"ISOLATION AND IDENTIFICATION OF PLASTIC DEGRADING BACTERIA FROM SOIL AND SEWAGE SAMPLE"

Project Work By

ANYA ELIZABETH SAM

AB21ZOO002

Under the guidance of

DR. MEERA JAN ABRAHAM, Associate Professor

Department of Zoology, St.Teresa's College (Autonomous),

Ernakulam

Kochi-683011

Submitted to St.Teresa's College (Autonomous), Ernakulam

Affiliated to Mahatma Gandhi University, Kottayam in partial fulfillment of requirement for the degree of Bachelor in Science in Zoology

2023-2024

CERTIFICATE

This is to certify that the project entitled “ISOLATION AND IDENTIFICATION OF PLASTIC DEGRADING BACTERIA FROM SOIL AND SEWAGE SAMPLE” submitted in partial fulfillment of the requirement for the award of the degree of Bachelor of Science in Zoology to the Department of Zoology, St.Teresa’s College affiliated to M.G University, Kerala, done by Ms.Anya Elizabeth Sam, Reg. No: AB21ZOO002 is an authentic work carried out by her at Primordia Lifesciences Pvt. Ltd, Kochi under my guidance and supervision during the period of September 2023-Novemeber 2023. The matter embodied in this dissertation has not formed the basis for the award of any Degree/Diploma/ Associateship /Fellowship to the best of my knowledge and belief.

Dr.Soja Louis ,

Head of the Department ,

St.Teresa's College ,

Ernakulam

Dr.Meera Jan Abraham ,

Associate Professor ,

St.Teresa's College ,

Ernakulam

Examiners

1.

2

DECLARATION

I hereby declare that project work titled "ISOLATION AND IDENTIFICATION OF PLASTIC DEGRADING BACTERIA FROM SOIL AND SEWAGE SAMPLE " submitted to St. Teresa's College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in the partial fulfillment of the requirements of Bachelor of Science degree in Zoology, is a record of original project work done by me under the guidance and supervision of Dr. Meera Jan Abraham, Associate Professor, Department of Zoology, St. Teresa's College (Autonomous), Ernakulam.

Name: Anya Elizabeth Sam

Reg.No: AB21ZOO002 Signature

ACKNOWLEDGEMENT

The success and final outcome of this project required guidance from many people and I am grateful to them for their assistance throughout the completion of my project work.

I respect and thank Dr. Soja Louis, HOD, Department of Zoology, St. Teresa's College (Autonomous), Ernakulam. I owe my gratitude to my project guide Dr. Meera Jan Abraham, Associate Professor, Department of Zoology, St. Teresa's College (Autonomous), who took keen interest in my project work and provided all the necessary information for developing a systematic schedule for my project.

I extend my sincere gratitude to Dr. Anu Yamuna Joseph, Principal Scientist, Primordia Lifesciences Pvt.Ltd, KINFRA Hi-TECH Park, Kalamassery, Kochi for providing the opportunity, the resources and for her valuable guidance which enabled the successful completion of my project on time. I am especially grateful to Ms. Anumol TA and Ms. Aswathy K B who dedicated their time to teach me the concepts and techniques required for my project.

I would also like to thank my parents for their unwavering support, for encouraging my scientific curiosity throughout this project and without whom this project wouldn't have been a success.

INDEX

ABSTRACT

The recalcitrant nature of plastics has caused the existing physical and chemical degradation methods to be of limited efficiency. Certain microbes have the built-in capacity to degrade plastic polymers and demineralize them to biomass CO_2 and water, helping to combat the persistent nature of plastics in environment. In this study, bacteria were isolated from plastic contaminated areas and were cultured in MSM to identify its plastic degrading potential. The microbes were biochemically characterized and identified as *Bacillus wiedmannii* and *Klebsiella pneumoniae* by 16s rRNA sequencing. These two microbes were also cultured for a period of 3 months to evaluate its plastic degrading capacity. The findings indicate that they can effectively degrades PE, with a significant weight loss rate of 17.13% and 21.44% (*Bacillus wiedmannii* and *Klebsiella pneumoniae* respectively) after 120 days. *K. pneumoniae* cannot be used commercially because of its virulence but further research can be focused on the screening of potential genes and thus enzymes that enable the bacteria to have plastic degradation capacity. *B. wiedmannii* proves to be a promising prospect for future elucidation of its genes, to attain an in-depth understanding into the process of plastic degradation. Large scale plastic waste treatment technology could be the most rewarding and efficient solution to the global plastic pollution problem.

INTRODUCTION

With the discovery of synthetic plastic in 1907 by Belgian chemist Leo Baekeland , the 20th century saw a revolution on plastic production : its excessive use because of its light weight, strength ,low cost etc . Since then, urban areas have been continually influenced by the anthropogenic stressor, ie ,plastic pollution, and today it plays a significant role in the worldwide plastic pollution problem .

Statistical reports show the global use of plastic growing at a rate of 12% per year .Around 0.15 billion tonnes of synthetic polymers are produced worldwide every year (Premraj and Doble 2005; Leja and Lewandowicz ,2010; Kumar & Das, 2014). Asian countries, in particular, have been highlighted by several published studies as contributing a considerable proportion of global macro- and microplastic release. In the context of India, it generates about 3.5 million tonnes of plastic waste annually and the per capita plastic waste generation has almost doubled over the last five years. The gap in waste management capacity and plastic consumption is called MWI and according to an EA report, India ranks fourth in the MWI, with 98.55 per cent of generated waste being mismanaged and fares poorly in the management of plastics waste.

Although plastic materials continue to be an integral part of the global economy, the issues associated with their extensive application cannot be ignored. Inevitably all forms of life are exposed to a large variety of toxic chemicals and microplastics through inhalation, ingestion, and direct skin contact. Over 10,000 chemicals in plastics have been identified, and data on more than 2,400 of these chemicals have classified them as substances of concern to our health and environment .To highlight a few of the numerous impacts on health and environment

Environment: Plastic contamination can affect water, soil, air by burning and has long term persistence in environment.

a) Recent modelling research has found that rivers worldwide discharge has approximately 1.2-2.4 MT of floating plastic contaminants from inland regions to oceans each year (Lebreton et al., 2018; Van Cauwenberghe et al., 2015). These accumulate in the riverbed and can negatively affect the flora and fauna, benthic organisms, and contribute to river water pollution (Maheswaran et al., 2022b).

b) Plastics contribute can indirectly contribute to warming temperatures and extreme weather events due to climate change. For instance, in its production from fossil fuels and by incineration, it releases greenhouse gases such as methane and CO_2 , contributing to global warming. Plastic burning results in the emission of toxic substances such as Nitrous Oxide, Sulphur dioxide,furans, dioxins and polychlorinated biphenyls. Plastic wastes, such as, microplastics has the ability to attract contaminants, such as, persistent organic pollutants, (Malhotra , 2021).

Research on the impact of plastic pollution on health suggests the following :

a) Plastic ingested by animals persists in the digestive system and can lead to decreased feeding stimuli, gastrointestinal blockage, decreased secretion of gastric enzymes and decreased levels of steroid hormones, leading to reproduction problems (Azzarello & Vleet, 1987). Research also revealed that microplastics ingested can act as vessels for pathogens (which have a particularly strong bind to plastic waste) to enter our system, increasing the spread of diseases.

b)It has been found that plastic associated chemicals, are those that can mimic, block or alter the actions of hormones, reduce fertility and damage the nervous system. Heavy metals, phthalates, bisphenol A, biocides, certain flame retardants, UV filters, or Per-and Polyfluoroalkyl Substances (PFASs) are known to bioaccumulate in exposed organisms . The high flow rates of many WWTPs are able to release large amounts of MPs, enter the marine ecosystem, eventually be ingested by aquatic organisms, and be consumed by humans (Lehtiniemi et al., 2018). Moreover, the high surface area and hydrophobicity features of MPs facilitate their ingestion by both land and sea creatures, enhance both risks of adsorption and desorption of toxic chemicals and pathogens in water, and eventually yield negative impacts not only for humans but also the holistic biodiversity (Amelia et al., 2021; Bhagat et al., 2021b).

c) In context of workers employed in plastic recycling industry ,over 95% of the e-waste is treated and processed in the majority of urban slums of the country, where untrained workers carry out the dangerous procedures without personal protective equipment, which are detrimental not only to their health but also to the environment (Annamalai ,2015).

Being naturally undegradable, microplastics together with plastics of other sizes i.e., megaplastics (more than 500 mm), macroplastics (50–500 mm), and mesoplastics (5–50 mm) accumulate to cause massive plastic pollution in both lands and oceans (Wang et al., 2018). Hence to combat this ,besides toxic methods like landfill dumping , incineration etc, research has been focused on understanding and enhancing degradation methods and mechanisms that exist in nature . Therefore, it is necessary to investigate the pathways of plastic degradation and their efficiency in the environment.

Degradation is reflected as changes in properties of material (mechanical, optical or electrical characteristics), in cracking, erosion, discoloration, phase separation and delamination. The changes include chemical transformation and formation of new functional groups (PospíŠil & Nešpůrek, 1997). Some of the degradation mechanisms :

I. Mechanical degradation: Mechanical degradation reduces the molecular weight of the polymer by subjecting the polymer to powerful stress ,ie, extrusion, agitation and grinding.A natural site for mechanical degradation is beaches .As a result ,experiments mimicking beach set ups, combining factors of sunlight and mechanical stress, by rotating the bottles containing the plastic strips for 24 h, at a constant speed, induced mechanical degradation and showed 14% weight loss . Thus a certain degree of depolymerization was observed (Arpia et al. , 2021) .But ,according to Enfrin et al,(2020) , even though mechanical abrasion is able to degrade MPs, the process is either enhanced or initiated by chemical degradation through

photooxidation, thermal oxidation, hydrolysis, and changes with salinity and alkalinity levels (Enfrin et al., 2020; Corcoran, 2021).

II. Thermal degradation: Degradation of polymers at elevated temperature or heat ,in the presence of air or oxygen. This process , though resulting in the generation of toxic volatile gases, is of rising importance because of its potential to be an alternative source of fuel. According to Chen et al. (2019) thermodegradation of MPs in supercritical water in an optimized environment is considered to be more energy-efficient and generates lower hazardous greenhouse gases (GHGs) as compared to incineration and mechanical recycling(Chen et al., 2019a).

III. Hydrolytic degradation: In cases where photooxidation, abrasion, thermal oxidation, and biodegradation cannot take place, i.e., in a landfill or the ocean floor, the aromatic polyesters undergo hydrolysis(Chamas et al., 2020). Plastic polymers bind and react with water molecules , in the process of which , the original chain breaks up into smaller segments , resulting in polymer degradation .

IV. Ultrasonic degradation: Ultrasound is employed for the degradation of polymers, wherein the most susceptible bond is targeted. This method is known to affect the mechanical, mechano-chemical, and morphological properties of the polymer (Mohod & Gogate, 2011c).

VI. Chemical degradation: Degradation of polymers assisted by chemical substances such as esters of H-phosphonic and phosphoric acids etc. to respective monomers or oligomers (Mitova et al. , 2013).

VII. Photochemical degradation: Certain chemicals and UV radiation cause polymer chains to disintegrate into monomers or oligomers.

V. Biological degradation: Decomposition or destruction of contaminant molecules by the action of the enzyme secreted by microorganisms is known as biodegradation .The polymers are degraded by microorganisms that specifically target certain functional groups and thereby, break the polymer structure into simpler molecules. These molecules are further mineralized completely with the aid of enzymes.

Natural plastics like Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and Polycaprolactone can be degraded by microbes such as *Clostridium botulinum* and *Clostridium acetobutylicum* (Abou-Zeid et al. ,2001). Some of the strains of microorganisms capable of depolymerizing synthetic plastics polyethylene include *Brevibacillus borstelensis*(Hadad et al. , 2005) *and Penicillium simplicissimum* YK (Yamada-Onodera et al., 2001) . PVC degrading microbes include *Pseudomonas chlororaphis* (Zheng et al. , 2005) and *Pseudomonas putida* (Anthony et al. ,2004).

