

Original Research Article

Degradation of Polylactide Plastic by PLA Depolymerase isolated From Thermophilic *Bacillus*

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A B S T R A C T

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In the present study, the compost thermophile isolated from plastic rich environment was used as a source for purification and characterization of PLA depolymerase. The depolymerase activity of *Bacillus licheniformis* was measured at 5 day interval and esterase activity of PLA depolymerase was confirmed by enzyme assay using p-nitrophenyl acetate (PNPA) as substrate. The esterase activity of PLA depolymerase markedly increased from day 5, reached maximum on day 20 and showed decreased trend in the esterase activity at later stages of degradation. PLA depolymerase was purified from the culture supernatant of *B.licheniformis* by using ammonium sulphate fractionation and ion exchange chromatography techniques. The PLA depolymerase was purified to homogeneity with a molecular mass of 44 KDa. The PLA depolymerase has optimal activity at temperature around 50°C-60°C and at pH 6 to 7. Ethyl acetate markedly inhibited enzyme activity, whereas all other solvents like hexane, isopropanol, DMSO, ethanol, methanol, acetonitrile and acetone showed only a minor inhibition in PLA depolymerase activity.

Introduction

Poly lactic acid (PLA) is the only thermoplastic polymer that is commercially produced to a relatively sizable scale at competitive price compared with petroleum based polymers. PLA is aliphatic polyester made from lactic acid ($\text{HOCH}_3\text{CHCOOH}$) monomer. PLA is a good material for production of textiles, carpet tiles, interior and outdoor furnishing, geotextiles, filtration systems etc. In addition, PLA is suitable for controlled drug delivery systems or surgical sutures (Laitinen et al., 1992). The mechanism by which PLA polymers

degrade depends upon the biological environment to which they are exposed. The environmental degradation of PLA occurs as a two-step process. During the initial phase of degradation, the water insoluble high molecular weight polyester chains hydrolyze to water soluble low-molecular-weight oligomers by attacking the chemical bonds in the amorphous phase. During the second step, the degradation process was continued through the conversion of low molecular weight components of PLA into carbon dioxide, water and humus (Kolstad et al., 2012). Biological processes by both

microbial and enzymatic activities are currently considered to be the most sustainable recycling methods for PLA degradation.

Enzymes play a significant role in the degradation of polymers, although they are not the only agents responsible for the hydrolysis of polymers. The enzymatic degradation of aliphatic polyesters by hydrolysis occurs through adsorption of the enzyme on the surface of the substrate through surface-binding domain and later hydrolysis of the ester bond (Tokiwa and Calabia.,2006). Enzymes such as lipases and PHA depolymerases cleave the ester bond of aliphatic polyesters including PLA. The hydrophobic domains of enzymes adhere to solid substrates by hydrophobic interactions before hydrolysis by catalytic domains. The activity of the enzymes like lipases is strongly dependent on the microorganism source, probably for the reason that the sequence structure of a binding domain is different, while enzymes have a common amino acid sequence around the active center regardless of microorganism species (Mochizuki and Hirami., 1997). Several enzymes like proteinase K, pronase and bromelain can degrade the PLA polymer and it has been reported that the degrading enzyme proteinase K can be derived from *T. Album* (Williams DF., 1981).

Enzymatic degradation of low molecular weight PLA is possible by using esterase type enzymes such as *Rhizopus delemere* lipase (Fukuzaki et al., 1989). The enzymatic degradation of L-PLA was examined at 50°C by using 56 commercially available proteases (Oda et al., 2000). They found that acid and neutral proteases have a little or no effect on L-PLA degrading activity but some alkaline proteases derived from *Bacillus* sp. showed appreciable L-PLA degrading activity. Considerably, little

information on the purification and characterization of LPLA degrading enzymes is available at present. Keeping in view of the present availability of limited literature about L-PLA degrading enzymes, the present study is designed to understand the role of PLA polymerase in the degradation process of polylactide.

Materials and Methods

The Compost samples were collected from various municipal solid waste dumping yards of Tirupati Municipal Corporation of Chittoor District, Andhra Pradesh, India. PolyLactide was kindly supplied by Purac Biomaterials, Netherlands. Plysurf A210G (a surfactant produced by DaiichiKogyo Seiyaku, Japan) was also a kind gift. DEAE-cellulose was obtained from Hi-Media. Standard molecular weight markers were procured from Genei, Bangalore, India. Protease inhibitors were acquired from Sigma. Unless otherwise stated, all the other chemicals used were of analytical grade.

