Different Types of Plastic Bioremediation Using Soil Bacteria and Superworm *Zophobas morio* Larvae

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of

Bachelor of Science in Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing the degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Ethics Statement

This research is done under proper supervision and it is the author's original work. No animal was harmed during experiments. The article is written in a manner that the writeup does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing. The experiment was done by maintaining all the rules and regulations of the Biotechnology and Microbiology laboratory of the Department of Mathematics and Natural Sciences, BRAC University.

Abstract

Plastic is a recalcitrant molecule that cannot be easily biodegraded. As a result, almost all the plastic that has been manufactured is still in the world. There are microplastics in everywhere like air, water, soil, and specialty foods. A recent discovery suggests we have microplastics in our blood and even microplastics can be transferred from mother to newborn child. There are many types of plastics, and in this research three types of plastics were used, Polyethylene (PE), Low-Density Polyethylene (LDPE), and Expanded Polystyrene (EPS). Two types of samples were used, soil and *Zophobas morio* larvae. From the soil sample, three types of bacteria were isolated and identified using biochemical tests and ABIS online software where *Prolinoborus fasciculus* was predominant. These bacteria remained alive for the last six months of incubation in minimal salt broth with only the carbon source being PE. On the other hand, *Zophobas morio*, a super worm, was used to find out the biodegradation capability in the larvae stage of the insect and also larvae feces bacteria that is responsible for aiding the larvae in digesting the plastics. The larvae consumed 47.07% LDPE, 30.51% EPS, and 26.32% PE in about two months, and in the duration of the experiment no larvae died and they were also seen to recycle their feces. From feces, four bacteria were isolated according to colony morphology that was incubated in minimal salt broth for two months with the sole carbon source being the aforementioned particular plastics, and by doing 16s rRNA sequencing, *Pseudomonas guariconensis*, and *Pseudomonas aeruginosa* were identified.

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CHAPTER 1

Introduction

1.1 Bioremediation

“Bioremediation is an ecologically sound and state-of-the-art technique that employs natural biological processes to eliminate toxic contaminants. Any process that uses microorganisms, fungi, green plants or their enzymes to return the natural environment altered by contaminants to its original condition” (Vidali, 2001). There is a rising level of concern regarding the rate of environmental pollution that is currently being observed all over the world. A substantial percentage of this concern is attributable to the increased production and consumption of fossil fuels (Adams et al., 2020). Due to the rising pollution in today’s world, bioremediation can play an important role in this situation. This is because bioremediation doesn’t involve any kind of chemical substance rather it uses microbes, fungi, or other green plants. Therefore, this technique can be used to remediate environmental pollution. A procedure of bioremediation is about detoxifying the hazardous pollutants into a less toxic state. However, there are two types of bioremediations, one is In-situ bioremediation and another one is Ex-situ bioremediation. Ex-situ bioremediation involves the removal of contamination from the site and relocating them to a different site for treatment whereas in-situ bioremediation involves treating the pollutant substance at the site of the pollution. It requires no excavation and little or no soil disturbance during construction. In terms of cost-effectiveness, in-situ bioremediation has far more advantages than ex-situ bioremediation (Sharma, 2020). To achieve world environmental sustainability, there is hardly any alternative to green technology to treat a variety of aquatic and terrestrial eco-system that has been polluted by human activities. The increasing level of human activity led to the deterioration of ecosystems around the world which made them unsuitable for the native species to survive. Furthermore, rapid industrialization to inefficient agricultural practice has caused the global climate change problem more difficult to face. There is also the concern of the unrestricted flow of chemical contaminants into the lands or water that have been severely damaging the world's ecosystems, as compounds like xenobiotics, and man-made products like the usage of plastic have increased daily which seriously damages our environment. Study shows every year 10 million tons of toxic chemicals are released into the environment around the globe. Due to the addition of dangerous toxic chemicals such as

polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCBs), soil and water systems have become contaminated (Juwarkar et al., 2010 & Arora, 2018).

Therefore, bioremediation can be the solution to fight against such kinds of problems as it approaches green technology rather than conventional chemical methods. However, bioremediation also has a set of disadvantages and so, it cannot be still implemented on a large scale. One of the primary factors that affected bioremediation is the availability of the right microorganism. This is because microorganisms are highly specific which means that only the correct microbes can interact with the specific compound to degrade it. There is also another concern is that sometimes the products of biodegradation can be more toxic than the actual contamination. Biodegradation also takes more time than conventional technology and is difficult to set up for field operations at a large scale (Abatenh et al, 2017).

Bioremediation technology is intriguing and has been proven to be an effective approach although additional study is required to comprehend the microbial process behind its degradation process. As bioremediation has minimal harmful consequences and has the better advantage to deploy at a contaminated place with minimal disruption therefore it can be a perfect technology to not only achieve sustainable development but also fight against the global climate problem (Chatterjee et al., 2008).

1.2 Plastics

The English word "plastic" comes from the Greek word "plastikos," which means "able to be molded into many shapes". Plastics are composed of carbon, hydrogen, silicon, oxygen, chloride, nitrogen etc. The primary raw materials for making plastics are oil, coal, and natural gas. Polythene, which is a linear hydrocarbon polymer made up of long chains of ethylene monomers (C_2H_4), makes up around two-thirds (or 64%, to be precise) of all plastic (Goosey, 1985). Due to their extensive use in agriculture, building and construction, and health, plastics play a crucial role in every aspect of the worldwide economy. They serve as the foundation of many companies since they can create a wide range of goods, from sanitary to other household items to defense components. Additionally, plastic is used in the packaging of cosmetics, detergents, and medications. The ecology of the world and human existence is seriously threatened by the excessive use of plastics. The buildup of plastics on land and in the ocean has sparked a lot of interest in the degradation of these polymers. Biodegradation methods must be employed to reduce the negative environmental impact of plastics. It is crucial to comprehend

how microbes and polymers interact to solve the problems associated with plastics. Numerous organisms, mostly bacteria, have developed strategies for the survival and breakdown of plastics (Oliveira et al., 2020). The current examination will concentrate on the introduction of several types of plastic, including LDPE (Low-Density Polyethylene), EPS (Expanded Polystyrene), and PE (Polyethylene), and how it is degraded by environmental microorganisms.

1.3 Microbial Degradation of Synthetic Plastics

1.3.1 Polyethylene (PE)

It is widely known that polyethylene has excellent resilience to deterioration as a material because it is chemically and biologically nonreactive and it is being used in a wide variety of products, such as plastic bags, pipes, fuel storage tanks, and other things. The production of plastics at an annual rate that is greater than 25 million tons is contributing to a growing environmental concern caused by the accumulation of rubbish made of plastic in the environment (Kumar et al., 2021).

Polyethylene is being used in the production of a rising number of items, which means that preventing the material from deteriorating is becoming an increasingly difficult problem. In the range of 500 billion to one trillion, polyethylene shopping bags has been used annually around the world. Extremely long-lasting, polyethylene can take up to a thousand years to disintegrate in the natural environment if left to its own devices (Sangale, 2012). Since its backbone chains are composed solely of C-C single bonds, PE is highly stable and resistant to hydrolysis and photo-oxidative degradation. As a result, PE can't be used for UV protection because it contains no chromophores, either visible or invisible. PE may also have a small number of unfilled carbon-carbon bonds in its main chain or at the ends of its chains (typically, vinyl groups in HDPE and vinylidenes in LDPE). These sites undergo rapid oxidation at the hands of O_3, NO_2, and other tropospheric radicals, frequently resulting in the creation of extremely unstable hydroperoxides, which are then transformed into UV-absorbing carbonyl groups that are more stable. Low-density polyethylene (LDPE) has a higher photo-oxidation rate than high-density polyethylene (HDPE) because it has more reactive branch points. When there is no sunlight,

the thermal-oxidative breakdown of PE at temperatures below 100 degrees Celsius does not occur at considerable rates (Chamas et al., 2020).

The biodegradation of polyethylene is challenging and is not completely understood. Close to the decaying PE, there have been detected eight distinct forms of *Aspergillus* fungi as well as five distinct types of gram-positive and gram-negative bacteria. The efficiency of various microbial species in the degradation of polyethylene was tested. *Pseudomonas* species were responsible for the breakdown of 20.54% of polythene and 8.16% of other plastics in one month. *Aspergillus glaucus* was responsible for the destruction of 28.80% of polythene and 7.26% of plastics over one month (Ali et al., 2023). According to the findings of this research, mangrove soil is home to a significant population of microorganisms that can break down polythene and plastic. Notably, several investigations concluded that waxworms, in addition to having the intrinsic ability to feed on and digest PE films, had this ability. After coming into touch with either the waxworm *Galleria mellonella* or the waxworm *Achroia grisella*, PE biodegradation was discovered (Ali et al., 2023).

1.3.2 Expanded Polystyrene (EPS)

Polystyrene was discovered in 1839 by a Berlin pharmacist called Eduard Simon, who dubbed it styrol. The English chemist John Blyth and the German chemist August Wilhelm von Hofmann synthesized the compound metastyrol in a vacuum in 1845 (Adeala & Soyemi, 2020). In the year 1845, Blyth and Hofmann published their theory. Polymerization of styrol was discovered in 1866, allowing for the controlled creation of metastyrol. Expanded polystyrene (EPS) is a lightweight foam made of tiny air bubbles suspended in a polymer matrix**.** EPS has a minimum lifespan of 500 years in nature since it is resistant to natural degradation. Current EPS disposal procedures result in the production of toxic chemicals such as furans, dioxins, and polychlorinated biphenyls. Plastic trash, such as expanded polystyrene foam agglomerates and degrades in landfills, producing leachate and microplastics that contaminate the groundwater, streams, and land itself, which in turn poisons humans and those who use water resources. This interaction has the potential to result in stunted growth, decreased reproduction, decreased feeding, and increased mortality rates. The toxicity of plastic can vary greatly depending on the particular components that make it up. In recent years, the EPS market has been experiencing difficulties as a result of some reasons, including worldwide oversupply, declining demand, and a persistently negative view of EPS products held by consumers. It is

anticipated that there would be a rise in worldwide demand over the next five years; yet, the total capacity restriction does not appear to be lessening. More than 5.5 million metric tons of excess EPS capacity will exist worldwide in 2020, with mainland China being home to 66% of that figure. Operating rates in Northeast Asia are near 52% right now because of the region's surplus of capacity. Despite the urgent need for reduction and rationalization, no formally planned capacity expansions are expected anywhere in the world during the next five years (Palmer et al., 2022). Based on the assumption that 48.2% of ingested EPS was biodegraded into carbon dioxide and biomass by mealworms into gut bacteria, the rates of EPS biodegradation were analyzed in the darkling beetle *Tenebrio molitor*. The microorganisms naturally present in the environment play a crucial role in the biodegradation process. Biodegradation of polymers calls for biochemical enzymes produced by microorganisms. To be ingested and digested by the microbe's own internal enzymes, long polymer chains must first be broken down into shorter ones. *Bacillus sp.* NB6, *Pseudomonas aeruginosa* NB26, and *Microbacterium sp*. NA23 are among the bacteria being studied for their potential to degrade EPS (Ho et al., 2018).

1.3.3 Low-Density Polyethylene (LDPE)

The original LDPE was created using high-pressure polymerization of ethylene. It has low density because there are only a small percentage of the carbon atoms in the chain that have branched off. With a yearly production of around 19 metric tons, LDPE is one of the most widely produced commodity polymers. Bags, food, films, and all manner of automobiles can be stored in them. It can be processed by a variety of methods, including extrusion, blowing, and injection. The molecular properties of the polymer, which in turn determine its processing and end-use features, are strongly influenced by the reactor's operating parameters. Although LDPE is chemically inert at room temperature, it can be gradually degraded by strong oxidizing agents, and some solvents can cause it to soften or swell (Selke & Hernandez, 2011). The packaging sector frequently uses linear low-density polyethylene film. Co-monomers, which link side groups to the central molecule and function as offshoots, play a key role in lowering the overall density of natural systems. Late in the 1940s, LDPE became the first plastic to find widespread commercial use in packaging. It is polymerized from ethylene, as opposed to HDPE, and has a highly branched structure with both long and short branches, which resists crystallization. Because LDPE has a lower crystallinity percentage than HDPE, it is softer,

more flexible, and has a somewhat lower barrier strength. It is also less dense than HDPE. The resistance of LDPE to chemicals and oils, in addition to its low cost, makes it an excellent material for flexible packaging. LDPE appears blurry while being cleaner than HDPE (Emblem, 2012). A study discovered that certain types of bacteria are capable of biodegrading LDPE. These bacteria species are found in the maritime environment and share similarities with *Cobetia sp, Halomonas sp, Exigobacterium sp*, and *Alcanivorax sp* (Chen et al., 2013). Other bacteria that can degrade LDPE include *Cupriavidus necator* H16, *Pseudomonas putida* LS46, and *Pseudomonas putida* IRN22. A study published in 2017 discovered that the wax moth, *Galleria mellonella*, can also destroy LDPE. *Aspergillus Niger*, *Aspergillus flavus*, *Penicillium*, white rot, and brown rot fungus were also capable of degrading LDPE. These bacteria species are found in the maritime environment, and they share many similarities with *Cobetia sp., Halomonas sp., Exigobacterium sp.,* and *Alcanivorax sp.* (Khandare et al., 2021).

1.4 *Zophobas morio*

Zophobas morio is better known as a superworm because of its large size, feeding potential, and biological and economical potential. Previous research has illustrated that it has a nutritional potential that can be used as a beneficial poultry feed as a valuable nutrient and antimicrobial source. Research is ongoing, and a recent study demonstrated that *Z. morio* has waste management potential as a bioremediation agent (Rumbos & Athanassiou, 2021).

There is confusion present about the taxonomy and classification of *Z. morio*. Very recent research has shown *Z. morio* as a conspecific with *Zophobas atratus* (F., 1775) formerly *Tenebrio atratus* (F., 1775); *Zophobas rugipes* (Tschinkel, 1984; Ferrer, 2006). Tenebrionidae is a large beetle family and *Z. morio* is a member of this family (Park et al., 2013). During the lifetime of *Z. morio*, generally, four defined stages are seen followed by Eggs, Larvae, Pupa, and Adults (Rumbos & Athanassiou, 2021). The female member of *Z. morio* lay a high number of eggs (about 2200) during their lifetime, eggs are oval and round edges, about 1.7 mm in length and 0.7mm in width (Fursov & Cherney, 2018). After that, larvae are generally 55 mm long, cylindrical in shape, and sclerotized exoskeleton with 7 to 9 abdominal segments (Friederich & Volland, 2004). The next stage is the pupal stage, and the duration is about 13-15 days depending on the temperature and the pupal weight. In this stage of life, they are mostly quiescent, but they can rotate the abdominal portion in a circular motion and exhibit other physiological responses primarily functioning as a defense mechanism (Ichikawa & Kurauchi

2009). At the end of the pupal stage, *Z. morio* reached its adult stage with a body length of 38-57mm, elongated body, and filiform antennae. An adult can survive for about 180 days (Fursov & Cherney, 2018).

The larvae stage of *Z. morio* proved to be biologically and commercially important (Rumbos & Athanassiou, 2021). They have potential economic importance as animal feed. It was reported that in some of the ethnic groups in Mexico, the *Zophobas* species are taken as food (Ramos-elorduy, 2009). In Brazil, *Z. morio* is considered a potential protein as well as a nutrient source for livestock animal feed and aquaculture (Araújo et al., 2019). The most important thing is that *Z. morio* has proven to be an excellent bioremediation agent. It has the capability of eating many types of plastic including polyethylene, polystyrene, low-density polyethylene, etc. (Miao & Zhang, 2010, Choi et al., 2020, Yang et al., 2020). A recent study on the plastic-degrading superworm *Z. morio* suggested that the gut microbiome of the larvae was primarily involved with its plastic degradation capability (Peng et al., 2020). When the gut microbiome was treated with antibiotics, plastic degradation of the superworm was significantly reduced and it proves the contribution of the plastic degradation capability of the superworm is coming from the gut microbiome (Yang et al., 2020, Peng et al., 2020). Some of the bacterial strains have been isolated which include *Pseudomonas* and research is ongoing to use *Z. morio* as a potential bioremediation agent.

