

BIODEGRADATION OF WASTE BY BIOREACTOR (ROTATIONAL SLURRY BIOREACTOR)

Submitted in partial fulfillment of the requirements for the award of a Bachelor of
Technology degree in Biotechnology

By

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**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**



SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade “A” by NAAC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of Gowshik T (39230017) who carried out the project entitled "BIODEGRADATION OF WASTE BY BIOREACTOR (ROTATIONAL SLURRY BIOREACTOR)" under my supervision from October 2021 to April 2022.


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DECLARATION

I, GOWSHIK T (39230017) hereby declare that the project report entitled **BIODEGRADATION OF WASTE BY BIOREACTOR (ROTATIONAL SLURRY BIOREACTOR)** done by me under the guidance of Dr.Sudha, Department of Bio-Technology, Sathyabama Institute of Science and Technology is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Bio-Technology.

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ABSTRACT

The study described the development of an effective bioaugmentation system for removing A-fuel oil from oil-defiled soil under low-temperature conditions using a rotational slurry bioreactor. The bioreactor was inoculated with mixtures of two *Rhodococcus* oil-degraders, strain A and C, and A-fuel oil was added to a final concentration of 2500 and 5000 mg/kg-slurry. The decomposition tests were conducted at 26 °C, which is the annual average temperature of India. The results of the study showed that after six days of treatment, more than 90% of the oil was removed in the inoculated samples, which was more than three times faster than a former degradation experiment without rotation. Likewise, a semi-continuous treatment was performed, which involved removing 85% of the treated slurry and then adding the same amount of contaminated slurry into the system without additional degraders. After six days of repeated treatment, 88% of A-fuel oil was successfully degraded, and this approach could reduce the cost of preparing the degraders. Interestingly, the study also found that the rotational slurry conditions could efficiently promote biodegradation by indigenous degraders, as the non-inoculated samples showed oil removal during semi-continuous treatment. This suggests that the rotational slurry bioreactor could accelerate the removal of oil impurities without adding further degraders and provides an efficient and cost-effective method for removing A-fuel oil using a semi-continuous system, which can be used in practical applications in areas with a cooler climate. Overall, the study highlights the potential of the rotational slurry bioreactor as an effective approach for the bioaugmentation of oil-impurities soil under low-temperature conditions. The results of this study could have significant implications for the development of cost-effective and sustainable strategies for the remediation of defiled spots in colder climates.

KEYWORDS:-Bioaugmentation, *Rhodococcus*, Rotational Slurry Bioreactor

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CHAPTER-1

INTRODUCTION

Soil contamination caused by oil spills is indeed a significant global problem, and bioaugmentation with oil-degrading bacteria has been proven effective in treating contaminated soil (Bejarano A.C. *et al.* 2016). However, as you mentioned, the success of bioaugmentation depends on several environmental conditions, including soil composition, water content, temperature, and indigenous microbes. (Ivshina I.B *et al.* 2015). Temperature is a crucial factor that affects the microbial process in bioaugmentation. In cooler or cold environments, microbial activity can slow down, making it difficult to achieve efficient bioremediation (Limbergen H.V. *et al.* 1998). Therefore, the isolation and use of microbial degraders adapted to cold environments have been performed in several studies. Two *Rhodococcus* strains, A and C, could effectively degrade A-fuel oil (500 mg/kg-soil) at lower temperatures (15 and 10 °C) as well as at 30 °C. Based on these results, according to guidelines for environmental risk assessment on bioremediation (bioaugmentation) to prefectural governments. This approval means that these strains could be used for bioaugmentation in cool areas, making the process more effective in cleaning up contaminated soil (Ławniczak Ł *et al.* 2014). Overall, the use of cold-adapted microbial degraders for bioaugmentation is a promising approach to treating soil contaminated by oil spills, and your findings provide valuable insights into the effectiveness of this method in different temperature conditions. (Afzal M *et al.* 2013) The water content of the soil is an important factor for bioaugmentation as it can affect the availability and mobility of contaminants and also influence the growth and activity of microbial degraders. In the case of slurry treatment, the addition of water can help to increase the contact frequency between bacterial cells and their substrate, which in turn can enhance the degradation of contaminants (Piotrowska-Seget Z *et al.* 2010). This is because the water can act as a carrier for nutrients and contaminants, promoting their diffusion and allowing for a greater distribution of microbes throughout the soil matrix. Moreover, water can also create an optimal environment for microbial growth, providing the necessary conditions for the development of a

diverse and active microbial community(Kostka J.E *et al.* 2019). The purpose of this study was to develop a lab-scale rotational slurry bioreactor that could be used to effectively remove contaminants from contaminated soils through bioaugmentation, even at low temperatures. The researchers used oil-degrading microorganisms that are known to be effective even in low-temperature conditions and compared the efficiency of removing A-fuel oil under different conditions using their lab-scale bioreactor. Rotational bioreactors are effective in accelerating the degradation of organic wastes by mixing the indigenous degraders and their substrates. However, they have traditionally been used at temperatures of 25-30°C, and low temperatures can negatively affect biodegradation rates. Therefore, the researchers aimed to test whether the slurry treatments and rotation could effectively work for bioaugmentation at low temperatures (Ribicic.D *et al.* 2018). Overall, the goal of this study was to develop a lab-scale bioreactor that mimics a concrete mixing system, which could be scaled up and used in the field to remediate contaminated soils. By using oil-degrading microorganisms that are effective even in low-temperature conditions, the researchers hoped to demonstrate the effectiveness of their lab-scale bioreactor for removing A-fuel oil under different conditions.

CHAPTER-2

LITERATURE REVIEW

2.1 Sources of Garbage

2.1.1 Municipal waste

All of the solid waste, or everyday goods that we use and then discard, such as product packaging, furniture, grass clippings, clothing, bottles, food scraps, newspapers, electrical equipment, paints, domestic debris, footwear, and batteries are collectively referred to as trash or garbage. This is produced in our residences, companies, stores, schools, and hospitals.

Disposable diapers frequently include germs, which are present in municipal garbage. Enteroviruses have been reported to be present in up to 10% of the fecal solid disposable diapers that are disposed of in landfills. Sewage bio-solids, which are disposed of together with other waste, are another major or primary source of pathogens. Food waste and household pet waste, such as a cat or dog litter, may potentially contain pathogens. According to research, residential municipal solid waste typically contains 7.7×10^8 coliform bacteria and 4.7×10^8 fecal coliform bacteria per gram. Additionally, home solid waste has been shown to contain Salmonella. Such bacteria or pathogens may be found in the leachate beneath landfills in unlined landfills (Kumar Sunil, Dhar Hiya, et al.,).

2.1.2 Medical/Clinical waste:

These include trash produced at healthcare facilities like hospitals and nursing homes, such as waste from immunizations, tests, diagnoses, and treatments, as well as culture dishes, gloves, bandages, glassware, needles, scalpels, swabs, and tissues. It has infectious components. Additionally known as biomedical waste (BMW). There are four major types of medical waste:

- i. **General garbage** is made up of normal office and domestic waste.
- ii. **Infectious waste** is any waste that can infect people, including bodily fluids, blood, and human tissues.
- iii. **Hazardous waste** is any waste that is toxic but not contagious, such as old chemicals or surgical instruments. They have some possible connections to the human race and the environment (Maczulak & Anne Elizabeth, et al.,).
- iv. **Radioactive waste**: trash produced by radioactive therapies including cancer therapy and laser surgery.

2.1.3 Agricultural waste

The waste products created exclusively by agricultural practices, such as pesticides, agricultural runoff, harvest trash, manures, and other waste from avian farms.

2.1.4 Industrial waste

Materials created during manufacturing operations like those of factories, mills, mining, etc., as well as trash from industrial activities including mining, manufacturing, coal combustion, oil and gas production, etc. It also contains garbage like paints, sandpaper, industrial debris, paper goods, metals, radioactive waste, and chemical solvents. These industrial wastes include harmful contaminants that must be carefully handled before being released into the environment (Why Can't I Put My Leftover Gyproc/Drywall in the Garbage, et al.,).

2.1.5 Electronic waste

Computers, VCRs, DVD players, TVs, stereos, copiers, fax machines, and other useless electronic gadgets are examples of e-waste or electronic garbage and are often dumped for recycling, reuse, or rehabilitation. Electronic trash has emerged as a significant issue for humanity. Mercury, lead, cadmium, and other harmful materials leak into the soil and water when these wastes are thrown into landfills, affecting both humans and animals.

2.1.6 Waste from constructions

A lot of garbage is produced whenever any form of construction, demolition, or renovation work is done on buildings, highways, flyovers, bridges, subways, etc. This comprises plasters, concrete, metal, wood, and plastics. It contains between 10 and 20 percent of municipal solid trash.

2.1.7 Commercial source

A significant quantity of garbage is produced daily by commercial organizations as a result of the largest improvements and modernization in cities, industries, and transportation. These might include things like food goods, single-use medical supplies, fabrics, and a lot more.

