ISOLATION AND CHARACTERIZATION OF POTENTIAL BACTERIA RESPONSIBLE FOR BIODEGRADATION OF LOW-DENSITY POLYETHYLENE (LDPE) COLLECTED FROM GARBAGE DUMP SITES IN DHAKA

Submitted by

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

Department of Mathematics and Natural Sciences

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Declaration

It is hereby declared that

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

Student's Full Name & Signature:

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Approval

The thesis titled “Isolation and Characterization of Potential Bacteria Responsible for Biodegradation of Low-density polyethylene (LDPE) Collected from Garbage Dump sites in Dhaka” submitted by Abida Nurun Nahar (18136030) and Maisha Farzana (18136069), has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor in Biotechnology on

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Ethics Statement

This material is an original work, which has not been previously published elsewhere. It is our own research and analysis in a truthful and complete manner. The paper properly credits all the sources used (correct citation).

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Sincerely,

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Abstract

Being a multifaceted polymer, the recalcitrant Low Density Polyethylene (LDPE) has slowly gained popularity for ease of access while simultaneously becoming a threat to nature. This is why investigating diverse bacteria and exploring their natural capability to biodegrade LDPE has become a pressing matter in environmental biotechnology research. This study explores the LDPE biodegradation potential of bacteria isolated from half-degraded plastics from garbage dump sites. Significant weight loss (20%-30%) was detected from five isolated bacteria from pure cultures. 16sRNA gene sequence of three of them revealed them to be *Bacillus* sp. (in: Bacteria) strain **JDMASP60**, *Bacillus atrophaeus* strain **MGB14**, and *Bacillus pumilus* strain **KD3** indicating their taxonomic novelty. Additional biodegradation examinations revealed their LDPE metabolization capability. Optical microscope analysis at 1000x magnification showed substantial amount of damage (cracks, grooves, and minor holes) on the polymer surface. FTIR showed a percentage increase in transmittance at the peaks 2918 cm^-1, 2852 cm^-1, 1468 cm^-1, 1373 cm^-1, 718 cm^-1, among many others indicating a decrease in native bonds. Moreover, a new peak corresponding to aromatic C=C bond at 1600cm^cm-1 was also reported. The study highlighted the biodegrading capability of naturally occurring bacteria at garbage dump sites while using LDPE as their sole carbon source.

1. Introduction

1.1. The Plastic Era

1.1.1. Terminology, history and characterization

From the routinely disposable coffee cup accumulating in the bin to flickering LED light bulbs, the versatility of plastics have revolutionized the world in more ways than imaginable. A material that can be extruded, shaped, cast, spun or used as a coating at any point in manufacturing is termed as “plastic”. Monomers derived from oil or gas are polymerized to form synthetic plastics in which numerous chemical additives are typically added [1]. Any synthetic or semi-synthetic material that consists of mainly monomers are grouped together to form plastics. While the first use of polymers can be traced back to the molding of natural rubber by Mesoamericans in approximately 1600 BC, the birth of the modern synthetic polymer began with Charles Goodyear’s invention of vulcanized rubber and discovery of polystyrene (PS) by Eduard Simon in 1839 [2]. The most ubiquitous plastic types are called commodity plastics that are used for products that do not require exceptional mechanical properties in contrast to engineering plastics [3]. Such commodity plastics are categorized into six major types - with each type being assigned a resin identification code (RIC) to indicate the type of resin it was produced from (Figure 1.1a) [4]:

1. Polyethylene terephthalate (PET)
2. High-density polyethylene (HDPE or PE-HD)
3. Polyvinyl chloride (PVC)
4. Low-density polyethylene (LDPE or PE-LD)
5. Polypropylene (PP)
6. Polystyrene (PS)

Types of plastics	Symbol	Applications
Polyethylene terephtalate (PET)		Beverage bottles, medicine jars, rope, clothing and carpet fiber
High-density polyethylene (HDPE)		Containers for milk, motor oil, shampoos and conditioners, soap bottles, detergents, and bleaches
Polyvinyl chloride (PVC)		All kinds of pipes and tiles
Low-density polyethylene (LDPE)		Cling-film, sandwich bags, squeezable bottles, and plastic grocery bags
Polypropylene (PP)		Lunch boxes, margarine containers, yogurt pots, syrup bottles, prescription bottles, plastic bottle caps, plastic cup
Polystyrene (PS)		Disposable coffe cups, plastic food boxes, plastic cutlery and packing foam
Polyethylene Acrylonitrile butadiene styrene (ABS) Polyamide (PA) or Nylons Polybutylene terephthalate (PBT)		Baby bottles, compact discs, and medical storage containers

Figure 1.1(a): The major types of plastic outlined along with their RIC and applications [4].

1.1.2. LDPE

LDPE is produced by a free radical polymerization method in pressures of 150–350 MPa and temperatures of 80–300°C [5]. Made up of long branches unable to be packed into crystallites, it is a tough and flexible polymer with a density of 0.910–0.925 g/cm3 [6]. LDPE demonstrates good resistance to chemicals and creeping alongside impact toughness and resistance at low-temperatures (Table 1) [6, 7]. Additionally, it is relatively translucent and has an oily surface [8]. Owing to its stability and toughness, LDPE is widely used in numerous products, especially packaging materials in food and beverages. The most common use of LDPE can be seen in plastic bags. Cheap, easily made and flexible are some of the advantages of using LDPE as packaging material [8]. In 2021, the Global LDPE market was estimated to be valued at $67.56 billion [9].

Table 1: Mechanical properties of LDPE [6, 7].

Mechanical Properties of Low Density Polyethylene (LDPE)	
Density	0.910-0.925 g/cm3
Melting temperature	109°C
Maximum using temperature	150°C
Ultimate tensile strength	60 Mpa
Module of elasticity	4500 Mpa
Hardness	275 HRB
Charpy impact toughness	1.4-1.6 kJ/m2

1.1.3. Soar in Global Plastic Production

Due to its lightweight nature, chemical and light resistance, stability at a wide range of temperatures, high strength, versatile application and low cost, plastics have garnered widespread use throughout the world [1, 10]. With rapid advances in agricultural, electronics, automotive, medical and food processing industries, demand for plastics as packaging and casing materials have skyrocketed [11]. In addition, the boom in global population has also increased the relentless consumption of plastics. Unsurprisingly, the global production of plastics has reached nearly 370 million metric tonnes in 2020 from just 2 million metric tonnes in 1950 - an increase of ~18,400% [12, 13]. The global annual plastic production from 2008 to 2020 is shown in Figure 1.1(b) [14]. In 2020, the production of plastic declined slightly with the pandemic slowing down the world economy [14]. However, the upward trend of production is only estimated to rise as dramatically in the future. It is projected that by 2050, the annual plastic production will reach 800 million metric tonnes [15].

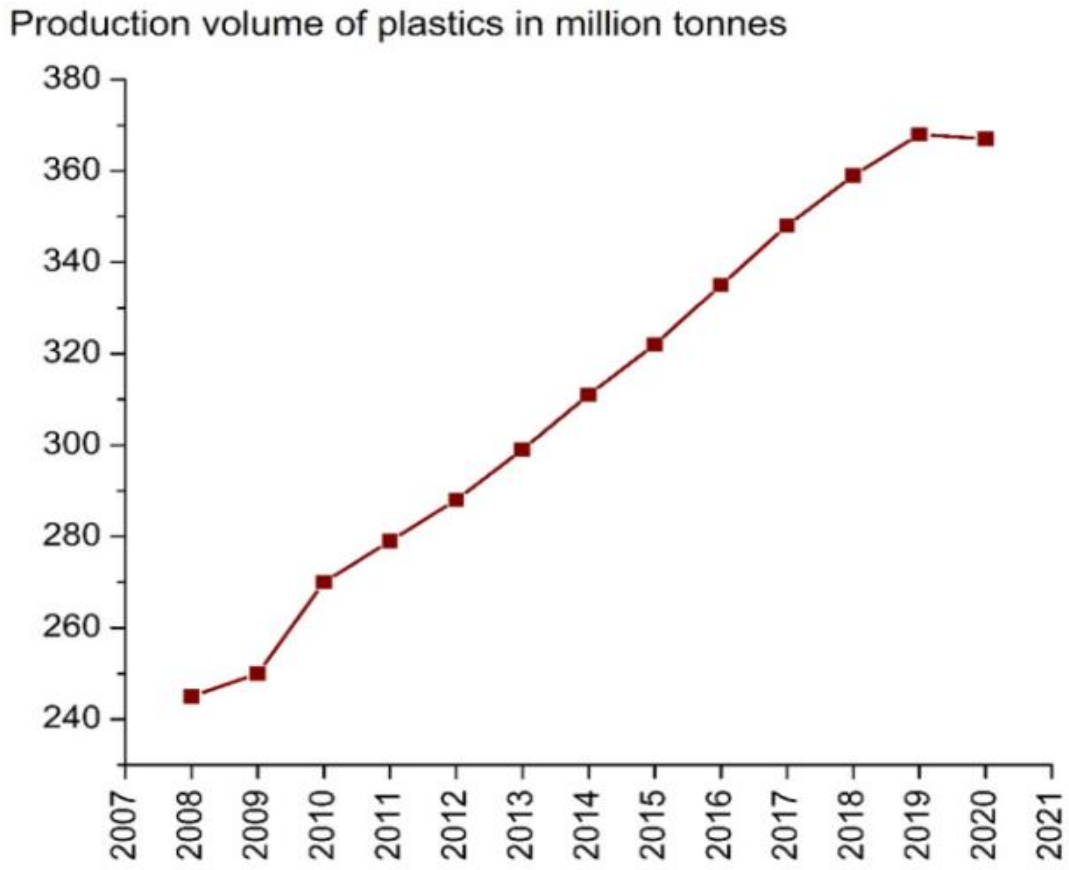

Figure 1.1(b): Production volume of plastics produced globally from 2008 to 2021 [14].

1.1.4. Bangladesh's Perspective

Consumption and production of plastics in Bangladesh has dramatically increased like the rest of the world. Plastic waste of the capital had skyrocketed from 178 tons per day in 2005 to 646 tons per day in 2020 - an increase of more than 3.5 times [16]. In particular, consumption of LDPE packaging materials rose fivefold from 2005 to 2020 [17]. Single-use plastics are the most mismanaged waste which has worsened in the COVID-19 pandemic due to mass usage of masks, gloves and Personal Protective Equipment. Additionally, Dhaka city's waste contributes to 10% of all the plastic waste in Bangladesh which highlights the massive daily plastic waste generated in the entire country [17]. In 2021, around 1700 tonnes of plastic waste had been generated daily in Bangladesh. Additionally, Bangladesh produces around 87,000 tonnes of single-use plastic waste every year from which 86% of the waste ends up in landfills [18]. In Dhaka alone, 310.7 tonnes of plastic waste ends up in landfills daily [19]. Consequently, such massive plastic waste has led to clogged drains, huge plastic dumps, bags floating around and overflowing plastic materials in rivers [18]. Although certain steps were taken to curb the plastic pollution such as banning plastic shopping bags and single-use plastic in coastal areas, plastic pollution has only increased. Furthermore, small amounts of plastic waste are recycled - only 37% in Dhaka [20].

1.2. Lack of a sustainable disposal method

1.2.1. Incineration

With the drastic increase in plastic consumption and production, there are numerous drawbacks associated with plastic wastes that pose threats to the human, vegetation, animal and environment alike. From the millions of plastic wastes generated annually, 9% of it are recycled, 12% are incinerated and the remaining 79% either dumped in landfills or released directly into the environment [21]. Incineration of plastic wastes generate toxic gases such as dioxins, mercury and furans which enter the atmosphere- harming neighboring human, animal, vegetation and environment [22]. To illustrate, 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) is a component of dioxins that causes cancer and neurological damage while disrupting thyroid, respiratory and reproductive systems. Moreover, burning PVC liberates hazardous halogens into the air that significantly contributes to climate change and depletion of the ozone layer.

1.2.2. Landfills

Most plastics end up in landfills around the world which also have many negative impacts on the environment and living organisms. Firstly, degradation of conventional plastics generates greenhouse gases such as methane and carbon dioxide amongst other toxic gases [23]. Approximately 5% of greenhouse gas emissions can be attributed to landfills [24]. Secondly, leachate from the deposited waste can seep into the groundwater or surface water that can endanger water quality and subsequently, human and animal lives. Next, landfills greatly downgrade the living conditions of surrounding inhabitants due to flies, smoke, noise and bad odors [23]. In a study in Bangladesh observed, people living close to landfills reported bronchial diseases, pneumonia, diarrhea, headache, itching and appetite loss [25]. Insect and animal infestations are also common near landfills that accelerate vector-borne diseases. Lastly, landfills are only a temporary solution to the growing plastic pollution since habitable land is a finite resource. Coupled with an ever-growing world population, landfills can no longer be the long-term solution for mitigation of plastic wastes.

1.2.3. Microplastics

Increasingly, plastic accumulation in the marine environment is becoming evident with adverse effects on multiple ecological aspects such as biodiversity and human health [26]. Predominantly, marine plastic pollution consists of microplastics which are defined as insoluble, synthetic solid particles sized from 1 μm to 5 mm of primary or secondary manufacturing origin [27]. Ingestion of microplastics by marine organisms such as zooplankton which can cause serious harm [28]. Negative health effects associated with ingestion include wounds, skin lesions, sores and blocked digestive tract [29]. Microplastics can travel through the food chain and enter human bodies where it causes numerous adverse effects (Figure 1.2a) [30]. Concerning additives such as Bisphenol A, heavy metals and phthalates have been held responsible for the harmful effects on humans [27]. Besides, entanglement with plastic debris may lead to starvation and debilitation of marine animals [29]. Also, plastic packing loops may tighten around animals' flesh causing cuts and eventually strangulation.

