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Microbial growth on Polyethylene

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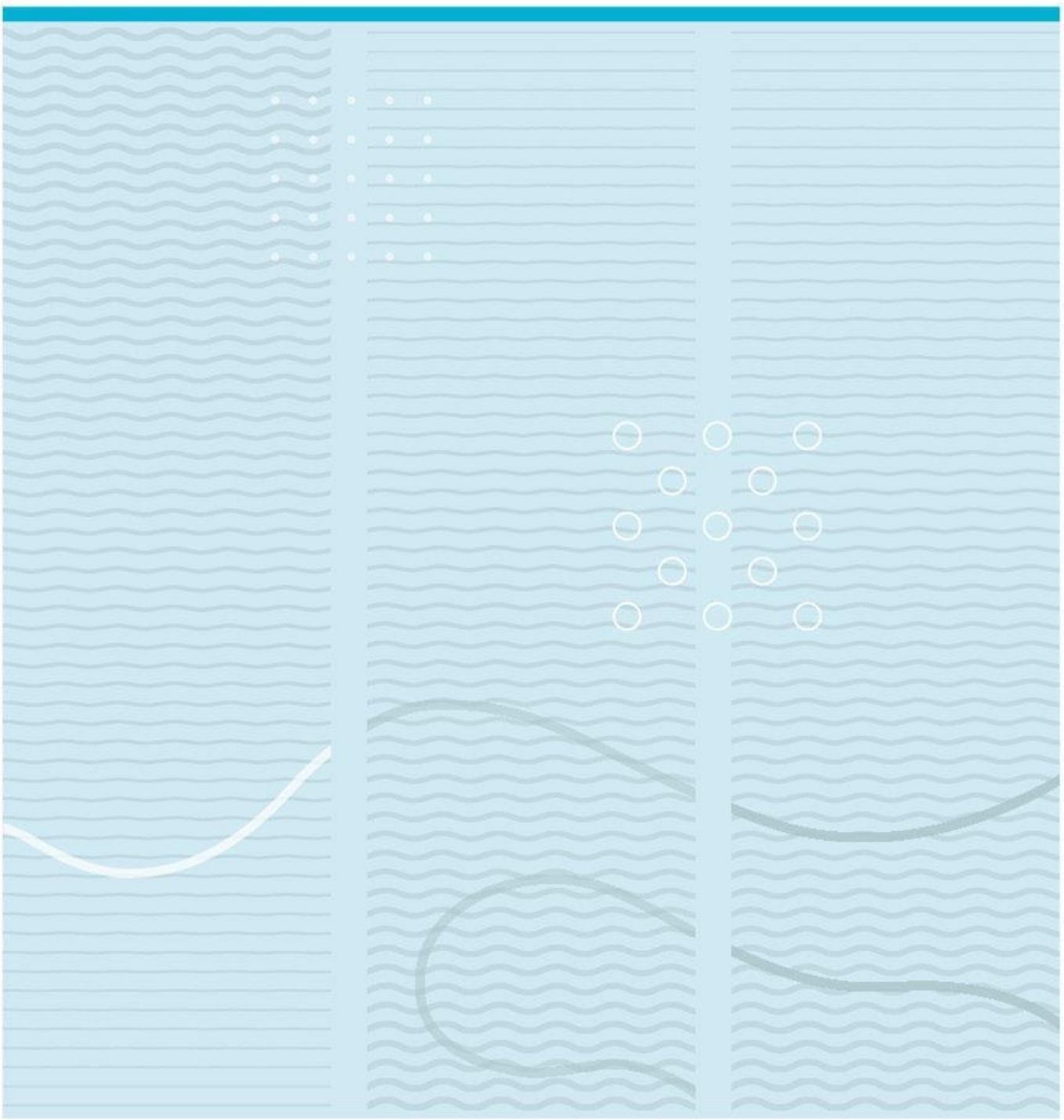

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This thesis is worth xx study points

Abstract

Polyethylene is a low-density polymer, widely distributed worldwide and resistant to biodegradation. Therefore, it is important to find microorganisms that carry out the process of biodegradation of polyethylene and to find chemicals that help these organisms in their work with biodegradation. Six bacterial strains were isolated from a piece of plastic waste. The ability of bacteria growth was studied in three different mediums. CO2 was measured for these strains after a 10-week incubation period. Strain ZZ-2 was able to grow better in the presence of PE than in culture without PE in two different media. CuSO4+FeCl2 supplement stimulated the growth of strains ZZ-1, ZZ-7, and ZZ-12,2 in culture containing PE, CuSO4+FeCl2 supplement stimulated the growth of strain ZZ-12,1 in culture containing PE slightly, but CuSO4+FeCl2 supplement inhibited bacterial growth of strain ZZ-6 in this study, this led to the conclusion that CuSO4+FeCl2 supplement is a stimulator or inhibitor of bacterial strains. By using Welch Two sample t-test, the two cultures (Test and Control) were compared for many tests. The CO2 measurement results were significant for strains ZZ-1 and ZZ-2 in the M9++ medium with PE as the only carbon source.

Contents

List of Abbreviations

PE	Polyethylene
PP	Polypropylene
PVC	Polyvinyl chloride
PS	polystyrene
PET	Polyethylene terephthalate
PC	Polycarbonates
HDPE	High-density polyethylene
LDPE	Low-density polyethylene
LLDPE	Linear low-density polyethylene
PBT	Polybutylene terephthalate
PI	polyimide
PTFE	polytetrafluoroethylene
PAI	polyamide-imide
PSU	polysulfone
ABS	Acrylonitrile butadiene styrene
POM	polyoxymethylene
PCBs	polychlorinated biphenyls
PA	polyamide
½ TSA	Half strength Trypticase Soy Agar
M9+	M9 salt and 2(1000×) trace element solution
M9++	M9 salt, 2(1000×) trace element solution and CuSO4+FeCl2 supplement
CFU	Colony Forming Unit
ATU	Allylthiourea
ATP	Adenosine triphosphate

Foreword

I would like to thank my Supervisor, Professor Andrew Jenkins for his guidance throughout the implementation of this project. I would like to thank the University of South-Eastern Norway to give me this chance to study in its educational system. Many thanks to my family and friends for their continued support.

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Lama Albasha

1 Introduction

Over the past century, plastic consumption has increased. Plastic has been used in all aspects of life (Zhao et al., 2022, 2019). Plastics have many advantages such as lightweight, low-cost, unbreakable, durable, transparent, and mouldable. These advantages made the production increase from year to year. The average growth rate of plastic production over the past 30 years has been 10%. About 57 million tons of plastic waste is generated annually in the world (Kumar et al., 2007). While one study has shown that in 1950 the world produced 2 million tonnes per year, annual production has increased nearly 200-fold, reaching 381 million tonnes in 2015 (Ritchie and Roser, 2018). Another study has estimated that in 2050, about 12000 million tons of plastic waste will accumulate in landfills (Tudor et al., 2019).

Plastic is a polymeric material that has large molecules. It is made up of an indefinitely long series of interconnected links (Chauhan and Wani, 2019). Common plastic consists of polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), polystyrene (PS, and expanded EPS), polycarbonate, polyethylene terephthalate (PET), etc (Millet et al., 2018). Plastic pollution is widespread all over the world, found in seas, oceans, rivers, soil, atmosphere, even within animals, and human food chains (Lau et al., 2020). Research on plastic pollution has suggested that many years from now, the seas and oceans will contain fewer fish than plastic, due to a possibility of fish injury and possibly death after exposure to plastic (Thakar, 2018).

Approximately 140 million tons of synthetic polymers have been produced, which do not degrade easily. Therefore, synthetic polymers constitute serious pollution to the environment. To address this problem, the biodegradation of plastic was studied.
(Shimao, 2001).

1.1 Categories of plastic

The two main groups of plastics are Thermoplastics and Thermosets

1.1.1 Thermoplastics

Thermoplastics are the most popular type of plastic and a plastic material that can be mechanically recycled. That is, it can be heated, shaped, and strengthened several times. They are classified according to the organization of the chemical structure of their molecules. According to the Capability by Temperature Index by Underwriter Laboratories, USA, thermoplastics are divided to Standard Plastics, Engineering Plastics, and High-Performance Plastics (Millet et al., 2018).

1.1.1.1 *High*-Performance Plastics

Plastics that have very high mechanical and chemical resistance. Used for specialized applications. Include Fluoropolymers (polytetrafluoroethylene (PTFE)), polyimide (PI), polyamide-imide (PAI), polysulfone (PSU), etc. (Millet et al., 2018).

1.1.1.2 Engineering Plastics

A tough kind of plastic. Temperature Index (100_150C) means that they have heat resistance, impact, chemical resistance, and mechanical strength. They are used in many applications such as electrical, electronic, transportation industry, etc. Acrylonitrile butadiene styrene (ABS) is the most used, followed by polyamide (PA), PET injection (PET), polyoxymethylene (POM), polybutylene terephthalate (PBT), polycarbonate (PC), etc. (Millet et al., 2018).

1.1.1.3 Standard Plastics

Polyolefins: (semi-crystalline structure) saturated hydrocarbons that are made by the polymerization of an olefinic monomer by a catalyst (Jones et al., 2021). The most common type of polyolefins; are polyethylene (PE) and polypropylene (PP) (Sauter et al., 2017). PE and PP are used in daily life, the most widely produced polymers in the world, low-cost to produce, less toxic, lightweight, and recyclable. Polyethylene is divided to Low-density polyethylene (LDPE), high-density polyethylene (HDPE), and linear low-density PE (LLDPE) (Millet et al., 2018).

Polyvinyl chloride PVC: (amorphous structure) It is almost one of the oldest types of plastic. It is made from salt (57%) and oil or gas (43%) (Millet et al., 2018).

Polystyrene (PS): (amorphous structure) Synthetic aromatic polymer. The formula ((C8H8) n) is made from the monomer styrene. PS exists in two states as a solid and foam and the monomer styrene is a liquid. The molecular weight is high. The melting point is low. Because of polystyrene's good mechanical properties and low cost, it is widely used in many aspects of everyday life and in the industries (packing industries, household appliances, etc.) (Ho et al., 2018).

Polyethylene terephthalate (PET): Polycrystalline semi-crystalline, durable, strong, thermoplastic, thermally and chemically stable. It is easily processed, a gas permeability (Rabek and Ranby, 1974). PET is consumed globally, and its waste constitutes 8% of the weight of global solid waste, recycled by applying mechanical, chemical, and thermal methods to PET waste (Glaser, 2015).

1.1.2 Thermosets

Thermosets are polymers that have a highly bonded chemical structure. The polymers in it cannot be melted and recycled after forming. Within this category of plastic polyurethane and phenol-formaldehyde (Alshehrei, 2017, 2019).

1.2 Plastic pollution

The significant growth of plastic consumption also led to increasing amounts of waste plastics. Plastic waste is a global threat, as it leads to environmental pollution (Gerdes et al., 2018). As plastic moves across borders to the most remote areas. It arrives at the alpine lakes, rivers, oceans, and arctic ice. (Farrelly and Green, 2020).

