ISOLATION AND CHARACTERIZATION OF LOW-DENSITY POLYETHYLENE DEGRADING AND BIOSURFACTANT-PRODUCING BACTERIA FROM SOILS

This thesis is submitted to BRAC University in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology

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March, 2019

IN DEDICATION OF MY PARENTS & SISTERS

GEORGE P. BAROI, LUCKY DAS, CLARA CAMELIA GEORGE & FABIAN PARSIA GEORGE

&

MY BEST FRIEND, MOHAMMED NIMEREE MUNTASIR

DECLARATION OF AUTHENTICITY

I, the undersigned, declare that the research work embodying the results reported in this thesis entitled “Isolation and characterization of Low-Density Polyethylene degrading bacteria from locally collected soils” is my original work, gathered and utilized for the sole purpose of fulfilling the objectives of this study. I confirm that the work has not been previously submitted to any other institution, in whole or in part, for a higher degree or diploma. I further declare that the thesis has been composed entirely by me under the supervision of Dr. Mahboob Hossain, Professor, Microbiology Programme, Department of Mathematics and Natural Sciences, BRAC University, Dhaka, except where stated otherwise by reference or acknowledgment.

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Certified

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ACKNOWLEDGMENT

I extend my sincerest gratitude to the Almighty, the Knower of the unseen and the witnessed for granting me health, wisdom, and patience in delivering this piece of work. First and foremost, I would like to thank my parents and my sisters without whose constant support and encouragement this thesis would not have been possible.

My deepest regards, gratefulness, and appreciation goes to my supervisor Dr. Mahboob Hossain, Professor, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University for his constant guidance, constructive critique and constant encouragement to pursue novel ideas throughout the course of this work.

I am grateful to Professor A F M Yusuf Haider, Professor, and Chairperson, Department of Mathematics and Natural Sciences, BRAC University for his benevolent guidance and encouragement.

My regards and gratitude go to Aparna Islam, Professor, Jebennesa Chowdhury, Assistant Professor, and Romana Siddique, Senior Lecturer, Department of Mathematics and Natural Sciences, BRAC University, for all their support and encouragement throughout the duration of my undergraduate degree.

I would like to extend my deepest gratitude and regards to Asma Binte Afzal for her constant guidance, support and wise words throughout the work in the lab.

I would like to thank Akash Ahmed, Rezwanul Kabir, Mahmudul Hassan, Shabnam Syeed, Raghib Mubassir Qazi, Shaeri Nawar, and Maliha Tabassum Rashid, Olia Najon Tuson, Phoebe A. William, Anika Nawar, Abu Nowroze Rafin, Saiful Islam Shanto and Nowrin Hosain for their words of encouragement and support and valuable company.

My regards to Ashiqe-E-Khuda, Tanzila Ahmmed Bonna, Shilpi Akter, Nadira Begum, Md. Furkan Mia, Md. Morshed-Al-Mamun for helping me out each time I asked.

Last but not the least, I would like to express my heartiest and warmest thanks to my biggest well-wisher and my best friend, Mohammed Nimeree Muntasir, without whose belief, motivation and hard-work, completion of this thesis would have been impossible.

Author

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ABSTRACT

Microorganisms play a very important role in the biological decomposition of various materials in the natural environment, this is called biodegradation. Synthetic materials including plastic and polyethylene waste accumulate in the environment and pose an ever-increasing ecological threat to humankind and the overall ecosystem on earth. Biodegradation of these plastic wastes using potent microbial strains could provide a solution to the problem. In the present study, the microorganisms responsible for biodegradation were isolated and characterized from local samples contaminated with hydrocarbon polymers. The experiment was conducted over a series of screening methods. The primary screening involved culturing collected samples for over 40 days to screen out low-density polyethylene (LDPE) non-degrading bacteria, followed by observation of polyethylene glycol (PEG) utilization by the formation of a clear zone. This led to the isolation of 43 different colonies. Secondary screening further allowed for selection of 14 different isolates that were able to form biofilm over the polymer surface. Finally, the tertiary screening allowed for the selection of 10 different isolates that are confirmed LDPE-degraders. From continuous culturing for 36 days, 8 out of 10 isolates were able to survive and propagate in minimal salt broth, namely, *Staphylococcus sp.*, *Acinetobacter sp., Clostridium sp*., *Bacillus sp*., and *Lysinibacillus sp*. Finally, organism able to produce anionic and cationic biosurfactant were identified as *Clostridium novyitype A* and *Staphylococcus massiliensis* respectively.

1. INTRODUCTION

1.1 Background

Plastics are organic polymers of high molecular mass that have adapted their name from the Greek word ‘plastikos’ which means ‘capable of being shaped or molded’ (Restrepo-Flórez, et al., 2014). Plastics have adopted widespread use in packaging industries such as food, pharmaceuticals, and cosmetics (Sharma, et al., 2014). As much as one-third of the plastics produced are being for packaging purposes (Raaman, et al., 2012). Piping, plumbing, automobiles, furniture, toys, etc. have all taken on the trend of using plastics in replacement of wood, stone, leather, horn, paper, metal, glass, ceramics, etc (Divyalakshmi, et al., 2016).

The most commonly used plastics are polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polybutyrene tetraphthalate (PBT) and nylon (Sharma, et al., 2014). The most common types of polyethylene are Low-Density Polyethylene (LDPE), High-Density Polyethylene (HDPE), Linear Low-Density Polyethylene (LLDPE) and Cross-Linked Polyethylene (XLPE). All these different subgroups of polyethylene differ in terms of the extensiveness of branching, the presence of functional groups on the surface and their density (Restrepo-Flórez, et al., 2014). Some may even have pro-oxidants and starch integrated into them as additives in order to facilitate the biodegradation of the substrate by microorganisms (Zheng, et al., 2008) (Koutny, et al., 2006).

RIC number	Plastic name	Abbreviated name	Product use	New products after recycling
	polyethylene terephthalate	PET or PETE	water, soft drink and juice bottles, carpet, polar fleece	food containers, carpet fibres, filling for jackets and cushions
	high-density polyethylene	HDPE	milk jugs, bottles, shopping bags	bins, pipes, new containers
	polyvinyl chloride	PVC	wrapping and packaging, pipes	pipes, traffic cones
	low-density polyethylene	LDPE	plastic bags, squeezable bottles	rubbish bin liners, compost bins, outdoor furniture
	polypropylene	PP	refrigerated food containers, dishware	tools, trays
	polystyrene	PS	disposable plates, cutlery, protective packaging	light switches, packaging, mouldings
	other	O	acrylic, nylon, composite plastics	low-grade bottles, outdoor products

Figure 1 Different plastics, their resin identification code (RIC) numbers and recycled reuses by *(Pūtaiao, 2017)*

1.2 LDPE

The most commonly used type of polyethylene is the Low-Density Polyethylene (LDPE) that comprises of approximately 60% of the total plastic production (Raaman, et al., 2012). It is a thermoplastic made from repeating units of the monomer ethylene and has SPI resin ID code 4 (Mahdiyah, et al., 2006). LDPE is widely used in the manufacture of containers, dispensing bottles, wash bottles, tubing, plastic bags for computer components and various molded laboratory equipment. Other common uses may include the making of trays and general purpose containers, corrosion-resistant work surfaces, very soft and pliable parts such as snap-on lids, six-pack rings, the inner and outer layer of juice and milk cartons, playground slides and plastic wraps.

Figure 2 A piece of packaging foam made from LDPE

Figure 3 LDPE packaging roll

In Bangladesh, the most common use of LDPE goes into the making of transparent plastic bags by vendors in grocery stores and street markets. A report in The Daily Star informed mentioned that single family uses four polythene bags every day and around 2 crore polythene bags are being used in Dhaka on a daily basis. The news article also stated that Prof Ahmad Kameuzzaman Majumder, chairman of Environment Science department of Stamford University Bangladesh, cited information in his keynote of Waste Concern to say that 3.5 kg plastic items were used by a person

in the country in 2014. The use of plastics in Bangladesh has increased by 80 times in the past 28 years, says the green group, Save the Environment Movement (POBA). Quoting a recent survey of POBA, the use of plastics in Bangladesh has grown to 1,200,000 metric tons in 2018 compared to just 15 thousand metric tons in 1990 (Tribune, 2018).

Figure 4 Plastic pollution in remote areas in Bangladesh by *(Tribune, 2018)*

1.2.1 Properties and Characteristics

The general properties of LDPE include the following. It has a density range of 0.917-0.930 g/cm^3. It is unreactive at room temperature and pressure (r.t.p.) with the exception of strong oxidizing agents, and some solvents cause swelling. It can withstand temperatures as high as 80°C continuously or 90°C temporarily. (n.d.). Also, the degree of branching in LDPE is a lot higher than HDPE, making former have fewer intermolecular forces as compared to the latter. This, in turn, coincides with the observation that LDPE has a lower tensile strength and higher resilience than HDPE, as is expected of it. It also further demonstrates that because of this extensive side

branching, LDPE is less tightly packed and contain fewer crystalline structure, resulting in a lower density as compared to that of HDPE (Royer, et al., 2018).

1.2.3 Business market of LDPE

Apart from plasticity, their other advantages involve ease of manufacture, low cost, versatility, high hydrophobicity (imperviousness to water), durability, etc (Divyalakshmi, et al., 2016). As a result, as has been observed, the worldwide LDPE market had reached a volume of about US$33 billion in 2013. The use of this synthetic polymer is growing at a rate of 12% per year, and while 140 million tons are produced worldwide, only 5.7% of this is being recycled each year according to EPA estimation (Mahdiyah, et al., 2006).

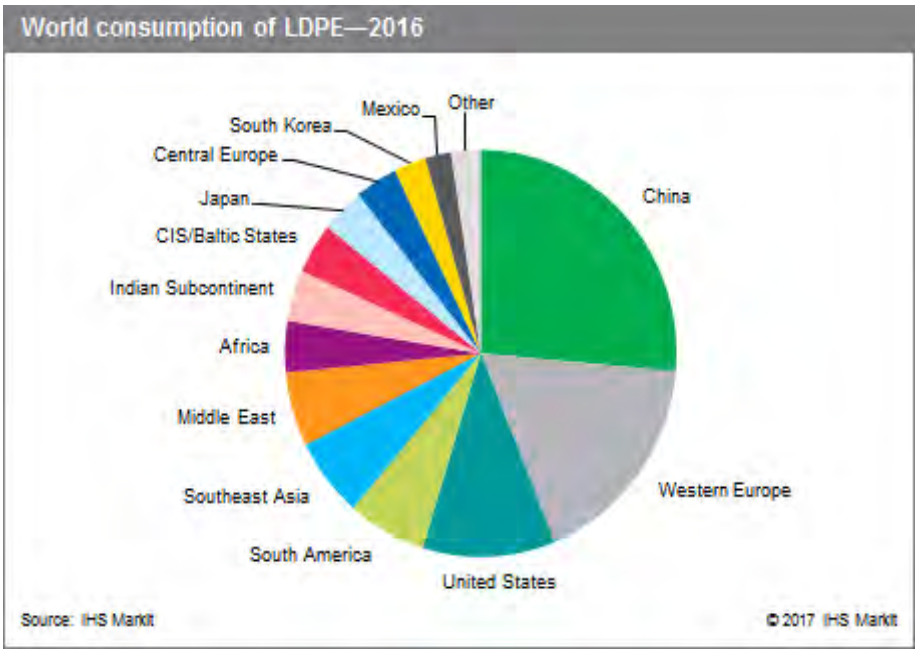


Figure 5 World consumption of LDPE in the year 2016 by *(Hakkarainen, et al., 2004)*

