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Use of Sugar Cane Molasses Enriched with Yeast Extract for the Production of Biopesticide from *Bacillus thuringiensis* var. *kurstaki* HD-1

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A B S T R A C T

Bacillus thuringiensis subspecies *kurstaki* HD-1 (*Btk HD-1*) has been widely used for five decades as a leading biopesticide for its ecological action. However, this biopesticide often has challenges related toits costs of production and its formulation. The purpose of this work was to evaluate a novel low-cost *Btk HD-1*-based biopesticide production method using sugar cane molasse enriched with yeast extracts. After determining their content, Four growth media containing variable proportion of molasses and yeast extracts were formulated, then inoculated with (*BtkHD-1*) and incubated to 30 ± 1 °C. The results showed that the cane molasses were enriched in minerals such as Mn (142.08 mg/kg), Cu (144.1 mg/kg), Fe (108.18 mg/kg), Ca (97.2 mg/kg), K (79.7 mg/kg), Mg (73.96 mg/kg), Na (51.24 mg/kg) and Zn(17.7 mg/kg). Moreover, the quantity of yeast extracts had an impact on the growth rate and sporulation of Bt. Overall, the media with 200 g of yeast extract was the most suitable growth medium *for Btk HD-1*.Sugar cane molasses-based culture medium enriched with yeast extract could replace soy flour for its availability and low-cost.

Introduction

Food safety remains a challenge for the countries in sub-Saharan Africa due to sustained population growth, overcrowded urban centers, and the age pyramids in which the youth dominates. Meeting the food needs requires among other things, cutting down on

production losses in order to increase agricultural yields. In this context, several approaches have been implemented to address the issue of food security in Africa. The main approach so far has been the use by farmers of pesticides. Deravel *et al.,* (2014) have shown that chemical safety issue demands an integrated strategy of crop pests and vectors of plant diseases control combining different techniques and methods. There are different types of biopesticides including anti-microbial pesticides (bacteria, viruses, protozoa, or fungi) and anti-vectoral pesticides that have less to no side effects on the environment. Chief among them is *Bacillus thuringiensis* (*Bt*). It is the most used biological toolin the control of pest type insects (Ndao *et al.,* 2017). A key feature of that bacterium is the secretion of biodegradable protein inclusion bodies (δ-endotoxins), and the secretion of other anti-virulence factors that are active against certain pest type insects and other plant pathogens (Bravo *et al.,* 2007; Zhou *et al.,* 2008; Arora *et al.,* 2013). Biopesticides derived from *Bt* are preparations obtained from the fermentation of organic matter in a bioreactor. And their use in agriculture appears particularly promising (Vu, 2009). Organic wastes used in the process of making these preparations contain enough carbon, and nitrogen, and mineral salts sources to sustain the growth and sporulation of *Bt* as well as the production of the δ-endotoxins (Mounsef *et al.,* 2014). The recycling of organic wastes into high value-added products is a solution to the puzzling agro-industrial waste management issues. Usually, *Bt*-based biopesticides are commercially produced using synthetic media containing soy flour, fish flour, glucose, yeast extracts, peptone, and oligo-elements. And sometimes other ingredients are added to improve the sporulation process during production. Despite their benefits of *Bt*-based biopesticides, their use in comparison to chemical pesticides remains limited due to the high production cost and the short shelf life (Mounsef *et al.,* 2014). Therefore, selecting less expensive growth media and/or raw materials seems acritical stepto commercially produced an affordable *Bt*-based biopesticide. Several raw materials (sub-industrial and agricultural products) were tested as alternatives to the synthetic growth media currently used. In a recent study, Gadji *et al.,* (2016) demonstrated that cacao tree pods could be used as growth medium for *Bacillus thuringiensis* var. *Kurstaki* HD-1 (*Btk HD-1*). Such use should it become widespread, could improve the income of local cacao producers. Sugar cane molasse (*Saccharum officinarum*) is abundantly produced by sugar cane processing facilities located in NorthernCôte d'Ivoire. It is considered an industrial byproduct of no value, and accordingly dumped as waste into the environment. This contributes to the pollution and degradation of aquatic ecosystems. In this study, this industrial by-product was tested as a growth medium with the aim of formulating a lower cost *Btk HD-1*-based biopesticides.

