PLASTIC DEGRADATION USING SOLID WASTE AND SOIL MICROFLORA

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“PLASTIC DEGRADATION USING SOLID WASTE AND SOIL MICROFLORA”

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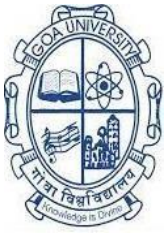

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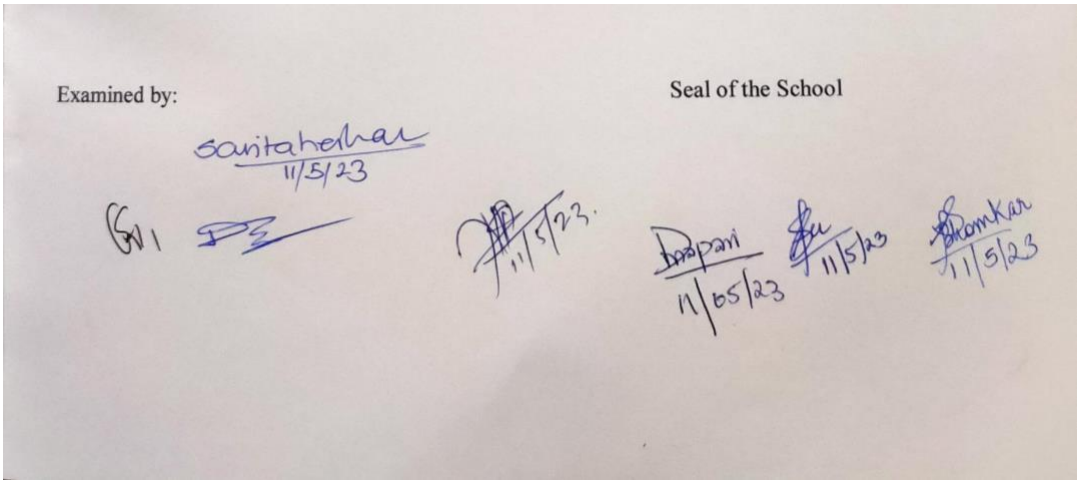

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation entitled, "Plastic Degradation Using Solid Waste and Soil Microflora" is based on the results of investigations carried out by me in the department of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision/Mentorship of Ms. Snesha Bhomkar and the same has not been submitted elsewhere for the award of a degree or diploma by me.

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COMPLETION CERTIFICATE

This is to certify that the dissertation / internship report "Plastic Degradation by Solid Waste and Soil Microflora" is a bonafide work carried out by Ms. Mrugnayni Mahesh Raikar under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of Master of Science in the Discipline Biotechnology at the School of Biological Sciences and Biotechnology , Goa University.

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Abbreviations

LDPE	Low Density Polyethylene
MDPE	Medium Density Polyethylene
HDPE	High Density Polyethylene
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinyl Chloride
PPE	Personal Protective Equipment
PE	Polyethylene
PET	Polyethylene terephthalate
PHA	Polyhydroxyalkanoates
PLA	Polylactic Acid
MSW	Municipal Solid Waste
GHG	Green House Gases
BP	Biodegradable Plastic
PUR	Polyurethane
PEA	Polyethylene adipate
PPL	Poly(β-propiolactone)
PBS	Polybutylene succinate
PCL	Polycaprolactone
O.D.	Optical Density
BHB	Bushnell Hass Broth
PEG	Polyethylene Glycol
MSM	Mineral Salt Medium
NA	Nitric Acid
MO	Mineral Oil
TSB	Trypticase Soy Broth
PBS	Phosphate Buffered Saline
SEM	Scanning Electron Microscopy
EDS	Energy Dispersive X-ray Spectroscopy

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1.Introduction

1.1 Emergence and popularity

Plastics are polymeric units made up of chemically linked monomers. Plastic is a generalized term that has been used to refer to a variety of synthetic or semi synthetic polymerized products (Vignesh et al., 2016). Plastics have emerged as a popular alternative to paper and other cellulose-based products for packaging due to their better tensile strength, light weight and resistance to water and microbial attack (Muhonja et al., 2018). The exceptional success of plastics in the material industry can be attributed to properties like low cost, versatility and durability (Viljakainen & Hug, 2021).

1.2 Types of plastics

Many forms of plastic have been identified and characterized, some of the commonly occurring forms include polyethylene (LDPE, MDPE, HDPE and LLDPE), polypropylene (PP), polystyrene (PS) and polyvinyl chloride (PVC), nylon etc. (Muhonja et al., 2018). Fig 1 illustrates the different types of commercially available plastics, their chemical formula,properties and applications.

Table 1 Commercially available plastics and their applications

Plastics	Chemical formula	Properties	Applications
PET (Polyethylene terephthalate)	$(C_{10}H_8O_4)_n$	• Thermoplastics • Resistance to aging • Barrier properties against gas and moisture • Lightweight	Used as an electronic component, as fibres in clothes, and in manufacturing drinking water bottles
PE, HDPE (High-density polyethylene), LDPE (Low-density polyethylene)	$(C_2H_4)_n$	• Thermoplastics • Good weathering resistance • Water repellent	Used as polyethylene bags, Milk carton bag lining
PVC (Polyvinyl chloride)	$(C_2H_3Cl)_n$	• Thermoplastic • Fire retarding properties • Resistance against acids, alkali, and inorganic chemicals • Easily bendable with other plastics	Used in the health care sector, automobiles, building constructions, and electronics
PP (Polypropylene)	$(C_3H_6)_n$	• Thermoplastic • High stiffness and low density • Heat resistance and transparency	Used in making syringes, Petri plates, and disposable cups and plates
PS (Polystyrene)	$(C_8H_8)_n$	• Thermoplastic • Impact resistance and toughness • Poor barrier against water and oxygen • Crystal-like appearance if unfilled	Used in thermal insulations, plastic cutlery, license plate frames, and plastic model assembly kits.

Fig. 1.2.1 Different commercial plastics and their properties and applications.
Image courtesy- (Shilpa, Basak, & Meena, 2022)

1.3 Threats associated with plastic usage

Plastics have now become an inseparable part of the day to day life of humans (Vignesh et al., 2016). This however has created a serious issue of plastic overaccumulation that poses a significant threat to the environment (Vignesh et al., 2016). During the global COVID-19 pandemic an exponential surge in plastic usage was observed due to plastic being used in the personal protective equipment (PPE). As a result, the plastic waste generation skyrocketed, reaching an estimated amount of 584 million tonnes (mt) globally in 2021 (Hossain et al., 2022).

Plastic wastes, which are typically single use, are generally discarded in landfills (Fachrul et al., 2021). This has significantly increased the percentage of the plastic solid waste in landfills consequently leading to an increase in the pollution level (Vignesh et al., 2016). In nature the plastic wastes can degrade into microplastics. The presence of such microplastics in the environment is a major threat. Plastics are persistent in nature and they can often contain substances that can be potentially toxic or carcinogenic. If these are consumed by organisms they can adversely affect them. The microplastic wastes can very easily enter the food chain and can be potentially harmful to both human and environmental health (Fachrul et al., 2021).

1.4 The need for solutions

Even if biodegradable plastics appear to be a healthier alternative for the environment, they cannot be considered as a solution to the plastic problem, as the already accumulated wastes of non biodegradable plastics are causing serious concerns. Therefore solutions are required for the degradation of existing plastic waste.

During recent studies several different microorganisms have been found to be producing enzymes responsible for the degradation of polyester. Some of the species associated with plastic degradation that have been identified include bacteria (*Pseudomonas, Streptococcus, Staphylcoccus, Micrococcus* and *Moraxella*), fungi (*Aspergillus niger, Aspergillus glaucus*), *Actinomycetes sp.* and*Saccharomonospora genus* (R. et al., 2011).

2.Review of literature

2.1 Introduction to plastics

The words "plastic" and "plastics" come from the ancient Greek term ‘plastikos’, which means "fit for molding," and the Latin term ‘plasticus’, which means "of molding" (Shrivastava, 2018). Plastics are derived from monomeric units. The vast majority of monomers used to make plastics, such as ethylene and propylene, are derived from fossil hydrocarbons (Geyer et al., 2017). A broad variety of soft, synthetic or semi-synthetic organic materials that can be molded into solid objects of various shapes make up plastic. Plastics often consist of other materials and are usually high molecular mass organic polymers. Most of them are synthetic, mostly made from petrochemicals, while many of them are partly natural (Verma et al., 2016).

The most prevalent linear hydrocarbon polymers are polypropylene (PP) and polyethylene (PE), both written as C_nH_{2n}.These polymers are versatile since they can be processed easily into a variety of goods and are produced from inexpensive petrochemical feedstocks using an effective catalytic polymerization process. PP has a methyl group on every other carbon in place of one of the hydrogens found in PE. There are three stereoisomeric types of PP: atactic, isotactic, and syndiotactic. PE is completely linear and is found in densities ranging from 0.91 to 0.97 g/cm3. In contrast to high density PE, which is more linear and has little branching, low density PE has random branching which results in low packing density of the polymer strands (Arutchelvi et al., 2008)

Polyethylene materials have become widely used and essential over the past decade in a variety of industries. They are ideal for a variety of uses and have a number of benefits over other materials, including versatility, light weight, low cost, strength, and the potential for transparency (Gajendiran et al., 2016).

Synthetic plastics are frequently used to package commodities like food, medications, cosmetics, cleansers, and chemicals. Due to their exceptional physical and chemical qualities, such as strength, lightness, and resistance to water and the majority of water-borne microorganisms, they have substituted paper and other cellulose-based goods for packaging (Shah et al., 2008). Production of personal protective equipment (PPE) using plastics is a significant application during the global COVID-19 outbreak. This resulted in skyrocketing plastic usage during the pandemic. In terms of municipal solid refuse, plastic waste (PW) is one

of the waste streams that is expanding the fastest globally. (Hossain et al., 2022).

2.2 Problems associated with plastic

The properties of plastic that make it the most valuable commodity like durability, light weight, and cheap cost, are also the biggest challenges during its disposal and waste management (Verma et al., 2016) Plastics are known to be resistant to microbial attack since evolution was unable to create new enzyme structures during the brief period that synthetic polymers were present in nature. Commercial plastics, which are used in packaging (for example, fast food), industry, and agriculture are not biodegradable, but their production has severely increased. This has drawn attention to a potentially serious environmental accumulation and pollution issue that could last for centuries.

An estimated 10% of household waste is plastic, and the majority of it is dumped in landfills (Verma et al., 2016). The common disposal methods for plastic garbage are landfilling, incineration, and recycling. However these methods generally prove inefficient. Several communities are now more aware of the effects of discarded plastic on the environment, including harmful effects on wildlife and on the aesthetic qualities of cities and woodlands, as a result of their persistence in our environment (Shah et al., 2008).

Discarded plastics are not only extremely visible, but they also make up a large portion of the solid waste in landfills and are resistant to biodegradation, which causes pollution and harms the ecosystem (Vignesh et al., 2016). Plastics build up in landfills and the ecosystem rather than breaking down. As a result, a growing concern is the near-permanent contamination of the environment with plastic waste. All major ocean basins have reported plastic debris, with an estimated 4 to 12 million metric tonnes (Mt) of plastic waste from land-based sources entering the marine ecosystem in just 2010 alone (Geyer et al., 2017).

Plastic is now pervasive in India. Burning of plastic waste is also very common. The majority of the time, toxic gases like dioxins, furans, mercury, and polychlorinated biphenyls are released into the atmosphere when municipal solid waste (MSW) that contains 10–12% plastic is burned. The second largest source of Green House Gases (GHG) emissions after fossil fuels is landfills. Currently landfills are overloaded with wastes, and burning waste and plastic bags together poses health risks. The need is for an immediate response to this issue (Verma et al.,

2016).

PW is made up of numerous valuable composite materials, harmful emissions, and residual ash, and as a result, handling waste is challenging because plastic is frequently contaminated with other types of waste, which reduces its ability to be recycled . Collection and segregation operations are carried out by multi-tier operators (the informal sector) in many developing nations like India, which makes it more difficult to track the flow of waste through the various waste streams (Hossain et al., 2022). According to studies, the increased use and production of plastic in developing and emerging nations is particularly concerning because their infrastructure for waste management may not be evolving at a pace that will allow it to handle the rising levels of plastic waste. The release of chemicals from plastic waste or the degradation of plastic into secondary microplastics may be impacted by changes in temperature and the atmosphere. The breakdown of larger plastic elements results in the formation of secondary microplastics (Verma et al., 2016).

The polymers require more than ten decades to mineralize under normal circumstances. Due to the fact that plastic bags contain a number of chemicals that are hazardous to human health and the environment, several countries have banned the distribution of plastic bags by supermarkets and other commercial establishments. Each year, it is believed that a million birds and 10,000 marine animals perish as a result of ingesting plastic (Gajendiran et al., 2016).

2.3 Possible solutions employed currently to tackle the plastic problem

Solutions to the plastic problem are urgently required in light of the enormous difficulties caused by plastic consumption. Some of the currently employed solutions include.

2.3.1 Measures to reduce the plastic waste generation

Two broad categories of policy tools are available to reduce the use of plastic. While some nations have completely prohibited the use of plastic bags and other products made of plastic (bans), others prefer to use economic policy tools like levies, taxes, or fees that are paid by either customers or the retail business upon usage of plastic (Heidbreder et al., 2019). More than 67 nations have so far implemented various bans on single-use plastics, including plastic bags and foamed plastic items like Styrofoam.

Kenya imposed the strictest plastic bag ban in the world in 2017, according to which anyone found in possession of a plastic bag may theoretically face up to four years in prison or a fine of USD 40,000. Alternately, levies are typically implemented to reduce the demand for plastics and/or to raise enough money to enable effective management of plastic waste. When the sum collected is significant enough to discourage unethical behavior, this strategy can often be effective (Nikiema & Asiedu, 2022).

A plastic ban has been introduced in India too. Given that 3.5 million tonnes of plastic were produced in 2020–21 and that India's recycling capacity is only half that amount, the country decided to stop producing, importing, stocking, distributing, selling, and using single-use plastic (SUP) products with low utility and a high potential for littering on July 1, 2022 (Nova, 2022).

2.3.2 Reuse of plastic

Durability is one of plastic's key attributes. In contrast, it is typically utilized in a single use manner, which is quite counterintuitive. Therefore, increasing the reuse of plastic products could be the answer to reducing the waste production of this persistent substance (Heidbreder et al., 2019).

2.3.3 Recycling of plastic

A significant amount of the plastic generated each year gets used to create disposable packaging materials and other short-lived goods that are thrown away within a year of production. One of the most crucial measures currently available for minimizing these effects is recycling, which also represents one of the most dynamic segments of the plastics business today. Recycling offers opportunities to cut down on the amount of waste that needs to be disposed off, carbon dioxide emissions, and oil use (Hopewell et al., 2009).

Both mechanical and chemical methods can be used to recycle plastic. The processes involved in mechanical recycling are: collecting, sorting, washing, grinding, compounding, and pelletizing. Chemical recycling can be broken down into thermochemical and catalytic conversion processes, which include chemolysis,

gasification, pyrolysis, fluid-catalyzed cracking, and hydrocracking (Bhatacharyya et al., 2019)

Consumers are now more aware about the negative impacts of plastic and are leaning towards recycling. Numerous private firms are impacted by the change in societal attitudes regarding plastic recycling. For instance, the Coca-Cola business has made a commitment to recycle 100% of its packaging by 2025, manufacture at least 50% of it's packaging from recycled materials by 2030, and to collect and recycle one bottle or can for every one sold by 2030 (Nikiema & Asiedu, 2022).

2.3.4 Incineration

According to Geyer et al. (2017), in 2015, roughly 26% of the plastic garbage generated globally was burned in an incinerator, frequently with other municipal and industrial solid wastes. Given that plastic has a calorific value that is comparable to that of fuel derived from hydrocarbons, plastic wastes offer a significant potential for energy production (Nikiema & Asiedu, 2022). Burning plastic garbage will release irritant gases (HCl) and greenhouse gases (CO2). Hazardous substances can be discharged into the environment through this disposal method of plastic waste (Shen et al., 2020). Waste incineration may result in the release of toxic compounds such as dioxins (for example, when processing chlorine-containing plastics like polyvinyl chloride), nitrogen oxides (typically 1.5 kg per MWh of energy generated and sulfur dioxide (typically 0.23 kg per MWh of energy generated), into the atmosphere, especially when incinerators are old or inefficient (Nikiema & Asiedu, 2022).

2.3.5 Biodegradable plastics

A type of plastic known as "Biodegradable plastic" is a material whose qualities may meet usage requirements, remain unaltered during storage, and, after use, decompose into environmentally sound substances in a natural setting. This brand-new class of plastics called BPs can biodegrade and disappear in the environment. According to its principle of degradation, it can be broken down into components (CO2, H2O, CH4, and biomass) by microbes (bacteria, fungi, algae, etc.) found in nature (Shen et al., 2020). Degradation of polymers is significantly influenced by factors including molecular

weight, crystallinity, functional groups, mobility, substituents present in the structure, and chemicals added to the polymers. There is still limited understanding about what happens to these organic polymers in the atmosphere or how long it takes for them to completely mineralize into CO_2 (Gajendiran et al., 2016).

