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Aegle marmelos: A novel low cost substrate for the synthesis of polyhydroxyalkanoate by *Bacillus aerophilus* RSL- 7

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ABSTRACT

Conventional plastics have created greater environmental concerns over the past few decades and in turn, have motivated researchers to find an interesting substitute for the petroleum based plastics. Polyhydroxyalkanoates could be valuable alternatives to conventional plastics. The current work presents the investigations carried out to produce PHA by *Bacillus aerophilus* (RSL- 7) isolated from oil contaminated site while utilizing Bael (*Aegle marmelos*) whole fruit hydrolysate as a low cost substrate. The organism was characterized by 16S rRNA sequencing and other biochemical tests. The produced PHA was extracted by solvent extraction method and chemical characterization was done using FT-IR and thermal characteristics were studied using TGA and DSC analyses, respectively. The highest amount of PHA produced was observed to be 2.47 g/L with a cell dry weight of 21.25 g/L at 72 h of incubation with the supplementation of 1% peptone and glucose, respectively to the fruit hydrolysate.

1. Introduction

Plastics have alleviated daily life with their usage increasing on ease in each and every sector. The annual production of plastic has augmented to a larger extent over the last 60 years. Fossil fuel or petroleum is a non-renewable resource, which is being depleted at high rate (Mathimani et al., 2015, 2017; Saravanan et al., 2018). The major sources for the production of plastics are petrochemicals, which are non-renewable in nature. A recent assessment done on the earth's mineral resources illustrated an alarming rate of depletion of these valuable natural resources. This has created a renewed impulsion to search for various other sustainable alternatives. The increasing cost and the awareness of consumers on the negative environmental impacts of the fluid mineral fuels and related products such as recalcitrance to biodegradation (Bera et al., 2015; Mohandas et al., 2018; Reddy et al., 2003), toxicity after incineration and massive waste accumulation into the landfills as well as growing water and land pollution problems have led to the concerns about plastics. With the wakefulness of this predicament and its consequences on the environment new curiosity has been arising in the area of financially viable and efficient sources of biodegradable polymers for the production of plastics, popularly known as the *"Bioplastics or Biopolymers"* (Divya et al., 2013). Full pledged use of bioplastics in daily life will solve the increasing problem of organic wastes and decrease the country's dependence on fossil fuels (Ceyhan and Ozdemir, 2011).

Polyhydroxyalkanoates are the sole 100% biodegradable polymers. They are polyesters of various hydroxyalkanoates (HAs), which are synthesized by numerous micro-organisms as energy reserve materials when an essential nutrient such as nitrogen or phosphorus is available only in limiting concentrations with an excess of carbon source (Anderson and Dawes, 1990; Zinn et al., 2001). They possess properties similar to various synthetic thermoplastics such as polypropylene (PP) and henceforth, can serve as an efficient alternative with superior properties such as biodegradability (Ho et al., 2002; Lenz and Marchessault, 2005; Lim et al., 2005), biocompatibility (Hazer and Steinbüchel, 2007; Zinn et al., 2001) and so on. They are completely degraded to water and carbon dioxide under aerobic conditions and to methane under anaerobic conditions by microorganisms in soil, sea, lake water and sewage (Khanna and Srivastava, 2005).

As cost being a great hindrance in the commercial production of PHA, different types of cheaper substrates were exploited as they contributed to about 40% of the total manufacturing cost of PHA. In the

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present work, a *Bacillus* strain (RSL- 7) isolated from oil contaminated soil was found to produce PHA utilizing bael fruit extract as a low cost substrate. *Aegle marmelos* (L.) commonly known as Bael, belongs to the family Rutaceae. It is a cosmopolitan distributed in the deciduous forests of India. *Aegle marmelos* (L.) is a very important fruit plant showing economic and nutritive values and a major source of medicine (Deokar). The organism was studied for its ability to produce PHA in the fermentation medium containing bael fruit as a substrate supplemented with simple nutrient components. The organism was capable of producing a high yield of PHA when bael fruit hydrolysate was used as a substrate, which in turn would introduce a novel low cost substrate for the effective production of PHA. The produced PHA was characterized to be polyhydroxybutyrate (PHB), which would host different application scopes. Hence, the present work has focused on the utilization of an unexplored low cost substrate for the production of PHB. Major focus was made on how novel substrates (renewable) could be cost effectively utilized for the synthesis of a biopolymer hosting a myriad of applications.

