SCREENING OF PLASTIC DEGRADING BACTERIA FROM DUMPED SOIL AREA

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MASTER OF SCIENCE IN LIFE SCIENCE

By

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CERTIFICATE

This is to certify that the thesis entitled "SCREENING OF PLASTIC DEGRADING BACTERIA FROM DUMPED SOIL AREA" submitted to National Institute of Technology; Rourkela for the partial fulfilment of the Master degree in Life science is a faithful record of bonafide and original research work carried out by POOJA THAKUR under my supervision and guidance.

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DECLARATION

I, Pooja Thakur, M. Sc. Life Science, 4th semester, bearing roll no. 410ls2079, Department of Life Science, NIT, Rourkela declare that my project work titled — **Screening of plastic degrading bacteria from dumped soil area,** is original and no part of this work report has been submitted for any other degree or diploma. All the given information and works are true to my sense and knowledge.

POOJA THAKUR

Date:

Place:

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A. ABSTRACT

Plastic wastes accumulating in the environment are posing an ever increasing ecological threat. Plastics that are biodegradable can be considered environment friendly, they have an increasing range of potential application and are driven by the growing use of plastics in packaging. In this study, the biodegradation of polythene bag was analyzed 1 month of incubation in liquid culture method. Microbial counts in the degrading materials were recorded up to 0.0278×109 per gram for total heterotrophic bacteria. The microbial species found associated with the degrading materials were identified as two Gram positive and five Gram negative bacteria. The microbial species associated with the polythene materials were identified as *Bacillus subtilis, Bacillus amylolyticus, Arthobacter defluvii*. The efficacy of microbes in the degradation of plastics were analyzed in liquid (shaker) culture method, among the bacteria *Bacillus amylolyticus* degrades plastic more in 1 month (30% weight loss/month) period compared to others and lowest degradation rate was observed in case of *Bacillus subtilis* (20% weight loss/month). This work reveals that *Bacillus amylolyticus* posses greater potential to degrade plastics when compared with other bacteria.

Key words: Biodegradation, plastics, degradation.

1. INTRODUCTION

Plastics are defined as the polymers (solid materials) which on heating become mobile and can be cast into moulds. They are non metallic moldable compounds and the materials that are made from them can be pushed into any desired shape and sizes (saymour, 1989). Commonly plastics are used in many purposes including packaging, disposable diaper backing, agricultural films and fishing nets. Plastics and their use has become a part in all sectors of economy. Infrastructure such as agriculture, telecommunication, building and construction, consumer goods, packaging, health and medical are all high growth areas that ensures present demand for plastics. Plastic is the mother industry to hundreds of components and products that are manufactured and used in our daily life like automobiles parts, electrical goods, plastic furniture, defense materials, agriculture pipes, packages and sanitary wares, pipes and fittings, tiles and flooring, artificial leathers, bottles and jars, PVC shoes and sleepers hundreds of household items.

Plastics are used in packaging of products such as food, pharmaceuticals, cosmetics, detergents and chemicals. Approximately 30% of plastics are used worldwide for packaging applications and the most widely used plastics used for packaging are polyethylene (LDPE, MDPE, HDPE, LLDPE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyurethane (PUR), polybutylene terephthalate (PBT), nylons. At present the industry is split into organized and unorganised sectors. The organized sector produce quality products whereas unorganized sector is not capable of producing quality products, it produces low quality, cheap products through excessive use of plastic scrap.

Almost invariably, organic polymers mainly comprise plastics. The majority of these polymers are based on chains of carbon atoms alone or with sulfur, oxygen or nitrogen as well. The backbone is the part of the chain on the main "path" linking a large number of repeat units together. In order To customize the properties of a plastic, different molecular groups "hang" from the backbone (usually they are "hung" as part of the monomers before linking monomers together to form the polymer chain). This property of the polymer by repeating unit's molecular structure has allowed plastics to become an indispensable part of the twenty-first century world.

Plastics are usually classified by their chemical structure of the polymer's backbone and side chains. Important groups in these classifications include acrylics, silicones, polyesters, polyurethanes, halogenated plastics. Plastics can be classified by the chemical process that is used in their synthesis.

Types of plastics

There are two types of plastics: thermoplastics and thermosetting polymers. Thermoplastics are plastics that do not undergo chemical change in their composition when heated and can be moulded again and again. Thermosets are assumed to have infinite molecular weight. These chains are made of many repeating molecular units, known as repeating units, derived from monomers; each polymer chain will have several thousand repeating units. Thermosets can melt and can be molded into various shapes. After they are solidified, they remain solid. In the thermosetting process, a chemical reaction occurs which is irreversible. Vulcanization of rubber is a thermosetting process. The polyisoprene is a tacky, slightly runny material, before heating with sulfur, but after vulcanization the product is rigid and non-tacky.

Other classifications are based on qualities that are relevant for manufacturing or product design. Plastics can also be classified depending on various physical properties, such as density, high tensile strength, and resistance to various chemical products.

Toxicity

Pure plastics generally have low toxicity due to their insolubility in water and relative chemical inertness. Some plastic products can be toxic due to the presence of some additives in them. For example, plasticizers like adipates and phthalates are often added to brittle plastics like polyvinyl chloride to make them pliable enough. Traces of these compounds can leach out of the product. The compounds leaching from polystyrene food containers have been proposed to interfere with hormone functions and are suspected human carcinogens. The finished plastic is non-toxic, the monomers that is used in the manufacture of the parent polymers may be toxic. But in some

cases, small amounts of these chemicals can remain trapped in the product unless a suitable processing is being employed.

Synthetic Fibres

Synthesis means to make and synthetic means man-made, so man-made fibers are known as synthetic fibers. A synthetic fibre is a chain of small units that are joined together. Each small unit is a chemical substance. Many such small units combine and form a large single unit called a polymer. The word 'polymer' comes from two Greek words; poly meaning many and mer meaning part/unit. So, many repeating units combine to form polymer.

Natural plastics

These are the products from renewable sources that totally biodegrade in their natural form and are components of plants, animals and algae. Natural plastics are biodegradable.

Many bacteria and archaea synthesize biodegradable plastics which are a group of biopolymers. Polyhydroxyalkanoates (PHA) are a good alternative to petrochemical plastics among the various biodegradable polymers because they are biodegradable, eco friendly and biocompatible. Non petroleum based biological polyesters are considered to be one of the most important next-generation polymers in the future in light of limiting natural resources. The properties of PHA are also similar to those of polyethylene (PE) and polypropylene (PP) (Kim and Lenz, 2001; Rehm, 2003). Many micro-organisms accumulate PHA as intracellular energy and storage of carbon inclusions when the carbon is in excess to the other nutrients such as nitrogen, sulfur, phosphorus and oxygen (Madison and Huisman, 1999; Reddy et al., 2003). PHA is produced by almost 250 organisms to be known, but only a few species can produce PHA at a high concentration e.g. Alcaligenes latus (Yamane et al., 1996), Pseudomonas oleovorans (Brandl et al., 1988), Cupriavidus necator (formerly Ralstonia eutropha) (Kim et al., 1994). Classification of PHA can be done into different types according to the number of repeating units in the polymers. Short-chain-length PHA (scl-PHA) is the polymer that contain monomers of C3 to C5 hydroxyl fatty acids e.g. polyhydroxybutyrate (PHB) and hydroxyvalerate (PHV). Similarly, the polymers composed of C6 to C16 hydroxyl fatty acids or aliphatic carbon sources are termed as

