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Evaluation of two different culture media for the development of biopesticides based on *Bacillus thuringiensis* and their application in larvae of *Aedes aegypti*

Samara Ernandes[1*], Vanildo Luiz Del Bianchi[2] and Iracema de Oliveira Moraes[3]

*[1]União das Faculdades dos Grandes Lagos, Rua Eduardo Nielsen, 960, 15030-070, São José do Rio Preto, São Paulo, Brazil. [2]Departamento de Engenharia e Tecnologia de Alimentos, Universidade Estadual Paulista "Julio de Mesquita Filho", São José do Rio Preto, São Paulo, Brazil. [3]Probiom Tecnologia, P&D Experimental em Ciências Físicas e Naturais Ltda., Campinas, São Paulo, Brazil. *Author for correspondence. E-mail: sernandes@ig.com.br*

ABSTRACT. The bacteria *Bacillus thuringiensis* var. *israelensis* (*Bti*) generates certain toxins with pesticide action, which can be used on the control of transmissible diseases by culicides, specially *Aedes aegypti*, the dengue vector. This biopesticide has been produced by submerged fermentation and, in Brazil, this production has been made by very little research centers and, more recently, by a unique small enterprise. For the implementation of a viable vectors control program through biopesticides, some studies about culture media are essential in order to join efficiency and low costs. In this way, agroindustrial wastes or by-products have been used as a nutrient source for the culture media production. In this study, corn steep liquor, a corn industrial processing by-product and tryptose, both with / without sugar addition, were compared as culture media. Cellular growth was evaluated by optical density at 620 nm, spore production by total viable cell count and LC_{50} by bioassays against 4th instar larvae. Among the four examined substrates, the medium composed by glucose plus corn steep liquor presented the best spore production and bioassay results.

Keywords: biological control, fermentation process, agroindustrial waste.

Avaliação de dois diferentes meios de cultura para o desenvolvimento de biopesticidas à base de *Bacillus thuringiensis* e sua aplicação em larvas de *Aedes aegypti*

RESUMO. Visando obter alternativas de meio de cultura de baixo custo para a produção de um biopesticida a partir de *Bacillus thuringiensis* var. *israelensis (Bti)*, que possua ação efetiva contra larvas de *Aedes aegypti (Ae. aegypti)*, vetor da dengue, foi feita uma comparação entre a eficiência de produtos à base de *Bti* obtidos através de um meio de cultura tradicionalmente utilizado em pesquisas (triptose) e um subproduto agroindustrial, proveniente de uma indústria de processamento de milho ("milhocina"). Para tanto, estes meios foram inoculados com 2 x 10^2 células mL^{-1} de *Bti* e incubados por 120 horas, retirando-se amostras periódicas, até 48 horas, para avaliar o crescimento celular (620 nm) e o pH do sistema. Ao final do processo, foi avaliada a quantidade de esporos (por contagem em placas) e a CL_{50} (com larvas de 4º instar), expressa como diluição do caldo final. Os resultados indicam uma melhor eficiência do meio "milhocina"-glicose (CL_{50} = 2,8 μg L^{-1}), em relação aos meios "milhocina" (5,2 μg L^{-1}), triptose-glicose (8,4 μg L^{-1}) e triptose (12,2 μg L^{-1}). Conclui-se que a "milhocina", suplementada com glicose, é um substrato potencialmente utilizável na preparação de um meio de cultura com um custo substancialmente inferior a um meio que utiliza a triptose.

Palavras-chave: controle biológico, processo fermentativo, resíduos agroindustriais.

Introduction

The control of insect vectors of important human diseases is carried out by chemical insecticides, which have raised several issues, including environmental pollution and an increase in the effects on human health, such as cancer and immune system disorders (BRAVO et al., 2011).

Bacillus thuringiensis var. *israelensis* (*Bti*) is known worldwide for producing toxins with biopesticide action, specifically for mosquitoes, including the *Aedes aegypti*, the dengue vector. Harmless to humans and other mammals, the main obstacle to its production is the cost in the process of obtaining it (CAPALBO et al., 2008).

In laboratorial scale, the culture medium is generally prepared using one of the commercial standard media. However, when large quantities of medium are required, one must find alternative

sources of nutrients, despite the dismissal of purity or standards related to their comercial products (ERNANDES; MORAES, 2001)

As an important surrogate for nutritional sources, these inputs are found among by-products, agricultural waste or industrial wastewaters can be used as substrates in order to minimize the cost of production (ADAMS et al., 1999).

