PURIFICATION AND CHARACTERIZATION OF POLY-HYDROXYBUTYRATE (PHB) IN CUPRIAVIDUS NECATOR

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ABSTRACT

Purification and characterization of biodegradable plastic namely Polyhydroxybutyrate (PHB) in Cupriavidus necator have been carried out. C. necator was grown on a Ramsay medium with fixed substrate conditions and optimized for time. Stepwise purification of PHB was carried out, by using hydrogen peroxide and chloroform. The effect of temperature, time, and hydrogen peroxide concentration on the purification were also evaluated. The extracted PHB was studied with XRD, FTIR and ^{1}H-NMR and ^{13}C-NMR to determine its structure and purity. Yield and crystallinity were also studied with HPLC and XRD, respectively. The results of the research showed that higher concentrations of hydrogen peroxide gave better yields, whereas higher temperatures and longer lysis times led to different results. Higher crystallinity was observed when purification temperatures were elevated, but higher hydrogen peroxide concentration and longer extraction time gave varying crystallinity. The highest yield ca 66.10 % DCW was reached by purification using H_2O_2 20 %, at 100 ^{0}C for 2 h. The results of TGA analysis indicated that the purity of the PHB obtained was about 75 % and by using DSC, it was found that the PHB showed good thermal properties.

Keywords: *PHB, recovery, hydrogen peroxide, characterization*

INTRODUCTION

The use of petroleum-based plastics places a heavy burden on the environment. Due to their persistent nature, these plastics either end up in the environment where they cause harm to wildlife or are incinerated, thus contributing to global warming through increased levels of carbon dioxide [1]. In order to overcome this problem, the use of biodegradable plastics offers a promising alternative [2]. Contrary to synthetic plastics, biodegradable plastics are broken down in the environment completely, leaving only carbon dioxide and water [3]. Thus, contrary to synthetic plastics, they do not leave behind an ecological footprint. A biodegradable plastic that has received much attention is polyhydroxybutyrate (PHB). It is produced intracellular by a number of soil living bacteria under unbalanced growth conditions.

Widespread production of PHB has far been limited due to high production costs. For PHB production to become more economically feasible, better bacterial strands as well as cheaper feedstock and purification methods are needed. Genetically modified bacteria will allow the use of cheap and abundant sources, such as household waste, agricultural and industrial waste, waste water, etc. for producing large amounts of PHB. Cheap, safe and efficient purification methods can then be used to recover PHB with high purity.

Among *known purification methods*, digestion by sodium hypochlorite followed by extraction with chloroform has received much more attention due to its simplicity and effectiveness. However, the chlorine given off by the reaction might cause health problems. To overcome this problem, in this research, the use of hydrogen peroxide as a digestion agent has been proposed to replace sodium hypochlorite. This method might prove to be a more environmentally benign, since hydrogen peroxide does not leave any traces in the product. In order to determine the most suitable condition for purification, the effects of temperature, hydrogen peroxide concentration and lysis time on yield and crystallinity of PHB have been evaluated. For producing PHB, *C. necator* bacteria was selected since it has well established biosynthesis pathways [4] and can produce PHB up to 80% of its cell weight [2].

EXPERIMENTAL SECTION

Microorganism and Culture Conditions

Cupriavidus Necator CCUG 52238 T was obtained from Chalmers University, Sweden. All media

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used were sterilised at 121 ^{o}C in an autoclave, prior to their use.

The strain was kept on agar slants in a medium of fish peptone, beef peptone, yeast extract and glucose (NGY) and stored at 4 ^{o}C. The fermentation process consisted of two pre-culture steps followed by fermentation. In the first pre-culture step a loopful of *C necator* was inoculated in 8 mL of an NGY medium and placed in an incubator shaker overnight. After the solution had become sufficiently cloudy, the optical density (OD) was measured at 600 nm with a UV-VIS spectrometer. When the OD was between 0.5 and 0.7 (with 10 times dilution) the solution was moved to a Ramsay medium with glucose as carbon source with a total volume of 80 mL to start the second pre-fermentation step. After 24 h in the incubator shaker, the solution was moved to the final fermentation medium which had a total volume of 800 mL. Bacterial growth was optimized so as to determine the optimum time for harvesting. The fermentation step was carried out in 2 l flasks with a 40 % working volume for sufficient oxygen levels.