Biodegradation is a specific enzymatic process. Certain enzymes break down certain substrates (Adamcová and Vaverková , 2014) . Research conducted by Danso et al (2017), shows the potential bacterial genera that are known to be involved in the breakdown of high molecular weight polymers . Polyethylene terephthalate can be degraded by PET esterases and cutinases

secreted by *Thermobifida* , *Ideonella* , *Bacillus* , *Pseudomonas* . Polyurethane has the potential to be depolymerized by cutinases , esterases and lipases secreted by *Comamonas* , *Pseudomonas* , *Fusarium* and others (Danso et al. , 2019).

The process of biodegradation produces not only non-toxic by-products but also provide energy to microorganisms or transform them into other useful products, causing a significant reduction in toxic plastic pollution. Biodegradation may effectively reduce the harm of additives to plastics and achieve a better ecological environment (Cai et al. , 2023). Thus biodegradation shows the potential to be one of the solutions to plastic pollution.

According to UNEP , global cumulative plastic production is predicted to reach 34,000 million tonnes between 1950 and 2050.The harmful chemicals released from plastic products throughout their entire life cycle can pose a serious risk to humans and the environment, including when waste is not properly managed, finding its way to air, water and soils. [https://www.uts.edu.au/isf/explore-research/projects/addressing-plastic-pollution-india]. This brings to attention the need for increased research work in not only the potential plastic degrading bacteria but also its practical application with least side effects to health and environment.

SCOPE, AIM AND OBJECTIVE

Currently the world has a plastic pollution crisis. After the discovery of plastics, uncontrolled use and irresponsible disposal created plastics to be a matter of great concern. The cumulative plastic waste is ~1–1.5 million tons annually which is contributing around 56% to global plastic waste (Ritchie and Roser,2018). Plastic can wreak slow but certain havoc on humanity and its environment. The theme for World Environment Day ,2023 (June 5) being 'Beat Plastic Pollution' calls the need to find solutions to the crisis a hand. The scientific realm of studies could contribute by finding ways to combat plastic pollution.

The recalcitrant nature of plastics has caused the existing physical and chemical degradation methods to be of limited efficiency. In most cases they either release toxic byproducts like harmful gases (eg: dioxins), create fragmented microplastics (that can make its way into the food chain), can affect soil fertility or other methods involved in degradation are too expensive and require constant monitoring.

Certain microbes have the built-in capacity to degrade plastic polymers and demineralize them to biomass CO_2 and water, helping to combat the persistent nature of plastics in environment. A lot of research is being conducted on plastic degrading microorganisms and the enzymes produced from them. Hence, they act as the most potent and beneficial key to finding an effective solution in reducing the negative impacts of plastics. Due to the great diversity in microbial communities, there lies scope in finding environment friendly, cost-effective solutions to the plastic pollution problems, with the discovery of several prospective strains.

This study aims to isolate and identify bacteria from plastic contaminated soil and sewage water.

OBJECTIVES:

1. Isolation of plastic degrading bacteria from plastic contaminated sewage water and soil using suitable media .
2. Morphological and biochemical characterization of bacteria.
3. Molecular characterization of bacteria.
4. Evaluation of plastic degrading capacity of isolated bacteria.

REVIEW OF LITERATURE

Plastics can be characterized into some of the essentials classes such as natural plastics, semi synthetic plastics, synthetic plastics, thermoplastics and thermosetting plastics. Plastics can also be classified as polythene (PE), propylene (PP), polystyrene (PS), polyurethane (PUR), nylon etc. Polyethylene can be either LDPE (low density polyethylene) or HDPE (high density polyethylene).

Low-density polyethylene is the most abundant plastic waste discarded in landfills in the form of plastic bags (69.13%). LDPE is mostly amorphous, with short branches (10–30 CH3 per 1,000 carbon atoms) .This branching system makes LDPE chains more accessible and the tertiary carbon atoms at the branch sites more susceptible to attack. Thus a lot of the research work finds LPDE degradation capacity of organisms.

Plastics are biodegraded aerobically in wild nature, anaerobically in sediments and landfills and partly aerobically and partly anaerobically in composts and soil. Carbon dioxide and water are produced during aerobic biodegradation and carbon dioxide, water and methane are produced during anaerobic biodegradation (Gu et al., 2000).

The sources of plastic degrading organisms from various environments including plants like microalgae ,bacteria ,actinomycetes etc.

The algal enzymes secreted into the liquid media interact and degrade the plastic surface. The polymer is then utilized by algae as carbon source (Chinaglia et al ,2018). Blue-green alga (Cyanobacterium), *Anabaena spiroides*, has shown the highest percentage of LDPE degradation (8.18%) followed by diatom *Navicula pupula* (4.44%) and green alga *Scenedesmus dimorphus* (3.74%) (Kumar et al , 2017) . A study by Sarmah and Rout concluded that freshwater nontoxic cyanobacteria (*Phormidium lucidum* and *Oscillatoria subbrevis*) which are readily available, fast-growing and easily isolable, are capable of colonizing the PE surface and biodegrading LDPE efficiently without any pretreatment or pro-oxidant additives (Sarmah & Rout, 2018).

Fungi have the capability to degrade plastic and its mechanism is similar to that of bacteria,ie, like bacteria , fungi attach to the plastic film, cause small-scale swelling and bursting, as the fungi penetrate the polymer solids .They secrete enzymes and grow on it by utilizing it as substrate and source of nutrition. It slowly gets depolymerized and degraded finally ending in mineralization process, where water, carbon dioxide, methane are formed (Montazer et al, 2019). *Phanerochaete chrysosporium* is fungal species that degrade high molecular weight polyethylene under nitrogen-limited and carbon-limited conditions (Shimao 2001). *Aspergillus, Cladosporium, Fusarium* are among others that have been reported for polyethylene degradation (Danso et al., 2019; Restrepo-Florez et al., 2014). *Penicillium oxalicum* NS4 (KU559906) was identified for degrading HDPE and LDPE (Ojha et al, 2017).

Microorganisms capable of hydrolyzing PE have been isolated from soil, sea water, compost and activated sludge (Montazer et al.,2019). Bacteria in the gut of the greater wax worm,

Galleria melonella have been found capable of hydrolyzing polyethylene (PE) (Yang et al., 2014; Bombelli et al., 2017; Cassone et al., 2020) . In 2016 , Japanese scientists ,after analyzing the bacteria *Idieonella sakaiensis* found that it produced digestive enzymes called hydrolyzing PET or PETase which interact with PET plastic to break it down into monomers called terephthalic acid and ethylene glycol. These monomers are then broken down further to release energy for growth of the bacteria. The biofragmentation and bioassimilation of LDPE into biomass by the bacterial species, *Pseudomonas putida* IRN22, P. putida LS46, *Acinetobacter pittii* IRN19, and *Micrococcus luteus* IRN20 has been reported. These bacterial species were able to utilize the untreated petroleum-derived LDPE as a sole source of carbon and energy for growth (Montazer et al. ,2019).

Aerobic degradation by bacteria occurs in the following stages:

(1) Biodeterioration, which is defined as formation of carbonyl-groups by the action of oxidative enzymes released by microorganisms or induced by exterior agents, like sunlight (ultra-violet) exposure. Subsequent oxidation reduces the number of carbonyl-groups and generates carboxylic acids 19) (Corcoran, 2020).So-called "weathering" and "photodegradation" are currently considered the main forces for initial depletion of plastics, and they mainly result in a modification of the chemical, physical, and mechanical properties of the plastics . The resulting particles have a much larger surface area, which makes them amenable to further degradation (Welzel K et al , 2002).

Photo-induced oxidative degradation of PE , PP and others results in decreased molecular weight and the formation of carbonyl end groups (Mohanan et al , 2020).

(2) Biofilm formation- microbes and other organisms colonize on the surface of the polyethylene films forming a biofilm. Once the organisms get attached to the surface, they start growing by using the polymer as the carbon source and performs biofragmentation, which involves hydrolysis and/or fragmentation of the polymer carbon chains and the release of intermediate products or smaller subunits (multimers, dimers) , mediated by enzymes secreted by microorganisms (Welzel K et al , 2002 ; Danso et al ,2019). The molecules of short chains, e.g., oligomers, dimers, and monomers, that are small enough to pass the semi-permeable outer bacterial membranes are utilized as carbon and energy sources. The process is called depolymerization. (Yabannavar et al , 1993 ; Hamilton et al., 1995). Poly(vinyl alcohol), poly(lactic acid), polycaprolactone, and polyamides are some examples of synthetic polymers along with oligomeric structures that biodegrade. (Huang et al., 1992).

(3) Bioassimilation, whereby small hydrocarbon fragments released by biofragmentation are taken-up and metabolized by bacteria or fungi. (Welzel K et al , 2002) ie, once in the cells , either the oligomers or the degradation products of biofragmentation are funnelled through the classical degradation pathways to yield energy and/or serve as building blocks for catabolism or metabolism (Dominick Danso et al , 2017)

During bioassimilation , when LDPE is used as a sole source of carbon and energy for growth, alkane hydrolysis products and biodegradable polymers polyhydroxyalkanoates are generated (Mohanan et al , 2020).

(4) Mineralization involves the intracellular conversion of hydrolysis products to microbial biomass with the associated release of carbon dioxide and water excreted out the cell (Albertsson & Karlsson, 1990; Ammala et al., 2011).

Generally, the breakdown of large polymers to carbon dioxide (mineralization) requires several different organisms, with one breaking down the polymer into its constituent monomers, one able to use the monomers and excreting simpler waste compounds as by products and one able to use the excreted wastes (http://en.wikipedia.org/wiki/Microbial_metabolism, Microbial metabolism, 2007) But it is important to note that the biodeterioration and degradation of a polymer substrate can rarely reach 100% and the reason is that a small portion of the polymer will be incorporated into microbial biomass, humus and other natural products (Ronald M. A ,1998).

On a molecular level , microbial action first yields primary or secondary alcohols by the hydroxylation of C-C bonds. These alcohols are further oxidised to produce ketones or aldehydes, which are then converted to hydrophilic carboxylic acids (Mohanan et al , 2020). Microbial oxidation results in the formation of carboxylic acids. Carboxylated n-alkanes which are analogous to fatty acids are then catabolized by bacteria via the β-oxidation system and subsequently to the TCA cycle . The oxidation products produced by enzymes action are digested and broken down by microorganisms (Yoon et al ,2012).

Biodegradation is governed by different factors that include polymer characteristics, type of organism, and nature of pretreatment. The polymer characteristics such as its mobility, tacticity, crystallinity, molecular weight, the type of functional groups and substituents present in its structure, and plasticizers or additives added to the polymer all play an important role in its degradation .(Artham & Doble, 2007; Gu et al., 2000b).

The biodegradation of plastics proceeds actively under different soil conditions according to their properties, because the microorganisms responsible for the degradation differ from each other and they have their own optimal growth conditions in the soil (Glass and Swift, 1990).

There are many ways in which plastic degrading bacteria can be isolated and characterized.

Plastic degrading bacteria from contaminated soil, were isolated using MSM media ,enriched with polyethylene and plastic powder (at a final concentration of 0.1% (w/v)) . Observation of growth showed 12 isolates that were able to utilize plastic and polythene as the sole source of carbon (Divyalakshmi and Subhashini,2016). Similar research methodology, as done by Vignesh et al (2016) ,showed 3 different plastic degrading bacteria characterized as *Streptococcus* sp, *Pseudomonas* sp. and *Bacillus* sp.

In an effort to find Polythene and plastics-degrading microbes from the mangrove soil , as done by Kathiresan (2003) ,the pour plate method was adopted using the Zobell's agar medium. The plates were then incubated at 30°C for 2-7 days and seven bacterial species were identified.

In another study to isolate LPDE degrading *Streptomyces* sp ,different soil samples from 5 areas were collected. After serial dilution of soil samples, 100µl of each dilution and cultivates were grown on soy bean agar plates (composed of g/l, soybean 20g, manitol 20g and agar 30g, at pH 7.0, enhanced with 0.25% of Nystatin) .Each colony was isolated on its own plate by standard

streaking method incubated at 30c for 14 days. Among the 15 isolates identified ,3 isolates (SSP 2, SSP 4, SSP14) of Streptomyces were able to degraded the LDPE powdered that add to media after incubation at 25-30ºC for 2 week 3 (Soud, 2019).

Two new strains, Pseudomonas *lini* JNU01 and *Acinetobacter johnsonii* JNU01, were specifically enriched in non-carbonaceous nutrient medium, ie, BSM, with 1 g/L PS powder as only source of carbon and was kept for incubation at 28 ◦C for 7 days .Their growth after culturing in basal media increased more than 3-fold in the presence of PS. These results provide significant insights into the discovery of novel functions of *Pseudomonas* sp. and *Acinetobacter* sp., as well as their potential as PS decomposers (Kim et al., 2021).