Poopathi and Abidha (2011) produced a culture medium with husk extract coffee, obtaining promising results concerning the larvicidal activity.

The same authors verified the viability of the residue from dairy industry, used in clarification of the butter, as a substrate for the fermentation process of *Bti*, obtaining results comparable to those obtained with conventional culture media, chemically defined (POOPATHI; ABIDHA, 2012).

In this sense, one can employ the corn steep liquor ('milhocina'), a concentrated solution obtained from soaking of corn grains, containing in its chemical composition soluble carbohydrates, amino acids and minerals (HULL et al., 1996; ERNANDES; MORAES, 2001).

Studying the history of the discovery and development of penicillin, it is observed that mass production in the 40's in Peoria, Illinois, was only possible with the use of corn steep liquor as substrate in the fermentation process, which allowed the use of penicillin as a 'miracle drug' for the treatment of wounded World War II (BHANDARI, 1996).

Hull et al. (1996) studied the biochemical composition of the soaking water in the varied stages of the corn processing, stressing that all the water existing in the process of maceration is evaporated to form a thick liquid, consisting of carbohydrates, amino acids, peptides, organic compounds, heavy metals, inorganic ions and phosphates. Microorganisms, particularly lactobacilli, are detected and contribute to fermentation of the remaining liquid, before the final stage that constitutes the concentration of the liquid obtained. As a result of fermentation, exists the production of some amino acids and lactic acid, which together with the presence of sulfur dioxide, used at the beginning of grinding, confers the pH around 3.5-4.3 to the final product.

In routine analysis of the then "Refinações de Milho Brazil S.A." (oral communication), in September 1997, it was obtained 96.2% insoluble and, in liquid phase, a composition of 7.5% of reducing sugar, 17% of lactic acid, 45% proteins, with a pH between 4.0 to 4.5 (ERNANDES; MORAES, 2001).

Capalbo et al. (2008) aiming to obtain an increase in cell yield of *Bacillus thuringiensis*, used 'milhocina' at 10 g L^{-1}, verified that the yield of medium with this residue was slightly superior to medium containing molasses, in the same concentration. The same author, in order to find, among these, a medium more economically viable, used increasing concentrations of 'milhocina', 20 to 100 g L^{-1}, achieving an optimal level of this residue around 25 g L^{-1} (CAPALBO et al., 2008).

This study aimed to determine the cell growth of *Bti* in submerged fermentation process, besides the production of spores and LC_{50} (median lethal concentration) of the preparations obtained, so comparing a standard medium containing a source of nitrogen, the tryptose and another containing a by-product of industrial processing of corn, the soaking corn water ('milhocina'). The same media were also compared, obeyed to the same assessment parameters, when added glucose.

Material and methods

Inoculum and fermentation

Assays were performed with *Bacillus thuringiensis* var. *israelensis* (serotype H-14). The strain was routinely cultivated in nutrient agar medium at 28°C for 72 hours and then at 4°C in a refrigerator. Fermented media were composed of four solutions, two containing tryptose at 20 g L^{-1}, NaCl 5 g L^{-1} and Na_2HPO_4 2,5 g L^{-1} and other two containing the same components, except the tryptose, which was replaced by 'milhocina'. In verification studies of the effect of nitrogen source on cell growth of *Bacillus thuringiensis*, Moraes et al. (2008) obtained higher yelds in dry weight with the tryptose, in concentration of 20 g L^{-1}, adopted in this work. In two of the media (one containing tryptose and another containing 'milhocina') was added glucose in concentration of 4 g L^{-1}.

Fermentations were performed in Erlenmeyer flasks of 250 mL with 50 mL of culture medium, packed in a stirrer-incubator, 150 rpm, 30°C for 120 hours. Were performed pre-fermentations using the same fermentation medium, lasting 15 hours, inoculating themselves with the microorganism withdrawn directly from tube with maintenance medium composed of Agar nutrient. The inoculum of fermentation consisted in 1 mL of the pre-inoculum, which corresponded to 2 x 10^2 cells mL^{-1}.

All assays were performed in two replicates.

Parameters monitored

Were checked cell growth, by optical density (620 nm), the pH variation, the emergence of spores and crystals, by microscopy, the quantification of

spores, by plating on nutrient agar and LC_{50} (median lethal concentration), through bioassays using dilutions of the final culture in *Aedes aegypti*.

