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# Molecular characterization of *Acinetobacter junii* CN1 PHB

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**ABSTRACT**

*The bacterial isolate Acinetobacter junii CN1, of cashew nut industrial site with the ability to convert large quantities of low - cost substrates of agro-industrial wastes / resources into PHB was exploited. PHB produced in the present investigation was characterized for its molecular properties with FTIR and GC. The IR spectrum of Acinetobacter CN1 PHB when read in the range 500 - 4000 $cm^{-1}$ revealed the peaks at wave number 3453, 2924, 2855, 1630, 1379, 1119 and 1028 $cm^{-1}$ which were seemed to be identical with the standard PHB. The chromatogram of characteristic ester of the extracted PHB exhibited distinct spot with a retention time 0.650 min and the concentration was estimated as 80.20%. As for the present study, the retention time of the peaks for the standard (0.612 min) also remained almost the same. Further, the molecular weight of PHB was also determined 3.9 x $10^{6}$ Da. The molecular properties of PHB have increasingly become an interest in forming a raw material for biodegradable plastics and can be employed as the most sustainable alternative to the petrochemical plastics.*

**Keywords:** *Acinetobacter junii* CN1 PHB, FTIR, GC, Molecular Weight.

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## INTRODUCTION

The allure of bioplastics can be linked with diminishing petrochemical reserves (Zagar *et al*., 2006) and the mounting apprehensive about their environmental effects have also prompted much interest in biologically derived polymers which are synthesized by microorganisms (Poirier *et al*., 1995) from the materials that can be readily eliminated from the biosphere in an ecofriendly fashion (Gross & Kalra, 2002). This practice would reduce non-degradable solid wastes (Sudesh & Iwata, 2008). Besides, the biodegradable polymers derived from renewable sources do have low environmental footprint (Patnaik, 2005).

In this context , it is worth mentioning the absolutely biodegradable (Hrabak, 1992) polyhydroxybutyrate (PHB) , the first discovered member of PHAs family, in *B. megaterium* ( Lemoigne , 1926). Macrae and Wilkinson (1958) have reported with the rapid biodegradability of PHB produced by *B.megaterium* and *B.cereus*. It is being produced intracellularly by various organisms *viz*., *Bacillus megaterium*, *Ralstonia eutrophus*, *Cupriavidus necator*, *Rhizobium* Spp., *Azotobacter* Spp., *Pseduomonas* Spp., etc. Besides, the bacterial strains which could produce PHB from waste products have been reviewed by Steinbüchel and Valentin (1995 ) and Song *et al*. (1999). Santimano *et al*. ( 2009) for their turn have assayed the ability of the microorganisms to utilize different carbon substrates which are generally found as one of the major components in agro-industrial wastes.

However, till date very limited research is being focused on PHB producing bacterial isolates with the ability to

convert large quantities of low - cost substrates of agro - industrial wastes. Hence, with a view to explore the best possibility of utilizing PHB of the bacterial isolate of cashew nut industrial site as an effective substitute to petrochemical plastics, the present investigation attempts to characterize the same for its molecular characteristics.

## MATERIALS AND METHODS

The PHB extracted from *Acinetobacter junii* CN1 ( Poornima, 2016) an isolate of cashew nut industrial waste was characterized for its molecular properties by subjecting it to Fourier Transform Infrared (FTIR) and Gas Chromatographic studies. Besides, the molecular weight of PHB was also derived from the viscosity ( Poornima, 2016) measurements .

### 2.1.FTIR Studies

One mg sample and standard PHB were ground well with 10 mg spectral pure anhydrous potassium bromide crystals and made into pellets for IR analysis. The relative intensity of the transmitted light energy was measured against the wavelength of absorption on the region 500 – 4000 $cm^{-1}$ using FTIR (Schimadzu - IR Tracer 100, Japan). IR spectra of the samples were then measured at ambient conditions (Ramsay *et al.*, 1994).

