Isolation and molecular identification of bacteria on surface of old pet bottle

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Abstract
The 16s rRNA gene sequence is commonly used for identification and classification of microbes. This study aimed at isolated and identified bacteria from soil dumped PET plastic surface. We determinate their 16s rRNA gene sequences as selected bacteria. Further, pairwise similarity were analysed with voucher specimen in NCBI.

The sequence was deposited with accession number KY026604 (Alcaligenes faecalis), KY026605 (Bacillus licheniformis) and KY051741 (Bacillus cecembensis). Bacterial Biofilm formation was further confirmed by Scanning Electron Microscope (SEM) as result rode shaped bacteria, clustered bacteria growth and mass colonization evince on bacterial treated PET plastic surface.

Keywords: 16s rRNA, PET degradation, SEM.

Introduction

Polyester PET strands are utilized in an extraordinary number of use territories, for example, clothing, home outfitting and inside materials, cleanliness and clinical materials.[5] PET is ordinarily utilized as binding films, synthetics strands, bottles for drink and nourishment and building plastic parts, inferable from their astounding warm and mechanical properties, high compound opposition and low gas permeability.[6,11,18,19] As of late there has been developing open worry over natural weakening related with the removal of synthetic plastics. Disposal of plastics, other than being exceptionally noticeable is a quickly expanding level of strong waste in landfills, impervious to biodegradation prompting contamination and destructive to the regular habitat.

These issues have made plastic waste a significant concentration in the administration of strong waste.[8] Increase of pollutants resulting from anthropogenic activities has become a major problem that made the requirement of new technologies for environmental cleansing more urgent. Polyethylene appears to be one of the most inert plastic materials. The ample applicability and wide availability of PET films have led to accumulation of PET waste and for this reason they should be generally recycled. Polyethylene terephthalate (PET) is widely used in multiple application such as fibers, films etc. The presence of PET in municipal solid waste has been increasing and it causes plastic waste disposal problem as consumption increases dramatically. But recycling is a complex process as the materials are degraded during normal usage and by the recycling process.[12]

These materials, due to high molecular weight and hydrophobicity, are resistant to environmental factors and after usage they become burdensome ballast to the environment.[9] The abundant accumulation of PET waste is of environmental concern due to its non-biodegradability, which is a major obstacle for disposal of PET by conventional methods such as land filling and incineration. The objective behind the present study was to assess the isolation and molecular identification of bacteria from PET plastic and bioflim formation conformed by SEM images.

Material and Methods

Isolation of bacteria from PET waste: Poly(ethylene terephthalate) waste bottles were collected from garbage dumped site at four places (Karumandapam, Subramanipuram, Yadamalaipatti pudur and TVS Nagar, Tiruchirappalli, Tamil Nadu, India). The soil particles on the surface of the PET waste were removed and washed with sterile distilled water and inoculated in the nutrient broth. After 24 hours of incubation, 100 µl of broth culture was inoculated into nutrient agar plates. After 24 hours of incubation the bacterial isolates were identified by the methods described in Bergeys Manual of Determinative Bacteriology.[14] Dominated three bacteria were selected for further study and 16srRNA sequences were analysed from Institute of Microbial Technology (CSIR-IMTECH), Chandigarh. Bacterial Gene sequences were submitted in NCBI to analyse pairwise similarity and submitted to Gene bank to receive accession number.

Incubation of PET flakes in MSM inoculated with bacteria: Purchased PET bottles were cut into small flakes, then washed with 70 % ethanol and again washed with distilled water and finally the samples were kept at 45 ^{o}C for drying. Then the samples were directly inoculated into Nutrient Broth Medium with *Bacillus cecembensis, Bacillus licheniformis* and *Alcaligenes faecalis*. They were kept in orbital shaker for a period of one month at 37 ^{o}C temperature with 120 rpm. After 30 day bacterial treated PET plastic was collected from nutrient broth medium for further analysis.

