Isolation of potential bacterial species from Hindon, Ghaziabad contaminated site for the degradation of HDPE microplastics

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I, Akanksha Saini, 2K20/IBT/02, student of M.Tech (Industrial Biotechnology), hereby declare that the project dissertation titled "Isolation of potential bacterial species from Hindon, Ghaziabad contaminated site for the degradation of HDPE microplastics" is submitted to the Department of Biotechnology, Delhi Technological University, Delhi, by me in partial fulfilment of requirement for the award of degree of Master of Technology (Industrial Biotechnology). This thesis is original work done by me and not obtained from any source without proper citation. This project work has not previously formed the basis for award of any degree, diploma, fellowship or other similar title or recognition.

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CERTIFICATE

I hereby certify that project dissertation titled "Isolation of potential bacterial species from Hindon, Ghaziabad contaminated site for the degradation of HDPE microplastics" submitted by Akanksha Saini, 2K20/IBT/02, Department of Biotechnology, Delhi Technological University, Delhi, in partial fulfilment of requirement for the award of degree of Master of Technology (Industrial Biotechnology), is a project done and carried out by the student under my supervision. To best of my knowledge and belief, this work has not been submitted in part or full for any degree or diploma to this university or elsewhere.

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ABSTRACT

Plastic use has been increased steadily in recent years owing to the urbanization and industrialization at global scale. Microplastic contamination in environment is facilitated through various sources including cosmetic products, drug carriers, glitters and disintegration of larger plastic products such as water bottles and fishing net. Due to their ubiquitous use in the environment, they possess serious threat to terrestrial and aquatic environments and human health. Therefore, it is necessary to degrade microplastics, like HDPE, that are nonbiodegradable and very stable in the environment by using an approach that has negative or no effects on the environment. Thus, in this study, we are isolating the bacterial strains, from the Hindon, Ghaziabad, contaminated site, to check their potential to degrade the HDPE microplastic. Two morphologically different bacterial isolates were selected for further study as they were able to revive in the microplastic containing media. After that, these bacterial isolates were used to study the biodegradation of HDPE microplastic, by growing them in a minimal salt media containing no carbon source. These samples were then maintained for 40 days to observe the growth of bacteria in the presence and absence of microplastic and the extend of degradation of HDPE microplastic by these bacteria. The reduction in weight of the microplastic before and after the incubation was analysed to check the extend of biodegradation of HDPE. The morphological changes of the microplastic were observed using the transmission electron microscope (TEM). The growth curve, total enzyme activity, changes in the pH, laccase activity and many other factors were analysed for this purpose. Therefore, this study could be helpful in assessing the ability of the bacterial isolates to degrade the HDPE microplastic and then further helpful in mitigating the HDPE microplastics pollution.

Keywords: Microplastics, pollution, environment, toxicology, human health, biodegradation, laccase.

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MPs	Microplastics
HDPE	High- Density Polyethylene
PE	Polyethylene
FDA	Fluorescein Diacetate
TEM	Transmission Electron Microscopy
PP	Polypropylene
PET	Polyethylene Terephthalate
MSM	Minimal Salt Media
ABTS	(2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid))
UV	Ultraviolet
T	Tons
MT	Metric tons
SCOD	Soluble Chemical Oxygen Demand
DSC	Differential Scanning Calorimeters
SDS	Sodium Dodecyl Sulphate
$Conc^n$	Concentration
&	And
mm	Millimetre
nm	Nanometre
°	Degree
%	Percentage

CHAPTER 1: INTRODUCTION

Over the past few years, accumulation of plastic is increasing in the environment due to its unfeasible use and disposal along with its low degradation rate. In 2018, approximately 360 million MT of plastics was produced globally and it is expected by the year 2050, the global production would be up to 33 billion tons (Fig. 1.1). Around the world, China is the largest producer of the plastics and it has the highly polluted water bodies also (Fig. 1.2) [1]. The Yangtze River, China, is the highly polluted river having approximately 310,000 tonnes of plastic, followed by the Ganga River, India, which is having 115,000 tonnes of the plastic waste [2]. Apart from that the current outbreak of Covid-19 has also increased the production of plastics as in the personal-protective equipment (including, masks and gloves), rubbers and plastics are the main components. Also, up until 2015, 6300 million tons of plastic was discarded as a waste and around 79% of waste was piled up in the landfills or in the natural environment and it is expected that the amount of waste would rise up notably in the coming years i.e., around 12,000million T by the year 2050, if management would not take immediate actions**.**

