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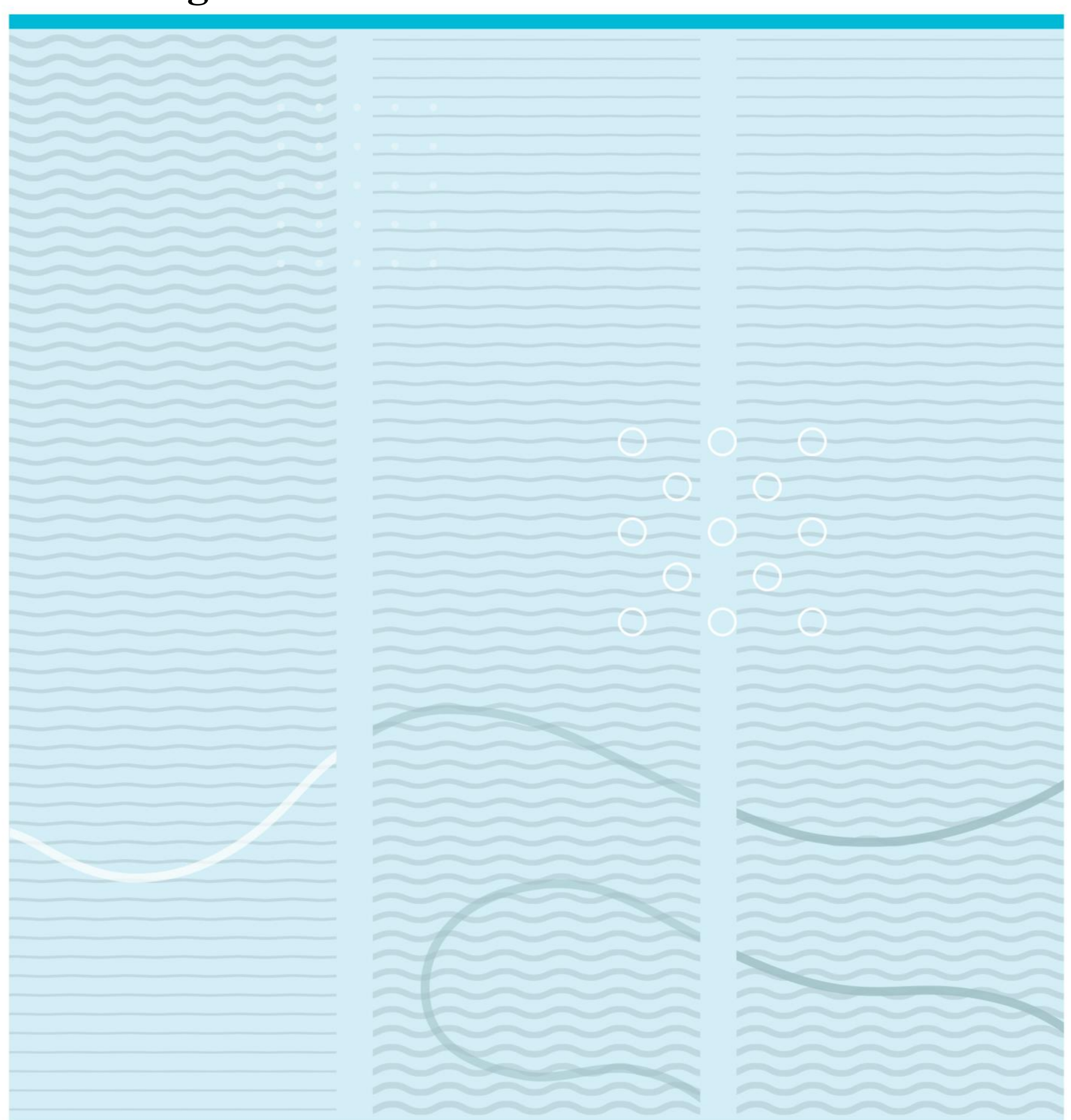
Isolation and Characterisation of Plastic-Degrading Microorganisms



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This thesis is worth 60 study points

Abstract

Different microorganisms from aged plastics wastes were sampled from various locations in previous studies. 14 strains were isolated and characterized by catalase, oxidase, gram and observational tests. After identification, the capability of these strains was investigated in the biodegradation of low-density polyethylene (LDPE). Three bacterial strains ZZ-12-2, ZZ-7 and ZZ-3 showed better possible biodegradability in comparison with other strains and probably belong to one of *Firmicutes* or *Proteobacteria* phyla. Some chemicals such as mineral oil, $(NH_4)_2SO_4$ and $MgSO_4$ were tested as biodegradation stimulators. Mineral oil inhibited bacterial growth in this study while $MgSO_4$ stimulated ZZ-2 strain biodegradation significantly and $(NH_4)_2SO_4$ stimulated ZZ-6 sample slightly which resulted in this conclusion that $MgSO_4$ in some strains of bacteria can serve as biodegradation stimulator. On the other hand, to determine the synergistic effect, different mixed cultures were investigated and only ZZ-2 +ZZ-3 and ZZ-11+ ZZ-12-2 suggested a potential synergistic effect after 22 days while it didn't observe after 112 days. So these mixed cultures cannot decisively be introduced as candidates with synergistic effect on biodegradation and more researches need to be carried out to confirm this statement.

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List of Abbreviations

PE	Polyethylene
PP	Polypropylene
HDPE	High-density polyethylene
LDPE	Low-density polyethylene
LLDPE	Linear low-density polyethylene
UHMWPE	Ultra-high molar weight polyethylene
ULDPE	Ultra-low-density polyethylene
PVC	Polyvinyl chloride
PVOH	Polyvinyl alcohol
PVAC	Polyvinyl acetate
PVDC	Polyvinylidene chloride
PVB	Polyvinyl butyral
PS	polystyrene
PC	Polycarbonates
PET	Polyethylene terephthalate
PBT	Polybutylene terephthalate
PTMT	Polytetramethylene terephthalate
PF	Phenolformaldehyde Resins
UF	Urea-Formaldehyde
PI	Polyimides
PCL	plasticspoly(e-caprolactone)
PBS/A	poly(butylene succinate/adipate)
PBA/T	poly(butylene adipate-co-terephthalate)
PLA	Polylactide
PHB	Polyhydroxybutyrate
PHBV	Polyhydroxybutyrate-co-valerate
PAHs	polycyclic aromatic hydrocarbons
OD	Optical density
ATP	Adenosine triphosphate

CFB	Cytophaga-Flavobacterium-Bacteroides
GPC	Gel Permeation Chromatography
DSC	Differential scanning calorimetric
FTIR	Fourier transform infrared spectroscopy
RIfS	Reflectometric interference spectroscopy
SEM	Scanning Electron Microscopy
GC-MS	Gas Chromatography-Mass Spectrometry
NMR	Nuclear magnetic resonance spectroscopy
½ TSA	Half strength Trypticase Soy Agar
MPs	Microplastics
CFU	Colony Forming Unit
$(NH_4)_2SO_4$	Ammonium sulfate
$MgSO_4.7H_2O$	Magnesium sulfate
HPLC	High Performance Liquid Chromatography
M9-PE	Minimal Salt
MPD	Maximum probable dilution
PHAs	Polyhydroxyalkanoic acids

Foreword

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1 Introduction

1.1 History of Plastics

Plastics are synthetic polymers with high molecular weight (Jansen, 2016) that consist of hundreds or thousands of monomer subunits and are linked together by strong covalent chemical bonds. With the invention of the first modern plastic “Bakelit” in 1907 by using a condensation reaction of phenol with formaldehyde, several non-expensive methods have been developed which has led to the growth of mass production of this durable and resistant synthesized product in the 50s (Chamas et al., 2020) and these characteristics caused the use of these products inevitably increased yearly (Andrady, 2011). From the 1940s up to now, mass production of plastics has been increased exponentially with 230 million tons produced worldwide in 2009 (Plastics Europe 2010). Plastics are non-degradable and convenient products with diverse use and low price which made them a favorable replacement for natural substances since 1960 (Jambeck et al., 2015; Ghatge et al., 2020). There is an increasing trend in the utilization of these synthetic polymers in different industries especially in the packaging industry which has had 30% consumption of plastics globally (Pathak & Navneet, 2017). According to Ritchie and Rose (2018) World plastics production was about 270 million tons per year in 2015 and 3% of these plastics directly poured into the oceans, 55% main amount dumped in landfills, 8% incinerated, and 6% only recycled and the remaining percentage was still in use (Ritchie & Roser, 2018; Taghavi et al., 2020). It is assumed that 5-13 million tons of plastics will be dumped into the oceans annually and this would have detrimental effects on human health, fauna and flora ecosystems (Geyer et al., 2017; Danso et al., 2019).

1.2 Plastics Classification and Synthesis

Plastics can be classified by their chemical structure and temperature behavior into thermoplastics and thermosets.

1.2.1 Thermoplastics

Thermoplastics are materials that can be melted and molded and include different groups as follow:

1- Polyolefins are synthetic polymers comprising olefinic monomers. Polyolefins or polyalkenes are produced by polymerization of alkenes (Zhang et al., 2017). They are polymers of simple alkenes such as ethylene, propylene, butenes, and pentenes. Polyethylene (PE) and polypropylene (PP) are two important Polyolefins. PE is divided into high-density polyethylene (HDPE), low-density polyethylene (LDPE), linear low-density polyethylene (LLDPE), ultra-high molar weight polyethylene (UHMWPE), and ultra-low-density polyethylene (ULDPE) (Kutz Myer, 2015). LDPE has a lower density, lower crystallinity, more flexibility, more transparency, odor-free, non-toxic, and these characteristics made it so favorable product in the food packaging industry. LDPE and HDPE have the highest rate of waste with 23 and 17.3 percent respectively in comparison with other types of polymers (Pathak & Navneet, 2017). Among all polymer types, PE and PP are the most common synthetic polymers which cover 60% of the total global plastic products with plenty usage of more than 116 million tons per year (Danso et al., 2019).
2- Vinyl polymers contain a great number of thermoplastics that are produced by polymerization of monomers containing vinyl groups include Polyvinyl chloride (PVC), Polyvinyl alcohol (PVOH), Polyvinyl acetate (PVAC), Polyvinylidene chloride (PVDC), Polyvinyl butyral (PVB), and Polyvinyl formal (PVF) (Oda & Shinke, 2021).
3- Polystyrene (PS): It is a synthetic aromatic polymer with high molecular weight made from styrene monomers. PS is mainly used in four types of products: GPPS, high impact polystyrene (HIPS), PS foam, and expanded polystyrene (EPS) foam (Ho et al., 2018).
4- Polyamide: Commonly known as nylon with High molecular weight. Polyamides are crystalline polymers typically produced by the condensation of a diacid and a diamine (McKeen, 2017)
5- Polycarbonates (PC): PC is an important thermoplastic polymer with a carbonate group in its structure. This group is divided into two chemical categories (1) straight-chain aliphatic (2) aromatic. Aliphatic is so beneficial thermoplastics which are made from CO_2 and epoxide. poly (bisphenol A carbonate) counts as the most important aromatic PC (Kausar, 2017).

6- Linear Polyester: Polyethylene terephthalate (PET), polybutylene terephthalate (PBT), and Polytetramethylene terephthalate (PTMT) belong to this group and are synthesized from ethylene glycol or butylene glycol (*Polymer Science and Engineering*, 1994).

1.2.2 Thermosets

Thermosets are a group of plastics that get harder by heating up, but remolding or reheating them would be impossible after the initial forming. Thermosets are divided into important subgroups such as (1) Unsaturated Polyester: Consist of two polymers which are a short-chain polyester and a vinyl monomer (Thomas et al., 2019). (2) Phenolformaldehyde Resins (PF): Produced by condensation process of phenol and formaldehyde. (3) Melamine Resins: Melamine, such as Urea-Formaldehyde (UF) or Melamine Formaldehyde (MF), are produced by the condensation of formaldehyde and urea or melamine. (4) Polyepoxides: There are several methods for producing epoxy resins. These methods comprise the condensation of epichlorohydrin with other compounds that have at least two hydrogens in their structure such as diacids, diamines, polyphenols (Hsissou et al., 2018). (5) Polyimides (PI) in the classic method are produced as the result of the reaction between a diamine and a dianhydride (Chen et al., 2017). (6) Polyorganosiloxanes: or silicones with repeating Si–O linkages backbone are the most popular silicon-based polymers. Silicones are produced in successive hydrolysis and polycondensation reactions of chlorosilanes with organic solvents or without them. An acid catalyzes The hydrolysis and polycondensation processes with an organic solvent because alkoxysilanes hydrolysis is lower than chlorosilanes (Ogawa et al., 2008)

1.3 Polymerization

Most plastics are produced as a result of polymerization of extracted monomers from oil or gas (Thompson et al., 2009), in different polymerization reactions i.e. addition and condensation. Addition polymerization reaction is an exothermic process in which polymers are formed from carbon-carbon double bond monomers. In this reaction, no atoms or molecules are lost from the reacting monomers. Polyethylene, polypropylene, polyvinyl (chloride), and polystyrene (thermoplastics) are produced in addition reactions. In contrast, condensation polymers are formed by a stepwise reaction of molecules with

different functional groups. The Condensation process consists of endothermic molecular reactions in which may some byproducts like water or methanol being eliminated. Thermoplastic Polyesters, polyacetal, polycarbonate, and polyamides are produced by this condensation process (Jansen, 2016).

1.4 Plastics Degradation

Since plastics degradation is a very slow process (Andrady, 2011; Hidalgo-Ruz et al., 2012) persistence and accumulation of plastics in the environment especially in the oceans seems to have been a crucial issue for many years (Barnes et al. 2009).

Polymer degradation is a process that is influenced by abiotic or biotic factors. Abiotic processes consist of parameters such as mechanical stress, light, and temperature while biotic processes are mediated by microorganisms such bacteria, fungi, and algae (Miranda Gabriela et al., 2020). Abiotic degradation pathways are divided into (1) physical degradation which includes structural changes such as cracking, embrittlement, and flaking, (2) chemical degradation which includes molecular changes such as oxidation of polymers with long chains or bond breakage that generates molecules with shorter chain lengths (Chamas et al., 2020). Chemical degradation can be in the forms of hydrolysis (requiring H_2O) or oxidation (requiring O_2) and acceleration of this process would be influenced by external factors such as microbial activities, heat, or light (Andrady, 2011). Biotic degradation or biological degradation is a process in which complex organic matters are transformed into carbon dioxide, methane, water, or minerals (mineralization) through the enzymatic activity of microorganisms such as bacteria and fungi. To achieve that, the surface of plastics should be colonized by microorganisms in order to secret certain enzymes which break down polymer chains into short-chain fragments (Kliem et al., 2020).

Plastics can be grouped as biodegradable plastics such as polycaprolactone (PCL), poly(butylene succinate/adipate) (PBS/A), poly(butylene adipate-co-terephthalate) (PBA/T), Polylactide (PLA), Polyhydroxybutyrate (PHB), Polyhydroxybutyrate-co-valerate (PHBV) and, Polyhydroxyalkanoic acids (PHAs) which can be completely degraded to carbon dioxide and water by natural microbial (bacteria, fungi, and algae) activities (Iwata, 2015). Polyhydroxyalkanoic acids (PHAs) are a typical form of biodegradable plastics while their properties are similar to conventional plastics. In addition, Starch-

based polymers are always favorable for microbial digestion by secreting hydrolytic enzymes which depletes their molecular weight. So in comparison with other polymers, starch-based or flax fiber structures present higher biodegradability (Kumar et al., 2011; Sen & Raut, 2015). Another group is synthetic plastics consists of polyethylene (PE), polypropylene (PP), and poly (ethylene terephthalate) (PET) and are typical oil-based non-biodegradable plastics (Iwata, 2015).

