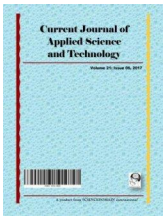

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Valorisation of Cassava Wastewater as Substrate for *Trichoderma virens* Production, Bio-control Agent Cocoa Black Pod Disease

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: In Côte d'Ivoire, several food products are made from cassava. However, this production generates effluents such as wastewater responsible for soil, as well as water and air pollution. Meanwhile, cassava wastewater can be used as an inexpensive substrate for the production of biopesticides, such as *Trichoderma virens*, a bio-control agent for black pod disease. This could address both the problems of cassava wastewater treatment and the use of conventional synthetic substrates for biopesticide production.

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Methodology: The experiments described here were conducted using the cassava wastewater, without any supplement, to produce spores of *T. virens* by liquid state fermentation, solid state fermentation on kaolin grains. The production of gliovirin in cassava wastewater was monitored by LC / MS analysis.
Results: The maximum production (≈ 1.7 x 10^8 spores / mL) was achieved at dilution 1/4 of the cassava wastewater while at dilutions 1 and 1/2 the concentrations were 6.3 x 10^6 and 7.2 x 10^6 spores / mL, respectively. Spores concentration increased when cassava wastewater was highly diluted. The cultures of *T. virens* on kaolin supplemented with the cassava wastewater recorded a concentration of 1.13 x 10^6 spores / g of kaolin. The presence of gliovirin was detected by LC/MS analysis of solid state fermentation of *T. virens* in cassava wastewater.

Keywords: Cassava wastewater; Trichoderma virens; fermentation; spore production; gliovirin.

1. INTRODUCTION

Cassava is one of the six main agricultural products including wheat, rice, corn, potatoes and oatmeal flour. It is a major source of dietary energy for more than 500 million people. This tuber represents an industrial interest for its starch richness [1]. In Côte d'Ivoire, the national production of cassava is about 5 millions of tons per year; its consumption comes above that of rice [2]. The cassava processing produces many highly valuable derivatives, the most important of which is a slightly acidic fermented semolina called "Attiéké". This homemade food, generally produced by women or women's associations, is increasingly on demand around the world. Thus industries began to settle with aim to meet this great need for "Attiéké". However, the production of this food as well as and many other foods obtained after several cycles of dewatering of fermented cassava paste, generates large amounts of starch-riched acidic wastewater. This untreated wastewater is directly discharged onto the processing sites, sometimes into watercourses, which causes unsanitary conditions like emission of gas, pollution of water and soil and, degradation of the immediate environment [3]. These wastewaters can be used as raw materials for fermentation in order to massively produce spores and metabolites of microorganisms of agricultural and biotechnology interest. Indeed, due to both constraints of low spore yield and very high costs of conventional synthetic media used for the production of biopesticides [4], many studies were undertaken on new residues as raw materials. Successful use of sewage sludge compost was reported [5], as well as wastewater from starch industries [4] for bio-control agent production. Besides, biological waste and cassava wastewater were tested as a substrate for the production of molecules as volatile compounds by *Geotrichum fragrans* [6]; or rhamnolipids by *Pseudomonas aeruginosa* [1]; and enzymes as cellulase by *Trichoderma asperellum* [7]; or cellulase and xylanase by *Trichoderma reesei* [8]. The present work carried out on the soil under cocoa farm in Côte d'Ivoire successfully isolated several strains of *Trichoderma* sp, of which three (*Trichoderma virens*, *Trichoderma asperellum* and *Trichoderma harzianum*) showed very high inhibitory powers against *Phytophthora palmivora*, the agent of cocoa black pods disease [9]. Chemical studies implemented on these three strains helped to purify and identify several active molecules, including gliovirin, a compound responsible for the inhibition of the mycelial growth of several species of *Phytophthora palmivora* and *Phytophthora megakarya* [10-11].

