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Screening and Isolation of Polypropylene Degrading Fungi from Waste Dumping Site, Kolhapur, India

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ABSTRACT

Polypropylene (PP) and other plastic wastes are found to accumulate in the environment, creating significant ecological issues. They are determined to be considered non-biodegradable, It has been established that once it enters the environment, it stays there permanently. The present investigation aims to biodegrade PP without physical treatment and exposing it to UV light and sunlight exposed to potential fungi isolated from the soil of solid waste dumping site based on 18SrRNA analysis and the isolated strains were identified as 98.54% similar to *Cladosporium* sp. The fungal strain was submitted with Gene Bank accession number ON024632 and registered as a *Cladosporium halotolerans* strain SUK PRAKASH. The degradation was performed for 8 months of incubation in the aqueous medium. The biodegradation of polypropylene FTIR spectroscopy was performed to further examine the sheets, and the results indicated that perhaps the bonds between the sheets were weakening and breaking. The biodegraded samples of without treated PP sheets, UV-exposed PP sheets, and sunlight-exposed PP sheets exhibit weight loss of 4.2%, 6.1%, and 8.6% respectively.

INTRODUCTION

Polymers made of monomers bound by chemical bonds are called plastics (Vignesh et al. 2016). In the previous several years, plastic output on a global scale has surpassed 330 million tonnes. Polypropylene (PP) is the material most in demand by the plastic converter sector out of all polymers. Because of its excellent processing capabilities and adaptability, PP is one of the most widely used and consumed polymers globally (Samper et al. 2018). It is used for numerous applications like commodities, medical applications, automotive, packaging plastics and packaging products, etc. (Samper et al. 2018). Nowadays, plastic is the major constituent of domestic solid waste and it is approximately 30-40% of the total volume of solid waste. When this polymeric substance is discarded, it is exposed to microorganisms for biodegradation, which leaves a deposit in the soil and landfills. Polymeric discard is a serious issue (Matos & Schalch, 2007). Plastics are strong, long-lasting, moisture-resistant carbon polymers with hydrogen, nitrogen, sulfur, and other organic and inorganic components produced from fossil fuel, a non-renewable resource. They are also lightweight, robust, and non-biodegradable (Kamble 2015), (Kumari et al. 2013).

Plastics have limitations since they don't biodegrade easily, which causes pollution and adverse effects on the environment. These resistive polymers strengthen and adsorb, which promotes the spread of unwanted and unwanted organisms (Ghosh et al. 2019). Furthermore, the harmful consequences involve swallowing by animals due to mistaken food which leads to entanglement (Yoshida et al. 2016). Therefore, there have been multiple attempts to minimize plastic waste. Plastic can take several centuries for natural degradation in the environment. There are several methods for addressing both physical and chemical degradation, including UV therapy, physical stress, oxidant methanolysis, hydrolysis, etc.

Microorganisms break down plastic by enzymatic activities that convert polymers into monomers and oligomers, followed by metabolic activity by microbial cells (Starnecker & Menner 1996). Microbes like bacteria, fungi, and actinomycetes deteriorate both synthetic and natural plastics (Ghosh et al. 2013). Using microbial enzymes, oxidation or hydrolysis of plastic resulted in chain cleavage of large compound polymers into small molecular monomers,

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which is the metabolic process that causes microbial degradation of plastic (Hugenholtz et al. 1998).

The purpose of the current study should be to identify and describe the high potential Polypropylene degraders using isolating microorganisms from soil and screening them. Through the serial dilution technique, soil samples from several areas where waste has been placed are used to accomplish the isolation. To find prospective PP degraders, the Clear Zone technique, an In-Vitro Biodegradation assay, and the calculation of the weight of PP were all utilized sequentially. The isolated strain was identified as the *Cladosporium halotolerans* strain based on an 18S rRNA study. PP deterioration was observed during an 8-month incubation period in an aqueous medium. Changes in PP weight and FTIR spectroscopy provided proof of the degradation.

MATERIALS AND METHODS

Soil Sampling: Soil samples were collected from solid waste dumping sites. The soil layer was dug up to 1 foot and collected soil samples in sample containers using a spatula. These sites are rich with microorganisms that can degrade plastic. Two sites were selected for soil collection, viz. Kasba Bawda and Jayanti Nala from Kolhapur City, Maharashtra, India.

