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Exploitation of Cocoa Pod Residues for the Production of Antioxidants, Polyhydroxyalkanoates, and Ethanol

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Abstract: Cocoa pod husks (CPH) and cocoa bean shells (CBS) are the main by-products of the cocoa industry and a source of bioactive compounds. These residues are not completely used and thrown in the fields without any treatment, causing environmental problems. Looking for a holistic valorization, the aim of this work was first to deeply characterize CPH and CBS in their chemical composition, amino acid, and fatty acid profiles, as well as their application as antioxidants. CBS had a high level of protein (17.98% DM) and lipids (16.24% DM) compared with CPH (4.79 and 0.35% DM respectively). Glutamic acid and aspartic acid were the predominant amino acids. The total phenolic compounds (TPC) detected in the ethanolic extracts of CPH and CBS were similar to pyrogallol as the main detected polyphenol (72.57 mg/L). CBS ethanolic extract showed a higher antioxidant activity than CPH. Both extracts increased the oxidation stability of soybean oil by 48% (CPH) and 32% (CBS). In addition, alkaline pretreatment of CPH was found suitable for the release of 15.52 ± 0.78 g glucose/L after subsequent saccharification with the commercial enzyme Cellic®. CTec2. Alkaline hydrolyzed and saccharified CPH (Ahs-CPH) was assessed for the first time to obtain polyhydroxy alkanoate (PHAs) and bioethanol. Ahs-CPH allowed the growth of both *Cupriavidus necator* DSM 545 and *Saccharomyces cerevisiae* Fm17, well-known as PHA- and bioethanol-producing microbes, respectively. The obtained results suggest that such agricultural wastes have interesting characteristics with new potential industrial uses that could be a better alternative for the utilization of biomass generated as million tons of waste annually.

Keywords: *Cupriavidus necator* DSM 545; *Saccharomyces cerevisiae* Fm17; polyhydroxyalkanoate; bioethanol; cocoa by-products; oxidative stability

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1. Introduction

Theobroma cacao is a perennial tropical tree, belonging to the Sterculiaceae family, native to the tropical forests of the upper Amazon region [1]. The cultivation of cocoa is of high economic importance. Indeed, according to the International Cocoa Organization (ICCO), over fifty million people depend on cocoa for their livelihood with a global production capacity of 68% in Africa, 17% in Asia, and 15% in the Americas [2]. In 2021, the world's annual cocoa bean production was approximately 4.2 million metric tons with the Ivory Coast, Ghana, Indonesia, Brazil, Nigeria, Cameroon, Ecuador, and Colombia as major producers [3,4]. After two years, the cacao tree produces large pod-shaped fruits, with cocoa beans contained in the cocoa pod, consisting of the shell, kernel (or cotyledon), and germ. Around 75% of the total weight of the fruit is due to pods [5].

The cocoa shell is removed along with the germ before or after roasting and the broken cotyledon fragments, called nibs, free from the shell are used in the production of chocolate [6,7]. Cocoa pod husks (CPH) and cocoa beans shells (CBS) are the main by-products of the cocoa industry. After cocoa beans are extracted from the fruit, CPH are generated. It has been estimated that for each ton of dry beans produced, 10 tons of wet CPH are spawned. CBS represent up to 20% of cocoa beans and are generally underutilized. As a result, the growing market has brought the cocoa industry to massive production levels, causing the excessive generation of waste [8–10].

Due to the large quantities, managing cocoa waste is challenging. Large amounts of the generated biomass are burned by farmers, simply chopped and incorporated into the soil as a fertilizer, or left directly into the ground until their decomposition, generating smelly odors, causing soil contamination and the emission of greenhouse gasses [11]. Additionally, under certain climatic conditions, this biomass can cause an excessive proliferation of fungi, including potential pathogens [1]. For this crop, Good Agricultural Practices (GAP) recommend at least shredding and composting [12]. Therefore, it is mandatory to explore alternative utilizations of CPH and CBS. These materials are composed mainly of fiber, carbohydrates, lignin, proteins, and minerals [13–15]. Furthermore, although considered as wastes, these residues are extremely rich in biologically active molecules, often with nutraceutical properties such as phenolic compounds.

The Sustainable Development Goals Report 2023 by the United Nations emphasizes the immense potential for the utilization of lignocellulosic biomass "contributing to create a brighter future for all" [16]. Indeed, after the development of effective pretreatment methods and biocatalytic systems, renewable lignocellulosic biomass could be abundant feedstocks for the sustainable production of a wide range of molecules in an integrated biorefinery platform, as well reviewed by Yadav [17] and Mujtaba et al. [18].

Thus, in the framework of the circular economy, CPH and CBS are attracting increasing attention as possible starting materials to obtain added value products in the food sector, as well as in other contexts. Thanks to their chemical characteristics, several studies proposed strategies for the exploitation of cocoa wastes, pods, and husks as soil fertilizers, sources of pectin and polyphenols, animal feed, and in the production of soap or activated carbon [10]. The nutritional and biotechnological applications of cocoa wastes, together with health benefits and possible therapeutic roles in cancer, have been considerably reviewed by Sanchez et al. [19] and Cinar et al. [20].

The search for new applications of cocoa wastes integrates well into the current concepts of bioeconomy as postulated also by the European Union "Green Deal", aimed to restructure the industrial sector, promoting the circular economy to minimize negative environmental impacts, drastically reducing plastic pollution and greenhouse gas emissions [21].

As also determined in the present work, the chemical composition of CPH and CBS is mainly cellulose, hemicellulose, fibers, amino acid, and fatty acids; therefore, these cocoa residues grant large volumes of lignocellulosic biomass, a green and cheap resource material to develop a wide portfolio of bioproducts. Thus, due to the growing interest in replacing synthetic food antioxidants with natural ones [22], dry samples of CPH and CBS were extracted by ultrasound-assisted extraction (UAE) to recover their total phenolic compounds (TPC), successively tested in soybean oil as natural antioxidants to delay lipid oxidation. Besides, this residual material could be a promising feedstock to obtain medium to high value-added molecules [13–15] such as bioethanol [13], other biofuels, and bioplastics of medical, pharmaceutical, agricultural, or food interest [5].

Specifically, bio-based bioplastics are numbered among the most promising products obtainable from renewable sources. Indeed, the indiscriminate use of conventional fossil-derived plastics generates significant environmental pollution, especially due to their extremely difficult degradation; for this reason, these commodities are considered as a potential alternative [23]. Polyhydroxyalkanoate (PHAs) can be included among the most promising bioplastics due to their high biodegradability, biocompatibility, and versatil-

ity [24]. PHAs are natural, biodegradable, and compostable polyesters accumulated by numerous microorganisms in the form of intracellular granules and are formed from 600 to 35,000 monomer units of hydroxyalkanoic acids. PHAs have functional characteristics similar to those of many of the most common fossil-based plastics; at the same time, they are completely biodegradable in soil, fresh water, and marine environments and are both industrially and domestically compostable. LCA (Life Cycle Assessment) studies estimate that the replacement of 1 kg of fossil plastic with PHA could reduce the amount of CO_2 emitted by 2 kg [21].