The main objective of this study is to promote the cassava wastewater, available in large amount, by using it as an inexpensive raw material to the preparation of culture medium for the mass production of spores and active molecules of *T. virens*, the agent for the bio-control of cocoa black pod disease. To this regards, fermentations of this microorganism were performed to determine the potential of the CW as ingredient of microbiological culture medium. Thus, two approaches have been developped for spore production: (i) liquid-state fermentation and (ii) solid-state fermentation on kaolin grains, and one for gliovirin production (iii) solid-state fermentation.

2. MATERIALS AND METHODS

2.1 Cassava Wastewater

The cassava wastewater was collected in Abidjan (South, Côte d'Ivoire) thanks to a group of women producers of "Attiéké". These women used to process an average of 200 kg of fresh cassava per week for 100 kg of Attiéké and for the same amount of untreated wastewater

discharged on site. A volume of 5 L of this fresh cassava wastewater is collected, sent to the laboratory and stored at 4°C to minimise any growth of microorganisms. The characteristics of the CW are the following: pH 4.3 ± 0.1; ratio C:N ≈ 34.25 and the following metals (Table 1).

Table 1. Metal characteristics of cassava wastewater

Parameter	Concentration (µM)
Cd	0.24
Cr	0.07
Mn	1.6
Fe	40.1
Ni	1.7
Cu	2.9
Zn	20.9

2.2 Fungal Material

T. virens strain (Fig. 1) was provided by the National Center for Agricultural Research (CNRA) in Ivory Coast [10]. It was cultured on agar of potato dextrose (PDA) in a 10 mL screw tubes for a period of 7 days. By the end of which, the tubes containing the culture of *T. virens* were stored at 4°C. The spore suspension was prepared as follows. On a 7-days-old mycelial cultures, 10 mL of sterilised distilled water were added with a sterile pipet. The surface of these cultures was scraped using a sterilised metal spatula. The solution obtained was homogenised in a test tube using a vortex to release spores in the water. This suspension was then filtered with muslin to remove the mycelial fragments. A spore suspension of 200 µL was taken and put in the wells of a Malassez hematimeter followed by a count of spores. This number was adjusted to 10^5 spores / mL per dilution with sterilised distilled water.

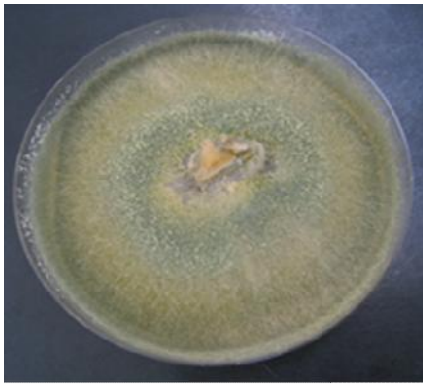


Fig. 1. *Trichoderma virens* cultured after 7 days on PDA

2.3 liquid-state Fermentation

Cultures media were obtained from CW after dilution (1, 1/2 and 1/4) with distilled water. The pH of these media was adjusted to 5.6 ± 0.2 before autoclave sterilisation. The fermentations were carried out in Erlenmeyer flasks (1L) containing 400 mL of culture medium (Table 2). After inoculation with 1 mL of the *T. virens* spore suspension (10^5 spores / mL) obtained as describe above, the flasks were incubated in a rotary shaker at 26°C ± 1 and 130 rpm for 120 hours. For each incubation, three samples (2 mL) were withdraw aseptically after 72 hours and 120 hours of incubation and then stored at 4°C for subsequent analyses.

Table 2. Method of dilution of cassava wastewater

Dilution	Water	Added volume (mL)
1	CW	400
	SDW	0
1/2	CW	200
	SDW	200
1/4	CW	100
	SDW	300

CW: cassava wastewater; SDW: sterilised distilled water

2.4 Solid-state Fermentation from Kaolin Grains

The kaolin bought on the market in Abidjan was pulverised with a grinder and sieved. A quantity of 500 g of the powder obtained was mixed homogeneously with a mixer in 500 mL of the CW at pH 5.6 ± 0.2. Grains were made from the paste obtained. These kaolin grains were placed in a container for 24 hours at room temperature to be dried. At the end of 24 hours, the kaolin grains are placed in petri dishes (50 g per petri dish) and then sterilised. These petri dishes are inoculated 4 mL of a spore suspension of *T. virens* at a concentration of 10^5 spores / mL. This spore suspension was prepared in sterile manioc waste water diluted 1/2. The dishes were subsequently incubated at 26°C ± 1 for 10 days. The tests were repeated 3 times.

