BIOREMEDIATION OF MICROPLASTIC USING MICROBES ISOLATED FROM MANGROVE SEDIMENTS

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BIOREMEDIATION OF MICROPLASTIC USING MICROBES ISOLATED FROM MANGROVE SEDIMENTS

ABSTRACT

The presence of microplastics and their continuous accumulation in the marine environment, poses a threat to the entire ecosystem. In this study, 22 bacteria were isolated from plastic/microplastic-inundated mangrove soil and were screened for the potential to degrade polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET) and polystyrene (PS) using the clear zone method. Nine isolates grew and demonstrated significant clear zones on synthetic media containing the different microplastics as carbon source. Shake flask experiments were carried out to further evaluate the biodegradability potential of the isolates individually and in consortia. Similarly, biodegradation experiments were carried out to evaluate the effect of different inoculum concentrations of the microbes on microplastic biodegradation. Degradation was monitored by recording the weight loss of the different microplastics and evaluating the growth response of the isolates to different times of exposition in Bushnell-Haas broth. The degradation extent was validated by assessment of the morphological, structural and surface changes through SEM and FTIR analyses. The results revealed that 6.2% of PE microplastics was reduced by *Bacillus gottheilii*. Maximum degradation of PP microplastics (6.4%) was attained by *Rhodococcus ruber*, while the maximum weight loss of PS (7.4%) and PET (6.6%) was recorded on treatment with *Bacillus cereus*. Engineering the microbes into consortia of different treatments (A, B, C, and D) formed from the combination of the microbes showed that maximum weight loss of PE (1.4%) and PET (1.2%) was attained on treatment with Treatment D (which consisted of all gram-positive bacteria) while maximum weight loss of PP (8.8%) and PS (21.4%) was attained by Treatment A (consisting of all nine bacteria). On treatment with different concentrations of the microbes, highest weight loss of PET microplastics by Treatment D

(7.2%) was achieved on treatment with 40% v/v inoculum concentration while highest weight loss for PS microplastics by Treatment A (30.8%) was attained on treatment with 10% v/v inoculum concentration. Bioremediation of soil contaminated with PET and PS microplastics and bioaugmented with 10% v/v of Treatment A was studied for a period of 90 days under field conditions in mangrove soil using the soil burial technique. At the end of 90 days, significant reduction in weight of both microplastics was observed in the microbially amended and the unamended soil portions. Maximum weight loss (17.8%) was observed in PET microplastics subjected to microbial treatment (microbially amended portion) while maximum weight loss of 19% was observed in PS microplastics buried in the unamended soil, suggesting that mangrove soil harbours a diversity of microbes with potential to degrade microplastics. SEM and FTIR analyses showed major structural, morphological, and surface changes and the formation of bacterial biofilm on the microplastic surfaces after degradation. The study thus, confirmed the ability of the marine microbes to utilize PE, PP, PET, and PS microplastics as carbon source and indicate positive potential towards remediation of microplastic-contaminated environments.

Keywords: microplastics, biodegradation, bacteria, marine, pollution

BIOREMEDIASI MIKROPLASTIK MENGGUNAKAN ISOLAT MIKROB DARI SEDIMEN PAYA BAKAU

ABSTRAK

Kehadiran dan akumulasi mikroplastik yang berterusan di persekitaran laut, mengancam keseluruhan ekosistem. Dalam kajian ini, 22 bakteria telah diasingkan daripada plastik/mikroplastik yang dikumpul dari kawasan paya bakau dan disaring untuk mengetahui potensi degradasi mikroplastik *polyethylene* (PE), *polypropylene* (PP), *polyethylene terephthalate* (PET) dan *polystyrene* (PS) dengan menggunakan kaedah *clear zone*. Sembilan isolat membiak dan menunjukkan *clear zones* yang signifikan pada media sintetik yang mengandungi mikroplastik yang berbeza sebagai sumber karbon. Eksperimen kelalang goncang telah dijalankan untuk penilaian lanjut terhadap potensi biodegradabiliti isolat secara individu dan di dalam konsortia. Eksperimen biodegradasi juga dijalankan untuk menilai kesan kepekatan inokulum mikrob yang berbeza terhadap biodegradasi mikroplastik. Degradasi telah dipantau melalui rekod penurunan berat microplastik yang berbeza dan dengan menilai tindak balas pertumbuhan isolat kepada eksposisi masa yang berbeza di dalam kaldu *Bushnell-Haas*. Tahap degradasi telah disahkan oleh penilaian perubahan morfologi, struktur dan permukaan melalui analisis SEM dan FTIR. Keputusan menunjukkan *Bacillus gottheilii* berjaya menurunka 6.2% mikroplastic PE (6.2%). Degradasi maksima mikroplastik PP (6.4%) dicapai oleh *Rhodococcus ruber*, manakala penurunan berat maksima PS (7.4%) dan PET (6.6%) dicatatkan oleh rawatan *Bacillus cereus*. Gabungan mikrob kepada konsortia rawatan yang berbeza (A, B, C, dan D), menunjukkan bahawa penurunan berat maksima PE (1.4%) dan PET (1.2%) tercapai oleh Rawatan D (yang mengandungi semua bakteria gram positif) manakala penurunan berat maksima PP (8.8%) dan PS (21.4%) telah dicapai oleh Rawatan A (yang mengandungi sembilan bakteria). Pada rawatan dengan kepekatan mikrob yang berbeza, penurunan berat mikroplastik PET tertinggi oleh Rawatan D (7.2%)

dicapai pada kepekatan inokulum 40% v/v manakala penurunan berat paling banyak untuk mikroplastik PS oleh Rawatan A (30.8%) telah dicapai pada rawatan dengan kepekatan inokulum 10% v/v. Ujian lapangan bioremediasi tanah yang tercemar dengan mikroplastik PET dan PS dan bioaugmentasi dengan 10% v/v Rawatan A dijalankan 90 hari menggunakan kaedah penimbusan. Pada akhir 90 hari, pengurangan jisim kedua-dua microplastik rekod di bahagian tanah yang diubahsuai dengan mikrob dan bahagian tanah kawalan tidak diubahsuai. Pengurangan berat maksima (17.8%) rekod pada mikroplastik PET yang dirawat dengan mikrob manakala penurunan berat maksima 19% diperhatikan pada mikroplastik PS kawalan. Ini menunjukkan bahawa tanah paya bakau mempunyai pelbagai mikrob yang berpotensi untuk mendegradasikan mikroplastik. Analisis SEM dan FTIR menunjukkan perubahan ketara terhadap struktur, morfologi, dan permukaan serta pembentukan *biofilm* bakteria pada permukaan mikroplastik selepas degradasi. Oleh itu kajian ini mengesahkan keupayaan mikrob marin untuk menggunakan mikroplastik PE, PP, PET, dan PS sebagai sumber karbon dan menunjukkan potensi yang positif ke arah pemulihan persekitaran yang tercemar dengan mikroplastik.

Kata kunci: mikroplastiks, biodegradasi, bakteria, marin, pencemaran

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LIST OF SYMBOLS AND ABBREVIATIONS

β	:	Beta
^{o}C	:	Degree centigrade
>	:	Greater than
∞	:	Infinity
<	:	Less than
%	:	Percentage
±	:	Plus-minus
AFM	:	Atomic force microscopy
Ag	:	Silver
Al	:	Aluminium
ASTM	:	American Society for Testing and Materials
ATR-FTIR	:	Attenuated total reflection-Fourier transform infrared
ATP	:	Adenosine triphosphate
BHB	:	Bushnell Haas broth
BPA	:	Bisphenol A
CFU	:	Colony forming units
DBP	:	Di-n-butyl phthalate
DDT	:	Dichlorodiphenyltrichloroethane
DEHP	:	Diethyl hexyl phthalate
EDS	:	Energy dispersive X-ray analysis
EQ	:	Equation
Fe	:	Iron
g	:	gram
GC	:	Gas chromatography

GESAMP	:	Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection
HDPE	:	High density polyethylene
HELCOM	:	Baltic Marine Environment Protection Commission-Helsinki Commission
HIPS	:	High input polystyrene
HPLC	:	High performance liquid chromatography
IF:	:	Inoculum fluid
kg	:	Kilogram
LDPE	:	Low density polyethylene
LMWPE	:	Low molecular weight polyethylene
MAP	:	Mediterranean Action Plan
meq	:	Milliequivalent
mg	:	Milligram
ml	:	Milliliter
Mn	:	Manganese
MSW	:	Municipal solid waste
NA	:	Nutrient agar
NB	:	Nutrient broth
NMR	:	Nuclear magnetic resonance
NGOs	:	Non-governmental organizations
OD_{600}	:	Optical density at 600 nm
OSPAR	:	Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic
PAHs	:	Polycyclic aromatic hydrocarbons
PBA	:	Poly-butyl acrylate
PBS	:	Polybutylene succinate
PBDEs	:	Polybrominated diphenyl ethers
PCA	:	Plate count agar

PCBs	:	Polychlorinated biphenyls
PCL	:	Polycaprolactone
PE	:	Polyethylene
PET	:	Polyethylene terephthalate
PLA	:	Polylactic acid
PLGA	:	Poly(lactic-co-glycolic) acid
PHB	:	Poly-B-hydoxybutyrate
PP	:	Polypropylene
ppm	:	Parts per million
ppt	:	Parts per thousand
PS	:	Polystyrene
POPs	:	Persistent organic pollutants
PU	:	Poly(urethane)
PVC	:	Polyvinyl chloride
3Rs	:	Reduce, reuse, recycle
rpm	:	Rotation per minute
sp	:	Species
SEC	:	Size exclusion chromatography
SEM	:	Scanning electron microscopy
TGA	:	Thermogravimetric analysis
UNEP	:	United Nations Environmental Program
UV	:	Ultra-violet
WWTP	:	Wastewater treatment plant
Zn	:	Zinc

CHAPTER 1: INTRODUCTION

1.1 Background of Study

Marine litter, especially plastics, has become a global problem that is affecting all parts of the marine ecosystem (Leslie *et al.*, 2017; Maes *et al.*, 2017). It arises mainly from activities on land and poses health, economic and environmental problems that emanates from indiscriminate human activities and behaviour, and poor waste management practices, and in some cases, lack of good infrastructure (Halstead *et al.*, 2018; Lwanga *et al.*, 2018). Additionally, marine litter has a negative impact on marine organisms, causing potential effects to human health through the transfer of chemicals/toxins up the food chain. This litter threatens the life in the marine ecosystem, overall public health, and the global economy. About 80 - 85% of marine litter is plastic (Essel *et al.*, 2015; Fossi *et al.*, 2017).

Plastics are man-made synthetic or semi synthetic long chain, malleable high molecular weight polymeric materials that are moisture resistant, durable, strong corrosion resistant, flexible, versatile, lightweight, inexpensive and persistent. Plastics are made from petrochemicals- although some are cellulose-based. They are insoluble in water and are resistant to many environmental influences (Gasperi *et al.*, 2015; Yoshida *et al.*, 2016; Zalasiewicz *et al.*, 2016). The unique properties of plastics favour their use in a great number of applications such as clothing, cosmetics, household goods, detergents, construction materials, personal goods, chemicals, food wrappings/packaging, disposable gloves, and medicine encapsulations (Van Cauwenberghe *et al.*, 2015; Zalasiewicz *et al.*, 2016). These unique properties of plastics also make them resistant against microbial attack (Shah *et al*., 2008). Approximately 30% of plastics are used globally for packaging applications (Sabir, 2004). The most widely used plastics in packaging include polystyrene (PS), polyethylene terephthalate (PET),

polylactic acid (PLA), polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), polyurethane (PUR), poly (butylene terephthalate (PBT), and nylons (Gewert *et al.*, 2015; Shah *et al.*, 2008). The widespread use of plastic materials has changed the nature of waste in the last three decades (Harshvardhan & Jha, 2013), and their disposal has become a major challenge to environmental protection as pollution by plastics constitutes a hazard to the entire ecosystem. It has attracted great public and media attention than any other component of the municipal solid waste (MSW), from scientists and the general public (Pettipas *et al.*, 2016). The accumulation of plastic waste in nature is one of the most recent ubiquitous and long-lasting changes to the surface of our planet, and one of the most pervasive environmental concerns at the moment.

The global demand for plastics yearly has continually increased over the recent years. In 1950, the total world demand for plastics was 35 million tonnes. In 2010, plastic production rose to 270 million tonnes. In 2015, world plastic production grew by 3.4% compared to 2014 (311 million tonnes). Presently, global plastic production stands at about 322 million tonnes as stated by (PlasticsEurope, 2016), and estimates has it that approximately 10 - 20 million tonnes of plastic waste end up in the ocean yearly due to improper disposal/inadequate management of waste (Boucher *et al.*, 2016). Large amounts of accumulated plastic waste in the marine environment has led to the emergence of a new type of contaminant in the environment, referred to as microplastics (Woodall *et al.*, 2014).

Microplastics, plastic particles with less than 5 mm particle size (Espinosa *et al.*, 2018; Fossi *et al.*, 2016; Kershaw, 2015), are ubiquitously present in marine ecosystems and makes up approximately 92.4% of plastic waste (Santana *et al.*, 2016). They consist mainly of PE, PP, PS, PVC, nylons and PET polymers (Auta *et al.*, 2017a; Carr *et al.*, 2016; Espinosa *et al.*, 2018). Microplastics are hardly degradable and therefore, persist in

the environment and are often very resistant to degradation induced by microorganisms (Yoshida *et al.*, 2016). Hence, microplastics are globally distributed across estuarine and marine ecosystems; occurring in surface waters, bottom sediments, water columns, along shorelines, and even in ice, from the Artic to the Antarctic (Avio *et al.*, 2017a; Espinosa *et al.*, 2018; Felsing *et al.*, 2018; Lusher, Tirelli*, et al.*, 2015; Peng *et al.*, 2018; Van Cauwenberghe *et al.*, 2015).

Pollution of the marine environment by microplastics has emerged as a global threat that is causing great concern and has captured great attention recently. Microplastics originate from different sources. Those that are intentionally produced in micro/smaller size as plastic pellets/nurdles, fibres, and microbeads, often manufactured for applications such as cosmetic products, toothpaste, exfoliating scrubs, and clothing, which are referred to as primary microplastics. Secondary microplastics are those that originate from the fragmentation of larger plastic debris by ultra violet (UV) radiation or turbulence (Auta *et al.*, 2017a; Ballent *et al.*, 2016; Felsing *et al.*, 2018). Microplastics enter into the ocean through several marine and terrestrial-based activities. Microbeads and fibres present in toothpaste, cosmetics and clothing enter into the aquatic environment from wastewater treatment plants and through domestic and industrial drainage systems. The degradation of larger plastic debris from waste dumps or landfills also serve as a source of microplastics into the oceans (Alomar *et al.*, 2016; Murphy *et al.*, 2016). When larger plastic particles fragment into smaller particles, the abundance of microplastics increases. Increase in the abundance of microplastics in the marine environment increases their encounter rate with marine biota (Cole *et al.*, 2013). Studies have demonstrated the increasing distribution of microplastics in marine environments worldwide. About 43,000 - 466,000 particles km^{-2} have been recorded in the Laurentian Great Lake (Eriksen *et al.*, 2014). Isobe *et al.* (2015) on the other hand, recorded high concentrations of about 1.72 billion pieces km^{-2} in the East Asian seas around Japan. Approximately 3000 pieces km^{-}

^{2} have been excavated from marine environments in Peninsular Malaysia (Fauziah *et al.*, 2015; Jayanthi *et al.*, 2014).

Microplastics have been consumed by a wide-range of marine organisms- from filter organisms, invertebrates, mammals, birds, and commercially important fish and shellfish, and the potential interference with the food chain has been demonstrated (Halstead *et al.*, 2018; Kärrman *et al.*, 2016). The pollutant is also known to adsorb and accumulate metals and persistent organic pollutants (POPs) from the surrounding environment thereby, serving as active carriers for heavy metal and POP contamination from the marine environment (Brennecke *et al.*, 2016; Fu *et al.*, 2018). The chemicals cause additional problems to the organisms. Some bacteria have been observed to colonize microplastics through biofouling indicating that microplastics can also serve as vehicles for the introduction of pathogenic bacteria into new environments (Bryant *et al.*, 2016; Jiang *et al.*, 2018).

Studies have demonstrated the increasing distribution of microplastics globally in aquatic ecosystems, especially in sediments. High concentrations of microplastics have been reported at the five oceanic gyres namely, North Pacific, South Pacific, South Indian, North Atlantic and South Atlantic (Nerland *et al.*, 2014), and they have ubiquitously permeated the marine environment including beaches, estuaries, rivers, and mangroves. The deposition of sediments into mangroves occur from different sources, and as with sediments in other aquatic environments, microplastics similarly accumulate in mangrove sediments (Jayanthi *et al.*, 2014; Smith, 2012).

Mangrove forests are among the most productive and biologically complex ecosystems on earth that plays a unique and crucial role in the socioeconomic well-being of coastal communities that depend on its harvested resources and services (Chan & Salleh, 1987). They play an important role in guarding coastal communities from storms,

flood and erosion, provide shelter and serve as a food resource. While mangrove forests have been known to be crucial to both animals, plants and humans, they have been under severe threat by anthropogenic activities. Studies have shown that more than 35% of mangrove forest areas have been destroyed in the past two decades (Bulow & Ferdinand, 2013) and have become the favorite dumping yards for solid waste. The lack of proper waste management/disposal system has made them favourable dumping yards for solid waste. Of more concern is the indiscriminate dumping of plastic waste in such areas, which has resulted in the pollution and loss of mangrove ecosystems globally. Despite the increasing awareness of the importance of mangrove forests, their destruction continues to occur for a variety of economic and political reasons. Mangrove environments and their microbial community are considered as areas of high biological productivity as they are known to harbour a variety of microorganisms (Thatoi *et al.*, 2012) which play significant roles in various environmental activities and applications. The high temperature, salinity, pH, and organic matter levels, as well as, low aeration and moisture makes it conducive for the distribution of diverse bacteria (Ghizelini *et al.*, 2012).

Since some microorganisms are capable of transforming and mineralizing plastics, microbial biodegradation is a viable method for remediation of contaminated soils (Ji *et al.*, 2013). Most microbes are opportunistic and have innate cability to adapt in every environment. Microbes also have the potential to transform a variety of compounds including plastic polymers. During polymer degradation, the microbes first of all adhere to the polymer surface which exposes it to microbial colonization (Dussud & Ghiglione, 2014). This is followed by extracellular enzymes secretion which bind to the plastic polymer and causes hydrolytic cleavage. The polymer is then degraded into monomers and oligomers and mineralized to carbon dioxide (CO_2) and water (H_2O) used by the microbe as energy source (Tokiwa *et al.*, 2009). Microplastic particles in the

microorganism pass through the cellular membrane and are broken down inside the cells of the microorganism by cellular enzymes that have the ability to utilize a particular polymer (Gewert *et al.*, 2015). Utilizing microorganisms to degrade microplastics will increase their biodegradation rate without causing harming the environment (Bhardwaj *et al.*, 2012). Therefore, identifying microbes that can degrade microplastics is a promising and environmentally safe strategy which could help the natural bioremediation process, and influence the remediation of natural ecosystems with no adverse impacts. Bioremediation accelerates natural attenuation by optimizing the limiting conditions, which is a non-destructive treatment, it is cost-effective as well as a logistically favorable remediation technology (Margesin *et al.*, 2007). Microorganisms possess special 'jumping genes' that enable them to develop biological resistance against toxic substances in the environment (Sinha *et al.*, 2009). Hence, while a number of them may not survive due to high toxicity, some develop resistance and survive and can be cultured for further use (Sinha *et al.*, 2009). When conditions are favorable, microbes can biodegrade/biotransform complex hazardous organic substances into simpler and harmless ones. Several success stories of the use of microbial technique in the clean-up of contaminated environments have been reported (Abioye *et al.*, 2010; Auta *et al.*, 2014; Emenike *et al.*, 2016). Whilst bioremediation is a well established technology for the removal of organic contaminants, the use of microbes to degrade contaminants like microplastics is still being investigated. A few research have been made on the biological degradation of microplastics using microrganisms due to their versatile metabolic abilities that enable them to degrade a diverse range of materials (Auta *et al.*, 2017b; Auta *et al.*, 2018; Paço *et al.*, 2017). Bioremediation, because of its cost-effectiveness and environmental acceptability, has been shown to be a practical method of remedying plastic-contaminated soil. Since bacteria are usually the agents in most bioremediation

processes, the evaluation of a polluted site before bioremediation often involves detection and enumeration of the quantity and activity of polymer-degrading bacteria (Jiang, 2013).

1.2 Problem Statement

Pollution of the environment with plastics is a growing problem and is expected to persist for hundreds and thousands of years. Malaysia is one of the largest producers of plastics in Asia with over 1550 manufacturers (Malaysia Investment Development Authority, 2011). There is an increasing evidence of extensive abundance and pollution of the marine environment, especially mangrove ecosystems, by microplastics in Malaysia (Jayanthi *et al*., 2014), as findings have provided a clearer understanding of the presence and distribution of plastic debris (Fauziah *et al*., 2015). Mangrove forest is the dominant coastal vegetation community in Malaysia that has been under threat from various human anthropogenic activities and indiscriminate dumping of garbage which has resulted in the pollution of the environment by macro and microplastics. Unlike macroplastics, microplastics are sometimes not visible to the naked eye and are globally distributed across estuarine and marine ecosystems, and their concentrations have increased significantly in the surface and benthic zones of the ocean in the last four decades. Concern about the potential impact of microplastics in the aquatic environment has significantly increased during the past few years and the number of scientific investigations has increased, along with public interest and pressure on decision makers to respond. Plastic waste can lead to suffocation and entanglement of marine animals and even starvation. Other impacts include decreased immune response, impaired reproductive activity, cancer and malformation in humans and animals (Cole *et al.,* 2013; Setala *et al*., 2016). Several studies have demonstrated the wide and increasing distribution of microplastics globally and their impact on a wide range of marine biota including shrimps, whales, birds, bivalves, zooplankton, and killing of commercial fish,

and reports have it that if action is not taken, the oceans will contain more tonnes of microplastics than of fish by the year 2050 (World Economic Forum, 2016). Microplastics can accumulate in the tissues of marine organisms and are also known to accumulate toxic contaminants such as PCBs, dioxins, DDT, and bisphenol A (Cole *et al*., 2011). This causes an additional impact on marine organisms and even terrestrial life such as humans. Plastic waste is the third highest waste generated in Malaysia (Agamuthu *et al.*, 2011), thus the potential of macro- and microplastics to contaminate the surrounding environment is very high making the disposal of plastic material a huge waste management concern. The small size and less visibility of microplastics make it extremely difficult to remove manually.

Numerous scientific studies have examined the distribution, ingestion, fate, behaviour, quantification, and effects of microplastics to fill knowledge gaps, but until now, clear methods for microplastic clean-up and/or remediation remains inconclusive. Microorganisms play a role as drivers of the global functioning of the aquatic biosphere and also serve as apparent mediators of the biodegradation of a variety of compounds including contaminants, plastic-associated additives or even the microplastics themselves. The excessive use of plastics and the high accumulation of microplastics in the marine environment necessitates the need to carry out investigation into the microbial biodegradation of microplastics, the structure, and the biodegradation activities of microorganisms in order to understand the responses of marine organisms to microplastics. This may possibly lead to solutions to the disturbing accumulation of microplastics, and to project management decisions aimed at remedying and keeping safe the ecological integrity of mangrove ecosystems in Malaysia, as well as, ensuring food security within the aquatic food supply chain.

1.3 Research Hypothesis

Bacteria isolated from mangrove sediments have bioremediation capability for microplastic contaminated mangrove soil. Therefore, the microbial consortium/cocktail produced will enhance the degradation of microplastics in the environment.

1.4 Research Objectives

The aim of this research work is to investigate the biodegradability potential of microbes isolated from mangrove area to degrade microplastics and to evaluate the potential of the microbes to remediate microplastic contaminated soil.

Therefore, the following are the objectives of the study:

i. To isolate and identify microbes from selected mangrove environments in Peninsular Malaysia.
ii. To screen the isolates for the ability to degrade microplastics.
iii. To conduct biodegradation studies on selected microplastics using individual microbes in monocultures and in consortia.
iv. To formulate potential microbial cocktail (bio-addition) that can efficiently degrade microplastics in contaminated soil.
v. To develop kinetic model for microplastic degradation during bioremediation experiments.
vi. To carryout bioremediation of microplastics in the field (*in situ*).

CHAPTER 2: LITERATURE REVIEW

2.1 Background of solid waste (MSW)

Solid wastes (most commonly known as trash, refuse or garbage) are wastes arising from the activities of humans and animals that are termed as useless or unwanted. They consist of everyday items that are used and discarded such as furniture, clothing, paint, appliances, batteries, bottles, plastic containers, food scraps, grass clippings, product packaging, newspapers, etc. (Shin, 2014). They are waste materials arising from agricultural, industrial, commercial, institutional, domestic, as well as public services (Johari *et al.*, 2014). These wastes inevitably place massive strain on natural resources and impairs sustainable and efficient development. Efficient management of solid wastes is one of the ways that can salvage the situation (Shin, 2014).

MSW management comprises all activities including waste generation, storage, collection, transportation, processing, treatment and disposal of waste materials in a manner that best addresses the range of aesthetic, public health, economics, conservation, engineering, and other environmental considerations (Emenike, 2013). Municipal solid waste management include planning, administrative, engineering, financial, and legal functions.

MSW management systems in developing nations display an array of challenges which include irregular collection services, low collection coverage, open dumping and burning without air and water pollution control, etc. this is as a result of urbanization coupled with lack of infrastructure, appropriate system and associated training, awareness, and commitment (Kawai & Tasaki, 2016; Marandi & Ghomi, 2016). These problems are as a result of technical, institutional, economic, financial, and social factors which impel the development of effective MSW management systems (Manaf *et al.*, 2009; Ogawa, 2008).

2.2 Solid waste generation in Asia

Out of the 12 billion tonnes of MSW generated worldwide, approximately 4.4 billion tonnes come from Asia (Agamuthu *et al.*, 2011). Population growth can increase the amount of municipal solid waste (MSW) significantly. Seasonal variation is another important factor that can influence the amount of waste generation (Agamuthu *et al.*, 2011). Waste generation rate increased from 0.7 kg/person in the 1990s to 1.2 kg/person in the year 2000 in developing countries like Malaysia (Agamuthu, 2001; Agamuthu *et al.*, 2011). In Malaysia, the generation of solid waste is expected to reach 1.5 kg/day in most cities. Hong Kong generated approximately 6.45 million tonnes of solid waste in 2009, which is twice that of 1990 levels (Agamuthu *et al.*, 2011). About 0.7 kg was generated in Vietnam and Laos, while Indonesia and Bangladesh recorded an average daily per capita waste generation of 0.75 kg and 0.25 kg, respectively. India and Pakistan on the other hand, generated 0.4 kg each (Agamuthu *et al.*, 2011). Variations and rise in per capita generation of waste is highly dependent on the country's socio-economic inclinations. The quantity of solid waste generated is much higher in urban areas than in rural areas. The generation rate in urban areas can reach up to 1.0 kg/cap/day while in rural areas, it can be as low as 0.15 kg/cap/day, (Agamuthu *et al.*, 2011).

2.3 Municipal solid waste composition

The waste composition of a country undergoes change as the country become more developed and urbanized. The most notable feature is the increase in the paper, plastic packaging, consumer products, multi-material packaging items. The composition and characteristics of MSW is influenced by the area (commercial, residential, industrial, etc.), the weather and season (amount of population during the year, tourists to places), the economic level (low-income and high-income levels), and the culture of the people living or doing business in the area (Wilson *et al.*, 2015). Low income areas produce more

of organic waste (e.g. leftover food), while high-income areas produce wastes that are usually more inorganic in nature such as paper and plastics (Abur *et al.*, 2014).

In low income areas, paper waste makes up an average of about 11% to 19% of the MSW, while in high income areas 24% of MSW is paper (Wilson *et al.*, 2015). These figures are in line with data on the annual per capita consumption of paper globally which ranges from 4 kg in Africa, 40 kg in Asia, 140 kg in Europe, and 240 kg in North America (Wilson *et al.*, 2015). Plastic on the other hand, makes up an average of 12 % to 20% of MSW globally. The levels of other waste materials like metals, textiles and glass are relatively low. Other components of MSW include hazardous substances often referred to as household hazardous wastes (HHW). Typical sources include waste electrical and electronic equipment (WEEE or e-waste), paints, batteries, vanishes, cleaning agents (disinfectants), solvents (nail vanish), pesticides (rat poison), motor oil (heating or roofing blankets), wood, preservatives, etc., where these make up 1%, but could be up to 5% if e-waste is included (Wilson *et al.*, 2015).

2.4 Municipal solid waste composition in Asian countries

In Cambodia, organic, paper and plastic wastes make up 63%, 6.4% and 16%, respectively. Philippines recorded about 33% of organic waste, 12% paper waste and 25% plastic waste. The Republic of Japan and Korea recorded an average of 26%, 21% and 8.9% of organic, paper and plastic wastes, respectively. China's waste stream is composed of 55.86% of organic wastes, 8.52% paper, 3.16% textiles, 2.94% wood waste and 11.15% plastic waste (Agamuthu *et al.*, 2011; Borongan & Okumura, 2010; Budhiarta *et al.*, 2012; Zhou *et al.*, 2014). Approximately 30,000 metric tonnes of MSW is generated in Malaysia on a daily basis. The dominant components are vegetable and food waste and these constitutes approximately 50% of the total waste stream, paper and

plastic waste accounts for 18% and 13%, respectively (Das, 2017). Table 2.1 shows the percentage composition of MSW in Malaysia for the year 2000 to 2012.

Table 2.1: Waste composition (%) in Malaysia from 2000 – 2012 (Emenike, 2013; Hamid *et al.*, 2015)

Waste composition	2000	2005	2010	2012
Organic	43.2	45.0	55.0	44.5
Paper	23.7	16.0	13.0	8.5
Plastics	11.2	24.0	20.0	13.2
Glass	3.2	3.0	2.0	3.3
Metal	4.2	3.3	3.0	2.7
Others	12.3	15.0	3.0	15.7

Approximately 6 million tonnes of MSW enters into the ocean on a yearly basis, 80% of which are plastics (Avio *et al.*, 2017a; Eagle *et al.*, 2016). Plastics are non-biodegradable and can therefore last for several decades.

2.5 Plastic and its composition

Plastics are long chain polymeric molecules (Scott, 1999; Shah *et al.*, 2008). They are synthetic or semisynthetic organic polymers that are durable, light weight, malleable, cheap, corrosion resistant, strong and extremely versatile (Li *et al.*, 2016; Thompson *et al.*, 2009). The word plastic comes from the Greek word "plastikos" meaning 'able to be moulded into different shapes' (Kale *et al.*, 2015). Plastics are made from organic and inorganic raw materials such as hydrogen, oxygen, nitrogen, carbon, silicon, and chloride (Kale *et al.*, 2015; Shah *et al.*, 2008). The term plastic covers a wide range of polymeric materials like rubbers, thermosets, textiles, technical elastomers, and technical fibres, with some 200 plastic families in production including polyvinyl chloride (PVC), polystyrene (PS), polyurethane (PUR), polypropylene (PP), nylon, polyethylene terephthalate (PET), acrylonitrile butadiene styrene (ABS), polyethylene (PE), polyvinyl alcohol (PVA), and polyamide (PA) (Shah *et al.*, 2008).

Synthetic plastics are used in several applications such as packaging products (food, cosmetics, detergents, pharmaceuticals, and chemicals), eating utensils, and trash bags (Gewert *et al.*, 2015). Approximately 30% of plastics are used globally for packaging applications. Their better physical and chemical properties, such as lightness, strength, resistance to water, cold, and heat, have made them to be more favored than cellulose-based products and paper (Shah *et al.*, 2008).

Basic materials used for the production of plastics are extracted from natural gas, coal, crude oil, salt, and biorenewable (Bowmer & Kershaw, 2010; IOC & Protection, 2010; PlasticsEurope, 2016). Plastics are principally made up of binders, fillers, plasticizers, pigments/dyes, and other additives (Fox, 2008). Binders gives plastic its main characteristics and its name. They may be natural materials such as casein, milk protein, or cellulose derivatives, but are more commonly synthetic resins. Binder materials contain very long chainlike molecules referred to as polymers. Synthetic resins are built up or polymerized from simple molecules called monomers. Plasticizers are additives, most commonly phthalate esters in PVC applications. They are added to a binder to increase toughness and flexibility. Almost 90% of plasticizers are used in PVC which gives the material durability and flexibility (Fox, 2008). Examples of plasticizers include phthalates, adipates, alkylphenols (Teuten *et al.*, 2009). Fillers are added to improve perfomance, reduce production cost, or to improve properties such as shock resistance or hardness. Examples of fillers include retardants, chalk, zinc oxide, ivory dust, wood flour, starch and cellulose. Pigments/dyes impart various colors to the plastics (Callister Jr & Rethwisch, 2012). Virtually, any desired shape or color, durability, elasticity, hardness, resistance to cold, heat and acid can be obtained in a plastic (Callister Jr & Rethwisch, 2012).

2.5.1 Types of plastics

Plastics are usually classified according to the chemical structure of the polymer's backbone and side chains. Examples include polyesters, silicones, acrylics, poly-urethanes and halogenated plastics. Plastics can also be classified based on the chemical process used in their production, they can be classified based on qualities that are relevant for manufacturing or product design, and on properties such as density, high tensile strength, and resistance to various chemical products (Lalit & Haripada, 2013; Shah, 2007). Different types of plastics exists which are grouped into thermosets and thermoplastics.

Thermoplastics are linear chain macromolecules that have their atoms and molecules joined end-to-end into a series of long, sole carbon chains (Lalit & Haripada, 2013). They do not undergo chemical change in their composition when heated and can therefore, be molded repeatedly. Thermoplastics soften on heating and harden on cooling. When frozen, however, thermoplastics becomes glass-like and subject to fracture. They can be reheated, reshaped, and frozen repeatedly; a quality that makes them mechanically recyclable. Examples of thermoplastics include PS, PET, PP, and PVC (PlasticsEurope, 2016). Thermoset plastics on the other hand are formed by polymerization under suitable conditions which allows bi-functional molecules to condense inter-molecularly with the production of small by-products such as H_2O and HCl (Lalit & Haripada, 2013). These polymers have infinite molecular weight. The chains are made of several repeating molecular units derived from monomers; with each polymer chain having several thousands repeating units. Thermosets are known to melt and can be molded into different shapes. When they are solidified, they become solid. Example of thermosetting process is the vulcanization of rubber. Examples include ureas, polylactic acid, poly-urethane, etc. (Lalit & Haripada, 2013; PlasticsEurope, 2016).

2.6 Global plastic production and associated plastic waste generation

Plastic production world-wide has been growing as the flexible, durable, primarily petroleum-based material gradually replaces metal and glass. For more than 50 years since plastic came into existence, global production has continued to rise (Figure 2.1). Global plastic production rose to 280 million tonnes in 2011, representing around 4% increase from 2010 when 270 million tonnes of plastic were produced (PlasticsEurope, 2016). In 2013, approximately 299 million tonnes were produced, also representing a 4% increase over 2012. In 2015 however, annual global plastic production ramped up from 211 million tonnes to 322 million tonnes, representing a 3.4% increase compared to 2014 (Felsing *et al.*, 2018; PlasticsEurope, 2015b). Of the plastics produced worldwide, about 8 million metric tonnes ends up in the waste stream on a yearly basis (PlasticsEurope, 2016).

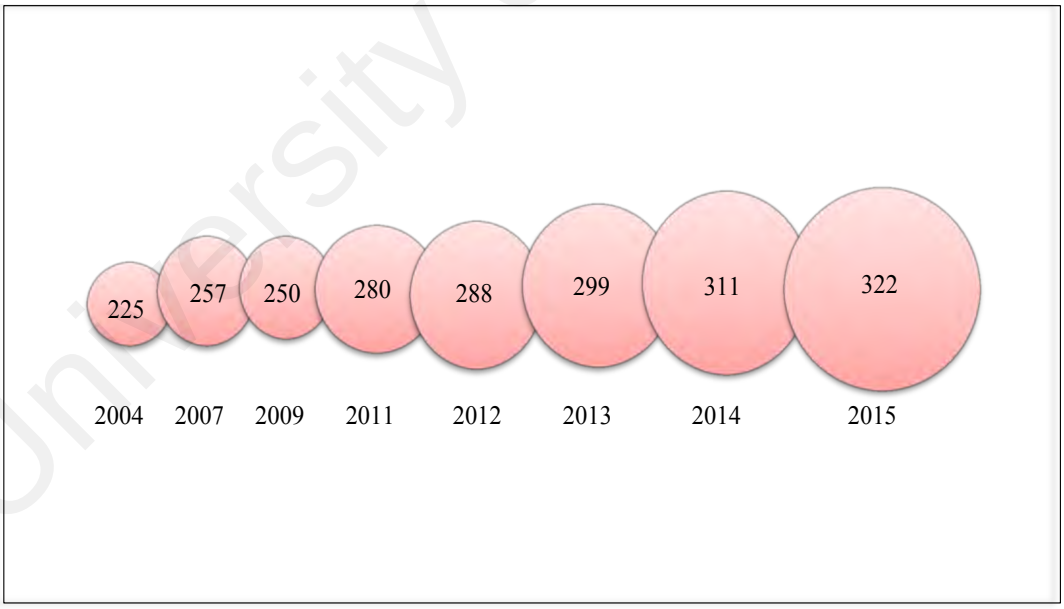

Figure 2.1: World plastic productions in million tonnes (Adapted from PlasticsEurope (2015b)

The global plastic market is governed by the end user industry application. Owing to the low cost, flexibility of use, availability of raw materials and ease of manufacture, plastics have displaced many conventional materials such as ceramic, metal, wood, paper,

glass, and leather. The heightened demand for durable and sustainable products in many end-user industries is driving the demand for different plastics globally. The plastic industry makes significant contribution to economic development and growth of various key sectors including electronics, healthcare, automotive, construction, textile, energy generation, aerospace, maritime, and packaging. None of these sectors would innovate and grow as much as they do without plastic materials and solutions (PlasticsEurope, 2015a).

Asia Pacific dominated the global market for plastics and accounted for more than 49% of worldwide production in 2015, with the leading country, China, accounting for 28% (Figure 2.2). Europe and NAFTA accounted for 18% and 19%, respectively. The Middle East and Africa accounts for 7%, Japan accounted for 4 %, Latin America 4%, and the rest of Asia accounted for 17% of world plastic produced (PlasticsEurope, 2015a). Europe produces about 47.8 million tonnes of plastics annually (PlasticsEurope, 2016). Approximately 26 million tonnes of post-consumer plastics were reported to have ended up in the waste stream, 39.5% and 29.7% was recovered through energy recovery processes and recycling, respectively, while 30.8% ended up in landfill. In the end, about 8 million tonnes of plastic waste were generated (PlasticsEurope, 2015a).

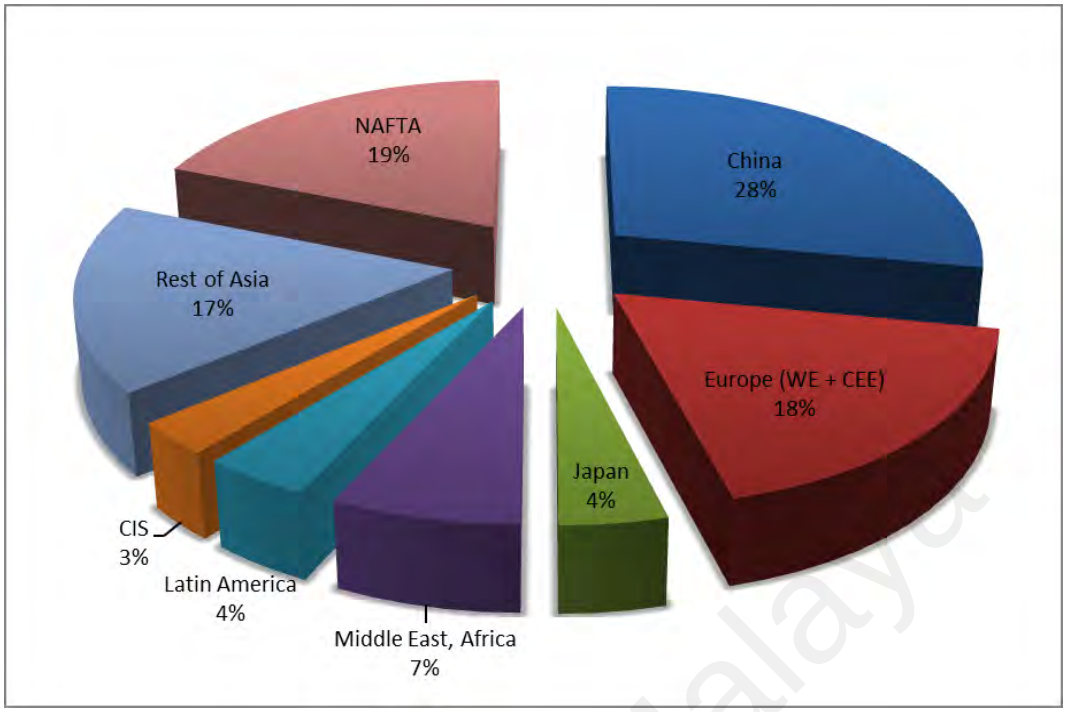

Figure 2.2: Global plastic productions 2015 (Adapted from PlasticsEurope (2015b)

When plastics waste is not properly disposed of, either intentionally or accidentally, it may end up in the environment and the oceans, seas, rivers, and lakes as plastic litter, and this poses a threat to the environment.

2.7 Plastic in the marine environment

The aquatic environment is of great importance to humanity and form an integrated and essential component of the Earth's ecosystem. In addition, the marine environment sustains more than half of the global primary production (Bergmann *et al.*, 2015) and support the greatest biodiversity on the planet. Marine environments are one of the largest carbon reservoirs in the Earth system and holds up to 54 times more carbon than the atmosphere (Bergmann *et al.*, 2015). Oceans and seas regulate the earth system, provide social and economic goods and services (tourism, marine transport, recreation, coastal protection, security) and supply living and non-living resources (marine biotechnology, fisheries, minerals and renewable energy). About 23% of the world's population live

within 100 km of the coast (Small & Nicholls, 2003), a figure that is expected to rise up to 50% by 2030 (Adger *et al.*, 2005).

Although the welfare of humankind is intricately linked with the sea and its natural resources, anthropogenic activities has substantially altered the face of the ocean within only a few centuries. Eutrophication, deep-sea hydrocarbon exploration, fisheries, ocean acidification, global ocean warming and pollution are prominent examples of pressures exerted on the ocean by humans, with severe ecological and socio-economic consequences (Bergmann *et al.*, 2015).

Recently, pollution of the marine ecosystems by anthropogenic litter has been recognized as a major global pollution problem. According to (Anderson *et al.*, 2015), marine litter is defined as "any persistent, manufactured or processed solid material discarded, disposed or abandoned in the marine and coastal environment" (Anderson *et al.*, 2015; Galgani *et al.*, 2010; Galgani *et al.*, 2015). This includes materials transported into the marine environment from land by rivers, storms, sewage systems, drainage or by wind and man-made items that have been used by people and deliberately discarded or unintentionally lost directly into the sea or on beaches. Such items consist of glass, paper, metal, fabric and plastic, with plastic considered as the majority, most persistent and problematic (Anderson *et al.*, 2015; Bergmann *et al.*, 2015). It has been estimated that approximately 10 – 20 million tonnes of plastic ends up in the marine environment on a yearly basis (Bergmann *et al.*, 2015). This is a serious amount in just one year.

Massive and increasing quantities of plastic materials, debris, and fragments are found in the open sea, in sea beds, on sea surface, sediments and coastlines (Barnes *et al.*, 2009), the sources of which come not only from dumping at sea, but also from terrestrial sources (Jambeck *et al.*, 2015). Most plastics disposed of poorly and/or indiscriminately get transported into the marine environment via rivers, currents and waves. Sewage effluents,

as well as, accidental spillage during transportation also serve as sources of plastics in the marine environment, so also does intentional dumping in seas, rivers, oceans and mangroves (Lithner *et al.*, 2011). Approximately 8 million tonnes of plastic litter enter the ocean yearly from 129 coastal countries around the world (PlasticsEurope, 2015b). More than half of the plastic waste that enter the marine environment comes from 5 countries namely; China (8.8 million tonnes), Philippines (1.9 million tonnes), Indonesia (2.4 million tonnes), Vietnam (1.8 million tonnes), and Thailand (1.0 million tonnes), contributing about 60% of all the plastic litter entering the oceans globally (Jambeck *et al.*, 2015).

Marine plastic pollution has significant economic, cultural, environmental and aesthetic costs (Cole *et al.*, 2011). It poses a complex and multidimensional challenge with significant implications for the coastal and marine environments, and human activities globally (Ten Brink, 2009). About 85% of marine litter is petroleum-based plastic (de Carvalho & Neto, 2016). Marine pollution by plastic is distributed across all oceans globally. Several studies have estimated the quantity and distribution of plastic litter on the sea floor, water column, beaches and the sea surface in different countries and oceans (Eriksen *et al.*, 2014; Jambeck *et al.*, 2015). The Great Pacific Garbage Island, located in North Pacific Gyre has been estimated to contain over 5 tons of plastic debris per square kilometer (Jambeck *et al.*, 2015). Eriksen *et al.* (2014) estimated a minimum of 5.25 trillion plastic particles weighing about 270,000 tonnes afloat in the world's oceans.

Plastic litter in the marine environment are readily visible and causes negative economic, social and ecological impact including entanglement of a wide range of marine biota (including fish, shellfish, turtles, seabirds, marine mammals and invertebrates) in fishing gear, to ingestion (Gündoğdu *et al.*, 2017). Juvenile animals in particular often

become entangled in plastic debris, which can result in serious injury as the organism grows or result in death, not to mention restriction of movement, preventing the animals from feeding properly and, in the case of mammals, breathing (Webb *et al.*, 2012). Plastic ingested by marine animals (especially marine birds) persist in the digestive system and can lead to gastrointestinal blockage, decreased feeding stimuli, decreased levels of steroid hormones and decreased production of gastric enzymes (Azzarello & Van Vleet, 1987). Plastic also cause the blockage of cooling water intakes on boats, requiring intervention by the rescue services (Anderson *et al.*, 2015; Bergmann *et al.*, 2015; Zettler *et al.*, 2013). Plastic debris in marine environments leach out toxic chemicals and additives, or adsorb persistent organic pollutants including dioxins, polychlorinated biphenyls (PCBs), nonylphenol (NP), polybrominated diphenyl ethers (PBDEs), dichlorodiphenyltrichloroethane (DDT) and polycyclic aromatic hydrocarbons (PAHs) that could be biomagnified throughout the food chain and may pose a direct risk to human health (Webb *et al.*, 2012).

Marine environments are continually becoming increasingly filled with plastic litter, where they float, or sink to the ocean depths and accumulate for decades (Auta *et al.*, 2017a). Most of the plastics that enter the marine environment do not fully "go away" but rather, they undergo a process of weathering and fragmentation into micro-sized particles called microplastics, mostly made up of PE, PS, PET, PP, and PVC (Auta *et al.*, 2017a; Caruso, 2015; Gewert *et al.*, 2015).

2.8 Microplastics

Larger plastic in the ocean breakdown into smaller fragments due to the action of ultra violet (UV) light from the sun, oxidation, low temperatures, and currents and waves. These smaller pieces of plastic are referred to as microplastics (Anderson *et al.*, 2015; Cole *et al.*, 2011). Microplastics are tiny ubiquitous plastic particles smaller than five

millimeters (5 mm) in size (Auta *et al.*, 2017a; Vroom *et al.*, 2017), and originate mainly from two sources; those that are manufactured purposely for a particular industrial or domestic application such as nurdles, exfoliating facial scrubs, toothpastes, and resin pellets used in the plastic industry (primary microplastics), and those formed from the breakdown of larger plastic items under ultraviolet radiation or mechanical abrasion (secondary microplastics) (Vaughan *et al.*, 2017). Microplastic particles enter the marine environment through a series of activities on land and in the marine environment. Microplastic beads present in facial cleansers, synthetic clothing, toothpaste, and scrubs reach the marine ecosystem through domestic effluent and industrial drainage systems and wastewater treatment plants (Cole *et al.*, 2011; Murphy *et al.*, 2016). Also, larger plastic particles from waste dumps that have been broken down into smaller fragments can be transported into seas which cause microplastic pollution (Alomar *et al.*, 2016). Microplastics are abundant and wide spread in marine habitats across the globe (Alomar *et al.*, 2016; Cole *et al.*, 2014; Reisser *et al.*, 2014).

The presence of microplastics in the ocean was first reported by Carpenter, and Smith, in the early 1970s. The researchers reported finding tiny beads and fragments of plastic, especially PS, in the ocean, and later of PE in fish (Carpenter *et al.*, 1972; Carpenter & Smith, 1972). The term 'microplastics' was first introduced in the mid-2000s. About 42 years have passed and the accumulations of these particles appear to have increased significantly in the surface waters of the ocean. Concern about the pollution and potential impacts regarding microplastics in the marine environment has gathered momentum over the past few years, and number of scientific investigations has increased, along with public interest and pressure on decision makers to respond (Andrady, 2011; Auta *et al.*, 2017a).

The enormous accumulation of microplastics in the marine environment has been recognized by scientists and authorities globally and studies have shown their ubiquitous presence (Löder & Gerdts, 2015). Microplastics have been regarded as a serious global environmental problem. Debris and microplastics can potentially be spreading globally since the ocean has no borders. A number of studies have reported the abundance of microplastic debris in different marine sites and ecosystems (Alomar *et al.*, 2016; Ferreira *et al.*, 2016). Microplastics are dispersed throughout the world's ocean. Often found in shorelines, seabed sediments (Peng *et al.*, 2018), beaches, wastewater effluents (Gallagher *et al.*, 2016) and even frozen ice (Bergmann *et al.*, 2016; Lusher *et al.*, 2015; Obbard *et al.*, 2014). Some have been found floating on surface waters (Eriksen *et al.*, 2014; Lusher *et al.*, 2015). Some are found within the Arctic and the Antarctic, transported by ocean currents, and wind (Cole *et al.*, 2011; Ferreira *et al.*, 2016; Setälä *et al.*, 2016; Van Cauwenberghe *et al.*, 2015). Pollution of the marine environment by microscopic plastic particles is regarded as a relatively "new" environmental problem (Ferreira *et al.*, 2016; Setälä *et al.*, 2016).

2.8.1 Sources of microplastics

2.8.1.1 Primary microplastics

Primary microplastics are microplastics that are manufactured purposely for domestic or industrial applications to be of microscopic size (Wright & Kelly, 2017). They include plastic particles used in facial cleansers, tooth paste, resin pellets and cosmetics like shower/bath gels, peelings, scrubs (Cole *et al.*, 2011), baby products, eye shadow, insect repellents, deodorant, nail polish, hair colouring, bubble bath lotions, blush powders, make up foundation, mascara, shaving cream, baby products, and sunscreen (Castañeda *et al.*, 2014; Duis & Coors, 2016; Fendall & Sewell, 2009), others include air-blasting media, abrasives found in cleaning products, drilling fluids, and synthetic clothing

(Alomar *et al.*, 2016; Gregory, 1996). These consumer products are characterized as "open use" since they are intended to be washed off and end up in drains (Castañeda *et al.*, 2014). The use of microplastics in medicine as vectors for drugs has also been reported (Patel *et al.*, 2009). Virgin plastic production pellets and nurdles (typically 2–5 mm in diameter) are also considered as primary microplastics, although their inclusion within this category has been criticized (Andrady, 2011; Costa *et al.*, 2010; Wagner *et al.*, 2014). Microplastic "scrubbers" used in exfoliating hand cleansers and facial scrubs, have replaced traditionally used natural ingredients, such as oatmeal, ground almonds, and pumice (Fendall & Sewell, 2009). The use of exfoliating cleansers containing plastics has risen dramatically since the patenting of microplastic scrubbers in cosmetics in the 1970s (Fendall & Sewell, 2009). For example, the presence of PS spheres (< 2 mm), and PE and PP granules (< 5 mm) in cosmetic products has been reported (Gregory, 1996). Typically marketed as "micro- beads" or "micro-exfoliates", the microplastics vary in composition, size and shape depending upon the product. The presence of microplastics in several cosmetic products and facial cleansers has been reported (Chang, 2013; Fendall & Sewell, 2009).

Primary microplastics are used in air blasting technology and involves blasting melamine, polyester or acrylic microplastic scrubbers at engines, boat hulls, and machinery to remove rust and paint. These scrubbers are used repeatedly until they diminish in size and their cutting power is lost, and they often become contaminated with heavy metals such as cadmium, chromium, and lead (Cole *et al.*, 2011).

2.8.1.2 Secondary microplastic

A culmination of physical, chemical and biological processes can reduce the structural integrity of plastic debris, thereby leading to fragmentation (Cole *et al.*, 2011; Vaughan *et al.*, 2017). Plastic materials on sea and on land over time, breakdown into smaller

fragments when exposed to the elements until they end up as microplastics. These types of microplastics are referred to as secondary microplastics (Auta *et al.*, 2017a; Vaughan *et al.*, 2017).

The disintegration of larger macro-size plastic debris into micro-size particles is influenced by a combination of factors (temperature and sunlight) and the properties of the polymer (size and density). Exposure of larger plastic debris to ultra violet (UV) radiation causes photo-degradation of plastics. The ultra violet radiation in the sun causes oxidation of the polymer matrix resulting in bond cleavage (Andrady, 2011; Shah *et al.*, 2008). Microplastic formation by fragmentation into smaller sizes is most effective on beaches due to physical abrasion by waves, oxygen availability, high UV light (Cole *et al.*, 2011), and turbulence (Barnes *et al.*, 2009). Subsequently, the plastic material turns brittle, forming cracks and "yellowing" (Andrady, 2011; Cole *et al.*, 2011). Once these fragments submerge into surface waters, or deep environments, cooler temperatures and reduced UV light renders the breakdown slow. Fragmentation continues until the material become smaller over time and become microplastic in size (Cole *et al.*, 2011; Rios *et al.*, 2007).

2.8.2 Types and composition of microplastics

Microplastics exist in different forms and are categorized into five major types (Figure 2.3).

a) **Nurdles**- Nurdles are small, lentil-sized plastic pellets or beads that serve as raw materials used to manufacture plastic goods (Ellison, 2007; Hagar, 2016). They are one of the main sources of primary microplastics and include microbeads found in detergents for cleaning, cosmetics as exfoliants, and in toothpaste. Nurdles are the raw materials used in the production of all plastic products and

are referred to as post-production plastic pellets. Nurdles are melted down and molded into plastic products such as lids to containers. Over 250 billion pounds of nurdles are manufactured and shipped around the world each year (Reddy *et al.*, 2014). Their small size renders them hard to contain and spills can occur during handling and in the manufacturing process (Maillard *et al.*, 2013).

b) **Fibres-** They are plastic particles ranging in size from 1 mm to 7 µm in diameter and make up 71% of the total microplastic pollution in the Great Lakes. Items such as diapers, cigarettes butts, netting, rope, and fleece clothing are made from fibres (Browne *et al.*, 2011). Washing of fleece clothing releases fibres into the environment. Washing of a fleece jacket is known to release about 2000 microfibres into waterways (Dris *et al.*, 2016; Mathalon & Hill, 2014). Fibres are made from synthetic materials like polyesters, and are non-biodegradable (Woodall *et al.*, 2014).

c) **Microbeads-** They are small spherical plastic particles with size ranging from 5 µm to 1 mm in diameter, often found in products like exfoliating soap products, toothpaste and facial cleansers (Tanaka & Takada, 2016). They are made from synthetic polymers including PS, PE, PET, PLA, or PP (Rochman *et al.*, 2015), Most body scrubs are usually made of PE, where they serve as exfoliants. They also add colour and texture to toothpaste, moisturizing creams, lip balm, and make up (Cheung & Fok, 2016). A tube of facewash can contain more than 300,000 of plastic beads (Institute, 2017b).

d) **Foams-** Microplastic also exist in the form of styrofoam (a kind of expanded polystyrene). Styrofoam is used in the production of packaging material, coffee cups, and food containers, and can fragment into smaller pieces. More than 25 billion cups made of styrofoam are used in America annually (Institute, 2017a).

e) **Fragments-** These are smaller pieces of plastic that fragment or are weathered from larger plastic debris (Tanaka & Takada, 2016). The fragmentation is brought about by UV radiation or the action of waves (Masura *et al.*, 2015). Examples include pieces of cutlery, plastic bags and lids.

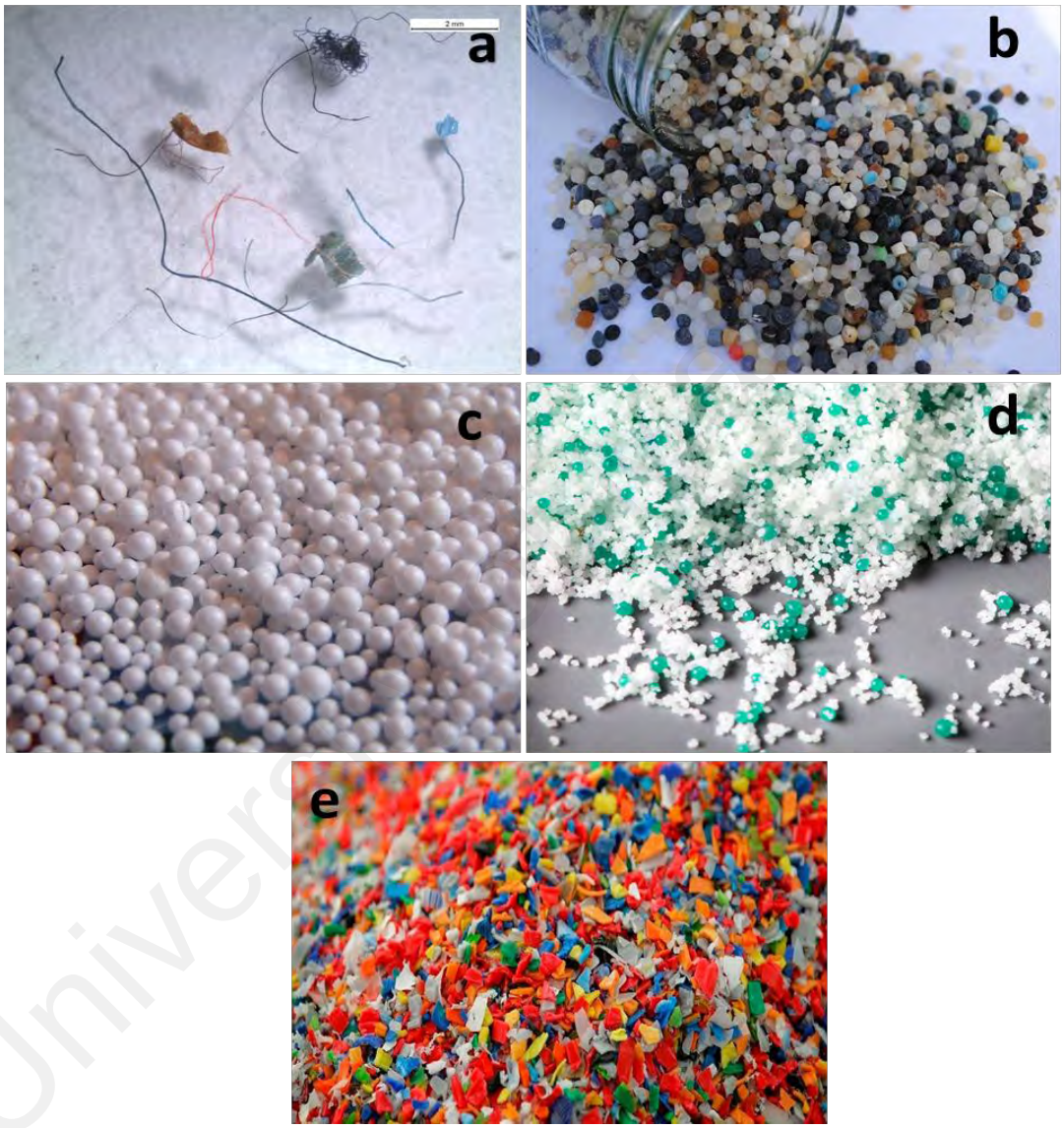

Figure 2.3: Microplastic forms (a) microfibre, (b) nurdles, (c) styrofoam, (d) microbeads (e) fragments (Sabir, 2014).

Polyethylene- PE is a semi-crystalline polymer with excellent resistance. It is the most common plastic polymer used in consumer products, and has a chemical formula of $(C_2H_4)_n$. The polymer has low strength, rigidity and hardness, but has high ductility and low friction. A molecule of PE is made up of a long chain of carbon atoms with two

hydrogen atoms attached to each of the carbon atom (Figure 2.4). PE is produced from the polymerization of ethylene, a gaseous hydrocarbon produced by cracking ethane.

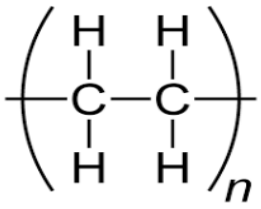

Figure 2.4: Polyethylene (PE)

The molecules are made up of two methylene units (CH_2) linked together by a double bond between the carbon atoms (CH_2=CH_2). Polymerization catalysts break the double bonds and the resultant extra single bond is used to link to a carbon atom in another ethylene molecule. PE has a melting point of 115-135 ^oC and a density of 0.91-0.96 g/cm^3 (Cheng, 2008).

Polypropylene- PP is a thermoplastic polymer made from polypropylene monomer and is usually resistant to many chemical solvents, acids and bases. It is the second most used plastic polymer globally. The polymer is similar to PE and consist of an additional methyl group which improves its mechanical properties and thermal resistance, and reduces its chemical resistance (Karger-Kocsis, 2012) (Figure 2.5).

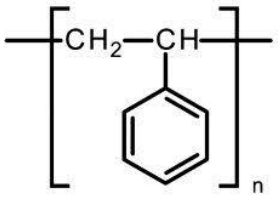

Figure 2.5: Polypropylene (PP)

The density of PP is between 0.85 and 0.92 g/cm^3, and the melting point is 171^oC, and consist of amorphous and crystalline regions (Tripathi, 2002). PP becomes tough and flexible when co-polymerized with ethylene. It also has a tertiary carbon which makes it

chemically less resistant than PE (Gewert *et al.*, 2015). PP is one of the polymers used in the production of plastic microbeads, and are present in products such as body wash and toothpaste (Barnes *et al.*, 2009).

Polyethylene terephthalate- PET is a thermoplastic polymer resin of the polyester family. It occurs both as a transparent material and as a semi-crystalline material. 60% of global PET production is for synthetic fibres (Fakirov, 2002). The polymer is made up of polymerized units of ethylene terephthalate monomer with repeating ($C_{10}H_8O_4$) units (Figure 2.6). PET exist as a semi-crystalline (particle size less than 500 nm) and amorphous (particle size up to a few μm).

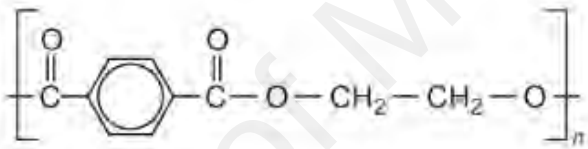

Figure 2.6: Polyethylene terephthalate (PET)

Terephthalic acid and ethylene glycol can synthesize the monomer bis(2-hydroxyethyl) terephthalate by esterification, producing H_2O as a by-product, or the monomer can be synthesized by transesterification reaction between ethylene glycol and dimethyl terephthalate with methanol as by-product. Polymerization of the polymer is through polycondensation reaction of the monomers, with H_2O as by-product. PET can be semi-rigid to rigid and is lightweight. The melting point of PET is 260 ^oC, density is 1.38 g/cm^3, and the specific heat capacity is 1.0 KJ/(Kg.K). PET is used in micro form in personal care products such as cosmetics, nail polish, enamel, hair colouring products, leg and body paint (Gupta *et al.*, 2002).

Polystyrene- PS is one of the polymers used in the production of microplastics. It is an aromatic polymer made from the monomer styrene. The chemical formula is $(C_8H_8)_n$

(Figure 2.7). PS can be solid or foamed and is made up of long chain hydrocarbons with alternating carbon centres attached to phenyl groups.

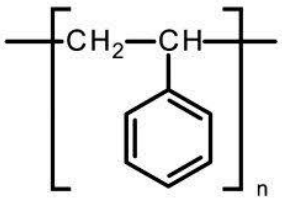

Figure 2.7: Polystyrene (PS)

PS is formed when monomers of styrene interconnect. In polymerization, the C – C bond of the vinyl group is cleaved and a new C – C bond is formed which attach the C of another styrene monomer to the chain. Depolymerization of PS is very difficult because of the stronger bonds that are formed. Each carbon backbone of the monomer has carbons that have a phenyl group (benzene ring) and a tetrahedral geometry attached to the chiral. PS has a density of 1.04 g/cm^3 and a melting point of 240 ^{o}C (Jinhua & Guangyuan, 2014).

Polyvinyl chloride- PVC comes in third after PE and PP as the most widely produced synthetic thermoplastic polymer with a chemical formula of $(C_2H_3Cl)_n$ (Figure 2.8). It can be rigid (with density of 1.3-1.45 g/cm^3) or flexible (with density of 1.1-1.35 g/cm^3), and has thermal conductivity of 0.14-0.28 and 0.14-0.17 W/m.K (Rahmah *et al.*, 2017).

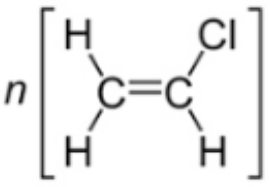

Figure 2.8: Polyvinyl chloride (PVC)

PVC is resistant to heat and water and is used in the production of umbrellas, raincoats, shower curtains and water pipes. The polymer has the same structure as PE except that

the hydrogen atoms on every other carbon in the backbone chain is replaced with a chlorine atom. PVC is synthesized by the free radical polymerization of vinyl chloride (Rahmah *et al.*, 2017). Pure PVC is a white brittle solid that is insoluble in alcohol but slightly soluble in tetrahydrofuran. PVC begins to decompose at 140 ^{o}C and has a melting temperature of 160 ^{o}C. It is chemically resistant to salts, bases, acids, alcohols, fats and some solvents (Handbook, 2005).

Both primary and secondary types of microplastics persist in the environment at high levels, particularly in marine ecosystems. Approximately 245 metric tonnes of different microplastics types are produced yearly, many of which enter into water bodies through different pathways (Bowmer & Kershaw, 2010).

2.8.3 Pathways of microplastics to the marine environment

A pollution source needs a name and an address but identifying and assigning a name and address of microplastic pollution so far out at sea is however, tedious. Microplastic particles entering in the ocean originate from a mix of several different sources and locations, released at different times and in different stages of deterioration. Microplastics enter the aquatic environment through storm sewers, wind, and currents (Murphy *et al.*, 2016; Zalasiewicz *et al.*, 2016). Some are transported out to sea via runoff (Cole *et al.*, 2011). Microplastics of these genera include those used in air-blasting (Cole *et al.*, 2011). those generated in ship-breaking industry (Reddy *et al.*, 2006), and industrial abrasives (beads of acrylic plastics and polyester) (Cole *et al.*, 2011).

Wastewater treatment plants are a significant point source for microplastic discharge into the marine environment (Kalčíková *et al.*, 2017; Mintenig *et al.*, 2017). Microplastic sheds released from synthetic fibres in clothing are washed into water or wastewater treatment plants as effluents. Wastewater treatment plants (WWTPs) located on the River

Clyde Glasgow has been reported to release about 65 million microplastic particles into the receiving water on a daily basis (Murphy *et al.*, 2016). Gouin *et al.* (2011) reported that the US population emits about 263 tonnes per year of PE microplastics, mainly from the usage of personal care products. They estimated the per capita consumption of microplastics to be 2.4 mg/person day^{-1}. This invariably makes up 25% of plastics in the North Atlantic subtropical gyre. Mintenig *et al.* (2017) on the other hand, reported the presence of large quantities of microplastics in the WWTPs in Lower Saxony, Germany. The microplastic consisted mainly of PE and polyester. Similarly, microplastic beads present in cosmetics such as scrubs, toothpastes, air-blasting media, and in clothing can enter the aquatic environment through industrial or domestic drainage systems (Murphy *et al.*, 2016). Microbeads are not biodegradable and are too small to be filtered out by water treatment plants. They travel through pipes, flow through rivers and sewers, and finally end up in seas and oceans. There, they are carried by currents or they enter the underwater food chain where they are ingested by marine organisms, thereby, poisoning the aquatic ecosystems. Leslie *et al.* (2012) estimated that exfoliate scrub is composed of 10.6% of microplastics. The 5 Gyres (an American NGO) observed that another similar product contains about 360, 000 microbeads. It is believed that cosmetics are the main source of microplastics pollution in the Walden Sea (Dubaish & Liebezeit, 2013). Sewage sludge is another possible source of microplastic pollution as it is known to contain more microplastics than that of effluent which are transported into the marine ecosystem. Its application in landfilling and as fertilizer in agriculture introduces microplastics to lakes, rivers and oceans (Alomar *et al.*, 2016; Leslie *et al.*, 2012). The small size and associated low density of microplastics contributes to their widespread transport and distribution across larger distances by waves and currents (Eerkes-Medrano *et al.*, 2015; Eriksson *et al.*, 2013). These small marine plastics are abundant and are widespread in all aquatic habitats across the world (Eerkes-Medrano *et al.*, 2015; Reisser *et al.*, 2014).

2.9 Factors affecting the bioavailability of microplastics in the marine environment

2.9.1 Size of microplastics

The main factor contributing to the bioavailability of microplastics is their small size, which make them readily available to lower trophic organisms since their small size means they can be mistaken for food by the smallest sea organisms, as well as, large animals such as seabirds and fish (Cole *et al.*, 2013). Many of these marine organisms apply limited selectivity between particles and capture anything of appropriate size. Alternatively, aquatic organisms, such as the blue whale, which feed on zooplankton or phytoplankton, could passively ingest microplastics during normal feeding behaviour or even mistake them for natural prey (Moore, 2008).

2.9.2 Density of microplastics

Density is one of the important factors that aid the distribution of microplastics in the marine environment (Löder & Gerdts, 2015). The density of a plastic material ascertains their bioavailability in the marine environment. For this reason, differences exist between the types of microplastic material that can be ingested among organisms. Microplastic particles with density lower than that of water will likely float on the surface while those with higher density will sink to the bottom. Therefore, organisms that inhabit the sea surface (suspension feeders, filter feeders and planktivores) will probably come across positively buoyant and lower density microplastics (Long *et al.*, 2015; Wright *et al.*, 2013). The ability of plastic to float on surfaces is influenced by biofouling (Löder & Gerdts, 2015), and the rate of biofouling is dependent on the surface energy, hardness of the polymer, and the water conditions (Andrady, 2011; Muthukumar *et al.*, 2011). As biofouling progresses, the density of the plastic material increases and once the density is

greater than that of the sea water, the plastic material sinks low into the bottom of the sea. Subsequent de-fouling by other mechanisms or organisms may occur which may cause the density of the microplastic to reduce thereby; enabling it return and float back to the surface of the water (Andrady, 2011). This cyclic pattern may make microplastics available to organisms occupying different depths of the water column at different times. Sediments seem to represent a sink for microplastics while beaches, as intermediate environments, can accumulate floating, neutrally buoyant as well as sinking plastics (Hidalgo-Ruz *et al.*, 2012).

2.9.3 Colour of microplastics

The colour of microplastics also contributes to the likelihood of availability and ingestion, due to their resemblance to prey item. Some commercially important fish and their larvae are visual predators as they prey on small zooplankton, and may unknowingly feed on microplastics which most resemble their prey i.e. yellow, tan and white plastic (Shaw & Day, 1994; Wright *et al.*, 2013). Microplastic ingestion due to food resemblance may also be applicable to pelagic invertebrates, which are visual raptorial predators (Wright *et al.*, 2013).

2.9.4 Abundance of microplastics

As the quantity and presence of microplastics in the aquatic environment increases, so does their bioavailability to organisms, and the possibility of an organism to encounter a microplastic particle increases. Therefore, the continuous fragmentation of macroplastic debris increases the amounts of particles available for ingestion to a wider range of organisms (Browne *et al.*, 2008; Thompson *et al.*, 2009).

2.10 Microplastic behaviour in the marine environment

Pollution of the environment by microplastics is a growing global problem that poses a threat to the marine biota and possibly human life and has attracted great attention and concern. Understanding the behavioural mechanism of microplastics in the environment will help to optimally comprehend the effects on the marine ecosystem. The behaviour of microplastics in the marine environment include accumulation, migration, sedimentation, adsorption, translocation, and ingestion (Wang *et al.*, 2016).

2.10.1 Accumulation

When macroplastic debris get into the sea, they are fragmented into microplastics and become distributed in surface waters, seabed sediments, and on shorelines. Cole *et al.* (2011) and Fauziah *et al.* (2015) reported that microplastics are persistent marine debris that has been accumulating in the marine environment since the 1940s, and that they concentrate at remote locations such as the mid ocean gyres and population centers (Kershaw, 2015).

The light weight of microplastics and their lesser density compared to that of seawater, allows them to accumulate on the surface of the oceans (Van Cauwenberghe *et al.*, 2015; Wang *et al.*, 2016). The density of microplastics could become higher than that of seawater (through biofouling), which make them sink and accumulate in sediments in the seafloor (Andrady, 2011; Zettler *et al.*, 2013). A study by Woodall *et al.* (2014) reported the massive build-up and accumulation of microplastics in the deep sea sediments, a number far greater when compared with the heavily contaminated surface waters, proving that the seabed is a possible sink for microplastics. Certain processes such as saline subduction, offshore breeze convection, and extreme coastal storms aid in settling microplastics in the ocean depth (Stabholz *et al.*, 2013; Wang *et al.*, 2016). Due to their

resistance to degradation, microplastics that have been ingested by organisms, bioaccumulate and can be carried to the food chain where they ultimately get to the higher trophic levels (Hall *et al.*, 2015; Rios *et al.*, 2007).

2.10.2 Migration

Once plastic debris enters the ocean, they begin to migrate to other sites. PE and PP float and are easily transported in seawater (PlasticsEurope, 2014). PVCs can also be carried by underlying currents, tsunamis, tides, and winds (Engler, 2012). Studies were carried out in Sweden about the transportation of microplastics in the river Göta älv and it was observed that microplastic concentration was 2.9 microplastics per m^3 during the rainy period but decreased to 0.9 microplastics per m^3 during the dry period. It was also observed that the large rivers that run into the eastern North Sea could be the routes for microplastics with low densities that hover on the surface of the sea or those suspended in water column to get into the Swedish Skagerrak and Kattegat coasts (Magnusson *et al.*, 2016).

2.10.3 Sedimentation

Microplastics in the water column rapidly become covered with biofilm and this makes them hydrophilic. As biofilms form, the density of the microplastics increases and become denser than that of seawater. This causes them to sink and accumulate in sediments (Green *et al.*, 2016; Lobelle & Cunliffe, 2011; Wang *et al.*, 2016). Reports on the presence of microplastics in sediments date back to the late 1970s. It was observed that industrial resin pellets of about 2-5 mm were present on beaches in Canada, Bermuda, New Zealand and Lebanon (Gregory, 1977, 1983; Shiber, 1979, 1982; Van Cauwenberghe *et al.*, 2015). The study on sediment samples collected from the field revealed that the sediment samples contained large quantities of microplastics (Van

Cauwenberghe *et al.*, 2015). Marine sediments serve as a long-term sink for microplastics and the concentration is expected to continue to increase globally in the coming years (Green *et al.*, 2016; Jambeck *et al.*, 2015).

2.10.4 Adsorption

Microplastics can adsorb toxic contaminants from the aquatic environment thereby serving as scavengers and transporters of organic pollutants (Bakir *et al.*, 2014). Adsorption is a physical and chemical behaviour which is dependent on the surface area and Van der Waal's force and the affinity of the organic pollutants for the hydrophobic surfaces of the microplastics (Wang *et al.*, 2016). The large surface area to volume ratio of microplastics makes them liable to water borne-contaminants such as persistent organic pollutants (POPs), metals (Ashton *et al.*, 2010; Cole *et al.*, 2011), and endocrine disrupting chemicals (Ng & Obbard, 2006). These chemicals are found in high numbers in the sea surface microlayer, where low density microplastics are also present in large numbers (Teuten *et al.*, 2009). Organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDTs), polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) can sorb unto the hydrophobic surface of the microplastics (Ogata *et al.*, 2009; Zhang *et al.*, 2017). The sorption capacity of microplastics is influenced by the type of polymer and its state (whether it is glassy or rubbery). Different polymers undergo different sorption mechanisms namely linear sorption isotherms (absorption to rubbery polymers), and non-linear sorption isotherms (absorption to glassy polymers) (Ogata *et al.*, 2009). Proof of toxic contaminant adsorption by microplastics has been reported. Hirai *et al.* (2011) and Ogata *et al.* (2009) reported the concentrations of POPs in marine plastic pellets to range from 1 to 10,000 ng/g plastic pellet globally. Marine microorganisms have been found to take in POPs that have been sorbed unto microplastics. For example, the assimilation of polybrominated

diphenyl ethers (PCDEs) from microplastics by *Allorchestes compresa* has been reported by (Chua *et al.*, 2014). The organisms were found to have ingested about ≤ 45 particles which got assimilated into the tissues. Wardrop *et al*. (2016) reported the assimilation of PCDEs by fish into tissues.

Aquatic sediments also serve as potential sinks for metals entering the aquatic environment where they sorb unto microplastics. Antifouling paints, fuel combustion, and industrial waste are major sources of heavy metals in the marine environment (Brennecke *et al.*, 2016; Deheyn & Latz, 2006). Studies have reported the ability of microplastics to sorb trace metals from the aqueous environment (Boucher *et al.*, 2016; Brennecke *et al.*, 2016; Rochman *et al.*, 2013). Potential toxic elements such as aluminium (Al), silver (Ag), zinc (Zn), lead (Pb), Iron (Fe), and manganese (Mn), have been detected on plastic pellets sampled from the seawater (Ashton *et al.*, 2010; Holmes *et al.*, 2012). Microplastics that have been covered with POPs and heavy metals may be carried across the oceans thereby contaminating other ecosystems (Zarfl & Matthies, 2010), or they can be ingested by marine biota which are transferred to higher marine trophic levels (Eerkes-Medrano *et al.*, 2015). Brennecke *et al.* (2016) examined the adsorption of Zn and Cu, that had been leached from an antifouling paint to PVC fragments and virgin PS beads in seawater.

2.10.5 Translocation

Laboratory studies have shown that microalgae (Sjollema *et al.*, 2016), fin whales (Fossi *et al.*, 2016), pelagic fish; herring and mackerel (Rummel *et al.*, 2016) copepod (Cole *et al.*, 2013), mussel (Avio *et al.*, 2017), sea turtles (Caron *et al.*, 2016), and brown shrimp (Devriese *et al.*, 2015), can ingest microplastic particles. Once ingested, they may be stored in the digestive tract, be excreted out when the organism passes out feces, be transported through the epithelial lining of the gut into the tissues of the body through

translocation (Von Moos *et al.*, 2012), or transported through the intestinal wall to the circulatory system (Van Cauwenberghe & Janssen, 2014). Experimental studies revealed an accumulation of microplastics in the gut of the filter-feeding mussels, *Mytilus edulis*, and within three days of ingestion, the microplastics were transferred to the circulatory system of the organisms where they persisted for more than six (6) weeks (Browne *et al.*, 2008). Studies carried out on rodents and humans showed that PVC and PS particles can translocate from the gut cavity to the lymph and circulatory system (Browne *et al.*, 2008). Smaller microplastic particles translocate more readily than larger particles (Browne *et al.*, 2008). A study conducted by Watts *et al.* (2014) revealed that the common shore crab, *Carcinus maenas*, took in microplastics (PS) through the gills by inspiration, as well as by ingestion and later translocated them to the body tissues. Studies have also shown the transfer of microplastics from zooplankton to the mysid shrimp (Setälä *et al.*, 2014).