2. Materials and methods

2.1. Substrate and sample collection

Oil contaminated soil was collected from the railway diesel loco shed, Erode, Tamil Nadu, India. The soil was transferred to the laboratory in sterile ziplock covers and stored at 4 °C till use. The dried fruits of Bael (*Aegle marmelos*) were collected from nearby temple complexes around Erode, Tamil Nadu, India. They were cut into small pieces, dried in a hot air oven for complete loss of moisture and crushed in a blender to get fine powder.

2.2. Bacterial isolation and identification

The soil sample was serially diluted and the organisms were isolated on nutrient agar plates. The pure cultures were maintained on agar slants for further analysis. The PHA positive organisms were identified by 16S rRNA sequencing using universal primers (Forward: GAGTTTG ATCGTGGCTCAG; Reverse: AGGGCTACCTTAGACTT) and subsequently, analyzing the sequences using multiple sequence alignment and BLAST.

2.3. Substrate preparation

The substrate was prepared using the modified method of Pumiput et al. (2008). The powdered substrate was steamed for 25–30 min and then, boiled for 1–2 h by adding sufficient water to soak it. It was then filtered and treated with concentrated hydrochloric acid (1%) and sterilized in an autoclave for 30 min under 121lb pressure. The hydrolysate was filtered again and pH was adjusted to 6.8 using sodium hydroxide (1M).

2.4. Primary and secondary screening

Nile blue agar plates were prepared and the pure culture colonies of bacteria were streaked onto them. The fluorescing colonies were considered to be PHA positive (Spiekermann et al., 1999) and taken for further screening procedures. The Nile blue positive strains were grown on 50 mL nutrient broth and incubated overnight. The cultures were then centrifuged and the pellets were washed twice with physiological saline, cultures with OD-1.0 were inoculated onto the Minimal Salt Media (MSM) NaCl – 1 % (w/v), Na_2HPO_4 - 0.37% (w/v), KH_2PO_4 - 0.1% (w/v), $MgSO_4.7H_2O$ - 0.05lb% (w/v), $(NH_4)2HPO_4$ - 0.02% (w/v), Glycerol - 2% (v/v) Peptone - 0.5% (w/v), Yeast extract – 0.05% (w/v) at pH – 7.5 ± 0.3 (Sun et al., 1994). The organisms grown in this media were subjected to PHA extraction after 72 h incubation and the highest producer was finalized for the fermentation process with bael fruit as the substrate.

2.5. PHA production using bael fruit substrate

The hydrolysate was supplemented with different concentrations of carbon (glucose) and nitrogen (peptone) sources to study the rate of PHA production. The PHA positive bacterial strain was inoculated onto the hydrolysate with 0, 1, 2 and 3% of glucose and peptone, respectively. The ability of the organism to produce PHA without any inorganic supplementation of nutrients was also analyzed by inoculating the culture onto the hydrolysate without the addition of any nutrients. The organisms were investigated at three different time intervals viz. 24, 48 and 72 h to understand the PHA accumulation property of the bacterium.

2.6. PHA extraction

The incubated cultures were centrifuged (10,000 rpm, 5 min), the collected pellets were dried and weighed to provide the cell dry weight (CDW). The pellets were then treated with sodium hypochlorite (4% w/v) solution and incubated at 37 °C for 1–2 h and again centrifuged at 10,000 rpm for 5 min. The supernatants were then discarded and the pellets were washed with acetone and diethyl ether (1:1) and suspended in hot chloroform (Dong and Sun, 2000). Gravimetric analysis of the extracted PHA was done to provide the dry weight of PHA (PDW). The percentage of PHA content was calculated using the concentration of PHA to CDW multiplied with 100 (Chien et al., 2007). All the experiments were performed in triplicates.

