Enhancement in biodegradation of thermoplastics using UV-pretreatment and biosurfactant

Navid Taghavi

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Abstract

Non-degradable plastics such as polyethylene (PE), polypropylene (PP), polystyrene (PS), and polyethylene terephthalate (PET) are among the most generated plastic wastes in municipal and industrial waste streams. The mismanagement of abandoned plastics and toxic plastic additives have threatened marine and land fauna as well as human beings for several decades. The available thermal processes can degrade plastic at pilot- and commercial-scale. However, they are energy-intensive and can generate toxic gases. Degradation of plastic waste with the help of live microorganisms (biodegradation) is an eco- and environmentally friendly method for plastic degradation, although the slow processing time and low degradation rate still hinder its applications at pilot- and large-scale.

The capability of different strains derived from New Zealand's soil, activated sludge, farm sludge, and worms' excreta were investigated for biodegradation of the above-mentioned plastics in unstimulated and stimulated conditions. Among several microorganisms *Penicillium raperi*, *Aspergillus flavus*, *Penicillium glaucoroseum* and *Pseudomonas* spp. were isolated as the most plastic degrading microbes. Maximum weight loss was seen by incubation of polyethylene with *A. flavus* (5.5 %) in unstimulated mix condition.

To enhance the biodegradation efficiency, UV-pretreatment (ageing) of plastics was conducted at various conditions. The findings of this research indicated that UV-pretreatment at optimum condition (UV dose of 7.02×10^{12} $\mu W.cm^{-2}.s$) (t_2 d_2 condition) resulted in a higher roughness, hydrophilicity, microbial viability, biofilm formation, surface degradation, and more significant physical and molecular weight reduction. The highest biodegradation happened for PE and PS with respective 7.8 and 5.13 % physical weight loss and 4.71 and 2.1 fold molecular weight reduction compared to the "un-pretreated & strain-free bio-treated" (control-3). The hydrophilicity of PS and PE were increased to the "UV-pretreated & bio-treated" samples with a reduction in water contact angle from 105° to 5° in PS and 102° to 60° in PE. Microscopic analysis indicated significant surface property changes or degradation (cracks and holes) on "UV-pretreated & bio-treated" samples. Chemical transformation with Fourier Transform Infrared Spectroscopy (FT-IR) also revealed new peaks in "UV-pretreated & bio-treated" samples, indicating the positive role of UV

in biodegradation efficiency. Statistics analysis showed between 45 and 90 days of biodegradation, 45 days was adequate to obtain optimum biodegradation efficiency ($p< 0.05$). The identification of short-chain by-products by gas chromatography - mass spectrometry (GC-MS) also confirmed that UV-pretreatment at optimum condition in presence of the identified strains was achievable.
The step further and increase the biodegradation rate, the capability of the identified strains was also evaluated by integration of the optimum-UV-pretreatment condition in presence of 160 mg/L rhamnolipid biosurfactant. The study found that the "UV-pretreated & bio-treated + biosurfactant" was the most effective condition for increasing the biodegradation rate of PS samples. The highest physical weight loss (7.47 %), surface degradation which was about 2.3 % more than condition with no biosurfactant. The higher wettability (< 5˚) and biofilm formation were also observed in this condition. In contrast, PE and PET had a higher biodegradation efficiency only in "UV-pretreated & bio-treated" condition. The utilisation of biosurfactant had negative effects on biodegradation and wettability of PE and PET, due to the consumption of rhamnolipid as food source rather than the plastic itself. Chemical transformation indicated new peak (C-O) in PS at both "UV-pretreated & bio-treated" and UV-pretreated & bio-treated + biosurfactant) conditions. However, the chemical transformation of PE and PET remained unchanged in all condition except "UV-pretreated & bio-treated". Thermogravimetric analysis showed 20 ˚C lower thermal stability of PS incubated at "UV-pretreated & bio-treated + biosurfactant" than other conditions. However, the pre-treated PE and PET had lower thermal stability where biosurfactant was not used.
This research proved that despite the main limitations in biodegradation and its low technology readiness levels (TRL), a path toward increasing the efficiency of plastic degradation was feasible upon using UV-pretreatment, biosurfactant and the combination of microbial strains.

Keywords: Biodegradation; Plastic waste; Pre-treatment; Waxworms; Mealworms; Hydrogen peroxide; Stimulation; Photo-oxidation; UV-pretreatment; Thermoplastic; Biosurfactant; Rhamnolipid

Dedication

This thesis is dedicated to:

All scientists pursuing their dreams for making a better environment

&

My beloved family

Who endlessly supported me during my education

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First of all, I am sincerely grateful to work under the supervision of Associate professor Saeid Baroutian, who not only was my supervisor, but also continuously supported me in my academic and personal life as a family member. His continued advices, motivations, and supervision helped me to be a professional researcher in my field. I was always encouraged by his feedback which strengthened my technical writing skills. My PhD education would have not been possible without his knowledge, professional comments, patients, motivation and assistance.

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Glossary

AFM:	Atomic force microscopy
AO:	Acridine orange
AS:	Activated sludge
ASTM:	American Society for Testing and Materials
ATP:	Adenosine triphosphate
C:	Carbon
C_4H_4O:	Furans
$C_4H_8O_2$:	Dioxins
CH_4:	Methane
CMC:	Critical micelle concentration
CO:	Carbon monoxide
CO_2:	Carbon dioxide
CV:	Crystal violet
ECM:	Extracellular matrix
Fe^{3+}:	Ferric iron
$FeSO_4$:	Iron (II) sulphate
FS:	Farm sludge
FT-IR:	Fourier-transform infrared spectroscopy
GC-MS:	Gas chromatography-Mass spectrometry
GHG:	Greenhouse gases
GPC:	Gel permeation chromatography
H:	Hydrogen
H_2:	Hydrogen gas
H_2O_2:	Hydrogen peroxide
H_2S:	Hydrogen sulfide
HDPE:	High-density polyethylene
Hg:	Mercury
HTL:	Hydrothermal liquefaction
K_2HPO_4:	Dipotassium phosphate

KH_2PO_4:	Monopotassium phosphate
kV:	Kilovolts
LDPE:	Low-density polyethylene
mA:	Milliampere
MFI:	Melt flow index
$MgSO_4$:	Magnesium sulphate
MJ:	Megajoule
mM:	Millimolar
mN/m:	Millinewtons per meter
$MnSO_4$:	Manganese (II) sulphate
MSM:	Mineral salt media
MSM-A:	Mineral salt media agar
MSM-B:	Mineral salt media broth
MW_e:	Megawatts electric
N_2:	Nitrogen gas
N_2O:	Nitrous oxide
NaCl:	Sodium chloride
NB:	Nutrient broth
NH_4NO_3:	Ammonium nitrate
NO_3^-:	Nitrate
O_2:	Oxygen gas
OD:	Optical density
OH^-:	Hydroxide
p:	The p-value in statistics
PAHs:	Poly Aromatic hydrocarbons
PBS:	Phosphate buffered saline
PDA:	Potato dextrose agar
PDB:	Potato dextrose broth
PET:	Polyethylene terephthalate
PI:	Propidium iodide
PP:	Polypropylene

PS:	Polystyrene
qPCR:	Quantitative polymerase chain reaction
SCOD:	Soluble chemical oxygen demand
SDS:	Sodium dodecyl sulphate
SEM:	Scanning electron microscopy
SMS:	Stimulated mixed system
SO_2:	Sulfur dioxide
SO_2^{-4}:	Sulphate
TGA:	Thermogravimetric analysis
TS:	Total solid
TTC:	Triphenyl tetrazolium chloride
UIS:	Unstimulated individual system
UMS:	Unstimulated mixed system
UV:	Ultraviolet
VS:	Volatile solid
wt %:	Weight per cent

Chapter 1

Introduction

Contents of this chapter have been published as follows:

Taghavi, N., I. A. Udugama, W.-Q. Zhuang and S. Baroutian (2021). "Challenges in biodegradation of non-degradable thermoplastic waste: From environmental impact to operational readiness." Biotechnology advances 49: 107731.

1.1. Background

Plastics are long-chain polymers that have strong water resistance, strength, durability and lightness, which made them ideal alternatives to cellulosic-based materials (Muhonja et al. 2018). Due to the superior properties of plastics, they are one of the most popular materials used in consumer and industrial products. The broad usage of plastics ranges from pharmaceuticals, food packaging, cosmetics, to beverage industries. The world production of plastic as of 2020 is about 367 million tonnes per year, of which about 3 % is dumped directly into the ocean, and from the remaining, 79 % is landfilled, 12 % is thermally incinerated and just 9 % is recycled (Plastics Europe 2020).

Plastics are categorised as thermoplastics and thermosets. Thermoplastics are a type of plastic that can be deformed upon heating and reform again once they are cooled. This characteristic of thermoplastic makes them be flexible and recyclable several times, nevertheless, the quality will reduce eventually (Cabanes Gil et al. 2019). Thermoset plastics, on the contrary, are stronger and brittle than thermoplastics, but they cannot be reformed after the initial heating (Sharma et al. 2019). Despite thermoset plastics being widely used in automotive, lighting and electrical industries, the scope of this review is more focused on the treatment and degradation of waste thermoplastics.

The most globally generated thermoplastic wastes which also are the main topic of this review are polyethylene terephthalate (PET), high-density polyethylene (HDPE), low-density polyethylene (LDPE), polypropylene (PP) and polystyrene (PS) with the proportion of about 11, 14, 20, 19 and 6 % in the municipal waste stream, respectively (Fig 1.1) (Shah et al. 2008, Parker 2018, Fotopoulou and Karapanagioti 2019).

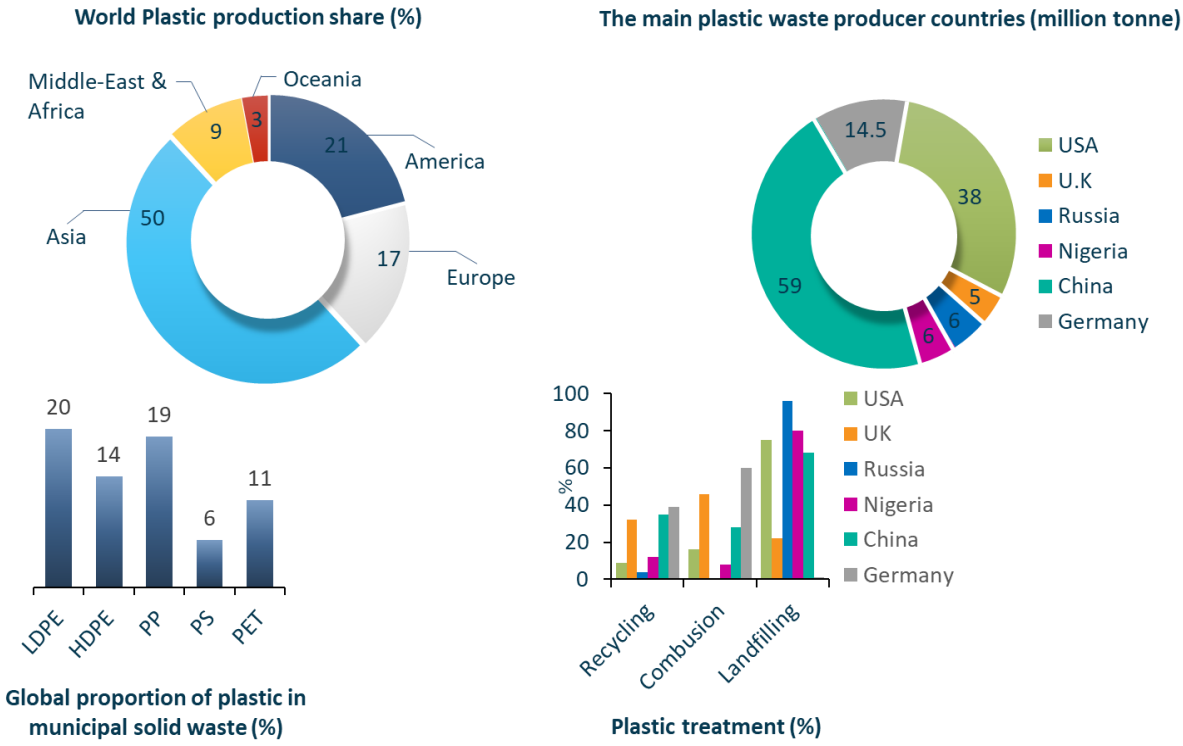


Fig 1.1 The estimated share of global plastic waste, and the used treatment method in main plastic waste producer countries (Ritchie and Roser 2018, Royer et al. 2018, Fedotkina et al. 2019, Olanrewaju and Oyebade 2019, Jiang et al. 2020)

Depending on the type of plastic and the environmental conditions, it is estimated that 10-1000 years is required for the natural degradation of thermoplastic wastes. The careless abandonment of plastic waste in the environment and deficiency of a proper waste treatment system would cause serious problems on land and marine animals. such as trapping, suffocation, and ingestion (Thevenon et al. 2014, Jambeck et al. 2015).

Plastic wastes are generated from post-consumer and post-industrial applications (Ignatyev et al. 2014). The post-industrial wastes are those plastic wastes that are disposed of at the end of a production line, and will never reach consumers. Such plastic wastes are easier to manage and handle due to their known types, sizes, and clean nature. On the other hand, post-consumer plastic wastes are mainly generated after a single use of a plastic product or plastic-containing product. It is noteworthy that post-consumer plastic wastes are usually mixed type and contaminated by other municipal solid waste streams; therefore, their separation, recycling and degradation require more energy and time (Hubo et al. 2016).

No matter where plastic waste stems from, its accumulation in the environment can cause serious issues. The presence of plastic debris in the aquatic system would release toxic compounds like plasticiser, bisphenol, phthalates, ethers, etc. into the soil, water system, and food chain (Meeker et al. 2009). These compounds can be harmful to the land and marine wildlife and endanger human health. Every year about 400,000 to one million people are killed in developing countries due to the burning of mismanaged municipal waste containing plastic debris and its associated diseases. It is also estimated that over 100 million just marine animals are killed every year from plastic pollution (Calderwood 2019, Harvey 2019, Ismail 2019). The production and treatment of plastic waste contribute to global warming because 3.8 % of the gasses produced from the processes are greenhouse gasses (methane, ethylene, and carbon dioxide) (Royer et al. 2018, Zheng and Suh 2019). It is estimated that increasing the population and quadrupling demand for plastic by 2050 will result in the emission of 56 billion tonnes of greenhouse gases (GHG), which is 13 to 15 % of the entire carbon budget (Lisa et al. 2019).

To degrade and deconstruct plastic wastes, biological and non-biological treatment methods can be used. The non-biological treatment includes photo-oxidation (ex. ultraviolet), thermal-oxidation (ex. incineration, pyrolysis, and gasification), hydrothermal liquefaction, and chemical hydrolysis (ex. enzymes, acid, base, and solvent). Mechanical processes while are not considered as a degradation method, are usually applied before each treatment to enhance the deconstruction efficiency. Recycling of plastic usually involves chemical or thermal processes that infuse with mechanical forces for the conversion of plastic waste into new products. Recycling is most feasible when the type of plastic is known, and plastic wastes all be in one type. Moreover, for some types of thermoplastics (e.g., PP and PS) which are coated with anti-oxidants, UV resistant, flame-retardant, and stabiliser to have a long-life usage in different applications (such as vehicle parts and electronic appliances) recycling can be the best eco and environmentally friendly method. However, recycling single-use plastics which are mostly mixed type and often contaminated with different municipal organic waste (e.g., food debris) may not be the proper method due to the high labour and energy requirement (e.g., collection, separation, and washing steps).

Thermal and chemical processes while effective in the degradation of plastic waste at a commercial scale within a short period, often have other limitations and drawbacks. These methods are labour and energy-intensive, release perilous chemicals and toxic gases into the environment, require a high volume of chemicals, and have difficulties in the recovery of precious by-products (Ragaert

et al. 2017, Moharir and Kumar 2019). Despite technological advances and serious efforts in plastic degradation, a gap in the utilisation of a standard and environmentally friendly method of plastic degradation remains. On the contrary, biodegradation will not leave hazardous gases or chemicals in the environment and is not energy-intensive in comparison to the current conventional methods (Schink et al. 1992, Urbanek et al. 2018).

1.2. Research Problems

The mass production of plastics and the lack of a proper environmentally friendly way of degradation damaged the environment and threatened many marine and life species. The current plastic waste degradation technologies are energy-intensive and often generate toxic gasses and by-products. Degradation of plastics with the use of live microorganisms (biodegradation) is the most environmentally friendly way of degradation. However, the slow processing time and low efficiency of biodegradation are its current main two limitations. Despite the identification of many strains able to degrade non-degradable plastics, the process is still in its infancy and cannot be implemented at a commercial scale.

1.3. Hypothesis

It is hypothesised that UV-radiation of plastics can form free-radicals within the plastic structures and improve hydrophilicity. Moreover, it is believed that biosurfactants can increase microbial colonisation by reducing the surface tension. Therefore, it is hypothesised that the integration of UV-pretreatment and the application of biosurfactants can increase the biodegradation rate and efficiency.

1.3. Objectives and scope

To date, there is no published evaluation on the effect of various UV-pretreatment conditions, the effect of rhamnolipid and the integration of both for improving the biodegradation efficiency of *P. raperi, A. flavus, P. glaucoroseum* and *Pseudomonas* spp. This study aims to provide implementable solutions for enhancing the biodegradation rate and accelerating its process by answering the two important questions in the biodegradation process:
For this purpose, several specific objectives were conceived and accomplished:

- Investigate the capability of isolated naturally-occurring strains in plastic biodegradation.

- Study the effect of hydrogen peroxide stimulated strains on biodegradation of plastic.
- Determine the effect of UV-pretreatment on biodegradation rate and efficiency.
- Study the effect of rhamnolipid biosurfactant and its integration with UV-pretreatment on biodegradation of plastics.
- Study the technology readiness level of biodegradation and its potentiality for large scale implementation.

1.4. Outline of Thesis

The thesis is comprised of six (6) chapters structured as a series of scientific papers that have been published or submitted for publication in International Scientific Indexing (ISI) journals. The contents of the chapters may overlap to some extent, specifically in the Introduction and the Experimental sections. Each chapter reports on different objectives in order to achieve the aim of the thesis. The publication-based chapters were followed by Chapter 7 "Conclusions and Recommendations for Future Work". An outline of the thesis chapters is described below.

Chapter 1. Introduction

This chapter gives a brief outlook on the adverse effects of plastic on the environment. The research problems, objectives, the scope of the study, and the outline of the thesis are also included in this chapter.

Chapter 2. Literature Review

In this chapter, the feasibility and limitation of available non-biological treatment methods on the degradation of non-degradable thermoplastic like PE, PP, PS, and PET are described in detail. Moreover, the capabilities of microorganisms, parameters affect biodegradation and the involved mechanisms are explained. Finally, the challenges in biodegradation and the environmental impact of plastic waste are described.

The contents in of chapter 1 and 2 have been published in the following article:
Taghavi, N., I. A. Udugama, W.-Q. Zhuang and S. Baroutian (2021). "Challenges in biodegradation of non-degradable thermoplastic waste: From environmental impact to operational readiness."Biotechnology advances 49: 107731.

Chapter 3. Degradation of plastic waste with stimulated and naturally occurring strains

This chapter explores the strains able to degrade non-degradable thermoplastics. The capability of identified strains is then compared together in both stimulated and unstimulated approaches.

The contents of this chapter have been compiled in the following article:
Taghavi, N., N. Singhal, W.-Q. Zhuang and S. Baroutian (2021). "Degradation of plastic waste using stimulated and naturally occurring microbial strains." Chemosphere 263: 127975.

Chapter 4. Enhanced biodegradation of non-biodegradable plastics by UV radiation

In this chapter, the effect of UV radiation conditions on PS, PE and PET is examined comprehensively. Analytical and statistical evaluations are conducted thereafter to determine the highest biodegradation efficiency and the most optimum condition.

The contents of this chapter have been compiled in the following article:
Taghavi, N., W.-Q. Zhuang and S. Baroutian (2021). "Enhanced biodegradation of non-biodegradable plastics by UV radiation: Part 1." Journal of Environmental Chemical Engineering 9(6): 106464.

Chapter 5. Enhancement in biodegradation of untreated and UV-pretreated non-degradable thermoplastic using rhamnolipid biosurfactant

This chapter aims to understand the biodegradation efficiency of mixed strains on UV-pretreated and un-pretreated PS, PE and PET plastic in the presence and absence of rhamnolipid (biosurfactant). Biodegradation efficacy then is examined by different analytical techniques to suggest the optimum condition for biodegradation of each plastic-type.

The contents of this chapter have been compiled in the following article:
Taghavi, N., W.-Q. Zhuang and S. Baroutian (2021). "Effect of rhamnolipid biosurfactant on biodegradation of untreated and UV-pretreated non-degradable thermoplastics: Part 2." Journal of Environmental Chemical Engineering 107033.

Chapter 6. Road to commercialisation

This chapter evaluates the technology readiness level of plastic degradation technologies and discusses the associated opportunities and limitations for the commercialisation of biodegradation.

The contents of Chapters 6 have been published in the following article:
Taghavi, N., I. A. Udugama, W.-Q. Zhuang and S. Baroutian (2021). "Challenges in biodegradation of non-degradable thermoplastic waste: From environmental impact to operational readiness."Biotechnology advances 49: 107731.

Chapter 7. Conclusions and Recommendations of Future Work

This chapter summarises the main findings with conclusions and the recommendations for future study.

Chapter 2

Literature Review

Contents of this chapter have been published as follows:

Taghavi, N., I. A. Udugama, W.-Q. Zhuang and S. Baroutian (2021). "Challenges in biodegradation of non-degradable thermoplastic waste: From environmental impact to operational readiness." Biotechnology advances 49: 107731.

2.1. Chapter Preface

The lack of proper plastic waste treatment systems has made the global generation of about 400 million tonne/year plastic. Although thermal technologies can degrade plastic waste, the left toxic by-products and gases endanger the environment. An energy-efficient and practical approach are required to solve one of the environmental issues. Biodegradation is a green and attractive method that can degrade plastic waste into its building blocks plastic in the most environmentally friendly way compared to conventional thermal approaches. This chapter critically reviews the advantages and drawbacks of the available plastic degradation technologies, and compare the conventional methods with biodegradation. The biodegradation mechanisms and the associated challenges are discussed in detail to suggest a proper method for enhancing biodegradation efficiency.

2.2. Current Treatment Methods of Plastic Degradation

2.2.1. Landfilling

It has been estimated that around 79% of plastic wastes are sent to landfill sites (Ritchie and Roser 2018). Generally, landfilling can be conducted in open or closed (sanitary) format. In the open-type landfilling, post-consumer plastic debris which is usually mixed with other organic compounds are accumulated on land without any engineering design of dumping site.

This type of disposal will cause critical environmental issues such as: i) soil and water pollution; ii) greenhouse gases emission; and iii) odour diffusion (Albright 2006). The abandonment of plastic waste in the open-type landfill would go under the natural photo-oxidation process. The UV and temperature from the Sun make plastic to be more brittle by defragmenting some of the C-H bonds within the polymeric structure (Andrady 2011, Raquez 2011). Consequently, the polymeric building blocks, additives and plasticizers are released into the environment over time (Hammer et al. 2012). With the addition of rainwater, these released toxic chemicals disperse into the soil and water sources (Asakura et al. 2004, Iskander Syeed Md 2016).

The emission of greenhouse gasses such as CH_4, CO_2, N_2, N_2O, SO_2 and H_2S is another drawback of the uncontrolled open-type landfill site. These gases not only are responsible for the diffusion of strong and unpleasant odours, but also have direct impacts on global warming and ozone depletion (Lamb D 2012, Royer et al. 2018). Eriksson and Finnveden (2009) reported 253 g CO_2 emitted per kg of disposed plastic in the open-type landfill in Northern Europe. In a study by Royer

et al. (2018) the amount of released CH_4 to the atmosphere from both virgin and aged non-degradable plastics were calculated, in which the amount of daily discharge CH_4 to the atmosphere was between 10- 4100 mol/g. Both CH_4 and CO_2 are prominent gases of landfill sites, however, the lighter molecular weight of CH_4 (16 g/mol) than CO_2 (44 g/mol) makes it quicker for transmission (Powell, 2016). Transmission of CH_4 from one spot to another can cause serious health problems such as nausea, dizziness, memory loss, vision problem etc. (Shen et al. 2012).

Emission of harmful greenhouse gases and release of toxic compounds in soil and water sources has resulted in the development of a safer method of landfilling, known as sanitary (Zafirakou A et al. 2013). This way of landfilling is designed to dump plastic and municipal solid waste in a pit with thick layers of protective plastic at the edges and bottom. Plastic waste in addition to the other municipal solid waste is buried in levels with clay layers in between. This can minimise the spread of leachate to soil and underwater resources by transferring it to leachate collector ponds (Patil et al. 2017). In sanitary landfilling plastic waste is buried in a deep pit with no light and oxygen transmission, degradation via photo and thermal oxidation is not feasible (Carballa et al. 2007). Although the lack of oxygen in sanitary landfilling, activate anaerobic strains, biodegradation of non-degradable plastic via this way is almost zero due to the very low energy production compared to aerobic condition. In the study conducted by Iwańczuk et al. (2015) the degradation rate as weight loss and mechanical properties of the buried PE in anaerobic conditions were not changed within 72 days of incubation.

Even though landfilling compared to other treatment methods is the easiest and common way of waste disposal, it is restricted in many countries due to environmental and land regulations. In the U.S and Australia, due to the vast sizes of ground and low-land tax policy, landfilling is still practised (Bolan et al. 2013). However, a higher labour and transportation cost in Europe, in addition to smaller land sizes compared to USA and Australia has caused many European countries to shift to other waste treatment methods (European Environment Agency 2016).

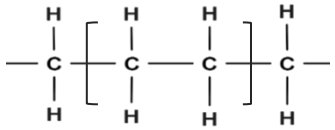
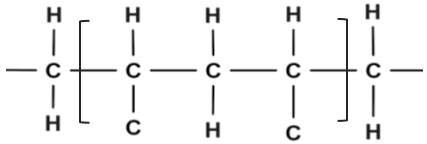
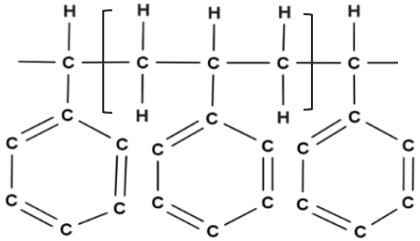
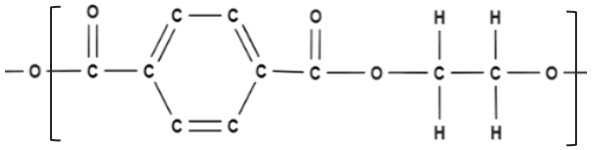
2.2.2. Incineration

Incineration is a thermal process in which air and high temperatures (>800°C) are used to reduce more than 90 % of plastic waste (Demirbaş 2000, Al-Salem et al. 2009). Incineration is also known as a waste-to-energy method that is used globally to reduce about three quarter million metric tonnes of solid waste per day to generate heat energy (Makarichi L et al. 2018). A study by Bujak

(2015) showed that an engineered incineration unit can significantly recover the produced heat energy from the combustion of plastic waste. As of 2019, 42% and 12.5% of solid waste including plastic is burned for energy recovery in Europe and the US, respectively (Elizabeth 2019). In the UK, 17% of electricity (700 MW_e) is generated from the incineration of municipal solid waste which consists of mixed plastic debris.

Energy recovery of incineration is dependent on the calorific value of the waste. Different properties of solid waste (paper, food, plastic etc.) will provide different calorific values and energy. The higher the calorific value, the higher the energy recovered from its combustion. Among other solid waste, oil-based plastic can provide higher calorific value upon combustion, which makes them a good source for energy recovery (Table 2.1) (Al-Salem et al. 2009). Morris et al., (1996) reported that effective and complete incineration of plastic waste can generate almost three times the energy compared to combustion of other municipal solid waste. Scott (1999) stated that mixed plastics have a calorific value between 30-40 MJ/kg which is almost three times higher than other municipal solid waste with a calorific value of 10 MJ/kg.

Table 2.1. Calorific value of plastic from combustion, emitted toxic gases, and GHG yield as CO2e (Wasielewski and Siudyga 2013, Zheng and Suh 2019)

Plastic type	Structure	Calorific value (MJ/kg)	CO_2e emission* (kg CO_2e/tonne polymer)	Toxic gases from combustion
LDPE		40- 44	3050	Dioxins Furans Carbon monoxide Carbon dioxide Benzene Formaldehyde Acrolein Polycyclic aromatic hydrocarbons
HDPE		37-40	3072	
PP		41-44	3349	
PS		38-40	4757	
PET		21-23	4137	
Mixed-plastic (Σ PE, PS, PP, PET)	-	35-40	3673	

* Over life cycle of plastic from production to its conversion.

Although incineration seemed to be an effective way of waste reduction and energy recovery, incomplete combustion of plastic and other municipal solid waste can create bottom ash, heavy metals, polyaromatic hydrocarbons (PAHs), and toxic gaseous such as dioxins ($C_4H_8O_2$), furans (C_4H_4O), carbon monoxide (CO), carbon dioxide (CO_2), hydrogen sulfide (H_2S)(Ecke H 2008, Verma et al. 2016).

It has also been reported that the emission of PAHs (>340 mg/kg) can cause cancer and other carcinogenic problems in humans (Valavanidis et al. 2008). In a study conducted by Li et al. (2001) the emission of PAHs from the incineration of different common plastic-type was analysed. This study found that PE, one of the most common plastic wastes, alone can produce 462.3 mg/kg PAHs. This has led countries like the Philippines and New Zealand to follow the Zero Waste Network regulation in order to ban incineration as a method of waste reduction (Ministry for the Environment 2009, Sonia 2009).

Reduction of the aforementioned gases and toxic compounds before their emission to the environment are currently among the main challenges in incineration technologies (Mikus et al. 2016). In some developed countries like Austria, Switzerland and Denmark incineration plants are optimised in a way to reduce or neutralise the creation of acidic gases, furan and dioxin with alkaline and activated carbon (Su et al. 2015, Mikus et al. 2016). However, designing and operating such incineration plants in the long term is costly and labour intensive.

2.2.3. Pyrolysis

Like incineration, pyrolysis is also categorised as a thermal treatment process which often is known as thermolysis (Anuar Sharuddin et al. 2016). In pyrolysis, complex materials are cleaved into smaller molecules in a non-oxidative condition under elevated temperatures (500-800 °C) at above atmosphere pressure (Miandad et al. 2016). Pyrolysis has been practised since the 19th century mainly for the conversion of biodegradable organic compounds like wood and coal (Sharma 2019). However, the mass use of plastic and its generated waste in the environment, has caused pyrolysis to be used as a method of plastic waste degradation (Anuar Sharuddin et al. 2016).

Pyrolysis highly depends on the physio-chemical composition of input, operating conditions (heating range, heating time, pressure) and pyrolysis type (catalytic, non-catalytic). Depending on the aforementioned factors the final product can be solid (coke, char and tar), liquid (liquid fuel) and gas (CO_2, CH_4, CO) (Williams and Slaney 2007, Chen et al. 2014, Tekin et al. 2014). Liquid

yield and its conversion to bio-oil/ fuel are one of the main aims of plastic waste pyrolysis (Scheirs and Kaminsky 2006). Polyolefin (PE and PP) and PS are good candidates for pyrolysis due to their higher calorific values and high yields of liquid oil. Production of liquid oil from pyrolysis of these plastics waste is well studied (Demirbas 2004, Kaminsky et al. 2004, Kim and Kim 2004, Wong et al. 2017). In most cases, more than 80 wt % liquid oil can be obtained from their pyrolysis (Williams and Slaney 2007). Ahmed et al. (2014) obtained about 81 wt % liquid from pyrolysis of PE at 350°C. Marcilla et al. (2009) also achieved about 85 wt % liquid oil from PE pyrolysis at 550°C (Marcilla et al. 2009). In other studies, 81 and 88 wt % liquid oil were produced by pyrolysis of PS and PP at 450 and 580°C, respectively (Miandad et al. 2016, Thahir et al. 2019).

In opposite to the high yield of liquid oil (>80 wt %) produced from PE, PP, and PS pyrolysis, generation of liquid oil from PET pyrolysis is much lower (23-40 wt %). In the study conducted by Cepeliogullar and Putun (2013), 23.1 wt % liquid oil was obtained from pyrolysis of PET at 500°C. Fakhrhoseini and Dastanian (2013), was able to produce slightly higher liquid oil (39.9 wt %) at almost similar conditions. The reason for the lower liquid oil is due to the different composition and lower calorific value of PET than other plastics. PET is composed of heteroatoms known as ethylene glycol and terephthalic acid. Therefore, for the complete degradation and liquid oil production more heating and time must be applied to break down each building block of PET. In a study conducted by Sogancioglu (2017) the amount of liquid oil from PET pyrolysis was zero due to the oligomer structure of the produced oil from PET compounds.

Pyrolysis is currently used in many countries for the production of bio-oil/fuel from plastic waste (Anuar Sharuddin et al. 2016). However, during pyrolysis, some condensable and non-condensable gases are also generated. The vapour as a routine product of pyrolysis can be added into the liquid product after its cooling and condensation, but other gases such as CO_2, CO, CH_4 and H_2 cannot be condensed and therefore remain in the gas phase, which is generally emitted to the atmosphere (Basu 2010). One of the main limitations of pyrolysis is the high operational cost. Due to the low thermal conductivity of plastic high-temperature range is required for the breakage of the polymeric structure. According to the 2013 U.S industry trade journal, a range between $8,000 to $11,500 per kilo waste is required to obtain 15 mega-watt output (Thomas 2014). The high operational and maintenance cost limit the use of pyrolysis as a green and eco-friendly method of degradation.

2.2.4. Gasification

Gasification also is a thermal treatment method to convert solid waste such as plastic into valuable syngas (H_2, CO). Depending on the use of an oxidising agent, plastic sort, gasifier type and operational conditions, the composition and application of the produced syngas are varied (Heidenreich and Foscolo 2015). The process occurs in presence of oxidising agents (air, steam or O_2) at elevated temperatures (500-1300^{o}C) (Young 2010, Dai et al. 2015). Based on the flow direction of gas, gasification is categorised into co-current (down-draft), counter-current (up-draft), cross draft, plasma and fluidized bed (Kumar 2009). One of the main advantages of gasification compared to incineration and pyrolysis is its flexibility in using mixed feedstock (ex. mixture of plastic with other solid waste). Moreover, the amount of required oxidising agent is lower than complete ignition as such less toxic gases (ex. dioxin and furan) are emitted to the environment (Thakare and Nandi 2016, Lopez et al. 2018).

The gasification of plastic waste is categorised into three main steps; drying, pyrolysis and cracking. The gasification process is initiated by the removal of external moisture in plastic waste before the pyrolysis step, where a series of complex destruction reactions take place. Gasification of plastic waste containing PE, PP, PS and PET results in almost complete conversion of plastic to volatile compounds (Sharuddin et al. 2016). However, in most cases, the municipal plastic wastes are mixed with other feedstock (ex. biomass, food residue, cardboard) which generates tar and char at the end of the process. One of the major challenges in gasification as mentioned above is the generation of char and tar, which reduces the overall syngas production (Wilk and Hofbauer 2013). To enhance the efficiencies of the process, an additional step (heterogeneous gasification) is often considered for the conversion of the formed char to syngas (Lopez et al. 2018). In the final step (cracking), the temperature is increased to about 800°C to convert volatile compounds to syngas (Artetxe et al. 2012).

The composition of produced syngas as mentioned earlier depends on operating conditions and the input feedstock. Gasification of plastic waste is currently conducted at an industrial scale; Texaco and AkzoNobel are two of the leaders of this technology, with the development based on the conventional gasification process for maximising energy recovery (Salaudeen et al. 2019). However, the complex behaviour of plastic scission during the operation, the tar and char formation and high operating temperature (< 500°C) are still the main obstacles of this treatment technology.

2.2.5. Hydrothermal liquefaction

Hydrothermal treatment is a green and promising technique due to almost > 90 % reduction of organic and solid waste in the aqueous media with almost no left toxic compounds compared to pyrolysis and gasification treatment. The operational process is also conducted at the high conditions known as subcritical (pressure: 15 to 100 bar, temperature: 150 to 250°C) or supercritical (pressure > 221 bar, temperature > 374 °C) (Baroutian et al. 2013, Baroutian et al. 2016, Munir et al. 2018). Hydrothermal liquefaction (HTL) is a subdivision of hydrothermal treatment occurring in the non-oxidative condition that has been used for depolymerisation and conversion of waste to oil (Behrendt F 2008, Toor 2011). The process occurs in presence of water which plays a key role in breaking down complex compounds at subcritical or supercritical conditions (Brunner 2009, Toor 2011, Hii et al. 2014) The process consists of three main steps known as; i) depolymerisation, ii) post-modification (ex. decarboxylation dehydration, deamination) where the oxygen content of the substrate is removed in the form CO_2, CO and H_2 gases (He et al. 2008) and iii) recombination of reactive fractions.

HTL is relatively less energy intensive in comparison to pyrolysis, gasification and incineration since a pretreatment step like drying is not required (Schindler 2015, Reißmann et al. 2018). Yang et al. (2017) studied the energy requirement between pyrolysis and HTL. As a result, they found that HTL required about 1.6 times lower energy consumption compared to pyrolysis. HTL also generates a lower tar and char compared to pyrolysis and incineration, which makes it a good substitute to conventional thermal waste degradation methods (Peterson AA 2008, Gollakota et al. 2018, Kumar 2018).

HTL is mainly used for biomass compounds, however, several studies were conducted so far on the conversion of plastic waste to oil by HTL technology (Shah et al. 1999, Zhang et al. 2007, Sugano et al. 2009, Wang et al. 2014, Raikova et al. 2019). The quantity and quality of produced oil in HTL is highly important and depends on several factors like the input feedstock, operating temperature, processing time and utilisation of homogenous or heterogeneous catalysts (Akhtar 2011). In the study conducted by Mansur et al. (2018) different parameters were examined for higher fuel production from plastic waste. Chen et al. (2019) obtained 91 wt % oil from liquefaction of PP plastic waste in supercritical conditions, which was about 10 wt % higher than oil yield in pyrolysis.

Despite relatively a lower energy requirement of HTL for breaking down the polymeric structure in comparison to the other aforementioned thermal technologies, HTL still is energy-intensive and this is considered as one of the important capital and operational limitations in comparison to biological degradation.

2.3. Biodegradation of Plastic

Biodegradation of plastics in this context defines as the utilisation of live microorganisms (mainly bacteria/ fungi) for depolymerisation of plastics and their consumption as a food source. Compared to thermal and hydrothermal degradation methods, the production of valuable products like bio-oil and syngas currently is not feasible in this method due to the low speed of biodegradation, and the nature of involved processes. However, biodegradation is relatively cheaper than the methods like pyrolysis and incineration due to not operating at high operating conditions. Moreover, biodegradation does not leave toxic gases and hazardous compounds in the environment which makes it an interesting eco and environmentally friendly way of plastic degradation.

The first sign of microbial colonisation on plastic dates back to the 1970s (Carpenter and Smith 1972). Since then, the search for identification of plastic degrading microorganisms has never stopped, and several strains from the genus of *Rhodococcus*, *Aspergillus*, *Pseudomonas*, *Penicillium*, *Bacillus*, *Serratia*, *Phanerochaete*, *Rhizopus* were reported to have the capabilities of degrading PE, PP, PS, and PET (Iiyoshi et al. 1998, Sivan et al. 2006, Kyaw et al. 2012, Yang et al. 2014, Yoshida et al. 2016, Lou et al. 2020, Zhang et al. 2020). As it can be seen from Fig 2.1, the number of biodegradation studies were increased from 33 in 1970-1980 to about 670 in 2010-2020. The studies were mainly focused on PE plastic which possibly could be due to the inert structure of PE that hardly degradation in the environment, and due to its higher production demand compared to other plastic types.

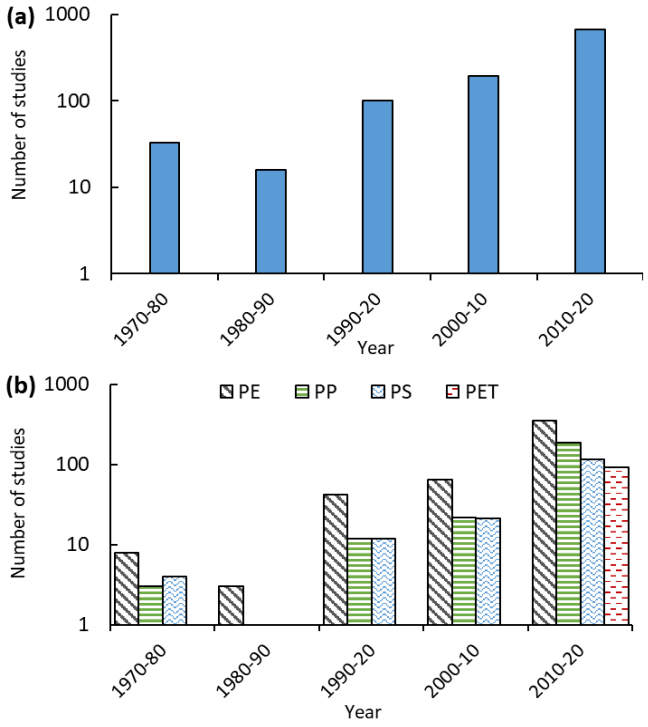


Fig 2.1. The number of biodegradation studies from 1970-2020 for (a) all non-degradable plastic types; (b) each plastic type. The data was obtained from Scopus database by limiting keywords in the abstract as "Biodegradation of plastic" AND "plastic type".

The increase in the number of studies in the last decade was due to the significant outcome, and identification of microbial capabilities in colonisation and secretion of enzymes to degrade plastic. For example, *Bacillus* spp. was reported to play an important role in plastic biodegradation due to their ability to secret surface-active compounds (biosurfactant) that results in higher plastic accessibilities and microbial colonisations (Mukherjee et al. 2016, Vimala and Mathew 2016, Tadimeti 2020). Recently, Yoshida et al. (2016), identified a novel strain *Ideonella sakaicnsis* 201-f6 that is able to degrade PET plastic into its building blocks via secretion of PETase and MHETase. Identification of the aforementioned new enzymes caused other researchers specifically in recent years to focus more on PET biodegradation via enzyme engineering (Wei et al. 2016, Knott et al. 2020). In Table 2.2 the capability of some of the highlighted strains in biodegradation of each plastic type is illustrated in more detail.