In 2022, PHAs consisted of only 3.9% of global bioplastic production [25] and the commercialization of PHAs has been slowed down by their high production costs, largely due to carbon substrates which represent 30–40% of the total [26]. Therefore, to reduce the price and make PHAs more economically sustainable, it is crucial to search for novel and low-cost carbon-rich substrates [27,28]. Thus, cocoa wastes might represent an attractive alternative to the pure sugars generally used to obtain PHAs.

Among the biofuels, bioethanol is obtained from renewable sources and can be used as a fuel, chemical, or solvent. To date, first-generation bioethanol is mainly obtained from feedstocks that contain simple sugars or starch, raising concerns related to the use of soil, the consumption of water resources, and the subtraction of the grain to the production of food or feed. The International Energy Agency (2010) [29] reports that in the coming years, it will be mandatory to produce ethanol from the waste of the food industry and agroforestry, such as those from the cocoa manufacturing.

In order to attain the above bio-products, both waste streams were chemically and enzymatically treated to obtain substrates suitable for the growth of suitable microbial strains. Particularly, *Cupriavidus necator* DSM 545 for the production of PHAs and *Saccharomyces cerevisiae* Fm17 for the production of bioethanol.

Although CBS has been has been successfully evaluated as a substrate for PHAs synthesis by *Bacillus thermus* after sulfuric acid thermal treatment [30] and CPH for ethanol production [13,31], to the best of our knowledge, this is the first account reporting the exploitation of CPH and CBS through the recovery of phenolic compounds and the sustainable microbial production of PHAs and bioethanol after efficient mild pre-treatments and the enzymatic hydrolysis of cocoa waste.

2. Materials and Methods

2.1. Feedstocks and Chemicals

CPH and CBS were supplied by the Servicio Nacional de Aprendizaje, Espinal, Colombia. After harvesting, CPH and CBS were washed with distilled water, cut into small pieces, dried at 48 °C for 24 h, ground by a professional mill (MF 10 basic Microfine grinder IKA-Werke, Staufen, Germany), finally sieved through a 500 µm sieve and stored at 4 °C. The material was derived from *T. cacao* clone IMC-67 (Iquitos Maranon Collection), cropped in Espinal, Tolima, Colombia, in 2010. The site is located at a latitude of 4°10′10″ N, a longitude of 74°55′52″ W, and an elevation of 348 m. Soybean commercial oil was purchased in a local market (Padova, Italy). All chemicals were of analytical grade and were obtained from Sigma-Aldrich, unless stated otherwise.

2.2. Chemical Analyses of CPH and CBS

CPH and CBS were analyzed in terms of ash, starch, hemicellulose, cellulose, lignin, and protein content, according to international standard methods, as described in [19]. In short, total ash was determined by calcinating the residues at 550 °C as described in methods 942.05 and 934.01 by Association of Official Analytical Chemists (AOAC). Cellulose, hemicellulose, and lignin were measured according to Van Soest et al. methodology [32]. Starch contents were calculated according to AOAC method 920.40. Total nitrogen was determined by the Kjeldahl method, followed by the protein calculation using the general factor of 6.25 (AOAC, Method 981.10). The dry matter (DM) content was obtained by drying triplicate samples for 48 h at 100 °C in an oven. The amino acid and fatty acid

profiles were determined as previously reported [20,21]. The dry matter (DM) content was obtained by drying triplicate samples for 48 h at 100 °C in an oven. The amino acid and fatty acid profiles were determined as previously reported [33,34].

2.3. Phenolic Extraction of Cocoa Pods and Shells

Two grams of dry CPH and CBS were solubilized in 10 mL of 70% v/v ethanol, mixed for 20 min using an orbital shaker at room temperature, and subjected to ultrasound for 2 min, at intervals of 30 s with SONOPULS ultrasonic homogenizer at 20 kHz ± 500 Hz frequency. The KE76 tip was used for the sonication. The samples were then centrifuged for 5 min at 9500× g at 4 °C (Hettich Zentrifugen, MOD: Universal 320R, Tuttlingen, Germany). The supernatants (ethanolic extracts) were recovered, filtered using Whatman paper No. 1, and stored at −18 °C until used.

2.4. Quantification of the Total Phenolic Compounds (TPC)

The TPC were quantified in the phenolic extracts using the FolinCiocalteu method [35]. Briefly, 1 mL of diluted ethanolic extract was mixed with 5 mL of $NaCO_3$ 10%, containing NaOH 1 M and 500 µL of Folin–Ciocalteau reagent previously diluted twice in distilled water. A blank solution with the dilution solvent was also set up. After 30 min under darkness, the samples were filtered using 0.45-µm Millipore acetate cellulose filters (Merk Life Science S.r.l., Milano, Italy). Hence, the absorbance was measured at 650 nm using a Varian Carry 50 Bio UV/Vis spectrophotometer. The results were expressed as mg of gallic acid equivalent per g of sample (mg GA/g).

2.5. Phenolic Compounds Identification

The phenolic profile of CPH and CBS was determined by HPLC analysis [35] using a Thermo Finnigan SpectraSystem UV6000LP (Thermo Finnigan, San Jose, CA, USA) HPLC system with diode array detector. Before injection in the column, the samples were filtered with a 0.22-µm cellulose acetate filter (Merk Life Science S.r.l., Milano, Italy). The phenols present in the sample were identified based on the retention time of the corresponding commercial standards (pyrogallol, hydroxybenzoic acid, protocatechuic acid, caffeic acid, syringic acid, and ferulic acid), previously solubilized in absolute methanol using the SupelcosilTM LC-18 column at the following operating conditions: mobile phase, 18 mL n-butanol (solvent A)/1.5 mL 50% v/v acetic acid (solvent B); flow rate, 0.6 mL/min; isocratic flow; wavelength, 214 nm, 275 nm, and 310 nm; temperature, 25 °C; and running time, 60 min.

2.6. Antioxidant Activity

The assay was performed by using the ferric reducing antioxidant potential (FRAP) method in agreement with Stratil et al. [36]. The FRAP reagent was prepared by mixing 2.5 mL of 0.01 M of TPTZ in HCl 40 mM, 2.5 mL of aqueous solution of 0.02 M $FeCl_3$, and 25 mL of 0.3 M of sodium acetate buffer (0.2 M sodium acetate/0.2 M acetic acid). A FRAP volume of 900 µL was mixed with 100 µL of sample and incubated at 37 °C for 30 min. Also, a blank solution was prepared with the dilution solvent. The absorbance was measured at 593 nm using a Varian Carry 50 Bio UV/Vis spectrophotometer. The results were expressed as mg of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent per g of sample (mg TE/g).