2.10.6 Ingestion

The small size of microplastics makes them readily accessible for ingestion by organisms in the marine environment. Marine organisms can ingest microplastics regardless of the route of entry into the marine environment (Lusher *et al.*, 2015). Bivalves, shore crabs, marine fish, fin whales, zooplankton, lugworm, amphipods barnacles, sea turtles, scleractinian corals, brown shrimps, microalgae, and marine birds and mammals, will all, in at least one life stage, ingest microplastics from the environment (Cole *et al.*, 2013). This is often with negative health consequences as the ingestion of microplastics can lead to depletion of energy reserves, reduce feeding, and decrease ecophysiological function as a result of false satiation and physiological stress (Cole *et al.*, 2013; Fossi *et al.*, 2016; Free *et al.*, 2014; Goldstein & Goodwin, 2013; Rehse *et al.*, 2016; Van Cauwenberghe & Janssen, 2014; Wright *et al.*, 2013).

Laist (1997), reported that over 250 marine species are believed to ingest microplastics with species of sea turtles making up about 86%, sea birds making up 44%, and 43% belonging to all marine mammal species. Microplastic debris has been found retained in the gut content of fish globally from estuaries, demersal habitats, and pelagic habitats (Lusher *et al.*, 2013). Hoshaw (2009) reported that the rainbow runner (*Elagatis bipinnulata*) was found to have 84 pieces of microplastics in its stomach.

2.10.7 Effects of microplastics

As the abundance of microplastics increases, its bioavailability to marine organisms also increases. The colour, density, shape, size, charge, aggregation and abundance of microplastic particles affect their potential bioavailability to marine organisms (Van Cauwenberghe *et al.*, 2015; Wright *et al.*, 2013). A good documentation of the harmful effects of microplastic debris on marine biota has been made and researchers worldwide have given reports on the uptake of microplastics by various marine organisms (Bergmann *et al.*, 2015; Browne *et al.*, 2008; Cole *et al.*, 2014; Ferreira *et al.*, 2016; Green *et al.*, 2016; Setälä *et al.*, 2016; Wright *et al.*, 2013), with most of the studies carried out in controlled laboratory experiments. These effects include leaching of toxic additives, physical harm from ingestion, and desorption of persistent, bio-accumulative and toxic chemicals, potentially fatal injuries such as blockages throughout the digestive system or abrasions from sharp objects (Desforges *et al.*, 2014; Wright *et al.*, 2013), which in contrast to macroplastics, mainly affect smaller invertebrates or larvae. In addition to these effects, the ecological implications can be more severe as microplastics can release toxic additives upon degradation and accumulate persistent organic POPs (Bakir *et al.*, 2014; Löder & Gerdts, 2015).

Studies have shown the consumption of microplastics by a wide range of marine biota representing different trophic levels including seabirds (Ryan *et al.*, 2009), commercial

fish (Batel *et al.*, 2016; Espinosa *et al.*, 2018; Lu *et al.*, 2016; Sjollema *et al.*, 2016), marine invertebrates (Cole *et al.*, 2013; Goldstein & Goodwin, 2013; Rehse *et al.*, 2016; Setälä *et al.*, 2016) and mammals (Batel *et al.*, 2016; Fossi *et al.*, 2016). Other marine biota such as sea cucumbers, mussels, lobsters, amphipods, lugworms and barnacles, ingest microplastics often with negative health implications (Avio *et al*., 2017; Caron *et al.*, 2016; Eerkes-Medrano *et al.*, 2015; Green *et al.*, 2016; Wright *et al.*, 2013). Ingestion of microplastics by organisms at the lower trophic level can interfere with the food chain as microplastics ingested by the lower tropic level could pass up the food chain when lower trophic organisms are fed upon by organisms at the higher trophic level (Hollman *et al.*, 2013).

The consumption of microplastics by marine organisms may cause mechanical effects such as attachment of the polymer to the external surfaces thereby, hindering mobility, and clogging of the digestive tract, or the effect could be chemical such as inflammation, hepatic stress, decreased growth, and endocrine disruption (Fendall & Sewell, 2009; Setälä *et al.*, 2016). When ingested, microplastics can block feeding appendages or stop food from passing through the intestinal tract (Tourinho *et al.*, 2010), and can be absorbed into the body upon passage through the digestive tract by a process called translocation. Translocation of PS microspheres has been demonstrated in humans, rodents and in mussels using fluorescence microscopy and histological techniques (Browne *et al.*, 2008; Cole *et al.*, 2011).

Microplastics have been reported to contain different kinds of toxic contaminants, either added during plastic production (Diethylhexyl phthalate, DEHP), or adsorbed from surrounding sea water (Bakir *et al.*, 2014). These organic contaminants, also hydrophobic in nature, have a high affinity for microplastics which serve as veritable sponges, adsorbs and concentrates them on their surfaces. In other words, the problem with microplastics

isn't just the plastic itself, but the complex mix of chemicals that plastics carry with them (Bakir *et al.*, 2014; Cole *et al.*, 2014; Hirai *et al.*, 2011). The toxic organic contaminants include PBDEs, PCBs, organo-chlorine pesticides (DDTs), BPA, and PAHs (Desforges *et al.*, 2014; Hollman *et al.*, 2013; Rios *et al.*, 2007; Teuten *et al.*, 2007). These organic compounds have a wide range of chronic effects which include mutation, cancer, birth defects, immune system problems, child development issues, and endocrine disruption (Bowmer & Kershaw, 2010; Setälä *et al.*, 2016; Teuten *et al.*, 2009). Furthermore, plastic additives (such as phthalates, BPA), which leach from plastics as they degrade induce toxic effects in aquatic organisms even at low concentrations (Boerger *et al.*, 2010; Wright *et al.*, 2013). This makes them even more harmful due to their potential transfer across the aquatic food chain to animals that consume them (Nobre *et al.*, 2015; Setälä *et al.*, 2016). Evidence has shown that microplastic can be taken up and stored in the tissues and cells of organisms when ingested, providing a possible pathway for accumulation of hydrophobic organic contaminants taken in from sea water with negative repercussions on the marine food web, humans and environment (Mato *et al.*, 2001; Wright *et al.*, 2013).

There is availability of microplastics to every level of the food web, from the primary producers (Oliveira *et al.*, 2013) to higher trophic-level organisms (Wright *et al.*, 2013). Individuals who ingest microplastics may suffer physical harm, such as internal abrasion and blockage. Impacts at the population-level are also possible as microplastics harbour the risk of transporting (POPs) into human food (Löder & Gerdts, 2015; Wright *et al.*, 2013). Because of their long residence time at sea, microplastics can travel long distances and therefore function as vectors for dispersal of pathogenic microorganisms like *Vibrio* sp. and toxic algae (Masó *et al.*, 2003), which colonize ocean plastics (Zettler *et al.*, 2013).

2.11 Global distribution of microplastics in the marine environment

Microplastics pollution is ubiquitous throughout the marine environment (Eriksen *et al.*, 2014). They are carried and dispersed throughout the oceans globally; in seabed sediments, beaches, water columns and on surface waters, from the Arctic to the Antarctic where they concentrate at remote locations. Several efforts at quantifying the level of microplastics in the marine environment have been carried out, the vast majority of which focus on the deposition of debris in sediments, on beaches and on surface waters and in water columns (Alomar *et al.*, 2016; Eriksen *et al.*, 2014; Fauziah *et al.*, 2015; Lusher *et al.*, 2015; Yu *et al.*, 2016).

Microplastics have been found distributed in the five sub-tropical gyres (North Pacific, South Pacific, North Atlantic, South Atlantic, and the Indian Ocean), the Bay of Bengal, Coastal Australia, and The Mediterranean Sea (Auta *et al.*, 2017a; Eriksen *et al.*, 2014). The distribution of microplastics in the marine environment is influenced by the density of the particles, location of the sources and conveyance with ocean currents and waves (Kukulka *et al.*, 2012; Magnusson *et al.*, 2016). The buoyant and persistent natures of microplastics allow them to become easily and widely dispersed via hydrodynamic processes and ocean currents (de Carvalho & Neto, 2016).

Investigations on the presence of microplastics in the marine environment started in the 2000s. Studies have demonstrated that microplastics have ubiquitously permeated the aquatic ecosystem, and even the Polar Regions are not left out (Lusher, Hernandez-Milian*, et al.*, 2015). Approximately 2 billion microplastic fragments have been reported to have entered the Californian coastal waters in just over a period of three days via two rivers (Moore *et al.*, 2005). Reddy *et al.* (2006) discovered a concentration of 81 ppm microplastics in sediments at an intertidal site near a shipwreck yard in India. Microplastic particles distribution on the surface and sub-surface areas of the Arctic waters, south and

southwest of Svalbard, Norway has been estimated to range between 0 to 1.31 particles m^{-3} and 0 to 11.5 particles m^{-3}, respectively (Lusher *et al.*, 2015). The composition of the particles suggested that they may have resulted from the breakdown of macro debris or from sewage and wastewater. High concentrations of microplastics (770 and 3,300 items kg^{-1} dry weight) have been reported in sediments in the Wadden Sea and the Rhine estuary, respectively, with about 400 items reported in the Coastal harbour sediments of Belgium. Investigations by Isobe *et al.* (2015) demonstrated a total particle count of about 1.72 million pieces km^{-2} (which is 10 times greater than in the North Pacific and 27 times greater than in the world oceans) in the East Asian Seas around Japan. In South Africa, microplastic densities in beach sediment ranged from 340.7 - 4757 particles m^{-2}, while those in the water column ranged between 204.5 – 1491.7 particles m^{-3}, which were governed by water circulation (Nel & Froneman, 2015). Studies were conducted to quantify microplastic debris in sand beaches in Peninsular Malaysia and a total of 2542 pieces (265.30 g^{-2}) of small microplastic debris were collected from six beaches (Fauziah *et al.*, 2015). Microplastic concentrations ranged from 8 - 9200 particles m^{-3} in offshore pacific waters, and increased to 6, 12, and 27-folds in West coast Vancouver Island, Straights of Georgia and Queen Charlotte Sound, British Columbia, Canada, respectively (Desforges *et al.*, 2014). An average microplastic density of 20, 264 particles km^{-2} has been recorded in Lake Hovsgol, Mongolia (Free *et al.*, 2014). Lusher *et al.* (2014) investigated the levels of microplastics in the Northeast Atlantic Ocean and the average microplastic abundance was calculated as 2.46 particles m^{-3}. The study was the first to report the ubiquitous nature of microplastic pollution in the North Atlantic Ocean. Study was carried out on the distribution, abundance and possible discharge of microplastics via treated municipal wastewater in urban estuaries. It was observed that the wastewater treatment facilities discharged about 7 million microplastic particles daily whereas, those found in the Midwest and Northeast US recorded a total of 2 million particles daily. In

total, 56 million microplastic particles were discharged into the San Francisco Bay. The distribution of microplastics in the surface waters of San Francisco bay ranged from 15,000 – 2,000,000 particles km^{-2} (Sutton *et al.*, 2016). Studies have reported that Denmark emits about 21,500 tonnes of microplastics on a yearly basis which arise from both primary and secondary sources, about which 2000 to 5600 tonnes are discharged into sewage from tyres and textiles (Lassen *et al.*, 2015). Norway on the other hand generates approximately 8000 tonnes yearly. Eriksen *et al.* (2013) found that in the Great Laurentian Great Lakes of the United States, particles greater than 1 mm were more easily identified as plastics. However, an average abundance of 43,000 microplastic particles km^{-2} were recorded and such included particles less than 5 mm. Antunes *et al.* (2013) reported that the average marine debris along the Portuguese coastline was 2421 items m^{-2} which computed to 362 gm^{-2}; 98 % were plastics (2397 items m^{-2}, 283 gm^{-2}) and were 4 mm in diameter. The most dominant class of plastic marine debris was resin pellets, representing 53 % of the total marine debris collected (1289 items m^{-2}, 30 gm^{-2}). Resin pellets are small cylindrical granules of about 2-7 mm in size (Andrady, 2011). As high as 127,500 tonnes of plastic pellets were collected on one 100 m-stretch of beach in Cornwall, and it was estimated that up to 53 billion tiny pellets escape into the UK's environment yearly, and about 230,000 tonnes are estimated to be entering the oceans in Europe annually (Berg, 2017). The range of microplastics abundance from 3 sample locations on the beach of the Chinese Bohai Sea was 63-201 items kg^{-1} most of which consisted of fragments and sheets and belonged to the PE (Yu *et al.*, 2016).

2.12 Microplastics in marine sediments

Marine sediments have the potential to accumulate microplastics (Nuelle *et al.*, 2014), and have been demonstrated to be long-term sinks for microplastics (Cózar *et al.*, 2014; Felsing *et al.*, 2018; Peng *et al.*, 2018). Very high concentrations of microplastics now

occur within marine sediments; such plastics can make up 3.3% of sediment weight on heavily impacted beaches (Boucher *et al.*, 2016; Van Cauwenberghe *et al.*, 2015). It is a fact that deep sea areas, submarine canyons, and marine coastal shallow sediments are sinks for microplastics (Alomar *et al.*, 2016; Pham *et al.*, 2014). As with sediments in other aquatic environments, microplastics similarly accumulate in mangrove sediments. Studies have demonstrated the prevalence of microplastics in mangrove habitats of Singapore (with concentrations of 12.0-62.7 particles per dry sediment) (Nor & Obbard, 2014). The presence of these different polymers of microplastics may be due to the degradation of marine macroplastic debris which could have accumulated in the mangroves. A total of 3349 items m^{-2} were recorded in mangrove dominated areas of Papua New Guinea out of these, 263 items were microplastic pieces (Smith, 2012). The distribution of microplastics in mangroves located in Peninsular Malaysia recorded approximately 418 items of different microplastic polymers ranging from plastic pellets to polystyrene foams (Barasarathi *et al.*, 2014). These statistics demonstrates that microplastics are prevalent in mangrove environments.

2.13 Degradation of plastic polymers

Degradation is any physical or chemical change that reduces the average molecular weight of a polymer and changes its properties. It is brought about due to environmental factors such as moisture, light, heat, UV radiation, air, chemical conditions and/or biological activity. Most plastics degrade in the terrestrial and aquatic environments (Andrady, 2011; Arutchelvi *et al.*, 2008; Shah *et al.*, 2008; Wang *et al.*, 2016). The disintegration of larger macro-size plastic debris is influenced by a mixture of environmental factors and the properties of the polymer and can proceed by biotic or abiotic pathways. Abiotic degradation usually precedes biodegradation and can be

initiated hydrolytically, mechanically, thermally or by ultra violet (UV) radiation in the environment (Gewert *et al.*, 2015; Lucas *et al.*, 2008; Yousif & Haddad, 2013).

2.13.1 Mechanisms of abiotic plastic polymer degradation

2.13.1.1 Photo-degradation

During abiotic degradation, UV radiation is one of the most important factors. Exposure of plastic debris to UV radiation from the sun induces photo oxidative degradation which results in the breaking of the C-H bonds on the polymer backbone, produces free radicals and reduces the molecular weight of the polymer (Andrady, 2011). As the molecular weight of the polymer is reduced, the material becomes brittle and become more susceptible to fragmentation (Andrady, 2011; Gewert *et al.*, 2015; Yousif & Haddad, 2013). Disintegration of plastics into smaller sizes is most effective on beaches due to high UV light, physical abrasion by waves, oxygen availability (Anderson *et al.*, 2015), and turbulence (Barnes *et al.*, 2009). With time, they become fragile, forming cracks and "yellowing" (Andrady, 2011; Cole *et al.*, 2011). Once these fragments submerge unto surface waters or deep environments, the breakdown is slowed down due to cooler temperatures and reduced UV light (Wang *et al.*, 2016). The breakdown continues until the fragments become smaller over time (Cole *et al.*, 2011; Rios *et al.*, 2007; Ryan *et al.*, 2009). By photo oxidation, most plastic polymers become fully converted into carbon dioxide (CO_2), water and inorganic molecules, a process that is very slow (Gewert *et al.*, 2015). Degradation can also occur thermo-oxidatively for some time without the need for further exposure to UV-radiation. As long as there is availability of oxygen to the system, autocatalytic degradation could begin. Due to UV deficiency and low temperatures in the marine environment, rate of degradation becomes slower than in the terrestrial environment (Ryan *et al.*, 2009).

2.13.1.2 Chemical degradation

Chemical degradation refers to the transformation that occurs when atmospheric pollutants and agro-chemicals interact with polymers, leading to changes in the properties of the macromolecule. Oxygen is one of the chemicals that provoke degradation of polymer materials. Oxygen (O_2 or O_3) attacks the covalent bonds leading to the formation of free radicals. Oxidative degradation depends on the structure of the polymer material. The oxidations can be synergic or concomitant to photo degradation to produce radicals (Lucas *et al.*, 2008). The peroxyl radicals formed by oxidative degradation can lead to chain scissions and/or crosslinking reactions (Briassoulis, 2005; Duval, 2009; Lucas *et al.*, 2008).

Plastic polymers can also undergo chemical degradation through hydrolysis (He *et al.*, 2004). The polymer must contain hydrolysable covalent bonds such as those in groups (anhydride, amide, ether, carbamide, ester and ester amide), to enable it become susceptible to splitting by H_2O (Lucas *et al.*, 2008). Hydrolysis is dependent on water activity, temperature, pH, and time. Hydrolytic and oxidative degradations on a given polymer material are more easily carried out within disorganized molecular regions (Lucas *et al.*, 2008).

2.13.1.3 Thermal degradation

Thermal degradation of plastic polymers is molecular deterioration that occurs due to overheating. At high temperatures, separation (molecular scission) of the components of the long chain backbone of the polymer begins. These components react with one another to change the properties of the polymer. The chemical reactions involved in thermal degradation lead to changes in the optical and physical properties relative to the initially specified properties (Olaosebikan *et al.*, 2014). Generally, thermal degradation involves

changes to the molecular weight of the polymer which include colour changes, reduced ductility, surface cracking, chalking, crazing, embrittlement, and disintegration (Gewert *et al.*, 2015; Olaosebikan *et al.*, 2014). The mechanism of degradation and the degree of resistance to degradation is dependent on the application and the polymer concerned. For example, PP is very liable to thermal degradation even at normal temperatures. Thermal degradation of PP causes chain breakage which leads to a decrease in the molecular weight of the polymer. PE is also susceptible to thermal degradation and this results in branching and cross-linking of the polymer chain which reduces the melt flow and produces cracking, colour change, chalking, and embrittlement (Olaosebikan *et al.*, 2014). Though plastics can linger in the environment for years, their degradation plays an important role in reusing or recycling these polymer wastes to control environmental pollution (Wang *et al.*, 2016).

2.13.1.4Mechanical degradation

Degradation by mechanical means takes place as a result of tension, compression, and/or shear forces. Examples include ageing due to load, water turbulences, air, material installation, bird damages, and snow pressure. Thermoplastic polymers can therefore, undergo a number of mechanical degradations (Briassoulis, 2005; Lucas *et al.*, 2008). Damages are not immediately visible at macroscopic levels, but degradation could start at molecular level. In field conditions, mechanical stresses act in synergy with other abiotic parameters such as chemicals, temperature, and UV radiations (Lucas *et al.*, 2008).

2.14 Biodegradation of plastics

Biodegradation is the disintegration of organic substances by the enzymatic machinery of living organisms. The term is often used in relation to waste management, environmental remediation (bioremediation), ecology, and to plastic materials due to their

long-life span. According to the ASTM Standard D- 5488-94 d, biodegradation is a process which is capable of decomposition of materials into carbon dioxide (CO_2), methane (CH_4), water (H_2O), inorganic compounds or biomass in which the predominant mechanism is the enzymatic action of microorganisms, that can be measured by standard tests in a specified period of time, reflecting the available disposal conditions (Singh & Sharma, 2008). Biodegradability is the tendency of a material to get fragmented into its molecular constituents by natural processes (often by microorganisms). The metabolites released by degradation are expected to be non-toxic to the environment and redistributed through the carbon, nitrogen and sulfur cycles. Biological degradation is chemical in nature but the source of the attacking chemicals (enzymes) is from microorganisms (Singh & Sharma, 2008). Plastic can be biodegraded aerobically or anaerobically. Plastics are degraded anaerobically in landfills and sediments, aerobically in wild nature, and partly anaerobically and aerobically in soil and compost. The end products of aerobic biodegradation are CO_2, and H_2O, while anaerobic biodegradation produce CO_2, H_2O, and CH_4 (Figure 2.9). Mineralization of polymers to CO_2 involves the action of different microbes. Those that break down the polymer into monomers, those that can use the monomers and in the process, excrete simpler waste compounds as by-products, and those that are able to utilize the excreted wastes (Andrady, 2011; Eubeler *et al.*, 2009; Kannahi & Sudha, 2013; Lalit & Haripada, 2013; Lucas *et al.*, 2008).

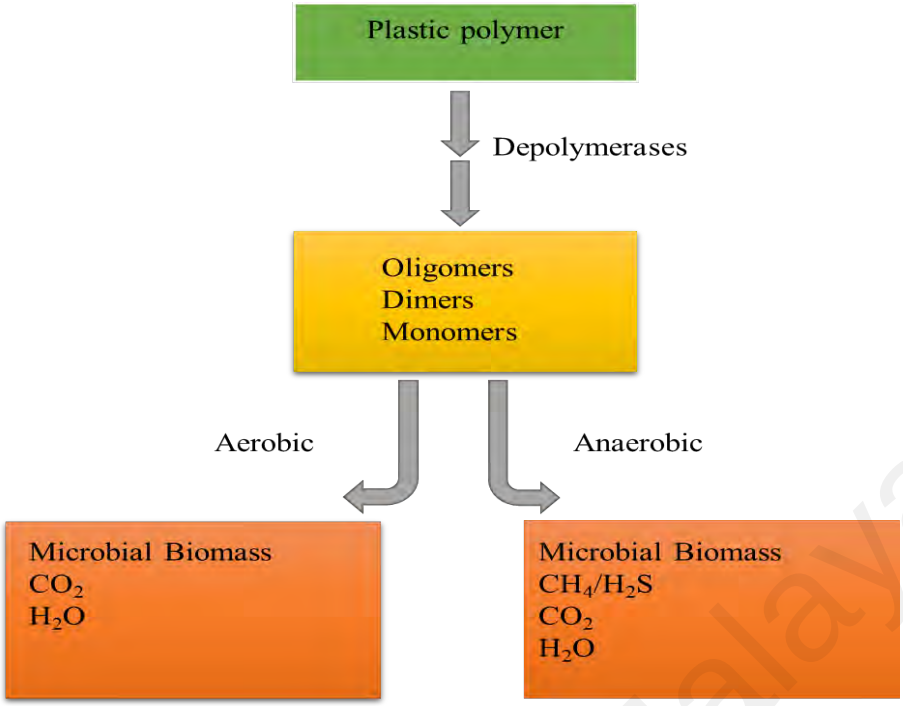

Figure 2.9: Schematic diagram of polymer degradation under aerobic and anaerobic conditions (Adapted from: Mohan and Srivastava (2010)

Bacteria have been utilized in the clean-up of PCBs (Michaud *et al.*, 2007), heavy metals (Emenike *et al.*, 2016), lubricating oil (Abioye *et al.*, 2010), polycyclic aromatic hydrocarbons (PAHs) (Mohd Radzi *et al.*, 2016), and crude oil (Auta *et al.*, 2014). Their metabolic diversity makes them a useful resource for remediation of pollution in contaminated environments (Iranzo *et al.*, 2001). Biodegradation is an attractive alternative to current practices for waste disposal as it is eco-friendly, less costly, more efficient and does not produce secondary pollutants such as those associated with landfilling and incineration (Webb *et al.*, 2012).

Microorganisms such as fungi and bacteria are involved in the degradation of both synthetic and natural plastics (Gu, 2003). They possess different characteristics and therefore, the degradation varies from one microorganism to another and each have their own optimal growth conditions. Polymers, especially plastics are potential substrates for

heterotrophic microorganisms. Microorganisms degrade polymers like PU, PS, PE, by using it as substrate for growth (Bhardwaj *et al.*, 2012; Shah *et al.*, 2008).

The degree of plastic polymer biodegradation is governed by various factors that include the organism characteristics (i.e. type of enzymes produced for biodegradation), kind of polymer, and the nature of pre-treatment (Artham & Doble, 2008; Bhardwaj *et al.*, 2012; Shah *et al.*, 2008). Polymer characteristics such as crystallinity, molecular weight, mobility, tacticity, type of functional groups and the substituents present in its structure, and additives or plasticizers incorporated into the polymer all play important role in its biodegradation (Artham & Doble, 2008; Shah *et al.*, 2008). Plastic degradation by microorganisms occur through several steps and are identified by specific terminologies namely biodeterioration, biofragmentation, bioassimilation and biomineralization.

2.14.1 Biodeterioration

A superficial degradation that modifies the physical, mechanical and chemical properties of plastic materials is termed deterioration. Biodeterioration result from the activity of microorganisms growing on the surface and/or inside a given material (formation of biofilm) (Lucas *et al.*, 2008; Walsh, 2001), and these microbes act on the polymer material by mechanical, chemical and/or enzymatic means (Gu, 2003; Lucas *et al.*, 2008).

The development of microbial biofilm is dependent on the composition and the properties of the plastic material, and also on environmental conditions such as weather, atmospheric pollutants and humidity (Lucas *et al.*, 2008; Lugauskas *et al.*, 2003). Diverse microorganisms are involved in biodeterioration of plastics and belong to fungi, protozoa, bacteria, algae, and lichnaceae (Wallström *et al.*, 2005). They form biofilms which work

in synergy and provoke serious physical and chemical damages on the polymer material (Bhardwaj *et al.*, 2012). The development of varying species of microorganisms in a specific order increases biodeterioration thereby, accelerating the production of simple molecules. These substances then act as nitrogen and carbon sources, as well as growth factors for the microbes (Lucas *et al.*, 2008). Several studies have shown that atmospheric pollutants are potential nutrients for some microbes. For example, the deposition of sulphur dioxide, aromatic and aliphatic hydrocarbons from urban air on several polymer materials have been reported to favour the colonization of polymer materials by other microbial species (Lucas *et al.*, 2008; Mitchell & Gu, 2000; Nuhoglu *et al.*, 2006; Zanardini *et al.*, 2000). Organic dyes have also been reported to serve as nutrients for the microorganisms (Faÿ *et al.*, 2007).

2.14.1.1 Physical deterioration

Microbial species adhere to the polymer surface and form a biofilm. The formation of biofilm is associated with the secretion of extracellular polymeric substances (EPS) made of proteins and polysaccharides (Cappitelli *et al.*, 2006). The EPS is a complex matrix that reinforces the cohesion of the microbial biofilm and adhesion to the plastic surface. It infiltrates the pores, alter the size and pore distribution, and changes the moisture degrees and thermal transfers, making the conditions favourable for the microbes (Dussud & Ghiglione, 2014). Filamentous microorganisms develop their mycelia framework within the material. The microorganisms then grow inside and in the process, increase the size of the pores and provoking cracks that weaken the physical properties (i.e. resistance and durability) of the plastic material (Bonhomme *et al.*, 2003; Lucas *et al.*, 2008).

2.14.1.2 Chemical deterioration

The microbial communities that develop on plastic material are highly diverse, with each contributing to chemical biodeterioration. The extracellular polymeric substances produced by microorganisms, act as surfactants which facilitate the exchange between hydrophobic and hydrophilic phases (Lucas *et al.*, 2008). These interactions favour the penetration of microorganisms. For example, the presence of slime increases the accumulation of atmospheric pollutants and this favours the development of the microbes thereby, accelerating the biodeterioration (Lucas *et al.*, 2008; Zanardini *et al.*, 2000). Each microbial flora developing successively on the polymer material contribute to chemical biodeterioration. Chemo-lithotrophic bacteria make use of ammonia, hydrogen sulphide, thiosulphates, elementary sulphur and nitrites as sources of electron and energy (Lucas *et al.*, 2008). They release active acidic compounds such as nitric acid (*Nitrobacter* sp.), nitrous acid (*Nitrosomonas* sp.) or sulphuric acid (*Thiobacillus* sp.) (Crispim & Gaylarde, 2005; Rubio *et al.*, 2006), while chemoorganotrophic microorganisms release organic acids including glyoxalic, glutaric, oxalic, fumaric, citric, oxaloacetic, and gluconic acids. Biotic and abiotic hydrolysis of polymers such as PLA, PBS and PBA, releases adipic acid, lactic acid and succinic acid, as well as butanediol (Lindström *et al.*, 2004; Tan *et al.*, 2008). Water gets into the polymer matrix which might be accompanied by swelling (Lucas *et al.*, 2008). Water intrusion initiates the hydrolysis of the plastic polymer, leading to the generation of monomers and oligomers. Continuous deterioration leads to changes in the microstructure of the matrix due to the formation of pores. The monomers and oligomers are then released. These degradation products (which normally have some acid-base characteristics (Göpferich, 1996), modify the pH inside the pores (Dussud & Ghiglione, 2014; Lucas *et al.*, 2008). Some sequestrate cations present into the matrix (e.g. Fe^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Al^{3+}, Si^{4+}), to form stable complexes, while others react with

the components of the material and increase the erosion of the surface (Lugauskas *et al.*, 2003).

2.14.2 Bio-fragmentation

Plastic polymers are molecules with high molecular weight that cannot cross the cell wall or/and the cytoplasmic membrane. Microorganisms therefore, secrete specific extracellular enzymes or generate free radicals that can catalyse reactions principally at the boundaries of the plastic material (Lugauskas *et al.*, 2003). The microbial vulnerability of plastic polymers is ascribed to the biosynthesis of enzymes including ureases, esterases, lipases, and proteases (Auta *et al.*, 2017a; Lugauskas *et al.*, 2003). These enzymes require the presence of cofactors such as coenzymes synthesized by microorganisms, and cations present in the polymer matrix (Pelmont, 2005). The enzymes decrease the level of activation energy of the molecules thereby, favouring chemical reactions (Lucas *et al.*, 2008). When released into the extracellular environment, enzymes are found as free catalysts, soluble within lipophilic or aqueous media or fixed on particles (sand, clay and soil organic matter). Fixed enzymes are stabilized and their catalytic activity is often increased (Mateo *et al.*, 2007).

The biodeterioration of thermoplastics polymers could proceed by two different mechanisms, namely bulk erosion and surface erosion.

- *Bulk (or homogeneous) erosion* - in which fragments are lost from the entire polymer mass and the molecular weight changes due to bond cleavage. This lysis is instigated by bases, acids, H_2O, radicals and transition metals, or by radiations but not by enzymes, because they are too large to penetrate throughout the matrix framework (Pepic *et al.*, 2008).

- *Surface (or heterogeneous) erosion* - in this case, matter is lost but the molecular weight of the polymer matrix does not change (Pepic *et al.*, 2008).

Bulk erosion for PLA and PLGA have been reported by Siepmann and Göpferich (2001), while surface erosion for PHB and polyanhydrides have been reported by Tsuji and Suzuyoshi (2002), and Göpferich and Teßmar (2002).

A polymer material is regarded as fragmented when low molecular weight molecules are found within the media (Lucas *et al.*, 2008). Gas chromatography (GC) and High-performance liquid chromatography (HPLC) are usually used to separate monomers and oligomers in a liquid or in a gaseous phase (Witt *et al.*, 2001) and intermediate molecules can be separated by mass spectrometry (MS) after separation (Beyler & Hirschler, 2002). The functional chemical changes are detected by Fourier transform infrared spectroscopy (FTIR) (Mohan *et al.*, 2016; Paço *et al.*, 2017). The structures of the monomers may be determined by nuclear magnetic resonance (NMR) (Lucas *et al.*, 2008; Marten *et al.*, 2005).

The limitation of bio-fragmentation is the stability of the plastic materials which are made up of long carbon and hydrogen chains that contain very balanced charges. Most bacteria that can breakdown plastics usually contain oxygenases which incorporate oxygen to a long carbon chain (Dussud & Ghiglione, 2014). For example, mono-oxygenases and di-oxygenases incorporate, respectively, one and two oxygen atoms, forming peroxyl or alcohol groups that are less recalcitrant to biodegradation. Subsequent transformations are then catalyzed by esterases and lipases after the formation of carboxylic groups, or by endopeptidases for amide groups (Dussud & Ghiglione, 2014; Lugauskas *et al.*, 2003).

2.14.3 Assimilation/mineralization

Assimilation refers to the integration of atoms from fragments of plastic polymers inside microbial cells. This integration brings to microorganisms the necessary sources of energy, elements and electrons such as carbon, phosphorus, nitrogen, sulphur, oxygen, etc., for the formation of the cell structure. The plastic monomers are oxidized through catabolic pathways to produce energy, cell structure, and new biomass. Assimilation allows microorganisms to grow and reproduce while consuming the plastic polymer substrate from the environment (Dussud & Ghiglione, 2014; Lucas *et al.*, 2008). For monomers surrounding the microbial cells to be assimilated, they must go through the cellular membranes. Some are easily carried inside the cell by specific membrane carriers while other molecules that are unable to pass through the membranes are not assimilated but can undergo biotransformation reactions yielding products that can be assimilated or unassimilated. Transported molecules are oxidized inside the cells via catabolic pathways leading to the production of adenosine triphosphate (ATP) and constitutive elements of cell structure (Lucas *et al.*, 2008).

Depending on the abilities of microbes to grow in aerobic or anaerobic environment, three essential catabolic pathways exist for energy production to maintain cellular activity, structure and reproduction. The three pathways are aerobic respiration, anaerobic respiration and fermentation (Dussud & Ghiglione, 2014). Assimilation results in the production of metabolites that can be transported outside the microorganisms that do not possess the metabolic capability to transform them or that do not need to metabolize or store it. The secondary metabolites produced could then be used by another to carry out further degradation or remain in the pool of non-assimilable compounds (Dussud & Ghiglione, 2014).

Mineralization on the other hand, is the complete degradation of primary and secondary metabolites that resulted in the excretion of complete oxidized metabolites (CO_2, H_2O, CH_4, N_2)) (Dussud & Ghiglione, 2014; Lucas *et al.*, 2008; Singh *et al.*, 2012). The different phases of polymer degradation are presented in Figure 2.10.

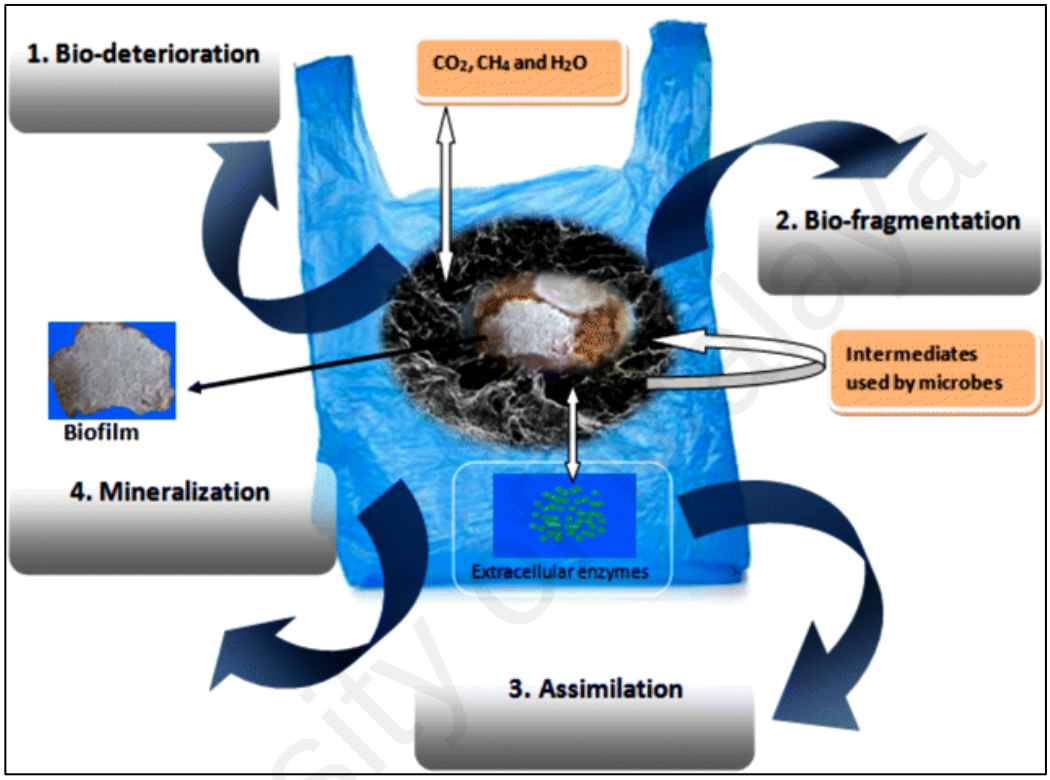

Figure 2.10: Different phases of polymer biodeterioration (Pathak (2017)

Numerous studies have investigated the biodegradation of a wide range of plastic polymers (Deepika & Jaya, 2015; Gewert *et al.*, 2015; Kavitha *et al.*, 2014; Mohan *et al.*, 2016; Paço *et al.*, 2017; Yoshida *et al.*, 2016). Gewert *et al.* (2015) reported that plastic polymers with carbon-carbon backbones (PE, PP, PVC and PS) are particularly resistant to biodegradation due to the high molecular weight and the lack of functional groups. They must therefore, be broken down by abiotic degradation to smaller pieces before biodegradation can take place. On the other hand, plastic polymers with heteroatoms in the main chain (polyamides, polyesters, PU, PET) show higher susceptibility to degradation (Gewert *et al.*, 2015).

2.15 Role of microorganisms in plastic polymer degradation

Microorganisms are ideally suited to the task of contaminant transformation or degradation because they are so small and are able to contact contaminants easily and because they are endowed with enzymes that allow them to use environmental contaminants as food (Devi *et al*., 2016).

Microbial biodegradation has become a widely accepted process of remediation and the bioremediation systems currently in operation rely on microorganisms native to the contaminated sites, boosting their efficiency by supplying them with the optimum levels of nutrients and other chemicals essential for their metabolism (biostimulation), or augmenting contained sites with non-native microbes (bioaugmentation), including genetically engineered microorganisms especially suited to degrading the contaminants of concern at a particular site (Devi *et al*., 2016; Raziyafathima *et al.*, 2016).

The transformation of organic contaminants by microorganisms normally comes about because the microbes can use the contaminants for their growth and reproduction. The organic contaminants provide carbon source which is one of the basic building blocks of new cell constituents. They also provide electrons needed by the organisms to obtain energy (Devi *et al*., 2016). Microorganisms obtain energy by catalysing energy-producing chemical reactions (oxidation-reduction reactions) that break chemical bonds and transfer electrons away from the contaminant. The energy gained is then "invested" along with some electrons and carbon from the contaminant to produce more cells Devi *et al*., 2016).

Microorganisms play very significant role in plastic degradation. Different types of microorganisms degrade different groups of plastics (Raziyafathima *et al.*, 2016). The biodiversity and occurrence of plastic-degrading microorganisms vary depending on the environment (such as sea, soil, sediment, compost, and activated sludge). Bacteria,

actinomycetes, and fungi degrade both natural and synthetic plastics (Deepika & Jaya, 2015; Gu, 2003; Mohan *et al.*, 2016; Paço *et al.*, 2017), and each have their own optimal growth conditions (Devi *et al*., 2016). Microbial degradation of plastics is brought about by hydrolysis or oxidation using microbial enzymes (Lucas *et al.*, 2008; Raziyafathima *et al.*, 2016). The adherence of microorganisms on the surface of the plastic polymer and the formation of biofilms is the major mechanism involved in microbial plastic degradation (Tokiwa *et al.*, 2009).

Oxidative degradation is the main mechanism involved in the degradation of plastics. The intracellular and extracellular enzymes that are produced by the organisms causes chain scission of the plastic material into smaller monomers, dimers and oligomers that are small enough to pass through the semi-permeable outer cell membranes to be utilized as carbon and energy sources (Devi *et al*., 2016; Raziyafathima *et al.*, 2016; Shimao, 2001). This initial process of polymer fragmentation is referred to as depolymerization and the degradation pathways associated with plastic degradation are often determined by environmental conditions. Hydrolysis is a type of enzymatic polymer cleavage in which peptide linkages, ester, and glycosidic bonds are subjected to hydrolysis through nucleophilic attack on the carbonyl carbon atom (Devi *et al*., 2016).

A microbe could constantly synthesize all enzymes required for degradation or they could activate the synthesis of the enzyme necessary to metabolize the plastic polymer when required, or is thermodynamically favourable (Devi *et al*., 2016). Microbial degradation of plastics has been reported by several studies which have addressed the abiotic and biotic degradation of a wide range of plastic polymers (Gewert *et al.*, 2015; Kale *et al.*, 2015). The different microorganisms that are responsible for the degradation of different groups of plastics are listed in Table 2.2.

Table 2.2: List of microorganisms associated with plastic polymer degradation

S/N	Plastic polymer type	Microorganism	Time of exposure (days)	Degradation efficiency (%)	Analytical technique	Observation	Reference
1.	Plasticizer PVC	*Aureobasidium pullulans* *Rhodotorula aurantiaca* *Kluyveromyces* sp.	720	6.8	SEM	Formation of colonies on polymer	Webb *et al.* (2012)
2.	Polyethylene bags & plastic cups	*Streptococcus* sp. *Staphylococcus* sp. *Micrococcus* sp. *Moraxella* sp. *Pseudomonas* sp. *Aspergillus niger* *Aspergillus glaucus*	270	2.19 & 1.07 16.39 & 0.56 6.61 & 1.02 7.75 & 8.16 20.54 & 8.16 17.35 & 5.54 28.8 & 7.26	Not specified	Not specified	Kathiresan (2003a)
3.	Polyethylene	*Rhodococcus ruber*	60	7.5	SEM	Biofilm formation	Sivan *et al*. (2006)
4.	LDPE	*Lynsinibacillus xylanilyticus* *Aspergillus niger*	126	15.8 29.5	FTIR, XRD, SEM	Appearance of new bonds, formation of ketone & aldehyde C=O groups, surface erosion, formation of cavities & pits	Esmaeili *et al*. (2013)

Table 2.2, continued.

S/N	Plastic polymer type	*Microorganism*	Time of exposure (Days)	Degradation efficiency	Analytical technique	Observation	Reference
5.	LDPE	*Kocuria palustris* M16 *Bacillus pumilus* M27 *Bacillus subtilis* H1584	30	1 1.5 1.75	FTIR	Formation of keto, vinyl, ester and internal double bonds	Harshvardhan and Jha (2013)
6.	Low molecular weight PE (LMWPE)	*Chelatococcus sp*. E1	80	Not specified	FTIR	Bond stretching, formation of alkenes, formation of new peaks, increase in peak intensity	Jeon and Kim (2013)
7.	LDPE	*Pseudomonas* sp. AKS2	45	6	SEM, AFM	Formation of biofilms, grooves & patches	Tribedi and Sil (2013)
8.	LDPE	Bacterial isolates 1 & 2	30	1.29 & 1.31	SEM, FTIR	Formation of hydroxylated, carboxylated compounds & ketone & aldehyde groups, broadening of peaks, formation of cavities & erosions	Kavitha *et al.* (2014)

Table 2.2, continued.

S/N	Plastic polymer type	Microorganism	Time of exposure (days)	Degradation efficiency (%)	Analytical technique	Observation	Reference
9.	Polyethylene -UV treated -autoclaved -surface sterilized	*Bacillus cereus*	90	 14 7.2 2.4	FTIR, SEM	Formation of aldehydes, carboxylic acids, alcohols, esters, ethers, alkenes, aromatics & phenol groups. Formation of holes.	
10.	PET & PS		Not specified	PET PS	Not specified	Not specified	Asmita *et al.* (2015)
		Bacillus subtilis		1.754 20			
		Pseudomonas aeruginosa		3.845 5			
		Staphylococcus aureus		0 4.762			
		Streptococcus pyogenes		3.922 8.33			
		Aspergillus niger		52.94 0			
11.	High Impact Polystyrene (HIPS)	*Pseudomonas* sp. *Bacillus* sp.	30	23	HPLC, NMR, TGA, FTIR, SEM	Changes in peaks, formation of halogenated compounds, release of bromine in form of methyl bromine, presence of phenyl ethanol	Mohan *et al.* (2016)

Table 2.2, continued.

S/N	Plastic polymer type	Microorganism	Time of exposure (days)	Degradation efficiency (%)	Analytical technique	Observation	Reference
12.	High Impact Polystyrene (HIPS)	*Enterobacter sp.* *Citrobacter sedlakii* *Alcaligenes sp.* *Brevundimonas diminuta*	30	12.4	FTIR, TGA, NMR, HPLC, SEM	Narrowing of absorption peaks, aromatic stretching, benzene ring formation, reduction in temperature, decrease in thermal stability, formation of aromatic & aliphatic protons, increase in peak	
13.	Polyethylene terephthalate (PET)	*Ideonella sakaiensis* 201-F6	Not specified	Not specified	FTIR, SEM, HPLC	Production of Mono (2-hydroxyethyl) terephthalic acid (MHET), TPA, bis(2-hydroxyethyl) TPA (BHET), pore formation	Yoshida *et al.* (2016)
14.	HDPE & LDPE	*Penicillium oxalicum* NS4 *Penicillium chrysogenum* NS10	90	55.34 & 36.60 and 58 & 34, respectively	FE-SEM, AFM, FTIR	Broadening of peaks, production of monomeric & oxidative forms of PE, formation of grooves & cracks	Ojha *et al.* (2017)
15.	polyethylene	*Zalerion maritimum*	28	Not specified	FTIR-ATR, NMR	Formation of hydroxyl, carboxyl, phenolic, esters, alcohol, carbonyl.	Paço *et al.* (2017)

2.16 General mechanism of plastic polymer biodegradation

The colonization of plastic marine debris by microorganisms was first reported in the 1970s, where studies noted the presence of diatoms and other microbes on the plastic debris (Carpenter & Smith, 1972; Colton *et al.*, 1974). Several studies have been carried out in which a few microbial strains capable of degrading PS (Asmita *et al.*, 2015; Atiq *et al.*, 2010; Mor & Sivan, 2008), PE (Asmita *et al.*, 2015; Deepika & Jaya, 2015; Harshvardhan & Jha, 2013; Kavitha *et al.*, 2014; Singh *et al.*, 2016; Sowmya *et al.*, 2014), and PVC (Shah *et al.*, 2008; Shimpi *et al.*, 2012) has been identified. Biodegradation is characterized with the development of biofilm on the microplastic surfaces by plastic-degrading bacteria (Deepika & Jaya, 2015), weight loss of the polymers, and the formation of pits on the surface of the microplastics (Wang *et al.*, 2016; Zettler *et al.*, 2013).

Once the organisms gets attached to the surface, it utilizes the plastic polymer as carbon source and begins to multiply (Caruso, 2015). PP and PE have CH_2 groups and are therefore hydrophobic (Arutchelvi *et al.*, 2008; Caruso, 2015). Initial chemical or physical degradation leads to the insertion of hydrophilic groups on the surface of the polymer, making it more hydrophilic for microbial attachment. The extracellular enzymes secreted by the microbes cleave the main chain and this lead to the formation of low molecular weight oligomers, dimers, and monomers. These low molecular weight compounds are then taken up by the microbes as carbon and energy sources (Arutchelvi *et al.*, 2008; Mueller, 2006). The mechanism of plastic biodegradation is presented in Figure 2.11.

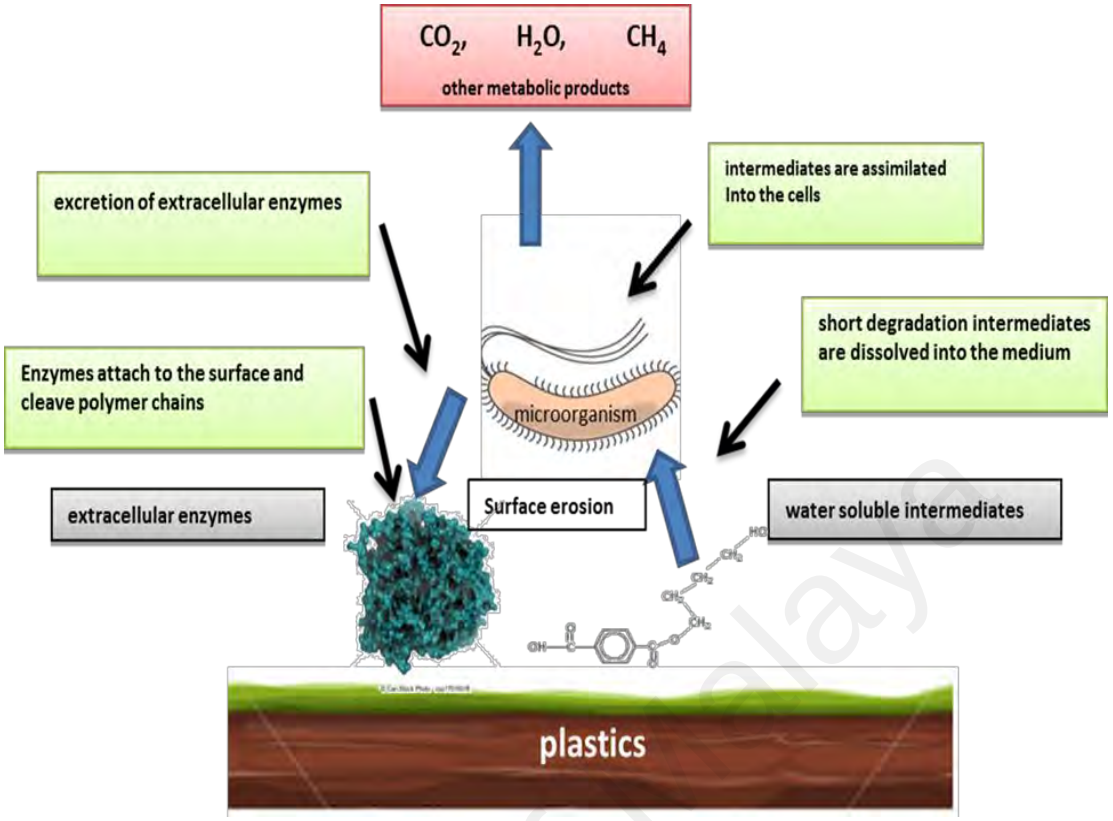

Figure 2.11: Summary of the mechanism of biodegradation of plastics (Adapted from Mueller, 2006)

Small oligomers may also diffuse into the organism and get assimilated. The final products of degradation are biomass, CO_2 and H_2O under aerobic conditions. Anaerobic microorganisms can also degrade plastic polymers under anoxic conditions leading to the production of end products such as biomass, CO_2, H_2O and CH_4 (under methanogenic conditions) and biomass, CO_2, H_2O, and H_2S (under sulfidogenic conditions) (Arutchelvi *et al.*, 2008).

2.17 Factors affecting plastic biodegradation

When investigating the biodegradability of a polymer material, the influence of the environment need to be taken into consideration. The different factors that influence microbial activity and biodegradation include oxygen, pH, polymer characteristics, molecular weight, melting temperature, chemical structure/composition, crystallinity,

hydrophobicity and hydrophilicity, additives/plasticizers/fillers/stabilizers and humidity/temperature.

2.17.1 Oxygen

Biodegradation of plastic polymers occur in basically two kinds of environments namely aerobic environment, in which oxygen is present and anaerobic environment, with no oxygen present (van der Zee, 2011). In the presence of oxygen, degradation of plastic polymers is brought about by aerobic microorganisms with microbial biomass, CO_2 and H_2O as end products. Whereas, under anaerobic conditions, polymers are degraded by anaerobic microorganisms leading to the formation of biomass, CO_2, H_2O, and methane (CH_4) as end products (Mohan, 2010; Muthukumar & Veerappapilli, 2015).

2.17.2 pH

Degradation rate of plastic polymers depends strongly on pH as it has crucial effect on the microbial population and enzyme activity (Gu, 2003). During the degradation process, chain scission transforms polymeric materials into oligomers, dimers and monomers which have different functional groups than the polymer (Mohan & Srivastava, 2010). Thus, esters and orthoesters are cleaved into alcohols, and carboxylic acids, and anhydrides into carboxylic acids. These degradation products influence the pH in the degradation medium, as well as, inside the pores (Göpferich, 1996; Mohan & Srivastava, 2010).

2.17.3 Polymer characteristics

Polymer characteristics refers to the complexity of the plastic materials with regard to their possible structures and compositions (Devi *et al*., 2016). The characteristics of plastic polymer materials such as modules of elasticity, crystallinity, tactility, chemical

structure, melting temperature, molecular weight, glass transition, surface conditions (hydrophilicity and hydrophobicity properties, surface area), and plasticizers/additives/fillers added to the polymer play important roles in the biodegradation process (Devi *et al*., 2016; Mohan, 2010; Muthukumar & Veerappapilli, 2015; Tokiwa *et al.*, 2009).

2.17.4 Molecular weight

Molecular weight is one of the factors that determine the biodegradation of plastic polymers. Low molecular weight is favourable for biodegradation (Tokiwa & Calabia, 2004). Degradation of plastics by microorganisms decreases with increase in molecular weight of the polymer. The higher the molecular weight the less soluble a polymer becomes, making it unfavourable for microbial attack as it needs to be incorporated into the cell membrane of microbes to be broken down into oligomers, dimers and monomers by enzymes (Tokiwa *et al.*, 2009). Abiotic hydrolysis, photo-oxidation and physical disintegration enhance the surface area of the plastic material and reduces its molecular weight thus, facilitating microbial degradation (Mueller, 2006; Mohan, 2010; Muthukumar & Veerappapilli, 2015; Tokiwa *et al.*, 2009; Yoon *et al.*, 2012).

2.17.5 Melting point

The melting temperature of plastic materials has significant effect on the enzymatic degradation of the polymer materials. The higher the melting point the lower the biodegradability of the polymer (Tokiwa & Calabia, 2004). Thus, the enzymatic degradation of plastic polymers decreases with increasing melting temperature. For example, low melting point polymers PPL, PBA and PCL were reported to have been effectively degraded by lipase produced by *Rhodococcus delemar*, but not polymer with high melting temperature like PHB (Tokiwa & Calabia, 2004; Tokiwa *et al.*, 2009).

2.17.6 Chemical structure/composition

Plastic polymers with side chains are not easily biodegraded when compared to plastic polymers with no side chains. Another structural characteristic of polymers is the cross-linking of polymers i.e., branching of chains or formation of networks. These varying structures of polymers can influence the accessibility of the plastic material to the enzyme-catalyzed polymer chain cleavage (Mueller, 2006).

2.17.7 Crystallinity

The degree of crystallinity is a critical factor affecting the biodegradability of polymer materials since the enzymes that catalyze biodegradation mainly attack the amorphous domains of polymers (Tokiwa *et al.*, 2009). Molecules in amorphous regions are loosely packed and are characterized with greater branching structures which make them prone to microbial attack (Tokiwa *et al.*, 2009). Amorphous polymers are more easily degraded as compared to crystalline polymers whose crystalline parts are rigid. The rigidity makes them more resistant to degradation (Singh & Sharma, 2008; Trivedi *et al.*, 2016; Wilkes & Aristilde, 2017). The high permeability of amorphous regions in a polymer are more labile to thermal oxidation (Singh & Sharma, 2008).

2.17.8 Hydrophobicity and hydrophilicity

The hydrophobic character of a polymer makes it less susceptible to biodegradation by microorganisms (Singh & Sharma, 2008). Hydrophilic degradation is therefore, faster than hydrophobic degradation (Alshehrei, 2017). PE is highly hydrophobic and therefore, interferes with the formation of biofilms and thus, reduces the extent of biodegradation (Singh & Sharma, 2008). Chemical or biological oxidation and hydrolysis of hydrophobic polymers increases the hydrophilicity of plastic polymers prior to microbial attack by providing functional groups such as carbonyl or alcohol groups that enhance bacterial

attachment and degradation (Arkatkar *et al.*, 2010; Sivan, 2011; Wilkes & Aristilde, 2017).

2.17.9 Additives/plasticizers/fillers/stabilizers

Non-polymeric impurities such as additives, stabilizers, plasticizers, pigments, antioxidants and fillers, added to a polymer during manufacture can lower the rate of degradation and may be toxic to microorganisms (Arutchelvi *et al.*, 2008), or can serve as good nutrients for the microbial assemblages that develop on polymer surfaces (Kyrikou & Briassoulis, 2007; Mohan & Srivastava, 2010). Yang *et al.* (2005) in their study reported that increase in the lingo-cellulosic filler loading in the polymer sample led to increase in the thermal stability and ash content in the sample. Additives can sometimes act as inhibitors (Kolvenbach *et al.*, 2014). For example, additives such as dibutyl tin dilaurate in PU-containing polymers act as antimicrobials (Cregut *et al.*, 2013; Wilkes & Aristilde, 2017). Other additives like glucose has been reported to affect degradation. This was demonstrated in the work of Tribedi *et al.* (2012), in which PES underwent a 45 % weight loss when acted upon by *Pseudomonas* sp. AKS2 in the absence of glucose whereas, with the addition of glucose the weight loss was reduced to 25 %.

2.17.10 Humidity/temperature

The extent of biodegradation can also be affected by abiotic factors such as humidity and temperature (Gu, 2003; Wilkes & Aristilde, 2017) which affect hydrolysis reaction rates during degradation. Increase in moisture and temperature leads to increase in hydrolysis reaction rates and microbial activity (Devi *et al*., 2016; Henton *et al.*, 2005). High-moisture conditions increases hydrolysis reactions which in turn, causes more chain cleavage, leading to increase in the available sites for microbial attack (Devi *et al*., 2016).

2.18 Biodegradation of synthetic plastic polymers

Synthetic plastics (polyolefins) are inert materials whose backbones are made up of long carbon chains. The characteristic structures such as high hydrophobicity, high molecular weight and lack of functional groups makes them resistant to biodegradation (Muthukumar & Veerappapilli, 2015). However, extensive study of the microbial degradation of synthetic plastic polymers has revealed that a number of microbes are capable of utilizing polyolefins especially those of low molecular weight (Muthukumar & Veerappapilli, 2015; Shah *et al.*, 2008). Generally, the degradation of synthetic plastics in nature is a very slow process that involves the action of environmental factors, followed by the action of microorganisms (Albertsson *et al.*, 1994; Devi *et al*., 2016; Gewert *et al.*, 2015). For synthetic plastics to be susceptible to biodegradation, they must undergo oxidation or hydrolysis by enzymes to create functional groups (e.g. carbonyl, alcohols or hydroxyl groups) that increases the hydrophilicity of the polymer and thus increase their availability for biodegradation (Devi *et al*., 2016; Gewert *et al.*, 2015; Muthukumar & Veerappapilli, 2015).

2.18.1 Polyethylene (PE)

PE is one of the synthetic polymers consisting of a backbone that is solely built of carbon atoms (Gewert *et al.*, 2015). It is a stable polymer with high molecular weight and high hydrophobicity makes the polymer resistant to degradation by microorganisms (Alshehrei, 2017; Muthukumar & Veerappapilli, 2015. Most packaging materials are made up of PE polymer (PlasticsEurope, 2015b), and as packaging materials are discarded after use, it has a higher potential to enter the environment in large quantities and to cause environmental problems (Gewert *et al.*, 2015; Shah *et al.*, 2008).

PE undergoes oxidation on exposure to UV radiation leading to the formation of oxygenated low molecular weight fragments like aldehydes, carboxylic acids, ketones, and alcohols (Vasile & Pascu, 2005). The presence of oxygen during the degradation process is essential. Random chain cleavage and cross-linking lead to a decrease or increase in the molecular weight, respectively. The degradation products formed include ethane, propene, propane, butene, hexane and ethene. The material becomes brittle and subject to fragmentation (Gewert *et al.*, 2015; Hakkarainen & Albertsson, 2004).

For PE to be biodegradable, the molecular weight, crystallinity level and mechanical properties that makes it resistant to degradation needs to be modified (Albertsson *et al.*, 1994; Shah *et al.*, 2008). This can be achieved through chemical or biological oxidation which increases the hydrophilicity of the polymer and makes it accessible for microbial attachment and degradation (Arkatkar *et al.*, 2010; Bikiaris *et al.*, 2007; Devi *et al*., 2016; Shah *et al.*, 2008). PE can also be made susceptible to microbial degradation by addition of pro-oxidants and starch. Addition of starch improves the hydrophilicity of the polymer and makes it susceptible to catalysis by amylase since microorganisms can easily access attack and remove this section (Alshehrei, 2017; Devi *et al*., 2016; Shah *et al.*, 2008). On addition of pro-oxidant (e.g. metal) to PE, the degradation is preceded by chemical and photo- degradation (Arkatkar *et al.*, 2010; Muthukumar *et al.*, 2011).

Microorganisms can attack PE at any terminal methyl group and the biodegradation is faster when the molecular weight is less than 500 Da (Gewert *et al.*, 2015; Vasile & Pascu, 2005). Smaller PE fragments are produced by abiotic reactions can be further biodegraded into more smaller fragments such as esters and acids. The ultimate end products after enzymatic action are humus, CO_2 and H_2O (Vasile & Pascu, 2005; Wilkes & Aristilde, 2017).

Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectra of virgin PE (Figure 2.12) shows vibrational peaks associated with C-H asymmetric stretching vibration at 2913 and 2846 cm^{-1}, C-H bending vibration at 1463 cm^{-1}, and C-C rocking vibrations at 729 and 719 cm^{-1}. During biodegradation, chain scission and hydrogen abstraction occurs on PE surface.

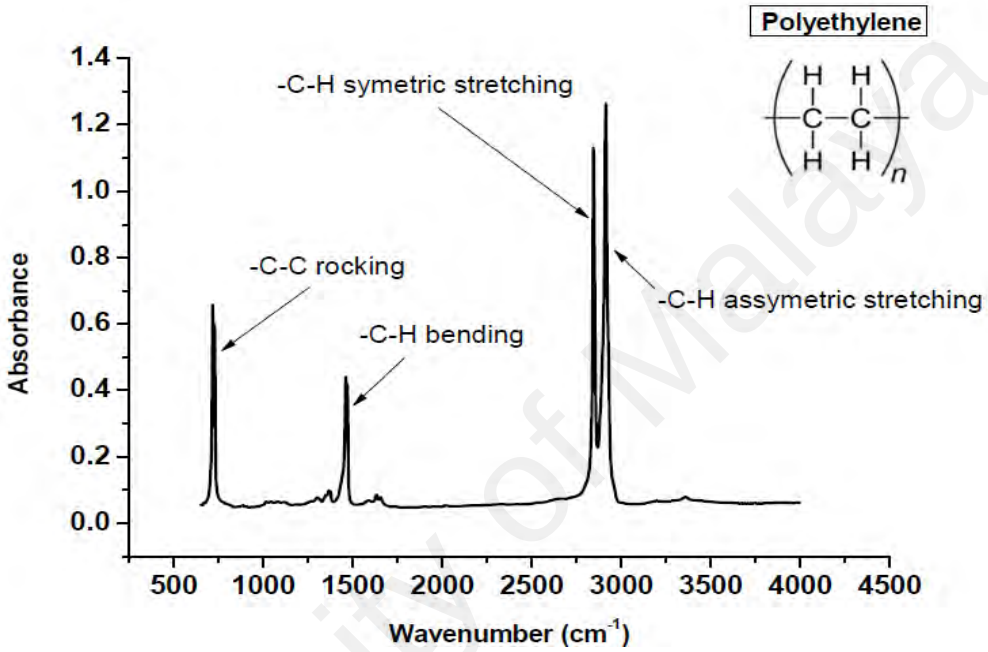

Figure 2.12: ATR-FTIR spectra of virgin PE (Kikani *et al*., 2015)

Studies have demonstrated the utilization of PE by microorganisms including *Desulfotomaculum nigrificans* and *Pseudomonas alcaligenes* isolated from soil (Begum *et al.*, 2015), marine bacteria such as *Kocuria palustris*, *Bacillus subtilis*, and *Bacillus pumilus* isolated from pelagic waters (Harshvardhan & Jha, 2013), *Bacillus* sp., *Staphylococcus* sp. and *Pseudomonas* sp. isolated from soil (Singh *et al.*, 2016), and the fungi *Streptomyces* sp., *Aspergillus niger*, and *Aspergillus flavus* isolated from garbage soil (Deepika & Jaya, 2015). Other PE degrading microbes include *Micrococcus* sp., *Moraxella* sp., and *Aspergillus* sp. isolated from mangrove soil (Kathiresan, 2003a), *Lysinibacillus xylanilyticus* isolated from soil (Esmaeili *et al.*, 2013), and *Penicillium*

oxalicum, and *Penicillium chrysogenum* isolated from plastic dumping soil (Ojha *et al.*, 2017). The degradation of PE in the marine environment is expected to be slow as the conditions are not optimized for polymer degradation. However, both abiotic and biodegradation are possible (Gewert *et al.*, 2015).

2.18.2 Polystyrene (PS)

PS is a synthetic plastic polymer that is hydrophobic, highly stable and has high molecular weight (Alshehrei, 2017). It is used in the production of disposable cups, laboratory ware, electronic equipment, and packaging materials. It is light weight and has excellent thermal insulation (Shah *et al.*, 2008). Under chemical or thermal degradation, products such as benzene, toluene, styrene, ethylbenzene, α-methylstyrene, phenol, ketones, acrolein, benzyl alcohol, benzaldehyde and benzoic acid are released (Alshehrei, 2017; Gewert *et al.*, 2015; Hoff *et al.*, 1982; Muthukumar *et al.*, 2011; Shah *et al.*, 2008). Few reports on the microbial degradation of PS exist, but the biodegradation of its monomer (styrene) have been reported. Mohan *et al.* (2016) and Sekhar *et al.* (2016) reported the biodegradation of high impact polystyrene (HIPS) by *Pseudomonas* sp., *Bacillus* strain, *Enterobacter* sp., *Alcaligenes* sp, *Citrobacter sedlakii*, and *Brevundimonas diminuta*. Other PS degrading microbes include *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pyogenes* *Aspergillus niger* (Asmita *et al.*, 2015), and *Rhodococcus ruber* (Mor & Sivan, 2008). PS is considered to be the most resistant thermoplastic polymer towards degradation by microorganisms (Mor & Sivan, 2008).

2.18.3 Polypropylene (PP)

Polypropylene is a thermoplastic commonly used in packaging materials, stationery, folders, plastic tubs, textiles, diapers, plastic parts and plastic mouldings (Alshehrei,

2017). PP plastic polymer is less stable than PE because every other carbon atom in the backbone is a tertiary carbon that is more prone to abiotic attack than the secondary carbons found in PE (Beyler & Hirschler, 2002; Gewert *et al.*, 2015). PP is liable to chain scission on exposure to UV radiation from sunlight and can be oxidized on exposure to heat (Alshehrei, 2017; Devi *et al*., 2016; Gewert *et al.*, 2015). PP is prone to oxidative degradation which usually occur in the tertiary carbon atom present. Free radicals are formed in the process which react with oxygen, followed by chain scission that lead to the production of lower molecular weight fragments and the formation of new functional groups like carbonyl and hydroperoxides groups (Chanda & Roy, 2006; Devi *et al*., 2016; Shyichuk *et al.*, 2001; Vasile, 2000). Products formed include aldehydes, carboxylic acids (Devi *et al.*, 2016b), pentane, 2-methyl-1-pentane and 2,4-dimethyl-1-heptane (Beyler & Hirschler, 2002; Gewert *et al.*, 2015).

Structural characteristics of PP (chain branching and the tertiary carbon present in the polymer) is reported to increase the resistance of PP to microbial degradation (Singh, 2004). Nevertheless, bacterial species such as *Vibrio* sp. and *Pseudomonas* sp., and fungal species such as *Aspergillus niger* have been reported to degrade PP polymer (Alshehrei, 2017; Arutchelvi *et al.*, 2008).

2.18.4 Polyethylene terephthalate (PET)

PET is a plastic polymer consisting of carbon and heteroatoms in the main chain and are therefore, more stable than polymers with solely carbon backbone (Gewert *et al.*, 2015). They are susceptible to hydrolytic cleavage of ester or amide bonds (Müller *et al.*, 2001).

Under marine environmental conditions, PET undergoes hydrolytic, photo and photo-oxidative degradation (Gewert *et al.*, 2015). During photodegradation, the ester bond is

cleaved leading to the formation of carboxylic and vinyl end groups directly, or radicals are formed which ultimately lead to the creation of carboxylic acid end group (Fagerburg & Clauberg, 2004; Gewert *et al.*, 2015). Like polymers with carbon-carbon backbone, PET can undergo photo-induced degradation by means of radical reactions, leading to the creation of a hydroperoxide at the methylene group. Photo-oxidation of PET is characterized by chain scission which lead to the formation of carboxylic acid group. The carboxylic acid end group formed influences thermos-oxidative and photooxidative degradations (Gewert *et al.*, 2015).

In water, PET undergoes hydrolytic degradation. During hydrolysis, carboxylic acid and alcohol functional groups are formed (Gewert *et al.*, 2015). The rate of hydrolysis is higher under acidic or basic conditions. Due to its compact structure, PET is highly resistant to microbial degradation. However, Zhang *et al.* (2004), in their study demonstrated weak degradation by microbes and lipase. Also, Yoshida *et al.* (2016), reported the degradation and assimilation of PET by a novel bacterium, *Ideonella sakaiensis*. The strain is reported to have produced two enzymes that were capable of hydrolysing PET and its intermediate (2-hydroxy ethyl) terephthalic acid. Other microorganisms that have been implicated in the degradation of PET include *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Aspergillus niger* (Asmita *et al*., 2015).

2.18.5 Polyvinyl chloride (PVC)

PVC is a strong plastic polymer that is resistant to abrasion and chemicals. It has low moisture absorption and is used in several applications including floor coverings, building pipes, fittings, electrical wire insulation, garden hoses, textiles, shoe soles, and synthetic leather products (Alshehrei, 2017; Muthukumar & Veerappapilli, 2015; PVC is the least

stable of the high tonnage polymer due to its sensitivity to UV radiation (Gewert *et al.*, 2015; Nicholson, 2017).

PVC monomer units contain only saturated chemical bonds and therefore, impurities are required for photo-initiation by UV radiation. Dichlorination of PVC is the first step that occur in PVC degradation when exposed to sunlight, this leads to the formation of conjugated double bonds in a polyene polymer and hydrochloric acid and little amounts of other products. The rate of photo-induced dichlorination of PVC is enhanced under aerobic conditions, humidity, mechanical stress, presence of other chemicals and high temperatures (Gewert *et al.*, 2015; Jakubowicz, 2003). The halogen component of PVC (chlorine) makes it highly resistant toward biodegradation. It is therefore, expected that abiotic degradation resulting in dichlorination of PVC will precede biodegradation (Gewert *et al.*, 2015). Reports exist on the degradation of PVC by *Aspergillus fumigatus*, *Phanerochaete chrysosporium*, *Lentinus tigrinus*, *Aspergillus niger*, and *Aspergillus sydowii* (Ali *et al.*, 2014; Devi *et al*., 2016).

Biodegradability tests are necessary to estimate the environmental impact of microplastics and to find solutions so as to avoid their disturbing accumulation in the environment (especially the marine environment). Microorganisms that can efficiently degrade plastic polymers can be explored for a better biodegradation of microplastic polymers. Low cost, efficient technology and eco-friendly treatments capable of reducing or eliminating microplastics are of great environmental interest, and biodegradation is an attractive option for environmentally friendly and efficient disposal of plastic waste. The microorganisms involved in plastic polymer degradation could be exploited for the clean-up of microplastic contaminated environments in bioremediation.

2.19 Bioremediation of microplastic contaminated soil

Environmental pollutants continue to be a major global concern and interest in the microbial degradation of pollutants has greatly intensified in recent years as mankind strives to search for sustainable means to clean up contaminated environments. Bioremediation technique has made it possible to diminish some of the damaging effects caused by pollutants in the environment and to effectively restore contaminated sites in an eco-friendly approach and at very low cost (Dadrasnia *et al.*, 2013).

Bioremediation is the process of using biological organisms (especially microorganisms), to degrade and detoxify hazardous substances into less toxic or non-toxic substances. It exploits biological activities for mitigation and complete elimination of the noxious effects caused by contaminants in the environment (Iturbe & López, 2015). Research has demonstrated that only few environments exist where microorganisms have not been able to survive, adapt and thrive. Microbes are able to utilize a wide variety of electron donors and electron acceptors (through oxidation and reduction reactions) to ferry their metabolism. In addition to this, they have also developed countless other strategies to enable them detoxify their environment. Bioremediation apply these principles to determine a suitable combination of the activities of the microbial assemblages, electron donor, pollutant concentration and other physical parameters to remediate or recover a targeted pollutant (Iturbe & López, 2015).

In bioremediation, the maximal possible growth of microorganisms is maintained until the pollutant (i.e. carbon source) diminishes and consequently, the population of the microorganisms decreases (Iturbe & López, 2015; Suthersan *et al.*, 2016). Bioremediation is a fast growing and promising remediation technique that is increasingly being explored and applied for clean-up of contaminants (Dadrasnia *et al.*, 2013). Numerous studies have demonstrated the effectiveness of bioremediation in the decontamination of surface and

subsurface soils, groundwater, contaminated land, and fresh water and marine ecosystems (Agamuthu *et al.*, 2013; Dadrasnia *et al.*, 2013). Various bioremediation techniques have been developed and modelled. However, no bioremediation technique serve as a "silver bullet" to restore contaminated environments due to the nature of the pollutants (Verma & Jaiswal, 2016).

Indigenous microorganisms that exist in microplastic-polluted environments hold the key to solving most of the challenges associated with biodegradation. Bioremediation of microplastic contaminants require that the environmental conditions are favourable for microbial growth and metabolism (Azubuike *et al.*, 2016; Verma & Jaiswal, 2016). Proper growth of the indigenous microorganisms requires sufficient oxygen supply, and nutrient content. Microorganisms from other location whose effectiveness have been tested can be added to contaminated sites that lack the presence of the biological activity required to degrade a particular contaminant (Azubuike *et al.*, 2016). The specific bioremediation technology to be used is determined by the site conditions, nature of the microbial populations present, and the quantity and toxicity of the pollutants. Different microorganisms degrade different types of compounds and survive under different conditions (Verma & Jaiswal, 2016).

The major advantages of bioremediation as compared to physical and chemical methods include:

- It is environmentally friendly,
- it is a cost effective and natural process,
- waste is permanently eliminated,
- many techniques can be conducted on site,

- uses naturally occurring microorganisms to breakdown hazardous substances into less toxic or non-toxic substances,
- pollutants can be treated on site, thus, reducing exposure risks,
- effluent levels generated are smaller when compared to standard practices, and
- often requires little to no residual treatment (i.e. microorganisms completely biodegrade the contaminant, whereas in physical or chemical treatment, the contaminants are often just separated (e.g. not actually remediating the contamination) (Azubuike *et al.*, 2016; Dadrasnia *et al.*, 2013).

Bioremediation has beneficial effects upon soil structure and fertility, but there are limitations on its effectiveness which include monitoring difficulties, space requirements, longer treatment time, difficult to determine whether contaminants have been destroyed (Azubuike *et al.*, 2016; Emenike, 2013).

The rate and extent of bioremediation of organic pollutants are influenced by a number of environmental factors, some of which can be manipulated whereas, others are difficult to modify within the contaminated site. The factors include temperature, pH, moisture, nutrients, contaminant availability, and contaminant concentration.

Temperature- influences the rate of microbial metabolism and hence, microbial activity (Adams *et al.*, 2015). The chemical and enzymatic reactions of the microbial cell increase with increasing temperature, and slows with decreasing temperature (Iturbe & López, 2015). Each organism has minimal temperature below which no growth occurs, an optimal temperature at which growth is faster, and a maximal temperature above which growth can occur. For example, heterotrophic aerobic bacteria have optimal temperature range between 20 ^{o}C to 35 ^{o}C (Iturbe & López, 2015; Yong & Mulligan, 2003).

pH- the intracellular pH value lies between pH 6.5 and 7.5. Hence, this is the required pH range for optimal microbial growth (Iturbe & López, 2015). Majority of bacteria depict optimal growth at neutral pH and in most cases, pH adjustment enhances the rate of biodegradation (Priyadarshini *et al.*, 2016).

Moisture- is the main nutrient of biological cells is water, and it constitute about 80% to 90% of the weight in the molecular composition. Water content in soils or sediments is an important factor that affect rate of biodegradation and bioremediation. The presence of water in the environmental matrix is vital for microbial growth. Microorganisms require about 12% to 25% of moisture for optimum growth and proliferation (Adams *et al.*, 2015; Mukherjee & Das, 2005).

Nutrients- nutrients are required by microorganisms for growth and division. The solid portion of the bacterial cell is composed of hydrogen, nitrogen, carbon, phosphorus, calcium, magnesium, potassium, iron, chlorides, etc., with carbon (the main component) making up 50% of the cell. Thus, the pollutant to be degraded must contain this element (Iturbe & López, 2015). Suitable amounts of trace nutrients for microbial growth are usually present but in almost all cases, addition of nutrients (via organic substrates) significantly increases the rate of bioremediation. The nutrients serve as electron donor to stimulate bioremediation (Adams *et al.*, 2015).

Contaminant availability- depends on the degree to which they sorb to solids or are sequestered by molecules in contaminated media. It also depends on the rate at which contaminants are diffused in macro-pores of soil or sediment, and other factors such as whether contaminants are present in non-aqueous phase liquid (NAPL) form. Pollutants that are more strongly sorbed to solids, enclosed in matrices of molecules in contaminated media, more widely diffused in macro-pores of soil and sediments, or are present in NAPL form are less available for microbial reactions (Adams *et al.*, 2015).

Contaminant concentrations- when pollutant concentrations are too high, the contaminants may have toxic effects on the microbial population. In contrast, low contaminant concentration may prevent induction of microbial degradation enzymes (Adams *et al.*, 2015).

2.19.1 Bioremediation approaches

Bioremediation technologies can be categorized into *in situ* and *ex situ*. *In situ* bioremediation technology involves treating the contaminated soil in the original location in which it was found and bringing the biological mechanisms to the contaminated soil. *Ex situ* bioremediation on the other hand, require excavation of the contaminated soil before it can be treated (Agamuthu *et al.*, 2013; Azubuike *et al.*, 2016; Pavel & Gavrilescu, 2008; Williams, 2006).

Bioremediation processes can thus, be classified into three different strategies namely bioattenuation, biostimulation and bioaugmentation.

Bioattenuation is a simple strategy that relies on natural degradation processes to dissipate contaminants through biological transformation. In bioattenuation process, indigenous microbial populations degrade pollutants based on their metabolic processes (Vásquez-Murrieta *et al.*, 2016). The natural degradation progress is characterized and monitored to ensure reduction in the pollutant concentrations with time at the desired contaminated site (Emenike, 2013; Rifai *et al.*, 1995).

Biostimulation- In some instances, the biological organisms attacking contaminants are extremely slow due to the lack of key nutrient needed for their growth and metabolism. In this case, manipulation of the biodegradation process such as provision of the needed nutrient can significantly enhance the rates of bioremediation. This is referred to as biostimulation as it primarily involves the addition of organic or inorganic

compounds to the contaminated environment in form of oxygen, substrates, vitamins, phosphorus, nitrogen, carbon, fertilizer and surfactants (Agamuthu *et al.*, 2013; Iturbe & López, 2015). Biostimulation techniques have been utilized in the removal of a wide array of environmental contaminants including herbicides (Kadian *et al.*, 2008), pyrene (Ghaly *et al.*, 2013), PAHs (Vattaso, 2014), petroleum hydrocarbons (Darmayati *et al.*, 2015; Simpanen *et al.*, 2016; Wu *et al.*, 2016), and diesel (Taccari *et al.*, 2012). However, the interest to introduce desired microbial strains into contaminated environments as an option of bioremediation has attracted significant attention.

