POLYETHYLENE BIODEGRADATION IN THE PRESENCE OF TITANIA (TiO_2) NANOPARTICLES

By

SALMA ALVI

(NUST-201362304-MSCEE65213F)

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

In

Environmental Science

Institute of Environmental Sciences and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

(2015)

POLYETHYLENE BIODEGRADATION IN THE PRESENCE OF TITANIA (TiO_2) NANOPARTICLES

By

SALMA ALVI

(NUST-201362304-MSCEE65213F)

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

In

Environmental Science

Institute of Environmental Sciences and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

(2015)

It is certified that the contents and forms of the thesis entitled

POLYETHYLENE BIODEGRADATION IN THE PRESENCE OF TITANIA (TiO_2) NANOPARTICLES

Submitted by

SALMA ALVI

Has been found satisfactory for the requirements of the degree of

Master of Science in Environmental Science

Supervisor: _____
Dr. Ishtiaq A. Qazi
Professor
IESE, SCEE, NUST

Member: _____
Dr. Muhammad Anwar Baig
Professor
IESE, SCEE, NUST

Member: _____
Dr. Muhammad Arshad
Associate Professor
IESE, SCEE, NUST

External Member: _____
Dr. Saadia Andaleeb
Assistant Professor
ASAB, NUST

Dedication

To my respected and loving parents,
Sajida Alvi & M. D. Alvi
For The reason of what I become today.
None of my success would be possible without their
amazing love, continuous support & care

ACKNOWLEDGEMENTS

This piece of work would not have been accomplished without the blessings, insight and power given to me by Allah Almighty.

I want to pay my utmost gratitude to my supervisor Dr. Ishtiaq A. Qazi (IESE) who provided his kind guidance and stood with me at every phase of my research work. I would also like to pay my sincere thanks to my Guidance and Examination Committee members Dr. Muhammad Anwar Baig (IESE), Dr. Muhammad Arshad (IESE) and Dr. Saadia Andaleeb (ASAB) who, through their kind guidance and encouragement made this research work possible. At the end, I wish to express my sense of gratefulness to my family members, friends and well-wishers who provided their moral support throughout my research work as well as tried to up lift my capabilities in this regard.

Salma Alvi

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

PE	Polyethylene
OD	Optical Density
CFU/ml	Colony Forming Unit per milli liter
EDS	Energy Dispersive Spectroscopy
FWHM	Full width of a diffraction line at half of maximum intensity
GPR	General Purpose Reagent
LI	Liquid Impregnation
SEM	Scanning Electron Microscopy
TiO_2	Titanium Dioxide
XRD	X - Ray Diffraction
λ	Wavelength
FTIR	Fourier Transform Infrared Spectroscopy
TNPs	Titania nanoparticles

LIST OF TABLES

LIST OF FIGURES

Chapter 2

Chapter 3

Chapter 4

ABSTRACT

Polyethylene (PE) is used for wide range of commercial purposes like carrying, storing and packaging in many ways. Because of its low degradation and continuous persistence in the environment, it poses long-term effects on all sectors of environment and public health as it is building up in to the environment in the form of dumps with passage of time. Biodegradation and degradation from nanoparticles both have proven to be useful for Polyethylene degradation in their own ways. This research collates the significance of nano-microbial interaction which can considerably influence the process of biodegradation. Soiled plastic samples were taken from four dumpsites of Islamabad and Rawalpindi and serial dilutions and streaking were performed to isolate dominant microbial strains. Further, identification process included gram staining, biochemical tests and gene sequencing. Using 16S rRNA analysis, potential Polyethylene degrading strain was identified as Pseudomonas aeruginosa. Concentration of titania nanopaticles (TNPs) for growth of microbes was optimized in nutrient broth media. Final experiment was performed in M9 media using polyethylene as carbon source with microbes and titania nanoparticles (TNPs). Results showed considerable enhancement in biodegradation of polyethylene in the presence of titania nanoparticles (TNPs). This study may lead to a better solution for PE deterioration in the form of nano-bioremediation in plastic sector as well as disposal sites.

Chapter 1

INTRODUCTION

Long chain monomers of ethylene constitute a polymer commonly known as Polyethylene. The worldwide consumption of Polyethylene is increasing at a rate of 12% on annual basis and global generation rate of polymer is about 140 million tons per annum. Polyethylene has lower tendency to degrade in the environment naturally so it has a potential to harm environment in different ways.

With continuous increasing production and slower natural degradation, this is leading towards a huge ecological issue. Polymer backbone which is mainly composed of carbon is utilized by microbes as energy source and process of Polyethylene degradation by bacteria is started by discharge of enzymes which convert polymer into monomer by breaking larger chains into smaller ones that can be easily metabolized by microbes Many bacterial strains have been identified which secrete enzymes, responsible for degradation of Polyethylene. Some of these microbes are (*Pseudomonas spp., Streptococcus spp., Staphylcoccus Micrococcus spp.* and *Moraxella spp.),* fungi (*Aspergillus niger, Aspergillus glaucus*), *Actinomycetes spp*. and *Saccharomonospora* genus. Among the microbes which are native to site of PE; *Pseudomonas spp., E.Coli , Klebsiella spp.* and *bacillus spp.* have been reported to be more efficient.

Titanium dioxide (TiO_2) is used as an efficient photo catalysts in many reactions. The competency of TiO_2 to degrade contaminants in many sectors of environment makes it a worthy applicant to practice for clean-up and purification. Though, most of its applications havebeen restricted to be performed under UV light because of its lights absorption limit which is lesser than 380 nm but many researches have been conducted to work in range of visible light (between 400-600 nm) (Pan *et al.*, 2006).

Degradation of PE by TiO_2 is one of the most promising approach as its higher degradation rate and lower cost (Deedar *et al*., 2009; He *et al.,* 2002; Hegde *et al.,* 2005). TiO_2 synthesized by different ways for its conversion into nanoparticles to increase its degradation ability. For this purpose, doping with different metals also performed to make degradation process more effective and faster in visible light (Ren *et al.,* 2007).

Nanoparticles like NanoBarium Titanate (NBT) assist the native microbial strains of Microbacterium species, Pseudomonas putida and Bacterium Te68R to degrade LDPE (Ahmann *et al.,* 2004).

In the present study, Polyethylene powder was prepared by dissolving beads into xylene. Microbial strains was isolated from PE waste soil by serial dilution method. TiO_2 nanoparticle concentrations were optimized for bacterial growth and 1% was found to be maximum concentration in which microbes can survive. Bacterial growth was monitored during experimentation using UV spectrophotometer by measuring optical density (OD), colony forming unit/ml (CFU/ml) and λmax. Final degraded powder was analyzed by Scanning electron microscopy technique (SEM) and Fourier transform infrared spectroscopy technique (FTIR).

1.1 Objectives

Main purpose of this study was to investigate the Polyethylene biodegradation in the presence of TiO_2 nanoparticles. More specifically, following were the objectives of this research:

- Isolation of indigenous microbes from PE waste soil and identification by biochemical and molecular characterization,

- Optimization of the TNPs doses for growth of PE degrading microbes to find out optimum concentration at which microbes can survive, and
- Examination of microbial degradation of PE in the presence of TNPs and final product characterization by SEM and FTIR.

Chapter 2

LITERATURE REVIEW

2.1 Polyethylene (PE)

2.1.1 Background

Polyethylene is the form of polymer which is commonly used for many years for applications like; packaging storing, carrying in all sectors of life. It is structured from ethylene monomers (CH_2=CH_2). Polyethylene was first time manufactured in 1934. Now a days, it is also known as low density polyethylene (LDPE) because of its tendency to float over surface of water and alcohol. Polyethylene is made up of loose and entangled strands of polymer so it is flexible in nature than other plastics (Mahalakshmi *et al.,* 2015). Due to its hydrophobic nature and lower molecular weight, it was fist time commercially used in World War II by allied troops to protect their tents and boots against water (Asghar *et al.,* 2010). Its production was exceeded that of steel by 1979 (Reazuddin *et al*., 2003).

Polyethylene is accumulating in environment because of its low degradability, mass manufacturing and highly persistent nature. Now it has become a major environmental and solid waste management issue as its demand and production is increasing with time (Kavitha *et al*., 2014).

2.1.2 Environmental impacts of PE

Annual production rate of Polyethylene is about 80 million tonnes globally (Piringer *et al.,* 2008). It is mostly used for packaging purposes so is rapidly discarded and piles up in the environment (Thompson *et al.,* 2009). Waste PE put massive burden on the natural environment, because its resistance to deterioration quickens the buildup in nature. Waste PE dumped in soil create obstruction to natural waterways under soil

surface and trigger agriculture toxicology. PE in the soil also interrupts the streaming of nutrients to the plant roots and disturb the natural penetration of sunlight. It also destroys the beneficial bacteria of soil and affects the soil fertility. It interferes with soil compaction, which in the long run, affects the construction of foundation of different civil structures. PE disposal in aquatic ecosystem causes death of marine animals by being trapped within PE debris or by ingesting it. When marine animals mistakenly ingest PE waste debris, it blocks their digestive tracts, harms stomach lining, reduces nurturing drives and leading to suffocation and subsequent death. It triggers ruinous effect on aquatic ecosystem by providing habitat for microbes (Usha *et al.,* 2011). PE bags also cause blockage of water pipes and drainage system and adding to formation of stagnant water ponds which breed flies and microbes and lead to spreading of harmful viral diseases (Bahri *et al.* 2007). Colored PE also considered as xenobiotic compounds as they are being used for wrapping food products and are not according to food-graded wraps. (Reazuddin *et al.,* 2003).

2.1.3 Conventional methods for PE degradation

PE usage and demand is increasing with time thus its waste accumulation in environment is unavoidable. Commonly, there are five methods which are in use from years to deal with PE: Landfill, Incineration, Thermal or catalytic degradation into fuel oil, Biodegradation and Photo catalysis (Sharma *et al*., 2003). All have their own innate limitation.

- **Burying in the landfill**

The primary downside of burying PE waste into a landfill is the wastage of useful land facility that could be better utilized by some other productive sector like farming (Zhang *et al.,* 2004). PE waste in landfill has been reported to persist for two

decades (Tansel *et al*., 2011). Mostly anaerobic conditions prevails in landfill which leads to lower degradation rate because absence of oxygen limits the existence of aerobic microbes for PE degradation (Massardier-Nageotte et al., 2006 ; Tollner *et al*., 2011). The reason behind slower degradation exhibited by many plastics is largely due to thermos-oxidative degradation (Andrady, 2011) and the unavailability of oxygen in landfills also further reduce degradation rates.

The remaining of PE waste in landfill contribute to many chemical reactions which leads to formation of secondary pollutants (Zhang *et al.,* 2004). Major pollutants comprise explosive organics, such as benzene, ethyl benzenes, xylenes, toluene, and trimethyl benzenes, discharged in the form of gases emissions and chemical leachate (Urase *et al.* 2008) and endocrine disturbing synthetic organic, like Bisphenol A (BPA) (Xu *et al.,* 2011; Svenson *et al.,* 2009). Along with its endocrine disabiling properties, BPA discharge from PE in landfill has also been reported to increase the generation of hydrogen sulphide by sulphate-reducing bacteria in soil populations. High concentrations of hydrogen sulphide are potentially harmful for surrounding environment (Tsuchida *et al.*, 2011).

- **Incineration**

Incineration, another conventional method which is usually used to get rid of PE waste is simply burning of it (Zhang *et al.,* 2004). Burning of PE waste is better than landfilling in the sense that it does not require much land to occupy for long time and it also has ability to recover energy in the form of heat (Sinha *et al.,* 2010). But, there is a significant drawback of PE incineration in that it leads to production of toxic pollutant which are mostly discharged into the atmosphere (Zhang *et al.,* 2004). When PE burns out it produces a large amount of CO_2 and oxygen based free radicals along with PCBs (Polychlorinated Biphenyls), PAHs (Polycyclic Aromatic Hydrocarbons),

and noteworthy amount of greenhouse gases (Astrup *et al.,* 2009; Khoo *et al*., 2010; Shen *et al*., 2010). The driving forces behind investigation of safer alternative disposal methods were major environmental issues of both landfilling and incineration.

- **Photo catalysis**

Photo catalysis, however, represents more promising and effective degradation approach for PE decomposition where a photo catalyst like TiO_2 is employed. It has been reported that composite films of PE with photo catalyst show more surface deterioration in open air under UV or solar exposure. Photo catalytic degradation methods for the disposal of plastics leave no toxic residue and is economically feasible as low heat process (Kim *et al.,* 2006; Zan *et al.,* 2004).

