

Degradation of Polylactide Film by Depolymerase from *Bacillus amyloliquefaciens*

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Abstract-: In the present study, the Poly Lactic Acid depolymerase was purified to homogeneity from the 20 day culture supernatant of *B. amyloliquefaciens*. The molecular weight of purified PLA depolymerase was 72.5 KDa as determined by SDS-PAGE. The purified PLA depolymerase shows significant activity towards PLA, casein and gelatin. On the other hand, specific activity was not significant with tributyrin and p-nitrophenyl acetate. Complete inhibition of PLA depolymerase was reported with PMSF and aprotinin demonstrates the presence of serine residue in the active site of PLA depolymerase. Differential Scanning Calorimetry (DSC) was used to analyze the thermal properties of the PLA. The glass transition (T_g) and melting temperature (T_m) of control PLA film were found to be 63.7°C and 177.28°C respectively whereas the PLA film degraded by *B. amyloliquefaciens* showed a glass transition temperature of 53.7°C and the melting temperature at 132.94°C. These results showed a significant decrease of 10°C (T_g) and 44.34°C (T_m) respectively. From the IR spectral data of PLA film control and PLA film (test) degraded by *B. amyloliquefaciens*, it was found that there was a shift of carbonyl (C=O) peak from 1756.05cm⁻¹ to 1719.50cm⁻¹ representing the degradation of PLA polymer to monomer.

Index Terms—*B. amyloliquefaciens*, compost mesophile, FT-IR analysis, PLA degradation, PLA depolymerase, poly lactide, serine protease.

1 INTRODUCTION

Polylactide or Poly lactic acid (PLA) is the only thermo-plastic polymer that is commercially produced on a relatively sizable scale on competitive cost compared with other petroleum based polymers. PLA is aliphatic polyester derived through bacterial or fungal fermentation of cassava, rice, corn and corncob. The global attention for PLA is high as it is used in different packaging materials, medical devices and also in agricultural applications as a substitute for synthetic polymers like poly ethylene and poly propylene [1]. The mechanism by which PLA polymers degrade depends on the biological environment to which they are being exposed. Biological processes by both microbial and enzymatic activities are currently considered to be sustainable recycling methods for PLA.

Literature about degradation of PLA is abundant, but understanding the mechanism of degradation is still inadequate. This is clearly due to the lack of information on the role of microorganisms in the mechanism of PLA degradation. Pranamuda *et al.*, [2] first reported the microbial degradation of PLA by actinomycetes *Amycolatopsis* strain. Majorly, the thermophilic bacteria have been reported to exhibit PLA-degrading ability such as *Brevibacillus* [3], *Bacillus smithii* [4] and *Geobacillus thermocatenulatus* [5] Mesophilic Gram-negative bacteria *Bordetella pettri*, isolated from domestic waste compost, found to be active in degradation of PLA film and showed 4% of weight loss after 40 days of incubation under mesophilic conditions [6]. Several enzymes like proteinase K, pronase and bromelain [7] can degrade PLA polymer. At first, Williams [7] reported the degradation of L-PLA by proteinase K derived from *T. Album*. Fukuzaki *et al.*, [8] showed enzymatic degradation of low molecular weight PLA using esterase-type enzymes such as *Rhizopus delemer* lipase. Oda *et al.*, [9] examined the enzymatic degradation of L-PLA at 50°C using 56 commercially available proteases. They found that acid and neutral proteases had a little or no effect on L-PLA degrading activity but some alkaline proteases derived from *Ba-*

cillus sp. showed appreciable L-PLA degrading activity. Little information on the purification and characterization of L-PLA degrading enzymes was available. Hence, the present study is designed for the purification and characterisation of PLA depolymerase from mesophilic bacterium *Bacillus amyloliquefaciens*.

2 MATERIALS AND METHODS

Polylactide bags used in the present study were provided by Nature-tech Ltd, Chennai, India. Plysurf A210G (a surfactant produced by Daiichi Kogyo Seiyaku, Japan) was a kind gift. DEAE-cellulose was purchased from Hi-Media. Standard molecular weight markers procured from Genei, Bangalore, India. Protease inhibitors were purchased from Sigma. Unless otherwise stated all the other chemicals used were of analytical grade.