Table 2.2. The capabilities of some highlighted strains in plastic biodegradation

Strains	Plastic type	Degradation time (day)	Weight loss (%)	Reference
Aspergillus niger	PE	30	17.35	(Kathiresan 2003)
Aspergillus niger	PE	30	4.32	(Wadood et al., 2018)
Aspergillus niger	PE	160	-	(Raghavendra et al. 2016)
Aspergillus niger	PE	30	5.8	(Raaman et al. 2012)
Aspergillus versicolor	PE	90	40.6	(Gajendiran et al. 2017)
Aspergillus flavus	PE	28	3.9	(Zhang et al. 2020)
Aspergillus flavus	PE	100	5.5	(Taghavi et al., 2021)
Aspergillus japonica	PE	30	11.11	(Raaman et al., 2012)
Aspergillus fumigatus	PE	30	2.49	(Wadood et al. 2018)
Aspergillus glaucus	PE	30	28.8	(Kathiresan, 2003)
Bacillus brevis borstelensis 707	PE	30	11	(Hadad et al. 2005)
Bacillus amyloliquefaciens	PE	60	11	(Das and Kumar 2015)
Bacillus subtilis UCP 999	PET	60	0.06	(Jara et al. 2009)
Bacillus subtilis	PE	30	9.26	(Vimala and Mathew 2016)
Bacillus subtilis	PE	60	17.7-23.1	(Ibiene et al. 2013)
Bacillus subtilis	PS	30	23	(Mohan et al. 2016)
Bacillus subtilis	PE	30	1.75	(Harshvardhan and Jha 2013)
Bacillus pumilus	PE	30	1.5	(Harshvardhan & Jha, 2013)
Bacillus mixture	PE	90	-	(Abrusci et al. 2011)
Bacillus cereus	PE	40	1.6	(Roager and Sonnenschein 2019)
Bacillus cereus	PP	40	12	(Auta et al., 2017)
Bacillus cereus	PS	40	7.4	(Roager and Sonnenschein 2019)
Bacillus cereus	PET	40	6.6	(Roager & Sonnenschein, 2019)
Bacillus gottheilii	PS	40	5.8	(Roager and Sonnenschein 2019)
Bacillus gottheilii	PE	40	6.2	(Roager and Sonnenschein 2019)
Bacillus gottheilii	PET	40	3	(Roager & Sonnenschein, 2019)
Bacillus circulans	PE	45	2.33	(Aly et al. 2018)
Bacillus mycoides	PE	60	10.5-11.3	(Ibiene et al., 2013)
Bacillus sphaericus	PE	365	3.5-10	(Sudhakar et al. 2008)
Bacillus thuringiensis	PE	9	-	(Ray 2019)
Bacillus sp. YP1	PE	28	10.7	(yang et al., 2014)
Ideonella sakaicnsis 201-f6	PET	42	58	(Yoshida et al., 2016)

Table 2.2. Continued

Strains	Plastic type	Degradation time (day)	Weight loss (%)	Reference
Penicillium simplicissimum	PE (treated)	90	38	(Sowmya et al. 2015)
Penicillium pinophilum	PE	942	-	(Volke-Sepúlveda et al. 2002)
Penicillium frequentans	PE	60	0.45	(Seneviratne et al. 2006)
Penicillium funiculosum	PET	84	0.21	(Nowak et al. 2011)
Penicillium chrysogenum NS10	PE (treated)	90	34.3- 58.5	(Ojha et al. 2017)
Penicillium oxalicum NS4	PE (treated)	90	36.6 – 55.3	(Ojha et al., 2017)
Pseudomonas aeruginosa	PE	120	20	(Kyaw et al. 2012)
Pseudomonas aeruginosa	PE	30	7.3- 8.5	(Badahit et al., 2018)
Pseudomonas syringae	PE	120	11.3	(Kyaw et al., 2012)
Pseudomonas putida	PE	120	9	(Kyaw et al., 2012)
Pseudomonas citronellolis	PE	4	17.8	(Bhatia et al. 2014)
Pseudomonas fluorescens	PE	30	7.8 -7.9	(Badahit et al. 2018)
Pseudomonas sp.	PE	30	20.54	(Kathiresan, 2003)
Rhodococcus rubber C208	PS	56	0.86	(Mor and Sivan 2008)
Rhodococcus rubber C208	PE (treated)	56	7.5	(Sivan et al. 2006)
Rhodococcus rhodochrous	PE	240	-	(Eyheraguibel et al. 2017)
Sporosarcina globispora	PP	40	11	(Auta et al. 2017)
Penicillium simplicissimum	PE (treated)	90	38	(Sowmya et al., 2015)

2.3.1. Biodegradation Mechanism

The mechanism of biodegradation ties to the microbial tactic to turn insoluble and long-chain molecules into soluble and small molecules through deterioration, depolymerisation, mineralisation, and assimilation steps.

The biodegradation begins with the adhesion and colonisation of living microorganisms (bacteria/ fungi) to the surface of plastics (deterioration). The surface deterioration would ease the penetration and colonisation of microorganisms internally. In the second stage, extracellular microbial exoenzymes further break down the deteriorated plastic into various compounds with different molecular weights (Mueller 2003).

In the assimilation step, some of the small-medium size oligomers (less than 600 Daltons) formed during the previous steps are now able to be transported or diffuse into the cytoplasm of microbes to be consumed for anabolism or catabolism. However, the remaining dimers and oligomers which could not be utilised formerly, are required to be further broken down by the microorganism's catalytic agents (exoenzymes / free-radicals) (Lucas et al. 2008). Now, some of the formed molecules are small and soluble enough to pass through the semi-permeable microbial membrane and be used as their energy sources. Upon complete degradation, the final products of the mineralisation step based on microbial respiration are CO_2, H_2O, and CH_4 (Gu 2003, Kolvenbach et al. 2014). Nevertheless, other metabolic products such as microbial biomass, salt, and organic compounds can also be formed as a result of biodegradation (Dommergues and Mangenot 1972, Muhonja et al. 2018). In Fig 2.2, the schematic of the plastic biodegradation mechanism is illustrated.

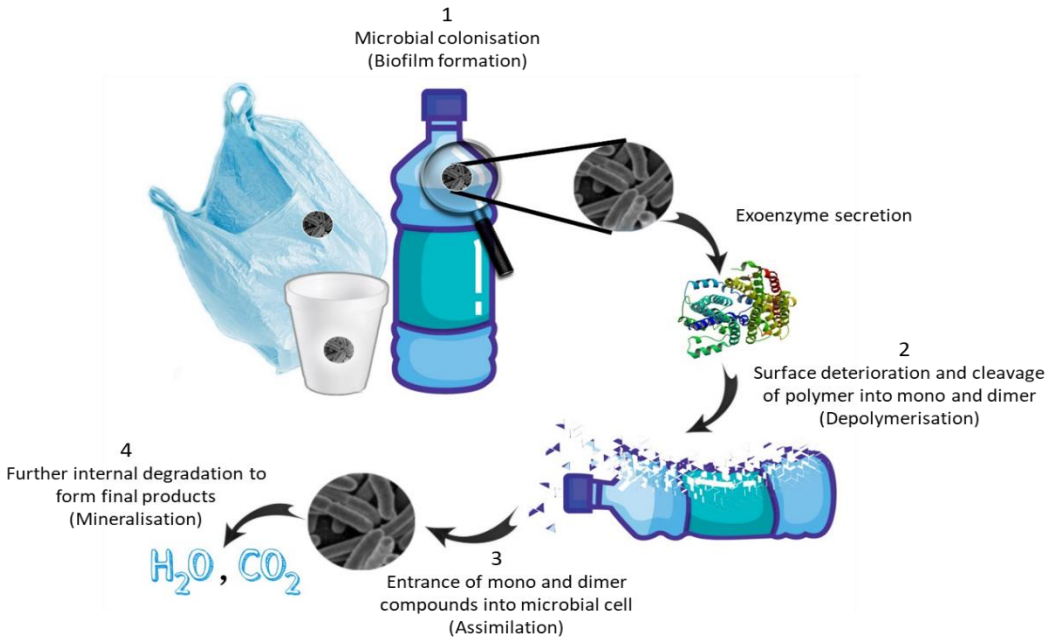


Fig 2.2. The mechanism of plastic degradation by microorganisms

Biodegradation is highly linked to the secretion of effective microbial exoenzymes on the surface of plastic (Donaruma 1991, Kamal M R and Huang B 1992). Indeed, microbial enzymes act as catalytic agents to break down long-chain and high-weighted polymers into smaller fragments. Their role is to reduce the activation energy for speeding the reactions (Blanco and Blanco 2017). Sometimes, for breaking down compounds, secretion of an enzyme from one strain is not effective and efficient enough, and instead, the synthesis of several enzymes from a group of strains (microbial consortia) is needed.

Enzymatic depolymerisation is followed by the catalytic triad mechanism sharing a set of three coordinated amino acids (aspartate, histidine, and serine). The aforementioned catalytic triad mechanism can be found in the α/β- hydrolase fold superfamily (Stryer L et al. 2002, Sun et al. 2014). An example of the serine-histidine-aspartate catalytic triad can be found in the study conducted by Yoshida et al. (2016). In their study, a novel enzyme, PETase, was identified from *Ideonella sakaiensis* 201-F6 which was able to degrade PET plastic into mono-2-hydroxyethyl terephthalate (MHET) and a low amount of bis (2-hydroxyethyl) terephthalate (BHET). Similar to the other previous PET degrading hydrolase enzymes (lipase, esterase, and cutinase), PETase also belongs to of α/β hydrolase superfamily that uses the triad serine-histidine-aspartate catalytic mechanism for depolymerisation (Liu et al. 2018).

The mechanism starts with the serine where its active site becomes receptive for the upcoming substrate in its cleft (Hedstrom 2002) (Fig 2.3). The serine then makes a nucleophilic attack on the substrate and discharges its hydrogen ion, which is later taken by the facilitated histidine (step 1). This nucleophilic attack would cause that serine to behave as an alkoxide group for making a covalent bond with the substrate carbonyl carbon (step 1) (Lucas et al. 2008, Alves et al. 2018). It is worth mentioning that the transition state complex is not formed without the cooperation of histidine and aspartate in the mechanism. Aspartate reduces the acidity level of imidazole nitrogen in histidine structure by increasing the pKa from 7 to 12; this makes histidine a strong base to take the hydrogen ion released from the nucleophilic attack of serine to the substrate (Gutteridge and Thornton 2005).

In the intermediate state, tetrahedral intermediates are produced which are then transformed into acyl-enzyme intermediate. This will be performed by a proton donation of histidine to the amide group of the substrate and in its cleavage at C-terminuses from the enzyme complex (steps 2 & 3) (Neitzel 2010). The next step in ternary catalytic hydrolases enzymes is in a correlation of water molecules. The hydroxide ion (OH^-) of the water binds to the ester functional group causing the removal of the covalent bond between serine and peptide (step 4). Subsequently, serine is turned into a very nucleophilic alkoxide group and takes the hydrogen (H^+) ion of water from histidine

(step 5) (Jiang et al. 2016). This brings separation of another peptide from the enzymatic mechanism and re-generation of serine peptidase for another set of the enzymatic cycle (step 6). In Fig 2.3, the catalytic mechanism of triad serine, histidine-aspartate upon reaching a compound having a carbonyl group in its structure was illustrated.

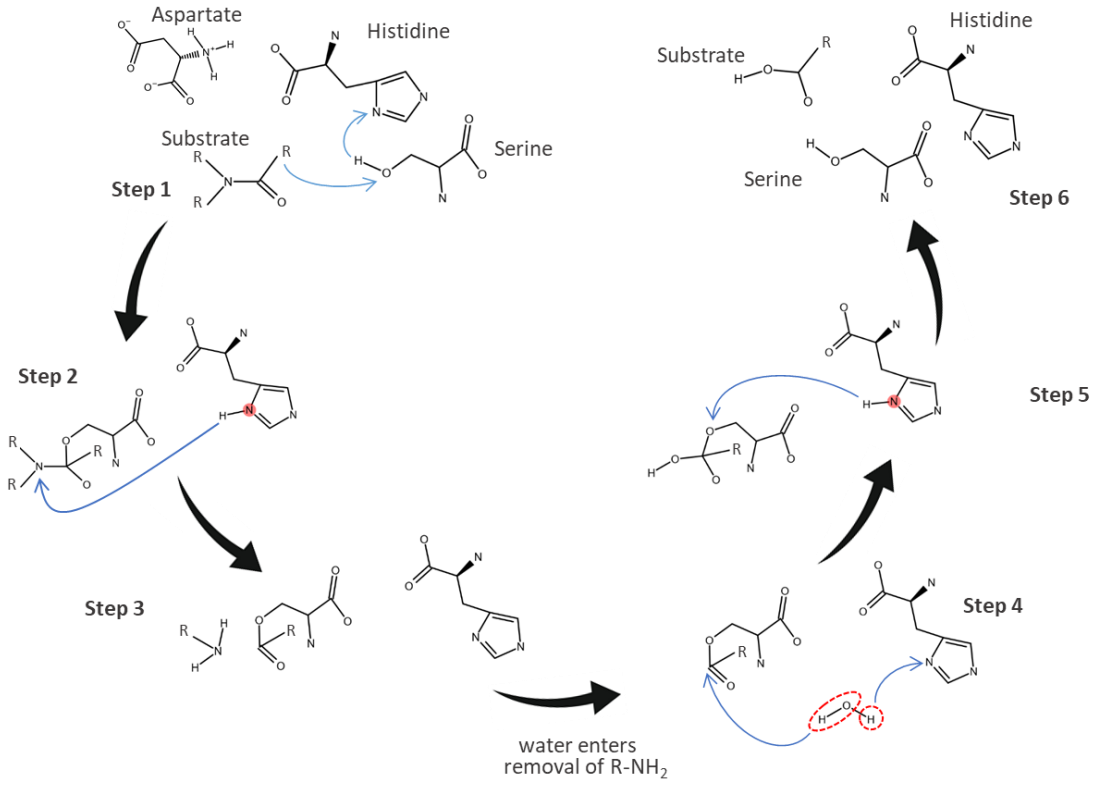


Fig 2.3. The catalytic mechanism of triad serine-histidine-aspartate; adapted from Cozma (2014)

It has been also reported that the degradation activity of selected strains as a mixed community is faster and more efficient than the degradation of monoculture (Sowmya et al., 2015) due to the participation of each strain in the microbial community and the diversity of secreted enzymes (Skariyachan et al. 2019). The utilisation of microbial consortia rather than a single strain for biodegradation enhancement was also reported by several researchers (Manzur et al. 2004, Roy et al. 2008, Mukherjee et al. 2016). The biodiversity of strains derived from landfills, soil, sludge, or wastewater can significantly affect the biodegradation process. In the following chapters, it will be shown that higher surface erosion and physical alteration occur on PS and PET plastic where these plastics will be incubated with a mixture of strains (*Penicillium raperi*, *Aspergillus flavus*, *Penicillium glaucoroseum*, and *Pseudomonas* spp.) rather than a single strain. Ogunbayo et al. (2019) showed that the incubated PE with a mixture of *Pseudomonas* sp. and *Aspergillus niger*

degraded up to 15 wt% compared to the individual incubation of PE with *Pseudomonas* spp (7.2 wt%) and *Aspergillus niger* (12.4 wt%).

Secretion of microbial enzymes is either constitutive or inductive. In the former case, enzymes exist constantly in the microbial pathways, while in the latter case the catalytic enzymes are involved in the reactions only if an inducing signal or external initiator reaches the cells. The synthesis of inductive enzymes is increased over time until the inducer is used completely (Lucas et al. 2008). A common example of an inductive enzyme is the synthesis of β-galactosidase for hydrolysing of lactose into D-glucose and D-galactose in *Escherichia coli*.

The production of β-galactosidase enzyme in *E. coli* was induced due to the presence of lactose in the microbial media as the sole carbon source. Indeed, the *E.coli* cells cannot utilise lactose to synthesize β-galactosidase when glucose is available insufficiently in the media (Juers et al. 2012). While degradation of a complex polymer such as plastic is different and not comparable to the degradation of simple sugar like lactose because stimulation can sometimes induce signals in microbial cells pathways to secret peroxidase enzymes in the benefit of polymer biodegradation, such as PE plastic.

Peroxidase is produced in most aerobic organisms to oxidise H_2O_2 to water and oxygen during aerobic respiration. It has been reported that stimulation by a non-lethal concentration of H_2O_2 can induce the microbial cells to secrete peroxidase for H_2O_2 removal. Moreover, Iiyoshi et al. (1998) showed that for breaking down the lignin in the media, fungal cells are required to secrete lignin peroxidase, which then was in benefit of PE biodegradation.

While the exact mechanism and role of peroxidase on PE degradation are not yet well understood, it is believed that the oxidation of H_2O_2 by peroxidase can cause the formation of free $OH^{\cdot}$ and $O^{\cdot}$ radicals. Since these radicals are not stable, they can interact with other organic compounds. In the presence of PE these radical species bind to the cleaved end or middle carbon of PE and make it more susceptible to microbial enzymatic attack. The free $OH^{\cdot}$ and $O^{\cdot}$ radicals can respectively make OH-C-C-C-C- and O=C-C-C-C- upon cleavage of PE from its end or can make -C-C-OH and =O-C-C-C when PE is cleaved from its middle part.

Despite the inductive or consecutive type of enzyme, the production of microbial enzymes can be extra or intracellularly. Intracellular enzymes (endoenzymes) associate with inner microbial pathways (tricarboxylic acid cycle, beta-oxidation, etc.) while extracellular enzymes (exoenzymes) secrete outside the microbial cell; i.e., within the aquatic media and/or among the organic matters (soil, sludge, sediment) for the initial breakdown of the polymeric structure. The exoenzymes are responsible for cutting the polymeric chains from the ends and edges (Moussard 2006, Mateo et al.

2007). Usually, the excreted exoenzymes react with molecules of 10-50 carbons; however, Yoon et al. (2012) reported a possible microbial enzymatic activity on molecules having 2000 carbons.

As mentioned earlier, biodegradation highly depends on the diversity of strains in the consortium, (landfill, soil, ocean, wastewater), the size of the microbial community, growth conditions (aerobic or anaerobic), colonisation on/in plastic, and secretion of different enzymes through the aforementioned mechanisms. For example, in aerobic conditions, microorganisms intake plastic as a carbon source and use oxygen molecules in their electron transport chain to reach the final product of CO_2, H_2O (Krzysztof and Magdalena 2012). This is how in anoxic conditions, nitrate (NO_3^-), ferric iron (Fe^{3+}), sulphate (SO_2^{-4}), and carbon dioxide (CO_2) are used as the terminal electron acceptors in the electron transfer chain to produce CH_4, CO_2, or H_2O as end-products. Compared to anaerobic respiration, more energy known as adenosine triphosphate (ATP) is produced in aerobic respiration, which indeed provides higher microbial survival, reproduction, and consequently a better efficiency.

The size of the microbial community (inoculum) also plays a key role in the biodegradation process. The higher number of microorganisms increases the chance of survival, colonisation, enzyme secretion, which can result in higher biodegradation. While no study measured the direct effect of inoculum size in biodegradation of the aforementioned thermoplastics (PE, PS, and PET), the effect of different inoculation sizes was studied in bioremediation of other tyre and toxic compounds like phenol and bisphenol-A (the usual toxic compounds derived from degradation of plastic). Beshay et al. (2002) showed that an increase in the size of *Acinetobacter* sp. from 0.05 g/L to 0.2 g/L could increase biodegradation of phenol by 8 folds. Similarly, Lao et al. (2019) also found that the maximum specific growth rate and shortest lag phases of *Bacillus* sp. on scrap tyre occurred when inoculum size increased from 5 to 20 %. In another study, Eltoukhy et al. (2020) found that the inoculum size lower than the optimum value reduced microbial survival, colonisation, and enzymes synthesis for degradation of bisphenol-A.

Besides the role of microorganisms in the biodegradation process, physiochemical properties of plastics (e.g., size, shape, thickness, and crystallinity) also can play crucial roles in benefit or against biodegradation. Chamas et al., (2020) described in detail that a plastic film could be degraded 260 and 1100 times faster than plastic fibre and bead of the same mass and crystallinity respectively. Increasing the number of branches in polymers structure results in higher molecular weight, toughness, viscosity, strength, and melting point, which indeed reduces the biodegradation efficiencies. Since microorganisms' transport systems are only able to take up low-mid range molecular weight into their intracellular membrane, high-molecular-weight polymers are required

to be defragmented into the smaller molecules before passing through the cell membrane (Shah et al. 2008, Kolvenbach et al. 2014).

Environmental factors such as UV, heat, weather conditions, and physical forces can cause surface cracking and erosion, chemical-bond cleavage, molecular weight, and tensile strength reduction. These physiochemical alterations make plastic more fragile and susceptible to being degraded by microorganisms. In the study conducted by Roy et al., (2008), the UV pretreated PE degraded higher than un-irradiated PE. Velrajan & Andrew (2012) also showed that applying heat as a way of PE physicochemical alteration could enhance the weight loss up to 5 %.

2.4. Biodegradation Challenges and Environmental Impact

2.4.1. Blending Plastics with Degradable Additives

In last recent decades, the research for the production of biodegradable plastic by proportional addition of degradable compounds like starch or corn into the structure of non-degradable thermoplastic was in high demand. Several studies stated that biodegradable plastics could be a green alternative to petroleum-based plastics, and degraded at a higher rate in the environment by the microbial community (Lefebvre 2012, Khoramejadian 2013, Mierzwa-Hersztek et al. 2019). Though the reported degradation efficiency of biodegradable plastics containing additives often misinterprets, and the stated degradation rates are related to the degradable part (starch or corn) rather than the plastic itself. In the study conducted by Dave et al. (1997) the blended PE with starch (70:30) showed only 6.3 % PE weight loss and 84.5 % degradation of starch. The addition of starch or corn into the plastic structure makes it more hydrophilic and results in higher microbial colonisation. Due to the gained microbial enzymes through the evolution, the biodegradable parts will degrade initially into simple sugars for consumption as a food source. However, after almost complete consumption of biodegradable parts, the degradation rate will be reduced due to the non-degradability of plastic itself. Rapa et al. (2010) examined the biodegradability of PE-starch blended (70:30) with soil microorganisms for three months. Their result showed that biodegradability is correlated to the consumption of starch and not the plastic itself. They also stated that the only benefit of starch blended PE was an improvement for the penetration of microbial into plastic through the formed holes in plastic after utilisation of biodegradable parts.

Besides the aforementioned reasons, the production of biodegradable plastics would not solve the current global plastic crisis. One of the current environmental issues, is the mass abonnement of plastics and micro and nanoplastics in soil, ocean, and water systems which can endanger several marine and land creatures. To solve the present plastic problem in the world, a green and efficient

method is required to degrade the current plastic waste rather than making biodegradable plastic for future use.

2.4.2. Environmental Impacts of Generated By-products

One of the main challenges in biodegradation is increasing degradation efficiency. Currently, the biodegradation rates of plastics are much lower than the conventional thermal treatment technologies. The low efficiency of plastic biodegradation stems from the lack of proper microbial enzymes, and the strong physiochemical structure of plastics making the biodegradation harder (Sánchez 2020).

On top of the low degradation efficiency, the effect of plastic degradation by-products on the environment is another concern. While biodegradation is considered the most environmentally friendly way of plastic waste reduction in comparison to thermal and landfilling technologies (Shah et al. 2008), the incomplete biodegradation process can also result in the formation of various by-products that are harmful to the environment. Therefore the toxicity of created by-products is needed to be carefully assessed for any new biodegradation processes (Shahnawaz et al. 2019).

Depolymerisation of plastic in the environment by heat, UV, or physical forces remains different un-degradable products that could affect the environment, animals, and humans. The nature of these generated by-products depends on different factors such as plastic type, strain specious, and microbial enzymatic system. In the study conducted by Shahnawaz et al. (2019) the diversity of generated by-products from two different bacteria were assessed. They found that even at the same operating condition each strain behaves differently on PE degradation, which results in the creation of different by-products. While the variety in the formation of by-products can broaden the chance of toxicity, most of the generated by-products were reported to have no or low-level toxicity to humans, animals, and the environment. Farzi et al. (2019) identified 20 different compounds from incubation of PET with *Streptomyces* sp; they found that except ethylbenzene and o-xylene the other identified by-products were safe for both humans and the environment.

In another study conducted by Sangale et al. (2019), the toxicity of derived by-products from biodegradation of PE with *Aspergillus terreus* strain MANF1/WL and *Aspergillus sydowii* strain PNPF15/TS were tested for the viability of a tiger shark and germination of sorghum seeds. In that study different compounds as 2, naphthalene carboxylic acid; 1,2 benzene dicarboxylic acid; octadecanoic acid; diethyl phthalate; dibutyl phthalate; 1,2-bis (trimethylsilyl) benzene; hexasiloxane; 2-cyclohexen-1; 7-Methylenebicyclo [3.2.0] hept-3-en-2-one; cyclohexane-1 and dodecahydropyrido [1,2-b] isoquinolin-6-one were formed with no death and germination

inhibition. Pramila and Ramesh (2015) also reported that the formed 2-buthene; 2- methyl, acetone, and ethene from biodegradation of PE with *Acinetobacter baumannii* were not toxic on germination, chlorophyll content, and physical morphology of *Vigna radiata*. In another study, analysis of formed by-product from incubation of *Brevibacillus* spp. and *Aneurinibacillus* spp. with PE and PP plastic within 140 days confirmed the formation of cis-2-chlorovinyl acetate; tri-decanoic acid, and octa-decanoic acid with no toxicity effect in the environment (Skariyachan et al. 2018).

Different bacteria and fungi strains were used to evaluate the effect of derived by-products from incubation mixed plastic (PE, PS, and PET) with soil, activated sludge and farm sludge microbial consortia. The toxicity of the derived by-products previously was evaluated colourimetrically by consumption of triphenyl tetrazolium chloride (TTC) in live and active strains and was shown that the produced by-product had no inhibition in microbial growth and activity compared to control.

Despite biodegradation is considered the most environmentally friendly way of plastic degradation (Sánchez 2020) with no left toxic and hazardous compounds, one of the important environmental impacts of plastic is the formation of micro (<5 mm) and nano size plastics (0.1 μm < in size). The small plastic particles can be formed as a by-product during biodegradation by the act of microorganisms or can be created after the abonnement of single-used plastics in the environment and exposure to UV, heat, or physical forces. In both cases, the formation of micro and nanoplastics could end up in the soil, food, and water systems which may bring several problems for marine and land species.

Napper and Thompson (2016) showed that about 700,000 microplastics particles were entered into our water system and then in the environment after just one cycle of washing machine contained synthetic clothes. While in many developed societies, these particles can be effectively filtered before entering into the water system, still there is a chance of contamination especially in developing countries where such advanced filtration may not even exist.

The toxicity and environmental pollutant of micro and nanoplastics may derive directly from ingestion of the plastic itself, or by contact with other chemicals that associate with microplastics in soil or ocean (Szymańska and Obolewski 2020). While the number of studies for toxicity effect of micro and nano size plastic is still very limited, the adverse effect of micro and nanoplastics were mainly documented in marine and land animals compared to humans except at a high concentration (Yong et al. 2020).

In the study conducted by Ziajahromi et al. (2018) the effect of PE microplastics on survival and growth of *Chironomus tepperi* was analysed and found that a high concentration of PE microplastics affected the growth and shape of *C. tepperi* body. Hwang et al. (2020) studied the

potential toxicity of micro and nano PS plastic particles at an approximate dosage of 500 μg/mL in humans at a molecular level. They found that PS microplastic particles at that dosage had no toxicity effect on humans' cells, however, the particles at nano size (460 nm- 1μm) were affected red blood cells and led them to hemolysis.

While the mass dispersion of micro and nanoplastics in ocean and soil is problematic, the exact effect of each plastic type at different concentrations is not yet well understood which require further investigation in both animals and humans. In Table 2.3, some of the highlighted generated compounds from the biodegradation of thermoplastics by different microbial strains are shown.

Table 2.3. The list of produced by-products as the result of plastic biodegradation

Plastic	Strain	Degradation time (day)	By-products		Reference
PET	*Streptomyces* sp.	18	1) Ethyl benzene 2) o-Xylene 3) 1,2- β Pinene 4) 1,8- Cineole 5) I-Menthone 6) p-Menthan−3-one	7) 2-(4′-Nitro−2′-Thienyl) Pyrimidine 8) Germacrene D 9) Hexamethyl Cyclotrisiloxane 10) 6-Methyl−2-Phenylindole 11) N-Methyl−1 Adamantaneacetamide 12) Cyclobarbital	(Farzi et al. 2019)
PS	*Exiguobacterium indicum* HHS31	60	1) 2-Pentanone, 4-hydroxy-4-methyl 2) (R)-(-)-2,2-Dimethyl-1,3-dioxolane-4-methanol 3) Oxalic acid, butyl 6-ethyloct-3-yl ester	4) Phenol, 2,4-bis(1,1-dimethylethyl) 5) 1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	(Yang et al., 2015)
PS	Cephalosporium sp.	56	1) Pyridine 2) 1,3,5 Cycloheptatriene 3) Benzene, chloro 4) Methane, tris(methylthio) 5) 1H-Indene,2,3-dihydro-1,1,3-trimethyl-3-phenyl 6) Benzene, 1,1′-(1,1,2,2-tetramethyl-1,2-ethanediyl) bis	7) 2,4-Diphenyl-4-methyl-2(E)-pentene 8) Pentadecanoic acid, 2,6,10,14-tetramethyl-, methyl ester 9) 13-Tetradece-11-yn-ol 10) 9-Octadecanoic acid, ethyl ester 11) Octadecanoic acid, ethyl ester	(Chaudhary and Vijayakumar 2020)
PS	*Mucor* sp.	56	1) *n*-Hexane *2)* Cyclohexane 3) Pyridine 4) 1,3,5 Cycloheptatriene 5) Benzene, chloro 6) Methane, tris(methylthio)	7) 2,4-Diphenyl-4-methyl-1-pentene 8) Benzene, (1,1-dimethyldecyl) 9) Phenol, 2,4-bis(1-methyl-1-phenylethyl) 10) 1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl	(Chaudhary and Vijayakumar 2020)
PE	*Streptomyces sp.*	90	1) 1,4-epoxynaphthalene-1(2h) -methanol,4,5,7-tris(1,1-dimethylethyl) −3 2) 2-t-butyl-5-chloromethyl-3-methyl-4-oxoimidazolidine-1-carboxylic 3) tetrapentacontane	4) Squalene 5) diethyl phthalate 6) benzene, 1,3-bis(1,1-dimethylethyl)	(Abraham et al. 2017)
PE	*Aspergillus nomius*	90	1) phenol 2) 3,5-bis(1,1-dimethylethyl) 3) 2-t-butyl-5-chloromethyl-3-methyl-4-oxoimidazolidine-1-carboxylic 4) dotriacontane	5) ethyl 14-methyl-hexadecanoate 6) diethyl phthalate 7) benzene, 1,3-bis(1,1-dimethylethyl) 8) dodecane, 1-fluoro 9) difluorophosphoric acid	(Abraham et al. 2017)

Table 2.3. Continued

Plastic	Strain	Degradation time (day)	By-products		Reference
PE	*Aspergillus sydowii* strain PNPF15/TS	60	1) 7- Methylenebicyclo [3.2.0] hept-3-en-2-one 2) Dibutyl phthalate 3) 1,4-Benzenediol	4) Dodecahydropyrido [1,2-b] isoquinolin-6-one	(Sangale et al. 2019)
PE	*Aspergillus terreus* strain MANF1/WL	60	1) 2 Naphthalene carboxylic acid 2) 2-Cyclohexen 3) Dibutyl phthalate	4) 1,2-Bis (Trimethylsilyl) benzene 5) Hexasiloxane 6) Hexadecanoic acid	(Sangale et al. 2019)
PE	*Lysinibacillus fusiformis* VASB14/WL	60	1) 1- Trimethylsilylmethanol 2) 1,2,3,4 Tetra methyl benzene 3) Hexadecanoic acid		(Shahnawaz et al. 2016)
PE	*Bacillus cereus* VASB1/TS	60	1) 1,2,3 Trimethyl benzene 2) 1 Ethyl 3,5-dimethyl benzene 3) 1,4 Di methyl 2 ethyl benzene	4) Dibutyl phthalate 5) Hexadecanoic acid	(Shahnawaz et al. 2016)
PE	*Pseudomonas aeruginosa* PAO1	140	1) Benzene, methyl 2) Tetrachloroethylene 3) Benzene,1,3-dimethyl 4) Octadecane 5) 7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione 6) Hexadecanoic acid 7) Eicosane 8) Octadenoic acid	9) Docosane 10) 3-Chloropropionic acid, heptadecyl ester 11) Tricosane 12) Octadecanoic acid, butyl ester 13) 1-Nonadecene 14) Hexacosane 15) Pentacosane 16) 1,2-Benxenedicarboxylic acid, diisoostyl ester	(Kyaw et al. 2012)
PE	*Aspergillus oryzae* strain A5	112	1) 4,4- Dimethy;-2-pentene 2) 4,6- Octadiyn-3-one,2-methyl		(Muhonja et al. 2018)
PE	*Enterobacter sp. D1*	31	1) 6-methyls-5-hepten-2-ol 2) Monobenzyl phthalate 3) N-Acetylglutamic acid	4) Ethyldodecanoate	(Ren et al. 2019)

2.5. Current Solution for Biodegradation Enhancement

2.5.1. Pretreatment

In pretreatment technologies (known as plastic ageing), plastic is exposed to chemicals (acid, base, solvent), heat, and/or ultraviolet (UV) radiation to enhance microbial colonisation and biodegradation efficiencies (Farzi et al. 2019). Regardless of the type of initiator in pretreatment technologies, the principal almost follows the same and would lead to the formation of free-radicals. The generated radicals will participate in different steps of the polymeric degradation pathways (initiation, propagation, and termination). Depending on the plastic type, random chain scission, autoxidation, and cross-linking reactions occur to create different short compounds (Gewert et al. 2015).

In the first step, UV or heat will cause the formation of free-radicals within the plastic backbone (step 1). It should be noted that for plastics such as PE and PP which lack unsaturated double-bonds in their structure, autoxidation may not occur as easily as PS and PET plastics. PE and PP are mainly produced in a transparent shape which means they lack chromophore for UV absorption. However, the addition of additives, dyes, pro-oxidant, impurities, or manufacturing defects is just enough to make PE and PP susceptible to autoxidation (Gewert et al. 2015, Chamas et al. 2020).

In the propagation step, the formed polymer radicals can join together via crosslinking or undergo autoxidation by the available environmental oxygen (step 2). An abstraction of H from another polymer can bind to the formed peroxy group in another polymer and make hydroperoxide. Chain scissions occur and the hydroperoxide converts into alkoxy radical and peroxyl radical by participation in different reactions (step 3). The propagation step repeats several times and water usually is generated as a by-product. In the final step, termination, two radicals join together to form short-chain and more stable compounds. Since alkoxy radical is less stable than peroxy radical, it undergoes random chain sessions and results in the formation of carbonyl and alkene (Vasile and Pascu 2005).

Photo-oxidation or thermal oxidation of PS (having styrene ring in its structure) as a way of pretreatment can lead to the formation of olefin, carbonyl, styrene, α-methyl styrene, benzene, benzoic acid, toluene, benzyl alcohol, phenol, benzaldehyde (Hoff et al. 1982). Oxidation of PS will cause a colour change from whitish to yellowish and makes PS be more brittle (Yousif and Haddad 2013). Despite PE, PP, and PS having C-C in their backbone, PET is made of heteroatoms by a combination of ethylene glycol and terephthalic acid. The existence of hydrolytic groups within the PET structure would lead PET to be hydrolysed from its ester or amide bond (Müller et al. 2001). Chain cession and hydrolysis of PET through the polymeric degradation pathway will create compounds mainly vinyl and carboxylic acid end-group (Thomas and P. M 2011). In Fig 2.4 the reaction mechanisms of lead to form shorter compounds are illustrated.

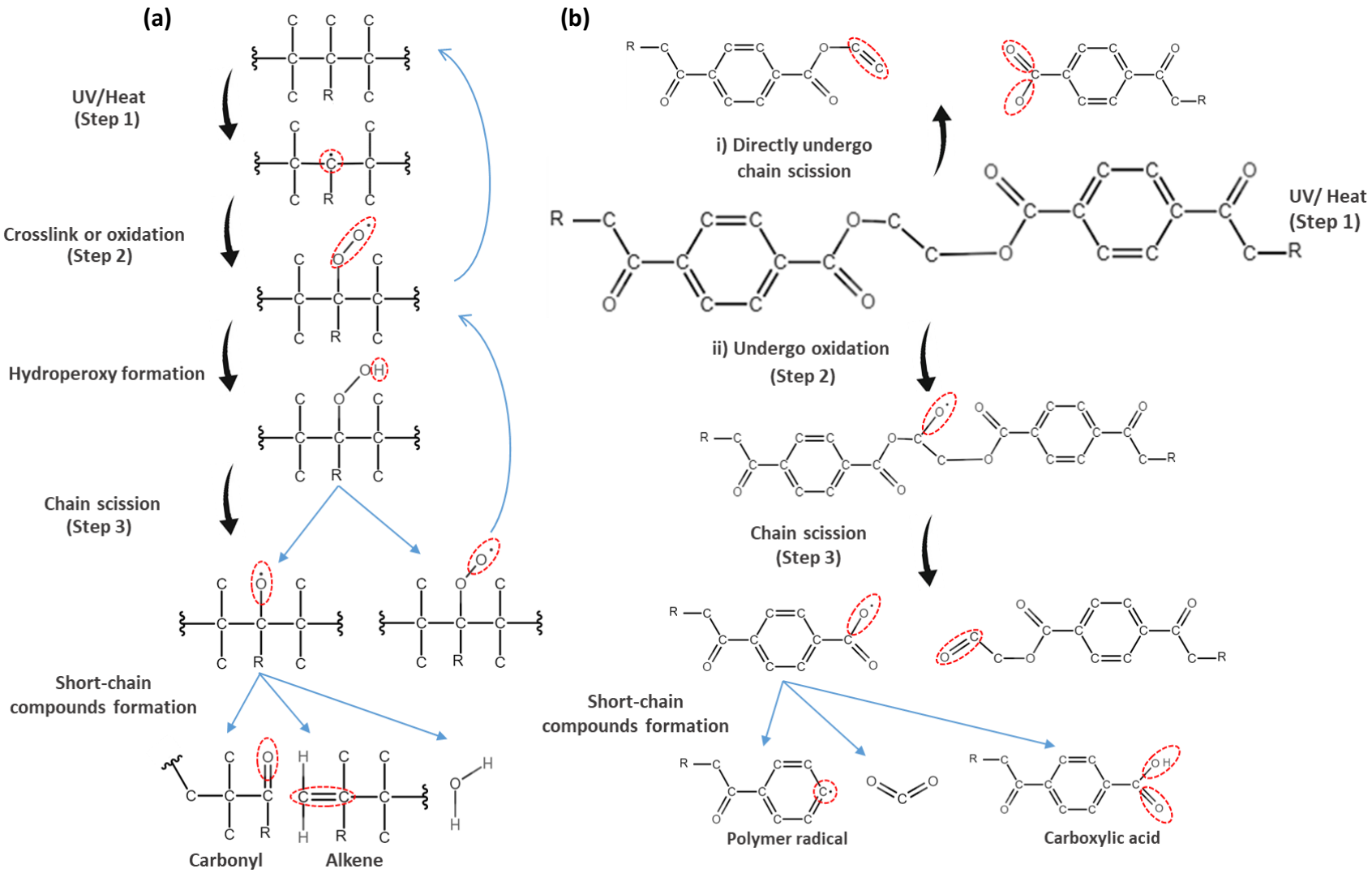


Fig 2.4. Degradation mechanisms of (a) PE, PP, and PS with carbon backbone structure and (b) PET with ethylene glycol and terephthalic acid; adopted from Gewert et al. (2015)

The creation of these short-chain compounds (carbonyl, carboxylic acid, and olefin) upon plastic pretreatment will increase hydrophilicity, microbial colonisation, and biodegradation efficiency (Erdmann et al. 2020). While the effect of pretreated PS and PET were not studied in biodegradation enhancement, there were many reports on the role of pretreated PE and PP in biodegradation improvement. Esmaeili et al. (2013), pretreated PE plastics under UV light in a laminar hood for 25 days and observed higher biodegradation (as mineralisation) in the UV pretreated PE plastic (29.5 %) than un-treated plastic (15.8 %). In another study conducted by Markandan et al. (2019), higher bacterial colonisation and growth rates were observed on the UV irradiated PET than un-irradiated. Chaudhary and Vijayakumar (2020) also reported that the chemically pretreated PE with nitric acid for 6 days can enhance the degradation efficiency up to 7.18 %. Rajandas et al. (2012) achieved 50.5 and 61% biodegradation after giving the pretreated PE in nitric acid to *Pseudomonas aeruginosa* and *Microbacterium paraoxydans*, respectively. In another study, Kittur and co-workers (2013) obtained weight loss up 27.33 % when pretreated PE with UV and nitric acid was given to microorganisms. Chemical pretreatment of plastic alone or in combination with other pretreatment methods while is effective in hydrolysing of plastic and degradation efficiency, it is not environmentally friendly. Indeed, the used chemical compounds (acid, base or organic solvent) is required to be recovered totally before their discharge into the environment, which is problematic. In Table 2.4, some of the used pretreatment methods for improving the biodegradation efficiency by particular strain is shown in detail.

Table 2.4. Previous studies on plastic pretreatment and biodegradation enhancement

Plastic	Strain	Pretreatment method	Pretreatment time (day)	Degradation time (day)	Weight loss %	Reference
PE	*Bacillus subtilis*	UV (< 300 nm)	3	30	9.26	(Vimala & Mathew, 2016)
PE	*Bacillus* spp. *(halodenitrificans, pumilus, cereus*)	UV (280-370 nm)	-	730	8.4	(Roy et al. 2008)
PE	*Penicillium simplicissimum*	UV and acid treated	-	90	38	(Sowmya et al., 2015)
PE	*Penicillium simplicissimum*	UV	20.8	90	-	(Yamada- Onodera et al., 2001)
	Penicillium simplicissimum	Nitric acid	6		-	
PE	*Penicillium* sp.	UV (< 300 nm)	10	180	~ 3.5	(Velrajan and Andrew 2012)
	Rhizopus arrhizus					
	Penicillium sp.				~ 3	
	Rhizopus arrhizus	Heat (100 °C)	30		~ 5	
	Penicillium sp.				~ 4.3	
	Rhizopus arrhizus	Nitric acid (concentrated)	10		~ 4.5	
					~ 4	
PE	*Acinetobacter pitti*	UV (245 nm)	16	28	26.8	(Montazer et al. 2018)
PE	*Curvularia lunata* SG1	UV (354 nm)	2.1	90	43.76	(Raut et al. 2015)
		Heat (80 °C)	5		36.20	
		UV (354 nm) + Heat (80 °C)	2.1 + 5		48.40	
PE	*Arthrobacter oxydans* + *Arthrobacter globiformis*	UV (312 nm) + Pro-oxidant (calcium stearate)	15 + 7	30	52	(Pereira et al. 2012)
PE	*Aspergillus niger* & *Lysinibacillus xylanilyticus* XDB9	UV (254 nm)	25	126	29.5	(Esmaeili et al., 2013)
PE	*Brevibaccillus borstelensis* strain 707	UV (312 nm)	2.5	30	11	(Hadad .D et al., 2004)
PE	*Fusarium* sp. AF4	UV (254 nm) + Nitric acid (99%)	16.4	90	-	(Hasan et al. 2007)
PE	*Microbacterium paraoxydans*	Nitric acid	10	60	61	(Rajandas et al. 2012)
	Pseudomonas aeruginosa				50.5	
PP	*Bacillus flexus*	Heat (100 °C)	8	365	0.65	(Arkatkar et al. 2010)
	Bacillus subtilis				0.6	
	Bacillus flexus	UV (225 nm)	6		2.5	
	Bacillus subtilis				1.5	
PP	*Bacillus flexus* + *Pseudomonas azotoformans*	UV (225 nm)	6	365	1.95	(Aravinthan et al., 2016)

2.5.2. Use of Surface-active Compounds

One of the main struggles in the enhancement of biodegradation efficiency is linked to the increase of microbial colonisation on the surface of the plastic. Previous studies showed that the utilisation of mineral-oil and surfactant were able to improve the biodegradation efficiencies. Surfactants are active compounds with an amphiphilic structure that can reduce the surface tension between the plastic and microbial cells. This causes plastics to be dispersed more in the media and results in a higher microbial attachment. Mor & Siven (2008) also reported that the utilisation of mineral oil as a surface-active agent increased the microbial biofilm formation on PS plastic. In the study conducted by Gong et al. (2019), higher microbial colonisation on PE plastic incubated with Tween 80 was observed.

Unlike synthetic surfactants which are non-degradable and toxic for the environment, biosurfactants (surfactants extracted from microorganisms) are completely biodegradable and can be used in bio-based applications. Several genera of bacterial species (ex. *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Rhodococcus*, *Mycobacterium*, *Serratia*, *Corynebacterium Flavobacterium*, *Clostridium*) were stated as biosurfactant producers for industrial-scale applications (Bodour et al. 2003, Sachdev and Cameotra 2013). However, the utilisation of synthetic and biosurfactants in plastic biodegradation has rarely been reported, which could stem from the complexity and effectiveness of the process. Almost all of the previously conducted studies were focused just on the enhancement of PE biodegradation and underestimated the role of surface-active compounds for a higher degradation of other plastic like PP, PS, and PET.

Among the previously mentioned strains, only the biosurfactants from *Bacillus* and *Pseudomonas* spp are known as surfactin and rhamnolipid respectively were studied in detail. It was reported they are efficient in biodegradation enhancement by reducing the water surface tension from 73 mN/m to less than 30 mN/m (Soberón-Chávez and Maier 2011). In the study conducted by Marajan et al. (2018) the surface tension of water reduced significantly from 73 to 30 after just 18 h growth of *Bacillus subtilis* in media.

Vimala and Mathew (2016) also showed that the secretion of surfactin from *B. subtilis* could increase the weight loss up to 9.2 %. From the reported results, *B. subtilis* has a great potential to be examined further in the enchantment of plastic biodegradation. Despite the potentiality of biosurfactants from *B. subtilis* in plastic biodegradation, there is still an extensive gap in the effect of many other surface-active compounds for maximising microbial attachment and biodegradation improvement of PP, PS, and PET plastic. Table 2.5, shows the studied surface-active compounds in plastic biodegradation.