Polyethylene (PE) shows considerable persistence and high durability to biodegradation among other plastics. The stability of covalent bonds in C-C and C-H is the first feature that has an important role in its resistance to biodegradation. Higher molecular weight makes their bonds impenetrable for microbial activities and also some other features such as a lack of functional group with great hydrophobic nature and inability of oxidation and hydrolysis can influence their biodegradation (Gautam et al., 2007). Different environmental parameters such as humidity, temperature, pH, salinity, the presence or absence of oxygen, sunlight, water, stress, and culture conditions have crucial effects on the microbial population and enzymatic activities besides biodegradation (Gu, 2003). Low molecular weight is favorable for biodegradation. The melting temperature (Tm) for polymers also has a tremendous influence on biodegradation. Polymers with higher melting point i.e. higher crystallinity has a higher resistance to biological attack hence the enzymatic degradability declines over time. Through time, properties of surface structure like crystallinity and value of elasticity will be changed which has a great effect on degradation. (Kale et al., 2015)

1.5 Biodegradation Pathways

Several steps occur in the plastic biodegradation process which was mentioned by Dussud and Ghiglione (2014). Bio-deterioration is a degradation of the surface layer in which the chemical and mechanical properties of plastics are changed by microorganisms. The next process is bio-fragmentation and consists of some catalytic reactions which leads to the breakage of polymers to smaller compounds e.g. oligomers, dimers, or monomers by exo-enzymes secreted by microorganisms. For polymers with high molecular weight, it is not possible to pass through cell walls. So microorganisms secret some extracellular enzymes (exo-enzymes) which catalyze reactions that lead to degrading plastics (Hamilton et el., 1995; Dussud & Giglione, 2014). Two groups of

enzymes play essential roles in depolymerization processes which are extracellular and intracellular depolymerase (Gu et al., 2000). More information about enzymatic processes can be seen in section (1.5.2). Assimilation is a process in which plastic monomers are absorbed by microbial cells. It leads to production of secondary metabolites that will be transferred out of microbial cells when the cells are no longer able to metabolize or store these metabolites and may be degraded by other active cells. Mineralization is the last process of degradation in which primary and secondary metabolites are completely degraded and as its result oxidized metabolites such as CO_2, H_2O or CH_4 are produced (Dussud & Giglione, 2014).

1.5.1 Aerobic and Anaerobic process

Since carbon is the main material in plastics, in aerobic conditions, microbes use plastics as a carbon resource and oxidize them. Subsequently, carbon dioxide and water are produced as final by-products in aerobic conditions. In reverse, in the anaerobic conditions of biodegradation in sediments and landfills, some organic acids and gases such as CO_2 and CH_4 are produced beside H_2O production. Also, biodegradation can be occurred in aerobically/anaerobically condition e.g. in soil and compost. The aerobic process is assumed to be more effective in comparison with the anaerobic process since in the anaerobic process CO_2 and SO_4 are used as electron receptors which are less efficient in comparison with O_2 (Dussud & Giglione, 2014, Bolhmann, 2006; Siracusa, 2019). In the biodegradation process, different types of microorganisms such as bacteria and fungi are involved in decomposing polymers macromolecules into their monomeric units. The Biodegradation rate directly depends on O_2 accessibility. For mineralizing the organic chemicals to smaller compounds in an anaerobic process, nitrate, sulfate, iron, manganese, and carbon dioxide are utilized as electron acceptors in some bacteria (Alshehrei, 2017).

1.5.2 Enzymatic processes

As it was mentioned, biodegradation of plastics includes different steps and begins with secretion of extracellular enzymes by the microorganisms, then attachment of these enzymes to the surface of plastics, hydrolysis them to short polymers, and finally microbial cells would assimilate these short polymers as the carbon source to release

CO_2. Alkane hydroxylases (AHs) are constant enzymes that have an essential role in the aerobic degradation of alkanes by bacteria. Hydroxylation of C-C bonds to release primary or secondary alcohols is the first step in degradation. These C-C bonds are oxidized to ketones or aldehydes, and ultimately to hydrophilic carboxylic acids (Mohanan et al., 2020). Monoxygenases are the most important enzymes in the alkane hydroxylase system (Jeon and Kim, 2016). The number and type of AHs are different in different bacteria e.g. in *Rhodococcus* sp. TMP2 genome encodes 5 AHs (alkB1, alkB2, alkB3, alkB4, and alkB5) while the *P. aeruginosa* genome encodes two AHs: alkB1 and alkB2 (Takei et al., 2008). The number of carbonyl groups can be reduced by microbial oxidation with formation of carboxylic acids and these carboxylated n-alkanes are analogous to fatty acids which are metabolized via the β-oxidation system pathway by the bacterial degradation process (Restrepo-Flórez et al., 2014; Mohanan et al., 2020).

1.6 Microorganisms' Role in Bio-degradation

Plastics colonization by different microorganisms was reported first in the 1970s and plastic surface seems to be a niche for microorganism and serves as buoyant particle with colonization and transportation ability especially in water (Dussud & Ghiglione, 2014). Different microorganisms are involved in biological degradation such as bacteria, fungi, and algae. Microorganisms can use polymers as carbon sources and secrete some enzymes that stimulate biodegradation (Gilan et al., 2004). Physical properties of polymers such as crystallinity, molecular weight, and functional groups in polymers are factors that have effects on biodegradation beside important role of organism species and pretreatment quality (Artham & Doble, 2008; Gu et al., 2000; Shah et al., 2008). A lot of researches have been carried out in order of determining different bacteria with biodegradable properties.

In Proteobacteria phylum, different bacteria get involved with biodegradation and the most important genera with colonization and degradation ability in the marine environment are in *Hyphomonadaceae, Rhodobacteraceae, Erythrobacteraceae families in Alphaproteobacteria* class and *Psuedomonadacea, Alcanivoraceae* and *Vibrionaceae* families in Gammaproteobacteria class. In Betaprotobacter class, the genus *Hydrogenophaga* belongs to *Comamonadaceae* family is reported as the plastic colonizer. *Erythrobacter* belongs to *Erythrobacteraceae* family in Proteobacteria phylum is a

significant plastic colonizer with positive catalase and oxidase properties. In Bacteriodetes phylum and Flavobacteriales order, *Flayobacteriaceae* family with two genera *Flavobacterium* and *Tenacibaculum* have been reported in different researches as plastics colonizers on PS, PE, PP, and PET (Roager & Sonnenschein, 2019). Furthermore, *Pseudomonas* is a well-known bacterium that a lot of researches show its property as a plastics-colonizer which belongs to *Pseudomonadaceae* family in Gammabacteria class and Proteobacteria phylum (Roager & Sonnenschein, 2019).

Gram-positive rod-shaped bacteria are most in Firmicutes, Actinobacteriota and Cyanobacteria phyla and divided into spore-forming bacteria such as *Bacillaceae* and *Clastridiaceae* families and most important genera such as *Bacillus* and *Clostridium* with plastic colonizing and degrading properties versus most important non-spore-forming bacteria families are *Corynebacteriaceae, Actenomycetaceae, Mycobacteriaceae, Nocardiaceae, Listeriaceae* and *Lactobacillaceae*. Some genera in Cyanobacteria phylum with different families such as *Phormidiaceae* (genus *Phormidium*) has been reported as biodegrading bacteria on PE, PET, and PP samples (Roager & Sonnenschein, 2019). *Rhodococcus* belongs to *Nocardiaceae* family and Actinobacteria phylum is known as plastics colonizer (Auta et al., 2017). Polyethylene can be degraded by a various number of bacteria species like Gram-negative genera *Pseudomonas*, *Ralstonia*, and *Stenotrophomonas* and several Gram-positive taxa such as *Rhodococcus*, *Staphylococcus*, *Streptomyces*, *Bacillus* (Sen & Raut, 2015, Resterepo-Florez et al., 2014; Danso et al., 2019). Also, Pathak and Naveen (2017) mentioned that some biological prokaryotic (bacteria) and eukaryotic (fungi, algae and plant) species cooperate in the bioremediation process such as *Pseudomonas, Streptomyces, Corynebacterium, Arthrobacter, Micrococcus, and Rhodococcus*. A lot of researches have been carried out to identify different microorganism species that get involve with biodegradation. Roager and Sonnenschein (2019) reviewed a lot of marine species with plastics biodegradability but still, a lack of a comprehensive reference for bacterial candidates for colonization and degradation of soil and landfill wastes is tangible. Puglisi et al, (2019) isolated different bacteria from different soil and landfills and concluded that the most important soil bacterial colonizers include: *Bacillus cereus, B. amiloliquefaciens, B. pumilus, B. mycoides, B. firmus, and B. marisfavi*. Also, they mentioned the role of *Rhodococcus ruber* in the biodegradation of polyethylene (Puglisi et al., 2019).

The most important fungi with high affinity in colonizing of polyethylene were identified by Zahra et al (2010) as *Acremonium flavum*, *Candida rugosa*, *Arthrographis kalrae*, *Aspergillus* sp., *Lichtheimia* sp., *Aspergillus fumigatus*, *Emericella nidulans*, *Aspergillus terreus,* and *Fusarium solanifrom* (Zahra et al., 2010). Yuan et al (2020) implied other fungi as fungal-mediated plastic degrading species such as *Aspergillus tubingensis*, *Aspergillus flavus*, *Penicillium simplicissimum*, *Penicillium pinophilum*, *Pestalotiopsis microspore*, *Zalerion maritimum, and Mucor rouxii* (Yuan et al., 2020). Taghavi et al, (2020) claimed that *Penicillium raperi, Aspergillus flavus, Penicillium glaucoroseum, and Psuedomans sp* were isolated as the most plastic degrading microbes (Taghavi et al., 2020).

1.7 Stimulation and Synergistic effect on Biodegradation

Some researches on plastics in recent years were based on the stimulation effect of some minerals which has an effect on microbial growth and ultimately plastic biodegradation. Many different types of metals associate with the metabolism of hydrocarbons include manganese, iron, cobalt, nickel, copper, zinc, molybdenum, and magnesium. Manganese is the substantial element in the structure of manganese peroxidase enzymes which involves in biotransformation of hydrocarbons. In the biodegradation system, hydrogen peroxide generates iron (Iv) which oxidizes manganese(II) to manganese(III). These compounds lead to the cleavage of dihydroxybenzene and dioxygenases rings. Iron plays a very important role in the biodegradation of hydrocarbons and the active site of mono- and dioxygenases. Monooxygenases have iron in two forms of non-heme iron or heme cofactor. Monooxygenases induce the formation of hydroxylate by adding one atom of molecular dioxygen to hydrocarbon. Also, iron is the main element in the structure of catalase and oxidase enzymes (Bertini & Rosaro, 2010). Cobalt is associated with catalyzing dehalogenation which produces some by-products. These by-products can serve as electron receptors in the respiration process. Cobalt has an important role in forming Co-C bonds by reducing dehalogenase. In attendance of nickel, the oxidation of methane is processed by prokaryotes under anaerobic conditions. Methane monooxygenase is one of the enzymes with copper in its membrane which converts methane to methanol in the co-metabolism process. Alcohol dehydrogenases are a large family of enzymes with zinc elements in their structures and catalyze reversible oxidation

of alcohols to aldehydes. ADHs are one of these enzymes which stimulate the growth of methylotrophs and oxidization of alcohols. Molybdenum is an important element in forming molybdopterin. This compound is essential in forming Moco cofactor in mitochondria which is the active catalytic site of all molybdenum-cofactor-containing enzymes. These enzymes catalyze the transferring of one atom of oxygen into water, based on two-electron redox reactions. Also, Moco cofactor cooperates in the synthesis of a cytoplasmic enzyme named aldehyde oxidase which oxidizes variant aromatic and non-aromatic aldehydes and forms carboxylic acid (Bertini & Rosaro, 2010). Magnesium (Mg2+) is another essential factor in bacterial growth rate and saturation density (Lusk et al., 1968) which is initially required in some enzymatic reactions in cells such as translation (Pontes et al., 2015; Nierhaus, 2014; Ashino et al., 2019). Also, in some bacteria, magnesium has an essential role in the cell membrane, phospholipids, lipopolysaccharide and polyphosphate compounds such as DNA, RNA and ribosome. This element is associated with the cell wall, nucleoside triphosphate and cytoplasmic molecules (Christiansen et al., 2017). Another crucial substance in living organisms seems to be ammonium ions (NH_4^+), especially in bacterial growth. Nitrogen in ammonium is an essential factor in nucleotides biosynthesis, DNA and RNA backbone and amino acid metabolism (Kanehisa & Goto, 2000; Kanehisa & Goto, 2017, Ashino et al., 2019). Nitrogen, phosphorous and magnesium are included in all microbial growth media and would be just as necessary if the bacteria had been growing on glucose. Ashino et al., (2019) worked on the stimulation effect of different compounds on bacterial growth and concluded that $(NH_4)_2SO_4$ and $MgSO_4$ stimulate bacterial growth.

On the other hand, one of the intriguing part of researches would be the bioaugmentation of microorganism which could have synergistic effect on plastic biodegradation. Synergy is the cooperative interaction of two or more bacterial species which cannot be achieved individually by each of them (Ori D Rotstein et al., 1985). Another process which is called ''Co-metabolism'' can influence the synergistic effect that always should be considered in the interpretation of the results. Co-metabolism is a pathway in which oxidation of hydrocarbons for instance is carried out without using the energy derived from the oxidation to aid microbial growth and is separated from the presence or lack of growth substrate during the oxidation (Horvath, 1972). In another definition, Co-metabolism is the simultaneous degradation process of two substances in

which degradation of the second compound or refractory material relies on the presence of the primary substance. Primary substances in biodegradation are micro-molecules that can be simply degraded by bacteria (Lu et al., 2016). Primary substances can play two essential roles; First, they can be counted as a carbon and energy source for the growth and activity of microorganisms; Second, they increase key enzymatic activities which accelerate secondary substrates degradation (Lu et al., 2016). Horvarth (1972) claimed that between 23–30 species of bacteria and actinomycetes, fungi and microalgae show the co-metabolism process in biodegradation such *as Achromobacter sp., Azotobacter chroococcum*, *Bacillus sp*., *Flavobacterium sp., Hydrogenomonas sp., Microbacterium sp., Pseudomonas sp., Vibrio sp., Xanthomonas sp., Nocardia sp., Aspergillus niger*, *Streptomyces aureofaciens*, *Trichoderma* and *Chlorella vulgaris* (Lu et al., 2016). Mikesková et al (2012) reviewed several works on antagonistic and synergistic effects of microbial consortia with various bacteria and fungi on biodegradation in different organic pollutants, like PAHs, synthetic dyes, TNT, phenol, atrazine, and pesticides and concluded these consortia showed a reasonable biodegradation efficiency in comparison with single microbial strains and stimulate this mixed potential of the consortia by catabolic enzyme activities. Trzesicka-Mlynarz and Ward (1995) worked on the synergistic effect of microorganism on biodegradation and mentioned that Bacterium *Stenotrophomonas maltophilia* in presence of pyrene can mineralize benzo(a) pyrene polymers efficiently and also fungus *Penicillium janthinellum* in nutrition broth media can degrade a limited amount of polycyclic aromatic hydrocarbons (PAHs) which has high molecular weight. On the other hand, it showed that mixed bacterial cultures of diverse bacteria like *Pseudomonas putida*, *Flavobacterium* sp and *Pseudomonas aeruginosa* have a coincident superior effect on (PAHs) biodegradation in comparison with single bacterial strains, which could result from the catabolic enzyme activity which stimulated by a high potential of combined consortia (Mikeskova et al., 2012). Aravinthan et al (2016) put forth the synergistic effect of biodegradation in some samples which were pretreated physically such as polypropylene (PP) were assessed by mixing two different microorganisms *Bacillus flexu*+ *Pseudomonas azotoformans* (B1) and *Bacillus flexus*+ *Bacillus subtilis* (B2), and the results showed that some combination had a positive synergistic effect on biodegradation such as B1 sample that was a combination of *Bacillus* and *Pseudomonas* in compared with only *Bacillus* samples. Mixed cultures and biofilms

perform differently in comparison with microbial monocultures. Interactions of microbes interspecifically are important for their metabolic cooperation in mixed cultures (Seneviratne et al., 2008). In another research, soil bacteria *P. aeruginosa* and *Rhodococcus erythropolis* were suggested to reduce laccase production by fungus *Trametes versicolor* during PAH degradation in soil. On the other hand, the mixture of these bacteria also causes of reduction in PAH degradation by mixed *T. versicolar and Irpex lacteus* while bacterial effects on these two fungi were different separately (Borras et al., 2010).