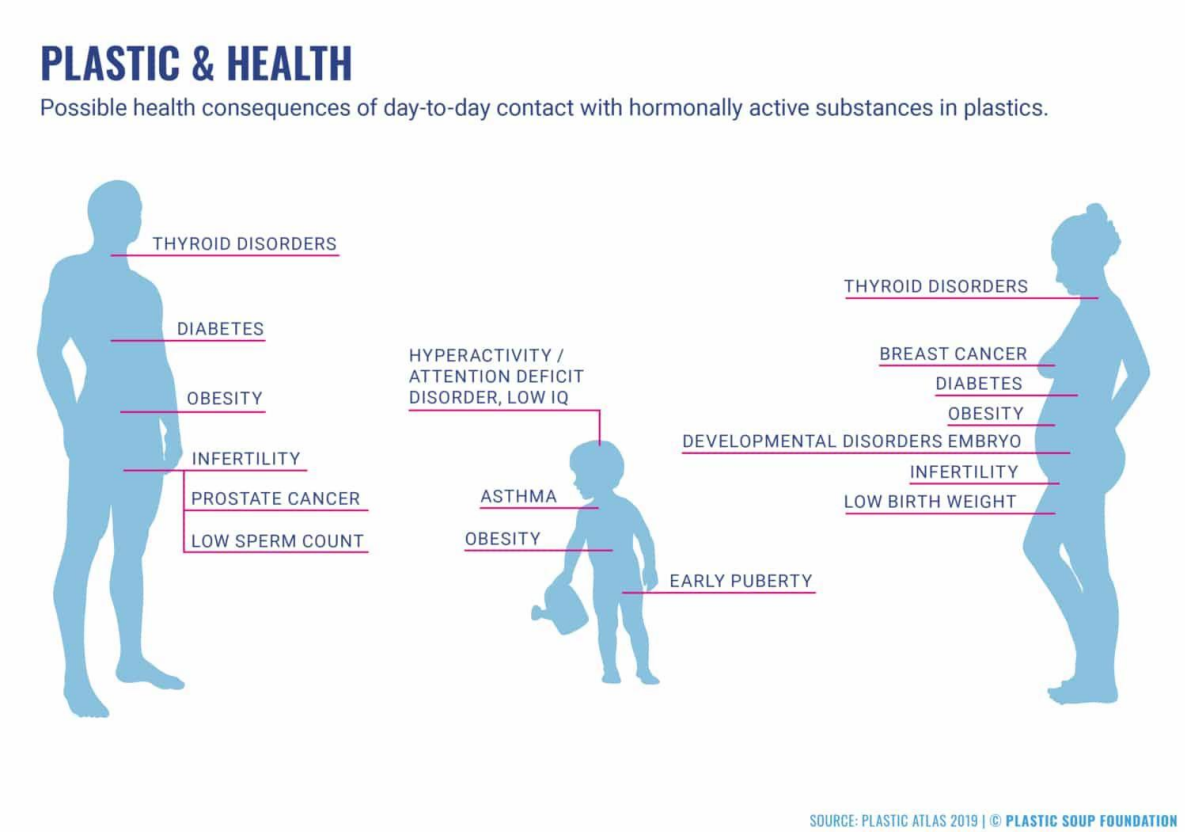

Figure 1.2(a): Negative health effects of microplastic ingestion on the human body [30].

1.2.4. Recycling

While recycling can reduce the plastic waste accumulated throughout the world, it has many significant challenges. Efficiency of recycling largely depends on the number of barriers that are subjected to individuals - physical barriers (waste bin infrastructure, proximity to recycling facilities, waste collection vehicle accessibility); socio-economic barriers (age, awareness, level of education, level of income); human behavioral factors; waste policy constraints; recycling service constraints [31]. Furthermore, recycling polymers severely reduces its functionality. As polymers are sensitive to very high temperatures and mechanical treatments, recycling results in inherent loss of many properties such as reduction in molecular mass [32]. Recycling is also carried out up to a greater extent with pre-consumer waste such as industrial packaging materials than post-consumer waste despite the latter being present in greater volumes [33].

1.3. Biodegradation

Biodegradation refers to the capability of microorganisms to successfully convert complex organic compounds into simple compounds [34]. By using these compounds as carbon and energy sources, microorganisms can better adapt to the contaminated environment as it gives them a selective advantage. Microbial metabolization of plastic results in an inert hummus-like substance which is less harmful to the environment [35]. Studies have revealed that biofilm formation on the plastic surface is a crucial step in the biodegradation process. As biofilms reduce hydrophobicity of the polymer, it facilitates the degradation rate [36]. While numerous biodegradation studies have taken place globally, scientists have discovered that the overall process involves the gradual conversion of whole plastic products into microbial biomass through fragmentation into microplastics, hydrolysis into soluble intermediate products and assimilation into biomass of microorganisms involved (Figure 1.3a) [37].

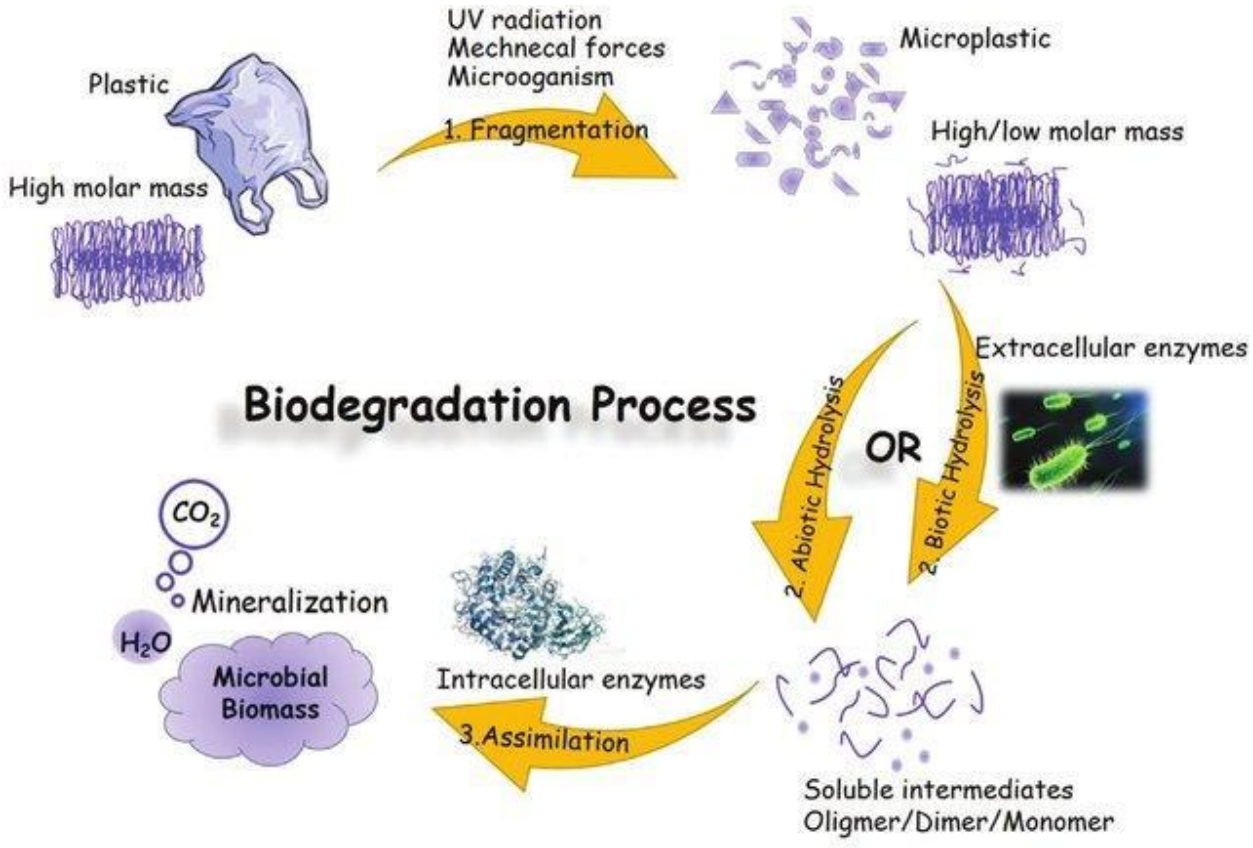

Figure 1.3(a): Schematic representation of the various steps involved in biodegradation [37].

1.3.1. Factors affecting biodegradation

Multiple factors control biodegradation. These factors can mainly be classified into two categories- polymer properties and exposure conditions [38].

1.3.1.1. Polymer properties

The chemical and physical properties of the polymer is vital for understanding the rate of biodegradation [39]. The aspects that affect biodegradability are mentioned below:

The presence or absence of functional groups plays a significant role. If a functional group responsible for hydrophobicity is present then the degradation rate will be slower in comparison to hydrophilic ones [40]. The molecular weight is also another important factor as it has a reciprocal relation with degradability, i.e. if the molecular weight increases, biodegradability decreases [39]. Furthermore, the morphology and degree of crystallinity also affects biodegradation rate [38]. The loosely packed amorphous region is first attacked by enzymes making them more susceptible to degradation compared to crystalline regions. Higher melting temperature (Tm) also lowers biodegradation rate [39]. Moreover, presence of easily breakable bonds like amide bonds or ester bonds also affects biodegradation [40]. Other aspects include additives, copolymers, cross-linking, blend, physical state etc. [38].

1.3.1.2. Exposure conditions

Similar to polymer properties, many biotic and abiotic factors act as stimulus for biodegradation.

1.3.1.2.1. Biotic factors

Biotic or biological factors like hydrophobicity, extracellular enzymes, nutrients, biosurfactants affect biodegradation [38]. The concentration of enzymes has a positive effect on the process as the rate of degradation increases with the increase of catalyst concentration. Other biological factors include mutation, horizontal gene transfer, biomass production, population size etc. [41].

1.3.1.2.2. Abiotic factors

Biodegradation also depends on the abiotic or environmental factors. In order to have a successful microbe-polymer interaction, the temperature, pH, moisture, UV radiation and many other factors need to be optimized [38]. Optimum moisture content ensures enhanced biodegradability via microbial growth and reproduction. On the contrary, an increase in temperature negatively affects biodegradation. Furthermore, the pH level also influences biodegradation rate as the acidic and basic conditions are altered which subsequently affects microbial growth [40]. Similarly, other abiotic factors like soil structure, water solubility, oxygen content, toxicity etc. directly affects the rate of biodegradation [41].

1.3.2. LDPE degradation pathway

LDPE biodegradation is initiated as soon as the microorganism attaches to the polymer surface and secrete extracellular enzymes [42]. The enzymes initiates depolymerization where the LDPE polymer chain into smaller monomers (oligomers, dimers, monomers) which pass through the semipermeable membrane of the microbial cells by carriers belonging to the Major Facilitor Superfamily (MFS) or harboring ATP binding cassettes (ABC) [43, 44]. The monomers are then utilized as the carbon source via mineralization producing carbon dioxide, water or methane as end products [45].

The beta-oxidation pathway allows the bacteria to produce its energy by decreasing the average molecular weight of the LDPE substrate [46]. As a result of oxidation, a radical reaction takes place which forms carboxylic groups, alcohols, ketones and aldehydes [44]. Radical reaction consists of 4 steps starting with radical initiation and de-propagation. Then comes intermolecular and intramolecular hydrogen transfer followed by beta scission where a new radical is formed by taking away a hydrogen atom from the reactant. Beta-scission is the breaking of the radical center to produce a molecule with double bond (with the carbon atom that had been at the radical center). Finally, the mechanism ends with radical termination [47]. The oxidation followed by fragmentation makes the otherwise hydrophobic polymer more hydrophilic in nature. This allows easier access of extracellular enzymes such as lipases, esterases, or endopeptidases [44]. The produced carboxylated n-alkanes then finally enter the beta-oxidation pathway where they are metabolized [48]. Based on the number of carbon atoms, the oxidized carboxylic molecules are

converted into two different molecules- Acetyl CoA when it is even and Propionyl CoA when it is odd [44]. Propionyl CoA is subsequently converted to Succinyl CoA using the enzyme propionyl-coA carboxylase [49]. The produced Acetyl CoA and Succinyl CoA then finally enters the tricarboxylic acid (TCA) cycle where ATP is produced. The bacteria uses the energy to replicate and create new microbial biomass [44, 48]. Figure 1.3(b) represents the metabolic pathways of depolymerized products of six different plastics [50].

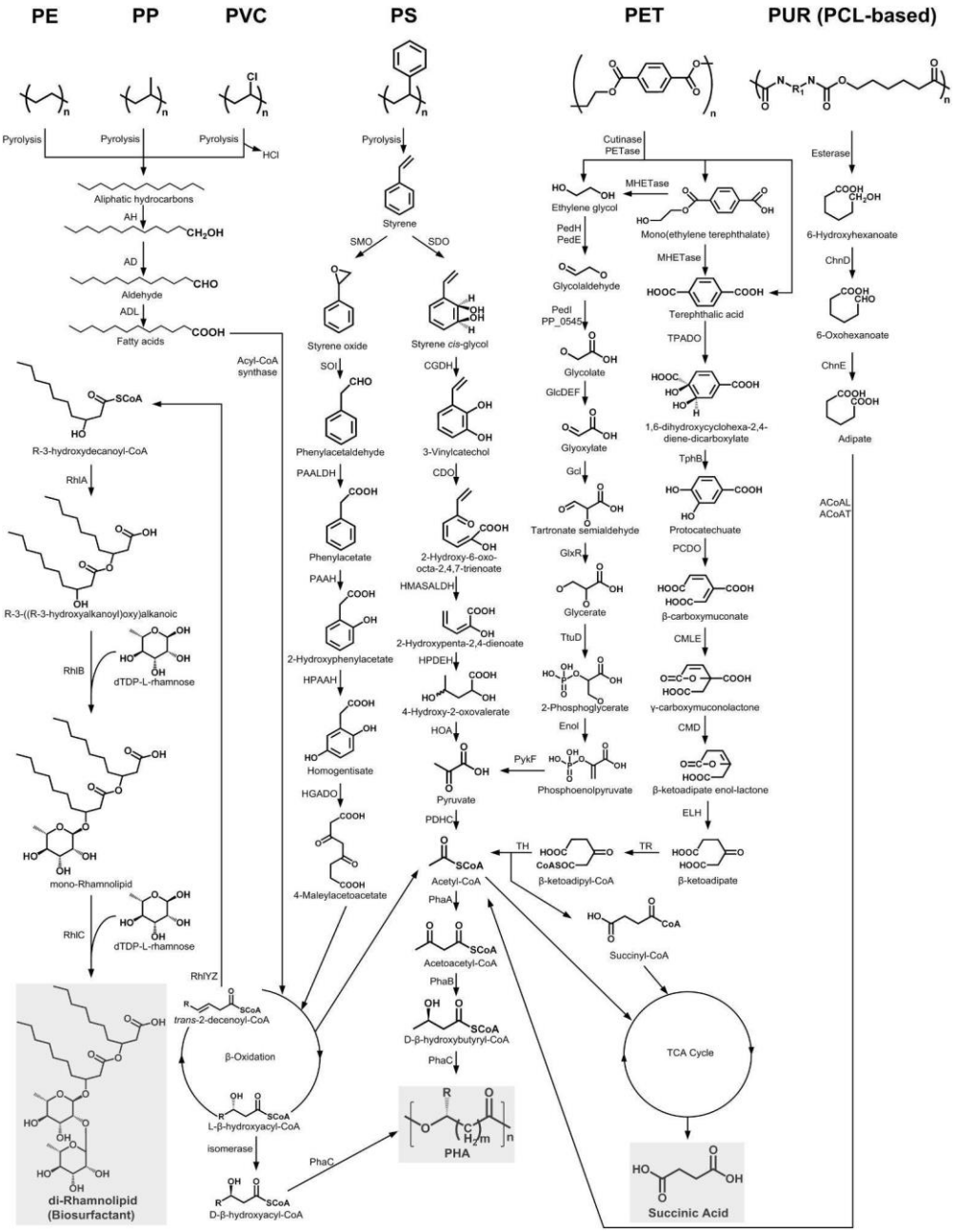

Figure 1.3(b): Metabolic pathways of biodegradation of six different plastic types [50].