2.5 Enumeration of Spores

Spore suspensions from the liquid state were obtained after filtering the culture medium with muslin so as to remove the mycelial fragments and spore concentrations were determinated according to the method described in 2.2. In the cases of solid state fermentation, spore

suspensions were obtained by homogenising a granule in 1 mL of distilled water. The concentration of spores was estimated by determining the average mass of a kaolin grain and given spores / g of kaolin. Three repetitions were made to perform the count.

2.6 Solid State Fermentation for Gliovirin Production and LC/MS Analysis

Three petri dishes (9 cm) containing 20 mL of sterilised solid culture medium prepared from CW (20 g of agar per 1 liter of cassava wastewater) were inoculated with 500 µL of spore suspension of *T. virens* at the concentration of 10^4 spores / mL. Petri dishes were incubated at 26°C ± 1 in the dark for 10 days. Controls for the production of gliovirin by *T. virens* were carried out on the PDA (20 mL per petri dish) inoculated and incubated as previously described. The tests were made in triplicate. After 10 days of incubation, cultures were extracted with ethyl acetate and the extracts obtained were dissolved in methanol to perform U-High Performance Liquid Chromatography (U-HPLC) coupled with mass spectrometry. Gliovirin (1 mg/mL) isolated from a culture of *T. virens* [11] was also analysed by LC/MS. Analysis was performed on an MAXIS II ETD © using a C18 column (Acclaim polar Advantage II PA2, ThermoScientific) equilibrated at 40°C. The mobile phase consisting of CH_3CN:H_2O (acidified with 0.1% formic acid) was used, starting at 5:95 for 2 minutes then increasing to 50: for 7 minutes, then at 90:10 in the space of 6 minutes, holding for 2 minutes, finally returning to the starting conditions in 4 minutes. For the mass spectrometry, the voltage was Es = 3500 V, the MS range: 100-1300 *m/z* and the acquisition speed of 2 Hz.

3. RESULTS AND DISCUSSION

3.1 Spore Production by Liquid State Fermentation and Solid State Fermentation on Kaolin Grains

Experiments were conducted in CW without any supplement. Under the 3 conditions of culture (dilution 1, 1/2 and 1/4), the growth and the production of spores by *T. virens* were observed. The results showed that after 120h of incubation, the fermented broths at dilutions 1 and 1/2 produced substantially the same concentration of approximately 10^6 spores / mL while a maximum concentration of approximately 1.73 x 10^8 spores / mL had been reached in the fermented broth at 1/4 dilution. In addition, the number of spores at this last dilution increased with the incubation time (Table 2) in contrast to broths fermented at the 1 and 1/2 dilution in which the number of spores did not vary. The rapid growth observed could be due to the fact that CW contains a source of carbon, easily metabolised by this fungus and a metal content necessary for its growth. Several parameters can influence the growth and spore production capacity of microorganisms in a broth. But the most important are the C: N ratio, the source of the carbon source and the glucose concentration [12]. CW ratio C:N of 34.25 could be responsible for conidiation ≥ 10^6 spores / mL. Indeed, studies on the variation of the C:N ratio made it possible to observe the decrease in the spore concentration with the decrease in the C:N ratio. In these studies, the ratios at 16 and 21 yielded concentrations of ≈10^6 and ≈10^8 spores / mL, respectively. High levels of spores may be due to cellulose, which is the main source of carbon in cassava wastewater. These results are in agreement with those of [13] which observed an increase in the number of spores when *Trichoderma viride* was grown on a substrate of cellulosic origin. The 1/2 dilution of CW did not give a significant effect on *T. virens* spore production, probably because of the high glucose concentration in the medium despite dilution. Glucose is easily used by *T. virens* as a carbon source. An increase in glucose in the culture medium can not only delay, but also decrease the conidiation due to high vegetative growth [4]. In contrast, a low glucose concentration could favor the production of spores. Thus, the fact that the maximum concentration was reached at a dilution of 1/4 could be due to a low concentration of glucose at this dilution.

