



BioTechnology

An Indian Journal

FULL PAPER

BTALJ, 11(9), 2015 [347-354]

Evaluation and characterization of polyhydroxybutyrate produced by *Azotobacter chroococcum*

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ABSTRACT

Production and characterization of biodegradable plastic namely polyhydroxybutyrate (PHB) in *Azotobacter chroococcum* have been carried out using different carbon sources such as: ethanol, sugar cane molasses in comparison with glucose leading to develop a low cost process of PHB production. The accumulation of PHB granule in cells of *A. chroococcum* was significantly dependent on the content of carbon sources in the medium culture. In the case of using ethanol, the PHB produced was high significant percentage (42 w/w %) in comparison with molasses and glucose (31 and 16 w/w %, respectively). Light, transmission and scanning electron microscopes (TEM and SEM) images of the staining cysts illustrated that different morphologies were obtained relative to that in the case of the vegetative one. Moreover, the physicochemical properties of the produced PHB were studied and evaluated using different analytical tools such as: Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance spectroscopy (¹H-NMR), X-ray diffraction pattern (XRD), high performance liquid chromatography (HPLC) and differential scanning calorimetry (DSC).
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KEYWORDS

A.chroococcum;
Cyst formation;
Sugar cane molasses;
Poly (hydroxybutyrate).

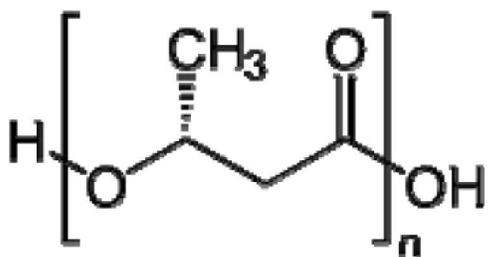
INTRODUCTION

Polyhydroxybutyrate (PHB), high molecular weight polyester, is accumulated as a storage carbon in many species of bacteria and is a biodegradable thermoplastic. To produce PHB by genetic engineering in plants, genes from the bacterium *Alcaligenes eutrophus* that encoded the two enzymes required to convert acetoacetyl-coenzyme A to PHB were placed under transcriptional control of the cauliflower mosaic virus 35S

promoter and introduced into *Arabidopsis thaliana*. Transgenic plant lines that contained both genes accumulated PHB as electron-lucent granules in the cytoplasm, nucleus, and vacuole; the size and appearance of these granules were similar to the PHB granules that accumulate in bacteria.

Cyst formation in nitrogen fixing free living bacterium such as *Azotobacter chroococcum* may be induced, and different shapes of the vegetative cells will be formed during the accumulation of the biopolymer

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(PHB). Each cyst could be produced from a single vegetative cell^[1-3]. Furthermore the presence of PHB is considered an important evidence for encystment in the *Azotobacter* genus^[4,5]. Polyhydroxyalkanoates (PHAs) are reserve polyesters that accumulated as intracellular granules in different bacterial genera including gram negative and gram positive micro-organisms^[6]. These materials are usually synthesized under unbalanced growth conditions also can be completely converted to CO₂ and energy by several microorganisms such as bacteria, fungi and algae^[7]. It has been using relatively cheap substrates (simple and complex carbon sources) such as methanol, ethanol, starch, cane molasses, wheat hydrolyzate and glucose or sucrose can be used to enhance the production of PHB^[8-13]. PHAs have gained recent industrial attention because they are naturally produced, biodegradable polyester thermoplastic (the melting temperature is approximately 180°C) with properties similar to isotactic polypropylene^[14]. PHB derived plastics have also surgical pins, plates, pages structures, implants for drug delivery, possibly mesh can be used as artificial skin materials^[15]. This work deals with production and characterization of PHB using *A. chroococcum* cyst and the physicochemical properties of the produced PHB including chemical structure verification were determined.

EXPERIMENTAL

Materials

Bacterial strain of *Azotobacter chroococcum* was isolated and produced by Microbial Biotechnol. Dept., Genetic Eng. & Biotechnol Inst., University of Sadat City, Minoufiya Governorate, Egypt. Crotonic acid (98 %), used as a standard for the poly β-hydroxybutyric acid assay, obtained from Sigma-Aldrich. All constituents of the bacterial growth were obtained from El Shark El-Awsat Chemicals Co.

