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Cocoa Bean Shell as Promising Feedstock for the Production of Poly(3-hydroxybutyrate) (PHB)

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Abstract: Cocoa bean shell (CBS), a by-product of the chocolate industry, has been employed as a substrate to obtain poly(3-hydroxybutyrate) (PHB) by fermentation with *Bacillus firmus*. With this aim, acid-thermal hydrolysis of CBS (20% *w*/*v*) was conducted at 135 °C for 10 min so that broths rich in fermentable sugars were obtained. These broths, both non-centrifuged and centrifuged, were employed as fermentation media. Significant polymer production was obtained from the broth with solids (non-centrifuged) with a yield of 107 mg of PHB/g dry matter. These results indicated that the presence of CBS solids played an important role in microorganism metabolism, with them being fundamental to the production of PHB. Experimental data were fitted by a model based on irreversible first-order reactions, and kinetic constants were obtained for solubilisation, hydrolysis, and sugar consumption. Although, several studies on obtaining PHB from other agri-food residues have been published, this is the first work on PHB production from CBS, with the study obtaining promising results with PHB concentrations similar or even higher than the others previously reported.

Keywords: cocoa bean shell; bioplastics; polyhydroxybutyrate; fermentable sugars; hydrothermal treatment

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1. Introduction

The growing environmental issues derived from organic waste generated during food processing make the development of new alternatives essential to transform these residues into value-added products [1]. In this sense, the EU Waste Framework Directive (European Parliament and Council, 2008, Directive 2008/98/EC) prioritizes residue prevention followed by recycling and valorisation and considers waste disposal as the most unfavourable option [2]. Additionally, the Circular Economy Action Plan adopted in 2020 by the European Commission constitutes one of the main building blocks of the European Green Deal, Europe's agenda for sustainable growth. Therefore, in the current context, industries are in need of the development of green technologies that allow the targets included in European policies to be achieved to move a sustainable society forward [2–4].

Almost 20 tons of waste are generated for each ton of dry cocoa beans produced; due to the rising demand of cocoa products in the last 10 years, nowadays, the cocoa industry has to address increasing environmental concerns [5]. Cocoa bean shell (CBS), the outer part that cover the cocoa bean, is a by-product, which is usually discarded as waste, generated during the cocoa roasting process. This residue means between 10–20% of the total cocoa bean weight, although these percentages can vary depending on the type of cocoa beans mboxciteB6-applsci-2132561,B7-applsci-2132561,B8-applsci-2132561. Although the composition of cocoa bean shell is also quite variable and depends on the origin and the processing of cocoa beans, among other factors, CBS is constituted by carbohydrates (including fructose, glucose, melibiose, sucrose, and raffinose), dietary fibre, theobromine, phenolic compounds, fats, and other components [1,9].

Due to environmental concerns related to plastic pollution and fossil resources depletion, the interest in biodegradable polymers as a novel and sustainable alternative is rapidly increasing [10,11]. Polyhydroxyalkanoates (PHAs), a class of biodegradable and biobased

polymers, are linear polyesters produced in nature by bacterial fermentation, which can be accumulated in microorganisms in form of intracellular granules in response to unstable environmental conditions [12]. These polymers are reducing-power storage materials in various microorganisms and act as a reserve for energy/carbon, so bacteria utilize them when the external supply is insufficient [13–16]. Poly(3-hydroxybutyrate) (PHB), the most common PHA, can be used as an alternative to synthetic polypropylene due to its similar structural and mechanical properties [13,17,18]. PHB is commonly produced intracellularly by microorganisms included in the genera *Bacillus*, *Azotobacter*, *Pseudomonas*, and *Rhizobium*; these bacteria can accumulate between 60–80% of their total weight as PHB [19]. *Bacillus* is one of the most employed genera to produce PHB, due to its high replication stability, predominance in nature, and lack of lipopolysaccharides, which makes PHB extraction much easier [20].

One of the major limiting factors that have to be faced for the industrial production of PHB is associated with carbon source expenditure, reaching in some cases 50% of the total production cost [18]. Therefore, it is necessary to explore potential alternatives to find new, easily available, as well as cheap, carbon sources. Lignocellulosic biomass, such as cocoa bean shell, after adequate pre-treatment of hydrolysis could be a promising alternative renewable source to be employed as a substrate of fermentation.

The aim of the present work has been to employ CBS as a carbon source to obtain PHB by means of *Bacillus firmus* CECT 14. CBS was hydrothermally treated, so that the resulting broths could be used as a substrate in the fermentation processes. To the best of our knowledge, this is the first research on PHB production from CBS, expanding the possible alternatives of valorisation of this lignocellulosic by-product.

2. Materials and Methods

2.1. Cocoa Bean Shell

The CBS was supplied by a local chocolate factory.

