UTILIZATION OF COCOA POD HUSK AS A SUBSTRATE FOR THE PRODUCTION OF HARZIANIC ACID BY *Trichoderma harzianum*

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**Corresponding author's email address: famoura@ufpa.br ABSTRACT*

ABSTRACT

The utilization of agricultural residues for the production of industrially relevant bioproducts is a growing field of study. This research investigates, for the first time, the feasibility of utilizing cocoa pod husk (CPH) as a substrate for the production of harzianic acid (HA) by the fungus Trichoderma harzianum. HA is a compound exhibiting antimicrobial properties with promising applications in various fields. The CPH was dried (60°C, for 4 days), ground (1.19 mm), and an aqueous extract was produced (40 g/L). The extract was supplemented with potato dextrose broth (PDB) at ratios of 1:1, 1:0.75, 1:0.50, 1:0.25, and 1:0.10 (v:v). A control with PDB (100%) was evaluated. *T. harzianum* spores at 10^5/mL were inoculated, and static fermentation was conducted in a 25°C greenhouse for 28 days. HA spectra produced were validated against the literature using NMR. HA quantification was performed via UHPLC. The average production of HA was 0.74 mg/L (14 days), 0.32 mg/L (21 days), and 0.50 mg/L (28 days), whereas the control exhibited an average concentration of 248 mg/L of HA. The study is pioneering in HA production by CPH, yet further research is needed to optimize production utilizing CPH.

Keywords: Agricultural residue, Static fermentation, Bioproduct.

1 INTRODUCTION

The agroindustrial production of cocoa (*Theobroma cacao* L.) generates cocoa pod husk (CPH) as its main agricultural residue, as it represents between 67% to 75% of the fruit.[1] The pursuit of biotechnological approaches has led to the study of using agroindustrial residues as substrates for the production of bioproducts, and recently, CPH has sparked interest as a raw material for producing various biomolecules of medium to high added value.[2] CPH is a nutrient-rich substrate (e.g., carbon, nitrogen, and minerals), capable of providing favorable conditions for fungal growth in both solid-state and liquid fermentations.[3] *Trichoderma harzianum* produces harzianic acid (HA), a biomolecule with antimicrobial properties that can be industrially exploited, as it has demonstrated potential in combating phytopathogens such as *Fusarium oxysporum*, in addition to gram-positive bacteria and antibiotic-resistant strains.[4,5] In this context, the aim of this study was to investigate, for the first time, the production of HA by *T. harzianum* using a CHP extract.

2 MATERIAL & METHODS

Cocoa fruits were kindly donated from Mr. Michinori Konagano's farm in Tomé-Açú, Pará state (2°28'34.46"S, 48°16'26.02"W). The fruits were transported to the Centre for valorization of amazonian bioactive compounds (CVACBA) at room temperature. They were sanitized with chlorinated water (200 ppm) for 15 minutes and subsequently washed with water (5X). The CPH was dried in an oven (60°C for 4 days) and ground in a knife mill until particles with 1.19 mm (16 mesh) were obtained. An aqueous extract of CPH was produced at a concentration of 40 g/L and supplemented with potato dextrose broth (PDB) in the ratios of 1:0.10, 1:0.25, 1:0.50, 1:0.75, and 1:1 (v/v). A control with PDB (100%) was evaluated. After sterilization, 100 mL of the extract was inoculated with 10^5 spores/mL of *T. harzianum*. Cultivation was carried out in Erlenmeyer flasks in an incubator (25°C), without agitation, for a period of 28 days.

The extraction and purification of HA were conducted according to previous studies.[6] Nuclear magnetic resonance (NMR) spectra of HA were recorded at room temperature and validated with literature data.[7] HA quantification was performed by UHPLC at 360 nm[6] based on retention time, spectrum, and calibration curve (y=0.5843x+1.4302; R^2=0.9935; 0.001-0.1 mg/mL) using purified HA from the work as an internal standard.

3 RESULTS & DISCUSSION

HA was produced for the first time using CPH as a substrate. Compound identification was performed by ^{1}H and ^{13}C NMR spectra of HA (Table 1), confirming the chemical nature of the compound 2-hydroxy-2-[4-(1-hydroxy-octa-2,4-dienylidene)-1-methyl-3,5-dioxo-pyrrolidin-2-ylmethyl]-3-methylbutyric acid (Figure 1), as described in the literature.[8,9] Chromatographic analysis of the isolated compound showed 79.5% purity.

Table 1: Comparison of ^{13}C and ^{1}H Nuclear Magnetic Resonance (NMR) signals at 400 MHz for harzianic acid produced by *T. harzianum* in cocoa pod husk extract as a substrate.

