MICROBIAL DEGRADATION OF HDPE AND NYLON 6,6 MICROPLASTICS: A POTENTIAL BIOREMEDIATION STRATEGY FOR CLEANER ECOSYSTEMS

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DECLARATION

This is to declare that the research work embodied in this thesis entitled "**Microbial degradation of HDPE and Nylon 6,6 microplastics: A potential bioremediation strategy for cleaner ecosystems***"* submitted to the Delhi Technological University is an original work and carried out by me for the degree of Doctor of Philosophy under the supervision of Prof. Jai Gopal Sharma, Department of Biotechnology, Delhi Technological University, Delhi and Co Supervision of Dr. Deenan Santhiya, Department of Applied Chemistry, Delhi Technological University, Delhi. This thesis is a contribution to my original research work. The extent of information derived from the existing literature has been indicated in the body of the thesis at appropriate places giving the source of information.Every effort has been made to make sure that the scientific contributions of others are appropriately cited. To the best of my knowledge, this research work has not been submitted in part or full for award of any degree or diploma in Delhi Technological University or in any other University/Institution.

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CERTIFICATE

This is to certify that the thesis entitled **"Microbial degradation of HDPE and Nylon 6,6 microplastics: A potential bioremediation strategy for cleaner ecosystems"** submitted to the Delhi Technological University, Delhi-110042, in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** has been carried out by the candidate, **Ms. Neha Tiwari**, (Reg. No. 2K18/PhDBT/502) under the supervision of Prof. Jai Gopal Sharma, Department of Biotechnology, Delhi Technological University, Delhi and Co-Supervision of Dr. Deenan Santhiya, Department of Applied Chemistry, Delhi Technological University, Delhi. It is further certified that the work embodied in this thesis has neither partially nor fully been submitted to any other university or institution for the award of any degree or diploma.

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Dedicated To

My Family…

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Neha Tiwari

Abstract

An alarming rise of micro-nano plastics (MNPs) in environment is currently causing the biggest threat to biotic and abiotic components around the globe. These pollutants, apart from being formed through fragmentation of larger plastic pieces and are also manufactured for commercial usage. MNPs enter agro-ecosystem, wildlife, and human body through the food chain, ingestion or through inhalation, causing blockage in the blood-brain barrier, lower fertility, and behavioural abnormalities among other problems. Hence, it becomes essential to develop novel procedures for remediation of MNPs. Among the numerous existing methods, microbial remediation promises to degrade/recover MNPs via a green route. Since microbial remediation processes mostly depend upon biotic and abiotic factors such as (temperature, pH, oxidative stress, etc.), it becomes easy to influence changes in the plastic pollutants. Hence, with the help of recent technologies, a complete degradation/removal of MNPs can be expected by utilizing the respective carbon content as energy sources for growth of microorganisms. In our study, considering the urgent environmental need, the degradation of micro-nano plastics with its corresponding degradation mechanisms has been brought out. Finally, the role of enzyme and metagenomics in remediation of MNPs are discussed.

Biodegradation is an eco-friendly strategy for removal of contaminants. Present investigation demonstrates bioremediation of nylon 6, 6 microplastics (NMPs) for the first time using a soil isolate called *Brevibacillus brevis* (*B.brevis*) by shake flask assay. Interestingly, 22 w/w% weight loss of NMPs were noticed after 35 days of incubation with *B.brevis*. Upon interaction with microplastics, rod to round shape change along with size reduction of the bacterium and irregular shapes of NMPs with cracks and holes were

visualized using SEM and TEM. TGA and FTIR analysis reported the disappearance of intermolecular hydrogen bonding of nylon 6, 6 after microbial interaction. The release of various organic acids and enzyme/enzymatic activities of the bacterium were found to be higher in the presence of NMPs. Interestingly, mass spectrometric analysis confirmed the release of adipic acid and hexamethylenediamine derivatives during the aerobic biodegradation. As a result, the degradation of NMPs by *B.brevis* as a sole carbon source was proven effectively in the environment.

The fragmentation of polyethylene plastics into polyethylene microplastics (PEMPs) due to biotic and abiotic factors affects the environment. Extensive investigations have shown its implications upon accumulation in the living systems. In this study, *B.brevis* was employed to degrade PEMPs. *B.brevis*-mediated degradation process has shown a reduction in microplastic's dry weight by 19.8% over 35 days of treatment. The biodegradation was achieved by releasing laccase enzyme and organic acids onto the surface of PEMPs, which were quantified by UP-HPLC and SEM analysis. The biodegradation was achieved by releasing laccase enzyme and organic acids onto the surface of PEMPs, which were quantified by UP-HPLC and SEM analysis. Further, the changes in the structural and functional composition of PEMPs were observed by TEM, DSC, FTIR and TGA analysis. Additionally, after the degradation, by-products were observed to contain a short polymer chain such as 2-hexadecanone, decanone. The products resulting from the biodegradation of PEMPs were further utilized for bacterial metabolism. These outcomes reveal the efficiency of *B.brevis* in PEMPs degradation.

The degradation of polyethylene microplastics (PEMPs) and nylon 6,6 microplastics (NMPs) were demonstrated first time using bacterial culture isolates along with degradation mechanism. Bacterial isolates were isolated from a municipal landfill site and identified through 16S rDNA and metagenomics techniques. The isolates identified as

Achromobacter xylosoxidans and mixed culture species in dominance of *Pulmonis sp.* were used to degrade PEMPs and NMPs. *Achromobacter xylosoxidans* assisted degradation process has resulted in a decrease in microplastic's dry weight by 26.7% (PEMPs) and 21.3% (NMPs) after 40 days of action. Mixed bacterial culture has shown weight reduction of 19.3% (PEMPs) and 20% (NMPs), respectively. Cell hydrophobicity test, SEM and TEM analysis revealed biodeterioration of microplastics through the attachment of bacterial cells onto the surface of microplastics. The release of enzymes, laccase and peroxidases revealed C-C bond cleavage and reduced polymer chain length. The thermal studies (TGA and DSC) revealed changes in the thermal stability and transition characteristics of microplastics over a period of time. The structural alterations on PEMPs and NMPs were recorded by FTIR analysis. Various byproducts such as alkanes, esters, aromatic compounds and carboxylic acids released were identified by GC-MS. The breakdown of microplastics produced small molecules, which were then employed for bacterial metabolism. These results conclude the effectiveness of isolated bacterial isolates in PEMPs and NMPs degradation and potentially leading to the development of more effective and sustainable solutions for managing plastic waste.

The major objectives of the present research investigation include:

1. Understanding the role of *Brevibacillus brevis* in the degradation of microplastics.
2. Elucidation of mechanisms involved in microbial degradation of microplastics.
3. Isolation and characterization of microbes that degrade microplastics.
4. Assessment of the bacterial cell interaction with plastic and corresponding metabolic products in modulating microplastic degradation.

This thesis is summarized in four chapters.

Chapter 1 discusses a brief introduction microplastics and its remediation technologies. It also talks about the sources and impacts of microplastics on human health and surrounding ecosystem.

Chapter 2 This chapter outlines the process of bioremediation of nylon 6,6 microplastics (NMPs) using a soil-derived strain called *Brevibacillus brevis (B.brevis)* via a shake flask assay. A novel biodegradation pathway is postulated in this chapter. Objective 1 and 2 covered in this chapter.

Chapter 3 The present chapter focuses on *B.brevis*-mediated degradation of polyethylene microplastics along with the degradation mechanism. Objective 1 and 2 covered in this chapter.

Chapter 4 The chapter focuses on the degradation of polyethylene microplastics (PEMPs) and nylon 6,6 microplastics (NMPs) along with degradation mechanism. The microbes were isolated from a municipal landfill site and identified through 16S rDNA and metagenomics techniques. A detailed study of degraded products and microplastics structural alterations were studied. The objectives 3 and 4 covered in this chapter.

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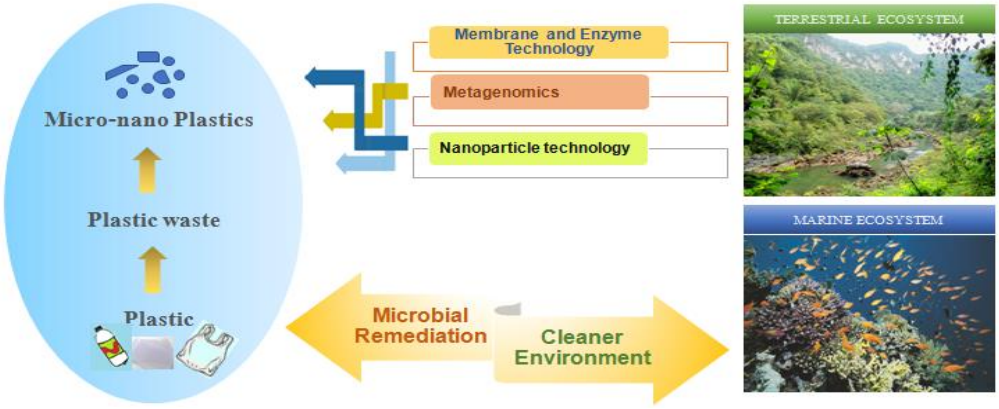
List of Abbreviations and Symbols

AR	Analytical reagent
B. brevis	*Brevibacillus brevis*
DSC	Differential scanning calorimeter
FDA	Fluorescein Diacetate assay
FTIR	Fourier transform infrared spectroscopy
HCl	Hydrochloric acid
HDPE	High density polyethylene
GC-MS	Gas Chromatography- Mass Spectroscopy
NMPs	Nylon 6,6 microplastics
MNPs	Micro-nano plastics
UPLC-MS	Ultra Performance Liquid chromatography-Mass Spectroscopy
PEMPs	Polyethylene microplastics
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinyl chloride
SEM	Scanning electron microscopy
TEM	Transmission electron microscope
TGA	Thermogravimetric analysis
XRD	X-Ray Diffraction
%	Percentage
Å	Angstrom
cm	Centimeter
gm	Gram

h	Hour
kV	Kilo Volts
M	Molar
mA	Milliampere
mg	Milligram
min	Minutes
mL	milliliter
mM	Millimolar
MW	Molecular weight
nm	Nanometer
°C	Degree Celsius
v/v	Volume per volume
w/v	Weight per volume
wt.	Weight
λ	X - ray wavelength
µg	Microgram
µm	Micrometer
ω	angular frequency
θ	Diffraction angle

Chapter 1

Introduction-Microbial remediation of micro-nano plastics: Current knowledge and future trends



Chapter 1

Introduction-Microbial remediation of micro-nano plastics: Current knowledge and future trends

1.1 Introduction

Plastic pollution is regarded as one of the most significant threats to ecosystem affecting biotic and abiotic components around the world. In 2018, global plastic production almost reached 360 million tonnes. As per the latest available data, the global production of plastic reached almost 360 million tonnes in 2018. Asia is the largest producer and consumer of World's plastics. Among global plastic production, China contributes the highest portion (30%), followed by Europe (17%) and altogether 18% contribution is from Canada, Mexico, and US. It is alarming that every year around 4.8 to 12.8 million metric tonnes of plastic debris are thrown into the ocean without proper waste management strategy and it is estimated to increase further in future (Jambeck et al., 2015; Lebreton et al., 2017). Highly polluted water bodies are from Asia. In China, the river Yangtze, the utmost polluted river, has an input plastic waste of approximately 310,000 tonnes, which is more than 4% of annual ocean plastic pollution. The second-largest polluted river with 115,000 tonnes of plastic waste is the river Ganges from India. It is pertinent to mention that petrochemical plastics contribute towards more than 80% of the worldwide plastic use. For example, polyethylene terephthalate (PET) Polyethylene (PE), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC) are the most used plastics (Urbanek et al., 2018). In spite of this, plastic materials form a fundamental part in worldwide economy; the issues related to its broad application can't be overlooked. Most of these plastics in environment are non-degradable and continue remaining in the environment for an extended period. Among the plastic remains, broken small particles have garnered much attention and are of greater concern (Eerkes-Medrano et al., 2015; Thompson et al., 2004).These particles are defined as microplastics (0.1 µm-5 mm) and nano plastics (0.001 µm -0.1 µm) based on size range(Gigault et al., 2018; Moore, 2008; Thompson et al., 2004; Waller et al., 2017). These are

leading contributors to plastic pollution in the ecosystem such as in agro-ecosystem, marine, and freshwater ecosystems (Chae and An, 2017; Ng et al., 2018; Thompson et al., 2004). Unwanted persistence and accumulation of nano plastic wastes are hazardous for the environment and human health. Recent evidences shows significant distortion of cells and aggregation of nano plastics in the organs (liver, kidney, and gut) (Deng et al., 2017; Mohr et al., 2014). Since micro-nano plastics are difficult to degrade, there is a need for proper waste management technology, which can serve as a potent tool to combat the adverse effects. From the last few years, researchers focussed on degradation of micro-nano plastics through microbial biotechnology approach (Pathak and Navneet, 2017). A large population of microbes (both bacteria and fungi) associated with the degradation of plastics are; namely, *Streptomyces setonii, Pseudomonas aeruginosa, Rhodococcusruber, Pseudomonasstutzeri, Streptomyces badius, Aspergillus niger, Aspergillus flavus, Fusarium lini* (Pathak and Navneet, 2017).

The present review highlights the impact of micro-nano sized plastic pollutants on the ecosystem and consequent remediation through microbial approaches. Also, a detailed understanding of the microbial degradation mechanisms of micro-nano plastics is brought out. Finally, the importance of novel approaches in the remediation of micro-nano plastics are discussed for future remedial aspect.

1.2 Sources of Micro-nano Plastic Pollutants

Based on sources, micro-nano plastics are categorized into two major divisions: primary and secondary MNPs. Primary micro-nano plastics originate from household products, cosmetics, and polymeric raw materials of plastic industries such as polyethylene (PE), polystyrene (PS) and polypropylene (PE). Secondary MNPs are formed due to fragmentation of extensive plastic waste from exposure to abiotic factors such as temperature, UV radiation, microbial degradation, and atmospheric pressure. Stress and reactive species, such as ozone, also play a

vital role in the polymer degradation. Interestingly, the physical abrasion of the plastic materials can also result in secondary MNPs due to oxidation (Arthur et al., 2009; Roex et al., 2013)(Fig. 1). In detail, materials made up of polymers such as polypropylene (PP), polyvinylchloride (PVC), and polybutadiene (PBD) are very susceptible to oxidative-thermal degradation, even at room temperature due to the respective chemical composition. These materials can only withstand deterioration when antioxidants and stabilizers are added. On the other hand, physical abrasion on these polymeric materials weakens molecular chains strong chemical bonds, leading to oxidative -thermal degradation.

Plastic is a manmade product that has spread all over land and water as a result improper usage and false waste management techniques; it is calculated to increase by 2050 (Geyer et al., 2017). The major part of plastics contaminants comes from single-use plastics food packaging such as polyethylene, polypropylene, and polyethylene terephthalate (Brooks et al., 2018). Micro-nano plastics used for a wide variety of applications in electronic, automobile, textile and paint industries have a chance for direct discharge into the river catchment areas, affecting the marine ecosystem (da Costa et al., 2016; Kay et al., 2018; Roex et al., 2013). Other well-known MNPs contaminants are from personal care products such as toothpaste, scrubs, cleaning materials, and cosmetics. However, the existing wastewater treatment plants have shown that tertiary treatment of water is not a source for micro-plastics pollution, as these pollutants are effectively removed by the skimming and settling treatment processes (Carr et al., 2016). Other sources include disintegration of synthetic fibres during washing of clothes, commercial activities like thermal cutting of polystyrene, agricultural activities such as the use of polyethylene, polyurethane, polystyrene foils, films as well as soil conditioners, ship-generated waste in water, drug delivery based on polymeric nano-particles (Liebezeit and Liebezeit, 2014; Tosin et al., 2012; Zhang et al., 2012). The current generation of secondary microplastics in the oceans around the world is a result of massive plastic production in the 1990s and earlier (Lebreton et

al., 2019).Even though we are trying to reduce the contaminant disposal in the environment, specialized expertise is needed to remove MNPs.

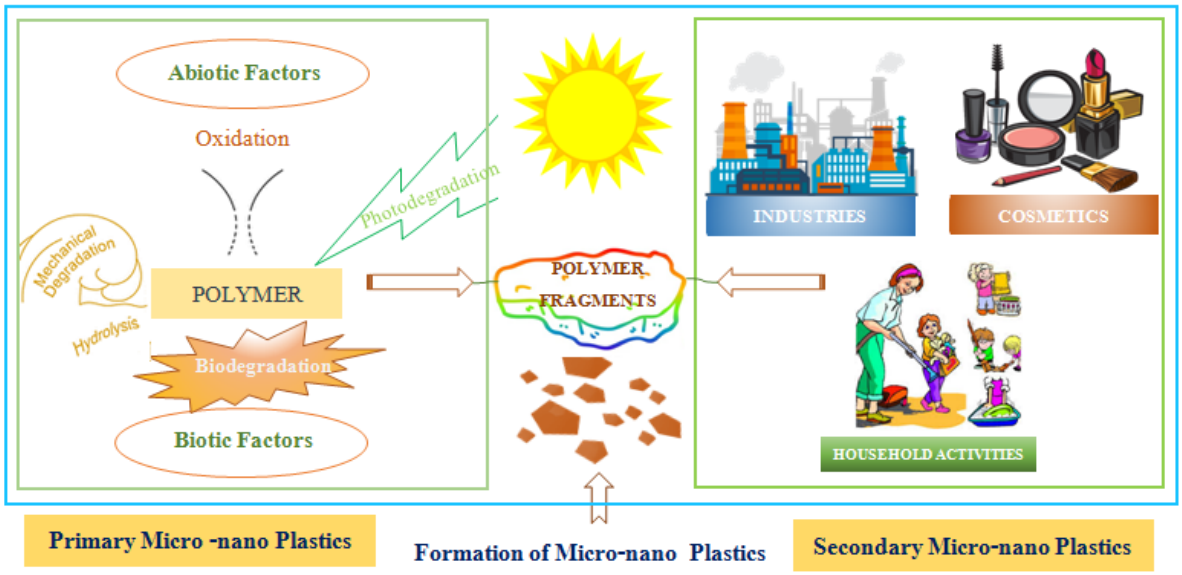


Fig. 1 Sources of micro-nano plastic pollutants (a) primary (b) secondary micro-nano plastics

1.3 Impact of Micro-Nano Plastics on Ecosystem

Current research shows the impact of micro-nano plastics as a new concern of plastic pollution in the ecosystem. These plastic particles may have immediate and circuitous toxicological effects on various organisms. Generally, plastics are considered as biochemically non-reactive because of their macromolecular structures (Hammer et al., 2012). On the other hand, MNPs might serve as carriers to transport other environmental contaminants (hydrophobic persistent organic pollutants (POPs) and pathogenic microbes from the encompassing media) into the body due to their high surface-area-to-volume ratio and hydrophobicity (Xu et al., 2019). Plastics cannot be removed easily and persist in the marine ecosystem longer than on the land ecosystem (Worm et al., 2017). The degradation of larger pieces into microscale and nanoscale plastics increases the bioavailability and associated severe impacts to ecosystem (Chamas et al., 2020). Micro-nano plastics can enter the human body through the food chain, ingestion, air

inhalation, and direct dermal exposure. These particles further can cause blockage in the digestive tract and abrasion as well as irritation in the mucosa (Barnes et al., 2009; Rehse et al., 2016) (Fig. 2.).

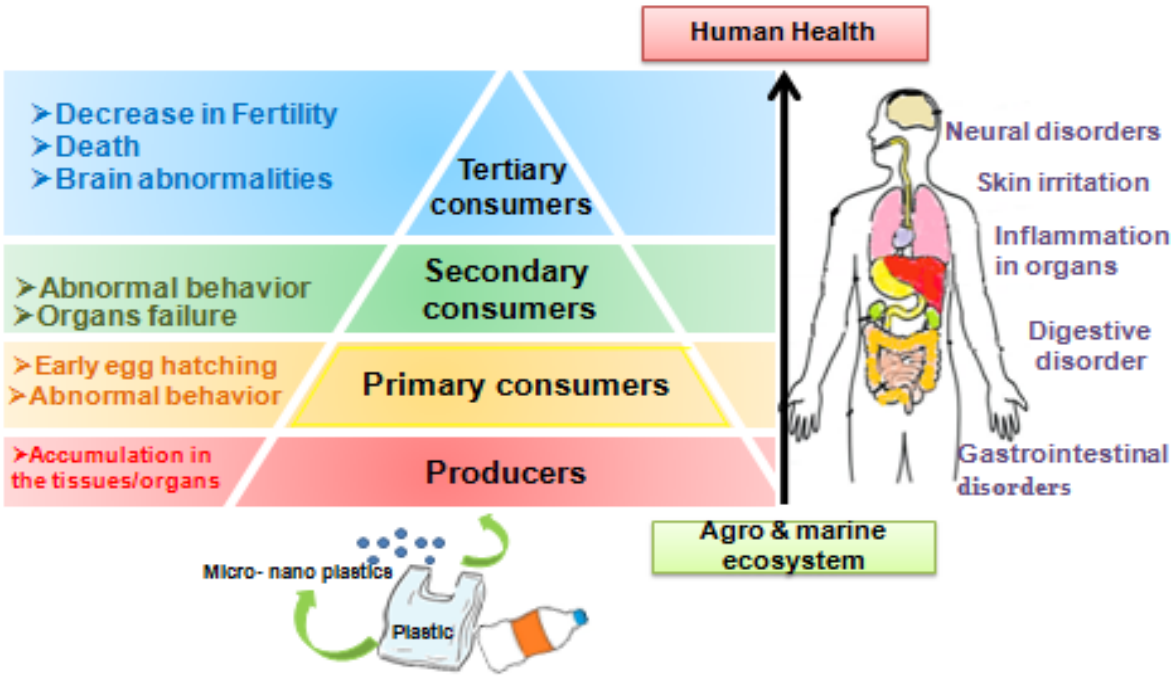


Fig. 2 Impact of micro-nano plastics on the ecosystem.

The potential cellular uptake of micro-nano plastic toxicity is still under observation. Nano-sized polystyrene (PS) particles can easily permeate into lipid membranes via different pathways such as phagocytosis, clathrin, caveolin–mediated pathway, and pinocytosis. These permeated PS particles not only affect the cellular activity but also disrupt membrane protein integrity. Finally, a drastic change in the structure of the membrane is brought out by PS with a consequent reduction in molecular diffusion and the fluidity of the membrane (Rossi et al., 2014).Recently, PS nano particles have been found to be present in the brains of various species. These observations suggest that nano-sized plastics are able to cross the blood-brain barrier, a highly selective permeability barrier required for the protection of brain from systemic toxins, and for retaining the balance for neuronal activity (Almutairi et al., 2016).

Several other effects of micro-nano PS particles on ecosystems are as follows: A decrease in the survival rate of *Oryziaslatipes* was observed due to the accumulation of latex micro-nano particles (39-500 nm) in the testis, gills, intestines, blood, and liver of the organism(Kashiwada, 2006; Manabe et al., 2011). PS particles of 20 nm size interfered in photosynthesis and promoted reactive oxygen species (ROS) production in *Chlorella sp.* and *Scenedesmus sp.*(Bhattacharya et al., 2010). Induced pro-apoptotic processes in *Mytilus galloprovincialis* resulted due to 200 nm-sized PS (Canesi et al., 2015). In *Mytilus edulis*, reduced filtering activity and production of pseudo faeces have been reported due to 30 nm of PS particles (Wegner et al., 2012). Accumulation of 100 nm PS particles has been observed in the digestive tract of *Mytilus edulis* and *Crassostrea virginica* (Ward and Kach, 2009).In the presence of 40 nm-sized PS, inhibited motility and influenced moulting phenomenon was observed in *Artemia franciscana* larvae(Bergami et al., 2016).A significant, more enormous neutrophil influx reported into the lung of *Rattus norvegicus* due to 64 nm PS(Brown et al., 2001). Recent investigations have shown that low-density polyethylene (LDPE) particles do not influence consecutive effects on zebrafish after the three-weeks of exposure (Karami et al., 2017; Rainieri et al., 2018). Whereas, more histological abnormalities were seen in clams when exposed to Polyvinylchloride and Polystyrene than to Polyethylene (Rochman et al., 2017). Accidental ingestion of micron-sized sphere-shaped PE particles by fishes as food particles causes reduced predacious performance and efficiency of common goby (*Pomatoschistusmicrops*) juveniles(de Sá et al., 2015). Further, the release of monomers and other added substances, during the deterioration of polymers can cause obstructive consequences on organisms. The vulnerability of micro-organisms to MNPs particles can lead to various serious causes, such as early death, inflammatory responses, slow/inhibited growth and development, decrease in energy, inhibited feeding activity, oxidative stress, low immunity, and neuronal inhibition and behavioural

deformity(Chen et al., 2017a, 2017b; de Sá et al., 2015; Ma et al., 2016; Rehse et al., 2016; Ziajahromi et al., 2018).

The presence of micro-nano plastics in agricultural soil is of vital concern as they impact environment and human health. Current research has shown that microplastic residues of polyethylene and biodegradable plastics have adverse effects on vegetation and reproduction of wheat, both above and below the ground(Cao et al., 2017)**.** High amounts of residual plastics also harm soil structure, water potential, and transportation of the nutrients, hence reducing crop production (Liu et al., 2014)**.** MNPs may persist in the soil, accumulate, and eventually affect the biodiversity and fitness of the soil organisms, which leads to the ecological risk in the terrestrial ecosystem (Ng et al., 2018; Rillig, 2012)**.** Furthermore, plastic particles increase carbon/nitrogen metabolism, which depletes soil organic matter, increase the release of greenhouse gases, and also soil water abomination(Steinmetz et al., 2016). The potential toxic effects of MNPs are observed in the gut of organisms and transferred through the food chain (Bouwmeester et al., 2015)**.**The mode of interaction of micro-nano plastics with organisms and characterization of the microscopic particles still remains a challenge (Ter Halle et al., 2017b). Undoubtedly, more research is required to understand the effects of MNPs pollutants on the agro-ecosystem, freshwater ecosystem, and human health**.** The connection between microplastics strength, exposure, and degradation pathways are the most exciting part of the research area.

1.4 Microbial Approaches for the Remediation of Micro-Nano Plastics

Microbial degradation is a practical, clean and affordable way to remediate micro-nano plastics contaminants. Multiple biochemical reaction pathways are involved in the microbial degradation of micro-nano plastics. Depending upon the chemical structure of MNPs, the degradation mechanisms also differ and need to be thoroughly understood to save our

environment (Velez et al., 2018; Yoshida et al., 2016). Bio-stimulation (the addition of limiting nutrients to support microbial growth) and bio-augmentation (the addition of living cells capable of degradation) are two crucial processes to enhance the biodegradation rate of contaminants in nutrient-limited environments (Yu et al., 2005). Microbial degradation of polymers is governed by many factors, which lead to changes in polymers' physicochemical properties. The degradation process is influenced by biotic (metabolic activity, the release of acids, enzymatic activity), abiotic factors (surface morphology, topography, surface hydrophobicity, electric charge distribution), and other environmental conditions (such as temperature, pH, salinity, oxygen level). The critical characteristics of MNPs, such as additive as well as hydrophobic nature and persistence of organic pollutants on the surface play a significant role in the remediation process(Min et al., 2020). Despite the fact, it is complicated to understand the degradation mechanisms of MNPs, plausible microbial plastic remediation mechanisms are portrayed schematically in Fig. 3. The microbial plastic degradation (Fig. 3.) occurs through the following consecutive steps (Dussud and Ghiglione, 2014): Biodeterioration (Changing the physical and chemical properties of the polymer by biological agents), bio-fragmentation (lytic cleavage of complex polymer into simpler forms through enzymes/acids, assimilation (Incorporation of the molecules by microorganisms) and mineralization (Oxidized metabolites produced by degradation such as CO_2, CH_4, and H_2O).Microbial degradation is reported to increase with abiotic factors such as UV radiation and photo-oxidation (Hadad et al., 2005; Sharma et al., 2015). Higher molecular weight plastic polymers hinder microbial degradation process due to the presence of large molecular fragments that are difficult for cellular uptake. Such large particles are depolymerized by microorganisms through two significant mechanisms called intracellular and extracellular degradation(Wilkes and Aristilde, 2017; Yuan et al., 2020). Intracellular degradation accumulates microbes on the surface of MNPs to hydrolyse the plastic into short chains. In extracellular degradation, bacteria secrete

some extracellular enzymes such as hydrolases which degrade complex polymers into simpler units, therefore, metabolized by the different metabolic pathways (Shah et al., 2008; Yuan et al., 2020). Further, microorganisms convert these short chains into end products (CO_2, H_2O, or CH_4) through aerobic or anaerobic metabolism by the process called mineralization. The final consumption of these end products as carbon and energy sources is called biological natural attenuation (Gu, 2003; Yoon et al., 1996; Yu et al., 2005).

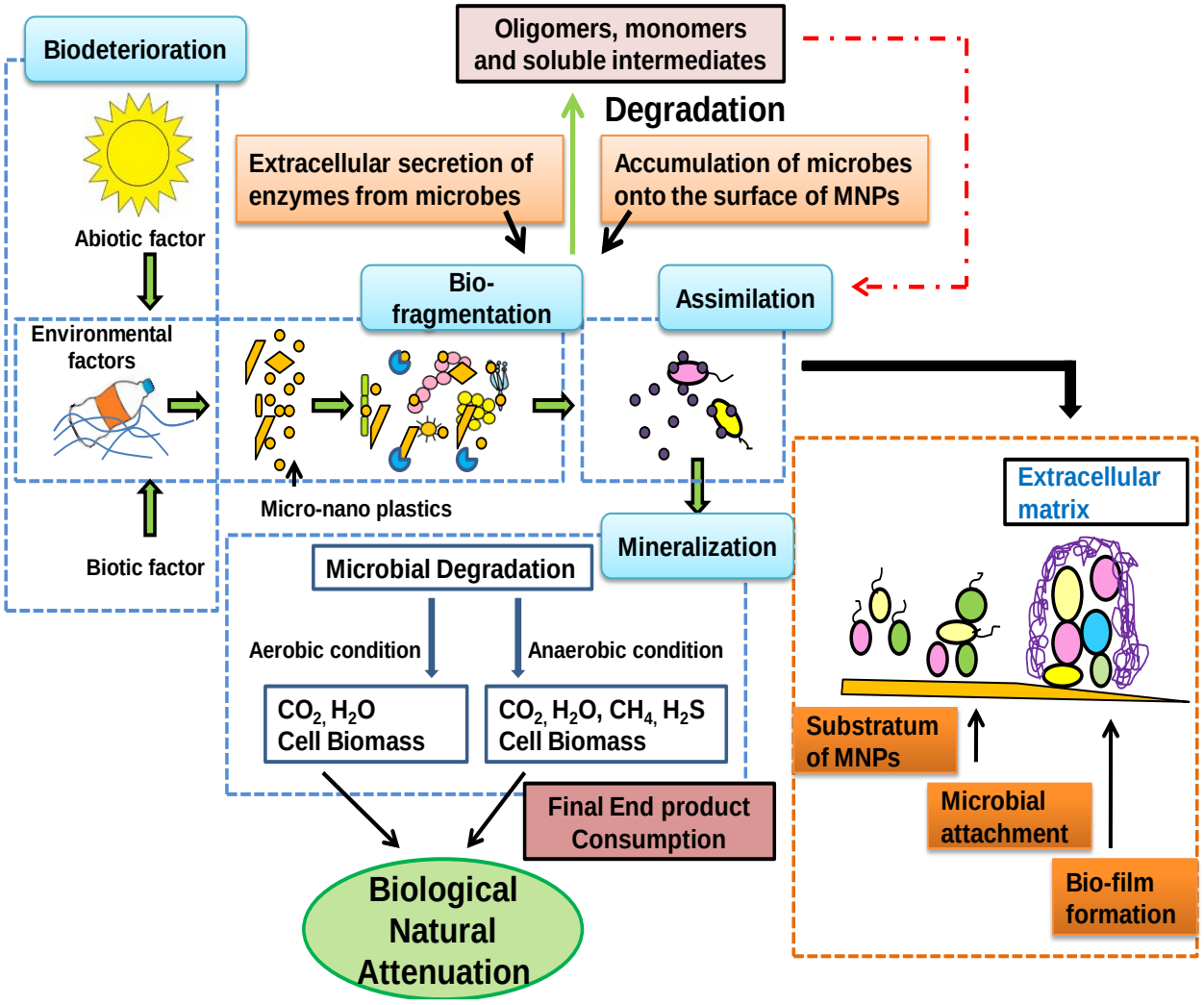
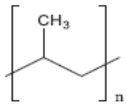
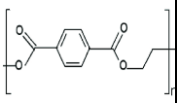
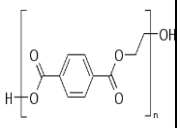
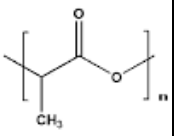
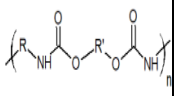
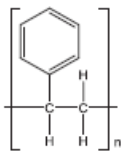
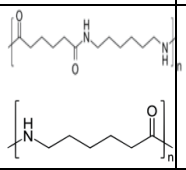
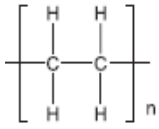


Fig. 3 Microbial remediation mechanisms of plastic pollutants.

Unfortunately, the detailed mechanisms of the bacterial attachment on the plastic surface are not known. But, the attachment of the microorganisms onto the plastic surfaces and biofilm

formation is a well-known strategy. Furthermore, to survive in the harsh or extreme environment, microbes form a protective layer to protect themselves from toxic substances and gathers nutrients by producing extracellular polymers (Junge et al., 2004, p. 200; Rummel et al., 2017).The bacterial and archaeal cells exist in the big deep oceanic subsurface, upper marine sediment, deep continental subsurface, soil and ocean, where it forms biofilms (Flemming and Wuertz, 2019). In seawater, bacterial colonization observed on low-density polyethylene (LDPE) plastic starts early. Immediately within few hours, microorganisms can attach assemblages over the surface of LDPE. During this stage, microbial assemblages might produce extracellular polymeric substances and other bacterial metabolites that can catalyze to breakdown the debris such as microplastic related compounds and could enhance the adsorption of other polymer/microbes (Harrison et al., 2011). Microbes involved in the degradation/ size reduction of plastic wastes can also accumulate MNPs. Microorganisms involved in micro-nano plastic degradation are listed in Table 1. The degradation/accumulation of MNPs can be well understood by analyzing changes in their physicochemical properties. For example, by identifying degraded products of plastics and corresponding cellular responses. The expected role of microorganisms can be understood through biodeterioration, bio fragmentation, assimilation, agglomeration, biosorption, and mineralization of MNPs.

Table 1: List of micro-nano plastic degrading microbial strains.