Preparation of Cell Free Filtrate

The thermophilic bacteria isolated from the compost samples were identified as *Bacillus licheniformis* based on biochemical and molecular characterization (PremaandUma Maheswari Devi.,2012). *Bacillus licheniformis* was grown under optimized conditions and the culture filtrate was collected, centrifuged at 10,000 rpm for 30min. The supernatant was collected and further utilized for the activity assay of PLA depolymerase. Esterase activity of PLA depolymerase was determined by using p-nitrophenyl acetate as substrate.

Effect of Inducers on Enzyme Production

In order 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. Thermophilic isolate was inoculated into the basal medium containing various inducers and incubated at 50° C for 1-20 days. At appropriate time intervals, the culture broth was harvested and centrifuged at 10,000 rpm for 30min. The supernatant was used for measuring the esterase activity.

Purification of PLA Depolymerase

The cell free extract of *Bacillus licheniformis* was subjected to 20-80% ammonium sulphate precipitation by adding ammonium sulphate crystals under continuous stirring at 4°C. The supernatant with no activity was discarded and the pellet was suspended in 10mM potassium phosphate buffer (pH 7.0). The suspension was dialyzed for 24h at 4°C. The dialysed (dialyzed) enzyme sample was applied carefully to the top of the DEAE-cellulose (Hi-Media) column and elution was performed using 10 to 100mM NaCl in 10mM potassium phosphate buffer (pH7.0) by linear gradient method and 30 fractions of 3 ml were collected at a flow rate of 15ml/h. All the fractions were assayed for esterase activity at 405nm in a UV-vis Spectrophotometer (Shimadzu UV-1601). The fractions showing esterase activity were pooled. The specific activity of the purified enzyme was compared with that of the crude enzyme and the purification fold was calculated (Sujatha et al., 2012).

Quantitative Assay for PLA Depolymerase Activity

Depolymerase activity against PLA was determined by microtitre plate method. The emulsion of polylactide (0.1% w/v) was obtained by treating with Plysurf A210G (0.01%) in 10mM potassium phosphate buffer (pH 7.0). The test was performed

with 5µl of enzyme solution (ranging from 1µg, 2.5µg, 5µg and 10µg) and 95µl PLA emulsion and the plates were incubated at 37°C for 30min. with continuous shaking at 500 rpm. The control was maintained by adding 5µl of distilled water and 95µl of PLA emulsion. The decrease in turbidity of PLA emulsion was measured at 630nm. One unit (U) of enzyme activity was defined as a change of 0.001 OD per minute (Wang et al., 2011).

Characterization of PLA Depolymerase

The molecular weight of the PLA depolymerase was determined by using Sodiumdodecylsulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (Laemmli.,1970). After the completion of electrophoresis, the gel was fixed in 7.5% acetic acid solution and later visualized by staining with Coomassie Brilliant blue R-250, in40% methanol and 7% aceticacid, followed by destaining in methanol: aceticacid: water(40:5:55)(v/v).

Effect of pH and Temperature on PLA Degrading Activity of the Purified PLA Depolymerase

The purified enzyme was assayed at various pH values (pH 4.0 -pH 10.0) using emulsified PLA as substrate. The buffers include citrate phosphate (pH 3.0 – pH 5.0), potassium phosphate (pH 6.0 – pH 7.0) and Tris – HCl (pH 8.0 – pH 10.0). The enzyme activity was also studied at various temperatures (30°C - 80°C).

Effect of Metalions and Solvents on PLA Depolymerase

To detect the effect of metal ions like (Na^+ , Ca^{2+} , Mg^{2+} , Cu^{2+} , Mn^{2+} , Fe^{2+} , K^+ , Zn^{2+}) and solvents like hexane, isopropanol,

dimethylsulfoxide (DMSO), ethanol, methanol, ethyl acetate, acetonitrile and acetone on purified enzyme, metallic salt (5mM) and solvents (0.5%) were added and the residual enzyme activity was determined.