Growth Optimization for Time

To determine the optimum harvesting time, dry cell weight (DCW), glucose and ammonia concentration was measured every 6 or 12 h over a period of 72 h. Culture samples (5 mL) were centrifuged (14.000 x g, 3 min) and the supernatant was stored at 4 ^{o}C for further analysis. To obtain the dry cell weight, the cell pellet was washed with distilled water, re-centrifuged and then dried to constant weight (90 ^{o}C, 24 h) and subsequently cooled in a desiccator and weighed. Using the supernatant stored, each sample was analysed for glucose using the *DNS* method [5] and the ammonia concentration was measured using the *Phenate* method [6].

Purification

After the fermentation process was halted, cells were centrifuged at 3000 g for 20 min. The supernatant was discarded and the cell mass was washed with a 0.85 % sodium chloride solution and centrifuged again. Finally, the cell mass was re-suspended and brought to a volume of 10 mL. From this suspension 1 mL aliquots were taken to undergo purification treatment. A simple 3-factorial design (2^3) with high and low was used for temperature (80 ^{o}C, 100 ^{o}C), hydrogen peroxide concentration (5 %, 20 %) and purification time (1h, 2h). After treatment with hydrogen peroxide solutions at given temperatures and time periods, the samples were washed with water and subsequently shaken with chloroform. Three layers appeared namely a watery layer on top, one of disrupted cells and heavier bottom layer. The top layer was removed with a pipette and the disrupted cells were removed through filtration. A fivefold volume of methanol – water mixture (7:3) was added for dry precipitation of PHB. The precipitate was centrifuged and washed twice with acetone and diethyl ether.

Analysis and Characterization

PHB produced was characterized with Shimadzu X-RD 6000X, Shimadzu FTIR spectrometer 8201 PC, and 500MHz JEOL ECA 500 H-NMR and C-NMR spectrometer to determine the structure. In addition, XRD and LC-10AD Shimadzu Liquid Chromatograph (HPLC) with CTO-10A Column Oven were used to measure crystallinity and yield, respectively of PHB purified under different conditions. For HPLC analysis, PHB was converted into crotonic acid by addition of concentrated H_2SO_4 [7]. In addition, thermal analysis was carried out with Perkin Elmer Pyris 1 DSC and TGA with Pyris Software for Windows version 5.

RESULT AND DISCUSSION

Culture Conditions

The dry cell weight (DCW) of *C. necator* measured every 6 to 12 h during growth, for a period of 72 h was displayed as Fig 1. It can be seen that after 66 hours the DCW had reached a maximum of 8.32 g.L^{-1} which dropped slightly to 7.88 g.L^{-1} after 72 h.

The glucose and ammonia concentrations left in the media due to be consumed by the bacteria were also measured during growth, and the results were presented as Fig 2 and 3, respectively. The figures showed that after 54 h the concentration of glucose had reached a level of 1.0 g.L^{-1} and the ammonium concentration had become depleted at around 42 h.

For an optimal yield of PHB, fermentation is usually halted during the exponential growth phase at the point where ammonia becomes the limiting factor. From Fig 2 and Fig 3 it can be seen that at 48 h, PHB was in the exponential growth phase, ammonia had become depleted, and thus further glucose consumption was due to PHB biosynthesis. Therefore, this time was taken as the most suitable to halt fermentation.

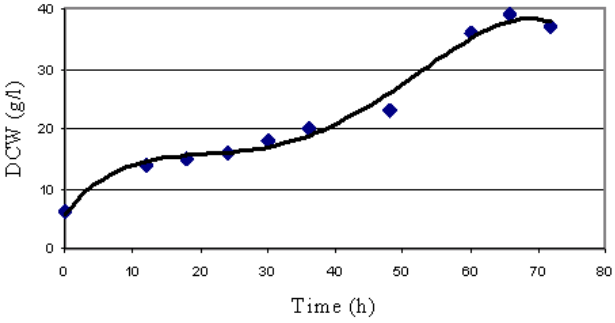

Fig 1. Growth curve of *C. necator*

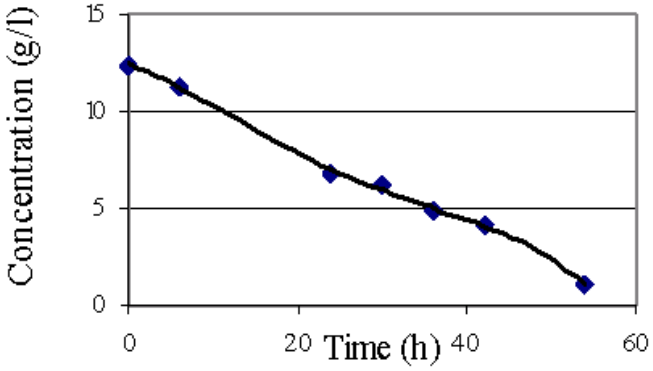

Fig 2. The concentration of glucose left in the media during growth of *C.necator*

