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Article

The Effects of Different Aquatic Environments on the Rate of Polyethylene Biodegradation by *Bacillus subtilis*

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SUMMARY

The proliferation of plastics in consumer products results in the release of countless tons of plastic waste into the environment. Because of their chemical composition, plastics take hundreds of years to decompose. The world today is overrun by plastic waste that occupies landfills, makes its way into rivers and oceans, and overwhelms several ecosystems. Current methods of handling the waste namely recycling, landfills and incineration, are inadequate, ineffective or harmful. In recent years, scientists have discovered that certain bacteria can degrade and assimilate polyethylene. Microbes have been shown to have the ability to "eat" petroleum-based products like natural gas and light sweet crude oil from oil spills. The ability of bacteria to decompose certain types of plastic makes them a potential bioremediation option. Current research indicates that the bacterium *Bacillus subtilis* (*B. subtilis*) is proficient at degrading polyethylene utilizing a biosurfactant called surfactin. Studies show that the bacterium is versatile and can thrive in various environments, while surfactin can withstand high concentrations of salinity. The aim of this study is to test the ability of *B. subtilis* to degrade high-density and low-density polyethylene in aquatic environments. Rates of degradation were studied and compared across fresh water, brackish water, and ocean water samples. Degradation occurred across all samples, although it was the highest in fresh water and lowest in ocean water. This study supports the hypothesis that *B. subtilis* can potentially be used to help degrade plastic in aquatic environments.

INTRODUCTION

Only 9% of plastic produced in the US is recycled – some of it is incinerated, a process that emits toxic gases into the atmosphere, and most of it is dumped in landfills (1, 2). Over 8 million metric tons of plastic waste enters the world's oceans annually (3). Marine animals are mistaking plastic waste for food; plastic has been found in more than 60% of all seabird and in 100% of sea turtle species (4). Besides being life threatening to animals and birds, this affects humans because we consume seafood that can contain chemicals from plastics. Diethylhexyl phthalate (DEHP), contained in some plastics, is a toxic carcinogen and other toxins in plastics are directly linked to cancers, birth defects, immune system problems, and childhood developmental issues. Bisphenol A (BPA), a known chemical that interferes with human hormonal function, is used in plastic bottles and food packaging materials. Over time the polymer chains of BPA break down and can enter the human body through drinking contaminated water or eating a fish that is exposed to the broken down toxins (5, 6).

Plastics are semi-organic materials that come from oil or petroleum. They are routinely labeled as polymers, as they are comprised of polymers which are large molecules made with a massive amount of smaller, identical molecules. Polymers have a different physical and chemical makeup than their monomers, and more uniquely, their properties can be tailored depending on their main purpose. Polyethylene is a polymer that is exceptionally versatile. About 80 million tons of this compound are produced each year for widespread usage in consumer products (7). It is a thermo-plastic created from the polymerization of ethylene, a process that produces very long, very straight chains of hydrocarbon monomers. By adjusting the polymerization process, the long chains can be made to branch, creating different kinds of polyethylene. The degree of branching determines what kind is produced. **(Figures 1A, 1B, 1C)**. Low-density polyethylene (LDPE) and high-density polyethylene (HDPE) are two of the most common types of polymers in commercial use. LDPE has the most long- and short-chain branching of any form of polyethylene, resulting in its lower density. LDPE is particularly useful for a range of applications, from rigid products like plastic bottles, buckets, and bowls, to filmy ones like plastic grocery bags and plastic cling-wrap. HDPE is characterized by minimal branching of the polymer chain, making it much denser and rigid. The added tensile strength of HDPE makes it useful for rigid applications such as milk and detergent jugs, garbage cans, water pipes and children's toys.

Plastics cannot dissolve in water and are estimated to require hundreds of years to naturally decompose (8). An environmentally-friendly solution to potentially help tackle plastic waste is biodegradation, which is the decay or breakdown of materials that occurs when microorganisms use an organic substance as a source of carbon and energy (9). Without releasing toxic byproducts into the atmosphere, these organisms can decompose the polyethylene present within plastics at rates significantly higher than natural degradation (10). Biodegradation is a two-step process. The first step is the cleavage of the large molecular chains, and the second step is the mineralization. External enzymes are

responsible for the first step, because the sizes of the polymer chains are considerably greater than the majority of the microorganisms. Once sufficiently reduced to a small size, monomeric fragments are transported into the cells where they mineralize. The products of this process are water, salts, minerals, gases and biomass (11).