Cell growth

The determination of cell growth was performed at regular intervals. The samples withdrawn (3 mL) were centrifuged at 10,000 rpm, three times, for 10 minutes, performing a washing with distilled water at least twice. Finally, the optical density of the suspension was determined in spectrophotometer at 620 nm (Ultropec 3000 Uv / Visible, Pharmacia Biotech).

pH

The pH was monitored (Analion PM 608) to check possible alterations during the process that could give rise some influence in the production of spores and crystals.

Verification of spores by microscopy

The slides were analyzed in the photomicroscope Olympus DX 60 in phase contrast system, with 100 times objective and the images were scanned in an image analyzer coupled (IMAGE - PRO - MEDIA PLUS®, Cybernetics). In this methodology, the spores are seen in ellipsoid shape and bright. The crystals are also bright in irregular shape and size between ¼ and ½ the size of the spore.

Quantification of spores

For quantification of spores, was performed a technique of plating with thermal shock, as Alves and Moraes (1998), in which the dilutions, after being prepared as usual, are inoculated in five or six points on the surface of the culture medium nutrient agar, in the volume of 5 µL point^{-1}, taking care to leave the plates opened in sterile environment, to dry up the suspension inoculated. Then, the plates are incubated at 30ºC for about 10 hours, after which we counted the colonies, preferably using a colonies counter. A good precision in count is obtained when one has, in average, between 20-40 colonies point^{-1}. For a better use of the media and materials, the plates were divided into two groups, being that each was relative to different dilutions.

Bioassays

The preparations were tested on fourth instar larvae of *Aedes aegypti* eggs collected by a Brazilian agency for control of endemic diseases (SUCEN - city of São José do Rio Preto, São Paulo State) through traps (ovitraps) installed and removed weekly in 150 households. The traps (Figure 1) were constituted of black plastic pots (acting as insect breeding) with 5 cm diameter by 12 cm depth, containing 300 mL of water in which were installed palettes of 12 cm long by 2 cm wide (Figure 2) and placed outdoors. On the sides, little below the top, were made three small holes so that the container would not be completely full of water. Thus, possibly *Aedes* had laid eggs on the palettes left partially submerged, once the mosquito does not lay them directly in water. The 150 pots were placed in homes of 14 sub-regions of the city and collected weekly. The traps positive for *Aedes* eggs were identified, stored in coolers (Figure 3) and placed for hatching in plastic containers containing water, about four days before the bioassays, in the entomology laboratory of SUCEN.

Figure 1. Ovitraps.

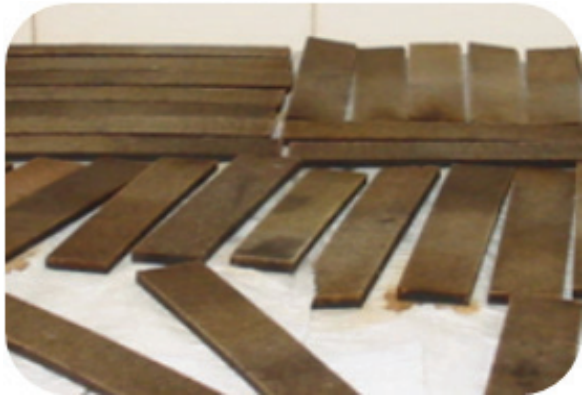

Figure 2. Eucatex palletes.

In order to ensure greater productivity, it was also maintained an insectary with adult mosquitoes, which fed blood of a mouse. A stock solution was prepared, pipetting 1 mL of fermented product in 99 mL of distilled water, totaling a dilution of 1:100. Plastic pots with a specific quantity for each test were then supplemented with 150 mL of distilled water and fifteen larvae were added to each pot with

a small sieve. To make up concentrations 2-28 μg L^{-1} (the range tested in all trials) were added volumes variables between 15 and 210 μg L^{-1} of stock solutions to the pots, of which withdrew, in advance, the same amount of water in that, in order do not verify dilutions errors. The LC_{50} was evaluated by Probit, version 1.5.

Figures 4, 5 and 6 display the steps of the bioassay.

Figure 3. Packaging of palettes in the laboratory after collection in households.

Figure 4. Separation of Aedes aegypti larvae of the same instar to use in bioassays.