Bacterial growth condition

One Strain (Az-4) of *A. chroococcum* was isolated and inoculated in medium containing (glucose 20 g/L, K₂HPO₄ 0.8 g/L, MgSO₄ 0.5 g/L, KH₂PO₄ 0.2 g/L FeCl₃ 0.1 g/L, CaCl₂ 0.05 g/L and NaMoO₄·2H₂O 0.05g/L) for enrichment of *A. chroococcum*^[16]. Erlenmeyer flasks 250 ml containing 100 ml of Atlas medium were stirred on rotary shaker (150 rpm /10 min) then streaked out on agar medium and incubated at 28°C for four days to be checked for purity. Bacterial culture grown stored in slope medium agar at 4°C. Cyst formation of *Azotobacter chroococcum* was induced by (1.6 %) aqueous ethanol and/or sugar cane molasses as a sole carbon source. pH was adjusted to 6.8-7 before sterilization at 121°C/15 min. Then, the flasks were incubated at 150 rpm and 30°C for 5 days. Complete Atlas medium was used as control treatment. At the end of incubation periods the microbial cultures were tested for cyst formation by accumulation of poly β-hydroxybutyrate (PHB). PHB was then precipitated by 95 % ethanol. Bacterial cells were harvested by centrifugation at 10000 rpm for 20 min at 10°C, and the pellets were washed twice with ethanol. The cells were then dried at room temperature for 24 h.

Characterization

Preparation of ultrathin sectioning of *A. chroococcum*

Embedding and ultra thin sectioning of *A. chroococcum* were done as follows: cells were pre-incubated in 0.5 w/v % ruthenium red for 30 min at ambient temperature. Fixation was done by addition of 1.25 v/v % gluteraldehyde to the media for 72 h at 4°C. After centrifugation, the fixed cells were resuspended in 0.1 M phosphate buffer (pH 7.4) and washed in three sedimentation resuspension cycles for 10 min. Washed cells were immobilized in 0.1 M phosphate buffered (pH 7.2), 2 w/v % agar and 1 w/v % osmium tetroxide over night at 4°C. Cells were dehydrated on ice with acetone series and embedded in epoxy resin. Ultrathin sections (120 nm thicknesses) were post-stained with lead citrate and kept for further analysis.

Morphologies examination using electron microscopes

The morphologies of vegetative and cyst forms of

A. chroococcum were examined by light, scanning and transmission electron microscopes (JEOL 1200-EXII) to investigate the surface of cells and cysts. The stained cysts were used to distinguish the morphological differences between vegetative, precyst forms and mature cyst of *Azotobacter* sp. This stain contained of the following: water (100 mL), glacial acetic acid (8.5 mL), sodium sulfate (3.25 g), neutral red (200 mg), light green SF yellowish (200 mg) and ethyl alcohol (50 mL).