medium- chain -length PHA (mcl-PHA) (Kim and Lenz, 2001; Sudesh et al., 2000). Heavy metals and antibiotics contaminate the environment from natural sources or directly and indirectly due to human activities and anthropogenic sources (Ware et al., 2006). The general use of antibiotics has been increased in many activities leaded by man, as agriculture, hospitals, animal husbandry, industry and prophylaxis. Heavy contamination from environment in the fermentor affects the production of PHA for industrial scale. Different scientists prefer to exploit the strains which are resistant to some antibiotics for controlling contamination (de Lima et al., 1999). Polyhydroxyalkanoates (PHA) are the biodegradable polyesters that are produced by bacteria in order to overcome the environmental stress. A large number of bacteria remains in contaminated environment which can accumulate PHA as their energy reserves. The polythene is the most typically found non-degradable solid waste that has been recently recognized as a major threat to marine life. The polythene might cause blockage in intestine of fish, birds and marine mammals (Spear et al. l995, Secchi and Zarzur l999). Degradation of polythene is a great challenge as the materials are increasingly used. A very general estimate of worldwide plastic waste generation is annually about 57 million tons (Bollag et al. 2000). These solid waste related problems pose threat to megacities especially the coastal ones. An attempt in this paper has been created to isolate the microorganisms that degrade the plastic materials potentially from the soil sediment.

Low density polyethylene is one of the major sources of environmental pollution. Polyethylene is a polymer made of long chain monomers of ethylene. The worldwide utility of polyethylene is expanding at a rate of 12% annum and approximately 140 million tones of synthetic polymers are produced worldwide each year (Shimao, M., 2001). With such huge amount of polyethylene getting accumulated in the environment, their disposal evokes a big ecological issue. It takes thousand years for their efficient degradation. Biodegradable polymers are designed to degrade upon disposal by the action of living organisms. Biodegradable polymers generally decompose in various medium in our environment. The depolymerisation results due to various physical biological forces. The physical forces such as temperature, moisture, pressure etc, deal with causing mechanical damage to the polymer. The microbial biodegradation is widely accepted and is still underway for its enhanced efficiency. Recently several microorganisms have been

reported to produce degrading enzymes. 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, respectively (Gu et al., 2000). The degradation leads to breaking down of polymers to monomers creating an ease of accumulation by the microbial cells for further degradation.

Biodegradation

Any physical or chemical change in polymer as a result of environmental factors such as light, heat, moisture, chemical conditions and biological activity is termed as degradation of plastic. Biodegradable polymers are designed to degrade upon disposal by the action of living organisms. Microbial degradation of plastics is caused by enzymatic activities that lead to a chain cleavage of the polymer into monomers. Microorganisms utilize polythene film as a sole source of carbon resulting in partial degradation of plastics. They colonize on the surface of the polyethylene films forming a biofilm. Cell surface hydrophobicity of these organisms was found to be an important factor in the formation of biofilm on the polythene surface, which consequently enhances biodegradation of the polymers. Once the organisms get attached to the surface, starts growing by using the polymer as the carbon source. In the primary degradation, the main chain cleaves leading to the formation of low-molecular weight fragments (oligomers), dimers or monomers. The degradation is due to the extra cellular enzyme secreted by the organism. These low molecular weight compounds are further utilized by the microbes as carbon and energy sources. The resultant breakdown fragments must be completely used by the microorganisms, otherwise there is the potential for environmental and health consequences.

The purpose of this study was to isolate microorganism from dumped soil area and screening of the potential polyethylene degrading microorganisms and indentifying the high potential microorganism that degrade the plastics.

2. REVIEW OF LITERATURE

PLASTICS

Plastics are man-made long chain polymeric units (Scott, 1999). Synthetic polymers started to substitute natural materials in almost every area more than half a century ago and nowadays plastics have become an indispensable part of our life. With time, the stability and durability of plastics have been improved continuously, and hence these groups of materials are considered as a synonym for materials that are resistant to many environmental influences. The word 'plastic' is derived from the Greek word “plastikos”, that means ‘able to be molded into various shapes and sizes’ (Joel, 1995). The plastics are made from inorganic and organic raw materials,used today, such as carbon, silicon, hydrogen, nitrogen, and oxygen. The basic materials are extracted from oil, coal and natural gas used for making plastics (Seymour, 1989).

Plastics are resistant to microbial attack, because their short time of presence in nature evolution could not design new enzyme structures capable of degrading synthetic polymers (Mueller, 2006). Nowadays, a wide variety of petroleum-based synthetic polymers are produced worldwide to the extent of approximately 140 million tons per year and remarkable amounts of these polymers are introduced in the ecosystem as industrial waste products (Shimao, 2001).

The synthetic plastics are used in packaging of products like food, medicines, cosmetics, detergents and chemicals. Approximately 30% of the plastics are used worldwide for packaging applications. The utilization is still expanding at a high rate of 12% per annum. They have replaced paper and other cellulose-based products for packaging because of they have better physical and chemical properties, such as strength, lightness, resistance to water and most water-borne microorganisms. The plastics used in packaging are polyethylene (LDPE, MDPE, HDPE and LLDPE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyurethane (PUR), poly(ethylene terephthalate) (PET), poly(butylene terephthalate) (PBT), nylons are widely used. The widespread applications of plastics are not only due to their favorable mechanical and thermal properties but also mainly due to the stability and durability (Rivard et al., 1995). Plastics (polymers) have attracted more public and media attention than any other

component of the solid waste stream because of their durability and visibility in litter. The total world demand for plastics was over 107 million tones in 1993 and it was estimated about 146 million tonnes in 2000.

The dramatic increase in production and lack of biodegradability of commercial polymers, mainly commodity plastics used in packaging (e.g. fast food), 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 landfilling, 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. Improperly disposed plastic play significant role in potentially harming life by causing environmental pollution. In addition to this, the burning of polyvinylchloride (PVC) plastics produces persistent organic pollutants (POPs) known as furans and dioxins (Jayasekara et al., 2005).

Synthetic plastics like polyester polyurethane, polyethylene with starch blend, can biodegrade, although most commodity plastics used now are either non-biodegradable or take decades to degrade. This growing concern about degradable polymers has raised and promoted research activity world wide to either modify current products to promote degradability or to develop new alternatives that are degradable by any or all of the following mechanisms: biodegradation, photodegradation, thermal degradation and environmental erosion (Kawai, 1995).

In 1980′s, scientists started to look if plastics could be designed to become susceptible against microbial attack, making them allowed to degrade in a microbial active environment. Biodegradable plastics has opened the way for new considerations of waste management strategies since these materials are designed to degrade under environmental conditions or in municipal and industrial biological waste treatment facilities (Augusta et al., 1992 and Witt et al., 1997).

Due to similar material properties to conventional plastics (Hocking and Marchessault, 1994 and Steinbuchel and Fuchtenbusch, 1998), polyhydroxyalkanoates (PHA), polylactides, polycaprolactone, polysaccharides and copolymer like biodegradable plastics (polyesters)or blend of these have been developed successfully over the last few years. The most important are poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate). Bioplastics (Biopolymers) obtained from growth of microorganisms or from plants which are genetically-engineered to produce such polymers are likely to replace currently used plastics at least in some of the fields (Lee, 1996). PHA's key properties are biodegradability, apparent biocompatibility, and also its manufacture from renewable resources . The global interest in PHAs is high because it is used in different packaging materials, medical purposes, disposable personal hygiene and also agricultural applications as a substitute for synthetic polymers like polypropylene, polyethylene etc. (Ojumu et al., 2004) (Lee, 1996).

