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DETERMINATION OF ESTERASE TYPE OF POLY LACTIDE DEPOLYMERASE FROM A COMPOST THERMOPHILE

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ABSTRACT
Compost samples were screened for isolation of PLA degrading thermophilic bacteria by the standard clear zone method at 50ºC and the degradation was observed on 20th day of incubation. The thermophilic isolate was identified as a Gram positive, rod shaped, non capsulated and spore forming bacterium. Based on biochemical characteristics and 16S rDNA gene sequence analysis the isolate was identified as *Bacillus licheniformis*. The PLA depolymerase was extracted and characterized as an esterase. The present study among the inducers tributyrin and PLA showed remarkable induction in esterase type of PLA depolymerase in *Bacillus licheniformis* on comparison with casein and gelatin

INTRODUCTION

Bio-based plastics are considered as preferable alternative to current petroleum based plastics to reduce the environmental pollution. Biodegradable polymers offer a viable substitution to commodity plastics in a number of applications. Among the biodegradable plastics, poly lactic acid (PLA) is gaining worldwide attention due to its synthesis from cornstarch by microbial fermentation or renewable resources such as cane molasses (Tsuji, 2005). Moreover, PLA is considered to be a very good polymer for various end use applications based on its physical and mechanical properties. In spite of its great demand in packaging industry, little information is available on the biodegradation of PLA. The enzymatic hydrolysis of PLA by commercial lipases was reported at 60º - 100ºC in organic solvents (Takahashi and Calabia, 2004). It is well known that PLA degrading microorganisms are not ubiquitous in the natural environment and thus PLA is less susceptible to degradation compared to other microbial and synthetic aliphatic polyesters (Tokiwa *et al.,* 2006). The degradation of PLA was first reported in actinomycete *Amycolatopsis* (Pranamuda *et al.,* 1997). Among the PLA degrading microorganisms, actinomycetes were found to be the predominant ones on comparison with bacteria and fungi. The fungal strains, *Penicillium roqueforti* and *Fusarium moniliforme* were showed to assimilate lactic acid and PLA degradation ability has been reported with *Tritirachium album* (Tokiwa and Calabia, 2006). The present study is designed to screen the compost samples for PLA degrading thermophiles, to determine the nature of PLA depolymerase and to identify the inducers of PLA depolymerase.

MATERIALS AND METHODS

The PLA used in the present study was a gift from Nature-tech Ltd, Chennai. Prior to utilization, the PLA film was rinsed with 70 % absolute alcohol and irradiated with ultraviolet light. PLA (Poly L lactide) was kindly supplied by Purac Biomaterials, Netherlands. Compost samples were collected from various places of Chittoor district, AP, India. Unless otherwise stated all the other chemicals used were of analytical grade.

Degradation of PLA by compost thermophiles

A basal medium containing PLA-1g/L, K_2HPO_4-2.34g/L, KH_2PO_4-1.33g/L, $MgSO_4.7H_2O$-0.2, $(NH_4)_2SO_4$-1g/L, NaCl-0.5,yeast extract-0.06, at pH 7.0 was used for isolation of PLA degrading thermophiles from the compost sample. The isolates forming clear zone around the colonies were isolated. The degradation of PLA by isolate 3 was further analyzed by measuring the growth of bacteria in the liquid culture with PLA film (2×4cm) as carbon source and by determining the concentration of lactic acid in the liquid culture.

Scanning electron microscopy

The morphology of the PLA film degraded by isolate 3 was examined using a SEM microscope (CARL ZEISS, model EVO MA 15). Prior to the analysis, the sample was dried overnight and coated with gold to protect the sample morphology against electron beam.

Identification of PLA degrading bacteria

The isolate 3 was characterized based on biochemical analysis (Mackie and McCartney, 2006; Sneath *et al.,* 1994) and 16S rDNA was amplified with universal primers and nucleotide

sequence was determined with an applied biosystems 3730 XL sequencer. The sequence was multiple aligned with representative sequences of selected genera using CLUSTAL W program. The phylogenetic tree for the datasets was utilized to access the relationship between the organisms.

