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Evaluation of the Efficacy of *Bacillus cereus* in the Biodegradation of High – Density Polyethylene (HDPE) for Sustainable Plastic waste Management

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Abstract

Plastic remains one of the fastest-growing pollutants in the twenty-first century. Among various plastic pollutants, high-density polyethylene (HDPE) cause drastic effects on the environment. However, only about 12 to 19% of plastic ever produced has been successfully incinerated by degradation methods such as thermal degradation, chemical degradation, and biodegradation. The usual breakdown of HDPE by thermal and chemical degradation produces secondary pollutants that are carcinogenic. Notably, in biodegradation, Bacillus cereus can degrade plastic without producing secondary pollutants. This makes Bacillus cereus a promising avenue for developing sustainable waste management strategies. By harnessing the natural capabilities of these bacteria, researchers hope to create environmentally friendly solutions that minimize the harmful effects of plastic waste on ecosystems. The Bacillus cereus uses polyethylene as a carbon source and traces cell growth over 60 days under supervised conditions. Likewise, the post-degraded polyethylene was analyzed with Fourier-transform infrared spectroscopy (FTIR) and scanning Electron Microscopy (SEM). Bacillus cereus effectively degrades HDPE. Hence Bacillus cereus potential biodegradation solution for efficient plastic waste management on this planet is promising. This approach not only highlights the potential of using microorganisms for environmental sustainability but also opens avenues for further research into enhancing biodegradation processes for various types of plastics.

Keywords: HDPE; FTIR; SEM; *Bacillus cereus*; Plastic Biodegradation.

1.INTRODUCTION

In the modern era, the usage of plastic greatly increased due to its durability and cost efficiency. Most of the people readily accepted the plastic-made articles such as plastic bottles, containers, bags, etc. Global share in plastic production occupies a market valued at about USD 560 billion, and the majority market share is held by Asia-Pacific, about 50% of the market share (Albertsson AC).

In the overall share of the plastic, high-density polyethylene represents a notable market share of approximately 44-47% of the total plastic production. Annual plastic production globally has surpassed 400 million tons in 2023. It almost doubled since 2000; the volume is expected to rise by 2040. The major production source to produce plastic is from fossil fuels such as coal, petroleum, and natural gas. The HDPE has a larger impact on the environment and biodiversity due to its fragmentation and persistence because HDPE is non-biodegradable naturally and leads to the creation of micro plastics and creates PCBs and DDT. It is involved in the bioaccumulation in marine biodiversity.

Polyethylene is created by the addition of ethylene monomers with the formula $(C_2H_4)_{n,}$ Polyethylene degradation can occur through physical, chemical and biological mechanisms. Physical degradation involves the breakdown of polyethylene due to environmental factors like sunlight, heat, and mechanical stress result in the fragmentation of the polymer into smaller pieces. Chemical degradation involves solvents or catalysts to break down the polymer chains, typically requiring harsh conditions and leading to the formation of unwanted byproducts

The breakdown of polyethylene by naturally existing microorganisms, on the other hand, is known as biological degradation and has a number of advantages over chemical and physical processes. Microbial deterioration has drawn a lot of interest.

Bacillus cereus has been capable to biodegrade the polyethylene. The Polyethylene used as a carbon source to their metabolism and the biological breakdown leads to the eco friendly substances unlike formation of secondary pollutants in thermal and chemical degradation.

In light of this, the main goal is to increase knowledge about PE degradation by investigating *Bacillus cereus*-assisted microbial breakdown of HDPE. polyethylene is examined in this study. Inoculating bacterial cultures into HDPE sheets as a carbon source and tracking their effectiveness in breaking down of HDPE over a period of 60 days under controlled conditions constitute the biodegradation treatment

process. The post-degradation HDPE is well characterized utilizing a variety of methods, such as scanning electron microscopy.

Fourier-transform infrared spectroscopy, and Scanning Electron Microscopy. These assays enable comparative investigation of degradation on both types of PE samples by offering insightful information about the changes in the Physicochemical characteristics of the polymer during the biodegradation process.

