

**Original Research Article**

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## **Valorization of the Mucilage Juice of Cocoa Beans for the Production of a Biopesticide based on *Bacillus thuringiensis* var.*kurstaki* HD-1**

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### **A B S T R A C T**

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The use of microbial pesticides in agriculture has some advantages, but their production in non-conventional experimental tests constitute a challenge. This study purpose is to evaluate the potential of cocoa mucilage juice. The mucilage juice of cocoa is a substratum, that has been used as an alternative growing test and associated to bacteria such as *Bacillus thuringiensis* var. *kurstaki* HD-1 (Btk HD-1) for the production of a biopesticide. After determining the physico-chemical characteristics of the mucilage juice, different combinations have been made with 2% (M2%), 4% (M4%), 6% (M6%) and 7% (M7%) cane molasses were established, then inoculated with 2% of the inocula and incubated at 30 °C for 48 hours under agitation. The results showed that the substratum contained nitrogen (1.68g/L) and carbon (9.9 g/L), which has an important mineral such as potassium (87.3 mg/L), phosphorus (38 mg/L) and magnesium (25.42 mg/L). The growth of Btk HD-1 in cocoa mucilage juice with 2% molasses (M2%) was the highest ( $p < 0.0001$ ) with average concentration of  $1.6 \times 10^9$  CFU/mL for cells and  $2.3 \times 10^9$  CFU/mL for spores. This combination therefore offers good prospects for the production of the Btk HD-1 biopesticide.

## Introduction

The emergence of pests that are resistant to certain chemical pesticides reduces the effectiveness and sustainability of chemical control. World wide, particularly in Côte d'Ivoire, these pests are the cause of a significant reduction in agricultural production, resulting in economic losses. However, since independence, Côte d'Ivoire economy has been lean back on agriculture. For decades, chemical control has been used to ensure the phytosanitary protection of plants and thus increase agricultural yields. Moreover, chemical pesticides are suspected of having considerable harmful side effects on crops, human health and the environment. Of this fact, the use of biopesticides in agriculture is strongly recommended. There are several types of biopesticides, but those based on Btk HD-1 are the subject of this study. These biological pesticides have several advantages. They are biodegradable and have very low toxicity on living organisms and the environment (Silverio *et al.*, 2009). Also, they have a broad spectrum of action on several pests (Mondéjji *et al.*, 2015).

Biopesticides based on Btk HD-1 are usually some suspensions formulated from mixtures of spores and protein crystals synthesized by the bacteria after cultivation (Adjallé, 2009). Its insecticidal activity resides in the production of a parasporal protein crystal called delta-endotoxin which is formed during sporulation (Osman *et al.*, 2015). Btk HD-1 grows in a medium which is important in carbon, nitrogen and mineral salts (Valicente *et al.*, 2008). However, the use of bioinsecticides remains very limited due to the relatively high production costs and the low stability of the product in the time (Mounsef *et al.*, 2014). Indeed, several authors have purposed different by-products agricultural and agro-industrial that are

important in carbon and nitrogen such as soybean meal, maize starch, yeast extract, peanut flour, fish meal, sugar cane molasses, hydrolyzed sludge and waste water treatment sludge that have been successfully substituted for expensive media (Satinder *et al.*, 2007; Valicente *et al.*, 2010; Zhuang *et al.*, 2011) and including kitchen waste (Zhang, *et al.*, 2013). Côte d'Ivoire is an agricultural country and with the advent of new technologies aimed at the valorisation of bulky residues from industrial, agricultural or domestic activities (Gadji, 2017). It is opportune that the research on crop protection to explore the use of certain local products that are not exploited for the production of low cost bioprotection agents. According to studies by Anvoh (2013), cocoa mucilage juice is one of the most important by-products of cocoa abandoned on farms by farmers each year with a volume of more than 300,000,000 L.

The objective of this work is to evaluate the nutrient potential of cocoa mucilage juice, a local substratum, as an alternative growing medium for the production of biopesticide based on Btk HD-1. This approach will add value to cocoa cultivation and increase the income of the farmer.