- **Microbial degradation**

Microbes are a useful resource for cleanup of PE pollution from environment because of diversity in metabolic pathways of microbes (Iranzo *et al.,* 2001). PCBs (Luigi et al., 2007) lead, arsenic, mercury and other heavy metals (De *et al.,* 2008; Takeuchi *et al.,* 2007) and Oil spills have been remediated by microbes (Rosa et al., 2006; Díaz et al., 2002; Hazen et al., 2010). Microbial degradation is competitive substitute to recent approaches for PE waste degradation because in competition to other approaches it does not produce harmful secondary pollutant and is economically cheaper (Takeuchi *et al*., 2007; Pieper *et al.,* 2000).

In some circumstances, end products generated by metabolic activity of microbes can be used as source of energy for another sector, for example, biogas from animal waste as biofuel. Complicated hydrocarbons and oily sludge poses practical limitations when remediated by microbes. Generally, microbes prefer nutrients in water medium but hydrocarbons and oils are incompatible to mix in water. Furthermore, hydrocarbons also lack many nutrients like potassium, phosphorus, nitrogen, which are

metabolically essential for microbes. (Rosa *et al.,* 2007). It has been reported that artificial addition of these nutrient in the form of fertilizers or broths can accelerate biodegradation process.

Complex hydrocarbons also poses threats to microbial life as they damage cell membrane by piling against phospholipid bilayer, yet many strains have been investigated which can survive with organic solvents like *P. aeruginosa, E. coli, Streptococcus* and different types of fungi (Pieper *et al*., 2000). Many researches have been carried out so far to investigate biological degradation of a wide range of polymers (Artham *et al.,* 2009; Kondratowicz *et al.,* 2009). Zheng and coworkers investigated that polymers with pure carbon structure carry limitations for many degradation approaches including biodegradation as it is safer but a slower process.

- **Thermo-catalytic conversion of PE into fuel oil**

Thermal/catalytic degradation of PE wastes, into other energy sources, has also been explored extensively. However, this approach is pricy as it works in high heating range and suitable catalysts which ensures the formation of required hydrocarbons. Problems such as coke formation, low-oil yield, poor-oil quality, and problems with the reuse of the catalyst, are also encountered with this technology (Ji et al., 2000; Dong et al., 2001).

2.2 Biodegradation of PE

Recently, the use of microbes for deterioration of PE waste has gained much attention because of the inability of other chemical and physical approaches to remediate as they pose many environmental complications. Microorganisms perform a substantial part in the biological decay of polymer (Shah *et al.,* 2008). Fungal species have also been investigated to secrete PE degrading enzymes (Shah *et al.,* 2008) and

multicellular plastics like as polysaccharides, which can assist to inhabit the plastic surface (Esmaeilia *et al.,* 2014) and the spreading and diffusion capability of the fungal hyphae is an additional benefit.

Biodegradation is an evergreen practice by which hydrocarbons are fragmented by living beings like microbes and fungus. Two major types of enzymes which take part actively in the degradation of Polyethylene are depolymerases which are either located within the cell wall or outside of the cell wall of bacteria. During degradation, extracellular enzymes breakdown larger polymer chains into smaller ones like dimer or monomer which can easily pass semi penetrable outer cell membranes, and then carbon and other nutrients are metabolically consumes and discharge end products such as CO_2 and H_2O.

PE biodegradation has been explored extensively over the last few decades (Albertsson *et al.,* 1987; Suresh *et al.,* 2011; Negi *et al.,* 2011) but these reports were mainly based on physically pre-treating of PE either thermally, under UV radiations or starch blended. Gilan *et al.* (2004) and Hadad *et al.* (2005) have been studied PE which was pre-degraded under UV radiation, and later introduced to *Rhodococcus ruber* and thermophilic bacteria *Brevibacillus parabrevis* at 37^{o}C. Sudhakar *et al*. (2008) and Harshavardhan and Jha (2013) have studied microbial strains isolated from marine environment and treated, thermally degraded and starch blended PE. Mahalakshmi *et al.* (2012) and Kyaw *et al.* (2012) have successfully worked on degradation unprocessed LDPE by *Pseudomonas* species.

2.2.1 Means of biological degradation

The biosphere is composed of many living agents like fungi, bacteria, mold which holds enzymes responsible for fragmentation of hydrocarbons complex structure.

They utilize carbon as energy source and convert raw complex structure into simpler one.

Polymers, composed of hydrocarbons are possible source of energy and carbon for heterotrophic microorganisms in metabolically different ways. The bacterial activities on polymeric compounds consist of two different modes:

1. Direct action: The deterioration of plastics which serve as a nutritive substance for the growth of the microorganisms

2. Indirect action: The influence of metabolic products of the microorganisms, e.g., discoloration or further deterioration (Mohan *et al.*, 2011)

Surface morphology of polymers disrupted by inhabiting sticky microbial colonies within hydrocarbon's (Pandey *et al.,* 2015).

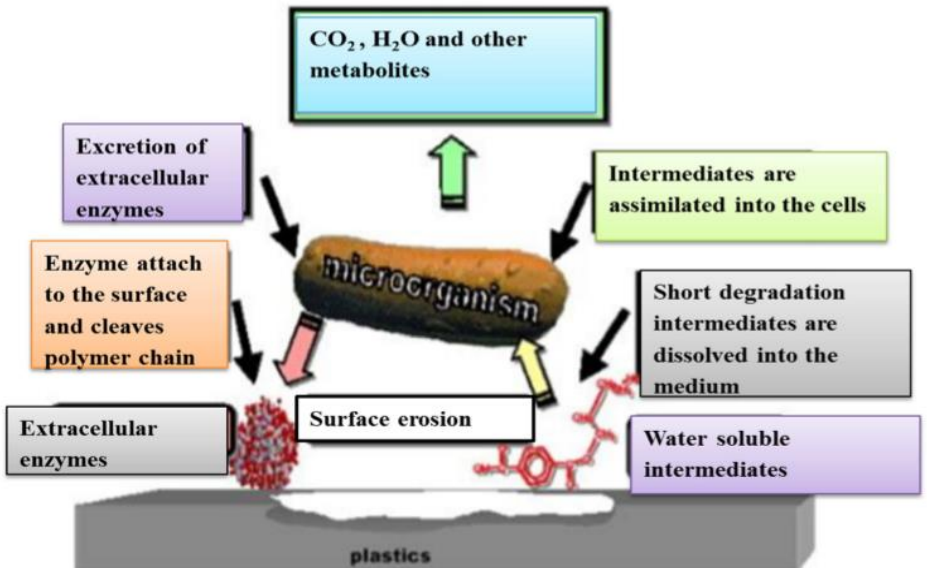

Figure 2.1. Mechanism of plastic biodegradation under aerobic conditions

2.3 TiO_2: As a Catalyst

2.3.1 Introduction

TiO_2 was first found, in the form of black soil, on the seashores of Cornwall, England in the 1790s. William Gregor, a Parish priest, explored that this black material can be attracted toward a magnet rather than silica. He first, mixed this black soil with HCl (hydrochloric acid) and then treated with the Fe_2O_3 (iron oxide), ended up into a white deposits which further introduced to H_2SO_4 (sulphuric acid). After calcination, on mixing with soda, final product was white powder, named as TiO_2 (titanium dioxide) (Reck *et al.,* 1999).

Indeed, William Gregor invented a formula to produce TiO_2 which was later extensively used for commercial purposes for up to 1960's. These days, two methods are in use for making TiO_2; either by using sulfate or chlorine. In the first one, a mineral named ilmenite, was used to convert into iron and titanium sulfates by treating with H_2SO_4 (sulfuric acid). Titanium hydroxide is synthesized by following methods of hydrolysis, filtration and calcination using high heat. In the chlorine method, crystals of seeds, made by basic hydrolysis, put on the reaction with chlorine to make titanium tetrachloride, to get pure TiO_2, titanium tetrachloride will undergo purification and re-oxidization (Reck *et al*., 1999).

TiO_2 has gained a lot of attraction essentially because of its low cost, non-toxic and chemically stable nature. Its major sectors of utilization are as white colorant in paints (51%), plastics (19%) and paper (17%) of total generation (Carp *et al.,* 2004).

2.3.2 TiO_2 Mineral forms

TiO_2 is usually found in three mineral shapes which are; Rutile, Anatase, and Brookite (Fig.2.2) (Puma *et al*., 2008). Anatase form of TiO_2 has a crystal-like structure that tallies to the tetragonal structure (with dipyramidal pattern) and mostly used as a

catalyst under UV radiation. Rutile form of TiO_2, with prismatic pattern, also exhibit a tetragonal crystalline arrangement. This form is mostly used in field of paints as white colorant. Brookite form of TiO_2 carries an orthorhombic crystal pattern. TiO_2, thus acquires multipurpose qualities which can be used a number of products like paint colorant, solar cells, sunscreen (SPF) creams, electrochemical electrodes, capacitors, and at the same time as a food dyeing pigment and in toothpastes (Meacock *et al.*, 1997). Generally, TiO_2 is mostly used in Anatase form due to its high photocatalytic activity in many reactions, meanwhile it has a more potential to conduct negative band energy (higher potential energy of photo forming electrons), non-toxicity, stable, high particular zone, and pretty low cost (Macwan *et al.*, 2011).

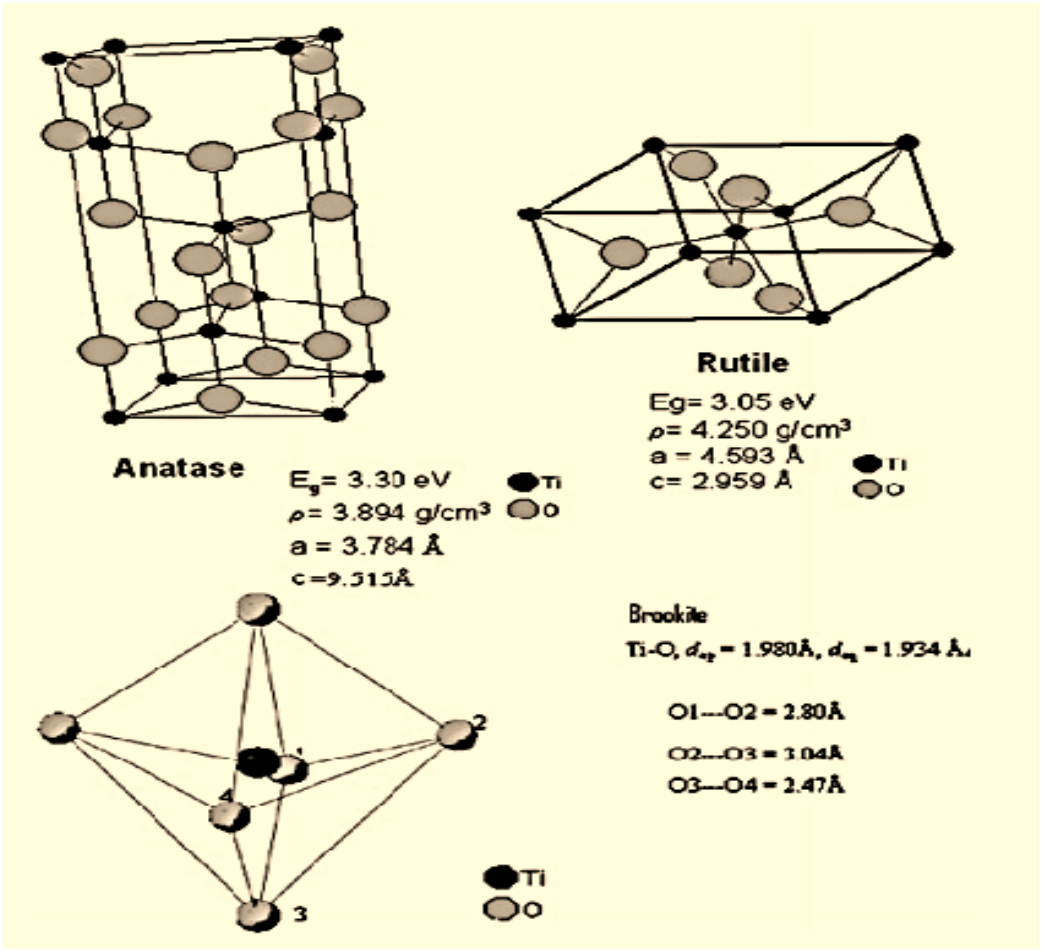

Figure 2.2. Crystalline patterns of (a) Anatase (b) Rutile and (c) Brookite

It has been cited that only Anatase and Rutile are generally utilized as photo catalysts, from the three forms. Though, it is attention-grabbing to notice that there exist conflicting reports regarding the photo catalytic ability for these two forms but normally, it has also been accepted generally that the anatase form (Carp *et al*., 2004; Beydoun *et al.,* 1999) has greater photo catalytic capability than rutile. It has also been reported that the rutile form has better photo catalytic capability (Neppolian *et al.,* 2002; Reck *et al.,* 1999) and even some reports claim that a combination of anatase (75-70%) and rutile (30–25%) attains more potential than pure ones (Sun *et al*., 2008; Giolli *et al*., 2007). The band gap value (Wunderlich, *et al.,* 2004) of the anatase structure is 3.3 eV, of rutile form is 3.05 eV and of brookite type is 2.96 eV. Thus, radiation of wavelength (λ) up to 380 nm, will stimulate an electron to jump from valence band to conduction band, producing an electron-hole (e–/h+) pair in case of anatase making it more effective for general applications.