2. MATERIALS AND METHODS

2.1 Collection of Plastic Samples

For the studies in this investigation, high-density polyethylene plastic sheets were chosen. For biodegradation analysis, 50 x 50 mm plastic sheets with a thickness of 20 µm were cut. The plastic sheets were cleaned using de ionized (DI) water to get rid of any surface impurities before the experiment. The sheets were then sterilized by immersing them in a 70% (v/v) ethanol solution for ten minutes. In order to prepare the plastic sheets for additional analysis, they were air-dried at room temperature for a whole night following sterilization.

2.2 Bacillus cereus and Growth Conditions

Bacillus cereus was the bacterium chosen for this polyethylene biodegradation investigation. This particular bacterium, which was obtained from the marine soil sample by Using sterile spatulas, soil samples were taken from plastic-contaminated locations and placed into sterile polypropylene containers in accordance with accepted environmental sampling procedures (Danso et al., 2019; Urbanek et al., 2021). an appropriate culture medium was made using tryptic soy broth to promote the growth of *Bacillus cereus*. Using aseptic methods, the *Bacillus cereus*. bacterial culture was streaked across the surface of a sterile agar plate to begin the inoculation process.

After the streaking process, the agar plate was incubated for 24 hours at a regulated temperature of 37ºC. The bacterium multiplied and created separate, isolated colonies on the surface of the agar plate throughout this incubation time. The Gram staining method was used to morphologically characterize the recovered microorganisms. OD595 was measured every 30 minutes to track the growth of a single colony that had been injected in broth at 37 °C. The growth pattern of the bacteria over time is shown by the resulting growth curve. A solid basis for investigating the microorganism's potential for biodegradation on

polyethylene materials is provided by the meticulous selection of Bacillus sp. and the ideal growth conditions.

2.3 Preparation of media

Very Little Media Preparation Continuous stirring was used to dissolve the compounds in de ionized (DI) water in order to prepare minimum media. The media contained 1 g K_2HPO_4, 1 g KH_2PO_4, 1 g NH_4NO_3, 0.2 g KCl, 0.2 g NaCl, 0.05 g $FeCl_3$, 0.02 g $CaCl_2$, 1 g Na_2HPO_4, 0.002 g $ZnSO_4 \cdot 7H_2O$, 0.002 g $FeSO_4 \cdot 7H_2O$, 0.002 g $CuSO_4$, and 0.002 g $MnSO_4$ per 1000 ml of DI water. Sigma-Aldrich supplied all of the chemicals needed for the MSM, guaranteeing consistency and quality. In order to prepare a homogenous solution, the required amounts of each component were dissolved in 1000 milliliters of DI water.

The solution successfully sterilized the media and prevented contamination by autoclaving it in a Market Forge Sterilmatic Sterilizer for 20 minutes at 121 °C and 15 pressure.

2.4 Biodegradation Treatment Process

The biodegradation treatment was conducted to assess the potential of Bacillus sp. in degrading HDPE plastics. Initially, the weight of sterilized plastic sheets was accurately measured, serving as the baseline for the experiment. The bacteria culture in its log phase (absorb ance of 0.952 at 595 nm) of growth was selected to ensure actively growing cells were used for inoculation. Bacterial culture suspension and minimal media were prepared in a 1:10 volume ratio. The initial pH of the mixture was 6.54. In 250 ml Erlenmeyer flasks, 0.1% polyethylene sheets by weight (3 sheets of each type) were added as the carbon source. Subsequently, the prepared bacterial culture-media mixture was added to the flasks. To facilitate biodegradation, the experimental flasks were incubated in a New Brunswick Gyratory Water Bath Shaker at 37ºC, with a shaking speed of approximately 120 rpm, under aerobic conditions. The experimental flasks were incubated in an aerobic New Brunswick Gyratory Water Bath Shaker at 37ºC with a shaking speed of about 120 rpm to promote biodegradation. A sample was kept free of polyethylene sheets, infected in minimum media, and exposed to comparable incubation conditions in order to create an appropriate control. Over the course of the 60-day degradation experiment, cell growth was routinely tracked using a spectrophotometer at an absorbance at 595 nm (OD_{595}) using minimum media as a blank. By using pH meter, the experiment's pH was regularly recorded at intervals of 10 to 15 days and compared to the original pH.