2.7. Analysis of thermal properties -differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA)

Differential scanning calorimetry was used to characterize the melting temperature (T_m) and glass transition temperature (T_g) for all polymer samples. DSC measures the amount of heat energy absorbed or released by a sample, as it is heated, cooled or held at a constant temperature. The sample was analyzed using a Mettler Toledo 822c instrument. The PHA samples were heated at a rate of 10 °C min^{-1} from 4 to 200 °C (Zhu et al., 2010). TGA (Perkin Elmer, Diamond, USA) offers complete details about the degradation temperature of the polymer and its thermal stability. The sample was scanned from 40 to 930 °C with a heating rate of 10 °C min^{-1} (Koller et al., 2007).

2.7.1. FTIR analysis

The chemical characteristics of the extracted PHA were inferred by Perkin Elmer Fourier Transform Infrared (FTIR) spectrophotometer (Jasco FTIR- 6100, Japan) using KBr disc (Shamala et al., 2003) and the spectrum was recorded in the wavenumber range of 400–4000 cm^{-1}.

2.7.2. Statistical analysis

All the experiments were done in triplicates and the data were analyzed statistically by means of standard deviation.

3. Results and discussion

Only a few reports have dealt with the industrial production of PHAs by potential bacterial strains (Chen, 2009; Reddy et al., 2003). The monomeric units of the PHA contribute to their varying physical and chemical characteristics, which are influenced by the type of microorganisms, media ingredients, fermentation conditions, modes of fermentation and the recovery process (Keshavarz and Roy, 2010). Hence, there is a need for screening large number of organisms that could accumulate PHA with varied combinations of monomers, which are high yielding and with the desirable traits. Isolation of diverse PHA producing bacteria from a niche was done wherein hydrocarbons were the only available nutrients for the bacteria; thus, making a stressful

Table 1
Bael fruit hydrolysate supplemented with different percentages of glucose.

Hydrolysate + glucose	24 h			48 h			72 h		
	CDW	PDW	%	CDW	PDW	%	CDW	PDW	%
0	7.75	0.47	6.12	12.75	0.9	7.05	21.25	2.47	7.61
1	3.26	0.25	7.66	10.02	0.52	5.18	5.5	1.18	6.91
2	5.37	0.5	9.31	10.75	0.9	8.37	15.15	1.07	7.06
3	7.13	0.32	4.48	12.11	0.6	4.95	24.25	1.22	5.03

environment for them to survive.

Serial dilutions of the soil sample yielded about 34 colonies of morphologically distinct bacterial isolates. The pure cultured strains of bacteria were streaked on Nile blue agar plates for primary screening, from which about 12 strains showed fluorescence under UV illumination. RSL- 7 was the strain, which exhibited intense fluorescence, explaining it to be a better PHA producer (Spiekermann et al., 1999) and it showed the highest production of PHA in the nutrient rich synthetic media (data not shown). RSL- 7 was subjected to 16S rRNA sequencing and from the multiple sequence alignment, the strain was identified to be *Bacillus aerophilus*. The sequence was deposited in the Gene Bank with the accession number SP KT364633.

As exploring a novel substrate for the reduction in the total cost of PHA production serves as a major aim, bael fruit hydrolysate prepared from simple acid hydrolysis method was supplemented with only two synthetic supplements (glucose and peptone), that too for the purpose of comparison to know whether the bacterial strain could produce PHA at different nutrient conditions.