Several biodegradable plastics have been introduced into the market in the past 10 years and none of them is found efficiently biodegradable in landfills. For this reason, none of the plastic products has gained widespread use (Anonymous, 1999). At present, biodegradable plastic represents just a tiny market as compared with the conventional petrochemical materials. The bioplastics will comparatively prove cheaper when oil prices will continue to hike up. The plastic shopping bags could be made from Polylactic acid (PLA) a biodegradable polymer derived from lactic acid although not in use today. This could be said as one form of vegetable-based bioplastic which biodegrades quickly under composting conditions without leaving toxic residue. But, bioplastic can have its own environmental impacts, depending on the way it is produced .There is an urgent need to develop efficient microorganisms and their products to solve this global issue (Kathiresan, 2003). This paper reviews the current research on the degradation of the biodegradable plastics

Degradation of plastics

Any physical or chemical change in polymer as a result of several environmental factors, such as light, temperature, moisture, chemical conditions or biological activity. Processes that induce changes in polymer properties due to physical,chemical or biological reactions resulting in

subsequent chemical transformations (formation of structural in homogeneities) are categorized as polymer degradation. Degradation are reflected as changes in properties of material (mechanical, optical or electrical characteristics), in cracking, erosion, discoloration, phase separation and delamination. The changes include chemical transformation and formation of new functional groups (Pospisil and Nespurek, 1997). The degradation will be photo, thermal or biological.

Sensitivity of polymers to photodegradation is related to the ability to absorb the harmful part of the tropospheric solar radiation. And this includes the UV-B terrestrial radiation (~ 295–315 nm) and UV-A radiation (~ 315–400 nm) responsible for the direct photodegradation (photolysis, initiated photooxidation). Visible part of sunlight (400–760 nm) accelerates polymeric degradation by heating. Infrared radiation (760–2500 nm) accelerates thermal oxidation (Gugumus, 1990 and Pospisil and Nespurek, 1997). The absorbance of high-energy radiation in the ultraviolet portion of the spectrum by most plastics, results in activation of their electrons to higher reactivity and that causes oxidation, cleavage, and finally process of degradation. Thermal degradation of polymers is 'molecular deterioration as a result of overheating'. The components of the long chain backbone of the polymer can begin to separate (molecular scission) at high temperatures and react with one another to change the properties of the polymer. Various chemical reactions involved in thermal degradation lead to physical and optical property changes relative to the initially specified properties. Thermal degradation of plastics generally involve changes to the molecular weight (and molecular weight distribution) of the polymer and typical property changes include; reduced ductility and embrittlement, chalking, color change, and general reduction in most other desirable physical properties (Olayan et al., 1996).

Oxo-biodegradation process uses two methods to start the biodegradation. The methods that are included are photodegradation (UV) and oxidation. The UV degradation process uses UV light to degrade the end product. The oxidation process makes use of time and heat to break down the plastic. Both the processes reduce the molecular weight of the plastic and allow it to biodegrade

Biodegradation is the process by which organic substances are broken down by living organisms. This term is often used in relation to ecology, waste management, bioremediation and to the

plastic materials, because of their long life span. Organic material can be degraded aerobically or anaerobically. Biomineralisation, is a term related to biodegradation, in which organic matter is converted into minerals.Plastics are biodegraded with oxygen (aerobically)in wild nature , without oxygen (anaerobically) in sediments and landfills and partly aerobically and partly anaerobically in composts and soil. During aerobic biodegradation carbon dioxide and water are produced and during anaerobic biodegradation, carbon dioxide, water and methane are produced (Gu et al., 2000a). Generally, the breakdown of large polymers to carbon dioxide (mineralization) requires several different organisms, one breaking down the polymer into its constituent monomers, with one able to use the monomers and excreting simpler waste compounds as by-products and one able to use the excreted wastes.

Biodegradation of plastics

Microorganisms such as bacteria and fungi are involved in the degradation of both natural and synthetic plastics (Gu et al., 2000a). The biodegradation of plastics proceeds actively under different soil conditions according to their properties, because the microorganisms that are responsible for the process of degradation differ from each other and they have their own optimal growth conditions in the soil. Plastics are potential substrates for heterotrophic microorganisms (Glass and Swift, 1989).

Biodegradation is governed by different factors that include characteristics of polymer, type of organism, and nature of pretreatment. The characteristics of polymer such as mobility, crystallinity, molecular weight, functional groups and substituents present in its structure, and plasticizers or additives when added to the polymer all play a significant role in its degradation (Artham and Doble, 2008; Gu et al., 2000).

The polymer is first converted to its monomers during degradation, after which these monomers are mineralized. Most polymers are too large that they can pass through cellular membranes, so for this they must first be depolymerized to smaller monomers before they can be absorbed and biodegraded within the microbial cells. The initial breaking down of polymers can result from a variety of physical and biological forces (Swift, 1997). Any of the physical forces, such as

heating, cooling, freezing, thawing, wetting or drying, can cause damage to the mechanical property such as the cracking of polymers (Kamal and Huang, 1992). The growth of many fungi on polymers can also cause small-scale swelling and bursting, as the fungi get penetrated in the polymer solids (Griffin, 1980). Synthetic polymers, such as poly(caprolactone) (Toncheva et al., 1996; Jun et al., 1994), can also be depolymerized by microbial enzymes, then the monomers are absorbed into microbial cells and biodegraded (Goldberg, 1995). The most important reaction for initiating the environmental degradation of synthetic polymers is the abiotic hydrolysis (Göpferich, 1997) like polycarboxylates (Winursito and Matsumura, 1996), poly(ethylene terephthalate)(Hiltunen et al., 1997; Nakayama et al., 1996), poly (α-glutamic acids) (Fan et al., 1996), and polydimethylsiloxanes, or silicones (Lehmann et al., 1995; Xu et al., 1998).

Generally, an increase in molecular weight results in a decline of polymer degradability by microorganisms. In contrast, a polymer's repeating units are monomers, dimers, and oligomers are much easily degraded and mineralized. There could be a sharp decrease in solubility due to high molecular weights of plastics making them unfavorable for microbial attack because bacteria require the substrate to be assimilated through the cellular membrane and then further degraded by cellular enzymes. There are at least two categories of enzymes that are actively involved in biological degradation of polymers: extracellular and intracellular depolymerases (Doi, 1990; Gu et al., 2000b). Exoenzymes from microorganisms break down complex polymers during degradation yielding smaller molecules of short chains, that are small enough to pass the semi-permeable outer bacterial membranes, and then utilized as carbon and energy sources. This process is called depolymerization. The degradation is called mineralization when the end products are Carbon dioxide, water, or methane (Frazer, 1994; Hamilton et al., 1995). It is important to note that the biodeterioration and degradation of a polymer substrate can rarely reach 100% and the reason is that a small portion of the polymer will be incorporated into microbial biomass, humus and other natural products (Atlas and Bartha, 1997 and Narayan, 1993). the dominant groups of microorganisms and the degradative pathways associated with polymer degradation are often determined by the environmental conditions. When Oxygen is available, aerobic microorganisms are mostly responsible for the destruction of complex materials, yielding microbial biomass, Carbon dioxide, and water as the final products.

Anaerobic consortia of microorganisms are responsible for polymer deterioration under anoxic conditions. The microbial biomass, Carbon dioxide, methane and water are the primary products under methanogenic (anaerobic) conditions (Barlaz et al., 1989) (e.g. landfills/compost) (Fig. 1).

