SCREENING OF PLASTIC DEGRADING *PSEUDOMONAS* SPP. FROM SOIL

A Dissertation Submitted to the **Central Department of Microbiology,** Tribhuvan University, Kathmandu, Nepal, in partial fulfillment of the Requirement for the Award of the Degree of Master of Science in Microbiology

(Public Health)

by

Govinda Badahit

2018

RECOMMENDATION

This is to certify that Mr. Govinda Badahit has completed this dissertation work entitled **"Screening of Plastic Degrading *Pseudomonas* spp. From Soil"** as a partial fulfillment of M.Sc. degree in Microbiology **(Public Health)** under our supervision. To **our** knowledge this is his original work and has not been submitted for award of any other degree.

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ABSTRACT

The plastics of various forms such as nylon, polycarbonate, polyethylene terephthalate, polyethylene, polypropylene, polystyrene, polytetraflouro ethylene, polyurethane and polyvinyl chloride are being continuously used in our day to day life. Polythene bags are made of polyethylene. Polythene constitutes 64% of the total synthetic plastic as it is being used in huge quantity for the manufacture of bottles, carry bags, disposable articles, garbage containers, margarine tubs, milk jugs and water pipes. Most of the plastic materials are generating as one of the major source of environmental pollution. Therefore, this research was conducted with an objective to find the screening of plastic degrading *Pseudomonas* spp. isolated from the different soil samples at different temperature. From the four sentinel sites Sisdol, Teku, Balkhu and Sanothimi, total of 60 soil samples were collected. The samples were processed in the Microbiology Laboratory form April to September 2017 at Central Department of Microbiology, Tribhuvan University, Kathmandu for the isolation and identification of the *Pseudomonas* spp. The organisms were identified by the conventional microbiological methods and biochemical reactions. The *Pseudomonas* spp., potency of degradation of plastic was screened. It was found that, the *Pseudomonas* spp. degraded 7.6% and 8.2% of plastic at 30°C and 37°C temperature during one month. Out of the total 24 isolates, *P. aeruginosa* degraded 7.3% and 8.5%, the *P. fluorescence* degraded 7.8% and 7.9% of the polythene at 30°C and 37°C temperature respectively during one month. This research shows the indigenous strain of *Pseudomonas* spp. has the potency of degradation of polythene and supportive for the way of municipality solid waste management.

Key words: Degradation, plastic, *Pseudomonas aeruginosa, Pseudomonas fluorescence*

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ABBREVIATION

D/W	: Distilled Water
EG	: Ethylene Glycol
FTIR	: Fourier Transformer Infrared Red Spectroscopy
HDPE	: High Density Polyethene
LDPE	: Low Density Polyethene
LLDPE	: Linear Low Density Polyethene
MDPE	: Medium Density Polyethene
MW	: Molecular Weight
PCL	: Polycaprolactone
PE	: Polyethylene
PEG	: Polyethylene Glycol
PES	: Polyrther Sulfone
PET	: Polyethylene Terephthalate
PHA	: Polyhydroxyalkanoates
POPs	: Persistent Organic Pollutants
PP	: Polypropylene
PS	: Polystyrene
PTFE	: Polytetraflouro Ethylene
PU	: Polyurethane
PVA	: Polyvinyl Alcohol
PVC	: Polyvinyl Chloride
SPI	: Society of Plastic Industry
TCA	: Tri-carboxylic Acid
TPA	: Terepthalic Acid

CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 Background

The word plastic comes from the Greek word “plastikos” which means able in molded into varied shapes (Joel 1995). Plastics are made of linking of monomers together by chemical bonds. Polyethene comprises of 645 of total plastic, which is a linear hydrocarbon polymers consisting of long chain of ethylene monomers (Sangale et al 2012). Plastic is the most useful synthetic ‘manmade’ substance made up of elements extracted from the fossil fuel resources. It has made possible most of the industrial and technological revolutions of the 19th and 20th centuries. The widely used packaging plastic mainly polythene constitutes about 10% of the total municipal waste generated around the globe (Barnes et al 2009).

The plastics of various forms such as nylon, polycarbonate, low density polyethylene, medium density polyethylene, high density polyethylene, linear low density polyethylene, polypropylene, polystyrene, polyurethane and polyvinyl chloride are being continuously used in our daily life (Begum et al 2015). Polythene bags are made of polyethylene. Polythene constitutes 64% of the total synthetic plastic finds a wide range of applications in human daily use because of its easy processing for various products used for carrying food articles, packaging textiles, manufacturing laboratory instruments and automotive components (Arutchelvi et al 2009).

With continuous growth for more than 50 years, global production in 2013 has increased to 299 million tons, meaning 3.9% increase in compared to 2012. Europe packaging applications are the largest application sector for the plastics industry and represent 39.6% of the total plastics demand. Electrical and electronic applications represent 5.6% of the plastics demand and are closely followed by agricultural applications which have a share of 4.3%. Other

application sectors such as appliances, household and consumer products, furniture and medical products comprise a total of 21.7% of the European plastics demand. In 2012, 25.2 million tones of post consumer plastics waste ended up in the waste upstream. 62% was recovered through recycling and energy recovery processes while 38% still went to landfill (Plastics Europe 2014/15).

The global use of plastic is growing at a rate of 12% per year and around 0.15 billion tones of synthetic polymers are produced worldwide every year (Premraj and Doble 2005). The synthetic plastics that constitute about 80% of total global plastic usage are polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), polyurethane (PU) and polyethylene terephthalate (PET) (Wilkes and Aristilde 2017). Accumulation rate of plastic waste in the environment is 25 million tons per year and is consequently considered a serious environmental danger (Kaseem et al 2012).

Rapid and uncontrolled urbanization, lack of public awareness and poor management by municipalities have intensified environmental problems in towns in Nepal including unsanitary waste management and disposal. The analysis of household, institutional and commercial waste composition indicated that the 66%, 22%, 43% organic waste, 12%, 21%, 22% plastic and 9%, 45%, 23% paper and paper products respectively. Municipality solid waste is composed of 56% organic waste, 16% plastics and 16% paper and paper products. From total solid waste in Kathmandu Metropolitan city, the Composition of plastic waste from household, institutional and commercial field is found 15.9%, 24.5% and 24.2% respectively. In aggregate, the composition of the plastic waste is 21.6% from the total solid waste in Kathmandu Metropolitan city (Banskota 2015).

In order to manage the utility of these polymers in the nature, there are two ways: one is to exploit the microorganisms in degrading polyethylene and the other is to develop artificial polymers susceptible to biodegradation. Subsequently, to gain large scale acceptance these manmade biodegradable polyethylene should retain

all the essential properties of utility by the consumer and when discarded in the environment should demonstrate their degradability more rapidly than the conventional ones (El-Shafei et al 1998).

Unlike most polymers, biodegradable polymers when disposed favorably in the environment e.g. compost, soil and waste water are acted upon and utilized by the indigenous microorganisms as sources of carbon and energy, thus are degraded (Starnecker and Menner 1996). As new biodegradable polymers and their packaging applications are emerging, there is a need to address their environmental performance particularly the time required for their complete disintegration in nature (Kale et al 2007). Less often it happens that the polymer may be safe before biodegradation but may turn toxic during degradation (Delgi-Innocenti et al 2001).

There is a growing interest in the development of biodegradable plastics that would enhance the degradability of other plastic products in landfills and composts under natural conditions (Pometto et al 1992). Chemical degradation is caused using certain chemicals like acids and alkalis etc. Usage of certain microorganisms and enzymes to degrade polymers are classified as the biodegradation method of polymers (Premraj and Doble 2005). The microbial species are associated with the degrading materials. Microbial degradation of plastics is caused by certain enzymatic activities that lead to a chain cleavage of the polymer into oligomers and monomers. These water soluble enzymatically cleaved products are further absorbed by the microbial cells where they are metabolized. Aerobic metabolism results in carbon dioxide and water (Starnecker and Menner 1996) and anaerobic metabolism results in the production of carbon dioxide, water and methane and are called end products (Gu et al 2003). The degradation leads to breaking down of polymers to monomers creating an ease of accumulation by the microbial cells for further degradation (Swift 1997).

Waste plastics lay enormous burden on the environment because their recalcitrance to degradation accelerates the accumulation in nature. Waste plastics

buried in soil cause the water clogging phenomena and devastate soil for agricultural cultivation. Many animals die of waste plastics either by being caught in the waste plastics trap or by swallowing the waste plastics debris to exert ruinous effects on the ecosystem (Usha et al 2011). The enzymatic degradation is most widely used methods for plastics waste treatment. This method of biodegradation by microbial enzymes increases the rate of degradation of plastics without causing any harm to the environment (Singh et al 2016).

The use of polythene is increasing every day and its degradation is becoming a great challenge. Plastic causes pollution and global warming not only because of increase in the problem of waste disposal and land filling but also release CO_2 and dioxins due to burning (Ali et al 2009). The burning of waste plastic material produces toxic gases posing health hazard by causing lung diseases and cancer after inhalation (Pramila and Vijaya 2011). For aquatic biota like mammals, sea turtles and seabirds, polythene waste is considered as a main risk that causes intestinal blockage when ingested unintentionally (Denuncio et al 2011). It also affects soil fertility, preventing degradation of other normal substances, which poses threat to whole world. The biodegradable polymers are designed to degrade quickly by the microbes due to their ability to degrade the organic and inorganic materials including lignin, starch, cellulose and hemicelluloses (Kumar et al 2013).

The problem of waste can be solved to some extent by using biodegradable plastics consequently there is growing attention in degradable plastics. Starch based degradable plastics is most commonly suggested for uses in composting of lawn, garden and shrub litter which could lessen the volume of material entering the landfills by up to 20% (Lee et al 1991). Attention in using biodegradable plastics for packaging, medical, agricultural and fisheries applications has increased in last decades (Orhan et al 2004). However, none of biodegradable of plastics was efficiently biodegradable in landfills. Therefore, none of the products has gained extensive use (Kathiresan 2003).

The *Pseudomonas* spp. was isolated and identified from the soil for the degradation of polyethene. The *Pesudomonas* spp. that can degrade the polythene is helpful for the reduction of the plastic waste.

1.2 Objectives

General objective:

To isolate and identify the plastic degrading *Pseudomonas* spp. from the soil.

Specific objective:

a) To screen the plastic degrading *Pseudomonas* spp.
b) To analyze the degradation of plastic by different *Pseudomonas* spp.
c) To compare the degradation of plastic by *Pseudomonas* spp. at different temperature.

CHAPTER II
LITERATURE REVIEW

The use of polyethylene growing worldwide at a rate of 12% per year and about 140 million tons of synthetic polymers are produced worldwide each year. Plastic waste is a global issue, rapidly escalating with approximately 311 million tons of plastic produced worldwide in 2014 (Neufeld et al 2016; Plastics Europe 2015). Polyethylene is a polymer made of long chain of monomers of ethylene. Polyethylene is highly hydrophobic, chemically inert and microbes on the earth surface have not yet been fully evolved to digest the artificially made plastics. A lot of research has been carried out to alleviate the environmental burden by improving degradability of the waste polyethylene (Kavitha et al 2014).