1.8 Biodegradation Determining Methods

To identify the rate of biodegradation in plastics, different methods can be used which depends on how much accuracy is needed and also how much sample is available. Since lack of a comprehensive reference was felt, all efforts were made in collecting a reference based on different literature reviews contain all methods were used in determining plastics biodegradation with their benefits and drawbacks and all are mentioned in Table (1-1). Different methods were used in this project which ATP measuring test, OD measurement, plate counting test and CO_2 evolution assay were the most important ones.

On the other hand, for isolation and characterization of microorganisms, catalase, oxidase, and gram staining methods were used. Catalase enzyme has an essential role in the aerobic process. This enzyme in the microorganism breaks down the hydrogen peroxide (H_2O_2) into oxygen and water and neutralizing oxidative stress caused by this substrate. Catalase enzyme has four porphyrin heme (iron) groups in its structure that cause the reaction of that with hydrogen peroxide which is a by-product of aerobic respiration (Kaushal et al., 2018).

In the respiratory system of many aerobic or facultative aerobic prokaryotes, the presence of mitochondrial cytochrome oxidase enzyme is essential which catalyzes transferring of an electron from cytochrome *c* to molecular oxygen and reducing that to water. In presence of this enzyme, the reagent 6-NNNN-tetramethyl-1-4 phenylen diammoniumdichloride is oxidized to indophenol which is purple and in absence of that the reagent would be colorless.

The principle of gram staining method is that in gram-positive bacteria, cell wall has thick mesh-like shape which consists of 50-90% peptidoglycan in its structure and remains purple in gram-staining procedure but cell wall in gram-negative bacteria is composed of a thinner layer consist of 10% peptidoglycan in which crystal violet-iodine will be rinsed during decolonization with alcohol (Thairu et al., 2014)

1.9 Aims of study

The main aims of this study are identifying and reviewing different aspects of biodegradation, isolating, assigning, and differing the bacterial strains with biodegradability property, investigating stimulation effect of some chemicals on bacterial growth which may result in accelerating and stimulating biodegradation and also introducing some bacterial mixed cultures with synergistic effect on biodegradation.

Table 1-1: Different popular techniques are used in determining plastics biodegradation

Methods	Methodology	Advantages	Disadvantages
1-Gravimetric determination of weight loss is based on the final determination of weight in an analyte. weight measuring is accurate and easy to compare with other properties.	determining the final weight of a material in quantifying manner % weight loss = [(initial weight – final weight)/initial weight] (Montazer et al., 2020)	1-Accurate and precise. 2-Reducing material use (solvent and sample)3-cheap 4-minimizing uncontrolled errors (Ratcliff, 2014)	1-Not precise for plastics with a high additive formulation like PVC.2-Interpretation can be influenced duo to chemical hydrolysis and fragmentation of plastics3- Weight loss is slow and biodegradation process is limited conduce to obtain imprecise data4- This technique always should be combined with other methods to gain better results (Raddadi & Fava, 2019)
2-Thermogravimetrical analysis (TGA) is based on monitoring the weight change in a sample that is heated at a constant rate by determining thermal stability in them and fraction of volatile components	TGA is a way to determine the thermal stability in polymers. A decrease in such stability is an indicator of polymer degradation. Mass of sample measure over time while the temperature is changed (Raddadi & Fava, 2019).	1-low-cost technique,2-needs a small sample, 3- Allows quantitative or qualitative analysis.	1-Not useful for plastics with a high amount of additives.2- TGA does not give the exact identification of the gases produced from the sample during heating. 3-Might not to be precise enough due to the presence of volatile chemicals in the sample (De Moraes et al., 2020).
3-Gel Permeation Chromatography (GPC) is based on separating analytes by size, typically in organic solvents for the analysis of polymers	In this method polymers with different sizes are separated by porous gels in which the larger particles subside through a short flow path while smaller particles go through a longer flow path and can access the deeper area of the porous filler (Ravin Narain, 2020).	1-Independent from temperature, PH and ionic strength, and buffer composition and just related to molecular weight. 2-Can be carried out in any conditions.3-Less tIme of analysis (Gel Filtration Chromatography, 2021)	1-Not accurate and sensitive enough if this method is carried out on a high volume of polymer especially when biodegradation occurs on the outer polymer surface at the primitive stage. (Raddadi & Fava, 2019)
4-Differential scanning calori **)SC) analysis**	This is a thermo-based method and the function of temperature is determined by changes in the amount of input heat which leads to increasing the temperature in the sample (Capitain et al., 2020)	1-High sensitivity 2-high accuracy 3-high-quality data (Spink., 2008) 4-carried out only with small amount of sample (few Milligram of the non-altered sample) 5-Pre-processing by high temperature or using some solvents for sterilization in not necessary 6-Save time 7- Save materials 8-multiple samples can be tested at the same time (Capitain et al., 2020).	The detector should be so sensitive to get reliable results.

Methods	Methodology	Advantages	Disadvantages
5- Fourier transforms infrared spectroscopy (FTIR) Useful technique to identify the functional groups by assessing the rate of infrared radiation over a range of wavelengths which is absorbed by these groups. It is used to detect oxidative products of physical plastic degradation (Montazer et al., 2019).	Fourier-transform infrared spectroscopy is a method that is used to gain an infrared spectrum of emission or absorption in a solid, liquid, or gas. FTIR monitors chemical changes and modifications in the polymer structure. Also, it detects the formation of chemical groups in polymers by microbial biodegradation (Raddadi & Fava, 2019).	1-Can give accurate information quantitatively and qualitatively without disturbing the sample 2- Can be used for different types of samples regardless of their physical status e.g. organic, inorganic, biological, polymer, etc. (András Gorzsás & Janice P L Kenney, 2012).	1- Not reliable when plastic has additives in its formulation 2- Removal of biofilm from plastic surface to avoid the interference in the identification of functional groups is compulsory which is a time-consuming process (Raddadi & Fava, 2019).
6- Radiolabeling	In this method carbon of polymer is labeled by carbon isotope ^{14}C and is used as the substrate for microbial activity and growth. Mineralization can be determined by measuring radioactive gases ($^{14}CO2$, $^{14}CH4$) which are produced by microbial activity (Raddadi & Fava, 2019).	Easy and sensitive to monitor without loss of material (Holtzhaue, 2006).	1-hard to employ radioactive labeled compounds and manage their waste 2-Producing labeled polymer with radioactive potential and similar molecular weight and same features is another obstacle in using this method (Lendlein & Sisson, 2011).
7- Enzyme Assays	In this method, several types of purified enzymes are kept in a buffered or pH-controlled system to which polymer substrate is added (Lendlein & Sisson, 2011).	1-Fast method 2- gives reasonable quantitative information (Lendlein & Sisson, 2011).	1-Mineralization rates cannot be determined by this test 2- If enzymes are not purified or stabilized properly, inhibitors can interfere in enzyme activity 3- If enzymes are paired to one polymer instead of pairing with different polymers as a screening tool it causes some problem in the test (Mayer & Kaplan, 1993).
8- CO_2 evolution test — In aerobic condition Sturm test and the laboratory-controlled composting test	Releasing carbon dioxide or methane can be an obvious result of mineralization. So carbon evolution test is a useful method to determine biodegradation. (Lendlein & Sisson, 2011).	1-Easy to carry out 2- sensitive enough. 3- A direct measurement for mineralization.4- Both water-soluble or insoluble polymers can be tested. 5-Test condition is flexible to the environment and condition where biodegradation is taking place (Lendlein & Sisson, 2011)	In complex environments is difficult to carry out with amount number of interfering compounds (Lendlein & Sisson, 2011).
8- CO_2 evolution test — In anaerobic biodegradation anaerobic sludge test anaerobic digestion test are used to determine CO_2 evolution			

Methods	Methodology	Advantages	Disadvantages
9- Plate test is based on determining cell number	This method is based on applying test material on the surface of salt agar in a petri dish that consists of no additional carbon source (Lendlein & Sisson, 2011).	1-Sensitive 2- only count living bacteria which is often the important issue 3- strains that grow on plates can often be easily isolated, characterized, and identified by traditional methods (Scow et al., 2001)	1-Positive result in this test won't indicate biodegradation and just shows microorganisms' growth which can be a cause of contamination or plasticizers, etc. which is still stuck in the polymer. 2-Complimentary tests should be performed for confirming the result 3- time consuming (Lendlein & Sisson, 2011).
10- Reflectometric interference spectroscopy (RIfS) is based on measuring bimolecular kinetic reactions.	This method is based on the emission of a white light beam onto a glass coated with a known layer. Interference patterns are formed and shifted because of the absorption of the analyte by layer. These shifts conduce to optical thickness change in the layer (Belmont et al., 2007)	1- very simple and successful approach 2- cheap 3-robust and reliable sensor elements 4- temperature dependency is low (Hänel & Gauglitz, 2002)	1-Needs good thermostats (below 0.1 K) 2-need to be referenced well using a dual-channel instrument (Mehlmann & Gauglitz, 2005).
11- Dynamic light scattering method is based on measuring the size of molecules and particles typically in the submicron region.	This method is used in measuring of Polyethylene terephthalate (PET) average nanoparticles, diameter, and its size distribution by applying a laser size scattering instrument by Wong et al (2018).	1- Measures particle sizes of 1nm 2- Short experiment duration (1-2min) 3-Reliable and repeatable analysis 4- No need for sample preparation 5- Low sample volumes (200uL) 6-Measures diluted samples 7-low cost (Stetefeld et al., 2016)	1- is highly sensitive to temperature and solvent viscosity 2- constant temperature and solvent viscosity are necessary to gain reliable results 3- differentiation of related molecules is challenging in this method 4- proper cleaning of the sample-holding cuvette before the measurement is essential (Jose et al., 2019)
12- Scanning Electron Microscopy (SEM) Can be used in examination and analysis of the morphology and chemical composition characterizations of molecules (Zhou et al., 2006) SEM images may show surface corrosion of polymers,	The surface morphology and dimensions of the samples are characterized by producing various signals as a result of interacting electrons in the beam over the surface but because of noise ratio, signal and the quality of the images can be affected and current resolutions of ~5nm are the best can get (Donald, 2003). Image formation in the SEM is related to the range of signal absorption produced from the electron and specimen interactions (Zhou et al., 2006)	1- Useful for complex samples. 2-Has vast application in different fields 3- Gives three-dimensional images. 4- Easy to operate and user-friendly method by using computer technology. 5- This method works fast. 6-Only needs minimal pre-preparation before placing in a vacuum chamber that is an advantage of this method to compare with others (Choudhary & Choudhary, 2017).	1-expensive 2-Needs a place far away from all-electric, magnetic, and vibration interference which is hard to establish 3-trained operators for carrying out and pre-preparing the samples are needed 4-Expertise researchers to operate 5-For solid and inorganic samples with a small size to be able of settling in a vacuum chamber and tolerated vacuum pressure 6-Risk of radiation exposure with electrons that scatter from the sample surface should be considered (Choudhary & Choudhary, 2017).7- SEM images alone cannot provide evidence for complete biodegradation of polyethylene (Montazer et al. 2019)

Methods	Methodology	Advantages	Disadvantages
13- High-Performance Liquid Chromatography (HPLC) Main components in this system contain a solvent, a high-pressure pump, a column, injector system, and the detector	Another form of column chromatography in a high pressure, a sample mixture in a solvent (mobile phase) pumps through a column consist of chromatographic material (stationary phase). Ultimately the sample is carried by Helium or Nitrogen stream(https://sciencing.com/disadvantages-advantages-hplc-5911530.html)	1- extremely quick and efficient 2- It uses a pump, rather than gravity, to force a liquid solvent through a solid adsorbent material 3- The process can be completed in roughly 10 to 30 minutes with high resolution 4- accurate and highly reproducible 5- largely automated with minimum training (https://sciencing.com/disadvantages-advantages-hplc-5911530.html)	1-costly, requiring large quantities of expensive organics 2-does have low sensitivity for certain compounds especially volatile substances are better separated by gas chromatography 3- relatively easy to use but can be complex to troubleshoot problems or to develop new methods
14- Gas Chromatography-Mass Spectrometry (GC-MS) combination of high-resolution capillary gas chromatography with mass spectrometry (GC/MS) in the analysis of volatile and semi-volatile organic molecules. Biofragmentation and the existence of saturated linear alkanes can be determined in culture media after biodegradation of polyethylene (Montazer et al., 2019).	Sample vaporized into the gas phase and separated to its components by capillary column coated with a stationary phase. Then compounds are moved by a gas such as helium, hydrogen, or nitrogen, and compounds based on their boiling point and polarity will be separated. After leaving the CG column, they are fragmented by ionization in the mass spectrometer.	1-vast application 2-sensitive (low detection limits), 3-highly quantitative and qualitative (specific) 4-fast 5-reliable 6-re-producible, 7-cheap 8-user friendly 9-portable 10-no waste 11- safe (Maštovská & Lehotay, 2003).	1- sample preparation with specific chemicals to increase thermal stability and volatility 2-time consuming 3- harsh ionization 4-limitation in analysis based on number of molecules (Want et al., 2005)
15-NMR or Nuclear magnetic resonance spectroscopy	In this method by placing a sample in a magnetic field, the NMR signal excites the electron of the nuclei with radio waves into nuclear magnetic resonance which can be assessed with sensitive radio receivers. All result in getting more information about functional groups and the electronic structure of molecules (Mohamed et al., 2020)	1- quickly measure analytes in bio fluids 2-accurate, 3-without the need for initial preparation. 4-Better resolution in recent years 5-lower instrument cost (Want et al., 2005)	1-poor sensitivity and dynamic range 2-some chemical classes cannot be detected (Want et al., 2005)

2 Materials and Methods:

The methodology was based on microorganism isolation and identification at the first step. The second Step was planned to detect microbial biodegradation by determining microbial growth on polyethylene in a liquid medium. Bacterial growth was assessed in all flasks in determined intervals over 3 months. The third step was testing stimulation and synergistic effects on biodegradation.

2.1 Isolation of plastics degrading microorganisms

2.1.1 Source of strains used

All strains were used in this project were prepared based on this protocol written by Andrew Jenkins.

Isolation on solid medium. Fragments of degrading plastic were placed on M9-LDPE agar plates and macerated in a drop of sterile PBS, which was then spread out on the surface of the plate. Plates were incubated for up to 12 months and examined periodically for signs of growth. Where microbial growth was observed, visually, or under ca. 50x magnification (Zeiss Discovery 2.0 stereomicroscope) This material was transferred to ½TSA, streaked to single colonies and colony purified by further streaking where necessary.

After 12 months of incubation, the plates had become dehydrated. They were rehydrated by the addition of 10 ml of M9 medium and gentle rotary shaking overnight. A further 10 ml of M9 medium was then added and a sterile plastic spreader was used to suspend any microbial growth on the surface. The suspension was pipetted off into 45 ml of M9 medium in a 250 ml Erlenmeyer flask and incubated for 3 months with gentle rotary shaking.

All cultures showed evidence of growth and they were spread on TSA plates and re-streaked to single colonies. These strains are the ZZ series and are the subject of Zahra Zollanvari's MSc project (Andrew Jenkins, Pers. comm.).

