CORN COB HYDROLYZATES USED FOR MICROBIAL BIOSYNTHESIS OF POLYHYDROXYBUTYRATE

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A new fermentation strategy to obtain polyhydroxybutyrate (PHB) with selected bacteria, using a sustainable raw material as the single carbon source, was analyzed. Corn cob dilute-acid hydrolyzates were obtained and used as a carbon source for a fermentative medium formulation to cultivate spore-forming bacteria in order to achieve biopolymer biosynthesis. Different methods for the hydrolysis of the raw material and for assessing the content of fermentescible sugars of the hydrolyzates were tested. The dilute-acid hydrolyzate with 35.84 g/L total sugars content assured the optimum fermentative conditions for microbial growth and PHB bioproduction by the selected strain *Bacillus* spp. BM 37. The intracellular polymer production and its structure were analyzed by HPLC-MS and NMR analyses. The yield of PHB produced after the cultivation of the selected bacteria, under submerged aerobic conditions, in liquid medium based on corn cob hydrolyzate, was 36.16% w/w dry biomass, which is comparable to the results reported in the literature. Our findings offer valuable perspectives regarding the use of corn cob agro-waste resources for the formulation of a fermentation medium by an economical biotechnological approach.

Keywords: corn cobs, dilute-acid hydrolysis, *Bacillus* spp., polyhydroxybutyrate (PHB)

INTRODUCTION

In the past two decades, the geopolitical and economic context, as well as the depletion of petroleum deposits, has directed increasing attention towards the production of bioplastics with selected microorganisms under controlled biotechnological conditions. Polyhydroxyalkanoates (PHAs) are one such family of biodegradable plastics. In accordance with the literature data, more than 250 different species of bacteria possess the ability to biosynthesize polyhydroxyalkanoates (PHAs) as intracellular compounds, when the carbon source is available in excess, so that it can ensure the energy resource under stress growth conditions.[1-3]

The most common polymer from the PHAs group is polyhydroxybutyrate (PHB), a high molecular weight linear polyester discovered by Lemoigne in 1926, which is accumulated in the intracellular granules.[2,4] Nowadays, this polymer is produced on an industrial scale by Zeneca Bio Products and Fine Chemicals Companies.[5]

PHB exhibits thermo-mechanical properties, similarly to petrochemical plastics, and possesses the advantages of being biodegradable and biocompatible. Because of its high production costs, the biopolymer is not used on a large scale.[6] However, due to its biocompatibility properties, it has found use in medicinal applications as an implant biomaterial and as a carrier in drug delivery systems.[6]

In the field of PHA bioproduction, several studies have been conducted in four main directions: (1) optimizing the bio-processing costs, which still remains the researchers' main objective;[8] (2) improving the conditions for polymer separation and purification; (3) increasing the yield of polymer production by strain selection and genetic engineering techniques; (4) obtaining new composites as a result of a mixture with other natural polymers to make them suitable for a wide range of applications.[4]

One possibility to reduce the production costs consists in using organic wastes, such as inexpensive fermentation substrates. Lignocellulosic wastes are the most abundant in nature, representing approximately sixty percent of all plant biomass.[9] Lignocellulosic wastes have a complex structure consisting of a mixture between lignin (polyphenols complex structure), cellulose (β-D-1,4 glucan) and hemicellulose fibers (carbohydrate polymers that contain D-xylose, D-arabinose, D-mannose, D-glucose, D-galactose and sugar alcohols).[9] The long term economical approach and sustainability concerns have encouraged the study of numerous possibilities to improve the value of these agro-industrial wastes by converting them into simple sugars that can be further used afterwards for different biotechnological purposes. The hydrolysis can be achieved by various methods, among them the most often used is the enzymatic and chemical processing.[9,10] These methods present advantages and disadvantages, but acid hydrolysis is still the preferred option due to its highest efficiency, even if some microbial inhibiting by-products may result.[9,10]

Corn cobs represent an agro-waste found in significant amounts, with a great potential to be used in different industrial areas, based on their rich carbohydrates composition.[11] Corn cobs are composed mainly of cellulose (35-55%), hemicelluloses (25-35%) and lignins (20-30%).[12,13] This renewable material represents the ideal feedstock that can be converted into value-added products using biotechnological routes.[10] In this context, the dilute-acid hydrolysis process of ground corn cobs releases remarkable amounts of sugar, which can be used for the biosynthesis of PHAs by microbial fermentation.