The bacteria caused the biodegradation ranging from 2.1% to 20.5% for polythene and from 0.5% to 8.1% for plastics. Polythene and plastic degraded to various extents by *Pseudomonas* spp. (37.1% and 28.4%), *Streptomyces* spp. (46.2% and 35.7%) and *Aspergillus* spp. (20.9% and 16.8%) in six months period in liquid (shaker) culture (Usha et al 2011). Degradation of plastic cups and polythene bags studied using bacteria and fungi for one month period. Among which bacteria *Pseudomonas* spp. degraded 20.5% of polythene and 8.1% of plastics while fungal species *A. glaucus* degraded 28.8% of polythene and 7.2% of plastics (Kathiresan 2003).

The soil bacteria were isolated from plastic contaminated soil sample. The bacterial isolates such as *Desulfotomaculum nigrificans* and *P. alcaligenes* were identified by morphological and biochemical characterization. The biodegradation efficacy of *D. nigrificans* and *P. alcaligenes* by using polythene bag were studied. The *P. alcaligenes* was found to be more effective than *D. nigrificans* in degradation of polythene bag at 30 days. An increase in incubation period there is a dramatic increase in weight loss of polythene bag (Begum et al 2015).

P. fluorescence was the most active of the tested microorganisms degrading approximately 18% and 16% of polythene at 9 and 12 months period respectively and 3.8% of plastics in twelve month period under field condition. Also 8% and 5.6% of polythene and plastics were respectively degraded in a month under laboratory condition. The biodegradation of the polythene material was relatively faster and earlier than that of the plastics with the polythene degrading for up to 12.9%, 16% and 15% at 9 months of analysis while only 2%, 3.8% and 4.8% of the plastic materials were degraded at 12 month by each of *Staphylococcus aureus, P. fluorescence* and *A. niger* respectively (Thomas 2015).

Pseudomonas spp. from sewage sludge dump (P1) was found to degrade polyethylene efficiently with 46.2% for natural and 29.1% for synthetic polyethylene. In contrast, *Pseudomonas* spp. from household garbage dump (P2) gave the lowest biodegradability of 31.4% and 16.3% for natural and synthetic polyethylene respectively. However, *Pseudomonas* spp. isolated from textile effluents drainage site gave biodegradability of 39.7% and 19.6% for natural and synthetic polyethylene respectively (Nanda 2010).

Maximum Degradation percentage was observed during 20 days intervals in case of isolate P1A (*Staphylococcus* spp.) which shows no degradation in 10 micron whereas 10% for 40 micron polythene and in case of isolate P1C (*Bacillus* spp.), degradation percentage for 10 micron polythene was 13.3% and 5% for 40 micron respectively and minimum degradation was shown by isolate P1C (*Bacillus* spp.) and P1B (*Pseudomonas* spp.) (Singh et al 2016).

The isolation of most efficient microorganisms using different soil samples were taken from three waste disposal sites such as industrial plastic waste dump area, leather industry waste and domestic waste dump area. The various microorganisms were isolated from the soil samples grown in an inorganic media (M9 media). There are some microorganisms that have the capacity to degrade plastic waste up to 51.5%. This result was achieved due to addition of starch as

additive in M9 media. This study reveals that *Pseudomonas* spp. posses greater potential to degrade polyethylene (Agrawal and singh 2016).

The biodegradation of plastic material was analyzed one month of incubation in liquid culture method. The microbial species found associated with the degrading materials were identified as three Gram positive and two Gram negative bacteria. The microbial species associated with the polythene materials were identified as *B. amylolyticus, B. firmus, P. putida, P. fluorescence* and *B. subtilis*. The efficacy of microbes in the degradation of plastics were analyzed in liquid (shaker) culture method, among the bacteria *P. putida* degrades plastic more in one month (30% weight loss/month) period compared to others and lowest degradation rate was observed in case of *B. subtilis* (22% weight loss/month). This work reveals that *P. putida* posses greater potential to degrade plastics when compared with other bacteria (Jumaah 2017).

P. putida S3A that has ability to degrade nylon6 film, crude nylon 6 and nylon 66 as sole source of nitrogen and carbon isolated from soil contaminated with plastic waste was included. This study was determined the ability of this isolate to degrade polyethylene as sole source of carbon. Some optimum conditions for degradation of polyethylene by this bacterium were studied. It was found that these conditions are growing *P. putida* S3A in mineral salt medium (pH 6.5) containing 0.5% of polyethylene and incubated with shaking (180 rpm) at 37°C for seven days. In addition, it has been found that this bacterium was able to survive with up to 0.9% of polyethylene. In order to ensure that this bacterium was found capable to degraded polyethylene. Results indicated that polyethylene was degraded by *P. putida* S3A, which used the (O-H, C-O and C-H) groups as carbon source (Jailawi et al 2015).

Microorganisms can degrade plastic over 90 genera from bacteria and fungi, among them *B. megaterium, Pseudomonas* spp., *Azotobacter, Ralstonia eutropha, Halomonas* spp., etc. (Chee et al 2010). Plastic degradation by microbes due to the activity of certain enzymes that cause cleavage of the polymer chains into

monomers and oligomers. Plastic that has been enzymatically broken down further absorbed by the microbial cells to be metabolized. Aerobic metabolism produces carbon dioxide and water. Instead of anaerobic metabolism produces carbon dioxide, water and methane as end products (Usha et al 2011).

Biodegradation resulting from the utilization of polyethylene as nutrient may be more efficient if the degrading microorganism forms a biofilm on the polyethylene surface (Shah et al 2009). The microbial species are associated with the degrading materials were identified as bacteria (*Pseudomonas, Streptococcus, Staphylococcus, Micrococcus* and *Moraxella*) fungi (*A. niger* and *A. glaucus*), *Actinomycetes* spp. and genus *Saccharomonospora* (Chee et al 2010; Swift 1997).

The term biodegradable plastics normally refer to an attack by microorganism on non water soluble polymer based materials. Plastics are resistant to the microbial attack because their short time of presence in nature evolution could not design new enzyme structures capable of degrading synthetic polymers. The term is often used in relation to ecology, waste management and environmental remediation and to plastic materials due to their long life span. Plastics can be classified by the chemical process that is used in their synthesis. Pure plastics generally have low toxicity due to their insolubility in water and relative chemical inertness (Vignesh 2016).

2.1 Factors affecting plastic Degradation

Environmental parameters such as humidity, temperature, pH, salinity, the presence or absence of oxygen, sunlight, water, stress and culture conditions not only affect the polymer degradation but also have a crucial influence on the microbial population and enzyme activity (Gu 2003). Maximum CO_2 evolution and optimal lignolytic activity occurred when fungi grow at lowest pH (Glass and Swift 1990).

The chemical and physical properties of polyester have a strong influence on its biodegradability. Molecular weight is one of the factors determining the biodegradation of plastics. Low molecular weight is favorable for biodegradation. The rate of enzymatic hydrolysis of polycaprolactone diol by *Rhizopus delemar* lipase was faster at the smaller molecular weight (Tokiwa and Suzuki 1977). The melting temperature (Tm) of a polymer has a great effect on enzymatic degradability. Generally, the higher the melting point of polyester, the lower the biodegradability tends to be. The enzymatic degradability decreases with increasing time. The higher order structure properties like crystallinity, modulus of elasticity and suppressed the polymer degradability (Tokiwa and Calabia 2004).

Additives, antioxidants and stabilizers used in manufacturing of polymer can slow down the rate of degradation and may be toxic to microorganisms. Besides all above mentioned factor structural (linearity and branching in polymer, type of bond like c-c, amide and ester), molecular composition and physical form of polymer like powder, films, pellets and fibres may also influences the biodegradability polymer. Ultimately the way and rate of polymer degradation depends on the mechanism of degradation and acceleration of process (Arutchelvi et al 2008).

2.2 Relevance of *Pseudomonas* spp. to plastic biodegradation

Polyethylene is the most widely produced synthetic plastic used in plastic bags, water and milk bottles, food packaging and toys (Shah et al 2016). A long chain polymer saturated with ethylene bonds. When polyethylene has branching chains that prevent tight packing into a crystalline structure, it is characterized as LDPE. On the other hand, when there is little to no branching and the molecules can stack and form strong intermolecular forces, PE is considered a HDPE. From the fifteen HDPE degrading bacterial species isolated from a marine ecosystem, *Pseudomonas* spp. was found to be the most efficient followed by *Arthrobacter* spp. (Balasubramanian et al 2010).

The addition of pro-oxidant additives was shown to increase the hydrophilicity of the long chain polymer of PE resulting in chain scission of the polymer and the generation of carbonyl functional groups and low molecular weight (MW) components (Chiellini et al 2006). After pretreatment with nitric acid, *P. aeruginosa* was able to degrade 0.25 gram of LDPE by 50.5% in 2 months (Rajandas et al 2012). However, no chemical pretreatment was needed for *Pseudomonas* spp. AKS2 to degrade LDPE films, albeit only 5% of the total mass of 300 mg was degraded within 45 days (Tribedi and Sil 2013c). Also without any pretreatment, an uncharacterized *Pseudomonas* spp. was found to degrade 28.6% of low MW PE (MW=1700 Da) in a sterilized compost condition after 40 days (Yoon et al 2012).

Therefore, the extent to which polyethylene (PE) and related plastics are biodegraded depends both on the structural arrangement of the plastic polymer and the type of *Pseudomonas* strains exposed to the polymer. Of particular interest to bioremediation strategies of PE are the mechanisms through which these strains can degrade PE containing plastics without pretreatment. Plastics with similar structures to PE but with more hydrolysable functional groups include PVA, PES and PEG. PVA has similar carbon–carbon linkages to PE, which is more water soluble than PE because of the presence of the hydroxyl functional group (Shimao 2001).

As a result, polyvinyl alcohol (PVA) was more easily degraded than PE. Since *Pseudomonas* spp. O3 was reported to degrade PVA (Suzuki et al 1973). The majority of reported PVA degrading bacteria belong to the *Pseudomonas* genus (Shimao 2001). Similar to PE in structure but with added hydrolysable ester bonds, PES is a plastic polymer that is considered more amenable to biodegradation. PES was typically found in shopping bags and agricultural films (Tribedi and Sil 2013a).

Pseudomonas spp. AKS2 can maximally degrade PES at a rate of 1.65 mg per day (Tribedi et al 2012). Furthermore, bio-augmentation of soil microcosms with *Pseudomonas* spp. AKS2 resulted in enhanced PES biodegradation (Tribedi and Sil 2013a). In addition to PVA and PES, PEG was more biodegradable than PE due to the presence of ether bonds and a hydroxyl end group. Complete biodegradation of PEG using *P. stutzeri* JA1001 has been demonstrated with PEG molecular weight up to 14000 Da in concentrations of 0.2% (w/v) after 30 hours (Obradors and Aguilar 1991). While PEG is not as commonly used as PE, it is still a pervasive plastic that is found in products such as pharmaceuticals, lubricants, cosmetics and inks (Gu 2003). Polystyrene is both light weight and stiff serves as an effective thermal insulation in disposable cups, packaging materials and laboratory equipment (Shah et al 2008).