2.4 Degradation of plastics

Plastic degradation by definition is a change in the material properties of plastic like mechanical properties, bond changes, and the formation of new functional groups (Fachrul et al., 2021). Biodegradation is defined as the process of decomposition of materials using microbial activity. Biodegradation is a complex process involving multiple steps.

1) Bio- deterioration- this involves the combined action of microbial population and abiotic factors resulting in fragmentation of materials into tiny fractions.

2) Depolymerization- during this step enzymes and free radicals are secreted by the microorganisms that cleave the polymer into oligomers, dimers and monomers.

3) Assimilation- in this step some molecules are identified by the receptors of the microbial cells and are allowed to go across the plasma membrane.

4) Mineralization- it is the release of simple molecules like CO_2 , N_2 , CH_4 , H_2O and different salts from the complete oxidation of intracellular metabolites (Muhonja et al., 2018).

The conventional plastics including polyethylene (PE), polypropylene (PP), polystyrene (PS) and polyethylene terephthalate (PET) are difficult to environmentally biodegrade. However, biodegradable plastics are a more environmentally friendly form of plastics that compose a small (<1%), but rapidly increasing fraction of plastic production. These can easily be metabolized by microorganisms to biomass, CO_2, and H_2O. Biodegradable plastics involve polyhydroxyalkanoates (PHAs), polylactic acids (PLA) or starch blends. The rate of biodegradation is strongly dependent on environmental factors like temperature, humidity, pH, and the microbial community and the enzymes encoded by them. Compostable plastics are defined as biodegradable plastics that are certified to decompose into CO_2 and H_2O within a specific time period when provided with ideal composting conditions (Viljakainen & Hug, 2021).

2.5 The need for better solutions

Annual increases in plastic consumption are expected to continue for at least a decade more. However, recycling only accounts for 9% of the 9 billion tonnes of plastic that have ever been manufactured. The majority of the remainder has been thrown into the environment, landfills, dumps, or rivers, lakes, and oceans (Nikiema & Asiedu, 2022). Synthetic plastics went from being basically non-existent to one of the major challenges confronting the globe today in just over 100 years due to their non-biodegradability and poor rate of reuse and recycling (Bhatacharyya et al., 2019). There is sufficient evidence that the current techniques of polymer degradation are not completely effective. Hence scientists are looking into innovative ways where microorganisms can be employed to degrade the long chain synthetic polymers into their monomers (Ghosh et al., 2013).

2.6 Microorganisms used in plastic degradation

The term "biodegradation" describes the breakdown of plastic over a predetermined time period with the help of enzymes or microorganisms Plastic deterioration is the product of a catabolic process. By selecting and isolating plastic-degrading microorganisms from environmental reservoirs, which are then further quantified using analytical methods, biodegradation of plastic can be studied in situ or under laboratory settings (Shilpa et al., 2022). According to recent studies, a significant amount of microorganisms, particularly some bacteria and fungi, have the ability to degrade these synthetic polymers much more rapidly than the natural process by employing various exo-enzymes when under stress (Ghosh et al., 2013). In theoretical terms, the question of how microbes may break down synthetic polymers is quite simple to address. Many enzymes that break down plastic have evolved from those that break down plant material. Key enzymes that can attack the polymer backbones of manufactured plastics naturally feed on plant polymers. For instance, cutin, an aliphatic polyester present in the cuticle of plants, can be hydrolyzed by cutinases. The ester linkages in PET and PUR can likewise be hydrolyzed by these enzymes. Low-density polyethylene (LDPE) is degraded by a number of the same enzymes that are involved in the metabolism of lignin (Jetkins et al., 2019).

Table 2.5.1 Previously studied plastic degrading microbes and their associated enzymes.

Plastic Type	Degrading microorganism species	Associated enzyme	Reference
Polyethylene adipate (PEA)	*Rhizopus arrhizus*	Lipase	(Ghosh et al., 2013)
	Rhizopus delemar	Lipase	
	Achromobacter sp.	Lipase	
	Candida cylindracea	Lipase	
poly(β-propiolactone) (PPL)	*Streptomyces sp.*	PHB depolymerase	(Ghosh et al., 2013)
Polybutylene succinate (PBS)	*Acidovoraxdelafieldii*	PBS depolymerase (lipase)	(Jenkins et al., 2019)
	Aspergillus oryzae	Cutinase	
	Moeszyomycesantarcticus (Pseudozyma antarctica)	Cutinase like enzyme	
Polycaprolactone (PCL)	*Streptomyces* sp. SM14	SM14est (PETase like enzyme)	(Almeida et al., 2019)
	Alcaligenes faecalis	PCL depolymerase (lipase)	(Jenkins et al., 2019)
	Fusarium moniliforme	PCL depolymerase (cutinase)	
	Fusarium solani	Cutinase	
	Aspergillus fumigatus	Cutinase	
	Pseudozyma japonica	Cutinase	
	Rhizopus oryzae (arrhizus)	Lipase	(Cobongela, 2021)

Polyurethane (PUR)	*Bacilus subtilis*	Polyurethanase-lipase	(Jenkins et al., 2019)
	Comamonasacidovorans TB-35	Polyurethane esterase	
	Pestalotiopsismicrospora	Serine hydrolase	
	Pseudomonas chlororaphis	Polyester polyurethanase (lipase)	
	Pseudomonas fluorescens	Polyurethanase-protease	
	Curvularia senegalensis	Esterase	(Cobongela, 2021)
Polyethylene (PE)	*Phanerochaete chrysosporium*	Manganese peroxidase	(Jenkins et al., 2019)
	Trametes versicolor	Laccase	
	Pseudomonas sp. E4	Alkane hydroxylase	
	Pseudomonas aeruginosa	Alkane monooxygenase, rubredoxin and rubredoxin reductase	
	Rhodococcusruber	Laccase	(Cobongela, 2021)
	Aspergillus fumigatus	Laccase	
Polyethylene Terephthalate (PET)	*Ideonellasakaiensis*	PETase (cutinase/ lipase) and MHETase (tannase/feruloyl esterase)	(Almeida et al., 2019)
	Thermobifidafusca	Polyester hydrolase (cutinase) and carboxylesterase	(Jenkins et al., 2019)
	Thermomyces (Humicola) Insolens	Cutinase	

	Moesziomycesantarcticus (Candida antarctica)	Lipase (CalB)	
	Saccharomonosporaviridis	Cutinase	(Cobongela, 2021)
	Thermobifidacellulosilytica	Cutinase	
	Thermomyceslanuginosus	Lipase	
	Triticum aestivum	Lipase	

2.7 Relevance of study

Since landfilling and incineration are not viable solutions for managing the ongoing accumulation of plastic garbage worldwide, a sustainable waste management model must be created. Even though numerous microorganisms have been studied in order to better comprehend the genes and enzymes involved in biochemical pathways involved in degradation, more research is still required on the complete degradation process. Therefore further studies on plastic degradation by bacteria are still essential (Shilpa et al., 2022).

3. Aims and Objectives

1. Enrichment of potential plastic degrading microorganisms.
2. Isolation of potential plastic degrading microorganisms
3. Screening of isolates for plastic degrading activity.
4. Partial biochemical characterization of potential plastic degrading isolates.

4.Materials And Methods

4.1 Sample collection

The samples were collected from two sites in the state of Goa.

i) Site 1- Sansoda, Margao- Goa

Fig. 4.1.1 Sample collection from Site 1

ii) Site 2- Goa University Campus

Fig. 4.1.2 Collected samples from Site 2

Two samples were collected from each of the sites.

a) Soil sample from the plastic polluted area.
b) Plastic sample from the area.

4.2 Enrichment of potential plastic

The collected Plastic samples and 1g soil sample were added to 100 ml normal saline (0.85%) and incubated on the shaker for 48 hours.

4.3 Isolation of bacterial strains from the samples.

1ml of the saline sample from the enrichment flask was inoculated into 50 ml nutrient broth and incubated for 24 hrs. Serial dilutions (10^{-1} - 10^{-6}) were prepared from the incubated nutrient broth using saline. The last three dilutions (10^{-1}, 10^{-2}, 10^{-3}) were used for plating. 100µl of the sample was spread plated on nutrient agar plates to obtain separate colonies. Plates were incubated for 24 hrs.

Colonies that appeared morphologically distinct were then isolated and purified by quadrant streak method.

The pure cultures so obtained were than maintained for further analysis.

4.4 Morphological Characterization of the obtained isolates

- Colony morphology of all the obtained isolates was studied.
- Gram's staining was performed for all obtained isolates.
- Motility testing using the hanging drop method was performed on the obtained isolates.

4.5 Determination of plastic degradation by weight loss method

Three different types of plastic samples (Blue, Black and Yellow) of LDPE (Low Density Polyethylene) were cut into 2cm x 2cm pieces. The weights of the plastic samples were recorded in mg using QUINTIX224- 10IN weighing balance. The pre-weighed plastic samples were sterilized with 70% ethanol and inoculated in 100 ml of Bushnell Hass Broth (BHB). The flasks were then inoculated with 1ml of bacterial isolates. The flasks were then incubated on the shaker for a period of 5.5 months. The O.D. of the broth in the flasks was recorded at 600nm initially after a period of 1.5 months and consecutively after every 30 days upto a period of 5.5 months. The plastic samples were recovered from the flasks after the incubation period and washed in normal saline. Further the plastic samples were washed with sterile distilled water. After a final rinse with 70% ethanol the plastic samples were air dried. The weight of the plastic samples was recorded in triplicates. The weight loss percentage of the plastic samples was calculated using the formula

$$\text{Percentage of weight loss} = \frac{Initial\ weight - Final\ weight}{Initial\ weight} \times 100$$

4.6 Determination of plastic degradation by using PEG powder.

The isolated bacteria were screened for their ability to degrade plastic by using mineral salt medium containing polyethylene glycol (PEG) powder at final concentration of 0.1% (w/v) after

filter sterilization. The medium was poured in sterile plates and allowed to solidify. Wells were made in the center of the plates using sterile gel puncture. 10µl culture suspension of each bacterial isolate was added to separate wells. The plates were then incubated at room temperature for 2-4 weeks and observed for growth around the wells. The diameter of the colonies was measured and the largest colonies showing most efficient growth were used for further experiments (Divyalakshmi & Subhashini, 2016).

4.7 Enhancing plastic degradation by the isolates via pretreatment of Plastic sample.

LDPE plastic sample was cut into 2cm x 2cm stripsand weighed. The pre-weighed plastic samples were exposed to various types of pretreatments.

4.7.1 Nitric Acid pretreatment

The plastic samples were exposed to 65% nitric acid solution for 10 days (Kotova et al., 2021). The weight of the samples was recorded after 10 days.

4.7.2 Mineral oil pretreatment

0.05% Mineral oil was added in the medium along with the plastic sample during with the culture (Bose & Olsen, 2020).

4.7.3 Xylene Pretreatment

The plastic samples were boiled with xylene for 15 minutes. The strips were then subjected to an ethanol wash (70%) followed by drying in the hot air oven at 60°C. The weights of the treated plastic samples were then recorded after pretreatment (Kalia &Dhanaya, 2021).

4.7.4 Thermal Pretreatment

The LDPE strips were treated thermally in a preheated hot air oven at 70°C for 10 days. After 10 days the weights of the thermally pretreated samples was recorded (Awasthi et al., 2017).

4.7.5 UV pretreatment

The LDPE strips were exposed to UV light in a laminar air flow (LAF) for a period of 1 week. The UV pretreated strips were weighed after exposure (Kalia &Dhanaya, 2021).

All the pretreated plastic samples were inoculated separately in 30 ml Bushnell Hass Broth along with the bacterial isolates after recording their post treatment weights. The optical density of the broth was recorded every 15 days at 600nm for a period of two months to check for the microbial growth. The weights of the plastic samples after the incubation was measured. The weight loss percentage was calculated.

4.8Crystal Violet Biofilm Assay

Crystal violet Biofilm assay was performed to analyze the biofilm formation capability of the best four isolates. 10ml of Trypticase soy broth (TSB) was inoculated with a loopful of 24 hr old culture and incubated overnight. The tubes were decanted and washed with PBS (Phosphate Buffered Saline)(pH- 7.3) and dried (Mathur et al., 2006). The dried tubes were stained with 1ml of 0.2% crystal violet solution. After 5 minutes, excess crystal violet was removed and the cell bound crystal violet was dissolved in 5 ml of 33% acetic acid. Biofilm growth was quantified by recording the optical density at 570nm (Shukla & Rao, 2017).

4.9 Biochemical tests

Carbohydate utilization tests of the best four isolates were performed using KB009 HiCarbohydrate kit (KB009A/ KB009B/ KB009C). write about inoculum preparation, OD checking and amount of inoculum added to each well

4.10 Enzyme assays

4.10.1 Lipase

Lipase activity of the isolates was screened using tributyrin nutrient agar plates supplemented with 1% (v/v) of tributyrin. The isolates were spot inoculated on the prepared plates and incubated overnight. The isolates showing clear zones of tributyrin hydrolysis were determined to be positive for lipase production (M. Veerapagu, 2013).

4.10.2 Protease

The protease production activity was determined on 25% nutrient agar plates supplemented with 1% (w/v) casein. The isolates showing zones of clearance by casein hydrolysis were determined to be positive for protease production.

4.10.3 Catalase

Loopful of a 24 hr old culture was transferred on a slide. Few drops of 3% hydrogen peroxide were added on the slide. The production of effervescence indicated the production of catalase enzyme.

4.10.4 Oxidase

Sterile Whatman filter paper was soaked with 1.5% of N- tetramethyl or oxidase reagent and air dried. 50 µl of 24 h culture was inoculated on the paper. Color change to deep blue or purple in 10-30 seconds indicated production of oxidase enzyme.

4.11 Confirmation of plastic degradation by SEM (Scanning Electron Microscopy) and EDS (Energy Dispersive X-ray Spectroscopy)

The plastic samples that showed the maximum weight loss percentage were analyzed using SEM/EDS to check for the presence of crack formation on the surface of the test samples (via SEM)and the change in carbon content (via EDS).

5. Results

5.1 Enrichment and Isolation

Areas with existing plastic waste contamination were used for sample collection.

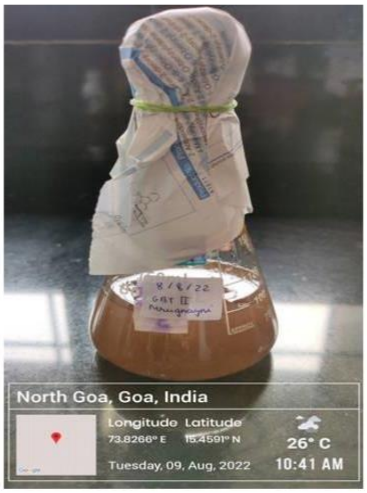


Fig. 5.1.1 Enrichment of Site 1 Plastic Sample

Fig. 5.1.2 Enrichment of Site 1 Soil Sample

Fig. 5.1.3 Enrichment of Site 2 Soil Sample

a) 3 cultures were isolated from Site 1 Plastic Sample

Fig. 5.1.4 Site 1 Plastic sample serial dilution plate (10^{-6})

Bacterial isolates obtained- MRP1, MRP2, MRP3

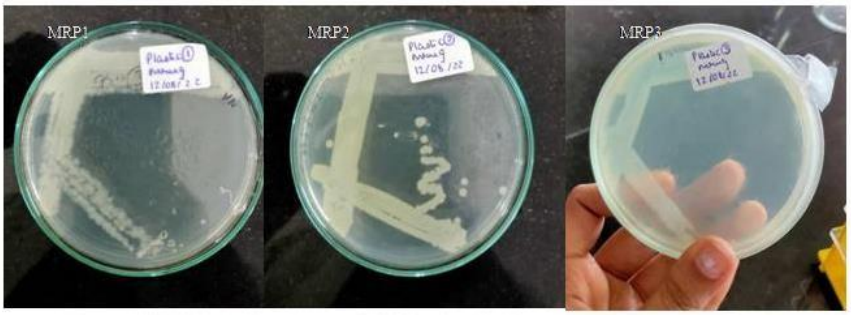


Fig. 5.1.5 Isolation of Bacterial isolates from Site 1 Plastic Sample

b) 4 cultures were isolated from Site 1 Soil Sample

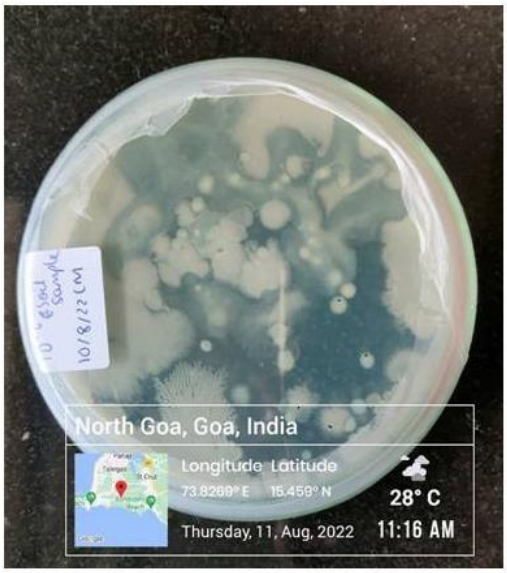


Fig. 5.1.6 Site 1 Soil Sample serial dilution plate (10^{-6})