Figure 5. Larvae into plastic pots with 150 mL of water.

Figure 6. Addition of dilutions of each product in pots with 150 mL of water.

Results and discussion

Experiments with tryptose, with and without glucose

Analyzing the results, the values of optical density increased until the time of 16 hours, for both media, observing that glucose positively influenced the growth (Figure 7).

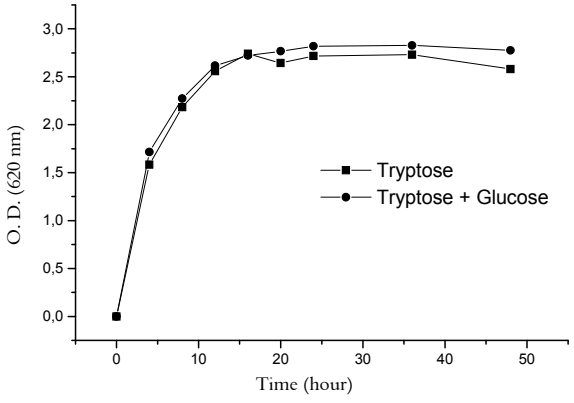

Figure 7. Growth kinetics of *Bti* in tryptose and tryptose plus glucose based media.

The pH varied differently in the medium with and without glucose. The medium without glucose showed a continuous and almost linear pH over 48 hours. The medium with glucose showed a sudden drop of pH during the first six hours, increasing progressively until achieving results after 48 hours similar to that without glucose (Figure 8). This may suggest the use of two metabolic pathways that lead to similar results in cell growth, with a slight advantage in media with glucose, as previously mentioned.

In microscopic monitoring, it was observed the appearance of spores and crystals, from 24 hours of the process. The Figures 9 and 10 exhibit the microscopic evaluation when the process ends, within 120 hours.

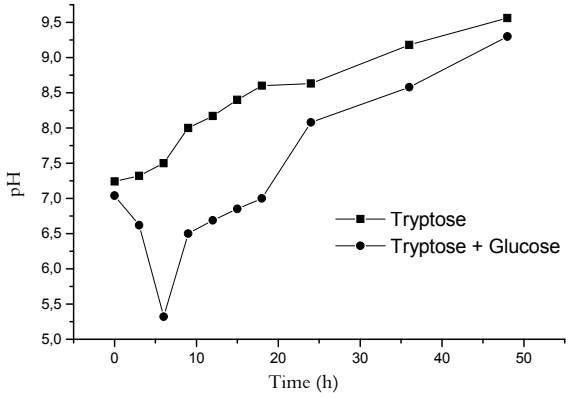

Figure 8. pH variation in tryptose and tryptose plus glucose based media.

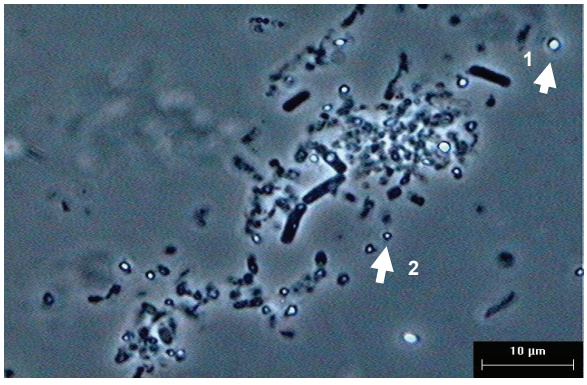

Figure 9. Spores and crystals in tryptose plus glucose based media as seen by microscopy *1-Spore 2-Crystal.

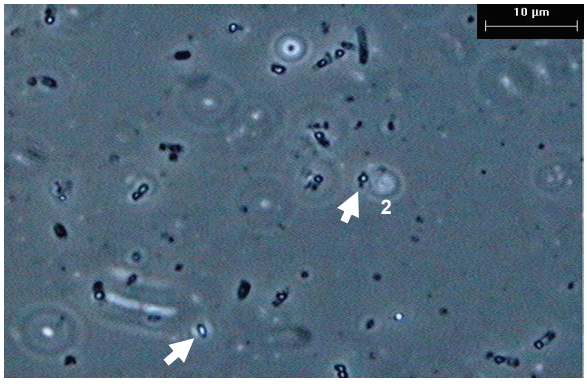

Figure 10. Spores and crystals in tryptose based media as seen by microscopy *1-Spore 2-Crystal.