Polypropylene: Polypropylene is a synthetic polymer synthesized from a hydrocarbon source and is synthesized by the polymerization of propylene. Among the various types of polypropylene Isotactic polypropylene was selected for the biodegradation study as these are used on a large scale in day-to-day life. PP sheets which have a thickness of 1 mm. and a size of 500×500 mm. were purchased from Sigma-Aldrich.

Preparation of enrichment culture: 1 g of soil from each site was put into a 500 mL conical flask containing 100 mL of synthetic medium (SM) that was sterilized. The preparation of the enrichment medium involved adding 0.1g of PP powder to it as the sole source of carbon. The prepared sets of flasks were incubated for a further week at room temperature on the shaker at 120 rpm.

Serial dilution method: One mL of enrichment culture was placed into a conical flask that contains 99 mL of saline. The mixture was serially diluted after shaking.

Isolation of PP degrading microorganisms: PP powder served as the primary source of carbon during the initial isolation of microorganisms using solid media (synthetic minimum medium-agar). Plating out on culture media and serial dilution (1:2, 1:5, 1:10) were performed to final enrichment cultures (FECs) (Esmaeili et al. 2013) for grams per liter was: NH_4NO_3 - 1g/L, $MgSO_4.7H_2O$ - 0.2g/L, K_2HPO_4 - 1 g/L, $CaCl_2.2H_2O$ - 0.1 g/L, KCl - 0.15 g/L, Yeast extract - 0.1 g/L and agar 8.0 g/L. Culture media was autoclaved at 121ºC for 15 minutes. After sterilization, the media was cool and then poured into a sterilized Petri plate. 0.2 mL of each dilution's suspension was streaked out on culture media. These plates were kept for 10 days at 37°C for incubation. After 10 days, the isolated colony, the number of colonies, and the characteristics of the colonies were observed (Burd 2008).

Purification of microbial strain: Colony was picked up by wire loop, re-suspended in 0.2 mL of 0.85% NaCl and streaked onto the same culture under sterile conditions. This suspension was streaked onto potato dextrose agar (PDA) for fungi and nutrient agar (NA) for bacteria to separate the bacterial and fungal strains. Plates were incubated for 36 hours and growth was observed. The subculturing was carried out to get a single isolated colony. As a result, a single colony was found and then suspended again on culture media with PP as the only supply of carbon (Burd, 2008).

The isolates were purified by streaking a pure culture on solid media, depending on the variations in morphology/ colony characteristics. These isolated strains were preserved on PDA slants at 4ºC for further identification and biodegradation study.

Identification of isolate 1: DNA was extracted and its purity was assessed on the 1.0 % agarose Gel, A single band of DNA with such a high molecular weight was shown. Fragments of the Internal Transcribed Spacer (ITS) region were amplified by PCR. When the PCR amplicon was resolved on agarose gel, only a single discrete band, approximately 700 bp, was observed. To remove contaminants, the PCR amplicon undergoes purification (Mandragutti et al. 2021). ITS1 and ITS4 primers using a BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer performed forward and reverse DNA sequencing reaction of PCR amplicon (Gudikandula et al. 2017). From the forward and reverse sequencing data, aligner software was used to create the consensus sequence of the PCR amplicon (Majeed et al. 2019). The ITS region sequence was used to perform BLAST with the database of NCBI Gene Bank. The first ten sequences were selected and aligned using Clustal W's multiple alignment software based on the maximum identity score (https://www.barcodebiosciences.com/genomics -services/microbial-identification/). Using MEGA 7, the distance matrix was generated, and the phylogenetic tree was constructed (Nanda, 2020).

***In-vitro* biodegradation assay:** The biodegradation of PP was investigated by exposing polypropylene sheets to a submerged cultivation process. In conical flasks, degradation using microorganisms was performed on shaker conditions.