The polystyrene structure is characterized by phenyl functional groups along the hydrocarbon chain. Although reports of PS biodegradation are scarce, the PS polymer can be eventually broken down to compounds such as styrene, toluene and benzene that are metabolizable in *Pseudomonas* spp. (Devi et al 2016). Additionally, it was shown that *Pseudomonas* spp. NCIM 2220 was able to degrade a heteropolymer made of PS with malefic anhydride anchored with minute amounts of lactose, sucrose and glucose (Galgali et al 2002).

Moreover, a high impact PS composed of a mixture of PS and polybutadiene can be degraded by an unclassified *Pseudomonas* spp., but there was only a 10% weight loss to the 200mg high-impact PS film (Mohan et al 2016). Polyurethanes are characterized by urethane bonds formed from the condensation of polyisocyanate and polyol, which have varied structures (aromatic, aliphatic, polycaprolactone, polyether and polyester type polyurathane) depending on their usage (Cregut et al 2013; Shah et al 2008). They were found in tires, sponges, refrigerator insulation, furniture cushions, gaskets, bumpers and paints (Shah et al 2008).

The presence of carbamate bonds in polyurethane (PU) renders it insoluble in common solvents including water, acetone and ethanol. Additionally, the durable properties of PU, which mediate its role as a flame retardant and antimicrobial reduce the degradation effects of temperature, pH, chemical agents and microorganisms (Biffinger et al 2015; Cregut et al 2013).

Despite these PU characteristics that are adverse to biodegradation, several *Pseudomonas* species including *P. fluorescens, P. aeruginosa, P. cepacia, P. protegens* and *P. chlororaphis* have been identified as PU degraders (Cregut et al 2013). It was reported that *P. aeruginosa* AKS9 can utilize both PU diol and Impranil DLNTM, a commercial variety of polyester PU as a sole carbon source (Mukherjee et al 2011). In addition, *P. aeruginosa* strain MZA-85 was found to degrade and metabolize polyester PU (Shah et al 2013). Extensive degradation of Impranil DLN was also accomplished by *P. protegens* Pf-5 and other *P. protegens* strains (Hung et al 2016).

The polymers with the least amount of reported biodegradation by *Pseudomonas* species were polypropylene, PVC and PET. Polypropylene is found in bottle caps, medicine bottles, car seats and disposable syringes and is the second most produced plastic after PE. Polyvinyl chloride is the third highest produced plastic and is commonly found in shower curtains, raincoats, bottles, garden hoses and shoe soles (PlasticsEurope 2015; Shah et al 2008).

Polyethylene terephthalate is also both thermally and chemically stable which has a structure amenable for use in water and soda bottles, electronics, automotive parts and textile fibers. The global production of PET is increasing and is approximated to be 50% of synthetic plastic products (Webb et al 2013). Both with and without chemical pretreatments, PP is difficult to be biodegraded. Following pretreatment with ultraviolet radiation, *P. azotoformans* and *P. stutzeri* were able to survive on PP as a sole carbon source but only minimal weight loss of the plastic polymer resulted after one year (Arkatkar et al 2009, 2010).

The polyvinyl chloride monomer and vinyl chloride was able to serve as a sole carbon source to support the growth of *P. putida* strain AJ (Danko et al 2004). *Pseudomonas* species have not been very effective for PET biodegradation. A lipase isolated from an unspecified *Pseudomonas* spp. was unable to catalyse PET degradation (Meuller et al 2005).

Likewise, an extracellular lipase from an unspecified *Pseudomonas* spp. was not effective at degrading a mixture with polycaprolactone and less than 50% PET (Jun et al 1994). However, a cutinase from *P. mendocina* had high affinity to low crystalline PET and reduced the weight of the film by 5%. The aforementioned studies highlight the diverse capabilities of *Pseudomonas* species to degrade and metabolize the different synthetic plastic polymers (Ronkvist et al 2009).

2.3 Importance of biofilms to biodegradation

The ability of bacterial cells to attach to and degrade plastic polymers is dependent on the structure of the polymer surface (Donlan 2002). The addition of hydrophilic functional groups to plastic polymers is often required to promote cell surface attachment due to the typical hydrophilic nature of cell surfaces which impair attraction to the hydrophobic polymers. Thus, greater surface roughness and hydrophilicity of the polymer was shown to facilitate both enhanced attachment of bacterial colonies and accessibility of secreted extracellular enzymes to polymer surface (Nauendorf et al 2016; Sanin et al 2003; Tribedi and Sil 2013c).

In cases of plastic polymers including PE have high hydrophobicity and molecular weight. The formation of a biofilm either requires the polymer to be altered by oxidation reactions or supplemented with chemicals in order to increase the surface interactions with bacterial cells (Shah et al 2008; Sivan 2011). Biofilm forming bacterial species with relatively high hydrophobic cell surfaces have

improved cell surface attachment to unmodified plastic polymers (Devi et al 2016; Gilan et al 2004; Tribedi and Sil 2013b).

Accordingly, biofilm adapted *Pseudomonas* spp. AKS2 cells were found to have greater cell surface hydrophobicity and LDPE-degrading ability than planktonic cells (Tribedi et al 2015). Furthermore, it was determined that the cells in biofilms secreted exopolysaccharides that aid in attachment to the plastic polymer (Tribedi and Sil 2013c). Although it was evident that the *Pseudomonas* spp. AKS2 has ability to form biofilms promoted the degradation of LDPE (Tribedi and Sil 2013c).

The ability to degrade LDPE may not be transferrable to other plastic polymers due to structural differences. Furthermore, the nutritional environment of the growth media may influence the extent of the biofilm formation (Sivan 2011).

The composition of extracellular polymer matrices can change depending on the growth conditions and can play an important role in the attachment properties of bacteria (McEldowney and Fletcher 1986; Sanin et al 2003). Cell surface hydrophobicity was found to be correlated positively with cell attachment, biofilm formation and PES weight loss (Tribedi and Sil 2013b).

In addition, low glucose content and high ammonium sulphate concentrations resulted in the greatest cell surface hydrophobicity for *Pseudomonas* spp. AKS2 grown on PES (Tribedi and Sil 2013b). In the presence of organic carbon rich marine sediments, biofilm formation was diminished and there was minimal to no degradation of PE (Nauendorf et al 2016). Therefore, environmental and nutritional conditions that favour the genesis of biofilms on plastic polymers are important stimuli for the degradation of synthetic plastics by *Pseudomonas* spp.

2.4 Plastic degradation by extracellular and intracellular enzymes

In general, enzymatic degradation involves two important processes that can be measured by weight loss and additions of functional groups. The reduction in molecular weight of the polymer enables the catalytic effects of enzymes that can only operate on smaller molecules and facilitates the transport of smaller molecules through the cell membrane (Shah et al 2008). Chemical or biological oxidation reactions are often necessary to increase the hydrophilicity of the polymer by providing a functional group such as alcohol or carbonyl groups that can enhance bacterial attachment and degradation (Albertsson et al 1995; Arkatkar et al 2010; Lucas et al 2008). Degradative products with carbonyl functional groups can be metabolized inside the cell through beta-oxidation and the TCA cycle (Restrepo-Florez et al 2014; Shah et al 2008).

Extracellular enzymes such as depolymerases and hydrolases act on large plastic polymers to break them down into smaller molecules (Shah et al 2008). Hydrolytic cleavage can occur either at the polymer chain terminus (exo-attack) or somewhere along the polymer chain (endo-attack). The two different modes of attack create different products. Exo-attack results in small oligomers or monomers that the bacteria can assimilate into the cell. On the other hand, endo-attack primarily reduces the molecular weight of the polymer, whereby the resulting products are not likely to be assailable without further degradation (Lenz 1993). An extracellular depolymerase from a *Pseudomonas* spp. was effective in breaking down a brominated high-impact polystyrene (Mohan et al 2016).

Degradation of PEG by *P. stutzeri* JA1001 involved a single intracellular PEG dehydrogenase that produced glyoxylic acid (Obradors and Aguilar 1991). Alkane hydroxylases from the AlkB family in *Pseudomonas* spp. E4 were involved in the degradation of PE with MW up to 27 000 Da (Yoon et al 2012). Furthermore, the extracellular PVA oxidase found in a number of *Pseudomonas* spp., including *Pseudomonas* spp. O-3, *P. vesicularis* PD and *Pseudomonas* spp. VM15C can oxidize PVA into a diketone structure (Kawai and Hu 2009).

Esterases, lipases and cutinases are hydrolases that are instrumental in plastic degradation (Mohan et al 2016; Novotny et al 2015; Ruiz et al 1999; Sangale et al 2012). Hydrolases are important for enzymatic polymer cleavage wherein ester bonds are broken through a nucleophilic attack on carbonyl carbon atoms (Devi et al 2016). The degradation of PES by *Pseudomonas* spp. AKS2 in a bioaugmented soil was facilitated by hydrolase and dehydrogenase activity as determined by enzyme assays (Tribedi and Sil 2013a).

Esterases can hydrolyse esters either already present in the polymer or produced through oxidation reactions into alcohols, phenols and acids. For instance, an esterase from *Pseudomonas* spp. AKS2 was able to break the ester bonds in PES to generate succinic acid, a TCA cycle metabolite (Tribedi et al 2012). Following the activity of a PVA oxidase introduced acetyl groups in the PVA. *P. vesicularis* was able to assimilate the altered PVA into the cell and hydrolyse it further with an intracellular esterase (Kawai and Hu 2009).

Polyurethane degrading enzymes are thought to be primarily extracellular esterases or proteases that are either membrane bound or secreted extracellularly (Cregut et al 2013; Mukherjee et al 2011; Shah et al 2013). The term polyurethanase is often used to describe enzymes responsible for the degradation of PU (Ruiz et al 1999; Stern and Howard 2000). However, this term is used without definitive confirmation of the hydrolysis of the carbamate bond. Therefore, it is recommended that polyurethanase should be reported as hydrolases or esterases. To this point, an extracellular enzyme from *P. chlororaphis* with esterase and protease activities was shown to degrade successfully polyester PU. This enzyme was also classified as a serine hydrolase because it could be inhibited by phenylmethane sulfonyl fluoride (Ruiz et al 1999). Polyurethane was degraded significantly by *Pseudomonas* spp. lipase but only partially degraded by a recombinant esterase from *P. fluorescens* (Biffinger et al 2014, 2015).

The production of high amounts of extracellular esterases and lipases in *P. aeruginosa* was reported to facilitate the degradation of aromatic, aliphatic polyesters and polyesteramides (Novotny et al 2015). An extracellular cutinase from *P. mendocina* acting on a PET film with 7% crystallinity caused a 5% weight loss of the film after 96 hours and produced TPA and EG as the sole products. These products can subsequently be incorporated into intracellular metabolism. However, much remains unknown regarding what happens intracellular to assailable plastic oligomers or monomers after they are transported across the bacterial cellular membrane (Ronkvist et al 2009).