Table 2.5. The utilisation of surfactant and biosurfactant for biodegradation enhancement

Plastic	Strain	Surface-active agent	Degradation time (day)	Weight loss (%)	Reference
PE	*Bacillus licheniformis* and *Lysinibacillus* sp.	Biosurfactant	30 days	2.97	(Mukherjee et al. 2016)
PE	*Bacillus subtilis*	Biosurfactant	30	9.26	(Vimala and Mathew 2016)
PE	*Bacillus licheniformis* and *Pseudomonas fluorescens*	Biosurfactant	30	7.13	(Mukherjee et al. 2018)
PE	*Streptomyces coelicoflavus* NBRC 15399T	Biosurfactant	28	30	(Midhun et al. 2015)
PE	*Pseudomonas aeruginosa*	Tween 80	60	-	(Albertsson et al. 1993)
PE	*Cupriavidus necator* H16	Tween 80	21	33.7	(Montazer et al. 2018)
PE	*Lysinibacillus fusiformis*	SDS (Sodium dodecyl sulphate)	30	7	(Mukherjee et al. 2017)
PE	*Rhodococcus ruber* C208	Mineral-oil	30	8	(Gilan et al., 2004)

2.5.3. Enzyme Engineering

One of the reasons that the biodegradation treatment of plastics is still in its infancy is due to the lack of proper microbial enzymes for complete degradation. Nevertheless, to date different enzymes (ex. esterase, catalase, hydrolase, lipase, tannase, cutinase, laccase, peroxidase, laccase, PETase, and MHETase) were discovered to degrade PE, PP, PS, and PET at a low rate. Among the aforementioned secreted enzymes, one of the highlighted examples is the discovery of PETase and MHETase from a novel bacterium *I. sakaiensis*. While these two enzymes are among the most powerful natural secreted enzymes for the deconstruction of PET into its building blocks, its low thermal stability at elevated temperature reduces its utilisation at any commercial scale.

To maximise stability and degradation efficiency, enzyme engineering can be beneficial for overcoming the associated challenges. In the study conducted by Austin and co-workers (2018), the engineered mutated PETase with a wider active site was synthesised which allows binding of larger compounds. Compared to the natural PETase, their mutant enzyme was able to improve the degradation activity of both PET and its bio-derived replacement (polyethylene-2,5-furandicarboxylate).

Wei et al. (2016) also showed that, by exchanging the cutinase enzyme sequence extracted from *Thermobifida fusca* KW3 (TfCut2) to the leaf-branch compost cutinase (LLC) from compost metagenome, their engineered enzyme was able to degrade PET more than 42wt% after 50 h of hydrolysis at 65°C. In a recent study conducted by Son et al. (2019) the engineered PETase not only exhibited higher stability by 8.8 °C, but also the degradation activity improved 14-fold relative to the wild type.

Knott and co-workers (2020) recently, engineered a super enzyme by linking PETase and MHETase (PETase-MHETase) together via a bridge, which could degrade PET into its building block up to six times faster than the naturally occurring enzyme when their secreted alone.

Despite significant improvement in the production of engineered PETase for PET degradation, there is not yet effort for synthesising the mutant enzyme for PE, PP, and PS degradation. While PE, PP, and PS are also among the most generated plastic wastes globally, the unavailability of proper natural enzymes to work with could be one of the main limitations in protein engineering. One of the reasons that biodegradation's efficiency of plastic in the environment is very low can stem from the fact that in the environment (e.g., landfill) plastics are not the only source of carbon for microorganisms, and they are usually mixed with other biodegradable organic wastes. Consequently, the chance of microbial habitation on plastic as the only available food source and synthesis of a new enzyme for its degradation will reduce. To identify a new plastic degrading enzyme from naturally occurring microbes, plastics should be introduced as the sole carbon source to microorganisms for a long period. The identification of two new plastic degrading enzymes (PETase, MHETase) from the novel strain,

I. sakaiensis was approve of the aforementioned statement. In which *I. sakaiensis* inoculated from the yard of a contaminated bottle-recycling factory which PET was the only source of carbon for five years.

2.6. Summary of Chapter and Conclusion

Mass production of plastics and the generation of plastic wastes undoubtedly are among the main environmental issues worldwide. Despite the technological advances in the thermal reduction of plastics at commercial scales, there are still thousands of tonnes that remained to be degraded. The unavailability of an efficient and environmentally friendly operational approach has even made the degradation process harder. Plastic biodegradation as an environmentally friendly method has received attention in the last decade, and several plastic degrading strains were identified in this regard. Nevertheless, the search for exploring new microbial enzymes and enzyme engineering is still in high demand. In addition to thermal processes and enzyme engineering, the effect of other green methods like UV radiation and surface-active compounds were also studied; however, these approaches alone were not effective enough in improving the degradation efficiency and integration of them is required for a higher biodegradation efficiency. In the following chapter, the capability of microbial strains for biodegradation of non-degradable thermoplastic in both naturally occurring and stimulated conditions are investigated.

Chapter 3

Degradation of plastic waste with stimulated and naturally occurring strains

Contents of this chapter have been published as follows:

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3.1. Chapter Preface

In this chapter, the biodegradation ability of different microbial strains from activated sludge (AS), farm sludge (FS), soil and worm's excreta will be evaluated individually and in the form of consortia (mixed) at both unstimulated and stimulated conditions. It is believed that oxidative condition such as H_2O_2 can stimulate strains for activation of new enzymes and metabolic pathways in benefit to biodegradation. Different analytical examination are then conducted to identify promising degrading strains.

3.2. Introduction

The lack or low activity of proper enzymes in microorganisms through the evolution has challenged the biodegradation. While microorganisms of one genus almost behave in a same way, the geographical differences always can be effective in mutation and activation of new enzymes. In 2016, a group of Japanese researchers searched a wide variety of native microorganisms for five years until they found a new strain *I. sakaicnsis* that could degrade PET bottle into its building blocks (Yoshida et al., 2016). Exploring a wider variety of microorganisms can increase the identification chance of novel strain(s) and enzyme(s) in benefit to biodegradation. Therefore, the first aim of this chapter is to evaluate the efficiencies of different microbial community from soil, activated sludge (AS), farm sludge (FS), and worms' excreta for biodegradation of plastic. The gut microbial community in mealworms (larvae of *Tenebrio molitor)* and waxworms (larvae of the *Plodia interpunctella*) have ability in biodegradation of plastic PS (Yang et al. 2014, Yang et al. 2015, Bombelli et al. 2017, Brandon et al. 2018, Lou et al. 2020). Upon ingesting the plastics, the living microbial strains in the gut system of such worms start to depolymerise plastic into small fragments by enzymatic activities. The excreta, which contains residues of undigested plastics and was mixed with gut's microorganisms can be used as an inoculum for biodegradation (Yang et al. 2015). Previous studies, identified strains from soil and worms' excreta in the natural occurring approach (Hadad et al. 2005, Esmaeili et al. 2013, Bettas Ardisson et al. 2014). However, the capability of strains in FS and AS are not studied globally and in New Zealand. Therefore, the capability of New Zealand's native microbial consortia derived from the aforementioned sources is worth to investigate. The second aim of this chapter is to test the most promising identified strains in both unstimulated (natural occurring) and stimulated conditions. Stimulation of the

strains by non-lethal concentration of H_2O_2 can induce microbial cells to secrete scavenging enzymes like peroxidase, which not only can help strains to survive in oxidative condition, but also can enhance the biodegradation efficiency by oxidation of plastic (Iiyoshi et al. 1998, Shimao 2001, Mukherjee and Kundu 2014). The outcome of this chapter can show that whether the native microbial community of New Zealand able to degrade non-degradable thermoplastics or not.
The following sections describe the experimental setup and various characterisation methods to confirm plastic biodegradation.

3.3. Experimental

3.3.1. Materials

The chemicals used in this study were KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, NH_4NO_3, NaCl, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, triphenyl tetrazolium chloride (TTC), crystal violet (CV), nutrient agar (NA), nutrient broth (NB), potato dextrose agar (PDA) and potato dextrose broth (PDB) and agar powder. These chemicals are analytical grade and were purchased from Sigma-Aldrich (New Zealand). Surface soil that was not mixed with any fertiliser or compost was collected from the garden of the University of Auckland. Live waxworms (the larvae of the *Plodia interpunctella*) and mealworms (the larvae of *Tenebrio molitor)* were obtained from local suppliers. Farm sludge and activated sludge were provided by Alfriston Valley Farm (Auckland), and Watercare wastewater treatment plant (Rosedale, Auckland), respectively.
Four plastic samples as HDPE (shopping bag), PP (sheet), PS (foam) and PET with yellow, milky, white and shiny-brown colour, and respective thickness of 0.14mm, 0.3 mm, 2 mm and 0.24 mm, were purchased locally. Two sets of plastic samples were prepared as 15 mm × 15 mm films and small pieces (~ L< 6 mm, W< 5 on average of 30 pieces). The reason behind using small pieces is to increase the surface area and better dispersion of plastic to microorganisms. Therefore, plastic samples were ground with a commercial grinder until fine particles were obtained. Prior to use, plastics were disinfected by 75 % ethanol for 1 h, rinsed 3 times with sterilised Milli-Q water and oven-dried at 60 °C overnight.

3.3.2. Media Preparation

Mineral salts media broth (MSM-B) was prepared as (per litre) 0.7 g of KH_2PO_4, 0.7 g of K_2HPO_4, 0.7 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of NH_4NO_3, 0.005 g of NaCl, 0.002 g of $FeSO_4 \cdot 7H_2O$, 0.002 g of $ZnSO_4 \cdot 7H_2O$, and 0.001 g of $MnSO_4 \cdot H_2O$ per 1000 mL of sterilised Milli-Q water according to ASTM G21-15 protocol (ASTM 2013) . The pH was adjusted to 7.2 with 1 M NaOH prior of usage. The mineral salts media agar (MSM-A) was prepared by adding 15 g agar powder to 1000 mL of MSM-B and autoclaved at 121 °C for 20 min. The microbial enrichment culture (NA, NB, PDA and PDB) were prepared according to the manufacture description and autoclaved at 121 °C for 20 min.

3.3.3. Inoculums

3.3.3.1. Worms

The preparation method was conducted according to (Yang et al., 2014) with some modifications. In brief, worms were subjected to 48 h starvation before having plastic films as a sole food source. Four containers containing 50 worms separately were fed with 8 pieces (15 mm × 15 mm) of each plastic type for 30 days. The survival rate of worms was checked every 3 days and dead worms were taken out from the containers. Every week live worms were transferred into other containers in order to clean the original containers and collect the excreta. The collected worms' excreta were stored at -20 °C before using, and the worms were moved back to their original clean container containing the same initial plastic. At the end of incubation, the excreta from the container with the highest mass loss in the given plastic was selected as microbial consortia for the experiment. The selected excreta were mixed with 0.9 % saline water, centrifuged at 12,000×g (6 min), and the pellet was mixed with 75 mL MSM-B to be used for the biodegradation experiment.

3.3.3.2. Sludge

Sludge samples were kept for 2 h at room temperature prior to characterisation and preparation. Prior of inoculation, the microbial consortia in the sludge samples were activated by constant aeration for 48 h with a small aquarium air pump. After the aeration, 300 mL of each sludge sample was transferred to a separate 50 mL falcon tube and centrifuged at 12,000×g for 5 min.

The supernatant was removed and pellet mixed with 75 mL MSM-B and used as microbial consortia in this study.

3.3.3.3. Soil

The soil sample (10 g) was crushed gently with a mortar and pestle prior to the experiment in order to obtain fine particles. The fine soil was mixed with sterilised Milli-Q water in a 0.3 mm porous stomacher bag and put in a stomacher apparatus for 15 s. The homogenous content was centrifuged at 12,000×g (6 min), and the obtained pellet was mixed with 75 mL MSM-B in order to be used as microbial consortia for the biodegradation experiment.

3.3.4. Biodegradation Design

3.3.4.1. Natural Occurring

The biodegradation was evaluated by surface cracking, weight loss, biofilm formation, decolourisation, chemical alteration and microscopy techniques. To understand the efficiencies of microbial strains under different conditions, a series of aerobic biodegradation experiments were conducted respectively as unstimulated mixed system (UMS), unstimulated individual system (UIS) and stimulated mixed system (SMS) within 270, 100 and 30 days, respectively. The mixed system here was defined as a condition where different microbial consortia inoculated with all four types of plastic, whereas in the individual system, single pure strains were inoculated individually with only one type of plastic. Unstimulated strains were inoculated in mixed and individual systems, while stimulated strains were only subjected to the mixed system.

To initiate the biodegradation process, each microbial suspension (75 mL) was mixed individually in 1000 mL conical flasks containing 175 mL of sterilised MSM-B. The flask containing mealworm excreta was incubated with 16 pieces of PE and PS plastic films (8 pieces of each type), and 4 g fine plastic particles of PE and PS films (2 g from each type). The flask containing waxworm's excreta was incubated with just 8 pieces of PS films and 2 g of PS fine plastic particle. All other microbial suspensions were incubated with 32 pieces of plastic films (8 pieces from each type), and 8 g of the fine plastic particle (2 g from each type) as a carbon source. Flasks containing MSM-B without any inoculum were used as a control. All experimental sets of solid and liquid cultures were performed in duplicate.

All flasks were incubated on a shaking incubator (110 rpm) at 30 °C for 270 days. After 45 days, 1 mL of enrichment culture containing microbial samples were removed from each flask and serially diluted to 10^{-4} to 10^{-6}. Diluted samples were individually spread (100 µL/ plate) on fresh sterilised MSM-A plates containing 8 pieces of sterilised plastic film (2 pieces from each type) as a carbon source. Agar plates were incubated at 30 °C for 28 days and the growth of microbial strains on plastic films were monitored every 3 days.

The grown bacteria and fungi on agar plates containing mixed plastic films were isolated and sub-cultured into fresh NA and PDA plates, respectively to obtain pure strains. To assure that the isolated strains were able to form biofilm and attach to the particular plastic type again, another set of experiment was performed. The pure strains were grown on MSM-A plates containing only the same type of plastic film, and were incubated at 30 °C for 28 days. To understand the efficiency of isolated pure strains in UIS biodegradation, each strain was inoculated aseptically in a 50 mL falcon tube containing 20 mL fresh sterilise MSM-B and the particular type of plastic they could grow on before. Tubes were incubated at 30 °C with constant shaking at 110 rpm for 100 days.

3.3.4.2. Stimulation

To investigate whether the microbial stimulation can enhance biodegradation efficiencies within a shorter time or not, different microbial consortiums knowing as FS, AS, soil, mixed pure strains (the mixture of all isolated pure strains), mix-consortia (the mixture of FS + AS + Soil) were prepared and subjected separately to an oxidative shock of non-lethal H_2O_2 concentration. The concept behind combining all of the microbial consortia, and the isolated strains was to understand whether a larger community of microorganisms in the unselected community (mixed consortia) behave differently to the selected community (mixed pure strains) in biodegradation or not.

Prior of the experiment, eight different bacteria and fungi were grown individually in 50 mL tubes containing nutrient-rich media for 48 h at 30 °C and 100 rpm agitation. After 48 h, 1 mL of each tube was removed and fungal and bacterial cells were adjusted to 4.3×10^4 spore/mL and OD_{600} = 0.4 - 0.5, respectively. To determine the non-lethal concentration of H_2O_2, 400 µL from the adjusted microbial cell suspension was transferred to 48-well microplates containing either NB or MSM-B depending on the microbial type. Microplates were appropriately incubated with 50 mM H_2O_2 to get a final concentration of 0.5, 1, 5, 10 mM. Media containing microbial inoculation with and without 1M H_2O_2 were used as negative and positive controls, respectively. All microplates

containing either bacteria or fungi were incubated for 7 days at 30 °C and 25 °C, respectively. All experiments were performed in duplicate.

Bacterial growth was measured via a direct reading of absorbance at 600 nm, while fungal growth was assessed colourimetrically via consumption of TTC according to Ghaly and Mahmoud (2006) with some modification. The TTC is a redox indicator that is mostly used to understand whether cells are metabolically activated and live or not. The white TTC powder will turn pink upon activation of dehydrogenases enzymes in live and metabolically activated cells.

In brief, after 7 days, microplates containing fungal cells were mixed appropriately with TTC at a final concentration of 10 mg/mL for 48 h at 25 °C. The formed TTC was extracted by 48 h incubation at 25 °C. At the end of incubation, the content of each well was transferred in 2 mL Eppendorf tubes and subjected to centrifugation at 10,000×g for 5 min. The supernatant containing extracted TTC was then filtered with 0.22 μm Whatman syringe filters and absorbance was read at 485 nm. The sample containing only 95 % ethanol was used as blank.

After identification of non-lethal H_2O_2 concertation, the stimulation phase began by preparing 5 different sets of microbial consortia as mentioned previously. The AS, FS and soil microbial samples were obtained by removing 5 mL aliquot directly from flasks used in UMS. Prior to the experiment, each microbial consortium was pre-cultured in NB until mid-log phase ($OD_{600\ nm}$= 0.3- 0.4); cells were centrifuged at 10,000×g, 15 °C for 6 minutes and pellets were suspended in either NB or Phosphate Buffered Saline (PBS) (pH= 7.4) for stimulation in the NB or MSM-B condition, respectively. Each obtained aliquot containing microbial consortia was added in an individual 250 mL conical flask containing sterilised plastics and the non-lethal concentration of H_2O_2. Flasks were incubated with constant agitation at 80 rpm, 30 °C for 30 days. Every 10 days, plastic samples were taken out for analysis. The experiments were carried out in duplicate and results were expressed as mean ± standard deviation of duplicate.

3.3.5. Analytical Methods

3.3.5.1. Sludge and Soil Characterisation

The sludge samples were characterised by measuring the soluble chemical oxygen demand (SCOD), total solid (TS), volatile solid/ total solid (VS/TS), moisture, ammonia-nitrogen content and pH. The pH of sludge was measured at room temperature by a pH meter (Orion Star A211, Thermo Scientific). The SCOD was determined by a test kit (Hach HR 25 to 1500 mg/L) and

evaluated by using a spectrophotometer (Hach, DR 3900) (Kolb et al. 2017). For this, 2 mL of each sludge sample was vacuumed filtered (MS2, 47 mm, MicroScience). Similarly, the ammonia content was also analysed by test kit (HacH HR 02.5 mg/L) followed by using a spectrophotometer (Hatch, DR 3900). Moisture content and total solid was fulfilled by drying sludge samples in the oven at 105 °C for 3 h. The difference between the weight of moist and weight loss of moisture indicates the moisture content (O'Kelly 2004).

To determine soil texture, 10 g of crushed soil mixed with 250 mL distilled water in a 250 mL measuring cylinder containing. The container was sealed with parafilm and shacked several times until soil was mixed fairly.

3.3.5.2. Biofilm Estimation

Biofilm formation was examined on the plastics incubated in UIS. Due to the strong attachment of microbial strains to the surface of plastic films, estimation of the formed biofilm was not feasible by using conventional direct methods or cell counting. Therefore, the evaluation of microbial population on the surface of plastic was assessed by CV assay according to Eich et al. (2015) with some modification. Crystal violet can stain the formed extracellular matrix (ECM) on the biofilm and can estimate the total biofilm existence (Chavez-Dozal et al. 2016).

In brief, plastic films were washed gently with sterilised Milli-Q water to remove unattached planktonic (free) cells. Samples then were immersed into 5 mL 3 % (w/v) CV for 15 min followed by a mild washing step. Plastic films then were subjected to an extraction step in a new plate containing 3 mL 95 % ethanol. The absorbance of the obtained aliquot at the end was measured at 590 nm in the microplate reader (PerkinElmer, MLD2300, USA).

3.3.5.3. Weight loss

Weight loss was examined on plastic films incubated in UIS and SMS. To determine weight loss in each period, two plastic films (known as sample 1 and sample 2) were gently washed 3 times with 75 % ethanol, sterilised Milli-Q water and then immersed in 30 mL 10 % SDS solution for 24 h. Plastic films were then oven-dried at 65 °C for 24 h and their weight was measured by a 6-digit balance.

Weight loss was calculated as: initial weight - final weight / initial weight × 100 (Muhonja et al.

2018). All tests were carried out in duplicate and results were expressed as mean ± standard deviation of duplicate.

3.3.5.4. Chemical Transformation

The chemical transformation of plastic samples was examined by FT-IR technique (PerkinElmer, USA). The clean and disinfected plastic films were scanned on both sides for 16 times at a resolution of 4 cm^{-1} from 4000 to 400 cm^{-1}.

3.3.5.5. Microscopy

Surface topography of treated and control plastic films was examined by optical microscope (Olympus BX51, Japan). Plastics were then cut in half to be scanned with atomic force (AFM, Cypher-ES, USA) and scanning electron microscope (SEM, Quanta 200f, USA). In AFM analysis, plastic films were mounted on the metal specimen discs Ø10 mm and scanned at 4 × 4 μm levels. For SEM analysis, samples were sputter coated with platinum (Quorum, Q150RS) for 1 min at 20 mA, and then were scanned under low vacuum at 10 kV.

3.3.5.6. Toxicity of By-products

Analysis of driven by-products in the media was an important factor to determine whether the produced compounds had negative impacts on microbial growth and biodegradation efficiencies or not. Due to the presence of a larger microbial community in UMS, and a higher chance of by-products formation by the microbial consortia activity, the evaluation was conducted in media obtained from AS, FS and soil flask. The toxicity of by-products was determined colourimetrically by utilization of TTC according to Moussa et al. (2013) with some modification. At the end of UMS incubation, 10 mL of microbial suspension derived from AS, FS and soil in UMS were removed and subjected to centrifugation at 12,000×g at 10 °C for 10 min. Each supernatant was filtered by using a 0.22 μm Whatman syringe, and the filtered cell-free supernatant was then used as the toxic compound to be tested on different microbial candidates. Prior to the experiment different bacteria and fungi (6 in total) were enriched 72 h in NB or PDB at 30 °C and 25 °C, respectively. After the incubation period, cells were harvested by centrifugation at 10,000×g for 6 min, washed with PBS (pH 7.4), and suspended in MSM-B. In each 48-well microplate, 150 μL

of each strain was individually incubated with 150 µL of extracted toxic compounds for 24 h. At the end of incubation, 100 µL of TTC (10 mg/mL) was mixed in each well and incubated for 48 h at either 30 °C for bacterial or 25 °C for fungal samples. The created red formazan was then extracted by 95 % fresh ethanol and the absorbance was read at 485 nm.

3.3.5.7. DNA Sequencing and Strain Identification

The most promising bacterial and fungal strains were analysed for genetic identification. Amplification of bacterial and fungal strains was conducted respectively on 16S rRNA and ITS regions by using universal primers. The used primers were as (27F) with the sequence of 5′-AGA GTTTGATCCTGGCTCAG-3′ and (1492R) with sthe equence of 5′-CGGTTACCTTGT-3′ for 16S rRNA, and (ITS1F) with the sequence of 5′ CTTGGTCATTTAGAGGAAGTAA-3′ and (ITS4) with the sequence of 3′ TCCTCCGCTTATTGATATGC-5′ for ITS regions (White et al. 1990). DNA was extracted from a single colony using the Geneaid Tissue DNA Mini Kit according to manufacturer's instructions and eluted in 100 µL elution buffer (10mM Tris). The qPCR was performed by using 2 µL extracted DNA along with PerfeCTa SYBR FastMix (Quantabio) on a Mic qPCR cycler with 95 °C (3 min), 95 °C (5 s), 55 °C (5 s) in 40 cycles, and 72 °C (30 s).

3.4. Results and Discussion

3.4.1. Natural Occurring Strains

3.4.1.1. Sludge and Soil Characterisation

Activated sludge characterised as 450 mg/L (SCOD), 2 % (TS), 40 % (VS/TS), 98 % moisture, 150 mg/L ammonia-nitrogen ratio and pH of 6.5. Farm sludge contained 133 mg/L SCOD, 1 % TS, VS/TS (50 %), moisture (99 %), ammonia-nitrogen ratio (130 mg/L) and pH of 6.8.

Soil texture was determined according to the standard from the United States Department of Agriculture (Milton Whitney 1911). The soil was found to be clay loam with 28, 32, 39 % sand, silt and clay, respectively.

3.4.1.2. Strain Isolation and Identification

Isolation and identification of candidate strains were conducted based on the microbial colonisation and biofilm formation on the surface, around or inside of plastic films. This was an important sign for the selection of the most plastic degrading strain in this study. Strains with significant growth on plastic were identified by the Sanger sequencing in both directions. The consensus made sequences were identified by using the blastn algorithm.

Among 17 candidates, there was significant growth of 4 strains on plastic films. These strains were identified as *Penicillium raperi* (derived from AS) grew on PS and PE; *Aspergillus flavus* (derived from, FS, soil, wax and meal worms' excreta) grew on PE and PS; *Penicillium glaucoroseum* (derived from AS) grew on PET and PS; and *Pseudomonas* sp. (derived from AS, FS and soil) grew on PET, PE and PS (Fig. 3.1i). Identified strains were in agreement with other studies (Sowmya et al. 2015, Kim et al. 2020, Zhang et al. 2020).

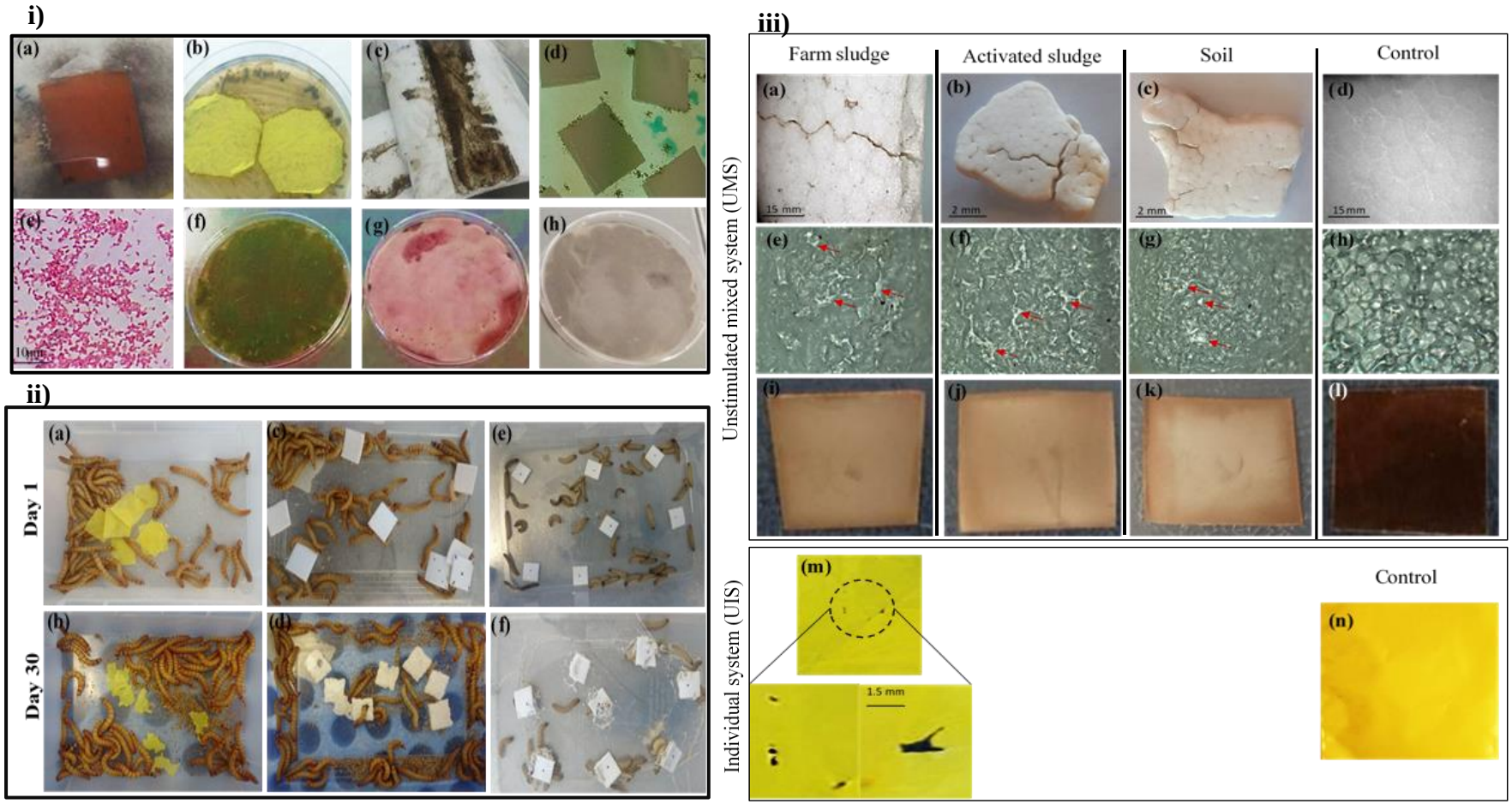

Fig 3.1. Visual observation and the isolated strains. **i)** Isolation of plastic degraders from (a) PET, (b) PE and (c-d) PS films on mineral salt media agar plates (MSM-A); (e-h) *Pseudomonas* sp; *Aspergillus flavus; Penicillium raperi*; *Penicillium glaucoroseum.* **ii)** Incubation of plastic films with live worms for 30 days with PE (a, b) and PS (c-f) films eaten by mealworms (a-d) and waxworms (e, f). **iii)** Physical alteration of treated plastics in unstimulated mixed system (UMS) and unstimulated individual system UIS; (a- l) incubated plastic films in UMS for 270 days; (a-c) PS cracking; (e-g) separated PS foam beads by stereo microscope at 55× magnification; (i-k) decolourisation of PET; (m) holes formation on PE films incubated with *A.flavus* in UIS for 100 days; (d, h, l, n) control.

3.4.1.3. Physical Changes

Among different plastic films given to worms, PS had the most physical changes compared to other plastic types. Mealworms, on the other side, consumed both PS and PE at almost the same level. On average 50 % weight reduction was observed for PS films that were given to the waxworms (Fig 3.1ii). Incubated PE and PS with mealworms had 57 and 66 % weight loss, respectively. That was the reason in the biodegradation test, the flasks containing meal and waxworm's microbial consortia were incubated with just PS + PE and PS plastic, respectively.

Due to the significant growth of the aforementioned strains on plastic in solid plates, it was assumed that the isolated strains will remain activated on plastic until the end of the experiment. Therefore, at the end of UMS experiment (270 days), plastic with significant physical changes were removed to understand whether the isolated strains still colonised on the particular plastic type or not.

Among the used plastics, PET and PS had the most physical changes in the flask containing either AS, FS or soil microbial consortia. PS films cracked significantly from different parts (Fig 3.1iii (a-c)), and the colour of PET changed from shiny-brown to matte-white (Fig 3.1iii (i-k)). A similar result was also reported by Syranidou et al. (2017) when marine consortia were used for PS degradation. The cracked PS and decoloured PET were removed individually from each flask, and washed gently two times with sterilised Milli-Q water in aseptic condition to detach the unbound cells. Plastic films were individually immersed in 15mL centrifuge tubes and vortexed. The aliquot of each tube was serially diluted to 10^{-5} and individually plated in NA and PDA, and the grown strains were identified genetically as described in the method section. After analysis, the isolated candidates were the same as the previously identified strains (*P.raperi, A.flavus, P. glaucoroseum* and *Pseudomonas* sp). This suggests that even after a long period (270 days) the isolated strains still able to survive on the plastic by colonising and consuming it as a carbon source.

The fewer physical changes of PE such as those were seen in PS (cracking) and PET (discolouration) in UIS is due to the high crystalline structure of HDPE, and the absence of hydrolytic groups in its backbone. The available commercial PS foam contained Atactic conformation, which makes it to be more amorphous. The amorphous structure of PS foam allows microbial strains and their metabolites to penetrate inside the PS films, and cause cracking. PET also has a semi-crystalline structure and contains hydrolytic functional groups in its backbone. The structure of PET has made it to be more susceptible to oxidation and catalytic activity of

microbial exoenzymes, which results in discolouration of PET.

Despite PS cracking and PET decolourisation in flask inoculated with AS, FS and soil microbial strains in UMS, there was no sign of PS cracking when this plastic was incubated in worms' flask containing just *A. flavus*. There were no physical changes on PS and PET when incubated individually with the characterised strains in UIS, except PE incubated with *A. flavus.* Incubation of PE with *A. flavus* caused the formation of several holes on the surface of PE as part of biodegradation (Fig 3.1iii (m)). Due to the obtained results, it is then believed that for decolourisation of PET, and cracking of PS, the presence of *Pseudomonas* sp. and *A. flavus* alone were not effective enough, and their cooperation with other microbial community in AS, FS and soil were essential.

While the exact mechanism of microbial cooperation is not yet understood in detail, it possibly follows metabolic cross-feeding via altruism or mutualism behaviour. This means that the absence of exoenzymes in one strain, can be found in other members of the community to break down the polymer into smaller compounds. Moreover, if the formed oligomers and monomers are not consumed by one strain, it can be taken by other microbial strains in the consortia for further degradation. In another word, if both actor and recipient uptake the formed compounds, they would go under mutualism behaviour and both benefit from this cooperation. However, if the created compound is just taken by the recipient, the cooperation is considered as altruism. In both cases, the cooperation of strains together in a larger community in the way explained above can improve the biodegradation efficiencies.

Despite significant microbial growth on PS, PE and PET, there was no microbial adhesion, physical changes and weight loss in PP, therefore, this plastic was taken out from other analytical and experimental tests.

3.4.1.4. Microscopy Examination

Surface deterioration, fissures and decolourisation are the initial and important signs of plastic degradation. Thus, plastic with no sign of physical changes was not further examined by optical, AFM and SEM microscopy.

Observation of untreated PS foam under an optical microscope revealed that this plastic was made from several attached union-shaped internal beads/ islands with a smooth surface. While the mentioned feature remained intact in control PS films (Fig 3.1iii (d, h)), the structure of the bead

of the incubated PS foam was mainly deformed (Fig 3.1iii (e-g)). Once pits and holes are formed on the surface of PS, microbial metabolites and exoenzymes will penetrate into these intracellular structures, and consequently will cause internal detachment and cracking of PS foam.

Surface deterioration of plastic films was further examined by AFM and SEM microscopy. Surface topography of control PS, PET and PE films remained smooth with some minor shallow pits and bumps, while surface deterioration and significant deep cavities were formed on the incubated plastics films (Fig.3.2 - Fig 3.4). This observation was constant with the work of other researchers (Syranidou et al. 2017).

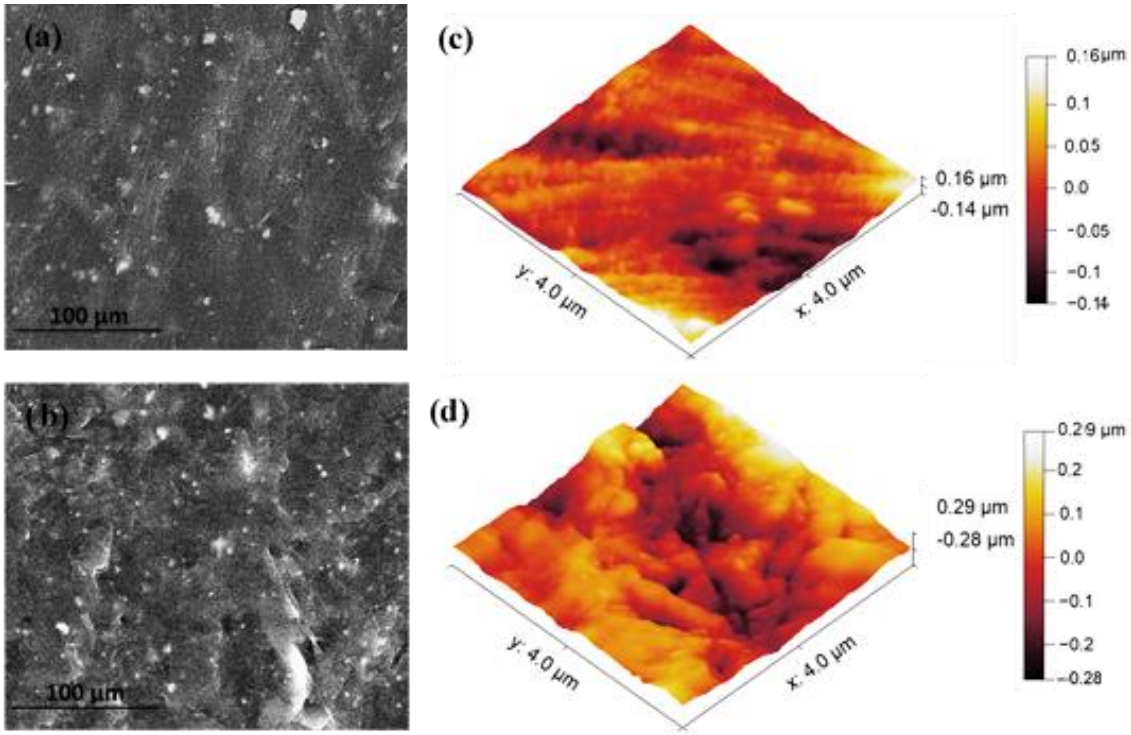

Fig 3.2. Surface topography of incubated PE films in unstimulated individual system (UIS) in 100 days. (a, b) SEM; (c, d) AFM; (a, c) control; (b, d) treated with *A. flavus*

The maximum height of the formed bumps and pits of the inoculated PE with *A. flavus* was about 0.6 µm, which was almost two times higher than what was identified on control samples (Fig 3.2c, d). This result was also higher than what was reported Yang et al., (2014) and Bombelli et al. (2017) with a maximum defect height of about 0.4 and 0.5 µm, respectively. Analysis the surface of control PE films by AFM and SEM, revealed the existence of several strip lines. This is how these strip lines were eroded and replaced with many deep cavities as enzymatic activity of *A.*

flavus on PE films in UIS (Fig 3.2). A similar result was also reported by Sangeetha Devi et al. (2015). The different surface morphology of treated and control PE films by AFM and SEM suggested that the shallow cavities and strip lines on the control PE were parts of its manufacturing processes.

Analysis of PS films with SEM and AFM indicated a smooth surface with minor pits on the surface. The maximum height of peaks and cavities of PS control was 0.16 μm, which was about 3.8 and 4.9 times lower than the treated PS with sludge (AS and FS) and soil inoculums (Fig 3.3e). Due to the colonisation of strains and penetration of microbial metabolites into the PS beads, surface deterioration and formed cavities on the incubated PS can clearly be seen on AFM and SEM images (Fig 3.3f-h). Similarly, the surface of control PET samples remained smooth in both AFM and SEM observations. The maximum height of the formed peaks and cavities in the control PET was 0.07 μm (Fig 3.4e). However, the surface of the incubated PET significantly eroded with FS, AS and soil microbial strains; having the maximum peaks and holes' height of 1.2, 0.68 and 0.83 μm, respectively (Fig 3.4f-h).

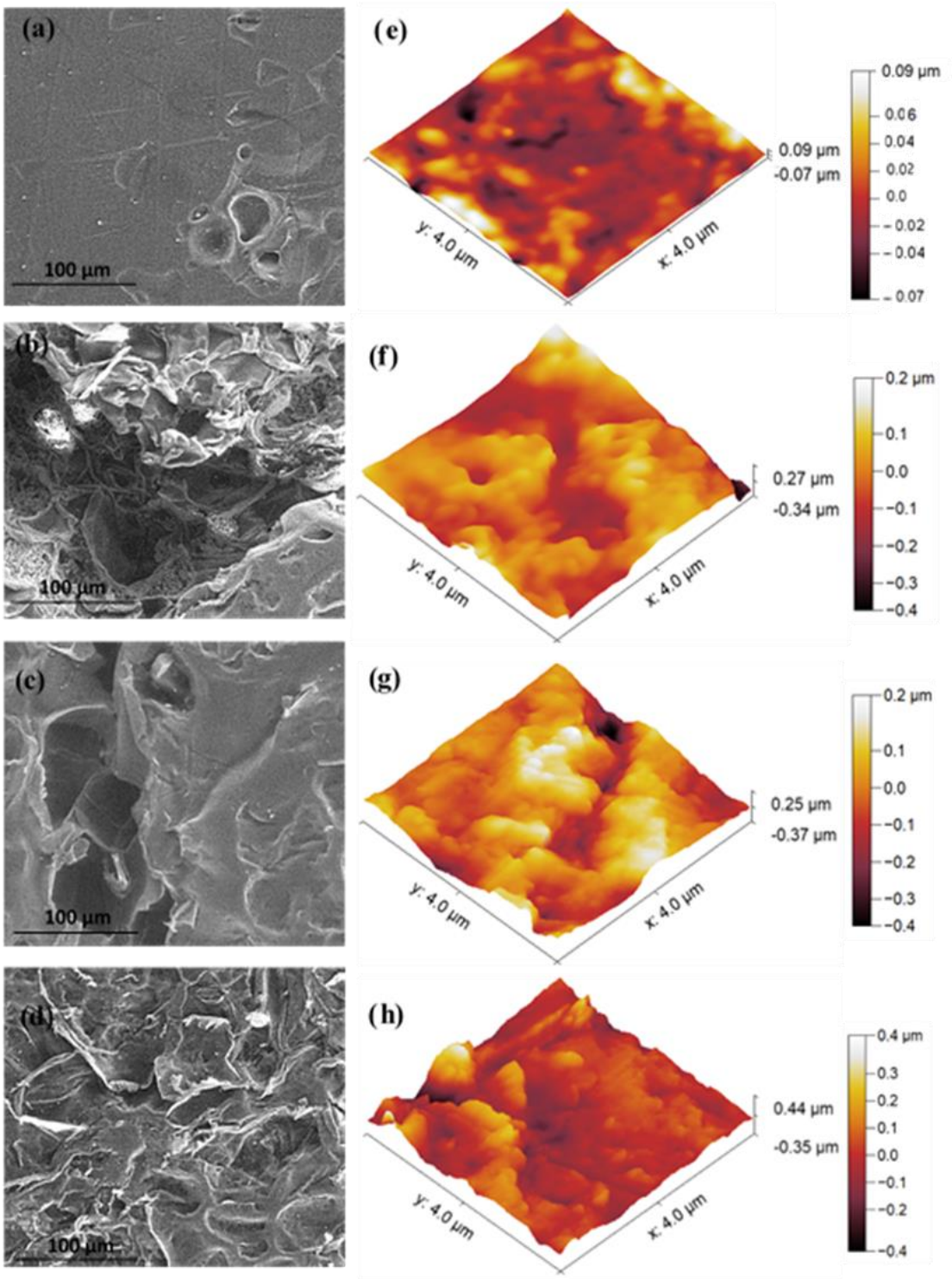

Fig 3.3 Surface topography of incubated PS films in unstimulated mixed system (UMS) in 270 days. (a-d) SEM; (e-h) AFM; (a, e) control. Cavities and surface deterioration can be seen at (b, f) farm sludge (FS); (c, g) activated sludge (AS); (d, h) soil

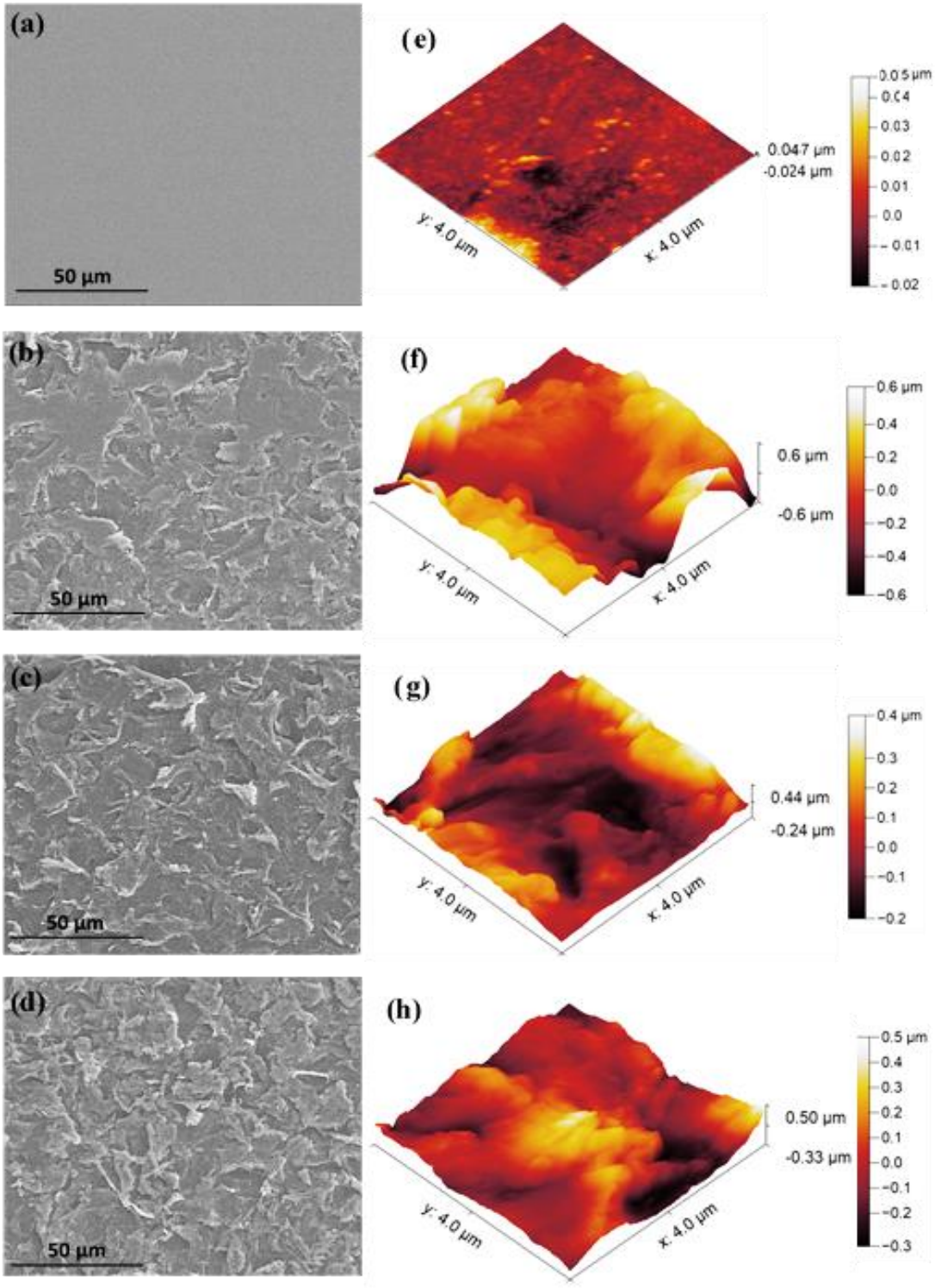

Fig 3.4. Surface topography of incubated PET films in unstimulated mixed system (UMS) within 270 days. (a-d) SEM; (e-h) AFM; (a, e) control. Cavities and surface deterioration can be seen at (b, f) farm sludge (FS); (c, g) activated sludge (AS); (d,h) soil

3.4.1.5. Weight loss and Biofilm Estimation

At the end of UIS incubation, the amount of formed biofilm on each of the incubated plastic was measured via the absorbance of CV, and then correlated to weight loss. It was found that in the same plastic type and same strain, the higher weight loss related to the higher absorbance of CV. For instance, the two incubated PE films with *A. flavus* had different values; plastic sample 1 had a weight loss and absorbance of 5.5 % and 0.41, respectively while plastic sample 2 had 2.5 % and 0.32, respectively. Recently, Zhang et al. (2020) also identified *A. flavus* from waxworms (*Galleria mellonella*) as a HDPE plastic degrader. However, the maximum weight loss that they achieved was 3.9 % which is 1.5 times lower than what was achieved in this study.