Screening of obtained isolates was carried out on M9 media enriched with Polyethylene glycol. Post incubatory plates showed seven bacterial isolates out of which two isolates were selected as elite plastic degraders viz., PDBH1 and PDBM 2 and were characterized morphologically (Rana & Rana, 2020).

MATERIALS AND METHODS

MATERIALS

1. Sample

Plastic contaminated soil sample and sewage water was collected .

2. Chemicals and biologicals

PEG , agar powder , Peptone water, Sodium chloride , Hydrogen peroxide, Oxidase discs , glycerine , Grams Staining Kit (Himedia) , Simmons citrate agar , Urea agar medium , Urea powder , Nitrate broth , Nitrate reagent (Solution A and Solution B), Triple sugar iron agar ,Kovacs reagent , MR-VP Broth, MR indicator ,Barritt's reagent A and Barritt's reagent B , Polyethylene pieces , Sodium Chloride , LB broth , Nucleosieve ®Bacterial DNA Extraction kit , Agarose powder , TAE buffer , Ethidium bromide , Bromophenol blue .

3. Glass wares

All glass wares and plastic wares were either of Borosil or Tarsons.

4. Instruments

Microscope (ZEISS Primo Star) , BIO-RAD T100^{TM} Thermal Cycler, Centrifuge (NEUATION) , Incubator(Rotek) , shaker incubator(Rotek) , water bath (genetix) , Gel electrophoresis unit (MEDOX) , GelDoc (BIO – RAD GelDoc Go Imaging System) , Vortex(Rotek).

5. Primers

16S rRNA uni F	TGCCAGCAGCCGCGGTA
16S rRNA uni R	GACGGGCGGTGTGTACCA

METHODS

1. Sample collection

Plastic contaminated soil was collected from South Kalamassery (10°02'58.8"N, 76°19'01.2"E) and sewage water was collected from the vicinity of St Teresa's college , Ernakulam (9°58'29.6"N, 76°16'43.4"E).

2. Isolation of organism

I. Preparation of MSM agar

- 0.18g of Dipotassium hydrogen phosphate , 0.01g of Sodium chloride, 0.4g of Ammonium chloride , 0.02g Magnesium sulphate and 0.001g Ferrous Sulphate were separately weighed out and added to 100ml dH_2O. 1.5g Agar and 0.5g PEG were weighed out separately and added to prepared 100ml MSM medium taken in a conical flask.
- It was sterilized in an autoclave.
- 20ml of sterilized media was poured into 2 plates and allowed to set.

II. Plating

- 0.1g of **soil** was weighed into a microcentrifuge tube and mixed with 1ml dH_2O. 100µl of prepared sample evenly spreaded using sterilized L – rod , onto prepared agar plate .
- 100µl of well shaken **sewage water sample** was evenly spreaded using sterilized L – rod , onto prepared agar plate .
- Plates were incubated at 37.0°C for 24hrs .

3. Weight loss experiment

i) Preparation of 100ml MSM media

- 0.18g of Dipotassium hydrogen phosphate , 0.01g of Sodium chloride, 0.4g of Ammonium chloride , 0.02g Magnesium sulphate , 0.001g Ferrous Sulphate and 1.5 g Agar were separately weighed out and added to 100ml dH_2O.
- It was sterilized in an autoclave.

ii) SAMPLE PREPARATION FOR INOCULATION

- **CONTROL**: 25ml MSM media and 83.5mg of polyethylene pieces were added into a conical flask .

- **SEWAGE WATER AND SOIL :** 25ml MSM media and 83.5mg of polyethylene pieces were added into two conical flasks . Onto MSM agar plate of sewage water and Soil, 1ml 0.9% saline solution was poured , rotated and collected with pipette and transferred into conical flasks.

- All 3 conical flasks were kept in shaker incubator at 37°C ,160rpm for 3months.

Figure 1- Soil sample bacterial isolate (Initial phase)

Figure 2- Sewage water(right) and soil sample(left) bacterial isolates (Initial phase)

Media was replenished in between.
After 3 months ,

- **CONTROL** : Culture was drained with a strainer .Plastic pieces were then transferred to a petri dish to dry in the incubator at 37.0 °C for 24hrs. After overnight drying , dry weight and thus Net weight loss was calculated.

- **SEWAGE WATER** : Culture was drained with a strainer .Plastic pieces were then transferred to a petri dish to dry in the incubator at 37.0 °C for 24hrs. After overnight drying , dry weight and thus Net weight loss was calculated.

- **SOIL** : Culture was drained with a strainer .Plastic pieces were then transferred to a petri dish to dry in the incubator at 37.0 °C for 24hrs. After overnight drying , dry weight and thus Net weight loss was calculated.

4. Identification of organism

a) Gram staining

- Grams Stains- Kit (From Himedia) was used .
- Procedure :
    - A thin smear of culture was prepared in clear dry glass slide .
    - It was air dried and fixed with gentle heat .
    - The smear was flooded with Gram's Crystal Violet for 1 minute and then washed off tap water .
    - The smear was then flooded with Gram's Iodine and kept for 1 minute.
    - Gram's Decolourizer was used to decolourize until the blue dye no longer flowed from the smear and then it was washed off with tap water.

- 0.5% w/v Safranin was used for counter staining for 20 seconds and rinsed off with water.
- The slide is allowed to air dry and examined under immersion objective.

b) Motility test

- Glycerine is dabbed onto 4 corners of a cover slip.
- A loopful of culture of culture is placed onto centre of coverslip.
- The concave depression of glass slide is placed upside down over the cover slip.
- The slide is then inverted to create the 'hanging drop' of the specimen.

c) Biochemical tests

Peptone broth inoculation:

- 0.5g of Peptone and 0.25g of NaCl were weighed out separately and added to 50ml dH_2O. It was sterilized in autoclave.
- 3ml of sterilized media was added to 2 boiling tubes.
- Colonies of soil and sewage water were picked and inoculated into media in each of the boiling tubes.
- Boiling tubes were kept in shaker incubator at 37°C ,160rpm for overnight.
- Cultures were used for biochemical tests.

➢ Oxidase test

Oxidase discs were placed into petri dish. The colony to be tested was picked and smeared over oxidase disc. Deep blue colour indicates positive result.

➢ Peroxidase test

A drop of Hydrogen peroxide was placed on a clean glass slide . Colony to be tested was picked from solid media and placed in Hydrogen peroxide drop. Effervescence indicates positive result.

➢ Indole production test

- 0.3g of Peptone broth was weighed and added to 20ml dH_2O and sterilized in an autoclave.
- 3ml was poured into a test tube into which a loopful of culture was inoculated into media .
- An uninoculated tube was kept as control.
- Tubes kept in shaker incubator at 37°C ,160rpm for overnight.
- After proper incubation, 1ml of Kovacs reagent was added to boiling tube, including control.

- The tubes were shaken gently after intervals of 10-15 minutes and then allowed to stand to permit reagent to come to top.
- Development of cherry red colour in top layer of tube indicates positive result.

- Methyl red test

  - Using sterile loop technique , colony to be tested was inoculated into labelled tubes containing MR broth by means of loop inoculation.
  - An uninoculated tube was kept as control.
  - Tubes kept in in shaker incubator at 37°C ,160rpm for overnight.
  - After proper incubation , 5 drops of MR indicator was added including control
  - It was mixed well and colour was observed. Red colour indicates positive result .Yellow colour indicated negative result.

- Voges-Proskauer test

  - Using sterile loop technique , colony to be tested was inoculated into labelled tubes containing VP broth by means of loop inoculation.
  - An uninoculated tube was kept as control.
  - Tubes kept in in shaker incubator at 37°C ,160 rpm for overnight.
  - After proper incubation , 3ml of Barritt's reagent A and 1ml of Barritt's reagent B was added into tubes including control.
  - The tubes were shaken gently for 30s with caps off to expose media to 0_2
    And the reaction was allowed to complete in 15-30minutes.
  - Red colour formation indicates positive result.

- Citrate utilization test

  - Using sterile loop technique , Simmons citrate agar slant cultures were inoculated with test organism by means of stab and slant inoculation.
  - An uninoculated tube was kept as control.
  - Tubes kept in in shaker incubator at 37°C ,160rpm for overnight.
  - Blue colour medium indicates positive result.

- Nitrate reduction test

  - Using sterile loop technique, test organism was inoculated into Nitrate broth by loop inoculation.
  - An uninoculated tube was kept as control.
  - Tubes kept in in shaker incubator at 37°C ,160rpm for overnight.

- After proper incubation, equal amount of Nitrate reagent (Solution A and Solution B) were added to nitrate broth cultures including control
- Red colour development indicates positive result.

➢ Urease test

- Using sterile loop technique test organism was inoculated into urea agar medium by loop inoculation.
- An uninoculated tube was kept as control.
- Tubes kept in in shaker incubator at 37°C ,160rpm for overnight.
- Pink colour development indicates positive result.

➢ Triple sugar iron agar test

- Using sterile loop technique test organism was inoculated into TSI agar medium by stab and streak inoculation.
- An uninoculated tube was kept as control.
- Tubes kept in in shaker incubator at 37°C ,160rpm for overnight.
- Tubes were observed for colour of broth , butt and slant . Observations of gas production by means of cracks/ gas bubbles/ blackness of butt were made.

d) Molecular identification

Preparation of LB broth cultures

- 1.25g of LB broth was weighed out and added to 50ml dH_2O. It was sterilized in autoclave.
- 3ml of sterilized media was added to 2 boiling tubes .
- Colonies of soil and sewage water were picked and inoculated into media in each of the boiling tubes.
- Boiling tubes were kept in shaker incubator at 37°C ,160rpm for 24 hours.
- Cultures were used for DNA isolation procedure.

➢ DNA Extraction

Bacterial DNA was extracted using Nucleosieve®Bacterial DNA Extraction Kit

- A water bath was set to 56°C.
- 1-2ml of bacterial cell is pelleted at 8000rpm for 2min and supernatant was discarded.

- Cell pellet was resuspended in 500µl DEB to which 5µl proteinase K was used to lyse the cells. It was mixed well by brief vortexing .
- Tube was incubated at 56°C with occasional vortexing for 15min
- To this 500µl Binding Buffer wad added and mixed well by vortexing to obtain a homogenous solution.
- The suspension was transferred to DNA spin column and centrifuged at ≥10000rpm for 1min, The filtrate is discarded and the spin column is placed into same collection tube.
- 500µl wash buffer WB_2 was added to column and centrifuged at ≥10000rpm for 1min. The flow though was discarded.
- Column is dry spinned ≥10000rpm for 1min to eliminate buffer carry over.
- DNA spin column was transferred into a fresh 1.5ml/2ml microcentrifuge tube into which 30µl Buffer EBA was added to the center of the spin column to elute the DNA , Care was taken not to contact the spin column membrane with pipette tip. It was then incubated for 1min at room temperature followed by centrifugation at ≥10000rpm for 1min. The column was discarded and purified DNA was stored at -20°C.

➢ Gel electrophoresis

- 0.48g agarose powder was weighed and added to 40ml TAE buffer in a conical flask. It was heated and to this 10µl EtBr was added , It was then poured into cast tray with comb and allowed to set for 15min. The band was observed under UV transilluminator.

➢ Polymerase Chain Reaction

- PCR reaction was carried out in 25µl reaction volume, containing Taq mix (12.5 µl) Template DNA (1 µl), forward and reverse primer (2 µl), and sterilized water (9.5µl).

- The cycling conditions consisted of an initial denaturation of 2minutes at 95°C , followed by 35 cycles of denaturation at 94°C for 30 seconds , annealing at 55°C for 30 seconds and extension at 72°C for 1minute . Final extension at 72°C for 5minutes was also performed.

➢ Gel electrophoresis

- The amplification of DNA was confirmed by agarose gel electrophoresis analysis .
- The DNA was electrophoresed on 1.5% gel in 1x TAE buffer. Ethidium bromide was added for the visualization of DNA bands. A 100bp DNA ladder(Primordia Lifesciences Pvt. Ltd) was loaded to compare the size of PCR product.
- The band was observed under UV transilluminator.

