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Screening and Identification of Ligninolytic Enzyme Producing Bacteria for the Degradation of Polyethylene Glycol

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ABSTRACT: The aim of the present study was to investigate the potential of bacteria producing ligninolytic enzymes, such as lignin peroxidase and laccase, to degrade plastic. Utilizing suitable biodegradable methods is imperative to alleviate the environmental impact of plastics. A comprehensive grasp of the interplay between microbes and a polymer is critical to address environmental issues associated with plastics. Numerous organisms, with enzymes playing a prominent role, have developed strategies to endure and break down plastics. The study at hand was shaped by the preceding ideas, seeking to explore how different organisms, especially those utilizing enzymes, have evolved approaches to endure and biodegrade plastics. Soil samples were collected from a landfill site and enriched in media containing low-molecular-weight polyethylene glycol (PEG). The isolates were then screened for their ability to degrade polyethylene strips and produce ligninolytic enzymes. Out of the 22 isolates obtained, 4isolates, namely Ia4, Ia12, Ia19, and Ia22, exhibited positive reactions for both laccase and lignin peroxidase. To assess the biodegradation potential of the isolates, a degradation test was conducted using polyethylene and plastic powder. Among the 4 isolates, Ia19 was highly degraded the plastic materials and based on the biochemical test, such isolates as Pseudomonas sp. Overall, the study highlights the potential of bacteria producing ligninolytic enzymes to degrade plastic. These findings contribute to the development of environmentally friendly methods for plastic degradation and provide insights into the role of microorganisms in mitigating plastic pollution.

Keywords: Polyethylene glycol (PEG), polyethylene glycol (PEG), lignin peroxidase, laccase, *Pseudomonas stutzeri*.

INTRODUCTION

For most of our history, humans crafted items using resources found in nature. However, the invention of plastic brought a complete revolution to our world. Its affordability, versatility, and sterility led to significant advancements across various industries. Nonetheless, this remarkable technological achievement has also posed considerable risks to mankind (Bhat *et al.*, 2021). Plastic products play a crucial role in our daily lives as essential consumer goods. Plastic materials, being derived from petrochemicals, present environmental hazards due to their non-biodegradable nature (Mahitha *et al.*, 2023).

Since 1950, the global production of non-fiber plastic products has reached an astounding 7300 tons, with Polyethylene (PE) constituting 36% of this total (Wong *et al.*, 1999). Polyethylene is a high molecular weight polymer composed of carbon-carbon single bonds. It possesses desirable characteristics such as chemical inertness, corrosion resistance, non-toxicity, recyclability, and reprocessability (Kong *et al.*, 2022).

Various forms of polyethylene, including high-density, low-density, and linear low-density polyethylene, find widespread applications in numerous aspects of our lives, such as toys, bottles, water tanks, shopping bags, construction tools, disposable cups, as well as electronic and medical consumer products (Mahmood *et al.*, 2022). Unfortunately, abandoned plastic products of all kinds end up in the natural environment, resulting in the formation of plastic particles of varying sizes. This phenomenon leads to severe water resource contamination and poses a grave threat to aquatic and marine life.

Currently, the predominant methods of disposing of PE waste are landfilling and incineration (Jiakang *et al.*, 2020). Shockingly, as of 2015, the global generation of plastic waste amounted to approximately 6.3 billion tons, with a mere 9% being recycled, 12% incinerated, and a staggering 79% accumulating in landfills or polluting the natural environment (Geyer *et al.*, 2017). Landfilling, while a straight forward approach, this was leaded to land occupation and groundwater contamination. Animals may inadvertently ingest

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plastic waste, resulting in a range of digestive tract ailments among livestock and, in severe cases, even death. Incineration, on the other hand, not only contributes to significant air pollution but also generates hazardous byproducts known as dioxins, which can persist in the soil for extended periods, taking at least a year to break down into degradable substances (Alshehrei, 2017; Royer *et al.*, 2018; Webb *et al.*, 2012; Chamas *et al.*, 2020). Consequently, there exists an urgent need to develop a highly efficient and environmentally friendly method to degrade PE plastics, which currently pose significant challenges in terms of degradation.

Microbial degradation of polyethylene (PE) occurs through the utilization of a diverse range of microorganisms, including bacteria such as Pseudomonas sp., Bacillus sp., and Enterobacter asburiae, as well as Actinomycetes sp (Waithaka et al., 2017)., and fungi including Fusarium solani and Penicillium citrinum PT1 (Tribedi et al., 2013; Yang et al., 2014; Liu et al., 2020). Especially ligninolytic enzyme producing bacteria are currently used. These microorganisms possess a remarkable enzyme system and intricate metabolic pathways that enable them to degrade PE. They secrete extracellular enzymes, such as manganese peroxidase, laccase, alkane hydroxylase, alkane monooxygenase, ligninolytic enzyme, and multicopper oxidase. This enzymatic activity allows for the breakdown of the complex structure of plastic, ultimately transforming it into carbon dioxide (CO₂) and water (H₂O).