2.1.2 Purification and Isolation of plastics degrading microorganisms

Nutrient media. Half-strength Tryptone Soya Agar (½TSA) was prepared for strain purification and routine culture. ½TSA consists of 10g TSA, 5g Bacto Agar in 500 ml deionized water. Then all the microorganisms were streaked out on TSA medium and kept at room temperature (21° C) for 5 days and let them grow. All samples were re-cultivated based on the shape, color, size and texture of colonies on new TSA media to gain purified cultures. Table (2-1) is a list of ingredients to make ½ TSA media

Table2- 1: ½ TSA media ingredients

½ TSA media half strength	
TSA	10 gr
Agar	5 gr
Water	500µl

2.1.3 Characterization of strains

After purification of colonies, the characterization of strains was tested by catalase, oxidase, and gram stain tests on purified samples to determine physical and chemical characteristics.

Then all samples were observed under a microscope with a resolution of 100X and photos of them were taken which can be seen in Figure (3-2).

2.1.3.1 Catalase Slide Method

The catalase test is one of the biochemical tests that shows the presence of catalase enzyme. For carrying out this test, the protocol by Reiner (2010) was implemented which is described below:

a. Use a plastics loop and take a small amount of growth colony and rub it on the surface of a clean, dry glass slide.
b. Place a drop of 3% H_2O_2 on the glass slide and mix it with bacterial colony
c. Observe the evolution of oxygen bubbles.

2.1.3.2 Oxidase test

First, 1% solution of oxidase reagent (6-NNNN-tetramethyl-1-4 phenylen diammoniumdichloride) was prepared and the method based on Shields & Cathcart (2010) protocol was carried out as below:

a. A filter paper is soaked with freshly made 1% solution of the reagent.
b. With a small loop, a speck of each bacterium sample is rubbed on the soaked paper.
c. An intense deep-purple hue is considered as a positive reaction appearing within 5-10 seconds, and a negative reaction by the absence of coloration or purple coloration after 60 seconds.

2.1.3.3 Gram staining

Gram staining test is a popular method is used to differentiate two large groups of bacteria based on their different cell wall components.

All reagents were used in gram staining are mentioned in Table (2-2) and the protocol is mentioned below as described by Moyes (2009).

Table2- 2: Reagents for gram staining identification test

Crystal violet (primary stain)
Iodine solution/Gram's Iodine (mordant that fixes crystal violet to the cell wall)
Decolorizer (e.g. ethanol)
Safranin (secondary stain)
Water (preferably in a squirt bottle)

For gram staining

a) Make a suspension of the bacterial culture and spread a loop of that on a glass microscope slide.
b) Allow it to dry.
c) Pass through a Bunsen flame three times.

   This fixes the bacteria to the slide and is a critical step. Insufficient heat will allow the cells to loosen from the slide. Too much will destroy the cell structure. The right speed is about the same as that used when using a handsaw.

d) Flood the slide with crystal violet. Wait 1 minute

e) Rinse the slides with a stream of water to remove additional crystal violet.

f) Then pour Gram's iodine over the slides and leave for 1 minute.

This agent fixes the crystal violet to the bacterial cell wall.

g) Rinse the Slides with acetone or alcohol for ~3 seconds and then rinse with a gentle stream of water.

h) Pour Safranin over the slides as a secondary stain and wait for 1 minute. Again wash all slides with a gentle stream of water for 5 seconds.

i) Observe bacteria under the microscope.

Samples with violet color are gram-positive while samples with red color are gram-negative bacteria.

2.1.4 Samples preservation

All samples were preserved in ½ TSA media with 25% glycerol in a freezer at -70°C for further researches.

2.2 Growth in liquid media

bacterial strains were tested for the ability to grow on polyethylene as a sole carbon source in a procedure as below. Polyethylene used for the whole project was low-density polyethylene (LDPE).

2.2.1 Inoculate purified bacteria in M9 medium

First, 1-gram LDPE added to 50 ml of M9 medium in 200ml Erlenmeyer flask. Then, one loop (include approximately 0.9 OD bacterial dilution) of each provided sample was added to prepared flasks and suspended completely. All flasks were covered with aluminum foil to inhibit contamination entrance. Lastly, all samples were incubated at room temperature (24°C) on a stirring shaker for 5 days and leave microorganisms to grow.

Table2- 3) is a list of Sigma M9 salt (Minimal salt) and trace element solution 2 (1000×) compositions. To prepare M9 (5×) media, 5.6 gr M9 salt added to 500 ml water consist of 500µl solution 2 (1000×) trace element solution.

In this study, 50 ml M9 medium + 2% w/v*[1] polyethylene was called M9-PE and this abbreviation is used in the whole project.

Table2- 3: Composition of M9 salt (5×) and 1000X trace elements solution

M9 salt 5×		1000X trace elements solution 2			
KH2PO4	15 g/L	MnSO4 - H2O	0.00017 mg/L	NiSO4 - 6H2O	0.00013 mg/L
NaCl	2.5 g/L	NaSiO3 - 9H2O	0.140 mg/L	SnCl2 (anhydrous)	0.00012 mg/L
Na2HPO4.7H2O	33.9 g/L	Molybdic Acid	0.00124 mg/L		
NH4Cl	5 g/L	Ammonium Salt NH4VO3	0.00065 mg/L		

2.3 Assessing microbial growth on Polyethylene

2.3.1 OD (Optical Density) measurement

This measurement is one of the main and prevalent measurement methods is used for detecting bacterial density and bacterial growth in a sample. Sutton (2011) used the same assay in measuring the microbial cells in his study.
After 7 and 13 days of bacterial inoculation in M9-PE, OD measurement was carried on all samples. To prevent the polyethylene interference on measurement results, 200 µl of all samples were filtered through nylon mesh with a mesh size of 50 µm. Then, 100µl of filtered samples were added to OD cuvette for reading and all samples were read by Eppendorf Bio photometer in wavelength of 600 nm.

2.3.2 ATP measurement

The ATP bioluminescence assay shows an estimation of bacterial metabolic activity in a bacterial suspension and is a rapid measurement (kounty et al., 2006). AquaSnap Total swab made by Hygiena company (www.hygiena.com) was used in all ATP measurements.

[1] weight/volume

Siro (1985) used ATP mesaurment in this study to measure bacterial growth. To carried out this test:

700 µl of fresh M9-PE (with Polyethylene and microbial inoculum) was filtered through nylon mesh with a mesh size of 50 µm and preserved in 1.5 Eppendorf. Then ATP AquaSnap total swap was completely soaked in filtered M9-PE culture and Read by an illuminometer device.

2.3.3 Viable plate count measurement

Viable plate count was carried out to determine whether the number of viable cells are increasing in the sample in time or not?

First, 100µl of M9-PE of all main samples were spread on ½ TSA plate media and incubated at room temperature for 10 days and viable cell density was measured after this time. For samples with a high CFU/ml rate, more serial dilutions were considered.

For viable plate count test by serial dilution: 100µl of M9-PE of all main samples were taken for serial. Then all dilutions spread on ½ TSA media, the number of colonies (viable cell density) were counted again after 16 days of inoculation to determine the effect of time on viable cell density.

The number of microorganisms in the particular test sample is determined by using this formula:

CFU[2]/mL= CFU x dilution factor /volume plated (ml)

2.3.4 CO_2 Evolution Test

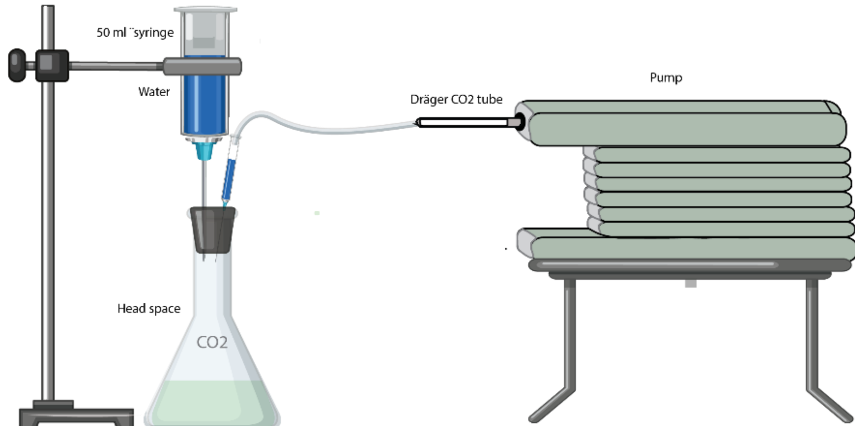
Because of using plastics as carbon sole resource, CO2 is released which can be a reasonable criterion in determining biodegradation of plastics. To prepare the samples for this test, in the first step, 500µl of 0.9 OD microbial dilution (to make all bacterial dilution equal) inoculated in the M9-PE medium in 150 ml Erlenmeyer which covered up with a rubber cap in order of prohibiting all gas transfer with circumference.

The system was designed by Andrew Jenkins for CO_2 evolution test and can be seen in figure (2-1). As the figure shows, two syringes were used in this system (1ml and 50 ml). At the end of the 1ml syringe, a tube was linked to a pump which a CO_2 100/a Dräger

[2] colony-forming unit" (CFU) is used for each colony

tube (Dräger company, Netherland) was installed at mid. Water was poured in the 50 ml syringe and it caused exotic pressure in the system and CO_2 went out by another syringe needle as the result of pumping pressure. Using these two syringes seems to be necessary because, in lack of them, there would be another pressure in the flask and since the pump may not be very powerful, it would not be able to draw a full 75 ml air in sample.
If the syringes were empty, atmospheric air would be drawn into the flask. This would mix with the headspace air and dilute the carbon dioxide, resulting in an underestimate of CO_2 concentration. The 50 ml syringe full of water (or fresh medium) allows pressure equalization while simultaneously preventing contamination of the headspace with ambient air.
If the targeted chemical(s) reacts with the reagent in the tube, the color of the Dräger tube is changed to violet which shows the presence of CO_2 and biodegradation. All results can be seen in Table (3-4).

Figure 2- 1: CO_2 measurement system designed by Andrew Jenkins



Some other complimentary tests were carried out on determining the chemicals with stimulation effect and bacterial synergistic effect on plastics biodegradation which can be used in further researches.

2.4 Stimulation effect experiment on bacterial growth

Since some chemical compounds have the stimulation effect on bacterial growth, three chemicals were tested to determine this effect on our bacterial growth which may

ultimately conduce to an acceleration in biodegradation. These chemicals were selected based on Ashino et al, (2019) works on bacterial growth by testing different chemicals.

2.4.1 Mineral oil

Since mineral oil (liquid paraffin) was largely used as a source of carbon and hydrogen (hydrocarbon) it was selected as stimulator for ZZ-6 strain (with higher results in TSA measurement). Firstly, this test was conducted without PE to just determine the effect of different proportions of oil on bacterial growth. So, different proportion of mineral oil based on Table (3-5) was added to 50 ml M9 medium without polyethylene and all flasks were autoclaved and covered with aluminum foil. Then, 0.1 OD dilution of ZZ-6 sample added to each flask and placed over spinning shaker to let bacterium grow. After 5 and 13 days, ATP measurement was conducted for each flask.

Based on observed results, to answer this question that ''Does the mineral oil inhibits bacterial growth and kill them instantaneously or simulates biodegradation?'' another test was carried out based on plate count measurement as follow:

Three (3) colonies of ZZ-6 strain were diluted in 1ml of M9 medium, named ''dilution 1'' and vortexed well to observe homogenous suspension. 100µl and 20 µl mineral oil in separated Erlenmeyers were added to 50 ml M9 consists of 100 µl of ''dilution 1'' without PE. Then after a short time, serial dilution was performed to 10^{-5} and all dilutions were spread on ½ TSA plates. All plates were incubated for 6 days and counted the colonies. An exposure time of mineral oil with bacterium was approximately 5-10 minutes in order of verifying the killing effect of mineral oil and simultaneously comparing the effect of different dilutions of oil on bacterial growth.

On the other hand, the effect of mineral oil on ZZ-6 bacterium in M9-PE was tested by ATP measurement and viable plate counting to see the possible effect of mineral oil on bacterial growth with polyethylene.

100µl mineral oil was added to 50ml M9-PE autoclaved medium consist of 100 µl suspension of ''dilution 1''. ATP measurements were performed in 8, 20, and 27 days after inoculation. Also, after 8 days of incubation, the sample was taken for the viable count to 10^{-4} and all dilutions were spread on ½ TSA plates and were incubated for 11 days before a new viable count was performed. The second viable count was carried out

18 days after inoculation and serial dilution was performed similar to the previous procedure.

2.4.2 Stimulation experiment with $(NH_4)_2SO_4$ and $MgSO_4$ chemicals on bacterial growth

In continuation of testing the stimulation effect of some chemicals on plastics biodegradation, another test was conducted with two compounds that were, $(NH_4)_2SO_4$ and $MgSO_4$. Ashino et al, 2019 used these chemicals in their work and showed their effective impact on bacterial growth. Since, ZZ-6, ZZ-7, ZZ-2, ZZ-12-2 strains had better results in ATP measurement, they were selected for stimulation experiment.

To test the stimulation effect of ammonium sulfate $(NH_4)_2SO_4$:

200 µl 1M $(NH_4)_2SO_4$ was added to separate flasks consist of 50 ml M9-PE inoculated by 100 µl of 0.1 OD dilution of ZZ-2, ZZ-6, ZZ-7 and ZZ-12-2 strains. ATP, CO_2 and plate count measurements followed the bacterial growth in all aforementioned samples. Negative control consists of no added $(NH_4)_2SO_4$ was prepared by inoculating only 100 µl of a 0.1 OD dilution of ZZ-2, ZZ-6, ZZ-7 and ZZ-12-2 bacteria to 50 ml M9-PE.

To test the stimulation effect of magnesium sulfate ($MgSO_4$)

200 µl 2M $MgSO_4$ was added to separate flasks consist of 50 ml M9-PE inoculated by 100 µl of a 0.1 OD dilution of ZZ-2, ZZ-6, ZZ-7 and ZZ-12-2 bacteria. ATP, CO_2, and plate count measurements again were carried out on these samples. Negative control consists of no $MgSO_4$ was prepared as the previous procedure for $(NH_4)_2SO_4$. All flasks were covered with cotton and aluminum foil and incubated at room temperature. The final concentration is 4 mM with respect to ammonium sulfate, but 8 mM with respect to ammonium.

*For preparing 1M $(NH_4)_2SO_4$, 13.2 gr of this compound was added to 50 ml distilled water (1 Molar=132 g/mol means 132 gr of substance is dissolved in 1000ml of water)

**For preparing 2M $MgSO_4.7H_2O$, 12.3 gr of this compound was added to 50 ml distilled water (2Molar= 246g/mol).

2.4.2.1 CO2 Evolution test for confirming stimulation effect on bacterial growth and biodegradation

After measuring the stimulation effect with ATP and plate counting assessment in order of identifying whether stimulated samples have more biodegradability or not, CO_2 evolution test was carried out on only ZZ-2 and ZZ-6 samples that showed better result in ATP measurements, The CO_2 evolution test was conducted as follow for $MgSO_4$:
500 µl of 0.9 OD dilution of ZZ-2 bacterium was added to 50 ml M-PE medium and 200µl 2M $MgSO_4$ was added to the flask and covered with a rubber cap. Also, a negative control was prepared for each sample by inoculating 500 µl of 0.9 OD dilution to M9-PE.
CO_2 evolution test for the stimulation effect of 1M $(NH_4)_2SO_4$ also was carried out on the ZZ-6 sample (with better results in ATP measurement). Like previous procedure 500 µl of 0.9 OD dilution of ZZ-6 bacterium was added to 50 ml M-PE medium and 200µl 1M $(NH_4)_2SO_4$ was added to the flask and covered with a rubber cap and also negative control of this sample was prepared similar to the $MgSO_4$ procedure.
A suspension, consists of 0.05 gr $FeCl_2$ and 0.05 gr $CuSO_4$ dilution in 20 ml water was prepared and 200µl of this suspension was poured in M9-PE samples inoculated with ZZ-2, ZZ-3, ZZ-6, ZZ-7, ZZ-12-2 and ZZ-13 strains and CO_2 evolution test was carried out on these samples one week later to determine the stimulation effect of these chemicals on biodegradation but because of limited time, integrating this part with the whole of this study was impossible.

