Biodegradation of polypropylene films by *Bacillus paralicheniformis* and *Lysinibacillus fusiformis* isolated from municipality solid waste contaminated soil

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Abstract
The fossil fuel or petroleum derived plastics are applied in our routine life because of their easy availability. Distribution and contamination of the plastics in the landfills are the major reasons for these biodegradation study. This current study reveals the biodegradation of polypropylene films and the growth of Bacillus paralicheniformis and Lysinibacillus fusiformis isolated from plastic contaminated soil collected from municipality solid waste management site.

The degradation rate of PP films was confirmed by the results of biodegradation analysis. The growth of Bacillus paralicheniformis and Lysinibacillus fusiformis had shown OD values at 600nm after the degradation period of 4 weeks increasing from 0.131 to 0.334 and 0.148 to 0.213 respectively. The viable cell count increased from 8×10^4cells/ml to 12×10^4cells/ml and 10.1×10^4 cells/ml to 15.2×10^4 cells/ml respectively. The physical and chemical changes of PP films were confirmed by FT-IR and XRD analysis. These analysis confirmed that the bacterial strains have the ability to change the chemical and physical nature of PP films and can utilize the PP films as sole carbon source.

Keywords: Polypropylene films, *Bacillus paralicheniformis*, *Lysinibacillus fusiformis*, Biodegradation.

Introduction

The synthetic plastics with beneficial characters and properties such as long lasting, elasticity, cheap production cost have been produced industrially over the past centuries and broad connections between customers.[20] Most of the plastic products are extremely accumulating in the environment due to the low activity of degradation character of microbes.[16] As of 2015 approximately 6300 million metric tons (MMTs) of plastic waste has been generated. From the total amount of wastes, 12% of plastic waste was incinerated, 7% was recycled and remaining 71% of wastes was accumulated in landfills and oceans.[8] 17 groundwater samples were collected from wells and springs, contaminated by micro plastic particles with minimum concentration of 15.2 particles per liter.[11] PP micro plastics were extracted from costal environments and digestive track of marine organisms[17] and also the micro plastics are affecting the natural ability of mussels to attach themselves to their surroundings.[10]

Polypropylene is one of the synthetic plastics. It is a thermoplastic polymer made up of carbon-carbon backbone chain.[7] Due to its good mechanical properties, the polypropylene is being applied for various purposes such as use and throw materials to life time applications and also in food packaging, textiles, lab equipment and automotive components.[12] After polyethylene, polypropylene is the second most produced and used plastic polymer globally.[13] The microorganisms such as bacteria and fungi can degrade the polymer by colonizing and adherence on the surface of the plastics and forms biofilm. They degrade by excretion of some extra cellular enzymes that can breakdown the polymers.[18]

However, novel strains such as *Aneurinibacillus aneurinilyticus*, *Brevibacillus sp*, *Brevibacillus brevis*, shows the highest weight reduction percentage for LDPE, HDPE and PP films treated with these consortia were 58.21±2, 46.6±3 and 56.3±2% respectively and pellets treated with consortia were determined to be 45.7±3, 37.2±3 and 44.2±3% respectively.[21]

Therefore, this current study was designed for the growth and degrading ability of bacteria (*Bacillus sp*. and *Lysinibacillus sp*) isolated from municipality solid waste management site for the degradation of polypropylene films.

Material and Methods

Preparation of polypropylene films and powder: The polypropylene films were bought from Rishaba Poly Packs, Coimbatore, Tamil Nadu, India. These polypropylene films were cut into small pieces and melted with xylene by heating for 3-4 min in microwave oven. The residue was grained by using mortar and pestle. The obtained powder was washed with ethanol to remove the traces of xylene and allowed to dry in hot air oven at 60^0C for overnight. These polypropylene films and powder were used as carbon source for the enrichment and isolation of polypropylene degrading bacteria.[5]

Sample collection, enrichment and isolation of bacteria: The plastic contaminated soil samples were collected from the municipality solid waste management site, Vellalore, Coimbatore, Tamil Nadu, India. The samples were collected

from different depths in the sediments at 1-5cm and 5-10cm and were named as sample 1 and sample 2 respectively. The soil samples were enriched to increase the activity of plastic degradation. The enrichment went for fifteen days in the plastic films containing Bushnell Hass broth. The enrichment medium was maintained at static condition in incubator for the biofilm formation. The bacterial strains were isolated on nutrient agar. Morphologically different colonies were subcultured and used for further PP degradation studies.