Scanning Electron Microscopy: Scanning electron microscope (SEM) (VEGA3 TESCAN) was used to determine the changes on the surface of PET flakes and colonisation of bacteria. Control and bacterial treated samples are generally sputter-coated with gold or some

metal ions before SEM examination. Analysis was carried out using low vacuum 0.68 Torr mode, 10 to 30 kv at different magnification 6.13 kx to 500 kx and LFD (large field Detector).[13]

Results

The dominant bacteria prevalent in the PET waste at different places are displayed in table 1. The dominant bacteria were *Alcaligenes faecalis, Acinetobacter sp.* and *Bacillus licheniformis,* in PET waste at Karumandapam. *Alcaligenes faecalis, Staphylococcus sp., Acetobacter sp., Bacillus licheniformis* and *Bacillus cecembensis* were the dominant bacteria present in the garbage dumped site at Subramanipuram. *Alcaligenes faecalis, Klebseilla pneumonia* and *Bacillus cecembensis* were the dominant bacteria in PET waste collected from Yadamalalipatti pudhur. The PET waste collected from garbage site at TVS nagar harboured diverse bacteria (*Staphylococcus aureus, Geobacillus stearothermophillus, Klebseilla pnemoniae, Alcaligenes faecalis, Bacillus subtillus, Acinetobacter sp.* and *Bacillus cecembensis*). *Alcaligenes faecalis, Bacillus cecembensis* and *Bacillus licheniformis* were selected for further studies and were confirmed by 16s rRNA gene sequences analysis.

SEM: Collection of the SEM images of control PET indicates no visible changes on the PET. In comparison to the smooth surface of the control PET flakes, *Alcaligenes faecalis* inoculated with PET flakes elicited rode shape cluster colonisation was evinced in the SEM images when compared to the control. Rode shaped and mass colonisation and rough surface of PET flake inoculated with *Bacillus licheniformis* were observed in the SEM image when compared to the control. Similar pattern of change on the PET surface was evident with respect to *Bacillus cecembensis* inoculated PET (Plate 1).

Table 1
Dominant bacteria prevalent in the PET waste collected from garbage dumped site at different places

S.N.	Karumandapam	Subramanipuram	Yadamalaipatti Pudhur	TVS Nagar
1	*Alcaligenes faecalis subsp. faecalis*	*Alcaligenes faecalis subsp. faecalis*	*Alcaligenes faecalis subsp. faecalis*	*Staphylococcus aureus*
2	*Acinetobacter sp.*	*Staphylococcus sp.*	*Klebseilla pneumonia*	*Geobacillus stearothermophilius*
3	*Bacillus licheniformis*	*Acetobacter sp.*	*Bacillus cecembensis*	*Klebseilla pnemoniae*
4		*Bacillus licheniformis*	*Bacillus licheniformis*	*Alcaligenes faecalis subsp. faecalis ,*
5		*Bacillus cecembensis*		*Bacillus subtillus*
6				*Acinetobacter sp.*
7				*Bacillus cecembensis*