2.2 Biodegradable and Non-Biodegradable Waste:

We can all agree that technology has improved living quality and sparked innovation across a range of industries. This has had an unpredictable effect on us, the ecosystem, and other life forms on the planet. Regarding the highly practical plastic water bottles, their disintegration is a test for nature because it might take thousands of years for them to break down. There is a significant quantity of trash and rubbish today that may be either degraded or not. In addition, all rubbish is hazardous to human life and a loss to the ecosystem, whether it is biodegradable or not. These are classified below:

2.2.1 Biodegradable waste

Biodegradable materials and substances are those that are quickly broken down by bacteria and other natural creatures without adding to pollution. It's simple to locate biodegradable garbage in Municipal solid waste, such as kitchen garbage, green waste, food waste, and paper waste, which are often broken down by abiotic factors including temperature, UV, oxygen, and other rays of light. They are converted into carbon dioxide, methane, water, and other fundamental natural mixtures by many different processes, including nourishing the soil, anaerobic processing, aerobic digestion, or comparable methods.

Additionally, it contains a few inorganic substances that can be broken down by bacteria, such as gypsum and its byproducts. The environment is only impacted by biodegradable trash when it is present in excess. Dumping grounds can act as a breeding ground for specific vectors or carriers like mosquitoes and rats which can ultimately cause various harmful diseases. They can generate a large quantity of microbial population around the waste which can cause many communicable diseases to humans, animals, etc., it can generate bad odor, release certain gas during the process of burning, and it can generate bad odor. By using anaerobic digestion or burning, biodegradable trash may also be used as a source of heat energy, electricity, and fills (Rachel Steffan, et al.).

2.2.2 Non-biodegradable waste

Non-biodegradable waste is defined as garbage or materials that cannot be broken down by biological processes, natural organisms, or biological degradation and contribute to pollution. It is impossible to manage this trash. It stays on the planet for a very long time without decomposing. They are therefore riskier than biodegradable garbage. Extreme application of these wastes, such as chemical fertilizers and pesticides, alters the soil's pH balance, which has an impact on plant development and soil fertility. These dangerous chemicals might leak from the fields into the neighboring water bodies, disrupting aquatic life and encouraging algal blooms that lead to eutrophication. The majority of nonbiodegradable garbage can enter biological cycles or food chains, and since people occupy the largest. The majority of the dangerous chemical concentrations are consequently found in the tropic levels during any of these cycles. people's bodies. Plastic is a typical illustration of this because it is present everywhere. To distribute plastics Better quality polymers are utilized because they are more durable and produce better results. Additional instances include metals, cans, industrial trash, chemicals from agricultural fields, etc (B. Z. G. Albertson A.C., et al.).

2.3 Plastics :

This wonder substance, which was created in the late 19th century, has made modern living simple and sustainable. Originally, "plastic" meant something that was "pliable and readily molded." It comes from a group of Materials known as polymers, which denotes

"of several pieces," which are formed of long chains of insoluble cellulose molecules, which are the primary component of plant cell walls and vegetable fibers like cotton. Polyethylene, PVC, nylon, and other materials are other types of polymers. The chemical alteration of natural materials like rubber, nitrocellulose, collagen, gallate, etc. was the next stage in the development of plastics. Finally, over a century ago, a wide variety of wholly synthetic materials that are now known as modern plastics began to emerge.

2.3.1 Categories of Plastics :

Numerous items, including water bottles, combs, beverage containers, bags, etc., require plastic as a key component. The types of plastics include :

- i. **Polyethylene Terephthalate (PETE/PET):** J. Rex Whinfield and James T. Dickson first introduced this in 1940. One of the most widely used plastics worldwide. Knowingly, it took about 30 years for it to be utilized for transparent bottles such as beverage cans.
- ii. **High-Density Polyethylene (HDPE):** created in 1953 by Karl Ziegler and Erhard Holzkamp. To manufacture high-density polyethylene, they employed a catalyst and low pressure. Today, a vast range of items are made using it. It was initially utilized for drain pipes, Mulches, etc. Under exposure to intense heat or cold, it does not break. Every year, around 12% of all HDPE is recycled.
- iii. **Polyvinyl Chloride (PVC):** One of the earliest artificial materials still in use in industrial manufacture. PVC was unintentionally discovered twice: once by a German chemist named Eugen Baumann in 1872 and secondly by a French physicist named Henri Victor Renault in 1838. They both discovered PVC inside vinyl chloride flasks that had been left outside in the sun. Less than 1% of PVC is recycled annually, making it one of the least recycled plastics overall. PVC, sometimes known as "poison plastic," is made up of several chemicals that are bad for both the environment and human health.
- iv. **Low-Density Polyethylene (LDPE):** As the first polyethylene to be made, LDPE is regarded as the material's ancestor. Due to its lower mass than high-density polyethylene, it is regarded as a distinct material for recycling.

Fortunately, a lot of more recent recycling initiatives are developing to handle these items.

- v. **Polypropylene (PP):** Polypropylene was discovered in 1951 by Philips Petroleum Company's Paul Hogan and Robert L. Banks. They were originally attempting to turn propylene into gasoline at the time, but instead, they found a novel catalytic method for producing plastic. Each year, just a small amount of this plastic gets recycled. Only 3% of polypropylene goods are recycled in the USA.
- vi. **Polystyrene or Styrofoam (PS):** German druggist Eduard Simon inadvertently discovered polystyrene in 1839 while manufacturing medicine. Without realizing it, he found it in natural resin. Later, Hermann Staudinger, a German chemist, improved on its application. Because polystyrene is light and simple to produce, it breaks easily and poses an increasing risk to the environment. About 35% of US landfill cover is made up of it.
- vii. **Miscellaneous Plastics:** The remaining plastics are polycarbonate, polylactide, acrylic, acrylonitrile, butadiene, styrene, fiberglass, and nylon. Additionally, there are a lot of variations among the plastics included in recycling programs. Due to their difficulty in decomposing, unless subjected to high temperatures, these polymers are almost hard to recycle.

2.4 Biodegradable plastics (BDP)

Because they are plastic, biodegradable plastics (BDPs) are not an alternative to plastic in the traditional sense. The sole positive is that they decompose naturally. Starch, milk, cellulose, and other substances that may decompose are categorized as biodegradable (or biodegradable), which decomposes into minute particles over many months, or bio-compostable, which can be composted with other organic materials. Both must be converted into inorganic mass, such as H₂O, CO₂, and CH₄, by microbes' enzymatic activity.

2.4.1 Types of Biodegradable Plastics

i. The **Bio-based** kind is made from organisms such as plants, animals, or bacteria that create proteins like gelatin, gluten, or casein as well as polysaccharides like starch, lignin, or cellulose. Also included are natural rubber, polyhydroxyalkanoates, poly-3-hydroxybutyrates, polylactic acid (PLA), which is derived from a bio-based monomer, lactic acid, etc.

ii. Aliphatic polyesters like polyglycolic acid and polycaprolactone (PCL) as well as aromatic copolyesters like polybutylene succinate terephthalate are examples of **petrochemical-based BDPs** that can biodegrade. An aliphatic polyester manufactured from fossil fuels called polycaprolactone can completely degrade after six weeks of production.

It is already employed in stitching for accuracy, and the primary barrier preventing its widespread use is the expensive cost of production. But if you mix it with cornstarch, these issues can be reduced.

Today, a lot of BDPs combine both categories for better performance or cost efficiency.

2.5 Plastic's Microbial Degradation

Modern biotechnological technologies are being used to address the drawbacks of traditional burning and landfilling procedures. These strategies include the creation of synthetic microbial consortiums, genetic engineering, and systems biology. Utilizing genetic engineering, it is possible to modify the genetic makeup of microorganisms to improve their capacity to break down plastic pollution.

Systems biology gives relevant data on the production of metabolites, RNA, and DNA as well as their expression. To create a synthetic microbial community, the natural microbial consortium's genetic potential is changed.

There are several stages to the microbial decomposition of plastics, and each stage maybe recognized by a specific terminology(Lugauskas A, et al., 2004).

2.5.1 Bio-deterioration:

This procedure affects all of the physical, chemical, and mechanical characteristics of plastic through superficial or surface degradation. The abiotic factors mostly aid in weakening the plastic's polymeric structure. These abiotic factors might occasionally function as a symbiotic element or as a catalyst for the biodegradation process. The process of bio-deterioration is started when a microbial biofilm forms. This biofilm develops both on the plastic's surface and inside of it. The circumstances of the surrounding environment and the structural characteristics of the plastic both affect how the biofilm forms. Hydrophobic plastics include PS and PE. Thus, the development of a stable biofilm will only occur if the bacterial surface is hydrophobic. We may use the very active biofilm of *Rhodococcus ruber* C208 that was produced on polyethylene as an example here. It stuck to the HDPE even after 60 days of incubation without any external carbon input (Brakstad O.G *et al.* 2006). The microbial biofilm is observed to produce a significant amount of chemical and physical deterioration:

- i. **Physical deterioration:** Extracellular polymeric substances highlight the biofilm's cohesiveness and adherence to the plastic's surface (EPS). In this way, these chemicals are connected to the microbial biofilm formation process. They then enter the pores, which allows bacteria to flourish there. As a result, fractures arise from an increase in pore size. The physical properties of the plastic are being weakened by these cracks (Bonhomme S *et al.*, n.d.).
- ii. **Chemical deterioration:** The biofilm that grows on plastic may harbor a variety of bacteria species and release acidic chemicals. For instance, nitric acid, nitrous acid, or sulfuric acid are produced by chemolithotrophic bacteria, such as *Nitrobacter* spp., *Nitrosomonas* spp., or *Thiobacillus* spp. Numerous organic acids, including glyoxalic, citric, fumaric, gluconic, glutaric, oxalic, and oxaloacetic acids, are also released by certain chemoorganotrophic groups. The pH is changed when all of these acids are released inside the pores, and this is followed by a slow deterioration that changes the microstructure of the plastic matrix (Zettler E *et al.*,).