From Table 1, it was evident that the medium with the hydrolysate containing 1% of glucose and peptone, respectively produced the highest amount of PHA 2.47 ± 0.19 g/L with a cell dry weight of 21.25 ± 0.03 g/L at 72 h of incubation. The highest amount of cell biomass was observed at 72 h with the hydrolysate media containing 3% glucose and 1% peptone, respectively (Table 1). Table 2 illustrates the highest accumulation percentage (11.70%) with the absence of nitrogen source (peptone) at 48 h incubation; the cell dry weight was 10.25 ± 0.06 g/L and the weight of PHA was 1.2 ± 0.15 g/L.

The organism inoculated into the hydrolysate medium without any nutritional supplementation produced about 1.27 ± 0.08 g/L of PHA at 72 h of incubation with 12.5 ± 0.26 g/L cell dry weight. Table 3 shows other details about the amount of PHA produced by *Bacillus aerophilus,* utilizing the hydrolysate alone. Figs. 1–3 demonstrate the chemical and thermal characteristics of the PHA extracted from *Bacillus aerophilus.* Galactose, arabinose, uronic acid and L-rhamanose are obtained on hydrolysis (Basak et al., 1982). The bael fruit contains about 24.1 ± 0.08 g/100 g of carbohydrates and 4.7 ± 0.13 crude protein (Singh et al., 2012) serving as a source of C: N, which has aided the organism to synthesize PHA without any other external nutrient supplementation (Table 3). The cell biomass had increased (24.25 ± 0.12 g/L) when the amount of glucose was high in the media but there was no considerable increase in the amount of PHA produced as the accumulation level was only 5.03%. This might have been due to the fact that the organism would have never undergone any stress because of high availability of nutrients and in general, most of the organisms accumulate PHA only under stress conditions.

Table 3
Bael fruit hydrolysate as sole substrate for PHA production by RSL-7.

Hydrolysate	24 h			48 h			72 h		
	CDW	PDW	%	CDW	PDW	%	CDW	PDW	%
Without glucose and peptone	7.17	0.37	5.16	9.02	0.92	10.19	12.5	1.275	10.16

3.1. FTIR, TGA and DSC analysis

From the FTIR spectra, it was comprehended that the organism was capable of producing polyhydroxybutyrate (PHB) as the FTIR spectrum was in accordance with the standard peaks of PHB (Sigma, India). Moreover, the results were concordant with the analysis data of Rohini et al. (2006) and Misra et al. (2000). The peak at 2928 cm^{-1} corresponded to C—H stretching and those at 1636 and 2928 cm^{-1} corresponded to the thioester (C═O) valence, which would serve as a predominant peak authenticating the production of PHB. Other peaks such as the one at 3433 cm^{-1} indicated OH stretching (Fig. 1).

For a better understanding of the thermal degradation of PHB-based systems and their related reaction mechanisms, the mass loss data obtained from the dynamic thermogravimetric analyses were used. Fig. 2 shows the weight loss curves (TG) for the PHB obtained from RSL-7. The weight loss (TGA) of PHA occurred during the heating treatment from 40 °C up to 930 °C under inert atmosphere (N_2). It was observed that the compound was stable until 276.21 °C.

TGA measures the amount and rate of change in the weight of a material as a function of temperature or time in a controlled atmosphere. In the present study, TGA showed that the polymer extracted from BP 25 was degraded at 276.21 °C, indicating that the biopolymer had a good thermal stability. The thermal stability of the PHB extracted was slightly higher than the thermal stability value of PHB reported in literature (Nair et al., 2014). Higher decomposition temperature provided a broader separation between the required melting temperature for injection molding of the polymers (Zhu et al., 2010).

The thermal behavior of the sample was investigated by DSC and the resulting thermogram is shown in Fig. 3. The results from DSC showed that the melting temperature of the polymer might be 163.99 °C. The polymer was degraded rapidly at 250.08 °C with a peak

Table 2
Bael fruit hydrolysate supplemented with different percentages of peptone.