There was 100 mL of the synthetic medium and PP sheets in each flask. Aliquots (100 mL) of synthetic media were added into a 500 mL conical flask and sanitized in an autoclave for 20 minutes at 121°C. After sanitizing aliquots (100 mL) of the synthetic medium in an autoclave for 20 minutes at 121°C. although it cooled, the medium was inoculated with 5 mL of fungal spore suspension made by suspending spores from the SM-agar plate in 20 mL sterile water. On a rotary shaker rotating at 110 rpm, the fermentation broth was kept for incubation. Sets were incubated for 8 months to evaluate PP degradation. The sole carbon in the medium originated from PP sheets.

Without inoculating any microbial strain, the control was preserved. The two sets were maintained in 3 groups sunlight-exposed PP sheets, UV-exposed PP Sheets, and PP sheets without treatment.

Determination of weight loss of polypropylene: To facilitate accurate measurement of weightless PP, biofilms were washed off the PP surface with ethanol.

$$\text{Weight loss} = Wi - Wf$$

$$\text{Weight loss \%} = \frac{Wi}{Wf} \times 100$$

Where, Wi - Initial weight

Wf - Final Weight

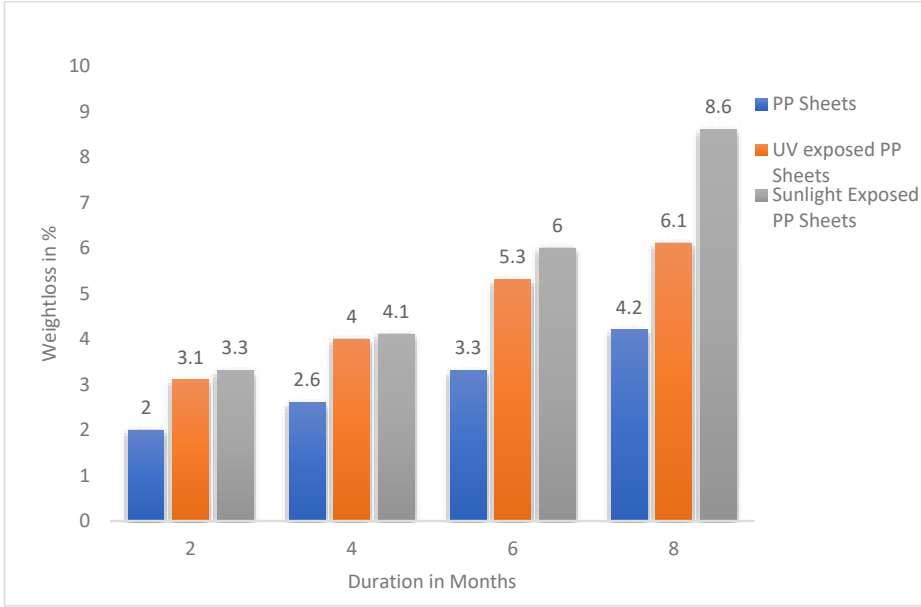
Characterization of identified isolate: Isolated pure fungal strains were sent to Eurofins Genomics India Private Limited, Banglore for identification of microbial strains by 18S rRNA technique. FTIR spectroscopy was used to investigate whether PP was degrading biologically. The data were recorded in the spectral range 4000-500 cm^{-1}.

RESULTS

Identification of Isolate-1: At Eurofins Genomics India Private Limited, Bangalore Isolate-1 was identified using

Table 1: Weight loss (%) of PP sheets, UV exposed PP sheets, Sunlight exposed PP sheets incubated with *Cladosporium halotolerans* strain SUK PRAKASH.

Sr. No.	Time (Month)	Weight Loss (%) on treated with Isolate-1		
		PP sheets	UV-exposed PP sheets	Sunlight-exposed PP sheets
1	0	0	0	0
2	2	2.0	3.1	3.3
3	4	2.6	4.0	4.1
4	6	3.3	5.3	6.0
5	8	4.2	6.1	8.6



Graph 1: Weight loss (%) of PP sheets, UV exposed PP sheets, Sunlight exposed PP sheets incubated with *Cladosporium halotolerans* strain SUK PRAKASH.

