



Original Research Article

Recovery and Characterization of Poly(3-Hydroxybutyric Acid) Synthesized in *Staphylococcus epidermidis*

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ABSTRACT

Keywords

Polyhydroxyalkanoates (PHA), poly (3-hydroxybutyric acid) (PHB), *Staphylococcus epidermidis*.

Polyhydroxyalkanoates are biodegradable polyesters accumulated intracellular as energy resources by bacterial species. In this study, fermentation process for production of PHA is carried out using sesame oil as carbon source. We studied recovery of poly(3-hydroxybutyric acid) (PHB) from *Staphylococcus epidermidis* by sodium hypochlorite digestion method. Recovered PHB sample was estimated by UV Spectrophotometer. PHB from *Staphylococcus epidermidis* was characterized and these findings, we examined purified PHB by differential scanning calorimeter (DSC), a thermo gravimetric analyzer (TGA), Thin layer chromatography(TLC) and Infrared Spectroscopy (IR).The results of our analysis of PHB while comparing with commercial source suggest that in DSC melting temperature of PHB was 173.36oC, TGA thermo grams of PHB sample was at 296.91oC, on TLC plate; Rf value was calculated as 0.71 and finally IR spectrum of the compounds showed characteristics bands for the groups CH, C=O and C-O, indicating the presence of PHB in the production medium.

Introduction

Often, research and media attention on the renewable bioproduct industry is focused specifically on fuel alternatives. This is logical since diminishing fossil fuels supply nearly 80% of the global energy demands, and it is predicted that the current demand will increase by 56% by 2040 (U.S. Energy Information Administration, 2013). Currently, though, the renewable energy industry is one of the two fastest-growing industries globally,

increasing at a rate of 2.5% per year (U.S. Energy Information Administration, 2013). By 2015, the demand for biodegradable plastics is estimated to reach 1.1 million tons (Metabolix, 2013a). To make environment free from plastics is one of the major interests to both decision makers and plastic industries (Chen et al., 2009). Poly (3-hydroxyalkanoic acid) (PHA) is a biodegradable polymer material that accumulates in numerous

microorganisms under unbalanced growth conditions (Ribera, et al., 2001). Poly β -hydroxybutyrate (PHB) is a biopolymer that can be used as biodegradable plastics; it is the most common natural microbial PHA (Singh and Parmar, 2013). In terms of molecular weight, brittleness, stiffness, melting point and glass transition temperature, the PHB homopolymer is comparable to some of the more common petrochemical derived thermoplastics such as polypropylene (Sayed et al., 2009). Although PHB was found to be at an advantage comparing to non-biodegradable plastics; its application is inevitably limited due to high production costs.

PHB is a microbial polyester produced by many bacteria and stored in their cells in the form of granules, about 0.5 μm in diameter. β -hydroxybutyrate is connected by ester linkage and forms PHB (Prasanna et al., 2011). PHB possesses only R (alkyl group) side chains (and lacks S (Sulfur) side chains) and hence reported as biodegradable materials (Anderson et al., 1990; Saito et al., 1996; Jung et al., 2001) e.g. Vulcanized rubber. PHB is an intracellular product; the method applicable for its effective separation from other biomass components is complex and expensive.

Number of different methods for the recovery of PHB has been suggested. There are some of the known, effective methods for separation of PHB from bacterial cells: Physical method using a bead mill (Kunasundari and Sudesh, 2011), Extraction method using an organic solvent (Ibrahim and Steinbuchel, 2009), Enzyme method (Kathiraser et al., 2007).

Finally, there must be a method which allows consistent recovery of the polymer

with high purity. There may be a different requirement of purity of biopolymer which depends on its intended application and which ultimately designs the recovery method of PHB extraction.

In this work we studied PHB recovery from *Staphylococcus epidermidis* which possesses a tendency to utilize sesame oil and it has been reported previously that plant oils are desirable feed stocks for PHA production because they are also inexpensive in comparison with other carbon sources, such as sugar (Akiyama et al., 2003). Hence, here *S. epidermidis*, a known PHA-producing bacterium is utilized for study of PHB production and recovery of PHB from *S. epidermidis* worthy of investigation. PHB was recovered through a dispersion of a sodium hypochlorite solution and chloroform. In this paper we also described characterization and determination of native PHB like granules which were recovered using various organic solvents.