2.2. Hydrothermal Hydrolysis

In order to obtain a broth with a high content in fermentable sugars and considering previous assays, the following procedure was employed. First, the CBS was milled in a professional blender (Braun 4041) during 2 min at room temperature, achieving between 1 and 2 mm of particle size, then 60 g of the milled CBS was introduced with 240 mL of 5% H_2SO_4 (Supelco) (20% *w*/*w*) in a 1-L Pyrex bottle. The mixture was autoclaved (AES 110 Raypa) at 135 °C for 10 min. Once autoclaved, two different broths were prepared to be employed in the fermentation processes. The broths were prepared under sterile conditions, as described below:

- Non-centrifuged medium: the content of the autoclaved bottles was adjusted to pH 6–7 with 5 M NaOH (VWR) and then, it was placed in a flask for subsequent inoculation.
- Centrifuged medium: solids were removed from the hydrolysate mixture with a sieve and the liquid phase was centrifuged for 10 min at 10,000 rpm and, after that, the pH of the supernatant was adjusted to 6–7 with 5 M NaOH (VWR). Finally, the broth was placed in a fermentation flask for inoculation.

2.3. Fermentation Process

Broths obtained from hydrothermal treatment of CBS as indicated in Section 2.2 were directly, i.e., without any supplementation, employed as a substrate to carry out fermentation in Erlenmeyer flasks. *Bacillus firmus* CECT 14 supplied by CECT (Spanish Type Culture Collection) was used and the initial microbial load was 2 × 104 CFU (Colony Forming Unit)/mL. Fermentations were conducted during 13 days in an incubator at 37 °C and 250 rpm. Samples (5 g of the mixture) were taken periodically every 48 h from the flasks, centrifuged (Heraeus Multifuge X1 Centrifuge Series) for 10 min at 10,000 rpm, and the pellet and the supernatant were separated. The liquid phase was frozen until

determination of carbohydrates, reducing sugars, and pH, and the pellet was frozen until analysis of the PHB content.

In addition, microbial growth was followed by means of taking 1 g of the broth with solids that was transferred to a Stomacher bag and homogenized with 9 mL of sterile saline solution (0.7% NaCl). In case of medium without solids, 1 mL of sample was homogenized with 9 mL of sterile saline solution (0.7% NaCl). After that, in both cases, serial decimal dilutions of the mixture were plated in triplicate onto nutrient broth agar medium and incubated at 30 °C for 24 h before counting.

Fermentations were performed at least in duplicate.

2.4. Morphological Analysis

In order to evaluate the possible effect of the presence of solids on the fermentation processes, a morphological analysis of the CBS particles was performed.

Samples of CBS, before and after hydrolysis, were dried overnight at 105 °C prior to the SEM analyses. Regarding samples of inoculated non-centrifuged medium, they were fixed overnight in 3% glutaraldehyde in 25 mM phosphate buffer (pH 6.8). Then, they were rinsed in two changes of buffer and were dehydrated in a graded ethanol series. Once 100% ethanol was achieved, they were moved in a graded acetone series, so when the samples were in 100% acetone, they were dried at room temperature. All of the samples were sputter-coated with a thin layer of gold in a Sputtering Balzers SCD 004 and were observed employing JEOL JMS-6610LV scanning electron microscopy (SEM) with a 0.3–30 kV voltage.

In addition, to observe the two different media employed in this work, a Leica TCS-SP8X Confocal Spectral Laser Microscope with the application of the 405 nm laser diode and white laser lines with a free selection of excitation lines between 470 and 670 nm was employed.

2.5. Analytical Methods

All analyses described below were carried out in triplicate.

2.5.1. Determination of Dry Extract

With the aim to express the results on a dry weight basis (*w*/*w*), a gravimetric method was employed to determine the moisture content of CBS. The sample (3 g) was weighed with sea sand washed and dried, thick grain technical grade (Panreac), in a stainless steel capsule. The mixture was dried in an oven at 105 °C for 24 h and was then was weighed again. Considering the difference between the initial and final weight, the moisture content and the dry extract were calculated.

2.5.2. Determination of Total Carbohydrates: Phenol–Sulfuric Acid Method

The amount of total carbohydrates in the samples was measured as reported in Sanchez et al. (2022) [3], using an adaptation of the phenol–sulphuric method described by Dubois et al. (1956) [21]. Briefly, 1 mL of sample was mixed with 0.5 mL of 5% phenol and 2.5 mL of 96% H_2SO_4 (Merck, Rahway, NJ, USA). The mixture was incubated at room temperature for 1 h and then, the absorbance was measured employing a spectrophotometer (DR/2500 HACH) at 492 nm. Glucose (Sigma Aldrich, St. Louis, MO, USA) was employed as standard.

2.5.3. Determination of Reducing Sugars: Dinitrosalicylic Acid (DNS) Method

Total reducing sugars were quantified using the Miller method (Miller, 1959) [22] as described in Díaz et al. (2017) [23]. For this assay, 0.5 mL of sample was mixed with 0.5 mL of DNS reagent (VWR) and the mixture was incubated at 95 °C in a water bath for 5 min. After that, the samples were cooled in ice and the absorbance was measured at 540 nm using a spectrophotometer (Thermo Scientific™ UV-Vis GENESYS™ 150, Waltham, MA, USA). Glucose (Sigma Aldrich) was employed as standard.

2.5.4. Determination of PHB

Extraction and quantification of PHB concentration was accomplished according to the Law and Slepecky method [24]. In this procedure, the pellet obtained, as has been described in Section 2.3, was digested with 10 mL of sodium hypochlorite solution 6–14% (Merk) at 37 °C in a water bath for 1 h. The mixture was centrifuged at 10,000 rpm for 30 min, the supernatant was discarded, and the pellet was subsequently washed with 10 mL of distilled water, acetone, and ethanol. After removing the supernatant, 10 mL of chloroform was added to the pellet, mixed, and filtered through a 20 µm cellulose filter (VWR), and finally, the filtrate was recovered.

