APPLICATION OF LACCASE PRODUCED BY MARINE ACTINOMYCETES IN ACCELERATING THE RATE OF BIODEGRADATION OF POLYETHYLENE

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ABSTRACT

Although plastic is very useful in modern life, its widespread use could impair human sustainability. Improper plastic waste management generates greenhouse gases and harmful waste. Plastics and its associated by-products, such as microplastics, accumulate on land and in the oceans causing harm to human and ecological health. An environmentally friendly solution, such as enzymes-mediated biocatalytic depolymerization, is required for efficient management of the large amount of waste generated around the world. Actinomycetes are less explored for commercial biodegradation processes but have attracted attention since they constitute a significant proportion of the soil and aquatic flora and because of their ability to degrade complex materials. This study involves identification of laccase-producing marine actinomycetes and examining the possibility of accelerating the rate of biodegradation of polyethylene by treating with laccase enzyme. The polyethylene test material treated with laccase enzyme for 30 days shows 9.36 percent rate of biodegradation, which was six times higher than the rate of biodegradation of an untreated one. The change in the chemical structure of the polyethylene was studied using Fourier transform infrared spectroscopy. After treatment with laccase enzyme, the carbonyl index of the polyethylene test material increased to 1.25 indicating that the polymer was oxidized, and post biodegradation study showed that the carbonyl index decreased to 0.66 which confirms the concept that oxidized polymer was utilized by the microorganisms. The laccase-producing isolate A-09 showed 99 percent identity as *Streptomyces rubiginosus* based on molecular fingerprinting.

Keywords: *actinomycetes, biodegradation, carbonyl index, environmentally friendly solution, laccase enzyme, microplastics, plastic waste, polyethylene, sustainability, Streptomyces*

INTRODUCTION

Plastics are materials artificially synthesized from a wide range of organic polymers, such as polyethylene, PVC, nylon, etc. These materials are widely used around the world and have become indispensable in modern society [1, 2]. The most used plastics in Southeast Asia are polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), and polyethylene (PE). Based on geography, Asia-Pacific had the largest market share of

49.3 % in 2017, with production of more than 50 million tons of polyethylene resins per year. PE is produced in three main forms: low density (LDPE) (< 0.930 g/cm^3) and linear low density (LLDPE) (ca. 0.915 - 0.940 g/cm^3) and high density (HDPE) (ca. 0.940 - 0.965 g/cm^3). HDPE and LDPE are the long chain polymers of ethylene with versatile nature, such as lightweight, inexpensive, durable, energy-efficient, and can be easily processed. Hence, they are widely used in the packaging industries. The LDPE or LLDPE form is preferred for film packaging (cling films, milk carton lining, stretch films, squeezable bottles, food boxes, etc.), cable coating, and electrical insulation. HDPE is blow-moulded to make containers for dustbins, crates, household chemicals such as detergent bottles, food packaging, and shopping bags for dry fruits and spices, for water pipes, and drums for industrial packaging [3 - 7].

Packaging material has a very short service life (typically around 6 months or less) due to which an excessive amount of plastic waste is generated [8]. HDPE generally remains in the environment due to its high durability and after being broken down into small particles by extrinsic factors, it can bioaccumulate into living organisms and the ecosystem. Chemical and physical methods for the disposal of these used plastic materials are very expensive and produce persistent organic pollutants (POP's) known as furans and dioxins. These pollutants have been reported to cause toxic irritant products which result in infertility of soil, prevent the degradation of the other normal substances and reduce the quality of underground water source [9 - 11]. At least 14 million tons of plastic enter the ocean each year, accounting for nearly 80 % of all marine debris found in surface waters to deep-sea sediments. Plastic waste is ingested or entangled by marine creatures, causing serious injury and death. In addition, the incineration of collected plastics causes huge emissions of carbon dioxide that can contribute to global warming and climate change. Environmental concerns over the accumulation of such plastics have led researchers to look for ways to solve the problem [12 - 14].

In order to reduce the bio risks caused by packaging waste, sustainable and cost-effective technologies are needed. Although the market for biodegradable packaging materials is growing, their use has not become popular, and they are not available to a large percentage of society.