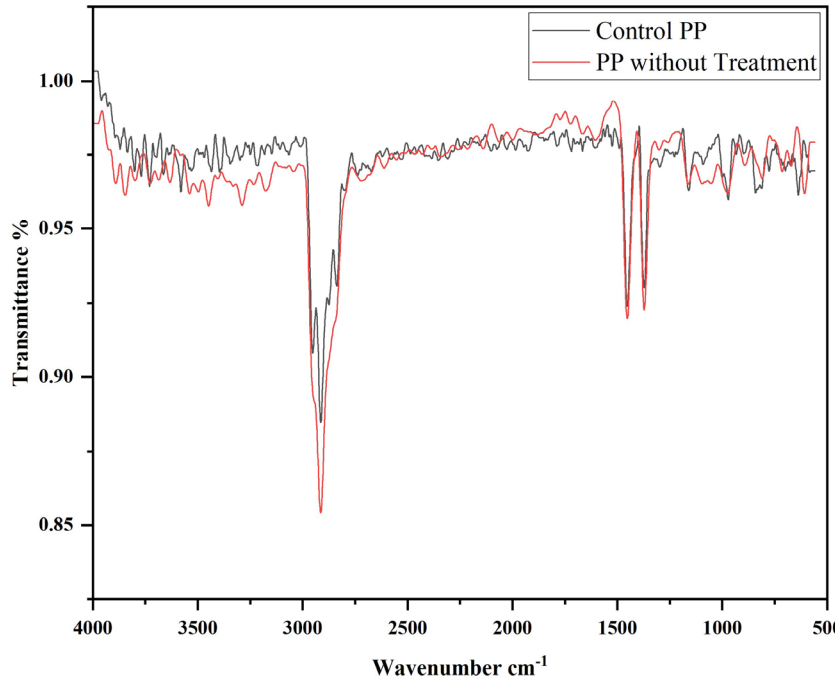


Fig. 1: FTIR spectrum of control PP Vs PP without treatment for the range 4000-550 cm^{-1}.

18SrRNA gene sequencing. With a maximum identification of 98.54% in the *Cladosporium sp*. phylogenetic tree, the identification report of Isolate-1 by 18SrRNA gene sequencing approach indicates the closest phylogenetic affiliation to *Cladosporium halotolerans* 18S ribosomal RNA gene partial sequence. The fungal strain, designated as *Cladosporium halotolerans* strain SUK PRAKASH, was deposited with Gene Bank accession number ON024632.

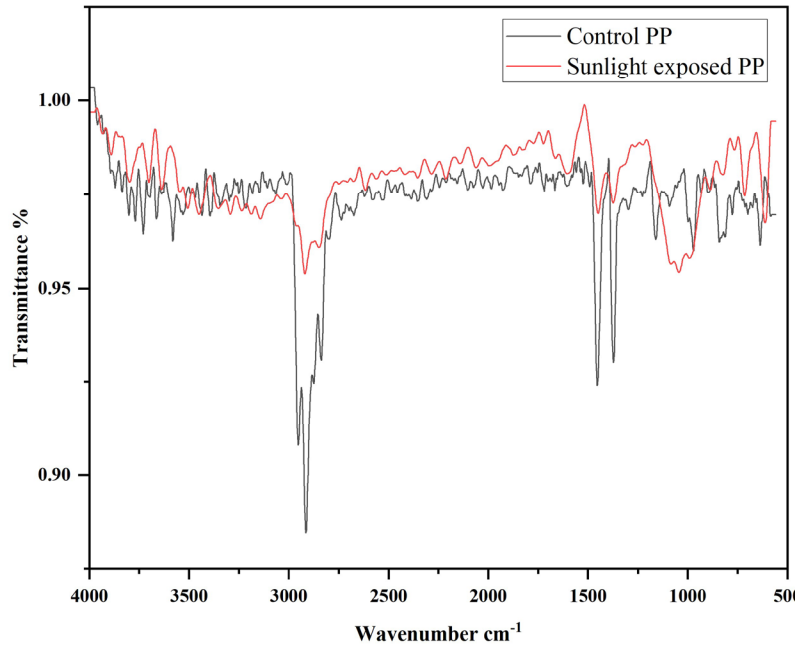


Fig. 2: FTIR spectrum of control PP Vs sunlight-exposed PP for the range 4000-550 cm^{-1}.