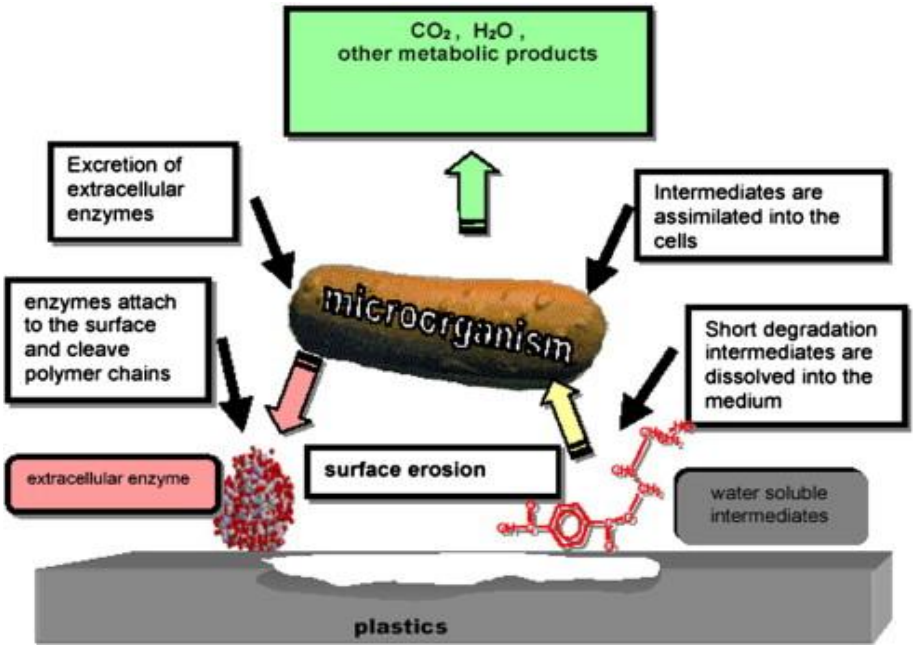


Fig.1 . General mechanism of plastic biodegradation under aerobic conditions (Mueller, 2003).

Biodegradation of natural plastics

Process of biodegradation of polyhydroxyalkanoates

Microorganisms that produce and store PHA under nutrient limited conditions may degrade and metabolize it when the limitation is removed (Williams and Peoples, 1996). The ability to store PHA does not guarantee the ability to degrade it in the environment (Gilmore et al., 1990). Individual polymers are larger enough to be transported directly across the bacterial cell wall. Therefore, bacteria must have evolved extracellular hydrolases in themselves to be capable of converting the polymers into corresponding hydroxyl acid monomers (Gilmore et al., 1990). R-3-hydroxybutyric acid is the product of PHB hydrolysis (Doi et al., 1992), while both 3-

hydroxybutyrate and 3-hydroxyvalerate are the products of extracellular degradation of PHBV (Luzier, 1992). The monomers are water soluble but they are smaller enough to passively diffuse through the cell wall, where the process of β-oxidation and tricarboxylic acid cycle (TCA) metabolizes them to produce carbon dioxide and water under aerobic conditions (Scott, 1999). Methane is also produced but under anaerobic conditions (Luzier, 1992). During PHA degradation, no harmful intermediates or by-products are produced. In fact, 3-hydroxybutyrate is generally found in all higher animals as blood plasma (Lee, 1996). For this reason, PHAs have been considered for medical applications, such as long-term controlled drug release, surgical pins, and bone and blood vessel replacement.

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 (Luzier, 1992). PHAs have attracted the industrial attention for long use in the production of biodegradable and biocompatible thermoplastics (Takaku et al., 2006). Sturm test has been used by many researchers to study the biodegradation of biodegradable polymers (Whitchurch and Terence, 2006), and the aliphatic and aromatic compounds (Kim et al., 2001).

Degradation of polyhydroxalkanoates by enzymes

At least two categories of enzymes are actively involved in biological degradation of polymer: extracellular and intracellular depolymerases (Doi, 1990;Gu et al., 2000b). the first category i.e extracellular PHB depolymerases are secreted from various microorganisms and play an important role in the metabolism of PHB in the environment. The enzymes are composed of two types of domains named substrate-binding domain and catalytic domain, and a linker region which connects the two domains. The substrate-binding domain plays role in binding to the solid PHB. And the catalytic domain contains the catalytic machinery which is further composed of a catalytic triad (Ser-His-Asp). Serine is a part of a lipase box pentapeptide Gly-X-Ser-X-Gly, which is found in all known hydrolases (lipases, esterases and serine proteases) (Jaeger et al., 1994). The PHB depolymerases properties have been studied extensively and share several biochemical properties such as: relatively small molecular weight, and most PHA depolymerases do not bind to anion exchangers such as DEAE but have strong affinity to hydrophobic materials

such as butyl-Toyopearl and phenyl-Toyopearl; optimum pH is between 7.5–9.8, only the depolymerase of Pseudomonas picketti and Penicillium funiculosum have optimum pH between 5.5 and 7; highly stable at a wide range of pH, temperature and ionic strength. Most PHA depolymerases are inhibited by serine esterase inhibitors such as diidopropyl-fluorylphosphate or acylsulfonyl compounds, bind covalently to the active site of serine hydrolases (Jendrossek, 1998).

Apparently, most PHA-degrading bacteria have been analyzed that produce only one PHA depolymerase. One of the best-studied PHA-degrading bacteria that is P. lemoignei, produces at least seven different extracellular PHA-depolymerases which differ slightly in their biochemical properties. The three PHA-depolymerases such as A, B and D, are specific for PHB and P(3HB-co-3HV) with a low 3-HV content. The other two PHA depolymerases i.e.(PHB depolymerase C and poly(d-3-hydroxyvalerate depolymerase) also degrade both PHB and PHV (Jendrossek and Handrick, 2002).

In 1990 the British-based company Imperial Chemical Industries (ICI) released a material called Biopol. Biopol is made from PHBV. This material is broken down by the microorganisms present in waste when thrown away and decomposed completely within a couple of months. The other versions of biodegradable plastics have also been developed in the United States. Procter and Gamble (In 1991), Du Pont, and Exxon worked on bacteria-based plastic at the University of Massachusetts at Amherst and in addition to this, Battelle (a private research company), produced a plastic that is completely biodegradable, from vegetable oils. Plastics can also be made from other glucose-intensive materials such as potato scraps, corn, molasses, and beets (Kings et al., 1992; Sharpley and Kaplan, 1976).

By 1997, compostable bags still sold for more than non-degradable bags. As marketed by Union Carbide, one synthetic polymer used in biodegradable plastic bags was aliphatic polyester called polycaprolactone. Another biodegradable plastic film called MaterBi contained corn starch and other proprietary ingredients. Corn was also used in making polylactic acid for Cargill's EcoPLA (a biopolymer). University of Michigan worked on a material called Envar that consists of an alloy of caprolactone and a thermoplastic starch. One of the research group at the University of

Utah at Salt Lake City in 1997, synthesized an injectable polymer that forms a non-toxic biodegradable hydro gel that acts as a sustained release matrix for drugs (Kings et al., 1992; Sharpley and Kaplan, 1976).

Biodegradation of synthetic plastics

The degradation of most synthetic plastics in nature is a very slow process that involves environmental factors, which follows the action of wild microorganisms (Albertsson, 1980; Cruz-Pinto et al., 1994; Albertsson et al., 1994). The oxidation or hydrolysis by enzyme to create functional groups that improves the hydrophylicity of polymer is the 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 and Karlsson, 1990; Albertsson et al., 1987; Huang et al., 1990). Poly(vinyl alcohol), poly(lactic acid), polycaprolactone, and polyamides are some examples of synthetic polymers along with oligomeric structures that biodegrade. The rate of degradation is affected by several physical properties such as crystallinity and orientation and morphological properties such as surface area (Huang et al., 1992).