2.5 Environmental degradation of plastic

In nature biotic and abiotic factors exist together. Therefore, the whole degradation mechanism of a certain material can be referred as "environmental degradation". The environmental degradation process of plastic was affected by its material properties such as molecular weight, optical purity, crystallinity, melting temperature and by environmental factors such as humidity, temperature and catalytic species (pH and the presence of enzymes or microorganisms) (Nishida and Tokiwa 1992; Tsuji 2010). When the molecular weight is low, plastic is brittle, cloudy and opaque while at higher molecular weights, plastic is stronger, more transparent and less susceptible to degradation (Ho et al 1999).

Crystalline regions within plastic hydrolyze much more slowly than the amorphous regions as water diffuses more readily into the less organized amorphous regions compared to the more ordered crystalline regions, causing greater rates of hydrolysis and increased susceptibility to biodegradation. In semi crystalline plastic degradation occurs first in the amorphous regions and more slowly in the crystalline regions. Therefore, the proportion of the crystalline regions within the plastic increases and the rate of degradation decrease with time (Henton et al 2005; Hoglund et al 2012). Increasing concentrations of D-units in plastic lowers optical purity and regularity leading to greater water diffusion

through the polymer matrix in the amorphous regions and accelerating hydrolysis (Hoglund et al 2012; Saha and Tsuji 2006).

The rate of plastic degradation is much greater above the glass transition temperature (Tg 55-62ºC) as polymer chains become more flexible and water absorption increases accelerating both hydrolysis and microbial attachment (Henton et al 2005; Kale et al 2007). Temperatures at or above Tg (55-62°C) and at high relative humidity (>60%), plastic hydrolysis is rapid. In its molten state, plastic can also undergo thermal denaturation (Lim et al 2008) as hydrolysis can occur in the presence of small traces of water or in the absence of water, plastic undergoes zipper like depolymerization, oxidative random chain scission, intramolecular transesterification to monomers and oligomers (Sodergard et al 2002).

Plastic degradation is generally accepted to be a two step mechanism involving first abiotic factors then biotic factors. The abiotic process is the chemical hydrolysis of plastic in the presence of water at elevated temperatures followed by biotic degradation in which microorganisms decompose polymer breakdown products generating carbon dioxide, water and biomass under aerobic conditions and methane, hydrocarbons and biomass under anaerobic conditions (Agarwal et al 1998; Copinet et al 2009; Henton et al 2005; Itavaara et al 2002; Kale et al 2007; Saadi 2012). While some studies have reported that microorganisms do not enhance plastic degradation (Agarwal et al 1998). Other studies have suggested that microbial enzymes exist that are capable of directly degrading high molecular weight plastic (Jarerat and Tokiwa 2001; Masaki et al 2005; Watanabe et al 2007).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

The materials, equipments and various reagents used in different stages of this research are listed in Appendix A.

3.2 Methodology

The research was conducted at Central Department of Microbiology, Tribhuvan University, Kirtipur from April to Sep 2017 to determine the degradation of polythene by *Pseudomonas* spp. isolated from soil.

3.2.1 Sample selection and Sample size

The soil sample surrounding the plastic waste from different place was selected. The plastic sample i.e. polythene bag was selected. Sixty different soil samples were collected (10 gram each) from different areas of the dumping site. The polythene bag of 13 micron thickness size was selected for this study.

3.2.2 Samples collection and transportation

3.2.2.1 Sample collection site

The soil sample was collected from the plastic waste dumped sites such as Sisdol dumping site, Teku dumping site, Balkhu dumping site and Sanothimi Bhaktapur household garbage dumped site. Polythene sample was purchased from local market.

3.2.2.2 Sample collection

The soil sample was collected from the soil surrounding the plastic waste by using sterile spatula and was put in sterile container. The plastic sample i.e. polythene bags was collected and transported in the laboratory.

3.2.3 Sample processing

3.2.3.1 Serial dilution of soil

One gram of the soil sample was added in 9 ml distilled water to make 1:10 dilution and 1 ml of 1:10 dilution into 9 ml distilled water to make 1:100 dilutions and so on. The diluted sample was inoculated on Pseudomonas Agar and Cetrimide Agar for the isolation and identification of *Pseudomonas* spp.

3.2.3.2 Pre-treatment of polyethylene

The polyethylene bags were cut in small strips and were transferred to a beaker with distilled water and stirred for 1 hour. Further, they were aseptically placed to ethanol solution 70% v/v for 30 minutes. Ethanol was used to disinfect the polyethylene and remove any organic matter adhering to its surface. Then, the polyethylene strips were transferred to a sterile Petri dish (El-Shafei et al 1998). Finally, the plastic strips were air dried and were weighted in fix mass.

3.2.4. Identification of the isolates

Identification of the isolates was performed according to their morphological, staining reaction, cultural and various biochemical characteristics by following Bergey's Mannual of Systematic Bacteriology (Kandler and Weiss 1986). Isolated colonies from the pure culture with oxidase positive were identified by performing the standard conventional biochemical tests. Furthermore, the isolates were subcultured on Cetrimide Agar (CA), a selective media for the isolation of *P. aeruginosa* and the plates were observed for bacterial growth after aerobic incubation in 37°C for 24 hours.

3.2.4.1 Identification with Gram staining

Gram staining was performed for the presumptive identification of the bacteria according to standard technique. *Pseudomonas* spp. was identified as gram negative rod.

3.2.4.2 Identification with Biochemical Tests

Typical colonies of bacterial isolates were subcultured on Nutrient agar and incubated at 37°C for 24 hours. After incubation, fresh culture of test organism was inoculated into different biochemical media. *P. aeruginosa* and *P. fluorescence* were characterized and identified using a combination of colony morphology, Gram stain characteristics and different biochemical tests. Catalase test, Oxidase test, Indole test, Methyl red test, Voges Proskauer test, Citrate utilization test, Oxidative/Fermentative test, Urease test, Gelatin hydrolysis test, Xylose and Glucose fermentation test, growth at 4°C temperature and Growth on 7% NaCl tests were performed for the identification of *P. aeruginosa* and *P. fluorescence*. Result interpretation was done based on the identifying characteristic of the isolates (Forbes et al 2007).

3.2.5 Inoculation of *Pseudomonas* spp. in Nutrient agar plate

The *Pseudomonas* spp. was inoculated by carpet culture method onto Nutrient agar plates containing polythene strips and incubated at 30°C and 37°C separately for one month. During one month, potency of degradation i.e. decrease in the weight of the plastic samples was determined. Negative control was maintained by adding the same quantity of plastic strips in the Nutrient agar plate without inoculation of the bacteria and incubated together with test at the same temperature.

3.2.6 Dry weight determination of recovered polyethylene

The residual polyethylene strips were recovered from the culture plates. The dry weights of recovered polyethylene from the culture media were taken in one month for accounting the rate of biodegradation. The bacterial cell mass adhering to the polyethylene surface was washed by a 2% (v/v) aqueous sodium dodecyl sulphate solution for 2 hours and finally with distilled water (Hadad et al 2005). The washed polyethylene particles were air-dried and weighed. The weight loss of the plastics was calculated by using the following formula.

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

3.3 Data analysis

All the results were entered in the worksheet of Microsoft Office Excel. The data was calculated and analyzed by using above formula.

CHAPTER IV

RESULTS

During six months period, total of 60 soil samples were collected and processed in the Microbiology Laboratory of Central Department of Microbiology. The soil samples were around the plastic waste from Sisdol, Sanothimi, Teku and Balkhu included and processed. The 24 isolates were isolated and identified as *P. aeruginosa* and *P. fluorescence* by the general microbiological techniques and biochemical reactions.

The average degradation of polythene by using *Pseudomonas* spp. was found to be 7.9% during one month. The *Pseudomonas* spp. was found to be degraded 7.6% and 8.2% of the polythene at 30°C and 37°C temperature respectively. The identified isolates i.e. *P. aeruginosa* was able to degrade 7.3% and 8.5%, the *P. fluorescence* degraded 7.8% and 7.9% of the polythene at 30°C and 37°C temperature respectively during one month.

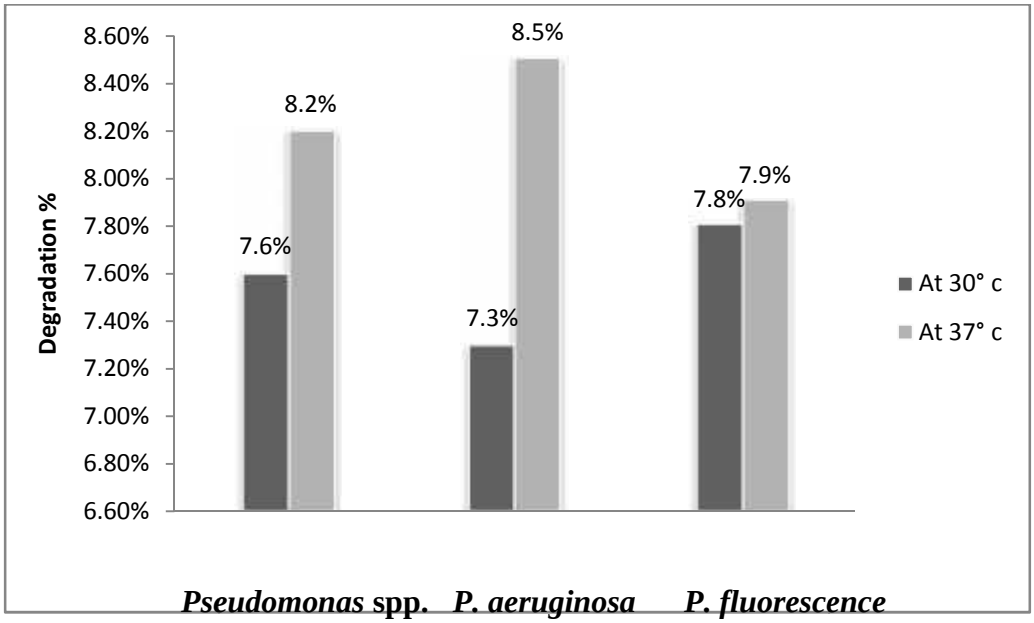
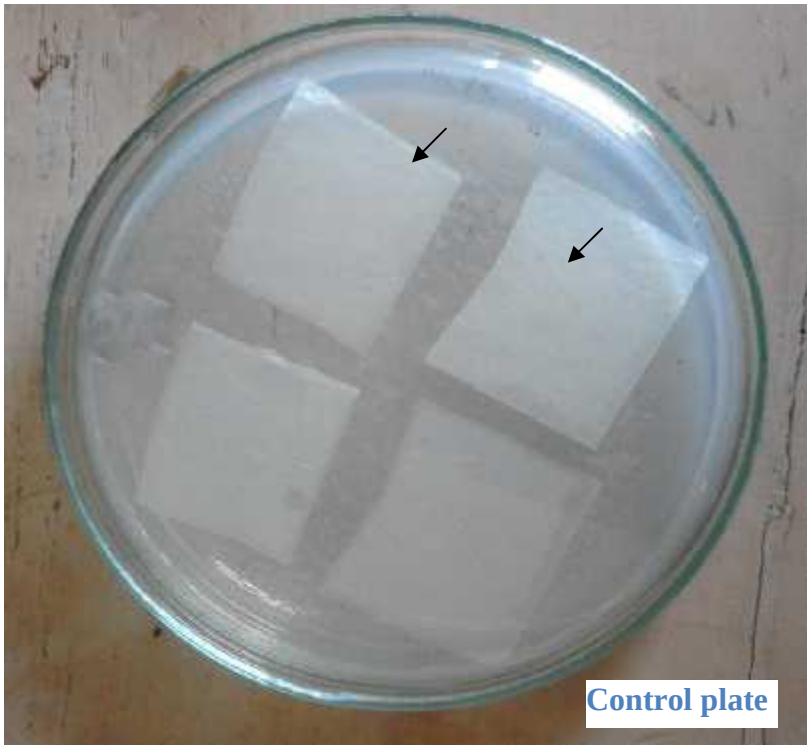