2.5.2 Bio-fragmentation:

Plastic polymers can break down into their constituent oligo- and monomers for a variety of reasons, including mechanical, thermal, UV radiation, chemical, and/or biological. Here, the biological component is the key concern. Because they are polymers, plastics have large molecular weights. They can't migrate over the cell wall. Exoenzymes, particularly extracellular enzymes released by microorganisms, target the plastic polymer's edge and catalyze the chemical processes there. Even though these enzymes are capable of performing many different chemical reactions, disintegration processes by these enzymes require an uneven electric charge. The primary disadvantage of biofragmentation is the lengthy chain of carbons and hydrogens that makes up polymeric polymers, which have well-balanced charges that make them stable. For the bacteria to affect them, the local electric charge must be upset. For this reason, bacteria have specific enzymes called oxygenases that add oxygen to a long carbon chain, creating unbalanced charges and enabling the degradation of the plastic by the bacteria. One or two oxygen atoms are present in mono-oxygenases and di-oxygenases, respectively, to generate alcohol or peroxy groups. These populations are easier to control for biodegradation. After the production of carboxylic groups, esterases and lipases catalyze the transformations other than these, or endopeptidases for amide groups (Lugauskas A, et al., 2013).

2.5.3 Assimilation and mineralisation:

The production of monomers does not guarantee assimilation by bacteria. They must employ this method to cross the cytoplasmic membrane and/or cell wall. Some carriers. Some of the monomers could float unrestrictedly about the microbial cells without being taken up. The oxidation of the plastic monomers via catabolic pathways within the cells results in the production of energy, cell structure, and fresh biomass. Depending on whether the microorganisms can thrive in anaerobic or aerobic settings, there are three key catabolic processes for the creation of the energy to maintain cellular activity, structure, and reproduction: fermentation, anaerobic respiration, and aerobic respiration (Dussud, et al.,) Even after the atoms have been absorbed into the microbial cells, there may still be some partial breakdown of the monomers. A large number of

secondary metabolites are created as a result of this fusion or integration. The metabolites are transported outside of bacteria that cannot metabolize or do not need to store these metabolites. The secondary metabolites that were previously expelled may subsequently be used by any other cells that are capable of performing further degradation. The entire degeneration of primary and secondary metabolites is indicated by mineralization, and this further leads to the excretion of totally oxidized metabolites (CO_2 , N_2 , CH_4 , and H_2O)(Urbanek A, n.d.).

2.6 Difficulties of Microbial Degradation:

Numerous variables, including molecular weight, surface area, functional groups, melting point, temperature, hydrophilic and hydrophobic properties, chemical structure, crystallinity, etc., affect how plastic pollutants degrade. By considerably lowering the solubility and the degradation rate, the molecular weight of the polymers tends to have an impact on the degradation process. Additionally, because the plastic polymer is often less soluble, it is typically less susceptible to microbial assault because bacteria digest them via the cell membrane. Additionally, it has been shown that when the crystalline nature of plastic polymers increases, it becomes less susceptible to microbial enzymatic action, which results in a reduction in the rate of plastic deterioration. In addition, the hydrophobic properties of plastic polymers limit microbial activity by preventing water absorption that can be recycled via the creation of biofilms. It follows that most polymers degrade depending on the right variables. Today, the manufacture of enzymes or the screening of bacteria both aid in the decomposition of polymers(Urbanek A, et al.,).

2.6.1 METHANE (CH_4):

Researchers have begun to pay attention to the methane emissions produced by the degradation of organic waste as a significant contribution to global warming since this greenhouse gas has a global warming potential 25 times larger than carbon dioxide over a time horizon of 100 years. Lack of oxygen (strictly anaerobic microorganisms), a redox potential below -200 mV, neutral pH, and the availability of nutrients and substrates rich in organic matter are the ideal growth conditions for methanogenic bacteria (Appuhamy, France, and Kebreab, 2016).

These circumstances are temporarily present in the early stages of degradation when the pile's oxygen content is reduced by the microbial activity that is sparked by the abundance of nutrients and readily accessible organic molecules. As a result, the majority of CH₄ emissions have been observed in the early stages of the process, at the start of the thermophilic phase (Miguel A. Sánchez-Monedero *et al.*, 2010). As a result of the process's early high temperatures, which make oxygen less soluble, anaerobic areas of the pile can more easily form. A high ammonia content, which may impede methanogen activity at pH levels higher than 9, or the presence of electron acceptors such as sulfates, which hinder their activity by competing with sulfate-reducing bacteria. According to (Sánchez-Monedero *et al.*, 2001), the degradation of olive mill waste was hampered by the high ammonia levels produced by the hydrolysis of urea, which was utilized as a nitrogen source. In addition to the biological activity of the pile, additional variables impacting gas transport from anaerobic regions to the pile surface, such as gas diffusion within the pile and the presence of methanotrophic bacteria, also influence the emission of CH₄ from degradation heaps. The ability to oxidize between 46 and 98% of the CH₄ produced in the pile is possessed by methanotrophs, aerobic bacteria that colonize the area around anaerobic zones and the pile surface (Aoki *et al.*, 2006). Other significant Greenhouse gases released during degradation, such as N₂O and CO, are produced and consumed in large part by methanotrophic bacteria. In addition to the biological activity of the pile, additional variables impacting gas transport from anaerobic regions to the pile surface, such as gas diffusion within the pile and the presence of methanotrophic bacteria, also influence the emission of CH₄ from degradation heaps. The ability to oxidize between 46 and 98% of the CH₄ produced in the pile is possessed by methanotrophs, aerobic bacteria that colonize the area around anaerobic zones and the pile surface (Jäckel, Thummes, and Kämpfer, *et al.* 2005). Other significant Greenhouse gases released during degradation, such as N₂O and CO, are produced and consumed in large part by methanotrophic bacteria. (Yasmin *et al.*, 2022) conducted a four-year interannual review of the GREENHOUSE GAS emissions from an olive mill waste degradation facility and discovered a decrease in CH₄ emissions linked to the facility's improved management

(watering and turning frequencies). The main factors governing CH₄ emissions during the process are the degradation feedstock, the height and shape of the pile, the control of moisture content, and turning frequency because these variables will affect both the oxygen availability and gas diffusion in the degradation pile. These factors were reviewed by (Kebreab *et al.*, 2006) in their reviews of the topic of GREENHOUSE GAS emissions from livestock and degradation operations. Due to the inclusion of anaerobic bacteria, the introduction of manure can also boost methane emissions, as was found by (Miguel A. Sánchez-Monedero *et al.*, 2010) in degradation heaps processing food and olive mill wastes, respectively.

2.6.2 NITROUS OXIDE (N₂O):

Denitrification is a primary source of N₂O emissions from degradation and is affected by various factors, such as the properties of the initial materials and amendments used, turning off the pile, solubility in the pile, and oxygen (O₂) availability at microsites (Ammonia and nitrous oxide interactions: Roles of manure organic matter management; Béline *et al.*, 1999; Hao *et al.*, 2001).

The study found low N₂O emissions in both non-lignite and lignite windrows, with lignite windrows accounting for 76.7% of total Greenhouse gas emissions. The cumulative N₂O emission was 1.16 (0.3) g kg⁻¹ DM in the lignite windrow and -0.52 (0.1) g kg⁻¹ DM in the non-lignite windrow, indicating a slow nitrification process. The addition of lignite to manure reduced nitrogen (N) loss as N₂O in degradation (Bai *et al.*, 2020). Similarly, the addition of bamboo biochar during poultry manure degradation significantly reduced N₂O emissions compared to degradation without biochar. Biochar promotes oxygenation and high temperatures, suppressing the nitrification process that produces N₂O by inhibiting the growth of nitrifying microorganisms and enzymes (Cui *et al.*, 2010; Sánchez-García, ... and 2015; Carbon dioxide concentration NASA global climate change). Excessive acidification (pH 6 and pH 5) accelerated the emission of N₂O by 18.6% and 17.6%, respectively. The emission of N₂O during denitrification increased as the pH value decreased from 7 to 5.5 (Russenes *et al.*, 2016). The study found that vermicomposting with agricultural waste resulted in lower N₂O emissions (5.76 kg CO₂-eq t⁻¹ DM) compared to thermophilic degradation (12.29 kg CO₂-eq t⁻¹ DM) due to the arrangement of the pile, which the earthworms destroyed during

vermidegradation, resulting in a 53% lower emission of N₂O. Denitrification occurred in the intestine of earthworms, resulting in lower N₂O emissions (Ma et al., Yang et al., 2017).