It takes about 1000 years for plastic buried in the Earth to break down. During the decomposition process, microplastic accumulates in the soil. Studies have indicated that soil organisms are negatively affected by microplastic that enters the organic matter and mineral substitutes in the soil (Tudor et al., 2019). 80% of plastic is transferred from the Earth’s surface to the aquatic environment (Tudor et al., 2019). When the plastic reaches the sea, it stays there. It may turn into small parts as a result of a collision with waves, UV exposure, and wind. Plastic is getting smaller and ready to be eaten by marine organisms (Nelms et al., 2016). Wabnitz et al., 2010 pointed out the harmful effects of eating plastic on many marine organisms, from plankton to seabirds (Wabnitz and Nichols, 2010). Some contaminants such as polychlorinated biphenyls PCBs and heavy

metals can build up on marine plastics, causing death to the organisms that eat plastic (Nelms et al., 2016). Marine plastic pollution is a gateway to ocean plastic pollution (Haward, 2018). A significant percentage of plastic waste ends up in the ocean. Only 1% of plastic floats on the surface of the ocean (Van Sebille et al., 2016). Plastic in the ocean poses many risks to the health and development of marine life, through entanglement, suffocation, ingestion, and transfer of non-native species. Other risks can also be that some plastics function as chemical carriers for the dangerous chemicals that stick in animals and marine life (De Frond et al., 2019).

The air is loaded with microplastic particles sourced from clothing, construction, and industry. A few of these particles may enter the human lung and may lead to various diseases such as chest fibrosis, asthma, and bronchitis. In addition, many diseases such as energy metabolism, inflammation, inhibition of acetylcholinesterase, reproductive failure, and growth restriction is also in part caused by microplastic particles. These diseases affect humans and animals (Lim et al., 2021).

1.3 Disposal of plastics

Until 2019, 7,3 billion tons of plastic were produced worldwide, a study estimated that 12% has been recycled, 15% has been incinerated, and 73% in landfill (Chauhan and Wani, 2019).

First recycled, an environmentally benign process that avoids the production of carbon dioxide, lowers the amount of fresh plastic generated, and decreases the amount of plastic trash in landfills (Sheldon and Norton, 2020). On the other hand, plastic recycling has many disadvantages: 1. Logistical (Sorting and collecting plastic from other waste and transporting it to recycling plants, needs infrastructure, educating the masses on how and the importance of sorting). 2. Chemical (Only thermoplastics are recycled, some plastics cannot be melted, some cannot be formed after melting, and the quality of plastics become poor with the age of plastics. 3. Financial challenges (Jenkins, 2020). There are many ways of recycling. Recycling of plastic materials with separation based on chemical analyses or vision, selective dissolution with separation, and without separation (Mølgaard, 1995).

Secondly, incinerated, although burning plastic residues results in energy that is used to generate electricity or heat. However, it has severe risks if the temperature and burning process are not monitored. As the burning of some plastic materials such as PVC results in volatile toxic substances and dioxins. Halogenated hydrocarbons can form gases such as hydrogen chloride and chlorine. In addition, burning leads to CO2 emissions. Thus, the burning leads to a rise in temperature (Ágnes and Rajmund, 2016).

Thirdly, a study showed that landfills are one of the cheapest and most environmentally friendly methods to dispose of plastic. Landfills allow the plastic to degrade in controlled conditions (Lema et al., 1988). Due to the slow degradation of the plastic, the amount of Co2 produced is small. Thus, it does not lead to an increase in the proportion of Co2 in the atmosphere. On the other hand, the landfill must be well built, especially from the sides. The landfill is rapidly filling up and the land is becoming unsuitable for anything else(Jenkins, 2020).

Fourthly, plastic degradation. There are four mechanisms basic for breaking down plastic: photodegradation, thermos oxidative degradation, hydrolytic degradation, and biodegradation (Webb et al., 2012).

1.4 Plastic degradation

Photodegradation is the first step of plastic degradation. UV radiation breaks polymer chains, produces radicals, and reduces the molecular weight of polymers, decreasing the plastic's mechanical properties(Yousif and Haddad, 2013). In thermal-oxidative degradation, oxygen atoms are incorporated into the polymer chains and cause a weakness in the plastic. When the polymer chains reach very low molecular weights, microorganisms metabolize them into smaller pieces (Webb et al., 2012).

1.5 plastic biodegradation

Microorganisms (fungi, bacteria, and algae)) break down natural materials and polymers well. But do microorganisms degrade synthetic polymers very efficiently?

The molecular weight, crystallinity, and physical shapes of polymers play a role in their biodegradability. When the molecular weight of the synthetic polymer is high, the

bacteria's ability to degrade it decreases. High molecular weights are slow to dissolve, which makes microbial attack difficult (Gu, 2003). Although microorganisms cannot degrade plastic easily, a small piece of plastic debris in the ocean or marine environment contains many types of microorganisms (Zettler et al., 2013). Microorganisms attach to the surface of the hydrophilic polymer after the environmental changes that occurred to the polymer due to (light, moisture, heat, and chemical conditions). These microorganisms begin to use carbon as a source of growth. Microorganisms secrete extracellular enzymes that break the polymer main chain. This stage is called the primary degradation stage and results in monomers, oligomers, or dimers (low molecular weight fragments). Microorganisms use these fragments as a carbon source (Alshehrei, 2017). Microorganisms and enzymes are responsible for the degradation of various polymers. Therefore, it is important to identify the types of microorganisms and to know their coenzymes and their impact on various environmental factors (Yasin et al., 2022).

1.5.1 Aerobic and Anaerobic biodegradation

Aerobic biodegradation occurs in the presence of oxygen. Microorganisms consume oxygen to break down organic matter into simpler compounds such as carbon dioxide, energy, and water. Anaerobic biodegradation happens without oxygen. Microorganisms break down organic matter into simpler compounds such as carbon dioxide, methane, and other organic acids. Anaerobic degradation is used to generate methanol gas and produce energy (Kale et al., 2007).

1.5.2 Enzymatic Degradation of Polymers

Enzymes are proteins with complex structures. The enzyme is a catalyst that assists in the biodegradation of polymers. Each enzyme possesses has a different mechanism of catalysis. 1. It changes the substrate through some free radical mechanisms. 2. Some of the enzymes follow different chemical methods. Most enzymes degrade the polymer by following one of the mechanisms of enzymatic hydrolysis or enzymatic oxidation (CATIA BASTIOLI, 2021).

One study investigated the oxidative degradation of water-soluble poly (ethylene glycol (PEG)) and the results were that several enzymes (ether-cleaving enzymes, aldehyde oxidising enzymes, and PEG-dehydrogenase act sequentially to catalyze the

oxidation of the R-O-CH2-CH2OH group (CATIA BASTIOLI, 2021). As a result of the production of 2-hydroxyacetic acid, the chain length of PEG is shortened by two CH2 units (CATIA BASTIOLI, 2021).

Enzymatic hydrolysis takes place in two steps 1. Binding of the enzyme to the polymer substrate. 2. Intracellular catalysis (hydrolysis of the internal carbon reservoir by accumulating microorganisms). The catalysis is extracellular (by using an external source of carbon possibly not by microorganisms). Both intracellular and extracellular stimulation in fungi and bacteria play a role in polymer removal. Extracellular hydrolysis of the polymer by enzymes gives water-soluble products. These products (carbon and energy sources) are used for growth (Alshehrei, 2017).

Recent research refers to the ability of natural catalytic enzymes that can degrade solid hydrocarbon bonds. Thus, helping microorganisms with plastic biodegradation. The enzymes like lipases excreted *by Rhizopus arrhizus, Candida cylindracea, esterases, Rhizopus Delmar*, and *Achromobacter sp* from hog liver have the potential to degrade polyethylene adipate and poly(ε-caprolactone) (Nadeem et al., 2021). A new novel strain *Ideonella sakaiensis 201-F6* was found to produce the protein ISF6_4381 that helps to degradation PET (Ojha et al., 2017). The role of both *cutinases, lipases*, and *esterases* in plastic degradation are mentioned in many studies (Tan et al., 2021).

1.5.3 Factors affecting plastic biodegradation

The physical and chemical properties of polymers have a major role in determining their biodegradability.1. Polymer density and molecular weight (lower molecular weight degrades faster than high molecular weight). 2. Structural complexity and crystallinity (crystalline polymer degrades slower than amorphous). The amorphous polymer is subjected to exogenous enzyme attack faster, which makes it susceptible to degradation. 3. The physical form of the polymer (films, powder, fibers, and pellets) and its hardness. Soft polymers degrade faster than hard. 4. The type of bonds in the polymer, the molecular structure, and the functional groups play an important role in biodegradation (Alshehrei, 2017); (Ullah, 2020).

There are biotic and abiotic environmental factors that effect on the biodegradation of polymers. Abiotic biodegradation is affected by many factors such as temperature, moisture, light, wind, and pH (Muthukumar and Veerappapillai, 2015). Biotic factors are

influenced by microorganisms, their growth, and their ability to metabolize plastics (Yasin et al., 2022).

1.5.4 Biodegradation of Polyethylene (PE):

Research indicated that there is not known mechanism of biodegradation of polyethylene until now. The research suggested that biotic and abiotic factors have an important role in the biodegradation of PE (Ghatge et al., 2020). Exposure to abiotic factors such as ultraviolet radiation (UV), chemicals in the environment, and heat can cause PE to oxidize. After the oxidation of polyethylene carbonyl groups are generated in the alkane chains of PE. Then it is degraded by microorganisms after a period of time (Montazer et al., 2020).

Several bacterial genera, including both Gram-positive and Gram-negative species, such as *Pseudomonas, Ralstonia, Staphylococcus, Stenotrophomonas, Acinetobacter, Rhodococcus, Streptococcus, Streptomyces, Klebsiella,* and *Bacillus*, have been found to degrade various types of PE (Ghatge et al., 2020). One study referred to the role of Actinomycetes such as a *Streptomyces* strain in the biodegradation of PE (KILDE). *Penicillium* and *Aspergillus* fungi were also experimented with in the biodegradation of PE (Alshehrei, 2017).

In addition to studying the biodegradation of polyethylene by bacteria and fungi, the ability of yeast to degrade PE was also studied (Elsamahy et al., 2023). This study isolated symbiotic yeast from the guts of termites as microbes to degrade LDPE. Specific species *Meyerozyma guilliermondii*, *Sterigmatomyces halophilus*, and *Meyerozyma caribbica* were incorporated to create DYC yeast. LDPE was used as the only carbon source, consortium LDPE-DYC showed high growth. The tensile strength of TS decreased by 63,4% and the LDPE mass decreased by 33,2% (Elsamahy et al., 2023).

