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Cocoa Pod Husk Valorization Through *Rhizopus stolonifer* Solid-State Fermentation: Enhancement in Antioxidant Activity

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Abstract: Cocoa pod husk (CPH), a significant agricultural byproduct of cocoa production, presents an opportunity for sustainable valorization through biotechnological methods. This study aimed to enhance the nutritional, antioxidant, and therapeutic properties of CPH using solid-state fermentation (SSF) with *Rhizopus stolonifer*. Physicochemical characterization confirmed CPH's suitability for SSF, providing a nutrient-rich and favorable environment for fungal growth. The fermentation process significantly improved protein recovery (11.327 ± 0.859 mg g^{-1}) and antioxidant activity, with ORAC (51.68 ± 0.35 mmol TE g^{-1}) and DPPH (7.09 ± 0.05 µmol TE g^{-1}) assays demonstrating marked increases in redox potential, particularly at 144 h and 96 h of fermentation, respectively. GC-MS analysis revealed the generation of bioactive compounds in fermented CPH (CPHF), including methyl 3-hydroxybutyrate, 10,12-Tricosadiynoic acid, and palmitic acid, which are known for their antioxidant, anti-inflammatory, and therapeutic properties. Additionally, phenolic compounds are biotransformed into more bioavailable forms, further enhancing the functional value of the material. This work demonstrates that SSF can effectively transform CPH from an agricultural waste product into a high-value biomaterial with potential applications in functional food, nutraceutical, and pharmaceutical industries. By addressing waste management challenges and promoting the development of innovative bio-based products, this study highlights the promising role of SSF in advancing sustainable and circular biotechnological solutions.

Keywords: cocoa pod husk; solid-state fermentation; sustainable biotechnology; food microbiology

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1. Introduction

Cocoa is an essential commodity in the world economic scenario, with a global gross production in 2023/2024 year (October–September) of almost 4.5 million tons [1] and an economic driving force for the major producing countries, such as Brazil, where processors handled over 220 thousand tons in 2023 and over 58 thousand tons in the first semester of 2024 [2]. However, the highly valuable cocoa beans used primarily in the chocolate industry constitute only 10% of the cocoa fruit's dry weight [3]. The remaining lignocellulosic constituents (e.g., cocoa pod husk and bean shell) are systematically generated at a 10:1 ratio and contribute significantly to the approximately 220 billion tons of residual biomass generated annually worldwide [4].

The cocoa pod husk (CPH) is a thick, oval-shaped external layer of the fruit. It is characterized by its constitution rich in non-starch polysaccharides (cellulose, hemicelluloses, and pectin), terpenoids, flavonoids, phenolic, and carboxylic acids (protocatechuic, salicylic, citric, and tartaric acids) [5]. Even though CPH is generally used as a fertilizer due to its high mineral and organic content, the incorrect disposal of this residual biomass presents an environmental concern. CPH may act as a reservoir of phytopathogens, such as *Phytophthora* sp., which are responsible for black pod rot disease [3,6].

In this sense, the biotechnological exploitation of CPH could improve its composition quality, support its valorization, and reduce its environmental impact [6]. The microbiological process of solid-state fermentation (SSF) offers a tangible alternative through the use of lignocellulosic wastes as support/substrate for the production of value-added products at feasible costs, showing significant advances in crucial fields, such as biofuels, enzymes, antibiotics, and organic acids production [7,8].

Fungi of the genus *Rhizopus* are generally saprophytes that colonize decaying or dead organic materials, such as leaves, husks, and soil. The most well-described member of the *Rhizopus* genus is *Rhizopus oryzae*, which is used to produce tempeh, a dish made from fermented soybeans. These microorganisms are instrumental in transforming agricultural and industrial residues into valuable bioproducts. *Rhizopus stolonifer*, commonly known as black bread mold, has extensive enzymatic capabilities, making it a promising candidate for biotechnological applications [9].

This study explores the potential of utilizing *Rhizopus stolonifer* to enrich CPH antioxidant properties and enhance its bioactive composition. By doing so, we seek to obtain a value-added product from this agricultural residue, contributing to its sustainable use and nutritional enhancement as a potential supplement to animal diets.

2. Materials and Methods

2.1. Cocoa Pod Husk Preparation

Cocoa fruits (*Theobroma cacao*, Forastero variety) were obtained in partnership with the Federal University of Pará (SisGen A4Ec257) from the Konagano cocoa farm (02°25′08″ S; 48°09′08″ W), located in the state of Pará, in the northern region of Brazil. Previously, the fruits were gently washed with distilled water to remove any debris after harvesting and shipment. CPHs were separated from the fruit pulp and dried in an oven with air circulation at 60 °C for 24 h. Subsequently, CPH was fragmented into smaller parts, ground in a knife mill, and separated into fractions using sieve sets ASTM No. 10 and No. 20, obtaining particles with sizes between 0.84–2.0 mm used in this study.

2.2. Physicochemical Characterization

Total and reducing sugars were determined using the 2-hydroxy-3,5-dinitrobenzooic acid reaction (DNS) and a glucose standard curve (R^2 = 0.9972) [10]. The Bradford method was used to determine the protein concentration using a bovine serum albumin (BSA) standard curve (R^2 = 0.9911) [11]. The presence of ions was determined using Ion Chromatography 761 Compact IC (Metrohm AG, Herisau, Switzerland). Cations were analyzed with a Metrosep C3–250/4.0 column maintained at 40 °C, using a mobile phase of 3.5 mM HNO_3 at a 0.9 mL min^{-1} flow rate. Anions were analyzed using a Metrosep A Supp 5–250/4.0 column at room temperature, with a mobile phase of 1 M sodium bicarbonate and 3.2 M sodium carbonate at a flow rate of 0.7 mL min^{-1}. AquaLab CX-2 (Decagon Devices, Inc., Pullman, WA, USA) was used to measure the water activity (Aw). The pH was measured in a mixture obtained by homogenizing 1 g of the sample in 10 mL of distilled water using a pH meter mPA 210 (MS Tecnopon, Piracicaba, Brazil). Ash content was determined following the method proposed by the National Renewable Energy Labo-

ratory [12], while humidity was analyzed using the methodology outlined by the Adolfo Lutz Institute [13].