Weight loss in other plastic types was not as significant as the PE films. Maximum weight loss and CV absorbance was 1.8 % and 0.49 in PS with *P. glaucoroseum* and 0.6 % and 0.16 for PET films with *Pseudomonas* sp (Fig 3.5).

The inconstancy between the acquired weight loss and absorbance from the same strain and same plastic sample could be explained as the difference surface availability of plastic for microbial strains. As several plastic samples of each type were incubated in a 50 mL Eppendorf tube, a chance of plastic bending, twisting or adhesion to each other was undeniable in such a small space. Therefore, some plastic samples could have a more available surface for microbial biofilm formation and some less. As results showed, plastic samples with a more available surface during the incubation had higher microbial colonisation (biofilm) on its surface, and consequently a higher chance of biodegradation as weight loss. The role of microbial biofilm formation and its correlation to biodegradation were well studied by several researchers (Gilan et al. 2004, Hadad et al. 2005, Tribedi and Sil 2013, Das and Kumar 2015, Ghosh et al. 2019).

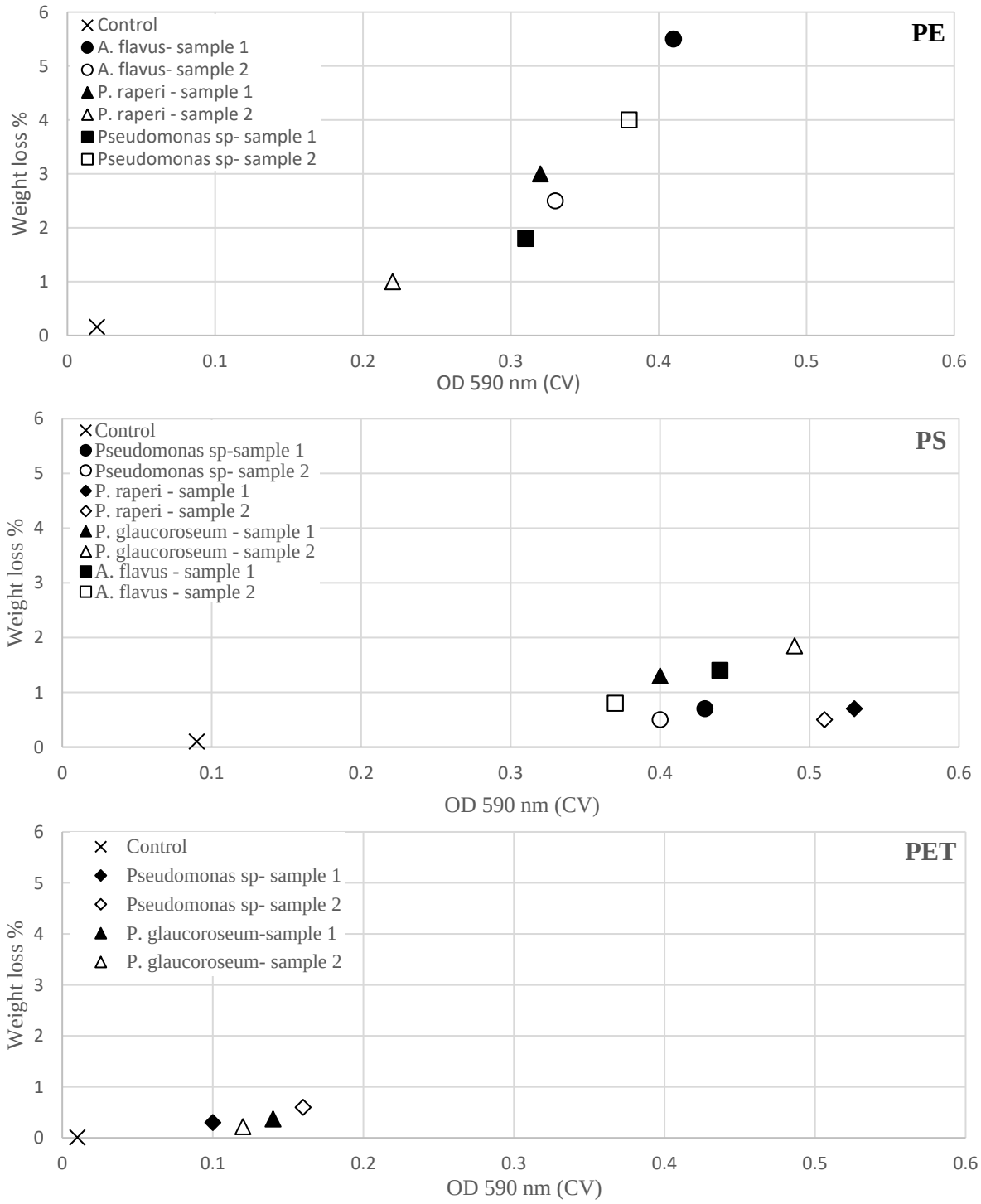

Fig 3.5. Correlation of weight loss and formed biofilm in unstimulated individual system (UIS) within 100 days. In each plastic type (PS, PE and PET), the highest weight loss corresponds to the highest crystal violet absorbance (CV) of the particular strain

3.4.1.6. Toxicity of By-products

To estimate the toxicity of formed by-products in the media, colourimetric analysis via utilisation of TTC was determined. TTC acts as an electron acceptor or oxidant in the electron-transport chain system and it turns from white to red compound in live and active microbial strains. It was found that the produced by-products of FS were less toxic in comparison to soil and AS in an orderly fashion (Fig 3.6). Also, the results showed that the growth and activity of strains remained the same or even higher than control samples. Suggesting that the produced by-products did not have negative effects on microbial growth and biodegradation efficiencies. It is believed that the higher growth of strains was due to the possible formed intermediate and short-chain compounds in the by-products and their consumption as a result of biodegradation.

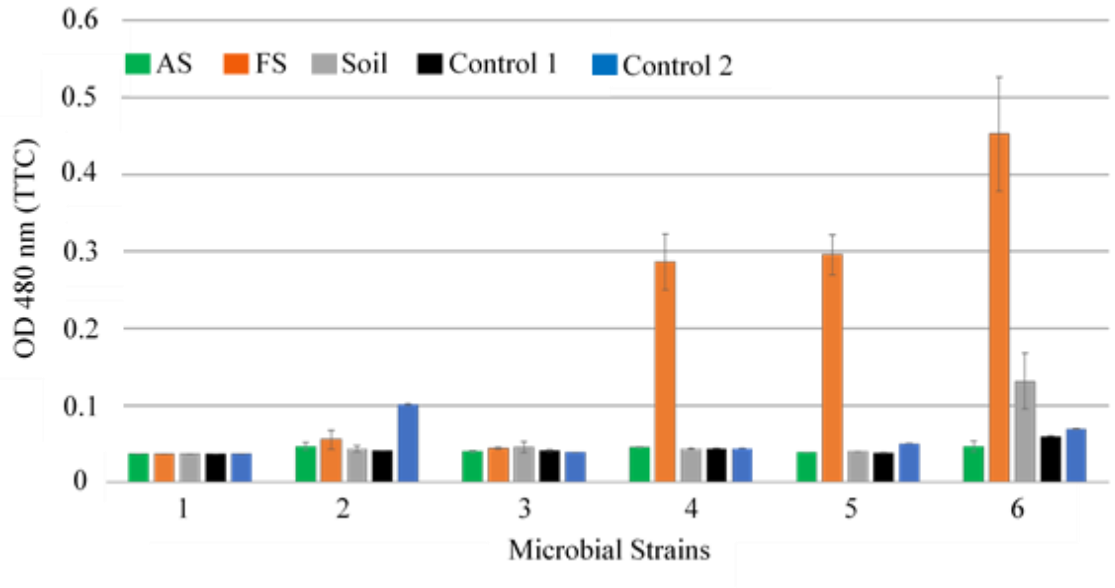

Fig 3.6. Viability of incubated pure strains in by products derived from activated sludge (AS), farm sludge (FS) and soil. The higher absorbance of TTC indicates the higher presence of live and active strain. Control 1 (strains without by-products); control 2 (strains in MSM-B)

3.4.1.7. Chemical Transformation

Chemical alteration of plastic samples was determined by spectroscopy techniques FT-IR. The analysis revealed the formation of new functional groups as hydroxyl (OH) at 3300 cm^{-1} in PS; CH stretch (alkyl (CH_3, CH_2)) at 2980 and 2880 cm^{-1} in PET; carbonyl (C=O) at 1740, 1710 cm^{-}

[1] in PS and PE; alkoxy (C-O) at 1020 cm^{-1} in PE and alkene (C=C) at 1650 cm^{-1} in PS and PE (Fig 3.7a-c). Similar FT-IR peaks were also identified by other studies (Esmaeili et al. 2013, Jeon and Kim 2013, Sangeetha Devi et al. 2015, Muhonja et al. 2018, Puglisi et al. 2019).

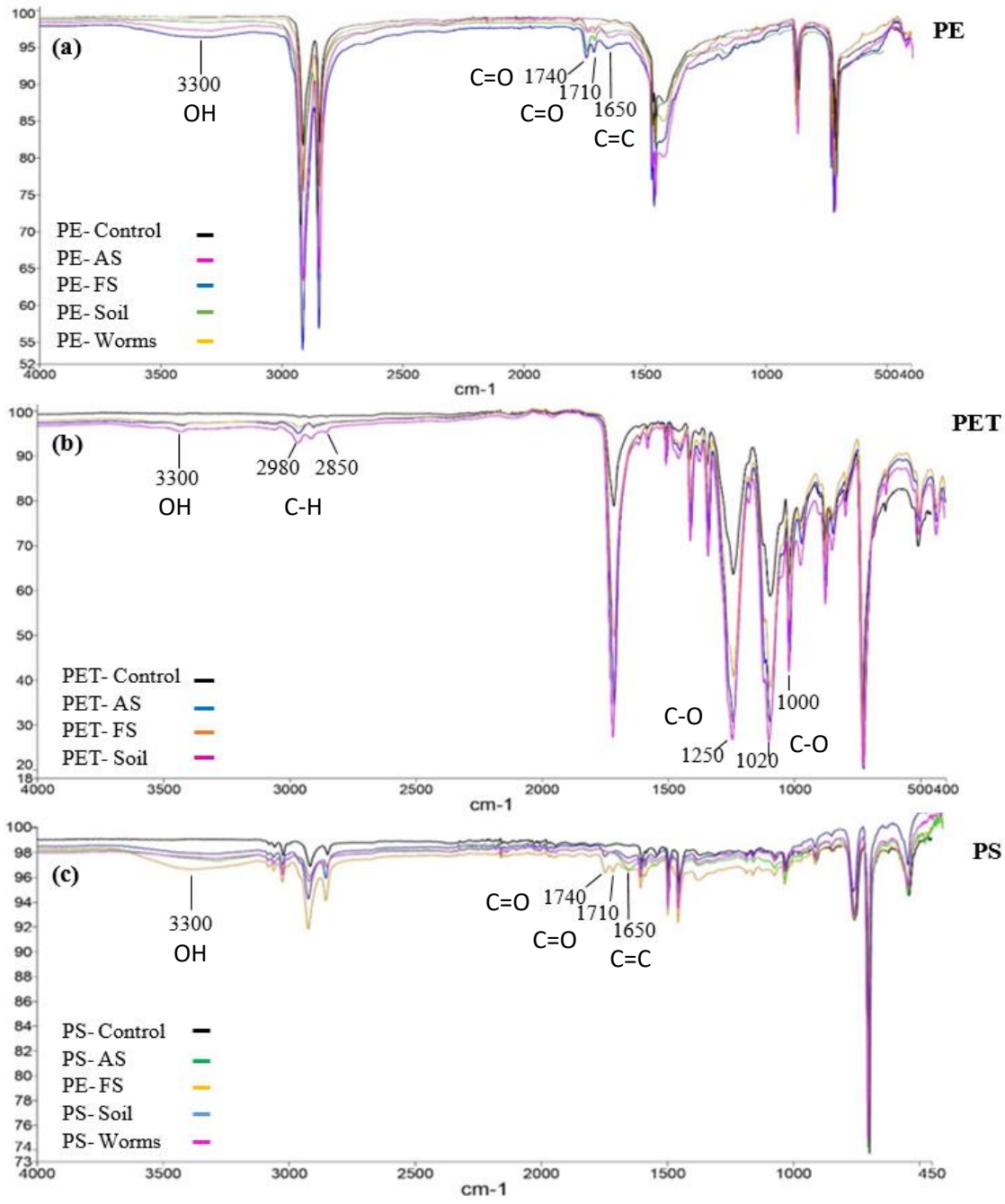

Fig 3.7. Chemical transformation of PE, PET and PS plastic films inoculated with unstimulated microbial strains. Various functional groups formed in incubated plastics films compare to controls

3.4.2. Stimulated Strains

3.4.2.1. Chemical Transformation

Chemical transformation of incubated plastic films in SMS was also determined by FT-IR spectroscopy. It was important to understand the possible role of media composition in the alteration of plastic's chemical structure. FT-IR analysis showed similar functional groups in the incubated plastics in both NB and MSM-B, therefore it was suggested that the media composition did not have an effect on the chemical transformation of plastic. It was also valuable to recognise the role of microbial enzymes in the formation of new bonds or removal of any functional groups compared to unstimulated conditions.

The FT-IR spectra of incubated plastics in SMS were almost similar to the identified peaks in unstimulated conditions, nevertheless, the peaks were sharper in general (Fig 3.8a-c). Sharper peaks (lower transmittance) were concluded as the higher existence of the corresponding functional group in that part of the spectra. The results indicate the formation of new intense peaks attributed to alkoxy groups (C-O) at 1250, 1020, 1000 cm^{-1} in PE films (Fig 3.8a), and 1250 cm^{-1} in PS (Fig 3.8c). The attributing peaks to the carbonyl bonds (C=O) at 1740 and 1710 cm^{-1} were not formed in the incubated PE in SMS, nevertheless, the alkene peak (C=C) at 1650 cm^{-1} was formed (Fig. 3.8c). There were similarities in the spectra of PET incubated in unstimulated and stimulated conditions (Fig 3.7b and Fig 3.8b). It was believed that the formation of hydrolytic bonds (hydroxyl, carbonyl and alkoxy, alkene) in plastic structures are crucial steps in the biodegradation process, which would simplify the biodegradation process by letting the microbial enzyme attack these functional groups. Without the formation of these functional groups, there would be no interaction between the microbial enzymes and plastic samples.

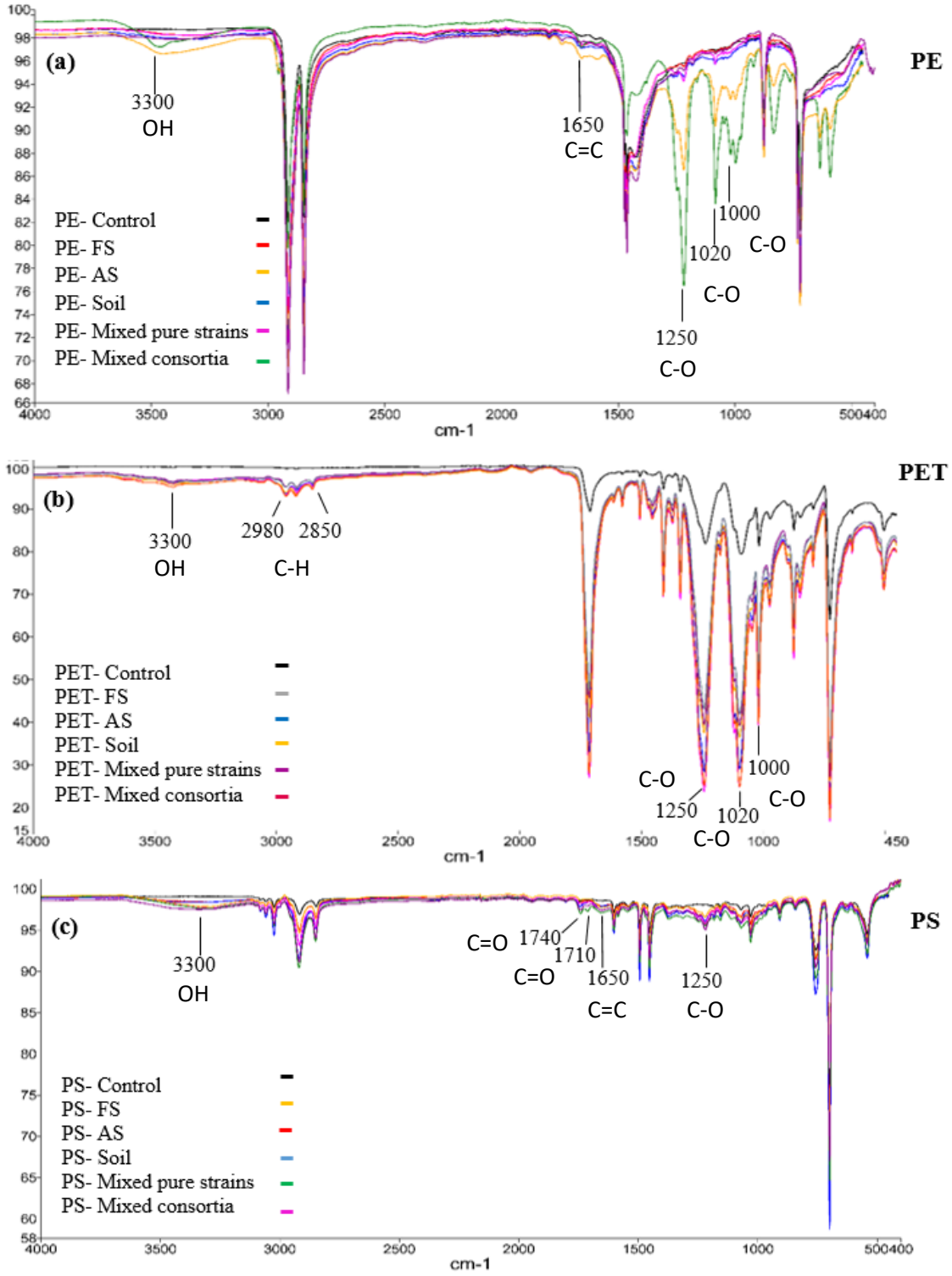

Fig 3.8. FT-IR analysis of PE, PET and PS plastic films inoculated with stimulated microbial strains. Various functional groups formed in the incubated plastics films compare to controls

3.4.2.2. Tolerable Concentration of H_2O_2

By the addition of H_2O_2 to the microbial suspension, simultaneously bubbles were formed as a sign of H_2O_2 degradation into water and oxygen. The result suggested that the used microbial isolates contain antioxidant enzymes such as catalase, peroxidase and superoxide dismutase.

The growth of microbial strains was monitored in both NB and MSM-B media during the experiment, and it was found that the strains in nutrient-rich media had a higher growth compared to those in MSM-B. This could be explained by the fact that more nutrients were available for their growth, repairing and activation of their exoenzymes.

The lack of nutrients in MSM-B reduced microbial growth and the chance of DNA repairing due to the suppression of antioxidant enzymes and stress-related proteins. Similar results were reported by Martins and English (2014) in H_2O_2 stimulation of yeast at nutrient-rich and synthetic media.

It was noticed that microbial growth and survivability in MSM-B were reduced after 72 h of incubation in 5 and 10 mM H_2O_2 (Fig 3.9). The 10 mM H_2O_2 was also found to be the lethal concentration for most incubated bacteria and fungi and led them into programmed cell death. In contrast, incubated strains at concentrations of 0.5 and 1 mM H_2O_2 were able to survive and grow for a longer period in both NB and MSM-B media (Fig 3.10). As the growth of strains was slightly higher in 1 mM H_2O_2, and the purpose of the stimulation experiment was to challenge strains in oxidative conditions for 30 days; therefore, the biodegradation test was carried out in 1 mM H_2O_2 solution.

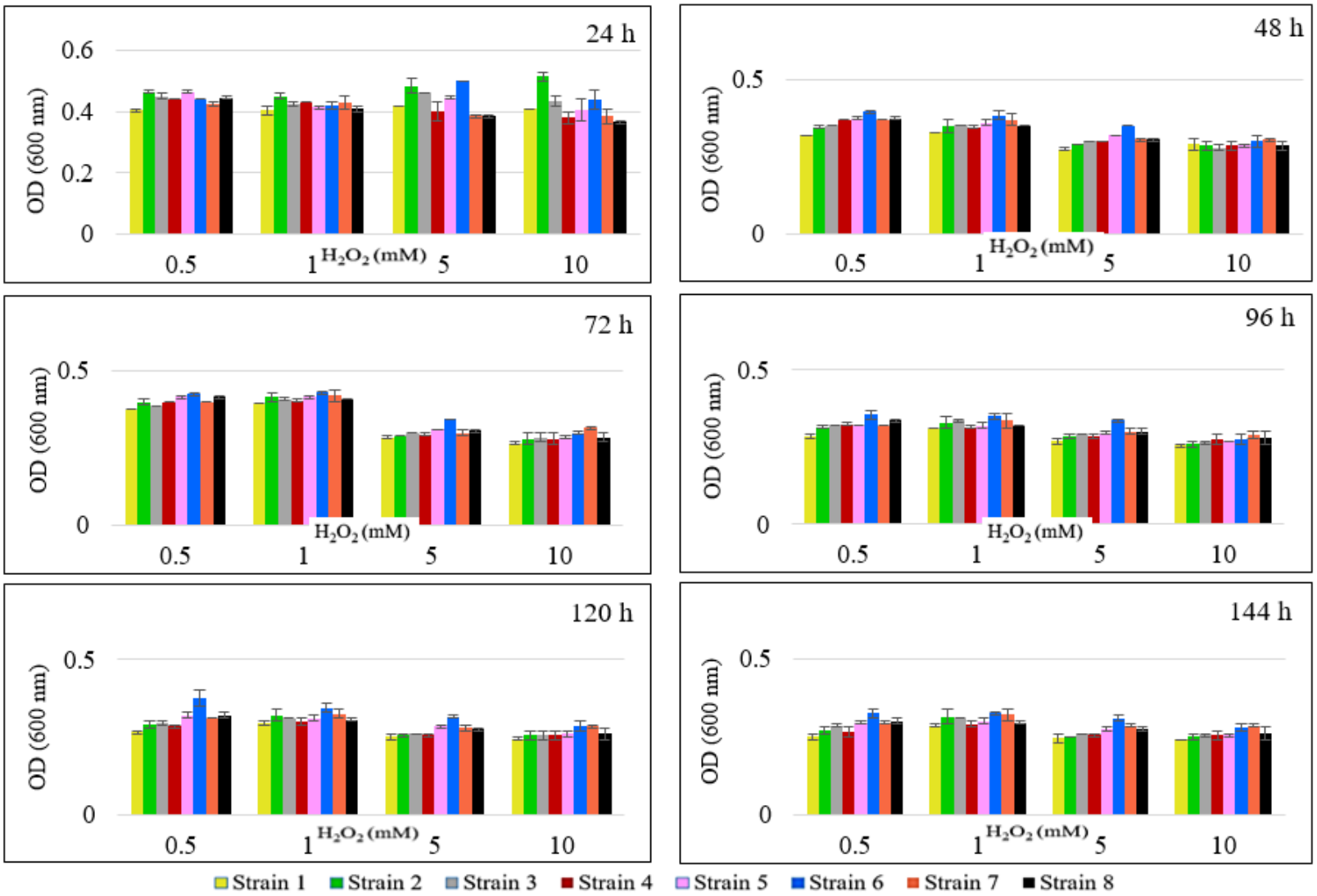

Fig 3.9. Comparison of bacterial growth in different concentration of H_2O_2 in mineral salt media (MSM-B) within 144 h

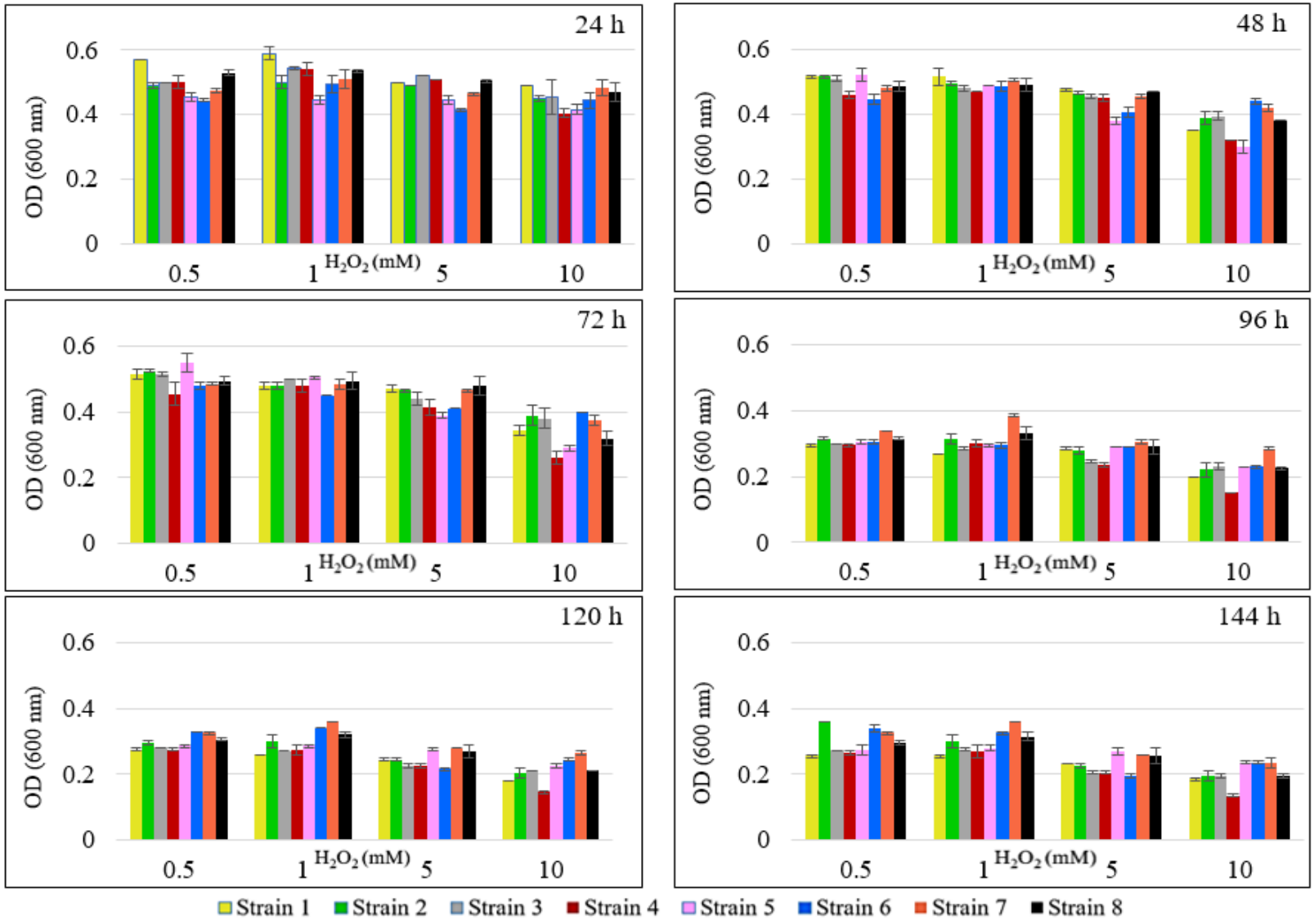

Fig 3.10. Comparison of bacterial growth in different concentration of H_2O_2 in nutrient broth (NB) media within 144 h

3.4.2.3. Physical Changes and Weight loss

In a constant interval (every 10 days) the incubated plastic films with stimulated strains were removed from each flask for examination. Observation of plastics showed the formation of some minor cracks and holes only on the surface of PS films; however, the cavities were not as significant as what was observed in UMS. There was a negative trend in weight loss of PS regardless of the media type. In the first 20 days, PS films gained weight instead of loss, however, the trend reduced noticeably in the last 10 days due to the formed cavities and holes on the PS surface (Fig 3.11). Previously it was discussed that PS films were made from several internal union-shaped islands/beads (Fig 3.1iii (d, h)). It was then believed that the weight gain of PS in the first 20 days was a result of microbial colonisation, and their secreted metabolites into the PS structure. The colonisation of strains into the PS bead would cause cracking, separation and weight reduction. The given reason can explain why the weight loss trend changed in the last 10 days. The weight gain phenomenon was also reported by El-Shafei et al. (1998). Therefore, weight loss evaluation of PS could not be accurately determined by the use of normal digital balance especially if there is no cracking on PS. To measure the exact weight loss of PS foam, other techniques such as melt flow index (MFI) or gel permeation chromatography (GPC) should be applied.

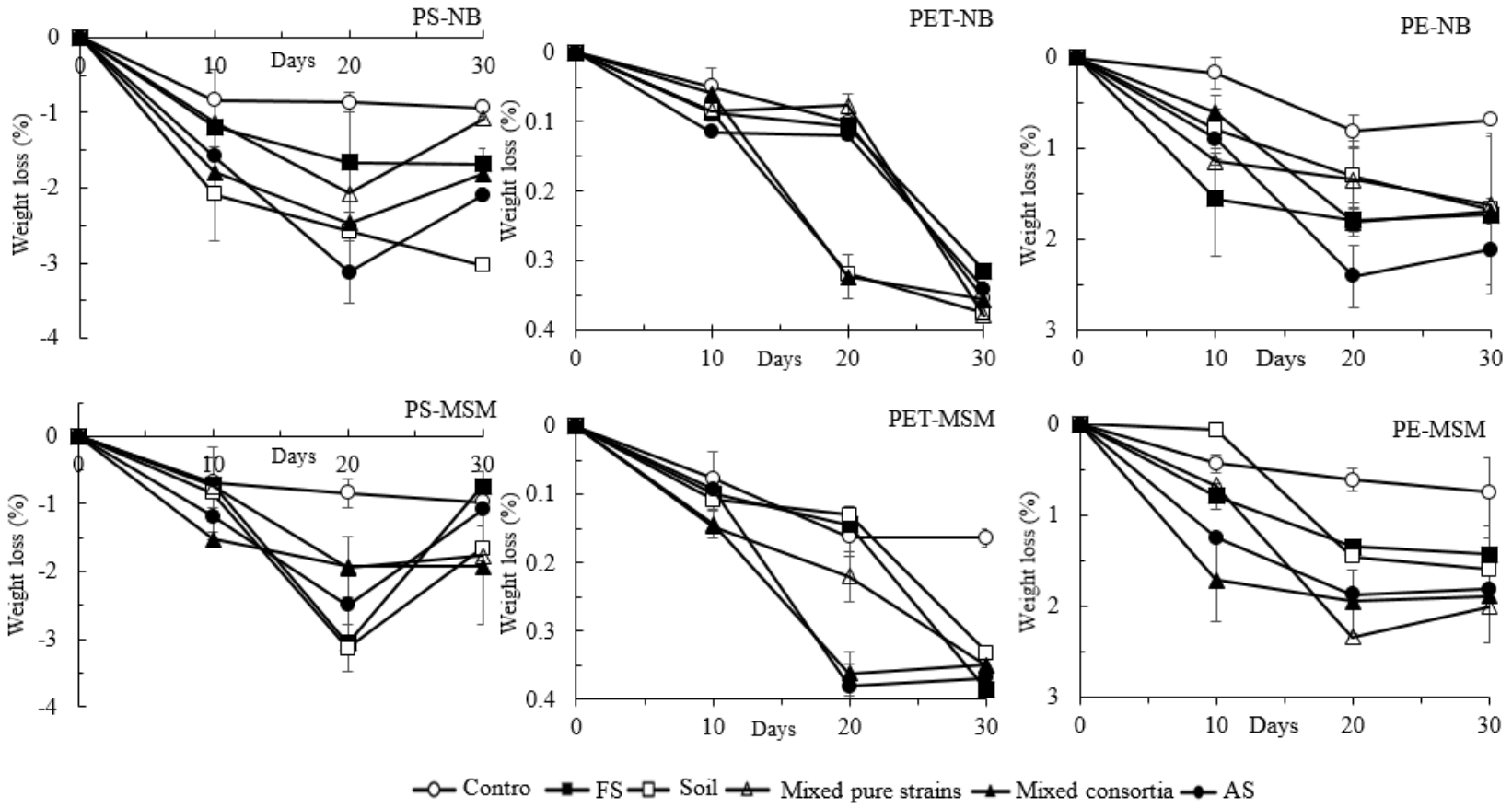

Fig 3.11. Weight loss of incubated plastic in stimulated mixed system (SMS) within 30 days. Plastic films were inoculated with five different microbial consortia separately in nutrient broth (NB) and mineral salt media broth (MSM-B)

There was no considerable weight loss in the incubated PET films in both NB and MSM-B media (less than 1 %) (Fig 3.11), and no decolourisation was observed in all flasks.

Maximum 2.5 % weight loss (day 20) was detected in the incubated PE films with pure strains in MSM-B, and the inoculated PE films with AS in NB media. There was almost a steady-state in weight loss trend in the last 10 days in both MSM-B and NB media (Fig 3.11). The highest degradation efficiencies of PE compared to other plastic was possibly due to the creation of scavenging peroxidase enzyme in the media. The utilisation of peroxidase for PE degradation was reported previously by other authors (Iiyoshi et al. 1998, Santo et al. 2013).

The no decolourisation of PET, nonexistence of major cracks in PS, and lower weight loss of PE in SMS condition could be explained as the lower incubation period compared to UMS (270 days) and UIS (100 days). Moreover, the oxidative stress by H_2O_2 may lead the strains into a programmed death cell cycle, which decreased their population and consequently the biodegradation efficiencies. Since the condition of each experiment (UMS, UIS and SMS) was different, therefore, the outcome of each study should not be compared together, and instead should be considered as a separate technique for biodegradation of plastic.

Stimulation of strains as a new approach in biodegradation can be considered to induce scavenging enzymes in the media in benefit of plastic deconstruction. In this study, only 2.5 % weight loss of PE was achieved within 30 days in SMS condition compare to UIS (5.5 % weight loss) within 100 days. However, stimulation can be an interesting option especially if PE is the main target of degradation and shorter operating condition are desired. This study showed that stimulation of strains by the non-lethal concentration of H_2O_2 can be used as a biodegradation strategy, and for the future optimisation different parameters such as pH range, temperature, type of stimulator and concentration) should be considered.

3.4.2.4. The Role of Plastic in Microbial Growth

In the preliminary experiment, it was realised that bacterial and fungal strains could not tolerate the oxidative condition more than 144 h where no plastic films were presented in MSM-B and NB. Therefore, it was important to understand the role of plastic as a carbon source in the extension of microbial growth and maintenance. For this purpose, strains were incubated for 30 days in flasks containing the same H_2O_2 concentration (1 mM) and same plastic type. At the end of the experiment, microbial growth was monitored by the direct spreading of incubated bacterial and fungal cultures on NA and PDA plates, respectively.

The results showed that the strains incubated with plastic films for 30 days were still alive and grew more on agar plates (plates were entirely covered with fungi and bacteria) compare to those incubated in plastic-free H_2O_2 media. This observation suggested that the strains could use plastic films as a source of carbon to extend their survivability and growth.

3.5. Summary of Chapter and Conclusion

Biodegradation was assessed in both mixed and individual systems by using unstimulated and stimulated strains. Among different candidates, *Penicillium raperi*, *Aspergillus flavus*, *Penicillium glaucoroseum* and *Pseudomonas* spp were isolated from AS, FS, soil and worms' excreta as the plastic degrading microbes. Analysis of plastics by FT-IR, AFM, SEM and optical microscopy revealed decolourisation, surface deterioration, physical and chemical alteration. In this study maximum of 5.5 and 2.5 % physical weight loss were observed in the incubated HDPE in UIS (100 days) and SMS (30 days), respectively. While there was a lower weight reduction in stimulation conditions, this approach can be considered if a lower operational cost is required due to the shortening of degradation time.

The next chapter studies the effect of UV-pretreatment on the biodegradation capacities of the identified strains in chapter 2, to suggest the optimum condition of biodegradation.

Chapter 4

Enhanced biodegradation of non-biodegradable plastics by UV radiation

Contents of this chapter have been published as follows:

Taghavi, N., W.-Q. Zhuang and S. Baroutian (2021). "Enhanced biodegradation of non-biodegradable plastics by UV radiation: Part 1." Journal of Environmental Chemical Engineering 9(6): 106464.

4.1. Chapter Preface

One of the ways to improve the biodegradation efficiency is to age plastics prior to biodegradation. UV-radiation can oxidise plastics by forming free-oxygen radicals within the plastic's structure. This chapter aims to understand the role of various UV-pretreatment conditions on the biodegradation efficiency of the identified strains in chapter 3. Evaluation is conducted comprehensively along with statistical analysis to determine the optimum biodegradation condition for non-degradable thermoplastics.

4.2. Introduction

The slow processing time and low efficiency reduce the potentiality of biodegradation as a green method of plastic degradation. While different approaches such as enzyme engineering and the use of surface-active agents can be used to improve the biodegradation rate, pretreatment (ageing) is one the most used technique to disorder the structure of plastic prior to the biodegradation process. Pretreatment can be performed chemically, thermally, and/or by UV radiation. Chemical pretreatment (ex. chromic acid, nitric acid, sulfuric acid) while can deteriorate the plastic structure prior to biodegradation (Yamada-Onodera et al. 2001, Velrajan and Andrew 2012), are not environmentally friendly and require recovery of the used chemicals. Pretreatment of plastic by heat or UV is a well-studied environmentally friendly technique and can be used for mixed and contaminated plastics waste, and capable of being implemented at a commercial scale due to their simplicity compared to other aforementioned methods.

It is believed that the exposure of plastic to UV or heat can generate free-radicals of oxygen within the polymeric structure of plastic (Gewert et al. 2015, Erdmann et al. 2020). The formed free-radicals can participate in the polymeric pathways (initiation, propagation and termination) to form shorter chain compounds such as ketone and olefin (Vasile and Pascu 2005, Gewert et al. 2015). The short-chain molecules formed can then be attacked more easily by microbial exoenzymes for further degradation and consumption. Moreover, UV radiation can also reduce the plastic hydrophobicity and results in higher microbial colonisation (Balasubramanian et al. 2011, Belmonte et al. 2016).

The application of sunlight-assisted degradation was studied previously by several authors (Abd El-Rehim et al. 2004, Yousif and Haddad 2013, Gong et al. 2019). Despite a general agreement in oxidation and weathering of plastics under sunlight, the low energy of emitted UV from the sun is not as efficient as artificial UV simulation with higher energy (Jones 2002, Doğan 2021). In the study conducted by Doğan (2021), the degradation efficiency of PE plastic was compared under sunlight and artificial UV, and it was shown that the emitted artificial UV-C resulted in a higher degradation in a shorter period compared to sunlight. Moreover, many abandoned plastics are floated on the ocean or sea, shielded from UV radiation by water, resulting in lower oxidation of plastics by sunbeam (Urbanek et al. 2018).

Therefore, focusing on just sunlight-assisted could not accelerate the biodegradation efficiency and solve the plastic problem. This study proposes an engineered method for a higher biodegradation rate within a shorter time. In this study, UV-C was used because of its higher energy than UV-A and UV-B. The UV-C can excite more photons in plastics in a much shorter period, resulting in lower energy consumption in the long term. The wavelength of UV-C was 245 nm which is at the middle of the UV-C spectrum (200- 280 nm). This wavelength is commonly used in most biological laboratories. This particular wavelength was also used for the pretreatment of plastics in another study (Montazer et al. 2018).

The mechanism of plastic oxidation by UV has been well studied by other authors (Yousif and Haddad 2013, Gewert et al. 2015). Previous studies focused mainly on UV-pretreatment of PE. However, the UV radiation of PS and PET plastic has not been widely studied yet. While many studies reported that UV-pretreatment could increase the biodegradation rate, the biodegradation efficiency of thermoplastics even after artificial pretreatment still is far below (Roy et al. 2008, Mahalakshmi and Andrew 2012, Esmaeili et al. 2013, Vimala and Mathew 2016, Montazer et al. 2018) (Table 1).

The pretreated PE plastics in other studies were mainly not examined for morphological, chemical, or structural changes before the biodegradation experiments. Moreover, no in-depth analysis was conducted in previous studies to understand how parameters (such as time, distance to UV source, UV dosage) can affect the polymer breakage, enhance microbial colonisation and improve biodegradation efficiency. To better understand the effect of UV-pretreatment in biodegradation enhancement, three different types of plastics (PE, PS and PET) were exposed to UV radiation for

two different durations and distances in this study. Based on the inverse square law, which describes the effect of lower UV dosage as the distance from the source increases (Goats 1988), it is assumed that UV radiation at a shorter distance to UV source can change the plastic structure and results in higher microbial colonisation and biodegradation. The UV radiation of non-degradable plastics was not conducted in detail in previous studies. While the principle of radiation was the same and a similar experimental set-up was used, there were some discrepancies between the current study and previous investigations. In this study, different analytical methods along with three control sets were used to determine whether the improvement in biodegradation was due to UV irradiation, microorganisms or the combination of both.

4.3. Experimental

4.3.1. Materials

The chemicals used in this study were KH2PO4, K2HPO4, MgSO4·7H2O, NH4NO3, NaCl, FeSO4·7H2O, MnSO4·H2O; dichloromethane; nutrient agar (NA); nutrient broth (NB); potato dextrose agar (PDA); potato dextrose broth (PDB) and agar powder. All used chemicals are analytical grade and were purchased from Sigma-Aldrich (New Zealand).

Three plastic samples, HDPE (shopping bag), PS (foam) and PET (drinking bottle), with pearl, white and shiny-brown colour and respective thickness of 0.12 mm, 2 mm and 0.24 mm were purchased locally. Two sets of plastic samples were prepared as 15 mm × 15 mm films (set-A) and small pieces (set-B) (about length< 10 mm, width< 5 mm). Set-B plastics were used in a separate biodegradation experiment only to determine the possible molecular weight reduction. Prior to the experiment, all plastic samples (including controls) were immersed in 75 % ethanol solution for one hour, rinsed with sterile Milli-Q water and oven-dried at 55 °C overnight (aseptic condition). There is a possibility of migration of plastic additives into the ethanol solution during washing steps and immersion of plastics into ethanol solution before biodegradation and at the end of the incubation period. However, all plastic samples (including controls) were treated similarly in all experimental conditions.