Identification of bacterial isolates: The bacterial colonies were cultured on LB broth and the genomic DNA was extracted by the standard phenol chloroform method. The bacterial 16S rDNA was amplified using the primers 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492 R 5' (TAC GGY TAC CTT GTT ACG ACT T)3'. The following PCR program was performed: initial denaturation at 94˚C for 2 min, denaturation at 94˚C for 1 min, annealing at 55˚C for 1 min, extension at 74˚C for 1 min, final extension at 72˚C for 8 min. Repeated the steps 2,3 and 4 for 4 cycles. The nucleotides were sequenced and the sequence similarities were searched by using BLAST database of NCBI. The phylogenetic tree and evolutionary analysis were identified by Mega software version7.

Preliminary screening of bacterial isolates for PP film degradation: The bacterial isolates were individually inoculated on the Bushnell Hass agar plate which contains plastic films. The plates were incubated at 37˚C for 24 h. The bacteria which were capable to colonize on the plastic films, were selected and used for secondary screening of PP film degradation.

Secondary screening of bacterial isolates for PP film degradation - Weight loss determination: Based upon the results of preliminary screening of PP film degradation, selected isolates were inoculated on the 50ml Bushnell Hass broth medium which contains 4×4cm size of preweighted PP film as carbon source. They were incubated on static condition at 27˚C for 4 weeks. After the period of incubation, the films were recovered and washed with ethanol, dried at 60˚C for overnight in hot air oven.

The percentage weight loss of PP films was measured by following equation:

$$\%\ weight\ loss = \frac{W_0 - W}{W_0}$$

where W_0 is the initial weight of the PP film and W is the weight of treated PP film.[19]

Determination of bacterial growth curve: The selected isolates were grown on nutrient broth medium at 37˚C for 8 h. The cells were collected by centrifugation at 4000 rpm for 10min. Then the pellets were washed with 0.1M phosphate buffer saline, pH 7.4 (PBS). Suitable amount(1ml) of dissolved pellets was inoculated with 50ml of Bushnell Hass broth which contains 4×4cm size of PP films (0.08g/50ml) as carbon source. The optical density (OD) values were measured at 600 nm from 0 hour to 4 weeks after each 1 week interval. The un-inoculated control was also maintained for comparison.[3,4]

Analysis of PP film degradation
After the incubation period, the treated PP films were recovered from the culture medium and analyzed by the following techniques. The untreated control was also analyzed for comparison.

Fourier Transform Infrared Spectroscopy: After the incubation of 15 days, to ensure the changes in its chemical structure and functional groups, the treated PP films were recovered and washed with ethanol and water. Then the films were allowed for air dry for overnight. Both treated and untreated control films were analyzed through FT-IR (Nicolet 6000 FT-IR Spectrometer). The spectrum ranges from 4500- 400cm^{-1}.

X Ray Diffraction: The treated and untreated control films were studied by XRD (PANalytical X' test pro MPD) with power -3KW (60KV and 60mA). Scan speed -0.001 to 1.27deg/sec with detector RTMS detection technology.

Results and Discussion

Bacterial identification and characterization: The municipality solid waste contaminated soil was rich in microbial community. In this present study, two different soil samples were collected and named as sample 1 and sample 2. There were 14 bacterial isolates screened from sample 1 and 12 bacterial isolates were screened from sample 2. Out of 26 strains, only two strains (S1B12 and S2B3) were capable to colonize on the surface of the PP films. The colonization of bacterial strains on the surface of PP film was represented on fig. 1(a) and (b).

