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ISOLATION AND CHARACTERIZATION OF POLYLACTICACID DEGRADING BACTERIA AND ITS DEGRADATION POTENCY

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ABSTRACT

Polylactic acid (PLA) is a bioplastic used as an alternative to synthetic thermoplastics. It is widely used in many applications and primarily in packaging, textiles and in biomedical field. Microbes play a vital role in the degradation of bioplastics. The present study aims to isolate PLA degrading bacteria from agriculture soil, garbage soil and composted coirpith.PLA degrading bacteria was isolated by spreadplate technique in minimal medium supplemented with emulsified PVA. The bacteria isolated was identified by 16S rRNA sequencing. The isolated bacteria was used to evaluate its degradation potential of PLAby soil burial test. PLA degrading bacteria was present only in agriculture soil and no bacteria was isolated from garbage soil and composted coir pith. PLA degrading bacteria isolated from agriculture soil was identified as Pseudomonas resinovorans. It degraded 11.36% of PLA in 30 days. FTIR spectrum of PLA film after 30 days of degradation by Pseudomonas resinovorans confirmed loss of ester bond. No loss of ester bond was observed in negative control maintained without Pseudomonasresinovorans.

Keywords: Polylactic Acid, FTIR, Pseudomonas resinovorans, biodegradation.

INTRODUCTION

Plastics constitute a major proportion of municipal waste. Plastic bags, bottles, carry bags etc. contribute to 10-13% of the total inorganic content of sewage waste (Kibria et.al.,2023). Sigle use plastic products account for 50% of the plastic wastes(Duru et.al.,2019). Pollution by plastics will increase beyond 300 million metric tons in five years (Millican and Agarwal, 2021).Plastics limit the aeration in the soil and pollute the environment (Paletta et al., 2019). To overcome the white pollution, PLA was introduced as an alternative to synthetic thermoplastics (Tokiwa and Calabia,2006).PLA is considered to be “double green plastics” (Gu, 2017).The properties of the bioplastic PLA is similar to the synthetic plastics (Sinclair,1996; Shah et.al.,2008). It possesses good mechanical property, transparent and biocompatible. All these desirable attributes make them suitable for packaging(Murariu and Dubois, 2016). The demand for PLA is expanding in all walks of life. It is also used textile industry, medicine, vehicle manufacturing, 3D printing.

PLA is a hydrolyzable aliphatic semicrystalline polyester produced by *Lactobacillus amylophilus, Lactobacillus bavaricus*etc (Singhvi et.al., 2019). Anaerobic glycolysis of carbohydrates produces lactic acid, which is then converted to poly-lactic acid (PLA) by condensation. PLA is degraded by mechanical (Chariyachotilert et.al.,2012) and chemical methods (Boonmee, et.al.,2022;Krause, and Townsend,2016). Bioremediation of plastics by microbes reduce their toxicity as well as their concentration (Janczak and Dąbrowska, 2018).In spite of its biological origin, biodegradationof PLA takes a longer period (58% in 60 weeks) to degrade due to its hydrophobicity. Degradation rate of PLA film was very slow accounting for the piling up of wastes in the environment (Janczak et al., 2020).

A lab investigation found that a single degrading bacterium (*Delftiatsuruhatensis*) has a limited ability to degrade PLA/PBAT films, with a degradation rate of only 6.87% in 7 days (Jia, et.al.,2020). Degradation of PBAT film by *Bacillus pumilus* was reported to be at the rate of 12.2 g/day/cm^2 at 30°C-40°C and 120 r/min (Muroi et al., 2017). Few bacteria reported to degrade PLA are *Pseudomonas* sp., *P. geniculate*, *Pseudonocardia* sp. and *Serratia* sp.

(Apinya et al., 2015; Bubpachat et al., 2018; Janczak et al., 2018). Yoshida et al. (2016) used the strain *Ideonellasakaiensis* for PET biodegradation. The source of xenobiotics degrading bacteria are soil (Hrynkiewicz et al., 2010).The present study was aimed to isolate a potent PLA degrading bacteria from soil and to evaluate its degradation efficiency.

MATERIALS AND METHODS

2.1 Materials

The PLAfilm was provided by ReyaPack, Thiruthangal, Sivakasi-626005, Tamil Nadu, India. To isolate PLA degrading bacteria, garbage soil at Sivakasi(9°29'49.9"N 77°49'25.4"E), outside the campus of Mepco Schlenk Engineering College, Sivakasi-626005, Tamil Nadu, andagricultural soil sample (9°32'07.9"N 77°51'16.0"E) and the composted coir pith within the College campus were collected. All chemicals used in the experiment were of analytical grade and purchased from Himedia, Mumbai, India.