2.7. Determination of the Oxidative Stability

Ethanol extracts from CPH and CBS at 40% were added to soybean oils and mixed for 20 min using an orbital shaker. After a sonication treatment for 2 min (4 intervals of 30 s), oxidative stability was evaluated in both oil mixtures using the official Rancimat method (AOCS, 2012), according to the procedure previously described by Tinello et al. [37]. As controls, soybean oil without supplementation was introduced in the Rancimat assay. A quantity of 3 g of samples (control or supplemented oil) was weighed in the Rancimat

apparatus (Metrohm, model 743, Herisau, Switzerland) and subjected to a stream of air at the rate of 20 L/h kept at a constant temperature of 110 °C, causing an accelerated oxidation process. The oxidative stability was expressed as the induction time (IT) corresponding to the time (h) at which the water conductivity (μS/min) starts increasing because of the production of 11 compounds involved in the lipid oxidation. The antioxidant activity index (AAI) was calculated by the following equation:

$$\text{AAI} = \frac{\text{IT of oil with GPP}}{\text{IT of oil without GPP}}$$

2.8. Pretreatments and Enzymatic Hydrolysis

Pretreatments of lignocellulosic substrates are needed to break the structure of lignin and remove the hemicellulose structure to increase exposure to cellulolytic enzymes in the subsequent hydrolysis phases. Therefore, before enzymatic hydrolysis, pretreatments with hydrogen peroxide and alkaline were evaluated:

To determine the best conditions for hydrogen peroxide pretreatment, 2.5 g of CPH in 100 mL flasks were treated with increasing doses of H_2O_2 (0, 2.5, 5, and 7.5% *v*/*w*). Water was added to a total volume of 25 mL. The flasks were incubated in an agitated bath (Mod. SW22 Julabo, Seelbach, Germany) at 55° C. After 4 h, the pH of the pretreated CPH were adjusted to 5.0 using concentrated HCl before the following saccharification phase.

For the alkaline pretreatment, 2.5 g CPH were incubated in a 100 mL flask with 25 mL of 4% NaOH. After boiling for 30 min, the pretreated CPHs were centrifuged at 5000 rpm for 15 min and the supernatant discarded. The pellet was washed twice with distilled water, the pH was adjusted with 0.5 M HCl at 7.0, washed again, dried at 100 for 16 h, and finally resuspended in 25 mL of 50 mM citrate buffer [38].

For the following enzymatic saccharification, Cellic® CTec2 (Novozymes, Bagsvaerd, Denmark) 12% *w*/*w* (g/g cellulose) was added to hydrogen peroxide or alkaline pretreated CPH, according to the supplier's instructions. The suspensions were stirred (100 rpm) at 50 °C. Samples (2 mL) were withdrawn after 0, 24, 48, and 72 h and boiled for 10 min to inactivate the enzymes. A blank without the enzyme addition was used to assess autohydrolysis. The suspensions were then centrifuged and the glucose content in the supernatants (pretreated CPH) was measured using the Megazyme glucose assay kit in a UV-Vis spectrophotometer (Megazyme International Ireland Ltd., Wicklow, Ireland) following instructions of the manufacturer. Optical densities were converted in g glucose/L.

On the basis of the glucose released from cellulose after treatment with Cellic® CTec2, the saccharification degree after alkaline or hydrogen peroxide pretreatment was calculated according to the following equation.

$$Ds_{glucan} = \frac{[\text{glucose g/L}] \times 0.9}{[\text{cellulose g/L}]} \times 100\%$$

In addition, reference experiments were performed using Cellic® CTec2 with non-pretreated CPH.

2.9. Microbial Strains

C. necator DSM 545, one of the most efficient PHAs producers and *S. cerevisiae* Fm17, outperforming bioethanol yeast, was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and the collection of DAFNAE (University of Padova, Italy), respectively [39,40].

2.10. PHAs Production by C. necator DSM 545 from Alkaline Pretreated CPH Hydrolyzate (Ahs-CPH)

Inocula of *C. necator* DSM545 were obtained in MM medium [41] amended with 30 g/L glucose in aerobic conditions at 30 °C under shaking (145 rpm) for 24 h. Bacteria were then centrifuged (5500 rpm for 15 min), washed twice with 0.9% NaCl to remove any carbon

sources, and re-suspended in sterile 0.9% NaCl. The experiments were conducted in 125 mL flasks containing 30 mL of Ahs-CPH, sterilized by 0.22 µm Whatman filters. In some flasks, the salts contained in MM medium were added to the supernatants. After inoculation, the flasks were incubated at 30 °C and agitated at 150 rpm. For an assessment of the cell growth, samples were withdrawn and OD_{600nm} was monitored by a spectrophotometer (Spectronic® Genesys™ 2PC, Vimercate, Italy). After 72 h, cultures were centrifuged, the pellets were frozen at −80 °C, and lyophilized for cell dry mass (CDM) determination and analysis of PHAs.

2.11. Cell Dry Matter and PHAs Analysis

To determine the cell dry matter (CDM), the freeze-dried bacterial pellets were weighed. PHAs were analyzed by Gas chromatography according to the protocol described by Braunegg et al. [42]. In brief, 10 mg of freeze-dried cells were treated for 4 h at 100 °C in a mixture of 2 mL of methanol containing 3% H_2SO_4 and 2 mL of chloroform. The resulting methyl esters of hydroxy alkanoic acids were analyzed by gas chromatography as previously described [43]. A Thermo Finnigan Trace GC gas chromatograph (Mundelein, Illinois, USA) was used with a AT-WAX fused silica capillary column (Alltech Italia s.r.l., Milan, Italy) and a flame ionization. The carrier gas was helium (He) and the operating temperatures during the analysis were: 250 °C for the injection chamber, 270 °C for the detector, and 150 °C for the oven. The internal standard was benzoic acid (2.5 g/L), while the external standard was 3-hydroxybutyric acid [43]. The results obtained were expressed as a percentage of PHAs of CDM.

2.12. Bioethanol Production by S. cerevisiae Fm17 from Ahs-CPH

S. cerevisiae Fm17 pre-cultures were obtained in 500 mL flasks containing 250 mL YPD medium incubated at 30 °C on a rotary shaker set at 130 rpm for 24 h. The pre-culture was centrifuged at 5500 rpm for 10 min and the pellet was washed twice in sterile demineralized water, re-suspended in 5 mL, and used as inoculum. Then, 50 mL of Ahs-CPH was transferred in 60 mL serum bottles and inoculated with yeast at a concentration of around 5×10^8 CFU/mL. Ampicillin and streptomycin (each 100 µg/mL) were added to avoid possible bacterial contamination. Rubber stoppers were used to set up oxygen-limited conditions and a needle was inserted for CO_2 removal. Bottles were then incubated at 150 rpm and 30 °C; 2 mL of samples was withdrawn at 0, 4, 24, 48, and 72 h for subsequent chemical analysis. Control fermentations were performed in YPD with 15 g/L glucose as a carbon source.