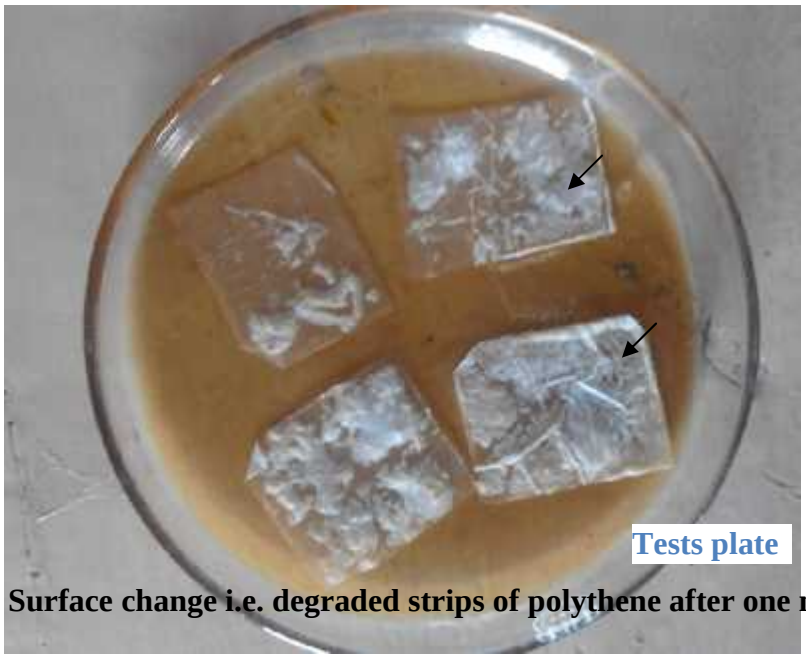
Figure 1: Polythene degradation by *Pseudomonas* spp.

4.1 Degradation based on surface change in plastic sample

The surface change of the plastic samples was observed after incubation with soil isolates. The surface of the plastic samples was turned from smooth to rough with cracking after one month represents the degradation of the polythene strips by *Pseudomonas* spp.



Photograph 1: Nutrient media containing polythene strips as control



Photograph 2: Surface change i.e. degraded strips of polythene after one month

4.2 Polythene degradation based on temperature

4.2.1 Degradation at 30°C

The polythene degraded by the *Pseudomonas* spp. isolated from the Sisdol dumping site soil was found to be 7.8%. From the same site, The *P. aeruginosa* and *P. fluorescence* were degraded 6.1% and 9.7% of polythene respectively at 30°C temperature during one month. The polythene degraded by the *Pseudomonas* spp. isolated from the Sanothimi household garbage site soil was found to be 8.9%. The *P. aeruginosa* and *P. fluorescence* isolated from same site soil were degraded 8.8% and 8.9% of polythene respectively.

At the same temperature and time duration, the *Pseudomonas* spp. isolated from the Balkhu dumping site soil was found to degrade 6.8% of polythene. The *P. aeruginosa* and *P. fluorescence* were able to degrade 8.0% and 5.7% of polythene from same site. The *Pseudomonas* spp. isolated from the Teku dumping site soil was found to degrade 6.3% of polythene. From the same site, the *P. aeruginosa* and *P. fluorescence* were able to degrade 6.2% and 6.6% of polythene respectively.

Table 1: Degradation of polythene by *Pseudomonas* spp. at 30°C

Soil from → Microorganism↓	Sisdol	Sanothimi	Balkhu	Teku
Pseudomonas spp.	7.8%	8.9%	6.8%	6.3%
P. aeruginosa	6.1%	8.8%	8.0%	6.2%
P. fluorescence	9.7%	8.9%	5.7%	6.6%

4.2.2 Degradation at 37°C

The polythene degraded by the *Pseudomonas* spp. isolated from the Sisdol dumping site soil, was found to be 7.5%. From the same site, the *P. aeruginosa* and *P. fluorescence* were degraded 8.1% and 6.9% of polythene respectively at

37°C temperature during one month. The polythene degraded by the *Pseudomonas* spp. isolated from the Sanothimi household garbage site soil was found to be 9.0%. The *P. aeruginosa* and *P. fluorescence* isolated from same site soil degraded 9.2% and 8.8% of polythene respectively after one month of incubation.

At the same temperature and time duration, the *Peudomonas* spp. isolated from the Balkhu dumping site soil was found to degrade 8.2% of polythene. The *P. aeruginosa* and *P. fluorescence* were able to degrade 8.9% and 7.6% of polythene from same site. The *Pseudomonas* spp. isolated from the Teku dumping site soil was found to be degraded 8.1% of polythene. From the same site, the *P. aeruginosa* and *P. fluorescence* were able to degrade 8.0% and 8.1% of polythene.

Table 2: Degradation of polythene by *Pseudomonas* spp. at 37° c

Place→ Microorganism↓	Sisdol	Sanothimi	Balkhu	Teku
Pseudomonas spp.	7.5%	9.0%	8.2%	8.1%
P. aeruginosa	8.1%	9.2%	8.9%	8.0%
P. fluorescence	6.9%	8.8%	7.6%	8.1%

4.3 Plastic degradation based on isolates isolated from different places of soil

4.3.1 Isolates from Sisdol dumping site soil

The polythene degraded by the *Pseudomonas* spp. isolated from the Sisdol dumping site soil was found to be 7.8% and 7.5%. The *P. aeruginosa* isolated from Sisdol dumping soil degraded 6.1% and 8.1% of polythene. The *P. fluorescence* isolated from the same site soil degraded 9.7% and 6.9% of the polythene at 30°C and 37°C temperature respectively during one month.

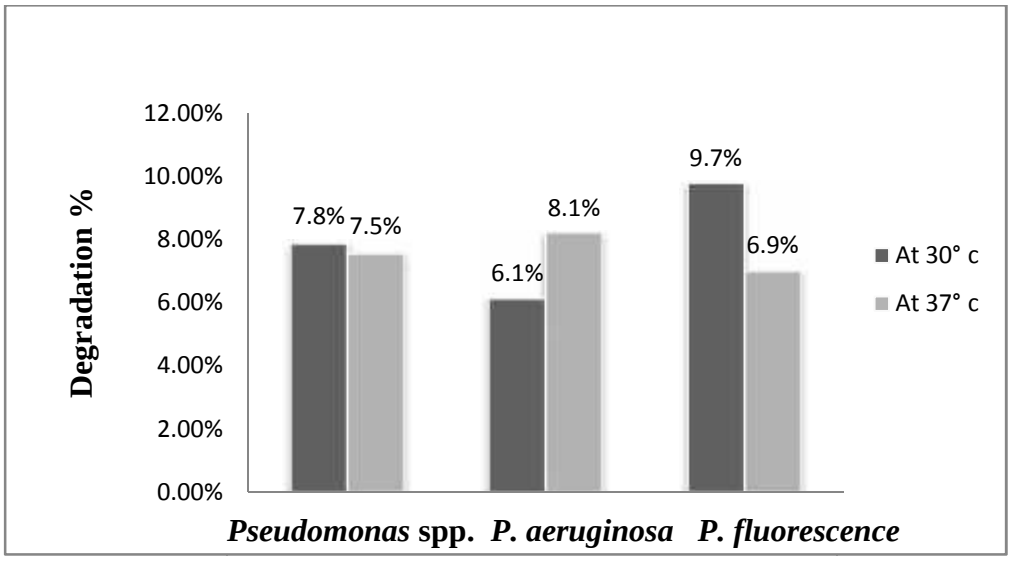


Figure 2: Polythene degradation by isolates from Sisdol dumping site soil

4.3.2 Isolates from Sanothimi household garbage site soil

The polythene degraded by *Pseudomonas* spp. isolated from the Sanothimi household garbage site soil was found 8.9% and 9.0% at 30°C and 37°C temperature. The *P. aeruginosa* isolated from same site soil degraded the 8.8% and 9.2% of polythene. And the *P. fluorescence* isolated from the same site soil degraded 8.9% and 8.8% of the polythene at 30°C and 37°C temperature during one month.

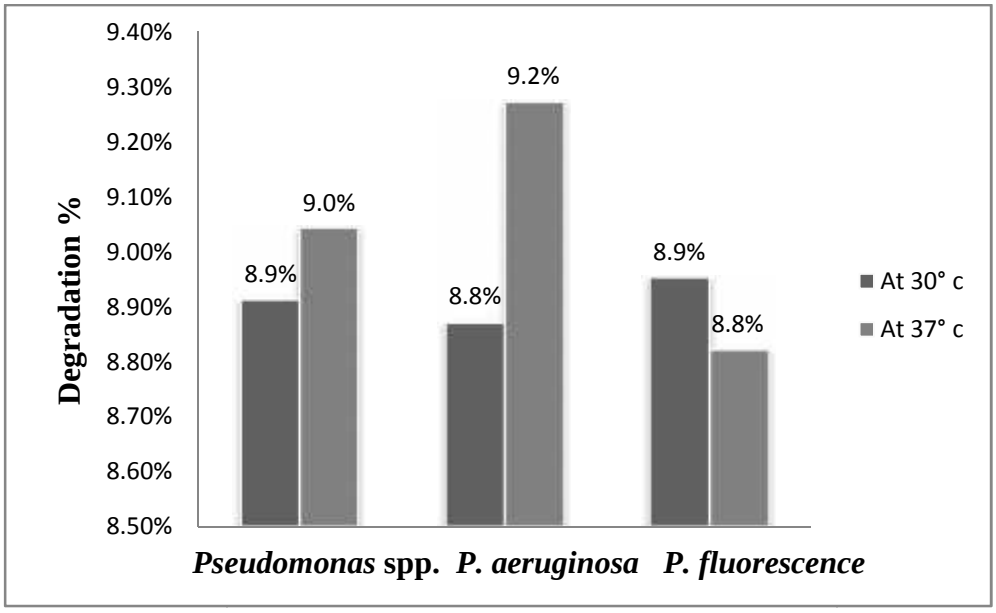


Figure 3: Polythene degradation by isolates from Sanothimi household garbage site soil

4.3.3 Isolates from Balkhu dumping site soil

The polythene degraded by the *Pseudomonas* spp. isolated from the Balkhu dumping site soil was found 6.8% and 8.2% at 30°C and 37°C temperature. The *P. aeruginosa* isolated from same site soil degraded 8.0% and 8.8% of polythene. The *P. fluorescence* isolated from the same site soil degraded the 5.7% and 7.6% of the polythene at 30°C and 37°C temperature respectively during one month.