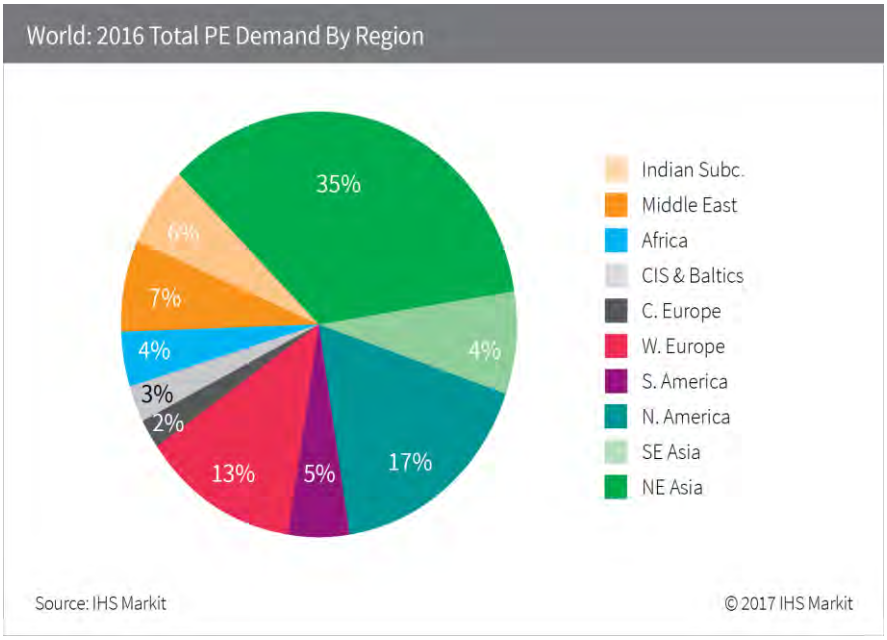


Figure 6 Total Polyethylene demand by region in 2016 by *(Hakkarainen, et al., 2004)*

1.2.3 Disadvantages

As for the remaining polyethylene, it takes thousands of years for their efficient degradation, which leaves with only two other options: incineration and landfilling. Incineration produces a massive amount of harmful greenhouse gases such as carbon dioxide and methane and requires the input of enormous energy to carry out such large projects, making the process both hazardous to the environment and expensive. On the other hand, landfilling requires the closing of landfill sites, requiring a lot of land space. It is also a major contributor of environmental pollution, as improperly disposed of plastic materials do not allow water and air to enter the earth, causing soil infertility (Restrepo-Flórez, et al., 2014). The leachate from the dumping site may seep into the soil and contaminate the groundwater, which could be the only source of water for drinking and other day to day purposes in some places. In the sea, the polythene may also cause blockage in intestines of fish, birds and marine mammals (Secchi, et al., 1999) (Spear, et al., 1996) (Starnecker,

et al., 1996). Plastics have been found to cover the sea-bed during deep-sea investigation using research submersibles (Fugikura, et al., 2008).

1.2.4 Extent of Inertness of LDPE

Compared to other methods, biodegradation ultimately remains as the only means of dealing with accumulating polythene. Albeit slow, the process is completely eco-friendly. The inertness of the polymer is demonstrated in the long-term study conducted by Albertsson and Karlson (1990). In the study, upon observation of the biodegradation of ^{14}C-labeled polyethylene, it was found that less than 0.5% of carbon (as CO_2) by weight was given off from an u.v.-irradiated polyethylene sheet after 10 years of incubation. Whereas, for nonirradiated polyethylene, only less than 0.2% of carbon was emitted for the same incubation period. Additionally, after incubation in moist soil for a decade, no further degradation was observed, and finally, after 32, partial degradation was observed for the same sheet of polyethylene in the soil burial (Otake, et al., 1995)

1.2.5 Factors contributing to its inertness

From a chemical perspective, one would expect polyethylene to be biodegradable since it is composed of linear alkene groups stacked side by side. However, in the case of polyethylene, the reason for its least susceptibility to biodegradation is the larger the molecular weight of the polymer. Macromolecules with molecular weight lower than 620 do support microbial growth, while those larger than 620 have low chances of supporting microbial growth (Haines, et al., 1974) (Potts, 1978). Furthermore, the polyethylene surface is hydrophobic, which make it impermissive and immiscible to hydrophilic solvents such as water. Due to its chemical nature, the polyethylene is unable to allow the microbial population to stick to its surface as most microorganisms have a hydrophilic surface. Therefore, it is widely accepted that the resistance of polyethylene to biodegradation stems from its high molecular weight, its hydrophobic nature, and its three-dimensional structure, all of which interfere with its availability to microorganisms (Arutchelvi, et al., 2008).

1.2.6 Enhancement of Biodegradation of LDPE

Given the circumstances of its inert nature, biodegradation can still be enhanced on exposure to biological agents like bacteria, fungi, enzymes etc., aided by initial pretreatment with abiotic factors such as u.v. irradiation (Cornell, et al., 1984), thermal treatment (Albertsson, et al., 1998) or oxidation with nitric acid (Brown, et al., 1974). All these abiotic factors work synergistically to increase the surface hydrophilicity of the polymer by introducing carbonyl groups by the process of oxidation which can be easily targeted by microorganisms (Albertsson, 1978) (Albertsson, 1980) (Cornell, et al., 1984). The biodegradability can be further improved by blending it with biodegradable additives, photo-initiators or copolymerization or using surfactants to reduce surface tension between hydrophilic and hydrophobic parts (G.J.L., 2007) (Hakkarainen, et al., 2004).

1.3 Microorganisms involved in polyethylene degradation:

Microorganisms able to degrade polyethylene has so far been limited to 17 genera of bacteria and 9 genera of fungi (Restrepo-Flórez, et al., 2014). Table 1 and 2 summarize the list of microorganisms responsible for colonization or degradation of polyethylene, or both. The more common microorganisms are *Bacillus megaterium, Pseudomonas sp., Azotobacter, Ralstonia eutropha, Halomonas sp* (Chee, et al., 2010).

Brevibaccillus borstelensis (30 days, 50°C) reduced its gravimetric and molecular weights by 11% and 30% respectively (Hadad, et al., 2005). Bacillus sp. showed 42.5% followed by Staphylococcus sp. 20%, Pseudomonas sp. 7.5% and consortium 5% degradation by weight loss in 40 days (Singh, et al., 2016). *Staphylococcus arlettae*, after 30 days of incubation at 37°C, caused loss of 13.6% of maximum weight loss (Divyalakshmi, et al., 2016). *Bacillus subtilis*, with the addition of its biosurfactant (surfactin), proved to cause a weight loss percentage of 9.26% in 30 days (Vimala, et al., 2016).

Table 1 Bacterial strains associated with polyethylene degradation by (Restrepo-Flórez, et al., 2014)

Genus	Species	Reference
Acinetobacter	*Baumannii*	Nowak et al., 2011
Arthrobacter	*spp*	Balasubramanian et al., 2010; Satlewal et al., 2008
	Paraffineus	Albertsson et al., 1995; Albertsson et al., 1998
	Viscosus	Nowak et al., 2011
Bacillus	*Amyloliquefaciens*	Nowak et al., 2011
	Brevies	Watanabe et al., 2009
	Cereus	Roy et al., 2008; Nowak et al., 2011; Satlewal et al., 2008; Sudhakar et al., 2008
	Circulans	Watanabe et al., 2009
	Halodenitrificans	Roy et al., 2008
	Mycoides	Nowak et al., 2011; Seneviratne et al., 2006
	Pumilus	Roy et al., 2008; Satlewal et al., 2008; Nowak et al., 2011
	Sphericus	Kawai et al., 2004; Sudhakar et al., 2008
	Thuringiensis	Nowak et al., 2011
Brevibacillus	*Borstelensis*	Hadad et al., 2005
Delftia	*Acidovorans*	Koutny et al., 2009
Flavobacterium	*spp*	Koutny et al., 2009
Micrococcus	*Luteus*	Nowak et al., 2011
	Lylae	Nowak et al., 2011
Microbacterium	*Paraoxydans*	Rajandas et al., 2012
Nocardia	*Asteroides*	Bonhomme et al., 2003;
Paenibacillus	*Macerans*	Nowak et al., 2011
Pseudomonas	*spp*	Balasubramanian et al., 2010; Yoon et al., 2012; Tribedi and Sil, 2013
	Aeruginosa	Koutny et al., 2009; Rajandas et al., 2012
	Fluorescens	Nowak et al., 2011
Rahnella	*Aquatilis*	Nowak et al., 2011
Ralstonia	*spp*	Koutny et al., 2009
Rhodococcus	*Ruber*	Gilan et al., 2004; Sivan et al., 2006; Santo et al., 2012
	Rhodochrous	Bonhomme et al., 2003; Koutny et al., 2006b; Fontanella et al., 2010
	Erythropolis	Koutny et al., 2009
Staphylococcus	*Epidermidis*	Chatterjee et al., 2010
	Cohnii	Nowak et al., 2011
	Xylosus	Nowak et al., 2011
Stenotrophomonas	*spp*	Koutny et al., 2009
Streptomyces	*Badius*	Pometto et al., 1992
	Setonii	Pometto et al., 1992
	Viridosporus	Pometto et al., 1992

Table 2 Fungal strains associated with polyethylene degradation by (Restrepo-Flórez, et al., 2014)

Genus	Species	Reference
Acremonium	*Kiliense*	Karlsson et al., 1988
Aspergillus	*Niger*	Raghavan and Torma, 1992, Volke-Sepulveda et al., 2002, Manzur et al., 2004
	Versicolor	Karlsson et al., 1988; Pramila and Ramesh, 2011b
	Flavus	Koutny et al., 2006b; Pramila and Ramesh, 2011a
Chaetomium	*spp*	Sowmya et al., 2012
Cladosporium	*Cladosporioides*	Bonhomme et al., 2003; Koutny et al., 2006b
Fusarium	*Redolens*	Albertsson, 1980; Karlsson et al., 1988; Albertsson and Karlsson, 1990
Glioclodium	*Virens*	Manzur et al., 2004
Mortierella	*Alpina*	Koutny et al., 2006b
Mucor	*Circinelloides*	Pramila and Ramesh, 2011a
Penicillum	*Simplicissimum*	Yamada-Onodera et al., 2001
	Pinophilum	Volke-Sepulveda et al., 2002; Manzur et al., 2004
	Frequentans	Seneviratne et al., 2006
Phanerochaete	*Chrysosporium*	Manzur et al., 1997; Orhan and Büyükgüngör, 2000; Manzur et al., 2004
Verticillium	*Lecanii*	Karlsson et al., 1988

Table 3 Weight loss percentage due to biological action for different types of polyethylene in various environments with pre-oxidative treatments by (Restrepo-Flórez, et al., 2014)

Substrate	Environment	Time	% Of weight loss	Reference
LDPE	Waste coal	225	−0.26	Nowak et al., 2011
	Forrest soil	225	−0.13	Nowak et al., 2011
	Crater soil	225	−0.28	Nowak et al., 2011
	Sea water	365	−1.9	Artham et al., 2009
	Soil + *Fusarium redolens*	3650	−0.2	Albertsson and Karlsson, 1990
	Soil	800	−0.1	Albertsson, 1980
	Mineral media + *Rhodococcus ruber*	56	−7.5	Sivan et al., 2006
	Mineral media + *Rhodococcus ruber*	30	−2.5	Santo et al., 2012
	Mineral media + *Brevibacillus borstelensis*	30	−2.5	Hadad et al., 2005
	Mineral media + *Pseudomonas* sp	45	−5	Tribedi and Sil, 2013
HDPE	Sea water	365	−1.6	Artham et al., 2009
	Soil	800	−0.4	Albertsson, 1980

1.3.1 Consumption of the polymer

The consumption of the polymer can be detected, although the process of its assimilation is very slow and difficult to detect. Some studies have used a reduction in the weight of samples determined by gravimetric analysis to monitor the corresponding degradation of the material *(Hadad, et al.*, 2005) (Sivan, et al., 2006) (Sudhakar, et al., 2008) . However, degradation can also be monitors by the evolution of CO_2 from the samples, which, out of the two techniques, is more widely accepted and used (Albertsson, 1980) (Karlsson, et al., 1988) (Pramila, et al., 2011). Although the latter is an indirect measurement, it does provide greater insight than the former. CO_2 evolution has the added benefit of not only monitoring the net degradation but also the rate of degradation over a given period of time (Albertsson, 1980) (Karlsson, et al., 1988).

1.3.2 Mechanism of microbial biodegradation of polyethylene:

Although there is enough evidence that proves biodegradation of polyethylene, there is still a lack of knowledge on the complete metabolic pathways involved in the process and the structure and identity of all the enzymes involved. The mechanism comprises of roughly three different steps:

1) microbial colonization of polymer
2) reduction of its molecular weight
3) oxidation of the molecules into CO_2 and H_2O.

