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Production of *Bacillus sphaericus* Entomopathogenic Biomass Using Brewery Residues

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Abstract

The use of brewery residues—yeast and trub—has been evaluated aiming to minimize the costs of the industrial production of *Bacillus sphaericus*-based bioinsecticide. Both brewery residues promoted growth and sporulation of the three *B. sphaericus* strains that were isolated from Brazilian soils (S1, S2, and S20). However, distinct growth and sporulation behaviors were observed in relation to the different nutritional conditions and strain used. The maximum sporulation percentage was obtained through the cultivation of S20 strain in brewery residual yeast. In general, the entomopathogenic biomasses produced showed good results for toxicity to *Culex* larvae. The minimum values of larvae population (LC50) were observed for the S20 strain grown on yeast brewery residue-containing media. After fermentation, a considerable decrease in the organic material of alternative media was verified, although the residual values were still higher than that considered appropriate for effluent discharge.

Index Entries: *Bacillus sphaericus*; bioinsecticide production; industrial residues; entomopathogenic biomass; spore/crystal toxins.

Introduction

The continuous and indiscriminating use of chemical pesticides to control insects and other vectors during several decades, in many cases, has resulted in environmental imbalance, particularly owing to the lack of specificity of the chemicals used (*1,2*). Among other deleterious effects, the application of pesticides, with long toxic residual action, may cause the development of resistant insect populations (*2*). For these reasons, the use of biological products is an attractive alternative, owing to their low toxicity and insect specificity. Furthermore, the use of biological insecticides should grow in the future as environmental protection agencies become

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more rigorous, in particular, those regulating developing countries, and also by launching of new, more efficient, and less expensive formulation products.

In the past few years, an increasing interest has been focused toward biological products with the ability to control tropical diseases. In Brazil, the goal is to eliminate specifically *Culex* (filariases vector), *Anopheles* (malaria vector), *Aedes* (dengue vector), and *Simulium* (oncocercosis vector) (3). Recently in Brazil, commercial microbial agents, such as *Bacillus thuringiensis* and *B. sphaericus*, are being applied to kill *Aedes*/*Simulium* and *Aedes* mosquitoes, respectively. Among those two bacilli, *B. sphaericus* toxins present several advantages such as high and long larvicidal activity and spore persistence, even in polluted environments. However, *B. sphaericus*, distinctly from the other species, is not able to utilize carbohydrates as carbon sources.

Considerable progress has been achieved in microbial insecticide production, either concerning the isolation of strains with high larvicidal activity against target organisms or through the development of new formulations, more stable, with longer environmental persistence (2). However, there is a need to reduce microbial insecticides' production costs, which are higher than that of synthetic insecticides. Hopefully, use of locally available and low-cost raw materials could make a bioinsecticide large-scale production more economically feasible (4,5). In Brazil, an industrial scale brewery normally produces 2.8 L residual yeast/hL beer with a standard production of 700,000 hL/mo, so the destination of such a waste is a problem of great magnitude to the industry. In addition, for each 1500 L of beer produced, approx 30–40 L of trub are accumulated, generating another problem for the company. Technically, trub is defined as the sludge consisting of proteins and hops that precipitate out of wort during the boiling and chilling processes of brewing production. This work aims to evaluate the substitution of the medium normally used for *B. sphaericus* entomopathogenic biomass production by brewery residues, attempting to make the biopesticide competitive with chemical pesticides production.

Methods

Microorganism

B. sphaericus S1 was isolated from a river sample from Vitoria (Espirito Santo, Brazil), *B. sphaericus* S2 from a soil sample from Brasilia (Distrito Federal, Brazil), and *B. sphaericus* S20 from a soil sample from Pantanal (Mato Grosso do Sul, Brazil).

Media

Culture growth has been performed on standard medium (medium S) containing: 1 g/L yeast extract, 3 g/L meat extract, 5 g/L peptone, 1 g/L KH_2PO_4, 0.15 g/L $CaCl_2$, 0.10 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.01 g/L $MnSO_4 \cdot H_2O$, and 0.01 g/L $ZnSO_4 \cdot 7H_2O$.

Table 1
Alternative Media Composition

Production medium	Residues (% [v/v])	
	Residual yeast	Trub
T	–	38.5
YT	46	19
Y	92	–

Table 2
Chemical Composition of Both Brewery Residues Used

Residue	Protein (g/L)	Fat (g/L)	Total carbon (g/L)
Residual yeast	7.6	0.7	36.9
Trub	18.2	2.3	32.2

Three other formulations consisting of the brewery residues (yeast and trub), isolated or in mixture, were tested for entomopathogenic biomass production (Table 1). The chemical compositions of both brewery residues are presented in Table 2. The protein content of the alternative media was established to assure an initial protein concentration of 7 g/L, according to previous studies *(6)*. Before use, the brewer's yeast residue has been heated to 60°C for 12 h in order to promote cell autolysis, filtered in qualitative paper no. 4 (Whatman plc, UK) for solid removal, and then stored at –20°C. The trub residue was filtered and stored using the same procedure.