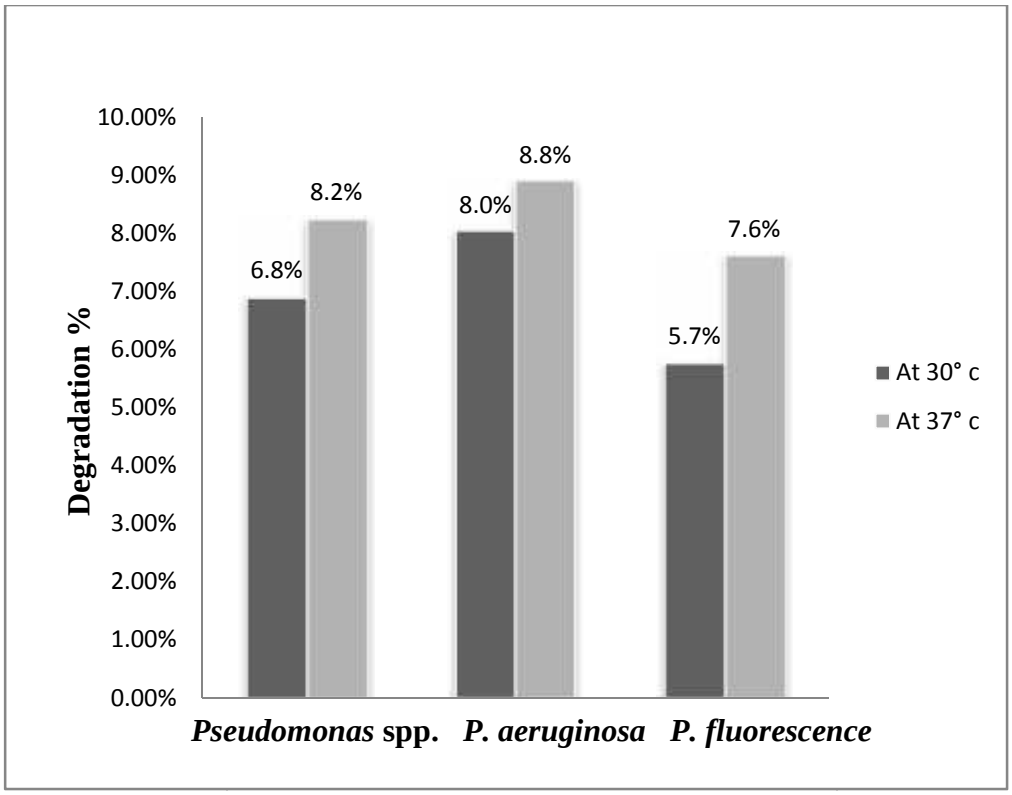


Figure 4: Polythene degradation by isolates from Balkhu dumping site soil

4.3.4 Isolates from Teku dumping site soil

The polythene degraded by the *Pseudomonas* spp. isolated from the Teku dumping site soil was found 6.3% and 8.0% at 30°C and 37°C temperature. The *P. aeruginosa* isolated from same site soil degraded 6.1% and 8.0% of polythene.

The *P. fluorescence* isolated from same site soil degraded 6.6% and 8.1% of polythene at 30°C and 37°C temperature during one month.

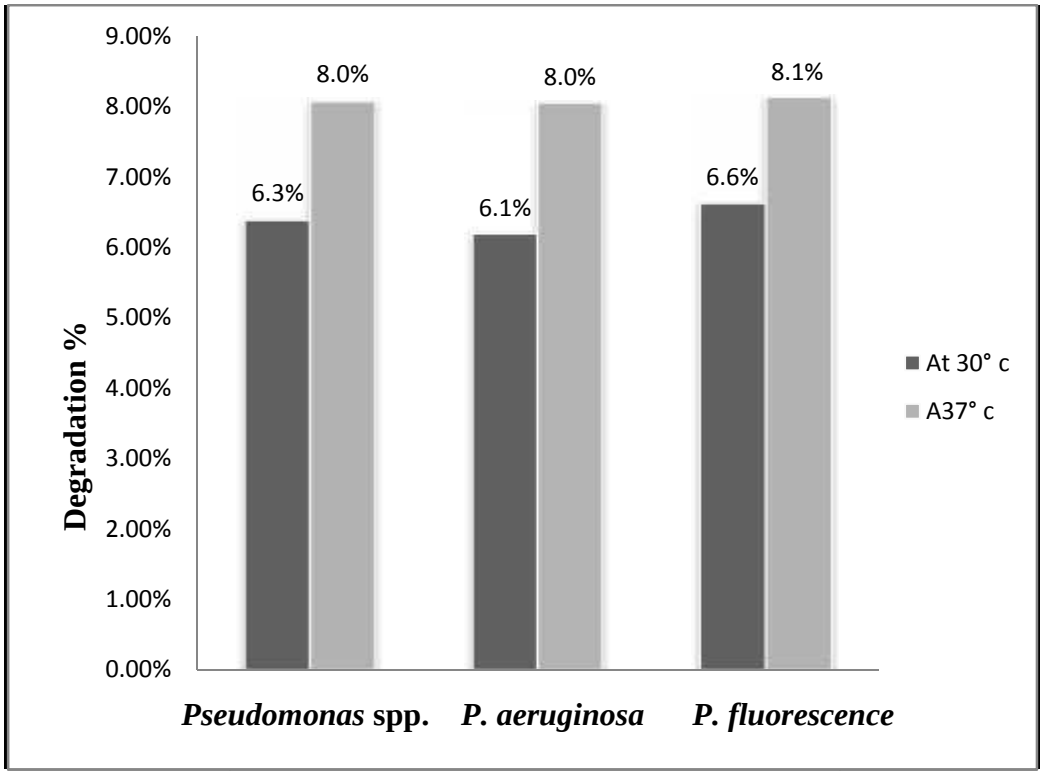


Figure 5: Polythene degradation by isolates from Teku dumping site soil

CHAPTER V
DISCUSSION

Solid waste is the global issue. Plastic waste is the major component of solid waste and source of environmental pollution of the world. The solid waste pollute the water, soil and air which has direct impact on human health, forest, animals and the fertile land. So, the solid waste management is necessary for the developing countries for the reduction of risk due to solid waste. In this research, the *Pseudomonas* spp. was isolated and identified and used for the screening of polythene degradation.

Polyethene bags are made of polyethylene. The worldwide utility of polyethylene is a rate of 12%, an approximately 140 million tones of synthetic polymers are produced worldwide each year. Accumulation rate of plastic waste in the environment is 25 million tons/year (El-Shafei et al 1998). With such huge amount of polyethylene getting accumulated in the environment and their disposal evokes a big ecological issue. It takes long time for their efficient degradation. Therefore, this research may supportive to the way of reduction of the polythene through biodegradation. The main dumping sites of Nepal are Sisdol, Pokhara and Karaute Dada Sanitary landfill sites. The composition of the wastes of these landfill sites are plastic, paper, glass, rubber, leather, metal, construction demolition waste, organic and other waste. Among them, the amount of plastic is high as an inorganic solid waste. Thus, the biodegradation of the plastic wastes in those landfill sites will help in the reduction of waste volume and area covered by inorganic wastes in the landfill sites (Thapa 2011).

In this research, the isolated *Pseudomonas* spp. was aseptically placed over the surface of the plastic strips containing Nutrient agar plate for the aerobic degradation. The growth and accumulation of the microbial cell on the surface of the plastic strips shows the rough and cracking of the plastic strips. It means the *Pseudomonas* spp. has the potency of the polythene degradation.

The soil bacteria were isolated from plastic contaminated soil sample. The bacterial isolates such as *D. nigrificans* and *P. alcaligenes* were identified by morphological and biochemical characterization. The biodegradation efficacy of *D. nigrificans* and *P. alcaligenes* by using polythene bag were studied. The *P. alcaligenes* was found to be more effective than *D. nigrificans* in degradation of polythene bag at 30 days. An increase in incubation period there is a dramatic increase in weight loss of polythene bag (Begum et al 2015). The bacteria caused the biodegradation ranging from 2.1% to 20.5% for polythene and from 0.5% to 8.1% for plastics. Polythene and plastic degraded to various extents by *Pseudomonas* spp. (37.1% and 28.4%) *Streptomyces* spp. (46.1% and 35.7%) and *Aspergillus* spp. (20.9% and 16.8%) in 6 month period in liquid (shaker) culture (Usha et al 2011).

In present study, the *Pseudomonas* spp. isolated from the different dumping sites of Kathmandu valley and Sisdol dumping site was degraded 7.6% and 8.2% of the polythene at 30°C and 37°C temperature during one month. This is due to the accumulation of microbial cell and production of enzymes by microorganism during the degradation process acted on the surface of polythene. The enzyme produced by the microorganism breakdowns the carbon, hydrogen bond of the polymer into monomer is easily utilizes the microbial cells (El-Shafei et al 1998).

Hadad et al (2005) reported the *D. nigrificans* degrade 10.2%, 13.2% and 16.2 % of polythene bag at 10, 20 and 30 days incubation respectively. At the same time, *P. alcaligenes* degraded 10.5%, 14.7% and 16.2 % of polythene bag at 10, 20 and 30 days incubation respectively. An increase in incubation period there is a dramatic increase in weight loss of polythene bag. Among the two isolates tested, *P. alcaligenes* was found to be more effective in degradation of polythene bag at 30 days. Previously, (Norman et al 2002; Tadros et al 1999) have reported on the biodegradability potential of *P. fluorescens* and *P. aeruginosa* on synthetic plastics.

In this study, the *P. aeruginosa* able to degrade 7.3% and 8.5%, the *P. fluorescence* able to degrade 7.8% and 7.9% of the polythene at 30°C and 37°C temperature respectively during one month. Among the two isolates tested, *P. aeruginosa* and *P. fluorescence* was found to be more effective in degradation of polythene bag at 37°C and 30°C temperature during one month respectively.

Degradation of plastic cups and polythene bags studied using bacteria and fungi for one month period, among which bacteria, *Pseudomonas* spp. degraded 20.5% of polythene and 8.1% of plastics, while fungal species, *A. glaucus* degraded 28.8% of polythene and 7.2% of plastics (Kathiresan 2003).