Materials and Methods

Collection of sugar cane molasse and *Bt*microbial strains

Sugar cane molasses was provided by the sugar cane processing facility of Borotou-Koro sugar complex that belongs to the SUCRIVOIRE firm. Located in the north-west of Côte d'Ivoire, this factory is 650 km from Abidjan. The strain of*Bacillus thuringiensis subspecies kurstaki* HD-1 (ATCC number 33679)was kindly provided by the laboratory of the Canadian Forest Service (CFS) in Sainte-Foy (Quebec, Canada).This variety of *Bt*is the commonly used strain in the biopesticides' industry due to its potential toxicity against a wide array of Lepidoptera. The strain is grown on trypticase

soy agar (TSA) at 30ºC for 12 h in an incubator (Imperial II incubator). The subculture is stored at 4ºC in the refrigerator for further use.

Physical and chemical analysis of the sugarcane molasse

Several parameters were measured in the sugarcane molasses using the methods highlighted in table1 before its use as a culture medium. Sugarcane molasses are loaded with organic materials. Therefore, a step of prior mineralization was required before analysis in order to limit the organic materials related-interference. The mineralization method was described in the ISO 5961 (1994) standard. It consists of introducing 100 mL of each homogenized sugar cane molasses sample into a 250 mL beaker. To this 100 mL sample, 1 mL of nitric acid (65 %) and 1 mL of hydrogen peroxide (32 %) were added. This mixture was heated on a hot plate at a temperature of 80 °C and evaporated to approximately 0.5 mL.

The sample must not be completely evaporated to avoid screwing up the sample. The residue of 0,5 mL is dissolved into 1 mL of fresh nitric acid (65 %) and 5 mL of ultrapure water. This mixture is transferred to a 100 mL bottle and made up to a volume of 100 mL with ultrapure water. This solution is then filtered through a 0.45 µm diameter filter. Finally, the filtrate is used for the determination of trace metal concentrations using a Varian AA20 Atomic Absorption Sspectrophotometer. The detection limit for traces of metals was 0.0005 mg/L.

Inoculum preparation and fermentation

Inan Erlenmeyer flask of 1L, 520mLof sugarcane molasse was diluted with 480mLof hot distilled water (60 ºC). The resulting mixture was put on a rotating plate to achieve a homogenized solution (Ammor, 2012) that was sterilized in a water bath at 80 ºC for 15 minutes to prevent the sugars from caramelizing and giving toxic vapors in the presence of phosphate(Kessas *et al*., 2012). A trypticase soy broth (TSB) was autoclaved at 121 ° C for 20 minutes, while sugar cane molasse was sterilized in a water bath at80 ºC for 15 minutes as described above. The pH of the sterile sugar cane molasse and the sterile TSB were adjusted to 7.0 using a solution of sodium hydroxide (NaOH) 1N and a pH indicator. A colony of the pure *Btk HD-1*strainwaslevied in sterile conditions and grown in 100 mL of trypticase soy broth (TSB)under agitation (300 rotations/min) at 30 °C for 8 to 12 h. Then 2 mL corresponding to 2% (v/v) of the culture was transferred in another Erlenmeyer flask containing 100mLof sterile sugar cane molasse. The latter was incubated under the same conditions to generate the *Btk HD-1*inoculum

Fermentation process

The fermentation process was achieved in 4Erlenmeyer flasks, labeled M_0, M_5, $M_{10}$and M_{15}, containing 50 mL of sterile sugar cane molasse each. Some yeast extracts were added to the content of M_5, $M_{10}$and M_{15} to the respective concentrations of 100 mg/L; 200 mg/L and 300 mg/L.M_0served as a no-yeast extracts control. The 4 flasks were inoculated with 2 % of the *Btk HD-1*inoculum, and incubated in a shaking incubator (Biobase) at 30 ± 1 °C for 48 h at 300 rotations/min.

Small aliquots of 2 mL of each of the fermentation media were taken every 3 hours for 48 h to assess the total number of spores and viable cells over time. Viable cell number and spores concentration data collected at each sampling time allowed the drawing of curves of evolution of these microbial products during the 48 hours of fermentation.