1.5 Anaerobic growth, Extracellular Lactase Production, and Antibiotic Production

1.5.1 Anaerobic Growth

Those microorganisms that can survive in a condition that does not have any oxygen are known as anaerobes. These bacteria can grow in an environment that does not have any oxygen. Anaerobic microorganisms use electron acceptors such as sulfate, nitrate, or inorganic electron acceptor to carry out their respiration. So, survival without oxygen is possible for anaerobes.

1.5.2 Extracellular Lactase Production

Lactase is an enzyme essential for humans or other mammals for the digestion of dairy products that contain lactose. It is mainly produced in the intestinal tract of animals by living microorganisms. Some bacteria can produce lactase as a secondary metabolite. Lactose is converted to glucose and galactose in the presence of lactase enzyme. Lactase can be commercially synthesized from microorganisms that are capable to produce extracellular lactase.

1.5.3 Antibiotic Production

Antibiotic is an essential element for the survival of human, animal, and agriculture as it is used to treat infectious diseases caused by bacteria. Antibiotic is produced by microorganisms such as bacteria, fungi, and actinomycetes as secondary metabolites and function as a defense system for their survival. Commercial antibiotics also depend on those naturally occurring bacteria involved in isolation and purification. Soil bacteria proved to be the biggest source of antibiotics.

1.6 Biochemical Tests

Xylose lysine deoxycholate (XLD) functions as the selective and differential culture medium. It is considered a selective medium because it allows the growth of gram-negative bacteria by inhibiting the growth of gram-positive bacteria and again for differentiation of *Salmonella* and *Shigella* species, the medium is considered as a differential culture medium.

Mannitol Salt Agar (MSA) functions as the selective and differential culture medium. The medium contains a high concentration of salt, and this salty environment inhibits the growth of most microorganisms. The organism capable of tolerating high concentrations of salt can grow in this media. The media is selective for some Gram-positive bacteria that can tolerate high concentrations of salt. The differential function of the medium comes from the mannitol, and it creates differentiation between the mannitol fermenter organism and the non-fermenter organism indicated by a color change in the medium. Phenol red is used in the medium as a color indicator and the medium color is changed based on the acid production because of the fermentation of mannitol.

MacConkey agar functions as the selective and differential culture medium. The medium is selective for Gram-negative microorganisms and enteric bacteria. The differential function of the medium comes from the lactose present in the medium and organisms can differentiate based on the fermentation of lactose. Lactose fermenter organisms change the medium color to red or pink and organism can be differentiated based on this color change. Neutral red is a pH indicator used in the medium responsible for this color change. Gram-negative lactose fermenter or no-fermenter microorganisms can be isolated from MacConkey agar medium

Citrate testing is used to determine the ability of an organism to use sodium citrate as the only source of carbon and inorganic ammonium hydrogen phosphate as a source of nitrogen. The test involves detecting the ability of an organism to utilize citrate as the main source of carbon and energy. Bacteria are inoculated on a medium containing sodium citrate and a pH indicator bromothymol blue. The medium also contains inorganic ammonium salts, which are utilized as the sole source of nitrogen. The utilization of citrate involves the enzyme citrate, which breaks down citrate into oxaloacetate and acetate. Oxaloacetate is further broken down into pyruvate and CO_2. Production of Na_2CO_3 as well as NH_3 from the utilization of sodium citrate and ammonium salt respectively results in alkaline pH. This results in a change of the medium's color from green to blue.

The triple Sugar Iron Test is a common biochemical test used to know microorganisms' ability to ferment sugars (glucose, lactose, and sucrose) and to produce hydrogen sulfide. It is a differential medium that can distinguish between several Gram-negative enteric bacteria. The medium contains 1.0% each of sucrose and lactose and 0.1% glucose. If only glucose is fermented, acid produced in the butt will turn yellow, but insufficient acid products are formed to affect the Phenol Red in the slant. However, if either sucrose or lactose is fermented, sufficient fermentation products will be formed to turn both the butt and the slanted yellow. If gas is formed during the fermentation, it will show in the butt either as bubbles or as cracking of the agar. If no fermentation occurs (as for an obligate aerobe), the slant and butt will remain red. The medium also contains ferrous sulfate. If the bacterium forms H_2S, this chemical will react with the iron to form ferrous sulfide, which is seen as a black precipitate in the butt (a black butt).

The Voges-Proskauer (VP) test is used to determine if an organism produces acetyl methyl carbinol from glucose fermentation. VP test detects butylene glycol producers. Acetyl-methyl carbinol (acetoin) is an intermediate in the production of butylene glycol. In this test two reagents, 40% KOH and alpha-naphthol are added to the test broth after incubation and exposed to atmospheric oxygen. If acetoin is present, it is oxidized in the presence of air and KOH to diacetyl. Diacetyl then reacts with guanidine components of peptone, in the presence of alpha naphthol to produce a red color. The role of alpha-naphthol is that of a catalyst and a color intensifier.

Methyl Red (MR) test is used to detect the production of sufficient acid during the fermentation of glucose and the maintenance of conditions such that the pH of an old culture is sustained below a value of about 4.5, as shown by a change in the color of the methyl red indicator which is added at the end of the period of incubation. This is to detect the ability of an organism to produce and maintain stable acid end products from glucose fermentation. Some bacteria produce large amounts of acids from glucose fermentation that overcome the buffering action of the system. Methyl Red is a pH indicator that remains red at a pH of 4.4 or less.

MIU is a well know biochemical test used to identify the motile and non-motile organisms, bacterial ability to the production of urease, and indole. The test is accomplished in a single test tube and phenol red is used in the medium function as a pH indicator. The test is mainly useful for the identification of gram-negative *Enterobacteriaceae* microorganisms.

The Oxidase test is a biochemical test used to know the bacterial ability to possess the cytochrome c oxidase enzyme. The test procedure is very simple and requires a very short time to accomplish.

Catalase test is a biochemical test used to identify obligate aerobic and anaerobic microorganisms. Anaerobic microorganisms do not have the enzyme and they show negative results.

Gram stain is a well-known differential staining procedure. The role of the Gram staining procedure is crucial in bacteriology. It is used to distinguish between Gram-positive and Gram-negative bacteria based on differential staining with a crystal violet-iodine complex (CV-I) and a safranin counterstain. The cell wall of gram-positive bacteria retain the CV-I complex after treatment with ethyl alcohol and appear purple, but gram-negative bacteria decolorize following such treatment and appear pink. Gram stain is an essential tool for the differentiation and classification of microorganisms.

1.7 DNA extraction, PCR, Gel Electrophoresis, 16s rRNA Sequencing

1.7.1 DNA Extraction

DNA extraction is the process used to isolate DNA from an organism by breaking down the cell wall and then the nuclear membrane. First, the cell is opened to release the nucleus and after that, the nucleus is opened to release DNA. After that, the isolated DNA must be separated from other cellular components and DNA must be protected from DNase, an enzyme that can degrade DNA. There are so many DNA extractions process available such as enzymatic, mechanical, and boiling method, and one's need to choose the suitable method based on the condition and need.

1.7.2 Polymerase Chain Reaction (PCR)

PCR is a DNA amplification method used to rapidly multiply the targeted DNA sequence into millions of copies by maintaining a thermal cycle. Primers are oligonucleotide sequences that give the PCR reaction specificity and DNA polymerase is used to synthesize the new copies of

the target sequence. All the necessary components are added to the PCR reaction for synthesizing new copies of the targeted region.

1.7.3 Gel Electrophoresis

Gel electrophoresis is a well-known laboratory method used to separate DNA, RNA, and Protein based on size and charge. Electric current is applied to the gel and molecules migrate to the positive electrode and become separated based on size. Larger stands migrated slower than the smaller stands and the DNA ladder of known sequence is used to know the size.

1.7.4 16s rRNA Sequencing

16s rRNA gene is known for encoding the small subunit ribosomal RNA molecules of ribosomes. It is about 1500 bp long gene sequence and there are nine variable regions interspersed throughout the region. The 16s rRNA gene sequencing can be used to identify and compare bacterial samples. It is possible to know the organism present in the sample.

CHAPTER 2
MATERIALS AND METHODS

2.1 Working Place for The Study

The present research work was performed in the Biotechnology and Microbiology Laboratory of the Department of Mathematics and Natural Sciences, BRAC University, Mohakhali, Dhaka 1212, Bangladesh.

2.2 Media, Solutions, and Reagents

Media, reagents, and solutions that were used in this thesis work were available as a reagent grade, and without further purification, those were used.

2.3 Handling of Laboratory Equipment

Detergents were used to wash all the glassware and rinsed 4-5 times with tap water. Autoclavable equipment was sterilized by autoclaving at 121ºC for 15 minutes at 15 psi. All the microbiological works were done inside the Biological Safety Cabinet. Larvae were kept in PET boxes which were in a hardboard box and safety was maintained so that no larvae could leave the box

2.4 Sample collection

2.4.1 Soil Sample

The soil sample was collected from “Matuail” Sanitary Landfill, in Dhaka, Bangladesh in a polyethylene bag. The soil that was collected was recently excavated from a deep layer that is almost 20 years old. The sample was quickly brought to the laboratory. The site was chosen because it was hypothesized that, the soil's endogenous microorganisms got enough time to evolve a plastic degradation pathway.

2.4.2 Larvae Sample

About 300 pieces of *Zophobas morio* larvae were bought from Sher-e-Bangla Agricultural University, Dhaka, Bangladesh in a PET box with some grains to feed them. In the laboratory, any organic food and feces were removed from the PET box, and a piece of EPS was given primarily to feed them.

2.5 Preparation for Soil Sample

2.5.1 Minimal Salt Broth 1 (MSB1)

This media was used to screen out plastic degrading bacteria as in this media, there was only salt and no carbon source. As a sole carbon source, plastics were added after inoculation. The media composition as follows, KH_2PO_4 (3g/L), K_2HPO_4 (0.1g/L), NaCl (5g/L), NH_4Cl (2g/L), $MgSO_4$. $7H_20$ (0.16g/L), $CaCl_2.2H_20$ (0.1g/L). pH was adjusted to 7.5. MSB1 was sterilized by autoclaving at 121 for 15 minutes at 15 psi. PE as the sole carbon source was added in two ways, shredded and square cut.

2.5.2 PE Preparation

Polyethylene bag was collected from nearby shops. Polyethylene was cut into (a) 1 x 1 cm film strips and (b) shredded pieces and transferred to a fresh solution having 70 ml Tween 20, 10 ml disinfectant (Dettol) and 920 ml distilled water and stirred for 30 minutes. After 30 minutes, the strip and the shredded PE were transferred into a beaker with distilled water and stirred for another 10 minutes. This step was repeated 3 times until both PE was ridden of any residual disinfectant or tween. Then, the strips and the shredded pieces were aseptically placed in a 70% ethanol solution for 30 minutes. Finally, the disinfected strips and shredded PE were transferred to a sterile petri dish and dried in the laminar hood, and put away for further use. Weight was measured for both strip and shredded pieces.

For the positive control, 0.1% glucose as the sole carbon source was added in another MSB1 flask, and for the negative control, no carbon source was added.

2.5.3 Inoculation

About 1g of soil sample was mixed thoroughly (vortexed) in 100 ml sterilized distilled water and then filtered using Whatman filter papers. 10^5-fold dilution was done using 9 ml 0.9% sterile NaCl solution. About 1 ml diluted sample was added into every Duran bottle which contained 250 ml MSB1 media with shredded or PE strips or glucose or no carbon source. After inoculation, media was vortexed and incubated in a shaker incubator at 37^o C at 120 rpm.

After every month, aseptically 10 ml of MSB1 was added to every Duran bottle containing the culture.

2.6 Preparation for Larvae Sample

2.6.1 Plastic Preparation

Three types of plastic were chosen, PE, LDPE, and EPS. These plastics were cut into square or rectangular shapes, measured by weight, and put in three different empty PET boxes. The same process was done for the main process after 14 days and for MSB2 media.

2.6.2 Larvae Preparation

For every type of plastic, about 20 larvae were chosen and put in the boxes. It was made sure, there was no organic or any type of food the larvae can feed on. This process is done for about two weeks (14 days) to clear out the digestive system of any organic food they ate before. Feces were removed every two days. After 14 days, all the plastics and feces were removed, and new three types of plastic were introduced, and from this moment data collection started for about two months.

In the meantime, five larvae were isolated in a different PET box to test the mortality rate without plastics as a food source, they were given no food during the period.

2.6.3 Minimal Salt Broth 2 (MSB2)

This media was used to screen out plastic degrading bacteria as in this media, there was only salt and no carbon source. As a sole carbon source, plastics were added after inoculation. The media composition as follows, KH_2PO_4 (3g/L), K_2HPO_4 (0.1g/L), NaCl (5g/L), NH_4Cl (2g/L), $MgSO_4$. $7H_20$ (0.16g/L), $CaCl_2.2H_20$ (0.1g/L). pH was adjusted to 6.5. MSB2 was sterilized by autoclaving at 121º C for 15 minutes at 15 psi. PE, LDPE, and EPS as sole carbon sources were added later.

2.6.4 Larvae Feces Preparation

After one month, feces were collected from each box and mixed. After that 0.1g of mixed feces sample was added in a 0.9% 10 ml NaCl solution, and vortexed. From that, a 10^4-fold dilution is made.

2.6.5 Inoculation

One milliliter of diluted feces sample was added in each Duran bottle containing 250 ml MSB2 media. Three different plastics were added to those three Duran bottles, and vortexed. They were placed in the shaker incubator at 120 rpm at 37ºC. For the positive control, 0.1% glucose

as the sole carbon source was added in another MSB2 flask, and for the negative control, no carbon source was added.

2.7 Media Preparation

- Nutrient Agar (NA) was used as a common growth medium, to find out the presence of bacteria and to find out CFUs
- Luria Bertani Broth, Miller was also used as a common growth medium.
- MAC, MSA, and XLD media to isolate different types of colonies
- MSB1 for soil sample
- MSB2 for larvae sample
- Premixed media for biochemical tests
- Lactose fermentation broth to test production of extracellular lactase
- Antibiotic production test broth to test antimicrobial agent production
- T1N1 media for storage

2.8 Assessment

2.8.1 Soil Sample Assessment

At every 7 days, about 100 µL MSB1 was spread on Nutrient Agar (NA) media to check for bacterial presence and colony count. After 6 months of incubation, from MSB1, bacteria were cultured on XLD, MAC, and MSA media to isolate individual colonies according to their colony morphology. Isolated colonies were then streaked on NA to get a pure culture from which the bacteria were again streaked on NA. These bacteria were subjected to first Gram staining and then biochemical tests to find out the probable genus of those bacteria.

After 8 months of incubation, the PE strip was separated from the MSB1 and seen under a microscope to see any difference. Then, the strip was washed thoroughly with running distilled water first. Next, the strip was aseptically placed in 70% ethanol solution for 30 minutes. After that, the strip was transferred into a beaker with distilled water and stirred for 10 minutes. This

step was repeated 4 or 5 times. Finally, the disinfected strip will be transferred to a sterile petri dish and dried in the laminar hood and weight was taken.

The bacteria isolated from MSB1 were also subjected to find out whether they can produce any antimicrobial agents and whether they produce extracellular lactase enzymes. They were also subjected to growing in an anaerobic condition to find out their oxygen demand.

These bacteria were then stored in T1N1 media for short-term storage.

2.8.2 Larvae Sample Assessment

Every 2 days, the weight of plastics and feces was taken to generate data on how much plastics were being eaten by the larvae. It was again made sure that no outside organic food source was in the boxes during the experiment time.

About 0.1g of feces was collected every 7 days and mixed with 10 ml dH_2O and vortexed to figure out the plastic presence in the feces.