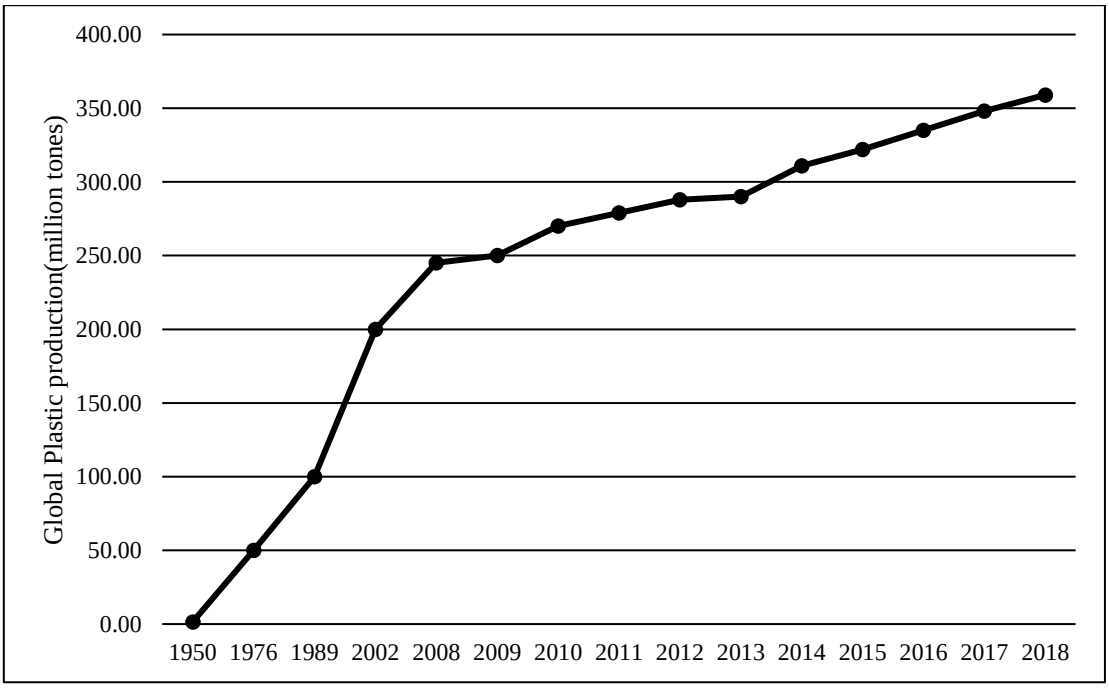


Figure 1.1: Global Plastic Production, 1950-2018

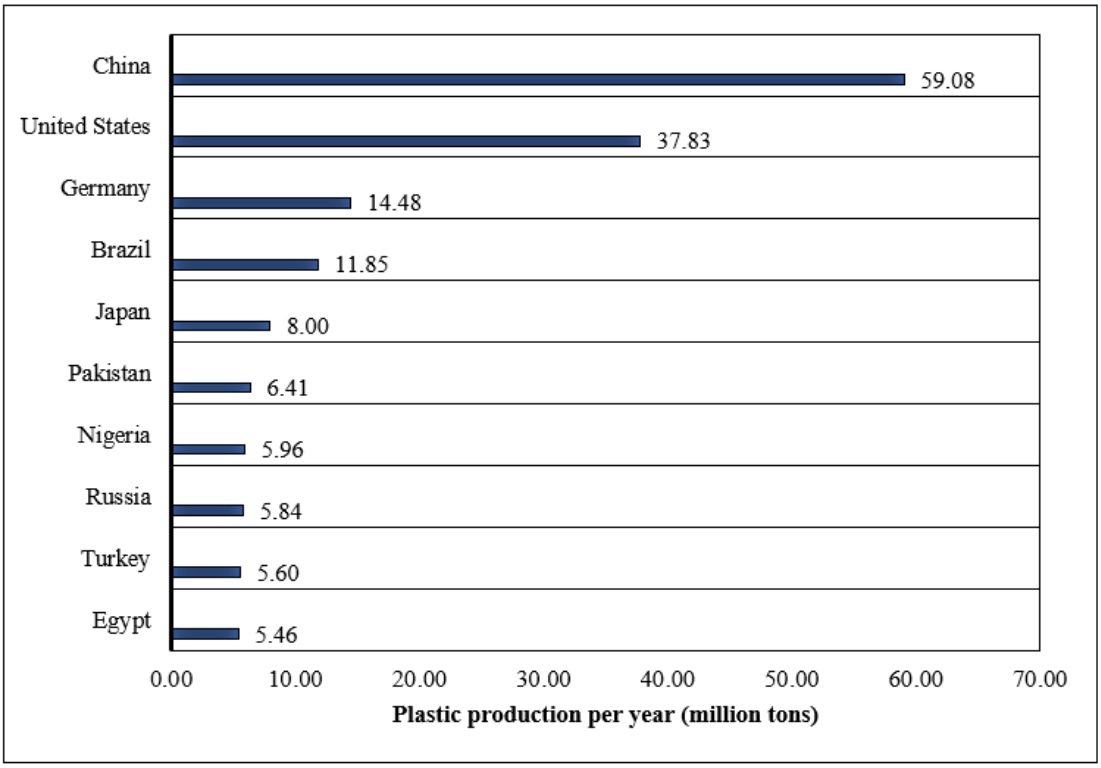


Figure 1.2: The countries with most plastic pollution

Microplastics can be defined as the plastics, having size ranges from 1µm to 5mm and irregular or regular in shape, which are basically insoluble in the water. These particle has been widely detected in a variety of shapes, like microbeads, fibres, nurdles, fragments and foam, and sizes that are based upon their continuous breakdown i.e., large MPs (range: 1-5mm), small MPs (range: 0.3-1mm) and nano plastics (<0.3mm) [3]. Various studies have been done which demonstrated the presence of MPs in different environments like marine environments, rivers, beaches, lakes, soil, air and other environments. For example, in the Dongting Lake, China, the concentration of MPs ranges from 900-2800 particles/ m^3 in concentration whereas in the North Atlantic Ocean it is 2.46 particles/m^3 [4, 5]. MPs can be generated from sources including effluents discharge, dumping of garbage, agricultural waste and human activities. Upon entering into the environment, its properties are influenced by its density as well as adsorption of biotic or abiotic substances onto its surface, that can also be responsible for the physical and physiological toxicity caused by it, to the organism upon its ingestion. Similarly, behavioural changes due to the exposure of microplastic pollutants in marine ecosystem

are also observed including physical, chemical and biological attributes [6]. Also, microplastic pollutants in the agricultural ecosystem effect the soil stability, molecular characteristics, plant growth parameters and adverse impact on soil microorganisms [7].

Microplastic pollution has detrimental effects on different countries with the most polluted being Maldives, reported in a recent study. The concentration of microplastic contaminants found in the Maldives is estimated to be around 55-1127.5 microplastic/kg. This value is approximately found to be greater than the microplastic pollution found at Tamil Nadu, India (3-611 microplastic/kg. Neighbouring countries, such as India, were also a major contributor to these pollutions. Apart from that poor wastewater and sewage system were also responsible. It is believed that speedy refinement in waste management and notable reduction in waste could help with the MPs pollutions in these small islands [8]. Therefore, MPs alone or with other pollutants can possess great impact on the ecosystem for longer duration due to its low rate of degradation.

Polyethylene (PE) amongst the most widely used type of polymer with a global annual production of around 116 million T in 2016 and is commonly used in industries, like food industry, beverage industry for the purpose of packaging [9]. It is a long-chain polymer of ethylene monomer, which is usually generated in two forms i.e., high-density and low-density polyethylene (HD/LDPE) polymers. HDPE polymer is a linear structured thermoplastic and with low water absorption and temperature resistance capability. PE polymer is considered as the least hazardous type of polymer and least harmful to humans [10]. Other than humans, it has been found that it has certain effects on other organisms also, for instance, Au et al.,2015, showed that exposure of PE to freshwater amphipods, *Hyalella azteca*, can cause alteration in their reproduction and growth after 46 days of exposure, whereas, Beiras et al.,2018, highlighted that zooplankton when exposed to PE didn't cause any harmful effects even after the 12 days of exposure [11,12]. Moreover, MPs of PE accounts for around 80% of the total MPs, that have been undergoing weathering in the marine environment. Many species of microorganisms, including *Pseudomonas*, *Bacillus*, *Streptomyces*, *Penicillium* and *Staphylococcus*, have also been reported for the degradation of PE [13]. Therefore, in order to restore

the natural ecosystem and mitigate the HDPE microplastics pollution, biodegradation of HDPE using microorganisms is necessary.

Therefore, in this study we are focusing on isolation of bacterial species from the contaminated site and then further study the ability of bacteria to degrade the HDPE microplastics. The biodegradation study of the HDPE microplastics is done by using various analytical methods like enzyme assays, TEM of microplastic, microbial growth under microplastic presence, SCOD analysis and DSC. These methods would help us in determining the extend of degradation of microplastics in the presence of bacteria and also the microbial growth in the presence of microplastics.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Microplastics and its chemical composition

Microplastics can be defined as the plastics, having size ranges from 1µm to 5mm and irregular or regular in shape, which are basically insoluble in the water. These particle has been widely detected in a variety of shapes, like microbeads, fibres, nurdles, fragments and foam, and sizes that are based upon their continuous breakdown i.e., large MPs (range: 1-5mm), small MPs (range: 0.3-1mm) and nano plastics (<0.3mm) [3]. The main components of the MPs are the polymeric raw materials like monomers, and the chemical additives. The basic units of plastic polymers commonly known as monomers, produce biochemically inert structure. Commonly used monomers include high- and low-density polyethylene (HD/LD-PE), polyethylene terephthalate (PET), polyvinyl chloride (PVC), polystyrene (PS) and polypropylene (PP). Lithner et al., 2011, in his study have ranked the polymers according to their hazardous properties and further found that the styrene polymer is a potential carcinogenic or mutagenic carrier and is one of the most hazardous polymers [14].

Other component of the MPs is the chemical additives that includes flame retardants, plasticizers, antioxidants, fillers, dyes, UV and heat stabilizers and lubricants. Flame retardants are basically used in order to protect or cool down the material in case of fire event [15]. Plasticizers are the complex chemical compounds having properties like low vapor pressure, chemical stability and are insoluble in the liquids and are used to enhance workability, distensibility or mobility of plastic. Fillers are of two types i.e., inert fillers, which are used for modifying the working, strength, shrinkage and flow properties, and reinforcing filler, that are superior to the base resin because of their strength. Soluble or insoluble dyes are used to give the desirable colour to the polymer. UV and heat stabilizers are added to protect the plastics from degradation by light, heat or UV radiations. By improving the flow characteristics of the plastic material, lubricants help in the facilitation of plastic processing. Table 1 depicts the examples of the additives that are commonly used for the production of the plastics.

Table 2.1 Examples of commonly used chemical additives for plastic production

Chemical Additives	**Examples**
Plasticizers	Phthalates, polymeric polyesters, carboxylic acid esters, etc.
Flame Retardants	Chlorine, Bromine, Aluminium hydroxide, phosphorus, etc.
Fillers (Inert or Reinforcing)	Clay, Chalk, glass, carbon black, carbon nanotubes, talc, etc.
Dyes	Heavy metals, azo dye, phthalocyanine dye, various chromophores, etc.
Lubricants	Calcium or manganese stearates.
UV or Heat stabilizer	Organic or inorganic barium, cadmium or lead salts.

2.2 Sources of MPs

Depending upon the source of the MPs, they could be categorized as primary MPs and secondary MPs. Primary MPs are mainly the MPs that are released from the products containing MP like plastic microbeads and nurdles that are the pre-production plastic pellets used for the production of the plastic products. The microbeads that are made up of polyethylene can be used as an exfoliants, in the cosmetics products, scrubs and toothpastes, and drug carrier, which makes them a potential source of primary MPs that are added in the environment after its use by the consumer [16]. Also, a recent study has highlighted that the glitters, used in crafts, textiles and cosmetics, are a great source of the primary MPs pollution. Another type of the MPs based on its source is the secondary MPs that are basically the fragments of the plastics that are generated upon the degradation of the larger plastics products, like rope, clothing and packaging products, via chemical, physical or/and biological processes. These plastics upon degradation are directly or indirectly ingested by the organisms leading to inflammatory responses and blockage in the gastrointestinal tract (GIT) [17]. Some common sources of secondary MPs include fishing nets, water bottles, tea bags & microwave containers.