4.3.2. Media Preparation

Mineral salts media broth (MSM-B) was prepared according to ASTM G21-15 protocol (ASTM 2013) as 0.7 g of KH_2PO_4, 0.7 g of K_2HPO_4, 0.7 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of NH_4NO_3, 0.005 g of NaCl, 0.002 g of $FeSO_4 \cdot 7H_2O$, 0.002 g of $ZnSO_4 \cdot 7H_2O$, and 0.001 g of $MnSO_4 \cdot H_2O$ per 1000 mL of water (pH 6.8). Other nutrient-rich media (NA, NB, PDA and PDB) were prepared according to the manufacture's descriptions. All media was autoclaved at 121 °C for 20 min.

4.3.3. UV-pretreatment

The pretreatment of plastic samples was conducted on both plastic sets and performed under four different radiation conditions in a closed metal chamber (length: 410 mm, width: 210 mm) equipped with a 35-watt power source, thermometer, and a mercury (Hg) lamp to generate 245 nm wavelength UV rays. Plastic samples were placed at the bottom of the UV chamber to be irradiated for 72 and 120 h at two different distances (24 and 12 cm) from the UV light source. At the end of each UV-pretreatment cycle, the plastic samples were flipped over, and the other sides were irradiated with the same conditions.

The UV-pretreatment period was chosen based on the average irradiation time conducted in previous studies, as shown in Table 4.1. The experiment was designed as; condition 1 (t_1 d_1): (PE t_1 d_1), (PS t_1 d_1), (PET t_1 d_1); condition 2 (t_1 d_2): (PE t_1 d_2), (PS t_1 d_2), (PET t_1 d_2); condition 3 ($t_1 d_2$): (PE t_2 d_1), (PS t_2 d_1), (PET t_2 d_1) and; condition 4 (t_2 d_2): (PE t_2 d_2), (PS t_2 d_2), (PET t_2 d_2) where t_1 and t_2 were irritating time (72 and 120 h) and d_1 and d_2 were distance from the light source (24 and 12 cm), respectively.

Before the experiment, important parameters such as intensity and dosage of UV light were calculated. The frequency of UV light was calculated initially using Equation 1:

$$\mathrm{C} = \lambda f \quad (1)$$

where C is the speed of light (3×10^8 m.s^{-1}), λ is the wavelength of UV (nm), and f is the frequency of UV (s^{-1}).

Following the determination of frequency, the energy of single photon, as well as total energy per exposure time, were calculated using Equations 2 and 3, respectively:

$$E = n\mathrm{h}f \quad (2)$$

where E is the energy of single photon (J), n is the number of photons, h is Planck's constant (6.626×10^{-34} J.s).

$$E_T = P\ t \quad (3)$$

where E_T is total energy (J), P is power (J.s^{-1}), and t is irradiation time (s).

Thereafter, the number of photons was calculated using Equation 4:

$$\text{Total number of photons} = E_T/E \quad (4)$$

Total number of photons for 72 and 120 h irradiation were 1.121×10^{25} and 1.87×10^{26}, respectively.

The intensity was then calculated using Equation 5:

$$I = \frac{n\ f\ \mathrm{h}}{a\ t} \quad (5)$$

where I is intensity of UV (μW.cm^{-2}), n is number of photons per exposure time, f is frequency (s^{-1}), and a is area of irradiation (cm^2).

The 72 and 120 h UV irradiation intensities at 24 cm from UV lamp were 4.063×10^5 and 4.064×10^6 μW.cm^{-2}, respectively.

Unlike mechanical waves (e.g. sound waves and water waves) with a constant intensity over time, in electromagnetic waves (e.g. UV radiation, light wave, microwave), intensity changes over time due to the difference in the generated number of photons. In mechanical waves intensity is defined as power per unit area, whereas in electromagnetic waves, intensity is defined as energy per square centimetre received per time unit. This explains the reason for different intensities between 72 and 120 h of irradiation.

Since intensity is proportional to the square of the distance from the UV light, the intensities of 72 and 120 h irradiation at 12 cm to the UV source were determined using the inverse square law as stated in Equation 6:

$$I_1/I_2 = (d_2)^2/(d_1)^2 \quad (6)$$

where d is distance from the UV lamp (cm).

Based on inverse square law, the intensities for 72 and 120 h of irradiation at 12 cm were 1.625×10^6 and 1.625×10^7 μW.cm^{-2}, respectively, suggesting the intensity at a shorter distance to UV source was higher than the intensity at a longer distance.

Finally, the dosage of UV was calculated according to Equation 7:

$$UV\ dose = It \quad (7)$$

Due to the direct relation between dosage and intensity, the dosage at a shorter distance to UV

lamp and at a longer exposure time (t_2 d_2) was higher than a condition with a longer distance to UV lamp and shorter irradiation time (t_1 d_1). Therefore, the UV dose for 72 and 120 h at 24 cm from the UV source was 1.051×10^8 and 1.755×10^{12} $\mu W.cm^{-2}.s$, while the irradiation dosage for 72 and 120 h at 12 cm from UV source were 4.2×10^{11} and 7.02×10^{12} $\mu W.cm^{-2}.s$, respectively.

To differentiate the effect of UV from biodegradation, three sets of control was used in this study. Control-1 contained un-pretreated plastics, which were incubated in MSM with microorganisms. Control-2 contained UV-pretreated plastic samples, which were incubated in MSM without microorganisms.

The reason that control-2 was pretreated at only t_2 d_2 condition was due to the fact that this condition generated a higher intensity and dosage, which then resulted in more significant physiochemical changes of plastic compared to t_1 d_1, t_1 d_2 or t_2 d_1 conditions. Therefore, it was more rational to use only this condition as a control for comparison. One of the aims of this study was to assure that the degradation efficiency of plastic with UV itself (at the highest condition, t_2 d_2) is lower than UV-pretreated & bio-treated condition (UV-pretreated plastics in the presence of microorganisms). Control-1 and control-2 were used to understand whether the changes of plastic samples at the end of the incubation period were due to microorganisms or UV radiation. Control-3 also contained un-pretreated plastics, which were incubated in MSM without microorganisms. Control-3 was used as a reference for comparison with the results of other conditions. The summary of the experimental design is shown in Table 4.1.

Table 4.1. UV-pretreatment and bio-treatment conditions

Sample name	Plastic type	UV-pretreatment Condition	
UV-pretreated	PS, PE, PET	t_1 d_1	72 h, 24 cm
	PS, PE, PET	t_1 d_2	72 h, 12 cm
	PS, PE, PET	t_2 d_1	120 h, 24 cm
	PS, PE, PET	t_2 d_2	120 h, 12 cm
Bio-treated (control-1)	PS, PE, PET		-
UV-pretreated & bio-treated	PS, PE, PET	t_1 d_1	72 h, 24 cm
	PS, PE, PET	t_1 d_2	72 h, 12 cm
	PS, PE, PET	t_2 d_1	120 h, 24 cm
	PS, PE, PET	t_2 d_2	120 h, 12 cm
UV-pretreated & strain-free bio-treated (control-2)	PS, PE, PET	t_2 d_2	120 h, 12 cm
Un-pretreated & strain-free bio-treated (control-3)	PS, PE, PET		-

After completion of UV-pretreatment, plastics were analysed for weight loss, hydrophobicity changes and chemical transformation. For set-B plastic, samples were first irradiated in a big sheet and then cut into small pieces (l < 10 mm, w < 5). All experiments were repeated four times, and the results were expressed as mean ± standard deviation.

4.3.4. Inoculums

The four different plastic degraders strains used in this study were *Penicillium raperi*, *Aspergillus flavus*, *Penicillium glaucoroseum* and *Pseudomonas* spp. Prior to the biodegradation, all strains were enriched individually on fresh NB or PDB for 72 h. Since the biodegradation experiment was conducted in two different flask sizes, (set-A (250 mL) and set B (2000 mL)), the amount of used inoculums after the enrichment period was adjusted accordingly based on the size of the flasks (5 mL for set-A and 40 mL for set-B plastics). The microbial suspension (5 mL or 40 mL) was removed after 48 h of enrichment and was subjected to centrifugation at 5000 × g (5 min). After that, the pellets were suspended individually in MSM-B (2 h) for media adaptation. The content was centrifuged at the same condition, and the obtained pellets were mixed to form a consortium. The consortium was vortexed and distributed 5 mL per 250 mL flask for set-A, or 40 mL per 2000

ml flask for set-B. Set-A contained 70 mL, and set-B contained 560 mL MSM-B. For a homogenous distribution of microbial strains in each flask, the content of made consortium was shaken before each addition. All experiments were conducted in duplicate, and the results were expressed as mean ± standard deviation.

4.3.5. Biodegradation

The biodegradation experiments were conducted on both pretreated set-A and set-B plastic sizes. For set-A, four pieces of each plastic type were added into the 250 mL flasks, and for set-B, five grams of each plastic type were added into the 2000 mL flasks. Both small and large flasks contained the same microbial consortium and MSM-B. Biodegradation of set-B plastics was conducted after understanding the optimum UV-pretreatment conditions and the biodegradation period from the results obtained in set-A plastics.

To keep the media level as the same as the initial experiments, freshly sterilised MSM-B was added accordingly in each flask when the level of media was reduced. The biodegradation was conducted according to the condition in Table 4.1. All flasks were incubated at 32 °C, 90 rpm for 90 days. All experiments were repeated two times, results then were compared against each other and expressed as mean ± standard deviation.

4.3.6. Analytical Methods

Different analytical examinations were performed on each plastic set. Plastics in set-A were examined for surface deterioration, weight loss, biofilm formation, decolourisation, wettability (water contact angle) and chemical alteration (FT-IR) prior to the experiment, after UV-pretreatment and after biodegradation (every 45 days).

Thermogravimetric (TGA), roughness, and microscopy (AFM, SEM) analyses were conducted on the samples with the most promising results. Set-B plastics were used to determine molecular weight reduction based on their melt flow index (MFI). After measuring the biofilm, each plastic sample was rinsed with sterilised distilled water, disinfected with 75 % (v/v) ethanol solution, immersed in SDS solution (2% w/v), followed by 24 h oven-drying for the other analytical analysis. The liquid culture was also analysed to determine the microbial viability, pH variation, and the identification of the possible by-product by GC-MS.

4.3.6.1. pH Variation

To determine whether the used microbial consortium remained metabolically activated during the biodegradation experiments, the pH of media in each flask that contained set-A or set-B plastics was aseptically measured every ten days. The results were compared with the control samples. The pH meter (Hanna, edge) first was disinfected under UV radiation (245 nm) of the laminar hood (15 min) followed by several times rinsing the pH electrode with 75% (v/v) ethanol and sterilise distilled water. The pH electrode was inserted in each flask, and the pH of each flask was measured two times. To minimise cross-contamination between flasks, the pH of control samples was measured first in each interval.

4.3.6.2. Biofilm Estimation

Biodegradation of plastic involves four main steps: colonisation, depolymerisation, assimilation, and mineralisation (Shah et al. 2008). The colonisation of microbial strains (biofilm) on the surface or inside plastic samples is one of the prerequisites in biodegradation (Gu 2007). Strains in the form of biofilm can behave differently compared to when they are in the planktonic form (Han et al. 2020). Strains in form of biofilm have a better ability to change plastic surface (ex. reduce hydrophobicity) and consume it as a food source. Therefore, estimation of the formed biofilm is vital to determine the effectiveness of biodegradation. The amount of biofilm in each plastic type for each pretreatment condition was monitored every 45 days.

To understand the efficiency of microbial colonisation on pretreated and untreated plastic samples, the formed biofilm on the surface of each plastic was assessed by crystal violet (CV) assay according to Eich et al. (2015). The evaluation was conducted on plastic films (set-A) having a larger surface for microbial colonisation. Crystal violet can stain extracellular matrix (ECM) on the formed biofilm and, therefore, indirectly estimate the total population of microorganisms on the surface of plastic (Chavez-Dozal et al. 2016). In brief, plastic films were washed gently with sterilised Milli-Q water to remove unattached planktonic (free) cells. Samples then were immersed into 1 mL 1% (w/v) CV for 15 min, and a washing step removed the excess stain. The stained plastic films were individually immersed in 3 mL 95% ethanol (5 min) for extraction. Finally, the absorbance of the extracted aliquot was measured at 595 nm in the microplate reader (PerkinElmer, MLD2300, USA).

4.3.6.3. Physical Weight loss and Melt Flow Index Determination

To determine physical weight loss, the initial weight of each plastic film (set-A) was measured three times prior to the experiment, after UV radiation, and at the end of each biodegradation cycle (every 45 days) by a 5- digit analytical balance (Precisa, EP 120A).

After the biodegradation test, the weight loss of plastic films was measured by removing two plastic films of each plastic type from each flask, followed by washing with distilled water and immersion in a 75% (v/v) ethanol solution. The plastic samples were then immersed in 30 mL of sodium dodecyl sulphate (SDS) solution (2% w/v) for 24 h followed by distilled water rinsing and oven drying at 55 °C for 24 h before measuring the weight loss. To assure the weight loss was not changing over time, the weight of cleaned and disinfected plastic samples was measured once after three days and once a week later. During that time, plastics were kept in the oven (50 °C). All measurements were repeated four times, and results were expressed as mean ± standard deviation.

The molecular weight loss was evaluated only on set-B plastics by melt flow index (MFI) technique. MFI is a well-used technique that can indirectly measure the molecular weight loss of thermoplastic by expressing the melt flow (in grams) after heating to the melting point and passing through capillary with a fixed applied pressure within ten minutes (Shenoy and Saini 1986, McKeen 2008). The high melt flow index corresponds to low molecular weight and vice versa. To differentiate the effect of UV radiation and biodegradation on molecular weight reduction of set-B plastic, different samples as: (i) bio-treated (control-1); (ii) "UV-pretreated plastics (at t_2 d_2 condition)" (control-2); (iii) "un-pretreated & strain-free bio-treated" (control-3); and (iv) "UV-pretreated (at t_2 d_2 condition) & bio-treated" were subjected to the MFI analysis and results were compared against each other.

The set-B plastics were removed from each flask and rinsed initially with distilled water, followed by washing with 75 % ethanol solution. Samples were then immersed in SDS (2 % w/v) for 24 h followed by rinsing and oven drying at 55 °C for 24 h. Before analysing the treated samples, parameters such as melting time, cutting time, melting temperature, and applied weight were optimised, and then samples were individually passed through the apparatus (Dynisco, LMI4000). The test was conducted according to ASTM D1238 – 10 in duplicate. The weight of each sample was recorded, and then MFI (g/10 min) was calculated automatically as; flow rate = ($600/t \times$ weight of extrudate) where t is the time of extrudate (s).

4.3.6.4. Microscopic and Roughness Analysis

Surface topography of plastic films was determined selectively only on plastic films with higher microbial growth, higher weight loss and lower water contact angle. Each plastic sample was examined with atomic force (AFM, Cypher-ES, USA) and scanning electron microscope (SEM, Quanta 200f, USA). For AFM analysis, plastic films were mounted on the metal specimen discs Ø10 mm and scanned at a 4 × 4 μm scale. For SEM analysis, the same samples were sputter coated with platinum (Quorum, Q150RS) for 5 min at 20 mA, and were scanned under low vacuum at 10 kV.

Roughness analysis was conducted at two different scales (4×4 μm and 15×15 mm). At the 4×4 μm scale, the taken images after AFM microscopy were analysed by Gwyddion software, and analysis was examined at the same position of the sample where AFM topography was performed. The roughness of samples at their full size (15×15 mm) was determined using optical profiler (Bruker Contour GT) and Vision-64 software. For plastic like PET with transparent characteristics, samples are first sputter coated with platinum.

4.3.6.5. Wettability

The water contact angle technique was used to assess hydrophobicity changes of plastic samples. The test was performed by placing a droplet of distilled water (4 μL) on the flat surface of each plastic sample. The angle between water and plastic film was calculated with Young-Laplace equation. Examination of each plastic sample was conducted two times at two various locations of plastic, and results were expressed as mean ± standard deviation.

4.3.6.6. Chemical Transformation

The chemical transformation of plastic films was examined by FT-IR technique (PerkinElmer, USA). Plastic films were scanned at both sides at a resolution of 4 cm^{-1} from a wavenumber range of 4000 to 400 cm^{-1}.

4.3.6.7. Thermogravimetric (TGA) analysis

To understand the changes in the thermal stability of samples, plastic films with the best

degradation efficiency was analysed using a thermogravimetric analyser (TGA-50, Shimadzu). Analysis was conducted at a temperature rate of 10 °C/min from 25 °C to 590 °C under an argon atmosphere.

4.3.6.8. Microbial Viability

Microbial viability was determined at the end of the biodegradation experiment. Due to the nature of the inoculum (mixture of bacteria and fungi), and because of the formation of hypha during the biodegradation, determining the microbial growth by normal techniques like plate counting or spore counting with hemocytometer was not feasible. Therefore, the viability of strains incubated with either set-A or set-B plastics was determined with double fluorescent staining using acridine orange (AO) and propidium iodide (PI). Acridine orange is a permeable stain that generates green fluorescent upon its binding to DNA/RNA of both live and dead cells, while PI can only pass through the compromised cells' membrane and stain the nucleated cells with a red fluorescent.
The viability test was conducted according to (Hussain et al. 2019) with some modifications. Briefly, 20 μL aliquots from each flask were removed and air-dried on a clean microscope slide, followed by cells fixation with 60 μL methanol. The air-dried slide then was incubated with a 60 μL filtered and fresh working solution of AO (1:50 mL in water) for 5 min followed by washing with water and staining with 60 μL of propidium iodide (PI) working solution (1 μL/mL in water) for 5 min. The excess dye was then washed with water, air-dried and analysed with a confocal laser microscope (Nikon H600L) at 420 nm and 450 nm excitation for AO and PI, respectively. To count the number of live and dead cells, the taken images were processed by Nikon software (NIS, Elements) in which the background was adjusted to black, and cell sizes in thresholding were limited to 0.5 to 3 μm. Moreover, due to the permeability of AO in both live and dead cells, the cells which counted twice (presented in both PI and AO staining) were marked in yellow and then were subtracted by the software from the total number of live or dead cells.

4.3.6.9. Identification of By-products by GC-MS

At the end of biodegradation (90 days), the culture media of each flask was removed for identification of the possible degraded compounds by chromatography- mass spectrometry (GC-MS) according to Skariyachan et al. (2018) with some modification. In brief, 20 mL of culture

media was removed and filtered through a 0.2 μm syringe filter. The obtained media was then mixed with dichloromethane in a 1:1 volumetric ratio followed by solvent evaporation for about 3 h. The extracted by-products were removed, filtered, and injected into the GC-MS equipped with a HP-5 column. The initial temperature was set to 60 °C and then increased at a 10 °C /min rate until it reached the maximum of 300 °C and was kept for 6 min. Helium was used as a carrier gas with a fixed flow rate of 1 mL/min, and the split ratio was set to 10:1. The biodegradation by-products were identified using the mass-spectrometry library.

4.3.6.10. Statistical Analyses

To understand the optimum biodegradation efficiency, each UV-pretreated and bio-treated condition was compared against each other and controls. The analysis was performed by one-way ANOVA using the SPSS software with a *p*-value of < 0.05 as the significant level. Analysis was repeated three times, and results were expressed as mean ± standard deviation.

4.4. Results and Discussion

4.4.1. Visual Examination

Physical changes such as cracking, change of colour, and brittleness indicate that degradation is happening. Therefore, plastics were observed for any visible changes after UV radiation and at the end of biodegradation. Analysis of PE samples after UV radiation showed that the structure of PE became very brittle compared to untreated samples (control-1 and control-3). For instance, untreated PE could be stretched upon elongation; however, UV-pretreated PE was torn easily. Pretreated PS samples also indicated physical changes, in which a yellowish powder-like compound was formed on its surface, and it became fragile. The formation of yellowish powder due to oxidation by UV, reported by (Yousif and Haddad 2013) (Fig 4.1). There were no physical and colour changes of PET after UV radiation and biodegradation.

It was observed that the UV-pretreated PS in set-B (where a higher amount of plastic (>5 grams) and smaller size (< 10 mm) were subjected to biodegradation), indicated more morphological changes in comparison to pretreated PS in set-A (where bigger plastic films (>10 mm) and lower amount (<1 gram) were used). The pretreated PS plastics in set-B after 45 days of biodegradation showed the formation of several surface cracks. This was not observed in the experiment where

untreated PS in set-A and set-B were subjected to biodegradation. The observation suggests that the smaller size and higher amount of pretreated PS plastic, increase the chance of plastic uptake by microorganisms. In the previous chapter, it was shown that more cracks were formed on untreated small-size PS after 270 days. However, in this chapter the pretreated small-sized PS was cracked just after 45 days of incubation, highlighting the positive effect of UV-pretreatment in biodegradation.

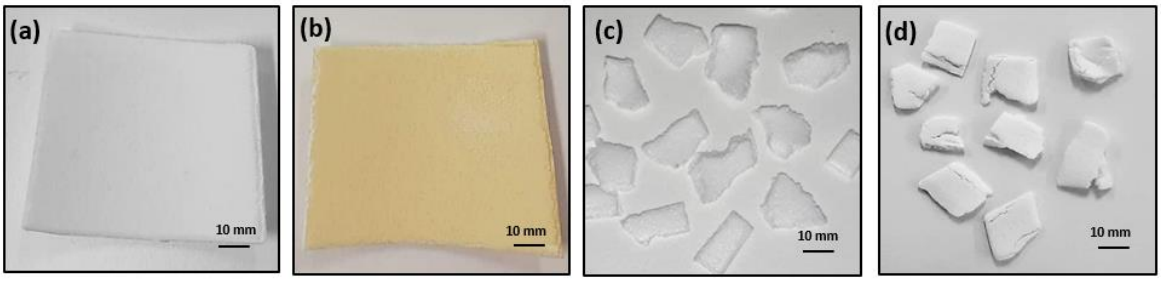

Fig 4.1. Polystyrene (PS) before UV-pretreatment (control-3) (a); after UV-pretreatment (b); and PS in set-B before UV radiation (c), and UV-pretreated and bio-treated after 45 days of experiment (d)

4.4.2. pH Monitoring

Since microbial strains excrete different metabolites and exoenzymes in media during the biodegradation, monitoring of pH can indicate whether the microbial strains are still active or not. There was a similarity in the pH condition between this study and others due to the usage of mineral salt media (carbon-free) with a pH range of ~ 6.8- 7. However, the variation in the microorganisms used in this study with other studies resulted in a different pH range.

The analysis shows that after 20 days of biodegradation, the pH of media in all flasks having different conditions of UV-pretreated plastic dropped from 6.8 to the lowest point of 6.4 (Fig 4.2). The fluctuation in the pH trend was due to the addition of fresh MSM-B with a pH of 6.8 to each flask to maintain the nutrient and media level at the beginning of the experiment. The drop in pH and similarity in the value of pH at day 20 and day 90 suggested that even after 90 days of incubation the microbial strains were still metabolically active, and allowed them to secrete their enzymes for depolymerisation of plastics and up-taking as a food source. Similar results were also

reported by Dey et al. (2020) as part of plastic biodegradation.

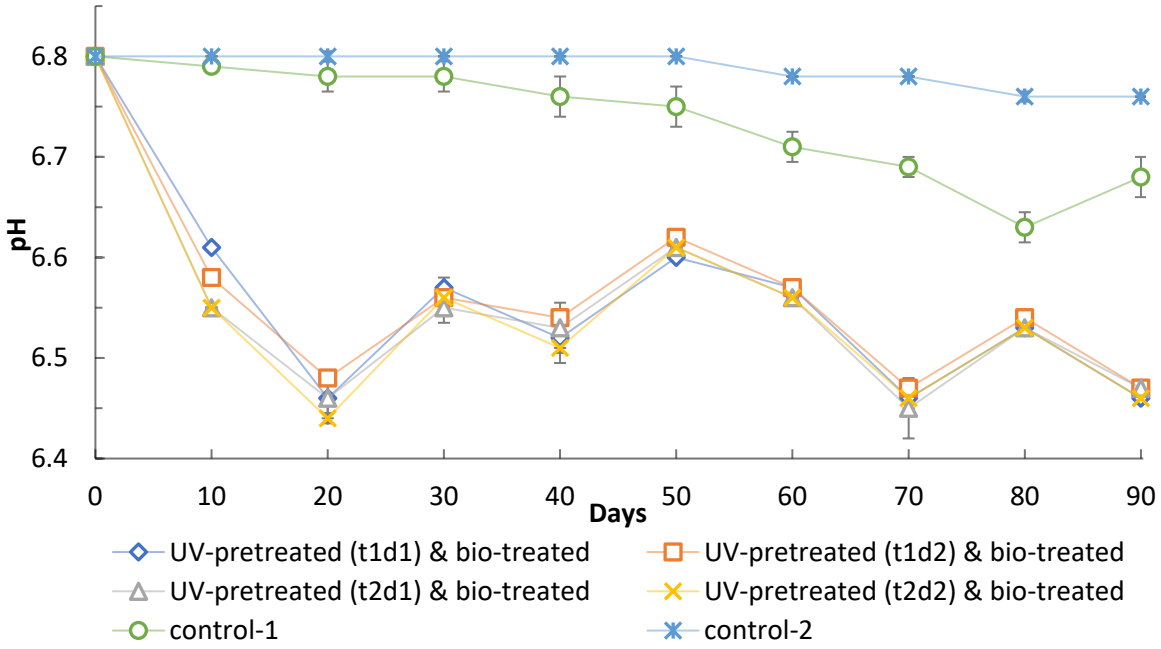

Fig 4.2. The pH changes of media in "UV-pretreated & bio-treated" and controls samples within 90 days of biodegradation

The pH of "bio-treated" (control-1), and "UV-pretreated & strain-free bio-treated" (control-2) samples was 6.68 and 6.76 after 90 days of incubation, respectively. The increase in pH of control-1 between days 80-90 was also due to the addition of MSM-B.

These results showed that UV-pretreated plastics were consumed easier by the microbial strains than the untreated samples.

4.4.3. Biofilm Formation

Analysis of plastic films during the biodegradation indicated noticeably visible colonisation on PS and PE (Fig 3a-c). After inoculating the formed biofilm on PS and PE, it was found that most of the created biofilms were related to *Aspergillus flavus* (Fig 4.3d). This observation was in agreement with the previous observation in chapter 3 where *A. flavus* was found attached to the PS and PE plastic films in mineral salt agar plates. Among the three plastic types, PS had higher biofilm colonisation compared to PET and PE. Polystyrene foam is made of several similar shape beads/islands where each bead/island is positioned compactly to the next one. Microscopic

analysis of the pretreated PS showed that the gaps (cavities) between beads/islands became wider compared to the controls (Fig 4.3e, f). These gaps improved microbial penetration and colonisation compared to PE and PET with an almost flat structure.

Results also showed that pretreated plastics had higher microbial colonisation in comparison to untreated plastics. Among the UV-pretreatment conditions, t_2 d_2 showed higher microbial colonisation regardless of plastic type and biodegradation period. It was found that biofilm formation was higher in the first 45 days compared to the second cycle of the experiment (90 days) (Fig 4.3g- h). This could be due to the reduction of the microbial population over the experiment and lower colonisation. The amount of formed biofilm (OD 595 nm) at t_2 d_2 condition for PS, PE and PET plastic were 1.2, 0.06, 0.16 and 0.9, 0.05, 0.05 respectively for 45 and 90 days.

Statistics analysis also showed a significant difference ($p< 0.05$) between the biodegradation periods (45 days and 90 days). Likewise, there is a significant difference ($p< 0.05$) between biodegradation conditions, in which the t_2 d_2 condition had a higher effect in microbial colonisation (biofilm).

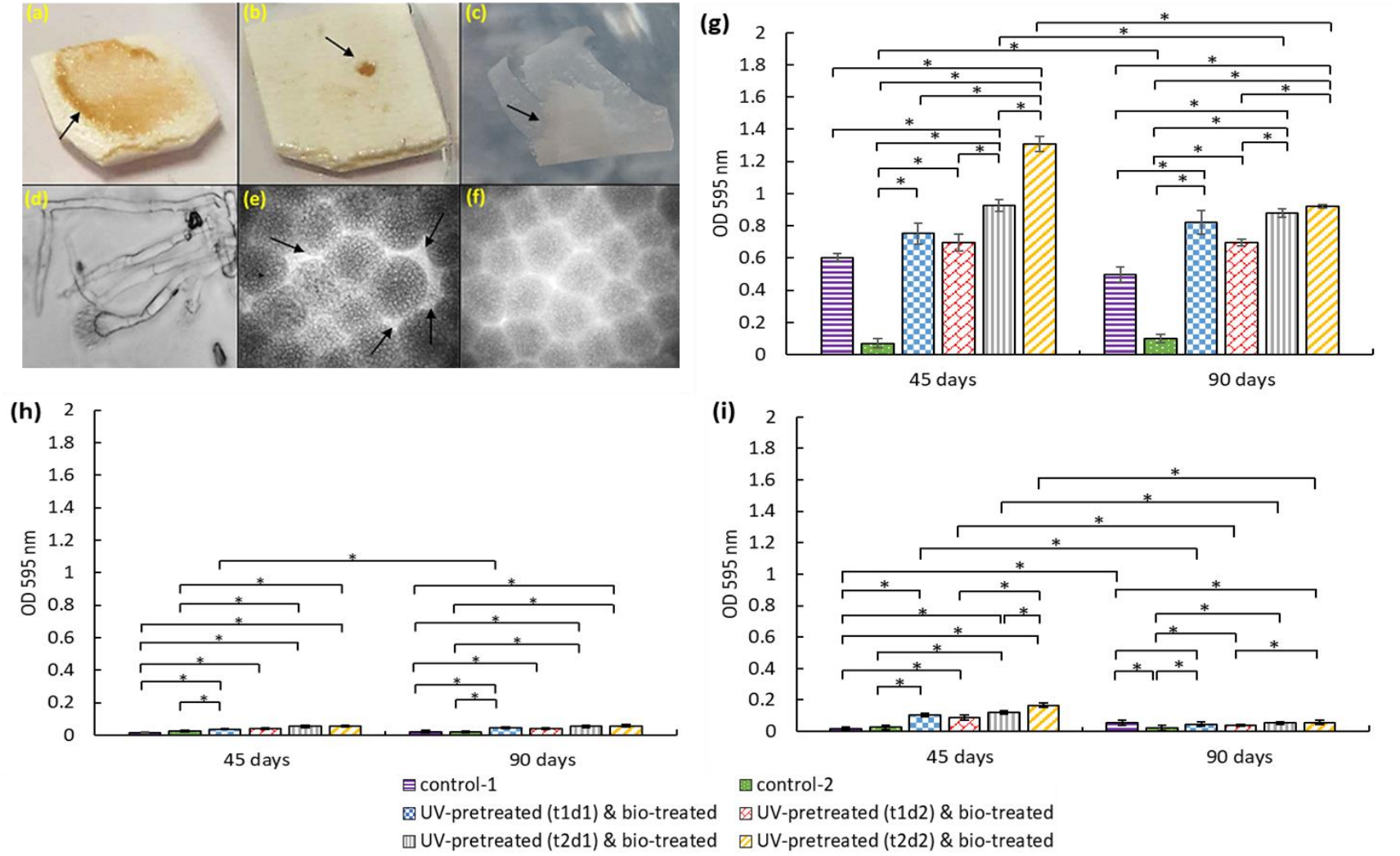

Fig 4.3. Biofilm formation on PS (a, b), and PE (c) during the biodegradation; *Aspergillus Flavus* colonised on plastics (d); gaps and structural differences between UV-pretreated PS (e); and untreated & strain-free bio-treated (control-3) PS (f); amount of formed biofilm within 45 and 90 days of biodegradation in PS (g), PE (h) and PET (i); * indicates $p < 0.05$

4.4.4. Physical and Molecular Weight Reduction

Analysis of plastics (set-A) after UV irradiation showed that PE, PET and PS samples at t_2 d_2 condition (the highest setting) had 5.2, 1.5 and 3.5 times higher physical weight loss compared to the pretreatment at t_1 d_1 condition (the lowest setting) (Fig 4.4a). The data suggested that the more extended pretreatment period with the closest distance to the UV light resulted in a higher plastic ageing and oxidation ($p< 0.05$). Due to the gain results in the pretreatment experiment, it was also expected to get a higher weight loss after the biodegradation test due to the mutual effect of UV and microorganisms.

As it was expected, t_2 d_2 was found the most effective condition in plastic weight reduction regardless of plastic type and biodegradation period. The observed weight losses for PE and PS after 90 days of biodegradation were 8.4 and 5.6 %, respectively. However, PET showed less than 1 % after 45 days and 90 days of biodegradation. The low rate of weight loss in PET in the 90 days of incubation suggested that the UV-pretreatment was not effective for enhancing the biodegradation efficiency of PET with the used microbial strains.

The statistical analysis showed no significant difference ($p< 0.05$) between 45 and 90 days of operation at t_2 d_2 condition in all three plastic types (Fig 4.4b-d). Therefore, it was concluded that 45 days of incubation at t_2 d_2 condition was enough to minimise the operation time and energy consumption.

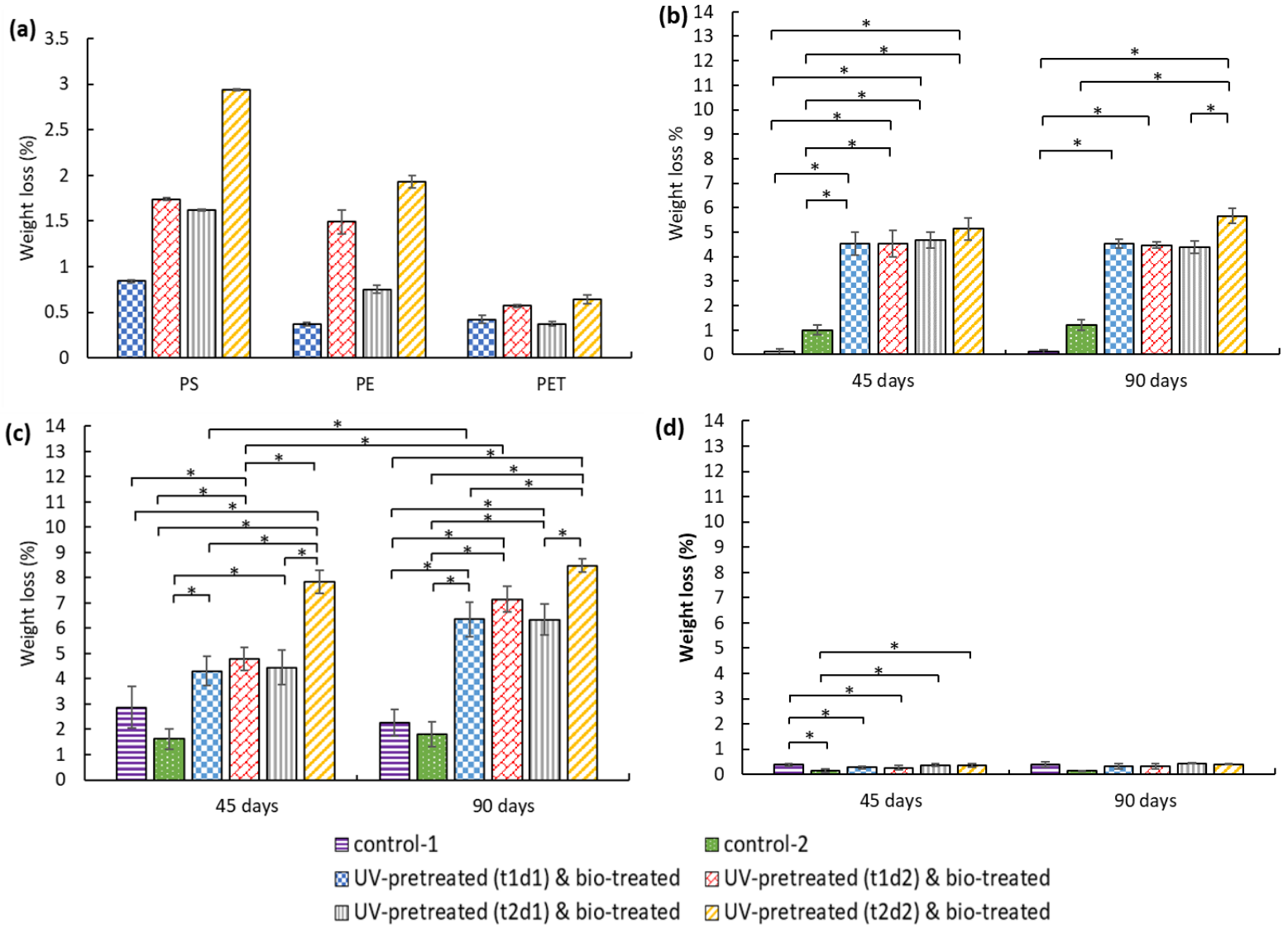

Fig 4.4. Physical weight loss right after UV-pretreatment (a); after biodegradation of PS (b), PE (c) and PET (d) within 45 and 90 days

The melt flow index analysis also showed that molecular weight reduction was higher for the "UV-pretreated (at t_2 d_2 condition) & bio-treated" PS and PE samples compared to the "UV-pretreated & strain-free bio-treated" (control-2), and "un-pretreated & strain-free bio-treated" (control-3) (Fig 4.5a, b). Nevertheless, there was a negligible difference between the MFIs of PET samples (Fig 4.5c), which was in agreement with the other obtained results of this study, as a higher MFI interpreted as a lower molecular weight or in other words a higher degradation. Changes in the molecular weight were because of the breakage of long polymer into shorter compounds which were due to the dual effects of microorganisms and UV. The results suggest that UV or microbes alone was not as effective as "UV-pretreated (at t_2 d_2 condition) & bio-treated" condition (Fig 4.5a, b).

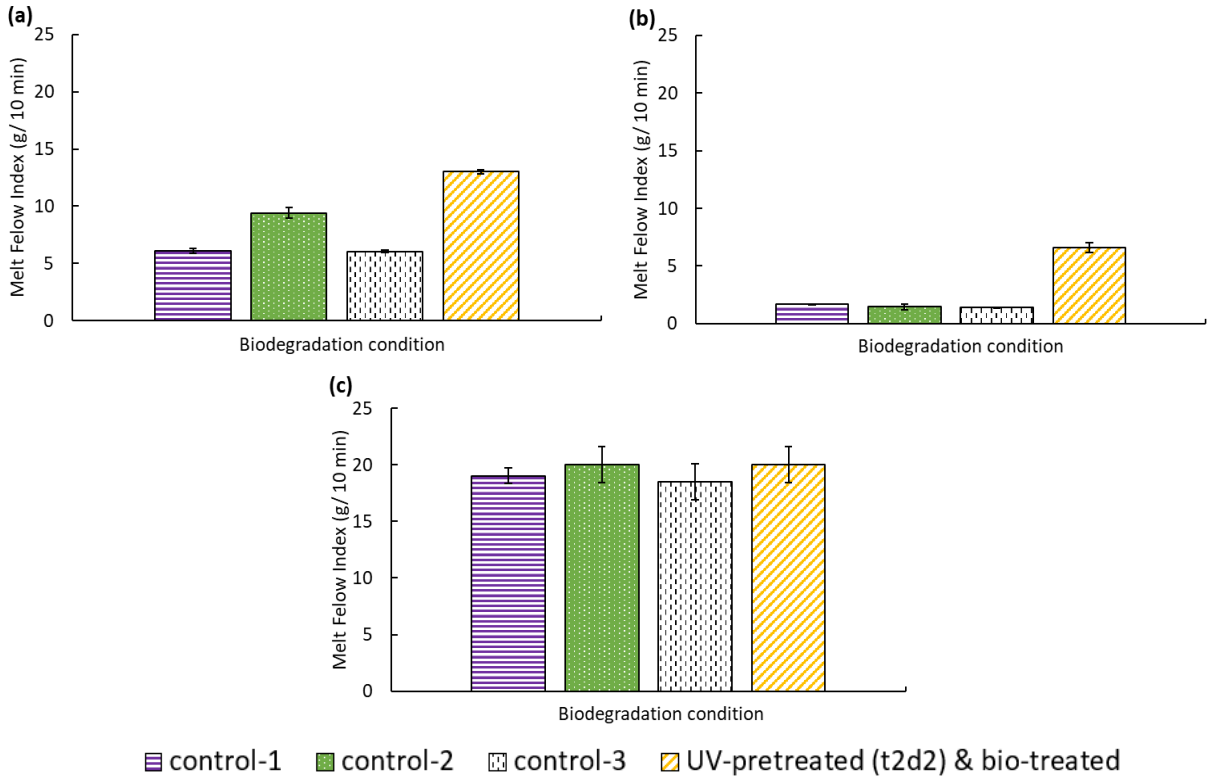

Fig 4.5. Molecular weight loss in PS (a), PE (b) and PET (c) after 45 days of biodegradation

4.4.5. Microscopy and Roughness

Microscopy analysis indicates the possible surface and texture degradation of plastic samples after UV-pretreatment and biodegradation. Atomic force microscopy (AFM) examination showed a higher surface degradation in the "UV-pretreated (at t_2 d_2 condition) & bio-treated" samples (Fig 4.6a, 4.7a, 4.8a) than "bio-treated" (control-1) (Fig 4.6b, 4.7b, 4.8b), "UV-pretreated (at t_2 d_2 condition) & strain-free bio-treated" (control-2) (Fig 4.6c, 4.7c, 4.8c), and "un-pretreated & strain-free bio-treated" (control-3) (Fig 4.6d, 4.7d, 4.8d). The maximum distance measured from the crest to the trough was 0.38, 0.31 and 0.25 μm for PS, PE and PET samples, respectively, in the "UV-pretreated (at t_2 d_2 condition) & bio-treated" samples (Fig 4.6a, 4.7a, 4.8a). Observation showed a negligible difference between control samples of PE. Likewise, there was not a noticeable difference between PET control samples. However, significant surface degradation (0.32 μm) was observed in PS control-1 compared to control-2 (0.17 μm) and control-3 (0.17 μm). The result suggested that while UV could change surface morphology and aid surface degradation, microorganisms were essential for further breakage and degradation.