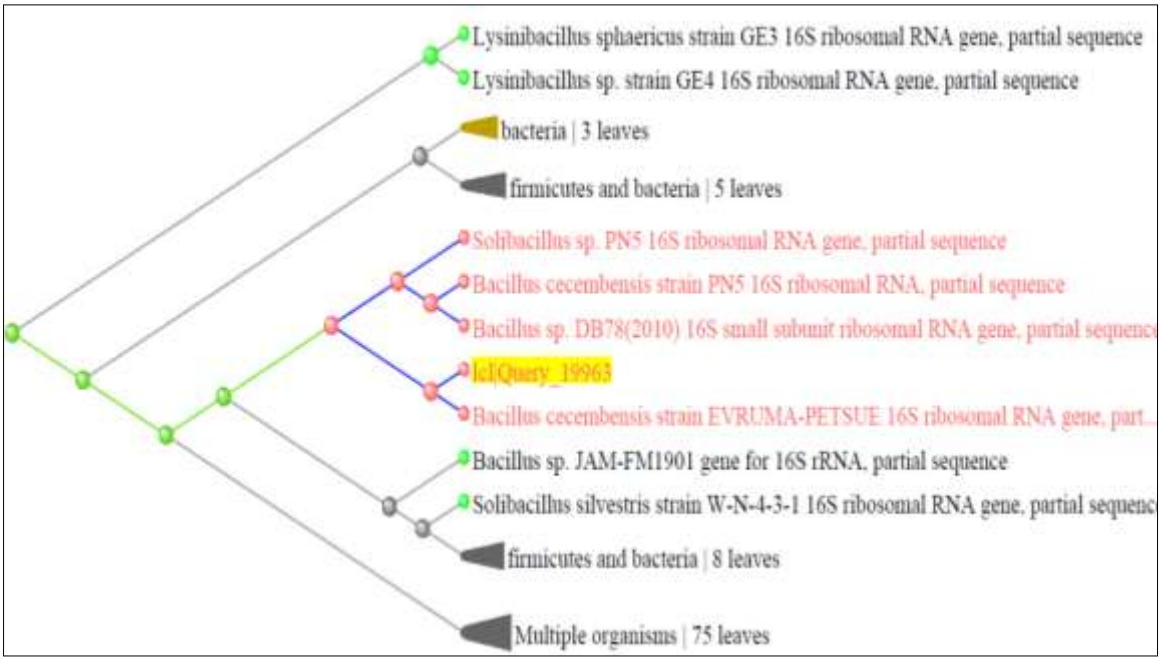
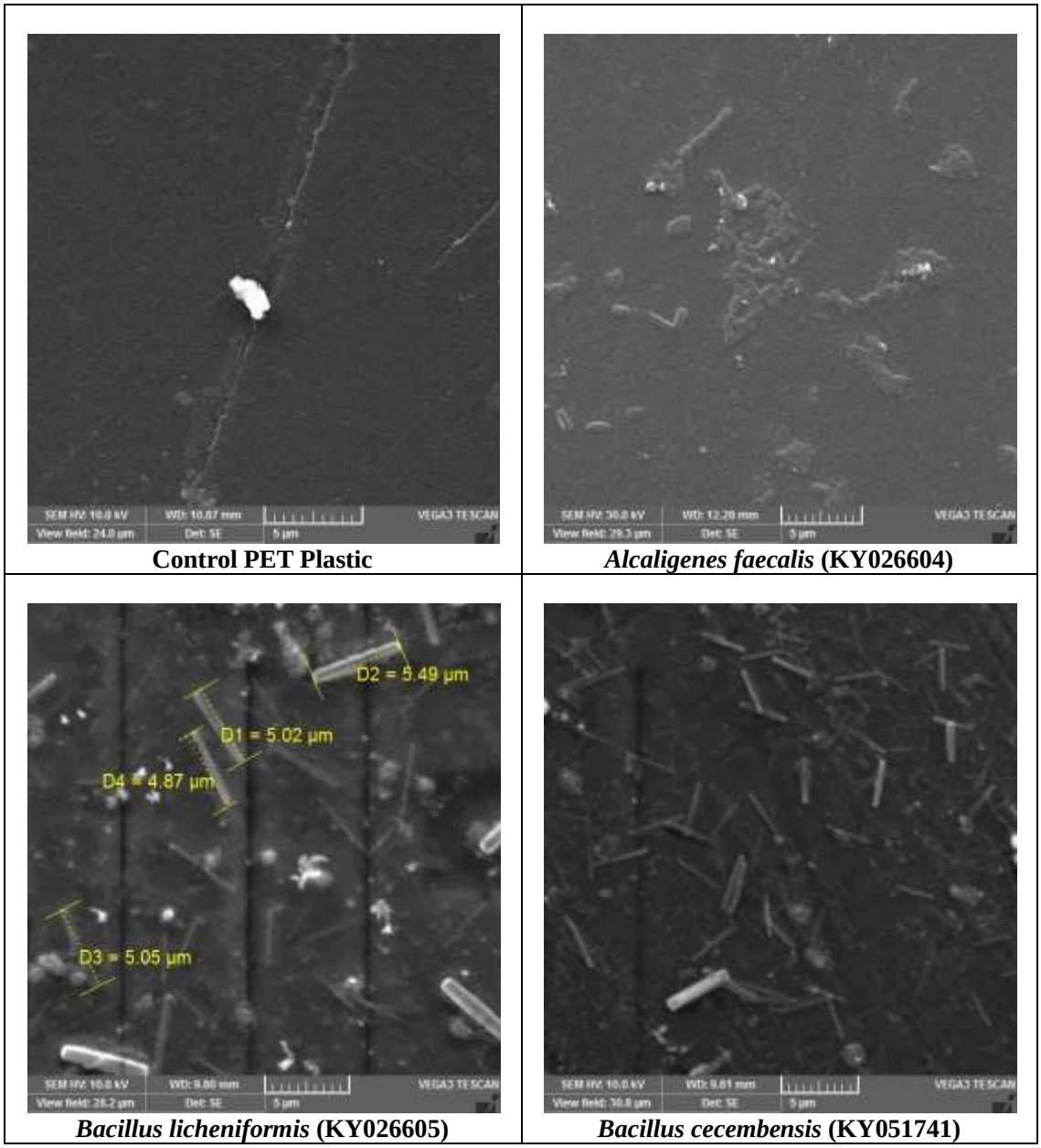