Bacterial isolates obtained- MRS1, MRS2, MRS3, MRS4

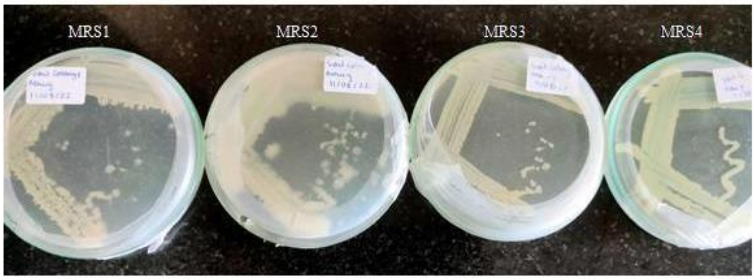


Fig. 5.1.7 Isolation of Bacterial isolates from Site 1 Soil Sample

c) 2 cultures were isolated from Site 2 Soil Sample

Fig. 5.1.8 Site 2 Soil Sample serial dilution plate (10^{-6})
Bacterial isolates obtained- MRS5, MRS6

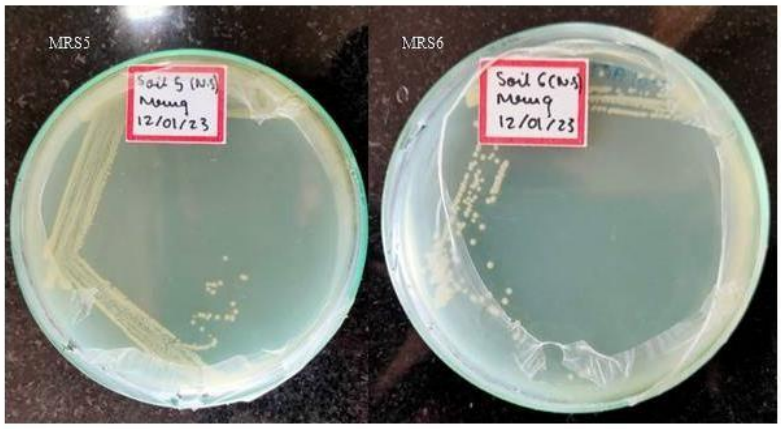


Fig 5.1.9 Isolation of Bacterial isolates from Site 2 Soil Sample

5.2 Colony characteristics of the isolated bacteria

Colony characteristics of the isolated colonies were noted.

Table 5.2.1 Colony characteristics of the isolated bacteria

Colony name	Colour	Size	Colony shape	Texture	Elevation	Margin
MRP1	Off White	2.2 cm	Irregular	Smooth	Flat	Undulate
MRP2	Off White	0.3 cm	Circular	Smooth	Flat	Entire
MRP3	Translucent	1.0 cm	Circular	Smooth	Flat	Entire
MRS1	Off White	0.7 cm	Filamentous	Smooth	Flat	Filiform
MRS2	Off White	0.9 cm	Irregular	Smooth	Flat	Lobate
MRS3	Off White	0.3 cm	Circular	Smooth	Flat	Entire
MRS4	Off White	0.4 cm	Circular	Smooth	Flat	Entire
MRS5	White	0.05 cm	Circular	Smooth	Flat	Entire
MRS6	Off White	0.2 cm	Circular	Smooth	Raised	Entire

5.3 Gram Characteristics and Motility

Gram character, morphology and motility of the isolated bacterial cultures was noted.

Table 5.3 Gram Character, morphology and motility of the isolated bacteria.

Colony name	Grams Character and morphology	Motility
MRP1	Gram -ve, Rods/ long chains	Non motile
MRP2	Gram -ve, Cocobacilli	Non motile
MRP3	Gram -ve, Tiny rods	Motile
MRS1	Gram -ve, Rods	Motile
MRS2	Gram -ve, Rods	Non motile
MRS3	Gram -ve, Short rods	Non motile
MRS4	Gram -ve, Cocobacilli	Non motile
MRS5	Gram -ve, Cocci	Motile
MRS6	Gram -ve, Cocci	Non motile

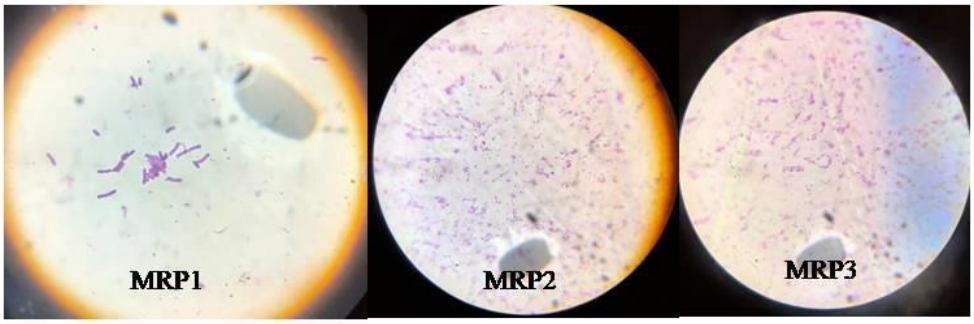


Fig. 5.3.1 Gram characteristics of isolates from Site 1 Plastic Sample

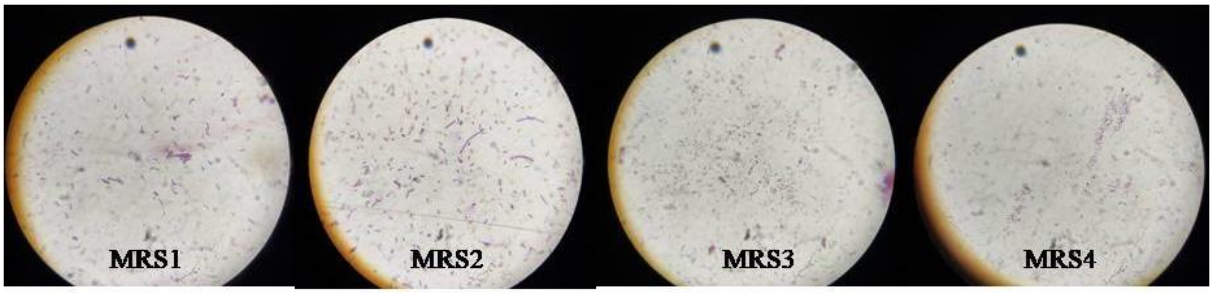


Fig. 5.3.2 Gram characteristics of isolates from Site 1 Soil Sample

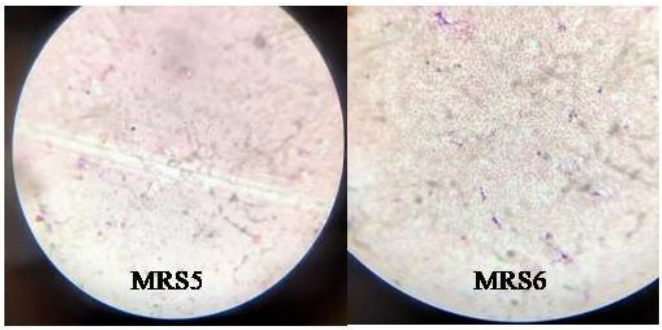


Fig. 5.3.3 Gram characteristics of isolates from Site 2 Soil Sample

5.4 PEG degradation test

After two weeks of incubation on mineral slat media supplemented with PEG the diameter of the bacterial colonies was recorded.

Table 5.4.1 Diameter of colonies during PEG degradation test

Isolate	Diameter in cm
MRP1	3.8
MRP2	1.5
MRP3	2.0
MRS1	1.8
MRS2	4.9
MRS3	2.6
MRS4	5.5

The highest growth in terms of diameter measured was shown by MRS4 followed by MRS2, MRP1 and MRS3 in descending order of growth. While the other isolates showed growth in the range of 1.5-2.0 cm. which was comparatively lesser.

Fig. 5.4.1 PEG degradation test of isolates from Site 1 Plastic Sample

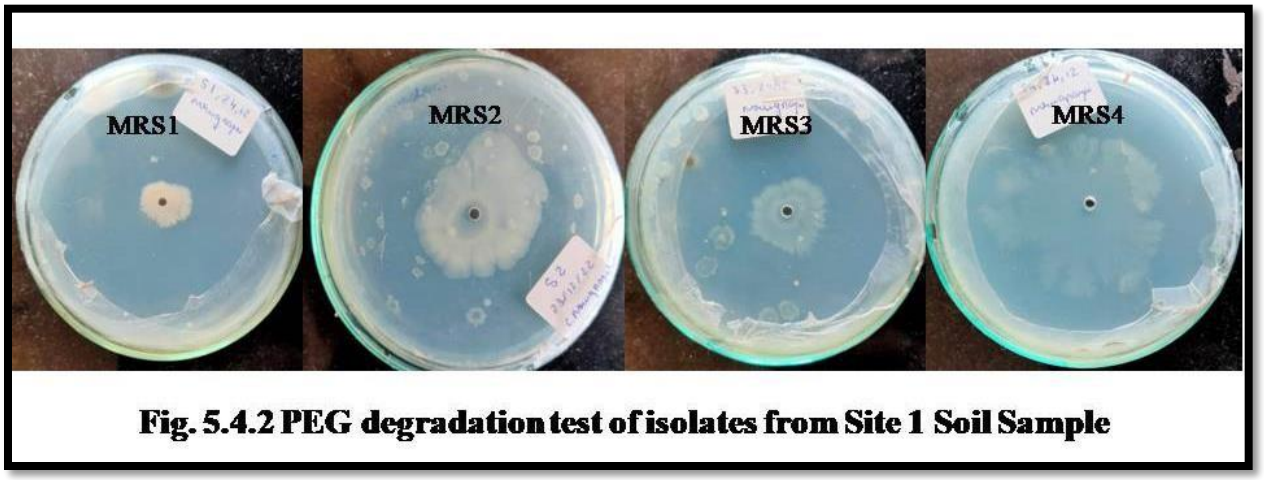


Fig. 5.4.2 PEG degradation test of isolates from Site 1 Soil Sample

5.5 Degradation of different types of plastics using the obtained isolates

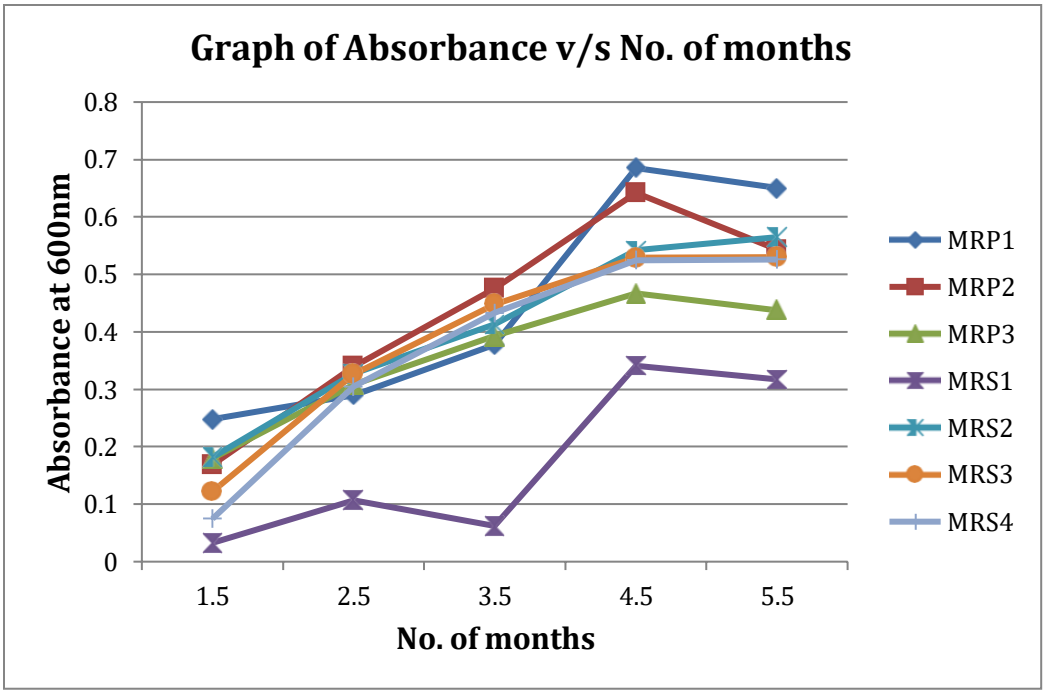
5.5.1 Growth monitoring

Monthly O.D. of the incubated cultures at 600 nm was recorded.

Table 5.5.1- Monthly O.D.. of the incubated cultures at 600 nm

Isolate	O.D. after 1.5 months	O.D. after 2.5 months	O.D. after 3.5 months	O.D. after 4.5 months	O.D. after 5.5 months
MRP1	0.248	0.290	0.377	0.685	0.650
MRP2	0.169	0.340	0.476	0.642	0.543
MRP3	0.180	0.308	0.393	0.467	0.438
MRS1	0.033	0.107	0.062	0.341	0.317
MRS2	0.182	0.327	0.413	0.542	0.565
MRS3	0.122	0.326	0.448	0.529	0.530
MRS4	0.075	0.305	0.433	0.525	0.526

At the end of 5.5 months as per the optical density recorded, the highest growth was shown by MRS2>MRS3> MRS4.



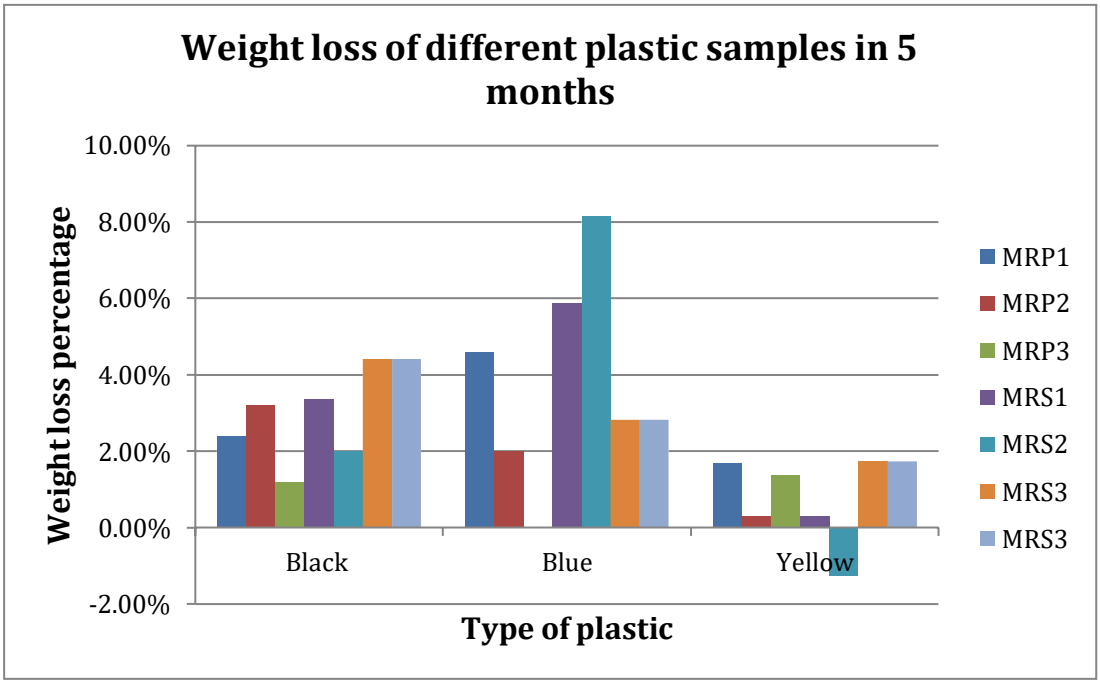
5.5.2 Weight loss in 5.5 months incubation

Weight loss of the incubated plastic samples was calculated after 5.5 months.

Table 5.5.2 Weight loss of the incubated Plastic samples in 5.5 months.