➢ Purification of PCR product from agarose gel

- The DNA fragment was excised from the agarose gel using a scalpel and transferred into a 1.5/2ml microcentrifuge tube .
- 2 volumes of Solubilizing buffer was added to each volume of agarose excised from the gel.
- It was incubated at 55°C for 10-15min until the gel slice was completely dissolved with intermittent vortexing during the incubation.
- The melted agarose solution was transferred to a spin column in the collection tube and centrifuged at 10000rpm for 1min . The flow through was discarded.
- To this 500µl of Wash buffer (WB_2) was added to the column and centrifuged at 10000rpm for 1min. The flowthrough was discarded and the column was placed into the same collection tube. This step was repeated again .
- The empty spin column was centrifuged at 1000rpm for 1min to remove residual Wash buffer and the collection tube was discarded.
- The spin column was placed in a clean 1.5/2 ml microcentrifuge tube to which 25µl of Elution buffer (EBA) was added. It was incubated for 1-3min at room temperature and then centrifuged at 10000rpm for 1min. The ultrapure DNA was then further used.

➢ Gel electrophoresis

- The Purified product was electrophoresed on 1.5% gel in 1x TAE buffer. Ethidium bromide was added for the visualization of DNA bands for visualization of bands . A 100bp DNA ladder (Primordia Lifesciences Pvt. Ltd) was loaded to compare the size of Purified product.
- The band was observed under UV transilluminator.

➢ Sequencing and sequence analysis

Purified DNA sample was sent for Sanger sequencing at Genespec Kakkanad. The sequence obtained was analyzed using NUCLEOTIDE BLAST for molecular identification of the isolated bacteria.

RESULT

1. Sample collection

Soil was collected from Plastic contaminated area from South Kalamassery (10°02'58.8"N, 76°19'01.2"E) and sewage water was collected from the vicinity of St Teresas college , Ernakulam (9°58'29.6"N, 76°16'43.4"E).

Figure 3 and 4 -Polluted soil from South Kalamassery area

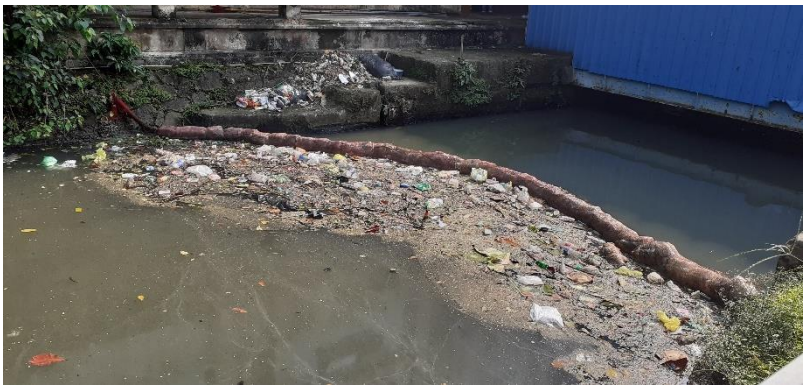

Figure 5- Polluted Sewage water area

2. Isolation of organism:

- Sewage water sample and soil sample were plated on MSM agar supplemented with PEG. Colonies were observed on both the plates (Figures 6,7).
- Morphological characters were given in the table (Table 1)

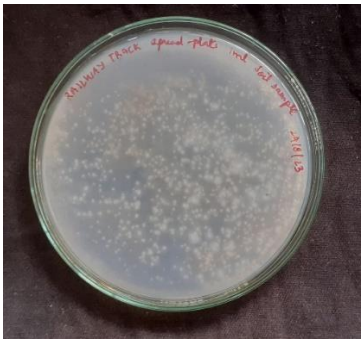

Figure 6- MSM agar plate of soil sample

Figure 7-MSM Agar plate of sewage water sample

MORPHOLOGICAL CHARACTERS

CHARACTERISTIC FEATURES	SOIL	SEWAGE WATER
SIZE	Pinpoint	Pinpoint
SHAPE	Round	Round
ELEVATION	Flat	Raised
MARGIN	Undulate with halo formation	Entire
CONSISTENCY	Mucoid	Dry

Table 1- Morphological characteristics of colonies

3. <u>Screening of bacteria: Weight loss experiment</u>

- As part of the weight loss experiment, weighed plastic pieces were incubated with the cultures isolated. After 120 days, the plastic pieces were taken from the culture flasks, dried and weighed. It was observed that there was a significant decrease in weight of the plastic after the incubation with the cultures (Table2). The plastic pieces incubated with cultures from the sewage water showed more weight loss than the ones incubated with the culture from the contaminated soil.
- The following picture shows the cultures during incubation for screening the bacteria of its degradation potential.

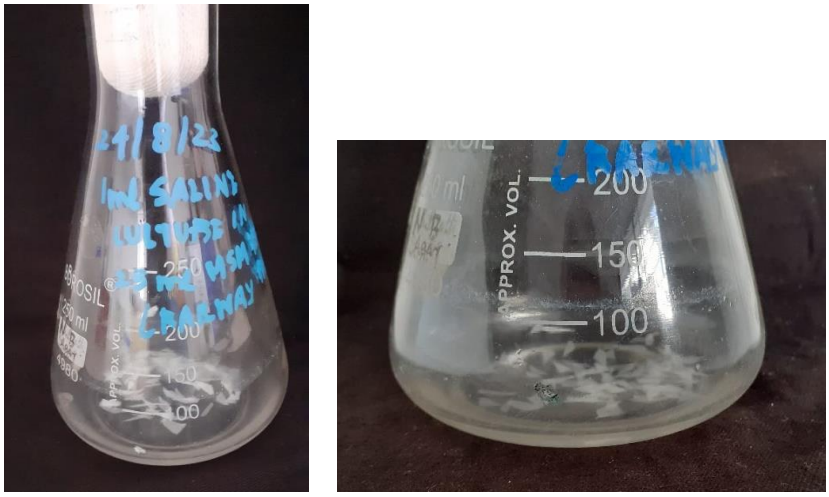

Figure 8- Plastic pieces incubated with bacterial isolates from soil sample (Intermediate phase)

Figure 9- Sewage water bacterial isolates (Intermediate phase)

Figure 10- Bacterial isolates (Final phase- after 3 months)

Figure 11- Plastic pieces (Final phase- after 3 months)

After 3 months,

	Initial weight (mg)	Final weight (mg)	Weight Loss(initial-final) (mg)
Control	83.5	83.5	0
Soil	83.5	69.2	14.3
Sewage water	83.5	65.6	17.9

Table 2- Weight differences in plastic pieces

Thus a significant weight loss of 17.13% in *Bacillus wiedmannii* and 21.44% in *Klebsiella pneumoniae* was seen after 90 days.

4. Identification of organism

e) Gram staining: After gram staining it was observed that bacteria from the soil sample showed purple rod like structures indicating that it is gram positive rod bacteria while the bacteria from sewage water sample showed pink rod like structures indicating that it is gram negative rod bacteria.

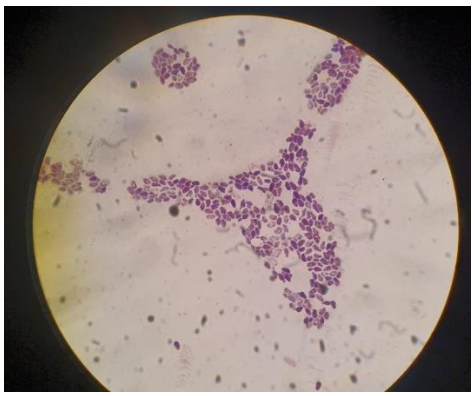

Figure 12- Soil sample

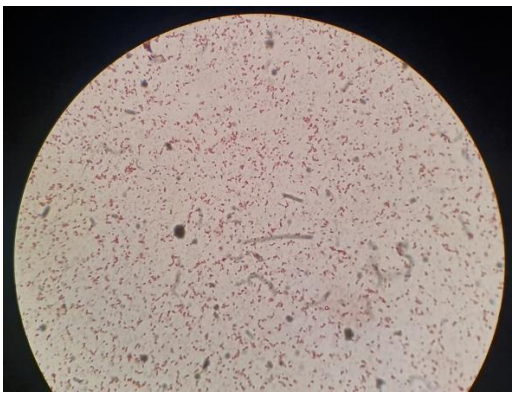

Figure 13- Sewage water sample

f) Motility test : Soil sample showed motile bacteria and sewage water sample showed non motile bacteria.

g) Biochemical tests

- Oxidase test : Soil sample and Sewage water sample showed deep blue colour indicating a positive result .

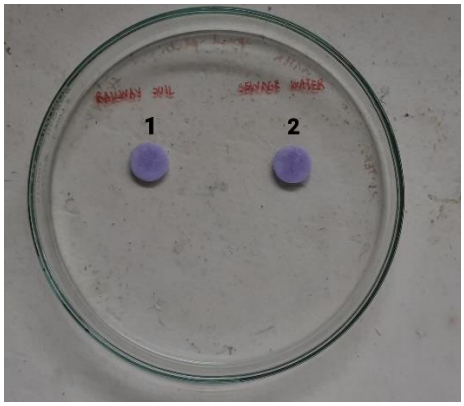

Figure 14- Oxidase test

1- Soil sample
2- Sewage water sample

- Peroxidase test : Soil sample showed slight effervescence indicating a slow positive result and Sewage water sample showed effervescence indicating a positive result.

Figure 15- Peroxidase test

- Indole production test : Soil sample showed absence of red colouration indicating a negative result and Sewage water sample showed cherry red colour in the top layer of the tube indicating a positive result.

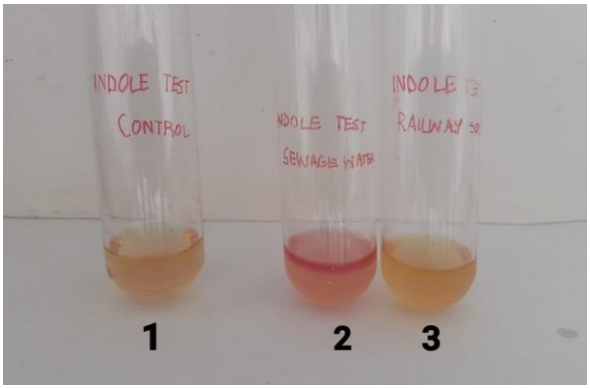

Figure 16- Indole test

Tube 1-Control
Tube 2-Sewage water sample
Tube 3-Soil sample

- Methyl red test : Soil sample showed yellow colour indicating a negative result and Sewage water sample showed yellow colour indicating a negative result.

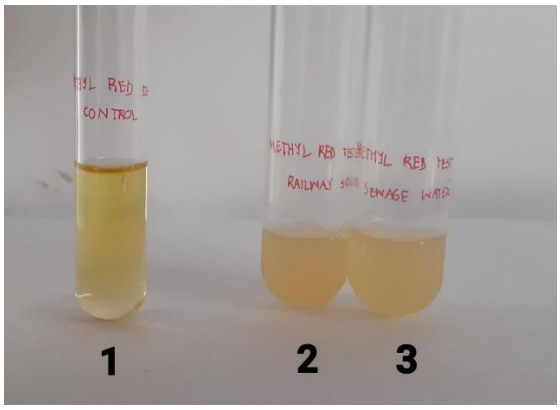

Figure 17- Methyl red test

Tube 1-Control
Tube 2- Soil sample
Tube 3- Sewage water sample

- Voges-Proskauer test: Soil sample showed no red coloration indicating a negative result and Sewage water sample showed red coloration indicating a positive result.

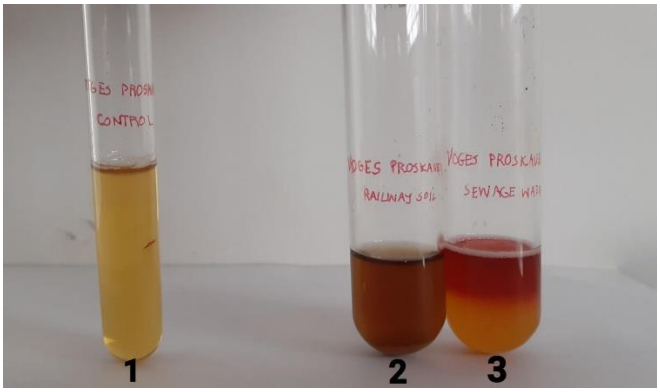

Figure 18- Voges-Proskauer test

Tube 1-Control
Tube 2- Soil sample
Tube 3- Sewage water sample

- Citrate utilization test : Soil sample showed remained green indicating a negative result and Sewage water sample showed blue colour indicating a positive result.