1.6 Aims of Study

This study aims to measure the ability of six bacterial strains to grow in media containing plastic and compare the ability to grow with media not containing plastic, check the effect of stimulating some chemicals on the growth of bacteria, and measure the ability of bacteria to metabolize and degrade plastics.

2 Methods

Bacterial strains used in this study, are provided by Andrew Jenkins and Zahra Zolanvari (Zolanvari, 2021).

The bacterial strains are called ZZ1- ZZ2- ZZ6- ZZ7- ZZ12,1- ZZ12,2.

The nutrient medium: Half-strength Tryptone Soya Agar (½TSA) was used in the whole study. The nutrient medium was prepared with the addition 5gr agar, 10gr TSA and 500ml distilled water, then it was sterilized by autoclave.

The liquid medium M9+ was prepared with the addition 5.6gr M9 salt, 500ml distilled water and 500µl solution 2(1000×) trace element solution, then it was sterilized by autoclave.

The liquid medium M9++ was prepared with the addition 5.6gr M9 salt, 500ml distilled water, 500µl solution 2(1000×) trace element solution, and 50µl CuSO4+FeCl2 supplement, then it was sterilized by autoclave.

Finely ground low density polyethylene (LDPE) was kindly provided by Hege Baan, Ineos Polyolefins that was used in this study.

Serial dilution method: A series of 5 tubes was prepared. 900µl of M9+ media was filled in tubes. 100µl of the culture flask was taken and placed over the first tube and the tube called 10^{-1}, then 100µl of the dilution 10^{-1} was taken to the second tube to prepare dilution 10^{-2}, then 10^{-3}, 10^{-4} and 10^{5}.

2.1 Assessing microbial growth on plastic (LDPE)

2.1.1 Growth in viable plate count measurement (liquid medium M9+ without CuSO4+FeCl2 supplement)

To test the ability of bacteria growth, a control culture and a test culture were prepared.

250ml Erlenmeyer flasks and caps were sterilized in the autoclave. Preparation of the homogeneous bacterial suspension; in the tube was filled 1ml media M9+ and a few fresh bacterial colonies and was shaken by a vortexer to homogenize.

In 250ml Erlenmeyer flask test and cultures were prepared as shown in the table (21). It was repeated for the six samples ZZ1- ZZ2- ZZ6- ZZ7- ZZ12,1- ZZ12,2. The flasks were covered with aluminium foil to avoid algae growth and placed on the A rotary shaker at a rate of 250rpm and at the room temperature.

By use of a serial dilution method, 100µl of all dilutions was spread on ½TSA agar, and it stayed at room temperature for several days. After that, the bacteria colonies were counted. The steps were repeated daily for many days for all strains, to monitor the growth of bacteria.

The number of microorganisms in this method is determined by using this formula:

$CFU.ml^{-1}$ = (Number of colonies x dilution factor) / volume of culture plated.

Table 2-1ingredients of test and control cultures to prepare test of growth in M9+ media.

Test culture	Control culture
2gr PE	-
500µl of the homogeneous bacterial suspension	500µl of the homogeneous bacterial suspension
50ml Liquid medium M9+	50ml Liquid medium M9+

2.1.2 Growth in viable plate count measurement (liquid medium M9++ with CuSO4+FeCl2 supplement):

Copper and iron play a role in bacterial growth and metabolism.

Many bacterial enzymes, such as cytochrome c oxidase, superoxide dismutase, and lysyl oxidase, depend heavily on copper and iron. The electron transfer processes that are necessary for bacterial metabolism also include copper and iron ions (Rosenbaum et al., 2011).

In this method, a liquid medium with CuSO4+FeCl2 supplement was used to test the ability of bacteria to grow in this medium.

The same steps in 3.1.1 were repeated. Only the liquid medium was exchanged in both cultures. In 250ml Erlenmeyer flask test and cultures were prepared as shown in the table (2-2).

Table 2-2ingredients of test and control cultures to prepare test of growth in M9++ media.

Test culture	Control culture
2gr PE	-
500µl of the homogeneous bacterial suspension	500µl of the homogeneous bacterial suspension
50ml liquid medium M9++	50ml liquid medium M9++

2.1.3 Growth in presence a nitrification inhibitor

Allylthiourea (ATU) has been used as a nitrification inhibitor that prevents microbes from using ammonium as an energy source. Therefore, plastic is now the only source of energy for the growth of bacteria in the medium.

Allylthiourea (ATU) is 1000× concentrate, (86mM, 1mg.ml^{-1}). PH= 8.

According to data obtained from viable plate count measurement (both mediums), growth was tested for strains ZZ-6, ZZ-12,1 and ZZ-12,2.

All instruments have been sterilized 250ml Erlenmeyer flask and caps in autoclaves. A homogeneous bacterial suspension (a lot of fresh bacterial colonies and M9+ media). Then four cultures for ZZ-6, ZZ-12,1, and ZZ-12,2 were prepared as shown in the table (2-3). Bacteria growth was measured daily by using viable plate count measure and serial dilution method.

Table 2-3 ingredients of four cultures to prepare test of growth in presence the nitrification inhibitor.

Test culture with ATU	Control culture with ATU	Test culture without ATU	Control culture without ATU
2gr PE	-	2gr PE	-
500µl the homogeneous suspension	500µl the homogeneous suspension	500µl the homogeneous suspension	500µl the homogeneous suspension
50µl Allylthiourea	50µl Allylthiourea	-	-
50ml M9+ media for strain ZZ-6. 50ml M9++ media for strains ZZ12,1 and ZZ-12,2	50ml M9+ media for strain ZZ-6. 50ml M9++ media for strains ZZ12,1 and ZZ-12,2	50ml M9+ media for strain ZZ-6. 50ml M9++ media for strains ZZ12,1 and ZZ-12,2	50ml M9+ media for strain ZZ-6. 50ml M9++ media for strains ZZ12,1 and ZZ-12,2

N. M9 media was chosen according to the best bacterial growth in the test culture.

2.1.4 Co2 production test

This method is based on the measurement of microbial degradation of plastic. The microbial strain is cultured with plastic in a sealed flask. After a period of growth, the CO2 concentration in the headspace is measured and compared to a negative control. A Dräger pump is used to draw a fixed volume of headspace gas through a glass tube containing hydrazine and a pH indicator, which reacts with CO2 to produce ammonia and turn the pH indicator purple. The length of the purple zone indicates the amount of CO2 present, and hence the amount of plastic metabolism. The metabolism of plastic is explained by an equation where 2CH2 + 3 O2 -> 2 CO2 + 2 H2O, indicating an increase in CO2 concentration as the plastic is metabolized.

The test system was designed by Andrew Jenkins for CO2 production test. The system needs two syringes; one syringes was 1ml to link a tube to pump which a CO2 100/a Dräger and another syringe was 50ml filled with water. The pressure was created in the system, and the CO2 is expelled through the needle of the other syringe as a result of the pumping pressure. The two syringes were used to depressurize the vial and draw 75mL of air from the sample. The 50ml syringe filled with water allows for pressure equalization and prevents contamination of the headspace with ambient air. So, the Co2 gets into the Dräger tube and reacts with the chemical existing. The color turns to violet in the Dräger tube. The violet color shows the presence of CO2 and biodegradation (Zolanvari, 2021).

The 250ml or 150ml Erlenmeyer flasks and self-sealing rubber caps are sterilized. A M9++ minimal medium supplemented with 500µl trace elements and 100µl of CuSO4+FeCl2 supplement are prepared and sterilized.

A homogeneous suspension of the strain in sterile M9+ media was made and diluted to A540= 0.5 to make all bacterial dilutions equal. To find out the ability of bacteria to metabolize plastic, three cultures were prepared as shown in Table (2-4). It was repeated for the six samples ZZ-1, ZZ-2, ZZ-6, ZZ-7, ZZ-12,1, and ZZ-12,2. The flasks were covered with aluminumm foil to avoid algae growth and placed on a rotary shaker at a rate of 250rpm and at room temperature.

Table 2-4 ingredients of cultures to prepare Co2 production test

Test culture	Control culture	Negative control culture
1gr PE	-	1gr PE
50ml M9++ media	50ml M9++ media	50ml M9++ media
1ml the homogeneous suspension	1ml the homogeneous suspension	1ml the homogeneous suspension

The test was conducted twice:

The first time: 250ml Erlenmeyer flasks were used, and the incubation period was 10 weeks.

The second time: 150ml Erlenmeyer flasks were used, and the incubation period was 11 weeks.

2.2 Statistical methods

By using the test of Normality in the RStudio program, the R cmdr (R commander) package for $CFU.ml^{-1}$ for bacterial growth in all cultures for all strains, the results were that the distribution was not normal, so the data was converted to Log10.

In R command NMBU version, the two cultures (test and control) were compared for many strains in each medium. Welch Two sample t-test was used to calculate the t and p-value.

The correlation coefficient between Log (CO2 measurements for the first time) and Log (CO2 measurements for the second time) was calculated by using the RStudio program, R cmdr (R commander NMBU version) package.

3 Results

3.1 Assessing microbial growth on plastic (LDPE)

3.1.1 Growth in viable plate count measurement

To test the ability of bacteria to grow on PE. Two cultures were prepared, a control culture without PE and a test culture with PE.

The growth of each strain was tested in two different mediums. In the first stage, the medium contains only M9+. In the second stage was added 50µl for 50ml liquid media (M9++). By using viable plate count and a serial dilution, the number of bacterial colonies in Petri dishes was counted.

3.1.1.1 Strain ZZ-1

In the M9+ medium, the bacteria in the test culture were unable to grow. As it began to die after the fifth day. while bacteria were able to grow after the fifth day in the control culture that did not contain PE. In the cultures with the CuSO4+FeCl2 supplement, the bacteria were able to grow and possibly metabolize PE in the test culture better than in the culture without PE.

The number of bacterial colonies for strain ZZ-1 was registered daily in Annex (1). The Logarithm (CFU.ml^{-1}) for strain ZZ-1 was drawn in Figure (3-1).

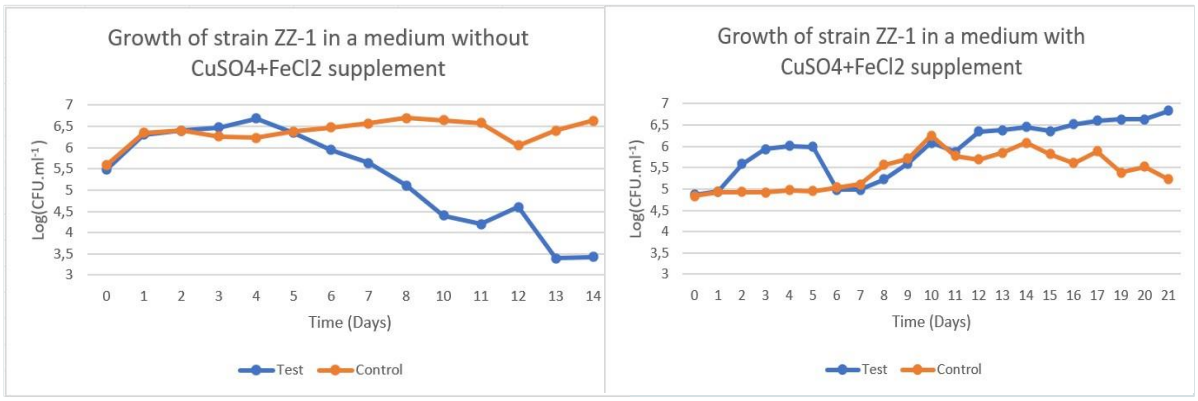

Figure 3-1 The growth curves of strain ZZ-1. The graph on the left shows the logarithm of the number of bacterial colonies in a medium without copper-iron per days. It was registered daily (except the 9th day) for 14 days. The Graph on the right shows the logarithm of the number of bacterial colonies in a medium with copper-iron per days. It was registered daily (except the 18th day) for 21 days.