After pretreatment with nitric acid, *P. aeruginosa* was able to degrade 0.25 gram of LDPE by 50.5% in 2 months (Rajandas et al 2012). However, no chemical pretreatment was needed for *Pseudomonas* spp. AKS2 to degrade LDPE films, albeit only 5% of the total mass of 300 mg was degraded within 45 days (Tribedi and Sil 2013c). Also without any pretreatment, an uncharacterized *Pseudomonas* spp. was found to degrade 28.6% of low MW PE (MW 1700 Da) in a sterilized compost condition after 40 days (Yoon et al 2012).

In this study, after pretreatment with 70 % ethanol, the plastic degraded by the *Pseudomonas* spp. isolated from the Sisdol dumping site soil was found 7.8% and 7.5% at 30°C and 37°C temperature. Among the two isolates tested isolated from the Sisdol dumping soil, *P. aeruginosa* able to degrade 6.1% , 8.1% and *P. fluorescence* degraded the 9.7% , 6.9% of the plastic at 30°C and 37°C temperature during one month.

The useful bacteria identified for the degradation of plastic are *Pseudomonas* spp., *Streptococcus* spp., *Staphylococcus* spp., *Micrococcus* spp. and *Moraxella* spp., *B. subtilis*, *B. amylolyticus* and *Arthobacter defluvii*. *B. amylolyticus* has more potential and *B. subtilis* has less potential to degrade plastic as compared to other

bacteria (Sharma 2013). There are no significant change in mass and appearance after 180 days of inoculation (Dey et al 2012).

In this research, the *Pseudomonas* spp. isolated from the Sanothimi household garbage site soil was found to be degrade 8.9% and 9.0% of polythene. Among them, the *P. aeruginosa* and *P. fluorescence* was able to degrade (8.8% , 9.2%) and (8.9% , 8.8%) of plastic at 30°C and 37°C temperature respectively during one month. The *Pseudomonas* spp. isolated from the Balkhu dumping site soil was found to degrade 6.8% and 8.2% of polythene. From there, the *P. aeruginosa* and *P. fluorescence* were found able to degrade (8.0%, 8.8%) and (5.7%, 7.6%) of plastic respectively. The polythene degraded by the *Pseudomonas* spp. isolated from the Teku dumping site soil was found to be 6.3% and 8.0%. The *P. aeruginosa* degraded the 6.1% and 8.0% of polythene. From the same site, the *P. fluorescence* degraded the 6.6% and 8.1% of the plastic at 30°C and 37°C temperature respectively during one month. Comparison among these isolates of all four sentinel sites, the highest amount of polythene was degraded by *P. fluorescence* at 30°C isolated from Sisdol landfill site soil and by *P. aeruginosa* at 37°C temperature isolated from Sanothimi household garbage site soil.

Pseudomonas spp. from sewage sludge dump (P1) from Tamil Nadu was found to degrade polyethylene efficiently with 46.2% for natural and 29.1% for synthetic polyethylene. In contrast, *Pseudomonas* spp. from household garbage dump (P2) gave the lowest biodegradability of 31.4% and 16.3% for natural and synthetic polyethylene, respectively. However, *Pseudomonas* spp. isolated from Tamil Nadu textile effluents drainage site gave an intermediate biodegradability of 39.7% and 19.6% for natural and synthetic polyethylene respectively (Nanda et al 2010).

P. fluorescence was the most active of the tested microorganisms degrading approximately 18% and 16% of polythene at 9 and 12 months period respectively and 3.8% of plastics in twelve month period under field condition. Also, 8.0% and 5.6% of polythene and plastics were respectively degraded in a month under

laboratory condition. The biodegradation of the polythene material was relatively faster and earlier than that of the plastics with the polythene degrading for up to 12.9%, 16% and 15% at 9 months of analysis while only 2%, 3.8% and 4.8% of the plastic materials were degraded at 12 month by each of *S. aureus*, *P. fluorescens* and *A. niger* respectively (Thomas et al 2015).

The isolation of most efficient microorganism using different soil samples were taken from three waste disposal sites such as industrial plastic waste dump area, leather industry waste and domestic waste dump area. The various microorganisms were isolated from the soil samples grown in an inorganic media (M9 media). There are some microorganisms that have the capacity to degrade plastic waste up to 51.5%. This result is achieved due to addition of starch as additive in M9 media. This study reveals that *Pseudomonas* spp. posses greater potential to degrade polyethylene (Agrawal and Singh 2016).

The biodegradation of plastic material was analyzed one month of incubation in liquid culture method. The microbial species found associated with the degrading materials were identified as three Gram positive and two Gram negative bacteria. The microbial species associated with the polythene materials were identified as *B. amylolyticus, B. firmus, P. putida, P. fluroscence* and *B. subtilis*. The efficacy of microbes in the degradation of plastics were analyzed in liquid (shaker) culture method among the bacteria *P. putida* degrades plastic more in one month (30% weight loss/month) period compared to others and lowest degradation rate was observed in case of *B. subtilis* (22% weight loss/month). This work reveals that *P. putida* posses greater potential to degrade plastics when compared with other bacteria (Jumaah 2017).

P. putida S3A that has ability to degrade nylon 6 film, crude nylon 6 and nylon 66 as sole source of nitrogen and carbon isolated from soil contaminated with plastic waste was included. It was found that these conditions are growing *P. putida* S3A in mineral salt medium (pH 6.5) containing 0.5% of polyethylene and incubated

with shaking (180 rpm) at 37°C for seven days. In addition, it has been found that this bacterium was able to survive with up to 0.9% of polyethylene. In order to ensure that this bacterium was capable to degrade polyethylene, the Fourier Transformer Infrared Red Spectroscopy (FTIR) was used. Results indicated that polyethylene was degraded by *P. putida* S3A used the O-H, C-O and C-H groups as carbon source (Jailawi et al 2015).

Microbial degradation of plastics is caused by certain enzymatic activities that lead to a chain cleavage of the polymer into oligomers and monomers. These water soluble enzymatically cleaved products are further absorbed by the microbial cells where they are metabolized. The degradation leads to breaking down of polymers to monomers creating an ease of accumulation by the microbial cells for further degradation (El-Shafei et al 1998).

Degradation of PEG by *P. stutzeri* JA1001 involved a single intracellular PEG dehydrogenase that produced glyoxylic acid (Obradors and Aguilar 1991). Alkane hydroxylases from the AlkB family in *Pseudomonas* spp. E4 were involved in the degradation of PE with MW up to 27 000 Da (Yoon et al 2012). Furthermore, the extracellular PVA oxidase found in a number of *Pseudomonas* spp., including *Pseudomonas* spp. O-3, *P. vesicularis* PD and *Pseudomonas* spp. VM15C can oxidize PVA into a diketone structure (Kawai and Hu 2009).

Different types of enzymes such as esterases, lipases and cutinases are hydrolases that are instrumental in plastic degradation (Mohan et al 2016; Novotny et al 2015; Ruiz et al 1999; Sangale et al 2012). Hydrolases are important for enzymatic polymer cleavage wherein ester bonds are broken through a nucleophilic attack on carbonyl carbon atoms (Devi et al 2016). For instance, an esterase from *Pseudomonas* spp. AKS2 was able to break the ester bonds in PES to generate succinic acid, a TCA cycle metabolite (Tribedi et al 2012).

Polyurethane degrading enzymes are thought to be primarily extracellular esterases or proteases that are either membrane-bound or secreted extracellularly (Cregut et al 2013; Mukherjee et al 2011; Shah et al 2013). The term polyurethanase is often used to describe enzymes responsible for the degradation of PU (Ruiz et al 1999; Stern and Howard 2000). However, this term is used without definitive confirmation of the hydrolysis of the carbamate bond. Therefore, it is recommended that polyurethanase should be reported as hydrolases or esterases (Biffinger et al 2014). To this point, an extracellular enzyme from *P. chlororaphis* with esterase and protease activities was shown to degrade successfully polyester PU. This enzyme was also classified as a serine hydrolase because it could be inhibited by phenylmethane sulfonyl fluoride (Ruiz et al 1999). Polyurethane was degraded significantly by *Pseudomonas* spp. lipase but only partially degraded by a recombinant esterase from *P. fluorescens* (Biffinger et al 2015).

The increase in production and lack of biodegradability of commercial polymers mainly commodity plastics used in packaging, industry and agriculture, has focused public attention on a potentially huge environmental accumulation and pollution problem that could persist for centuries (Albertsson et al 1987). Plastic waste is disposed off through the process such as land filling, incineration and recycling. Several communities are now more sensitive to the impact of discarded plastic on the environment because of their persistence in our environment, including deleterious effects on wildlife and on the aesthetic qualities of cities and forests. In addition to this, the burning of PVC plastics produces persistent organic pollutants (POPs) known as furans and dioxins. Many polymers like PVC and other halogen and nitrogen-containing polymers can form corrosive and toxic substances upon burning and can cause health hazards or pollute the environment. Also, the manufacturing of plastics often creates large quantities of chemical pollutants. Non-biodegradable polymers also have the capacity to act as disease foci because they persist in the environment for a very long period of time enabling organisms to accumulate (Jayasekara et al 2005).

In Nepal, Municipality Solid Waste is composed of 56% organic waste, 16% plastics and 16% paper and paper products. From total solid waste in Kathmandu Metropolitan city, the Composition of plastic waste from Household, Institutional and Commercial field is found 15.9%, 24.5%, 24.2% respectively. In aggregate, the composition of the plastic waste is 21.6% from the total solid waste in Kathmandu Metropolitan city. This is due to the rapid and uncontrolled urbanization, lack of public awareness and poor management by municipalities has intensified environmental problems in towns in Nepal including unsanitary waste management and disposal (Banskota 2015).

The problem of waste can be solved to some extent by using biodegradable plastics. Starch based degradable plastics is most commonly suggested for uses in composting of lawn, garden and shrub litter which could lessen the volume of material entering the landfills by up to 20% (Lee et al 1991). Attention in using biodegradable plastics for packaging, medical and agricultural applications has increased in last decades (Leja and Lewandowicz 2010; Orhan et al 2004). However, none of biodegradable of plastics was efficiently biodegradable in landfills. Therefore none of the products has gained extensive use (Kathiresan 2003).

The degradation of most synthetic plastics in nature is a very slow process that involves environmental factors which follows the action of wild microorganisms. The oxidation or hydrolysis by enzyme to create functional groups that improves the hydrophylicity of polymer is the primary mechanism for the biodegradation of high molecular weight polymer. Consequently, the main chain of polymer is degraded resulting in polymer of low molecular weight and having feeble mechanical properties which makes it more accessible for further microbial assimilation (Albertsson et al 1987).

Because a microbial environment is required in the process of degradation, therefore PHA is not affected by moisture alone and is indefinitely stable in air.

PHAs have attracted the industrial attention for long use in the production of biodegradable and biocompatible thermoplastics. Sturm test has been used by many researchers to study the biodegradation of biodegradable polymers and the aliphatic and aromatic compounds (Kim and Rhee 2001).