1.4. Objectives

In our literature review, we have noticed that a comprehensive evaluation of biodegradation of LDPE by bacteria isolated from the soils of Bangladesh was lacking. Through our research, we attempt to design a valid experiment that provides a sustainable solution to plastic waste management in our country. Particularly, our objectives include the following:

- Evaluation of biodegradation of LDPE by soil microorganisms
- Identification of microorganisms responsible for biodegradation
- Determination of chemical bond modifications through biodegradation
- Study of plastic surface topology changed after biodegradation
- Provide a sustainable plastic waste management system for Bangladesh

2. METHODS AND METHODOLOGY

2.1. Sample site and collection

Partially degraded plastic samples were aseptically collected from 3 different garbage dumping sites within Dhaka City- Shahjahanpur Railway Colony, Mirpur and Jashohara Railway. To maintain aseptic condition sterile forceps were used and gloves were worn while picking up the plastic samples. Necessary steps were taken to ensure that the chosen plastic samples were buried well deep into the soil. The hypothesis behind this being the soil adhered to the sample contained bacteria that allowed degradation. The collected samples were immediately taken to the laboratory in an airtight zip lock bag for further processing.

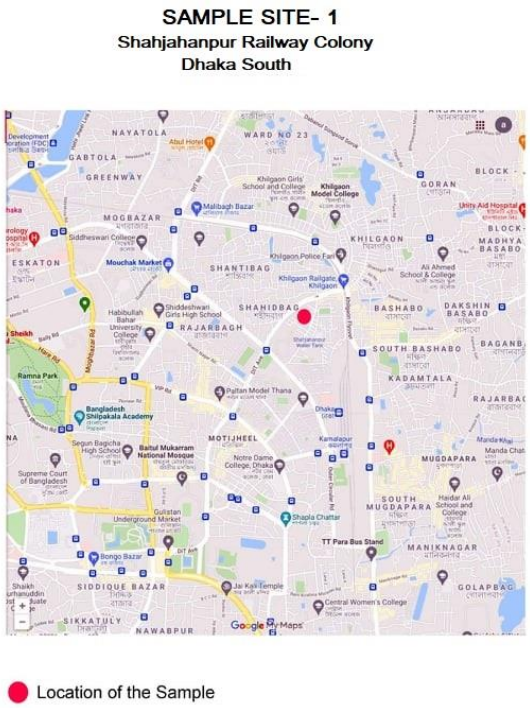

Figure 2.1 (a): Sample Site-1 (Shahjahanpur Railway Colony)

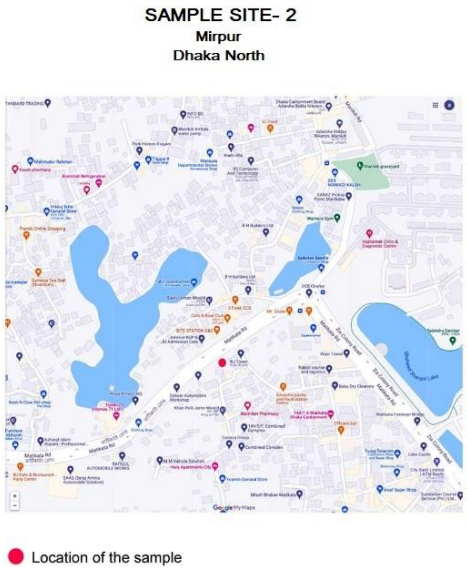

Figure 2.1 (b): Sample Site- 2 (Mirpur)

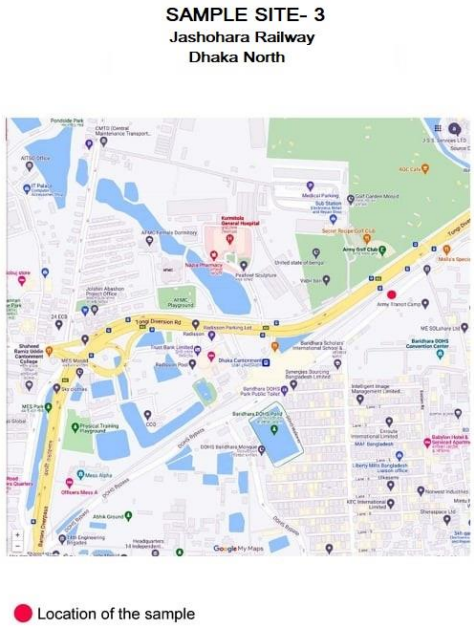

Figure 2.1(c): Sample Site- 3 (Jashohara Railway)

2.2. Initial screening and Isolation of LDPE Degrading Bacteria

The collected samples were carefully inspected and the parts of plastic samples that showed partial degradation were thinly cut using sterile scissors and forceps. Each of the samples were mixed separately with 0.9% saline in different test tubes. Serial dilution up to 10^-6 dilution factor was done for each of the samples. Among them, dilution factor 10^-1, 10^-5 and 10^-6 were cultured on Trypticase Soy Agar (TSA) media using spread plate technique. 10^-1 dilution of each sample was also cultured in Nutrient Agar (NA) media to get an idea regarding the unique microbiota present on each of the sample and its surrounding soil.

After 24 hours of incubation, morphologically distinct colonies were selected from plates of 10^-5 and 10^-6 dilutions and sub-cultured on Nutrient Agar (NA) plates to get pure isolates. A total of 15 colonies were selected and labelled from A through O. Further screening was done with naked eyes, and distinct colonies were selected from the pure isolates and labelled (A1, A2, A3 and so on).

Selected colonies from NA were cultured on Mannitol Salt Agar (MSA) media, selective for gram positive bacteria and incubated for 24hours at 37^{o}C.Selected colonies were similarly cultured on MacConkey Agar (MAC), selective for gram negative bacteria and incubated for 24 hours at 37^{o}C.

MSA and MAC were finally sub-cultured in NA again to get pure isolates and those were used for incubation with polyethylene film for incubation.

2.3. Preparation of Low Density Polyethylene (LDPE) film for incubation

For this experiment we used polyethylene film eventually collected from commercially available plastic wrap as it is a kind of LDPE. 1cm^2 of plastic film was cut with sterile scissors, sprayed with 70% ethanol [51] under laminar air flow while wearing gloves to ensure zero contamination.

2.4. Incubation for 1 month

18 Morphologically distinct colonies were selected from. Initially they were inoculated into Nutrient Broth (NB) and incubated overnight at 37^{o}C for 24 hours. 1ml inoculum was collected from overnight incubated Nutrient Broth. It was then inoculated into Minimal Salt Media (MSM)

: (g/l: K2HPO4 1.0, KH2PO4 0.2, NaCl 1.0, CaCl2.2H2O 0.002, H3BO3 0.005, NH4 (SO4)2 1.0, MgSO4.7H2O 0.5, CuSO4.5H2O 0.001, ZnSO4.H2O 0.001, MnSO4.H2O 0.001, Fe2 (SO4)3.6H2O 0.01, Agar 15) using spread plate technique [52]. The prepared plastic film was placed in the middle of the plate and incubated for 30 days at 37°C.

2.5. Analysis of LDPE Degradation

2.5.1. Determination of dry weight (Weight loss)

After 1 month of incubation, the plates were taken out of the incubator and the LDPE films were washed with 2% Sodium dodecyl sulfate (SDS). It was then placed on a filter paper and allowed to dry at 60°C overnight [53]. The films were then weighed on a Weight Scale to measure the weight loss using the following formula:

Percentage of weight loss = [(Final weight – Initial weight) / Initial weight] * 100 [53]

2.5.2. Surface topology analysis of LDPE films by Optical microscope

1000x magnification of the treated LDPE films was carried out at Bangladesh University of Engineering and Technology using an Optical Polarizing Microscope. The resulting pictures were analyzed to observe changes in surface topography. [51].

2.5.3. Changes in chemical bonds by Fourier Transform Infrared Spectroscopy (FTIR) of LDPE films

Fourier transform infrared (FTIR) spectroscopy was done at Bangladesh University of Engineering and Technology (BUET) using the Shimadzu IRSpirit spectrophotometer. The obtained data was then plotted using Microsoft Excel to obtain the necessary graph and analyzed.

Formation of new bonds or disappearance of old ones of the incubated LDPE can be assessed using FTIR [54]. Incorporation of chemical moieties like branches, co-monomers and unsaturation can be determined through this [55].

2.6. Characterization of Bacteria

2.6.1. Gram Staining

Gram staining of the 18 selected bacterial colonies was done in order to further distinguish between the gram positive and gram negative bacteria as well as to observe their morphology.

24 hours fresh bacterial culture was smeared onto a glass slide with a few drops of distilled water using a loop. After allowing it to air dry, it was heat fixed by holding over a flame. First, a few drops of the primary dye (crystal violet) were added for 1 minute and then washed with distilled water. This was followed by a few drops of Grams iodine which was kept for 45 seconds before washing with distilled water. The slide was then washed with 95% of ethanol for 20 seconds. Finally, a few drops of safranin was added which was kept for 1 minute and later washed with distilled water. The slides were allowed to air dry. The dried slides were then observed under 100x microscope with immersion oil to confirm their morphology.

2.6.2. Biochemical tests

A total of 7 biochemical tests were carried out in order to characterize the 18 isolates using 24 hour fresh culture.

2.6.2.1. Triple Sugar Iron Test

Triple sugar iron test is a differential biochemical test where the bacteria is distinguished based on their ability to metabolize glucose, sucrose, or lactose, during fermentation from the changes in color of butt and slant.

The media was inoculated and incubated at 37^{o}C for 24 hours after which the results were observed.

2.6.2.2.Methyl Red Test

Methyl Red test allows bacterial characterization based on their ability to produce stable acids or mix acids as end products from glucose fermentation. For this broth was used which contained dextrose as a source of glucose.

The broth was inoculated and incubated at 37ºC for 48 hours. After incubation, 5 drops of methyl red (indicator) was added to observe the result.

2.6.2.3. Voges Proskauer Test

This test allows the identification of bacteria that are capable of producing acetoin or acetyl methyl carbinol in buthylene glycol pathway during glucose fermentation.

MRVP broth was inoculated and incubated at 37ºC for 48 hours. After incubation, 6 drops of Alpha-napthol (Barrit's A reagent) followed by 2 drops of 40% KOH (Barrit's B reagent) was added. The result was observed after 1 hour.

2.6.2.4. Citrate Test

Citrate test allows the identification of bacteria that are able to utilize citrate as their sole source of carbon by changing the color of the media which contains the indicator bromothymol blue.

Simmons Citrate Agar was inoculated with isolated bacteria and incubated at 37ºC for 48 hours after which change in the media color was observed.

2.6.2.5.Motility Indole Urease Test

MIU test allows the identification of bacteria based on three characteristics- motility, urease enzyme production, and indole production. Motility is indicated by the spiraling growth away from the stab line. A change in the media color from orange to pink indicates urease production and formation of red ring after adding Kovac's reagent implies indole production using the tryptophanase enzyme.

40% urea solution was added to autoclaved MIU media and then transferred to sterile test tubes. The media was allowed to set and then inoculated by stabbing method by leaving 1/3 of the bottom. It was then incubated at 37ºC for 24 hours and observed for results.

2.6.2.6. Oxidase test

Oxidase test allows detection of the presence of a cytochrome oxidase system which catalyzes the electron transport between donors as well as reduces the redox dye- tetramethyl-p-phenylene-diamine into deep purple color.

A strip of Whatman filter paper is soaked with freshly made 1% oxidase reagent on which a little culture of isolated bacteria is rubbed with a toothpick. Change in color is observed for results.

2.6.2.7.Catalase test

Catalase test detects the presence of catalase enzyme which breaks down hydrogen peroxide (H2O2) into hydrogen and water.

A drop of 3% H2O2 is taken on a slide on which a little culture of isolated bacteria is mixed with a toothpick. Formation of bubbles is observed for results.

2.7. Molecular analysis of bacterial isolates

2.7.1. DNA Extraction

DNA of the selected colonies was extracted using the Boil Extraction Method. At first, 24-hour fresh culture was inoculated in Luria Bertani Broth (LB) and incubated for 24 hours at 37^{o}C in an incubator shaker at 135 rpm. 700 µL of the broth was collected in an Eppendorf tube and centrifuged at 13000 rpm for 10 minutes. After discarding the supernatant, the pellet was washed with 300 µL of Phosphate Buffered Saline (PBS and centrifuged again at 13000 rpm for 5 minutes. 200 µL Tris-EDTA (TE) Buffer was added after discarding the supernatant and heated at 100^{o}C for 15 minutes in a water bath followed by 10 minutes of cold shock in ice. Finally, after 10 minutes of centrifugation at 13000 rpm, the supernatant was collected.

2.7.2. Polymerase Chain Reaction (PCR)

The extracted DNA was amplified using 27F (5'AGAGTTTGATCCTGGCTCAG-3') as forward primer and 1492R (5'-CGGTTACCT TGTTACGACTT-3') as reverse primer [54]. 10x Primer was prepared as working solution by adding 10 µL stock solution of primer to 90 µL of distilled water.

25 µL mixture was prepared for each of the sample. The components of the mixture were as follows: mastermix-10 µL, 10x 26F (forward primer) – 2.5 µL, 10x 1496R (reverse primer) – 2.5 µL, Dnase free water- 5 µL, DNA template- 5 µL.

The PCR tubes were then gently spun before putting into the Thermal Cycler [56]. The following conditions were maintained during PCR: initial denaturation at 95°C for 5 min, followed by 30 cycles (amplification) of 95°C for 30 s (denaturation), 52°C for 45 s (annealing), 72°C for 1min 30 s (extension) and final extension at 72°C for 10 min. The final product was stored at -20°C for further analysis.

2.7.3 Agarose gel electrophoresis

Agarose gel electrophoresis allows us to assess the success of DNA extraction and amplification. DNA, which is negative in nature [56].

0.8% of agarose was prepared by mixing 0.64g agarose with 80ml of 1x TAE buffer. The solution was heated in a microwave until it was clear. The agarose solution was then poured into a previously assembled gel casting unit and an appropriate comb was used to create the wells after which the gel was allowed to completely polymerize before removing the combs. The gel was then put into a gel electrophoresis tank in which 1x TAE buffer was poured until the gel was submerged. 4 µL of each PCR product was loaded into the wells along with 1kb ladder. 100 volts was applied for 60 minutes after which the gel was observed under UV light.

2.7.4. 16sRNA Sequencing

16sRNA sequencing was carried out at International Centre for Diarrheal Disease Research, Bangladesh (icddr,b). DNA Baser Assembler software was used to retrieve the FASTA file of the received data. The results were then submitted to the National Centre for Biotechnology Information (NCBI) gene bank and BLAST (nucleotide) was used to obtain the species with highest similarity along with their accession number.

3. Results

3.1. Initial screening and Isolation of LDPE Degrading Bacteria

All of the NA plates showed overgrowth and unique colonies that overlapped with each other. While no colony was subcultured from the individual NA plates, they represented an overview of the unique soil microbiota present on each sample and in the surrounding soil (Figure 3.1a).