Isolate	Plastic	Initial weight (mg)	Final Weight (mg) 1	2	3	Average final weight (mg)	Difference	Percentage of weight loss
MRP1	Black	11.3	11.0	11.1	11.0	11.03	0.27	2.39%
	Blue	5.0	4.7	4.9	4.7	4.77	0.23	4.6%
	Yellow	10.1	10.0	9.9	9.9	9.93	0.17	1.68%
							Avg=	2.89%
MRP2	Black	10.3	10.0	10.0	9.9	9.97	0.33	3.20%
	Blue	5.0	4.8	5.0	4.9	4.9	0.1	2%
	Yellow	10.1	10.0	10.1	10.1	10.07	0.03	0.3%
							Avg=	1.83%

MRP3	Black	11.0	10.8	10.9	10.9	10.87	0.13	1.18%
	Blue	4.8	4.8	4.8	4.8	4.8	0	0%
	Yellow	9.5	9.4	9.4	9.3	9.37	0.13	1.37%
							Avg=	0.85%
MRS1	Black	11.0	10.7	10.6	10.6	10.63	0.37	3.36%
	Blue	4.6	4.2	4.5	4.3	4.33	0.27	5.87%
	Yellow	9.9	9.8	9.9	9.9	9.87	0.03	0.3%
							Avg=	3.18%
MRS2	Black	11.5	11.3	11.3	11.2	11.27	0.23	2%
	Blue	4.9	4.5	4.6	4.4	4.5	0.4	8.16%
	Yellow	10.2	10.2	10.5	10.3	10.33	-0.13	-1.27%
							Avg=	2.96%
MRS3	Black	11.3	10.8	10.8	10.8	10.8	0.5	4.42%
	Blue	4.6	4.5	4.5	4.4	4.47	0.13	2.83%
	Yellow	9.8	9.6	9.7	9.6	9.63	0.17	1.73%
							Avg=	2.99%
MRS4	Black	10.7	10.4	10.7	10.4	10.5	0.2	1.87%
	Blue	5.3	5.0	5.0	5.1	5.03	0.27	5.09%
	Yellow	8.9	8.6	8.5	8.6	8.57	0.33	3.71%
							Avg=	3.56%



On incubation of the plastic samples with the isolates in minimal media for 5.5 months it was observed that isolates MRS4>MRS1>MRS3> MRS2 showed highest percent of weight loss.

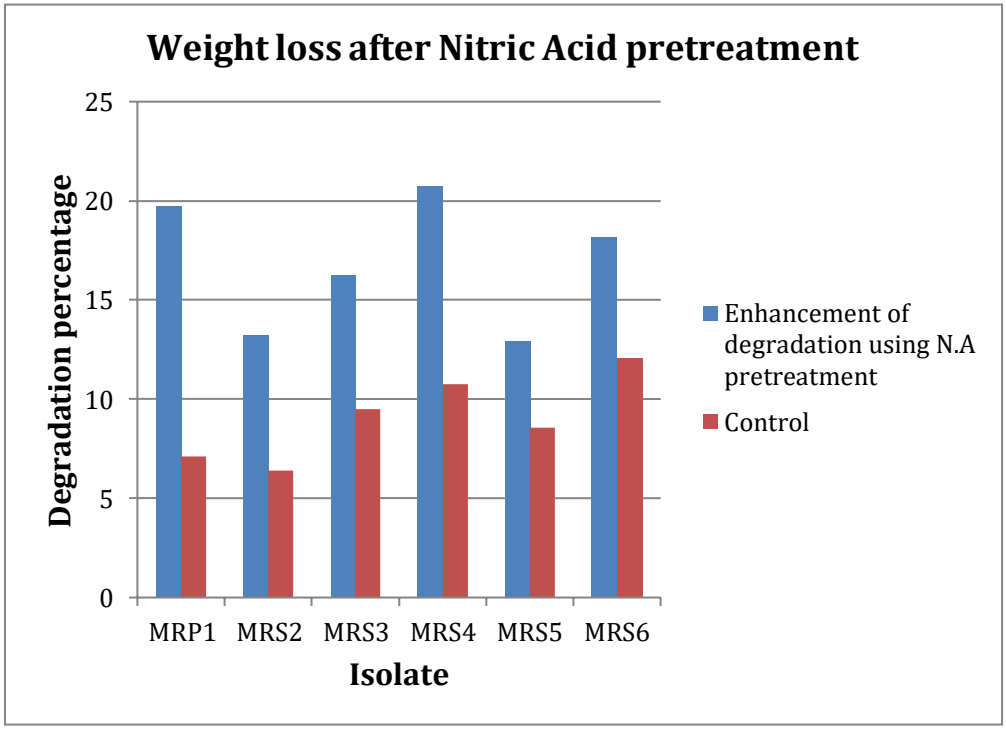
5.6 Enhancement of degradation using pretreatments

5.6.1 Nitric acid (NA) pretreatment

Table 5.6.1.1 Degradation and weight loss by Nitric acid pretreatment

Isolate	Weight before pretreatment (mg)	Weight after pretreatment (mg)	Weight after 2 months incubation (mg) 1	2	3	Average	Percentage of weight loss using pretreatment (%)	Control Weight loss (%)	Enhancement of weight loss% (enhanced weight loss-Control weight loss)
MRP1	3.9	3.4	3.1	3.2	3.1	3.13	19.74	7.1	12.64
MRS2	4.0	3.7	3.4	3.5	3.5	3.47	13.25	6.39	6.86
MRS3	4.3	3.9	3.6	3.6	3.6	3.6	16.27	9.49	6.79
MRS4	4.2	3.6	3.4	3.3	3.3	3.33	20.71	10.75	9.96

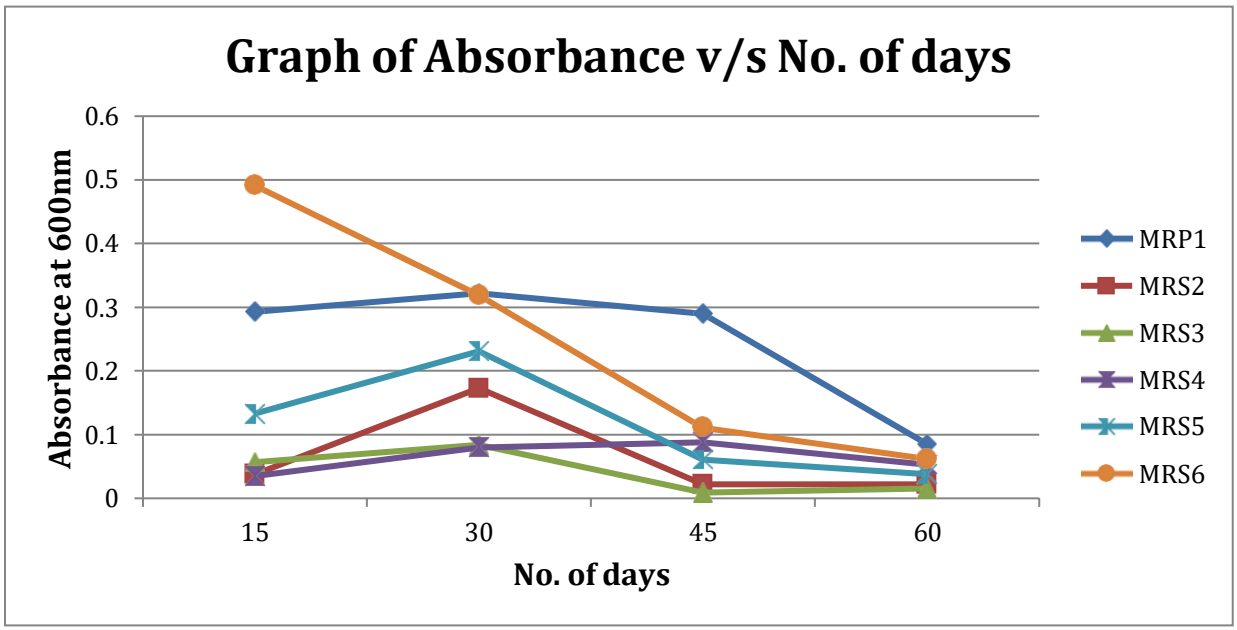
MRS5	4.1	3.6	3.5	3.6	3.6	3.57	12.92	8.57	4.35
MRS6	4.4	3.7	3.6	3.6	3.6	3.6	18.18	12.05	6.13
Control	3.6	3.0	3.0	2.9	2.9	2.93	18.61	15.56	3.05



5.6.1.2 Growth monitoring using Nitric Acid Pretreatment

Table 5.6.1.2 O.D. of the incubated cultures with Nitric Acid pretreatment at 600 nm

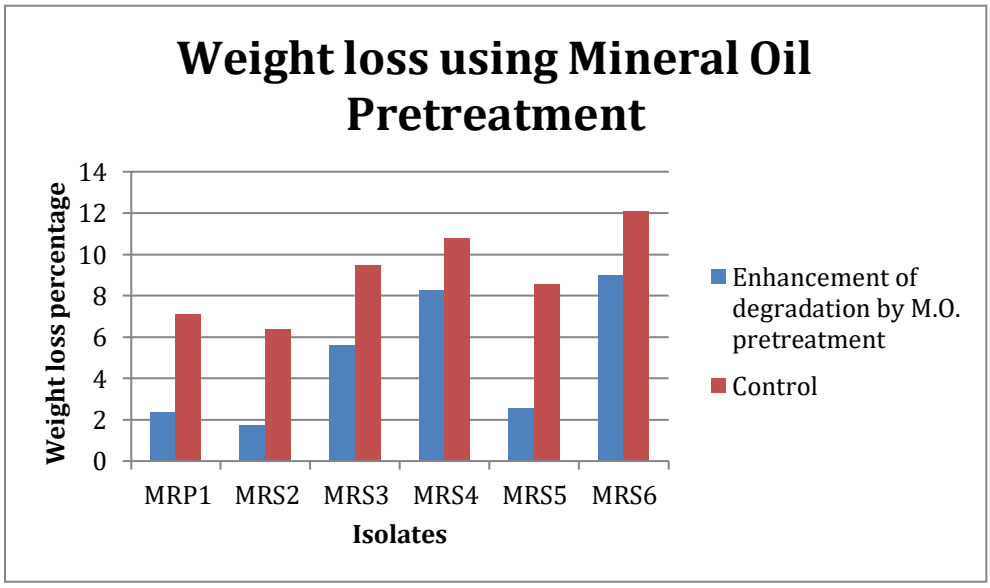
Isolate	15 days	30 days	45 days	60 days
MRP1	0.293	0.322	0.290	0.085
MRS2	0.038	0.173	0.022	0.022
MRS3	0.057	0.084	0.009	0.016
MRS4	0.035	0.080	0.088	0.053
MRS5	0.133	0.231	0.061	0.038
MRS6	0.491	0.319	0.111	0.062



5.6.2 Mineral oil (MO) pretreatment

Table 5.6.2.1 Degradation and weight loss by Mineral Oil pretreatment

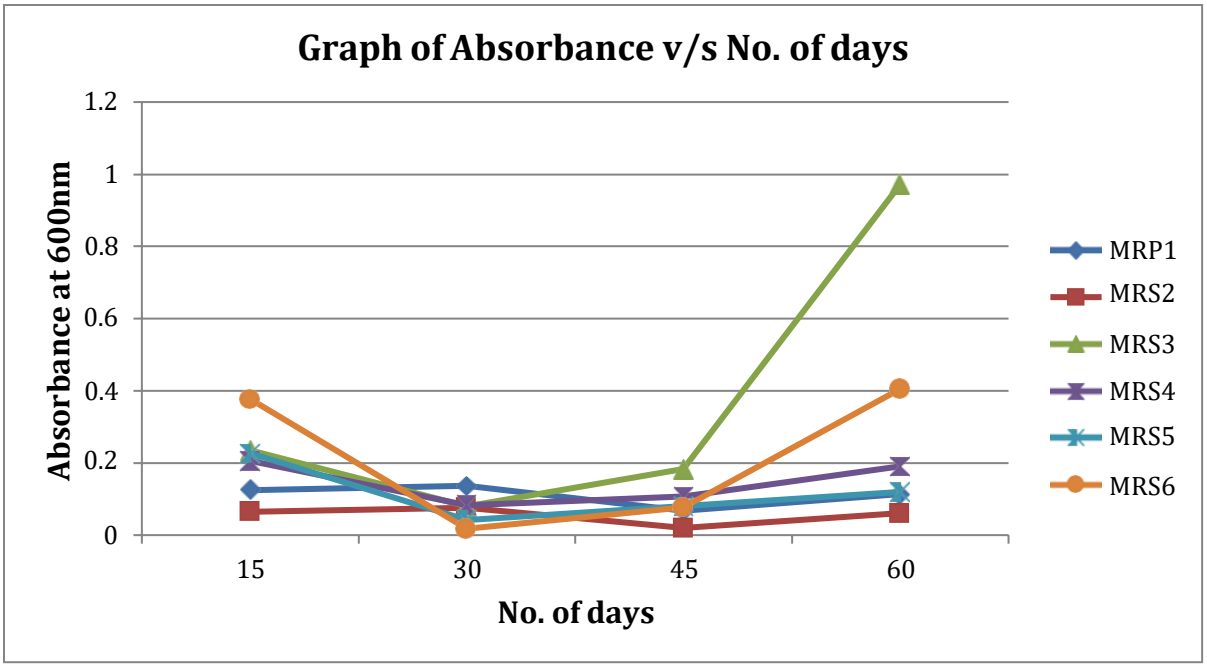
Isolate	Weight before pretreatment (mg)	Weight after 2 months incubation (mg) 1	2	3	Average	Percentage of weight loss using pretreatment (%)	Control weight loss (%)	Enhancement of weight loss% (enhanced weight loss-Control weight loss)
MRP1	4.2	4.1	4.2	4.0	4.1	2.38%	7.1	-4.72
MRS2	4.1	4.1	4.0	4.0	4.03	1.71%	6.39	-4.68
MRS3	4.3	4.1	4.1	4.0	4.06	5.58%	9.49	-3.91
MRS4	4.0	3.7	3.7	3.6	3.67	8.25%	10.75	-2.5
MRS5	3.9	3.7	3.8	3.9	3.8	2.56%	8.57	-6.01
MRS6	4.8	4.5	4.3	4.3	4.37	8.96%	12.05	-3.09
Control	4.4	3.9	4.1	4.1	4.03	8.41%	15.56	-7.15



5.6.2.2 Growth monitoring using Mineral Oil Pretreatment

Table 5.6.2.2 O.D. of inoculated cultures with Mineral Oil pretreatment at 600 nm

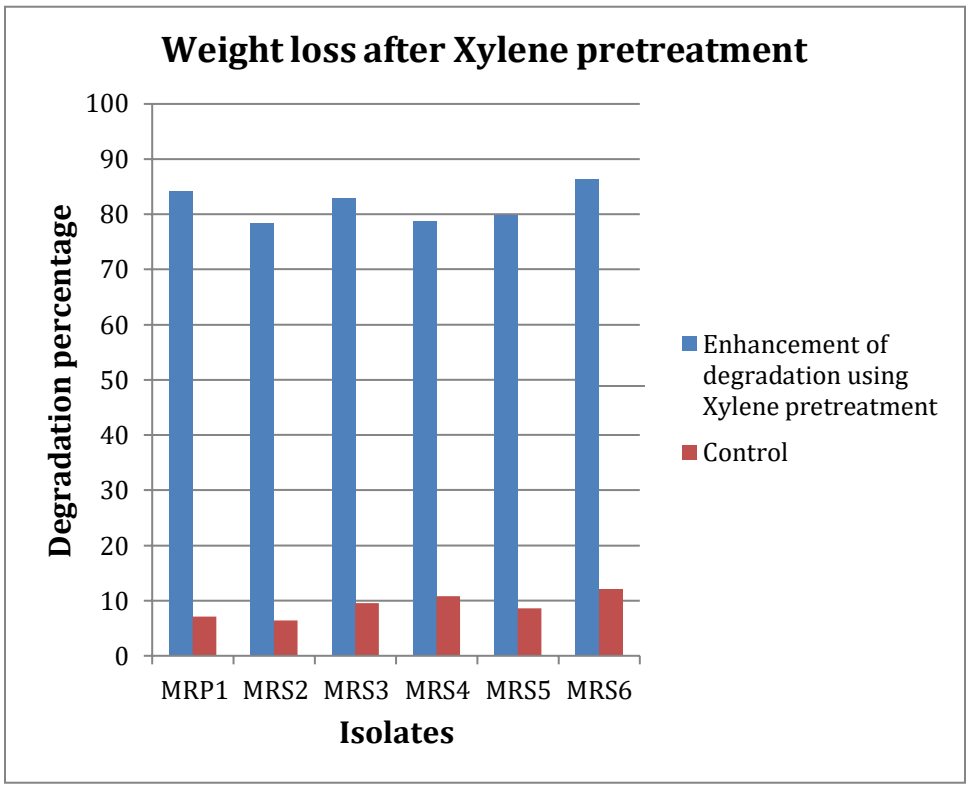
Isolate	15 days	30 days	45 days	60 days
MRP1	0.125	0.137	0.068	0.114
MRS2	0.065	0.076	0.020	0.061
MRS3	0.235	0.080	0.183	0.972
MRS4	0.206	0.083	0.107	0.190
MRS5	0.227	0.042	0.080	0.120
MRS6	0.376	0.018	0.077	0.405