For the cultures on kaolin supplemented with the CW, growth of *T. virens* was observed on all grains (Fig. 2). The count of spores showed a concentration of 1.13 x 10^6 spores / g of kaolin. This concentration was substantially the same as that obtained with the fermentation in the liquid state at dilutions 1 and 1/2.

T. virens was grown directly on kaolin grains supplemented with CW. In contrast to several studies in which kaolin was used in formulations as a carrier, the present study revealed that kaolin could serve as an inorganic carrier for spore production [14-15]. For growth on kaolin grains, *T. virens* metabolised the carbon source of CW and used the kaolin minerals. In addition to being a support for the formulation, kaolin could constitute a growth support for biopesticides.

Table 3. Spore concentration as a function of time and different dilutions of cassava wastewater

Dilution of cassava wastewater	Number of spores (spores / mL)	
	72 h	120 h
1	$1.8 \times 10^6 \pm 0.05$	$6.3 \times 10^6 \pm 1.09$
1/2	$2.8 \times 10^6 \pm 0.11$	$7.2 \times 10^6 \pm 0.87$
1/4	$1.6 \times 10^6 \pm 0.11$	$1.73 \times 10^8 \pm 0.24$

± SD Three repetitions were made to perform the count

Fig. 2. Aspect of growth of *T. virens* on kaolin grains supplemented with cassava wastewater

3.2 Solid State Fermentation for Gliovirin Production and LC/MS Analysis

Growth of *Trichoderma virens* was observed on both PDA (Fig. 3**A**) and CW agar medium (Fig. 3**B**). LC/MS analysis of these extracts revealed the production of gliovirin by *T. virens* grown on the CW agar. This production was demonstrated by the presence of the molecular ion peak *m/z* ($[M+H]^+$ 481.073) that corresponds to gliovirin in comparison to the control of gliovirin (Fig. 4**C**) and the production of gliovirin by *T. virens* on PDA (Fig. 4**B**). However, the peak intensity of gliovirin on CW agar medium (Fig. 4**A**) was not as high as that of gliovirin on PDA medium. The production of secondary metabolites by fungi is influenced by a set of stimuli among which, the source of carbon and, nitrogen [16]. As a diketopiperazin [17], gliovirin needs a source of nitrogen from amino acids. Yet, CWs are very poor in amino acids [18]. This could explain the low intensity of gliovirin in cassava wastewater.