Assessment of the number of viable cells and spores

Time course aliquots from the fermentation step were serially diluted (10-fold dilutions) by adding 0.5 mL of the corresponding sample to 4.5 mL of sterile saline water (NaCl0.85%) as described by Gadji *et al.*, (2016)to generate 10 tubes of dilutions. The dilutions were thoroughly mixed with a vortex between two consecutive dilutions. From the last 4 dilution tubes, 0.1 mL of each dilution was directly spread on 3 TS agar (TSA) plates per dilution. The Petri dishes were incubated at 30 °C for 20 hours. Only dishes with a number of colonies ranging from 30 to 300 colonies were selected to assess the total number of viable cells per mL also called Colony Forming Units (CFU/mL) (Barnabé, 2000). To count the number of spores, the remaining part of the last 4 dilutions was heated in a water bath at80°C for 15 min (Barnabé, 2000). An aliquot of 0.1 mL of these heated dilutions was spread on TSA plates. Per dilution, 2 TSA plates were made, and incubated at 30 °C for 16 hours. Only TSA plates containing 30 to 300 colonies were selected to assess the number of spores CFU/ml (Barnabé, 2000).

Statistical analysis

The data obtained at the end of fermentation were subjected to a single-factor ANOVA analysis for paired multiple comparison tests for the different culture media. Xlstat version 2016 software was used to perform these tests. Thus, the Tukey method withlevel of significance 5% was applied to all possible pairs of differences.

Results and Discussion

Physical and chemical features of the sugarcane molasse

Total phosphorus concentration was 580 mg/kg. The carbon (C) and nitrogen (N) contents of the sugar cane molasse used in this study were respectively 564,000 mg/kg and 46,300 mg/kg (total indicated values). The pH of the molasse before the fermentation process was 5.1, and the C/N ratio was 12.8 (table 2). These results revealed the presence of minerals such as manganese (142.08 mg/kg), copper (114.10 mg/kg), iron (108.18 mg/kg), calcium (97.2 mg/kg), potassium (79.7 mg/kg), magnesium (73.96 mg/kg), sodium (51.24 mg/kg), and zinc (17.7 mg/kg) (table 3).

According to Tukey's test (table 4), all pairs of culture media seem to be significantly different; (M_0, M_5); (M_0, M_{10}); (M_0, M_{15}); (M_5, M_{10}); (M_5, M_{15}); (M_{10}, M_{15}); (M_0, M_5); (M_0, M_5). These culture media containing the sugar cane molasses and having received different quantities of yeast extract do not have the same content of spores and cells at the end of fermentation.

Figure 1shows the growth rate of*BtkHD1* in the different growth media tested. *Btk HD1* showed good growth and good use the nutrients available in all the media used. The lag phase of the growth curve was missing for all five media. The bacterium multiplied much faster in M_{10} medium compared to the other media up to 21 hours of fermentation. The cell concentration was 7.1 $\pm 3.5\times10^{11}$ CFU/mL. For the M_5 medium, the cell concentration was $9.9 \pm 4.9\times10^{10}$ CFU/mL for the same fermentation time. However, samples containing 300 mg/L of yeast extract and no yeast extract recorded cell concentrations of $3 \pm 1.5\times10^{7}$ CFU/mL and $7.9 \pm 3.9\times10^{10}$ CFU/mL respectively after 21 hours of fermentation. At the end of the first day of fermentation, a decrease in the cell concentration was observed for media containing 100 mg/L; 200 mg/L; 300 mg/L of yeast extract with recorded values of $1.5 \pm 0.7\times10^{10}$ CFU/mL; $7.1 \pm 3.5\times10^{9}$ CFU/mL; $2.8 \pm 1.4\times10^{8}$ CFU/mL, respectively. For the control medium, the variation in cell