Santo demonstrated that enzymes secreted by microorganisms can catalyze the breakdown of polyethylene macromolecules. In particular, it was observed that ligninolytic enzyme of laccase has the ability to interact with polyethylene molecules, leading to the formation of carbonyl groups. This process results in a reduction of the molecular weight of polyethylene molecules. However, there are very few studies that have found lignolytic properties in plasticdegrading bacteria. Based on this information, the present study aimed to investigate whether bacteria producing enzymes such as Lignin peroxidase, Manganese peroxidase, and Laccase have the ability to degrade plastic.

MATERIALS AND METHODS

Collection of Soil Samples. Twenty soil samples were collected from a landfill site in Namakkal, Tamil Nadu, where plastic and waste materials were deposited. The selection of this dump yard was based on the assumption that the microbial communities present would have adapted to the plastic waste over time, making it a potentially abundant source of microbes capable of degrading pre-treated plastic materials.

Enrichment of Isolates. One gram of collected soil samples were inoculated into enriched media The composition of the enriched media included 0.1g yeast extract, 0.25g MgSO₄.H₂O, 5.8g KH₂PO₄, 3.7g K₂HPO₄, and 2.0g KNO₃, 100mg of LMWPE (Low-Molecular-Weight Polyethylene) strips in 1000ml of

distilled water. After autoclaving the media, all flask were incubating for seven days.

Screening of Plastic Degrading Isolates. In this study, two types of substrate-containing media were utilized to screen for effective microbes capable of degrading polyethylene strips. For assessing the formation of clear zones around bacterial colonies, a synthetic medium was prepared by combining a mineral salt medium with polyethylene glycol and plastic powder (prepared separately) at concentrations of 0.2% (w/v) and 15% (w/v) agar. Following sterilization and solidification of the media, cultures from enriched media were obtained and inoculated onto the prepared plates using an inoculation loop. The plates were then incubated at 30 °C for duration of 2 weeks.

After the 2-week incubation period, the plates were subjected to staining with a 0.1% Coomassie Brilliant Blue (CBB) solution followed by destaining to visualize the presence of a clear zone surrounding the bacterial colonies. The CBB solution was prepared by dissolving 0.1% (w/v) CBB in a mixture of 40% (v/v) methanol and 10% (v/v) acetic acid. The destaining solution was prepared by adding 40% (v/v) methanol to 10% (v/v) acetic acid. The agar plates were flooded with a 0.1% solution of Coomassie Blue R-250 for duration of 20 minutes. Subsequently, the CBB solution was drained, and the plates were flooded with the destaining solution for an additional 20 minutes. Bacteria that displayed a clear zone against a blue background were identified as polyethylene degraders (Gupta et al., 2016).

Screening of Ligninolytic Isolates. To identify bacteria capable of producing ligninolytic enzymes, including laccase, lignin peroxidase and manganese peroxidase, a selection protocol based on their ability to decolorize synthetic dyes, specifically the ligninolytic indicator dye Phenol Red, was employed. The indicator dyes were filter sterilized and added to autoclaved media under aseptic conditions. These dyes possess structural similarities to lignin. Minimal salts medium (MSM) agar plates, supplemented with 0.1 g/100 ml of Phenol Red and methylene blue were streaked with bacterial isolates and subsequently incubated at 37°C. After 24-48 hours of incubation, the plates were examined for the presence of a decolorized zone around the bacterial colony. The appearance of a decolorized zone indicated the presence of ligninolytic enzyme activity in the bacterial isolates (Hooda et al., 2015).

Screening of Lignin Peroxidase Producing Isolates. The isolated bacteria that tested positive in the dye decolorization assay were further assessed for qualitative lignin peroxidase activity, following the method described by Falade *et al.* (2017). Each positive isolate was cultivated on separate nutrient agar plates and incubated at 30°C for 48 hours. Afterward, a mixture of hydrogen peroxide (0.4%; 30 μ l) and pyrogallol (1%) was added to the grown colonies on the plates. A positive result was indicated by the presence of yellow-brown colored colonies.