2.3.3 Synthesis of TiO_2 nanoparticles

Titanium dioxide (TiO_2) nanoparticles is a most beneficial material, can be extensively utilized in many application because of its high photo catalytic ability (Allen *et al.,* 2008), excellent gas-sensitive properties (Chen *et al.,* 2007), dielectric properties (Cao *et al.,* 1995), chemical stability, cheap cost and non-toxic nature. Chemically stable and unique optical nature of titanium dioxide makes it more favorable for splitting of water (Sugimoto *et al.,* 2003) and in the photo-oxidation (Rao *et al.,* 1980) processes. This material in the nano range, shows a wide range of UV absorption, an attractive approach that has been successfully applied in sunscreens products. TiO_2 nanostructures can be synthesized by Hydrothermal, Sono-chemical, Microwave, Electro deposition, Chemical Vapor deposition, direct oxidation, Sol-gel, Solvo-thermal (Byranvand *et al.,* 2013) and Liquid impregnation method.

Liquid Impregnation (LI) method involves the mixing of TiO_2 in water,followed by the slow addition of a dopant metal solution. Only stirring is required during and after the addition process. Liquid impregnation method is a low cost technique, shows very good reproducibility and requires simple apparatus to synthesize TiO_2 (Behnajadyet al., 2008).

2.4. Nanoparticles as PE biodegradation enhancers

It has been reported that effect of Super Paramagnetic Iron Oxide Nanoparticles (SPION) in different sizes on the growing pattern of bacterial strains responsible for Low-Density Polyethylene (LDPE) degradation including *Microbacterium spp*., *Pseudomonas putida* and *Bacterium Te68R* whose growth was examined in M9 broth (Davis without iron and dextrose). These nanoparticles improve log phase span to 36 h thus accelerating the bacterial growth. Furthermore, the additional influence of sonication and shifting in lag-period was also reported on growth patterns. SPION with nano size of 10.6 nm were documented to considerably uplift the microbial degradation process efficacy of bacterial strains as analyzed by λ-max changes, Fourier transform infrared spectroscopy (FT-IR) and simultaneous thermogravimetric-differential thermogravimetry-differential thermal analysis (TG-DTG-DTA). It highlights the worth of bacteria and nanoparticle collaborations which can radically effect basic metabolic pathways like microbial degradation (Kapri *et al.,* 2010).

To influence the growth cycle of LDPE degrading bacterial consortia Nanobarium titanate (NBT) was supplemented in minimal broth. It influenced lag phase, exponential phase and stationary phase and act by reducing the duration of lag phase and increasing the duration of exponential and stationary phase. For the accelerated growth of bacterial consortia NBT was a supportive nutritional component and thus assist the consortia in

plastic waste biodegradation. The preferred particle size is 38nm with bacterial consortia of *Microbacterium* species strain MK3, *Pseudomonas Putida* strain MK4 and *Bacterium* Te68R strain PN12 (Kapri *et al.,* 2009).

Fullerene-60 used at 0.01% (w/v) concentration due to the fact that they are detrimental to bacterial growth with increasing concentrations from 0.25 to 1%. LDPE used with Fullerene 60 at a concentration of 5 mg/ml. The polymeric structure got changed making a shift in λmax was observed from 209 nm, constant for 2 days, to 220 nm, after 3 days and to 223 nm after 4 days, in absence of Fullerene 60 nanoparticles. Whereas, in the samples with fullerene-60 λmax was found to shift from 209-224.97 nm on 1st day itself, showing an improved rate of degradation (Sah *et al.,* 2010).

Chapter 3

MATERIALS AND METHODS

Biodegradation studies were carried out in lab scale orbital shaker placed in virology lab of Atta-Ur-Rahman School of Applied Biosciences, National University of Science and Technology.

3.1 Materials and chemicals

Polyethylene beads manufactured by QAPCO Petrochemical Corporation, Qatar were purchased from the local market. Titanium Dioxide (GPR, BDH Chemicals Ltd. England) and Ethanol (C_2H_5OH) (BDH AnalaR, England) were used for the synthesis of pure TiO_2 nanoparticles. Solidified agar petri plates were prepared with the help of Nutrient Agar (Merck, VM 100650 943). Xylene (AR grade, Sigma-Aldrich, USA) to dissolve PE beads was purchased from local market.. Pyrex glassware and double distilled water was use in all experiments.

3.2 Composition of the media

Sterilized glassware and distilled water were used in each experiment. All the objects were autoclaved at 121^oC for 20 min to make sure the sterilized conditions. Mineral Salt media composition mentioned in Table 3.1 and it was prepared to use in biodegradation studies.

Table 3.1. Composition of mineral salt media

S.No.	Component	Quantity (g/L)
1	Potassium Phosphate Dibasic (K_2HPO_4)	1.5
2	Ammonium Nitrate (NH_4NO_3)	1.5
3	Potassium Monobasic Phosphate (KH_2PO_4)	0.5
4	Sodium Chloride (NaCl)	0.5
5	Magnesium Sulphate Heptahydrate ($MgSO_4.7H_2O$)	0.2

3.3 Methods

3.3.1 Preparation of Polyethylene powder

PE beads were converted into powdered form by boiling with xylene at 120^{o}C and 300 rpm and to get moisture free powder, solvent kept to evaporate at room temperature (28-30^{o}C) for three days. The powdered PE was consecutively washed by 70% ethanol to remove xylene residues from powder, subsequently dried, and used as key carbon source for biodegradation process. Furthermore, the powder was dried in oven at 40-50°C for 24 hours. The above procedure was done so that the surface area of PE can be increased enough for better biodegradation (Sohni *et al.*, 2009, Sah *et al.*, 2010).

3.3.2 Synthesis of Titania nanoparticles (TNPs)

For TNPs use in degradation studies, Liquid Impregnation (LI) method was used in IESE laboratory to synthesized TNPs. Specifically, a mixture of 6 g TiO_2 (general purpose reagent, purity>99%), 240 mL distilled water and 12 mL absolute ethanol was prepared and placed on a magnetic stirrer to get homogeneous solution at 325 rpm by stirring vigorously (model: Stuart SB162). After 48 hours of stirring, the sonication of solution was done at room temperature (28-30^{o}C) for 40 minutes. Sonication was performed using JAC Ultrasonic 1505, JINWOO.

After complete precipitation, the slurry was kept overnight to settle down. The pH of TNPs was found to be 6.44 (acidic). The final solution was allowed to dry in hot air oven at 105ºC for 12 hours. Finally, the dried material was grounded in mortar pestle and then calcined in muffle furnace (NEY M-525 series II) at 500ºC for 5 hours (Khan *et al.,* 2013).

3.4 Characterization of Titania nanoparticles (TNPs)

- **X ray diffraction (XRD) analysis**

The crystal structure of powdered TNPs was characterized by X-ray diffraction spectroscopy (XRD) technique and the average crystallite size and lattice strain was determined by Scherrer's calculator using X'Pert Highscore (plus) software. The XRD pattern of TiO_2 was attained under CuKα radiation with wavelength of 0.1540 nm in the position 2Θ, and scanned in the range of 20^o-80^o with a fixed step of 0.5^o using STOE, Scintag Theta-Theta X-ray Diffractometer system (Rafiq *et al*., 2014.

- **Scanning electron microscopy (SEM)**

The surface features of TNPs were analyzed by SEM (JSM-6490A, JEOL) with 3nm resolution at 20 kV. Before micro scoping, the grounded TNPs were diluted in distilled water and then homogeneous solution was prepared through sonication for 30 minutes. Then, diluted solution of 10 µL was allowed to settle on a carbon counterfoil and air dried. The moisture free powder was splutter covered with gold to increase surface conductivity. Atomic Ion Sputtering Device was use to layer TNPs, JEOL, JFC-1500, Gold $250A^o$ (Vernon-Parry, 2000).

- **Electron dispersion microscopy (EDS)**

Energy dispersive X-ray spectroscopy (EDX) measurements coupled with SEM and used for determination of elemental composition. It operates in device known as

compact detector unit (DCU) assimilated in SEM and helps to find out quantitative as well as semi-quantitative measurements. The EDX spectrum was graphed at an increasing energy of 20 kV and work for 50s. Energy emissions from chemical element were graphed in Pseudo-colors to exhibit two dimensional spatial variations in sample powder (Hollerith *et al.*, 2004).

3.5 Samples collection from plastic waste disposal sites

Six samples were collected from 3 waste disposal sites (two from each site) in the premises of Islamabad and Rawalpindi containing small pieces of Polyethylene bags. Samples were collected successfully from depth of 5-8 cm using spatula, packed in air tight polybags and brought to laboratory.

3.6 Moisture content (%) and pH measurement of waste soil samples

After moving back to lab, Fresh samples were placed on filter paper and the initially weighed. Subsequently 10 g of air dried soil was taken in petri plate and dried overnight in oven at 105°C with loosely fit glass cover and then allowed to cool in desiccator for half an hour. The weight of samples was again recorded a number of times until a constantly same weight was attained (Olsen *et al.,* 1954). Moisture of soil samples was calculated by applying the following formula obtained from AWPA (1986).

$$\mathrm{MC}\ (\%) = \frac{\mathrm{W} - \mathrm{w}}{\mathrm{w}} \times 100$$

Where, MC is moisture present in soil sample, W is the initial weight right after sampling and w is that persistent same weight coming after continuous oven drying. pH

was calculated by using pH meter by Electrometric approach using assembled glass electrode (Kousar *et al.,* 1990; Saha *et al.,* 2014).

3.7 Isolation and identification of dominant microbial culture

A composite sample was prepared by mixing all collected samples. Microbial isolation from collected soil samples was done using serial dilution, spreading and streaking method in which soil samples were diluted from 10^1 to 10^7 dilutions. Spreading of diluted soil sample on sterilized agar plates was done and inoculated plates were incubated at 37^{o}C for 24 hours. Incubation resulted in mixed culture on plates. After 24 hours, dominant colony was successively streaked on sterilized nutrient agar plates and further identification was done by morphological, biochemical and molecular characterization.

3.7.1 Morphological characterization

- **Colony morphology**

Observation of the single colony plays a vital role in explanation and identification of unknown microbial strain. The single colony was selected and picked gh wire loop and streaked on fresh nutrient agar plate repetitively until the pure culture was achieved. Microbial colony was examined, by naked eye as well as under microscope, at each step of purification to check color, margin, shape, size, elevation (height), texture, and secretions.

- **Cell morphology**

Bacterial culture was mixed into a drop of distilled water on a clean glass slide by gentle stirring with wire loop to prepare a smear. The slide was air dried and then heat-fixed by passing over flame thrice. After solidifying, smear was introduced to four stains

- The first stain; crystal violet was sprayed over the smear for about one minute and rinsed with distilled water. The gram-negative bacterial cell wall is composed of a thin layer of about 10% of peptidoglycan which allows crystal violet to rinse away when successively splashed with ethanol (Roberto *et al.,* 1993).
- Then the iodine solution was applied for one minute followed by washing with distilled water.
- After this, the slide was engulfed with decolorizing solution for about twenty seconds. After treating with decolorizing solution, the glass slide was air-dried and
- Finally, safranin was applied on the smear for 40-45 seconds. The gram- negative cell wall of anonymous strain took Safranin and appeared pink.

- **Motility test**

Two techniques are generally used for motility test; one is hanging drop and second is agar stabbing method. Hanging drop approach was used to determine whether strain is motile or non-motile. A drop of autoclaved distilled water was placed on the clean cover slip, and fresh microbial suspension was prepared by using sterile toothpick. Then the cover slip was carefully turned over the cavity and the drop hanged from the cover slip into the centrally hollowed glass slide. Successively, the slide was examined under the microscope first at 50X and then at 100X to visualize the motility of bacteria.

3.7.2 Biochemical characterization

Biochemical description is a vital stage in identification of isolated unknown bacterial strain through different reactions performed by bacterial strain during incubation. In the current study, API 20E (Analytical Profile Index 20 Enterobacteria) kits were used for biochemical identification of microbe. The API 20E kits contain twenty microtubes having dry reagents. The microbial culture was inoculated in the

cupules and change in colors indicates the positive or negative reaction. Microbial suspensions were prepared in sterilized saline solution (0.85%). Micropipettor was used to fill the microtubes with the microbial suspension. Mineral oil was added on the microtubes labeled as LDC (Lysine decarboxylase), ADH (Arginine dihydrolase), H_2S (Hydrogen sulphide production), ODC (Ornithinine decarboxylase) and URE (Urease) in order to create the anaerobic conditions to check ability of isolated microbes to use material as the source of carbon and energy or not in the absence of oxygen. After this process, the strips were incubated at 37° C for 24 hours and examined visually.