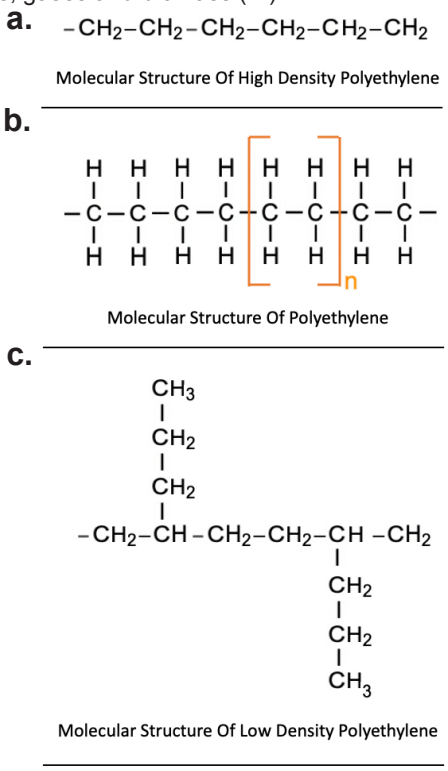

Figure 1: Visual depiction of the molecular structure of (A) HDPE, (B) polyethylene, and (C) LDPE.

All living organisms are able to produce diverse antimicrobial compounds (12). Soil bacteria and fungi produce antibiotics to gain an advantage when competing for food, water, and other limited resources in a particular habitat, as the antibiotics kill off competition (13). Lipopeptides, one category of such compounds, represent a unique class of cyclic peptides and exhibit remarkable therapeutic and biotechnological properties (14). Surfactin, one of the principal representatives of the lipopeptide family, is produced by *Bacillus subtilis* (*B. subtilis*), a rod-shaped gram-positive bacterium typically found in the upper layers of soil as well as in oceans. Gram-positive bacteria are important to the field of biotechnology since these bacteria produce enzymes critical for industry such as proteases, amylases, and lipases, both reliably and at low cost (16). *B. subtilis* secretes proteins in the range of grams per liter, making *B. subtilis* an excellent candidate to study for this application (17).

Surfactants are substances that reduce the interfacial tension between water and oil and adsorb at the interface to stabilize emulsions (18). They have an amphipathic molecular structure – having both hydrophilic and hydrophobic parts – and exhibit properties like formation of emulsions, foaming and detergency. Because of these properties, surfactants lower the surface energy and tension of a medium and oxidize the polyethylene. This oxidation converts carbonyl groups into unsaturated hydrocarbons, breaking down the structure of polyethylene. Through this process, described as depolymerization, the large polymer chains are broken into smaller, water-soluble fragments that can pass through microbial membranes, where they are biodegraded by cellular enzymes and used as carbon and energy sources (19).

Microorganisms, possessing a large surface area-to-volume ratio, produce a variety of surfactants referred to as biosurfactants (20). Biosurfactants have advantages over their synthetic counterparts because they are biodegradable, less toxic, and effective at extreme temperatures and pH (21). One of the most powerful biosurfactants, surfactin lowers the surface tension of water, even at very low concentrations (22). Surfactin is a highly potent agent with diverse commercial applications, including subsurface pollution remediation and the enhancement of the availability of hydrophobic compounds, thus increasing the potential for biodegradation by microbes (23).

Current research indicates that certain biosurfactants are proficient at degrading polyethylene, utilizing surfactin, which isn't degraded itself, to initiate plastic decomposition (24). *B. subtilis* has been found in different oceans; it is known to be versatile and can thrive in various environments. Its polyethylene-degrading biosurfactant can withstand high concentrations of salinity, supporting the theory that the bacteria can survive in underwater environments and decompose the polyethylene, even in situations where it is exposed to high concentrations of chlorine. We therefore utilized *B. subtilis* in this study to determine if it would biodegrade polyethylene in different aquatic environments because of its production of a biosurfactant and ability to withstand extreme environments.

The tests were conducted across three different levels of salinity of water: fresh water, brackish water and ocean water. Bacteria grow slower in ocean water than in freshwater (25, 26). Accordingly, we hypothesized that the reduction of plastic will be the lowest in ocean water and highest in freshwater, with the mass reduction in brackish water falling in between the two.

RESULTS

To test the effect of salinity on the ability of *B. subtilis* to degrade plastic, we placed bacteria and a square piece of polyethylene into test tubes mimicking different aquatic environments. Specifically, the test tubes contained a growth medium consisting of nutrients that support bacterial growth, and we filled each test tube with freshwater, brackish water or ocean water to model the conditions present in various bodies of water. We placed the test tubes in an incubator to allow the bacteria to grow. After 30 days of incubation, we measured the mass of each polyethylene piece. We decided

to run the experiment over a 30 day period as we deemed it to be a reasonably long period for observable degradation to occur. Future iterations of the experiment may be run using different time periods based on results.