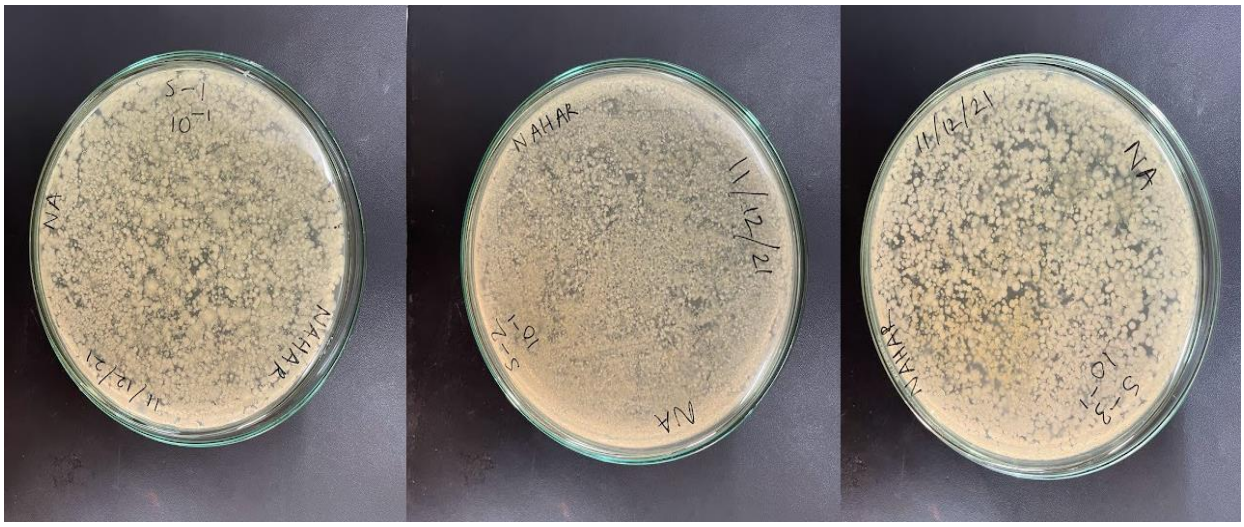

Figure 3.1(a): Microbial colonies isolated on Nutrient Agar (NA) with Dilution factor 10^-1 with S-1, S-2 and S-3 going from left to right.

The colonies were isolated on another enrichment media, TSA, which provided a different growth pattern than the NA plates. Although serial dilution was conducted thoroughly, the diluted solutions still demonstrated a high number of distinct colonies which indicates the rich diversity of the soil microbiota present in the three samples. In Sample 1, 10^-1 plate showed overgrowth colonies, 10^-5 plate showed a lot more discernible yellow colonies and 10^-6 demonstrated growth of mainly two types of yellow colonies (Figure 3.1b).

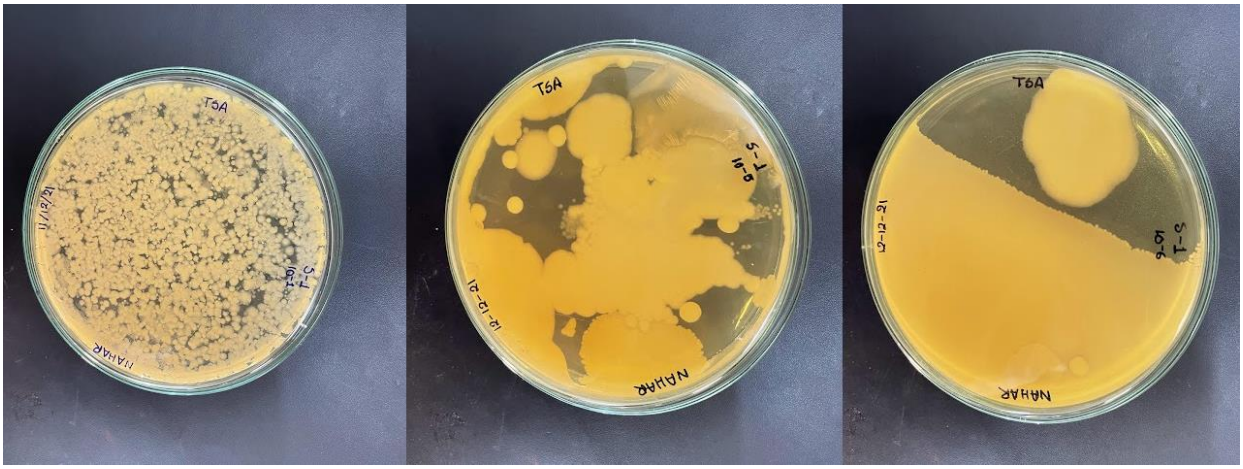

Figure 3.1(b): Microbial colonies isolated from Sample-1 on TSA with Dilution factors 10^-1 (left), 10^-5 (center) and 10^-6 (right).

A similar situation was observed in colonies from Sample 2 where the plate with the 10^-1 dilution factor showed a uniform spread of very similar looking colonies, the plate with the 10^-5 dilution factor showed colonies of different sizes and morphology and the plate with the 10^-6 dilution factor showed widespread growth of a particular colony (Figure 3.1c).

Figure 3.1(c): Microbial colonies isolated from Sample-2 on TSA with Dilution factors 10^-1 (left), 10^-5 (center) and 10^-6 (right).

Sample-3 demonstrated the most unique colonies out of the three samples. While the plate with the 10^-1 dilution factor showed similar results to their counterparts in samples 1 and 2, the plates with the 10^-5 and 10^-6 dilution factors showed growth of many different unique colonies from which most of our colonies were later subcultured onto NA (Figure 3.1d).

Figure 3.1(d): Microbial colonies isolated from Sample-3 on TSA with Dilution factors 10^-1 (left), 10^-5 (centre) and 10^-6 (right).

Selected colonies were subcultured onto the selective media Mannitol Salt Agar (MSA) and MacConkey Agar for primary identification of the bacterial colonies. MSA was used to identify the Gram positive colonies while MacConkey Agar was used to identify the Gram negative colonies. Surprisingly, all of the colonies showed more or less growth on MSA while MacConkey Agar reported no visible growth of the colonies. Amongst the growth on MSA, both pink and yellow colonies were observed. Mannitol fermenting bacteria showed up as yellow colonies while non-mannitol fermenting bacteria showed up as pink colonies (Figures 3.1).

Figure 3.1 (e): Microbial colonies on MSA from colony A1 up to M2 (the cultures N and O were grown later).

3.2. Subculture of bacterial colonies

The distinct colonies from MSA were subcultured again onto NA and a total of 65 colonies were seen (Figure 3.2). While most of the colonies were similar to each other, a total of 18 distinct colonies were identified from visual growth alone. From the 18 colonies, we had chosen 5 colonies at the end of our experiment for genetic sequencing as they had demonstrated the most dramatic biodegradation rates. These 5 colonies are described in Table 2.

Table 2: Microbial colonies ultimately selected for genetic sequencing, along with their source and descriptions of colony morphology.

Microbial colonies selected for sequencing, their source plates and colony morphology							
Colony label	Source plate of colony	Colony morphology					
		Size	Colour	Translucency	Shape	Surface Appearance	Elevation
B5b	S-3, 10^-5	Small	Pale-yellow	Slightly translucent	Circular	Smooth	Umbonate
C2	S-3, 10^-5	Medium	Dark yellow	Opaque	Circular	Glistening	Convex
F3a	S-3, 10^-5	Large	Yellow	Opaque	Circular	Wrinkled	Raised
H4	S-2, 10^-6	Medium	Off-white	Opaque	Irregular	Rough	Raised
N	S-3, 10^-6	Large	Off-white	Translucent	Filamentous	Wrinkled	Raised

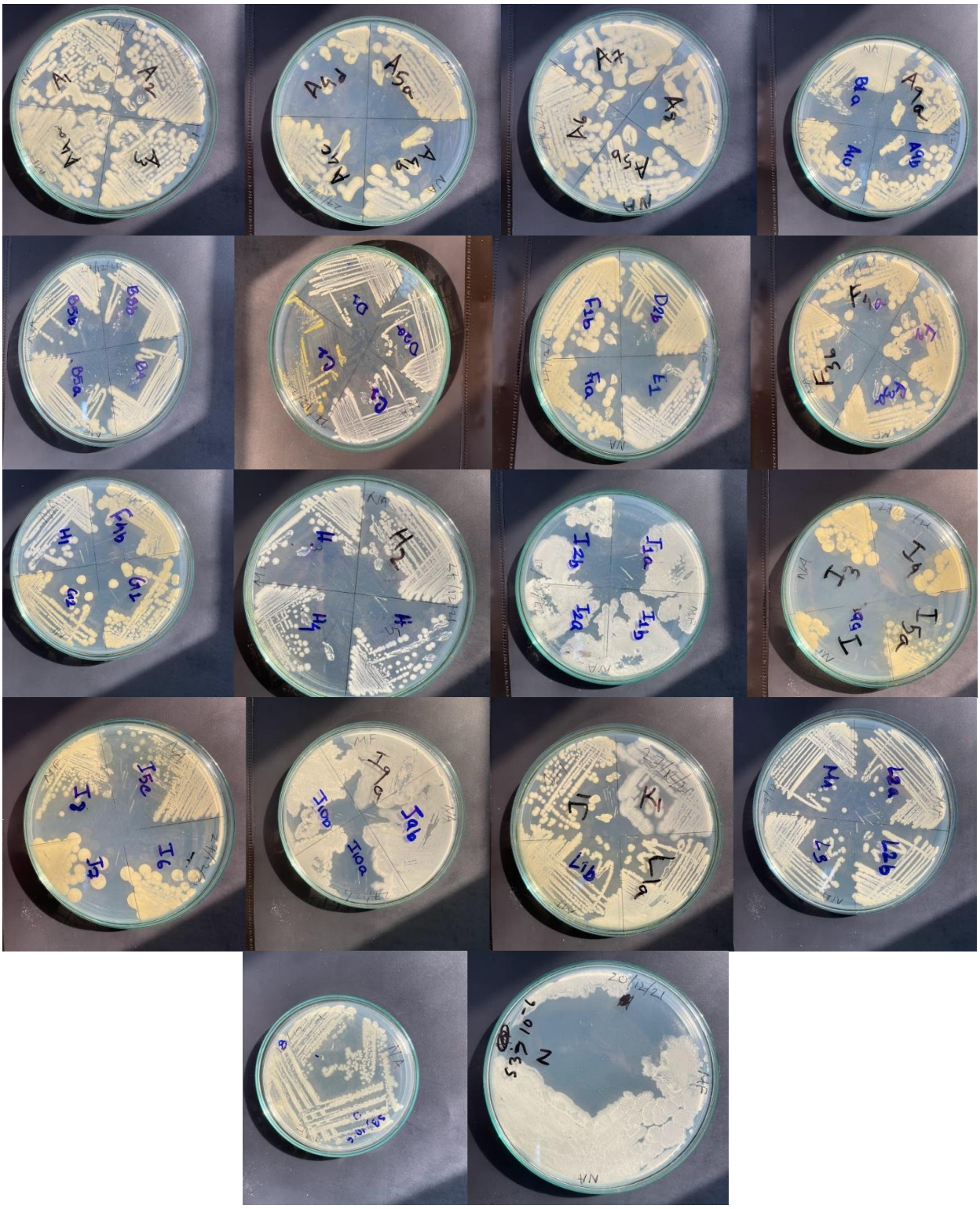

Figure 3.2: Microbial colonies A1 up to O on Nutrient Agar (NA) media.

3.3. Preparation of Low Density Polyethylene (LDPE) film for incubation

MSM media were prepared followed by bacterial inoculum spread on it through the spread plate method. LDPE films were sterilized and placed onto the middle of the MSM plates (Figure 3.3a and b). Since the bacterial inoculum made the surface of the media sticky, the LDPE films could be placed more rigidly onto the media.

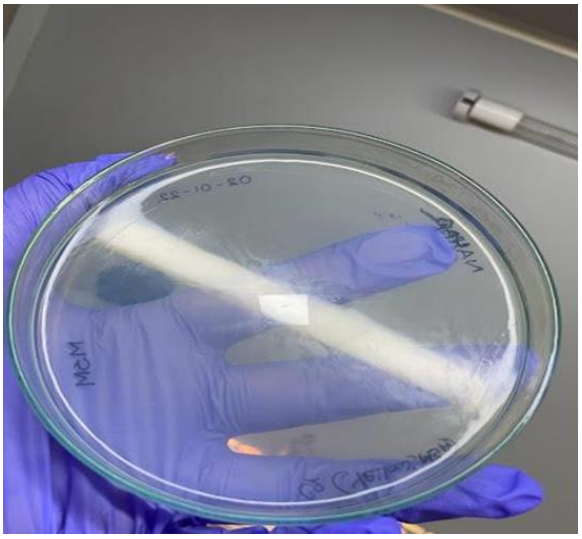

Figure 3.3(a): A sterile LDPE film (1 cm x 1 cm) placed in the middle of a Petri plate containing bacterial inoculum and MSM media.

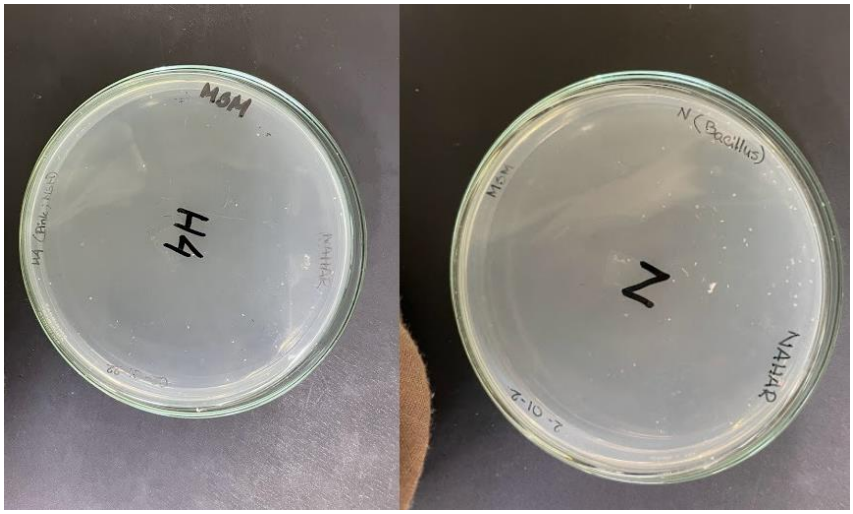

Figure 3.3(b): Petri plates containing MSM and bacterial inoculum with LDPE films are shown before the start of the 1-month long incubation.

3.4. Analysis of LDPE Degaradtion

3.4.1. Loss of dry weight after 1-month incubation

The loss in dry weights of the LDPE films were calculated and summarized in Table 3.

Table 3: Loss in Dry Weights of LDPE films seen in plates inoculated with different bacterial colonies.

Change in Dry Weights of LDPE films			
Colony label	Initial Weight after incubation, g	Final Weight after incubation, g	Percentage of weight loss, %
B5b	0.0010	0.0008	**20**
C2	0.0010	0.0008	**20**
F3a	0.0010	0.0008	**20**
H4	0.0010	0.0007	**30**
N	0.0010	0.0008	**20**

3.4.2. Change in Chemical Bonds seen through Fourier Transform Infrared Spectroscopy (FTIR)

FTIR graphs of the colonies are provided in Figures 3.5a, b, c, d and e. FTIR graph of the Negative Control (NC) is provided in Figure 3.5f. All of the changes recorded in the FTIR graphs are considered with NC as a reference graph. The peaks in the graphs indicate the vibration of a specific chemical bond which are labelled in all graphs. We will notice the transmission % of five specific wavelengths representing native bonds and additional new peaks.