To determinate the concentration of PHB, 100 µL of the filtered extract was added to 10 mL of 96% H_2SO_4 (Merk) and the mixture was incubated in a water bath at 95 °C for 10 min. Then, the samples were cooled in ice and the absorbance was measured at 235 nm by employing a spectrophotometer (Thermo Scientific™ UV-Vis GENESYS™ 150). Crotonic acid (Sigma-Aldrich) was used as standard.

2.5.5. Determination of Fermentation Inhibitors

Acetic acid, HMF, and furfural were analyzed by high efficiency liquid chromatography (HPLC), following the method described by Sánchez et al. (2022) [3]. To quantify the concentration of acetic acid, an ICSep ICE-ION (Tecnokroma) column with a refractive index detector (RID) was employed. The mobile phase was sulfuric acid (0.45 Mm, pH 3.1) with a column temperature of 75 °C and a flow of 0.3 mL/min. For the detection of 5-hydroxymethylfurfural (HMF) and furfural, a Gemini-NX 5 µm C18 110 Å column (Phenomenex) was used with a diode detection system (DAD). The mobile phase was methanol/water (10:90) with a flow of 1mL/min and the temperature of column was 30 °C. Chemical standards of acetic acid, HMF, and furfural were supplied by VWR.

2.5.6. Elemental Analysis

Concentrations of total nitrogen and total phosphorous in the hydrolysate broths were obtained as described by Pola et al. (2021) [25], i.e., total nitrogen was determined with a CNHS elemental analyzer, Vario EL cube (Elemetar, Langenselbold, Germany), whereas total phosphorous was determined by quantitative analysis with a Thermo Scientific's Neptune plus ICP-MS (Inductively Coupled Plasma Mass Spectrometer, Waltham, MA, USA).

2.5.7. Determination of Total Suspended Solids

To determine the evolution of total suspended solids during the fermentation processes, a gravimetric method was used.

3. Results and Discussion

3.1. Characterization of Hydrolysed Broths and CBS Solids

In order to hydrolyze the complex carbohydrates and to extract as many fermentable sugars as possible, CBS was treated by means of the acid hydrothermal procedure [3] for 10 min at 135 °C. These conditions were selected according to previous tests carried out in order to maximize the extraction of fermentable sugars. According to the methodology previously described, two different hydrolyzed media were used as fermentation broths, one without CBS solids (centrifuged) and one with them (non-centrifuged). Therefore, just after the hydrothermal treatment, both media differ only in the solid content. Table 1 shows the composition of the two media used as fermentation broths in terms of the concentration of total suspended solids, soluble total and reducing sugars, total nitrogen, total phosphorus, and pH. In addition, the dry extract of the different batches of CBS employed in this work was quite similar, between 92.3 and 93.0%.

Table 1. Composition of the two different broths used in the fermentation process, expressed in g/L.

	Centrifuged Medium	Non-Centrifuged Medium
Soluble total sugars	34.5 ± 0.05	35.9 ± 0.6
Soluble reducing sugars	26.8 ± 0.02	31.5 ± 0.7
Soluble total nitrogen	2.21	2.14
Soluble total phosphorus	0.45	0.64
pH	6.8	6.8
Total suspended solids	~0	152 ± 0.03

At the beginning of fermentation (time 0), the concentrations of soluble total carbohydrates and reducing sugars in both media ranged between 34–36 and 27–32 g/L, respectively. Reducing sugars accounted for approximately 80% of the total sugars. As expected, the liquid fractions of both broths were quite similar regarding the amount of sugars and the total nitrogen concentration. However, the concentration of total phosphorus was slightly higher in the non-centrifuged medium with respect to the broth without solids.

The composition of the hydrolyzed broths is similar to those described in the literature for other media used to obtain PHB by fermentation. For instance, Sukruansuwan and Napathorn (2018) [26] employed a hydrolysate of pineapple peels with a concentration of fermentable sugars of 28 g/L for the production of PHB. Likewise, a broth with a concentration of 30 g/L of glucose and fructose, used for bioplastic production, was obtained by Yustinah et al. (2019) [27] from palm-oil fruit treated by acid hydrolysis. PHB, a semicrystalline biopolyester that bacteria accumulate as a reserve material within their cells, is a secondary metabolite produced in response to environmental stress [20]. Specifically, it has been reported that this polymer is generated through a fermentation process under restricted growth conditions for nitrogen, phosphorus, sulfur and/or oxygen in the presence of an excess carbon source [28]. Hence, knowing the nitrogen and phosphorus concentrations in the growth media is fundamental. Some authors have reported that the optimized medium conditions to maximize PHB production were 22–30 g/L of carbon, 2 g/L of nitrogen, 0.5 g/L of phosphorous, and a pH of 7, values quite similar to those obtained in this study in the initial media [29–31]. Therefore, according to bacterial nutritional requirements reported in the literature data, the resulting broths from CBS hydrolysis seems to be an optimal fermentation medium for the production of PHB by *B. firmus*.