We cut all the HDPE and LDPE pieces into similar shapes with a uniform mass of roughly 17 mg. We set up control samples of HDPE and LDPE by placing the HDPE and LDPE pieces in test tubes containing water representing the different aquatic environments, the growth medium, and no bacteria. We ensured that *B. subtilis* was the only agent causing the degradation of the polyethylene. As expected, the control samples, which were not exposed to any bacteria, remained at approximately the same mass, having an average of a 0.04 percent decrease in mass over the 30 day period across all three types of water environments. Some control samples increased in mass over the 30 day period, which could be due to either mass measurement errors or improper cleaning of the samples to remove excess chemicals or bacteria.

To test whether the 0.1M HCL wash and water caused any degradation of the plastic, additional LDPE and HDPE pieces of shape, size, and mass similar to the test tube samples were weighed before and after a HCl wash; no significant amount of mass degradation was noticed. No significant polyethylene degradation occurred during the heating (verified by a separate, dry, control).

Samples exposed to *B. subtilis* in freshwater had the highest amounts of degradation relative to the controls. The average mass of the HDPE pieces had a 5.79% decrease, while the average mass of the LDPE pieces had a 5.77% decrease over a period of one month, when compared to the controls **(Figure 2)**.

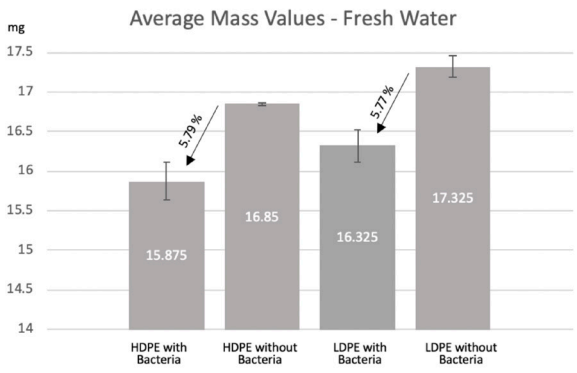

Figure 2: The HDPE and LDPE samples exposed to *B. subtilis* in freshwater over a 30 day period showed degradation when compared to the samples that were not exposed to the bacteria.

Samples exposed to *B. subtilis* in brackish water underwent a smaller decrease in mass. The average mass of the HDPE pieces had a 5.08% decrease, while the average mass of the LDPE samples had a 5.30% decrease over one month, compared to their controls **(Figure 3)**.

Samples exposed to *B. subtilis* in ocean water experienced the least amount of degradation. The average mass of the HDPE pieces decreased 3.61%, while the average mass of the LDPE samples decreased 2.47% over one month, compared to their controls **(Figure 4)**.

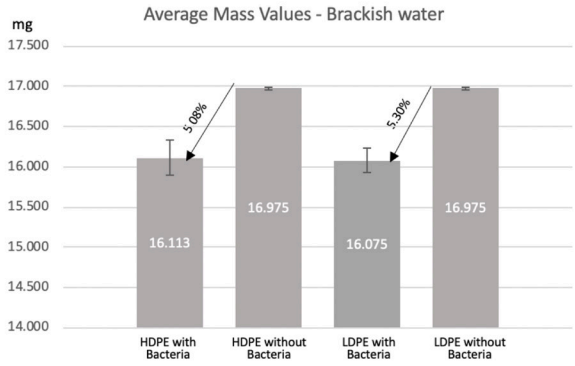

Figure 3. The HDPE and LDPE samples exposed to B. subtilis in brackish water over a 30 day period showed degradation, although it was less than the bacteria-exposed samples in freshwater.

The amount of degradation between HDPE and LDPE pieces was not consistent. We did not explore the implication of this outcome.

The results appear to support the hypothesis that the presence of *B subtilis* accelerates the rate of degradation of HDPE and LDPE. Based on available research findings, this outcome can be attributed to the depolymerization of the large molecules into smaller monomers with the help of the biosurfactant surfactin, and the subsequent passing of the monomers through the cell membranes of the bacteria to be converted into energy.