## Materials and Methods

### *Bacillus thuringiensis* var. *kurstaki* HD-1

The bacterium, *Bacillus thuringiensis* var. *kurstaki* HD-1 (Btk HD-1), was isolated from the fermented broth of wastewater from starch industries. This fermented broth is obtained by bioreaction of this waste water and packaged in plastic bottles at the Laboratory of Bioconversion of Waste water and Sewage Sludge into High Value Added Products, of the National Institute for Scientific Research (INRS Eau-Terre-Environment), University of Quebec, Canada. The strain in the form of broth was provided under ATCC number

33679 by the Canadian Forest Service (CFS) Laboratory in Sainte-Foy (Quebec, Canada).

### **Collect and physico-chemical characterisation of the Mucilage Juice from Cocoa Beans (JMFC)**

An empty 50 kg filtsac branded nylon bag placed on pieces of wood was used to collect the beans surrounded by their white solid mucilage. After liquefaction the solid mucilage into liquid matter, the juice has been collected in a basin of capacity 10 L placed under the bag. It was transferred to a cooler of capacity 2.5 L hermetically sealed and then the sample obtained was placed in a big cooler (5 L) in which there was some ice. The sample has been transported to the Central Environmental Laboratory (LCE) of the Ivorian Anti-Pollution Centre (CIAPOL). It has been filtered with cotton wool to remove the suspension materials, pod debris and spine.

The physicochemical parameters of the mucilage juice were measured according to different methods recapitulate in Table 1 before their utilization as culture medium. The minerals were determined by atomic absorption spectrometry, using the slightly modified method described by Toundou (2016). Indeed, 10 mL of cocoa mucilage juice was mixed with 6 mL of a solution of chloridric acid (2N HCl) and 3 mL of sulphuric acid. The mixture was heated for 2 hours at 150°C. The solution (mineralise) obtained was then filtered and the concentrations of the minerals (Cu, Fe, Zn, Pt, Mg, Na, K, Mn, Ca) were quantified.

### **Biopesticide production based on Btk HD-1**

#### **Preparation of culture media**

The media for the production of Btk HD-1 have been conceived in the laboratory

according to the following principle: The mucilage juice was divided into different batches of 50 mL which were supplemented with different proportions particularly 2, 4, 6 and 7% of cane molasses (M) as an additional source of carbon, to constitute the media M2%: 50 mL JMFC + 2% M, M4%: 50 mL JMFC + 4% M, M6%: 50 mL JMFC+6% M, M7%: 50 mL JMFC + 7% M respectively. The mucilage juice (JMFC) without the addition of molasses was used as a control.

#### **Preparation of inocula and fermentation process**

The isolation of Btk HD-1 colonies was realized by streak seeding technique on trypticase soy agar (TSA) after 12 hours of culture at 30°C in 9 mL of trypticase soy broth from 1 mL of fermented starch industry waste water. The inocula were then prepared from isolated colonies of Btk HD-1 using the slightly modified technique of Mourin *et al.*, (2015). Indeed, four 250 mL glass bottles containing 50 mL JMFC each were supplemented with 2, 4, 6 and 7% of cane molasses. The different media (Control, M2%, M4%, M6% and M7%) were adjusted to pH 7.2 with sodium hydroxide and chloridric acid (NaOH, 1 N/ HCl 2N), sterilised at 121 °C for 15 min. They were each inoculated with an isolated colony of strain Btk HD-1. The preparations obtained were homogenized by vortexing and cultured in a shaker incubator (Biobase, China) at 250 rpm and 30°C during 12 hours. The mucilage juice without cane molasses, treated under the same conditions, served as a control.

The fermentation process was carried out according to the method of Valicente *et al.*, (2010). Indeed, five glass bottles of 250 mL capacity were prepared as previously. They were inoculated with 1 mL (2% v/v) of each of the media inocula. They were incubated at 30°C in orbital shaker (shaking incubator) at

250 rpm during 48 hours. Aliquots were collected regularly every 3 hours for the first 24 hours and every 6 hours until the end of the experiment to count viable cells and spores.

### Enumeration of viable cells and spores

The evaluation of the cells (vegetative form) and viable spores was done in accordance with the technique of direct seeding on agar medium carried out by Gadji *et al.*, (2016). This technique consisted of spreading 0.1 mL of the aliquots taken from the dilutions on trypticase soy agar (TSA) medium in a Petri dish. Then, the Petri dishes were incubated an oven at 30°C for 24 hours. After incubation, only results between 30 and 300 colonies were used to estimate the total number of viable cells in colony forming unit per millilitre (CFU/mL).