2.6.3 CARBON DIOXIDE (CO₂):

One of the main greenhouse gases that trap heat and are mainly responsible for global warming is CO₂ (Riebeek: NASA earth observatory - Google Scholar). The biogenic CO₂ was produced by biological processes like litterfall decomposition or soil respiration (Harris, Milner, and Taylor, 2018). As CO₂ released during decomposition is already taken as food by plants and is therefore considered to be biogenic in origin, the Global warming potential (GWP) of carbon dioxide gas is not analyzed for organic waste (Jungbluth, Hartung and Brose, 2001; He, Han and Huang, 2020). Changes to the factors that contribute to emissions can lower GREENHOUSE GAS emissions. The increase or decrease in Greenhouse gas emissions is significantly influenced by the insertion of modifications. (Wang, Awasthi, et al., 2018) revealed that using 10% of the recommended amount of biochar during the degradation of pig manure might reduce CO₂ emissions by a proportion equal to 26.06%. The early thermophilic phase and the final maturation, or mesophilic phase, of degradation, are the two stages of waste stabilization.

Aerobic and anaerobic thermophiles consume and degrade readily available OM during the thermophilic phase. High temperatures (50–70°C) and increased microbial activity during this phase, which causes OM to volatilize and CO₂ and NH₃ to be released, make this phase distinct. Temperature decreases (40–50°C) during the last maturation phase show a full breakdown of degradable OM in the trash and minimal to no Greenhouse gas emissions as a result. (Awasthi et al., 2019) noted that CO₂ emissions were very high in the beginning and subsequently dropped. According to what many other researchers discovered, the beginning of the degradation or vermidegradation process employing substrates such as municipal solid waste, green waste, and sewage sludge resulted in the largest CO₂ emissions, which then gradually decreased over the latter stages (Lv et al.,). For the first two weeks of degradation food waste, (Hwang et al., 2020) discovered a considerable initial CO₂ emission of around 9.23g kg⁻¹ hr⁻¹, which significantly declined in the following stages. A temperature rise has been seen

by several other researchers as well. The temperature can increase up to 55°C in the early stages before dropping throughout the following stages. As a result, the early phases' high temperatures probably are what caused the early phases' increase in CO₂ emissions. CO₂ is produced by the breakdown of plant matter, the CO₂ emitted during degradation is thought to have a neutral Global warming potential.

2.7 IMPACTS ON THE ENVIRONMENT:

Organic waste may be managed very well by degradation without creating any garbage. Greenhouse gases (Greenhouse gases), which are a factor in the greenhouse effect, are nevertheless also released as a result of it. Greenhouse gases are essential to preserving the Earth's habitable climate for all living things. Sadly, the excessive amount of Greenhouse gases poses a threat of substantial changes that might have a negative influence on life on this planet (Nunez *et al.*, 2019). The effects of greenhouse gas emissions on the environment include:

2.7.1 Global warming

Human activities have been proven to make a major contribution to the effect of climate change during the last 200 years (Moumen *et al.*, 2019). The chemical composition of the atmosphere has been modified as a result of these human activities. Besides, the two key reasons contributing to global climate change are Greenhouse gases and the Earth's magnetic field. The quantity of radiation impacting the earth's surface is less likely to be reflected as greenhouse gas emissions grow. To balance the anomaly and restore the equilibrium between incoming and outgoing radiation, the Earth's climate changes. More radiation is trapped within the Earth's surface when greenhouse gas emissions grow. These greenhouse gases enhance the temperature of the earth's surface by allowing sunlight to enter and blocking heat from escaping. This creates a rise in air temperature, as well as irreversible implications such as global warming and climate change (Huang *et al.*, 2016). Many researchers and experts worldwide have done countless experiments and have evaluated to uncover several techniques to decrease Greenhouse gases and thus minimize the effect of global warming and maintain the balance of life on Earth. According to several studies, in the last 100 years, the Earth's global temperature has risen by 0.5-1°C. Although the rise may look tiny, even a slight gain in global temperatures generates some important impacts such as

cloud cover and wind patterns. In its third assessment, the "Intergovernmental Panel on Climate Change" (IPCC) anticipated that global mean temperature would grow from 1.4°C to 5.8°C by the end of 2100, resulting in more catastrophic scenarios rapidly (Yasmin et al., 2022c).

2.7.2 Melting of Glaciers and ice sheets

Ice is a great indicator of changes in the climate. Glaciers and ice sheets tend to reflect the radiations of the sun. However, melting of the ice decreases the ice cover, which leads to increased absorption of these radiations instead of reflecting them. Climate change is most likely to occur as of global warming since it has a significant potential for melting icemasses on our globe, such as ice sheets and mountain glaciers. The melting of glaciers and permafrost has had a considerable influence on their stability. These are often perceived as quicker melting, greater glacier flow, the creation of supra-glacial lakes, and a rise in permafrost deterioration. Even in the absence of future temperature changes, some glaciers are projected to retreat, while the number of rising glaciers will expand (Ding et al., 2018).

2.7.3 Rise in sea level

According to (Latake et al., 2016), the emission of greenhouse gases is causing a rise in sea level, which is predicted to climb by 0.09 to 0.88 meters between 1990 and 2100. This elevation is connected to two independent processes: thermal expansion and melting of mountain glaciers and ice caps. Thermal expansion occurs due to higher temperatures, culminating in a rise in sea level while melting Greenland and Antarctica glaciers and ice sheets would add water to the ocean. These two processes are projected to contribute the most major contributions to sea level rise. Additionally, the ocean absorbs roughly 80% of the heat contained by greenhouse gases, causing the water temperature to rise, leading to water expansion. The basic engine of sea level rise over the millennia is thermal expansion, according to many researchers. Since 1990, the sea level has risen at a pace of 0.14 inches per year, largely attributable to thermal expansion. As the temperature increases, snow and ice melt more rapidly, leading to a rise in sea levels (Latake et al., 2016). According to (H. Riebeek's research studies), this problem is worsened.

2.8 EMISSION FACTORS:

2.8.1 Temperature

The temperature is a strong predictor of microbial activity throughout the degradation process and the maturity of the final product collected (Duan et al., 2019). The release rate of greenhouse gases is roughly related to temperature in both open and closed degradation systems. The concentrations of CH₄ and NH₃ are greatest when the temperature is between 40°C and 50°C, but N₂O is only generated when the temperature is below 40°C. Due to temperature fluctuations and changes in the microbial population, the degradation process is seen as a succession of different sub-processes, such as mesophilic and thermophilic (Vergara et al., 2019). Researchers have noted a rapid spike in temperature during the first few days of degradation. Following the thermophilic phase, the temperature progressively decreased to ambient levels in all treatments, suggesting that the mature state had been obtained. (Ren et al., 2020) reported that N₂O-N emissions in the two treatments were lower in the starting stage but peaked throughout the cooling stage, corresponding to Jiang et al.'s (2016) findings during pig manure degradation. This pattern was created by high temperatures. Vergara et al. (2019) revealed that as temperatures hit 65°C, methane fluxes surged more rapidly. (Arora, Rajpal, and Kazmi, 2016) indicated that as the temperature rose, treatment parameters such as Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) declined. They also observed that the optimal temperature for earthworms fluctuates between 25-27°C.

2.8.2 Aeration

The aeration process plays a vital role in establishing the aerobic state of degradation. Studies have demonstrated that continual aeration during degradation may reduce greenhouse gas emissions (Keener et al., 2001). Therefore, it is crucial to provide continuous aeration not only after the thermophilic stage but also during the maturation phase to decrease the environmental impact of N₂O emissions (Pereira *et al.*, 2018). The creation of anaerobic pockets is the major source of CH₄ production, which is created by methanogens deoxidizing carbon dioxide/hydrogen and acetic acid under anaerobic settings. The occurrence of these pockets signifies a lack of oxygen supply or

aeration in the composter. (Scaglia et al., 2011) suggest that by adding O₂ to the compost pile, the production of these anaerobic pockets may be decreased, as previously observed by (Osada *et al.*, 2000), who displayed a reduction in CH₄ and N₂O emissions resulting from reduced anaerobic zone growth. (Zeng *et al.*, 2018) have revealed that the amount of GREENHOUSE GAS emissions is largely dependent on the duration of aeration. GREENHOUSE GAS emissions were lower during the aeration period compared to the aeration interval, i.e., the time without aeration. Therefore, prolonging the duration of the aeration interval may aid in effectively lower GREENHOUSE GAS emissions. (Amlinger et al., 2008) advise enhanced air supply and removal of NH₃ during the early stages of thermophilic decomposition to prevent N₂O production. However, it has been found that higher aeration in open compost may modestly augment NH₃ production due to increased material and turning frequency. The equivalent effect may be seen in closed compost, backed by improved NH₃ removal and evaporation. Furthermore, higher aeration may reduce CH₄ emissions. Proper maintenance greatly minimizes gas release. Well- managed and operated composters release less methane and N₂O throughout the degradation process (Dhamodharan et al., 2019).