As per manufacturer's instructions, reaction was completed by addition of further reagents for some tests (indole, tryptophan deaminase, nitrite production, and voges proskauer) before recording the final result. For the nitrogen production, zinc dust (2-3 mg) was added in the same microtube of GLU and examined after five minutes. The changed color of microtube i.e. yellow indicated positive result in this case. In contrast, if tube turned orange-red, it is an indication of negative result (Reynolds, 2009). API 20E strip is shown in Figure 3.1. Pure culture was also streaked on cetrimide agar to confirm identification of pseudomonas aeruginosa.

Figure 3.1 API 20E strip for bacterial identification

- **.Catalase test**

Catalase enzyme is secreted by aerobic microbes or many of facultative anaerobes. In this reaction, catalase enzymes simply break down H_2O_2 (hydrogen peroxide) into H_2O (water) and O_2 (oxygen).

$$2H_2O_2 + \text{Catalase} \longrightarrow H_2O + O_2$$

Microbial strain was streaked on fresh agar plate and kept in incubator for 24 hours. A single colony was picked using wire loop and transferred on glass slide. Then, one drop of prepared 3% H_2O_2 solution was smeared on slide. Prompt bubble formation indicated test is catalase positive while if there were no bubble forms then test would be catalase negative.

- **Oxidase test**

Aerobic microbes generally have cytochrome c oxidase and cytochrome c. For this test, an artificial electron acceptor holding chemical, N, N, N', N'-tetramethyl-p-phenylenediamine I (TMPD) was prepared. Redox dependent this electron acceptor converts itself into some pigmented product i.e. purple or dark blue.

For this purpose, Filter paper was first fragmented and autoclaved. Autoclaved strips of filter paper strips were dipped in the TMPD solution and air dried. Inoculating loop holding sufficient bacterial culture was scrubbed smoothly on paper strips. A change in color to blue indicated that isolated strain is able of oxidase production while there were no change in color then it would be oxidase negative.

3.7.3 Molecular characterization

- **16s rRNA gene sequencing**

Isolated bacteria were further preserved and fresh bacterial inoculum was prepared for gene sequencing. Inoculum was successively poured into autoclaved Eppendorf. Eppendorf was centrifuged at 2000 rpm for 10 minutes to allow bacterial culture to make a thin separate film and supernatant was discarded afterwards. 1 ml of 50% glycerol and 3 ml of 30% nutrient broth was poured in Eppendorf and refrigerated at -20ºC. For further 16S rRNA sequencing the preserved isolate was sent to Genome Analysis Department Macrogen Inc. Korea.

Once identified sequence was processed through BLAST nucleotide search from databases of National Center for Biotechnology Information (NCBI). After proper detection, sequence was further submitted to gene band for accession number.

3.8 Optimization of microbial tolerance for titania nanoparticles (TNPs)

Pure culture was revived on agar plates to get fresh culture after incubation. The maximum concentration of titania nanoparticles tolerated by pure culture was tested by inoculating pure culture (300µl) into 250-ml Erlenmeyer flasks placed in orbital shaker at optimum pH of (7.0±0.4) and temperature (37^{o}C) and constant shaking (160 rpm). Different concentrations of titania nanoparticles were added with increasing trend from 0.05%, 0.1%, 0.25%, 0.5%, 1% and 1.5% respectively. After the regular interval of 24 hours, samples were collected to measure optical density (OD) at 600 nm and colony forming unit per milliliter (CFU/ml) and mean generation time (t_{gen}) of microbial colonies was calculated using following formula (VLab, 2011);

$$\mathrm{k} = \frac{\log 10[\mathrm{Xt}] - \log 10[\mathrm{Xo}]}{0.301 \times \mathrm{t}} = \frac{\mathrm{gen}}{\mathrm{hr}}$$

Where, X_t = higher CFU/ml value, X_o = lower CFU/ml value

$$t_{gen} = \frac{1}{\mathrm{k}} = \frac{\mathrm{min}}{\mathrm{gen}}$$

3.9 Comparative Polyethylene biodegradation analysis with titania nanoparticles (TNPs)

For the degradation experiment, 200 ml of mineral salt media (pH 7.0±0.04) was added to 250-mL Erlenmeyer flasks holding PE powder at a concentration of 20

mg/ml. 600 µl of active inoculum was introduced to flasks. The experiment got started with controls lacking TNPs and containing 1% of titania nanoparticles, respectively. The flasks were kept in incubator at 37ºC with constant shaking (150 rpm). The trends were monitored for bacterial growth by measuring the OD at 600 nm after regular intervals of 1 day. The λ max was also determined for monitoring changes in the broth due to polymer dissolution. Degraded products were recovered from the media after 5^{th} day of incubation.

3.10 Retrieval of degraded powder

Degraded powder samples were collected from the media after passing through filter paper and following filtrate evaporation. The remaining left was gathered, and centrifuged at 2000 rpm for 40 min to get rid of microbial biomass. The supernatant was placed in an oven to dry at 60ºC for 24 hours to remove moisture and the lasting dry sample was collected and examined by SEM and FTIR, taking pure PE powder as the control.

- **SEM Analysis**

Scanning Electron Microscope was used to provide images of high resolution. The focused beam of electrons produces images which provide us information like appearance, form, and size, elemental composition of polyethylene powder before and after degradation.

- **Fourier Transform Infrared (FT-IR) Spectroscopy**

The degraded samples recovered, from media when the experiment was completed, were examined by FTIR spectra and different peaks relative to CH_2 deformation, formation, bending, and stretching and carbonyl bond were compared

with reference (control). Perkin Elmer FTIR Spectrophotometer was used to document FTIR spectra in potassium bromide (KBr). Following formula was used for CI calculation (Salem, 2001; Roy *et al.*, 2007);

$$\text{Carbonyl Index (C. I.)} = \frac{A_{1720}}{A_{1465}}$$

Chapter 4

RESULTS AND DISCUSSION

4.1 Characterization of TNPs

4.1.1 X-ray diffraction (XRD) analysis of TNPs

Objects are attributed to nano size when their dimensions lie within range of 100 nm. XRD determines the crystalline phase of the nanoparticles by analyzing diffraction of X-rays. Scherer's formula was used to determine average crystallite size of TNPs (Younas *et al.*, 2011).

$$L = \frac{k\lambda}{\beta \cos\theta}$$

Where,

L = Average particle size

k = 0.891, a shape factor of spherical particles

λ = 0.1542, wavelength of X-Rays

β = Full width of a diffraction line at half of maximum intensity (FWHM)

θ = Diffraction angle of crystal phase

Crystallite size was calculated as 42.51 and lattice strain 0.0039. Peaks of XRD results reveal that nanoparticles have crystalline structure (see Figure 4.1). Strong diffraction peaks at 25.271^{o} and 47.980^{o} confirm that synthesized TNPs are in anatase phase using range of diffraction angle 20^{o}-80^{o} (Ba-Abbad *et al.*, 2012). Sample was compared with card no. JCPDS 01-089-4921. The crystal structure was found to be tetragonal which was in agreement of that reported in literature. Peaks obtained at

25.271 º, 37.013, 37.847, 38.644, 48.145, 53.974, 55.186, 62.812, 68.879 and 75.203 were of (101), (103), (004), (112), (200), (105), (211), (204) and (215) planes respectively.

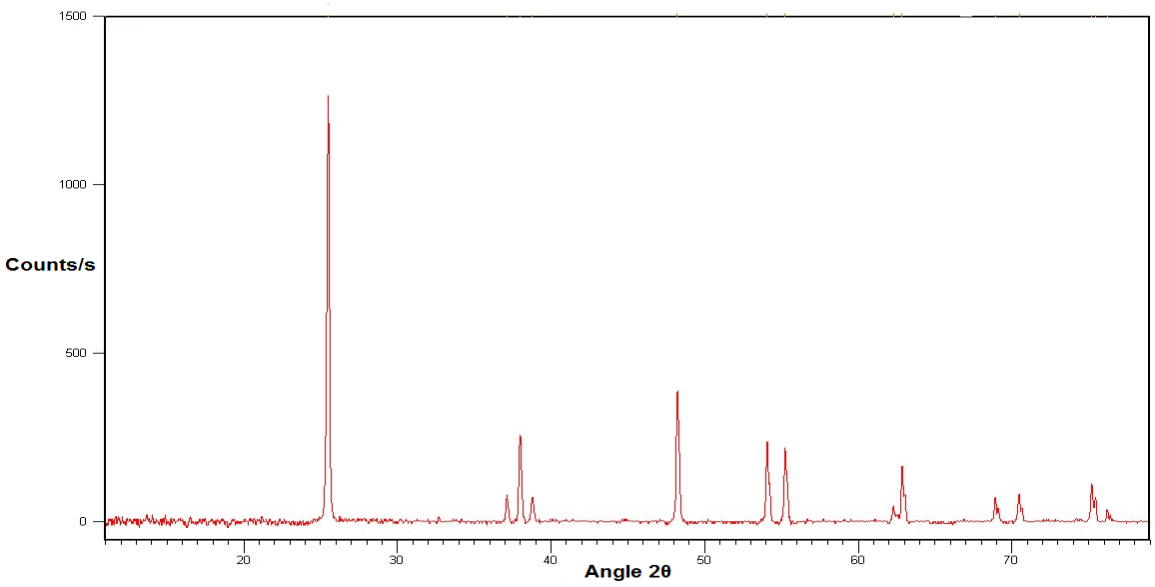

Figure 4.1 XRD Pattern of pure TNPs

Table 4.1 X-ray Diffractometer Results of Pure TNPs

S. No.	Peak Angle	Spacing	CPS	FWHM
1	25.1	3.52	3867	0.2
2	37.7	2.38	838	0.3
3	47.95	1.89	1037	0.3
4	55	1.67	617	0.35

4.1.2 Scanning electron microscopic (SEM) images

The surface morphology of TNPs was observed by JEOL JSM-6460 SEM at 20,000 magnification. The image of the pure titania shows that particles are spherical in shape and distributed in the range of 50.00-72.11 nm (Fig. 4.2). Image of undoped

TNPs (Figure 4.2) affirmed the existence of a pervious and spongy structure of great gruffness and complexity. This type of structure confirms the high surface area which has been reported to be very effective for the catalytic degradation processes (Gaya *et al.*, 2008).

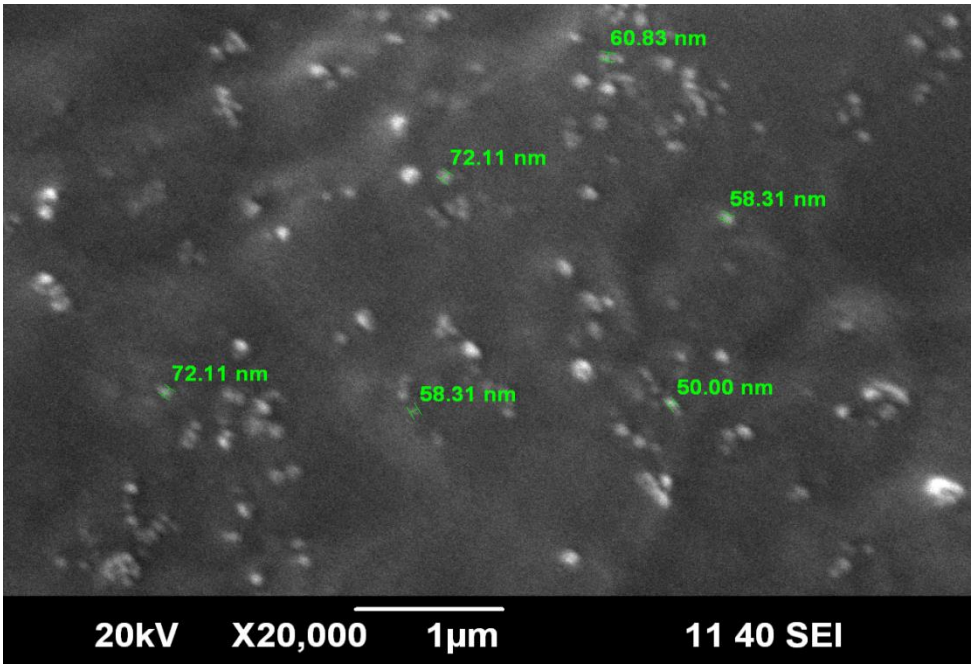

Figure 4.2 SEM image of pure TNPs at X20,000

4.1.3 Energy dispersive spectroscopy (EDS)

EDS examined elemental composition of pure TiO_2 nanoparticles. Figure 4.3 and Table 4.2 shows the presence of Ti and O elements in the representative sample i-e about 57.06% Ti and 42.04% O in TNPs indicating the sample to be pure.