3. AIMS AND OBJECTIVES

1. Isolation of microorganisms from plastics dumped in soil.

2. Identification of plastic degrading bacteria isolated from plastics.

3. To study the biodegradative ability of these microorganism.

4. MATERIALS AND METHODS

1) Sample collection

Plastic sample was collected from the dumped soil of hostel garden, NIT Campus.

2) Isolation

a] Serial dilution:

After the collection of plastic sample, these were taken and 1gm of this sample was cut into pieces and added to 9 ml of sterile water to make 1:10 dilution, adding 1ml of the 1:10 dilution of 9ml of sterile water makes a 1:100 dilution and so on.

b] Total heterotrophic count:

C.F.U. /g= Number of colonies/ inoculum size (ml) X dilution factor

3) Identification

Identification of the isolates were performed according to their morphological, cultural and biochemical characteristics by following Bergey's Mannual of Systematic Bacteriology (Kandler and Weiss, 1986). All the isolates were subjected to Gram staining and specific biochemical tests.

1]Morphological-

GRAM STAINING METHOD:

A clean grease free slide was taken and a smear of the bacterial culture was made on it with a sterile loop. The smear was air-dried and then heat fixed. Then it was subjected to the following staining reagents:

(i) Flooded with Crystal violet for 1 min. followed by washing with running distilled water.

(ii) Again, flooded with Gram's Iodine for 1 min. followed by washing with running distilled water.

(iii) Then the slide was flooded with Gram's Decolourizer for 30 seconds.

(iv) After that the slide was counter stained with Safranin for 30 seconds, followed by washing with running distilled water.

(v) The slide was air dried and cell morphology was checked under microscope.

COLONY MORPHOLOGY:

This was done to determine the morphology of selected strains on the basis of shape, size and colour.

2)Biochemical tests:

Biochemical identification of the isolated strains were done by using Biochemical identification kit (Hibacillus identification kit, HIMEDIA) and some manual biochemical methods. Biochemical Identification test kit is a standardized colorimetric identification system utilizing conventional biochemical tests and carbohydrate utilization tests. The test is based on the principle of change in pH and substrate utilization. Organisms undergo metabolic changes on incubation which are indicated by a colour change in the media that is either interpreted visually or after addition of a reagent.

3.1] CATALASE TEST:

The catalase test was performed to detect the presence of catalase enzyme by inoculating a loopful of culture into tubes containing 3% of hydrogen peroxide solution. Positive test was indicated by formation of effervescence or appearance of of bubbles, due to the breaking down of hydrogen peroxide to o_2 and h_2o.

3.2] OXIDASE TEST:

The oxidase test was done with the help of commercially available disc coated with a dye N-tetramethyl paraphenylene diamine dihydrochloride (Himedia), to detect the presence of

cytochrome ‘c’ oxidase which is responsible for the oxidation of the dye. Rubbing a small quantity of bacterial culture by means of a sterile toothpick on the disc causes formation of purple colour within 10-30 sec indicating positive reaction whereas no colour change indicates a negative reaction.

3.3] MANNITOL TEST:

This experiment is generally performed to determine whether the bacteria is capable of fermenting mannitol sugar or not. Whenever organisms ferment mannitol agar, the pH of media becomes acidic due to production of acids. The fermentation of the media form red to yellow which shows positive test result.

3.4] MOTILITY TEST:

The motility test was done to determine the motility of the organism. Bacterial cultures were stabbed into the motility test medium (Himedia) and were incubated at 37 C for 48 hrs. Turbitity and observation of growth besides the stab line indicated a positive reaction whereas clear visibility with growth indicated a negative reaction.

3.5] MALONATE UTILISATION:

Malonate utilisation test was performed to observe the utilisation of malonate present in the malonate test medium (Himedia). Malonate test medium contains Bromothymol blue as indicator. Sodium malonate is the carbon source and ammonium sulphate is the nitrogen source. Organisms, which are able to utilise malonate, release sodium dioxide. The resulting alkaline conditions cause the indicator to change from light green to blue. Colour of the medium changes from light green to blue if the test is positive. Medium remains in light green colour if the test is negative.

3.6] NITRATE REDUCTION TEST:

This test was done to test if microorganisms are able to convert nitrate to nitrite or not by adding 1-2 drops of sulphanilic acid and 1-2 drops of N,N-Dimethyl-Napthylanine reagent to the kit

medium. Immediate development of pinkish red colour there on addition of reagent indicates positive reaction. Negative reaction could be observed if there is no change in the colour.

3.7] CITRATE UTILISATION TEST:

This test determines the ability of bacteria to convert citrate (an intermediate of the Kreb's cycle) into oxaloacetate (another intermediate of the Kreb's cycle). Citrate is the only carbon source available to the bacteria in this media. If bacteria cannot use citrate, it will not grow. Positive result is seen if the bacteria grows and the media turns into bright blue colour as a result of an increase in the pH of the media.

3.8] GAS PRODUCTION FROM GLUCOSE:

Gas production from glucose was assessed by inoculating the isolated strains in MRS broth containing glucose containing Durham tube in inverted condition and incubated at 37^{o}C for 48-72 hrs. The upward movement of inverted Durham tube indicates positive reaction (gas production).

3.9] CARBOHYDRATE UTILIZATION TEST:

For carbon utilisation pattern HiCarbo Kit(Part A, Part B, and Part C) (Himedia catalog no. KB009) was used. Bacteria produce products that are acidic in nature when they ferment certain carbohydrates. The carbohydrate utilisation tests are designed to detect the change in pH that occurs if fermentation of the given carbohydrate occurred. Acids lower the pH of the medium which causes the pH indicator (phenol red) to turn yellow. If the given carbohydrate is not fermented by bacteria then the media remains red.

4) <u>Microbial Degradation of Plastics in Laboratory Condition</u>:

Determination of Weight Loss:

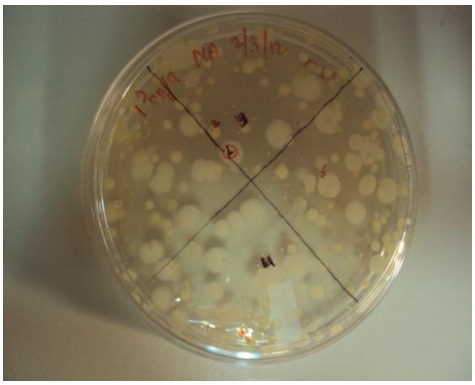
Pre-weighed discs of 1-cm diameter prepared from polythene bags were aseptically transferred to the conical flask containing 50 ml of culture broth medium, inoculated with different bacterial

species. Control was maintained with plastic discs in the microbe-free medium. Different flasks were maintained for each treatment and left in a shaker. After one month of shaking, the plastic discs were collected, washed thoroughly using distilled water, shade-dried and then weighed for final weight. From the data collected, weight loss of the plastics was calculated.

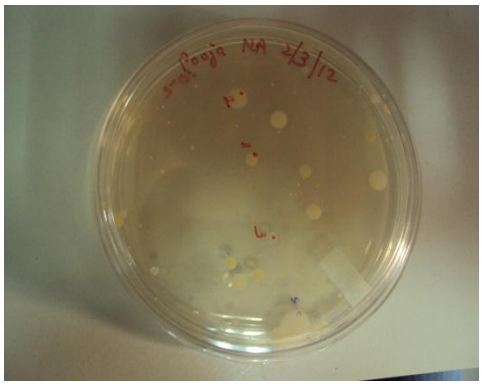
5. RESULTS

Table no. 1: Colony morphology of the bacterial strain on the basis of serial dilution.