2.8.3 Moisture content

In the vermicomposting process, moisture content has a major influence on CH₄ and N₂O emissions. Due to constraints in their dermal respiratory systems, extreme water content (either high or low) may trigger earthworm death in vermicomposting systems. Moreover, it may boost N₂O production by concurrently promoting nitrification and denitrification in mesophilic settings. In addition, waste substrates with a high-water content led to anaerobic conditions in the vermis, which preferred methanogenic bacteria for CH₄ emission (Swati and Hait, 2018). Several research has revealed that the ideal range for the water content for vermicompost is between 70 to 90% (Amouei, Yousefi, and Khosravi, 2017), yet a water level of 25 to 30% in vermicompost resulted in increased microbial activity. (Velasco-Velasco et al., 2011) reported that water content of 80% at 15°C created less NH₃ than 70% water content at 22°C in vermicomposting. (Ermolaev et al., 2019 a) observed that elevating the feedstock water content (WC) from 43 to 66% contributed to an increase in CH₄ production by about 1000-fold in

degradation done in a small-scale reactor with the regulated temperature at 55°C, forced aeration, and frequent mixing. Raising WC by 43-59% lowered N₂O emissions by roughly 77%. The greatest CH₄ and N₂O were emitted by oversaturated compost with 66% WC. The significant amount of water in residential composters, as well as the reported temperatures, may result in excessive N₂O emissions. Increasing the water content reduces the diffusion of oxygen, which impacts Greenhouse gas emissions.

2.8.4 Carbon and Nitrogen ratio

Insufficient nitrogen availability hampers microbial metabolism when the C/N ratio is extremely high. The degradation and end product maturity is determined by the C/N ratio (Chen et al., 2019). Raw material quality generates differences in the C/N ratio, with greenwaste having the largest C/N ratio, followed by cow dung and chicken manure. A C/N ratio of 12 demonstrates compost maturity and stability, and according to (Jjagwe et al., 2019a), the vermicompost reached entire maturity with a C/N ratio of 10 in week 12. The C/N ratio is also significant for determining whether the compost is suitably stabilized. The optimal C/N ratio range is 15 to 30, but degradation may occur in a larger range of values. CO₂ emissions are influenced by the total carbon (TC) and total nitrogen (TN) content. Greater carbon and nitrogen levels are more beneficial to microorganism respiration. For microbial metabolism, TC and TN are important to offer a carbon and nitrogen source as well as the energy needed for life. (Qin et al., 2010) discovered that changes in TC concentration were strongly related to CO₂ emissions. CO₂ generation was also demonstrated to be substantially linked with temperature. This may be connected to the rapid breakdown of organic molecules at higher temperatures (Patty et al., 2005). Moisture content was inversely related to CO₂ emissions, as demonstrated by component analysis (PCA). Increased moisture content enhances microbial mass metabolism but may also extend the heap's anaerobic region. Less NH₃ production is promoted by high aeration and high heating when the feedstock has a low C/N ratio. A C/N ratio of more than 35 will result in incorrect decomposition and humification of the process, but a C/N ratio of more than 25 may aid restrict NH₃ and N₂O formation. In microbial communities like soil, the C: N ratio is a key indicator as it describes a balance between energetic foods

(represented by carbon) and material to build protein with (represented by nitrogen). An optimal C: N ratio of around 24:1 provides for higher microbial activity.

2.8.5 Ph

The quickest degradation rates were discovered when CO₂ production reached its peak two days after the initiation of the therapy, and again when the temperature soared. As previously noted in their reactor testing. (Ermolaev et al., 2019) showed that the initial peak in CO₂ production corresponded with a fall in pH, indicating the synthesis of organic acids during the fermentation of readily available carbohydrates. The pH is changed by the application of bulking agents. Supplying 12% biochar to treatments with 10%, 15%, and 30% zeolite generated appropriate buffering capacity in the compost matrix to maintain a neutral pH throughout the degradation process, which was acceptable for Malt Extract Agar (MEA) degradation and Total Organic Matter (TOM) breakdown. Conversely, the pH of compost treated with Biochar and lime decreased from 8.58 to 6.87 on day 10, and this acidic state continued for a short time before steadily recovering, with a slightly alkaline pH observed near the finish of degradation. Recent pH investigation has proven that it has an effect on the discharge of odors. Additionally, earthworms may modify the original properties of substrates by accelerating the degradation of organic components and causing an acidic pH. It has been shown that higher pH values restrict earthworm generation rates because they facilitate the passage of nano toxin particles to the worm (Vergara, Letters and 2019, 2019). (Mahbub et al., 2017) observed that these toxins decreased the number of harmful chemicals in the earthworm. N₂O emissions were negatively related to substrate pH, and low pH may restrict NH₄⁺-N conversion to NH₃ while concurrently promoting the nitrification/denitrification process. As a result, the greater N₂O emission reported in Verm degradation may be due to a lower pH than in control treatments.

CHAPTER - 3

3.1 AIM:-

To degrade the oil-defiled contaminated soil under low-temperature conditions using a rotational slurry bioreactor.

3.2 OBJECTIVES:-

To overcome this issue, isolated and used microbial degraders adapted to cold environments. These adapted degraders are capable of functioning efficiently even in low-temperature conditions, making them a useful tool for bioremediation in areas with cooler climates. They use two *Rhodococcus* oil-degrading bacteria strains, A and C, to enhance the degradation process in low-temperature conditions. The effectiveness of bioremediation largely depends on using appropriate microbial degraders that are adapted to specific environmental conditions. Microbial degraders are microorganisms that can break down or transform pollutants into less harmful substances.

CHAPTER - 4

MATERIALS AND METHODS

4.1 Bacterial Strains and Culture Conditions

The growth conditions and carbon source for two strains of *Rhodococcus erythropolis* (strain A and strain C), which are permitted as microbial degraders for bioaugmentation.

- The strains were grown overnight at 30 °C in 1/3 LB medium, which contains tryptone, yeast extract, and NaCl. The minimal salt medium used for the experiments is called W medium and contains various salts, including potassium phosphate, sodium phosphate, ammonium sulfate, magnesium sulfate, ferrous sulfate, magnesium oxide, calcium carbonate, zinc sulfate, copper sulfate, cobalt sulfate, and boric acid, as well as concentrated hydrochloric acid.
- In the experiments, the A-fuel oil, which is a heavy oil with low viscosity, was used as the sole carbon source for the bacteria. It appears that the goal of the experiments was to investigate the ability of these strains to degrade the A-fuel oil, potentially for use in bioremediation applications.

➤ 4.2 Preparation of Model Slurry

The slurry samples were prepared using a modified method, where two different commercial horticultural soils were used: Akadama soil and horticulture sand. The Akadama soil was not heat-treated volcanic ash soil, and the horticulture sand was dried sand which was heat-treated. To prepare the slurry samples, 180g of Akadama soil and 120g of horticulture sand were mixed. The texture of the model soil was loamy sand, and the percentages of sand, silt, and clay in the model soil were 85.3%, 1.40%, and 13.3%, respectively. The total carbon and total nitrogen content of the soil samples were 3.0 g/kg and 0.30 g/kg, respectively. To prepare the slurry, 200 mL of distilled water medium was added to the dry soil, which was then mixed with A-fuel oil at concentrations of either 2500mg/kg-slurry or 5000 mg/kg-slurry. It is worth noting that the slurry and A-fuel oil were not sterilized.

TABLE 1:-

No. of Carbon	Conc. In 2500mg A-fuel oil (mg)
10	22
11	56
12	78
13	126
14	219
15	292
16	327
17	378
18	296
19	259
20	205
21	187
22	156
23	102

Composition of n-alkanes in 2500-mg A-fuel oil

4.3 A Rotational Slurry Bioreactor

A 5 L rotational bioreactor made of an acrylic cylinder with three baffles and a 15-mm air hole in the lid. The bioreactor was rotated using a Plate Mix Mill PMM-20 at 12 rpm and a 10° angle. The model slurry was mixed with A-fuel and incubated at 15°C for 24 hours before the test of biodegradation. The slurry sample was inoculated with 10–10 colony forming units (CFUs)/g-slurry of A-fuel oil degraders and mixed for 30 minutes before being collected. The degraders were cultured in 0.8 L of 1/3 LB.

