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Extraction and NMR Determination of PHB from *Azospirillum brasilense* Sp7

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Abstract: Microbial polyhydroxyalkanoates (PHAs) are a family of natural polyesters structurally diverse produced by many bacteria trough fermentation. They are accumulated intracellularly as energy storage compounds and they can be produced from renewable resources. PHAs have been attracting considerable attention as biodegradable substitutes for conventional polymers and these have gained major importance due to their structural diversity and close analogy to plastics. B*acillus megaterium*, *Alcaligenes eutrophus* and others bacteria are known to produce a biodegradable homopolymer, poly(3-hydroxybutyrate) (PHB). *Azospirillum brasilense* has not been widely studied for PHB production. This paper describes its isolation from *Azospirillum brasilense* Sp7. Also, its spectroscopic characterization by Nuclear Magnetic Resonance spectra (NMR) of protons ^{1}H and carbon ^{13}C. These techniques confirmed its purity. In the same way, its infrared spectrum (FTIR) allowed to corroborate the absence of another compound different to the PHB and also calculate de Crystallinity Index. From this work, it may be concluded that *Azospirillum brasilense* Sp7 is a suitable microorganism for bioproduction of the PHB.

Key words: PHB, *Azospirillum brasilense*, NMR characterization, FTIR spectroscopy, Crystallinity Index

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a group of bacterial polyesters. The ability of numerous bacteria to synthesize PHAs as storage material in intracellular granules has been extensively studied[1-3]. These carbon storage molecules are synthesized by many microorganisms due to limitation of one or more environmental or nutritional factors such as nitrogen, phosphorus, potassium or oxygen[4]. The bacterial polyesters are biodegradable green plastics. One of the most well-studied members of the PHAs family is poly (3-hydroxybutyrate) (PHB)[5], (Fig.1), homopolymer that contains monomer units of 3-hydroxybutyrate[6].

PHB was discovered in *Bacillus megaterium* by Lemoigne[7] in 1926 [(9)]. Since its discovery more than 90 genera of archaea and eubacteria ($Gram^+$ and $Gram^-$) have been detected in aerobic and anaerobic habitats able to produce PHAs[8,9].

Nowadays, a wide variety of petroleum based synthetic polymers are produced worldwide and remarkable amounts of these polymers are introduced in the ecosystem as industrial waste products[10]. In recent years biopolymers have attracted more and more interest due to increasing environmental concern and decreasing fossil resources. This evolution motivates academic and industrial research to develop novel materials labelled as "environmentally-friendly", materials produced from alternative resources, biodegradable and non-toxic to the environment[11].

PHAs have been attracting considerable attention as biodegradable substitutes for conventional polymers like polypropylene and polyethylene.

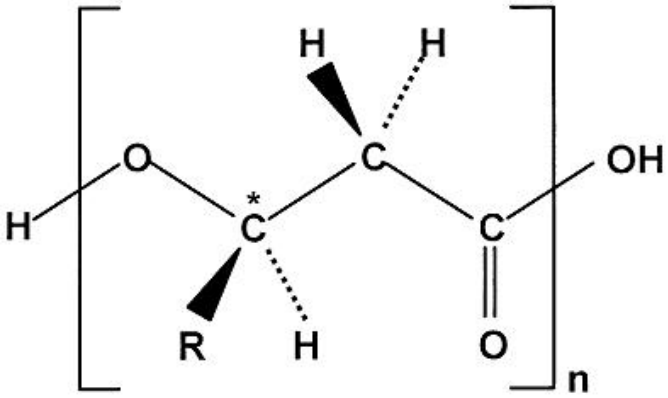

Fig.1: Chemical structure of poly (3-hydroxybutyrate) (PHB)

Azospirillum brasilense has been studied for a long time as a plant growth promoting *bacteria*. To the best of our knowledge, *Azospirillum brasilense* has not been widely studied for PHB production. Microbiological generation of PHB could be an important economic alternative in polymer industries.

The objective of this work is to demonstrate that *Azospirillum brasilense* Sp7 is able to produce the homopolymer PHB in a high purity, as is verified by its Nuclear Magnetic Resonance spectrum and Fourier Transform Infrared spectrum too.