2.5 Synergy Experiment

To determine the bacterial synergistic effect on polyethylene biodegradation, another experiment was planned based on different bacterial consortium based on Mikesková et al, 2012 works.
Different bacterial mixed cultures were prepared based on locations where bacteria were collected and ATP results. Two criteria were considered to make a mixed culture:

1. The Combination of two bacteria that were collected from the same place (same plastic host for degrading) to decrease the effect of different factors on the test.
2. Each bacterium showed a different growth rate based on ATP measurement (one with low bacterial growth and another with high bacterial growth results) to investigate their synergistic effect on biodegradation

For ATP measurement, in the first step, pure culture of all bacteria was prepared to compare their results with mixed cultures. 100µl of 0.1 OD bacterial dilution was inoculated to 50ml M9-PE medium in 150 ml Erlenmeyer and covered with cotton and aluminum foil.

For preparing bacterial mixed cultures, 50 µl of each suspension with 0.1 OD dilution was added to 50ml M9-PE media. All the results after 7, 24 and 114 days were registered in Table (3-11).

2.5.1 Plate counting test to determine the synergistic effect

As a comparison to the results of ATP measurement assay for synergistic effect, direct plate counting was conducted on only three samples (ZZ-2, ZZ-3, and ZZ-2+ZZ-3). 100 µl of inoculated samples prepared in ATP measurement were directly poured over ½ TSA media and kept at room temperature and the plated were counted after 6 days.

2.5.2 CO_2 evolution test for determining the synergic effect

To test the synergistic effect of ZZ-1+ZZ-2, ZZ-2+ZZ-3, and ZZ-11+ZZ-12-2 mixed culture on biodegradation, the CO_2 evolution test was conducted after 14 days for both pure (as negative control) and mixed cultures.

250µl of 0.9 OD dilution of ZZ-1 bacterium was mixed with 250µl of 0.9 OD dilution of ZZ-2 bacterium and added to 50 ml M9-PE medium. Simultaneously, pure cultures of ZZ-1 and ZZ-2 as negative control were prepared by adding 500 µl of 0.9 OD bacterial dilution to 50 ml M9-PE medium. The same procedure as explained was carried out for other mixed cultures.

2.6 Statistical methods

To calculate the correlation coefficient between ATP with OD and plate count measurement RStudio program, Rcmdr (Rcommander) package were used.

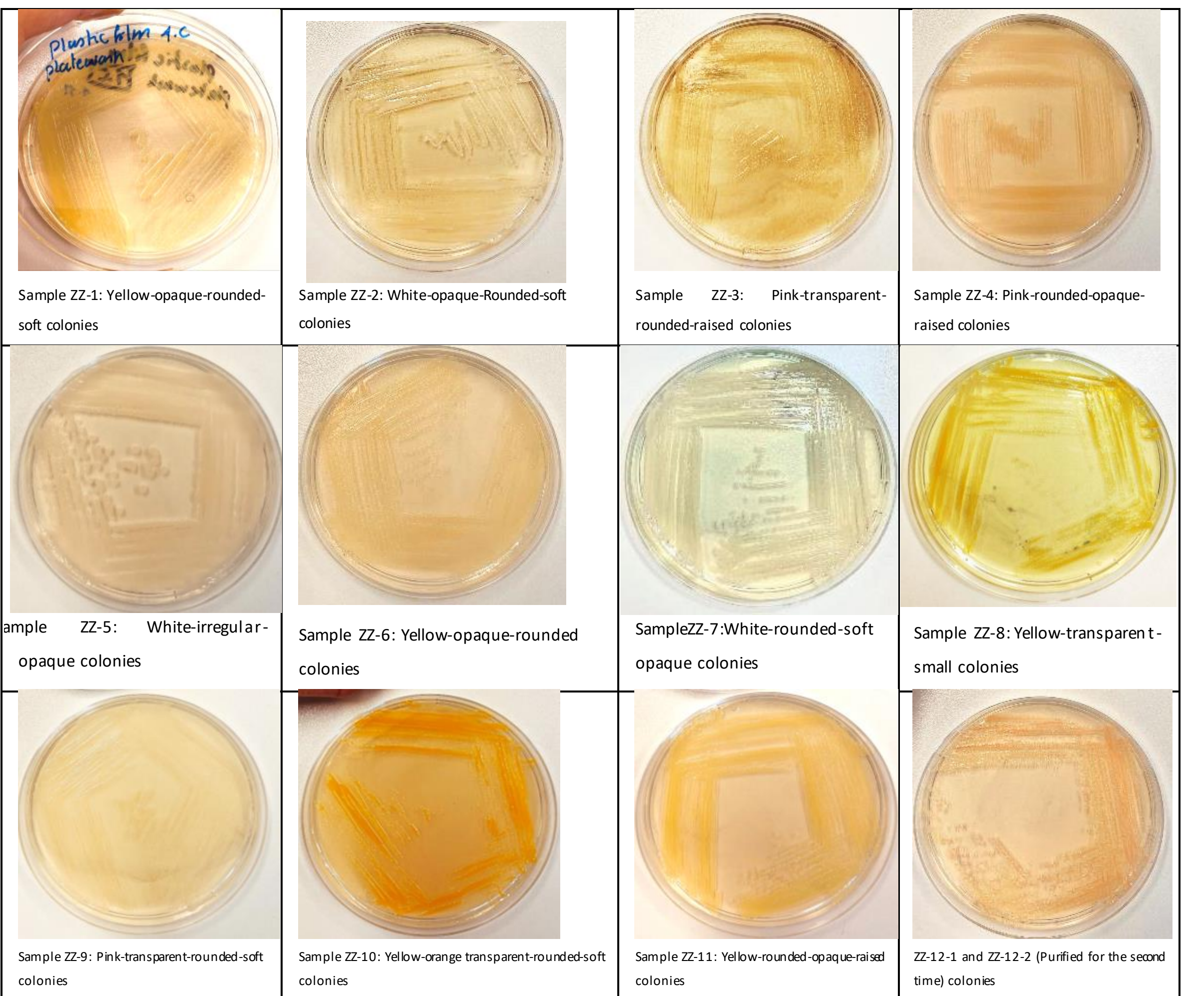
3 RESULTS

Here the results will be separated into Isolation of plastic-degrading microorganisms and assessing the growth of microorganisms on Polyethylene sections and ultimately we will depict the result as complimentary tests to investigate the stimulation effect of three chemicals on bacterial growth and synergistic effect of mixed cultures.

3.1 Isolation of plastics degradable microorganism

In the isolation of microorganisms, different strains of bacteria with different apparent characteristics were observed by chemical tests such as catalase, oxidase, and gram staining. Purification of samples was repeated by streaking out the colonies of microorganisms on ½ TSA media to obtain pure cultures for further biodegradation test. all the results were depicted based on color, the shape of colonies, density, and stickiness in Figure (3-1).

Figure3- 1:Morphological characteristics of colonies after 2times purification



Sample ZZ-1: Yellow-opaque-rounded-soft colonies

Sample ZZ-2: White-opaque-Rounded-soft colonies

Sample ZZ-3: Pink-transparent-rounded-raised colonies

Sample ZZ-4: Pink-rounded-opaque-raised colonies

ample ZZ-5: White-irregular-opaque colonies

Sample ZZ-6: Yellow-opaque-rounded colonies

SampleZZ-7:White-rounded-soft opaque colonies

Sample ZZ-8: Yellow-transparent-small colonies

Sample ZZ-9: Pink-transparent-rounded-soft colonies

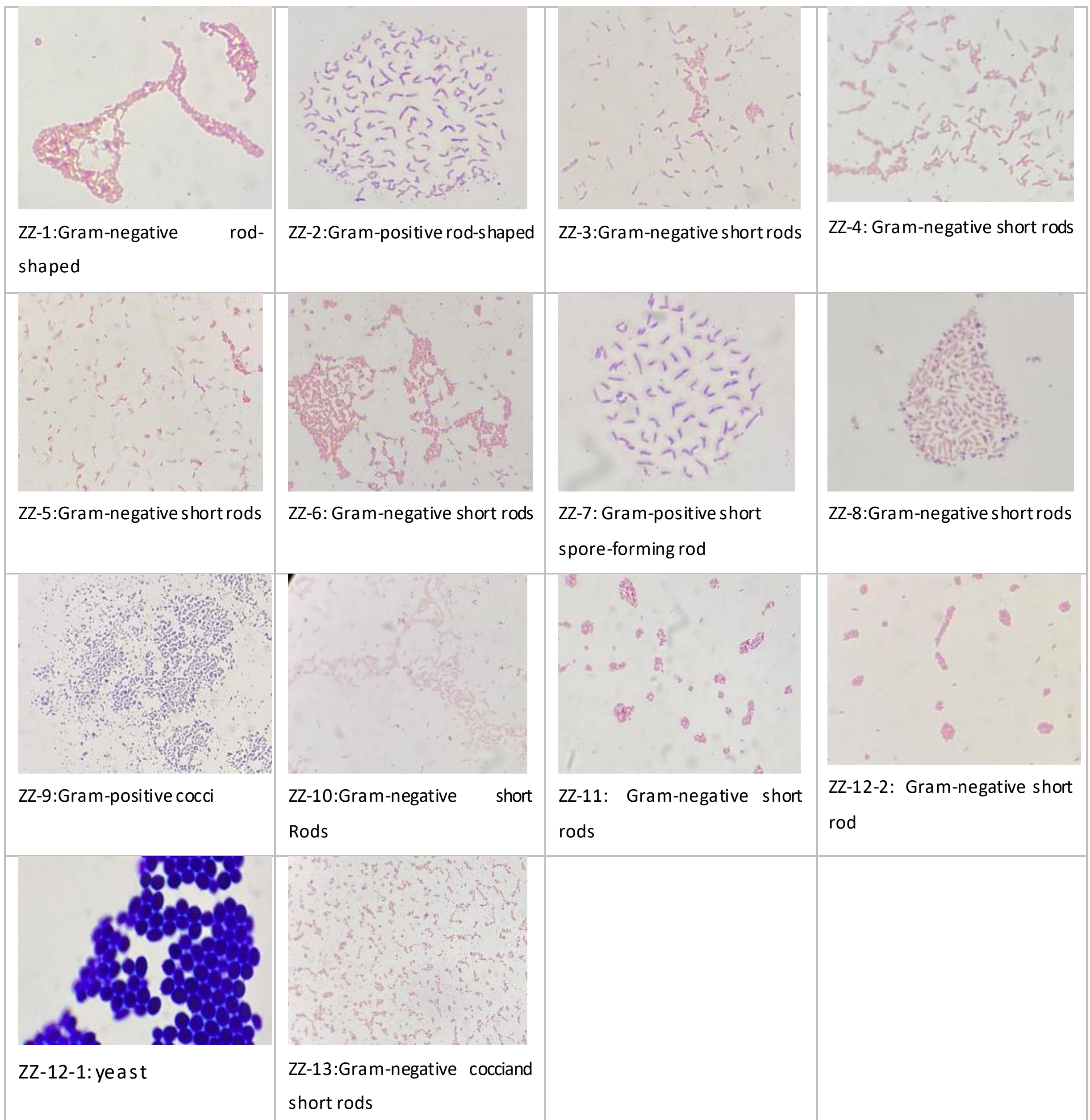
Sample ZZ-10: Yellow-orange transparent-rounded-soft colonies

Sample ZZ-11: Yellow-rounded-opaque-raised colonies

ZZ-12-1 and ZZ-12-2 (Purified for the second time) colonies

After determining apparent characteristics such as the color of the colonies, size of colonies and the shape of colonies, all samples were observed under microscope with resolution 100X and photos of them were taken which can be seen in Figure (3-2).

Figure3- 2: Microscopic images after Gram staining with 100X magnificent resolution of all samples.



ZZ-1:Gram-negative rod-shaped

ZZ-2:Gram-positive rod-shaped

ZZ-3:Gram-negative short rods

ZZ-4: Gram-negative short rods

ZZ-5:Gram-negative short rods

ZZ-6: Gram-negative short rods

ZZ-7: Gram-positive short spore-forming rod

ZZ-8:Gram-negative short rods

ZZ-9:Gram-positive cocci

ZZ-10:Gram-negative short Rods

ZZ-11: Gram-negative short rods

ZZ-12-2: Gram-negative short rod

ZZ-12-1: yeast

ZZ-13:Gram-negative cocciand short rods

Based on chemical tests and apparent microscopic observations, all strains were classified in Table (3-1) with their characteristics. Possible plastotroph is an expression we used for some samples based on their turbidity observed at first glance in the M9-PE medium.

Table3- 1: Morphology, Microscopic, Catalase/Oxidase/Gram characteristics of collected samples

Strain	Colony Morphology	Oxidase	Catalase	Gram	Cell shapes	Plastotrophy	Source
ZZ-1	Yellow-opaque-rounded-soft	-	+	-	Rod	yes	PF
ZZ-2	White-opaque-Rounded-soft	-	+	+	Rod	yes	PF
ZZ-3	Pink-transparent-rounded-raised	+	+	-	Short Rod	NA (maybe grow slowly)	PF
ZZ-4	Pink-rounded-opaque-raised	-	+	-	Rod	NO	PT
ZZ-5	White-irregular-opaque-	+	+	-	Dual Rod	NO	PT
ZZ-6	Yellow-opaque-rounded-high consistancy	-	+	-	Short Rod	YES	PT
ZZ-7	White-rounded-soft-opaque	-	+	+	Spore forming, rod	YES	PS
ZZ-8	Yellow-transparent-small colonies	+	+	-	Short Rod	NO	PS
ZZ-9	Pink-transparent-rounded-soft	+	+	+	Cocci	NA(maybe grows slowly)	PF
ZZ-10	Yellow-orange transparent-rounded-soft	+	+	-	Short rod shape	NO	PF
ZZ-11	Yellow-rounded-opaque-raised.	-	+	-	Short rod	NA	PF
ZZ-12-1	Pink-opaque-small (yeast)	+	+	+	Big cells	YES(Yeast)	PF
ZZ-12-2	Yellow-transparent-small	-	+	-	Short rod	YES	PF
ZZ-13	Yellow-rounded-opaque-big colonies	+	+	-	Cocci and short rods	NO	PS

PS: Polystyrene foam, ' Fjelldalen, Skien' PF: Plastic film, 'Fjelldalen, Skien' PT:Plastic tray, 'Fjelldalen, Skien'

3.2 Assessing the growth of microorganism on Polyethylene

3.2.1 ATP and OD measurements

As it was mentioned in the method sections, all the results were registered in 7 and 13 days intervals for OD measurement after inoculating pure cultures in M9-PE. ATP measurement was carried out in 7, 13, 29, 40, and 63 days after inoculation. According to data obtained from OD measurement, some fluctuation results were seen through carrying out this test that could be due to growth, death and lysis, or attachment to the plastic particles or plastics buoyancy and filtration improperly; So alternative methods such as plate counting and CO_2 evolution test were considered instead of OD measurement on samples with high ATP results. Only measurements continued with ATP assay later and OD reading was omitted because of poor accuracy. For OD and ATP measurement all the results are registered in Table (3-2).