Materials and Methods

Cultivation of Bacteria: *Staphylococcus epidermidis*, isolated from edible oil contaminated sites and was used for PHB production (Marjadi and Dharaiya, 2011). *S. epidermidis* was grown and maintained in a modified mineral salts medium (MSM) (Marjadi and Dharaiya; 2011).

The production of PHB or copolymer was carried out by two-stage cultivation (Hartmann et al., 2010). First stage organisms were cultivated in the nutrient broth medium without any nutrient limitation, at 37 °C and 150 rpm for 24 hrs. In second stage, after incubation, 2 ml of culture was taken to inoculate according to their dry cell weight (Marjadi and

Dharaiya, 2011) the flask containing 200 ml of sterile production medium, and all the isolates were first grown for 72 hrs. at 37 °C with shaking at 150 rpm in a carbon – rich MSB medium containing sesame oil (1% w/v) as a sole carbon source and cells accumulating PHB were cultivated in 250 ml of modified mineral salts basal medium (MMSB) as described by Marjadi and Dharaiya(2012).

Production and storage of PHB-containing biomass: To recover and characterize PHB produced in *S. epidermidis* after fermentation, the cell broth was concentrated by centrifugation at 4,000 RPM for 15 min at 25°C, washed twice with distilled water, and then freeze dried. The resulting cell powder was stored at 4°C until they used further.

PHB recovery: After 96 hrs of incubation at 37 °C, 10 ml of culture was taken into clean polypropylene centrifuge tubes which had been previously washed thoroughly with ethanol and hot chloroform to remove plasticizers. In each tube, 5 drops of formaldehyde were added in order to stop all biological activity and then centrifuged at 8000 rpm for 15 min. The supernatant was discarded and collected pellet was washed twice with 5 ml cold water and 2 ml cold hexane (Loba®) twice to remove hydrophobic residual oil (Kahar et al., 2004). The remaining pellet was dried in oven until obtaining the constant weight. The dried pellet was treated with equal to the original volume of culture medium (10ml) of 30 % (v/v) sodium hypochlorite (NaOCl) (Loba®) and the mixture was incubated at 37 °C for 1 hr. After incubation, the mixture containing the lipid granules was centrifuged at 6,000 rpm for 15 min and was washed with water, and then with 5 ml 96% cold acetone (Loba®) and followed by ethanol

(Loba®) (1:1). The precipitates thus formed were allowed to dry to obtain PHB crystals.

PHB extraction: Extracted PHB crystals were re-dissolved in 5mg in 5ml (Sayyed et al., 2009) of chloroform in a test tube in water bath at 100 °C for 20 min and filtered through Whatman No.1 filter paper and chloroform was evaporated by pouring the solution on sterile glass petri plate and then kept at 4 °C in deep fridge. After some time, powder was collected from petri plates by slowly scratching for further analysis (Kuniko et al., 1989; Bowker, 1981; Ishizaki and Tanaka, 1991).

Estimation of PHB concentration: PHB concentration was estimated as suggested by Law and Slepecky, (1961). Extracted PHB powder was transferred to clean test tube (Figure-1) and 10 ml of concentrated H₂SO₄ was added to the tube which was capped and heated for 20 minutes at 100 °C in a water bath. PHB crystals were converted in to crotonic acid by dehydration (Aslim et al., 2002). The resultant brown colour crotonic acid solution was cooled, and after thorough mixing, a sample was transferred to a quartz cuvette and the absorbance was measured at 235 nm in UV Spectrophotometer against a sulfuric acid blank. Standard curve of pure PHB (Sigma®, USA) was prepared by the modified method as suggested by Slepecky and Law (1960).

Characterization of PHB: The chemical structure and the thermal properties of PHB were used as parameters for qualitative analysis of PHB. Characterization and determination of native PHB like granules involved precise measurements to analyze their physical

properties and were characterized mainly by four methods in the present study: TLC, IR, DSC and TGA.