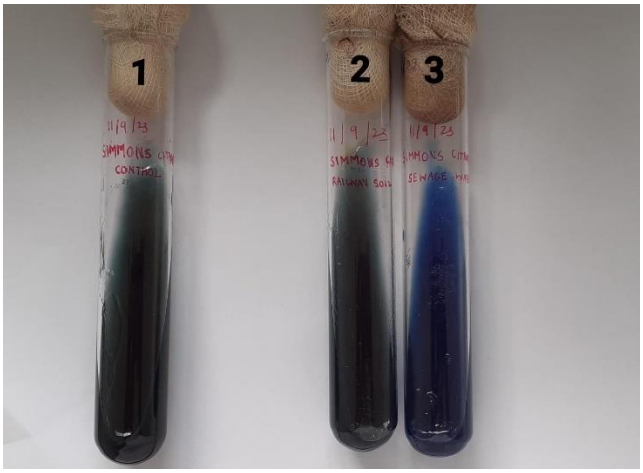

Figure 19- Citrate utilization test

Tube 1-Control
Tube 2- Soil sample
Tube 3- Sewage water sample

- Nitrate reduction test : Soil sample showed no colour change indicating a negative result and Sewage water sample showed red colour indicating a positive result.

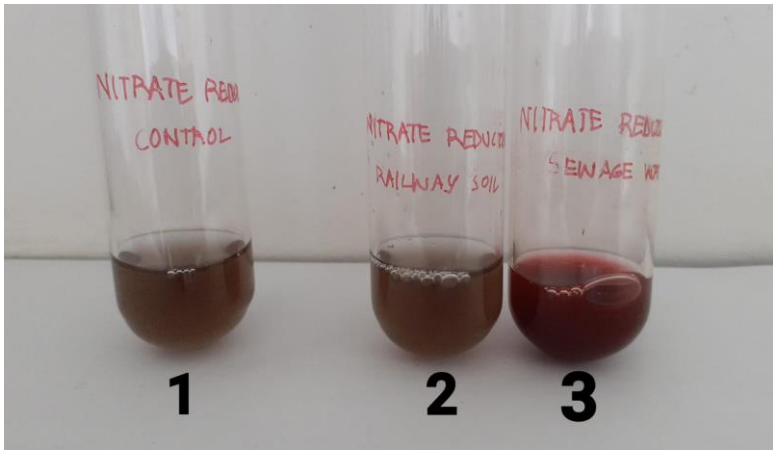

Figure 20 - Nitrate reduction test

Tube 1-Control
Tube 2- Soil sample
Tube 3- Sewage water sample

- Urease test : Soil sample and Sewage water sample showed partial pink indicating a slow positive result.

Figure 21 - Urease test

Tube 1-Control
Tube 2- Soil sample
Tube 3- Sewage water sample

- Triple sugar iron agar test : Soil sample showed alkaline slant, alkaline butt, no gas production and Sewage water sample showed acid slant, acid butt, gas production

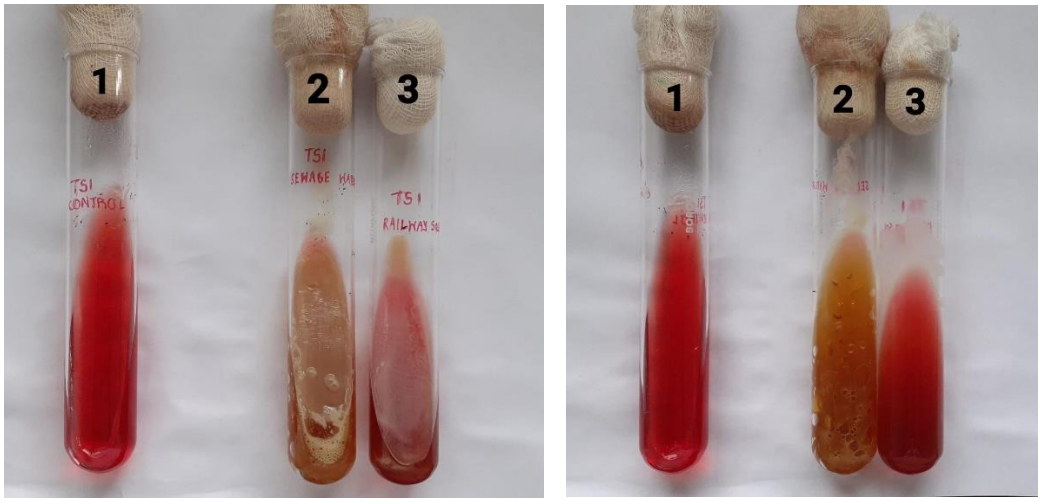

Figure 22 – TSI test

Tube 1-Control
Tube 2-Sewage water sample
Tube 3-Soil sample

S.NO	BIOCHEMICAL TEST	SOIL SAMPLE	SEWAGE WATER SAMPLE
1.	Peroxidase test	Slow positive	Positive
2.	Oxidase test	Positive	Positive
3.	Indole production test	Negative	Positive
4.	Methyl red test	Negative	negative
5.	Voges -Proskauer test	Negative	Positive
6.	Citrate utilization test	Negative	Positive
7.	Nitrate reduction test	Negative	Positive
8.	Urease test	Slow positive	Slow positive
9.	Triple sugar iron agar test	Alkaline slant, Alkaline butt, No gas production	Acid slant, Acid butt, Gas production

Table 3-Biochemical tests results

Molecular identification

- DNA Extraction

DNA was extracted from both soil and sewage water bacterial cultures using Nucleosieve® Bacterial DNA Extraction Kit. DNA Bands were observed on Agarose Gel.

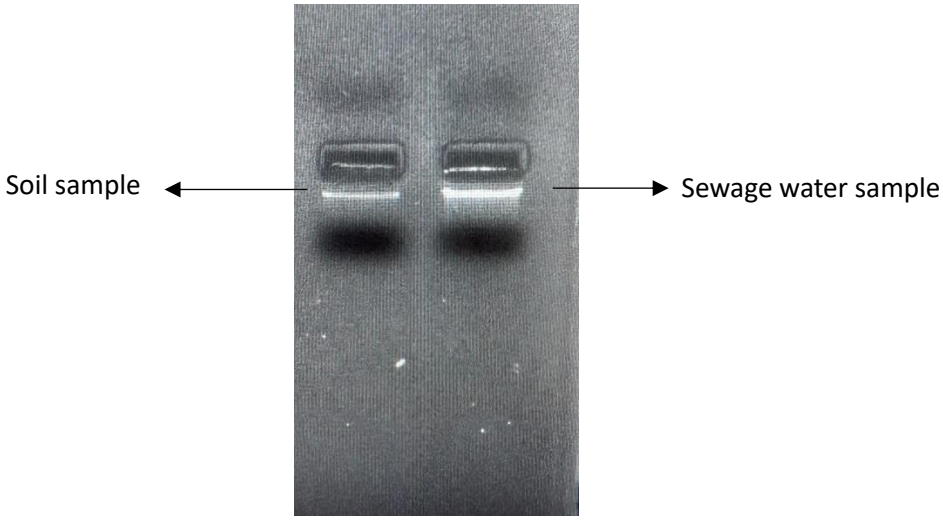

Figure 23 – Agarose gel electrophoresis of isolated DNA

➢ Polymerase Chain Reaction

PCR reaction was carried out using 16S forward and 16S reverse RNA primers.

Band was observed on Agarose Gel. The size of Amplicon was 900bp ,which was compared using 100bp ladder (Primordia Lifesciences Pvt. Ltd).

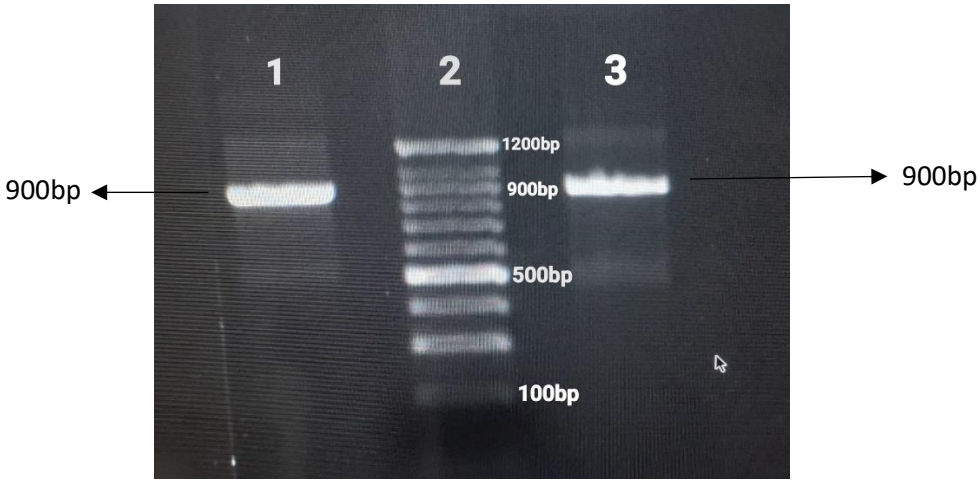

Figure 24 – Agarose gel electrophoresis of PCR amplified DNA

Lane 1 – PCR product of soil sample (900bp)

Lane 2 – 100 bp ladder (Primordia Lifesciences Pvt. Ltd)

Lane 3 – PCR product of sewage water sample (900bp)

➢ Purification of PCR product from agarose gel

PCR product was purified and extracted using Nucleosieve® Gel extraction and PCR Product Purification Kit. A clear band of approximately 900bp was observed on agarose gel ,which was compared to 100 bp ladder(Primordia Lifesciences Pvt. Ltd).

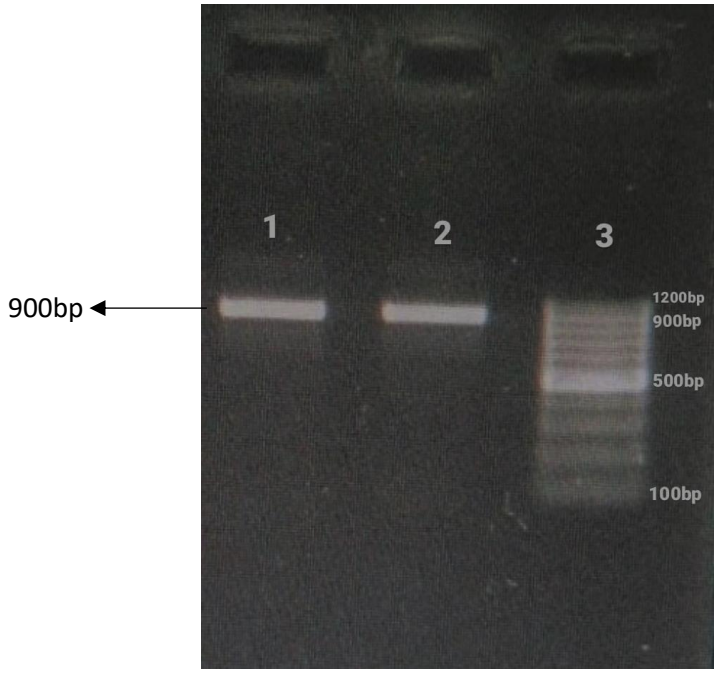

Figure 25 – Agarose gel electrophoresis of purified PCR product

Lane 1 – PCR product of soil sample (900bp)

Lane 2 – PCR product of sewage water sample (900bp)

Lane 3 – 100 bp ladder (Primordia Lifesciences Pvt. Ltd)

- Sequencing

  The 16srRNA sequence of both cultures were obtained by Sanger sequencing (Fig.26a, 26b) on blast analysis. It was observed that the sequences from contaminated soil showed 99.19% similarity was with ***Bacillus weidmannii*** while the sequences from sewage water showed 100% similarity was with ***Klebsiella pneumoniae*** (Fig.27a, 27b).

TATTGGGCGTAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAA
CCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAT
GTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGG
TCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAG
TTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCT
TACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAG
TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGG
CTACACACGTGCTACAATGGACGGTACAAGAGCTGCAAGACCGCGAGG

Figure 26a - 16s rRNA sequence of bacteria isolated from soil

GCGTAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGG
AACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAG
CGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAG
ACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGC
GTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGG
TCTTGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGG
TGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGAT
AAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACAC
ACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAA
AGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGT
AATCGTAGATCAGAATGCTACGGTGAATACGTTCCC

Figure 26b - 16s rRNA sequence of bacteria isolated from sewage water sample

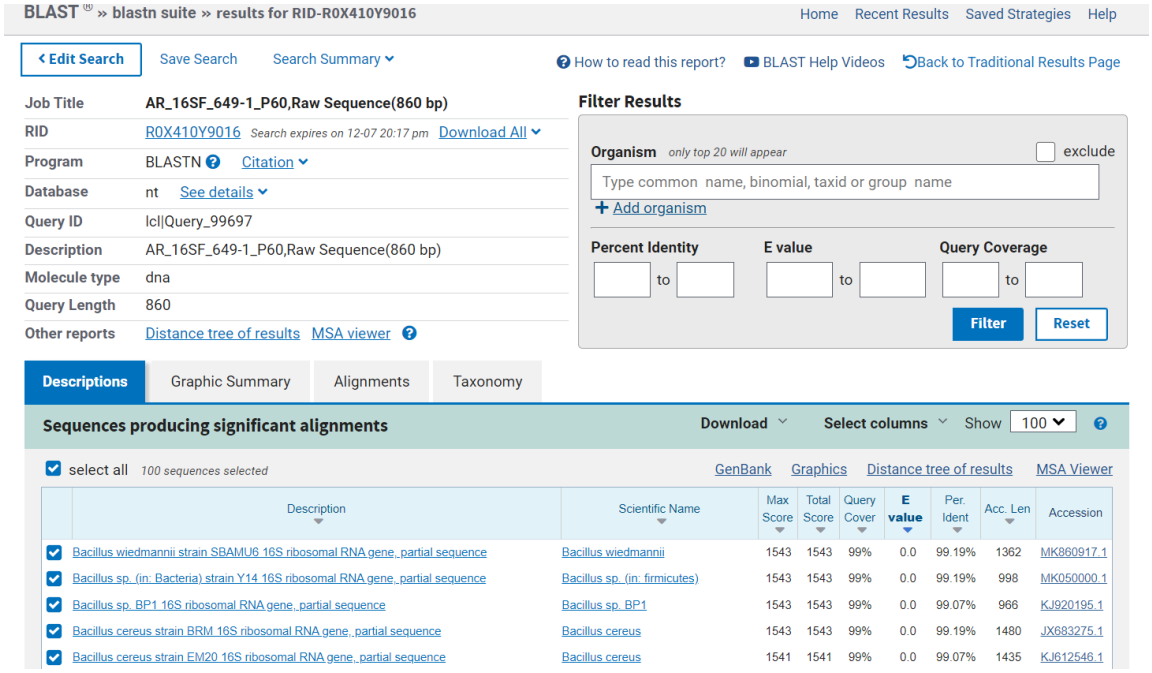

Figure 27a – BLAST analysis (Soil sample)

Sequence was checked using BLAST analysis . 99.19% similarity was observed with ***Bacillus wiedmannii.***

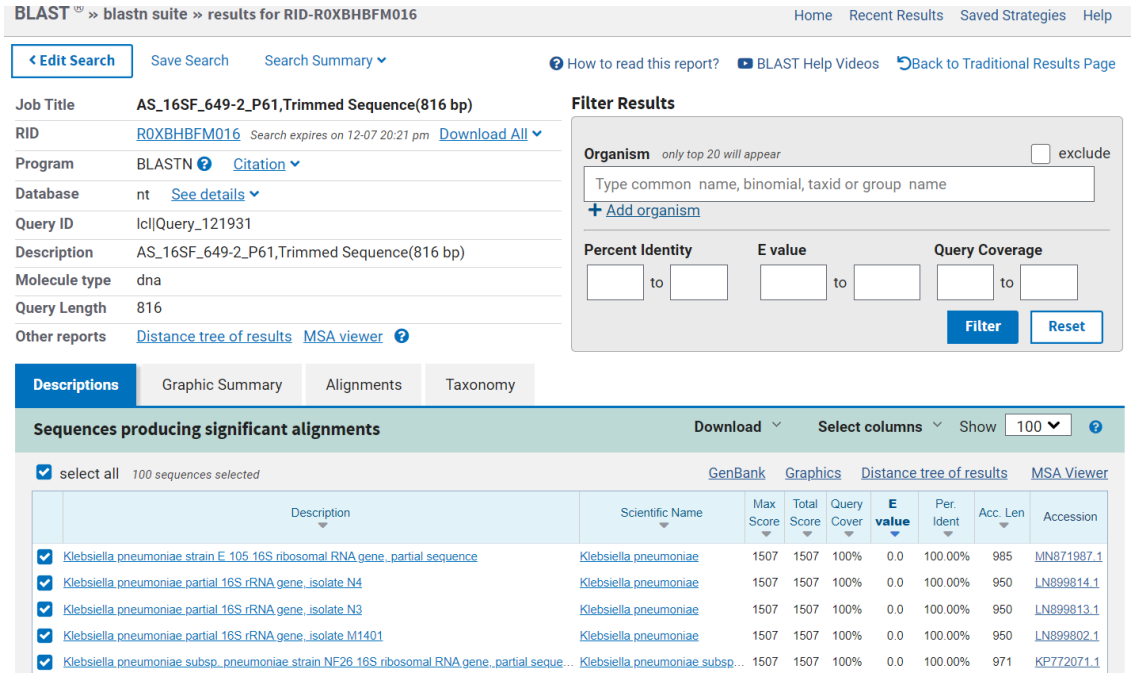

Figure 27b- BLAST analysis (Sewage water sample)

Sequence was chekced using BLAST analysis . 100% similarity was observed with ***Klebsiella pneumoniae.***

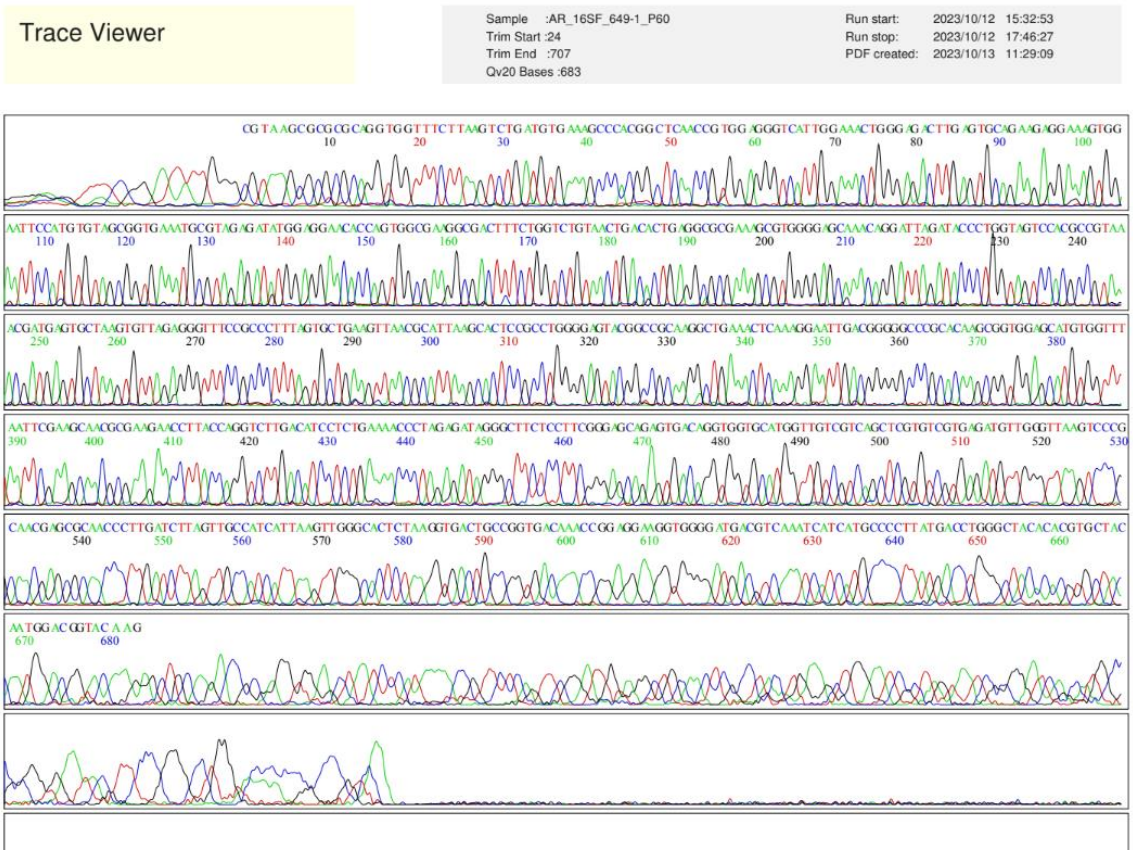

Figure 28– Chromatogram of DNA Sequence of *B.weidmanii* from Soil sample bacteria

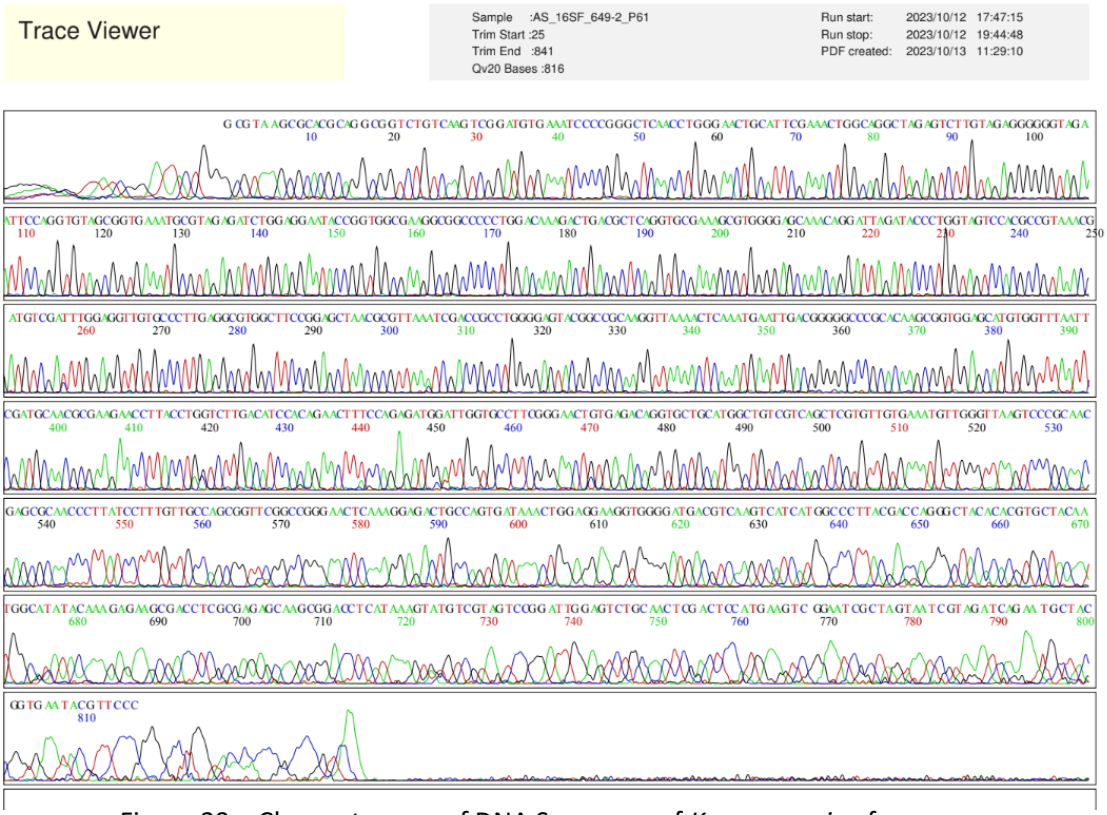

Figure 29 – Chromatogram of DNA Sequence of *K.pneumoniae* from sewage water sample

DISCUSSION

Plastic debris is now prevalent in the environment, with concerns being raised for micro-, sub-micro- and nano-sized plastic interactions with the environment and the life it holds. In recent years, microplastics (particles less than 5 mm in size) have gained much attention as they are causing damage to the ecosystem by, for example , accumulating in water bodies and entering food webs. (Huerta Lwanga et al, 2017).