2.2 Isolation of PLA Degrading Bacteria

The three different soil sources of PLA degrading bacteria used in the current study namely agricultural soil, contaminated garbage soil and composted coir pith (5g) were suspended in100mL of sterile saline kept in shaking at 250rpm overnight at 30°C. Minimal medium was prepared in 100mM potassium phosphate buffer (pH-7) using NaCl ($1gL^{-1}$), NH_4Cl ($1gL^{-1}$), $MgSO_4.7H_2O$ ($0.25gL^{-1}$) and agar ($20\ gL^{-1}$). The samples were diluted to $10^{-3}$with sterile waster and 100µl of each sample was spread on to minimal media supplemented with 1% of emulsified PVA-PBAT film.The plates were kept in an incubator at 30°C. Individual colonies observed in the plates were pure cultured and used for characterization and degradation of PLA film.

2.3 Identification of Isolated Bacteria

The PLA degrading bacterial isolate was identified by 16S rRNA gene sequencing analysis. Genomic DNA was isolated from the pure cultured bacterial isolate. Fragment of 16S rRNA gene was amplified by 27F (5`-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose gel. Sequencing of PCR amplicon was carried out using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The 16S rRNA gene sequence was used to BLAST with the NCBI Genbank database. Based on the maximum identity score first ten sequences were selected and aligned using the multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

2.4 Biodegradation Test of PLA film by Bacterial Isolates

The PLA degradation efficiency of the isolate was assessed by soil burial test. To ensure that the degradation of PLA occurs in soil under natural conditions, the agriculturesoil was sterilized and sterilization was confirmed by zero colony in viable plate count. The soil used for burial test was amended with 50mL of PLA degrading isolate with the absorbance of 0.3 at 600nm.Moisture in the soil sample was adjusted to 45% with sterile distilled water. The PLA film wascut into a size of 5*5cm and sterilized with 70% ethanol. The samples were aseptically buried in the soil at 7-8 cm depth. The rate of degradation of PLA film was determined from the weight loss with time done by using the formula, Weight loss(%) = (Initial weight - Final weight)/Initial weight *100. The experiment was replicated thrice and the data represented are the mean of three replicates. FTIR spectrum of the PLA film before and after degradation by the isolate was recorded.

RESULTS AND DISCUSSION

3.1 Isolation and Identification of PLAFilm Degrading Bacteria

The minimal media supplemented with emulsified PLA plates used for isolation of PLA degrading bacteria from garbage soil and composted coirpith showedno colonies. Agriculture soil showed the presence of large number of colonies of single type of bacteria. Isolation of PLA degrading bacteria using emulsified PLA agar plates was supported by Nishida and Tokiwa (1992). Studies by Suyama et al. (1998) Documentation that PLA degrading bacteria are not widely distributed. Ikura and Kudo (1999) also reported that out of 50 different samples, only two

strains degrading PLA was identified. The genomic DNA and the amplicon of the 16S rRNAisolated from the PLA degrading bacterial isolateare shown in Figure 1.