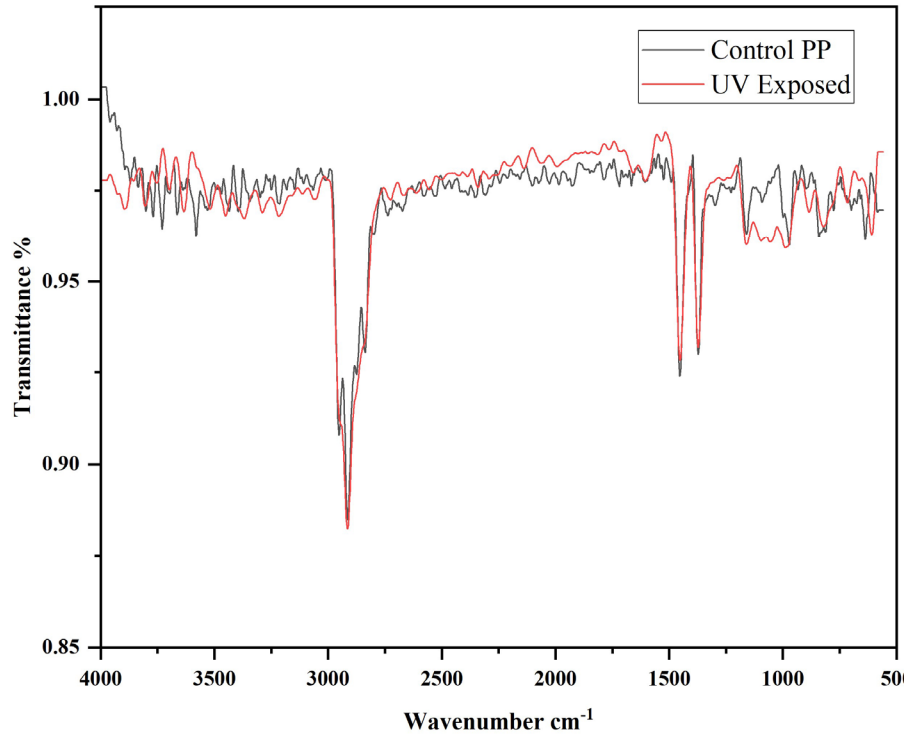


Fig. 3: FTIR spectrum of control PP Vs UV-exposed PP for the range 4000-550 cm^{-1}.

Evaluation of weight loss: The polypropylene sheets were treated with *Cladosporium halotolerans* strain SUK PRAKASH and showed weight loss after 8 months. The highest weight loss is in sunlight-exposed PP sheets i.e. 8.6%, UV-exposed PP sheets, and without treated PP sheets showed weight loss of 6.1%, and 4.2% respectively (Table 1, Graph 1)).

FTIR analysis: Many biodegradation studies employ FTIR spectroscopy as an analytical technique. It is a useful instrument to examine the development of new functional groups and the disintegration of existing functional groups (Das 2015). The presence of additives like antioxidants, as well as degradation products, chemical moieties attached to polymer molecules like branches, co-monomers, and unsaturation, may all be detected using this approach. When the test materials were incubated with microorganisms, such as untreated PP sheets, UV-exposed PP sheets, and sunlight-exposed PP sheets, there was a variance in the intensity of bands in the different locations. For the control spectrum, the specific absorption bands were allocated at 2951cm^{-1}, 2837 cm^{-1} (C-H stretch), 1491cm^{-1} (phenol ring), 1371 cm^{-1} (O-H bend), 1159 cm^{-1} (alkyl amine) and 971 cm^{-1}, 897 cm^{-1} (C-H bend mono) (Fig. 1, Fig. 2, Fig. 3).