2.5 Imaging and Quantification of Biofilms

The HDPE sheets were carefully removed from the degradation media in order to examine the development and measurement of biofilm on the degraded HDPE sheets. To get rid of any last bits of debris, they were then carefully cleaned with deionized water. Crystal Violet dye was applied to one sheet in order to observe the development of biofilm. The biofilm, which shows up as characteristic deep purple -colored clusters on the PE sheet's surface, was visible thanks to the staining procedure. Another sheet was subjected to mild water bath sonication in 1 ml of 0.85% saline for 5 minutes using Ultrasonic Cleaner in order to measure the biofilm.

The biofilm was removed off the PE surface with the use of sonication, and it was subsequently gathered in the saline solution. The Bradford Assay was then used to detect the protein concentration in the saline solution using a spectrophotometer.

The amount of biofilm on the sheet was quantitatively estimated by this measurement. Crystal Violet staining was applied to sonicated sheets in order to emphasize the variations in biofilm growth.

An imager was used to make the observations, allowing for a comparative study of the HDPE surface. Additionally, a Nikon TMS inverted phase contrast microscope was used to examine both sheets in order to verify that the biofilm had been removed during sonication. The biofilm and its absence following the sonication process could be clearly seen thanks to the microscope. After two minutes of washing the sheet of both samples in sterile phosphate buffer (pH 7), a tiny piece was cut to look at the surface morphology and see any structural alterations brought on by the degradation process.

Calculating Half-Life and Weight Loss Following biofilm analysis and removal from the degradation medium, the degraded PE sheets were rinsed with a 2% v/v sodium dodecyl sulfate (SDS) solution for five hours. After rinsing the PE sheets with Milli-Q water to get rid of any contaminants on the polyethylene surface, they were dried at 35 °C for the entire night. With an accuracy of 0.1 mg, the weight of dried PE polymers following the microbial treatment was determined using an analytical balance. The following formula was used to calculate the percentage of weight loss in PE polymers compared to their starting weight before to the biodegradation process in order to assess degradation: $(W_i - W_f) \times 100\ W_i$ (1) is the weight loss percentage.

2.6 Characterization of Biodegradation

The High-density polyethylene samples underwent a variety of characterization procedures following the biodegradation process in order to evaluate changes in their structure and characteristics.

2.6.1 Calculating Half-Life and Weight Loss

Calculating Half-Life and Weight Loss Following biofilm analysis and removal from the degradation medium, the degraded PE sheets were rinsed with a 2% v/v sodium dodecyl sulfate (SDS) solution for five hours. After rinsing the PE sheets with Milli-Q water to get rid of any contaminants on the polyethylene surface, they were dried at 35 °C for the entire night. With an accuracy of 0.1 mg, the weight of dried PE polymers following the microbial treatment was determined using an analytical balance. The following formula was used to calculate the percentage of weight loss in PE polymers compared to their starting weight before to the biodegradation process in order to assess degradation:

$(Wi - Wf) \times 100 / Wi$ (1)

is the weight loss percentage.

where the weights of the PE sheets prior to and following the microbial treatment are denoted by Wi and Wf, respectively.Using the first-order kinetic model, the findings were examined to calculate the half-life of residual PE and the rate constant of PE decrease, as indicated below.

$K = -1 (\ln .Wf/Wi)$

where t stands for time in days and K is the rate constant for PE consumption per day.

