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Mangrove Rhizosphere Microflora in Polythene Biodegradation

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ABSTRACT

Mangrove rhizosphere now days are seen to play a very important role in the degradation of polythene. Polythene – a versatile manmade synthetic material, play a both useful and harmful role in our day to day life. Being non biodegradable it affects the soil as well as the marine organisms. Many of the polythene degrading micro flora including bacteria like *Rhodococcus ruber, Moraxella* sp., *Streptococcus* sp., *Staphylococcus* sp., etc. along with some fungal species, heterotrophic bacteria, gram positive and gram negative bacteria has been identified today. This review mainly focuses on the works done on some of these mangrove rhizosphere microbes and their activities that contributes to polythene degradation which can create a positive impact on our environment.

1. Introduction

Forests of Mangrove are unique and diverse coastal ecosystems located in the tropical and subtropical regions. These forests are both ecologically and economically important. In addition to protecting coastal areas from erosion, mangroves also reduce the impact of natural calamities and serve as critical nurseries for juvenile fishes [1, 2]. Mangroves also provide a unique ecological niche to different types of microbes which help in nutrient recycling and various other environmental activities. These highly potential microbes living in the mangrove soil helps in continuously transforming nutrients from dead mangrove vegetation and converting them into sources of nitrogen, phosphorous etc. [3]. Nitrogen fixation in sediments is likely to be limited by insufficient energy sources, the mangrove rhizosphere sustains high rates of nitrogen fixing activity [4, 5], which may contribute significantly to the health and sustenance of the ecosystem by supplying most of its nitrogen requirements [6, 7, 8].

Mangrove forests thrive near the mouths of large rivers, where deltas provide lots of sediments. Mangrove roots collect sediments and slow the water flow hence preventing soil erosion. Mangrove forests are of global, ecological and economic importance but are also of the world's most threatened ecosystems. This ecosystem plays an important role as refuge, feeding and breeding region for many organisms and help in maintaining an extensive food web based on detritus. This ecosystem also helps in giving nutrients to additional marine ecosystems that includes coral reefs and sea grass communities. The conditions that prevail in the mangrove rhizosphere might be helping in denitrification and also allow the sustenance of a denitrifying population in that particular habitat [9].

Mangrove ecosystem is also found to be rich in bacterial flora. The microbial decomposition of organic matter and nutrient recycling resulted in the fertility of mangrove ecosystems. On comparison it was found that the bacterial population dominated the fungal population in the mangrove soil. The bacteria remained as symbiont with plants and animals, saprophytes on dead organic matter and as parasites on living organisms. Different activities such as photosynthesis, nitrogen fixation, methanogenesis, magnetic behaviour, production of antibiotics, human pathogens and enzymes such as arylsulphatase, L-glutaminase, chitinase, L-asparaginase, cellulase, protease, phosphatase, etc. was found to be performed by bacteria in the mangrove soil. In the degradation of litter and a live woody species of mangrove ecosystem, marine fungal population was found to play a key role. Over 50 % of these total 450 species of obligate marine fungi was found to accommodate the lignicolous marine fungi, whereas about 150 species were found to be exclusively magnicolous fungi [10].

Micro organisms play an important role in biological decomposition of materials which included the synthetic polymers found in the natural environment. This phenomenon is termed as biodegradation. The two types of commonly used synthetic plastics are the low density and high density polyethylene. Both these were found to be slowly degrading in the natural environment, which results in many serious environmental hazards. This has led to the growing interest in the synthetic polymer biodegradation using potential microorganisms. The microbial degradation of solid polymers such as polythene requires the formation of a biofilm on the surface of polymer which enables the microbes to efficiently utilize the non soluble substrate by enzyme activity. The development of multicellular microbial community called biofilm, which on attachment to the surface of synthetic wastes have been found to be an effective degrading agent which showed an increased resistance to antimicrobial agents [11]. Biodegradation is a process in which naturally occurring microorganisms like bacteria, fungi, or algae act on the material. Bio degradable plastics break down completely into non-plastic and non-toxic constituents like water, carbon dioxide, methane and other biological materials [12]. Indeed, there is a good proof to support the hypothesis that polythene fragments produced as a result of photo degradation are found to be more easily degradable [13].

It is, however, estimated that more than over three fourth of India's commercial fishing in the Ganga-Brahmaputra

estuary is mainly dependent on the health of the mangroves [14]. The total area under mangrove along the Indian coast is estimated as 4460 km sq. [15]. The dynamics of mangrove ecosystem is complemented by its soil organisms. All these benthic organisms form an important part of the detritus food chain of coastal area [16].