Every 7 days, about 100 µL MSB2 from three different plastics were spread on Nutrient Agar (NA) media to check for bacterial presence and colony count. After 2 months of incubation, from MSB2, bacteria were cultured on XLD, MAC, and MSA media to isolate individual colonies according to their colony morphology. Isolated colonies were then streaked on NA to get a pure culture from which the bacteria were again streaked on NA. These bacteria were subjected to first Gram staining and then biochemical tests to find out the probable genus of those bacteria.

Genomic DNA was extracted from these isolated bacteria and PCR was done with 27F and 1492R universal primers to amplify the 16s rRNA segment to proceed through 16s rDNA sequencing by which the genus of those bacteria was confirmed.

These bacteria were then stored in T1N1 media for short-term storage.

2.9 Media and Reagent Preparation for Different Tests

2.9.1 Gram Staining

This test was done for both MSB1 and MSB2 isolated bacteria. About one drop of dH_2O was placed on microscope glass slides. Every bacteria sample had different slides. Half loopful of bacteria from 24-hour culture cultured on NA was taken and placed onto the slides to make a smear by heat fixing. After heat fixing, the following reagents were added accordingly- Chrystal violet for 1 minute then Grams iodine for 1 minute, then acetone for 10 seconds, and lastly safranin for 45 seconds. This procedure distinguishes between gram-positive and gram-negative groups whether they give purple or pink color. The morphology of those bacteria was also checked during this method.

2.9.2 Endospore Staining

This test was done for both MSB1 and MSB2 isolated bacteria. About one drop of dH_2O was placed on microscope glass slides. Every bacteria sample had different slides. Half loopful of bacteria from 7 days culture cultured on NA was taken and placed onto the slides to make a smear by heat fixing. After heat fixing, 5-6 drops of malachite green were added over top. Then the slide was carefully held over a flame until the dye started to steam and bubble. After the bubble formed, the slides were removed from the flame and allowed to cool then washed with dH_2O. After that, 5-6 drops of safranin were added. The dye was allowed to sit for 3 minutes before the slides were again washed with dH_2O and allowed to air dry. Finally, they were seen under a microscope for any endospore formation.

2.9.3 Biochemical Tests

There were different types of biochemical tests were done to classify the presumptive genus of isolated bacteria. Microbiology: a laboratory manual by Cappuccino and Welsh was followed to carry out all tests using fresh, 24-hour cultures from NA plates. These tests were done for both MSB1 and MSB2 isolated bacteria. All tests had negative control where no inoculation was done and *E.coli* was used as another control. The following tests were performed:

2.9.3.1 Methyl Red (MR)

Loopful of isolated 24-hour cultured bacteria were inoculated in test tubes containing 6 ml MRVP broth and incubated for 48 hours at 37^{o}C. After incubation, 5 drops of MR reagent were added to the tube without shaking. For the result, a cherry red color was taken as positive for MR, while orange was taken as inconclusive, and yellow was taken as a negative result.

2.9.3.2 Voges Proskauer (VP)

Loopful of isolated 24-hour cultured bacteria were inoculated in test tubes containing 6 ml MRVP broth and incubated for 48 hours at 37^{o}C. After incubation, 6 drops of Barritt's reagent A were added and vortexed. After that, 2 drops of Barritt's reagent B were added and vortexed, and observed for up to 30 minutes. For results, a pink ring formation was taken as VP positive while negative results were indicated by brownish rings.

2.9.3.3 Motility, Indole, and Urea Test

This test was performed using an MIU medium. Using a needle, bacteria were stabbed carefully one time in the test tube containing MIU media which contained 40% urea, and incubated for 24 hours at 37^{o}C. After incubation, results can be seen for motility and urease. For a motility-positive result, a diffuse zone of growth flaring from the line of inoculation was seen, and otherwise, it was motility-negative. For urease positive, a color change from yellow-orange to pink-red was seen, and otherwise, it was urease negative. For indole, a positive result was found when it gave a red color after the addition of 5-10 drops of Kovac's reagent, otherwise, it was indole negative.

2.9.3.4 Citrate Test

Using a needle, bacteria were spread carefully on the slant in the vial containing 3 ml citrate media and incubated for 48 hours at 37^{o}C. For a positive result, growth with a color change to blue was seen, and for a negative result, growth was seen but no color change.

2.9.3.5 Triple Sugar Iron Test

Using a needle, bacteria were stabbed and spread carefully on the slant in the test tube containing 6 ml TSI media and incubated for 24 hours at 37^{o}C. For results, black coloration was taken as H_2S positive, an alkaline/acid which was red slant and yellow butt reaction was taken as dextrose fermentation only, an acid/acid which was yellow slant and yellow butt reaction was taken as the fermentation of dextrose, lactose and/or sucrose and an alkaline/alkaline which was red slant and red butt reaction was an indicator of the absence of carbohydrate fermentation, and bubble or cracks formation in the agar was taken as gas positive.

2.9.3.6 Oxidase Test

On a Whatman paper, one drop of oxidase reagent was put, and using a toothpick, bacteria were streaked on the dropped reagent. Purple to blue coloration in 30 seconds was taken as oxidase positive.

2.9.3.7 Catalase Test

On a microscope glass slide, one drop of 30% H_2O_2 solution was put and using a toothpick, bacteria were mixed with the solution. Immediate bubble formation was taken as catalase positive.

2.9.4 Lactose Fermentation Test

This test was done only for MSB1 bacteria. For this test, Phenol Red Lactose broth was used which consists of protease peptone (10.00g/L), beef extract (1.00g/L), sodium chloride (5.00g/L), lactose (5.00g/L), phenol red (0.018g/L). Loopful of isolated 24-hour cultured bacteria were inoculated in test tubes containing 5 ml Phenol Red Lactose broth and vortexed and incubated for 24 hours at 37^{o}C. The media color turning yellow was an indication of lactase positive.

2.9.5 Antimicrobial Production Test

This test was done only for MSB1 bacteria. For this test, a media consisting of glucose (30g/L), $NaNO_3$ (6g/L), KH_2PO_4 (1g/L), KCl (5g/L), $MgSO_4$ (0.2g/L), $FeSO_4$ (0.1g/L), Peptone (50g/L), Beef Extract (30g/L), H_2O (1000 ml) was prepared. In a test tube, 3ml of the media was taken and a loopful of bacteria was inoculated and incubated for 48 hours at 37°C. after incubation, the media was vortexed and centrifuged to separate the supernatant. In the MHA media plate, known pathogenic bacteria *Vibrio cholerae,* and *E. coli* were spread. Wells were made on the media, and about 200 µl of supernatant was pipetted. After incubation for 24 hours at 37°C, the MHA plates were analyzed. A clear bacteria colony-free zone was taken as the indicator of the presence of antimicrobial agents.

2.9.6 Anaerobic Growth Test

This test was done only for MSB1 bacteria. Bacteria were streaked on NA and Petri dishes were put into the anaerobic jar containing AnaeroGen 3.5L from Thermo Scientific. They were incubated at 37°C for 24 hours.

2.9.7 T1N1 Media

The media consists of tryptone (10g/L), NaCl (10g/L), and agar (10g/L). Using a needle, bacteria were stabbed multiple times in the vial containing the media and incubated for 24 hours at 37°C. After incubation, 200µl sterile paraffin oil was pipetted into the vials, and lids were secured with parafilm.

2.9.8 Bacterial Genomic DNA Extraction

Bacteria from the larvae sample (MSB2) were subjected to genomic DNA extraction, PCR, gel electrophoresis, and 16s rRNA sequencing. Bacteria were cultured in NA, and from the 24-hour culture, a loopful of bacteria was mixed with 150 µl TE buffer in a 2ml microcentrifuge tube and vortexed. The water bath machine was set at 95°C. The microcentrifuge tubes were put in the water bath machine for 20 minutes. After 20 minutes, the tubes were taken out of the water bath machine and centrifuged at 10,000 rpm for 10 minutes. After centrifugation,

supernatants from each microcentrifuge tube were collected in another 2 ml microcentrifuge tube and stored at -20ºC. These tubes contained the template DNA.

2.9.9 PCR

For PCR, the total volume was selected 25µl for each sample, where template DNA was 5µl, the forward primer was 2.5 µl, the reverse primer was 2.5 µl, PCR master mix was 12.5 µl and nuclease-free water was 2.5 µl. As the target sequence was the bacteria 16s rRNA gene, 27F forward primer and 1492R reverse primer were selected as universal primers. PCR cycle temperatures were, initial denaturation at 95ºC for 2 minutes, denaturation at 95ºC for 30 seconds, annealing at 50ºC for 30 seconds, elongation at 72ºC for 2 minutes, and the final cycle was at 72ºC for 7 minutes. A total of 30 cycles were run. The PCR products were then stored at -20ºC.

2.9.10 Gel Electrophoresis

This part of the experiment was done to make sure there were PCR products of the intended site. 1% agarose gel was made for this experiment. 1g agarose was mixed with 100 ml TAE buffer which was boiled and mixed. Then it was cooled to semi-warm and added 5 µl 0.5 microgram/ml EtBr and mixed. The casting tray was prepared with combs, as there were many samples, and two combs were placed on the casting tray. The mixture was then poured into a casting tray and let to solidify. After solidification, the combs were removed gently, the placed the tray into the gel electrophoresis machine. TAE running buffer was added. 6µl 100 bp Ladder was put into the first well in each row, the PCR products were put in the other wells at 6µl per well. The gel was run at 110 voltage for 40 minutes. After gel electrophoresis was done, gel bands were seen with UV light.

2.9.11 16s rRNA Sequencing

About 50 µl of the PCR product of all the samples were sent to International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) for 16s rRNA sequencing.

CHAPTER 3

Results

This research aimed to find bacteria that are capable of degrading different types of plastics, namely PE, LDPE, and EPS efficiently. As there were two types of samples, soil sample and larvae sample, different approaches had been made to screen out our bacteria of interest. For the soil sample, only PE was the plastic choice and for this type of sample, the soil was measured, diluted, and inoculated into MSB1 medium alongside shredded and square-cut PE. After incubation, bacteria were isolated from MSB1 and different biochemical tests were done to find out possible species of those bacteria. They were also subjected to an antibiotic production test, lactase production test, and also anaerobic growth test. For the larvae sample, first, any organic food was put away and larvae only ate particular plastics for 2 weeks. After that, data collection was started with fresh plastics. The feces sample was diluted and mixed with MSB2 media containing different plastics. After 2 months of incubation, bacteria were isolated from MSB2 and different biochemical tests were done to find out possible species of those bacteria. Bacterial genomic DNA was also isolated and was done PCR to amplify the 16s rRNA gene with the intention of sequence that gene.

3.1 Soil Sample

The sample was diluted in a conical flask first in MSB1. It was shifted to a Duran bottle due to volume issues. After 7 days of incubation, bacteria were spread on LBA to see how much bacteria were present at that time, and CFU/ml was found to be 2.85×10^6 for shredded PE and 2.73×10^6 for cut PE. For the first four months, CFU/ml decreased rapidly for both, but after four months, CFU/ml was steady. However, negative control showed growth for only one week, and after one month no growth was seen. Positive control had growth for over 2 months.

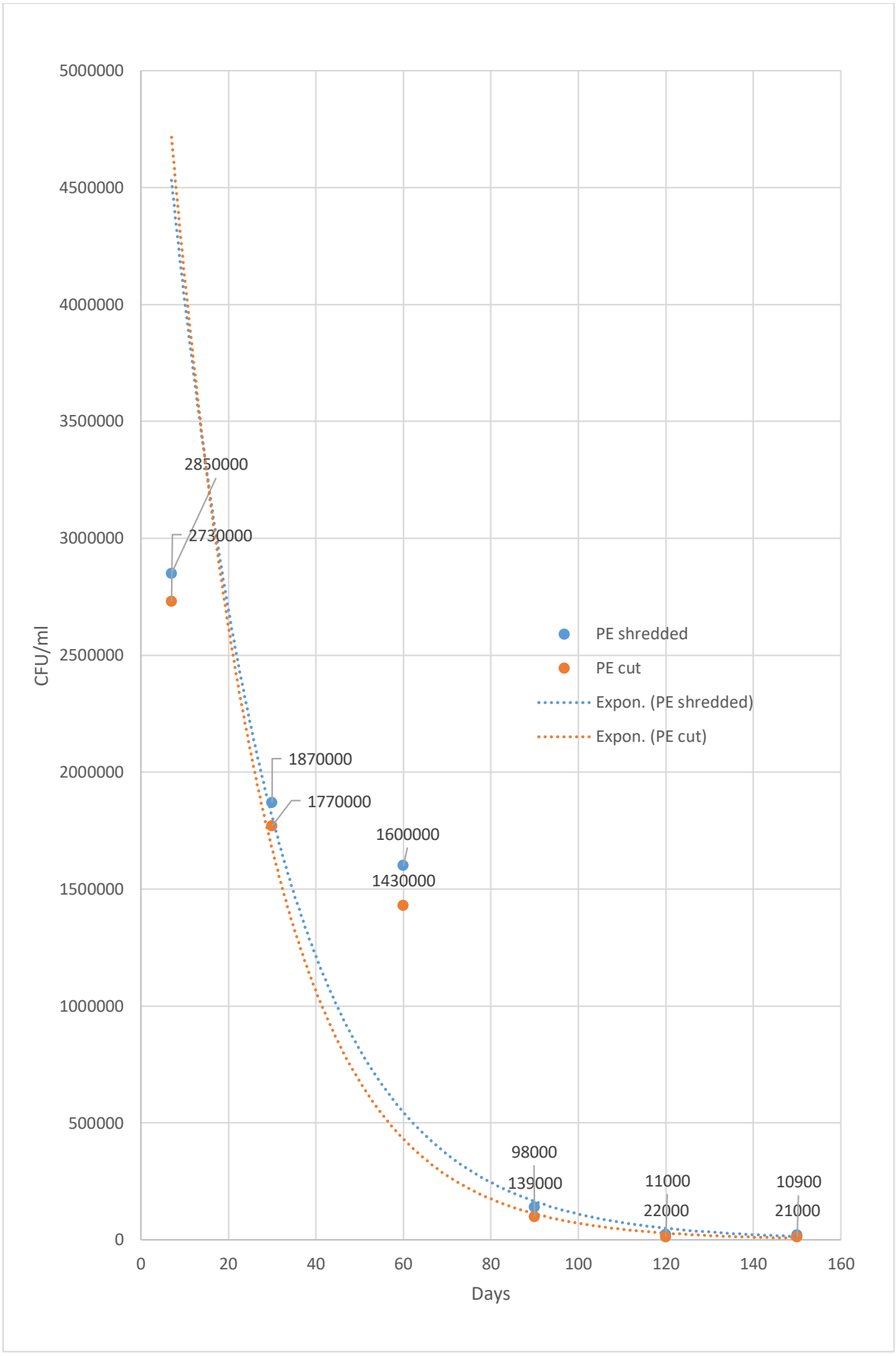

Figure 1: **The graph showing the steep decline in bacterial population from MSB1 every month**

After 6 months of incubation, bacterial presence was confirmed and isolated from MSB1 using XLD, MAC, and MSA media, and found 19 different bacteria according to their colony morphology. These bacteria were subjected to biochemical tests, antibiotic production tests, lactase production tests, and anaerobic growth tests.