Polyethylene is a hydrophobic, high molecular weight molecule, and therefore it is commonly accepted that biofilm colonization is the first step for degradation of this polymer (Gilan, et al., 2004). Biofilms are sessile communities of microorganisms developed on a surface that can be composed of individuals from the same or different species (Donlan, 2002). Studies on microorganisms' attachment to polyethylene have identified that the main limitation of the colonization process is the relatively high hydrophobicity of the polymer in contrast to the regularly hydrophilic surfaces of most microorganisms (Gilan, et al., 2004) (Tribedi, et al., 2013). It has been proposed that strains with more hydrophobic surfaces can play an important role in the initial colonization of the polymer. The other metabolic adaptation that can be important in

polymer colonization is the production of surfactants, molecules that can mediate the attachment process of microorganisms to the hydrophobic surface (Karlsson, et al., 1988) (Tribedi, et al., 2013). Theoretically, polyethylene can be used as a carbon source for microorganisms similar to many other hydrocarbons; however, its high molecular weight limits its use as a substrate for enzymatic reactions to take place.

Once colonization is done, there are two key reactions that follow, the first one being the reduction of its molecular weight and the second being the oxidation of the molecules. (Restrepo-Flórez, et al., 2014). Reduction of molecular weight is required for two reasons, firstly to enable transport of molecules through the cell membrane, and secondly because enzymatic systems present in the microorganisms are only able to attack certain molecular weights, usually in the range of 10-50 carbons, though there has been a report of enzymatic activity up to 2000 carbons (Yoon, et al., 2012). Once the size of the molecule is reduced, oxidation is required in order to transform the hydrocarbon into a carboxylic acid that can be metabolized by means of b-oxidation and the Krebs cycle (Albertsson, et al., 1987). Fig. 7 presents the proposed mechanisms of biodegradation for polyethylene.

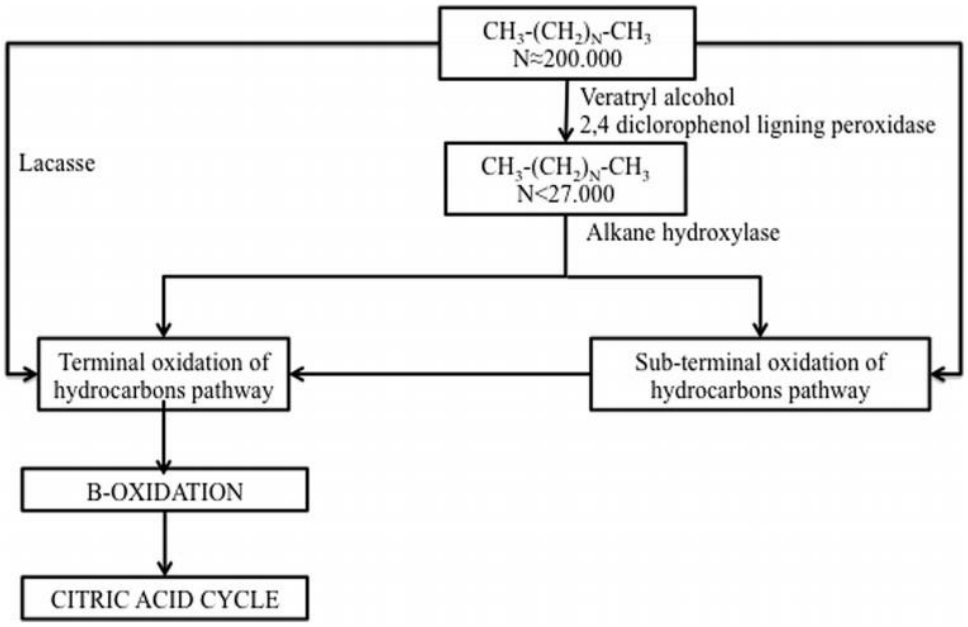


Figure 7 Hypothetical mechanisms of polyethylene degradation. This mechanism is the result of the adaptation of the works presented by different authors by *(Albertsson, et al., 1998) (Rojo, 2010) (Santo, et al., 2012)*

1.3.4 Enzymes associated with degradation

There are very few works devoted to studying the enzymes involved in these processes. Breaking down large polyethylene molecules can be accomplished by enzymatic action, as proven by (Santo, et al., 2012), who found that by incubation with the enzyme laccase the molecular weight of polyethylene was reduced and its keto-carbonyl index increased. These two factors were felt to indicate that both scission and oxidation reactions were taking place by the same enzyme. In regards to the oxidation process, there was another important work, this one by (Yoon, et al., 2012) that isolated an alkane hydroxylase from the AlkB family that was active to polyethylene samples with molecular weights up to 27,000 Da. In general, it is accepted that alkane hydroxylase performs the first oxidation that leads to the subsequent degradation of a hydrocarbon (Rojo, 2010)

13.5 Effect of microbial activity on the degradation of polyethylene:

One may wonder what happens to the LDPE films during and after the microbial attack. Upon colonization by microorganisms on polyethylene surfaces, the properties of the synthetic polymer undergo diverse changes. The extent of biodegradation takes a toll on seven different characteristics of the polymer: functional groups on the surface, hydrophobicity/hydrophilicity, crystallinity, surface topography, mechanical properties, molecular weight distribution and mass balance (Restrepo-Flórez, et al., 2014). The modifications to the surface chemistry of LDPE are evidence of interactions by microorganisms with the surface.

- **1.3.5.1 Functional groups on the surface**

  The presence and nature of functional groups on the surface of polyethylene are determined by FTIR spectroscopy. Whenever there are biological activities occurring on the surface of a synthetic polymer, changes in following functional groups are observed: carbonyls (1715 cm^{-1}) esters (1740 cm^{-1}), vinyls (1650 cm^{-1}) and double bonds (908cm^{-1}) (Albertsson, et al., 1987) (Gilan, et al., 2004) (Hadad et al., 2005) (Santo, et al., 2012) (Sudhakar, et al., 2008). Some studies claim that there is a corresponding decrease in the concentration of carbonyl indices and increase in double bonds (Albertsson, et al., 1987) (Raghavan, et al.,

1992) (Gilan, et al., 2004) (Hadad, et al.) while others claim an increase in the concentration of carbonyl group and decrease in double bonds during biodegradation (Gilan, et al., 2004) (Raghavan, et al., 1992) (Manzur, et al., 2004). The indifference in these reports suggests that the biodegradation is rather a complex process that can differ for different microorganisms and what holds true is the fact that the surface of LDPE most certainly undergoes a change in surface chemistry.

- **1.3.5.2 Hydrophobicity/Hydrophilicity**

  The hydrophobicity/hydrophilicity of a surface depends on the nature, concentration, and exposition of the functional groups present in the material. If the degree of oxidation by the abiotic factors such as UV or activity of enzymes is higher than the degree of consumption of functional groups, then there will be an increase in the net concentration of hydrophilic functional groups, hence the hydrophilicity of the material will increase. On the contrary, if the degree of oxidation by the abiotic factors is lower than the degree of consumption of functional groups, then the net concentration of hydrophilic functional groups will tip to hydrophobicity. Therefore, the material will become more hydrophobic (Restrepo-Flórez, et al., 2014). It is important to maintain this ratio of oxidation versus consumption as hydrophilic surfaces facilitate degradation but hydrophobic surfaces do not unless the microbial colony has hydrophobic surface too.

- **1.3.5.3 Crystallinity:**

  Comprising of crystalline microstructures surrounded by amorphous regions, polyethylene is a semi-crystalline polymer. Experimentally, it has been corroborated that amorphous regions are consumed first due to their accessibility before they move on to consume smaller crystals (Manzur, et al., 2004), resulting in an increase in the proportion of larger crystals (Albertsson, et al., 1998) (Sudhakar, et al., 2008).

- **1.3.5.4. Molecular weight distribution**

  Just as was with crystallinity, the average molecular weight seems to increase after a microbial attack as a result of consumption of lower molecular weight chains. (Hadad et al., 2005) (Santo, et al., 2012).

- **1.3.5.5 Surface topography**

  Surface colonization by microorganisms causes substantial changes in the surface topography. For fungi, hyphal structures may penetrate the surface (Gilan, et al., 2004) (Sivan, et al., 2006) (Koutny, et al., 2006) (Pramila, et al., 2011) (Tribedi, et al., 2013). Cracking and pitting in the polymer surface after biodegradation process can be observed, causing superficial damage to the film.

2. MATERIALS AND METHODS

2.1 Working place for the study

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

2.2 Media, Solutions, and Reagents:

Most of the required solutions, reagents and media available in the laboratory were of reagent grade and were used without further purification.

2.3 Handling of Laboratory Apparatus, Glassware and Analysis Equipment

All glassware was washed with detergents, rinsed 4-5 times with tap water and sterilized in hot air oven (Binder ED23, Germany) at 160 for 2 hours. McCartney bottles, Durham bottles, conical flasks, micropipette tips, glass spreader, glass test tubes, falcon tubes, and microfuge tubes were sterilized by autoclaving at 121 for 15 minutes at 15 psi (Sturdy, SA-300VF, Taiwan). All the microbiological works were done inside the Biological Safety Cabinet (LabTech, LCB-1803B-A2, Korea). For the measurement of absorption in the UV-Visible region, a high-performance compact split beam spectrophotometer (PG Instruments, T60, UK) was used.

2.4 Sample Collection:

2.4.1 Soil samples: Samples were collected in sterile test tubes from 7 different sources listed below. The soil collected was dug out from approximately 10 centimeters deep from the surface

using sterilized forceps and spatula. Marine water was collected from an approximate 10 centimeter deep from surface water. The soils collected from petrol pump stations were hypothesized to harbor bacteria able to breakdown larger and complex hydrocarbons like gasoline and diesel. Garbage dump sites were ravaged for soil sample as the majority of plastic wastes would be piled up at the sites. Marine water from sea-shore was nearer to the large dump sites of plastics and polythenes, and one would expect the leachates from the dump site to wash into the water near the shore with tidal waves. Table 4 lists the details of sample collection.

2.4.2 LDPE films: Low-Density polyethylene bags were collected from local markets

Table 4 Tabulation describing the sample number, material and location of sample collection throughout Bangladesh

Sample number	**Material collected**	**Location**
S1	Soil	Shyamoli petrol pump station
S2	Soil	Shyamoli garbage dump site
S3	Soil	Farmgate garbage dump site under City Corporation
S4	Soil	Mirpur petrol pump station
S5	Soil	Gabtoli petrol pump station
S6	Marine water	Cox's Bazar sea-shore
S7	Marine water	Moheshkhali sea-shore

2.5 Media and LDPE emulsion/films preparation:

2.5.1 Agar media and broth:

- Minimal salt broth (MS 1) was used for the primary screening of LDPE degrading bacteria where only pieces of LDPE films were used as carbon source along with inorganic. The composition follows: KH_2PO_4 (3.0 g/L), K_2HPO_4 (0.1 g/L), NaCl (5.0 g/L), NH_4Cl (2.0 g/L), $MgSO_4$ (0.2 g/L) and $CaCl_2.2H_2O$ (0.15 g/L). The medium was supplemented with 0.3% LDPE films as a carbon source.

- Nutrient agar was used as a common bacteriological growth media. As an alteration, LDPE strips were placed on the media after the spread plate method, as a method of secondary screening
- Minimal salt agar (MSA) media was used for the tertiary screening of LDPE degrading bacteria supplemented with LDPE powder as carbon source made from scratch. The media composition follows: (per liter of distilled water) K_2HPO_4 , 1g; KH_2PO_4, 0.2g; NaCl, 1g; $CaCl_2.2H_2O$, 0.002g; boric acid, 0.005g; $(NH_4)_2SO_4$, 1g; $MgSO_4.7H_2O$, 0.5g; $CuSO_4.5H_2O$, 0.001g; $ZnSO_4.7H_2O$, 0.001g; $MnSO_4.H_2O$, 0.001g and $FeSO_4.7H_2O$, 0.01g. Polyethylene (LDPE) emulsion was added to the mineral salt medium at a final concentration of 0.1% (w/v) respectively.

- Minimal salt broth (MS 2) was used to observe the colony-forming unit (CFU) of those bacteria that tested positive for LDPE degradation in order to evaluate whether the specific species will be able to sustain in MS 2 broth on its own without needing to add additional inoculum periodically. This adaptation was employed to study the degradation of LDPE films over 30 days' incubation which is not covered in this dissertation. The MS 2 broth has the same composition as above, only that the instead of emulsified LDPE, LDPE strips were used at a final concentration of 0.1% (w/v) respectively. The pH of the broth was adjusted to pH 6, pH 7 and pH 8 to check for optimal conditions for growth.