This paper presents an approach to the biosynthesis of polyhydroxybutyrate (PHB), consisting in formulating a minimal liquid medium, based on corn cob acid hydrolyzate, with a high content of monosaccharides (20 g/L), as the single carbon source, suitable for the cultivation of aerobic spore-forming bacteria (*Bacillus megatherium* BM 37 selected strain) under submerged cultivation conditions. The yield and structure of the produced polymer were analysed by HPLC-MS and NMR analyses.

EXPERIMENTAL

Materials

Corn cobs were received from a local farm, in Galati, Romania. They were collected during the harvesting period of the mature corn crop. The cobs were ground in a grinding mill and then sifted with RETSCH AS 200 basic sieving equipment, with four sieves, to separate four different fractions: Fraction F1: 15.5%, particle size > 1.6 mm; Fraction F2: 41.71%, 1.6 mm > particle size > 800 µm; Fraction F3: 8.83%, 800 µm > particle size > 630 µm; Fraction F4: 33.96%, particle size < 630 µm. These four corn cob fractions were subjected to three different acid hydrolysis assays.

Acid hydrolysis of corn cob fractions

Three methods to obtain corn cob acid hydrolyzates were performed as follows:

Method I: 0.2, 0.5 and 1 g of ground corn cob fractions and 10 mL of 5.2% (v/v) sulfuric acid solution were subjected to acid hydrolysis at 90 °C, for 2 hours. After cooling at room temperature, in order to neutralize it up to a pH of 5.5, 5 mL of water and 1 g of calcium carbonate were added to each hydrolyzed fraction.

Method II: 0.2, 0.5 and 1 g of ground corn cob fractions and 10 mL of 2.8% (v/v) sulfuric acid solution were subjected to acid hydrolysis at 121 °C, for 45 minutes. After cooling at room temperature, in order to neutralize it up to a pH of 5.5, 10 mL of water and 1 g of calcium carbonate were added to each hydrolyzed fraction. At the same time, 1 g of non-separated fractions of ground corn cobs was subjected to hydrolysis.

Method III: 1 g of ground corn cob fractions and 9 mL of 4.8% (v/v) sulfuric acid solution were subjected to acid hydrolysis at 90 °C, for 2 hours, after a previous pretreatment with 1 mL of 0.2% (v/v) sulfuric acid solution at 135 °C, for 15 minutes. After cooling at room temperature, in order to neutralize it up to pH of 5.5, 10 mL of water and 1 g of calcium carbonate were added. After centrifugation at 6000 rpm, for 10 min, the supernatants were analyzed by HPLC-MS for determining sugar composition and for sugar quantification.

Pretreatment of a corn cob hydrolyzate

The corn cob hydrolyzate previously treated with calcium carbonate was further neutralized with sodium hydroxide until the pH reached the value of 7.0 (Diagram 1). In the next step, the corn cob hydrolyzate was subjected to another pretreatment with a mixture of dipotassium hydrogen phosphate and monopotassium dihydrogen phosphate, in order to buffer it until pH 7.2, which was afterwards filtered to remove any precipitate and sterilized at 121 °C, for 15 minutes.

Physical, chemical and biological characterization of hydrolyzates

Thermogravimetric analysis of ground corn cob fractions

Thermogravimetric analysis (TGA) and differential thermal analysis (DTG) of ground corn cob fractions

were performed using STA 449F1 Jupiter NETZSCH equipment (Germany). Approximately 10 mg of samples was used for the analyses. Measurements were carried out in the temperature range of 30-700 °C, by applying a heating rate of 10 °C/min. Nitrogen purge gas was used as inert atmosphere, at a flow rate of 50 mL/min. The samples were heated in open Al_2O_3 crucibles. The device was calibrated for temperature and sensitivity with indium, according to the standard procedure as recommended by the producer.

Quantification of sugars by High Performance Liquid Chromatography (HPLC)

HPLC analysis was carried out using a Perkin Elmer HPLC system with a Flexar Refractive Index LC Detector (USA). For the separation, an amino column (3 µm, 150 mm x 4.5 mm) was used. The temperature was maintained at 35 °C and a mobile phase consisting of a mix of 75:25 v/v HPLC grade acetonitrile and double distilled water was used, at a flow rate of 0.98 ml/min. All the sample dilutions were conducted in the mobile phase.[15] The content of individual sugars in the corn cob hydrolyzates was calculated based on a calibration curve, using glucose, xylose and arabinose as standards. For the calibration curve, several concentrations of 8, 6, 4, 2, 1, 0.8, 0.6, 0.4, 0.2 and 0.1 mg/mL were prepared from each type of sugar, using the same injection volume as for the sample.

