Augmentation of a Microbial Consortium for Enhanced Polylactide (PLA) Degradation

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Abstract Bioplastics are eco-friendly and derived from renewable biomass sources. Innovation in recycling methods will tackle some of the critical issues facing the acceptance of bioplastics. Polylactic acid (PLA) is the commonly used and well-studied bioplastic that is presumed to be biodegradable. Considering their demand and use in near future, exploration for microbes capable of bioplastic degradation has high potential. Four PLA degrading strains were isolated and identified as *Penicillium chrysogenum*, *Cladosporium sphaerospermum*, *Serratia marcescens* and *Rhodotorula mucilaginosa*. A consortium of above strains degraded 44 % (w/w) PLA in 30 days time in laboratory conditions. Subsequently, the microbial consortium employed effectively for PLA composting.

Keywords Biodegradability · Polylactic acid · Enzymatic degradation · Microbial degradation

As a result of rapid technology growth and global population explosion, the necessity of plastic materials enormously increased in every discipline of life and industries.

The increasing accumulation of non-biodegradable synthetic plastics in the environment has been a menace to the earth. The bio-degradable nature of PLA makes it most acceptable to resolve this issue.

A very few actinomycetes and thermophilic bacteria such as *Brevibacillus*, *Bacillus smithii*, *Geobacillus*, and *Bacillus licheniformis* has manifested PLA-degrading ability [1–4]. Jarerat et al. [5] reported that most of the degraders were restricted to the family *Pseudonocardiaceae* and related genera such as *Saccharothrix*, *Amycolatopsis*, *Lentzea*, *Streptoalloteichus* and *Kibdelosporangium*.

PLA biodegradation takes place by the hydrolysis of the ester bonds. The ester linkages in PLA are vulnerable to both chemical hydrolysis and enzymatic cleavage. Microorganisms are devoid of transporters to uptake the plastic polymers directly into the cells due to water insolubility and size of the polymer molecules. The extracellular enzymes secreted by the microbes helps to depolymerize the polymer [6]. An interesting report was that enzymes like Proteinase K secreted by *Tritirachium album* can degrade PLA [7]. Lee et al. [8] proposed a mechanism of enzymatic degradation for PLA nonwovens fabrics and could be very useful for waste management in the textile industry. Earlier we reported that incorporation of relatively cheap and eco-friendly polymer such as thermoplastic starch, to PLA enhances its biodegradability [9]. The present work intends to demonstrate the rapid biodegradation of PLA by employing a consortium of few new microbial isolates.

A commercial grade PLA with molecular weight of 85,000–160,000 was obtained from Sigma-Aldrich (India).

Approximately 300 soil samples from different sources (Tropical rain forest areas of Western Ghats of Kerala, composting grounds in local markets, solid waste treatment plant, mangrove soil, activated sludges and dairy sludge

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from milk processing plant) were screened to isolate PLA degrading microorganisms.

PLA solution (1 g PLA in 40 mL chloroform) was emulsified into mineral medium (1 L) with 100 mg surface active agent (plysurf). The composition of the mineral medium was as follows (g L^{-1}): $(\text{NH}_4)_2\text{SO}_4$ (1), K_2HPO_4 (1.6), KH_2PO_4 (0.2), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2), FeSO_4 (0.005), ZnSO_4 (0.005), MnSO_4 (0.005), CaCO_3 (1), Yeast extract (0.1) and pH 7. After sonication, the emulsion was evaporated at 80 °C for 3–5 h to remove chloroform. Soil samples (1 g) were aseptically transferred to the mineral medium, thoroughly mixed and kept for incubation at 30 °C and 150 rpm for 2 weeks. After desired period of incubation, 0.1 mL of the suspensions was plated onto the surface of PLA emulsified plates by spread plate technique.

PLA films of thickness 20 μm were prepared by casting PLA using chloroform as the solvent (1 g/50 mL). The resulting transparent films were dried overnight and surface sterilized using sodium hypochlorite solution (5 %) for 10 s. After washing with sterile distilled water, PLA film (100 mg) was added to 100 mL basal mineral medium. Individual isolates of a biomass density (2×10^8 colony-forming units (CFU) per mL) was obtained from 50 mL of mid-exponential phase cultures. It was inoculated into a fresh mineral medium with PLA film and gelatin as a carbon source. The mineral medium containing PLA film without microorganism served as film control. The culture inoculated medium without film set as culture control. It was incubated at 30 °C in shaking condition (200 rpm).