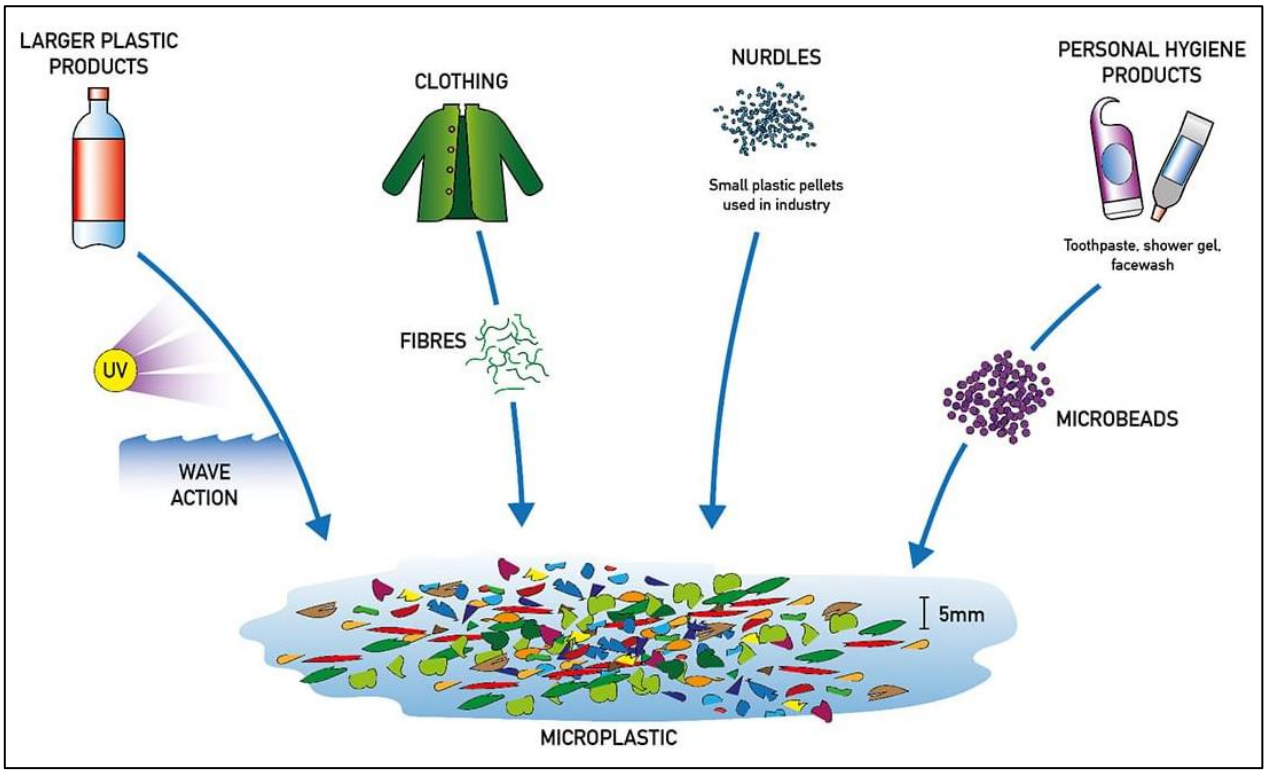


Figure 2.1 Sources of microplastics

MPs have been widely used in industries including textile, automobiles, electronics and paints, from there they can be directly discharged into the water and thus affects the aquatic ecosystem. Another source of the MPs is the plastic fibres that are used in the textile industry, as several clothes are made up of these fibres which upon washing can release its ultrafine particles in the environment. Other well-known sources include the plastic film that is widely used in the agricultural sector, tires, paint particles, polymeric materials used for drug delivery, waste generated by the ship in the water and plastics food packaging products. [2,18]. In addition, causes of the MPs in the air are industrial emissions, degradation of larger plastics material, particles that are released from the traffic, waste disposed in the landfills, and resuspension of road dusts.

2.3 Toxicological effects of MPs and its additives

Long chain organic polymers are the building block of the plastics and these are not considered very hazardous to the environment because of its large molecular size which makes them biochemically inert. Many hazardous substances like monomers, chemical additives and its degradation product or

by product could be released throughout the life cycle of the plastics. Based on the composition of the monomers, the most hazardous type of polymers are polyurethanes (PUR), polyvinylchlorides (PVC) and polyacrylonitriles (PAN). On the other hand, polyvinyl acetate (PVAc), polypropylene (PP), polyethylene (PE) and ethylene-vinyl acetate (EVA) are considered as the least hazardous type of the polymers. Some monomers have harmful effects on humans like vinyl chloride and styrene monomers both have shown mutagenic and carcinogenic effects, whereas BPA monomer disrupts the endocrine function. Contrary to that, monomers like ethylene and propylene are considered as least harmful to humans [19]. In one of the studies, it is found that air pollution can be caused by the volatilization of chemicals that are released from the polymers like styrene, formaldehyde and vinyl chloride [20]. Food and beverage industry commonly used polymers are polyethylene terephthalate (PET), polycarbonate and high-density polyethylene. Some studies have shown that BPA is released from the polycarbonate polymer is associated with various human health problems like type-2 diabetes, obesity, reduced sperm production, cardiovascular diseases and increases the chances of breast cancer and prostate cancer [21]. On the other hand, uptake of PET by the human can lead to health problems like chronic pneumonia, allergy, asthma, gastrointestinal obstruction, etc. In case of PVC, phthalates are used to make them flexible, which are harmful to the human health as they can cause skin disease, ulcer, vision failure, genetic abnormalities, birth defects etc [22].

Additives are added to the plastics, during its manufacturing to improve its properties like strength, workability, UV or heat resistance, etc. These additives have several toxicological effects also, as they are not always bound to the polymer of the plastics [18]. In case of MPs, this release of additives is more effective due to its large surface area to volume ratio. In addition, various additives are highly toxic, like PBDEs (heat resistant) and nonylphenol (antioxidant) [24]. Also, additives are released more often in areas where there is more concentrated MPs, where plastics and its components are exposed to UV and areas having higher temperatures [13]. Phthalates (plasticizers), due to its ability to create changes in the endocrine function of the body, can affect the development of the amphipods and crustaceans, reproduction of animals and can also induce genetics aberrations [25]. Plasticizers

are highly toxic to plants also, for example, tetra chlorophenol, a heat-resistant thermoplastic, is directly toxic to the phytoplankton. The toxicological effects on human health are still in its initial stage. Many studies have highlighted that humans that consume fish containing MPs have health problems like inflammation, cell necrosis, etc. [26]. Also, ingestion of the food contaminated with MPs can cause serious problems to the humans as well as to the organism positioned at the top of food chain.

2.4 Occurrence of MPs in various environments

2.4.1 Marine Water

Presence of MPs have been reported in marine systems globally. About 80% of the plastic waste that is present in the ocean is the land-based plastic debris that enters through shipping, activities related to fishing, and aquaculture as well as via rivers, beach littering and atmospheric transport [27]. In addition to those natural disasters like hurricanes, strong sea and tsunamis can also cause the transport of large number of MPs to the marine environment. WWTPs can also contributes to MPs pollution into the ocean by either releasing the effluents or by releasing it into the sea via river**.** In North-western Pacific Ocean, the surface waters are polluted with the MPs with concentration ranging from 640-42000 items /km^2 whereas, in the Arabian Bay, the surface waters accounts for the MPs concentration ranges from 4.38 × 104 to 1.46× 106 items/km^2 [28, 29].

2.4.2 Freshwater

The release of MPs in freshwater occurs due to incomplete MPs retention in the sewage sludge or due to MPs being filtered out during sewage treatment process. MPs in the freshwater are the plastic resin powder originated from the industries, personal care products containing microbeads, pellet spillage from the air blasting machine, as well as the secondary MPs. In one of the study it was found that, storm water ponds in Denmark that receive water from the urban runoff were reported to have MPs concentration of up to 22,849 MPs/m^3 [30]. Moreover, a recent study has found that even the higher

removal efficiency of the WWTPs cannot offset the number of MPs that are released into the freshwater via WWTPs and thus making WWTPs a source of MPs [31].