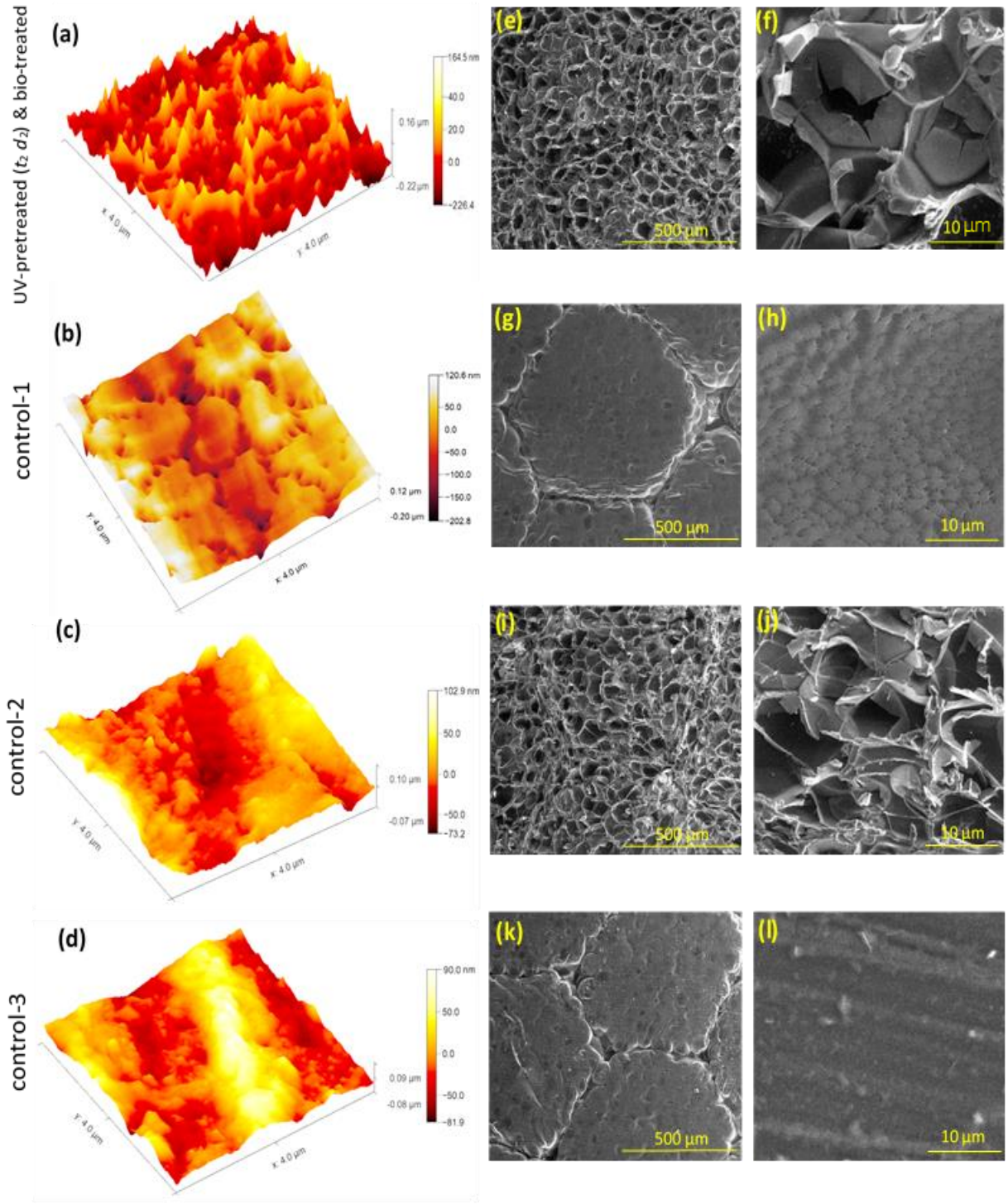

Fig 4.6. Microscopy analysis of PS after 45 days of biodegradation (at t_2 d_2) and its comparison with control samples. Images (a-d) represent AFM and (e-l) illustrate SEM

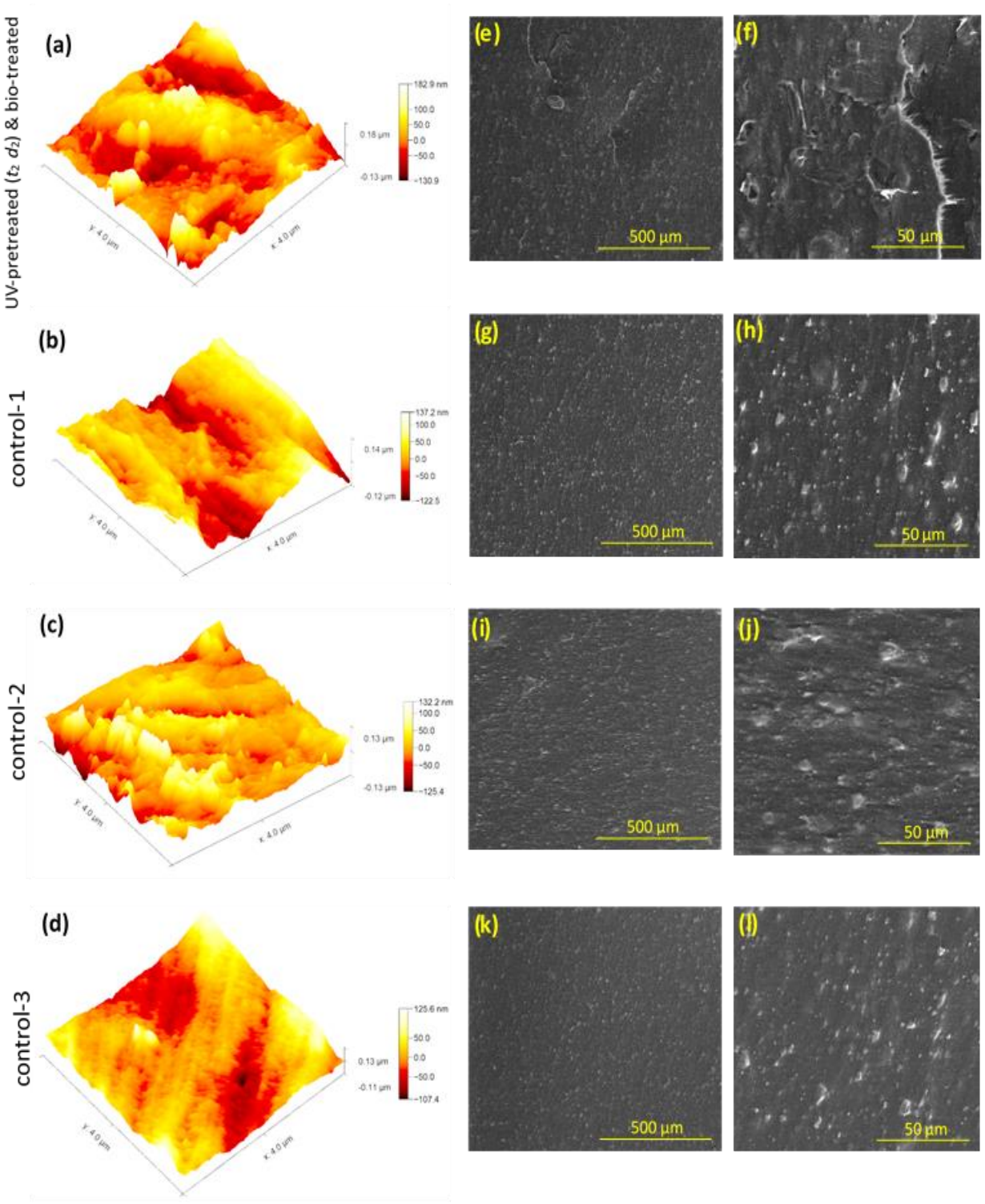

Fig 4.7. Microscopy analysis of PE after 45 days of biodegradation (at t_2 d_2) and its comparison with control samples. Images (a-d) represent AFM and (e-l) illustrate SEM

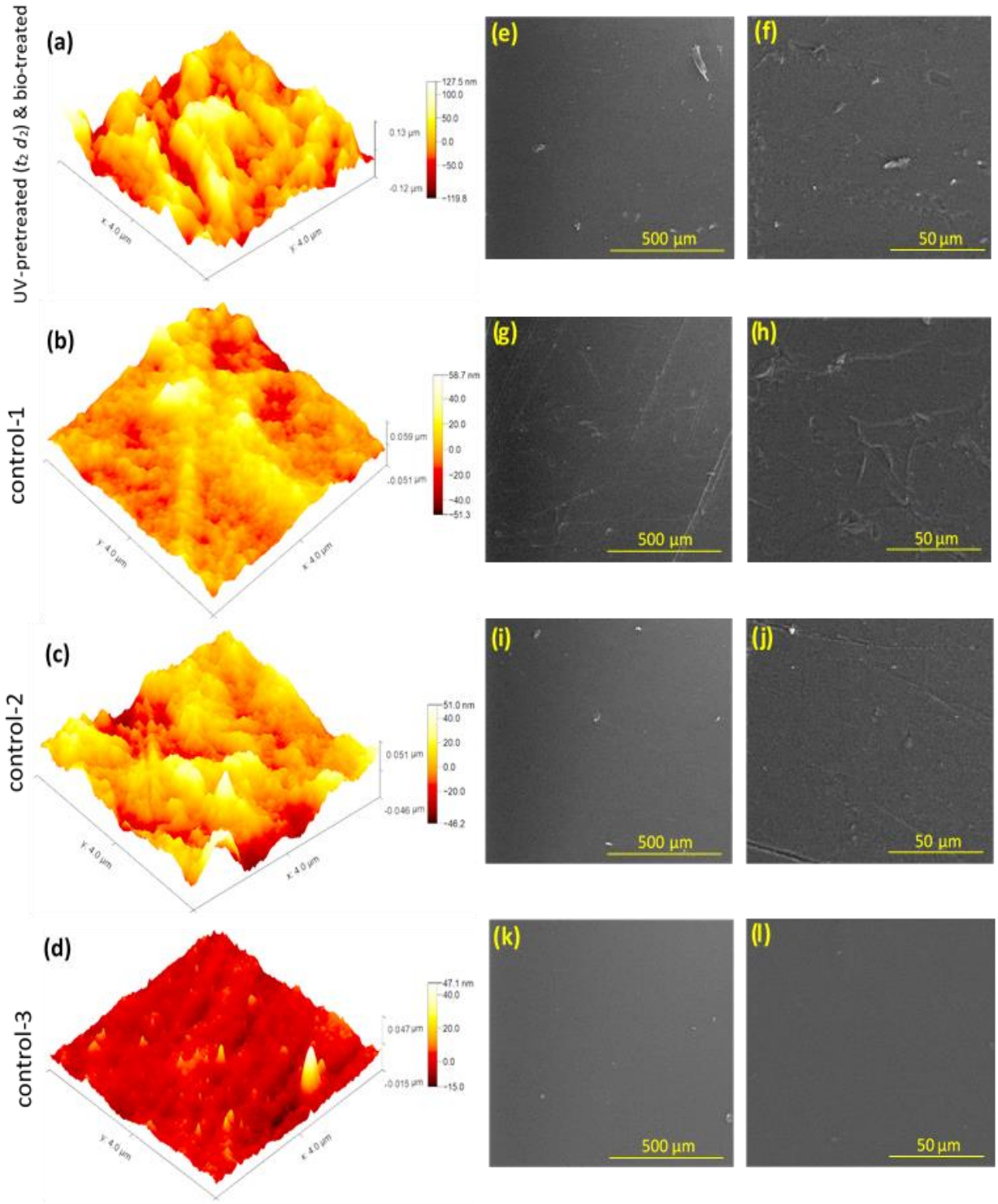

Fig 4.8. Microscopy analysis of PET after 45 days of biodegradation (at t_2 d_2) and its comparison with control samples. Images (a-d) represent AFM and (e-l) illustrate SEM

Analysis of SEM microscopy also confirmed the results obtained in AFM topography and showed that a higher surface degradation occurred in the "UV-pretreated (at t_2 d_2 condition) & bio-treated" samples (Fig 4.6e-f, Fig 4.7e-f, Fig 4.8e-f) than other UV-pretreatment conditions and controls. Results also showed surface deformation and the removal of boundaries between PS beads. Each PS bead was torn significantly and became porous in the samples subjected to UV radiation (Fig 4.6e, i). This structural change was not observed in the control-1 and 3 (Fig 4.6g, k). However, analysis at higher magnification (8000×) revealed a partial degradation around PS beads in control-2 (Fig 4.6h). This can be concluded as the positive role of microorganisms in PS deconstruction.

Surface morphology of PE samples with SEM showed that integration of UV-pretreatment with microbial degradation) resulted in the formation of a fibrous-like structure and several holes on the surface (Fig 4.7f). These changes were not seen in any other biodegradation conditions and control samples (Fig 4.7h, j, l).

Electron microscopy analysis of PET samples illustrated surface degradation in "UV-pretreated (at t_2 d_2 condition) & bio-treated", control-1 and control-2 samples. Nevertheless, there was a negligible difference in terms of PET surface degradation. Analysis of PET at a higher SEM magnification showed that the surface of the control-3 (Fig 4.8l) remained smooth. In contrast, a noticeable surface deterioration was observed for control-1 (Fig 4.8h) and control-2 (Fig 4.8j).

Based on these microscopy analyses, it can be concluded that surface degradation was due to the combination of UV radiation, microbial enzymatic activities, colonisation, and penetration of their metabolites within the plastic structure.

Roughness analysis of plastics at both full-scale (15 × 15mm) (Fig 4.9a) and micro-scale (4 × 4 μm) (Fig 4.9b) also indicated a higher surface roughness for the "UV-pretreated (at t_2 d_2 condition) & bio-treated". Those samples previously showed higher microbial colonisation (biofilm). Nauendorf et al (2016) observed a similar relationship and concluded that higher surface roughness is due to more significant microbial colonisation (Nauendorf et al. 2016).

Results indicated that the changes in roughness were not only due to the effect of UV radiation. The roughness values of control-2 in all three plastic types were lower than control-1. This can confirm the positive role of strains in biodegradation and roughness changes.

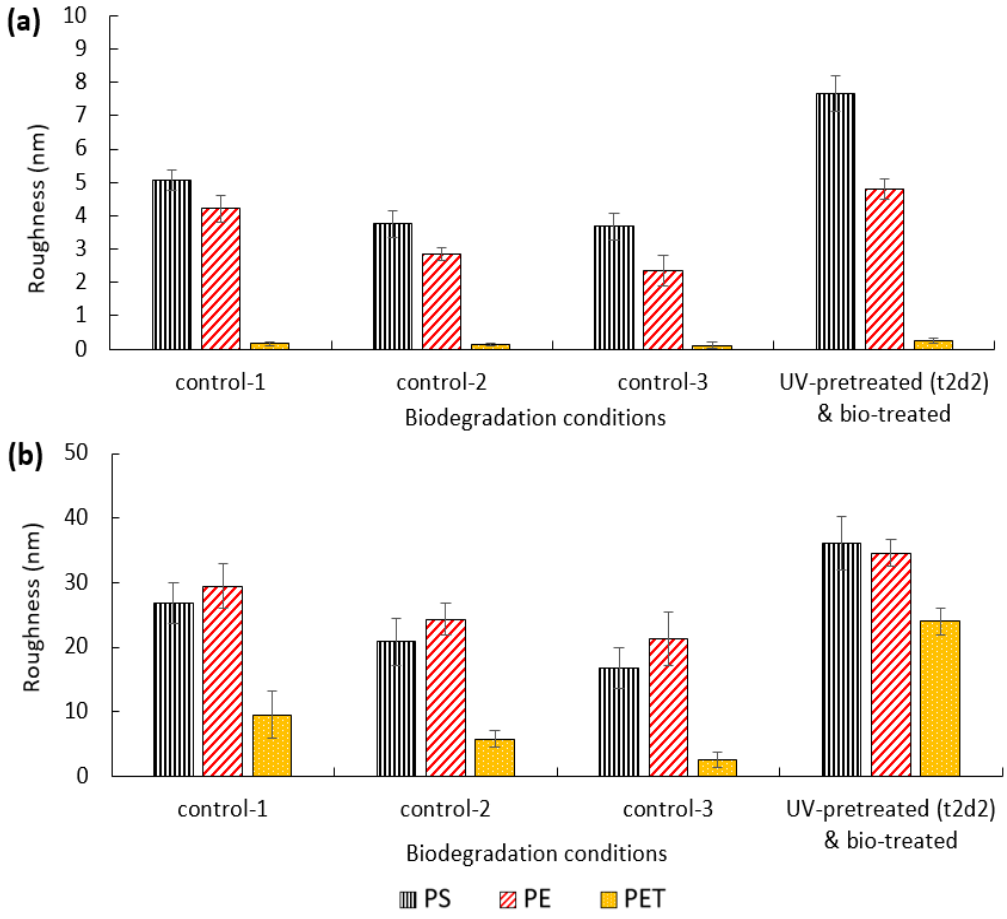

Fig 4.9. Roughness analysis of samples at (a) their full-scale (15 × 15mm) and (b) at (4 × 4 µm) plastic size

4.4.6. Water Contact Angle

One of the main obstacles in biodegradation is the hydrophobicity nature of plastics. Due to this physicochemical property of plastic, microorganisms hardly can attach and colonise on the plastic surface. In this study, the hydrophobicity of each plastic type was examined before and after

biodegradation (every 45 days). Analysis of samples before biodegradation showed that utilising UV as a pretreatment technique made plastic more hydrophilic which explained as the possible formation of free-radicals (oxygen radicals or polymer radicals) within the plastic structure. However, the PS samples became more hydrophobic after UV radiation (Fig 4.10a). This observation could be explained by the formation of a yellowish powder-like compound on the surface of PS, derived from its oxidation (Fig 4.10c, d). Sakti et al. (2017) reported a similar finding. They reported that the hydrophobicity of PS was increased after exposure to UV. Despite the reverse effect of UV radiation on PS samples prior to biodegradation, analysis of PS samples after biodegradation showed a significant decrease in hydrophobicity (< 5˚) regardless of the UV-pretreatment conditions (Fig 4.10b, e). The reduction in hydrophobicity of the PS samples after biodegradation was due to the removal of yellowish powder from PS surface after rinsing with water and ethanol before incubation, which resulted in better accessibility to the microorganisms.

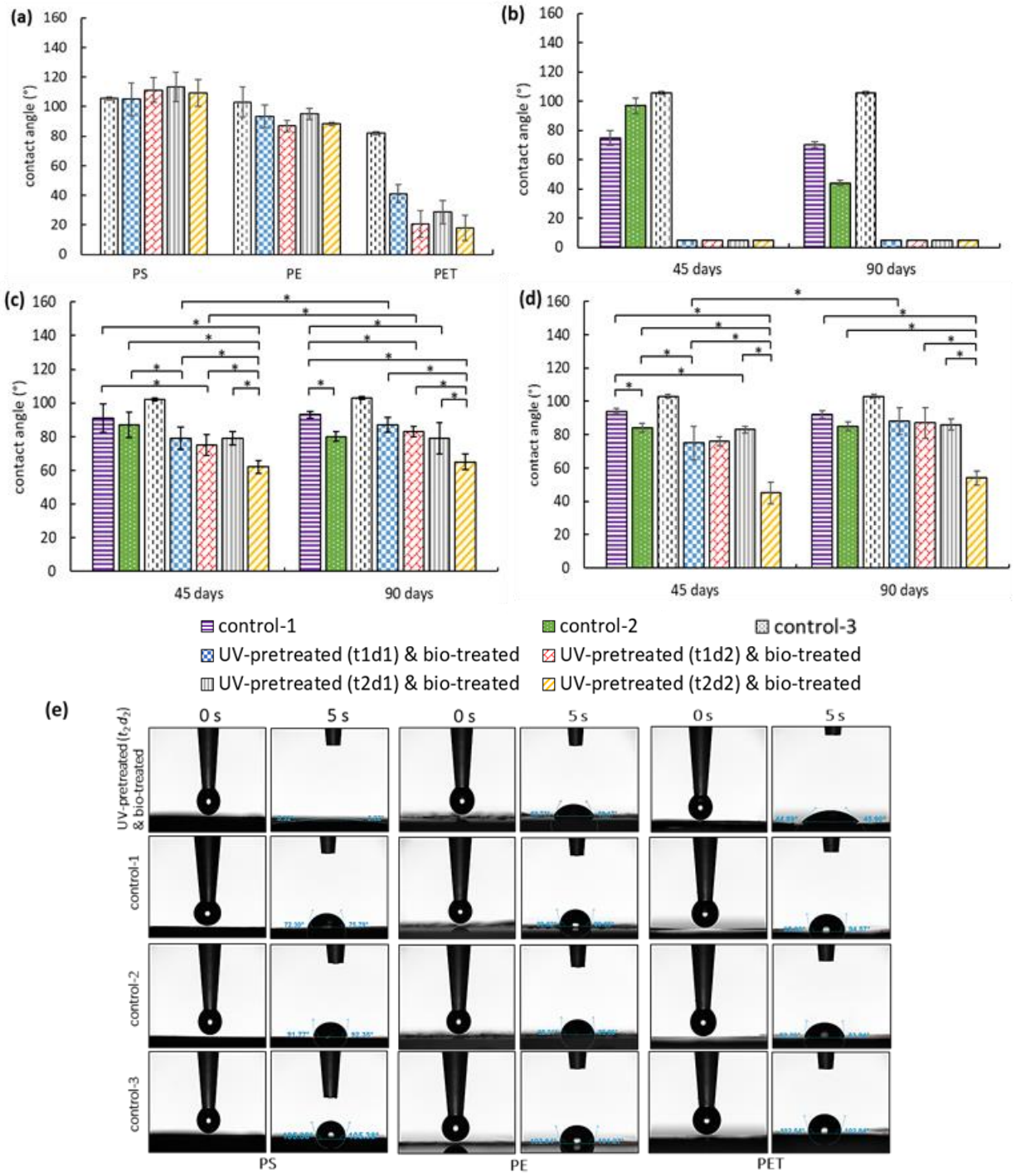

Fig 4.10. Water contact angle of plastics before biodegradation (a), and after 45 and 90 days of biodegradation (b-d) at different conditions. Images (b-d) represent PS, PE and PET respectively. Image (e) illustrates wettability of plastics after 45 days of biodegradation at $t_2\,d_2$ condition and comparison with controls at 0 s and 5 s of analysis

Similar to PS, UV-pretreated PE after biodegradation also showed reductions in hydrophobicity. The lowest contact angle of (59˚) observed in the PE samples pretreated under $t_2\,d_2$ condition (Fig 10b, e), suggesting that the hydrophobicity reduction was not just because of the UV effect.

It was also found that there was a contrast in hydrophobicity of PET before and after the biodegradation. Prior to biodegradation, UV radiation helped to reduce the hydrophobicity of PET from 88˚ to below 40˚. However, after biodegradation, hydrophobicity of pretreated PET ($t_1\,d_1$, $t_1\,d_2$, $t_2\,d_1$ conditions) was increased noticeably to about 85˚ (Fig 4.10a, b). This observation was due to the phenomena known as hydrophobic recovery, which is common in plastics like PET (Gotoh et al. 2011). Indeed, UV treated PET can become hydrophobic again after contacting with air or rinsing with water (Gotoh et al. 2011). Long radiation time could be another reason for the reverse effect in the wettability of plastics (Jaganathan et al. 2015). This is the reason why a lower wettability was observed for the PE and PET samples pretreated under $t_2\,d_1$ condition than those pretreated under $t_1\,d_1$ condition. However, the lower contact angle values of PE and PET pretreated under $t_2\,d_2$ condition was due to the higher chance of UV absorption in the shorter distance, which outweigh the adverse effect of longer exposure time at $t_2\,d_1$ condition.

Statistics analysis showed that there was not a significant difference (p> 0.05) between the biodegradation periods (45 and 90 days) for the samples pretreated under $t_2\,d_2$ condition (Fig 10b-d). In both periods, the maximum wettability occurred for the samples pretreated under $t_2\,d_2$ condition regardless of the plastic type. Thus, it could be concluded that biodegradation of the pretreated plastics ($t_2\,d_2$ condition) within 45 days was enough to make plastic more hydrophilic.

4.4.7. Chemical Transformation

The results from FT-IR analysis showed that the spectra pattern of all UV-pretreated PS prior to biodegradation was almost the same regardless of pretreatment condition. However, a comparison of “un-pretreated & strain-free bio-treated” (control-3) with “bio-treated” (control-1), and “UV-pretreated & strain-free bio-treated” (control-2) indicated noticeable changes. For instance, the intensity of peak assigned to C-H stretch (2950 cm^{-1} and 2850 cm^{-1}), C=C stretch (1650 cm^{-1}), C-H out-of-plane bend (755 cm^{-1} and 695 cm^{-1}) in the PS control-3 were reduced compared to pretreated PS (at $t_2\,d_2$ condition) before and after biodegradation (Fig 4.11a). The higher value of transmittance (lower intensity) was interpreted as a lower absorption and less availability of that chemical bond in the sample (Chaudhary and Vijayakumar 2020). This could be due to the

oxidation and degradation of polymer into shorter compounds through polymeric chain reactions and their consumption by microorganisms.

It was observed that the peaks at 1150- 1300 cm^{-1} were disappeared in all PS samples subjected to pretreatment (at t_2 d_2 condition). Also, the peak attributed to C=C (in aromatic at 1530 cm^{-1}) was removed from spectra of control-2, and "UV-pretreated (at t_2 d_2 condition) & bio-treated" samples (Fig 4.11a).

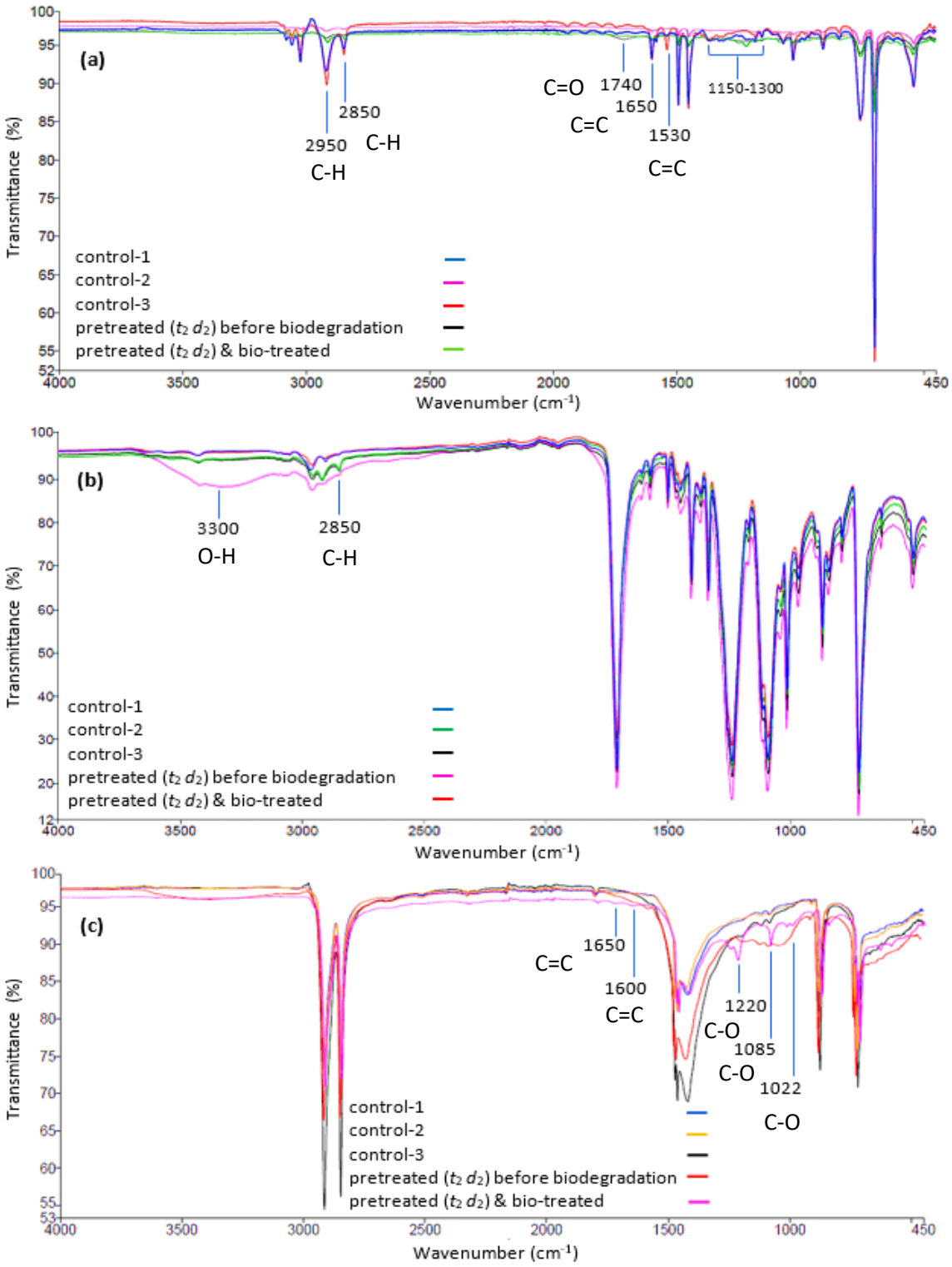

Fig 4.11. Chemical transformation of UV-pretreated (at t_2 d_2) and control samples in PS (a), PE (b), and PET (c) after 45 days of biodegradation

Formation of a new peak at 1740 cm^{-1} (C=O) was identified only in the UV-pretreated PS samples before bio-treatment, this peak was removed in UV-pretreated (at t_2 d_2 condition) & bio-treated" samples, and instead a new peak at 1250 cm^{-1} (C-O) was formed, suggesting the biodegradation of PS (Fig 4.11a). Analysis of "UV-pretreated (at t_2 d_2 condition) & bio-treated" samples at 45 and 90 days also showed that the spectra of samples were almost the same with a similar peak intensity. Comparison between pretreated PS samples after bio-treatment showed almost the same spectra except for the formation of peak attributed to C-O at 1250 cm^{-1} in pretreated (t_1 d_2) & bio-treated and pretreated (t_2 d_2) & bio-treated, suggesting that pretreatment was more efficient where plastics were exposed to UV sources at a lower distance.

Comparison of pretreated PET samples before biodegradation with control samples showed the formation of a new peak at 3300 cm^{-1} assigned to the OH group, which was due to PET oxidation by UV and formation of carboxylic end-group via chain scission reactions (Fig 4.11b). This peak was not seen in control-1, control-2, control-3 and "UV-pretreated (at t_2 d_2 condition) & bio-treated", which also could explain the reason for low wettability in PET samples after biodegradation (Fig 4.10d). Analysis of "UV- pretreated (at t_2 d_2 condition) & bio-treated" samples also showed the removal of the C-H peak at 2850 cm^{-1} from PET spectra regardless of their pretreatment conditions. The removal of C-H peak in control-1 samples suggested the capability of strains in breakage and removal of C-H from PET structure (Fig 4.11b).

Analysis of PE samples showed the formation of new peaks at 1650-1600 cm^{-1} attributed to C=C, and 1220, 1085, 1022 cm^{-1} attributed to C-O in the spectra of "UV-pretreated (at t_2 d_2 condition) & bio-treated" samples, and resulted in a shift in the spectra (Fig 4.11c). The absence of the aforementioned peaks in control-1, control-2 and control-3 resulted as the degradation capability of the strains in the utilisation of pretreated plastic as a carbon source. Comparison between pretreated samples after bio-treatment showed the formation of C-O peak at 1220 and 1085 cm^{-1} in just pretreated (t_2 d_2) & bio-treated conditions. Results suggested that t_2 d_2 condition could have a higher efficiency in changing the chemical structure of PE plastic.

4.4.8. TGA

Thermogravimetric analysis (TGA) was conducted to understand the possible change in the thermal stability of samples. Analysis showed that all three plastic types had one step thermal degradation. The results also showed a noticeable shift in degradation temperature of "UV-

pretreated (at t_2 d_2 condition) & bio-treated" samples compared to all other conditions. The "UV-pretreated (at t_2 d_2 condition) & bio-treated" samples in PS, PE and PET respectively started to degrade at much lower temperatures 345, 375 and 375 ˚C compared to their control-3 with the value of about 370, 440, 395 ˚C (Fig 4.12).

The reduction in thermal stability of plastic samples was due to the combined effects of UV-pretreatment and microbial enzymatic activity. As SEM and AFM microscopy analysis showed earlier, UV radiation could change the structure of plastics, which can result in better penetration of microorganisms. Upon surface degradation and separation of polymeric structure, lower thermal stability and shift in degradation temperature would result.

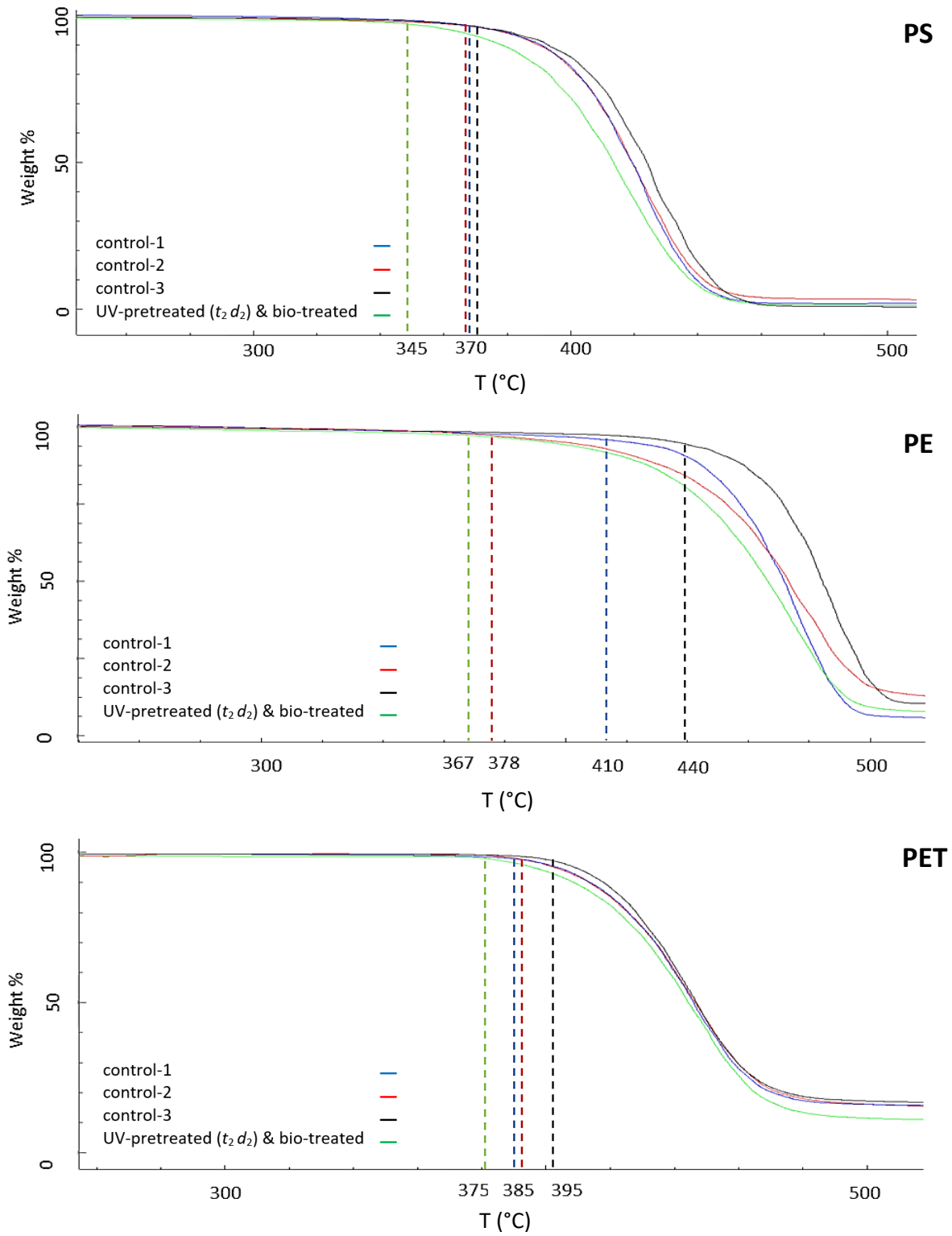

Fig 4.12. TGA analysis of "UV-pretreated (at t_2 d_2 condition) & bio-treated" plastic samples and comparison with control samples

4.4.9. Identification of By-products by GC-MS

The media of set-A and set-B flasks having "UV-pretreated (at t_2 d_2) & bio-treated", and "bio-treated" (control-1) plastic samples were removed after 45 days of biodegradation and subjected to GC-MS analysis individually. Analysis showed that each plastic set (set-A or set-B) had the same compounds in the media containing "UV-pretreated (at t_2 d_2) & bio-treated" and control-1 samples (Table 4.2) (Fig 4.13). The lack of compounds in control-2 suggests that the produced by-products were due to the microbial enzymatic activities and not just because of UV-pretreatment. Nevertheless, the percentage of most identified peaks were slightly higher in UV-pretreated (at t_2 d_2) & bio-treated, than bio-treated in both plastic sets, suggesting the higher efficiency of degradation after UV-pretreatment. Analysis between set-A and set-B showed the formation of more compounds in the media of set-B flask, implying that the smaller plastic size and a larger quantity resulted in higher biodegradation of plastic into shorter compounds (Table 4.2).

The higher percentage of peak area for the same compounds in set-A than set-B also was due to the lower degradation of plastics into shorter compounds in set-A. In other words, the accumulation of larger molecule compounds that did not degrade into shorter molecule compounds resulted in a higher percentage of peak area in set-A samples.

The identified compounds were mostly alkane (octane, decane, dodecane, nonane, tetradecane, heptadecane, hexadecane, icosane, hexacosane) which was possibly derived from PE biodegradation. The formation of 2,4-Dimethyl-1-heptene (alkene) was in parallel with FT-IR results showing the formation of C=C at 1650 and 1600 cm^{-1} (Fig 4.11b).

In both set-A and set-B the compounds "2,4-di-tert-butylphenol" and "benzene, 1,3-bis(1,1-dimethylethy)" were derived from the biodegradation of PS incubated in "UV-pretreated (at t_2 d_2) & bio-treated" and control-1 conditions. While the compound "2,4-di-tert-butylphenol" generally have been using as an antioxidant agent in the plastic industry, its identification in control-1 suggested the positive role of strains in PS degradation. The identified by-products were in agreement with other studies (Bortoluzzi et al. 2008, Bohrer 2012, Kyaw et al. 2012, Panda 2018, Medić et al. 2019, Chaudhary and Vijayakumar 2020, Tsochatzis et al. 2020). There were no compounds related to PET biodegradation, which was in parallel to the other obtained results of this study showing a very low biodegradation rate.

Table 4.2. Identified by-products derived from plastic biodegradation by GC-MS

Compounds	**Retention time (min)**	**Peak area (%)**				
		Set-A (UV-pretreated (t_2 d_2) & bio-treated)	Set-A (Control-1)	Set-A (Control-2)	Set-B (UV-pretreated (t_2 d_2) & bio-treated)	Set-B (Control-1)
Heptane, 2,4-dimethyl-	2.482	2.24	2.14	*ND*	0.97	0.88
2,4-Dimethyl-1-heptene	2.727	2.99	2.75	3.10	1.27	1.02
Decane	6.458	5.06	4.8	5.64	1.90	1.91
Decane, 4-methyl	6.804	*ND*	*ND*	*ND*	1.62	1.07
Nonane, 2,5-dimethyl-	6.974	*ND*	*ND*	*ND*	1.26	1.3
Octane, 6-ethyl-2-methyl	7.995	*ND*	*ND*	*ND*	0.97	0.34
Decane, 3,7-dimethyl-	8.842	1.98	1.8	2.06	1.48	1.15
Dodecane	10.669	14.75	13.6	14.76	5.58	5.29
Benzene, 1,3-bis(1,1-dimethylethy	11.567	10.27	10.14	*ND*	7.09	5.84
2,4-Dimethyldodecane	11.714	*ND*	*ND*	*ND*	1.25	0.88
Nonane, 5-butyl-	11.968	*ND*	*ND*	*ND*	1.16	0.76
Dodecane, 4-methyl	12.694	*ND*	*ND*	*ND*	2.33	1.07
Tetradecane	13.683	16.30	16.10	15.81	6.33	5.84
2,4-Di-tert-butylphenol	15.147	5.80	5.65	*ND*	3.96	2.70
Icosane	15.500	3.05	3.02	3.59	1.79	2.29
Hexadecane	16.195	11.98	12.20	12.05	3.37	3.78
Heneicosane	16.745	*ND*	*ND*	*ND*	1.87	0.78
Heptadecane	17.334	2.34	2.52	7.27	3.03	2.9
Hexacosane	19.705	*ND*	*ND*	*ND*	1.74	1.10
Octadecanal	20.636	4.16	4.16	4.30	3.28	2.81

ND: not detected

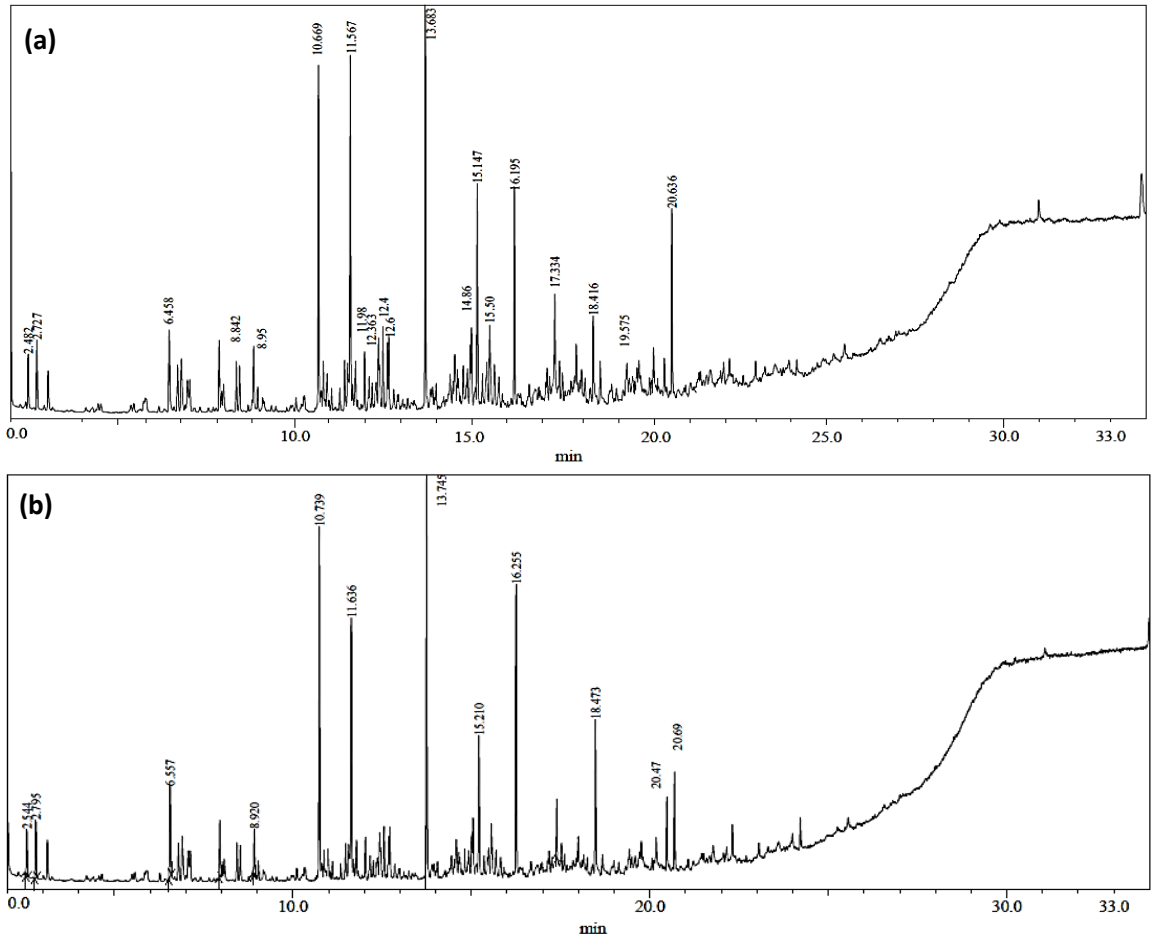

Fig 4.13. The identified compounds in the media contained UV-pretreated (at t_2 d_2) & bio-treated" and "bio-treated (control-1)" samples in set-A (a) and set-B (b) plastic size

4.4.10. Microbial Viability

The viability of strains was examined at the end of biodegradation (90 days) to understand the most promising pretreatment condition for microbial survival. Biodegradation of "UV-pretreated (at t_2 d_2 condition) & bio-treated", and "UV-pretreated (at t_1 d_2 condition) & bio-treated" with respective dead: live ratio of 0.33 and 4.13 were found the best conditions in terms of microbial survivability (Fig 4.14, Fig 4.15). The results also revealed that the viability of strains used in the biodegradation of t_1 d_1 and t_2 d_1 pretreated samples Therefore, UV-pretreatment at a shorter distance to UV light resulted in a higher plastic deterioration and, indeed, better microbial survivability.