Structure elucidation

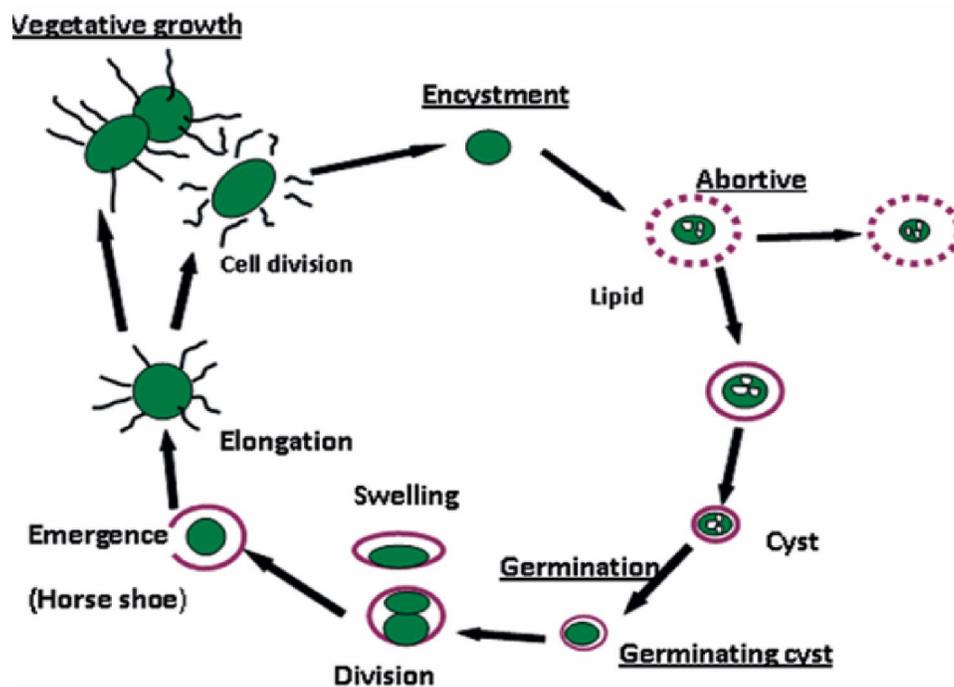
The structure elucidation and the physicochemical properties of the produced PHB were determined using different analytical instruments such as: Shimadzu XRD 6000X, Perkin-Elmer FTIR spectrometer and JEOL-ECA 500MHz nuclear magnetic resonance spectroscopy ($^1\text{H-NMR}$). In addition, thermal analysis was carried out with Perkin-Elmer differential scanning calorimetry (DSC). Moreover, high performance liquid chromatography (HPLC Agilent 1100 series) was recorded under the following condition: mobile phase 0.014 N H_2SO_4 , flow rate 0.7 ml/min, quaternary pump (G1311A), variable wave length detector (G1314A) and Zorbax 300SB C_{18} column. Injection was carried out at wave lengths 214 nm for separation. A crotonic acid was used as a standard for PHB acid assay be-

cause of the quantitative conversion of PHB to crotonic acid by heating in concentrated sulfuric acid and the solution was measured using Shimadzu UV-visible spectrophotometer at wavelength 235 nm^[17]. The particle size and zeta potential measurements were recorded using Zeta-seizer Malvern instrument.

RESULTS AND DISCUSSION

Production of PHB in different carbon sources

As expected from some previous works^[18,19], the homopolymers (β -hydroxybutyrate) could be formed by *A. Chroococcum* strain Az-4. In the most of the investigated organisms, PHB was synthesized from acetyl coenzyme A (acetyl-CoA) by a sequence of three reactions catalyzed by 3-ketothiolase (acetyl-CoA acetyltransferase), acetoacetyl-CoA reductase (hydroxybutyryl-CoA dehydrogenase and poly (3-hydroxybutyrate) synthase. Figure 1 shows the effect of ethanol and molasses, as a sole carbon source, on the biomass of cells and the content of PHB, in comparison with glucose (control). It can be observed that in the case of using ethanol as a sole carbon source, the biomass of *A. chroococcum* and PHB were significantly enhanced (0.85 and 1.75 g/L, respectively) in



Schematic Illustration of the vegetative and cyst formation condition cycle of *Azotobacter* sp.

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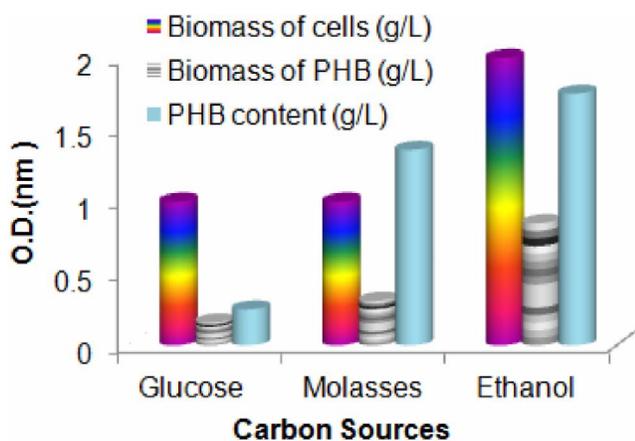