Samples from bioethanol production were analyzed for ethanol and residual glucose through liquid chromatography using a Shimadzu Nexera HPLC system equipped with a RID-10A refractive index detector (Shimadzu, Kyoto, Japan), and a Phenomenex Rezex ROA-Organic Acid H+ (8%) column (300 mm × 7.8 mm) was used as described in Cagnin et al. [44]. The column temperature was set at 65 °C and the flow rate was 0.6 mL/min using isocratic elution, with 0.005 M H_2SO_4 as a mobile phase. The ethanol yield (g of ethanol/g of used glucose equivalent) was calculated based on the amount of glucose utilized during the fermentation and compared to the maximum theoretical yield of 0.51 g of ethanol/g of utilized glucose.

2.13. Statistical Analysis

The results are presented as mean ± standard deviation of three independent experiments (n = 3) by using an analysis of variance (ANOVA) one way, followed by the post-hoc Duncan test ($p < 0.05$) using Statgraphics Centurion XIX (StatPoint Inc., Rockville, MD, USA).

3. Results and Discussion

3.1. Chemical Composition of Cocoa Pods Husk

Table 1 shows the proximate composition of the CBS and CPH. This step was crucial to trigger a possible valorization project of these by-products as potential sources of ingredients which can be used in food, as well as animal feed, and as cheap substrates for microbial fermentations. The dry matter content of the CPH and CBS was found to be 91.84 and 94.88% respectively. Both by-products are rich in non-starchy polysaccharides with cellulose contents of 22.32% and 12.30% of DM, respectively. Hemicellulose was around 10% of dry matter for both residues and lignin 21.15 and 14.80% for the CPH and CBS, respectively. All values are in agreement with those previously described by de Souza [15] and Mendoza- Meneses [45].

Table 1. Chemical composition (% of dry matter) of the CPH and CBS. Results of chemical analyses are the means of three replicates with standard deviation below 5%.

	CPH	CBS
Cellulose	22.32	12.30
Hemicellulose	10.10	10.07
Acid Detergent Lignin (ADL)	21.15	14.80
Neutral Detergent Fiber (NDF)	53.85	37.91
Acid Detergent Fiber (ADF)	43.75	27.85
Acid Insoluble Ash (AIA)	0.28	0.66
Protein	4.79	17.98
Ashes	10.41	7.28
Lipids	0.35	16.24

The CPH here studied contained low amounts of lipids (0.35%), while other authors reported contents reaching values of 1.5–2.34%.

A high ash content (10.41% and 7.28%) was detected in both residues. Other authors reported that the ash content could be referred as mineral content in some foods [46], suggesting an additional possible utilization of these residues as a mineral boost. Regarding other components, the moisture (8.16% for the CPH) and protein (4.79% for the CPH) contents are in agreement with previous reports [46,47]. The total fiber, measured by the NDF method, showed values of 53.85 and 37.91%, for the CPH and CBS, respectively. Martinez et al. [47] account total dietary fiber (TDF) values for cocoa pods in congruence with this study (55.99–56.10%). Vrismann et al. [8] reported a lower TDF for cocoa pods (36.6%), and lignin values similar to those found in this study (21.4%). Regarding the CBS, the NDF, ADF, and ADL values are similar to those found by Campione et al. [48].

In conclusion, although the chemical composition of the cocoa wastes can vary according to the plant varieties, growing conditions, soil, and type of material, the results here obtained were comparable to those shown in previous studies [7,38,49].

Regarding protein, the values here found are in the range presented by other authors (15.59–20.9%) [46,50,51] with the highest content in the CBS (18%), which could therefore represent a potential cheap source of sustainable vegetal protein.

Results concerning amino acids and fatty acid profiles are reported in Tables S1 and S2, respectively. Table S1 indicates that, compared to the CBS, CPH has a lower content of acidic (aspartic and glutamic acid) and basic (arginine and lysine) amino acids. Proline and valine are more present in the CBS as well as other aromatic amino acids (phenylalanine, tyrosine, histidine, and tryptophan). These results are consistent with those of other authors that found aspartic and glutamic acids as predominant in the CPH [52,53]. The CBS contains higher quantities of essential amino acids than the CPH with leucine as the most concentrated, reaching values of 243.17 and 870.03 mg/100 g for the CPH and CBS, respectively.

Regarding the lipid component (Table S2), the predominant fatty acids are palmitic, stearic, oleic, and linoleic acids in both residues. The saturated fatty acid (myristic, palmitic,

and stearic) content is similar in both the CPH and CBS. Regarding unsaturated fatty acids, the CBS has a higher content of oleic, while the CPH contains more linoleic acid. These results are similar to those of the literature [50,54,55], which reported that the main fatty acids found in cocoa bean shells were oleic, stearic, and palmitic acids. Further studies showed that linoleic acid was the predominant component of the oil from cocoa pods [56] or in the CPH powder extract [57].

3.2. Quantification and Identification of Total Phenolic Compounds (TPC) and Antioxidant Activity

The amounts of TPC in the ethanolic extracts of the CPH and CBS are shown in Table 2. According to various authors, the TPC extracted from the CPH with different solvents and conditions range between 2.07 and 107.3 mg GAE/g [58–62]. The values found in the present study are within the mentioned ranges and are not significantly different ($p > 0.05$) between the CPH and CBS (10.08 ± 1.40 mg GA/g and 13.04 ± 0.10 mg GA/g, respectively).

Table 2. Total phenolic compounds (mg GA/g) and antioxidant activity (mg TE/g) from the CPH and CBS. Mean values (n = 3) and standard deviations are presented. Different letters within the same column indicate significant differences, according to ANOVA (one-way) and the Duncan test ($p < 0.05$).

Residue	TPC (mg GA/g)	Antioxidant Activity (mg TE/g)
CPH	10.08 ± 1.40 [a]	9.93 ± 0.38 [b]
CBS	13.04 ± 0.10 [a]	16.24 ± 0.61 [a]

TPC: total phenolic compounds, GA: gallic acid, TE: Trolox.

However, higher or lower values were recorded by several previous studies, clearly affected by cultivation area, cocoa variety, postharvest processes, extraction methods, used solvent, etc., [46,58,60,63,64]. For example, Sotelo et al. [61] extracted significantly ($p < 0.05$) more phenols using ultrasound (23.0 ± 0.9 mg GAE/g) in comparison to a conventional method (16.4 ± 0.41 mg GAE/g).