2.3. Solid-State Fermentation

Rhizopus stolonifer NRRL 28169 was acquired from the National Center for Agricultural Utilization Research (NRRL, Peoria, IL, USA) and maintained in the Department of Bioprocess Engineering and Biotechnology of the Federal University of Paraná. The strains were inoculated in medium Potato Dextrose Agar (PDA) for five days at 30 °C in Erlenmeyer flasks. Spores were harvested by submerging the culture in sterile distilled water containing 0.1% Tween 20. Spore concentrations were determined using a Neubauer chamber.

The physical conditions of SSF were based on the natural needs of the *Rhizopus* strains [14]. Erlenmeyer flasks containing 5 g of autoclaved CPH were inoculated with 5 mL of a spore suspension at a concentration of 10^7 spores/mL. The initial pH of the CPH was 5.5 ± 0.1, with no adjustment. The moisture content was adjusted to 50% using distilled water, and the flasks were incubated and maintained at 30 °C.

2.4. Extraction Methods

In this study, two different extraction methods were utilized to obtain different fractions of compounds. An aqueous ethanol extract (EAE) was obtained by mixing 5 g of sample from each flask in 50 mL of a 50% ethanol/DI water solution and agitated for one hour at 25 °C and alongside a method previously proposed by Conet et al. (2003) [15] and modified by Gonçalves et al. (2016) [16], where an acidified acetone (AAE) solution composed of acetone, water, and acetic acid (70:29.5:0.5 v/v) was used in three continuous steps on 5 g of sample in a total of 50 mL. After extraction, the suspension was filtered on the Whatman paper. All solvents from both extraction methods were evaporated in a vacuum kiln at 40 °C, and the samples were resuspended in ultrapure water for analyses.

2.5. Total Phenolic Content

Total phenolic content (TPC) was measured using the Folin-Ciocalteu method by Zheng and Wang (2001) [17], modified by Gouveia and Castilho (2011) [18]. The samples were dissolved in methanol to achieve a concentration (w/v) of 10 mg/mL. Aliquots of 50 µL were mixed with 1.25 mL of Folin-Ciocalteu reagent (diluted 1:10) and 1 mL of 7.5% sodium carbonate solution. After 30 min at room temperature, the decrease in absorbance was measured at 765 nm using a spectrophotometer. The results were quantified using a gallic acid standard curve (R^2 = 0.9994) and expressed in gallic acid equivalent (GAE) per gram of sample (mg GAE g^{-1} sample).

2.6. Oxygen Radical Absorbance Capacity Assay and TEAC Calculation

The oxygen radical absorbance capacity (ORAC) was determined as described by Zulueta et al. (2009) [19]. The reaction was allowed to proceed for 30 min at 37 °C, and the absorbance was measured every minute to generate a decay curve of the absorbance value. The obtained values were then related to the Trolox standard curve, providing an ORAC result in µmol Trolox equivalence. The following equation was used for the calculations:

$$ORAC\ (\mu mol\ TE)\frac{C \times k \times (AUCs - AUCb)}{(AUCt - AUCb)} \quad (1)$$

where C is the Trolox concentration (50 µM), K is the sample dilution factor, AUC is the area below the fluorescence decay curve of the sample ($AUCs$), blank ($AUCb$), and Trolox ($AUCt$) that is calculated by the following equation:

$$AUC = \left(0.5 + \frac{f5}{f0} + \frac{f10}{f0} \ldots + fn + \frac{5}{f0}\right) \times 5 \quad (2)$$

where $f0$ is the initial fluorescence and fn is the fluorescence at time n.

2.7. DPPH Radical Scavenging Activity and TEAC Calculation

The radical scavenging activity (RSA) of the extracts was measured according to the methodology presented by [20] with modifications. The reaction mixture consisted of 100 µL of sample (diluted to 1:20 w/v) added to 1.4 mL of DPPH methanol solution (100 µM). Absorbance was reset with distilled water and then read at 517 nm after 30 min of reaction using a UV-VIS spectrophotometer. The following equation was used to determine the scavenging activities of the samples:

$$RSA\ (\%) = \left(\frac{\left[Abs_{control} - Abs_{sample}\right]}{Abs_{control}}\right) \times 100$$

where $Abs_{control}$ is the absorbance of the sample eluent in DPPH solution. Abs_{sample} is the absorbance of the sample in DPPH solution after 30 min of reaction.

A Trolox standard curve was prepared to estimate the sample's Trolox equivalent antioxidant capacity (TEAC). Known concentrations of Trolox were subjected to the DPPH assay under identical conditions. The RSA values of Trolox were plotted against their concentrations to generate a standard curve (R^2 = 0.9967).

2.8. Volatile Compounds Determination by GC-MS

The analysis of volatile compounds from the CPH and CPHF was performed using a headspace vial coupled to a solid phase microextraction (SPME) fiber (5% Carboxen [CARB]/95% Polydimethylsiloxane [PDMS], df 75 µm, partially cross-linked, Supelco, St. Louis, MI, USA). For each determination, 1 g of the sample was stored in a 20 mL HS vial. The flask was heated at 70 °C for 10 min without shaking, followed by 15 min of fiber exposure in the COMBI-PAL system to balance the volume in the vial. The compounds adsorbed by the fiber were desorbed into the gas chromatograph injection system gas phase (CGMS-gun TQ Series 8040 and 2010 Plus GC-MS Shimadzu, Tokyo, Japan) at 250 °C. The compounds were separated on a column containing 95% PDMS and 5% phenyl (30 m × 0.25 mm, 0.25 µm film thickness). The GC was equipped with an HP 5972 mass selective detector (Hewlett Packard Enterprise, Palo Alto, CA, USA). Helium was used as the carrier gas at a 1.0 mL/min rate. Mass spectra were obtained by electron impact at 70 eV. The compounds were identified by comparing the mass spectra those with in the library database (Nist'98 and Wiley7n) [21]. The same procedure was followed to perform the GC-MS analysis of the CPH and CPHF extracts (EAE and AAE). The extracts were completely dried and resuspended in ethanol P.A. HPLC grade. For each determination, 1 mL of the sample extract was stored in a 20 mL HS vial. The flask was heated at 50 °C for 10 min without shaking, followed by 15 min of fiber exposure in the COMBI-PAL system to balance the volume in the vial. The fiber exposure was reduced to 50 °C due to the ethanol boiling temperature (78 °C) to avoid fiber saturation.

