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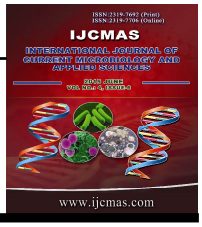

Original Research Article

Production, Inexpensive cultivation and Optimization of copolymer biosynthesis by *Brevundimonas* sp OU6T from rice bran

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Keywords

Medium chain length polyhydroxy alkanoates (MCL- PHA), Copolymer, Rice bran

A B S T R A C T

Brevundimonas sp.OU6^{T} was isolated from polluted water was characterized to evaluate its ability to accumulate MCL- PHA(co-polymer) using rice bran, a low cost agro industry residue. The culture is able to produce 1.72 g/L of polymer after 40 h at 30°C.Growth of the isolate OU6^{T} was assessed in mineral media containing glucose, starch, bagasse, whey and rice bran without adding any precursors. Strain OU6^{T} grew well on hydrolyzed rice bran at pH7 and accumulated 61.86 PHA%(wt).Optimization studies were done at different temperatures, pH, incubation periods and different carbon sourses. *Brevundimonas* sp.OU6^{T} was able to utilize a wide range of carbon sources with good enzymatic potentials, The purified polymer sample from cells was confirmed as MCL-PHA by FTIR, ^{1}H NMR, ^{14}C NMR analysis. The GC-MS spectra analysis confirmed that strain was able to produce Octanoic acid, Decanoic acid and Do-decanoic acid along with Poly hydroxyl butyrate. Polymer shows flexibility and toughness comparable to conventional thermoplastics. In addition, the utilization of rice bran as raw material, will achieve further cost reduction along the effective utilization of waste material.

Introduction

Polyhydroxyalkanoates (PHA) are a family of polyesters accumulated as intracellular granules of reserve materials by several bacteria under unbalanced growth conditions such as limitation of nitrogen and carbon source excess (Brandl,1992). PHA are classified according to the number of carbons of each repeating units in the polymer. Polymer containing monomers composed of C3 to C5 (e.g. polyhydroxybutyrate (PHB) and hydroxyvalerate (PHV)) hydroxyl fattyacids are referred to as short chain lenth PHA (SCL PHA). In contrast, those composed of C6 to C16 hydroxyl fatty acids oraliphatic carbon sources are termed as medium chain length PHA (MCL PHA) (Matsusaki *et al*., 1998, Tian *et al*., 2005).*Cupriavidus necatar* is the most intensively studied bacteria to synthesize PHB (Vandamme *et al*., 2004). Another species common for its ability to synthesize MCL PHA is Pseudomonas

oleovarans (Timm *et al*., 1990) MCL PHA consists of monomers of 3 hydroxy-hexanoate (HHx), 3-hydroxyoctanoate (HO), 3-hydroxydecanoate (HD), 3-hydroxydodecanoate or even higher chain length monomers (Ouyang *et al*., 2007). Properties of MCL PHA are completely different from polyhydroxybutyrate (PHB). MCL- PHA is amorphous and viscoelastic and has low *T*m, weaker tensile strength, and longer elongation rate compared with PHB.

Since the first report on Polyhydroxyalkanoates (PHA) biodegradability, numerous studies have focused both on the isolation of new microorganisms with PHA synthesis capacities and the use of substrates cheaper than glucose as a source for PHA production (Cerrone *et al*.,2010). From an economical point of view, the cost of substrate for PHA production can be decreased if a waste product is used (Choi *et al.*, 1997). This strategy could make PHA production more economical, which at the same time treating wastes without extra disposal cost.

The ability to produce biodegradable polymers from inexpensive and renewable carbon sources may improve the economics of the process and lower production cost. There has been considerable interest in the use of cheap carbon substrates for PHA production since the cost of the carbon source accounts for about 50% of the production costs (Kim., 2000). The various cheap carbon sources used include whey, wastewater from olive mills, molasses, corn steep liquor; starchy wastewater and palm oil mill effluent (Lapointe *et al.*, 2002; Marangoni *et al.*, 2002; Pozo *et al*., 2002).There are only a few reports on the use of rice bran by bacteria to produce PHA. Van-Thuoc *et al*., (2007) reported that in order to use the agro-industrial residues as fermentation substrates, these should be subjected to hydrolysis for the release of easily metabolizable sugars. In this study, we hydrolyzed the rice bran by using water and not by acids and have studied the cultural conditions on the synthesis and accumulation of PHA by *Brevundimona*s sp $OU6^T$ .The potential of the isolate to accumulate copolymer (MCL- PHA) using rice bran andanalysis of the copolymer has also been evaluated.