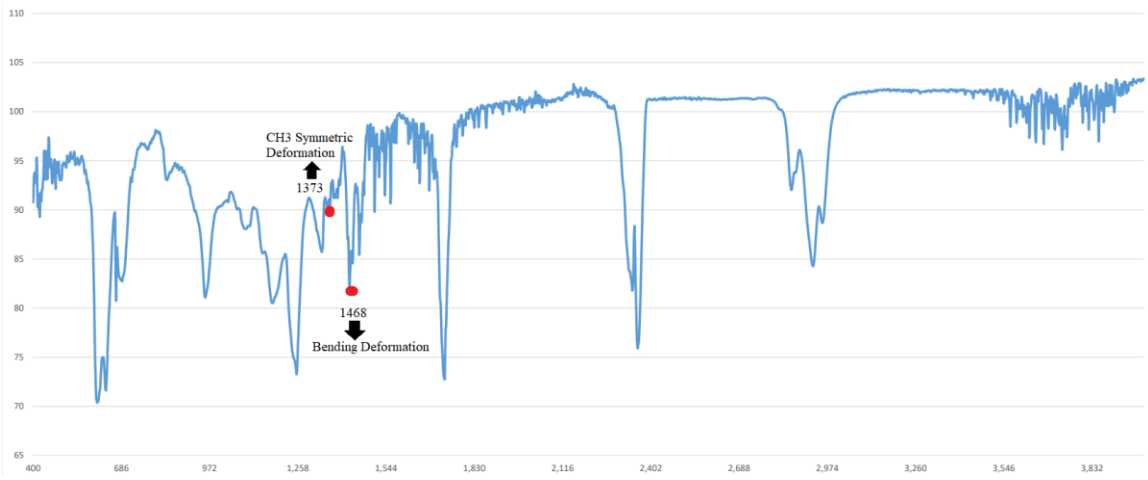

Figure 3.4(a): FTIR Graph of colony B5b.

At first, we look at the FTIR graph for B5b (Figure 3.5a). At 1468 cm^-1 and 1373 cm^-1, there is a greater % in transmission when compared to the peaks in NC.

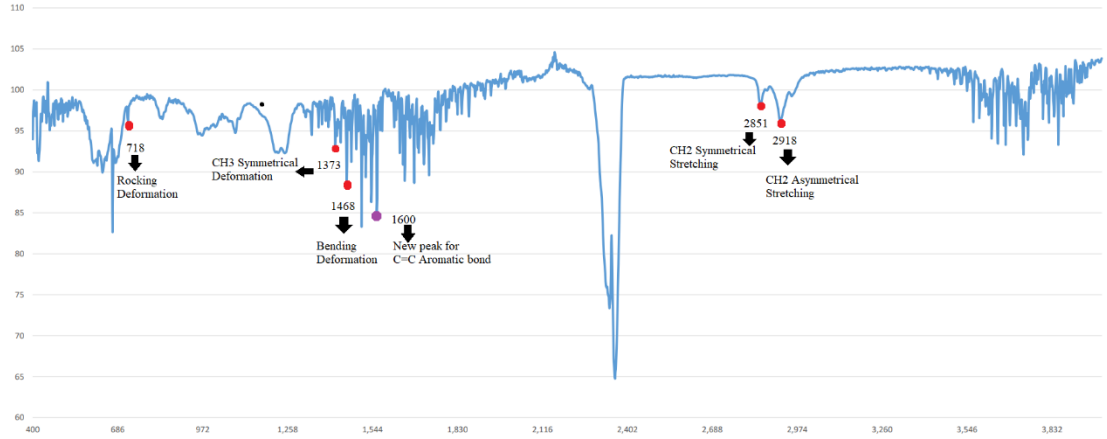

Figure 3.4(b): FTIR Graph for colony C2.

Secondly, we analyze the FTIR graph of C2 (Figure 3.5b) and compare it with NC. Greater % in transmission is seen at the following wavelengths compared to NC: 718 cm^-1, 1373 cm^-1, 1468 cm^-1, 2851 cm^-1 and 2918 cm^-1. Importantly, one new peak is seen around 1600 cm^-1 for C=C bond.

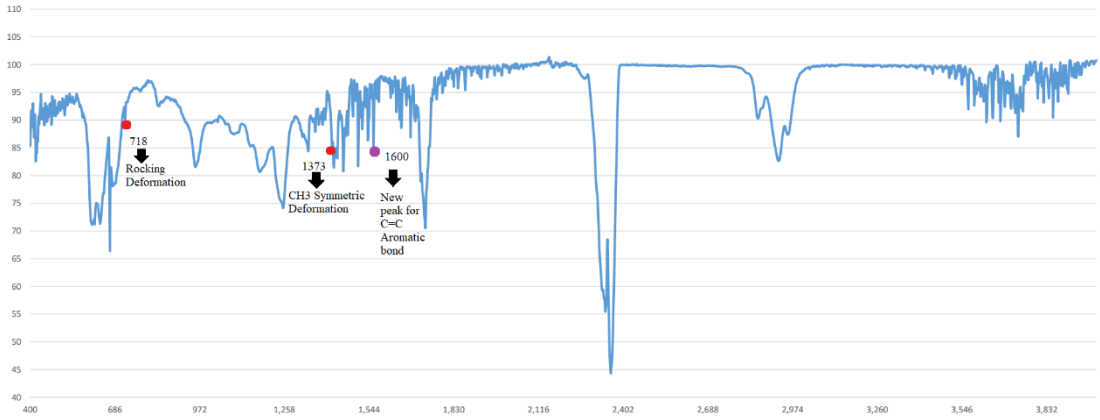

Figure 3.4(c): FTIR Graph for colony F3a.

Thirdly, we analyse the FTIR graph of F3a (Figure 3.5c) and compare it with NC. Noticeably, there is higher % in transmission for the peaks at wavelengths 1468 cm^-1, 1373 cm^-1 and 718 cm^-1 compared to NC. Additionally, a new peak around 1600 cm^-1 is seen for aromatic C=C bond.

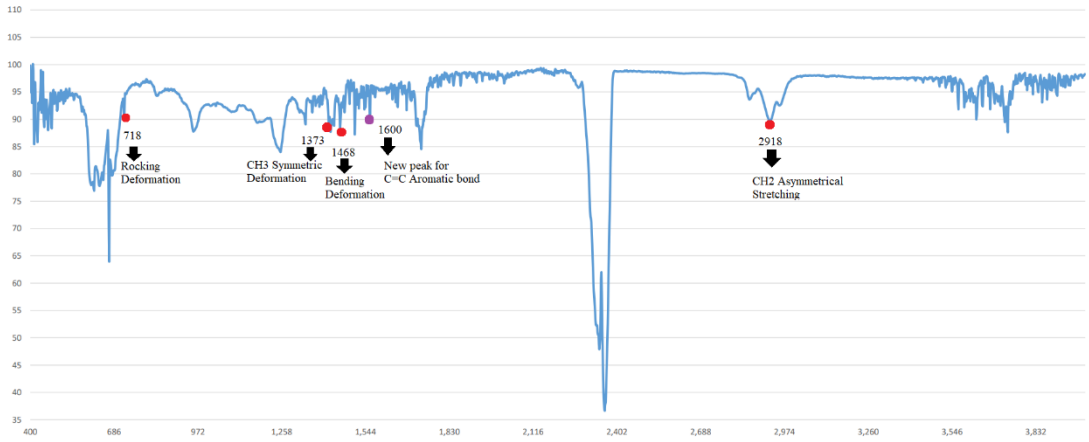

Figure 3.4(d): FTIR Graph for colony H4.

Next, we analyse the FTIR graph of H4 (Figure 3.5d) and compare the peaks with NC. Out of all the graphs, the graph for colony H4 showed the most drastic visual change with less steep curves than NC. Compared to NC, greater % in transmission is seen for peaks at wavelengths 718 cm^-1, 1373 cm^-1, 1468 cm^-1 and 2918 cm^-1. We also notice a peak at 1600 cm^-1 for C=C bond.

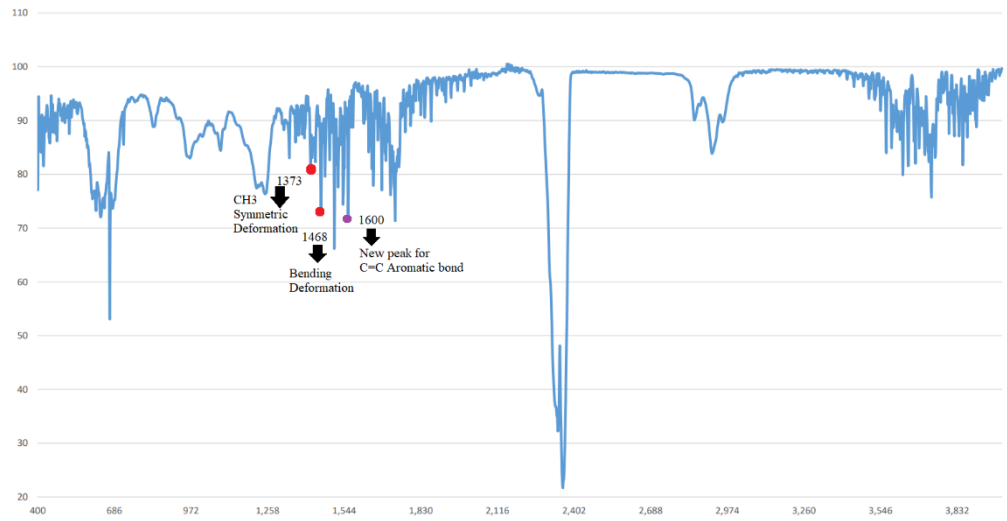

Figure 3.4(e): FTIR Graph for colony N.

Finally, we analyse the peaks for colony N and compare them with NC. Compared to all other graphs, the graph for colony N is the most similar to the graph for NC. However, there are still changes seen in the peaks for N. Higher percentage in transmission is seen for peaks at wavelengths 1468 cm^-1 and 1373 cm^-1 compared to NC while there is a new peak seen around 1600 cm^-1 for C=C bond.

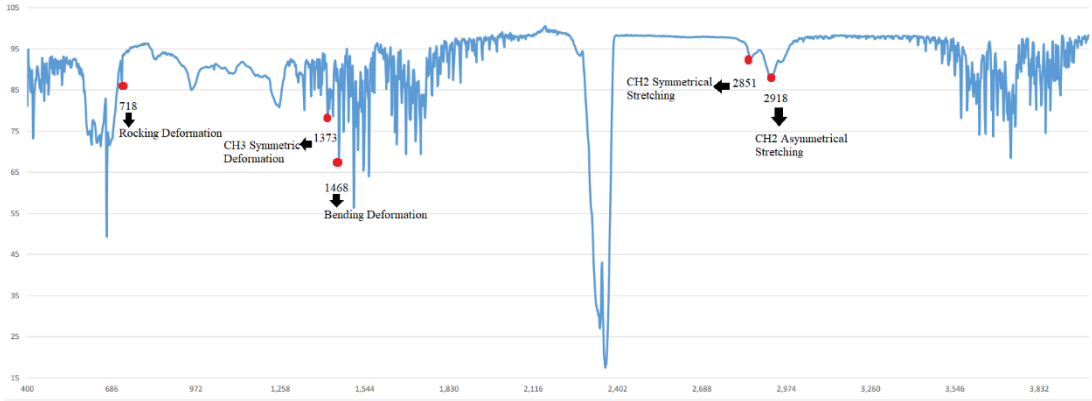

Figure 3.4(f): FTIR graph for Negative Control (NC).

3.4.3. Surface Topography Changes seen through Optical Magnification

To observe changes in the surface topography of the LDPE films, they were subjected to optical magnification at 1000x. Figures 3.11 (a) through 3.11 (e) shows changes in the

surface topography of the LDPE films and figure 3.11 (f) shows the negative control for comparison.

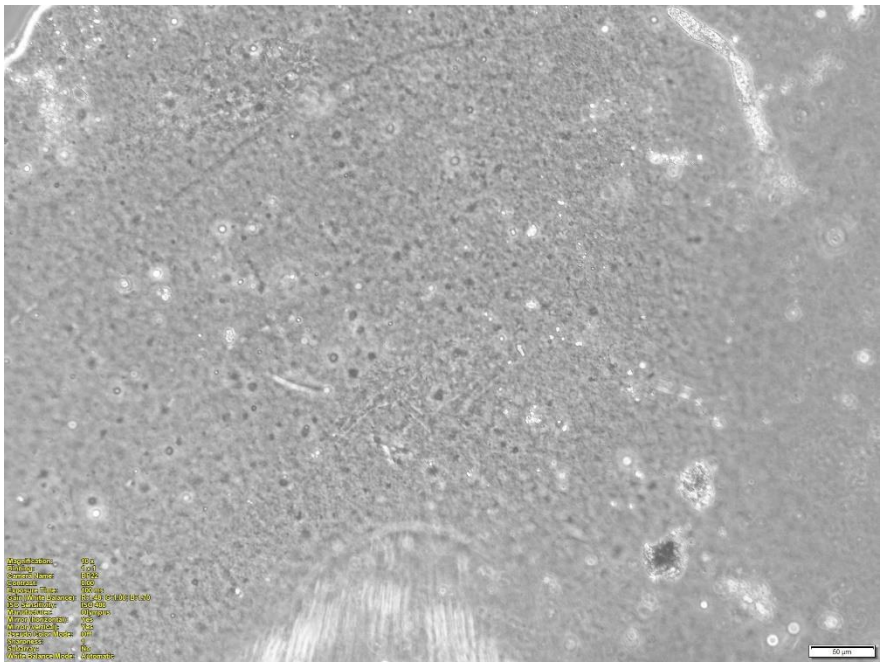

Figure 3.4(g): Changes in surface topography of LDPE film treated with bacterial isolate B5b.

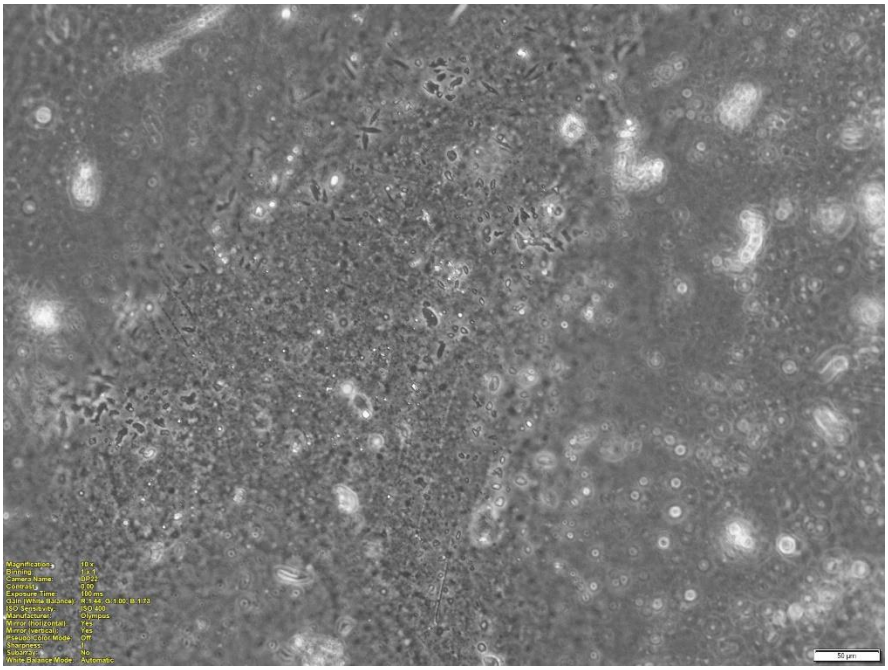

Figure 3.4(h): Changes in surface topography of LDPE film treated with bacterial isolate C2.