Figure 1 : Biomass of cells and PHB content after 5 days of *A. chroococcum* growth in the different carbon sources in comparison with glucose (control)

comparison with molasses (0.31 and 1.36 g/L, respectively) and glucose (0.16 and 0.25 g/L, respectively). Figure 2 shows PHB percentages (w/w %) after 5 days of *A. chroococcum* growth in the different carbon sources (ethanol and molasses) in comparison with glucose. In the case of using ethanol, PHB produced with high significant percentage (42 w/w %) in comparison with molasses and glucose (31 and 16 w/w %, respectively). The ability of the *A. chroococcum* Az-4 strain to reserve PHB was markedly affected by the source of carbon, which serves as the carbon and/or energy source, for the encystment process. Consequently, the polymer production (PHB) and its formation rate were also affected by the type of the carbon source. It can be expected that *A. chroococcum* strain Az-4 taken up the carbon source from ethanol and stored it after its conversion to bacterial polyester. In other words, at high concentration of ethanol, the PHB accumulation was increased relative to that in the case of glucose. This may be due to the significant availability of the carbon in the growth media accompanied by the poor intracellular PHB degradation. On the other hand, the encystment of *A. chroococcum* was induced during replacement of glucose by ethanol and molasses. It can be concluded that, using high concentration of ethanol led to enhancement in the rate and extent of encystment and accumulation of PHB owing to the high carbon/nitrogen ratio content in the growth medium that favoring PHB production.

Morphology of *A. chroococcum* vegetative and cyst cells

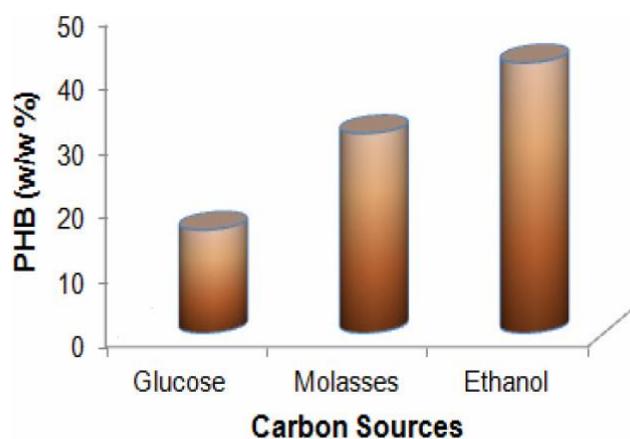


Figure 2 : PHB percentage (w/w %) after 5 days of *A. chroococcum* growth in the different carbon sources in comparison with glucose (control)

The formation of PHB in *A. chroococcum* was investigated by light, scanning and transmission electronic microscopes to show the PHB granules at the early stages of formation, as seen in Figure 3 (A, B and C, respectively). Cyst germination of *A. chroococcum* was accompanied by shedding of the cyst exine and the central body grows into a small bulge in the exine with eventual rupture of the coat with liberation of large vegetative cells. This observation was previously confirmed by Bisset *et al.*^[20] and Abdel-hamid M.S. *et al.*^[21] that reported about the formation of horse shoe cells after germination the exine. Furthermore, it can be seen that the cell periphery of ovoid-shaped cells. Furthermore, after 4 days of inoculation, PHB granules were found to be larger in agreement with the previously results reported by Hermawan *et al.*^[22], which indicated that PHB granule formation in *A. vinelandii* apparently clearly located in the cytoplasm of the cell. Cyst germination of *A. chroococcum* was accompanied by shedding of the cyst exine and the central body grows into a small bulge in the exine with eventual rupture of the coat with liberation of large vegetative cell mentioned that after germination the exine, appeared as horse shoe (Figure 3A). SEM images showed that the cyst morphology differs from the vegetative cell and precyst (Figure 3B). The vegetative cells appear as long rod-shaped or peanut-shaped cells with a fairly homogenous cytoplasm and a relatively much thinner than cell wall while precyst became rounded. Figure 3C shows TEM image of the *A. chroococcum* vegetative and cyst forms. It can be seen that the existence of ultrathin section of the cyst and the cell wall composed of two distinct lay-