Another aspect that must be taken into account is the possible presence of inhibiting compounds of fermentation. Acetic acid, furfural, or 5-hydroxymethylfurfural (HMF) can be formed when severe conditions in hydrolysis processes are employed and these species may affect cell viability due to the physiological stress generated in bacteria [32]. For acetic acid, concentrations of 1–10 g/L have been reported to inhibit sugar fermentation by *Bacillus*, whereas it has been found that the growth of this genus was inhibited at concentrations of furfural and HMF between 0.05–5 g/L [33,34]. In this work, both broths employed for fermentation contained initially concentrations of acetic acid and HMF of 2.8 and 0.03 g/L, respectively. Hence, the presence of these compounds could affect bacterial growth, which would affect the production of PHB. In the case of furfural, for both centrifuged and non-centrifuged media, the concentration of this inhibitor was below the detection limit (<1 mg/L).

The morphology of CBS powder was determined by SEM. Xylem conductive cells known as tracheid were observed in cocoa powder next to sclerenchyma cells fibers (Figure 1A). According to the literature, CBS is also composed of fat that could reside inside cells or be deposited in fat globules as shown in Figure 1B (see yellow arrows). The solids resulting from milling CBS were observed by SEM, before and after hydrothermal treatment. As can be observed in the micrographs shown in Figure 1, in general, in both cases the particle size was lower than 50 µm. However, several modifications of the surface morphology in the treated samples with respect to the control (untreated CBS) were assessed. In the control samples, the surface structure (Figure 1C) of the milled CBS particles was found to be little porous and smooth, whereas in the hydrolyzed samples (Figure 1D),

a notable number of pores (see yellow arrows) and surface ruptures could be observed. These changes could facilitate the diffusion of sugars to the liquid phase making them available for microorganisms.

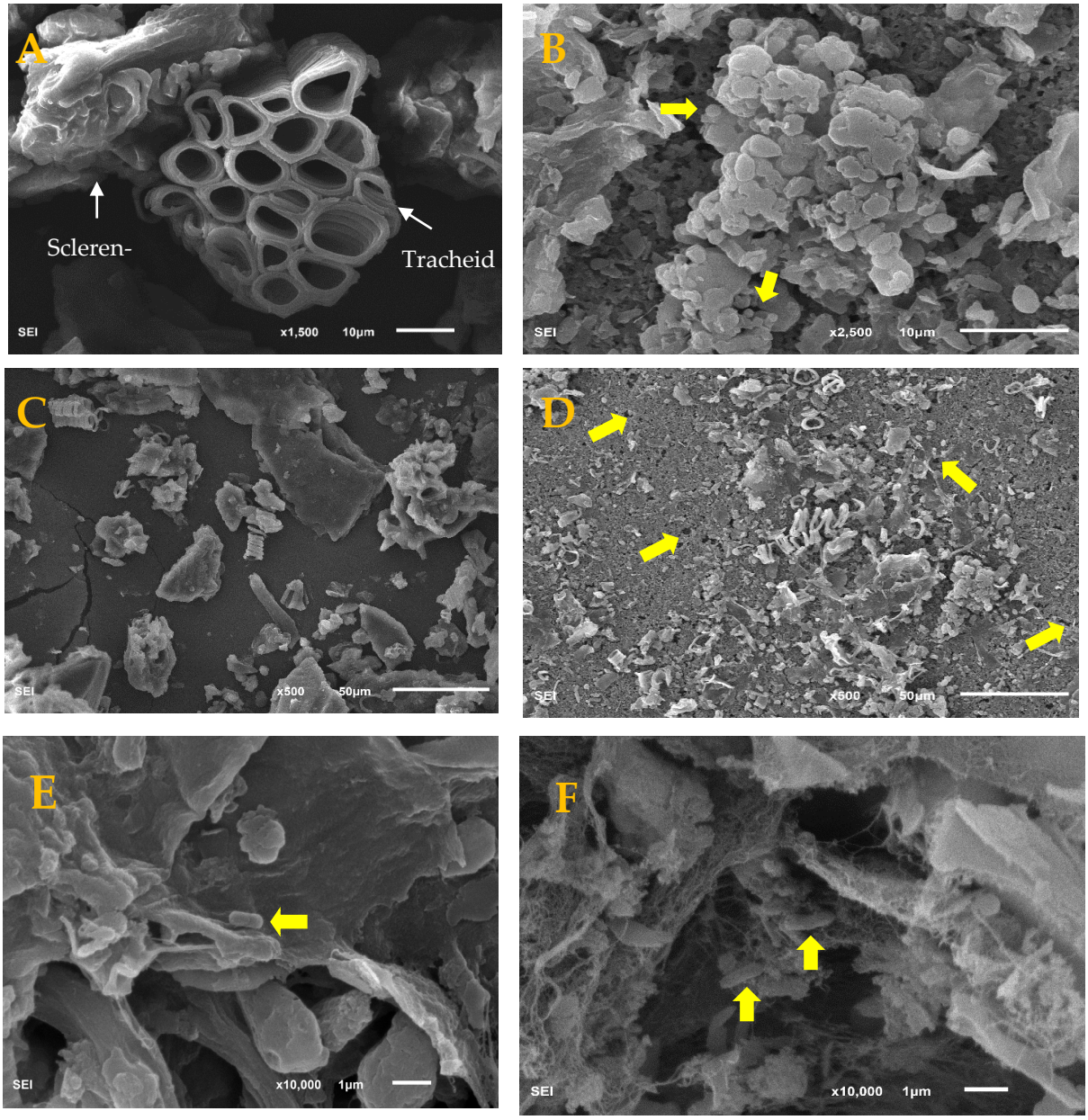

Figure 1. SEM micrographs of CBS powder: (**A**–**C**) untreated, (**D**) just after hydrolysis, and (**E**,**F**) hydrolyzed after 48 h of fermentation (non-centrifuged medium). Magnification indicated in each picture.