Table 3 shows the phenolic compounds identified in the CPH and CBS by HPLC. In this study, the main detected polyphenols in both substrates were pyrogallol (72.567 mg/L) and vanillic acid (7.207 mg/L). p-hydroxybenzoic acid (2.467 mg/L) and syringic acid (0.765 mg/L) were found only in the CPH. The Antioxidant activity (AOA) was 9.93 ± 0.38 and 16.24 ± 0.61 mg TE/g for the CPH and CBS, respectively (Table 4). However, the concentration of the solvent may deeply affect the results [59,65]. In this work, 70% ethanol was used as a solvent and high concentrations of pyrogallol were found in the CBS (164.474 mg/L) and in CPH (72.567 mg/L). The higher antioxidant capacity of the CBS could be therefore attributed to this molecule.

Table 3. Phenolic compounds characterization of the CBS and CPH by HPLC. The data are expressed in mg/L.

Compound	CBS	CPH
Pyrogallol	164.47	72.57
Syringic acid	8.59	0.77
p-hydroxybenzoic acid	-	2.47
Vanillic acid	-	7.21

Table 4. Induction time (IT) and Antioxidant Activity index (AAI) of the CPH and CBS. Mean values (n = 3) and standard deviations are presented. Different letters within the same column indicate significant differences. according to ANOVA (one-way) and the Duncan test ($p < 0.05$). IT refers to the time (h) at the break point of the two extrapolated straight parts of the curve obtained by Rancimat apparatus. AAI = IT of corn oil with antioxidant/IT of soybean oil (control).

Sample	IT (hours)	AAI (mg TE/g)
CPH	9.46 ± 0.04 [a]	1.48
CPS	8.45 ± 0.10 [b]	1.32
Control (soybean oil)	6.40 ± 0.06 [c]	1.00

3.3. Oxidative Stability

As reported in Table 4, the oxidative stability of soybean oil supplemented with the ethanolic extracts of the CPH and CBS was evaluated by measuring the induction time (IT) and the antioxidant activity index (AAI). The IT of the cocoa pod's extract (9.46 ± 0.04 h) was significantly longer than the cocoa shell's extract (8.45 ± 0.10 h), both being significantly longer than the control, consisting of soybean oil without supplementation (6.40 ± 0.06 h).

Indeed, according to the AIA values, the CPH achieved an increase of 48% and the cocoa shell of 32%, as compared to the control ($p < 0.05$).

Similarly, Boungo Teboukeu et al. [65] found that the CPH phenolic extract was effective in delaying the oxidation of palm oil during heating at 180 °C and reported a maximum value of AAI of 1.20 when the extract was added at 200 ppm. Other similar results were obtained with ethanolic extracts from by-products such as rapeseed [35] and red chicory powder [66] on soybean oil or grape pomace powder on corn oil [67].

These findings further support that the exploitation of raw materials of residual origin as sources of cheap and renewable proteins and antioxidants plays an essential role in the emerging eco-products and healthy ingredients market. Although additional research is necessary to determine the optimal conditions for an efficient extraction of antioxidant compounds from these by-products, the ultrasound application resulted to be a green solution for the environmentally friendly recovery of these molecules.

3.4. Pretreatment of Cocoa Residues

Based on the analyses reported in Table 1, the amount of glucose that could be released from the hydrolysis of cellulose in the CBS would be too low to support microbial growth. Therefore, pretreatment trials were conducted exclusively on the CPH.

To better expose the feedstock cellulose to the successive enzymatic hydrolysis, two pretreatments of the CPH were attempted: increasing amounts of hydrogen peroxide or 4% *w*/*v* NaOH. After pretreatments, samples were enzymatically saccharified with Cellic® CTec2 and the released glucose was then determined. This hexose is one of the preferred carbon sources for both the microorganisms used in this study (*C. necator* and *S. cerevisiae*) and both microbes cannot use pentoses.

3.4.1. Hydrogen Peroxide (H_2O_2) Pretreatment of CPH

For the reason discussed above, increasing amounts of H_2O_2 were applied to maximize the release of glucose. The largest amount of this sugar (6.5 g/L) was obtained with 7.5% hydrogen peroxide and 24 h of incubation, with a percentage of saccharification of 21.27 ± 0.29% (Figure 1). A lower concentration of H_2O_2 resulted in quantities of released sugar only slightly above those obtained from the non-treated samples. A kinetic model was developed to describe the dynamics of glucose release after treatment with H_2O_2 and the maximum yield for glucose was 43.49% [68]. However, the H_2O_2 pretreatment conditions were very different; these authors operated in a reactor at 150 °C for 6 h while, in this work, H_2O_2 incubations were conducted at 55 °C for 4 h in a thermal bath. High temperatures seem to favor the release of sugars from the CPH and additional studies would

be necessary to determine whether in harsh conditions inhibiting substances that may affect subsequent fermentation processes are generated. Indeed, recent studies reported that high temperature pretreatments of lignocellulose can generate furfurals and 5-hydroxymethylfurfural, which are inhibitory compounds for a variety of microorganisms [69]. In addition, high temperatures for extended periods could be considered uneconomical and not environmentally friendly. Thus, although raising H_2O_2 resulted in increasing, but still low, amounts of enzymatically released glucose, the H_2O_2 pretreatment was not considered suitable for a subsequent sustainable biotechnological use of CPHs as feedstocks.