2.9. Statistical Analysis

All measurements were performed in triplicate, and the data are presented as mean ± standard error of the mean. Statistical analysis was performed using two-way analysis of variance (ANOVA) to evaluate the effects of extraction method (EAE vs. AAE) and time on the DPPH values. Tukey's Honestly Significant Difference (HSD) test was used for

post-hoc comparisons to identify specific differences between the groups. The differences between the EAE and AAE methods at each time point were assessed using Student's *t*-test. All statistical tests were conducted at a significance level of $\alpha = 0.05$. Differences were considered statistically significant at $p < 0.05$. The analysis was performed using GraphPad Prism version 9.0.

3. Results

3.1. CPH Characterization

The physicochemical analysis of cocoa pod husk (Table 1) revealed a slightly acidic pH (6.18 ± 0.01), low water activity (0.283 ± 0.001), and minimal moisture content (3.4 ± 0.003%), indicating its stability and suitability for solid-state fermentation. CPH exhibited high nutritional potential, with notable levels of protein (18.30 ± 0.51 mg g^{-1}), total sugars (17.347 ± 0.26 mg g^{-1}), and reducing sugars (11.67 ± 0.15 mg g^{-1}), which provide essential substrates for microbial metabolism. Its mineral composition, including sulfate (0.649 ± 0.029 mg g^{-1}), phosphate (0.335 ± 0.010 mg g^{-1}), and magnesium (0.440 ± 0.021 mg g^{-1}) supports enzymatic activity and fungal growth. These results suggest that CPH is a nutritionally rich and stable substrate, making it an effective material for fermentation with *Rhizopus stolonifer* to enhance its functionality and nutrition.

Table 1. Physicochemical composition of CPH.

Analysis	Value in CPH
pH	6.18 ± 0.01
A_w	0.283 ± 0.001
Ashes Content (%)	8.2 ± 0.04
Humidity (%)	3.4 ± 0.003
	Value (mg g^{-1})
Total sugar	17.347 ± 0.26
Reducing sugar	11.67 ± 0.15
Protein	18.30 ± 0.51
F^-	0.114 ± 0.009
Cl^-	0.318 ± 0.015
Br^-	0.198 ± 0.008
$NO_2{}^-$	0.359 ± 0.005
$SO_4{}^{-2}$	0.649 ± 0.029
$PO_4{}^{-3}$	0.335 ± 0.010
Na^+	0.022 ± 0.003
$NH_4{}^+$	0.080 ± 0.010
K^+	0.200 ± 0.006
Mg^+	0.440 ± 0.021
Ca^+	0.032 ± 0.009

3.2. SSF Kinetics

CPH soluble protein content (18.30 ± 0.51 mg g^{-1}) was reduced due to sterilization in autoclave, starting the SSF at 11.100 ± 0.417 with a content decrease to 8.707 mg at 72 h, but regaining soluble protein levels over 11 mg, reaching 11.327 ± 0.859 mg per g of CPHF at 144 h (Figure 1). Sugar content (total sugar: 17.347 ± 0.26 mg g^{-1}, reducing sugar: 11.67 ± 0.15 mg g^{-1}) provides essential nutrients for *R. stolonifer* metabolism. The slight variation in pH from 5.57 ± 0.05 (0 h) to 4.91 ± 0.18 (144 h) is ideal for *Rhizopus* spp. spore germination [22].

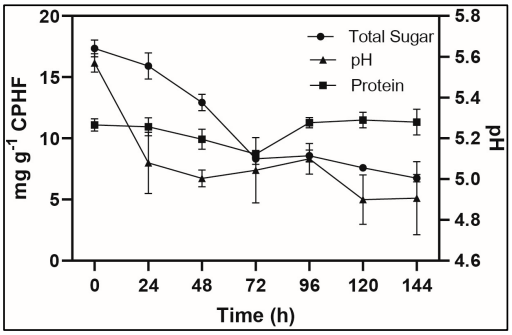


Figure 1. Kinetics of solid-state fermentation. Total sugar consumption, protein, and pH profile. Error bars represent the standard error values (n = 3).

3.3. TPC

The TPC varied significantly across fermentation times and between the extraction methods (EAE and AAE). For dried CPH, the TPC was significantly higher with AAE (204.48 ± 1.27 mg GAE g^{-1}) compared to EAE (125.00 ± 0.53 mg GAE g^{-1}, $p < 0.05$), and both methods show how rich CPH is in its composition. At 0 h of SSF, the TPC decreased markedly for both methods due to sterilization in the autoclave, with EAE yielding 73.17 ± 0.84 mg GAE. g^{-1} and AAE yielding 82.39 ± 2.64 mg GAE. g^{-1}, with no statistically significant difference between them. Throughout fermentation, the TPC fluctuated significantly. At 24 h, a peak in AAE was observed (134.18 ± 2.11 mg GAE. g^{-1}) compared to that in EAE (87.69 ± 1.06 mg GAE. g^{-1}, $p < 0.05$). This was followed by a substantial increase at 72 h for both methods, with AAE achieving 188.21 ± 0.53 mg GAE. g^{-1} and EAE achieving 114.70 ± 0.23 mg GAE. g^{-1} ($p < 0.05$). At 96 h, AAE maintained high TPC levels (122.84 ± 2.32 mg GAE. g^{-1}) compared to EAE (71.08 ± 1.16 mg GAE. g^{-1}, $p < 0.05$). The TPC plateaued at 120 h and declined slightly at 144 h for both methods, with AAE (92.69 ± 0.74 mg GAE. g^{-1}) continuing to exhibit significantly higher values than EAE (75.90 ± 0.11 mg GAE. g^{-1}) (Table 2). These results suggest that the AAE method consistently yielded higher TPC across all fermentation time points, with notable variations linked to time, likely due to the differential efficiency of phenolic compound release and degradation during extraction.

Table 2. Total phenolic content (TPC) of cocoa pod husk for AAE and EAE methods during fermentation (values with an asterisk (*) indicate a statistically significant difference between AAE and EAE, $p < 0.05$).