Polythene or polyethylene is the most widely used plastic which is the most versatile synthetic man made material made of fossil fuel that revolutionized industrial and technological areas of the nineteenth and twentieth century. They are both a "boon and bane" – they are advantageous over other materials as they are light weighted, cost effective, highly durable and relatively unbreakable. At the same time they are disadvantageous as they are non biodegradable and that because of their buoyant nature they possess long term persistence in the marine environment and thereby affect the marine life.

In the recent years, the quest for clean environment has created a great eagerness in finding microbes related techniques of waste disposal which incorporates degradation of hydrocarbons including petroleum, plastic wastes etc. A large number of pollutants and wastes that contains hydrocarbons are found to get deposited to a great extent now days. It has been estimated that approximately $6x10^6$ chemical compounds are being synthesized annually. Almost most 60,000 to 95,000 hydrocarbon containing chemicals are commercially being used. General method for the isolation of microbes from plastic / polythene like materials are done by the collection of polythene samples followed by washing and scrapping them so as to remove the soil particles on their surface. For the isolation of polythene loving bacterial colonies, small pieces of polythene was inoculated on Thornton's agar medium at 28±2ºC for 2 to 7 days till new colonies appeared. These colonies were further streaked on agar medium to get pure culture of polythene loving bacteria [17]. The growing demand and supply of fuel oil and new chemicals by the industrialized society of the twentieth century has placed increasingly higher stress on the natural environment [18]. The damaging effects are due to suffocation and toxicity of the crude oil [19]. Bacteria which exist ubiquitously in the environment have a great potential to degrade these crude oil and other petroleum products [20].

This review mainly focus on some polythene degrading soil microflora of mangrove soil and their polythene degradation activity. Some of the different types of soil micro and mycoflora identified from the mangrove soil included – heterotrophic bacterial species, fungal species such as *Aspergillus* sp.*, Fusarium* sp., *Phanerochaete* sp., and some bacterial species such as *Rhodococcus ruber, Streptomyces* sp., *Bacillus* sp., *Staphylococcal* sp. etc.

2. The polythene degrading activities of the following microbes from the mangrove rhizosphere were referred

Rhodococcus ruber is a genus of aerobic, non motile, non sporulating, gram positive bacteria. A strain of *Rhodococcus ruber* designated C208 was isolated, which was found to use polyethylene as sole carbon source of energy which thereby degraded the polymer [21]. The above isolated strain was found to be highly hydrophobic which thereby enabled the formation of a dense biofilm on the polythene surface and hence enhanced the biodegradation capacity. Branched low density (0.92 g cm^{-3}) polyethylene with an average molecular weight of 191,000 was used. The bacterial culture C208 of *R. ruber* was maintained in nutrient broth (NB) or nutrient agar (NA) media. The synthetic medium (SM) used for polythene degradation assay and formation of biofilm contained the following elements (in distilled water)–1.0 g l^{-1} NH_4NO_3 ; 1.0 g l^{-1} K_2HPO_4; 0.2 g l^{-1} $MgSO_4.7H_2O$; 0.15 g l^{-1} KCL; 0.1 g l^{-1} $CaCl_2.H_2O$ and each of the following microelements: 1.0 mg l^{-1} $FeSO_4.6H_2O$; 1.0 mg l^{-1} $ZnSO_4.7H_2O$ and 1.0 mg l^{-1} $MnSO_4$. The assay followed by the scientists to study the biodegradation of *Rhodococccus ruber* was performed with polythene films (3x3cm) that was dried overnight at 60℃ , which was weighed and disinfected by putting in ethanol for 30 minutes. This polythene films about three samples in each (replicates) were then added to about 100 ml of SM and then this was inoculated with 2ml of the *Rhodococcus ruber* (C 208) culture. Followed by this, the cell density of the inoculii was adjusted to 1.5 x 10^8 colony forming units (CFU) per ml, and was precipitated in a micro centrifuge at 1000 rpm, this was then again washed and resuspended in SM.

For the accurate determination of the dry weight of the residual polythene, the bacterial biofilm was removed from the polythene surface by incubating the samples in flasks containing 2 % (v/v) aqueous sodium dodecyl sulphate solution for 4 hrs. Followed by this the polythene samples were collected in a filter paper, rinsed with distilled water and dried overnight at 60℃ and was then finally weighed. The initial weight of the polymer was taken before in the same above way. It was observed that at the initial stage after 16 days polythene started showing degradation. It was concluded that during prolonged incubation (8 weeks) with C208 strain, 7.5 % of the initial weight of polythene was found to be lost and later completely deterioted. The kinetics of polythene degradation showed a linear pattern due to *Rhodococcus ruber* degradation activity [22].