The centrifuged medium and the non-centrifuged broth were observed using a confocal laser microscope. In Figure 2, it can be seen that the non-centrifuged broth presented, as expected, a greater number of particles than the centrifuged medium (Figure 2A). In addition, the size of these particles was significantly larger in the non-centrifuged medium, with particles close to 50 µm, whereas in the centrifuged broth solids, they did not exceed 10 µm in general.

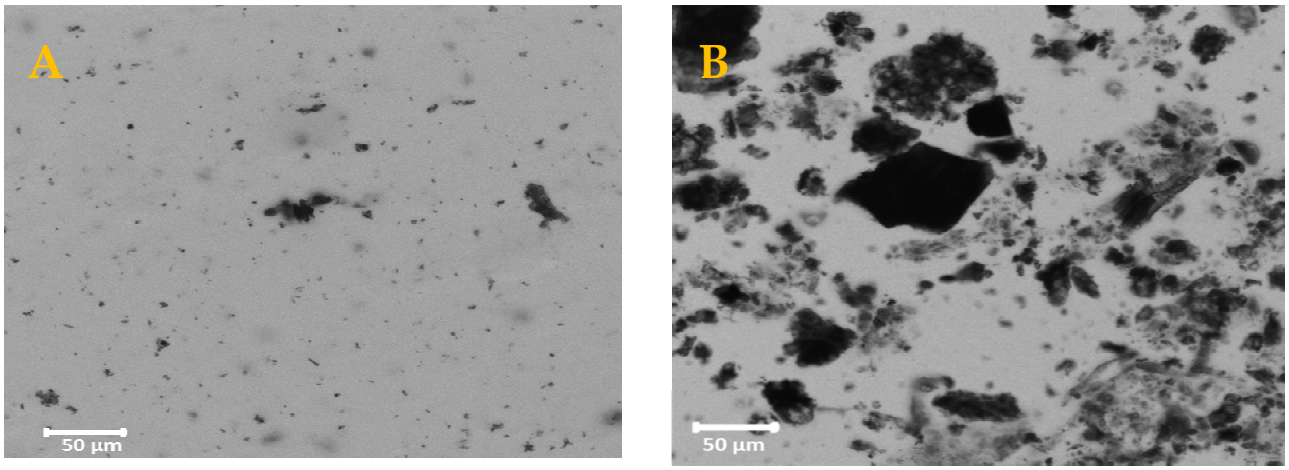

Figure 2. Confocal microscopy images of centrifuged (**A**) and non-centrifuged (**B**) media employed as fermentation broths (×20 magnification).

3.2. Fermentation with B. firmus

3.2.1. Centrifuged Medium

The results obtained from fermentation employing the centrifuged broth (without solids), including the evolution of PHB, the total and reducing sugars, and the growth of bacteria, are shown in Figure 3.

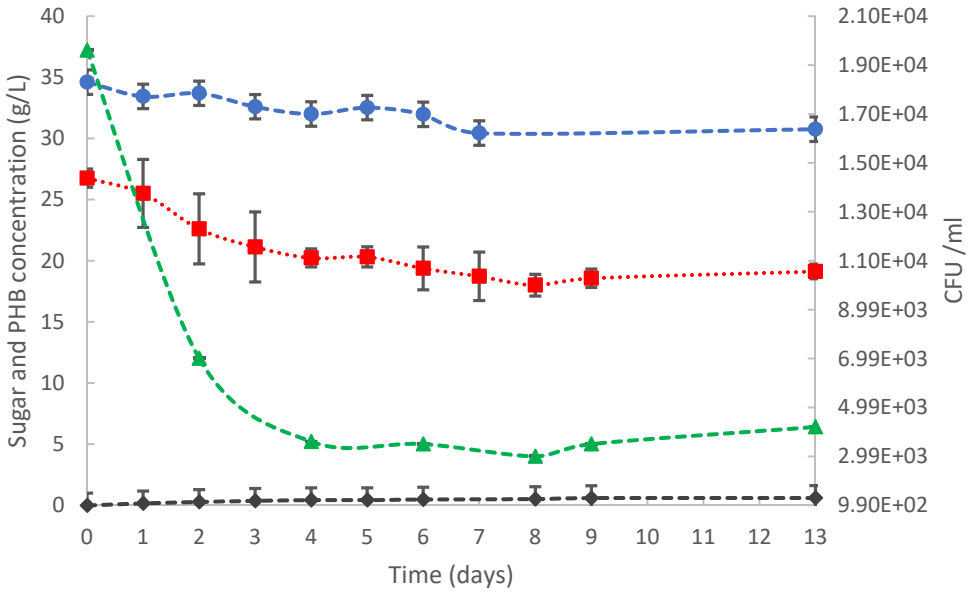

Figure 3. Evolution of the concentration of total sugars (●), reducing sugars (■) and PHB (♦), and bacteria viability (▲) during the fermentation of the centrifuged medium obtained from CBS hydrolyzed at 135 °C during 10 min.