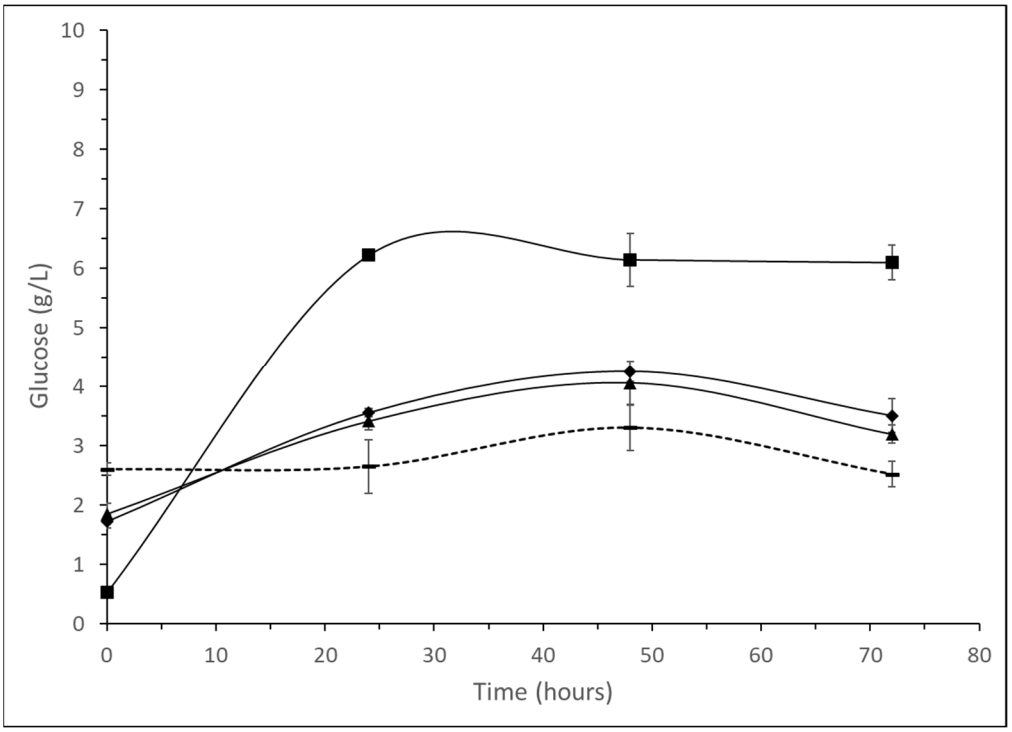

Figure 1. Effect of H_2O_2 pretreatments of CPH on the release of glucose after enzymatic hydrolysis with Cellic® CTec2. H_2O_2 2.5% *v*/*w* (▲). H_2O_2 5.0% *v*/*w* (♦). H_2O_2 7.5% *v*/*w* (■). No H_2O_2 added (-). Mean values (n = 3) and standard deviations are presented.

3.4.2. Alkaline (NaOH) Pretreatment of CPH

Alkaline pretreatment has been demonstrated as suitable to solubilize lignin and, partially, the hemicellulose, and it is traditionally used in pulp processing. This application increases the internal surface of cellulose, contributes to reduce its crystallinity, and, thus, makes the polysaccharide more accessible to further enzymatic attack by cellulases [70]. When the CPH was pretreated with NaOH and the subsequent enzymatic hydrolysis was applied, a higher amount of glucose (15.52 ± 0.78 g/L) was found after 72 h of saccharification; this value corresponds to a degree of saccharification of 62.26%. Non-pretreated samples did not show a spontaneous glucose release (Figure 2). Moreover, comparing the yields with those obtained on H_2O_2 pretreated substrates, alkaline pretreatment revealed to be more effective in making cellulose more accessible to further enzymatic attack by cellulases.

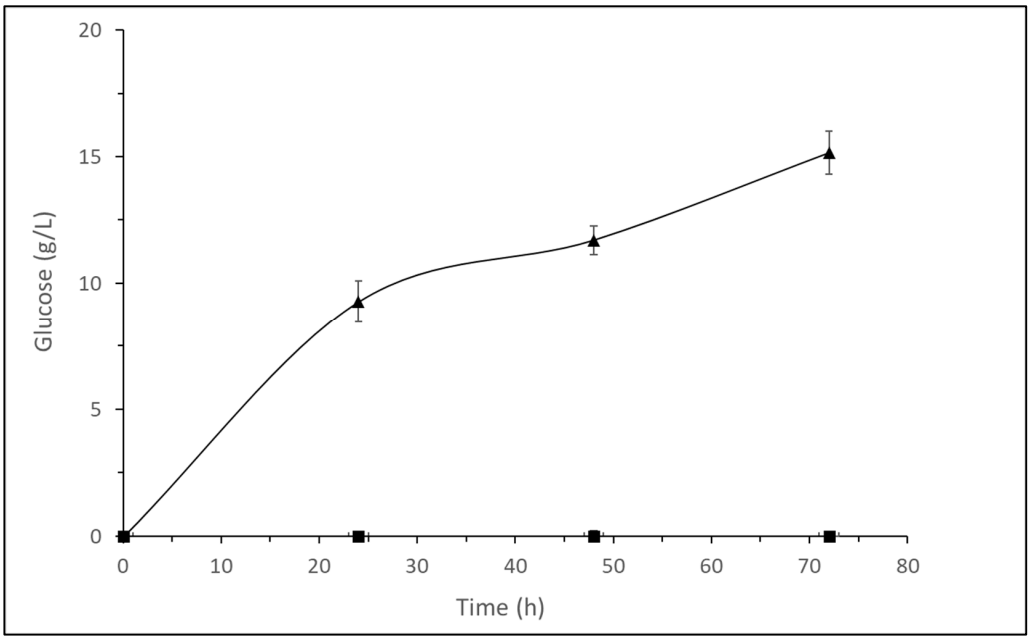

Figure 2. Effect of NaOH pretreatments of the CPH on the release of glucose during enzymatic hydrolysis with Cellic® CTec2. NaOH 4% *v*/*w* pretreated (▲). No NaOH added (■). Mean values (n = 3) and standard deviations are presented.

These results are partially in agreement with those of other authors that used the harsher NaOH autoclave-assisted hydrolysis. For example, with this pretreatment, Sarmiento-Vasquez et al [71] found a higher maximum concentration of 60.5 g/L of glucose and a yield of 275 mg glucose/g of CPH. Hernández-Mendoza et al. [38] obtained a syrup with 66.80 g/L of the reducing sugars.

Other authors used acidic hydrolysis on cocoa residual biomass but with lower results. For example, Shet et al. [72] carried out an acidic optimized pretreatment with HCl 3.6 M), reporting an initial sugar concentration of only 4.09 g/L.

Overall, in this work, around 160 g of glucose was recovered per g of CPH. Non-pretreated samples did not show spontaneous glucose release (Figure 2), thus confirming that a mild alkaline pretreatment of the CPH is effective in making cellulose more accessible to further enzymatic attack by cellulases.

3.5. PHAs and Bioethanol Production from Alkaline Pretreated Saccharified CPH (Ahs-CPH)

3.5.1. PHAs

C. necator DSM 545 growth and PHAs accumulation were assessed on media containing Ahs-CPH with or without MM salts (Figure 3). Reference growths were performed with amounts of glucose comparable with those contained in the Ahs-CPH. Although pure CPH saccharified hydrolysate contains around 15 g/L glucose (Figure 2), it poorly sustains the development of *C. necator*. On the other hand, the amendment of the hydrolyzate with MM salts resulted in bacterial growth similar to that obtained with MM + 15 g/L glucose, indicating that the hydrolyzate is lacking the essential nutrients contained in MM.