Heterotrophic Bacteria and Some Fungal Species: Heterotrophic bacteria are those that feed on other bacteria or on other organisms for their food. Fungi, is a member of a large group of eukaryotic organism that includes microorganisms like yeast and molds etc. These are different from the cellulosic cell wall containing plants as they have their cell wall made of chitin. They can be pathogenic, saprophytic, endophytic etc.

Mangrove soil samples from three different sites were collected. These samples were serially diluted and pour plated into sterile nutrient agar and potato dextrose agar for the estimation and isolation of heterotrophic bacteria and fungi respectively and then incubated at 37℃ for 48 hours. After incubation, total bacterial and fungal count was expressed in CFU (per gram of soil). After the isolation and characterization, the bacterial and fungal species were identified till genus level using the Bergey's manual of determinative bacteriology [23] with the help of staining and biochemical reactions such as

gram staining, spore staining, motility, oxidation, fermentation test, Kovac oxidase and catalase test.

The evaluation of the hydrolytic enzymes produced by the heterotrophic bacteria from the mangrove soil was done with the help of 15–20 ml of sterilized (15lb for 15min) media in sterile petri plates of respective media (nutrient agar supplemented with 1% starch, 1% gelatin and 1% tween 80 to get starch agar, gelatin agar and lipid agar respectively). These plates were then surface dried overnight and spot inoculated with isolated bacteria (4 isolates per plates) and then incubated at 37 ℃ for 48 hrs. After this, the starch agar plates were flooded with mercuric chloride solution. Appearance of clear zone around the cultures on starch agar and gelatin agar plates was considered positive for amylase and gelatinase ativity respectively. The change in the opacity of the lipid agar around positive culture was considered positive for lipase activity.

The biodegradation of polythene by heterotrophic bacteria and some fungal species was also done by the scientists by creating a duplicate environment as that of mangrove in the lab with 59cm (length) x 36cm (width) x 2cm (depth) plastic crate. The water level was managed so as to maintain the submerged condition. Three classes of polythene such as class 1 – LDPE, class 2 and class 3 as HDPE based on the melting point curves using differential scanning calorimeter (OSC) were made. Fifteeen pieces of each of the samples were taken along with their mean weight estimated. These samples were removed after 4^{th} and 8^{th} week, and then washed; dried and finally mean weight was recorded.

The experiment revealed that the fungal load in mangrove soil that degraded polythene was comparatively lower than the total heterotrophic bacterial load (in CFU). Also the characterization of the bacterial isolates revealed that *Bacillus* sp. was the dominant genus in mangrove soil followed by *Micrococcus* sp., *Listeria* sp. and *Vibrio* sp. Results also indicated that the class 2 polythene (HDPE) held soil had a comparatively high bacterial load than the other two classes. Finally from the enzyme activity assessment it was revealed that the bacterial isolates from class 2 polythene had a lower gelatinase and amylase activity, at the same time an improvement in lipolytic activity. Simultaneously the isolates from class 1 and 3 had an increased ability to produce gelatinase and amylase enzymes. The final conclusion arrived was that class 3 polythene (HDPE) had undergone higher biodegradation (in terms of %) as compared to other two [24].

Gram Positive Bacteria (*Streptococcus* sp., *Staphylococcus* sp., *Micrococcus* sp.) Gram Negative Bacteria (*Moraxella* sp., Pseudomonas sp.) and fungal species (*Aspergillus glaucus, Aspergillus niger)*: An experimental set up was made to study the polythene degradation under the soil conditions of two different mangrove species *Rhizophora* sp. and *Aviccinia* sp. Seven microbial species were tested in the laboratory for their ability of degrading polythene. The species tested included *Moraxella* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Micrococcus* sp., *Streptococcal* sp. and two fungal sp. *Aspergillus glaucus* and *Aspergillus niger*. These seven different species were allowed to degrade polythene. The polythene was found to be degraded after the inoculation of 6 and 9 months. The biodegradation activity was found higher in *Avicinnia* zones (4.2%) as compared to *Rhizophora* zones (3.77%) after 9 months of analysis. The bacterial strains caused biodegradation of about 2.19 – 20.54 %. Among the five bacterial genera *Pseudomonas* sp. and *Moraxella* sp. was found most active in polythene degradation showing 20.54% of degradation. Among the fungal strains, *Aspergillus glaucus* was found more active by showing polythene degradation of 28.8% as compared to *Aspergillus niger*.

Finally it was concluded from the analysis that the above bacterial and fungal strains caused polythene degradation of 28.8% in a month. The surface of plastic materials turned from smooth to rough with cracking. This may be due to compounds secreted extracellularly by microbes that may break the complex structure of plastics [25].