Hydrolysate + Peptone	24 h			48 h			72 h		
	CDW	PDW	%	CDW	PDW	%	CDW	PDW	%
0	8.25	0.42	5.09	10.25	1.2	11.70	12	1.35	11.25
1	8.75	0.9	10.28	10.5	1.075	10.19	11.5	1.22	10.60
2	5.75	0.57	9.913	7.25	0.77	10.62	9.5	1.075	11.31
3	5.5	0.52	9.45	9.15	0.72	7.86	10.25	0.9	8.78

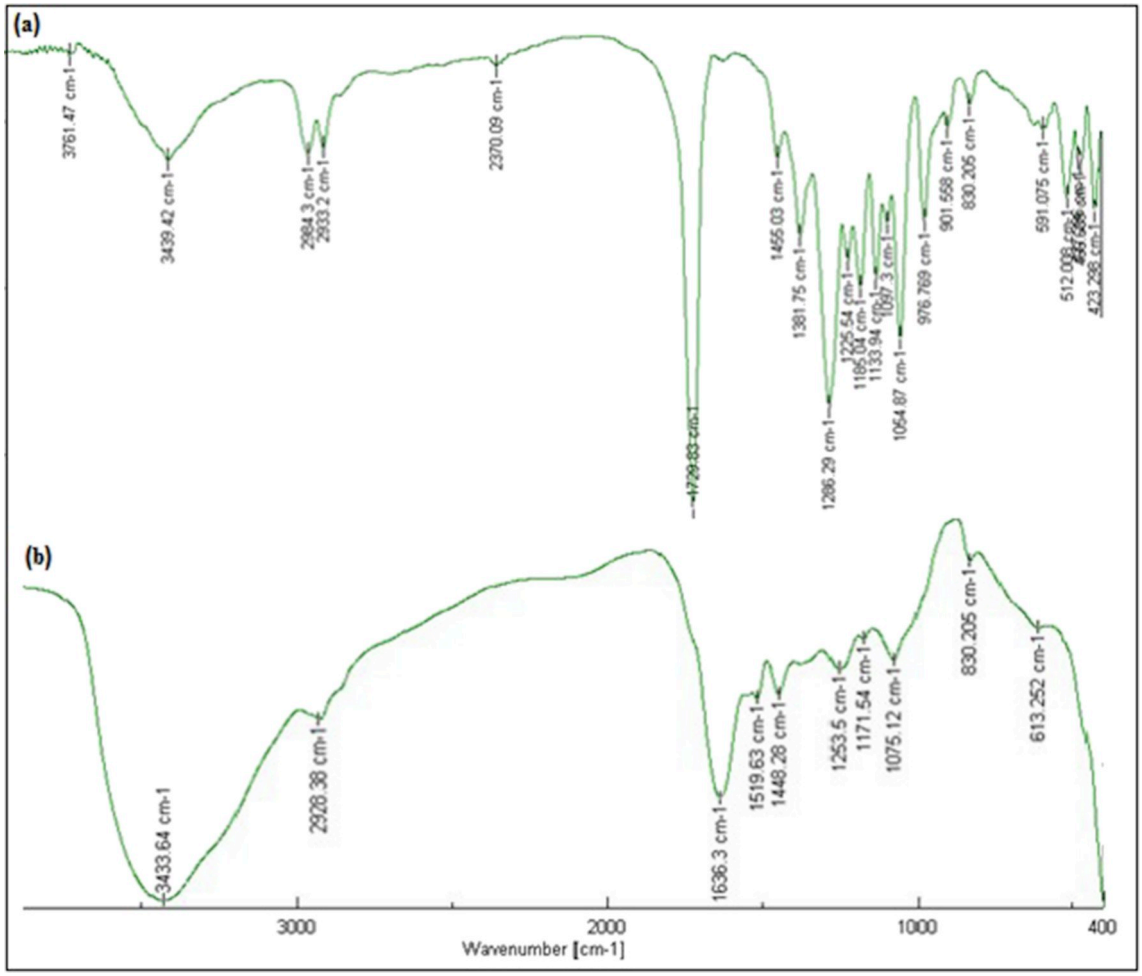

Fig. 1. FTIR spectrum of a) PHB extracted from RSL-7; b) Standard PHB.