Results and Discussion

In the present study, the compost samples collected from plastic rich environment were used as a source for isolating the PLA degrading microorganisms. Based on the preliminary screening of compost samples for PLA degradation, the isolate which showed significant degradation at 50°C was selected for further study and was identified as *Bacillus licheniformis* based on biochemical and phylogenetic analysis. During the course of degradation, the PLA depolymerase activity was measured at 5 day interval for 25 days from day1 onwards. The enzymatic activity of PLA depolymerase was determined at 275nm and 405nm using casein and p-nitrophenyl acetate as substrates respectively. The PLA depolymerase exhibited esterase activity with the liberation of p-nitrophenol from p-nitrophenyl acetate. The esterase activity markedly increased from day 5 onwards and reached to maximum on day 20. Decreased trend was noticed in the esterase activity at later stages of degradation from day 21 onwards (Figure 1). The PLA depolymerase activity was not detected with casein as substrate.

This results inferred that the PLA depolymerase was an esterase. Esterase activity of *B. licheniformis* was qualitatively monitored on tributyrin agar plate due to the formation of clear zone around the colonies after two days incubation at 50°C (Figure 2). It can be easily extracted from the result that the isolate *B. licheniformis* could able to produce an extracellular enzyme that is responsible for hydrolysis of tributyrin and

can hydrolyze ester bonds with short chain. PLA depolymerase exhibited an esterase activity in *Rhizopus delemar* (Fukuzaki et al., 1989) and lipase activity with *Pseudomonas sp.* DS04-T (Wang et al., 2011). The extensively studied thermophilic depolymerase can be derived from *B. smithii* (Sakai et al., 2001). Inducers enter into the microbial cell and can signal the presence of the substrate and continually provide stimulus for the accelerated synthesis of the enzyme which is capable of degrading PLA continually. The effects of various inducers on PLA depolymerase enzyme production were investigated and summarized in Table 1. Among the inducers, tributyrin and PLA showed remarkable increase in esterase type of PLA depolymerase induction in *B. 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).

The PLA depolymerase was purified from the 20-day old culture supernatant of *B. licheniformis*, by using ammonium sulphate fractionation and ion exchange chromatography techniques. PLA depolymerase was purified with an overall purification fold of 1.8 with specific activity of 1.6U/mg. A typical purification profile of PLA depolymerase of *Bacillus licheniformis* is provided in Table 2. The active fractions were collected and analyzed through SDS-PAGE and the PLA depolymerases showed the presence of a single band with relative molecular mass of about 44KDa (Figure 3). It has been reported that the PLA depolymerase, purified from *Bacillus amyloliquefaciens* have a molecular mass of 72.5kDa (Prema and Uma maheswaradevi, 2014). In addition, the PLA depolymerase with lower molecular weight were also reported. For example, *Amycolatopsis* PLA depolymerase has a molecular mass of 24 kDa (Nakamura et al., 2001).

Table.1 Effect of Inducers on Activity Profile of PLA Depolymerase from *B.licheniformis*. The Data Indicates the Mean of Three Independent Experiments. Mean \pm SEM, p<0.01

Inducer	U/mg
Yeast extract	15 \pm 0.32
Peptone	19 \pm 0.64
Glycine	20 \pm 0.53
Alanine	25 \pm 0.26
Tributyrin	45 \pm 0.12
Polylactide	40 \pm 0.78
Casein	10 \pm 0.87
Gelatin	15 \pm 0.43

Table.2 Purification Profile of PLA Depolymerase from *B.licheniformis*

Sample	Total protein (mg/ml)	Total activity(U)	Specific activity (U/mg)	Purification fold	Protein recovery%
Crude	112	98.6	0.88	1.0	100
Ammonium sulfate (20-80%)	32	46.0	1.43	1.62	46.6
Dialysate	21	27.0	1.3	1.47	27.3
DEAE-column	7.8	12.3	1.6	1.8	12.4

Table.3 Degradation Activity of PLA Depolymerase

S.No	Concentration of PLA depolymerase in μ g	Degradation activity U/mg
1	1	3 \pm 0.04
2	2.5	29.3 \pm 0.02
3	5	42.5 \pm 0.06
4	10	52.8 \pm 0.03