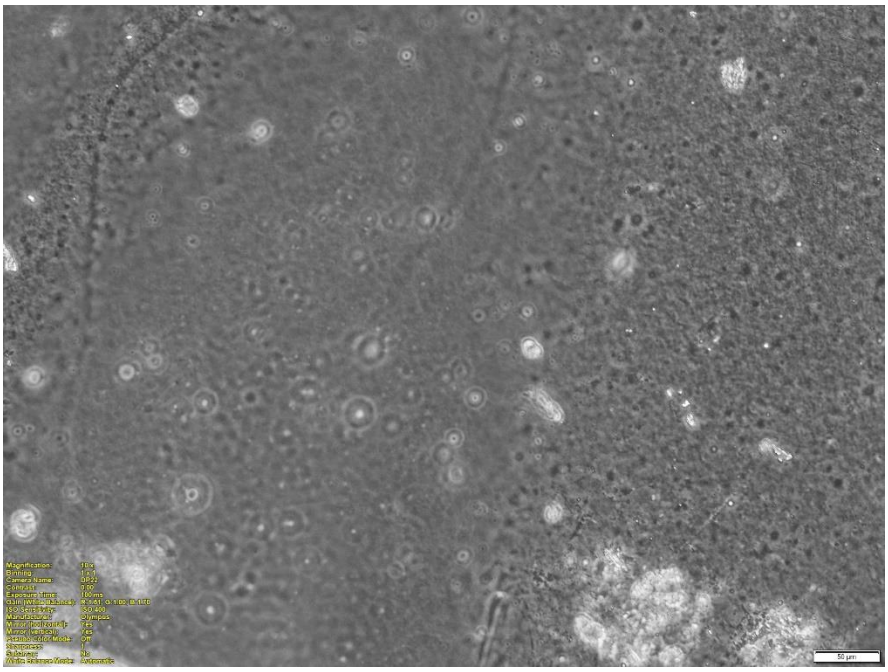

Figure 3.4(i): Changes in surface topography of LDPE film treated with bacterial isolate F3a.

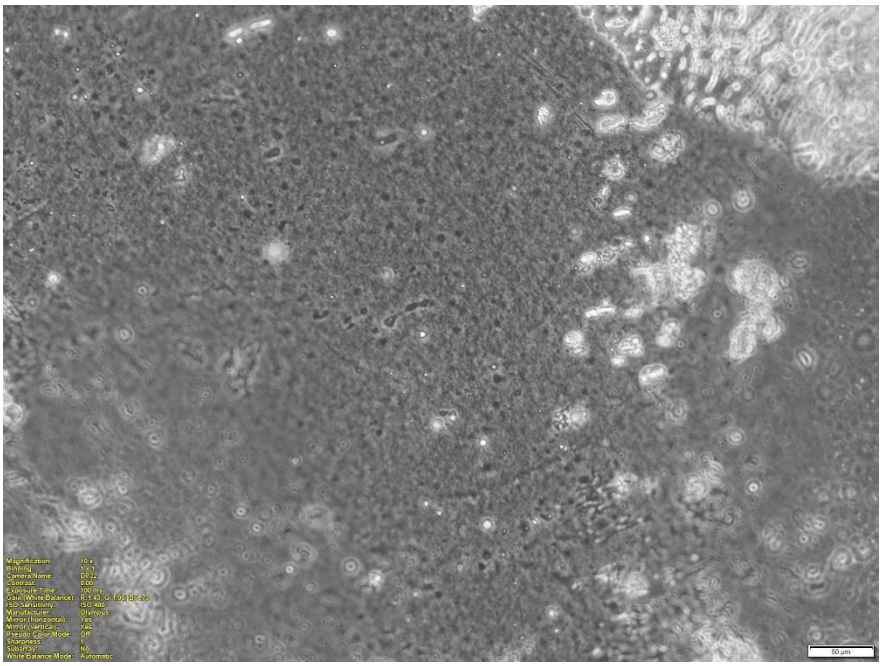

Figure 3.4(j): Changes in surface topography of LDPE film treated with bacterial isolate H4.

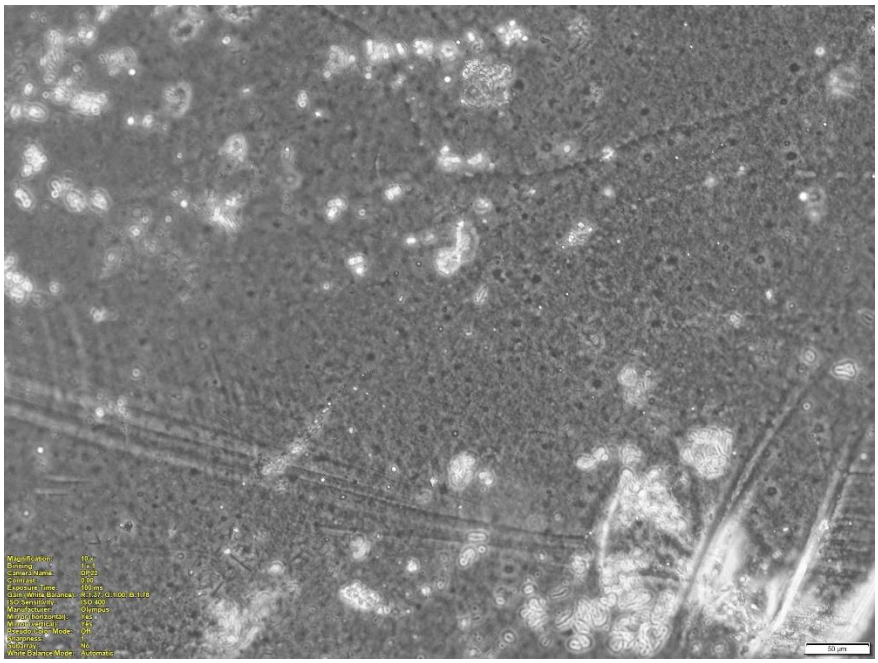

Figure 3.4(k): Changes in surface topography of LDPE film treated with bacterial isolate N.

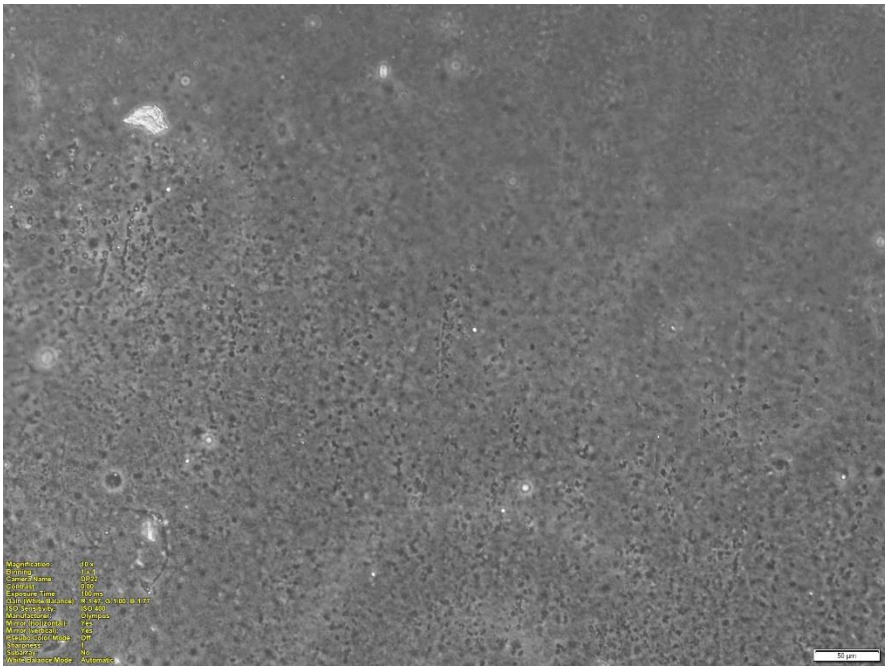

Figure 3.4(l): Surface topology of untreated LDPE film (control).

3.5. Gram Staining

Gram staining results demonstrated that all of the colonies isolated were Gram-positive bacteria. Almost all of the colonies showed that bacteria were rod-shaped which is expected as rod-shaped bacteria are ubiquitous in the environment, particularly in the soil. Figures 3.6 a, b, c, d and e show the different Gram stains of the five colonies that were finalized at the end.

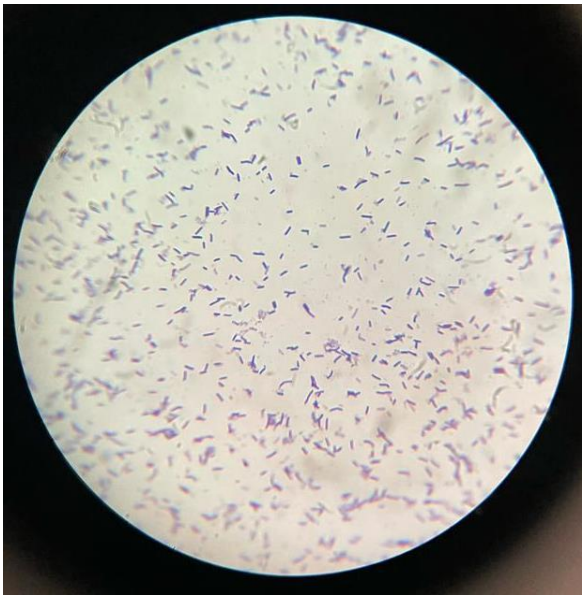

Figure 3.5(a): Gram-stain of Colony B5b shows bacteria is Gram +ve (purple stain) and rod-shaped (termed “Bacillus”).

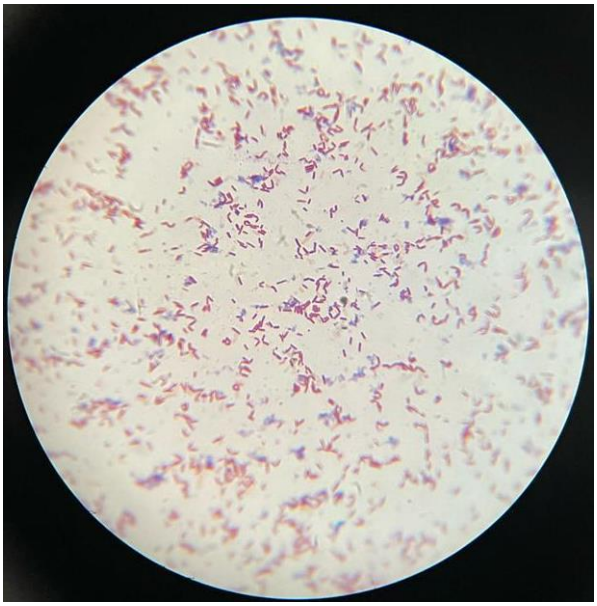

Figure 3.5(b): Gram-stain of Colony C2 shows bacteria is Gram +ve (purple stain) and rod-shaped (termed “Bacillus”).

Figure 3.5(c): Gram-stain of Colony F3a shows bacteria is Gram +ve (purple stain) and rod-shaped that are rounded on the sides (termed “Coccobacillus”).

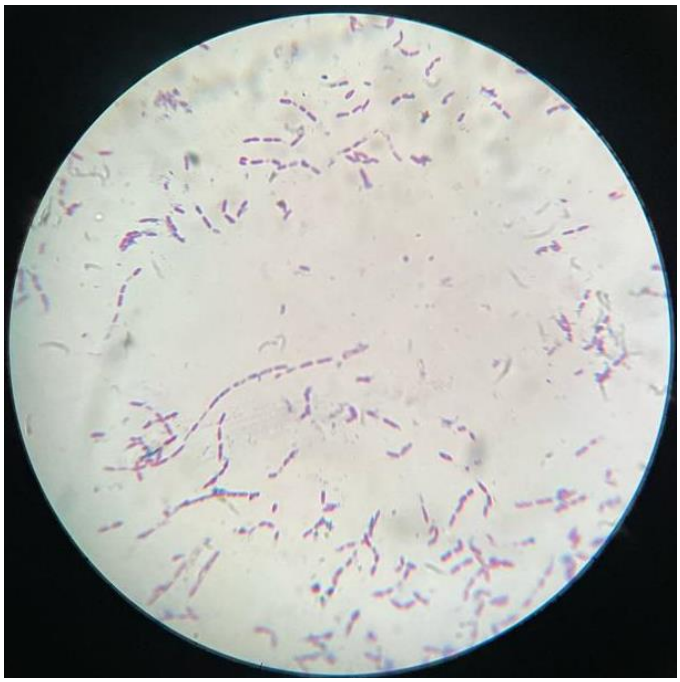

Figure 3.5(d): Gram-stain of Colony H4 shows bacteria is Gram +ve (purple stain) and rod-shaped that are rounded on the sides and arranged in chain-like structures (termed “Coccobacillus”).

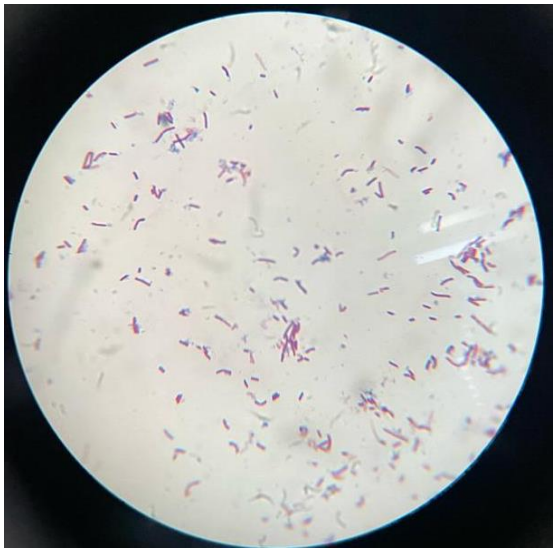

Figure 3.5(e): Gram-stain of Colony N shows bacteria is Gram +ve (purple stain) and rod-shaped (termed “Bacillus”).

3.6. Biochemical Test Results

The biochemical results of the selected colonies are summarized in Table 4. Additionally, Figures 3.5 a, b, c, d, e, f and g help to visualize the results of the colonies.

Table 4: Biochemical test results of the 5 selected colonies are summarized.