The process of bioaugmentation involves the application of native or tailored microbial consortium to polluted environments in order to accelerate the removal of undesired compounds (Mrozik & Piotrowska-Seget, 2010). In most bioaugmentation processes, cultured microbes used for remediation are 'specialists' in degrading specific target pollutants. Scientific advances have allowed the isolation and mass-production of standardized pro-biotic microorganisms into concentrated inoculums of multiple strains which can act on specific contaminants. The standardized formulations of high concentration of microbial populations allows the bioaugmentation process to remediate contaminants at a rate which far exceeds that of natural indigenous microbes (Nzila *et al.*, 2016).

Some pollutants are either resistant to biodegradation or their chemical structures may be so complex that consortia of different microbes may be necessary for their biodegradation (Jasmine & Mukherji, 2014). Bioaugmentation can overcome these challenges since treatment can be tailored to a specific contaminant that is dominant in the environment. Moreover, a mixed consortium may display diverse modes of pollutant uptake (Jasmine & Mukherji, 2014). Bioaugmentation is therefore, an attractive approach

for addressing the increasing number of emerging pollutants, as well as, pollutants that are present at high concentrations (Nzila *et al.*, 2016).

For bioaugmentation to be successful in the field, the microbial inoculum formulations must be able to degrade the pollutant, maintain genetic stability and viability during storage, have the ability to survive in foreign, hostile, and stressful environment, effectively compete with indigenous microbes, and penetrate pores of the sediment to the pollutant (Adams *et al.*, 2015; Goldstein *et al.*, 1985). When screening for microorganisms to be applied in bioaugmentation process, the chemical structure and concentration of the pollutant, nature and size of the microbial population, and the physical environment (i.e. temperature, moisture, pH) are factors that should be taken into consideration (Adams *et al.*, 2015).

Bioaugmentation has been proven successful in remediation of environments contaminated with hydrocarbons (Benyahia & Embaby, 2016; Malina & Zawierucha, 2007), DDT (Gao *et al.*, 2015), PCBs (Federici *et al.*, 2012), and diesel oil (Lee *et al.*, 2011; Mariano *et al.*, 2009). Other pollutants include 2,4-dichlorophenoxyacetic acid (Chang *et al.*, 2015), and endosulfan (Bhalerao, 2012). However, the technology still faces many challenges such as survival of strains introduced into the contaminated soils. It has been demonstrated that a number of exogenous microorganisms reduces shortly after inoculation, and that a number of both abiotic and biotic factors influence the effectiveness of bioaugmentation (Bento *et al.*, 2005; Mrozik & Piotrowska-Seget, 2010; Wolski *et al.*, 2006). In a study that was aimed at evaluating the merits of bioaugmentation and biostimulation strategies of soil contaminated with crude oil, bioaugmentation reduced 77% of the contaminant in 156 days which was more while compared with the biostimulation option (23% reduction) (Benyahia & Embaby, 2016). Fan *et al.* (2014) studied the bioaugmentation ability of *Candida tropicalis* on total petroleum hydrocarbon

(TPH), and they observed that at optimum pH, 96% and 42% of TPH were degraded by the organism at initial diesel oil concentrations of 0.5% and 5% v/v respectively.

2.20 Management strategies for microplastic pollution (challenges and possible solutions)

Plastic production has followed an exponential increase for several decades and it seems inevitable that the abundance of microplastic particles will continue to increase in years to come. As an emerging pollutant of great concern, the possible detrimental dangers posed by microplastics is comparable to macro-plastics. To decrease the entry of microplastics into the aquatic environment, the original sources and classes of plastics and microplastics entering the marine environment need to be identified. Also, creating public awareness through education at the public, private, and government sector is necessary. Concern about microplastics has led to the development of management guidelines by several organizations. For example, The United Nations Expert Panel of the United Nations Environmental Programme (UNEP) has called for immediate action to rid the oceans of microplastics as they have noted that microplastics are consumed by a large number of marine organisms, and that this inflicts both physical and chemical harm on them (Caruso, 2015). Therefore, UNEP has come up with a program engaging over 40 million people from 120 countries and has set up educational measures to create awareness and promote the decrease of plastic use, encourage recycling, and evaluate disposal facilities (Caruso, 2015). Similarly, the United Nations Environment Program/Mediterranean Action Plan (UNEP-MAP), the Oslo/Paris convention (for the protection of the marine environment of the North-East Atlantic (OSPAR), and the Baltic Marine Environment Protection Commission-Helsinki Commission (HELCOM) have developed guidelines for assessing marine litter including microplastics. The plan includes organizing several workshops to encourage capacity building and spreading of

good practices among individuals. The plastic industry in 2011 came up with a Joint Declaration of the Global Plastics Associations for solutions on marine litter which comprised of programs to reduce litter and the commitment to support a number of litter assessment (Kershaw, 2015). Non- Governmental Organizations (NGOs) have also come up with programs aimed at raising awareness and help to quantify the extent of microplastics pollution and the effects at the national, regional and international scale. All are aimed at creating a safe environment for marine life and for humans. The Plastic Disclosure Project (PDP) intends to reduce the environmental impact of plastic wastes by encouraging companies to use plastics more efficiently and intelligently and creating awareness on methods for daily use of plastic materials. The Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) advocates for all nations to lead urgent efforts on decreasing the amount of plastics entering the ocean by adopting the reduce reuse-recycle (3R) circular economy as this will represent a cost-effective way to reduce the quantity of plastic objects and microplastics particles entering and gathering in the ocean (Kershaw, 2015). In 2015, the California Microbead Ban, AB 888 was approved (Casebeer, 2017). The ban is to provide the strongest protections from plastic microbead pollution, which include the banning of all types of plastic microbeads and encourages the use of natural alternatives such as walnut husks, sea salt and apricot pits in toothpastes, facial scrubs and body washes. AB 888 plans to ban the sale of products containing plastic microbeads by the year 2020 (Casebeer, 2017).

Pollution of the marine environment by microplastics has become so widespread, and their persistence continues to increase due to the difficulty to remove them since they are small and less visible. Also, the rate at which microplastics enter the environment supersedes the rate of removal. Hence, identifying the possible origins of sea and land-based sources for plastics and microplastics is crucial to understand the treatment options. However, a more promising approach could be provided by utilizing microbes that are

able to degrade microplastic polymers through bioaugmentation as a bioremediation option. Bacteria are very opportunistic and can invade and adapt in any environment. Several bacteria species have been reported to degrade plastic polymers. These microbes could thus, be harnessed as an environmentally safe way to degrade microplastics. Such microbes could then be applied to the treatment of sewage wastewater as this could limit inputs from domestic uses or they could be exploited for the remediation of contaminated environments.

CHAPTER 3: MATERIALS AND METHODS

3.1 Biodegradability studies of PE, PP, PET and PS microplastics

This chapter describes the biodegradation studies of different microplastics by microbes isolated from mangrove sediments. PE, PP, PET and PS were selected for the study as they are among the commonly found in the mangrove ecosystem.

3.1.1 Polymer characterization

Microplastics used in this study were purchased from Sigma Aldrich Chemical Co. (Product of USA) and some were used plastics picked up from the sampling sites and consisted of;

i. Polyethylene (PE) powder (white), ultra-high molecular weight, surface modified powder with 53-75 µm particle size, CAS: 9002-88-4 and density of 0.94 g/mL at 25 ^{o}C.

ii. Polypropylene (PP) pellets (white, spherical), isotactic, with a density of 0.9 g/mL at 25 ^{o}C (lit.), average molecular weight (Mw): 250,000, and number average molecular weight (Mn): 67,000, CAS no: 9003-07-0.

iii. Polystyrene (PS) granules (white/spherical) with density of 1.59 g/mL at 25 ^{o}C, average molecular weight (Mw): 192,000, CAS number: 9003-53-6, n20/D 1.59.

iv. Polyethylene terephthalate (PET) granular (milky white), containing 30% of glass particles as reinforcer, density of 1.68 g/mL at 25 ^{o}C, melting point (mp): 250-255 ^{o}C. CAS number: 25038-59-9 (Plate 3.1).

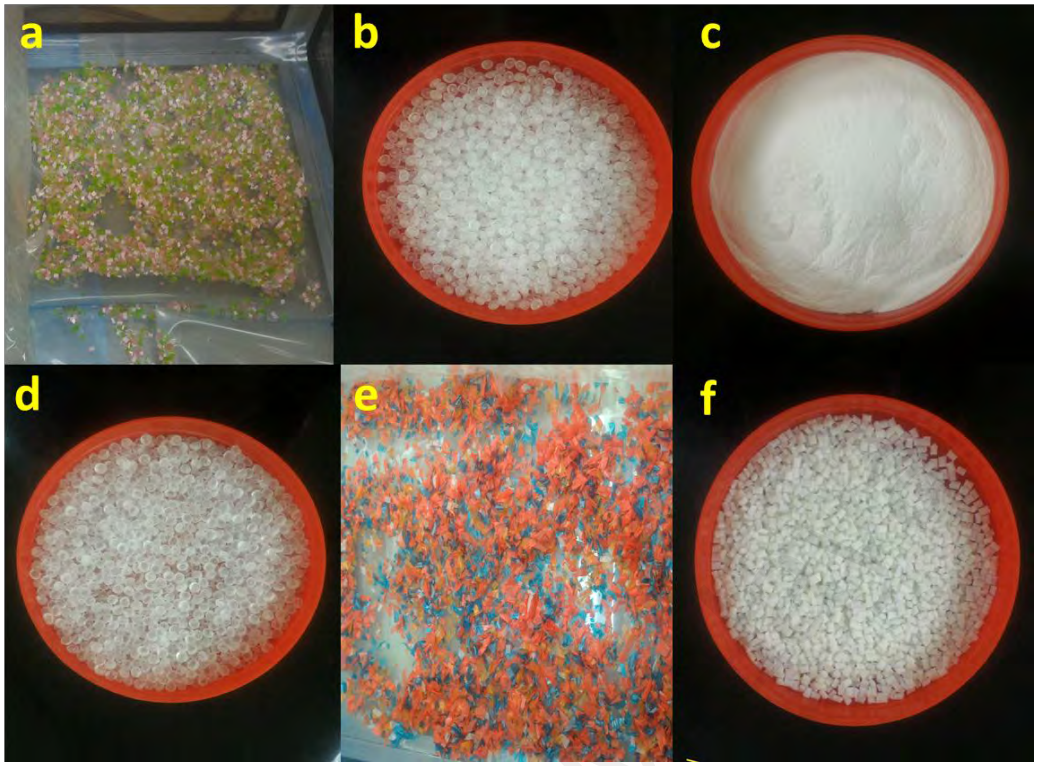

Plate 3.1: Microplastics used in the study: (a) PP & PET fragments, (b) PS pellets, (c) PE powder, (d) PP pellets, (e) PE fragments, (f) PET granules

For degradation experiments, microplastic were obtained by grating/cutting commercial plastic materials obtained from plastic producing industries made of PE, PP, PET, and PS, using a bastard-cut hand file and scissors. The grated plastics obtained were passed through sieves (mesh size 2 mm & 5 mm) (mesh No. 60, Chunggye Industrial MFG Co., Seoul, Republic of Korea) to screen off larger debris. Each was irradiated for 2 days under ultra violet rays and stored for further use. The sizes of the prepared plastic debris were measured using an optical microscope (IX71, Olympus, Japan) equipped with 4 × lens (Olympus) (Auta *et al.*, 2018).

3.2 Soil sample collection and characterization

The mangrove sites were randomly selected in this study to collect sediment to provide the indigenous microbes for the biodegradation and bioremediation study. The soil samples were collected bi-monthly from different locations at mid tidal zones through a period of twelve (12) months to observe the seasonal variations in the diversity of the microbes. Samples were collected from Matang mangrove in Perak (4°50'25.80"N, 100°38'9.60"E), Cherating mangrove in Pahang (4°7'36.15"N, 103°23'29.46"E), Tanjung Piai in Johor (1°16'5.20"N, 103°30'31.36"E), Sekam mangrove in Melaka (1°19'37.84"N, 103°26'30.61"E), Sedili Besar in Johor (1°55'54.39"N,104°7'27.25"E), and Pasir Puteh mangrove in Kelantan (5°50'0.79"N, 102°25'41.07"E), in Peninsular Malaysia. The map of different sampling locations and geographical coordinates is presented in Figure 3.1 and some of the sampling environments are presented in Plate 3.2. Soil samples were collected from 0 cm to 4 cm depths in the sediment from 3 different points with a quadrat of 0.5 m x 0.5 m placed 2 m apart from high tide in undisturbed areas as described by Nor and Obbard (2014), filled into sterile containers and transported to the laboratory for further analysis. All sample collections were collected in replicates to accommodate variability and ensure homogeneity. The excavated samples were analyzed for pH, salinity, and temperature using a multi probe meter (YSI Professional Plus, USA). All assessments were carried out in triplicates. Sequel to sample collection, microbial study became a routine component of the study. Preliminary investigation and assessment of the mangrove sites; including visual observation, topographical outlay, and soil testing, determined the degree of heterogeneity and siting of the sampling spots.

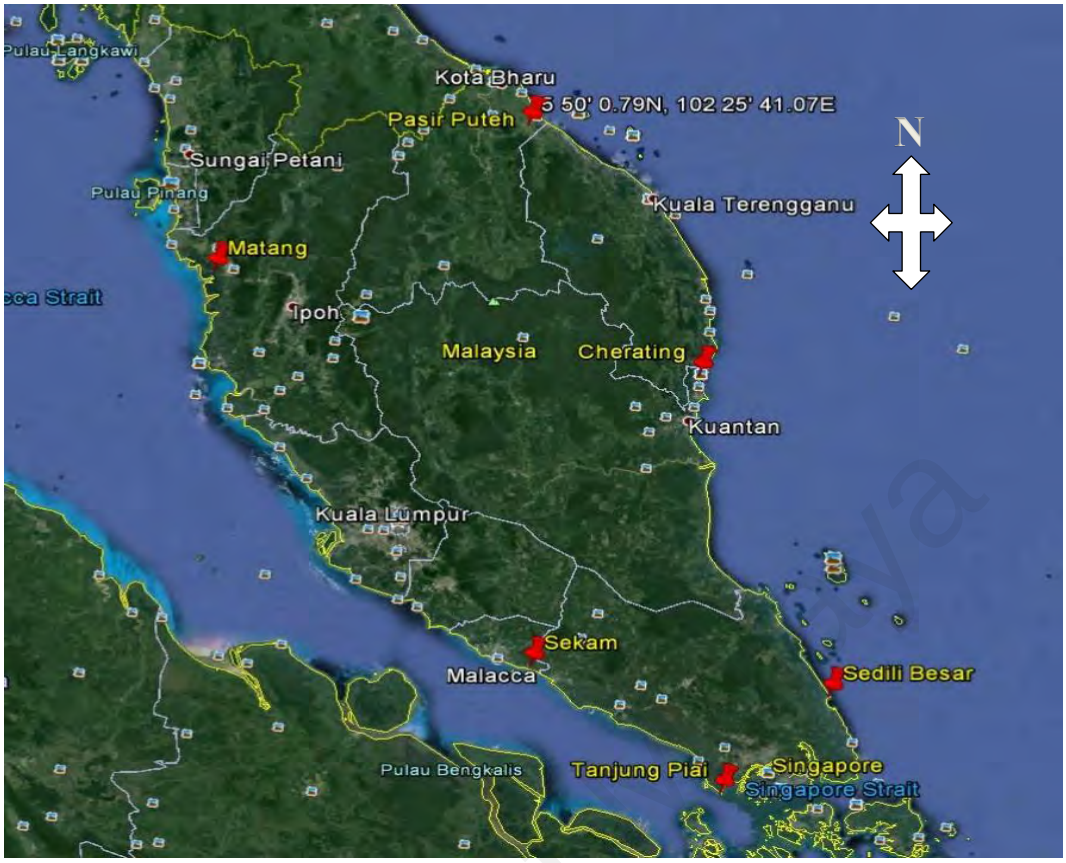

Figure 3.1: A geographical map showing the sampling sites (marked red) and locations

Plate 3.2: Soil collection points (a) Sekam mangrove, Melaka (b) Tanjung Piai mangrove, Johor

3.2.1 Media

For the isolation and screening process, routine culture media including Nutrient agar and Bushnell Haas media (BH) were used. The composition of BH media was as follows (g/ml): $MgSO_4$ (0.20), $CaCl_2$ (0.02), KH_2PO_4 (1.00), K_2HPO_4 (1.00), NH_4NO_3 (1.00), $FeCL_3$ (0.05), agar (20.00), and pH (7.0 ± 0.2).

3.3 Bacteria isolation from mangrove sample sites

Bacteria species were isolated by mixing 1 g of soil samples from Matang, Cherating, Tanjung Piai, Sekam, Sedili Besar, and Pasir Puteh mangroves with 10 ml of normal saline water (0.9 % NaCl) as stock. The mixture was vigorously shaken for 3 h at 150 rpm using Lab-Line 3521 orbit shaker (LabLine Instruments, Inc., Maharashtra, India). The resulting suspension was subjected to 20 times serial dilution. 0.1 ml dilutions were dispensed on freshly prepared nutrient agar (NA) under aseptic conditions (Kauppi *et al.*, 2011). The inoculated media plates and associated replicates were incubated at 37 ^{o}C for 24 h. Colonies that developed were further sub-cultured on freshly prepared NA in triplicates to obtain discrete individual pure cultures and ensure the purity of the samples prior to identification (Plate 3.3).

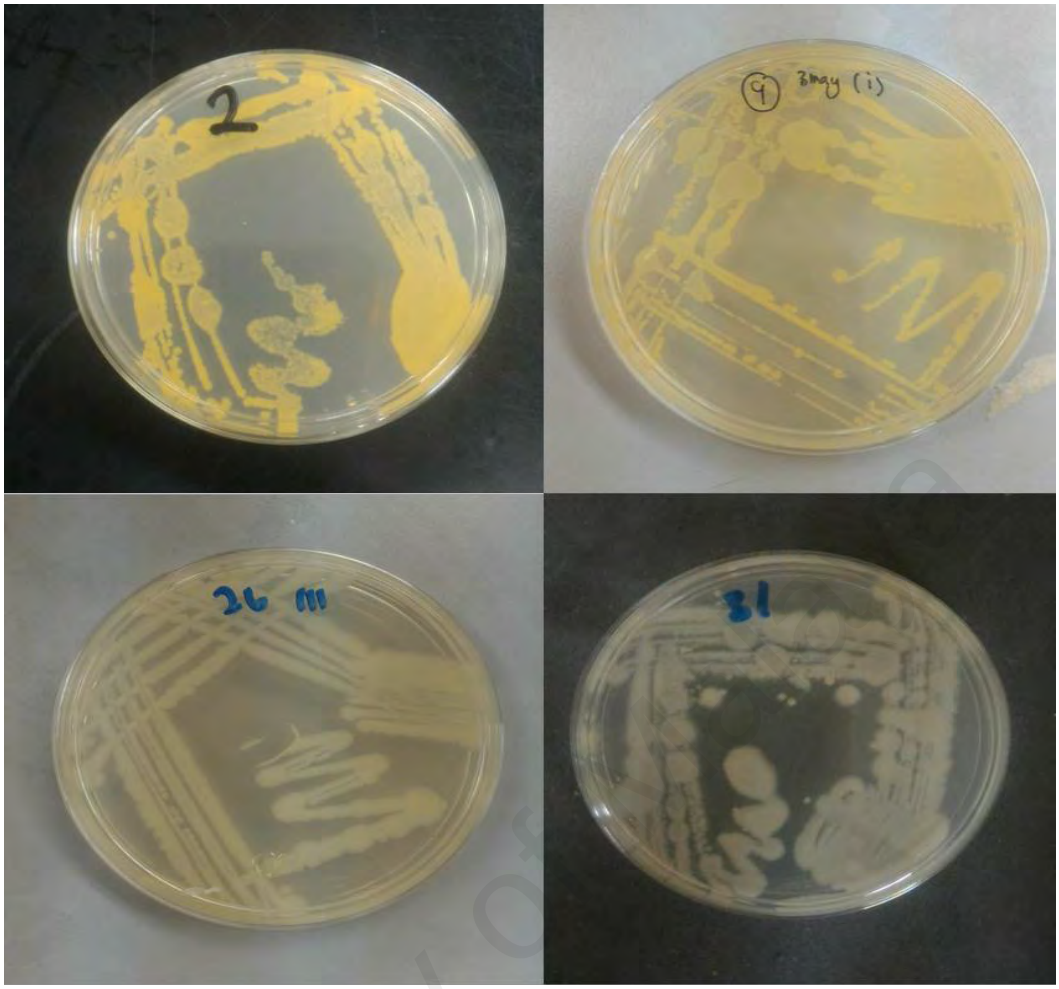

Plate 3.3: Samples of bacteria isolated from mangrove soil

3.4 Identification of microbes

Isolated bacteria were identified using the Biolog GEN III microplate protocol. A standardized micromethod to profile and identify a broad range of Gram-positive and Gram-negative bacteria using 94 biochemical tests is provided by the GEN III MicroPlate™ test panel (Bochner, 1989). It is comprised of 71 carbon source utilization assays and 23 chemical sensitivity assays. The test panel provides a "Phenotypic Fingerprint" of the microorganism that can be used to identify the microbe at the species level. Omnilog® Data Collection (Biolog's microbial identification system software) was used to identify each isolate from its phenotypic pattern in the GEN III MicroPlate.

For identification purpose, the cells were freshly regrown on agar in order to avoid loss of viability and metabolic vigour which is typical of most organisms when in the stationary phase. Using the inoculum fluid (IF), inoculums of each cell was prepared using Protocols A (IF-A Catalog no. 72401) and B (IF-B Catalog no. 72403) at turbidity range of 95 – 98%T. This was done using a cotton-tipped inoculator swab (Catalog no. 3321) to pick up 3 mm diameter area of cell growth from the surface of the agar plate, and eventually dipping it into the desired IF. To ensure uniform suspension, any cell clump was carefully crushed against the tube wall. The resulting cell suspensions were poured into a multichannel pipette reservoir.

An 8-channel automated pipettor was used to dispense 100 μL of the suspension into each of the wells in the MicroPlate (Catalog no. 1030). The wells (Table 3.1) contained 71 carbon source utilization assays (Column 1 – 9) and 23 chemical sensitivity assays (Columns 10 – 12), hence, the isolates can be identified at the species levels based on the "Phenotypic Fingerprint" of the microorganisms provided by the test panel. All the wells start out colourless when inoculated. During incubation, there is increased respiration in the cells where the cells can utilize a carbon source and/or grow. Increased respiration causes reduction of the tetrazolium redox dye, forming a purple colour. Negative wells remain colourless as does the negative control well (A-1) with no carbon source. A positive control well (A-10) is also present and is used as reference for the chemical sensitivity assays in columns 10-12. After incubation, the phenotypic fingerprint of the purple wells is compared to Biolog's extensive species library. If a match is found, a species level identification of the isolate is made. These MicroPlates were placed in Omnilog reader which identified the bacteria species contained in the Biolog's Microbial Identification Systems Software. The identified microbes were recorded. The microbes isolated/identified were then formulated for biodegradability tests.

Table 3.1: Layout of assays for MicroPlate (GEN III)

A1 **Negative control**	**A2** **Dextrin**	**A3** **D-Maltose**	**A4** **D-Trehalose**	**A5** **D-Celloblose**	**A6** **Gentiblose**	**A7** **Sucrose**	**A8** **D-Turanose**	**A9** **Staychose**	**A10** **Positive control**	**A11** **pH 6**	**A12** **pH 5**
B1 **D-Rafflinose**	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic acid	B10 1% NaCl	B11 4% NaCl	B12 6% NaCl
C1 **α-D-Glucose**	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 **D-Sorbitol**	D2 D-Mannitol	D3 D-Arabitol	D4 Myo-inositol	D5 Glycerol	D6 D-Glucose-6-PO_4	D7 D-Fructose-6-PO_4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 **Gelatin**	E2 Glycl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidne	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 **Pectin**	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 **p-Hydroxy-Phenlyacetic Acid**	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Gglutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 **Tween 40**	H2 γ-Amino-Butryic Acid	H3 α-Hydroxy-Butryic Acid	H4 β-Hydroxy-D,L-Butryic Acid	H5 α-Keto-Butryic Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

3.5 Screening bacterial isolates for PE, PP, PET and PS microplastics degradation

Isolates were screened for microplastic degradation using Bushnell-Haas Media (BH) as described by Kannahi and Sudha (2013) and Harshvardhan and Jha (2013), with modifications. The media contained all nutrients except a carbon source necessary for bacterial growth. All bacteria isolated were assayed for the potential to utilize PE, PET, PP, and PS microplastics as source of carbon and energy for growth. Isolated bacteria were aseptically re-grown by inoculating each species onto NA. All pure colonies obtained were transferred unto freshly prepared BH agar plates supplemented with 0.5 g of microplastics at pH 7 and incubated for a period of four weeks at room temperature. Control sets were maintained (inoculation on media without polymer) for each sample simultaneously, and the media were observed for growth. All experiments were carried out in triplicates. Polymer degrading activity of the isolates was screened by formation of clear halo zones around the colonies. The diameter of clear zones was measured and recorded after nine days.

3.6 Biodegradation experiment setup using individual isolates (shake flask experiment)

The degradation of PE, PP, PET and PS microplastics in this study by specific microorganisms was determined according to the ASTM D5257 Standard for biodegradation studies. The microorganisms used in the study were selected on the basis of their ability to degrade a specific microplastic as obtained from the screening tests and are presented in Table 3.2. Zero point five grams (0.5 g) of each microplastic were dispensed into 300 ml Erlenmeyer flasks containing 270 ml BH broth.

Table 3.2: Treatment formulations for shake-flask biodegradation experiment using individual isolates

Treatment	Description
PE control	NB
PE 1	*Bacillus cereus* + Polyethylene
PE 4	*Bacillus vietnamensis* + Polyethylene
PE 5	*Sporosarcina globispora* + Polyethylene
PE 7	*Staphylococcus epidermidis* + Polyethylene
PE 8	*Bacillus flexus* + Polyethylene
PE 10	*Bacillus gottheilii* + Polyethylene
PP control	NB
PP 3	*Bacillus sonorensis* + Polypropylene
PP 5	*Sporosarcina globispora* + Polypropylene
PP 9	*Rhodococcus ruber* + Polypropylene
PP 10	*Bacillus gottheilii* + Polypropylene
PET control	NB
PET 1	*Bacillus cereus* + Polyethylene terephthalate
PET 10	*Bacillus gottheilii* + Polyethylene terephthalate
PS control	NB
PS 1	*Bacillus cereus* + Polystyrene
PS 10	*Bacillus gottheilii* + Polystyrene

NB = No bacteria addition

3.6.1 Microbial inoculum preparation for individual isolates

Each of the bacteria isolated and identified as microplastic-degrading microbes was formulated for biodegradation assay (Plate 3.4). The isolates were individually grown on freshly prepared NA to obtain pure cultures at 33 ^{o}C for 24 hours before inoculation in nutrient broth, and grown to a stationary phase in rotating shaker at 29 ^{o}C at 150 rpm (Auta *et al.*, 2017b). Individual inoculum attained similar physiological phase (1.2 OD_{600}). The cell densities of the inoculums were adjusted to 1.2 x 10^7 colony-forming units (CFU/ml) for the biodegradability experiment.

Plate 3.4: Formulation of treatments for biodegradation experiment (inoculum preparation)

3.6.2 Biodegradation experiment set-up with 10 % inoculum of individual isolates

Ten percent (10 %v/v) of the pure cultures of each individual microplastic degrading bacteria from mid-exponential phase having approximately 1.2 x 10^7 CFU/ml cells were inoculated into 270 ml Bushnell-Haas broth (BHB) in flasks containing 0.5g of PE, PP, PET and PS microplastics. The flasks containing non-inoculated BHB media supplemented with PE, PP, PET and PS microplastics served as control (negative control). Triplicates were maintained for all experiments and were left on a shaker (150 rpm) at 30 ^oC under anaerobic conditions for a period of 40 days (Plate 3.5). Growth of the isolates was monitored by measuring the turbidity of the culture at OD_{600}, pH and microbial counts (CFU/ml) were monitored at every ten (10) days interval for a period of 40 days (Auta *et al.*, 2018).

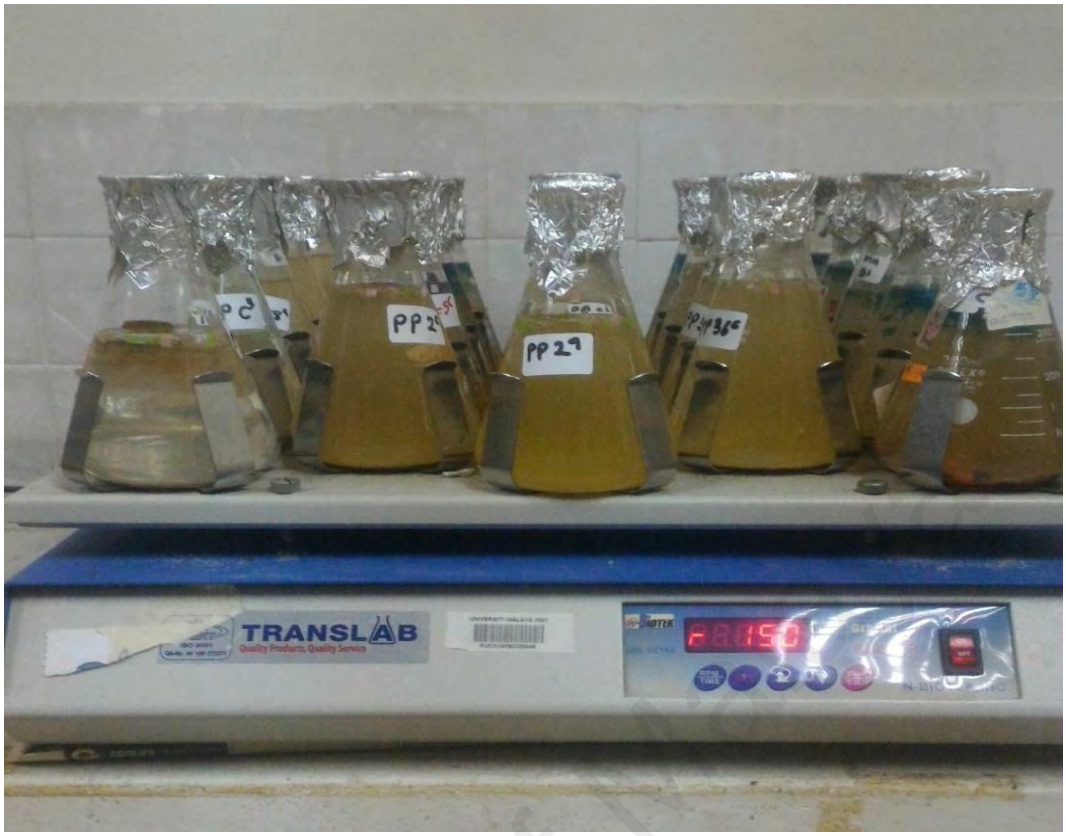

Plate 3.5: Shake flask experiment set-up (flasks containing microplastics on shaker for biodegradation studies)

3.6.2.1 Growth kinetics of isolates in microplastic-infused BHB media

The flasks containing the different isolates in BHB were kept in shaker incubator at 30 ^{o}C and 150 rpm. Absorbance (OD) readings were taken at 600 nm using UV-Visible Spectrophotometer (Spectroquant® Pharo 300, Germany) during the biodegradation studies. The growth pattern of the isolates under controlled conditions in the presence of the different microplastics were estimated by measuring the OD at intervals of 10 days. OD represents the logarithm of the number of microorganisms. Aliquot was drawn from the flasks for quantification of bacteria i.e. at time of inoculation (at 0 hr) and at intervals of 10 days for the 40 days incubation period. All experiments were carried out in triplicates.

3.6.2.2 Bacteria count (colony forming unit) measurement

The colony forming unit (CFU) measurement was employed to monitor growth of bacteria using different microplastics as carbon source in microplastic-infused BHB media. 1 ml of sample from each setup was collected at the time of inoculation and at intervals of every 10 days. These samples were serially diluted with sterile distilled water to make 10^{-3}, 10^{-5} and 10^{-7} dilutions. 100 µl of culture from 10^{-3}, 10^{-5} and 10^{-7} dilutions were then transferred to plate count agar (PCA) plates and spread using spreader. The plates were kept in incubation at 30 ^{o}C for 24 hrs and distinct bacterial colonies were carefully counted and multiplied with the dilution factor to get the representative bacterial counts in 1 ml culture of each setup (Nayak *et al.*, 2012). All experiments were carried out in triplicates.

3.6.2.3 Determination of dry weight of residual microplastics

After 40 days of incubation, all microplastics were recovered from the BHB broth through filtration and sieving. The bacterial films colonizing the microplastics were removed by washing the microplastics with 70% ethanol (4-step washing, with incubation time of 2 minutes for each step). These were kept on filter paper and then dried in hot air oven at 50 ^{o}C overnight prior to weighing. Residual microplastic weight was determined to measure the extent of degradation using Sartorious analytical balance ENTRIS 224-1S (accuracy ± 0.0001 g) (Auta *et al.*, 2018; Mohan *et al.*, 2016). The initial weights of the pre-incubated microplastic samples were also measured following the same technique mentioned above. The degradation of the microplastic polymers was evaluated in terms of percentage weight loss using Eq. 1 as follows:

$$\% \text{ weight loss} = \left(\frac{W_0 - W}{W_0} \right) \times 100 \quad (1)$$

Where W_0 = Initial weight of microplastics (g), W = residual weight of microplastics (g)

3.6.3 Determination of the rate of reduction of PE, PP, PET and PS microplastics

The data were further processed to determine the rate constant of PE, PP, PET and PS microplastics reduction using the first-order kinetic model based on the parameters assessed; initial and final weights along specific intervals (10 days) (Alaribe & Agamuthu, 2015) in Eq. 2 as follows:

$$K = -\frac{1}{t}\left(\ln\frac{W}{W_O}\right) \quad (2)$$

Where k = first-order rate constant for polymer uptake per day, t = time in days, W= weight of residual microplastics (g), W_O = initial concentration of microplastics (g).

Following the generation of the microplastic removal rate constant, the Half-life ($t_{1/2}$) was calculated according to Eq. (3) as shown:

$$(t_{1/2}) = \frac{1n\ (2)}{k} \quad (3)$$

Where t = time, 1n (2) = 0.69, k = rate constant.

3.6.4 Analytical methods for monitoring biodegradation

3.6.4.1 Fourier transform infrared analysis of microplastic polymers

Changes in the structure of the microplastic polymers with subsequent bacterial inoculation were analysed by Attenuated total reflectance-Fourier transform infrared spectroscopy, ATR-FTIR (Perkin-Elmer 400) at the frequency range of 4000 – 450 cm^{-1}.

This was carried out to in order to determine the formation of possible functional groups on the microplastic surfaces which could be attributed to biological degradation. The different microplastic samples were placed in a transmission cell fitted to a Nicolet 510 FTIR spectrophotometer (DTGS detector) with air purge. Spectra were made up of 50 scans with a resolution of 2 cm^{-1}. Samples were also measured by total internal reflection using an ATR accessory. This was carried out on the PE, PP, PET, and PS microplastics inoculated with the individual bacterial strains. The results obtained for degraded individual microplastics were compared with the uninoculated (control) PE, PP, PET and PS microplastics (Auta *et al.*, 2017b; Mohan *et al.*, 2016).

3.6.4.2 Scanning electron microscopy (SEM) of PE, PP, PET and PS microplastics

The morphology of the degraded microplastic particles were monitored after 40 days of incubation with bacterial isolates using the scanning electron microscopy, SEM (Leica EM SCD005, Austria), magnification 100x – 10,000x. This was done to get insight into the small-scale changes of the microplastic surfaces. The samples were removed from the culture medium and gently washed with distilled water to remove excess medium and most of the biofilms without damaging the microplastic surfaces. This was followed by washing with 70 % ethanol and then rewashed with distilled water for elimination of most surface-adhered cells. The samples were dried and sputter-coated with a gold layer at 25 mA under Argon (Ar) atmosphere at 0.3 MPa and visualized under the SEM at 3,500X magnification (Auta *et al.*, 2017b; Sekhar *et al.*, 2016).

3.7 Biodegradation experiment set-up with 10 % inoculum of blended isolates

3.7.1 Microbial formulation with blended isolates

The formulation of bacteria into consortia is expected to display increased metabolic potential and to enhance the degradation of microplastics. Microbial formula used for

biodegradability and bioaugmentation experiments composed of nine different strains of bacteria isolated from plastic/microplastic inundated mangrove sites in Peninsular Malaysia. The strains were grouped into four different treatments according to the characteristics of the microbes, including control as presented in Table 3.3.

Table 3.3: Bacteria formulation for biodegradation experiments using blended isolates

	Treatment			
Control	**A**	**B**	**C**	**D**
NB	*B. cereus*	*B. cereus*	*S. globispora*	*B. cereus*
NB	*B. sonorensis*	*B. sonorensis*	*A. faecalis*	*B. sonorensis*
NB	*B. vietnamensis*	*B. vietnamensis*	*S. epidermidis*	*B. vietnamensis*
NB	*S. globispora*	*B. flexus*	*R. ruber*	*B. flexus*
NB	*A. faecalis*	*B. gottheilii*		*B. gottheilii*
NB	*S. epidermidis*			*S. globispora*
NB	*B. flexus*			*S. epidermidis*
NB	*R. ruber*			*R. ruber*
NB	*B. gottheilii*			

NB = No bacteria addition, Treatment A = All microbes, Treatment B = Bacilliceae group, Treatment C = Non Bacilliceae, Treatment D = All gram positives

3.7.2 Microbial inoculum preparation for blended isolates

Each strain was grown as a pure culture as described in Section 3.5.1. The isolates were inoculated in nutrient broth and grown to a stationary phase in rotating shaker at 29 ^{o}C at 150 rpm. Individual suspensions at the same physiological phase (1.75 OD_{600}) were pooled in equal proportions to set up inoculums for biodegradation. The cell densities of the inoculums were adjusted to 1.76 x 10^{11} colony-forming units per ml (CFU/ml) for the biodegradability experiment.

3.7.3 Biodegradation experiment set-up with blended isolates

Ten percent (10 %v/v) of the blended isolates having approximately 1.76 x 10^{11} CFU/ml cells were inoculated into 270 ml Bushnell-Haas broth (BHB) in flasks containing 0.5g of PE, PP, PET and PS microplastics. The flasks containing non-

inoculated BHB media supplemented with PE, PP, PET and PS microplastics served as control (negative control). Triplicates were maintained for all experiments and were left on a shaker (rpm 150) for a period of 40 days. The optical density (OD_{600}), pH and microbial counts (CFU/ml) were monitored at every ten (10) days interval for a period of 40 days. The weight loss, rate of reduction and half-lives of residual PE, PP, PET and PS microplastics were determined as earlier described in Sections 3.6.3 and 3.6.4, respectively. The microplastic samples were also subjected to analytical procedures for monitoring biodegradation (FTIR and SEM) analyses as described in Sections 3.6.41 and 3.6.42, respectively.

3.8 Biodegradation set-up to study the effect of different inoculum concentrations of blended microbes on PE, PP, PET and PS microplastics

The effect of different inoculum concentrations on PE, PP, PET and PS microplastics degradation was tested. Similar to 10 % v/v inoculum concentration set-up, the experiment was carried out with 10 %, 20 %, 30 %, 40 % and 50 % v/v of microbial inoculum concentrations, with microbial cell counts of 7.61 x 10^{11} CFU/ml. the experiment was conducted in triplicates for all treatments. The growth of the microbes, pH, microbial counts, rate of reduction, half-life, FTIR and SEM analysis were carried out as earlier described.

3.8.1 Site selection and design

Since the study was based on remediating microplastic contaminated mangrove environments, Sementa mangrove located in Klang (2º54’38 N 101º21’06” E), Selangor State, was selected for the field tests bioremediation set-up. A preliminary visit to the mangrove was made to identify a suitable site based on the tidal zones. The experiment was designed to investigate the degradation of microplastics buried under sand kept wet

with sea water hence, a mid-tidal zone was selected for the experiment. The best treatments from the laboratory scale biodegradation experiment for PET and PS microplastics were chosen for the set-up. The mangrove soil used in the field biodegradation tests had the following characteristics: total organic carbon (3.8%), total alkalinity (12 ppm), organic matter content (11.4%), percentage of chlorides (0.02%), moisture content (51.6%), total nitrogen (15 ppm), salinity (1.99 ppm), and cation exchange (12.2 meq/100 g). The pH of the soil was 7.4. This pH was found to be near optimal for hydrocarbon biodegradation and it was therefore assumed that this pH would favour microplastic degradation (Yabannavar & Bartha, 1994).

The plain selected for the experimental set-up was divided into two portions: portion A (amended portion) and portion B (non-amended control portion), with dimensions of 1.82 m (6ft.) by 1.2 m (4 ft.) each, with a gap of 0.6 m (2 ft.) as illustrated in Figure 3.2. Injection wells made of perforated PVC pipes were installed (at 30 cm depth), linearly at 30 cm intervals in portion A. No installation of PVC pipes was made in portion B.

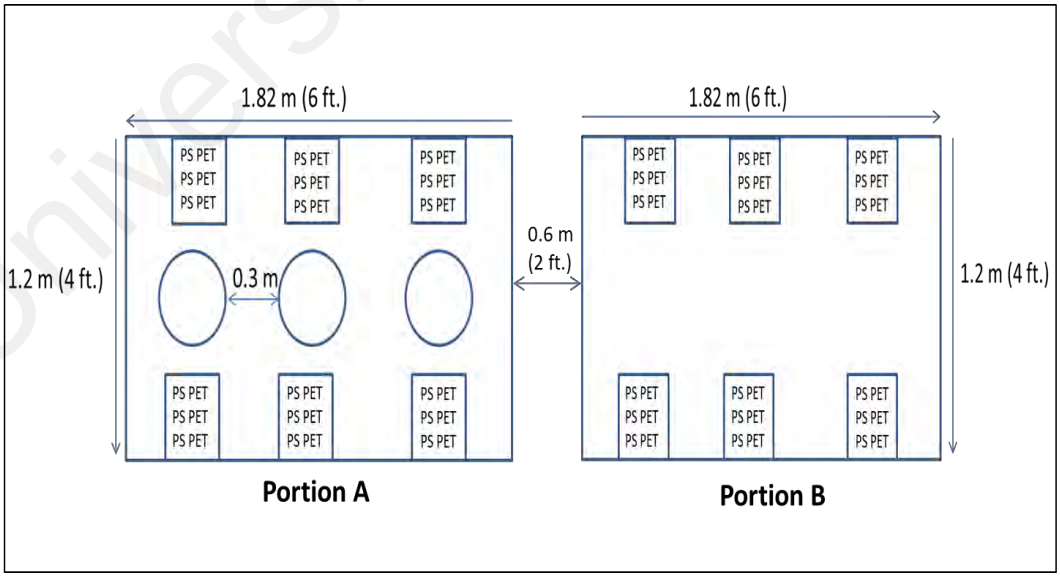

Figure 3.2: Schematic diagram of in situ experiment design

3.8.2 Inoculum preparation for in situ bioremediation experiment set-up

Inoculum for the experiment was prepared as described earlier. Individual suspensions at the same physiological phase (1.163 OD_{600}) were pooled in equal proportions to set up inoculums for bioremediation. 18 litres of inoculum were generated for application. The study assumes this volume to be approximately relative to the soil compartment of the selected areas. Hence, the microbial cell concentration used for remediation was 3.49 x 10^{11} CFU/ml.

3.8.3 *In situ* bioremediation set-up

For both amended and non-amended portions, microplastics were sewn into small bags made to prevent eventually forming microplastic fragments from falling apart. The material was non-biodegradable with a 1.8 mm x 1.6 mm mesh. The bags were buried in triplicates at 10 cm depth in the mangrove soil (Plate 3.6), with a distance of approximately 2 cm between each bag and covered with soil (Tosin *et al.*, 2012). Consequently, the prepared inoculum was introduced into the injection wells (9 litres) and the other 9 litres was applied directly to the soil surface and allowed to penetrate/percolate the soil core. No inoculum was applied in the control portion (portion B). Monitoring activities was carried out at 15 days interval for a period of 90 days. For sampling, mesh bags with the microplastic specimens were taken out (in triplicates) to monitor biodegradation. Basic soil environmental properties (such as pH, salinity, temperature and redox potential.) were monitored as well. The microbial population in the amended and the non-amended control portion was measured every 15 days. The microbial consortium formulated was introduced to the amended portion on each monitoring day.

Plate 3.6: Setup for field (*in situ*) experiment in Sementa mangrove, Klang

3.9 Statistical analysis

Statistical analysis of all data was carried out using analysis of variance (ANOVA) in the SPSS software 21.0., with the LSD post-hoc test at p-value = 0.05. This was done to compare the means of variance, that is, to test the differences between the means.

CHAPTER 4: RESULTS AND DISCUSSION

This study investigated the potential of marine bacteria isolated from mangrove sediments to degrade polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET and polystyrene (PS) microplastics. Hence, this section detailed the findings of the research activities. This includes the baseline properties of mangrove sites that served as the habitat of the isolated microbes selectively used for remedial activities, and evaluation of the isolates with respect to degradation/remediation potentials on microplastics and microplastic inundated environment.

4.1 Site characterization of mangrove sites

The mean values of the environmental parameters of the mangrove soil/water during sampling are presented in Table 4.1. Variations existed in the parameters during each of the sampling days.

Table 4.1: Site characterization of each mangrove site across months

	Parameter		
Sample site	**Temperature (ºC)**	**pH**	**Salinity (ppt)**
Matang mangrove (Perak)	28.90 ± 0.24	6.75 ± 0.10	9.56 ± 4.22
Sekam mangrove (Melaka)	28.80 ± 0.08	7.44 ± 0.46	29.51 ± 0.49
Tanjung Piai mangrove (Johor)	29.20 ± 0.36	5.99 ± 0.18	30.83 ± 2.37
Cherating mangrove (Pahang)	28.60 ± 0.16	6.32 ± 0.23	21.50 ± 0.85
Sedili Besar mangrove (Johor)	29.40 ± 0.36	7.50 ± 0.40	8.99 ± 1.97
Pasir Puteh mangrove (Kelantan)	28.80 ± 0.08	7.23 ± 0.39	19.39 ± 0.72

n = 3

The temperature during sampling were similar for all mangrove sites and ranged from 28.6 ºC to 29.4 ºC, with the highest temperature (29.4 ºC) recorded in Sedili Besar mangrove in Johor and the least temperature (28.6 ºC) recorded in Cherating mangrove in Pahang. The pH on the other hand, ranged from pH 5.99 to pH 7.50. Tanjung Piai

mangrove (Johor) recorded the highest salinity level (30.83 ppt), while the lowest (5.99 ppt) was recorded in Sedili Besar mangrove in Johor.

The temperature of mangrove water bodies varies with the amount of sun that hit the area. Malaysia is a tropical country and so, such temperature ranges were expected. The lower ambient temperatures recorded could have been as a result of reduced light intensity in the mangroves during sample collection (samples were collected at morning hours when the tides were low).

Mangrove soils are neutral to slightly acidic and the acidity is usually due to the presence of acidic clays and sulphur-reducing bacteria. The pH of the different sediments of the mangroves were within neutral levels (which is the stable pH for seawater), except for Tanjung Piai mangrove in Johor that recorded a slightly acidic pH (5.9). Some mangroves in Malaysia have been reported to have very acidic brackish waters (Kelvin *et al.*, 2001). Some researchers recorded pH of mangrove soil ranging from 2.87 – 6.40 (Empi *et al.*, 2010; Moreno & Calderon, 2011) while others have recorded mangrove soil pH ranging from 7.0 – 8.22 (Das *et al.*, 2012; Hossain & Nuruddin, 2016; Hossain *et al.*, 2012).

Variations in salinity levels of the different mangroves can be observed from the salinity readings in Table 4.1. The variations that existed could have resulted from the relative amounts of precipitation or evaporation in the mangrove (Ranjan *et al.*, 2006; Shiau *et al.*, 2017). Hence, the degree of salinity of Matang and Pasir Puteh mangroves can be categorized as mesohaline waters of intermediate salinity (ranging from 5 – 18 ppt), while Sekam, Tanjung Piai, Cherating, and Pasir Puteh mangroves can be categorized as polyhaline waters of high salinity (ranging from 18 – 30 ppt.) (Kelvin *et al.*, 2001). Mangrove environments are one of the most adverse environments owing to their varying nature of pH, salinity, currents, precipitation regimes, temperature and wind

patterns. Due to the constant variation of environmental conditions, the microbes present in the environment are more suitably adapted to the adverse conditions, hence, possessing complex characteristic features of adaptation. The bacteria isolated from the plastic inundated mangrove soils can hence, be better utilized in the biodegradation of microplastics through biofilm formation and production of extracellular polymeric substances.

Furthermore, the site characterization was not limited to the evaluation of the fore mentioned physical conditions. Rather, the study assessed the microbial distribution of the mangrove sites which is a part of the biological component of the area and is expected to play significant role in the overall biodegradation of microplastics.

4.2 Microbial isolation and identification

The study carried out isolation of microbes (bacteria) from six different mangrove soils in Peninsular Malaysia. Some of the mangrove environments from observation, served as dumping sites for solid waste and most of the waste stream were comprised of plastic materials which could probably become fragmented into microplastics with time. Also, the mangroves were characterized by plastic bags hanging on the mangrove shrubs and plastic materials washed ashore from the ocean. Table 4.2 contains the list of twenty-two marine bacteria isolated from the different mangrove soils.

Table 4.2: List of bacteria isolated from the different mangrove environments and the sites of isolation

		Mangrove location					
S/N	**Microbe**	**Matang**	**Cherating**	**Tanjung Piai**	**Sekam**	**Sedili Besar**	**Pasir Puteh**
1	*Bacillus cereus*	√	√	-	-	-	-
2	*Bacillus cibi*	√	√	√	-	√	-
3	*Acinetobacter schindleri*	-	√	-	-	-	-
4	*Serratia entomophila*	-	-	-	√	-	-
5	*Bacillus thuringiensis*	√	√	-	√	√	√
6	*Alcaligenes faecalis*	√	√	√	-	√	-
7	*Enterococcus faecium*	-	√	-	-	-	-
8	*Bacillus stratosphericus*	√	-	-	-	-	-
9	*Exiguo bacteria sp.*	√	-	√	-	√	√
10	*Bacillus flexus*	-	-	-	-	-	√
11	*Bacillus ruris*	-	√	-	-	-	√
12	*Bacillus pseudomycoides*	√	√	-	√	√	√
13	*Bacillus sonorensis*	√	-	-	-	-	-
14	*Staphylococcus epidermidis*	-	√	-	-	-	-
15	*Bacillus vietnamensis*	√	√	-	√	-	-
16	*Rhodococcus ruber*	-	-	-	√	-	-
17	*Pseudomonas stutzeri*	√	-	√	-	√	√
18	*Bacillus aquimaris*	√	√	√	√	√	-
19	*Sporosarcina globispora*	√	√	-	-	-	-
20	*Bacillus gottheilii*	-	√	-	√	√	-
21	*Stenotophomonas maltophila*	√	√	-	-	-	-
22	*Bacillus pumilus*	√	√	-	-	-	√

The list demonstrated diverse genera of microbes that included aerobic gram-positive and gram-negative bacteria. The growth patterns were distinctive enough to enhance identification and differentiation into individual isolates. The isolated species belonged to 16 genera of *Bacilli*, 5 genera of Proteobacteria, and one genera of Actinobacteria. The microbes isolated reflected the native bacteria community found in mangrove environments (Basak *et al.*, 2016; Saimmai *et al.*, 2012). Akpan-Idiok and Solomon (2012), Behera *et al.* (2014), and Behera *et al.* (2016), also isolated *Bacillus* sp., *Pseudomonas* sp. (sulphate-reducing bacteria), and *Staphylococcus* sp. from mangrove soils. *Bacillus* was the most abundant genus isolated in this investigation. The result corroborates the results obtained by Castro *et al.* (2014) who reported the predominance of *Bacillus* sp. in their study of the bacterial diversity of Brazilian mangrove ecosystem, and the results obtained by Ando *et al.* (2001) who isolated vast number of *Bacillus* sp. from mangrove sediments in Japan. The microbes isolated were halophilic bacteria hence, their presence in mangrove soil was not surprising. The microbes have been reported to possess useful antibiotics, proteins, enzymes and salt tolerant genes, all of which have biotechnology significance (Castro *et al.*, 2014; Thatoi *et al.*, 2013). However, in order to identify the strains that possess degradation/deterioration potential on microplastics, bioassay screening on the isolates was carried out.

4.3 Screening of isolates for microplastic degradation

Nine bacterial isolates out of the 22 were capable of growing on Bushnell Haas (BH) media and utilizing the PE, PET, PP, and PS polymers as carbon source. The isolates grew and indicated significant clear zones on the media within 5 to 9 days after incubation. This indicated that the microbes could depolymerize the polymer, which is usually the first step of biodegradation as reported by (Shah *et al.*, 2008). The clear zones observed could have been due to the hydrolysis of the polymer materials by the microbes

as a result of the extracellular enzymes excreted by the microbes which diffused through the agar and degraded the polymers into water soluble materials. The screening assay for microplastic degradation is presented in Table 4.3.

Table 4.3: Screening test results for different microplastic degradation by bacterial isolates

Organism	Control (C)	PS	PE	PET	PP
B. cereus	--	+++	+++	+++	++
B. sonorensis	--	+	+	--	+++
B. thuringiensis	--	++	+	+	+
B. vietnamensis	--	+	+++	+	++
B. ruris	--	+	++	+	++
S. globispora	--	++	+++	++	+++
B. cibi	--	+	+	+	+
A. schindleri	--	+	--	+	++
E. faecium	--	--	+	++	++
S. maltophilia	--	+	+	+	--
B. pseudomycoides	--	--	+	+	--
A. faecalis	--	--	+	--	+++
B. pumilus	--	+	+	+	+
B. stratosphericus	--	+	+	--	+
Exiguo bacterium sp.	--	+	+	+	++
B. aquimaris	--	--	+	--	+
S. epidermidis	--	+	+++	++	++
B. flexus	--	++	+++	++	++
P. stutzeri	--	--	+	--	+
R. ruber	--	--	+	--	+++
S. entomophila	--	--	--	++	+
B. gottheilii	--	+++	+++	+++	+++

Strong (+++) = diameter ≥ 2.5 mm, moderate (++) = diameter between 1.0 – 2.5 mm, weak (+) = diameter < 1.0 mm, no growth (--)

From the Table 4.3, *B. cereus*, *A. faecalis*, *B. flexus*, *B. sonorensis*, *S. epidermidis, B. vietnamensis*, *R. ruber*, *S. globispora*, and *B. gottheilii* demonstrated significant clear zone and were therefore, selected for the biodegradation study. Also observed is the ability of *B. gottheilii* to degrade all four different microplastic types as compared to other isolates. All microbes with the ability to degrade microplastics in this study are gram-positive, except for *A. faecalis* which is a gram-negative organism. Most of these bacterial

species have been reported to be potential producers of biosurfactants which facilitate the assimilation of pollutants especially those of hydrocarbon origin (Saimmai *et al.*, 2012), and as such, their utilization in polymer biodegradation was expected to show metabolic potential.

B. cereus is a gram-positive, motile, rod-shaped, spore forming bacteria that is widely distributed in nature. It is found in food, decaying organic matter, soil, and marine waters (Balakrishnan *et al.*, 2015; Vilain *et al.*, 2006). The spores of *B. cereus* have high metabolic dormancy and tough physical structure, and thus, is resistant to environmental stress. Previous studies have demonstrated the application of *B. cereus* in the degradation of PE (Sowmya *et al.*, 2014; Suresh *et al.*, 2011). Therefore, the isolation of *B. cereus* in the plastic-contaminated mangrove soil portrays its relevance as a possible bioremediation agent.

Another microbe that was isolated from the plastic-contaminated mangrove soil is *A. faecalis.* It is a gram-negative, rod-shaped bacterium commonly found in soil, water and environments associated with humans. It is non-pathogenic, though, opportunistic infections may occur.

Similarly, *B. flexus,* as found in the plastic contaminated soil, is also suggestive of the organism as a common microbe in polluted environments. The microbe is a gram-positive, aerobic, motile, endospore forming, salt-tolerant, soil bacterium. Other reports of its isolation from mangrove soils have been reported (Kannan *et al.*, 2009; Sukhdhane *et al.*, 2016). The organism is capable of secreting enzymes such as cellulases, esterases and depolymerases (Kumar *et al.*, 2007; Oda *et al.*, 1997), characteristics that could have endowed it with polymer-degrading ability. Nayak *et al.* (2012) reported the degradation of polypropylene by *B. flexus*.

Also isolated from the plastic contaminated mangrove soils is *B. sonorensis*- a gram-positive, rod-shaped, motile, microbe. The bacterium is salt tolerant (a characteristic that could have enabled its survival in mangrove environments) (Palmisano *et al.*, 2001).

S. epidermidis is another soil microbe isolated from the plastic contaminated mangrove soil. A non-motile, gram-positive, cocci, that is part of the normal human skin flora. The organism has been reported to possess well equipped genes that provide it with protection in harsh conditions encountered in its natural habitat. It is able to cope with extreme salt concentrations and osmotic pressure (Otto, 2009). Its ability to tolerate high salt concentrations may have contributed to its survival in the mangrove environments. *Staphylococcus* sp. isolated from mangrove soils has been demonstrated to degrade PE (Kathiresan, 2003b).

Another important organism belonging to the genus *Bacillus* isolated was *B. vietnamensis*. It is a gram-positive, aerobic, endospore forming, moderately halo-tolerant bacterium. *B. vietnamensis* has been isolated from marine and mangrove sediments (Sukhdhane *et al.*, 2016).

The bacterial community isolated from the plastic inundated mangrove soil also had *R. ruber*, an actinomycetes belonging to the genus *Rhodococcus*, closely related to *Corynebacterium* and *Mycobacterium* (Gibson *et al.*, 2003). It is an aerobic, non-sporulating, gram-positive bacterium. Several studies have demonstrated the ability of *R. ruber* to colonize and degrade PE polymer (Chandra & Rustgi, 1997; Orr *et al.*, 2004; Sivan *et al.*, 2006), and hence, its possible ability to survive in the plastic contaminated mangrove soil.

Previously known as *Bacillus globisporus*, *S. globispora*- a gram-positive, aerobic, round-shaped, spore forming, halophilic soil and marine *Bacillus*- was also isolated from

the plastic-inundated mangrove soils. Its application in bioremediation has not been reported in literature but its presence in the plastic-contaminated mangrove soil is suggestive of the organism as a common microbe in polluted soil.

Last of the bacteria isolated from the plastic inundated mangrove soil was a rod-shaped, motile, strictly aerobic, endospore-forming bacterium designated *B. gottheilii*. It commonly occurs in soil but has also been isolated from mangrove water. Though not widely investigated, the microbe has been demonstrated to produce the enzyme tannase on inert polyurethane foam support (Subbalaxmi & Murty, 2016). *B. gottheilii* was the only organism among all the potential microplastic-degraders that could degrade all the four different microplastic types. This ability could be due to the fact that the organism may possess copious amounts of enzymes that is characteristic of *Bacillus* species.

Microplastics are a great concern for waste/pollution management due to their accumulation in natural habitats, especially the marine environment (Auta *et al.*, 2017a). Hence, a suitable method for disposal that is eco-friendly must be searched for. In this regard, microbial degradation is one of the best options. Several reports on the biodegradation of plastics by organisms indicate that it could be a viable proposition for microplastics disposal when suitable microorganisms are utilized (Shah *et al.*, 2008; Singh & Sharma, 2008).

Considering the potential application of the microbes, the study further assessed the biodegradability potential of each of the isolates, individually and in consortium, in aqueous medium containing PE, PP, PET and PS microplastics as source of carbon. Therefore, biodegradation studies on the individual isolates became necessary in order to evaluate the actual impact of the microbes on various microplastic exposure.

4.4 Biodegradation experiments using individual isolates (shake-flask experiment

From the screening tests, six microbes depicted the ability to degrade PE microplastics and were therefore, selected for the study.

4.4.1 Determination of weight loss of PE microplastics

PE microplastic degradation was initially monitored after 40 days of incubation with the individual isolates, the weight loss in percentage was calculated and the results are presented in Figure 4.1.

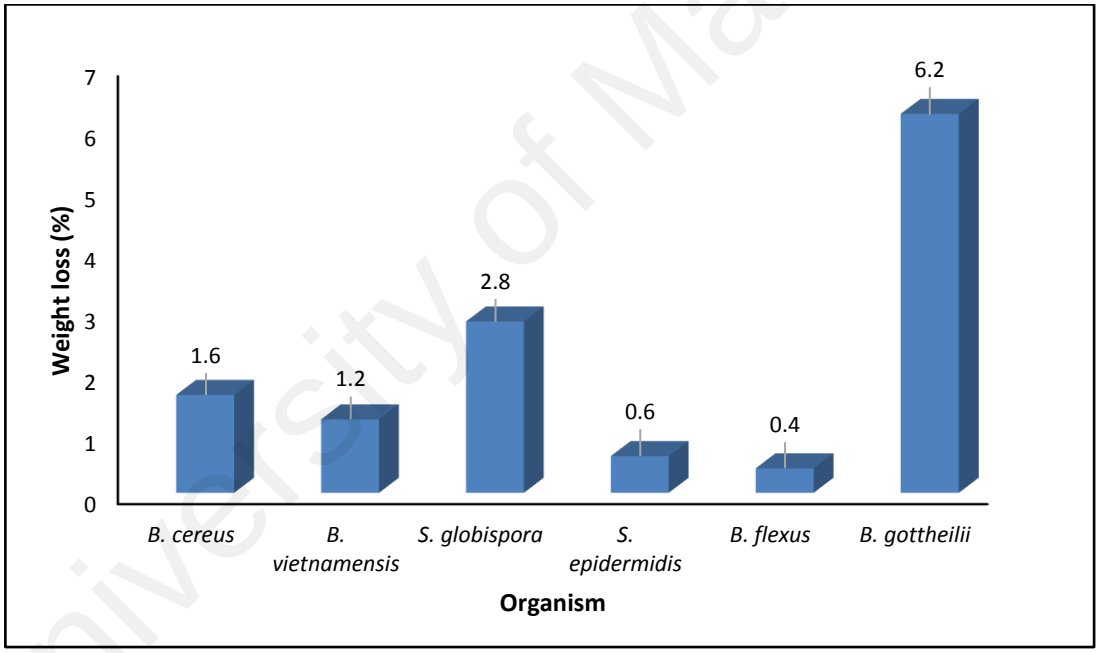

Figure 4.1: Weight loss of microbially treated PE microplastic after incubation for 40 days. Maximum weight loss (6.2% w/v) was obtained for *B. gottheilii* treated PE microplastic

The weight loss was 1.6% for PE microplastic inoculated with *B. cereus*, 1.2% in *B. vietnamensis*, 2.8% in *S. globispora*, 0.6% in *S. epidermidis*, 0.4 % in *B. flexus* and 6.2% in *B. gottheilii* (F-value = 2.169; P = 0.126). There was no weight reduction in the control. The weight reductions of the PE microplastics on inoculation with the microbes can be attributed to the breakdown of the carbon chain of PE due to enzymatic degradation by

the isolates. It was observed that maximum weight loss was seen for PE microplastic incubated with *B. gottheilii* while the least weight loss was recorded by *B. flexus*. Furthermore, the comparison of the weight loss of PE microplastics by the different isolates after 40 days revealed statistical differences in the weight loss between *B. gottheilii* and *B. cereus* (P = 0.049), *B. gottheilii* and *B. vietnamensis* (P = 0.033), *B. gottheilii* and *S. epidermidis* (P = 0.020) and *B. gottheilii* and *B. flexus* (P = 0.016). But no significant difference existed in the weight loss between *B. gottheilii* and *S. globispora* (P = 0.131), even though the former recorded highest weight loss. The reason for the different weight reductions by the different organisms could be due to their genetic makeup or the discrete potential of the organisms to impact the bonds of PE microplastics. Microorganisms possess different characteristics, hence, the degradation varies from one microbe to another (Bhardwaj *et al.*, 2012).

Microbial degradation of PE polymer requires the formation of biofilm on the surface of the polymer to enable the formation of biofilm on the surface so as to enable effective colonization and utilization of the polymer by microorganisms. The lower PE microplastic weight loss recorded by *S. epidermidis* and *B. flexus* could probably mean that the microbes were less hydrophobic, and therefore, may not have been able to produce significant biofilm on PE microplastics, and were therefore, less efficient in degrading PE microplastics (Orr *et al.*, 2004).

4.4.2 Growth pattern of individual isolates in PE microplastic-infused media

Polymer chain length can be reduced by oxidation and this can be assessed by microbial growth. Biodegradation of PE microplastics by the isolates was assessed in liquid synthetic medium. When inoculated into the media, the isolates were able to colonize the microplastics surface within a few days. This was evident by the biofouling of the PE microplastics that was observed which caused most of the microplastics to sink

to the bottom. The growth of the microbes on exposure to the microplastic samples in the media was monitored at several intervals (days 0, 10, 20, 30 and 40). Figure 4.2 shows the growth profile of the individual microbes upon exposure to PE microplastics.

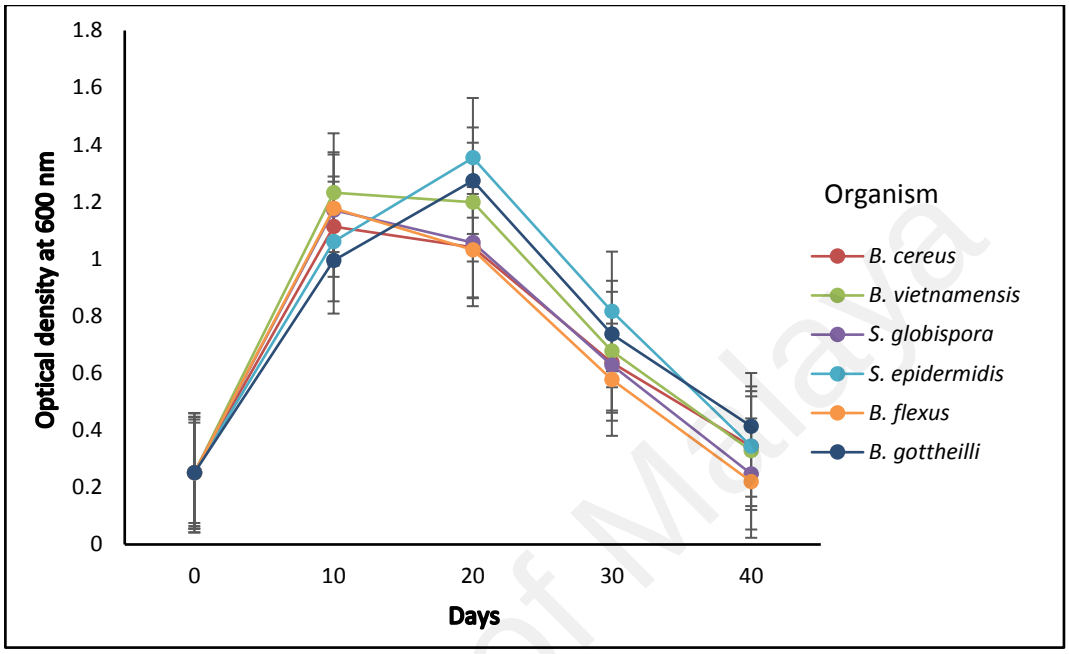

Figure 4.2: Growth profile of individual isolates in PE microplastic-infused media during biodegradation studies

The growth profile revealed that all microbes were able to grow in the BH medium containing PE microplastics as carbon source. The growth curve reflected varied growth patterns of the isolates characterized by significant growth phase, followed by mild, stable and decline phases. It was observed that the initial period of growth of all isolates was rapid, with a significant growth phase indicating rapid increase in microbial cell load, indicating that the bacterial isolates were capable of breaking the bonds of PE microplastics and utilizing it as carbon source. A steady, continuous increase that reached a peak on the 10th day was observed for *B. cereus* (1.113 OD_{600}), *B. vietnamensis* (1.232 OD_{600}), *S. globispora* (1.171 OD_{600}), and *B. flexus* (1.177 OD_{600}). This period recorded the optimum growth for all these isolates. Furthermore, the growth comparison after 10 days of exposure revealed varying absorbance values. *B. vietnamensis* recorded highest

absorbance value (1.232 OD_{600}) whereas, the least growth response was found with *B. gottheilii* (0.99 OD_{600}). However, these variations in absorbance values did not imply statistical differences across the exposed isolates as the p-values were more than 0.05. Hence, it reflects similarity in growth response and potentials to metabolize when exposed to the microplastics. On the other hand, *S. epidermidis* and *B. gottheilii* depicted optimum growth of 1.354 OD_{600} and 1.274 OD_{600}, respectively, on the 20^{th} day. No significant differences were observed in the growth of the organisms ($p < 0.05$) on this day. A decline phase was attained by the organisms on the 30^{th} day, eventually revealing statistical differences in the growth responses at $p < 0.05$ between *B. cereus* and *S. epidermidis* ($p = 0.039$), *S. epidermidis* and *S. globispora* ($p = 0.032$) and *S. epidermidis* and *B. flexus* ($p = 0.009$). Further decline in growth of the isolates was observed on the 40^{th} day with OD readings of 0.343 OD_{600}, 0.329 OD_{600}, 0.247 OD_{600}, 0.344 OD_{600}, 0.220 ABS and 0.414 OD_{600} for *B. cereus*, *B. vietnamensis, S. globispora, S. epidermidis, B. flexus* and *B. gottheilii*, respectively. However, no significant difference, existed between the growth response of the isolates on the 40^{th} day.

The enhanced growth observed through the measured OD on the 10^{th} and 20^{th} days as mentioned earlier did not depict optimal performance/response of the isolates upon exposure to the microplastics, rather, it may mean the duration of most favourable period of interaction between the microplastics and the bacterial cells that allow for rapid metabolism. This is because the overall responses across the 40 days of exposure showed that the microbes, when exposed to the PE microplastics, accelerated toward positive growth patterns. Hence, it may be important to deduce that the isolates potentially exhibited more survival potential between the 10^{th} and 20^{th} days of exposure to PE microplastics. Generally, assessment of the growth responses of the isolates upon exposure to PE microplastics showed that no significant differences existed across the varying days of experimental monitoring with p values ranging from 0.093 – 0.646 ($p <$

0.05). This possibly justifies the ability of the isolates to favourably metabolize in the presence of PE microplastics.

Population count of the bacteria during the biodegradation studies revealed a fluctuating distribution across the organisms and biomonitoring days. On the 10^{th} day high increase in bacteria counts was observed in all treatments as compared to the initial counts (at start of experiment) which coincided with the logarithmic increase in the number of bacterial cells during the same period. This served as an indication that more PE microplastics were utilized due to increase in number of cells. The results are presented in Figure 4.3. The counts ranged within 3.8×10^8 CFU/ml – 4.7×10^{11} CFU/ml and 3.8×10^8 CFU/ml – 4.8×10^{11} CFU/ml for PE microplastics treated with *B. cereus* and *B. vietnamensis*, respectively, while PE microplastics treated with *S. globispora* recorded highest bacterial counts that ranged within 3.8×10^{11} CFU/ml – 6.3×10^{11} CFU/ml. Counts ranging between 3.8×10^{11} CFU/ml – 5.4×10^{11} CFU/ml were recorded for PE microplastic inoculated with *S. epidermidis*. PE microplastics inoculated with *B. flexus* and *B. gottheilii* recorded counts ranging within 3.8×10^8 CFU/ml – 5.3×10^{11} CFU/ml and 3.8×10^8 – 5.7×10^{11} CFU/ml, respectively.

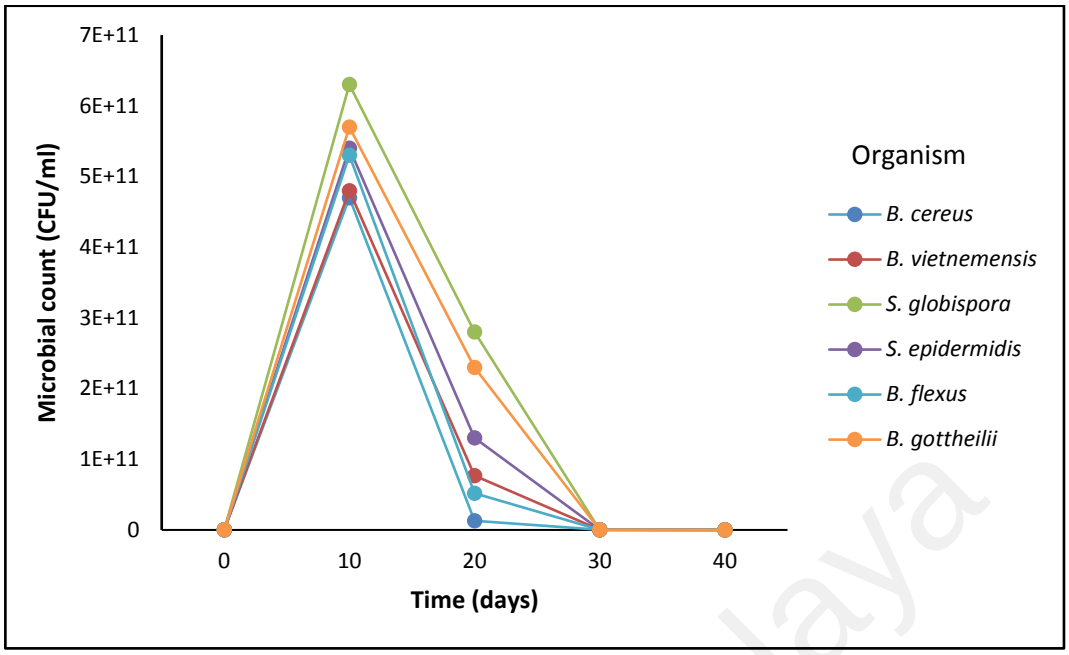

Figure 4.3: Bacterial counts of isolates inoculated in PE microplastic-infused media during 40 days biodegradation.

Highest counts of all the isolates were recorded on the 10th day, with highest count recorded PE microplastics treated with *S. globispora* (6.3 x 10^{11} CFU/ml) and least count recorded in PE microplastics inoculated with *B. cereus* (4.7 x 10^{11} CFU/ml). This was followed by a decline in microbial count on Day 20, with *S. globispora* and *B. cereus* still having the highest and lowest counts of 2.8 x 10^{11} CFU/ml and 1.3 x 10^{10} CFU/ml, respectively. Further degree of reduction in counts were observed that went below the initial counts recorded at the start of the experiment on Day 30. After 30 days, *S. epidermidis* had the highest counts of 3.5 x 10^{8} CFU/ml while *S. globispora* had the least count (2.6 x 10^{7} CFU/ml). This might be that the cells of *S. globispora* were no longer duplicating as before due to stress associated with the biodegradation process, hence, mortality or inhibition of the bacterial cells occurred. This is agreeable to findings by Emenike *et al.* (2016). 40th day witnessed further decline in bacterial counts with *S. globispora* having the highest count (1.5 x 10^{8} CFU/ml) and lowest counts recorded by *B. vietnamensis* (1.2 x 10^{7} CFU/ml).

Comparing the bacterial load on each day with the growth curve, it was observed that highest counts for *B. cereus*, *B. vietnamensis*, *S. globispora* and *B. flexus* recorded on the 10th day corresponded with the optimum absorbance attained by the microbes on same day but did not correspond with the OD (absorbance) readings recorded by *S. epidermidis* and *B. gottheilii* whose optimum OD readings were on the 20th day. This depicts that highest absorbance recorded for both isolates could have been due to the presence of degradation metabolites that may have been produced by the isolates during biodegradation, and not due to microbial multiplication.

4.4.3 pH changes of PE microplastic-infused media during biodegradation studies with individual isolates

The periodic measurements of pH of the BH culture media infused with PE microplastics during the biodegradation studies are presented in Figure 4.4. The pH increased early in the biodegradation process from 6.7 at the start of the experiment and levelled off to 9 for all microbes with values ranging between pH 8.42 – 8.59, pH 8.79 – 9.06, pH 9.22 – 9.45 and pH 9.15 – 9.55 on 10, 20, 30, and 40 days of biomonitoring, respectively. No changes in pH was observed in the control samples. Similar pH values were found to be near optimal for hydrocarbon biodegradation by bacteria isolates (Das & Chandran, 2011), and it was assumed that the pH ranges could have also favoured the survival of the microbes and the biodegradation of PE microplastics. The changes in pH of the media observed could have been due to the metabolic reactions of the microbes during degradation of PE microplastics. Similar changes in pH were reported by Zahra *et al.* (2010) during the biodegradation of low density polyethylene (LDPE) by fungi isolated from solid waste medium. Statistical analysis revealed that there was no significant difference in the pH changes between the isolates at $p < 0.05$ on day 10 ($p =$

0.121) and day 20 (p = 0.448). Statistical differences however, existed in the pH values between the isolates on day 30 (p = 0.000) and day 40 (p = 0.029).

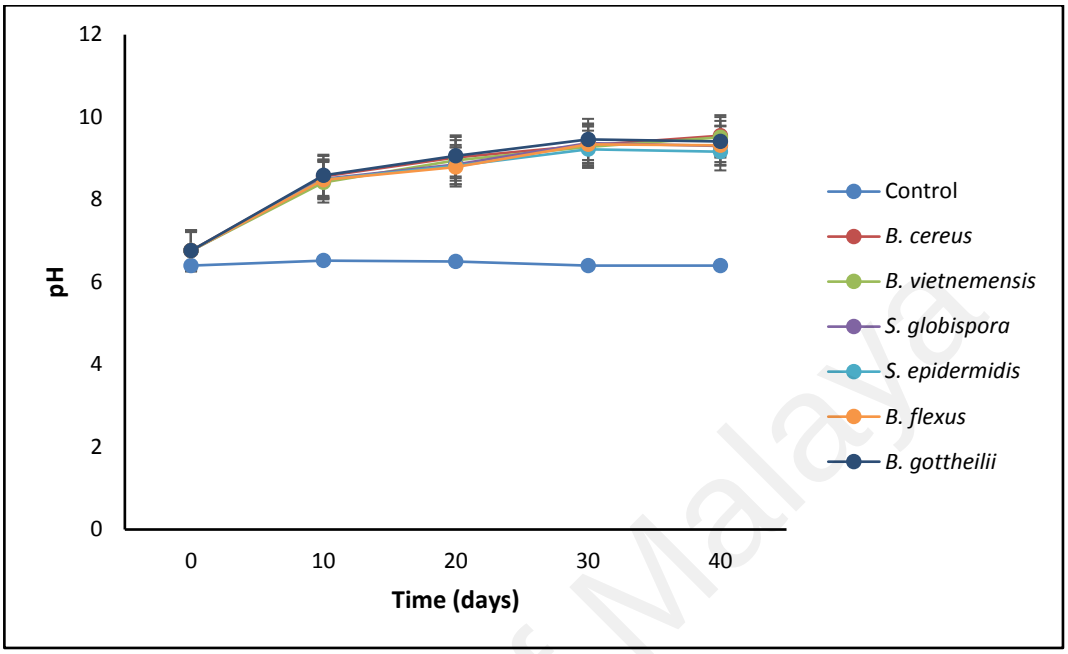

Figure 4.4: pH changes of PE microplastic infused media inoculated with individual organisms during biodegradation studies. Each data point represents the average of three replicates ± SD

4.4.4 Biodegradation rate constant and half-life of PE microplastics treated with individual isolates (kinetic model)

The study further determined the removal rate constant of the PE microplastics per day by the isolates and the time it will take for half of the PE microplastics to reduce by half using the first order kinetics model (*k*) and the half-life ($t_{1/2}$). The rate constant and half-life calculation were previously employed by several researchers to calculate the rate removal constant and half-lives of other contaminants such as fenamiphos` (a nematicide) (Cáceres *et al.*, 2008), benzo[a]pyrene (Aziz *et al.*, 2017), heavy metals (Emenike *et al.*, 2016; Fauziah *et al.*, 2017) , pyrene (Ghaly *et al.*, 2013), and di-*n*-butyl phthalate ester (Li *et al.*, 2006). Table 4.4 revealed that the PE microplastics inoculated with *B. gottheilii* showed the highest degradation/removal rate of 0.0016 day^{-1} and half-life of 431.25 days.