Type of MNPs	Structure	Microorganisms	Source	Applications	References
PP (Polypropylene)		*Bacillus Rhodococcus, Bacillus gottheilii*	Mangrove sediment	Medical, Clothing, Repairing, Aircraft, Wearable items	(Auta et al., 2018, 2017)
PE (Polyethylene)		*Bacillus gottheilii Ideonella sakaiensis*	Contaminated samples, Mangrove ecosystems	Packaging, Automotive, Electrical, Plastic parts, Laminates etc.	(Auta et al., 2017; Palm et al., 2019; Yoshida et al., 2016)
PET (polyethylene terephthalate)		*Bacillus gottheilii Zalerion maritimum Ideonella sakaiensis, Muricauda sp. and Thalassospira sp.*	Contaminated samples, Marine, Mangrove ecosystems	Packaging (bottles, jars, containers).	(Auta et al., 2017; Debroas et al., 2017; Paço et al., 2017; Palm et al., 2019; Yoshida et al., 2016)
PLA (Polylactic Acid)		*Paenibacillus amylolyticus*	Soil samples	Plastic films, Bottles, and Biodegradable medical devices (e.g. screws, pins, rods, and plates)	(Teeraphatpornchai et al., 2003)
PUR (Polyurethane)		*Comamonas acidovorans, Bacillus subtilis*	Soil samples	Durable elastomeric wheels and tires, Automotive suspension bushings, Electrical potting Compounds, Seals, Gaskets, Carpet underlay, and Hard plastic parts	(Nakajima-Kambe et al., 1997; Shah et al., 2013)
PS (Polystyrene)		*Bacillus gottheilii*	Mangrove ecosystems	Lining refrigerators, Packaging, Construction, and in medical sector.	(Auta et al., 2017)
Nylon 66 and nylon 6		*Bacillus cereus* and *Bacillus sphaericus*	Marine	Textiles, Fishing line and Carpets	(Sudhakar et al., 2007)
HDPE (High density polyethylene)		*Aspergillus tubingensis*VRKPT1, *Aspergillus flavus*VRKPT2 *Bacillus spp*. and *Pseudomonas spp*. *Brevibacillus borstelensis*	Marine coastal area	Laundry, Shampoo, Conditioner, Household Cleaning products, Motor oil, Antifreeze and Recycling bins.	(Devi et al., 2019, 2015; Mohanrasu et al., 2018)
LDPE powder (Low-density polyethylene)	Less branched same as HDPE	*Penicillium pinophilum Kocuriapalustris, Bacillus pumilus and Bacillus subtilis*	Purchased from center, Pelagic waters	Used in food packaging materials, Rigid containers and Plastic film	(Harshvardhan and Jha, 2013; Volke-Sepúlveda et al., 2002)

Though several researchers have shown the potential role of microorganisms and their enzymes involved in the degradation of MNPs, there still remains toxic plastics in the environment waiting to be degraded. Further, isolation and cultivation of plastic specific microbes and their enzymes need to be identified and commercialized in a big way. Hence, a collaborative research approach with various technologies is required to get a concrete solution in the remediation of micro-nano plastics.

1.5 New Approaches in the Degradation of Micro-Nano Plastics

1.5.1 Membrane and enzyme technology

The major microplastics that prevailed in the water system are polyethylene terephthalate, polypropylene, and polyethylene microplastics. PET microplastics load constitutes more than 79 percent of other plastics present in the wastewater treatment plants. The un-treated microplastic fibers and particles are discharged from WWTP(wastewater treatment plant) to the aquatic ecosystem(Lares et al., 2018). Hence, wastewater treatment plants become one of the major routes of micro-nano plastics contamination in the environment.

A few researchers reported on the elimination of microplastics from the freshwater and drinking water by conventional treatment methods. It includes granular activated carbon and sand filtration, coagulation-flocculation, precipitation and sedimentation (Pivokonsky et al., 2018). The major disadvantage of these conventional treatments' method is the formation of a large amount of chemical sludge, which is associated with several complications such as an increase in turbidity and reduction in disposal potential (Zinicovscaia, 2016). Currently, membrane technology is widely used to purify water in wastewater treatment plants (WWTP) and is majorly categorized into ultrafiltration, nanofiltration, and reverse osmosis (Baker, 2012). Importantly, membrane ultrafiltration is well established for eradicating contaminants such as microbes, small organic acids, and soluble microbial products from wastewater treatment (Wu

et al., 2019). The porosity of membranes used for water treatment is in the same size range of microplastics. Hence, the membrane also has high stability and improved selectivity to remove microplastic contaminants and its by-products (Baker, 2012; Parks et al., 2017). The advancement in membrane technology resulted in membrane bioreactor technology with better removal efficiency of contaminants than conventional methods(Lares et al., 2018). The membrane bioreactor technology is a combination of conventional biological sludge treatment and micro-ultrafiltration system. MBR technology has provided many advantages over conventional methods such as a high removal rate of contaminants, low energy consumption, and low rate of sludge production (Zinicovscaia, 2016). MBR technology is reported to remove microplastic contamination more efficiently than conventional methods in the wastewater treatment plants (Talvitie et al., 2017). The nano-sized particles such as polyvinyl chloride, polyethylene terephthalate, polystyrene and polyethylene observed even after ultrafiltration of contaminants (Ter Halle et al., 2017a). Historically, removing nano-sized contaminants is challenging, but advancement like membrane technology provides appreciable solutions. With breakthroughs in membrane technology, membranes for nanofiltration, a blend of reverse osmosis and ultrafiltration is currently emerging. Herein, the removal of contaminants may vary with the membranes' constituents. Immobilization of nano-materials on or within the matrix or membrane can serve as a promising approach in the membrane technology. Depending upon the contaminant, the nano-material can be selected, which may exhibit better stability and improved performance. The emerging nanotechnology-based membrane material concepts intended for use in water purification such as zeolite-coated ceramic membranes, inorganic organic thin film nanocomposite (TFN) membranes, and hybrid protein polymer biomimetic membranes(Hoek et al., 2014). The composites material for membrane technology represents next generation of materials, through innovative methods and understanding the interactions between the contaminant and membrane dynamics (Ref 37). Nanofiltration in membrane

technology has shown significant success in the desalination and removal of boron from seawater (Roth et al., 2009). Interestingly, membranes with nanopores have the promising capabilities to serve as fine filters and allow only water molecules to pass through their structure (Atkinson, 2017).Though membrane technology provides a successful solution for the removal of MNPs from water bodies, further degradation/utilization of these toxic contaminants is crucial for pollution-free environment. Interestingly, enzyme technology can be explored for production, isolation, purification, and providing enzymes for the degradation of plastics. These enzymes are non-toxic and biodegradable. In the last decade, a few polymer plastics chains (PE, PP, PS, and PVC) are subjected to degrade by a distinct group of enzymes. Several enzymes, such as esterases, protease, cutinase, and laccase (Table 2), have shown promising results in the degradation of plastics. Interestingly, a bacterium Ideonella *sakaiensis*is can utilize polyethylene terephthalate (PET) as its primary energy and carbon source (Yoshida et al., 2016). This bacterium converts PET into its monomers terephthalic acid and ethylene glycol by two actively participating enzymes (PETase and MHETase) (Palm et al., 2019). Though a handful of enzymes and their activity on the plastic degradation are understood, the problem has been hardly addressed. This is due to the lack of technologies to improve the efficiency and the commercial production of these plastic degrading enzymes.

Recent studies on the enzymatic degradation of plastics have generated considerable interest in improving enzyme activity through protein/enzyme engineering. An engineered PETase mutant from *Ideonella sakaiensis,* exhibits an increase in the three mutants *(*R61A, L88F, and I179F) by 1.4fold, 2.1 fold, and 2.5 fold, in comparison to wild type strain. This study suggests that enzyme activity can be improved by rational protein engineering and by modifying key hydrophobic grooves of substrate binding sites (Ma et al., 2018). Interestingly, a recent report has shown the successful role of protein-engineered enzymes in degradation of microplastics (Islam et al., 2019).The report states that the degradation of polyurethane microplastics

commences by the polymer binding peptides, such as anchor peptides, which serve as a binding tool of synthetic polymers. The observed reduction in the shelf life of polyurethane from 41.8 to 6.2 h, approximately 6.7 folds is due to the improved degradation efficiency of the protein engineered enzyme, Tachystatin A2 (anchor peptide) of polyurethane as compared to Tcur1278-WT (wild type) (Islam et al., 2019).These remarkable results suggest that protein/enzyme engineering also is one of the potential techniques to remove micro-nano plastics in a better way. Another modern approach is strain improvement or strain engineering of microbial cells. A study has shown that under high pH conditions, alkaline microbial strain reduces the size of PET polymer and increases the by-product concentration of PET compared to enzymes under neutral condition. The microbes can utilize products generated in the medium for the growth, and feedback inhibition process can be hindered(Gong et al., 2018).

Interestingly, extensive research has been done to develop enzyme immobilization methods and their wide applications in environmental remediation. These immobilized enzyme techniques are eco-friendly in the eradication of micropollutants (such as dyes, Phenolic endocrine-disrupting chemicals, pharmaceuticals, etc.) from the environment (Shakerian et al., 2020).Bisphenol A (BPA), a monomer of polycarbonate plastics, is one of the largest produced chemicals worldwide(Hacıosmanoğlu et al., 2019). Laccase enzyme was reported as the most used enzyme to degrade BPA in immobilized systems (Brugnari et al., 2018; Piao et al., 2019). Immobilization of oxidative enzymes such as laccase and horseradish peroxide showed high stability, durability, reusability, and cost-effectiveness compared to free enzymes (Shakerian et al., 2020).Hence, the amalgamation of membrane and enzyme/microbial technology is expected to provide a promising future in the remediation of micro-nano plastic from the environment. As a result, it becomes essential to develop these existing methods to immobilize plastic degrading enzymes in large amounts, at least in the pilot-scale level for water purification. To

address this challenge, it is also essential to develop new methods that produce large amounts of enzymes in a cost-effective and simpler ways.

Table 2: List of plastic degrading enzymes.

Enzyme	Microbe	Polymer	Reference
Polyurethane esterase	*Comamonas acidovorans*	Polyester PUR	(Akutsu et al., 1998)
Cutinases Glycoside Hydrolases, PETase and MHETase	*Thermobifidafusca* *Ideonella sakaiensis* *Zalerion maritimum* *Bacillus gottheilii*	PET	(Auta et al., 2017; Paço et al., 2017; Palm et al., 2019; Ronkvist et al., 2009; Yoshida et al., 2016)
Serine hydrolases	*Pestalotiopsis microspore*	Polyester polyurethane (PUR)	(Russell et al., 2011)
Putative polyurethanases	*Pseudomonas chlororaphis*, *Candida rugosa*	Polyester PUR	(Russell et al., 2011)
Recombinant Alkane hydroxylases (AH)	*Pseudomonas sp.* E4 expressed in *Escherichia coli* BL21	PE (polyethylene)	(Yoon et al., 2012)
Alkane monooxygenase, Rubredoxin and Rubredoxin reductase	` *Pseudomonas aeruginosa* E7	PE (polyethylene)	(Jeon and Kim, 2015)
Laccase	*Rhodococcus ruber* C208, *Bacillus cereus*	PE (polyethylene)	(Santo et al., 2013; Sowmya et al., 2014)
Manganese peroxidase (MNPs)	*Penicillium simplicissimum*, *Bacillus cereus*	PE (polyethylene)	(Sowmya et al., 2015, 2014)
Protease	*Brevibacillus spp.* *Bacillus spp.*	Low molecular weight PLA	(Bhardwaj et al., 2012)
Esterase	*Delftiaacidovorans* *Comamonas acidovorans* *Bacillus subtilis*	Polyurethane (PUR)	(Nakajima-Kambe et al., 1997; Shah et al., 2013; Wei and Zimmermann, 2017)
Carboxylesterases	*Bacillus licheniformis*, *Bacillus subtilis* and *Thermobifidafusca*	PET oligomers	(Wei and Zimmermann, 2017)

1.5.2 Metagenomics

Metagenomics has been playing a promising part in the investigation of "hidden" genetic features. The discovery of novel DNA sequences, enzymes, metabolic pathways, and metabolically active molecules with new or better metabolic functions help in the development of biotechnological applications for polymer degradation (Alves et al., 2018). The identification of novel genes and DNA sequences can be divided into a sequence-based as well as functional-based screening methods (Schmeisser et al., 2007) while the functional-based screening methods can uncover these novel functions of identified enzymes(Uchiyama and Miyazaki, 2009). A significant example of functional screening metagenome is using tributyrin agar plates for LC cutinase activity from the fosmid library of a leaf branch compost metagenome. LC cutinase then further modified through protein engineering and termed as LC cutinase*.LC cutinase* not only serve as a good model for mechanistic study, but also has a potential role in surface modification and the degradation of PET fibers(Sulaiman et al., 2012). In the view of better metabolically active catalyst, high-throughput screening methods explicitly developed to examine polyester hydrolase activities in the hydrolysis of PET(Wei et al., 2012). The discovery of new microbial polyester hydrolases and the isolation/constructing an assembly of metabolically active enzymes remains a big question for the generation of a metabolic process to remove polyethylene waste (Wei and Zimmermann, 2017).

Micro-nano plastics have assembled on coastal areas and deep oceans at an anomalous rate. Various microbial communities of bacteria, archaea, and single-celled eukaryotes have colonized the plastisphere that plays a crucial role in environment and human health (Jacquin et al., 2019; Zettler et al., 2013). This cultivation impediment has altered our view of the microbial diversity and limited our appreciation of the microbial world through screening throughput and computational techniques (Parks et al., 2017). Hence, it is essential to understand the genetic diversity, population structure, and ecological roles of the bulk of

microorganisms using a culture-independent approach. Recent reports on shotgun metagenomic sequencing of biofilms fouling plastic and bioplastic microcosms have revealed that plastic biofilms are influenced by sulfate-reducing microorganisms (SRM) (Pinnell and Turner, 2019). Investigation of bio-plastic exposed enhancement of activity of esterases, depolymerases, adenylyl sulfate reductases (aprBA), and dissimilatory sulfite reductases (dsrAB). The metagenomic reconstruction of genomes recognized novel and new species of *Desulfovibrio, Desulfobacteraceae,* and *Desulfobulbaceae* among the huge SRM, and these genomes carry genes are fundamental to both bioplastic degradation and sulfate reduction (Pinnell and Turner, 2019). These outcomes show that bioplastic can promote a rapid and remarkable shift in benthic microbial diversity and genomes, selecting for microbes that highly participate in the degradation of bioplastic and sulfate reduction. Metagenomics, or the culture-independent genomic analysis of an assemblage of microorganisms, has enabled large-scale investigations of complex microbiomes and also answered some metabolic pathways of polymer degradation.

1.5.3 Nanoparticle Technology

The fundamental distinction between plastic and microplastics is particular size range. As explained previously, micro-nano plastics either originate from various sources or are generated extensively from large plastic pieces due to biotic and abiotic factors. Day by day increase of MNP pollutants in ecosystem and the corresponding toxicological impact on various organisms cautions to limit plastic production as well as consumption. A better way to reduce MNPs in the ecosystem is to design environmentally benign plastics in order to accelerate their natural degradation. Nanotechnology could be a promising area to explore suitable methods to clean up the micro-nano plastics in the ecosystem. There are a few exciting approaches by which nanotechnology contributes to plastic degradation. The biodegradation of plastics is enhanced by the addition of nanoparticles in the microbial cultures. For example, the presence of SiO_2

nanoparticles at different concentrations was reported to influence the growth of the plastic degrading bacteria (Pathak and Navneet, 2017). Herein, SiO_2 nanoparticles provide better growth by reducing the lag phase and dramatically enhancing the plastic degradation rate of bacterial strains through bacteria–nanoparticle interactions(Pathak and Navneet, 2017). Fullerene 60 nanoparticles generally considered toxic in higher concentrations. But when supplemented at a minimal level, it shows an increase in the plastic degradation process. Supplementation of fullerene 60 nanoparticles in the minimal media of bacteria performs well and degrades LDPE by weakening its hydrocarbon chain and forms various by-products(Sah et al., 2010). Another report suggests that the Supplementation of nanoparticles, such as superparamagnetic iron oxide(SPION) and nano barium titanate (NBT), enhances the degradation of LDPE through bacterial metabolic interaction(Kapri et al., 2010). Importantly, the presence of nanoparticles in the bacterial culture media promotes their growth, by an early rise of the exponential phase but also increases the biodegradation efficiency of a potential polymer-degrading microbial consortium (Kapri et al., 2010). These studies suggest the importance of nanoparticles and bacterial interactions. Exploring other nanoparticles for its ability to promote microbial remediation process can help in the commercialization of plastic degradation.

1.6 Conclusion

Micro-nano plastic pollution is regarded as a significant pollutant among others, which persists in the environment for a longer duration. Microbial mediated remediation of MNPs can provide a platform to degrade or remediate the MNPs by the activity of enzymes on plastics. It is still not well understood how microbes work on the degradation of MNPs. Furthermore, utilization of microbial enzymes that help in the degradation and restructuring of plastics for better biodegradation is a future challenge. More importantly, future research should address the identification of organisms that can act on a broad spectrum of plastics and also on high

molecular weight polymers. Monomers, dimers, and oligomers formed after the degradation of polymers can be reused to build new materials. Since the identification and isolation of highly active and functional enzymes is challenging to achieve, the metagenomics approach could help identify the non-cultivated microorganisms, offering potential help to setup function-based assays. Though an integrative role of membrane as well as enzyme technology, nanoparticle technology, and metagenomics is promising in the remediation of MNPs, it is important to explore "micro-nano plastics active" or "micro-nano plastics degrading enzymes" and its pathway. Extensive research in this respect should considerably reduce our global micro-nano sized plastic pollutants and rend good health for future generations.

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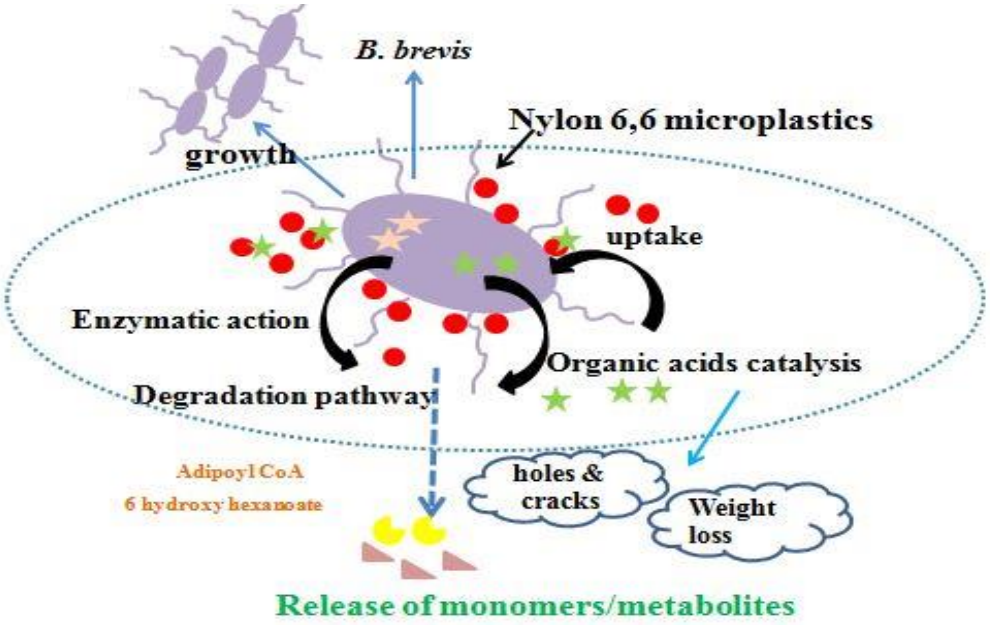
Chapter 2

Biodegradation of micro-sized nylon 6, 6 using *Brevibacillus brevis* a soil isolate for a cleaner ecosystem



Chapter 2

Biodegradation of micro-sized nylon 6, 6 using *Brevibacillus brevis* a soil isolate for a cleaner ecosystem

2.1 Introduction

Microplastics range in size from 0.1 µm to 5 mm and are either manufactured in the industries (Primary microplastics) or result from fragmentation of bulk plastic through biotic and abiotic factors (Secondary microplastics) (Auta et al., 2017a; Ritchie and Roser, 2018). These plastic contaminants accumulate widely onshore affecting the ecosystem at an alarming rate. Massive amount of plastic contaminants is released into the ocean from various land-based activities(Jambeck et al., 2015). For instance, out of the total plastic waste generated throughout the world, 18.3% is dumped into the oceans by automotive industries in India and other Southeast Asian nations. North America dumping plastic in oceans constitutes 17.2% of worldwide plastic waste generated (Ritchie and Roser, 2018). Recently, the International Union for Conservation of Nature (IUCN) has reported that yearly 15 % to 31 % of primary microplastics out of 9.5 Mt of plastic contaminants enter into oceans("Marine plastic pollution," 2018).

Micro-nano plastics (MNPs) are challenging contaminants for remediation due to their inertness, high surface-area-to-volume ratio and hydrophobicity(Tiwari et al., 2020). Various microplastics like polypropylene (Browne et al., 2011), low density polyethylene (LDPE) (Dey et al., 2021)and polystyrene (PS) (Liu et al., 2021) have been isolated from seashores, sewage discharges and effluents with their effects being monitored on various ecosystems (Manabe et al., 2011; de Sá et al., 2015). In literature, biodegradation of microplastics has been scarcely reported and includes few candidates like PS, polyethylene (PE), LDPE and PP (Mohanan et al., 2020; Savoldelli et al., 2017). A recent study demonstrated the role of a *Pseudomonas sp.* ADL15 and *Rhodococcus sp.* ADL36 on

degradation of polypropylene (PP) microplastics after 40 d of treatment with weight loss observed at 17.3 % and 7.3 %, respectively (Habib et al., 2020). Similar studies have been reported on PS degradation by *Pseudomonas* sp. DSM 50071 (Kim et al., 2020) and polyethylene microplastics degradation by *Zalerion maritimum*(Paço et al., 2017). All of these prove that microplastic degradation through microbiota can be an effective approach and more research into the field is warranted keeping in mind the importance of the strategy in today's world.

Among many plastics which act as source of environmental pollutants, nylon 6, 6 constitutes a primary source. Nylon 6, 6 is a thermoplastic polymer and has versatile nature as fibre and plastic. It is made up of two monomers each of which contains six carbon atoms namely hexamethylenediamine and adipic acid (Feldmann, 2020). Due to its high tensile strength and resistivity to chemical and heat- induced degradation; nylon 6, 6 is widely used in both industrial (textile, cosmetic, packaging and automotive) and household applications (Bilad et al., 2018; Kale et al., 2021).The presence of micro-sized nylon 6, 6 particles/fibres mostly originating from synthetic textiles have been recently identified in wastewater from domestic washing machines (Fan et al., 2022; Hamidian et al., 2021) The worldwide market for nylon was calculated at around USD 24.29 billion in 2019 and is expected to increase by 6 % towards the end of 2027 majorly through its application in automobile industries("Nylon Market Size, Share, Report | Global Industry Trends [2020-2027]). Extensive usage of nylon increases its accumulation in the environment and poses significant threats to land and aquatic life. When compared to other classes of microplastics, degradation of nylon 6, 6 microplastics (NMPs) requires several harsh chemicals, extensive thermal and physical processing in order to overcome intermolecular hydrogen bonding between nylon molecular chains (Tomita et al., 2003).

The exposure to NMPs were noticed to influence usual developmental pattern (e.g., growth) in an ecologically essential cold water copepod *Calanus finmarchicus* (Cole et al., 2019). Micro-sized nylon were also reported to serve as a vector to another pollutant namely bisphenol A in fresh water and induced toxicity in organisms (Rehse et al., 2018). Polyamide microplastics resulting from nylon 6, 6 due to photoaging have demonstrated negative effects on food digestion and nutrient assimilation in fish. Further, these polyamide microplastics have been reported to have resulted in down-regulation of genes related with triglyceride resynthesis and transportation which in turn led to lipid malabsorption and growth hindrance in the fish (Zhang et al., 2022). Micro-sized contaminants can enter human body during inhalation, through direct dermal exposure or through food chain thus causing severe health effects (Bansal et al., 2021). All of these suggest that there is an urgent requirement to explore more degradation pathways of nylon 6, 6 microplastics so as to promote its removal from the earth's environment. During microbial degradation of plastics, microorganisms adhere to surface of polymers and begin degrading the same through secretion of some exoenzymes or metabolites, thus altering the polymer structure (Deng et al., 2021; Sowmya et al., 2015). (Sudhakar et al., 2007a) demonstrated the role of marine bacteria (*Bacillus cereus, Bacillus sphericus, Vibrio furnisii, and Brevundimonas vesicularis)* in degradation of nylon 6, 6 by reporting 7 % weight loss along with 42 % decrease in the average molecular weight over a period of three months. Whereas, plastic degradation by *B. cereus* resulted into 1.6 %, 6.6 % and 7.4 % weight loss in 40 d for PE, PET, and PS respectively. Similarly, *B.gottheilii* also showed 6.2 %, 3.0 %, 3.6 % and 5.8 % weight loss after 40 d for PE, PET, PP, and PS, respectively (Auta et al., 2017c). To our knowledge, apart from the study by Sudhakar *et al* on nylon 6, 6 degradations through microbiota, very few studies have focussed on the same. This gap in knowledge has encouraged us to choose nylon 6, 6 microplastic as a substrate for

biodegradation. Further, our study involves *B.brevis* as the bacterial candidate for promoting degradation of nylon 6, 6 which has hitherto been unreported in any study as per our literature review.

The genus *Brevibacillus* (family *Paenibacillaceae* and class *Bacilli*) was initially described as *Bacillus brevis* in 1900 by Migula (Nakamura, 1993) . The bacterium was observed in diverse habitats, including agricultural soil, wastewaters as well as hot springs (Khalil et al., 2018). *B.brevis* is a potential microbe to degrade hydrocarbons. It is reported to break down over 80 % of naphthalene at room temperatures (25 °C) (Al-Thukair et al., 2020). The role of *B.brevis* in the degradation of pyrene, polyethylene and polypropylene (Skariyachan et al., 2018; Wei et al., 2017) has also been well reported. These findings suggest that *B.brevis* growing in presence of plastic contaminants can prove to be an exceptional candidate for degradation of the same (Khalil et al., 2018). Thus, *B.brevis* was chosen to study the degradation of MNPs synthesized from nylon 6, 6 pellets in our study. NMPs in our case can be considered as a representative material for understanding the mechanism involved in microbial mediated plastic degradation under laboratory conditions through simple experimental design. The novelty of the present manuscript focuses on the direct interaction of NMPs and *B.brevis* without any surface additives. Microplastics degradation property and cellular metabolic activity of *B.brevis* under NMPs stress were observed systematically by shake flask assay. Changes in the structure of microplastics as well as morphology of the bacterium were monitored and the compounds released in the metabolite were identified. The biodegradation pathway for NMPs degradation by *B.brevis* has also been proposed the first time.

2.2 Material and Methods

Nylon 6, 6 pellets and all other chemicals used throughout this study were of AR-grade and obtained from standard manufacturers. The organism *B.brevis* (MTCC NO. 7822) was purchased from the Institute of Microbial Technology (IMTECH), Chandigarh, India. As per the information provided by MTCC, the bacteria were isolated from soil in Dehradun and Haridwar in the state of Uttarakhand in India. The culture was revived on nutrient plates and was sub-cultured for three generations in minimal salt medium (MSM). The MSM contains 0.02 % $MgSO_4$, 0.02 % $CaCl_2$, 0.1 % KH_2PO_4, 0.1 % K_2HPO_4, 0.1 % NH_4Cl, 0.05 g $FeCl_3$, and 0.1 % NaCl along with 1.8 g glucose per litre of distilled water. When NMPs are used as carbon source, glucose is not added in MSM (Kowalczyk et al., 2016; Sowmya et al., 2015)

Positive (inoculation on MSM without NMPs, *B.brevis*_MSM) and negative control (without inoculation on MSM with NMPs, NMPs_MSM) sets were maintained simultaneously and the media were observed for bacterial growth.

2.2.1 Synthesis of nylon 6, 6 microplastics

Nylon 6, 6 microplastics (NMPs) was synthesized using a method described by Crespy *et al* (Crespy and Landfester, 2007). A solution containing 1 g of nylon 6, 6 and 20 ml of formic acid was stirred on a magnetic stirrer for 1 h until completely dissolved. Then in 100 ml deionized water, the nylon 6, 6 solution was added slowly while carrying out probe sonication at 70 % amplitude (Branson sonifier W450 Digital, tip size 6.5 mm) for 30 s under ice-cooling conditions. Obtained solution is then centrifuged, washed with water followed by ethanol and finally stored after air-drying.

2.2.2 Microbial inoculums preparation and aqueous phase degradation study

The bacterial culture was revived on nutrient agar plates. The bacteria grew in nutrient broth to a stationary phase in a rotating shaker at 30 ºC and 120 rpm. Bacterial growth in

the culture medium was monitored by observing its absorbance at wavelength of 600 nm using Eppendorf UV-vis Spectrophotometer (Bio Spectrometer basic model). The third-generation culture utilized for the biodegradation study was reported for a log phase with an absorbance of 0.8 at 600 nm. In our bio-degradation study, we considered applying third generation bacterial cultures which were identified to be in log phase on observation of absorbance values of 0.8 at 600 nm using the UV-visible spectroscope. The culture was grown in MSM without any carbon source and was used in the degradation experiments. Prior to degradation studies, the synthesized NMPs were UV-treated post washing with ethanol to ensure sample sterility. The bacterial culture was inoculated into 100 ml of MSM broth with (*B.brevis* _NMPs) and without (*B.brevis*_MSM) 0.5 g of UV treated NMPs (Auta et al., 2018, 2017c) . Different parameters such as pH, Optical density (OD) and metabolite contents were monitored every five d for 35 d.

2.2.3 Preparation of samples for various characterization methods

After incubation of NMPs in absence (Negative Control, NMPs_MSM) and presence of *B.brevis* (*B.brevis*_NMPs) for 35 d, the contents were filtered with 0.22 µm polytetrafluorethylene (PTFE) Millex filters. Both filtrate and residue of each sample were preserved for further characterisation.

Chemical analysis of metabolites

2.2.4 High Pressure Liquid Chromatography (HPLC)

Organic acids were estimated (malic acid, citric acid and oxalic acid) from the bacterial culture after every 5 d of treatment with NMPs. 1 ml of both the cultures (*B.brevis*_MSM and *B.brevis*_NMPs) were extracted separately and filtered through 0.22 µm polytetrafluorethylene (PTFE) Millex filters to obtain cell free metabolites (*B.brevis*_MSM and *B.brevis*_NMPs metabolites). Presence of organic acids were analysed in these two metabolites using High-Performance Liquid Chromatography (HPLC) equipped with

photodiode array detection (PDA) detector at wavelength 210 nm. The Shimadzu allure organic acid 5 µm analytical column with 250 mm x 4.6 mm (id) in dimension was employed with an optimum temperature of 25 ºC and an injection volume of 10 mL. Methanol and potassium dihydrogen phosphate (10 mM, pH 2.8) were mixed at a ratio of 10:90 to act as the mobile phase (flow rate at 1 mL/min) (Han et al., 2019; Xiao et al., 2006) .

Metabolites of *B.brevis*_NMPs along with appropriate control samples were also analysed for presence of hexamethylenediamine and adipic acid using Dionex model HPLC (Thermo Fisher make) equipped with a PDA detector at wavelength 254 nm and 425 nm. The Thermo fisher C18 column (3 µm, 150 x 4.6 mm) functioned as the stationary phase. The mobile phase was a mixture of acetonitrile with 0.1% formic acid and water (50:50 v/v) for hexamethylenediamine whereas a ratio of 0.1% formic acid and acetonitrile (60:40 v/v) was considered for adipic acid. Separation was performed at a flow rate of 1 mL/min at 30 ºC temperature (Bui and Frost, 2012; "LC/MS Analysis of Organic Acids (DE-213) | Shodex/ HPLC Columns, Detectors, Standards,").

2.2.5 Total enzyme activity

Fluorescein diacetate (FDA) hydrolase activity was assayed based on hydrolytic cleavage of FDA into fluorescein. 2 mL of each culture (*B.brevis*_MSM and *B.brevis*_NMPs) was incubated with 20 mL of phosphate buffer (pH = 7.4) and 0.2 mL FDA stock solution (1000 µg mL^{-1}) at 30 °C for 40 min in a water bath shaker (150 rpm) along with respective positive and negative controls (only culture, no FDA and only FDA, no sample). 20 mL of chloroform/methanol in ratio of 2:1 was added into each sample and the suspension was centrifuged for 7 min at 6000 rpm. Fluorescence intensity of the supernatant was measured at excitation wavelength of 490 nm and emission wavelength of 519 nm (Adam and Duncan, 2001; Schnürer and Rosswall, 1982).

2.2.6 Laccase activity

Activity of laccase enzyme was determined from the culture media of *B.brevis* using ABTS(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid))as a substrate. 100 µL of culture media were mixed with 100 µL of 10 mM ABTS in 800 µL of 50 mM sodium acetate buffer (pH 5) and incubated for 5 min at 30 °C. The formation of green color is due to oxidation of ABTS and was measured spectrophotometrically at A420 (ε = 36000 $Lmol^{-1}{\cdot}cm^{-1}$). The production of enzymes was monitored at different time durations starting from the 5^{th} d to the 35^{th} d. One unit of activity of laccase was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per minute at 30 °C (More et al., 2011)

2.2.7 Ultra-performance Liquid chromatography-Mass Spectrometry (UPLC-MS)

After 35 d of bacterial degradation of nylon 6, 6 microplastics, the culture was extracted in acetonitrile for carrying out MS. 1 mL of aliquot was centrifuged at 12000 rpm for 15 min to obtain cell free supernatant. Acetonitrile is the most preferred solvent to extract the water-soluble metabolites to maintain free from salts (Rabinowitz and Kimball, 2007).The supernatant was used for the analysis of metabolites. UPLC-MS analysis of the acetonitrile extracted sample was performed using Synapt G2 (Waters ACQUITY QSM) with BEH C18 Column. The Waters acquity C18 column (130Å, 1.7 µm, 2.1 mm X 50 mm, 1/pk) functioned as the stationary phase while the MS system using positive electron spray ionization (ESI) operated at 0.75 mL/min flow rate over a period of 20 min(Kalsi et al., 2021) .

2.2.8 Thermogravimetric analysis (TGA)

The TGA of the control and *B.brevis*_NMPs microplastics were performed using Perkin Elmer thermogravimetric analyzer TGA 4000. All samples were heated from room

temperature to 800 °C at a heating rate of 10 °C min^{-1} in presence of nitrogen flow of 10 ml min^{-1} ("Characterization of Polymers using TGA," 2011).

2.2.9 Fourier transform infrared (FTIR) spectroscopy.

The changes that occurred in nylon 6, 6 microplastics after interaction with *B.brevis* (*B.brevis*_NMPs) were analysed by FTIR spectroscopy (Perkin-Elmer 400 FT-IR/FT-FIR) operating in the frequency range of 2000 cm^{-1}- 450 cm^{-1} with appropriate control samples. The pellets of required samples were prepared using KBr and scanning was carried out at a resolution of 4 cm^{-1} (Sudhakar et al., 2007b).

2.2.10 Morphological Analysis

2.2.11 Scanning Electron Microscopy (SEM)

The changes in bacterial morphology before and after degradation of nylon 6, 6 microplastics were investigated after 35 d of incubation with *B.brevis* under Scanning Electron Microscope (SEM, Model: EVO18 Zeiss, Germany) at an accelerating voltage of 5 kV at 10,000 magnifications. The culture was centrifuged and washed with Milli-Q water. Fixative (Karnovsky's fixative) was used to fix the bacterial cells before examination. The bacterial cells were sputter-coated with a gold layer at 25 mA under Ar atmosphere at 0.3 MPa and subsequently examined under SEM (Auta et al., 2017c).