Thin Layer Chromatography (TLC)

TLC was carried out in a glass plate (10X5cm²) coated with silica (3g/15ml of chloroform), prepared using a spreader. About 50 µl of propanolysed organic phase which involve propanolysis of PHB in a tightly sealed vial (10 ml) to which 2 ml of dichloroethane and 2 ml of a solution of propanol-hydrochloric acid (4:1 [vol/vol]) (Panda et al; 2008) of sample was loaded on the TLC plate and allowed to run in the solvent system consisting of ethyl acetate and benzene (SRL®) (1:1) mixture for 40 min. The plate was left to dry after run and for staining 50 ml of iodine solution (Hi-media®) was vaporized in water bath at 80-100°C. TLC plate was kept over the beaker containing iodine solution for 5-10 min in order to get it saturated with iodine vapour. The R_f values of the spots were calculated using standard formula and compared with the standard chart (Rawte and Mavinkurve, 2002).

Infrared Spectroscopy (IR)

IR analysis of PHB-like granules was performed using a commercial customer service, Aarti Industries, Tarapur, India. Briefly, extracted sample and standard PHB from Sigma® was separately made in to solid pellet by making an intimate mixture of a powder sample with potassium bromide for IR analysis. The relative intensity of transmitted light was measured against the wavelength of absorption on the region 800 to 4000cm⁻¹ using IR double beam Spectrophotometer (Shimadzu®). IR spectra of samples were measured at ambient condition.

Differential scanning calorimetry (DSC)

DSC analysis of PHB-like granules was performed using a commercial customer service, Center of Excellence, Vapi, India. Briefly, Differential scanning calorimetry was used to characterize the melting temperature (T_m) of samples which was done in a range of 30 °C air to 450 °C air at 10 °C air /min. The melting temperature (T_m) and melting enthalpy (ΔH) were determined from DSC endothermal peaks.

Thermo gravimetric analysis (TGA)

TGA analysis of PHB-like granules was performed using a commercial customer service, Center of Excellence, Vapi, India. Briefly, Thermo gravimetric analysis was used to determine the decomposition temperature (T_{decomp.}) of PHB. Ten milligrams of PHB film were folded into a platinum tray and subjected to a heating rate of 20 °C air/min from ambient to a final temperature of 500°C air.

Results and Discussion

Lipid inclusion granules were stained black whereas the bacterial cytoplasm was stained pink in color confirming the presence of lipid inclusion granules inside the bacterial cell. PHB production was found to be influenced by the utilization of carbon from sesame oil.

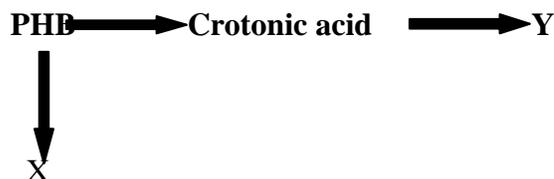
PHB was isolated from the production medium by solvent extraction technique. The sodium hypochlorite digestion process enables in the digestion of cells and release of the PHB granules outside the cells for easy extraction of PHB. As present work involves utilization of edible oil as carbon source, extraction of PHB with a pre treatment of hexane helps in efficient removal of edible oil from fermentation broth.

After achieving constant weight of cell biomass at 105°C after 24 hrs in an oven, the biomass is treated with the hypochlorite solution, based on the fact that it can dissolve nearly all components of cell except PHB granules (Yu et al., 2006). The solvent extraction is widely used to recover PHB with high purity. Sodium hypochlorite breaks the cell wall of bacteria and facilitates elimination of Non-PHB Cellular Material (NPCM) resulted in the lysis of cells without affecting the PHB (Jacquel et al., 2008). The solvent system consisting of 1:1 mixture of ethanol and acetone washings serves to remove water which interferes with the extraction of the polymer into chloroform. It also proves to be specific and efficient for lysing the NPCM without affecting PHB. The present system also assists in extraction of cell lipid content and other molecules (except PHB). (Rawte and Mavinkurve, 2002).

The conversion to crotonic acid by hot concentrated sulfuric acid proved to be about one-third time more sensitive than alkaline hydrolysis of PHB extraction and analysis (Marjadi and Dharaiya, 2012). The protocol that was most efficient in

determining PHB, i.e. β -elimination of crotonic acid; a brown colored compound effected by concentrated sulfuric acid, was described by following reaction.

The reaction can be roughly described by Reaction 1 as under.