Materials and Methods

Bacterial Strain and media

The bacterial strain ***Brevundimonas sp* OU6** CCM 7839^T was isolated from polluted pond, was used study for the production of PHA. The strain was maintained on E2 mineral medium (Lagveen *et al.*, 1988) to check the PHA production. Cultures were incubated at 30^0 C for 48 h on a rotatory shaker at 150 rpm/min. Glucose and rice bran (each 20 g l^{-1}) were used separately as sole as carbon source. The pH of the medium was adjusted to 7.2 before sterilization. LB agar was used as a solid medium for colony purification and for maintenance of the strain at 4^0C.

Preparation of rice bran hydrolysate

Rice bran is a byproduct of the rice milling process and its hydrolysate was prepared by suspending 20 gms of finely powdered rice bran (2%) in 1 liter of water. pH was adjusted to 7.0 and heated to 80^0 C for 30 min. The content was filtered and the volume was made up to 1 liter. This was used directly with the E_2 mineral medium.

Determination of total sugar content

The carbohydrate content of raw materials in the culture broth was measured by phenol sulphuric acid method (Dubios *et al.*, 1956) using standard graph.

Optimization studies

Different parameters were selected for optimization. These include different concentrations of rice bran, effect of various carbon source concentrations, incubation periods, pH and temperature. The carbon sources (2%), glucose, lactose, galactose, sucrose and maltose were added to the E_2 mineral medium. Flasks with E_2 mineral medium were incubated at varying temperature between 15-40 ^{0}C with a difference of 5 ^{0}C. For the analysis of other parameters, *Brevundimonas sp* OU6^T was grown on E2 media with 2% glucose as sole source of carbon, for 48h on an orbital shaker at 150 rpm and 30 °C. Time dependent studies were carried out by withdrawal of a pair of flasks at regular intervals (4 h). The growth in the liquid medium was monitored in terms of absorbance of the culture at 420 nm using visible range of a UV spectrophotometer. The effect of variation in the pH of medium was recorded by growing the isolates at different pH values on E_2 mineral media. The rpm of the shaker was adjusted at different speeds, to study the growth and PHA accumulation of the isolates at different variations of rpm. The analysis was also done by different concentrations of glucose.

Fluorescence microscopy

The presence of cytoplasmic inclusions were seen with Sudan black B staining (Norris and Swain., 1971), further confirmed by Nile blue staining (Ostle and Holt., 1982) and observed the cells under the fluorescence microscope.

Analytical methods

After incubation, each sample was used for the determination of the cell dry weight (cdw) and PHA content in the culture supernatant fluid. The cell concentration was determined by measuring cdw as follows: 5 ml culture broth were centrifuged, pellet obtained was washed and dried at 105°C until the weight did not decrease further. PHA estimation was carried out according to Law and Slepecky (1961). PHA (%) was defined as the percentage of the ratio of PHA to cdw. PHA extraction was performed according to the hypochlorite method (Ramsay *et al*., 1990).

FTIR

Extracted polymer from strain *Brevundimonas sp* OU6^T was subjected to FTIR analysis. Intense absorptions at 1724–1740 cm−1, which corresponds to ester functional groups primarily from lipids, fatty acids and PHA were evident.

^{1}H NMR

The ^{1}H NMR analysis of the polymer sample was carried out on Varian-300 spectrometer (USA). The 300 MHz ^{1}H NMR spectra were recorded at 24°C in CDCl3 solution of polyester (50 mg/ml) with a acquisition time of 2.0480 seconds, sweep width of 4000 Hz. Tetra methyl saline was used as an internal chemical shift standard. The spectra was recorded for commercial PHA (Sigma-Aldrich, USA) and for the polymer extracted from test strains.

^{14}C NMR

The 100-MHz 13C NMR spectra were recorded at 23°C on a CDCl3 solution of polyester (50 mg mL−1) with an 18.5-μs pulse width, 90° pulse angle, 5 s repetition and 24,154 Hz spectral width. The chemical shifts (δ) were referenced against tetra methyl silane internal standard.