2.2.12 Transmission Electron Microscopy (TEM)

The morphology of microplastics NMPs_MSM as well as *B.brevis*_NMPs after treating with NMPs was analyzed using TEM (Tecnai G2 200 KV HRTEM SEI HOLLAND). Before examination, particles/bacterial cells were washed thoroughly ,lyophilized and subjected to TEM at an accelerating voltage of 200 kV at 2550 magnifications (Yang et al., 2021).

2.2.13 Dry weight determination of residual nylon 6, 6 microplastics

After 35 d of incubation, nylon 6, 6 microplastics was recovered from the broth through filtration (0.22 µm polytetrafluorethylene (PTFE) Millex filters). Plastic particles were washed with 70 % ethanol and dried in hot air oven at 50 ºC overnight. Residual polymer weight was determined to measure the extent of degradation (Auta et al., 2017b; Mohan et al., 2016;). The initial weights of the pre incubated microplastic were measured following the same technique mentioned above. The plastic polymer degradation was evaluated in terms of percentage weight loss using the following formula:

$$\%\, weight\ loss = \frac{(initial\ weight\ of\ polymer - Final\ weight\ of\ polymer)}{initial\ weight\ of\ polymer} * 100$$

2.2.14 Statistical Analysis

All experiments were carried out in triplicates. Statistical analysis on experimental data was carried out using Origin2019b software and analysis of variance (ANOVA) was done in the SPSS software 21.0 with the LSD post-hoc test at *p-value* = 0.05. All values have been expressed as mean ± standard deviation with triplicate samples (n = 3).

2.3 Results and discussion

2.3.1 Biodegradation assays for nylon 6, 6 microplastics

In this study, microbial degradation of NMPs was investigated by estimating growth of *B.brevis* through observation of parameters like optical density, pH and presence of various organic acid contents of the metabolite in absence (*B.brevis*_MSM) and presence of NMPs (*B.brevis* _NMPs).

2.3.1.1 Optical density

B.brevis was assessed for its growth through optical density measurements at wavelength of 600 nm after acclimatizing it to MSM for three generations (Fig. 4(a)). In absence of nylon 6, 6 the lag phase was observed from 0 d (0.0155 OD) to 5 d (0.044 OD). An

exponential growth response was recorded after the 5th d to the next five d till the OD at wavelength of 600 nm reaches 1.33. In presence of *B.brevis*_NMPs, the lag phase was observed from 0 d (0.05 OD) to 5 d (0.155 OD). Exponential growth phase (1.67 OD) was observed to have attained slowly in 15 d compared to the control (*B.brevis*_MSM). The increased number of days in case of *B.brevis* _NMPs may have been due to interaction between the bacterial cell membrane and NMPs. The stationary phase was observed to be longer for *B.brevis*_NMPs when compared to *B.brevis*_MSM. This implies that the growth of *B.brevis* in the presence of NMPs ceased after 15 d although the cells may have still been metabolically active as observed from the prolonged stationary phase. A sharp decline in the growth of the *B.brevis* was observed on the 25th d lasting till 35th d on exposure to nylon 6, 6 microplastics indicating decline/death phase. The decline phase of *B.brevis* in presence and absence of nylon 6, 6 may have been due to cell lysis as a result of nutrient depletion and presence of excessive metabolites in the culture media.

2.3.1.2 pH

One of the most critical factors in the growth of microbial population for its survival, enzyme activity and stability is the pH of its growth medium. Media pH plays vital influence on the growth of microbes, activity of enzymes and the degradation rate(Gu, 2003; Xu et al., 2011). It is well known that *B.brevis* produces organic acids such as malic acid, citric acid and oxalic acid in lower concentrations for its metabolism. The rate of production of these organic acids by *B.brevis* has been reported to increase degradation of contaminants such as tricresyl phosphate (Liu et al., 2019). Fig. 4(b) represents changes in pH of the bacterial culture in the presence and absence of NMPs during 35 d of biodegradation assay. For the first 5 d, similar pH changes were observed in case of *B.brevis*_MSM and *B.brevis*_NMPs. However, starting from 5 d till 35 d, decrease in pH could be observed (pH 6.5 to 5.8) for *B.brevis*_NMPs. This implies that the pH for

optimum growth of *B. brevis* is 6 ± 0.2 and is in good agreement with the lag phase observed through optical density measurements (Fig. 4(a)). The rapid decrease in pH observed in case of *B.brevis*_NMPs when compared to growth of bacteria without microplastics may have led to the decline of microbes as revealed by OD measurements (Fig. 4(a)). The physiochemical characteristics in microenvironment such as pH is greatly affected by the presence of low molecular weight organic acids (Xiao and Wu, 2014) .The microbial growth and enzyme activity are hampered by even a slight change of optimum pH during degradation due to the accumulation of metabolites and degraded pollutants(Auta et al., 2018). The pH decline of bacterial culture containing NMPs microplastics indirectly indicates presence of organic acids in the metabolite which may in turn lead to its degradation.

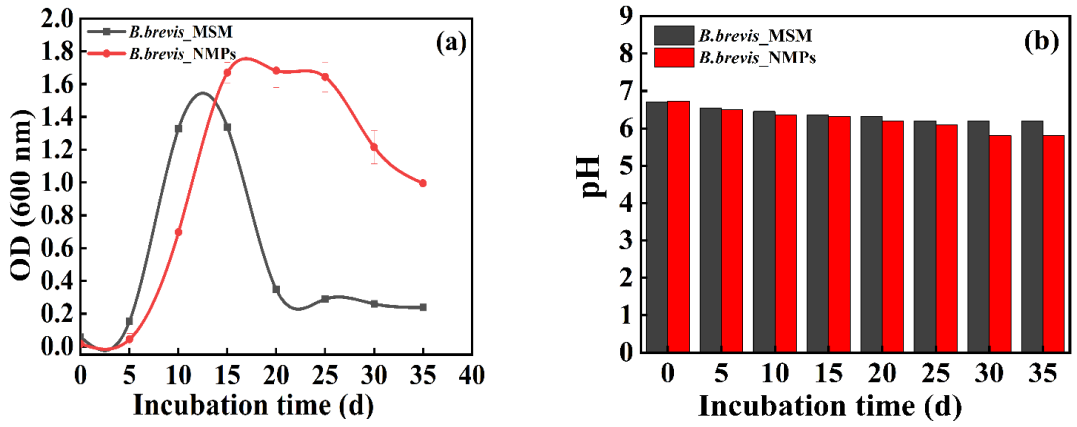


Fig. 4. (a) Growth curve of *B.brevis* (b) A comparison of pH changes during the growth of *B.brevis* in the absence and presence of NMPs. Error bars denote standard errors.

2.3.1.3 Organic acids

Organic acids participate in biodegradation of several micro-pollutants and are involved in metabolic processes such as TCA (tricarboxylic acid cycle) (McNamara et al., 2014; Xiao and Wu, 2014). As shown in Fig. 5, *B.brevis* produced more organic acids like malic acid, citric acid, adipic acid and oxalic acid in the presence of NMPs when compared to the control. Out of the produced organic acids, amount of citric acid (Fig. 5(c)) and adipic acid

(Fig. 5(a)) was higher than malic acid (Fig. 5(b)) and oxalic acids (Fig. 5(d)) in the nylon 6, 6 treated bacterial cultures. In presence of NMPs, citric acid content in the metabolite could be detected for up to 25 d and declined in the later stages. The observation indicates increased utilization of citric acid in the TCA metabolic process of the cell for its growth and cellular activities in presence of microplastics as observed in an earlier study (Xiao and Wu, 2014). Adipic acid concentration increases relatively from 10 d in presence of microplastics than in its absence. The reported excess adipic acid might be due to bacterial secretions in addition to the release of monomer due to the microbial degradation of nylon 6, 6.

An increase in concentration of malic and oxalic acid was observed in presence of nylon 6, 6 microplastics from 10 d. Oxalic and malic acids are intermediate molecules formed through mineralization of pollutants such as Dichlorodiphenyltrichloroethane (DDT), polycyclic hydrocarbons and heavy metals, mainly from anthropogenic activities (Xiao and Wu, 2014). Organic acids influence the changes in the microenvironment such as altering the chemical composition of pollutant and activity of microorganisms upon degradation (Strobel, 2001). The presence of oxalic acid, malic acid and adipic acid in this case in increased concentrations infer that these acids have a significant role in degradation of nylon 6, 6 microplastics which were not further utilized by the microbe. These findings are in good agreement with earlier reports ((Yi et al., 2017) and (Liu et al., 2019)) where an increase in oxalic acid content with biodegradation of pollutants (triphenyltin and tricresyl) could be observed.

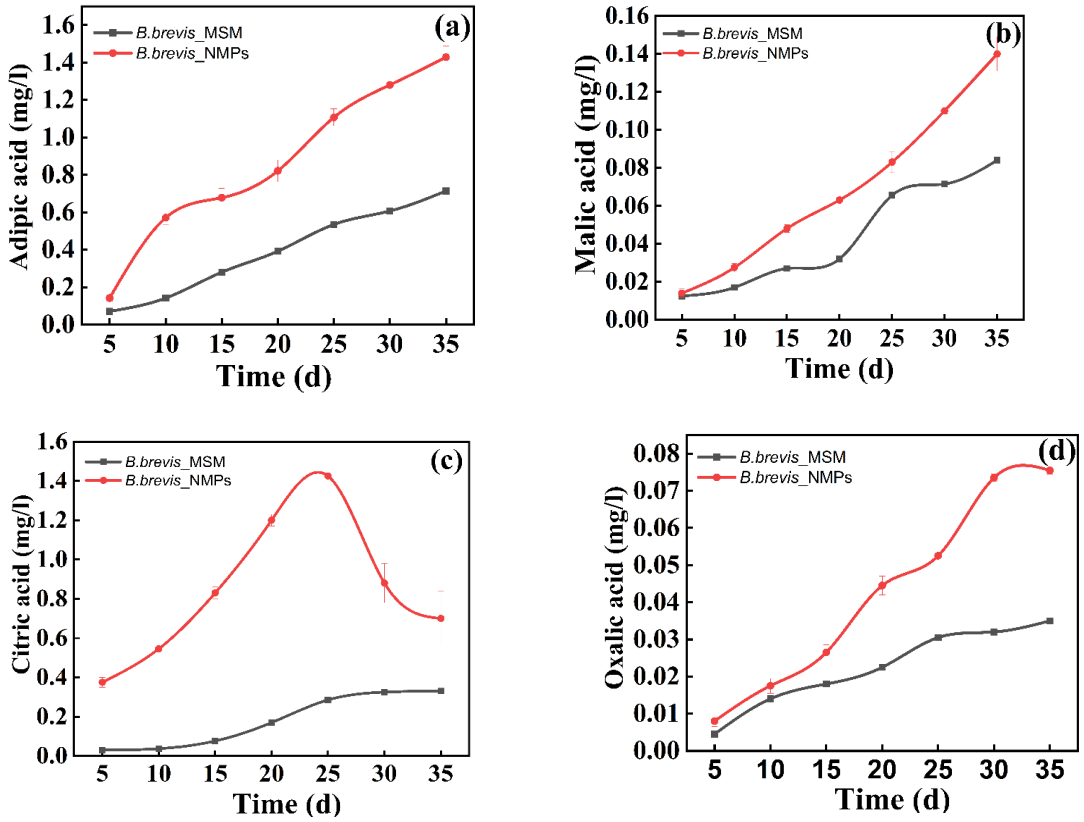


Fig. 5. Release of organic acids by *B.brevis* under the stress of NMPs (a) Adipic acid (b) Malic acid (c) Citric acid and (d) Oxalic acid. Error bars denote standard errors

2.3.1.4 Total enzyme activity of *B.brevis* in *in-vitro* biodegradation flask

Total enzyme activity as a measurement of cellular viability was performed by monitoring the FDA hydrolyzed (hydrolysis of FDA). Upon FDA treatment, the bound and unbound enzymes hydrolyzed to a yellowish green colored end product called fluorescein. The MSM samples were subjected to an FDA reaction as in which the enzyme activity was expressed as FDA hydrolyzed per ml. Since the biodegradation study was carried out in strict sterile conditions, both positive and negative controls showed negligible amount of FDA hydrolysis (Schnürer and Rosswall, 1982). Fig. 6(a) represents relative increase in the total enzyme activity of *B.brevis* in presence of microplastics (*B.brevis*_NMPs) than in its absence (*B.brevis*_MSM). These findings confirm both growth of bacteria and secretion of enzymes required for nylon degradation. It was observed that with increase in time of exposure to NMPs, enzyme secretion of *B.brevis* correspondingly increased and showed

maximum activity by 25 d (41 µg FDA hydrolyzed/ml of media in 40 min reaction time) and decreased thereafter. An important reason for the higher enzyme activity of the microbe in the presence of microplastics can be due to the release of enzymes such as protease, esterase and laccase responsible for its degradation (Kaushal et al., 2021). This pattern was found to be similar to the growth of *B.brevis* monitored in our case (Fig. 4(a)).

2.3.1.5 Laccase enzyme activity

Fig. 6(b) represents relative increase in activity of laccase in presence of NMPs (*B.brevis*_NMPs) than in the absence of microplastics (*B.brevis*_MSM). It was observed that the laccase secretion of *B.brevis* increased as interaction time increased with NMPs. The maximum laccase activity was reported at 25 d (5.7 µmol/min in 5 min reaction time) and decreased thereafter. This pattern was found to be similar to the total enzyme/enzymatic activity of *B.brevis* (Fig. 6(a)). The important role of laccase enzyme in biodegradation of plastics such as polyethylene has been demonstrated by (Santo et al., 2013). Secretion of laccase enzyme induces depolymerization of polyethylene via oxidation of the polymer backbone (Santo et al., 2013). Laccase (Phenol Oxidases) is secreted extracellularly and its action is induced by specific substrate such as copper (Santo et al., 2013)

2.3.1.6 Dry weight loss analysis of nylon 6, 6 microplastics upon microbial degradation

The changes that occurred due to microbial action on nylon 6, 6 were assessed quantitatively by measuring the weight loss of the nylon 6, 6 microplastics after inoculation with *B.brevis strain*. The results are presented in Table 3. No weight loss of NMPs was observed for NMPs_MSM. The weight loss of *B.brevis*_NMPs was 22 % after 35 d of biodegradation assessment with *B.brevis*. These findings confirm the ability of the bacterial isolate to excrete some enzymes/acids (Fig. 5 and Fig. 6(a-b)) that can degrade

NMPs and utilize the degradation products for its own metabolism. The microbial degradation of nylon 6, 6 possibly results from biochemical reaction pathways (bio-fragmentation and bio-assimilation). In an earlier study, the weight loss of nylon 6, 6 pellets were recorded as approximately 7% and 4% when exposed to *B.cereus* and *B.vesicularis* organisms respectively for over a period of three months (Sudhakar et al., 2007b). Only about 2% weight loss is observed for the same organisms with nylon 6 after 3 months period(Sudhakar et al., 2007b). The weight loss of NMPs after the incubation could be an outcome of microbial activity and presence of its metabolites. Further, the weight loss indicates a possible change in the physiochemical (surface properties, thermal properties and crystallinity) and physical (mechanical and tensile strength) nature of the microplastics (Zhang et al., 2021) Microbes behave differently in different conditions to adapt to their surroundings. The observed weight loss hints at the biological breakdown of microplastics and the utilization of carbon source for its survival (Bhardwaj et al., 2012).

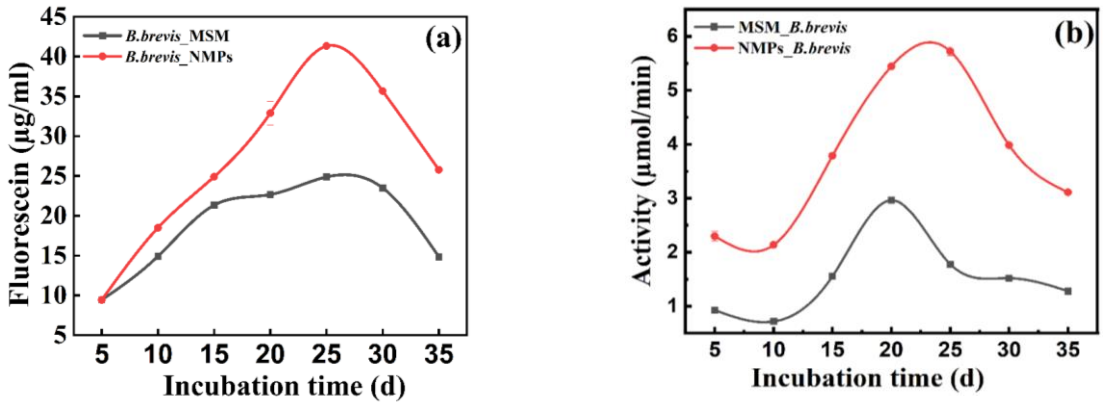


Fig. 6. (a) Total enzyme and (b) Laccase activities of *B.brevis* in the presence and absence of NMPs. Error bars denote standard errors.

Table 3. Dry weight analysis of NMPs in the presence of *B.brevis*

Organism	Initial weight (g)	Final weight (g)	Weight loss (%)	F-value	Sig.	Removal constant (K) d^{-1}	Half-life (d)
Control	0.5	0.5	0				∞
B.brevis	0.5	0.387±0.01	22.60%	6012.84	<0.01	0.0073	94.93

2.4 TGA analysis on nylon 6, 6 microbeads, NMPs, NMPs_MSM and *B.brevis*_NMPs treated for 35 d were depicted in Fig. 7(a). In case of the nylon 6, 6 beads, initially, 9% weight loss was observed at 389 °C due to the loss of surface water. 50% weight loss was observed at 450 °C in NMPs and 50% weight loss at 448 °C in NMPs_MSM (Negative control). Maximum weight loss of 80% was observed at 468 °C in all three samples (beads, NMPs and NMPs_MSM). This signifies reduction in size from beads to NMPs. At about 57 ˚C, the initial onset of degradation, starts the release of moisture in both the nylon 6, 6 beads (as received sample) and synthesized microplastics. A maximum weight loss of 80 % was observed at 468 °C in all two samples (beads and NMPs). This confirms a reduction in size from beads to NMPs does not change the thermal and structural properties of the nylon 6, 6. The nylon 6, 6 beads and synthesized nylon 6, 6 microplastics have the same end degradation rate approximately at 500 °C as described by handbook of PerkinElmer TGA which signifies the purity of microplastics ("Characterization of Polymers using TGA," 2011.). In case of *B.brevis*_NMPs, 35% weight loss was observed at 100 °C and 50 % at 290 °C. The maximum weight loss was observed as 70% at 407 °C which requires lesser temperature compared to untreated nylon 6, 6. The observation confirms some

structural changes such as reduction in the molecular weight as well as intermolecular cohesive forces of the nylon molecules due to the bacterial activity.

2.5 FTIR analysis of nylon 6, 6 microplastics

FTIR spectra of NMPs, NMPs_MSM and *B.brevis*_NMPs post 35 d treatment is shown in Fig. 7 (b, c, d). The presence of amide groups in NMPs (Fig. 7 (b)) is well reflected as a strong band at 1648 cm^{-1} due to the –C=O stretching vibrations (amide I). The band at 1536 cm^{-1} is assigned to $–NH_2$ deformation (amide II) of polyamide chain. The –C-H stretching vibrations of $–CH_2$ axial groups close to carboxyl groups of the polymer chain and –C-N stretching of the amide III band appeared at 1196 cm^{-1} and 1263 cm^{-1}, respectively.

In Fig. 7(c) and 7(d), FTIR spectra of NMPs_MSM and *B.brevis*_NMPs have been portrayed respectively. A shift towards higher wave number was observed for the amide I band (from 1637 $cm^{-1}$to 1643 cm^{-1}) and on the other hand amide II band slightly shifted to lower wave number (from 1544 cm^{-1} to 1533 cm^{-1}) in case of *B.brevis*_NMPs compared to NMPs_MSM. A higher wave number shift of amide III bond from 1262 cm^{-1} to 1273 cm^{-1} denotes a possible change in the crystalline peak for *B.brevis* treated nylon 6, 6 compared to control samples.

The changes observed in the characteristic vibrational peaks (amide I (strengthened), amide II (weakened) and amide III (strengthened) of *B.brevis*_NMPs compared to NMPs_MSM confirmed breaking of intermolecular hydrogen bonding between –C=O and –N-H groups of nylon 6, 6 molecules due to microbial enzymatic degradation. The observed alterations in the chemical structure of nylon 6, 6 particularly in amide (-CONH-) functional groups after microbial interaction confirm breaking of intermolecular cohesive forces between nylon molecules degradation. Similar changes in the chemical structure of

nylon 6, 6 upon enzymatic degradation (lipase and protease) were reported in an earlier study (Parvinzadeh Gashti et al., 2013).

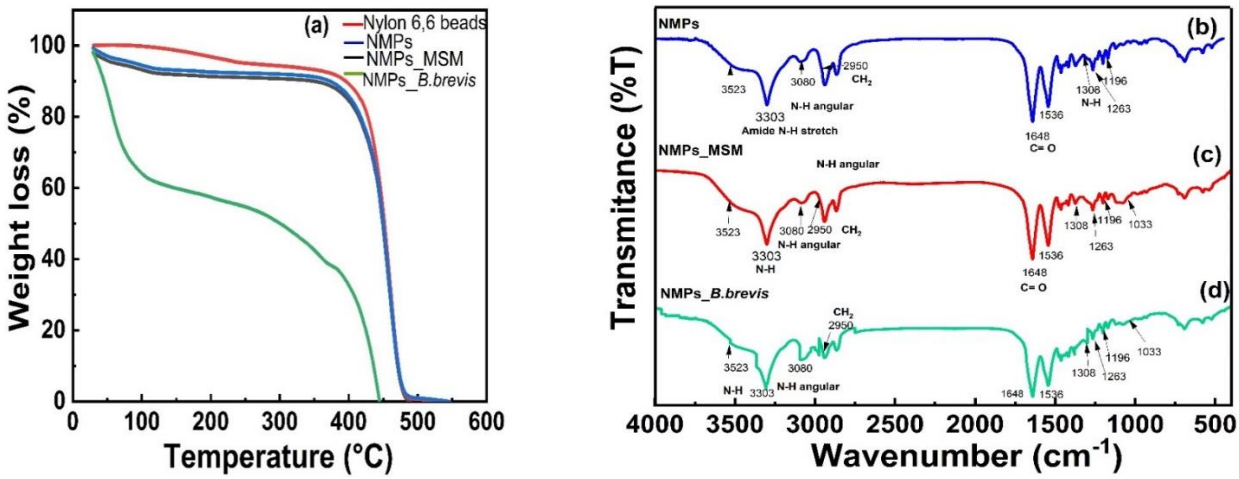


Fig. 7. (a) TGA thermograms (Nylon 6, 6 beads, NMPs, NMPs_MSM, NMPs_*B.brevis)* and FTIR spectra of (b) NMPs (c) NMPs_MSM and (d) NMPs_*B.brevis*.

2.6 Morphological Analysis

SEM: *B.brevis* cells were rod shaped with 2 μm size at lag phase growth in MSM (Fig. 8(a)). The observed lag phase morphology was retained even after 35 d of incubation with MSM (Fig. 8(b)). On incubation of the bacteria with NMPs for 35 d, size of the bacterial cells were observed to have reduced from 2 μm to 0.5 μm while the shape of *B.brevis* cells changed from rod to round. (Fig. 5(c)). These observations clearly exhibited the attachment and colonization of *B.brevis* onto the microplastics for the requirement of carbon source from the plastics. These findings were supported by a recent report on the most degraded polyethylene in soil which hosted a bacterial community(Puglisi et al., 2019).

TEM: The biodegradation of microplastics was validated by the observed morphological changes on both *B.brevis* as well as NMPs after interaction with each other using TEM. The size of NMPs before interaction with *B.brevis* was observed to be 200 nm (Fig. 9(a)). Various cracks, holes and irregular structure of microplastics were observed on NMPs after 35 d of treatment with *B.brevis* due to microbial activity (Fig. 8(b) and Fig. 8(c)). The

adherence of ≈200 nm sized microplastic particles on the surface of *B.brevis* (Fig. 8(d)) and the observed surface deposits on the microplastics (insert of Fig. 8(d)) clearly indicate the role of microbes and their enzymes (intracellular and extracellular) in the degradation of the microplastics. The released enzymes could also be responsible for the generation of cracks and holes as observed on the microplastics which may have further weakened the structure of the polymer for degradation as evidenced by TGA (Fig. 7 (a)) and FTIR (Fig. 7 (d)) analysis. A similar colonization of microbes on plastic films resulting into the formation of cracks, holes/pores and cavities have been reported for HDPE(Sowmya et al., 2014) and PS nanoparticles(Awet et al., 2018).The authors further reported substantial degradation of the candidate polymers in the respective studies. Further, TEM analysis showed a change in the size and shape of the microplastics. It is in good agreement with a report showing colonization of microbes on the surface of polyethylene plastics and eventually leading to the formation of pits/erosion of plastic film after the biodegradation (Bonhomme et al., 2003).

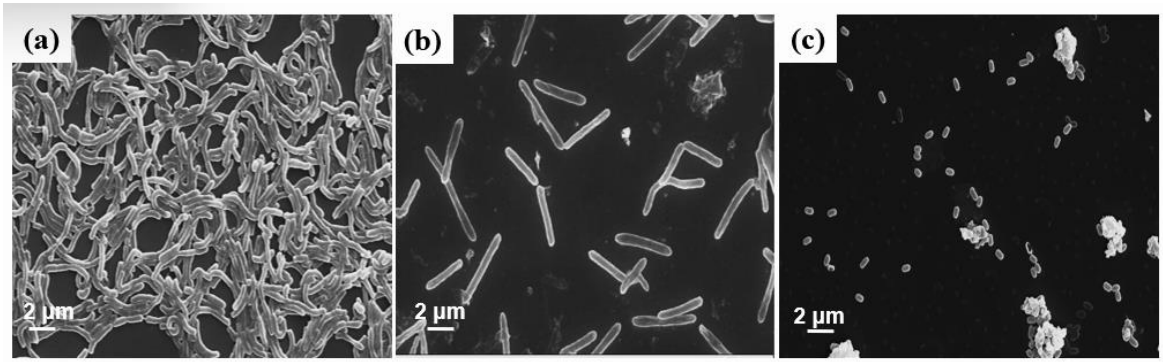


Fig. 8. SEM micrographs (magnification 2550 X) of *B.brevis*_MSM (a) at lag phase as well as (b) after 35 d and (c) *B.brevis*_NMPs after 35 d.

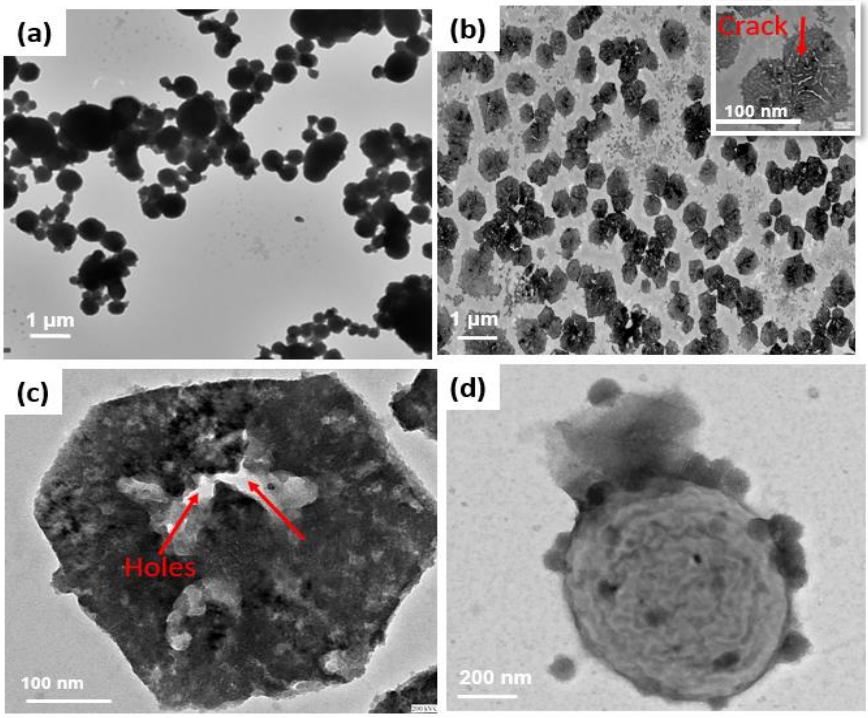


Fig. 9. TEM micrographs of (a) NMPs (magnification X2550/1µm) before interaction with *B.brevis*, (b) NMPs (magnification X2550/1µm and X7000/100 nm (insert)), (c) NMPs (magnification X 29000/100 nm) with cracks and holes after 35 d interaction with *B.brevis* and (d) *B.brevis* with NMPs attachment (magnification X15000) after 35 d interaction with NMPs.

2.7 MS data analysis

Biodegradation of plastics is a promising approach to convert polymers into monomers for recycling or to mineralize them into simpler compounds such as carbon dioxide and water. Biodegradation involves release of enzymes/acids, attachment/absorption of microbes onto the surface of plastics and hydrolysis of longer chains into shorter monomers which is further assimilated by the microbes. Based on this theory, a degradation pathway has been proposed as follows. Identification of the bacterial mediated plastic degraded products, the *B.brevis*_NMPs metabolite was subjected to MS using positive electron spray ionization (ESI). The mass spectrum (Fig. 10) reported two important peaks at m/z = 116 and m/z = 175 and confirmed the formation of 1, 6-hexanediamine and dimethyl adipate. The resulting 1,6-hexanediamine and Dimethyl adipate indicate breakdown of amide bonds in nylon 6, 6 due to the depolymerization activity of enzymes followed by acidolysis. It is

pertinent to recall that the total enzyme and laccase activities of *B.brevis* were found to be higher in the presence of NMPs (Fig. 6(a) and (b)). The observed higher laccase activity in presence of NMPs might have induced depolymerization of nylon 6, 6 via oxidation of the polymer backbone as reported by Santo et al., 2013 in case of polyethylene degradation. Acidolysis of organic acids in tricarboxylic acids cycle, glycolysis and glyoxylate cycle is quite common in these organisms(Drincovich et al., 2016; Papagianni, 2011). The observation is also supported by recent findings (Jehanno et al., 2018), which revealed the formation of 1, 6-hexanediamine and dimethyl adipate due to the acid catalyzed degradation of polyamides 66. It is a crucial first step of nylon degradation, which involves the breakdown of amide group present in the structure of nylon 6, 6 (Matsumoto et al., 2017). The existence of the peak at m/z 131 shows the presence of 1, 6-hexanediaol. 1, 6-hexanediaol is reported as a subsequent hydrolysis product of hexamethyldiamine (Jehanno et al., 2018). The existence of hexamethyldiamine and adipic acid in the *B.brevis*_NMPs metabolite were also evidenced by HPLC analysis after 35 d of treatment and reported as 0.956 mg/l ± 0.005 and 1.428 mg/l ± 0.005 respectively. The conversion of 1,6-hexanediaol to adipic acid is expected through ω-oxidization as per (Kunz and Weimer, 1983). The reported intermediate 6-hydroxyhexanoate (fragment at m/z = 131) in our case confirmed the ω-oxidization of 1, 6-hexanediaol (Fig. 7). The observation is also supported by the latest report by Capece *et al*(Capece et al., 2020) which showed formation of adipic acid (m/z = 145) through intermediate, 6-hydroxyhexanoate from 1,6-hexanediaol by an aerobic oxidation process. The resulting adipic acid is utilized by *B.brevis* to form Adipoyl CoA. The peaks corresponding to m/z values = 131, 359 , 428, and 866 as per(PubChem, n.d.) evidenced the existence of Adipoyl CoA in the *B.brevis*_NMPs metabolite. The intermediate compound peak of hexanoyl Coenzyme A at m/z = 896 indicated its presence in the mass spectrum (Fig. 10). Hexanoyl Coenzyme A

is a dicarboxylacyl CoA and is reported to result from the formal condensation of the thiol group of coenzyme A with one of the carboxy groups of adipic acid("adipoyl-CoA (CHEBI:34528)," n.d.). Based on above findings, the following degradation pathway has been proposed for nylon 6, 6 microbial degradation using *B.brevis* in Fig. 11.