3.1.1.2 Strain ZZ-2

In both M9+ and M9++ mediums, bacteria showed their ability to grow and possibly metabolize PE in the test cultures better than the control cultures that did not contain PE. In Annex (2) was registered daily the number of bacterial colonies for strain ZZ-2. The Logarithm (CFU.ml^{-1}) for the strain ZZ-2 was drawn in Figure (3-2).

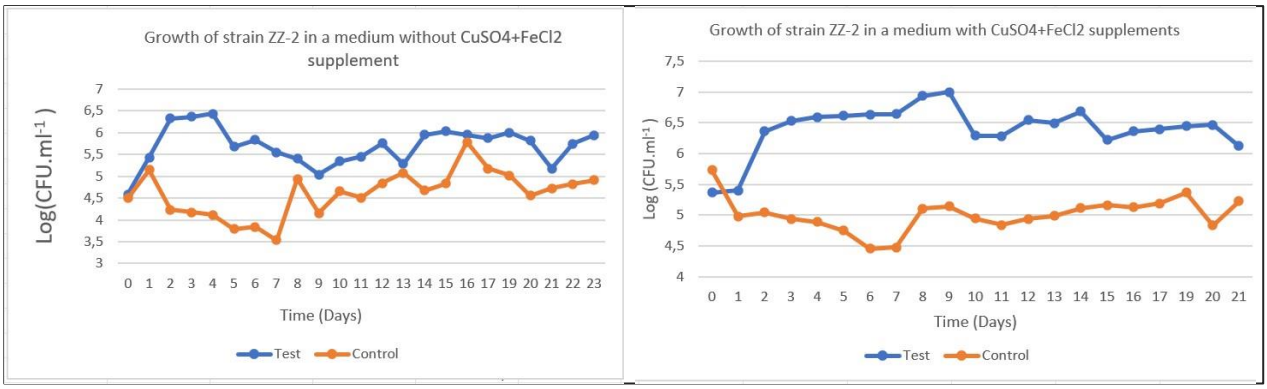

Figure 3-2 The growth curves of strain ZZ-2. The graph on the left shows the logarithm of the number of bacterial colonies in a medium without copper-iron per days. It was registered daily (except the 18th day) for 23 days. The Graph on the right shows the logarithm of the number of bacterial colonies in a medium with copper-iron per days. It was registered daily (except the 18th day) for 21 days.

3.1.1.3 Strain ZZ-6

Based on the results in Annex (3) and growth curves Figure (3-3). In M9+ medium, it seems clear that there is no difference between the test and control. This indicates that the strains are growing on M9+ medium and are probably not metabolizing PE. In the M9++ medium, it seems that bacteria cannot grow in both test and control cultures.

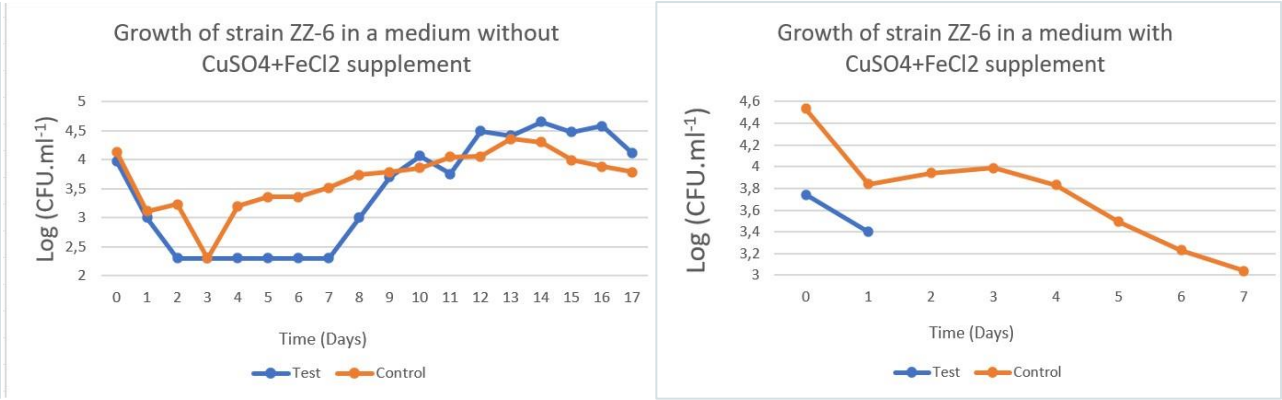

Figure 3-3 The growth curve of strain ZZ-6. The graph on the left shows the logarithm of the number of bacterial colonies in a medium without copper-iron per days. It was registered daily for 17 days. The Graph on the right shows the logarithm of the number of bacterial colonies in a medium with copper-iron per days. It was registered daily for 7 days.

3.1.1.4 Strain ZZ-7

As can be seen in Annex (4) and growth curves Figure (3-4) in the M9+ medium, the bacteria in the test culture couldn't grow in a medium containing PE. As it began to die after the fourth day. while bacteria were able to grow after the fourth day in the control culture that did not contain PE. In the cultures with the CuSO4+FeCl2 supplement, the bacteria were able to grow and possibly metabolize PE in the test culture better than in the culture without PE.

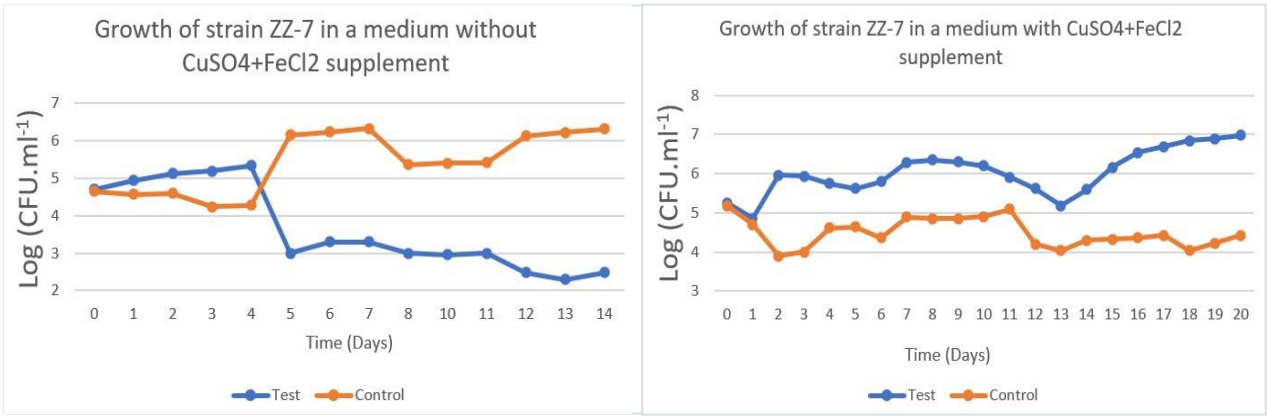

Figure 3-4 The growth curve of strain ZZ-7. The graph on the left shows the logarithm of the number of bacterial colonies in a medium without copper-iron per days. It was registered daily (except the 9th day) for 14 days. The Graph on the right shows the logarithm of the number of bacterial colonies in a medium with copper-iron per days. It was registered daily for 20 days.

3.1.1.5 Stain ZZ-12,1

By looking at Annex (5) and the growth curves Figure (3-5), bacteria in the M9+ medium did not grow in the test culture. But it did grow in the control culture after the fourth day of incubation.

When preparing cultures of M9++, old bacteria were used (spread on agar for a long time ago). Therefore, it was difficult for bacteria to grow in the test culture with PE. The bacteria began to die gradually until 4th day Then it began to grow slowly. As for the culture of control, it grew gradually.

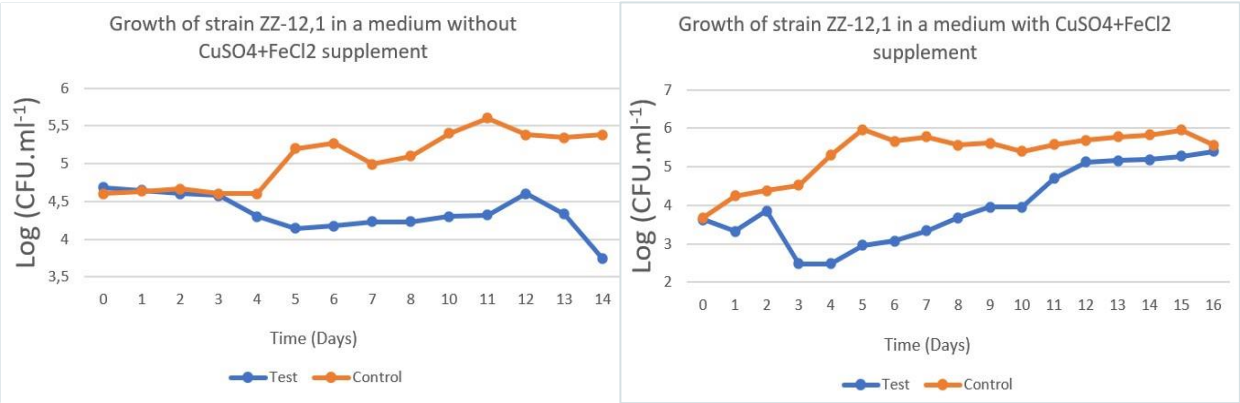

Figure 3-5The growth curve of strain ZZ-12,1. The graph on the left shows the logarithm of the number of bacterial colonies in a medium without copper-iron per days. It was registered daily (except the 9th day) for 14 days. The Graph on the right shows the logarithm of the number of bacterial colonies in a medium with copper-iron supplement per days. It was registered daily for 16 days.

3.1.1.6 Strain ZZ12,2

In both test and control cultures, the bacteria were unable to grow in the M9+ medium. When cultures were prepared with CuSO4+FeCl2 supplement, old bacteria were used (spread on agar for a long time ago). In the M9++ medium, the bacteria began to grow until the second day and then began to die gradually. The opposite happened in the control culture, as the bacteria began to die until the seventh day and then returned to grow. Annex (6) and growth curve Figure (3-6) explain the results.