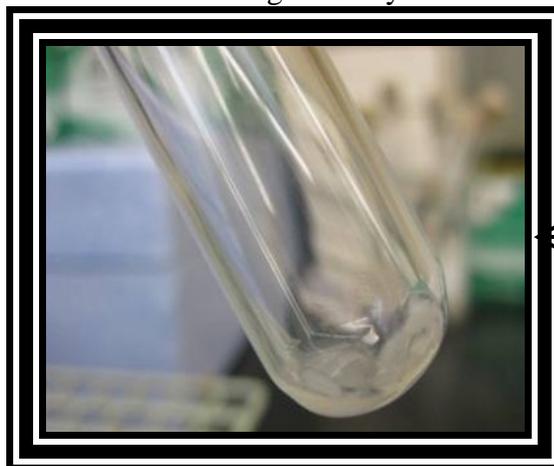


Reaction 1

Where X refers to other degradation products of PHB and Y refers to degradation products of crotonic acid. (After Huang and Reusch; 1996)

The amount of PHB in the extracted samples was determined with UV spectrophotometer at 235 nm with reference to the standard graph of 3-hydroxy butyric acid (Data not shown here).

Figure.1 Test tube containing PHB crystals and Chloroform



Mixture of PHB crystals and chloroform

Table.1 IR spectrum of sample and standard PHB

Sample	Peak regions	Comments
PHB Sample	1635	Carbonyl group (C=O)
	3097	Methine groups (CH)
	1089	Ester group (C–O)
	3578.55	Intramolecular H bond
	3415.06	H bond
PHB (Sigma)	1673	Carbonyl group (C=O)
	2928	Methine groups (CH)
	1076	Ester group (C–O)
	3330.13	Intramolecular H bond
	3417.70	H bond