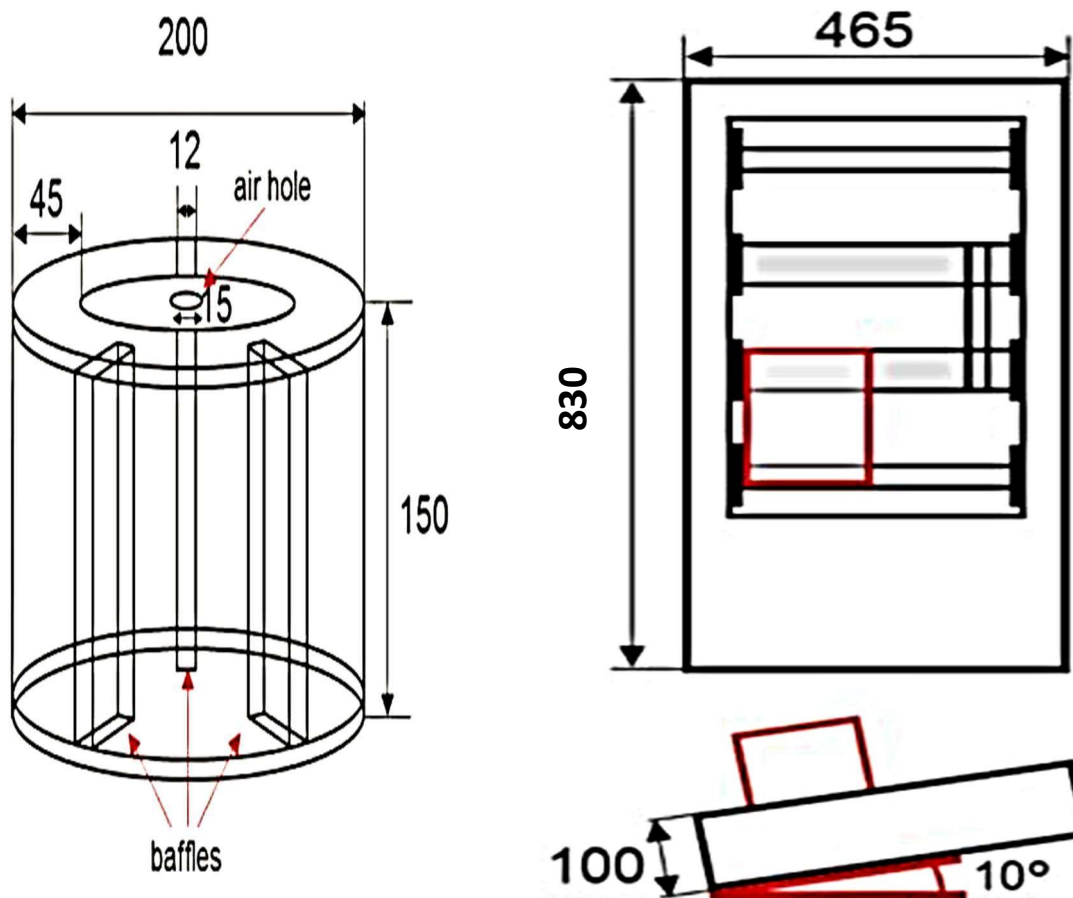


Figure-4.1

A 5 L rotational slurry bioreactor panel and rotator and reactor with the model slurry panel. The digits indicate the size of the reactor (mm). The system was put in a 15 °C incubator with a 10° tilt. The red box indicates the reactor.

4.4 Biodegradative Assays in the Rotational Slurry Bioreactor

Preparing three different soil slurry samples and testing the biodegradation of A-fuel oil from these samples over a period of 6 days. The first sample was rotated at a standard rate of 12 rpm, without inoculating any degraders. The second sample was also rotated at 12 rpm but strains A and C were inoculated into the sample. The third sample was not rotated but strains A and C were inoculated.

The slurry samples were incubated at 15°C, and the remaining A-fuel oil was extracted from each sample at 0, 2, 4, and 6 days after starting the test. The amount of slurry sample collected differed depending on the sampling point (1 g at 0, 2, and 4 days, and 3 g at 6 days). Hexane was added to each sample in capped 15 mL glass test tubes, and the A-fuel oil was extracted by shaking at 200 rpm for 60 minutes. After centrifugation and removal of water and debris by Na₂SO₄ addition, the resultant sample was analyzed using a gas chromatograph system equipped with an FID detector and an autosampler. The GC column was DB-1, and helium was used as a carrier gas. The resulting chromatography data was analyzed using CDS-Lite ver.5 software. Finally, a standard curve was made using 250, 500, and 1000 mg/L of A-fuel oil in hexane to analyze the concentration of A-fuel oil in the samples. To determine the numbers of total bacteria and degraders of A-fuel oil in the model soil slurry, one gram of soil slurry sample was collected on each sampling day and suspended with 3 mL of sterilized phosphate buffer saline (PBS) containing 3 µL of Tween-80. The suspension was mixed by vortexing, and the supernatant was serially diluted by PBS. The diluted samples were spread onto 1/3 LB plates and W plates with A-fuel oil supplemented as a vapor by placing 10 µL of the oil on the lid of the plate. The plates were then incubated at 30°C for a few days, and the colonies on the plates were counted to determine the numbers of total bacteria and degraders of A-fuel oil in the sample.

4.5 Semi-Batch Degradative Assays in the Rotational Slurry Bioreactor

In the semi-batch degradative assays, a total of 500 grams of model slurry was used. After the 6-day treatment of biodegradation with A-fuel oil degraders, 10%(50g) of the treated slurry was left in the reactor, and 90% (450 g) of fresh slurry was added. The slurry was then mixed for 24 hours before A-fuel oil was added at a final concentration of 2500 mg/kg-slurry. No degraders were inoculated at this point. As a control, 100% of the slurry was removed, and 100% fresh slurry was added. After 24 hours of mixing, the fresh A-fuel oil at a concentration of 2500 mg/kg-slurry and its degraders (10 cells/g-slurry) were inoculated.

The remaining A-fuel oil was then measured using the extraction and gas chromatography method described above.

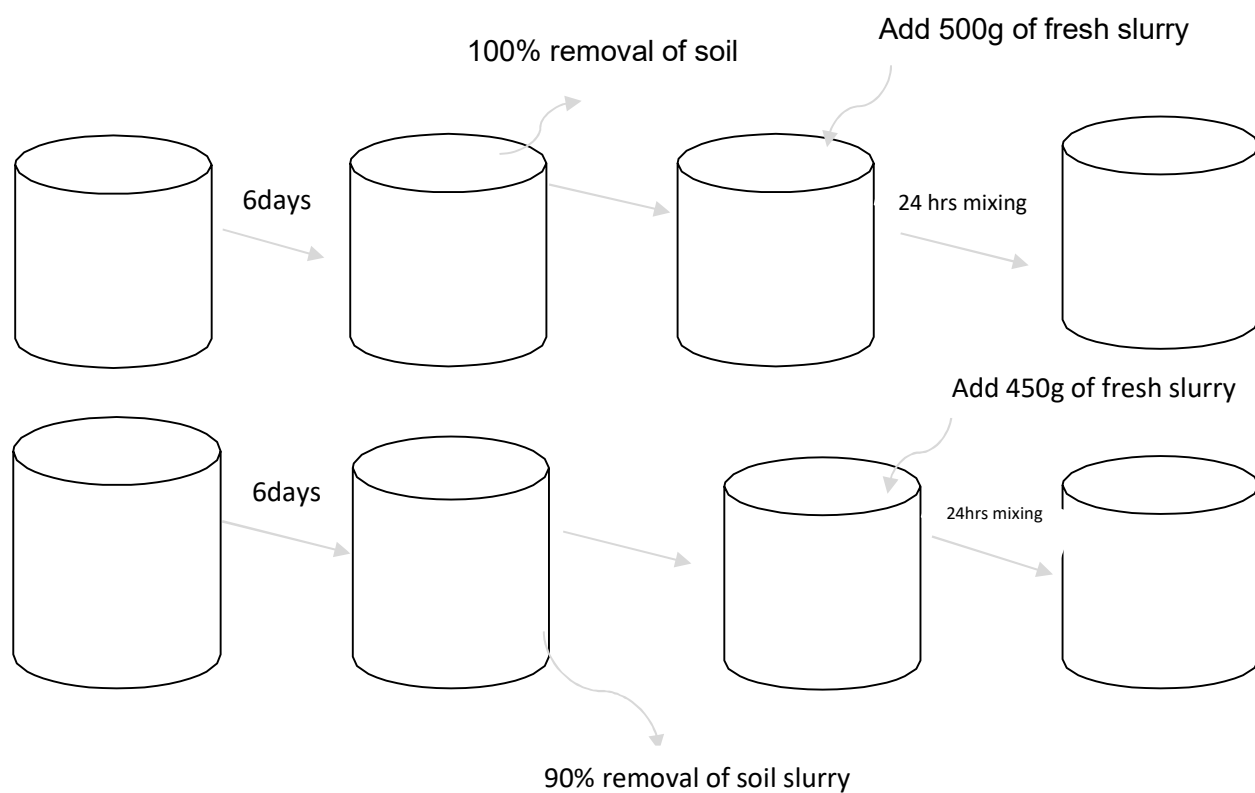


Figure 4.2

Semi-batch degradative experiments were performed in a single cycle in a rotating slurry bioreactor. Semi-batch tests (lower) and control assays (upper) with 100% soil slurry removal following a 6-day treatment. Three cycles were completed.