Assay of PLA depolymerase

Isolate 3 was inoculated into the basal medium containing PLA film (2×4 cm) and incubated at 50ºC for 20 days. The culture broth was taken at appropriate time intervals of cultivation and was centrifuged at 10,000 rpm for 30 min, and then the supernatant is used for enzyme assay. Esterase and protease activities were determined using p-nitrophenyl acetate and casein as substrates respectively.

Esterase activity of PLA depolymerase was determined as per the method of Kay *et al*. (1993), whereby the liberation of p-nitrophenol from p-nitrophenyl acetate was monitored spectrophotometrically at 405nm. One unit is defined as the amount of enzyme required to liberate 1μ mole of p-nitrophenol per minute at 30ºC. Similarly protease activity of PLA depolymerase was measured at 275 nm using UV-VIS spectrophotometer (UV 1801 Schimadzu) (Kunitz, 1947). The typical reaction mixture contained 50 mm Sodium phosphate buffer (pH 7.0) with 0.6 % casein and the reaction was initiated by the addition of 0.3mL PLA depolymerase and incubated at 30ºC for 1h. One unit of protease activity was defined as the amount of enzyme required to liberate 1μ mole equivalent of L-tyrosine per min. Protein concentration of PLA depolymerase was determined using bovine serum albumin as standard. (Lowry *et al.,* 1951)

Effect of inducers on enzyme production

To determine the effect of inducers on PLA depolymerase production different inducers like yeast extract, peptone, glycine, alanine, tributyrin, PLA, casein and gelatin were added at a concentration of 0.1% to the basal medium. Isolate 3 was inoculated into the basal medium containing various inducers and incubated at 50ºC for 1-20 days. At appropriate time intervals, the culture broth was harvested and centrifuged at 10,000rpm for 30min. The supernatant was used for measuring the esterase activity.

RESULTS AND DISCUSSION

In the present study, compost samples were screened for isolation of PLA degrading thermophilic bacteria by the standard clear zone method at 50ºC. Four PLA degrading strains were successfully isolated based on their ability to form clear zone around the colonies on emulsified PLA agar plates. The isolates varied in their morphological and physiological properties. Among the four isolates, the isolate 3 was found to be effective PLA degrading bacteria due to its ability to form a large hydrolytic zone (14mm) on PLA emulsified agar medium (Fig. 1). The clear zone method is the most widely used method for isolating polymer-degrading microorganisms (Nishida and Tokiwa, 1993). The extracellular hydrolyzing enzymes secreted by the target organism hydrolyze the suspended polyesters in the turbid agar medium into water soluble products thereby producing zones of clearance around the colony.

As shown in Fig. 2 the PLA stimulated the growth of isolate 3

Table 1: Biochemical characteristics of *Bacillus licheniformis* PLA3

Characteristics	*Bacillus licheniformis* (Isolate 3)
Colony morphology	Irregular
Cells- shape	Rod
Gram stain	+
Growth in enrichment media	
Nutrient agar	+
Spores	+
Capsule	-
Growth at 50ºC	+
Growth at pH 5.7	+
Growth at 7% NaCl	+
Catalase	+
Oxidase	+
Indole	-
Methyl red	-
Citrate	-
Voges Proskauer	+
Triple Sugar Iron Agar	Alkaline slant acid butt, No H2S production.
Urease	+
Nitrate reduction	+
Arginine dihydrolase	+
Levan sucrose production	+
Hydrolysis of	
Starch	+
Casein	-
Gelatin	+
Tributyrin	+
Utilization of sugars	
Dextrose	+
Maltose	+
Xylose	-
Arabinose	+
Mannitol	+
Galactose	-
Lactose	+
Sucrose	-
Sorbitol	-

Figure 1: Clear zone formation due to hydrolysis of PLA by *Bacillus licheniformis* on PLA emulsified agar plate

