 26).

4.6.4 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy is an effective method to quantify the contents of carbonyl, double bonds and other functional groups during biodegradation. Changes in polyethylene film after incubation with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) were analyzed by FTIR after

30 days of incubation. To obtain the quantitative information of structural modifications of HDPE after microbial treatment, carbonyl and double bond indexed were estimated. The FTIR spectrum of thermally treated polyethylene film showed a typical carbonyl peak at 1715 cm^{-1} which was absent in untreated sample. Incubation of polyethylene films with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) for 30 days resulted in a marked reduction in the amount of carbonyl residues (Fig. 27, 28 and 29). Percentage reduction in the carbonyl index was 0.089, 0.092 and 0.073 for HDPE films incubated with *A. flavus, A. fumigatus* and *A. niger* respectively. Carbonyl index of thermally treated and incubated polyethylene film was calculated to quantify this reduction (Table 12). Significant increase in the double bond index (DBI) was observed for all the three isolates with values of 0.0429, 0.0532, and 0.0291 for *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) respectively (Table 12).

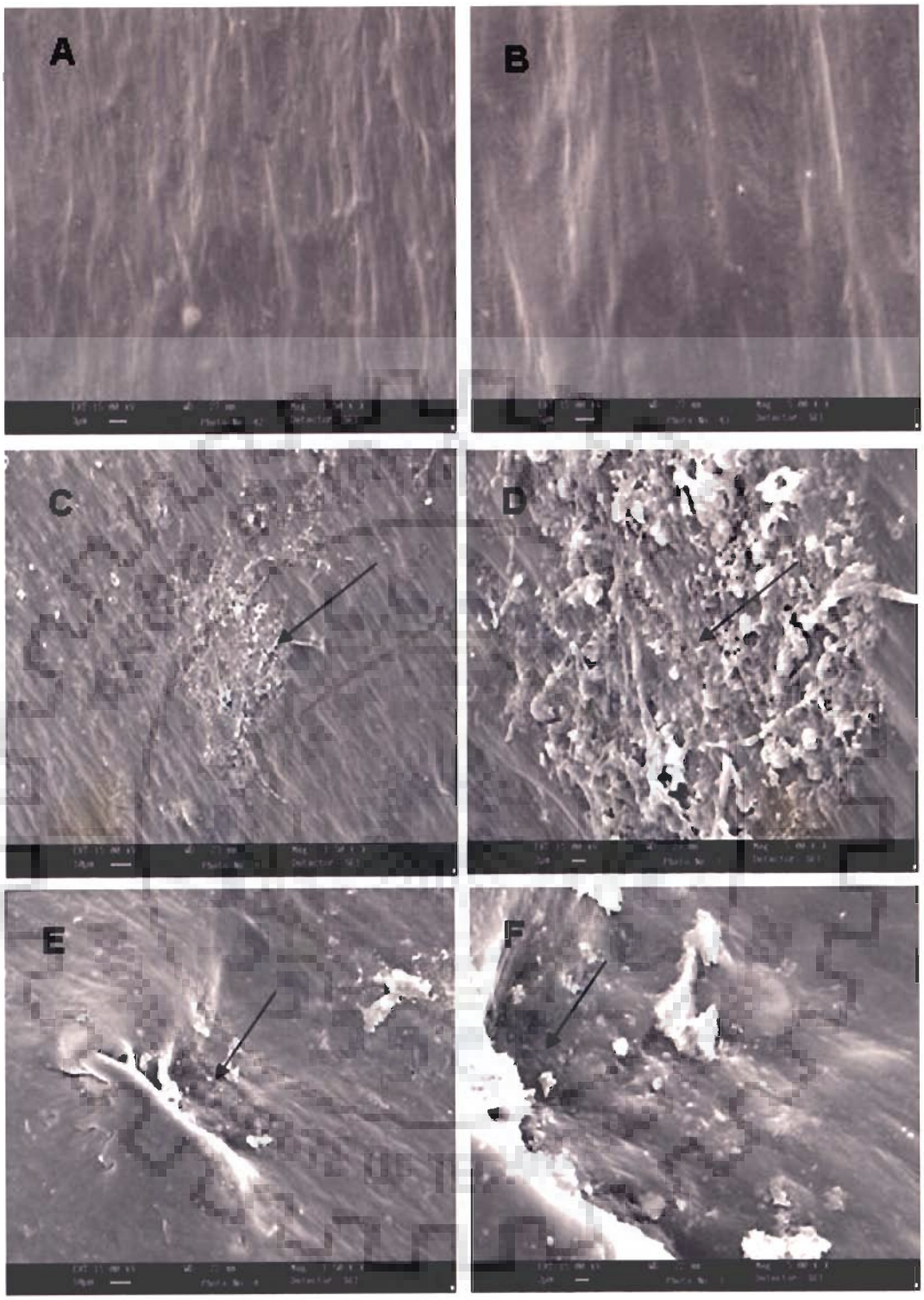


Figure 24. Scanning electron micrographs of high density polyethylene (HDPE) film surface showing changes and deformity after 30 days of incubation with *A. flavus* (ITCC No. 6051) **A & B** Control (1.5 KX & 5 KX), **C & D** *A. flavus* treated film (1.5 KX & 5 KX respectively) arrows showing spore and hyphal growth on polyethylene surface; **E & F** *A. flavus* treated film (1.5 KX & 5 KX respectively) arrows indicating damage and deformity on the polyethylene surface. Bar = 2µm.

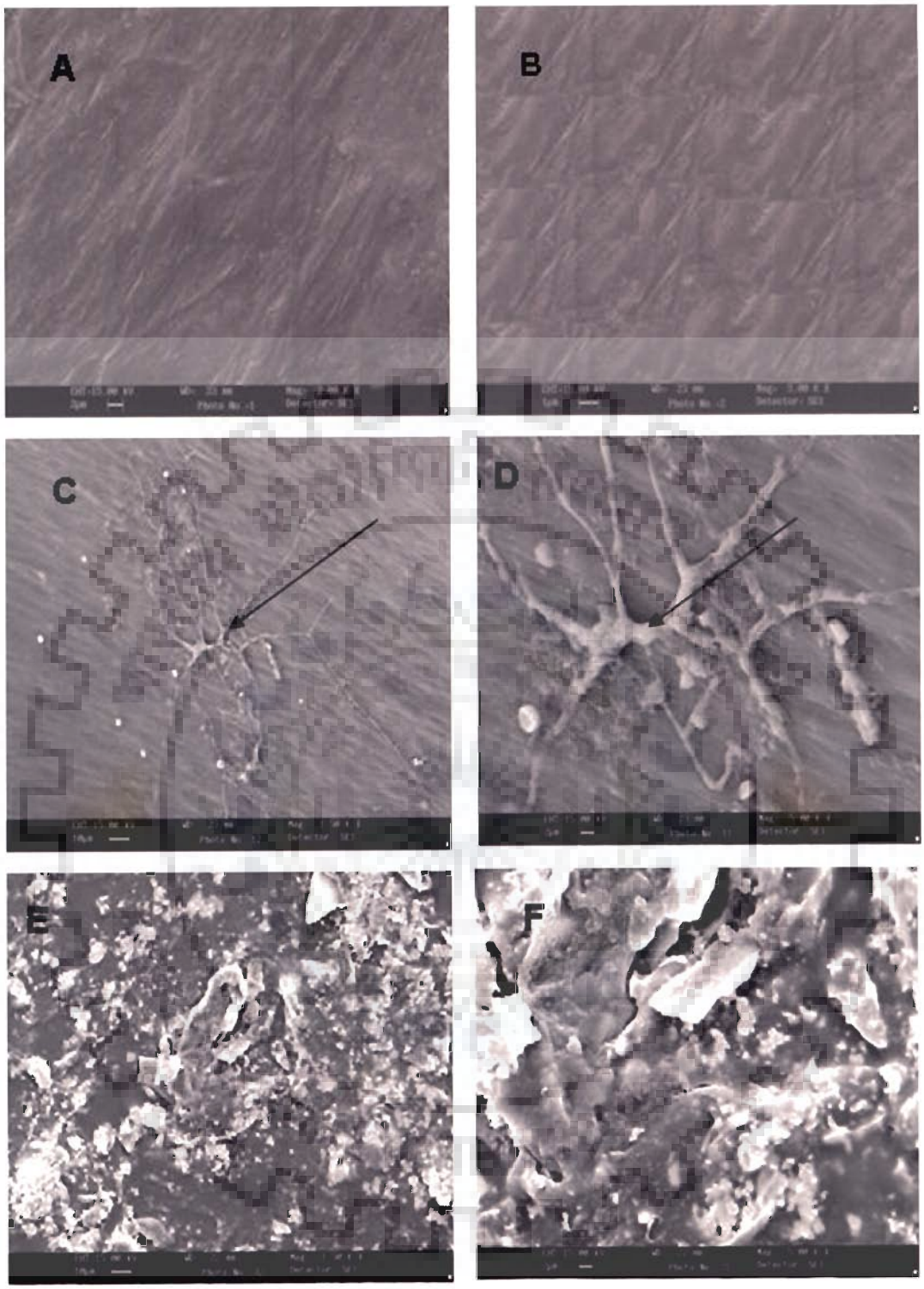


Figure 25. Scanning electron micrographs of high density polyethylene (HDPE) film surface showing changes and deformity after 30 days of incubation with *A. fumigatus* (ITCC No. 6050). **A & B** Control (1.5 KX & 5 KX) , **C & D** *A. fumigatus* treated film (1.5 KX & 5 KX respectively) arrow showing spore and hyphal growth on polyethylene surface; **E & F** *A. fumigatus* treated film (1.5 KX & 5 KX respectively) arrow indicating damage and deformity on the polyethylene surface. Bar = 2µm.

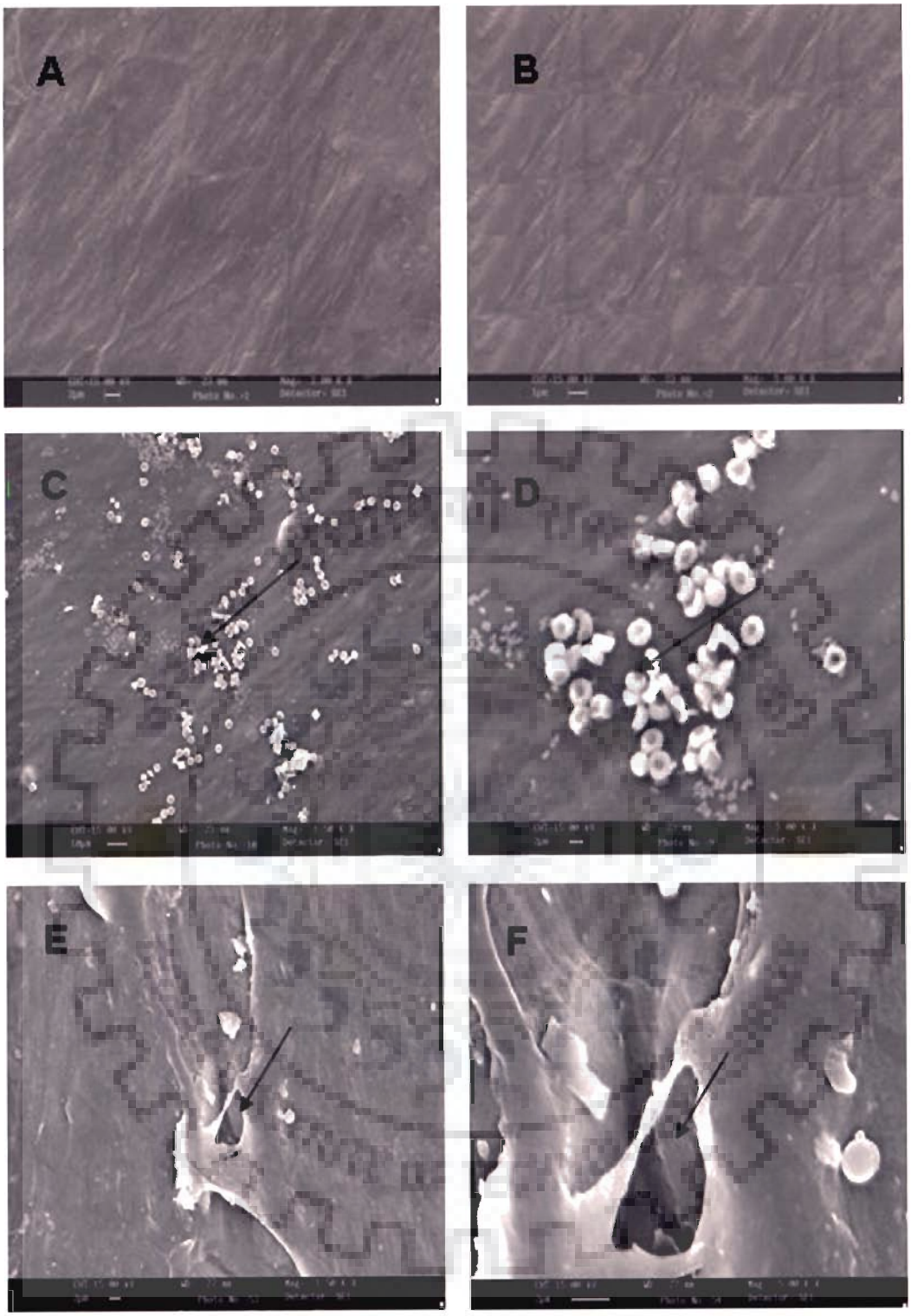


Figure 26. Scanning electron micrographs of high density polyethylene (HDPE) film surface showing changes and deformity after 30 days of incubation with *A. niger* (ITCC No. 6052). **A & B** Control (1.5 KX & 5 KX) , **C & D** *A. niger* treated film (1.5 KX & 5 KX respectively) arrow showing spore and hyphal growth on polyethylene surface; **E & F** *A. niger* treated film (1.5 KX & 5 KX respectively) arrow indicating damage and deformity on the polyethylene surface. Bar = 2μm.