Experiments with 'milhocina', with and without glucose.

Figures 11 and 12 show, respectively, the cell growth and pH variation in the medium based on 'milhocina'.

Analyzing the Figure 11, there was cell growth until 18 hours, for the medium with glucose and 21 hours in the medium containing only 'milhocina'. Once again glucose influenced positively the cell growth. The medium containing tryptose and glucose was more efficient in cell production, since the absorbance of the medium 'milhocina' and glucose was about 20% lower than the absorbance of the medium tryptose and glucose (Figure 7).

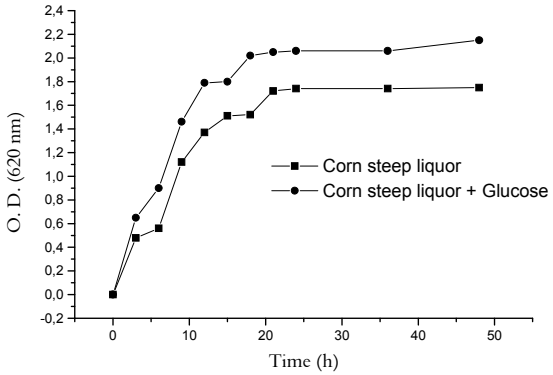

Figure 11. Growth kinetics of *Bti* in corn steep liquor and corn steep liquor plus glucose based media.

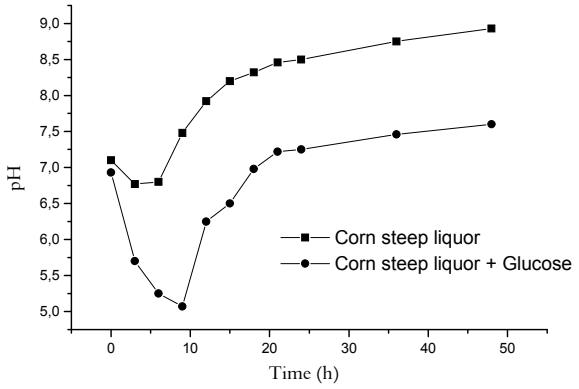

Figure 12. pH variation in corn steep liquor and corn steep liquor plus glucose based media.

Regarding pH, Figure 12, becomes evident again the behavior commented in previous study, that the medium with glucose presented, during the first nine hours, a remarkable drop of pH due to acid production, in relation to the medium without glucose, returning to assume increasing results until the end of the process. The medium without glucose presents a slight drop of pH in the first six hours. From this point, there is an expressive elevation, till the end of 48 hours. Unlike the medium tryptose-glucose, the values of pH of the medium 'milhocina'-glucose do not reach high values (pH 9.0), remaining below 7.5.

Spores and crystals are present in medium 'milhocina' with and without glucose, in the end of the fermentation process. In general and visually, the medium corn steep liquor without glucose present amount of spores and crystals slightly lower than corn steep liquor with glucose (Figures 13 and 14).

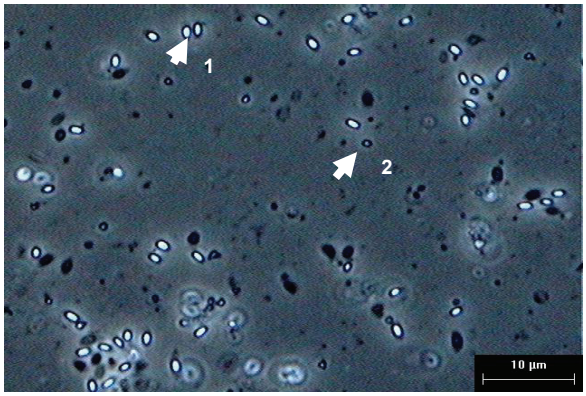

Figure 13. Spores and crystals in corn steep liquor plus glucose based media as seen by microscopy *1-Spore 2-Crystal.

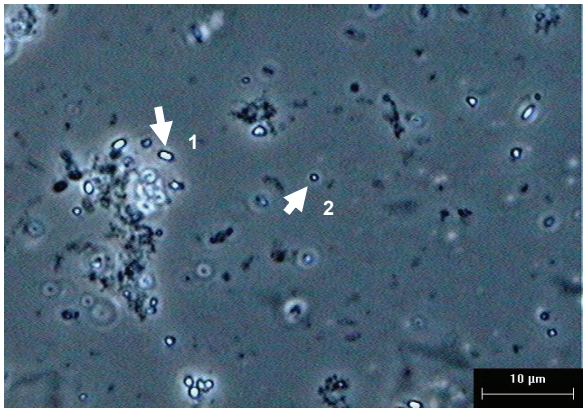

Figure 14. Spores and crystals in corn steep liquor based media as seen by microscopy *1-Spore 2-Crystal.