This might be from the microbe that may discretely have PE microplastic polymer removal capacity. The lowest biodegradation rate of 0.0001 day^{-1} and half-life of 6930 days was recorded in PE microplastics inoculated with *B. flexus*.

Table 4.4: Removal rate constant (*k*) and half-life of PE microplastics across individual isolates

Treatment	**Removal constant (*k*) day^{-1}**	**Half-life ($t_{1/2}$) (days)**
Control	0	∞
B. cereus	0.0004	1725
B. vietnamensis	0.0003	2300
S. globispora	0.0007	985.714
S. epidermidis	0.00015	4600
B. flexus	0.0001	6930
B. gottheilii	0.0016	431.25

n = 3

The highest degradation rate and the lower half-life recorded by *B. gottheilii* was expected since the organism recorded the highest weight loss of PE microplastics. The calculated microplastic removal rate constant and the corresponding half-life further buttressed the degree of activities within the aqueous media. The results depicted that 0.0016 g of PE microplastic was removed or taken up by *B. gottheilii* on a daily basis during the biodegradation studies and that it would take the organism approximately 431 days to reduce the polymer to its half.

The uninoculated control sample that did not record any loss in weight and as such, it recorded a zero-biodegradation rate and half-life of infinity (∞), depicting that it will take forever for PE microplastics to degrade if left untreated.

4.4.5 Fourier transform infrared (FTIR) spectroscopy analysis of PE microplastics inoculated with individual isolates

Structural analysis is an important parameter to identify the structural changes that appear during degradation responsible for the weight loss. FTIR is sensitive to local molecular environment and was therefore, applied to investigate the interactions between the macromolecules during the PE microplastic degradation. Hence, the structural changes in the biologically treated microplastics were analysed using FTIR, and the results are presented in Figures 4.5 – 4.11.

For PE microplastic inoculated with *B. cereus***,** the peak 1798 cm^{-1} in uninoculated PE microplastic (Figure 4.5) assignable to C=O carbonyl band was absent in the FTIR spectra of PE microplastic inoculated with *B. cereus* (Figure 4.6). New absorption bands appeared at 3738 cm^{-1} and 3419 cm^{-1}, attributed to N–H and O–H bonds, respectively. This could have possibly been due to the formation of amino and hydroxylated compounds. The N–H band split into two, showing the presence of a primary amine and the formation of a new peak at 1460 cm^{-1} attributed to C=C of an aromatic compound. This reflects the intrinsic constituents of *B. cereus* which is chemically complex, especially in the protein level, and the amino and neutral polysaccharides (Ma *et al.*, 2014; Matz *et al.*, 1970). Hence, the replacement of the carbonyl band with amine bands indicated favourable metabolism of the strain in PE microplastic-induced environment and possible evidence of gradual interference with the chemical structure of the PE microplastic which could cause degradation. Furthermore, elongation of the peak at 730 cm^{-1} was observed, so also was the disappearance of the peak at 848 cm^{-1} and the phenolic peak C–O at 1038 cm^{-1} after the action of the microbe. This can be attributed to the oxidation of the PE microplastic by the isolate.

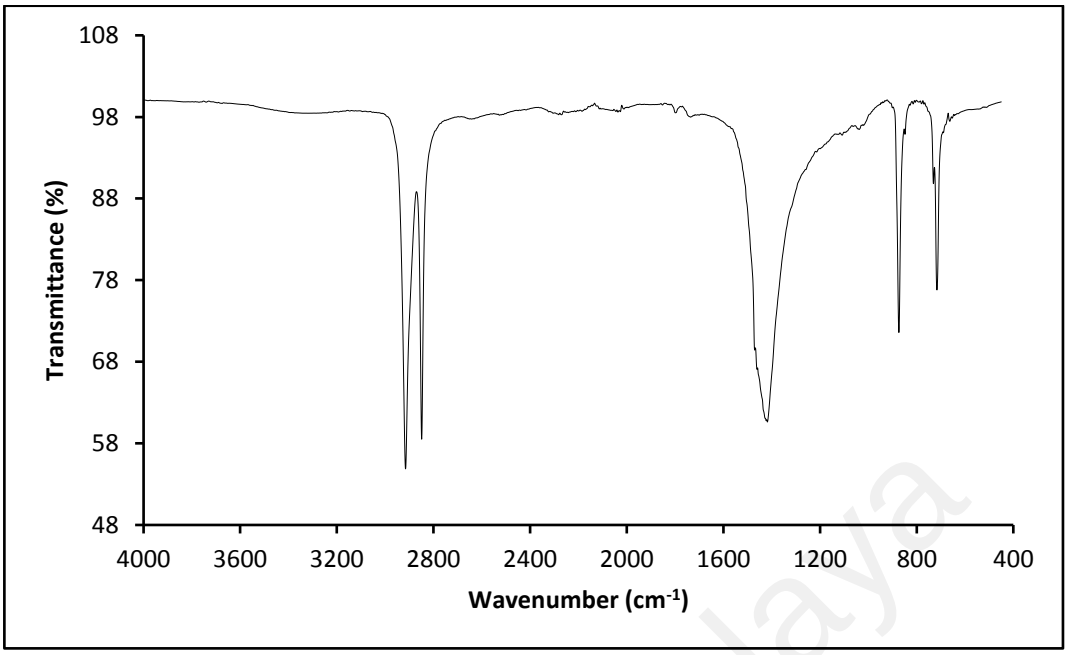

Figure 4.5: FTIR spectrum of control (uninoculated) PE microplastic

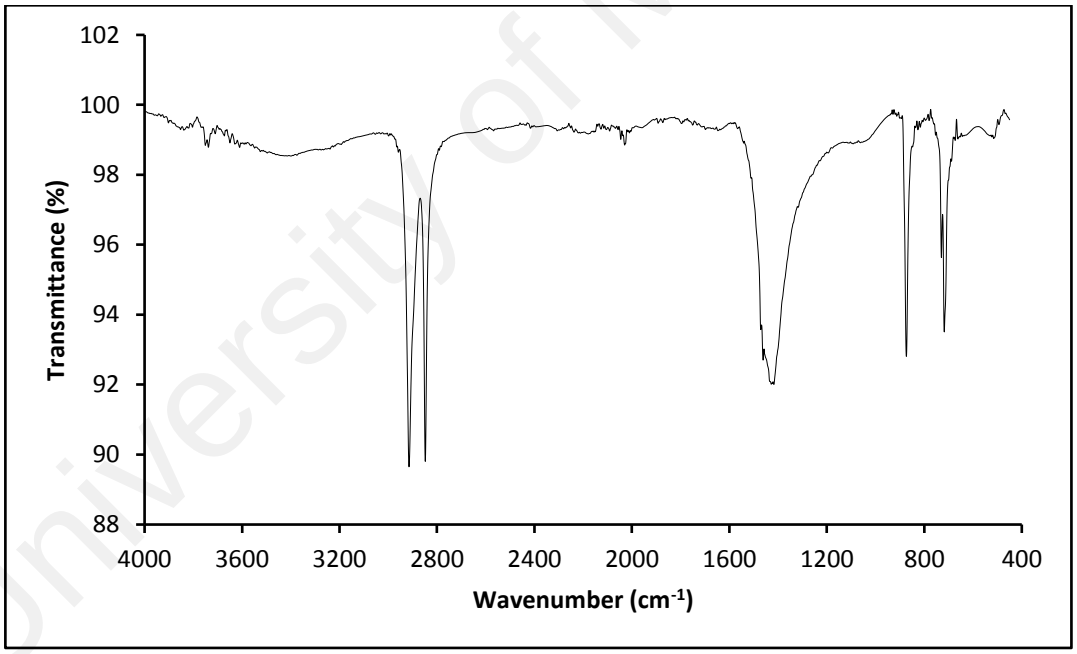

Figure 4.6: FTIR spectrum of PE microplastic inoculated with *B. cereus*

Figure 4.7 Shows the FTIR spectra of PE microplastic inoculated with *B. vietnamensis*. As observed in the figure, new absorption peaks appeared at 3745 cm^{-1} and 3330 cm^{-1} in the PE microplastic, assignable to N–H and O–H hydroxyl bands of a hydroxylated compound, respectively. The observation of these peaks was probably due to the

vibrations in the stretching of the O – H bond in alcohols and phenols. An absorbance range of 3500 – 3200 cm^{-1} corresponds to the presence of phenols and alcohols. Similar peaks were observed by Ojha *et al.* (2017) in the degradation of PE by fungi species. Reduction of the C–H aliphatic stretching bands at 2915 cm^{-1} and 2849 cm^{-1} were observed, so also was the disappearance of the carbonyl band at 1798 cm^{-1} that was present in the uninoculated PE microplastic. Absorbance range of 3000 – 2800 cm^{-1} corresponds to C – H stretch and the presence of alkanes (Shah *et al.*, 2007). Furthermore, a reduction of the absorption peaks at 1426 cm^{-1} (C=C aromatic stretching), 874 cm^{-1} (C=C bending), and 731 cm^{-1}, were also observed, however, the peak at 730 cm^{-1} was elongated. There was also a disappearance of the peak at 663 cm^{-1} and the formation of a new peak at 533 cm^{-1}. Absorbance range of 1500 – 1400 cm^{-1} corresponds to – CH_2 stretching and the presence of aromatics (Negi *et al.*, 2011), while the absorbance range of 700 – 900 cm^{-1} corresponds to – C = C – stretching and the presence of alkene group (Esmaeili *et al.*, 2014)

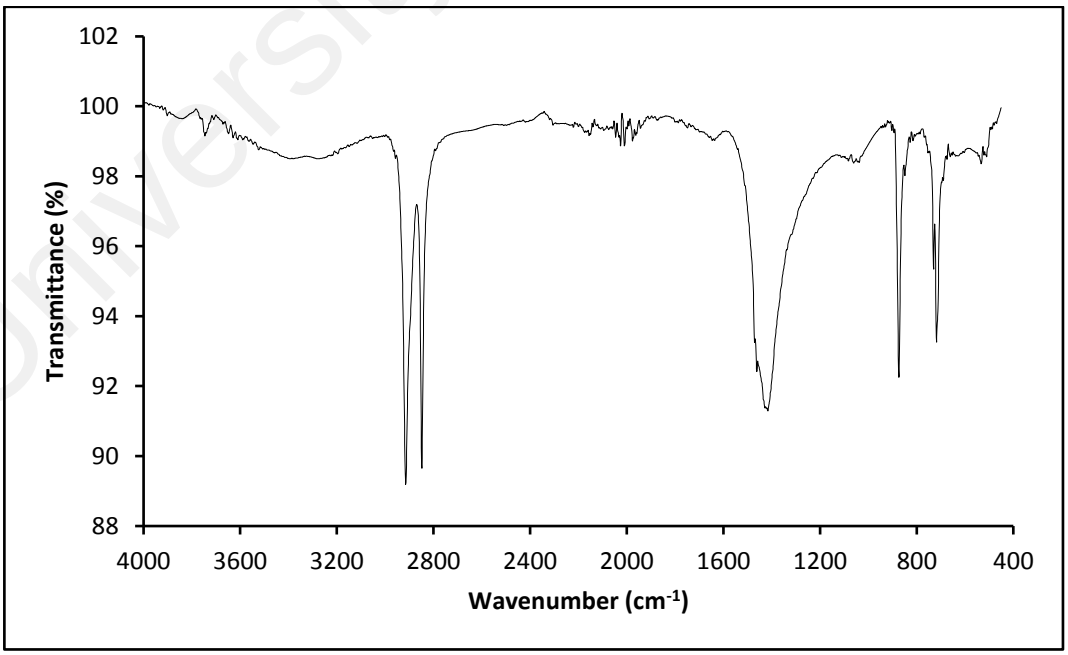

Figure 4.7: FTIR spectrum of PE microplastic inoculated with *B. vietnamensis*

The FTIR spectra of PE microplastic inoculated with *S. globispora* is presented in Figure 4.8. The formation of new absorption peak assignable to O–H hydroxyl band at 3394 cm^{-1} and the reduction of the C–H aliphatic stretching peaks at 2914 cm^{-1} and 2848 cm^{-1} were observed in the FTIR spectra. The C–O carbonyl band at 1798 cm^{-1} present in uninoculated PE microplastic shifted to 1645 cm^{-1} and appeared stronger in PE microplastic inoculated with *S. globispora*. Harshvardhan and Jha (2013) also reported similar results in their study of the biodegradation of PE by marine bacteria. The C=C aromatic stretching absorption peak at 1426 cm^{-1} was reduced and a stronger, broader and elongated C–O phenolic band was observed at 1048 cm^{-1} which appeared at a lower wavelength (1038 cm^{-1}) in the uninoculated PE microplastic. Additionally, the peaks at 874 cm^{-1} (C = C bending) and 718 cm^{-1} were reduced while the peak at 730 cm^{-1} became elongated. New absorption peaks were formed at 526 cm^{-1} and 468 cm^{-1}.

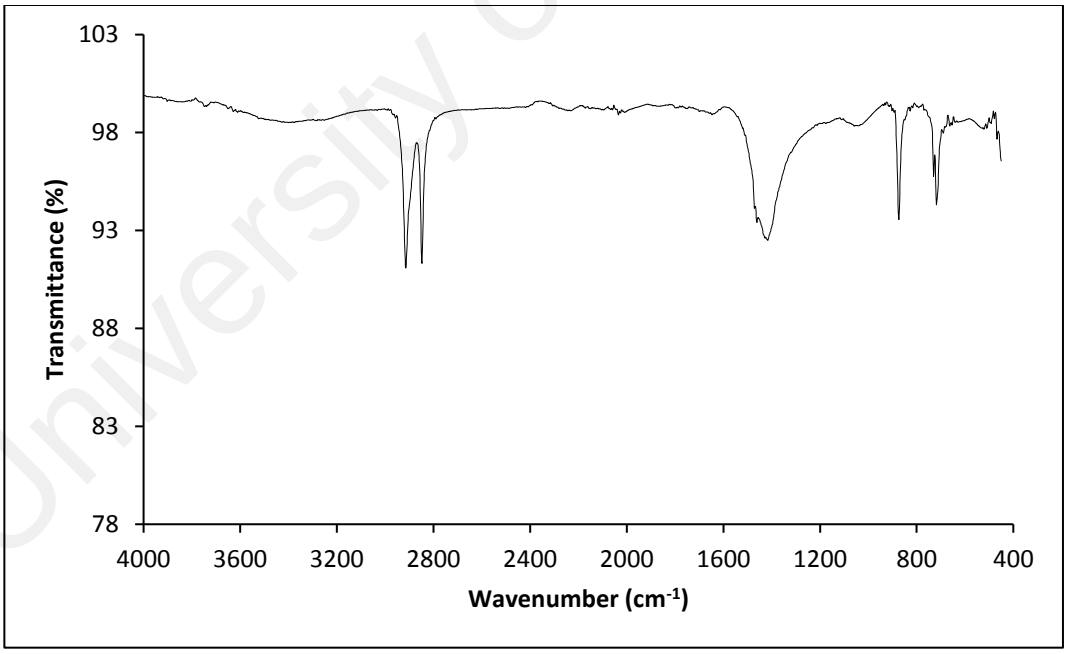

Figure 4.8: FTIR spectrum of PE film inoculated with *S. globispora*

The PE microplastic treated with *S. epidermidis* showed the formation of O–H hydroxyl peak at 3406 cm^{-1} and the removal of C=O carbonyl band at 1798 cm^{-1} (Figure 4.9). Reduction of the C–H aliphatic stretching peak at 2916 cm^{-1} and 2848 cm^{-1}, the C=C

aromatic stretching at 1415 cm^{-1}, the C=C bending at 873 cm^{-1} and 718 cm^{-1}, due to the action of the microbe were also observed. The peak at 730 cm^{-1} became elongated and a new peak was formed at 508 cm^{-1}.

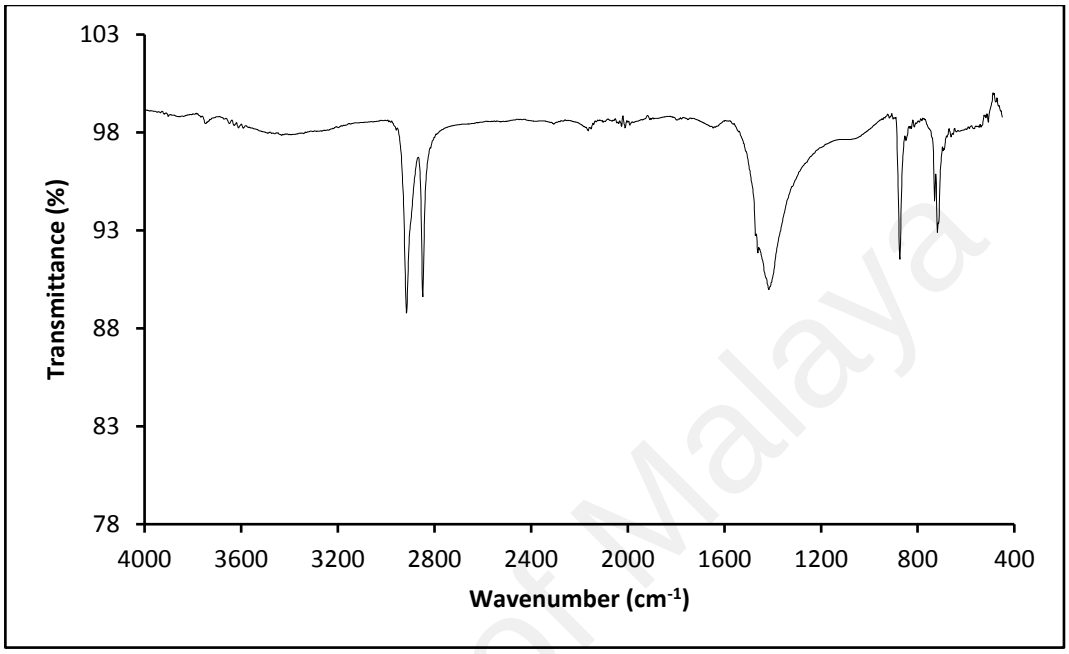

Figure 4.9: FTIR spectrum of PE film inoculated with *S. epidermidis*

From Figure 4.10, the peak at 3387 cm^{-1} in the FTIR spectra of PE microplastic treated with *B. flexus* attributed to O–H hydroxyl band is absent in uninoculated PE microplastic. This depicts the formation of a hydroxyl group by bacterial action. The C–H aliphatic stretching at 2916 cm^{-1} and 2848 cm^{-1} became reduced compared to uninoculated PE microplastic. Also reduced were the peaks at 1419 cm^{-1} (aromatic stretching), 874 cm^{-1} (C=C bending), 716 cm^{-1}, while the peak at 731 cm^{-1} became elongated.

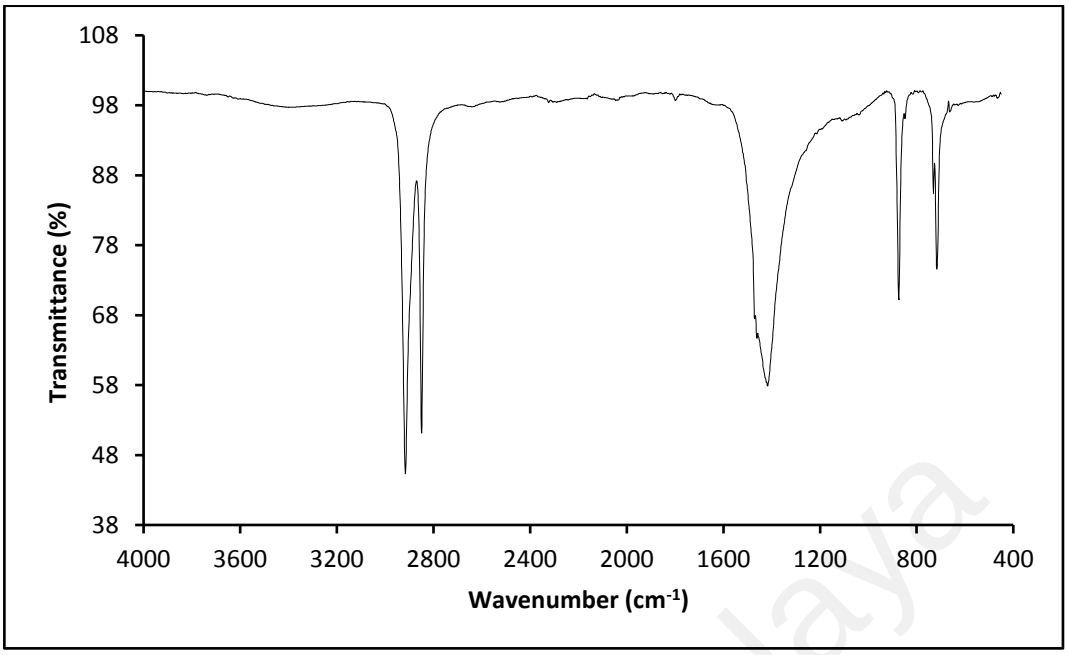

Figure 4.10: FTIR spectrum of PE microplastic inoculated with *B. flexus*

New absorption bands appeared at 3419 cm^{-1} attributed to O–H bonds in PE microplastic treated with *B. gottheilii* (Figure 4.11). This could probably be due to the formation of hydroxylated compounds. Elongation of the peak at 730 cm^{-1}, disappearance of the peaks at 848 cm^{-1} and the phenolic peak C–O at 1038 cm^{-1} were observed which is attributed to the oxidation of the PE microplastic by the isolate.

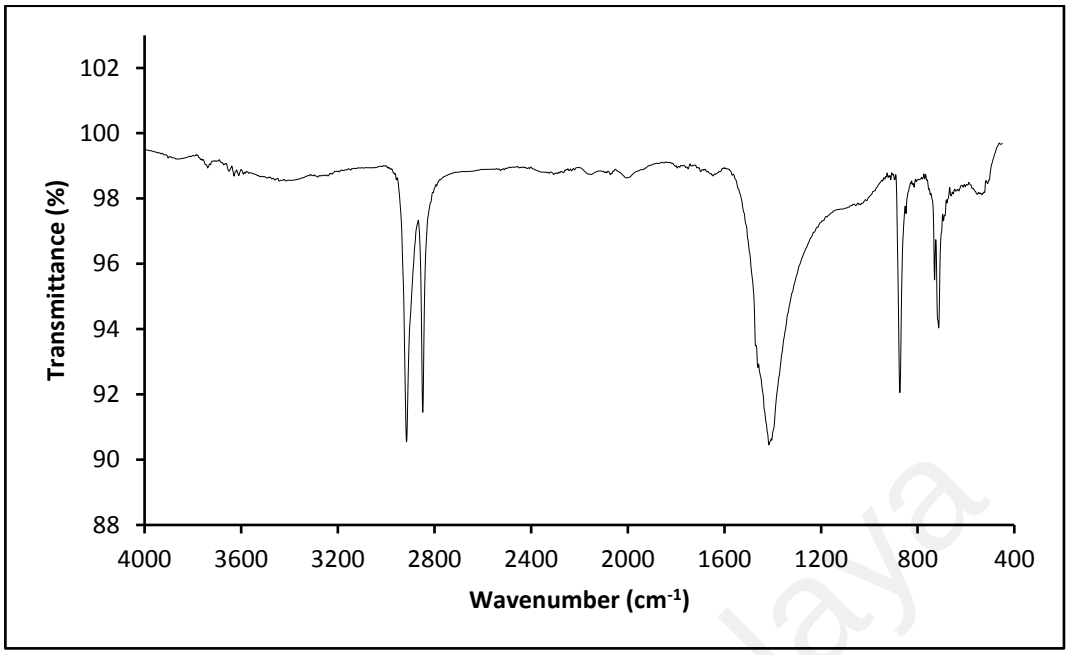

Figure 4.11: FTIR spectrum of PE film inoculated with *B. gottheilii*

Overall, the degradation of PE microplastics by individual bacterial isolates revealed shift in peaks and the formation of carbonyl and hydroxyl groups, esters, aldehydes, alcohols, phenolic, and aromatic compounds at different frequencies, indicating PE microplastics degradation by oxidation reactions. According to Sivan (2011) and Wilkes and Aristilde (2017), the formation of biofilms on PE alters the polymer by oxidation reactions. Oxidation of the polymer by the isolates may have increased its hydrophobicity by producing functional groups such as alcohol, carbonyl, phenolic and hydroxyl groups which enhanced adherence and biodegradation. The shift in peaks and the formation of oxidation products observed in this study reflect changes to the chemical structure of the PE microplastics. This is probably due to adherence of the microbes which may have altered the PE microplastics through oxidation reactions and supported the conformational changes on the PE microplastic samples. The carbonyl, hydroxyl, and alcohol functional groups produced are easily metabolized in the bacterial cells through β-oxidation and the tricarboxylic acid (TCA) cycle, thereby, facilitating growth and biofilm formation (Mehmood *et al.*, 2016). The findings of this study were consistent

with the study by Gajendiran *et al.* (2016) and Esmaeili *et al.* (2013), where the spectra of PE film showed several new bands at same wavelength. Similar patterns were equally observed by Kavitha *et al.* (2014), where LDPE degradation by bacteria isolated from oil contaminated soil were analyzed through ATR-FTIR spectrum. Das and Kumar (2014) demonstrated the formation of new and disappearance of functional groups in their studies of LDPE degradation by *Bacillus amyloliquefaciens* strain.

The study of the chemistry of PE microplastic surface turns out to be very important, because oxidized groups are more easily degraded by microorganisms (Albertsson *et al.*, 1995) and because oxidized groups modulate microbial attachment by increasing the hydrophilicity of the surface (Tribedi & Sil, 2013). This implies that polyethylene degradation will be boosted if a more oxidized surface is used as substrate.

4.4.6 Scanning electron microscopy (SEM) analysis of PE microplastics treated with individual isolates

The changes to the surface morphology of PE microplastics after bacterial action were investigated under SEM after 40 days of exposure (after removal of biofilm). The results are presented in Figures 4.12 - 4.17. After 40 days of incubation, the surfaces of the PE microplastics treated with the individual microbes became rough with numerous erosions, pits, holes/pore, grooves (Figure 4.13) and cavities when compared to uninoculated PE microplastics shown in Figure 4.12, which had no defects.

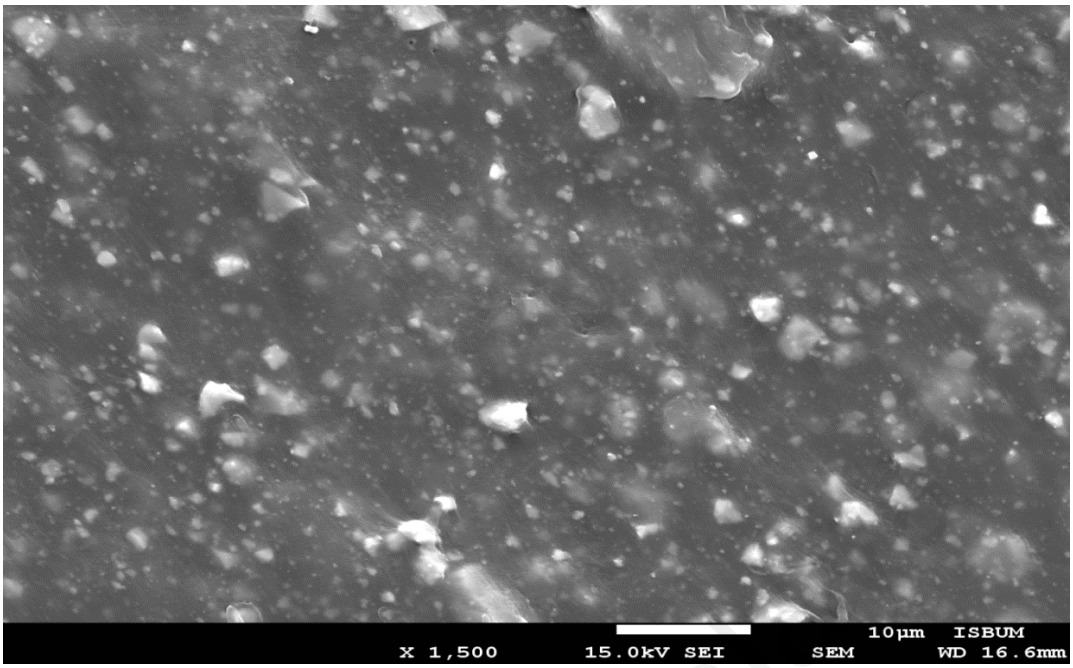

Figure 4.12: SEM micrograph of control PE microplastic (uninoculated)

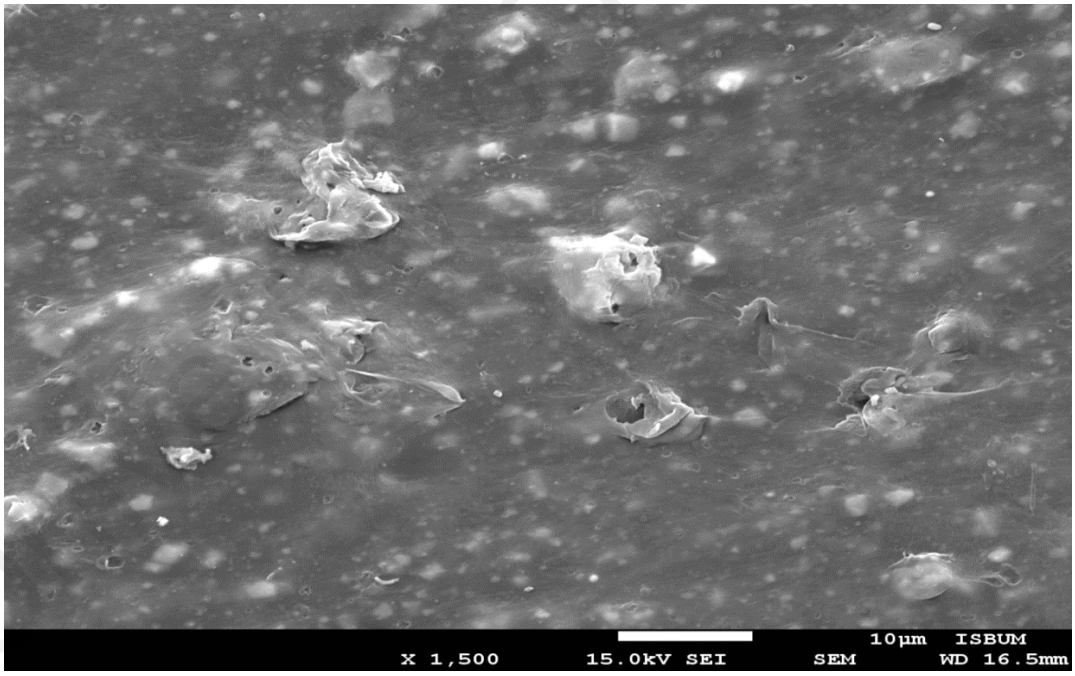

Figure 4.13: SEM micrograph of PE microplastics inoculated with *B. cereus*

This provided evidence of the deterioration of the microplastics due to microbial action on the microplastic surface. Surface bio-erosion is the primary cause of mass loss from polymer surface. As a cross reference to earlier studies on PE polymer biodegradation, several researchers have reported similar morphological changes on PE degradation by

bacteria isolated from oil contaminated soil (Kavitha *et al.*, 2014), degradation of PE by *Pseudomonas* species (Kyaw *et al.*, 2012), and the degradation of PE by *Bacillus cereus* (Sowmya *et al.*, 2014), biodegradation of low density PE by marine bacteria isolated from pelagic waters (Harshvardhan & Jha, 2013).

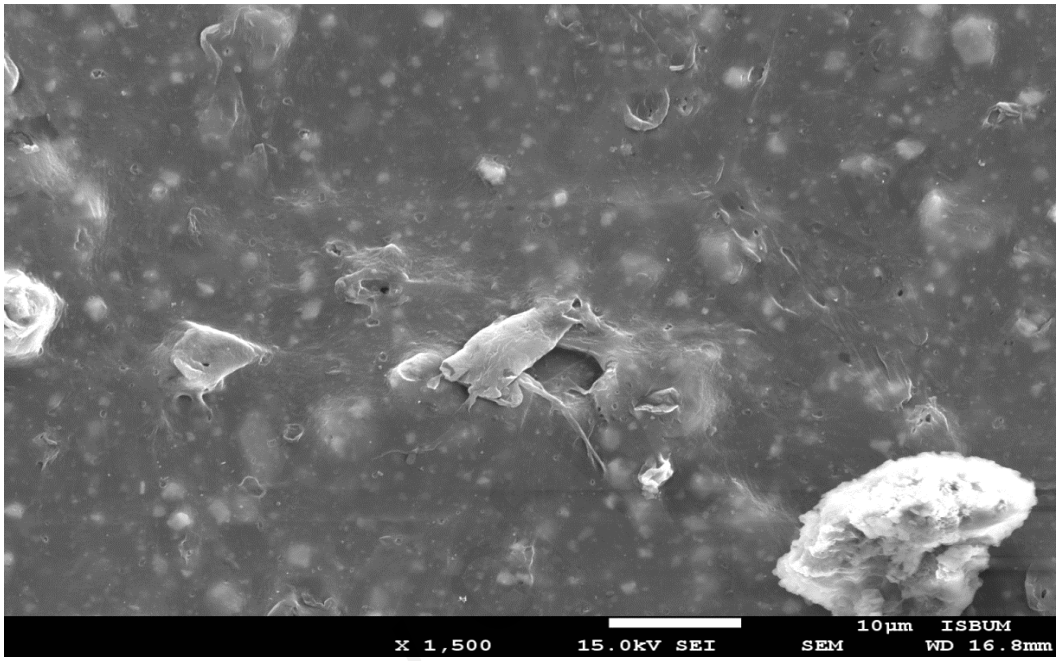

Figure 4.14: SEM micrograph of PE microplastics inoculated with *S. globispora*

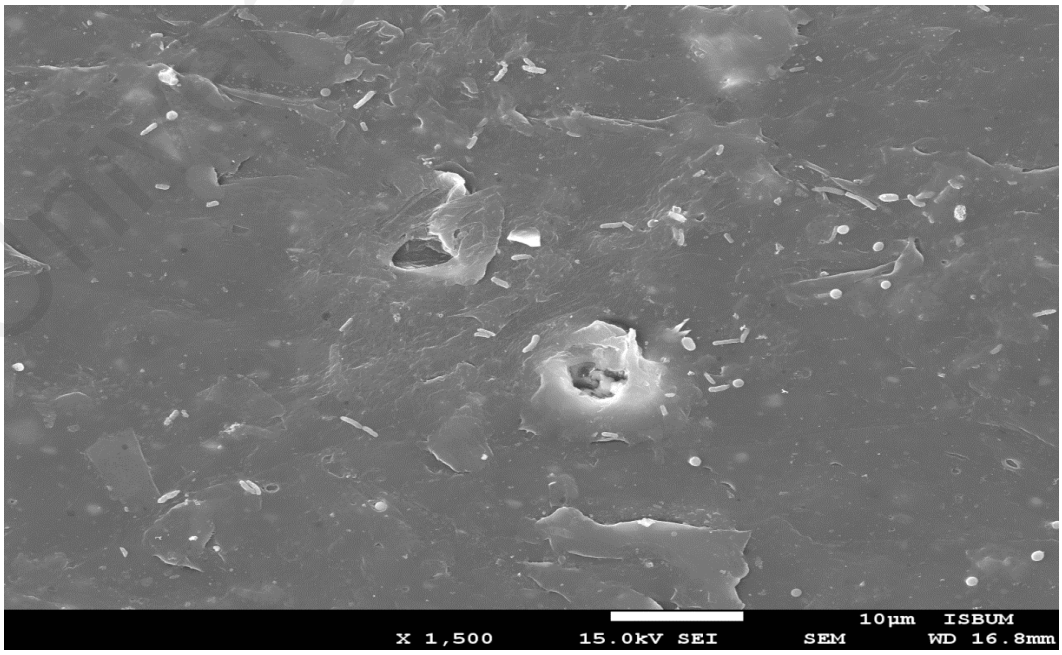

Figure 4.15: SEM micrograph of PE microplastics inoculated with *S. epidermidis*

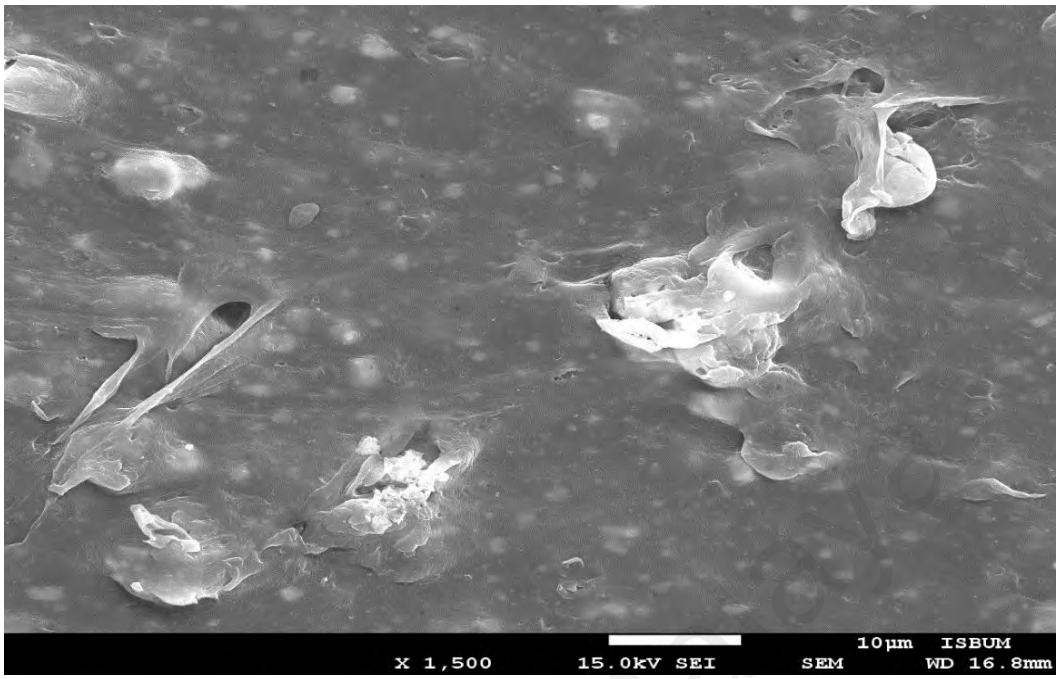

Figure 4.16: SEM photograph of PE microplastics inoculated with *B. flexus*

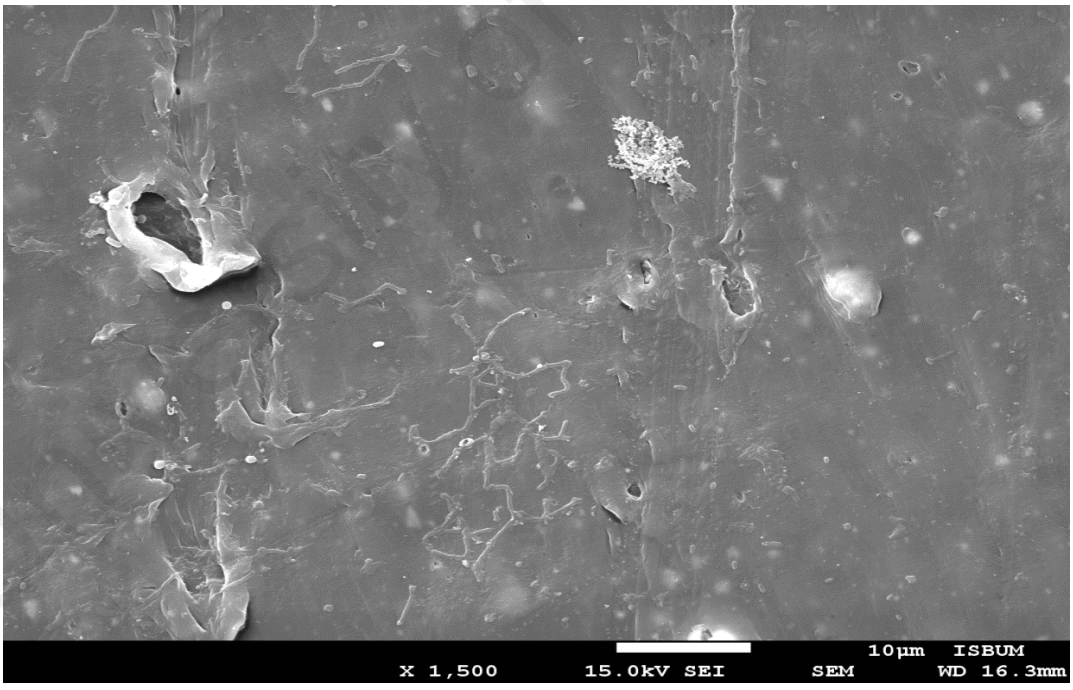

Figure 4.17: SEM micrograph of PE microplastics inoculated with *B. gottheilii*

The study supported enzymatic activity on PE microplastics as SEM micrographs of PE microplastics showed some localized degradation around the bacterial cells. Four different mechanisms are reported to exist for the study of plastic degradation by

microorganisms namely: solubilization, hydrolysis, enzyme-catalysed degradation, and charge formation (Harshvardhan & Jha, 2013). The loss of dry weight in this research work supports solubilization, and the SEM images support enzyme-catalysed degradation.

PE microplastics are accumulating in the environment, especially the marine environment and creating serious environmental concern. The inert nature of PE that confers it the ability to resist deterioration and degradation. This in vitro biodegradation study of PE microplastics suggests the suitability of six marine bacteria: *B. cereus*, *B. vietnamensis*, *S. globispora*, *S. epidermidis*, *B. flexus*, and *B. gottheilii*, isolated from mangrove sediments in the degradation of PE microplastics. Based on the weight loss and rate of removal ability, *B. gottheilii* was more efficient in PE microplastic degradation than other bacteria.

4.5 Biodegradation of polypropylene (PP) microplastics

From the screening assay, only five of the isolates namely: *B. sonorensis*, *S. globispora*, *A. faecalis*, *R. ruber* and *B. gottheilii* showed degradation ability for PP microplastics and were selected for this study.

4.5.1 Determination of weight loss of PP microplastics

Changes that occurred as a result of microbial degradation were assessed qualitatively by measuring the weight loss of the PP microplastics after inoculation with selected isolates. The residual weight reduction of PP microplastics by the selected isolates that possessed the potential to degrade PP microplastic after 40 days is presented in Table 4.5.

Table 4.5: Average mass reduction of PP microplastics after 40 days of incubation with individual isolates

Treatment	Initial weight (g)	Residual weight (g)	Weight loss (%)
PP Control	0.500	0.500	0
B. sonorensis	0.500	0.480	4.0
S. globispora	0.500	0.497	0.6
A. faecalis	0.500	0.489	2.2
R. ruber	0.500	0.468	6.4
B. gottheilii	0.500	0.482	3.6

n = 3

Highest weight loss of 6.4% was achieved by *R. ruber*, followed by *B. sonorensis* which recorded a weight loss of 4.0%. *B. gottheilii* and *A. faecalis* also showed good degradation of 3.6% and 2.2%, respectively. This may imply the isolates ability to excrete specific enzymes that could putatively attack PP microplastics. This indicates that the isolates may have catalysed metabolic reactions that contributed to the adsorption, desorption, and breakdown of PP microplastics (Auta *et al.*, 2018). *S. globispora* recorded the least weight loss of 0.6%. The isolates may have catalysed metabolic reactions that might have contributed to the adsorption, desorption and breakdown of the PP microplastics. No weight loss of PP microplastics was observed in the control (uninoculated) flask. The weight loss of PP microplastics after incubation could be as a result of microbial activity and indicate not only the extent of decrease in weight but also the loss of certain properties, hence, hinting at the physical breakdown of PP microplastic and its degradation (Board, 2006). The analysis of variance indicated no significant differences with $p < 0.05$ at the end of 40 days. However, the difference in PP reduction between the isolate that gave highest weight loss (*R. ruber*) and the least (*S. globispora*) was significant at p (0.024) < 0.05. Jeyakumar *et al.* (2013) in their study, recorded gravimetric weight loss of 18.8% in UV-treated Mi-PP inoculated with *Engyodontium album* and 9.42% when inoculated with *Phanerochaete chrysosporium.* 10% weight loss

was recorded in starch blended PP after direct exposure to sunlight, and 0.5% weight loss was recorded with unblended PP in marine waters for a period of 6 months.

4.5.2 Growth pattern of individual isolates during PP microplastic degradation studies

The growth profile of the isolates on exposure to PP microplastics revealed that all five isolates exhibited varied growth patterns on exposure (Figure 4.18). *B. sonorensis* depicted an exponential increase in growth after Day 0, and the peak of growth was noticed on the 10th day with OD reading of 1.376 OD_{600}. This was followed by a sharp decline in growth on the 20th day, and a gradual decline on the 30th and 40th days. A similar growth pattern was demonstrated by *B. gottheilii* on exposure to PP microplastic, with optimal growth (1.497 OD_{600}) that reached a peak on the 20th day. Furthermore, a sharp decline phase was observed until the 40th day. *S. globispora* and *R. ruber* demonstrated an exponential growth response from Day 0 (0.251 OD_{600}) until Day 10 (1.05 OD_{600} and 0.903 OD_{600}, respectively). However, the increase in growth observed on the 10th day did not portray optimal response on performance of the microbes upon exposure to PP microplastics; rather, it may signify that the period was favourable for interaction between the bacterial cell and the microplastics which could have allowed for rapid metabolism.

The growth of *S. globispora* and *R. ruber* accelerated towards a positive growth pattern from 0.251 OD_{600} to 1.259 OD_{600} and 0.251 OD_{600} to 1.05 OD_{600}, respectively, that reached a peak on the 20th day. This was followed by a decline in growth up to the 40th day. *A. faecalis* on the other hand demonstrated slow growth that was characterized by a significant growth phase that experienced highest growth on the 10th day (0.993 OD_{600}). This was accompanied by a mild and stable decline phase on Day 20, followed by a sharp decline phase on Day 30 after which the organism experienced a reduced growth up to

the 40th day. This may have been as a result of bacterial death due to waste accumulation in the synthetic medium (Hall *et al.*, 2013). Increase in OD indicates the utilization of the PP microplastics as the carbon source. Highest increase in OD was found in PP microplastic inoculated with *B. gottheilii* and the least was recorded by PP microplastic inoculated with *A. faecalis*. Statistically, no significant difference existed in the growth response between the isolates on the 10th day of monitoring at p (0.104) < 0.05 as F = 2.557. There was however, significant differences in the growth response of the isolates on the 20th, 30th and 40th days of monitoring with p-values ranging from 0.01 – 0.02.

Furthermore, the PP microplastic-tolerance of *B. sonorensis* and *A. faecalis* was clear along the decline phase because their reduction in measured OD was not as steep as that demonstrated by *B. gottheilii*, *S. globispora* and *R. ruber* upon exposure to the PP microplastics. The varying response patterns depicted by the isolates could have been as a result of the different genetic characteristics of the microbes. Growth kinetics in synthetic medium is known to depict the growth pattern of microorganisms as well as their survival potential in the medium. Proportional increase in growth shows that degradation must have taken place.

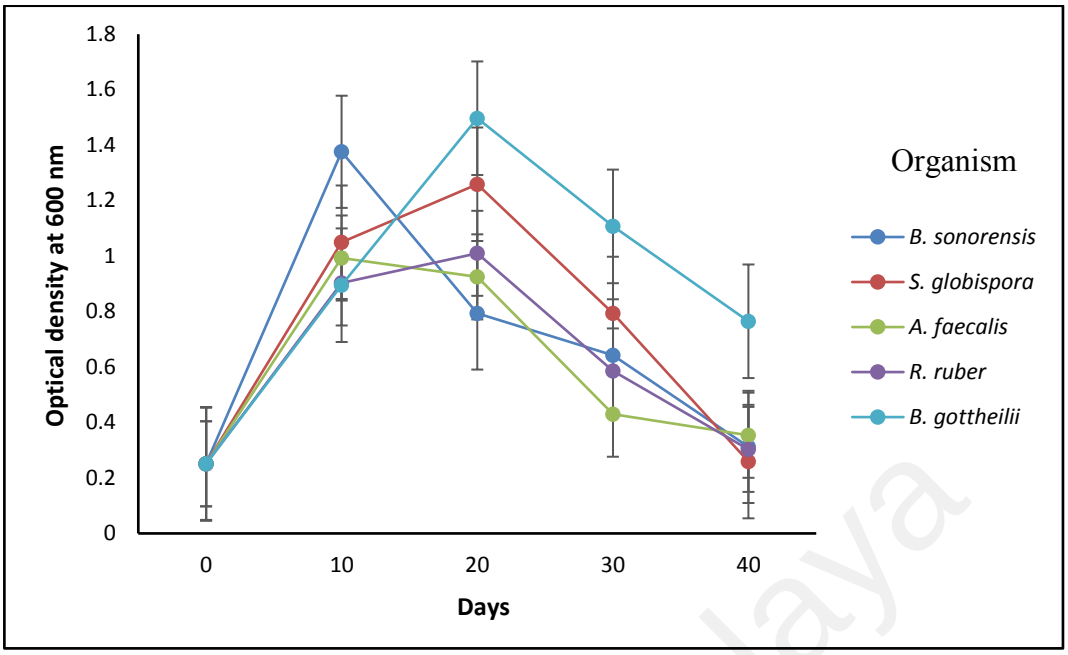

Figure 4.18: Growth profile of individual isolates in PP microplastic-infused media during biodegradation studies

Counts of bacteria inoculated in media containing PP microplastics demonstrated similar pattern of growth as depicted by the growth curve of all isolates; characterized by a log (exponential) phase from Day 0 to Days 10 and 20 for the isolates in which rapid growth of the microbes was observed. The log phase is usually the period at which the cells are metabolically more active. The log phase was accompanied by mild, stable and decline phases for the isolates depicting cell death. Figure 4.19 shows the counts of bacteria in PP microplastic synthetic liquid media inoculated with PP degrading bacteria. Counts in PP microplastic media inoculated with *B. sonorensis* ranged between 3.8 x 10^8 CFU/ml and 7.3 x 10^{11} CFU/ml. Counts in PP microplastic inoculated with *S. globispora* ranged between 3.8 x 10^8 and 3.7 x 10^{11}, while counts in PP microplastics treated with *A. faecalis*, *R. ruber* and *B. gottheilii* ranged between 3.8 x 10^8 (initial counts for all three isolates) and 5.1 x 10^{11} CFU/ml, 4.3 x 10^{11} CFU/ml, 3.1 x 10^{11} CFU/ml, respectively. Highest counts were recorded in PP microplastic-infused media inoculated with *B. sonorensis*.

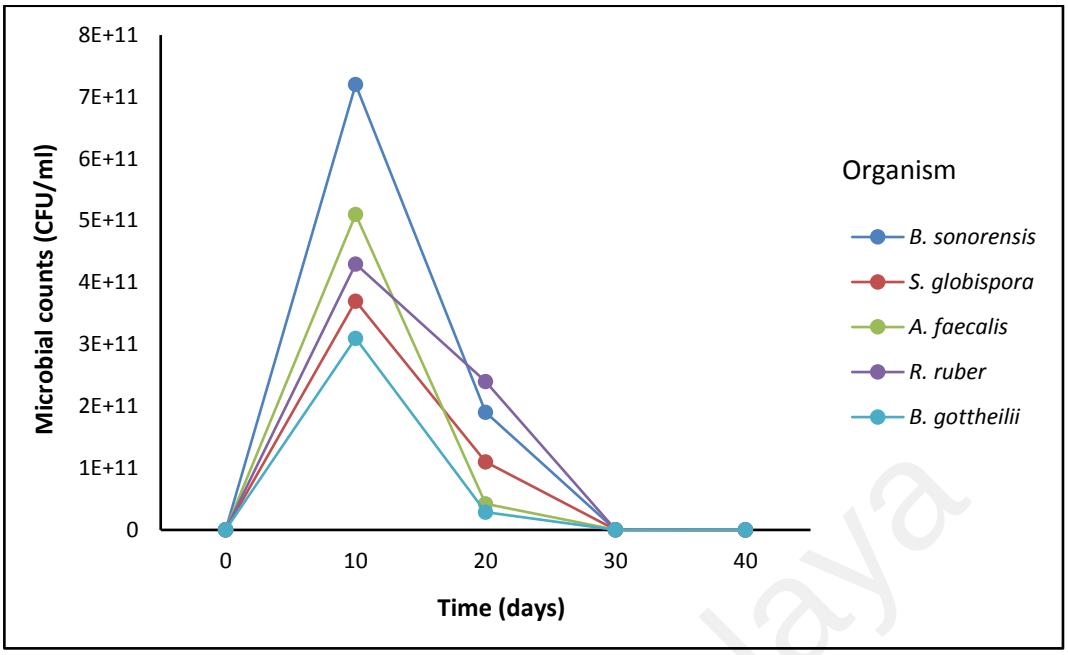

Figure 4.19: Bacterial counts of isolates inoculated in PP microplastic-infused media during 40 days biodegradation. Bars indicate standard error (n =3)

All the isolates recorded highest bacterial counts on the 10th day with maximum count of 7.2 x 10^{11} CFU/ml recorded by *B. sonorensis* and lowest count of 3.1 x 10^{11} CFU/ml recorded by *B. gottheilii*. This was followed by decline in counts on Day 20 up to the 40th day, with highest counts on these days recorded by *R. ruber*. The decline in cell counts on Days 30 and 40 went below the starting (initial) counts. The decline in cell counts might be that the cells were no longer duplicating as before due to stress associated to the biodegradation process, hence, mortality or inhibition of cell duplication occurred. The highest counts recorded by *R. ruber* depict that the organism had the potential to significantly impact the bonds of PP microplastics and utilize it for growth as has been shown by the highest weight loss.

The highest counts recorded by *B. sonorensis* and *A. faecalis* on the 10th day were consistent with the highest OD readings recorded by the microbes on same day but such was not the case for *S. globispora*, *R. ruber* and *B. gottheilii* that recorded highest counts on the 10th day but recorded highest OD readings on the 20th day. This demonstrated that

increase in OD on the 20th day by the isolates could not have been due to increase in cell number, rather, it could have been as a result of degradation metabolites in the medium produced by the microbes. This could have caused the culture media to become turbid, and hence, the higher absorbance readings.

4.5.3 Changes in pH of PP microplastic-infused mineral salt media during biodegradation studies

Figure 4.20 depicts the changes in pH of PP microplastic-infused BH media by individual isolates during the 40 days biodegradation studies. Degradation of PP microplastics by *B. sonorensis*, *S. globispora*, *A. faecalis*, *R. ruber* and *B. gottheilii* increased the pH of the aqueous media. Similar pH variations were observed for all microbes. The pH progressed from the initial neutral (pH 6.76) towards alkalinity after 10 days of incubation with the isolates (ranging from pH 8.44 to pH 8.51), with highest growth of the isolates attained at this pH. This may imply that the pH could have been conducive for the growth of the organisms. However, continued increase in pH was observed up to the 40th day (ranging from 9.37 to 9.55) just as the study observed decline in growth of the microbes. There was no significant difference in the pH changes across the monitored days at $p < 0.05$. The changes in pH from neutral to alkalinity may be attributed to the metabolic products excreted by the microbes in the BH media. This may suggest that pH modulating metabolites could have been produced by the isolates during the biodegradation assay which support the evidence of degradation in the media. Previous studies by Xu *et al.* (2011), observed rapid microbial degradation of polylactic acid (PLA) at pH 8. The pH of the control PP microplastic sample however, remained same.

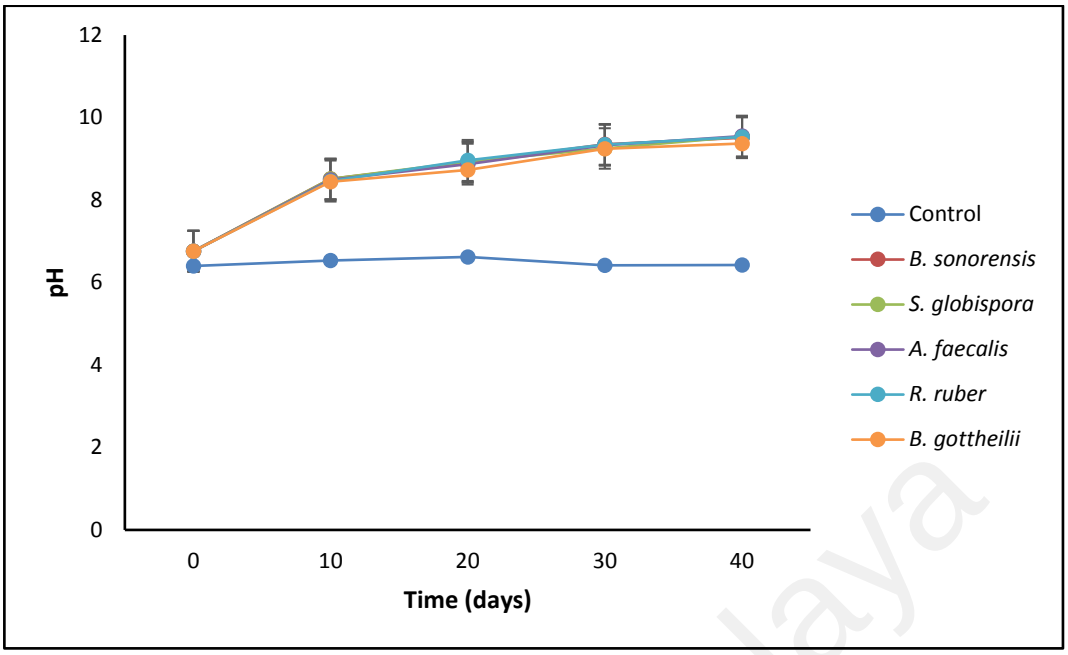

Figure 4.20: pH changes of PP microplastic-infused BH media inoculated with individual organisms during biodegradation studies.

4.5.4 Biodegradation rate constant and half-life of PP microplastics inoculated with individual isolates

Table 4.6 shows the rate of uptake of PP microplastics by individual isolates within the period of the study (40 days). PP microplastic inoculated with *R. ruber* recorded highest uptake rate of 0.002 day^{-1}. This was followed by *B. sonorensis* which recorded 0.001 day^{-1}, then *B. gottheilii* which recorded 0.00091 day^{-1}, whereas, *A. faecalis* and *S. globispora* recorded 0.00055 day^{-1} and 0.00015 day^{-1}, respectively. No reduction was observed in the uninoculated PP microplastic sample. This result shows that the highest PP microplastic uptake rate was by *R. ruber*. The reason for this higher PP microplastic uptake might be due to the differences in the organism's physiological make up. Therefore, the results observed that lower mass of PP microplastics in the synthetic medium corresponds to enhanced half-life. PP microplastic inoculated with *R. ruber* recorded half-life of 431 days, *B. sonorensis* recorded 690 days, 758 days (*B. gottheilii*),

1254 days (*A. faecalis*), 4600 days (*S. globispora*) and infinity (∞) for uninoculated control PP microplastic sample.

Table 4.6: Removal rate constant (*k*) and half-life of PP microplastics across individual isolates

Treatment	Removal constant (*k*) day^{-1}	Half-life ($t_{1/2}$) (days)
Control	0	∞
B. sonorensis	0.001	690
S. globispora	0.00015	4600
A. faecalis	0.00055	1254.54
R. ruber	0.0016	431.25
B. gottheilii	0.00091	758.24

4.5.5 Fourier transform infrared (FTIR) spectroscopy analysis of PP microplastics

The spectrum of PP microplastics incubated with individual isolates for 40 days contrasted with the corresponding control (uninoculated PP microplastic samples). The absorption peak at 3310 cm^{-1} attributed to O–H of a hydroxyl band, the peak at 1739 cm^{-1} assignable to C=O of a carbonyl stretch of an ester, and 1102 cm^{-1} assignable to the C–O bond stretch of an ester are all present in the spectrum of the uninoculated PP microplastic (Figure 4.21).

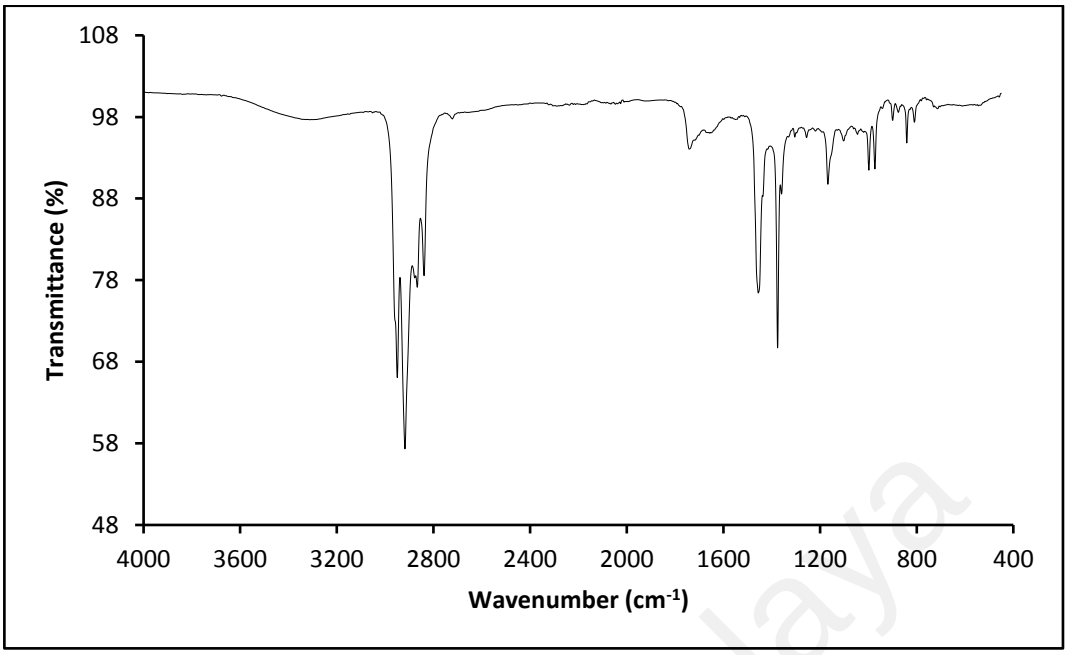

Figure 4.21: FTIR spectrum of control (uninoculated) PP microplastics

These bands which indicate the presence of esters and hydroxylated compounds disappeared in the spectra of PP microplastic inoculated with *B. sonorensis* (Figure 4.22). This signifies the oxidation of ester and reduction of hydroxylated compounds in PP microplastic by the bacteria. Elongation of the C–H aliphatic stretching peaks at 2950 cm^{-1} – 2839 cm^{-1}, C–H aliphatic bending peak at 1376 cm^{-1}, C=C aromatic stretching peak at 1455 cm^{-1} and C=C bending peak at 841 cm^{-1} – 810 cm^{-1} were detected in the spectra of PP microplastic inoculated with the isolate. Furthermore, the peak at 715 cm^{-1} in PP microplastic inoculated with *B. sonorensis* disappeared and the bend around 460 cm^{-1} appeared broader. Similar peaks were also detected by Jeyakumar *et al.* (2013) in their study of the synergistic effects of pre-treatment and blending on the biodegradation of i-PP by fungi.

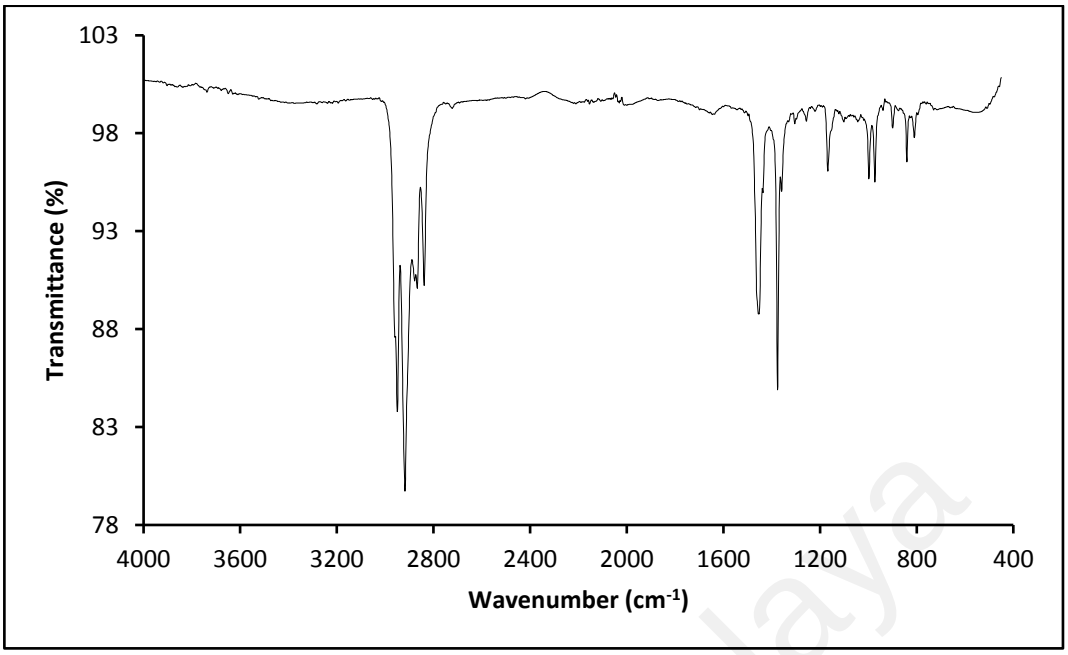

Figure 4.22: FTIR spectrum of PP microplastics inoculated with *B. sonorensis*

On treatment with *S. globispora* (Figure 4.23), the disappearance of the absorption peaks at 3310 cm^{-1} due to O–H hydroxyl band, and 1739 cm^{-1} for C=O carbonyl band in control (uninoculated) PP microplastics was observed. This signified effective biodegradation of the microplastic by the microbe. The elongation of peaks at 2950 cm^{-1} – 2839 cm^{-1} (C–H aliphatic stretching), 1455 cm^{-1} (C=C aromatic stretching), 1376 cm^{-1} (C–H aliphatic bending), 99 cm^{-1}, 973 cm^{-1} and 900 cm^{-1} (=C–H bending), and 809 cm^{-1} (C=C bending) were also observed in the spectra of PP microplastic inoculated with *S. globispora* when compared with the uninoculated PP microplastic. Additionally, the peak at 876 cm^{-1} present in the spectra of the uninoculated PP microplastics disappeared on treatment with the isolate, and the emergence of a new peak at 536 cm^{-1} was observed in the spectra of the *S. globispora* treated PP microplastic.

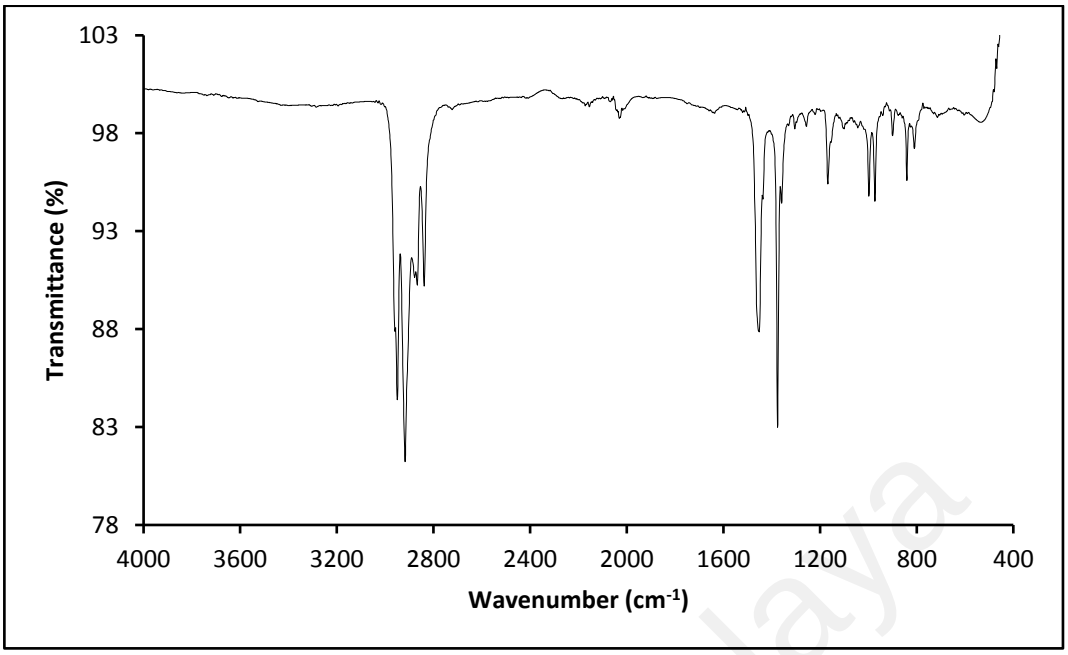

Figure 4.23: FTIR spectrum of PP microplastics inoculated with *S. globispora*

When PP microplastics were inoculated with *A. faecalis*, the hydroxyl absorption peak at 3310 cm^{-1} in uninoculated PP microplastics shifted to 3392 cm^{-1} in PP microplastics inoculated with the microbe, and the peak also became elongated (Figure 4.24). The C=O carbonyl peak at 1739 cm^{-1} shifted to 1648 cm^{-1} and became reduced. This may probably have been due to the release of short chain carboxylic acids in the form of degradation products as observed in PE where the carboxyl functionalized fragments can undergo β-oxidation by co-enzymatic action. Similar reduction in carbonyl peak at same frequency have been reported by Pandey and Singh (2001) on the degradation of PP by microorganisms.

There was elongation of the C–H aliphatic stretching peaks at 2951 cm^{-1}, 2918 cm^{-1} and 2838 cm^{-1}. The C–H aliphatic stretching peak at 2867 cm^{-1} in spectra of uninoculated PP microplastics shifted to 2873 cm^{-1} and became elongated in PP microplastics inoculated with *A. faecalis* and did not split as it did in the uninoculated control PP microplastic spectra. Also observed in the spectra of PP microplastic inoculated with the isolate is the elongation of the peaks at 1168 cm^{-1}, 1102 cm^{-1} (C–O phenolic bands) and

997 cm^{-1} (=C–H bending), and the formation of new absorption peaks at 559 cm^{-1} and 459 cm^{-1}.

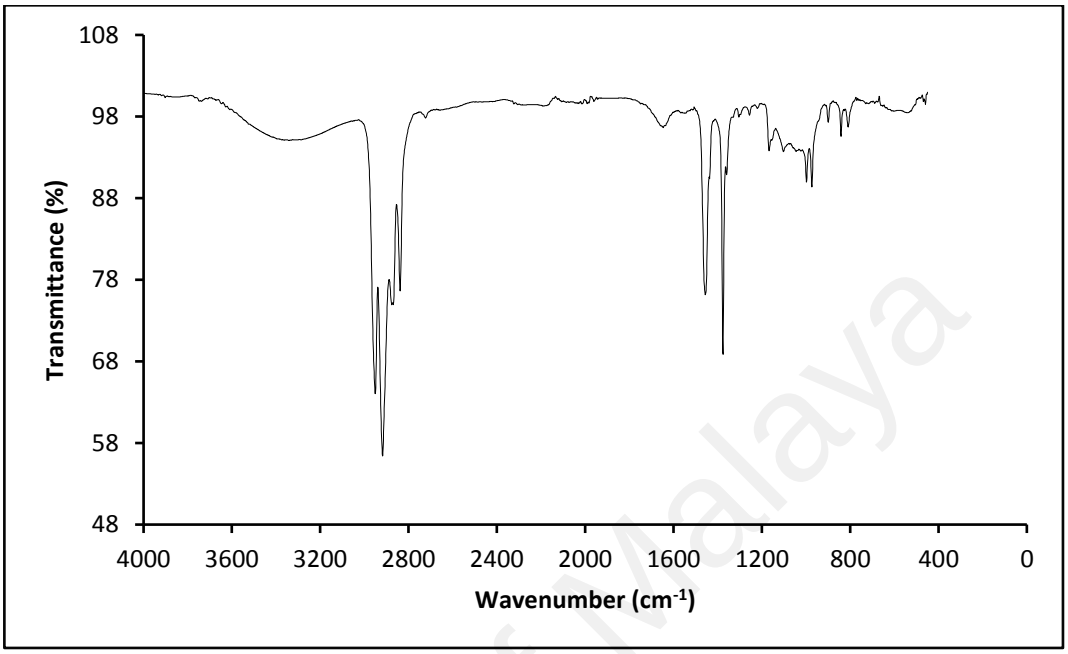

Figure 4.24: FTIR spectrum of PP microplastics inoculated with *A. faecalis*

The disappearance of the peaks at 3310 cm^{-1} for O–H hydroxyl band, 1739 cm^{-1} for C=O carbonyl band and the peak at 876 cm^{-1} present in the spectra of uninoculated control PP microplastic sample was observed in PP microplastics inoculated with *R. ruber* (Figure 4.25). This may indicate effective biodegradation. Elongation of the peaks at 900 cm^{-1}, 2950 cm^{-1} – 2839 cm^{-1} for C–H aliphatic stretching and 1376 cm^{-1} for C–H aliphatic bending was observed in the spectra of PP microplastics inoculated with *R. ruber*. Also observed was the reduction of the C–O peak at 1103 cm^{-1} in the spectra.

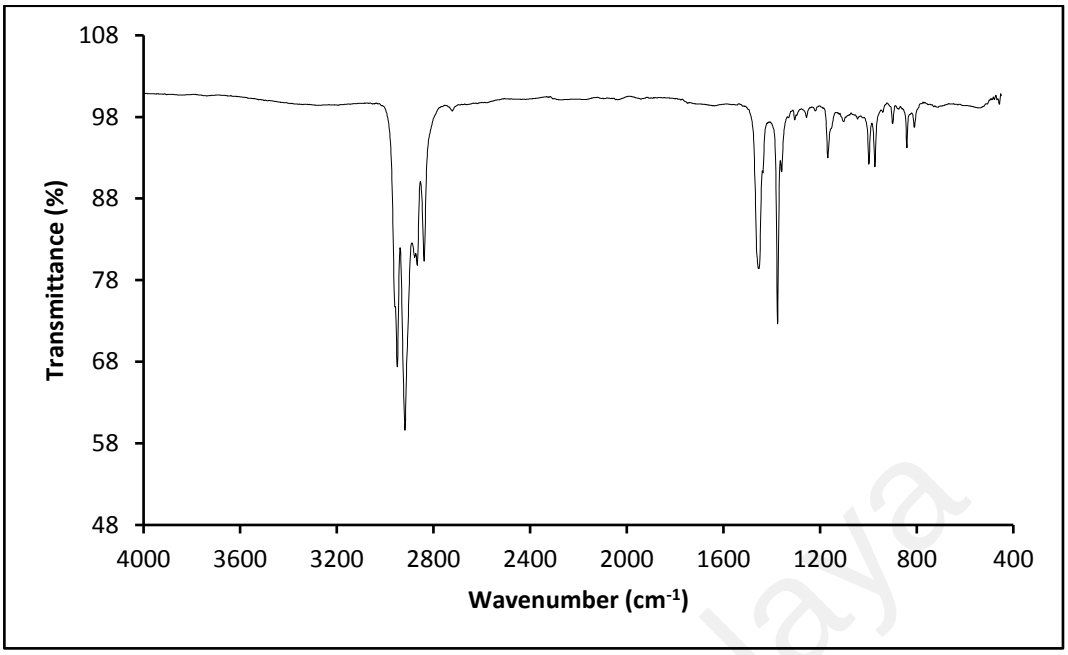

Figure 4.25: FTIR spectrum of PP microplastics inoculated with *R. ruber*

Figure 4.26 shows the FTIR spectra of PP microplastic inoculated with *B. gottheilii*. The formation of a broader and stronger peak at 3300 cm^{-1} assignable to O–H hydroxyl band was observed in the spectra when compared to the spectra of uninoculated PP microplastic. This band could have been due to the neighbouring intramolecular hydrogen bonded hydroperoxide and alcohols. Similar band formation was observed by Pandey and Singh (2001) in their studies of isotactic PP (i-PP) biodegradation by microbial cultures. The carbonyl band C=O at 1739 cm^{-1} in spectra of uninoculated PP microplastic shifted to 1645 cm^{-1} in PP microplastic inoculated with *B. gottheilii*, with stronger and elongated appearance. The formation of a strong C–O phenolic absorption band at 1014 cm^{-1} was also observed in the spectra. Furthermore, there was reduction in the C–H aliphatic stretching peaks at 2950 cm^{-1} – 2839 cm^{-1} and C–H aliphatic peaks at 1376 cm^{-1} in PP microplastic inoculated with the isolate. The reduction of the peak at 1455 cm^{-1} assignable to C=C aromatic stretching and the elongation of the absorption peaks at 998 cm^{-1} and 973 cm^{-1} attributed to =C–H bending was equally observed in the spectra of PP microplastic inoculated with *B. gottheilii*.

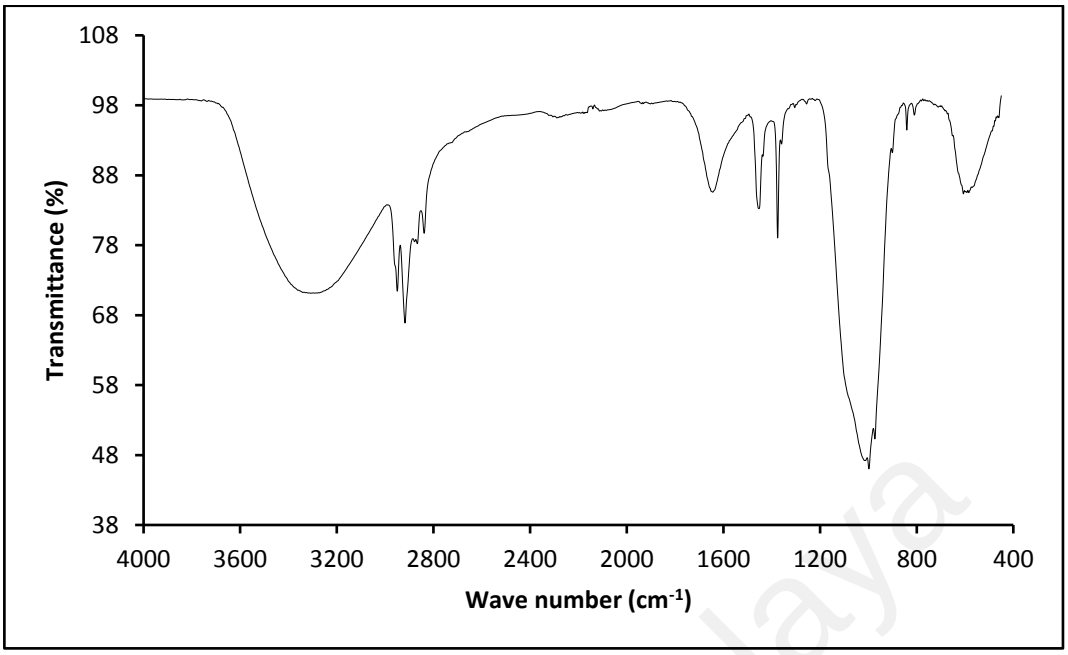

Figure 4.26: FTIR spectrum of PP microplastics inoculated with *B. gottheilii*

From these results, it is suggestive that substantial changes in the functional groups of PP microplastic test samples occurred after 40 days incubation with the selected microbial isolates. Formation of new groups such as hydroxyl and carbonyl were also observed by Iwamoto and Tokiwa (1994) and Alariqi *et al.* (2006) during the degradation of PP by microorganisms. Additionally, studies have suggested that monitoring the disappearance or formation of carbonyl peaks using FTIR is essential in understanding the mechanism of plastic polymer biodegradation process (Sheik *et al.*, 2015).