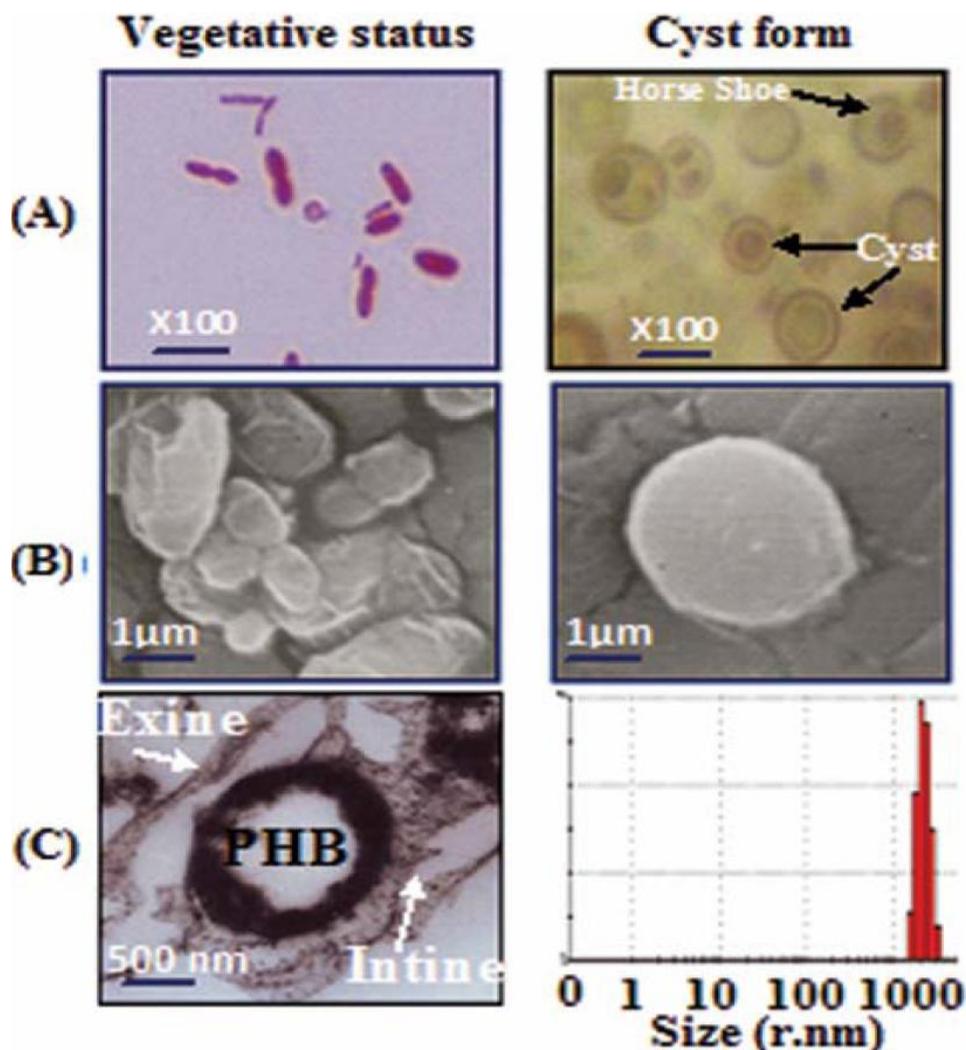


Figure 3 : Particle size distribution by number of the produced PHB, A) Light microscope images (X100) of *A. chroococcum* stained with Gram stain and different stages of stained cyst formation. B) SEM-micrographs of vegetative and cyst cells. C) TEM image of mature cyst form showing the various coats (Intine and Exine) of cyst and cytoplasmic inclusions (PHB)

ers, the outer layer (exine) had rigid structure that offered considerable degree of physical protection. While, the inner wider layer (intine) appeared more homogenous. The cyst showed the various coats and cytoplasmic inclusions. In other words, PHB accumulated as distinct white granules that are clearly visible in the cytoplasm of the cell and is bounded by an internal coat (intine). These results proved that cysts are composed of a central body containing PHB surrounded by capsule like exine and intine layers.