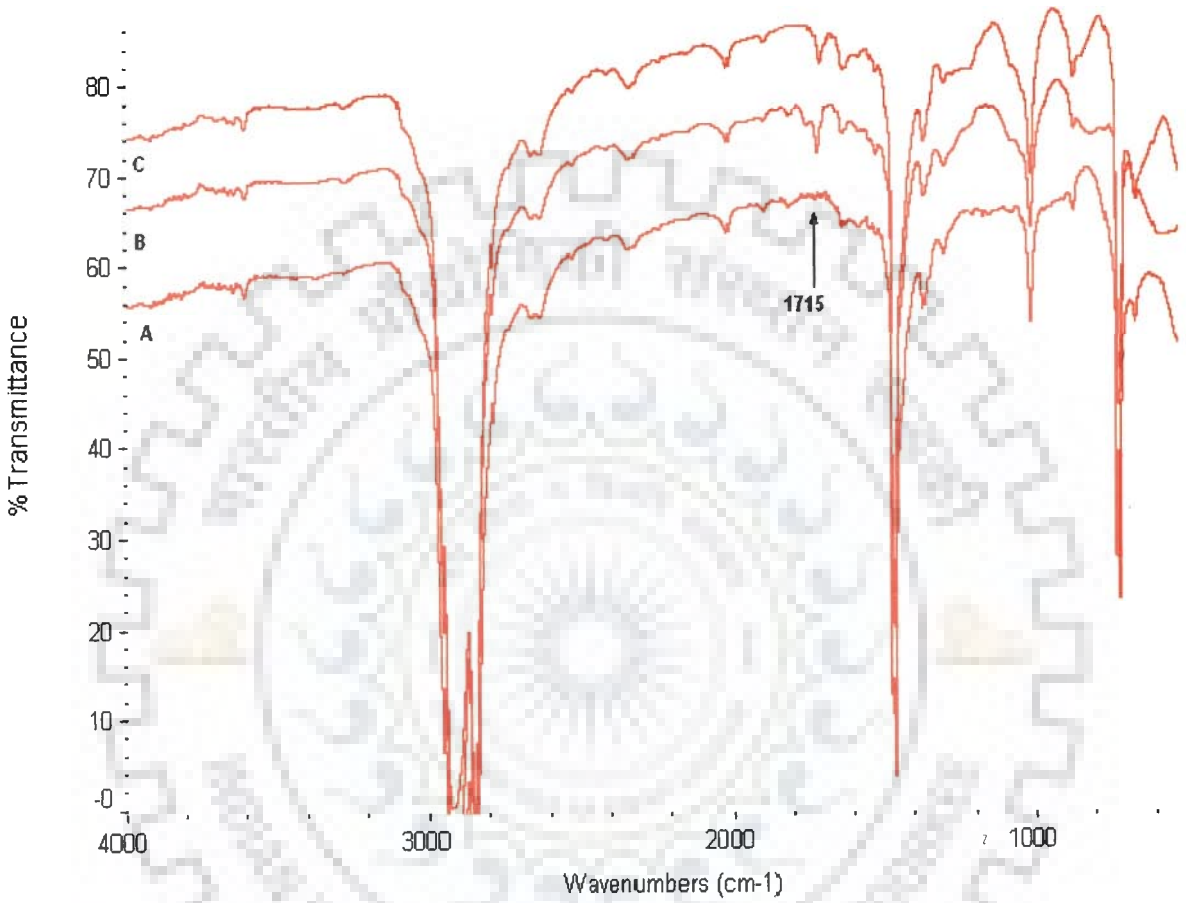


Figure 27: Fourier Transform Infrared Spectroscopy (FTIR) of high density polyethylene (HDPE) films. **A**-Control, **B**-Heat treated and **C**-*A. flavus* (ITCC No. 6051) treated film. Arrow indicates the carbonyl peak at 1715 cm^{-1}.

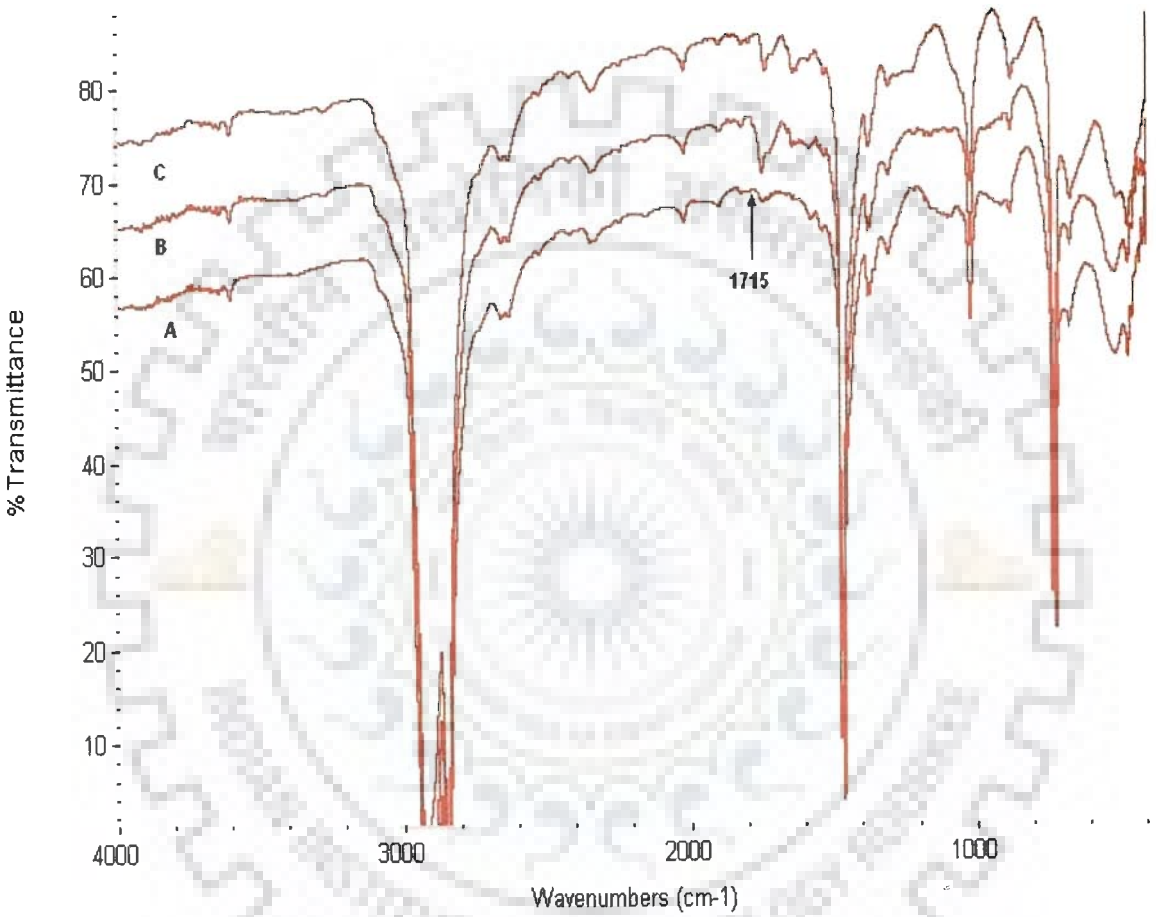


Figure 28: Fourier Transform Infrared Spectroscopy (FTIR) of high density polyethylene (HDPE) films. **A**-Control, **B**-Heat treated and **C**-*A. fumigatus* (ITCC No. 6050) treated film. Arrow indicates the carbonyl peak at 1715 cm^{-1}.

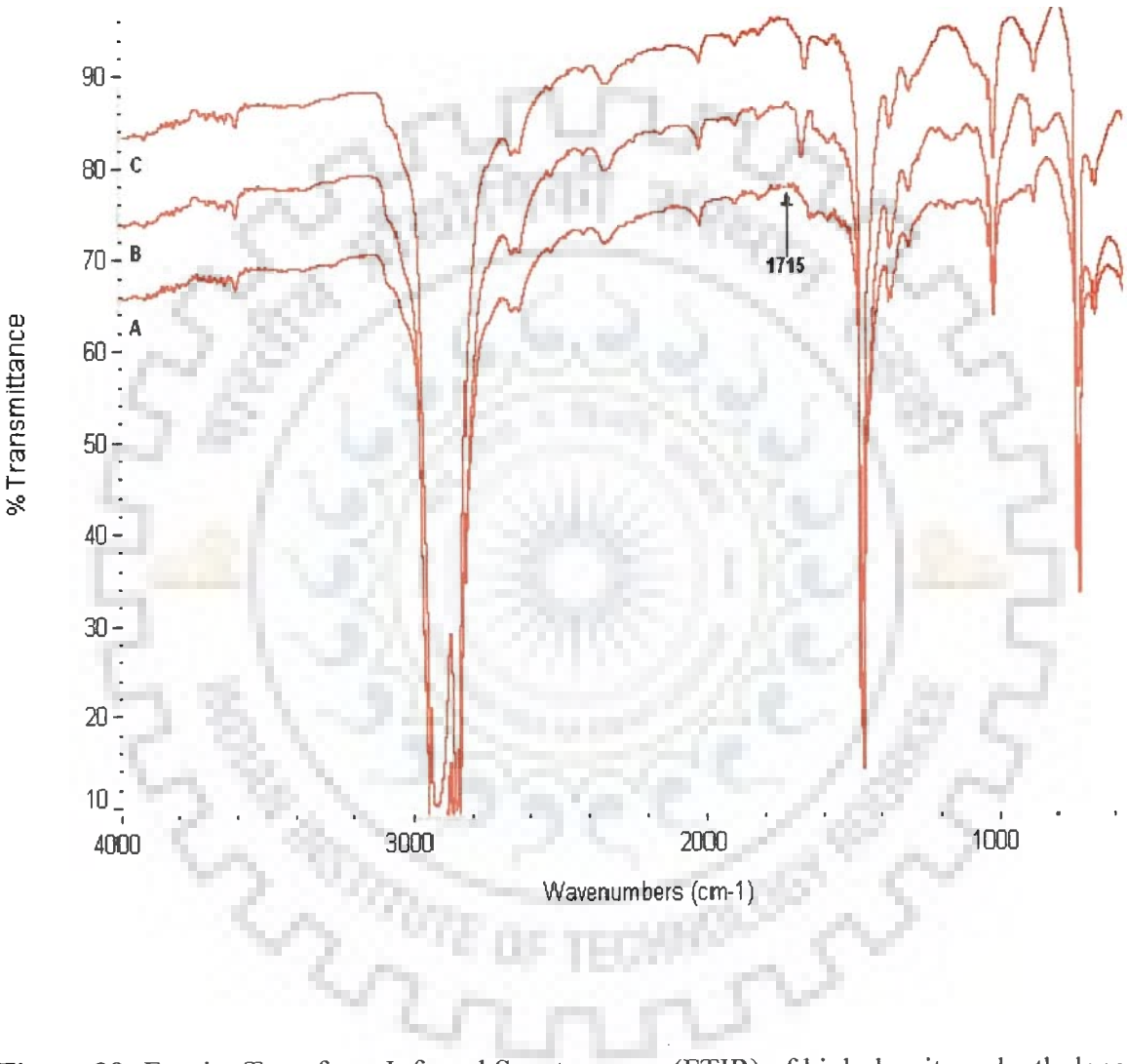


Figure 29: Fourier Transform Infrared Spectroscopy (FTIR) of high density polyethylene (HDPE) films. **A**-Control, **B**-Heat treated and **C**-*A. niger* (ITCC No. 6052) treated polyethylene film. Arrow indicates the carbonyl peak at 1715 cm^{-1}.