METHODS

Azospirillum brasilense Sp7 was cultivated in minimum medium. The pH of the medium was adjusted to 6.8 with 0.1 M KOH. Culture Sp7 was prepared in a 250 ml Erlenmeyer flask containing 50 ml media and was placed in a rotary shaker with 150 rpm at 32ºC.

A fermenter (SEV) of 5 L containing 3 L culture media was sterilized at 121ºC for 20 min, cooled and then inoculated with 10% inoculum (v/v) of Sp7. Cultivation was carried out at 32ºC in the fermenter for 72 h.

The recovery of the polymer was carried out using a sodium hypochlorite solution (5.25% w/v), at 37°C, during 1:30 h. for the cellular material digestion. Cells were separated by centrifugation at 12000 rpm during 10 minutes at 4ºC, and PHB was extracted by means of chloroform at room temperature and the dissolved PHB was separated from chloroform by evaporation (Fig.2).

The NMR and FTIR techniques have been used to characterize the composition and structure of PHAs after extraction from cells[12]. The ^{1}H and ^{13}C NMR analyses of the polymer samples were carried out on a Bruker spectrometer of 500 MHz and 125 MHz, respectively.

The FTIR spectroscopy was done on Perkin Elmer Spectrum One. It was used under the following conditions: spectral range, 4000-650 cm^{-1} to confirm the functional groups of the extracted polymer. The spectra for PHB extracted from *Azospirillum brasilense* Sp7 were compared against the spectra of commercial PHB (Sigma-Aldrich Chemicals, USA).

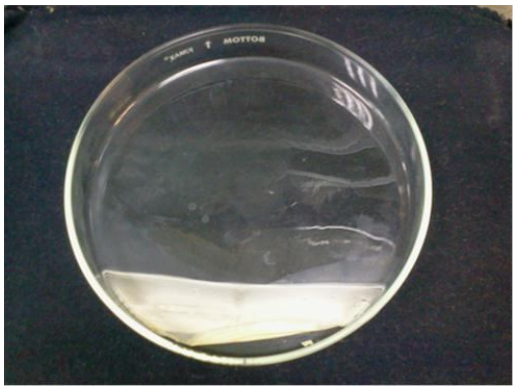

Fig.2: PHB separated from chloroform by evaporation.

RESULTS

A selected ^{1}H NMR spectrum of crude extract (Fig.3, spectrum in $CDCl_3$-TMS) shows the presence of three main groups of signals, characteristics for PHB: a doublet at 1.29 ppm which is assigned to the methyl group, two doublet of doublets, at 2.45 and 2.60 ppm, for the methylene protons and a doublet of quadruplets at 5.25 ppm, characteristic of the methine group.

The structure of the isolated PHB was confirmed by comparison with a ^{1}H NMR spectrum of a commercial PHB sample.

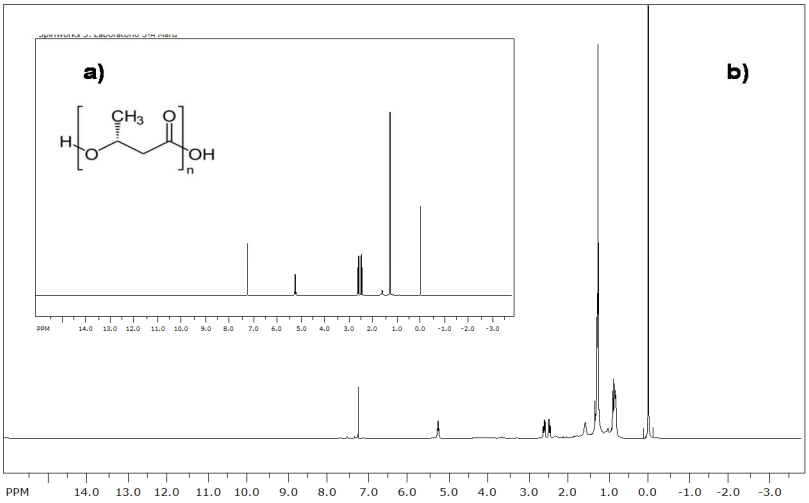

Fig.3: [1]H NMR spectra of the PHB/Sigma-Aldrich Chemicals, USA **(a)** and of the PHB isolated from *Azospirillum brasilense* Sp7 **(b)**