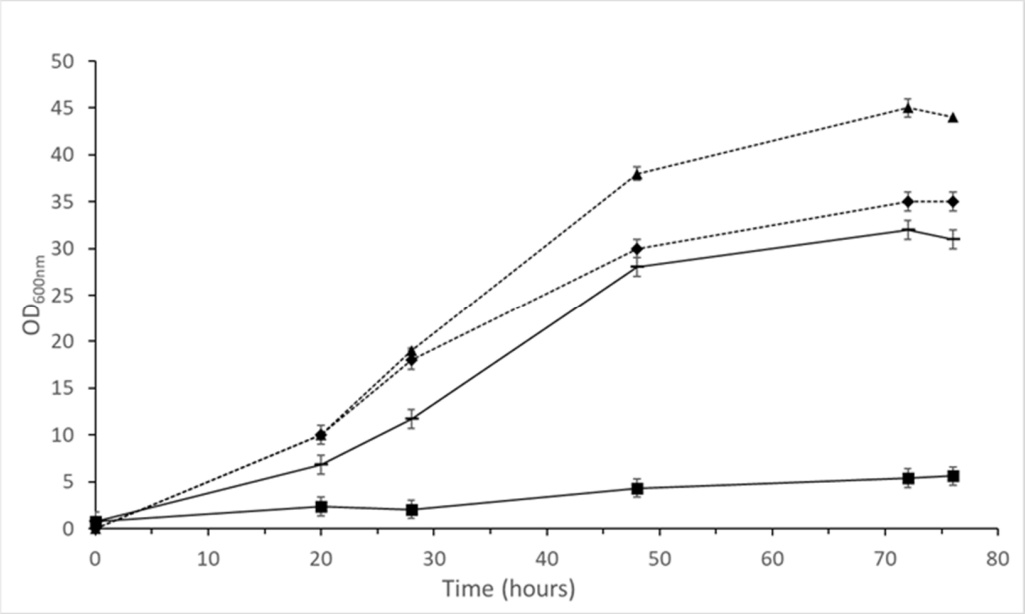

Figure 3. Growth of *C. necator* DSM 545 on media containing Ahs-CPH (solid line) or glucose (dashed line) as a carbon source. MM + 20 g/L glucose (▲). MM + 15 g/L glucose (♦). Ahs-CPH (■). Ahs-CPH + MM salts (-). Mean values (n = 3) and standard deviations are presented.

After 76 h, the culture broths were centrifuged, the pellets collected, and the PHAs content determined (Table 5). As previously reported [73], the PHAs accumulated by *C. necator* DSM 545 on glucose ranged from 72.00 to 74.60% of CDM, confirming the ability of this strain to efficiently accumulate PHAs from this monosaccharide. On Ahs-CPH, the PHAs reached 51.30% of CDM. When the MM salts were supplemented to Ahs-CPH, the percentage of the PHAs increased to 58.60% showing once again that essential nutrients were not present in the substrate.

Table 5. PHAs accumulation of *C. necator* DSM 545 on Ahs-CPH, MM + Ahs-CPH, MM + 15 g/L glucose, and MM + 20 g/L glucose after 76 h incubation. Mean values (n = 3) and standard deviations are presented.

Medium	PHAs (% CDM)
Ahs-CPH	51.30 ± 2.83
MM+ Ahs-CPH	58.60 ± 4.95
MM+ glucose 15 g/L	72.00 ± 1.00
MM + glucose 20 g/L	74.60 ± 0.28

CDM: cell dry matter.

Though studies specifically focused on PHAs production from the CPH are not available in the literature, thus limiting the discussion, the PHAs accumulated by *C. necator* on the Ahs-CPH are comparable with those obtained from other agro-industrial substrates and microorganisms.

For example, Brojanigo et al. [39] reported PHB values up to 44% of CDM using *C. necator* DSM 545 and enzyme-treated broken rice and, for the first time, reported on the Consolidated Bioprocessing of PHB from broken rice (43% CDM) and purple sweet potato waste (36% CDM) [74] by using a specifically engineered *C. necator* DSM 545 strain. *C. necator* H16 was screened for PHAs production from bagasse hydrolyzate and wheat bran hydrolyzate by Brodin et al. and Annamalai & Sivakumar, who found PHAs contents of 54 and 66% of CDM, respectively [75,76]. De Souza et al. [77] found 57.8% PHAs accumulated by *Bacillus megaterium* using pretreated corn husk hydrolyzate as a carbon source. Up to

62% PHAs of CDM produced by *Burkholderia cepacia* from sugarcane bagasse hydrolyzate were also reported [78].

Although further studies are required to optimize the processes, CPH could be considered a promising substrate for the growth of and PHAs synthesis by *C. necator*.

3.5.2. Bioethanol

To test the possible exploitation of CPHs for bioethanol production, the growth and ethanol performances by *S. cerevisiae* Fm17 were assessed for 72 h in a medium with Ahs-CPH (Figure 4A). A glucose (15 g/L) broth was included as benchmark.