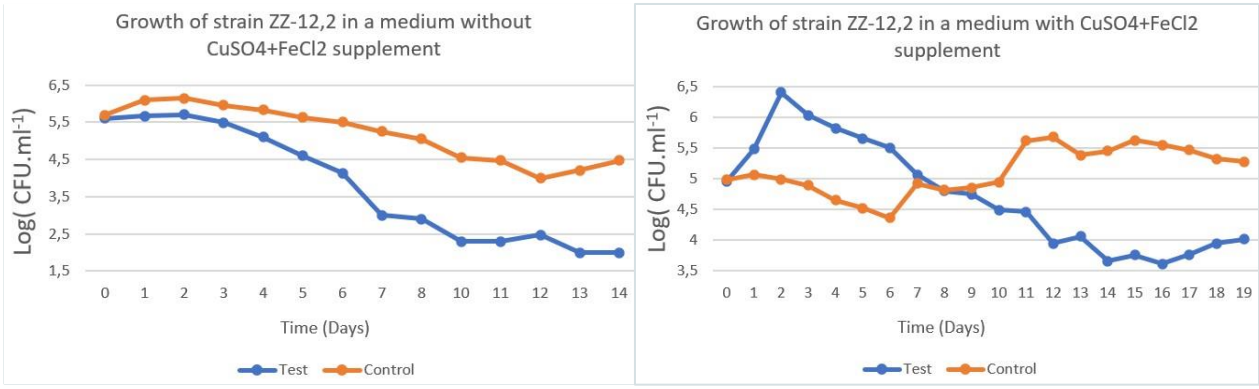

Figure 3-6The growth curves of strain ZZ-12,2. The graph on the left shows the logarithm of the number of bacterial colonies in a medium without copper-iron per days. It was registered daily (except the 9th day) for 14 days. The Graph on the right shows the logarithm of the number of bacterial colonies in a medium with copper-iron supplement per days. It was registered daily for 19 days.

3.1.2 Bacterial growth in presence a nitrification inhibitor

Four cultures were prepared (test culture with ATU and PE, control culture with ATU and without PE, test culture with PE, and control culture without PE and without ATU) to test the ability of bacteria to grow in the presence or absence of PE as the only carbon source and ATU inhibitor.

3.1.2.1 *Strain* ZZ-6

By looking at Annex (8) and the growth curve Figure (3-7), it was found that the growth of bacteria in the four cultures was variable (between growth and death of bacteria). Noting that growth in the two control cultures are better than growth in the two test cultures that contain PE.

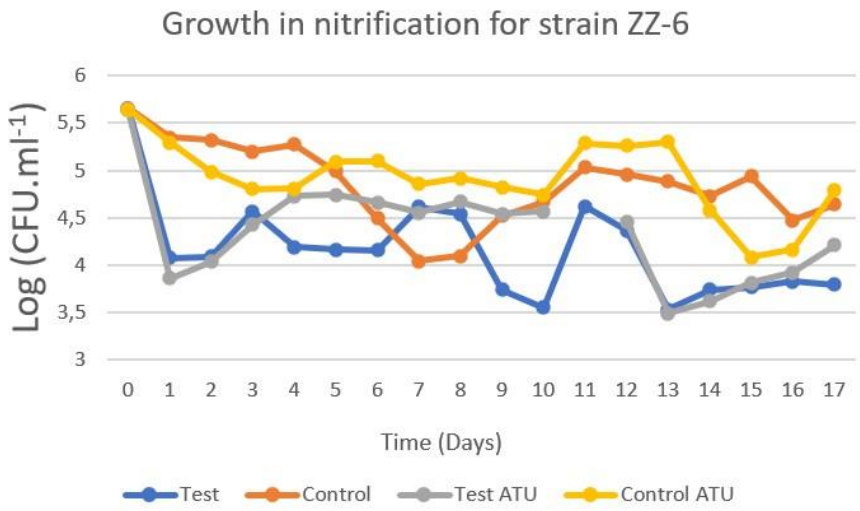

Figure 3-7 The growth curves (Nitrification) of ZZ-6 strain show the logarithm of the number of bacterial colonies in a medium without copper-iron per days. For four cultures (Test, Control, Test ATU, and Control ATU). It was registered daily (except the 11th day for Test ATU) for 17 days.

3.1.2.2 Strain ZZ-12,1

By observing the growth of bacteria in the four cultures for a period of 17 days, it was noted that the growth of bacteria was variable from day to day.

Due to the ability of bacteria to grow in a medium containing CuSO4+FeCl2 supplement. Nitrification was tested in this medium. Newly grown bacteria on agar plates were used. Bacteria in the test culture without the presence of ATU grew better than the rest of the cultures. The Annex (8) and growth curve Figure (3-8) show that.

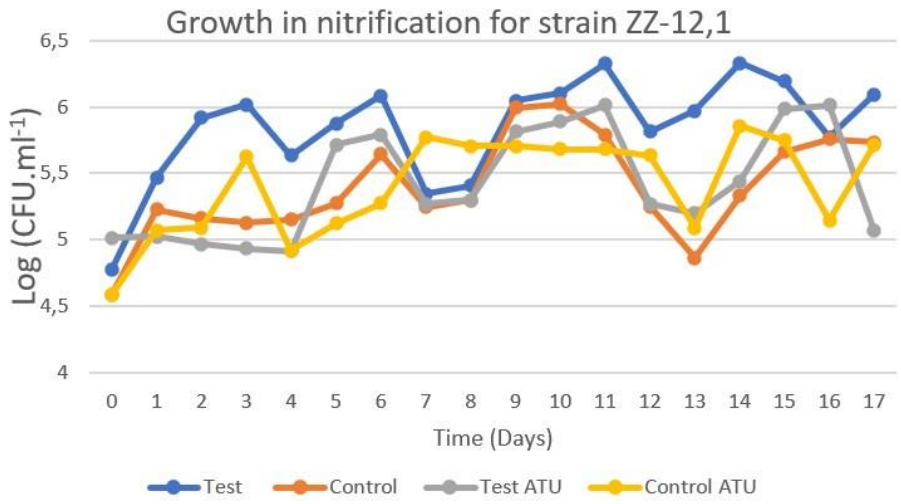

Figure 3-8 The growth curves (Nitrification) of strain ZZ-12,1 Shows the logarithm of the number of bacterial colonies in a medium with copper-iron per days. For four cultures (Test, Control, Test ATU, and Control ATU). It was registered for 17 days.

3.1.2.3 Strain ZZ-12,2

Bacteria in the test culture without ATU (which grew significantly after the second day of incubation) grew better than control groups. Also, the bacteria in the test culture with ATU grew better than the control culture with ATU. Annex (9) and the growth curves (3-9) show the results.

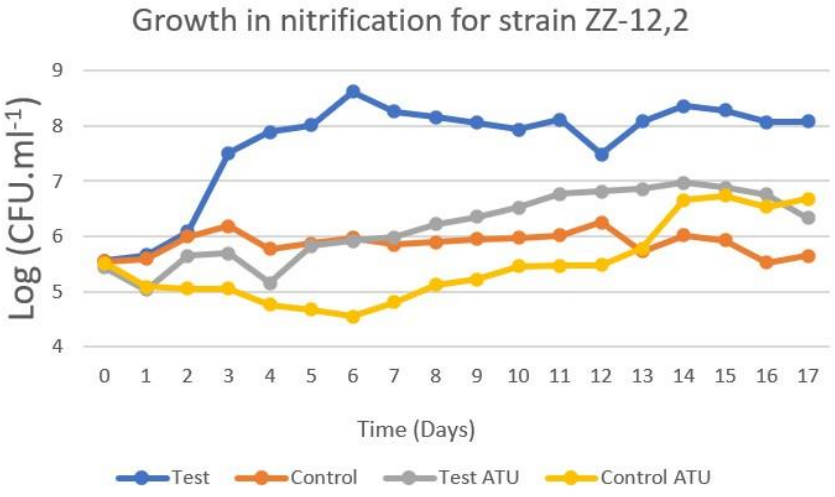

Figure 3-9 the growth curves (Nitrification) of strain ZZ-12,2 shows the logarithm of the number of bacterial colonies in a medium with copper-iron per days. For four cultures (Test, Control, Test ATU, and Control ATU). It was registered for 17 days.

3.1.3 CO2 production test

The biodegradation of the plastic was determined by a Co2 production test. Where the percentage of CO2 was measured in isolated flasks containing plastic (polyethylene) and bacteria and compared with isolated flasks containing bacteria and growth media. It was also compared to a negative control culture containing PE and growth media.

The test was repeated twice.

Based on the results in Figures (3-10) (3-11) and Table (3-1),

ZZ-1 and ZZ-2 samples showed an increase in CO2 production in the test culture over other cultures of the same strain. As for the ZZ-6 sample, CO2 production in the test culture was low compared to the rest of the cultures. Samples ZZ-7, ZZ-12,1 and ZZ12,2, CO2 production in the test culture was a little higher than the rest of the cultures of the same strains.

Table 3-1results of CO2 production test on all samples to show their biodegrading ability.

Strain		CO2 measurements for the first time	CO2 measurements for the second time	Biodegradation ability
ZZ-1	Test	200 ppm	100ppm	Probably Yes
	Control	105ppm	50ppm	
	Negative control	175ppm	25ppm	
ZZ-2	Test	200ppm	75ppm	Probably Yes
	control	150ppm	50ppm	
	Negative control	10ppm	25ppm	
ZZ-6	Test	300ppm	50ppm	No
	control	300ppm	75ppm	
	Negative control	300ppm	25ppm	
ZZ-7	Test	210ppm	90ppm	No
	control	150ppm	80ppm	
	Negative control	200ppm	25ppm	
ZZ-12,1	Test	200ppm	60ppm	No
	control	200ppm	50ppm	
	Negative control	100ppm	25ppm	
ZZ-12,2	Test	150ppm	60ppm	No
	control	100ppm	40ppm	
	Negative control	200ppm	25ppm	

Figure 3-10 CO2 measurements for the first time.

Neg. co refer to Negative control culture, t refers to test culture and co refer to control culture.

ZZ-1	ZZ-2	ZZ-6	ZZ-7	ZZ-12,1	ZZ-12,2
Neg.co- t- co	co- t- Neg.co	co- t- Neg.co	Neg.co-t-co	co- t- Neg.co	co-t-Neg.co

Figure 3-11CO2 measurements for the second time.

ZZ-1 control- test ZZ-2 control- test ZZ-6 control- test ZZ-7 control- test ZZ-12,1 control- test ZZ-12,2 control- test negative control.

3.2 Statistical methods

The two cultures were compared to test the growth of both ZZ-2 in M9+ and M++ media, and ZZ-1, and ZZ-7 in the M9++ media to make sure there are differences between the two cultures or not. Welch Two sample t-test (confidence level is 95) results are shown in Table (3-2). As for the rest of the growth tests for the rest of the strains, the statistical analysis is not important.