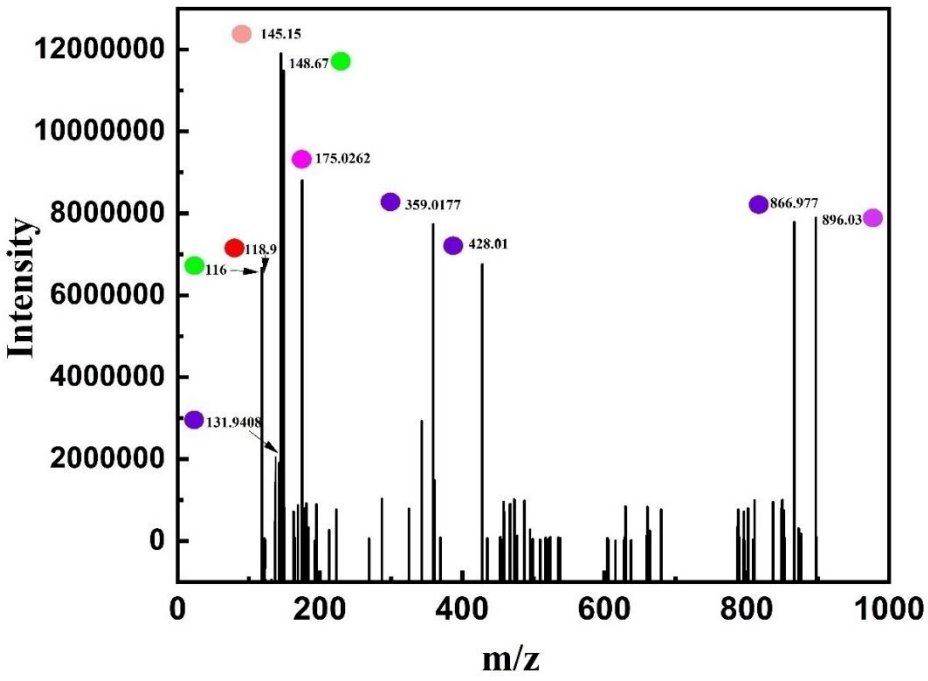


Fig. 10. Mass spectrum of metabolite *B.brevis*_NMPs obtained after 35 d (m/z 116 1, 6-hexanediamine, m/z 175 Dimethyl adipate, m/z 131 1,6 hexanediaol, m/z 145 adipic acid, m/z 131, 359, 428 and 866 Hexanoyl Coenzyme A, m/z 896 Adipoyl CoA)

Pathway

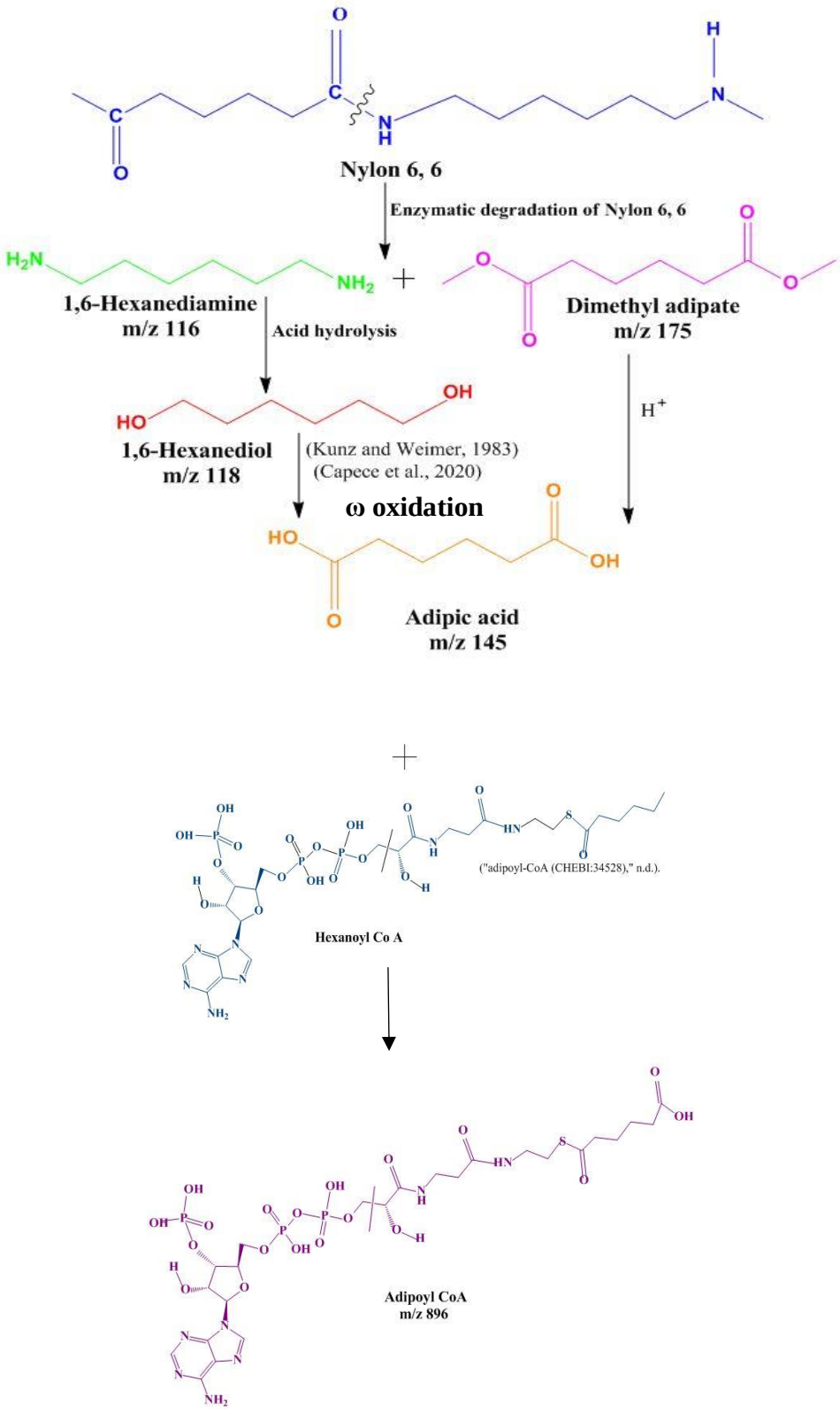


Fig. 11. Possible Pathway of nylon 6, 6 microplastics degradation by *B.brevis*

2.8 Conclusion

The main objective of the present study was to understand the mechanism of microbial degradation of NMPs. We demonstrated the growth of *B.brevis* in presence of NMPs. The changes observed in characteristic FTIR peaks of NMPs before and after treatment with *B.brevis* confirmed cleavage of intermolecular cohesive forces between –C=O and –N-H groups of nylon 6, 6 molecules. Significant reduction in the dry weight of NMPs and morphological changes visualized on the microbe as well as plastic surfaces through SEM and TEM analyses indicate nylon 6, 6 degradations. The enhanced release of adipic, malic, oxalic and citric acids and the monomers (adipic acid and hexamethylene diamine) of nylon 6, 6 were first time reported supporting the degradation mechanism via enzymatic activity and acid hydrolysis. These new findings are further confirmed by analysis of metabolic responses through UPLC-MS and a degradation pathway is proposed as a novel outlook into the mechanism of microplastics degradation. The observations confirm that the *B.brevis* itself is capable of degrading nylon 6, 6 microplastics by breaking amide bonds to release nylon 6, 6 monomers in a shorter duration compared to existing reports on microbial degradation of plastics.

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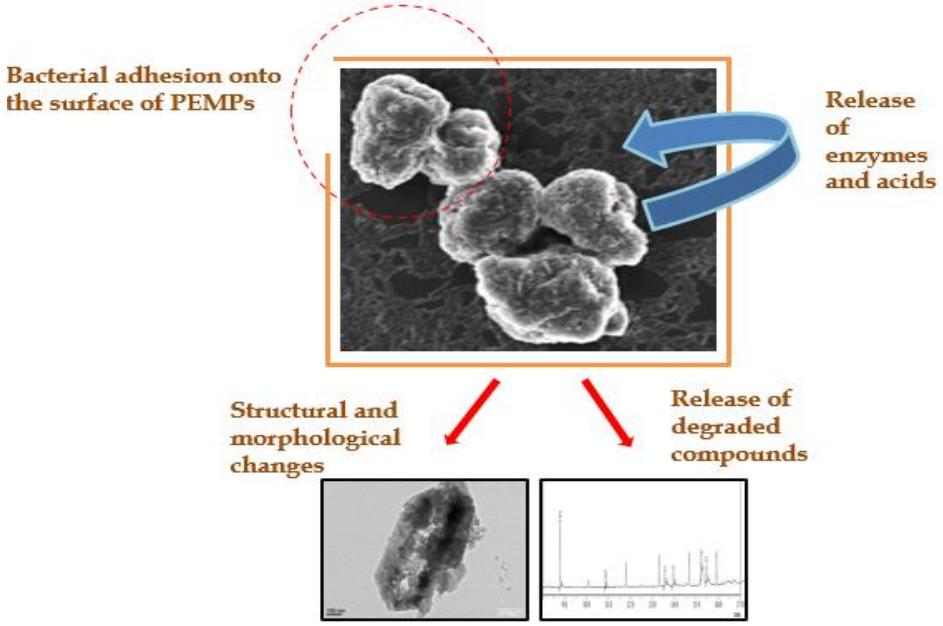
Chapter 3

Degradation of polyethylene microplastics through microbial action by a soil isolate of *Brevibacillus brevis*



Chapter 3

Degradation of polyethylene microplastics through microbial action by a soil isolate of *Brevibacillus brevis*

3.1 Introduction

Global production and utilization of plastics have increased from 335 Mt in 2016 to 367 Mt in 2020 ("Plastics - the Facts 2021). Plastic pollution in the ecosystem results from the use and production of plastics on a worldwide scale, endangering both the environment and human health (Ritchie and Roser, 2018). It is estimated that around 3 % of the world's plastic waste enters the ocean, while most remains in landfills (Jambeck et al., 2015). Most of these plastics in the environment are non-biodegradable and persist for longer in various size ranges. Among these plastic fragments, particles of the size range (0.1 µm – 5 mm) are called microplastics. There are two types of microplastics: primary microplastics, which are microscopic particles that enter the environment as a direct result of being used as industrial abrasives or in cosmetic items, and secondary microplastics, the majority of which microplastics are found in the environment and are created by the ageing of greater pieces into microplastics due to biotic and abiotic causes (Wang et al., 2020). These microplastics are hazardous to the ecosystem and have attracted the scientific community's attention for their remediation.

Microplastics are circulated globally in the world's water bodies, including water columns, water surfaces, and deep inside sediments (Chaudhry and Sachdeva, 2021). By different mechanisms, microplastics can be ingested by lower-level organisms to higher-level organisms, which also transfer associated pollutants, pathogens, and additives (Sunitha et al., 2022). Polyethylene (PE) is a majorly manufactured polymer required in different industries, such as packaging products, containers and coating material (Beg et al., 2016). Due to PE's strong recalcitrance and inert properties, it is exceedingly difficult to break down in the environment, even after being buried for a long time in a landfill (Otake et al.,

1995). The microbial degradation by the fungus *Aspergillus flavus* named PEDX3 degraded high-density polyethylene (HDPE) microplastics into microplastics with a lower molecular weight after 28 days of treatment (Zhang et al., 2020). Thermo-oxidative pre-treated low-density PEMPs degradation by *Achromobacter denitrificans* Ebl13 showed 12.3 % weight loss (Maleki Rad et al., 2022). Li et al., showed 30 days of cultivation of the marine bacterium *Microbulbifer hydrolyticus* IRE-31 strain is responsible for the biodegradation of linear low-density PE particles by morphological changes of the polymer surface(Li et al., 2020a). These findings emphasize that the microbes which can grow in the presence of PEMPs are expected to degrade the plastics much more efficiently. As per our knowledge, highly active microbial enzymes responsible for the PEMPs degradation are not yet identified. Knowledge on the degradation mechanism and identification of the degraded products from plastics are limited. This information is very much essential for providing sustainable interdisciplinary solutions for rapid remediation of plastic from the environment.

In this investigation, we have emphasized the interaction of PEMPs with *B.brevis* without any previous pre-treatment and discuss consequent morphological and structural changes of the microplastics. The chosen strain, *B.brevis* is reported to be a high-potential candidate in hydrocarbon degradation such as pyrene, naphthalene degradation, nylon 6, 6 and polypropylene degradation (Skariyachan et al., 2018; Tiwari et al., 2022; Wei et al., 2017). The PEMPs degradation and metabolic response of *B.brevis* under microplastic presence were followed periodically by shake flask study. The role of enzymes and organic acids in the bacterial degradation of PEMPs was studied to understand the degradation mechanism. Morphological changes of *B.brevis* and PEMPs are monitored using SEM and TEM respectively, and the degradation products of PEMPs are identified through GC-MS.

3.2 Materials and Methods

Polyethylene (ultra-high molecular weight) 40-48 µm particle size (PE) (CAS no: 9002-88-4) and all additional chemicals utilized throughout this experiment were of analytical reagent grade. Before degradation study, particles were sterilized by immersing in 70 % ethanol in a glass Petri dish and dried for 16 h in an air oven at 55 °C. The organism *B.brevis* (MTCC NO. 7822) was purchased from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The obtained bacteria were isolated from soil in Dehradun and Haridwar in Uttarakhand in India. As received, lyophilized bacterial culture was revived on nutrient medium plates. Minimal salt medium (MSM) containing 0.025% $MgSO_4$, 0.025% $CaCl_2$, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.1% NH_4Cl, 0.05 g $FeCl_3$, 1.8 g glucose and 0.1% NaCl per L of distilled water, was used to sub-culture for three generations.

3.2.1 Microbial inoculums preparation and *in vitro* PEMPs degradation study

The bacteria grew in nutrient broth to a stationary phase in a rotating shaker at 37 ºC at 120 rpm. The bacterial growth in the culture medium was monitored by observing its absorbance at 600 nm using Eppendorf UV-vis Spectrophotometer (Bio Spectrometer basic model). The third-generation culture utilized for the biodegradation study was reported for a log phase with an absorbance of 0.7. The bacterial culture was grown in MSM without any carbon source and was used in the *in vitro* degradation experiments. The culture was inoculated into 100 mL of MSM broth with *B.brevis* (*B.brevis*_MSM/ Experimental flask and without inoculum but only microplastics (PEMPs_MSM/ negative control) 0.5 g of UV treated PEMPs. The parameters such as pH, optical density (OD), secretions of organic acids and enzyme activity were monitored every 5 d for 35 d.

3.2.2 Characterization studies

After the incubation of PEMPs in the absence of *B.brevis* (negative control, PEMPs_MSM), *B.brevis* in MSM (Positive control, *B.brevis*_MSM) and the presence of *B.brevis* (*B.brevis*_PEMPs) for 35 d, the contents were filtered with 0.22 µm polytetrafluorethylene (PTFE) Millex filters. Both filtrate and residue of each sample were taken for further characterization as follows:

Metabolic analysis

3.2.3 Ultra Performance High-Pressure Liquid Chromatography (UP-HPLC)

After every 5 d of incubation, 1 mL of sample from each flask of *B.brevis*_MSM, PEMPs_MSM and *B.brevis*_PEMPs was pipetted out and filtered through 0.22 µm polytetrafluorethylene (PTFE) Millex filters for UP-HPLC analysis. Using UP-HPLC with a photodiode array detection (PDA) detector at wavelength 210 nm, organic acids in *B.brevis*_MSM, *B.brevis*_PEMPs and PEMPs_MSM metabolites were analyzed. Methanol and potassium dihydrogen phosphate (10 mM, pH 2.8) were combined at a ratio of 10:90 to serve as the mobile phase and added to the 250 mm x 4.6 mm (id) Shimadzu Allure Organic Acid 5 m Analytical Column at the optimal temperature of 25 ºC and flow rate of 1 mL/min.

3.2.4 Total enzyme activity

The hydrolytic cleavage of fluorescein diacetate (FDA) into fluorescein served as the basis for the hydrolase's assessment. Along with the corresponding positive and negative controls, 2 mL of each culture media (*B.brevis*_MSM and *B.brevis*_PEMPs) was incubated with 20 mL phosphate buffer (pH = 7.4) and 0.2 mL FDA stock solution (1000 µg mL^{-1}) at 30 °C for 40 min in a water bath shaker for 150 rpm (only culture, no FDA and only FDA, no sample). For each sample, 20 mL of a 2:1 chloroform/methanol solution was added before centrifugation for 7 min at 6000 rpm. The fluorescence intensity of the supernatant

was measured at 490 nm using Eppendorf UV-vis Spectrophotometer (Bio Spectrometer basic model) (Adam and Duncan, 2001; Schnürer and Rosswall, 1982).

3.2.5 Laccase activity

The activity of laccase from *B.brevis* culture media was monitored using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as a substrate. In 100 µL of culture media, 100 µL of 10 mM ABTS was added to 800 µL of 50 mM sodium acetate buffer (pH 5). After that, the mixture was incubated at 30 ℃ for 5 minutes. Eppendorf UV-vis Spectrophotometer (Bio Spectrometer basic model) was used to determine the presence of the green color that comes from ABTS oxidation at A420 (ε = 36000 $Lmol^{-1}cm^{-1}$). The secretion of enzymes was monitored at various time points from the 5 d to the 35 d. The quantity of enzyme necessary to oxidize 1 mol of ABTS per minute at 30 °C was defined as one unit of laccase activity (More et al., 2011).

3.2.6 Gas Chromatography-Mass Spectrometry (GC-MS)

The metabolite was obtained after continuous shaking of 35 d, the PEMPs were filtered from the bacterial culture. Further, the obtained liquid was centrifuged at 12000 rpm for 7 min to remove any cell debris or microplastics. The supernatant was filtered with filter paper (Whatman no. 2). The degraded products were treated using solvent, diethyl ether. Diethyl ether is often used for analyzing polyethylene degraded compounds in GC-MS due to its efficiency to dissolve polymer and its degraded products. Each filtrate was dissolved in an equal volume of diethyl ether (10 mL) using a separating funnel. 1 mL of the dissolved degraded products was subjected to GC-MS (Rxi-5Sil MS Gas chromatogram Mass Spectrometer)(Shahnawaz et al., 2016). Helium was used as carrier gas with a flow rate of 1 mL/min. The GC-MS column dimensions (length 30 m; 0.25 mm ID.; df = 0.25 µm). The GC-MS real-time analysis program conducted the data processing, peaks integration, and

quantifications. Major peaks were identified using a NIST- 14 and WILEY-8 library search.

Structural analysis on PEMPs

3.2.7 Differential Scanning Calorimetry (DSC)

Thermograms of (control, PEMPs_MSM and *B.brevis*_PEMPs) were obtained using DSC (Perkin Elmer DSC 8000 instrument). The samples were placed in an aluminium crucible (approx. 10 mg). The samples were heated at a rate of 10 ºC per min from 10 to 200 ºC under a nitrogen atmosphere (Raghavan and Torma, 1992; Sudhakar et al., 2007).

3.2.8 Thermogravimetric analysis (TGA)

The TGA of the control, PEMPs_MSM and *B.brevis*_PEMPs were performed using Perkin Elmer thermogravimetric analyzer TGA 4000. All the samples were heated from room temperature to 800 °C at a heating rate of 10 °C min^{-1} and a nitrogen flow of 10 mL min^{-1}.

3.2.9 Fourier transform infrared (FTIR) spectroscopy.

The structural changes that occurred in PEMPs after interaction with *B.brevis* (*B.brevis*_PEMPs) were analyzed by FTIR spectroscopy (Perkin-Elmer 400 FT-IR/FT-FIR) in the frequency range of 2000 cm^{-1}- 450 cm^{-1} with appropriate control samples. The PEMPs interacted with *B.brevis* were washed three times with milliQ water and dried below 100 ºC to remove bacterial debris. KBr was used to prepare the appropriate sample pellets, which were then scanned at a resolution of 4 cm^{-1}.

3.2.10 X-ray diffraction (XRD) analysis

The XRD analysis of, *B.brevis*_PEMPs and PEMPs_MSM was conducted with BRUKER D4 X-ray diffractometer operating at 20 kV and 10 mA using Cu-Kα radiation at room temperature. For analysis, 2θ range of 5º to 40° with a step size of 0.01 and counting time of 6 s per step was followed. The crystallinity index (CI) was determined using formula:

$$CI = \frac{I}{(I_C + Ia)} \times 100$$

Where, I_c represent crystalline area of diffraction peaks and $I_c + I_a$ represents the total diffractogram area (crystalline and amorphous peaks) (Jeon et al., 2021; Park et al., 2010).

Morphological Analysis

3.2.11 Scanning electron microscopy (SEM)

The changes in bacterial morphology before and after the degradation of PEMPs were investigated after 35 d of incubation with *B.brevis* under SEM (SEM, Model: EVO18 Zeiss, Germany) at an accelerating voltage of 5 kV at 10,000X magnifications. The culture media was centrifuged and washed with Milli-Q water. The fixative (Karnovsky's fixative) was used to fix the bacterial cells before the examination. The bacterial cells were sputter-coated with a gold layer at 25 mA under Ar atmosphere at 0.3 MPa and subsequently examined under SEM.

3.2.12 Transmission electron microscopy (TEM)

The morphology of microplastics PEMPs_MSM as well as *B.brevis*_PEMPs was analyzed using TEM (Tecnai G2 200 KV HRTEM SEI HOLLAND). Before the examination, the samples were washed and dried to ensure the removal of bacterial cells attached on to the surface of microplastics (as explained in section 2.13) and then subjected to TEM at an accelerating voltage of 200 kV at 2550X magnifications.

3.2.13 Dry weight determination of residual PEMPs

After 35 d of incubation the PEMPs were filtered using vacuum filtration system with polycarbonate filter paper (pore size: 0.22 µm). The samples were transferred on to the filter paper, carefully. Gentle suction pressure was applied to initiate the filtration process, which allows the media to pass through while keeping the microplastics onto the filter paper. Further the microplastics were washed with sterile water and 70% ethanol to ensure sterile particles. The particles were dried in the oven at 60 ^{o}C for 12. Residual polymer

weight was determined to measure the extent of degradation (Auta et al., 2017; Mohan et al., 2016; Mor and Sivan, 2008). The initial weights of the pre-incubated microplastic were measured as discussed above. The plastic polymer degradation was evaluated in terms of percentage weight loss using the following formula:

$$\%\ weight\ loss = \frac{(initial\ weight\ of\ polymer - Final\ weight\ of\ polymer)}{initial\ weight\ of\ polymer} * 100$$

3.2.14 Statistical Analysis

The experiments were conducted in triplicates to ensure reproducibility and reliability of the results. Statistical analysis of the experimental data was performed using Originpro2022 software, along with analysis of variance (ANOVA) using SPSS software version 21.0. To determine significant differences between groups, the LSD post-hoc test was employed at a significance level of P-value = 0.05. All values are reported as mean ± standard deviation, based on triplicate samples (n = 3), providing a comprehensive representation of the data. To calculate the rate constant and half-life the following formula was used (Auta et al., 2018):

$$\text{Rate Constant } K = -\frac{1}{t}(ln\frac{W}{W0})$$

where K is the rate constant for polymer uptake, t is the time in days, W is the weight of residual polymer (g), and W_O is the initial weight of polymer (g).

$$\text{Half-life } (t_{1/2}) = \ln(2)/K$$

Where ln is the natural logarithm, and K is the Removal Constant.

3.3 Results and Discussion

3.3.1 Biodegradation study for PEMPs

Microbial degradation of PEMPs was investigated by evaluating the growth parameters of *B.brevis* such as growth curve, pH, enzyme activity as well as various organic acid

contents of the metabolite both in the absence (*B.brevis*_MSM) and presence of PEMPs (*B.brevis* _PEMPs).

3.3.1.1 Growth curve

After three generations of adaptation to MSM media, optical density (OD) measurements at 600 nm were carried out to monitor the growth of *B.brevis* (Fig. 12(a)). In the absence of PEMPs the lag phase was recorded from 0 d (0.035) to 5 d (0.24). The OD increased exponentially over the following 5 d, reaching 1.31 on the 5 d. Whereas, in the presence of PEMPs the lag phase was noticed from 0 d (0.046) to 5 d (0.074). In contrast, exponential growth (1.78) was reached gradually in 10 d compared to the control (*B.brevis*_MSM). The increase in incubation days corresponds to the interaction between the bacterial cell membrane and PEMPs. The stationary phase attained a longer duration for *B.brevis*_PEMPs as compared to *B.brevis*_MSM. This suggests that the growth of *B.brevis* in the presence of PEMPs terminated after 25 d, but the bacterial cells were still metabolically active. A major decay in the growth of the *B.brevis* was observed on the 25 d up until the 35 d on interaction with PEMPs. The decline phase of *B.brevis* in the presence and absence of PE suggests cell lysis due to nutrient depletion and increased metabolite content in the culture medium.

3.3.1.2 pH

For the survival, enzyme activity, and stability of the microbial population, pH is one of the most important elements in its growth. It has a critical impact on the development of microorganisms, the activity of enzymes, and the pace of pollutant degradation (Gao et al., 2010; Xu et al., 2011). Organic acids such as malic acid, citric acid, and oxalic acid are released in lesser amounts for microbial metabolism which regulates the pH. The amount of organic acids increases after the breakdown of pollutants such as tricresyl phosphate (Liu et al., 2019a). The presence of organic acids plays a crucial role in the

biodegradation of contaminants such as in the case of 14C phenanthrene (Vázquez-Cuevas et al., 2020) Fig. 12(b) signifies the changes in the pH of the bacterial cell culture in the presence and absence of PEMPs during 35 d of biodegradation study. Initial pH changes were recorded in the *B.brevis*_MSM and *B.brevis*_PEMPs for up to 5 d. A decline in pH was observed from 5 d to 35 d (pH 6.7 to 5.7) for *B.brevis*_PEMPs. This observation suggests the ideal pH for the growth of *B.brevis* is pH 6 ± 0.3. A sudden decrease in the pH was observed for *B.brevis*_PEMPs when compared to *B.brevis*_MSM due to the production of organic acids in the medium with lower pH. Also, reduction in pH with the duration of time suggests a suitable pH is required for the growth and metabolism of the bacterial cells for efficient degradation of contaminants. Another study suggests the culture medium pH should be neutralized periodically to enhance the rate of biodegradation of contaminants (Nievas et al., 2005).

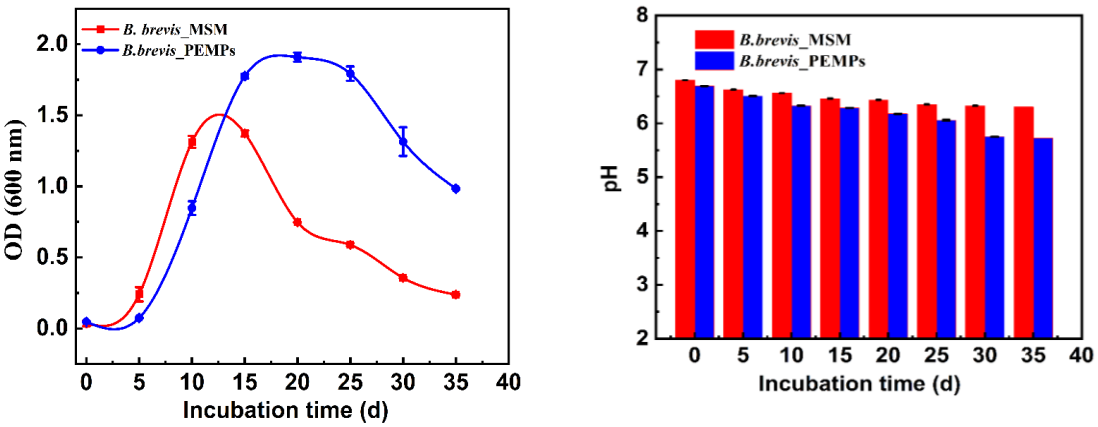


Fig. 12. (a) Growth curve of *B.brevis* and (b) pH comparison of *B.brevis* growth culture in the presence and absence of PEMPs

3.3.2 Release of Organic acids

Organic acids (OAs) are mono-, di-, and tricarboxylic acids that naturally occur as intermediates in several intracellular metabolic pathways, including the tricarboxylic acid cycle (TCA), the development of neurotransmitters, and the formation of cholesterol

(Kölker, 2014). The release of organic acids accelerates the microbial breakdown of the pollutants and aid to biodegrade a variety of micropollutants (Gibson and Suflita, 1990; McNamara et al., 2014; Xiao and Wu, 2014). As shown in Fig. 13 (a, b, c), When PEMPs were present, *B.brevis* excreted more organic acids than the control, including malic acid, citric acid, and oxalic acid. Remarkably, citric acid (Fig 13(a)) content was higher than malic acid (Fig 13(b)) and oxalic acid (Fig 13(c)) in the PEMPs treated with bacteria. It is also notable that increased citric acid content in the metabolite was seen in the presence of PEMPs for up to 25 d, with a subsequent reduction in a later stage. The observation supports that increase in the concentration of citric acid increases the bioavailability of phenanthrene and pyrene in the soil, which is used for growth and other cellular processes more frequently in their TCA metabolic path way (Ling et al., 2009; Xiao and Wu, 2014). Malic and oxalic acid concentrations increased in presence of PEMPs beginning on 5 d. Additionally, research shows that the intermediate molecules oxalic and malic acids are generated by the mineralization of contaminants, primarily those from anthropogenic activity (Xiao and Wu, 2014). Here, the higher levels of oxalic acid and malic acid suggest that these acids play a substantial role in the breakdown of PEMPs, which the microbe did not use further. These results are reasonably consistent with past studies (Yi et al., 2017), (Liu et al., 2019b), (Gao et al., 2010) and (Ling et al., 2009) where an increase in the oxalic acid content with the biodegradation of pollutants (triphenyltin, tricresyl, phenanthrene, and pyrene) could be detected.

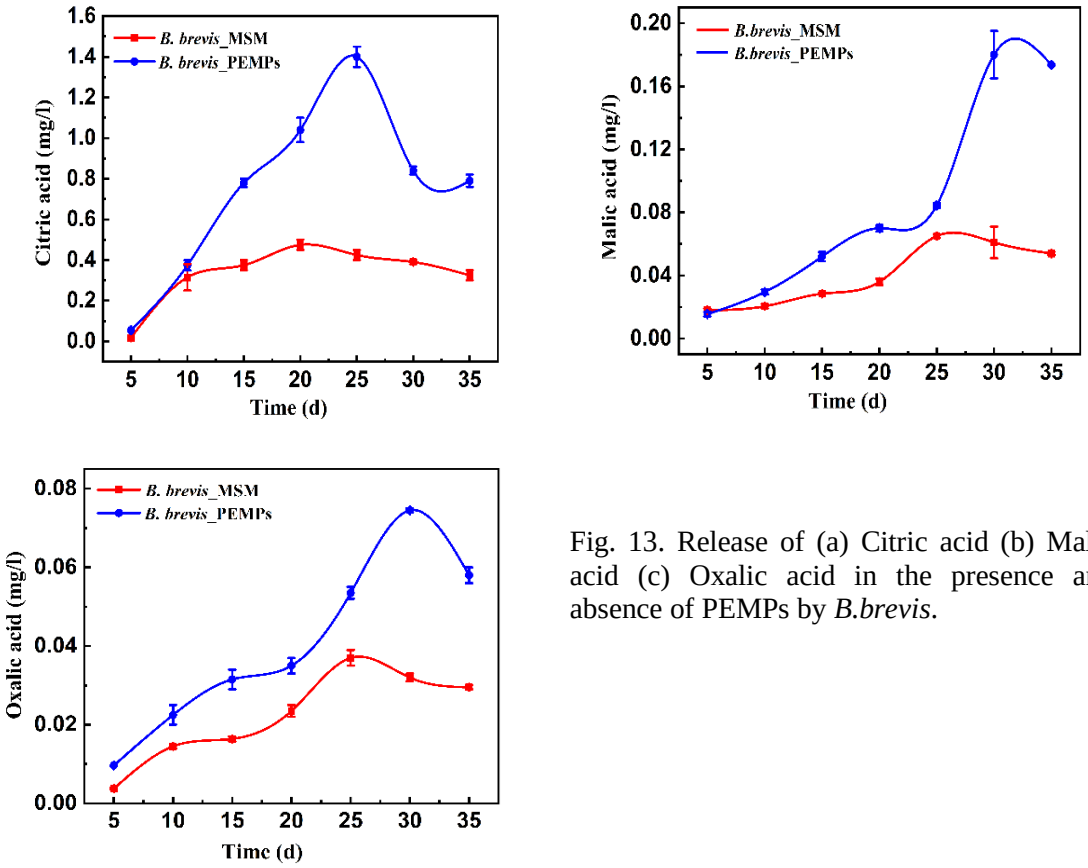


Fig. 13. Release of (a) Citric acid (b) Malic acid (c) Oxalic acid in the presence and absence of PEMPs by *B.brevis*.

3.3.3 Enzyme Assays of *B.brevis* during degradation of PEMPs

3.3.3.1 Total Enzyme Activity

Fluorescein diacetate is the method used for the measurement of microbial cell activity in a wide range of environmental samples. During the FDA process, the bound and unbound enzymes hydrolyzed to produce fluorescein, a substance with a yellowish-green color. The control samples were subjected to an FDA reaction in which the enzyme activity was calculated as FDA hydrolyzed per mL. The biodegradation study was done in strict sterile conditions, and both positive and negative controls exhibited a negligible amount of FDA hydrolysis (Schnürer and Rosswall, 1982). This method has been extensively used to study the impact of pollutants on microbial activity (Mahu et al., 2018; Muter et al., 2012). Fig. 14(a) shows a significant increase in the total enzyme activity of *B.brevis* in the presence of microplastics (*B.brevis*_PEMPs) than in its absence (*B.brevis*_MSM). Such results confirm bacteria' growth and the excretion of enzymes for PE degradation. It was detected

that with the increase in incubation time to PEMPs, enzyme release of *B.brevis* subsequently increased and exhibited its highest activity by 20 d (39 μg FDA hydrolyzed/mL of media in 40 min reaction time) and lessened after that. A crucial reason for the higher enzyme activity of the microbe in the presence of microplastics can be due to the release of enzymes such as protease, esterase and laccase responsible for its degradation (Kaushal et al., 2021). Such a pattern was observed to be similar to the growth of *B.brevis* monitored in our case (Fig. 12(a)). The role of a well-known enzyme i.e, laccase (Mohy Eldin et al., 2022; Sowmya et al., 2015), and its activity was observed at different time intervals and results are discussed in the subsequent section.

3.3.3.2 Laccase Activity

Laccase plays a significant role in the biodegradation of PEMPs. It is widely well-known enzyme related to PE degradation (Santo et al., 2013). Laccase enzyme is considered under the oxidase group enzyme, which is known to depolymerize the polymer chain via oxidative cleavage of the amorphous region of PE. The cleavage provides an accessible carbonyl region within the polymer chain for degradation (Kang et al., 2019). Fig. 14(b) shows a comparative increase in the activity of laccase in presence of PEMPs (*B.brevis*_PEMPs) than in the absence of microplastics (*B.brevis*_MSM). It was detected that the laccase secretion of *B.brevis* enhanced as interaction time increased with PEMPs. The highest laccase activity was detected at 25 d (5.4 μmol/min in 5 min reaction time) and decreased thereafter. This similar pattern was found in the total enzyme/enzymatic activity of *B.brevis* (Fig. 14(a)). The most significant role of laccase enzyme in the biodegradation of plastics such as polyethylene was demonstrated by Santo *et al* (Santo et al., 2013). Secretion of laccase enzyme induces depolymerization of polyethylene via oxidation of the polymer backbone(Santo et al., 2013). Santo et al. demonstrated that thermostable laccase can degrade the PE, after two days of the incubation period. The specific substrate also

induces the activity of laccase such as copper. Treatment with Copper also affected the laccase activity during the PE degradation and found an enhanced rate of biodegradation by 75% C208 cultures containing polyethylene (Santo et al., 2013).

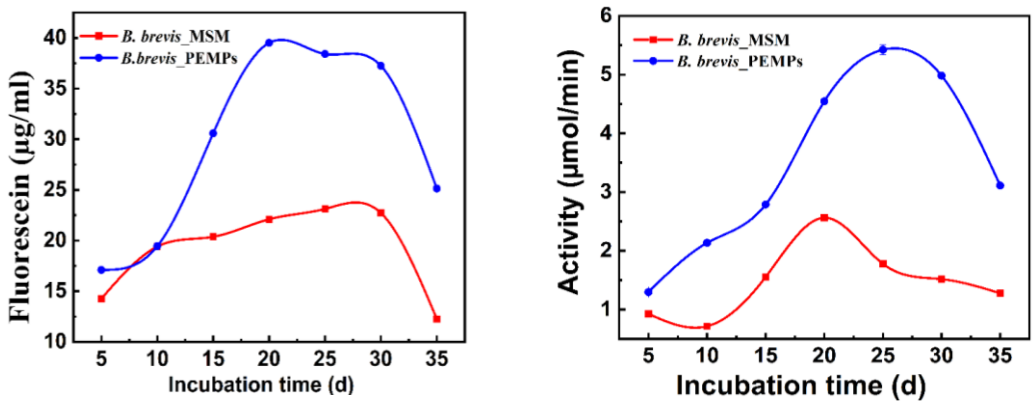


Fig. 14. (a) Total enzyme activity and (b) Laccase enzyme activity of *B.brevis* in the presence and absence of PEMPs

3.3.4 Weight Loss of PEMPs upon microbial-assisted degradation

Upon microbial interaction on PEMPs, changes that occurred on plastic were quantified by the weight loss percentage of PEMPs. The results are represented in Table 4. The weight loss of PEMPs was not detected for PEMPs_MSM. The weight loss of *B.brevis*_PEMPs was 19.8% after 35 d of biodegradation. These outcomes confirm the capability of the bacterial isolate to excrete some crucial enzymes/acids (Fig. 13 and Fig. 14(a-b)) that can utilize the carbon source of PEMPs for microbial survival. Furthermore, the microbial degradation of the PEMPs conceivably results from biochemical pathways (Bio-fragmentation and Bio-assimilation). In a similar study, the weight loss of PEMPs was recorded as 14.7% when exposed *to Bacillus sp.* and *Paenibacillus sp. vesicularis*, over 60 days (Park and Kim, 2019). Whereas, about 12% weight loss is observed for the pre-treated PE with *Achromobacter denitrificans* (Maleki Rad et al., 2022). Observed changes in the weight loss percentage could be the outcome of microbial activity upon PEMPs. Further, the weight loss specifies possible alterations in the chemical composition of the

microplastics (Zhang et al., 2021). Biodegradation can lead to weight loss as the polymer chains are broken down into smaller molecules, including carbon dioxide, water, and other by products. This fragmentation process ultimately results in a reduction in the weight of the plastic particles, as the plastic is broken down into smaller molecules and compounds that can be used by the microorganisms for energy and growth (Restrepo-Flórez et al., 2014) . Most microbes adapt to their surroundings based on the availability of nutrients. Hence, this weight loss suggests that *B.brevis* utilize carbon from the backbone of the PEMPs by attacking its chain (Bhardwaj et al., 2012).