Fig.4 shows the [13]C NMR spectra of PHB/Sigma-Aldrich Chemical, USA and PHB isolated. The following chemical shifts confirmed the chemical structure of the isolated PHB. Four narrow lines appeared at 19.6934 ppm assignable to the methyl, 40.8740 ppm to the methylene, 67.6739 ppm to the methine, and 169.2542 ppm to the carbonyl.

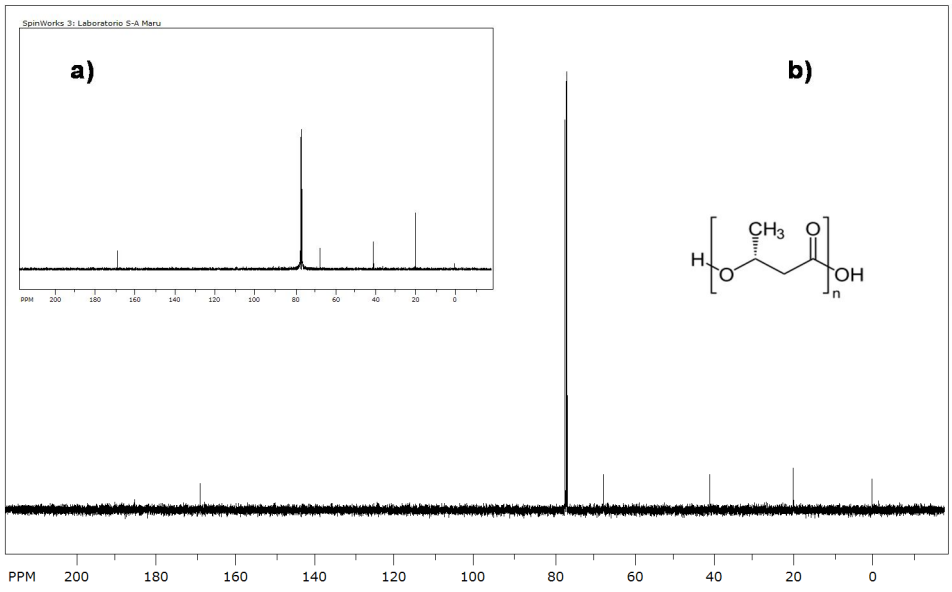

Fig.4: [13]C NMR spectra of the PHB/Sigma-Aldrich Chemicals, USA **(a)** and of the PHB isolated from *Azospirillum brasilense* Sp7 **(b)**

Table 1 shows the chemical shifts of poly(3-hydroxybutyrate)/Sigma-Aldrich, PHB isolated by Doi *et al*.[13] from *Alcaligenes eutrophus* and PHB isolated from *Azospirillum brasilense* Sp7. It is observed from this data that the four signals of the PHB isolated from *Azospirillum brasilense* Sp7 are in very good agreement with those obtained for Doi-PHB and Sigma-Aldrich-PHB.

Table 1: Chemical shifts and assignments to ^{13}C NMR for PHB

	^{13}C NMR (ppm)		
Carbon type	**Doi *et al.*[(4)]**	**Sigma-Aldrich**	**This work**
CH_3	19.79	19.7832	19.6934
CH_2	40.82	40.7875	40.8740
CH	67.64	67.6258	67.6739
C = O	169.16	169.1796	169.2542

Fig.5 shows the FTIR spectra of PHB isolated from *Azospirillum brasilense* and Sigma-Aldrich PHB. The FTIR spectrum of isolated PHB presented almost identical peak positioning when compared to the spectrum obtained from Sigma-Aldrich-PHB. The most prominent marker band for the identification of PHB is the carbonyl band at 1740-1720 cm^{-1}, ester groups at 1330-1050 cm^{-1} and methyl group at 1450-1375 cm^{-1}, these corresponding to the main bands of PHB.