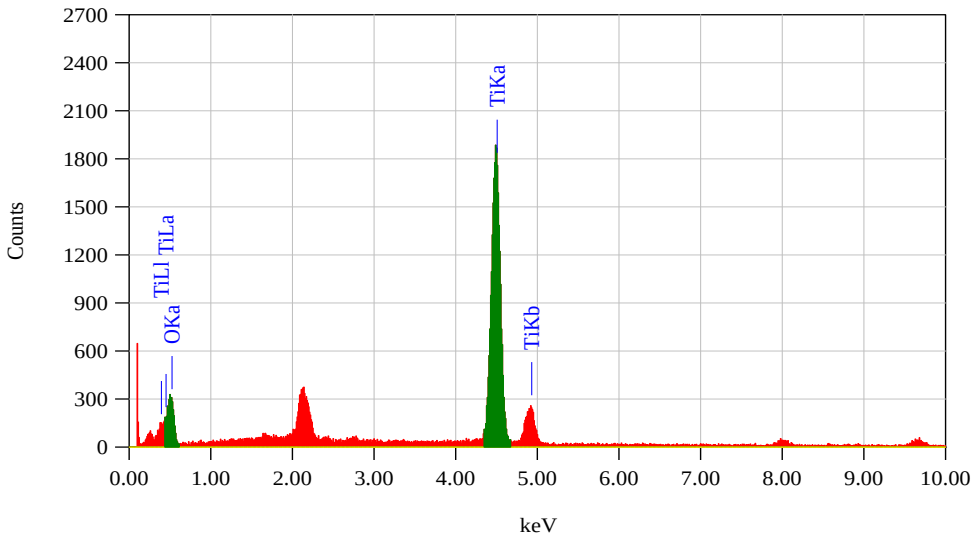

Figure 4.3 EDS analysis of TNPs

It changes from point to point indicating diverse composition of the synthesized TNPs that support the SEM results. It also indicates that nanoparticles contained oxygen and titanium only while no alien element or impurity was introduced during the synthesis process.

Table 4.1 EDS Results of TNPs

Material	Ti		O	
	Expected	Found	Expected	Found
TNP (Pure)	60	57.06	40	42.94

4.2 Physical characteristics of waste soil

A broad range of moisture can allow microbes to survive. In this study, the moisture content in collected waste soil samples were found between ranges of 61.24-67.77%. Microbial growth closely associated to moisture content of native soil. The

maximum microbial biomass has been determined in soils of relatively high moisture percentage and the favorable environment for aerobes survival mostly is a 50-75% of the soil moisture carrying capability (Alexander, 1977).

In this study the pH of soil samples were optimized for culturing bacterial strains. Before growing microbes in artificial media, measurement of pH is a main factor to provide microbes a survivable environment. For present study, pH was acclimatized in collected waste soil samples. Four samples at pH of 6.9. 7.2, 7.4 and 7.6 were taken as suitable for the optimal growth of the microbial strains. Bacteria can bear pH from 4 to 10 during soil reactions, but the recorded suitable pH for the common microbes is slide between basic and neutral (Berkeley et al. 1972).

4.3 Identification of isolated strain

For identification of isolated strain, results were conducted from morphological, biochemical characterization and gene identification as well.

4.3.1 Morphological characterization

Isolated bacterial strain was studied for form, color, opacity, elevation, margin and surface. The isolated strain was assigned name as SA1 in Tables 4.3 and 4.4 representing colony and cell morphology of isolated bacteria respectively.

Table 4.3 Colony and cell morphology of bacteria isolated from waste soil

Bacterial isolate	**Visual color**	**Form**	**Size (mm)**	**Elevation**	**Texture**	**Margins**
SA1	Green	Circular	2	Convex	Pasty	Entire smooth
	Arrangements	**Shape**	**Motility**		**Gram reaction**	
	Pairs	Bacilli	Very fast		Negative	

The strain SA1 turned out to be gram negative when observed under microscope after gram staining. SA1 was bacilli, appeared in pairs and was motile when observed under microscope at 100X.

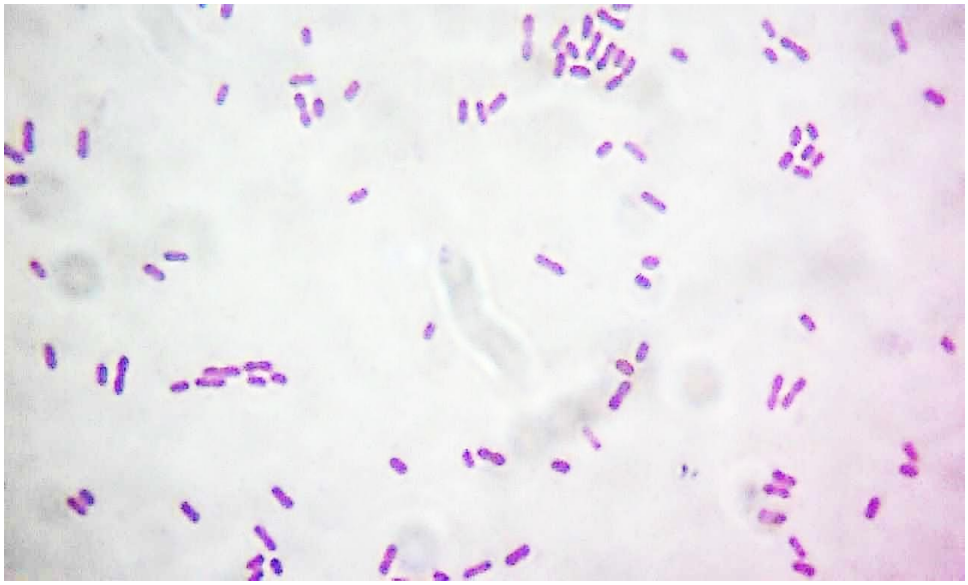

Fig. 4.4 Image of isolated strain SA1 under microscope at 100X

4.3.2 Biochemical characterization

Biochemical characterization was performed using API20E and results were determined through analyzing the codes in API 20E software. The biochemical Characterization of SA1 is listed in Table 4.4.

Table 4.4 Biochemical characterization results of isolated strain SA1

S.No.	*API tests*	*SA1 response*
1	Ortho-Nitrophenyl-βgalactoside (ONPG)	Negative
2	Arginine Dihydrolase	Positive
3	Lysine Decarboxylase	Positive
4	Ornithine Decarboxylase	Positive
5	Citrate utilization	Positive
6	H_2S production	Negative
7	Urease	Positive
8	Tryptophan Deaminase	Positive
9	Indole production	Positive
10	Acetoin production (Voges Proskauer)	Negative
11	Gelatinase	Positive
12	Oxidation/fermentation of Glucose	Positive
13	Oxidation/fermentation of Mannitol	Negative
14	Oxidation/fermentation of Inositol	Negative
15	Oxidation/fermentation of Sorbitol	Negative
16	Oxidation/fermentation of Rhamnose	Negative
17	Oxidation/fermentation of Sucrose	Negative
18	Oxidation/fermentation of Melibiose	Negative
19	Oxidation/fermentation of Amygdalin	Negative
20	Oxidation/fermentation of Arabinose	Positive
21	Nitrate Reduction	Negative
22	Nitrogen Production	Negative

4.3.3 Catalase and oxidase tests

Selected bacterial isolates were capable of catalase production. The results are mentioned in Table 4.5 along with identification of SA1 through API 20E. The biochemical Characterization indicated the SA1 as Pseudomonas aeruginosa. Selected bacterial isolate was capable of catalase and oxidase production.

Results of present study coincide with numerous studies where indigenous polyethylene degrading bacterial strains were isolated from plastic waste dumpsite. Gilan and his co-workers (2004) isolated *Rhodococcus ruber* (C208) from soil to degrade polyethylene and reported that indegenous strain utilized polyethylene films as sole carbon source. Usha and co-workers (2011) also found microbes as PE biodegraders and associated microbes recognized as *Pseudomonas sp, Staphylococcus sp, Aspergillus flavus, Aspergillus nidulans, Bacillus sp.* and *Streptomyces sp.*

Prosun and Alok (2012) reported that polyethylene breakdown by *Pseudomonas sp.* AKS2 is quite fast as it can degrade about 5 % of the initial material in 45 days deprived of preceding oxidation.

4.3.4 Gene sequencing of SA1 strain

SA1 Gene sequencing of SA1 was performed at Genome Analysis Department Macrogen Inc. Korea. The strains were screened and noise was removed manually. Strain was identified through BLAST search available at National Center for Biotechnology Information (NCBI) databases revealing up to 100% similarity to *Pseudomonas aeruginosa*. Furthermore, nucleotide sequence was assigned accession number as KU198667.

4.4 Optimization of microbial tolerance for Titania nanoparticles (TNPs)

4.4.1 Microbial growth profiling concerning of Optical density (OD)

Determination of the maximum concentration level for TNPs that could allow the growth of microbes was required in order to study its effect upon Polyethylene biodegradation. Of the six concentrations 0.05%, 0.1%, 0.25%, 0.5%, 1% and 1.5% (w/v) taken to perform assay, microbial culture exhibited a progressive decrease in

bacterial OD (at 600 nm) with the addition of nanoparticles at increasing concentrations (Fig. 4.5).

This increase became detrimental in maximum concentration of 1.5%. However, the concentration of 1% (maximum after 1.5%) was found to moderately affect the bacterial growth trend because all growth phases were successfully completed with regressive growth. Hence, 1% was selected as the optimum concentration for further experimentations.

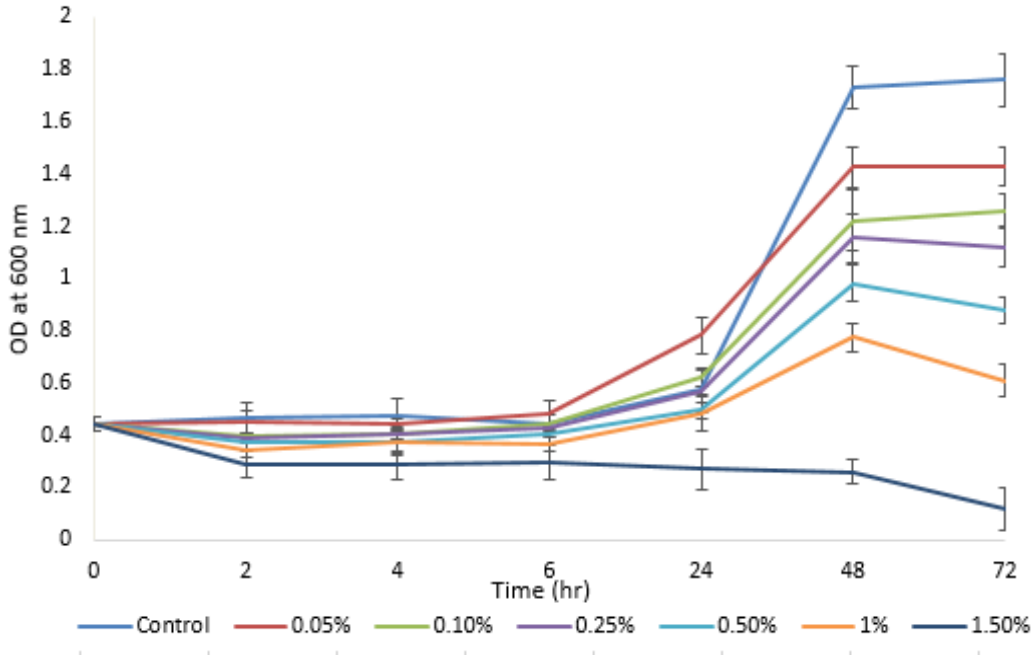

Fig. 4.5(a) Tolerance level of the Polyethylene degrading strain against TNPs concentrations

An optimum concentration of 0.01% nanobarium titanate (NBT) nanoparticles has previously been reported to enhance LDPE biodegradation [29]. Fullerene-60 and Super magnetic iron oxide nanoparticles (SPION) have also been studied to support microbial growth trend in progressive way (Sah *et al.*, 2010).

4.4.2 Microbial growth profiling concerning of Colony Forming Unit/ml

The CFU counts also showed a reduction when the cultures were grown with gradual increase of TNPs as compared to control (Fig. 4.6). Distinguished microbial colonies were appeared at 7^{th} dilution factor so it was the used to count CFU/ml.

Till the concentration of 0.1% TNPs, there was no significant effect on the growth of microbes. Even at concentration of 1%, a gradual decrease was noted on log and lag phase of microbial cycle but microbial colonies were growing productively till 48 A deadly decrease in microbial growth was observed with concentration of 1.5%. So, concentration of 1% was taken to continue with furthur experiment.hours.