### 2.2.Gas Chromatography

The samples *viz*., the extracted microbial PHB, in chloroform as well as the standard PHB (Aldrich,USA) taken in crimp top vials were evaporated. The polymers were then esterified with propanol (4 parts) and hydrochloric acid (1 part). Trichloroethylene was used as a solvent and the reaction was allowed to continue in a tightly sealed crimp top vial at 100 °C for 24 hrs. After cooling down to room temperature, the esterified samples were supplemented with 1 ml water for phase separation. The separated ester (organic) phases of the sample as well as the standard were injected into GC (GC - 8205-, Systronics, India) for further analyses (Bhabatarini *et al.*, 2008). The description of the GC is as follows. Column 3 %; OV: 1,352 M; Detector: FID; Injecting volume: 0.4 µl ; Injecter : 220 °C, FID temperature: 220 °C; Oven : 90 - 200 °C, 10 °C /min; Run time :10 min (Kashma, 2004).

### 2.3.Molecular Weight Determination

The molecular weight (Mw) of the extracted PHB was derived from the viscosity measurements by employing the following Mark - Hauwink –Sakuradea's equation (Quaggliano *et al*., 2001). Where , the viscosity( ת ) of *Acinetobacter junii* CN1 PHB was determined as 0.00037 cP (Poornima , 2016) .

ת $= K^1 ( Mw)^a$

$K^1 = 7.7 \times 10^{-5}$ dl/g and a = 0.82

## RESULTS AND DISCUSSION

The IR spectrum in its turn seems to be very much useful to elucidate chemical and physical structures, hydrogen bonding , end group detection, degradation reactions, cross linking behaviour of molecules and copolymer composition ( Bhargava *et al*., 2003). Hence, the extracted PHB of the present investigation was read in the range 500 - 4000 $cm^{-1}$ and recorded with the characteristic bands for the groups CH, C=O and C-O ( Figure 1 & 2 ; Table 1). The IR spectrum of *Acinetobacter* CN1 PHB revealed the peaks at wave number 3453, 2924, 2855, 1630, 1379 , 1119 and 1028 $cm^{-1}$which were seemed to be identical with the standard PHB. Thus the IR spectrum obtained for the biopolymer extracted from *Acinetobacter* CN1 was confirmed as PHB.

Further, the functional group of PHB granules was identified as C=O. Similar trend has been reported with *B. subtilis* G1S1 strain (Shah, 2014) as well. Charen *et al*.( 2014) for their turn have recorded the peaks at 1724.04 $cm^{-1}$ and 1280.69 $cm^{-1}$ corresponding to C= O and C-O stretching group respectively. The IR spectrum obtained by Anjali *et al*. (2014) has revealed the presence of marked peaks at wave numbers 3440.77 $cm^{-1}$, 1600 $cm^{-1}$ and 1724.10 $cm^{-1}$ corresponding to the hydroxyl (OH) stretching, C=O (Reis *et al*., 2010) and aliphatic stretching of carbonyl group, RCOA of the polymer ( Oliveira *et al*., 2007) respectively.