5.6.3 Xylene pretreatment

Table 5.6.3.1 Degradation and weight loss by Xylene pretreatment

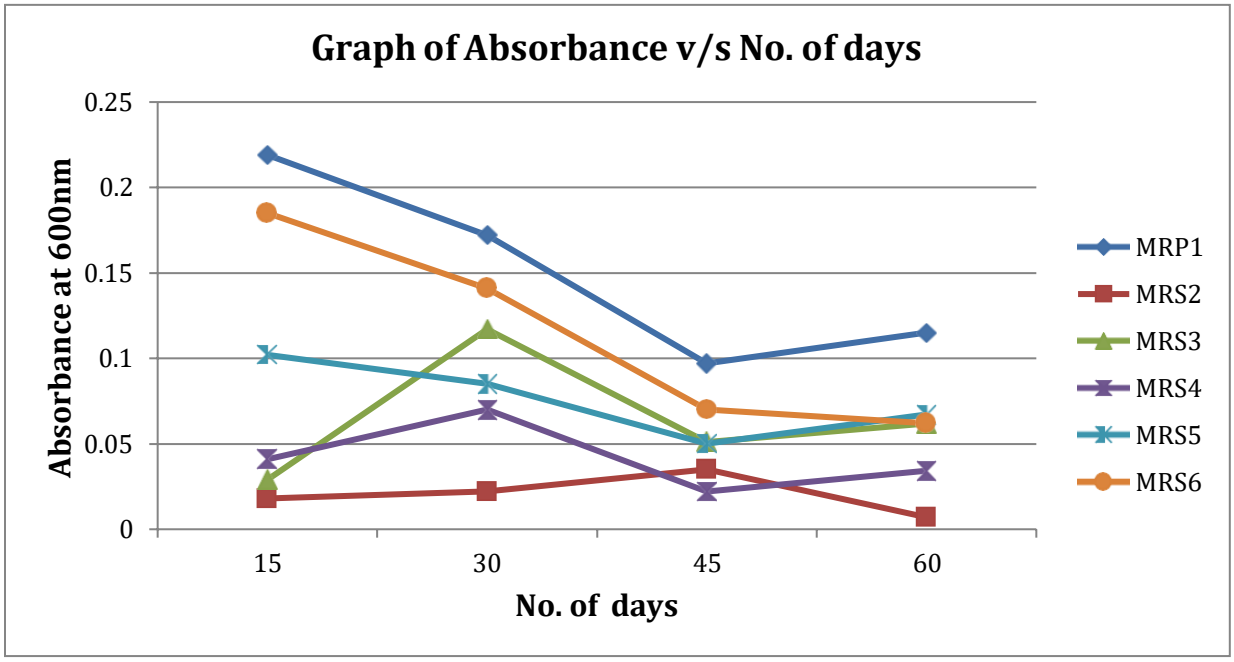
Isolate	Weight before pretreatment (mg)	Weight after pretreatment (mg)	Weight after 2 months incubation (mg) 1	2	3	Average	Percentage of weight loss using pretreatment (%)	Control weight loss (%)	Enhancement of weight loss% (enhanced weight loss-Control weight loss)
MRP1	4.0	0.9	0.7	0.5	0.7	0.63	84.25	7.1	77.15
MRS2	3.7	1.2	0.8	0.8	0.8	0.8	78.37	6.39	71.99
MRS3	3.9	0.8	0.7	0.7	0.6	0.67	82.82	9.49	73.33
MRS4	3.9	1.2	0.8	0.9	0.8	0.83	78.72	10.75	67.97
MRS5	3.8	0.9	0.8	0.7	0.8	0.77	79.74	8.57	71.17
MRS6	3.9	0.7	0.5	0.4	0.7	0.53	86.41	12.05	74.36
Control	3.8	1.4	1.4	1.2	1.1	1.23	67.63	15.56	52.07



5.6.3.2 Growth monitoring with Xylene pretreatment

Table 5.6.3.2 O.D. of inoculated cultures with Xylene pretreatment at 600 nm

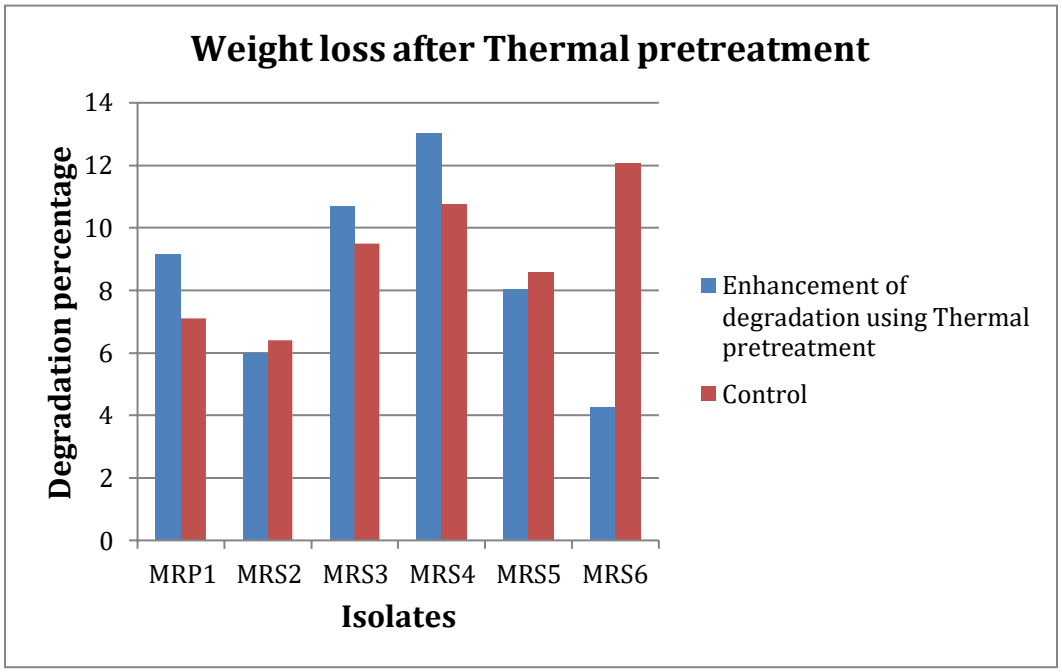
Isolate	15 days	30 days	45 days	60 days
MRP1	0.219	0.172	0.097	0.115
MRS2	0.018	0.022	0.035	0.007
MRS3	0.029	0.117	0.051	0.062
MRS4	0.041	0.070	0.022	0.034
MRS5	0.102	0.085	0.050	0.067
MRS6	0.185	0.141	0.070	0.062



5.6.4 Thermal pretreatment

Table 5.6.4.1 Degradation and weight loss by Thermal pretreatment

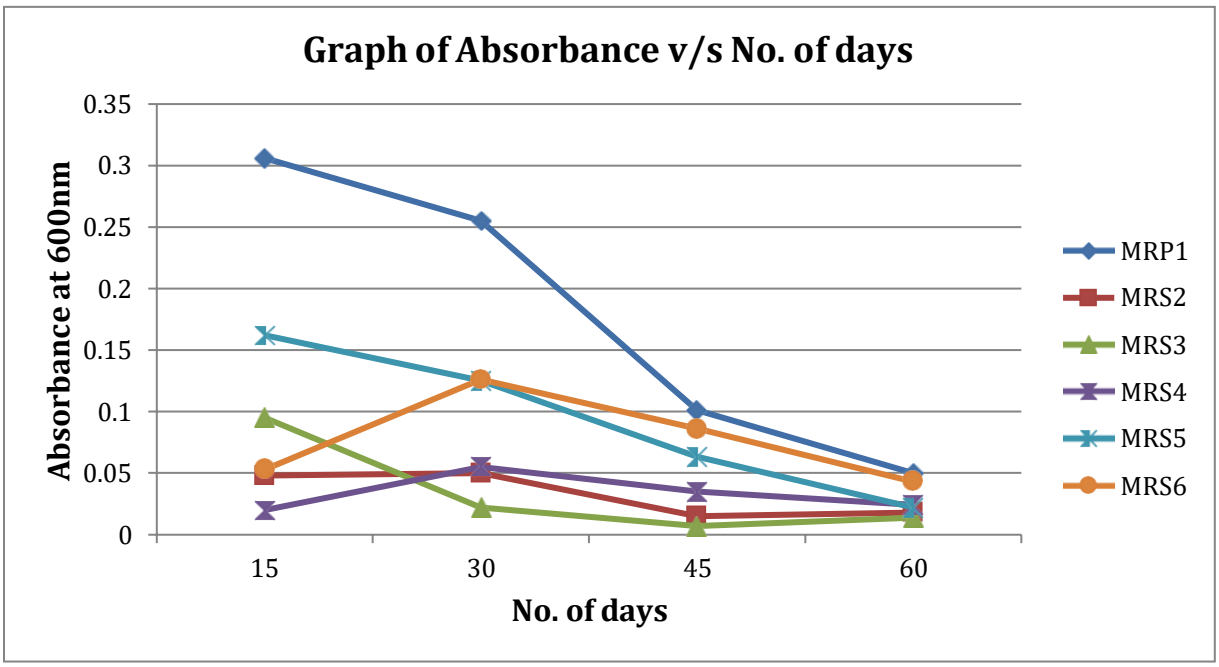
Isolate	Weight before pretreatment (mg)	Weight after pretreatment (mg)	Weight after 2 months incubation (mg) 1	2	3	Average	Percentage of weight loss with pretreatment (%)	Control weight loss %	Enhancement of weight loss% (enhanced weight loss-Control weight loss)
MRP1	4.7	4.6	4.3	4.2	4.3	4.27	9.15	7.1	2.048936
MRS2	4.5	4.3	4.2	4.3	4.2	4.23	6	6.39	-0.39
MRS3	4.4	4.3	3.9	3.9	4.0	3.93	10.68	9.49	1.191818
MRS4	4.6	4.4	4.0	4.0	4.0	4.0	13.04	10.75	2.293478
MRS5	4.6	4.6	4.2	4.2	4.3	4.23	8.04	8.57	-0.52652
MRS6	4.7	4.6	4.5	4.5	4.5	4.5	4.25	12.05	-7.79468
Control	5.3	4.7	4.7	4.7	4.6	4.67	11.89	15.56	-3.67321



5.6.4.2 Growth monitoring with Thermal pretreatment

Table 5.6.4.2 O.D. of the inoculated cultures with Thermal pretreatment at 600nm

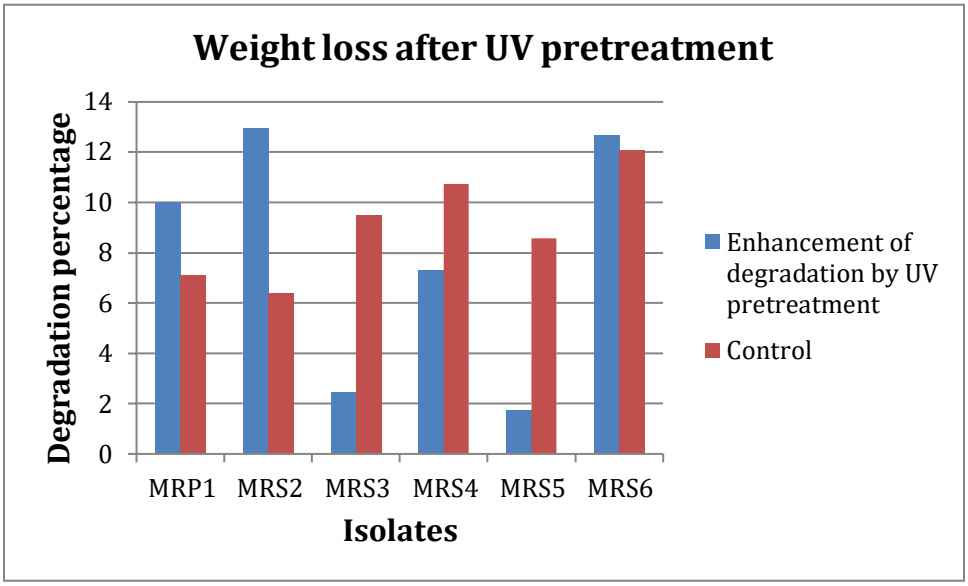
Isolate	15 days	30 days	45 days	60 days
MRP1	0.306	0.255	0.101	0.050
MRS2	0.048	0.050	0.015	0.018
MRS3	0.095	0.022	0.007	0.014
MRS4	0.020	0.055	0.035	0.024
MRS5	0.162	0.125	0.063	0.022
MRS6	0.053	0.126	0.086	0.043



5.6.4 UV pretreatment

Table 5.6.5.1 Degradation and weight loss by UV pretreatment

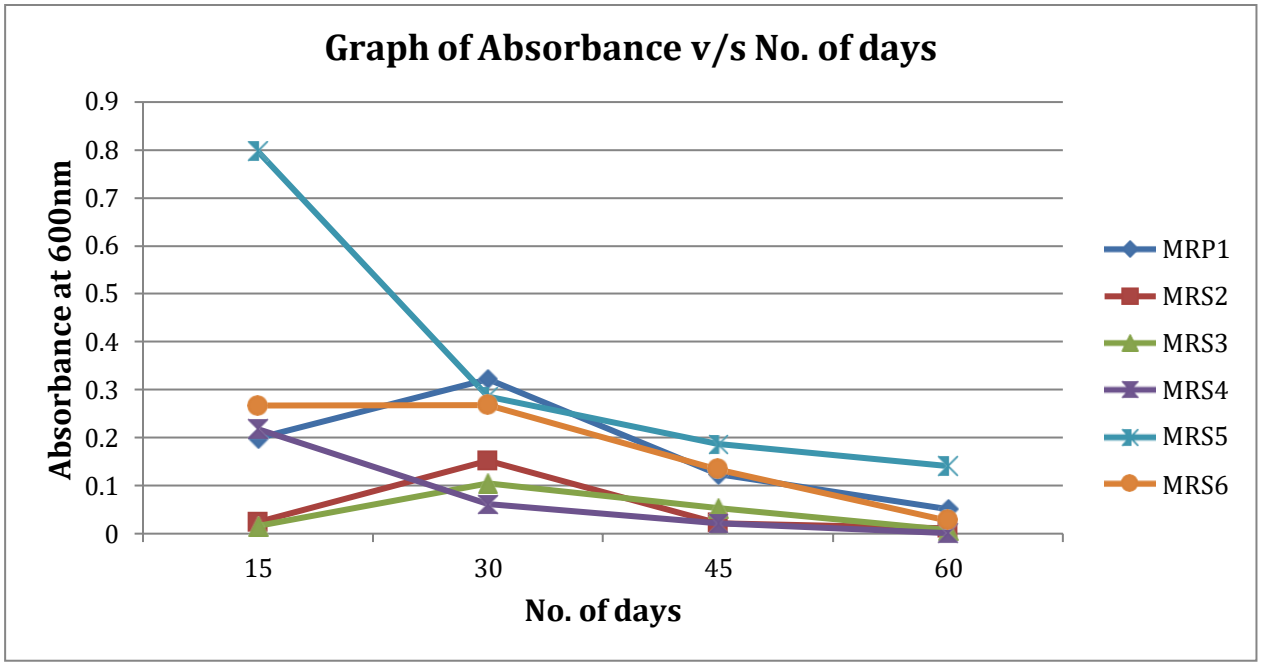
Isolate	Weight before pretreatment (mg)	Weight after pretreatment (mg)	Weight after 2 months incubation (mg) 1	2	3	Average	Percentage of weight loss with pretreatment (%)	Control weight loss %	Enhancement of weight loss% (enhanced weight loss-Control weight loss)
MRP1	4.3	4.3	3.9	3.9	3.8	3.87	10	7.1	2.9
MRS2	4.1	3.9	3.5	3.6	3.6	3.57	12.93	6.39	6.54
MRS3	4.1	4.1	4.0	4.0	4.0	4.0	2.44	9.49	-7.05
MRS4	3.7	3.6	3.5	3.4	3.4	3.43	7.29	10.75	-3.45
MRS5	4.0	4.0	3.9	3.9	4.0	3.93	1.75	8.57	-6.82
MRS6	4.5	4.3	4.0	3.9	3.9	3.93	12.67	12.05	0.62
Control	4.9	4.7	4.2	4.2	4.5	4.3	12.24	15.56	-3.32



5.6.5.2 Growth monitoring with UV pretreatment

Table 5.6.5.2 O.D. of the incubated cultures with UV pretreatment at 600 nm.