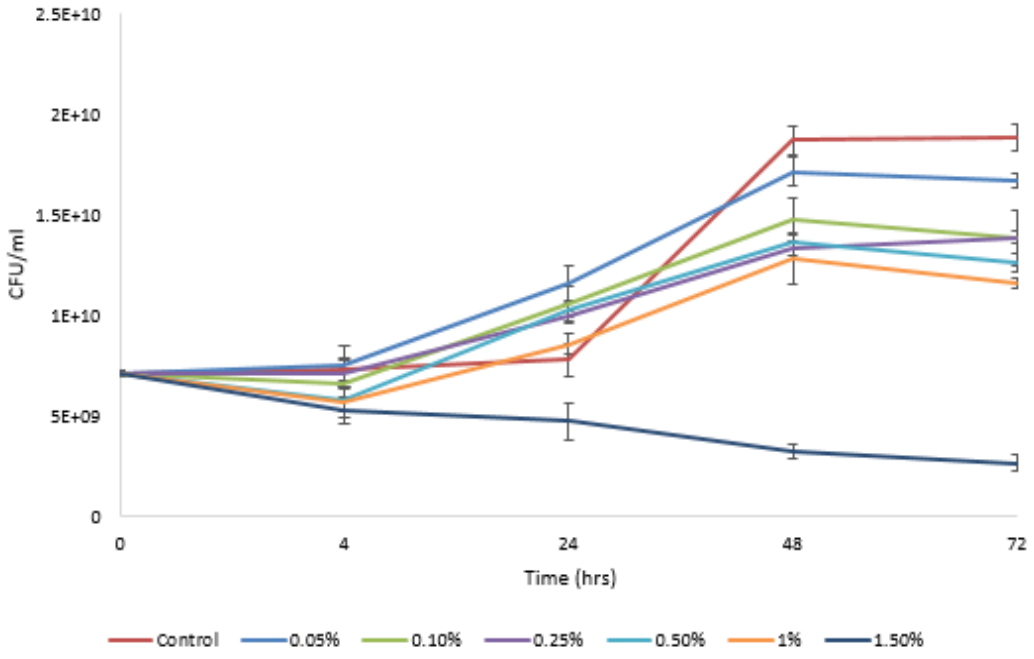

Fig. 4.5(b) Tolerance level of the Polyethylene degrading strain against TNPs concentrations

Mean generation time for isolated strain is reported in table 4.5. Microbial colonies showed reverting growth with increasing concentrations of TNPs.

Table 4.5 Comparative microbial growth and t_{gen} in the presence of TNPs

Sample Name	k (gen/hr)	t_{gen}(min/gen)
Control	30.3	2
0.05%	14.9	4
0.1%	11.5	5.2
0.25%	10.2	5.9
0.5%	9.8	6.1
1%	13.9	4.3
1.5%	-13.5	-4.5

Control exhibited maximum generaton per hour in the absence of TNPs while with 1.5% concentration of TNPs, k value showed decay of isolated strain during log phase. With concentration of 1%, generation per hour was 13.9 and generation time was 4.3 which were most optimal to use in biodegradation of Polyethylene when compared to lowest and highest concentration of TNPs.

Elo and co-workers (1972) found that titanium dioxide levels of up to 0.1M do not affect the growth of oral bacteria (e.g *E.coli*) but above this 0.1 M mark, it does have some affect. So, final experiment was carried out to analyze effect of TNPs on performance of Polyethylene degrading microbes.

4.5 Polyethylene biodegradation analysis in the presence and absence of Titania nanoparticles (TNPs)

During the process of biodegradation, optical density (OD) and λmax were assessed comparatively using spectrophotometer and evaluated in Fig. 4.6.

In many studies it is reported that TNPs have bactericidal/ antibacterial effects (Saman et al. 2013) which are obvious in the results but somewhat there is a possibility of microbial survival in the presence of higher concentration of TNPs as they are

showing continuous persistent growth assay in nutrient broth and minimal media with Polyethylene as carbon source. Optical density at 600nm was found with shorter lag phase and early log phase in the presence of TNPs as compared to control without TNPs.

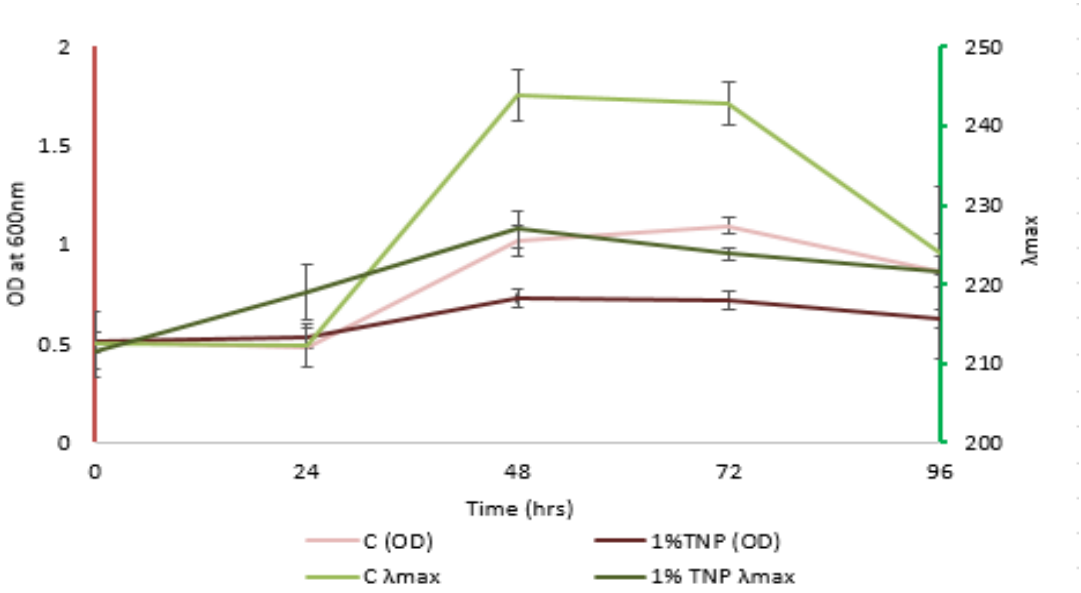

Fig. 4.6 Comparative growth profiling in the presence and absence of TNPs using *P. aeruginosa*

In case of λmax of media, control was found to be constant at 212 nm for the initial 2 days, which thereafter lifted to 243 nm after 3 days, finally reaching a value of 242 nm after 4 days. The shift in λmax suggests changes taking place in the polymer backbone between 2 to 4 days of incubation as a result of microbial action. On the other hand, in the presence of TNPs, a λmax shift from 211 nm to 219 nm was observed within 1 day, suggesting rapid changes occurring in the polymer backbone during the log phase. The value of λmax showed slight decrease during the stationary phase, suggesting no significant changes in the chemical structure of PE during this period.

CFU/ml, to some degree, showed similar trend like OD and λmax in the presence and absence of TNPs showed viability of P. aeruginosa in degradation

experiment. The CFU/ml calculation revealed that the introduction of PE to microbes has supported their growth rate, proposing the adaptation of the polymer as a source of energy. CFU/ml was initially noted to be 84×10^5 CFU/ml and 102×10^5 CFU/ml in absence and presence of TNPs after 24 hours of incubation.

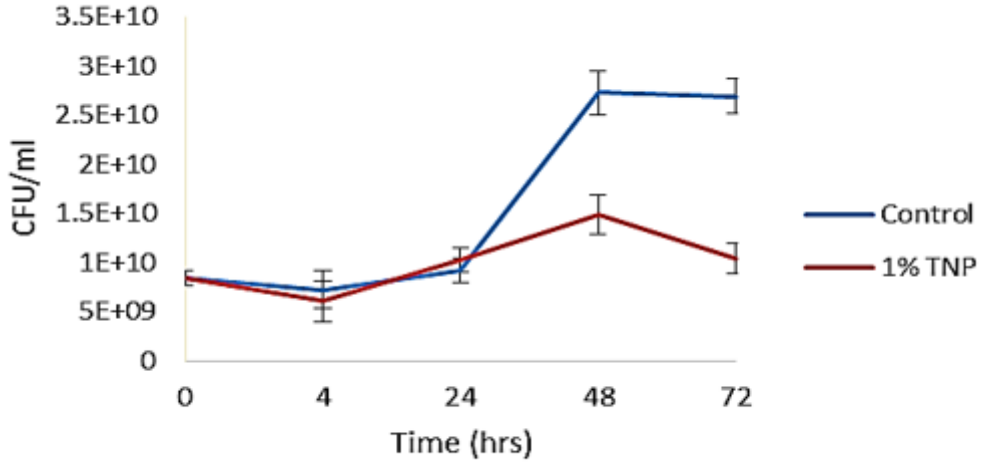

Fig. 4.7 Microbial counts trend in the presence and absence of TNPs in biodegradation

Growth trend was sustained throughout incubation period with regressive growth in the presence of TNPs.

4.6 Polyethylene degradation analysis

4.6.1 Scanning electron microscopy (SEM) analysis

To analyze morphological changes in Polyethylene powder before and after degradation, SEM was conducted. Before degradation, there are comparatively smooth and clear surface than bio-degradation and bio-nano-degradation. After biodegradation, there are comparatively rough surface with some holes as the same condition can be seen in the image after nano-degradation. But after nano-biodegradation, a significant degradation can be observed. Many researches have been carried out till date to degrade

PE films photocatalytically well and their SEM results revealed high surface deformations due to nano-activity (Thomas *et al.*, 2013), (Asghar *et al*., 2011).

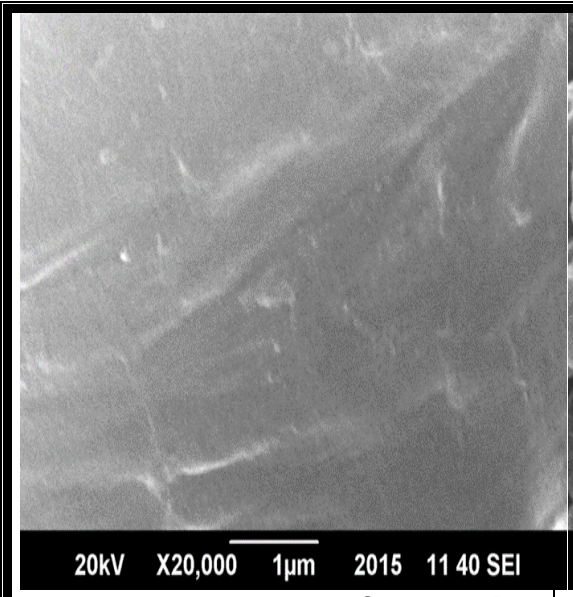

Fig. 4.8(a) SEM image of PE powder before degradation

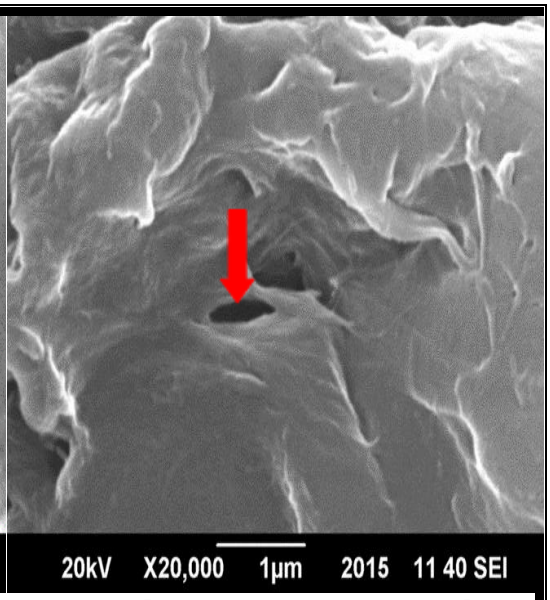

Fig. 4.8(b) SEM image PE powder after biodegradation

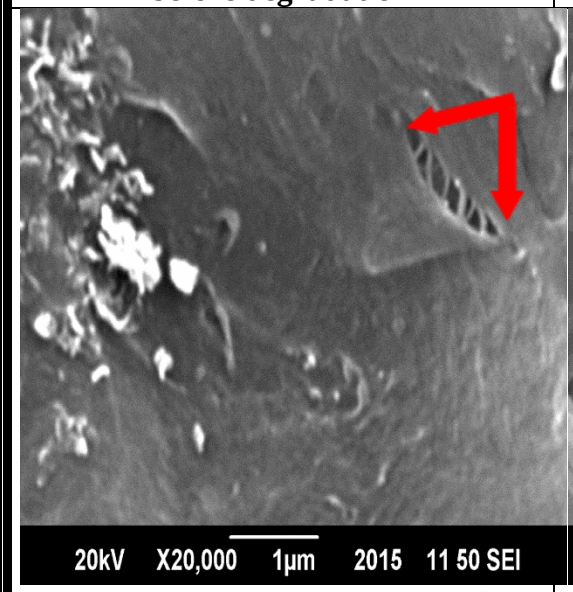

Fig. 4.8(c): SEM image PE powder after nanodegradation

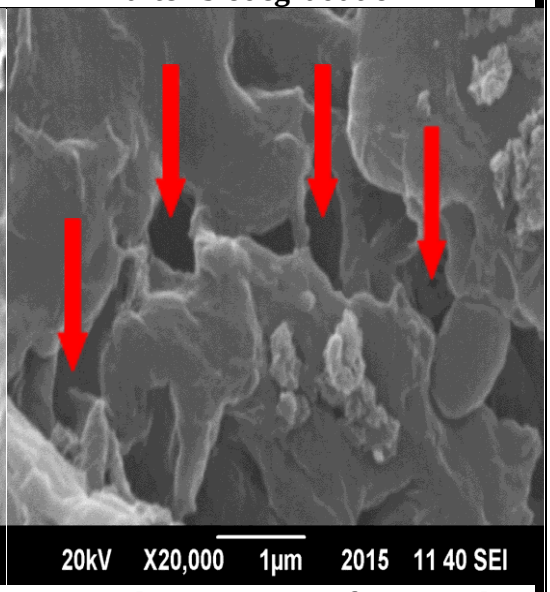

Fig. 4.8(d) SEM image of PE powder after Nano-biodegradation

4.6.2 Fourier transforms infrared spectroscopy FTIR

Pure Polyethylene has shown FTIR absorptions (KBr, cm^{-1}) corresponding to rocking CH_2 (720.2), bending CH_2 (1,465.2), CH_2 deformation (1720), CH_2 (2844), CH_2 stretching asymmetrically (2916), and stretching CH (3624) (Fig. 4.12) (Lyon et al. 2008). Introduction of C-O frequencies (1,077) was observed in the biodegraded and nano-biodegraded samples owing to inclusion of O atoms into the hydrocarbon polymer