The genomic DNA was extracted from the PLA degrading bacterial and fungal cultures using standard DNA extraction procedures [10]. 16S rRNA gene amplification was performed using the set of primers 27F (*Escherichia coli* position 8–27, 5'-AGAGTTTGA TCCTGGCTCAG-3') and 1492R (*E. coli* position 1510–1492, 5'-GGCTACCTTGTTACGACTT-3'). ITS1 forward primer (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 reverse primer (5'-TCCTCCGCTTATTGATATG C-3') were used for amplifying fungal DNA. Genomic DNA amplification was carried out using a thermal cycler (Eppendorf-Mastecycler, Germany). PCR conditions included 4 min at 96 °C, 30 cycles (20 s at 95 °C, 15 s at 55 °C, 2 min at 72 °C) and 20 min at 72 °C for 16S rRNA amplification and 4 min at 96 °C, 32 cycles (20 s at 96 °C, 20 s at 54 °C, 1 min 30 s at 72 °C) and 10 min at 72 °C for ITS amplification. PCR products were purified and sequenced in an ABI 3500 Series Genetic Analyzer (USA). Sequence analysis was done using BLAST (blastn) search against the nucleotide database at NCBI (<http://www.ncbi.nlm.nih.gov/blast>).

The culturing of microorganisms in combination is essential for improving biodegradation [11]. Four potent PLA degrading strains were used for consortium

development. The biocompatibility of these strains was investigated by cross-streaking and parallel streaking methods. The viability of cells in consortium broth was checked at regular intervals. The strains were used in different combinations such as combinations of 3 or 4 [12]. For the inoculums of strains as consortia, each strain was added in equal proportion for the fixed final inoculums such as 2×10^8 CFU/mL as different strains have different growth profile.

The biodegradation was performed by adding biomass of active consortium harvested from 50 mL of a mid-exponential phase culture, that were maintained in basal mineral medium with PLA powder and gelatin as carbon source, into 100 mL of minimal broth containing PLA film as sole source of carbon. The flasks were kept in a shaker (150 rpm) at 37 °C. After the consortium had attained its stationary growth phase, degraded PLA film samples were recovered from the broth. The supernatant was analyzed for lactic acid production and pH. The recovered films were monitored to calculate the residual film weight and to check the morphological changes using SEM imaging.

Lactic acid concentration in the medium was estimated before and after inoculation at regular intervals by Baker and Summerson [13]. Changes in pH during the degradation process were analyzed at various time intervals during incubation.

The bacterial film colonized on the polylactide surface was washed out with 2 % (v/v) aqueous sodium dodecyl sulfate (SDS) solution and further washed with sterile distilled water several times. The polylactide film samples were collected on filter paper and then dried at 50 °C. Residual film weight was taken to determine the extent of degradation using microbial consortium.

The biodegradation of plastic film was calculated in terms of percentage of film weight loss using the following formula:

Percentage weight loss of film

$$= \left(\frac{\text{Initial weight of film} - \text{weight of film after degradation}}{\text{Initial weight of film}} \right) \times 100$$

Initial weights of the pre-incubated polylactide samples were measured using the same procedure mentioned above.

A compost pile made of soil (70 %) and kitchen waste comprising vegetable waste, wood chips, fruit peels, etc. (30 wt%) were used for assessing the biodegradability of the PLA sheets. The formulation was sprayed and mixed thoroughly with the compost pile and incubated for a week by mixing it at regular intervals. For composting in controlled laboratory conditions, the compost pile was autoclaved prior to the fixing of PLA sheets to ensure that the degradation is solely due to the consortium. The temperature, moisture

content, and pH of the pile were in the range of 40–60 °C, 45–50 % and 6 respectively. But composting under natural conditions was done as an outdoor experiment, which provided a realistic environment with climatic changes. In parallel, a blank without any added inoculum was kept as a reference. PLA films supported by petri dishes and PLA films as such were both placed inside the pile after the conditioning of the pile for a week. The composting process was continued for 30 days after the addition of the test samples. Triplicates of each sample were run, and aeration was provided by mixing the sample at regular intervals. The PLA sheets were visually inspected for color and shape after they were removed from the compost.

Two types of PLA samples were analyzed, one incubated with the consortium and the other was the un-inoculated film control. The polylactide films were removed from the culture medium at a pre-scheduled time to observe bacterial colonization and surface erosion. The samples were washed for 2 min in 10 mM phosphate buffer (pH 7.2) to release excess medium. In contrast to the procedure for the examination of surface erosion, polylactide samples were washed with a 2 % SDS followed by warm distilled water to thoroughly remove surface adhered cells. The samples were dried in a vacuum, gold coated at 25 mA in Argon (Ar) atmosphere at 0.3 MPa and scanned in a Joel JEE 10000 electron microscope (Japan).