Thermal analysis

The thermal properties of the homopolymers, copolymers were investigated by DSC. Sample OU6 DSC (Universal V4.2E TA Instrument) runs were made at a heating rate of 10_C/m between 10 and 500_C under 50 mL/m nitrogen in a dynamic mode of operation. The Tg was determined from the second heating cycle.The polymer film weighing 5-10 mg was encapsulated in aluminum pans and sealed in a press. The reference pan was sealed empty. Two holes were bored in the pan lids to allow gaseous nitrogen to create an inert atmosphere in the DSC chamber and the sample. The pans were uniformly heated with a temperature increment of 10°C/min up to 190°C and rapidly quenched thereafter. The sample was reheated till 190°C to check the stability of the polymer. A peak was obtained at the Tm of the sample. In case of two peaks obtained, the higher temperature was considered as the Tm.

GC-MS analysis

For the peak analysis of OU6, Capillary GC-MS was performed on a Shimadzu GC-MS –G1110/MS data system. Samples were ionized by electron impact (70 eV). Column and temperature were as follows: DB wax column (polar, 30m, 0.32µm, 0.25µm thickness), temperature of the injector was 250° C; the initial oven temperature was increased at the rate of 15° C/min from 50 ° C up to 200° C. The helium gas used as carrier gas, with a split less injection (80:1), 1 µl of sample was injected and Hewlett-Packard model 5972 was used.

Results and Discussion

Polymer analysis

Florescence microscopy revealed the presence of polymer granules present in side the bacterial cells (fig 1). Polymer production was observed from 20h to 60h incubation period. Maximum growth was at 40h and polymer content was 61% (w/v) of CDW with rice bran. Maximum biomass was observed at pH 7.0. It was also observed that biomass production was fastest between 32h to 40h of growth. Strain produced 1.722 PHA g/Lwhen E2 Mineral medium was supplemented by 2% crude rice bran as carbon source, promoted PHA accumulation and utilized rice bran completely(Fig.2).

Polymer was extracted and IR spectra was recorded for the polymer dissolved in chloroform. Spectra showed two intense absorption bands at 1,727.19 and 1,277.07 cm^{-} 1, corresponding to C = O and C–O stretching groups, respectively. Other absorption bands at 1,379, 1,459, 2,922 and 3,410cm- 1corresponding to -CH3, -CH2, -CH and O–H groups are shown in Figure 3.

The ^{14}C NMR spectrum of PHA from $OU6^{T}$ is shown in Figure .4. The resonance of the monomers at 19.73, 40.75, 67.58, and 169.13 ppm were assigned by data comparison to 3HB according to the previous report (Doi *et al.*, 1986), The spectrum has 10 major and minor peaks for the carbon atoms of the individual monomer units, although the peaks corresponding to carbon atoms 6 and 7 can be resolved only when an expansion of the side-chain methylene carbons is examined.

Expansion of the carbonyl, side-chain methylene, and methyl carbon regions showed minor peaks due to 3HO monomer units, but these peaks were poorly resolved from the major peaks in all cases. The chemical shift data of individual monomers are consistent with those described for other PHA. Very small peaks corresponding to 3HHx monomers were observed in the spectrum.

Fig.1 Flourascence micrograph of . Brevundimonas sp.OU6T showing polymer inclusions