Fermentation Time (h)	TPC (mg GAE. g^{-1} Sample)	
	EAE	**AAE**
Dried CPH	125.00 ± 0.53 ^{e*}	204.48 ± 1.27 ^{g}
0 h	73.17 ± 0.84 ^{a*}	82.39 ± 2.64 ^{a}
24 h	87.69 ± 1.06 ^{c*}	134.18 ± 2.11 ^{e}
48 h	85.26 ± 2.10 ^{c*}	115.52 ± 0.63 ^{c}
72 h	114.70 ± 0.23 ^{d*}	188.21 ± 0.53 ^{f}
96 h	71.08 ± 1.16 ^{a*}	122.84 ± 2.32 ^{d}
120 h	82.09 ± 1.48 ^{bc*}	137.61 ± 1.16 ^{e}
144 h	75.90 ± 0.11 ^{ab*}	92.69 ± 0.74 ^{b}

Note: Values are presented as mean ± standard error. Different superscript letters (a–g) within each column indicate statistically significant differences ($p < 0.05$) based on two-way ANOVA, followed by Tukey's HSD test. Asterisks (*) indicate significant differences between EAE and AAE methods for each time point ($p < 0.05$).

3.4. ORAC

The oxygen radical absorbance capacity (ORAC) assay results revealed a significant enhancement in antioxidant activity throughout the fermentation process. The dried cocoa pod husk (CPH) had an initial ORAC value of 47,723.04 ± 2796.90 µmol TE. g^{-1} sample, representing the SSF baseline. After 48 h, the value increased significantly to

50,312.11 ± 485.19 ($p < 0.05$). A steady upward trend was observed at 72, 96, and 120 h, showing progressive improvements, although not always statistically significant. At 144 h, the ORAC value peaked at 51,676.48 ± 347.99 µmol TE. g^{-1} sample, which was significantly higher than that of dried CPH ($p < 0.05$) (Table 3). These results suggest that SSF enhances the antioxidant capacity of the CPH.

Table 3. Oxygen radical absorbance capacity (ORAC, Trolox equivalent—TE) of EAE from cocoa pod husk during fermentation (48 to 144 h).

Fermentation Time (h)	ORAC (µmol TE. g^{-1} Sample)
Dried CPH	47,723.04 ± 2796.90 ^{a}
48 h	50,312.11 ± 485.19 ^{b}
72 h	50,391.40 ± 4501.17 ^{bc}
96 h	50,535.57 ± 800.70 ^{bc}
120 h	51,645.00 ± 76.93 ^{cd}
144 h	51,676.48 ± 347.99 ^{d}

Note: Values are presented as mean ± standard error. Different superscript letters (a, b, c, d) indicate statistically significant differences ($p < 0.05$) based on one-way ANOVA, followed by Tukey's HSD test.

3.5. TEAC

The antioxidant activity of cocoa pod husk (CPH) was assessed during solid-state fermentation at various time points using both EAE and AAE methods. Initial measurements at 0 h showed similar antioxidant activity, with 6.55 ± 0.05 µmol TE. g^{-1} sample for EAE and 6.72 ± 0.01 for AAE. Both extraction methods showed increased antioxidant activity as fermentation progressed, peaking at 96 h, with EAE measuring 7.09 ± 0.05 and AAE measuring 7.25 ± 0.04. A paired *t*-test analysis revealed that the differences in antioxidant activity between EAE and AAE were statistically significant at all time points ($p < 0.05$) (Table 4). These findings suggest that both extract fractions have an overall increase in antioxidant activity during SSF, confirming the findings observed in ORAC results. At the same time, AAE consistently reported higher values compared to EAE, with statistically significant differences observed throughout the process.

Table 4. Trolox equivalent antioxidant capacity (TEAC) measured by DPPH Assay in µM TE per gram of cocoa pod husk for AAE and EAE methods during fermentation.

Fermentation Time (h)	TEAC (µmol TE. g^{-1} Sample)	
	EAE	AAE
Dried CPH	6.52 ± 0.03 ^{a*}	6.60 ± 0.03 ^{a}
0	6.55 ± 0.05 ^{a*}	6.72 ± 0.01 ^{b}
24	6.56 ± 0.05 ^{a*}	6.95 ± 0.02 ^{c}
48	6.64 ± 0.08 ^{ab*}	7.10 ± 0.02 ^{d}
72	6.73 ± 0.09 ^{bc*}	7.15 ± 0.01 ^{de}
96	7.09 ± 0.05 ^{e*}	7.25 ± 0.04 ^{f}
120	6.98 ± 0.01 ^{de*}	7.17 ± 0.05 ^{ef}
144	6.93 ± 0.09 ^{cd*}	7.09 ± 0.03 ^{d}

Note: Values are presented as mean ± standard error. Different superscript letters (a–f) within each column indicate statistically significant differences ($p < 0.05$) based on a two-way ANOVA followed by Tukey's HSD test. Asterisks (*) indicate significant differences between EAE and AAE methods for each time point ($p < 0.05$).

3.6. GC-MS

Volatile profile analysis of CPH and CPHF revealed several bioactive compounds with significant potential applications in the food, medical, and pharmaceutical industries. Notably, compounds such as α-methylbenzyl alcohol, 2-acetylpyrrole, aristolene, and rosefuran were exclusively identified in CPHF, along with methyl 3-hydroxybutyrate (M3HB), palmitic acid, and 10,12-tricosadiynoic acid. These bioactive compounds exhibit diverse biological activities, including antioxidant, anti-inflammatory, and therapeutic effects (Table 5).

Table 5. Volatile compounds identified in non-extracted CPH and CPHF after 96 h of fermentation, acidified acetone extract (AAE), and ethanol aqueous extract (EAE) from cocoa pod husk and cocoa pod husk After 48, 96, and 144 hours of fermentation by gas chromatography coupled to mass spectrometry (GC-MS).