Isolate	15 days	30 days	45 days	60 days
MRP1	0.199	0.322	0.124	0.051
MRS2	0.024	0.152	0.022	0.011
MRS3	0.016	0.105	0.053	0.008
MRS4	0.218	0.061	0.021	0.001
MRS5	0.797	0.286	0.187	0.141
MRS6	0.267	0.268	0.134	0.027



5.6.6 Control of Untreated plastic samples

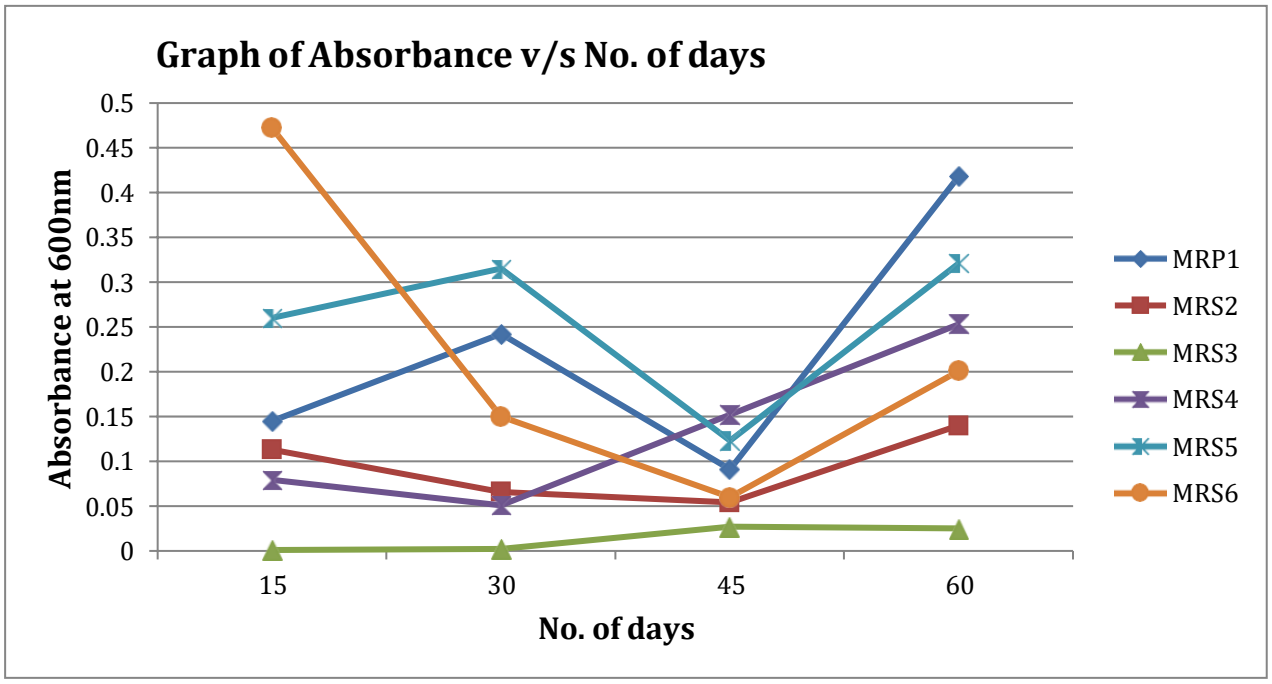
Table 5.6.6.1 Degradation and weight loss of Untreated plastic sample

Isolate	Weight before pretreatment (mg)	Weight after 2 months incubation (mg) 1	2	3	Average	Percentage of weight loss after incubation
MRP1	3.8	3.5	3.5	3.6	3.53	7.10%
MRS2	3.6	3.4	3.3	3.4	3.37	6.39%
MRS3	3.9	3.5	3.6	3.5	3.53	9.49%
MRS4	4.0	3.5	3.6	3.6	3.57	10.75%
MRS5	3.5	3.3	3.1	3.2	3.2	8.57%
MRS6	3.9	3.5	3.5	3.3	3.43	12.05%
Control	4.5	3.8	3.8	3.8	3.8	15.56%

5.6.6.2 Growth monitoring of Untreated plastic samples

Table 5.6.6.2 O.D.. of the incubated cultures with no pretreatment at 600nm.

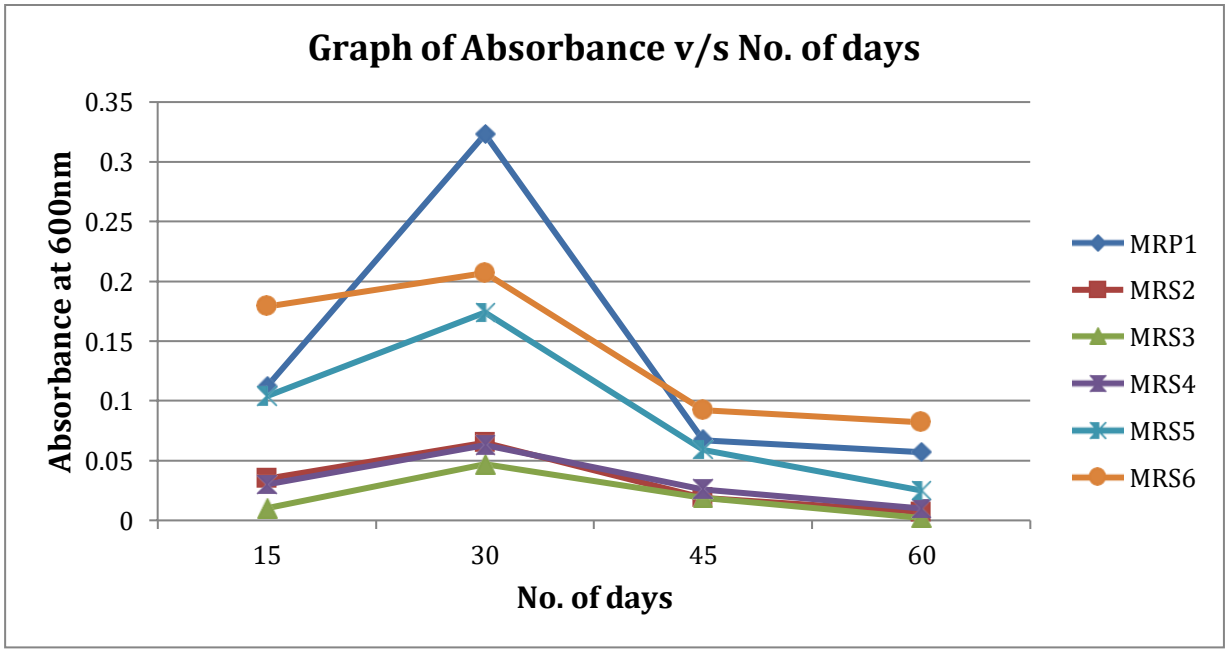
Isolate	15 days	30 days	45 days	60 days
MRP1	0.145	0.242	0.091	0.418
MRS2	0.113	0.066	0.054	0.140
MRS3	0.001	0.002	0.027	0.025
MRS4	0.079	0.051	0.152	0.253
MRS5	0.260	0.315	0.123	0.321
MRS6	0.472	0.150	0.060	0.201



5.7 Incubation of control of Bushnell Hass Broth with the isolate and no carbon source.

Table 5.7.1 O.D. at 600 nm of isolates without carbon source

Isolate	15 days	30 days	45 days	60 days
MRP1	0.112	0.323	0.067	0.057
MRS2	0.035	0.065	0.019	0.007
MRS3	0.010	0.047	0.019	0.002
MRS4	0.030	0.063	0.026	0.010
MRS5	0.104	0.174	0.059	0.025
MRS6	0.179	0.207	0.092	0.082



5.8 Biofilm Assay

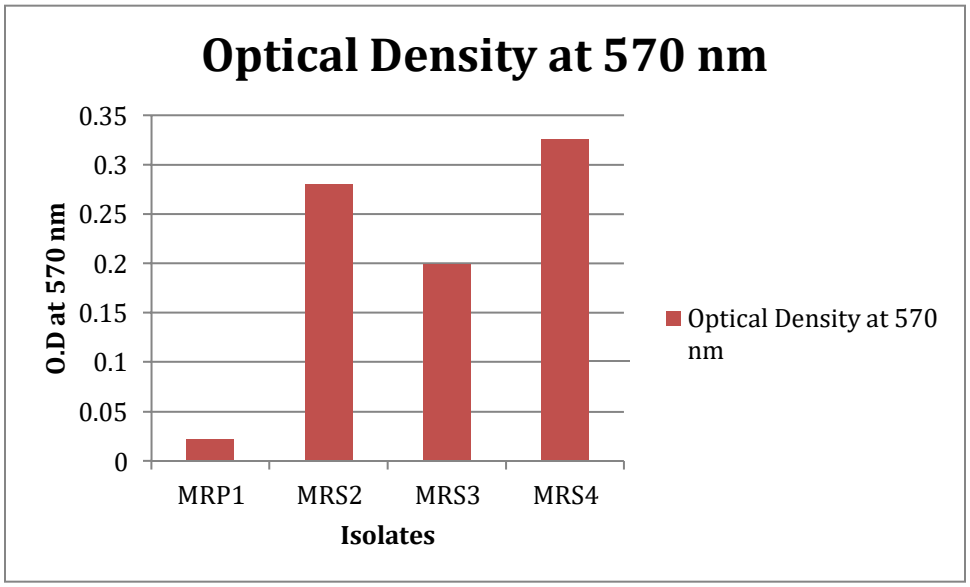
Best four isolates that showed highest growth in the PEG supplemented medium as was as highest weight loss percentage of the 5.5 month incubation were selected for the biofilm assays. The selected isolates were MRP1, MRS2, MRS3, MRS4.

After performing the biofilm assay the optical density was recorded at 570nm.

Table 5.8.1 Optical density for the biofilm assay.

Isolate	Optical density at 570 nm
MRP1	0.022
MRS2	0.280
MRS3	0.199
MRS4	0.326

The Optical Density recorded was in the order MRS4>MRS2>MRS3>MRP1.



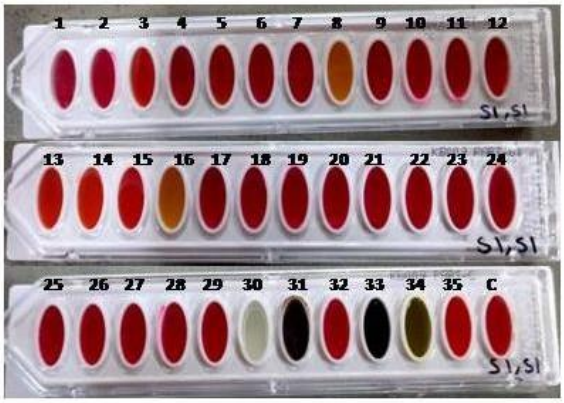
5.9 Biochemical tests

The best four isolates were selected to check their ability to utilize a wide range of carbohydrate substrates. The selected isolates were MRS1, MRS2, MRS3, MRS4.

Table 5.9.1 Biochemical tests of the selected isolates

No.	Test	MRS1		MRS2		MRS3		MRS4	
		24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
1	Lactose	-	-	-	-	-	-	-	-
2	Xylose	-	-	-	-	-	-	-	-
3	Maltose	-	-	-	-	-	-	-	-
4	Fructose	-	-	-	-	-	-	-	-
5	Dextrose	-	-	-	-	-	-	-	-
6	Galactose	-	-	-	-	-	-	-	-
7	Raffinose	-	-	-	-	-	-	-	-
8	Trehalose	+	+	-	-	-	-	-	-
9	Melibiose	-	-	-	-	-	+	-	-
10	Sucrose	-	-	-	-	-	-	-	-
11	L- Arabinose	-	-	-	-	-	+	-	-
12	Mannose	-	-	-	-	-	-	-	-
13	Inulin	-	-	-	-	-	-	-	-
14	Sodium gluconate	-	-	-	-	-	-	-	-
15	Glycerol	+	-	-	-	-	-	-	-
16	Salicin	-	+	-	-	-	-	-	-

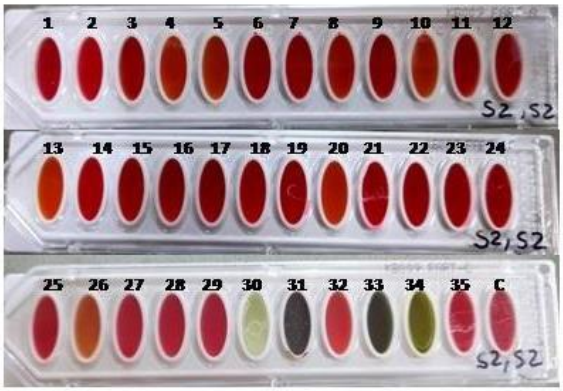
17	Dulcitol	-	-	-	-	-	-	-	-
18	Inositol	-	-	-	-	-	-	-	-
19	Sorbitol	-	-	-	-	-	-	-	-
20	Mannitol	-	-	-	-	-	-	-	-
21	Adonitol	-	-	-	-	-	-	-	-
22	Arabitol	-	-	-	-	-	-	-	-
23	Erythritol	-	-	-	-	-	-	-	-
24	α-Methyl-D-glucoside	-	-	-	-	-	-	-	-
25	Rhamnose	-	-	-	-	-	-	-	-
26	Cellobiose	-	-	-	-	-	-	-	-
27	Melezitose	-	-	-	-	-	-	-	-
28	α-Methyl-D-mannoside	-	-	-	-	-	-	-	-
29	Xylitol	-	-	-	-	-	-	-	-
30	ONPG	-	+	-	+	-	+	-	+
31	Esculin hydrolysis	+	+	+	+	+	+	+	+
32	D-Arabinose	-	-	-	-	-	-	-	-
33	Citate utilization	-	+	-	-	-	+	-	+
34	Malonate utilization	-	-	-	-	-	+	-	-
35	Sorbose	-	-	-	-	-	-	-	-



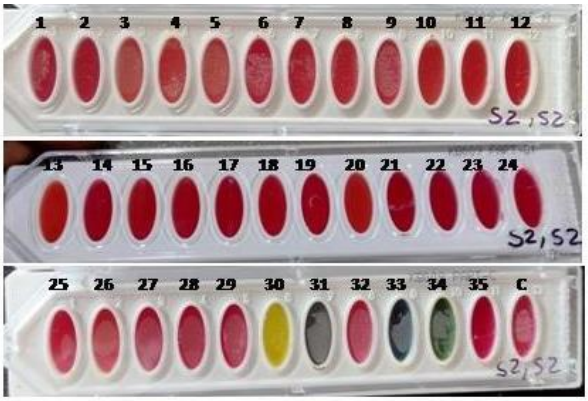
24 hrs

48 hrs

Fig. 5.9.1.Carbohydrate utilization test results of isolate MRS1

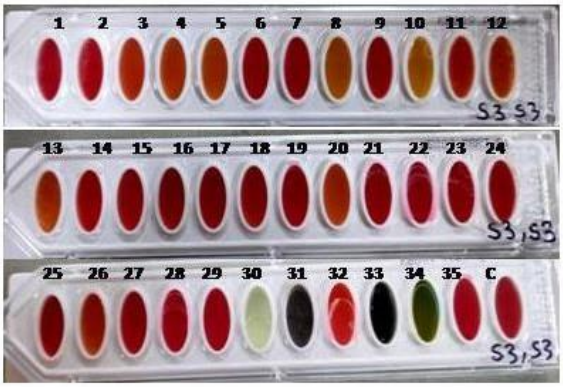


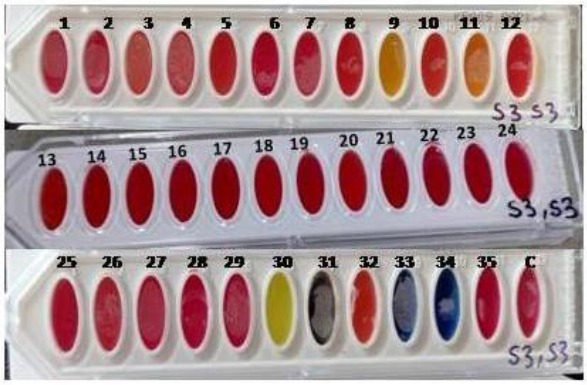
24 hrs



48 hrs

Fig. 5.9.2Carbohydrate utilization test results of isolate MRS2

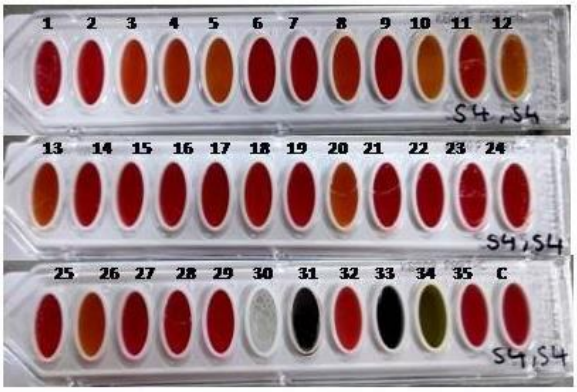




24 hrs

48 hrs

Fig.5.9.3. Carbohydrate utilization test results of isolate MRS3



24 hrs

48 hrs

Fig.5.9.4. Carbohydrate utilization test results of isolate MRS4

5.10 Enzyme assays

Enzyme assays were carried out with the best isolates (MRS1, MRS2, MRS3, MRS4).