Pseudomonas species, which have been touted historically for contaminant remediation due to their ability to degrade oil contaminants, also have the potential to degrade and metabolize plastic wastes. Biodegradation of structurally different plastics and their associated byproducts is species dependent due to the required set of enzymes. A comprehensive understanding of the enzymes involved in the degradation of different plastics as well as the identification of their extracellular versus intracellular localization will inform bioengineering approaches for optimizing plastic biodegradation. For instance, the hydrocarbon degradation genotype of *P. putida* was used to transform a native marine bacterium in order to confer capabilities to degrade hydrocarbons (Latha and Lalithakumari 2001).

Plastic polymers can be broken down to varying degrees both physically and biologically with minimal generation of compounds amenable to metabolism inside the cells. Intermediate products produced from the first steps of biodegradation can interfere with future steps needed for uptake and subsequent intracellular metabolism (Barth et al 2016; Kolvenbach et al 2014).

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Plastic waste is the major problem of the environmental pollution. The degradation rate of polythene is slow process. This study showed that the degradation of plastic by *Pseudomonas* spp. during one month differs by temperature and isolates from different places.

The rate of degradation of polythene by *Pseudomonas* spp. isolated from Sanothimi household garbage site soil showed higher than isolates from the Sisdol dumping site and Balkhu and Teku dumping site soil. The *P. fluorescence* and *P. aeruginosa* isolated from Sisdol landfill site and Sanothimi household garbage site soil isolates were found to degrade 9.7% and 9.2% of polythene at 30°C and 37°C temperature respectively. These percentages of polythene degradation were found higher amount than other isolates of them from same site and from different sites soil isolates during one month. Therefore, the *Pseudomonas* spp., the degradation of polythene was found to be different potential.

In this study, the degradation of the polythene by *Pseudomonas* spp. is comparable to the other Asian and Indian research journal regarding degradation of polythene. This research showed degradation rate of polythene is higher during one month. The degradation of polythene by *Pseudomonas* spp. was found to be significantly potential during one month. In this way this study helps the supportive guideline for the plastic waste management and has future research needs.

6.2 Recommendations

1) *Pseudomonas* spp. was found to be potential of degradation of polythene of 13 micron size plastic can be useful for general purpose.

2) Continuous degradation of the polythene by *Pseudomonas* spp. should be done in order to reduce, manage and control the solid waste.

3) Enzymatic degradation of plastic also can be done by following this study.

4) The degradation of ploythene can be done by using *Pseudomonas* spp. at different time range.

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APPENDIX A

LIST OF EQUIPMENTS AND MATERIALS

A. Equipments

Autoclave, Incubator, Hot air oven, Microscope, Refrigerators, Weighing machine, Bunsen burner and inoculating loops.

B. Glass wares

Beakers, Conical flask, Glass rods, Measuring cylinder and Petri-dishes.

C. Microbiological media

All the media was used Himedia Laboratories.

Nutrient Agar	Nutrient Brooth
Pseudomonas Agar	Cetrimide Agar
MRVP Broth	Simmons Citrate Agar
Urea Agar Base	Sulphur Indole Motility Media
Triple Sugar Iron Agar	

D. Chemical reagents

Ethanol, Catalase reagents (3% H_2O_2), Oxidase reagents (1% tetramethyl p-phenylenediaminedihydrochloride), Kovac's reagent, Barritt's reagent (40% KOH, 5% ά– napthol in a ratio of 1:3) Conc. H_2SO_4, 7% NaCl, Glycerol, Gram's reagents, glucose and xylose.

APPENDIX B

COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA

A. Composition and preparation of different culture media

a. Pseudomonas Agar

Ingredients	Gms / Litre
Peptic Digest of Animal Tissue	20.0
Magnesium Chloride	1.4
Potassium Sulphate	10.0
Agar	15.0
Final pH (at 25°C)	7.0±0.2

Directions: 46.4 grams of the Pesudomonas Agar was suspended in 1000 ml distilled water containing 10 ml glycerol. The medium was dissolved completely and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The medium was aseptically poured into the sterile Petri plates.

b. Cetrimide Agar (HIMEDIA)

Ingredients	Gms / Litre
Pancreatic digest of gelatin	20.0
Magnesium chloride	1.4
Potassium sulphate	10.0
Cetrimide	0.3
Agar	15.0
Final pH (at 25°C)	7.2±0.2

Directions: 46.7 grams of medium was Suspended in 1000 ml distilled water containing 10 ml glycerol. The medium was heated, to boiling, to dissolve the medium completely. The medium was sterilized by autoclaving at 15 lbs pressure

(121°C) for 15 minutes. If desired, rehydrated contents of 1 vial of Nalidixic Selective Supplement (FD130) may be added aseptically to 1000 ml medium. The medium was mixed well and poured into sterile Petri plates.

c. Nutrient Agar (HIMEDIA)

Ingredients	**Gms / Litre**
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
Final pH (at 25°C)	7.4±0.2

Directions: 28 grams of medium was suspended in 1000 ml distilled water. The medium was heated to dissolve the medium completely. It was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Then the medium was mixed well before pouring and dispensed into the Petri plates.

B. **Composition and reparation of different biochemical tests media**

a. MR-VP Medium (HIMEDIA)

Ingredients	**Gms / Litre**
Buffered peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0
Final pH (at 25°C)	6.9±0.2

Directions: 17 gram of medium was suspended in 1000 ml of distilled water and heated to dissolve the medium completely. The medium was distributed in test tubes in 10 ml amounts and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

b. Simmons Citrate Agar (HIMEDIA)

Ingredients	**Gms / Litre**
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothymol blue	0.8
Agar	15.0
Final pH (at 25°C)	6.8±0.2

Directions: 24.28 grams of medium was suspended in 1000 ml distilled water and boiled to dissolve the medium completely. The medium was mixed well and distributed in tubes or flasks. The media was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. After then the tubes containing medium were tilted to form slant.

c. SIM (Sulphur Indole Motility) Agar

Ingredients	**Gms / Litre**
Beef extract	3.0
Peptic digest of animal tissue	30.0
Peptonized iron	0.2
Sodium thiosulphate	0.025
Agar	3.0
Final pH (at 25°C)	7.3±0.2

Directions: 36.23 grams of media was suspended in 1000 ml distilled water. The media was boiled to dissolve the medium completely and dispensed into tubes.

The media was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Then the medium was allowed the tubes to cool in an upright position.

d. Urea Agar Base (HIMEDIA)

Ingredients	**Gms / Litre**
Dextrose	1.0
Peptic digest of animal tissue	1.5
Sodium chloride	5.0
Monopotassium phosphate	2.0
Phenol red	0.012
Agar	15.0
Final pH (at 25°C)	6.8±0.2

Directions: 24.51 grams of media was suspended in 950 ml distilled water. The media was heated to boil to dissolve the medium completely. The media was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The media was cooled to 50°C and aseptically added 50 ml of sterile 40% Urea Solution and mixed well. The 5 ml media was dispensed into sterile tubes and allowed to set in the slanting position.

C. Composition and preparation of different staining and test reagents

For Gram stain

a. Crystal Violet Solution

Crystal Violet	20.0 g
Ammonium Oxalate	9.0 g
Ethanol or Methanol	95.0 ml

Direction: In a clean piece of paper, 20 gram of crystal violet was weighted and transferred to a clean brown bottle. Then, 95 ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 9 gm of ammonium oxalate dissolved in 200 ml of D/W was added. Final volume was made 1000 ml by adding D/W.

b. Lugol's Iodine

Potassium Iodide	20.0 g
Iodine	10.0 g
Distilled Water	1000 ml

Directions: The 250 ml of D/W, 20 gm of potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it was dissolved completely. Final volume was made 1000 ml by adding D/W.

c. Acetone- Alcohol Decolorizer

Acetone	500 ml
Ethanol (Absolute)	475 ml
Distilled Water	25.0 ml

Direction: The 25 ml of D/W, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 500 ml acetone was added to the bottle and mixed well.

d. Safranin (Counter stain)

Safranin	10.0 g
Distilled Water	1000 ml

Direction: In a clean piece of paper, 10 gm of safranin was weighted and transferred to a clean bottle. Then 1000 ml D/W was added to the bottle and mixed well until safranin dissolved completely.

D. Composition and preparation of biochemical test reagents

a. Catalase Reagent (For Catalase Test)

Hydrogen Peroxide	3.0 ml
Distilled Water	97.0 ml

Direction: The 97 ml of D/W, 3 ml of hydrogen peroxide was added and mixed well.

b. Oxidase Reagent (impregnated in a Whatman's No.1 filter paper) (For Oxidase Test)

Tetramethyl *p*-phenylenediaminedihydrochloride (TPD)	1.0 g
Distilled Water	100 ml

Direction: This reagent solution was made by dissolving 1 gm of TPD in 100 ml D/W. To that solution strips of Whatman's No.1 filter paper were soaked and drained for about 30 seconds. These strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

c. Kovac's Indole Reagent (For Indole Test)

Isoamyl alcohol	30 ml
p- dimethyl aminobenzaldehyde	2.0 g
Conc. Hydrochloric acid	10.0 ml

Direction: In 30 ml of isoamyl alcohol, 2 grams of *p*-dimethyl aminobenzaldehyde was dissolved and transferred to a clean brown bottle. Then to that, 10 ml of conc. HCl was added and mixed well.

d. Methyl red solution (For Methyl Red Test)

Methyl red	0.05 g
Ethyl alcohol (absolute)	28.0 ml
Distilled Water	22.0 ml

Direction: To 28 ml ethanol, 0.05 g of the methyl red was dissolved and transferred to a clean brown bottle. Then 22 ml D/W was added to that bottle and mixed well.

e. Barritt's Reagents (For Voges- Proskauer Test)

Solution A

ά–napthol	5.0 g
Ethyl alcohol (absolute)	100 ml

Direction: The 25 ml ethanol, 5 g of ά–napthol was dissolved and transferred into a clean brown bottle. The final volume was made 100 ml by adding D/W.

Solution B

Potassium hydroxide	40.0 g
Distilled Water	1000 ml

Direction: The 25 ml D/W, 40 gm of the KOH was dissolved and transferred into a clean bottle. The final volume was made 100 ml by adding D/W.

APPENDIX C

IDENTIFICATION OF *P. aeruginosa* AND *P. fluorescence*

Characters	***P. aeruginosa***	***P. fluorescence***
Indole	Negative	Negative
Methyl Red	Negative	Negative
Oxidative/Fermentative	Oxidative	Oxidative
Citrate	Positive	Positive
Triple Sugar Iron Agar	Alk/no change	Alk/no change
Catalase	Positive	Positive
Oxidase	Positive	Negative
Urease	Negative	Positive
Motility	Positive	Positive
Morphology	Rod	Rod
Glucose Fermentation	Positive	Positive
Xylose Fermentation	Negative	Negative
Growth at 4°C	Negative	Positive
Growth on 7% NaCl	Negative	Positive
Gelatin Hydrolysis	Positive	Positive