			Solid-State		EAE				AAE				Bioactivity	Reference
Alcohol	**Formula**	**Mol Wt**	**CPH**	**CPHF**	**CPH**	**48 h**	**96 h**	**144 h**	**CPH**	**48 h**	**96 h**	**144 h**		
2,3-Butanediol	$C_4H_{10}O_2$	90	-	+	-	-	-	-	-	-	-	-	Fuel additive	[23]
1-Hexanol	$C_6H_{14}O$	102	+	-	-	-	-	-	-	-	-	-	Aroma	[24]
(2S,4S)-(+)-Pentanediol	$C_5H_{12}O_2$	104	+	+	-	-	-	-	-	-	-	-	N/A	
1,2-Pentanediol	$C_5H_{12}O_2$	104	-	+	-	-	-	-	-	-	-	-	N/A	
1-Methoxy-2-butanol	$C_5H_{12}O_2$	104	+	+	-	-	-	-	-	-	-	-	N/A	
2-Ethoxy-1-propanol	$C_5H_{12}O_2$	104	-	-	-	-	-	-	+	-	-	-	N/A	
Benzyl alcohol	C_7H_8O	108	-	+	-	-	-	-	-	-	-	-	Antimicrobial	[25]
3,4-Dimethylpent-2-en-1-ol	$C_7H_{14}O$	114	-	+	-	-	-	-	-	-	-	-	N/A	
5-Methyl-2-hexanol	$C_7H_{16}O$	116	-	+	-	-	-	-	-	-	-	-	Aroma	[26]
2-Methyl-3-hexanol	$C_7H_{16}O$	116	-	+	-	-	-	-	-	-	-	-	N/A	
2-Ethyl-3-pentanol	$C_7H_{16}O$	116	+	-	-	-	-	-	-	-	-	-	N/A	
Hexylene glycol	$C_6H_{14}O_2$	118	-	-	+	-	+	+	-	-	-	+	Antibacterial & Antifungal	[27]
Phenylethyl Alcohol	$C_8H_{10}O$	122	+	+	-	-	-	-	-	-	-	-	Antifungal & Aroma	[28]
α-Methylbenzyl alcohol	$C_8H_{10}O$	122	-	+	-	-	-	-	-	-	-	-	Aroma	[29]
1-Octen-3-ol	$C_8H_{16}O$	128	+	-	-	-	-	-	-	-	-	-	Food additive	[30]
2,6-Dimethylcyclohexanol	$C_8H_{16}O$	128	+	-	-	-	-	-	-	-	-	-	Anesthetic	[31]
2-Ethyl-1-hexanol	$C_8H_{18}O$	130	+	-	-	-	-	-	-	-	-	-	Aroma	[32]
1-Nonanol	$C_9H_{20}O$	144	+	-	-	-	-	-	-	-	-	-	Antifungal & Aroma	[33]
1-Phenoxy-2-propanol	$C_9H_{12}O_2$	152	-	-	-	-	+	-	-	-	-	-	Anesthetic	[34]
3-Phenoxy-1-propanol	$C_9H_{12}O_2$	152	-	-	-	-	+	-	-	-	-	-	Aroma	[35]
Linalool	$C_{10}H_{18}O$	154	+	-	-	-	-	-	-	-	-	-	Anti-inflammatory & Anticonvulsant	[36]
α-Terpineol	$C_{10}H_{18}O$	154	+	-	-	-	-	-	-	-	-	-	Antifungal	[37]
[5-Hydroxymethyl)-1,3-dioxolan-4-yl]methanol	$C_5H_{10}O_4$	134	-	-	-	-	-	+	+	-	-	-	N/A	
Cis-Linalool oxide	$C_{10}H_{18}O_2$	170	+	+	-	-	-	-	-	-	-	-	Aroma	[38]
Linalool oxide pyranoid	$C_{10}H_{18}O_2$	170	+	+	-	-	-	-	-	-	-	-	Aroma	[39]

Table 5. *Cont.*

			Solid-State		EAE				AAE				Bioactivity	Reference
	Formula	**Mol Wt**	**CPH**	**CPHF**	**CPH**	**48 h**	**96 h**	**144 h**	**CPH**	**48 h**	**96 h**	**144 h**		
Trans-furanoid linalool oxide	$C_{10}H_{18}O$	170	+	-	-	-	-	-	-	-	-	-	Aroma	[40]
11-Methyldodecanol	$C_{13}H_{28}O$	200	+	-	-	-	-	-	-	-	-	-	N/A	
1-Tridecanol	$C_{13}H_{28}O$	200	+	-	-	-	-	-	-	-	-	-	N/A	
1-Methoxy-3-(2-hydroxyethyl)nonane	$C_{12}H_{26}O_2$	202	-	-	+	-	-	-	-	-	-	-	Antioxidant & Antifungal	[41,42]
2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1-perhydrol	$C_{14}H_{24}O_4$	256	-	-	-	-	-	-	-	-	+	-	Antioxidant, Anti-inflammatory, Antidiabetic, Antitumor, etc	[43,44]
Aldehyde														
(E,E)-2,4-Heptadienal	$C_7H_{10}O$	110	+	-	-	-	-	-	-	-	-	-	Antifungal & Aroma	[45]
Heptanal	$C_7H_{14}O$	114	+	-	-	-	-	-	-	-	-	-	Antifungal	[46]
Methoxycitronellal	$C_{11}H_{22}O_2$	186	-	+	-	-	-	-	-	-	-	-	Aroma	[47]
Nonanal	$C_9H_{18}O$	142	+	+	-	-	-	-	-	-	-	-	Antifungal	[48]
Octanal	$C_8H_{16}O$	128	+	+	-	-	-	-	-	-	-	-	Antifungal	[49]
(E)-2-hexenal	$C_6H_{10}O$	98	+	-	-	-	-	-	-	-	-	-	Antifungal	[50]
(E)-2-Octenal	$C_8H_{14}O$	126	+	-	-	-	-	-	-	-	-	-	Antifungal	[51]
Phenylacetaldehyde	C_8H_8O	120	+	+	-	-	-	-	-	-	-	-	Aroma	[52]
β-Cyclocitral	$C_{10}H_{16}O$	152	+	-	-	-	-	-	-	-	-	-	Antibacterial	[53]
Ketone														
2-Heptanone	$C_7H_{14}O$	114	+	-	-	-	-	-	-	-	-	-	Neuromodulation	[54]
Geranylacetone	$C_{13}H_{22}O$	196	+	-	-	+	-	-	-	-	-	-	Aroma	[55]
Acetophenone	C_8H_8O	87	+	-	-	-	-	-	-	-	-	-	Aroma & Food additive	[56]
2-Dodecanone	$C_{12}H_{24}O$	184	+	-	-	-	-	-	-	-	-	-	Insecticidal & Repellent	[57]
1,3-Diacetylbenzene	$C_{10}H_{10}O_2$	162	-	-	-	-	+	+	+	-	-	+	N/A	
1,4-Diacetylbenzene	$C_{10}H_{10}O_2$	162	-	-	+	-	+	+	+	-	+	+	N/A	