backbone. Pure PE did not show stretching C-O frequencies. Degradation in the absence of microbes with titania nanoparticles also brought about significant shifts in the FTIR absorption frequencies of PE as compared with the pure PE control. The degraded samples also recorded absorptions (cm^{-1}) corresponding to bending of CH_2 (1,465), and CH_2 deformation (1,720) along with a pair of combination bands due to bending of CH_2 and rocking of CH_2 at 2,844 and 2,916 respectively.

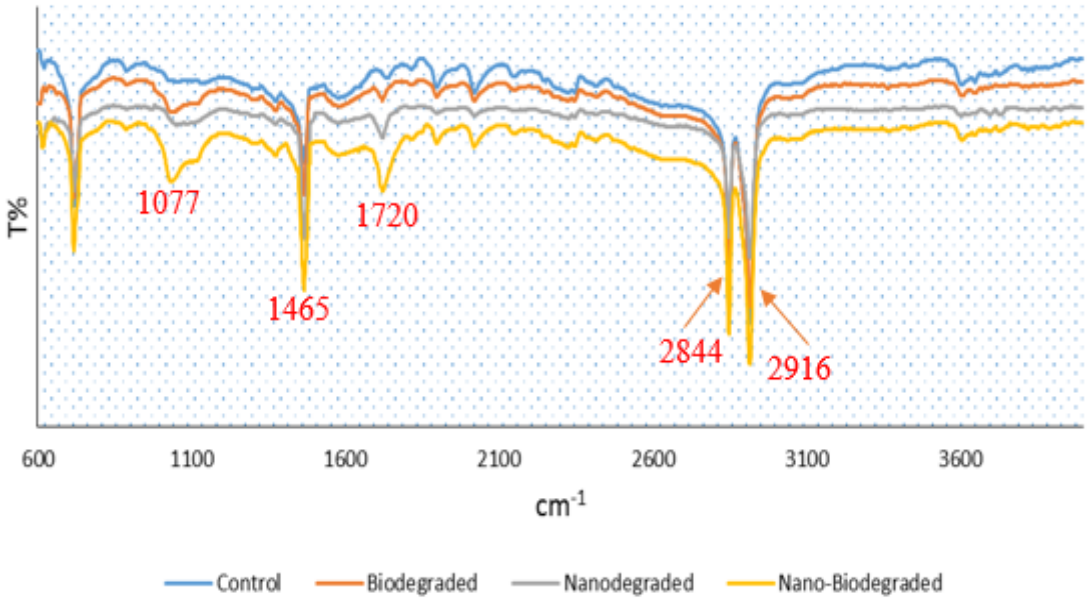

Fig. 4.9 FTIR spectra of PE degraded by microbes in the presence and absence of TNPs

Furthermore, inclusion of O atoms into PE, due to microbial action, introduced stretching C-O frequencies corresponding to 1,077 cm-1. In addition to this, deletion of frequencies corresponding to CH_2 bending, CH_3 symmetrical bending, symmetrical stretching of CH_2, and CH_2 asymmetrical stretching were also observed. Microbes were also found to significantly affect bending and rocking of CH_3 and CH_2 group frequencies respectively.

FT-IR assignments revealed the presence of C60 in these assays from the absorption at 1,465 and 1720 cm-1. Furthermore, the biodegradation of LDPE was

ascertained by the presence of C-O group frequencies at 1077 cm^{-1}. The C-O frequencies appear as a result of the introduction of O atoms into the hydrocarbon backbone of the polymers by the action of mono- and dioxygenases (Hayaishi, 2005).

These bonds are more susceptible to attack by the microbial enzyme systems and might be responsible for the dissolution of polymers. Changes in the FTIR absorption frequencies have also been reported in the case of various polymers like LDPE, HDPE, and epoxy by different bacterial consortia (Satlewal et al. 2008). With the addition of titania nanoparticles, the FTIR profiles of degraded PE exhibited significant deformation along with inclusion of C-O frequencies.

- **Carbonyl Index (CI)**

An obvious reduction in CI was observed after incubation of samples. After degradation, CI value was increased to 1.10 in samples exposed to *Pseudomonas aeruginosa*, to 1.115 in samples with TNPs as compared to pure Polyethylene as control (1.08). Maximum CI (1.12) was observed in sample encountered by both TNPs and *P. aeruginosa.*

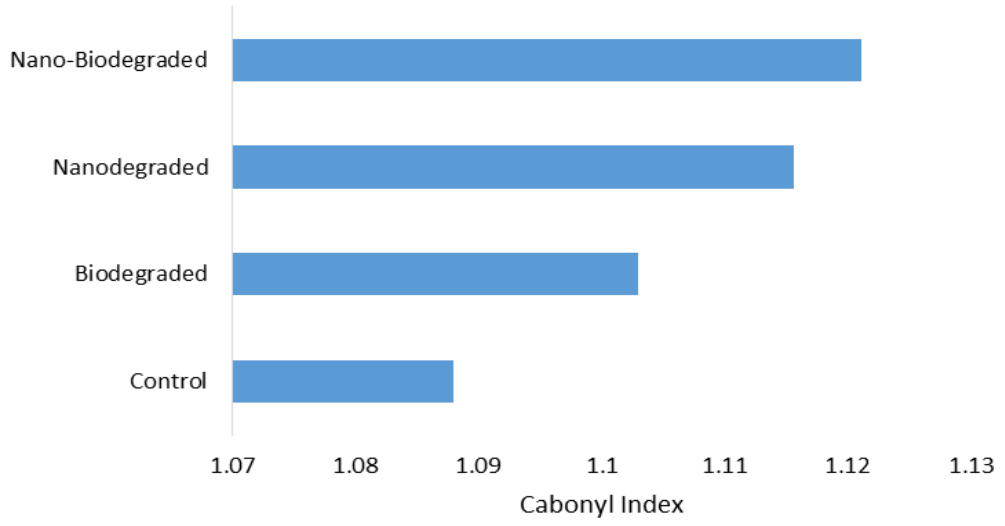

Fig. 4.10 Change in carbonyl index in the presence and absence of TNPs

It has been reported that both microbes and TNPs have ability to degrade Polyethylene by increasing CI though TNPs attain more tendency to enhance CI (Kyaw *et al.*, 2012; Asghar *et al*., 2011).

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Study reveals that microbial strain which was isolated from PE waste soil sample and later identified as *Pseudomonas aeruginosa* was dominant microbes among other indigenous microbes in dumpsite to degrade PE. 1% TNP was found to be maximum concentration at which microbes can survive and this concentration enhanced biodegradation process of PE.

5.1 Recommendations

Based on current research work, following recommendations are made:

- This work can be evaluated to check growth pattern of beneficial microorganisms other than *Pseudomonas aeruginosa*.
- This idea can be implemented on large/commercial scale to check its effectiveness
- Research study can be extended by using different metal (Fe, Pt, Cu, etc.) doped nanomaterials.

Chapter 6

REFERENCES

Albertsson, A. C., Andersson, S. O. & Karlsson, S. (1987). The mechanism of biodegradation of polyethylene. Polym. Deg. Stab. 18:73-87.

Allen, N. S., Edge, M., Verran, J., Stratton, J., Maltby, J. & Bygott, C. (2008). Photocatalytic titania based surfaces: environmental benefits. Polym. Deg. Stab. 93(9): 1632-1446.

Asghar, W., Qazi, A. I., Ilyas, H., Khan, A. A., Awan, A. M. & Aslam, R. M. (2011). Comparative solid phase photocatalytic degradation of Polyethylene films with doped and undoped TiO_2 Nanoparticles. J. Nanomat. 2011:8

Astrup, T., Møller, J. & Fruergaard, T. (2009). Incineration and co-combustion of waste: Accounting of greenhouse gases and global warming contributions. Waste Manag. Res. 27:789–799.

Altschul, S., Madden, T. I., Schäffer, A. A., Zhang, J., Miller, W. & Lipman, D. J. (1997). Gapped blast and psi-blast: a new generation of protein database search programs. Nucleic Acids Res. 25(17):3389-402.

AWPA, American Wood Preservers Association Standards, Books of standards, Stevensville, Maryland, USA, 1986.

Bahri, & Doan, A. (2007). Africa wages war on plastic bags. Retrieved from: http://plasticbaglaws.org/plastic-bag-waste-in-developing-countries/

Berkeley, R. C. W. & Campbelt, R. (1972). Microbial nutrition and the influence of environmental factors on microbial growth and other activities. In: Microorganism: function, form and environment. Hawker LE & Linton AH (Eds.), Edward Arnold Publisher limited, London.

Behnajady, M. A., Modirshahla, Shokri, M. & Rad, B. (2008). Enhancement of Photo Catalytic Activity of TiO2 Nanoparticles by Silver Doping: Photo Deposition versus Liquid Impregnation Methods. Glob. NEST J. 10: 1-7.

Byranvanda, M., Kharata, N., Fatholahib L. & Beiranvandc, M. Z. (2013). A review on synthesis of nano-TiO_2 via different methods. J. nanostruc. 1-9.

Carp, O., Huisman, & Reller, A. (2004). Photo induced reactivity of titanium dioxide. Prog. Sol. State Chem. 32: 33-177.

Chen, X. & Mao, S. S. (2007). Titanium dioxide nanomaterials: synthesis, properties, modifications, and applications. Chem. Rev. 107(7):2891-2959.

Cao, F., Oskam, G., Searson, P. C., Stipkala, J. M., Heimer, T. A., Farzhad, F. & Meyer, G. J. (1997). The open circuit photovoltage in dye sensitized nanostructured TiO_2 films. J. Phys. Chem. 99:11974-11980.

Díaz, P., Boyd, K.G., Grigson, S.J.W. & Burgess, J.G. (2002). Biodegradation of crude oil across a wide range of salinities by an extremely halotolerant bacterial consortium MPD-M, immobilized onto polypropylene fibers. Biotechnol. Bioeng. 79:145–153.

De, J., Ramaiah, N. & Vardanyan, L. (2008). Detoxification of toxic heavy metals by marine bacteria highly resistant to mercury. Mar. Biotechnol. 10:471–477.

Dong, D., Tasaka, S. & Inagaki, N. (2001). Thermal Degradation of Monodisperse Polystyrene in Bean Oil. Polym. Degrad. Stab. 72: 345-351.

Elo, k., Maatta, E., Uksila & Arstila. (1972). Pulmonary deposits of titanium dioxide in man, J. Arch. Path. 417-424.

Esmaeilia, A., Pourbabaeea, A.A., Alikhania, H.A., Shabanib, F. & Kumar L. (2014). Colonization and biodegradation of photo-oxidized low-density polyethylene (LDPE) by new strains of *Aspergillus sp*. and *Lysinibacillus sp.* Biorem. J. 213-226.

Gaya, U. I., & Abdullah, A. H. (2008). Heterogeneous photocatalytic degradation of organic contaminants over titanium dioxide: A review of fundamentals, progress and problems. J. Photochem. Photobio. C: Photochem. R. 9(1):1-12.

Giolli, C., Borgioli, A. Credi, & Fabio (2007). Characterization of TiO2 Coatings Prepared by a Modified Electric Arc-Physical Vapor Deposition System. Surf. Coat. Tech., 202: 13-22

Gilan, I., Hadar, Y. & Sivan, A. (2004). Colonization, biofilm formation and biodegradation of polyethylene by a strain of *Rhodococcus ruber*. In: Appl Microbiol Biotech. Pg. 97–104.