Microorganism

The spore-forming bacterial strain, belonging to *Bacillus* genus, coded BM 37, was isolated from a Brasilia soil sample using a minimal growth medium that contained (g/L): sucrose, 10; Na_2HPO_4, 2.5; KH_2PO_4, 2.5; $(NH_4)_2HPO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot 7H_2O$, 0.007, with a final pH adjusted to 7.0. Usually, a stock culture of the pure strain is preserved at -80 °C in 20% glycerol.[3] This strain was previously identified as *Bacillus aryabhattai* (93.95% similarity index by 16S rDNA sequence using molecular analysis), which belongs to the *Bacillus megaterium* taxonomic group (unpublished data).[14] The pure stock culture is part of the collection of microorganisms of "Petru Poni" Institute of Macromolecular Chemistry (ICMPP), Iaşi, Romania.

Submerged fermentation for PHB biosynthesis

For preparing the *Bacillus* spp. BM 37 strain inoculum, the cultivation was carried out in a minimal liquid medium with the following composition (g/L): glucose, 20; KH_2PO_4, 2.5; Na_2HPO_4, 2.5; $(NH_4)_2HPO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot 7H_2O$, 0.007) and pH 7.0.[16] The cultivation took place on a GFL 30-31 rotary shaker (GFL Gesellschaft fur Labortechnik GmbH Germany), at 200 rpm, 37 °C, for 48 h. The PHB biosynthesis was performed in a medium containing pretreated sterilized corn cob hydrolyzate, supplemented with 20% sterilized solution of microelements, with the following composition (g/L): Na_2HPO_4, 12.5; KH_2PO_4, 12.5; $(NH_4)_2HPO_4$, 25; $MgSO_4 \cdot 7H_2O$, 2.0; $FeSO_4 \cdot 7H_2O$, 0.1; $MnSO_4 \cdot 7H_2O$, 0.05 and pH 7.0. The mineral solution was sterilized separately and added to the sterilized pretreated corn cob hydrolyzate before starting the fermentation.

A volume of 100 mL of the fermentation medium was inoculated with 2.5% of 48-hour old inoculum ($OD6_{00\ nm}$ = 2.0). The cultivation took place under similar conditions as the one described above. For comparison, a fermentation conducted under similar biotechnological conditions was performed in a medium with the following composition (g/L): glucose, 30.0; Na_2HPO_4, 2.5; KH_2PO_4, 2.5; $(NH_4)_2HPO_4$, 5.0; $MgSO_4 \cdot 7H_2O$, 0.4; $FeSO_4 \cdot 7H_2O$, 0.02; $MnSO_4 \cdot 7H_2O$, 0.01; the pH was adjusted to 7.0-7.2. After 48 h of submerged cultivation, the biomass was harvested by centrifugation using a Sigma 2-16 KL centrifuge (SIGMA Laborzentrifugen GmbH, Germany) at 6000 rpm, 4 °C, for 15 minutes. The cell dry weight (CDW) was evaluated to express the biosynthesis yield.

Polyhydroxybutyrate biosynthesis, extraction and quantification

Highlighting the PHB granules inside the cells

Heat-fixed smears of bacterial cells were stained with Nile red solution and a gas burner was used until the total amount of water was removed from the stain solution. The smears were examined with an Olympus BX 41 microscope (Olympus America Inc., USA). The Nile red solution was prepared from Nile blue by dissolving 1 g in 500 mL H_2SO_4 and boiling it under reflux for 2 hours. After cooling down, the Nile red was separated by extraction into equal volumes of xylene until the organic phase was colorless. The organic solvent was removed completely by evaporation, leaving a dark purple residue,[18] and then dissolved in 50 ml of distilled water in order to prepare the staining solution for microscopy analysis.[17]

PHB extraction

Prior to the PHB extraction, the wet biomass was freeze-dried in an Alpha 2-4 LSC Plus freeze dryer (Martin Christ, Germany). For breaking cell walls, approximately 1 g of lyophilized cells was suspended in 10 mL of 1% (w/v) lysozyme in water solution and incubated under stirring at 100 rpm and 40 °C, for 60 minutes. The PHB was extracted from the cell suspension with 10 volumes of chloroform for 15 minutes. After separation, the chloroform phase was concentrated at 70 °C until a final volume of 10 mL was reached. The precipitation of PHB from the concentrated chloroform phase was done with 12 volumes of chilled ethanol at 4 °C for 24 h. Finally, the PHB precipitate was collected by centrifugation at 10 °C, 10.000 rpm, for 20 minutes.[18]