4.5.6 Scanning electron microscopy (SEM) analysis of PP microplastics inoculated with individual isolates

Figures 4.27 – 4.32 shows the SEM micrographs of uninoculated (control) and inoculated PP microplastics surfaces after 40 days incubation with microbes. The uninoculated (control) PP microplastic sample showed smooth surface with no defects (Figure 4.27). In addition, no special features were detected in the SEM micrograph of uninoculated PP microplastics.

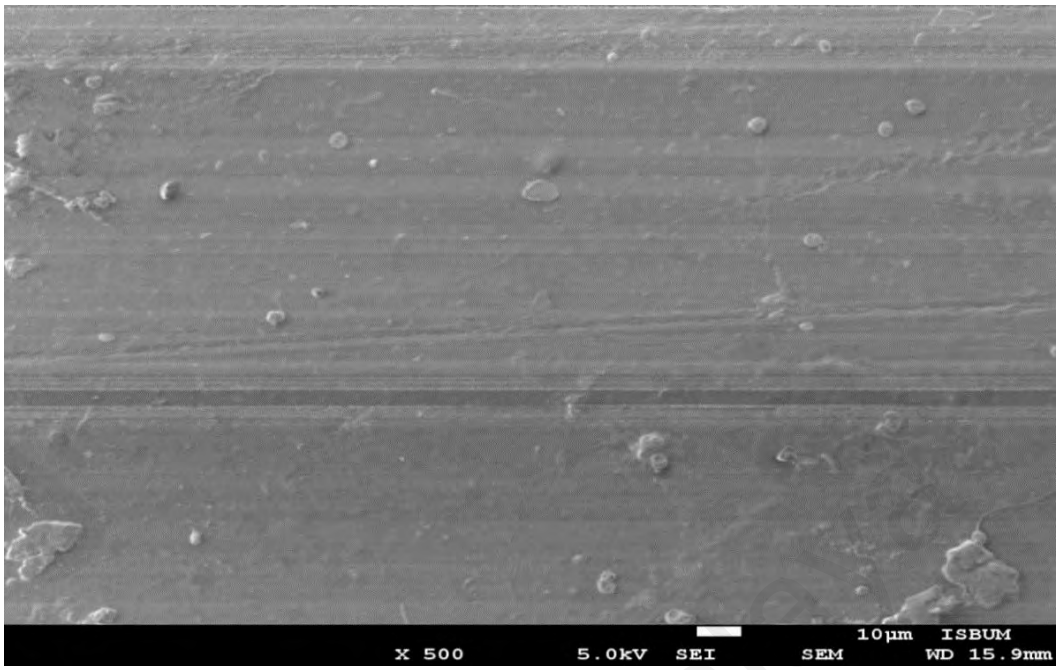

Figure 4.27: SEM micrograph of control (uninoculated) PP microplastics

However, after 40 days incubation with selected individual microbes roughening as well as the presence of bacteria colonies (biofilms) of the isolates on the surface of the PP microplastics was observed (Figure 4.28), which was considered to be as a result of the surface moistness (Zahra *et al.*, 2010). Such colonization and adhesion by the microbes are a fundamental prerequisite for the biodegradation of PP microplastics.

Surface erosion, formation of pores, cracks, pits and cavities on the surface of the microplastics could be observed (Figures 4.29-4.32). This might be due to the absence of uniform distribution of short branches or degradable products in the polymer matrix (Manzur *et al.*, 2004; Pandey & Singh, 2001), suggesting that the organism penetrated into the PP microplastics matrix during degradation. Furthermore, the microbes may have released extra-cellular metabolites and enzymes in response to stress that could have resulted in the deformation of the PP microplastics. Surface bio-erosion is known to be the primary cause of mass loss from polymer surfaces. As a cross reference to earlier studies on biodegradation, same morphological changes have been reported on PP polymer by microorganisms (Khoramnejadian, 2013; Pandey & Singh, 2001).

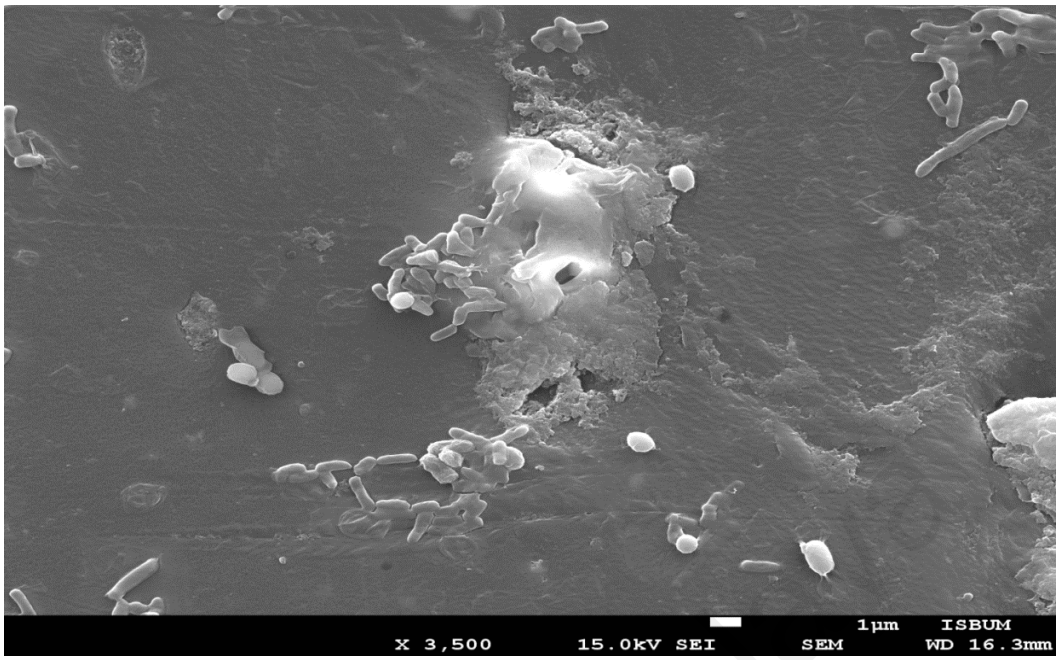

Figure 4.28: SEM micrograph of PP microplastics inoculated with *B. sonorensis*

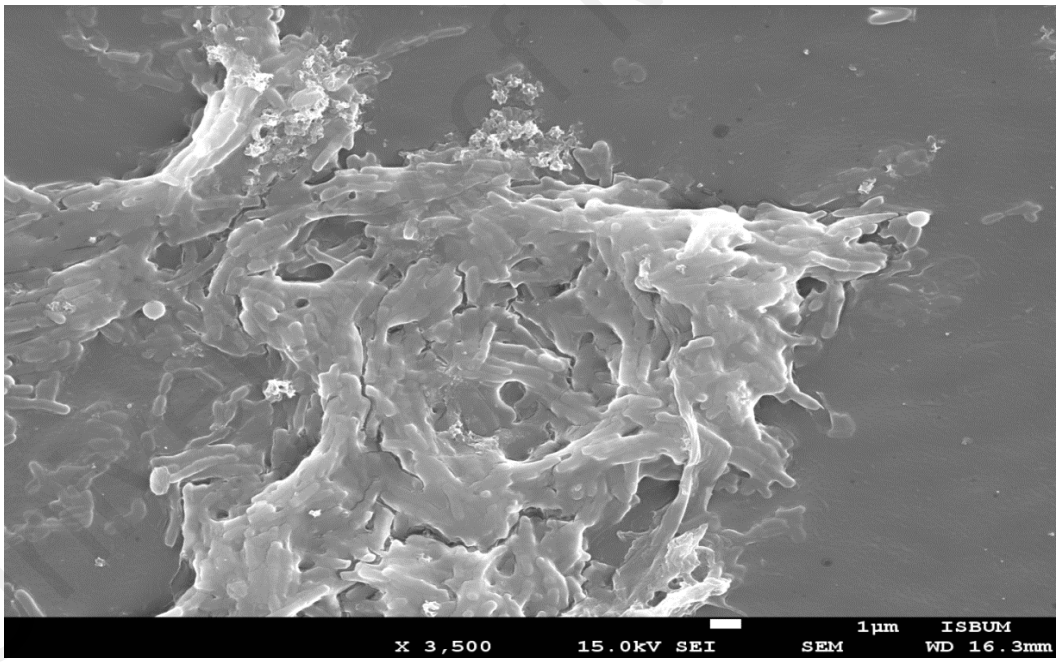

Figure 4.29: SEM micrograph of PP microplastics inoculated with *S. globispora*

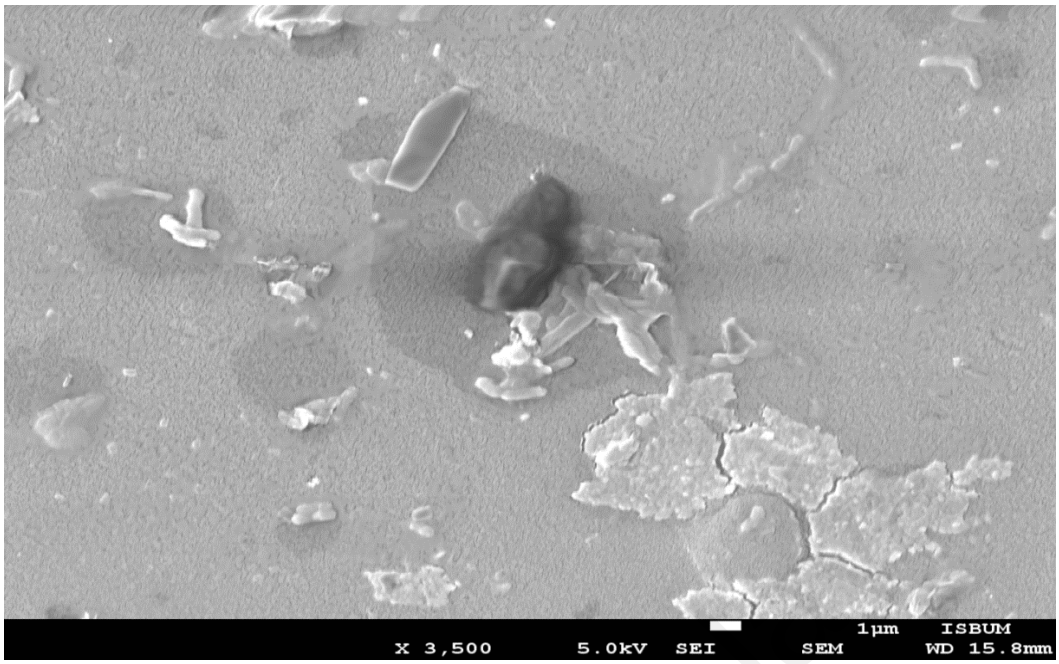

Figure 4.30: SEM micrograph of PP microplastics inoculated with *A. faecalis*

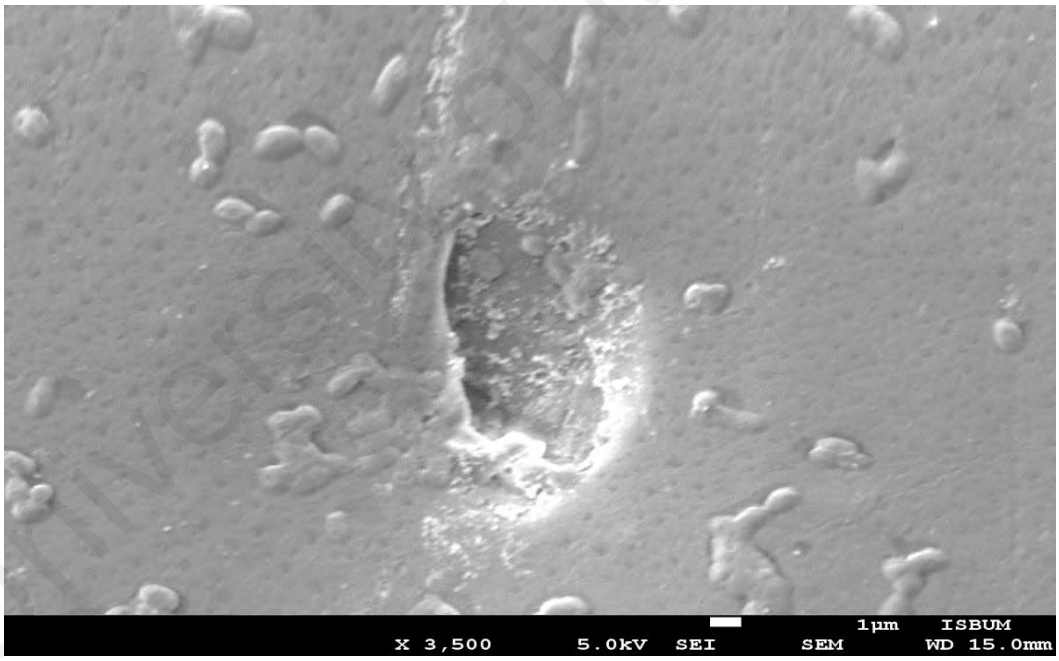

Figure 4.31: SEM micrograph of PP microplastics inoculated with *R. ruber*

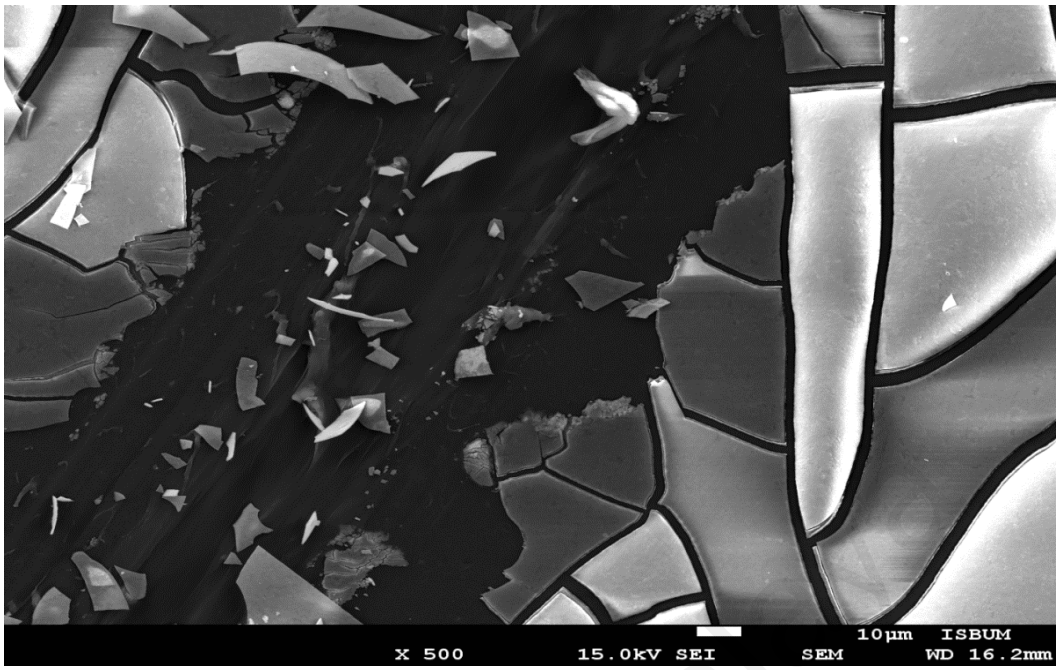

Figure 4.32: SEM micrograph of PP microplastics inoculated with *B. gottheilii*

4.6 Biodegradation of polyethylene terephthalate (PET) microplastics

Only B. cereus and B. gottheilii demonstrated the ability to degrade PET microplastics and were therefore, used for the present study.

4.6.1 Determination of weight loss of PET microplastics by individual isolates (*B. cereus* and *B. gottheilii*)

The residual weight and percentage weight loss of PET microplastics after 40 days incubation with *B. cereus* and *B. gottheilii* was calculated. The results for the weight loss are presented in Table 4.7. Highest mass reduction of PET microplastics after the 40 days incubation was recorded by *B. cereus* with a mass loss of 0.467 g and weight loss of 6.6%. while *B. gottheilii* recorded a mass reduction of 0.485 g and weight loss of 3.0% (F-value = 0.595; p = 0.484).

Table 4.7: Average mass of PET microplastics after 40 days incubation with individual isolates

Treatment	Initial weight (g)	Residual weight (g)	Weight loss (%)
Control	0.500	0.500	0
B. cereus	0.500	0.467	6.6
B. gottheilii	0.500	0.485	3.0

n = 3

No weight loss was observed in uninoculated (control) PET microplastics. It can therefore, be stated that the percentage weight loss of PET microplastics observed when inoculated with the isolates could have been as a result of biological process and not as a result of the chemicals in the BH medium. Similarly, Marqués-Calvo *et al.* (2006), in their study of the degradation of PET, recorded no weight loss in PET when subjected to hydrolytic degradation.

4.6.2 Growth pattern of *B. cereus* and *B. gottheilii* in PET microplastic-infused media

Figure 4.33 shows the growth profile of *B. cereus* and *B. gottheilii* in PET infused culture media during the 40 days biodegradation assay. The growth profile revealed that both isolates had ability to grow in BH media containing PET microplastics.

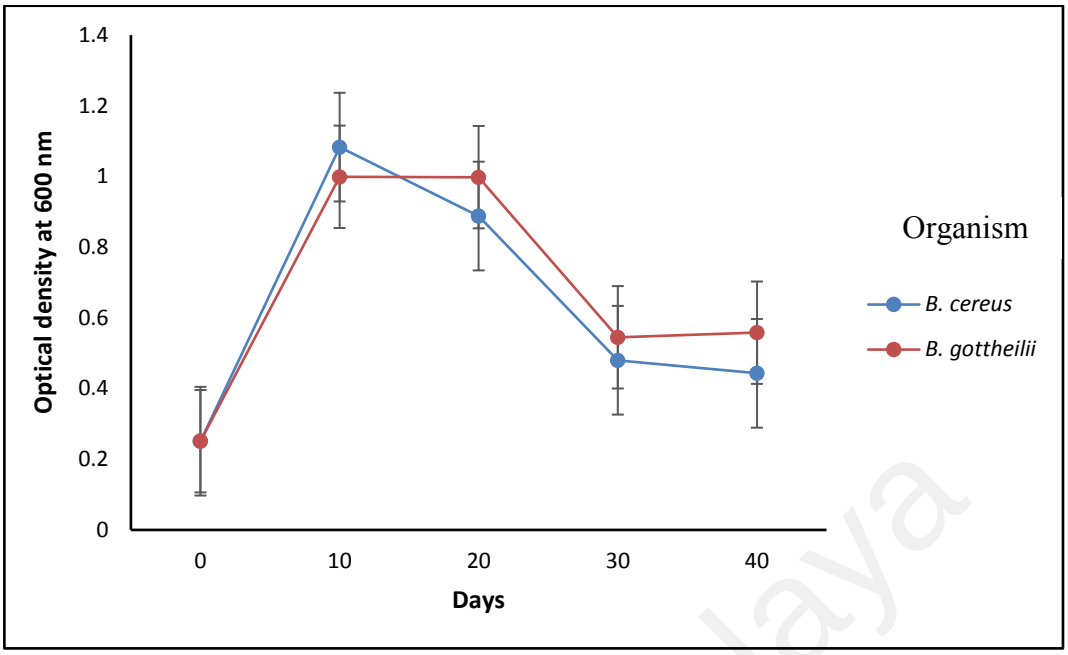

Figure 4.33: Growth profile of *B. cereus* and *B. gottheilii* in PET microplastic-infused media during biodegradation studies

A sudden decrease in transmittance, i.e. increase in absorbance, was observed after 10 days for both isolates. *B. cereus* recorded highest absorbance value (1.083 OD_{600}) while *B. gottheilii* recorded the least absorbance value (0.999 OD_{600}). This coincided with the logarithmic increase in the number of cells during the same period (Figure 4.34).

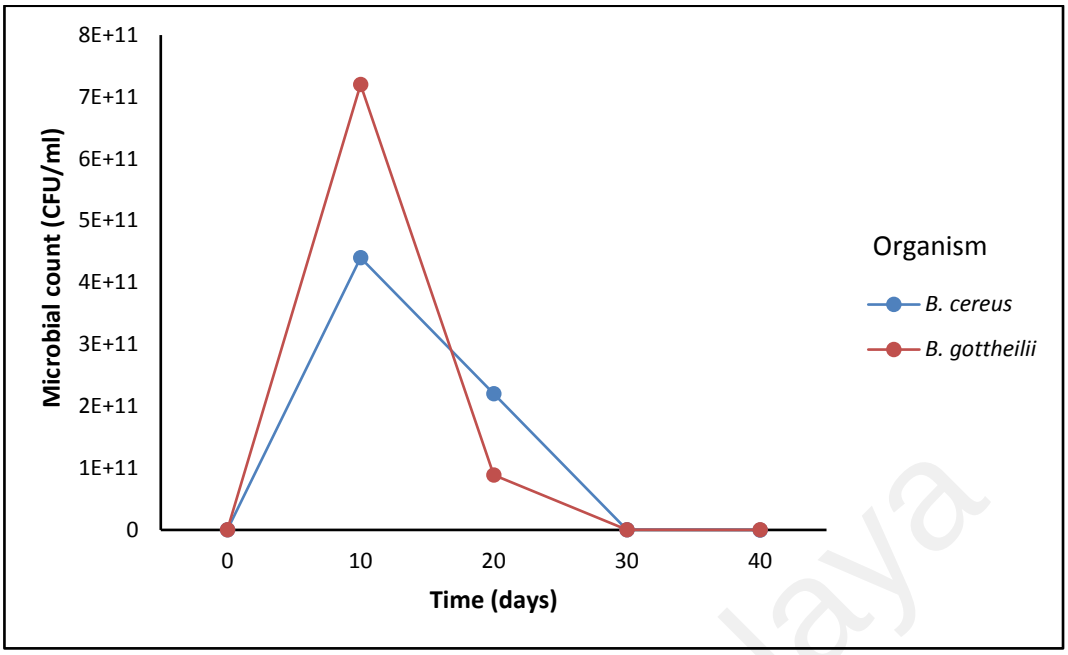

Figure 4.34: Bacterial counts of individual isolates inoculated in PET microplastic infused media during 40 days biodegradation. Bars indicate standard error (n =3)

This could be an indication that the conditions in the medium were favourable or that more PET microplastics were utilized which led to the increase in cell number during this period. Similar results were reported by Kavitha *et al.* (2014).

The growth of *B. cereus* was observed to have declined on the 20th day, with the isolate recording the least absorbance value of 0.888 OD_{600} while absorbance of *B. gottheilii* remained same (0.998 OD_{600}). The organism attained an almost stationery phase of growth on the 20th day. The stationary phase attained however, did not correspond with the increase in cell counts recorded on same day demonstrating that degradation metabolites formed in the media during biodegradation could have been responsible for the increased absorbance readings and not microbial growth. Continued decline in growth was observed on Day 30 (0.48 OD_{600} for *B. cereus* and 0.545 OD_{600} for *B. gottheilii*). On the 40th day however, the absorbance value of *B. gottheilii* increased (0.558 OD_{600}) while that of *B. cereus* experienced a continuous decrease with an absorbance value of 0.443 OD_{600}.

Both *B. cereus* and *B. gottheilii* inoculated in BH media infused with PET microplastics showed similar growth trend. The counts ranged between 3.8 x 108 to 4.4 x 10^{11} CFU/ml for *B. cereus*, with higher counts (4.4 x 10^{11}) recorded on Day 10. A gradual decrease in the cell counts were observed on Day 20 (2.2 x 10^{11} CFU/ml), after which an abrupt decrease in cell number occurred on Days 30 and 40 (1.0 x 10^{8} and 2.2 x 10^{7} CFU/ml, respectively). These counts were lower than the initial counts at the start of the experiment. *B. gottheilii* on the other hand, recorded cell counts that ranged between 3.8 x 10^{8} CFU/ml and 7.2 x 10^{11} CFU/ml, with maximum counts (7.2 x 10^{11} CFU/ml) recorded on Day 10. This high increase in cell number was accompanied by a decrease in cell counts on Day 20 (8.9 x 10^{10} CFU/ml). Further decrease in cell number continued on the 40th day that dropped below the initial value of cell counts. This might be that the cells began to die off due to stress associated to the biodegradation process. Highest counts for both isolates were recorded on Day 10. Even though *B. cereus* recorded the highest percentage weight loss of PET microplastic, the microbe recorded the least counts (4.4 x 10^{11} CFU/ml) as against the highest, 7.2 x 10^{11} CFU/ml recorded by *B. gottheilii*. However, increase in the counts of *B. gottheilii* did not indicate higher weight loss of PET microplastic by the microbe, rather, it might be that the environment might have been made favourable for cell multiplication and growth. This may indicate that the environment could have favoured the growth of *B. gottheilii* and not its ability to degrade the PET microplastic.

4.6.3 pH changes of PET microplastic-infused mineral salt media during biodegradation studies with individual isolates

Figure 4.35 shows the pH changes that occurred in PET microplastic-infused culture media at the 40 days biodegradation period. Increase from neutral pH to alkalinity was depicted by both *B. cereus* and *B. gottheilii*. Highest counts of the isolates were recorded

on Day 10 when it reached pH 8.59 and pH 8.46 for *B. cereus* and *B gottheilii*, respectively. This depicted the optimal pH for the growth of the isolates as further increase in pH observed on Days 30 and 40 led to the decline in growth of the microbes. Similar pH was recorded by Ruiz e*t al*. (2011) who reported pH 8.5 to be the optimal value for polyurethane degrading enzyme from *P. chlororaphis*.

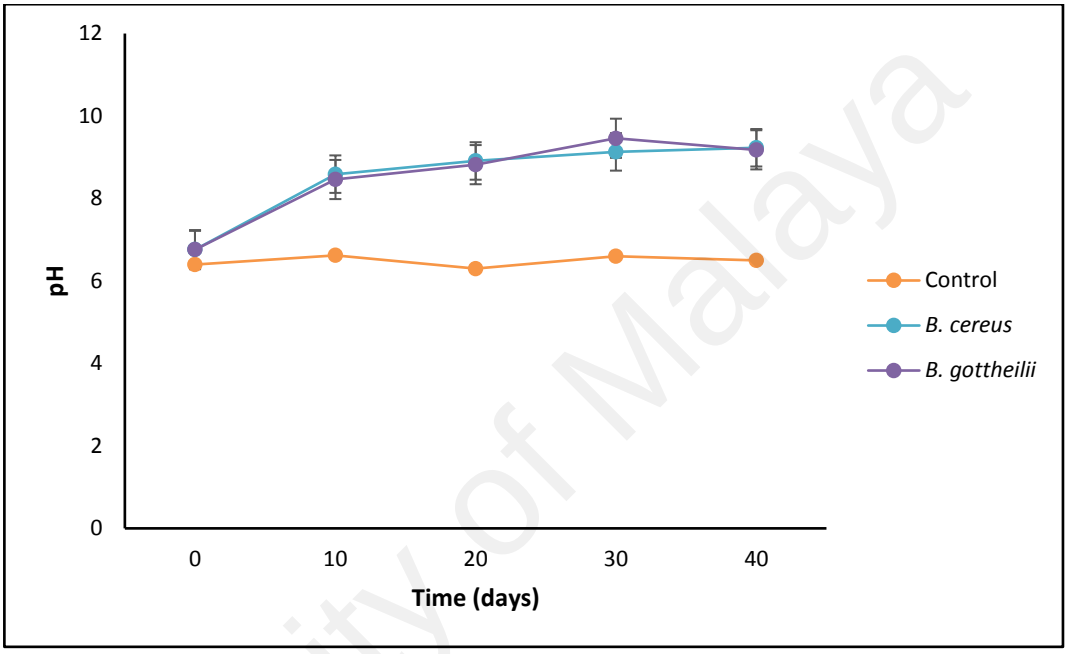

Figure 4.35: pH changes of PET microplastic-infused media inoculated with individual organisms during biodegradation studies. Each data point represents the average of three replicates ± SD

4.6.4 Biodegradation rate constant and half-life of PET microplastics inoculated with individual isolates

Table 4.8 shows the rate of PET microplastic uptake by *B. cereus* and *B. gottheilii* within the 40 days period. The rate of uptake of PET microplastics by *B. cereus* at the end of the 40-day period of biodegradation studies was 0.0017 day^{-1} with a half-life of approximately 405.88 days. *B. gottheilii* on the other hand, recorded an uptake rate of 0.00076 day^{-1} and a half-life of 907.89 days. The rate of PET microplastic removal by *B. cereus* was relatively higher than the uptake rate recorded by *B. gottheilii*. The reason for

the higher rate of PET uptake by *B. cereus* may be due to the physiological make-up of the microbe and its intrinsic ability to reduce PET microplastic polymer.

Table 4.8: Removal rate constant (*k*) and half-life of PET microplastics across individual isolates

Treatment	Removal constant (k) (day^{-1})	Half-life ($t_{1/2}$) (days)
Control	0	∞
B. cereus	0.0017	405.88
B. gottheilii	0.00076	907.89

n = 3

4.6.5 Fourier transform infrared (FTIR) spectroscopy analysis of PET microplastics inoculated with *B. cereus* and *B. gottheilii*

Figure 4.36 shows the FTIR spectra of uninoculated PET microplastics after the 40 days experimental period.

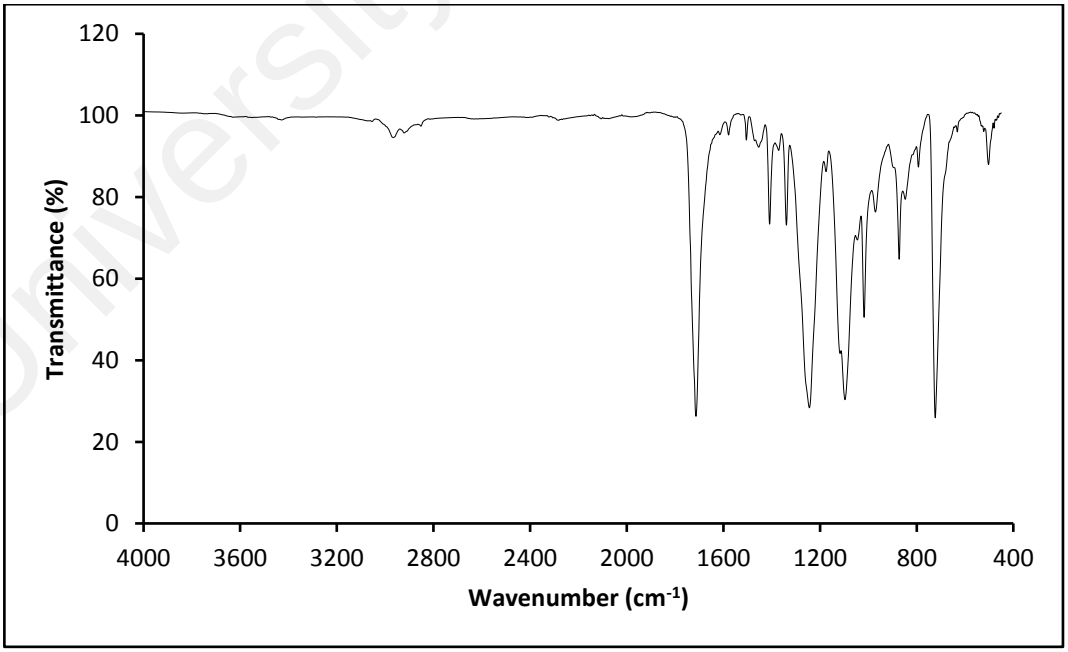

Figure 4.36: FTIR spectrum of control (uninoculated) PET microplastic

The aliphatic stretching peak at 2966 cm^{-1}, C=O carbonyl band at 1714 cm^{-1} (ketone), C=C aromatic stretching at 1409 cm^{-1}, C–H aliphatic bending at 1338 cm^{-1}, C–O phenolic aromatic at 1247 cm^{-1}, C – O phenolic aliphatic at 1097 – 1018 cm^{-1}, and C=C bending at 873 – 848 cm^{-1}. Other peaks at 722 cm^{-1}, 522 cm^{-1}, 503 cm^{-1} and 480 cm^{-1} were observed in spectra of uninoculated control PET microplastics.

All of these peaks were also present in spectra of PET microplastic inoculated with *B. cereus* but were more elongated (Figure 4.37). This could have occurred probably due to oxygen build up in the aliphatic chains and the degradation of PET microplastics. The C–H aliphatic stretching peaks at 2968 cm^{-1} and 2908 cm^{-1} in PET microplastic inoculated with the microbe became elongated. Also observed in the spectra was the disappearance of the peak at 1046 cm^{-1} assignable to the phenolic C–O band. respectively.

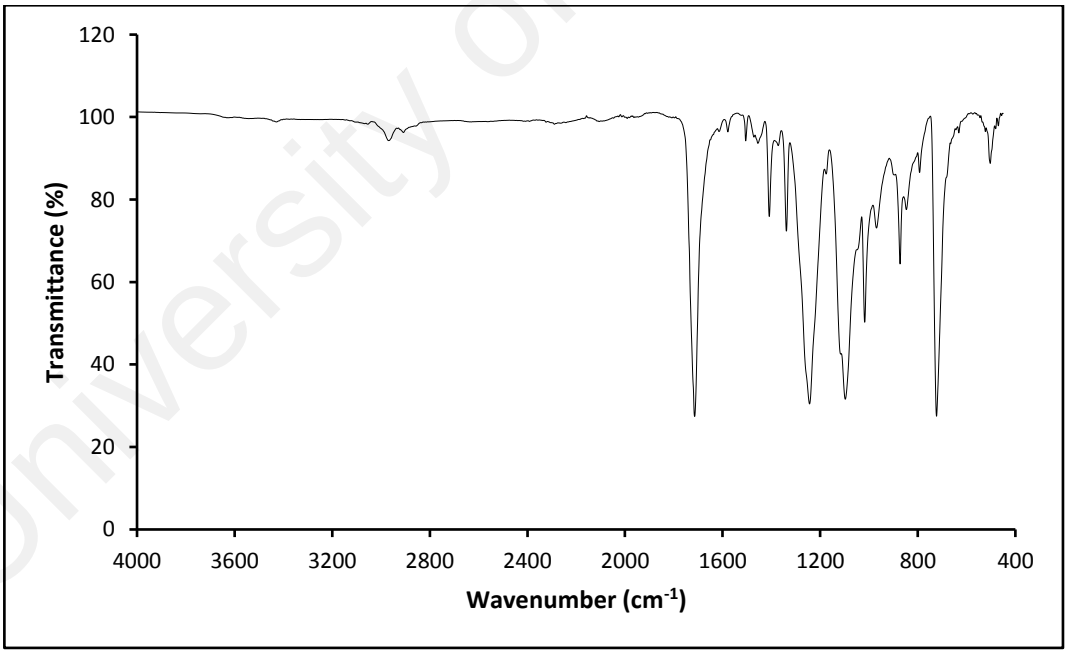

Figure 4.37: FTIR spectrum of PET microplastic inoculated with *B. cereus*

Furthermore, there was a shift of the peak at 470 cm^{-1} in spectra of uninoculated PET microplastic to 480 cm^{-1} in spectra of PET microplastic inoculated with *B. cereus*, and the elongation of the same peak. These expressed the action of biodegradation of the

microbe on PET microplastic. Similar changes in peaks were observed by Ioakeimidis *et al.* (2016) and Nowak *et al.* (2011) in the spectra of PET bottles degraded in marine environments and PET modified with "Bionolle(e)" polyester, respectively.

The FTIR spectra of PET microplastic inoculated with *B. gottheilii* demonstrated the disappearance of the peak at 1046 cm^{-1} assignable to the phenolic C–O band in uninoculated PET microplastic was observed in spectra of PET microplastic inoculated with the isolate (Figure 4.38). It can therefore, be deduced that *B. cereus* and *B. gottheilii* were able to induce chemical-structural changes in the PET microplastics.

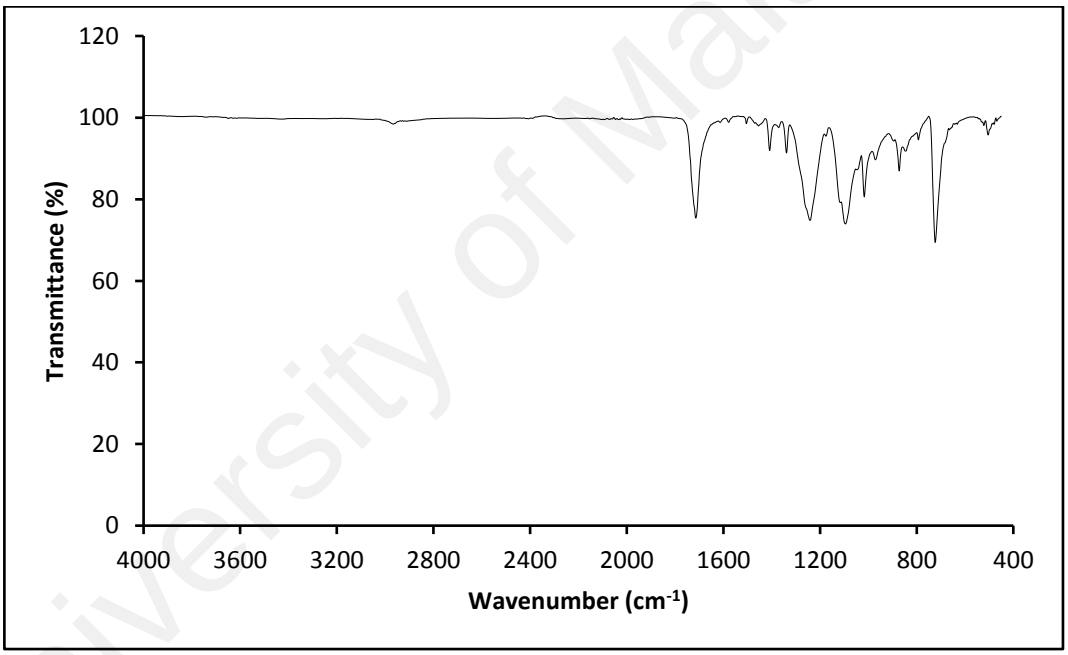

Figure 4.38: FTIR spectrum of PET microplastic inoculated with *B. gottheilii*

4.6.6 Scanning electron microscopy (SEM) analysis of PET microplastics inoculated with *B. cereus* and *B. gottheilii*

Degradation impact by *B. cereus* and *B. gottheilii* on the structure of PET microplastics were observed by scanning electron microscopy (SEM) micrographs. The surface of the uninoculated PET microplastics remained smooth, with no distortions observed (Figure 4.39).

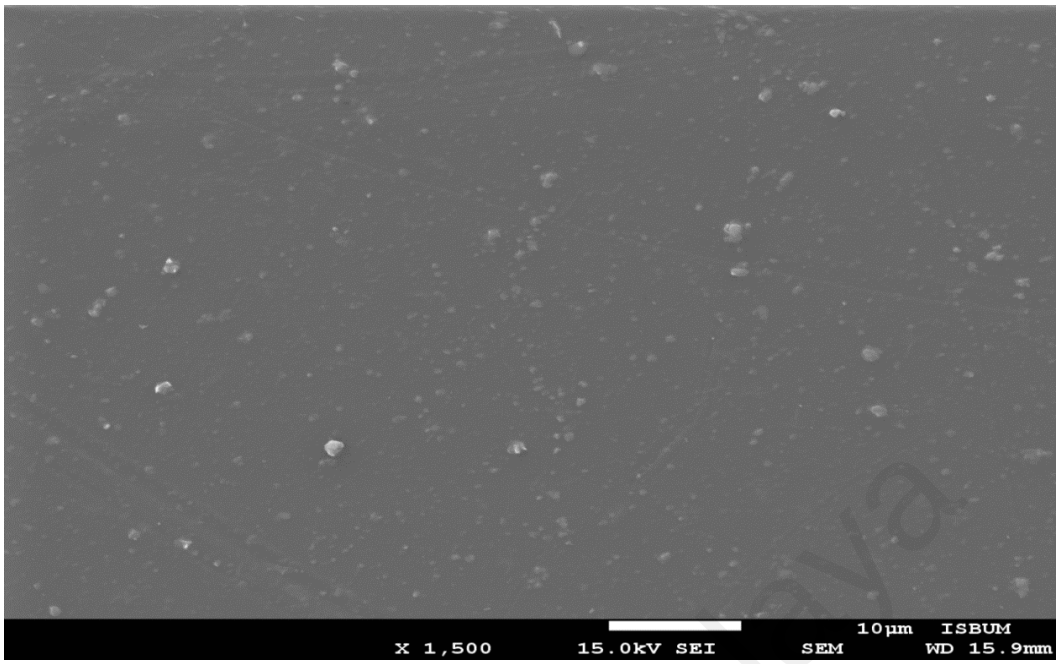

Figure 4.39: SEM micrograph of control (uninoculated) PET microplastic

On treatment with *B. cereus*, the development of cracks, grooves, and distortions on the surface of PET microplastics were observed (Figure 4.40). Also observed were the presence of abundant colonies of the isolate scattered on the microplastic surface, and the roughening of the surface of the PET microplastics.

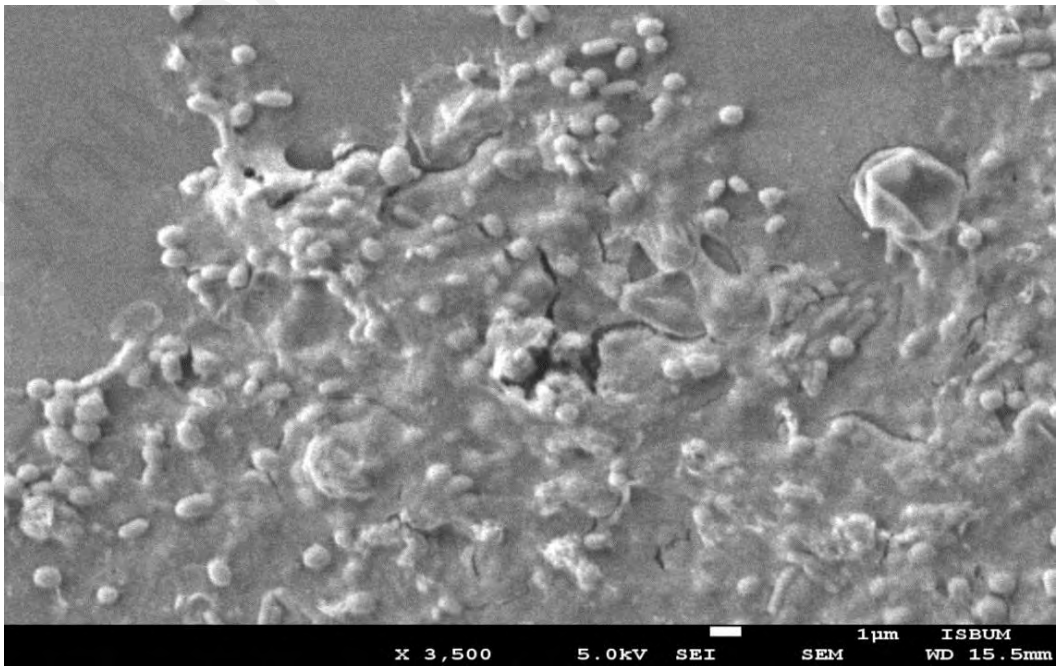

Figure 4.40: SEM micrograph of PET microplastics inoculated with *B. cereus*

The SEM micrograph of PET treated with *B. gottheilii* demonstrated roughening and distortion of the surface of PET microplastics (Figure 4.41).

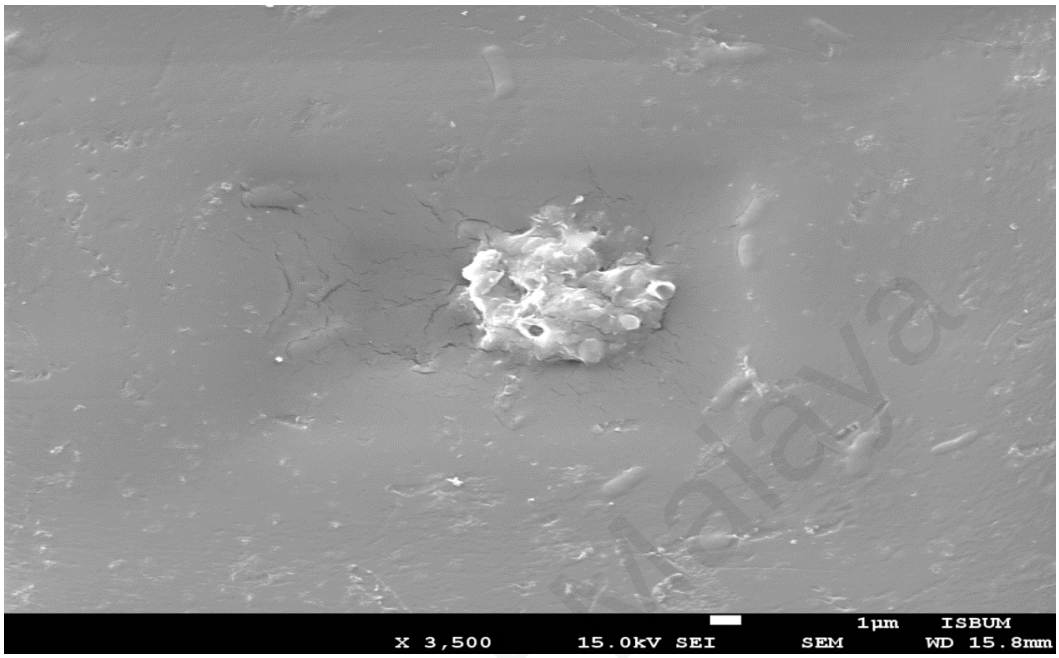

Figure 4.41: SEM micrograph of PET microplastics inoculated with *B. gottheilii*

The cracks and holes demonstrated might have resulted due to the adherence of the microbes on the surface of the microplastics. The microbes could have secreted extracellular polymeric substances that possibly entered the pores, allowing the microbes to grow inside which increased the sizes of the pores and provoked cracks that weakened the physical properties of the microplastics (Bonhomme *et al.*, 2003).

4.7 Degradation of polystyrene (PS) microplastics

4.7.1 Determination of percentage weight loss of PS microplastics by individual isolates (*B. cereus* and *B. gottheilii*)

Table 4.9 shows the weight loss of PS microplastics inoculated with *B. cereus* and *B. gottheilii*. The results show that *B. cereus* recorded 0.463 g reduction in mass whereas, *B. gottheilii* recorded a weight reduction of 0.471 g after 40 days of biodegradation period.

The percentage weight reduction was 7.4% for PS microplastic inoculated with *B. cereus* and 5.8% for PS microplastic inoculated with *B. gottheilii*. No weight loss was observed in the uninoculated PS microplastic.

Table 4.9: Average mass of PS microplastic after 40 days incubation with individual isolates

Treatment	Initial weight (g)	Residual weight (g)	Weight loss (%)
Control	0.500	0.500	0
B. cereus	0.500	0.463	7.4
B. gottheilii	0.500	0.471	5.8

n = 3

The results demonstrated greater degradability of *B. cereus* than that of *B. gottheilii*. This indicated that *B. cereus* was capable of degrading PS microplastics more efficiently. Studies have demonstrated the weight loss of PS after 28 days by *Pseudomonas aeruginosa* to range between 5.7% - 25%. (Shimpi *et al.*, 2012) and the weight loss of PS film by *R. ruber* to be 0.8% (Sekhar *et al.*, 2016).

4.7.2 Growth curve of *B. cereus* and *B. gottheilii* in PS microplastic-infused media

Figure 4.42 presents the growth profile of *B. cereus* and *B. gottheilii* on exposure to PS microplastics during the 40 days incubation period.

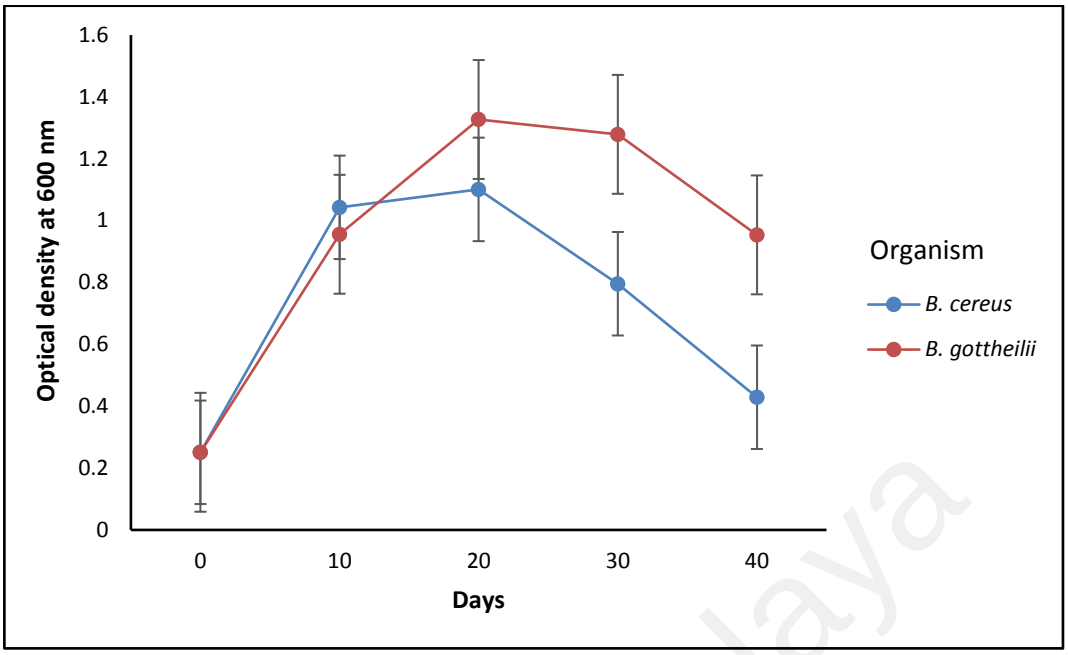

Figure 4.42: Growth profile of *B. cereus* and *B. gottheilii* in PS microplastic-infused media during biodegradation studies

B. cereus recorded an exponential increase in growth on Day 10 (0.251 OD_{600} – 1.043 OD_{600}) with maximum growth was attained on Day 20 (1.101 OD_{600}). Growth of the isolate dropped after the 20th day, and further decline in growth was observed on the Days 30 and 40, depicting decline phase. Same pattern of growth was demonstrated by *B. gottheilii.* The microbe attained an exponential growth phase that reached a peak on the 20th day (1.327 OD_{600}). This was followed by a gradual decrease in growth of the isolate on the 30th day (1.279 OD_{600}). Growth of the microbe continued to decline up to the 40th day (0.954 OD_{600}). The decline phases attained by the isolates might be due to lysis of the cells. Similar growth pattern was also recorded by Shimpi *et al.* (2012) in their study of the biodegradation of polystyrene and polylactic acid by *Pseudomonas aeruginosa*. Overall assessment showed significant difference in the growth of *B. cereus* and *B. gottheilii* across the monitoring days with p-values ranging from 0.000 – 0.012 ($p < 0.05$).

Figure 4.43 shows a comparison of the counts of bacteria during the monitored days of the biodegradation studies.

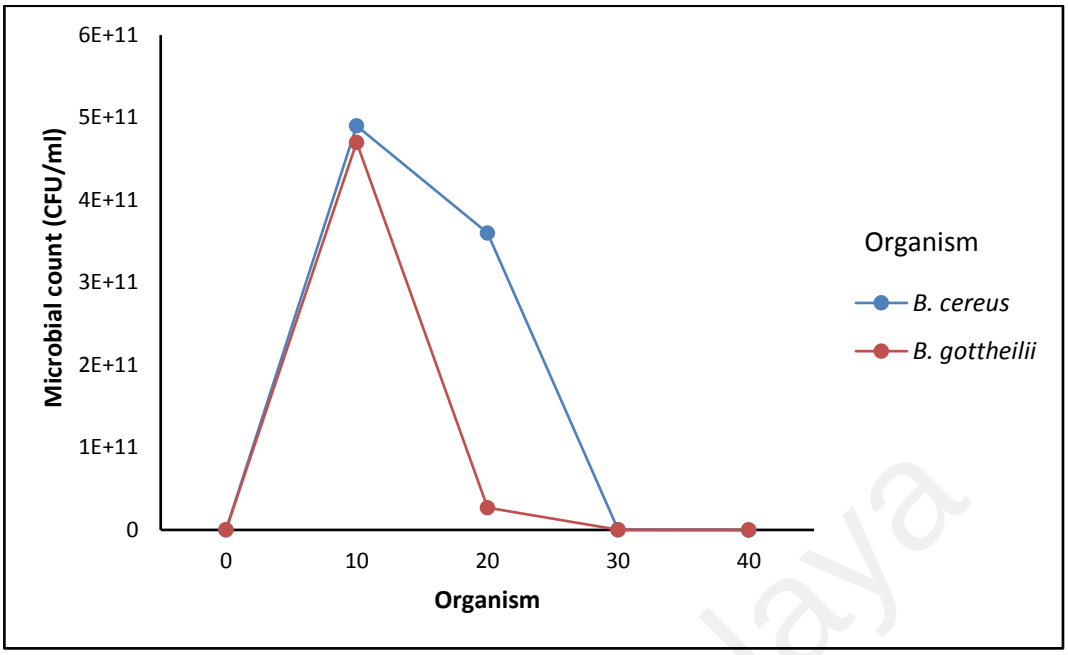

Figure 4.43: Bacterial counts of isolates inoculated in PS microplastic-infused media during 40 days biodegradation. Bars indicate standard error (n =3)

Counts for *B. cereus* on Day 0 and those on Day 10 ranged between 3.8 x 10^8 CFU/ml to 4.9 x 10^{11} CFU/ml. Highest growth was recorded at this period while the counts for *B. gottheilii* ranged between 3.8 x 10^8 CFU/ml to 4.7 x 10^{11} CFU/ml. This indicate that the organisms could have been utilizing the PS microplastics and this resulted in the fast division of the cells in the PS microplastic-infused media. However, the 20th day of biomonitoring witnessed the decline in bacterial cell counts (3.6 x 10^{11} CFU/ml and 2.7 x 10^{10} CFU/ml for *B. cereus* and *B. gottheilii*, respectively). This may imply depletion in available nutrient required for the survival of the microbes as confirmed by Lin *et al.* (2010). The reduction was very drastic (2.7 x 10^{10} CFU/ml) for *B. gottheilii* but somewhat gradual (3.6 x 10^{11} CFU/ml) for *B. cereus*. This highlighted that *B. cereus* had more tolerance to PS microplastics than *B. gottheilii*. Further reduction in cell counts was observed after 30 days (3.8 x 10^8 CFU/ml and 2.6 x 10^8 CFU/ml for *B. cereus* and *B. gottheilii*, respectively) while the counts of *B. cereus* reached the value of the initial counts.

Day 40 witnessed decrease in cell counts for both microbes that went lower than the counts recorded at the start of the experiment (1.3 x 10^7 and 4.5 x 10^7 CFU/ml for *B. cereus* and *B. gottheilii*, respectively). This reduction in cell counts is likely due to the rapidly diminishing nutrient supply or the isolates may have undergone stressed conditions that led to the inhibition of cell growth and mortality. These stresses could be due to the metabolic process required for the cleavage of PS microplastic. However, the higher counts recorded by *B. cereus* on Days 10 to 30 may be a reflection of higher tolerance of the microbe which might have added to the organism's ability to degrade higher percentage of PS microplastics and even higher removal rates.

4.7.3 pH changes of PS microplastic-infused mineral salt media during biodegradation studies with individual isolates

The changes in pH of PS microplastic infused BH media inoculated with *B. cereus* and *B. gottheilii* are shown in Figure 4.44. Both organisms recorded similar pH changes during biodegradation assay which varied from neutral to alkaline. This is in line with the findings of Zahra *et al.* (2010). The increase in pH to alkaline level could have been due to the ammonification of nitrogen components in the culture media (Zahra *et al.*, 2010).

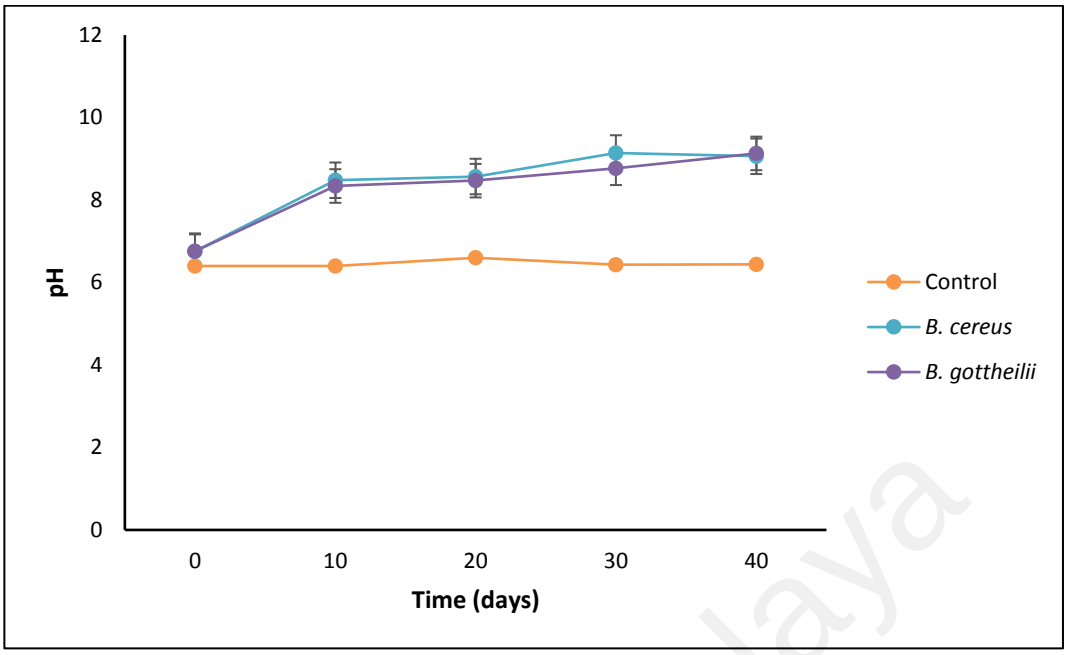

Figure 4.44: pH changes of PS microplastic-infused media inoculated with individual organisms during biodegradation studies. Each data point represents the average of three replicates ± SD

4.7.4 Biodegradation rate constant and half-life of PS microplastics inoculated with *B. cereus* and *B. gottheilii*

The removal rate constants per day and half-life of PS microplastics inoculated with *B. cereus* and *B. gottheilii* were generated using the first-order kinetic model. This was to show the estimated daily PS microplastic degradability by the microbes. Table 4.10 revealed that the removal rate constant of PS microplastic inoculated with *B. cereus* within the 40 days of the study was higher than the removal rate by *B. gottheilii*. This implies that optimal removal rate for the studied microplastic was achieved with *B. cereus*. Reason for the higher reduction observed may be linked to a very specific but complex interaction. It could also be that the microbe was able to impact the important absorbance peaks of PS microplastics as observed in the FTIR spectra of the isolate.

Table 4.10: Removal rate constant (*k*) and half-life of PS microplastics across individual isolates

Treatment	Removal constant (*k*) (day^{-1})	Half-life ($t_{1/2}$) (days)
PS control	0	∞
B. cereus	0.002	363.157
B. gottheilii	0.0014	460

n = 3

In general, it is evident that PS microplastics inoculated with microbes was more efficiently biodegraded as compared to the uninoculated negative control. However, the isolates differentially reduced the PS microplastics. *B. cereus* removed 0.002 day^{-1} and recorded a half-life of 363.16 days while *B. gottheilii* removed 0.0014 day^{-1} and recorded a half-life of 460 days.

4.7.5 Fourier transform infrared (FTIR) spectroscopy analysis of PS microplastics inoculated with *B. cereus* and *B. gottheilii*

The FTIR spectrum of the uninoculated PS microplastics after 40 days experimental period is presented in Figure 4.45.

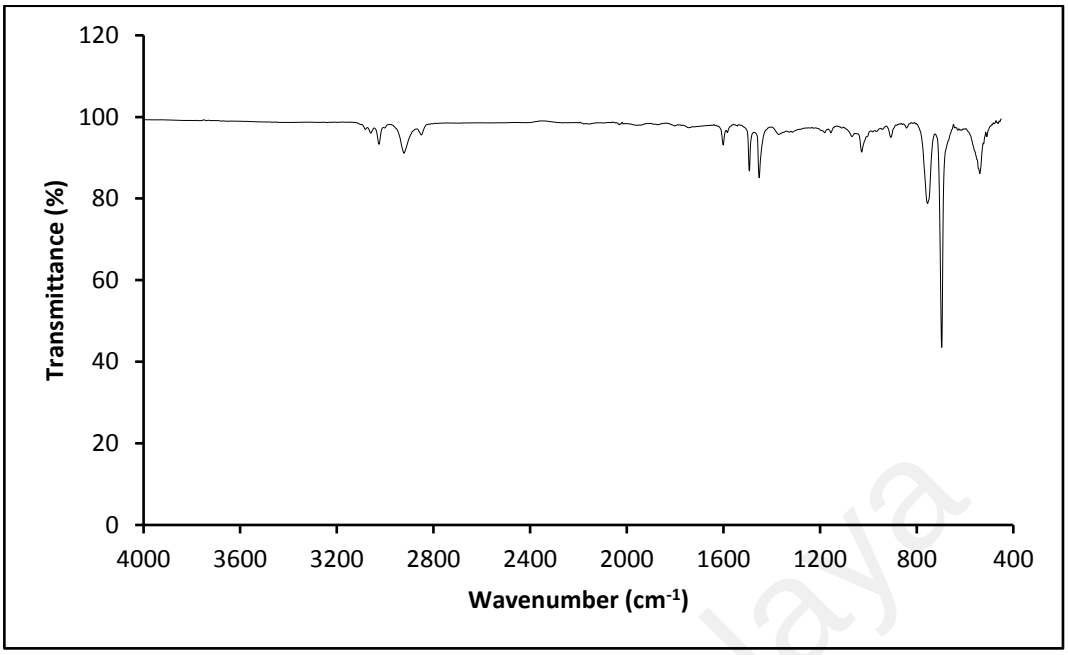

Figure 4.45: FTIR spectrum of control (uninoculated) PS microplastic

Evident elongations and reductions were observed in almost all the peaks in spectra of PS microplastics inoculated with *B. cereus* and *B. gottheilii*, respectively. The elongations observed in spectra of PS microplastic inoculated with *B. cereus* (Figure 4.46), when compared with the peaks present in the spectra of uninoculated PS microplastics are C–H (aromatic) peaks at 3082 – 3026 cm^{-1}, C–H (aliphatic) peaks at 2922 – 2850 cm^{-1}, C=C (aromatic) peaks at 1601 cm^{-1} and 1492 – 1452 cm^{-1}, the phenolic band, C–O at 1027 cm^{-1}, C–H (aliphatic bending) peaks at 1370 cm^{-1}. Allen *et al.* (2004) had reported that the important absorbance peaks of PS are CH_2 asymmetric and symmetric stretching around 2924 cm^{-1} and 2852 cm^{-1}, 3026 cm^{-1} (aromatic C–H stretches), 756 cm^{-1} (out-of-plane C–H bending mode of aromatic ring), 698 cm^{-1} (ring bending vibration) and 1600 cm^{-1} and 1491 cm^{-1} (benzene ring).

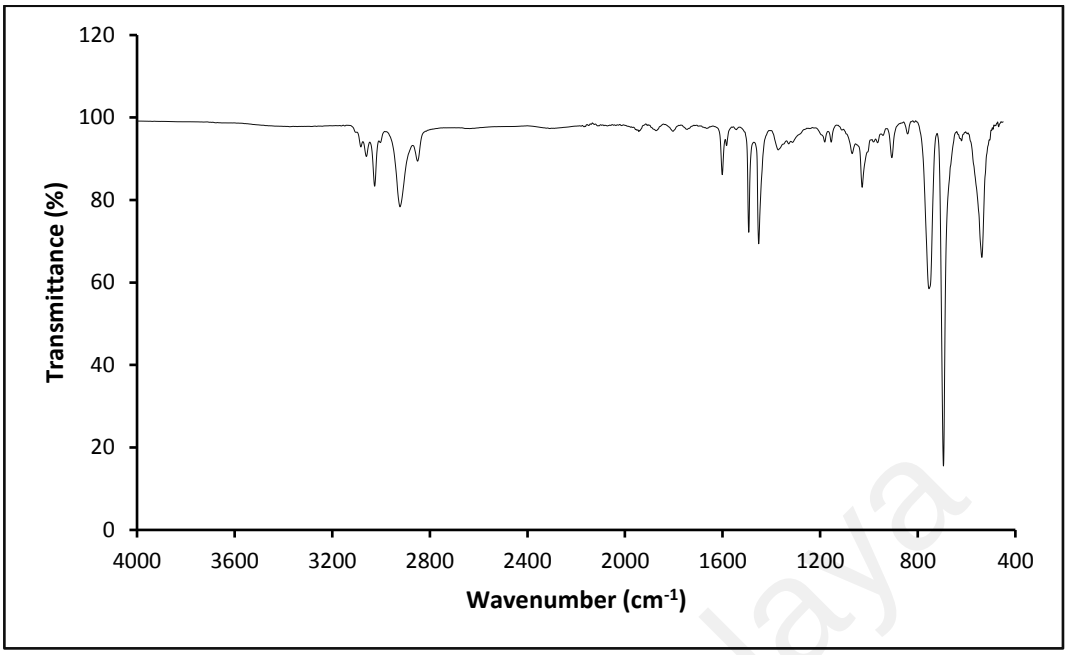

Figure 4.46: FTIR spectrum of PS film inoculated with *B. cereus*

For PS microplastic inoculated with *B. gottheilii* (Figure 4.47), reduction of the sizes of the peaks were observed in the spectra. The elongations and reduction in peaks occurred as a result of microbial degradation. Similar elongation and reduction in peaks were reported by Shimpi *et al.* (2012) and Mohan *et al.* (2016).

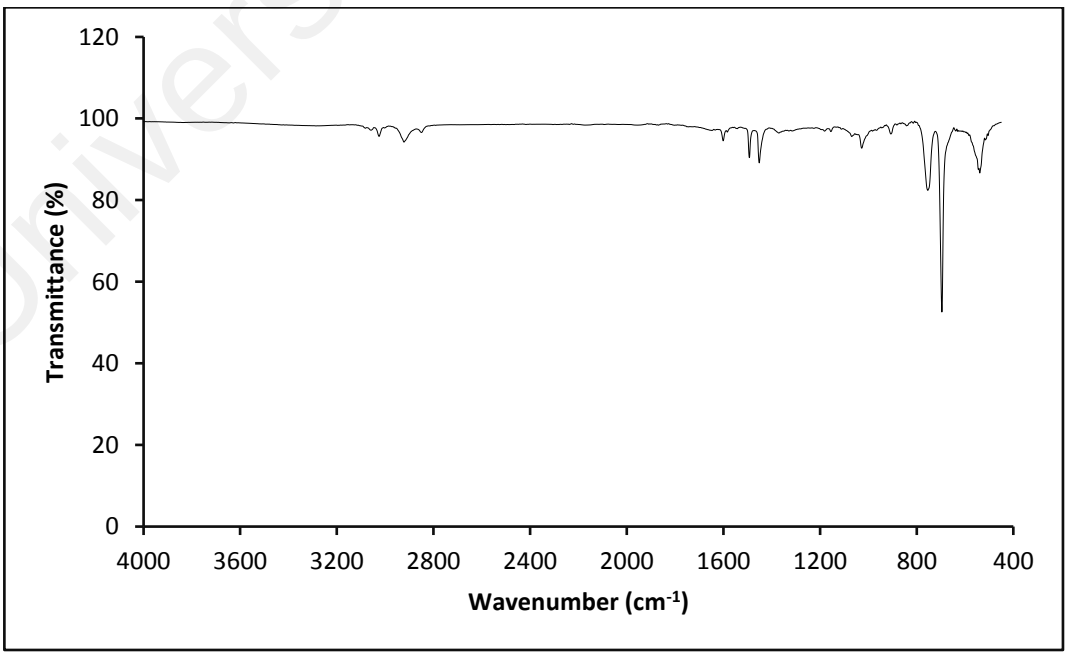

Figure 4.47: FTIR spectrum of PS film inoculated with *B. gottheilii*

4.7.6 Scanning electron microscopy (SEM) analysis of PS microplastics inoculated with *B. cereus* and *B. gottheilii*

The SEM micrographs indicated that PS microplastics before biodegradation (Figure 4.48) had no characteristic defects.

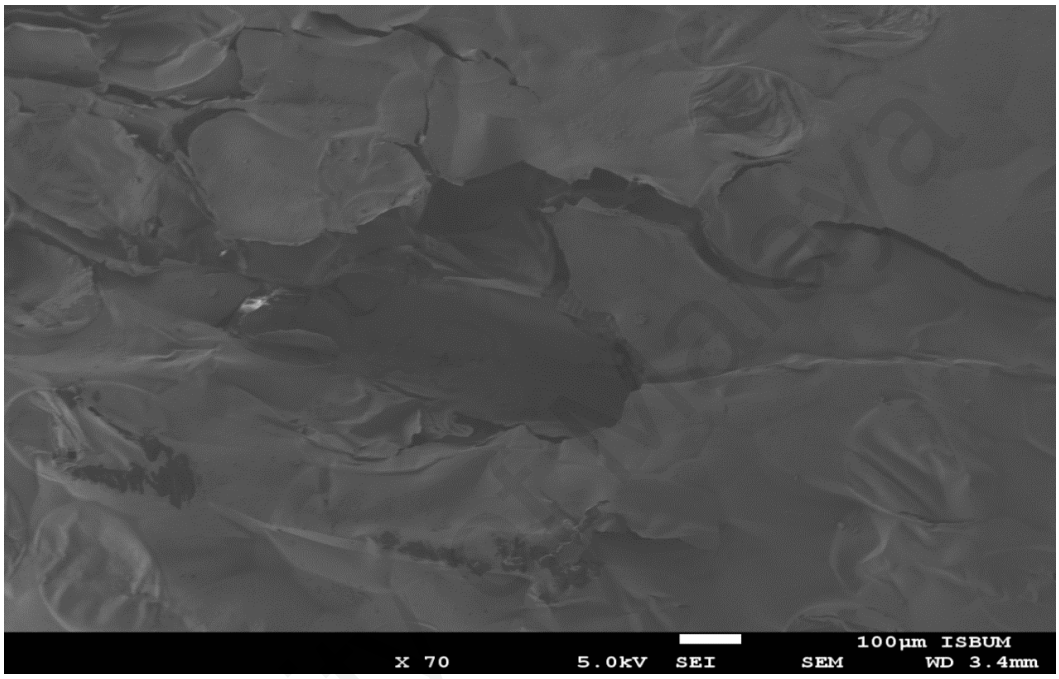

Figure 4.48: SEM micrograph of control (uninoculated) PS microplastic

However, after 40 days of incubation, with *B. cereus* and *B. gottheilii*, the surfaces of the PS microplastics became rough with numerous pores and grooves (Figures 4.49 and 4.50) which provide evidence for the deterioration of the microplastics due to the action of the microbial PS degrading enzymes secreted by the microbes. Similar biodegradation studies made use of SEM micrographs as a tool to confirm degradation and indicated grooves, pores and cavities formed on PS as evidence of induced impact of microbial degradation (Mohan *et al.*, 2016; Sekhar *et al.*, 2016; Shimpi *et al.*, 2012).

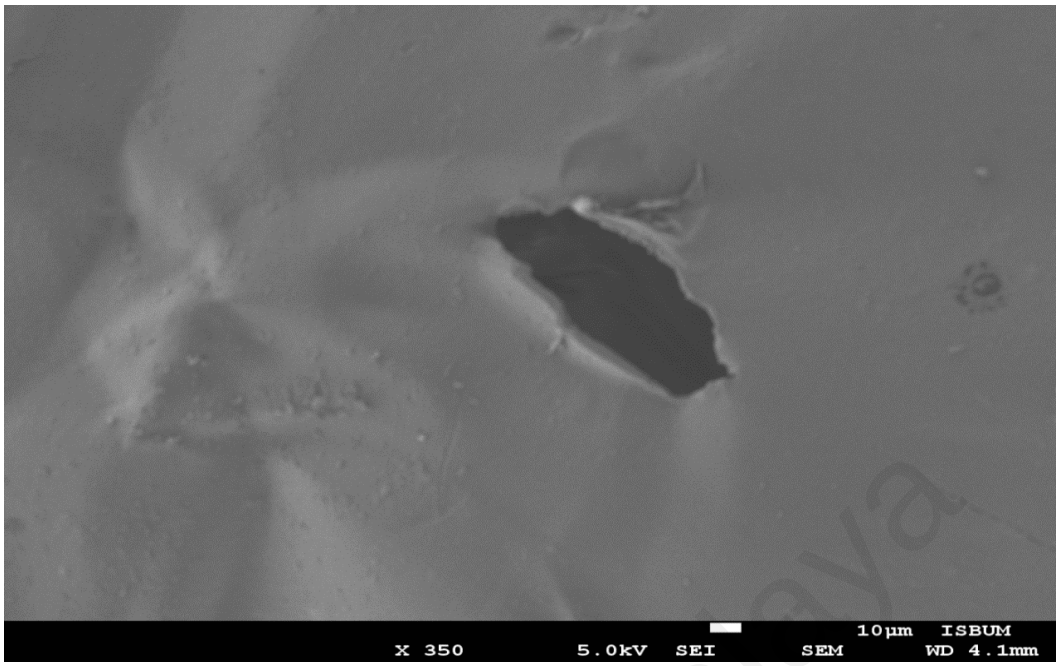

Figure 4.49: SEM micrograph of PS microplastics inoculated with *B. cereus*

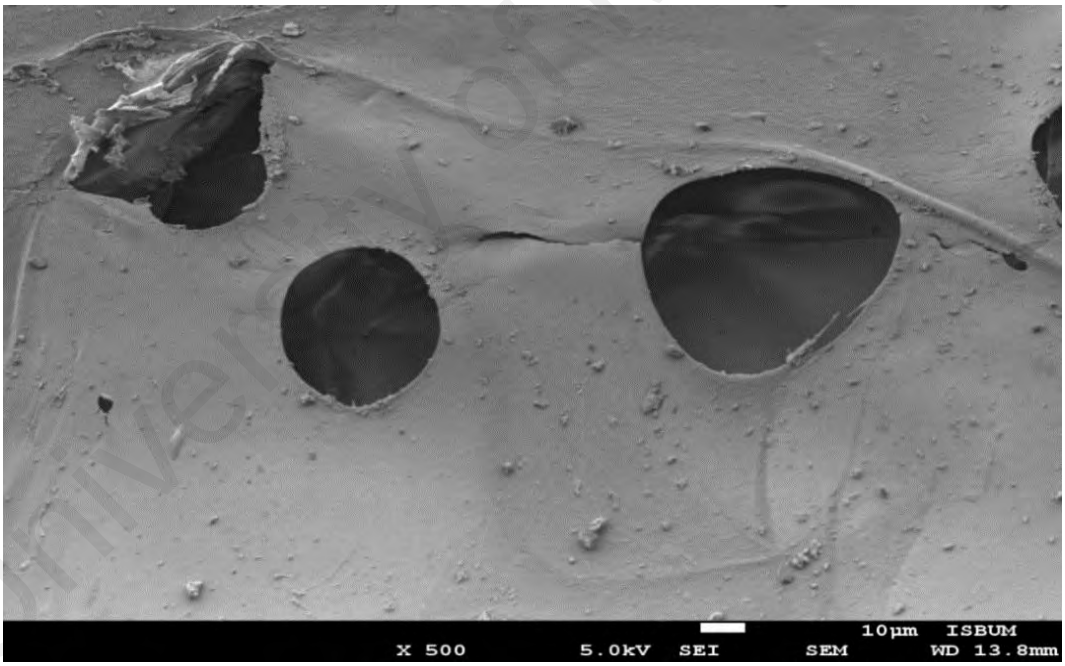

Figure 4.50: SEM photograph of PS microplastics inoculated with *B. gottheilii*

Generally, the results showed that among all nine isolates, the degradation of PE, PP, PET and PS microplastics varied among the isolates, but *B. gottheilii* was the only microbe that had the potential to degrade all four different microplastic types. Highest weight loss of PE microplastic was recorded by *B. gottheilii* (6.2%), highest weight loss

of PP microplastic loss (6.4%) was recorded on treatment with *R. ruber* whereas, highest weight loss of PET and PS were on treatment with *B. cereus* (6.6% and 7.4%, respectively).

Engineering microbes into consortia is expected to display increased metabolic potential for microplastic degradation as interactions of multiple microbial populations have the ability to perform tasks that are more complicated, difficult or even impossible for individual populations and can endure more changeable environments. Members of a consortium communicate with each other via trading of metabolites or through exchange of dedicated molecular signals (Brune & Bayer, 2012). Studies have demonstrated that the interaction that occur between different microbes in consortia enhances the rate of plastic polymer degradation (Skariyachan *et al.*, 2015; Tribedi *et al.*, 2012). Hence, studies were conducted to formulate efficient microbial consortia for effective biodegradation of microplastics. The results are discussed in the consecutive sections.

4.8 Biodegradation experiments using blended isolates on different microplastics

4.8.1 Degradation of PE microplastics using blended isolates

After 40 days of incubation, PE microplastics were recovered and the weight loss was analyzed

4.8.1.1 Determination of weight loss of PE microplastics using blended isolates

Table 4.11 displays the initial weight, final weight and weight loss of PE microplastics with the different treatments.

Table 4.11: Average mass of PE microplastics after 40 days incubation with blended isolates

Treatment	Initial weight (g)	Residual weight (g)	Weight loss (%)
A	0.500	0.498	0.4
B	0.500	0.497	0.6
C	0.500	0.499	0.2
D	0.500	0.493	1.4

n = 3

Highest weight loss was achieved by Treatment D (1.4%), followed by Treatment B (0.6%), whereas, Treatments A and C recorded weight losses of 0.4% and 0.2%, respectively, after 40 days of incubation. The results showed greater degradation ability of Treatment D towards PE microplastics. No weight loss was observed in the control sample. Statistically, there was no significant difference in the weight loss of all the treatments (F-value = 1.765; P = 0.231). Normally, increased diversity of microbes would yield better degradation yet, Treatment D with fewer number of microbes showed more degradation than Treatment A with all nine bacteria. A comparison with the highest weight loss of PE microplastics recorded, higher weight loss (6.2%) was achieved when PE microplastics were inoculated with individual isolates as against the 1.4% recorded when inoculated with Treatment D. Reasons could be that some of the microbes in the formulated consortia may have secreted metabolic products that could have led to poor performance of the microbes. Also, some of the isolates may have produced metabolites that caused other isolates in the consortia to reduce their degradation performance. These results disagreed with any hypothesis that projects the highest level of biodegradation/bioremediation to be the performers of large numbers of microbes than lesser microbes. In as much as increased diversity may allow for synergy of various metabolic potentials of microbes towards reduction of a pollutant, yet it is possible that some microbes do not exhibit optimal performance when in association with non-

compatible organisms. Hence, it is clear that selective grouping may yield best performance with respect to specific pollutant as shown by Treatment D on PE microplastics.

4.8.1.2 Growth pattern of blended isolates during PE microplastic degradation studies

Growth pattern of the different treatments in PE microplastic-infused media was assayed for 40 days. The results are presented in Figure 4.51.