Colony	TSI	MR	VP	Citrate	MIU			Oxidase	Catalase
					Motility	Indole	Urea		
B5b	A/A	-ve	-ve	+ve	+ve	-ve	+ve	-ve	+ve
C2	K/K	+ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve
F3a	K/K	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve
H4	K/A	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve
N	K/K	-ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve

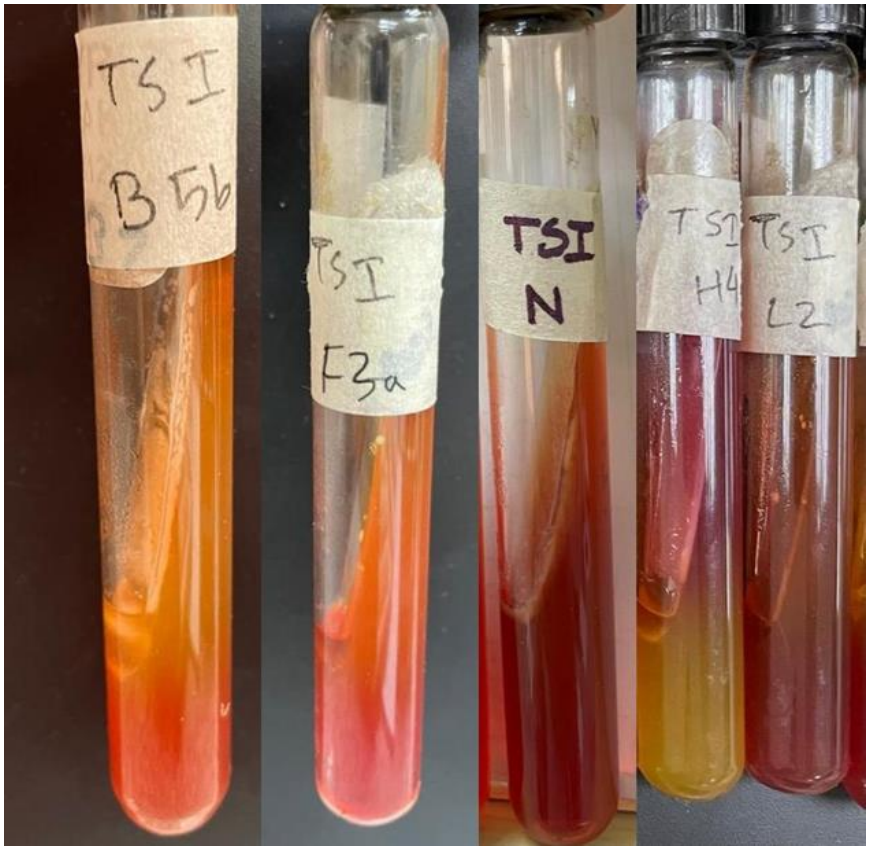

Figure 3.6(a): TSI test results of the colonies.

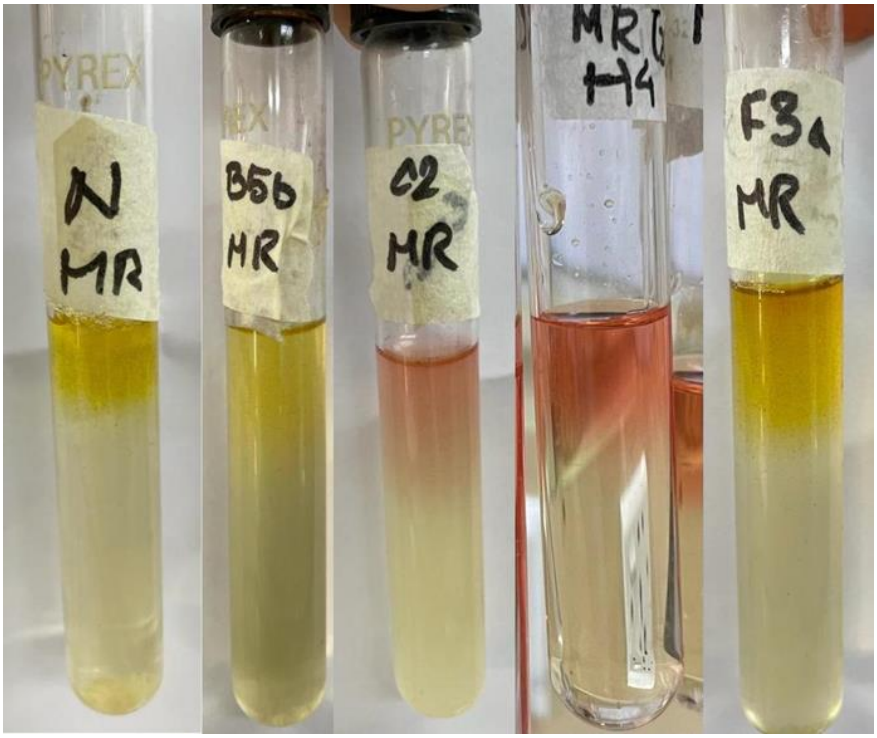

Figure 3.6(b): MR test results of the colonies.

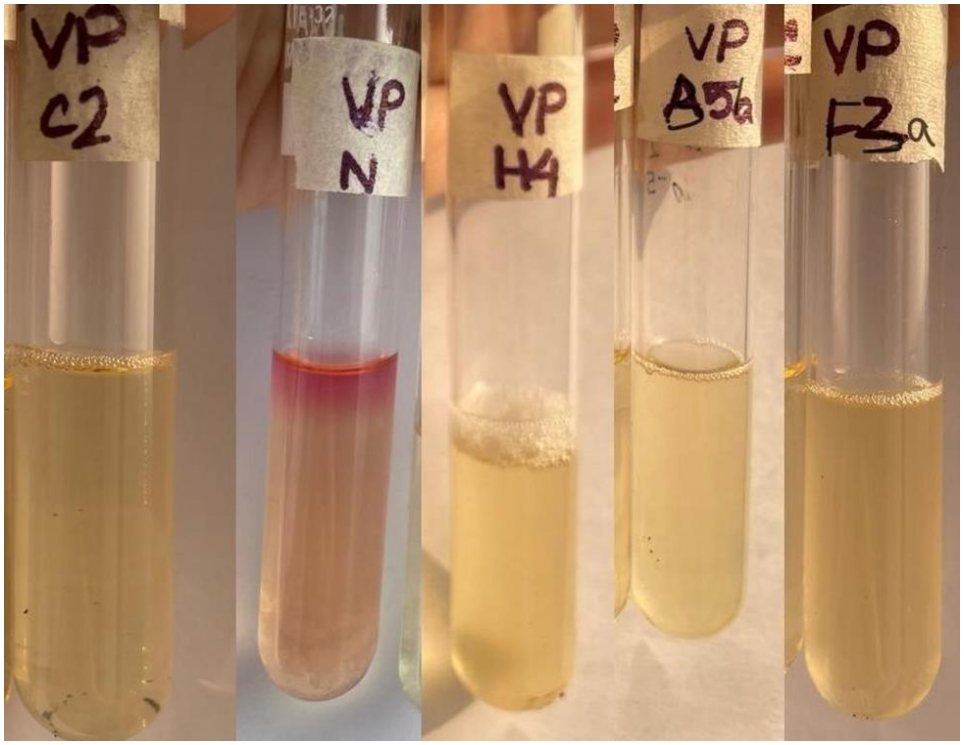

Figure 3.6(c): VP test results of the colonies.

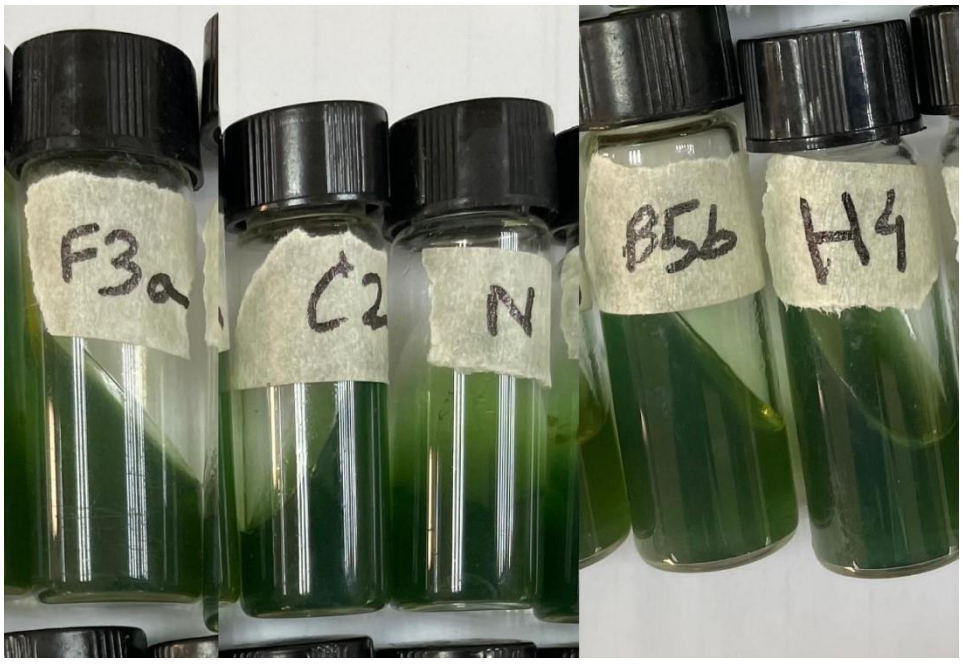

Figure 3.6(d): Citrate test results of the colonies.

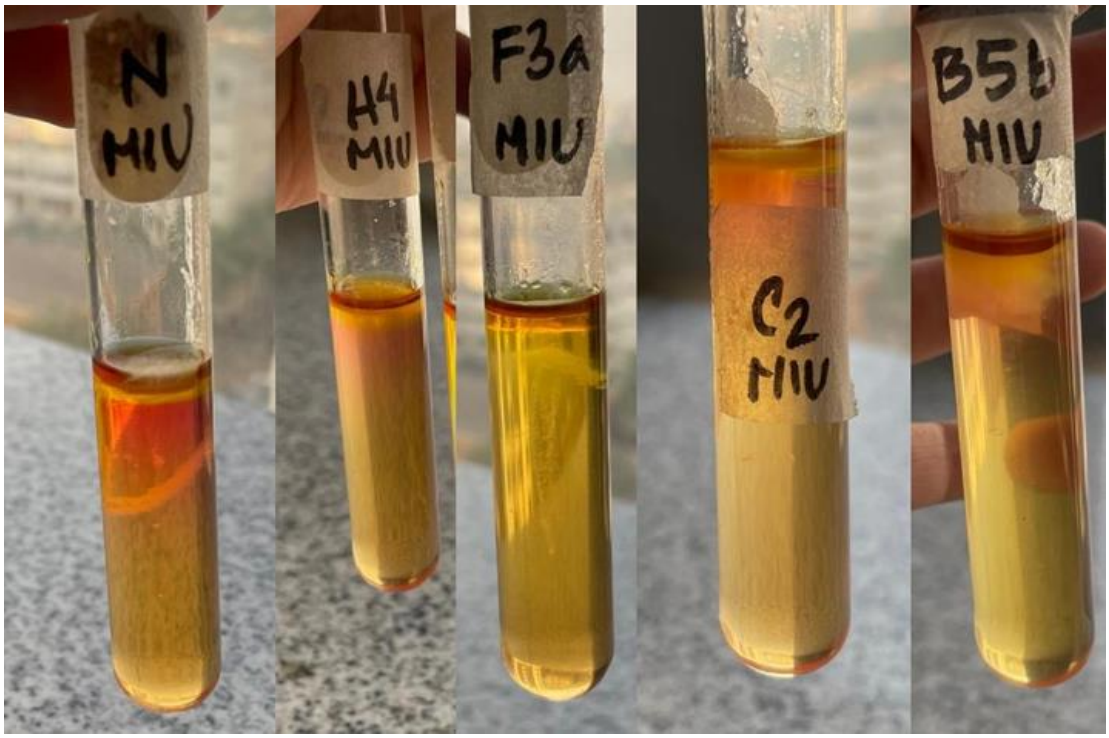

Figure 3.6(e): MIU test results of the colonies.

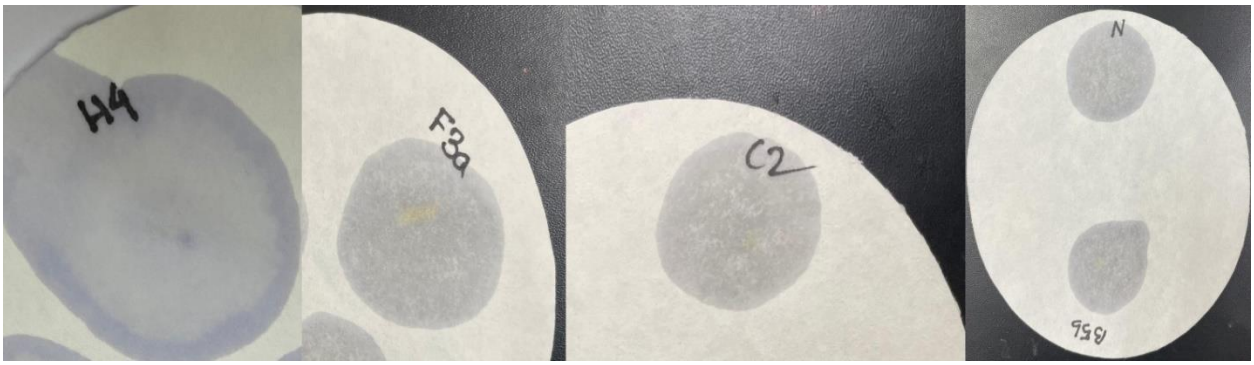

Figure 3.6(f): Oxidase test results of the colonies.

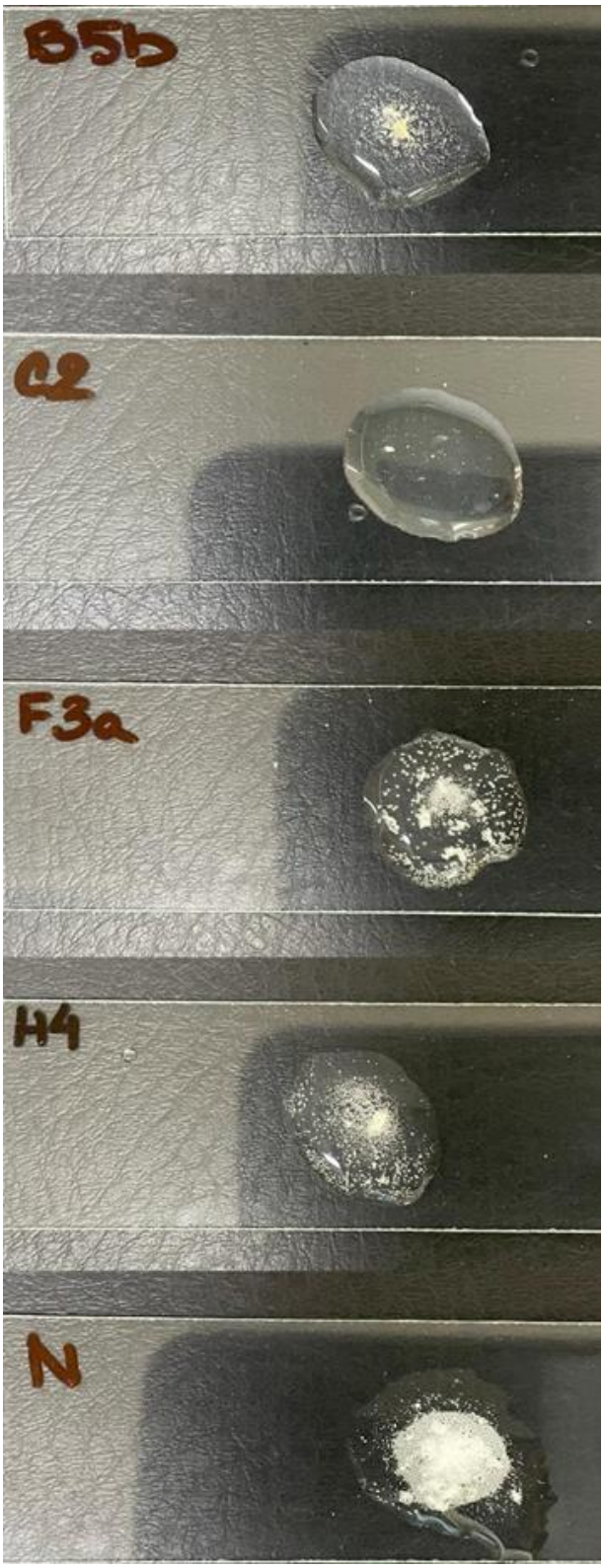

Figure 3.6(g): Catalase test results of the colonies.