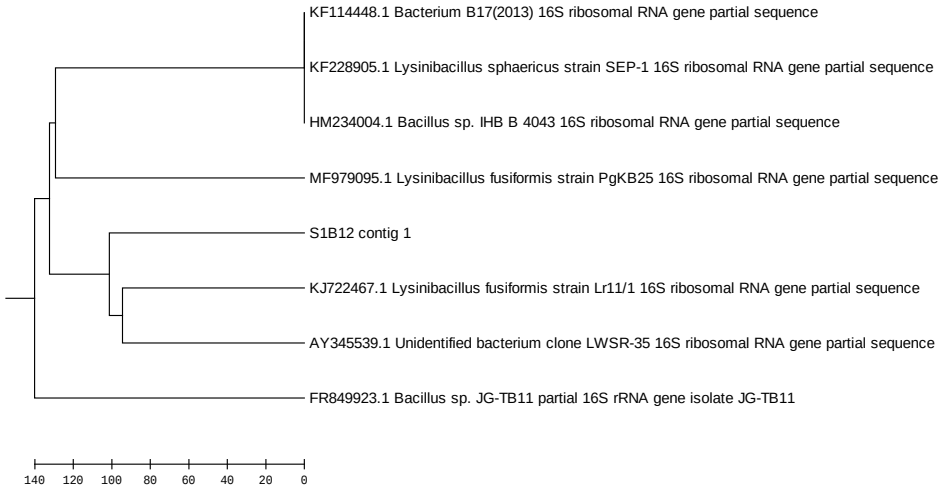
These two strains were considered for further studies. In NCBI BLAST, the strain S1B12 shows 99% similarities with *Lysinibacillus fusiformis* and S2B3 shows 99% similarities with *Bacillus paralicheniformis*. The 16s rRNA nucleotide sequences were submitted on NCBI, the accession numbers of S1B12 and S2B3 are MK559524 and MK559529 respectively.

The phylogenetic tree of the bacterial strains S1B12 and S2B3 was represented on fig. 2(a) and (b). Mukherjee et al[15] reported the *Lysinibacillus fusiformis* has the ability to form biofilm on polyethylene bags degradation study.

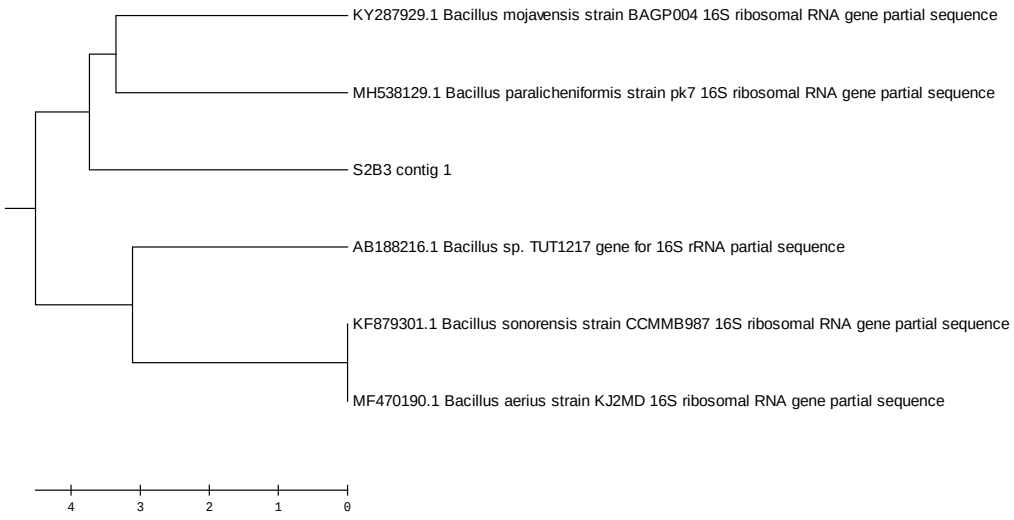
Determination of weight loss of PP films: The method suggested by Auta et al[3] was followed to measure the percentage weight loss and degradation of PP films by *Lysinibacillus fusiformis* and *Bacillus paralicheniformis*. The results are represented in table 1 and fig. 3.

(a) **(b)**

Fig. 1: Colonization of bacteria on the PP films (a) containing agar plate and (b) bacterial colonies attachment on the edge of PP films



(a)



(b)

Fig. 2: The phylogenetic dentodrogram of the relationship between the 16s rRNA sequences retrived from NCBI BLASTn and the16s rRNA sequences of S1B12 and S2B3 represented in fig. 2(a) and (b) respectively. The evolutionary history was inferred using the UPGMA method. The evolutionary distances were computed using the Maximum Composite Likelihood method. The analysis involved 6 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7