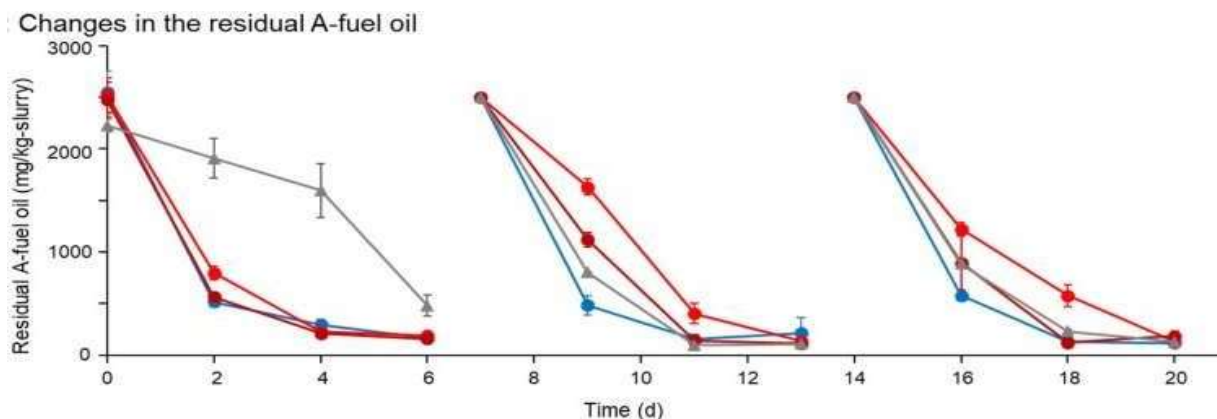
CHAPTER - 5

RESULTS AND DISCUSSION

5.1 Biodegradation of A-Fuel Oil was Much Faster in a Rotational Slurry Bioreactor

Inoculation of *Rhodococcus erythropolis* strains A and C significantly enhanced the biodegradation of A-fuel oil in the rotational slurry bioreactors, leading to the removal of 98% of the oil in just 6 days. In contrast, the non-inoculated samples with rotation or the inoculated samples without rotation had much lower levels of biodegradation, with over 48% of the oil remaining after 6 days. The level of total bacteria in the inoculated samples remained constant at 10^7 – 10^8 CFUs/g slurry throughout the assays, whereas the sample without inoculation had a similar level of bacteria.

Similarly, 90% of A-fuel oil was removed in rotational slurry bioreactors in 6 days, even when the concentration of A-fuel oil increased to 5000 mg/kg-slurry, whereas more than half of them remained in the rotated sample without inoculation with degraders or the inoculated sample without rotation. The numbers of total bacteria in the samples inoculated with degraders were around 10^7 – 10^8 CFUs/g slurry, whereas those in the sample without inoculation increased from 10^5 to 10^7 cells/g slurry in 6 days. We performed these experiments several times, and similarly, the A-fuel oil removal was always faster with the inoculation with degraders and rotation of the bioreactor. However, the number of total bacteria was different at the end of the various experiments.



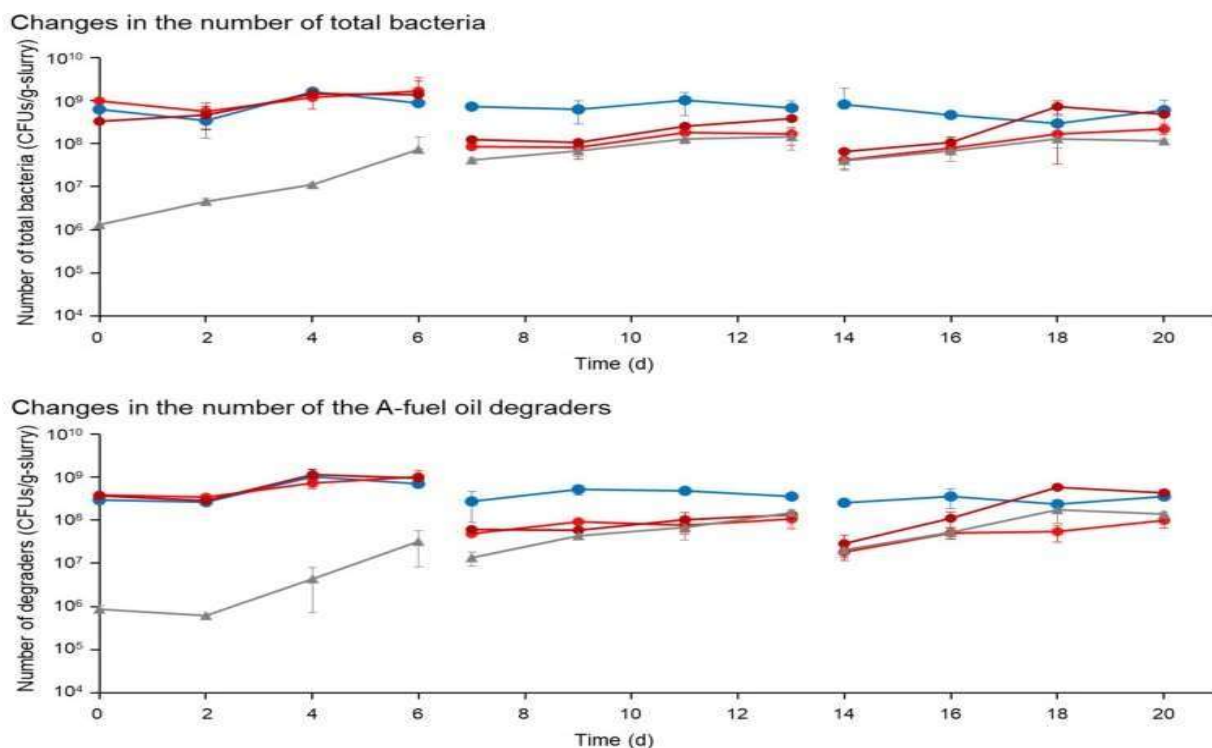


Figure-5.1

Rotational slurry bioreactors with 10 CFUs/g-slurry of degraders were used for semi-batch degradative experiments of 2500 mg/kg-slurry of A-fuel oil. The remaining A-fuel oil was subjected to three cycles of treatment, each lasting six days, as shown in Panel). Between the two cycles, there was a mixing period of 24 hours. The numbers of total bacteria (panel) and A-fuel degraders (panel), respectively, have changed. With a single inoculation of the degraders, two separate tests were run on the samples. Data in triplicate with standard deviations are displayed.

5.2 Semi-Batch Degradative Assays in the Rotational Slurry Bioreactor

In rotational slurry bioreactors, semi-batch degradative experiments of A-fuel oil (2500 mg/kg-slurry) were carried out. More than 95% of the oil was eliminated during the first cycle for the samples injected with degraders. In comparison to semi-batch samples, the sample with degrader inoculation had a quicker removal rate in the second and third cycles. However, both samples had more than 95% of the oil removed after the six-day treatment in each cycle. It's interesting to note that 80% of the oil was removed from the sample during the first cycle without inoculation with degraders. In those samples without

inoculation, the second and third cycles eliminated more than 95% of the oil. Regarding the variations in the total number of bacteria or oil degraders, the samples infected with degraders in the first cycle contained 10^6 CFUs/g-slurry of total bacteria and 10^8 CFUs/g-slurry of oil degraders. These numbers were present in the second and third cycles in the infected samples. The total number of bacteria and oil degraders in the semi-batch samples was around 10^6 CFUs/g-slurry, and 10^8 CFUs/g-slurry at the start of the cycle. During the six-day treatment period, they grew to 10^6 - 10^8 CFUs/g-slurry.

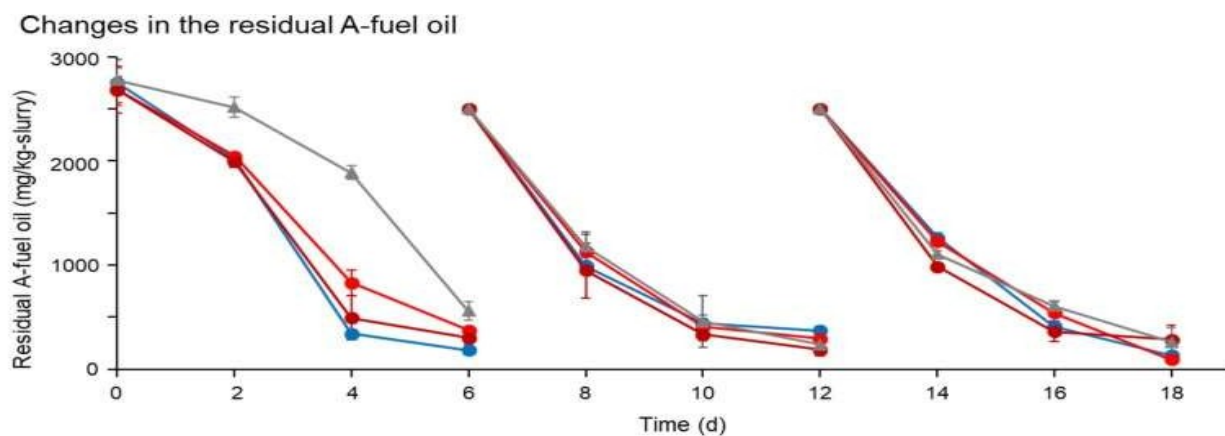
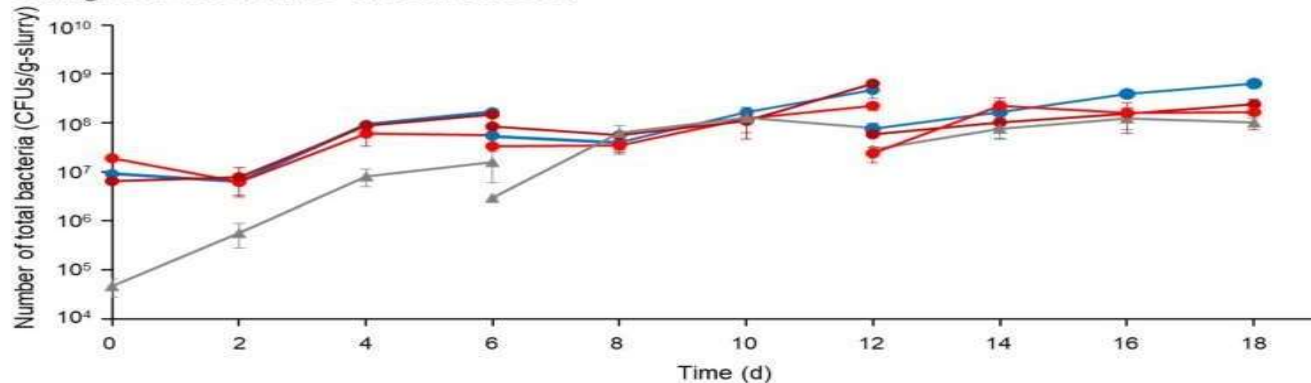