Table 4. Dry weight analysis of PEMPs in the presence of *B.brevis*

Organism	Initial weight (g)	Final weight (g)	Weight loss(wt %)	F-value	Sig.	Removal constant (K) day^{-1}	Half-life (days)
Control	0.5	0.5	0				∞
B.brevis	0.5	0.387±0.001	19.80 %	96.032	<0.001	0.007	94.93

3.3.5 Morphological Analysis

SEM: The morphological analysis of *B.brevis* cells indicates a rod-shaped structure of bacteria with 2 μm in size at lag phase growth in MSM medium (Fig. 15(a)). After 35 d of incubation with MSM, *B.brevis* cells undergo morphological changes including, the formation of loosely connected bacteria cells that resemble chains (Fig. 15(b)). Another observation made abundantly clear that *B.brevis* adherence and colonization onto the microplastics is due to its need for a carbon source from the plastics (Fig. (c)). These findings were in good agreement with a recent report on the bacterial colonization of PEMPs for biodegradation (Park and Kim, 2019).

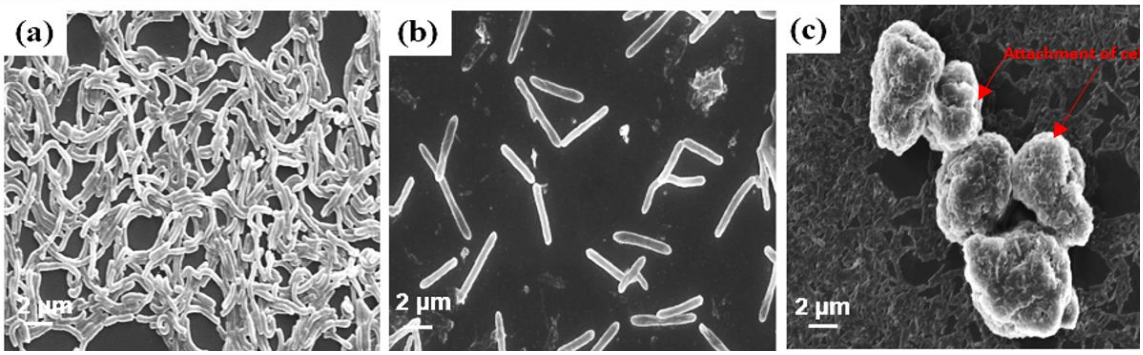


Fig. 15. SEM Images (magnification 2550X) of (a) *B.brevis* in log phase (after 10 d) (b) *B.brevis* in death phase without microplastics (after 35 d) (c) attachment of microbe onto the surface of microplastics (after 35 d).

TEM: By employing TEM to study morphological changes in PEMPs after interacting with *B.brevis*, the biodegradation of microplastics was confirmed. The size of PEMPs before interaction with *B.brevis* is 40-48 µm and no holes/cracks with smooth surface were observed (Fig. 16(a)). Whereas Fig. 16(b) represents one of the broken pieces of microplastic (*B.brevis*_PEMPs) along with some holes with thinned edges. Fig. 16(c) is magnified image (43000X) represented a portion of Fig. 16(b) indicating prominent morphological changes on PEMPs due to bacterial degradation. The resulted morphological changes of *B.brevis*_PEMPs are in good agreement with FTIR (Fig. 17), DSC (Fig. 18 (a) TGA (Fig. 18 (b)), and XRD (Fig. 18(c)) analyses. The adherence of microbes on plastic films results in the formation of cracks, holes/pores and cavities, depending upon the extent of degradation that was well reported on HDPE ("Biodegradation of polyethylene by Bacillus cereus," n.d.), nylon 6, 6 (Tiwari et al., 2022) and PS nanoparticles(Awet et al., 2018).

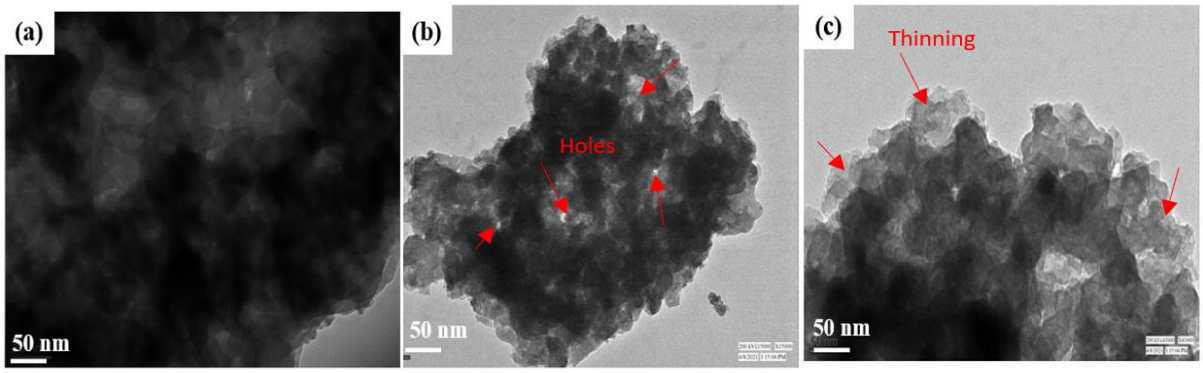


Fig. 16. TEM Images of (a) untreated PEMPs (magnification 43000X/50 nm) (b) fragmented PEMPs (magnification 15000X/50 nm) and (c) fragmented PEMPs (magnification 43000X/50 nm) upon *B.brevis* mediated degradation

3.3.6 Characterization of PEMPs_MSM and *B.brevis*_PEMPs

3.3.6.1 FTIR analysis

The FTIR spectra of PEMPs before and after interaction with *B.brevis* were shown in Fig. 17.The major characteristic absorption bands were assigned at 2916 cm^{-1} (-CH_3 stretching), 2846 cm^{-1} (-CHO stretching), 1476 cm^{-1} (C=C double bond stretching), 1377 cm^{-1} (-O-H stretching), and 718 cm^{-1} (C=C-H stretching) in PE (Park and Kim, 2019). The FTIR analysis has shown that the *B.brevis*-treated PEMPs formed new functional groups such as hydroxyl groups (O-H stretch at 3450 cm^{-1}) and carbonyl groups (conjugated ketone or aldehyde R-C=O stretch at 1640 cm^{-1}) ("Interpretation of Infrared Spectra, A Practical Approach - Coates - Major Reference Works - Wiley Online Library,") , indicating the oxidation on the surface of the polymer. In a study, the soil microbial community employed for the degradation of PE and it has been observed that the formation of hydroxyl groups (-OH) and the C=C bond occurred at 3428 and 1629 cm^{-1} as well as the formation for a new peak at 1733 cm^{-1} expressing the generation of carbonyl bands (Huang et al., 2021). This observation is consistent with prior research studies (Li et al., 2020b; Zhang et al., 2021). The concluding observation indicates the presence of hydroxyl and carbonyl groups (conjugated ketone or aldehyde) and supports the evidence of bio-oxidation of PE. Through

bio-oxidation, microorganisms can break down the complex structure of polyethylene into simpler compounds that can be further degraded by other microorganisms or assimilated into the metabolic cycle (Zhang et al., 2021).

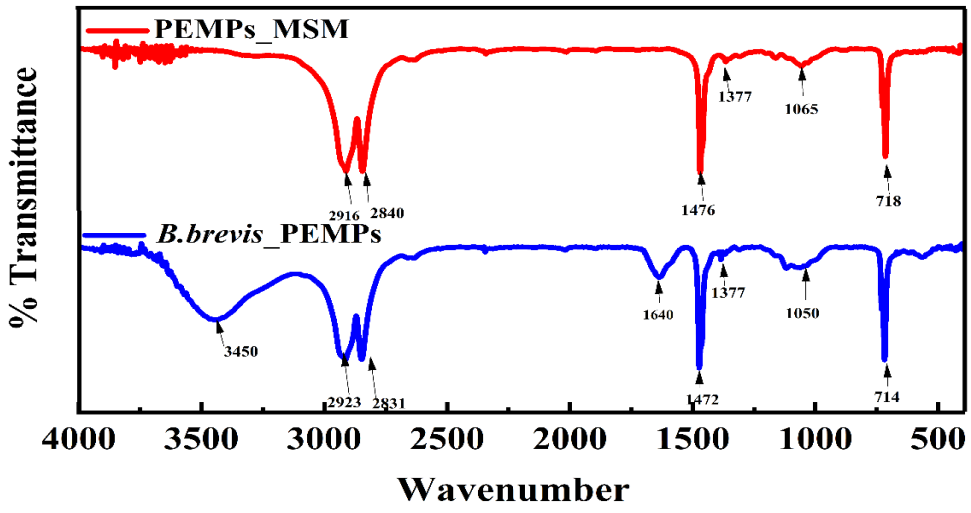


Fig. 17. FTIR spectra of *B.brevis*_PEMPs and PEMPs_MSM (PEMPs were washed with MilliQ water and dried below 100 ºC)

3.3.6.2 DSC analysis

The DSC thermograms of *B.brevis* treated and untreated microplastics indicate the difference between characteristics of the sample, Fig. 18(a). Untreated PEMPs (Control/PEMPs_MSM) show a single melting step around 139 °C. Besides this significant melting step of control sample, the treated sample (*B.brevis*_PEMPs) showed an additional step around 109 °C, which is due to the plastic degradation. The corresponding enthalpies of melting in the DSC thermograms have been noticed to change from ΔH= 77.07 J/g (Control, PE) to ΔH= 82.98 and 66.96 J/g treated sample (*B.brevis*_PEMPs). A study conducted the degradation of polyethylene waste by bacterial colonization, observed the emergence of the additional peak in the treated polyethylene which is attributed to the degradation process that resulted in chain scission, which further contributed to the decrease in the melting point (Puglisi et al., 2019). Similar results were observed in the thermogram of degraded polyethylene by *A.niger* (Volke-Sepúlveda et al., 2002). The

result depicts the degradation is due to the bio-oxidation, which causes the reduction in the size of polymer chain and form oxidized groups such as carboxyl, carbonyl and hydroxyl, on the surface of the amorphous polyethylene units (Othman et al., 2021; Raghavan and Torma, 1992).

3.3.6.3 Thermogravimetric analysis of PEMPs

Thermogravimetric analysis represents the thermal stability of PEMPs which were characterized by using TGA, as shown in Fig. 18 (b). For the degraded particles, the temperature at which 9% weight loss of *B.brevis*_PEMPs occurred, was the temperature of 115 °C whereas the control loses 3% at the same temperature. 50% weight loss was observed at 460 °C in treated PEMPs whereas 50% weight loss at 468 °C in Control. Upon increasing the temperature to more than 495 °C, the control completely lost weight. *B.brevis*_PEMPs still maintained its weight to some extent regardless of incubation time indicating the existence of small molecules due to microbial degradation. Similar results were also observed in the PEMPs degradation by *Bacillus sp*. and *Paenibacillus sp*..(Park and Kim, 2019). The observation concludes few changes in the structure changes such as reduction in the molecular weight and loosening of intermolecular cohesive forces of the PEMPs is due to the bacterial mediated degradation.

3.3.6.4 XRD analysis

Crystallinity is an important factor to predict the degree of biodeterioration of the polymer, which is measured with the help of DSC. The amorphous region of the polymer is easy to degrade by microorganisms, which results in an initial increase in crystallinity of the polymer (Puglisi et al., 2019). The degree of crystallinity of PEMPs_MSM and *B.brevis*_PEMPs were analyzed using XRD (Fig. 18 (c). The XRD pattern of PEMPs_MSM, showed prominent diffraction maxima at 21.48º and 23.98º of the angular

position 2θ, which indicate the crystalline structure of PEMPs before and after microbial degradation (Jeon et al., 2021). The crystallinity index of PEMPs_MSM (control) was calculated to be 27%, while that of *B.brevis*_PEMPs was found to be 37%. The increase in crystallinity can be attributed to the initial microbial attack on the susceptible amorphous region of the polymer (Jeon et al., 2021; Restrepo-Flórez et al., 2014; Santo et al., 2013). This observation is consistent with previous reports in the literature for low-density polyethylene (LDPE) and polypropylene (PP), by bacterial degradation (Jeon et al., 2021). Microbes consume the easily accessible amorphous portion of HDPE, which significantly increases the crystallinity index, leading to the breakdown of the larger plastic into smaller fragments (Yuan and Xu, 2023). The changes observed in the PEMPs post degradation through DSC, TGA and XRD analysis confirm the structural changes of PEMPs due to *B.brevis* degradation.

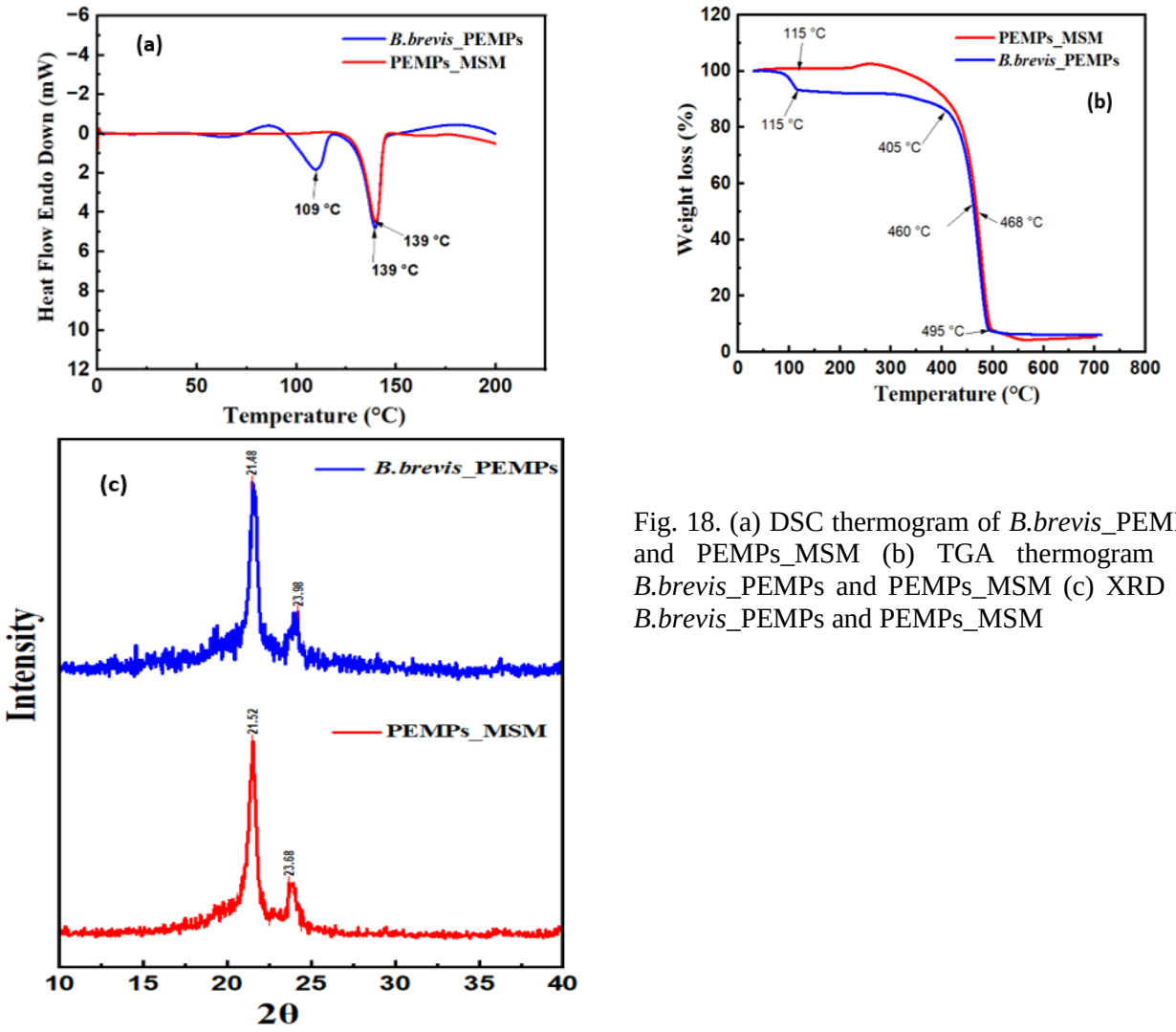


Fig. 18. (a) DSC thermogram of *B.brevis*_PEMPs and PEMPs_MSM (b) TGA thermogram of *B.brevis*_PEMPs and PEMPs_MSM (c) XRD of *B.brevis*_PEMPs and PEMPs_MSM

3.3.7 GC-MS of the metabolite

The biodegradation of PEMPs with *B.brevis* was observed in a 100 mL flask of MSM medium kept at regular shaking for 35 d at 37 ºC and filtered. After filtration the metabolite was subjected to the GC-MS analysis. During the biodegradation of PEMPs, alkanes and aromatic compounds are formed by depolymerization through enzymatic hydrolysis, such as laccase and release of organic acids which are then consumed by *B.brevis* via β-oxidation and TCA cycle metabolism (Ji et al., 2013). In GC–MS analysis of the metabolite revealed various aromatic and hydrocarbon compounds as listed in Table 5. This observation is in good agreement with the results observed by (Jeon et al., 2021; Muhonja

et al., 2018; Shahnawaz et al., 2016) in the biodegradation of PEMPs. Further, the obtained results confirm the biodegradation of PEMPs into simpler compounds. The release of degraded compounds is likely due to the action of the laccase enzyme (a multi-copper polyphenol oxidoreductase laccase) on PEMPs from the *B.brevis.* The role of copper binding enzyme laccase is well studied and shows the higher degradation rate in the presence of enzyme. As previously reported, by Santo et al., the *Rhodococcus ruber* strain is capable in the biodegradation of polyethylene through enzymatic hydrolysis (Santo et al., 2013).

Another study revealed several homologous gene sequences identified as copper-binding oxidases from *Lysinibacillus sp.* JJY0126 by blast search (NCBI) estimated to have an ability to degrade, the polyethylene, by oxidizing CH_2 bonds (Jeon et al., 2021).

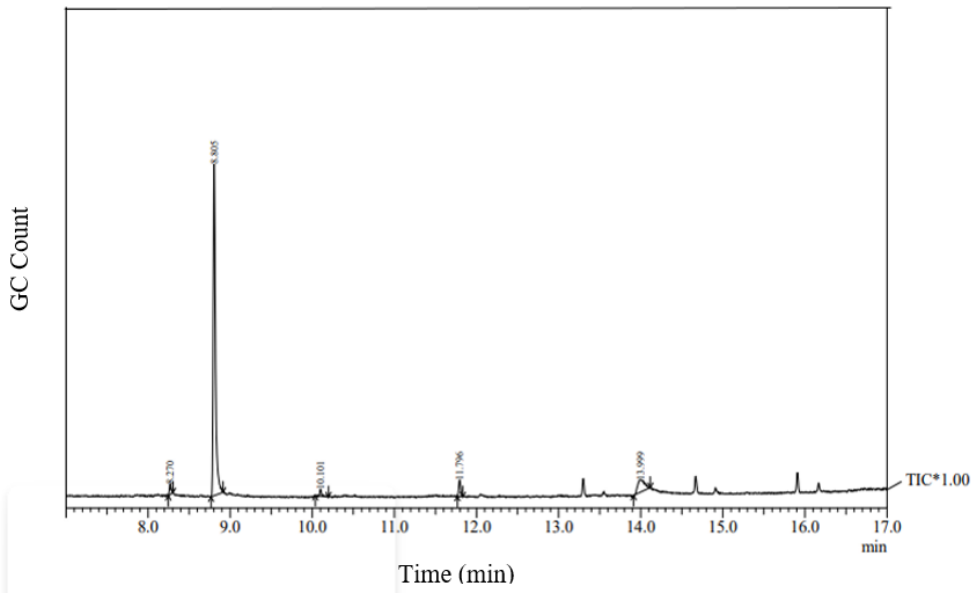


Fig. 19. Gas chromatography-mass spectrometry chromatogram of metabolite (*B.brevis*_PEMPs) after 35 d of incubation

Table 5. List of compounds released after biodegradation of PEMPs

Peak	Retention Time	Area	Area %	Chemical compound name
1	8.270	50296	2.13	5 (bis(1, 1-dimethyl ethyl)-4 hydroxy-)
2	8.805	1970106	83.16	4 Di-tert butylphenol
3	10.101	31543	1.34	Benzaldehyde 2, 5-dimethyl
4	11.796	80444	3.41	Dodecane
5	13.999	223975	9.51	2-hexadecanone

The proposed mechanism of *B.brevis*-mediated degradation of PEMPs represents a promising approach towards depolymerizing the polymer chain into small molecules, which can be further mineralized into carbon dioxide, water, and new biomass compounds. Based on the experimental observations, a hypothetical mechanism of PEMPs degradation is proposed (Fig. 20). PEMPs is a semi-crystalline polymer with both crystalline and amorphous phases (Restrepo-Flórez et al., 2014). It is evidenced by XRD analysis (Fig. 18(c)) the amorphous region is susceptible to microbial degradation and increases the crystallinity by 10%. The release of laccase enzyme by *B.brevis* (an oxidoreductase enzyme) is responsible for the breakage of carbon-carbon bonds and oxidation of terminal ends of hydrocarbon (Guengerich and Yoshimoto, 2018; Santo et al., 2013). Bio-fragmentation is a process by which lytic cleavage of complex polymers occurs, breaking them down into simpler forms through enzymes(Tiwari et al., 2020). Through GC-MS analysis, the C-C bond breakage was identified to release of dodecane as one of the fragments. Bio-transformation is a biochemical modification with enzyme-catalyzed reactions or whole-cell catalysis, through which one chemical compound is transformed into another that may be less or more toxic (Albertsson et al., 1987; Kaur and Gosal, 2021). Similarly, the release of 4 di-tert butylphenol, dodecane, benzaldehyde 2,5-dimethyl, and 2-hexadecanone was observed due to the biodegradation of PEMPs through GC-MS analysis. Additionally, terminal oxidation caused the addition of a carboxylic acid group to the polymer chain (Santo et al., 2013). Co-enzyme A is a crucial enzyme necessary for the

oxidation of fatty acids and the citric acid cycle (Albertsson et al., 1995). During the beta-oxidation of an acyl-CoA molecule, it cleaves at the β-carbon atom and forms a molecule that is two carbons shorter than it was at the beginning of the process[9] . Further, the molecule is taken for the Tricarboxylic acid cycle , which provides energy for cellular metabolism (Albertsson et al., 1995, 1987; Restrepo-Flórez et al., 2014) .

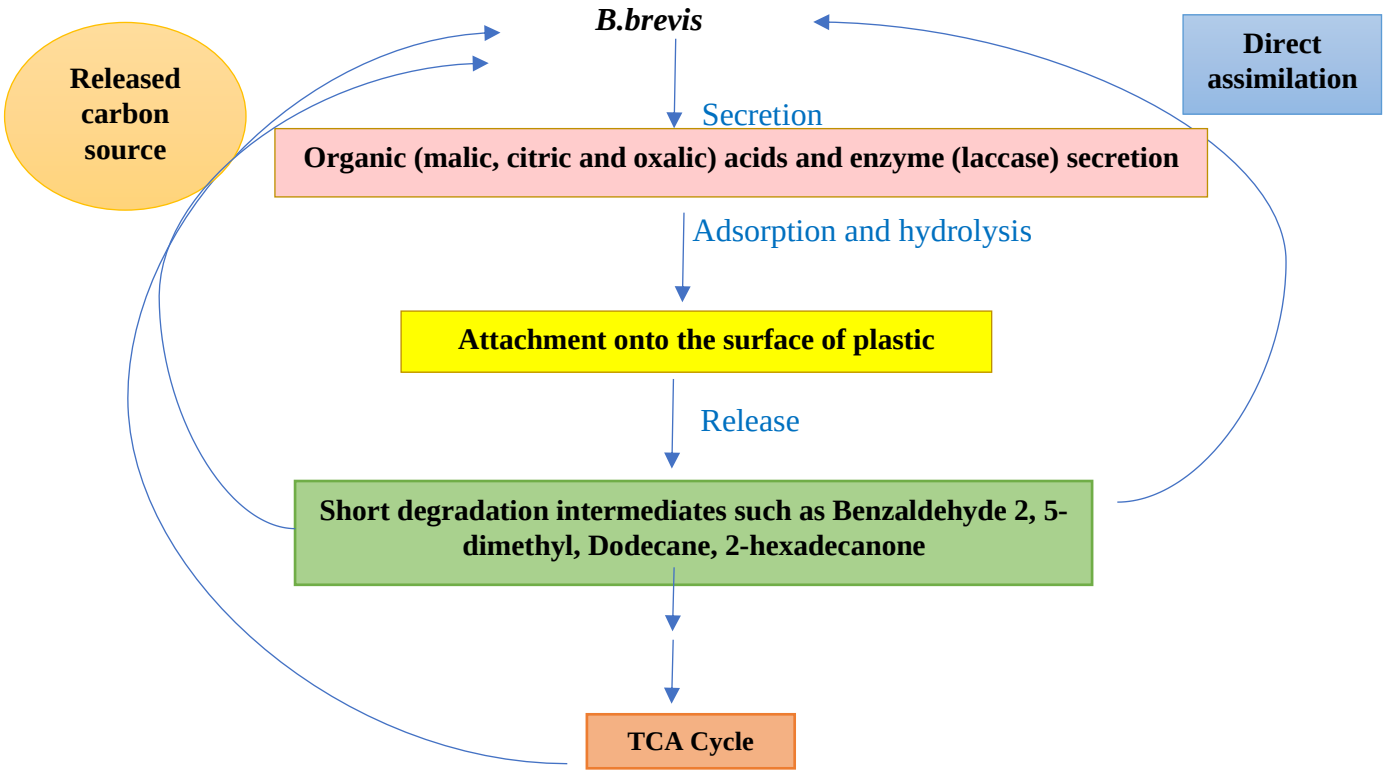


Fig. 20. The hypothetical mechanism of PEMPs degradation by *B.brevis*.

3.3.8 Conclusion

In recent years, extensive research has been conducted to identify bacteria capable of degrading plastics. This study focuses on the microbe-assisted degradation analysis of polyethylene microplastics (PEMPs) by *B.brevis*, a promising strain that exhibits the ability to degrade microplastics without requiring pre-treatment processes. The degradation

process was found to involve the presence of specific enzymes that played crucial roles in the breakdown of PEMPs. Moreover, the growth of *B.brevis* in the presence of microplastics led to notable physical and chemical changes in the microplastic structure. These changes were confirmed through various analytical techniques, including FTIR, TGA, DSC, XRD, and GC-MS. FTIR analysis revealed the formation of new functional group peaks resulting from the bio-oxidation of PEMPs during degradation. This bio-oxidation process led to the fragmentation of PEMPs into shorter polymer chains, as evidenced by GC-MS analysis. Furthermore, an increase in crystallinity indicated the utilization of the amorphous regions of the polymer as a carbon source for the nutritional requirements of *B.brevis*. SEM images provided visual evidence of microbial attachment onto the polymer surface, suggesting that *B.brevis* specifically targets polyethylene for its carbon source, essential for its survival. The observed reduction in weight and the presence of holes in the PEMPs further indicated that microplastics serve as a nutritional (carbon) source for *B.brevis*, providing energy for its metabolic activities. Collectively, these findings confirm the successful biodegradation of PEMPs by *B.brevis.* Although the biodegradation process has not yet reached a large or commercial scale, it has the potential to significantly reduce environmental plastic waste pollution. Finding or developing microorganisms that can efficiently break down untreated plastic and degrade it quickly with great efficiency is important to attain an industrial scale. These observations on bacterial degradation of PEMPs can also serve as a foundation for future research in areas like establishing the degradation mechanisms of a specific enzyme that breaks down polyethylene. Further to identify the degradation products of the plastics, as well as investigating the economics of plastic biodegradation.

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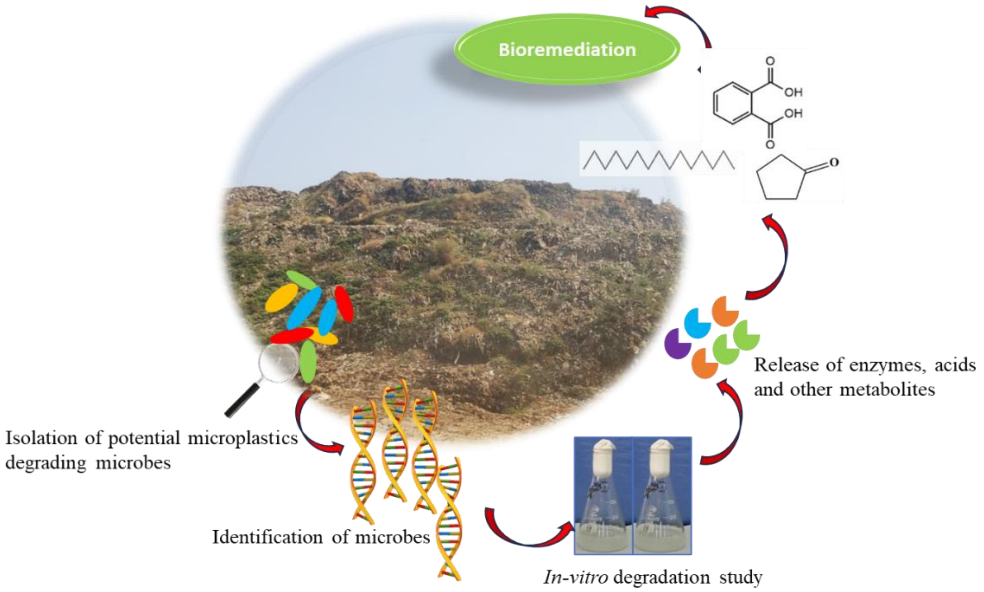
Chapter 4

Significance of landfill microbial communities in biodegradation of polyethylene and nylon 6,6 microplastics



Chapter 4

Significance of landfill microbial communities in biodegradation of polyethylene and nylon 6,6 microplastics

4.1 Introduction

Increasing global waste production anticipated that the production of municipal solid waste would reach 3.4 billion metric tons by 2050 in worldwide, representing 70% over growth (Hossain et al., 2022;). Between 1950 and 2015, the cumulative production of plastics, synthetic fibers, and additives amounted to 8.3 billion metric tons, of which 30% of primary plastics were still in use (Geyer et al., 2017). According to Geyer et al., approximately 55% of total plastic waste ended up in landfills, while 8% was incinerated, and only 9% was recycled (Geyer et al., 2017). Furthermore, waste is often disposed of in hazardous open dumpsites, particularly in developing nations (Kaza et al., 2018). In 2019, nearly half of the global plastic waste was landfilled, with an estimated 82 million metric tons being mismanaged and littered. The unregulated landfills of Asia, where the majority of plastic waste is disposed lead to significant contamination of groundwater. India, for instance, generates 15 million metric tons of plastic waste annually, of which only one-quarter undergoes recycling (Rafey and Siddiqui, 2021). Compared to other countries such as China (10%), Japan (12%), Europe (7%), the USA (10%), and South Africa (16%), India's recycling rate for plastic waste is relatively higher, at approximately 60% (Hossain et al., 2022; Rafey and Siddiqui, 2021).

Mismanaged plastic waste frequently finds its way into rivers, oceans, and other water bodies, causing severe damage to marine life and ecosystems (Ritchie and Roser, 2018). Consequently, investigation on the origin, distribution, fate, and environmental implications of plastics and microplastics as emerging environmental contaminants has significantly expanded in recent years (Tiwari et al., 2020). Microplastics (< 5 mm)

originate from various sources, being generated through the fragmentation of larger plastic debris due to weathering and degradation (known as primary microplastics) or being present as microbeads (referred to as secondary microplastics) (Gigault et al., 2018). The most common types of microplastics contributing to environmental pollution are polystyrene (PS), polyethylene (PE), polypropylene (PP), nylon 6,6, and polyethylene terephthalate (PET) (Urbanek et al., 2018)

The backbone structures of HDPE and nylon 6,6 are considered to be resistant to any degradation due to their hydrophobicity, inertness and crystallinity. Moreover, the degradation rate of polymers is primarily influenced by key factors such as chain length, mechanical properties, and molecular weight distribution (Restrepo-Flórez et al., 2014). However, in addressing the challenges of plastic removal, both abiotic and biotic mediated degradation methods have been explored as potential results. In abiotic degradation, chemical and mechanical methods of plastic degradation have shown non-specific of remediation and also the release of harmful byproducts which further causes environmental damage (Chamas et al., 2020). Recently, there have been studies of identifying microorganisms with the abilities of consuming plastics as their carbon and energy sources. Bioremediation holds significant promise in addressing the challenge of microplastics contamination, through the biological processes involved in microplastic degradation may require substantial time.

The diversity of microorganisms capable of degrading plastics remains relatively restricted, but advancements in sensitive isolation and characterization techniques, particularly those based on rDNA sequencing and metagenomics approaches, hold the promise of expanding the current knowledge. Traditional microbiology methods are limited in detecting the non-culturable microorganisms, which can comprise as much as 90% of the true biodiversity in an ecosystem (Hugenholtz et al., 1998). However, with

the advent of rDNA sequencing and metagenomics, researchers can now gain insights into the vast and previously hidden potential of microorganisms involved in plastic degradation, thus paving the way for future breakthroughs in biodegradation strategies (Restrepo-Flórez et al., 2014). Isolated *Lysinibacillus sp. JJY0216* from a grove's soil, capable of degrading LDPE by 7.1% and polypropylene by 3.1% in 28 days (Jeon et al., 2021). *Aspergillus sp.* and *Lysinibacillus sp*. from landfill soils reduced UV-irradiated LDPE weight by 29.5% and non-UV-irradiated by 15.8% in 128 days. Moreover, *Achromobacter denitrificans Ebl13* demonstrated 12.3% and 6.5% degradation of PVC and LDPE, respectively in 6 weeks (Maleki Rad et al., 2022). Microbes isolated from municipal landfill site (*Bacillus sp. And Paenibacillus sp*.) thrived in a non-carbonaceous nutrient medium and became dominant species upon exposure to PE microplastics. These isolated bacterial strains showed 14.7% degradation of PE microplastics in 60 days. (Park and Kim, 2019). Marine bacteria such as *Bacillus cereus, Bacillus sphericus*, *Vibrio furnisii*, and *Brevundimonas vesicularis* were shown to contribute to the degradation of nylon 6, 6, resulting in weight loss and a decrease in average molecular weight in three months(Sudhakar et al., 2007). The degradation of plastics such as HDPE and nylon 6,6 and the identification of microorganisms capable of degrading these microplastics have been areas with limited research available till today. However, recent advancements in rDNA sequencing methods have facilitated the discovery of numerous new microbial species with potential biodegradation capabilities of such inert plastics.