- Bushnell-Haas supplemented CTAB/SDS-methylene blue agar was used to screen anionic/cationic biosurfactant producing bacteria. The media composition follows: $MgSO_4$ (0.200 g/L) $CaCl_2.2H_2O$ (0.020 g/L), KH_2PO_4 (1.000 g/L), K_2HPO_4 (1.000 g/L), NH_2NO_3 (1.000 g/L), $FeCl_2$ (0.050 g/L), glucose (20 g/L), methylene blue (0.2 g/L) and Agar 20.000 (g/L). To screen for anionic biosurfactant, 0.5 mg/mL CTAB was added to the media.

Alternatively, for screening of cationic biosurfactant, 0.5 mg/mL SDS was added to the media.

- Peptone water used as enrichment broth for all microorganisms. It is composed of peptone (10 g/L) and NaCl (5 g/L).

- T1N1 agar was used for preservation or storage of organisms for over 6 months in RTP. It is composed of trypticase or tryptone, 10g/L; NaCl, 10g/L, and Agar, 20g/L.

2.6 LDPE films preparation:

Polyethylene sheets were cut into 2x2 cm. Pre-weighed strips were transferred to a fresh solution having 70 ml Tween 20, 10 ml disinfectant (Detol) and 983 ml distilled water and stirred for 60 minutes. The strips were transferred into a beaker with distilled water and stirred for another 60 minutes. This step was repeated 4 or 5 times until the LDPE films were ridden of any residual disinfectant or tween. Finally, the strips were aseptically placed in 70% ethanol solution overnight. Finally, the disinfected strips were transferred to a sterile petri dish and dried in the laminar hood and put away for further use. The whole process was carried out inside laminar hood using autoclaved distilled water, autoclaved beaker, and sterile forceps/spatula.

2.7 LDPE emulsion preparation

One gram of packaging LDPE foam was dissolved in 100 ml of benzene. The solution was emulsified in 1000 ml of M2 broth using a sonicator. 20 g of agar was added to the emulsion in 1L Erlenmeyer flask and stirred continuously while heating for at least 30 minutes to evaporate the benzene completely. The medium was autoclaved resulting in homogenous opaque plates before being plated.

2.8 Staining and Destaining solutions:

Staining solutions were prepared by mixing 40% methanol, 10% acetic acid and 0.1% Coomassie blue R250 mixed in 50% distilled water. Destaining solutions were prepared the same way, omitting the addition of coomassie blue.

2.9 Starter inoculum preparation

1 gram or mL of soil/water from sample was added to 9 ml peptone water in test tubes, vortexed and incubated overnight.1 ml of the solution was transferred to 9 ml nutrient broth (NB), vortexed and incubated for 24 hours. The solution was transferred to falcon tubes and centrifuged at 15,000 rpm for 10 minutes to obtain a cell pellet. The supernatant was discarded and the pellet was resuspended in 0.9% NaCl solution. The above steps were done for S1 through S7.

2.10 Isolation of LDPE degrading organisms:

2.10.1 Primary screening

For primary screening, 1 ml of initial inoculum was added to 100 ml of MS 1 broth in 250 ml Erlenmeyer flask containing 0.3% (w/v) LDPE strips. The inoculated broth was incubated at 37°C at 150 rpm for 1 week. After 1 week of incubation, 1 ml of the old broth was into a fresh MS 1 broth supplemented with polyethylene films as a carbon source. Every 1st and 7th day of each week, the OD of the broth was taken using a spectrophotometer, with an increase in OD corresponding to an increase in growth. Positive and negative controls were maintained at all times where the positive control had 1 g/L of glucose supplemented as a carbon source instead of polyethylene strips and the negative control was maintained where no carbon source was present at all. The above steps were repeated for samples S1 through S7. The surviving bacteria were obtained as single colonies were obtained by streaking on Nutrient Agar media.

2.10.2 Petri plate method- secondary screening:

A single colony of each isolate was resuspended in 9 ml of 0.9% NaCl solution and 100 µl of the solution was spread onto NA. Sterilized LDPE strips were placed aseptically onto the NA plates using sterilized forceps. The NA plates were incubated for 1- 2 weeks at 37℃ (Kowalczyk, et al., 2016) (Urbanek, et al., 2017) (Singh, et al., 2016).

2.10.3 Clear zone method: Tertiary screening

For the final step of screening, the 24hr old culture of each isolate was streaked onto the MSA medium. The medium was incubated for 7 days at 37℃. After incubation, the plates were stained with a staining solution for 15 minutes. The stain was decanted and destained with a destaining solution. Isolates that gave a clear zone were positive for LDPE degradation. Isolates that rendered positive for clear zone method were preserved on T1N1 agar in small vials.

2.10.4 Screening for bacterial survival

For the final part of the experiment, the 24-hr culture of LDPE degrading organisms (obtained after tertiary screening) were inoculated in 3 ml of MS 2 (pH7) broth in vials. The turbidity of the culture was referenced against McFarland 2.0 standards. The 3ml of the culture was added to 50 ml of MS 2 broth in 100 ml Erlenmeyer flask containing 0.1% (w/v) sterile LDPE films. The broth was incubated at 37℃ at 150 rpm in shaker-incubator. After every 9 days, 1 ml of fresh MS 2 broth was added to the existing broth and 100 µl of the culture was spread onto NA and incubated for 24 hours at 37℃. The CFU was measured to monitor the survival and reproduction of each isolate in extreme conditions. The above steps were repeated again for MS2 broths of lower pH, i.e., pH 6 and added 250 µl of Tween 20. This was done to see if lowering the pH and adding a little bit of biosurfactant would enhance or aid the survival of specific bacteria. The whole process

took place over a span of 36 days in total in continuous incubation in the shaker-incubator. Isolates that were still TNTC (too numerous to count) were preserved in T1N1 for storage.

2.10.5 Screening of Biosurfactant producing bacteria:

Isolates obtained after the primary screening were tested for their ability to produce biosurfactants. A single colony of each isolate was picked up and inoculated in 0.9% saline solution. To test for production of anionic surfactants, Bushnell-Haas mediated CTAB-methylene blue agar was used. Well was cut into the agar using a cork-borer and 50 µl of the liquid inoculum was micropipette into the wells. The plate was then incubated at 37℃ for 72 hours. To test for production of cationic surfactants, Bushnell-Haas mediated SDS-methylene blue agar was used and step 4 was repeated for this medium as well. Formation of a dark blue ring around the wells signified formation of cationic or anionic surfactant by the concerned organism.

2.11 Gram's staining

Gram staining is a common technique that is used to differentiate two large groups of bacteria based on their different cell wall constituents. The Gram stain procedure distinguishes between gram-positive and Gram-negative groups. The morphology of the bacteria can also be checked using this method.

Endospore staining Endospores were stained via modified Schaeffer-Fulton method using malachite green and safranin dyes. A loopful of the culture was smeared onto a glass slide with a few drops of distilled water. The smear was heat fixed before adding 5-6 drops of malachite green over top. The slide was carefully held over a flame until the dye started to steam and bubble, at which point it was removed from the flame and allowed to cool. Cooled slides were washed thoroughly with distilled water before the addition of 5-6 drops of safranin. The dye was allowed to sit for 3 minutes before the slides were again washed with distilled water and allowed to air dry. The dried stained slides were then observed 19 under 100x magnification of microscope with immersion oil to confirm presence or absence of endospores. Isolates were only designated

negative for spore formation if there were no visible spores under the microscope after 7 days of incubation

2.12 Biochemical tests:

A range of biochemical tests was carried out in order to further classify the presumptive species of isolates bacteria. All tests were carried out according to Bergey's Manual of Systematic Bacteriology [21] using fresh, 24 hour cultures from nutrient agar plates. The following tests were performed:

- MR
- VP
- Indole
- Citrate
- Catalase
- Oxidase
- Motility
- Urea
- TSI
- Nitrate Reduction
- Starch hydrolysis

2.12.1 Methyl Red (MR): Half a loopful of culture was inoculated into a test tube containing 5ml of MRVP broth, and the tube was incubated at 37°C for 24 hours. For observation, 5 drops of methyl red dye were added to the tube without shaking. A cherry red color indicated positive for mixed pathway fermentation of glucose, while orange indicated inconclusively, and yellow indicated negative results.

2.12.2 Voges Proskauer (VP): Half a loopful of culture was inoculated into a test tube containing 5ml of MRVP broth, and the tube was incubated at 37°C for 24 hours. After incubation, 6 drops of Barritt's reagent A were added and the tube was shaken. After that, 6 drops of Barritt's reagent

B were added to the tube and observed up to 1 hour. Formation of a pink ring indicated the presence of acetoin, 2while a brownish ring indicated negative results.

2.12.3 Indole: A loopful of culture was inoculated into a test tube containing 5ml of tryptophan broth, and the tube was incubated at 37°C for 24 hours. After addition of 5 drops of Kovac's reagent to the broth, a red color indicated positive tryptophan hydrolysis while a yellow color was taken as negative.

2.12.4 Citrate: Sterile slants of Simmon's Citrate Agar were prepared in vials and the surface was streaked heavily with a loopful of culture. The vials were incubated at 37°C for 48 hours and observed for bacterial growth and color change. Growth on the slant with the color change of the medium to blue indicated positive for utiliza5tion of citrate as a carbon source, while growth without color change was taken as a negative result.

2.12.5 Catalase: 3 drops of 3% hydrogen peroxide were taken on a clean glass slide. Half a loopful of bacterial culture was mixed with the hydrogen peroxide on the slide and observed for bubble formation. Immediate, sustained formation of bubbles was taken as a positive indicator of catalase production by the sample bacterium, while the slow or delayed formation of a small number of bubbles was taken as negative.

2.12.6 Oxidase: On a sterile petri dish lid, the filter paper was soaked in oxidase reagent. Using a sterile inoculating loop, a heavy inoculum of bacteria was smeared onto a section of reagent soaked filter paper and observed for color change. Changing of the smear to a pink color indicates a positive test for the presence of cytochrome c oxidase enzyme, while no color change indicates a negative result.

2.12.7 Motility and Urea: Both motility and urease tests were carried out using MIU agar. The agar was prepared first, sterilized in test tubes, and then cooled to approximately 50°C before adding 5% (v/v) of 40% syringe filtered urea solution. Once solidified, the agar was inoculated via a single stab of heavy inoculum using a sterile inoculating needle and incubated at 37°C for 24 hours. Change of the orange media to pink indicated positive for the presence of urease enzyme while spiraling growth away from the stab line was indicative of motility.

2.12.8 Triple sugar iron test: Triple sugar iron agar is a differential medium used to determine H2S production and the type of carbohydrate fermentation from the discoloration of butt and slant.

Gas from carbohydrate metabolism can also be detected. To conduct the test, an isolated colony was inoculated in the TSI medium. The results were observed after 24 hours of incubation at 37°C.

2.12.9 Nitrate reduction test: Many gram-negative bacteria use nitrate as the final electron acceptor. Nitrate reduction test is a test that determines the production of an enzyme called nitrate reductase, which results in the reduction of nitrate (NO3). Bacterial species may be differentiated on the basis of their ability to reduce nitrate to nitrite or nitrogenous gases. Positive Test corresponds to the development of a cherry red coloration on the addition of reagent A and B and the absence of red color development on adding Zn powder. Negative Test corresponds to the development of red color on the addition of Zn powder

2.12.10 Starch Hydrolysis: Pure cultures were streaked onto starch agar plates and incubated at 37°C for 24 hours. After incubation, the plates were flooded with Gram's iodine. Clear zones around bacterial colonies against a now dark blue medium indicated positive results for hydrolysis of starch.