Conventional physicochemical techniques like incineration and landfilling release poisonous gases and hazardous materials into the environment, posing serious ecological problems. On the other hand, many living microbes have evolved strategies to survive and degrade plastics (Zeenat et al, 2021). Biodegradation research is most environmentally significant as microbes contribute to soil fertility, decrease plastic accumulation in the environment, and reduce the cost of waste management. Further research thus should be focused on finding solutions to help control the growing global problem of plastic pollution, by speeding up the natural decomposition process. The screening of plastic-degrading microorganisms is crucial for identifying the depolymerases and other key enzymes involved in plastic degradation.

In this study, two different bacteria were isolated from plastic contaminated sewage and soil. One of these bacteria was a gram positive rod while the other one was a gram negative rod.

Various enzymes are produced by bacteria to meet their distinct metabolic demands.As a result different bacterial species have distinct enzymatic profiles by which they can be characterized. Hence the presence of catalase, gelatinase, oxidase, urease, for example, can be used to identify the species of bacteria. Biochemical tests are designed to measure the levels of bacterial enzymes ,which in turn can be interpreted to accurately identify the species of bacteria they have been produced by.As a part of this study , a series of 9 biochemical tests were conducted and the results were tabulated.

Bacteria which can make the catalase enzyme, degrade the hydrogen peroxide added and the resulting O_2 production produces effervescence, indicating a positive test. The soil sample and sewage water bacteria both showed effervescence indicating the presence of catalase activity.The oxidase test used to test ability of bacteria to synthesize cytochrome c oxidase enzyme, indicated by a positive blue colour as a result of indophenol formation. The soil sample and sewage water bacteria both showed positive results.To determine the ability of the organism to convert tryptophan into indole, Indole production test is done. This on reaction with Kovac's reagent ,produces an affirmative cherry red colour .The soil sample bacteria showed negative result and sewage water bacteria showed positive result. Methyl Red test helps analyse the fermentation pathway used by bacteria to utilize glucose and produces acid end products which give a red colour on addition of MR indicator. The soil sample sewage water bacteria showed negative result.Voges-Proskauer test helps confirm the presence of acetoin. On addition of Baritt's reagent A and B , a red colour is produced. The soil sample bacteria showed negative result and sewage water bacteria showed positive result.The citrate test screens bacteria for the ability to utilize citrate as its carbon and energy source, creating alkaline end products which turn the bromothymol blue into blue colour. The soil sample bacteria

showed negative result and sewage water bacteria showed positive result.The nitrate reduction test helps detect nitrate which in the presence of nitrate reductase and further reaction with sulfanilic acid and α-naphthylamine gives a red complex, showing a positive result. The soil sample bacteria showed negative result and sewage water bacteria showed positive result.The urease test characterize bacteria capable of hydrolysing urea to produce ammonia(alkaline) This changes phenol red indicator in media to pink colour, designating a positive result. The soil sample bacteria showed negative result and sewage water bacteria showed slow positive result.The triple sugar- iron agar is designed to differentiate among organisms based on the differences in carbohydrate fermentation patterns and hydrogen sulfide production. An acid butt,acid slant and cracks in the agar indicates the fermentation of dextrose, lactose and/or sucrose and the production of CO_2 . An alkaline butt ,alkaline slant and cracks in agar indicates absence of carbohydrate fermentation by bacteria.They break down peptones,followed by the release of ammonia (alkaline) resulting in the pH indicator, phenol red, turning from pink to red.

The results of biochemical characterisation were inconclusive. Molecular characterisation using 16S rRNA sequencing was done to confirm the identity of the isolates. BLAST analysis of the sequences confirmed that the bacteria isolated from soil was *Bacillus wiedmannii* and the one isolated from sewage was *Klebsiella pneumoniae*. The plastic degrading capability of these two microbes were analysed by a weight loss experiment in which these bacteria were inoculated in a MSM medium containing plastic material for a period of 120 days. *Bacillus wiedmannii*_decreased the weight of plastic pieces by 17.13% from the initial weight while *Klebsiella pneumonia* decreased 21.44% weight confirming their plastic degrading property.

As facultative anaerobes, the majority of strains of the Klebsiella species can live on ammonia as their only source of nitrogen and on citrate and glucose as their only sources of carbon.Previous studies have concluded that high adhesive potential is a key factor in the formation of a biofilm (Lenchenko et al ,2020) and the results in another study confirm that a significantly higher proportion of K. pneumoniae strains produced biofilm with a biofilm formation index (BFI) levels <2 (with lower values indicating increased presence of biofilm (range = 0-21) (Surgers et al ,2019).

It was previously shown that different strains of *Klebsiella pneumoniae* effectively degrades PE. SEM analysis found that there were obvious rough and uneven, with cracks and creases on the surface of the PE film which prove to be favourable for the attachment and colonization of microbes on PE, which further accelerates the degradation rate of PE. FTIR (Fourier-transform infrared spectroscopy) results found that new functional groups (carbonyl groups) were generated on the surface of the PE film, and the appearance of carbonyl groups indicated that the PE film was oxidized (Zhang et al , 2023; Awasthi et al , 2017).Analysis at a molecular level revealed that the most susceptible functional groups on the surface of PET were the vinyl, carbonyl, and carbon–oxygen groups, as obtained from functional group indices. The formation of carbonyl and vinyl groups clearly indicates that the degradation process as a result of chain scission and deterioration by photooxidation increase the formation of these groups (Saygın & Baysal, 2020).

Overall, these results indicated that K. pneumoniae can be used as potential candidate for HDPE degradation in eco-friendly and sustainable manner in the environment

One of the characteristic features of the bacterium *Klebsiella pneumoniae* is that once it enters the body, it can display high degrees of virulence and antibiotic resistance. It is resistant to the environment and action of disinfectants as well as many antibiotics, which makes it lethal. (Lenchenko et al , 2020) .These microorganisms can cause pneumonia, acute intestinal infections, urogenital infections, conjunctivitis, meningitis, and sepsis in lambs . Hence this species cannot be used commercially as such (for eg, by enhancing its natural degradation process) but can be screened for potential genes and thus enzymes that enable the bacteria to have degradation capacity. Such studies have helped identify the key gene of PE degradation , which may be a laccase- like multi-copper oxidase gene (KpMco) .It was successfully expressed in E.coli and it laccase activity confirmed by having certain degradation effect on PE film, indicating its potential for to be used for the biodegradation of PE (Zhang et al , 2023).

Bacillus wiedmannii is commonly found in various sources such as soil, wastewater, sewage sludge, and cattle manure (Danial et al ,2021a). It has also been characterised as a psychrotolerant and cytotoxic species isolated from dairy foods and dairy environments (Miller et al, 2016).Among the taxa that biodegrade LDPE, Bacillus is the most commonly recognised and numerous studies have been conducted to examine its efficiency, though limited literature exists on *Bacillus wiedmannii*'s capacity to degrade plastic. A few studies have shown the plastic degrading capability of this species. It has been established that the biodegradation by selected *B.weidmannii* isolates is limited to surface of LDPE films and is a relatively slow but continuous process. The LDPE degradation process is molecularly explained by the presence of LDPE degrading genes in these isolates, which encode the enzymes laccase and alkane hydroxylase (Maroof et al , 2020; Zerhouni et al , 2018). In the future, novel ways need to be developed to increase the expression of these enzymes and to make the most of their degradation potential.

Investigations into the metagenomics of microbial communities may prove to be a valuable, technique in the search for new strains , inherent genes and enzymes ,involved in the breakdown of plastics(Wani et al, 2023). The microbes can be used as such (by enhancing its natural degradation process) or the specific genes responsible for degradation of plastic can be cloned and expressed for degradation-specific enzymes ,to enable effective plastic breakdown.

CONCLUSION

In this study, two organisms showing plastic degradation capacity, namely *Bacillus wiedmannii* and *Klebsiella pneumoniae* , were isolated .Due to the diversity and richness in the composition of the microbial communities, there are a myriad of organisms with untapped potential , having useful properties such as plastic degradability. In addition, proteomic and molecular investigations are needed to assess the genes involved and to optimize effective enzymatic conditions for plastics degradation (Cai et al , 2023). These organisms can be used to manage many of our environmental problems and further studies needs to be done to evaluate the utility of such organisms in handling plastic pollution.

BIBLIOGRAPHY

References (hyperlinks)

- http://en.wikipedia.org/wiki/Microbial_metabolism, Microbial metabolism, 2007
- https://economictimes.indiatimes.com/industry/environment/india-generates-3-5-million-tonnes-plastic-waste-annually-environment minister/articleshow/90668558.cms?utm_source=contentofinterest&utm_medium=text&utm_campaign=cppst

References (papers)

1. Abou-Zeid, D., Müller, R., & Deckwer, W. (2001). Degradation of natural and synthetic polyesters under anaerobic conditions. *Journal of Biotechnology*, *86*(2), 113–126.
2. Adamcová, D., & Vaverková, M. (2014). Degradation of biodegradable/degradable plastics in municipal solid-waste landfill.
3. Albertsson, A., & Karlsson, S. (1990). The influence of biotic and abiotic environments on the degradation of polyethylene. *Progress in Polymer Science*, *15*(2), 177–192.
4. Amelia, T. S. M., Khalik, W. M. a. W. M., Ong, M. C., Shao, Y. T., Pan, H., & Bhubalan, K. (2021). Marine microplastics as vectors of major ocean pollutants and its hazards to the marine ecosystem and humans. *Progress in Earth and Planetary Science*, *8*(1).
5. Ammala, A., Bateman, S., Dean, K., Petinakis, E., Sangwan, P., Wong, S., Yuan, Q., Yu, L., Patrick, C. R., & Leong, K. H. (2011). An overview of degradable and biodegradable polyolefins. *Progress in Polymer Science*, *36*(8), 1015–1049.
6. Annamalai, J. (2015). Occupational health hazards related to informal recycling of E-waste in India: An overview. *the Indian Journal of Occupational & Environmental Medicine/the Indian Journal of Occupational and Environmental Medicine*, *19*(1), 61.
7. Arpia, A. A., Chen, W., Ubando, A. T., Naqvi, S. R., & Culaba, A. B. (2021). Microplastic degradation as a sustainable concurrent approach for producing biofuel and obliterating hazardous environmental effects: A state-of-the-art review. *Journal of Hazardous Materials*, *418*, 126381.
8. Artham, T., & Doble, M. (2007). Biodegradation of aliphatic and aromatic polycarbonates. *Macromolecular Bioscience*, *8*(1), 14–24.
9. Awasthi, S. K., Srivastava, P., Singh, P., Tiwary, D., & Mishra, P. K. (2017). Biodegradation of thermally treated high-density polyethylene (HDPE) by Klebsiella pneumoniae CH001. *3 Biotech*, *7*(5).
10. Azzarello, M., Van Vleet, E. S., & University of South Florida. (1987). Marine birds and plastic pollution. In *Marine Ecology Progress Series* (Vol. 37, pp. 295–303).
11. Bhagat, J., Nishimura, N., & Shimada, Y. (2021). Toxicological interactions of microplastics/nanoplastics and environmental contaminants: Current knowledge and future perspectives. *Journal of Hazardous Materials*, *405*, 123913.
12. Bombelli, P., Howe, C. J., & Bertocchini, F. (2017). Polyethylene bio-degradation by caterpillars of the wax moth Galleria mellonella. *CB/Current Biology*, *27*(8), R292–R293.
13. Cai, Z., Li, M., Zhu, Z., Wang, X., Huang, Y., Li, T. J., Gong, H., & Yan, M. (2023). Biological degradation of plastics and microplastics: A recent perspective on associated mechanisms and influencing factors. *Microorganisms*, *11*(7), 1661.
14. Cassone, B. J., Grove, H. C., Elebute, O., Villanueva, S. M. P., & LeMoine, C. M. R. (2020). Role of the intestinal microbiome in low-density polyethylene degradation by caterpillar larvae of the greater wax moth, Galleria mellonella. *Proceedings - Royal Society. Biological Sciences/Proceedings - Royal Society. Biological Sciences*, *287*(1922), 20200112.