For the enumeration of viable spores, the dilutions were first heated in a water bath at 80°C for 15 min. An aliquot of 0.1 mL of these dilutions was spread on solid TSA medium in petri dishes. Each dilution was spread in triplicate. The petri dishes were then incubated at 30°C. Enumeration was realized after 24 hours incubation in the oven and results between 30 and 300 colonies were retained and expressed as CFU/mL (Zhang *et al.*, 2013).

### Determination of pH

The pH of each culture media, adjusted before sterilisation to 7.2 with 1N NaOH / HCl 2 N was determined and adjusted until the end of fermentation (48 hours) in the different media according to Valicente *et al.*, (2010). Each pH was determined in three repetitions for each medium and at different times.

### Statistical analysis of the data

The variability of cell and spore concentrations in each culture medium was

studied by an analysis of variance using the ANOVA method of Xlstat version 2016 software. Comparisons of the cell and spore averages of the five culture media (control, M2%, M4%, M6% and M7%) were realized by Tukey test with level significance 95%.

## Results and Discussion

### Physico-chemical characteristics of the mucilage juice of cocoa beans

Table 2 presents the physico-chemical characteristics, in particular the concentrations of total organic carbon (Ct), total nitrogen (Nt) and the C/N ratio of the cocoa bean mucilage juice (JMFC). Indeed, this juice contains 9.90 g/L of Ct and 1.68 g/L of Nt. His pH before fermentation is 3.4 and C/N ratio is 5.89. This juice also contains high levels of potassium (87.3 mg/L), total phosphorus (38.00 mg/L) and magnesium (25.42 mg/L). Sodium, calcium, iron, copper and zinc contents are 15.21, 5.47, 3.31, 0.80, 0.29 mg/L and manganese contents are less than 0.02 mg/L (Table 3).

### pH evolution during the production of Btk HD-1

Fig. 1 shows the pH changes during the production of Btk HD-1 in mucilage juice supplemented with cane molasses. The results showed a variation of pH in the different media after 48 hours of fermentation. A decrease of pH was observed in all media during the first 6 hours of fermentation with decreases of 0.75, 1.16, 1.24, 0.95, 1.08 respectively. After this period, and until the end of the process, a progressive evolution of pH was observed in the M2% and M4% culture media to reach 8.47 and 8.07 respectively and a pH decrease in the M6% and M7% media (6.3 and 6.2 respectively); the pH in the control stayed constant and near of the neutrality. The Tukey test at the 5% level shows a significant difference with p <

0.001 between the control, M2%, M4% and the M6% and M7% media.

### Production of BtkHD-1 cells in different media during fermentation

In this study, different cell growth profiles were observed in the different media tested, the results showed a variation of cells during the 48 hours of fermentation (Fig. 2). During fermentation, cell production by the Btk HD-1 increase in the different media (Control, M2%, M4%, M6% and M7%) between 0 and 3 hours. The respective average were  $3.4 \pm 0.056.10^6$  CFU/mL for the control,  $3.3 \pm 0.054.10^6$  CFU/mL for M2%,  $4.2 \pm 0.068.10^6$  CFU/mL for M4% and  $3.2 \pm 0.05.10^6$  CFU/mL for M6% at 3 hours of fermentation. After this period, cell production by the Btk HD-1 seemed to be stable in the five media until 18 hours of fermentation, at this time the medium M2% has reached a maximum production with a respective average of  $1.7 \pm 0.03.10^8$  CFU/mL. After 18 hours, the cell production of Btk HD-1 in the M2% medium increased slightly compared to the other four media (Control, M4%, M6%, M7%), which decreases until the end of the fermentation process. Moreover, the media with lower cell production were M6% and M7%. The respective average were  $1.6 \pm 0.026.10^9$  CFU/mL for M2% compared to  $9 \pm 0.15.10^6$  CFU/mL for the control,  $3.3.10^7 \pm 0.05.10^7$  CFU/mL for M4%,  $2.2 \pm 0.04.10^5$  CFU/mL for M6% and  $2 \pm 0.03.10^5$  CFU/mL for M7%.

The statistical analysis of the data by a Tukey test at the 0.95 level showed a significant difference with  $P < 0.0001$  for cell production by Btk HD-1 in the different media tested.