2.4.3 Soil

Soil acts as a key reservoir for the MPs. Various studies have demonstrated that MPs can be found in sewage sludge and compost fertilizers that are commonly used for the agricultural purposes. Common sources of MPs in the soil includes disintegration or fragmentation of plastic waste in the landfills, use of sewage sludge as a fertilizer, car tyre debris, flooding of wastewater and atmospheric deposition. A study was conducted by Scheurer and Bigalke, 2018, in which they found that conc^{n} of MPs in the soil of 26 floodplain sites in Switzerland, was about 55.5 mg/kg [32]. In another study, Corradini et al., 2019, have highlighted that the conc^{n} of MPs in the agricultural fields of Chile applied along with sludge was about 0.57 to 12.9 mg/kg [33].

2.4.4 Air

Due to the low density and small size, MPs can travel easily in the wind and can be observed commonly at the downwind sites in large quantity. The common sources of the MPs in air are the urban dust, erosion of the synthetic rubber tires and synthetic textiles. A study was done by Dris et al., 2016, in which they found that the suburban fibre fallout was about 50% of the observed urban fallout i.e., 53 particles/m^2/day as compared to 110 particles/m^2/day and thus they concluded that the fallout of fibre is lower in suburban areas comparatively [34]. In further study, Dris et al., 2017, showed that the concentration of MPs can be detected more in the indoor air i.e., 1-60 fibres/m^3, as compared to outdoor air i.e., 0.3-1.5 fibres/m^3 [35]. The concentration of MPs in indoor air is more because there is more release of particles by the sources inside the house as well as there is lower removal rate of particles by dispersal mechanisms [36].

2.5 Impact of MPs on the environment

2.5.1 Soil

2.5.1.1 Soil Properties

MPs can interact with the multiple properties of soil as these particles can integrate into the soil aggregates and incorporate into the soil clumps with varying degree i.e., loosely in case of fragmented type whereas more tightly in case of linear-type. Furthermore, a study was conducted by de Souza Machado et al., 2018, in which he highlighted that the polyester fibres can enhance the water-holding capacity & reduce the water-stable aggregation and bulk density; although, no change was observed in the water holding capacity in case of polyethylene and polyacrylic acid [37]. Moreover, MPs can also modify the permeability & water-retention of soil which further affects the water evaporation, for example, Wan et al., 2019, conducted a study in which he observed that the addition of MPs can enhance desiccation cracking and water evaporation in two clay soils [38]. Furthermore, Huang et al., 2019, observed that the MPs can have a notable effects on the enzymatic activity of various soil enzymes, like urease, phenol oxidase, catalase activities and FDAse (fluorescein diacetate hydrolase), that can cause short-term effects on soil quality [39].

2.5.1.2 Soil Microorganisms

Also, Changes in the soil properties like soil moisture and soil porosity could change the relative distribution of the anaerobic & aerobic microorganisms due to the alteration in the flow of oxygen caused by the addition of MPs. Liu et al., 2017, observed that PP particles (7% and 28%) have positive impact on the activity of soil microorganisms while de Souza Machado et al., 2018, have reported that polymers like polyester (0.05-0.4%), polyacrylic (0.05-0.4%) and polystyrene particles (1mg/kg) have negative impact on it [40]. Wang et al., 2020 and de Souza Machado et al., 2019, observed that presence of MPs in the soil can also affect the properties of soil fungus like root colonization rate of AMF at different degrees [41,42]. Chen et al., 2020, reported that PLA MPs could affect the interaction between the microbial species present in the soil and thus further affects the microbial assisted nitrogen fixation rates and mineral absorption.

2.5.1.3 Soil Animals

Along with microorganisms, soil animals are also affected by the MPs. Ingestion of MPs by animals is accidental in most of the cases as animals consider MPs as food. These ingested MPs can then cause false satiation, which leads to reduction in carbon biomass ingestion, that further leads to decreased growth, energy depletion and in some cases, death. Song et al., 2019, investigated the toxic effects of PET fibre on snail (*Achatina fulica*) by exposing it to MPs contaminated soil at a concentration of 0.014-0.71g/kg for 28 days and observed that these fibres could reduce the excretion and food intake, influence oxidative stress and induce villi damages in the walls of GIT and other adverse effects on snails [63]. Moreover, X. Jiang et al., 2020, reported that the presence of PS particles could induce DNA damage in the earthworms (*Eisenia fetida*) [43]. Moreover, MPs can cause intestinal obstruction, oesophagus damage, reduced reproduction and some biochemical responses like metabolism disorders and reduced immune response, to soil animals. Also, MPs adhered to the outer surface of the animals can directly arrest their mobility [44]. In general, MPs can cause various effects on properties of soil and thus further effects the soil microorganisms and animals which leads to variation in structure, community and diversity, and evolutionary consequences.

2.5.2 Plants

Upon exposure to MPs due to plastic mulching, organic manures and sewage sludge as fertilizer, the plants that are grown in it get subjected to MPs. MPs that are bigger in size (100nm -5mm) can affect the plants by modifying or disrupting the soil structure and fertility or by clogging the seed pores. Qi et al., 2018, performed a study on wheat plant (*Triticum aestivum*) and found that both the vegetative and the reproductive growth of the plant was affected in the presence of the LDPE MPs (1%w/w) [45]. In another study, Wang et al., 2020, performed an experiment on Maize (*Zea mays*) plants and found that PLA caused reduction in chlorophyll content and maize biomass and stronger phytotoxicity and PLA along with PE caused alteration in AMF community diversity and structure and increase the pH and Cd concentration in the soil [46]. Boots et al., 2019, studied the exposure of Perennial ryegrass

(*Lolium perenne*) to biodegradable PLA and virgin HDPE microplastic clothing fibres and observed that there was a reduction in biomass and shoot height and also fewer seeds were geminated after the exposure [47]. However, recent studies relatively focused on effects of MPs on smaller plants like, wheat (*Triticum aestivum*), cress (*Lepidium sativum*) and spring onion (*Allium fistulosm*) [48]. Therefore, there is a need to conduct more research in an effort to understand the impacts of MPs on higher plants, as the concept is still very unclear.

2.5.3 Aquatic Organisms

Recent researches on the impact of MPs mainly focus on the marine & freshwater organisms. For instance, copepod (*Centropages typicus*), the marine jacopever (*Sebastes schlegelii*), the diving beetle (*Cybister japonicas*) and the crab (*Carcinus maenas*) when exposed to the MPs experienced reduction in the ingestion rate, assimilation efficiency, feeding capacity and swimming speed. Also, some species are able to egest the MPs rapidly, whereas other might be unable to do so and thus MPs retain and accumulate in their system. For example, a large amount of microbeads was egested by tadpoles of *Xenopus tropicalis* after they were transferred to clean water with having 95% depuration rate after 6 days [49]. In case of zebrafish (*Danio rerio*), PS microbeads can cause inflammation, oxidative stress, accumulation of lipid in the liver and accumulation of the microbeads in the liver, gut and gills [50]. MPs can also impact small sized planktons due to their small size by acting as substitute of nutrients that are required by planktons and thus resulting in loss of energy and eventually leading to death of the organism. Other studies have also demonstrated the negative impact of MPs on microalgae which includes reduction in growth rate, photosynthetic activity and chlorophyll content [51]. Additionally, MPs can affect the organisms at molecular level also, by altering their genes, for example, in case of *Dicentrarchus labrax* and *Mytilus galloprovincialis*, MPs were found to alter the expression of genes that are responsible for DNA repair, biotransformation, immunity, lipid metabolism signalling pathways and stress response [49]. Also, toxicological effects are caused by additives in MPs as they enter into the organisms along with the MPs and then during desorption

process they are released and cause carcinogenic, mutagenic or endocrine-disruptive effects in the aquatic organisms [50].

2.5.4 Human Health

Due to the omnipresence of MPs in the environment, its exposure to humans is completely unavoidable. Humans are mainly exposed to the MPs via three different routes: inhalation, ingestion and dermal exposure. The MPs that are inhaled by humans mainly originates from the sources including urban dust, rubber tyres and synthetic textiles, while in case of ingestion, MPs contaminated seafoods and other food items and drinking water are the main sources. Although in case of dermal exposure, it is not possible for the MPs to pass through the skin membrane as it is too fine for the particles to pass through it, but it can enter through other possible routes such as sweat glands, open wounds or hair follicles [51]. In general, exposure of MPs to humans results in particle toxicity, with inflammatory lesions, enhanced uptake and oxidative stress and also since immune system cannot discard the MPs, so it might cause increase risk of neoplasia and chronic inflammation. In the study conducted by Prata, 2018, they reported that inhalation of MPs at a concentration of around 26-130 MPs/d can cause respiratory problems like dyspnea and can also induce other inflammatory responses, mainly in case of industrial workers that are exposed to MPs for longer period of time [52]. Phthalate esters can potentially cause harmful effects to humans upon exposure including abnormal sex development and birth defects. Other researches have demonstrated that the chemical compounds that is present in the plastic or is adsorbed on the MPs can become mutagenic and carcinogenic upon their exposure. To understand the risk of MPs to humans, further studies are needed to be conducted.