Figure.2 IR spectra comparison between sample and standard PHB

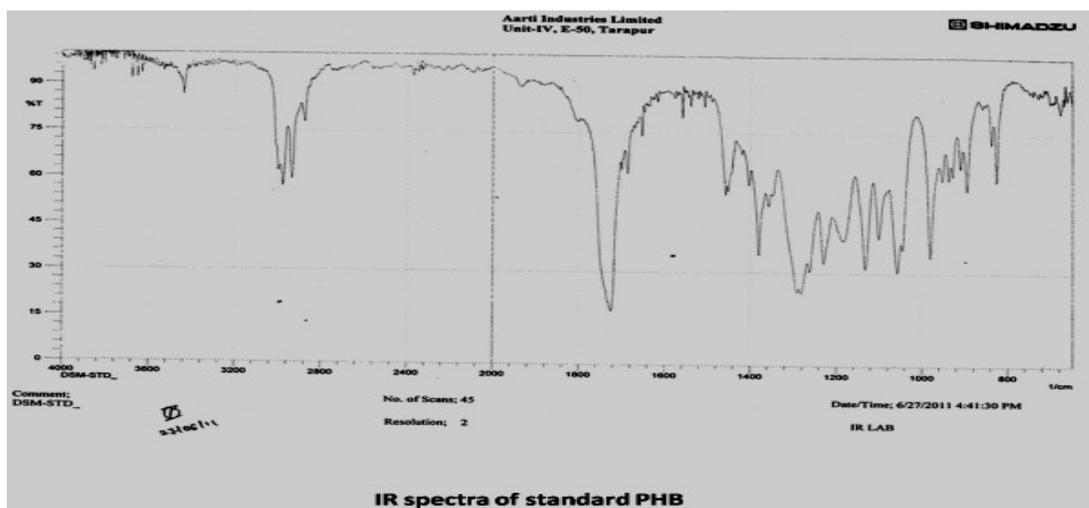
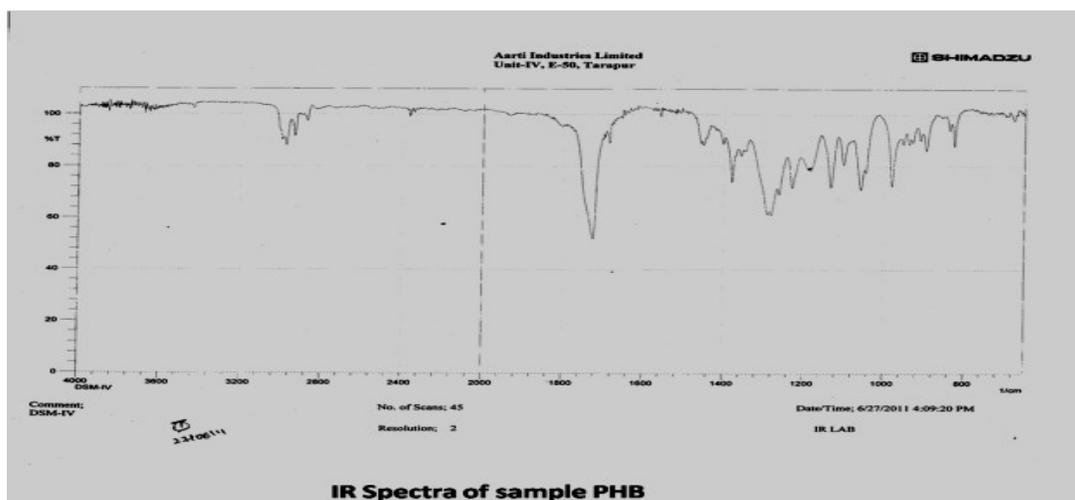
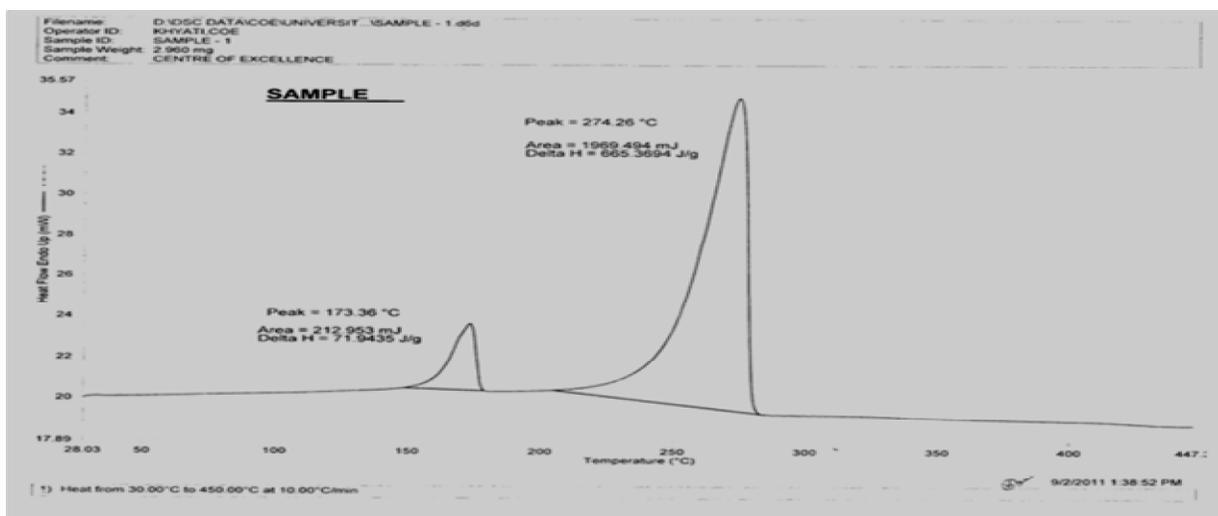