Table3- 2: All data gained from OD and ATP measurement are registered on different dates to show the rate of growth in all samples

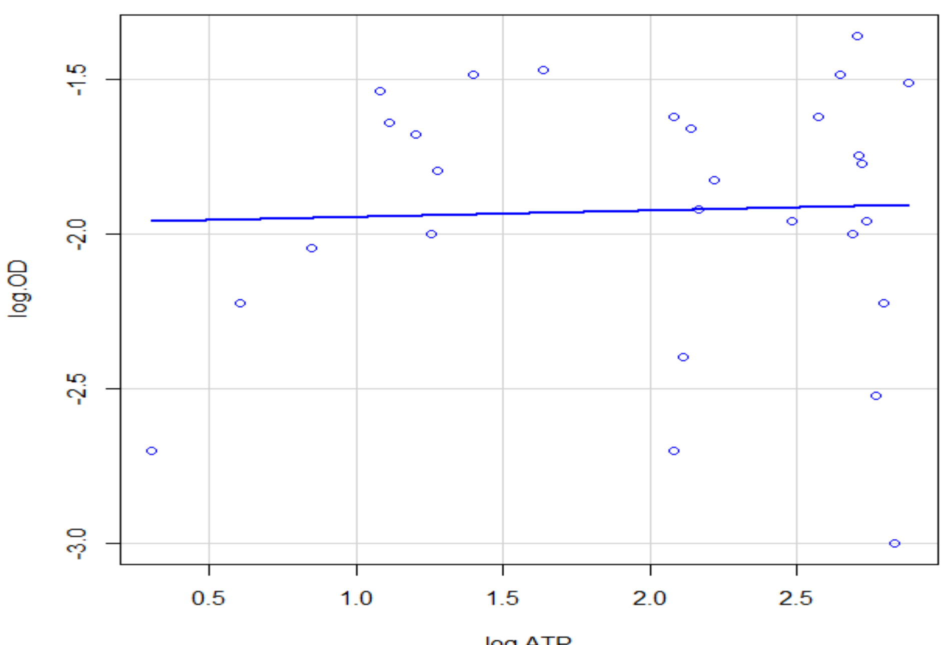
Strain	OD (Optical density) 7 days after inoculation	OD (Optical density) 13 days after inoculation	ATP 7 days after inoculation	ATP 13 days after inoculation	ATP 29days after inoculation	ATP 40 days after inoculation	ATP 63 days after inoculation
ZZ-1	0.011	0.024	304	372	388	315	204
ZZ-2	0.006	0.001	621	679	940	972	1038
ZZ-3	0.022	0.015	137	166	240	11	203
ZZ-4	0.006	0.002	4	2	0	0	ND
ZZ-5	0.023	0.029	13	12	11	7	2
ZZ-6	0.011	0.031	548	755	936	711	830
ZZ-7	0.018	0.003	513	584	898	933	1057
ZZ-8	0.016	0.021	19	16	12	8	0
ZZ-9	0.024	0.004	121	129	173	179	174
ZZ-10	0.034	0.009	43	7	8	9	ND
ZZ-11	0.012	0.002	146	121	164	167	137
ZZ-12-1	0.017	0.010	527	486	545	608	671
ZZ-12-2	0.044	0.033	507	444	540	530	561
ZZ-13	0.033	0.010	25	18	23	100	3
Negative	0.007	0.006	3	3	3	3	3

Based on Table (3-2), ATP measurement results were plotted in line comparative graphs to determine the samples with positive growth in Figure (3-3).

Figure3- 3: Rate of growth by ATP measurements in all samples

It was observed that ZZ-2, ZZ-7, ZZ-6, ZZ-3, ZZ-12-1, ZZ-12-2 strains have the best bacterial growth respectively in ATP measurement and are used in further complimentary tests i.e. stimulation and synergy tests. On the other hand, based on the results achieved, the correlation between ATP measurement versus OD measurement was investigated in the scatter plot in Figure (3-4). Considering to high difference between data gained in ATP and OD measurements, the logarithm of all data was calculated first. Here, the correlation coefficient is approximately 0.039 which is a very low rate and it is close to zero and there is no correlation between these two measurements.

Figure3- 4: Correlation between ATP and OD measurement



3.2.2 Viable plate count

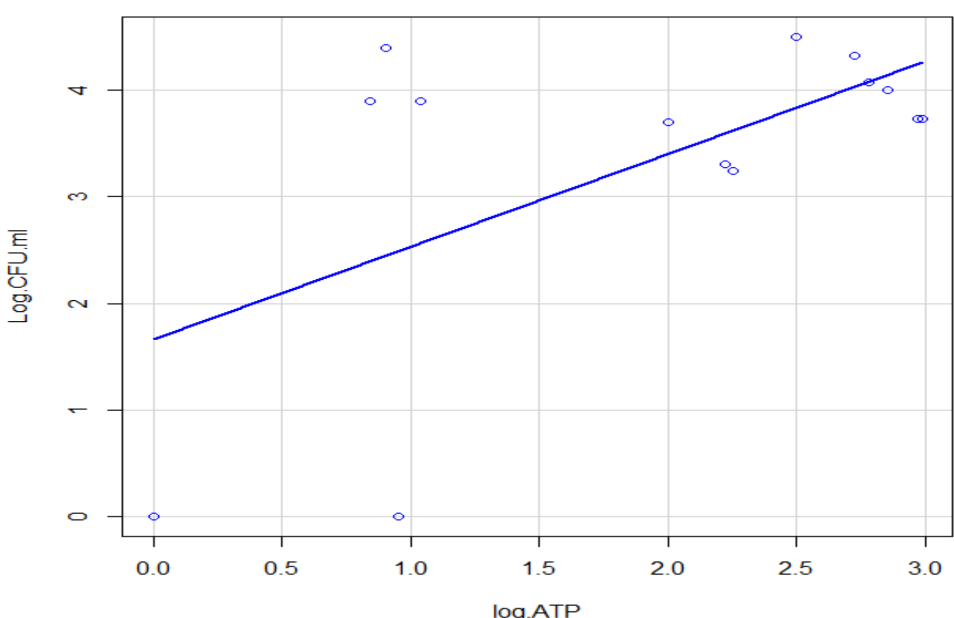
100 µl of all M9-PE source samples were spread out on ½ TSA media for direct plate counting test and the number of colonies was counted after 10 days. After first plate counting, viable serial dilution was carried out for all samples to figure out whether the number of viable cells increase in time or not? As it can be observed in the table, the number of viable cell density had a significant increase in ZZ-1, ZZ-8, ZZ-13 samples in viable plate count in 16 days on polyethylene which can be a good reason to claim that PE could be consumed as a sole carbon resource in these samples and in ZZ-12-2 after 6 days negligible decrease was observed that can be disregarded and was considered as biodegrading sample. As it can be observed, the results in ATP measurement and viable counting method are not in the same agreement even in some samples such as ZZ-2 with the high rate of ATP measurement, very low viable plate counting was observed or conversely for ZZ-13 sample viable plate counting unexpectedly was high to compare with its ATP result that it may returns to different essence of these two methods.

Table3- 3: Viable plate count after 10 and 16 days of incubation for all samples

Strain	Viable count after 10 days (CFU/ml)	Average viable plate count after 16 days by serial dilution (CFU/ml)
ZZ-1	3.2×10^4	29×10^4
ZZ-2	0.542×10^4	0.41×10^4
ZZ-3	0.795×10^4	0.43×10^4
ZZ-4	0	0
ZZ-5	0.793×10^4	0.45×10^4
ZZ-6	0.997×10^4	0.15×10^4
ZZ-7	0.544×10^4	0.12×10^4
ZZ-8	2.5×10^4	9×10^4
ZZ-9	0.176×10^4	0.18×10^4
ZZ-10	0	0
ZZ-11	0.203×10^4	0.07×10^4
ZZ-12-1	1.2×10^4	0.33×10^4
ZZ-12-2	2.1×10^4	1.9×10^4
ZZ-13	0.501×10^4	17×10^4

Also, the correlation between ATP measurement and plate count assay was investigated. Considering to high difference between data gained in ATP and Plate count measurements, the logarithm of all data was calculated and the correlation between them was plotted in Figure (3-5). Based on RStudio analytic program Correlation coefficient is approximately 0.59. Here since the correlation coefficient is between 0.5 and 0.7 it indicates that variables can be moderately correlated.

Figure3- 5: Correlation between ATP and Plate counting



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3.2.3 CO_2 Evolution test

To provide a measure of plastic degradation, CO_2 evolution tests were conducted. After 14 days of inoculating bacteria on polyethylene in isolated flasks, CO_2 Evolution results were registered in Table (3-4) for all samples to confirm their positive or negative biodegrading property. Based on the results in Figure (3-6) and Table (3-4), samples ZZ-3, ZZ-7, ZZ-12-2, ZZ-11 showed an increase in CO_2 emission in inoculated M9-PE after this interval and is in agreement with samples ZZ-2, ZZ-7, ZZ-6, ZZ-3, ZZ-12-2 which indicated higher results in ATP measurement.

Table3- 4: results of CO_2 Evolution test on all samples to show their biodegrading ability

Strain	CO_2 Positive	CO_2 Negative	CO_2 rate After 14 days	CO_2 rate with $CuSO_4$+$FeCl_2$ after 7 days	Biodegradation ability
ZZ-1		×	<50 ppm	<50 ppm	No
ZZ-2	×		<50 ppm	200 ppm	Probably yes (in presence of $FeCL_2$)
ZZ-3	×		80 ppm	100 ppm	Probably Yes
ZZ-5		×	<50 ppm	<50 ppm	No
ZZ-6	×		<50 ppm	80 ppm	weakly yes (in presence of $FeCH_2$)
ZZ-7	×		100 ppm	120 ppm	Probably Yes
ZZ-8		×	<50 ppm	-	No
ZZ-9		×	<50 ppm	-	No
ZZ-11	×		110 ppm	-	Probably yes
ZZ-12-1	-	-	-	-	ND
ZZ-12-2	×		≈150 ppm	250 PPM	Probably Yes
ZZ-13	×		<50 ppm	250 ppm	Probably yes (in presence of FeCL2)
Negative control		×	<50 ppm	<50 ppm	

*ZZ-10 and ZZ-4 were omitted based on getting non-positive result in ATP measurement

Figure3- 6: CO_2 evolution test on pure cultures, probable stimulators, and synergistic samples

CO2 evolution test with adding 200µl CuSO4+FeCl2

ZZ-6 with (NH4)2so4

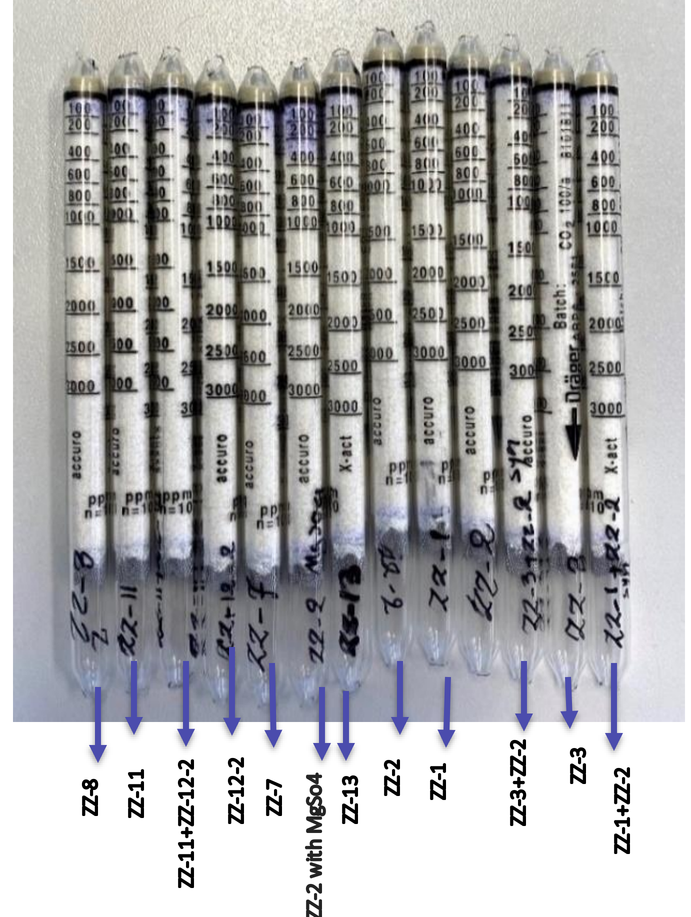
ZZ-13

ZZ-2

ZZ-7

ZZ-11+ZZ-12-2

CO_2 evolution test without adding



Other complementary tests

3.3 stimulation effect experiment on bacterial growth

The aim of these tests is to determine the positive effect of three chemicals which may have a stimulation effect on bacterial growth and probably biodegradation.

3.3.1 Mineral oil

To determine the chemical stimulation effect, first, the effect of different proportions of mineral oil on ZZ-6 sample without adding PE was tested with ATP illuminator measurement assay and all results were registered in Table (3-5) after 5 and 13 days. ATP measurement assay show very low stimulation effect of mineral oil on microbial growth.

Table3- 5: ATP results of mineral oil effect with different proportion on ZZ-6 bacterial growth after 5 and 13.

Proportion (amount of oil µL /50 ml M9)	ATP measurement after 5 days	ATP measurement after 13 days
1/10000	30	56
1/1000	4	1
1/500	20	13
1//250	11	4
1/125	30	15
1/62.5	34	0
1/31.25	24	21

Mineral oil has an inhibition effect on ZZ-6 which is increased with mineral oil concentration and the viability of ZZ-6 is decreased, approximately 7-fold at 0.1% oil concentration after 5 days and approximately 14-fold at 0.4% oil concentration after 13 days to compare with negative controls in M9 media.

After measuring mineral oil effect on ZZ-6 bacterium with ATP measurement, another test was carried out in order of determining the direct effect of mineral oil with different dilution in M9-ZZ-6 suspension without PE to confirm the agreement of ATP assay with plate counting measurement and also to verify whether mineral oil instantaneously kills bacterium or not? In this test, as can be seen in Table (3-6), samples were divided into three groups: the first group is negative control and two other groups with different

additional oil dilutions to see the direct effect of mineral oil on ZZ-6 sample growth. All results were gained after 6 days with plate counting measurement.

Table3- 6: Viable plate counting of mineral oil stimulation effect on ZZ-6 sample in different mineral oil dilution after 6 days

Dilution	Negative control (CFU/ml)	Viable plate count 20µl oil+ dilution 1 [3]+ 1ml M9 (CFU/ml)	Viable plate count 100µl oil+ dilution 1+50ml M9 (CFU/ml)
10^{-1}	uncountable	uncountable	Approximately 0.1×10^5
10^{-2}	uncountable	uncountable	0.53×10^5
10^{-3}	uncountable	23.1×10^5	0.3×10^5
10^{-4}	97×10^5	12×10^5	1×10^5
10^{-5}	110×10^5	0	0

It can be seen that negative controls with no mineral oil have better results in viable plate counting measurements in comparison with samples stimulated with mineral oil. Also, it is observed that with increasing mineral oil concentration the growth efficiency is declined. Other results in Table (3-7) are obtained from the effect of mineral oil on ZZ-6 bacterium inoculated in M9-PE to see whether mineral oil can stimulate polyethylene-degrading bacterium or not? ATP measurement was carried out 3-times after the first inoculation while plate counting was carried out 2-times.

Table3- 7: ATP and Plate counting measurement on stimulation effect of mineral oil on ZZ-6 sample

Time	ATP	plate count assay CFU/ml
After 9 days	40	ND
After 11 days	ND	1.4×10^4
After 18 days	ND	0.38×10^4
After 20 days	31	ND
After 27days	9	ND

ND: Not-determined

All the results above in determining mineral oil effect on bacterial growth and consequently on polyethylene biodegradation both with ATP and plate counting

[3] Three (3) colonies of ZZ-6 sample were diluted in 1ml of M9 medium and called "dilution 1"

measurement show after passing approximately 7-days interval between first and second plate counting, 2.2 times decrease in bacterial growth is observed in 10^{-1} dilution. Also, a significant decrease can be seen in other dilutions. A similar decrease can be observed in ATP measurement which proves that mineral oil has an inhibition effect on bacterial growth instead of stimulation.