Position	This work ^{13}C NMR (ppm)	This work ^{1}H NMR (ppm)	Healy et al., 2015 [7] ^{13}C NMR (ppm)	Healy et al., 2015 [7] ^{1}H NMR (ppm)
1	13.63	0.93	13.9	0.94
2	21.71	1.48	21.9	1.49
3	35.42	2.23	35.6	2.23
4	149.76	6.34	150.1	6.39
5	129.59	6.34	129.8	6.39
6	147.41	7.53	147.7	7.58
7	119.05	6.96	119.2	6.99
12	64.11	3.91	64.2	3.62
13	33.87	1.87	33.9	1.88
15	35.99	2.02	36.1	2.01
16	17.41	0.97	17.6	0.98
17	16.17	0.97	16.4	0.98
18	26.52	2.94	26.7	2.96

Solvent used: deuterated chloroform

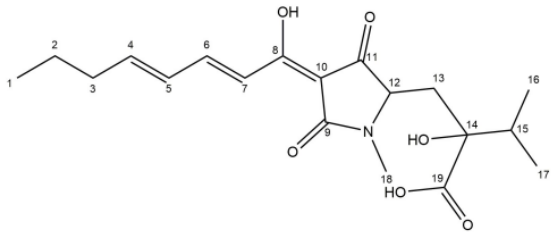

Figure 1: Structural formula of harzianic acid.

Table 2 presents the results of HA production by *T. harzianum* using CPH as a nutrient source over 28 days. It was noticeable that the CPH extract at a concentration of 40 g/L played an inhibitory role in fungal growth. The highest concentration of HA was 2.3 mg/L after 14 days of fermentation. However, HA production in PDB reached a maximum value of 376.6 mg/L after 28 days of fermentation. It was observed that the strategy of supplementing CPH extract with PDB was not effective for HA production. Literature data indicate HA production in PDB medium ranging from 67.5 to 190 mg/L.[10]

Table 2: Production of harzianic acid by *T. harzianum* in cocoa pod husk extract over 28 days of fermentation.

Formulation	Harzianic acid (mg/L)		
	14 days	21 days	28 days
CPH / PBD (1:1, v:v)	2.30 ± 0.64^{bA}	0.92 ± 0.50^{bA}	1.94 ± 1.10^{bA}
CPH / PBD (1:0,75, v:v)	0.58 ± 0.12^{cA}	0.38 ± 0.09^{bcA}	0.26 ± 0.05^{cB}
CPH / PBD (1:0,50, v:v)	0.58 ± 0.01^{cA}	0.13 ± 0.01^{cB}	0.14 ± 0.00^{cB}
CPH / PBD (1:0,25, v:v)	0.14 ± 0.03^{cA}	0.10 ± 0.01^{cA}	0.11 ± 0.01^{cA}
CPH / PBD (1:0,10, v:v)	0.13 ± 0.02^{cA}	0.09 ± 0.01^{cB}	0.09 ± 0.01^{cB}
Control PBD (100%)	207.54 ± 4.73^{aB}	159.98 ± 32.45^{aB}	376.65 ± 4.69^{aA}

PDB: Potato dextrose broth, CPH: Cocoa pod husk extract at 40 g/L. Different letters indicate significant differences ($p < 0.05$) for the same day (lower case) and formulation (uppercase).

The best results for HA production occurred within the first 14 days of static fermentation. Further studies optimizing cultivation conditions should be conducted to assess the optimal amount of CPH to enhance HA production by *T. harzianum*. Additionally, it is possible that the presence of inhibitors in CPH may interfere with the fungus's metabolism, diverting its metabolic pathway away from HA production. Therefore, strategies such as physical-chemical and/or enzymatic pretreatments on CPH could be explored to reduce inhibitor presence and increase the availability of fermentable sugars.

4 CONCLUSION

The pioneering study demonstrated that CPH, a commonly underutilized byproduct of the cocoa processing industry, has potential for the production of HA, a high-value biocompound, by *T. harzianum*. The outcome achieved in this study promotes bioeconomy by emphasizing the importance of sustainable valorization of agricultural residues. However, in light of the positive prospects observed, it is crucial to emphasize the importance of further studies to optimize the fermentative processes for HA production by *T. harzianum* using CPH as a substrate.

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ACKNOWLEDGEMENTS

The author acknowledges CAPES (Coordination for the Improvement of Higher Education Personnel) for the scholarship provided. Aditionally, the author expresses gratitude to the Graduate Program in Biotechnology at the Federal University of Pará (PPGBiotec/UFPA) and the National Council for Scientific and Technological Development (CNPq) for their support. Special thanks are extended to Dr. Wandson Braamcamp de Souza Pinheiro from the Central Extraction Laboratory (ICEN/UFPA) for his valuable contribution to the NMR analysis. The author would also like to express gratitude to the Pro-Rectory of Extension (PROEX) at the Federal University of Pará for their support.