Table 5.10.1 Enzyme assays

Isolate	Lipase enzyme production	Protease enzyme production	Catalase enzyme production	Oxidase enzyme production
MRS1	+ve	-ve	+ve	-ve
MRS2	+ve	+ve	+ve	-ve
MRS3	+ve	+ve	+ve	-ve
MRS4	+ve	+ve	-ve	-ve

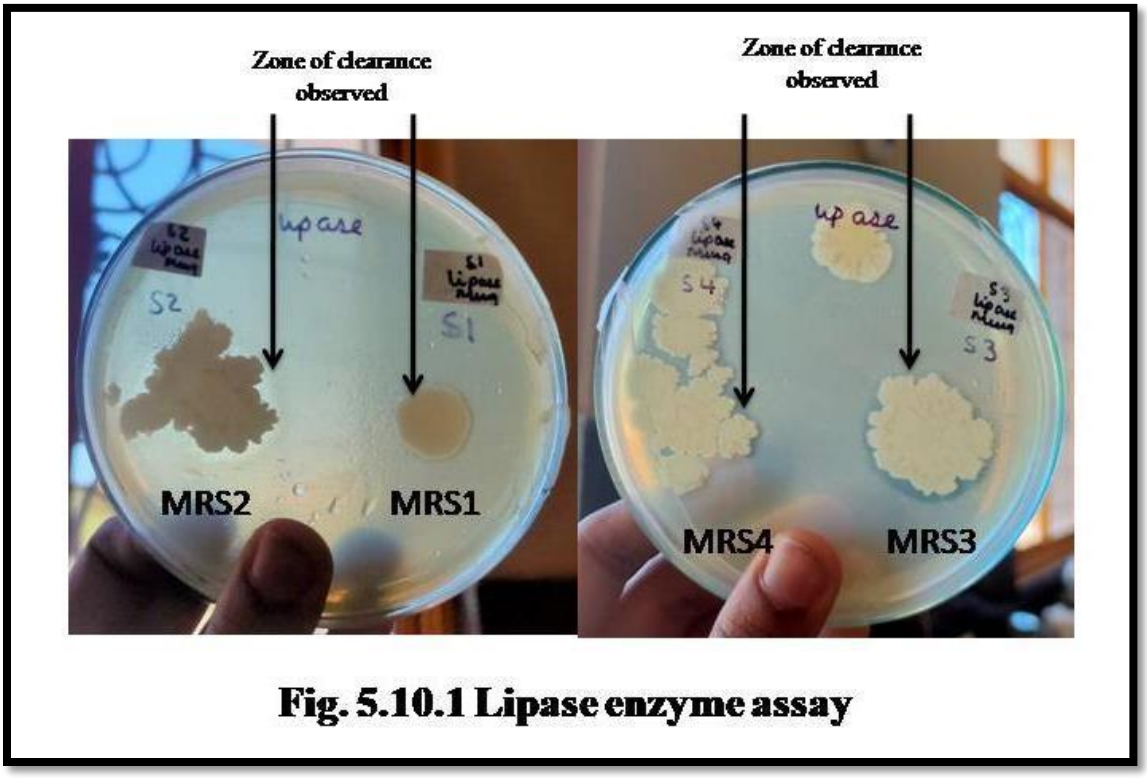


Fig. 5.10.1 Lipase enzyme assay

Fig. 5.10.2 Protease enzyme assay

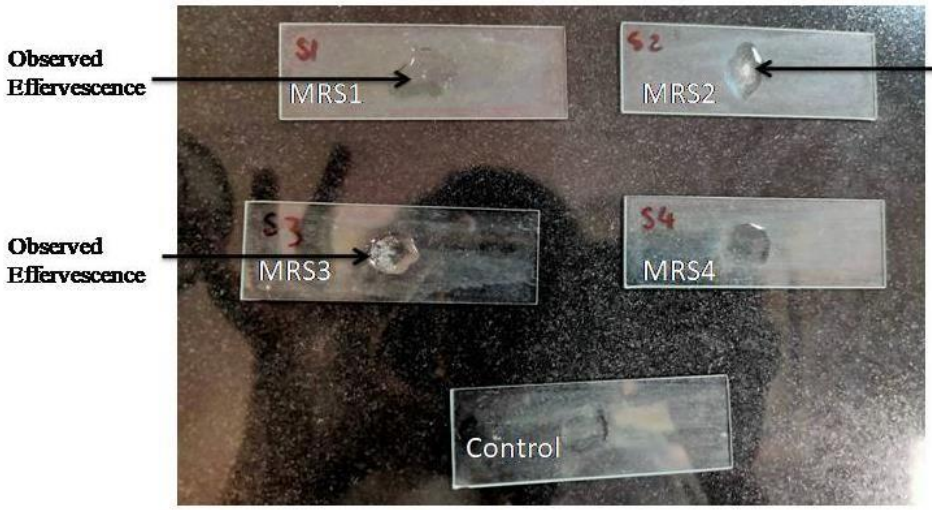


Fig. 5.10.3 Catalase enzyme assay

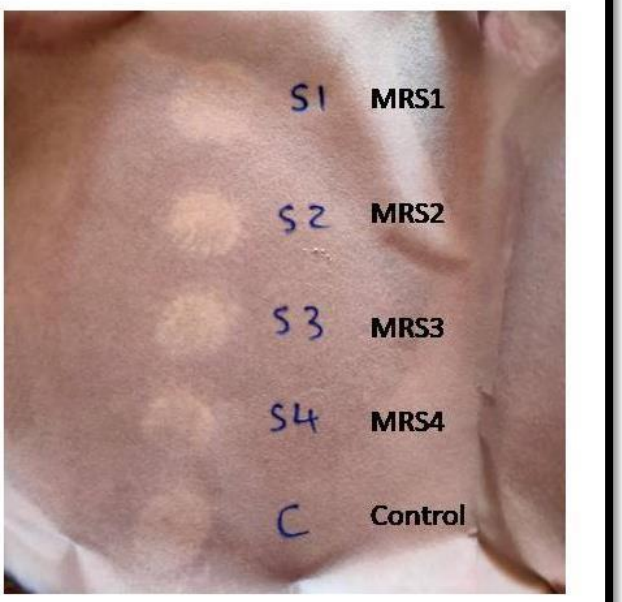


Fig. 5.10.4 Oxidase enzyme assay

5.11 SEM Analysis

SEM analysis of the degraded plastic samples showing maximum weight loss percentage (MRS1, MRS4) was carried out. The analysis showed evidences of cracks and degradation on the plastic surface.

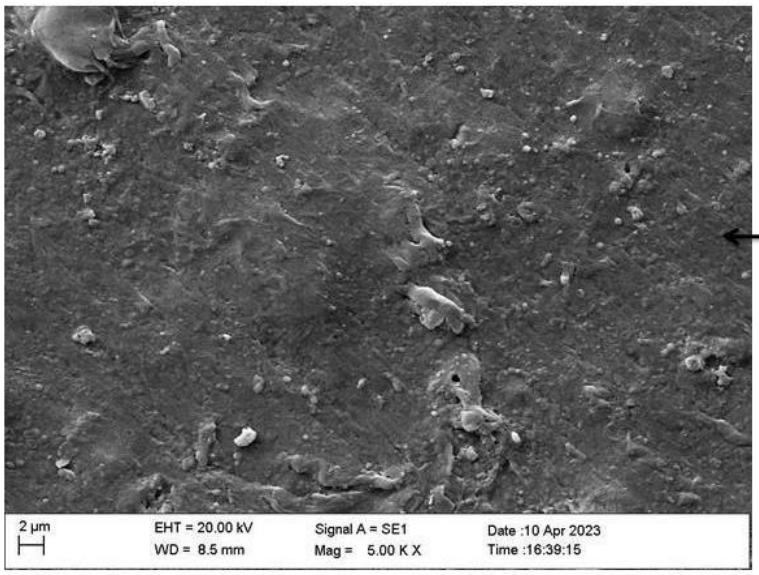


Fig. 5.11.1 SEM image of Control

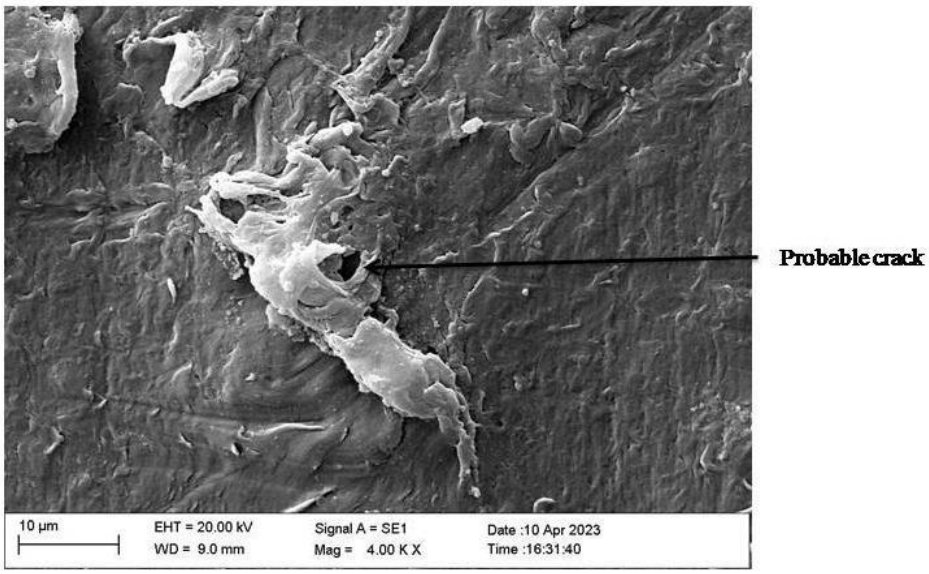


Fig. 5.11.2 SEM image of MRS1

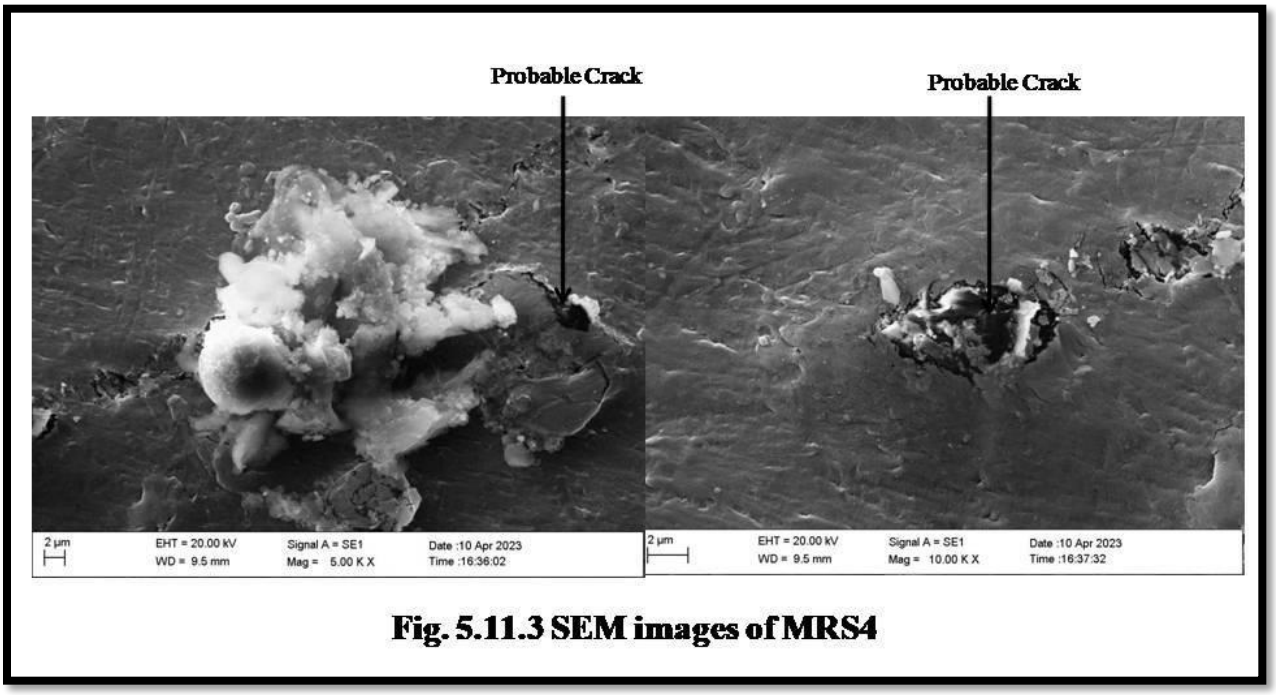


Fig. 5.11.3 SEM images of MRS4

5.12 EDS analysis

The EDS analysis was performed on two test plastic samples that showed maximum weight loss percentage during the 5 months incubation (i.e. blue coloured LDPE degraded with isolate MRS1 and blue coloured LDPE degraded with isolate MRS4) along with a control. The resulting graph gave an estimation of the carbon content in the samples. The EDS analysis revealed that the carbon content of the test plastic samples was lower than the control.

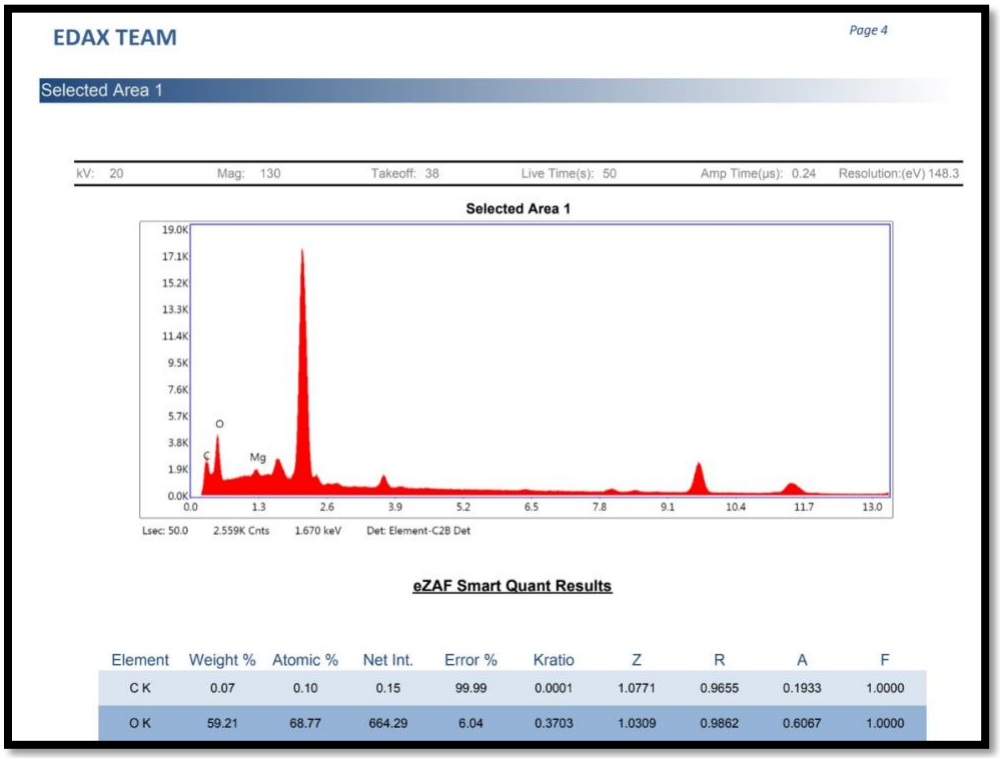


Fig. 5.12.1 EDS analysis of Control

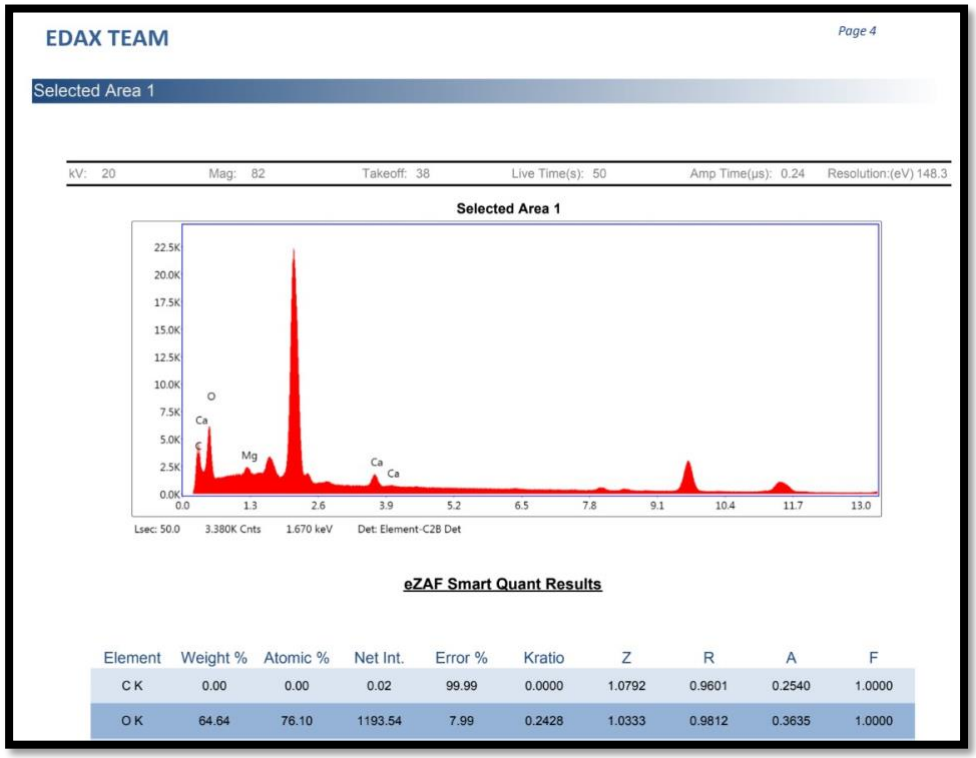
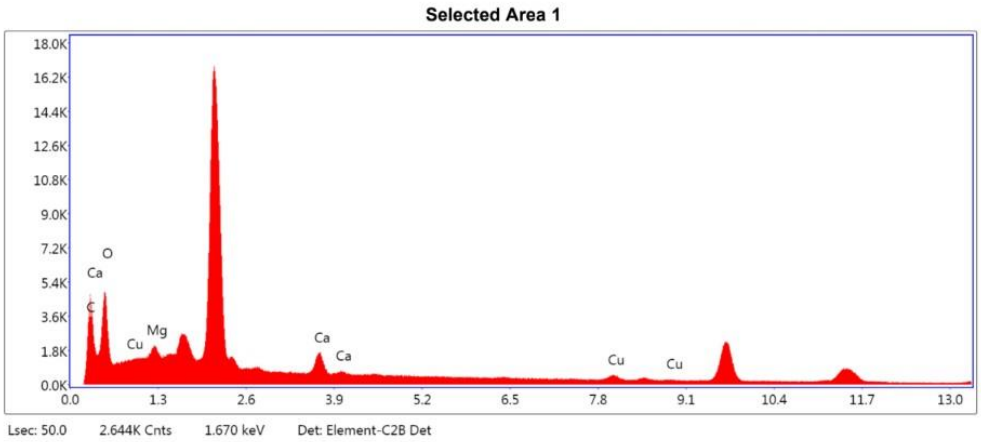


Fig. 5.12.2 EDS analysis of plastic sample incubated with isolate MRS1

Selected Area 1

kV: 20 Mag: 153 Takeoff: 38 Live Time(s): 50 Amp Time(µs): 0.24 Resolution:(eV) 148.3



eZAF Smart Quant Results

Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	R	A	F
C K	0.00	0.01	0.02	99.99	0.0000	1.1012	0.9457	0.2404	1.0000
O K	56.93	72.56	861.21	8.24	0.2096	1.0554	0.9677	0.3488	1.0000

Fig. 5.12.3 EDS Analysis of plastic sample incubated with isolate MRS 4

6 Discussion

6.1 Isolation and enrichment

The plastic sample and the plastic associated soil sample were inoculated in minimal media for 48 hrs. since there was no additional source of carbon except for the plastic, organisms that potentially could utilize plastic as carbon source were isolated. A total of 9 bacterial cultures were obtained and purified from the two sites and named MRP1, MRP2, MRP3, MRS1, MRS2, MRS3, MRS4, MRS5 and MRS6.