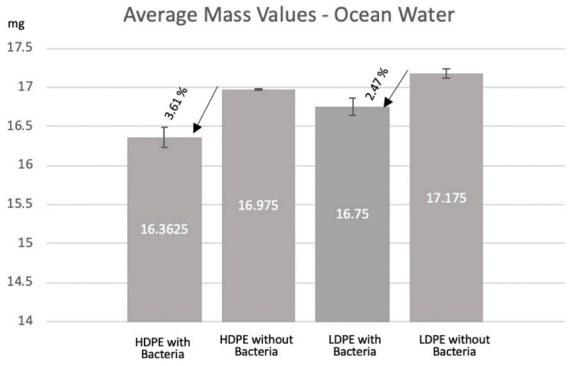

Figure 4: The HDPE and LDPE samples exposed to B. subtilis in ocean water over a 30 day period showed degradation, although it was the lowest when compared to samples in fresh or brackish water.

DISCUSSION

The main goal of our study was to test whether *B. subtilis* can degrade polyethylene in aquatic environments and to compare the rate of degradation across varying levels of salinity that simulate real-world bodies of water. The degradation as measured by the reduction in mass of the

HDPE and LDPE samples after 30 days of incubation was found to be the highest in freshwater samples and the lowest in ocean water samples. This supported the hypothesis that *B. subtilis* can degrade polyethylene in salt water and that the amount of degradation decreases with increase in salinity; our findings are consistent with at least one previous study that identified *B. subtilis* as an agent of degradation in freshwater (27).

The ability of *B. subtilis* to degrade polyethylene in aquatic environments offers the possibility that this bacteria may be deployed as a solution to plastic pollution in various bodies of water. The superior degradation in freshwater suggests that bacterial degradation of polyethylene can tackle plastic pollution in lakes and rivers. The degradation in brackish water suggests that biodegradation can be used in areas like estuaries, large lakes, and at the mouths of rivers. Finally, degradation in more ocean water suggests that bioremediation can be a viable solution to tackle plastic pollution in our oceans. The long-term goal of this study is to develop a cost effective and environmentally safe solution to degrade plastic at scale, using natural organisms like *B. subtilis* at temperature ranges where it is active.

Research shows that bacterial growth is inhibited with increases in salinity, which corresponds to the decreased degradation in the ocean water samples (28). It is not clear from this experiment whether the reduced degradation is due entirely to the slower growth of bacteria or if surfactin's effectiveness is also reduced in ocean environments. The rates of degradation observed may correspond to the temperature and salinity at which the experiment was conducted. To further validate the differences in the rates, the experiment should be repeated at varying temperature and salinity levels.

Further research is required in several areas to establish *B. subtilis* as a practical option for the degradation of plastic waste in our water bodies. The lower rate of degradation implies either that the bacteria are not as productive in ocean environments or that surfactin is less effective in the presence of salts and other chemicals seen in ocean water. There is adequate research to show that *B. subtilis* is a common inhabitant of ocean habitats, so the bacteria's viability in natural, marine environments is well established (29,30). Additional research into where in the oceans the bacteria thrive the most may suggest the regions of the oceans where *B. subtilis* can be deployed. Future studies should also focus on the effects of ocean water on the detergency of surfactants with the end goal of creating conditions that may enhance their effectiveness.

Studying the productivity of *B. subtilis* in expressing surfactin as well as absorbing the degraded smaller monomer molecules when in ocean environments would be key next steps for this research. The optimal temperature for the growth of *B. subtilis* is 25-35°C, while the optimal conditions for high surfactin production in the laboratory are a slightly acidic pH (6.5–6.8) and an incubation temperature of 30°C (31). Ocean temperatures vary from -2°C to 35°C which is well within the range for *B. subtilis* to thrive, though colder areas may see less productivity from bacteria (32). However, the pH of oceans is typically at an average of 8.1 and therefore less acidic than ideal incubation conditions (33). We need to find ways to overcome the pH difference and encourage bacterial growth; otherwise, *B. subtilis* may only be a viable option in fresh and brackish water, and not in oceans.

Under laboratory conditions, research has shown that pretreatment of polymer films with UV radiation aids its accessibility as food for the microorganisms, enabling a much faster rate of biodegradation (27). Therefore, there is reason to believe that in natural conditions, the UV radiation from the sun can aid biodegradation by *B. subtilis*. However, to be a commercially available solution, the rate of degradation by the bacteria may have to be much faster than what was observed in the experiment.

So far, much of the laboratory research points to the use of microbes in situ to produce surfactants. An alternative method to harness *B. subtilis* could be to produce surfactin under optimal industrial conditions, transport it to the site of plastic pollution, and spray it to stimulate biodegradation. However, it is important to note that studying the effectiveness of surfactants to fight hydrocarbon pollution in the open sea remains a challenge, and research is still ongoing in this area (34, 35). Studying formulations that are inherently more vulnerable to biodegradation and promoting the use of such plastics can help with the natural degradation process. Research shows that additives such as pro-oxidants and starch make plastics biodegradable. Starch-blended polyethylene makes the material hydrophilic and allows it to be catalyzed by amylase enzymes; microorganisms are then able to attack and remove this section thus degrading the plastic (36).

