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# Gene analysis and Molecular characterisation of low-density polyethylene degrading bacteria from Municipal dumpsite

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**Introduction**

Polyethylene (PE) is man-made polymers used in many aspects of human life. They are made of carbon and hydrogen, with nitrogen, sulphur, and other various organic and inorganic materials derived from fossil fuels (Kumari *et al*., 2013 & Angga Puja *et al*., 2021). It is widely used due to its light weight, inexpensive, strong and durable nature. Most of the plastic waste is non-biodegradable which takes thousands of years to be decomposed (UNEP 2015) The worldwide production rate of synthetic polymers is about 140 million tons and its utility is increasing at the rate of 12% per annum (Sekhar *et al*.,2016 & Lalina Maroof *et al*., 2021). Although plastic products are reusable, they are still one of the main factors causing environmental pollution (Drzyzga *et al*., 2018) Plastics has become an omnipresent part of environment and it seems that their biodegradation is extremely slow, often burns in the open area leads to air pollution (Jyothika *et al*.,2020)

Microorganisms such as *Brevibacillus spp*, *Aneurinibacillus spp* (Skariyachan et al., 2018) bacillus cereus, *Brevibacillus borstelensis* (Ndahebwa Muhonja 2018), *Pseudomonas aeruginosa* (Jeon &Kim 2015) and *Enterobacter spp* (Skariyachan, Manjunath *et al*., 2016) have been reported in recent studies to degrade polymer. Various studies have been done to investigate the efficacy of genus Bacillus and are most frequently identified among biodegradation.

Landfills are the oldest and most common method of plastic waste disposal. Several studies have been reported the polyethylene degrading microorganisms from these waste disposal sites (Ndahebwa Muhonja 2018 and Gajendiran *et al.,* 2016). This is the first study on isolation of bacteria from waste dumpsites, Raichur, Karnataka for polyethylene degradation and we found *Bacillus firmus* can degrade polyethylene very efficiently. The degradation of polyethylene was confirmed that *Bacillus firmus* producing gene which is the molecular explanation for the degradation of polyethylene.

**Materials & Methods:**

**Soil sample collection:**

Soil samples were collected from municipal waste dumpsite in Raichur, this dumpsite was selected since the plastic waste/ bags were dumped for long time i.e., 30-40years, which has the probability of potent polyethylene degrading bacteria. One gram of soil was brought to the soil in sterilised zip lock cover and stored at room temperature and used for isolation of microorganisms within 24h.

**Isolation and identification of bacteria:**

Serial dilution and spread plate technique was performed on Nutrient agar and Enrichment media and the plates were incubated at 37℃ until bacterial growth appeared, grown bacterial cultures were identified based on microscopic observations, further these bacterial growth colonies were sub-cultured to get pure isolates and preserved for further use (Ojha *et al*.,2017 and Lalina *et al.,* 2021).

**Screening for degrading bacteria**

**Plate assay method:**

Screening media was prepared without the addition of agar. Polyethylene was added to the media and was placed on the rotary shaker for about an hour there after agar was added to the media and sterilized then the media was poured onto the plates and allowed for solidification. Bacteria was inoculated to the sterilized media and incubated for 24-48h, isolates showed clear hallow zone around the colonies.

**Biodegradation studies:**

LDPE strips (2 cm X 2 cm) were weighed, surface sterilized with sodium hypochlorite, washed in water, placed in ethanol and then washed, this process was followed for 3-5 times and then these strips were placed in 100ml MSM media and then a loop full of bacterial culture was dropped into the media. The flasks were incubated at 37℃ in shaking incubator for 90 days (Kyaw *et al.,* 2012 and Lalina *et al.,* 2021)

**Molecular identification of the isolates:**

Genomic DNA was extracted from fresh culture. Cells were lysed using lysis buffer, amplification was performed using universal primers 27F ($5^{l}$ AGAGTTTGATCTGGCTCAG $3^{l}$) and 1492R ($5^{l}$ TACGGTACCTTGTTACGACTT $3^{l}$) 25µL of Taq Master Mix, 1.5 µL of each primer, DNA polymerase and 5µL of deionised water. PCR was conducted at following conditions: Initial denaturation at 95℃ for 5min, followed by 25 cycles of 95℃ for 30S, 55℃ for 30S, 72℃ for 2 min and final extension at 72℃ for 10 min. The amplified product was analysed and sequenced (Applied Biosystems). The nucleotide sequences were analysed and aligned using MUSCLE 3.7 (Edgar 2004) program, the resulting aligned sequences were cured using the program Gblocks 0.91b in Fig 1. The phylogenetic tree was constructed using the Neighbor-joining method.