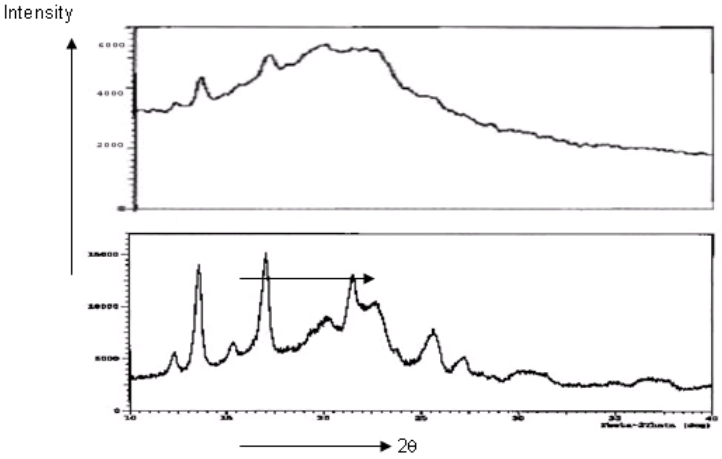

Fig 4. XRD patterns of*:* PHB recovered from *C. necator* (Top*), and of* standard PHB (Bottom)

Identification and determination yield of PHB

To identify the PHB produced in *C. necator* its XRD pattern along with that of standard PHB were recorded from 20-40 of 2theta, as seen in Fig 4. It can be seen that the characteristic peak values of 2θ of 12.1, 13.4, 15.2, 16.8, 20.0, 22.4 and 25.4 found in standard PHB were also found for extracted PHB. The sharper peaks and higher peak intensities found in analytical grade PHB were due to its higher crystallinity.

FTIR spectrum of PHB was presented as Fig 5 that revealed characteristic peaks at 1288.4 cm^{-1} and 1728.1 cm^{-1} which correspond to the C–O and C=O stretch respectively. The data was in agreement with XRD, confirming the presence of PHB. An additional peak found at 3440 cm^{-1} did not belong to PHB and was believed to be caused by the presence of water traces in KBr used for preparing the samples.

Furthermore, ^{1}H-NMR spectrum of PHB produced is seen as Fig 6. The figure showed a number of characteristic PHB peaks at δ = 5.2, 2.5, and 1.2 which correspond to a ─CH doublet, ─CH_2 multiplet, and –CH_3 doublet respectively [8]. Two small additional peaks at

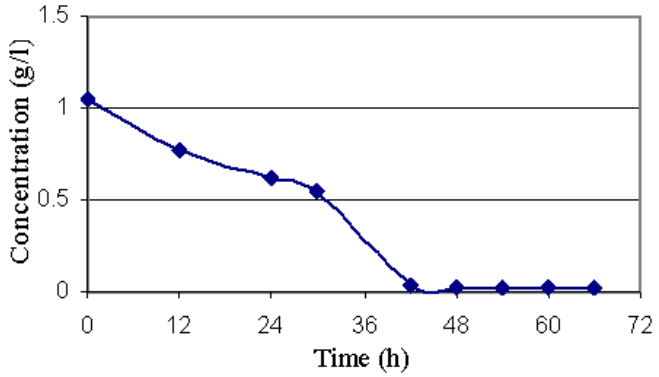

Fig 3. The concentration of ammonia left in the media during growth of *C. necator*