2.6 Microbial Degradation of MPs

Microbial degradation method is the reliable, easy and clean method for the remediation of MPs pollution. This process is controlled by various factors which can lead to modification in the physiochemical properties of the polymers. The process of degradation is affected by factors like abiotic factors, including surface hydrophobicity and surface morphology, biotic factors, such as

activity of enzymes, release of acids and metabolic pathways, and other common environmental factors, such as temperature, pH and oxygen level [2]. The microbial degradation occurs via the following steps: Biodeterioration (using a biological agent to change the chemical and physical properties of MPs); bio-fragmentation (cleavage of the complex polymeric unit into simpler units via acids/enzymes); assimilation (microorganism incorporate the molecules); & mineralization (degradation produces the oxidised metabolites like carbon dioxide, water and methane). It is reported that abiotic factors like UV radiation and photooxidation, can enhance the microbial degradation process [53]. Also, degradation is hindered by the high molecular weight polymers as they have larger fragments of molecules that are difficult to uptake by cells. Therefore, two different mechanisms called intracellular and extracellular degradation are used for the high molecular weight polymers. Intracellular degradation involves aggregation of microbes onto the surface of the MPs to hydrolyse the complex polymer into smaller units whereas, extracellular degradation involves degradation of complex unit into simpler unit via the extracellular enzymes like hydrolases that are secreted by the bacteria [54]. Various microorganisms have been found to have a potential to produce an enzyme which can result in the degradation of MPs polymer, for instance, *Bacillus sphericus* and *Bacillus cereus* strains are found to help in the degradation of PE polymer, whereas, enzyme like hydrolase produced by the *Thermobifida fusca*, is capable of degrading the PET polymer (megha maam paper ref). Laccase enzyme produced by the *Rodococcus rubber* strain have also shown promising results in the degradation of PE polymer [55].

CHAPTER 3: METHODOLOGY

3.1 Materials and sample collection

All the chemicals & HDPE microplastics that were used throughout the study are of analytical and gradient grade and were obtained from standard manufactures. The samples were collected from the Hindon, Ghaziabad, contaminated site at a depth of 3m.

3.2 Isolation of bacterial species

The bacterial culture was prepared by mixing 20g of soil sample in an isotonic solution. The mixture was then kept on vortex for 2hrs, followed by settling for 30min. After that 10ml of the supernatant was inoculated in the NB broth and then incubated in a rotating incubator for 24hrs at 34°C and 120rpm.

3.3 Screening of isolated bacteria for microplastic degradation

The screening of bacterial isolates was done to check the ability of these isolates to use MPs as their sole carbon source. For this purpose, a mixed bacterial culture was inoculated in the Minimal Salt Media, in which 0.4g of sterilized HDPE microplastic was further added. The mixture was then incubated on the shaker incubator for 15 days at 34°C and 120rpm. After that, the incubated microbial cultures were plated on the Nutrient Agar (NA) plate for 24hrs at 30°C. Then the growth and the colonies of the bacteria were observed. Later, NA plates of each of the morphologically different colony were prepared and then incubated for 24hrs at 30°C.

3.4 Microbial culture preparation for biodegradation study

Two bacterial isolates (bacterial isolate 1 and bacterial isolate 2) that were identified as the microplastic degraders were then isolated and inoculated into the fresh minimal salt media and then incubated in a shaker incubator at 30°C until their population density reached to log phase i.e., absorbance of 1.00 ± 0.05 at 600nm. After that, 500µl of the bacterial strains were inoculated into the flask (labelled: A1 bacterial isolate 1 with microplastic and A2 bacterial isolate 2 with microplastic) containing 100ml of minimal salt media (media containing all the required nutrients except glucose)

and 0.4g of sterilized HDPE microplastics. Two negative control was prepared. For the negative control 1 (labelled: A3 bacterial isolate 1 without microplastic and A4 bacterial isolate 2 without microplastic), 500µl of bacterial inoculum was added into 100ml of minimal salt media and 1ml of glucose in the absence of HDPE microplastic and for the negative control 2 (labelled: A5 microplastic without bacterial isolates), an uninoculated 100ml of minimal salt media supplemented with the 0.4g of HDPE microplastic was prepared. Duplicates were prepared for all the experiments. All the flasks were then left on shaking incubator at 30°C and 120 rpm for 21days. The pH, optical density (OD), total enzyme activity and laccase activity of each of the flask were evaluated at every 5 days for a period of 40days. The OD at 600nm was taken in order to check the growth of microbes in the designed experimental flask.

3.5. *In vitro* biodegradation experiments

3.5.1 Determination of total enzyme activity

The FDA (fluorescein diacetate) hydrolysis assay was performed to check the total enzyme activity of the microbial population and could also provide an overall estimation of microbial activity in the sample. This assay is considered non-specific because of its sensitivity towards the activity of several enzymes, which eventually results in the hydrolytic-cleavage of FDA (colourless) into fluorescein (fluorescent yellow-green). For this, 5ml of 60mM Phosphate-buffered saline (PBS) buffer was added to the 2ml of each sample in a tube and an additional tube was prepared as a control in which only PBS was added and no sample was there. After that, briefly vortex the solution in order to suspend the samples. Add 25µl of 2mg/ml FDA to each of the tubes and shake again briefly by hand. Place the tubes in a water bath shaker (200rpm) and incubate there for 60min at 30°C. After the incubation, carefully add 5ml of 2:1 chloroform/methanol to each tube and shake it briefly by hand. The tubes were then spined at 5000 x g for 5min to separate the aqueous(buffer) and organic (chloroform/ methanol) phases. After that, 5ml of the resultant supernatant was collected in another clean tube. The intensity of the resulting yellow-green colour would indicate the amount of enzymatically cleaved FDA molecule and total enzyme activity in the sample. The intensity of the colour was

measured at 490nm and then compared to a standard curve to determine the relative microbial activity in each of the samples.

3.5.2 Determination of Laccase enzyme activity

Laccase activity was evaluated by using the ABTS method. In this method, ABTS is oxidised by the laccase enzyme to a more preferable and stable state of cation radical. The concentration of this cation is responsible for the intensity of blue-green colour which can be correlated with the enzyme activity and can be evaluated by measuring the OD at 420nm. The reaction mixture was prepared by adding 100µl of 10mM ABTS, 800µl of 50mM sodium acetate buffer (pH 5) and 100µl of culture supernatant. The reaction mix was then incubated for 25mins at 30°C. Then measure the absorbance at 420nm.

3.5.3 Determination of dry weight

After the end of incubation periods, the HDPE MPs in the samples were filtered out by using the 0.45µm cellulose-acetate membrane filter. This filtrate can be used for the purpose of SCOD analysis also. The filters were then washed with 2% (w/v) SDS solution for 4hrs, in order to remove the bacterial films that were colonized around the surface of the microplastic particles, and then dried overnight in an oven at 50°C. The amount of microplastic degraded was evaluated by comparing the dried polymer weight before and after the test using an analytical balance. Further, the percentage of weight loss of HDPE microplastics due to the degradation was evaluated by using the following equation:

$$\%\text{weight loss} = ((W_i - W_f) / W_i) \times 100\ \%$$

where W_i is the initial weight of the HDPE microplastic in grams and W_f is the residual weight of the HDPE microplastics after degradation period in grams.

3.5.5 Determination of soluble oxygen demand (SCOD)

The soluble oxygen demand (SCOD) analysis was done to determine the degree of bio-decomposition of samples before and after the incubation period of 40 days. For this, 5g of soil sample was suspended in 20ml of distilled water, which is followed by filtration using Whatman filter paper**.** After that, 5ml

of filtrate was added in the mixture of 7ml sulfuric acid (95% purity) and 3ml of potassium dichromate (0.01667M) and then heated at 150°C in an oven for 2hrs. After heating for 2hrs, the samples were cooled down and then add ferroin indicator was added to each sample. Then titrate the samples with ferrous ammonium sulphate solution (FAS, 0.02M) until its colour turned to reddish brown. After that, the COD can be calculated using the following equation:

$$\textbf{COD mg } O_2 \textbf{ } L^{-1} \textbf{ = ((A-B) x M x 8,000) / V}$$

where A and B are the titration volume of FAS that was consumed for the blank and sample, respectively, M is the molarity of FAS, 8,000 is the milliequivalent weight of oxygen and V is the volume of sample used for the purpose of COD analysis.