Fig. 1: Phylogenetic tree of *Bacillus cecembenis* bacteria



Control PET Plastic | ***Alcaligenes faecalis*** **(KY026604)**

Bacillus licheniformis **(KY026605)** | ***Bacillus cecembensis*** **(KY051741)**

Plate 1: SEM images of PET plastic after biofilm formation of Bacteria

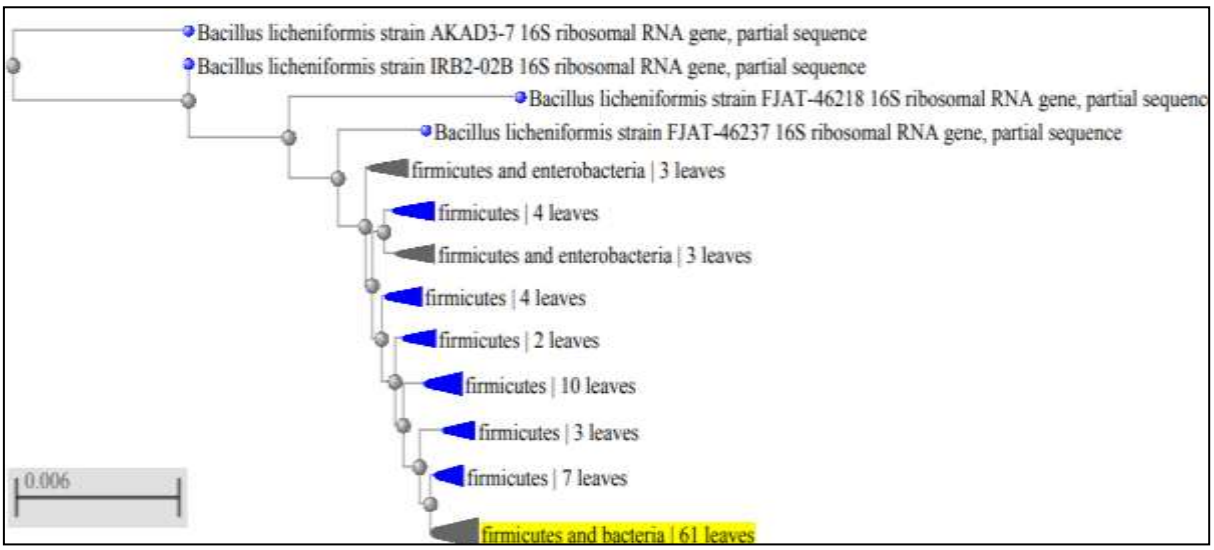


Fig. 2: Phylogenetic tree of *Bacillus licheniformis* bacteria

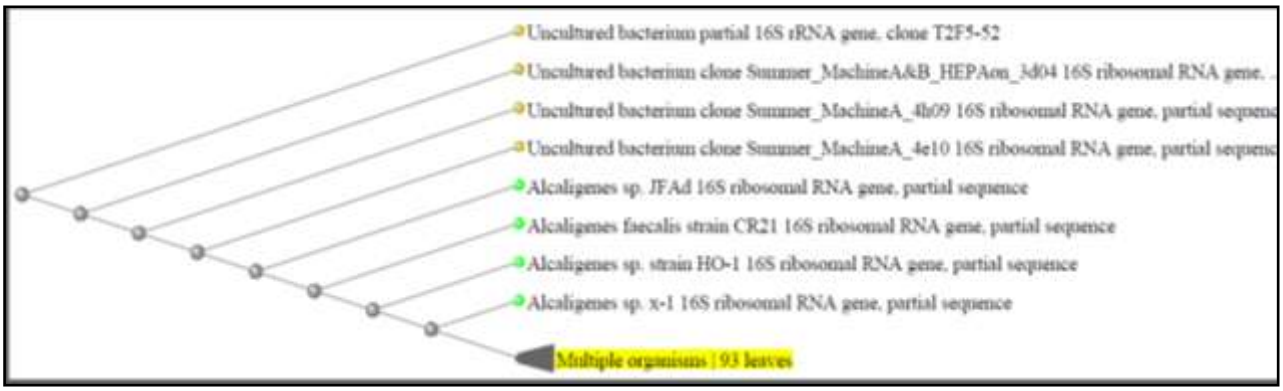


Fig. 3: Phylogenetic tree of *Alcaligenes faecalis* bacteria

In the figure 3 phylogenetic trees were built by utilizing greatest miserliness criteria with closest neighbour optimization. Sequences with 98% similarity to some voucher bacterial specimen sequences were checked from nucleotide alignment tools of BLAST in NCBI and received accession number of identified bacterial species of *Alcaligenes faecalis* (KY026604), *Bacillus licheniformis* (KY026605) and *Bacillus cecembensis* (KY051741) (Fig. 1, Fig. 2, Fig. 3).

Discussion

The PET waste associated bacterial species evinced in this study agrees with that of Vijaya and Reddy[17] who have isolated and identified microbial species harbouring in the polyethylene carry bags and cups naturally buried in the municipal compost of Kavali town, India (*Bacillus sp., Staphylococcus sp., Streptococcus sp., Diplococcus sp., Micrococcus sp., Pseudomonas sp., Moraxella sp., Aspergillus niger., A.ornatus, A. nidulans, A. cremeus, A. flavus,A candidus and A. glaucus*). They have reasoned out that these microorganisms utilize polyethylene films as sole source of carbon resulting in partial degradation of plastics and colonise on the surface of the polyethylene films or plastic cups forming a biofilm.

In addition, they have stated that cell surface hydrophobicity of these organisms was an important factor in the formation of biofilm on the polyethylene surface, which consequently enhanced biodegradation of polymers. Barratt et al[3] have demonstrated that fungi *Nectria gliocladioides* (white colonies), *Geomyces pannorum* (peach colonies) and *P.ochrochloron* (green colonies) in the soil were the predominant microorganism responsible for degradation of soil buried polyester polyurethane over range of soil water holding capacity.

The present result is in good accord with the findings of Balasubramanian et al[2] have who isolated bacteria from plastic wastes dumped sites in the Gulf of Munnar region, India. They have further screened 15 bacteria competent of HDPE (high density polyethylene) degradation.