Hazen, T.C., Dubinsky, E.A., DeSantis, T.Z., Andersen, G.L., Piceno, Y.M., Singh, N., Jansson, J.K., Probst, A., Borglin, S.E., Fortney, J.L., Stringfellow, W.T., Bill, M., Conrad, M.E., Tom, L.M., Chavarria, K.L., Alusi, T.R., Lamendella, R., Joyner, D.C., Spier, C., Baelum, J., Auer, M., Zemla, M. L., Chakraborty, R., Sonnenthal, E. L., D'haeseleer, P., Holman, Y.N., Osman, S., Lu, Z., van Nostrand, J. D., Deng, Y., Zhou, J., & Mason, O. U. (2010). Deep-sea oil plume enriches indigenous oil-degrading bacteria. NCBI Sci. 330:204–208.

Hadad, D., Geresh, S. & Sivan, A. (2005). Biodegradation of polyethylene by the thermoplhilic bacterium *Brevibacillus borstelensis*. J. Appl. Microbiol. 98:1093-1100

Harshavardhan, K. & Jha, B. (2013). Biodegradation of low density polyethylene (LDPE) by marine bacteria from pelagic water, Arabian Sea, India. Mar. Pollut. Bull. 77:100-106.

Hayaishi, O. (2005). An odyssey with oxygen. Biochem. Biophys. Res. Commun. 338: 2-6.

Iranzo, M., Sainz-Pardo, I., Boluda, R., Sánchez, J. & Mormeneo, S. (2001). The use of microorgansims in environmental remediation. Ann. Microbiol. 51:135–143.

Ji. X., Qian, J. L., Wang, J. Q., Li, S. Y. & Guo, S. H. (2000). Prospect and current situation of technologies for converting plastic waste to oils in china. Env. Prot. Chem. Ind. 20: 18-22.

Jakubowicz, I., Yarahmadi, N. & Arthurson, V. (2011). Kinetics of abiotic and biotic degradability of low-density polyethylene containing pro-degradant additives and its effect on the growth of microbial communities. Polym. Degrad. Stab. 96: 919–928.

Kyaw, B.M., Champakalakshmi, R., Sarkharkar, M.K., Lim, C.S. & Sarkharkar, K.R. (2012) Biodegradation of LDPE by *Pseudomonas* species. Ind. J. Microbiol. 52(3):411-419

Kim, S. H., Kwak, & Suzuki, T. (2006). Photo catalytic degradation of flexible PVC-TiO2 nano-hybrid as an eco friendly alternative to the current waste landfill and dioxine - emitting incineration of post-use PVC. Polym. 47: 3005–3116.

Kavitha, R., Mohanan, A. k., & Bhuvaneswari, V. (2014). Biodegradation of low density polyethylene by bacteria isolated from oil contaminated soil. Int. J. Plant Animal Env. Sci. 4(3): 601- 610.

Khan, S., Qazi, I. A., Hashmi, I., Awan, M. A. and Zaidi, N-u-S, S. (2013). Synthesis of silver-doped titanium TiO2 powder-coated surfaces and its ability to inactive *Pseudomonas aeruginosa* and *Bacillus subtilis*. J Nanomater. 2013:1-8.

Khoo, H. H. & Tan, R. B. H. (2010). Environmental impacts of conventional plastic and bio-based carrier bags: Part 2: End-of-life options. Int. J. Life Cyc. Assess. 15:338–345.

Kausar, M. A., Tahir, M. & Hamid, A. (1990). Comparison of three methods for the estimation of soil available boron for maize. J. Sci. Ind. Res. 33: 221 – 224.

Kapri, A., Zaidi M. H., Satlewal, A. & Goel, R. (2010). SPION-accelerated biodegradation of low-Density Polyethylene by indigenous Microbial Consortium. Int. Biodet. Biodeg. 64: 238–44.

Kapri, A., Zaidi, M. H. & Goel. (2009). Nanobarium titanate as supplement to accelerate plastic waste biodegradation by indigenous bacterial consortia. AIP Conf. Proc. 1147: 469-474.

Lyon, D. Y., Brown, D. A. & Alvarez, J. J. (2008). Implications and potential applications of bactericidal fullerene water suspensions: Effect of nC60 concentration, exposure conditions and shelf life. Water Sci. Technol. 57(10): 1533-1538.

Luigi, M., Gaetano, D. M., Vivia, B. & Angelina, L. G. (2007). Biodegradative potential and characterization of psychrotolerant polychlorinated biphenyl-degrading marine bacteria isolated from a coastal station in the Terra Nova Bay (Ross Sea, Antarctica). Mar. Pollut. Bull. 54:1754–1761.

Mahalakshami, V., & Abubakar Siddiq, S. (2015). Enhanced biodegradation of polyethylene by development of a consortium. Adv. Appl. Sci. Res. 6(4):183-189.

Mahalakshmi, V., Abubakker, S. & Niran, A.S. (2012). Analysis of polyethylene Degrading potentials of microorganisms isolated from compost soil. Int. J. Pharm. Biol. Arch. 3(5):1190-1196

Macwan, D. P. & Dave, P. N. (2011). A review on nano-TiO_2 sol–gel type syntheses and its applications. J. Mater. Sci. 46:3669–3686.

Massardier-Nageotte, V., Pestre, C., Cruard-Pardet, T., & Bayard, R. (2006). Aerobic and anerobic biodegradability of polymer films and phsyico-chemical characterization. Polym. Degrad. Stabil. 91:620–627.

Mohan, K. & Srivastava, T. (2011). Microbial deterioration and degradation of Polymeric materials. J. Biochem. Tech. 2(4):210-215

Negi, H., Gupta, S., Zaidi, M.G.H. & Goel, R. (2011). Studies on biodegradation of LDPE film in the presence of potential bacterial consortia enriched soil. Biologia. 57:141-147.

Prosun, T., and Alok, K. (2012). Low-density polyethylene degradation by *Pseudomonas sp.* AKS2 biofilm. Env. Sci. Poll. Res. 4146–4153.

Piringer, Otto G., Baner, & Lawrence A. (2008). In: Plastic packaging: interactions with food and pharmaceuticals. 2nd Ed.

Puma, G.L., Bono, A., Krishnaiah, D., Collin, J.G., Haz, J. & Mat. (2008). Preparation of titanium dioxide photocatalyst loaded onto activated carbon support using chemical vapor deposition: a review paper. J. Haz. Mat. 209–219.

Pieper, D. H. & Reineke, W. (2000). Engineering bacteria for bioremediation. Curr. Opin. Biotechnol. 11, 262–270.

Pandey, P., Swati, Harshita, Manimita, Shraddha, & Yadav M. (2015). Nanoparticles accelerated in-vitro biodegradation of LDPE: A review. Adv. Appl. Sci. Res. 6(4):17-22

Rao, M. V., Rajeshwar, K., Pal, Verneker, V. R. & DuBow, J. (1980). J. Phys. Chem. 84:1987-1991.

Rafiq, R., Arshad, M., Khokhar, F. M., Qazi, A. I., Hamza, A. & Virk, N. (2015). Growth response of wheat to titania nanoparticles application. NUST J. Eng. Sci. 7(1):42-46

Rosa, A.P. & Triguis, J.A. (2007). Bioremediation process on Brazil shoreline. Laboratory experiments. Environ. Sci. Poll. Res. 14:470–476.

Reazuddin, M. D. (2003). Banning Polyethylene shopping bags: A step forward to promoting environmentally sustainable development in Bangladesh. Bangla. Cen. Adv. Stud.

Reynold, R. (2009).API 20e. Lab manual. Richland College.

Roy, P. K., Titus, S., Surekha, P., Tulsi, E., Deshmukh, C., & Rajagopal, C. (2008). Degradation of abiotically aged LDPE films containing pro-oxidant by bacterial consortium. Polymer Degradation and Stability. 93(10): 1917-1922.

Shang, J., Chai, & Zhu (2003). Solid phase photo catalytic degradation of polystyrene plastic with TiO_2 as photo catalyst. J. Solid State Chem. 174: 104-110.

Shah, A. A., Hasan, F., Hameed, A. & Ahmed, S. (2008). Biological degradation of plastics: A comprehensive review. Biotech. Adv. 26: 246-265.

Sohni, R., Kapri, A., Zaidi, M.G. H., & Goel, R. (2009). Comparative biodegradation studies of non-poronized and poronized LDPE using indigenous microbial consortium. J. Polym. Env, 17(4): 233-239

Suresh, B., Maruthamuthu, S., Palanisamy, N., Ragunathan, R., Navaneetha, K. & Muralidharan, V.S. (2011). Investigation of biodegradability of polyethylene by *Bacillus cereus* strain Ma-Su isolated from compost soil. Int. Res. J. Microbiol. 2(8):292-302.

Sah, A., Kapri, A. & Zaidi, M. H. (2010). Implications of fullerene-60 upon in-vitro LDPE biodegradation. J Microbiol biotechnol; 20(5): 908–16

Sinha, V., Patel, M. R. & Patel, J. V. (2010). PET waste management by chemical recycling: A review. J. Polym. Env. 18: 8–25.

Saha, A. & Santra, C. S. (2014). Isolation and characterization of bacteria isolated from municipal solid waste for production of industrial enzymes and waste degradation. J. Micro. Exp. 1(1):2014

Svenson, A., Sjöholm, S., Allard, A. S. & Kaj, L. (2009). Antiestrogenicity and estrogenicity in leachates from solid waste deposits. Env. Toxicol. 26: 233–239.

Satlewal, A., R. Soni, M. G. H. Zaidi, Y. Shouche, & R. Goel. (2008). Comparative biodegradation of HDPE and LDPE using an indigenously developed microbial consortium. J. Microbiol. Biotechnol. 18(3): 477-482.

Salem, M. A. (2001). Mechanical properties of UV-irradiated low-density polyethylene films formulated with carbon black and titanium dioxide. Egypt. J. Sol. 24(2): 141150.

Shen, C., Tang, X., Yao, J., Shi, D., Fang, J., Khan, M.I., Cheema, S.A. & Chen, Y. (2010). Levels and patterns of polycyclic aromatic hydrocabons and polychlorinated biphenyls in municipal waste incinerator bottom ash in Zhejiang province, China. J. Haz. Mater. 179:197–202.

Tollner, E.W., Annis, P.A. & Das, K.C. (2011). Evaluation of strength properties of polypropylene-based polymers in simulated landfill and oven conditions. J. Env. Eng. 137: 291–296.

Tsuchida, D., Kajihara, Y., Shimidzu, N., Hamamura, K., & Nagase, M. (2011). Hydrogen sulfide production by sulfate-reducing bacteria utilizing additives eluted from plastic resins. Waste Mang. Res. 29:594–601.

Thomas, T. R. & Sandhyaranin, N. (2013). Enhancement in the photocatalytic degradation of low density polyethylene-TiO2 nanocomposite films under solar irradiation. RSC Adv. 3(33).

Thompson, R.C., Moore, C.J., VomSaal, F.S. & Swan, S. H. (2009). Plastics, the environment and human health: current consensus and future trends. Int. J. Biol.Sci. 364:2153-2166.

Tansel, B., & Yildiz, B.S. (2011). Goal-based waste management strategy to reduce persistence of contaminants in leachate at municipal solid waste landfills. Env. Dev. Sus. 13:821–831.

Urase, T., & Okumura, H., Panyosaranya, S., & Inamura, A. (2008). Emission of volatile organic compounds from solid waste disposal sites and importance of heat management. Waste Mang. Res. 26:534–538.

Usha, R., Sangeetha, T., & Palaniswamy, M. (2011). Screening of Polyethylene degrading Microorganisms from Garbage Soil. Libyan Agric. Res. Cen. J. Intl. 2 (4): 200-204.

Vlab.amrita.edu. (2011). Bacterial Growth Curve. Retrieved 22 January 2016, from vlab.amrita.edu/?sub=3&brch=73&sim=1105&cnt=1

Vernon-Parry, K. D. (2000). Scanning Electron Microscopy (SEM): An introduction. 13(4), 40-44.

Xu, S. Y., Zhang, H., He, P. J. & Shao, L.M. (2011). Leaching behavior of bisphenol A from municipal solid waste under landfill environment. Env. Technol. 32:1269–1277.

Younas, H., Qazi, I. A., Hashmi, I., Awan, A. M., Mahmood, A. & Qayyum, H. A. (2014). Visible light photocatalytic water disinfection and its kinetics using Ag-doped titania nanoparticles. Environ. Sci. Poll. Res. 21: 740-752.

Zhang, J., Wang, X., Gong, J., & Gu, Z. (2004). A study on the biodegradability of polyethylene terephthalate fiber and diethylene glycol terephthalate. Int. J. Appl. Polym. Sci. 93:1089–1096.