Inoculum

A loopful of bacterial growth from nutrient agar (Merck 1.05450.0500) was used to inoculate a 500-mL Erlenmeyer flask containing 100 mL of medium S. After incubation of the flask on a rotatory shaker (New Brunswick, US) at 250 rpm and 30°C for 24 h, volumes corresponding to approx 0.15 g/L of exponential-phase cells were used as inoculum for production media. The cell concentration was determined by dry weight *(7)*.

Bioinsectide Production

Batch cultures were performed in triplicate on 500-mL Erlenmeyer flasks containing 100 mL of production medium. After 48 h of incubation, at 29 ± 1°C and 250 rpm, samples of fermented media were withdrawn aseptically for microbiological analysis. Other samples were centrifuged for cell removal, lyophilized, and the toxicity of spore/crystals produced was determined by bioassays. Supernatants were submitted to chemical analysis.

Analytical

Total viable cell was quantified by serial plating technique as described by Moraes and Alves *(8)*. For spore count, samples were heated at 80°C for 12 min before plating. Total organic carbon quantity was measured in media, before inoculation, and after cultivation process, using a Shimatzu TOC 5000 analyzer. Total protein and lipid content was measured in media before inoculation and was determined according to the micro-Kjeldahl *(9)* and the Bligh and Dyer methods *(10)*, respectively. The pH values were determined using a potenciometer (DMPH-1 DA Digimed). In order to determine the entomotoxic activity of *B. sphaericus* strains, the biomasses produced were assayed against third-instar larvae of *Culex quinquefasciatus* according to the protocol of Monnerat et al. *(11)*. The lethal concentration necessary to reduce 50% of larvae population (LC50) was calculated by Probit analysis *(12)*. All samples were taken in duplicate and the counts were averaged.

Results and Discussion

The tested brewery residues—yeast and trub—were both able to enhance the growth and sporogenesis of the three selected strains (Fig. 1). However, distinct growth and sporulation behaviors were observed related both to the strain and to the composition media used. In most cases, these results were superior to those found for the production of *B. sphaericus* bioinsecticide using the medium S. In general, the cultivation of the S1 strain produced the largest growth in the tested alternative media. However, regarding the sporulation percentage, the S20 strain proved to be, overall, the most efficient of the studied strains. It must be emphasized that all presented data are linked to a fermentation time of 48 h, as longer periods did not lead to best results (data not shown).

Among all media, that which constituted exclusively of trub (medium T) was the most appropriate for *B. sphaericus* strain growth and S1 in particular. On the other hand, in the case of S1 strain in medium T, the sporulation percentages were not so satisfactory in comparison with the cultures grown in the other alternative media. The medium consisting of both brewery residues (medium YT) promoted a different growth profile for each of the studied strains. In particular, S2 was the only one to present maximum sporulation in this medium, which was even greater than that observed in medium S. Mainly, in the media containing residual brewery yeast, media Y and YT, S20 achieved the maximum sporulation frequencies of 92% and 66%, respectively. Those results were even higher than those evidenced for the other strains. According to Pelczar et al. *(13)*, autolyzed yeast extract solution is a recommendable nitrogen source. This product is added to culture media to stimulate microbial growth, as it contains vitamins, in particular B-complex vitamins, as well as amino

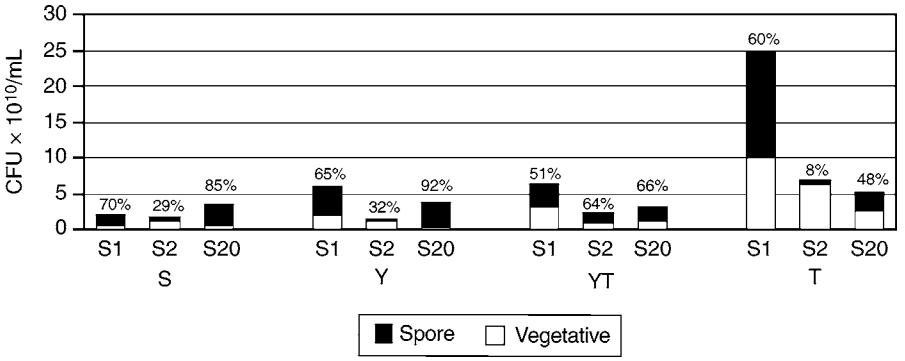

Fig. 1. Vegetative and spore counts (CFU) and sporulation percentage of *Bacillus sphaericus* strains (S1, S2, and S20) on different media (S, standard medium; Y, 100% residual yeast; YT, 50% residual yeast plus 50% trub; and T, 100% trub) after 48-h incubation.

acids and other stimulating substances that are important to the metabolic activity of these microorganisms. Trub residue also has high protein content, but its polyphenol content (11–26%) and lack of growth factors can interfere with bacteria metabolism *(14)*.