As can be seen in Figure 3 and Table S1, with an initial reducing sugar concentration of 26.7 g/L, after 13 days of fermentation, a concentration of only 0.6 g/L of PHB (3.7 mg PHB/g dry matter of CBS) was obtained. The maximum productivity took place during the first 4 days with an average value of 0.005 g/L/h. The consumption of sugars during the process was very low, with a reduction in the concentration of approximately 5 g/L. With respect to bacteria growth, it is noticeable that during the whole assay, not only was an increase in *B. firmus* growth not observed, but also a decrease in microbial viability (from 10^4 to 10^3 CFU/mL) could be appreciated from the first day of fermentation. Despite of

the low concentration of here achieved here, similar concentrations have been reported by other authors using different substrates. For example, Martinez-Herrera et al. (2020) [35] obtained a PHB concentration of 0.67 g/L from agave syrup using *Bacillus cereus* as a fermentative microorganism, whereas Kovalcik et al. (2020) [36], who employed grape pomace hydrolysates as a fermentation substrate, achieved a PHB concentration between 0.5–1.8 g/L using *Cupriavidus necator* as microorganism.

When hydrolysis processes are carrying out in severe conditions, inhibitory compounds from the lignocellulose structure can be released [3]. The inhibitory effect of weak acids, such as acetic acid, is based on their rapid dissociation due to the neutral intracellular pH, leading to a decrease in internal pH and, therefore, a reduction in cell viability. In addition, it has been suggested that this internal acidification could cause the enzyme inhibition of cells [33]. Regarding furan derivates, their toxic effects generate several disturbances in microorganisms such as a drop in the cell growth rate and cell membrane permeability, interference with fermentative enzymes and biological activity, inhibition of protein synthesis, and the breakdown of DNA [37,38]. As has been commented on in Section 3.1, both acetic acid and HMF were found in the hydrolysate broths in concentrations that might be considered as inhibitory. Thus, the decrease in the microbial viability during the fermentation process could be related to the presence of these inhibitors.

3.2.2. Non-Centrifuged Medium

The evolution of the concentration of PHB, total and reducing sugars, and bacterial growth during fermentation of the non-centrifuged hydrolysate (with CBS solids) is shown in Figure 4. As can be seen, the behaviour was completely different from the case with the centrifuged hydrolysate. During the first two days, a mild lag phase was observed that was accompanied by the moderate consumption of reducing sugars and the fast production of PHB. Then, a period of exponential growth was observed that extended until the 10th day. The maximum specific rate of growth was calculated obtaining a value of 1.18 $days^{-1}$. This value is within the range given (0.2–2.1 $days^{-1}$) when *B. firmus* was used for aniline degradation [39]. During the exponential phase of growth, a fast consumption of sugars took place and the production of PHB continued more slowly achieving a maximum of PHB concentration of 20 g/L (107.5 mg PHB/g dry weight) after 8 days of fermentation. The simultaneous increase in both PHB concentration and biomass would confirm that PHB production is related in some way to the growth of *B. firmus*. In addition, PHB production ceased when bacterial growth approached the stationary phase.

The consumption of sugars by the bacterium was much more noticeable in this case than in the fermentation of the centrifuged medium and, at the end of the experiment, the concentrations of total and reducing sugars were about half of the initial concentrations. It can be observed that after 10 days of fermentation, there was no consumption of sugars by *B. firmus* and the PHB concentration began to decrease. Sarmiento-Vásquez et al. (2022) [40], who tested the use of soybean hull hydrolysed to obtain PHB, observed that after 48 h of fermentation, there was no sugar consumption by *C. necator* and the concentration of PHB remained stable. They explained that this fact could be due to the microorganism's preference for a specific type of simple sugar. Thus, those sugars not consumed by microorganisms remained in the broth at the final stages of fermentation.