concentration was different. Indeed, an increase in cell biomass was recorded up to 36 hours of fermentation ($9 \pm 4.5\times10^{11}$ CFU/mL). It then decreased until the end of fermentation ($4.1 \pm 2\times10^{4}$ CFU/mL). Viable cells peaked at 36 hours incubation ($8.9 \pm 4.5\times10^{11}$ CFU/mL) for the medium without yeast extract (M_0) and much slower than the M_{10} medium ($7.1 \pm 3.5\times10^{11}$ CFU/mL) at 24 hours incubation. These two media contained the highest viable cell loads compared to M_5 medium ($4.5 \pm 2.2\times10^{10}$ CFU/mL) at 30 hours of fermentation and M_{15} medium ($2.8 \pm 1.4\times10^{8}$ CFU/mL) at 24 hours of fermentation. At the end of fermentation, the M_5 medium recorded the highest cell turnover ($8.8 \pm 4.4\times10^{8}$ CFU/mL) while the M_{10} medium recorded $2.1 \pm 1.0\times10^{7}$ CFU/mL. Viable cell concentrations were $3.3 \pm 1.6\times10^{5}$ CFU/mL and $4.1 \pm 2\times10^{4}$ CFU/mL for M_{15} and M_0 media.

In all media, sporulation began 6 h after the start of fermentation. From 9 h until the end of fermentation, the spore concentration of the M_{10} medium increased steadily (from $1.4 \pm 0.7\times10^{6}$ CFU/mL to $10^{12} \pm 0.5\times10^{12}$ CFU/mL). However, for the other media (M_0; M_5; M_{15}), a significant variation in spore concentration was observed over time. Indeed, from 12 h to 24 h of fermentation, the M_{15} medium experienced a decrease in spore concentration (between $1.7 \pm 0.8\times10^{8}$ CFU/mL and $2.8 \pm 1.4\ 10^{9}$ CFU/mL). At 24 h of fermentation, the control medium M_0 recorded a spore concentration of $4.3 \pm 2.1\times10^{11}$ CFU/mL while the spore concentration of the medium M_5 was $8.5 \pm 4.2\times10^{9}$ CFU/mL at 30 h of fermentation. At the end of the fermentation (48 h), the media M_{15} ($4.6 \pm 2.3\times10^{12}$ CFU/mL), M_0 ($1.3 \pm 0.6\times10^{12}$ CFU/mL) and M_{10} ($1 \pm 0.5\times10^{12}$ CFU/mL) sporulated much more than the medium M_5 ($8.6 \pm 4.3\times10^{11}$ CFU/mL) (Figure 2).

Table.1 Methods for the physical and chemical analysis of the sugarcane molasses

Parameters	Methods	References
pH	Electrometric Method	(CEAEQ, 2003)
Total Organic Carbon (CT)	Method of Walkley-Black	(Martinez-Chois, 2012)
Total nitrogen (NT)	Colorimetric Method	(CEAEQ, 2014)
Total phosphorus (P)	Ion chromatography	(Toundou, 2016)
Cu, Fe, Zn, Mg, Mn, Na, K, Ca	Atomic Absorption Spectrometry (AAS)	ISO 5961 (1994)

Table.2 Physical and chemical features of the sugarcane molasses

Parameters	The values
pH	5.1
Total carbon (mg/kg)	564,000
Total nitrogen (mg/kg)	46,300
Total phosphorus (mg/kg)	580
Ratio carbon / nitrogen (C/N)	12.8

Table.3 Mineral contents of the sugarcane molasses

Minerals	Levels (mg/kg)
Calcium	97.2
Potassium	79.7
Magnesium	74.0
Sodium	51.2
Copper	114.1
Iron	108.2
Manganese	142.1
Zinc	17.7

Table.4 Comparison of environments (M_0; M_5; M_{10}; M_{15})

Mid / Tukey (HSD) / Analysis of the differences between the terms with a 95% confidence interval (H-48):						
Contrast	Difference	StandardizedDifference	Critical value	Pr >Diff	**Significant**	
M_5 vs M_0	4,33	35,35	3,20	< 0.0001	**Yes**	
M_5 vs M_{15}	3,43	27,96	3,20	< 0.0001	**Yes**	
M_5 vs M_{10}	1,62	13,27	3,20	< 0.0001	**Yes**	
M_{10} vs M_0	2,70	22,08	3,20	< 0.0001	**Yes**	
M_{10} vs M_{15}	1,80	14,70	3,20	< 0.0001	**Yes**	
M_{15} vs M_0	0,90	7,39	3,20	0,00035288	**Yes**	
Critical value of d of Tukey:			4,53			