A PLA-degrading mesophile, isolated from compost sample, was identified by 16S rDNA sequences analysis and designaed as *Bacillus amyloliquefaciens* MS2. The sequence of was deposited in the GenBank nucleotide sequence database under accession number JF825309. For the production of PLA depolymerase, *Bacillus amyloliquefaciens* MS2 was grown under optimized conditions and culture filtrate was collected, centrifuged at 10,000 rpm for 30min. The supernatant was collected and concentrated by ammonium sulphate precipitation initially to 20% saturation and further to 20-80% saturation by addition of ammonium sulphate. The concentrated enzyme sample was dialyzed against 10mM potassium phosphate buffer (pH 7.0). The dialysed enzyme sample was applied carefully to the top of the DEAE-cellulose (Hi-Media) column, previously equilibrated with potassium phosphate buffer (pH 7.0). Adsorbed proteins were eluted with a linear gradient of 0—1 mol/L NaCl in the same buffer. The active fractions were collected and were assayed for

esterase and protease activities at 405nm and 275nm in a UV-vis Spectrophotometer (Shimadzu UV-1601).

PLA depolymerase was separated by electrophoresis on a 12% (w/v) SDS-PAGE and it was stained to locate the protein bands. The molecular weight of the PLA depolymerase was determined on comparison with the standard molecular weight marker. In order to detect the activity of purified PLA depolymerase, the samples were mixed with 5x Laemmli sample buffer containing SDS without reducing agent (DTT) and were kept at 45°C for one hour for binding of SDS to protein and then loaded on to the gel. The activity of the enzyme was detected on native polyacrylamide gel (12%) containing 0.1% PLA as substrate [10]. The purified enzyme was loaded onto the gel and electrophoresis was done at 4°C. Finally, the gel was stained using Commassie Brilliant Blue R-250 to observe the formation of clear zone on blue background.

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. *B.amyloliquefaciens* was inoculated into the basal medium containing various inducers and incubated at 37° 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 protease activity. To evaluate the effect of inhibitors on PLA degrading activity of the purified enzyme phenylmethylsulfonyl fluoride (PMSF), aprotinin, pepstatin, β -mercaptoethanol and ethylenediamine tetraacetic acid (EDTA) were added at a concentration of 100 μ m and residual enzyme activity was measured.

In order to study the structural changes carried out during the degradation by *B.amyloliquefaciens*, the FTIR spectra of PLA films were recorded. Degraded PLA film and control PLA film were analyzed and spectra were recorded using Perkin Elmer Spectrum GX FT-IR spectrometer of He-Ne laser in the frequency range of 400 to 4000cm⁻¹. Thermal properties of the control (PLA film) and PLA film degraded by *B.amyloliquefaciens* were investigated by a Differential Scanning Calorimetry (DSC) using Mettler Toledo having model DSC1/700/414 under nitrogen atmosphere. Approximately 1.36mg of control PLA film and 1.36mg of degraded PLA film were encapsulated in a 40 μ l aluminum standard cap with pin. The reference pan weights taken as 48.61mg. Samples were heated under a nitrogen gas flow of 40 μ l/min from 0°C to 220°C at a rate of 10°C/min.

Degradation products of polylactide were analyzed directly with Gas chromatography - Mass spectrometry (Thermo TRACE GC ULTRA model gas chromatography coupled to a Thermo DSQ II Quadrapole Analyzer mass spectrometer (Electron Impact Ionization mode – EI). The GC oven was programmed from 40°C to 300 at 5°C/min then held for 5min at 300°C. The injector temperature was 240°C.

Statistical analysis of the data was performed with In-Stat statistics software.

3 RESULTS

The culture supernatant of *B. amyloliquefaciens* containing an initial activity (126U) was concentrated by ammonium sulphate precipitation. The optimum ammonium sulphate fractionation (80 % w/v saturation) showed 2.8 fold increases in specific activity compared to crude enzyme. The protein pellet obtained after 80% saturation with ammonium sulphate was dialysed and passed through DEAE ion exchanger and was equilibrated with 10mM potassium phosphate buffer (pH 7.0) to elute PLA depolymerase. The purification fold obtained was 4.4 with an overall yield of 11.6 and specific activity of 8.16U/mg protein (Table 1).

SDS-PAGE was performed using 12% gel. The result indicates the presence of single band with a molecular weight of 72.5 KDa (Fig. 1A). The activity of the isolated protein band was detected by clear zone formation on native polyacrylamide gel containing 0.1% PLA as substrate for testing enzyme activity. The purified sample was applied on native polyacrylamide gel. After staining, clear zone was observed on blue background indicates the activity of PLA depolymerase (Fig. 1B).