Microorganisms that can secrete enzymes for degradation when found on the surface of plastic are usually the cause of plastic biodegradation. In the biodegradation process, microorganisms help the process by secreting PE-degrading enzymes that oxidize or degrade the PE polymer chains into smaller fragments and ultimately utilize these fragment releasing by-products like carbon dioxide and water. These enzymes mainly include laccase, lipase, or dehydrogenase, which attack the polymer substrate. As a result, the polymer is broken down into smaller molecules (such as oligomers, dimers, and monomers), which microbial metabolism eventually converts into carbon dioxide or water [15 - 17].

Compared to the conventional technique, microbe-based processes are more environmentally friendly. After initial adherence to material surfaces, bacteria can create large biofilms and subsequently change the physico-chemical characteristics of polymeric films, such as changes in functional groups, hydrophobicity/hydrophilicity, crystallinity, surface morphology, and molecular weight distribution. After the attachment of microorganism, measurements show that the surfaces of film become more hydrophilic [18].

Terrestrial bacteria produce valuable antibiotics and metabolites amongst which about 99 % are known microbial compounds and these habitats are exhaustively studied. On the other hand, the sea, which covers more than 70 % of Earth's surface, has an incredible diversity of life and makes up more than 95 % of the biosphere. The microbes that survive in these habitats are important because they can survive the extreme conditions present in the ocean, and therefore the metabolites they produce are vital. Recently, it has been proven that the ocean floor is an ecosystem with many

unique forms of actinomycetes. They are active components of marine microbial communities and form stable, persistent populations in various marine ecosystems. Although there are many advantages and applications of microbial enzymes derived from actinomycetes, they have not yet been utilized as biocatalysts to their optimum capacity [19 - 22].

Major research efforts are being made worldwide to develop processes and innovative solutions for the degradation of petroleum-, fossil-, and bio- based polymers to ascertain new environmentally friendly applications and waste control plans. Many researchers have reported the degradation of HDPE by fungal enzymes (e.g., laccase, and peroxidases). Fungal species of Ascomycota (e.g., *Aspergillus*) reportedly degrade HDPE in a liquid medium under laboratory conditions [23 - 24]. Basidiomycota are also widely known to produce extracellular oxidative enzymes together with laccase and peroxidases, and many of those enzymes are said to be able to degrade plastics. Very few studies have been carried out on laccase produced by actinomycetes and their application in plastic waste remediation. Rapid biodegradation is the only environmentally acceptable approach that can tackle the problem of plastic waste management [25 - 26].

Sustainable material choices and innovative product development, such enzyme-mediated waste reduction and biological process for degradation of plastic waste, are the main focus of today`s industry. The aim of this study is to investigate the possibility of increasing the rate of biodegradation of polyethene in natural environmental conditions using laccase enzyme. The laccase enzyme produced by marine actinomycetes will be used for primary treatment of the polyethene before undergoing biodegradation in natural environment.

MATERIALS AND METHODS

Isolation of actinomycete strains

The state of Maharashtra has a 720 km long indented coastline defined by significant estuaries and tiny creeks. Thane, Raigad, Mumbai, Ratnagiri, and Sindhudurg are the coastal districts. Mumbai itself has numerous creeks with close to 71 km^2 of creeks and mangroves along its coastline. These ecosystems are enriched with diverse flora and fauna. For this study, 32 samples of seawater, mangroves and sediment and sand were collected from different geographical locations within Maharashtra. 20 samples were collected in autumn and winter season between November 2018 and February 2019 in Thane & Mumbai district, while 12 samples were collected from Raigad and Ratnagiri district in March 2019. Sediment sand samples were collected using an alcohol-sterilized spatula and stored in zip-lock bags, and water samples were collected and stored in sterile screw-cap bottles. Water samples were stored at 4 ± 1 ˚C and processed within 24 - 48 hours. All the mangroves and sediment and sand samples were air-dried for 24 hours, homogenized using mortar and pestle and sieved through 2 mm sieve to obtain fine soil particles.