Analyzing Figures 15, 16, 17 and 18, where are shown the results of bioassays with fermented broths studied, it was found a 100% mortality at concentrations of 24 and 28 μg L^{-1} for all products tested. The fermented broth-based on 'milhocina' plus glucose showed a higher efficacy, and mortality at very low concentrations, followed by the medium composed only by 'milhocina'. Comparing the products based on tryptose medium, the addition of glucose was a differential factor for effectiveness on the larval mortality.

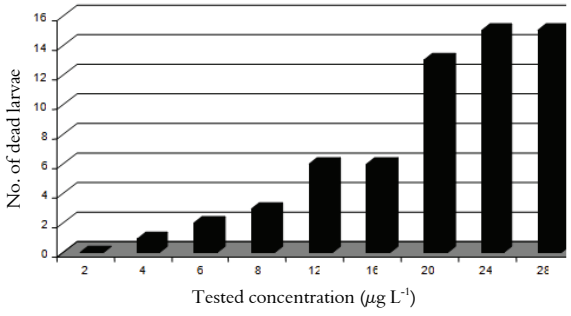

Figure 15. Number of dead larvae at different concentration of fermentation broth-based tryptose.

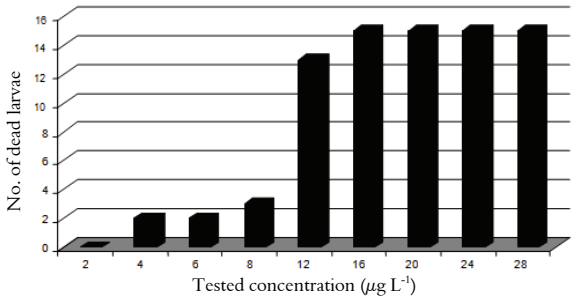

Figure 16. Number of dead larvae at different concentrations of fermentation broth-based glucose and tryptose.

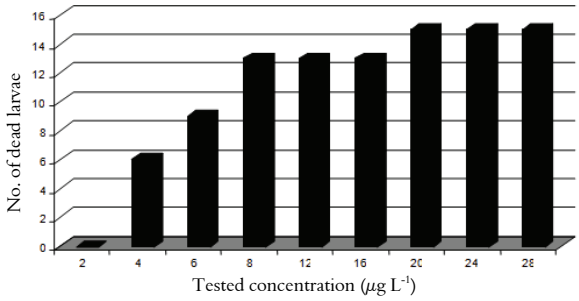

Figure 17. Number of dead larvae in different concentrations of fermented broth-based "milhocina".

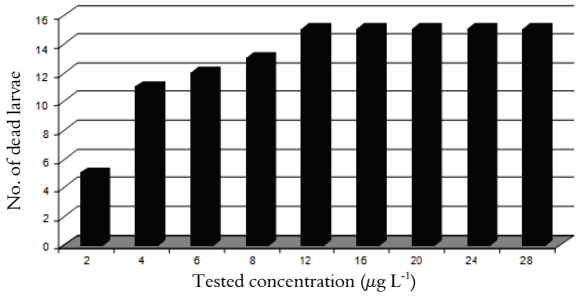

Figure 18. Number of dead larvae in different concentrations of fermented broth-based "milhocina" and glucose.

Through the Probit program, version 1.5, we calculated the LC_{50} of each product (Table 1). The results corroborated the previous discussion, whereby the lowest LC_{50} was obtained by means of corn steep liquor, emphasizing its superiority, especially when supplemented with glucose for the production of toxins. In contrast, the highest LC_{50} was obtained with the medium with tryptose, not viable as a culture medium for production of *Bti* in large scale and, indirectly, strengthens the role of glucose as an important nutrient, on the media studied, to the effectiveness of the biopesticide.