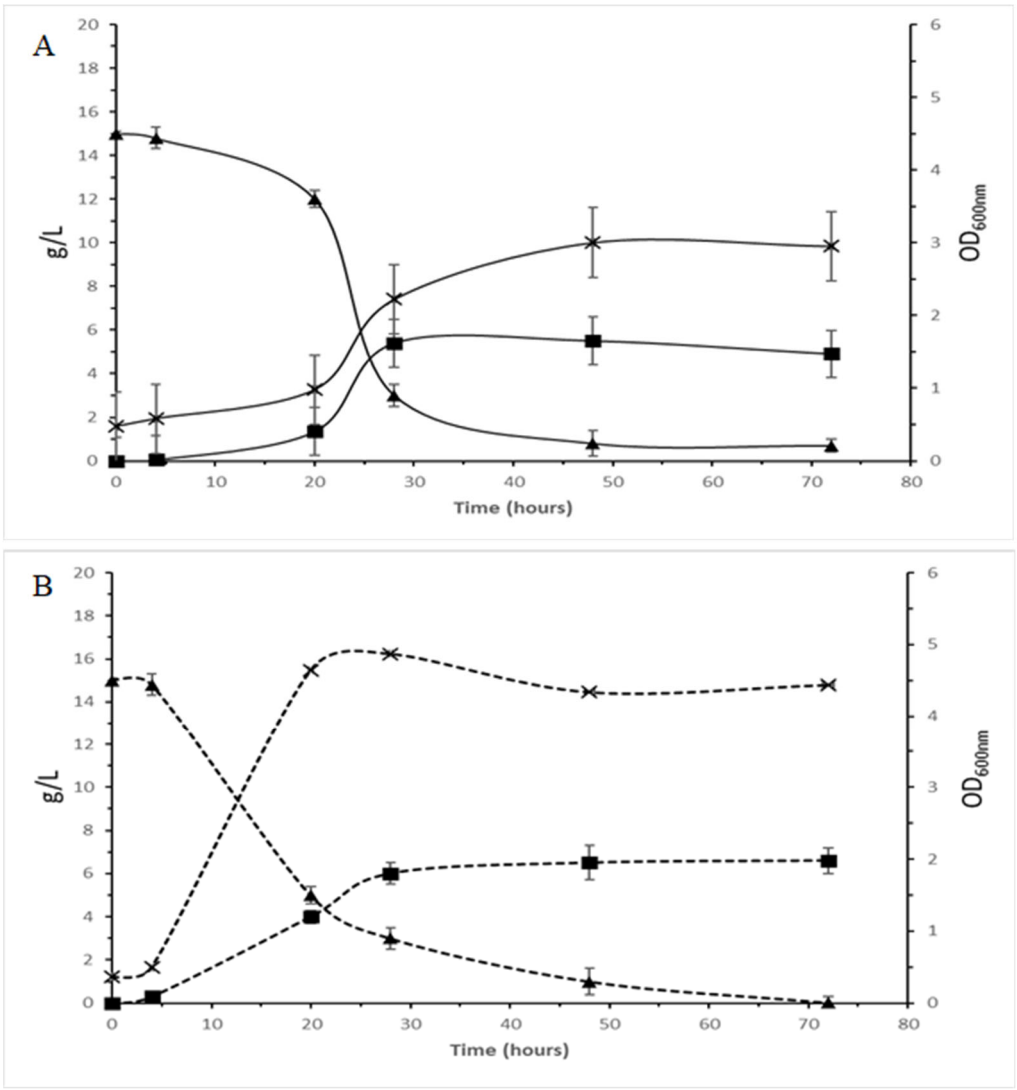

Figure 4. Growth (X), glucose consumption (▲) and ethanol production (■) by *S. cerevisiae* FM17 in Ahs-CPH ((**A**) solid line) and glucose benchmark broth ((**B**) dashed line). Results are the means of three replicates and the standard deviations are reported.

Ethanol production started within the first twenty hours of fermentation in both media. With Ahs-CPH, *S. cerevisiae* Fm17 metabolized 95.7% of released glucose (Figure 4A, Table 6 within the first 50 h of incubation; the ethanol concentration increased, reaching a steady state approximately 30–40 h after inoculation, with a maximum value of 5.50 g/L at 48 h.

Table 6. Consumed sugars. Ethanol and ethanol yields by *S. cerevisiae* Fm17 in glucose and Ahs-CPH. $Y_{EtOH/S}$ is the ethanol yield per gram of consumed substrate calculated on the highest ethanol production. Results are the means of three replicates and, when relevant, the standard deviations are reported.

Parameter	Growth on Glucose	Growth on Ahs-CPH
Sugars concentration (g/L)	15.00 ± 0.08	15.15 ± 0.85
Consumed sugars (%)	100.0	95.3
Highest Ethanol (g/L)	6.60 ± 0.60	5.50 ± 0.30
$Y_{EtOH/S}$	0.44	0.38
% theoretical yield	86	74

In pure glucose with a concentration similar to the amount used in fermentation with Ahs-CPH, the ethanol level (6.00 g/L) was slightly higher than that of Ahs-CPH (5.50 g/L) (Figure 4B and Table 6), probably due to the presence of inhibitors or to the lack of some nutrients in the hydrolyzate. While with pure glucose 100% of sugars were consumed by yeast, with the hydrolyzate, a small amount of sugar remained in the exhausted broth. The maximum ethanol yield (% of the theoretical) and the ethanol yield/consumed carbon (g ethanol/g glucose) obtained with the hydrolyzate were 74% and 0.38 g/g respectively, which is slightly lower if compared with those obtained with pure glucose (86% and 0.44 g/g) (Table 6).

Other authors found a bioethanol yield of 13.66 g/L by *S. cerevisiae* but using a CPH hydrolysate by 1 M HCl, representing a bioconversion efficiency of 87% at 26 h of fermentation [31].

In this work, the pretreatment was performed with 100 g of CPHs per liter and, after saccharification and fermentation, 5.5 g/L of ethanol was obtained. As such, the yield of bioethanol per unit of feedstock should be 0.055 g of ethanol per g of CPH.

Similar results were obtained by Valladares-Diestra et al. [13] using a CPH's hydrothermal pretreatment assisted with citric acid. This author reports an overall yield of 0.07 g of ethanol per g of CPH, but only 0.042 g of ethanol was raised by *S. cerevisiae*.

In optimized conditions, Hernandez-Mendoza et al. [38] obtained from CPHs 18.06 g/L of ethanol, but after a harsher treatment with 5% NaOH and 30 min at 120 °C in autoclave.

Cocoa by-products are generated in large amounts and theoretically could be used as a potential substrate for bioethanol production. The synthesis of bioethanol from CPHs via direct fermentation using *Zymomonas mobilis* was successfully obtained by Yogaswara et al. [79], but with a low value of conversion and a small maximum reaction velocity. The microbial production of bioethanol from lignocellulosic cocoa residues is in fact hardened by the accessibility of fermentable sugars contained in the recalcitrant structure of cellulose and hemicellulose polymers. Thus, pretreatments aimed to facilitate the enzymatic saccharification of biomass are necessary [5]. The acid pretreatment of CPH is easy, cheap, and efficient, but generates fermentation inhibitors such as furfural and hydroxymethylfurfural, which are toxic to the metabolism of fermentative microorganisms [73]. On the contrary, the use of alkali on CPHs in mild conditions, like those used in this work, seems to generate glucose, minimally affecting yeast fermentation. Nevertheless, the glucose concentrations obtained in this study should be considered as a proof-of-concept and need specific investigations dealing with substrate loadings, NaOH concentrations, mixing, as well as enzyme dosage optimization. As such, ethanol levels would become more and more profitable with concentrations higher than 4% (*v*/*v*) [80].

4. Conclusions

This paper demonstrated for the first time that both the CPH and CBS can be efficiently converted into a cluster of valuable products. This biorefinery approach, indeed, resulted in the valorization of cocoa by-products into bioactive compounds, such as phenolic compounds with promising industrial traits, as well as PHAs and bioethanol. Ultrasound-assisted extracts from the CPH and CBS showed a relevant antioxidant ability on the

soybean oil here used as a model substrate; this activity is probably due to pyrogallol revealed by the chemical analyses. Furthermore, a mild alkaline pretreatment with 4% NaOH and the subsequent enzymatic saccharification of the CPH and CBS resulted in the release of glucose to support the synthesis of ethanol and PHAs by *S. cerevisiae* Fm17 and *C. necator* DSM 545, respectively.

Further insights are needed to fully exploit both tested waste streams and will deal with up-scaling, the optimization of process parameters, as well as techno-economical analyses. This perspective will be of great impact to boost bioeconomy applications also in developing countries.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9090843/s1, Table S1. Total amino acid profile of CBS and CPH (mg/100 g DM). Results of chemical analyses are the means of three replicates with standard deviation below 5%. Table S2. Fatty acid profile of cocoa pod and cocoa shell (mg/100 g DM) Results are the means of three replicates with standard deviation below 5%.

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