Actinomycete Isolation Agar at pH 8 (M490 HiMedia laboratories) was used for the isolation of actinomycetes from the sediment samples. As suggested in various research, in order to minimize the growth of bacteria and fungi, the antibiotics Cycloheximide - 50 µg/ml and Streptomycin 100 µg/ml were added to the medium. Incubation period varied for various strains from 1 to 3 weeks at 35 ± 2 ˚C. Actinomycete colonies were selected according to their morphological characteristics with Labomed Czm 4 binocular zoom stereo microscope. The preliminary identification was based on appearance of hyphae and spore chains by microscopic examination. A potential strain was identified using 16S rRNA sequencing.

Identification of potential strain by phylogenic analysis using 16S rRNA sequencing

Molecular identification of actinomycete cultures was done using 16S rRNA gene sequence. Molecular phylogeny of actinomycete was determined by amplification of the genomic 16S rRNA region. Two primers specific to 16S rRNA region used in this study were 235F and 878R to amplify approx. 643bp sequence of actinomycete 16S rRNA gene. DNA extraction was carried out using HiPurA Bacterial Genomic DNA Purification Kit (MB505 HiMedia laboratories). The DNA isolated from both the cultures was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler (T-Personal 48). After sequencing, the DNA sequences were analysed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI).

Screening for laccase producers

Qualitative laccase plate assay was carried out using basal mineral medium (M1588 HiMedia laboratories) with trace solution and inducers such as 0.05 % guaiacol, 0.02 % 2,5-xylidine dissolved in 95 % ethanol (filtered) and Tween 80 dissolved in 95 % ethanol. 50 % artificial seawater was used as a diluent. Composition of artificial seawater was (g/L): $(NH_4)_2SO_4$ 2.6 g, K_2HPO_4 1g, KH_2PO_4 0.5 g, $MgSO_4$ 0.2 g, yeast extract 1 g, maltose 20 mM, asparagine 2. Asparagine was added as the source of organic nitrogen. A sterile trace solution of 0.1 % v/v was added to the basal medium after autoclaving. Composition of the trace solution was (g/L): $CuSO_4 \cdot 7H_2O$ 0.025, $CaCl_2 \cdot 2H_2O$ 1.5, $ZnSO_4 \cdot 7H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.25, $MnCl_2 \cdot 5H_2O$ 0.2, $COCl_2 \cdot 6H_2O$ 0.05, $MgSO_4 \cdot 7H_2O$ 2.5. Positive laccase activity was indicated by the reddish-brown halo formed around the colony when guaiacol was incorporated in the medium, by an orange pigment when 2,5-xylidine was incorporated in the medium. Laccase activity was determined spectrophotometrically (Perkin Elmer) at 470 nm using 5mM guaiacol as a substrate.

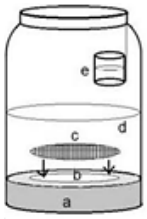
Treatment of test material with laccase producer prior to the biodegradation study

The test material polyethene bag (PE bag) with a thickness of 65 micron was used for the study. The effects of laccase were studied on the tested material by treating it with laccase-producing isolates for 30 days. Disc-shaped test material weighing about 200 mg/L was placed in 250 ml screw-cap glass bottles with 100 ml of mineral medium and laccase-producing isolates of about 10^8 CFU/ml previously induced with 5mM of guaiacol. The test material was then subjected to a biodegradation study. Similarly, the test material without treating with laccase was also subjected to biodegradation study as a control sample for comparative analysis.

Preparation of biodegradation setup according to ISO 19679:2020 standard

The rate of biodegradation of PE material when deposited on marine sand sediments at the interface between seawater and seabed was measured by the CO_2 evolved. The environment condition for the study was diffused light at 28 ± 2 ˚C and the study was conducted for 180 days under these conditions. The inoculum used for the study was natural seawater and sediment sand collected during the daytime at Juhu beach in Mumbai during the autumn season in October 2021. Plate Count Agar (M091 HiMedia laboratories) was used to perform viable count of the inoculum. For the study, the inoculum strength was maintained at 10^8 CFU/ml. A positive control (readily biodegradable cellulose disc) was used to validate the study. A 250 ml reactor flask with holders for placing a 50 ml glass beaker was used for the research (Figure 1). A total 12 reactor flask were used in the study, 3 for positive control, 3 for inoculum control, 3 for test sample treated with laccase and 3 for control samples without any treatment. A 50 ml glass beaker in each reactor flask had a CO_2 absorber (25 ml of 0.0125 $Ba(OH)_2$). The amount of CO_2 produced was determined by titrating the remaining $Ba(OH)_2$ with 0.05 mol/l HCl to a phenolphthalein end point. These titration was performed at regular