Dilution no.	Sl. No.	Colony morphology	Source	Code
10^{-1}	1	Large round white	Dumped plastic material from hostel garden.	PLRW
	2	Small round yellow	Dumped plastic material from hostel garden.	PSRY
	3	Small round white	Dumped plastic material from hostel garden.	PSRW
	4	Large irregular white	Dumped plastic material from hostel garden.	PLIW
10^{-2}	1	Large round pale yellow	Dumped plastic material from hostel garden.	PLRP
	2	Small round yellow	Dumped plastic material from hostel garden.	PSRY
	3	Small round transparent	Dumped plastic material from hostel garden.	PSRT
	4	Large irregular white	Dumped plastic material from hostel garden.	PLIW
10^{-3}	1	Large round white	Dumped plastic material from hostel garden.	PLRW
	2	Small irregular yellow	Dumped plastic material from hostel garden.	PSIW
10^{-4}	1	Large irregular white	Dumped plastic material from hostel garden.	PLIW



(a)



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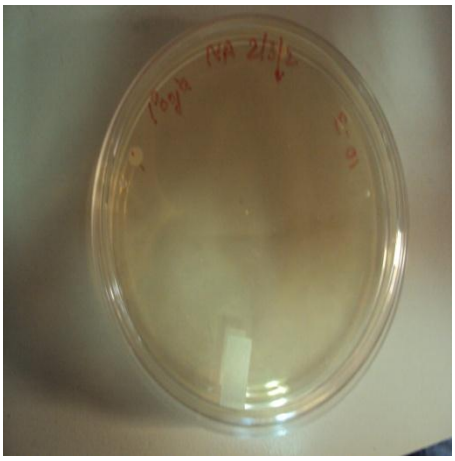
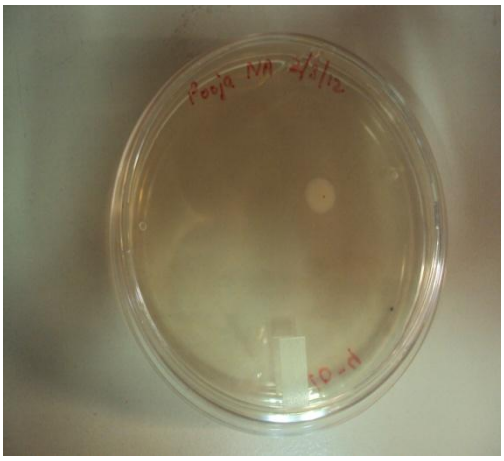


(c)



(d)

Fig 2: colony morphology of the strains on the basis of serial dilution

(a):10^{-1}, (b):10^{-2}, (c):10^{-3}, (d):10^{-4}

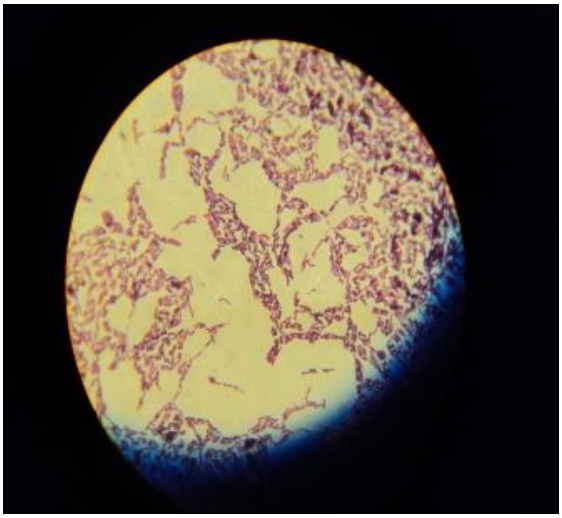
Table no.2:

GRAM STAINING: The bacterial strains are identified from the seven selected strains.

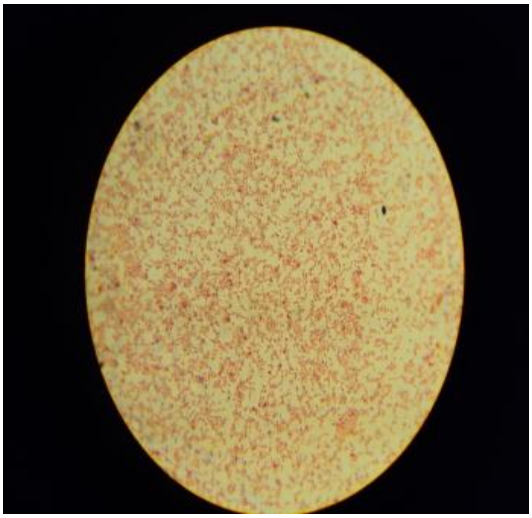
Bacterial Strain no	Strain	Shape of the organism	Colour	Characteristic
1	PLRW	Rods in chain	Purple	Gram +ve, bacillus
2	PSRY	Coccus in chain	Pink	Gram –ve, coccus
3	PSRW	Coccus in chain	Pink	Gram –ve, coccus
4	PLIW	Rods in chain	Purple	Gram +ve, bacillus
5	PLRP	Rods in chain	Pink	Gram –ve, bacillus
6	PSRT	Rods in chain	Pink	Gram –ve, bacillus
7	PSIY	Rods in chain	Pink	Gram –ve, bacillus

- The table contains the bacterial strains which are gram +ve & Gram –ve. bacterial strains like 1 and 4 were found to be Gram +ve & strain 2,3,5,6 and 7 were Gram –ve. Bacterial strains like 1,4,5,6 abd 7 were bacillus & strains 2 and 3 were coccus.

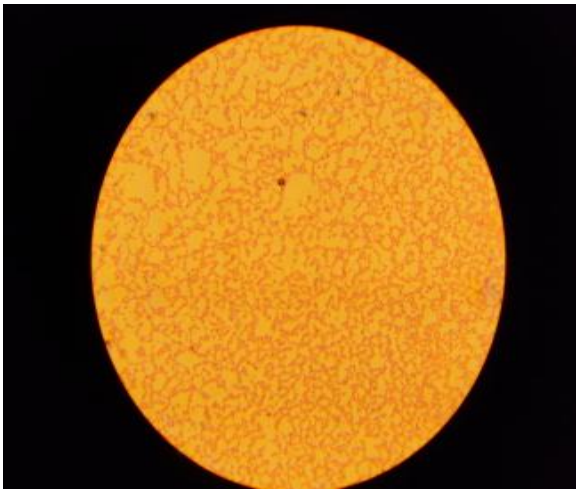
- The code stands for the morpholohical characteristics of the bacterial strain: **LRW**- Large Round White, **SRY**- Small Round Yellow, **SRW**- Small Round White, **LIW**- Large Irregular White, **LRP**- Large Round Pale, **SRT**- Small Round Transparent, **SIY**- Small Irregular Yellow.