3. RESULTS

The main objective of the experiment was to isolate bacteria that were able to degrade a type of polyethylene known as Low-Density polyethylene or LDPE. For that purpose, samples were taken from multiple oil or plastic contaminated soil and water from places all over within and outside Dhaka city. In total of 7 different samples were collected from petrol pump stations and garbage dumping sites that have a high probability of harboring high molecular weight polymer degrading bacteria. The samples were collected in autoclaved sterile tubes from a depth of no less than 10 centimeters from the surface as polymer degrading bacteria mainly favor higher temperatures for optimum growth. The samples were subjected to a primary screening where they were incubated in minimal salt broth supplemented with LDPE films as a carbon source for over 40 days, which was followed by growth of isolates on 1% PEG media and looking for a subsequent zone of the clearance formed by them. Next, the isolates were subjected to secondary screening or the Petri plate method where pure colonies of bacteria were spread on NA agar plate and were checked to see if their growth extended onto the hydrophobic surface of LDPE. For the final part of the screening method, LDPE emulsion plates were used for clear zone formation method by organisms that degrade LDPE. The experiment was concluded by growing positive isolates in 36 days' of continuous incubation in minimal salt broth (pH 7 and 6) supplemented in 0.3% LDPE film as a carbon source to observe CFU throughout the incubation period. Last but not the least, to check for biosurfactant producing ability by isolates, Bushnell-Haas media SDS/CTAB methylene blue agar plates were inoculated with isolates and left for incubation for 48 hours to observe the formation of the dark blue ring or precipitate around wells.

3.1 Primary screening

Primary screening involved the inoculation of bacterial pellet isolated from S1, S2, S3, S4, S5, S6 and S7 into minimal salt broth (MS 1 broth) and incubating for 7 days at 37°C at 150 rpm before transferring the old inoculum into the fresh broth. During each periodic transfer of inoculum from old to fresh MS1 broth, the growth was monitored by taking the OD immediately after inoculation and 7 days later (Table 5). The interpretation of the rising/falling OD reading follows in the

Discussion section (figure 11 & 12). It was an indirect method of monitoring the increase in bacterial growth alongside observing the changing of the color of broth from transparent to milky white (Fig 8). A negative and positive control was taken at all times for each sample. The positive control had glucose as carbon source and the negative control was transparent colorless (Fig 9 and 10). The tabulation (table 5) below lists the recorded OD.

Figure 8 Semi-milky color of broth containing LDPE films after 7 days incubation

Figure 9 Milky color of broth supplemented with 1% glucose after 7 days incubation (positive control)

Figure 10 Transparent color of broth containing inoculum but no carbon source after 7 days incubation (negative control)

Table 5 The initial and final OD recorded periodically after every 7 days in MS broth supplemented with LDPE films

Sample	Day 1	Day 7		Day 14		Day 21		Day 27		Day 35		Day 42
	I_1/nm	F_7/nm	I_7/nm	F_{14}/nm	I_{14}/nm	F_{21}/nm	I_{21}/nm	F_{27}/nm	I_{27}/nm	F_{35}/nm	I_{35}/nm	F_{42}/nm
1	0.315	0.572	0.120	0.275	0.100	0.118	0.000	0.052	0.000	0.048	0.000	0.045
2	0.302	0.505	0.100	0.198	0.056	0.068	0.000	0.020	0.000	0.012	0.000	0.010
3	0.276	0.455	0.089	0.180	0.054	0.066	0.000	0.020	0.000	0.010	0.000	0.010
4	0.303	0.512	0.112	0.252	0.076	0.090	0.000	0.037	0.000	0.026	0.000	0.022
5	0.299	0.499	0.095	0.200	0.080	0.102	0.000	0.040	0.000	0.027	0.000	0.024
6	0.310	0.568	0.118	0.262	0.089	0.111	0.000	0.050	0.000	0.023	0.000	0.019
7	0.250	0.423	0.070	0.160	0.042	0.056	0.000	0.017	0.000	0.009	0.000	0.005

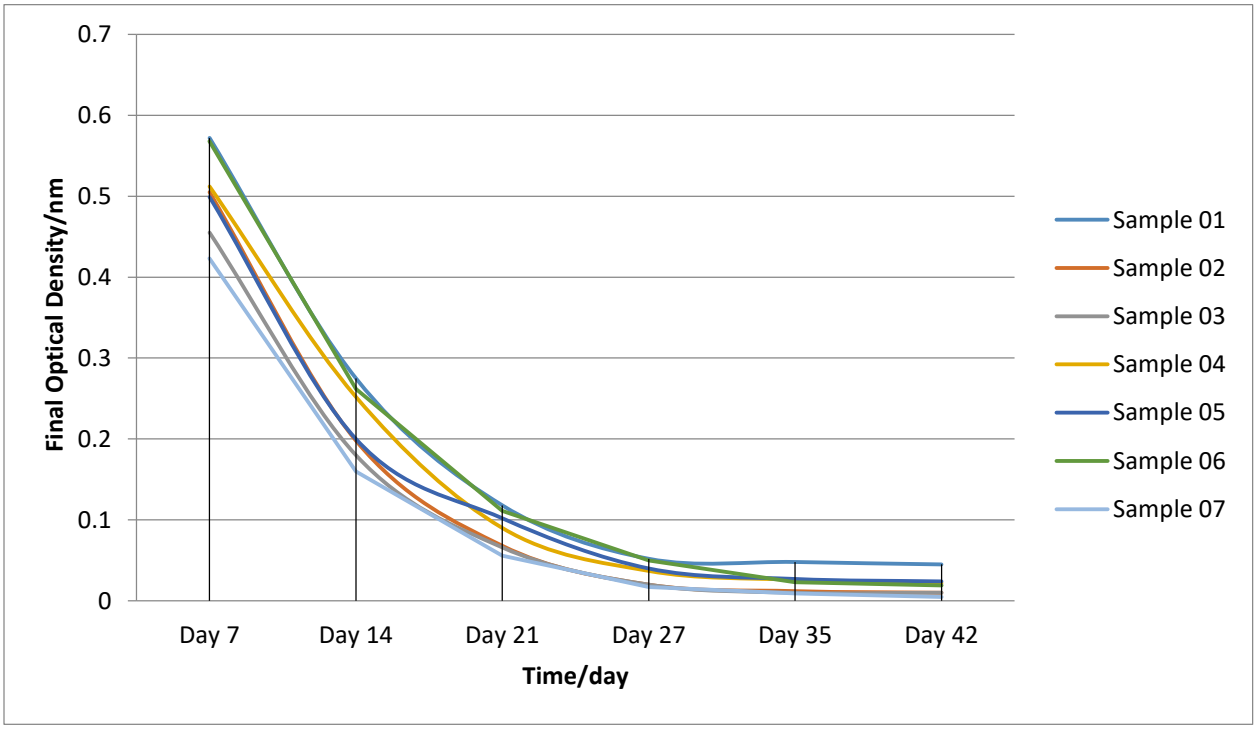


Figure 11 A graphical representation of the declining microbial population in primary screening as a result of the absence of simple sugars as a carbon source

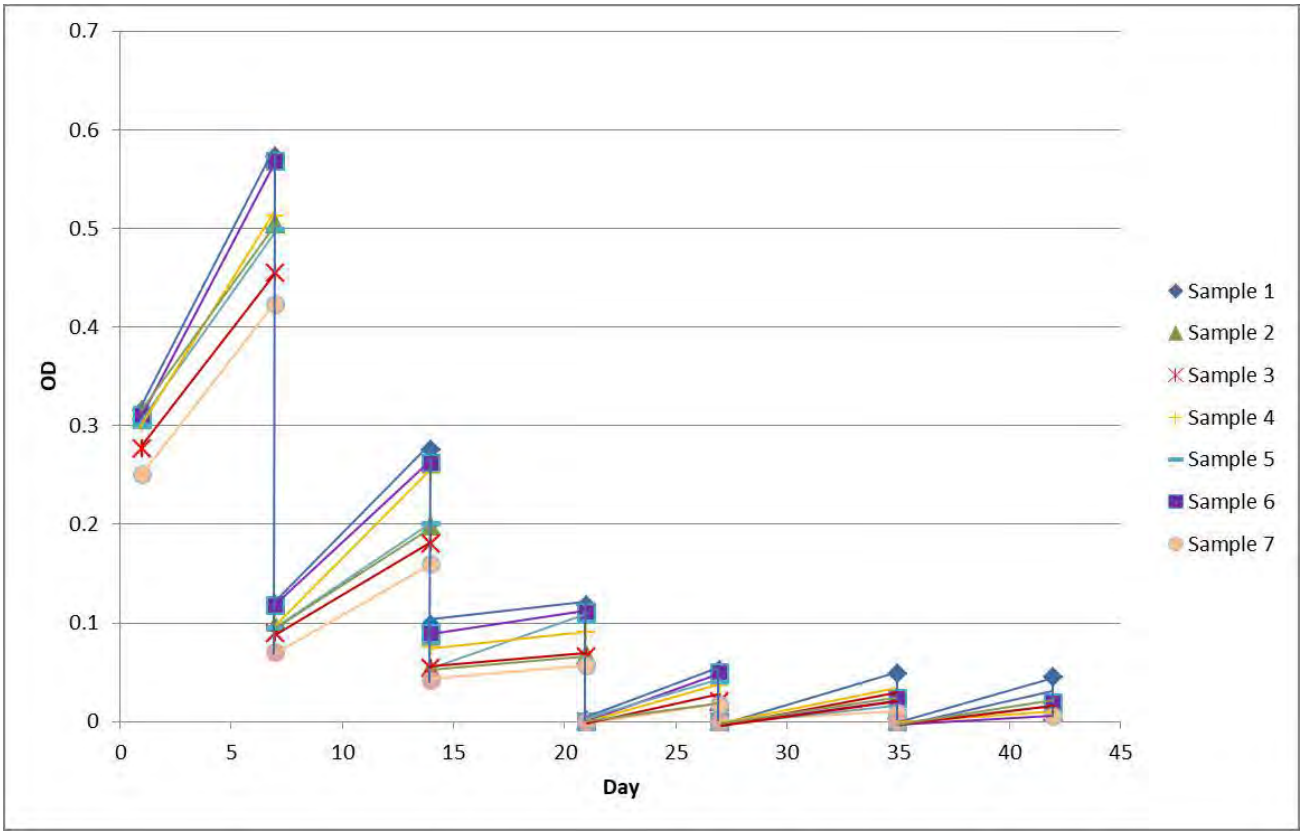


Figure 12 Graphical representation of increasing differences between final OD and Initial OD

3.3 Secondary Screening: Petri plate screening

After obtaining 43 different isolates from primary screening, the isolates were observed after 14 days incubation to check for biofilm formation over the hydrophilic polyethylene surface placed on top of agar surface. The 14 isolates that did were considered positive for the screening were A.a, B.a, B.b, B.c, E1, E2, F, G, H, J.a, J.b, L, N9, N53a. *Escherichia coli* was taken as the negative control.

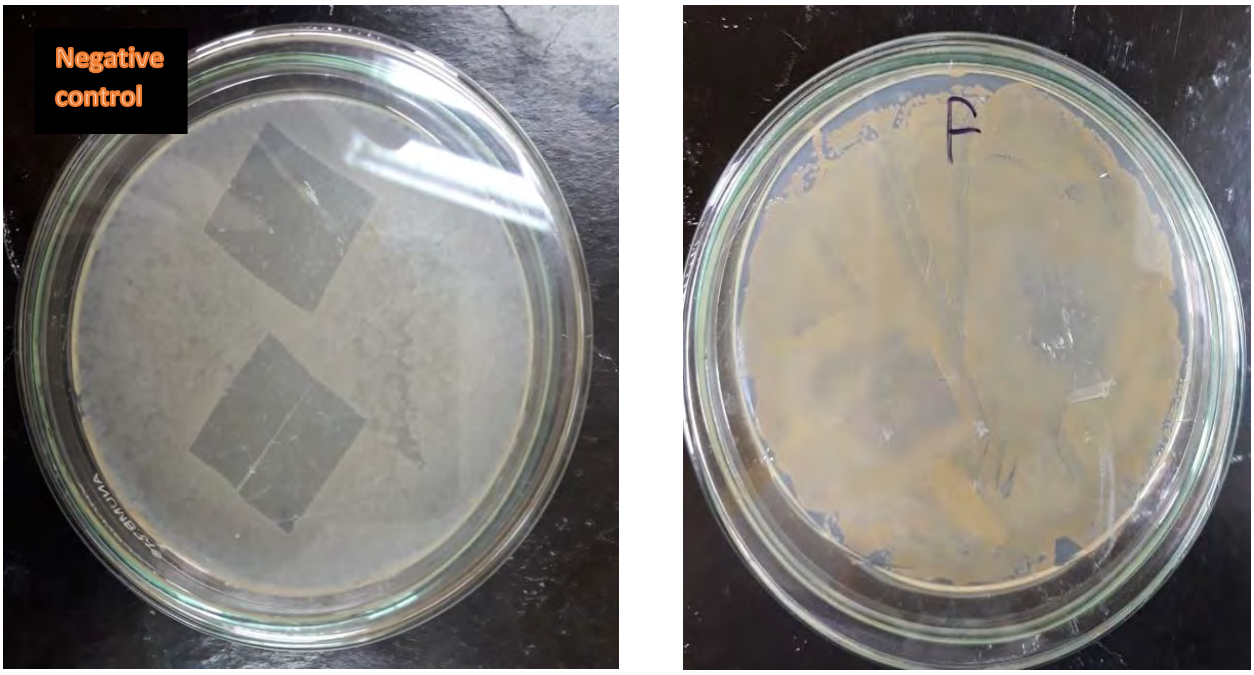


Figure 14 Growth of bacteria on the surface of the polyethylene. Negative control (top left) was maintained by E. coli

3.4 Clear zone method: Tertiary screening

For the final screening, the 14 positive isolates were streaked on LDPE turbid agar where the LDPE foam was boiled in benzene, emulsified in MS2 broth by sonication and boiled till evaporation of benzene. After streaking and 7 days' incubation, the plates were stained with Coomassie blue staining dye and destained with the destaining dye. A zone of clearance around isolates was taken positively for LDPE degradation. The isolates that gave clear zones were: B.c, E1, E2, F, G, H, J.a, J.b and N53a (figure 13). The zone of clearance was ranked in order of prominence with '+++++' being the biggest zone while '+' being the smallest. Table 6 lists the results obtained from the tertiary screening.