interval of 3 to 5 days, and after titration, a freshly prepared solution of $Ba(OH)_2$ was added into the beakers.



a- Marine Sediment
b- Test material to be placed
c- Sample holder
d- Marine water
e-CO_2 trapping $Ba(OH)_2$ solution.

Figure 1. Apparatus for the study of biodegradation

The change in physical structure and properties of the tested material was studied. Also, the possibility of increasing the rate of biodegradation of the tested material treated with laccase was studied. The tensile strength and percent elongation of original test material, after laccase treatment and after biodegradation, were studied using Tinius Olsen H10KS according to ISO 527-1. Perkin Elmer FTIR was used to study the changes in the chemical structure of the examined material (polyethene bag). Microscopic examination of the test sample for surface characteristics was performed using a Zeiss Discovery V8 microscope.

RESULTS AND DISCUSSION

Isolation and screening of laccase producer

Forty-two strains of actinomycetes were isolated from various locations and numbered from A-01 to A-42. Out of the 42 isolates screened for laccase production using different inducers, only 3 isolates showed laccase activity and were graded on the scale from fair to very good, A-09 (very good), A-16 (good) and A-20 (fair) (Figure 2). After 72 hours of incubation, activity was visible on the medium containing the three inducers for all three isolates. Various research confirmed that laccase enzyme can be overexpressed in presence of inducers such as aromatic inducers guaiacol, xylidine, Tween 80 [27].

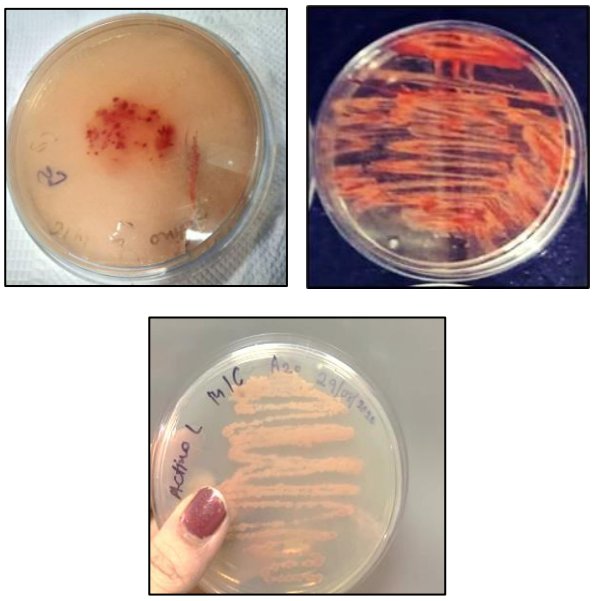


Figure 2. Screening of laccase enzymes by Agar plate assays techniques

Laccase activity of the of A-09 isolate was found to be 11.78 U/ml. Similar results were recorded where laccase enzyme activity of *Streptomyces psammoticus* was 5.1 U/ml and crude laccase enzyme activity from marine *Streptomyces lydicus* was 1.69 U/ml [28].

Identification of potential strain by phylogenic analysis using 16S rRNA sequencing

The morphological characteristics of A-09 isolate colony showed similarity with *Streptomyces* sp. Relative abundance at phylum, class, order, family, genus and species levels was found to be 99.99 % for Acitnobacteria, 99.99 % for Streptomycetaceae family and 99.88 % for *Streptomyces* genus (Figure 3). The laccase-producing isolate A-09 showed 99 % similarity with *Streptomyces rubiginosus* strain with maximum number of hits. This species is known for production of sugarcane molasses for biofuels and is considered a promising source for biofuels production [29].