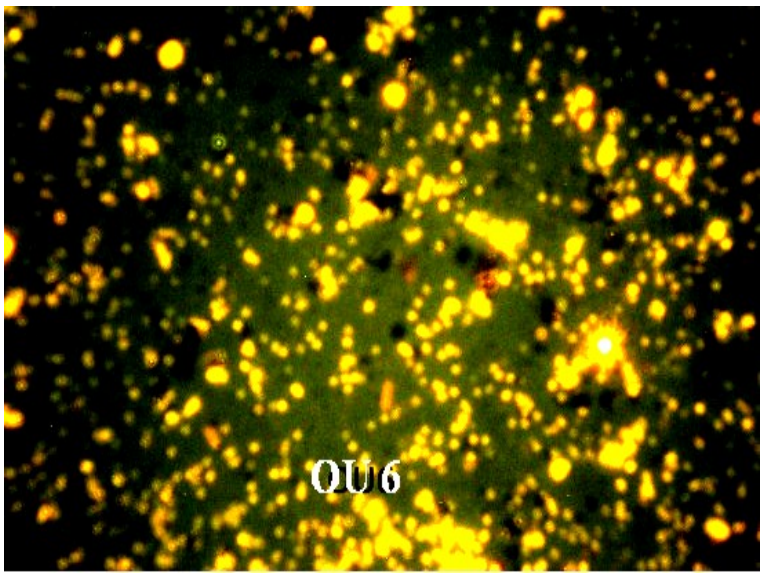


Fig.2 Graphical representation showing the optimization of various parameters of *Brevundimonas* sp.OU6^{T}