The current investigation was conducted to evaluate the biodegradation of nylon 6,6 and polyethylene (PE) microplastics, ranging in size, 200 nm and 45 µm . These microplastics were exposed to a bacterial strain isolated from a municipal dumpsite located in the Bhalswa region of Delhi, where solid waste is disposed of in a landfill. To our current understanding, this study represents the first attempt to isolate and characterize a bacterial

consortium with the capacity to degrade nylon 6,6 microplastics and PE microplastics, employing metagenomics techniques. In addition, a range of analytical instruments, including a Scanning electron microscope (SEM), Transmission electron microscope (TEM), Fourier transform infrared (FTIR) spectrometer, gas chromatography-mass spectrometer (GC-MS), X-ray diffraction (XRD), differential scanning calorimetry (DSC), and thermogravimetric analyzer (TGA), were also employed to compare the attributes of the degraded microplastics with the control samples. The enzymes participated in the degradation of microplastics were revealed through enzyme activity analysis. Further, the degraded compounds from plastics were identified through GC-MS analysis.

4.2 Materials and Method

4.2.1 Microplastics

Nylon 6, 6 microplastics (NMPs) was synthesized using a method defined by Crespy et al. (Crespy and Landfester, 2007) with slight modifications detailed in (Tiwari et al., 2022). The PE microplastics used in this study were composed of ultra-high molecular weight with a particle size of 42-48 µm. All supplementary reagents employed in this investigation were of analytical grade. Prior to the degradation study, the microplastics underwent sterilization by submersion in 70% ethanol within a glass Petri dish for a duration of 15 minutes. Subsequently, they were dried for 12 h in a hot air oven set at 50 °C.

4.2.2 Soil Sample Collection

Soil sediment samples were taken from a landfill site, located in Bhalswa, Delhi (Site A 28°44'25"N, 77°9'6"E; Site B 28°44'30"N, 77°9'12"E;), as shown in Fig. 1, where plastic wastes had previously been dumped. They were taken from different depths at 1 and 3 m below the landfill cover soil from which 1 kg of sediment sample was then contained in

amber bottles without headspace and transferred to a laboratory in ice-packed storage(Park and Kim, 2019) .

4.2.3 Microplastic degrading bacteria isolation and identification

The bacterial cultures were prepared by agitating 4 g of sediment samples in an isotonic saline solution for 3 h followed by settling for 40 min, after which the subsequent supernatant was inoculated in LB broth medium. Next, they were cultivated at 30 °C in a rotating incubator (140 rpm) for 48 hrs. Fifteen single colonies were obtained from bacterial cultures using the pour plate technique onto LB agar plates. Subsequently, the microplastic-degrading bacteria were screened among these bacterial strains. The screening process involved assessing the bacterial isolates' ability to utilize PEMPs and NMPs-as the only carbon source. To accomplish this, bacterial consortium was cultivated in Minimal Salt Medium (MSM) supplemented with, 0.25% $MgSO_4$, 0.025% $CaCl_2$, 1% KH_2PO_4, 2.3% K_2HPO_4, 1% NH_4Cl, 0.05 g $FeCl_3$, and 0.5% NaCl with 1 mL of trace element solution (20 mg L^{-1} $CoCl_2.6H_2O$, 18 mg L^{-1} $NiCl_2.6H_2O$, 24 mg L^{-1} $CuSO_4.5H_2O$, 0.5 g L^{-1} $CaCl_2$, 1.62 g L^{-1} $FeCl_3.6H_2O$) per liter of distilled water. The medium was further amended with 0.5 % (w/v) sterilized microplastics consisting of PEMPs and NMPs. The incubation of the microorganisms was done aerobically at 30 °C for 15 d. Following incubation, the cultures were streaked onto LB agar plates and incubated for 24 hours at 30 °C. Subsequently, a single colony from each plate was selected and subjected to the same incubation conditions as described earlier. The identification of the microorganisms was performed by 16S rDNA and Illumina sequencing methods (Park and Kim, 2019; Puglisi et al., 2019a).

16 S rDNA Sequencing for pure strain: Isolation of the genomic DNA from each of the single colonies was performed using a HiGeneTM Genomic DNA Prep Kit 156 (BIOFACT, Korea). The bacterial 16S rDNA was amplified using the primer sets of 27F

(5`- 157 AGA GTT TGA TCM TGG CTC AG-3`) and 1492R (5`-TAC GGY TAC CTT GTT ACG 158 ACT T-3`). Amplifications were carried out using a thermal cycler instrument (Proflex PCR systems, thermofisher Scientific, Germany) in a final volume of 25 µL, with each containing 2 × Taq Reaction Buffer, 1 mM of dNTPs, 1 mM of each primer, 1.5 U of Taq polymerase, and 3 µL of template DNA. The PCR conditions were 1 cycle (95 °C for 14 min) for initial denaturation, 30 cycles 163 (95 °C for 30 s, 50 °C for 40 s, and 72 °C for 5 min) for denaturation, annealing, and extension, and 1 cycle (72 °C for 5 min) for the final extension of the amplified DNA. Next, the PCR products were purified and sequenced with forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. A similarity search was conducted from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7 (Auta et al., 2017).

Bacterial communities' molecular identification of soil sample and isolated microbes. Microbial DNA extraction was carried out from 0.5 mL of microbial culture using the Nucleospin soil kit. The quality of isolated metagenomic DNA sample was specific region were synthesized and amplification of bacterial 16S region was carried out (Puglisi et al., 2019a). The primer pairs used in the study were 16S rRNA F and 16S rRNA R. 3 µL of PCR product was resolved on 1.2% agarose gel at 120V for60 min. Further, the amplicons with the illumine adapter were amplified using i5 and i7 primers that a dd multiplexing index sequences and common adapters required for cluster generation (P5 and P7) as per standard Illumina protocol. The amplicons libraries were purified by AMPure XP beads

and quantified using Qubit Fluorometer. The amplified library was analyzed on 4200 Tape station system (Agilent Technologies) using D1000 Screen tape. After obtaining the mean peak size from Tape station profile, libraries were loaded onto MiSeq at (10-20pM) concentration for cluster generation and sequencing. QIIME is comprehensive software comprising of tools such as FastTree for heuristic based maximum-likelihood phylogeny interference (Estaki et al., 2020; Raiyani and Singh, 2020), the RDP classifier using naïve Bayesian classifier (Lan et al., 2012). High quality clean reads were obtained using Trimmomatic v0.38 to remove adapter sequences, ambiguous reads and low-quality sequences along with sliding window of 10 bp and a minimum length of 100 bp. Further, stitching the PE data into single ends reads and picking Operational Taxomonic Units (OTUs) based on sequence similarity within the reads, and picks a representative sequence for 16S bacteria from each OTUs against Greengrenes database (version 13_8). Moreover, assinging the OTUs a taxonomic identity using a reference databases the diversity metrics for each sample were calculated (Puglisi et al., 2019a).

4.2.4 Microbial inoculum preparation and *in vitro* degradation assay

In the present study, a degradation assay was performed using MSM composition of0.25% $MgSO_4$, 0.025% $CaCl_2$, 1% KH_2PO_4, 2.3% K_2HPO_4, 1% NH_4Cl, 0.05 g $FeCl_3$, and 0.5% NaCl with 1 mL of trace element solution (20 mg L^{-1} $CoCl_2.6H_2O$, 18 mg L^{-1} $NiCl_2.6H_2O$, 24 mg L^{-1} $CuSO_4.5H_2O$, 0.5 g L^{-1} $CaCl_2$, 1.62 g L^{-1} $FeCl_3.6H_2O$) per liter of distilled water, supplemented with 0.5% (w/v) PEMPs and NMPs microplastics. Bacterial growth was initiated by inoculating the microorganisms into nutrient broth and allowing them to reach the stationary phase in a rotating shaker at 30 ºC and 140 rpm. The growth progress was monitored by measuring the absorbance at 600 nm using an Eppendorf UV-vis Spectrophotometer (Bio Spectrometer basic model). For the biodegradation study, the third-generation cultures, exhibiting a log phase with an absorbance of 0.8, were selected.

The culture was transferred to MSM broth with microplastics as a sole carbon source and served as the inoculum for the *in vitro* degradation experiments. Subsequently, the cultures were inoculated into 200 mL of MSM broth supplemented with PEMPs and NMPs, and assigned as PEMPs_MSM (PE microplastics with minimal glucose), PEMPs_B1 (PE microplastic with *Achromobacter xylosoxidans)*, PEMPs_B2 (PE microplastic with mixed bacterial *sp.*), MSM_B1 (*Achromobacter xylosoxidans* with minimal salt medium), MSM_B2 (mixed bacterial *sp.* with minimal salt medium), NMPs_MSM (Nylon 6,6 with minimal glucose), NMPs_B1 (Nylon 6,6 microplastic with *Achromobacter xylosoxidans*), and NMPs_B2 (Nylon 6,6 microplastic with mixed bacterial *sp.*). Over a period of 40 d, the pH, optical density (OD), release of organic acids, and enzyme activity (total, peroxidase, and laccase) were monitored at 5-day intervals.

4.2.5 Cell-surface hydrophobicity measurement

Cell-surface hydrophobicity of both cell cultures was measured by following the bacterial adhesion to the hydrocarbon (BATH) test as previously described by (Rosenberg et al., 1980). The bacterial cultures in the logarithmic growth phase were collected through centrifugation at 7000 g for 12 minutes. The resulting cell pellets underwent three rounds of washing before being re-suspended in a phosphate urea magnesium sulfate (PUM) buffer (22·2 g $K_2HPO_4 \cdot 3H_2O$; 7·26 g KH_2PO_4; 1·80 g Urea; 0·20 g, $MgSO_4$, pH: 7·2) to OD600 of 1·0 ± 0·05. 3 mL of this cell suspension was transferred to a glass test tube, and then 1 mL of hydrocarbon was added. Four types of hydrocarbons, namely Toluene (a monopolar aromatic solvent), Xylene (an apolar aromatic solvent), Chloroform (a monopolar aliphatic solvent), and n-Hexane (an apolar aliphatic solvent), were subjected to testing. Test Tubes were incubated at 37 °C for 12 min for temperature equilibrium, followed by vortex for 20 s. Cell suspensions were kept undisturbed at 30 °C for 20 min for phase separation (Arkatkar et al., 2010). Lower aqueous phase was collected carefully

and OD600 was recorded. Percentage of Cell-surface hydrophobicity of the organism was measured using the following equation:

$$Cell\ surface\ hydrophobicity\ (\%) = \frac{(initial\ OD - Final\ OD)}{initial\ OD} * 100$$

4.2.6 Dry weight calculation of residual PEMPs and NMPs

After 40 d of incubation, the PEMPs and NMPs were recovered from the broth through filtration (0.22 µm polytetrafluorethylene (PTFE) Millex filters). Plastic particles were washed with 70% ethanol and dried in a hot air oven at 60 ºC for 12 h. Residual polymer weight was determined to measure the extent of degradation (Auta et al., 2017; Mohan et al., 2016; Mor and Sivan, 2008). The weights of the pre-incubated microplastic were measured. The plastic polymer degradation was evaluated in terms of percentage weight loss using the following formula (Auta et al., 2018):

$$\%\ weight\ loss = \frac{(initial\ weight\ of\ polymer - Final\ weight\ of\ polymer)}{initial\ weight\ of\ polymer} * 100$$

To calculate the rate constant and half-life the following formula was used (Auta et al., 2018):

$$\text{Rate Constant K} = -\frac{1}{t}(\ln\frac{W}{W0})$$

where K is the rate constant for polymer uptake, t is the time in days, W is the weight of residual polymer (g), and W_O is the initial weight of polymer (g).

$$\text{Half-life } (t_{1/2}) = \ln(2)/K$$

Where ln is the natural logarithm, and K is the Removal Constant.

4.2.7 Ultra Performance High-Performance Liquid Chromatography (UP-HPLC)

Organic acids were calculated (malic acid, citric acid and oxalic acid) from the bacterial cultures after every 5 d of treatment with microplastics. 1 mL of the cultures were extracted

separately and filtered through 0.22 µm polytetrafluorethylene (PTFE) Millex filters to obtain cell free metabolites. Presence of organic acids were analyzed using UP-HPLC equipped with photodiode array detection (PDA) detector at wavelength 210 nm. Methanol and potassium dihydrogen phosphate (10 mM, pH 2.8) were mixed at a ratio of 10:90 to act as the mobile phase (flow rate at 1 mL/ min)(Han et al., 2019).

4.2.8 Total enzyme activity

Hydrolase activity assessment was conducted through the hydrolytic cleavage of fluorescein diacetate (FDA) to fluorescein. Each culture medium (2 mL) was incubated with phosphate buffer (pH = 7.4, 20 mL) and FDA stock solution (0.2 mL, 1000 µg mL^{-1}) at 30 °C for 40 min. in a water bath shaker operating at 150 rpm. Three distinct conditions were considered: culture only (without FDA), FDA only (without the sample), and culture with FDA.

Following the incubation period, a 2:1 chloroform/methanol solution (20 mL) was added to each sample, and the mixture was centrifuged at 6000 rpm for 7 min. The fluorescence intensity of the resulting supernatant was measured at 490 nm using an Eppendorf UV-vis Spectrophotometer (Bio Spectrometer basic model). For comprehensive analysis, corresponding positive and negative controls were also included in the experimental setup (Adam and Duncan, 2001; Schnürer and Rosswall, 1982).

4.2.9 Laccase enzyme activity

Laccase enzyme activity in the bacterial culture media was evaluated by employing ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) as the substrate. In a 100 µL volume of the culture media, 100 µL of 10 mM ABTS was combined with 800 µL of 50 mM sodium acetate buffer (pH 5). This mixture was then incubated at 30 °C for 5 minutes. To determine ABTS oxidation, the appearance of a green color was assessed at A420 using an Eppendorf UV-vis Spectrophotometer (Bio Spectrometer basic model) (with an

extinction coefficient, ε, of 36000 $Lmol^{-1}cm^{-1}$) (Arkatkar et al., 2010). The secretion of laccase enzymes was monitored at various time points, spanning from day 5 to day 40 .For quantification, the amount of enzyme required to oxidize 1 mol of ABTS per minute at 30 °C was defined as one unit of laccase activity (More et al., 2011).

4.2.10 Peroxidase enzyme activity

Peroxidase enzyme activity was assessed in the culture media using pyrogallol as the substrate. A reaction mixture of 3 mL was prepared, consisting of 2.1 mL of Ultra-pure water, 0.32 mL of phosphate buffer, 0.16 mL of peroxide solution, 0.32 mL of pyrogallol solution, and 0.1 mL of culture media (Sowmya et al., 2015).The resulting solution was thoroughly mixed, and the formation of a brown color was measured spectrophotometrically at A420 (with an extinction coefficient, ε, of 36000 $Lmol^{-1}\cdot cm^{-1}$). For quantification, one unit of peroxidase activity was defined as the amount of enzyme required to produce 1.0 milligram of purpurogallin from pyrogallol in 20 seconds, under standard conditions of pH 6.0 and 20 °C. This measurement serves as a reliable indicator of the peroxidase enzyme's catalytic efficiency under the specified experimental conditions.

4.2.11 Gas Chromatography-Mass Spectrometry (GC-MS)

Following 40 d of continuous shaking, the metabolites were extracted from the bacterial culture, and the PEMPs and NMPs were removed via filtration. The resulting liquid underwent centrifugation at 13,000 rpm for 10 minutes to remove any remaining cell debris or microplastics. The supernatant was further filtered using Whatman filter paper (No. 2). To process the degraded products, diethyl ether was utilized as the solvent. Each filtrate was dissolved in an equal volume of diethyl ether (10 mL) using a separating funnel (Shahnawaz et al., 2016).

For analysis, a portion of 1 mL from the dissolved degraded products was subjected to Gas Chromatography-Mass Spectrometry (GC-MS) using an Rxi-5Sil MS Gas Chromatogram Mass Spectrometer . Helium was employed as the carrier gas at a flow rate of 1 mL/min. The GC-MS column dimensions were as follows: length 30 m, inner diameter 0.25 mm, and a film thickness of 0.25 μm. Data analysis, peak integration, and quantification were performed using GC-MS real-time analysis software. The identification of major peaks was achieved through a NIST-4 and Wiley library search, providing crucial insights into the composition and characteristics of the degraded products.

4.2.12 DSC

DSC was employed, utilizing the Perkin Elmer DSC 8000 instrument, to generate thermograms for both the control and all the treated sample materials. Each sample, weighing approximately 10 mg, was carefully positioned in aluminum crucibles. The heating process was conducted under a nitrogen atmosphere, with a controlled heating rate of 10 ºC per minute, spanning the temperature range from 10 ºC to 300 ºC (Raghavan and Torma, 1992; Sudhakar et al., 2007).

4.2.13 TGA

The TGA was performed on both the control and all the treated microplastics utilizing the Perkin Elmer TGA 4000 thermogravimetric analyzer. The analysis involved subjecting the samples to a controlled heating process, from room temperature and to upto 800 °C. The heating rate was set at 10 °C min^{-1}, and a constant nitrogen flow of 10 mL min^{-1} was maintained throughout the experiment (Tiwari et al., 2023).

4.2.14 FTIR spectroscopy

The experiment aimed to explore the structural changes in microplastics resulting from their interaction with bacterial cells. To achieve this, FTIR spectroscopy using the Perkin-

Elmer 400 FT-IR/FT-FIR instrument was employed, covering the frequency range of 4000 cm^{-1} to 400 cm^{-1}.

To prepare the microplastics that had undergone interaction with bacterial cells, a thorough washing process with milliQ water was performed to remove any adhered bacterial debris. Subsequently, the samples were dried at a temperature below 100 ºC. Sample pellets were prepared using KBr and the scanning resolution for the FTIR spectra was set to 4 cm^{-1} (Maleki Rad et al., 2022).

4.2.15 SEM

The investigation aimed to explore the impact of microplastic degradation on bacterial morphology over a 40-d incubation period. To achieve this, bacterial cells were subjected to analysis prior and subsequent after the incubation. SEM with the EVO18 Zeiss model from Germany was employed, operating at an accelerating voltage of 5 kV and a magnification of 10,000. Sputter-coating with a gold layer at 25 mA under an Ar atmosphere at 0.3 MPa was performed, followed by the examination of the specimen's using SEM (Park and Kim, 2019).

Before examination, the samples underwent preparation by subjecting the culture media to centrifugation and washing with Milli-Q water. Subsequently, the bacterial cells were fixed using Karnovsky's fixative. Finally, the prepared specimens were meticulously examined using SEM to study the alterations in bacterial morphology induced by microplastic degradation.

4.2.16 TEM

The examination of microplastic morphology was conducted using a TEM equipped with the Tecnai G2 200 KV HRTEM SEI HOLLAND system. Prior to analysis, the particles and bacterial cells were subjected to rigorous washing and confirming the removal of bacterial cells. Following the preparation, the specimens were meticulously examined

using the TEM, operational at an accelerating voltage of 200 kV and a magnification of 2550X.

4.2.17 Statistical Analysis

The experimental data underwent statistical analysis and graphical representation using Originpro2023 software. ANOVA was performed with SPSS software version 21.0, and the LSD post-hoc test at a significance level of P-value = 0.05 was applied. . All values were presented mean ± standard deviation, with triplicate samples (n = 3).

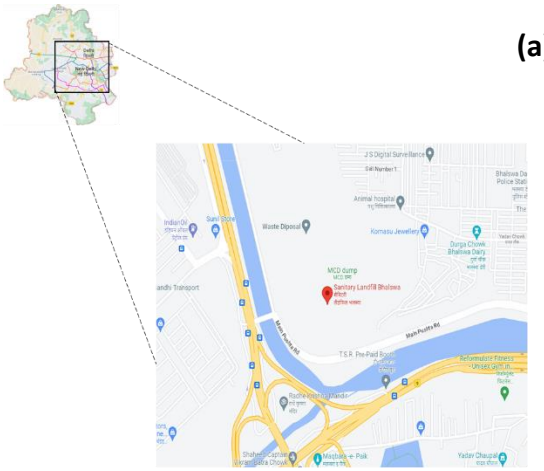
4.3 Results and Discussion

4.3.1 Isolation and identification of bacterial strains

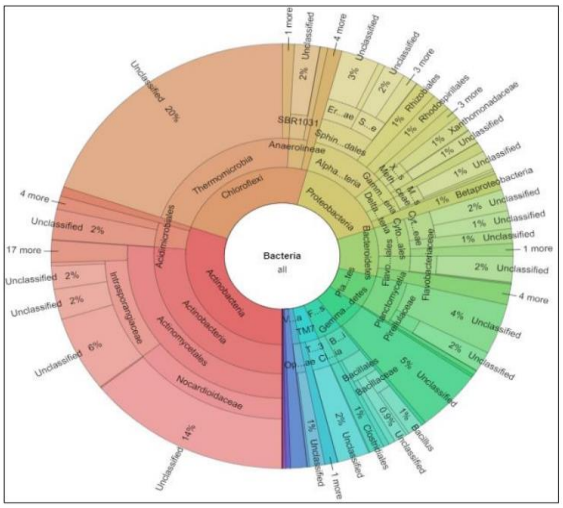
The soil sample taken from landfill site of Delhi region in India depicted in Fig. 21(a) where industrial and household waste materials were disposed. The soil sample was subjected for metagenomic sequencing for the identification of whole bacterial community present in the soil, represented in Fig 21(b) . The soil bacteria distribution at taxonomic phylum level depicted in Fig. 21(c). The phylum level bacterial diversity was analyzed through the high throughput 16S rRNA metagenomic approach. The distribution shows dominance of *Actinobacteria* (30.08%) in soil sample. The second most abundant bacteria was *Chloroflexi* (23.72%) and third one was *Proteobacteria* (15.65%). The soil was mixed in isotonic saline solution for 3 h followed by settling for 40 min and the resulted supernatant was inoculated in LB broth. Fifteen bacterial strains obtained from soil sample were screened for microplastics-degrading potential, specifically PEMPs and NMPs. The selectively isolated bacteria from the LB agar plates were then inoculated into a basal medium containing 0.5% (w/v) PEMPs and NMPs, respectively. Subsequently, all single colonies were picked and assessed for their capacity to degrade microplastics in the basal medium in the presence of microplastics. Out of 15

single colonies two colonies were identified to degrade PEMPs and NMPs on the basis of logarithmic growth in the presence of microplastics. These two different cultures were incubated for 15 d under aerobic conditions to determine their plastic degradation abilities. Further analysis through 16S rDNA gene sequencing revealed that one of the bacterial cultures was identified as *Achromobacter xylosoxidans*, based on nucleotide homology analysis. Additionally, for another strain, the 16S rDNA gene sequencing approach could not work out due to the presence of mixed strains. 16S rRNA Metagenomics approach (amplicon sequencing using Illumina MiSeq platform) identified the dominance of *Proteobacteria* genus (78.08%), specifically *Pulmonis sp.,* (fig. 21(e and f)), abundance which displayed significant PEMPs and NMPs degradation capabilities in the MSM medium.

Subsequent biodegradation experiments were performed using the two bacterial strains. The first strain, identified as *Achromobacter xylosoxidans,* was labeled as B1, while the second mixed strain, consisting of *Pulmonis sp.,* was labeled as B2. Both strains were tested for their microplastics degradation potential individually for 40 d, with B1 and B2 targeting PEMPs and NMPs, individually. The goal of this experiment was to understand and document characteristic changes that occurred in both bacterial strains (physiological and biochemical) and microplastics (physicochemical) during the degradation.



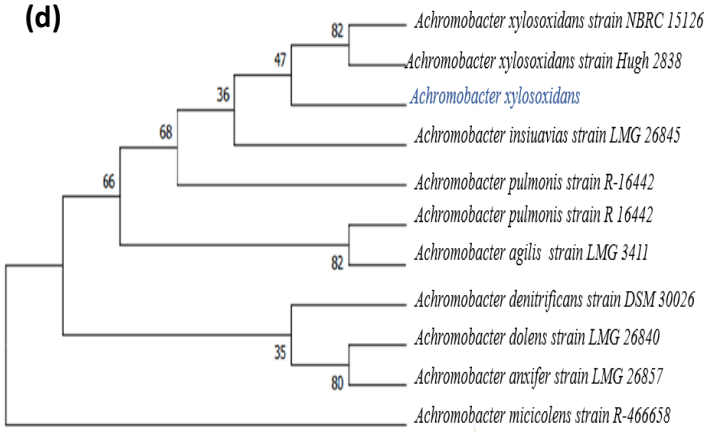
(a)



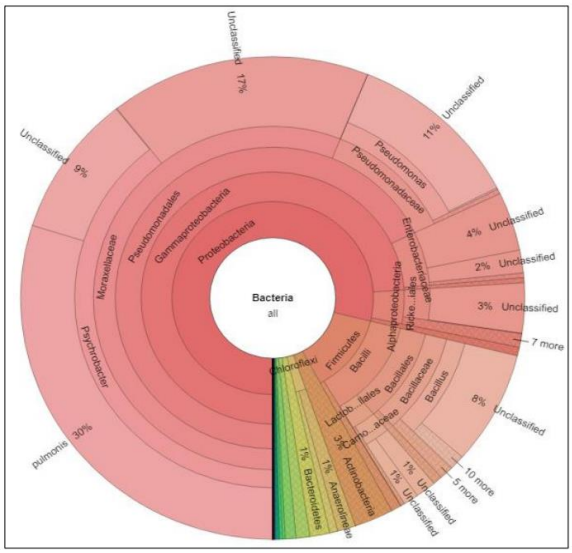
(b)

Legends	Taxonomy	Abundance
	k__Bacteria;p__Actinobacteria	30.08%
	k__Bacteria;p__Chloroflexi	23.72%
	k__Bacteria;p__Proteobacteria	15.65%
	k__Bacteria;p__Bacteroidetes	8.69%
	k__Bacteria;p__Planctomycetes	5.94%
	k__Bacteria;p__Gemmatimonadetes	4.94%
	k__Bacteria;p__Firmicutes	4.2%
	k__Bacteria;p__TM7	3.13%
	k__Bacteria;p__Verrucomicrobia	1.24%
	k__Bacteria;p__[Thermi]	1.12%
	k__Archaea;p__Euryarchaeota	0.69%
	k__Bacteria;p__Acidobacteria	0.33%
	k__Bacteria;p__Fibrobacteres	0.08%
	k__Bacteria;p__Chlorobi	0.08%
	k__Bacteria;p__BRC1	0.07%
	k__Archaea;p__Crenarchaeota	0.02%
	k__Bacteria;p__OD1	0.01%
	k__Bacteria;p__Nitrospirae	0.01%
	k__Bacteria;p__Spirochaetes	0.01%

(c)



(d)



(e)

Legends	Taxonomy	Abundance
	k__Bacteria;p__Proteobacteria	78.08%
	k__Bacteria;p__Firmicutes	13.18%
	k__Bacteria;p__Actinobacteria	2.48%
	k__Bacteria;p__Chloroflexi	1.67%
	k__Bacteria;p__Bacteroidetes	1.27%
	k__Bacteria;p__Planctomycetes	0.87%
	k__Bacteria;p__Acidobacteria	0.68%
	k__Archaea;p__Crenarchaeota	0.63%
	k__Archaea;p__Euryarchaeota	0.36%
	k__Bacteria;p__Verrucomicrobia	0.18%
	k__Bacteria;p__Nitrospirae	0.17%
	k__Bacteria;p__Chlorobi	0.07%
	k__Bacteria;p__Gemmatimonadetes	0.07%
	k__Bacteria;p__WS3	0.06%
	k__Bacteria;p__OP8	0.05%
	k__Bacteria;p__NC10	0.04%
	k__Bacteria;p__TM7	0.03%
	k__Bacteria;p__GAL15	0.02%
	k__Bacteria;p__Tenericutes	0.02%
	Others	0.1%

(f)

Fig. 21. (a) Soil sample collection point at landfill Bhalswa, Delhi, India (b) Krona chart of soil bacteria (c) Taxonomic distribution of soil bacteria at Phylum level (d) 16S rDNA based Molecular Phylogenetic analysis of *Achromobacter xylosoxidans* by Maximum likelihood method (e) Krona chart of plastic degrading consortium (f) Taxonomic distribution of plastic degrading consortium at Phylum level

4.3.2 Growth Curve

The isolated bacterial strains were adapted to MSM medium for three generations. Thereafter, optical density (OD) recorded at 600 nm to monitor the bacterial growth in the presence and absence of microplastics and results are displayed in Fig. 22 (a). In the absence of microplastics, the lag phase was observed from 0 d (0.08 OD) to 5 d (0.65 OD) for MSM_B1. Similarly, for MSM_B2, the lag phase was reported from 0 d (0.07 OD) to 5 d (1.1 OD). The bacterial growth exponentially increased over the period of 25 d, reaching 1.68 OD for MSM_B1 and declined to 1.55 OD after 30 d. The maximum lag phase was achieved at 2.35 OD after 30 d for MSM-B2 and further decline phase was observed after 35 d (1.95 OD). In the case of PEMPs_B1 and PEMPs_B2the lag phase was observed from 0 d to 5 d with more or less similar ODs. Further, the maximum exponential growth was observed at 30 d (2.35 OD and 2.48 OD) for PEMPs_B1 and PEMPs_B2 respectively. In contrast, 5 days delay in death phase was observed for PEMPs_B2 (1.55 OD at 40 d) compared to PEMPs_B1 (2.28 OD at 35 d). In the case of NMPs_B1 and NMPs_B2 the lag phase was observed from 0 d to 5 d with more or less similar OD. Further, the maximum exponential bacterial growth was observed after 25 d for NMPs_B1 (2.81 OD) and NMPs_B2 (2.61 OD). In both the cases, the death phase was observed after 35 d for NMPs_B1 (1.7 OD) and NMPs_B2 (1.2 OD). Interestingly, in the presence of microplastics, the stationary phase of bacterial growth exhibited an extended duration compared to conditions without microplastics. This indicates that although the growth of bacterial cells ceased in the presence of microplastics, the cells remained metabolically active (Tiwari et al., 2022). Notably, a significant decline in bacterial growth was observed between 35 d and 40 d when interacting with microplastics, implying cell death as a result of nutrient exhaustion and accumulation of

metabolites in the culture medium. In general, both B1 and B2 grow well in the presence of microplastics compare to control due to the availability of carbon source.

4.3.3 Cell surface hydrophobicity percentage

Cell hydrophobicity is a critical determinant in plastic degradation studies as it offers insights into bacterial cells' potential to adhere to and interact with plastic surfaces. Bacteria with higher hydrophobicity display increased adhesion to plastic surfaces, which facilitates the formation of biofilms and enhances plastic degradation through mechanisms like enzymatic breakdown or biofilm-mediated degradation (Arkatkar et al., 2010; Hadad et al., 2005). The microbial adhesion interaction involves a blend of electrostatic and short-range hydrophobic interactions between the surfaces. To understand the hydrophobic, hydrophilic, acidic, and basic nature of the cell surface components of B1 and B2, their affinity for solvents with different physicochemical properties (aliphatic/aromatic/apolar/polar/acidic/basic) was assessed (Fig. 22 (b)). The highest affinity of B1 and B2 strains towards hexane and chloroform (non-polar aliphatic hydrocarbon and monopolar aliphatic solvents, respectively) indicates the existence of low electron acceptor/acidic surface components on their cellular surface. On the other hand, xylene and toluene show low affinity for attachment of B1 and B2 strains. It is reported that production of higher carbohydrate and protein on the surface of Polypropylene (PP) makes *P. azotoformans* and *B. subtilis*, relatively more hydrophobic in nature (Arkatkar et al., 2010).

4.3.4 pH

pH is a crucial determinant of microbial growth, influencing the structure, function, and stability of proteins and biomolecules by affecting their ionization state. Microorganisms have specific pH requirements for optimal growth, enzyme activity, and stability, and the

pH of the environment can either promote or inhibit their growth (Yao et al., 2022). The biodegradation study showed changes in the pH of bacterial cell cultures (B1 and B2) during a 40 d incubation in the presence and absence of microplastics. In the presence of NMPs, pH changes were observed more or less similar for NMPs_MSM, NMPs_B1, and NMPs_B2 in first 5 d. However, after 5 d until 40 d, a noticeable decrease in pH occurred (pH 6.8 to pH5.7) for NMPs_B1 and (pH 6.75 to pH 5.8) for NMPs_B2. These observations suggest that the optimum pH for the growth of the B1 strain is 6 ± 0.1, and for the B2 strain, it is 6.2 ± 0.2 in the presence of NMPs and aligning well with the lag phase observed through optical density measurements (Fig. 22 (c)). In contrast, for PEMPs, a decline in pH was observed after 5 d until 40 d (pH 6.5 to pH 5.7) for PEMPs_B1 and (pH 6.8 to pH 5.81) for PEMPs_B2 (Fig. 22(d)). The rapid pH decrease in the presence of microplastics was observed compared to the control (NMPs_MSM), likely contributed to the decline of microbes, as revealed by OD measurements (Fig. 22 (a)).

The physicochemical properties of the microenvironment are notably affected by low molecular weight organic acids, including pH (Tiwari et al., 2022). Even a minor deviation from the optimal pH during degradation might impede microbial growth and enzyme activity due to the accumulation of metabolites and degraded pollutants (Auta et al., 2018). The reduction in pH observed in the bacterial culture containing NMPs and PEMPs microplastics indirectly indicates the presence of organic acids in the metabolites, potentially influencing the degradation process.