Screening of Laccase Producing Isolates. The culture was inoculated onto guaiacol agar plates containing 0.01% guaiacol in CDA (complete agar medium) and

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subsequently incubated at a temperature of 25° C. The growth of the culture was monitored over a period of 2 weeks. A positive reaction was indicated by the formation of a brown zone surrounding the bacterial colony. This positive result signifies the production of the laccase enzyme, which oxidizes guaiacol and leads to the observed brown coloration (Sharma *et al.*, 2017; Dayal *et al.*, 2023).

Isolation of Potential Isolates. To isolate potential strains, positive isolates were selected based on the aforementioned parameters. For the biodegradation test, LDPE films measuring 1.5×1.5 cm were utilized at a concentration of 0.2% (w/v). Prior to the test, the films were dried overnight at 60°C, weighed, disinfected by immersing them in 70% ethanol for 30 minutes, and airdried for 15 minutes in a laminar airflow chamber. The dried and disinfected films, weighing 0.2 g each, were added under aseptic conditions to Erlenmeyer flasks containing 100 ml of sterile mineral salt medium supplemented with 0.01% (w/v) yeast extract. To inoculate the flasks, 1 ml of a 24-hour-old culture grown in a nutrient broth medium was added. The flasks were then incubated on a rotary shaker at 35°C and 120 rpm for duration of 60 days. A sterile control flask without inoculation was included for comparison. After the 60-day incubation period, the extent of LDPE film biodegradation was determined using the weight loss method. The weight loss percentage was calculated using the following formula:

Weight loss (%) = (Initial Weight - Final Weight) / Initial Weight * 100.

RESULT AND DISCUSSION

In response to the urgent environmental challenge posed by plastic pollution, current research has focused on isolating soil bacteria, such as Pseudomonas sp and Bacillus sp that demonstrate remarkable abilities to degrade extensively utilized polymers. Microbial degradation, harnessing of the capabilities microorganisms to break down both organic and inorganic molecules, offers a viable solution to reducing plastic pollution. Soil, being a vital natural resource, serves as a rich reservoir of diverse microorganisms that can contribute to this degradation process.

The isolation of bacteria was conducted on a minimal agar medium, resulting in the obtainment of a total of twenty-two bacterial isolates. Screening of these isolates for their ability to degrade PEG and plastic powder was performed using the clear zone method. Fig. 1 and 2 provides the results of plastic degradation using the soil isolates. Among the 22 isolates tested, seven isolates displayed a distinct clear zone of inhibition on the plate containing PEG (polyethylene glycol), indicating their degradation activity. Additionally, five isolates showed a zone of inhibition on the plate containing plastic powder, suggesting their potential for degrading this type of plastic.



Fig. 1. Isolation of plastic degrading ligninolytic enzyme producing isolates.

Screening of plastic degrading isolates with using PEG



Screening of plastic degrading isolates with using plastic powder



Fig. 2. Isolation of plastic degrading isolates.

Bakht *et al.* (2020) conducted a study where they isolated bacterial strains from soil samples contaminated with plastic waste. The researchers employed media supplemented with polyethylene glycol (PEG) and plastic powder to selectively

determine the plastic-degrading capabilities of these isolates. This approach of isolating bacteria from plastic-dumped soil samples is supported by numerous studies, as it offers several advantages for selecting indigenous bacteria for plastic degradation. These

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advantages include their ability to adapt to the local environment, their specificity in targeting plastic waste, a reduced risk of ecological disruption, a sustainable approach aligned with biodiversity conservation and cost-effectiveness.

The microbial degradation of polyethylene (PE) is primarily attributed to the remarkable enzyme system and intricate metabolic pathways possessed by microorganisms. These microorganisms are capable of secreting extracellular enzymes that can effectively break down complex structures. A significant breakthrough in this field was made by Santo, who demonstrated that enzymes produced bv microorganisms, particularly laccase, can catalyze the cleavage of polyethylene macromolecules, leading to the formation of carbonyl groups and a subsequent reduction in the molecular weight of polyethylene (Santo et al., 2013). Ligninolytic enzymes have emerged as particularly crucial in the degradation process. In a study conducted by (Sowmay et al., 2015)

it was observed that *Trichoderma harzianum*, a fungal species, exhibited degradation activity towards polyethylene due to the presence of laccase and manganese peroxidase. While numerous studies have reported the effectiveness of fungal ligninolytic enzymes in degrading plastic materials, the occurrence of plastic degradation by bacteria producing ligninolytic enzymes is relatively rare.