PHB quantification by HPLC-MS analysis

An amount of 50 mg of freeze-dried biomass was dissolved in 3.5 mL of 96% sulfuric acid and heated at 100 °C for 1 h. After hydrolysis, the PHB was converted to crotonic acid, which was further analyzed.[19] For quantification of the crotonic acid, the microbial biomass hydrolyzate was subjected to HPLC and electrospray (ESI) mass spectrometry analysis, using Thermo Scientific HPLC/MSQ Plus (Thermo Scientific, SUA) equipment. A Hamilton PRP-X300 ion exclusion column filled with polystyrene/divinylbenzene copolymer with sulfonic acid groups (250 x 4 mm, 7 µm particle size, 1-13 pH range, 24 MPa and 5-60 °C temperature range) was used. For the crotonic acid assay, UV detection was carried out at the wavelength of 210 nm, a flow rate of 1 mL/min, at 25 °C. The dilution factor of the samples in 1 mM sulfuric acid solution was 250 and the analysis was performed using a gradient phase with the following procedure: phase A) 70% of 1 mM sulfuric acid: 30% acetonitrile in 1 min; phase B) linear gradient 10% of 1 mM sulfuric acid: 90% acetonitrile in 2 min and held for 5 min; phase C) linear gradient 70% of 1 mM sulfuric acid: 30% acetonitrile in 2 min and held for 11 min. The retention time of crotonic acid is in the range of 2.3-2.4 min and both mass spectra were recorded with ESI positive signal at 400 °C and maximum pressure of 70 eV. The quantification of crotonic acid was done based on a calibration curve for the concentration range of 0.01-0.1 mg/mL, upon a linear correlation with R^2 of 0.9992. Standard deviation errors were also presented.

Nuclear Magnetic Resonance spectroscopy (NMR)

For NMR analysis, the PHB extracted from freeze-dried biomass was used. The PHB NMR spectra were recorded with a Bruker Avance III 400 MHz spectrometer (Bruker BioSpin Corporation, Germany), equipped with a 5 mm inverse detection z-gradient probe, operating at 400.1 and 100.6 MHz for ^{1}H and ^{13}C nuclei. The PHB sample was dissolved in deuterated chloroform $CDCl_3$ and the spectra were recorded at room temperature with TopSpin 2.1 PL6 processing software (Bruker BioSpin Corporation, Germany). For the spectra of each proton, 16 scans were acquired, whereas for the carbon spectra, 1024 scans were obtained. Calibration spectra were made on residual solvent signal as follows: in ^{1}H NMR, $CDCl_3$ produced a signal at 7.3 ppm and in ^{13}C NMR, at 77.1 ppm.

RESULTS AND DISCUSSION

In order to complete our goals, we separated four fractions from the ground corn cobs and subjected them to dilute-acid hydrolysis by three different methods, according to the protocols presented in Diagram 1. The difference between these fractions is the particle size, so that we expected to obtain different amounts of sugars after the hydrolysis processes.

Thermal analysis of ground corn cob fractions

TGA analysis was applied to highlight the difference in stability and mass loss attributed to hemicellulose degradation (as a peak) among different corn cob fractions. We thought that we could relate this information with the sugar release yield during the acid hydrolysis of each fraction.

The obtained fractions of ground corn cobs were subjected to thermal analysis to identify the thermal stability and the residual composition, given the versatility of the method to measure mass loss as a function of temperature for a wide variety of compounds.[20]

Figure 1 and Table 1 show that all the studied structures follow a similar thermal decomposition trend in three stages. The physical dehydration process ends around 130 °C and a maximum mass loss occurs between the temperatures of 300 and 355 °C. The studied samples exhibit two main DTG peaks, corresponding to the hemicelluloses degradation, both between 228 °C and 320 °C, and also in the 316 °C and 500 °C range, which is attributed to the degradation of α-cellulose with lignin, respectively.[21] The most stable fraction is F1, with a maximum mass loss of 41.84% at 340 °C. The values of the remaining residue vary in the range of 26.84 and 29.31%, which corresponds to the remaining inorganic material, similar to other reports described in the literature.[22] The mass loss associated to peak II (Table 1) is almost the same for all the corn cob fractions.

Acid hydrolysis and chemical characterization of hydrolyzates

In the literature, the influence of several factors, such as acid concentration, temperature, hydrolysis time and solid to liquid ratio, on the dilute-acid hydrolysis kinetics of lignocellulosic wastes was emphasized.[10] Our study takes into consideration all of these factors and furthermore shows the impact of the raw material's particle size. On top of that, the most important aspect is to integrate this carbon source into a medium that contains other different chemicals and to remove the inhibitory by-products in order to use it as a nutrient in the fermentation process.