6.2 PEG degradation test

The isolated cultures were incubated in wells on MSM media plates along with PEG powder as the sole source of carbon for 2-4 weeks. The growth on PEG supplemented media indicates that the isolates have the ability to utilize the polyethylene as the sole source of carbon (Divyalakshmi & Subhashini, 2016). The diameter of the growing colonies are directly proportional to their potential ability to utilize and hence degrade polyethylene. According to this screening technique the polyethylene degradation potential of the isolates was in the descending order MRS4, MRS2, MRP1, MRS3, MRP3, MRS1, MRP2, with MRS4 showing the maximum ability. In accords with this test the isolates MRS4, MRS2, MRP1 and MRS3 were used for further analysis like degradation with pretreatments along with the previously screened isolated MRS5 and MRS6.

6.3. Degradation of different types of plastics using the obtained isolates

The low density polyethylene (LDPE) is one of the most widely used and commercially available plastic which can make up one of the major components of the generated plastic waste. LDPE plastics show the presence of major chain branching and long side chains; hence it is less dense and crystalline. This makes it more flexible and a thinner form of plastic. However this kind of plastic is generally single use and is quite difficult to recycle (Bahraini, 2018). However even in

LDPE various different types are available in the market. Three of the commonly available LDPE types were used for this experiment (Blue coloured LDPE, Black coloured LDPE and Yellow coloured LDPE). These different types of plastics showed a difference in their weights and densities with the Black coloured LDPE being the heaviest and the Blue coloured LDPE being the lightest.

O.D. measurements of the incubated isolates revealed that the isolates MRS2, MRS3, MRS4 showed more growth in the order MRS2>MRS3> MRS4. The other isolates entered the stationary phase at 4.5 months.

The weight loss percentage of the inoculated plastic samples was calculated using the formula:

$$\text{Percentage of weight loss (\%)} = \frac{Initial\ weight - Final\ weight}{Initial\ weight} \times 100$$

It was observed that on an average the blue coloured LDPE showed the maximum percentage of weight loss (4.08%), followed by the black coloured LDPE (2.63%) and Yellow coloured LDPE showed the least degradation (1.12%). The light weight of the weight of the Blue coloured LDPE could have been allowed its easy degradation.

The maximum degradation was caused by the isolate MRS4, followed by MRS1, MRS3, MRS2, MRP1 and MRP2 respectively.

The degradation of the yellow coloured LDPE with the isolate MRS2 showed an increase in weight by 1.27%. this could however be due to the bacterial biofilm still being attached to the degraded plastic sample during the weight measurement after the incubation period.

6.4 Enhancement of degradation using pretreatments

6.4.1. Nitric acid pretreatment.

The graph of O.D. at 600 nm for the Nitric Acid pretreatment showed that the isolates MRP1, MRS5, MRS2 and MRS3 peaked at 30 days before entering the stationary phase. However MRS6 peaked at 15 days before entering the stationary phase. The isolate MRS4 showed the longest log phase peaking at 45 days before entering the stationary phase.

According to the weight loss percentage calculations after Nitric acid pretreatment, the pretreatment enhanced the degradation by 7.79% on an average. The isolate MRP1 showed the maximum degradation using this pretreatment followed by MRS3, MRS4, MRS2, MRS6 and MRS5 respectively.

6.4.2. Mineral Oil Pretreatment

According to the graph of O.D. at 600nm isolates MRP1 and MRS2 peaked at 30 days before entering the stationary phase. The isolates MRS3, MRS4, MRS5 and MRS6 entered stationary phase after peaking at 15 days.

According to the weight loss percentage calculations the average weight loss by the mineral oil pretreatment was 4.91%. Which was lower compared to the control of untreated plastic which showed an average degradation percentage of 9.06%. Hence the Mineral oil pretreatment was not found to be enhancing degradation. However the isolate MRS6 showed the maximum degradation using Mineral oil pretreatment.

6.4.3 Xylene Pretreatment

The graph of O.D. at 600 nm for the Xylene pretreatment showed that the isolates MRP1, MRS5, and MRS6 peaked at 15 days before entering the stationary phase. However MRS3 and MRS4

peaked at 30 days before entering the stationary phase. The isolate MRS2 showed the longest log phase peaking at 45 days before entering the stationary phase.

The Xylene pretreatment showed a very significant enhancement in the degradation of plastics with an average percentage of 72.66% degradation after the pretreatment. The isolate MRS6 showed the maximum degradation of plastic with the Xylene pretreatment followed by MRP1, MRS3, MRS5, MRS4 and MRS2 respectively.

6.4.4. Thermal Pretreatment

According to the graph of O.D. at 600nm isolates MRP1, MRS3 and MRS5 peaked at 15 days before entering the stationary phase. The isolates MRS2, MRS4 and MRS6 showed the longest log phase and peaked at 30 days before entering the stationary phase.

The thermal pretreatment enhanced the degradation only for the isolates MRP1, MRS3 and MRS4. While for the other isolates the degradation percentage was lower than the control. The isolate MRS4 showed the highest degradation with the thermal pretreatment.

6.4.5 UV pretreatment

The graph of O.D. at 600 nm for the UV pretreatment showed that the isolates MRS4 and MRS5 peaked at 15 days before entering the stationary phase. However MRP1, MRS2, MRS3 and MRS6 showed a longer log phase with a peak at 30 days before entering the stationary phase.

The UV pretreatment only enhanced the degradation with the isolates MRP1, MRS2 and MRS6 with the highest degradation occurring with the isolate MRS2.

On an average over all the pretreatments the isolate MRS6 was the most efficient in plastic degradation capability.

Overall the Xylene pretreatment was observed to be the most efficient in the process of enhancement of plastic degradation.

6.5 Incubation of Broth with isolates

A control of Bushnell Hass Broth along with the isolates and no carbon source was maintained to check the survival of the isolates. The graph of the O.D. at 600 nm showed the peak for all isolates (MRP1, MRS2, MRS3, MRS4, MRS5 and MRS6) at 30 days. The log phase however was observed to be shorter for certain isolates in some of the pretreatments mentioned above. This could be due to the accumulation of the degradation products of plastic degradation.

6.6 Biofilm Assay

The O.D. at 570 nm was used to quantify the biofilm production in the crystal violet biofilm assay used. The ability of the isolates to adhere to a substratum correlates with their ability to form a biofilm. Higher adhesion results in higher intake of crystal violet resulting in an higher absorbance value. Thus indicating that higher the absorbance value, better is the biofilm formation ability of the isolate. According to this assay the biofilm formation ability was in the order MRS4, MRS2, MRS3 and MRP1 with MRS4 having the maximum biofilm formation. The biofilm forming ability of the isolate MRS4 could have been responsible for it's good performance across all the experiments.

6.7 Biochemical tests

The HiMedia KB009 kit Part A, B1 and C were used for the carbohydrate utilization tests. It was observed that the isolates were not utilizing some of the very commonly found carbohydrates like Lactose, Maltose, Fructose, Dextrose, Sucrose etc. This was one of the unique features observed in the obtained isolates. The inability of the isolates to utilize the common carbohydrates as a carbon source could have resulted in them utilizing plastic as a carbon source, hence resulting in it's degradation.

6.8 Enzyme assays

Potential plastic degrading bacteria have the ability to produce several catabolic enzymes that are involved in plastic degradation. These enzymes are particularly hydrolases or oxidation enzymes. It is difficult to degrade plastics due to their hydrophobicity. The enzymes involved in plastic degradation are commonly surface modifying (eg. Lipases, proteases) and help in increasing the hydrophilicity of the plastic surface resulting in better degradation (Cobongela, 2021).

6.8.1 Lipase assay

The isolates were screened for lipase enzyme production by spot inoculating on tributyrin agar plates with 1%w/v tributyrin. The positive result indicating the production of lipase enzyme was determined by formation of clear zones around the growing colonies (M. Veerapagu, 2013). The isolates MRS1, MRS2, MRS3, MRS4 were determined to be positive for lipase production.

6.8.2 Protease assay

The isolates were screened for production of protease by using Casein (1% w/v) agar plates. The isolates that are able to produce the protease enzyme form a zone of clearance and a visible ring around the growing colony due to the hydrolysis of casein in the media. The isolates determined to be positive for protease enzyme production were MRS2, MRS3 and MRS4. MRS1 was negative for protease enzyme production.

6.8.3 Catalase assay

The production of catalase enzyme was determined by the formation of effervescence on the addition of hydrogen peroxide to the isolates. The isolates MRS1, MRS2 and MRS3 were positive for the production of catalase while MRS4 was negative.

6.8.4 Oxidase assay

A whatman filter paper soaked in the oxidase reagent is used for screening for oxidase production. A colour change to deep blue or purple upon addition of the culture indicates positive result. However all the tested isolates were negative for oxidase production.

6.9 SEM analysis

The surface of the degraded plastic samples was analyzed using scanning electron microscopy. The SEM images of the degraded plastics using isolates MRS1 and MRS4 clearly showed cracks and evidences of biodegradation, compared to the control image showing no evidences of degradation.

6.10 EDS analysis

The degraded Blue coloured LDPE plastic samples using isolates MRS1 and MRS4, along with a control, were subjected to EDS analysis for the determination of carbon content. The carbon content in the Control was determined to be at 0.07% weight percentage and 0.10% atomic percentage.

The carbon content of the plastic sample that was inoculated with the isolate MRS1 was 0.00% of weight percentage and atomic percentage.

The carbon content of the plastic sample that was inoculated with the isolate MRS4 was 0.00% of weight percentage and 0.01% atomic percentage.

The carbon content of both the test plastic samples was lower than the control, indicating that that the plastic sample was being used as a carbon source by the bacterial isolates during degradation.

7 Summary And Conclusion

Plastics are one of the most widely used materials around the world due to properties like their durability, low cost, flexibility etc. However this has led to overaccumalation of plastics and in recent years has been causing drastic environmental impacts. Recycling and reducing the plastic use are some of the options employed to combat the plastic problem, however, the already accumulated plastic needs to be degraded. Many microorganisms have been identified that are involved in the biodegradation of plastics.

In this study bacterial cultures were isolated from two plastic contaminated areas of Goa. A total of nine isolates were obtained namely MRP1, MRP2, MRP3, MRS1, MRS2, MRS3, MRS4, MRS5 and MRS6.

Degradation of three different LDPE samples (Blue coloured LDPE, Black coloured LDPE and Yellow coloured LDPE) were degraded using the obtained isolates over a period of 5.5 months. The isolate MRS4 was found to be most efficient in the plastic degradation.

Different types of pretreatments were carried out to understand their role in enhancing the plastic degradation ability of the isolates. The pretreatments included Nitric Acid pretreatment, Mineral Oil pretreatment, Xylene pretreatment, Thermal pretreatment and UV pretreatment. The Xylene pretreatment was found to be the most efficient in enhancing the degradation of plastics. And considering an average of all the pretreatments the isolate MRS6 was the most efficient in plastic degradation.

Overall the isolate MRS6 along with the Xylene pretreatment was the best combination for plastic degradation amongst the various pretreatments and isolates studied.

The isolates MRS4 and MRS6 could be potentially used in a consortium for enhanced degradation of plastics.

The best selected isolates were screened for biofilm production. The isolate MRS4 showed the maximum biofilm formation ability.

Biochemical tests were performed on the selected isolates for characterization. And they were screened for enzymes like Lipase, Protease, Catalase and Oxidase.

SEM analysis was done to study the surface morphology of the plastic samples degraded using the isolates MRS1 and MRS4. The SEM images showed evident cracks on the surface and evidence of degradation.

EDS analysis was performed for carbon content analysis of the plastic samples degraded with the isolates MRS1 and MRS4. Both the samples showed lower carbon content than the control, hence proving that carbon from the plastic sample was utilized during degradation.

This study determined the isolate MRS4 to be the most efficient and useful in the plastic degradation experiments. It could potentially be of immense assistance in solving the plastic waste accumalation problem.

8 Future Prospects

1. To study enhancement of plastic degradation using co-degradation with added enzymes.
2. To study the genes involved in plastic degradation.
3. To apply the isolated culture for infield biodegradation of plastic waste and to assess the effects and scale up potential.
4. To study the effects of physiological parameters like temperature, pH etc on plastic degradation.
5. Isolation of more potential plastic degrading bacteria from other plastic contaminated sites.
6. Improvement of the current strain obtained for plastic degradation.

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APPENDIX – I (MEDIA)

1) Nutrient Agar

Components	Concentration (Gram/litre)
Peptone	5
Meat extract	5
Sodium Chloride	3
Agar	15
Distilled water	1000 ml
pH	5 ± 0.2

2) Bushnell Haas Broth

Components	Concentration (Gram/litre)
Magnesium Sulphate	0.2
Calcium chloride	0.02
Monopotassium phosphate	1.0
Dipotassium phosphate	1.0
Ammonium nitrate	1.0
Ferric chloride	0.05
Distilled water	1000 ml
pH	6 ± 0.2

3) Tributyrin Agar

Components	Concentration (Gram/100 ml)
Yeast extract	0.5

Peptone	0.3
Tributyrin	1 ml
Distilled water	100
pH	7

4) Casein Agar

Components	**Concentration (Grams/100 ml)**
Nutrient Agar	100 ml
1 % Casein	1
Distilled water	100 ml
pH	7

5) Mineral Salt Medium

Components	**Concentration (Grams/litre)**
K_2HPO_4	1
KH_2PO_4	0.2
NaCl	1
$CaCl_2 .2H_2O$	0.002
Boric acid	0.005
$(NH_4)2SO$	1
$MgSO_4 .7H_2O$	0.5
$CuSO_4 .5H_2O$	0.001
$ZnSO_4 .7H_2O$	0.001
$MnSO_4 .H_2O$	0.001
$FeSO_4 .7H_2O$	0.01

APPENDIX – II (BIOCHEMICAL REAGENTS)

1) Crystal Violet

Components	Concentration (Gram/100 ml)
Iodine	2
Absolute alcohol	20
Ammonium monohydrate	0.8
Distilled water	80

2) Destaining Solution

95% ethanol

3) Saffranine

Components	Concentration (Gram/100 ml)
Saffranine	0.25
Ethanol	10
Distilled water	90

4) Catalase reagent

3% Hydrogen Peroxide

5) 3% H_2O_2

Components	Concentration

	(ml)
Hydrogen Peroxide	3
Distilled Water	100

6) Oxidase reagent

Components	**Concentration(Gram/litre)**
Tetramethyl-p-phenylenediamine dihydrochloride	1
Distilled water	1000 ml

SOLUTIONS

1) 0.8% Saline

Components	**Concentration (Grams/100 ml)**
Sodium Chloride	0.8
Distilled water	100 ml

2) Phosphate buffered saline

Components	**Concentration (Grams/litre)**
Sodium Chloride	8
Potasssium chloride	0.2
Disodium hydrogen phosphate	1.44
Potassium dihydrogen phosphate	0.22
Distilled water	1000 ml
pH	7.4