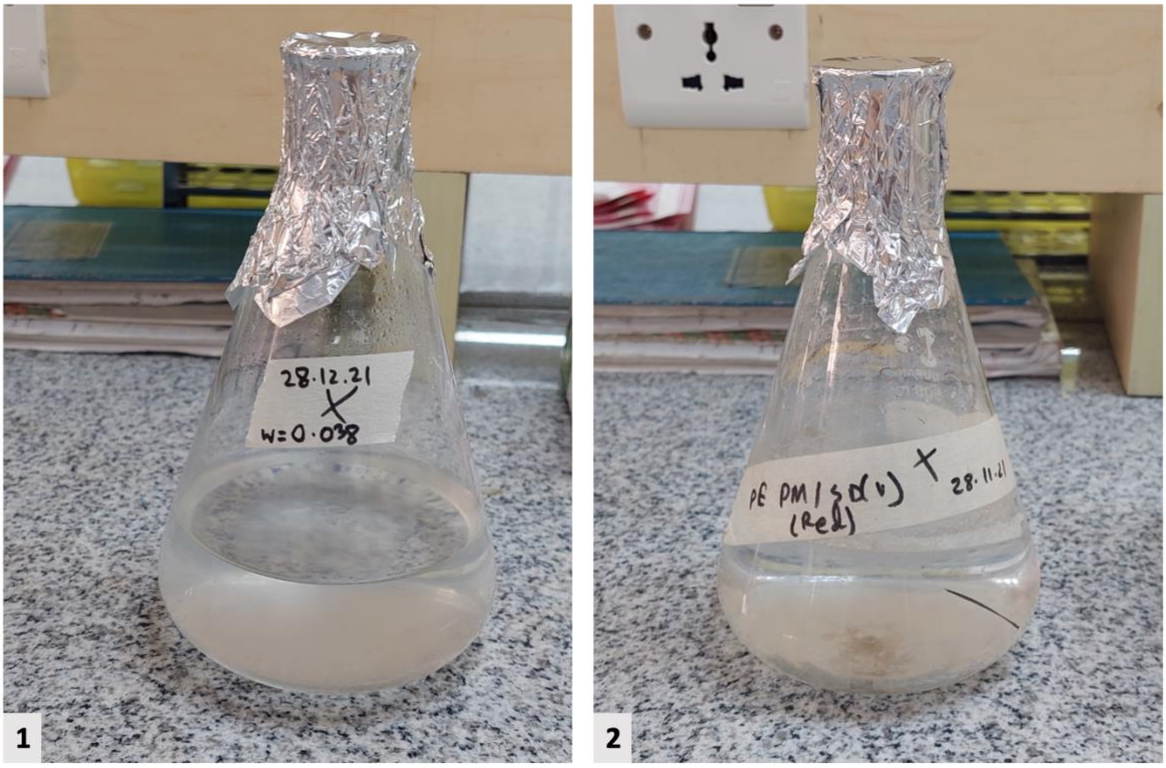

Figure 2: **MSB1 broth with soil sample and PE (1) Cut , (2) Shredded**

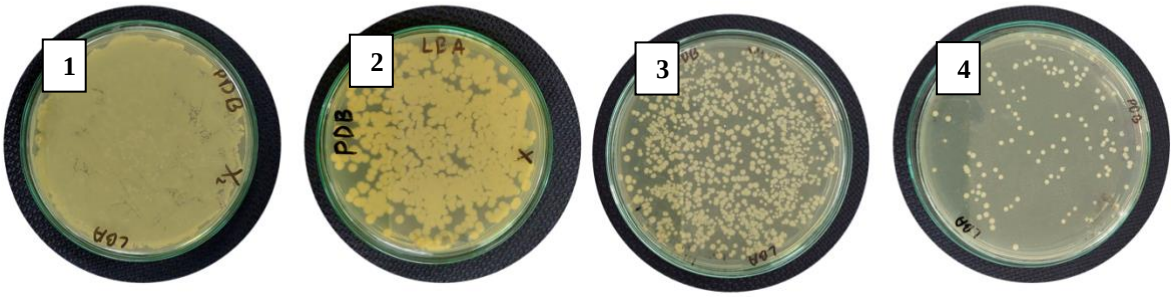

Figure 3: **Decreasing CFU for cut PE. (1) 1st month, (2) 2nd month, (3) 3rd month, (4) 4th month**

3.1.1 Biochemical Tests

All 19 isolated bacteria were subjected to the biochemical tests 4 times, and the average result was counted (Table 1). No bacteria produced endospores. Results are shown in Table 1.

Table 1: Isolated bacteria and their biochemical test results

Bacteria Sample ID	**Medium**	**Colony morphology**	**TSI**				**MIU**			**Citrate**	**MR**	**VP**	**Oxidase**	**Catalase**	**Gram Staining**	
			SLANT	BUTT	H2S	GAS	MOTILITY	INDOLE	UREA						P/N	SHAPE
SP-1	MAC	LARGE PINK	A	A	-ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	COCCI
SP-2	MAC	PINK CENTER	B	B	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	ROD
SP-3	MSA	PINK	B	A	-ve	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	ROD
SP-4	XLD	YELLOW	A	A	-ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	-ve	+ve	-ve	COCCI
SP-5	XLD	SMALL YELLOW	B	B	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	ROD
SP-6	XLD	PINK	B	B	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	ROD
SP-7	MAC	PINK CENTER	B	B	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	ROD
SP-8	MAC	LIGHT PINK	B	B	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	ROD
SP-9	MAC	TRANSPARENT	B	B	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	ROD
SP-10	MSA	PINK	B	B	+ve	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	ROD
SP-11	XLD	MUCOID PINK	B	B	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	ROD
SP-12	XLD	TRANSPARENT	B	B	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	ROD
SP-13	XLD	SMALL YELLOW	B	B	+ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	ROD
SP-14	XLD	SMALL PINK	B	B	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	ROD
SP-15	MAC	DARK PINK	B	B	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	ROD
SP-16	MAC	LIGHT PINK	B	B	+ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	ROD
SP-17	XLD	LARGE YELLOW	A	A	-ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	-ve	COCCI
SP-18	XLD	SMALL YELLOW	B	B	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	COCCI
SP-19	XLD	SMALL PINK	B	B	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	ROD

A= Acidic B= Basic

3.1.2 Antibiotic Production Test

From the 19 bacteria isolated, only 7 gave zone, (Table 2) but those zone had bacteria growing. Though bacteria were growing, concentration was subsequently low, which means there was some kind of antibiotic agent present there. This experiment was run 3 times to find a reproducible result.

Table 2: **Antibiotic production by various bacterial isolates collected from soil**

Bacteria Sample ID	Zone	Bacteria number	Zone
SP-1	Yes	SP-11	No
SP-2	No	SP-12	Yes
SP-3	Yes	SP-13	No
SP-4	No	SP-14	Yes
SP-5	No	SP-15	Yes
SP-6	Yes	SP-16	No
SP-7	No	SP-17	No
SP-8	No	SP-18	No
SP-9	Yes	SP-19	No
SP-10	No		

3.1.3 Lactase Production Test

Of the 19 bacteria, only 3 bacteria were found to ferment lactose. Bacteria Sample SP-3, SP-10, and SP-17 gave yellow media color. This experiment was run 4 times to find a reproducible result.

3.1.4 Degradation of PE

The change in the weight of PE was not measurable, which means the cut PE and the shredded PE had the same weight as the initial weight. However, there is a chance of any nano-fracture on the PE body but to measure that Scanning Electron Micrograph (SEM) needs to be done.

3.1.5 Anaerobic Growth Test

Only 3 bacteria did not grow in the absence of oxygen, which means they are strictly aerobic. Others are facultative anaerobic. (Table 3)

Table 3: **Growth of bacteria in anaerobic condition**

Bacteria Sample ID	Growth	Bacteria number	Growth
SP-1	No	SP-11	Yes
SP-2	Yes	SP-12	Yes
SP-3	No	SP-13	Yes
SP-4	No	SP-14	Yes
SP-5	Yes	SP-15	Yes
SP-6	Yes	SP-16	Yes
SP-7	Yes	SP-17	Yes
SP-8	Yes	SP-18	Yes
SP-9	Yes	SP-19	Yes
SP-10	Yes		

Using ABIS online software, bacteria from soil sample was identified according to their biochemical test result and aerobic/anaerobic growth (Table 4)

Table 4: **Probable bacteria, search result from ABIS online based on biochemical and anaerobic growth test**

Bacteria Sample ID	Possible organism
SP-1	Not matched
SP-2	*Prolinoborus fasciculus*
SP-3	*Salinicoccus albus*
SP-4	Not matched
SP-5	*Prolinoborus fasciculus*
SP-6	*Prolinoborus fasciculus*
SP-7	*Prolinoborus fasciculus*
SP-8	*Prolinoborus fasciculus*
SP-9	*Prolinoborus fasciculus*
SP-10	Not matched
SP-11	*Prolinoborus fasciculus*
SP-12	*Prolinoborus fasciculus*
SP-13	*Prolinoborus fasciculus*
SP-14	*Prolinoborus fasciculus*
SP-15	*Prolinoborus fasciculus*
SP-16	*Prolinoborus fasciculus*
SP-17	Not matched
SP-18	*Aquaspirillum*
SP-19	*Prolinoborus fasciculus*

3.2 Larvae Sample

There are two parts of the result for this section, larvae itself and bacteria isolated from its feces.

3.2.1 Plastic Degradation by Larvae

Larvae were given 3 different types of plastics, PE, LDPE, and EPS, and different results were seen for each plastic. Firstly, after two months, no larvae died because of starvation. The molting process was seen in the meantime in most of the larvae. 47.07% LDPE, 30.51% EPS, and 26.32% PE were consumed by the larvae in about two months. Only feces-fed larvae also showed growth (Molting) and showed recycling of microplastics from feces. After about 2 months, the larvae became weak and started to die. Cannibalistic behavior was found after the initial two months.

The best result came for LDPE, the rate was better than the others. EPS had an average remediation rate and lastly, the lowest remediation rate was for PE.

Table 5: Degradation of particular plastics by the larvae of *Zophobas morio*

Plastic-type	Period (days)	Initial weight (mg)	Final weight (mg)	The amount is eaten (mg)	The amount is eaten (%)	The amount ate (mg) 100 $larvae^{-1}$ day^{-1}
LDPE	36	512	271	241	47.07	33.47
EPS	69	3310	2300	1010	30.51	73.19
PE	69	133	98	35	26.32	2.54

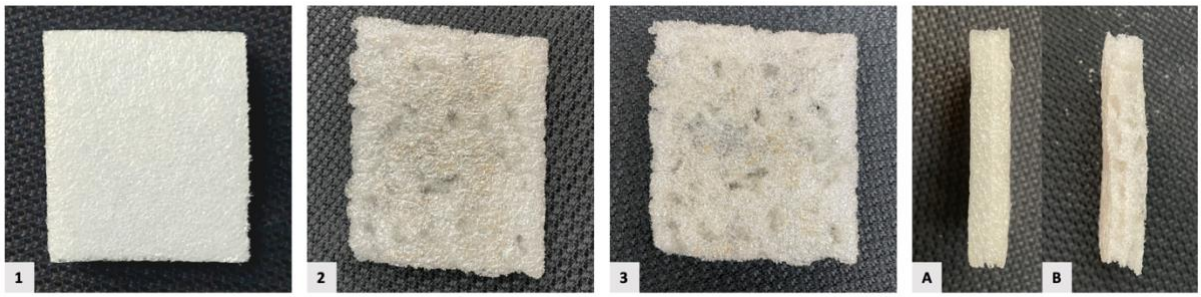

Figure 4: **LDPE eaten by larvae. (1) Control , (2) after 15 days. (3) after 36 days. (A) side view of initial stage and (B) side view of after 36 days**

Figure 5: **EPS eaten by larvae. (1) initial , (2) after 69 days. (3) after 90 days**

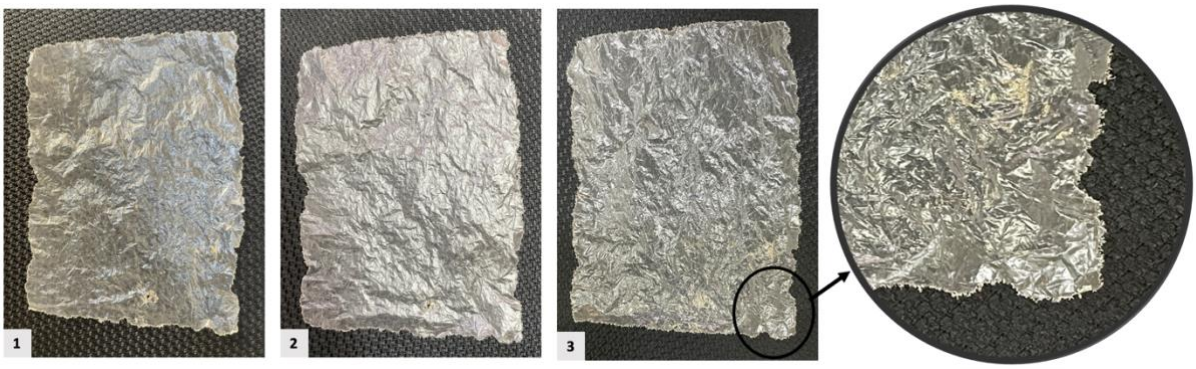

Figure 6: **PE eaten by larvae. (1) after 15 days , (2) after 30 days. (3) after 69 days. Number 3 is zoomed for clear indication of eating by larvae**

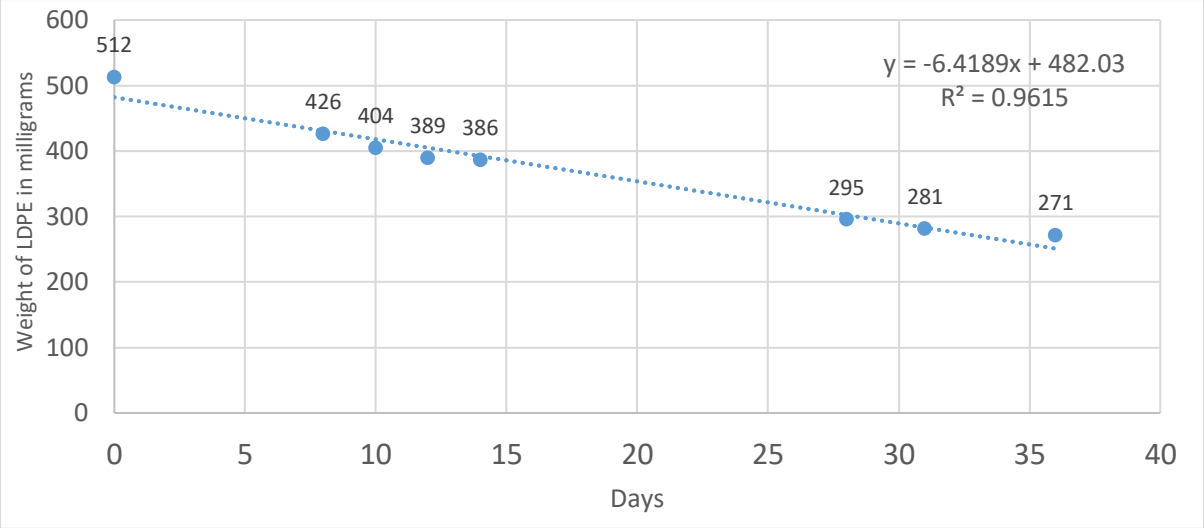

Figure 7: Consistent consumption of LDPE by the larvae

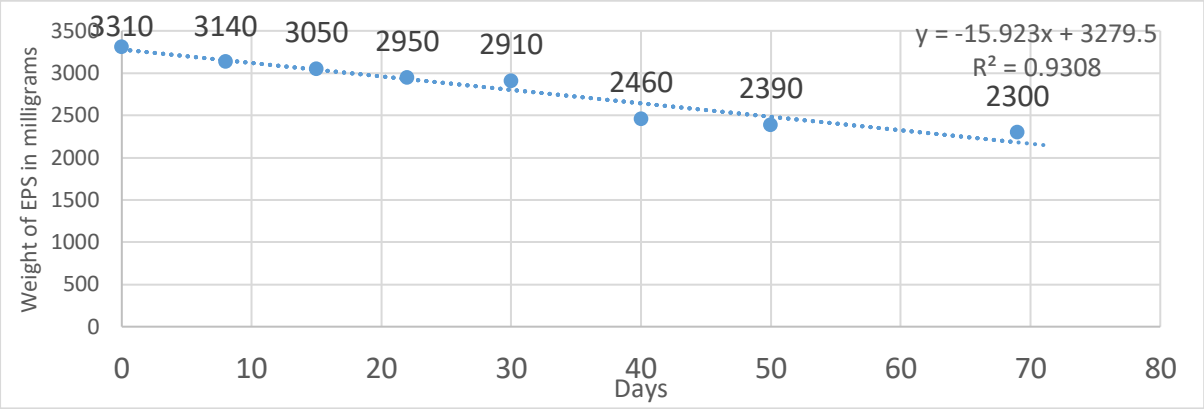

Figure 8: Gradual consumption of EPS by the larvae

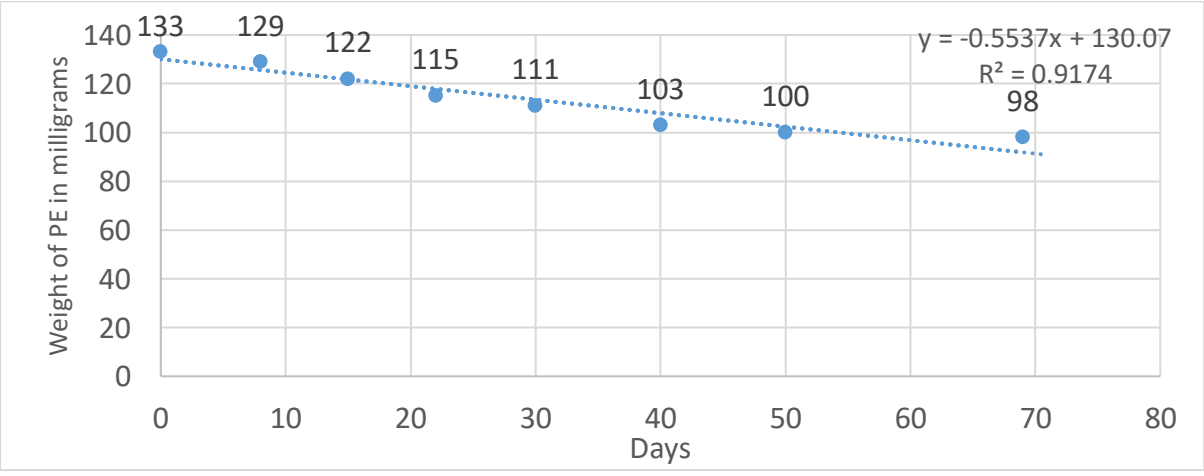

Figure 9: Consistent consumption pattern for PE by the larvae which has the lowest eating rate among all the plastics