The half-life (t 1/2) was then calculated using Eq. $t_{1/2} = \ln(2)/K$

2.6.2 Scanning Electron Microscopy

The surface morphology of HDPE samples during deterioration was examined using scanning electron microscopy (SEM). The primary goals were to observe bacterial colonization on the damaged PE surfaces and to visualize and examine any structural alterations brought about by the degradation process. SEM analysis was also performed on untreated PE samples as controls for comparison. Before being mounted on SEM stubs, the PE sheets were meticulously cleaned with phosphate buffer and dried. The mounted samples were coated with a 25 nm layer of gold-palladium (Au/Pd) using a Leica EM ACE600 device with a current of 5.0E-2 mA prior to imaging. After that, the sputter-coated samples were put on the SEM holder and inspected.

Employing a high-resolution scanning electron microscope (FEI QUANTA 600F) at different magnifications. This analysis allowed for a thorough assessment of the microbial interactions and structural

changes brought about by the biodegradation process, as well as important insights into the physical changes and surface characteristics of the deteriorated PE samples.

2.6.3 Fourier-transform infrared (FTIR) spectroscopy

To look into possible chemical changes brought on by biodegradation in both control HDPE and treated HDPE samples, Fourier-transform infrared (FTIR) spectroscopy was used. All PE samples were thoroughly dried after being cleaned with 2% SDS, 70% ethanol, and distilled water prior to analysis. Attenuated total reflection (ATR) mode FTIR spectra were obtained using the Perkin-Elmer Frontier FTIR instrument. The FTIR spectra were obtained at room temperature in the 4000–400 cm^{-1} region with a resolution of 1 cm^{-1}. The HDPE samples that were cultured with the bacteria and the untreated control samples were examined.

3. Results and Discussion

3.1 Identification of BACTERIAL strain

The streak plate method was used to revive pure *Bacillus sp*. culture, producing unique, isolated colonies that were described by their round shape and pale golden hue.
The existence of *Bacillus sp.,* a Gram-positive rod-shaped bacterium with an estimated length of ~ 10 µm, was confirmed by Gram staining the isolated bacteria under an optical microscope, which displayed a unique purple coloration Verifying the existence of Bacillus species. The isolate's capacity to manufacture indole, carry out mixed-acid fermentation, and incorporate citrate as a carbon source is demonstrated through the IMViC biochemical profile, which exhibited Tryptophan (Indole) positive, Methyl Red positive, Voges–Proskauer negative, and Citrate positive. The catalase test was strongly positive, with vigorous bubble formation with the addition of hydrogen peroxide, proving the organism's ability to detoxify reactive oxygen species, nevertheless the urease test was negative, displaying no color change.

The effective isolation of highly purified genomic DNA needed for replication was confirmed by the distinct, whole DNA band obtained from genomic DNA extraction on agarose gel electrophoresis. A distinct ~350 bp band was generated via PCR-based amplification of the 16S rRNA gene, indicating effective amplification of the target area. After the isolated amplicon was sequenced, BLAST analysis showed a high degree of sequence similarity with reference strains in the NCBI database, guaranteeing precise species identification. This identification was further confirmed by phylogenetic analysis, which

showed that the isolate tightly grouped with similar sequences in the neighbor joining tree that was created. A unique amplicon of about 1,500 bp was produced by PCR amplification of the 16S rRNA gene. Significant sequence similarity (>99%) with *Bacillus cereus* strain NZ_CP017060.1 from the NCBI GenBank database was found by BLASTn sequence analysis. The acquired sequence was added to the GenBank database with accession number PZ028803. The isolate's taxonomic position within the *Bacillus* cereus clade was further validated by phylogenetic analysis using the Neighbor-Joining method.

All things considered, this investigation verified the isolated HDPE-degrading gram-positive bacterium's morphological, biochemical, and molecular identification.