Figure.1 Activity Profile of PLA Depolymerase of *B.Licheniformis* During Degradation with p-Nitrophenyl Acetate as Substrate. The Data Indicates the Mean of Three Independent Experiments Mean \pm SEM. p<0.001

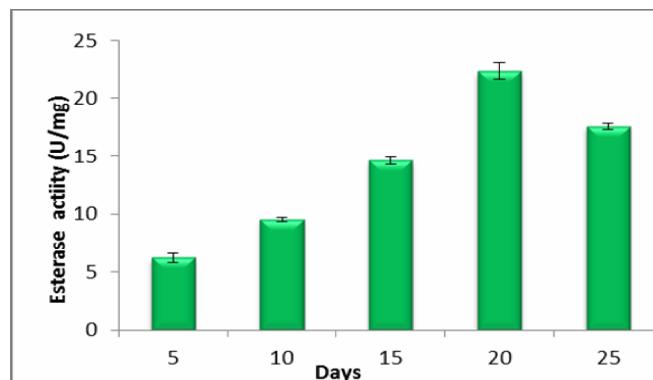


Figure.2 Hydrolysis of Tributyrin by *B.Licheniformis*

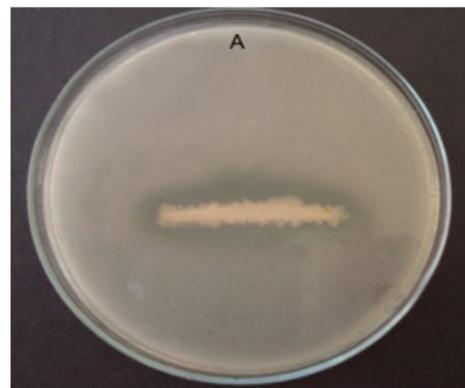
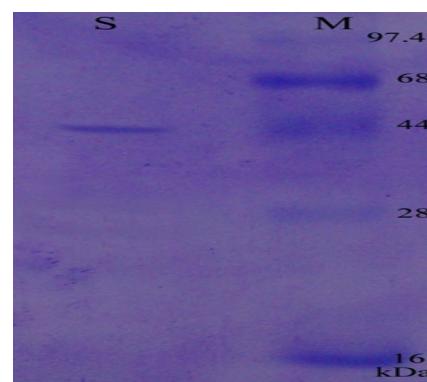


Figure.3 Molecular Weight of Purified PLA Depolymerase from *B.Licheniformis*



Lane M: Molecular Weight Markers; S: Purified Enzyme with 44kDa

Figure.4 Degradation of Emulsified Polylactide by PLA Depolymerase Obtained from *B.amyloliquefaciens*

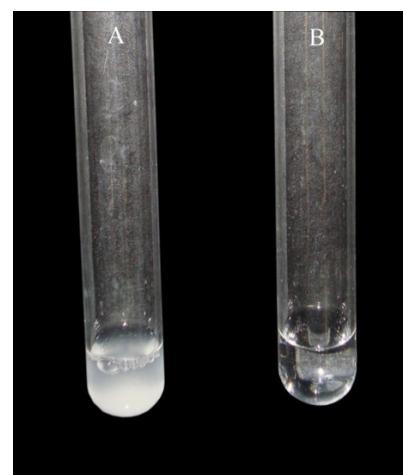


Figure.5 Effect of Ph on PLA Degrading Activity of PLA Depolymerase. The Data Represent the Mean \pm SEM from Three Independent Experiments p<0.01

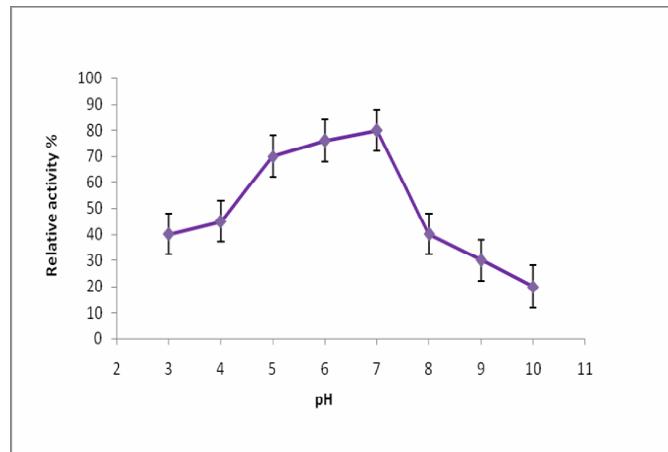


Figure.6 Effect of Temperature on PLA Degrading Activity of PLA Depolymerase. The Data Indicates the Mean \pm SEM from Three Independent Experiments p<0.001