Production of laccase enzyme from this species has not been recorded. This would be the first report of laccase enzyme from this species in marine environment.

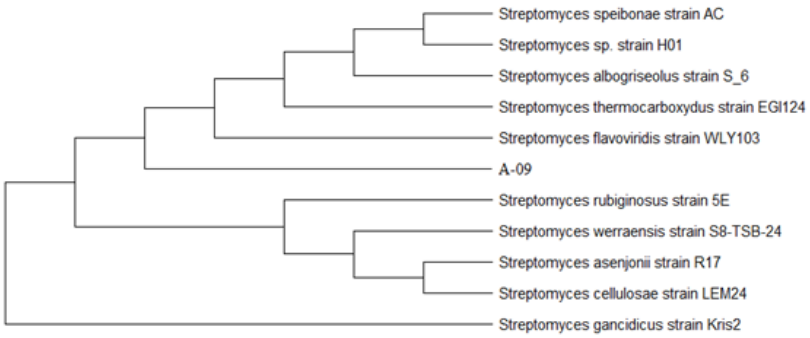


Figure 3. Cladogram of top 50 hits from NCBI 16S rRNA database with consensus sequence of A-09

Effects on physical properties of the polyethene test material

The extent of change in physical properties was studied on the original sample before exposure to enzyme and biodegradation. Tested material with and without laccase enzyme treatment was subjected to biodegradation for 180 days. In the FTIR spectra (Figure 4a and 4b) a shift in the peak at 3200 - 3400 cm^{-1} was observed due to the vibrations in the stretching of the O-H bond in alcohols and phenols. The stretching vibration of carbon-hydrogen (CH) group of the main chain at 2800 - 3000 cm^{-1}, the wagging and rocking vibration of methylene (CH_2) at 1440 - 1490 cm^{-1} stretching and presence of aromatics, bending of CH at 1367 - 1363 cm^{-1} was observed. Absorbance centered at 717 cm^{-1} consistent with methylene (CH_2) rock observed in polyethylene. The absorbance range of 700 – 900 cm^{-1} corresponds to $-C=C-$ stretching and the presence of alkene group.

The carbonyl index of the tested polyethylene material was calculated and shown in Table 1. The carbonyl index of original tested material without any treatment was 0.05 and after treatment with laccase enzyme for 30 days it reached 1.25, which indicates the formation of carbonyls (C=O) and vinyl (CH2=CH) groups, and, finally, changes in the conformation and crystallinity of the polymer.

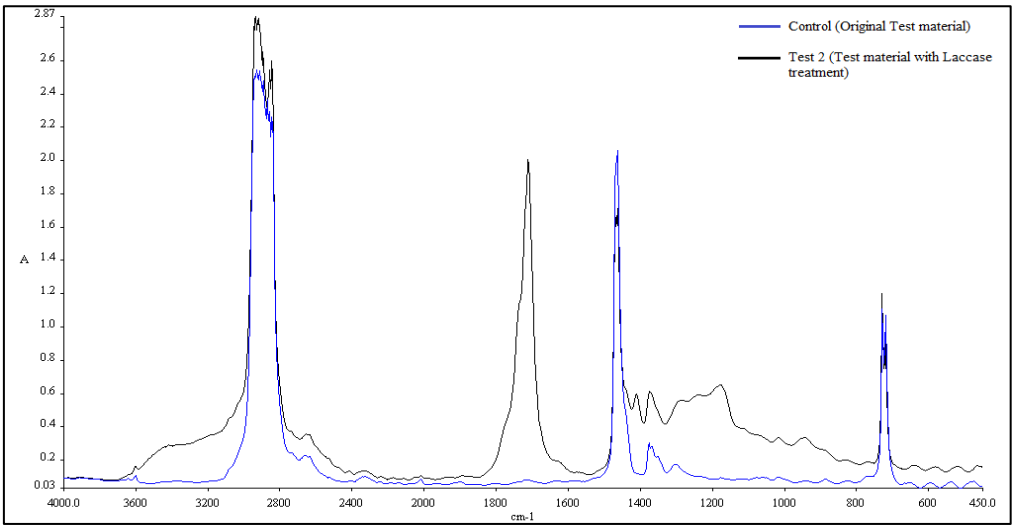
FTIR analysis was observed to help show that laccase-mediated system added new oxygen-containing functional groups, such as -OH, -C=O and C=C [30]. The carbonyl index of the tested material treated with laccase and subjected for 180 days of biodegradation was reduced to 0.66. The decrease in the carbonyl index confirms the concept that microorganisms have utilized the oxidized polymers.