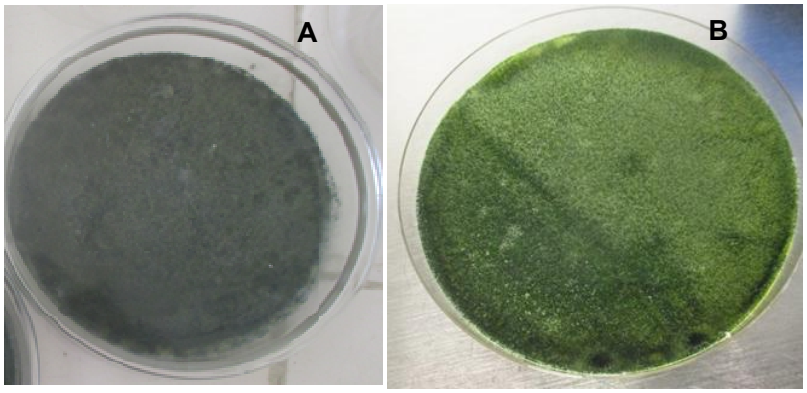
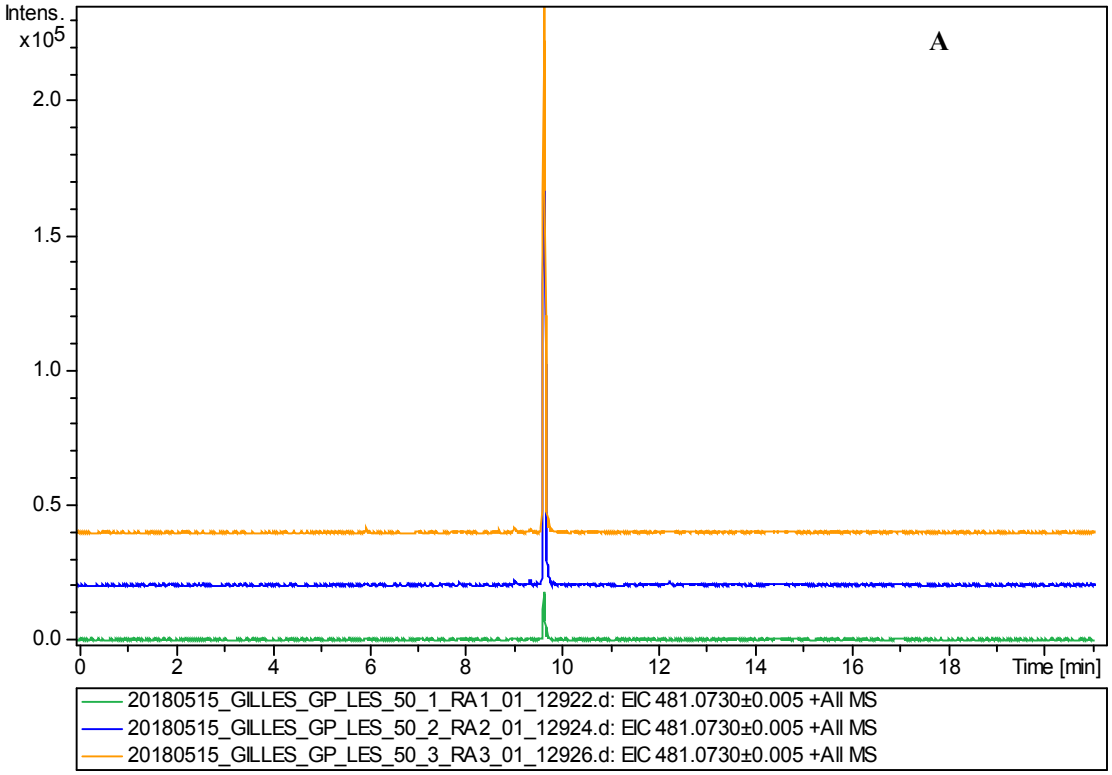