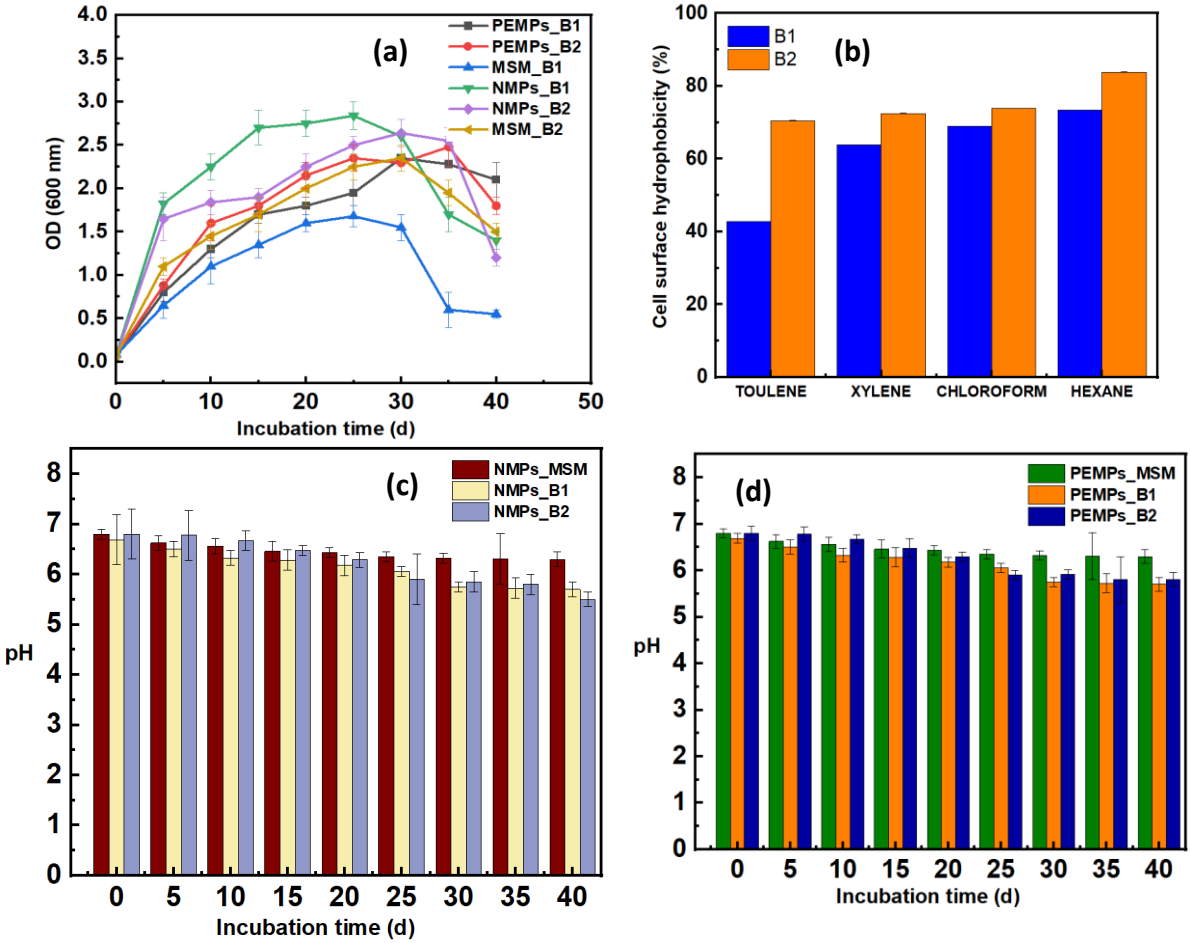


Fig. 22. (a) Growth curve (b) Cell hydrophobicity percentage test (c) pH comparison of bacterial cells in the presence and absence PEMPs (d) pH comparison of bacterial cells in the presence and absence NMPs

4.3.5 Organic acid analysis

Organic acids play a pivotal role in the biodegradation of various micro-pollutants and participate in essential metabolic processes, such as the tricarboxylic acid cycle (TCA), within cellular activities to bolster the microbial degradation of said pollutants (Xiao and Wu, 2014). As illustrated in Fig. 23 (a, b, and c), the entities labeled as PEMPs_B1, PEMPs_B2, NMPs_B1, and NMPs_B2 exhibited increased secretion of organic acids—namely malic acid, citric acid, and oxalic acid—in the presence of microplastics, in contrast to the control counterparts (MSM_B1 and MSM_B2). The levels of citric acid surpassed those of malic and oxalic acids in PEMPs_B2 and NMPs_B2. This intriguing observation suggests that the presence of microplastics stimulates the production of citric acid by mixed

culture microbes. Notably, when NMPs were present, an augmented citric acid content was noticeable in the metabolite for up to 25 days, after which it declined. This pattern aligns with an increased utilization of citric acid within the TCA metabolic pathway for cellular growth and activities in the presence of microplastics, as previously documented.

An upsurge in oxalic acid concentration was visible in the presence of PEMPs_B2. While there was a gradual rise in oxalic acid concentration across all treated samples, a sudden decrease was observed subsequently, suggesting its metabolic utilization. Conversely, the concentration of malic acid increased in all samples, peaking at 40 d. Existing literature points to oxalic and malic acids as intermediate molecules formed during the mineralization of pollutants, primarily arising from anthropogenic sources (Ling et al., 2009; Xiao and Wu, 2014). The elevated presence of oxalic and malic acids suggests their significant role in the degradation of PEMPs and NMPs. However, it is apparent that these acids were not entirely consumed by the microbes. These findings are in congruence with prior reports (Liu et al., 2019; Tiwari et al., 2023, 2022), where an augmentation in oxalic acid content coincided with the biodegradation of pollutants—triphenyltin, nylon 6, 6, polyethylene and tricresyl.

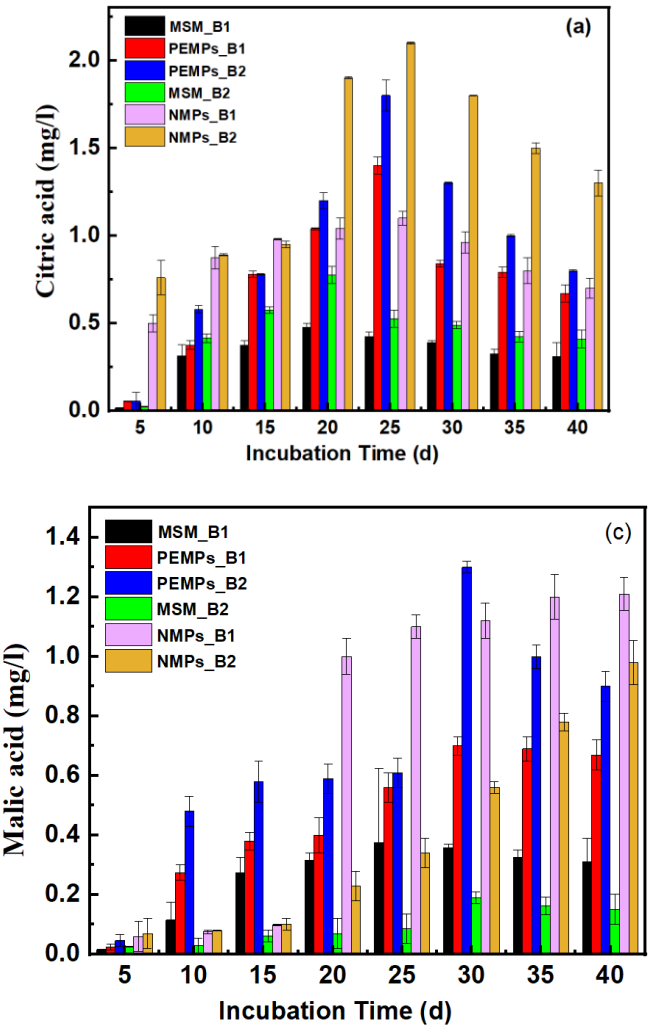


Fig. 23. Secretion of organic acids (a) Citric acid (b) Oxalic acid and (c) Malic acid in the presence and absence of PEMPs and NMPs

4.3.6 Weight loss percentage

The impact of bacterial interaction on PEMPs and NMPs was evaluated by determining the weight loss percentage of these microplastics. The results of this investigation are presented in Table 6 and 7. Notably, negligible weight loss was observed for PEMPs_MSM and NMPs_MSM during the study period. However, the biodegradation of PEMPs_B1 and PEMPs_B2 resulted in weight losses of 24.7 wt.% and 19.3 wt.%, respectively, after 40 d. Similarly, the weight loss percentages for NMPs_B1 and NMPs_B2 were 21.3 wt.% and 20 wt.% after 40 d of biodegradation, respectively.

These findings demonstrate the bacterial isolate's capacity to produce critical enzymes/acids (as illustrated in Fig. 24(a, b, and c)) that effectively utilize the carbon

content of PEMPs and NMPs for microbial survival. The observed weight loss percentages underscore the ability of these bacteria to metabolize and degrade these microplastics, implying their potential role in mitigating microplastic pollution through biodegradation processes. The microbial degradation of the PEMPs and NMPs conceivably results from biochemical pathways (Bio-fragmentation and Bio-assimilation). In a similar study, the weight loss of polyethylene were recorded as 9%, 24% and 3% when exposed *to Penicillium sp., A. Terreus* and *Bacillus sp. + Penicillium sp vesicularis*, over 105 days (Mohy Eldin et al., 2022). Whereas, about 22.06% weight loss is observed for the NMPs with *Brevibacillus brevis* (Tiwari et al., 2022). The observed changes in weight loss percentage of plastic particles can be attributed to the activities of microorganisms acting upon PEMPs and NMPs. This weight loss phenomenon signifies potential alterations in the chemical composition of microplastics, as a consequence of biodegradation (Zhang et al., 2021). Biodegradation entails the breakdown of polymer chains into smaller molecules, such as carbon dioxide, water, and other byproducts. The fragmentation process leads to a reduction in the weight of plastic particles as they are broken down into smaller compounds, which microorganisms can utilize for their energy and growth (Auta et al., 2018). Microbes possess the ability to adapt to their environment based on the availability of nutrients. Therefore, the observed weight loss implies that microbial cells effectively utilize carbon from the backbone of PEMPs by degrading their chain structure (Sowmya et al., 2015).

Table 6. Dry weight analysis of PEMPs in the presence of B1 and B2 bacterial cells

Organism	Initial weight (g)	Final weight (g)	Weight loss (wt%)	F-value	Sig.	Removal constant (K) day^{-1}	Half-life (days)
Control PEMPs	0.5	0.5	0				∞
PEMPs_B1	0.5	0.37±0.025	24.66	111.677	<0.001	0.00326	92.33
PEMPs_B2	0.5	0.41±0.020	19.33			0.00215	140

Table 7. Dry weight analysis of NMPs in the presence of B1 and B2 bacterial cells

Organism	Initial weight (g)	Final weight (g)	Weight loss (wt%)	F-value	Sig.	Removal constant (K) day^{-1}	Half-life (days)
Control	0.5	0.5	0				∞
NMPs_B1	0.5	0.39±0.025	21.33	120.87	<0.0024	0.0269	111.89
NMPs_B2	0.5	0.40±0.026	20			0.0024	125

4.3.7 Enzymatic Assays (Total enzyme, laccase and peroxidases activity)

The Fluorescein Diacetate (FDA) assay is a method used to assess cell viability in a wide range of environmental samples. Within the FDA process, both bound and unbound enzymes undergo hydrolysis, resulting in the production of fluorescein, a substance exhibiting a yellowish-green color. The samples undergoes an FDA reaction wherein the enzyme activity was quantified as the amount of FDA hydrolyzed per mL. The study was done in strict sterile conditions, and both positive and negative controls exhibited a negligible amount of FDA hydrolysis (Schnürer and Rosswall, 1982). This method has been extensively used to study the impact of wide range of anthropogenic pollutants on microbial activity (Mahu et al., 2018; Muter et al., 2012). Fig. 24 (a) demonstrates a notable elevation in total enzyme activity in the presence of microplastics (PEMPs_B1, PEMPs_B2, NMPs_B1, and NMPs_B2) compared to its absence (MSM_B1 and

MSM_B2). These results suggest bacterial growth and the secretion of enzymes for microplastics degradation. The release of enzymes in PEMPs increased as the incubation time advanced, reaching its peak activity at 25 d in PEMPs_B1 and PEMPs_B2 (31.5 µg and 19.9 µg FDA hydrolyzed/mL of media in 40 min reaction time) and subsequently declining. Similarly, the maximum activity of NMPs_B1 and NMPs_B2 occurred after 25 d (29.3 µg and 20.7 µg FDA hydrolyzed/mL of media in 40 min reaction time) in the presence of NMPs.

The increased enzyme activity in bacterial cells in the presence of microplastics can be attributed to the secretion of specific enzymes such as protease, esterase, and laccase, responsible for microplastics degradation. Notably, the growth pattern of bacterial cells (Fig. 22(a)) mirrors these enzyme activity trends. The well-known enzymes, laccase, and peroxidase (Mohy Eldin et al., 2022), so exhibited varying activities at different time intervals, which will be further discussed in the subsequent section.

Laccase is a crucial player in the biodegradation of plastics, particularly in relation to PE degradation (Santo et al., 2013; Thurston, 1994). Belonging to the oxidase group of enzymes, laccase is renowned for its ability to initiate the depolymerization process by oxidatively cleaving the amorphous region of PE. This cleavage creates an accessible carbonyl region within the polymer chain, facilitating its subsequent degradation (Kang et al., 2019). Peroxidase also contributes a significant role in plastic degradation processes. When present in microorganisms or other organisms involved in biodegradation, peroxidase enzymes can actively contribute to breaking down of plastics (Sowmya et al., 2015). These enzymes facilitate the breakdown of polymer chains, enabling the degradation of plastics into smaller, more manageable components (Mohy Eldin et al., 2022).

Fig. 24(b) represents a comparative increase in the activity of laccase in PEMPs_B1 at 20 d and PEMPs_B2 at 25 d (14.6 µmol/min and 14.6 µmol/min) than in the absence of PEMPs at 20 d and 25 d (MSM_B1/7.7 µmol/min and MSM_B2/ 7.4 µmol/min). Further, the production of laccase enzyme enhanced in NMPs_B1 at 20 d (9.1 µmol/min) and NMPs_B2 at 25 d (10.5 µmol/min) in the presence of NMPs. It was observed that the secretion of laccase enzyme enhanced during the degradation process of microplastics. Moreover, the maximum peroxidase activity (fig. 24 (c)) observed at 25 d in PEMPs_B1, PEMPs_B2, NMPs_B1and NMPs_B2 (7.7, 8.7, 7.03 and 8.9 µmol/min). It has been observed that the laccase enzyme production is higher than peroxidase enzyme. It also concludes that laccase and peroxidase act synergistically to degrade the plastics. Further it has been reported the laccase enzyme exhibits the ability to initiate reactions by catalyzing a one-electron oxidation process in the substrate, resulting in the release of CO_2 from the polymer (Schlosser and Höfer, 2002; Sowmya et al., 2015). Subsequently, this enzyme proceeds through a series of successive steps, leading to the reduction of an O_2 molecule and the generation of an oxygen free radical ($O^{\cdot}$). These events culminate in the formation of hydrogen peroxide (H_2O_2), which then serves as an initiator for peroxidase reactions, ultimately leading to the production of two molecules of water (H_2O) (Mohy Eldin et al., 2022). Due to these interconnected enzymatic reactions, the activities of both enzymes demonstrate fluctuating patterns in an alternating manner during the course of this investigation.

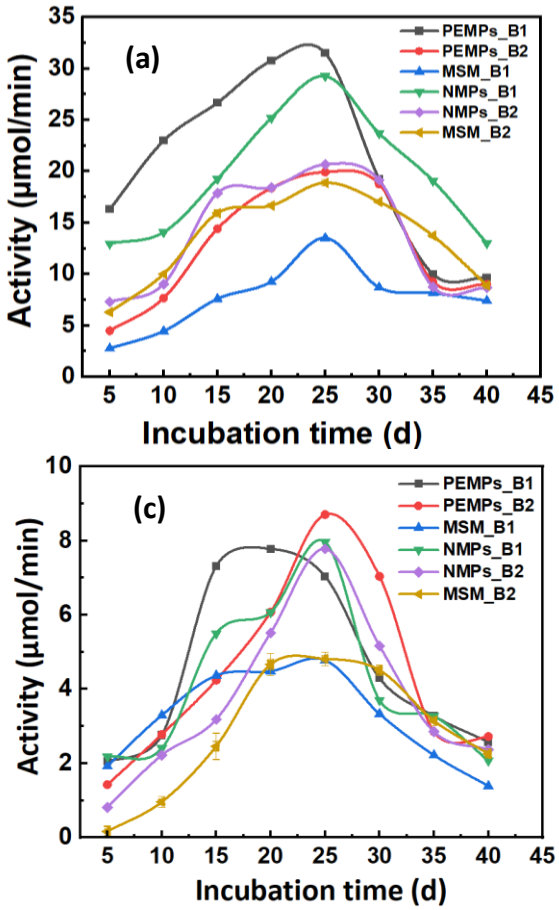


Fig. 24. (a) Total enzyme activity and (b) Laccase enzyme activity (c) Peroxidase enzyme activity of bacterial strains in the presence and absence of PEMPs and NMPs

4.3.8 DSC

DSC measures the heat flow in a sample as it undergoes temperature changes, such as melting or crystallization. The enthalpy change (ΔH) represents the amount of heat absorbed or released during these transitions (Raghavan and Torma, 1992). The DSC thermograms of the samples and control microplastics represents the change between characteristics of the polymer, Fig. 25 (a). PEMPs, PEMPs_MSM, PEMPs_B1, PEMPs_B2 demonstrates a single melting step around 139 °C but upon degradation the enthalpy change was observed. The recorded ΔH for PEMPs (ΔH = 77.1 J/g) and PEMPs_MSM (ΔH = 77 J/g) remains the with a slight difference, whereas a major change observed in PEMPs_B1 (ΔH = 61.04 J/g) and PEMPs_B2 (ΔH = 66.7 J/g). Further, a single melting step observed at 218 °C in all NMPs and recorded ΔH for NMPs and NMPs_MSM

(40.1 J/g) remains the same whereas a change observed in NMPs_B1 (ΔH = 29.7 J/g) and NMPs_B2 (ΔH = 32.9 J/g) (Fig. 25 (b)). A decreased ΔH value during the melting transition of a microplastics indicates that it requires less heat to change from its solid state to its molten state which imply that the treated microplastic is susceptible for further degradation (Puglisi et al., 2019b). In a study reported, micro-sized polyethylene (PE) degradation was examined using mesophilic mixed bacterial cultures derived from a municipal landfill sediment. Notably, *Bacillus sp.* and *Paenibacillus sp.* were isolated. Thermal gravimetric analysis revealed enhanced thermal stability (temperatures > 570°C) in aged particles compared to controls. This points towards enzymatic chain scission as the mechanism behind microplastic degradation, possibly due to higher refractory fractions in aged particles, which persist at elevated combustion temperatures. (Park and Kim, 2019).

4.3.9 TGA

TGA represents the change in the thermal stability and decomposition behavior of materials including plastics as depicted in Fig. 25 (c and d). The temperature at which control microplastics, PEMPs_MSM and NMPs_MSM showed 10% weight loss at temperature 390 °C and 365 °C, respectively. In the case of PEMPs_MSM and NMPs_MSM, 50% weight loss achieved at temperature 469 °C and 448 °C. Moreover, maximum weight loss approximately 92% obtained at temperature 497 °C and 481 °C, respectively. For the PEMPs_B1 and PEMPs_B2 treated microplastics of polyethylene, the temperature at 10% weight loss observed, was 426 °C and 290 °C. Upon increasing the temperature, PEMPs_B1 and PEMPs_B2 observed 50% weight loss change at 480 °C and 460 °C, and maximum weight loss of 90% and 80% recorded at the temperature 512 °C and 499 °C respectively. In the case of NMPs_B1 and NMPs_B2 10% weight loss recorded at 100 °C and 266 °C. Further, 50% weight loss recorded at temperature 423 °C and 465 °C in the treated samples of NMPs_B1 and NMPs_B2. Maximum weight loss attained at

temperature 486 °C (92%) and 523 °C (83%) in NMPs_B1 and NMPs_B2. The observed results concludes that treated microplastics still maintained some of its weight regardless the degradation indicates the presence of small molecules which could be the degradation products after bacterial degradation. The outcomes suggested that few alterations in the thermal stability is due to the antioxidants. Antioxidants serve as scavengers, neutralizing free radicals in polymers and preventing their harmful chain reactions. By deactivating free radicals, antioxidants create a barrier against degradation, thus significantly increasing the thermal stability of the polymers (Suresh et al., 2011). Similar thermal degradation reported for micro-polyethylene degradation by bacterial colonies of a mixed microbial consortium and for LDPE and PVC microplastics by *Achromobacter denitrificans Ebl13* (Maleki Rad et al., 2022; Park and Kim, 2019).

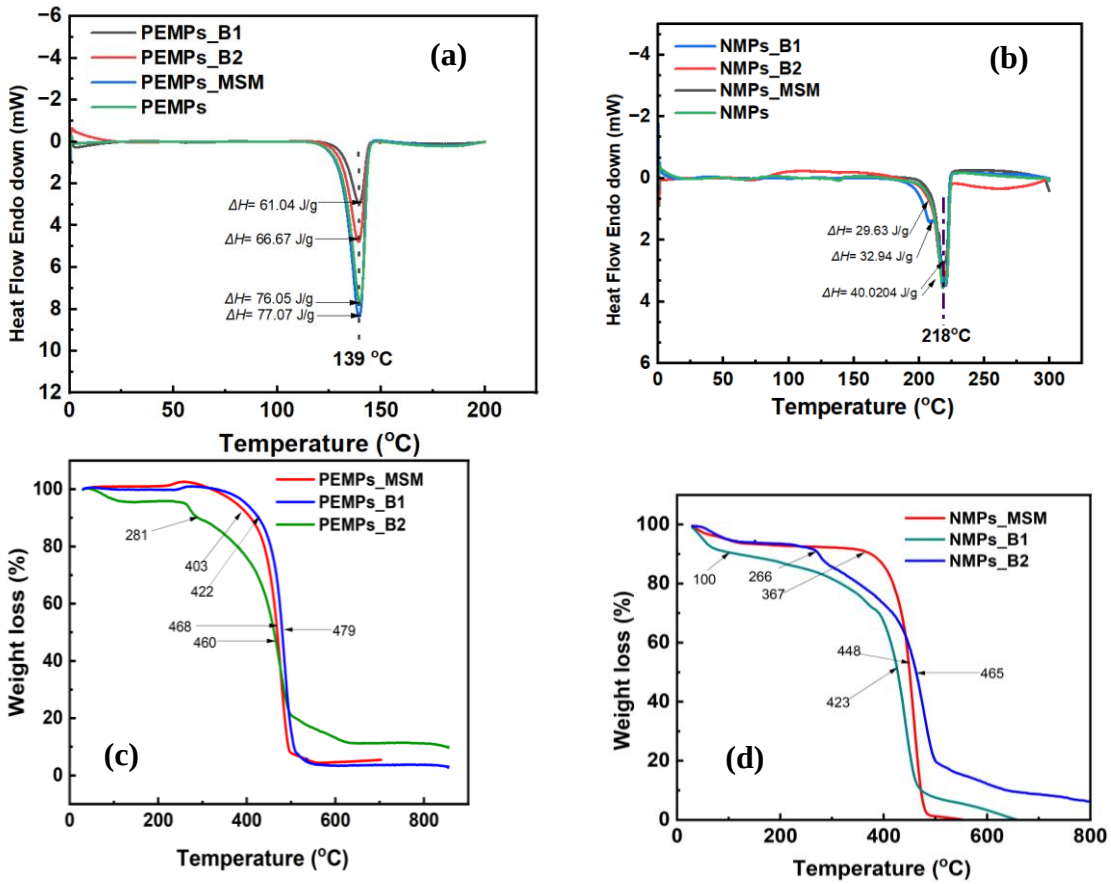


Fig. 25. (a) DSC thermogram of PEMPs, PEMPs_MSM, PEMPs_B1 and PEMPs_B2 (b) DSC thermogram of NMPs, NMPs_MSM, NMPs_B1 and NMPs_B2 (c) TGA thermogram of PEMPs_MSM, PEMPs_B1 and PEMPs_B2 (d) TGA thermogram of NMPs_MSM, NMPs_B1 and NMPs_B2

4.3.10 FTIR Investigation

The FTIR spectra of PEMPs both prior to and subsequent interaction with isolated microbes were represented in Fig. 26 (a and b).The major characteristic absorption bands were assigned at 2920 cm^{-1} (-CH_3 stretching), 2831 cm^{-1} (-CHO stretching), 1473 cm^{-1} (C=C double bond stretching), and 721 cm^{-1} (C=C-H stretching) in PE (Park and Kim, 2019). The FTIR analysis has indicated that the PEMPs_B1 formed new functional groups such as hydroxyl groups (O-H stretch at 3429 cm^{-1}), methyl deformation (1473 cm^{-1}), carbonyl groups (conjugated ketone or aldehyde R-C=O stretch at 1628 cm^{-1}), 1367 cm^{-1} occurring in the end of methyl groups indicating the bio-oxidation and polymer chain breakage on the surface of the PEMPs (Huang et al., 2021; Li et al., 2020). Similarly, in the PEMPs_B2, functional groups at 3395 cm^{-1} exhibits hydroxyl groups (O-H stretch), 1045 cm^{-1} (C-O-C

stretch) , and 1732 cm^{-1} carbonyl band formation are crucial signs of PE degradation (Puglisi et al., 2019b). In a study, microbial consortium employed for the degradation of PE for 40 and 60 days of incubation, and it has been observed that the formation of hydroxyl groups (-OH) expressing the breakage of PE polymer (Park and Kim, 2019). Through bio-oxidation, microorganisms can break down the complex structure of polyethylene into simpler compounds that can be further degraded by other microorganisms or assimilated into the metabolic cycle (Zhang et al., 2021).

Figure 26 (b) represents the FTIR range of NMPs, NMPs_MSM, NMPs_B1, and NMPs_B2 after a 40 d treatment. The existence of amide peaks in NMPs is evident from the sharp band/peak at 1640 cm^{-1}, attributed to the C=O stretch (amide I), and the strong band at 1540 cm^{-1}, designated to NH_2 bend (amide II) of the nylon 6,6 chain. Additionally, the C-H stretch of CH_2 axial groups near the carboxyl groups of the polymer chain, and the C-N stretch of the amide III band, were observed at 1197 cm^{-1} and 1268 cm^{-1}, respectively.

A noticeable move towards greater wavenumber was observed for the amide I band (from 1640 cm^{-1} to 1635 cm^{-1}), while the amide II band slightly shifted to lower wavenumbers (from 1540 cm^{-1} to 1535 cm^{-1}) in the case of NMPs_B1 and NMPs_B2 compared to NMPs_MSM. Additionally, a higher wavenumber shift of the amide III band from 1268 cm^{-1} to 1272 cm^{-1} indicates a potential change in the crystalline peak for NMPs_B1 compared to the control samples. Moreover, NMPs_B1 (at 2937 cm^{-1} and 2866 cm^{-1}) and NMPs_B2 (at 2929 cm^{-1} and 2842 cm^{-1}) displayed moderate C-H stretching. The observed changes in the characteristic vibrational peaks of the treated samples, including the strengthened amide I, weakened amide II, and strengthened amide III, in comparison to NMPs_MSM, serve as evidence confirming the microbial enzymatic degradation of nylon 6,6. These alterations in the structural chemistry, particularly affecting the amide (-CONH-) functional groups, provide insight into the disruption of intermolecular hydrogen bonding

between C=O and N-H groups within nylon 6,6 fragments due to degradation mediated by enzymes. This degradation-induced phenomenon, as demonstrated by previous research involving lipase and protease (Parvinzadeh Gashti et al., 2013), underscores the impact of enzymatic processes on the modification of nylon 6,6's molecular cohesion.

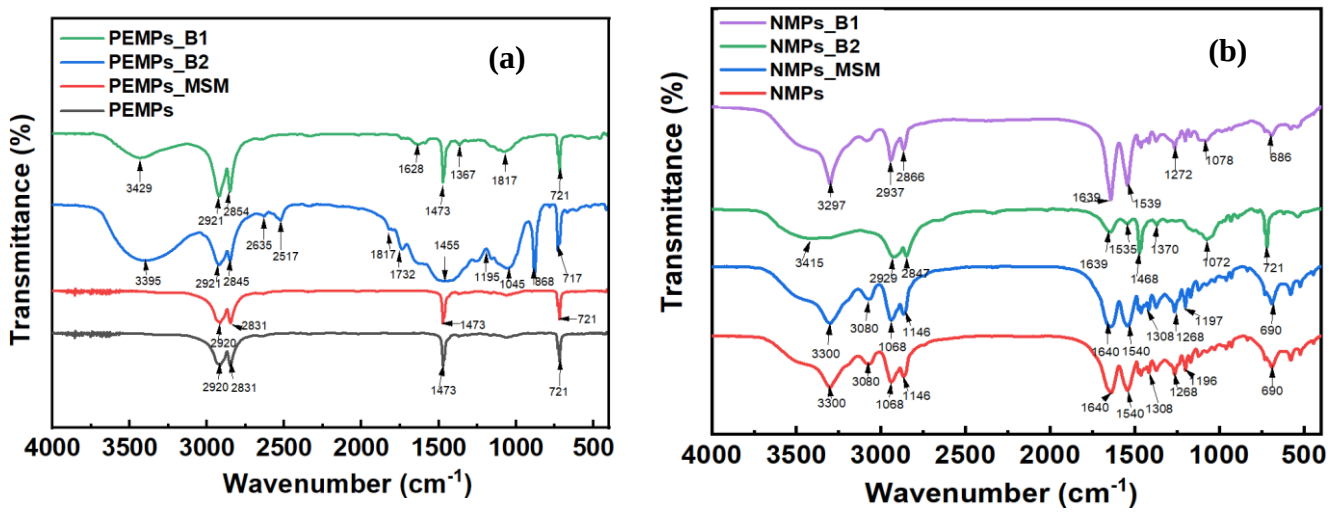


Fig. 26. (a) FTIR spectra of PEMPs, PEMPs_MSM, PEMPs_B1 and PEMPs_B2 (b) FTIR spectra of NMPs, NMPs_MSM, NMPs_B1 and NMPs_B2

4.3.11 Morphological analysis

SEM: The investigation of microbial interaction and attachment onto plastic surfaces (PEMPs and NMPs) was conducted using SEM. In Fig. 27 (a and b), B1 and B2 bacterial cells are shown in the log phase before any interaction, displaying healthy cellular morphology with intact structures. However, Fig. 27 (c and d) illustrates the same B1 and B2 cells after 40 d of incubation in the MSM medium, where the cells' shape has changed from a regular structure to a shrunken appearance due to cellular starvation, ultimately leading to cell apoptosis.

Examining Fig. 27 (e), it is evident that B1 cells adhered to the surface of NMPs, indicating their consumption as a carbon nutrient source. Similarly, in Fig. 27 (f), B2 cells are observed attached to the NMPs surface, further confirming their preference for plastic surfaces as a

carbon nutritional requirement. Moreover, Fig. 27 (g and h) reveal the attachment of B1 and B2 cells onto the surface of PEMPs, with an agglomeration of cells, suggesting their affinity towards the plastic surface as a preferred carbon source. This corroborates that the presence of microbial attachment is indicative of the cellular demand for the available carbon from the plastic surfaces. Similar studies have shown the attachment of microbes on to the surface of the polymer for the carbon requirement (Huang et al., 2021; Mohy Eldin et al., 2022).

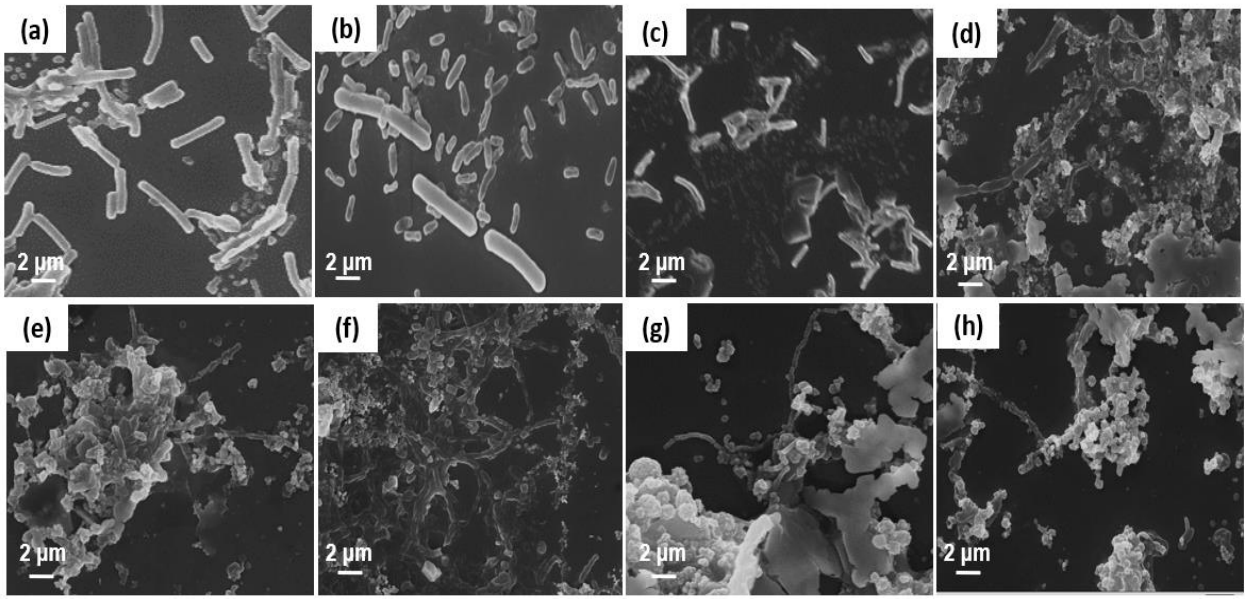


Fig. 27. SEM Images (magnification 5000X) of (a) B1 in log phase (after 5 d) (b) B2 in log phase (after 5d) (c) death phase after 40 d, B1 without microplastics (d) after 40 d B2 without microplastics (e) aggregation of B1 in NMPs after 40 d (f) B1 after 40 d in PEMPs (g) B2 after 40 d in PEMPs and (h) B2 after 40 d in NMPs

TEM: TEM was utilized to investigate the morphological alterations in PEMPs and NMPs subsequent to their interaction with microbes, enabling the confirmation of microplastic biodegradation. Prior to microbial interaction, the size of NMPs and PEMPs were measured at 200 nm and 40-48 µm, respectively, with no observable changes in the plastics' morphology (see Fig. 28 (a and e)). Upon examining NMPs treated with B1 bacterial cells (Fig. 28 (b)), thinning of microplastics was evident at the corners. In contrast, Fig. 28 (c) revealed an irregular and distorted structure of NMPs following treatment with B2

microbes. Additionally, analysis of PEMPs microplastics treated with B1 showcased a broken PE microplastic fragment with several holes, Fig. 28 (f), while treatment with PEMPs_B2 caused in a microplastic with a less compact structure and smaller holes, indicating a weakened polymer chain due to microbial interaction, fig. 28 (g). These secretions have made the polymer's structure more susceptible to degradation, as shown by DSC and TGA(Fig. 25) and FTIR (Fig. 26) analyses. The adherence and microbial secretions on plastic particles results in the formation of porous structure, holes/pores and cavities, depending upon the extent of degradation that was well reported on LDPE (Mohy Eldin et al., 2022), HDPE (Jeon et al., 2021) , nylon 6, 6 (Tiwari et al., 2022) and PS nanoparticles(Awet et al., 2018).