Table 3-2 Results of Welch Two sample t-test

Strain	t	P-value
ZZ-2 in M9+ media	7,5986	$0,1791e^{-8}$
ZZ-2 in M9++ media	13,066	$3,557e^{-15}$
ZZ-1 in M9++ media	3.0457	0.004362
ZZ-7 in M9++ media	10,298	$5,176e^{-12}$

The correlation between Log (CO2 measurements for the first time) and Log (CO2 measurements for the second time) was investigated, to find out if there is a correlation between the size of the flask and the amount of CO2 produced. By using RStudio program the correlation coefficient is approximately R=0,312 which is a low rate. The correlation coefficient is draw in the scatter plot in Figure (3-12)

Fant ingen figurlisteoppføringer.

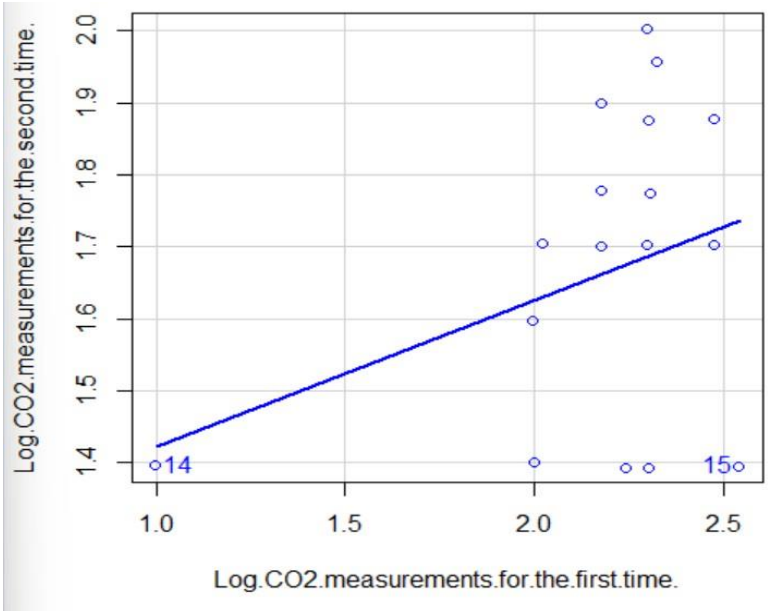

Figure 3-12 The scatter plot illustrating the correlation between Log (CO2 measurements for the first time) and Log (CO2 measurements for the second time), n= 18, R=0,312.

4 Discussion

The ability of six strains of bacteria to grow in a medium containing polyethylene was tested. Testing occurred in several mediums. The bacteria's ability to metabolize plastic was also tested by measuring the production of CO2.

4.1 Growth of strain ZZ-1 and growth of strain ZZ-7

The growth curves of both strains were similar. The results (Figure (3,1) on the left and Figure (3-4) on the left) in this study showed that the M9+ medium was not suitable for the growth of bacteria in the presence of PE, due to the gradual death of bacteria after many days of incubation, so the presence of PE inhibited the growth of bacteria (Kim et al., 2022). The evidence is that the bacteria grew well in the control culture that did not contain plastic after many days of incubation. There is a possibility that the bacteria obtained the CO2 needed for growth in control culture from the surrounding air when growth was measured daily or from the nutrients in M9+ medium(Eide, 2023). Another possibility is that the lack of nutrients in M9+ led to the bacteria's inability to form biofilms on PE and thus affected on the amount of CO2 present in the medium (Qurashi and Sabri, 2011). This indicates the negative effect of PE on the growth of bacteria in this medium.

In the M9++ medium, bacteria were able to grow and possibly metabolize plastics in the presence of polyethylene as a carbon source. The growth of bacteria indicates the role of the CuSO4+FeCl2 supplement in the growth of bacteria. Due to the role of copper and iron in stimulating oxygenase enzymes that carry out the primary oxidative attack on PE (Lubben, 1994). This could be an explanation for the growth of bacteria in this M9++ medium.

Welch's test for ZZ-1: t= 3.0457 and P-value= 0.004362, Welch's test for ZZ-7: t= 10,298, P-value= $5{,}176e^{-12}$ also showed that there was a difference between the two cultures. The growth curves (Figure (3-1) on the right and Figure (3-4) on the right) prove that the best growth was for the bacteria present with PE. The ATP test was carried out to estimate the metabolic activity of the same strain ZZ-1, the result was that the metabolic activity of these bacteria was low (Zolanvari, 2021), but the bacterial suspension did not contain CuSO4+FeCl2 supplement. This showed the importance of

this supplement for the growth of this strain. As for the ATP test on strain ZZ-7, the metabolic activity of this strain was high (Zolanvari, 2021), which may indicate another reason that led to the cessation of the growth of this bacterium in the test culture in medium M9+.

4.2 Growth of strain ZZ-2

Strain ZZ-2 grew better in the test culture in both M9+ and M9++ media Figure (3-2). This referred to the bacteria may have the ability to degrade PE.

Welch's test supports this observation. t=7,5986, P-value= $0,1791e^{-8}$ in the M9+media. t=13,066, P-value= $3,557e^{-15}$ in M9++ media.

Welch's test showed a very low p-value, indicating that the difference observed between the test and control cultures is highly significant. As for the ATP test on strain ZZ-2, the metabolic activity of this strain was high (Zolanvari, 2021), these results indicate good activity and growth of bacteria in the presence of PE.

On the anther hand, many organisms form a biofilm on the surface of the plastic without breaking the plastic bonds, so the number of bacteria and the number of biofilms do not indicate plastic degradation (Eide, 2023). However, the growth of bacteria in a medium containing plastic is the first step in the biodegradation of plastic.

4.3 Growth of strain ZZ-6

The results (Figure (3-3) on the left) showed the ability of this strain to grow just in the M9+ medium without using carbon as an energy source. Bacterial metabolic activity in the ATP test of this strain was high, indicating the ability of the bacteria to grow in the M9+ medium (Zolanvari, 2021).

On the other hand, the bacteria could not grow in the M9++ medium in both cultures (Figure (3-3) on the right). This could be because the bacteria did not adapt to the ironcopper supplement, or because the iron-copper supplement inhibited bacterial growth (Johnson et al., 2017).

Due to the bacteria's ability to grow in the M9+ medium without using the carbon as a source of energy, there was a possibility that the bacteria convert ammonium into nitrite and nitrate and thus derive energy for growth from this conversion (Ebeling et al.,

2006). A test was conducted to inhibit the action of ammonium in the medium by adding ATU to the medium and forming four cultures (Test, Control, Test ATU, and Control ATU). The result was that there was no difference in the growth curves between the four cultures (Figure 3.7). The bacteria derive the energy needed for growth from the surrounding medium only, and after some time, the bacteria will die. The bacterial strain ZZ-6 is gram-negative (Zolanvari, 2021) therefore, there is a possibility that it contains the enzyme *lysine oxidase*, which causes the dispersion of biofilms in some gram-negative bacteria (Landini et al., 2010). In other words, the presence of the *lysine oxidase* enzyme does not allow bacteria to attack plastic to form biofilms on it, but bacteria only grow in the medium.

4.4 Growth of strain ZZ-12,1 and growth of strain ZZ-12,2

Based on the provided information from this study (Figures (3-5) on the left and (3-6) on the left), the result appears that PE negatively affected bacterial growth in the M9+ medium. The evidence is that the bacteria were able to grow the M9+ medium in the control culture (growth assumptions were explained in para 4.1).

Strain ZZ-12,1 in the beginning, the bacteria could not grow in the M9++ medium containing PE, which could be attributed to the use of aged bacteria. After some days, the bacteria began to multiply again due to the presence of nutrients in the M9++ media. The opposite happened in strain ZZ-12,2, whereby the bacteria grew for two consecutive days after incubation in the test culture and then began to die gradually. This case is explained in this way, many of the enzymes in the cell may be degraded, except enzymes that utilize of using energy. When nutrients and energy are available, the cell recombines enzymes from existing DNA, and the bacteria return to grow and reproduce (Morita, 1990). But the bacteria in strain ZZ-12,2 could not rebuild the enzymes.

To ensure that the energy derived for the growth of bacteria is from Co2 or through the nitrification process, a nitrification test was performed. From Figures (3-8) and (3-9), The result was shown that young bacteria grow in the M9++ medium that contained PE and not containing ATU nitrification inhibitor. Strain ZZ-12,1 might be chemoorganotrophic organisms that derive energy from carbon and the nitrification process according to an explanation about organic organisms with nitrogenous chemical

nutrition (Sayler et al., 2013). The bacterial strain ZZ-12,2 was able to grow in the test culture containing ATU inhibitor and PE better than the growth in a medium that contained the ATU inhibitor and did not contain PE Figure (3-9). This shows the role of CO2 as the only source of energy for bacterial growth in this strain.

4.5 CO2 production test

When polymers degrade, products such as H2O, CH4, and CO2 are released. Measuring these products helps measure the rate of biodegradation of plastic (Anjana et al., 2020). The CO2 production test is a measure of the degradation of polyethylene, as it is the only source of carbon in a closed flask. Strains ZZ-1 and ZZ-2 samples showed a higher measurement of CO2 in the test culture than in the negative control and control cultures according to two tests done. For a single polymer material, must 60% of the organic carbon converted to CO2 for satisfactory biodegradation, and 90% for segmented copolymers, and the additives are of low molecular weight (Kale et al., 2007). Strains ZZ-6, ZZ-7, ZZ-12,1, and ZZ-12,2 The measurements of Co2 in the test culture were low compared to the rest of the cultures.

The lack of CO2 production in strains ZZ-7 and ZZ-12,2 (which gave positive growth in this study in M9++ medium) indicates that the bacteria may grow without using carbon as an energy source, or that the percentage of complement in the CO2 test was less than its percentage in the growth test. Where used 100µl per 500ml M9+ media in the Co2 production test but used 50µl per 50ml M9+ media in test growth.

In the first CO2 measurement, strains ZZ-6 and ZZ-7 were covered with cellulose caps and another plastic cap with airtight seals. In strain ZZ-6, high measurements of CO2 were obtained in the three cultures, cellulose caps must have been directly or indirectly affected on these measurements. But the measurement is the same in all cultures(300ppm), so these measurements can be ignored.

On the other hand, the level of CO2 in the atmosphere is about 420 ppm and by compared to the measurements of CO2 in the two tests, it was found that the measurements did not exceed 420 ppm, which indicates that there is no exaggeration in the measurements.

When the Erlenmeyer flasks were covered with self-sealing rubber caps, there was a possibility that some air may remain inside the flasks. In this study, two different sizes of

Erlenmeyer flasks (with the same amounts of components and approximately the same incubation period) were used to measure the production of CO2, the measurements were very different, so the correlation coefficient was measured between Log (CO2 measurements for the first time) and Log (CO2 measurements for the second time) to find out whether the amount of CO2 produced was affected by the size of flasks used.