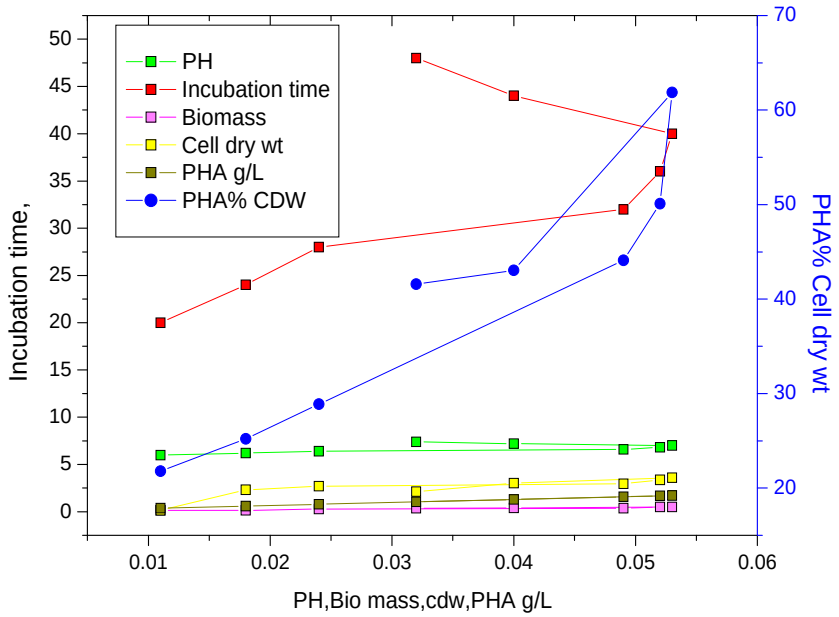


Fig.3 IR spectra of polymer from isolate OU6, grown on rice bran

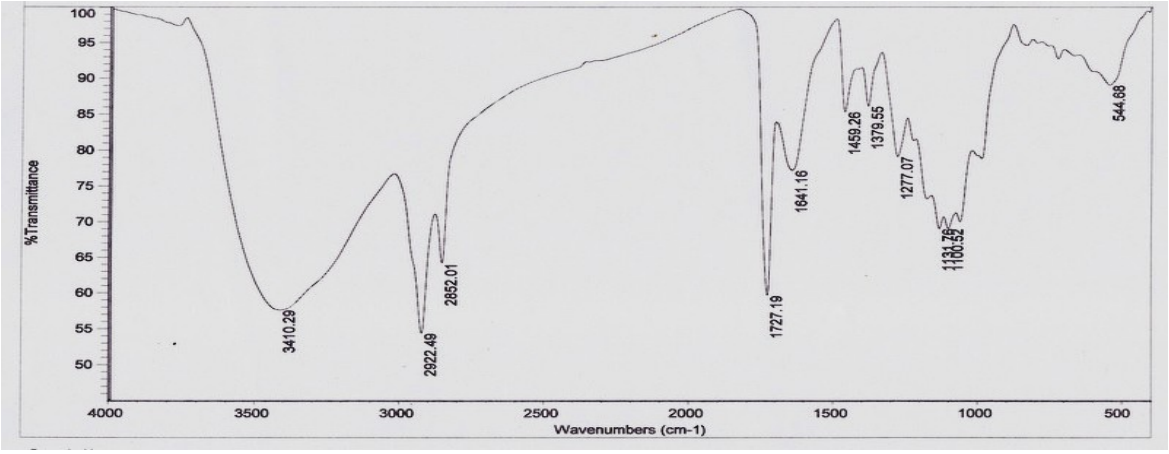


Fig.4 ^{14}C NMR spectra of polymer from isolate OU6

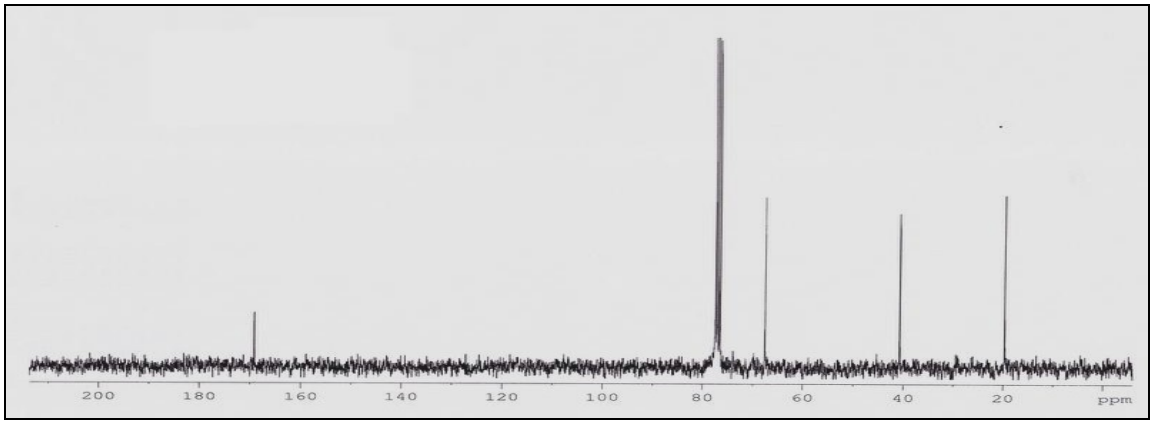


Fig.5 ^{1}H NMR spectrum of the polymer isolated from OU6

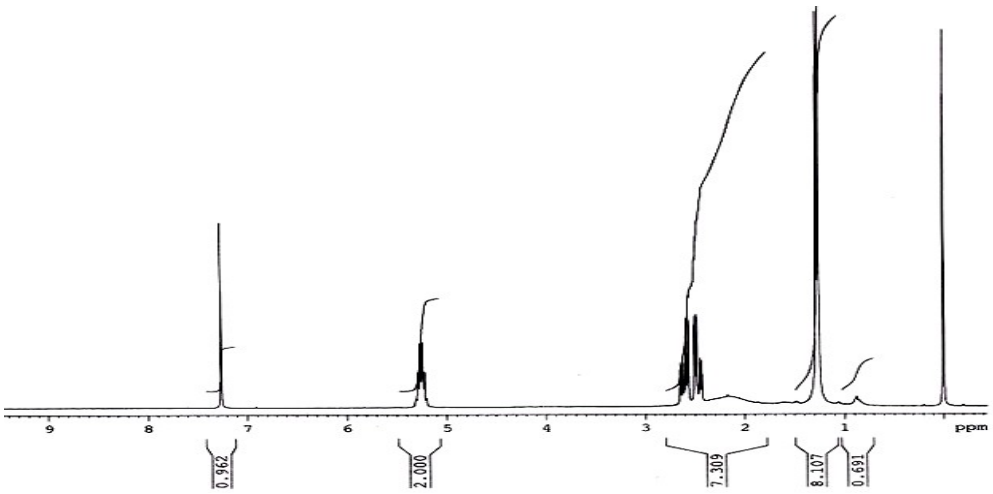


Figure.6 GC spectra of polymer from isolate OU 6 with rice bran; 6^{I} -3-hydroxy butyrate ; 10^{I} - benzoic acid; 23^{I}-dodecanoic acid