Tributylin agar plate screening method was employed for isolating esterase producing micro-organisms. The zone formation was monitored after 48 h of incubation at 37 °C. Lipase producing bacteria can be specifically detected using rhodamine-olive oil-agar plate assay and the positive strains were selected by orange fluorescent halos formation around bacterial colonies under UV irradiation [14].

Out of the 300 samples screened, a total of 45 morphologically different microbial growths appeared on emulsified plates. Some of them gave zone of clearance (S. Figure 1).

Four of them turned out to be potent isolates that utilized polylactide films as a sole source of carbon. The percentage of film weight loss in each case determined after 15 days of incubation were given in Table 1. The presence of lactic acid in the supernatant is an important indication of PLA depolymerisation since the lactic acid concentration in film control flasks was null. Assimilation of initial lactic acid might have led to the continued growth of the microbes on the film surface. After 3 weeks of incubation, most of the lactic acid was removed from the medium that clearly depicts the maximum growth of microbes and utilization of PLA monomers during this period of incubation (Fig. 1).

Based on 16S rRNA and ITS sequence homology, isolates IS102, IS121, IS143 and IS148 showed 99 % homology with *Cladosporium sphaerospermum*, *Rhodotorula mucilaginosa*, *Penicillium chrysogenum* and *Serratia marcescens* respectively. These strains were selected for formulating a consortium for PLA degradation (S. Figure 2). Biocompatibility of these four isolates was tested so that they can be used as a formulation or consortium to degrade PLA. In flask level studies, consortium of four strains proved more effective than when inoculated individually and combinations of three strains with PLA films as the sole source of carbon (S. Figure 3).

The mechanism involved in the biodegradation of plastics in a composting environment is predominantly hydrolysis incorporated with aerobic and anaerobic microbial degradation. Every 10 days, PLA sheets (100 mg each) were visually inspected for color and shape after they were removed from the compost. Small white colored spots on the surface of the PLA sheet could be visually observed. The PLA sheets were deformed and changed from clear to white opaque. We obtained 27 % film weight loss after 15 days of incubation. After 20 days of incubation, they became brittle and started breaking apart. Only a few pieces of PLA were observed after a month of incubation. After that, it was hard

Table 1 Film weight retained after 15 days of biodegradation by individual isolates and consortium

Sample	Inoculum type	Residual film weight after 15 days (mg)
1	Film control	99.6
2	<i>Rhodotorula mucilaginosa</i>	96.6
3	<i>Cladosporium sphaerospermum</i>	93.9
4	<i>Penicillium chrysogenum</i>	84.8
5	<i>Serratia marcescens</i>	82.0
6	<i>P. chrysogenum</i> + <i>C. sphaerospermum</i> + <i>R. mucilaginosa</i>	81
7	<i>C. sphaerospermum</i> + <i>R. mucilaginosa</i> + <i>S. marcescens</i>	82
8	<i>R. mucilaginosa</i> + <i>S. marcescens</i> + <i>P. chrysogenum</i>	75
9	Microbial consortium	73.1
Initial film weight—100 mg		

Fig. 1 Lactic acid concentration in the medium during the time course study using PLA films. A film control, B *Serratia marcescens*, C *Cladosporium sphaerospermum*, D *Penicillium chrysogenum*, E *Rhodotorula mucilaginosa*

to detect any PLA residues through visual inspection (S.Fig. 4 and S.Fig. 5). Toshinori et al. (2002) investigated that PLA soft film samples degraded within 3 weeks [15]. A microbial consortium fragmented PLA films after 35 days of incubation in the natural environment [16].

SEM images revealed that at first the surface of PLA films was smooth and was significantly eroded when subjected to composting with the microbial consortium. Many bacteria/actinomycetes and mycelial structures of fungi adhered on the film surface were visible. Surface erosion patterns and cracks after the growth of microbes were also seen on PLA film samples when washed to remove the microbial growth (S.Fig. 6).

The hydrolysis involving enzymes is affected by the process of interaction between enzymes and plastic polymers [17]. All four isolates were esterase and lipase positive (S. Figure 7). The esterase enzyme from *B. smithii* exhibited PLA degradation [2]. Liu et al. [18] monitored polymer degradation (i.e. PLA and poly- ϵ -caprolactone) with the combination of proteinase K and *Pseudomonas* lipase.

Biodegradation studies to explore the ability of mixed microbial population for PLA degradation succeeded in formulating a consortium comprising four potent PLA degraders. The formulation effectively degraded PLA films in the compost within 15 days. Enzyme profile of the microbes in the consortium was vivid and active. Thus, applying this consortium or individual cultures can help broadly in degrading PLA at a much faster rate which otherwise a rate limiting step in the natural degradation.

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