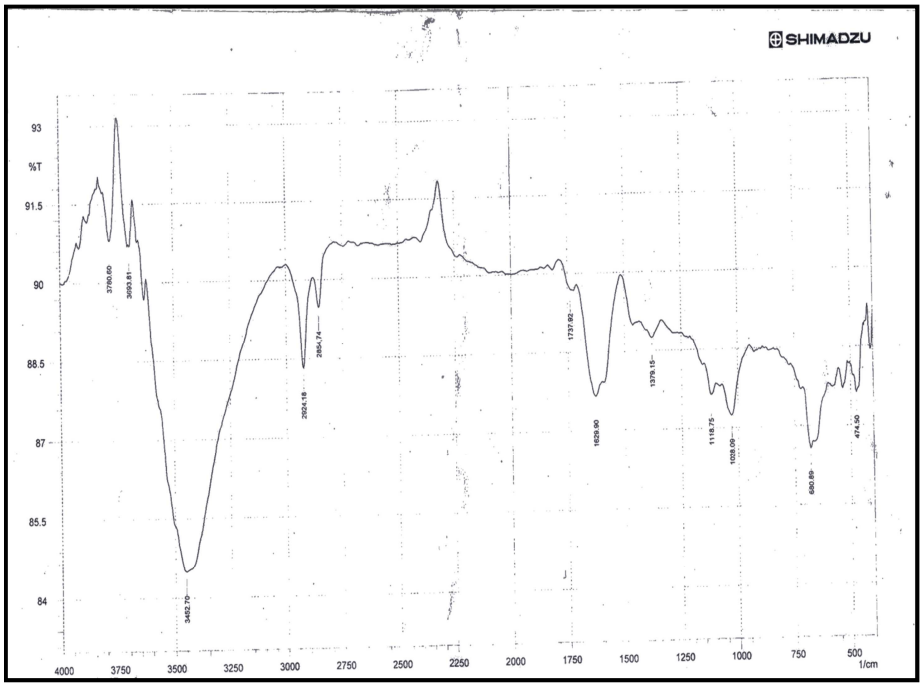



**Figure 1: IR spectrum of the standard PHB**

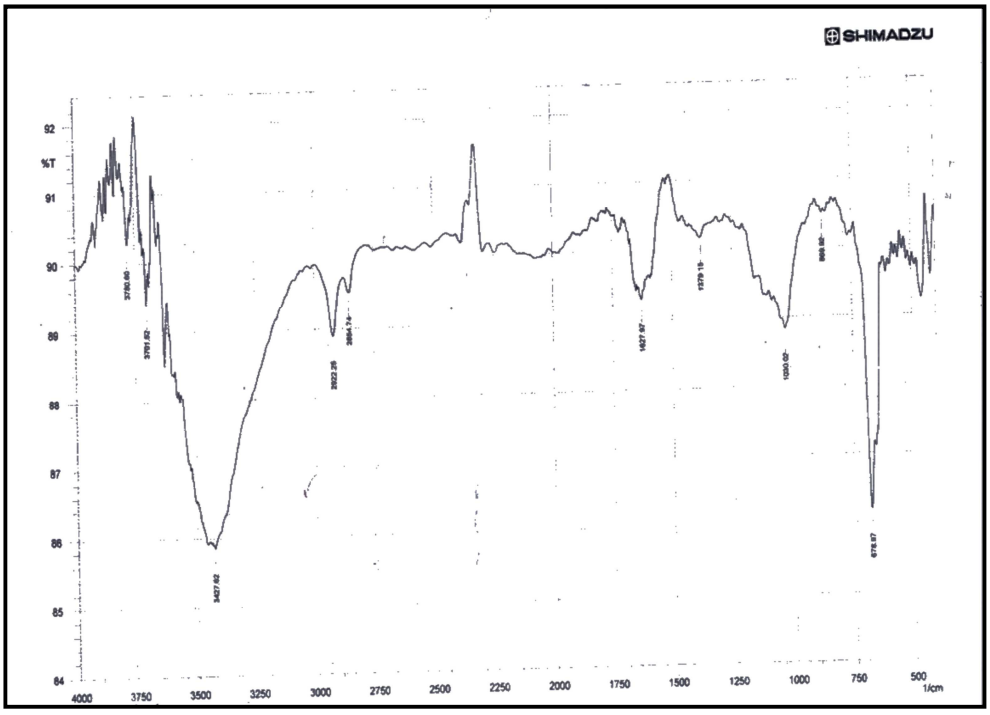



**Figure 2: IR spectrum of *A.junii* CN1 PHB**

The IR spectrum obtained for PHAs samples of *R. meliloti* 14 (Kashma, 2004) has shown characteristic absorption bonds for esters. Further, the author has also reported with C=O and C-O bonds at 1724 $cm^{-1}$ and 1281 $cm^{-1}$ respectively. Similar results are also reported by Misra *et al*. (2006) as well. Apart from the peaks obtained by Kashma (2004) an additional peak has also been observed at 1377 $cm^{-1}$ which might probably be due to the CH3

(or) methyl bending. Besides, the peaks due to methyl stretching have also been observed at 2975 $cm^{-1}$ and 2926 $cm^{-1}$. Further, $CH_2$ (or) methylene group has been observed at 1450 $cm^{-1}$ and methane or CH peak at 3434 $cm^{1}$. Interestingly, an increased shift to left / higher frequency (1739 $cm^{-1}$) has been observed in some samples due to the presence of higher alkanoates (Kashma, 2004). Besides, the most prominent marker band for PHB i.e. C=O stretching has been obtained at 1720 – 1740 $cm^{1}$ (Kashma, 2004). Whereas, in the present investigation, C=O stretching was reported at 1630 $cm^{-1}$. Similar trend has also been reported by Arun *et al*. (2006) and Ramsay *et al*. (1994).