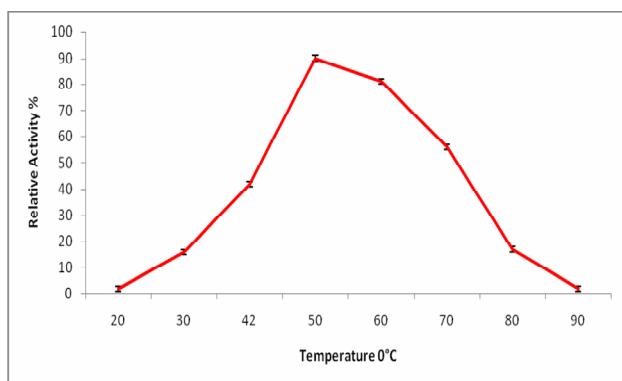


Figure.7 The Effect of Metal Ions on the Activity of PLA Depolymerase. Data Indicates the Mean \pm SEM of Three Independent Experiments, p<0.01

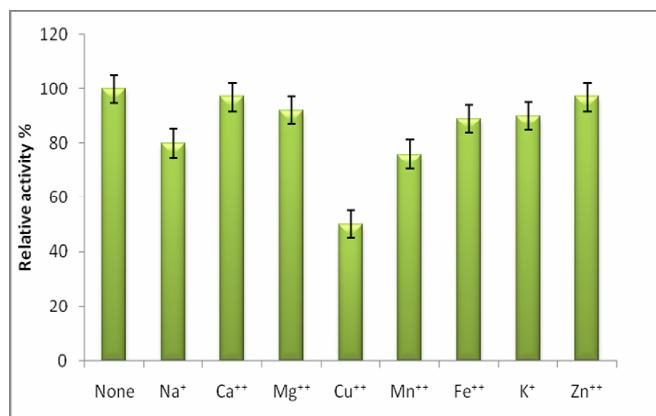


Figure.8 Effect of Solvents on the Activity of PLA Depolymerase. Data Indicates the Mean ± SEM of Three Independent Experiments, Mean ± SEM, p<0.01

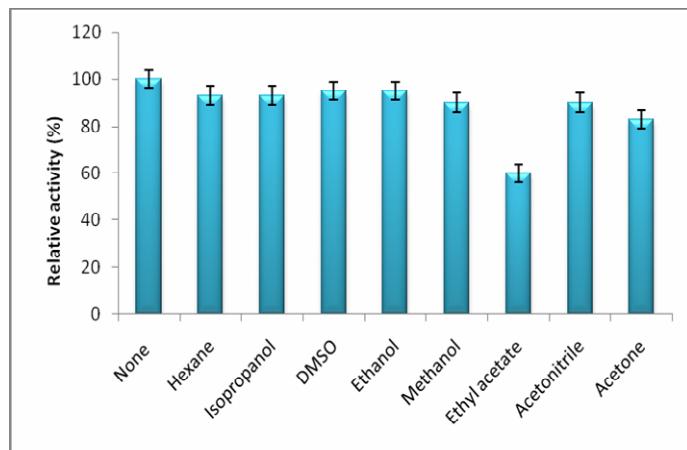
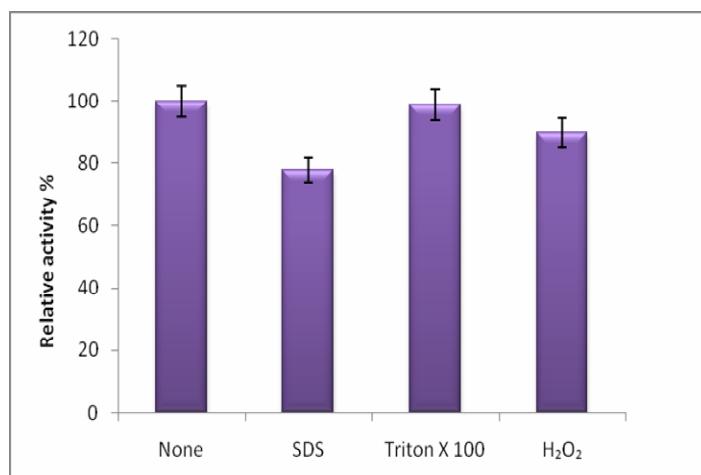