Table 5. *Cont.*

			Solid-State		EAE				AAE				Bioactivity	Reference
	Formula	**Mol Wt**	**CPH**	**CPHF**	**CPH**	**48 h**	**96 h**	**144 h**	**CPH**	**48 h**	**96 h**	**144 h**		
2-Acetylpyrrole	C_6H_7NO	109	-	+	-	-	-	-	-	-	-	-	Antioxidant & Hepatoprotective	[58]
Sulcatone	$C_8H_{14}O$	126	+	-	-	-	-	-	-	-	-	-	Antimicrobial	[59]
trans-3-Octen-2-one	$C_8H_{14}O$	126	+	-	-	-	-	-	-	-	-	-	Aroma	[60]
β-Ionone	$C_{13}H_{20}O$	192	+	-	-	-	-	-	-	-	+	-	Aroma, Antimicrobial & Insecticidal	[61]
Ester														
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	278	-	-	-	-	-	-	+	-	-	-	Antioxidant & Antimicrobial	[62]
1,2-Dimethylpropyl acetate	$C_7H_{14}O_2$	130	+	-	-	-	-	-	-	-	-	-	N/A	
1-Ethylpentyl acetate	$C_9H_{18}O_2$	158	-	+	-	-	-	-	-	-	-	-	Antimicrobial	[63]
2-(Heptyloxycarbonyl)benzoic acid	$C_{15}H_{20}O_4$	264	-	-	-	-	+	+	+	+	-	-	Antiobesity & Anti-hyperlipidemic	[64]
Triethylene glycol dimethacrylate	$C_{14}H_{22}O_6$	286	-	-	-	-	-	-	+	+	-	-	Cytotoxic (mammalian cells)	[65]
Cinnamic acid, 4-hydroxy-3-methoxy-, (5-hydroxy-2-hydroxymethyl-6-[2-(4-hydroxy-3-methoxyphenyl)ethoxy]-4-(6-methyl-3,4,5-trihydroxytetrahydropyran-2-yloxy)tetrahydropyran-3-yl) ester	$C_{31}H_{40}O_{15}$	652	-	-	+	+	+	+	+	+	-	+	Antioxidant & Antiviral	[66]
Dimethyl phthalate	$C_{10}H_{10}O_4$	194	-	-	+	-	+	+	-	-	-	+	Environmental Contaminator	[67]
Dodecanoic acid, 2,3-bis(acetyloxy)propyl ester	$C_{19}H_{34}O_6$	358	-	-	+	-	-	-	-	+	-	-	Antiviral	[68]
Ethyl iso-allocholate	$C_{26}H_{44}O_5$	452	+	+	+	+	+	+	+	+	+	+	Antiangiogenic	[69]

Table 5. *Cont.*

			Solid-State		EAE				AAE				Bioactivity	Reference
	Formula	**Mol Wt**	**CPH**	**CPHF**	**CPH**	**48 h**	**96 h**	**144 h**	**CPH**	**48 h**	**96 h**	**144 h**		
Fumaric acid, 2-isopropyl phenyl dodec-2-en-1-yl ester	$C_{25}H_{36}O_4$	400	-	-	-	-	-	-	-	-	+	-	Antioxidant, Immunomodulating, and Anti-inflammatory	[70]
Methyl salicylate	$C_8H_8O_3$	152	+	+	-	-	-	-	-	-	-	-	Anti-inflammatory & Analgesic agent	[71]
Octacosanoic acid, methyl ester	$C_{29}H_{58}O_2$	438	-	-	-	-	-	-	-	+	-	-	N/A	
Oxalic acid, bis(6-ethyloct-3-yl) ester	$C_{22}H_{42}O_4$	370	+	-	-	-	-	-	-	-	-	-	N/A	
Methoxy-Phenyl-Oxime	$C_8H_9NO_2$	151	+	+	-	+	+	+	+	+	+	+	Antibacterial	[72]
Methyl 3-hydroxybutyrate	$C_5H_{10}O_3$	118	-	+	-	-	-	-	-	-	-	-	Therapeutic effect on Alzheimer's disease & Inhibition of apoptosis	[73]
γ-Butyrolactone	$C_4H_6O_2$	86	+	-	-	-	-	-	-	-	-	-	Regulate Antibiotic production in Streptomyces	[74]
Pantolactone	$C_6H_{10}O_3$	130	+	-	-	-	-	-	-	-	-	-	Food additive	[75]
Mevalonolactone	$C_6H_{10}O_3$	130	-	+	-	-	-	-	-	-	-	-	Mevalonate pathway precursor (3-Hydroxy-3-methylglutaryl Coenzyme A Reductase inhibitor)	[76]
3,3,5-trimethylcyclohexyl methacrylate	$C_{13}H_{22}O_2$	210	-	-	+	+	-	-	-	-	-	-	N/A	
Organic acid														
10,12-Tricosadiynoic acid	$C_{23}H_{38}O_2$	346	-	+	-	-	-	-	-	-	-	-	ACOX1-specific inhibitor	[77]
Isovaleric acid	$C_5H_{10}O_2$	102	+	-	-	-	-	-	-	-	-	-	Colonic Smooth Muscle Relaxation	[78]
Palmitic acid	$C_{16}H_{32}O_2$	256	-	-	-	-	-	-	-	+	-	-	Diet Supplement for animal	[79]
Tetradecanoic acid	$C_{14}H_{28}O_2$	228	-	-	-	-	-	-	-	+	-	-	Larvicidal & mosquito repellent	[80]