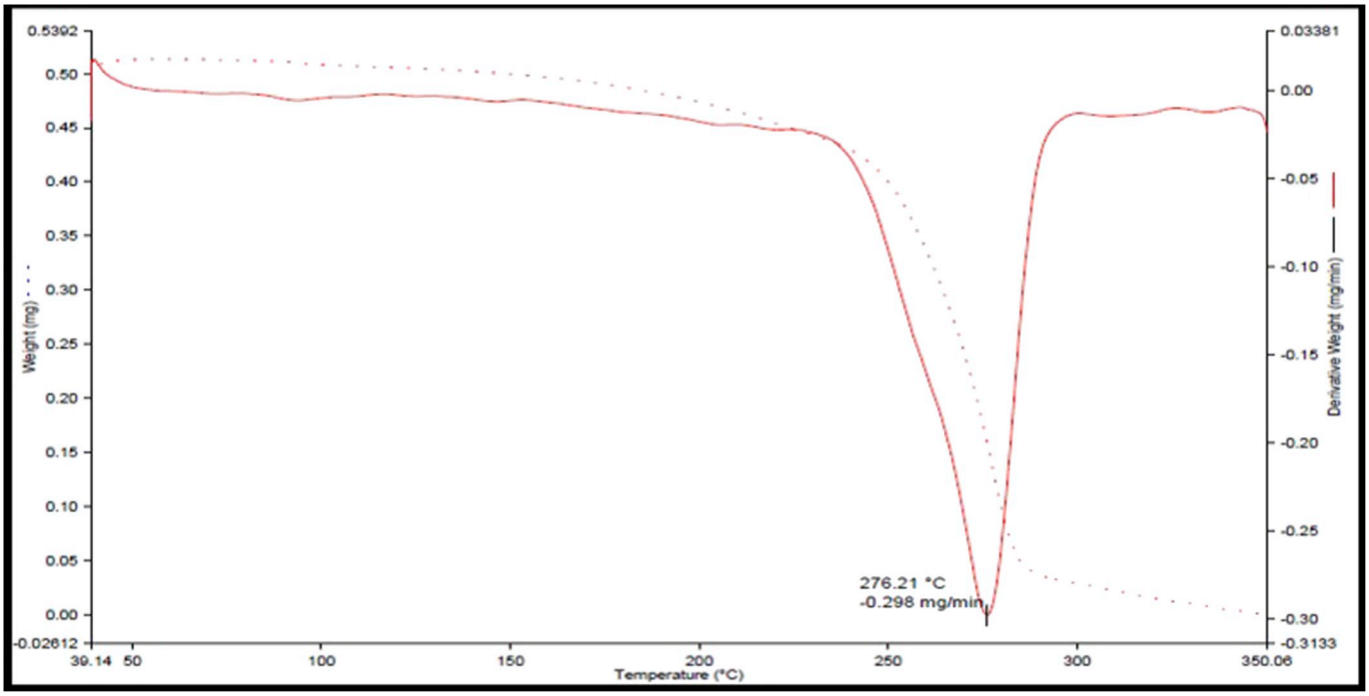

Fig. 2. Thermogravimetric analysis of the extracted PHB.