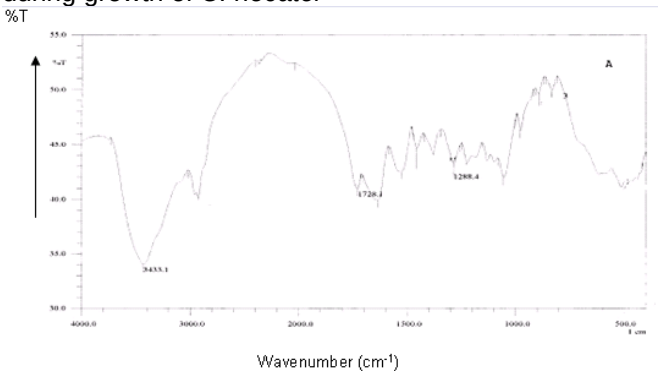

Fig 5. FTIR spectra of *:* PHB recovered from *C. necator*

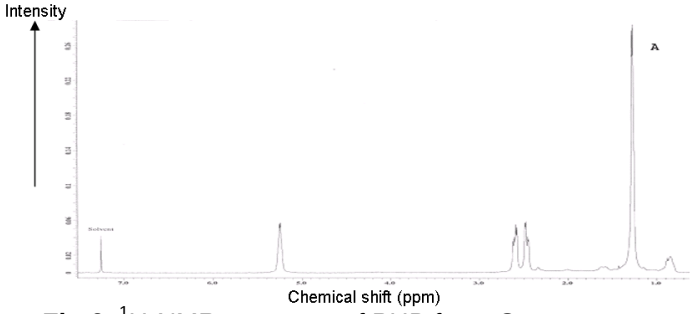

Fig 6. ^{1}H-NMR spectrum of PHB from *C. necator*

δ = 0.9 and δ = 1.6 were found may be due to impurities present.

Following the identification, the yield of PHB was determined for different recovery conditions using HPLC and the results are given in Fig 7.

It was found that higher concentrations of hydrogen peroxide gave better yields. This was due to higher concentrations of hydroxyl radicals formed, which led to increased cell disruption, allowing PHB to be extracted more easily by chloroform. The hydroxyl radical is produced from hydrogen peroxide through an iron-based enzyme [9]. The reactive hydroxyl radical is known to damage macromolecules, including DNA, proteins and lipids. This is initiated by a process called lipid peroxidation, which occurs when hydroxyl radicals attack unsaturated fatty acids, the main component of

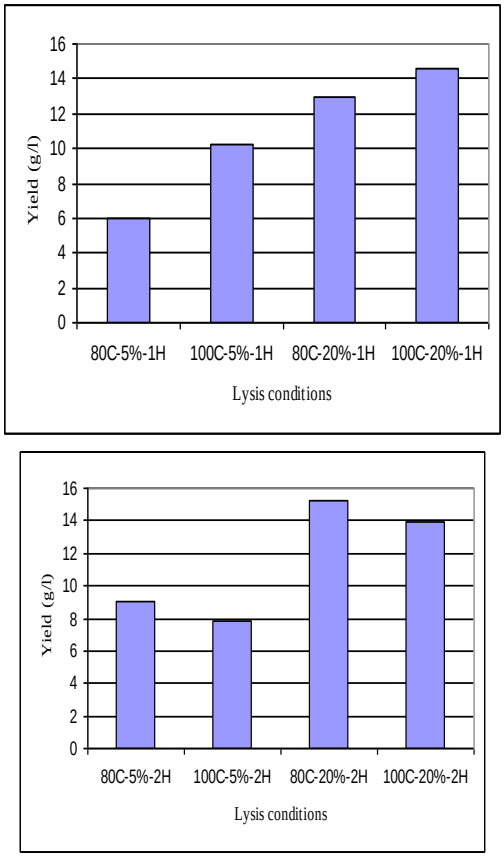

Fig 7. Yield of PHB recovered from *C. necator* with hydrogen peroxide and chloroform under different conditions.