The optical density (OD) of the media with glucose reached a higher value than the media without sugar. Comparing the cell growth on the medium based on tryptose with the 'milhocina' medium, the first reached higher values (Figures 7 and 11). Lee and Seleena (1991) drew a comparison

in the OD (600 nm) of a medium containing soybean residue and a standard medium containing nutrient broth, yeast extract, $CaCl_2.2H_2O$, $MnCl_2.4H_2O$ and $MgCl_2.6H_2O$. The maximum number of viable cells and endospores in the first (1.15) was 3.5 times lower than of the standard medium (3.99). The pH was monitored, since according to Abdel-Hameed et al. (1990) in experiments using waste for the growth of *Bti*, the toxin production is not affected when the pH remains at 6.5 and 7.0. But when it reaches values above or equal to 8.0, the bacterial growth and sporulation are affected, leading to a significant decrease in the production of toxins. Therefore, the greater or lower efficacy of a biopesticide may be justified by the pH values reached during the fermentation process. This behavior was observed in environments with high pH at the end of the process, and where the LC_{50} was high too, indicating to be less effective with respect to insecticide activity (Table 1, Figures 8 and 12). With regard to production of spores (Table 1), the yield of corn steep liquor was similar to that obtained by Abdel-Hameed et al. (1990), between 1 and 2 x 10^8 using legume seeds, agro-products and molasses for sporulation and toxin production by *Bti*. The same results were obtained with *Bacillus thuringiensis kurstaki* (*Btk*) in a study using activated sludge from a wastewater treatment unit (LACHHAB et al., 2001). However, it was superior when compared with the results of Salama et al. (1983), which reported 10^7 spores in a medium composed of 2% of corn steep liquor into basal medium (BM), also for production of *Btk*.

Table 1. LC_{50} and spores production in tested media.

Media tested	LC 50 ($\mu g\ L^{-1}$)	Spores (CFU mL^{-1})
Tryptose	12.2	9 x 10^7
Tryptose + Glucose	8.4	2 x 10^8
'Milhocina'	5.2	2 x 10^8
'Milhocina' + Glucose	2.8	3 x 10^8

Through the values presented in Table 1, it was observed the absence of a similar proportion between the LC_{50} and the concentration of spores. This reinforces the assertion of Dulmage (1970a and b), about the lack of correlation between the number of spores and insecticidal activity, and Abdel-Hameed et al. (1991), who verified a good sporulation rate for *Bti*, although a low toxin production. Moraes et al. (2008) found that increasing concentrations of glucose in the culture medium favored the increase in protein content of the spore-crystal protein complex, and obtained the best yields with 6 g L^{-1} glucose. Also, when using tryptose at 20 g L^{-1}, among other sources of nitrogen for the growth of *Bacillus thuringiensis*, it was obtained a higher yield by dry weight, compared with the other standardized sources.

The LC_{50} obtained in all studied media were lower and therefore, more effective than those obtained by Lee and Seleena (1991), who obtained 0.2 to 0.032 mg L^{-1}, using residues to produce *Bti*, Melo-Santos et al. (2001) reached 0.3 to 0.01 mg L^{-1}, through the assessment of an experimental media formulated by FIOCRUZ, Amalraj et al. (2000) achieved 0.06 mg L^{-1}, with Vectobac AS (suspension) for *Aedes aegypti*, in laboratory and Ejiofor and Okafor (1991) 0.056 mg mL^{-1}, using local waste to produce a prepared designated CMPC-2.

Using a association of *Bti* and *Bs*, Zhuang et al. (2011) conducted fermentation with wastewater from sewage, obtaining promising results superior to those obtained in this study for the production of spores and median lethal concentration, validating once again the feasibility of using waste in obtaining effective biolarvicides at low cost.

Poopathi and Abidha (2012) have used dairy industry waste for the production of *Bti* and obtained larvicidal activity of 0.0036 mg L^{-1} (LC_{50}) against *Culex quinquefasciatus*, value superior and therefore, less effective compared to that obtained in this study, with *Aedes aegypti*.

Conclusion

Although the tryptose has had a better performance in the generation of cells, by optical density, for both the generation of spores and the efficiency of bioassays, the 'milhocina' proved to be the best culture medium. Likewise, glucose, in both media, provides better results. When comparing the costs of a culture medium with tryptose ($ 282 to $ 758 per kg) with the cost of 'milhocina' ($ 0.30 per kg), the use of this by-product, besides being more efficient, brings huge savings in the amount spent on vector control, especially against *Aedes aegypti*, vector of dengue, a disease that constantly worries the public health authorities.

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