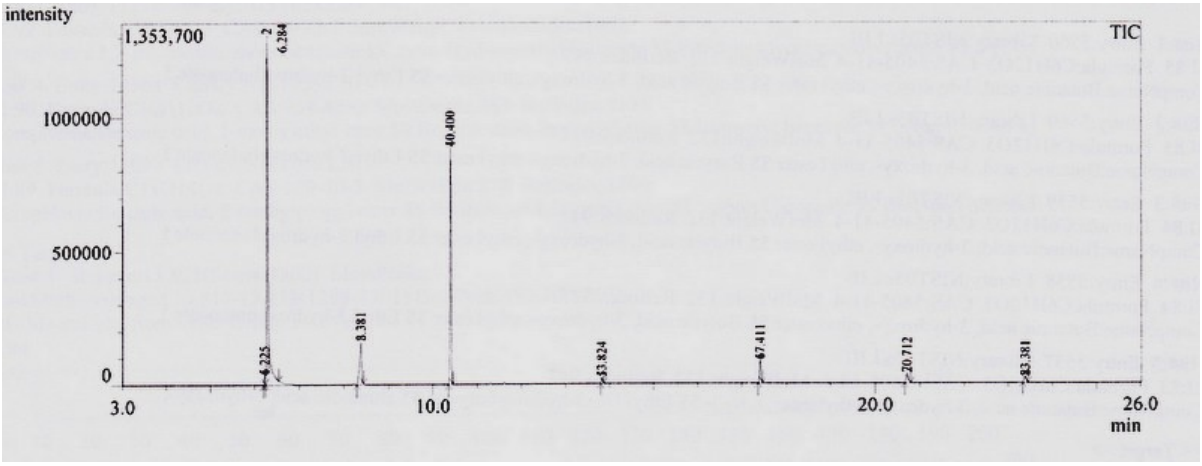


Figure.7 a and b.MS of the polymer extracted from the isolate OU6 with rice bran

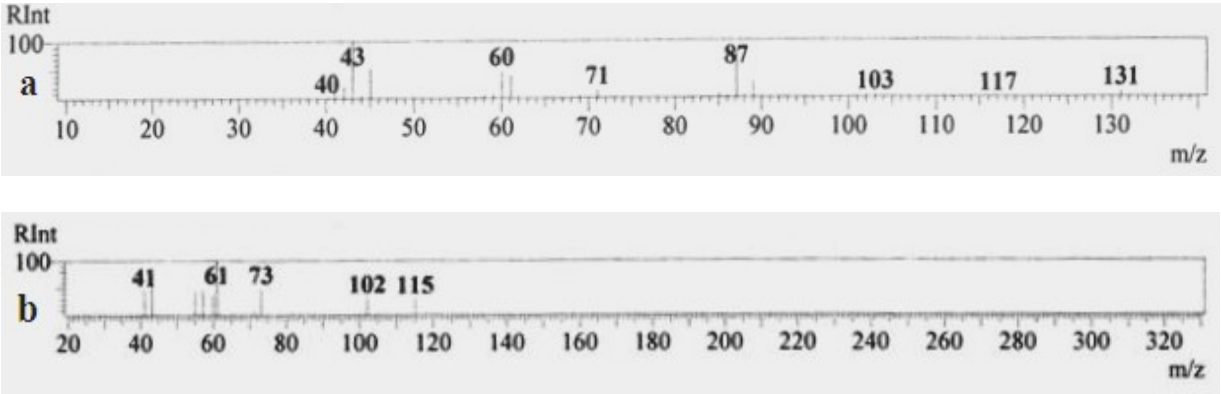
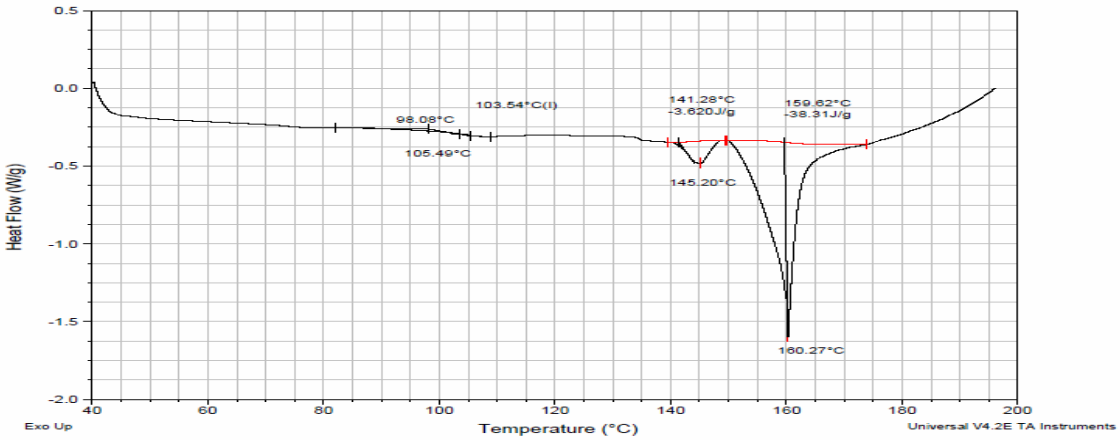