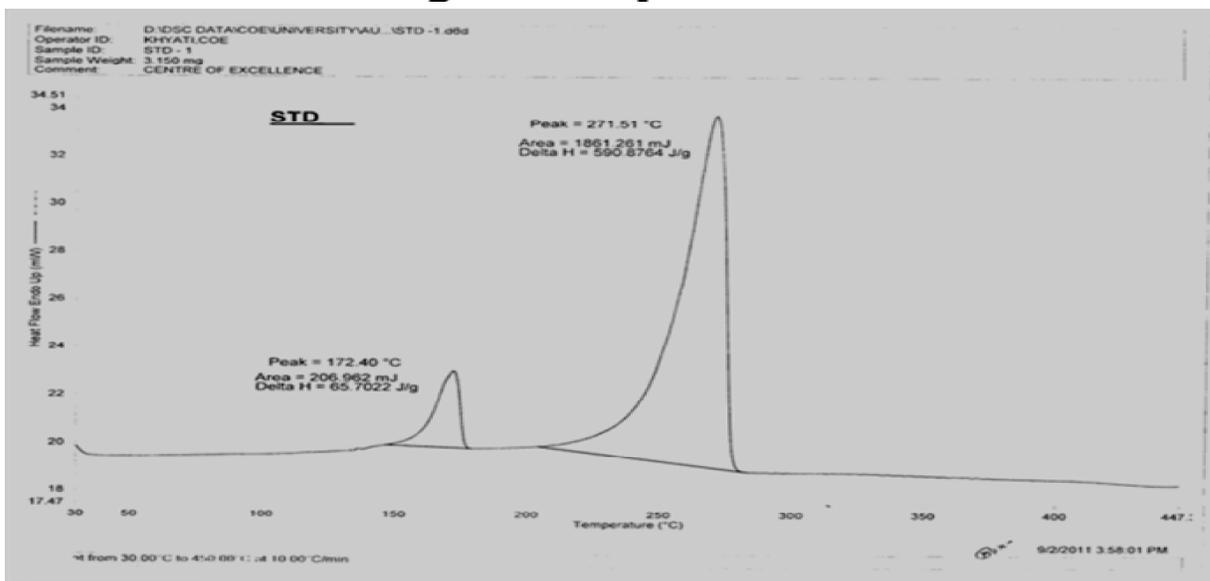
Table.2 Thermal properties of PHB

Sample	T_m ($^{\circ}\text{C}$)	ΔH (J g^{-1})
PHB Sample	173.36	69.54
PHB (Sigma)	172.40	65.70