***Phanerochaete* sp. and *Streptomyces* sp.:** *Phanerochaete* is a genus of fungi, several species in this genus are plant pathogens. This genus includes white rot fungi that are able to degrade lignin to carbon dioxide. *Streptomyces* sp. are soil dwelling bacteria that break down plant biomass and they are among a selected group of micro organisms that accumulate triacylglycerols (precursor of biodiesel).

In this analysis lignin degrading microorganism's ability to degrade polythene with pro oxidant additive and 6% starch was analysed using shake flask method. Enhancement of pro oxidant activity was done by oven drying the plastic at 70 ℃ and given air flow of 0, 4, 8, 12 . . . 20 days. The effect of 2, 4 and 8 week long wave UV irradiation at 365 nm on plastic biodegradability was also found. During shake flask culture method, plastics were chemically disinfected, incubated and then shaken at 125 rpm at 37 ℃ in 6% yeast extract medium (p^H = 7.1) for *Streptomyces* sp. and at 30 ℃ for the fungal species in malt extract medium (p^H = 4.5) for 4 weeks along with uninoculated controls for each treatment. The weight loss data was not fully convincing due to cell mass accumulation.

Prominent reduction was observed in the 4^{th} and 8^{th} day heat treated films by all the three bacteria. Heat treated film incubated with *P. chrysporium* gave an higher percentage of elongation and molecular weight average than the corresponding uninoculated controls, but they were lower than the corresponding controls which were heat treated without 4 weeks incubation, whereas 2 and 4 weeks UV treated films showed higher biodegradation by all the three bacteria, and no fungal degradation was observed [26].

Staphylococcus epidermis: - *Staphylococcus* is a genus of gram positive bacteria. This genus includes at least forty species, of these 9 have 2 sub species each and 1 has 3 sub species. Most of them are harmless and reside normally on the skin and mucous membranes of humans and other organisms. They form a small component of soil microbial flora.

Locally available LDPE films were used for this experimental work. The chemical components required for this included- nutrient medium, inorganic salts such as KH_2PO_4, K_2HPO_4, NH_4NO_3, $MgSO_4.H_2O$, $ZnSO_4.7H_2O$, and

CuSO4.5H2O, $FeSO_4.7H_2O$, and MnSO4.6H2O, solvent benzene, PEG 20000, alcohol and protein molecular weight standards.

The first step done for the study of polythene biodegradation by *Staphylococcus* sp. was the preparation of polymer overplayed and inoculation. The polymer over layer method used was the modified ion of ASTM-D-2676 (American Society for Testing and Materials). In this method, a glass petridish was covered with an LDPE film of 20 micron thickness, which was pretreated with benzene and alcohol so as to remove if so any plasticizers, coloring agents, or filters with a different chemical composition than the polyethylene. These plates were then autoclaved and then enriched nutrient medium comprising of 5% tryptone, 5% NaCl and 1% yeast extract with 2% agar was carefully poured and then covered with another autoclaved petridish. The whole process was done in a laminar air flow chamber so as to maintain sterility. 50µl bacterial culture was then spreaded on the polythene films and then incubated at 37 ℃ for 2-3 days in an inverted condition.

Followed by this was the preparation of PEG and Polyethylene Based Liquid Culture. Minimal nutrient medium composed of inorganic salts supplemented with 2% 20 k Da molecular weight PEG 20000 was prepared and then filtered (nutrient medium). The same inorganic salt minimal medium was then supplemented with 2% of autoclaved shredded polyethylene (nutrient medium) which acted as a sole carbon source. Overnight liquid culture of *S.epidermis* (BP/SU1) in the enriched medium [27] was centrifuged at 500 rpm for 5 minutes to remove the nutrient medium. The centrifuged cell suspended in the same volume of sterile water was then added in the ratio 1:10 (v/v) in the shredded polyethylene based liquid culture and incubated at 39 ℃ at 180 rpm under shaking condition. Microscopic characterization was done using the scanning electron microscope.

Testing the viability Of BP/SU1 in the Polyethylene-based liquid culture: After the interval of 15 days of incubation at 37 ℃, 50µl culture was removed and then spreaded on an enriched medium agar plate overnight at 37℃. After 18 hours, the plates were observed for BP/SU1 colony formation. The purity of the culture was ascertained through biochemical testing and characteristic morphology determination [28]. 50µl O/N liquid culture of BP/SU1 in enriched media which has been supplemented with 2% glucose was spread on the polymer overlaid nutrient agar medium and incubated at 37℃ . The same had been repeated in the case of 50 ul O/N liquid culture of *E. coli*. After18 hours, growth was observed only BP/SU1 plate alone, the *E. coli* inoculated polyethylene overlaid plates did not show any bacterial growth. Hence it was concluded that commercially available LDPE packaging material can be degraded by BP/SU1 strain of *S. epidermis* found in mangrove soil [29].