The correlation coefficient between Log (CO2 measurements for the first time) and Log (CO2 measurements for the second time) was approximately 0.312, this means that there is no correlation between the size of the flasks used and the amount of CO2 produced.

5 Conclusion

This study investigated the ability of six strains isolated from a piece of waste plastic to grow in the presence and absence of PE under laboratory conditions.

The ZZ-2 strain showed its ability to grow in the presence of PE in the presence and absence of the CuSO4+FeCl2 supplement.

ZZ-1, ZZ-7, and ZZ12,2 strains were able to grow in the presence of CuSO4+FeCl2 supplement and PE, which refer to the role of the supplement to help bacteria attack PE. Bacterial growth in strain ZZ-6 was inhibited by the CuSO4+FeCl2 supplement.

Bacteria in strain ZZ-12,2 could grow by using carbon as an energy source after the addition of the ATU inhibitor.

The Co2 production test was also positive for strains ZZ-p1 and ZZ-2. Studies of the biodegradation of plastics should not be confined to laboratory conditions, they should be studied in the field.

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Annexes

Annex 1: Table 1 The number of bacterial colonies for the strain ZZ-1.

Different concentrations of bacteria were taken in the two different mediums on the day of incubation

Day	CFU.ml^{-1} ZZ-1 Test Medium without CuSO4+FeCl2	CFU.ml^{-1} ZZ1 Control. Medium without CuSO4+FeCl2	CFU.ml^{-1} ZZ-1 Test Medium with CuSO4+FeCl2	CFU.ml^{-1} ZZ1 Control. Medium with CuSO4+FeCl2
0	30 ×10^4	38 ×10^4	7,4 ×10^4	6,8 ×10^4
1	202 ×10^4	220 ×10^4	8,7 ×10^4	8,5 ×10^4
2	250 ×10^4	250 ×10^4	38 ×10^4	8,5 ×10^4
3	300 ×10^4	185 ×10^4	85 ×10^4	8,3 ×10^4
4	490 ×10^4	170 ×10^4	103 ×10^4	9,4 ×10^4
5	220 ×10^4	240 ×10^4	98 ×10^4	8,9 ×10^4
6	88 ×10^4	300 ×10^4	9,5 ×10^4	198 ×10^4
7	43 ×10^4	370 ×10^4	9,7 ×10^4	12,8 ×10^4
8	13 ×10^4	500 ×10^4	16,9 ×10^4	37 ×10^4
9	-	-	39 ×10^4	52 ×10^4
10	2,5 ×10^4	440 ×10^4	120 ×10^4	180 ×10^4
11	1,6 ×10^4	380 ×10^4	75 ×10^4	59 ×10^4
12	4 ×10^4	112 ×10^4	220 ×10^4	49 ×10^4
13	0,25 ×10^4	250 ×10^4	240 ×10^4	71 ×10^4
14	0,27 ×10^4	430 ×10^4	280 ×10^4	121 ×10^4
15	-	-	225 ×10^4	65 ×10^4
16	-	-	330 ×10^4	40 ×10^4
17	-	-	400 ×10^4	76 ×10^4
18	-	-	-	-
19	-	-	430 ×10^4	24 ×10^4
20	-	-	430 ×10^4	33 ×10^4
21	-	-	680 ×10^4	17 ×10^4

- : Bacterial colonies were not counted for this day.

Annex 2: Table 2 The number of bacterial colonies for the strain ZZ-2strain.

Different concentrations of bacteria were taken in the two different mediums on the day of incubation.

Day	CFU.ml^{-1} ZZ-2 Test Medium without CuSO4+FeCl2	CFU.ml^{-1} ZZ2 Control. Medium without CuSO4+FeCl2	CFU.ml^{-1} ZZ-2 Test Medium with CuSO4+FeCl2	CFU.ml^{-1} ZZ2 Control. Medium with CuSO4+FeCl2
0	$3,8 \times 10^4$	$3,1 \times 10^4$	23×10^4	54×10^4
1	$2,7 \times 10^4$	14×10^4	25×10^4	$9,6 \times 10^4$
2	210×10^4	$1,7 \times 10^4$	230×10^4	$11,1 \times 10^4$
3	230×10^4	$1,5 \times 10^4$	340×10^4	$8,7 \times 10^4$
4	270×10^4	$1,3 \times 10^4$	390×10^4	$7,7 \times 10^4$
5	47×10^4	$0,62 \times 10^4$	410×10^4	$5,6 \times 10^4$
6	68×10^4	$0,69 \times 10^4$	430×10^4	$2,86 \times 10^4$
7	35×10^4	$0,34 \times 10^4$	440×10^4	$2,99 \times 10^4$
8	25×10^4	$8,6 \times 10^4$	86×10^4	$12,7 \times 10^4$
9	$10,8 \times 10^4$	$1,41 \times 10^4$	990×10^4	$13,8 \times 10^4$
10	22×10^4	$4,5 \times 10^4$	196×10^4	$8,8 \times 10^4$
11	28×10^4	$3,2 \times 10^4$	192×10^4	$6,9 \times 10^4$
12	56×10^4	$6,9 \times 10^4$	350×10^4	$8,6 \times 10^4$
13	19×10^4	12×10^4	310×10^4	$9,7 \times 10^4$
14	88×10^4	$4,7 \times 10^4$	480×10^4	$12,9 \times 10^4$
15	106×10^4	$6,7 \times 10^4$	165×10^4	$14,6 \times 10^4$
16	89×10^4	60×10^4	230×10^4	$13,4 \times 10^4$
17	74×10^4	15×10^4	250×10^4	$15,4 \times 10^4$
18	-	-	-	-
19	99×10^4	$10,4 \times 10^4$	280×10^4	23×10^4
20	65×10^4	$3,6 \times 10^4$	290×10^4	$6,8 \times 10^4$
21	15×10^4	$5,3 \times 10^4$	133×10^4	$16,8 \times 10^4$
22	55×10^4	$6,6 \times 10^4$	-	-
23	86×10^4	$8,1 \times 10^4$	-	-

- : Bacterial colonies were not counted for this day.

Annex 3: Table 3 The number of bacterial colonies for the strain ZZ-6.

Different concentrations of bacteria were taken in the two different mediums on the day of incubation

Day	CFU.ml^{-1} ZZ-6 Test Medium without CuFe	CFU.ml^{-1} ZZ-6 Control. Medium without CuFe	CFU.ml^{-1} ZZ-6 Test Medium with CuFe	CFU.ml^{-1} ZZ-6 Control. Medium with CuFe
0	$9,6 \times 10^3$	$13,6 \times 10^3$	$5,5 \times 10^3$	34×10^3
1	1×10^3	$1,3 \times 10^3$	$2,5 \times 10^3$	$6,9 \times 10^3$
2	$0,2 \times 10^3$	$1,7 \times 10^3$	0	$8,7 \times 10^3$
3	$0,2 \times 10^3$	$0,2 \times 10^3$	0	$9,7 \times 10^3$
4	$0,2 \times 10^3$	$1,6 \times 10^3$	0	$6,8 \times 10^3$
5	$0,2 \times 10^3$	$2,3 \times 10^3$	0	$3,1 \times 10^3$
6	$0,2 \times 10^3$	$2,3 \times 10^3$	0	$1,7 \times 10^3$
7	$0,2 \times 10^3$	$3,3 \times 10^3$	0	$1,1 \times 10^3$
8	1×10^3	$5,5 \times 10^3$	-	-
9	5×10^3	$6,1 \times 10^3$	-	-
10	$11,7 \times 10^3$	$7,2 \times 10^3$	-	-
11	$5,7 \times 10^3$	$11,3 \times 10^3$	-	-
12	31×10^3	$11,5 \times 10^3$	-	-
13	26×10^3	23×10^3	-	-
14	45×10^3	20×10^3	-	-
15	30×10^3	$9,8 \times 10^3$	-	-
16	38×10^3	$7,7 \times 10^3$	-	-
17	13×10^3	$6,1 \times 10^3$	-	-

- : Bacterial colonies were not counted for this day.

Annex 4: Table 4 The number of bacterial colonies for the strain ZZ-7.

Different concentrations of bacteria were taken in the two different mediums on the day of incubation

Day	CFU.ml^{-1} ZZ-7 Test Medium without CuFe	CFU.ml^{-1} ZZ-7 Control. Medium without CuFe	CFU.ml^{-1} ZZ-7 Test Medium with CuFe	CFU.ml^{-1} ZZ-7 Control. Medium with CuFe
0	5×10^4	$4{,}5 \times 10^4$	$18{,}1 \times 10^4$	15×10^4
1	$8{,}8 \times 10^4$	$3{,}7 \times 10^4$	$7{,}2 \times 10^4$	$5{,}1 \times 10^4$
2	$13{,}5 \times 10^4$	4×10^4	91×10^4	$0{,}79 \times 10^4$
3	$15{,}4 \times 10^4$	$1{,}76 \times 10^4$	85×10^4	$0{,}99 \times 10^4$
4	22×10^4	$1{,}88 \times 10^4$	55×10^4	$4{,}1 \times 10^4$
5	$0{,}1 \times 10^4$	145×10^4	42×10^4	$4{,}3 \times 10^4$
6	$0{,}2 \times 10^4$	174×10^4	62×10^4	$2{,}32 \times 10^4$
7	$0{,}2 \times 10^4$	210×10^4	191×10^4	$7{,}7 \times 10^4$
8	$0{,}1 \times 10^4$	23×10^4	222×10^4	$7{,}1 \times 10^4$
9	-	-	199×10^4	$7{,}2 \times 10^4$
10	$0{,}09 \times 10^4$	25×10^4	156×10^4	$7{,}9 \times 10^4$
11	$0{,}1 \times 10^4$	26×10^4	82×10^4	$12{,}2 \times 10^4$
12	$0{,}03 \times 10^4$	133×10^4	42×10^4	$1{,}57 \times 10^4$
13	$0{,}02 \times 10^4$	167×10^4	$15{,}2 \times 10^4$	$1{,}09 \times 10^4$
14	$0{,}03 \times 10^4$	206×10^4	39×10^4	2×10^4
15	-	-	143×10^4	$2{,}1 \times 10^4$
16	-	-	350×10^4	$2{,}3 \times 10^4$
17	-	-	490×10^4	$2{,}7 \times 10^4$
18	-	-	690×10^4	$10{,}9 \times 10^4$
19	-	-	780×10^4	$1{,}7 \times 10^4$
20	-	-	940×10^4	$2{,}7 \times 10^4$

- : Bacterial colonies were not counted for this day.

Annex 5: Table 5 The number of bacterial colonies for the strain ZZ-12,1.