Fig.8 DSC of polymer from isolate OU6 grown on rice bran



All carbon lines are sharp singlets, ^{14}C NMR spectrum of OU6 revealed the presence of a copolymer with rice bran, containing PHB, along with decanoic acid and do decanoic acid which was confirmed by GC-MS (Figures 6).

The ^{1}H NMR spectrum of the polymer isolated from OU6 cells grown on ricebran showed major peaks corresponding to P (3HB) at 1.26 ppm (Figure.5). A doublet was recorded at 2.57and 2.50 ppm corresponding to the methylene group (-CH2-), while a multiple signal at 5.22 ppm corresponded to the methyne group (-CH-). Another doublet signal at 1.28 ppm corresponded to the methyl group (-CH3-). The methyl esters showed a sharp signal at 5.20 ppm, corresponding to the CH3-O group of the esters. These results suggest the major components of polymer to be poly-β-hydroxybutyrate. In addition a major peak at 0.8 ppm indicated the incorporation of monomers in the polymer sample. This characteristic signal indicates that the polymer accumulated by OU6 grown on rice bran contained greater than 90 mol% 3HB monomer units with a trace of octanoate and decanoate .From the spectra ,it is evident that OU6 produced a considerable amount of copolymer with rice bran.

In GC-MS, three major ester peaks were found for the PHA isolated from the strain OU6,with retention times of 6.2, 23.3,23.4 min(fig 6).These monomers were identified as, 3-hydroxybutyricacid, 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD) respectively. Thepeak at m/z 43 represented the 3-hydroxy butyrate (3HB), the peak at m/z 105.10 represented the standard benzoic acid, and the peak at m/z 61.05 represented the 3- hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD)(fig.7). These mass spectra were confirmed by comparison with those of authentic standards. The composition of PHA synthesized by OU6 from rice bran was found to consist of 95mol% 3HB, 2.5 mol%3HO and 2.5 mol%3HD.

DSC traces showing the melting temperature for the PHA isolated fromOU6, when grown on rice bran is shown in Fig 8. The melting point of copolymer (160.27^{0} C) is with in the range of those reported for PHA (Yuji *et al.*, 2008). The temperature at which the peak was obtained was considered as the temperature of melting (Tm), however, if two peaks were obtained; the higher temperature value was considered as the Tm. The calorimetric scan for OU6 is showing two peaks, one small peak is formed at 145.20^{0} C and the other large peak was at 160.27^{0} C. The polymers were found to be melt stable, when heated above their Tm till 190°C, was re obtained on second heating, suggesting their stability. When heated beyond the Tm till 210°C and quenched to 50°C, the peak at Tm was lost on subsequent heating. Melting behaviour and crystallization of PHA have been studied by Gunaratne *et al*. (2005). The polymer with highest value of T_m (160^{0} C) was of relatively high crystallinity, resulting in high stiffness and brittleness. On the other hand, the lowest value of T_m (145^{0} C) was that of copolymer with the highest other than butyrate molecules.

Production of co-polymer P-3(HB-co-HV) by *Bacillus sp*.40 using various inexpensive carbon sources including rice bran was reported by Nagamani *et al.* (2013). The biosynthesis of PHA by *Brevundimonas vesicularis* LMG P-23615 and *Sphingopyxis macrogoltabida* LMG 17324 using acid-hydrolyzed sawdust as the carbon source was carried out by Johanna A *et al*.(2007). Huang *et al.* (2006) have successfully used inexpensive extruded rice bran and corn

starch in PHA production from *Haloferax mediterranei*. Typical mcl PHA commonly produced by *Pseudomonas spp*. are sticky amorphous materials without useful thermal and mechanical advantages (Fiedler *et al.*, 2000). Copolymers of scl and mcl monomers combine the strength of PHB and the flexibility of mcl PHA; they are more promising for at least packaging applications (Qiu *et al.*, 2004;).

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