**Table 1: IR peak region of the standard and *Acinetobacter* CN1 PHB**

| Sample | Peak region$cm^{-1}$ | Comments |
|---|---|---|
| Standard PHB | 3427. 62 | H bonds |
| | 2922. 25 | $CH_2$ Stretching |
| | 2854. 74 | $CH_2$ Stretching |
| | 1627. 97 | C= O |
| | 1379. 15 | O - H tertiary alcohol |
| | 1030. 02 | C - O Stretching |
| *Acinetobacter* CN1 PHB | 3452.70 | Intra molecular H bonds |
| | 2924.18 | $CH_2$ Stretching |
| | 2854.74 | $CH_2$ Stretching |
| | 1629.90 | C= O Stretching |
| | 1379.15 | O - H tertiary alcohol |
| | 1118.75 | C- O Stretching |
| | 1028.09 | C- O Stretching |

Kashma (2004) has identified GC as a very efficient method not only for quantitative estimation but also for characterization of PHAs. This method employs accurate procedure which could be completed within 4 hrs (Hong *et al*., 1999) and enable to read the concentrations even as low as 10 µm.

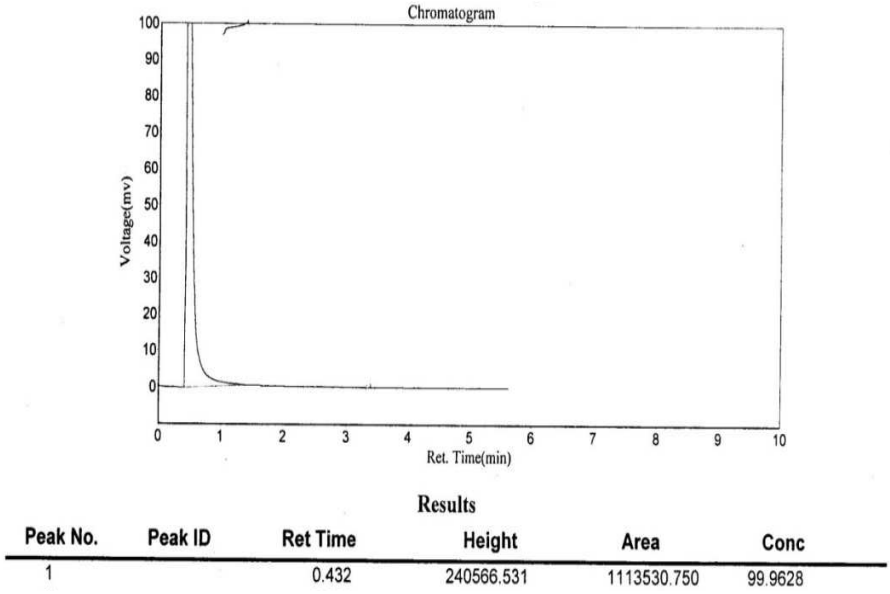
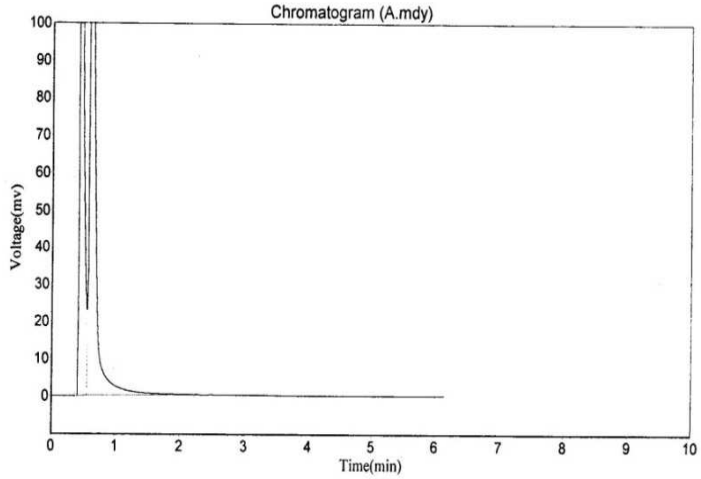



**Figure 3: Chromatogram of the solvent**

In a routine practice of GC , 50 -100 µg PHAs sample shall be used for the analysis (Hong *et al*., 1999). Hence, the extracted *Acinetobacter* CN1 PHB was subjected for the analysis with GC for more accurate results. In this context, 50 µg chloroform extracted *Acinetobacter* CN1 PHB was pyrolysed at 220 °C for 10 min. The volatile products were then subjected to GC. The chromatogram of characteristic ester of the extracted PHB exhibited distinct spot with a retention time 0.650 min and the concentration was estimated as 80.20%.