The percentage weight loss of PP films observed after 4 weeks of incubation was 4.0% and 2.5% by *Lysinibacillus fusiformis* and *Bacillus paralicheniformis* respectively. In a different study, the *Bacillus* sp. and *Rhodococcus sp*. degraded the PP microplastics. They caused 6.4% and 4.0% weight loss after the incubation of 40 days respectively.[4] and 2.97±0.05% of polyethylene degradation was achieved with *Lysinibacillus fusiformis* in one month.[14]

In another study, the *Aspergillus niger* and *Lysinibacillus xylanilyticus* showed 29.5% and 15.8% of degradation for UV irradiated and non UV irradiated LDPE films[6] and based upon the results of weight loss, we conclude that the bacterial strains have an ability to degrade the PP films. The weight loss of the PP films also considered as an evidence to degradation.

Growth measurement of PP film degrading strains: The evaluated growth of PP films degrading strains was represented in fig. 4. The growth of *Lysinibacillus fusiformis* (S1B12) and *Bacillus paralicheniformis* (S2B3) was observed from 0 day to 4 weeks. On the 0 day, the OD values of the uninoculated medium, S1B12 containing medium and S2B3 containing medium were 0.076, 0.131 and 0.148 respectively.

After 4 weeks of incubation, the OD values of the S1B12 containing medium and S2B3 containing medium were increased to 0.334 and 0.213 respectively at 600 nm and there was no change in the uninoculated medium. At the same time the bacterial cell counts of S1B12 and S2B3 were increased on the 4th week from 0 day.

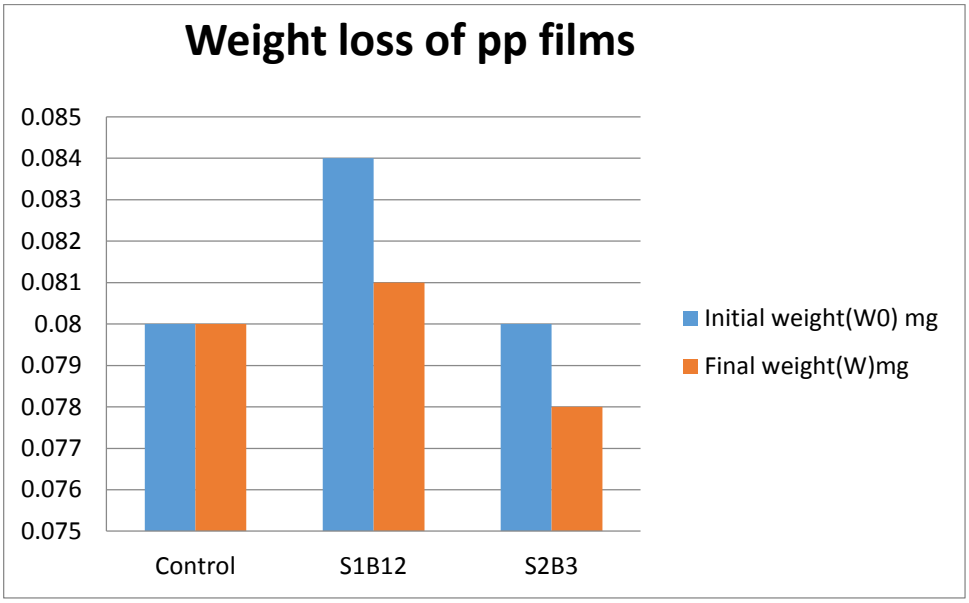


Fig. 3: % weight loss of bacterial treated PP films after the incubation of 4weeks. The maximum weight loss (4%), 0.003mg obtained for PP film inoculated with *Lysinibacillus fusiformis* (S1B12). The minimum weight loss (2.5%), 0.002mg obtained for PP film inoculated with *Bacillus paralicheniformis* (S2B3).