In the present study, all 22 isolates were subjected to determine the production of ligninolytic enzymes. Among them, 45.4% isolates were positive for laccase production, while 18.1% of were showed positive results for lignin peroxidase. Based on the overall results of the aforementioned parameters, four isolates, namely Ia4, Ia12, Ia19, and Ia22, were considered potential candidates. These isolates exhibited positive reactions for both laccase and lignin peroxidase and, notably, a zone of clearance was observed around their colonies in media containing PEG and plastic powder (Fig. 3).

Isolation of laccase

producing isolates



Clear zone of decolorization around colony indicate the positive for ligninolytic isolates positive for lignin peroxidase positive for ligninolytic enzymes producing bacterial isolates. Brown colored colonies Fig. 3. Isolation of ligninolytic enzymes producing bacterial isolates.

Lopez *et al.* (2006) have established that the application of lignin as a carbon source for the selection of ligninolytic microorganisms is widely recognized as one of the most suitable methods worldwide. However, the ability of the isolates to utilize and degrade lignin is further supported by their capacity to utilize lignin model compounds, such as veratryl and guaiacol alcohols, as well as their demonstrated capability to decolorize industrial dyes. These findings align with previous studies by (Husain 2006; Bandounas *et al.*, 2011; Falade *et al.*, 2017), which also provide evidence of the colonies' potential to effectively utilize and degrade lignin.

However, it is important to note that growth on polymeric lignin or lignin monomers does not necessarily provide a comprehensive measure of the ligninolytic potential. To assess the ligninolytic potential independently from lignin utilization, the decolorization of synthetic lignin-like dyes was studied, as described by Falade *et al.* (2017). The selected organisms in this study demonstrated the ability to effectively decolorize phenol red and methylene blue. It should be noted that while 2 lignin-modifying enzymes (LMEs) have the capacity to decolorize 2 dyes, previous studies (Hooda *et al.*, 2015) have specifically associated Azure B, phenol red and methylene bluedecolorization with high redox potential agents such as lignin peroxidases (LiP). The majority of the ligninolytic isolates exhibited significant potential for degrading lignocellulosic waste. In this study, the biodegradation of a degradable plastic film composed of linear low-density polyethylene with pro-oxidants was investigated using a lignin-degrading isolates. A previous paper by one of the authors demonstrated that the lignin-degrading fungus IZU-154 displayed substantial degradation capabilities towards nylon-66 and nylon-6 membranes under ligninolytic conditions, indicating the potential for polyethylene degradation. Therefore, the present study aimed to assess the degradation of polyethylene (PE) and plastic powder by lignin-degrading bacteria under specific nutritional conditions, while also investigating the enzymes associated with this degradation process. Totally 4 isolates were selected and subjected to analysis the degradation level with weight loss of plastic strip. Among them Ia 19 was resulted in maximum weight loss which was selected as potential degraders for further analysis. The variations in the plastic degradation capabilities of different microorganisms can be attributed to the variations in their respective environmental origins (Ali et al., 2021).

According to the biochemical analysis, the potential isolates Ia19 was identified as Pseudomonas sp. In a study conducted by Jumaah *et al.* (2017), plastic-degrading isolates of *Bacillus sp. and Pseudomonas sp.* were observed in samples from municipality waste and

plastic dumped soil. Since the initial report in 1973 by (Suzuki *et al.*, 1973) which demonstrated the ability of *Pseudomonas sp.* to degrade PVA, a significant number of documented PVA-degrading bacteria have been identified within the *Pseudomonas genus* (Shimao, 2001). Like those authors found the plastic degrading isolates from soil samples, nevertheless very few authors correlated the ligninolytic isolates and it's degrading the plastics. Kavith and Bhuvaneshwari (2021) were found the ligninolytic enzymes of laccase, lignin peroxidase producing *Bacillus* sp., and degraded the polyethylene. Recently, Salinas *et al.* (2023) were degraded the LLDPE with using ligninolytic enzyme producing *Pseudomonas* sp.

CONCLUSION,

The ligninolytic isolates demonstrated the ability to degrade polyethylene glycol (PEG). The ligninolytic isolates, which possess enzymes capable of breaking down lignin, showed promise in breaking down PEG as well. The degradation of PEG by ligninolytic isolates is significant as it offers a potential solution for the remediation of PEG-contaminated environments. The ability of these isolates to break down PEG suggests that they have the potential to be utilized in bioremediation strategies or waste treatment processes involving PEG-contamination. Further research is needed to fully understand the mechanisms by which ligninolytic isolates degrade PEG and optimize the degradation process. Overall, the findings of this study highlight the potential of ligninolytic isolates as a natural and efficient means for the degradation of polyethylene glycol, contributing to the development of eco-friendly solutions for PEG waste management.

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