The purified PLA depolymerase obtained from *B.amyloliquefaciens* was tested for its hydrolytic activities against various substrates such as polylactide, casein, gelatin, p-nitrophenyl acetate and tributyrin. Interestingly, the enzyme exhibited relatively higher proteolytic activity against casein and PLA (Fig. 2). Although the purified enzyme can degrade both peptide bond and ester bond, it seems to be more adaptable to catalyze the hydrolysis of ester bond between lactate units. The PLA depolymerase was strongly inhibited by PMSF and aprotinin (serine protease inhibitor). From these results, it can be concluded that PLA depolymerase as serine protease. On the other hand β -mercaptoethanol and pepstatin (metallo protease inhibitor) showed least inhibition (Table 2).

PLA film degraded by *Bacillus amyloliquefaciens* was subjected to FTIR spectroscopy for observing the structural changes in the PLA film. Analysis of the spectra showed the formation of some new bands and decrease in the intensity of existing bands in the PLA film degraded by *B.amyloliquefaciens* on comparison with the control PLA film. The IR spectrum of the control PLA film showed strong bands at 2995.66 cm⁻¹, 2945.43 cm⁻¹, 2880.74 cm⁻¹ which indicates C-H stretching vibration i.e., asymmetric, symmetric stretchings of CH₃ and CH respectively. A broad band at 1756.05 cm⁻¹ assigned to (C=O) stretching of the polymer. The spectrum showed the presence of band at 1455.67 cm⁻¹ assigned to C-H deformation of CH₃. The CH deformation asymmetric band appeared at 1383.34 cm⁻¹, and 1362.14 cm⁻¹. An IR band at 2168.29 cm⁻¹ assigned to C-O stretching of the ester group. C-O-C asymmetric mode appears at 1091.90 cm⁻¹. A weak band at 956cm⁻¹ indicates CH₃ rocking (Fig. 3A).

The IR spectrum of PLA film degraded by *B.amyloliquefaciens* exhibited a weak band at 3053.43 cm⁻¹ which may be due to the CH stretching vibration of an alkene functional group. This observation together with a change in appearance of bands due to CH stretching of CH₃ indicates the cleavage of the polymer. The shift of C=O peak from 1756.05

cm^{-1} to 1719.50 cm^{-1} indicates the conversion of polymeric alkyl ester (PLA) to α , β unsaturated esters such as acrylates. The band around 3200 cm^{-1} is related to stretching of OH group and this increase from the polymer to the monomer is due to degradation of polyester which is manifested in the releases of the OH groups from the acids by breaking ester bond (Fig. 3B).

Further the cleavage of the polymer was supported by the appearance of -C=C- stretching vibration (weak) at 1578.11 cm^{-1} and strong bands at 729.04 cm^{-1} and 669.99 cm^{-1} due to out of plane CH bending vibrations of alkene functional group. The spectrum also exhibited peaks responsible for CH deformation of CH_3 at 1458.14 cm^{-1} and other peaks responsible for ester linkage that appeared in the region of 1268.50 cm^{-1} and 1024.00 cm^{-1} . From the above observations, it can be concluded that the PLA polymer was cleaved to different open chain and cyclic oligomers.

Thermal properties of the material were analysed with DSC (Fig. 4). The analysis was carried out from 0°C to 220°C with 10°C rise in temperature. About 5 mg of control and test sample were enclosed into standard $40 \mu\text{L}$ aluminium cups. During experiment, a physical distinct phase transition was observed at 63.7°C (for control which is having integral value of -6.82 mJ and at -216.42 mJ for degraded sample). The curve maxima correspond to inflection points on the row curves which give sharper depiction of glass transition temperature. The melting peaks were observed at 132.94°C for degraded and 177.2°C for control PLA samples. The area under the melting curve represents the enthalpy that comprises the transition and can also be useful as a quantitative measurement of the phase.