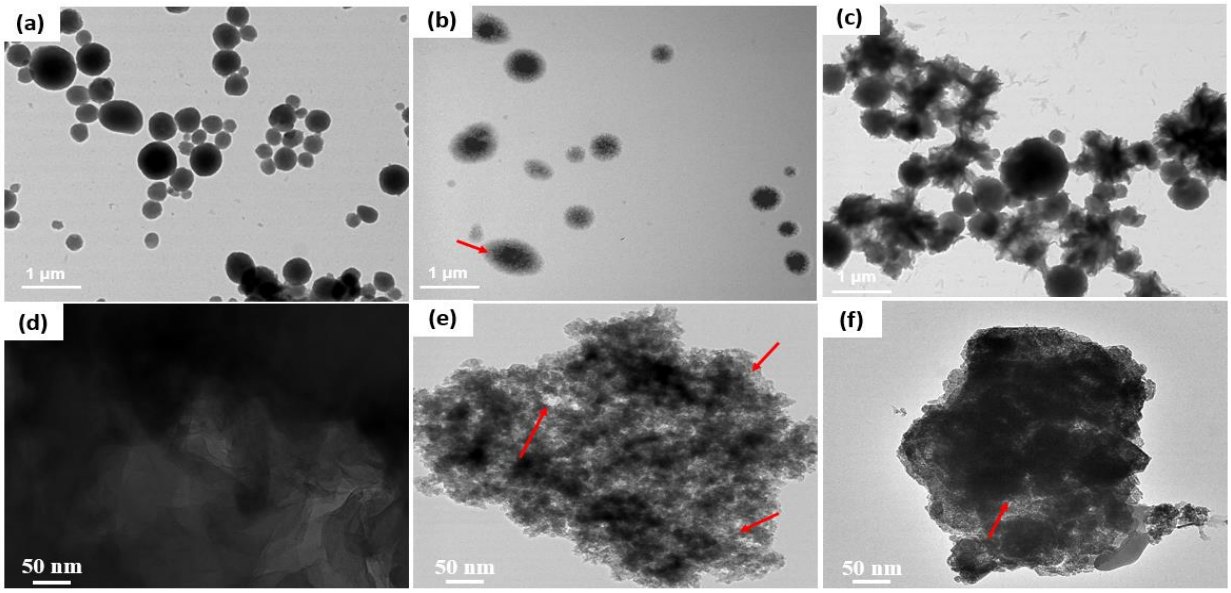


Fig. 28. TEM Images of (a) untreated NMPs (b) B1 treated NMPs (c) B2 treated NMPs (magnification 2550X/1 µm) / (d) untreated PEMPs (magnification 44000X/50 nm) (e) B1 treated PEMPs fragmented into pieces (magnification 16000X/50 nm) and (c) B2 treated PEMPs fragment (magnification 16000X/50 nm)

4.3.12 GC-MS of metabolites and degradation pathway

In the biodegradation of PEMPs and NMPs, alkanes (such as Undecane, Pentadecane, Undecane, etc., ketone (cyclopentanone), esters (Diethyl Phthalate, Butyl 8-methylnonyl ester, 1,6-dimethyl hexanedioate ,Di-n-octyl phthalate), carboxylic acids (1,2-

Benzenedicarboxylic acid) and aromatic compound (Phenol, 2,4-bis (1,1-dimethylethyl)) produced by depolymerization through the catalytic activity of enzymes like laccase and peroxidase. enzymatic action, such as laccase and peroxidase enzymes. The resulted compounds serve as substrates for microbial metabolism through β-oxidation and Tricarboxylic Acid (TCA) cycle metabolism for energy and growth (Fujisawa et al., 2001; Ji et al., 2013).

GC–MS study of the metabolites recorded several compounds as listed in Table 8. These analysis corroborates findings, confirming the presence of various metabolites, a result aligned with significant studies on the biodegradation of PEMPs and NMPs (Jeon et al., 2021; Muhonja et al., 2018; Smith et al., 2012). The attained results indicate the biodegradation of PEMPs and NMPs into less complex compounds. However, many degraded products of plastics identified in GC-MS remain unclarified. The obtained degraded compounds are expected due to the synergistic action of the laccase and peroxidase enzymes on PEMPs and NMPs. The action of laccase (copper binding enzyme) is well explored and displays the higher degradation efficiency in the presence of enzyme in the degradation of both PEMPs and NMPs (Fujisawa et al., 2001). Moreover, the synergistic effect of laccase and peroxidase enzymes mediated degradation of polyethylene bags were reported.

Table 8. List of identified chemical compounds in GC-MS analysis of control (PEMPs_MSM and NMPs_MSM) and treated samples (PEMPs_B1, PEMPs_B2, NMPs_B1 and NMPs_B2). (Y or N denotes Yes or No)

Retention Time	Hydrocarbon compounds	PEMPs_MSM	PEMPs_B1	PEMPs_B2	NMPs_MSM	NMPs_B1	NMPs_B2
	Alkane group						
4.346	Undecane, 2-methyl-	Y	Y	Y	Y	Y	Y
4.47	Dodecane	N	Y	y	N	N	Y
4.472	Undecane	N	N	N	N	Y	N
4.824	Pentadecane	N	N	N	N	Y	N
4.977	Undecane, 2,5-dimethyl-	N	Y	Y	N	Y	Y
5.422	Tridecane	Y	Y	Y	Y	N	N
5.345	Undecane, 4,7-dimethyl-	N	N	N	N	Y	N
5.503	Benzene, 1,3-bis(1,1-dimethylethyl)	N	Y	Y	N	Y	Y
5.592	Decane, 2,3,4-trimethyl-	N	Y	Y	N	N	N
5.632	Undecane, 2,4-dimethyl-	N	Y	Y	N	Y	Y
5.8	Dodecane, 2,6,11-trimethyl-	N	N	Y	N	N	Y
5.92	Nonane, 5-(2-methylpropyl)-	N	N	Y	N	Y	Y
6.233	Decane, 2,3,7-trimethyl-	N	Y	Y	N	Y	Y
6.42	Tetradecane	Y	Y	Y	N	N	N
6.427	Dodecane, 4,6-dimethyl-	N	Y	N	N	Y	Y
6.55	Dodecane, 2,6,10-trimethyl-	N	N	Y	N	N	Y
6.557	Nonane, 5-butyl-	N	Y	N	N	Y	Y
6.67	Nonane, 5-(2-methylpropyl)-	N	N	Y	N	N	Y
6.679	2,6,10-Trimethyltridecane	N	Y	N	N	Y	Y
6.998	Undecane, 2-methyl-	N	Y	N	N	N	N
6.99	Tridecane, 2-methyl-	N	N	Y	N	Y	Y
7.103	Decane	Y	Y	Y	Y	N	Y
7.61	Tridecane, 2,5-dimethyl-	N	N	Y	N	N	Y
7.614	3,5-Dimethyldodecane	N	Y	N	N	N	Y
7.671	Octadecane	Y	Y	Y	N	N	N
7.744	Tetradecane, 4-methyl-	N	Y	Y	N	Y	Y
8.113	Pentadecane	Y	Y	Y	N	N	N
8.115	Pentadecane, 2,6,10-trimethyl-	N	Y	Y	N	Y	N
8.27	Decane, 2,3,5,8-tetramethyl-	Y	N	Y	N	N	N

8.35	2,6,10-Trimethyltridecane	N	N	Y	N	Y	N
8.53	Heptadecane	Y	Y	Y	N	N	N
9.151	Hexadecane	Y	Y	Y	Y	Y	Y
9.52	Pentadecane, 2-methyl-	N	N	Y	N	N	N
10.1	Hexadecane, 4-methyl-	N	N	Y	N	N	N
10.264	Heneicosane	Y	Y	Y	Y	Y	Y
10.5	Pentadecane, 2,6,10-trimethyl-	N	Y	Y	N	Y	N
11.4	Tridecanol, 2-ethyl-2-methyl-	N	N	N	N	N	Y
11.584	Eicosane	N	Y	Y	N	Y	Y
12.975	Hexadecane, 2,6,10,14-Tetramethyl-	N	Y	N	N	N	N
15.358	Dotriacontane	N	Y	Y	N	Y	N
17.2	Tetrapentacontane	N	Y	Y	N	N	Y
22.89	6,6-Diethylhoctadecane	N	N	Y	N	Y	N
	Aromatic groups						
8.911	Phenol, 2,4-bis (1,1-dimethylethyl)-	N	Y	Y	N	Y	Y
	Ester groups						
9.86	Diethyl Phthalate	N	N	Y	N	N	Y
13.8	Butyl 8-methylnonyl ester	N	N	N	N	Y	N
16.08	1,6-dimethyl hexanedioate	N	N	N	N	Y	Y
19.3	Di-n-octyl phthalate	N	N	N	N	Y	N
	Carboxylic group						
13.836	1,2-Benzenedicarboxylic acid,	N	Y	N	N	Y	Y
	Ketone group						
8.76	cyclopentanone	N	N	N	N	Y	Y

A hypothetical mechanism of PEMPs and NMPs degradation is elucidated, based on the experimental observations, Fig. 29. The suggested degradation of PEMPs and NMPs showcases a promising strategy for breaking down the polymer chain into smaller molecules. These molecules can subsequently undergo mineralization, foremost to the formation of carbon dioxide, water, and novel biomass compounds. It is shown by DSC analysis (Fig. 25 (a and b) that decreased ΔH value during the melting transition of a microplastics indicates that it requires less heat to change from its solid state which imply

that the treated microplastics were loosen and the degradation process. TGA analysis complements this by illustrating the presence of residual weight in treated microplastics, hinting at the existence of small molecules, potentially representing degradation byproducts. The release of laccase and peroxidase enzymes by microbes are responsible for the splitting of carbon-carbon bonds and terminal oxidation at the ends of PEMPs and NMP (Guengerich and Yoshimoto, 2018; Santo et al., 2013).

Bio-fragmentation is a complex process of breaking down complex substances into simpler compounds through enzymatic reactions (Tiwari et al., 2020). The Carbon-carbon bond breakage was recognized to release of dodecane, pentadecane etc. through GC-MS study (listed in table 8.). Bio-transformation is a process facilitated by enzyme-catalyzed reactions or entire cellular systems, leading to the conversion of a given chemical compound into an altered form. This alteration can result in the new compound possessing a lower or higher degree of toxicity compared to the original compound (Kaur and Gosal, 2021). Similarly, the release of 1,2-Benzenecarboxylic acid, Phenol 2,4-bis (1,1 dimethylethyl), etc detected due to the biodegradation of PEMPs and NMPs through GC-MS study. Further, oxidation of terminal ends caused the addition of a carboxylic acid group to the polymer chain (Santo et al., 2013). As Section 4.3.7 explained the release of laccase and peroxidase enzyme work synergistically, by catalyzing a one-electron oxidation process in the substrate, resulting in the release of CO_2 from the polymer. Further the peroxidase enzyme proceeds through a series of successive steps, leading to the reduction of an O_2 molecule and the generation of an oxygen free radical ($O^{\cdot}$) which causes the formation of hydrogen peroxide (H_2O_2), ultimately serves as an initiator for peroxidase reactions, ultimately leading to the production of water molecules. Coenzyme A plays a vital role in facilitating the oxidation of fatty acids and in the progression of the citric acid cycle (Mohy Eldin et al., 2022). Beta-oxidation of an acyl-CoA molecule initiates a

cleavage at the β-carbon atom. Consequently, this cleavage leads to the creation of a molecule that possesses a length shortened by two carbon units compared to its initial state (Restrepo-Flórez et al., 2014). This enzymatic cascade ultimately fuels cellular metabolism by providing energy through the Tricarboxylic Acid (TCA) cycle (Albertsson et al., 1995).

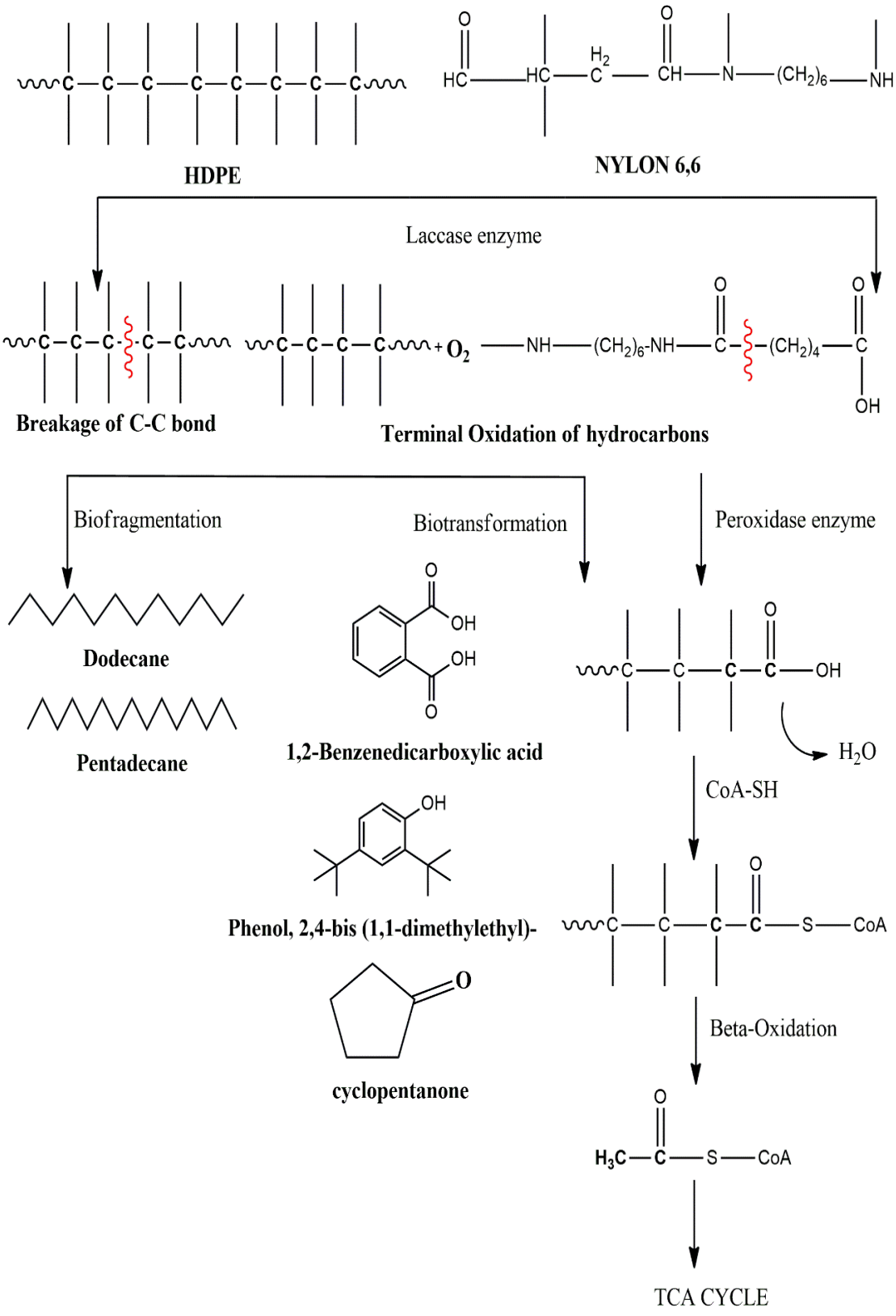


Fig. 29. PEMPs and NMPs degradation mechanism based on experimental outcomes

4.4 Environmental significance of PEMPs and NMPs bioremediation

Microplastics, comprising materials like PEMPs and NMPs, present notable environmental concerns due to their enduring nature, widespread dispersion, and potential adverse effects on ecosystems. The small size of microplastics renders them easily consumable by a diverse array of organisms, spanning from plankton to larger marine creatures, resulting in potential disruptions within the food chain and the accumulation of harmful substances. The identification and isolation of distinct microorganisms for plastic remediation play a crucial role in addressing the escalating predicament of plastic pollution.

Microbes possess a distinctive capacity to break down various kinds of plastics through enzymatic activities and metabolic processes. The isolation and characterization of these specialized microorganisms aid in comprehending the mechanisms behind plastic degradation, thereby customizing their applications for efficient plastic remediation. This isolation process also facilitates the evaluation of how these microbes interact with different plastic varieties, their adaptability to diverse environments, and their potential implications for ecosystems. By studying and harnessing the inherent capabilities of these microbes, it becomes possible to devise targeted strategies, such as identifying and modifying enzymes, to expedite the degradation of plastics. Enzymes are pivotal due to their specific functions, and their vital role and selective effectiveness offer substantial potential for mitigating environmental harm, achieved by facilitating a less toxic breakdown of plastics. Furthermore, exploring into the microbial communities related to plastic degradation can shed light on the complex ecological connections and associations within environments contaminated by plastics. Tackling this concern mandates a comprehensive approach, encompassing the reduction of plastic production and consumption, enhancements in waste management practices, the development of sustainable alternatives, and the promotion of education and awareness regarding the

impact of microplastics on the environment. Collaborative endeavors involving governments, industries, researchers, and communities are essential to curbing the release of microplastics into the environment and safeguarding the delicate equilibrium of our ecosystems.

4.5 Conclusion

This detailed study demonstrated the potential of the isolated bacterial strains, *Achromobacter xylosoxidans* (B1) and a mixed bacterial strain (B2), to effectively degrade PEMPs and NMPs. The growth curve analysis revealed that both B1 and B2 strains exhibited exponential growth in the presence of microplastics, indicating their metabolic activity and ability to utilize microplastics as a carbon source. Cell surface hydrophobicity analysis indicated that both B1 and B2 strains possessed hydrophobic characteristics, which could facilitate adhesion to plastic surfaces and enhance degradation through biofilm-mediated mechanisms. Changes in pH during the degradation process of microplastics highlighted the influence of organic acid release on microbial growth. The decline in pH suggested the production of organic acids as metabolic byproducts, impacting microbial growth and enzyme activity. Enzymatic assays demonstrated increased enzyme activity, play a crucial role in initiating the degradation of plastic polymers, leading to the breakdown of their complex structures. Physical characteristics of microplastics tested via thermal analysis (DSC and TGA) which indicated alterations in the thermal stability of microplastics after interaction with bacterial strains. The decrease in enthalpy change and weight loss percentage indicated the susceptibility of treated microplastics to further degradation. FTIR analysis showed changes in functional groups and chemical structure of treated microplastics, confirming enzymatic degradation. The weakening of characteristic peaks suggested enzymatic breakdown of polymer chains. Morphological analysis using SEM and TEM provided visual evidence of bacterial

attachment to microplastics and their role in causing structural changes, including the formation of pores and cavities on the plastic surfaces. Moreover, the release of plastic byproducts evidenced by GC-MS study concludes the breakdown of plastics and suggested possible solutions. Our findings contribute to understand the interdisciplinary actions between biological activity and alterations in the chemical structure of the microplastics upon degradation process. Also, microbial-induced biodegradation offers valuable understandings into the plastic waste management and environmental impacts, shedding light on the role of microorganisms in mitigating plastic pollution.

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Future Scope

- The future of identifying highly active enzymes for plastics degradation holds immense potential. Advances in bioinformatics and high-throughput screening techniques will accelerate the discovery of novel enzymes with better plastic-degrading capabilities. Enzymes can be engineered to efficiently break down various types of plastics, contributing to eco-friendly waste management. This field's growth will drive sustainable solutions and reduce the environmental impact of plastic pollution.
- The identification of water-soluble intermediates, monomers, and oligomers resulting from plastics degradation presents a promising avenue for the future.
- Identifying uncultured microbes involved in polymer degradation can have far-reaching applications. Techniques such as metagenomics helps in the identification of microbes in the environment and subsequently utilized in the bioremediation of plastic waste in natural environments or for the development of biotechnological processes for polymer recycling.
- Nano-technology could be a promising area to explore suitable methods to clean up the micro plastics in the ecosystem

Research Publications

- **Tiwari, N.,** Santhiya, D. and Sharma, J.G., 2023. Significance of landfill microbial communities in the biodegradation of Polyethylene and Nylon 6,6 microplastics. Journal of hazardous materials, p.110436. **Impact factor- 13.6**
- **Tiwari, N.,** Santhiya, D. and Sharma, J.G., 2023. Degradation of polyethylene microplastics through microbial action by a soil isolate of Brevibacillus brevis. *Polymer Degradation and Stability*, p.110436. **Impact factor- 5.9**
- **Tiwari, N.,** Santhiya, D. and Sharma, J.G., 2022. Biodegradation of micro sized nylon 6, 6 using Brevibacillus brevis a soil isolate for cleaner ecosystem. *Journal of Cleaner Production*, *378*, p.134457. **Impact factor- 11.072**
- **Tiwari, N.,** Bansal, M., Santhiya, D. and Sharma, J.G., 2022. Insights into microbial diversity on plastisphere by multi-omics. *Archives of Microbiology*, *204*(4), pp.1-16. **Impact factor- 2.667**
- Kumar, N., Sharma, J., Kumar, G., Shrivastav, A.K., **Tiwari, N**., Begum, A. And Chakrabarti, R., 2021. Evaluation of the nutritional value of prickly chaff flower (Achyranthes Aspera) as a fish feed ingredient. *Indian Journal of Animal Sciences*, *91*(3), pp.239-244. **Impact factor- 1.07**
- **Neha Tiwari**, Deenan Santhiya, Jai Gopal Sharma 2020. Microbial remediation of micro-nano plastics: Current knowledge and future trend. Environmental pollution 265 (2020) 115044. **Impact factor- 9.988**

Conferences

- Participated in Platinum Jubilee Conference on Perspective in Material Research (PMR-2022)" organized by Department of Materials Engineering, IISc, Bengaluru on 21-23rd December, 2022.
- Participated and won the **Third-best Poster Presentation** award in the 5th International Conference on (AAEBSD-2021)" on 05-07 August 2021.
- Participated and on **Third best short talk award** in the 26th international conference of the international academy of Physical sciences, Mahatma Gandhi University, Kottayam, Kerala, India, December 18- 20, 2020
- Participated and won **Third-best Poster Presentation award** at the First International Online conference on Blends, Composites, Bio-Composites and Nanocomposites (ICNC–2020) 9th - 11th October 2020 Kottayam, Kerala, India.
- Participated in DTU Sponsored One-week International E-Workshop on Bioinformatics, Dept. of Biotechnology, Delhi Technological University, Delhi, December 14-18,2020.
- Participated in Virtual International Conference on Surface Chemistry held on 27th and 28th August 2020 at Annamalai University, Annamalai Nagar, India

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Microbial remediation of micro-nano plastics: Current knowledge and future trends☆

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ABSTRACT

An alarming rise of micro-nano plastics (MNPs) in environment is currently causing the biggest threat to biotic and abiotic components around the globe. These pollutants, apart from being formed through fragmentation of larger plastic pieces and are also manufactured for commercial usage. MNPs enter agro-ecosystem, wildlife, and human body through the food chain, ingestion or through inhalation, causing blockage in the blood-brain barrier, lower fertility, and behavioural abnormalities among other problems. Hence, it becomes essential to develop novel procedures for remediation of MNPs. Among the numerous existing methods, microbial remediation promises to degrade/recover MNPs via a green route. Since microbial remediation processes mostly depend upon biotic and abiotic factors such as (temperature, pH, oxidative stress, etc.), it becomes easy to influence changes in the plastic pollutants. Hence, with the help of recent technologies, a complete degradation/removal of MNPs can be expected by utilizing the respective carbon content as energy sources for growth of microorganisms. In this review, considering the urgent environmental need, the impact of micro-nano plastics on ecosystem along with its corresponding degradation mechanisms has been brought out. Also, importance of the various recent research approaches in MNPs remediation is highlighted. Finally, the role of enzyme and membrane technology, nanoparticle technology, and metagenomics in remediation of MNPs are discussed for the first time in detail to bring out a novel remedy for the environment.

1. Introduction: micro-nano plastics

Plastic pollution is regarded as one of the most significant threats to ecosystem affecting biotic and abiotic components around the world. In 2018, global plastic production almost reached 360 million tonnes. As per the latest available data, the global production of plastic reached almost 360 million tonnes in 2018. Asia is the largest producer and consumer of World's plastics. Among global plastic production, China contributes the highest portion (30%), followed by Europe (17%) and altogether 18% contribution is from Canada, Mexico, and US (https://www.plasticseurope.org/application/files/9715/7129/9584/FINAL_web_version_Plastics_the_facts2019_14102019.pdf). It is alarming that every year around 4.8 to 12.8 million metric tonnes of plastic debris are thrown into the ocean without proper waste management strategy and it is estimated to increase further in future (Jambeck et al., 2015; Lebreton et al., 2017). Highly polluted water bodies are from Asia. In China, the river Yangtze, the utmost polluted river, has an input plastic waste of approximately 310,000 tonnes, which is more than 4% of annual ocean plastic pollution. The second-largest polluted river with 115,000 tonnes of plastic waste is the river Ganges from India. It is pertinent to mention that petrochemical plastics contribute towards more than 80% of the worldwide plastic use. For example, polyethylene terephthalate (PET) Polyethylene (PE), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC) are the most used plastics (Urbanek et al., 2018). In spite of this, plastic materials form a fundamental part in worldwide economy; the issues related to its broad application can't be overlooked. Most of these plastics in environment are non-degradable and continue remaining in the environment for an extended period.

Among the plastic remains, broken small particles have garnered much attention and are of greater concern (Eerkes-

☆ This paper has been recommended for acceptance by Eddy Y. Zeng.

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MINI-REVIEW

Insights into microbial diversity on plastisphere by multi-omics

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Abstract

Plastic pollution is a major concern in marine environment as it takes many years to degrade and is one of the greatest threats to marine life. Plastic surface, referred to as plastisphere, provides habitat for growth and proliferation of various microorganisms. The discovery of these microbes is necessary to identify significant genes, enzymes and bioactive compounds that could help in bioremediation and other commercial applications. Conventional culture techniques have been successful in identifying few microbes from these habitats, leaving majority of them yet to be explored. As such, to recognize the vivid genetic diversity of microbes residing in plastisphere, their structure and corresponding ecological roles within the ecosystem, an emerging technique, called metagenomics has been explored. The technique is expected to provide hitherto unknown information on microbes from the plastisphere. Metagenomics along with next generation sequencing provides comprehensive knowledge on microbes residing in plastisphere that identifies novel microbes for plastic bioremediation, bioactive compounds and other potential benefits. The following review summarizes the efficiency of metagenomics and next generation sequencing technology over conventionally used methods for culturing microbes. It attempts to illustrate the workflow mechanism of metagenomics to elucidate diverse microbial profiles. Further, importance of integrated multi-omics techniques has been highlighted in discovering microbial ecology residing on plastisphere for wider applications.

Keywords Plastisphere · Microbes · Metagenomics · Next generation sequencing · Bioremediation · Multi-omics

Introduction

Ocean plastics

Since 2014, usage of plastic materials has been increasing worldwide with over 300 million tons in production every year (Eriksen et al. 2014). As a result, plastic waste has become a serious environmental concern in seas and oceans, posing immense threat to marine life and human health (Bansal et al. 2021). Despite widespread recognition of these threats, plastics are still abundantly released into oceans through various pathways like rivers, atmospheric transmission, beach littering, shipping, aquaculture and oil spills (Kershaw and Rochman 2015). These plastic wastes accumulate in marine ecosystems because of various characteristics such as prolonged durability, lower rate of recycling, improper waste management and prolonged use (Barnes et al. 2009). Figure 1 portrays an estimate of global plastic wastes across various ocean surfaces (adapted from Eriksen et al. 2014).

Plastic wastes disposed of in oceans can be classified into three categories based on size: macroplastics (having

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Biodegradation of micro sized nylon 6, 6 using *Brevibacillus brevis* a soil isolate for cleaner ecosystem

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ABSTRACT

The increasing accumulation of microplastic constitutes pathogens and hazardous chemicals posing a great threat to the ecosystem. Biodegradation is an eco-friendly strategy for the removal of these contaminants. The present investigation demonstrates bioremediation of nylon 6, 6 microplastics (NMPs) for the first time using a soil isolate called *Brevibacillus brevis* (*B.brevis*) by shake flask assay. 22 w/w % weight loss of nylon 6, 6 microplastics was noticed after 35 d of incubation with *B.brevis*. Upon interaction with microplastics, a change in the shape of the bacterium (rod to round) could be observed along with the size reduction of the microorganism. In the case of NMPs incubated in presence of this bacterium, irregular shapes of NMPs with cracks and holes could be visualized using SEM and TEM. TGA and FTIR analysis reported the disappearance of intermolecular hydrogen bonding of nylon 6, 6 posts microbial interaction. The release of various organic acids and enzyme/enzymatic activities of the bacterium was found to be higher in the presence of NMPs. The mass spectrometric analysis confirmed the release of adipic acid and hexamethylenediamine derivatives during aerobic biodegradation, indicating NMPs being the only carbon source for the growth of *B.brevis* in the reaction environment. The study addresses *B.brevis* as a viable bacterial source for the degradation of NMPs while at the same time attempting to understand the mechanism involved. A biodegradation pathway for *B.brevis* induced NMP degradation has been also proposed for the first time. This finding promises a potential approach for reducing the accumulation of nylon 6, 6 in the environment.

1. Introduction

Microplastics range in size from 0.1 μm to 5 mm and are either manufactured in the industries (Primary microplastics) or result from fragmentation of bulk plastic through biotic and abiotic factors (Secondary microplastics) (Auta et al., 2017a; Ritchie and Roser, 2018). These plastic contaminants accumulate widely onshore affecting the ecosystem at an alarming rate. Massive amount of plastic contaminants is released into the ocean from various land-based activities(Jambeck et al., 2015). For instance, out of the total plastic waste generated throughout the world, 18.3% is dumped into the oceans by automotive industries in India and other Southeast Asian nations. North America dumping plastic in oceans constitutes 17.2% of worldwide plastic waste generated (Ritchie and Roser, 2018). Recently, the International Union for Conservation of Nature (IUCN) has reported that yearly 15%–31% of primary microplastics out of 9.5 Mt of plastic contaminants enter into oceans("Marine plastic pollution," 2018).

Micro-nano plastics (MNPs) are challenging contaminants for remediation due to their inertness, high surface-area-to-volume ratio and hydrophobicity(Tiwari et al., 2020). Various microplastics like polypropylene (Browne et al., 2011), low density polyethylene (LDPE) (Dey et al., 2021)and polystyrene (PS) (Liu et al., 2021) have been isolated from seashores, sewage discharges and effluents with their effects being monitored on various ecosystems (Manabe et al., 2011; de Sáet al., 2015). In literature, biodegradation of microplastics has been scarcely reported and includes few candidates like PS, polyethylene (PE), LDPE and PP (Mohanan et al., 2020; Savoldelli et al., 2017). A recent study demonstrated the role of a *Pseudomonas* sp. ADL15 and *Rhodococcus* sp. ADL36 on degradation of polypropylene (PP) microplastics after 40 d of treatment with weight loss observed at 17.3% and 7.3%, respectively (Habib et al., 2020). Similar studies have been reported on PS degradation by *Pseudomonas* sp. DSM 50071 (Kim et al., 2020) and polyethylene microplastics degradation by *Zalerion maritimum*(Paço et al., 2017). All of these prove that microplastic

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Degradation of polyethylene microplastics through microbial action by a soil isolate of *BreviBacillus brevis*

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ABSTRACT

The fragmentation of polyethylene plastics into polyethylene microplastics (PEMPs) due to biotic and abiotic factors affects the environment. Extensive investigations have shown its implications upon accumulation in the living systems. In this study, *B.brevis* was employed to degrade PEMPs. *B.brevis*-mediated degradation process has shown a reduction in microplastic's dry weight by 19.8% over 35 days of treatment. The biodegradation was achieved by releasing laccase enzyme and organic acids onto the surface of PEMPs, which were quantified by UP-HPLC. Further, the changes in the morphological and structural changes on of PEMPs were observed by TEM, SEM, DSC, TGA, XRD, and FTIR analysis. Additionally, after the degradation, by-products were observed to contain small molecules such as 2-hexadecanone, decanone etc. The small molecules were resulted from the biodegradation of PEMPs and were further utilized for bacterial metabolism. These outcomes reveal the efficiency of *B.brevis* in PEMPs degradation.

1. Introduction

Global production and utilization of plastics have increased from 335 Mt in 2016 to 367 Mt in 2020 [1]. Plastic pollution in the ecosystem results from the use and production of plastics on a worldwide scale, endangering both the environment and human health [2]. It is estimated that around 3% of the world's plastic waste enters the ocean, while most remains in landfills [3]. Most of these plastics in the environment are non-biodegradable and persist for longer in various size ranges. Among these plastic fragments, particles of the size range (0.1 μm – 5 mm) are called microplastics. There are two types of microplastics: primary microplastics, which are microscopic particles that enter the environment as a direct result of being used as industrial abrasives or in cosmetic items, and secondary microplastics, the majority of which microplastics are found in the environment and are created by the aging of greater pieces into microplastics due to biotic and abiotic causes [4]. These microplastics are hazardous to the ecosystem and have attracted the scientific community's attention for their remediation.

Microplastics are circulated globally in the world's water bodies, including water columns, water surfaces, and deep inside sediments [5]. By different mechanisms, microplastics can be ingested by lower-level organisms to higher-level organisms, which also transfer associated pollutants, pathogens, and additives [6]. Polyethylene (PE) is a majorly manufactured polymer required in different industries, such as packaging products, containers and coating material [7]. Due to PE's strong recalcitrance and inert properties, it is exceedingly difficult to break down in the environment, even after being buried for a long time in a landfill [8]. The hydrophobicity of PE due to the linear carbon atom backbone, degree of crystallinity, and large molecular weight are the causes of its recalcitrance [9,10]. Polyethylene foils/wraps are used in agricultural activities such as to regulate the temperature, hold irrigation water in the soil, and shield the crops, but over time they disintegrate and break down into micro-sized and cause problems [11]. A study has shown that PEMPs change the behavioural responses of an insect herbivore, such as the attraction of fungus gnats toward a plant-soil system [12]. Exposure to PEMPs in human cells shows the cytotoxic and genotoxic impact on human peripheral lymphocytes even at the slightest concentration (25 μg/mL) of PEMPs [13]. A Similar study showed the accumulation of PEMPs in the tissues of crayfish caused histopathological changes and oxidative stress [14]. Tong et al., showed that PEMPs provide a surface for the attachment of *Helicobacter pylori* which promotes gastric injury and severe inflammation in the mice's intestinal tissue [15].

Microbial-assisted degradation of plastics has the potential to play an

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Significance of landfill microbial communities in biodegradation of polyethylene and nylon 6,6 microplastics

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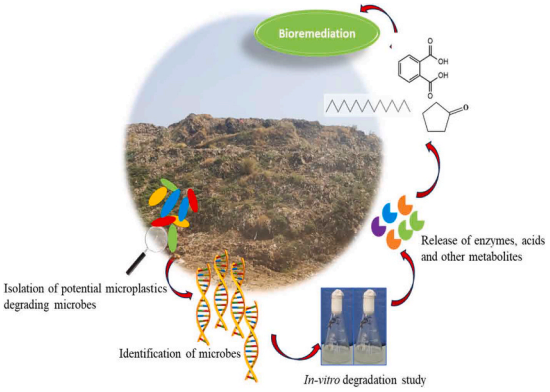
HIGHLIGHTS

- Metagenomics reveals plastic degrading soil bacterial community.
- Laccase and peroxidase plays significant role in microplastic degradation.
- Thermal studies demonstrated thermal stability and degradation of PEMPs and NMPs.
- Identification of alkanes, esters and aromatic, byproducts through GC-MS analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

Plastic pollution, particularly microplastics, poses a significant environmental challenge. This study aimed to address the urgent need for sustainable solutions to manage plastic waste. The degradation of polyethylene microplastics (PEMPs) and nylon 6,6 microplastics (NMPs) were investigated using bacterial culture isolates, isolated from a municipal landfill site and identified through 16 S rDNA as well as metagenomics techniques.The study demonstrated for the first time along with degradation mechanism. The isolates identified as *Achromobacter xylosoxidans* and mixed culture species in dominance of *Pulmonis sp.* were used to degrade PEMPs and NMPs. *Achromobacter xylosoxidans* reduced microplastic's dry weight by 26.7% (PEMPs) and 21.3% (NMPs) in 40 days, while the mixed culture achieved weight reductions of 19.3% (PEMPs) and 20% (NMPs). The release of enzymes, laccase and peroxidases revealed C-C bond cleavage and reduced polymer chain length. The thermal studies (TGA and DSC) revealed changes in the thermal stability and transition characteristics of microplastics. The structural alterations on PEMPs and NMPs were recorded by FTIR analysis. Byproducts such as alkanes, esters, aromatic compounds and carboxylic acids released were identified by GC-MS. These results suggest the effectiveness of bacterial isolates in degrading PEMPs and NMPs, with potential for sustainable plastic waste management solutions.

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