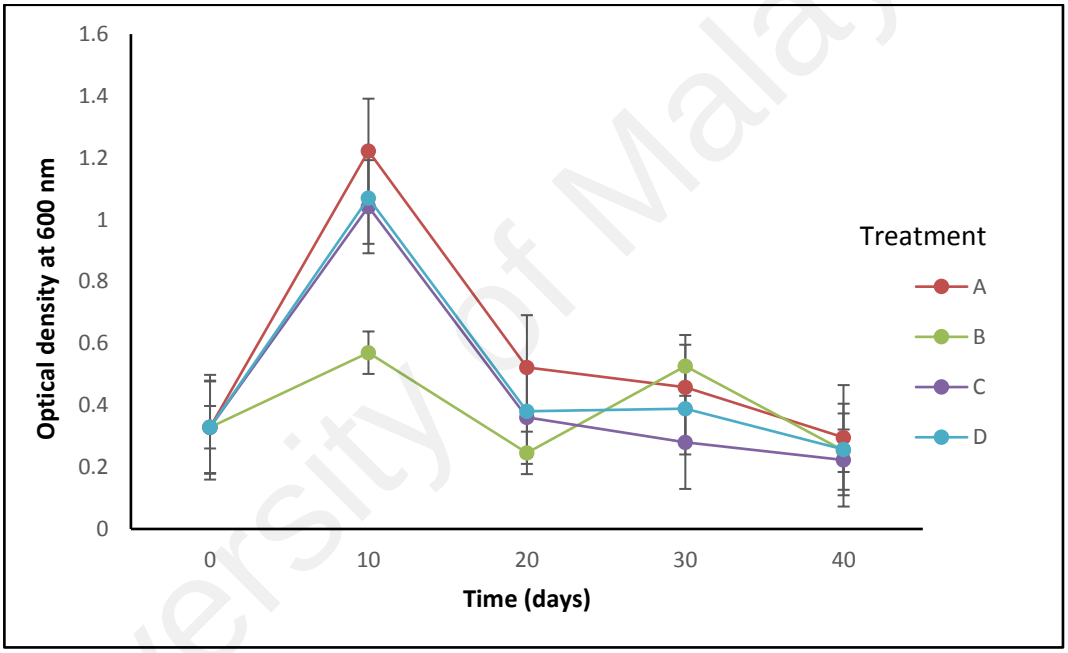

Figure 4.51: Growth pattern of blended microbes in PE microplastic-infused media during biodegradation studies

The treatments exhibited varied growth patterns on exposure to PE microplastics. Significant growth response from the treatments were observed as the measured optical density across the microplastic-induced media increased from the initial 0. 329 OD_{600}. A common observation from the growth response is the similarity in growth pattern as the treatments responded exponentially on the 10^{th} day (Treatment A = 1.222 OD_{600}, Treatment B = 0.57 OD_{600}, treatment C = 1.042 OD_{600} and Treatment D = 1.07 OD_{600}). This probably is the most favourable period of interaction between PE microplastics and

the bacterial cells. When exposed to PE microplastic, all treatments showed acceleration towards a positive growth pattern on the 10^{th} day. This was accompanied by a sharp decline in growth on Day 20 (treatment A = 0.522 OD_{600}, Treatment B = 0.246 OD_{600}, treatment C = 0.361 OD_{600} and treatment D = 0.381 OD_{600}), which could have been as a result of cell death due to depletion of nutrients or the presence of inhibitory degradation products in the media. Further decline in growth was observed on the 30^{th} and 40^{th} days for treatments A, C and D whereas, Treatment B picked up growth and recorded an absorbance value of 0.527 OD_{600} on Days 10 and 30 before experiencing a further sharp decline on Day 40. There was significant difference in the growth response of the microbes ($P < 0.05$). The culture conditions may have been made favourable for the microbes and hence, increase in growth occurred.

Counts of bacteria during the biodegradation studies fluctuated across the different treatments and the incubation time. Figure 4.52 shows that the initial counts and counts on the first 10 days ranged within 1.8×10^{11} CFU/ml – 4.6×10^{11} CFU/ml, with Treatment B recording the highest counts among all the treatments.

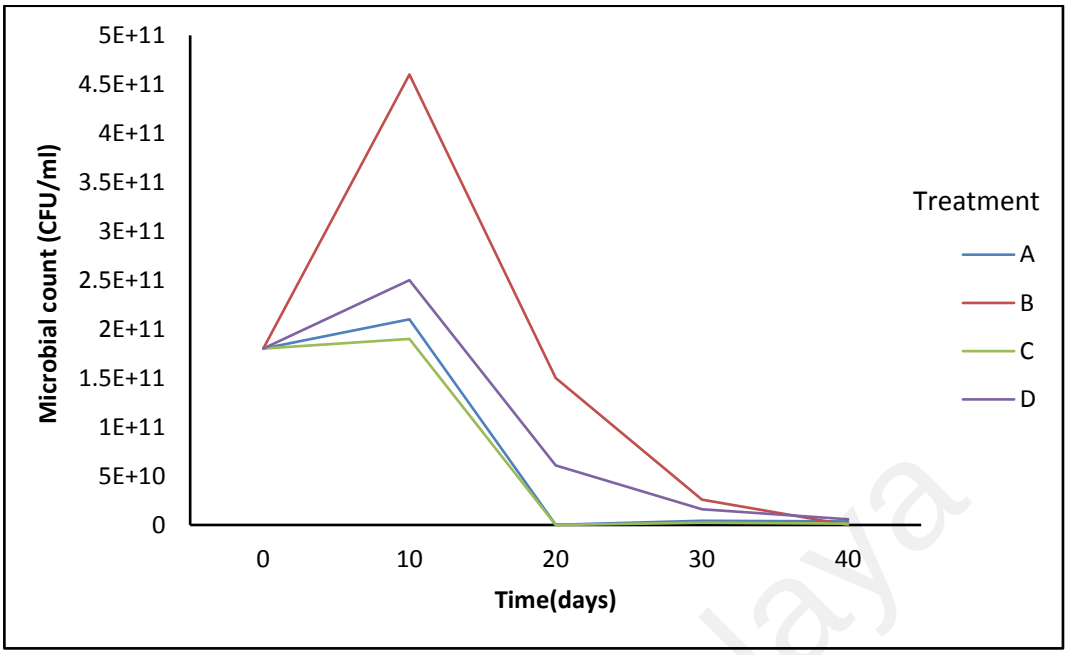

Figure 4.52: Bacterial counts of blended microbes in PE microplastic-infused media during 40 days biodegradation assay. Bars indicate standard error (n = 3).

Highest growth of all four treatments was recorded at this period, with least counts recorded by Treatment C (1.9 x 10^{11} CFU/ml). These counts were expected since Treatment C contained only four microbes as against Treatments A, B, and D which contained 9, 5, and 8 microbes, respectively. However, decline in bacterial counts across all treatments was observed on the 20th day, with least counts still recorded by Treatment C (1.7 x 10^{6} CFU/ml) and highest counts by Treatment B (1.5 x 10^{11} CFU/ml). The decline in cell number may have been a result of stress associated with biodegradation or it could have been due to accumulation of toxic products during biodegradation. These may have slowed down growth or led to cell death. Further decline in counts were observed on the Day 30 and Day 40, with Treatment B still recording highest counts on Day 30. Counts of Treatment A however, increased from 1.9 x 10^{8} CFU/ml to 4.4 x 10^{9} CFU/ml on the 30th day. This increase in cell numbers may have been due to the production of degradation products that favoured bacterial multiplication. 40th day of biodegradation witnessed a decline in counts in all treatments with Treatment B recording the least counts (1.9 x 10^{7} CFU/ml) and Treatment D showed the highest (5.8 x 10^{9}

CFU/ml). This continuous decline in counts that were demonstrated on days 20 to 40 could most likely have been as a result of stress associated with the degradation process. However, highest counts found in Treatment D may be a reflection of higher tolerance by the combined microbes that promote the microbial ability to degrade more PE microplastics at high removal rates.

4.8.1.3 pH changes of PE microplastic-induced media during biodegradation studies with blended isolates

Changes in pH that occurred in PE microplastic-infused BH media during the 40 days biodegradation studies are shown in Figure 4.53.

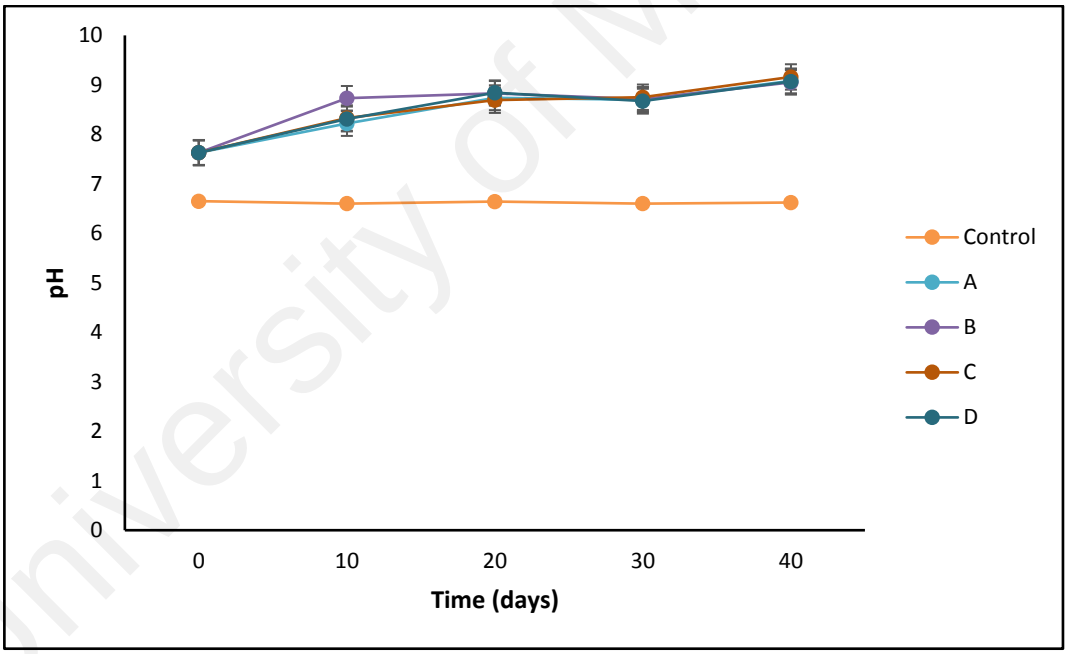

Figure 4.53: pH changes of PE microplastic-infused media inoculated with blended microbes during biodegradation studies. Each data point represents the average of three replicates ± SD

The pH increased from the initial neutral pH 7.6 to alkaline range (pH 8.2) for Treatment A, pH 8.7 for Treatment B, pH 8.3 for Treatment C and pH 8.8 for Treatment D. The increase in pH could have emanated from the metabolic activities of the growing bacteria in the medium as reported by Lucas *et al.* (2008). The culture media experienced

further increase in pH that ranged from pH 8.6 to pH 9.1 It was observed from the study that increase in pH led to the decline in cell count. Hence, it implies that the changes in pH that occurred may have contributed to the differences in community composition. Significant differences occurred in the pH changes between the different treatments on Day 10, but no significant differences existed in the pH changes on the 20^{th}, 30^{th} and 40^{th} days ($P > 0.05$). Similar increase in pH was also reported in a study by Esmaeili *et al.* (2013), in which the biodegradation of LDPE by a mixed culture of *Lysinibacillus xylanilyticus* and *Aspergillus niger* was evaluated.

4.8.1.4 Biodegradation rate constant and half-life of PE microplastics inoculated with blended isolates

The results of rate of biodegradation and the half-life of PE microplastics after treatment with the blended isolates are presented in Table 4.12.

Table 4.12: Removal rate constant (*k*) and half-life of PE microplastics across treatments

Treatment	Removal constant (k) day^{-1}	Half-life (days)
Control	0	∞
A	0.00010	6900
B	0.00015	4600
C	0.00005	13800
D	0.00035	1971.42

n =3

PE microplastics inoculated with Treatment D showed highest biodegradation rate of 0.0035 day^{-1} and half-life of 1971.42 days. Treatment A recorded biodegradation rates and half-life of 0.00015 day^{-1} and half-life of 4600 days, respectively. Treatment B recorded 0.0001 day^{-1} and half-life of 6900 days while Treatment C recorded the least biodegradation rates of 0.00005 day^{-1} and half-life of 13800 days. This implies that the highest removal rate for PE microplastic was achieved with Treatment D. Reason for the

foregoing observation maybe linked to the complex interaction that existed between the microbes that is very specific. This is because Treatment A which had a combination of all the microbes was expected to show higher removal rate but it might be that in the presence of some microbes, some degree of antagonistic reaction took place which invariably impaired optimal performance of the discrete organisms especially those that individually possess degradation capabilities. Further explanation on this can be observed in Treatment D where *B. gottheilii* was present and may have availed the performance of *B. cereus* that have PE microplastic degradation potential (Sowmya *et al.*, 2014; Suresh *et al.*, 2011), and *R. ruber* which can be a good degrader of PE and other plastic polymers (Gilan & Sivan, 2013; Orr *et al.*, 2004).

4.8.1.5 Fourier transform infrared (FTIR) spectroscopy analysis of PE microplastics inoculated with blended isolates

Figure 4.54 shows the FTIR spectrum of uninoculated (control) PE microplastics. The absorption peak at 2916 cm^{-1} and 2848 cm^{-1} are attributed to C−H of an alkyl, while the peaks at 1470 cm^{-1} and 1462 cm^{-1} are assigned to C−H of methylene (CH_2) group bending. The peaks at 875 cm^{-1}, 730 cm^{-1} and 719 cm^{-1} are assigned to C−H bending.

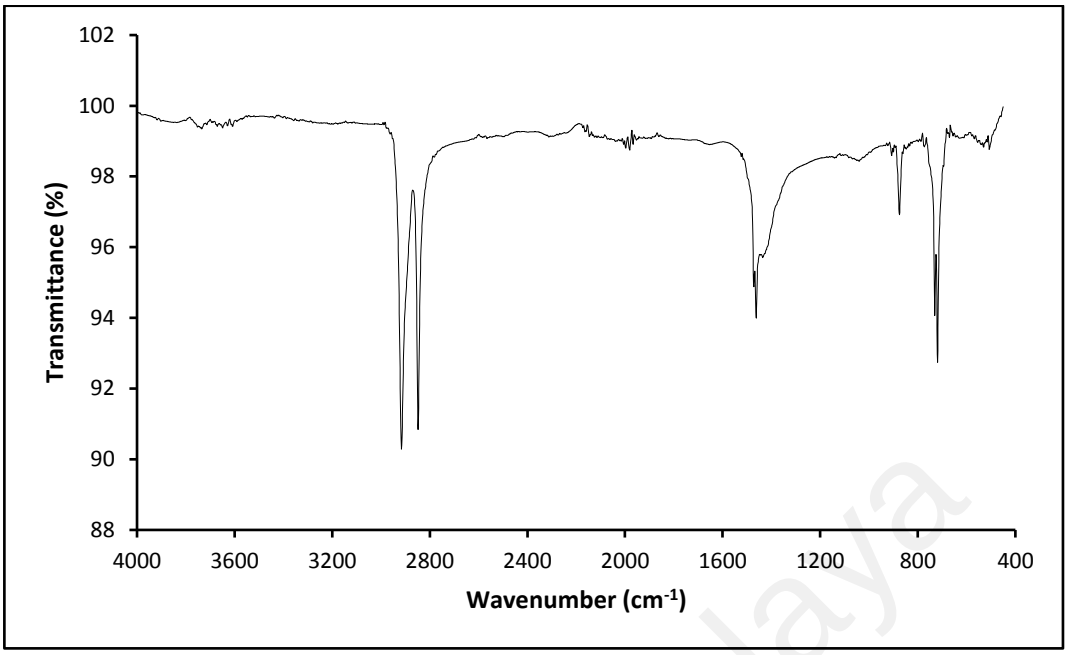

Figure 4.54: FTIR spectrum of uninoculated (control) PE microplastic

There are C−H alkyl absorption peaks in spectrum of PE microplastic with Treatment A at 2916 cm^{-1} and 2848 cm^{-1} (Figure 4.55) as in uninoculated PE microplastics.

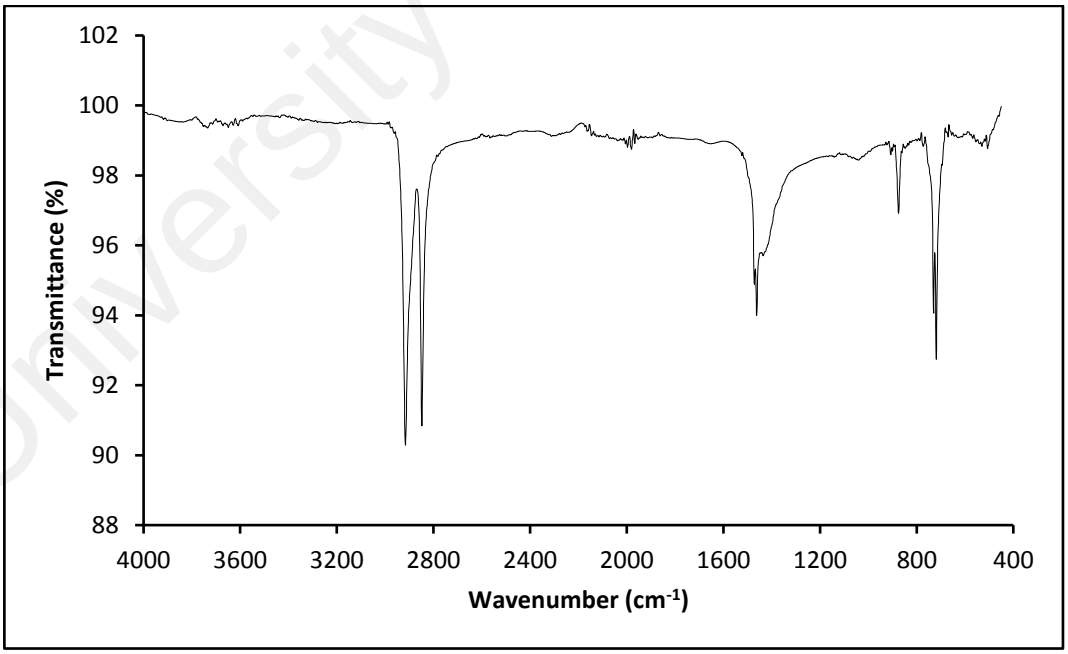

Figure 4.55: FTIR spectrum of PE microplastic inoculated with Treatment A

The peaks at 1470 cm^{-1} however, became reduced and shifted to 1472 cm^{-1} when inoculated with Treatment A. The peak is attributed to C−H of methylene (CH_2) group

bending. The absorption peaks at 875 cm^{-1}, 730 cm^{-1} and 719 cm^{-1} are assigned to C−H bending. The peak at 509 cm^{-1} shifted to 460 cm^{-1} and became elongated. Bhatia *et al.* (2014) had carried out a similar work on PE with a bacterial consortium and similar reduction and shift in the peaks were observed.

PE microplastics inoculated with Treatment B showed absorption peaks at 2914 cm^{-1} and 2848 cm^{-1} assigned to C−H stretch of an alkyl (Figure 4.56). There are absorption peaks at 1471 cm^{-1} and 1463 cm^{-1} attributed to C−H bending of methylene (CH_2) group, but the peak at 1462 cm^{-1} in control PE microplastics which appeared at 1463 cm^{-1} in PE microplastic inoculated with Treatment B was much reduced.

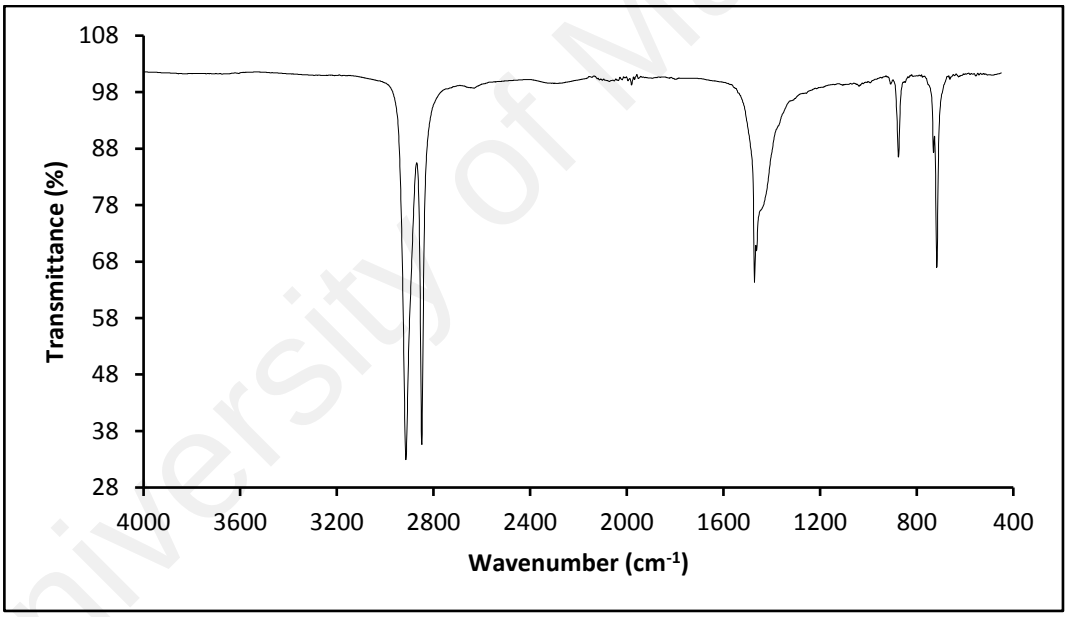

Figure 4.56: FTIR spectrum of PE microplastic inoculated with Treatment B

The C−H bending peaks are observed at 731 cm^{-1} and 717 cm^{-1}. The peak at 731 cm^{-1} became reduced, while the peak at 508 cm^{-1} in spectrum of control PE microplastics disappeared. Das and Kumar (2015), and Ojha *et al.* (2017) in their work on the biodegradation of PE, reported similar shift and reduction in peaks upon treatment with *Bacillus amyloliquefaciens* and *Penicillium* sp., respectively.

The FTIR spectrum of PE microplastics inoculated with Treatment C is presented in Figure 4.57. The absorption peaks at 2915 cm^{-1} and 2849 cm^{-1} are for C−H stretch of an alkyl. The peaks at 1472 cm^{-1} and 1462 cm^{-1} are assigned to C−H bending of methylene (CH_2) group. However, the peak at 1462 cm^{-1} became reduced as compared to that of the control experiment. The C−H bending peaks at 731 cm^{-1} and 718 cm^{-1} also became reduced, while the peak at 508 cm^{-1} disappeared.

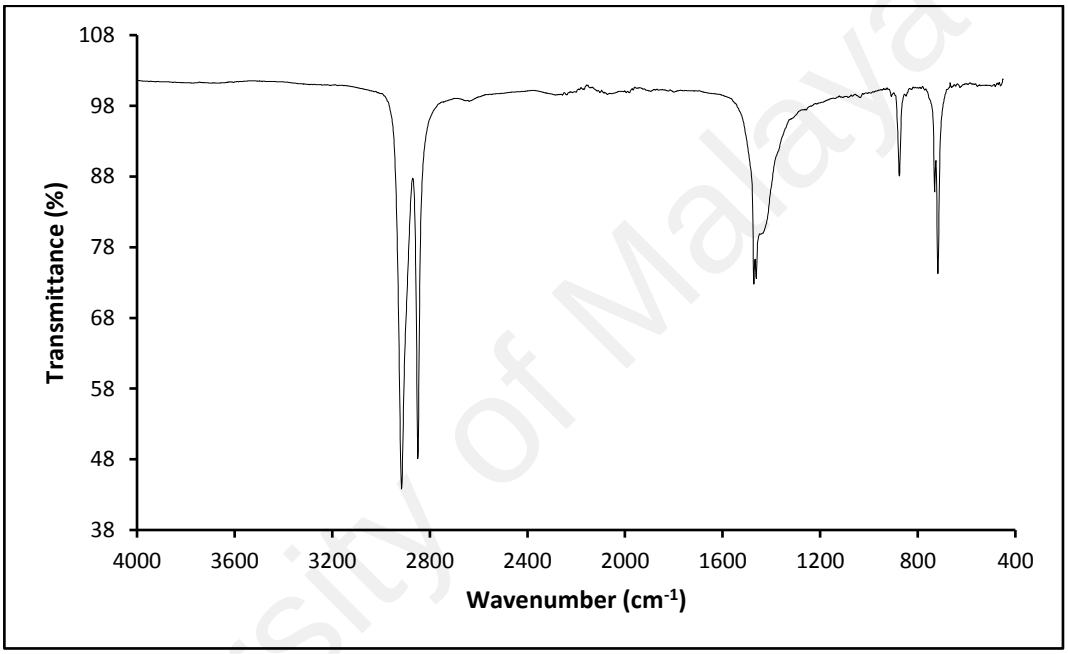

Figure 4.57: FTIR spectrum of PE microplastic inoculated with Treatment C

The spectrum of PE microplastics inoculated with Treatment D has absorption peaks at 2916 cm^{-1} and 2848 cm^{-1} assigned for C−H aliphatic stretching (Figure 4.58).

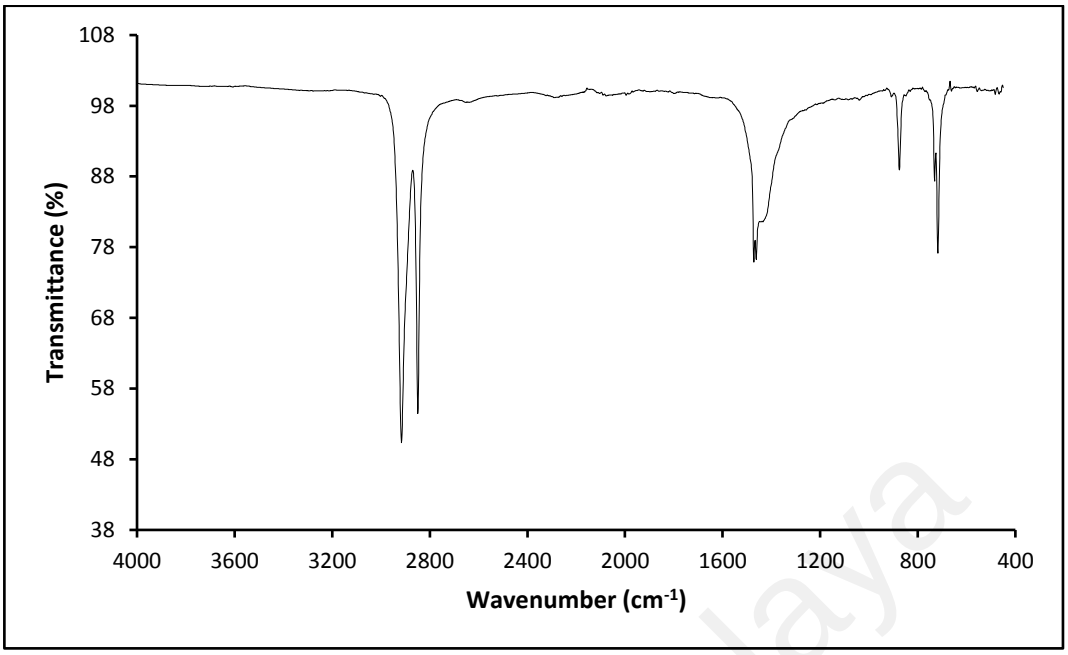

Figure 4.58: FTIR spectrum of PE microplastic inoculated with Treatment D

Peaks at 1472 cm^{-1} and 1462 cm^{-1} are assigned to C−H bending of methylene (CH_2) group. The C−H bending peak at 1462 cm^{-1} became reduced compared to that in control experiment while the peak at 1981 cm^{-1} disappeared. Absorption peaks at 731 cm^{-1} and 718 cm^{-1} and 875 cm^{-1} are for C−H bend of an alkyl. The peak at 509 cm^{-1} present in the spectrum of control microplastics shifted to 482 cm^{-1} and was reduced.

4.8.2 Biodegradation of PP microplastics using blended isolates

4.8.2.1 Determination of weight loss of PP microplastics inoculated with blended isolates

The weight loss of the PP microplastics were measured after 40 days of incubation and the results are presented in Figure 4.59.

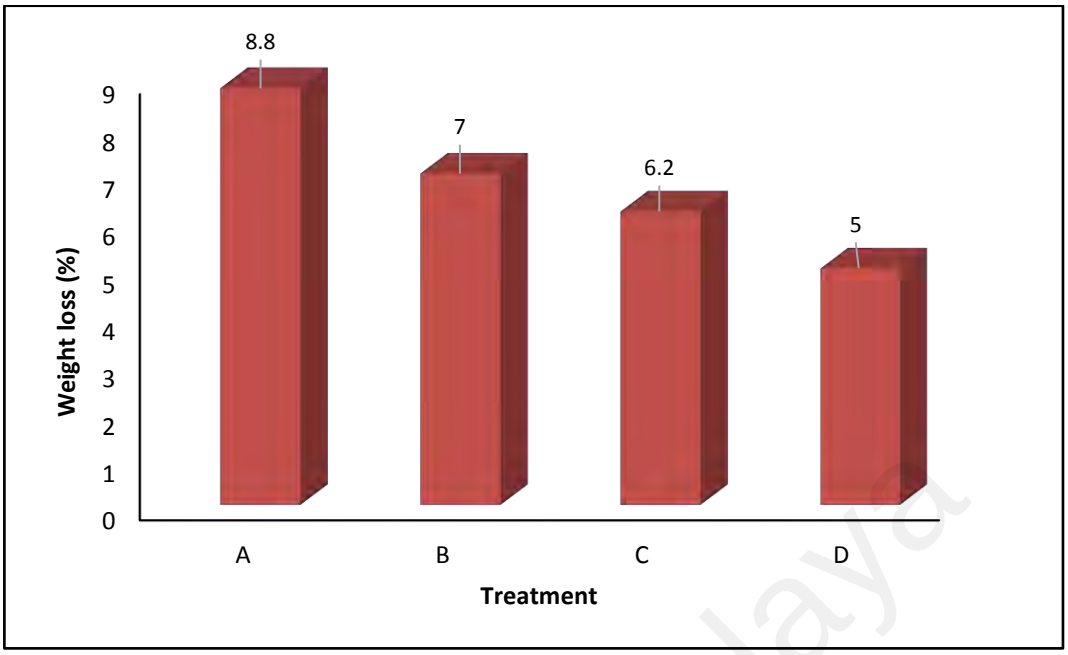

Figure 4.59: Weight loss of microbially treated PP microplastic after incubation for 40 days. Maximum weight loss (8.8%) was obtained for PP microplastic treated with Treatment A.

The weight loss of PP microplastics after 40 days of incubation with the treatments were 8.8% for Treatment A, 7.0% for Treatment B, 6.2% for Treatment C, and 5% for Treatment D. Treatment A recorded the highest weight loss among all treatments. The results indicated that all four treatments were capable of degrading PP microplastics (F-value = 0.576; P = 0.647). Engineering microbes into consortia was expected to display increased metabolic potential for PP microplastic degradation as multiple interacting microbial populations have the potential to perform complicated functions that monocultures cannot do. This was observed in the higher weight loss recorded when the microbes were blended; 8.8% maximum for treatment A, as compared to a maximum of 6.4% by *R. ruber* alone. Furthermore, treatment A contained more number of diverse microbes and was expected to show higher PP reduction rate as it is assumed that degradation potential will increase with increased microbial diversity.

Generally, the mechanism behind the significant bio-reduction in all treatments might be due to imbued interactions that exist among microbes upon manipulation of diversity

and cell concentrations. Anwar *et al.* (2013) and Skariyachan *et al.* (2015) also reported that consortia consisting of *Pseudomonas* sp., *Bacillus* sp. and *Mycobacterium* sp. showed successful degradation of LDPE.

4.8.2.2 Growth pattern of blended microbes in PP microplastic-infused media

The curves depicted by the different treatments were essentially congruent; characterized by an exponential phase of growth with maximum growth attained by all four treatments on Day 10 and absorbance readings of 0.967 OD_{600}, 1.318 OD_{600}, 1.138 OD_{600}, and 0.904 OD_{600} for Treatments A, B, C and D, respectively (Figure 4.60).

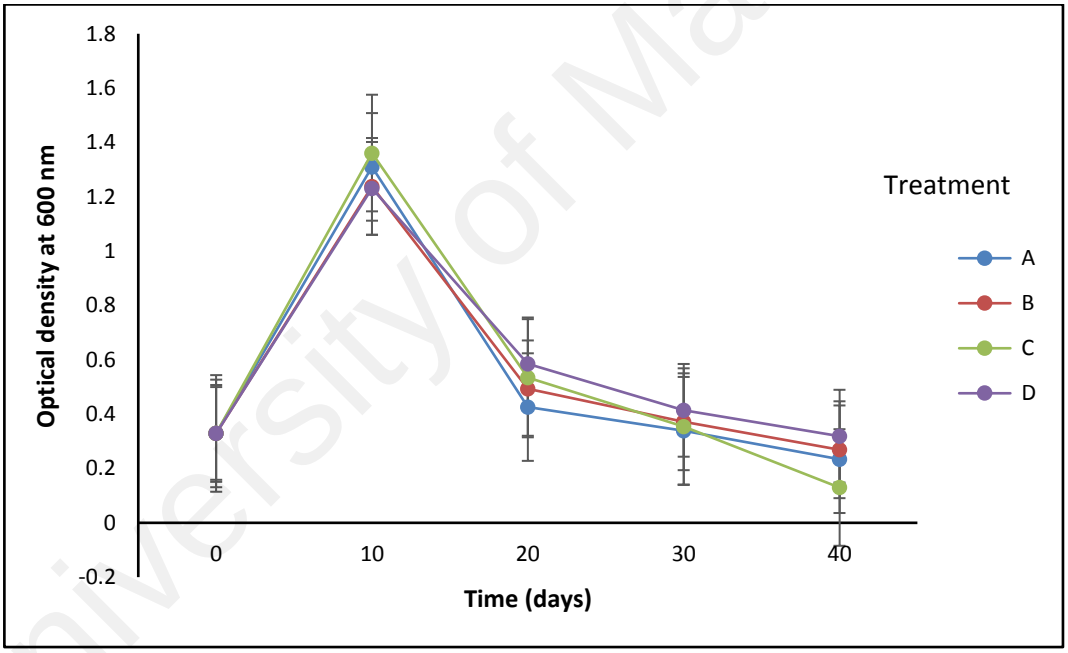

Figure 4.60: Growth pattern of blended microbes in PP microplastic-infused media during biodegradation studies

Highest counts of the microbes were also recorded on the 10th day, with cell counts of 4.9 x 10^{11} CFU/ml, 5.9 x 10^{11} CFU/ml, 1.5 x 10^{11} CFU/ml, and 6.3 x 10^{11} CFU/ml for Treatments A, B, C, and D, respectively. After Day 10, the 20th day witnessed a sharp decline in growth. Continued decrease in growth was observed up to the 40th day in all the treatments (Figure 4.61). This might probably have been as a result of bacterial cell

lysis. The growth curves demonstrated by the treatments were quite supportive of the fact that the microbial associations were good enough to utilize PP microplastics for cell growth and multiplication. No significant difference existed between the growth of the treatments on Days 10, 20, and 30. Significance difference however existed on Day 10 (F-value = 5.766; P = 0.021). Similar growth profile studies have earlier been reported to increase bacterial biomass on exposure to LDPE and HDPE by Bhatia *et al.* (2014).

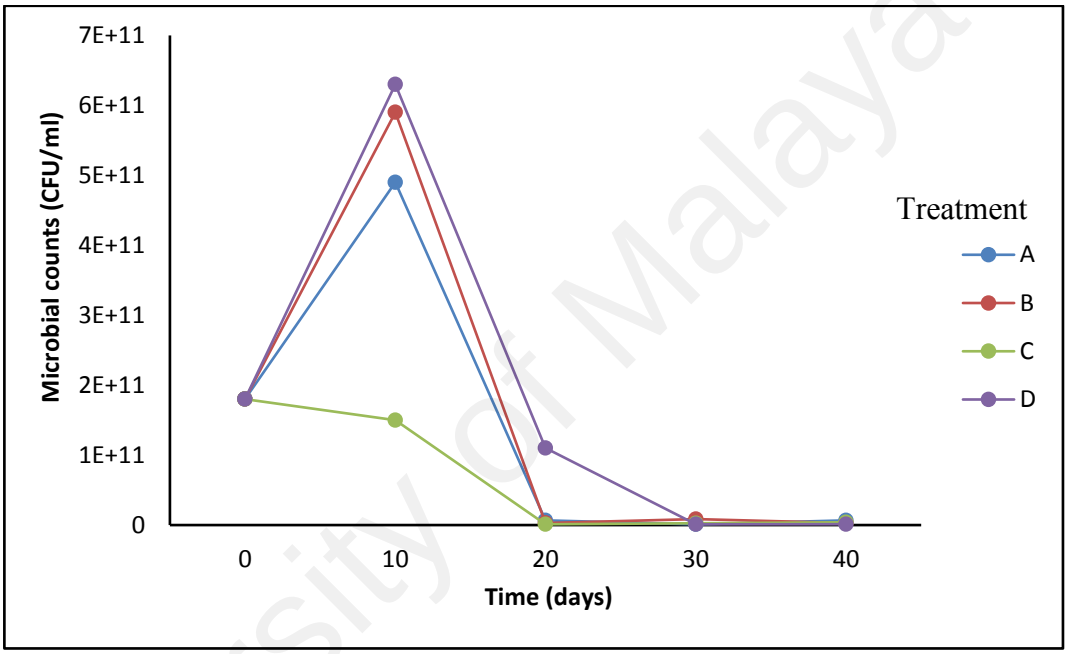

Figure 4.61: Bacterial counts of blended microbes inoculated in PP microplastic-infused media during 40 days biodegradation assay. Bars indicate standard error (n = 3).

The counts in PP microplastic-infused culture medium inoculated with Treatments A, B, C and D recorded highest counts on the 10th day with Treatment D recording highest counts that ranged between 1.7 x 10^{11} CFU/ml and 6.3 x 10^{11} CFU/ml. This was followed by Treatment B which recorded counts of 5.9 x 10^{11} CFU/ml. Treatment A recorded 4.9 x 10^{11} CFU/ml. This is an indication of the growth and survival of the microbes in the minimal media by utilizing PP microplastics as nutritional source. Treatment C however, recorded the least count of 1.5 x 10^{11} CFU/ml, counts that were less than the counts recorded at the start of the experiment. The lower count recorded by Treatment C could

probably have been as a result of the inability of the microbes to adapt well to the environment and hence, the population counts decreased even though the cells were metabolically active as has been proven by the weight loss demonstrated by the treatment. 20^{th} day witnessed further decline in counts of bacteria across all treatments, with Treatment D and C still displaying highest counts and least counts of 1.1 X 10^{11} CFU/ml and 1.1 x 10^{9} CFU/ml, respectively. The decline in growth could probably be due to the progressive death of cells as a result of nutrient exhaustion or due to the presence of toxic metabolites in the culture media that may have caused cell death. Further decline in growth of the microbes continued up to the 40^{th} day with highest growth recorded by Treatment B while Treatment A had the least count. The counts of Treatment C however, began to increase on the 30^{th} and 40^{th} day (2.4 x 10^{9} CFU/ml and 3 x 10^{9} CFU/ml), so also did counts of Treatments A and D on Day 40 when compared to counts recorded by the treatments on the 20^{th} day. This depicts that conditions of the culture medium became favourable for cell multiplication during the period as some living microbes may have probably made use of the breakdown products of dead bacteria as nutrient and remain as 'persister'.

4.8.2.3 Changes in pH of PP microplastic culture media during 40 days biodegradation studies using blended microbes

Figure 4.62 describes the changes in pH of PP microplastic media during the 40 days biodegradation.

The pH of the culture medium increased from neutral (pH 7.6) to alkaline range (pH 8.2) for Treatments A, C and D, and pH 8.3 for Treatment B on the 10^{th} day, with maximum growth of the microbes attained at these pH ranges.

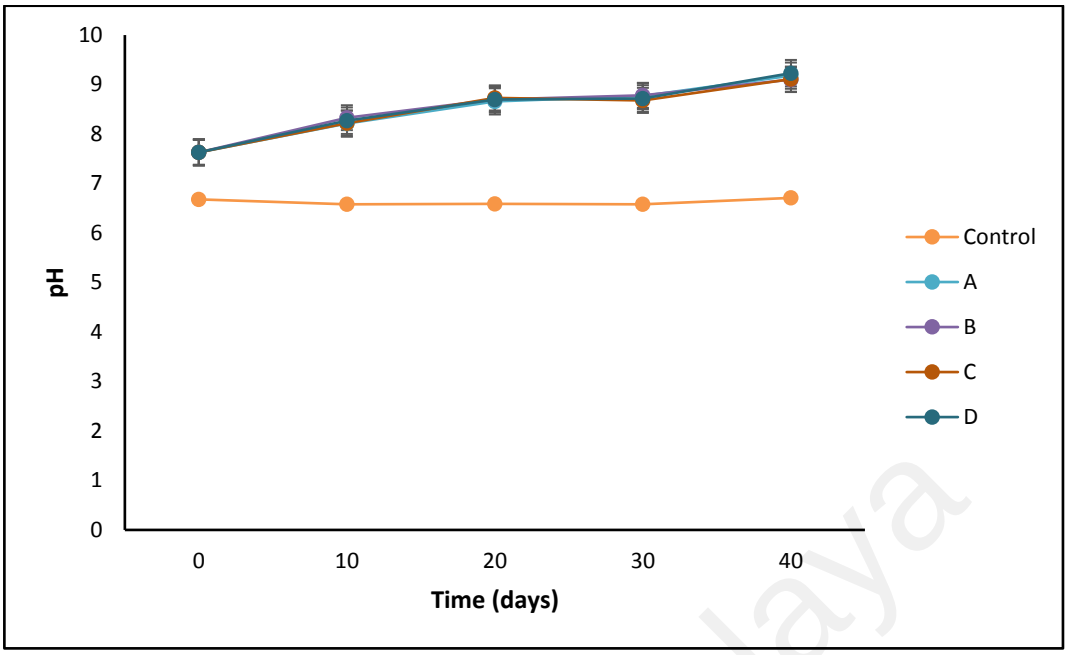

Figure 4.62: pH changes of PP microplastic-infused media inoculated with blended microbes during biodegradation studies. Each data point represents the average of three replicates ± SD

As the pH progressed towards alkaline conditions after the 10th day, decline in growth of the microbes began. This could probably mean that the pH ranges attained by the microbes on the 10th day were the most optima pH for growth and survival. Furthermore, it can be observed that the pH changes that occurred in all treatments experienced continuous increase from the 10th to the 40th day, except for the culture medium inoculated with Treatment C which demonstrated slight decline (pH 8.6). This decline in pH may probably have been responsible for the increase in counts on same day. Similar results were recorded by Rao *et al.* (2016) on the biodegradation of polycyclic aromatic hydrocarbons (PAHs) by *Bacillus* sp. Decline in growth of the microbe was observed when alkalinity increased.

4.8.2.4 Biodegradation rate constant and half-life of PP microplastics inoculated with blended microbes

Table 4.13 reveals that the rates of uptake of PP microplastics and their corresponding half-life values on inoculation with the different treatments for 40 days was higher in PP microplastics inoculated with Treatment A (0.0023 day^{-1} and half-life of 300 days).

Table 4.13: Removal rate constant (*k*) and half-life of PP microplastics across treatments

Treatment	Removal constant (*k*) day^{-1}	Half-life (days)
Control	0	∞
A	0.0023	300
B	0.0018	383.33
C	0.0016	431.25
D	0.0012	575

n = 3

The reason for the higher uptake might be due to the fact that Treatment A contained all microbes and therefore, had higher number of diverse organisms and was therefore, expected to show better removal rate that other treatments hence, it showed better degradation competence. Another reason could be that, maybe a specific synergy existed when a blend of all microbes was used on PP microplastic, because such impact was not found when the microbes were exposed to PE.

4.8.2.5 Fourier transform infrared (FTIR) analysis of PP microplastics inoculated with blended microbes

The results of the FTIR analysis of PP microplastics displayed a number of peaks reflecting the complex structure. Control PP microplastics (Figure 4.63) showed O−H, hydroxyl peak around 3700 cm^{-1} and N−H stretching of an amine around 3300 cm^{-1}.

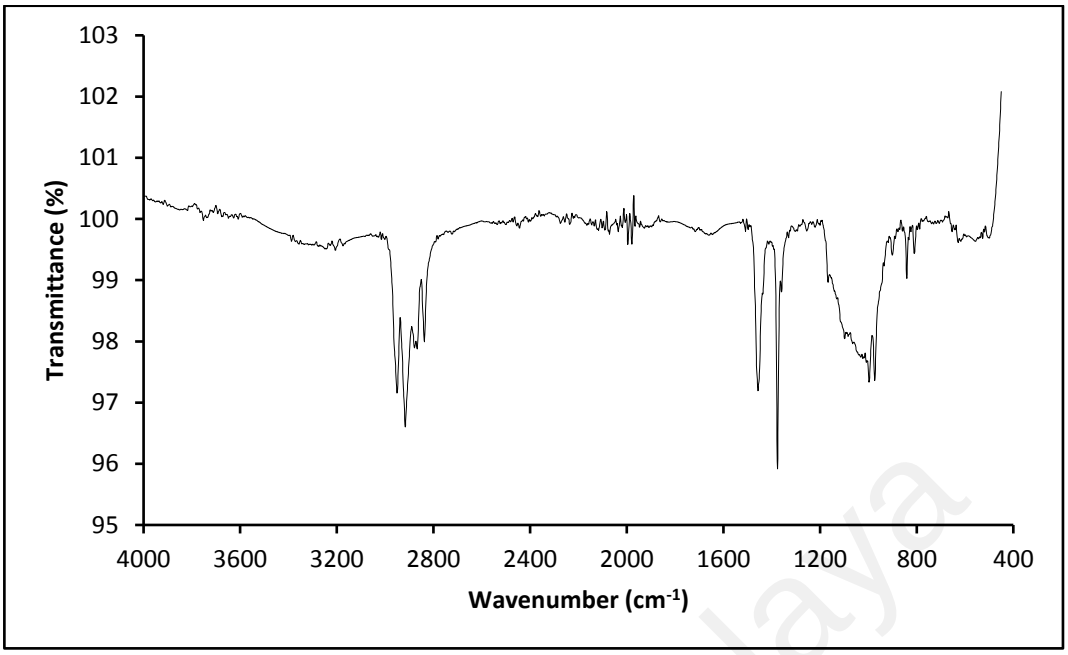

Figure 4.63: FTIR spectrum of uninoculated (control) PP microplastics

The absorption peaks at 2838-2951 cm^{-1} are attributable to C−H, alkyl stretch while the peak around 1650 cm^{-1} was assigned to N−H bend. The peaks at 1456 cm^{-1} and 1376 cm^{-1} are assigned to C−H bend of methylene (CH_2) group and C−H bend of methyl group (CH_3), respectively. The peaks at 998 cm^{-1}, 899 cm^{-1}, 841 cm^{-1}, and 809 cm^{-1} can be assigned to C−H alkyl bend.

The O−H, hydroxyl peak around 3700 cm^{-1} in the control disappeared on inoculation with Treatment A, so also the peak at 622 cm^{-1} (Figure 4.64). These demonstrated the action of the microbes on PP microplastics. Furthermore, there was formation of a new absorption peak at 1167 cm^{-1} assignable to C−O phenolic stretch. Every other peak obtainable in control PP microplastics were present. However, the peaks at 997 cm^{-1}, 973 cm^{-1}, 898 cm^{-1}, 841 cm^{-1}, 810 cm^{-1} became reduced.

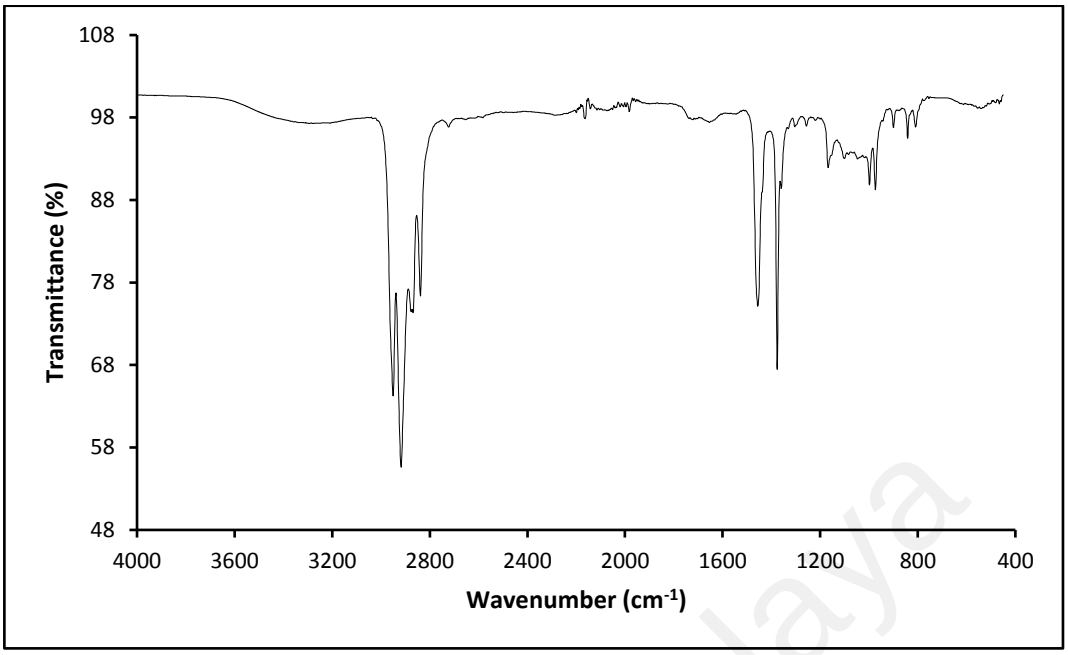

Figure 4.64: FTIR spectrum of PP microplastic inoculated with Treatment A

On inoculation with Treatment B, the O−H, hydroxyl peak around 3700 $cm^{-1}$and the peak at 622 cm^{-1} in the control disappeared. A new absorption peak was formed at 1168 cm^{-1} assignable to C−O phenolic stretch (Figure 4.65). Also observed were the reduction of the peaks at 998 cm^{-1} and 973 cm^{-1} and the elongation of the peaks at 900 cm^{-1}, 841 cm^{-1} and 809 cm^{-1}. These revealed the action of the biodegradation bacteria on PP microplastics.

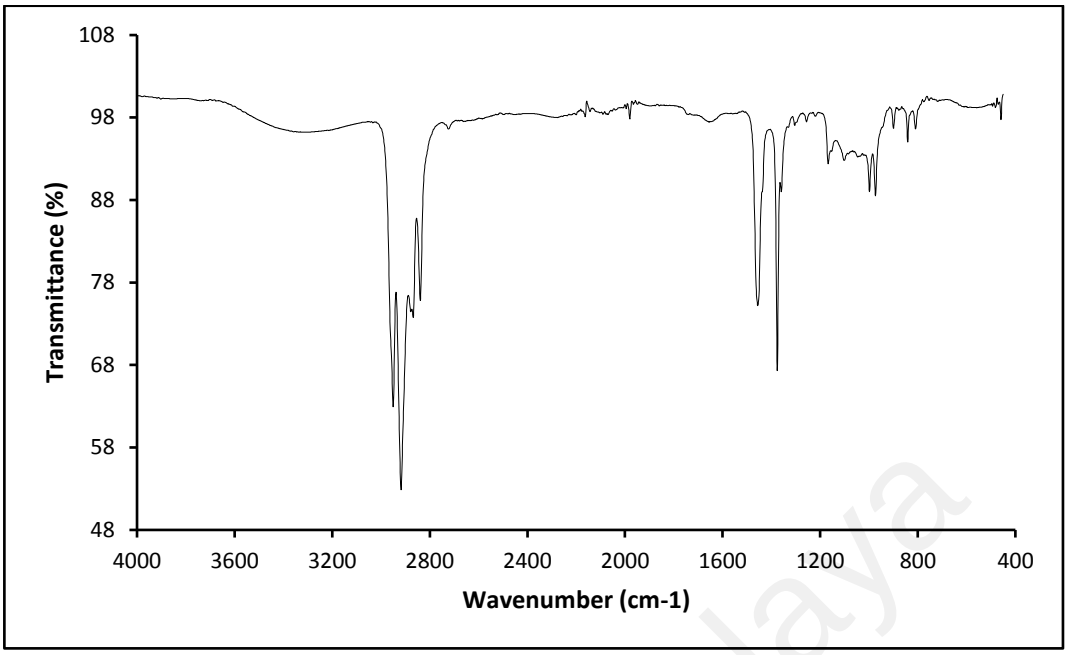

Figure 4.65: FTIR spectrum of PP microplastic inoculated with Treatment B

The O−H, hydroxyl peak around 3700 cm^{-1} and the peaks at 622 cm^{-1} disappeared with Treatment C (Figure 4.66). A new peak was formed at 591 cm^{-1}. The N−H stretching absorption peak of an amine at 3330 cm^{-1} and its bending peak at 1651 cm^{-1} became elongated. The peaks at 998 cm^{-1} and 973 cm^{-1} also became elongated while the C−H bending peaks at 900 cm^{-1}, 841 cm^{-1}, and 809 cm^{-1} became reduced.

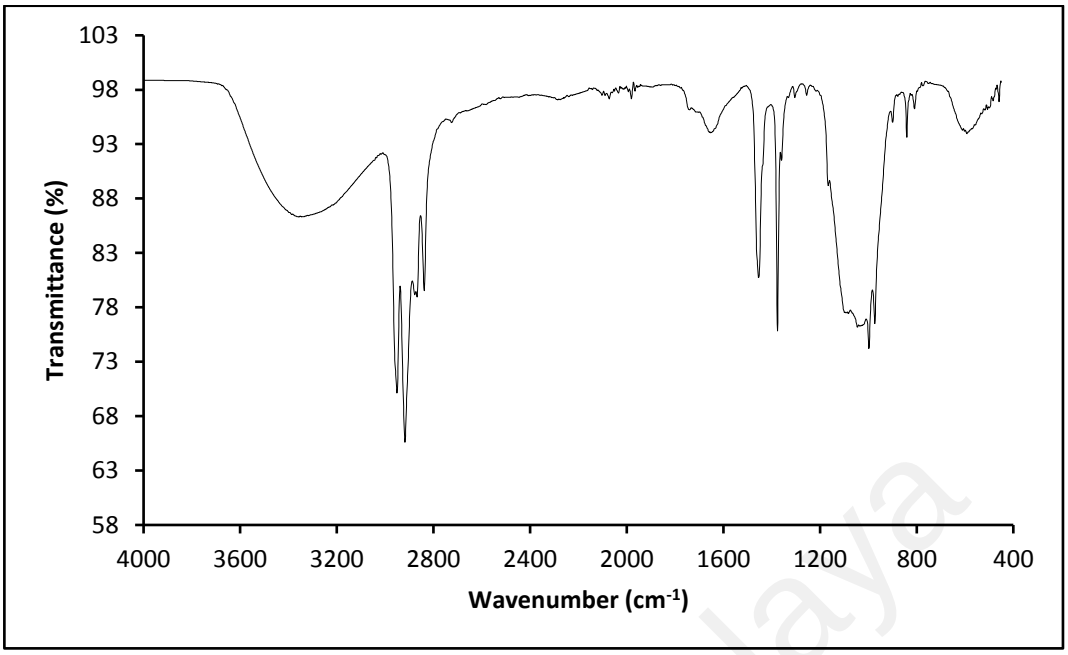

Figure 4.66: FTIR spectrum of PP microplastic inoculated with Treatment C

The O−H, hydroxyl peak around 3700 cm^{-1} and the peak at 622 cm^{-1} disappeared in PP microplastics subjected to degradation by Treatment D (Figure 4.67). The N−H stretching absorption peak of an amine at 3330 cm^{-1} and its bending peak at 1651 cm^{-1} became elongated while the C−H bending peaks at 1456 cm^{-1} and 1376 cm^{-1} assigned to C−H of methylene (CH_2) group and C−H bend of methyl group (CH_3), respectively, became reduced. The same reduction was applicable to the C−H bending peaks at 900 cm^{-1}, 841 cm^{-1}, and 809 cm^{-1}. New absorption band at 583 cm^{-1} was formed.

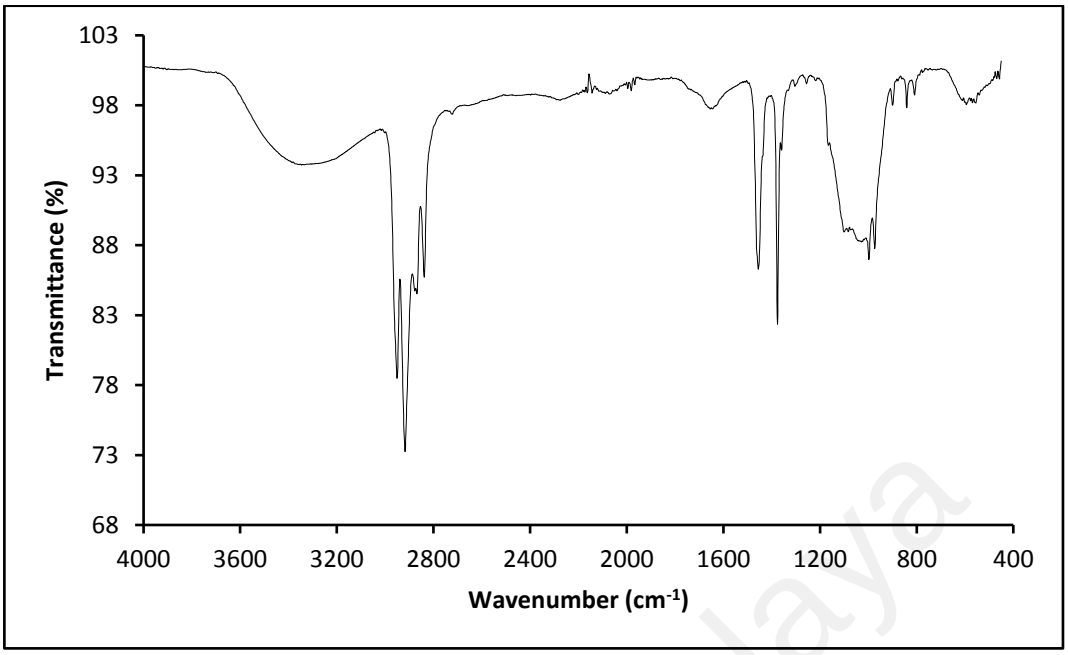

Figure 4.67: FTIR spectrum of PP microplastic inoculated with Treatment D

4.8.3 Biodegradation of PET microplastics using blended isolates

4.8.3.1 Determination of weight loss of PET microplastics inoculated with blended isolates

Figure 4.68 shows the extent to which PET microplastics were degraded by the different treatments. It demonstrated that while about 1.2% PET microplastic was degraded by Treatment D in 40 days, Treatment B recorded only 0.4%. The higher degradation by Treatment D was not surprising since all microbes that made up the treatment were all gram positives. Several studies have also demonstrated significant degradation of plastic polymers by gram positive bacteria (Balasubramanian *et al.*, 2010; Kathiresan, 2003b). Treatment A came in second, recording a weight loss of 1.0%. The treatment consisted of all nine isolates and was therefore, expected to show good degradation potential as a result of increased diversity.

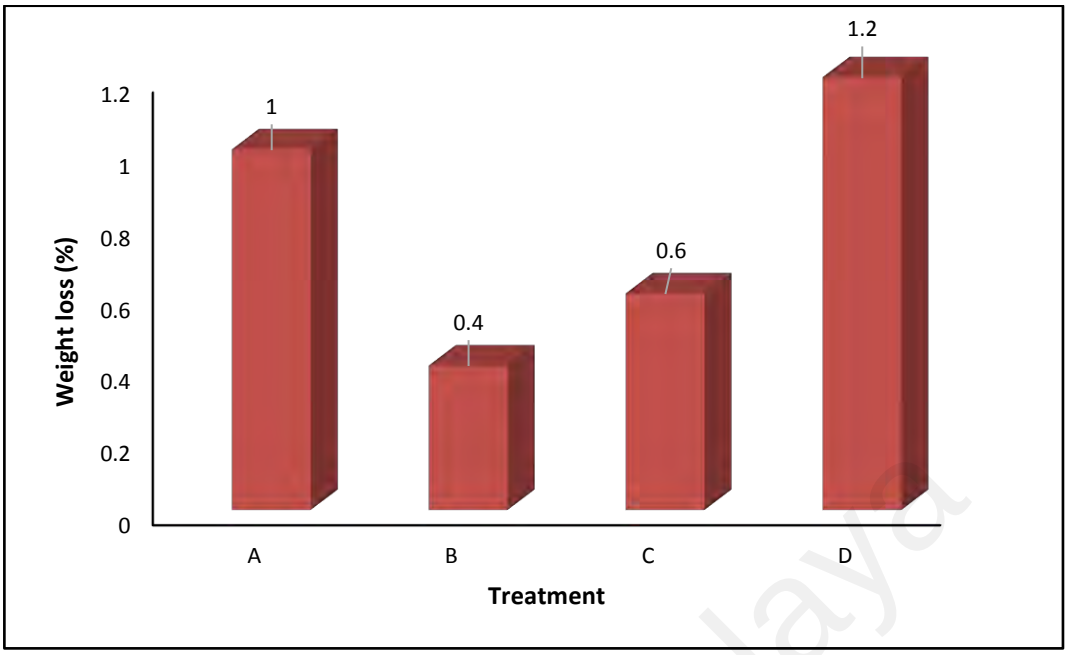

Figure 4.68: Weight loss of microbially treated PET microplastic after incubation for 40 days. Maximum weight loss (1.2%) was obtained for PET microplastic treated with Treatment D.

Treatment C however, came in third, recording a weight loss of 0.6%, while Treatment B recorded the least weight loss of 0.4%. No significant difference was obtained between the treatments. The degradation mechanism behind the result may be due to the interactions that occur among microbes when diversity is manipulated. *B. cereus* and *B. gottheilii*, present in Treatments A, B, and D are good degraders of PET microplastics as recorded when the isolates were screened for PET microplastic degradation. Hence, this degradation ability of the isolates may have enhanced PET microplastic degradation when inoculums were used as treatment agents. Despite the presence of *B. cereus* and *B. gottheilii* in Treatments B, removal was more pronounced in Treatments A and D: hence, the order of PET microplastic degradation across treatments was B<C<A<D.

Although degradation of PET microplastics was observed on treatment with blended microbes, higher degradation PET microplastics was however, achieved with individual isolates (*B. cereus* = 6.6% and *B. gottheilii* = 3.0%). The release of inhibitory substances or the competitive action between the enzymes secreted by the different isolates in the

consortia for single substrate site might be the reason for the reduced degradation recorded by the blended isolates as compared to individual isolates. The conditions of the culture media may have become unfavourable and as such, reduced the degradation ability of the potential PET microplastic degraders. The results obtained were in contrast with the results recorded by Syranidou *et al.* (2017) who in their study, recorded higher weight loss of PE on treatment with tailored indigenous marine microbial consortium. The results contrasted with the study of Patowary *et al.* (2016) who also, observed higher degradation of hydrocarbons with mixed culture of microbes.

4.8.3.2 Growth pattern of blended microbes in PET microplastic-infused media

Figure 4.69 shows the optical density/incubation time curve of the different treatments on exposure to PET microplastics. All four treatments demonstrated similar growth patterns characterized by a log phase that reached a peak and attained maximum OD readings on the 10^{th} day; 0.967 OD_{600}, 1.318 OD_{600}, 1.138 OD_{600} and 0.904 OD_{600} for Treatments A, B, C and D, respectively, which suggest increase in bacterial growth. The log phase was accompanied by a sharp decline in growth on the 20^{th} day and a gradual decline from the 20^{th} day to the 40^{th} day which seem to depict cell death. Statistical analysis showed no variance in the growth of the different treatments on exposure to PET microplastics ($P < 0.05$).

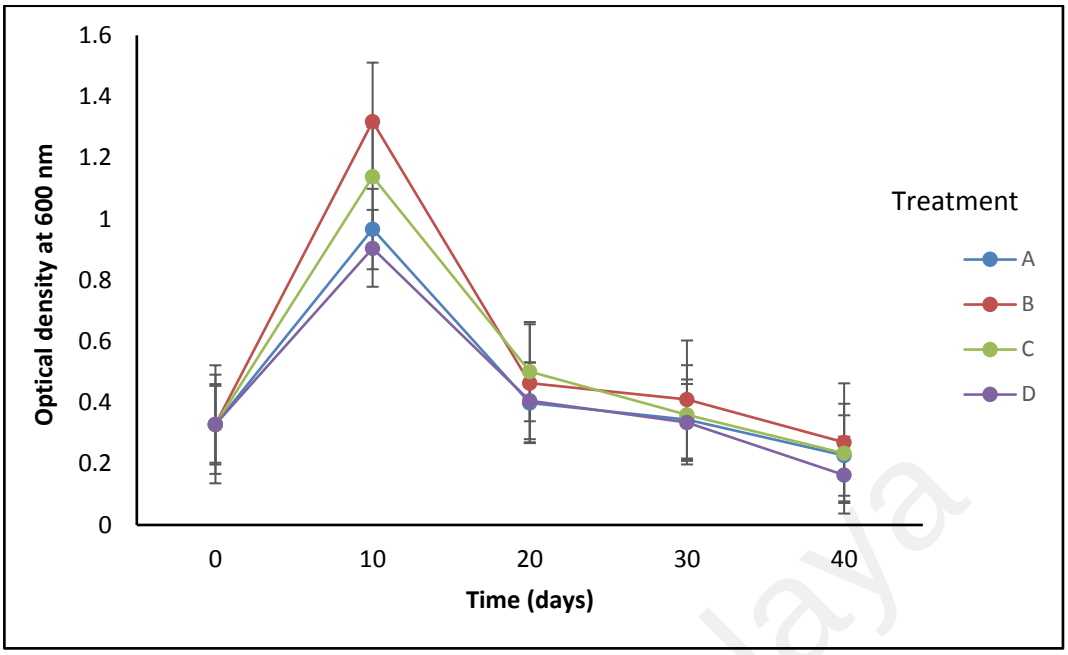

Figure 4.69: Growth pattern of blended microbes in PET microplastic-infused media during biodegradation studies

The bacterial counts during the 40 days incubation period is presented in Figure 4.70. On Day 10, Treatments A and B recorded highest counts of 4.6 x 10^{11} CFU/ml and 2.1 x 10^{11} CFU/ml, respectively. This depicts that the microbes may have adjusted and were utilizing the PET microplastics for growth. Highest OD values of both treatments were also recorded on this day. Treatments C and D recorded highest OD values on Day 10, the counts, however, were observed to have dropped on same day. The greater OD values measured probably indicated the presence of other metabolic materials and the decline in counts could have probably been due to stress associated with PET microplastic degradation. Further decline in counts were recorded in all treatments on the 20th day, with Treatment D recording the highest counts of 8.1 x 10^{10} CFU/ml while Treatment C recorded the least counts of 1.2 x 10^{9} CFU/ml. In Treatment A, a drastic decline in counts on the Day 30 recorded a count of 8.8 x 10^{4} CFU/ml. The counts of Treatments B and D were also observed to have declined while an increase in count was observed in Treatment C (1.2 x 10^{8} CFU/ml – 2.6 x 10^{9} CFU/ml). The count was observed to have however, increased across all treatments on the 40th day, with Treatment A recording maximum

counts of 2.9 x 10^9 CFU/ml. This could probably be due to the production of metabolites that could have favoured microbial growth. Additionally, some spore formers could have persisted beyond the death phase and thus, regenerated when the conditions in the culture media became favourable.

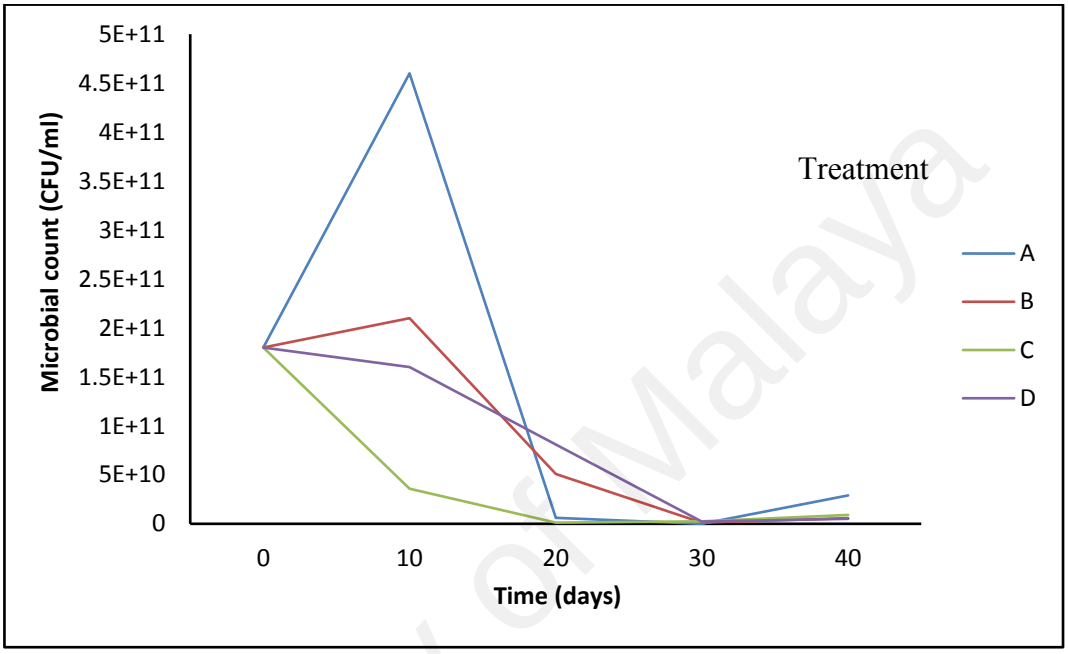

Figure 4.70: Bacterial counts of blended microbes inoculated in PET microplastic-infused media during 40 days biodegradation assay.

4.8.3.3 Changes in pH of PET microplastic culture media during 40 days biodegradation studies using blended microbes

Changes in pH of PET microplastic-infused media possibly occurred due to extracellular metabolite production by the microbes. The pH changed towards alkalinity after 10 days, recording pH of 8.5, 8.4, 8.3, and 8.8 for Treatments A, B, C and D, respectively (Figure 4.71), which are suitable pH that favours plastic degradation (Arutchelvi *et al.*, 2008).

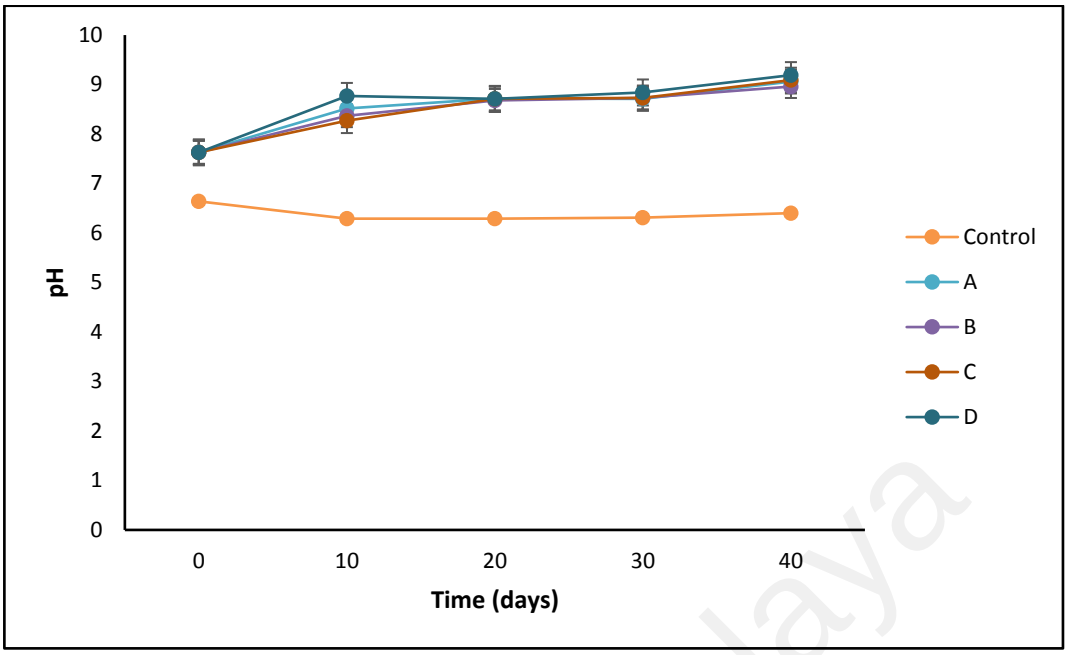

Figure 4.71: pH changes of PET microplastic-infused media inoculated with blended microbes during biodegradation studies. Each data point represents the average of three replicates ± SD

This may have influenced optimum performance of the microbes. Continuous increase in pH was observed up to the 40th day of experiment. The changes in pH from neutral to alkaline could be due to the metabolic activities of the growing bacteria in the PET microplastic-infused media. It can therefore, be concluded that degradation of PET microplastics may have drastically increased the pH of the aqueous media.

4.8.3.4 Biodegradation rate constant and half-life of PET microplastics inoculated with blended microbes

Table 4.14 shows that the rate of PET microplastics uptake by the different treatments within 40 days was higher when PET microplastics were inoculated with Treatment D. This recorded removal rate of 0.0003 day^{-1} and half-life of 2300 days. This was followed by Treatment A which recorded removal rate of 0.00025 day^{-1} and half-life of 2760 days. Treatment C recorded removal rate of 0.00015 day^{-1} and half-life of 4600 days while Treatment B had 0.0001 day^{-1} and half-life of 6900 days. No removal was recorded in

control PET microplastic. Therefore, it implied that the rate of daily reduction was more prevalent in Treatment D than in other treatments. Hence, it supports the total weight reduction by Treatment D found at the end of the experiment.

Table 4.14: Removal rate constant (*k*) and half-life of PET microplastics across treatments

Treatment	Removal constant (*k*) day^{-1}	Half-life (days)
Control	0	∞
A	0.00025	2760
B	0.0001	6900
C	0.00015	4600
D	0.0003	2300

n = 3

4.8.3.5 Fourier transform infrared (FTIR) analysis of PET microplastics inoculated with blended microbes

Control PET microplastics showed absorption peaks around 2965 cm^{-1} assigned to C−H alkyl stretching, C=O, carbonyl band of an ester at 1715 cm^{-1}, C=C aromatic stretching peak at 1408 cm^{-1}, C−H bending peaks at 1456 cm^{-1} and 1372 cm^{-1} assigned to C−H of methylene (CH_2) group and C−H bend of methyl group (CH_3) respectively. C−O, phenolic of an ester were at 1238 cm^{-1}, 1091 cm^{-1} and 1016 cm^{-1}, and C=C aromatic bending at 723 cm^{-1} (Figure 4.72).

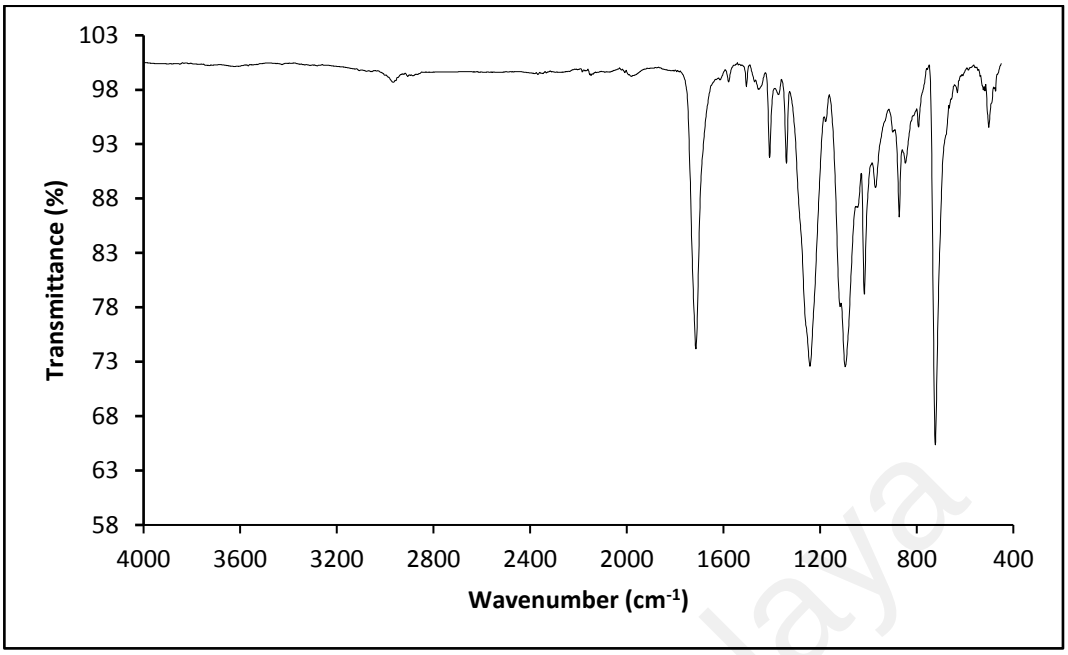

Figure 4.72: FTIR spectrum of uninoculated (control) PET microplastic

Figure 4.73 shows the changes that occurred in PET microplastics subjected to degradation by Treatment A. It resulted in the elongation of the absorption peaks of C=O, carbonyl band of an ester at 1715 cm^{-1}, C=C aromatic stretching peak at 1408 cm^{-1}, C−H bending of methylene (CH_2) group at 1454 cm^{-1}, and C−H bending of methyl group (CH_3) at 1372 cm^{-1}. Also observed were the elongation of C−O, phenolic of an ester at 1238 cm^{-1}, 1091 cm^{-1} and 1016 cm^{-1}, and C=C aromatic bending at 873 cm^{-1}. The peak at 470 cm^{-1} present in the spectrum of control PET microplastics disappeared.

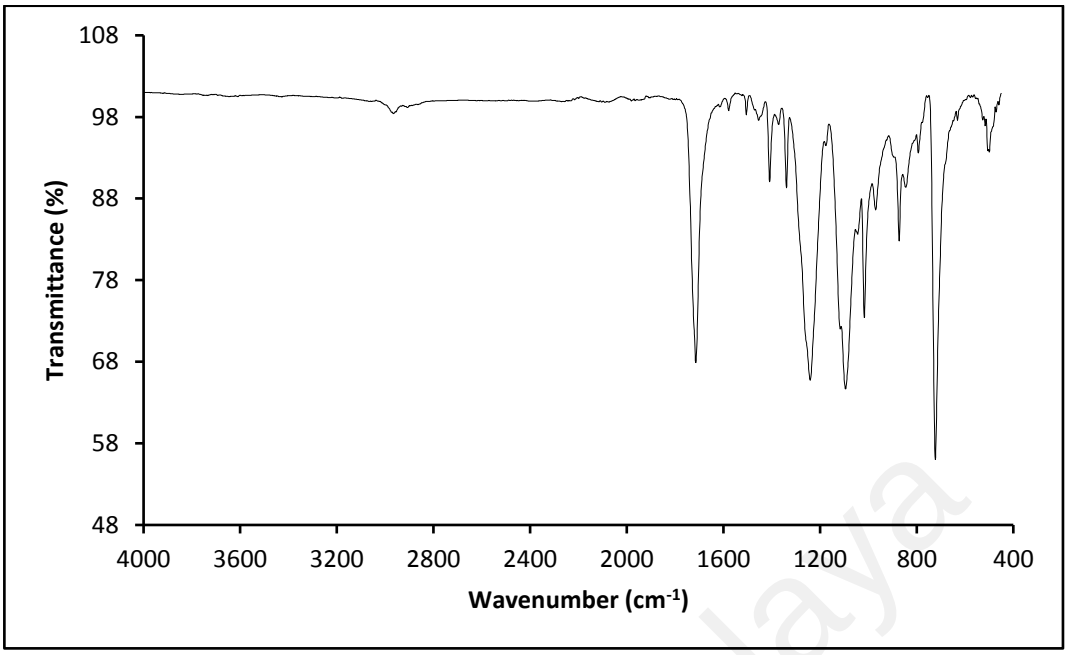

Figure 4.73: FTIR spectrum of PET microplastic inoculated with Treatment A

New peaks were formed at 1177 cm^{-1} and 1045 cm^{-1} assignable to C−O, phenolic of an ester when PET microplastics were inoculated with Treatment B (Figure 4.74). Also observed was the disappearance of the peak at 470 cm^{-1}. Furthermore, elongation of the absorption peaks of C=O, carbonyl band of an ester at 1714 cm^{-1}, C=C aromatic stretching peak at 1408 cm^{-1}, C−H bending of methylene (CH_2) group at 1454 cm^{-1}, C−H bending of methyl group (CH_3) at 1372 cm^{-1}, C−O, phenolic of an ester at 1238 cm^{-1}, 1091 cm^{-1} and 1016 cm^{-1} and C=C aromatic bending at 873 cm^{-1} were also observed.