The GC-MS spectra of polylactide emulsion and polylactide emulsion incubated with depolymerase (degraded PLA) revealed that the enzyme is able to cleave the polymer into various cyclic oligomers. GC-MS spectrum of polylactide emulsion was shown in Figure 5A. GC-MS spectrum of degraded PLA showed similar pattern, with polylactide emulsion however there was a difference in the relative abundances of the molecular fragments between PLA emulsion and degraded PLA. The spectrum of degraded PLA at the retention time $34.21\text{-}34.43 \text{ min}$ revealed peaks for molecular fragments having $m/z = n.72\text{-}88$ with approximately high relative abundance when compared to polylactide emulsion (Fig. 5B). The spectrum of degraded polylactide also reveals the presence of molecular fragments at $280.81, 206.82, 72.92$ similar to polylactide emulsion but with low relative abundance indicates that the PLA may be degraded or cleaved by the enzyme to various cyclic or acyclic oligomers.

The second major difference between polylactide emulsion and degraded polylactide was also observed. The relative abundance of spectral peaks with m/z at 128, 200, and 272 of polylactide emulsion dropped in the case of degraded PLA sample. The similar trend was observed with m/z values of 206 and 280. From the present data, it was deduced that depolymerase was involved in alkyl oxygen homolysis of polylactide polymer into cyclic oligomers.

4 Discussion

In the present study, single PLA depolymerase was purified to homogeneity from the culture supernatant of *B.amyloliquefaciens*. The molecular weight of the purified PLA depolymerase was 72.5 KDa as determined by SDS-PAGE. The molecular mass of PLA depolymerase from *Paenibacillus amylolyticus* TB-13 was 22 KDa [11], depolymerase from *Cryptococcus* sp. strain S-2 was reported to be 22 KDa [12]. The molecular masses of three PLA depolymerases derived from *Amycolatopsis* were $24.0, 19.5$ and 18.0 kDa , with the pH optima being $9.5, 10.5$ and 9.5 respectively [13]. PLA depolymerase purified from *Amycolatopsis* strain 41, with a molecular mass of 44 KDa , was found to degrade casein, silk fibroin succinyl (L-alanyl-L-alanyl L-alanine)-p-nitroanilide but not PCL and PHB. The PLA depolymerase of *B.amyloliquefaciens* was analyzed for its hydrolytic activities against for several substrates. PLA depolymerase hydrolysed casein and gelatin. On the other hand, the enzyme showed no significant hydrolytic activity for tributyrin and p-nitrophenyl acetate. Thus PLA depolymerase of *B.amyloliquefaciens* was found to be a protease with caseinolytic activity. Most of the PLA degrading enzymes are classified as serine protease from *Amycolatopsis* sp. [14], [15], [16] and lipase *Bacillus* sp. [17].

The PLA depolymerase from *B.amyloliquefaciens* was significantly inhibited by the addition of phenylmethylsulfonyl fluoride (PMSF), indicating the presence of serine residues in its active site. The enzyme activity was inhibited by ethylenediamine tetraacetic acid (EDTA) and β mercaptoethanol. Based on the hydrolyzing activity against casein, gelatin and sensitivity to PMSF, aprotinin suggests that PLA depolymerase produced by *Bacillus amyloliquefaciens* is a serine protease, which acted on both peptide and ester bond. It is well known that some serine proteases could hydrolyze L-PLA and DL-PLA but not D-PLA [18], [19].

Fourier transform infrared spectroscopy (FTIR) analysis is a useful tool to determine the formation of new and disappearance of functional groups. FTIR spectroscopy is used as a major analytical technique in many biodegradation studies [20], [21], [22], [23]. From the IR spectral data of PLA film control (without isolate) and PLA film (test) degraded by *B.amyloliquefaciens*, it was found that there was a shift of carbonyl (C=O) peak from 1756.05 cm^{-1} (in control) to 1719.50 cm^{-1} (degraded film). The peak observed at 1759 cm^{-1} corresponding to C=O stretching vibration of polymer was shifted towards 1719.50 cm^{-1} representing the degradation of polymer to monomer. A weak band at 3053.43 cm^{-1} was appeared in degraded sample spectrum indicates the OH stretching vibration of an alkene functional group. The bands in the range of $2800\text{-}3000 \text{ cm}^{-1}$ are related to stretching vibrations of hydrocarbon groups i.e. CH_2 and CH_3

characteristic of lactic and glycolic acid. Further, the cleavage of the polymer was supported by the appearance of $-C=C-$ stretching vibration with weak band at 1578.11cm^{-1} and strong bands at 729.04cm^{-1} and 669cm^{-1} due to out of plane CH bending vibration of alkene. The spectrum of degraded PLA film also exhibited peaks responsible for CH deformation of CH_3 at 1458.14cm^{-1} and other peaks responsible for ester linkage appeared in the region of 1268.50cm^{-1} and 1024.00cm^{-1} . From these results, it can be concluded that the PLA polymer was cleaved to different acyclic, cyclic oligomers and α , β unsaturated esters such as acrylates.