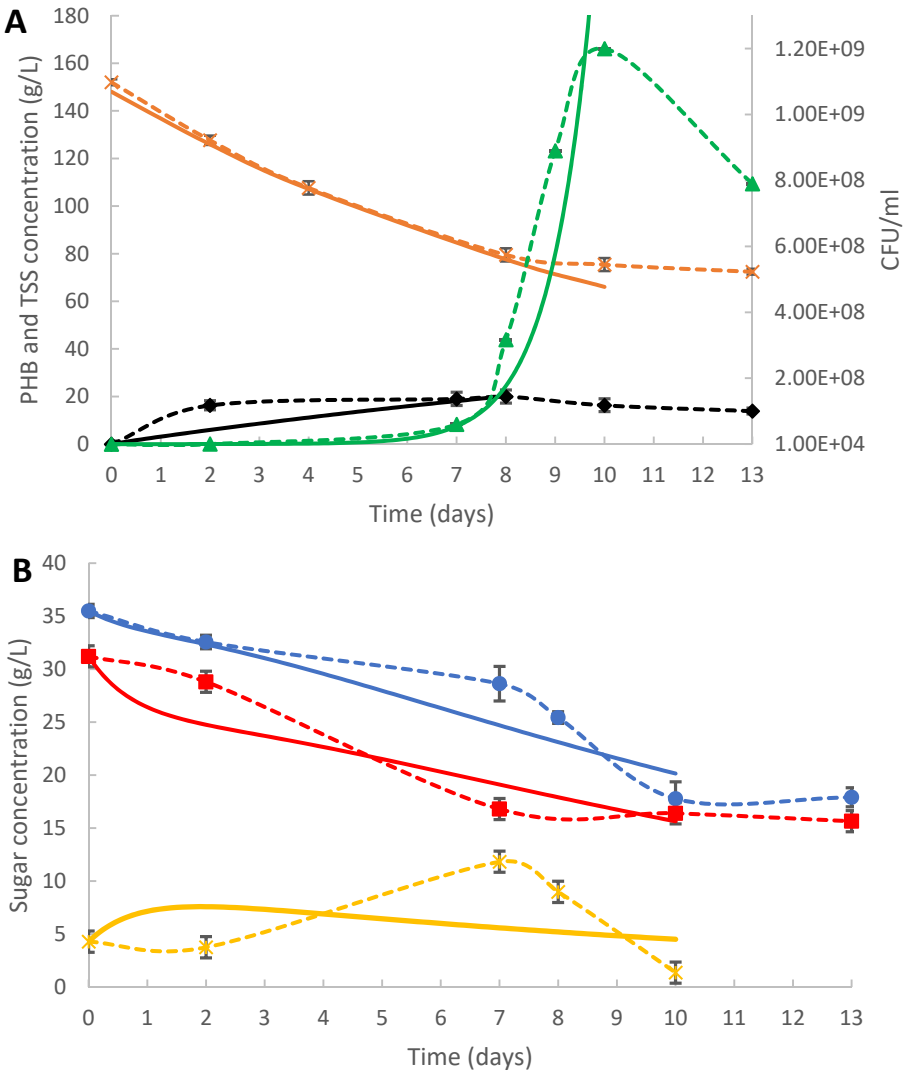

Figure 4. Evolution of (**A**) the concentration of TSS (x), PHB (♦), and bacteria viability (▲), and (**B**) the concentration of total sugars (●), reducing sugars (■), and non-reducing sugars (*) during fermentation of the non-centrifuged medium obtained from CBS hydrolyzed at 135 °C for 10 min. The symbols and dashed lines correspond to experimental data and continuous lines correspond to model results.

It is necessary to consider that in this fermentation, as the CBS solids are in the broth, it seems quite possible that some sugars were being dissolved at the same time as the fermentation was being conducted with the participation of enzymes released by the bacterium. For this reason, the evolution of total suspended solids (TSS) was also followed during fermentation. TSS included the CBS solids and the microorganisms. However, considering the concentrations of *B. firmus* during the fermentation and the average dry weight of bacterium, it was probed that the weight of the bacteria was always lower than that of 0.3% of the TSS measured, so it was considered that the TSS measured corresponded with the concentration of CBS solids. As can be seen in Figure 4A, the concentration of CBS solids decreased during all of the experiment. Therefore, sugars were dissolving from the solid at the same time as they were being consumed by the microorganism. To describe the transformations that occurred, a model based on irreversible first-order reactions was used [3]. It considers that CBS solids are dissolved by enzyme activity into non-reducing sugars that are subsequently hydrolysed to reducing sugars. Thus, *S* is the solid matter, *D* is the dissolved non-reducing-sugars (estimated as dissolved total

sugars minus dissolved reducing sugars), and M is the dissolved reducing sugars that are being consumed by microorganisms. Model reactions are represented below; k_1 is the solubilisation rate of solid matter, k_2 is the hydrolysis rate of soluble non-reducing sugars into reducing sugars, and k_3 is the consumption rate of reducing sugars by microorganisms; FP indicates fermentation products.

$$S \xrightarrow{k_1} D \xrightarrow{k_2} M \xrightarrow{k_3} FP \quad (1)$$

$$\frac{dS}{dt} = -k_1 S \quad (2)$$

$$\frac{dD}{dt} = k_1 S - k_2 D \quad (3)$$

$$\frac{dM}{dt} = k_2 D - k_3 M \quad (4)$$

Microsoft Excel software was used to obtain the kinetic constants' values by fitting the model to experimental data. Only experimental data obtained before the stationary phase of growth were used (until 10th day). The comparison between experimental and model data is shown in Figure 4A,B. The tendency is well predicted by the model, although certain discrepancies were observed for reducing sugars' concentrations, specifically during the first hours. This is probably due to the existence of a lag phase of growth that is not considered by the model. In Table 2, the values of the kinetic parameters are shown. The hydrolysis constant was considerably higher than the solubilisation and the consumption constants, which is in agreement with the fact that at the end of fermentation, almost all of the remaining sugars were reducing sugars.

Table 2. Kinetic constants calculated from experimental data.