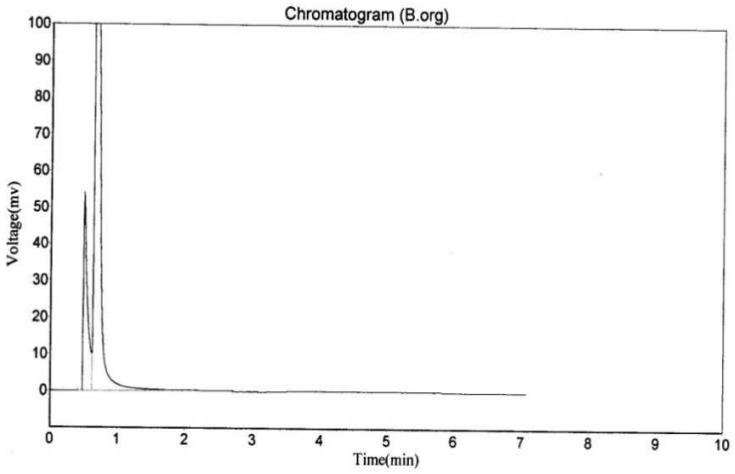
Similarly, Riis and Mai (1988) have reported the PHB content in the dry preparations of microbial biomass grown in synthetic and sugar beet juice media as 80.15 and 65.60% respectively. Nevertheless, Riis and Mai (1988) have obtained the peak for characteristic ester of the extracted PHB at 0.778 min. As for the present study, the retention time of the peaks for both the standard (0.612 min) and extracted PHB (0.650 min) remained almost the same (Figure 3-5).




**Results**

| Peak No. | Peak ID | Ret Time | Height | Area | Conc. |
|---|---|---|---|---|---|
| 1 | | 0.447 | 192615.375 | 734622.813 | 44.3768 |
| 2 | | 0.612 | 135405.875 | 920797.563 | 55.6232 |

**Figure 4: Chromatogram of standard**




**Results**

| Peak No. | Peak ID | Ret Time | Height | Area | Conc. |
|---|---|---|---|---|---|
| 1 | | 0.498 | 53020.121 | 214214.266 | 19.7287 |
| 2 | | 0.650 | 158431.078 | 871584.250 | 80.2713 |

**Figure 5 : Chromatogram of *A.junii* CN1 PHB**

Bacteria produce PHAs with average molecular mass, up to 4.6 x $10^6$ Da with polydispersity (Mw/Mn) of around 2.0 (Agus *et al*., 2009). The molecular weight of the PHAs shall vary with the microorganisms and their growth conditions (Keshavarz & Roy, 2010). Further, the impact of substrate on the degree of polymerization and the

influence of culture conditions on the molecular mass of PHAs have also been revealed by Chen and Page (1994) and Gerhart *et al*. (1998).

Senior and Dawes (1973) opine that the method of isolation also cause severe damage to the PHB granules and thereby reduce the molecular mass of the polymer. Fabiane *et al.* (2007), while extracting PHB with chloroform from bacterial isolates, have reported with the molecular weight 5.2 x $10^5$ Da. Whereas, very high molecular weight of 4 x $10^6$ Da has been reported by Chen and Page (1994) with PHB extracted from the biomass using commercial bleach (30% $Na_2CO_3$; pH 10). However, PHB of the present study, extracted by blending the cells with chloroform had shown the molecular weight 3.9 x $10^6$ Da .

Supportingly, the molecular properties of pure PHB have increasingly become an interest in forming a raw material for biodegradable plastics and serve as the most sustainable alternative to the petrochemical plastics. It can be made better with improved physical and mechanical properties by blending / copolymerization with 5 % valerate (Aldor & Keasling, 2003). In general, copolymerization enables the polymers to become more flexible and tougher than PHB. Further, they do facilitate easier degradation when discharged into natural environment (Vasile, 1993). Besides, the spectrum of possible applications also expands with the modified physical properties of the polymer blends. With the wide applications of PHB, the problems associated with solid waste disposal can be solved.

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