Changes in the thermal properties of PLA film were examined by DSC as a function of the biodegradation. The glass transition (T_g) and melting temperature (T_m) of control PLA film were found to be 63.7°C and 177.28°C respectively where as in the PLA film degraded by *B.amyloliquefaciens* showed the glass transition temperature as 53.7°C and the melting temperature at 132.94°C . These results showed a significant decrease of 10°C (T_g) and 44.34°C (T_m) respectively. The correlative results were reported by Li, [24] while analyzing the PLA degradation and reported 10°C decrease in glass transition and 15°C decrease in melting temperature. Also, the PLA melting temperature decreased by 6.5°C , as compared to initial temperature, after 4 weeks of degradation in biotic environment [25].

The GC-MS spectra of polylactide emulsion and polylactide degraded by depolymerase, purified from *B.amyloliquefaciens* exhibited several peaks at almost similar retention time value. The peak present at a RT value 15.18 min, 15.16min for PLA emulsion and degraded polylactide showed a strong and characteristic fragment at m/z 56. This may be due to methylketene which is a common fragment of all the oligomers. The spectra of various chromatographic peaks for PLA emulsion with different RT values also exhibited characteristic fragment at m/z 56 with 100% relative abundance. The spectra of degraded polylactide with RT value 31.45-31.59 min and 34.21-34.43 min showed prominent mass fragments at $m/z = n.72-88$ (128, 200, 272, 344, and 416). These peaks were mass fragments of cyclic oligomers [26]. These spectra also exhibited mass fragment at $m/z = n.72$. The spectra of PLA emulsion with RT value 31.36-31.60 min and 34.20-34.40 min showed mass fragments at $m/z = n.72-88$ (i.e. at 128, 200, 272 etc.). The relative abundances of these mass fragments were less than that of mass fragments of degraded polylactide observed with almost same RT value. This indicates that the PLA depolymerase causes alkyl oxygen homolysis. Kopinke *et al.*, [26] also reported that the formation of cyclic oligomers is because of alkyl oxygen homolysis.

The second major difference between control and degraded PLA sample mass spectra was also observed. The relative abundances of mass fragments of PLA emulsion at $m/z = 206, 280$ were higher than the degraded polylactide. These fragments were representatives of acyclic dimer and

trimer coupled with acetaldehyde. Another observation was the difference in the relative abundances of mass fragment at m/z value 72. This may be due to acrylic acid. The relative abundance of these peaks in degraded polylactide was less on comparison with PLA emulsion. These observations indicates the degradation of PLA through intra- and intermolecular ester exchange which results in the formation of methylketene, acetaldehyde, CO_2 and carbon monoxide.

Figures and Tables

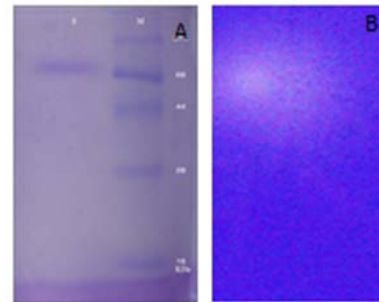


Fig.1. (A) SDS-PAGE of PLA depolymerase purified from *B.amyloliquefaciens*. Lane M: Molecular weight markers, Lane S: Purified PLA depolymerase with 72.5 KDa. (B) Zymogram analyses of purified PLA depolymerase obtained from *B.amyloliquefaciens*. Clear zone indicates PLA depolymerase activity.

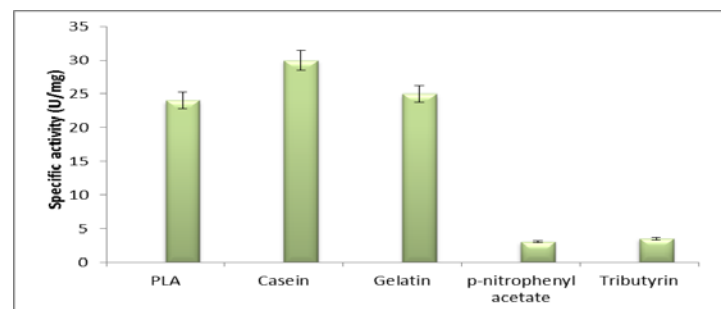


Fig. 2. Substrate specificity of PLA depolymerase obtained from *B.amyloliquefaciens*. Each bar represents the mean \pm SEM of three independent values. $p < 0.1$