Figure.3 DSC thermo gram comparison between standard and sample PHB



DSC thermo gram of sample PHB

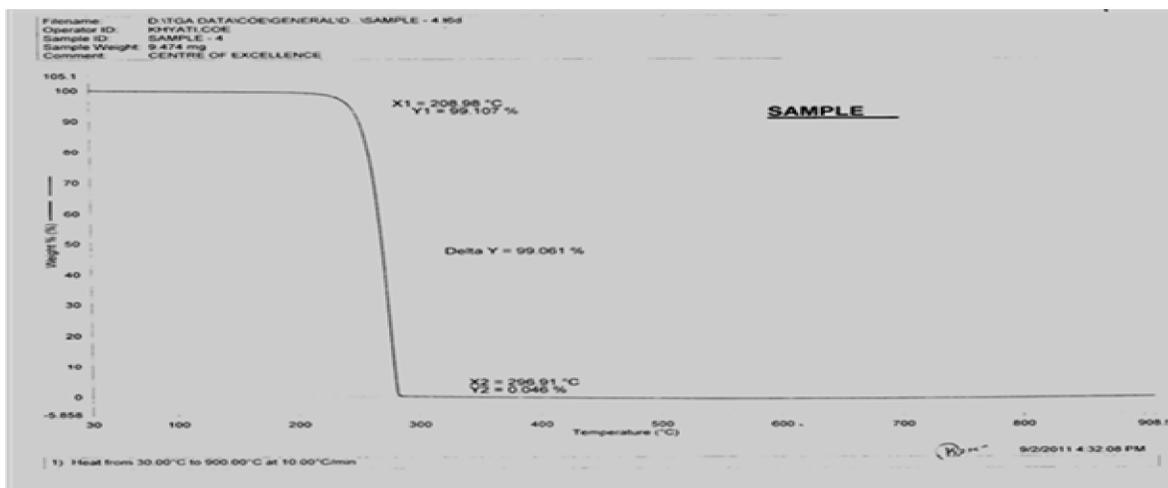


DSC thermo gram of standard PHB

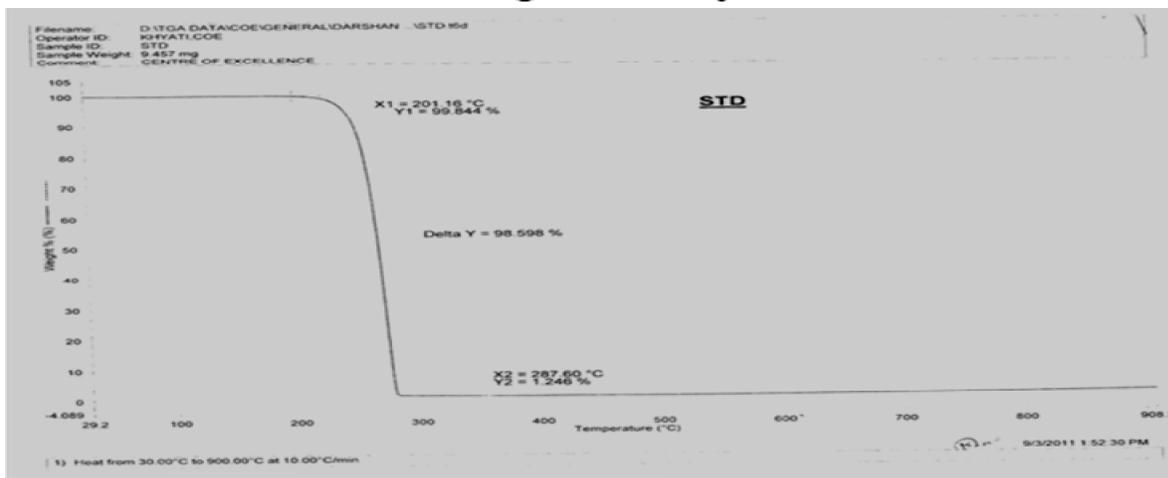
Table.3 Initial and maximum decomposition temperatures evaluated from TGA

Sample	Ti (°C)*	T max (°C)
PHB Sample	208.98	296.91
PHB (Sigma)	201.16	287.60

Figure.4 TGA thermo gram comparison between sample and standard PHB



TGA thermo gram of sample PHB



TGA thermo gram of standard PHB

Thin Layer Chromatography (TLC)

As per the procedure described earlier, when the TLC plate sprayed with iodine vapour, PHB appeared as greenish-black spot surrounded by brown colour on white background. The comparison between

standard and sample was performed using solvent system of ethyl acetate and benzene on TLC plate; Rf value (0.71) indicated the presence of PHB in the production medium by comparing with standard PHB.

Infrared Spectroscopy (IR)

IR spectrum of the compounds were recorded in the range of 800-4000 cm⁻¹ and showed characteristics bands for the groups CH, C=O and C-O (Sindhu, Raveendran et al. 2011). The methine groups (CH) gave strong band in the range of 1360-1416 and 2914-3097. These frequency values were higher than the normal values because of polymerization. The carbonyl group (C=O) gave strong band in the range of 1636-1673. These frequency values were lower than the normal value because of polymerization. The (C-O) group showed strong and broad absorption in the range of 1047-1089.

Differential Scanning Calorimetry (DSC)

The thermal properties of PHB samples and commercial PHB were investigated by differential scanning calorimetry (DSC). The data illustrated in figure 3. Since the melting temperature of PHB is around 170-180°C (Matko et al., 2005), while that of the PHB sample is within the range (173.36°C), which is close to that of the commercial PHB.

Thermo gravimetric analysis (TGA)

Figure 4 shows the TGA thermo grams of PHB sample and standard. The thermal degradation of extracted PHB proceeds by a one-step process with a maximum decomposition temperature at 296.91°C. This thermal degradation at maximum decomposition temperature of approximately 300 °C is mainly associated with the ester cleavage of PHB component by β-elimination reaction (Choi et al., 2003). The temperature of 296.91°C was found to be the maximum decomposition

temperature for biopolymer made with extracted PHB and it was almost similar with that of the standard PHB from Sigma (287.60 °C).

Further, the characterization of PHB like granules by various methodologies and their comparison with standard PHB as described earlier shows that the extracted polymer from the microbial isolate possess almost similar properties and was finally confirmed to be PHB (Poly-β-hydroxybutyrate) and of good quality.

Polyhydroxybutyrate (PHB) was successfully produced through biosynthesis in *S. epidermidis* and recovered appropriately. The method of PHB extraction was also influence the quality of polymer. Therefore, bacterial cells were blended with chloroform using high speed homogenizer for a short time to cause lower damage of PHB the molecular weight. The identical PHB sample was verified to commercial PHB and other PHB data that reported in literatures. The obtained PHB has the same thermal properties as commercial PHB with higher molecular mass (approx. 3.9 x 10⁶ Da) and lower degree of crystallinity.

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