For *B. subtilis* to be a practical option to tackle the enormous amount of plastic waste, it should be deployable at scale. Further experimentation is required to study the optimal methods to incubate, transport and deploy large amounts of this bacterium, while also evaluating any potential adverse impact on the aquatic ecosystems when doing so. Surfactin, produced by *B. subtilis*, is indeed commercially produced and sold, although at present it is not cost effective to produce at scale. One reason for the high cost is due to the substrates employed for their production and the purity level required for application in the fields of pharmaceutics and medicine as well as the small batch sizes (37). Recent research into industrial scale production of surfactin at lower cost has been driven by the desire to clean up oil spills using biosurfactants and shows promise (38, 39).

Further research and experimentation is required to assess whether the introduction of additional surfactin can enhance the ability of *B. subtilis* which is naturally present in oceans to act as a viable agent of polymer degradation. As an alternative, the possibility of introducing adequate amounts of *B. subtilis* to ocean environments to help degradation has to

be evaluated along with a study of the impact of introducing such vast amounts of the bacteria on marine ecosystems.
The experiment showed that the presence of *B. subtilis* accelerates the rate of degradation of HDPE and LDPE in aquatic environments. The results offer the promise that microbial biodegradation of plastics can be an option to pursue in our efforts to mitigate the vast amounts of plastic pollution in water bodies.

MATERIALS AND METHODS

Bacterial Culture

B. subtilis (sourced from Carolina Biological by the Harker School wet lab) was obtained in a sterile, 20 x 150 mm test tube, approximately one-third filled with agar. Colonies of *B. subtilis* were visible as small bumps on the surface of the agar **(Figure 5A)**. Bacteria were streaked onto a petri dish containing agar using a cotton swab **(Figure 5B)**. Bacteria in petri dishes were left at room temperature to culture and placed in a refrigerator for preservation.

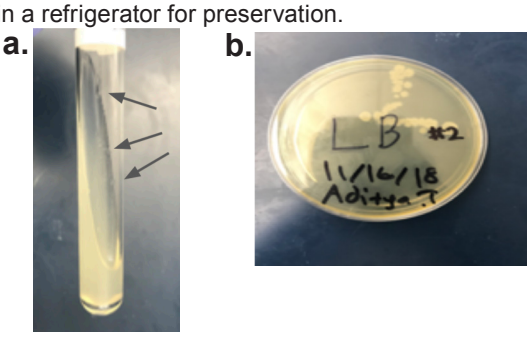

Figure 5. A) *B. subtilis* is visible as small bumps in the test tube with agar. B) *B. subtilis* colonies are visible as small white spots in the petri dish with agar.

Test Tube Setup

Out of the 72 test tubes, 36 contained LDPE and 36 contained HDPE. Each polyethylene piece was cut in roughly the same square shape, with a uniform mass of approximately 17 mg. Within both groups, 24 test tubes contained bacteria, while the remaining 12 tubes served as controls without bacteria. Both control groups and bacteria groups were split into three subgroups, containing freshwater, ocean-water, or brackish water.

Water Setup and Incu-shaker

Sterilized water was added to each test tube. For test tubes in the ocean water group, a premade powder called Instant Ocean was added to achieve a salt concentration of roughly 35 parts per thousand, which is the average salinity of oceans (40). For test tubes in the brackish water group, Instant Ocean powder was added to achieve a salt concentration of roughly 15 parts per thousand. An identical amount of salt medium of very low concentration was added to all test tubes to support the bacteria's growth. All concentrations remained the same with the addition of the growth medium (2 g $NaNO_3$, 0.5 g $MgSO_4$, 0.5 g KCl, 0.01 g $Fe_2(SO4)_3$, 0.14 g KH_2PO_4, 1.2 g K_2HPO_4, 0.02 g yeast extract, 1 L water). All test tubes were then placed inside a shaking incubator set at 35°C.

Mass Measurement and Analysis

After 4 weeks, each polyethylene piece was removed with tweezers and cleaned of bacteria/salt using a 0.1M HCl wash and water. Each piece was placed on a paper towel and heated to 40-50ºC. Then, the mass of the piece was measured on a scale and recorded. The data was analyzed utilizing the statistical and graphing functions of Microsoft Excel.

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