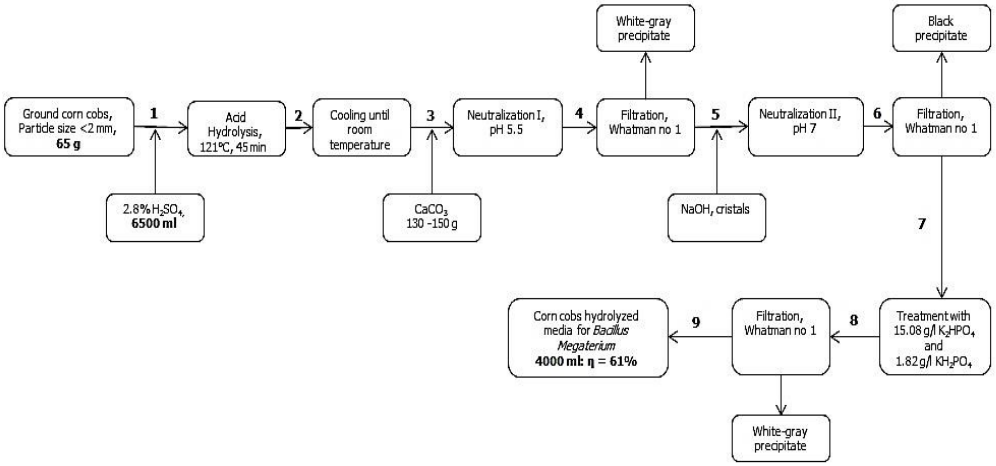

Diagram 1: Production and pretreatment of corn cob hydrolyzates

Table 1
Thermal characteristics of corn cob raw material

Sample	F1				F2				F3				F4			
	T_i, °C	T_{max}, °C	T_f, °C	Δ_w, %	T_i, °C	T_{max}, °C	T_f, °C	Δ_w, %	T_i, °C	T_{max}, °C	T_f, °C	Δ_w, %	T_i, °C	T_{max}, °C	T_f, °C	Δ_w, %
Peak I	66	96	128	4.23	48	81	113	4.71	48	82	114	5.32	49	78	113	4.75
Peak II	247	292	295	25.58	243	289	320	26.5	240	285	315	25.92	228	278	316	29.16
Peak III	316	340	351	41.84	318	338	353	37.92	320	338	352	38.82	322	337	355	36.74
W_{rez}, %	26.84				29.11				29.31				27.18			

T_i – initial thermal degradation temperature; T_{max} – temperature corresponding to the maximum rate of decomposition for each stage, evaluated from the peaks of DTG curves; T_f – final thermal degradation temperature; Δw – weight loss rate corresponding to T_{max} values; W_{rez} – percentage of residue at the end of thermal degradation (700 °C)

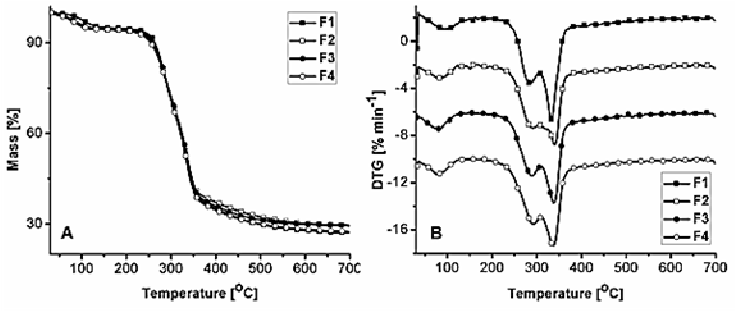

Figure 1: TG (A) and DTG (B) analysis of the fractions

Corn cob biomass was chosen due to its high content of hemicellulose, of 34.3% (w/w of dry material), according to K. Chandel *et al.*,[10] and to its availability – of millions of tons every year due to the large corn crops, according to D. Zych.[23]

Dilute-acid hydrolysis is the fastest method for the depolymerization of hemicelluloses into a sugars mixture, consisting mainly of xylose and small amounts of glucose, arabinose, galactose and mannose. The effectiveness of this method is reduced by its non-selectivity and high temperature requirements, leading to inhibitory by-products.[9] Because the number of microorganisms that metabolize pentoses, besides hexoses, is rather limited, sometimes a two-step hydrolysis is necessary in order to depolymerize the cellulose fraction that was not broken down during dilute-acid hydrolysis.[9] Most probably, only a few microorganisms that present endo and exo-cellulase enzymatic activity can grow on such hydrolyzates.