Fig. 3. *Trichoderma virens* cultured after 10 days on PDA (A)and on CW agar medium (B)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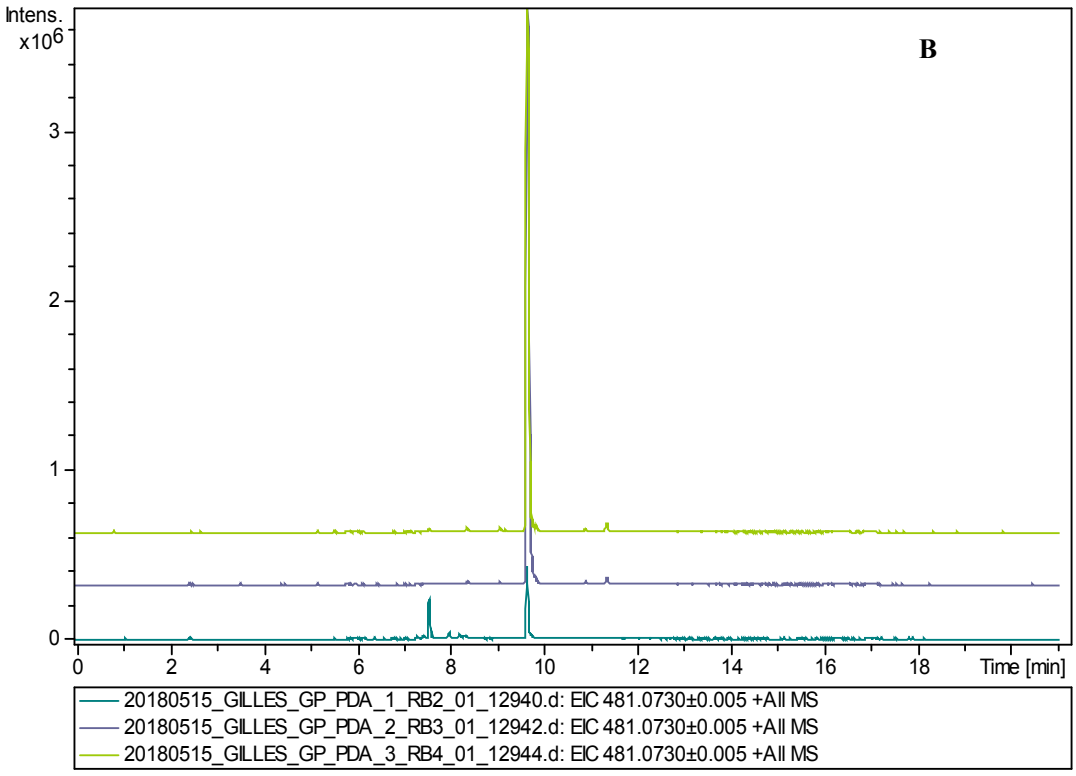
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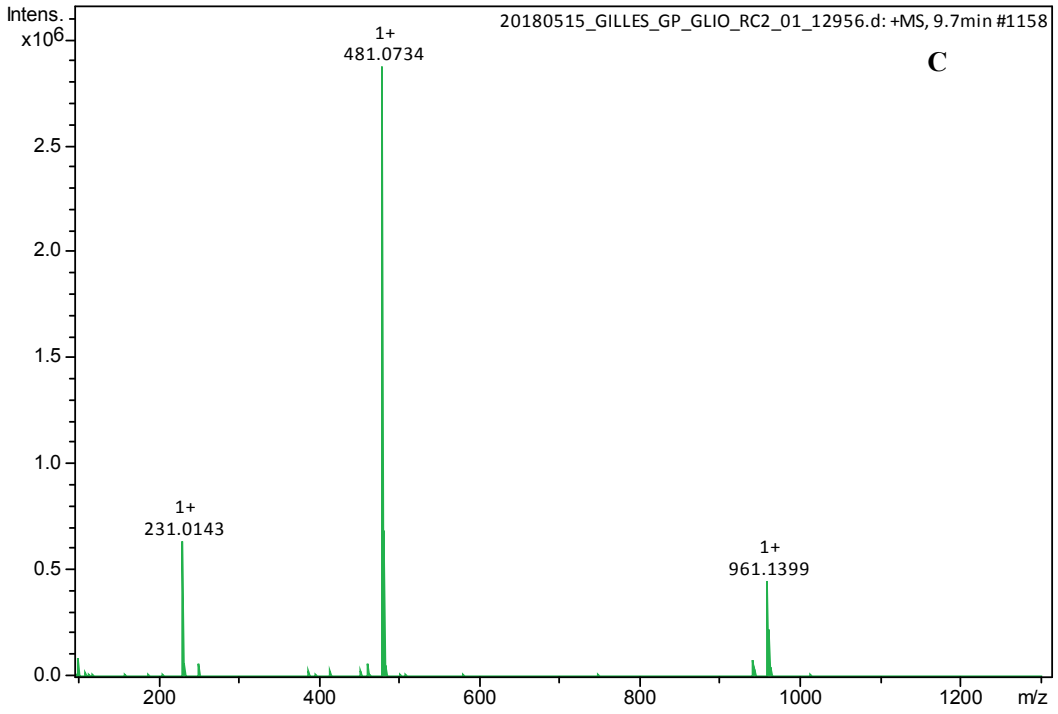

Fig. 4. Spectra showing gliovirin Extraction Ion Chromatogram (EIC) from CW agar medium (A), from PDA (B) and control gliovirin mass spectrum (C)

4. CONCLUSION

In this study the maximum concentration (≈10^8 spores / mL) indicated that cassava wastewater could be directly used for *T. virens* conidia production of in formulations. This work showed that wastewater could be recycled by using it as a very low cost substrate for cocoa black pods disease biocontrol agent production.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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