DISCUSSION

Weight loss is a simple and rapid approach to assess the biodegradation of polymers. Since biodegradation often begins at the polymer's surface, an increase in weight caused by growing microorganisms within the polymer is related to accumulation, whereas a loss is proportional to the surface area. The pretreatment technique typically consists of physicochemical oxidation with a time-consuming, intricate UV exposure process (Jeon et al. 2021). The highest weight loss is in sunlight-exposed PP sheets i.e. 8.6%, UV-exposed PP sheets, and without treated PP sheets showed weight loss of 6.1%, and 4.2% respectively. The pre-treated PP with UV and sunlight has increased biodegradation by facilitating microbial growth than without treated PP. The constraints of the plastic biodegradation process might therefore be addressed by using microorganisms or enzymes that have inadequate biodegradation activity without pretreatment (Jeon et al. 2021). Ahmed & Swrgiary (2021) isolated *Enterococcus cloacae* as a PE degrader for 30 days of incubation weight loss was recorded at 59.02%. As the sample was untreated the microbial colonization requires time to initiate the consuming LDPE (Sudhakar et al. 2008). In biological systems, large molecules are broken down through oxidation and hydrolysis. It is essential that there

are hydrolyzable and oxidizable functional groups along the polymer chain for biodegradation. For the degradation of polyethylene, findings of *Cladosporium, Fusarium, Aspergillus, Penicillium*, and *Phanerochaete* have been highlighted (Munuru et al. 2022). *Pseudomonas* are among the most sought-after bioremediation agents for hydrophobic polymers, according to Wilkes & Aristilde (2017) review of the genus. This is because of their distinctive cell-surface attachment, numerous catalytic enzymes, and extensive metabolic pathways customized for plastic polymers (Habib et al. 2020). *Pseudomonas* and *Vibrio* bacteria as well as the fungus *Aspergillus niger* have been identified to degrade PP (Cacciari et al. 1993 & Mohanan et al. 2020). Mukherjee & Chatterjee (2014) have observed 32% weight loss by *Bacillus weihenstephanensis* for the degradation of thick plastics after six months of incubation. Chonde et al. (2012) noticed comparable weight loss patterns of Nylon 6 sheets on fungus *Phanerochaete chrysosporium* NCIM 1073 incubation and observed the weight loss of Nylon 6 sheets reduced from 0.013 gm to 0.006 gm after 75 days. Ingavale et al. (2018) observed similar results with LDPE and HDPE biodegradation with *Bacillus weihenstephanensis* for 6 months of incubation and recorded weight loss was 7.02% and 7.08% respectively. Pretreated PP has been used in most of the research. The preliminary treatments involved UV-irradiation (Kaczmarek et al. 2005), -irradiation (Iwamoto & Tokiwa 1994), or thermal treatment (Ramis et al. 2004) and have been shown to reduce the polymer's hydrophobicity or add groups like C=O or -OH, which are more susceptible to degradation (Mohanan et al. 2020). During the biodegradation process, new groups (carboxyl and hydroxyl) are formed as well as a decrease in viscosity (Iwamoto & Tokiwa 1994, Sameh et al. 2006). Significant and similar changes were found in all incubated samples the peaks at 2951 cm^{-1}, 2837 cm^{-1} correspond to C-H stretch, 1491 cm^{-1} corresponds to phenol ring and 1371 cm^{-1} corresponds to O-H bond was slightly weakened in PP without treatment but in the sunlight exposed PP and UV exposed PP observed peak were close to disappearing. After incubation for 8 months, some peaks disappeared and a few new peaks are formed which indicates depolymerization occur or starts in the polymer. Peaks at 1720-1730 cm^{-1} and 1640 cm^{-1} areas might be caused by carboxylic ester, aldehyde, ketone, or even double bond groups (Mahlberg et al. 1998). According to Arkatkar et al. (2010), the oxidation of the polymer is indicated by the development of keto carbonyl and ester carbonyl groups. The biodegradation process is initiated by the microorganisms submerged in the landfill using sample oxidation (1723 cm^{-1}) to form carbonyl groups.

CONCLUSION

PP degradation by oxidation or microbial enzymes' principal method of hydrolysis activity for the biodegradation of high molecular weight polymers to produce functional groups that can significantly enhance their hydrophilicity. The FTIR technique estimates the change in transmittance of the native bonds found in PP, which serves as an indication of biodegradation. For the economically and environmentally favorable degradation of PP, many more effective laboratory studies with PP-degrading microbes are needed to explore. Biodegradation of PP without any pretreatment or addition of chemicals to tackle the problem of commercially available plastic.

ABBREVIATIONS

Polypropylene (PP), Fourier transform spectroscopy (FTIR), Final enrichment culture (FEC), Internal Transcribed Spacer (ITS)

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