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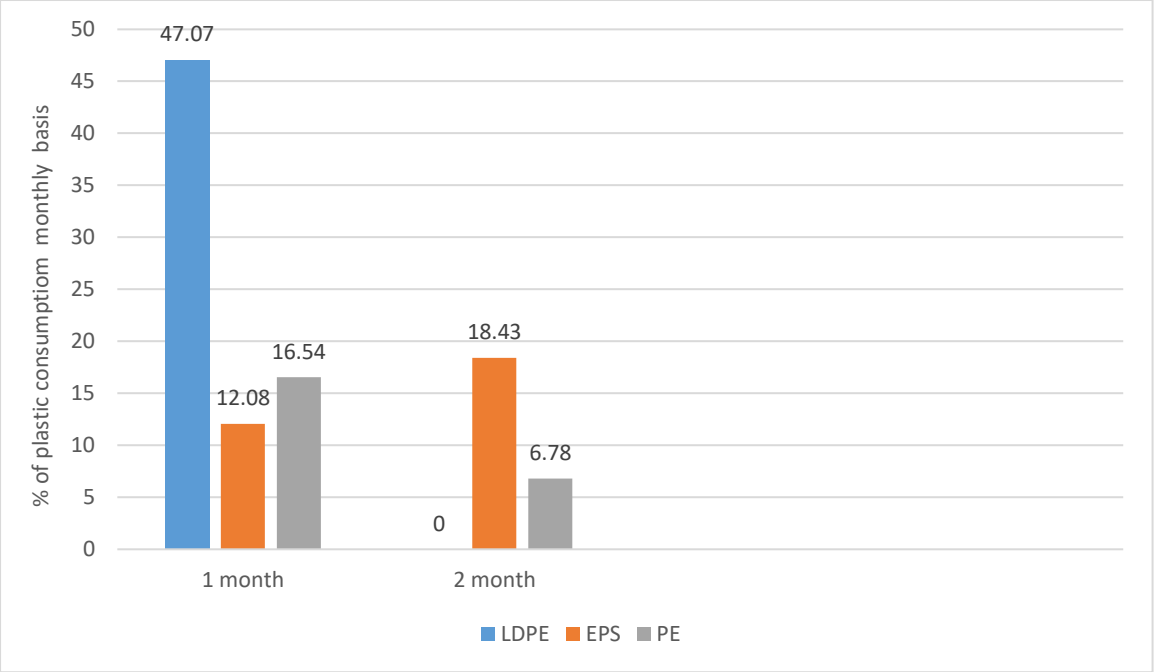

Figure 10: **From the graph, after the first month, larvae consumed more EPS but for PE, larvae consumed less. So, EPS consumption increased after one month whereas PE consumption decreased. For LDPE, as data was collected for only 1 month, there was no data for the 2nd month**

For larvae itself, every stage of the insect was found in the duration of the experiment, from larvae to adult beetle (Figure 11). This is important because the larvae that became beetle had no food but plastics. So, the larvae that will come from the beetle should have more efficiency in eating plastics.

Figure 11: **Different stages of *Zophobas morio* seen during the experiment. (1) larvae, (2) after molting, (3) Pre-pupae, (4) Pupae, (5) Beetle, (6) Male and female beetle**

3.2.2 Plastic Degradation using Larval Fecal Bacteria

Only a total of 4 bacteria were isolated using XLD and MAC from three different plastics, however, there was no bacterial growth on MSA. From PE, one bacteria were isolated from MAC, from LDPE, one bacteria were isolated from XLD, and from EPS, two bacteria were isolated from both media.

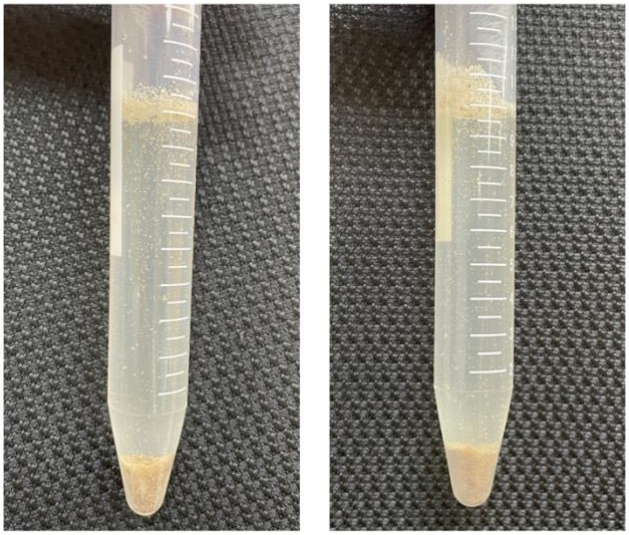

Figure 12: **Larval feces in dH_2O after centrifugation. Upper layer is the non-digested plastic of the feces**

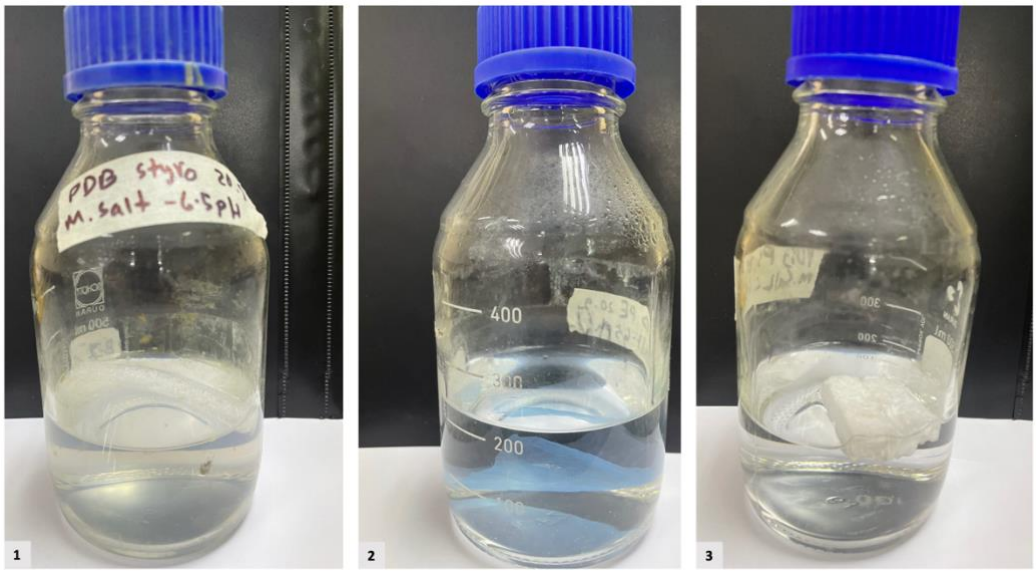

Figure 13: **Plastics in MSB2 broth inoculated with feces. (1) LDPE, (2) PE, (3) EPS**

3.2.2.1 Biochemical Test Results

Table 6: **Biochemical test results of the bacterial samples isolated from larvae feces**

Bacteria Sample ID	Medium	TSI				MIU			Citrate	MR	VP	Oxidase	Catalase	Gram Staining	
		Slant	Butt	H2S	Gas	Motility	Indole	Urea						+/-ve	Shape
SL-1	MAC	B	B	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	ROD
SL-2	MAC	B	B	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	ROD
SL-3	XLD	B	B	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	ROD
SL-4	XLD	B	B	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	ROD

B= denotes basic pH

Table 7: List of probable organisms identified by using ABIS online software

Bacteria Sample ID	Probable Organism
SL-1	*Pseudomonas sp*
SL-2	*Pseudomonas sp*
SL-3	*Pseudomonas sp*
SL-4	*Pseudomonas sp*

3.2.2.2 PCR and Gel Electrophoresis Result

According to the gel run, the 16s rRNA gene was successfully amplified. Though there was some minor non-specific amplification that can be seen, it was negligible.

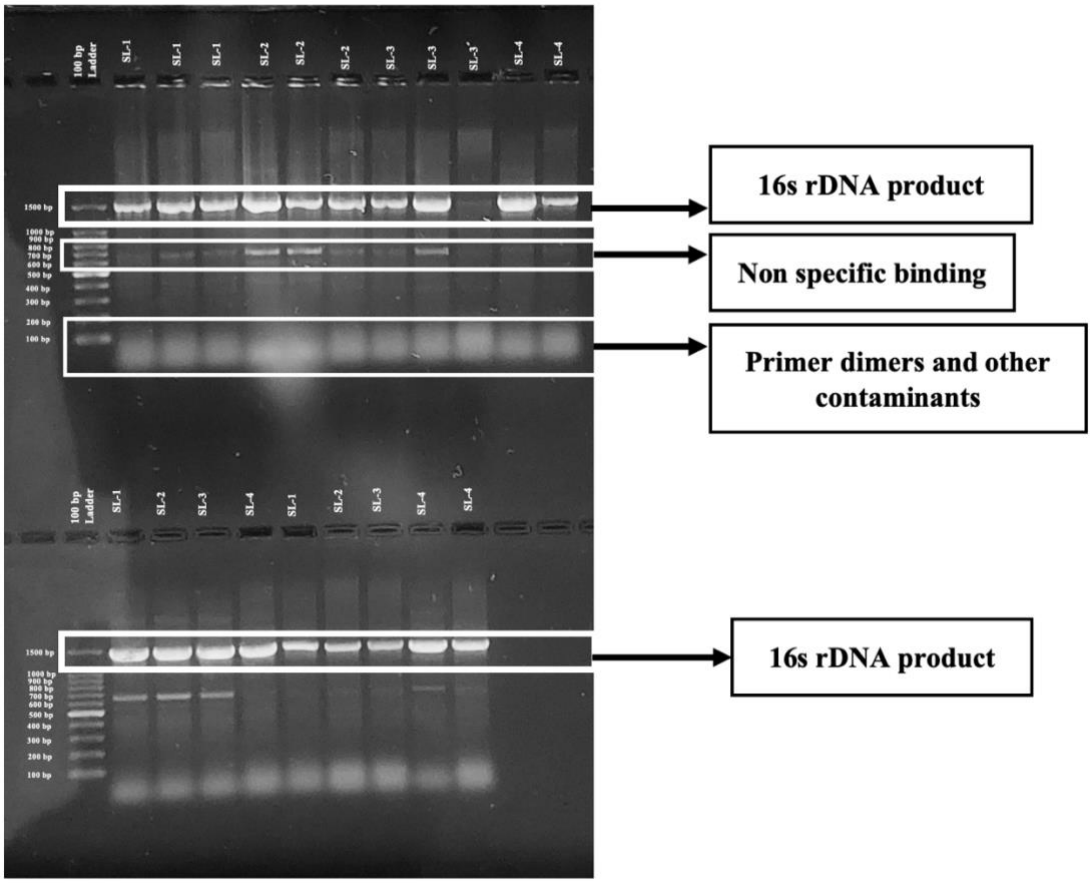

Figure 14: **Gel electrophoresis after PCR with 27F and 1492R universal primer. The ladder is 100 bp. The top band formed at 1500 bp which is the approximate length of the 16s rRNA gene**

3.2.2.3 16s rRNA Sequencing Result

The following result was found after 16s rRNA sequencing,

Table 8: **Using NCBI BLAST tool, these species were identified**

Bacteria Sample ID	Probable Organism
SL-1	*Pseudomonas aeruginosa* strain DSM 50071
SL-2	*Pseudomonas guariconensis* strain PCAVU11
SL-3	*Pseudomonas guariconensis* strain PCAVU11
SL-4	*Pseudomonas aeruginosa* strain DSM 50071