### Production of viable spores of Btk HD-1 in the different media during fermentation

The results showed a variation of spores in the five media during the 48 hours of fermentation (Fig. 3). Spore production in the different media did not start at the same time. It started at 9 hours of fermentation for the control, 12 hours for the media (M2%, M4%) and finally 15 hours for the media (M6% and M7%). The respective average were  $2.1 \pm 0.035.10^6$  UFC/mL for the control,  $2.3 \pm 0.038.10^6$  UFC/mL for M2%,  $2.4 \pm 0.04.10^6$  UFC/mL for M4%,  $2.7 \pm 0.044.10^5$  UFC/mL for M6% and  $2 \pm 0.031.10^5$  UFC/mL for M7%. After these periods, spore production by Btk HD-1 in the media (Control, M2%, M4%, M6%, M7%) was similar until 30 hours of fermentation. After this period, only the M2% medium show high spore production until the end of the experiment (48 hours), with an average  $2.3 \pm 0.037.10^9$  CFU/mL. At the same moment, the spores production was decrease in the media (Control, M4%, M6%, M7%). The statistical analysis of the data by Tukey test with level significance 0.95 ( $P < 0.0001$ ) showed a significant difference in spore production by Btk HD-1 in the different media tested.

**Table.1** Methods for the analysis of the physico-chemical parameters of the mucilage juice of the Cocoa beans (Toundou, 2016; Gadji *et al.*, 2016)

Parameters	Methods	References
pH	Electrometric method	CEAEQ (2003)
Total organic carbon (Ct)	Walkley-Black method	Martinez-Chois (2012)
Total nitrogen (Nt)	Colorimetric method	CEAEQ (2014)
Cu, Fe, Zn, Pt, Mg, Mn, Na, K, Ca	Dosing by spectrometry atomic absorption	Toundou (2016)

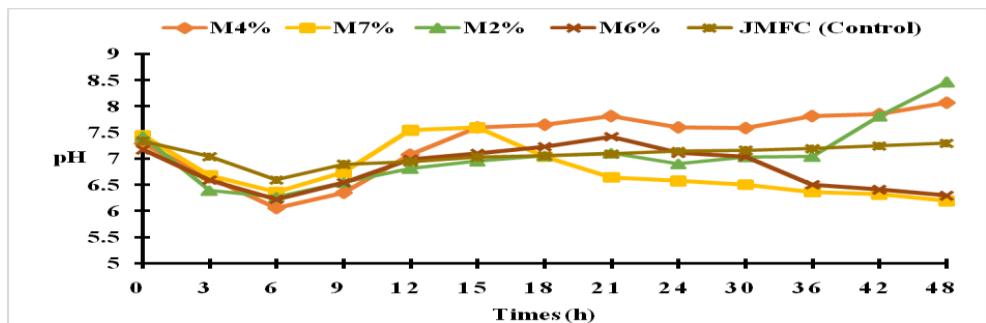
**Table.2** Physico-chemical characteristics of the mucilage juice of cocoa beans

Parameters	Values
pH	3,4
Total organic carbon (g/L)	9.90
Total nitrogen (g/L)	1,68
C/N ratio	5,89

**Table.3** Content in minerals (mg/L) of the mucilage juice of cocoa beans

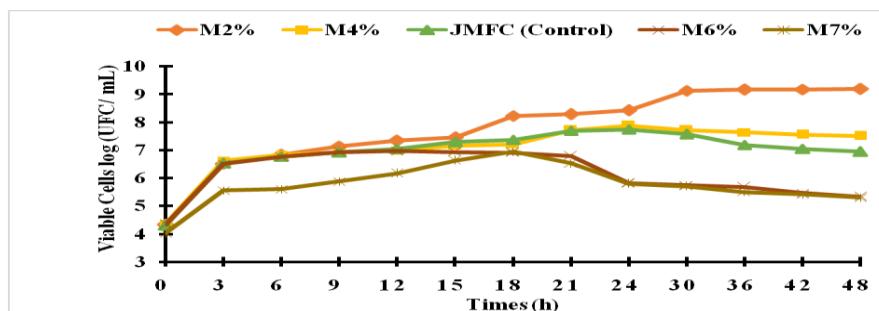
Parameters	Teneur (mg/L)
Copper (Cu)	0,80
Iron (Fe)	3,31
Zinc (Zn)	0,29
Total phosphorus (Pt)	38,00
Magnesium (Mg)	25,42
Sodium (Na)	15,21
Potassium (K)	87,30
Manganese (Mn)	< 0,02
Calcium (Ca)	5,47