Table 12: Carbonyl index ($A_{1715/1465}$) and Double Bond Index ($A_{16531465}$) obtained from FTIR spectra of heat treated high density polyethylene (HDPE) films incubated for 30 days with different fungal isolates and non-inoculated polyethylene film.

Treatment	Carbonyl index ($A_{c=o}$:A_{CH2})	Double bond index(DBI) ($A_{1715/1465}$))
Non treated polyethylene	0.044	0.00021
Heat treated (70°C)	0.295	0.0217
Heat treated + *A. flavus* (ITCC No. 6051)	0.089	0.0429
Heat treated + *A. fumigatus* (ITCC No. 6050)	0.092	0.0532
Heat treated + *A .niger* (ITCC No. 6052)	0.073	0.0291

4.6.5 Morphological changes (DSC and XRD)

The morphological changes of thermally treated HDPE film after incubation with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) were evaluated as the changes in melting temperature (Tm), onset temperature (T_o) and percentage of crystallinity (% C_{XRD}). Control HDPE film was used as the reference sample for the changes produced. The values of T_m, T_o and % C_{XRD} are listed in Table 13 and 14. Films incubated with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) showed 1.49, 1.86 and 0.47 degrees decrease in melting temperature, 1.62, 1.47 and 0.52 degrees decrease in onset melting temperature (Fig. 30) and a 4.3, 3.9 and 1.441 units reduction in %C_{XRD}, respectively. The difference between T_m and T_o can be taken as measure of polydispersity in the crystalline size. Since T_o represents the temperature at which the bigger or less perfect crystals start to melt,

reduction in T_m and T_o may show some relationship with reduction of crystallinity. The reduction in T_m shows the presence of imperfect crystals as compared to original.

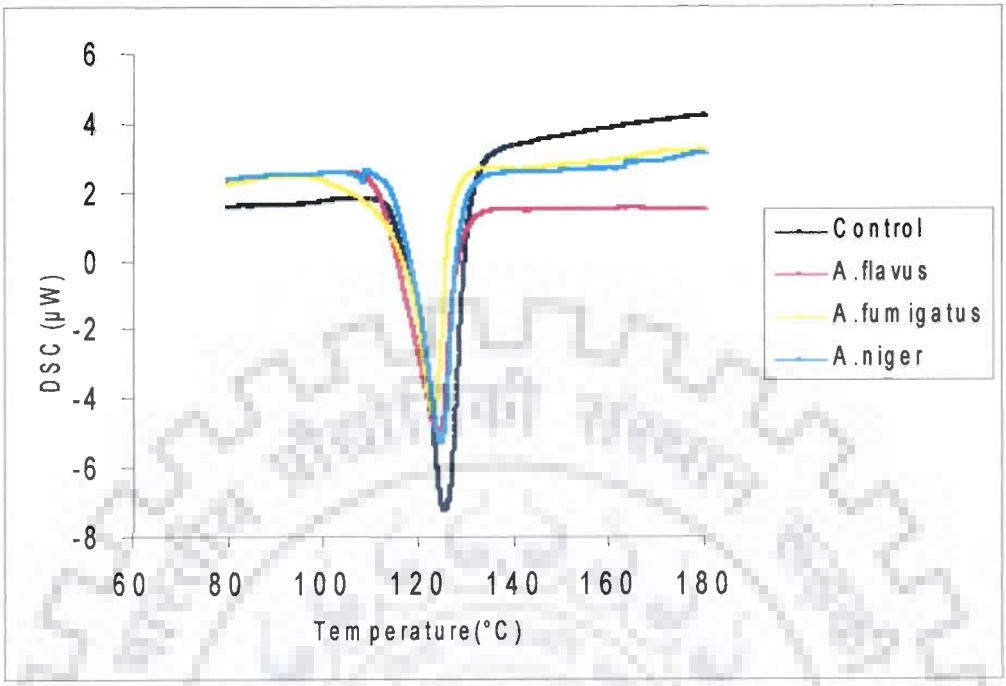


Figure 30. Differential Scanning Calorimetric (DSC) study of control, *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) treated HDPE films. A significant shift in melting and onset temperature could be observed after treatment with fungal isolates.

Table 13: Morphological changes in control and fungal treated HDPE films using DSC

Treatment	$T_m(^0C)$	$T_o(^0C)$
Control	126.31	110.45
A .flavus (ITCC No. 6051)	124.82	108.83
A. fumigatus (ITCC No. 6050)	124.45	108.98
A. niger (ITCC No. 6052)	125.84	109.93

Table 14: X-Ray diffraction (XRD) study of control and fungal treated HDPE films

Treatment	% C_{XRD}
Control	60.081
A .flavus (ITCC No. 6051)	55.781
A. fumigatus (ITCC No. 6050)	56.18
A. niger (ITCC No. 6052)	58.64

4.6.6 GC-MS analysis of degradation products

Culture broth was analyzed for the presence of degradation products of HDPE by GC-MS and identification of compounds was done by comparison with NBS database. Fig. 31 displays the GC-MS chromatogram of products formed during degradation. Table 15 shows degradation products identified after biodegradation of HDPE. Major products identified were toluene, 1,2-Benzene Dicarboxylic acid, Diisooctyl ester, Propanoic acid, Phenol, 4,6-Di (1,1-Dimethylethyl)-2-Methyl, Methyl carbamate, Phenol,2,6-Bis (1,1-Dimethylethyl)-4-Methyl-,Methylcarbamate, 2,6-Di-T-Butyl-4-Methylphenol acetate (ester). Carboxylic acid was identified in control HDPE sample (Table 15). The acid formed in abiotic environment was totally assimilated after incubation of HDPE samples with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052). During biodegradation, microorganisms can assimilate the abiotic degradation products.

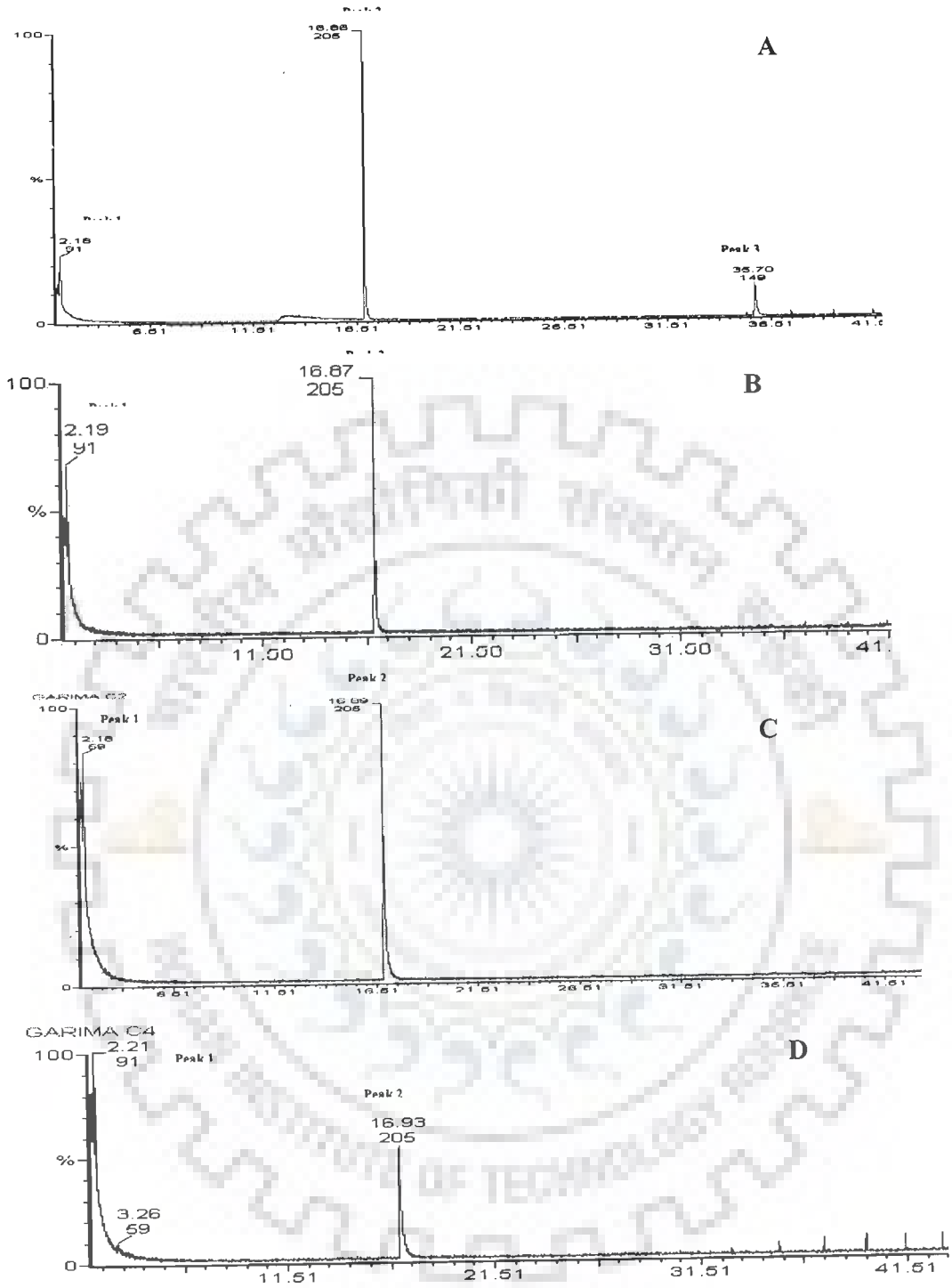


Figure 31: GC-MS chromatograms of the products extracted from heat treated HDPE **A:** Control; **B:** after inoculation with *A. flavus* (ITCC No. 6051); **C:** *A. fumigatus* (ITCC No. 6050); **D:** *A. niger* (ITCC No. 6052).

Table 15. GC-MS analysis of 1 month old culture broths of heat treated HDPE (Control) and heat treated HDPE inoculated with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052)

Figure no/Peak no.	Compound	Control	*A. flavus*	*A. fumigatus*	*A. niger*
A,C,D/1	Toluene ((NBS 9397)*	+	-	+	+
B/1	1,3,5-cycloheptatriene (NIST 37276)*	-	+	-	-
C/2	Phenol, 4,6-Di(1,1-Dimethylethyl)-2-Methyl, Methyl carbamate (NIST 84223)*	-	-	+	-
B/2	2,6-Di-T-Butyl-4-Methylphenol acetate(ester)(NIST 84385)*	-	+	-	-
A/2	Phenol,2,6-Bis (1,1-Dimethylethyl)-4-Methyl-,Methylcarbamate (NIST 84225)*	+	-	-	-
D/2	Propanoic acid, 2-Methyl-3-[4-Butyl] Phenyl-(NIST 84391)*	-	-	-	+
A/3	1,2-Benzene Dicarboxylic acid, Diisooctyl ester (NIST 67063)*	+	-	-	-

*Identification by mass spectroscopy by comparison with the NBS database

D. DEGRADATION STUDY OF POLYURETHANE

4.7.1 Polyurethane degradation rate

PU degradation was monitored by measuring the weight of polyurethane films before and after incubation with different fungal isolates (Fig. 32). There was found to be 60.6, 56.67 and 31.96 % reduction in weight of polyurethane films after 30 days of incubation with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052), respectively (Table 16).

4.7.2 Scanning Electron Microscopic (SEM) study

Fig. 33, 34 and 35 represents the SEM photomicrographs showing the surface topography of the polyurethane sample before and after incubation with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052). For the control sample, it was observed that sample surface was smooth, however after exposure to different fungal isolates, the surface became rough, having a large number of erosion regions showing that degradation has taken place. Large number of fungal spores and hyphae can be seen dispersed over the polyurethane surface. Heavy colonization of polyurethane surface by all the fungal isolates was observed though the extent of surface colonization and erosion varied with each fungal isolate. The attachment of the spores and hyphae to the surface suggests that the development of a biofilm may be an important step in the biodegradation of polyurethane.