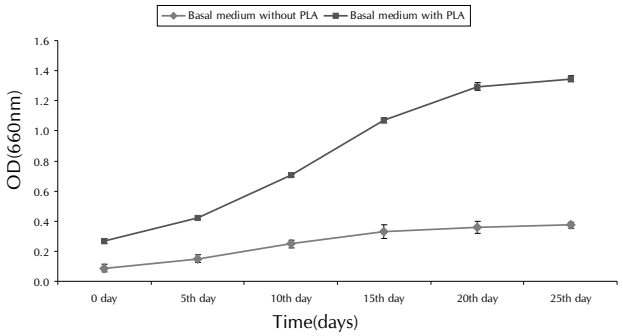

Figure 2: Growth profiles of *Bacillus licheniformis* in the presence and absence of PLA. The data indicates the mean of three independent experiments Mean ± SEM. p < 0.001

from the initial stage and progressed, but in control (without PLA) the absorbance increased only at the initial stage and decreased gradually. This indicates that initially the growth relied upon a small amount of soluble carbon source in the medium and then later the microorganism utilized PLA as carbon source. Isolate 3 showed remarkable degradation of PLA film after 20 days of growth at 50ºC. The present strain differs from other thermophiles like *Bacillus smithii* (Sakai *et al.*, 2001) and *Bacillus licheniformis* (Kim *et al.,* 2008) which showed optimal growth and degradation at 58ºC and 60ºC respectively. The PLA degrading thermophilic bacteria might be scattered throught out the *Bacillaceae* family (Tomita *et al*., 2004). The concentration of lactic acid in the medium increased gradually from 15th to 20th day of degradation and pH dropped to 4.5. The PLA film gradually became opaque and its structure being markedly modified due to the growth of *Bacillus licheniformis*.

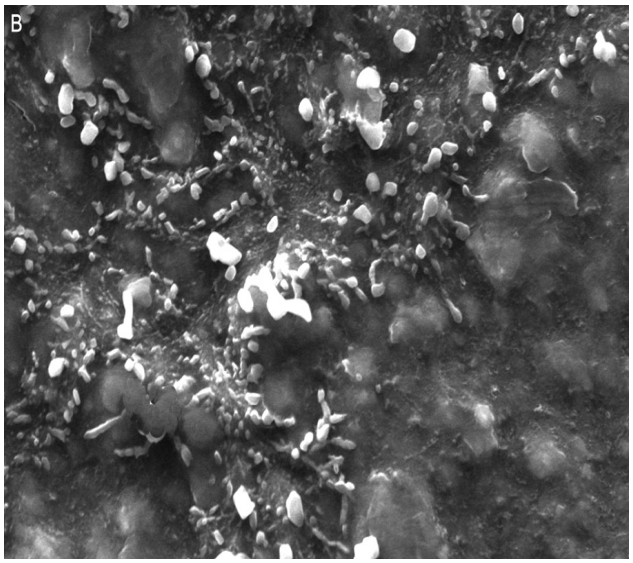

Figure 3: Scanning electron microscope image (5.00 KX at 20.00 kV) of PLA film degraded by *Bacillus licheniformis*

The surface of the PLA film appears to be smooth in the absence of PLA degrading bacteria on comparison with the rough eroded structure of the film in the presence of isolate 3 (Fig. 3).The isolate 3 was identified as a Gram positive, rod shaped, non capsulated and spore forming bacterium. Its biochemical characteristics are summarized in Table 1

Table 2: Effect of inducers on activity profile of PLA depolymerase (esterase) *Bacillus licheniformis*. The data indicates the mean of three independent experiments, Mean ± SEM. p < 0.001

Inducer	U/mg
Yeast Extract	15
Peptone	19
Glycine	20
Alanine	25
Tributyrin	45
PLA	40
Casein	10
Gelatin	15

Sequence analysis of the 640 bp of 16S rDNA gene of isolate 3 showed 98.87% homology with *Bacillus licheniformis* and its 16S rDNA gene was deposited in the GenBank nucleotide sequence database under accession number JF 825308. The phylogenetic tree was drawn by comparing *Bacillus licheniformis* with other PLA degrading bacteria. As indicated in figure: 4, it is evident that the present strain phylogenetically belongs to a quite different line from the others. Consequently, isolate 3 not only showed different behaviour toward PLA, but also different phylogenetic characteristics. Most of the PLA degrading strains phylogenetically belong to *Pseudonocardiaceae* family including *Amycolatopsis*,