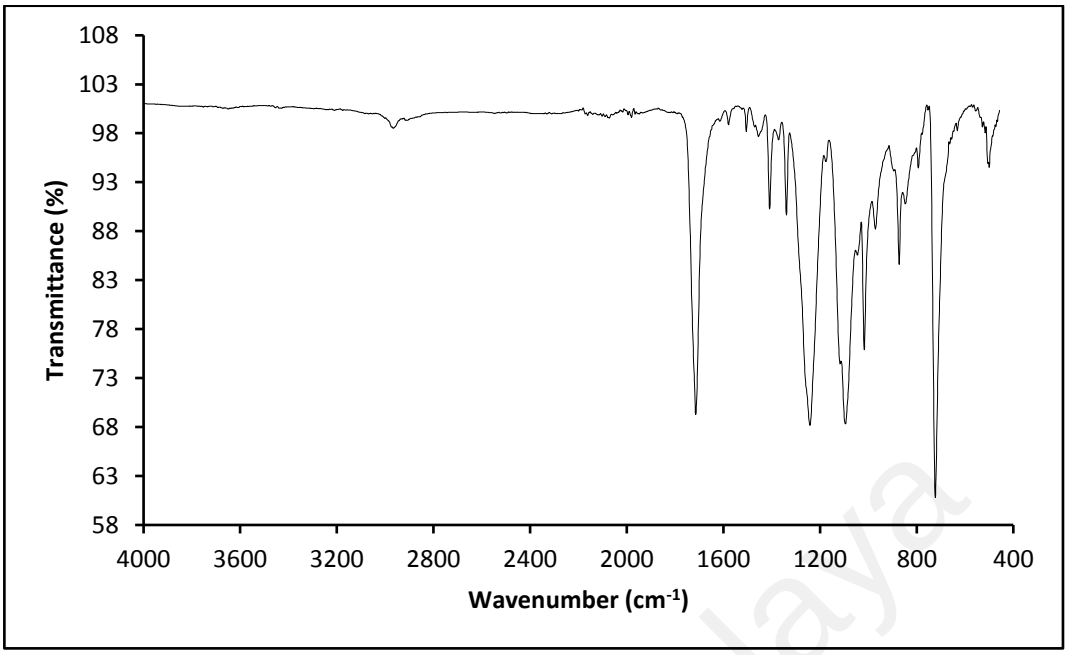

Figure 4.74: FTIR spectrum of PET microplastic inoculated with Treatment B

When PET microplastics were inoculated with Treatment C (Figure 4.75), new peaks were formed at 1175 cm^{-1} and 1043 cm^{-1} assignable to C−O, phenolic of an ester. The peak as that at 470 cm^{-1} in control PET microplastics disappeared. There was elongation of the absorption peaks of C=O, carbonyl band of an ester at 1714 cm^{-1}, C=C aromatic stretching peak at 1409 cm^{-1}, C−H bending of methylene (CH_2) group at 1454 cm^{-1}, C−H bending of methyl group (CH_3) at 1372 cm^{-1}, C−O, phenolic of an ester at 1238 cm^{-1}, 1091 cm^{-1} and 1016 cm^{-1} and C=C aromatic bending at 873 cm^{-1}.

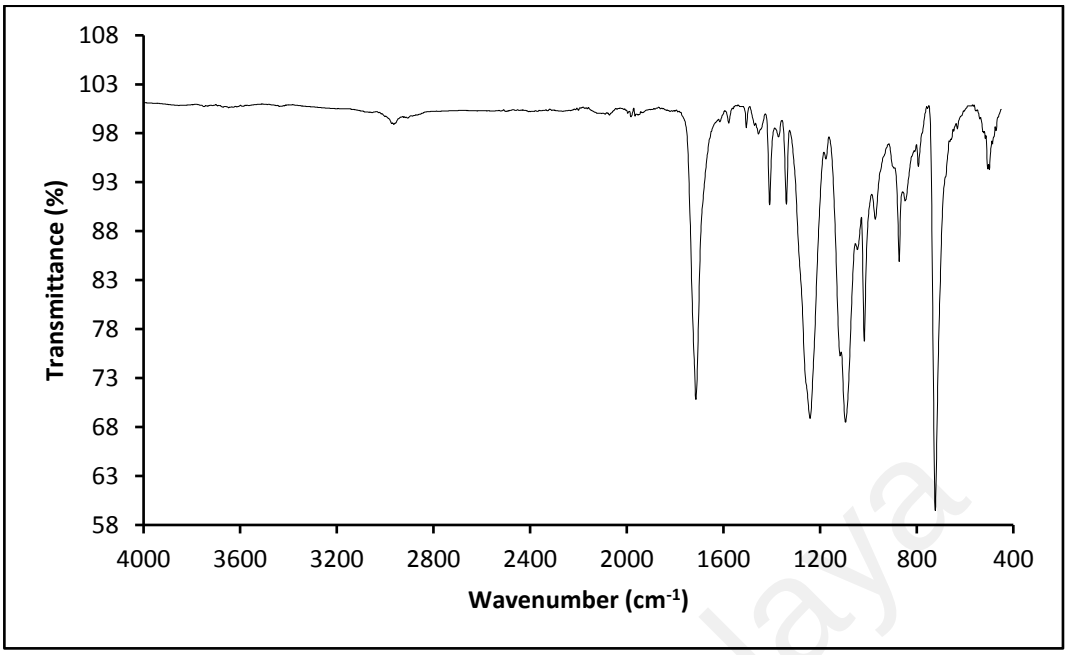

Figure 4.75: FTIR spectrum of PET microplastic inoculated with Treatment C

Compared to the control, new peaks were formed at 1177 cm^{-1}, 1116 cm^{-1} and 1045 cm^{-1} assignable to C−O, phenolic of an ester in the spectrum (Figure 4.76). Elongation of the absorption peaks of C=O, carbonyl band of an ester at 1714 cm^{-1}, C=C aromatic stretching peak at 1408 cm^{-1}, C−H bending of methylene (CH_2) group at 1454 cm^{-1}, and C−H bending of methyl group (CH_3) at 1372 cm^{-1} occurred. Additionally, the elongation of the absorption peaks of C−O, phenolic of an ester at 1238 cm^{-1}, 1091 cm^{-1} and 1016 cm^{-1} and C=C aromatic bending at 873 cm^{-1} were also observed.

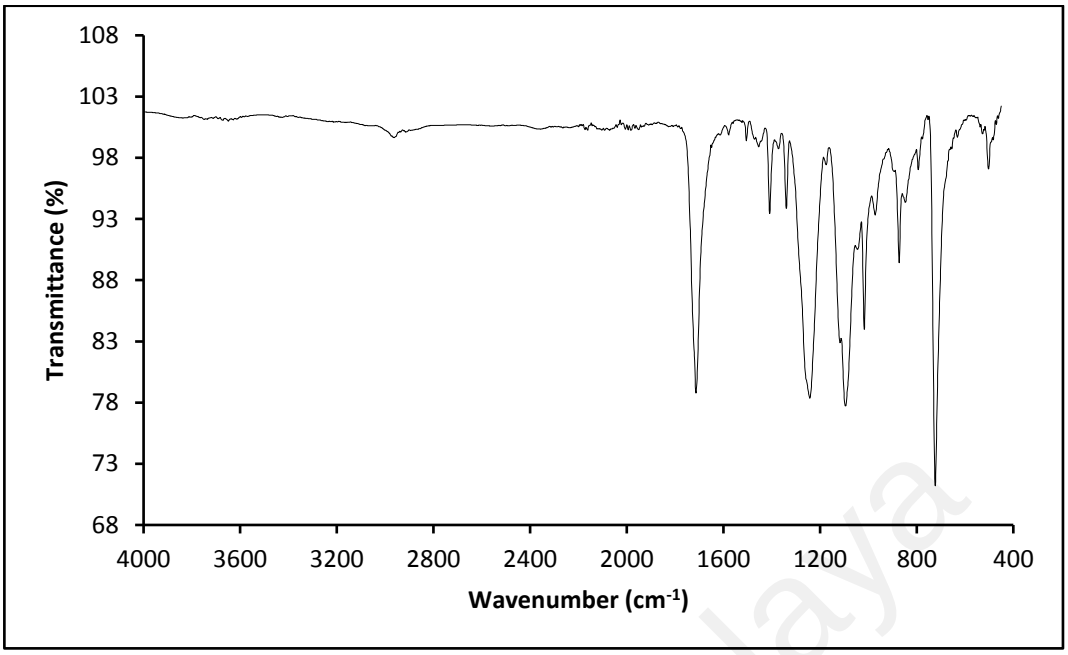

Figure 4.76: FTIR spectrum of PET microplastic inoculated with Treatment D

4.8.4 Biodegradation of PS microplastics using blended isolates

4.8.4.1 Determination of weight loss of PS microplastics inoculated with blended isolates

The weight loss in percentage after 40 days of biodegradation assay is presented in Figure 4.77. Treatment A recorded highest weight loss of 21.4%, this was followed by Treatment D with weight loss of 20.8%. Treatment B came in third with weight loss of 19.8% while Treatment C recorded the least weight loss of 19.6%. No weight loss was recorded in the uninoculated control PS samples. Analysis of variance indicated no significant difference existed between the degradation by the different treatments ($P < 0.05$).

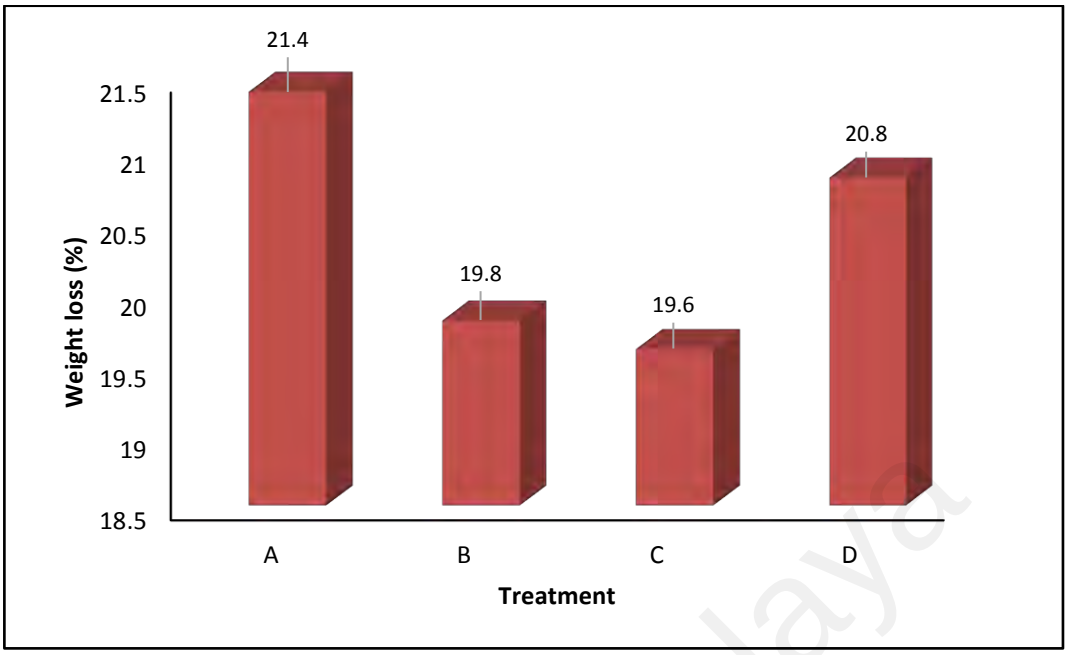

Figure 4.77: Weight loss of microbially treated PS microplastic after incubation for 40 days. Maximum weight loss (21.4%) was obtained for PS microplastic treated with Treatment

The weight loss of PS microplastic can be attributed to the breakdown of the carbon backbone of PS microplastic polymer as a result of enzymatic degradation by the microbial combinations. Burd (2008) also recorded higher degradation of PE when two strong PE degraders were combined. It can be concluded that the degree of PP microplastic degradation was significantly increased when the microbes were blended into consortia.

4.8.4.2 Growth pattern of blended microbes in PS microplastic-infused media

The growth pattern of the different treatments in mineral salt media containing PS microplastics is presented in Figure 4.78. All four treatments exhibited similar growth pattern upon exposure to PS microplastics. This was characterized by several major phases that followed one after the other for all treatments.

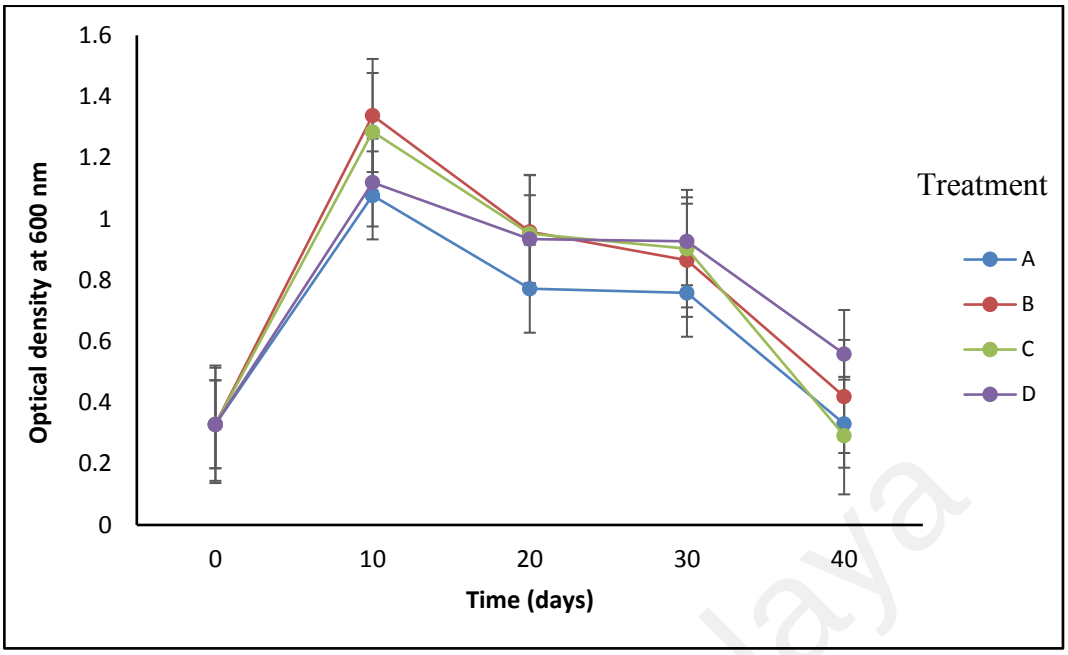

Figure 4.78: Growth pattern of blended microbes in PS microplastic-infused media during biodegradation studies

A log phase pattern was observed in the first 10 days which recorded the highest growth. Treatment B recorded the highest OD reading of 1.338 OD_{600}, before Treatment C with 1.285 OD_{600}, and Treatment D with 1.119 OD_{600}. Treatment A recorded the least OD reading of 1.077 OD_{600}. This corresponded with highest cell counts recorded on same day by the microbes. This possibly implied an increase in metabolic activity in the treatments. This was however, followed by a phase of declining growth observed on the 20th day for all treatments which could have been due to depletion of nutrients or the subsequent accumulation of metabolic waste products and other toxic chemicals in the culture media. Continued decline in growth was observed for Treatments B and C on the 30th and 40th days. Treatments A and D however, observed a stationary phase of growth on Days 20 and 30 (0.772 OD_{600} and 0.759 OD_{600} for Treatment A and 0.934 OD_{600} and 0.927 OD_{600} for Treatment D, respectively), indicating an almost balance between growing cells and dying cells a condition that might have been caused by stress associated with the biodegradation process such as waste build-up. Inhibition of cell growth due to waste build-up has been reported to occur in cultures with high density (Maier *et al.*,

2009). A decline in growth was observed for Treatments A and D on Days 30 and 40 indicating continuous cell death due to unfavourable conditions or decline in nutrient of the culture media.

Total bacterial counts ranged from 6.4 x 10^6 CFU/ml to 1.7 x 10^{11} CFU/ml for treatment A, 7.2 x 10^8 CFU/ml to 3.5 x 10^{11} CFU/ml for treatment B, 7.2 x 10^4 CFU/ml to 3.9 x 10^{11} CFU/ml for treatment C and 9.6 x 10^6 CFU/ml to 1.76 x 10^{11} CFU/ml for treatment D (Figure 4.79).

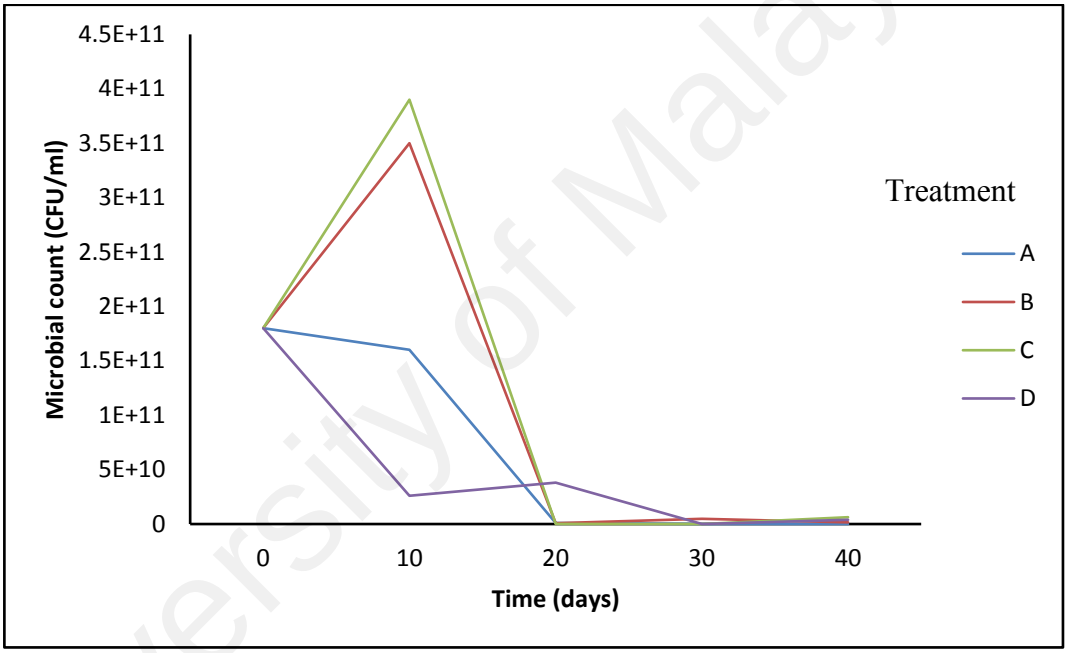

Figure 4.79: Bacterial counts of blended microbes inoculated in PS microplastic-infused media during 40 days biodegradation assay. Bars indicate standard error (n = 3).

The highest count (3.9 x 10^{11} CFU/ml) was recorded in Treatment C on Day 10, followed by Treatment B (3.5 x 10^{11} CFU/ml). On Day 10, the counts for Treatments A and D dropped below the initial counts recorded at the start of the experiment. This is probably due to the inability of the microbes to completely adapt to the culture conditions of the media. Studies have reported that when a population is introduced into a foreign environment, it tends to decrease with time due to abiotic or biotic factors such as

temperature and pH changes (Vásquez-Murrieta *et al.*, 2016). Day 20 witnessed a sharp decline in counts for all treatments except Treatment D which experienced an increase in cell counts from 2.6 x 10^{10} CFU/ml to 3.8 x 10^{10} CFU/ml. This increase could have been due to favourable conditions in the culture media. A decline in counts of Treatments A, C and D was observed on Day 30 while the counts of Treatment B increased. Treatment A maintained a count of 6.4 x 10^{6} CFU/ml on the 30th and 40th days, indicating that the cells had attained a stationary phase of growth where the number of living cells equals the number of dying cells; a condition that may have been brought about due to limited resources or build-up of bacterial waste products. The counts in Treatment B declined from 4.7 x 10^{9} CFU/ml on Day 30 to 1.7 x 10^{9} CFU/ml on Day 40. The counts of Treatment C and D however, increased on Day 40, with Treatment C recording highest counts of 6.3 x 10^{9} CFU/ml, indicating that the conditions may have been made favourable for the growth of the microbes.

4.8.4.3 Changes in pH of PS microplastic culture media during 40 days biodegradation studies using blended microbes

The periodic pH measurements of PS microplastic culture media are shown in Figure 4.80. It was observed that the alkalinity of the PS medium inoculated with the treatments increased over the incubation time across all treatments whereas, the pH of control remained unchanged.

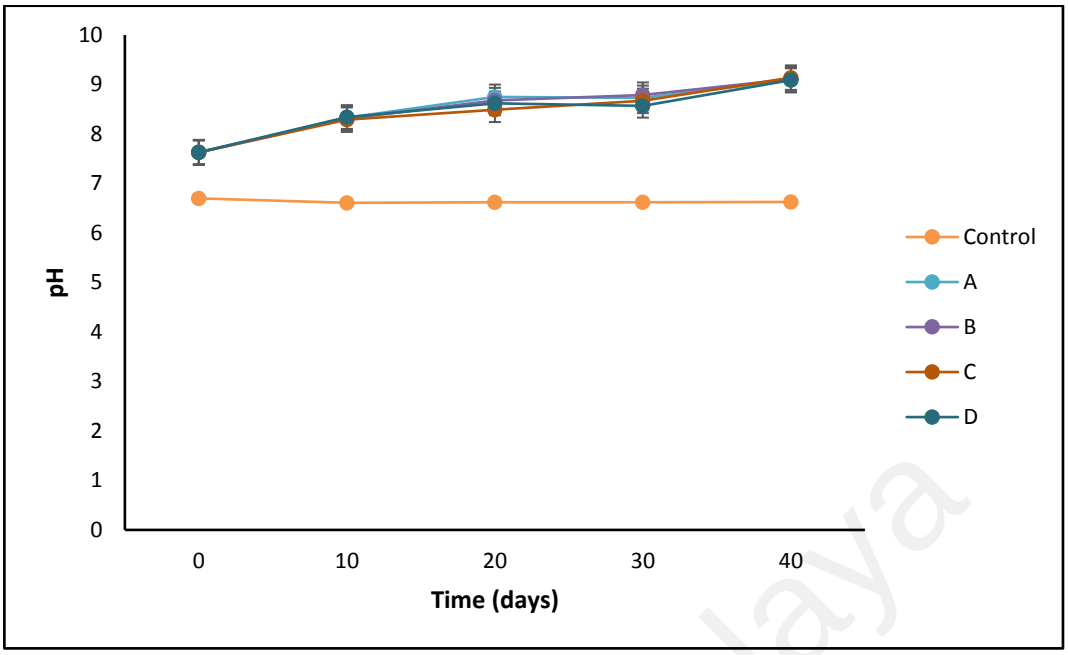

Figure 4.80: pH changes of PS microplastic-infused media inoculated with blended microbes during biodegradation studies. Each data point represents the average of three replicates ± SD

On the 10th day, Treatments A, B, C and D recorded pH 8.33, pH 8.31, pH 8.29 and pH 8.34, respectively. The culture media experienced continuous elevation in pH that reached pH 9.09, pH 9.11, pH 9.14 and pH 9.09 for Treatments A, B C and D, respectively. The increase in pH of the culture medium media towards alkalinity indicated the metabolic activities of the microbes in the mineral salt medium containing PS microplastics as carbon source. Similar pH changes were reported by Muhsin and Hadi (2002) on the alkalinization of culture medium during keratin degradation by fungi.

4.8.4.4 Biodegradation rate constant and half-life of PS microplastics inoculated with blended microbes

The removal rate constant (k) and half-life of PS microplastics across treatments is presented in Table 4.15.

Table 4.15: Removal rate constant (*k*) and half-life of PS microplastics across treatments

Treatment	Removal constant (*k*) day^{-1}	Half-life (days)
Control	0	∞
A	0.00601	114.6
B	0.0055	125.45
C	0.00543	156.81
D	0.00583	118.35

n = 3

The highest rate of removal calculated and least half-life were obtained in PS microplastics inoculated with Treatment A with removal rate and half-life of 0.006 day^{-1} and 114.6 days, respectively. This could have resulted to the removal ability of the microbes in Treatment A. This was followed by Treatment D which recorded removal rate of 0.0058 day^{-1} and half-life of 118.35 days. Treatment B recorded removal rate of 0.0055 day^{-1} and half-life of 125.45 days while the least removal rate was observed in Treatment C (0.0054 day^{-1} and half-life of 156.81 days). The low bio-removal rate and subsequent higher half-life recorded by Treatment C could be from the reduction in the activity of the microbes in the culture media. This has been reported in other studies (Adesodun & Mbagwu, 2008). The highest removal rate of Treatment A is expected since the treatment recorded highest weight loss of PS microplastics among all treatments. No removal was observed in control sample.

4.8.4.5 Fourier transform infrared (FTIR) analysis of PS microplastics inoculated with blended microbes

The FTIR analysis of PS microplastics recovered in the shake flask experiment after 40 days incubation period with blended isolates showed changes in peak intensities in different regions of the spectra, demonstrating changes in the structure of PS

microplastics as a result of microbial treatment. Figure 4.81 shows the FTIR spectrum of control PS microplastics.

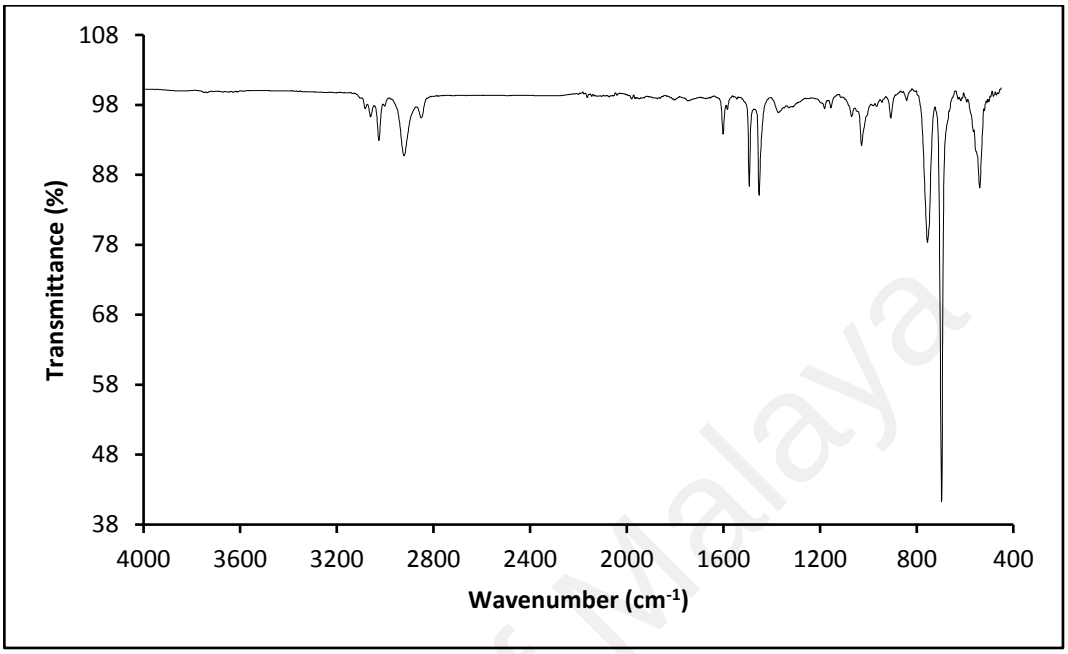

Figure 4.81: FTIR spectrum of uninoculated (control) PS microplastic

Control PS microplastics showed absorption peaks at 3060 cm^{-1} and 3026 cm^{-1} for C−H aromatic stretching, and peaks at 2922 cm^{-1} and 2850 cm^{-1} for C−H aliphatic (alkane) stretching. The peaks at 1601 cm^{-1}, and 1493 cm^{-1} are attributable to C=C stretch of an aromatic. The peaks at 1452 cm^{-1} and 1370 cm^{-1} are attributable to C-H bend of a methylene (CH_2) group and methyl (CH_3) group, respectively. The absorption peaks at 754 cm^{-1}, 677 cm^{-1} and 616 cm^{-1} are assigned to C−H bending of a benzene derivative compound. An absorption peak is also present at 539 cm^{-1}.

In Treatment A, the C−H aromatic stretching peak at 3026 cm^{-1} became reduced compared to uninoculated PS microplastics (Figure 4.82). The peaks at 2921 cm^{-1} and 2850 cm^{-1} assigned to C−H aliphatic stretching also became reduced. The C=C aromatic stretching peaks at 1601 cm^{-1}, 1493 cm^{-1} and 1452 cm^{-1} also reduced due to microbial action. The peaks at 1452 cm^{-1} and 1370 cm^{-1} are attributed to C–H bend of a methylene

(CH_2) group and methyl (CH_3) group, respectively. The C−H bending of benzene derivative compounds at 752 cm^{-1} and 695 cm^{-1}, and the peak at 539 cm^{-1} however, became elongated.

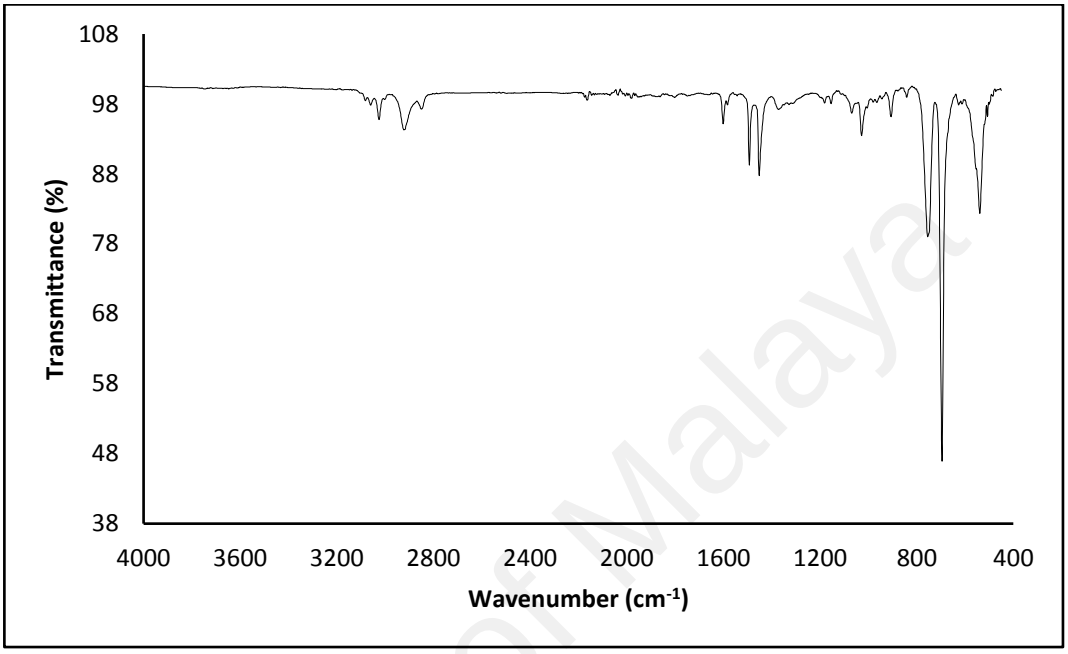

Figure 4.82: FTIR spectrum of PS microplastic inoculated with Treatment A

Figure 4.83 shows the FTIR spectrum of PS microplastics in Treatment B. The spectrum showed reduction in C−H aromatic stretching peak at 3026 cm^{-1} and C−H aliphatic stretching at 2921 cm^{-1}. The C=C stretch aromatic peaks at 1601 cm^{-1} and 1493 cm^{-1} became reduced. Reduction of peaks at 1452 cm^{-1} and 1370 cm^{-1} attributed to C–H bend of a methylene (CH_2) group and methyl (CH_3) group, respectively, were similarly observed.

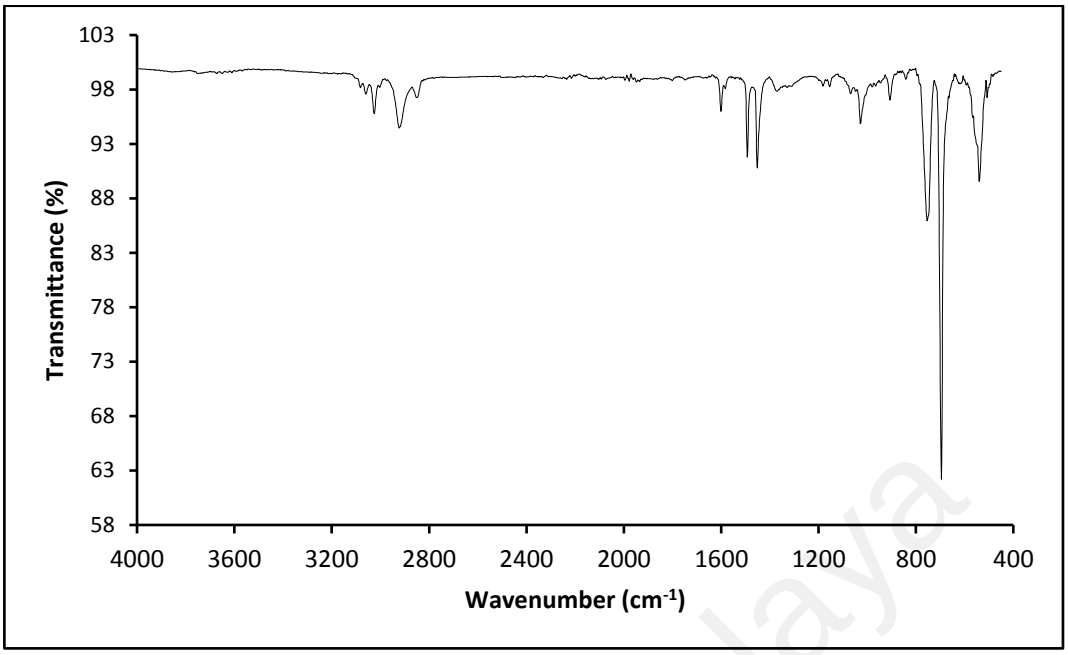

Figure 4.83: FTIR spectrum of PS microplastic inoculated with Treatment B

For PS microplastics in Treatment C, the C−H aromatic stretching peak at 3026 cm^{-1}, and the C−H aliphatic stretching peak at 2921 cm^{-1} became reduced (Figure 4.84). Moreover, the peaks at 1601 cm^{-1} and 1493 cm^{-1} attributed to C=C aromatic stretching peaks also became reduced. Similarly, reduction in the peaks at 1452 cm^{-1} and 1370 cm^{-1} attributed to C−H bend of a methylene (CH_2) group and methyl (CH_3) group respectively, also became reduced.

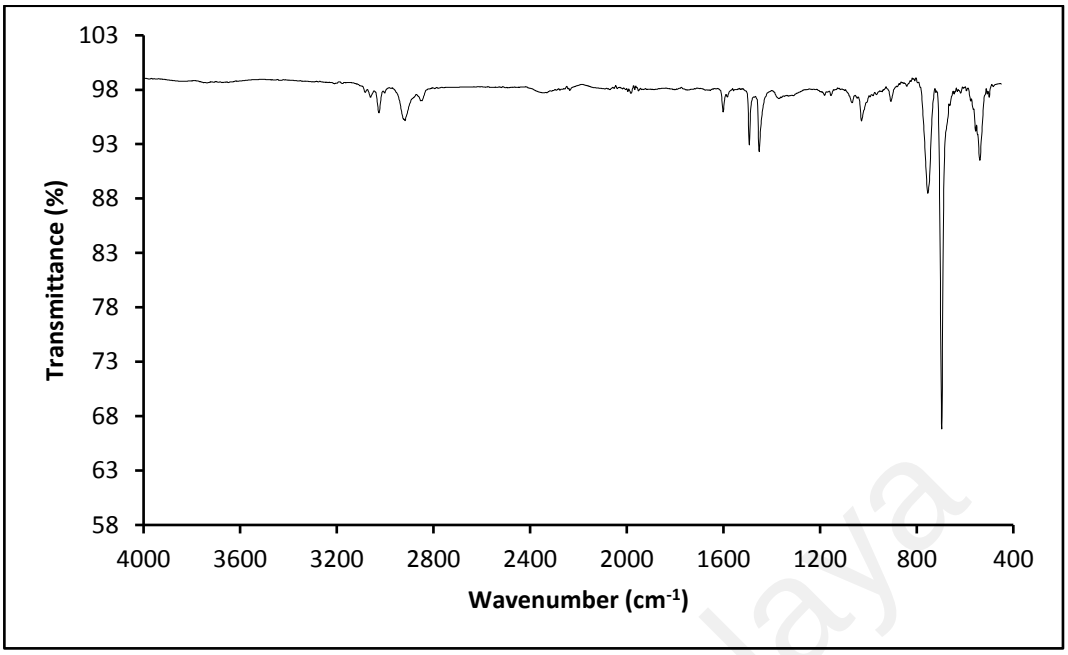

Figure 4.84: FTIR spectrum of PS microplastic inoculated with Treatment C

The aromatic C−H stretching peak at 3026 cm^{-1} and C−H aliphatic stretching at 2921 cm^{-1} both became reduced in Treatment D as compared to control PS microplastics (Figure 4.85).

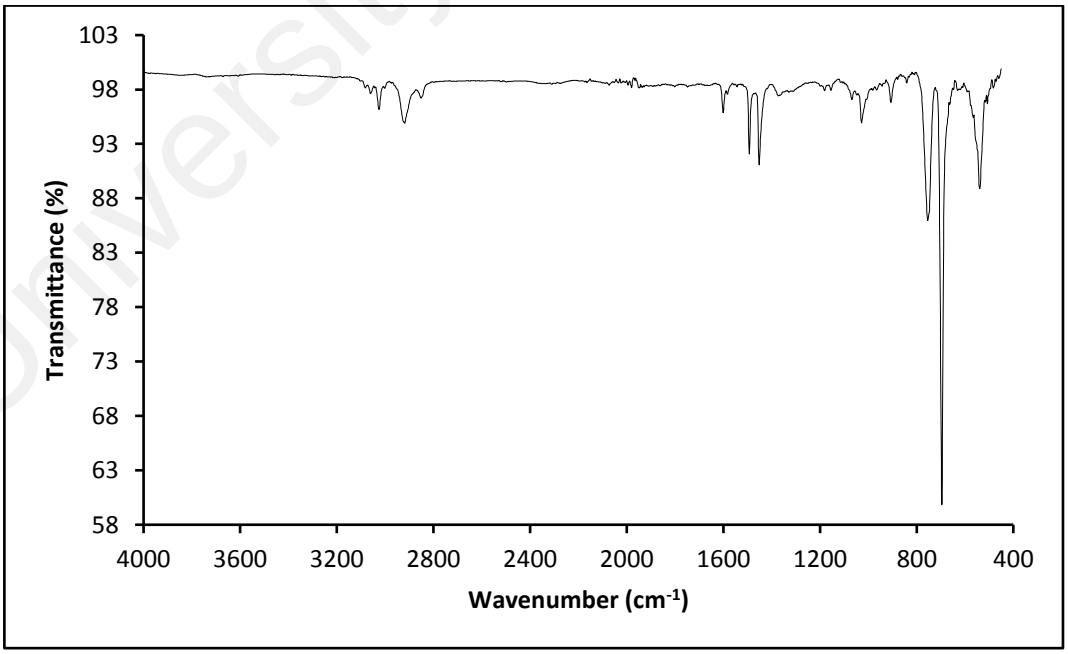

Figure 4.85: FTIR spectrum of PS microplastic inoculated with Treatment D

The aromatic C=C stretch peaks at 1601 cm^{-1} and 1493 cm^{-1} all became reduced. Reduction of the C−H peaks at 1452 cm^{-1} and 1370 cm^{-1} attributed to C-H bend of a methylene (CH_2) group and methyl (CH_3) group, respectively, occurred. Similar reduction in peaks were observed by Atiq (2011) in the study of the degradation of polystyrene and styrofoam by *R. oryzae*, *A. terreus*, and *P. chrysosporium*.

4.8.5 Effect of inoculum concentration on the degradation of PET and PS microplastics

4.8.5.1 Determination of weight loss of PET microplastics inoculated with different inoculum concentrations of Treatment D

Figure 4.86 illustrates the weight loss in percentage of PET microplastics with different inoculum concentrations.

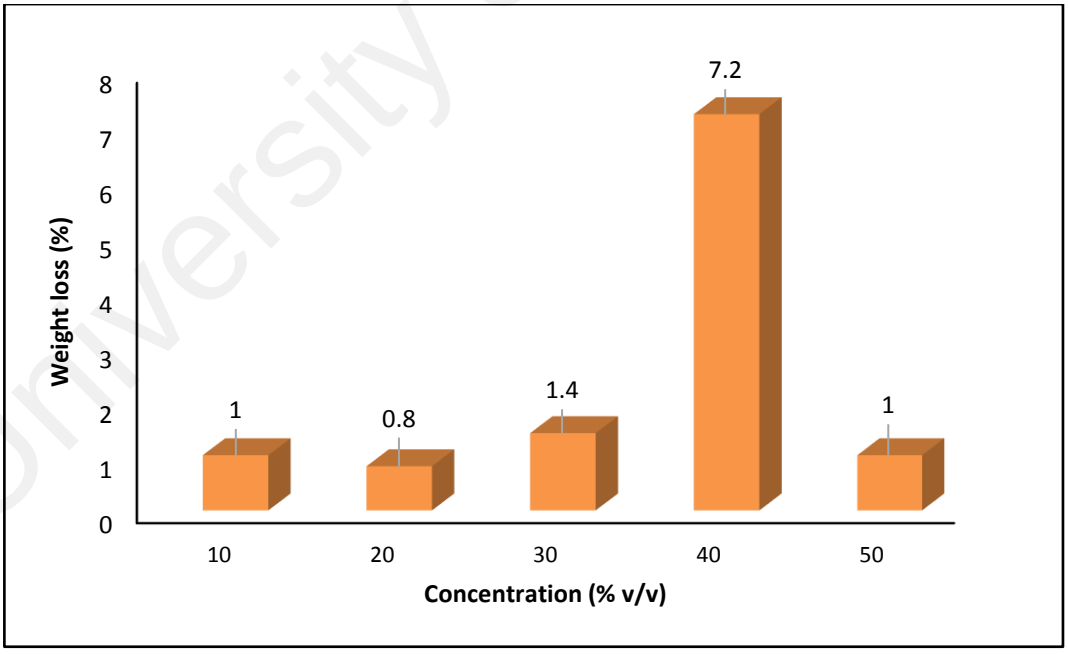

Figure 4.86: Weight loss of microbially treated PET microplastics after incubation for 40 days. Maximum weight loss (7.2%) was obtained for PET microplastics treated with 40% v/v inoculum concentration

At inoculum concentration of 10% v/v, the weight loss was 1.0%. While increasing the concentration to 20% gave a reduced weight loss of 0.8%. The weight loss slightly increased when the inoculum concentration was 30% v/v. Highest weight loss of PET microplastics was observed with 40% v/v inoculum concentration with weight loss of 7.2%. However, increase in inoculum concentration to 20% v/v resulted in decrease in weight loss of PET microplastics (0.8%). This could be an effect of physiological change (not monitored) which did not enhance metabolic interaction of the bacterial cells to induce higher degradation impact. With 30% v/v of inoculum concentration, a slight increase in weight loss (1.4%) was recorded. Further increase in inoculum concentration beyond 40% v/v resulted in decrease in weight loss (1.0%) (F-value = 3.229; P = 0.06). The results depict that inoculum concentration of 40% v/v was the optimal concentration for PET microplastic degradation with Treatment D. The low weight loss for PET microplastics treated with lower inoculum concentrations (10, 20, and 30% v/v) maybe due to the lower number of cells as compared to the number of cells in 40% v/v inoculum concentration. However, the higher weight loss recorded with 40% v/v inoculum concentration could have been due to the high cell concentration and the unique nature of diverse microbial cells and their metabolic activities in the PET microplastic culture media. High cell concentrations have been reported to cause increased activities of microbes (Dada *et al.*, 2012). Wolski *et al.* (2006) and Kauselya *et al.* (2015) also reported increase in degradation of pentachlorophenol (PCP) and benzene with increasing inoculum concentration, respectively.

It was observed in this study that higher inoculum concentration beyond 40% v/v resulted in lower degradation rate of PET microplastics.

4.8.5.2 Microbial growth pattern at different concentrations in PET microplastic-infused media

In order to understand the extent of physiological and metabolic changes within the microcosm, growth response monitored showed variations along the inoculum concentrations for PET microplastics degradation (Figure 4.87).

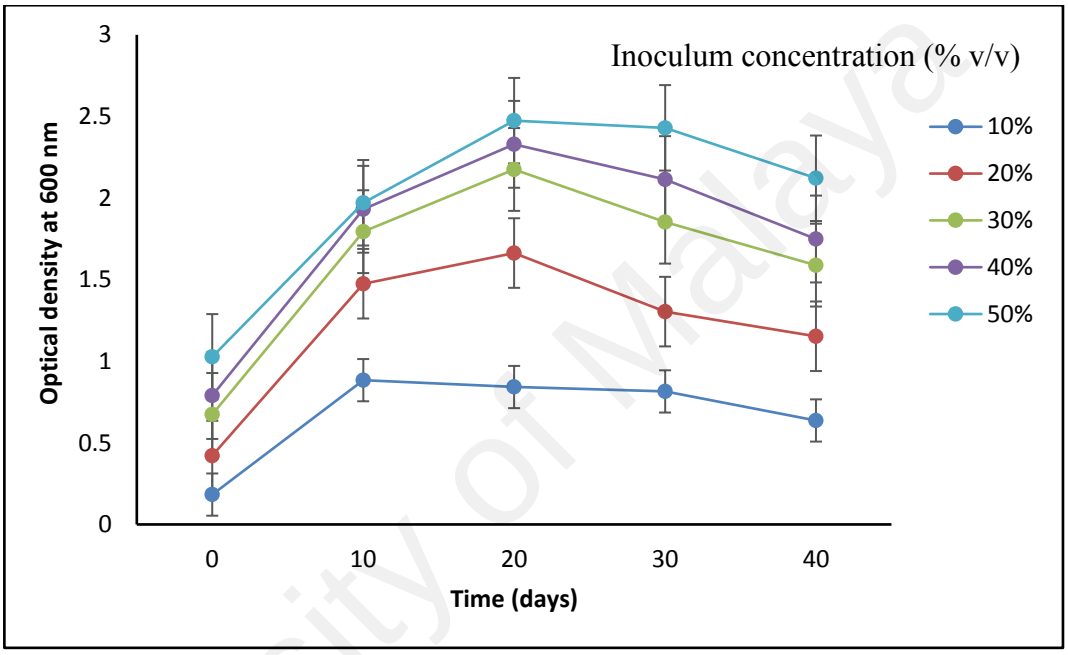

Figure 4.87: Growth pattern of different concentrations of treatment D in PET microplastic-infused media during biodegradation studies

To assess possible growth in the experiment, the absorbance for PET laced with 10% v/v microbes recorded 0.884 OD_{600} on the 10th day (the maximum attained in the treatment across 40 days). This was accompanied by a gradual decline phase on Day 20 with absorbance reading of 0.842 OD_{600}. Growth of microbes in 20%, 30%, 40% and 50% v/v inoculum concentrations however, recorded higher absorbance readings of 1.663 OD_{600}, 2.175 OD_{600}, 2.329 OD_{600}, and 2.474 OD_{600}, respectively on the 20th day of biomonitoring. A gradual decline in growth was exhibited by the microbes on the 30th day, recording absorbance readings of 0.815 OD_{600}, 1.852 OD_{600}, 2.113 OD_{600} and 2.430 OD_{600} for 10%, 20%, 30%, 40% and 50% v/v inoculum concentrations, respectively. The

40th day showed further decline in growth of microbes in all inoculum concentrations. Statistical analysis showed significant difference between the growth of the microbes during the different days of monitoring ($P < 0.05$). The optimal OD readings observed for the different inoculum concentrations on Day 10 (10%v/v) and Day 20 (20%, 30%, 40% and 50% v/v) probably depict the duration for the most favourable period of interaction between the bacterial cells and PET microplastics. It is therefore, important to presume that among all inoculum concentrations, the 10% v/v concentration exhibited more survival potential between Days 10 to 30 of exposure to PET microplastics. However, light absorbance may not reflect sign of absolute growth in the flask experiment because microbes readily generate metabolites which may increase turbidity to give higher OD_{600} readings.

Hence, the suspected growth became justifiable with bacterial cell counts while monitoring the experiment. The population count of the microbes during the biodegradation period revealed variations across the different inoculum concentrations and biomonitoring days. Figure 4.88 demonstrates that the counts ranged within 5.5×10^9 CFU/ml to 1.4×10^{11} CFU/ml with 10% v/v inoculum concentration. 20% v/v inoculum concentration recorded counts that ranged within 1.9×10^{10} CFU/ml and 2.7×10^{11} CFU/ml while counts of 30% inoculum concentration ranged within 3.4×10^9 CFU/ml and 2.0×10^{11} CFU/ml, while 40% v/v and 50% v/v inoculum concentrations recorded counts that ranged within 3.4×10^9 CFU/ml and 3.9×10^9 CFU/ml, and 6.4×10^9 CFU/ml and 1.9×10^{11} CFU/ml, respectively. The highest counts of 2.7×10^{11} were recorded with 20% v/v inoculum concentration on Day 10. This was followed by 50% v/v inoculum concentration, with counts of 1.9×10^{11} CFU/ml.

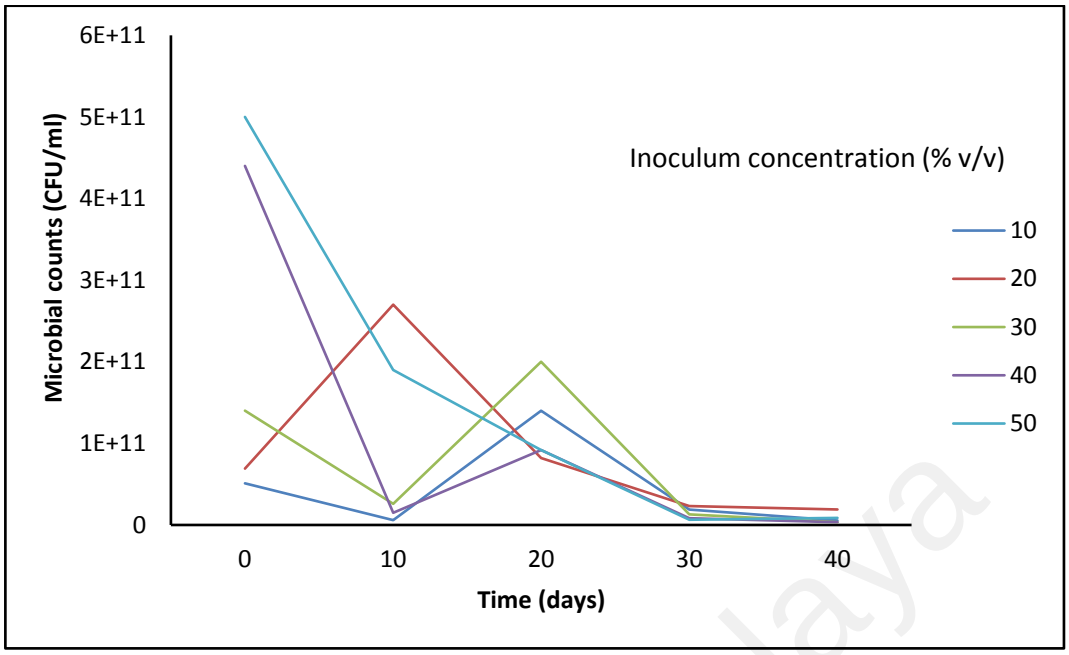

Figure 4.88: Bacterial counts of PET microplastics treated with different concentrations of treatment D during 40 days biodegradation assay.

30% v/v and 40% v/v inoculum concentrations recorded counts of 2.6 x 10^{10} CFU/ml and 1.5 x 10^{10} CFU/ml, respectively. These counts were lower than the initial counts recorded at the start of the experiment. Least count of 5.8 x 10^{9} CFU/ml was recorded with 10% v/v inoculum concentration. This study inferred that the changes in bacterial cell count did not only indicate metabolic interaction that ensured survival within the microplastic-infused culture media, rather, it showed that increase in population density of the microbes does not give a direct impact on degradation alone. Instead, formation of metabolites (not analysed) could reduce growth concentration while enhancing microplastic degradation due to physico-chemical interaction between the metabolites and carbon structure of the microplastics.

4.8.5.3 Changes in pH of PET microplastic culture media during 40 days biodegradation studies using different concentrations of Treatment D

Figure 4.89 illustrates the changes in pH in PET microplastics with different inoculum concentrations.

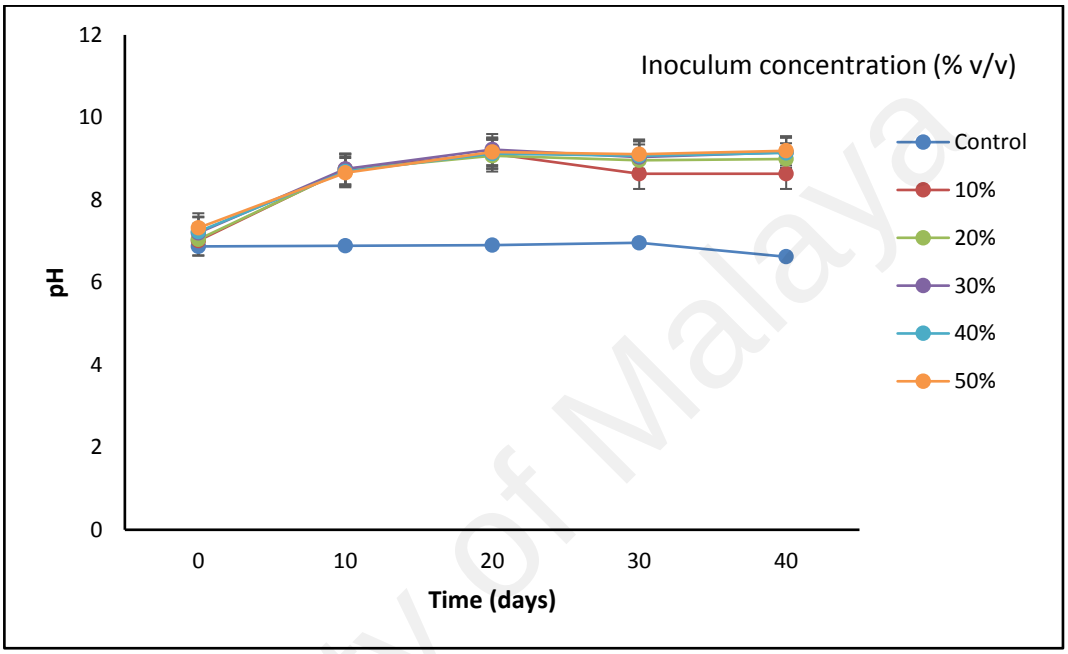

Figure 4.89: pH changes of PET microplastic-infused media inoculated with different concentrations of Treatment D during biodegradation studies. Each data point represents the average of three replicates ± SD

On the 10th day of biomonitoring, increase in pH were observed. This recorded an increase from 7.01 to 8.74 for 10% v/v inoculum, pH 7.04 to 8.73 for 20% v/v, 7pH .2 to 8.75 for 30% v/v, pH 7.23 to 8.69 for 40% v/v, and pH 7.32 to 8.66 for 50% v/v inoculum. This may be due to the release of metabolic products such as ammonia or other basic components into the culture media during the biodegradation process that caused the pH to drift towards alkalinity. Further increase in pH was observed on the 20th day; pH 9.12, pH 9.07, pH 9.22, pH 9.12 and pH 9.16 recorded for 10% v/v, 20% v/v, 30% v/v, 40% v/v and 50% v/v inoculums, respectively. Similar increase in pH was observed by Unmar and Mohee (2008), in their evaluation of the effect of Mater-Bi, a biodegradable plastic

made from corn starch, on composting system. Leejarkpai *et al.* (2011) in their study of the biodegradation kinetics of plastics under controlled composting conditions, also recorded pH increase during biodegradation.

Conversely, the pH levels gradually decreased to pH 8.63, pH 8.96, pH 9.04, pH 9.07, and pH 9.11 for 10% v/v, 20% v/v, 30% v/v, 40% v/v and 50% v/v inoculums, respectively, on the 30th day. Such could indicate the probable formation of organic acids which might have been responsible for the slight reduction in pH levels. Ghorpade *et al.* (2001) also observed similar decrease in pH and reported that lactic acid generation occur during PLA biodegradation which result in pH decrease. On the 40th day of biodegradation, the pH of PET culture media treated with 10% v/v inoculum remained as that on the 30th day (pH 8.63), while the pH increased in 20%, 30%, 40% and 50% v/v to pH 8.99, pH 9.15, pH 9.14, and pH 9.19, respectively. Statistical analysis demonstrated that no significant difference existed between the pH changes of the different inoculum concentrations on the 10th and 20th days of incubation. However, there was a significant difference in the pH changes on the 30th and 40th days ($P < 0.05$). It can thus, be concluded that the pH changes observed supported the metabolic activity of the strains on PET microplastics degradation.

4.8.5.4 Biodegradation rate constant and half-life of PET microplastics inoculated with different inoculum concentrations

The biodegradation rate constant (k) and the corresponding half-life values ($t_{1/2}$) of PET microplastics treated with the different inoculum concentrations are presented in Table 4.16.

Table 4.16: Removal rate constant (*k*) and half-life of PET microplastics across different inoculum concentrations of Treatment D

Concentration (% v/v)	Removal constant (*k*) (day^{-1})	Half-life ($t_{1/2}$) (days)
Control	0	∞
10	0.00025	2772
20	0.0002	3465
30	0.00035	1980
40	0.0013	533.07
50	0.00025	2772

It showed that PET microplastics treated with 40% v/v inoculum recorded the highest reduction rate of 0.0013 day^{-1}. The 40% v/v inoculum treated PET microplastics recorded the least time (half-life) of 533 days. The 30% v/v inoculum had removal rate of 0.00035 day^{-1} and half-life of 1980 days while 10% v/v and 50% v/v inoculums had similar result with removal rate of 0.00025 day^{-1} and half-life of 2772 days. However, treatment with 20% v/v inoculum recorded the least removal rate of 0.0002 day^{-1} and half-life of 3465 days. No removal/reduction was observed in the control PET microplastic samples. The variations in the degradation rate constants among the different inoculum concentrations may have been due to the different numbers of bacterial populations. Increase in inoculum has been reported to have significant effect on degradation (Dada *et al.*, 2012). In the present study, it was observed that when the bacterial cells were increased to 40% v/v in the system, higher PET microplastics removal was observed, which indicate that bacterial strains or enrichment can degrade PET microplastics at higher inoculum concentrations. Hence, it can be concluded that inoculum concentration influenced PET degradation, and 40% v/v of inoculum concentration was the best concentration for PET microplastics degradation in the study.

4.8.5.5 Fourier transform infrared (FTIR) analysis of PET microplastics inoculated with different concentrations of Treatment D

Figure 4.90 shows the FTIR spectrum of control PET microplastics. The spectrum showed absorption peaks at 2965 cm^{-1} assigned to C−H alkyl stretching, 1714 cm^{-1} assigned to C=O, carbonyl band of an ester, 1408 cm^{-1} assigned to C=C aromatic stretching peak, 1452 cm^{-1} and 1339cm^{-1} assigned to C−H bending peaks of methylene (CH_2) group and C−H bend of methyl group (CH_3) respectively, 1240 cm^{-1}, 1095 cm^{-1} and 1017 cm^{-1} assigned to C−O, phenolic of an ester, and 723 cm^{-1} attributable to C−H aromatic bending.

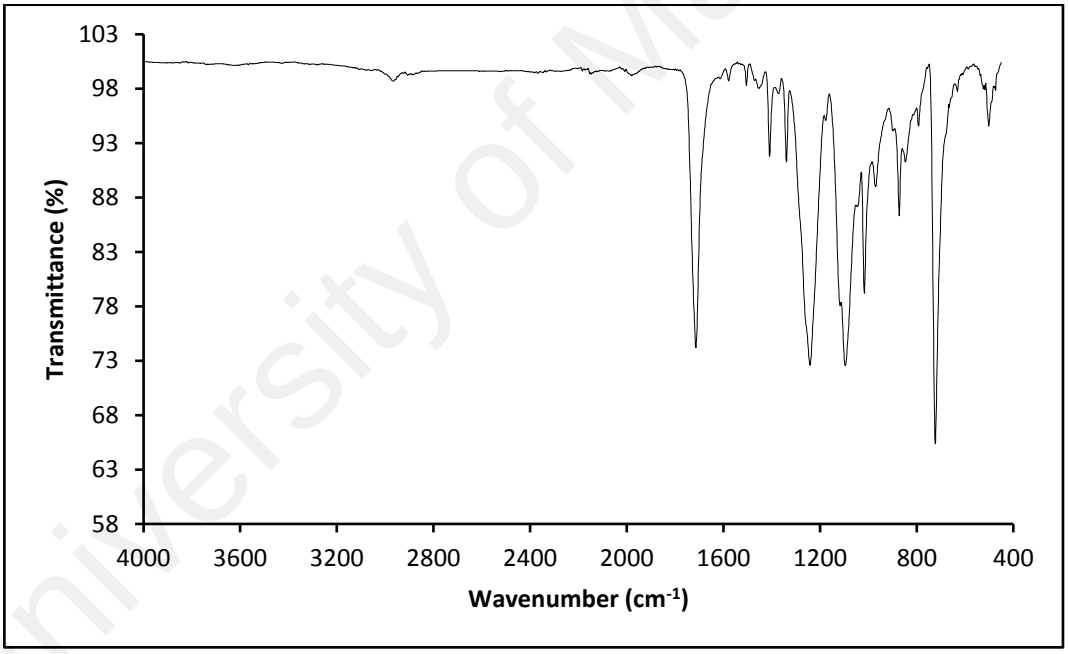

Figure 4.90: FTIR spectrum of control (uninoculated) PET microplastics

Inoculation with 10% v/v of inoculum resulted in the formation of a new peak at 1175 and 1045 assignable to C−O, phenolic of an ester which was absent in the control (Figure 4.91). The absorption peaks of C−H alkyl stretching at 2965 cm^{-1}, C=O, carbonyl band of an ester at 1713 cm^{-1}, C=C aromatic stretching peak at 1408 cm^{-1}, and C−H bending of methylene (CH_2) group at 1453 cm^{-1}, became elongated. Also observed was the

elongation of the C−H bending of methyl group (CH_3) at 1339 cm^{-1}, C−O, phenolic of an ester at 1238 cm^{-1}, 1091 cm^{-1} and 1016 cm^{-1} and C−H aromatic bending at 723 $cm^{-1}$and 632 cm^{-1}.

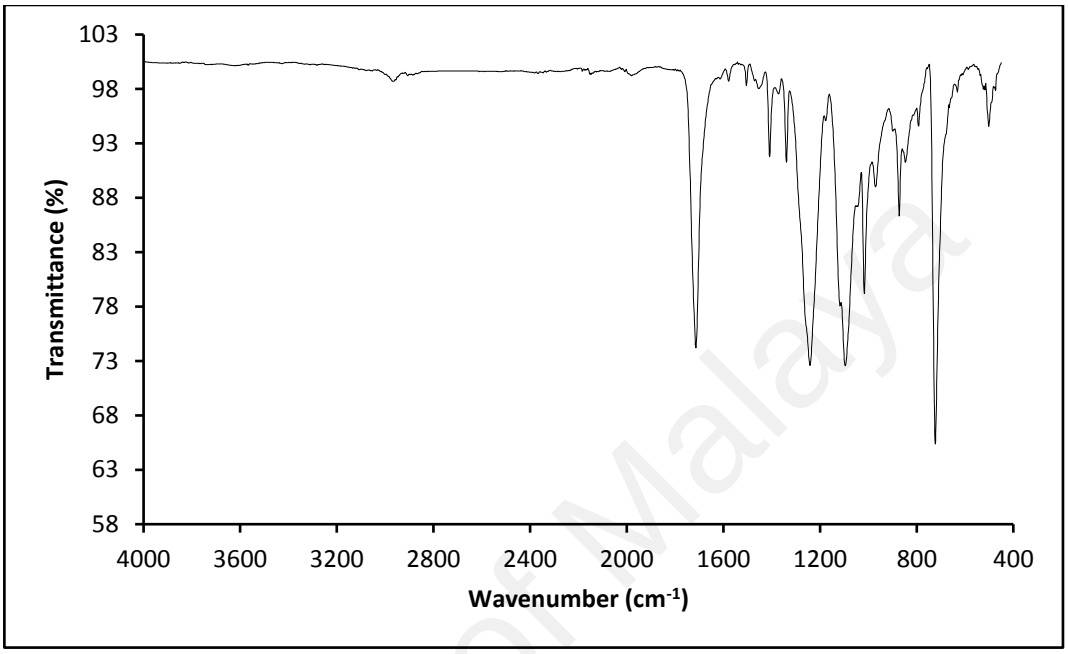

Figure 4.91: FTIR spectrum of PET microplastic treated with 10% v/v of inoculum concentration of Treatment D

The formation of a new peak around 3700 assignable to O−H, hydroxyl stretching occurred when PET microplastics were subjected to degradation with 20% v/v inoculum (Figure 4.92). There was reduction of the absorption peaks of C−H alkyl stretching at 2965 cm^{-1}, C=O, carbonyl band of an ester at 1712 cm^{-1}, C=C aromatic stretching peak at 1408 cm^{-1}, C−H bending of methylene (CH_2) group at 1454 cm^{-1}, C−H bending of methyl group (CH_3) at 1340 cm^{-1}, C−O, phenolic of an ester at 1244 cm^{-1}, 1092 cm^{-1} and 1016 cm^{-1} and C−H aromatic bending at 723 cm^{-1}.

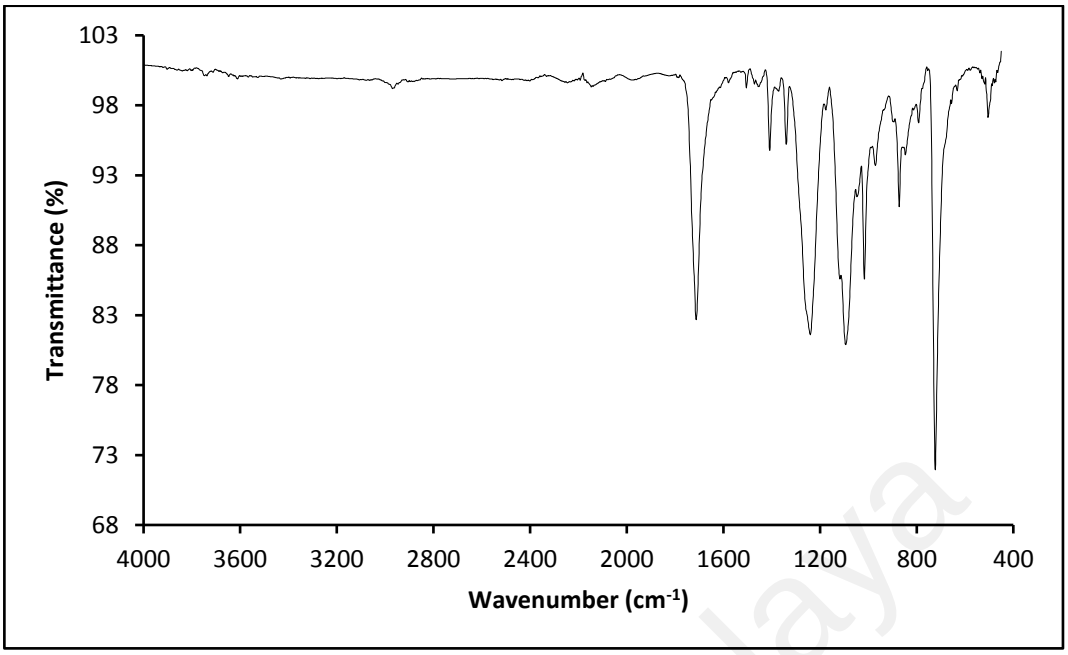

Figure 4.92: FTIR spectrum of PET microplastic treated with 20% v/v of inoculum concentration of Treatment D

In spectrum of PET microplastics inoculated with 30% v/v (Figure 4.93), new peaks were formed around 3700 cm^{-1} assignable to O−H, hydroxyl stretching, at 1116 cm^{-1} and 1045 cm^{-1} assignable to C−O, phenolic of an ester. There were reduction of the absorption peaks of C−H alkyl stretching at 2968 cm^{-1}, C=O, carbonyl band of an ester at 1712 cm^{-1}, C=C aromatic stretching peak at 1408 cm^{-1}, C−H bending of methylene (CH_2) group at 1471 cm^{-1}, C−H bending of methyl group (CH_3) at 1340 cm^{-1}, C−O, phenolic of an ester at 12346cm^{-1}, 1092 cm^{-1} and 1016 cm^{-1} and C−H aromatic bending at 722 cm^{-1} .

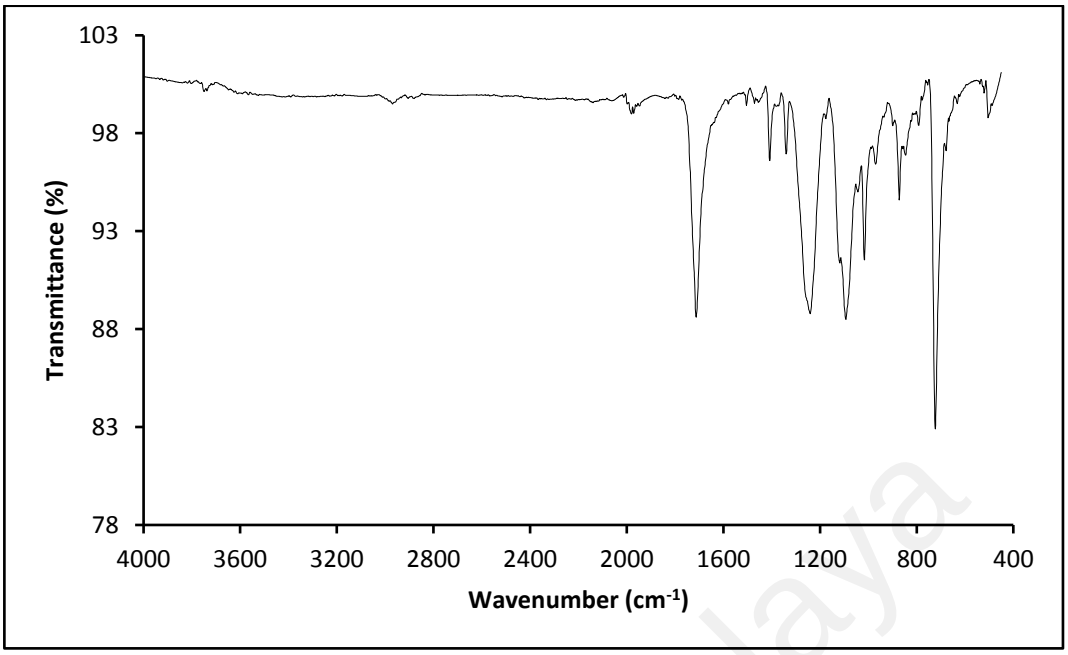

Figure 4.93: FTIR spectrum of PET microplastic treated with 30% v/v of inoculum concentration of Treatment D

A new peak was formed in at 3736 cm^{-1} assignable to O−H, hydroxyl stretching in PET microplastics subjected to degradation using 40% v/v inoculum (Figure 4.94). The absorption peaks of C=O, carbonyl band of an ester at 1715 cm^{-1}, C=C aromatic stretching peak at 1408 cm^{-1}, C−H bending of methylene (CH_2) group at 1454 cm^{-1}, C−H bending of methyl group (CH_3) at 1338 cm^{-1}, C−O, phenolic of an ester at 1241 cm^{-1}, 1092 cm^{-1} and 1016 cm^{-1} and C−H aromatic bending at 722 cm^{-1} all became reduced.

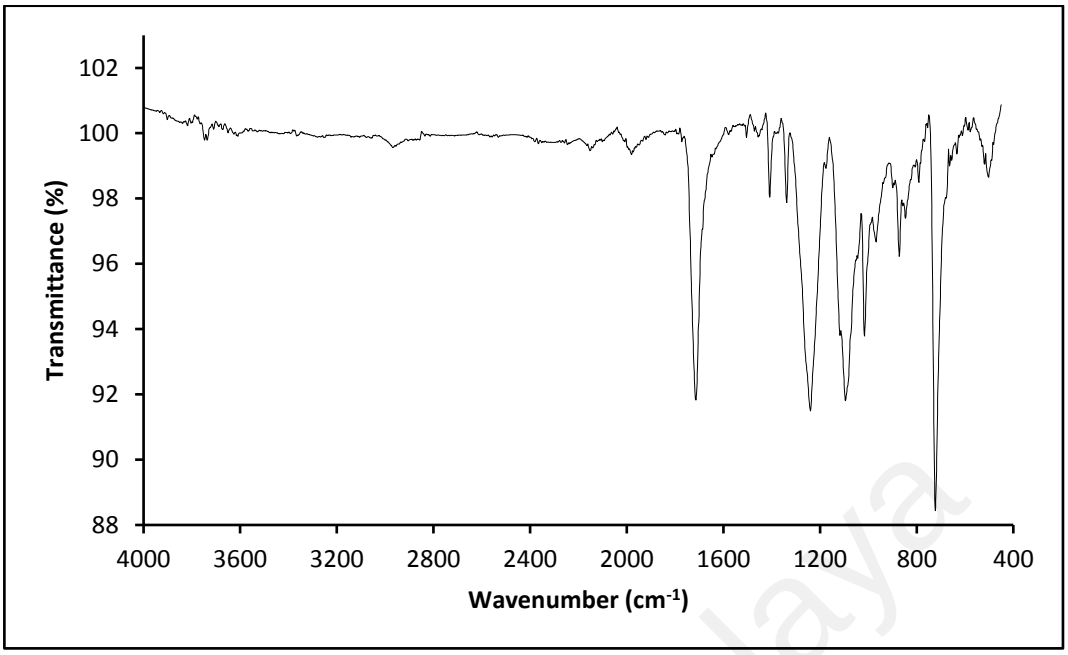

Figure 4.94: FTIR spectrum of PET microplastic treated with 40% v/v of inoculum concentration of Treatment D

The structural changes that appeared when PET microplastics were treated with 50% v/v of inoculum are presented in Figure 4.95. A new peak also appeared at 3736 cm^{-1} assignable to O−H, hydroxyl stretching. Reduction of the absorption peaks of C=O, carbonyl band of an ester at 1712 cm^{-1}, C=C aromatic stretching peak at 1404 cm^{-1}, C−H bending of methylene (CH_2) group at 1457 cm^{-1}, C−H bending of methyl group (CH_3) at 1339 cm^{-1}, C−O, phenolic of an ester at 1242 cm^{-1}, 1092 cm^{-1} and 1016 cm^{-1} and C−H aromatic bending at 723 cm^{-1} were observed.

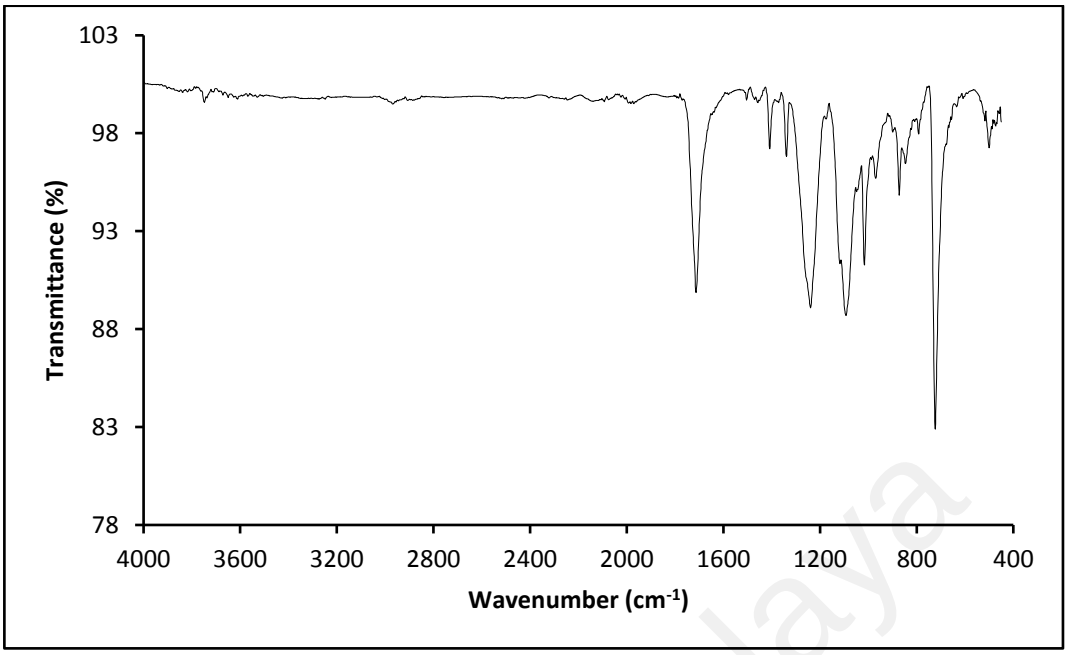

Figure 4.95: FTIR spectrum of PET microplastic treated with 50% v/v of inoculum concentration of Treatment D

Degradation of PET polymer occur by oxidation and hydrolytic degradation processes which causes chain scission and reduction in molecular weight. This results in increase in carboxylic acid and alcoholic end groups. FTIR results revealed increase in peak areas of the hydroxyl stretching which is associated with increase in hydroxyl groups as revealed in the FTIR spectra.

4.8.6 Biodegradation of PS microplastics using different inoculum concentrations

4.8.6.1 Determination of weight loss of PS microplastics inoculated with different inoculum concentrations

Figure 4.96 illustrates the average weight loss in percentage of PS microplastics treated with 10%, 20%, 30%, 40% and 50% v/v inoculum. The treated PS microplastics after 40 days in different inoculum concentrations showed considerable microplastic loss in all. It was observed that the highest weight loss (30.8%) of PS microplastics was attained with 10% v/v inoculum. The mechanism behind the significant reduction of PS microplastics

might be due to the ingrained interactions that exist among bacterial strains upon diversity manipulation and cell concentrations. At the same time, 20%, 30%, 40% and 50% inoculum concentrations degraded 16.2%, 16.6%, 12.8% and 11.2% of PS microplastics, respectively. The weight loss depicted by the different inoculum concentrations differed statistically ($P < 0.05$). It was observed from the study that the inoculum concentrations studied showed good degradation of PS microplastics and that increase in inoculum concentration led to a dramatic decrease in weight loss of PS microplastics. This probably is due to the competition for available nutrients by larger number of microbes in the consortia (Cai *et al.*, 2013). However, such assertion may be peculiar to PS exposure because PET exposure showed otherwise. Among all five concentrations, 10% v/v was found to be more effective in the degradation of PS microplastics to make it the best treatment option for PS microplastic degradation in this study. Similar results were reported by Liu *et al.* (2011), in which the highest weight loss was achieved by 10% v/v inoculum concentration and further increase in inoculum concentration beyond 10% resulted in decrease in biodegradation. Based on the results, the order of biodegradability of PS microplastics by the different inoculum concentrations ranked from 10% > 30% > 20% > 40% > 50% v/v inoculum.