Table 1. Carbonyl index of the tested material

	Control (Original test material)	Test 2 (Test material with laccase treatment for 30 days)	Test 2 (Test material with laccase treatment after 180 days of biodegradation)
Carbonyl index (CI)	0.05	1.25	0.66

Biodegradation of polyethylene test material

The test material with and without laccase enzyme treatment was subjected to aerobic biodegradation for 180 days according to ISO 19679:2020 standard (Figure 5). The reference material (cellulose) was used for inoculum validation and biodegradation of 91.36 was achieved % in 180 days. The test material without laccase treatment directly subjected to biodegradation process in the marine environment showed biodegradation of 1.48 %, while the test material treated with laccase for 30 days before biodegradation showed biodegradation of 9.36 %. The rate of biodegradation after laccase treatment increased 6 times compared to that without treatment.



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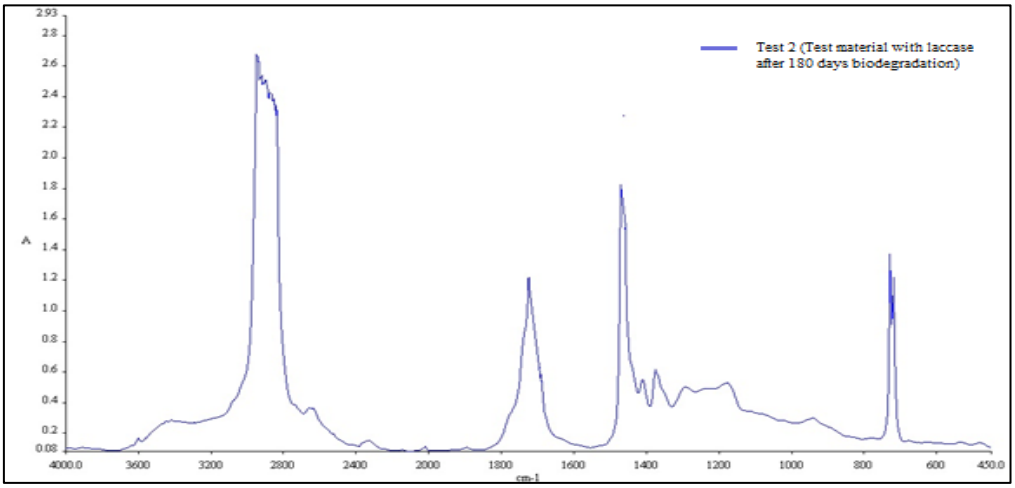


b)

Figure 4. a) FTIR spectra of control and test 2 (test material after laccase treatment), b) test 2 (test material with laccase treatment after180 days of biodegradation)