3.2 WEIGHT LOSS:

After 60 days of incubation, *Bacillus cereus* was observed to significantly destroy 18.4% of the thermally treated HDPE (Fig. 1). Similar observations were also reported by Sudhakar et al. (2008), who discovered that thermal pretreatment increased HDPE degradation when incubated with *B. cereus* (i.e., 9% against 3.5% in the case of untreated HDPE). Therefore, compared to untreated HDPE, more weight loss was seen following thermal treatment. Similarly, following 30 days of incubation with Arthrobacter sp. and Pseudomonas sp., Balasubramanian et al. (2010) likewise noted a corresponding loss of 12 and 15% in HDPE weight. likewise, it was discovered that after fifteen days of incubation with *bacillus cereus* a biofilm grew on the surface of the HDPE films. It could be explained by the fact that thermal pretreatment oxidizes the polyethylene chain, resulting in the creation of carbonyl groups that promote the growth of biofilms on HDPE. The current study's breakdown rate increased as a result of the biofilm's reduction of the polymer's hydrophobicity. However, the synthesis of protein that permits the creation of a stable biofilm may be responsible for the robust adherence and comparatively high survival rate of *b. cereus* biofilm to the polyethylene surface. These characteristics validate its effectiveness in using polyethylene as a carbon and energy source despite the limited carbon availability.

3.3 FTIR Analysis of HDPE Degradation

FTIR spectroscopy was used to identify chemical changes that occurred during the breakdown of high - density polyethylene, such as the polymer's fragmentation into shorter chains. FTIR has also been used in earlier studies to identify the emergence or loss of functional groups, giving information on the chemical changes and breakdown products in polyethylene. The FTIR spectra of the untreated (control) and samples following the biodegradation treatment of HDPE were obtained in this work using the ATR mode of FTIR

(Fig.1). control HDPE showed distinctive peaks at 1634.33 cm^{-1}, 1459.89 cm^{-1}, 1353.70 cm^{-1}, 1165.18 cm^{-1}, 997.66 cm^{-1}, 972.59 cm^{-1}, 898.74 cm^{-1}. Stable ester bonds and a structurally intact polymer backbone are indicated by these distinct, sharp peaks. Stable (C-C) bonds and a structurally intact polymer backbone are indicated by these distinct, sharp peaks. The bacteria-treated HDPE, on the other hand, showed discernible changes in these distinctive peaks. The C=O peak (~1623 cm^{-1}) showed a notable decrease in intensity, indicating breaking of the bonds, which are the primary structural connections in HDPE. The hydrolysis of ester groups was further confirmed by the broadening and decreased intensity of the C–O stretching peaks (~1294–723.50 cm^{-1}).