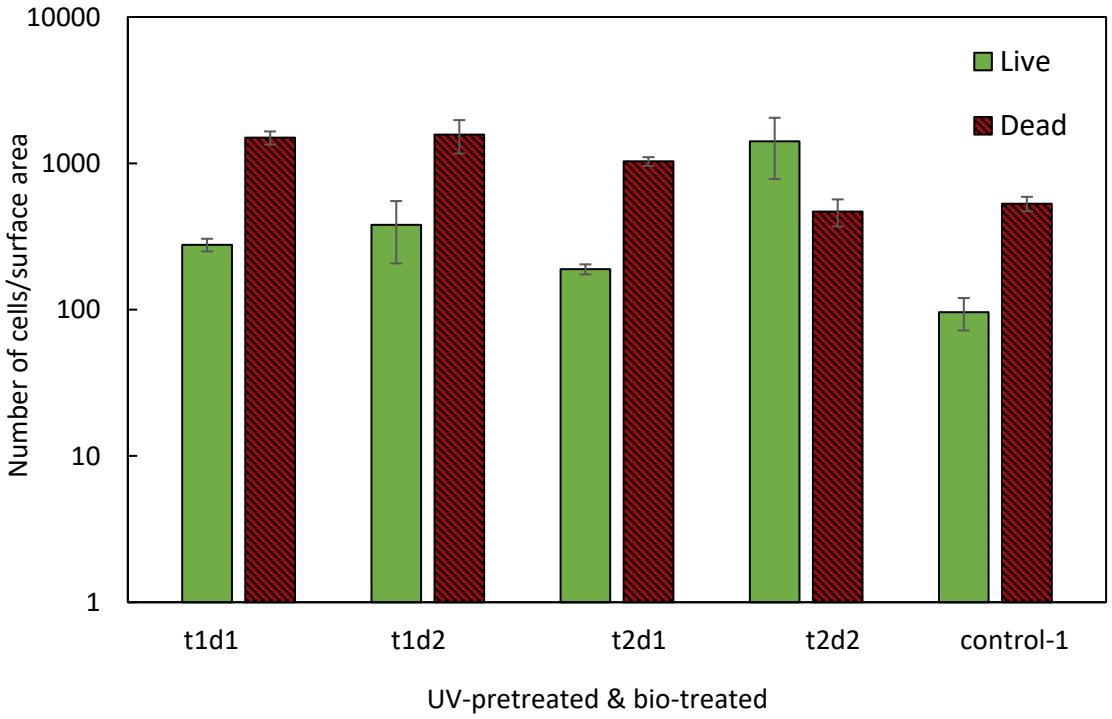

Fig 4.14. The number of microbial survivals in each condition

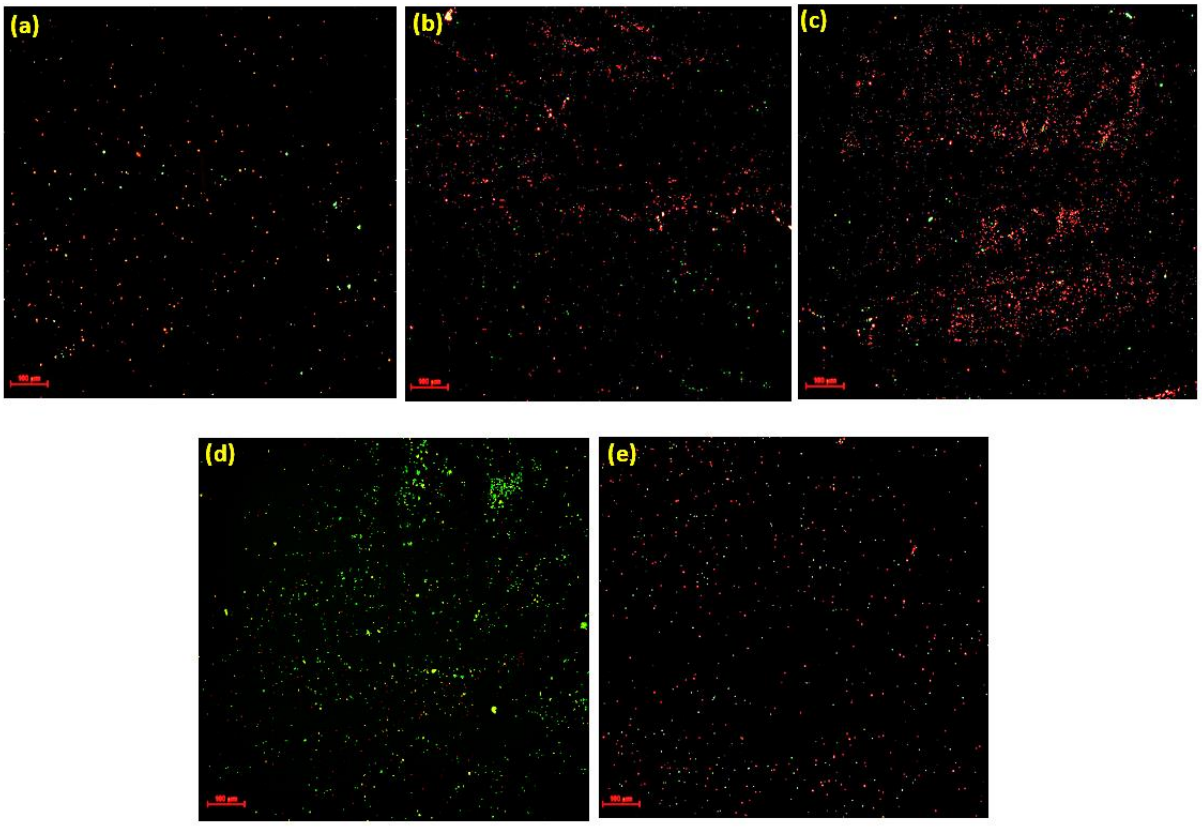

Fig 4.15. The microbial viability after 90 days of biodegradation with double fluorescent stain. (a-e) illustration of live and dead cells at $t_1 d_1$, $t_1 d_2$, $t_2 d_1$, $t_2 d_2$ and bio-treated samples (control-1)

4.5. Summary of Chapter and Conclusion

Among different UV-pretreatment conditions, It was found that a longer incubation time and shorter distance to UV source ($t_2 d_2$ condition) (UV dose of 7.02×10^{12} $\mu W.cm^{-2}.s$) resulted in a higher roughness, hydrophilicity, microbial viability, biofilm formation, surface degradation, and a more significant physical and molecular weight reduction. Moreover, higher microbial colonisation and viability were detected on pretreated plastic at $t_2 d_2$ condition. Comparison of two biodegradation periods (45 and 90 days) at the optimum condition ($t_2 d_2$) suggested no significant statistical difference in terms of biodegradation efficiency as weight loss, and 45 days of incubation

was enough to minimise the operational cost. Analysis of by-products by GC-MS also showed the formation of short-chain compounds mainly alkane as part of PE and PS biodegradation. While UV-pretreatment could affect the plastic structure, its utilisation alone was not sufficient for plastic degradation, and the presence of microbes was essential for further breakage of plastic.

Despite the observation of surface degradation with AFM and SEM in all three-plastic types, the highest biodegradation efficiency within 45 days of incubation at t_2 d_2 condition occurred on PE and PS with 7.8 and 5.13 %, respectively. It was also found that size and amount of used PS were essential to obtain more significant morphological changes. Indeed, the pretreated small-size PS (<10 mm) with a higher quantity (>5 g) indicated several fissures on their surface as a result of microbial consumption. Overall, it can be concluded that UV-pretreatment can improve the biodegradation efficiency of the selected microorganisms within the given time.

The following chapter studies the effect of biosurfactants in biodegradation enhancement of un-pretreated and UV-pretreated plastics.

Chapter 5

Enhancement in biodegradation of untreated and UV-pretreated non-degradable thermoplastic using rhamnolipid biosurfactant

Contents of this chapter have been published as follows:

Taghavi, N., W.-Q. Zhuang and S. Baroutian (2021). "Effect of rhamnolipid biosurfactant on biodegradation of untreated and UV-pretreated non-degradable thermoplastics: Part 2."Journal of Environmental Chemical Engineering 107033.

5.1. Chapter Preface

Surface-active compounds such as surfactants and biosurfactants with the amphiphilic structure are able to increase the accessibility of plastic to microorganisms by reducing the surface tension of media. Rhamnolipid is among the well-studied biosurfactants in the removal of organic pollutants. In this chapter, the capability of the selected strains from chapter-3 is studied in the presence and absence of rhamnolipid on both un-pretreated and UV-pretreated plastics. The results will demonstrate whether integration of rhamnolipid and UV-pretreatment can be a proper approach for biodegradation enhancement or not.

5.2. Introduction

As mentioned previously in chapter 2, the biodegradation of plastics is categorised into four main steps; colonisation, depolymerisation, assimilation and mineralisation. One of the ways to improve biodegradation efficiency is to increase microbial colonisation, the very first step of biodegradation. Upon colonisation and biofilm formation, cell to cell signalling and communication will occur, resulting in a more coordinated microbial tactic for biodegradation (Shrout et al. 2011). To increase microbial colonisation, surface-active compounds like biosurfactants can be added to the media. Biosurfactants are a class of surfactants that are produced by microorganisms and are environmentally friendly compared to synthetic surfactants. The amphiphilic structure of biosurfactant causes the attachment of hydrophobic compound (like plastic) to its non-polar part and the attachment of its polar part to water (Fig 5.1). Indeed, biosurfactants can increase microbial colonisation by increasing the substrate availability to microorganisms and cell permeability.

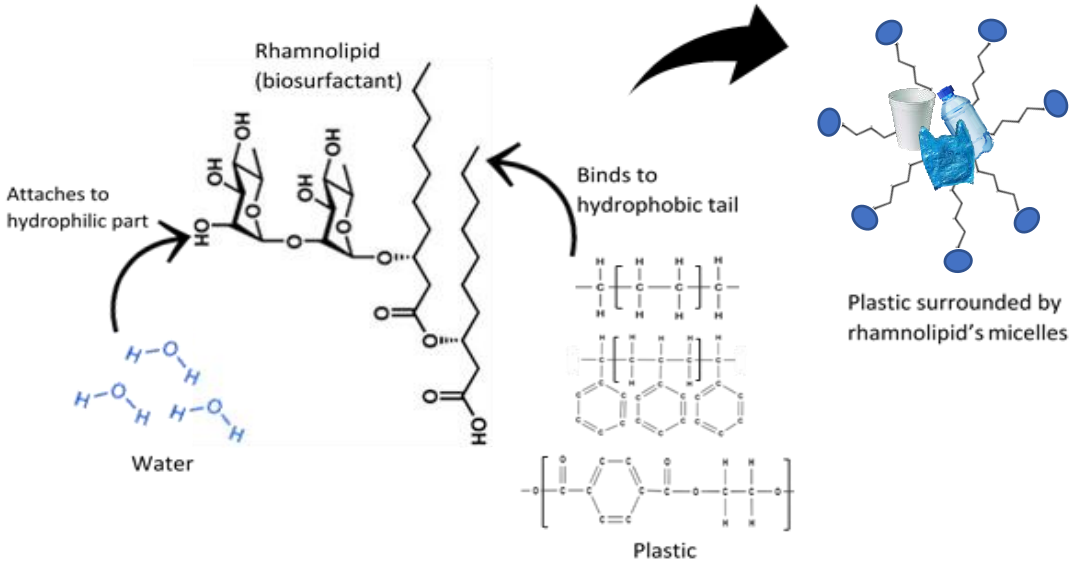

Fig 5.1. The structure of rhamnolipid (biosurfactant) and its attachment to plastic

Biosurfactants are divided into five main categories: glycolipids, phospholipids, lipopeptides, fatty acids, and polymeric compounds. Lipopeptides and glycolipid biosurfactants such as surfactin (from *Bacillus subtilis*) and rhamnolipid (from *Pseudomonas aeruginosa)* are two important examples in bioremediation and biodegradation applications. The above-mentioned biosurfactants can reduce the surface tension of water from 73 to 25-28 mNm^{-1} at a very low critical micelle concentration (CMC) in comparison to synthetic surfactant such as Triton X-100 (2000 mg/L), (Mohan 2007, Wei et al. 2007, Khoshdast et al. 2012). The utilisation of rhamnolipid and surfactin in the removal of environmental pollutants such as heavy metals, petroleum derivatives, phenols and polycyclic aromatic hydrocarbons are widely reported (Mulligan 2005, Massara et al. 2007, Liu et al. 2010, Liu et al. 2018). However, the application of biosurfactant for biodegradation of non-degradable thermoplastics is not well-studied, and among the previous studies, only the effect of surfactin was reported (Mukherjee et al. 2016, Vimala and Mathew 2016). In the study conducted by Vimala & Mathew (2016), the surfactin biosurfactant, which was obtained from B. subtilis was introduced into the biodegradation process to enhance the biodegradation efficiency of PE. The author reported that the pretreated PE with the thickness of 18µm in the presence of surfactin resulted in 9.26 % physical weight within 30 days of incubation. However, detailed and adequate analytical evaluations, as well as comparisons between samples and controls, were

lacking in the study. For example, no surface morphology was evaluated to understand the efficacy of surfactin in PE biodegradation.

In this chapter, the addition of exogenous rhamnolipid to mineral salt media was studied for the first time to understand its effect on microbial colonisation and biodegradation efficiency of both UV-pretreated and untreated non-degradable thermoplastics. The reason behind choosing rhamnolipid as the biosurfactant was due to the fact that in the preliminary results one of the strains *Pseudomonas* sp produced this biosurfactant in the media. However, the concentration of produced rhamnolipids was not enough to accelerate biodegradation. Rhamnolipids are a glycolipid-type biosurfactant with rhamnose head and β-hydroxyalkanoic acid tail. This structure of rhamnolipids helps to increase cell motility and biofilm formation (Davey et al. 2003, Chrzanowski et al. 2012, Nickzad and Déziel 2014). To investigate the biodegradation efficacy, different analytical techniques were used in detail to suggest the optimum condition for biodegradation of each plastic type.

5.3. Experimental

5.3.1. Materials

The chemicals used in this study were analytical grade and were purchased from Sigma-Aldrich (New Zealand). The chemicals in this experiment were KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, NH_4NO_3, NaCl, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, rhamnolipid (95 %), nutrient agar (NA), nutrient broth (NB), potato dextrose agar (PDA), potato dextrose broth (PDB) and agar powder.

Three types of plastic samples, HDPE (shopping bag), PS (foam), and PET (drinking bottle), were purchased locally and cut by a scissor to make 15 mm × 15 mm films. The PE, PS and PET plastic samples were pearl, white, and shiny-brown in colours and had thicknesses of 0.12 mm, 2 mm, and 0.24 mm, and original melt flow index (MFI) of 2, 6 and 17 g/10min, respectively. Each plastic sample was soaked in 75 % ethanol solution for one hour, rinsed with sterile Milli-Q water and oven-dried at 55 °C overnight (aseptic condition).

5.3.2. Media Preparation

The mineral salts media broth (MSM-B) was prepared according to ASTM G21-15 protocol (ASTM 2013) as 0.7 g of KH_2PO_4, 0.7 g of K_2HPO_4, 0.7 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of NH_4NO_3,

0.005 g of NaCl, 0.002 g of FeSO4·7H2O, 0.002 g of ZnSO4·7H2O, and 0.001 g of MnSO4·H2O per 1000 mL of water (pH 6.8). Other nutrient-rich media (NA, NB, PDA and PDB) were prepared according to Sigma-Aldrich's description. The optimum concentration of rhamnolipid was evaluated and then mixed well in MSM-B. All prepared solutions after that were autoclaved at 121 °C for 20 min before the experiment.

5.3.3. Critical Micelle Concentration of Biosurfactant

Understating the critical micelle concertation of biosurfactant is highly important. Above the critical point, the surface tension of water would not change any further (Lu et al. 1993), and an excess amount of the surfactants can become toxic to microbes (Efroymson and Alexander 1991, Stelmack et al. 1999). To avoid the mentioned issue, different concentration of rhamnolipid (0, 10, 20, 40, 80, 160, 320 mg/L) was mixed with MSM-B before biodegradation. Samples were then analysed individually to determine the critical micelle concentration (CMC) required for the lowest surface tension. The experiment was conducted in duplicate ± standard deviation.

5.3.4. UV-pretreatment

The pretreatment of plastic samples was conducted under the optimum condition, according to chapter 4. In brief, plastic samples (15 mm × 15 mm films) were placed in a closed metal chamber (length: 410 mm, width: 210 mm) equipped with a 35 W power source, thermometer, and a mercury (Hg) lamp to generate 245 nm wavelength. Plastics were placed at the bottom of the UV chamber with 12 cm from UV light and then irradiated at a dosage of 7.02×10^{12} $\mu W.cm^{-2}.s$ for a constant of 120 h on each side. Plastics were flipped over for irradiation of the other side at the same condition and UV dosage. It should be mentioned that changing the chemical structure of plastics requires a high energy beam. While UV-A and B do not have enough energy to penetrate non-degradable thermoplastic, UV-C, with its high energy beam, can penetrate plastics, excite more photons and generate free-radicals within the plastic structure. In this study, UV- C at 245 nm was used because this wavelength is almost at the middle of the UV-C spectrum (200- 280 nm). This wavelength is commonly used in most biological laboratories and also has been used previously for the pretreatment of plastics (Montazer et al. 2018).

After completing the UV-pretreatment, plastics were removed to be analysed for weight loss, hydrophobicity changes and chemical transformation. All experiments were conducted in triplicates, and the results were expressed as mean ± standard deviation.

5.3.5. Inoculums

In this study, *Penicillium raperi*, *Aspergillus flavus*, *Penicillium glaucoroseum* and *Pseudomonas* spp were used which were isolated and identified previously in chapter 3. Bacteria were cultivated in the nutrient broth (NB) for 48 h, and fungi were grown in the potato dextrose broth (PDB) for 72 h prior to inoculation. After the cultivation, each strain was centrifuged at 5000 × g (5 min) and the pellet of each strain was suspended individually in the MSM-B (2 h) for media adaptation. The content was centrifuged again at the same condition, and the obtained pellet of each strain was mixed to form a consortium of the four strains.

5.3.6. Biodegradation

The biodegradation test was initiated after adding the made consortium of the four strains (5 mL) into each 250 mL flask that contains four pieces of previously disinfected 15 x 15 mm plastic from each type (12 pieces in total per 250 ml flask), and a mixture of biosurfactant solution in MSM-B. All flasks were incubated at 32 °C, 90 rpm for 45 days. To maintain the media strength, sterile MSM-B without biosurfactant was added proportionally. To understand the biodegradation efficiency, different experiments were designed (Table 5.1). All experiments were conducted in duplicate, and the results were expressed as mean ± standard deviation.

Table 5.1. The experimental conditions of plastic biodegradation

Condition	Strain	UV-pretreatment (120 h, 12 cm)	Biosurfactant	Plastic type
UV-pretreated & bio-treated + biosurfactant	+	+	+	PS, PE, PET
Bio-treated + biosurfactant	+	-	+	PS, PE, PET
UV-pretreated & strain-free bio-treated + biosurfactant	-	+	+	PS, PE, PET
Un-pretreated & strain-free bio-treated + biosurfactant	-	-	+	PS, PE, PET
UV-pretreated & bio-treated	+	+	-	PS, PE, PET
Bio-treated	+	-	-	PS, PE, PET
UV-pretreated & strain-free bio-treated	-	+	-	PS, PE, PET
Un-pretreated & strain-free bio-treated (control)	-	-	-	PS, PE, PET

\+ included
\- excluded

5.3.7. Analytical Methods

5.3.7.1. pH Variation

Monitoring pH is one of the common methods to understand the metabolic activity of strains. Therefore, the pH of media in each flask was monitored before biodegradation and during the experiment (every five days) with a pH meter (Hanna, edge). Before analysis, the pH electrode was disinfected under UV radiation (245 nm) in a laminar hood (15 min), followed by several rinsing the pH electrode with 75 % ethanol and sterilised distilled water. The pH of strain-free samples was measured initially in each interval to reduce the possible chance of cross-contaminations between flasks. On day 20, fresh MSM media was added once to the flasks to keep the level of media as same as the beginning of the experiment.

5.3.7.2. Biofilm Estimation

The formation of biofilm on the surface of each plastic was assessed by crystal violet (CV) assay according to Eich et al. (2015). Crystal violet can stain extracellular matrix (ECM) on the formed biofilm and indirectly indicate the microorganisms' colonisation on the surface of plastic (Chavez-Dozal et al. 2016). In brief, plastic films were washed gently with sterilised Milli-Q water to remove unattached planktonic (free) cells. Samples then were immersed into 1 mL (1% w/v) CV solution for 15 min. After several gentle washing with distilled water, the excess stain was removed, followed by immersion for 5 min into ethanol solution (95% v/v) to extract the stain. There were no Gram-positive bacteria used in this study, so almost all CV could be extracted from stained cells. The absorbance of extracted aliquot after the extraction step was measured at 595 nm in the microplate reader (PerkinElmer, MLD2300, USA).

5.3.7.3. Wettability

Hydrophobicity is one of the important factors in biodegradation. The higher hydrophilicity resulted in a higher chance of microbial colonisation and subsequent biodegradation. Since biosurfactant was used in this study, it was expected that the hydrophilicity of plastics increase compared to samples without biosurfactant. To understand the wettability, each of the disinfected plastic samples (as previously explained) was individually placed on the apparatus holder, and a droplet of fresh distilled water (4 μL) was added to the flat surface of the plastic sample. The angle between water and each plastic film was calculated with the Young-Laplace equation.

5.3.7.4. Physical Weight loss

Weight loss was used as an indication of biodegradation. Samples were measured three times before the experiment, after UV radiation, before biodegradation, and at the end of biodegradation by a 5-digit analytical balance (Precisa, EP 120A) weight loss calculation.

After the biodegradation test and biofilm measurement, plastic samples were cleaned and disinfected by rinsing with sterilised distilled water and immersed in 75% (v/v) ethanol for 15 min, and sodium dodecyl sulphate (SDS) solution (2% w/v) for 24 h. Plastic samples were then rinsed multiply times gently with sterilised distilled water and oven drying at 55 °C for 24 h.

5.3.7.5. Chemical Transformation

To understand the possible changes within the chemical bonds of polymer, each clean and disinfected plastic sample (as explained previously) was examined by Fourier-transform infrared spectroscopy (FT-IR, PerkinElmer, USA) before the experiment, after UV-pretreatment, and at the end of the biodegradation period. Samples were scanned at both sides at a resolution of 4 cm-1 and a wavenumber of 4000 to 400 cm-1. To ensure the intensity of peaks was not because of the variation in gage force, each plastic type was analysed with a fixed apparatus gage force as PS: 30, PE: 50 and PET:80.

5.3.7.6. Thermogravimetric (TGA) Analysis

The thermal behaviour and stability of each cleaned and disinfected plastic sample (as explained previously) was examined by a thermogravimetric analyser (TGA-50 Shimadzu), and thermal stability was determined by T onset. Analysis was performed under argon atmosphere at a heating rate of 10 °C/min from 25 °C to 590 °C. TGA analyses were conducted by using eight replications for each plastic type to validate the results statistically.

5.3.7.7. Microscopy

At the end of the biodegradation period (45 days), the plastic samples having more significant signs of biodegradation were examined by atomic force (AFM, Cypher-ES, USA) and scanning electron microscope (SEM, Quanta 200f, USA). The AFM surface topography was carried out by mounting each plastic sample on the metal specimen discs Ø10 mm. Samples then were scanned at 4 × 4 μm. In SEM analysis, samples were sputter-coated with platinum (Quorum, Q150RS) for 5 min at 20 mA, and were scanned under low vacuum at 10 kV.

5.3.7.8. Statistical analysis

The biofilm estimation, FT-IR and TGA analyses were conducted using eight replications for each plastic type to validate the results statistically. For the wettability analysis, the examination was also performed using eight replicates in addition to the examination of each plastic piece at two different locations. Physical weight loss was also performed in eight replicates plus reading of

each plastic piece's weight three times. The results were expressed as mean ± standard deviation.

5.4. Results and Discussion

5.4.1. Critical Micelle Concentration

Among the different concentrations of rhamnolipid (biosurfactant) used in this study, it was found that 160 mg/L was the optimum concentration for a maximum surface tension reduction of water from 73 to 29.9 mN/m (Fig 5.2). Therefore, this concertation was used in the biodegradation experiment.

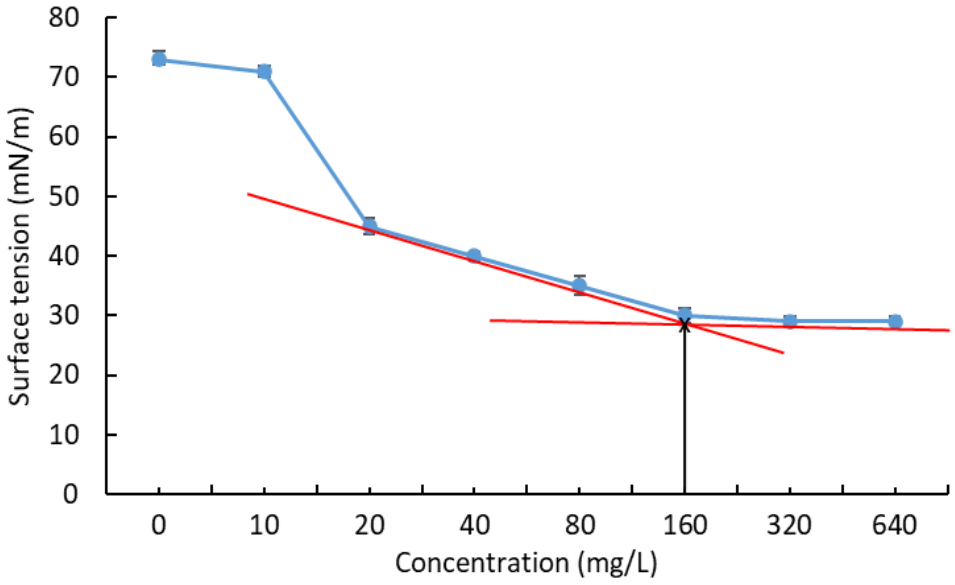

Fig 5.2. Determination of critical micelle concentration (CMC)

5.4.2. Change in pH

Variation of pH could be used as an indication of whether microorganisms still metabolically activate during the biodegradation or not. In this study, pH was monitored at different biodegradation conditions. Based on the results, it was found that UV-pretreated samples could be

taken more as a food source by microorganisms compared to untreated plastic samples. The lowest pH was seen at day 20 which corresponded to (i) "UV-pretreated & bio-treated" treated plastics (pH= 6.44) followed by (ii) "UV-pretreated & bio-treated + biosurfactant" (pH= 6.51) (Fig 5.3). The increase in pH after day 20 was due to the addition of fresh MSM-B (pH= 6.8) to keep the level of media as same as the beginning of the experiment.

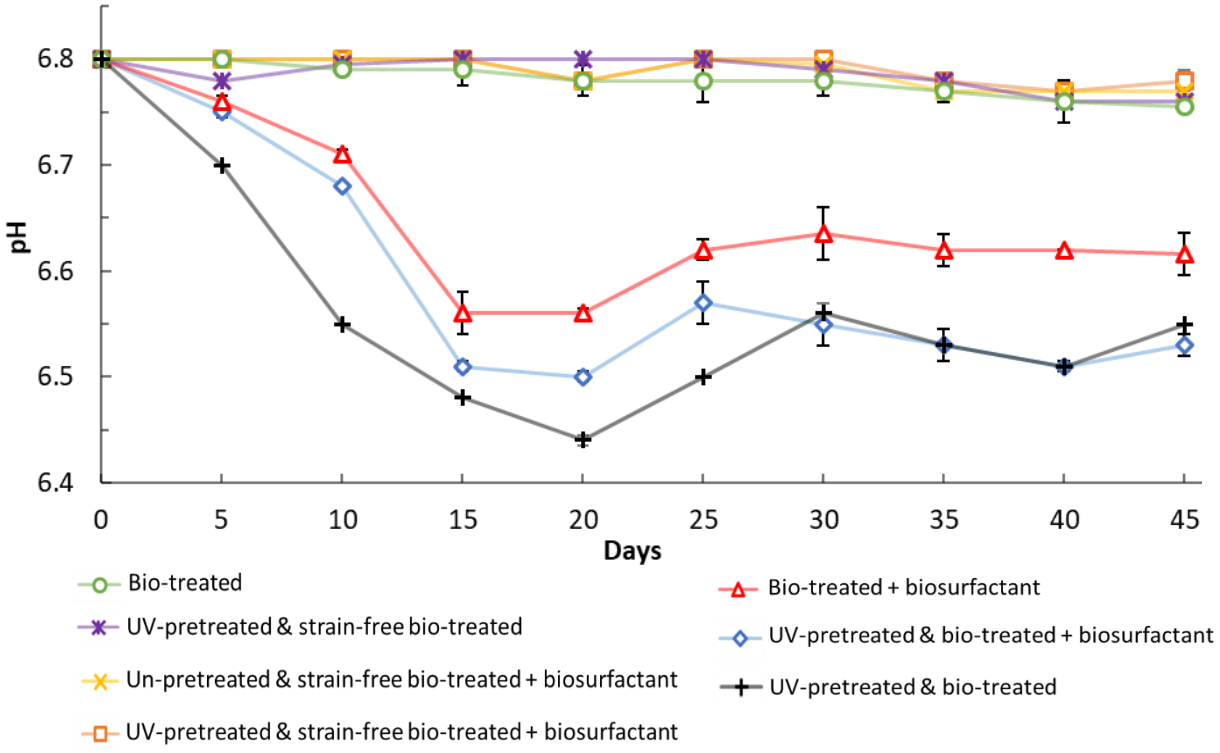

Fig 5.3. The variation of pH after 45 days of biodegradation at different conditions

Despite the lower value of pH in (i) "UV-pretreated & bio-treated + biosurfactant" and (ii) "Bio-treated + biosurfactant", the pH trend was almost similar in both conditions. The sudden drop in pH by day 20 in (i) "Bio-treated + biosurfactant", and (ii) "UV-pretreated & bio-treated + biosurfactant" was probably due to the consumption of biosurfactant as a food source rather than plastics. Once strains consumed biosurfactants, the pH did not change noticeably afterwards, and the trend remained almost constant for the last ten days (Fig 5.3). In contrast, in samples with no biosurfactant (UV-pretreated & bio-treated), the drop in pH by day 20 was due to plastic

consumption as the only available carbon source. The difference in pH value of (i) "Bio-treated + biosurfactant", and (ii) "UV-pretreated & bio-treated + biosurfactant" after day 20 was also due to easier consumption of UV-pretreated plastic as a food source compared to untreated plastics.
It was believed that in the presence of two different carbon sources in media, strains would take the carbon source with easier digestion; this metabolic behaviour is known as diauxie (Görke and Stülke 2008, Hermsen et al. 2015, Wang et al. 2019). In this case, rhamnolipid (biosurfactant) was taken first by strains as an easy food source for the first 20 days. To consume plastic as the second carbon source, especially in "Bio-treated + biosurfactant", possibly a longer biodegradation period (more than 45 days) might be needed. The longer biodegradation period can give a higher chance to strains to secrete their enzymes for plastic degradation and its consumption as a food source.

5.4.3. Biofilm

Analysis of plastic after biodegradation showed that a combination of biosurfactant and UV radiation (UV-pretreated & bio-treated + biosurfactant) enhanced microbial colonisation for PS. In contrast, the biofilm formation on PE and PET at this condition remained almost as same as "Bio-treated + biosurfactant" (Fig 5.4). The higher biofilm formation in PS could be due to its structure. The PS foam is made from several beads that each bead joints tightly next to each other. The UV radiation of PS changed its structure and made it more porous than un-treated samples.

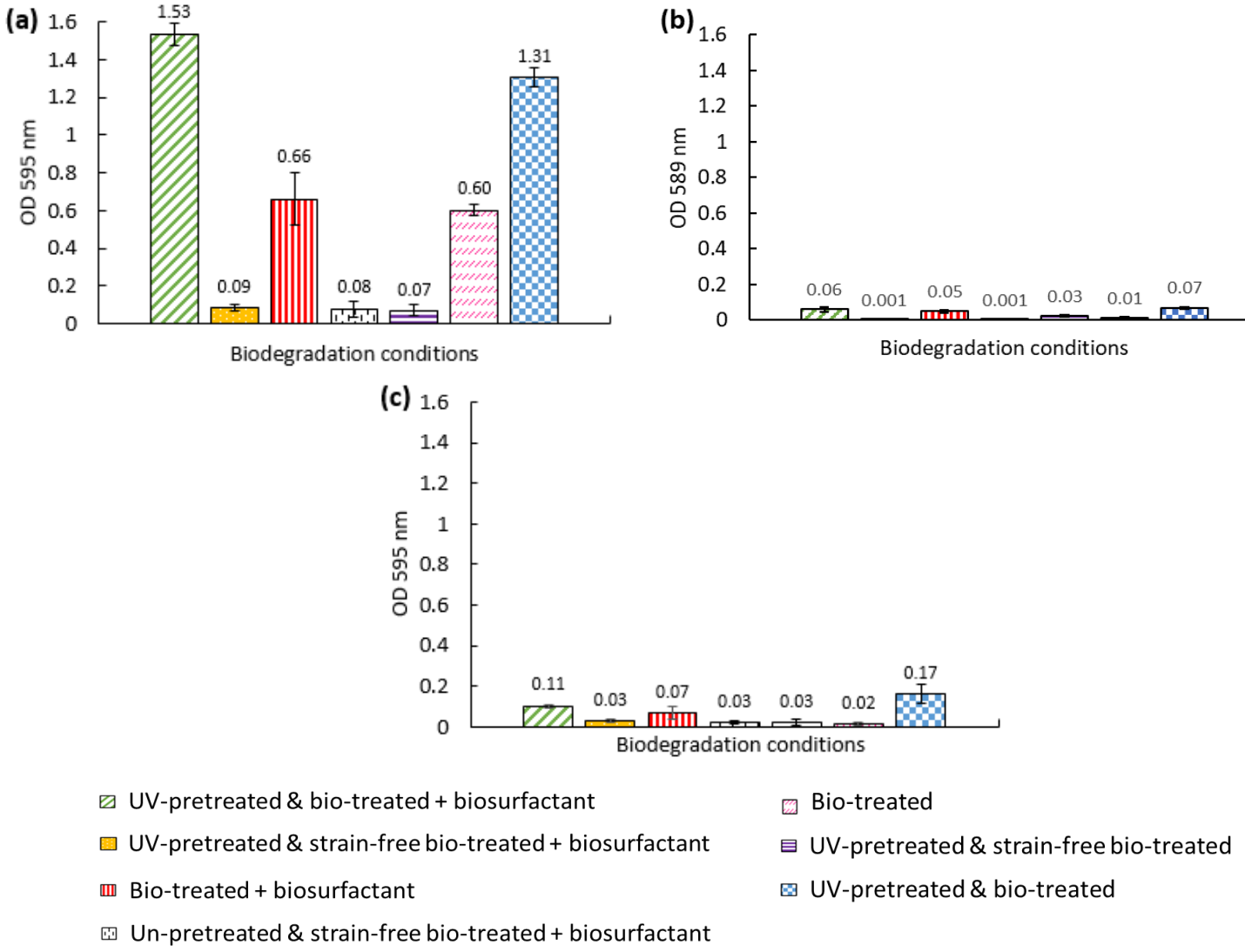

Fig 5.4. The amount of formed biofilm in PS (a), PE (b) and PET (c) in different biodegradation conditions

Previous studies have shown that biosurfactants can influence cells' hydrophobicity and facilitate microbial colonisation on different surfaces or compounds. (Czaczyk et al. 2008, Kaczorek et al. 2018, Subbiahdoss and Reimhult 2020, Sharma et al. 2021). Czaczyk et al. (2008) found that the biosynthesis of lipopeptide biosurfactant reduced *Bacillus* spp cell's hydrophobicity to increase its attachment on stainless still. In this study, UV-pretreatment of plastics resulted in a more hydrophilic structure, which then the utilisation of biosurfactant assists microorganisms in changing their cell hydrophobicity for a higher attachment to plastics samples.

Among the used plastic types, it was expected to see even a higher biofilm formation in PS due to PS porous structure. To consume the adsorbed biosurfactant within PS porous beads, strains needed to penetrate and colonise inside PS, resulting in a higher biofilm formation. This is how less biofilm formation was observed in "Bio-treated + biosurfactant" and "Bio-treated" samples where UV-pretreatment was not conducted (PS was not porous) (Fig 5.4a). Results suggest that un-treated PS with unchanged structure could not facilitate microbial colonisation even in the presence of biosurfactant compared to samples that underwent UV radiation.

Comparing the un-treated PS, PE and PET samples in "Bio-treated + biosurfactant" and "Bio-treated" conditions showed the effectiveness of biosurfactant in microbial colonisation. However, the amount of biofilm was lower than "UV-pretreated & bio-treated + biosurfactant" and "UV-pretreated & bio-treated" conditions, which suggests the higher efficiency of UV-pretreatment than using biosurfactant (Fig 5.4b, c). Moreover, analysis between "UV-pretreated & bio-treated + biosurfactant" and "UV-pretreated & bio-treated" also showed that UV had a better effect in microbial colonisation of PE and PET than conditions where biosurfactant presented. The lower microbial colonisation on PE and PET was due to their flat surface structure than PS with the porous structure.

5.4.4. Wettability

It is generally believed that hydrophobic surfaces result in a higher microbial attachment. However, microbial attachment depends on several physicochemical and molecular parameters e.g. roughness and presence of functional groups, microbial cells hydrophobicity, surface charge, and spreading of extracellular polymeric substances on hydrophilic surfaces. In the study conducted by Kuyukina et al. (2016) it was found that different bacteria had different affinities to the polystyrene surface.

Bhagwat et al. (2021) reported that plastic with higher hydrophilicity such as polylactic acid had a higher chance of microbial colonisation due to a higher chance of biomolecules accumulation from the surrounding environment. In another study, Sudhakar et al. (2008) reported microbial colonisation and surface degradation once pretreated polyethylene became more hydrophilic. Therefore, higher hydrophilicity can also result in better plastic accessibility and a higher biodegradation efficiency (Suresh et al. 2011). Based on the results obtained in biofilm formation, it was expected to see higher wettability for PS than PE and PET. According to the results, the highest wettability occurred for PS samples in "UV-pretreated & bio-treated + biosurfactant", and "UV-pretreated & bio-treated" conditions**.** It was found that the integration of biosurfactant and UV-pretreatment was only effective in hydrophobicity was reduction of PS. The contact angle of PS in both conditions reduced to less than 5 degrees (< 5°) (Fig 5.5a).

Due to the very low contact angle of PS in both mentioned conditions, it was not feasible to determine which condition had the most significant effect on the PS wettability. However, by comparing "UV-pretreated & bio-treated + biosurfactant", "Bio-treated + biosurfactant", "UV-pretreated & bio-treated", and "Bio-treated" conditions it was concluded that the reduction of PS wettability was mainly due to the effect of UV and strains rather than biosurfactant.

Analysis of PE and PET samples showed that the highest wettability occurred in "UV-pretreated & bio-treated" condition, and biosurfactant did not play a key role in this process (Fig 5.5b-d). The reason why a lower wettability of PE and PET was seen in "UV-pretreated & bio-treated + biosurfactant" despite the use of UV-pretreated samples could be explained due to the consumption of biosurfactant as a food source rather than plastic. Moreover, the lower contact angle of "Bio-treated" compare to "Bio-treated + biosurfactant" suggesting the negative effect of biosurfactant in biodegradation and wettability of PE and PET plastic. It should be mentioned that the higher wettability of PS compared to PE and PET in "UV-pretreated & bio-treated + biosurfactant" condition was due to the morphological structure of PS as explained previously.

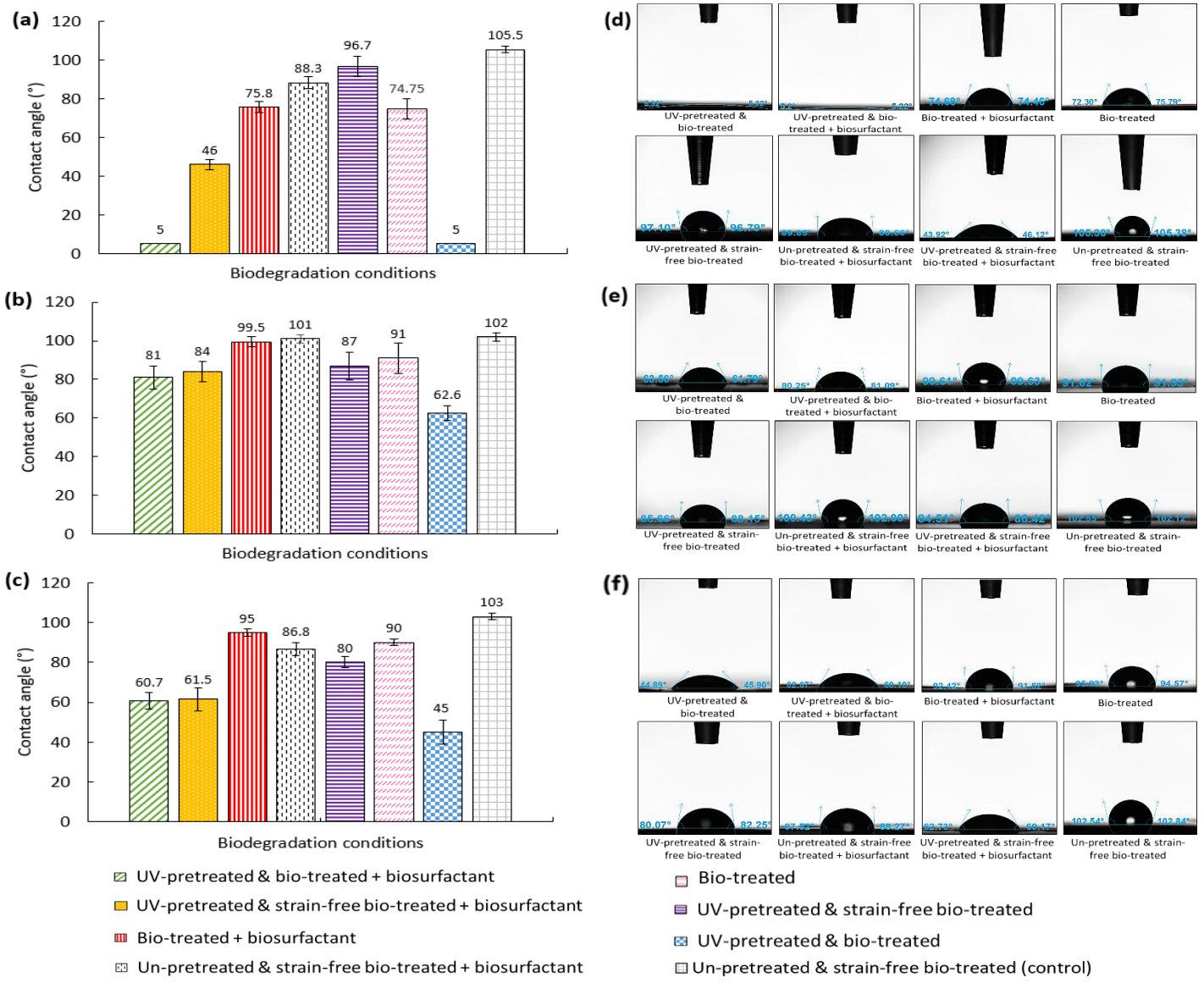

Fig 5.5. Wettability of PS (a, d), PE (b, e) and PET (c, f) after different biodegradation conditions. A lower contact angle represents a higher hydrophilicity (wettability)

5.4.5. Weight loss

Biodegradation efficiency was determined based on the physical weight loss of the plastic samples. The results showed that the combination of biosurfactant and UV-pretreatment in "UV-pretreated & bio-treated + biosurfactant" enhanced PS weight loss up to 7.5 % compared to "UV-pretreated & bio-treated" with just 5.1 % (Fig 5.6a). It was believed that the higher weight loss of PS in "UV-pretreated & bio-treated + biosurfactant" than "UV-pretreated & bio-treated" correlated to the higher hydrophilicity and biofilm formation observed previously. As mentioned previously, the penetration and settlement of biosurfactant through pretreated PS with porous morphology increased microbial colonisation, affecting hydrophilicity and biodegradation by consuming PS as the second food source.

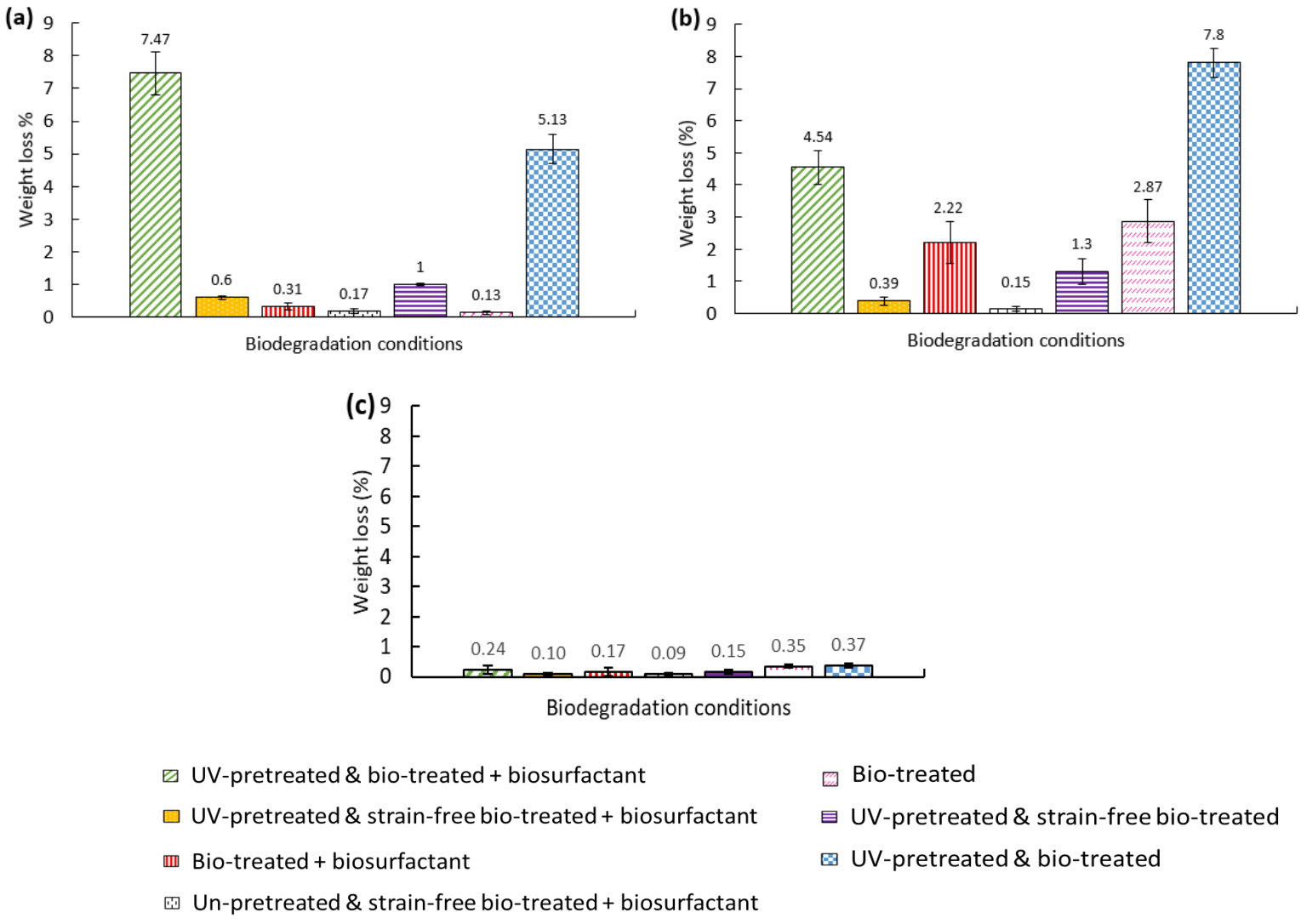

Fig 5.6. Weight loss of PS (a); PE (b), and PET (c) after 45 days of biodegradation in different conditions

In contrast to PS, there was no increase in weight loss of PE and PET samples incubated in "UV-pretreated & bio-treated + biosurfactant" compared to "UV-pretreated & bio-treated" where biosurfactant was not used. The maximum weight loss of PE occurred in "UV-pretreated & bio-treated" with 7.8 % followed by "UV-pretreated & bio-treated + biosurfactant" with 4.5 % (Fig 5. 6b). Comparison of PE samples incubated in "UV-pretreated & bio-treated + biosurfactant" and "Bio-treated + biosurfactant" suggested that the higher weight loss of PE in "UV-pretreated & bio-treated + biosurfactant" was because of UV effect and not biosurfactant. Among all plastic types, PET samples had the lowest weight reduction (<1 %) regardless of biodegradation conditions. Based on the results, it was concluded that the lower weight reduction of PE and PET in "UV-pretreated & bio-treated + biosurfactant" was due to be the consumption of biosurfactant as a food source instead of plastic, which is in agreement with the other results obtained in this study.