Fig.1 Evolution of the number of viable *Btk HD-1* cells grown on M_0; M_5. M_{10}; M_{15} during the fermentation process

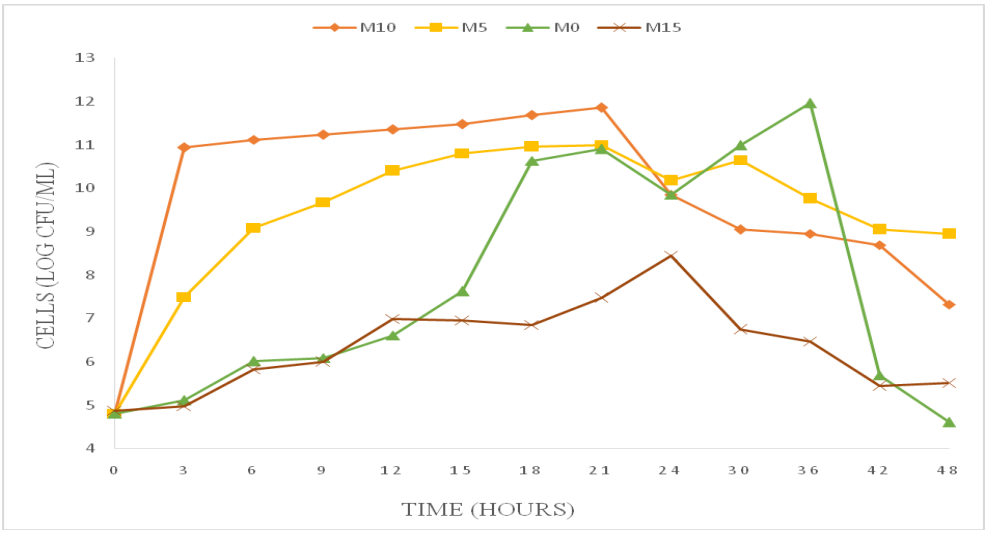
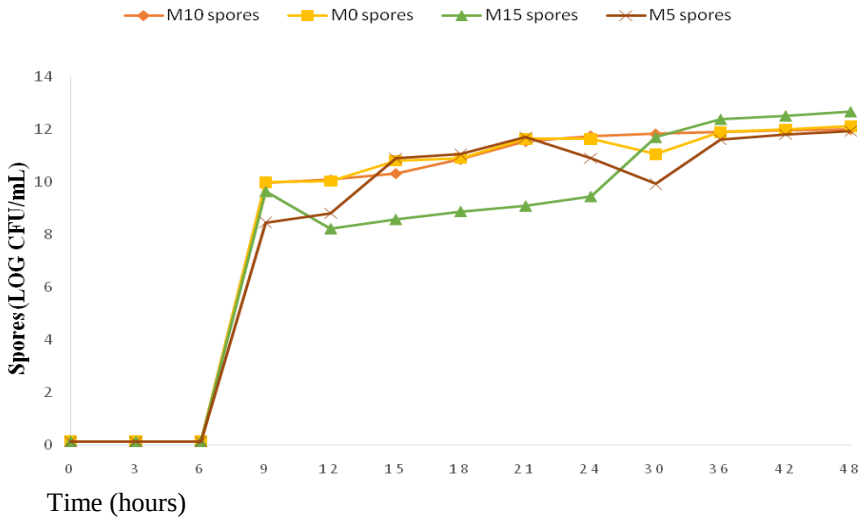


Fig.2 Evolution of the concentration of spores of *(Bt)* in environments M_0; M_5. M_{10}; M_{15} during the fermentation