3.5.6 Transmission Electron Microscopy (TEM) of microplastics

The ultra-structure of HDPE microplastics before and after treating it with the bacterial culture was characterized by using TEM (Tecnai G2 200 KV HRTEM SEI HOLLAND).

3.5.7 Differential Scanning Calorimetry (DSC) analysis

The DSC measurements were performed on a Perkin Elmer 4 instrument to study the variation in the crystallinity of polyethylene during biodegradation. The instrument was calibrated for a temperature range of 25°C to 170°C with an indium standard sample. Polyethylene samples were placed in an aluminium DSC pan (approx. 15 mg) and were run on the DSC under a free flow of nitrogen between 75°C and 150°C. The heating rate was approximately 10ºC/min. Heat of reaction was determined from the peak temperature.

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 Screening of Bacterial isolates

Two bacterial isolates, bacteria 1 and 2, were isolated from the sample as they were capable to grow in the MPs-infused media and were used for the further study.

4.2 Weight loss % of HDPE microplastic by bacterial isolates

The activity of bacterial strains on the HDPE MPs showed a significant decrease in the weight loss of the HDPE MPs. The decrease in the weight of microplastic was found to be approx. 18% and 16%, after the incubation for 40 days with the bacterial isolate 1 and 2 respectively. This result depicts that both the isolates have the capability to release the enzymes that are capable of degrading the HDPE MPs and cause reduction in its weight. While, in case of control i.e., media having only the microplastic, weight loss of the microplastic was less than 5%. Moreover, out of these two bacteria, bacteria 1 is more capable of degrading the HDPE MPs as compared to the bacteria 2. Weight loss (%) of both the bacteria are as follows:

Bacteria 1 Treatment= ((0.4g – 0.322)/0.4) x 100% = ~18%

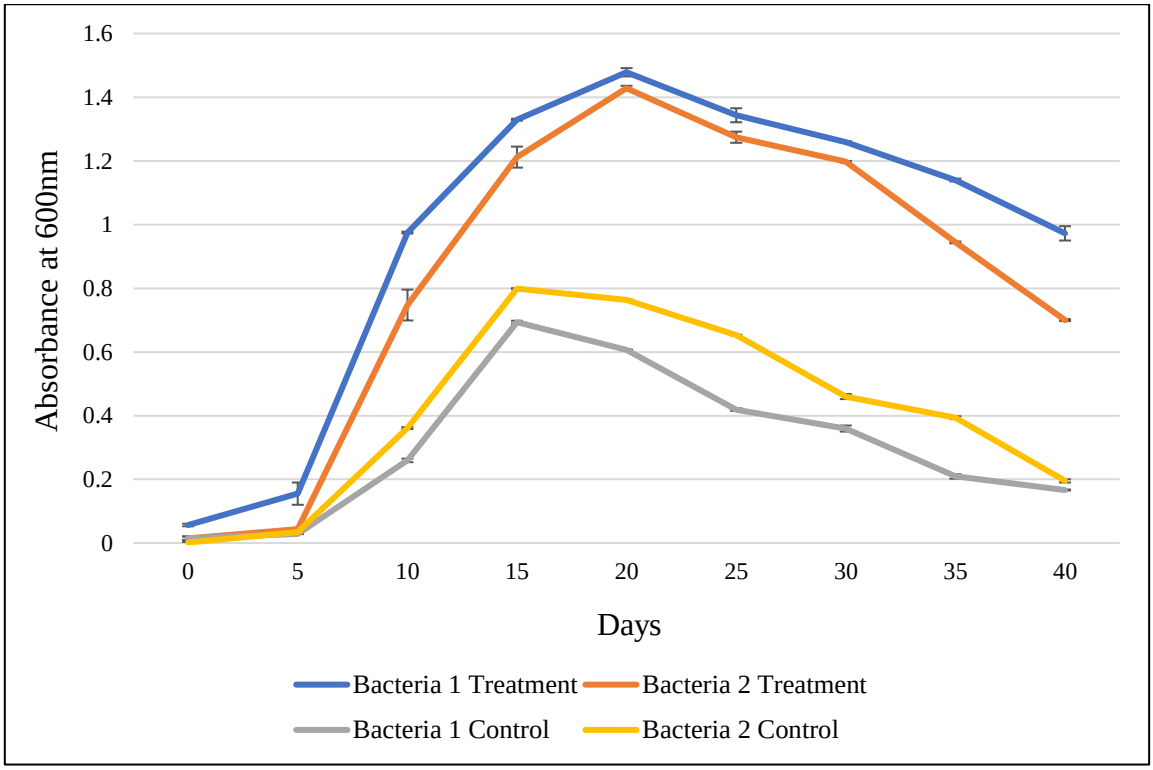
Bacteria 2 Treatment= ((0.4g – 0.339)/0.4) x 100% = ~16%

Control= ((0.4g – 0.391)/0.4) x 100% = <5%

4.3 Growth pattern of bacteria 1 and 2 upon HDPE MPs exposure

Graph 1. depicts the growth pattern that was exhibited by both the bacterial isolates in the presence and absence of HPDE MPs during the 40days of period. A lag phase was observed for all the samples from day 0 to day 5 after that there was an exponential increase in the growth of bacteria. An exponential increase in the growth from 5^{th} to 20^{th} day was observed in case of bacteria 1 and 2 treatment whereas, it was from 5^{th} to 15^{th} day in case of bacteria 1 and 2 control. This increase in the treatments could be due to the interaction between the bacterial membranes and the microplastics. The maximum growth of both the bacteria was observed on 20^{th} day in the presence of microplastic while in case of control it was on 15^{th} day. After that a gradual decrease in the growth was observed

till the 40th day and this could be due to the cell lysis, depletion of nutrients or release of the growth inhibitory products in the media. This decrease could also be due to the inability of bacteria in adapting the changing condition of the culture media due to the release of the degradation products. Also, the results show that microplastic was a better source of carbon for bacteria 1 and bacteria 2 as compared to the glucose as more growth was observed in the presence of microplastic as compared to controls. Also, bacteria 1 exhibited more tolerance to the HDPE microplastics as compared to the bacteria 2 throughout the degradation period.