A



B



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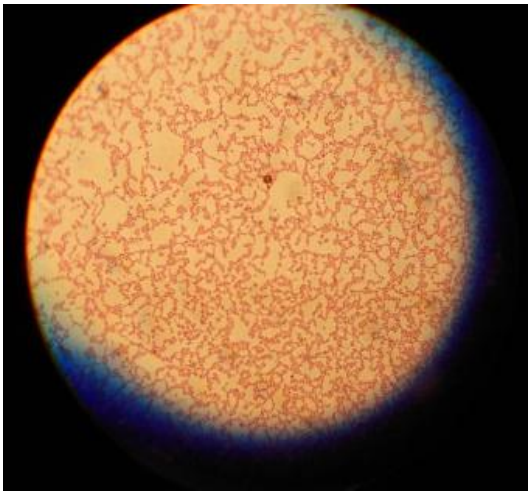

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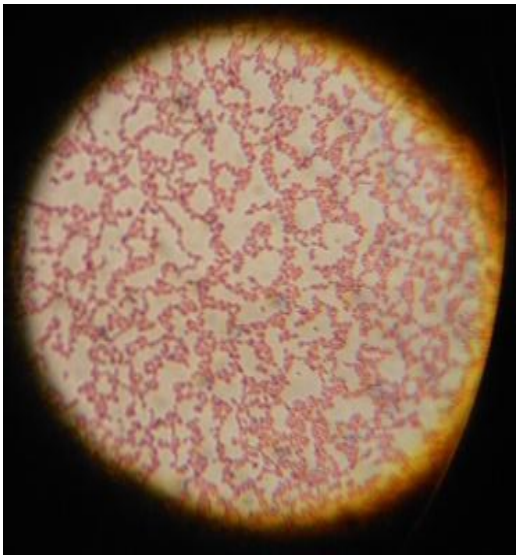

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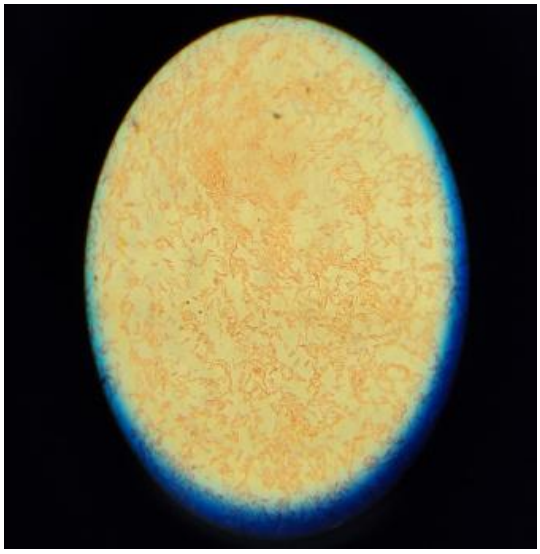

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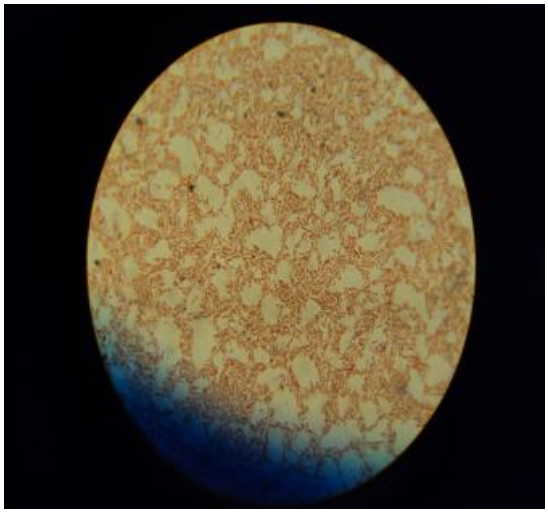

G

Fig 3: Gram staining of seven selected strains (A-G) on the basis of colony morphology.

Bacterial count

Table no 3: Total heterotrophic bacterial count:

Dilution	Number of colonies	Inoculums size (in mL)	CFU/g
10^{-3}	278	0.1	0.0278×10^{9}

Table no. 4: RESULT OF BIOCHEMICAL TEST

Sl no:	Catalase test	Oxidase test	Mannitol test	Motility test	Citrate utilisation test	Nitrate reduction test	Malonate utilisation test	Gas production from glucose
1	+ve	+ve	+ve	Non-motile	+ve	-ve	-ve	-ve
2	+ve	+ve	+ve	Non-motile	-ve	-ve	+ve	+ve
3	+ve	+ve	+ve	Non-motile	+ve	-ve	-ve	+ve
4	+ve	+ve	+ve	Non-motile	-ve	+ve	-ve	+ve
5	+ve	+ve	+ve	Non-motile	-ve	-ve	-ve	-ve
6	+ve	+ve	-ve	Non-motile	-ve	+ve	+ve	-ve
7	+ve	+ve	+ve	Non-motile	-ve	-ve	-ve	-ve

- Biochemical tests shows, catalase and oxidase test result of all the strains were found to be positive.
- Mannitol test of the strains were also found positive excluding strain no.6, Motility test shows all the strains are non-motile.
- Citrate test of strain no 1 and 4 were found positive and rest of them showed a negative result.
- Nitrate reduction test of strain no.4 was found positive and rest of them showed a negative result.
- Malonate test shows only strains 4 and 6 gave a positive result.
- The test named gas production from glucose shows strains 2, 3 and 4 showed a positive result.

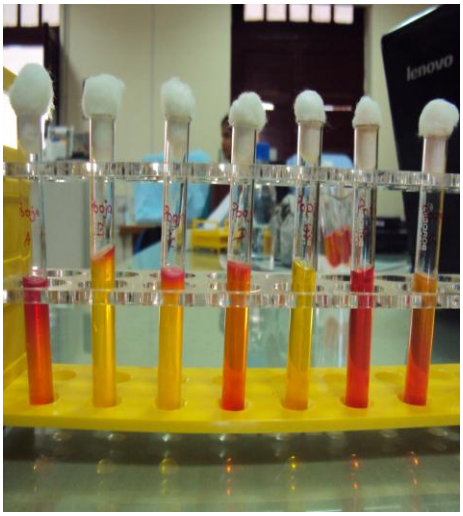

Fig 4: Mannitol-Motility test.

Fig 5: Citrate utilisation test.

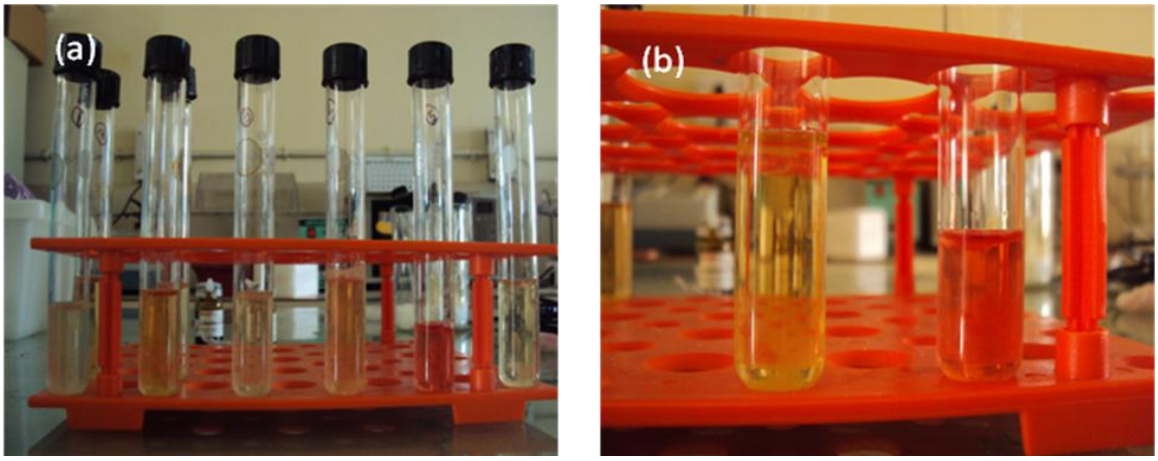

Fig 6 a and b: Nitrate reduction test.