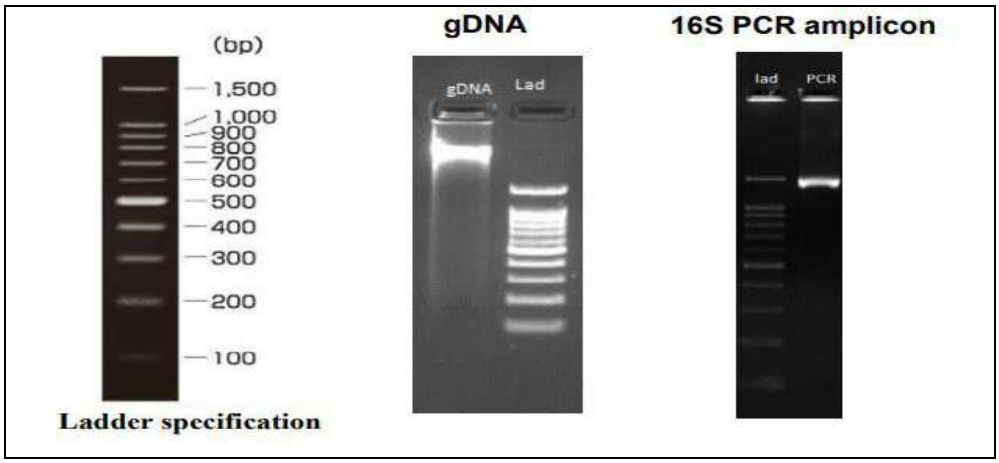


Figure 1. Genomic DNA and 16S rRNA of PLA Degrading Bacteria

Based on the 16S rRNA sequence, PLA degrading bacterial isolate was identified as *Pseudomonasresinovorans.* The evolutionary history of the PLA degrading *Pseudomonas resinovorans*was inferred using the Maximum Likelihood method based on the Kimura 2- parameter model (Kimura,1980). Figure 2 represents the phylogenetic tree of *Pseudomonas resinovorans*

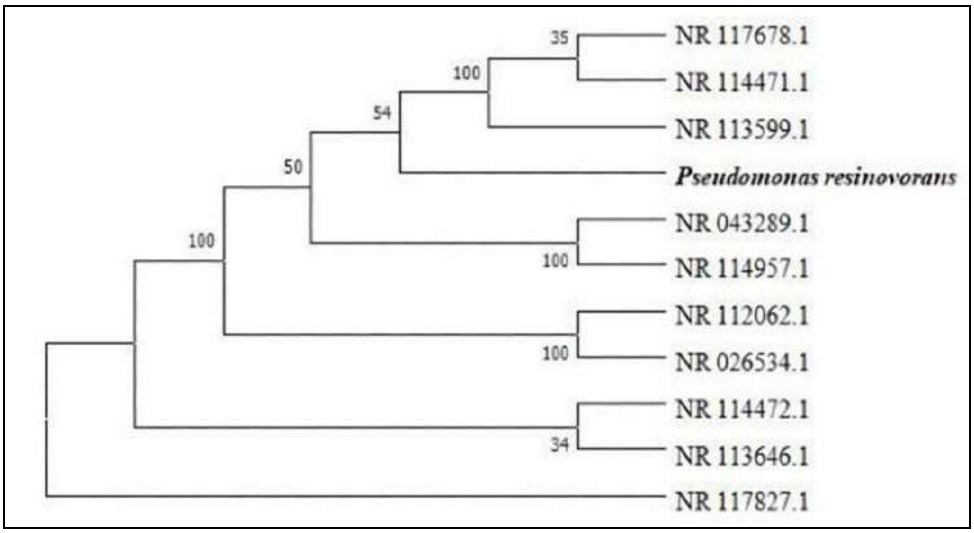


Figure 2. Phylogenetic Tree of PLA degrading *Pseudomonas resinovorans*

Soil is a great source for the presence of dynamic array of different microbial population with varying potential for degradation of xenobiotics. Knowing the ecological distribution and taxonomy of polymer degrading bacteria enables to develop a consortium of PLA degrading bacteria and unravel the mechanism of degradation.

3.2 Biodegradation of PLA Film

3.2.1. Soil Sterilization

To ascertain the degradation of PLA film by the isolated *Pseudomonas resinovorans*and not by the bacteria present in the soil, the soil samples were autoclaved. The results of sterilization is shown in Figure 3. Figure 3A represents the bacterial colonies present in the unsterilized soil and Figure 3B represents sterilized soil without any bacteria. The absence of colonies in the sterilized soil confirmed the absence of live bacteria in the soil.