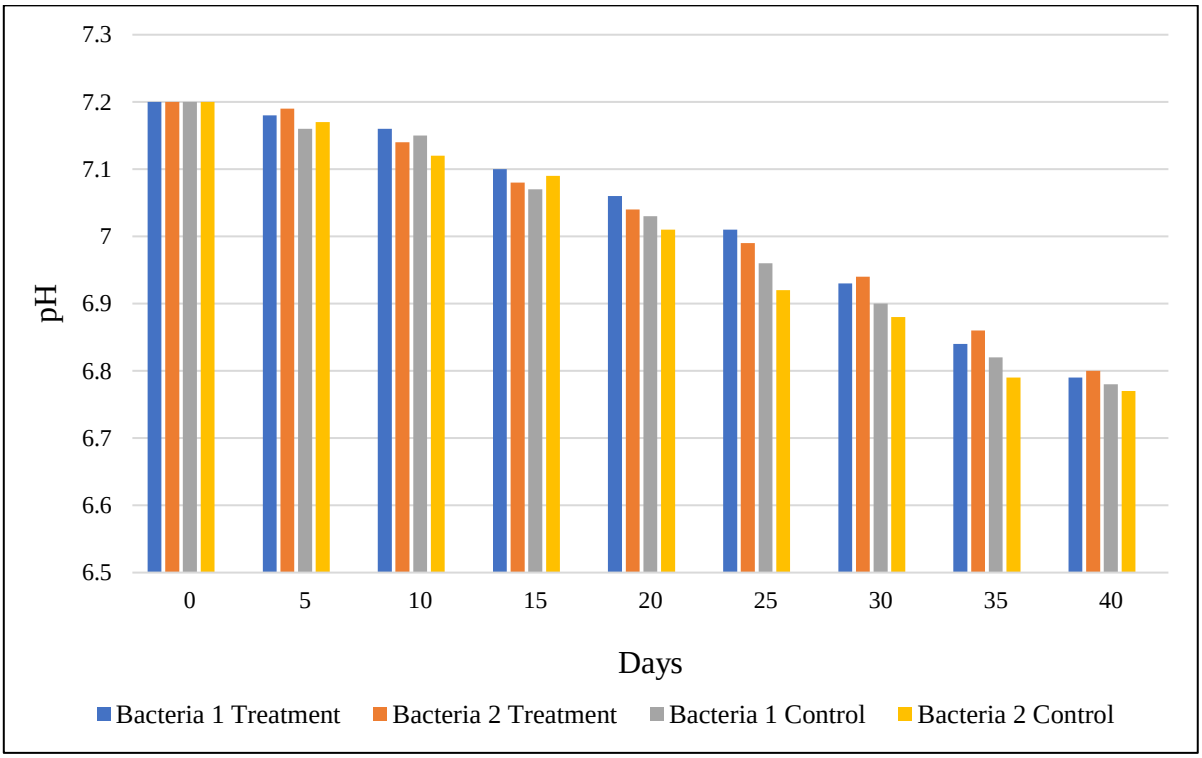


Graph 4.1 Growth curve of bacteria 1 and 2 treatment and control

4.4 Change in pH throughout the incubation period

pH is considered as one of the important factors that is needed for the activity and survival of the microorganisms. Graph 2. demonstrates the change in pH of the HDPE MPs and bacterial isolates inoculated culture media during the period of 40days for the study of biodegradation of MPs. The results shows that the degradation of the MPs by the isolates significantly effects the pH value, as the pH was constantly shifting towards the acidity throughout the incubation period of 40days. Also, in

case of both the bacteria controls and treatments, similar variations in the pH were observed. The decrease in the pH could be due to the production of metabolites by the isolates, which further suggests that these bacterial isolates had formed pH modulating metabolites, while degrading the microplastic. This changing trend in the pH values shows the potential of bacteria 1 and 2 for degrading the HDPE microplastics.

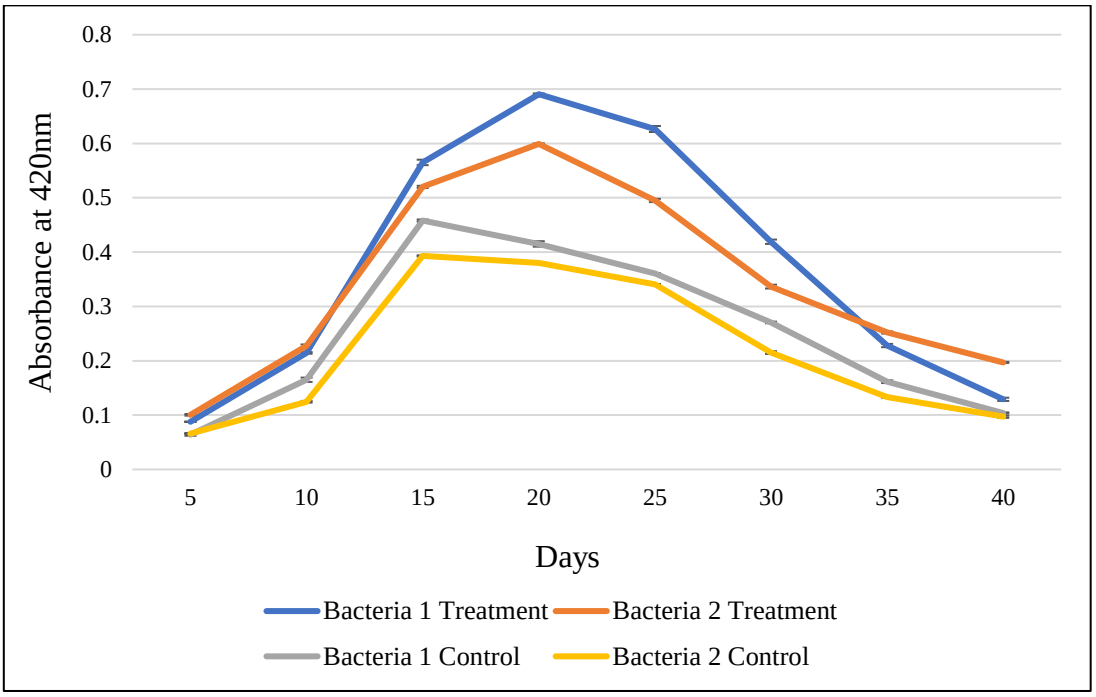


Graph 4.2 pH change upon exposure to Bacteria 1 and 2 treatment and control

4.5 Total enzyme activity throughout the incubation period

Microbial degradation of the microplastic involves the release of enzymes by the microorganisms, that are responsible for the degradation of microplastic polymer. Therefore, to check the release of enzymes by the microbes and the overall activity of these enzymes in the media, FDA was done. Graph 3. demonstrates the overall activity of the enzymes during the degradation period. After 5th day, a significant increase in the activity of enzyme was observed and both the isolates in the presence of microplastic showed maximum enzyme activity on 20th day while the control showed the maximum activity on 15th day. This depicts that both the bacterial isolates were able to release the enzymes in the media and also, the enzyme activity was increasing or decreasing with the increase or

decrease in the growth of bacterial isolates. After 20th day and 15th day a significant decrease in the overall activity was observed till the 40th day in case of both the treatments and the controls respectively. This decrease could be due to the depletion of substrate, changing pH conditions of the media or formation of inhibitory products. Also, it was observed that bacteria 1 has higher enzyme activity as compared to bacteria 2 and controls.

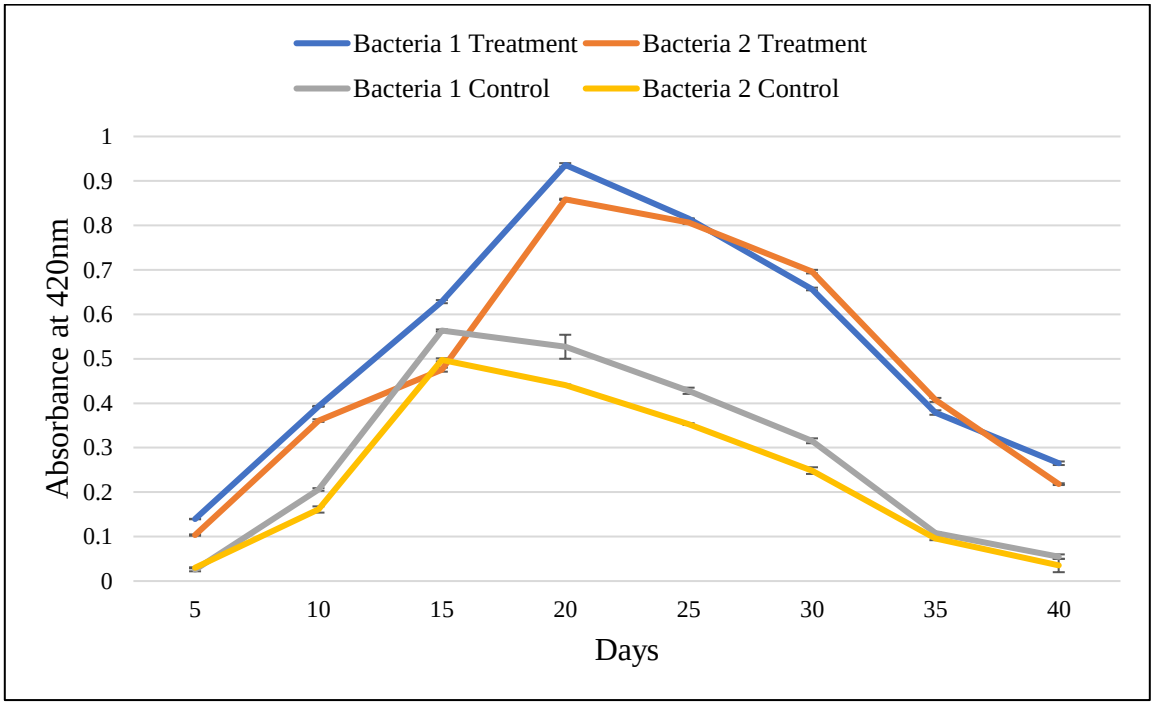


Graph 4.3 Total enzyme activity in the presence of Bacteria 1 and 2 treatment and control

4.6 Laccase activity throughout the incubation period

Laccase is one of the enzymes that are produced by various bacterial and fungal strains and has shown promising results in the degradation of HDPE microplastics. Laccase activity using ABTS method was done to check the activity and of laccase in the sample. Graph 4. demonstrates the activity of laccase for the 40days of degradation period. It was found that bacteria 1 and 2 both were able to produce laccase in the sample with or without microplastics. Also, bacteria that are treated with the microplastic demonstrated higher laccase activity on 20th day, whereas in case of controls it was on 15th day. Both the bacteria were able to produce more laccase in the presence of microplastic and this could be due to more growth of microorganisms and enzymes activity were observed when treated with microplastic and could be due to more formation of products during the degradation of

microplastic, that might be essential for laccase activity. After 20th day and 15th day the activity of laccase enzyme started declining in case of treatment and control respectively. This decreased trend in the activity could be due to the unsuitable conditions of the media for the enzyme activity, deficiency of the substrate, formation of inhibitory products or decrease in the population of the microorganisms. Also, bacteria 1 has shown higher laccase activity as compared to the control and the bacteria 2 treatment, which depicts that bacteria 1 is comparatively a good source for laccase production when using microplastic as a carbon source.