Many other works also have been carried out on mangrove rhizosphere by different environmentalists. In some cases for example The Great Nicobar islands, was investigated for the presence of arbuscular mycorrhizal fungus, phosphate solubilising bacteria (PSB) etc. The percentage of AMF colonization in mangrove rhizoshere was estimated to be between 0-17 percent. The presence of AMF in the aerenchymatous corex revealed that the mangroves helped the existence of AMF by giving them the provision of oxygen supply. The PSB also plays an important role in mobilizing insoluble phosphate [30]. Other plastic degradation works are also being studied which resulted in the finding that the plastic degrading activity of *Aspergillus oryzae* found in the mangrove rhizosphere was due to the cutinase enzyme, as the fungus could grow fairly well under culture conditions that contained emulsified poly (butylene succinate co adipate) PBSA and poly (butylene succinate) PBS as a sole carbon source and could digest them easily [31]. Polythene degradation is highly influenced by the ligninolytic activity of lignin degrading fungi. It is also estimated that MnP (manganese peroxidase) is the key enzyme that activate polythene degradation as in case of lignin degrading fungi [32]. Eventhough hydrocarbons undergo processes like photolysis, chemical oxidation, volatilization and other physical and chemical processes, microbial degradation is a major process affecting their fate in the environment [33, 34]. Some of the hydrocarbon pollutants serve as the carbon and energy source for micro organisms, whereas others might disturb the microbial population depending on the pollutants, and their concentrations present in the sediment.

The hydrocarbon pollutants like polythene in the sediments have both useful and harmful function on the microbial population, also the presence of hydrocarbon degrading microbes can effect the concentration of the hydrocarbon present in the sediment [35]. The high concentration of hydrocarbons such as (PAH– Poly cyclic aromatic hydrocarbon) in the mangrove soil indicates the bioaccumulation of pollutants in the plant tissue [36]. Mangroves form the highly productive ecosystem next to the coral reefs and provide energy to marine habitats through production and decomposition of detritus materials [37]. Not only this but also from the above results made from this review it has been cleared that the mangrove ecosystem plays an important role in polythene degradation to the maximum extent. The mangrove soil maintains moisture by tidal water flood during high tide and the soil gets heated during low tide when exposed to exothermic reaction of biological compounds in the soil [38]. The polythene degradation by microbes might be due to the compounds secreted extracellularly by these microbes that may break the complex molecular structure of plastics [39]. The degradation of different polymers by micro organisms under laboratory conditions was supported by different workers in this field [40].

Biodegradation of polythene is found to occur by two mechanisms– 1) Hydro biodegradation and 2) Oxo biodegradation [41]. Both of these mechanisms occurs due to the modification of the two additives, starch and pro oxidant, that are used in the synthesis of biodegradable polyethylene. Polyethylene blended with starch has a continuous starch phase that makes it hydrophilic and hence gets catalyzed by amylase activity. Oxo biodegradation makes use of two methods such as UV degradation that uses UV light to degrade (photo degradation) and oxidation, which utilizes time and heat for the breakdown of plastic. Both these methods reduce the molecular weight of plastic and allow it to be degraded [42].

3. Conclusions and Applications

As all the above analysis referred helped us in understanding the polythene degradation properties of mangrove rhizosphere, it can be concluded that polythene materials would be degraded by the mangrove soil irrespective of the mangrove zones. This reveals that the mangrove soil can be a good source of factors responsible for the degradation of polythene materials. The factors included microbes besides moisture, heat, etc. Besides these abiotic conditions, microbial counts are also high favouring the degradation of polythene.

Even though from the above mentioned references we could find that some fungal species, *Rhodococcum ruber,* gram negative bacteria, *Streptomyces* sp. mainly helps in the polythene degradation in the mangrove soil, yet the mechanism of degradation of polythene is not fully known. Several workers had reported that the enzymatic activity of the microorganisms was responsible for the degradation of polythene and several biodegradable plastics. Biodegradable plastic is an innovative means of solving the plastic disposal problem from the standpoint of development of new materials. For a cleaner environment, a massive awareness camp and knowledge about the dangerous impact of introducing hydrocarbon containing compounds, plastics etc. into our environment should be made available to the public by the government. The Government and the individuals should join hands for the recycling and degradation of plastics / polythene for a good eco friendly livelihood of our present as well as future generations.

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