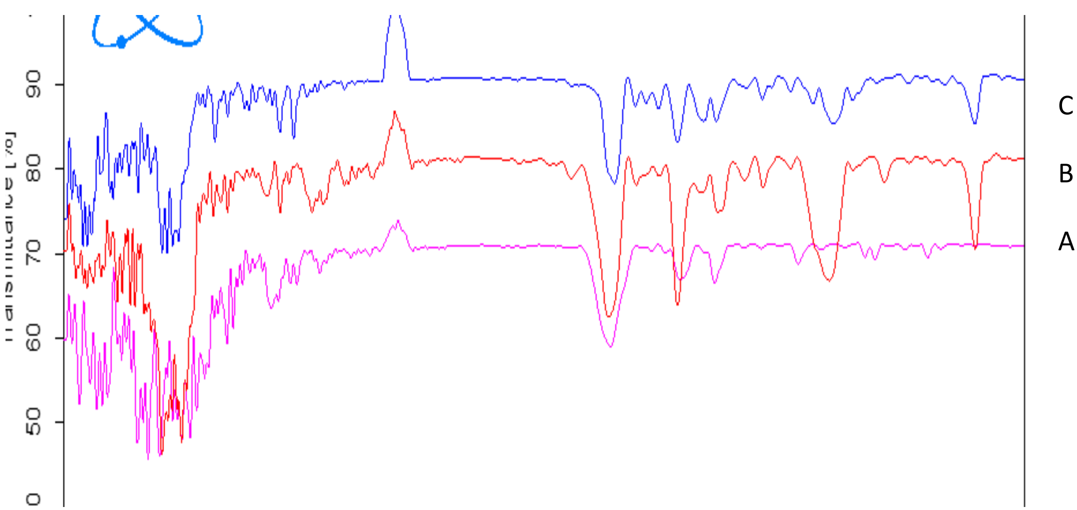

Figure 1: FTIR- A-Control and B-Thermal, C-bacterial treated

3.4 SEM Analysis of HDPE Degradation

We observed some patchy polyethylene degradation surrounding the bacterial cells in the biofilm in the current investigation, as shown in the SEM micrographs (Fig 2). In the polyethylene, the bacterial biofilm displayed a sculpted pattern resembling a cell. These forms have previously been observed for biodegradable polymers, such as poly-β-hydroxybutyrate. SEM images revealed a similar change in surface structure for HDPE films treated with bacteria. After sixty days of incubation period, the treated HDPE film had a rough surface with several cracks and grooves. Conversely, under the same circumstances, the untreated film maintained a smooth surface. It shows that the *bacillus cereus* secretes enzymes that can break down polyethylene and cause grooves to appear.

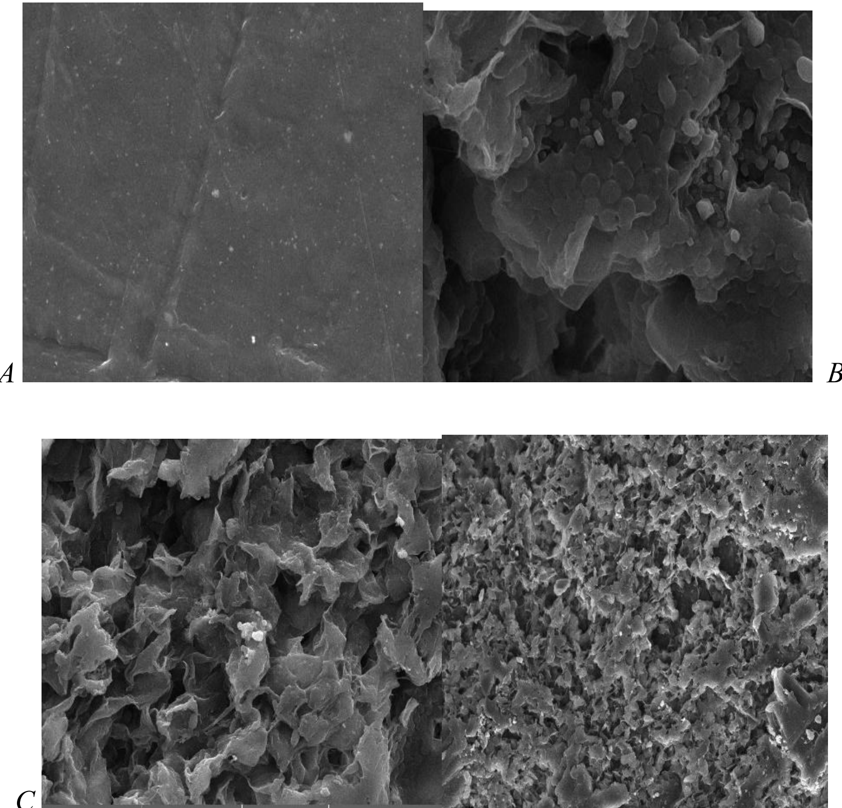

Figure: 2 A-Control HDPE; B and C- bacterial treated HDPE

4. CONCLUSION

HDPE biodegradation has drawn a lot of attention lately, but there hasn't been much research done on it yet. Compared to the other microbial species reported for HDPE breakdown in the literature, we found that a unique *Bacillus cerous*, isolated from a marine soil sample, was incredibly efficient. Our isolated strain's comparatively strong breakdown capacity in this investigation suggests that microorganisms may be naturally evolving to exploit HDPE as a source of carbon and energy. Significant chemical alteration of HDPE was confirmed by FTIR data, which showed bond breaking and the creation of new hydroxyl groups. however, the SEM image reveals significant surface erosion, polymer cracking, and structural collapse. All of these results show unequivocally that *Bacillus cereus* can efficiently depolymerize HDPE via oxidative and hydrolytic processes. This work identifies *Bacillus cereus* as a promising candidate for future biotechnological applications in HDPE bioremediation and provides important evidence for the development of microbial techniques for sustainable plastic waste degradation.

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