SL-1 Sequencing Results

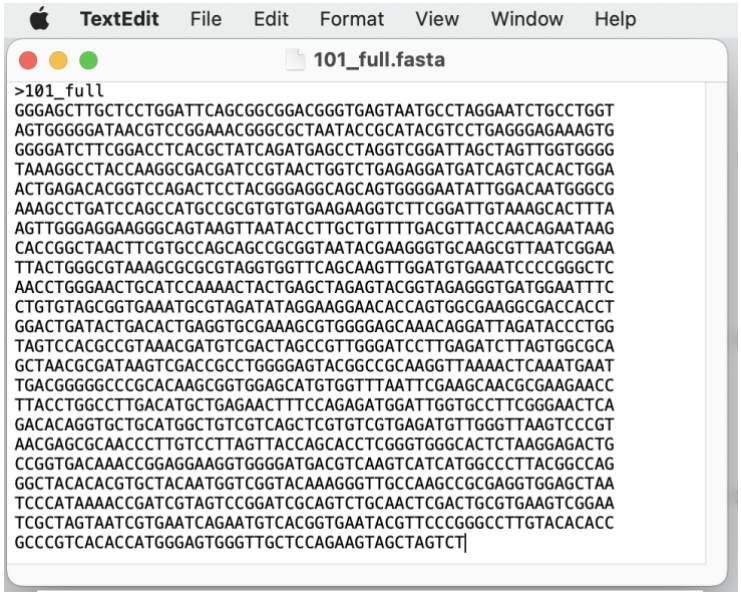

Figure 15.1: FASTA file for SL-1

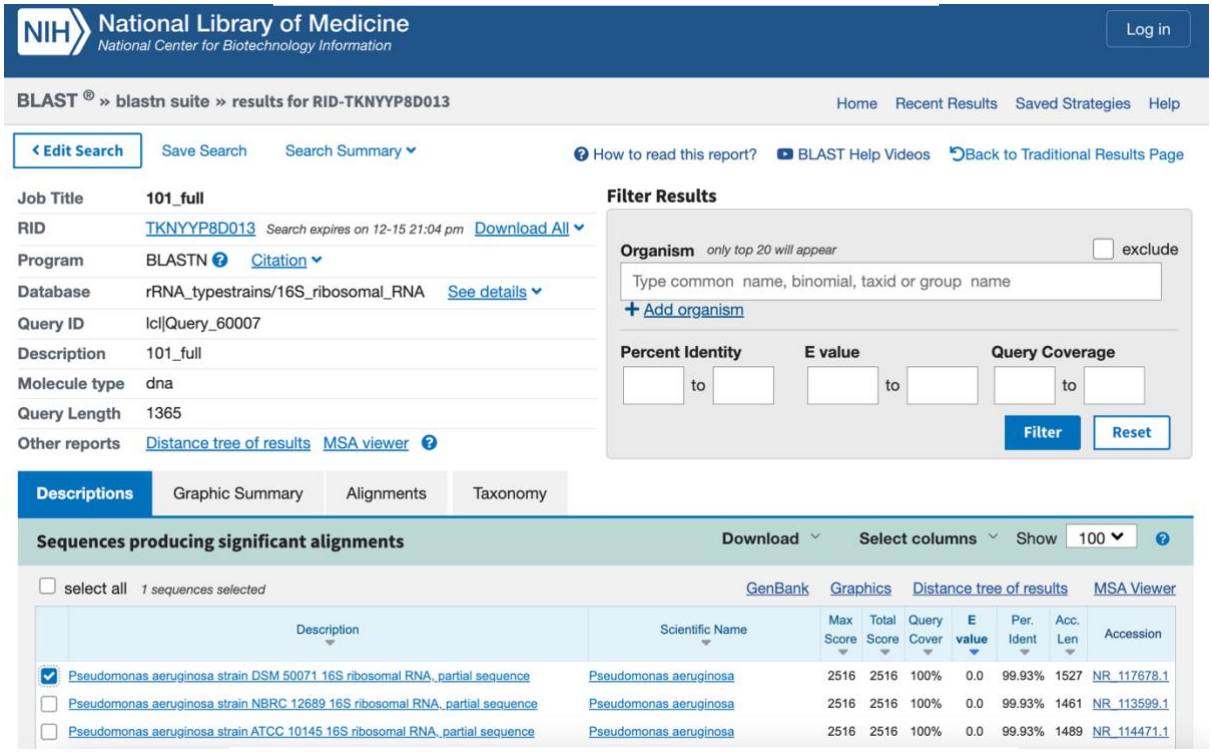

Figure 15.2: Species identification for SL-1 using BLASTn

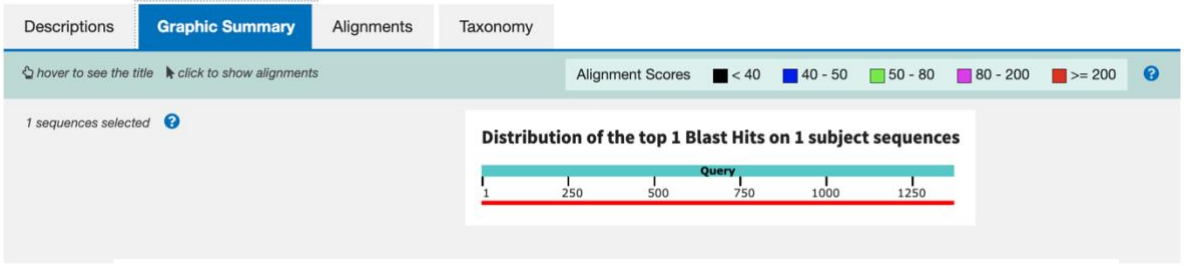

Figure 15.3: Species identification for SL-1 using BLASTn (Graphic

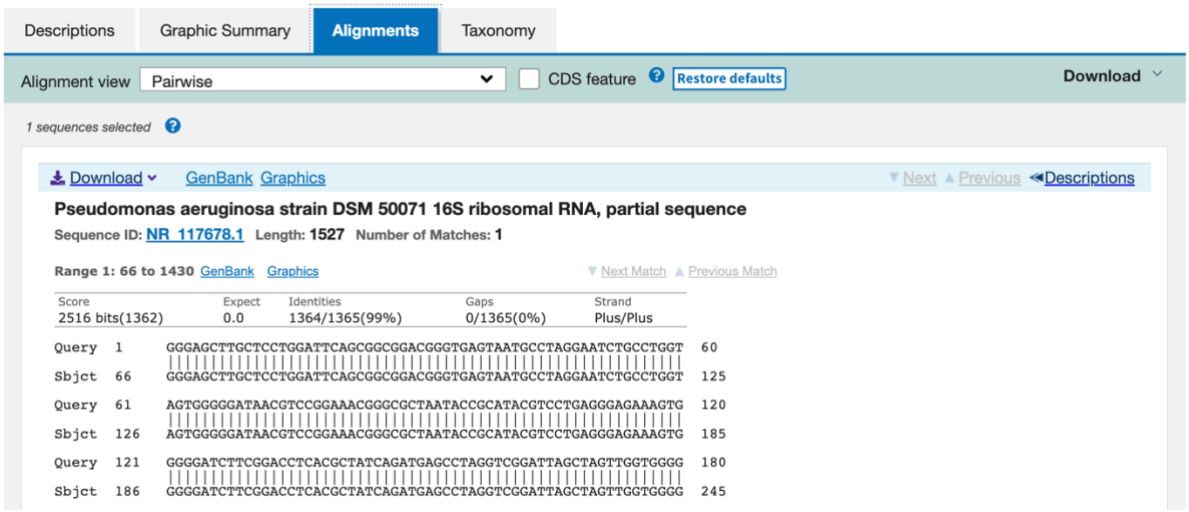

Figure 15.4: Species identification for SL-1 using BLASTn (Alignments)

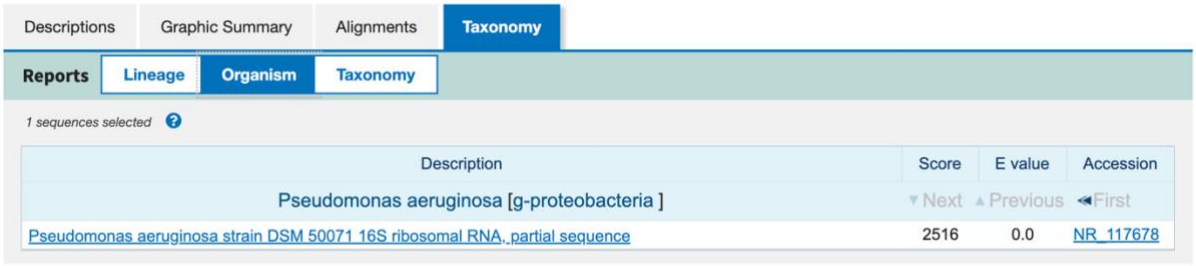

Figure 15.5: Species identification for SL-1 using BLASTn (Taxonomy)

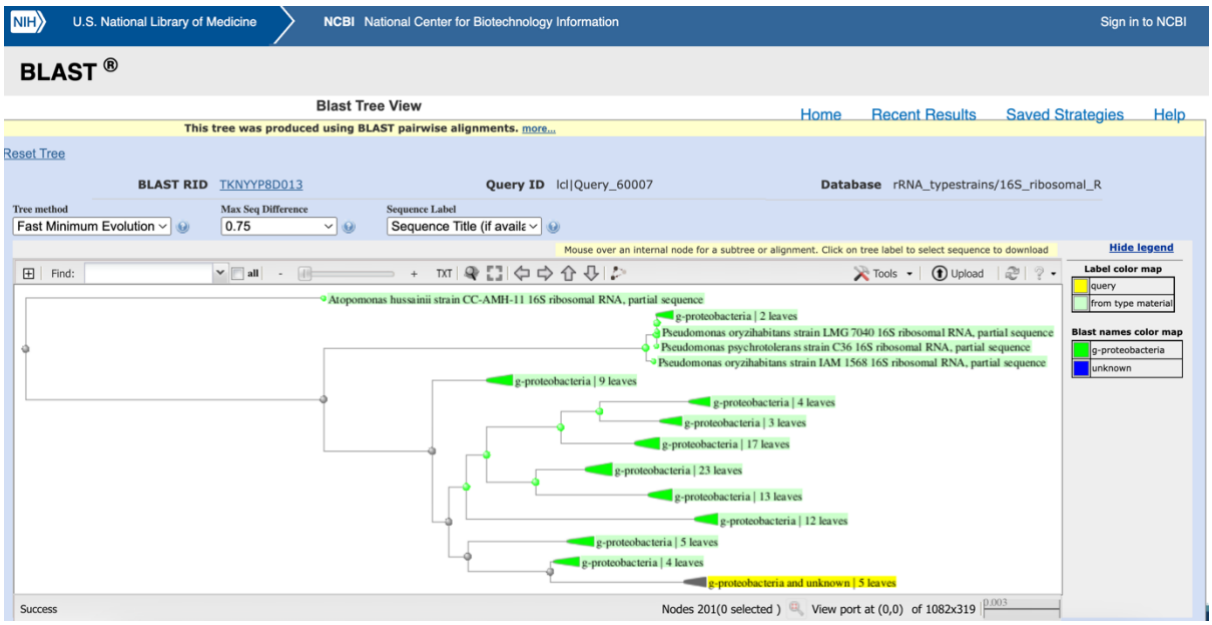

Figure 15.6: Species identification for SL-1 using BLASTn (Blast tree View)

SL-2 Sequencing Results

Figure 16.1: FASTA file for SL-2

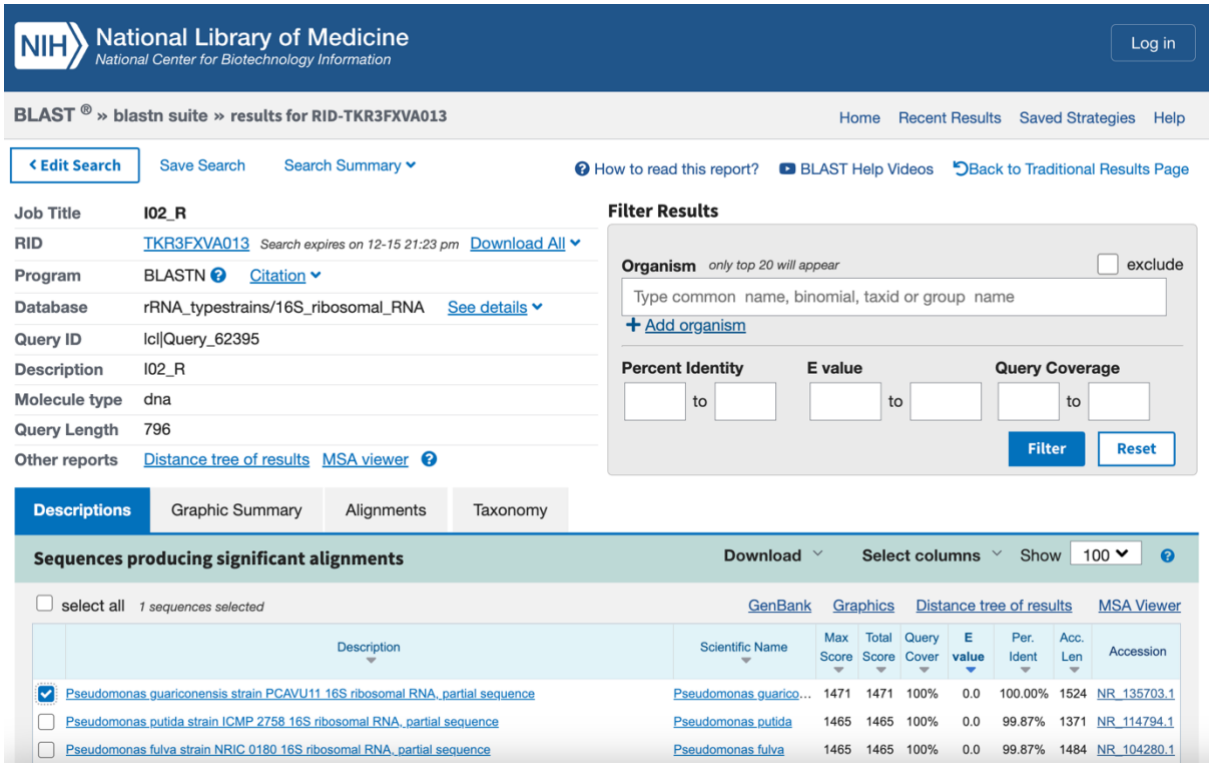

Figure 16.2: Species identification for SL-2 using

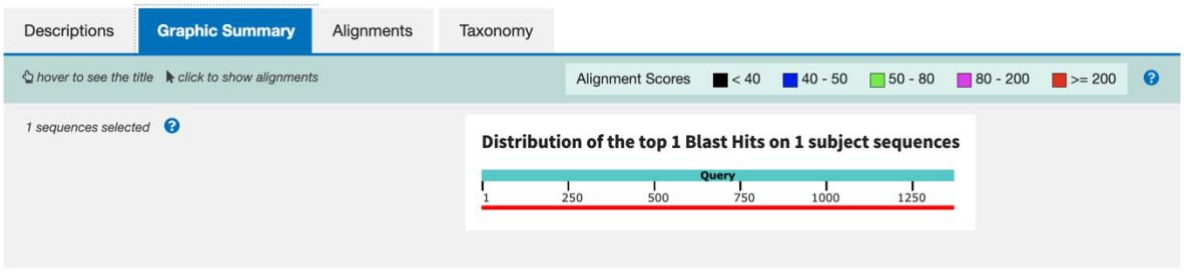

Figure 16.3: Species identification for SL-2 using BLASTn (Graphic

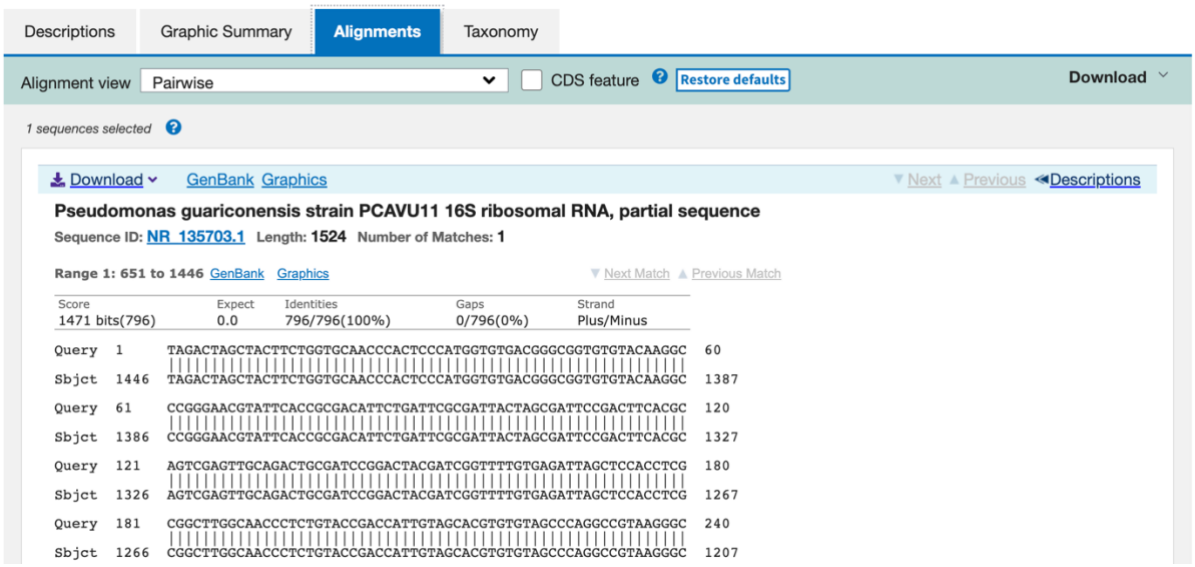

Figure 16.4: Species identification for SL-2 using BLASTn (Alignments)