3.7. DNA Extraction of Bacterial Isolates

The DNA extracted from all colonies were conducted and an agarose gel run was conducted at 0.8% concentration. Figure 3.8(a) indicates the results of the DNA extraction where almost all colonies indicate presence of DNA.

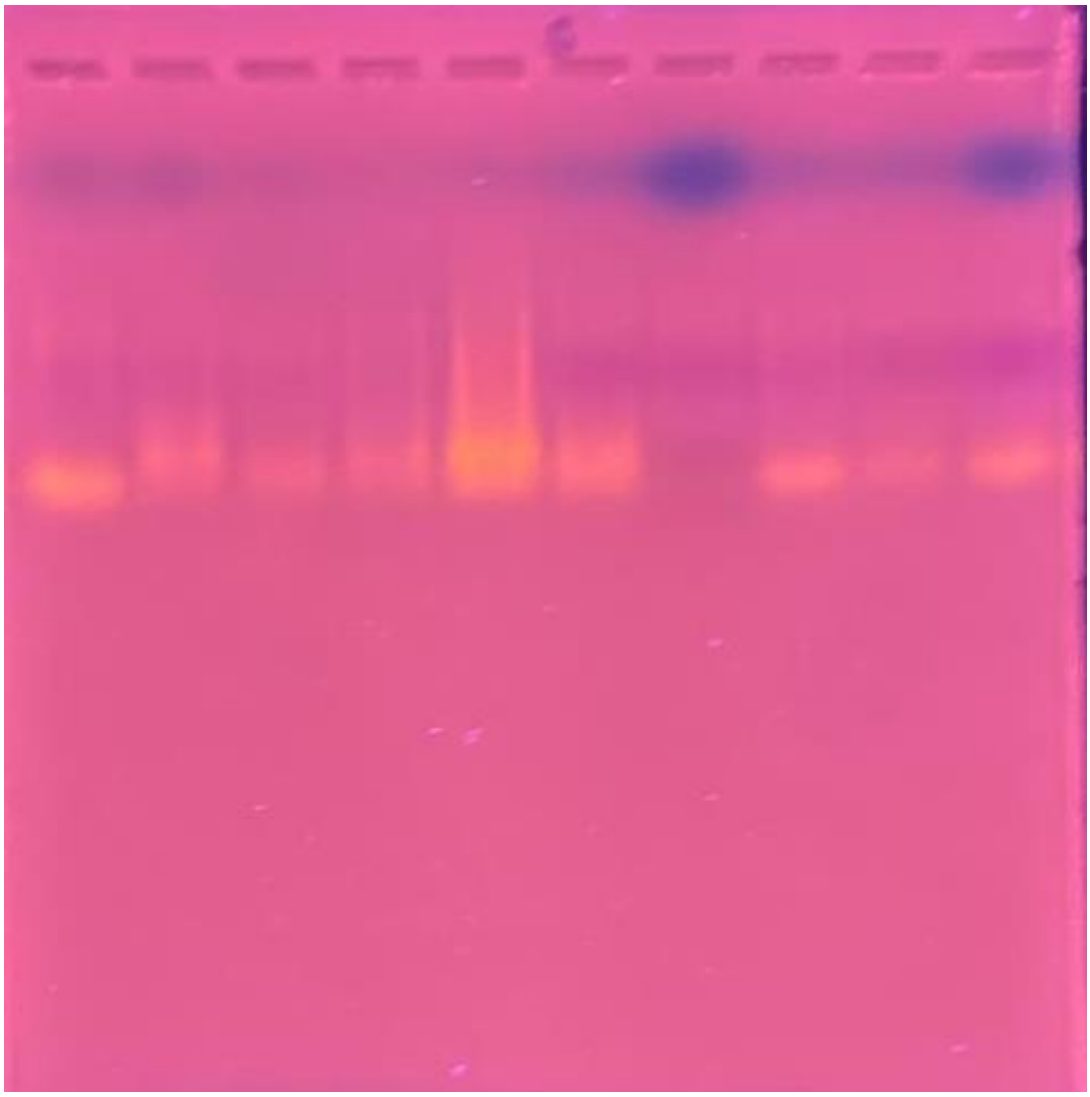

Figure 3.7: The DNA bands seen after gel run at 0.8%.

3.8. PCR Bands of Bacterial Isolates

After the PCR, a gel electrophoresis was conducted at 0.8% concentration and a 1kb plus ladder was loaded into a well. Figure 3.9(b) shows the PCR bands of all the colonies which were amplified in the PCR run. All wells show clear PCR bands with adequate DNA in it.

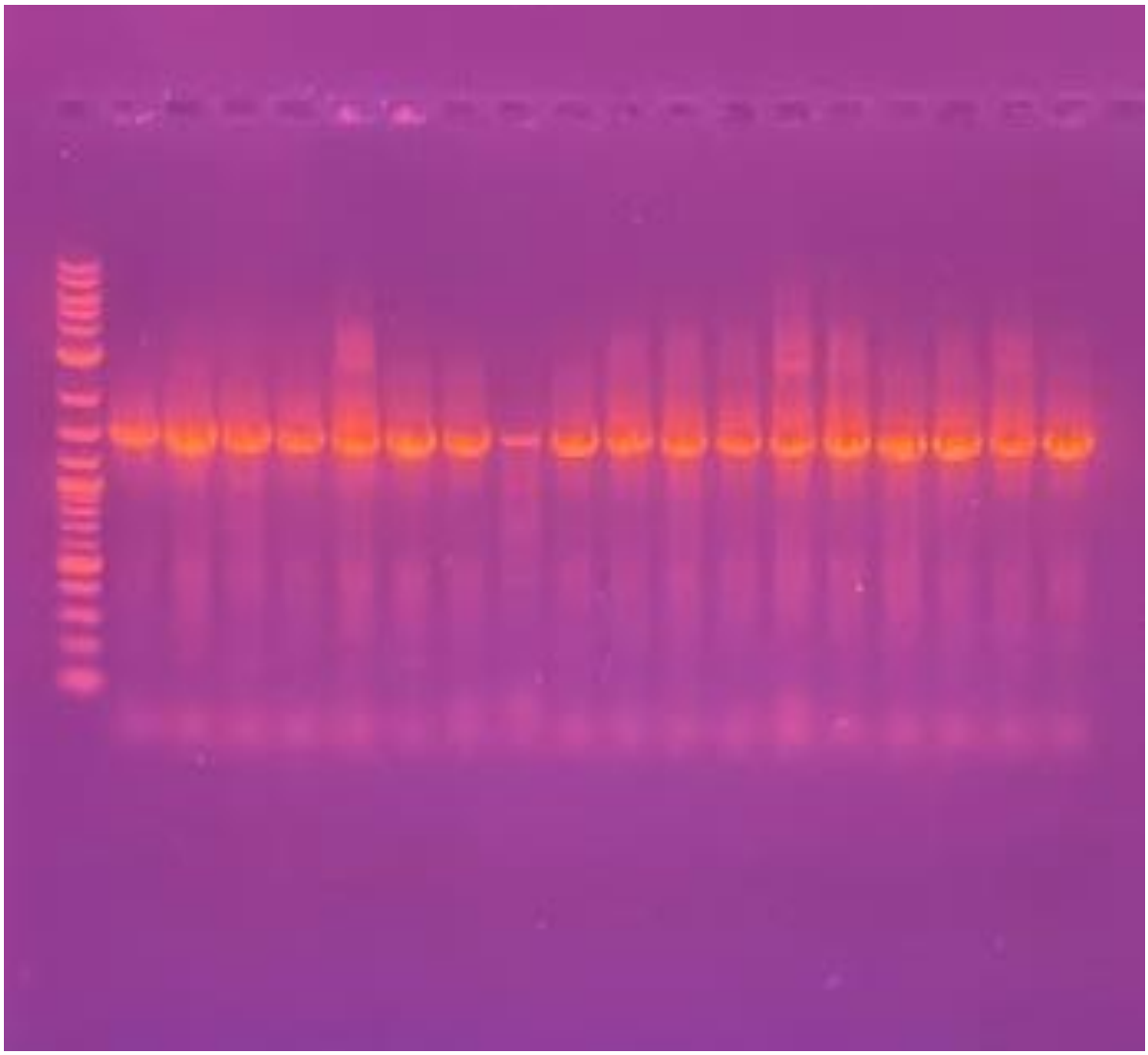

Figure 3.8: The PCR bands seen after gel run was done at 0.8% alongside a 1kb+ ladder.

3.9. Identification of isolates via 16sRNA sequencing:

After conducting 16sRNA sequencing of the PCR products, the isolates were identified to be *Bacillus* sp. (in: Bacteria) strain **JDMASP60**, *Bacillus atrophaeus* strain **MGB14**, and *Bacillus pumilus* strain **KD3**. The sequencing results are summarized in Table 5 below.

Table 5: Identified Bacterial Isolates

Colony isolate	NCBI Accession Number	Organism	Percentage Identity	E value
B5b	EU500930.1	*Bacillus pumilus* strain **KD3**	100%	0.0
F3a	KX817964.1	*Bacillus* sp. (in: Bacteria) strain **JDMASP60**	100%	0.0
N	KR815994.1	*Bacillus atrophaeus* strain **MGB14**	95.54%	0.0

4. Discussion

Due to an alarming rise in plastic pollution worldwide, microbial biodegradation of plastics including LDPE has been extensively researched for the last few decades [57]. However, recent studies that focus primarily on biodegradation in Bangladesh have been lacking which warranted the need for this study. In this study, we chose to test on samples from Dhaka as the metropolitan generates relatively a larger proportion of plastic waste - around 14 to 15 million polyethylene bags are discarded after their first-use every day in Dhaka [58]. The sample sites were all highly concentrated with partially-degraded plastic wastes which provided an ideal opportunity to test the soil bacteria that were responsible for long-term degradation. Initial screening and isolation involved separate testing for Gram-positive and Gram-negative bacteria which surprisingly resulted in no Gram-negative bacteria found. Following incubation of the morphologically distinct colonies with LDPE film as a sole carbon source, visible growth in the MSM media confirmed the successful enrichment of LDPE degrading bacteria.

Afterwards, the rate of degradation was measured through three primary methods -determination of dry weight loss, surface morphology analysis and change in chemical structure. Firstly, substantial loss in dry weight was recorded, with the highest being in colony H4. Unsurprisingly, greater loss in dry weight is suggested to be evident with a longer incubation time [59-61]. Secondly, the surface topography analysis at 1000x magnification showed cracks, minute holes, and other disruptions along with bacterial adhesion on the surface of all the samples as seen from Figure 3.4(g) through Figure 3.4(k) in comparison to the control [Figure 3.4(l)]. This colonization indicates bacterial attack leading to biodegradation [6]. The photographs also showed the adhesion to be scattered and mostly concentrated around the fissures. These findings correspond with studies that suggest bacterial susceptibility in the amorphous region of the LDPE samples [62]. All of these outcomes confirm the degradation capacity of the bacterial isolates.

Finally, the structural changes occurring due to induced degradation is an important parameter for measuring the degree of degradation in a molecular level [63]. FTIR has been used in numerous degradation studies to determine the decrease in native bonds that provides evidence of the polymer being fragmented into shorter chains. Additionally, generation of new or loss of functional groups further indicates the degradation process [64]. In a complete biodegradation pathway, the

polymer is oxidized by the oxygen in the air to form carbonyl groups which then forms carboxylic groups, Ultimately, the intermediates undergo β-oxidation and enter the citric cycle which form CO_2 and H_2O [64]. Native bonds in polyethylene correspond to the following peaks: 2918 cm^-1 (CH_2 asymmetrical stretching), 2851 cm^-1 (CH_2 symmetrical stretching), 1468 cm^-1 (bending deformation), 1373 cm^-1 (CH_3 symmetric deformation) and 718 cm^-1 (rocking deformation). These peaks are seen in the FTIR for the control LDPE (NC) [65]. In the FTIR spectroscopy of all LDPE samples incubated with bacterial colonies, there is an increase in transmittance % at these peaks that indicates more light is captured at those wavelengths i.e. less of that native bond is present. To specify, there is an increase in % transmittance at the following peaks: 1468 cm^-1 and 1373 cm^-1 for B5b; all 5 peaks for C2; 1468 cm^-1, 1373 cm^-1 and 718 cm^-1 for F3a; all peaks except 2851 cm^-1 for H4; 1468 cm^-1 and 1373 cm^-1 for N. Therefore, we can see there is a decrease in native bonds in all samples that shows the polymer is being fragmented. A new peak around 1600 cm^-1 is seen for C2, F3a, H4 and N that corresponds to absorbance of aromatic C=C bonds. As we can see in Figure 1.3(b), many intermediates contain aromatic functional groups.

The bacteria isolated in our study were confirmed to be *Bacillus* sp. (in: Bacteria) strain **JDMASP60**, *Bacillus atrophaeus* strain **MGB14**, and *Bacillus pumilus* strain **KD3** respectively. Multiple studies have shown different species of *Bacillus* to be a major genus possessing biodegrading capabilities [66]. A weight loss of 1.75% after 30 days [67] and 10.7% after 60 days [68] of incubation with *Bacillus* sp. were also reported in some studies. Another study showed 18.9% weight loss after 180 days of incubation with *Bacillus pumilus* [69]. The variation in weight loss and incubation period in our study with these studies is monumental as we have observed 20-30% weight loss after only 30 days of incubation. Moreover, to the best of our knowledge, we are reporting *Bacillus atrophaeus* as an LDPE degrading bacteria for the first time.

5. Conclusion

The present study highlights the potential use of the soil bacteria of Bangladesh in the successful biodegradation of LDPE. If the appropriate microorganisms are isolated, they can be utilized to tackle the growing plastic pollution that haunts Bangladesh. Three different strains of *Bacillus* sp. were found to be capable of using LDPE as the sole carbon source alongside two more unidentified strains that will be identified later on. While most studies have been carried out for longer durations, this study has shown relatively promising degradation rates for one month. Degradation has been proved through several different methods which strengthens the findings of the study and confirms our hypothesis. Fragmentation of the polymer chain into smaller intermediates through microbial enzymes is the key principle used to explain the biodegradation process.

While the pathways for microbial degradation have been suggested, additional research on exactly how these particular strains carry out degradation are needed. Moreover, this study warrants further investigation using more sample sites, LDPE powder and a longer incubation time. Furthermore, higher magnification of surface morphology changes in the tested LDPE is needed through Scanning Electron Microscopy (SEM) which is not possible to carry out in Bangladesh currently.

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