On the other hand, the bands present at 1220 cm^{-1}, 1256 cm^{-1} and 1280 cm^{-1} are bands sensitive to crystallinity and are characteristic of C-O-C.

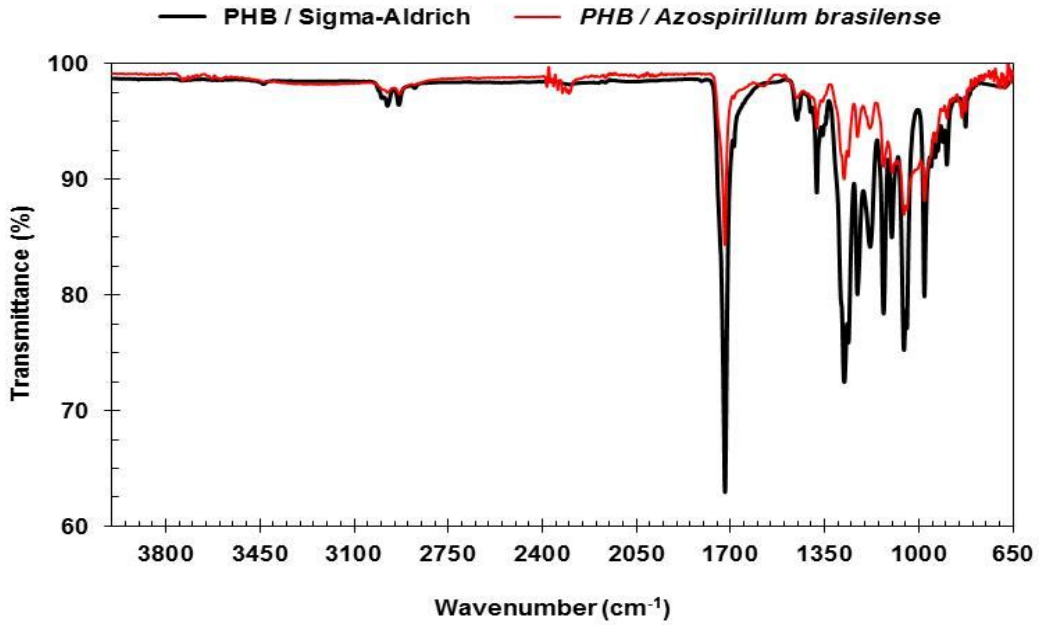

Fig.5: FTIR spectra of PHB extracted from *Azospirillum brasilense* Sp7 compared to the spectrum of Sigma-Aldrich Chemicals, USA.

Randriamahefa *et al.*[14] reported that FTIR can also be used to evaluate the Crystallinity Index (CI) of PHB quantitatively. A relative measure of the degree of crystallinity can be obtained by calculating the CI and can be used to compare the relative crystallinity obtained from the various FTIR spectra. In the literature, the CI is defined as the absorbance ratio of the reference band at 1379 cm^{-1} which is insensitive to the degree of crystallinity to the crystallinity sensitive[14,15] band at 1278 $cm^{-1.}$ The equation for calculating the CI is the following

$$CI = \frac{A_{1379}}{A_{1278}}$$

The CI obtained for Sigma-Aldrich-PHB was 0.34, which was lower compared to the value obtained for PHB isolated from microorganism cells with value of 0.55. Greater CI correlates to a higher % crystallinity. This CI is not to be confused with an absolute degree of crystallinity but is useful for comparison.

CONCLUSIONS

Azospirillum brasilense Sp7 is a suitable microorganism for bioproduction of poly (3-hydroxybutyrate). The isolated PHB from *Azospirillum brasilense* Sp7 has a high purity. The methine and methylene protons show a unique coupling pattern indicating that the 3-hydroxybutyrate moiety was obtained as a chiral compound, being the methine group the chiral centre. The FTIR spectra for isolated PHB from *Azospirillum brasilense* Sp7 cells showed identical peak positioning and also confirmed the structure of the PHB isolated.

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