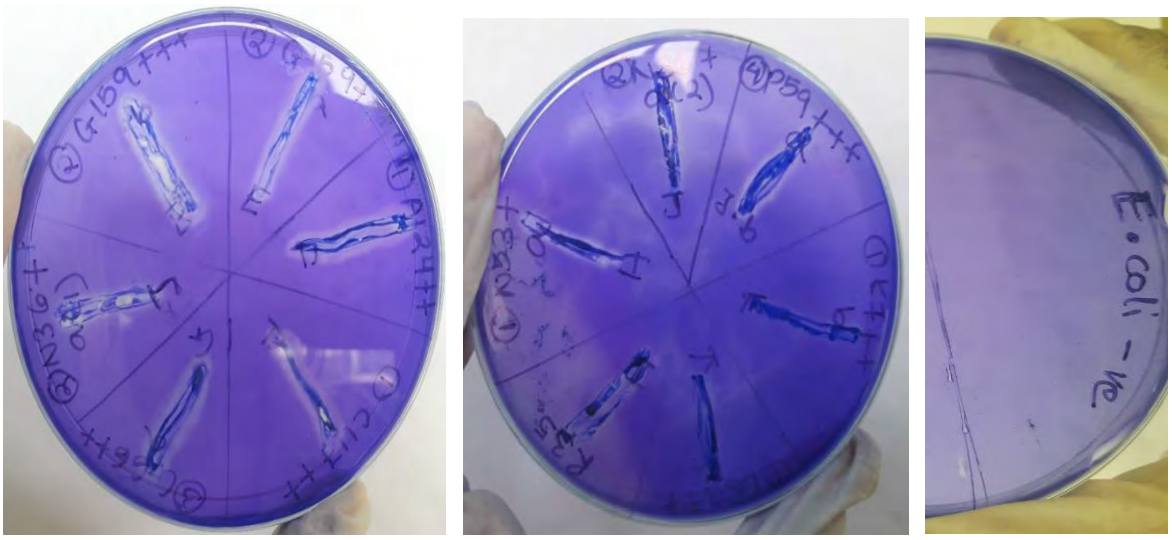


Figure 15 Clear zone formed by isolates on LDPE emulsified turbid agar plates

Table 6 Clear zones formed by isolates

Serial Number	Isolate number	Zone of clearance
1	B.c	++++
2	F	++++
3	G	++++
4	A.a	+++
5	E1	+++
6	E2	+++
7	J.b	++
8	J.a	+
9	H	+
10	N53a	+
11	B.b	-
12	B.a	-
13	L	-
14	N9	-

3.5 Bacterial survival screening:

The 10 different isolates found positive in the tertiary screening were further subjected to survival in MS2 broth supplemented with LDPE films yet again. The isolates were grown in 50 ml broth in 100 ml conical flasks grown at 37°C at 150 rpm (Figure 16). After every 9 days, 100 µl of broth is spread on Nutrient Agar (NA) and the colony count is observed after 24-hour incubation. Furthermore, 1 ml of fresh broth was added to each flask. After 36 days of continued incubation, out of 15 different isolates, 8 out of 10 isolates resulted in TNTC (too numerous to count) colonies namely: A.a, B.c, E1, E2, G, H, J.a and N53a (figure 18). These colonies were also observed to form biofilm over the film surface. The remaining isolates (J.b) and (F) ultimately gave lesser colonies until none over the incubation period (figure 19). A negative control was maintained at all times. For the same isolates, the experiment was conducted for pH 6 adjusted MS2 broth supplemented with 250 µl of Tween 20 to reduce surface tension between two phases (figure 17). The result did not vary from that obtained in pH 7.

Figure 16 Growth of isolates in MS2 broth in presence of 0.1% LDPE strips

Figure 17 Growth of isolates in MS 2 broth (pH 6 with 250 ul Tween 20) with 0.1% LDPE

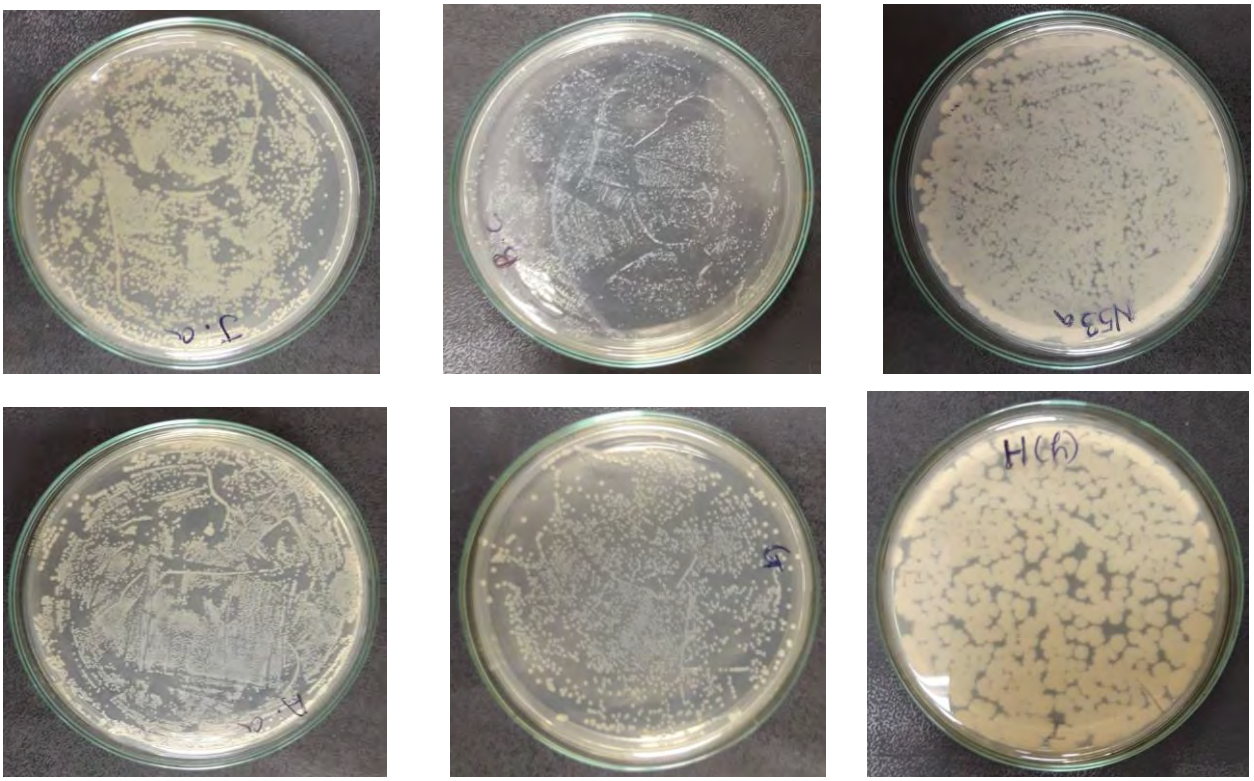


Figure 18 CFU of isolates after 36 days of continued growth in minimal media

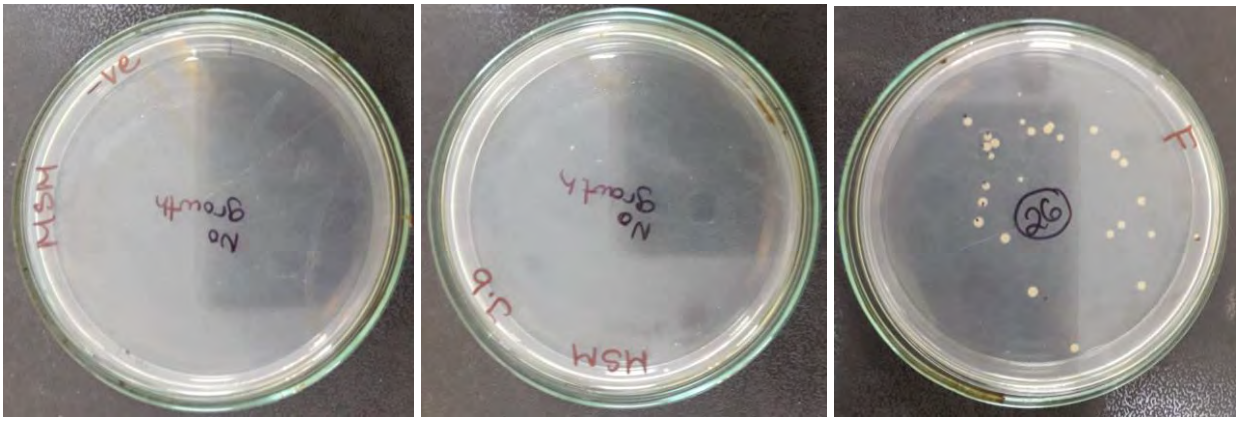


Figure 19 Minimal or no growth is observed for some isolates after 36 days of incubation; negative control at left

3.6 Biosurfactant production:

43 isolates obtained from primary screening were subjected to testing for biosurfactant production. 2 different media were used. Both the media had similar composition, except that CTAB media had CTAB supplemented in it while SDS media had SDS supplemented in it (figure 17). The zone observation can be aided by making a well in the agar and pipetting liquid inoculum into the well. A blue halo around the well or blue precipitate is an indication of positive biosurfactant production. The plate was then incubated for 48 hours at 37℃. The isolates that produced anionic biosurfactants were B76a, E133, and B103. The organisms that produced cationic biosurfactants were G159 which is also LDPE degrader.