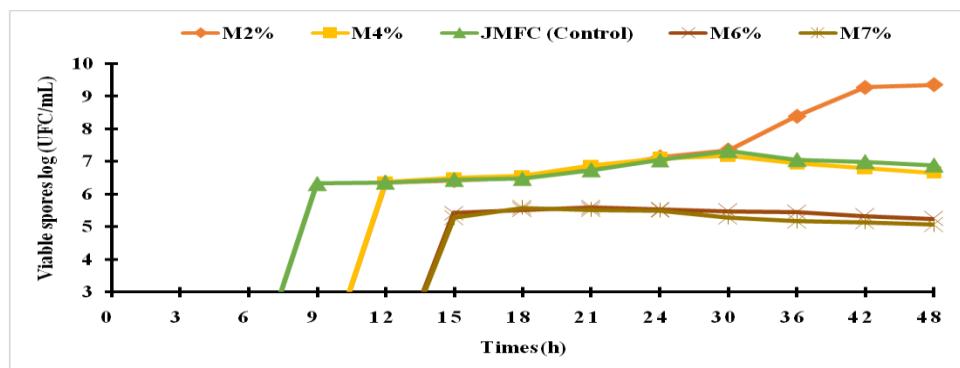
**Fig.1** pH variation during fermentation of Btk HD-1



JMFC (50 mL): Control, M2%: JMFC + 2%M, M4%: JMFC + 4%M, M6%: JMFC + 6%M, M7%: JMFC + 7%M

**Fig.2** Growth evolution profiles of Btk HD-1 cells during fermentation in media



**Fig.3** Growth evolution profiles of Btk HD-1 spore during fermentation in media

### Physico-chemical characteristics of the mucilage juice of cocoa beans

The mucilage juice of cocoa beans contains elements that can be used as a substratum for the culture of the Btk HD-1 bacteria. These elements are nitrogen, carbon and minerals (Mg, Na, Fe, Ca, K, P) which are important for growth and sporulation of this bacteria, as indicated by Gadji *et al.*, (2016). The results obtained in the present study are different from those of Anvoh (2013).Indeed, the concentrations of potassium (87.3 mg/L), total phosphorus (38 mg/L), magnesium (25.42 mg/L) obtained in the cocoa mucilage juice tested in this study are much lower than those obtained by this author who found some concentrations of 950 mg/L for potassium, 62.47 mg/L for total phosphorus and 82 mg/L for magnesium. The differences in concentrations would certainly be due to the use of agricultural inputs, soil type, the cocoa clones present and the region of origin of the cocoa samples. This was also reported by Gadji *et al.*, (2016) in their study on cocoa pericarp. In addition, the carbon-nitrogen ratio (C/N) plays an important role in growth, sporulation and protein (delta-endotoxin) production by Btk HD-1(Stephanie *et al.*, 2014).The value obtained in this study is similar to the value (5.74) of Vidyarthi *et al.*, (2002)in unenriched sewage sludge.

However, this value is extensively below of those reported by several other authors in different sources, particularly 10 in soy flour (Stephanie *et al.*, 2014), 12.87 from waste water from starch industries(Vu *et al.*, 2009)and finally, 56.90 in cocoa pericarps (Gadji *et al.*, 2016).

### pH evolution during the production of Btk HD-1

The pH evolution profiles studied in the different media showed a variation, she would due to the consumption of the mucilage juice from the cocoa beans and to changes in metabolic activity. Indeed, the decrease in pH observed in the media from their initial pH of around 7 to 6 during the first 6 hours could be explained by the use of carbohydrates by the Btk HD-1 bacteria causing an acidification of the culture media (Barje *et al.*, 2012; El fels *et al.*, 2014). The values obtained during our work were similar in terms of the pH decrease at the beginning of the fermentation process in the different media to those of Valicente *et al.*, (2008), in the context of their work on the evaluation of carbon and nitrogen-rich media for biopesticide production. Also, they were similar to those of Zhang *et al.*, (2013) in their study on kitchen waste as a fermentation substrate and finally to those of Salazar-Magallon *et al.*, (2015)in their study on the

use of industrial by-products for the production of Btk HD-1. These authors observed a drop in pH ranging from 7 to 6 during the first 10 hours of fermentation. After this period, and until the end of the process, the progressive increase in pH in the M2% and M4% culture media would due to a bacteria hydrolysis of nitrogen with production of ammonia associated with the degradation of proteins and the decomposition of organic acids. The values obtained were similar to those of Montiel *et al.*, (2001) and Vidyarthi *et al.*, (2002). These authors found pH 8 and 8.5 at the end of fermentation in the case of using sewage sludge for the growth of Btk HD-1. The pH stabilization observed until the end of fermentation in the control could be explained by an equilibrium between the sources of carbon and nitrogen in this medium. This result (7) is similar with that of Valicente *et al.*, (2008) in the different media (1.0% corn juice + 3.0% soybean powder and Luria Bertani + salts).