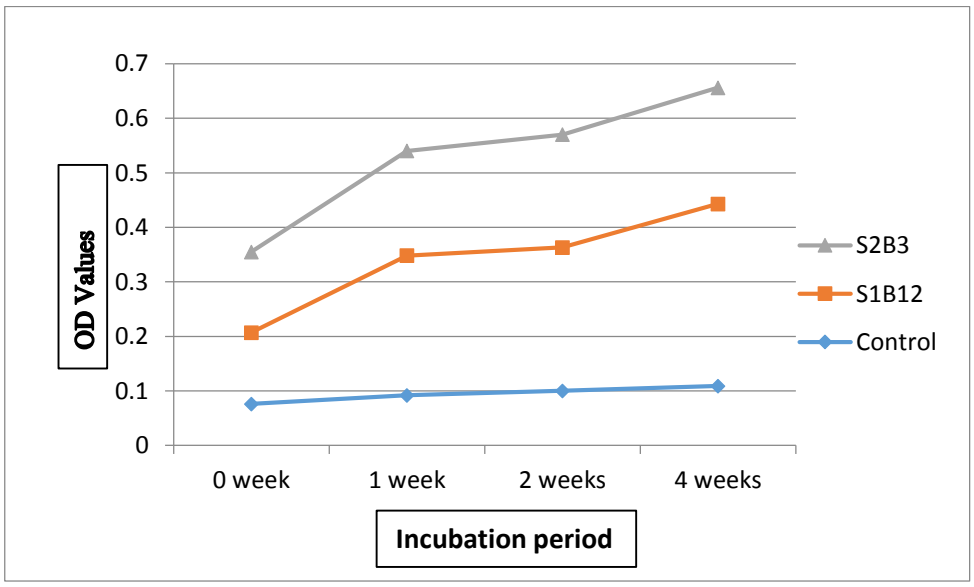
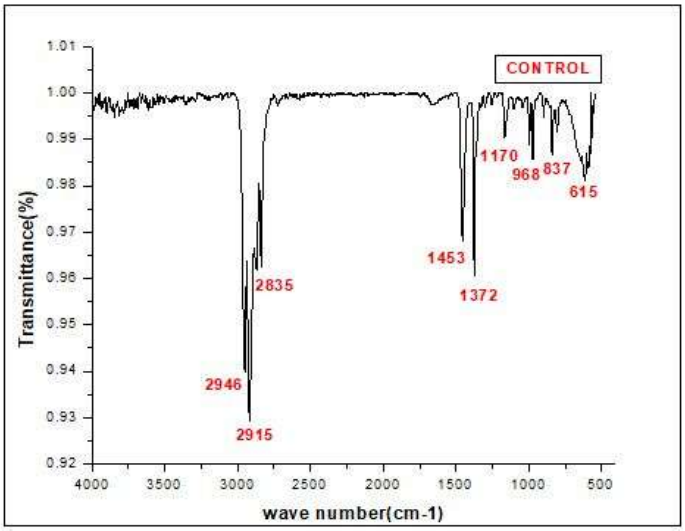
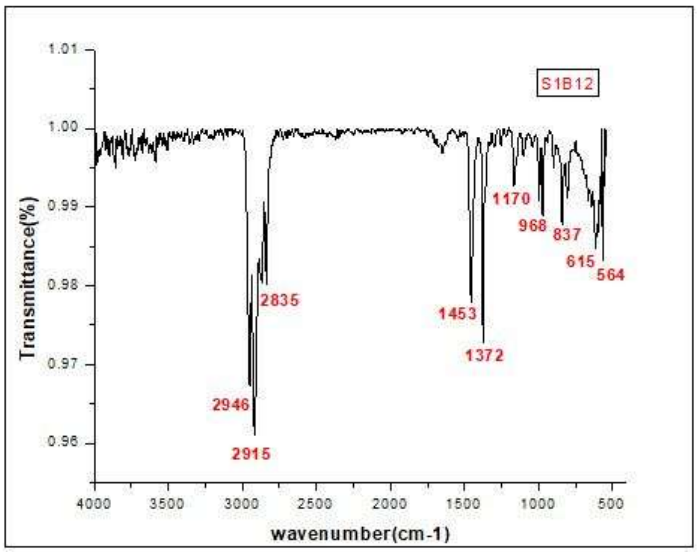


Fig. 4: growth curve of *Lysinibacillus fusiformis* (S1B12) and *Bacillus paralicheniformis* (S2B3).