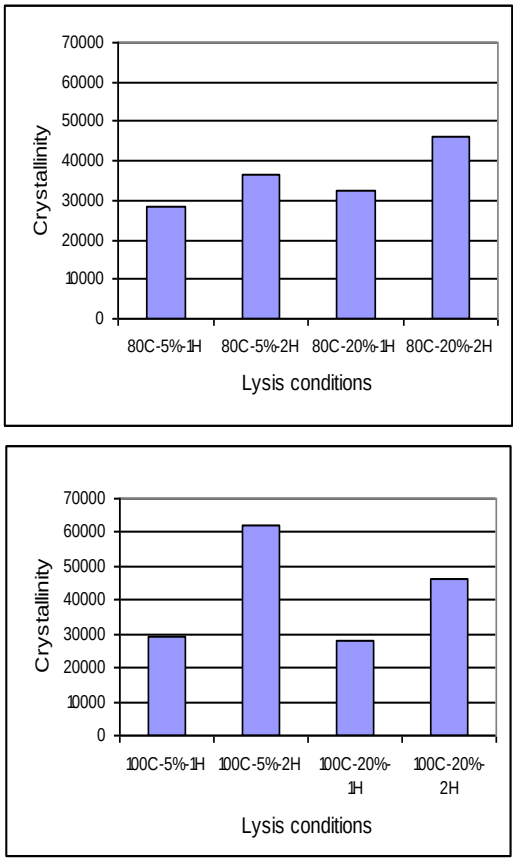

Fig 10. Crystallinity of PHB recovered under different conditions

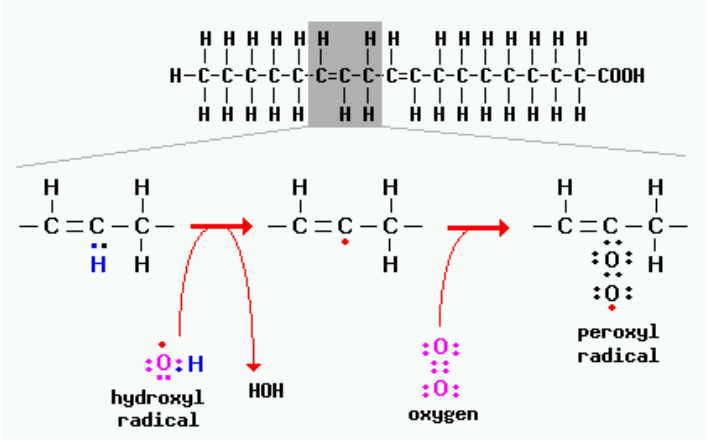

Fig 8. Lipid peroxidation by the hydroxyl radical [9].

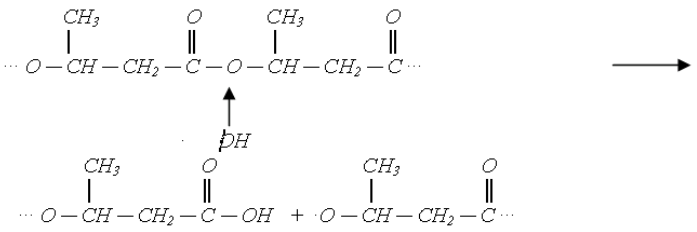

Fig 9. Reaction of OH radical with PHB

cell walls. Fig 8 shows how a hydroxyl radical abstracts a methylene hydrogen leading to the formation of another radical. The radical formed reacts with oxygen to give a peroxyl radical which in turn, can react with other fatty acids or proteins, resulting in a chain reaction.

In addition, higher temperatures and longer lysis times led to different results, which indicated that there was an interaction between both factors. Lower yields at longer times or higher temperatures were the result of PHB degradation by hydroxyl radicals. Degradation of PHB through hydrogen peroxide is thought to take place according to Fig 9.

A hydroxyl radical attacks the polar carbonyl bond in PHB, which leads to the formation of another radical. This radical in turn can attack another carbonyl bond; hence scission of the polymer chain occurs, causing degradation of PHB. The highest yield was found for recovery at 80 ^{o}C, concentration of 20 % H_2O_2 and a recovery time of 2 h.

Characterizations

In a similar way as for yield, the crystallinity of extracted PHB was also measured for different

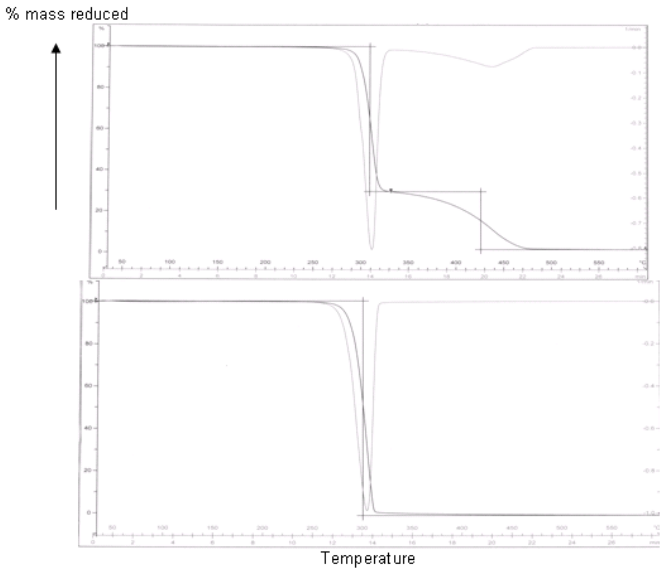

Fig 11. Thermogram of PHB. Top: *Top*: PHB recovered from *C.necator. Bottom*: Analytical grade PHB

recovery conditions, and the result is presented as Fig 10. The trend of PHB crystallinity obtained by varying conditions was similar as that of the yield. It is clear that the crystallinity degree is proportional with the yield. The highest crystallinity was found for recovery at 100 ^{o}C with 5 % H_2O_2 at 2 h as can be seen.