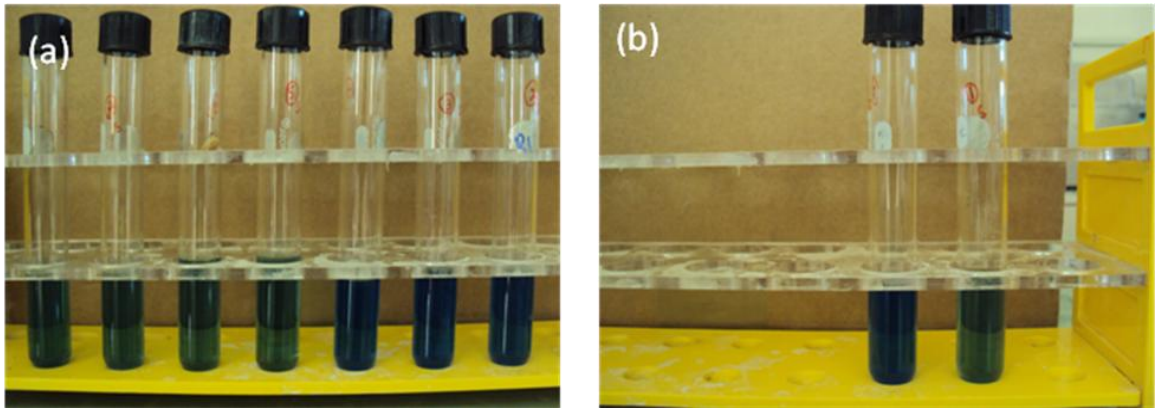

Fig 7 a and b:Malonate utilisation test.

Fig 8: Gas production from glucose.

Table no. 5: Result of carbohydrate test: (a)

Sl. No.	Carbohydrate	Isolate 1	Isolate 2	Isolate 3
1	Lactose	+ve	-ve	-ve
2	Xylose	+ve	-ve	-ve
3	Maltose	+ve	-ve	-ve
4	Fructose	+ve	-ve	-ve
5	Dextrose	+ve	-ve	-ve
6	Galactose	+ve	-ve	-ve
7	Raffinose	+ve	-ve	-ve
8	Trehalose	+ve	-ve	-ve
9	Melibiose	+ve	-ve	-ve
10	Sucrose	+ve	-ve	-ve
11	L-Hrabinose	+ve	+ve	-ve
12	Mannose	+ve	+ve	**+ve**

(b)

Sl. No.	Carbohydrate	Isolate 1	Isolate 2	Isolate 3
13	Inulin	-ve	-ve	-ve
14	Sodium galactose	-ve	+ve	-ve
15	Glycerol	-ve	-ve	-ve
16	Salicin	+ve	+ve	+ve
17	Dulcitol	-ve	-ve	+ve

18	Inositol	-ve	-ve	+ve
19	Sorbitol	-ve	-ve	+ve
20	Mannitol	-ve	-ve	+ve
21	Adonitol	-ve	-ve	+ve
22	Arabitol	-ve	-ve	+ve
23	Erythritol	-ve	-ve	+ve
24	α methyl D glucoside	-ve	-ve	+ve

(c)

Sl. No.	**Carbohydrate**	**Isolate 1**	**Isolate 2**	**Isolate 3**
25	Rhamnose	-ve	-ve	-ve
26	Cellobiose	+ve	-ve	-ve
27	Melezitose	-ve	-ve	-ve
28	α methyl- D mamoside	-ve	-ve	-ve
29	Xylitol	+ve	-ve	-ve
30	ONPG	+ve	+ve	+ve
31	Esculin hydrolysis	+ve	+ve	+ve
32	D-Arabinose	-ve	-ve	-ve
33	Citrate utisation	+ve	+ve	-ve
34	Malonate utilisation	+ve	+ve	-ve
35	Sorbose	-ve	-ve	-ve

(A)

(B)

(C)

Fig 9: <u>Carbohydrate test</u> : (A) Isolate-1, (B) Isolate-2, (C) Isolate-3.

Table no.6:

Result of degradation of plastic sample by bacteria after 1 month:

Strain no.	**Initial wt (mg)**	**Final wt (mg)**	**Difference**	**Weight loss/month (in %)**
1	50	40	10	20
2	50	35	15	30
3	50	38	12	24
4	50	37	13	26
5	50	39	11	22
6	50	37	13	26
7	50	38	12	24

6. DISCUSSION

This study has covered the major concerns about the natural and synthetic polymers, their types, uses and degradability also it has looked at the disposal methods and the standards used in assessing polymer degradation. Another area examined has been the biodegradation of plastics by the liquid culture method. It is clear that most recalcitrant polymers can be degraded to some extent in the appropriate environment at the right concentration.

The present study deals with the isolation, identification and degradative ability of plastic degrading microorganisms from soil. Different types of changes are produced by the microorganism during morphological and biochemical analysis. Synthetic plastic sample was collected from the dumped soil of hostel garden was used in this study. This plastic was used to study their biodegradation by microorganisms isolated from them.

Microbial degradation of a solid polymer like polyethylene requires the formation of a biofilm on the polymer surface to enable the microbes to efficiently utilize the non-soluble substrates by enzymatic degradation activities. Development of multicellular microbial communities known as biofilm, attached to the surface of synthetic wastes have been found to be powerful degrading agents in nature.

When the total biodegradation process of any organic substrate is considered the formation of microbial colony is critical to the initiation of biodegradation. Thus, the duration of the microbial colonization is an important factor that effects total degradation period.

Microbial counts in the degrading materials were recorded up to 0.0278×10^{9} per gram for total heterotrophic bacteria. The microbial species found associated with the degrading materials were identified as two Gram positive and five Gram negative bacteria.

In the present study pieces of plastics were inoculated in the liquid culture medium containing bacterial isolates and kept for 1 month to observe the percentage of weight loss by bacteria. The result shows the degradative ability of the microorganisms after one month of incubation. The

percentage of weight loss due to degradation was found more by *Bacillus amyloliticus.* This shows it has the greater potential of degradation compared to other bacteria.

PIBWIN (Probabilistic identification of bacteria) programme provides probabilistic identification of unknown bacterial isolates against identification matrices of known strains. This programme has following three major functions:

- It identifies an unknown isolate.
- Selects additional tests in order to distinguish between possible strains if identification is not achieved.
- The storage and retrieval of results.

The program makes use of Excel (2007) files to store identification matrices. The program is designed to use probabilistic identification matrices that have either published in the literature or created by the user.

The bacteria which are identified from the above biochemical tests are *Bacillus Subtilis* (strain-1), *Bacillus Amylolyticus* (strain-2) *and Arthobacter defluvii* (strain-3) by the software PIBWIN (Probabilistic identification of bacteria). These three bacterial species were also found on the basis of common morphological characteristics.

7. CONCLUSION

- The bacteria were identified to be *Bacillus Subtilis, Bacillus Amylolyticus and Arthobacter defluvii.*
- *Bacillus amylolyticus* degrades plastic more than that of other bacteria.
- *Bacillus subtilis* has less capacity to degrade plastic as compared to other bacteria.
- The isolated microbes were native to the site of polyethylene disposal and shown some degradability in natural conditions, yet they also exhibited biodegradation in laboratory conditions on synthetic media.

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