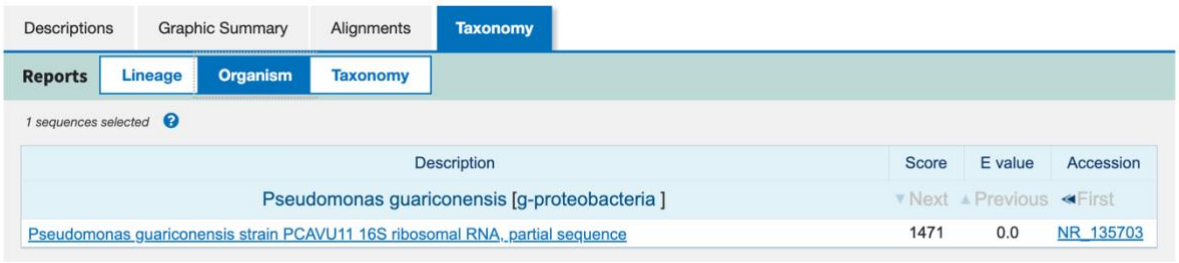

Figure 16.5: Species identification for SL-2 using BLASTn (Taxonomy)

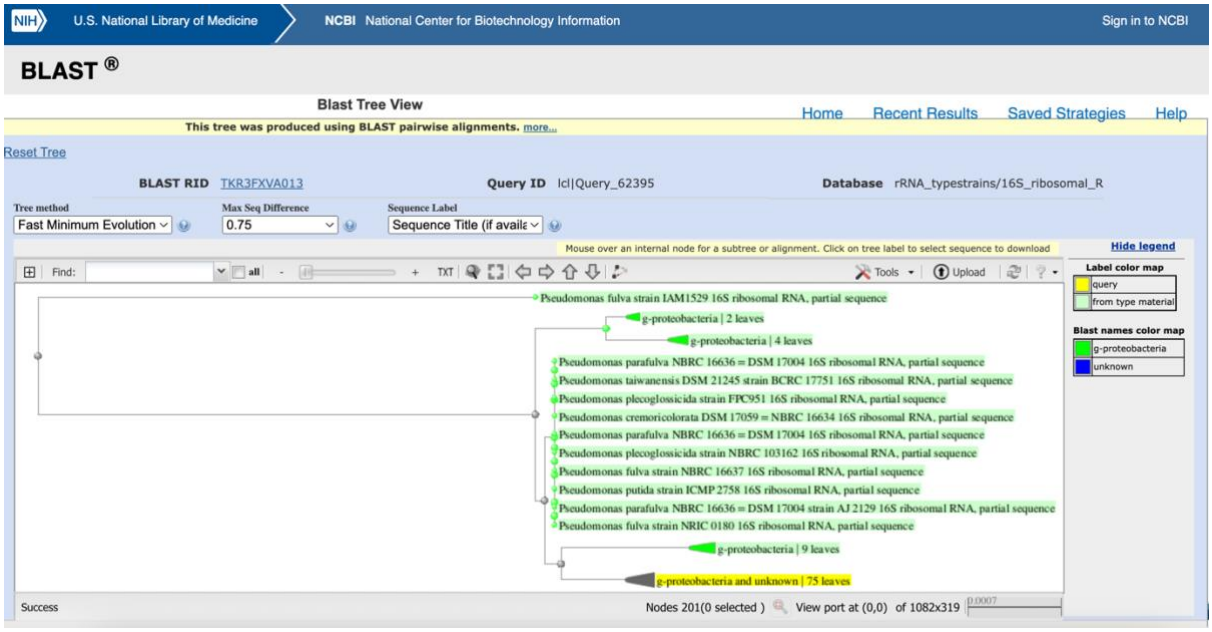

Figure 16.6: Species identification for SL-2 using BLASTn (Blast tree View)

SL-3 Sequencing Results

TextEdit File Edit Format View Window Help

103_FULL.fasta

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>103_full
GGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGT
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Figure 17.1: FASTA file for SL-3

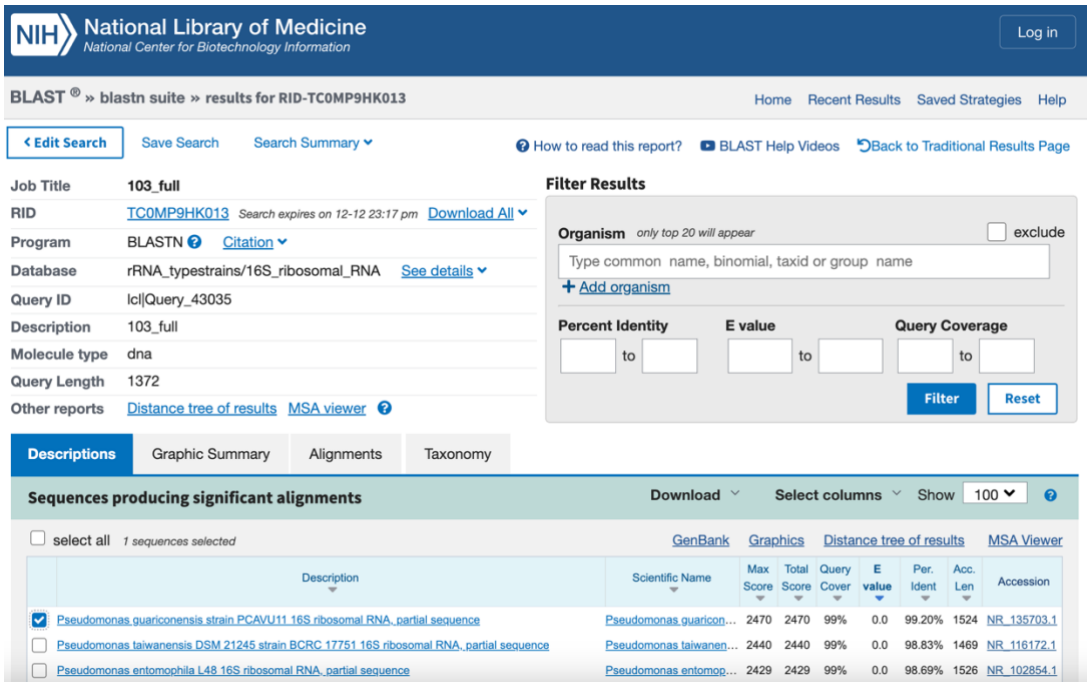

Figure 17.2: Species identification for SL-3 using

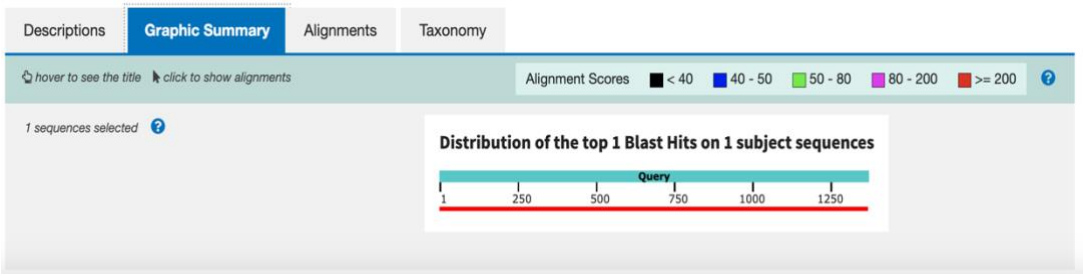

Figure 17.3: Species identification for SL-3 using BLASTn (Graphic

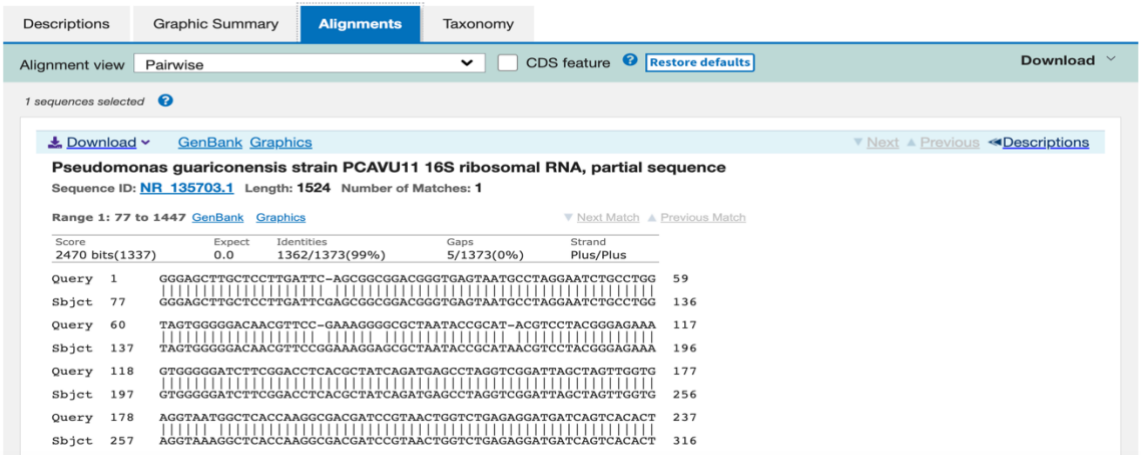

Figure 17.4: Species identification for SL-3 using BLASTn

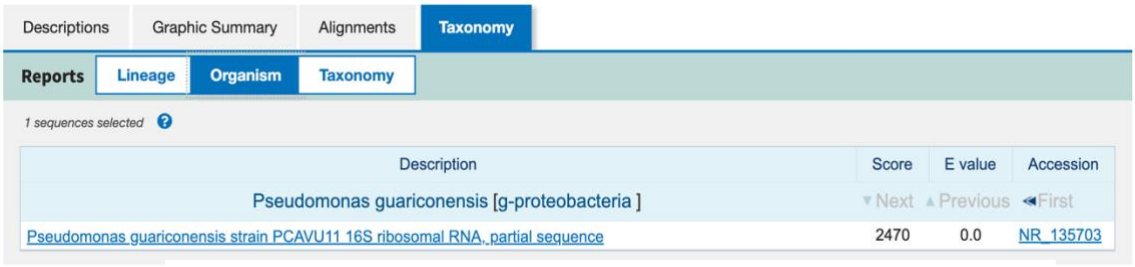

Figure 17.5: Species identification for SL-3 using BLASTn

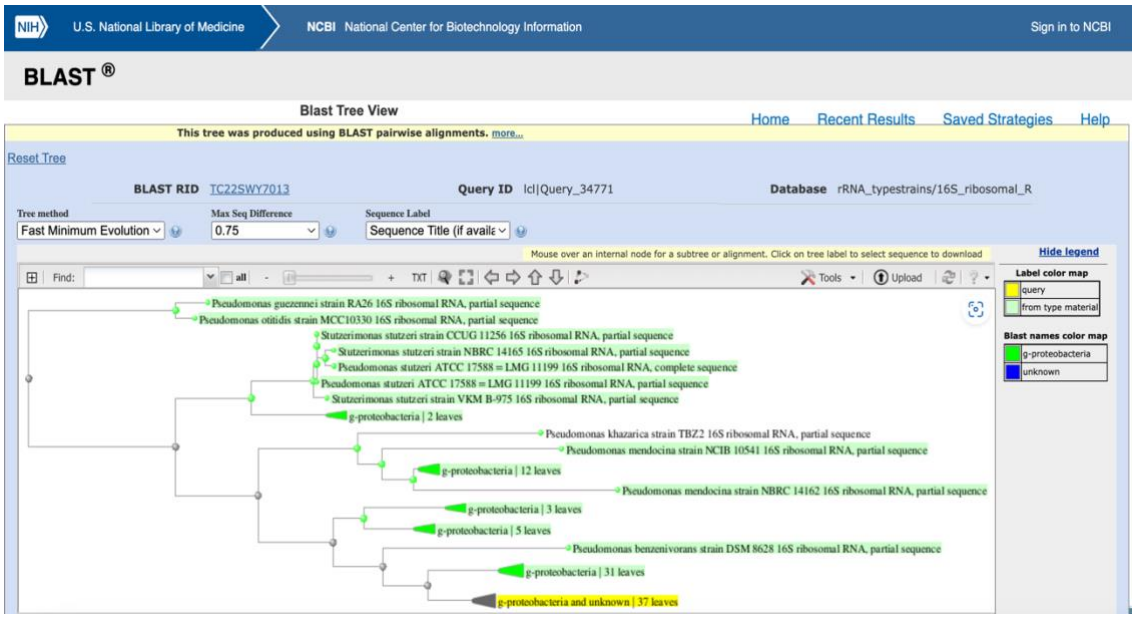

Figure 17.6: Species identification for SL-3 using BLASTn (Blast Tree

SL-4 Sequencing Results

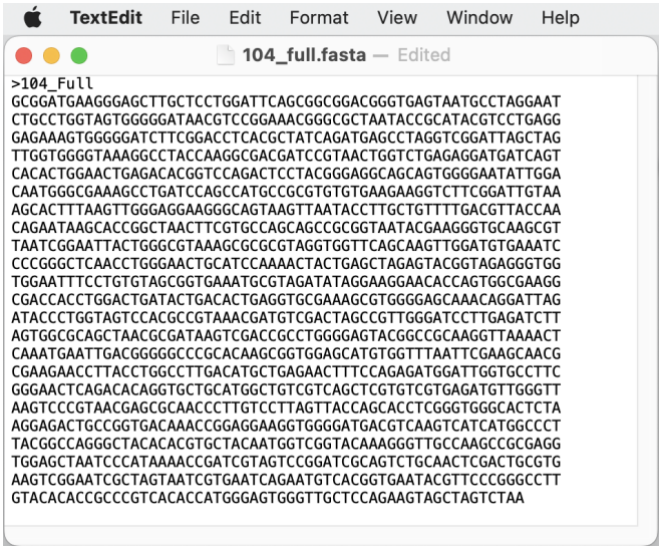

Figure 18.1: FASTA file for SL-4

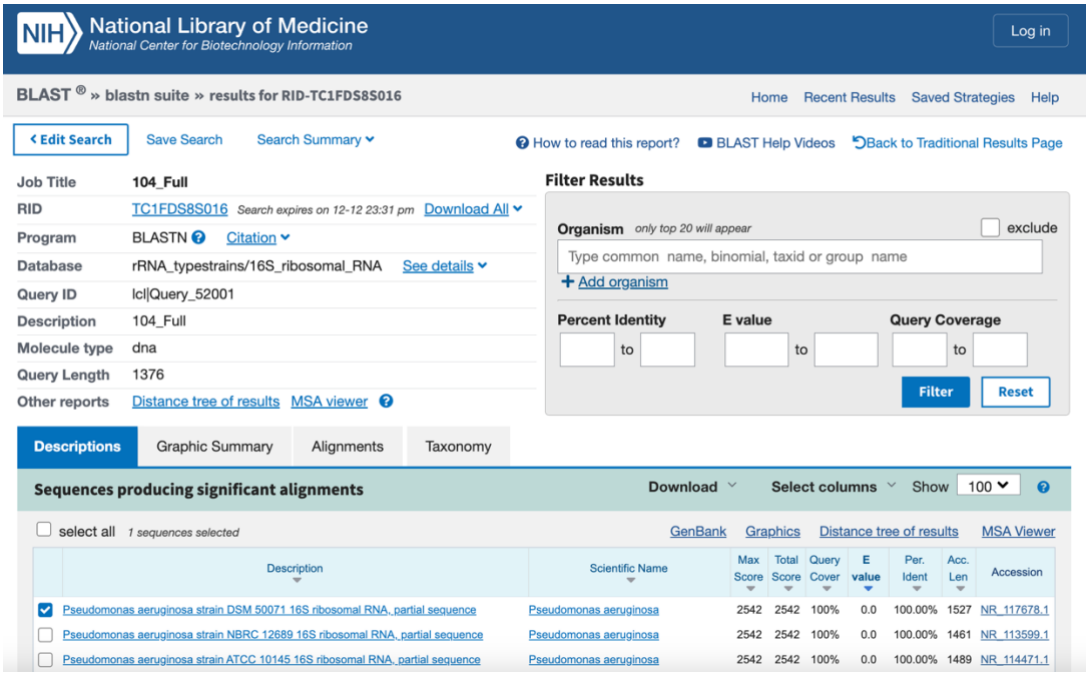

Figure 18.2: Species identification for SL-4 using

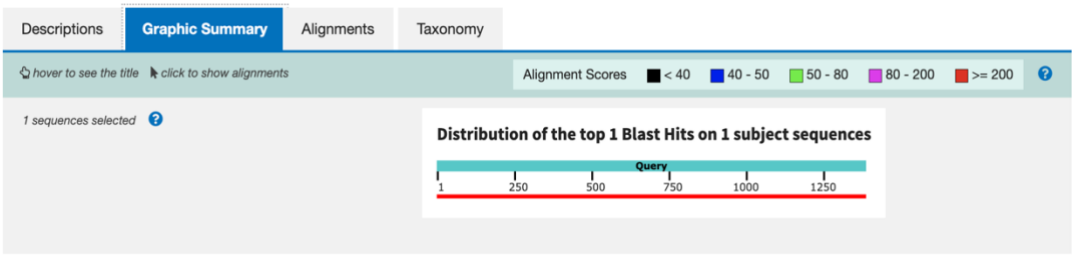

Figure 18.3: Species identification for SL-4 using BLASTn (Graphic Summery)