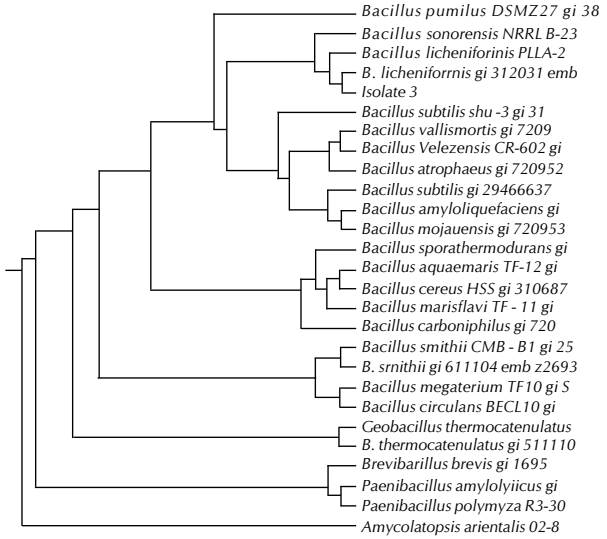

Figure 4: Phylogenetic tree based on 16S rDNA sequences comparing the isolated strain with the previously reported PLA degrading bacteria. Distances were established by using the neighbour joining method

Streptoalloteichus and *Kibdelosporangium* (Jarerat *et al.,* 2002, Tokiwa and Calabia,2004).

PLA depolymerase play a significant role in the degradation of PLA. The esterase and protease activity of PLA depolymerase was studied at 405 nm and 275 nm respectively. The crude enzyme showed only esterase activity (Fig. 5) but not the protease activity. The present findings indicate the esterase type of PLA depolymerase. Similarly, thermophilic depolymerase purified from *Bacillus smithii (Sakai et al., 2001) and Rhizopus delemar* (Fukuzaki *et al.*, 1989) showed esterase activity. *Amycolatopsis* exhibited the protease type PLA depolymerase (Nakamura *et al.,* 2001) and lipase activity was

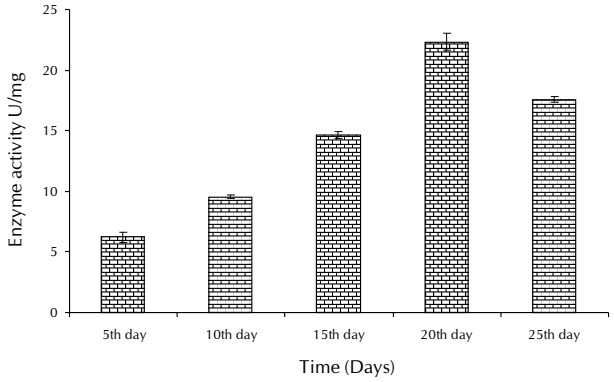

Figure 5: Enzyme activity levels PLA depolymerase (esterase) during degradation of PLA film by *Bacillus licheniformis*. The data indicates the mean of three independent experiments Mean ± SEM

reported with PLA depolymerase of *Pseudomonas* sp. DS04-T (Wang *et al.,* 2011).

The effects of various inducers on PLA depolymerase enzyme production were investigated and summarized in Table 2 Among the inducers tributyrin and PLA showed remarkable increase in esterase type of PLA depolymerase induction in *Bacillus licheniformis* on comparison with casein and gelatin. *Amycolatopsis* showed higher levels of PLA degrading activity in the presence of proteins or amino acids (Jarerat *et al.,* 2004). Inducers enter into the microbial cell and can signal the presence of the substrate and continually provide the stimulus for the accelerated synthesis of the enzyme capable of degrading PLA continually. This results clearly indicates the esterase type of PLA depolymerase rather than protease.

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