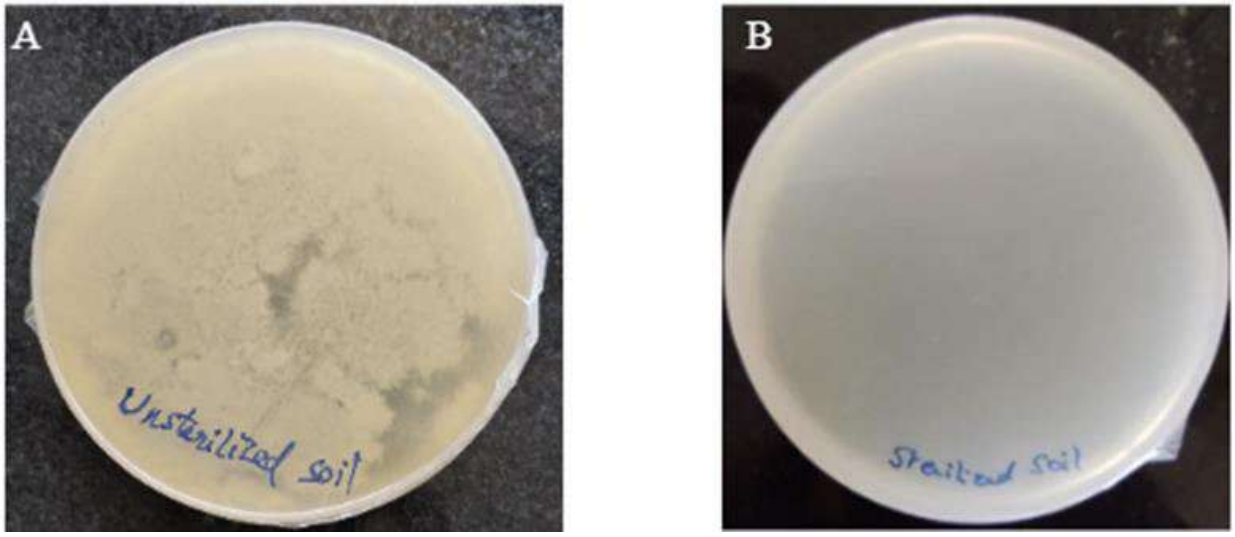


Figure 3: Unsterilized SoilSterilization of soil

3.2.2. Biodegradation of PLA Film

The degradation of PLA film in soil was assessed for 30 days. The results of biodegradation of PLAby natural auto degradation and degradation by the isolated *Pseudomonas resinovorans*are represented in Figure 4.*Pseudomonas resinovorans*degraded 6.65% on 10th day and it increased to 8.98 and 11.36% on 20th and 30th day respectively.