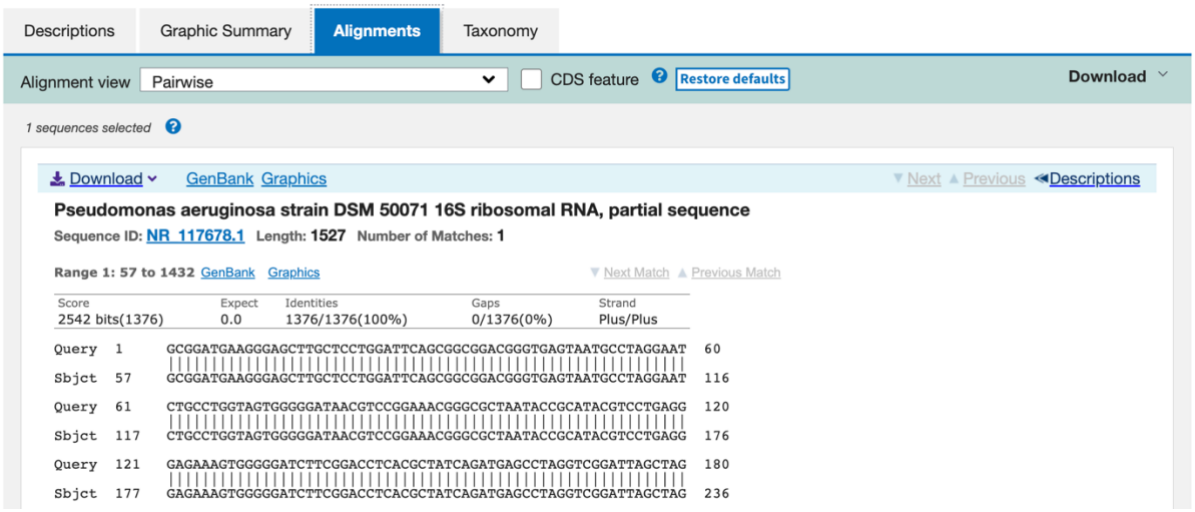

Figure 18.4: Species identification for SL-4 using BLASTn (Alignment)

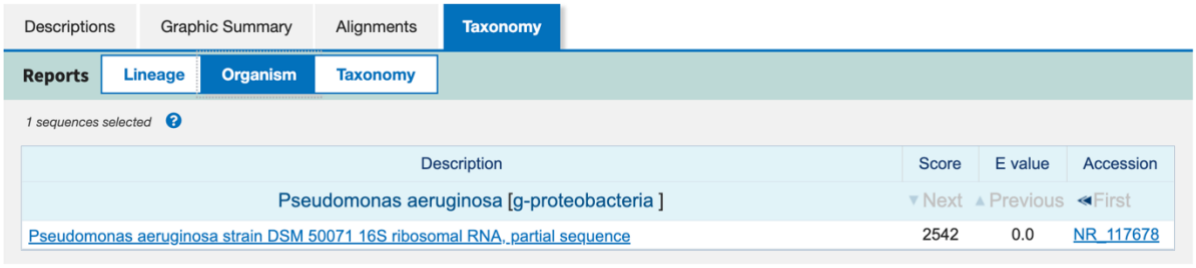

Figure 18.5: Species identification for SL-4 using BLASTn (Taxonomy)

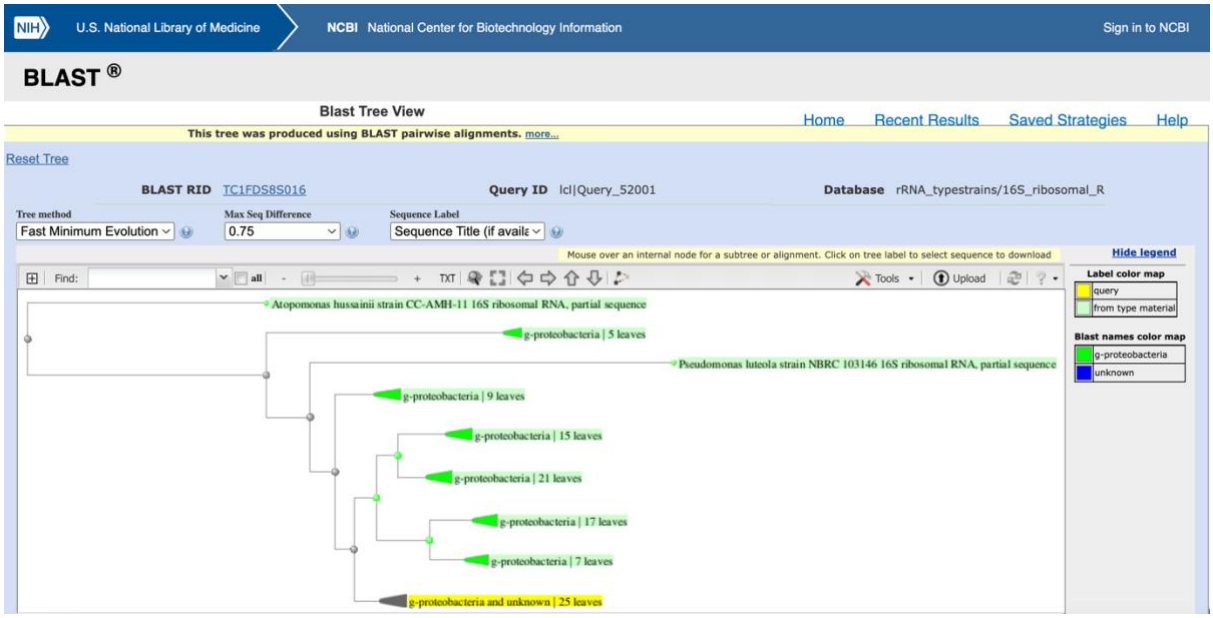

Figure 18.6: Species identification for SL-4 using BLASTn (Blast Tree view)

CHAPTER 4

Discussion

4.1 Soil Sample

"Matuail" Sanitary Landfill was the chosen sample location as it has layers of garbage dating back to 50 years. The soil which was collected was under several layers for almost 20 years, which is sufficient for bacterial evolution. This landfill gets garbage from all the Dhaka city and near Dhaka city, which results in a mixture of different types of garbage. As a result, there is much probability of bacterial evolution for bioremediation of different compounds, plastic is one of them. When the soil was collected, the soil had plastics, mainly polyethylene, all mixed up, so indigenous microorganisms had the opportunity to consider plastics as their food source. Again, as the layer of the soil was almost in anaerobic condition, many bacteria may have evolved to be facultative anaerobic, which our anaerobic growth test revealed. Out of 19 isolates, only three did not grow in an anaerobic condition, which means they are obligate aerobes as the anaerobic jar had no oxygen left in there because of the kit. Though there were plastics in the soil layer, a drawback can be the presence of organic material in the soil which can slow down evolution. In our result, the isolates were incubated for over 6 months, and though most of the bacteria died out, only a handful of microorganisms survived.

The minimal salt broth was used in the experiment because it had all the necessary nutrients except carbon sources, which made it particularly easy to add different types of carbon sources, in this case, plastics. The broth for a soil sample, which was MSB1 had a pH of 7.5, whereas the broth for the larvae sample, which was MSB2 had a pH of 6.5. The difference was made because the soil sample which was collected had a pH of 7.7. On the other hand, the middle midgut pH for insect larvae averages 5-6, or slightly alkaline, which was why the pH was set to 6.5 as a middle ground. (Erban and Hubert, 2010) Now, both MSB1 and MSB2 had carbon sources only from plastics, MSB1 had polyethylene, and MSB2 had PE, EPS, and LDPE. All the plastics were put in different Duran bottles. Every month about 10 ml of MSB (without carbon source) was aseptically added into each bottle because of the probability of the need for nutrients.

From the soil sample result, shredded PE had more bacteria in MSB1 than cut PE. The reason might be the surface area as shredded PE had more surface area covered than rectangularly cut PE, which resulted in more PE availability to the bacteria. In the 6 months of incubation, the

first four months had a declining bacterial population. The reason might be the other species of bacteria that were in the diluted sample started dying off as they were unable to utile PE as a food source. Though the sample was diluted, there was the probability of a small amount of organic material being present, which was why after 7 days of incubation, data collection started because, in the meantime, bacteria present in MSB1 should have utilized the organic materials. Figure 1, showed that after four months population decline slowed down and became stable. The reason might be only those bacteria that could utilize PE was present after four months. As the degradation of PE was slow and not efficient, which means not much carbon source for many bacteria, the bacterial population did not increase much.

The biochemical tests and anaerobic growth test gave three types of probable organisms that might be present in the MSB1, *Prolinoborus fasciculus*, *Salinicoccus albus*, and *Aquaspirillum*. *Prolinoborus fasciculus* was the dominant bacteria in the media, and it was already published that, this species can degrade plastic (Torre et al., 2018 & Atanasova et al., 2021). Though these bacteria were termed extremophiles, the current research suggests there is a high probability it can also be found in soil in a much moderate environment. *Salinicoccus albus* has hydrolytic enzymes importantly DNase, inulinase, and cellulase (Babavalian et al., 2013). As these bacteria were present in the soil for quite a long time, there is a high probability they evolved to make hydrolytic enzymes for PE. As no 16s rRNA sequence or whole genome sequence has been done on these isolates, there is also a probability that the probable species might change when the sequence will be done, because only ABIS online tool and biochemical tests cannot give 100% correct results.

Though the PE did not visually degrade, the bacteria should have utilized them. Because for six months without a carbon source and without making any endospores, bacteria should not survive. Also, in the negative controls, there were no bacteria after one month.

In addition to plastic degradation, the isolates from the soil sample were also subjected to antibiotic production and extracellular lactase production test. As the world slowly loses potent antibiotics because of antimicrobial resistance, new compounds are in high need. Though the result was not satisfactory, some bacteria gave some type of zone. Now, from the broth, the supernatant which contained an antimicrobial compound was separated using centrifugation. But those compounds were highly diluted. As there was no purification step involved, the diluted compounds were used for the well-diffusion method, which resulted in the low-quality

zone. As the diluted sample gave some kind of zone, it is safe to assume that if the concentration was higher, it would have given a much better result.

Lactose intolerance affects many people around the world, so many foods are supplemented with lactase, and even there are lactase tablets for daily use, as a result, there is a market for lactase (Kies, 2014). The three bacteria that produce extracellular lactase could not be identified by biochemical methods using ABIS software.

Of the bacteria that were grown in minimal salt broth, all the bacteria are halophilic, as there were six types of salt, nothing else other than plastics. Though the weight of PE did not decrease, there is a high chance of nano-fracture on the films, which needs SEM to quantify. More research is needed, mainly 16s rRNA sequencing of the isolates that have a probability of being *Prolinoborus fasciculus*. Enzymatic tests also need to be done, to find out enzymes, and their pathways.

4.2 Larvae Sample

Zophobas morio is called the superworm because of its nutritional value, size, and its plastic degradation capability, and for this reason, this particular larva was chosen for the experiment. For the larvae sample, there were two parts of the experiment, larvae themselves and bacteria from its feces. *Zophobas morio* eats different types of plastic in its larvae stage. In the experiment, among the three different types of plastics used, the larvae were found to be most efficient in LDPE consumption, with about 47.07% consumed in just one month. But for PE, the larvae only consumed 26.32% of the PE film in two months. Though the larvae are most known for their EPS eating capability, they performed average, about 30.51% consumed in two months. Now, for all the types of plastic, there were only 20 larvae to consume them. From the calculation, it can be seen that for the amount of plastic in milligrams eaten 100 $larvae^{-1}$ day^{-1}, EPS had the highest value, almost 73.19 mg. The larvae performed better than the research by Wang et al. in 2022, where the survival rate for those larvae that ate solely EPS and PE are 78.33 ± 5.67% and 68.33 ± 2.88% respectively in 45 days, whereas in our research, the survival rate for all the plastics were 100% for over two months. In another study by Peng et al. in 2020, the survival rate of larvae eaten solely PE was 94.0 ± 1.0% and 96.5 ± 0.5% for EPS which is almost similar to our findings. Their survival rate for unfed was also similar to our result, 60.5 ± 1.5% over one month compared to 55% of ours. As both the studies mentioned, cannibalism was seen in each plastic, but after 75 days, their population decreased and no dead body was

found. Interestingly, for those larvae who were unfed, no cannibalism was seen for 2 months. In most of the larvae, the molting process was seen, which indicates the food availability, in this case plastics. The hypothesis is that they digest the plastic and transform them into simple carbohydrates, and they do this using their gut microbiome.

Now for the bacteria isolated from feces, only 2 types of the colony were found, and according to 16s rRNA sequencing, all are different species of *Pseudomonas*. These 4 isolates were incubated in three different plastics, SL-1 was in PE, SL-2 and SL-3 were in EPS and SL-4 was in LDPE. Though two isolates (SL-2 and SL-3) were identified as *Pseudomonas guariconensis* PCAVU11, there are insufficient studies on it which prove they degrade plastic. This research might be one of the first to prove this species' biodegradation capability. On the other hand, *Pseudomonas aeruginosa* has been already published to have plastic biodegradation capability (Lee et al., 2020 & Kyaw et al., 2012). Research by Wang et al in 2022 identified *Spiroplasma* and *Rhodotorula* for PE degradation, *Issatchenkia* for both PS and PE degradation, and *Pseudomonas* for PS degradation in superworms where we got different species of *Pseudomonas* only. The reason might be the use of Minimal Salt Broth where these bacteria were incubated. Even if initially those bacteria were present, during MSB2 incubation they died out and only *Pseudomonas* persisted, which might prove these species of *Pseudomonas* were more evolutionarily stronger than those other bacteria.

For 16s rRNA sequences, Sl-1 had hit for 3 strains of *Pseudomonas aeruginosa* and as they all had the same percent identity which was 99.93%, the first result was chosen because the first result is considered the best result. The same process was done with SL-4 which had percent identity of 100% for three strains. Whole genome sequencing of these bacteria can unearth their true identity and the enzymes which are used for plastic degradation.

Conclusion

In conclusion, there is much possibility for plastic biodegradation from soil and the superworm. Lots of research is needed to find out enzymes and pathways of degradation. The isolates from the soil sample need to have a 16s rRNA sequencing, preferably the whole genome because with the sequencing we can find out the species as well as enzymes. It will help to find plastic degradation pathways also.

Moreover, *Zophobas morio* is better at consuming LDPE than EPS and PE. The molting process suggested they got food as if there was no food, there would be no growth. They can also recycle their own feces, which is important to decrease microplastic pollution. As there was no proper nutrient, only plastics were given which are mostly hydrocarbons, the lack of nutrients made them weak after three months and they started to die. As the sample size was small, it can be concluded that they are much more efficient in various plastic degradation than other species of larvae. The bacteria isolated from their feces have good potential to degrade different types of plastic. More research is needed to find more efficient bacteria, or even other bioremediation techniques to remediate plastics because plastic pollution is getting out of hand and it already invaded our human body.

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