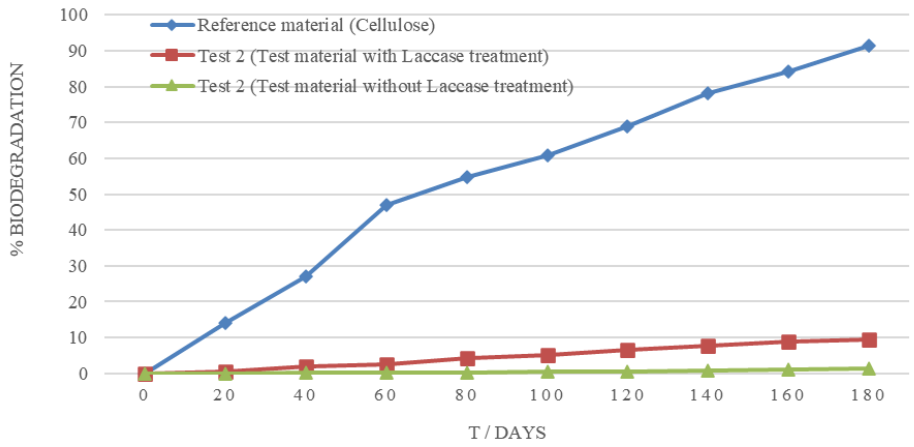


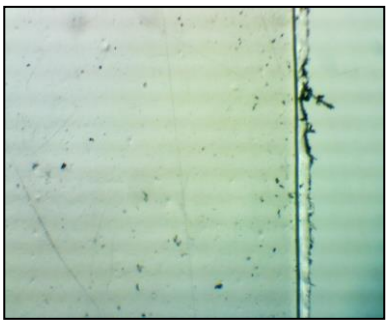
Figure 5. Graphical representation of the biodegradation rate of reference material and test material with and without laccase enzyme treatment

Biodegradation of plastic and polyethylene takes place with the help of enzymes produced by various microorganisms present in the environment. Many studies have been published on the enzymes responsible for degradation of polyethylene. Fungal enzymes from *Aspergillus japonicus* isolated from polyethylene polluted sites around Chennai shows 12 % increase in LDPE degradation rate [31]. The rate of biodegradation of plastic (BP) mulch films PBS and PBSA was increased by 6 % by spray treatment with *Paraphoma*-related cutinase-like enzyme [32]. Polyethylene films exposed to extracellular laccase showed an increase in the carbonyl peak during FTIR examination, indicating that laccase's enzymatic oxidation plays a significant role in the biodegradation of polyethylene [33].

The process of biodegradation had begun on the surface of the tested material, which can be seen by colonization of microbes, cracks and pitting on the tested material after 180 days of exposure (Figure 6).

CONCLUSION

This study shows that microbial enzymatic activity plays a vital role in the degradation of polyethylene. The change in the chemical structure, such as bond scission, chemical transformation, and formation and disappearance of any functional group of PE after enzymatic action was determined with the help of FTIR analysis. As the rate of biodegradation increases over time, the peaks widened as more monomeric and oxidative forms of polyethylene were formed. The PE test material treated with laccase enzyme for 30 days showed a six times higher rate of biodegradation than the untreated one. After 180 days, the laccase-treated PE test material showed a biodegradation of 9.36 %, while untreated one showed only 1.48 % biodegradation. The reference material (cellulose) was used for inoculum validation and achieved 91.36 % biodegradation in 180 days. Biodegradation was high without any photooxidation or chemical treatments, thus eliminating the possibility of formation of microplastics. These findings support the extensive research on polyethylene biodegradation using various microbial strains and enzymes-mediated systems. Therefore, the information obtained from this research serves as evidence that the laccase enzyme plays a major role in improving the biodegradation process of polyethylene and can be further used on large scale to treat plastics waste before it is released into the natural environment. An environmentally friendly solution that could be a sustainable solution for managing plastic waste around the world in the future.



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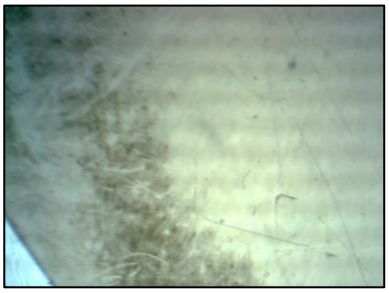

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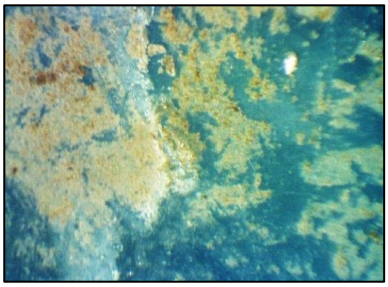

c)

Figure 6. Microscopic examination: a) test material - control sample, b) test material directly subjected to biodegradation, c) test material subjected to laccase-producing isolates for 30 days and then subjected to biodegradation

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