Figure-5.2

Rotational slurry bioreactors with 10^6 CFUs/g-slurry of degraders were used for semi-batch degradative experiments of 2500 mg/kg-slurry of A-fuel oil. The 24-hour mixing process in between was skipped. The remaining A-fuel oil was subjected to three cycles of treatment, each lasting six days, as shown in the Panel.

Changes in the number of total bacteria



Changes in the number of the A-fuel oil degraders

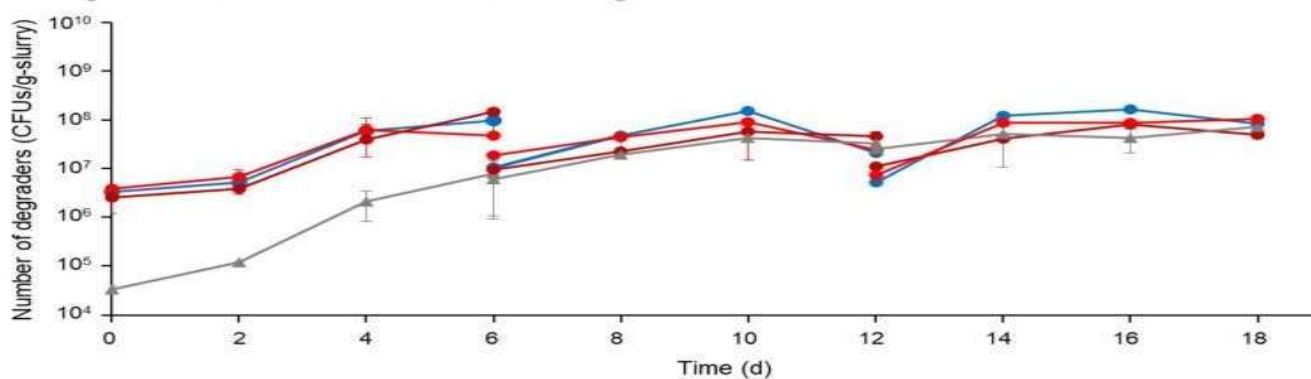


Figure-5.3

There were two cycles separated by 24 hours of mixing time. The numbers of total bacteria (panel) and A-fuel degraders (panel), respectively, have changed. When the de-graders were vaccinated once, two separate analyses were run on the samples. Data in triplicate with standard deviations are displayed.

CHAPTER - 6

CONCLUSION

The study presented discusses the biodegradation of A-fuel oil from soil using *Rhodococcus erythropolis* strains A and C under low-temperature conditions. The authors found that using a rotational slurry treatment, they were able to remove more than 95% of the oil in just 6 days, a significant improvement over previous studies that took more than 40 days.

The rotational slurry treatment was effective due to the increased contact between the degraders and their substrate, as well as the supply of oxygen in the slurry. This treatment method has been previously reviewed and shown to be effective for bioaugmentation, but this study is the first to report its effectiveness at low temperatures. One-time inoculation with the degraders was enough for the removal of A-fuel oil from the soil, and the initial inoculation amount could be reduced to at least 1/100, reducing the time and energy costs for the cultivation of degraders. This reduction in inoculates in the soil also reduces the impact of inoculating on the soil, making these degraders suitable for use in bioaugmentation in open system

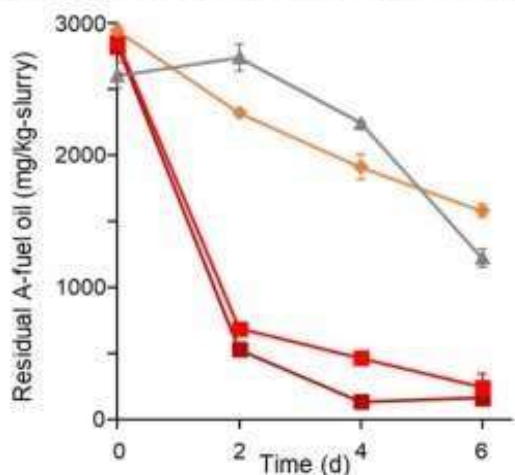
Overall, this study presents a promising method for the biodegradation of A-fuel oil from soil using *Rhodococcus erythropolis* strains A and C under low-temperature conditions with a rotational slurry treatment. This method has the potential to significantly reduce the time and energy costs associated with traditional bioaugmentation methods and could be used in open systems with reduced environmental impact. Even though no degraders were injected, the elimination of A-fuel was seen in batch and semi-batch biodegradative experiments. The non-inoculated samples had variations in the amount of leftover oil according to the batch analysis. These occurrences were caused by the A-fuel oil's volatility, which might be increased by rotation. Because the slurry and A-fuel oil were not sterilized, the number of native degraders for A-fuel oil slightly increased in our system. Because non-sterilized slurry and oil were used, the total bacterial counts varied between experimental lots. The native degraders significantly increased their abundance in the semi-batch assays, and the non-inoculated samples likewise removed A-fuel oil effectively.

Therefore, even though we have not yet identified these degraders, the native degraders may increase their abundance by rotation. This observation suggests that slurry treatment and rotation might boost local degrader(s) activity and abundance, which were also advantageous aspects of our system. The methods suggested in the current study, which have two degraders, might be employed in applications using trucks that transport concrete mix in regions with colder environments.

The detailed process behind why and how the slurry treatment and rotation may hasten the elimination of the A-fuel oil was still unknown in the current investigation. Alkane hydroxylase typically starts the aerobic breakdown of alkanes by converting alkanes to

alkanols while consuming oxygen. Recently, it was discovered that *R. jostii* strain RHA1, one of the alkane degraders that is close to strains A or C, has a three-component alkane hydroxylase made up of alkane 1-monooxygenase, rubredoxin, and rubredoxin reductase. Although the molecular basis for strains A and C's ability to degrade A-fuel oil was still unknown, the two degraders stood out due to the homologous gene sets found in their genome sequences. Therefore, it is likely that the degradative pathways used by these two degraders are similar to those already known, and oxygen may play a significant role in the biodegradation of A-fuel oil. The molecular processes by which our two strains may break down the A-fuel oil in the slurry at lower temperatures will be revealed by further research on our two strains, including transcriptional levels of *alkB* genes at various temperatures. In cooler temperatures, these factors will be crucial for more successful bioaugmentation.

Changes in the residual A-fuel oil (2500 mg/kg-slurry)



Changes in the numbers of total bacteria (2500 mg/kg-slurry)

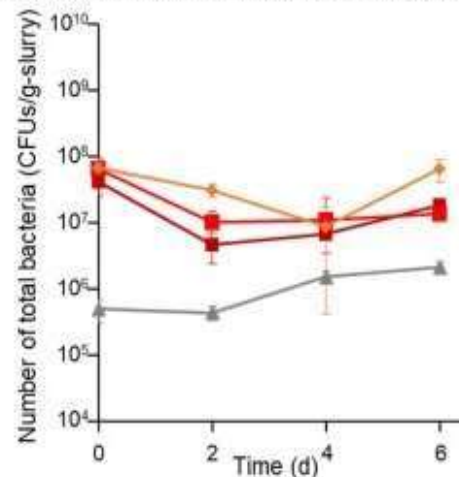


Figure-6.1

Rotational slurry bioreactors for the biodegradation of 2500 panels of A-fuel oil. The residual A-fuel oil has changed in the six days, as shown in the Panels. The numbers of total bacteria and A-fuel oil degraders (CFU/g-slurry) have changed over six days, as shown in the panels. For the samples infected with degraders (strains A and C) at 12 rpm rotation, two separate experiments were carried out. The standard deviations of data in triplicate are displayed.

Pictures of Project Result

Rotational Slurry Bioreactor



Mixture of Akadama Soil with Horticulture Sand



A mixture of Heavy oil with the Above Sand



Nearly 85% of oil removed from Sand



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