(a)



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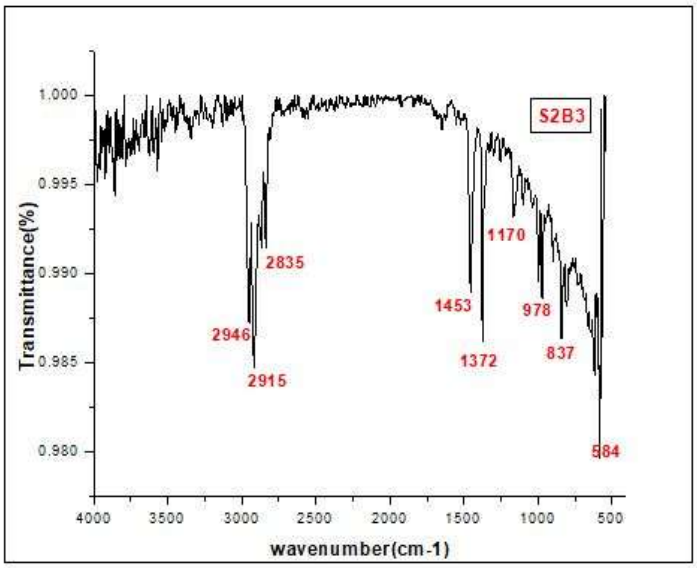
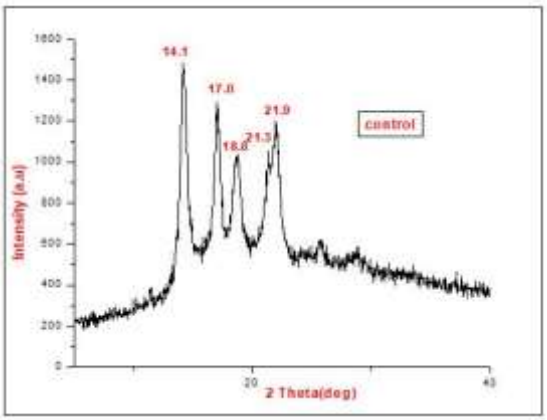


(c)

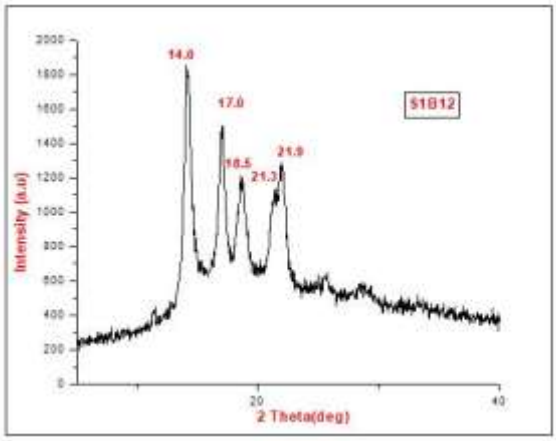
Fig. 5: (a) FT-IR spectrum of untreated control PP film, (b) S1B12 treated PP film and (c) S2B3 treated PP film

The viable cell counts were increased from 8×10^4cells/ml to 12×10^4cells/ml and from 10.1×10^4 cells/ml to 15.2×10^4 cells/ml for S1B12 and S2B3 respectively. The increase in the OD values and bacterial cell count proves the capability of the survival of bacterial isolates on the PP film containing medium. Auta et al[3] determined the growth patterns of the isolates in microplastic-infused media and observed the subsequent changes in the weight loss in microplastics.

Fourier Transform Infrared Spectroscopy analysis of PP film: The biodegradation and the changes in chemical bonds of PP films were determined after the incubation of 4 weeks with *Lysinibacillus fusiformis*(S1B12) and *Bacillus paralicheniformis* (S2B3) by FTIR analysis. The FT-IR spectra of untreated control PP film and PP film treated with S1B12 and PP film treated with S2B3 were represented in fig. 5 (a), (b) and (c) respectively. The FT-IR spectra have been recorded and the peaks were assigned by comparing their wavenumbers with previous literature[2,13]. In the untreated control film, the absorption peaks existed at 2946 cm^{-1}, 2915cm^{-1} and 2835cm^{-1} which were recognized to C-H stretch. The peaks at 1453cm^{-1} and 1372cm^{-1} were attributed to the CH_2 bend and CH_3 bend respectively.