3.3.2 Stimulation experiment for $(NH_4)_2SO_4$ on bacterial growth

Since mineral oil didn't show a positive effect on stimulation, $(NH_4)_2SO_4$ and $MgSO_4$ chemicals based on Ashino et al, (2019) work on bacterial growth were selected for stimulation experiment and all results for the effect of $(NH_4)_2SO_4$ on samples ZZ-6, ZZ-7, ZZ-2, ZZ-12-2 are depicted in Table (3-8) and for the effect of $MgSO_4$ are registered in Table (3-10). ATP measurement was carried out in 7 days, 24 days, and 114 days after inoculation of samples in M9-PE with 200 µl added chemicals. It can be seen that $(NH_4)_2SO_4$ has a modest positive effect on ZZ-6 growth in comparison with its negative control and other samples. Also, ammonium sulfate seems to have a negative effect on ZZ-12-2 and ZZ-2 samples after 7 and 24 days. Neither $(NH_4)_2SO_4$ nor $MgSO_4$ showed any positive stimulatory effect on ZZ-7 sample and measuring this sample on other dates was ignored.

Table3- 8: ATP measurement for $(NH_4)_2SO_4$ stimulation effect on bacterial growth

Strain	ATP results in the negative control			ATP results for $(NH_4)_2SO_4$		
	7 days	24 days	114 days	7 days	24 days	114 days
ZZ-2	83	311	359	52	150	ND
ZZ-6	39	54	94	75	134	225
ZZ-7	27	143	ND	35	99	ND
ZZ-12-2	81	116	188	43	76	ND

ND: Not-determined

3.3.3 Stimulation experiment for $MgSO_4$ on bacterial growth

Also, the stimulation experiment was conducted on $MgSO_4$ in parallel to the previous exam to compare the results with each other. After 7, 24, and 114 days of inoculation, ATP measurement was implemented. Based on Table (3-9), it is observed that $MgSO_4$ has

a significant stimulatory effect on ZZ-2 growth and a slight stimulatory effect on ZZ-12-2 and ZZ-6 samples in comparison with their negative controls. In the ZZ-7 strain, $MgSO_4$ shows a non-positive stimulatory effect.

Table3- 9: ATP measurement for $MgSO_4$ stimulation effect on bacterial growth

Strain	ATP results in the negative control			ATP results for $MgSO_4$		
	7 days	24 days	114 days	7 days	24 days	114 days
ZZ-2	83	311	359	205	552	1857
ZZ-6	39	54	94	56	86	ND
ZZ-7	27	143	ND	89	152	ND
ZZ-12-2	81	116	188	67	144	229

ND: Not-determined

To verify the stimulation effect of $(NH_4)_2SO_4$ or $MgSO_4$ on biodegradation, CO_2 evolution test was carried out on ZZ-2 and ZZ-6 samples and all data are registered in Table (3-10) and can be compared with pure cultures.

Table3- 10: CO_2 Evolution test on stimulation of $(NH_4)_2SO_4$ and $MgSO_4$

Strain	Negative control	$(NH_4)_2SO_4$	$MgSO_4$
ZZ-2	< 50 ppm	ND	300 ppm
ZZ-6	<50 ppm	80 ppm	ND

ND: Not-determined

Data from CO_2 evolution test shows approximately 300 ppm CO_2 emission in ZZ-2 sample in result of MgSO4 stimulation effect on bacterial growth which conduces to PE biodegradation by using that as sole carbon source. Also, ZZ-6 sample with added $(NH_4)_2SO_4$ as stimulator has slightly positive bacterial growth in comparison with its negative control.

3.4 Synergy test

Synergy test was carried out by using ATP, plate count and CO_2 measurements. Mixed cultures of two bacteria were prepared to determine their synergistic effect on biodegradation. ATP measurement in assessing the synergistic effect of bacteria on biodegradation shows that a significant synergistic effect is observed only in ZZ-3+ZZ-2 mixed cultures after 22 days to compare with their pure cultures. The synergistic effect can be calculated as follow:

ZZ-2: 311 RLU
ZZ-3: 39 RLU
ZZ-2 + ZZ-3 = 311 + 39 = 350 (anticipated)
ZZ-2+ZZ-3: 462 RLU (observed after 22days in Table 3-11)

Here the anticipated synergistic effect of mixed cultures is equal to the sum of its parts and is approximately 350 RLU while the observed result in Table (3-11) is 462 RLU that is more than additive and a synergistic effect is possible, although it does not seem to be visible after 112 days.

But for ZZ1 + ZZ2 after 22 days by ATP measurement
ZZ-1: 233 RLU
ZZ-2: 311 RLU
ZZ-1+ZZ-2: 544 RLU (anticipated)
ZZ-1+ ZZ-2 = 520 (observed after 22days in Table 3-11).

Here the anticipated synergistic effect of mixed cultures is approximately 544 RLU while the observed result in Table (3-11) is 520 RLU that is less than additive and a synergistic effect is far-fetched.

On the other hand, in ZZ-11+ZZ-12-2 sample a slightly positive synergistic effect is considered After 22days which cannot be considered after 112 days by ATP measurement.

ZZ-11: 125 RLU
ZZ-12-2: 116 RLU
ZZ-11+ZZ-12-2: 241RLU (anticipated)
ZZ-11+ZZ-12-2 = 385 (observed after 22days in Table 3-11)

In plate counting measurement as it is depicted in the table below, ZZ-1, ZZ-2 and ZZ-3 pure cultures show a high population density of cells (> 10000 CFU/ml) after 6 days to compare with ZZ-3+ZZ-2 mixed culture with less than 6000 CFU/ml. These results depicted that ATP assay is not in agreement with plate counting measurement which is not exactly possible to claim which mixed culture has a better effect on biodegradation. To check the synergistic effect of mixed cultures on biodegradation other complimentary tests i.e. CO_2 evolution test was conducted only on ZZ-1+ZZ-2, ZZ-2+ZZ-3 and ZZ-11+ZZ-12-2 mixed cultures with better results in ATP measurement. Based on the results obtained from CO_2 measurement in Table (3-11) only ZZ-11+ZZ-12-2 with approximately 150 ppm CO_2 emission shows a better result in comparison with other samples but in comparison with its pure culture, not any significant synergistic effect is observed. The number of colonies were formed on the plate in plate count test shown in Figure (3-7).

Table3- 11: Results of synergy test on pure and mixed cultures by ATP, plate count, and CO_2 evolution measurements

Strains	ATP after 7 days of inoculation	ATP after 22 days of inoculation	ATP after 112 days of inoculation	CO2 Evolution test after 14 days of inoculation	plate count test (CFU/ml) after 6 days
ZZ-1 (-) control	76	233	250	<50 ppm	> 10000
ZZ-2	83	311	359	<50 ppm	> 10000
ZZ-3	6	39	37	≈ 100ppm	> 10000
ZZ-1+ZZ-2	285	520	560	80 ppm	7800
ZZ-3+ZZ-2	143	462	346	<50 ppm	< 6000
ZZ-7	154	368		100ppm	
ZZ-8	16	4		<50 ppm	
ZZ-7+ZZ-8	155	12			
ZZ-9		1			
ZZ-10		1			
ZZ-11	ND	125	181	100ppm	
ZZ-12-2		116	188	150ppm	
ZZ-9+ZZ-12-2		122			
ZZ-10+ZZ-12-2		106			
ZZ-11+ZZ-12-2		385	360	150 ppm	

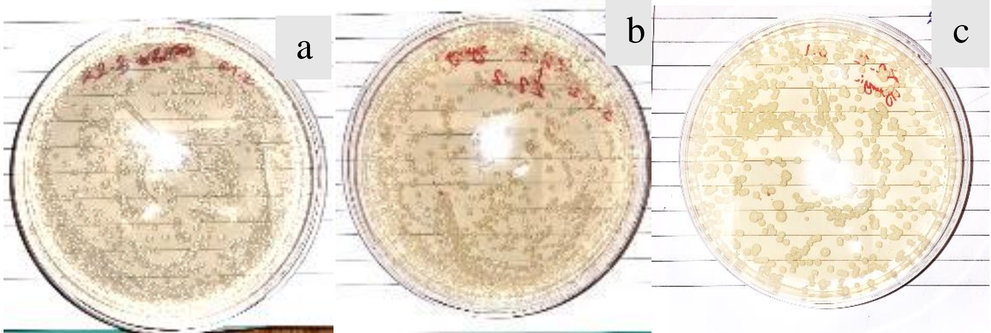


Figure3- 7: Plate count measurement on ZZ-3 and ZZ-2 negative controls and ZZ-3+ZZ-2 mixed culture in order of verifying synergistic effect (a) Number of colonies are more than 1000 in pure ZZ-3 culture on TSA media (b) Number of colonies in ZZ-2 are more than 1000 but less than ZZ-3 sample on TSA media (c) Number of colonies in ZZ-3+ZZ-2 sample are less than 600 colonies which cannot confirm our results from ATP measurement.

4 Discussion

Albeit, Plastics durability is one of the important criteria for practical usage of them on the other hand their recalcitrant properties made them a big concern for environmental pollution. So all attempts are in direction of degrading plastics with microorganisms in an eco-friendly manner.

In this study, different microorganisms were first collected from aged different plastics wastes and then identified and isolated. In the last step, the biodegrading characteristics of them were investigated by using different measurements.

4.1 Isolation and characterization of bacteria on TSA media

Nine strains of bacteria were gram-negative rod-shaped and one of them was gram-negative cocci, two strains were gram-positive rod-shaped and one is gram-positive cocci. Here only one fungus (yeast) was identified as budding yeast.

The evidence shows:

ZZ-7 and ZZ-2 both are aerobic gram-positive, rod-shaped, catalase-positive, oxidase-negative spore-forming

ZZ-9 is an aerobic gram-positive spherical-shaped (cocci), catalase-positive and oxidase-positive bacterium.

ZZ-12-2 is an aerobic gram-negative spherical-shaped, catalase-positive and oxidase-negative bacterium.

ZZ-1, ZZ-4, ZZ-6 and ZZ-11 all are aerobic gram-negative rod-shaped, catalase-positive and oxidase-negative bacterium.

ZZ-3, ZZ-5, ZZ-8 and ZZ-10 are aerobic gram-negative rod-shaped, catalase-positive and oxidase-positive bacteria.

ZZ-13 is an aerobic gram-negative spherical-shaped, catalase-positive and oxidase-positive bacterium.

ZZ-12-1 seems to be a fungus by identification tests which exact detecting type needs more molecular assessments.

4.2 Identification of plastics-degrading bacteria

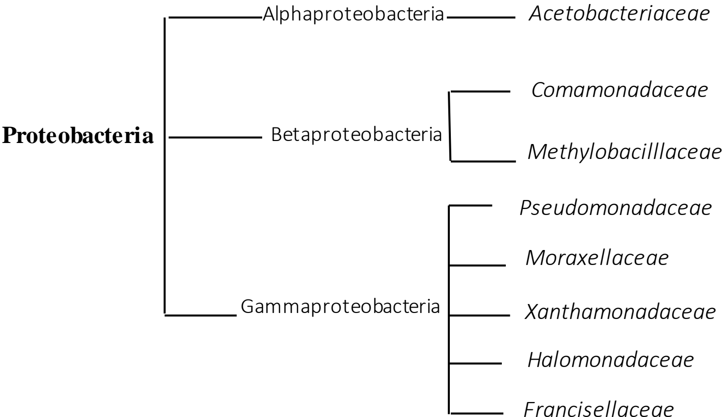
At least six of the bacterial strains tested in this study were able to degrade polyethylene on its material surface. ZZ-2, ZZ-3, ZZ-6, ZZ-7, ZZ-12-1 and ZZ-12-2 had the best response in ATP measurement. ZZ-1, ZZ-8, ZZ-12-2 and ZZ-13 showed better results in viable plate counting. Furthermore, ZZ-3, ZZ-7, ZZ-11, ZZ-12-2 showed positive results in CO_2 evolution test after 14 days and ZZ-2 and ZZ-13 after 21 days in attendance of $FeCl_2$ and $CuSO_4$ showed drastically an increase in their CO_2 evolution test results that indicate biodegradation. Based on all the results, only ZZ-12-2 bacterium in all assessments depicted the best results and latter to a lesser extent ZZ-7 and ZZ-3 respectively suggested better results in CO_2 and ATP measurements, with a high possibility they can be counted as plastic-biodegrading bacteria. Four strains didn't show any evidence of biodegradation in none of the measurements which were ZZ-4, ZZ-5, ZZ-9 and ZZ-10.
Since limited researches carried out on soil bacterial strains with biodegradability, here based on morphological characterization of bacteria showed positive results in both ATP measurement and CO2 Evolution test, we aim to determine these bacteria systematically to detect possible phyla and families they belong to.
ZZ-7 is an aerobic gram-positive rod-shaped, catalase-positive and oxidase-negative spore-forming bacterium. Based on *Bergey's Manual of Determinative Bacteriology* (Bergey & Holt, 1993; Don J et al., 2005) this bacterium with this characteristic possibly belongs to:

Phylum BXIII. *Firmicutes*

Order III. *Bacilli*

Family I. *Bacillaceae*

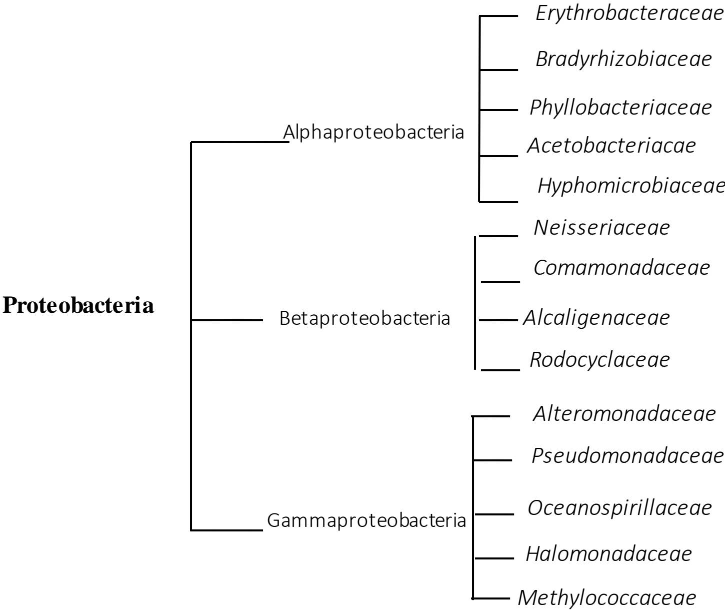
Since our sample was collected from soil, this result completely agrees with Puglisi et al, (2019) who mentioned the most important soil plastics colonizers are different species of *Bacillus*.
ZZ-12-2 is an aerobic gram-negative coccus, catalase-positive and oxidase-negative bacterium. With these characteristics, this bacterium probably belongs to one of these families mentioned below that all of them are subdivisions of Proteobacteria phylum.



All properties of ZZ-11 seem to be so similar to sample ZZ-12-2 and possibly belongs to Proteobacteria phylum, and one of Alphaproteobacteria, Betaproreobacteria, or Gammaproteobacteria Classes.

ZZ-3 is an aerobic gram-negative rod-shaped, positive-catalase and oxidase-positive bacterium.

Based on Bergey's Manual of Determinative Bacteriology this bacterium belongs to one of these phyla: Proteobacteria or Bacteriodetes: In the Bacteriodetes phylum *Flavobacterium* species from the *Flavobacteriaceae* family have similar characteristics. In the Proteobacteria phylum, some families have some similarities include:



Roager and Sonnenschein, (2019) implied that in Proteobacteria, marine plastic-biodegrading bacteria are most in the *Hyphomonadaceae, Rhodobacteraceae, Erythrobacteraceae, Psuedomonadacea, Alcanivoraceae*, *Vibrionaceae*, *Comamonadaceae,* and *Flavobacteriaceae* families. On the other hand, Puglisi et al, (2019) mentioned that soil plastic-biodegrading bacteria are most in *Bacillaceae* and *Nocardiaceae* families which *Nocardiaceae* belongs to Actinomycetales order and Actinobacteria phylum with substrate mycelium. With referring to these studies, all our results show that two samples with a high percentage of biodegradation in this study have possibly belonged two one of *Erythrobacteraceae, Psuedomonadacea, Comamonadaceae*, *Flayobacteriaceae,* or *Bacillaceae* families which for determining exact species molecular assessments such as PCR amplification of the bacterial 16S rRNA hypervariable regions.