Physicochemical properties of the produced PHB

To identify the PHB produced by *A. chroococcum*, different analysis tools were recorded in comparison with standard and the previous reports. The produced PHB has particle size about 2.24 μm with negative sur-

face charge density in an aqueous media (ζ - 22.7 mv) (Figure 3). FTIR spectrum of the produced PHB was recorded as Figure 4. It can be observed that characteristic peaks at 3438, 2945, 2925, 2825, 1645, 1416 and 1032 cm^{-1} which correspond to the -OH, -CH₃, -CH₂, -CH, -C=O, -C-O, respectively. H-NMR spectrum of the produced PHB is seen as Figure 5. It showed a number of characteristic PHB peaks at δ = 3.9, 1.6 and 1.2 ppm which correspond to CH, -CH₂ and -CH₃ groups, respectively. While, small additional peak at δ = 0.8 ppm was found may be due to impurities present. XRD pattern of the produced PHB was recorded from 5-60 of 2 Theta, as seen in Figure 6. It can be seen that the characteristic peak values of 2 θ 13.3, 16.8, 25.4 were found in the extracted PHB.

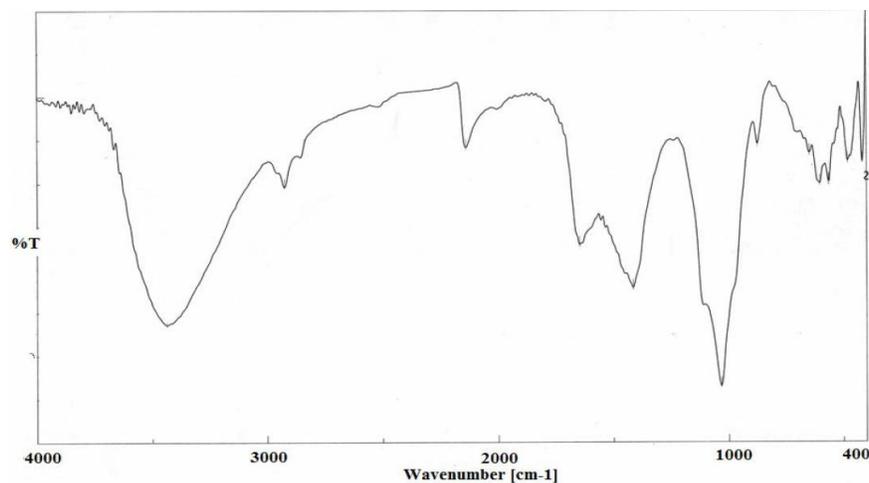


Figure 4 : FTIR spectrum of the produced PHB

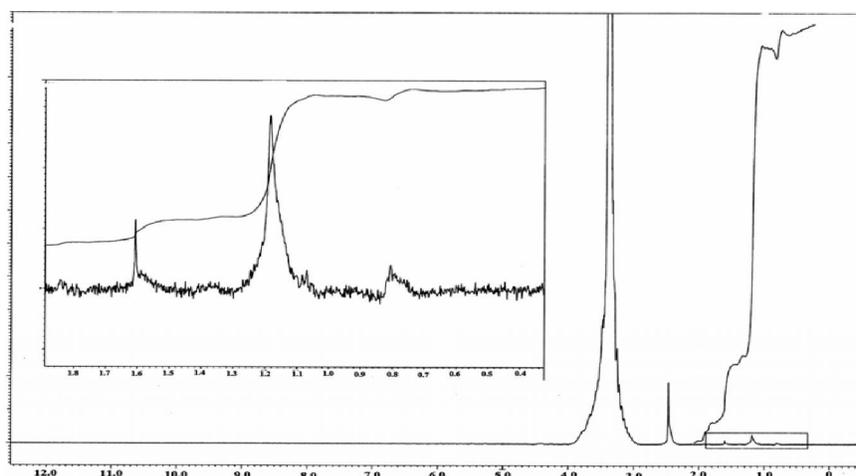


Figure 5 : ¹H-NMR spectrum of the produced PHB

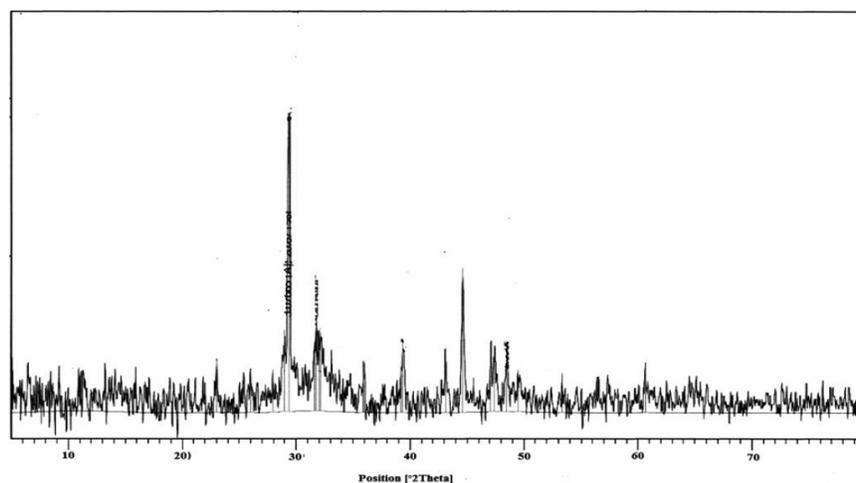


Figure 6 : XRD patterns of the produced PHB