Table 5. *Cont.*

			Solid-State		EAE				AAE				Bioactivity	Reference
	Formula	**Mol Wt**	**CPH**	**CPHF**	**CPH**	**48 h**	**96 h**	**144 h**	**CPH**	**48 h**	**96 h**	**144 h**		
Terpenes														
Aristolene	$C_{15}H_{24}$	204	-	+	-	-	-	-	-	-	-	-	Biopesticidal, Anti-inflammatory, Antidiabetic, Anti-urolithic, and Tyrosinase inhibitory potentials	[81,82]
1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene	$C_{15}H_{24}$	204	+	+	-	-	-	-	-	-	-	-	Antioxidant & Antibacterial	[83]
Isoledene	$C_{15}H_{24}$	204	+	-	-	-	-	-	-	-	-	-	Antiviral & Anti-leishmania	[84]
Valerena-4,7(11)-diene	$C_{15}H_{24}$	204	+	-	-	-	-	-	-	-	-	-	Sedative effect	[85]
α-Copaene	$C_{15}H_{24}$	204	+	+	-	-	-	-	-	-	-	-	Antimicrobial	[86]
γ-Cadinene	$C_{15}H_{24}$	204	+	-	-	-	-	-	-	-	-	-	Antioxidant & Anti-inflammatory	[87]
γ-Muurolene	$C_{15}H_{24}$	204	+	-	-	-	-	-	-	-	-	-	Antioxidant & Anti-inflammatory	[88]
D-Limonene	$C_{10}H_{16}$	136	+	+	-	-	-	-	-	-	-	-	Anti-inflammatory & Antibiofilm	[89]
Rosefuran	$C_{10}H_{14}O$	150	-	+	-	-	-	-	-	-	-	-	Antioxidant	[90]
Hydrocarbon														
1,2,4-Trimethylcyclopentane	C_8H_{16}	112	-	+	-	-	-	-	-	-	-	-	N/A	
13-Phenylpentacosane	$C_{31}H_{56}$	428	-	+	-	-	-	-	-	-	-	-	N/A	
1-Heptadecene	$C_{17}H_{34}$	238	+	-	-	-	-	-	-	-	-	-	N/A	
2,6,10,15-Tetramethylheptadecane	$C_{21}H_{44}$	296	+	-	-	-	-	-	-	-	-	-	Multiple therapeutic potentialities	[91]
2-Cyclopropylidene-1,7,7-trimethylbicyclo [2.2.1]heptane	$C_{13}H_{20}$	176	-	+	-	-	-	-	-	-	-	-	Antimicrobial	[92]
2-Ethylhexene	C_8H_{16}	112	-	+	-	-	-	-	-	-	-	-	N/A	

Table 5. *Cont.*

			Solid-State		EAE				AAE				Bioactivity	Reference
	Formula	**Mol Wt**	**CPH**	**CPHF**	**CPH**	**48 h**	**96 h**	**144 h**	**CPH**	**48 h**	**96 h**	**144 h**		
2-Methyl-n-hexacosane	$C_{27}H_{56}$	380	+	-	-	-	-	-	-	-	-	-	Anti-Carcinoma Cell Growth	[93]
Eicosane	$C_{20}H_{42}$	282	+	-	-	-	-	-	-	-	-	-	Antifungal, Antioxidant, and Anti-inflammatory	[94,95]
Heneicosane	$C_{21}H_{44}$	296	+	-	-	-	-	-	-	-	-	-	Antimicrobial	[96]
Nitrile														
Octanenitrile	$C_8H_{15}N$	125	+	-	-	-	-	-	-	-	-	-	Aroma	[97]
Amine														
Putrescine	$C_4H_{12}N_2$	88	+	-	-	-	-	-	-	-	-	-	N/A	
2-Hydrazino-2-imidazoline	$C_3H_8N_4$	100	-	-	-	-	-	-	-	+	-	-	Antioxidant	[98]
Heterocyclic														
Benzothiazole	C_7H_5NS	135	-	-	+	-	+	+	+	-	+	+	Antimicrobial, Anticonvulsant, Neuroprotective, Anti-inflammatory, and Antitumor	[99]
Caffeine	$C_8H_{10}N_4O_2$	194	-	-	-	-	-	-	+	-	-	-	Alleviates fatigue and drowsiness	[100]
Amide														
Caprolactam	$C_6H_{11}NO$	113	-	-	-	-	-	+	-	-	-	-	Caspase-1 inhibitor	[101]
Ether														
β-Hydroxyethyl phenyl ether	$C_8H_{10}O_2$	138	-	-	-	-	+	-	-	-	+	-	Antibacterial & Antifungal	[102]

Note: (+) Presence or (-) Absence. N/A—Bioactivity not found in the literature.

4. Discussion

The physicochemical composition of CPH highlights its suitability for solid-state fermentation (SSF) by *Rhizopus stolonifer*. The slightly acidic pH (6.18 ± 0.01) and low water activity (Aw = 0.283 ± 0.001) create a favorable environment for fungal growth, requiring minimal adjustments [14]. The mineral profile, including sulfate (0.649 ± 0.029 mg g^{-1}), phosphate (0.335 ± 0.010 mg g^{-1}), and magnesium (0.440 ± 0.021 mg g^{-1}), supports enzymatic activity and fungal growth [14]. These characteristics make CPH an ideal substrate for R. stolonifer to enhance its nutritional and functional properties through fermentation, demonstrating its potential for sustainable biotechnological applications (Table 1).

The TPC results indicated a higher phenolic content in the AAE fraction compared to theEAE fraction, with significant differences between the EAE and AAE methods across all time points ($p < 0.05$) (Table 2). AAE consistently yielded higher TPC values compared to EAE. After the CPH passed through sterilization at 120 °C in an autoclave for 20 min, all these physical factors, such as high pressure and temperature during thermal pretreatment, generated an approximate reduction of 59% in EAE and 40% in AAE from the initial TPC value [103,104]. The EAE fraction of dried CPH was 125.00 ± 0.53 mg GAE g^{-1}, then fluctuated during SSF from 73.17 ± 0.84 at 0 h to its peak at 72 h, reaching 114.70 ± 0.23 mg GAE g^{-1}. The AAE fraction showed a 204.48 ± 1.27 mg GAE g^{-1} of CPH before sterilization, starting SSF at 82.39 ± 2.64 mg GAE g^{-1}, and it showed a similar pattern of fluctuation, with its peak at 72 h (188.21 ± 0.53 mg GAE g^{-1}).

Previous work using the CPH showed lower values, reaching a range of 46–69 mg GAE g^{-1} [105]. Vriesmann et al. (2011) showed the presence of 46.0 ± 0.40 mg GAE g^{-1} of CPH from farms in Bahia, Brazil [106]. Yapo et al. (2013) registered 69.0 mg GAE/g CPH of fresh CPH from Côte d'Ivoire using four sequential solvents [107]. Even considering all the compositions of CPH from various areas and countries, the two methods presented in this work showed their efficiency in extracting phenolic compounds and support the hypothesis proposed in this study on how SSF using *Rhizopus stolonifer* can generate biomolecules with redox potential.

This study employed two complementary methods to assess antioxidant activity, ORAC and DPPH assays, to confirm the redox potential changes associated with biotransformation during SSF.

The ORAC assay revealed a significant increase in antioxidant activity due to SSF (Table 4). The initial ORAC value for CPH was (47.70 ± 0.28) × 10^3 µmol TE g^{-1}, which increased significantly to (51.70 ± 0.35) × 10^3 µmol TE g^{-1} after 144 h ($p < 0.05$). Significant differences were observed between the CPH and all subsequent time points ($p < 0.05$). The ORAC values showed a gradual increase, with significant differences also noted between 48 h and both 120 h and 144 h ($p < 0.05$). The highest ORAC value was observed at 144 h, indicating a continuous increase in antioxidant activity during the extraction period.