(a)



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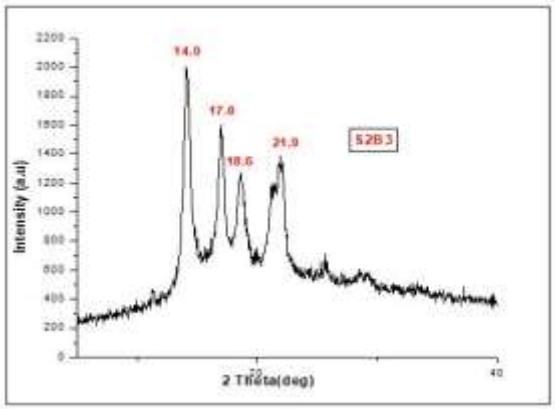

(c)

Fig. 6: (a) XRD spectrum of untreated control PP film, (b) XRD spectrum of S1B12 treated PP film and (c) XRD spectrum of S2B3 treated PP film showing 2θ angle values.

Table 1
Weight loss and percentage weight loss of control PP film and treated PP films with *Lysinibacillus fusiformis* (S1B12) and *Bacillus paralicheniformis* (S2B3).

S.N.	Culture Id	Initial weight(mg) W_0	Final weight (mg) W	Percentage weight loss%
1	Control	0.080	0.080	0%
2	S1B12	0.084	0.081	4%
3	S2B3	0.080	0.078	2.5%

The peak at 1170cm^{-1} stands for the C-H bend. The peaks observed at 968cm^{-1} and 837cm^{-1} are assigned to CH_3 rock and CH_2 rock respectively. When the PP films were treated with S1B12 (*Lysinibacillus fusiformis)*, a new peak seemed at 564cm^{-1} was assigned to the CH out of plane band, C-Br stretch. On treatment with S2B3 (*Bacillus paralicheniformis*), a new peak was detected at 584cm^{-1} which was attributed to the C-Br stretch. The rest of the peaks which were observed on the S1B12 and S2B3 treated PP films were similar to the peaks present in the untreated control PP film. The transmittance percentage of the peaks of S1B12 and S2B3 treated PP films was decreased from the control value. It may occur due to biodegradation effects.

X Ray Diffraction analysis: The changes in the crystallinity structure of the PP films were identified by using XRD analysis. The X Ray Diffraction patterns of the untreated control, S1B12 treated and S2B3 treated PP films were represented in fig. 6. (a), (b) and (c) correspondingly. The sample spectrum peaks of the untreated control PP films are at 2θ= 14.1°,17.0°, 18.8°, 21.3°, 21.9° while the S1B12 treated PP films spectrum peaks appeared at 2θ= 14.0°, 17.0°, 18.5°, 21.3°, 21.9° and the spectrum peaks at 2θ= 14.0°, 17.0°, 18.6° and 21.9° seemed in S2B3 treated PP film.

These spectrum values were almost similar as Abiona and Osinkolu[1] have observed before, In their research, the peaks seemed at 2θ= 14.0°, 17.0°, 18.6°, 21.1°, 21.8°, 22.5° and 28.6°. and the sample is identified as α (monoclinic) isotactic polypropylene because the XRD peaks are similar with the spectrum previously by Grady et al.[9] When compared with control PP film with treated films, the changes in the intensity values of PP films were observed. The reduction in the crystallinity of the PP films happened due to the microbial activities which decreased the crystallites of the PP film.

Conclusion

The present study showed the addition of potential micro-organisms especially soil bacteria namely *Bacillus paralicheniformis* (S1B12) and *Lysinibacillus fusiformis* (S2B3) isolated from municipality solid waste contaminated soil in the degradation of PP films. This study proves that the bacteria can grow in Bushnell Hass broth medium which contains PP films as carbon source. The results of FT-IR and XRD evidenced the changes in the functional groups and their crystallinity of the PP films by the biodegradation activity of bacteria.

The results of weight and growth curve ensure the ability of the bacteria to utilize the PP film as carbon and energy sources. The continuity of this work will provide possible solution for the degradation of plastics and will clean up the environmental plastic pollution.

Acknowledgement

The authors express their gratitude to Tamil Nadu State Council for Science and Technology (TNSCST), Tamil Nadu, India, for funding this research work under Student Project Scheme 2018. The authors also thank Department of Science and Technology (DST) for supporting Department of Microbial Biotechnology, Bharathiar University under DST – FIST scheme to have necessary facilities for the successful execution of this research work.

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(Received 30th May 2020, accepted 10th August 2020)