According to Rooy *et al.*^[23], these values were also found in the standard PHB. Moreover, some other sharper and higher peak intensities were observed in the produced PHB in the range of 2θ 29-45. This may

be due to its higher crystallinity. Figure 7 shows HPLC diagrams of the standard crotonic acid and the produced PHB. It can be observed that the produced PHB and the standard crotonic acid had nearly the same

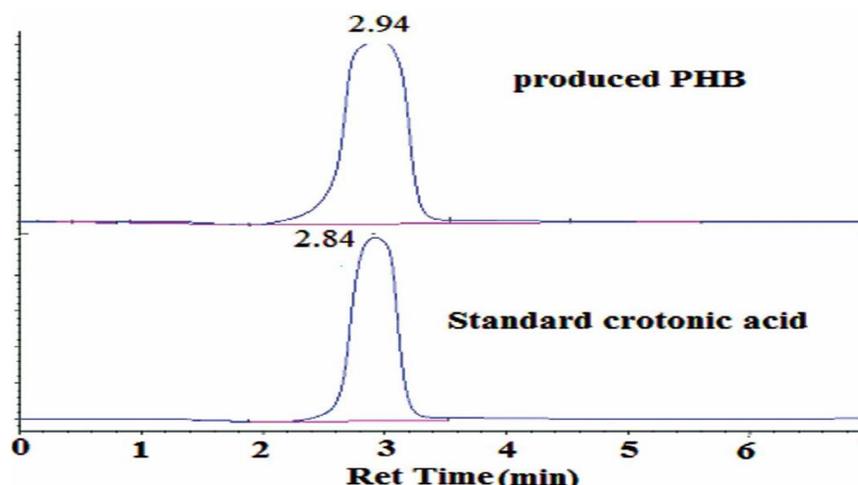


Figure 7 : HPLC diagrams of the standard crotonic acid and the produced PHB

retention time (2.94 and 2.84 min, respectively).

CONCLUSIONS

A. chroococcum was used due to its several benefits, acts as nitrogen fixing bacterium, producing plant growth promoting substains, as well as recently produced one of the most important biopolymer (PHB) providing novel substrate for different biomedical applications. PHB production can be induced in *A. chroococcum* using the different carbon sources, ethanol and sugarcane molasses, in comparison with glucose. The results demonstrated that using of ethanol produced high significant percentage of PHB (42 w/w %) in comparison with that in the case of molasses and/or glucose (31 and 16 w/w %, respectively). Moreover, the data illustrated that carbon source and microorganism types might be affected the PHB accumulation and its molecular weight.

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