5.4.6. Chemical Transformation

Analysis of plastic samples showed that the intensity of major peaks in PS, PE and PET subjected to UV-pretreatment reduced about 30, 20 and 10 % respectively compared to un-pretreated samples (Fig 5.7). The reduction of intensity suggests a lower presence of that compound within the plastic structure and vice versa (Chaudhary and Vijayakumar 2020). Results showed that the chemical transformation's pattern of PS samples was almost identical in all conditions, except the formation of a new peak at 1250 cm^{-1} in "UV-pretreated & bio-treated" and "UV-pretreated & bio-treated + biosurfactant" samples. Since this peak was not observed in "UV-pretreated & strain-free bio-treated", and "Un-pretreated & strain-free bio-treated + biosurfactant" samples, it was suggested that the formation of C-O was due to the effect of microbes and not UV or biosurfactant.

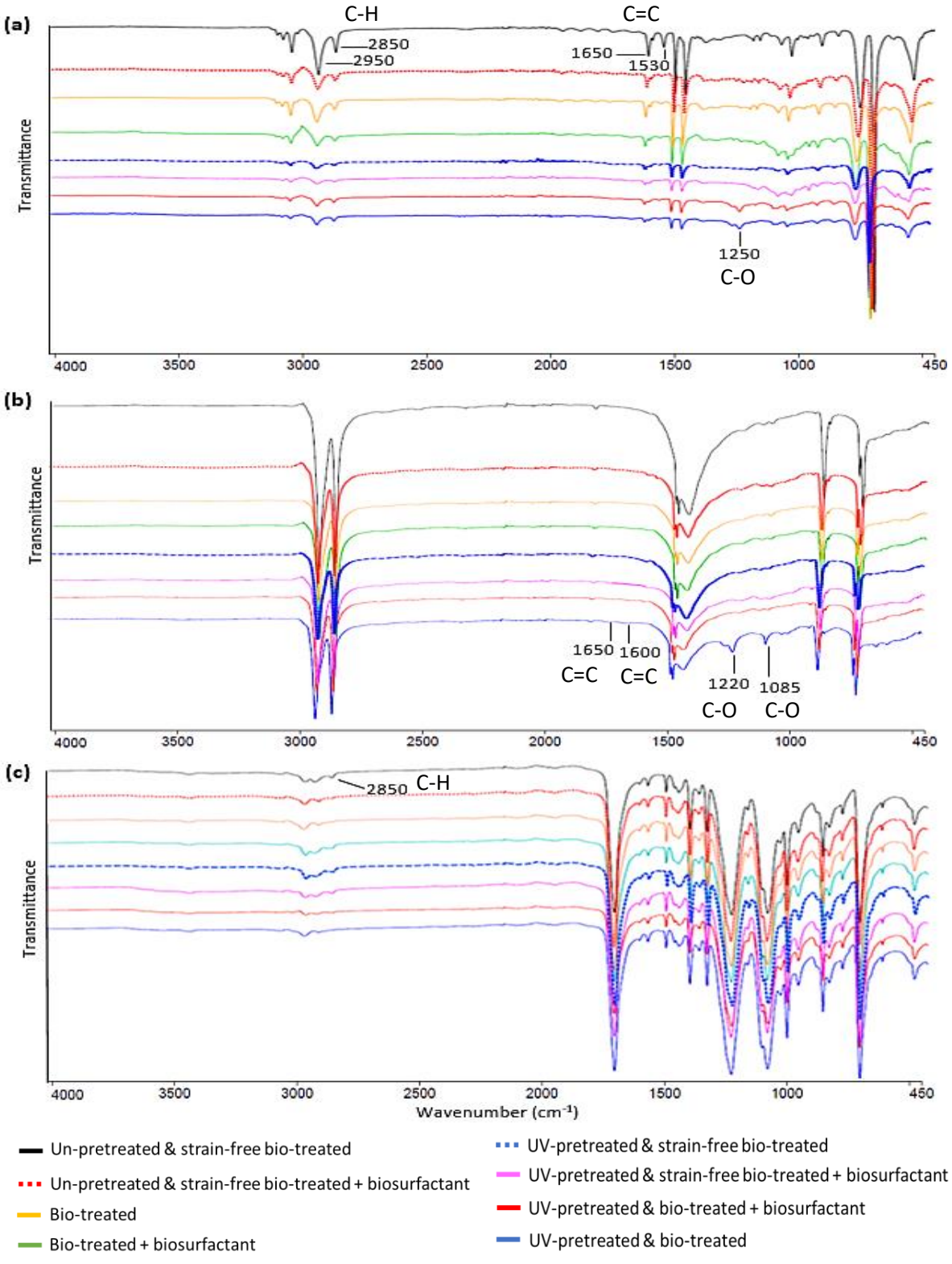

Fig 5.7. The chemical transformation of PS (a), PE (b), and PET (c) samples ad different treatment conditions

Besides the formation of the new peak, the intensity of signature peaks of PS (3050-2850 cm^{-1}, and 1650-1480 cm^{-1}) reduced noticeably in samples where UV radiation was used (Fig 5.7a).

Analysis of PE indicated the formation of new peaks attributed to C=C (1650 and 1600 cm^{-1}) and C-O (1220 and 1085 cm^{-1}) in "UV-pretreated & bio-treated" samples (Fig 5.7b). These peaks were not seen in other conditions. The lack of the mentioned peaks in "UV-pretreated & bio-treated + biosurfactant" suggested the negative effect of biosurfactant in biodegradation efficacy of PE, which was in parallel to the other results of this study.

There was no removal or addition of peaks in any conditions for PET samples. However, the intensity of the C-H bond (2850 cm^{-1}) was reduced significantly in samples subjected to UV-pretreatment (Fig 5.7c). The similarity between the FT-IR patterns in all conditions could explain the low biodegradation rate of PET samples.

5.4.7. TGA

Thermogravimetric analysis of all three plastic types indicated one-step thermal degradation regardless of biodegradation conditions (Fig 5.8). Results showed a reduction in thermal stability of PS incubated in "UV-pretreated & bio-treated + biosurfactant" in comparison to "UV-pretreated & bio-treated" condition. At "UV-pretreated & bio-treated + biosurfactant" condition, PS samples started to thermally degrade at 320 ˚C, which was about 20 ˚C and 65 ˚C lower than "UV-pretreated & bio-treated" and "Un-pretreated & strain-free bio-treated" (control), respectively (Fig 5.8a).

Comparatively, in another study conducted by Monsores et al. (2021), the decomposition temperature onset of pretreated (399.6 ˚C) and un-pretreated PS (392.9 ˚C) was not significantly different. This could be due to the utilisation of a lower energy UV beam (UV-B) compared to the higher energy beam (UV-C) used in this study.

In contrast to PS, the efficiency of "UV-pretreated & bio-treated + biosurfactant" condition for changing the thermal stability of PE and PET was almost the same as "UV-pretreated & bio-treated" and "UV-pretreated & strain-free bio-treated" conditions. At all of the mentioned conditions, PE started to degrade at about 365 ˚C and PET at 375 ˚C (Fig 5.8b, c). The comparison between "UV-pretreated & strain-free bio-treated", "UV-pretreated & bio-treated", "Bio-treated + biosurfactant", "Bio-treated" and "UV-pretreated & bio-treated + biosurfactant" suggested that the reduction in thermals stability was due to the mutual effect of UV radiation and microorganisms rather than biosurfactant (Fig 5.8b).

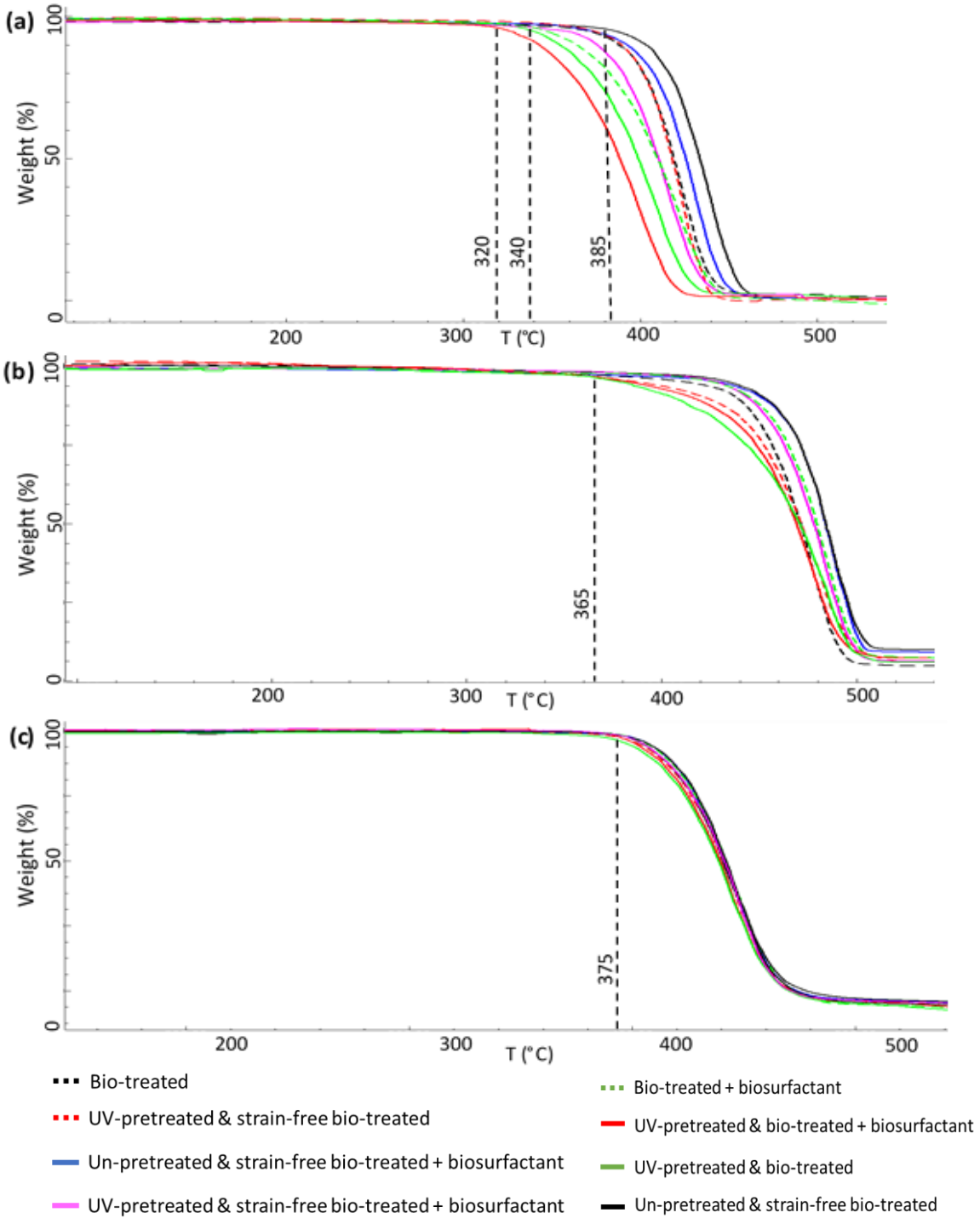

Fig 5.8. TGA analysis of PS (a), PE (b) and PET (c) after 45 days of biodegradation in different conditions

5.4.8. Microscopy

Analysis of PS by AFM showed that pretreated samples had more severe surface degradation in "UV-pretreated & bio-treated" and "UV-pretreated & bio-treated + biosurfactant" conditions. The AFM topography showed that the maximum distance from the crest to the trough of both "UV-pretreated & bio-treated" and "UV-pretreated & bio-treated + biosurfactant" was about 0.38 μm (Fig 5.9a, b), which was almost 1.6 and 2 times higher than "Bio-treated + biosurfactant" and "Un-pretreated & strain-free bio-treated" (control) samples, respectively (Fig 5.9c, d).

Examination of PS samples by SEM also showed significant surface deformation in both "UV-pretreated & bio-treated" and "UV-pretreated & bio-treated + biosurfactant" conditions (Fig 5.9e-h). There was no similar surface deformation in any other conditions where UV was not used (Fig 5.9i-l), suggesting that the deformation and the porous structure of PS were due to UV radiation and not the presence of biosurfactant. Similar results were also obtained where pretreated PS films were exposed to UV-C radiation. Monsores et al. (2021) also reported significant changes in properties of PS after irradiation with UV-B for a maximum of 575 h.

Analysis of un-pretreated PS at "Bio-treated + biosurfactant" condition showed a grade of surface degradation at high magnification (Fig 5.9) suggesting the degradation ability of microbes even in presence of biosurfactant.

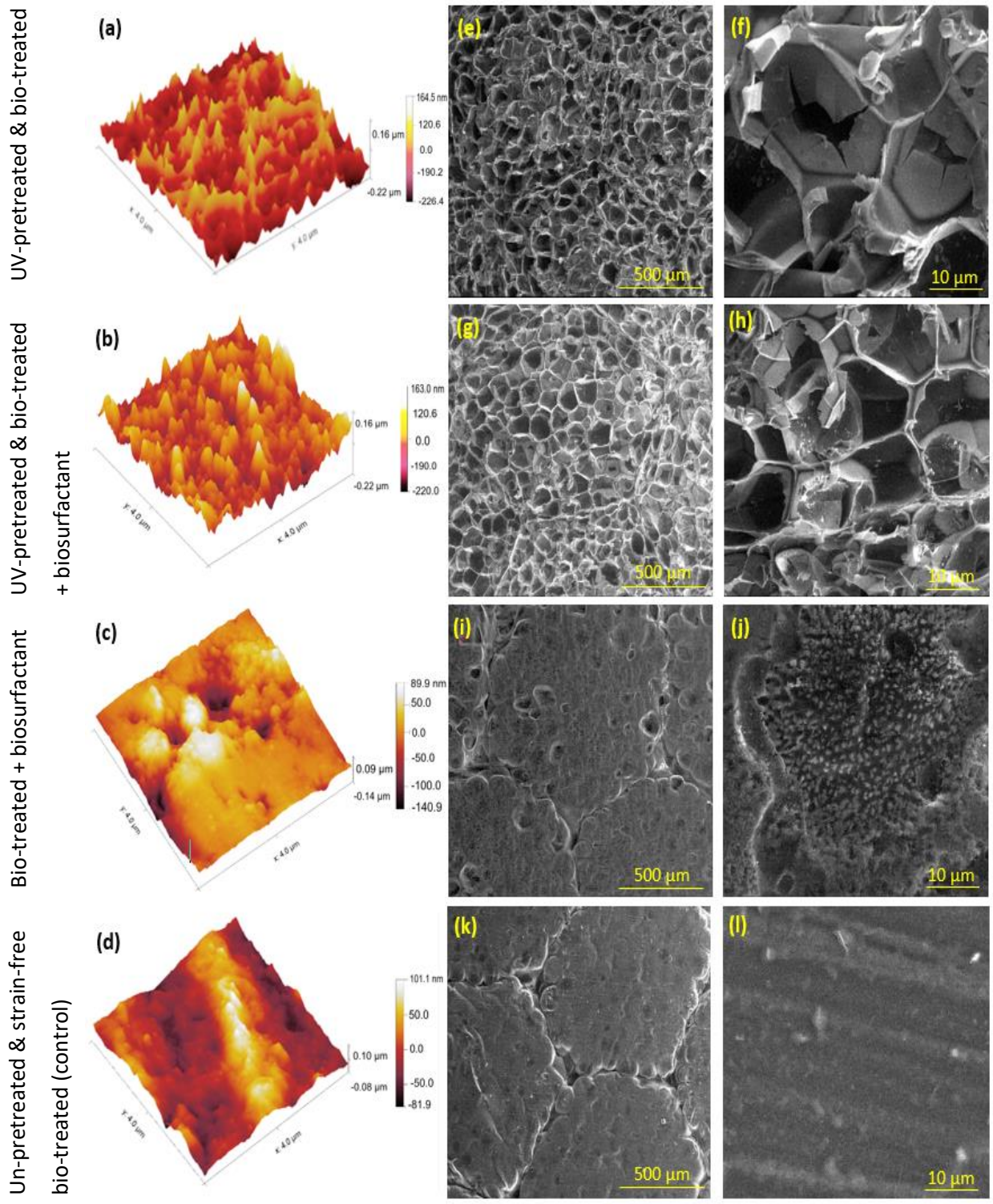

Fig 5.9. Surface morphology of PS after 45 days of biodegradation at different conditions by AFM (a-d) and SEM (e-l)

The SEM analysis also showed more attachment (biofilm) of strains within the formed porous structure of PS (Fig 5.9h). The observation was in agreement with the biofilm estimation result of this study. The use of biosurfactants could enhance microbial colonisation within PS plastic, especially if PS became porous after UV-pretreatment.

Surface topography of PE samples by AFM and SEM examination showed that higher surface degradation occurred in pretreated samples in the absence of biosurfactant (UV-pretreated & bio-treated), followed by PE in "UV-pretreated & bio-treated + biosurfactant" conditions. The AFM analysis showed that the maximum distance from the crest to the trough in "UV-pretreated & bio-treated" and "UV-pretreated & bio-treated + biosurfactant" was 0.31 and 0.27 μm, respectively (Fig 5.10a, b). The surface of both "Bio-treated + biosurfactant" and "Un-pretreated & strain-free bio-treated" (control) samples remained smooth and the highest distance from the deepest cavity to the peak was almost the same (0.21 μm) (Fig 5.10c-d).

Analysis of PE samples at "UV-pretreated & bio-treated" condition by SEM indicated the formation of major holes and fibrous-like structure due to PE biodegradation. These surface morphological changes were not seen in any other conditions. This confirms that the presence of biosurfactant reduced the biodegradation capability of the used microbial strains even if PE samples were UV-pretreated before biodegradation. The observation was in parallel with the other results of this study.

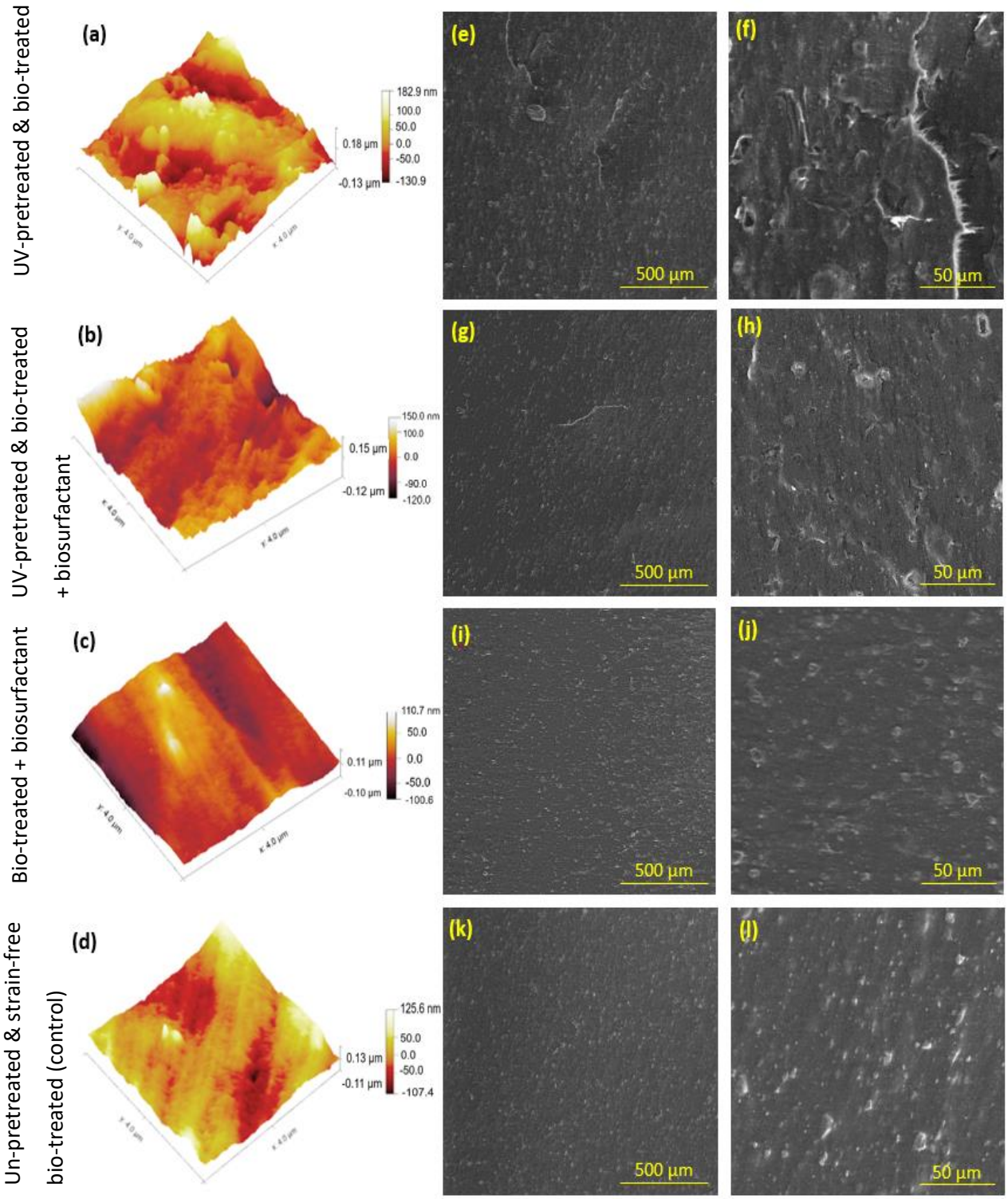

Fig 5.10. Surface morphology of PE after 45 days of biodegradation at different conditions by AFM (a-d) and SEM (e-l)

Similar to the PE samples, the AFM and SEM microscopy analysis of PET was also indicated a higher degree of surface degradation in the "UV-pretreated & bio-treated" condition. At this condition, the total distance from the crest to the trough of PET in the AFM analysis was about 0.25 μm. This value was almost two times higher than samples in "UV-pretreated & bio-treated + biosurfactant" and four times higher than "Bio-treated + biosurfactant" and "Un-pretreated & strain-free bio-treated" (control) samples (Fig 5.11a-d).

Surface topography of PET samples by SEM showed minor degradation in "UV-pretreated & bio-treated + biosurfactant" samples compared to "UV-pretreated & bio-treated" where more cracks and surface deformation occurred (Fig 5.11e-h). The SEM analysis of PET samples in "Bio-treated + biosurfactant" and control showed unchanged surface morphology even at higher magnification (Fig 5.11i-l). Therefore, it was believed that the utilisation of rhamnolipid reduced biodegradation efficiency.

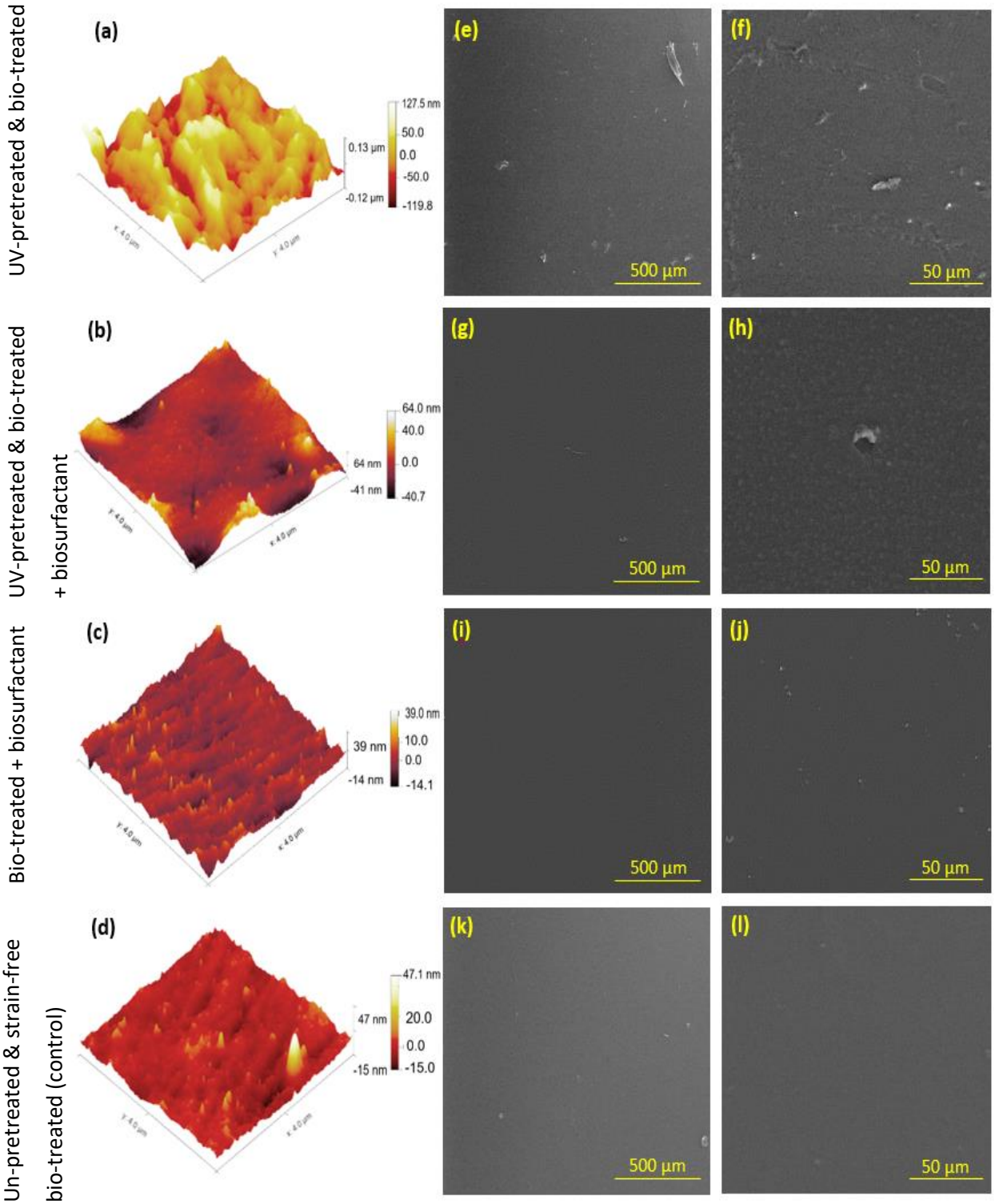

Fig 5.11. Surface morphology of PET after 45 days of biodegradation at different conditions by AFM (a-e) and SEM (e-l)

5.5. Summary of Chapter and Conclusion

The capability of a microbial community with four strains for plastics biodegradation was evaluated in different conditions, including UV-pretreatment, biosurfactant and the combination of both. This study indicated that the combination of UV-pretreatment at a dosage of 7.02×10^{12} $\mu W.cm^{-2}.s$ and rhamnolipid (160 mg/L) could be used to increase the biodegradation rate of PS by 2.3% weight loss. However, for higher PE biodegradation, UV-pretreatment should be only considered when using the same microbial strains. The very low weight loss (< 1%) of PET, also suggested that neither UV-pretreatment nor rhamnolipid and the combination of both could increase the biodegradation efficiency of the used microbial strain in this study.

The highest attachment of microorganisms was also seen on the PS samples at "UV-pretreated & bio-treated + biosurfactant" and "UV-pretreated & bio-treated" conditions with OD 595nm of 1.53 1.31, respectively.

Chapter 6

Road to Commercialisation

Contents of this chapter have been published as follows:

Taghavi, N., I. A. Udugama, W.-Q. Zhuang and S. Baroutian (2021). "Challenges in biodegradation of non-degradable thermoplastic waste: From environmental impact to operational readiness."Biotechnology advances 49: 107731.

6.1. Chapter Preface

Implementation of effective system for the degradation of plastic in the most environmentally friendly way is challenging. The limitations present in the biodegradation process reduce its capabilities to be commercially employed worldwide. In this chapter technology readiness level of plastic degradation technologies as well as opportunities and limitations associated with biodegradation are discussed to provide solutions for the commercialisation of biodegradation.

6.2. Technology readiness level

One of the associated challenges in the biodegradation of plastic is the implantation of an effective system to enhance biodegradation efficiencies. Technology readiness levels (TRL) is a well-established system initially developed by NASA to monitor the development of space-based technologies. Since then, the TRL concept has been widely used in other domains including in the areas of resource recovery. TRL matric runs from level 1 to 9 and can be categorised into three main scales in terms of the development i) lab-scale (level 1-3); ii) pilot-scale (level 4-6); and iii) full-scale (level 7-9). Fig 6.1 and Table 6.1 summarised the TRL levels in each degradation technology and the list of available companies involved in thermal and hydrothermal degradation of plastic was shown. In this instance, the number of applications in TRL 1-3 and 4-6 was determined based on the number of studies in Scopus with the search keywords of technology + plastic + degradation. The number of studies in biodegradation and HTL was determined as biodegradation + plastic; "Hydrothermal liquefaction" + plastic. TRL 7-9 was found based on the number of available companies in the deconstruction of plastic at a commercial (full) scale and the number of registered landfills in the world.

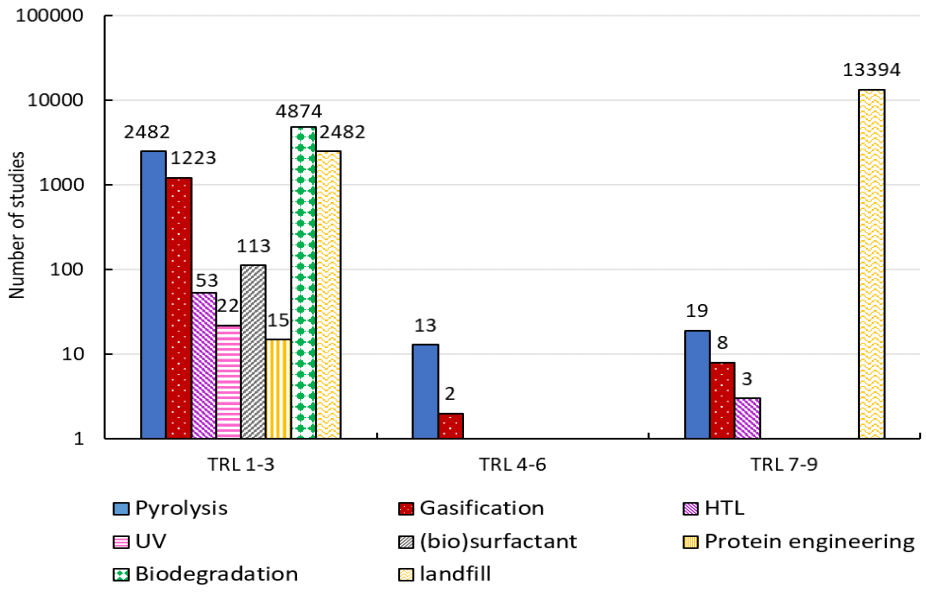

Fig 6.1. TRL level in different plastic degradation technologies

Table 6.1. Example of commercial processes in thermal and hydrothermal plastic degradation

Company	Process	Product	Input	Feedstock capacity	Reference
Climax Global Energy	Pyrolysis	Pyrolytic oil	Mixed, post-consumer plastics	-	(Steve Russell 2012)
RES polyflow	Pyrolysis	Diesel, naphtha blend stocks, wax	Mixed, medical automobile, post-consumer plastics	90,000 tonne/year	(Dan Sandoval 2018)
JBI (plastic2oil)	Pyrolysis	Naphtha, fuel oil	Rigid and Film Plastics (LDPE, HDPE, PP, PS)	1.8 tonne/hour	(Plastic2Oil 2013)
Agilyx	Pyrolysis	Pyrolytic oil	Discrete polymers, mixed	3,600 tonne/year	(Alexander H. Tullo 2019)
Braven Environmental	Pyrolysis	Diesel	Mixed plastics	59,000 tonne/year	(Axel Barrett 2020)
Klean Industries Inc	Pyrolysis	Pyrolytic oil	Mixed plastics	-	(Klean Industries 2020)
Natural State Research	Pyrolysis	Diesel, natural gas	Mixed plastics	-	(Imogene Tyler 2020)
Nexus Fuels	Pyrolysis	Gasoline, kerosene, diesel, heavy oils, waxes	Clean plastics (LDPE, HDPE, PS, PP,)	45 tonne/day	(Nexus Fuels 2019)
Rational Resource	Pyrolysis	Pyrolytic oil	-	-	(ReSource plastic 2020)
Vadxx Energy	Pyrolysis	Diesel, naphtha, waxes	Mixed post-consumer and industrial plastics	54 tonne/day	(Waste today 2020)
Axion Group	Pyrolysis	-	Separated and individual plastic batch	-	(Axion group 2020)
Plastic Energy	Pyrolysis	Diesel, naphtha, pyrolytic oil	Mixed, contaminated, multi-layered plastics	-	(Plastic energy 2020)
Entech - Renewable Energy Solutions	Pyrolysis	Diesel, gasoline, wax, pyrolytic oil	Mixed plastics	-	(Energy expert 2020)
Integrated Green Energy Solutions	Pyrolysis	Diesel, naphtha, marine oil	Waste plastics	31,000 tonne/year	(Sustainable world ports 2018)

Table 6.1. Continued

Company	Process	Product	Input	Feedstock capacity	Reference
Kanzler Verfahrenstechnik GmbH (KVT)	Pyrolysis	Diesel, gasoline	Sorted and unsorted mixed plastics (PP, PS, LDPE, HDPE)	13,600 tonne/year	(KVT Process Technology 2020)
GEWA Technology	Pyrolysis	High quality fuel	Waste plastics	-	(Environmental expert 2020)
Beston	Pyrolysis	Fuel oil	Waste plastics (LDPE, HDPE, PP, PS)	6-24 tonne/day	(Beston 2020)
Cynar Plc	Pyrolysis	Diesel, pyrolytic oil	Mixed (LDPE, PP, PS)	-	(Process industry Match 2020)
Reaktor ApS	Pyrolysis	Diesel	Contaminated plastics	-	(Denova 2020)
Waste2Tricity	Gasification	High-grade hydrogen to be used as transport fuel	All unrecyclable waste plastics	25 tonne/day	(Thomas Parker 2019)
Showa Denko	Gasification	Obtaining hydrogen for the synthesis of ammonia	Crushed & moulded used plastic	63,000 tonne/year	(Showa Denko 2020)
Texaco	Gasification	Syngas	Mixed plastics	150 tonne/ day	(A. Tukker et al. 1999)
Joint cooperation (Shell, Air Liquide, AkzoNobel, Enerkem, Nouryon)	Gasification	Syngas and clean methanol for using in the chemical industry	Unrecoverable plastics mixed with waste streams	325,000 tonne/year	(Jim Lane 2019)
ReNew ELP	HTL	Valuable oil, naphtha, heavy waxes	Contaminated, multi-layer flexible and rigid plastics	-	(ReNew ELP)
Neste	HTL	Fuel and new plastics	Waste plastics	907,000 tonne/day (by 2030)	(Licella 2018)
Licella	HTL	Valuable fuels and chemicals	End-of-life plastics	-	(Licella 2020)

Based on this information the following conclusions can be made:

1) Landfilling is a commercially used technology hence a TRL level of 9 can be assigned
2) Similarly, Gasification, Pyrolysis, and HTL of plastic is commercially available technology hence a TRL of 9 can be assigned
3) All other methods of plastic degradation are still at a TRL level of 1-3.

As such, from a practical point of view, only the thermal-based plastic degradation processes are currently available as competitive and implementable technologies to the current practice of landfilling.

Although, thermal (ex. pyrolysis and gasification) and hydrothermal approaches (HTL) can currently deconstruct plastic wastes at commercial and pilot scales, the high requirement of energy input, operational expenditures, and high generated toxic gases are the main drawbacks and can make them unattractive options for long-term degrading operation.

6.3. Biodegradation Opportunities and Limitation

Degradation of plastic with the use of microbial strains (biodegradation) is economically and environmentally beneficial. However, the slow degradation rate due to the lack of proper microbial enzymes is currently the main limitation. Previous studies were mainly focused on the biodegradation of one plastic type (PE), while in the environment a mixture of plastic wastes is exposed for degradation. Enzyme engineering and surface-active agent utilisation have also been reported as potential ways of biodegradation enhancement, our current knowledge and performance in biodegradation are far behind to carry out biodegradation at the pilot and commercial levels.

While biodegradation is a promising technology in its own right for dealing with plastic wastes and perhaps represents the most advanced and eco-friendly manner to deal with these plastic wastes there are several key limitations and unknowns that must be discussed. The key technical limitation of biodegrading currently is the long periods required for degradation to occur, and the fact that total degradation does not occur. In an attempt to remedy these limitations, there has been a drive toward enhancing natural biodegrading mechanisms and/or engineered solutions. In both approaches, enzymatic products specifically are designed to “consume” certain types of plastics, and genetically modified organisms (GMO’s) are being developed with the same purpose.

However, the use of such GMO's and even modified enzymes means future biodegradation plants and processes systems need to ensure no accidental release of these substances (in particular GMO's) take place. On the other hand, the economic costs of designing and developing such organisms will inevitably increase the cost of developments that are needed to migrate from a low TRL to a medium-high TRL for these pathways. As such, efficiently and economically addressing these shortcomings is an ongoing technical limitation. Another key limitation is the fact that biodegrading of non-degradable thermoplastics mainly occurs on PET due and the availability of discovered enzymes, and it is still limited to the other plastics such as PE, PP, and PS.

There have been questions raised in academic circles about the overemphasis of the results achieved in the field of biotechnological plastic degradation, which must also need to be given due consideration. Indeed, the current form of biodegradation technology is unable to deal with the broad spectrum of plastic waste. It is also important to note that, if a specific microorganism is found to have excellent PET degrading capabilities and mediocre for PS, as an example, then commercialisation of such technology would implicitly require the sorting of the plastic waste prior to biodegradation. Alternatively, further development work needs to be done of developing mixed cultures (similar to that of wastewater treatment) that have a combination of microorganisms either in engineering or natural occurring format to degrade different types of plastic at the same time.

6.4. Role of Process Integration

Taking the above-mentioned limitations into account, concepts of process integration from the domain of process systems engineering (PSE) can be applied to somewhat remedy these inherent limitations. One possible approach is to improve the plastic ageing process before exposing to biodegradation. This can be done with the help of surface-active compounds (mineral oil, surfactant, biosurfactant), short hydrothermal treatment at low operating conditions or with the help of UV radiation. As mentioned previously UV has been used as a green pretreatment technology for ageing plastic prior to biodegradation (Roy et al. 2008, Vimala and Mathew 2016, Montazer et al. 2018). To maximise photo-oxidation by this method, low-wave length (< 300 nm) radiation should be used to obtain maximum penetration in the plastic structure. Irradiation on both sides of plastic also is an important factor, which can enhance the again performance. Most of the conducted studies pretreated plastic samples at a higher wavelength (> 300 nm) and irradiated just

one side of the plastic. Irradiation on both sides of plastic generates more free-radicals and short-chain compounds in the polymeric structure, which then can increase the chance of microbial attachment and colonisation on the surface of the plastic.

Chapter 7

Conclusions and Recommendations for Future Work

7.1. Conclusions

The mass generation and careless abonnement of plastics have become one of the leading environmental issues globally, with an annual generation of about 360 million tonnes. While the current thermal degradation methods like pyrolysis, gasification and hydrothermal liquefaction can convert plastic waste into energy, these methods are often energy and labour intensive due to plastic sorting prior to treatment and removal of generated toxic by-products. Biodegradation is the most environmentally friendly way of plastic degradation, and previous studies have shown that microorganisms were able to degrade plastics. However, the low degradation efficiency of biodegradation limits its capability at large scale applications.

In this study, the capabilities of *P. raperi*, *A. flavus*, *P. glaucoroseum* and *Pseudomonas* spp were evaluated for biodegradation of four types of thermoplastics at different conditions. From the given results it can be confirmed that the isolated strains were able to degrade non-degradable plastics in both stimulated and unstimulated conditions within the given period and biodegradation was feasible. The oxidative shock of strains by H_2O_2 while could be an effective method for reducing the operational cost and accelerating the process due to the production of new enzymes within the media, was not as effective as unstimulated conditions.

The findings of this study showed that UV-pretreatment (245 nm) at a longer incubation period and shorter distance to UV light source (7.02×10^{12} $\mu W.cm^{-2}.s$) (t_2 d_2) can maximise the biodegradation capability of the used strains within the given period. It was also concluded that the UV-pretreated plastics were consumed more when they were presented in a smaller size and higher quantity

The integration of optimum UV-pretreatment with rhamnolipid was shown to be effective in the biodegradation rate of PS. However, it was shown that its consumption as a food source rather than plastics limits the capability of strains and resulted in lower biodegradation for PE and PET. Therefore, rhamnolipid should not be used with UV-pretreated PE and PET samples if a higher biodegradation efficiency is required.

7.2. Recommendations for Future Work

This research as well as previous studies showed that increasing the biodegradation rate is feasible. The question which remains is how and when biodegradation can be implemented commercially. While the slow processing time and low biodegradation rate are the current main limitations of this process, optimisation of the system by following what other researchers have found so far can accelerate the process.

In this study, it was shown that pretreatment can increase biodegradation efficiency. However, the current research only investigated the effect of UV-radiation as a way of plastic pretreatment. The effect of other green pretreatment methods such as hydrothermal and cold plasma at mild operating conditions should also be investigated. Moreover, understanding the chemical reactions and mechanisms of UV radiation with plastics can be studied in the future.

It is also recommended that for large and bulk plastics, mechanical forces like chopping or shredding should be applied in advance to make plastics into smaller fragments; allowing microbial enzymes or UV to penetrate more easily into the plastic structure.

While this study has shown that increasing the biodegradation efficiency of PS in the presence of rhamnolipid was feasible, the effect of other important biosurfactants like sophorolipids on biodegradation enhancement of PE, PS and PET and surfactin on biodegradation improvement of PS and PET should also be studied in detail.

Despite the mentioned approaches of biodegradation enhancement in chapter 2, a recommendation for future studies can be the combination of identified strains with the highest capability of plastic degradation. Based on previous reports and the results of this study, the consortium should include the combination of: *I. sakaicnsis, B. subtilis, P. aeruginosa, A. niger, A. Flavus, A. glaucus, A. versicolor*. The combination of the most promising strains could facilitate the biodegradation process due to the availability of different enzymes in the aforementioned strains. The generated by-products from the enzymatic activity of one strain can be consumed by the other strains and result in higher degradation of plastics.

It should be reminded that complete biodegradation of all plastic types may not be feasible in the near future; therefore, it is recommended to design a procedure in a way to produce valuable by-products. For example, a new study found that the breakage of PET into its basic unit (terephthalic acid) can be used as the main component for vanilla extract production. This can be very interesting for many industries since no large farm fields or growing vanilla beans are needed. Instead, waste PET plastic can be turned into vanilla extract and this can enhance the commercial profit.

Another recommendation for future researches is understanding the effect of plastic additives compounds such as anti-oxidant and plasticizers in the biodegradation efficiency and viability of microorganisms.

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