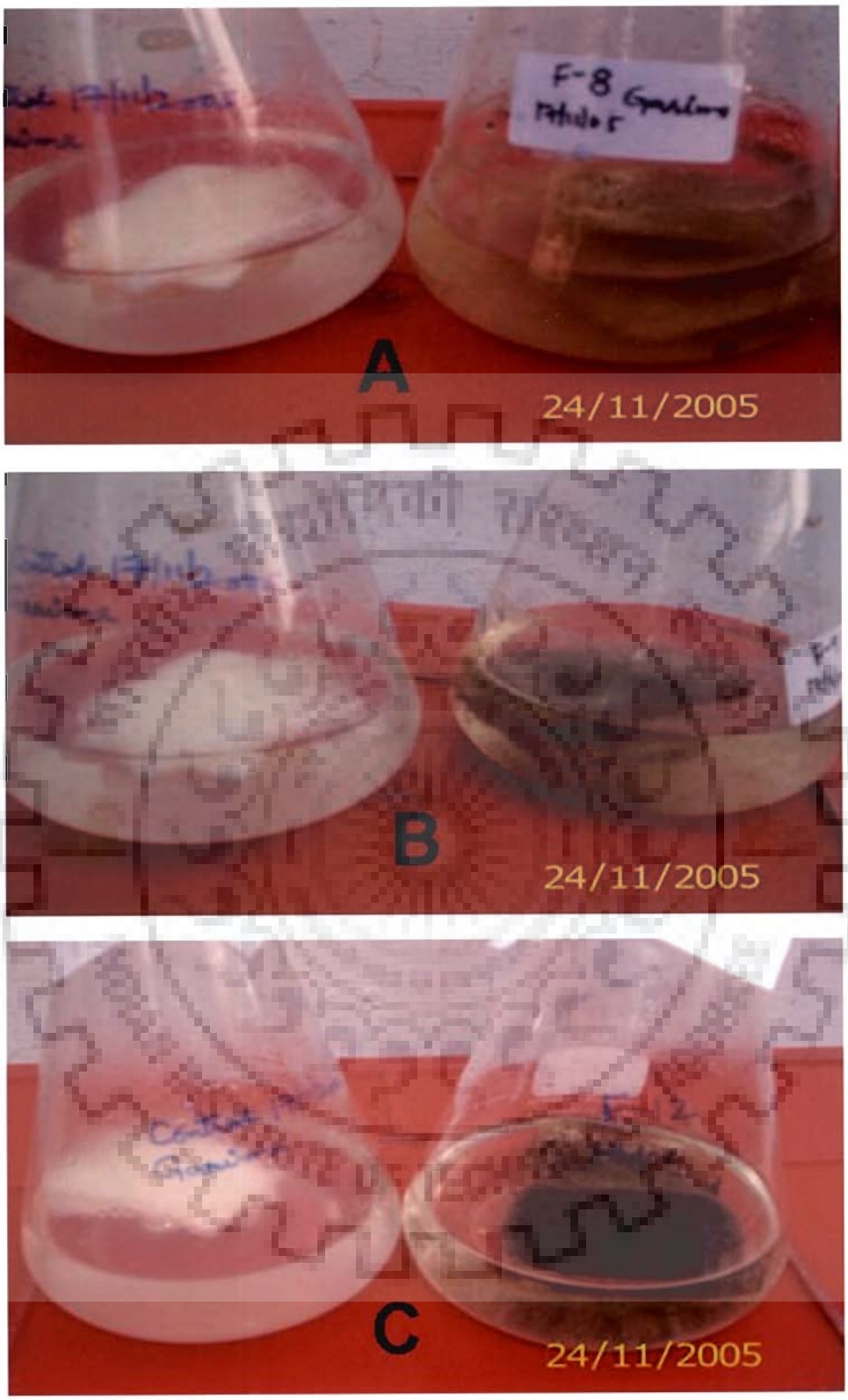


Figure 32. Pure shake flask culture set up for biodegradation studies of Polyurethane. Growth of different fungal isolates in minimal media containing polyurethane as carbon source could be seen. **A**- *A. flavus* (ITCC No. 6051); **B**- *A. fumigatus* (ITCC No. 6050); **C**- *A. niger* (ITCC No. 6052).

Table 16. Percentage reduction in weight of polyurethane film after 30 days of incubation with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) in minimal media containing polyurethane as carbon source.

Sample	Percentage reduction in weight
Control	0.00
A .flavus (ITCC No. 6051)	60.6
A. fumigatus (ITCC No. 6050)	56.67
A. niger (ITCC No. 6052)	31.96

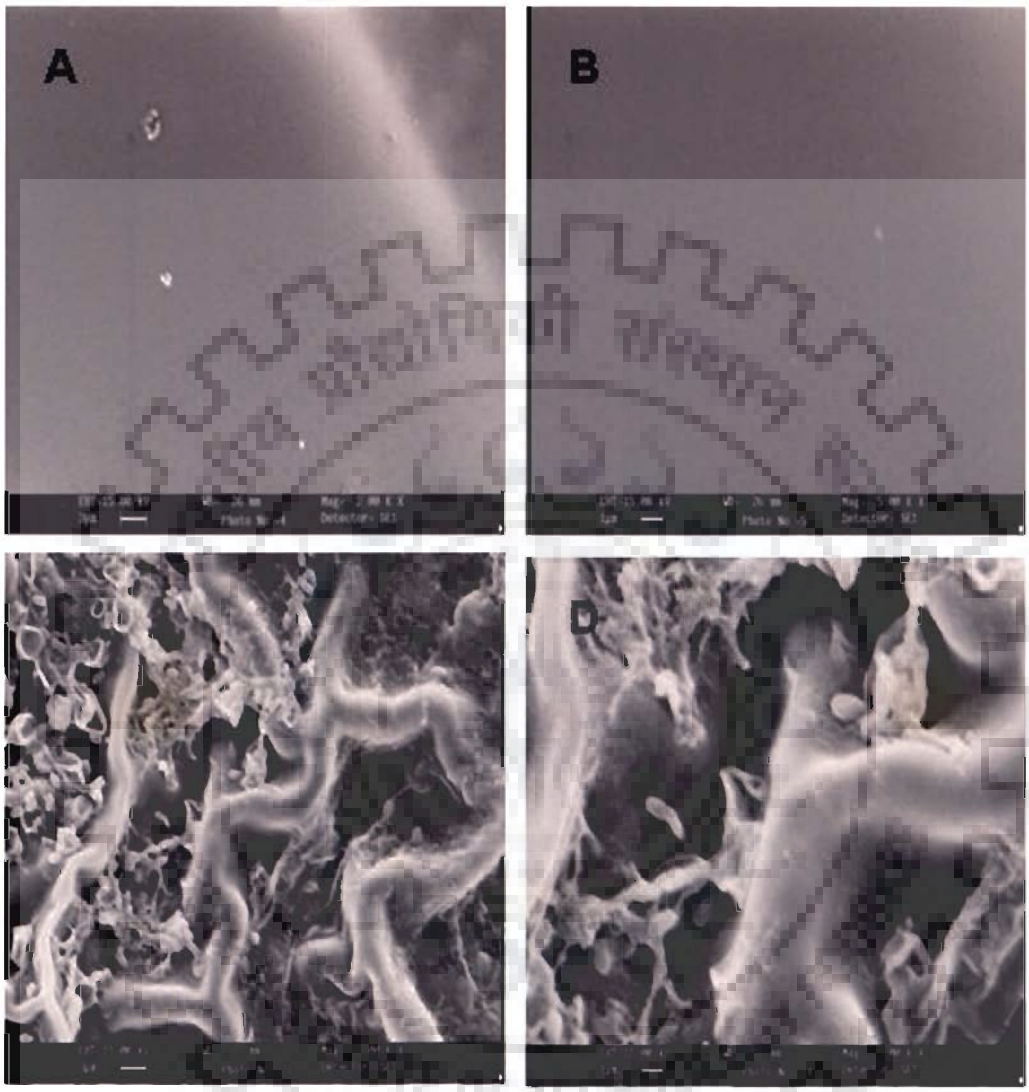


Figure 33. Scanning electron micrographs of polyurethane showing changes and deformity after 30 days incubation with *A. flavus* (ITCC No. 6051). **A & B**-Control (2 KX & 5 KX), **C & D** *A. flavus*(ITCC No. 6051) treated film (2KX & 5 KX respectively). Spore and embedded hyphae could be seen on polyurethane; Bar = 2µm.

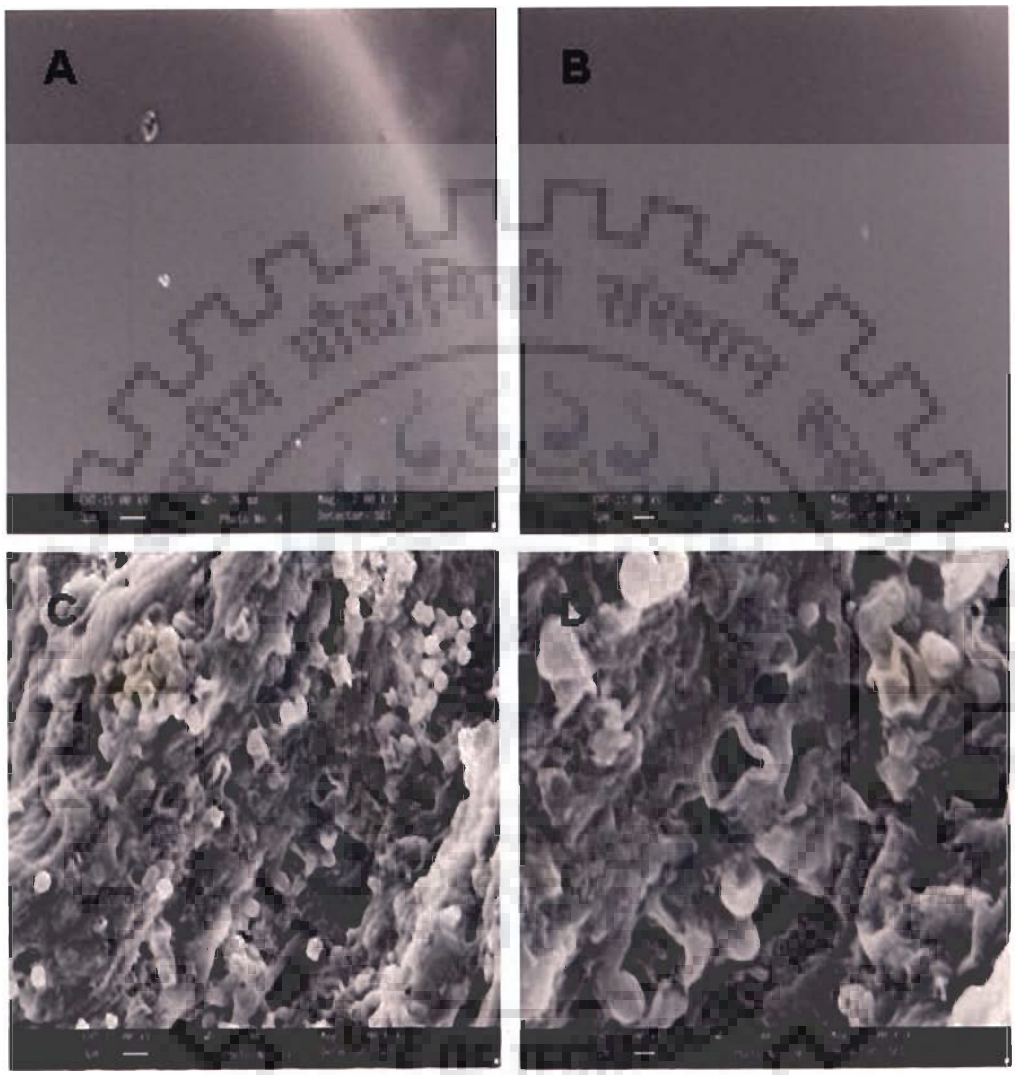


Figure 34. Scanning electron micrograph of polyurethane showing changes and deformity after 30 days incubation with *A. fumigatus* (ITCC No. 6050). **A & B**-Control (2 KX & 5 KX), **C & D** *A. fumigatus* (ITCC No. 6050 treated film (2KX & 5 KX respectively). Spore and embedded hyphae could be seen on polyurethane; Bar = 2µm