Cane molasses is the waste of the sugar manufacturing process from sugarcane. It is considered to be an inexpensive carbon source for mineral fermentation. The results of analyses carried out on sugar cane molasse show that this agro-industrial residue is rich in minerals. This strong presence of minerals could be explained by the cultivation of sugarcane on soils rich in these minerals (Yao, 2019). This chemical composition depends on the type of soil, the variety of sugarcane, the transformation process used and the yield of the sugar mill (Orobinski and Serboulov, 1989). This agrees with data published by Archimedes *et al*., (2011) on the same fermentation substrate. The present study data demonstrate that local sugarcane molasse contains five metal ions (Mg^{2+}; Mn^{2+}; Fe^{2+}; Zn^{2+}; Ca^{2+}) that are considered particularly important for growth, sporulation, and δ-endotoxins synthesis (Mounsef, 2015; Gadji, 2016). In addition to key metal ions, the balance between carbon and nitrogen sources is key to avoid decreasing pH values below 5.6 during bacterial growth, which can affect cell growth and sporulationefficiency[22]. The pH value found (5.1) is consistent with that obtained by Curtin(1983), whose pH value for sugarcane molasses varies between 4 and 6.In this work, the C/N ratio was 12.8 in sugarcane molasse alone (M_0 medium). This ratio is similar to the one found by Vu *et al.,* (2012)from wastewater produced by starch industries, which is about 12.9.It remains higher than the 7.5 ratio needed to obtained the highest growth yield and glucose consumption, compared to C/N ratiosequal to 5 or 11(Mounsef, 2015). The high C/N ratio in sugarcane molasse is due to its high sucrose and low nitrogen contents (Archimède *et al.,* 2015). According to the work of Seid *et al.,* (2016), molasses is particularly low in nitrogen: 25 g of N per kilo of raw molasses. Adding yeast extracts supplements the molasse with another nitrogen source, and also provides vitamins to improve cell growth and sporulationas in M_5; M_{10} and M_{15} media. The data indicated that with low quantities of the yeast extracts, sporulation significantly was improved. The yeast extract, which contains proteins and vitamins, probably met some of the physiological needs of *Bacillus thuringiensis* in contrast to the M_0 medium. Similar observations were reported by Tirado-Montiel *et al.,* (2001). Comparison of the number of

cells and spores of Btk HD-1 cultured in four media, namely M_0; M_5; M_{10}; M_{15}, shows that M_{10} medium provides better cell growth and spore production compared to other media. These observations could be related to the quality of the substrate used. Indeed, sugarcane molasse is a source of carbon easily assimilated by bacterial cells that possess a β-glucosidase activity (Mounsef, 2015). Also, the yeast extract also contains proteins that modify the nutritional quality in the fermentation medium(Milne *et al.,* 1990). It therefore improved the C/N ratio by enriching the medium with nitrogen. However, low or very high quantities of the yeast extracts as in the M_5 and M_{15} medium did not promote efficient sporulation during fermentation. Thus, it seems that a concentration of more than 200 mg/L of yeast extracts might cause was metabolic disruption that was probably resulting in enzyme inhibition. The M_{10} medium yielded a greater biomass in terms of viable cells and spores when compared to all other media (M_0; M_5 and M_{15}). This medium would contain the nutrients required for biopesticide production. Indeed, during sporulation, each *Bacillus thuringiensis* cell sporulates and releases a spore and a crystal of δ-endotoxin. Both crystal toxins (not determined in this work) and spores play an important role in insect mortality. It has been reported that high spore concentration is proportional to high toxicity (Vu *et al.,* 2012).

In conclusion this study demonstrated that sugar cane molasse from food processing plants in Côte d'Ivoire enriched with yeast extracts could be used as a suitable substrate for Btk HD-1 growth. The physical and chemical features of this agro-industrial residue showed that it contains the necessary minerals (Ca, K, Mg, Fe, Na, Cu, Mn, Zn) for the growth and sporulation of Btk HD-1. The comparison of three culture media (M_5; M_{10} and M_{15}) enriched with yeast extract with the control medium (M_0) without yeast extract showed that the M_{10} medium seemed the most favorable. It was capable of supporting the growth and sporulation of Btk HD-1. The formulation of the biopesticide using sugarcane molasses reduced the cost of the growing medium. The valorization of sugarcane molasses from the Borotou-koro agro-industrial complex represents an attractive economic option for SUCRIVOIRE, since it reduces the cost of disposing of residues, while generating a second income. The knowledge of industrial techniques for the valorization and use of this molasses can help avoid waste and preserve the environment.

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