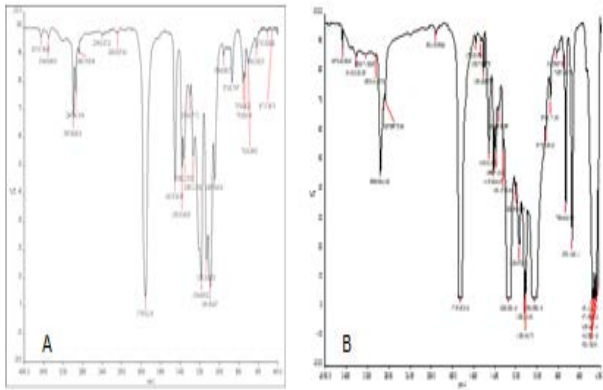


Fig. 3A: FTIR spectrum of (A) undegraded PLA film (control). (B) Degraded PLA film.

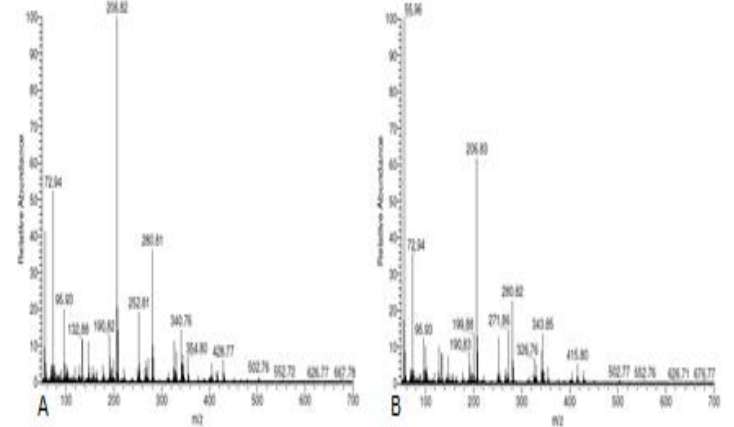


Fig. 5: GC-MS spectra of (A) polylactide emulsion. (B) polylactide emulsion (degraded) incubated with enzyme

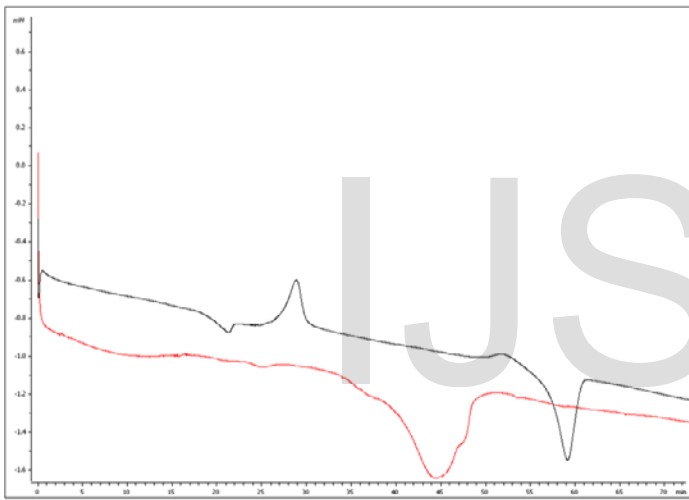


Figure 4: DSC thermograms of control and degraded PLA film

Table 1
 Purification profile of PLA depolymerase from *B. amyloliquefaciens*

Sample	Total protein (mg/ml)	Total activity (U)	Specific activity (U/mg)	Purification fold	Protein recovery %
Crude	68	126.0	1.85	1.00	100
Ammonium sulfate	11.0	55.0	5.0	2.78	43.6
Dialysed	4.0	29.0	7.25	3.9	23.8
DEAE-column	1.8	14.7	8.16	4.41	11.6

TABLE 2
 Effect of inhibitors on the activity of PLA depolymerase.

Inhibitor	Residual activity of PLA depolymerase (%)
PMSF	5.2±0.07
Aprotinin	10.9±0.03
Pepstatin	81±0.04
β-Mercaptoethanol	85.0±0.09
EDTA	47.2±0.02

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