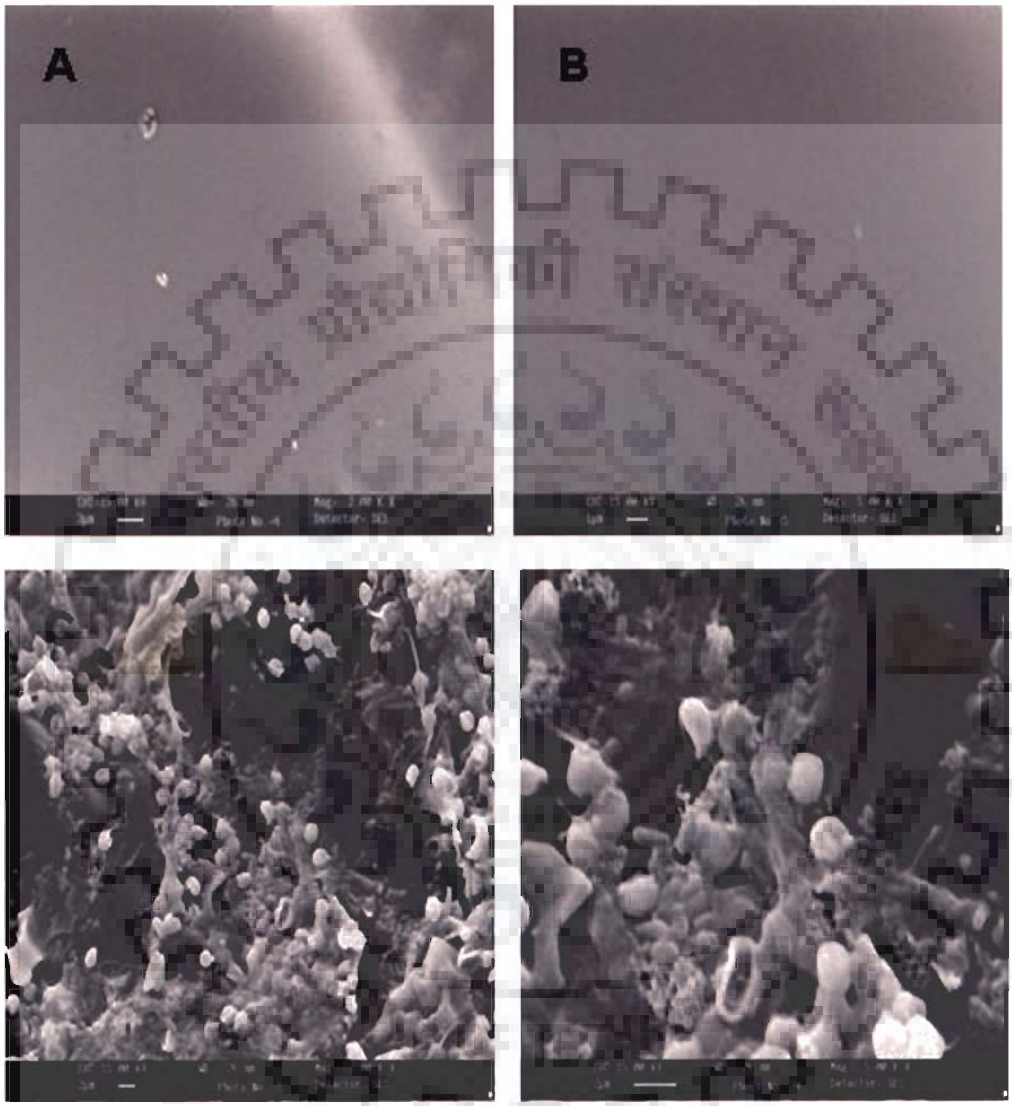


Figure 35. Scanning electron micrographs of polyurethane showing changes and deformity after 30 days of incubation with *A. niger* (ITCC No. 6052). **A & B** Control (2 KX & 5 KX), **C & D** *A. niger* (ITCC No. 6052) treated film (2KX & 5 KX respectively). Spore and embedded hyphae could be seen on polyurethane; Bar = 2µm

4.7.3 Fourier Transform Infrared spectroscopy (FTIR) study

In order to find out whether the chemical composition of polyurethane changes due to biodegradation, it is important to monitor specific peaks at each wavelengths and ether index that constitutes the chemical signature of PU. FTIR spectroscopy was used to monitor changes in the composition on the surface of polyurethane due to microbial degradation. FTIR analysis of the PU films after treatment with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) showed a marked reduction in ether index which mainly happened due to microbial attack (Fig. 36, 37 and 38). The reduction in the ether index was 1.0866, 1.0712 and 1.0427 and reduction in carbonyl index was 0.947, 0.886 and 0.951 respectively for *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) (Table 17).

Table 17. Carbonyl ($A_{1715/1465}$) index and ether index ($A_{1105/1413}$)obtained from FTIR spectra of polyurethane film incubated for 30 days with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) and control.

Treatment	Carbonyl index ($A_{C=O}$:A_{CH2})	Ether index ($A_{1105/1413}$)
Control	1.117	1.0988
A. flavus (ITCC No. 6051)	0.947	1.08622
A. fumigatus (ITCC No. 6050)	0.886	1.0712
A. niger (ITCC No. 6052)	0.951	1.0427

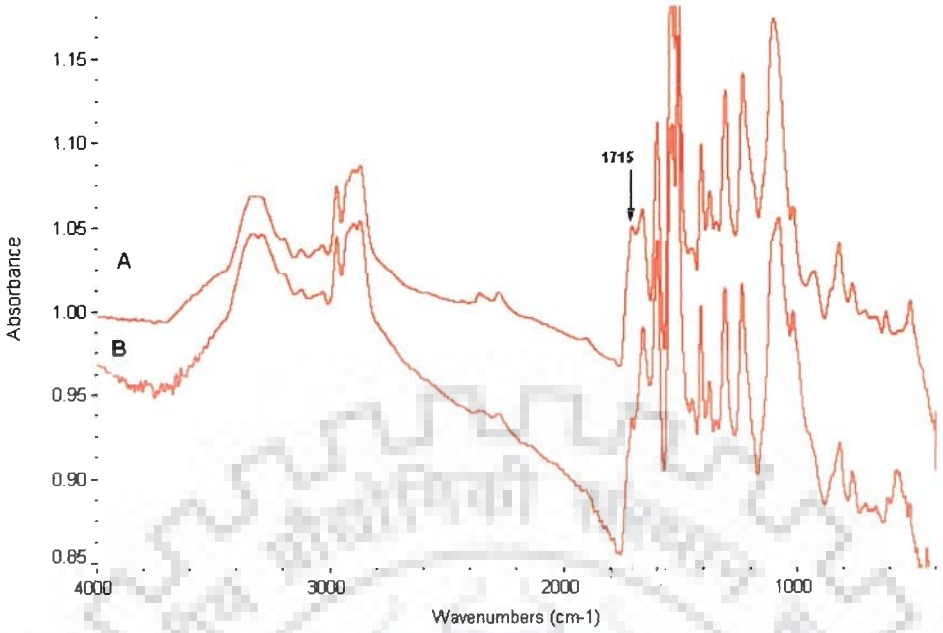


Figure 36. Fourier transform infrared spectroscopy (FTIR) of polyurethane. **A**-Control, **B-** *A. flavus* (ITCC No. 6051) treated film. Arrow indicate the carbonyl peak at 1715 cm^{-1}

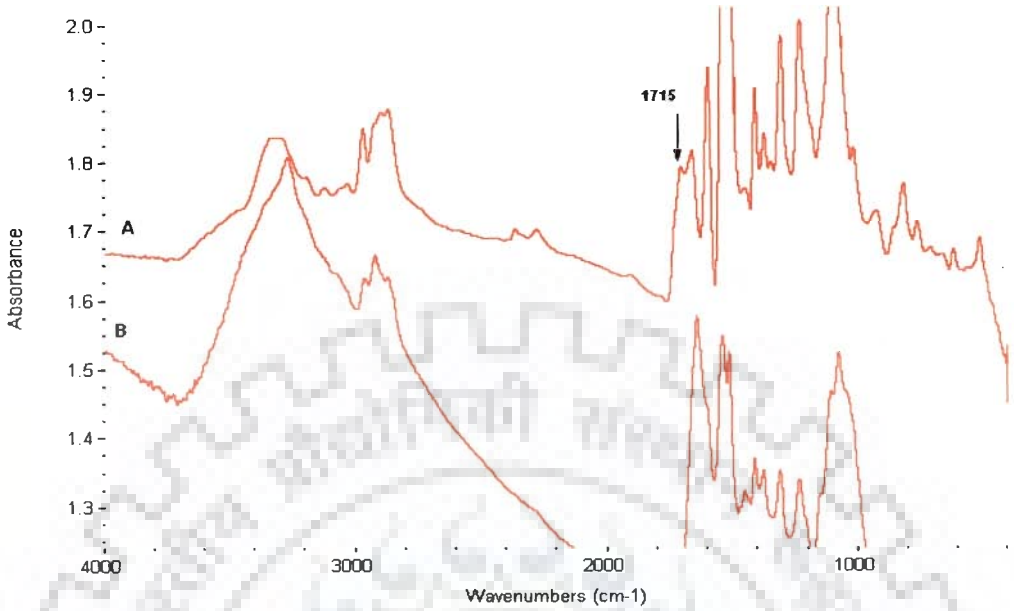


Figure 37. Fourier transform infrared spectroscopy (FTIR) of polyurethane. **A**-Control, **B-** *A. fumigatus* (ITCC No. 6050) treated film. Arrow indicate the carbonyl peak at 1715 cm^{-1}

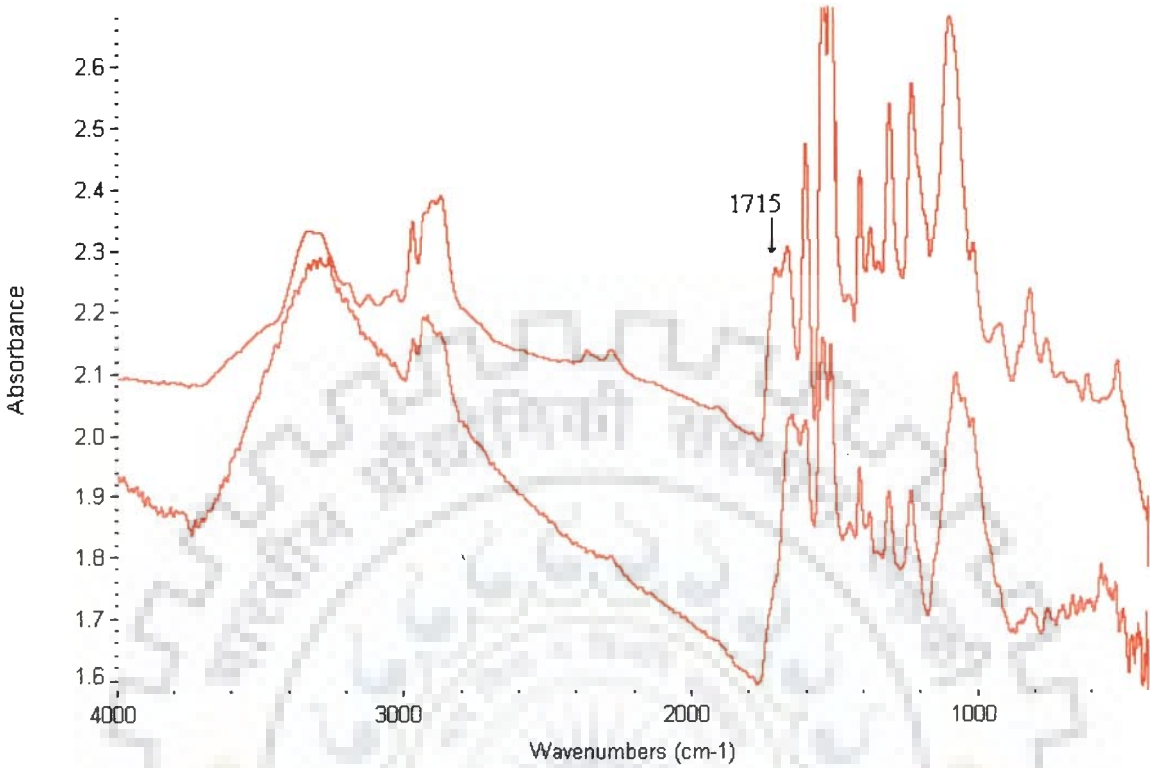


Figure 38. Fourier transform infrared spectroscopy (FTIR) of polyurethane. **A**-Control, **B-** *A. niger* (ITCC No. 6052) treated film. Arrow indicate the carbonyl peak at 1715 cm^{-1}

4.7.4 Thermogravimetric analysis

An important application of the thermogravimetric method is to study the thermal behaviour of the polymer. The TG curves provide information about the decomposition of different kind of polymers. In reference to the study of biodegradation of PU, a controlling TGA curve of the control sample that was exposed to culture media only, was made (Fig. 39). It was found that decomposition process takes place in three steps. The first step in the decomposition which takes place at 100-300°C is related to the loss of the volatile compounds such as additives used during the synthesis. Second decomposition (300-400°C) is related to the breakdown of urethane linkages and third step (400-500°C) is related to the rupture of ester bonds. Comparing the thermogravimetric curves of the samples exposed to different fungal isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) with control, third decomposition step corresponding to ester links disappeared after microbial attack.