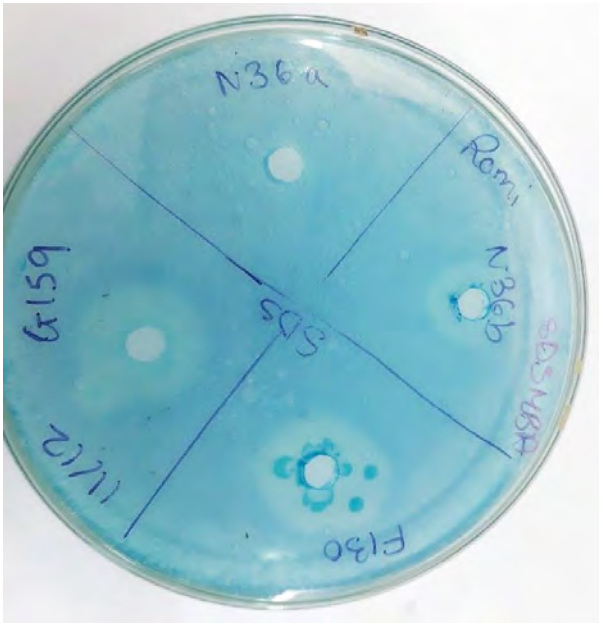


Figure 20 SDS agar giving dark blue halo around isolate G159 (E1) showing cationic biosurfactant production

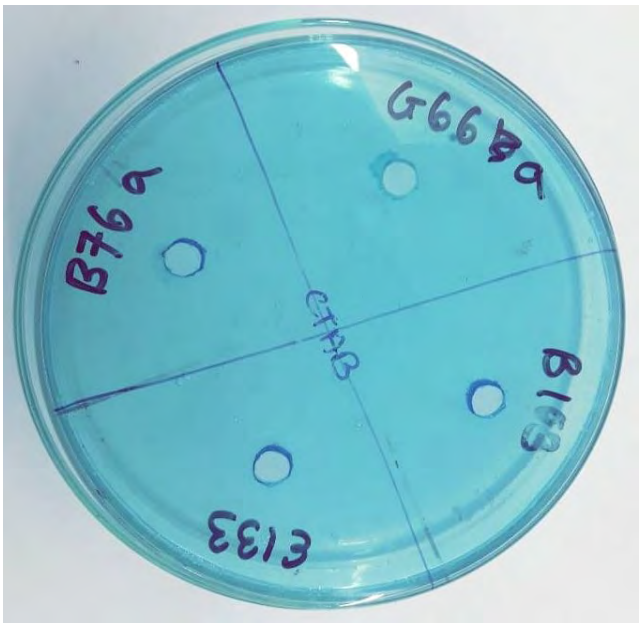


Fig 21 CTAB agar giving dark blue precipitate around well for B76a, E133 & B103 showing anionic biosurfactant production

3.7 Gram's staining

The Gram's staining was done twice for the same isolates, once before and once after 36 days of checking for bacterial survival in MS2 broth. The results were the same both times. The smears were observed under 100x oil immersion lens. The color and morphology of the isolates were

observed as seen in figure 22. The chart below lists the color, morphology, and structure of all the isolates (table 7).

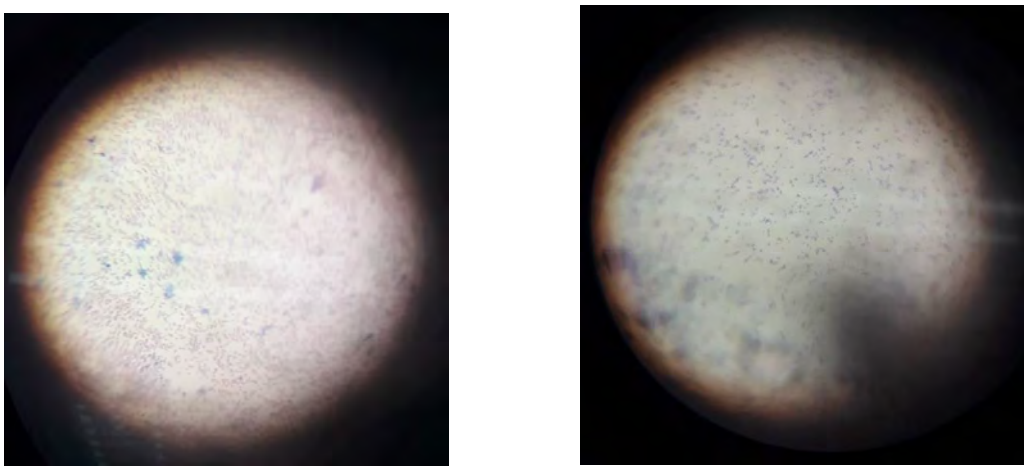


Figure 22 Gram-negative (left) and Gram-positive (right) isolates

3.8 Endospore staining:

The Endospore staining was done twice for the same isolates, once before and once after 36 days of checking for bacterial survival in MS2 broth. The results were the same both times. The smears were observed under 100x oil immersion lens. The spore was observed as green structures as seen in figure 9. The chart below lists the presence or absence of all the isolates (table 7).

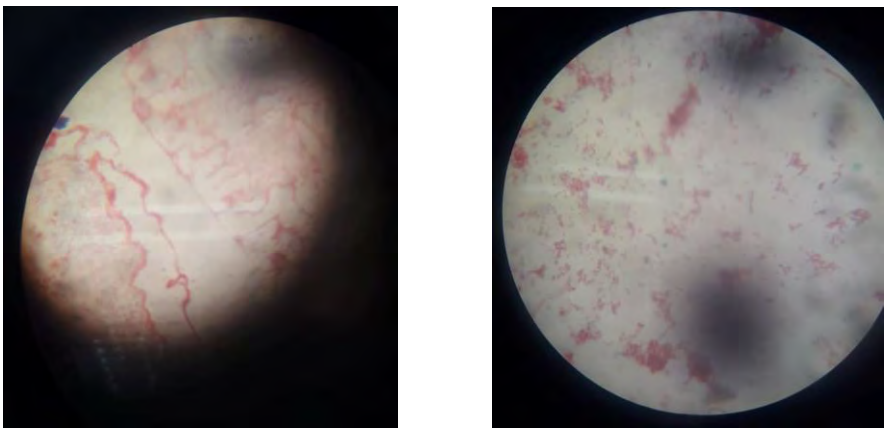
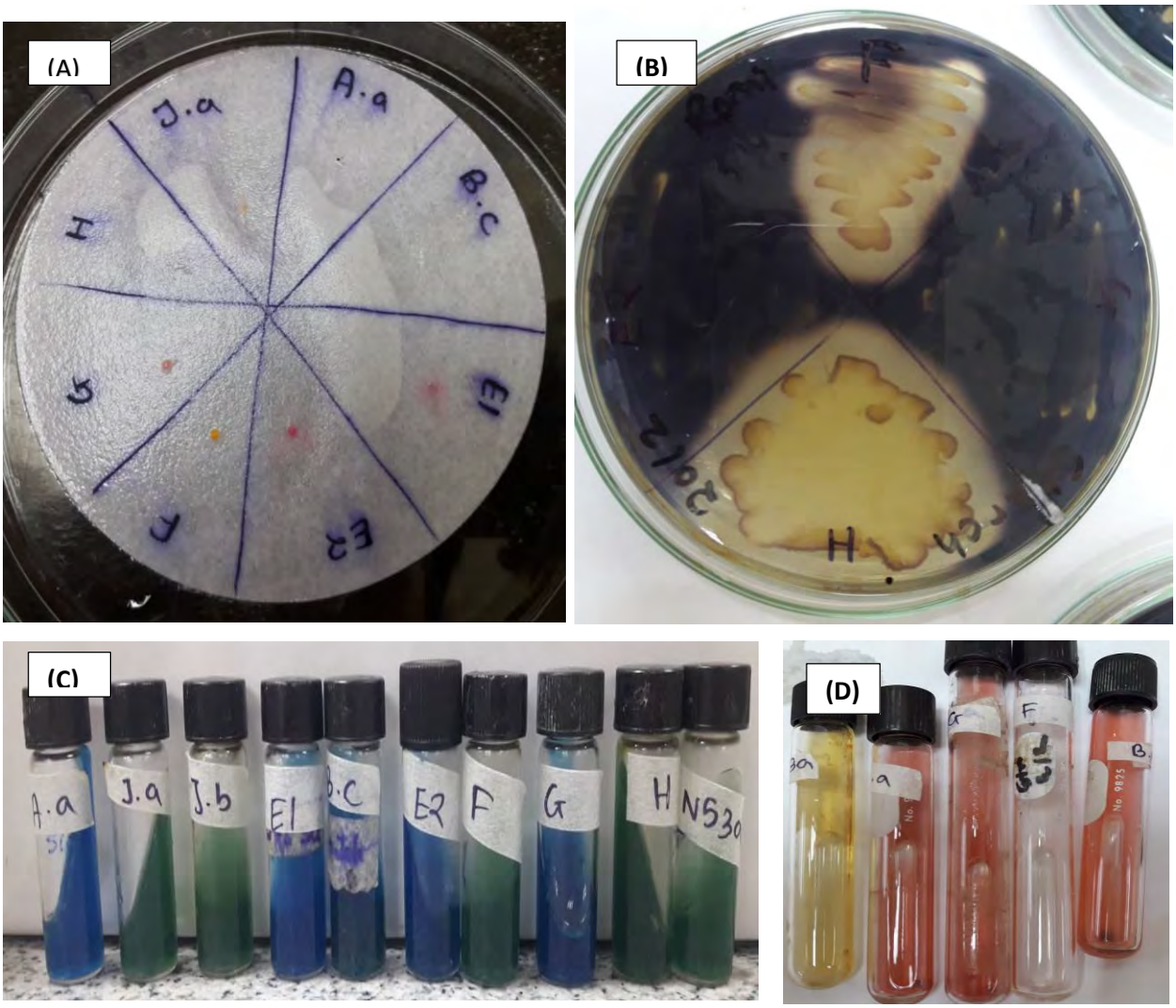


Figure 23 Non-sporing bacteria (left) and Sporing bacteria (right)

3.9 Biochemical tests

The biochemical tests were done twice for the same isolates, once before and once after 36 days of checking for bacterial survival in MS2 broth. The results of biochemical tests were the same for both times (figure 24) (Table 7). The probable organism was identified using ABIS Bacterial Identification online software.



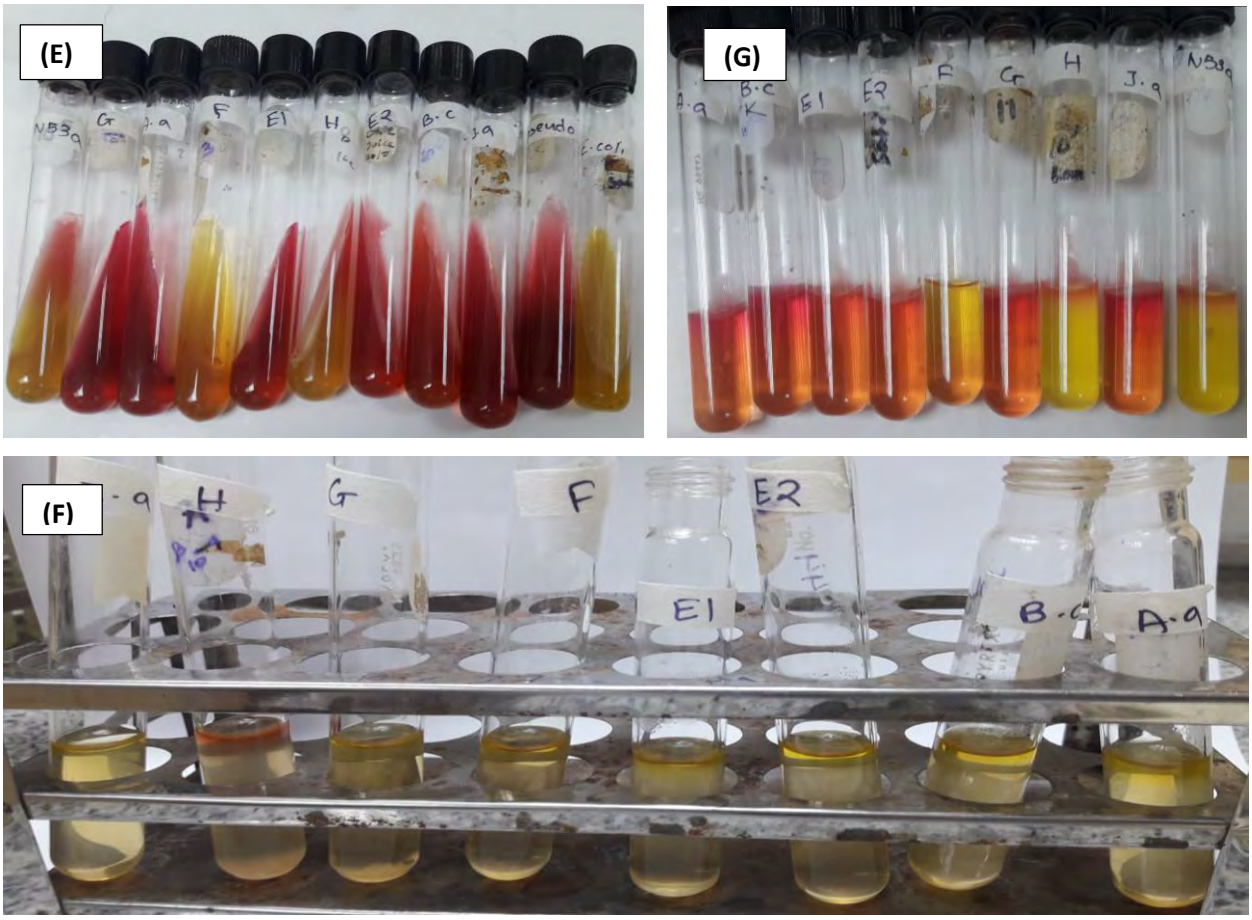


Figure 24: (A) Oxidase test: pink color for oxidase positive reaction and no color change for negative reaction, (B) Starch hydrolysis test: presence of amylase detected by clear zone around colony, (C) Citrate test: Citrate fermention positive gives green color while negative reaction gives no color change, (D) Nitrate Reduction: Nitrate reduction by formation of red color while nitrate reduction negative gives no color change, (E) TSI: for glucose, fructose sugar fermentation, (F) MIU: for motility and indole reaction, (G) MR: MR positive gives red ring on the surface