Different concentrations of bacteria were taken in the two different mediums on the day of incubation

Day	$CFU.ml^{-1}$ ZZ-12,1 Test Medium without CuFe	$CFU.ml^{-1}$ ZZ-12,1 Control. Medium without CuFe	$CFU.ml^{-1}$ ZZ-12,1 Test Medium with CuFe	$CFU.ml^{-1}$ ZZ-12,1 Control. Medium with CuFe
0	$4,8 \times 10^4$	4×10^4	$0,42 \times 10^4$	$0,48 \times 10^4$
1	$4,4 \times 10^4$	$4,3 \times 10^4$	$0,21 \times 10^4$	$1,75 \times 10^4$
2	4×10^4	$4,6 \times 10^4$	$0,71 \times 10^4$	$2,4 \times 10^4$
3	$3,8 \times 10^4$	4×10^4	$0,03 \times 10^4$	$3,3 \times 10^4$
4	2×10^4	4×10^4	$0,03 \times 10^4$	20×10^4
5	$1,4 \times 10^4$	$15,9 \times 10^4$	$0,09 \times 10^4$	92×10^4
6	$1,5 \times 10^4$	$18,6 \times 10^4$	$0,12 \times 10^4$	46×10^4
7	$1,7 \times 10^4$	$9,8 \times 10^4$	$0,22 \times 10^4$	60×10^4
8	$1,7 \times 10^4$	$12,6 \times 10^4$	$0,47 \times 10^4$	37×10^4
9	-	-	$0,91 \times 10^4$	41×10^4
10	2×10^4	25×10^4	$0,91 \times 10^4$	25×10^4
11	$2,1 \times 10^4$	40×10^4	$4,9 \times 10^4$	38×10^4
12	4×10^4	24×10^4	$13,3 \times 10^4$	49×10^4
13	$2,16 \times 10^4$	22×10^4	$14,6 \times 10^4$	59×10^4
14	$0,55 \times 10^4$	24×10^4	$15,5 \times 10^4$	68×10^4
15	-	-	$18,9 \times 10^4$	89×10^4
16	-	-	25×10^4	37×10^4

- : Bacterial colonies were not counted for this day.

Annex 6: Table 6 The number of bacterial colonies for the strain ZZ-12,2.

Different concentrations of bacteria were taken in the two different mediums on the day of incubation

Day	CFU.ml^{-1} ZZ-12,2 Test Medium without CuFe	CFU.ml^{-1} ZZ12,2 Control. Medium without CuFe	CFU.ml^{-1} ZZ-12,2 Test Medium with CuFe	CFU.ml^{-1} ZZ12,2 Control. Medium with CuFe
0	40×10^4	49×10^4	$8,9 \times 10^4$	$9,5 \times 10^4$
1	47×10^4	125×10^4	30×10^4	$11,5 \times 10^4$
2	50×10^4	138×10^4	250×10^4	$9,7 \times 10^4$
3	31×10^4	89×10^4	108×10^4	$7,8 \times 10^4$
4	$12,7 \times 10^4$	67×10^4	66×10^4	$4,5 \times 10^4$
5	4×10^4	42×10^4	45×10^4	$3,3 \times 10^4$
6	$1,36 \times 10^4$	32×10^4	32×10^4	$2,3 \times 10^4$
7	$0,1 \times 10^4$	18×10^4	$11,5 \times 10^4$	$8,4 \times 10^4$
8	$0,08 \times 10^4$	$11,3 \times 10^4$	$6,3 \times 10^4$	$6,5 \times 10^4$
9	-	-	$5,6 \times 10^4$	$7,1 \times 10^4$
10	$0,02 \times 10^4$	$3,5 \times 10^4$	$3,1 \times 10^4$	$8,8 \times 10^4$
11	$0,02 \times 10^4$	3×10^4	$2,9 \times 10^4$	41×10^4
12	$0,03 \times 10^4$	1×10^4	$0,89 \times 10^4$	47×10^4
13	$0,01 \times 10^4$	$1,6 \times 10^4$	$1,15 \times 10^4$	24×10^4
14	$0,01 \times 10^4$	3×10^4	$0,46 \times 10^4$	28×10^4
15	-	-	$0,57 \times 10^4$	42×10^4
16	-	-	$0,41 \times 10^4$	35×10^4
17	-	-	$0,58 \times 10^4$	29×10^4
18	-	-	$0,88 \times 10^4$	21×10^4
19	-	-	$1,04 \times 10^4$	19×10^4

- : Bacterial colonies were not counted for this day.

Annex 7: Table 7 the number of bacterial colonies for the strain ZZ-6 in the presence of ATU inhibitor

Day	CFU.ml^{-1} ZZ-6 Test Medium without ATU	CFU.ml^{-1} ZZ-Control. Medium without ATU	CFU.ml^{-1} ZZ-6 Test Medium with ATU	CFU.ml^{-1} ZZ-6 Control. Medium with ATU
0	45 ×10^4	45 ×10^4	43 ×10^4	44 ×10^4
1	1,19 ×10^4	22,2 ×10^4	0,73 ×10^4	19,7 ×10^4
2	1,23 ×10^4	20,9 ×10^4	1,09 ×10^4	9,7 ×10^4
3	3,7 ×10^4	15,8 ×10^4	2,67 ×10^4	6,4 ×10^4
4	1,55 ×10^4	18,7 ×10^4	5,4 ×10^4	6,5 ×10^4
5	1,47 ×10^4	9,8 ×10^4	5,5 ×10^4	12,3 ×10^4
6	1,45 ×10^4	3,1 ×10^4	4,6 ×10^4	12,5 ×10^4
7	4,1 ×10^4	1,1 ×10^4	3,6 ×10^4	7,2 ×10^4
8	3,5 ×10^4	1,25 ×10^4	4,7 ×10^4	8,3 ×10^4
9	0,55 ×10^4	3,3 ×10^4	3,5 ×10^4	6,7 ×10^4
10	0,36 ×10^4	4,7 ×10^4	3,7 ×10^4	5,5 ×10^4
11	4,2 ×10^4	10,7 ×10^4		19,5 ×10^4
12	2,32 ×10^4	9,1 ×10^4	2,89 ×10^4	18,2 ×10^4
13	0,34 ×10^4	7,6 ×10^4	0,31 ×10^4	19,9 ×10^4
14	0,55 ×10^4	5,4 ×10^4	0,42 ×10^4	3,8 ×10^4
15	0,59 ×10^4	8,6 ×10^4	0,65 ×10^4	1,21 ×10^4
16	0,67 ×10^4	2,96 ×10^4	0,84 ×10^4	1,46 ×10^4
17	0,62 ×10^4	4,4 ×10^4	1,63 ×10^4	6,2 ×10^4

Annex 8: Table 8 the number of bacterial colonies for the strain ZZ-12,1 in the presence of ATU inhibitor

Day	$CFU.ml^{-1}$ ZZ-12,1Test Medium without ATU	$CFU.ml^{-1}$ ZZ-12,1 Control. Medium without ATU	$CFU.ml^{-1}$ ZZ-12,1 Test Medium with ATU	$CFU.ml^{-1}$ ZZ-12,1 Control. Medium with ATU
0	$5,9 \times 10^4$	$3,8 \times 10^4$	$10,3 \times 10^4$	$3,8 \times 10^4$
1	$29,5 \times 10^4$	$16,8 \times 10^4$	$10,5 \times 10^4$	$11,7 \times 10^4$
2	83×10^4	$14,4 \times 10^4$	$9,2 \times 10^4$	$12,3 \times 10^4$
3	104×10^4	$13,4 \times 10^4$	$8,6 \times 10^4$	42×10^4
4	43×10^4	$14,2 \times 10^4$	$8,2 \times 10^4$	$8,3 \times 10^4$
5	75×10^4	$18,8 \times 10^4$	52×10^4	$13,3 \times 10^4$
6	122×10^4	44×10^4	62×10^4	$18,7 \times 10^4$
7	$22,2 \times 10^4$	$17,6 \times 10^4$	$18,6 \times 10^4$	59×10^4
8	$25,4 \times 10^4$	$19,9 \times 10^4$	$19,9 \times 10^4$	51×10^4
9	111×10^4	99×10^4	65×10^4	51×10^4
10	127×10^4	106×10^4	78×10^4	48×10^4
11	212×10^4	62×10^4	103×10^4	48×10^4
12	65×10^4	$17,8 \times 10^4$	$18,6 \times 10^4$	43×10^4
13	94×10^4	$7,3 \times 10^4$	$15,8 \times 10^4$	$12,3 \times 10^4$
14	214×10^4	$21,5 \times 10^4$	$27,5 \times 10^4$	72×10^4
15	156×10^4	46×10^4	97×10^4	56×10^4
16	59×10^4	57×10^4	103×10^4	14×10^4
17	125×10^4	54×10^4	$11,6 \times 10^4$	52×10^4

Annex 9: Table 9 the number of bacterial colonies for the strain ZZ-12,2 in the presence of ATU inhibitor

Day	CFU.ml^{-1} ZZ-12,2 Test Medium without ATU	CFU.ml^{-1} ZZ-12,2 Control. Medium without ATU	CFU.ml^{-1} ZZ-12,2 Test Medium with ATU	CFU.ml^{-1} ZZ-12,2 Control. Medium with ATU
0	$3,6 \times 10^5$	$3,5 \times 10^5$	$2,76 \times 10^5$	$3,3 \times 10^5$
1	$4,6 \times 10^5$	$3,9 \times 10^5$	$1,08 \times 10^5$	$1,23 \times 10^5$
2	$12,3 \times 10^5$	$9,9 \times 10^5$	$4,5 \times 10^5$	$1,14 \times 10^5$
3	320×10^5	$15,5 \times 10^5$	$4,9 \times 10^5$	$1,13 \times 10^5$
4	780×10^5	$5,9 \times 10^5$	$1,42 \times 10^5$	$0,58 \times 10^5$
5	1020×10^5	$7,4 \times 10^5$	$6,7 \times 10^5$	$0,47 \times 10^5$
6	4100×10^5	$9,4 \times 10^5$	$8,2 \times 10^5$	$0,36 \times 10^5$
7	1840×10^5	$7,2 \times 10^5$	$9,8 \times 10^5$	$0,65 \times 10^5$
8	1450×10^5	$7,8 \times 10^5$	$16,7 \times 10^5$	$1,34 \times 10^5$
9	1140×10^5	$8,9 \times 10^5$	$22,5 \times 10^5$	$1,68 \times 10^5$
10	860×10^5	$9,5 \times 10^5$	$33,2 \times 10^5$	$2,91 \times 10^5$
11	1290×10^5	$10,5 \times 10^5$	59×10^5	$2,99 \times 10^5$
12	307×10^5	$17,8 \times 10^5$	65×10^5	$3,07 \times 10^5$
13	1190×10^5	$5,3 \times 10^5$	72×10^5	$6,1 \times 10^5$
14	2320×10^5	$10,5 \times 10^5$	94×10^5	45×10^5
15	1890×10^5	$8,6 \times 10^5$	76×10^5	54×10^5
16	1180×10^5	$3,4 \times 10^5$	58×10^5	34×10^5
17	1210×10^5	$4,5 \times 10^5$	$21,5 \times 10^5$	48×10^5

List of Tables

List of Figures