Broth	k_1 (Days^{-1})	k_2 (Days^{-1})	k_3 (Days^{-1})
Non-centrifuged	0.072	1.223	0.4213

Regarding PHB production, a maximum of 20 g/L of PHB (107.5 mg PHB/g CBS dry weight) was obtained after 8 days of fermentation. The highest rate of biopolymer production occurred between 0 and 2 days (lag phase of growth) with an average PHB productivity of 0.34 g/L h. PHB production continued during the exponential phase of growth but with a significantly lower productivity (0.04 g/L h). Although it seems clear that PHB is only produced when the bacterium grows, its production is not totally associated with the microorganism's growth. In fact, the maximum production rate occurred during the first stage of growth, when the bacterium was adjusting to the new environment. On the contrary, Mohapatra et al. (2017) [20], who conducted a review on PHAs' production by *Bacillus* species, reported that the bacteria synthesize PHAs inclusions in the late log phase of the growth cycle. This disagreement can be explained due to the particular characteristics of the CBS medium. *Bacillus* genus can produce biofilms in response to stress; in addition, CBS solids seem to be an accurate support to stimulate biofilm formation. Khyvami et al. (2011) [41] described that when *Bacillus* SA was grown in palm syrup by means of forming biofilms on plastic composite supports, PHA production decreased after 30 h, which is in accordance with the results found in the present work. Moreover, the former authors obtained a maximum PHA of 70 g/L in optimal conditions. SEM micrographs showed the presence of bacillary formations, identified as *B. firmus*, on CBS particles of the non-centrifuged broth (Figure 4). It should be noted that the presence of biofilms was not observed, since they were probably degraded during the preparation of inoculated samples, which included several washes, to be observed through SEM.

If only poly(3-hidroxybutyrate) (PHB), which is a polyhydroxyalkanoate (PHA), is considered, the concentration of PHB achieved in this work was higher than the other concentrations reported in the literature that used other substrates and/or microorgan-

isms. For instance, Manikandan et al. (2020) [42], who studied the use of carob pods as raw materials to produce PHB, with an initial sugar concentration of 40 g/L, obtained a maximum of 12 g/L of PHB. Valdez-Calderón et al. (2022) [43] obtained a maximum of 24 g/L of PHB from fruit peel residues employing *Klebsiella pneumoniae* as a bacterial producer. Saratale et al. (2019) [44], employing kenaf hydrolysates as feedstock, obtained a maximum of 10 g/L of PHB using *Ralstonia eutropha* as a fermentative microorganism. Sindhu et al. [15] who employed pretreated rice straw and *B. firmus* obtained a maximum PHB concentration of only 7.7 g/L.

Comparing both media, with similar initial characteristics in the liquid medium, it PHB production 95% higher when using the non-centrifuged medium with respect to the centrifuged one was observed. Hence, this indicates that the presence of solids plays an essential role in the growth of the microorganism and, therefore, the production of PHB. The consumption of sugars was much higher when the CBS solids were in the broth. In the centrifuged medium, the consumption of total sugars was lower than 10 g/L, whereas in the non-centrifuged medium it was almost 100 g/L (considering the initially dissolved sugars and those dissolved during fermentation). Thus, it seems possible that this continuous supply of sugars and/or other micronutrients dissolved from the CBS solids are responsible for the observed difference in cell growth and PHB production. In addition, some authors have demonstrated that the presence of solids in fermentation broths can improve the formation of fermentation products due to the presence of growth-promoting compounds on the solid surface, resulting in an increase in the nutrients uptake by microorganisms and, hence, leading to an acceleration in the cell growth rate [45,46].

Although the concentration of inhibitors (acetic acid and HMF) is the same in the centrifuged and non-centrifuged broths (Section 3.2.2), the CBS solids could be employed by *B. firmus* as a support, forming a biofilm on their surface. It is well known that microorganisms forming biofilms are more resistant to adverse environmental conditions, thus this could be an explanation for the different results obtained [47,48]. In addition, according to the literature, CBS can be employed as an alternative bio-adsorbent, with it being effective in removing some organic pollutants, including phenolic species [49–52]. Thus, it could be possible that acetic acid and HMF are partially adsorbed by CBS solids, decreasing their toxic effects on *B. firmus*.

4. Conclusions

CBS was used for the first time as feedstock to obtain PHB, a sustainable alternative to synthetic polypropylene, by fermentation with *B. firmus*. Two different broths resulting from CBS hydrothermal hydrolyses (centrifuged and non-centrifuged) have been tested. Although very low PHB production was observed with the centrifuged medium, excellent results were obtained with the non-centrifuged medium, with a maximum of PHB of 20 g/L. In addition, it was observed that, in this last case, *B. firmus* increased four orders of magnitude (from 10^4 to 10^8 CFU/mL), whereas in the centrifuged medium, the microbial viability decreased since the first fermentation day. It is evident that the presence of CBS solids is fundamental for bacterium growth and PHB production. This could be explained by the additional source of nutrients that is the presence of the solids that continued dissolving during fermentation. In addition, the formation of biofims on the solids surface may cause certain changes in bacterium metabolism and even protect them from inhibitory compounds. The kinetic constants for the solubilization, hydrolysis, and sugar consumption steps were estimated and values of 0.072, 1.223, and 0.4213 $days^{-1}$ were obtained, respectively.

The results of this work highlight the potential of CBS residues to be employed as substrates for fermentation with the aim of producing value-added products and demonstrate, in particular, the interest of using CBS as a source for PHB production. As future work, fermentation conditions (i.e., inoculum size, temperature, agitation, time, etc.) should be optimized in order to consider the possible scaling of the process. An array of research

areas from engineering and biotechnology to food chemistry has to be brought together to achieve significant advances in this field.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13020975/s1, Table S1. Evolution of concentration of total sugars, reducing sugars, PHB, and bacteria viability during the fermentation of the centrifuged medium obtained from CBS hydrolyzed at 135 °C during 10 min.

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