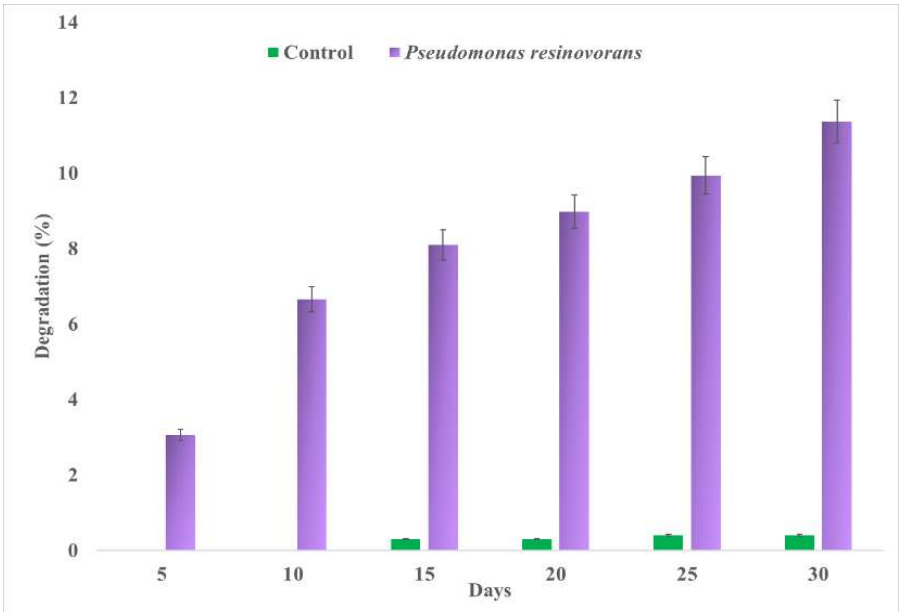


Figure 4: Biodegradation of PLA Film

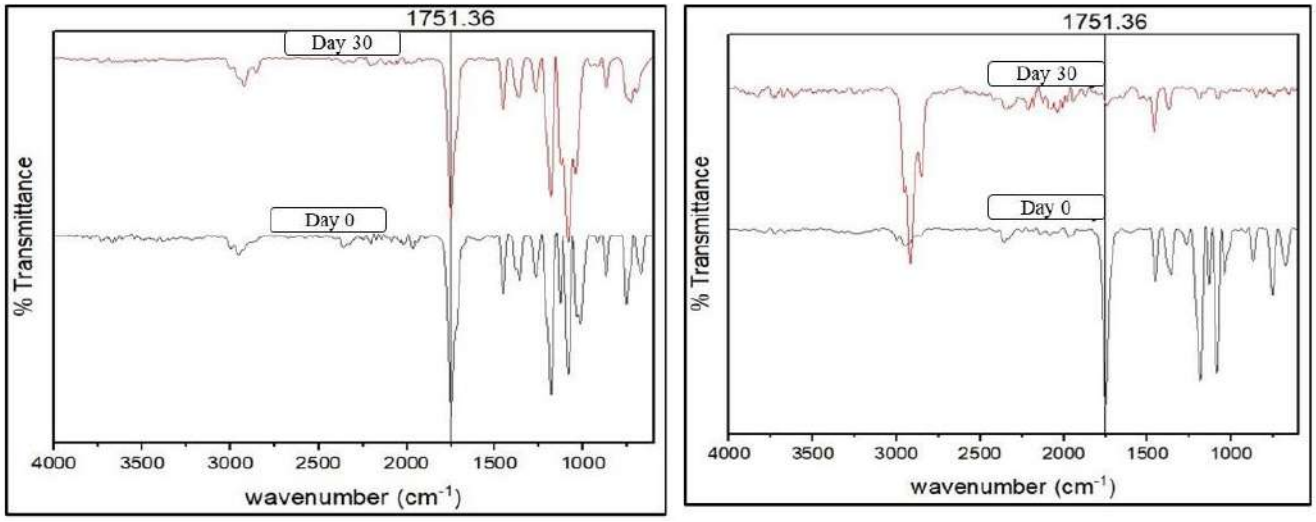
Biodegradation of PLA film is determined by many factors (Ranakotiet al., 2022). The molecular weight of the PLA film, additives used during the manufacturing of the film, degrading environmental conditions and the microbial growth rate (Haider et.al.,2018).

The present rate of degradation was significantly more than what is reported in earlier studies. Reports by Ohkitaand Lee (2006) documented that degradation of PLA was initiated only after six weeks. SimilarlyUrayama et al. (2002) showed that the molecular weight of PLA decreased only after 20 months in the soil. During

degradation of the polymers, the bacteria secrete depolymerases to break the larger molecules to short chained oligomers, dimers and monomers which can easily penetrate into the bacteria(Lunt et.al.,1998; Shahar et.al.,2022).

FTIR spectrum of PLA film buried in the soil without and with *Pseudomonas resinovorans*areshowedin Figure 5A and 5B respectively. PLA film before degradation showed peaks at 675 cm^{-1}, 756.10 cm^{-1}, 869.90 cm^{-1}, 1012.63, cm^{-1} 1085.92 cm^{-1}, 1130.29 cm^{-1}, 1184.29 cm^{-1}, 1269.16 cm^{-1}, 1359.82 cm^{-1}, 1452.40 cm^{-1}, 1751.36 cm^{-1}, 1963.53 cm^{-1}, 2144.84 cm^{-1}, 2360.87 cm^{-1}, 2953.02 cm^{-1} and 2997.38 cm^{-1}. The peak at 1751.36 cm^{-1} represents the ester bond. Peak at 2953.02 cm-1 is due to symmetric CH_3groups and 1085.92cm^{-1} is ascribed to symmetric CH_3group (Chieng et.al.,2013). The peak at 1184.29 cm^{-1}, corresponds to C–O–C stretching (Mofokeng et.al.,2011), peak at 2995 cm^{-1}, corresponds to $CH_2$and 1184 is due to bending of C=O (Tyagi et.al.,2006).

FTIR spectrum of PLA film buried in the soil without *Pseudomonas resinovorans* termed as negative control (Figure 5A) showed the peaks same as that of neat PLA film. FTIR spectrum of PLA degraded by *Pseudomonas resinovorans*(Figure 5B) showed the loss of peak at 1751.36 cm^{-1} indicating the hydrolysis of esterbond. Simultaneously, the intensity of the peak at 2997.38 cm^{-1} was increased due to the release of monomer lactic acid from the degraded PLA. Small peak at 1652 cm^{-1} alsorepresents the presence of monomeric lactic acid. Peak at 1184.29 cm^{-1}, the characteristics of PLA was not observed in FTIR spectrum of PLA degraded by*Pseudomonas resinovorans*in 30 days.Many peaks present in PLA between 1000 $cm^{-1}$and 1500 cm^{-1}were absent in the FTIR spectrum of PLA degraded by *Pseudomonas resinovorans*.



A) Control **B) Degraded by *Pseudomonasresinovorans***

Figure 5: FTIR Spectrum of PLA

CONCLUSION

The PLA degrading bacteria isolated from the agricultural soil was identified as *Pseudomonas resinovorans*. Itdegraded 11.36% of PLA film in30 days.

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