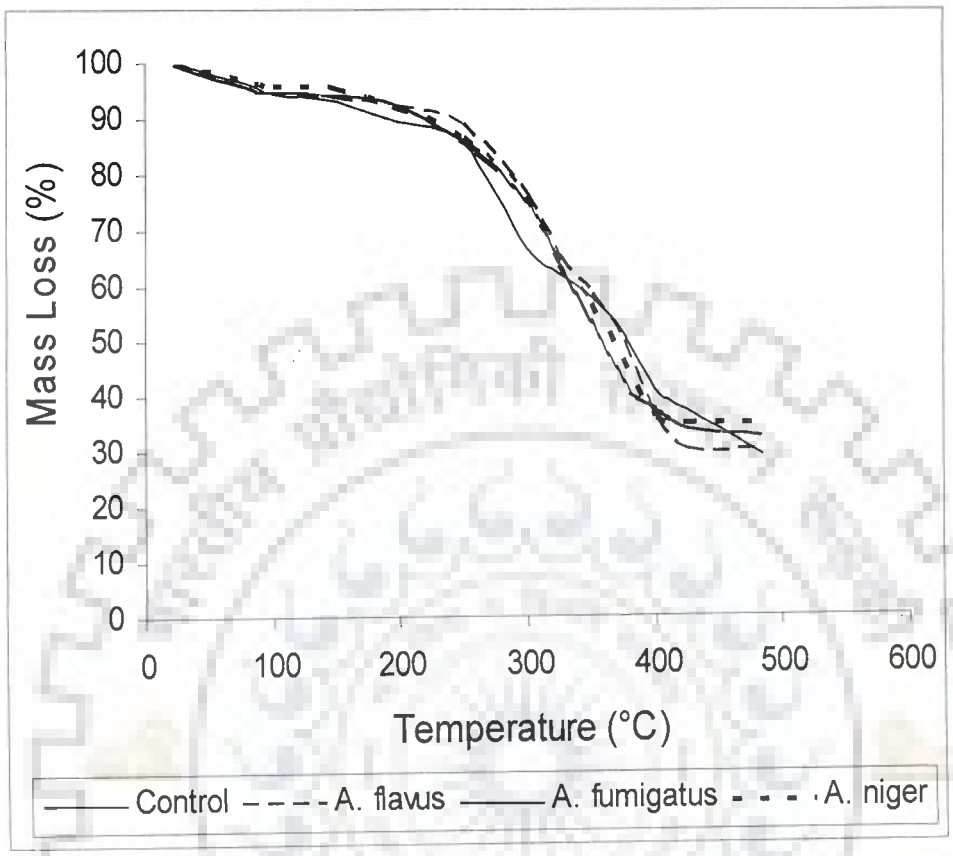


Figure 39. Thermogravimetric curve for Polyurethane after treatment with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052)..

E. HYDROLYTIC ENZYMES PRODUCING ABILITY OF FUNGAL ISOLATES AND POSSIBLE ROLE IN PLASTIC DEGRADATION

It is hypothesized in the earlier studies that the plastic degradation ability of microbes is associated with their hydrolytic particularly esterases, LiP and MnP enzyme secretion. Therefore, extracellular esterase, LiP and MnP activities were measured in the culture broths inoculated by *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) containing polyethylene or polyurethane as carbon source (Table 18).

4.8 Esterase enzyme activity

Since the esterases are found to play a major role in biodegradation of polymer, fungal inoculated culture broths were analyzed for their esterase activity when they were grown in minimal media with HDPE or PU as carbon source. Esterase activity in the culture broth containing HDPE or PU inoculated by three fungal isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) are shown in Table 18. Significant high esterase activity was observed in both HDPE and PU containing culture broths. However, it was much high in PU culture broth. There was a difference of 20-30 units when the fungal isolate was grown in the presence of polyurethane as carbon source. *A. fumigatus* (ITCC No. 6050) showed the highest esterase activity as compared to *A. flavus* (ITCC No. 6051) *and A. niger* (ITCC No. 6052) having values of 152 U/50ml for HDPE and 164 U/50ml for PU. This higher esterase producing ability in the presence of polyurethane may be attributed to the presence of ester linkages in polyurethane.

Table 18. Extracellular Esterase, Manganases peroxidase (MnP) and Lignin peroxidase (LiP) activities in 30 days incubated culture broths inoculated with by *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) containing either HDPE or PU as carbon source. Each value represents mean ± SE of triplicates.

Enzyme activity(Total U/50ml)	High Density Polyethylene (HDPE)			Polyurethane		
	A. flavus	*A. fumigatus*	*A. niger*	*A. flavus*	*A. fumigatus*	*A. niger*
Esterase	136.2±10.01	152.65±9.62	130.5±10.21	158±5.003	164±7.1093	141±5.1002
Manganese Peroxidase (MnP)	18.98±2.32	21.549±1.78	8.25±2.03	1.94±0.071	1.6±0.062	1.32±0.0402
Lignin Peroxidase (LiP)	9.02±0.08	8.25±1.01	6.35±0.81	3.95±0.182	3.35±0.192	3.02±0.151

4.9 Manganese Peroxidase activity

There was a significant difference in the MnP activity of all the three isolates when they were grown in the presence of HDPE and PU. MnP activity was maximum when the fungal isolates were grown in the presence of HDPE as carbon source. On the other hand, PU containing culture broth inoculated with these three fungal isolates showed very low activity, almost negligible compared to HDPE broth. The HDPE containing culture broth inoculated with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) showed MnP activity of 18, 21 and 14 U/50ml respectively while in the case of polyurethane containing cell broth it was almost negligible with 1.9, 1.6 and 1.32 U/50ml respectively after 30 days of incubation (Table 18).

4.10 Lignin Peroxidase activity

Lignin Peroxidase activity was found to be higher in case of HDPE as compared to polyurethane. There were significant differences in the values of LiP units when fungal isolates were incubated with HDPE or PU as carbon source. There was approximately two to three fold increase in the values of LiP activity of all the three isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) when they were grown in the presence of HDPE (Table 18), while observed negligible in case of polyurethane containing culture broth.

4.11 SDS-PAGE and Esterase in gel assay

Since high esterase activity was observed in the culture broth inoculated by these three isolates containing HDPE or PU, attempt was made to get the idea about the molecular weight of the esterase broth of these three fungal isolates. Crude culture broth and 30% acetone precipitated fraction of the broth were resolved on 10% SDS-PAGE. The esterase band was visualized by coomassie blue stain and by in gel activity assay. SDS-PAGE (10%) of culture broth obtained from the 30 days grown culture inoculated with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) showed an esterase band having a range of 45-48 kDa (Fig. 40, 41 and 42).

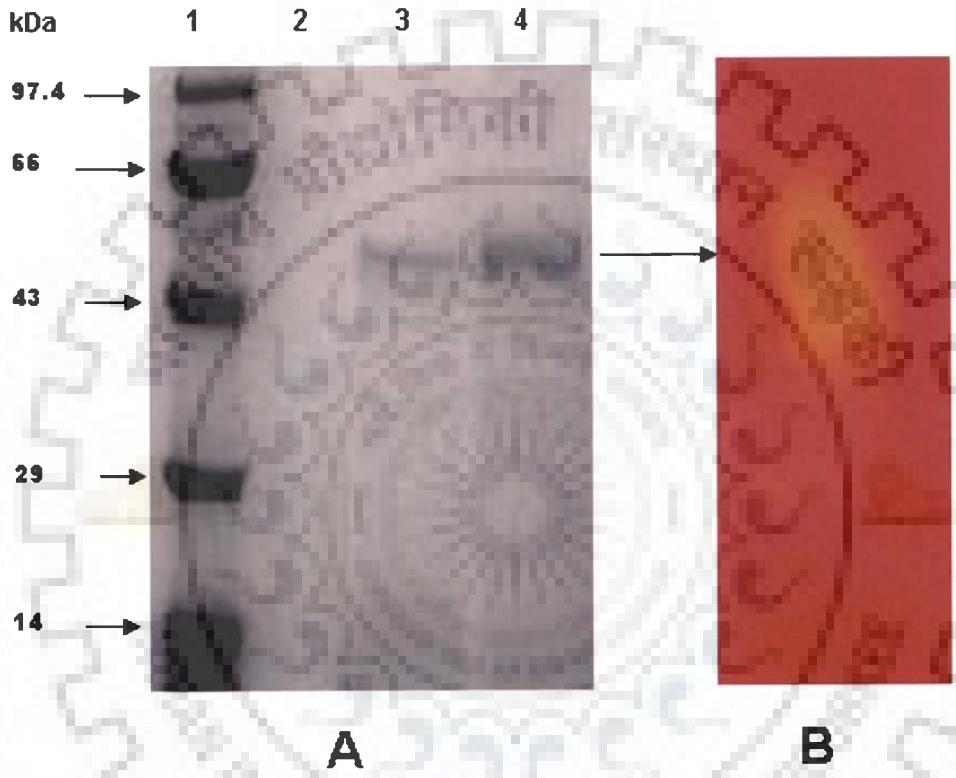


Figure 40. SDS-PAGE (10%) of culture broth obtained from the 30 days grown culture inoculated with *A. flavus* (ITCC No. 6051). A-Coomassie blue stained gel, lane -1-Marker, lane-2-control without fungal inoculation, lane 3-crude broth of inoculated culture and lane 4- 30% acetone enriched fraction. B-Esterase in gel assay of the crude broth.

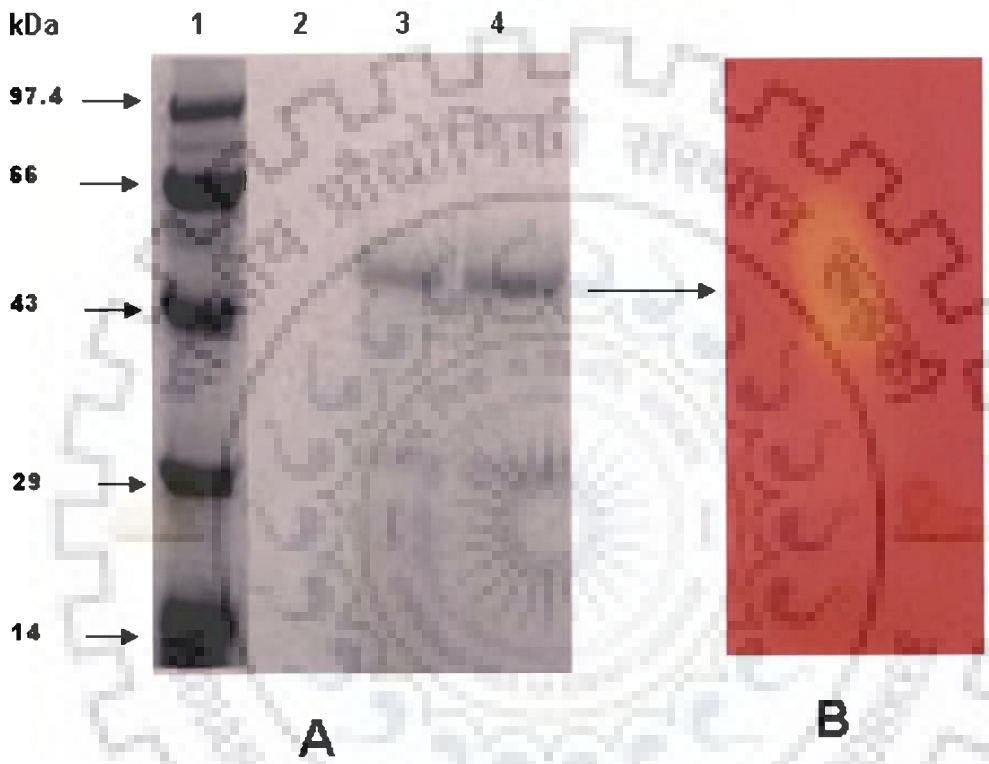


Figure 41. SDS-PAGE (10%) of culture broth obtained from the 30 days grown culture inoculated with *A. fumigatus* (ITCC No. 6050). **A**-Coomassie blue stained gel, lane -1-Marker, lane-2-control without fungal inoculation, lane 3-crude broth of inoculated culture and lane 4- 30% acetone enriched fraction. **B**- Esterase in gel assay of the crude broth.