Sugars content of corn cob hydrolyzates

The impact of hydrolysis conditions and corn cob particle size on the content of mono-sugars released during acid hydrolysis performed according to three methods was studied. Among these methods, the first one used a higher acid concentration (5.2% v/v sulfuric acid) at a lower temperature (90 °C) and at a longer hydrolysis time (2 h) (Fig. 2); the second used the lowest acid concentration (2.8% v/v sulfuric acid) and the highest temperature (121 °C) with the shortest hydrolysis time (45 min) (Fig. 3), and the third one consisted in a pretreatment of the lignocellulose material with 0.2% v/v sulfuric acid at 135 °C, for 15 min, followed by acid hydrolysis with 4.8% v/v sulfuric acid at 90 °C, for 2 h (Fig. 4).

For the first two methods listed above, three ratios of the corn cob fractions and the acid solution were analyzed (0.2 g: 10 mL, 0.5 g: 10 mL and 1 g: 10 mL). To avoid the dilution of the corn cob hydrolyzate, calcium carbonate was used for neutralization. To achieve a hydrolyzate with a mono-sugars content higher than 20 g/L, the optimum ratio between the corn cob fraction and the sulfuric acid solution for methods I and II was 1 g: 10 mL. The HPLC analysis revealed three types of mono-sugars in the hydrolyzate compositions: two pentoses (xylose and arabinose) and one hexose (glucose). Differences in the content of mono-sugars as a function of the corn cob fraction and the hydrolysis method used can be observed in Figures 1-3.

The highest amount of sugars was obtained from fractions F1 and F3 using method I, with a mono-sugars content higher than 40 g/L (Fig. 2). A concentration of mono-sugars higher than 30 g/L was obtained for fractions F1 and F4, when method II was used (Fig. 3). A total mono-sugars mixture of 25.75 mg/mL of xylose, 5.09 mg/mL of glucose and 6 mg/mL of arabinose was obtained when 1g of whole ground corn cobs was subjected to acid hydrolysis using method II. This hydrolyzate presents a slightly higher mono-sugars yield than all the separate corn cob fractions (Fig. 3). This can be explained by the large chemical composition changes that corn cobs undergo during grinding. The pretreatment used in method III did not show any positive impact on the sugars yield (Fig. 4).

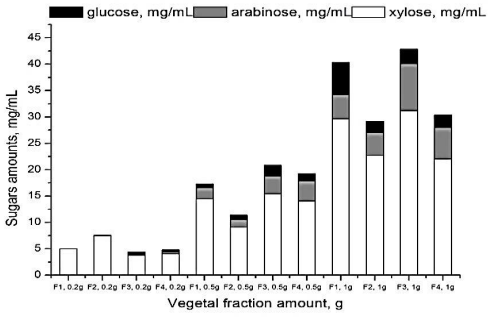

Figure 2: HPLC analysis of sugars in hydrolyzates obtained by method I

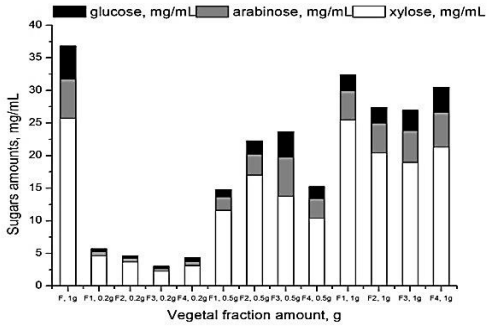

Figure 3: HPLC analysis of sugars in hydrolyzates obtained by method II

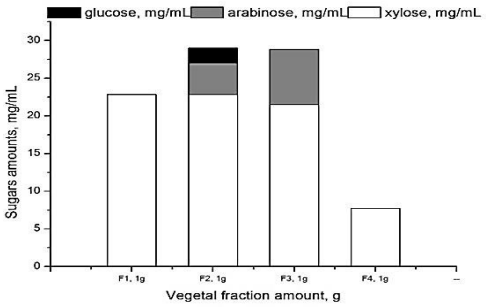

Figure 4: HPLC analysis of sugars in hydrolyzates obtained by method III

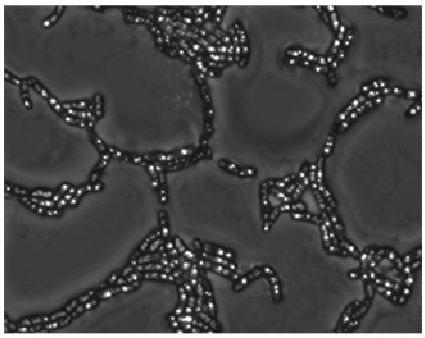

Figure 5: Polyhydroxybutyrate granules within cells (100x)