Figure.9 Effect of Additives on the Activity of PLA Depolymerase. Data Indicate the Mean ± SEM of Three Independent Experiments p<0.01



PLA-degrading enzymes produced namely PLAase I, II and III, with molecular masses of 24.0, 19.5 and 18.0kDa respectively were purified from *Amycolatopsis orientalis* (Li et al., 2008). The molecular mass of PLA depolymerase from *Paenibacillus amylolyticus* TB-13 was 22kDa and that of depolymerase from *Cryptococcus* sp. strain S-2 was reported to be 22kDa (Masaki et al., 2005). It is also reported that PLA-degrading enzyme was produced by *A. keratinilytica* strain T16-1 in liquid medium (Sukkhum et al., 2009). The degradation

potential of PLA depolymerase was assessed by the treatment of PLA emulsion with the different concentrations of purified enzyme for 30 min. As shown in the Table 3, the turbidity of the PLA emulsion decreased with the increase in the concentration of PLA depolymerase. Thus, the depolymerase enzyme purified from *B. licheniformis* degraded the PLA polymer into soluble products (Figure 4). The turbidity of emulsion decreased due to the hydrolysis of polylactide by depolymerase. The PLA depolymerase exhibited the highest activity

in the pH range of 5.0 to 7.0 with maximum activity at pH 7.0 (Figure 5). The effect of temperature on the activity of purified esterase is represented in Figure 6. The highest enzyme activity recorded in the temperature range of 50°C-60°C, indicate the thermophilic nature of the PLA depolymerase. The relationship of an esterase with degradation of PLA has been reported only for PLA depolymerase from a thermophile such as *Bacillus smithii* (Shigeno et al., 2003). The activities of PLA depolymerase from *Amycolatopsis* sp. strain K104-1 for PLA, PCL, PBS and PHB can be calculated by measuring the turbidity of solution (Matsuda et al., 2005). Metal ions were added at 5 mM concentration and the relative activity of the enzyme was determined. Mn²⁺, Cu²⁺, Na⁺ showed a marked inhibition of depolymerase activity while K⁺, Mg²⁺, Fe²⁺ showed slight inhibition of enzyme activity on comparison with other metal ions (Figure 7). The enzyme activity of PLA depolymerase was studied in the presence of solvents at a concentration of 0.5 % v/v. Ethyl acetate markedly inhibited the enzyme activity whereas, all other solvents like hexane, isopropanol, DMSO, ethanol, methanol, acetonitrile and acetone show only a minor inhibition in PLA depolymerase activity (Figure 8).

In order to detect the effect of various additives on PLA depolymerase, additives like SDS, triton X-100 and H₂O₂ were added and the residual enzyme activity was measured. SDS inhibited the enzyme activity upto 20% whereas triton X-100 and H₂O₂ showed a slight inhibition (Figure 9) only. The characterization experiments yielded more information about the biochemical nature of PLA depolymerase derived from *B.licheniformis*. The PLA depolymerase has optimal activity at temperatures around 50°C-60°C and pH 6.0

to pH 7.0. Similar results were reported with PLA depolymerases which showed highest activity at pH 6.0 and at temperature range 37°C-45°C (Pranamuda et al., 2001). PLA depolymerase produced from *Amycolatopsis* had optimum pH and temperature in the ranges of 9.5-10.5 and 55°C -60°C respectively (Nakamura et al., 2001). Three novel purified PLA degrading enzymes from *Amycolatopsis orientalis* were reported and which showed maximum activity in pH range of pH 9.5-10.5 and temperature range of 55°C -60°C respectively.

With this study, it can be concluded that depolymerase isolated from compost thermophile was an esterase and can degrade PLA under neutral pH and thermophilic conditions. The physicochemical properties of esterases derived from *B.licheniformis* are different from that of other esterases with reference to the molecular weight and optimal pH and temperature conditions.

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