### **Production of BtkHD-1 cells in different media during fermentation**

The high cell production during the first hours of fermentation (0 and 3 hours) is below that of Gazali *et al.*, (2019) in their study of cell production by Bt berliner in natural media. These authors obtained average included between  $5.9 \cdot 10^{11}$  to  $9.8 \cdot 10^{11}$  cell/mL for the media tested. This difference in value would probably be related to the Bt strain used and the composition of the culture media. Indeed, the rapid production of cells after 3 hours of fermentation in both cases demonstrates the influence of the composition of the medium on cell production. This constat was also reported by Gazali *et al.*, (2019). The exponential cell growth time in this study was shorter than that of Ndao *et al.*, (2019) using industrial wastewater. These authors obtained an exponential growth that lasted 4 times longer than ours. The stability of cell

production observed after this period until 18 hours of fermentation would certainly be due to a decrease in nutrients in the culture media. At this time, the peak observed in the M2% medium would be due to a medium that is still more favourable to the growth of Btk HD-1 than the others. After this period, the decrease in cell production observed until the end of the experiment in almost all media would be strongly due to a total poverty of nutriments in the media or to cell lysis. Moreover, the results obtained at the end of fermentation in the media (Control, M4%, M6%, M7%) were lower than those of Vidyarthi *et al.*, (2002) in terms of the number of viable cells. These authors obtained  $8 \cdot 10^8$ ,  $6.2 \cdot 10^8$ ,  $5.8 \cdot 10^8$  UFC/mL in three different media (Soy yeast extract, soybean meal, waste water sludge).

### **Production of viable spores of Btk HD-1 in the different media during fermentation**

Concerning the production of spores, it did not all start at the same time in the media. In the control it began during the first hours of fermentation (9 h) compared to the M2%, M4% and M6%, M7% (12 and 15 h) media. This difference would be due to a more rapid consumption of the bacteria (Btk HD-1) from carbon (C) and nitrogen (N) sources present in small quantities. This constat was also reported by Barnabé (2004) in his study of the use of sewage sludge as medium of fermentation. Spore production is stable in the media from 9 hours to 30 hours of fermentation. It could be due to a poverty of the media in nutriments. After 30 hours of fermentation, the M2% medium was the most favourable for spore production by Btk HD-1. This indicates that the sporulation process worked more efficiently in this medium than in the others. The result obtained for M2% ( $2.3 \pm 0.037 \cdot 10^9$  CFU/mL) was high compared to Yezza *et al.*, (2005) and Mourin *et al.*, (2015). The first authors obtained  $5.4 \cdot 10^8$  and  $7.9 \cdot 10^8$  CFU/mL in the untreated

and treated wastewater as fermentation substrate and the second  $7.1 \cdot 10^6$  CFU/mL in the molasses-enriched soy yeast extract medium. This difference would probably be due to the amount of inoculum used for fermentation.

In conclusion he stands out of this study that the JMFC contains the necessary nutriments to support the growth and sporulation of Btk HD-1. Enrichment of this substrate with (2% v/v) molasses improved the cellular and sporulation performance of Btk HD-1. The number of cells and spores obtained at the end of the 48 hours of fermentation was  $1.6 \pm 0,026 \cdot 10^9$  CFU/mL and  $2.3 \pm 0,037 \cdot 10^9$  CFU/mL respectively. In view of these results, and until the efficacy of the fermented broths is evaluated on plant pests, the JMFC is a suitable substratum for the culture of this bacterium. Thus, the use of this juice for the production of *Bacillus thuringiensis kurstaki* var. HD-1 (Btk HD-1) represents a new alternative for the valorisation of this cocoa waste and can contribute to reduce the production costs of Btk HD-1-based biopesticide.

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