As evinced in this study Gupta et al[7] have reported the presence of *Pseudomonas sp., Xanthomonas, Flavobacterium, Agrobacterium* and *Bacillus sp.,* as the most associative bacterial flora with the samples of old polyethylene / plastic wastes collected from various organic matter rich sites of four districts at Chhattisgarh, India.

Lee et al[10] have studied the fungal communities associated with *in situ* degradation of PU in natural soils and degradation was extensive in both soil tested (neutral and acid soil) with the polyester polyurethane losing up to 95% of its tensile strength after 5 months. Similar to the present result, Nanda et al[15] have isolated *Brevibacillus, Pseudomonas spp.* and *Rhodococcus* spp., from a waste disposal site dumped with polyethylene bags and plastic products.

Smooth surface of PET evinced in the SEM image is in good accord with the observations of Umamaheswari et al[16] who have also visualised smooth surface of PET in the SEM image. They also noticed no visible structural changes on the surface of UV treated PET. In addition, they also noticed *Pseudomonas sp.,* colonies on the surface as well as inside PET on inoculation of UV treated PET with *Pseudomonas sp*. These observations are in line with the present findings.

Arkatkar et al[1] have detected surface changes in the SEM images of short UV treated polypropylene films after 12 months of incubation with *Bacillus flexus*. Sharon and Sharon[4] have detected in the SEM image of polyethylene terephthalate sheet kept in a closed drawer untouched for nearly a year, crystals of degraded products as well as microbial colony growing inside as well as on the surface of the crystals.

Conclusion

The observations of the present study reveal that PET waste dumped in soil is exposed to a variety of organism, especially bacteria. *Bacillus licheniformis, Alcaligenes faecalis and Bacillus cecembensis* isolated from PET waste were reinoculated under laboratory conditions. The augmentation on PET surface was evidenced in the SEM images.

Thus, these findings permits us to conclude that *Bacillus licheniformis, Alcaligenes faecalis and Bacillus*

cecembensis are able to colonise and form biofilms on PET surface which indicate that bacteria are able to utilize PET as a carbon source for their growth. However, the involvement of Bacteria in degrading PET has to be confirmed by further studies.

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