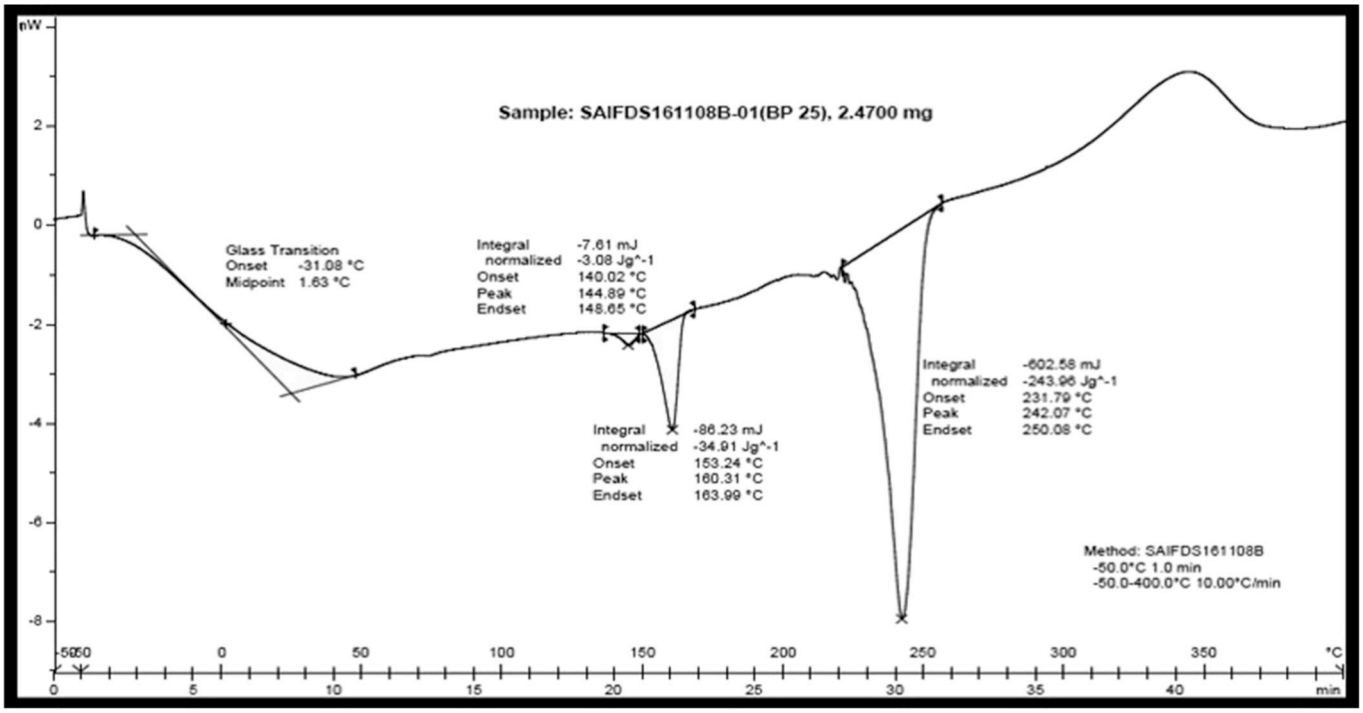

Fig. 3. DSC thermogram of the extracted PHB.

at 250 °C. The melting temperature of PHB (164 °C) in the present study was nearer to the PHB obtained from *B. cereus* (170 °C) (Labuzek et al., 1994). (Abd-El-Haleem et al. (2007); Gunaratne et al., 2004) showed that the melting point of PHB extracted from yeast ranged from 152.84 °C to 168.33 °C. Thermal degradation occurred around 230.07 °C and 269.90 °C with a single weight loss step. Wang et al. (2013) determined the melting temperature of the polymer produced by *Alcaligenes latus* using sugar beet juice to be 151.46 °C, which was a lower value compared to the present study. Thus, characterization by the thermal analyses confirmed the purity of the product.

4. Conclusion

The present study has shed valuable information about the utilization of bael fruit hydrolysate as a novel and renewable source of substrate for the production of the biopolymer PHB. The highest amount of PHA produced was observed to be 2.47 ± 0.19 g/L with a cell dry weight of 21.25 ± 0.03 g/L at 72 h of incubation. A simple and less time consuming methodology was incorporated for the substrate preparation, which serves as an added advantage. This shall be further optimized to increase the yield so that industrial level production of PHB shall be feasible. This shall pave way for the reduction in the overall cost of biopolymer production; thus, aiding in the utilization of biodegradable polymers on large scales.

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