Considering that a lot of parameters, such as concentration, particle size of the raw material and the solid/liquid ratio, have an influence on the concentration of the sugars released, the hydrolysis processes present high variability. Findings similar with our results were also reported by other researchers, *e.g.* an amount of reducing sugars higher than 35-40 g/L was obtained from rice bran treatment, under similar acid hydrolysis conditions, *i.e.* 3.5% sulfuric acid, at a temperature of 120 °C, solid:liquid ratio of 1:10 for 60 min, according to A. K. Chandel *et al*.[10]

Production of PHB and yield of biosynthesis

Microscopic smears obtained from a 48 h culture in a stationary cultivation system were stained with Nile red dye in order to highlight intracellular PHB granules as refractive orange color spots inside the cells. Nile red, known as Nile blue oxazone (lipophilic stain), stains intracellular neutral lipid droplets in red. In most polar solvents, Nile red will not present fluorescence, however, in a lipid-rich environment, it can become a strong fluorescent dye with colors varying from deep red to strong yellow gold.[17,24] Black and white photomicrographs (Fig. 5) show the presence of PHB granules (white spots) under contrast-phase microscope analysis of the cells.

After 48 h of fermentation, the cell dry weight (CDW) obtained in a glucose medium is slightly higher than that for the medium based on corn cob hydrolyzate. The PHB yield was 36.16% (w/w CDW) by cultivation in the medium based on corn cob hydrolyzate, compared to 28.6% (w/w CDW) by cultivation on the medium with glucose. Thus, the ability of *Bacillus* spp. BM 37 strain to grow well under minimal fermentation conditions was demonstrated.

HPLC-MS analysis of the biomass extract obtained by cultivation on the medium based on corn cob hydrolyzate (method II) revealed the presence of crotonic acid. Both mass spectra presented two main molecular fragments with a molecular mass of 42 and 83 (Fig. 6). The 42 mass fragment of a lower intensity is the result of fragmentation characteristic of unsaturated molecules, as well as carboxylic acids. These fragments are obtained after breaking the allylic rearrangement of the molecule and eliminating the carboxylic acid moiety, according to Diagram 2.

The highlighted amount of PHB expressed as a CDW percent is very close to other published results (Table 2).[25,26]

The ^{1}H NMR spectrum obtained for the extracted PHB showed the characteristic signals of the biopolymer. The peaks in the 5.24-1.23 ppm region were assigned to aliphatic protons. The CH proton appeared as a multiplet at 5.24-5.20 ppm.

Additionally, the observed peaks at 2.42-2.60 ppm occurred due to CH_2 linked to the carbonyl group, whereas the doublet centered at 1.23-1.25 ppm was attributed to the methylenic protons of the compound. The diastereotopic CH_2 protons appeared to be coupled due to their specific configuration, the coupling constant being 5.6 (Fig. 7) (^{1}H NMR (400 MHz, CDCl3: 7.3 ppm, CH: 5.24-5.21 ppm (m, J_1 = 5.6 Hz, J_2 = 6.0 Hz), CH_2: 2.6-2.4 ppm (dd, J_1 = 7.6 Hz, J_2 = 5.6 Hz), CH_3: 1.25-1.23 ppm (d, J = 6.4, 1.6)). The ^{13}C NMR spectra also confirmed the structures. Thus, for all the compounds, the signals of the quaternary carbon of carbonyl groups were found at 168.98 ppm, while the signals of the CH were observed at 67.44 ppm.

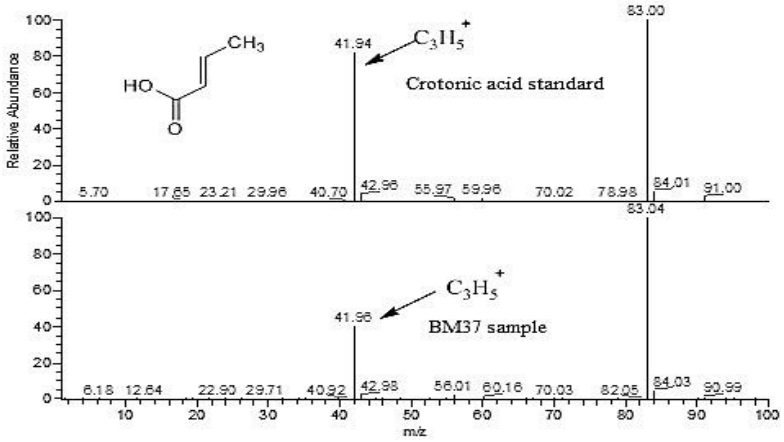

Figure 6: HPLC-MS spectrum of crotonic acid synthetized by *Bacillus* spp. BM 37 strain