The result of TGA analysis was presented as Fig 11. The data obtained showed that thermal degradation of PHB occurred at 300 ^{o}C, indicating that the PHB produced has good thermal stability in comparison to analytical grade PHB.

It was seen however that PHB produced was degraded in two steps, with the second step occurring at 400 ^{o}C. After the first step, a residue of 29.6 % was found and after the second step 3.6 % remained. This was due to impurities present which might be proteins or lipoproteins from non-extracted cell mass.

To confirm TGA data with regard to the thermal stability of the extracted PHB, Differential Scanning Calorimetry (DSC) was also employed. The second run in Fig 12, after quenching and re-heating, showed a glass transition point (T_g) between 4 ^{o}C and 5 ^{o}C, a crystallization during heating of around 40 ^{o}C or 50 ^{o}C and a melting point (T_m) at around 170 ^{o}C. The cooling curve showed a crystallization peak close to 85 ^{o}C. The data found were in agreement with values as reported by Janigova *et al.* [10]. Thus, from the results obtained, it is clearly indicated that recovered PHB was of good quality.

CONCLUSION

Polyhydroxybutyrate (PHB) was successfully produced through biosynthesis in *C. necator* bacteria harvested at 48 h as the optimum growth time. Purification results showed that longer lysis time gave lower yields due to degradation of PHB. Higher yields of PHB were obtained when higher concentrations of hydrogen peroxide at different temperatures and lysis times were employed. Highest yields was reached by using purification at temperature of 80 ^{o}C, concentration of 20 % H_2O_2 and time of 2 h, that was 66.1 % DCW, while the highest crystallinity of PHB produced was obtained with purification at 100 ^{o}C, 5 % H_2O_2 and 2 h. Characterization results showed that PHB produced had good thermal stability. However, the PHB produced still contained impurities around 25 %. To increase the purity, longer extraction times and or multiple extraction steps is suggested to be further studied.

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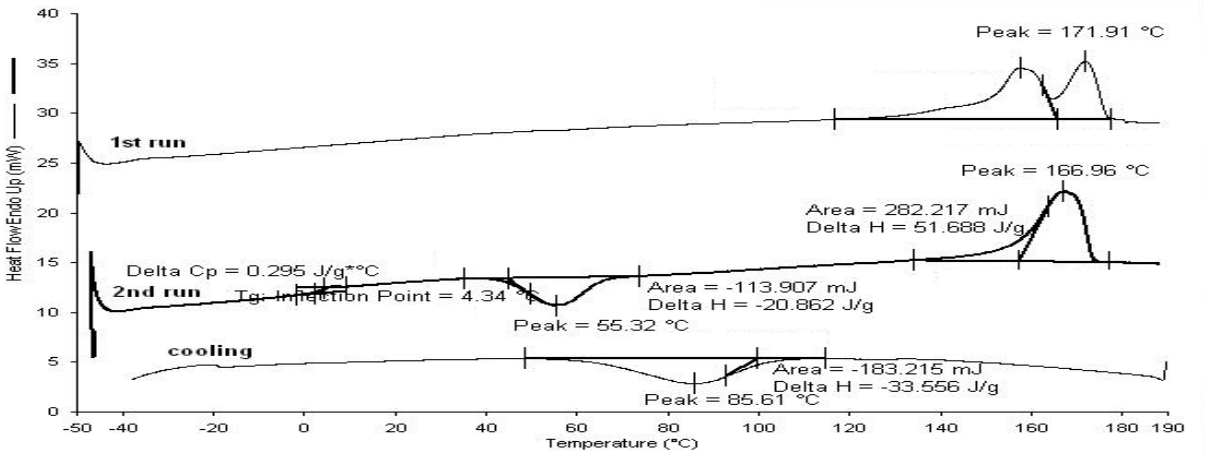

Fig 12. DSC curve of PHB from *C. necator*

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