The DPPH assay demonstrated significant differences between the EAE and AAE extraction methods across all time points ($p < 0.05$) (Table 3). For EAE, the DPPH values increased from 6.52 ± 0.03 µM TE g^{-1} at CPH to a peak of 7.09 ± 0.05 µM TE g^{-1} at 96 h before slightly decreasing. AAE showed a more consistent increase, starting at 6.60 ± 0.03 µM TE g^{-1} (CPH) and reaching a maximum of 7.25 ± 0.04 µM TE g^{-1} at 96 h after inoculation. Two-way ANOVA revealed significant main effects for both extraction method and time ($p < 0.001$), as well as a significant interaction between these factors ($p < 0.01$). AAE consistently yielded higher DPPH values compared to EAE across all time points.

The antioxidant activity observed in CPH is similar to that reported by Yapo et al. (2013), where approximately 85% of antioxidant activity was reported using fresh cocoa pod

husk from the Ivory Coast [108]. The TEAC results obtained using the DPPH assay revealed significant differences between the EAE and AAE extraction methods. Consistently higher DPPH values for AAE at all time points indicate that this method may be more effective in extracting antioxidant compounds than EAE. This difference could be attributed to the varying selectivity of the two extraction methods for different antioxidant compounds in the sample [109].

For both extraction methods, the general trend of antioxidant activity peaked at 96 h of fermentation, followed by a slight decline. This pattern suggests an optimal fermentation duration during which antioxidant compounds can be degraded or reused. The increase in TEAC values confirmed the potential of *Rhizopus stolonifer* to enhance the antioxidant activity of CPH through SSF.

Analysis of the volatile profiles provided deeper insights into the antioxidant activity of CPH and CPHF and identified bioactive compounds of interest. The analysis revealed the presence of several bioactive compounds with potential application in food, medical, and pharmaceutical industries, such as α-methylbenzyl alcohol, 2-acetylpyrrole, aristolene, rosefuran, benzyl alcohol, 1-ethylpentyl acetate, 2-cyclopropylidene-1,7,7-trimethylbicyclo[2.2.1] heptane, β-hydroxyethyl phenyl ether, 2-hydrazino-2-imidazoline, methyl 3-hydroxybutyrate (M3HB), caprolactam, palmitic acid, and 10,12-tricosadiynoic acid which were all exclusively found in CPHF samples (Table 5). Beyond the antimicrobial, antioxidant, and anti-inflammatory activities of most of these compounds, SSF is justified by enhancing CPH with compounds like M3HB, which is known as an alternative energy source for cells with impaired metabolic function and has recently been reported to have therapeutic effects on Alzheimer's disease and inhibition of apoptosis [73]. In addition, 10,12-tricosadiynoic acid, a specific Acyl-CoA Oxidase-1 inhibitor, enhances hepatic lipid metabolism and reduces oxidative stress by boosting mitochondrial fatty acid oxidation [110–112], and palmitic acid, which has shown anti-inflammatory potential and is an impactful animal diet supplement for the dairy industry [79,113].

Compounds known for their bioactive potential such as 2-methyl-n-hexacosane, γ-muurolene, isoledene, linalool, α-terpineol, 1-methoxy-3-(2-hydroxyethyl)nonane, heptanal, 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester, were inevitably lost or metabolized by the *R. stolonifer* as most of these compounds present antifungal activities (Table 5). Furthermore, compounds like α-copaene, D-limonene, benzothiazole, β-ionone, and ethyl iso-allocholate were identified in CPH and CHPF, and are recognized for their anti-cancer effects, further highlighting the health-promoting potential of the fermented cocoa pod husk [66,99,114,115]. D-limonene is known for its antioxidant and antimicrobial properties [116].

Detection of complex compounds like cinnamic acid, 4-hydroxy-3-methoxy-(5-hydroxy-2-hydroxymethyl-6-[2-(4-hydroxy-3-methoxyphenyl)ethoxy]-4-(6-methyl-3,4,5-trihydroxytetrahydropyran-2-yloxy)tetrahydropyran-3-yl) ester, a derivative of ferulic acid (4-hydroxy-3-methoxycinnamic acid). This common plant-based phenolic acid is found in cell walls, bound to polysaccharides [66]. It has a sugar-like glycosylated structure with multiple hydroxyl and methoxy groups, which is typical of phenolic glycosides. This sugar-like structure attached to ferulic acid can influence its solubility, stability, and bioavailability, potentially increasing its antioxidant properties. These molecules can be transformed during fermentation, where microorganisms release bound ferulic acid and convert it into bioactive forms with enhanced antioxidant properties [117]. The presence of these compounds highlights the potential of CPH and CPHF as sources of bioactive compounds with therapeutic applications and as enriched nutritional supplements for animal feed.

5. Conclusions

This research demonstrates that solid-state fermentation with *Rhizopus stolonifer* can transform CPH, an agricultural waste product, into a valuable biomaterial with enhanced nutritional, antioxidant, and potential therapeutic properties, making CPHF a promising candidate for applications in food, animal feed, and bio-based industries. The physicochemical characterization of CPH revealed its suitability as a substrate for SSF, with fermentation improving protein recovery and significantly increasing antioxidant activity, as confirmed by ORAC and DPPH assays. These results highlight the redox potential of the biotransformation process, with the highest antioxidant activity observed at 144 h and 96 h.

GC-MS analysis identified a range of bioactive compounds, including methyl 3-hydroxybutyrate (M3HB), 10,12-Tricosadiynoic acid, and palmitic acid, which have applications in the food, pharmaceutical, and medical industries. Additionally, the preservation and transformation of phenolic compounds during SSF further enhanced the antioxidant profile of the fermented CPH.

This approach offers a sustainable solution for agricultural waste management and opens new avenues for developing functional food supplements, nutraceuticals, and potentially pharmaceutical ingredients. These findings underscore the potential of SSF to valorize agricultural byproducts, contributing to sustainable biotechnological innovations and the production of high-value biomaterials. Future studies will focus on pilot-scale experiments to evaluate production efficiency, product consistency, and potential commercial applications, while also incorporating kinetic modeling approaches to better understand and optimize the solid-state fermentation process.

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