15. Chamas, A., Moon, H., Zheng, J., Qiu, Y., Tabassum, T., Jang, J. H., Abu-Omar, M. M., Scott, S. L., & Suh, S. (2020). Degradation rates of plastics in the environment. *ACS Sustainable Chemistry & Engineering*, *8*(9), 3494–3511.
16. Chen, W., Jin, K., & Wang, N. L. (2019b). Use of Supercritical Water for the Liquefaction of Polypropylene into Oil.
17. Corcoran, P. L. (2020). Degradation of microplastics in the environment. In *Springer eBooks* (pp. 1–12).
18. Danial, A. W., Hamdy, S. M., Alrumman, S. A., El-Rab, S. M. F. G., Shoreit, A. a. M., & Hesham, A. E. (2021b). Bioplastic Production by Bacillus wiedmannii AS-02 OK576278 Using Different Agricultural Wastes. Microorganisms, 9(11), 2395.
19. Danso, D., Chow, J., & Streit, W. R. (2019). Plastics: Environmental and biotechnological perspectives on Microbial degradation. *Applied and Environmental Microbiology*, *85*(19).
20. Degli-Innocenti, F. (2022). Biodegradation rate of biodegradable plastics by molecular level [Biodegradation Study]. *Journal of Bioremediation & Biodegradation*, *6–6*, 513–513.
21. Divyalakshmi, S. (2016). Screening and Isolation of Polyethylene Degrading Bacteria from Various Soil Environments. *IOSR Journal of Environmental Science*, 10, 1-07
22. Enfrin, M., Lee, J., Gibert, Y., Basheer, F., Kong, L., & Dumée, L. F. (2020). Release of hazardous nanoplastic contaminants due to microplastics fragmentation under shear stress forces. *Journal of Hazardous Materials*, *384*, 121393.
23. Glass, J. E., & Swift, G. (1990). *Agricultural and synthetic polymers: Biodegradability and utilization*.
24. Hadad, D. J., Geresh, S., & Sivan, A. (2005). Biodegradation of polyethylene by the thermophilic bacterium Brevibacillus borstelensis. *Journal of Applied Microbiology*, *98*(5), 1093–1100.
25. Hamilton, J. D., Reinert, K. H., Hagan, J., & Lord, W. (1995). Polymers as solid waste in municipal landfills. *Journal of the Air & Waste Management Association*, *45*(4), 247–251.
26. Kathiresan K. (2003). Polythene and plastic-degrading microbes in an Indian mangrove soil. *Revista de biologia tropical*, 51(3-4), 629–633.
27. Kawai, F. (1995). Breakdown of plastics and polymers by microorganisms. In *Advances in biochemical engineering, biotechnology* (pp. 151–194).
28. Kim, H., Jo, J. H., Kim, Y., Le, T., Cho, C., Yun, C., Seok, W., & Yeom, S. (2021). Biodegradation of polystyrene by bacteria from the soil in common environments. *Journal of Hazardous Materials*, *416*, 126239.
29. Kumar, R. V., Gopal, R. K., & Elumalai, S. P. (2017). Biodegradation of polyethylene by green photosynthetic microalgae. *Journal of Bioremediation & Biodegradation*, *08*(01).
30. Kumar, S., & Das, M. P. (2014). Microbial Deterioration of Low Density Polyethylene by Aspergillus and Fusarium sp. ResearchGate.
31. Lehtiniemi, M., Hartikainen, S., Näkki, P., Engström-Öst, J., Koistinen, A., & Setälä, O. (2018). Size matters more than shape: Ingestion of primary and secondary microplastics by small predators. *Food Webs*, *17*, e00097.
32. Leja K, Lewandowicz G (2010) Polymer Biodegradation and Biodegradable. *Pol J Environ Stud 19* (2):255-66
33. Lenchenko, E., Blumenkrants, D., Sachivkina, N., Shadrova, N., & Ibragimova, A. (2020). Morphological and adhesive properties of Klebsiella pneumoniae biofilms. *Veterinary World/Veterinary World*, *13*(1), 197–200.
34. Lwanga, E. H., Mendoza-Vega, J., Ku-Quej, V., De Los Angeles, J., Del Cid, L. S., Chi, C., Escalona-Segura, G., Gertsen, H., Salánki, T., Van Der Ploeg, M., Koelmans, A. A., & Geissen, V. (2017). Field evidence for transfer of plastic debris along a terrestrial food chain. *Scientific Reports*, *7*(1).

35. Maheswaran, B., Karmegam, N., Al-Ansari, M. M., Subbaiya, R., Al-Humaid, L., Raj, J. S., & Govarthanan, M. (2022). Assessment, characterization, and quantification of microplastics from river sediments. *Chemosphere*, *298*, 134268.
36. Malhotra, M. (2021). Photocatalytic degradation of plastic. In *Materials research foundations* (pp. 163–178).
37. Maroof, L., Khan, I., Yoo, H. S., Kim, S., Park, H., Ahmad, B., & Azam, S. (2020). Identification and characterization of low density polyethylene-degrading bacteria isolated from soils of waste disposal sites. *Environmental Engineering Research/Enviromental Engineering Research*.
38. Miller, R. W., Beno, S. M., Kent, D. J., Carroll, L. M., Martin, N., Boor, K. J., & Kovač, J. (2016). Bacillus wiedmannii sp. nov., a psychrotolerant and cytotoxic Bacillus cereus group species isolated from dairy foods and dairy environments. *International Journal of Systematic and Evolutionary Microbiology*, *66*(11), 4744–4753.
39. Mitova, V., Grancharov, G., Molero, C., Borreguero, A. M., Troev, K., & Rodríguez, J. F. (2013). Chemical degradation of polymers (Polyurethanes, polycarbonate and polyamide) by esters of H-phosphonic and phosphoric acids. *Journal of Macromolecular Science. Pure and Applied Chemistry/Journal of Macromolecular Science. Part a. Pure & Applied Chemistry,* 50(7), 774–795.
40. Mohan, S., Jr., & Srivastava, T. (2011). Microbial deterioration and degradation of polymeric materials. In Sevas Educational Society, *J Biochem Tech* (Vol. 2, Issue 4, pp. 210–215).
41. Mohanan, N., Sharma, P., & Levin, D. B. (2020). Characterization of an intracellular poly(3-hydroxyalkanoate) depolymerase from the soil bacterium, Pseudomonas putida LS46. *Polymer Degradation and Stability*, *175*, 109127.
42. Mohod, A. V., & Gogate, P. R. (2011). Ultrasonic degradation of polymers: Effect of operating parameters and intensification using additives for carboxymethyl cellulose (CMC) and polyvinyl alcohol (PVA). *Ultrasonics Sonochemistry*, *18*(3), 727–734.
43. Montazer, Z., Najafi, M. B. H., & Levin, D. B. (2019). Microbial degradation of low-density polyethylene and synthesis of polyhydroxyalkanoate polymers. *Canadian Journal of Microbiology*, *65*(3), 224–234.
44. Ojha, N., Pradhan, N., Singh, S., Barla, A., Shrivastava, A., Khatua, P., Rai, V., & Bose, S. (2017). Evaluation of HDPE and LDPE degradation by fungus, implemented by statistical optimization. *Scientific Reports*, *7*(1).
45. PospíŠil, J., & Nešpůrek, S. (1997). Highlights in chemistry and physics of polymer stabilization. *Macromolecular Symposia*, *115*(1), 143–163.
46. Premraj, R., & Doble, M. (2005b). Biodegradation of polymers. ResearchGate.
47. Rana, K., & Rana, N. (2020). Isolation and Screening of Plastic Degrading Bacteria from Dumping Sites of Solid Waste. *International Journal of Current Microbiology and Applied Sciences*, *9*(7), 2611–2618.
48. Restrepo-Flórez, J., Bassi, A., & Thompson, M. R. (2014). Microbial degradation and deterioration of polyethylene – A review. *International Biodeterioration & Biodegradation*, *88*, 83–90.
49. Ritchie, H., Roser, M., 2018, 'Plastic Pollution', Our World in Data.
50. Ronald M. A (1998). *Microbial ecology*. Google Books
51. Sarmah, P., & Rout, J. (2018). Efficient biodegradation of low-density polyethylene by cyanobacteria isolated from submerged polyethylene surface in domestic sewage water. *Environmental Science and Pollution Research International*, *25*(33), 33508–33520.
52. Saygın, H., & Baysal, A. (2020). Insights into the Degradation Behavior of Submicroplastics by Klebsiella pneumoniae. *Journal of Polymers and the Environment*, *29*(3), 958–966.
53. Shimao, M. (2001). Biodegradation of plastics. *Current Opinion in Biotechnology*, *12*(3), 242–247.

54. Soud, S. A. & College of Science, AlKarkh University of Science. (n.d.). Biodegradation of Polyethylene LDPE plastic waste using Locally Isolated Streptomyces sp. *J. Pharm. Sci. & Res.*, *11*(4), 1333–1339.
55. Surgers, L., Boyd, A., Girard, P., Arlet, G., & Decré, D. (2019). Biofilm formation by ESBL-producing strains of Escherichia coli and Klebsiella pneumoniae. *International Journal of Medical Microbiology*, *309*(1), 13–18.
56. Van Cauwenberghe, L., Devriese, L., Galgani, F., Robbens, J., & Janssen, C. R. (2015). Microplastics in sediments: A review of techniques, occurrence and effects. *Marine Environmental Research*, *111*, 5–17.
57. Vignesh, R., Deepika, R. C., Manigandan, P. and Janani, R. 2016. Screening of plastic degrading microbes from various dumped soil samples. Int. Res. J. Eng. Tech., 3(4): 2493-2498.
58. Vogel, K., Wei, R., Pfaff, L., Breite, D., Al-Fathi, H., Ortmann, C., Estrela-Lopis, I., Venus, T., Schulze, A., Harms, H., Bornscheuer, U. T., & Maskow, T. (2021). Enzymatic degradation of polyethylene terephthalate nanoplastics analyzed in real time by isothermal titration calorimetry. *Science of the Total Environment*, *773*, 145111.
59. Wang, J., Zheng, L., & Li, J. (2018). A critical review on the sources and instruments of marine microplastics and prospects on the relevant management in China. *Waste Management & Research*, *36*(10), 898–911.
60. Wani, A. K., Akhtar, N., Naqash, N., Rahayu, F., Djajadi, D., Chopra, C., Singh, R., Mulla, S. I., Sher, F., & Américo-Pinheiro, J. H. P. (2023). Discovering untapped microbial communities through metagenomics for microplastic remediation: recent advances, challenges, and way forward. *Environmental Science and Pollution Research International*, 30(34), 81450–81473.
61. Yabannavar, A., & Bartha, R. (1993). Biodegradability of some food packaging materials in soil. *Soil Biology and Biochemistry/Soil Biology & Biochemistry*, *25*(11), 1469–1475.
62. Yamada-Onodera, K., Mukumoto, H., Katsuyaya, Y., Saiganji, A., & Tani, Y. (2001). Degradation of polyethylene by a fungus, Penicillium simplicissimum YK. *Polymer Degradation and Stability*, *72*(2), 323–327.
63. Yang, J., Yang, Y., Wu, W., Zhao, J., & Jiang, L. (2014). Evidence of Polyethylene Biodegradation by Bacterial Strains from the Guts of Plastic-Eating Waxworms. *Environmental Science & Technology*, *48*(23), 13776–13784.
64. Yoon MG, Jeon HJ, Kim MN (2012) Biodegradation of Polyethylene by a Soil Bacterium and AlkB Cloned Recombinant Cell. *J Bioremed Biodegrad* 3:145.
65. Zeenat, Elahi, A., Bukhari, D. A., Shamim, S., & Rehman, A. (2021). Plastics degradation by microbes: A sustainable approach. *Journal of King Saud University. Science/Mağallaï Ǧāmi'aï Al-malik Sa'ūd. al-'Ulūm*, *33*(6), 101538.
66. Zerhouni, K., Abbouni, B., Kanoun, K., Daouadji, K. L., Tifrit, A., Benahmed, M., & Chaouche, T. M. (2019). Isolation and identification of low density polythene-degrading bacteria from soil of North West of Algeria. *South Asian Journal of Experimental Biology*, *8*(3), 76–82.
67. Zhang, X., Feng, X., Yuan, L., Gou, H., Zhang, Y., & Yang, L. (2023). Degradation of polyethylene by Klebsiella pneumoniae Mk-1 isolated from soil. *Ecotoxicology and Environmental Safety*, *258*, 114965.