**Purification of PCR production:**

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR clean up kit (Millipore). The PCR product was sequenced using the primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

**Results and Discussion**

**Identification and screening of bacterial isolates capable of LDPE biodegradation:**

Many distinct colonies were observed on nutrient agar and enrichment plates after incubation at 37℃ for 24-48h. About forty morphologically distinct colonies were selected and sub-cultured for further use. All the bacterial isolates were screened for their degradation ability using LDPE strips and MSM media. Growth was observed on

the surface of LDPE strips after 60d of incubation at 37℃ (Fig---). Out of forty isolates only few showed biofilm formation revealing their capacity to utilise LDPE as a carbon source and was further characterised. Biofilm formation on LDPE strips determine its biodegradation potential because strips causes the bacteria to efficiently utilize the insoluble polymer substrate (Ott IG *et al.,* 2004). No growth was seen on control with LDPE strips without inoculation Isolated and identified bacteria in the current study were: - *Alcaligenes faecalis*, *Bacillus firmus*, *Kleibsiella aerogenosa* and *Alcaligenes faecalis* strain. According to Applied Biosystems and 16SrRNA gene sequencing, Phylogenetic tree showed related species as per 16SrRNA sequencing as illustrated in Fig 1.

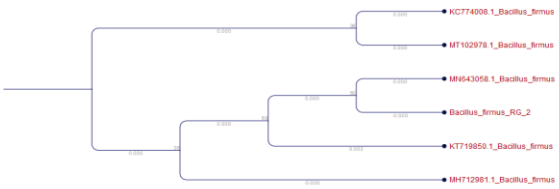


**Fig 1. Phylogenetic Tree**

**Investigation of LDPE degradation:**

Biodegradation of LDPE was monitored by weight loss percentage.

**Percent weight loss determination:**

Identified isolates were tested for their degradation capacity that was measured in terms of percentage weight loss (Table 1)

| S.No | Sample | Initial Weight(mg) | Final Weight(mg) | Weight loss (%) |
|---|---|---|---|---|
| 1 | Control | 100 | 100 | 0 |
| 2 | *Bacillus Firmus* | 100 | 93.3 | 9.33 |

The highest percent weight loss was presented by bacteria and fungi. Bacterial isolates such as *B.siamensis* (8.46 ± 0.3%) followed by *B cerus* (6.33 ± 0.2%), *B wiedmannu*

(5.39 ± 0.3%) and *B subtilis* (3.75 ± 0.1%). Among bacterial isolates *P aeruginosa* (1.15 ± 0.1% and *A iwoffi* (0.76 ± 0.1%) percent weight reduction was negligible.

Bacillus species were more efficient in degrading LDPE strips in this study as compared to other isolates. Several studies in literature also identified the potential of *Bacillus spp*

for polyethylene degradation via percentage weight loss, such as Harshavardhan and Jha 2013 reported 1.75% weight loss in 30 days, Yang *et al*., 2014 observed10.7 ± 0.2% in 60 days of incubation and Sowmya, observed 1 4% weight loss for UV treated polyethylene in 90 days. In the current study *Bacillus firmus* showed 9.33 % of weight loss of surface sterilized LDPE strips, while the previous study reported percent weight loss of 35.2% in 30micron film after four months of incubation (Muhonja *et al.*,2018). In present study, *B firmus* resulted 9.33 % is comparatively higher than 1.85% weight loss in 30 days of incubation (Vimala P *et al.,* 2016). However, another work reported 23.15% reduction in 60 days of treatment with *B subtilis* (Ibiene *et al.,* 2013). The variation in percent weight loss in our study with comparison to literature might be attributed to the origin of bacterial isolates, LDPE thickness and cultural conditions such as incubation time. In this study *B firmus* resulted in the weight loss of 9.33 ± 0.3% respectively. To the best of our knowledge, we report *B firmus* for the first time in LDPE degradation which exhibits the ability of utilizing LDPE as s sole carbon source. Bacterial genes were amplified in the selected bacterial isolates. Amplified segments were subjected to agarose gel electrophoresis *B firmus* was amplified and bands appeared in the isolates indicating their presence in them (Fig 2). Moreover, two set of primers were used for amplification.

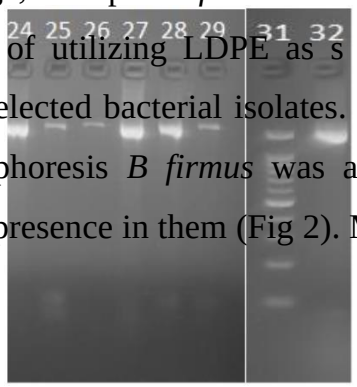


**Fig 2. PCR image**

| Sl.No. | Well No. | Sample |
|---|---|---|
| 1 | 31 | Ladder |
| 2 | 27 | *B firmus* |

**Conclusions:**

In this study we isolated bacterial species from municipal dumpsites of Raichur, Karnataka for LDPE degradation. We hereby conclude that the Bacillus species were capable of utilizing LDPE as a sole carbon source as evidenced by weight loss determination. *Bacillus firmus* exhibited effective LDPE degradation. The confirmation of biodegradation not only by the attachment of the bacteria to the LDPE strips and weight loss percentage along with physical and chemical changes. The biodegradation by selected bacterial isolate was limited to surface of LDPE strips, relatively slow and continuous process.

**Author contributions**

Geeta Diddigi conducted all the experiments in the Bioprocess and Fermentation technology laboratory, Department of Microbiology, Davangere University, Davangere under the guidance of Dr. Ramalingappa B Professor. Yaazh Xenomics helped in Gene sequencing and constructing phylogenetic analysis of the isolate.

**Conflicts**

Author do not receive any funding or financial assistance for this work.

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