Since ZZ-12-2 indicated the highest biodegradability and in a lesser extend ZZ-7 and ZZ-3 suggested biodegradation property, these isolates probably have a propensity for attachment to PE film although this has not been tested.

Bacterial surfaces are hydrophilic While hydrophobicity of PE surface can prohibit bacterial attachment to the surface although after attachment its surface can be the perfect place for the establishment of biofilm (Bardaji et al., 2019). Attached microbes can secrete degradation enzymes near the PE surface which conduce to their high concentration around the polymer and trigger the degradation process (Han et al., 2020). As a consequence of the microbial biodegradation process, the material loses its mechanical properties and disintegrates into small fragments. Microorganisms can colonize the surface of polyethylene and have different effects on surface and molecular properties. Changes in surface characteristics may consist of a reduction in tensile strength, roughness of the surface, or molecular changes such as an increase in carbonyl index, molecular weight reduction and modification in some functional groups –i.e. ester or ketones (Fotopoulou & Karapanagioti, 2017). So determining the growth of microbes is a useful procedure to identify latter biodegradation properties. Microbes can metabolize plastics insoluble compounds by producing some bio-surfactant which make the bacterial surface more hydrophobic and lead to adhere them to the hydrophilic surface of the substrate and increase substrate molecules accessibility for enzymes (Lang & Philp, 1998).

4.3 Growth measurements

4.3.1 Viable plate count

Agar plate count is used to estimate the viable number of cells that exist colonies. Metabolically active cells are typically quantified by using viable plate count assay in a streak or spread plate method. As it can be seen in Table (3-3), the CFU/ml of ZZ-1, ZZ-8 and ZZ-13 had a significant increase in growth which is the apparent evidence of viable cells increase in time which can be a good reason to claim that PE could be consumed as a sole carbon resource for these strains while in ZZ-12-2 modest decrease was observed that was ignored. However, it should be noted that ZZ-13 showed very low results in ATP and OD measurements but unexpectedly showed high CUF/ml which would be explained by the different essence of these two methods. OD measurement seemed to be reliable in assessing plastic-degrading while ATP measurement is based on the energy that is released by bacteria due to metabolic activities. Viable plate count measurement seems to have its limitations but in determining biodegradation the main issue would be that it only detects cells that are in suspension. Cells attached to plastic surfaces will not be reliably counted. If a plastic particle is small enough to be pipetted, it will form a single colony, although there may be thousands of cells attached to it. Similarly, if the cells are growing in clumps one colony may represent many cells. Therefore, plate counts tend to be underestimated for cells growing in the biofilm.

4.3.2 ATP measurement

ATP is an energy currency that provides energy to drive many processes in living cells. The essence of ATP assay is based on luciferase enzyme catalyzes luciferin to oxyluciferin in the presence of Mg^{2+} ions and emitting a luminescent signal. Principle differences in the essence of this method with OD and viable plate count measurements may explain why some samples such as ZZ-8 or ZZ-1 indicated low ATP results but high CFU/ml rate in the viable count method or conversely samples such as ZZ-2, ZZ-6 and ZZ-7 had high ATP results but low CFU/ml viable density on TSA media based on Table (3-2 and 3-3). Albeit, none of these methods can confirm microbial biodegradation in polyethylene but they are reliable methods to confirm bacterial growth.

ATP measurement is an easy and rapid method for assessing bacterial growth based on metabolic activity. In ATP measurement, our results were in accordance with Kounty et al (2006) that mentioned in the presence of PE as the only carbon source for metabolic activities, at the beginning of incubation a period of rapid bacterial growth occurred which probably caused by the consumption of extracted compounds with low molecular weight in the result of biodegradation and oxidation of PE and most often terminated with carboxylic groups. After this rapid phase, a reduction in metabolic activity occurs, and detecting biodegradation became complex. Here, ATP measurements suggested that microorganisms still obtained energy from PE oxidation at a relatively low rate several months after the initial rapid growth period.

Also, all data from ATP measurement suggested that although biodegradation progress is slow, microorganisms still acquire energy from PE as the sole carbon resource and it is not possible to make an exact estimation of biodegradation in limited time under laboratory condition and also ATP assay sensitivity is influenced by pipetting replicate samples. Another limitation of ATP measurement is the high cost of swaps and interpretation of the results in complex samples is sophisticated. Nonetheless, ATP measurement in assessing biodegradation is the fastest cell viability assay and sensitive, and is less prone to artifacts than other viability assays (Aslantürk, 2018).

4.3.3 Optical density (OD) measurement

The essence of this method is based on determining bacterial turbidity in the presence of 540-600 nm light. Optical density (OD) measurement seems to be a not reliable test for biodegradation assessments according to Table (3-2) and fluctuations in the results. plastics buoyancy and not accurate filtration or also this fluctuation could be due to growth, death and lysis, or attachment to the plastic particles. Since some plastics particles are still floated in filtered samples and incline to come up to the surface which can improperly influence OD reading results. Large plastic particles will lead to optical distortions of the light beam, resulting in unstable measurements. Also, Small particles scatter light much as bacteria do and contribute to turbidity. Compared with optical density measurements at 600 nm, the viable plate count method is more effective for measuring living microbes. The most important issue in OD measurement is that it also measures dead cells and If many dead cells are present in the culture it causes the wrong

OD in the results of dead cell precipitation. Furthermore, suspended plastic particles were a big issue in this study which influenced negatively the results. Also attached bacteria to plastic particles will distort the results (Andrew Jenkins, Pers. comm.).

4.3.4 CO_2 evolution test

CO_2 gas is the main by-product during the biodegradation of polythene. CO_2 evolution test is the most direct measure of PE degradation as the sole carbon source. In CO_2 evolution test ZZ-3, ZZ-7, ZZ-11, and ZZ-12-2 samples indicated the best results after 14 days of inoculation respectively and subsequently ZZ-2, ZZ-3, and ZZ-13 samples suggested better results after 21 days by adding mixed component consist of $FeCl_2$ and $CuSO_4$ which all results can be seen in Table (3-4). High growth after adding Fe compound can be described in different ways. First, that is the result of giving more time to bacteria to grow or because of Fe compound as stimulator which needs to spend work to resolve. Second, another possibility is that iron/copper may be needed by the oxygenase enzymes that carry out the initial oxidative attack on the PE polymer (Andrew Jenkins, Pers. comm.).

On the other hand, it should be considered that the CO_2 atmospheric level is almost 420 ppm but by comparing this with our negative result, it seems that this CO_2 hasn't had any issues with our interpretation. Nonetheless, it seems ZZ-12-2 with highest probability and ZZ-3 and ZZ-7 strains with less confidence level can be claimed as possible plastic-biodegrading bacteria based on positive results obtained in different experiments.

CO_2 evolution test has some limitations that should be aware of and include; leakages in the complicated system may conduce to low carbon dioxide values during the test. Also, impurities adhere to the PE surface or incorporated to PE chain may consist of compounds that bacteria consume them as carbon source and interfere with consumption of PE as a carbon source. On the other hand, in the study of carbon dioxide evolution measurement, all factors such as biomass, volatile compounds, and the proportions of dissolved and undissolved parts of the polymer should be determined but assessing biomass and the number of insoluble residues has some obstacles to achieve (Itävaara & Vikman, 1996).

4.4 Correlation coefficient results

Correlation between ATP and plate counting measurement and also ATP and OD measurement were carried out. The correlation coefficient between ATP and OD measurement was approximately 0.039 which shows there is no correlation between these two methods. Since these two methods include different mythologies, ATP is based on energy consumption and optical density is based on determining bacterial turbidity in the sample in presence of light, it can be justified that why no correlation can be observed between ATP and OD. Furthermore, the correlation coefficient between ATP and Plate count measurement was approximately 0.59 and shows that they moderately correlated and these results are in agreement with the results obtained by Deininger and Jiyound (2001) which claimed a high correlation between rapid ATP assay and the Direct viable count method.

4.5 Complimentary tests

4.5.1 Stimulation effect

Three chemicals were tested in this study. Liquid paraffin, ammonium sulfate and magnesium sulfate.

Paraffin is a hydrocarbon compound used by Fuhs (1961) and he mentioned that several microorganisms can consume that as a carbon source. The theoretical reason for expecting PE-degraders to be stimulated by paraffin oil is that both PE and paraffin are saturated hydrocarbons. They are very chemically similar, although paraffin has a much lower molecular weight. The biochemical pathways needed to activate paraffin and PE are likely to be similar.

Based on the results paraffin inhibited growth and caused a reduction in viable counts and it can be described in two aspects: **a)** Different microorganisms have the different capability of growing on complex media which make them heterotrophs or chemoorganotrophs. These organisms gain their nutrition from other carbon resources or use chemical bonds in organic compounds or O_2 as their energy source. These organisms can all grow in the presence of oxygen, making them aerobes/facultative anaerobes (conceivably aerotolerant anaerobes, but this seems less likely). They are all also capable of growing at 20 C. In presence of paraffin, the accessibility of

microorganisms to O_2 is inhibited and aerobic microorganisms completely lost their growth rate in presence of O_2 while other facultative anaerobes still have less capability of growth by using organic compounds (TSA medium) as the sole carbon source for growth. So we cannot imply that paraffin has a toxic effect on all microorganisms and better to put forth it inhibits bacterial growth.

b) In some samples, no growth means that the oil caused the killing of the cells by disrupting their cell membranes, more or less instantaneously. If it merely inhibited growth, in process of performing dilutions the inhibitory effect would decrease with increasing dilution. If there is the instantaneous killing of the cells, this cannot be due to preventing access to oxygen, because cells that are deprived of oxygen, do not die instantaneously, but suffocate slowly as their energy production runs down and they run out of ATP. It might be that the adaptations that allow ZZ-6 to assimilate the molecular fragments of PE also make their membranes sensitive to higher molecular weight oils (Andrew Jenkins, Pers. comm.).

4.5.1.5 Stimulation effect of $(NH_4)_2SO_4$ and $MgSO_4$ on bacterial growth

Nitrogen in ammonium ions (NH_4^+) and ammonium sulfate, accelerates bacterial growth and is an essential element in the biosynthesis of nucleotides and microbial activities (Ashino et al., 2019). On the other hand, magnesium (Mg^{2+}) in bacterial growth involves significantly in the enzymatic reactions in cells, such as translation. Since a lot of researches were based on the stimulation effect of organic compounds consist of nitrogen and magnesium on bacterial growth, two compounds $(NH_4)_2SO_4$ and $MgSO_4$ were selected as chemical stimulators in this study to survey their effect on bacterial growth and subsequently on biodegradation.

The results in Table (3-9) indicated that $MgSO_4$ had a stimulation effect on bacterial growth in the ZZ-2 sample by ATP measurement. Subsequently the effect of $MgSO_4$ as stimulator on biodegradation was investigated on inoculated polyethylene sample with ZZ-2 bacterium by CO_2 evolution test and it was observed that stimulated sample with $MgSO_4$ showed high CO_2 emission rate in compare with its negative control. Here, it suggested that $MgSO_4$ is probably a stimulator for this bacterium strain. That why these chemicals haven't had good effect on other samples can be described by that, 1M $(NH_4)_2SO_4$ and 2M $MgSO_4$ were used in this study in high concentration that may cause

osmolarity effect conduce to toxicity of ammonium at high concentrations (>500 mM) (Müller et al., 2006). Ashino et al., (2019) mentioned that for the best growth performance, the and NH_4^+ concentration is required to be from 63.2 to 282, and for Mg^{2+} from 0.1 to 22.4 mM.

On the other hand, a slight positive result on the ZZ-6 sample was considered with $(NH_4)_2SO_4$ as a stimulator while it had an inhibiting effect on ZZ-2 and ZZ-12-2 samples by ATP test and can be seen in Table (3-10). To test the positive effect of $(NH_4)_2SO_4$ on ZZ-6 biodegradation (with better results in ATP measurement in comparison with other samples), CO_2 evolution test was conducted and the results suggested that a slight positive bacterial biodegradation on this sample. So it cannot be claimed certainly that $(NH_4)_2SO_4$ is able to stimulate bacterial growth which ultimately leads to acceleration biodegradation in Table (3-10).

Based on these results, it can be concluded, determining the type of chemicals with stimulation effect on bacterial growth depends on their species and their requirements in metabolic pathways (Jurtshuk, 1996) and different chemicals may have different stimulation effects on microbial growth but not necessarily can influence biodegradation. Two weeks before submitting this study, considering the compositions of minimal salt and solution 2 (1000×) trace element solution, lack of essential minerals such as Fe, Zn, Si for growth of microorganisms was observed by chance and a suspension consisting of 0.05 gr $FeCl_2$ and 0.05 gr $CuSO_4$ dilution in 20 ml water was prepared and 200µl of this suspension was poured in M9-PE samples inoculated with ZZ-2, ZZ-3, ZZ-6, ZZ-7, ZZ-12-2 and ZZ-13 strains and one week later CO_2 evolution test carried out on these samples and all results can be seen in Table (3-4). All results indicated that after adding this suspension to the aforementioned samples an accelerant bacterial growth was observed approximately 2 times raise in ZZ-3, 4times raise in ZZ-2 and 5-times raise in ZZ-13, respectively and suddenly in ZZ-6 with no CO_2 emission in pure culture measurement (without $FeCl_2$ suspension) 80 ppm raise was observed; Unfortunately, in consequence of limited time, it was not possible to integrate all the results into this study but after the submission due date, working on aforementioned observations will be continued and all the results will be added to reviewed version.

4.5.2 Synergy test

Different definitions and terms are made to explain consortium. Kull (2010) concluded that a consortium is a group of microorganisms that cooperate together to complete a certain biochemical transformation of the substrate (Kull, 2010). Here, our aim was to make an artificial environment similar to a consortium to investigate the synergistic effect of bacteria on biodegradation. Mechanism of synergy effect is that interaction or cooperation of two or more microorganisms makes greater combined effect than the sum of all separate effects. The principle of making mixed culture in this study was different locations that bacteria were collected to limit the ropable factors effect and ATP results.

No synergistic effect was observed in none of the mixed cultures with a positive effect on biodegradation based on ATP, viable plate count, and CO_2 evolution measurements. Here, the anticipated synergistic effect of mixed cultures was equal or a bit higher than the sum of their parts and only ZZ-2+ZZ-3 and ZZ-11+ZZ-12-2 suggested a possible synergistic effect after 22 days, although it did not seem to be visible after 112 days and cannot decisively assert that these samples have a synergistic effect on biodegradation and introduce them as synergistic mixed cultures.

5 Conclusion

Between 14 microorganisms were isolated and identified, three bacterial samples ZZ-12-2, ZZ-7 and ZZ-3 suggested the highest possible biodegradability property. For biodegradation assessment different experimental methods such as ATP, Plate counting and CO_2 measurement were conducted. ATP and plate count tests determine the growth and metabolic state of the bacteria while CO_2 is the most direct measure of PE degradation but a standard reference experiment that would be able to determine biodegradation specifically seems to be required. On the other hand, some chemicals can stimulate biodegradation that in this study $MgSO_4$ demonstrated drastic stimulation effect on ZZ-2 growth which led to high CO_2 emission in the result of biodegradation and modest stimulation effect of $(NH_4)_2SO_4$ on ZZ-6 bacterium was observed. Here, it could be claimed that $MgSO_4$ can stimulate biodegradation but no strong evidence can prove the stimulating effect of $(NH_4)_2SO_4$ on biodegradation. Although in this study none of the mixed bacterial cultures showed a positive synergistic effect, finding effective consortia, can help in eco-friendly remediation in biodegradation searches. It is suggested that in continuation of this study, molecular identifications conduct on some strains with biodegradability property especially on genomes of the plasmids to compare and identify the loci that involve in biodegradation. Also because of shortage of time, more workings on determining simulation effect of $FeCl_2$ and $CuSO_4$ on biodegradation had not been achievable and it is suggested to consider for further researches.

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