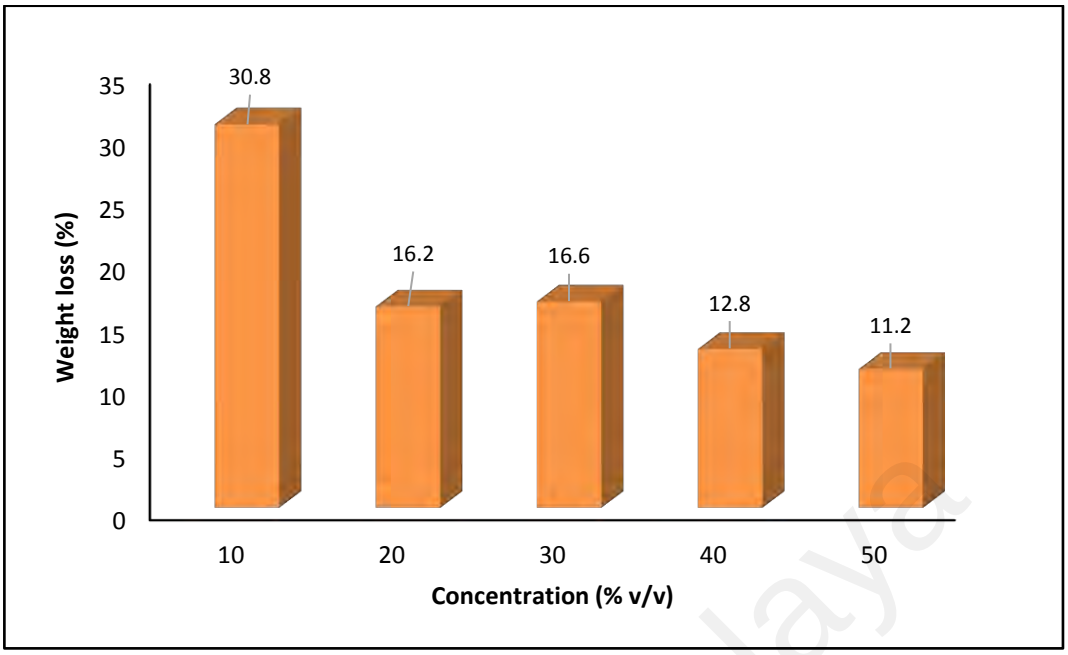

Figure 4.96: Weight loss of microbially treated PS microplastics after incubation for 40 days. Maximum weight loss (30.8%) was obtained for PS microplastics treated with 10% v/v inoculum concentration of treatment A

4.8.6.2 Growth pattern of different concentrations of blended microbes in PS microplastic-infused media

Figure 4.97 illustrates the growth curve pattern of the different inoculum concentrations of Treatment A upon exposure to PS microplastics. The growth curve was characterized by several distinct growth phases including exponential, stationary, and decline/death phases; representing distinct period of growths that were associated with typical physiological phases in cell cultures. In 10% v/v and 40% v/v inoculums, significant growths were recorded on the 30th day with absorbance readings of 1.015 OD_{600} and 2.233 $OD_{600,}$ respectively. This was followed by gradual decline phase on the 40th day (0.964 OD_{600} and 1.933 OD_{600}) for 10% v/v and 40% v/v inoculum, respectively.

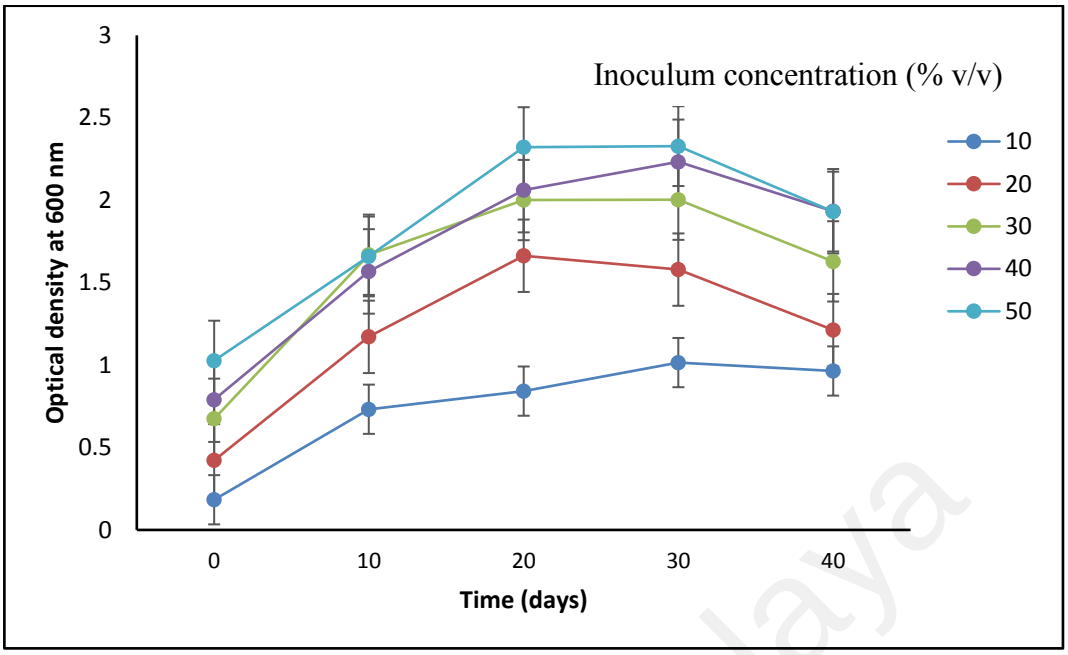

Figure 4.97: Growth pattern of different concentrations of Treatment A in PS microplastic-infused media during biodegradation studies

For 20% v/v inoculum, optimum growth of 1.663 OD_{600} was attained the 20th day and was accompanied by a decline phase on the 40th day (1.212 OD_{600}). The growth curves for 30% v/v and 50% v/v inoculum concentrations were characterized by log phases which were accompanied by stationary phases of growth, attained on the 20th and 30th days with 2.001 OD_{600} and 2.003 OD_{600} for 30% v/v inoculum. 50% v/v inoculum recorded 2.322 OD_{600} and 2.328 OD_{600}. Phases of decline then followed on the 40th day. Statistical analysis demonstrated significant difference in the growth response of the microbes across the different days of biomonitoring period ($P < 0.05$).

Bacterial population count in different inoculum concentrations fluctuated throughout the monitoring period (Figure 4.98).

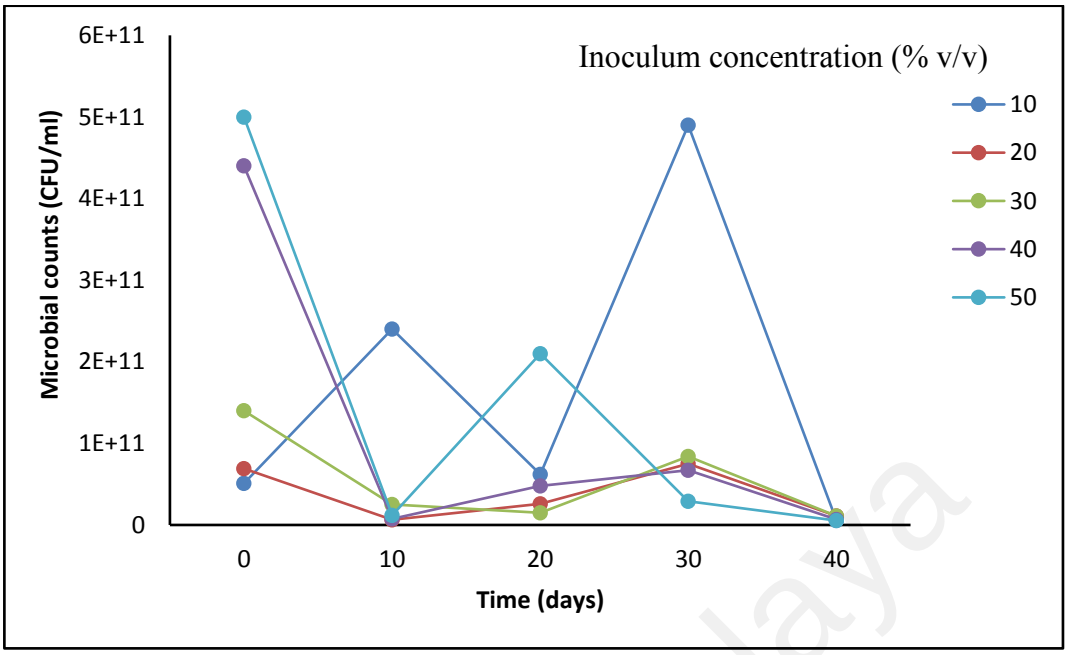

Figure 4.98: Bacterial counts of PS microplastics treated with different concentrations of Treatment A during 40 days biodegradation assay. Bars indicate standard error (n = 3).

At the start of the experiment, initial counts were 5.1 x 10^{10} CFU/ml, 6.9 x 10^{10} CFU/ml, 1.4 x 10^{11} CFU/ml, 4.4 x 10^{11} CFU/ml and 5.0 x 10^{11} CFU/ml for 10%, 20%, 30%, 40% and 50% v/v inoculum concentrations, respectively. On the 10^{th} day, counts for all inoculum concentrations, except 10% v/v decreased to 6.4 x 10^{9} CFU/ml, 2.5 x 10^{10} CFU/ml, 7.3 x 10^{9} CFU/ml and 1.2 x 10^{10} CFU/ml for 20%, 30%, 40% and 50% v/v inoculum concentrations, respectively.

This was not reflected in the growth curve, which may indicate that possible metabolite formation by the microbes may have increased the turbidity in the culture media. However, the count for 10% v/v inoculum was significantly increased to 2.4 x 10^{11} CFU/ml. The increase in count might have been due to the ability of the microbes to breakdown and utilize the hydrocarbons backbone in PS microplastics as carbon source which creates conditions favourable for multiplication. Day 20 witnessed increase in counts for 20%, 40% and 50% v/v inoculum, recording counts of 2.6 x 10^{10} CFU/ml, 4.8 x 10^{10} CFU/ml and 2.1 x 10^{11} CFU/ml, respectively. The counts of 10% and 30% v/v

inoculum however, decreased. Highest count at this period was recorded by 50% v/v inoculum. Increase in counts of 10% v/v inoculum (4.9 x 10^{11} CFU/ml), 20% v/v (7.5 x 10^{10} CFU/ml), 30% v/v (8.4 x 10^{10} CFU/ml) and 40% v/v (6.7 x 10^{10} CFU/ml) were observed on the 30th day. This is probably due to the availability of nutrients in the culture media.

The population counts decreased on the 40th day across all the different inoculum concentrations, with the highest count (1.1 x 10^{10} CFU/ml) recorded by 20% v/v inoculum and the least count (5.4 x 10^{9} CFU/ml) recorded again by 50% v/v inoculum. Cells probably were dying due to the stress associated with PS microplastics degradation or due to depletion of nutrients in the PS microplastic culture media.

4.8.6.3 Changes in pH of PS microplastic culture media during 40 days biodegradation studies using different concentrations of Treatment A

Figure 4.99 shows the variations in pH of PS infused culture media during the biodegradation studies. The pH of PS microplastic culture media increased towards alkalinity across all inoculum concentrations. The pH of 10% v/v inoculum increased from 7.01 to 8.52 while those of 20%, 30%, 40% and 50% v/v inoculums increased from pH 7.04 to pH 8.48, pH 7.2 to pH 8.24, pH 7.23 to pH 8.39, and pH 7.32 to pH 8.1, respectively. This indicate the possible release of basic metabolites by the microbes into the culture media or the probable increase in the number of hydroxyl radicals during PS microplastic degradation which caused the pH to turn alkaline.

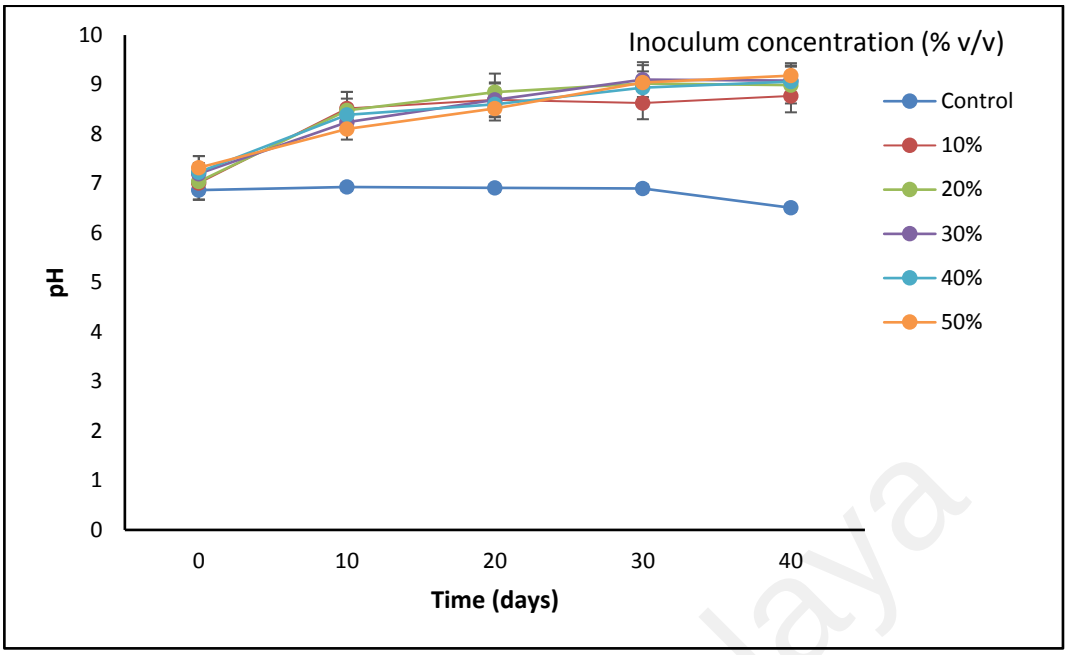

Figure 4.99: pH changes of PS microplastic-infused media inoculated with different concentrations of Treatment A during biodegradation studies. Each data point represents the average of three replicates ± SD

At the end of the experiment, the pH of the culture media was pH 8.77, pH 8.99, pH 9.08, pH 9.06 and pH 9.18 for 10%, 20%, 30%, 40% and 50% v/v inoculums, respectively. pH is known to influence many enzymatic processes and transport of various components across the cell membrane and large pH changes are often associated with microbial activities together with changes in nutritional sources (Das & Kumar, 2015). The pH changes that characterize the different inoculum concentrations in the culture media during PS biodegradation may suggest the production of enzymes and metabolites, supporting the overall metabolic activity of the strains on PS microplastics and also its degradation.

4.8.6.4 Biodegradation rate constant and half-life of PS microplastics inoculated with different inoculum concentrations

PS microplastics culture media treated with 10% v/v inoculum recorded the highest biodegradation rate of 0.009 day^{-1} and the shortest half-life of 77 days, whereas, treatment with 30% v/v inoculum recorded 0.005 day^{-1} and half-life of 154 days. PS treatment with 20% v/v inoculum gave degradation rate of 0.004 day^{-1} and half-life of 157.5 days, while 40% v/v inoculum recorded 0.0034 day^{-1} and 202.82 days (Table 4.17).

Table 4.17: Removal rate constant (*k*) and half-life of PS microplastics across different inoculum concentrations

Inoculum concentration (% v/v)	**Removal constant (*k*) (day-)**	**Half-life ($t_{1/2}$) (days)**
Control	0.000	∞
10	0.009	77
20	0.004	157.5
30	0.005	154
40	0.0034	202.82
50	0.00296	238.96

The least biodegradation rate (0.002 $day^{-1)}$ and highest half-life (238.96 days) was obtained upon treatment with 50% v/v inoculum. The increase in counts of bacterial population recorded in PS microplastic media treated with 10% v/v inoculum on the 10^{th} and 30^{th} days may have contributed to the reduction of PS microplastics as the large number of organisms may have caused increased activities such as proliferation of biomass and enzyme synthesis which increases degradation. Similar findings were also reported by Rodrigues *et al.* (2008).

4.8.6.5 Fourier transform infrared (FTIR) analysis of PS microplastics inoculated with different concentrations of Treatment A

The FTIR spectrum of uninoculated PS microplastics is presented in Figure 4.100. Control PS microplastics showed absorption peaks at 3060 cm^{-1} and 3026 cm^{-1} assignable to C−H aromatic stretching, and C−H alkyl stretching peaks at 2921 cm^{-1} and 2849cm^{-1}. Peaks at 1601 cm^{-1} and 1492 cm^{-1} are attributable to C=C stretching of an aromatic. While the peaks at 1452 cm^{-1} and 1370 cm^{-1} are assigned to C−H bend of a methylene (CH_2) group and methyl (CH_3) group respectively. The peaks at 754 cm^{-1}, 677 cm^{-1} and 616 cm^{-1} are assigned to C−H bending of a benzene (aromatic) derivative compounds. An absorption peak is also observed at 539 cm^{-1}.

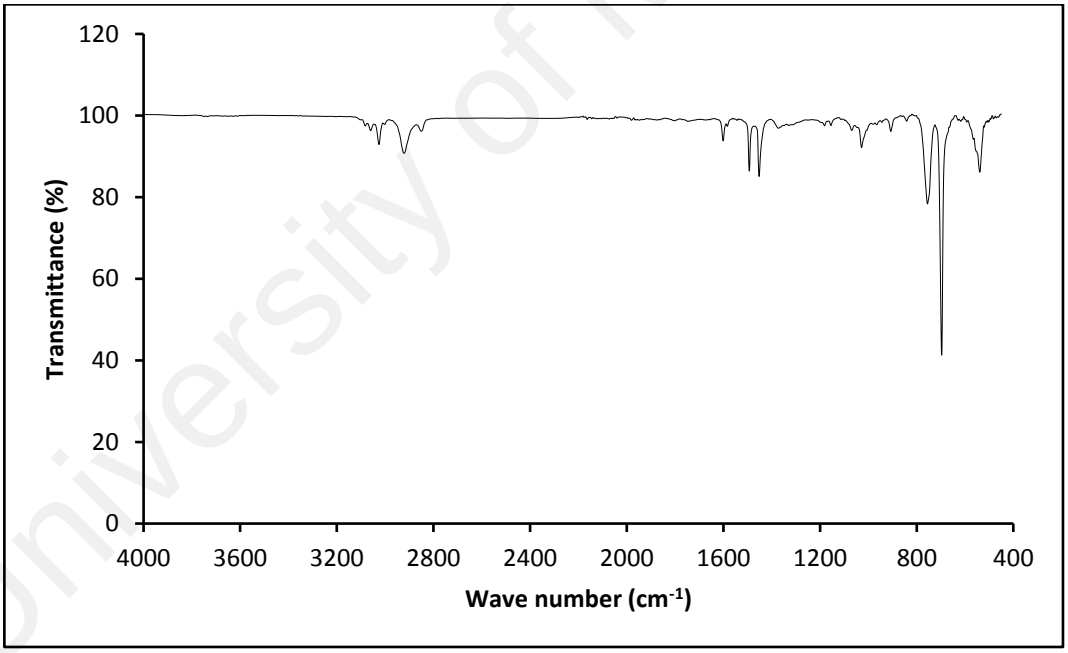

Figure 4.100: FTIR spectrum of control (uninoculated) PS microplastics

The C−H aromatic stretching peak at 3026 cm^{-1}, 3060 cm^{-1}, 3026 cm^{-1} in PS microplastics inoculated with 10% v/v became elongated (Figure 4.101), compared to control. The peaks at 2922 cm^{-1} and 2851 cm^{-1} assigned to C−H aliphatic stretching also became elongated. C=C aromatic stretching peaks observed at 1601 cm^{-1}, 1492 cm^{-1}

became elongated unlike in control PS microplastics. Elongation of the peaks at 1452 cm^{-1} and 1370 cm^{-1} assigned to C−H bend of a methylene (CH_2) group and methyl (CH_3) group, respectively, were also observed. Furthermore, the C−H bending of benzene derivative compounds at 752 cm^{-1} and 695 cm^{-1} became elongated, and a new peak appeared at 621 which is attributable to C−H bending of benzene (aromatic) derivative compounds.

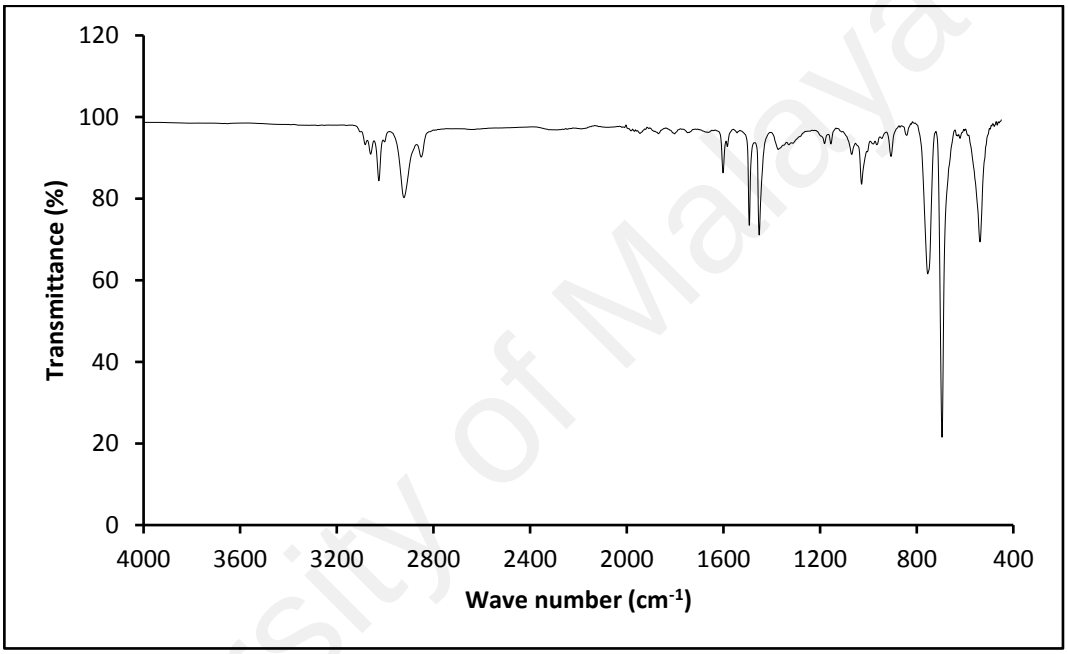

Figure 4.101: FTIR spectrum of PS microplastic treated with 10% v/v of inoculum concentration of Treatment A

Treatment with 20% v/v inoculum showed C−H aromatic stretching peak at 3025 cm^{-1} and C−H aliphatic stretching at 2920 cm^{-1}. These peaks became reduced (Figure 4.102) compared to control PS microplastics. The C=C stretch aromatic peaks at 1601 cm^{-1}, and 1492 cm^{-1}. Elongation of the peaks at 1452 cm^{-1} and 1370 cm^{-1} assigned to C−H bend of a methylene (CH_2) group and methyl (CH_3) group respectively were also observed.

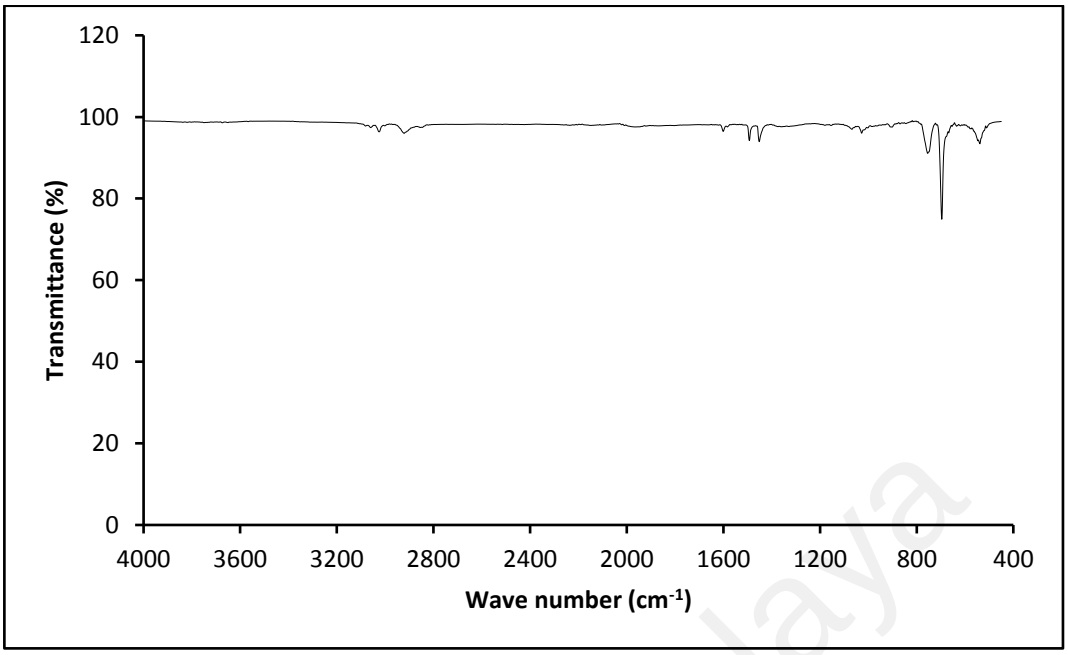

Figure 4.102: FTIR spectrum of PS microplastic treated with 20% v/v of inoculum concentration of Treatment A

The C−H aromatic stretching peak at 3026 cm^{-1} in PS microplastics treated with 30% v/v inoculum became reduced compared to PS control microplastics, so also did the C−H aliphatic stretching peak at 2921 cm^{-1}. Moreover, the peaks at 1601 cm^{-1}, and 1492 cm^{-1} attributed to C=C aromatic stretching peaks also became reduced. The peaks at 1451 cm^{-1} and 1370 cm^{-1} are attributed to C−H bend of a methylene (CH_2) group and methyl (CH_3) group respectively, became reduced too. The C−H bending of benzene derivative compounds at 753cm^{-1} and 696 cm^{-1} however, became elongated (Figure 4.103).

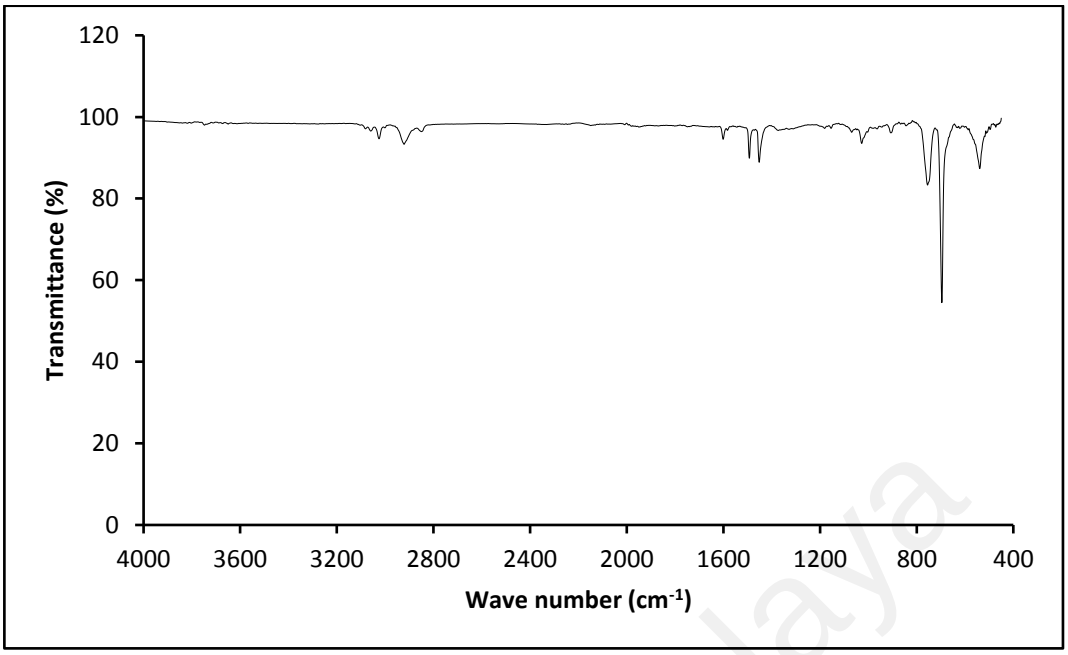

Figure 4.103: FTIR spectrum of PS microplastic treated with 30% v/v of inoculum concentration of Treatment A

For PS microplastics inoculated with 40% v/v inoculum concentration, the aromatic C−H stretching peak at 3060 cm^{-1} and 3026 cm^{-1} and C−H aliphatic stretching at 2921 cm^{-1} and 2850 cm^{-1} both became reduced (Figure 4.104) compared to control. Reduction of the aromatic C=C stretch peaks at 1601 cm^{-1}, and 1492 cm^{-1} were also observed, so were the reduction of the C−H peaks at 1452 cm^{-1} and 1370 cm^{-1} attributed to C−H bend of a methylene (CH_2) group and methyl (CH_3) group respectively, are observed while the C−H bending of benzene derivative compounds at 753cm^{-1} and 696 cm^{-1} became elongated.

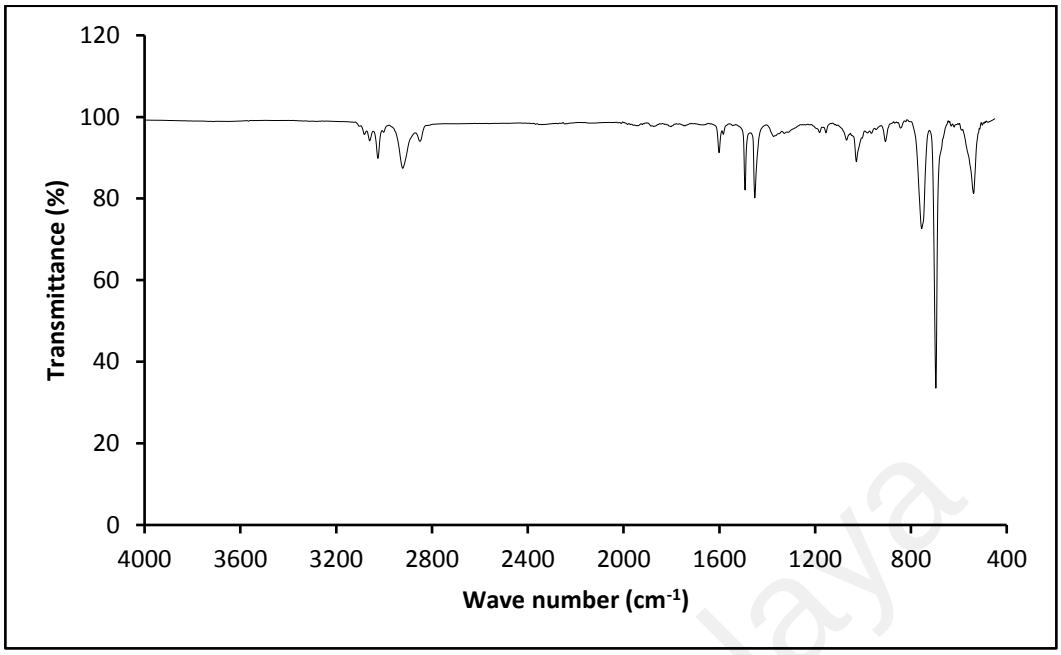

Figure 4.104: FTIR spectrum of PS microplastic treated with 40% v/v of inoculum concentration of Treatment A

Inoculation with 50% v/v inoculation led to the formation of a new absorption peak at 3700 cm^{-1}. The aromatic C−H stretching peak at 3025 cm^{-1} and C−H aliphatic stretching at 2972 cm^{-1} however, became reduced compared to control. Furthermore, the aromatic C=C stretch peaks at 1601 cm^{-1}, and 1493 cm^{-1} also became reduced. Reduction of the C−H peaks at 1452 cm^{-1} and 1370 cm^{-1} attributed to C−H bend of a methylene (CH_2) group and methyl (CH_3) group respectively, were similarly observed. The C−H bending of benzene derivative compounds at 753cm^{-1} and 696 cm^{-1} became elongated (Figure 4.105).

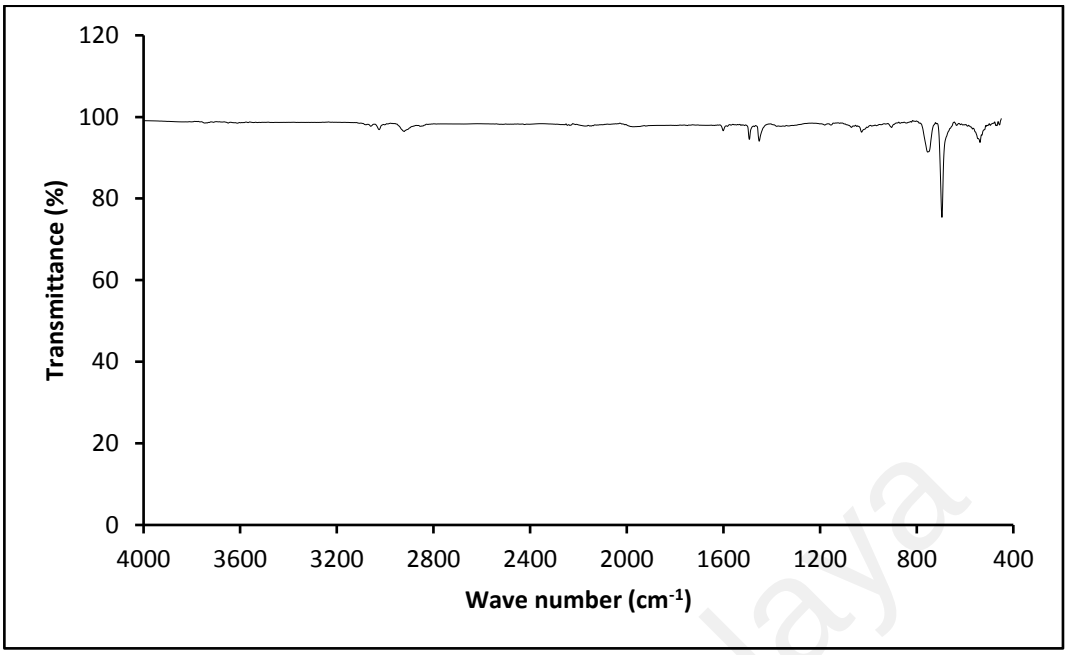

Figure 4.105: FTIR spectrum of PS microplastic treated with 50% v/v of inoculum concentration of Treatment A

4.9 *In situ* (field) experiment

The laboratory scale biodegradation studies (shake-flask experiment) discussed in the preceding sections demonstrated that the microbial formula (consortium) consisting of all the nine isolates (Treatment A) gave the highest reduction of the microplastics studied as against the use of other consortia formulated. Hence, the study further investigated an *in-situ* remediation of mangrove soil that was artificially contaminated with PET and PS microplastics using Treatment A. The microbes formulated in the Treatment are indigenous mangrove bacteria that had been enhanced in the laboratory and were therefore, expected to thrive well give better degradative performance when introduced back into the mangrove environment. This study is the first report of a project that is aimed at investigating the degradation of microplastics in natural marine environments and gaining information on the behaviour of the tested microplastics in the presence of physical strength due to tidal inundation and waves.

4.9.1 Bioremediation of PET and PS microplastics

4.9.1.1 Determination of weight loss of PET and PS microplastics

The overall results of the microplastic degradation at the marked plots showed varied degree of weight loss after 90 experimental days (Figure 4.106). Even the control experiment showed bio-reduction for PET and PS, demonstrating that Sementa mangrove soil exhibited a natural ability to remediate itself from microplastic pollution. The degradation trend exhibited by the microplastics differed between PET and PS under *in situ* conditions.

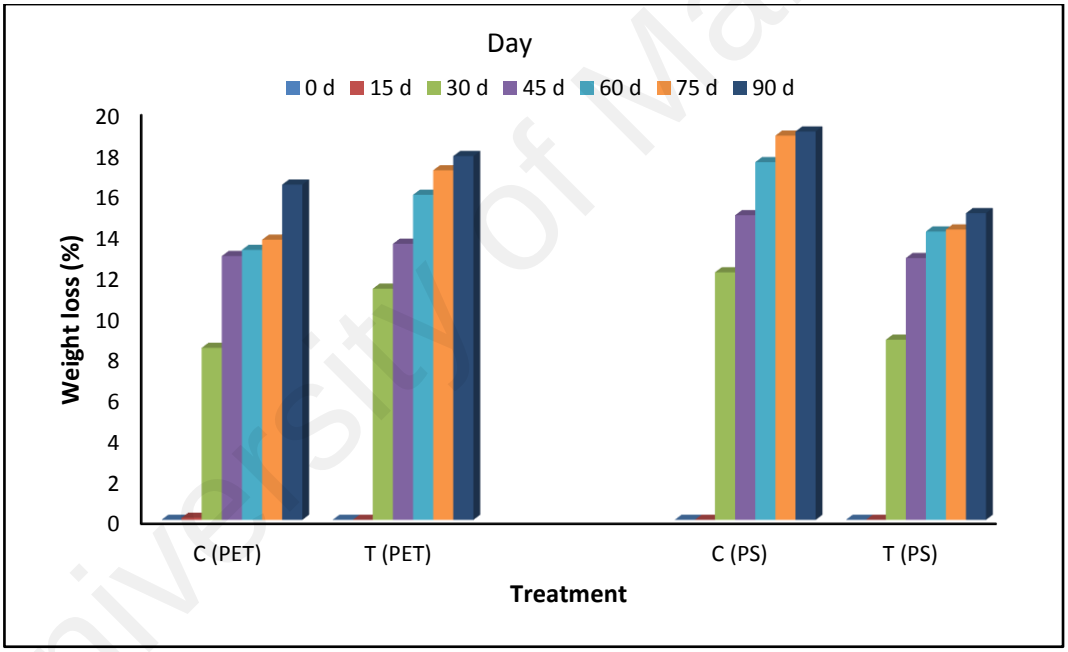

Figure 4.106: Weight loss of PET and PS microplastics across days during in-situ bioremediation studies in mangrove soil (C is unamended control soil, T is microbially augmented soil)

PET microplastics assessment, the weight loss for control was 16.4% while the treated plot recorded 18% weight loss. The reduction was 2.0% higher compared to control. Though it was just 1.6% difference, yet, significant difference existed ($P < 0.05$). Such reductions could have probably been induced by microbial interaction with the microplastics. Considering the augmentation done on the experimental plot, some

synergy between the introduced consortia and indigenous microbes seemed to have induced a degradation effect on PET than the level found in the control experiment. Similarly, the isolates used to formulate the consortia were originally isolated from mangrove soil. This may justify the reason for the reduction of PET microplastics recorded in the control plot, but in some cases, microbes in the polluted environment may not have enough metabolic strength to remedy the ecosystem, except when amended with respect to diversity and concentration (Brenner *et al.*, 2008). Furthermore, some other environmental factors possibly influenced the results outcome, especially pH.

Similar evaluation of bio-reduction of PS microplastics in the same environment did not yield exact results and findings for PET microplastics, possibly due to microbial specificity with environmental conditions. With respect to overall weight loss, the control experiment showed better performance in PS microplastics degradation. The control experiment recorded 19% while only 15% was recorded in the amended portion. This implies 4% difference in weight loss. It therefore, became clear that environmental conditions may influence microbial concentration in a unit area, yet, may differently direct their metabolic complex interactions, especially with pollutants. The reason for this finding is subjected to the fact that PET microplastics were buried in the same plot with PS and were expected to experience the same effect from the prevalent microbial concentration. Therefore, the lesser degradation recorded for PS suggested the influence of some other factors. The observed weight loss within the control plot could be attributed to prevalent microbial community that were indigenous to the mangrove environment. The population distribution during the 90 days showed fluctuation often due to some other associated environmental factors including pH, salinity, DO, and other factors that were not monitored in this study such as nutrient enrichment, tidal distribution, and fauna capacity. The existing indigenous microbes probably had more selective activity on the carbon content of PS microplastics and hence, were utilizing more PS microplastics.

Statistical assessment indicated significant difference in the overall weight loss of PS microplastics after 90 days ($P < 0.05$; F-value = 2293.2). However, there was no statistical difference between the weight loss of PET microplastics ($P > 0.05$; F-value = 0.005). It was interesting to note that Sementa mangrove environment had the ability to degrade PET and PS microplastics naturally over the three months period of the study. This high intrinsic ability might have been caused by the environmental conditions, possible presence of microplastic degrading microbes in the mangrove soil, and the microplastics characteristics.

Bioaugmentation to degrade PET microplastics was faster and higher in the treated plot than in the control experiment. This was demonstrated by the reduction rate which was expressed as *k* value (Table 4.18) and the weight loss of PET microplastics.

Table 4.18: Rate constant (*k*) in each treatment at different periods of the bioremediation experiment, n = 3

Treatment	**K value at certain periods of time (day^{-1})**					
	0-15 days	0-30 days	0-45 days	0-60 days	0-75 days	0-90 days
Control (C) (PET)	0.000001	0.001	0.0018	0.0016	0.00163	0.0019
Bioaugmented (T) (PET)	0.0	0.0013	0.0016	0.0019	0.0021	0.0022
Control (C) (PS)	0.0	0.0014	0.0018	0.0021	0.00231	0.0024
Bioaugmented (T) (PS)	0.0	0.0010	0.0015	0.0016	0.0017	0.0018

The *k* value on the 90th day of bioremediation studies was 0.0022 day^{-1} which was higher than the 0.0019 day^{-1} of PET in Control. On the other hand, bioaugmentation treatment recorded lower *k* values (0.0018 day^{-1}) than that recorded by the control in PS degradation (0.0024 day^{-1}), demonstrating that natural degradation of PS microplastics is higher.

4.9.1.2 Microbial growth

The bacterial population was measured at every monitoring day during the bioremediation period. The counts across the monitoring days are presented in Figure 4.107.

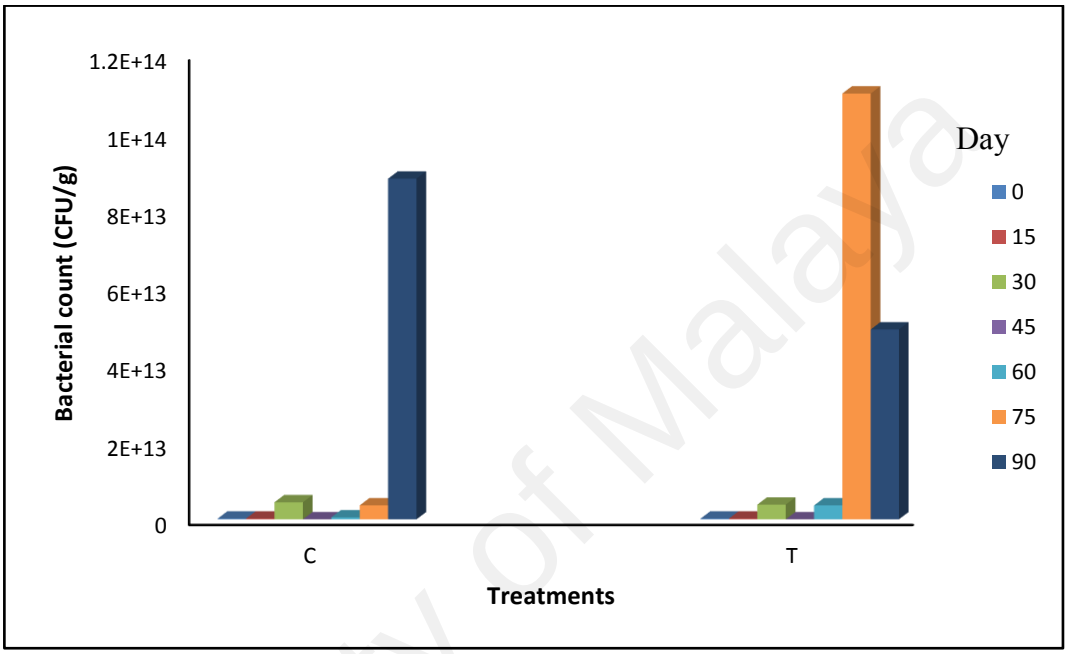

Figure 4.107: Microbial counts across days for in-situ bioremediation of microplastic contaminated mangrove soil in Sementa, Klang (C represents un-amended soil (control) and T represents microbial amended soil)

The results revealed that the microbial population density in both treatments varied during the experimental period. Total cell counts in the control portion ($1.2 \times 10^{10} – 8.8 \times 10^{13}$ CFU/g) were lower than the counts in the bioaugmented portion ($1.8 \times 10^{10} – 1.1 \times 10^{14}$ CFU/g). A lag phase was observed in both treatments in the first 15 days. This probably indicated a period of acclimatization of the microbes and their inability to adhere and colonize the PET and PS microplastics hence, no weight loss was recorded at this period except for PET in the control portion which recorded a weight loss of 0.1%. An exponential phase of growth occurred between 15-30 days in both treatments and at this period, reduction in weight of PET and PS microplastics was observed indicating that after acclimatization, utilization of the microplastics by the microbes began and this

allowed the synergy between the microbial consortium and the existing indigenous microbes. Higher count (4.4 x 10^{12} CFU/g) was recorded in the control plot than in the augmented plot indicating that the microbes were probably utilizing the microplastics for growth and despite the addition of inoculum, the microbes were still trying to establish a defined interaction with the existing indigenous microbes. The growth of the bacterial cells was concomitant with the reduction of PET and PS microplastics. The correlation between PET and PS microplastic degradation and the population density of the microbes in the control and augmented soil is presented in Figure 4.108. The results revealed that there was a strong positive correlation between the degradation of PET and PS microplastic and the microbial population density in both the control and augmented soil during the experimental period with R^2 values ranging from 0.83 – 0.87. Highest correlation (R^2 = 0.87) was recorded between PET microplastic degradation and microbial growth in the control soil, indicating that the percentage correlation was about 87%. On Day 45, the cell densities from both treatments were reduced to 1.1 x 10^{10} CFU/g and 1.8 x 10^{10} CFU/g for control and augmented portions, respectively. This possibly is due to changes in environmental conditions in the mangrove soil.

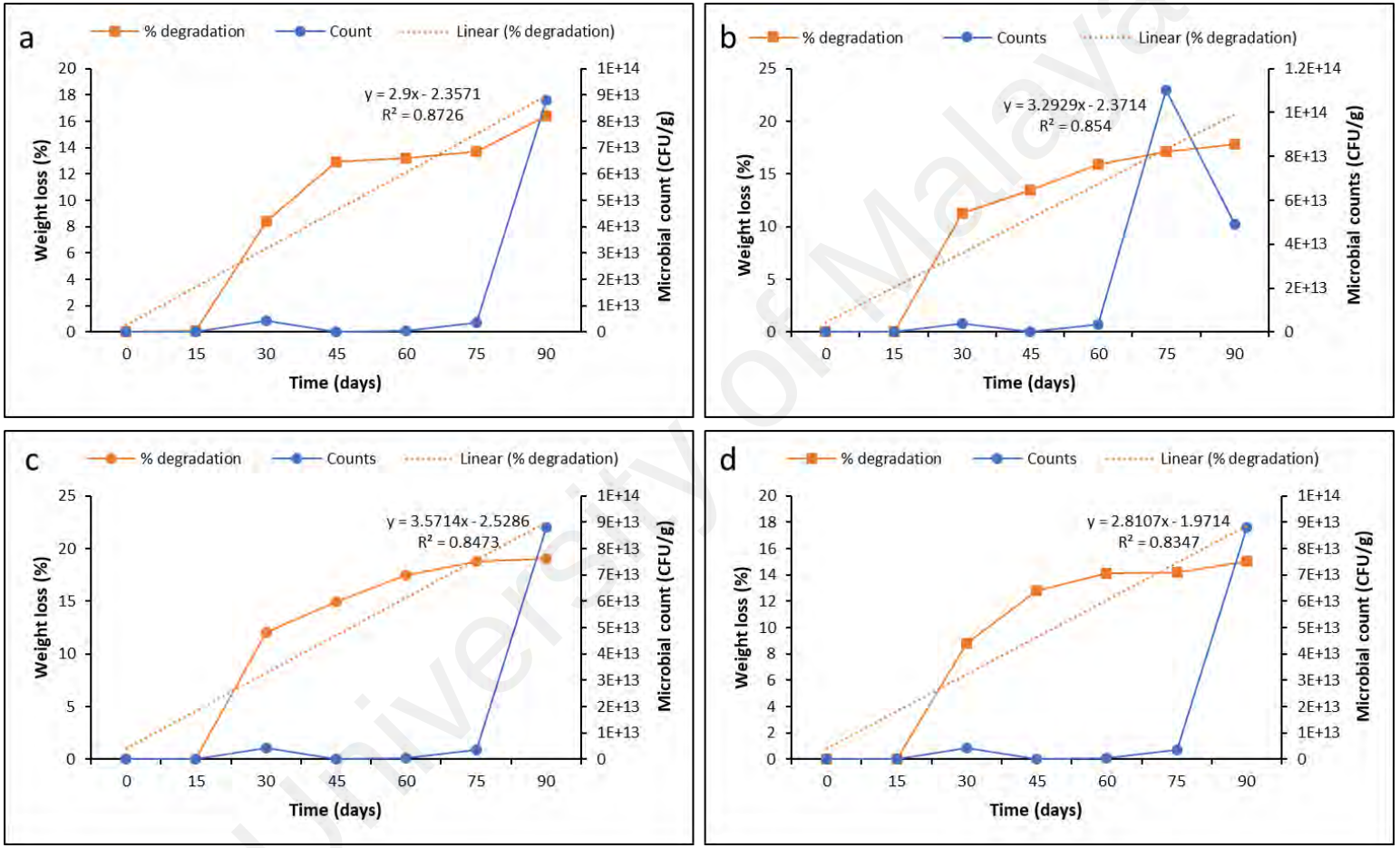

Figure 4.108: Relationship of microplastic degradation (%) and bacterial cell numbers in each treatment during the 90 days bioremediation study. (a) PET microplastics in control (unamended) soil, (b) PET microplastics in amended soil, (c) PS microplastics in control (unamended) soil, (d) PS microplastics in amended soil

The bacterial population in the augmented and the control treatments exhibited highest numbers of colonies on the 75th and 90th days, respectively. These counts were higher than those recorded by Kumar *et al.* (2007) and Kathiresan (2003b) in mangrove soils from Suva, Fiji Islands and in India, respectively. However, such variations can occur between different geographical locations owing to differences in the environmental parameters. The high cell load reported in this study is possible as Sementa mangrove soil was rich in nutrients and organic matter, the content of which was about 11% and might have contributed to the proliferation of the microbes. Organic matter in soil has been reported to influence the activity of microbes as well as enzyme production (Nowak *et al.*, 2016; Salazar *et al.*, 2011).

4.9.1.3 Environmental conditions

The temperature of the mangrove soil across the monitoring days ranged from 25.9 – 28.4 ^{o}C in the control plot and 26.5 – 28.4 ^{o}C in the bioaugmented plot, while the salinity ranged between 1.72 – 3.4 ppt and 1.99 – 3.4 ppt, respectively. The availability of oxygen in the soil declined during the experiment, not only in the bioaugmented treatment mesocosm, but also in the control mesocosm, although, the depletion rate in the augmented plot was lower than the control plot.

Fluctuation in dissolved oxygen (DO) values in both control and augmented soil plots was observed throughout the experimental period. The DO became stable (0.08 mg/L) in the augmented plot on the 30th and 45th days. The control soil recorded lowest average DO value of 0.07 mg/l on the 30th day while the augmented soil had its lowest DO values (0.08 mg/L) recorded on the 30th and 45th days (Figure 4.109).

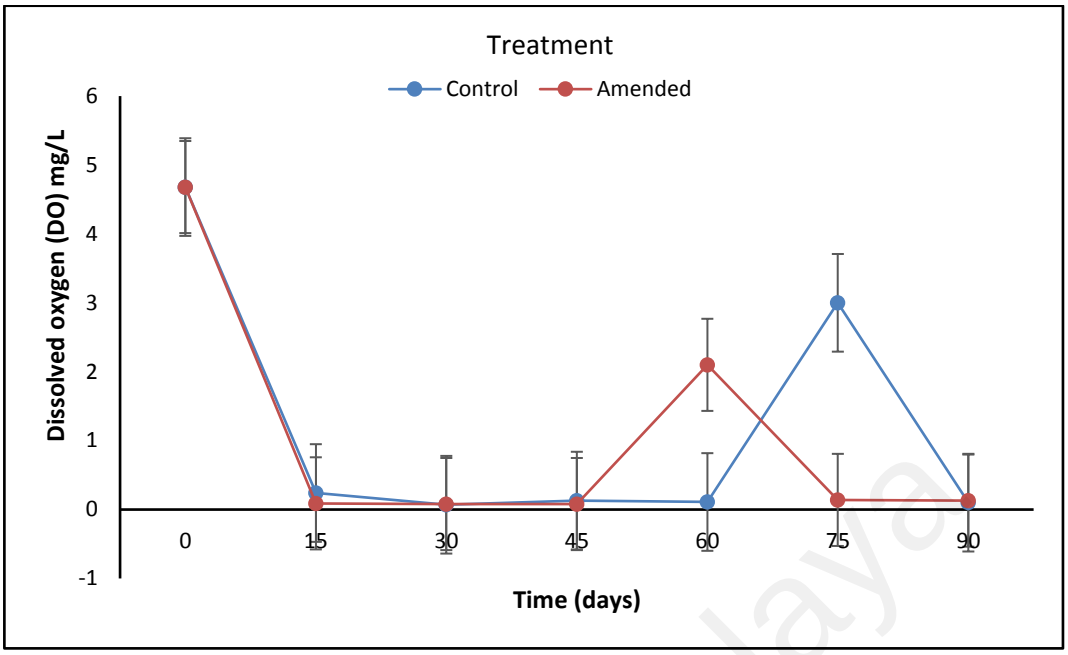

Figure 4.109: Changes in dissolved oxygen (DO) content in each treatment across the experimental monitoring days (n = 3 for each sampling time)

DO is necessary for the survival and proliferation of microbes in the aquatic environment (Spietz *et al.*, 2015). The DO values of control and augmented soils increased on the 75th and 60th days, recording DO values of 3.0 mg/L and 2.1 mg/L, respectively.

The increase in DO values on these monitoring days might have been as a result of wave action and might have contributed to the increase in cell counts recorded on same days. Darmayati *et al.* (2015) recorded similar fluctuation in DO in the study of the effect of biostimulation and biostimulation-bioaugmentation on oil degradation on sandy beaches using mesocosms.

Salinity of the soil in the control and amended portions varied during the experiment. Salinity in the control and amended soil were in the range of 1.72 – 3.4 ppt and 1.99 – 3.4, respectively (Figure 4.110).

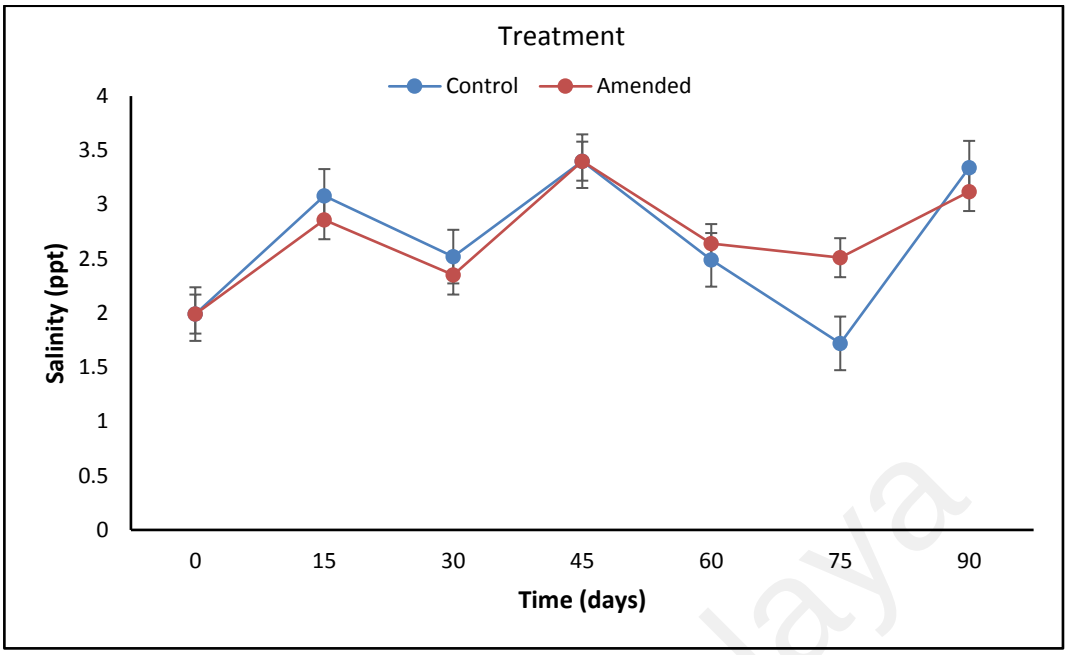

Figure 4.110: Changes in mangrove soil salinity across days in amended and un-amended (control) mangrove soil during bioremediation studies (n = 3 for each sampling time)

The lowest salinity value was observed on the 75th day for control while the augmented soil recorded lowest salinity levels on the initial day of the experiment. This low salinity values recorded might have resulted due to rainfall. Higher salinity levels in both treatments were recorded on the 45th day. This increase in salinity might have been responsible for the decrease in bacterial growth observed in the growth profile (Ryan, 2017). The decrease however, did not affect PET microplastics degradation as the rate of degradation of the microplastics in the augmented plot increased on the 45th day as compared to the control. The variations in salinity observed during the experimental period could have resulted from the relative amount of precipitation (by rain), or evaporation in the mangrove environment. Other factors that might have contributed to the variations include tidal fluctuations and freshwater runoff into the mangrove environment. The salinity changes in this study were under the tolerable level for marine bacteria. This can be explained by the significant growth demonstrated by the microbes during the experimental period.

pH is one of the major selective environmental factors that affect microbial growth and activity, nutrient availability, transport process and enzyme activity (Dhote *et al.*, 2010). The periodic pH changes that occurred in the control and augmented soil across the monitoring days during the bioremediation experiment are presented in Figure 4.111.

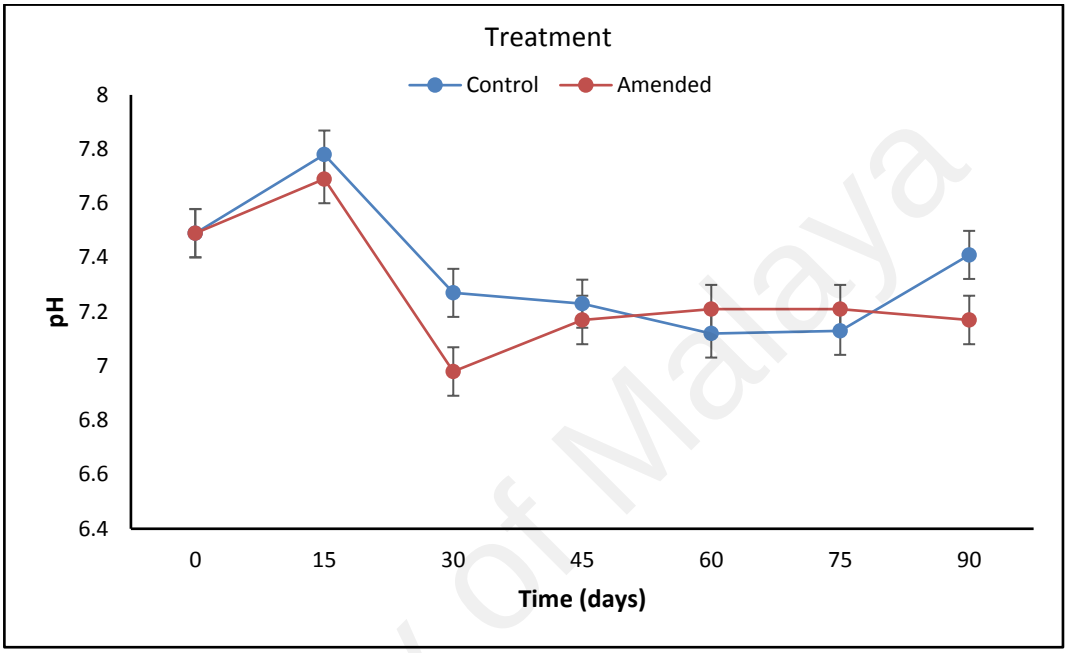

Figure 4.111: pH changes across days in amended and un-amended (control) mangrove soil during bioremediation studies

After 15 days, the pH values of both treatments increased (pH 7.49 – pH 7.78) for control soil and (pH 7.49 – pH 7.69) for amended soil without a corresponding change in the weight of the buried microplastics. The changes in pH might have been as a result of ammonification of nitrogenous components present in the soil by the microbes (Esmaeili *et al.*, 2013). The 30th day witnessed a drop in pH values and significant weight loss of the PET and PS microplastics were recorded in both treatments, with the highest loss of PET recorded in the augmented plot. The decrease in pH values observed could be attributed to the production of organic acids during microplastic degradation.

Rate of PET hydrolysis has been reported to be higher under acidic or basic conditions, and results in the formation of alcohol functional groups and carboxylic acid end groups

(Gewert *et al.*, 2015). Darmayati *et al.* (2015) recorded similar changes in pH values in their study of the degradation of oil in oil polluted sandy beaches by microbial consortium. The decrease in pH values might have also favoured microbial proliferation as the number of microbes in both treatments increased on the 30th day, depicting that the pH attained was possibly optimum for the degradation of the microplastics in both treatments. The pH in both treatments became stable on the 60th and 75th days after which the pH in the control soil increased (pH 7.41) while the pH in the augmented soil was observed to have decreased towards neutrality (pH 7.17). It appears that for the microbes to degrade PS microplastics, they require a more alkaline condition as against PET microplastics which appeared to have been responding to neutrality. This can be justified by the fact that higher rate of PS microplastics degradation was observed on the 15th – 45th day when the pH values drifted towards alkalinity while higher rate of PET degradation was observed when the pH declined towards neutrality.

Scanning electron microscopy coupled with energy-dispersive X-ray spectroscopy (SEM/EDS) was used to analyze the elemental composition of the mangrove soil before and after bioremediation. The EDS spectra showing peaks corresponding to different elements in the control and treated soil samples before and after bioremediation are presented in the Figure 4.112 while the elemental composition in terms of weight is presented in Table 4.19. It is evident from the analysis that both soils before treatment were enriched with organic and inorganic elements such as carbon (C), oxygen (O), silicon (Si), aluminium (Al), potassium (K), magnesium (Mg), sodium (Na), chlorine (Cl), sulfur (S), and iron (Fe). Figures 4.112a and 4.112b demonstrated that when the elemental compositions were compared before and after bioremediation, the concentration of C, Mg and Cl in the control soil increased while the concentrations of O, Na, Al, Si, S, K and Fe decreased at the end of the experiment. The augmented soil on the other hand, recorded increased concentrations of Si, S and Fe while the concentrations

of C, O, Na, Mg, Al, Cl and K decreased after bioremediation (Figure 4.112d) compared to the concentrations recorded in the soil before bioremediation (Figure 4.112c). The decreased Si and O content possibly indicated increased microbial biomass and gives insight into the ability of the microbes in changing the elemental composition of the microplastic contaminated soils during biodegradation (Varma *et al.*, 2017). The concentrations of alkaline earth metals such as Na and K decreased in both treatments after bioremediation while the concentration of Mg in control soil increased as compared to augmented. Conclusively, the study demonstrated that there was little change in the concentration of most of the elements in both the control and augmented soil after the bioremediation process.

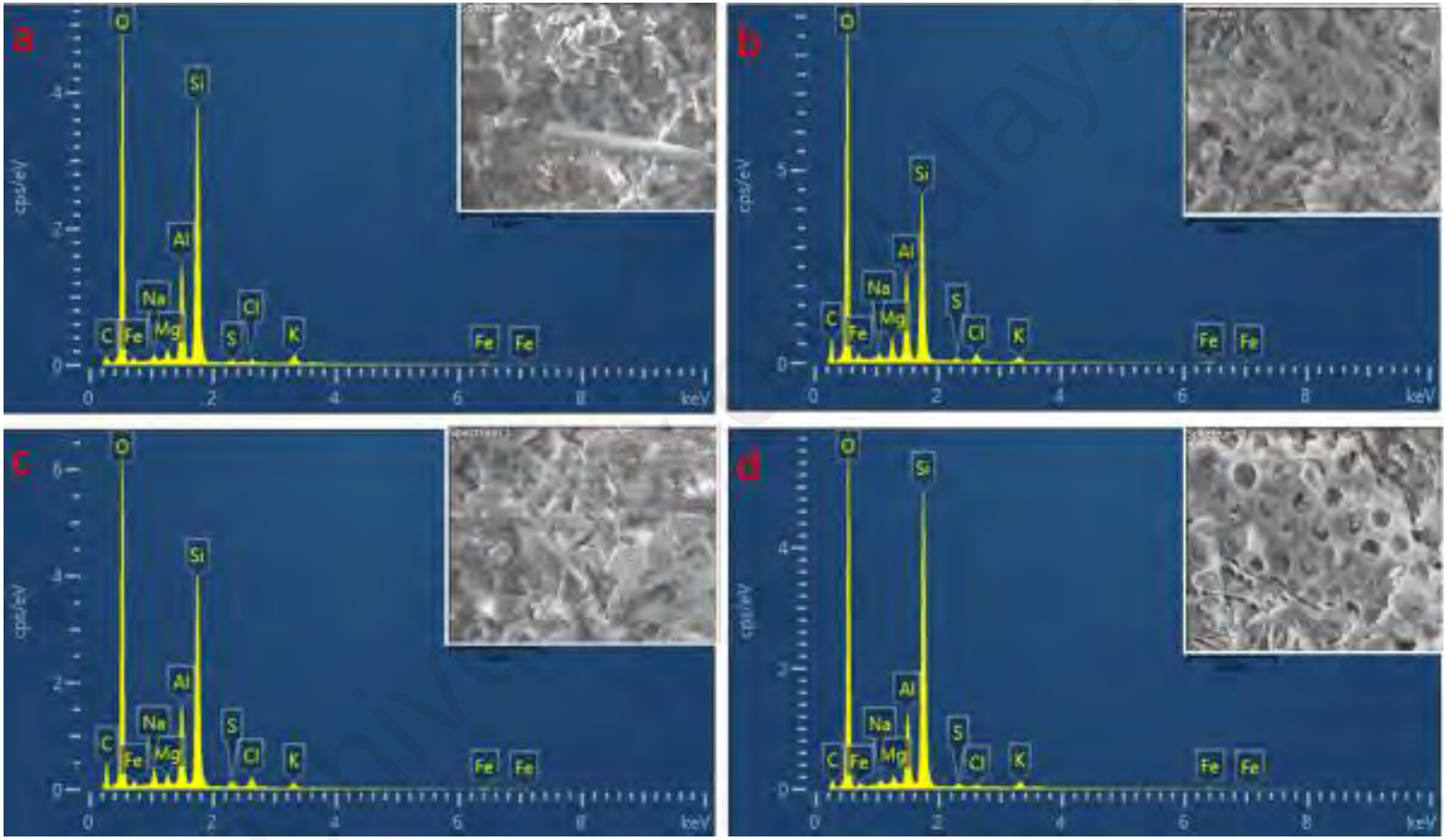

Figure 4.112: SEM photograph and corresponding EDS spectrum of (a) control soil before bioremediation, (b) control soil after bioremediation, (c) microbially amended soil before bioremediation, (d) microbially amended soil after bioremediation

Table 4.19: EDS analysis of mangrove soil before and after bioremediation

Elemental composition	Control soil		Amended soil	
	Before bioremediation (Wt %)	After bioremediation (Wt %)	Before bioremediation (Wt %)	After bioremediation (Wt %)
C	7.18	17.34	10.47	6.42
O	48.08	42.72	43.99	43.79
Na	0.75	0.69	1.46	1.37
Mg	0.91	1.32	0.99	0.64
Al	10.54	9.05	9.59	9.29
Si	26.16	24.70	24.26	28.67
S	0.57	0.50	0.93	3.82
Cl	0.75	1.09	2.21	0.53
K	2.26	1.98	1.97	1.81
Fe	2.82	2.05	4.14	4.42

The scanning electron microscopy (SEM) micrographs of PET and PS microplastics before and after the 90 days of the *in situ* bioremediation period is presented in Figure 4.113. Before experiment, the samples had smooth surfaces, with no defects observed (Figure 4. 113 a, b c and d intercepts). However, after 90 days bioremediation period, formation of cavities, distortions, cracks, irregularities, surface erosion and fissures were observed on the surface of the microplastics, revealing the disruption of the surface texture of both microplastics. The micrographs similarly demonstrated the occurrence of several non-uniformly-scattered whitened and eroded areas (Figures 4.113a and b) illustrating surface erosion mechanism involved in the degradation of the microplastics which might have been due to the catalytic action of the enzymes produced by the microbes. SEM characterization by Bhatia *et al.* (2014) in their study of the degradation of LDPE also revealed similar results.

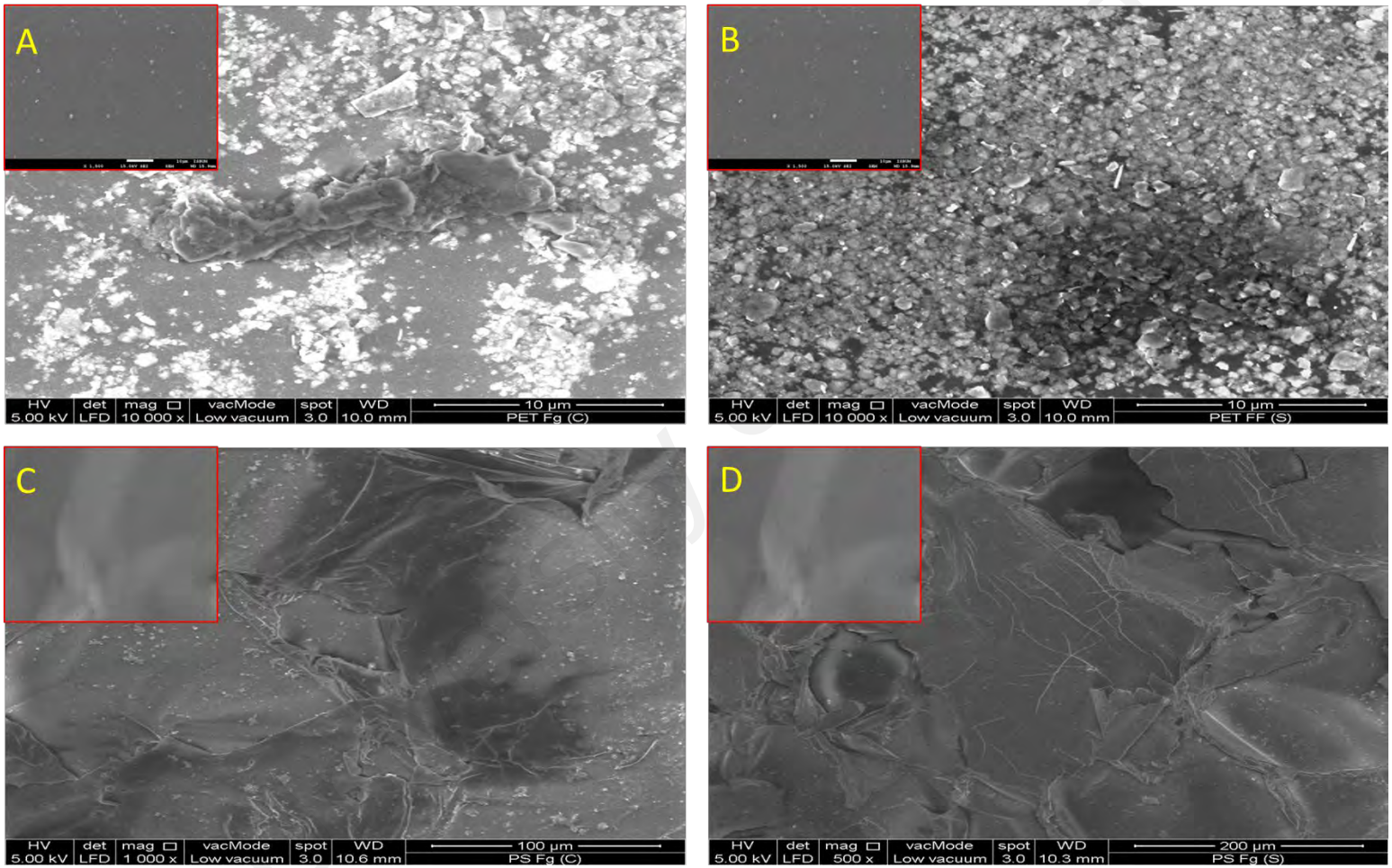

Figure 4.113: SEM micrographs of PET and PS microplastics before and after bioremediation in mangrove soil. (a) Un-amended (control) PET microplastics (b) amended PET microplastic (c) un-amended PS microplastics (d) amended PS microplastics

In conclusion, it was observed that weight reduction of PET and PS microplastics had taken place indicating degradation activity. The reduction could have been due to oxidative or hydrolytic cleavage of the ester or amide bonds. Surprisingly, in the laboratory (shake flask) biodegradation experiment using aqueous medium, PET microplastics degradation by the mixed culture was lower (7.2%) than its degradation in mangrove soil under natural conditions (17.8%) for microbially amended and 16.4% for control) which suggests the ecological nature of mangrove soil in comparison with liquid synthetic medium. It also reveals that mangrove soil can be a source of factors responsible for good PET microplastic degradation. Such factors may include moisture, heat, microbes and salinity. Mangrove soil becomes heated during the low tides on exposure to sunlight as well as due to exothermic reactions of biological compounds in the soil and maintains moisture by tidal water flooding during the high tides (Kathiresan, 2003).

PET is hygroscopic, meaning that it absorbs water from its surrounding and when heated, the water hydrolyses the polymer, decreasing its resilience. This unique property of PET might have enhanced its degradation in the mangrove soil which was demonstrated by yellowing of the microplastics due to the formation of chromophoric systems (Adhikari *et al.*, 2015). The high microbial counts observed in the study might have also favoured PET microplastic degradation. Similarly, the pH conditions of the soil observed in both soil portions during the *in situ* experimental period might have played a role in promoting better degradation of PET microplastics in the mangrove soil. Though the control experiment indicated almost neutral pH, yet it recorded higher pH that was directed towards alkalinity. Hence, this could have promoted the metabolism of the pollutants in the experimental portion leading to the production of high concentrations of potential degrading enzymes (not measured in this study), and eventually higher PET microplastics reduction. A positive correlation existed ($r^2 = 0.64$) when the pH level was compared with weight loss of PET microplastics in the experimental portion. This could

possibly justify the higher reduction of *in-situ* PET microplastics remediation than laboratory experiment with aqueous system. The pH under the laboratory evaluation was more alkaline (≈ 8 – 9) and reduced about 7.2% of PET microplastics unlike the 18% recorded in *in-situ*.

PS microplastics degradation on the other hand was higher under laboratory conditions. About 30.1% of PS microplastics was reduced in aqueous medium under laboratory conditions as against 15% recorded *in situ* upon exposure to the same consortia. The study found that the pH level attained under laboratory experiment was more alkaline (pH 9) whereas, the maximum pH recorded during *in situ* experiment was pH 7.78 which dropped to pH 7.41 after 90 days.

CHAPTER 5: CONCLUSION

5.1 CONCLUSION

Twenty-two bacteria were isolated from different mangrove soil in Peninsular Malaysia using enrichment technique. Distribution of microbes in the plastic-inundated mangrove soils included both gram-positive and gram-negative bacteria belonging to 16 genera of *Bacilli*, five genera of proteobacteria, and one genera of Actinobacteria. The results revealed that *Bacilli* were the dominant genera in the mangrove soil. Microbes isolated were those with either known biotechnological/bioremediation potential or with clinical relevance, and reflected the native bacteria community found in mangrove ecosystems. Nine isolates out of the 22 were capable of degrading PE, PP, PET and PS microplastics suggesting that mangrove soil harbours potential microplastic degraders. The study revealed that the selected microplastics can be biodegraded provided the right microbial strain(s) is employed under appropriate conditions. Culture enrichment methods were found effective for enhancing the abilities of the isolates in utilizing PE, PP, PET and PS microplastics as carbon and energy source, and the microbes demonstrated varied metabolic responses to the different microplastics.

Experimental data of the biodegradation studies using single isolates revealed that *B. gottheilii* demonstrated highest (6.2%) PE degradation in terms of weight loss and therefore, recorded the highest reduction rate (0.0016 day^{-1}) and lowest half-life (431.25 days). Although biodegradation of PE was very minute, *S. epidermidis* (0.6%) and *B. flexus* (0.4%) were able to survive in PE microplastic-infused media and thus, demonstrated little degradation. For PP microplastics, highest weight loss (6.4%) was achieved by *R. ruber* while *B. cereus* caused the highest weight loss for both PET (6.6%) and PS (7.4%) microplastics. Biodegradation studies with blended isolates revealed lower weight loss of PE and PET microplastics when compared to the weight loss recorded

when individual isolates were employed. Higher weight loss of PP and PS microplastics was however recorded when the organisms were blended demonstrating that engineering the microbes into consortia increased metabolic potential for PP and PS microplastics degradation. The study also showed that inoculum size affected the rate of PET and PS microplastics degradation by the blended microbes. The inoculum concentrations of 10% v/v and 40% v/v were the optimal conditions to obtain the maximal degradation of PS and PET microplastics, respectively in the shake flask experiments.

In-situ bioremediation experiments demonstrated significant degradation of PET and PS microplastics in both unamended and microbially amended soil. This indicated that the consortium formulated exhibited great potential for microplastics biodegradation under natural conditions such as those found in mangrove environments. The results also demonstrated that Sementa mangrove contains diverse potential microplastic degraders and has a natural ability to remediate itself of microplastics contamination. The study therefore, proposed the application of first order kinetic model and half-life for the generation of PE, PP, PET and PS microplastics removal rate for biodegradation and bioremediation of microplastic contaminated soil.

FTIR and SEM results showed changes in the chemical and morphological structure of the microplastics. Formation and disappearance of carboxylic acids, esters, aldehydes, alcohols, phenols, aromatic, ether and alkene groups at different frequencies indicating the degradation of the different microplastics by the isolates. SEM analysis showed the formation of cavities, pits, pores, erosions, irregularities and roughening, and colonization of the microplastic surfaces demonstrating the ability of the isolates to colonize, adhere and modify the surface of the microplastics.

This study underlines the potential of mangrove bacteria in future biodegradation/bioremediation strategies aimed at curtailing the increasing presence and

accumulation of microplastics in the environment, thus responding to the current and urgent need for alternative routes to minimize and abate the presence of microplastics in the aquatic environment.

5.2 RECOMMENDATION

1. This study is the first report on the biodegradation of microplastics in a mangrove environment. Therefore, studies on the reaction pathway and potential degradation products of microplastics under these environmental conditions should be carried out. The findings would provide more understanding on the degradation rates in the natural system.

2. Isolated microbes should be further investigated for their potential to degrade microplastics. This include the use of technologies such as genomics, proteomics, metabolomics, and transcriptomics.

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