Table 7 List of Gram staining, Endospore staining and Biochemical Test

Serial Number	1	2	3	4	5	6	7	8	9	10	11	12	13
Sample Number	A.a	B.c	E1	E2	F	G	H	J.a	J.b	N53a	B76a	E133	B103
Gram staining	Positive, coccus	Negative, Rod	Positive, rod	Positive, rod	Positive, coccus	Positive, rod	Positive, rod, chain	Negative, rod	Negative, rod	Positive, rod, chain	Positive, coccus	Positive, coccus	Positive, coccus
Spore staining	-	-	-	-	-	-	+	-	-	+	-	-	-
Catalase	+	+	-	-	+	+	+	+	+	+	+	+	+
Oxidase	-	-	+	+	-	+	-	-	-	-	-	-	-
motility	-	-	-	-	-	-	+	-	-	+	-	-	-
Urease	+	+	+	+	-	+	+	+	+	+	+	+	+
citrate	+	+	+	+	-	+	-	-	-	-	+	+	+
Butt/Slant	K/K	A/K	A/K	A/K	A/A	K/K	A/K	K/K	A/K	A/K	K/K	K/K	K/K
H2S Production	+	-	-	-	-	-	-	+	-	-	+	+	+
Gas Production	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl Red	-	-	-	-	-	-	+	-	-	+	-	-	-
Voges Proskeur	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate Reduction	-	-	+	+	-	-	+	-	+	+	-	-	-
Starch Hydrolysis	-	-	-	-	+	-	+	-	-	+	-	-	-
Probable Organism	*Staphylococcus massiliensis*	*Acinetobacter baumannii*	*Clostridium novyitype A*	*Clostridium novyitype A*	*Staphylococcus arlettae*	*Lysinibacillus sphaericus*	*Bacillus psychrosaccharolytius*	*Acinetobacter lwoffii*	*Citrobacter rodentium*	*Bacillus psychrosaccharolyticus*	*Staphylococcus massiliensis*	*Staphylococcus massiliensis*	*Staphylococcus massiliensis*

4. DISCUSSION

The soil samples were mainly collected from petrol pumps and garbage sites (Albertsson, et al., 1998). Garbage sites have greater chances of harboring polyethylene degrading bacteria as these sites contain huge dumps of plastic wastes. On the other hand, petrol pumps deal with diesel, kerosene, petrol, etc, a large amount of which is spilled when oil trucks are emptied into reservoir tanks. These oils seep into the soil, for which reason, these soils are thought to be rich in hydrocarbon-degrading bacteria. As both oil and polyethylene are both macromolecular hydrocarbons, bacteria able to degrade oil are also suspected to be able to release a complex array of enzymes that may degrade polyethylene too (Albertsson, et al., 1987). That is why petrol pump sites are targeted in this experiment. Furthermore, water bodies around garbage dump sites have also been targeted in this experiment. These water bodies are along the shoreline where huge piles of plastic wastes stand. Rainfall causes leachates from these sites to trickle down into a water body. This water then, too, becomes a reservoir for polyethylene degrading bacteria. The samples (soil and water) have been collected from a depth of nearly 10 centimeters because hydrocarbon degrading bacteria usually have a higher optimum temperature, therefore, chances are these bacteria would be found in layers deeper than the surface where the temperatures are a bit higher (Brown, et al., 1974).

Primary screening screens out bacteria unable to survive on a synthetic polymer such as LDPE from the ones that can. The broth that has been used in the screening procedure is selective in the sense that it only has LDPE films as a carbon source. Periodic inoculation of the previous inoculum into fresh broth ensures that the organisms do not run out of nutrients to survive (Mahdiyah, et al., 2006). In other words, the only component that is limiting in the broth is the carbon source itself. Nevertheless, some bacteria unable to degrade polyethylene may still survive in this selective broth, the reason being that breakdown of polyethylene produces by-products that include ketones, aldehydes, acids, alkalis, etc (Raaman, et al., 2012). These by-products are fed upon by non-degraders that ensure their survival even in selective broth. Therefore, primary screening is not enough alone (Tribedi, et al., 2013).

The spectrophotometer readings that were taken throughout the incubation period is an indirect measure of the falling bacterial population in the broth. The graph (figure 11) below is a visual

demonstration of the falling final OD with increasing time, i.e., the final OD is inversely proportional to time. The final OD is indicative of the total microbial population in the culture, which suggests that over the increasing period of time, the organisms are dying for not being able to breakdown LDPE (Mahdiyah, et al., 2006). On the other hand, Figure 12 shows a graph that demonstrates the increasing OD between the initial OD and final OD. The positive difference between the two ODs is indicative of the presence of bacteria that are able to degrade LDPE as a carbon source, hence, their numbers are rising. The difference between the two values keeps decreasing over time, which further signifies that undesired organisms are dying off from lack of food source (Mahdiyah, et al., 2006).

Secondary screening is targeted towards organisms that can perform bacterial colonization over the surface of LDPE films because the first step in the mechanism of LDPE degradation is biofilm formation. Without proper attachment of bacteria to the surface of polyethylene, degradation will not occur. Therefore, the experiment set out to observe and isolate organisms that were able to grow over the polyethylene films placed on Nutrient Agar (NA). Furthermore, those that grow on polyethylene surfaces were most likely to secrete the complex array of enzymes that can degrade the polymer (Gilan, et al., 2007). One drawback to this technique is that some non-degrading organisms may overgrow on the far end of the plastic corners. Therefore, these organisms were further analyzed in the tertiary screening. Out of 43 isolates obtained as PEG degraders, only 14 isolates were able to form microbial colonization or biofilm over LDPE surface.

Tertiary screening of organisms is a final method of interpretation of polymer-degrading organisms. Here, the LDPE (low-density polyethylene collected from the market) is incorporated into the solid agar media, resulting in a turbid agar plate. Organisms that are able to breakdown the LDPE in the media will secrete LDPE degrading enzymes around it. As the enzymes use the LDPE as a carbon source, they cause a clearing of the turbidity around the colony, giving a clear zone in its surrounding area (Pramila, et al., 2011). To make a clear zone prominent, a staining solution followed by the destaining solution is added to the media (Tribedi, et al., 2013). This causes the zone to become more prominent. The media is also selective and the growth on the media is very slow. However, very few organisms are able to grow on the media, though they do not give a clear zone. It is because these bacteria are able to use the agar as a carbon source and survive on the media. Nevertheless, this method is an accurate screening method as it gives visual

confirmation of the degrading organisms. Out of the 14 isolates retrieved from secondary screening, 10 were able to form a zone of clearance in the tertiary screening.

For the final part, the isolates were grown in MS2 broth supplemented with 0.1% LDPE as a carbon source. The purpose of growing isolates in a continuous incubation was to observe whether the isolates were able to sustain growth in salt broth by itself by visually witnessing the CFU (Colony Forming Units) after every 9 days in a condition with a limited carbon source. The broth culture was replenished with 1 or 2 milliliters of salt media after every 9 days so that organisms do not run out of nutrients to grow and divide. Out of a total of 11 isolates retrieved from primary screening, 8 isolates were able to survive in continuous culture method over 36 days period, namely *Staphylococcus massiliensis* (A.a), *Acinetobacter baumannii* (B.c), *Clostridium novyitype A* (E1), *Clostridium novyitype A* (E2), *Lysinibacillus sphaericus* (G), *Bacillus psychrosaccharolyticus* (H), *Acinetobacter iwoffii* (J.a) *and Bacillus psychrosaccharolyticus* (N53a). Theoretically, all the 10 isolates should have been able to survive in this limiting conditions as all the 10 isolates were able to form clear zones in LDPE media. The slight derivation by two isolates, namely, organism *Staphylococcus arlettae* (F) and *Citrobacter rodentium* (J.b), may be due to the fact that these organisms survive in SM (Synthetic Media) rather than MSM (Minimal Salt Media) (Sen, et al., 2015). MSM media are composed of all salts whereas, SM media are composed of fewer salts and yeast or peptone extracts (Gilan, et al., 2007). The added yeast or peptone acts as a co-substrate that act as electron donors and enhance the reductive cleavage of the polymer (Santo, et al., 2012). In recent literature, (Brandon, et al., 2018), mealworms that were fed polyethylene were found to degrade the polymer by 60.1% of weight loss. It was found that the gut microbiome consisted of the bacteria *Citrobacter sp* and one other organism. This shows that *Citrobacter* propagates in rather complex media than simple minimal salt media, which further goes on to explain why the organism did not grow in MSM2 broth. The experiment was repeated in pH 6 and the addition of Tween 20 as a surfactant. The purpose of this method was to see if the bacterial count of isolates F and J.b increased with lower pH. A lower pH is often time the case found in liquid media as the CO_2 emitted mixes with the aqueous solution to cause a drop in the pH (Tribedi, et al., 2013). Addition of Tween 20 was carried out to enhance to jump-start the degradation and increase CFU number. However, all these factors did not bring any change to the final result and isolates F and J.b showed decreasing CFU number with increasing time.

Finally, in the case of biosurfactant production screening, the CTAB media were used for the screening of anionic biosurfactant producing organisms while SDS media were used for screening of cationic biosurfactant producing organisms (Karlsson, et al., 1988). The basic mechanism follows. CTAB itself is a cationic surfactant. When the CTAB (cationic) is incorporated into the media, and an organism produces an anionic biosurfactant, the CTAB (cationic) and anionic biosurfactant produced by the microorganism form an insoluble complex with the methylene blue in the media giving a dark blue halo zone around the colony (Aouseoud, et al., 2011). The opposite is true for SDS media, however, the mechanism remains the same. The 3 isolates that produced anionic surfactants namely B76a, E133, and F103 were identified to be *Staphylococcus massiliensis* in ABIS. The G159 or E1 isolate was identified to be *Clostridium novyitype A* as mentioned earlier.

Gram's staining, endospore staining, biochemical tests, and clear zone screening were done twice, once before the 36 days incubation in continuous culture, and once after the culture. This was done so as to ensure that the organisms that were present in 36 days culture were the same organisms that were identified after the primary, secondary and tertiary screening, and not contaminants. This step was a necessary precaution as it is very likely that conical flasks may easily get contaminated when cultured in continuity for such a long period of time.

4. CONCLUSION

In conclusion, the microbial strains able to degrade synthetic market-grade low-density polyethylene were successfully isolated from locally collected soil samples. After the primary, secondary and tertiary screening, the microorganisms that were discovered to as potential degraders are *Staphylococcus massiliensis, Acinetobacter baumannii Clostridium novitype A, Staphylococcus arlettae, Lysinibacillus sphaericus, Bacillus psychrosaccharolyticus, Acinetobacter iwoffii, Citrobacter rodentium.* The process of degradation is, as of now, a slow process, hence, the isolated biosurfactant producing bacteria *Staphylococcus massiliensis* and *Clostridium novyitype A*could be used to enhance the degradation. Nevertheless, degradation of up to 60.1% has been achieved in earlier experiments, albeit over a 30 days' incubation period. The process can be paced by performing the pretreatment of polyethylene before subjecting them to biodegradation, using microbial consortium to aid in the process, or a more modern approach would be to perform protein engineering in order to increase the efficiency of enzymes responsible for degradation. The isolation is a part of an even bigger experiment, where the next step is the determination of the rate of polyethylene degradation, observation of the presence of enzyme gene responsible for degradation, isolation of crude enzyme and determines its effect on degradation. These findings have important applications in solving the plastic waste problem through bioremediation where modern approach developed for remediation can be combined and applied with these organisms.

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