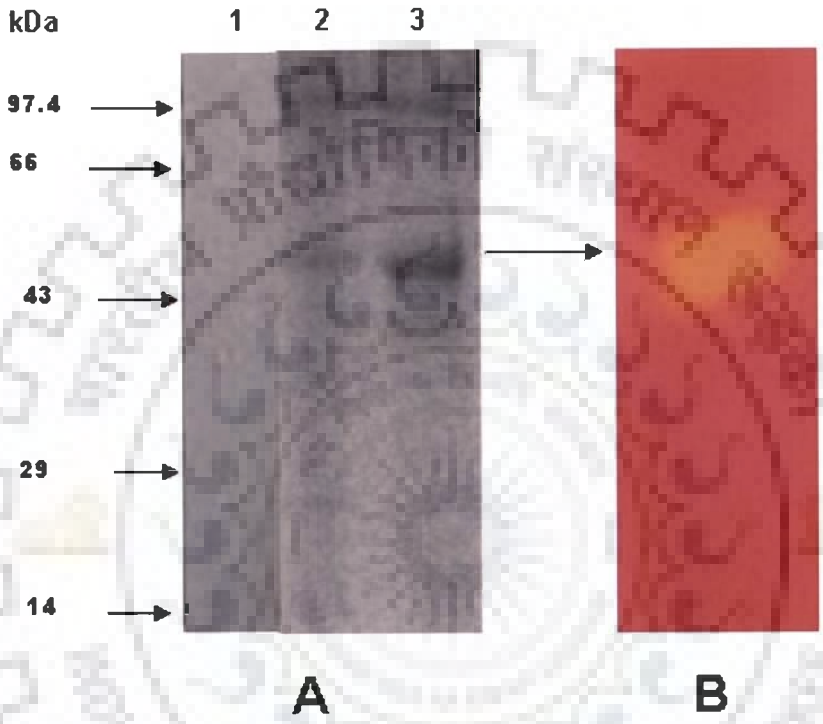


Figure 42. SDS-PAGE (10%) of culture broth obtained from the 30 days grown culture inoculated with *A. niger* (ITCC No. 6052). **A**- Coomassie blue stained gel, lane -1-Marker, lane-2-control without fungal inoculation, lane 3-crude broth of inoculated culture and lane 4- 30% acetone enriched fraction. **B**- Esterase in gel assay of the crude broth

CHAPTER 5

DISCUSSION AND CONCLUSION

Degradation of inert synthetic plastics particularly polyethylene has been of major concern presently as it is the major contributor to the emerging problem of environmental pollution by plastic waste. Biodegradation of polyethylene has been suggested to be safe and ecofriendly approach for the plastic waste disposal compared to the conventional method. But the polyethylene is relatively resistant to microbial attack and thus presumed to be non-biodegradable till recent past. However, the observation that many microorganisms can utilize paraffin as carbon source provided the soul basis to the idea that such microbes could also utilize polyethylene (Fuhs, 1961). This led to the search for microorganisms which can degrade polyethylene. In last few years, much emphasis has been given on the isolation and screening of naturally occurring microorganisms with inherent ability to utilize polyethylene as carbon and energy source and ultimately causes its degradation. A number of bacteria (*Rhodococcus ruber*, *Brevibacillus borstelensis*) and fungi (*Penicillium lilanicum, Aspergillus* sp., *Penicillum simplicissimum* YK) have been isolated which were able to utilize polyethylene as carbon source and cause degradation of polyethylene (Cuevas and Manaligod, 1997; Gilan *et al.*, 2004; Hadad *et al.*, 2005; Yamada *et al.*, 2001). It has been hypothesized that if the right kind of microorganism is isolated, then so called non-biodegradable polyethylene could be degraded.

In present work, an attempt has been made to isolate potential plastic degrading fungi with main emphasis on high density polyethylene degradation. The fungal isolates were obtained from the surface of the buried polyethylene film or plastic waste dumpsites. These isolates were screened for their cell surface hydrophobicity, ability to utilize HDPE or PU as carbon source and extracellular hydrolytic enzymes particularly

esterase producing ability. Out of the twelve fungal isolates, three of the isolates namely F-8, F-9 and F-12 have high cell surface hydrophobicity, utilized HDPE or PU as carbon source when grown in minimal media. The other isolates were found to have very poor cell surface hydrophobicity and could not utilize HDPE as carbon source as they could not grow in minimal media containing HDPE as carbon source. Since the resistance of polyethylene to microbial attack is mainly due to its high surface hydrophobicity, it is logical that microorganism with high cell surface hydrophobicity and ability to utilize HDPE as carbon and energy source could be the potential degraders. This is evident from recent studies of polyethylene degradation by bacteria and fungi (Gilan *et al.*, 2004; Yamada *et al.*, 2001). Thus on the basis of high cell surface hydrophobicity, ability to utilize HDPE as carbon source and extracellular esterase producing ability, in the present study fungal isolates F-8, F-9 and F-12 were selected as potential candidate with ability to degrade HDPE. The isolates F-8, F-9 and F-12 were identified as *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) respectively. These isolates were further used for detailed degradation study.

Biofilm formation by microorganisms on the surface of polymer is a prerequisite for their degradation. In most of the cases, it is detrimental to polymer material but it could be advantageous by degrading inert synthetic polyethylene. Mechanism of biofilm formation by bacteria on polymer material is well understood and there are several excellent reviews highlighting bacterial biofilm formation (O'Tootle *et al.*, 2000; Watnick and Kolter, 2000). According to consensus model of bacterial biofilm formation (O'tootle *et al.*, 2000), it is a complex multistage process which initiates with cell

adhesion to polymer surface followed by colonization and cell growth and ultimately forming a cellular scaffold due to production of extracellular polysaccharides.

In contrast, the biofilm formation by fungi on polymeric material is poorly understood that is mainly due to the lack of suitable model system for fungal biofilm formation study and complex growth pattern of fungi. In last few years, a number of model systems have been developed and the biofilm formation by *Candida* and other fungi has been studied (Blakenship and Mitchell, 2006; Reynolds and Fink, 2001). Attempt has been made to develop a consensus model for fungal biofilm formation study. According to which the presence of spores, hyphae on polymer surface along with increase in EPS production, increase in biofilm protein content and biofilm biomass has been assumed as a clear sign of fungi biofilm formation and these parameters are commonly used to access the fungal biofilm formation.

Major hurdle in biodegradation of polyethylene is its hydrophobicity, which interferes with fungal adhesion to the surface since most microbial cells are hydrophilic. Biofilm formation on polyethylene surface enables the microbes to utilize it efficiently thus leads to degradation. Therefore, the colonization and biofilm formation on HDPE surface by fungal isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) was studied in minimal media containing polyethylene film as carbon and energy source. All the three isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) showed preference for adherent mode of growth over planktonic growth as there was found to be constant increase in adherent cell population and reduction in planktonic cell population with increasing time (Fig. 12, 13

and 14). Since as per the consensus model for fungal biofilm formation, the presence of spores and hyphae on polymer surface is an indication of biofilm formation. All the three isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) showed clear sign of biofilm formation on polyethylene surface as spores and hyphae could be clearly seen as revealed from SEM study (Fig. 20, 21 and 22). This was also supported by increasing EPS content, biofilm protein content and biofilm fungal biomass (dry weight) during 30 days of incubation (Fig. 15). Since the development of biofilm is closely associated with generation of matrix which is made up of extracellular material mainly of exopolysaccharides (Baillie and Douglas, 2000; Chandra *et al.*, 2001). This complex extracellular material is suggested to be seen as scaffold for maintaining integrity of biofilm, to limit the diffusion of toxic substances into biofilm or in some combination of above (Blankenship and Mitchell, 2006).

Colonization of polyethylene and biofilm formation by all the three isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) were as per consensus model of biofilm formation (Blakenship and Mitchell, 2006; Watnik and Kolter, 2000) and in agreement with earlier studies of colonization and biofilm formation by fungi on plastics surfaces where the above parameters were seen as sign of biofilm formation (Chandra *et al.*, 2001; Peciulyte, 2002; Webb *et al.*, 2000; Ramage *et al.*, 2001; Reynolds and Fink, 2001). Most of the studies related to biofilm characterization are often performed with readily available carbon sources. In contrast the biofilm formed by three isolates in the present study was under nutrient limiting conditions and HDPE served both as carbon source as well as substratum on which biofilm grow. It has been reported that microorganisms can resist environmental

conditions adverse to microbial life such as nutrient starvation and others by switching from planktonic growth to biofilm form (Sivan *et al.*, 2006). Similar trend was observed in present study, where all the three isolates showed preference for biofilm mode of growth compared to planktonic when grown in minimal medium containing HDPE as carbon source.

The adhesive properties of the cells changed under nutrient limiting condition and there is tendency to adhere to the substrate by hyphae or exopolymers secretion, which give rise to biofilm development (Abrusci *et al.*, 2005). In one of the study, it was observed that adherence of yeast to plastic was enhanced as the glucose concentration was lowered, but it was reduced in the complete absence of glucose, suggesting that there is a requirement for small amount of glucose initially for active metabolism (Reynolds and Fink, 2001). In agreement with the observation made in earlier studies mentioned above, it is likely that the preference for biofilm mode of growth over planktonic growth in minimal medium containing HDPE film as carbon source by *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) are presumably due to increase in the adhesive properties of the fungal cells and addition of 0.1% malt extract in the minimal media was needed initially for survival of fungal cells before they could colonize and use polyethylene as carbon source. Similar observation has been made in one of the recent studies where biofilm formation on low density polyethylene (LDPE) surface by bacteria (Gilan *et al.*, 2004; Hadad *et al.*, 2005; Sudhakar *et al.*, 2007) and high density polyethylene (HDPE) surface by fungi (Alariqi *et al.*, 2006; Liyoshi *et al.*, 1998; Yamada *et al.*, 2001) were reported under nutrient limiting conditions having LDPE and HDPE as sole carbon source respectively. The microorganisms were able to

form an active biofilm on polyethylene surface and were able to utilize the polyethylene as carbon source and provide the clear evidence of polyethylene degradation by microbe.

There are several reports demonstrating the correlation between cell surface hydrophobicity and carbon starvation. The *Saccharomyces cerevisiae* cells in lack of glucose become more hydrophobic and adhesive and cause invasive growth or biofilm formation (Cullen and Sprague, 2000; Reynolds and Fink, 2001). Increased adhesion of *Candida albicans* to plastics has been associated with increased hydrophobicity both of the fungal surface (Miyake *et al.*, 1986; Panagoda *et al.*, 1998) and of the substratum (Klotz *et al.*, 1985). It is found that microbial cell surface becomes more hydrophobic and adhesive in carbon-starved cultures than with nonstarve cultures (Sakharovaski *et al.*, 1999; Sanin *et al.*, 2003). It is logical that similar conditions prevailed in cultures of these three isolates, where low carbon availability may enhance the cell surface hydrophobicity and biofilm formation and consequently, improve the biodegradation of polyethylene. A higher extent of hydrophobicity of the interacting cells always promotes the adhesion of cells and consequent biofilm formation which causes adverse effect to the physical or chemical integrity of the material and ultimately leads to its degradation (Morton and Surman, 1994). Therefore, it is likely that the biofilm formation on HDPE surface by the isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) in the present study is due to their high cell surface hydrophobicity and the culture conditions.

In present study the HDPE films were heat treated prior to incubation. It has been reported that physical treatment of the polyethylene film facilitates biodegradation

(Volke-Sepulveda *et al.*, 2002). This is mainly due changes in the surface properties of polyethylene particularly formation of carbonyl bonds which make hydrophobic surface relatively hydrophilic and thus make it accessible to microorganisms and is helpful in biofilm formation. Development of biofilm is closely associated with the generation of matrix, the majority of which is extracellular material (Chandra *et al.,* 2001). This complex extracellular material might function to serve as a scaffold for maintaining biofilm integrity, to limit diffusions of toxic substances into the biofilm, or in some combination of above (Blakenship and Mitchell 2006). With numerous reports showing that biofilm extracellular polymeric material constitutes mainly polysaccharide (Chandra *et al.,* 2001), the significantly high level of EPS at 30 days of incubation showed the biofilm formation. Analysis of protein content of the biofilm and dry weight was done to monitor biomass. The increase in protein content of the biofilm with increase in time suggests that there was an increase in biomass density of biofilm formed by *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052).