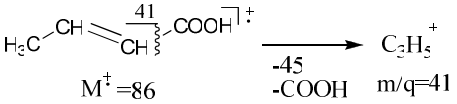

Diagram 2: 83 mass fragment, the maximum intensity is specific to the molecular peak [M-3H$^+$]

Table 2
PHB biosynthesis yield of *Bacillus* spp. BM 37 strain in correlation with literature data

Strain	Fermentation time, h	Carbon source	CDW,* g/L	PHB, g/L	PHB productivity, g/L·h	PHB yield, % on CDW
Bacillus spp. BM 37	48	3% dextrose	2.82	0.81	0.017	28.6
Bacillus spp. BM 37	48	Corn cob hydrolyzate (3% xylose and glucose)	2.65	0.96	0.02	36.16
Rastolnia eutropha MTCC 1472[26]	120	Straw hydrolyzate	-	-	-	41.77
Bacillus megaterium ATTC 6748[4]	45	4% molasses, 4% corn steep liquor	6.3	2.52	0.056	43.00

*CDW – cell dry weight

At higher fields (~40 ppm), the signals for the CH_2 groups appeared close to the carboxyl group, whereas the signals at ~19.58 ppm could be attributed to the methylenic carbon (Fig. 8) (^{13}C NMR (400 MHz, $CDCl_3$ 77.1 ppm, 168.98 ppm (C=O); 67.44 ppm (CH); 40.61 ppm (CH_2), 19.58 ppm (CH_3).

In conclusion, the NMR data confirm that the PHB polymer was biosynthesized.

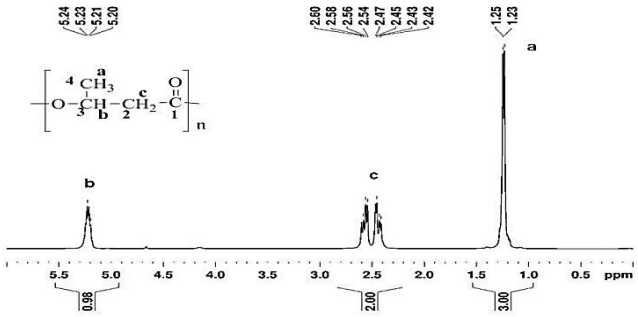

Figure 7: ^{1}H NMR spectrum in $CDCl_3$ corresponding to PHB

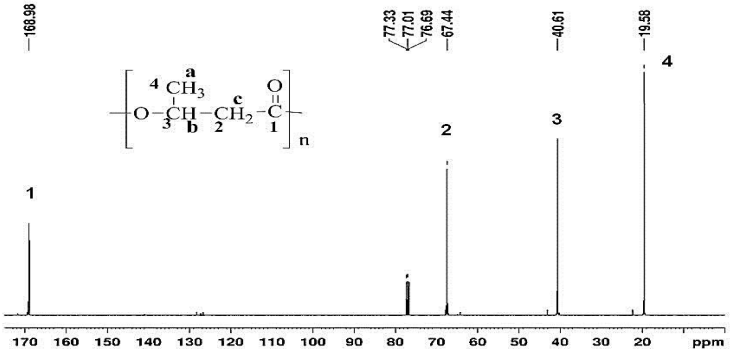

Figure 8: ^{13}C NMR spectrum in $CDCl_3$ corresponding to PHB

CONCLUSION

The present study demonstrated the efficiency of using corn cob dilute-acid hydrolyzates with high sugars content as the single carbon source for *Bacillus* spp. growth and PHB biosynthesis. The PHB production by *Bacillus* spp. BM 37 selected strain was confirmed by contrast-phase microscopy, HPLC-MS and NMR analyses. The biosynthesis yield was comparable between the cultivation of spore-forming bacteria under submerged aerobic conditions on a minimal medium based on corn cob acidic hydrolyzate (3% xylose and glucose) and the cultivation on a medium containing 3% dextrose, as the only carbon source. This study offers an approachable alternative to increase the economic efficiency of the fermentation process, especially on an industrial level, by reducing the costs of nutritional components for the culture medium formulation.

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