Graph 4.4 Laccase activity in the presence of bacteria 1 and 2 treatment and control

4.7 SCOD Analysis

During the incubation period, the organic matter content of the samples was measured as SCOD. For the bacteria control 1 and 2, the values of the SCOD were observed as 680 mg O_2 L^{-1} and 664 mg O_2 L^{-1} respectively. After the incubation period of 40 days, it was observed that the SCOD was decreased to 62 mg O_2 L^{-1} and 53 mg O_2 L^{-1} in case of bacteria 1 and 2 respectively. therefore, the results clearly depicts that both the bacterial isolates were able to biologically attack the structure of the HDPE MPs particles, and produce products which were further decomposed by some biological or chemical reactions.

4.8 Transmission Electron Microscopy (TEM) of HDPE MPs

TEM was done to observe the morphological changes in the MPs before and after the treatment with the bacterial isolates. Fig 4a show the microplastic before the treatment with the bacterial isolates whereas, fig 4b and 4c depicts the microplastic after it was treated with the bacterial isolate 1 and 2 respectively. The results show that both the bacterial isolates were able to degrade the microplastic debris and had successfully formed the cracks and holes in the microplastics, as shown in fig b and c, which indicates its degradation by the bacteria.

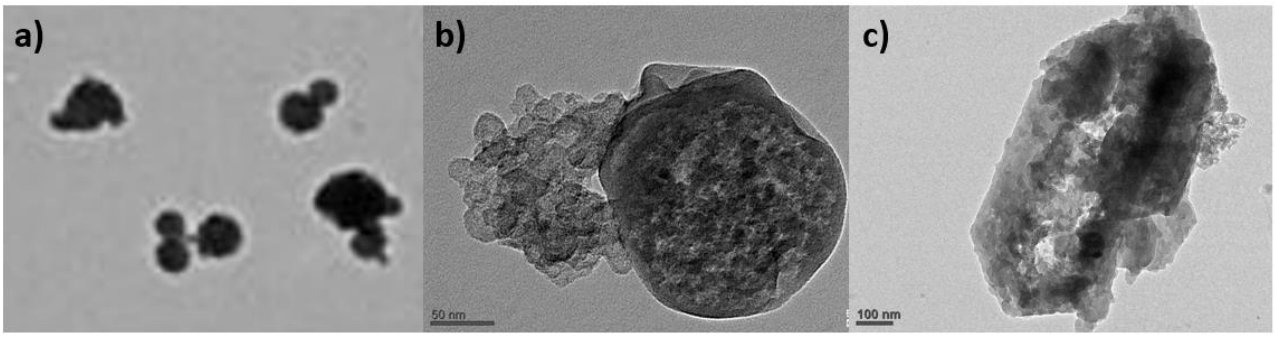
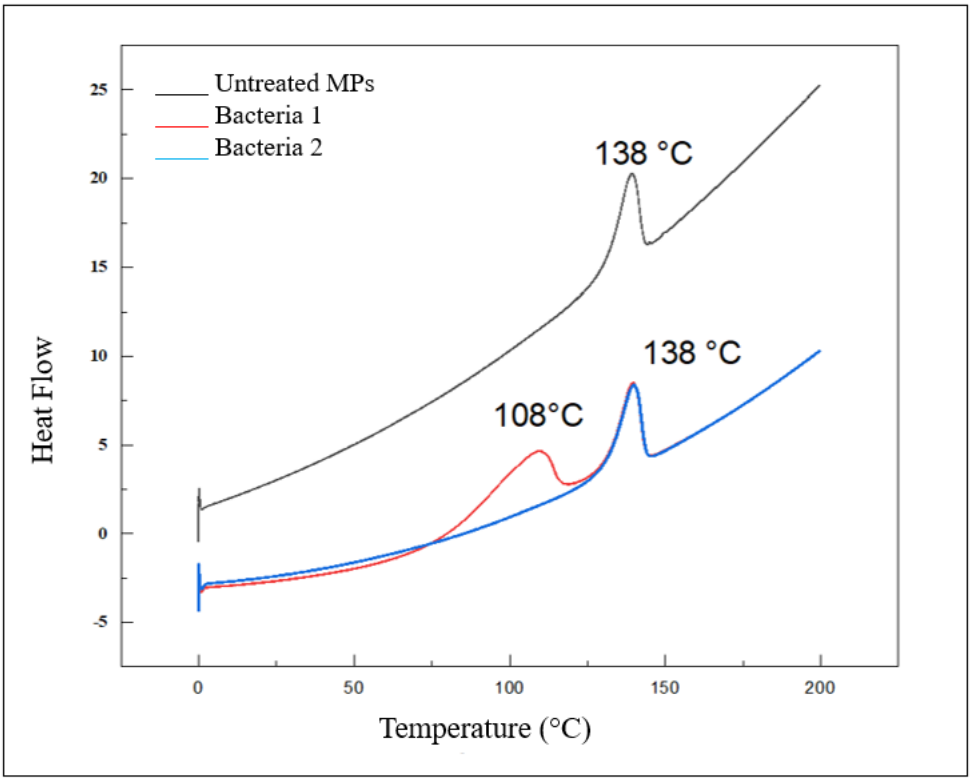


Figure 4.1 HDPE microplastics a) before degradation; b) after degradation by bacteria 1; and c) after degradation by bacteria 2

4.9. DSC thermogram of degraded microplastics



Graph 4.5 DSC thermogram of untreated MPs & bacteria 1 and 2 treated MPs

The DSC thermograms of Untreated microplastics of HDPE (Control) show a single melting step around 138 °C. Besides this significant melting step of control sample, the treated sample (Bacteria 1) showed an additional step melting peak around 108 °C, which is because of the degradation. In order to quantify the degree of degradation of these samples, the enthalpies of melting in the DSC thermograms have been changed from Delta H= 75.0680 J/g (Untreated, PE) to Delta H= 89.98 and 65.96 J/g treated sample (Bacteria 1) and Delta H= 90.56 J/g (Bacteria 2).

CHAPTER 5: CONCLUSION

Microplastic pollution has drastically increased in recent years owing to the urbanization, industrialization and population expansion creating negative impacts globally. Because of MPs contamination in air, water and soil, most of the organisms and humans are being affected having prolonged impacts. Eco-friendly remediation measures, like microbial remediation, to eliminate microplastics from environment also need to be utilized for enhancing the habitat of aquatic species, soil organisms, plants, soil structure, air and water. This study exhibited the potential of bacterial isolates that were isolated from the Hindon, Ghaziabad, contaminated site to degrade the HDPE microplastics. The in vitro study of HDPE microplastics bio-degradation showed the potential of bacterial isolates, 1 and 2, in degrading the HDPE microplastics. Growth pattern of both the bacterial isolates demonstrated a significant increase in the presence of microplastics and slight pH change was observed in all the samples during the degradation period. Also, the biodegradation efficacy was further confirmed by the TEM images. Enzyme and laccase activity were also observed in the samples and were significantly high in the presence of microplastics. DSC analysis further facilitated the potential of the bacterial isolates in the degradation of HDPE microplastics. Therefore, this study is considerably helpful in identifying the bacteria that have the potential to degrade the HDPE microplastics. The utilization of microorganisms for the remediation of microplastic provides a new strategy for sustainable environment.

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