Since all the three isolates namely *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) were able to colonize and form biofilm on HDPE surface, their effect on degradation was evaluated. Degradation in the present study by three isolates were carried out by axenic cultures conditions. Degradation study by axenic culture is advantageous over the studies carried out in environmental conditions such as compost (soil) as it enables the better understanding of biodegradation mechanism. In case of degradation studies under soil, mixed microbial flora and many abiotic factors play a role in biodegradation which makes it difficult to determine which part of degradation is due to chemical degradation and which is due to biological

degradation. A number of studies on biodegradation of polyethylene have been carried out by axenic cultures (Byungtae *et al.*, 1991; Ei-Shafei *et al.*, 1998). Degradation of polyethylene is a complex process and both abiotic and biotic factors contribute in it (Volke-Sepulveda *et al.*, 2002). The initial degradation takes place by abiotic factors which is later taken over by biotic factors. Biofilm formation on polyethylene surface by these biotic factors further causes changes in physical properties and cause deterioration and degradation. In nature, degradation of polyethylene is a very slow process. However, it has been reported that physicochemical treatmemts like thermal oxidation (Albertsson *et al.*, 1998; Volke-Sepulveda *et al.*, 2002), UV photooxidation (Cornell *et al.*, 1984; Gilan *et al.*, 2004) and chemical oxidation (Brown *et al.*, 1974) of the polyethylene films facilitates the biodegradation. These physicochemical treatments causes the oxidation of polyethylene and bring changes in the surface properties of polyethylene particularly by formation of carbonyl bonds which make hydrophobic surface relatively hydrophilic and thus making them susceptible to microbial attack which causes deterioration and degradation of the material and brings further changes in the surface properties. The changes in the physical properties are determined by various physical methods Scanning electron microscopy (SEM), Fourier Transform infrared spectroscopy (FTIR), Differential scanning calorimetry (DSC), X-Ray diffraction (XRD) and Gas Chromatography-Mass spectrometry (GC-MS) order to study the extent of degradation.

In the present study HDPE films were thermally treated prior to incubation with fungal isolates. The thermal treated HDPE films were incubated with fungal isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) for 30 days. Degradation of HDPE was evaluated by weight loss measurement, reduction

in tensile strength, Scanning electron microscopy (SEM), Fourier Transform infrared spectroscopy (FTIR), Differential scanning calorimetry (DSC), X-Ray diffraction (X-RD) and Gas Chromatography- Mass Spectrometry (GC-MS). There was reduction in the weight of HDPE films after incubation with all three isolates. Weight loss observed by these isolates was in range of 1.16-4.41%. The maximum weight loss (4.41%) was observed with *A. fumigatus* (ITCC No. 6050). Although the loss level seems low, however this is significant keeping in mind the inert nature of HDPE and short incubation time of 30 days and it is comparable to weight loss observed in earlier studies (Gilan *et al.*, 2004; Hadad *et al.*, 2005). Degradation was more clearly evident from tensile strength data. A significant reduction in tensile strength of HDPE films 61.3, 60 and 58.77 %, respectively after treatment with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) were obtained. This is in well agreement with the earlier studies where a tensile strength reduction of 60-65% and reports showing similar observations (Byungtae *et al.*, 1991; Ei-Shafei *et al.*, 1998).

Colonization and biofilm formation on the HDPE surface in the present study by *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) (Fig.19, 20 and 21) can be seen in SEM micrographs by the presence of superficial growth of hyphae and spores on HDPE surface. The deterioration and degradation of surface was evident by formation of cavities on the polyethylene film surface and penetration of fungal hyphae. Similar observations have been made in earlier studies related to biodegradation of polyethylene (Albertsson and Karlsson, 1993; Manzur *et al.*, 2004; Volke-Sepulveda *et al.*, 2002).

FTIR is found to be an accurate method to detect structural changes occurring in the polymer due to thermal and biological treatment (Siesler, 1993). There found to be significant difference in carbonyl residues in fungal treated and untreated HDPE films (Fig. 27, 28 and 29). The observed decrease in the amount of carbonyl residues of HDPE film after incubation with *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) in the present study is likely due to its utilization by fungi. Thermal treatment of polyethylene prior to their exposure to microbe generates carbonyl groups, which make polyethylene surface susceptible to microbial attack (Gilan *et al.*, 2004). Reduction in carbonyl index (CI) and an increase in double bond index (DBI) after incubation with these isolates showed a clear sign of degradation. Reduction in carbonyl index and increase in double bond index after fungal treatment has been reported in earlier studies related to polyethylene degradation (Hadad *et al.*, 2005; Volke-Sepulveda *et al.*, 2002). The carbonyl index (CI) reduction in fungal treated HDPE film can be explained according to the propsed mechanism for polyethylene biodegradation (Albertsson *et al.*, 1987). Once formed carbonyl groups along the polymer chain, these can be attacked by microbes, leading to decrease in CI. Oxidized polyethylene molecules can be hydrolyzed by extracellular enzymes. The molecule is then transformed to a fatty acid and metabolized by means of β-oxidation.

The degradation was also studied by determining the changes in the physical properties of HDPE film before and after incubation with fungal isolates. DSC has been used to study the thermal behaviour of polymer. It has been reported that the thermal profile of polyethylene changes after treatment with microbes (Volke-Sepulveda *et al.*, 2002). A reduction in T_m and T_o was observed after incubation of HDPE films with all

these isolates in minimal media. This decrease in T_m and T_o reflects the presence of smaller or imperfect crystals compared to originally existing one. It has been reported that once the polymer is exposed to the biological treatment, the fungi attack mainly the amorphous phase causing the separation of crystalline block and thus small crystals are obtained (Manzur *et al.*, 2004). The observed reduction in $\%C_{XRD}$ in HDPE film after fungal treatment might be attributed to the smallest or imperfect crystalline fraction. Thus changes in the crystallinity (reduction) after fungal treatment, as observed from DSC and $\%C_{XRD}$ confirmed the degradation of HDPE by these isolates. A reduction in $\%C_{XRD}$ as a measure of degradation of polyethylene has earlier been reported (Albertsson and Karlsson, 1993; Volke-Sepulveda *et al.*, 2002).

The degradation of HDPE was also confirmed by the analysis of product profile of broth of control and fungal inoculated cultures using GC-MS. The degradation mechanism governs the type of degradation products obtained. During biodegradation, microbes can assimilate abiotic degradation products which are formed during the physicochemical treatment of polyethylene. Carboxylic acids have previously been reported as main product category formed during abiotic oxidation process (Albertsson *et al.*, 1995) and complete disappearance of the shortest chain acids has been reported in biotic environment as an evidence for biodegradation. Similar to the earlier observations, In the present study, there was found to be complete disappearance of carboxylic acids after incubation with all the three fungal isolates (Fig. 31). The utilization of low molecular weight carboxylic acids as a carbon source by fungi has been reported (Albertsson *et al.*, 1995). The complete disappearance of dicarboxylic acid after incubation with these fungal isolates is in agreement with biodegradation mechanism

proposed where subsequent lowering of the carbonyl index occurs simultaneously with the assimilation of soluble dicarboxylic acids in biodegraded LDPE samples (Albertsson *et al.*, 1987; Albertsson *et al.*, 1995). Thus it is clear that all the three isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) have potential to degrade HDPE under axenic culture conditions.

The ability of all the three fungal isolates to degrade PU was also studied. The degradation study was performed in pure shake flask culture in axenic culture conditions similar to HDPE. Polyurethanes are polyesters and susceptible to microbial attack. There was found to be fast degradation of PU as compared to HDPE by the fungal isolates as expected. All the fungal isolates resulted in loss of weight after incubation with fungi. This loss in weight of PU was relatively higher as compared to HDPE which is expected as polyurethane is susceptible to microbial attack. Degradation was also studied by SEM, FTIR and thermogravimetric study. All these confirmed the PU degradation ability of these three isolates and the rate of PU degradation by these isolates was faster as compared to HDPE degradation by these isolates. which was as per expectation due to PU susceptibility to microbial attack. Due to its susceptibility to microbial attack, the biodegradation of PU has been extensively studied. Biodegradation of polyurethane by a number of bacteria *Corynebacterium* sp. (Kay *et al.*, 1991), *Pseudomonas aeruginosa* (Kay *et al.*, 1991), *Comamomas acidovorans* (Nakajima-Kambe *et al.*, 1995) and fungi *Chaetomium globosum* (Boubendir, 1993), *Aspergillus terrus* (Boubendir, 1993), *Fusarium solani* (Crabbe *et al.*, 1994), *Aureobasidium pullulans* (Crabbe *et al.*, 1994) has been reported. The mechanism of PU biodegradation is well understood and hydrolytic enzymes particularly esterases are found to play a major role in PU degradation by

attacking the ester linkages (Evans and Levisohn, 1968; Flilip, 1978; Griffin, 1980; Hole, 1972).

Plastic degrading ability of the microorganisms are attributed to their hydrolytic enzymes producing ability particularly esterase, Lignin Peroxidase (LiP) and Manganese peroxidase (MnP) (Kounty *et al.*, 2006a; Liyoshi *et al.*, 1998). Hydrolytic enzyme producing ability of all the three isolates was investigated. All the three isolates showed high extracellular esterase activity in the respective culture broths both in the presence of HDPE or PU (Table 18). Relatively higher esterase activity was observed in PU culture broth compared to HDPE as per expectation because PU is a polyester. On the other hand, LiP and MnP activity was observed only in culture broths inoculated with these isolates containing HDPE films, almost no activity was observed in PU culture broths. Since all the three isolates showed potential to degrade HDPE and PU, their HDPE or PU degradation ability is attributed to their extracellular esterase producing ability. As it is well established that esterase play important role in the PU degradation (Allen *et al.*, 1999; Black and Howard, 1998; Evans and Levisohn, 1968). The efficient PU degradation by these isolates *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) in the present study is due to their high extracellular esterase activity.

However, unlike PU, esterase does not play major role in degradation of HDPE, but, it play critical role in HDPE degradation at initial stages by attacking the ester bonds of stabilizers and plasticizers such as phthalate esters, thus making them susceptible to microbial attack by reducing the HDPE surface hydrophobicity. (Feldman and Banu, 2005) Once the attack has been initiated, further role in biodegradation of HDPE is played by LiP and MnP by causing

oxidative breakage of the alkene bond. It has been reported in one of the earlier studies that the microorganism producing extracellular lignolytic enzymes may play an important role in the polyethylene degradation (Anthony *et al.*, 1992). This was further substantiate with the fact that some fungi and bacteria with ability to produce various peroxidases and other enzymes, as a consequence of their common action, were able to oxidize and break the structure of normally very recalcitrant insoluble high molecular lignin (Kirk *et al.*, 1984). Besides, lignolytic enzymes are produced in conditions of nutrient limitation (Cancel *et al.*, 1993) and thus their presence in a PE degrading culture further supports their role in HDPE degradation.

Conclusion

Thus it is concluded that three potential fungal isolates with ability to utilize HDPE or PU as carbon source were isolated and identified as *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052). All the three isolates were able to colonize and form a biofilm on HDPE surface in nutrient limiting medium. This is attributed to their high cell surface hydrophobicity or culture conditions which increases its adhesive properties, colonization and biofilm formation. Prolong survival of biofilm by these isolates even after 60 days in low carbon availability shows the efficiency of fungi in utilizing HDPE as carbon source. These isolates showed ability to degrade both inert synthetic high density polyethylene (HDPE) and polyurethane (PU) films in axenic culture conditions. The HDPE or PU degradation potential of these isolates are due to their efficient extracellular esterase, MnP and LiP producing ability. Though PU degradation was mainly due to esterases, while the HDPE degradation ability was attributed to both esterase and xylolytic enzymes (LiP and MnP) enzyme activities. This

is the first report showing the degradation of inert synthetic HDPE and PU by *A. flavus* (ITCC No. 6051), *A. fumigatus* (ITCC No. 6050) and *A. niger* (ITCC No. 6052) in axenic culture conditions. This supports the hypotheses that if right kind of microbes are selected, then so called inert synthetic polymer like HDPE can be degraded.

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