The results obtained are in agreement with those reported by other authors. According to Dias *(15)* the growth and sporulation of *B. sphaericus* 2362 were different as a function of the composition medium. The author obtained maximum growth and sporulation of 9.1×10^{10} cell/mL and 84.7%, respectively, through the use of brain heart infusion (BHI) medium *(16)*. A similar sporulation frequency was obtained in NYSM (a peptone-based broth) medium *(17)* although, under this condition, a considerably lower cell concentration (1.4×10^8 cell/mL) was determined *(18)*.

The spores/crystals of *B. sphaericus* strains produced from tested media and bioassayed against third-instar larvae of *C. quinquefasciatus* are given in Fig. 2. Our results show LC50 values for alternative media (Y, T, and YT), varying from 8 to 7815 μg/L. It should be highlighted that the smaller the LC50 value, the greater the lethal power of the toxin produced by the microorganism. Again, the dependence of larvicidal activity on the nutritional condition was confirmed: *B. sphaericus* S2 has shown greater entomotoxic activity, of 88 and 87 μg/L, when cultivated on media T and YT, respectively. On the other hand, the S1 strain has shown the poorest lethal larvae activity when cultivated on media YT. The toxicity of *B. sphaericus* S20 proved to be, overall, the most efficient of the studied strains. This strain showed good larvicidal activity in all alternative production media in comparison with medium S cultives. The best LC50 values of 8 and 10 were obtained for S20 on media Y and YT, respectively.

The fermentation of *B. sphaericus* 2362 on protein-based media supplemented with glycerol, a byproduct of yeast metabolism, led to an almost

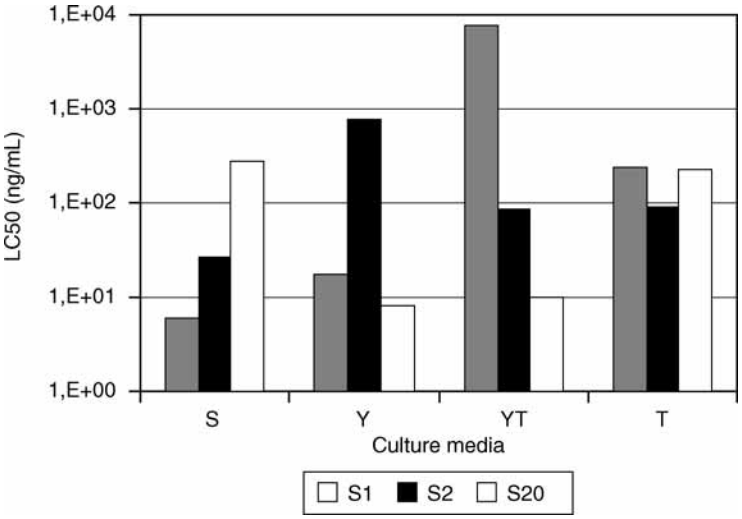

Fig. 2. Effect of growth of different strains (S1, S2, and S20) on different media (S, standard medium; Y, 100% residual yeast; YT, 50% residual yeast plus 50% trub; and T, 100% trub) after 48-h incubation in entomotoxic analysis (LC50, lethal concentration necessary to kill 50% of larvae).

complete sporulation and a considerably improved larvicide production *(19)*. Also, Nigerian agricultural products provided good sporulation (53–80%) of *B. sphaericus* 1593, whose powders were effective against *C. quinquefasciatus* larvae with LC50 of 193 μg/L *(20)*. The culture of this same strain on defatted groundnut cake and defatted milk powder led to 85% sporulation and a LC50 value of 52.4 μg/L *(21)*.

Distinct behaviors concerning the pH were observed depending on the strain and the alternative media used for bioinsecticide production (Fig. 3). In some conditions, a slight increase of the pH was observed. All fermented media by the *B. sphaericus* S20 strain presented this profile. This is probably a reflection of the protein consumption, mainly as a carbon source. According to Arcas *(22)*, the pH gradually increases during the fermentation process, reaching values varying from 8.0 to 9.0, and it is possibly owing to the consumption of amino acids as the carbon source.

According to Yousten *(23)*, the need to control the pH during the fermentation process seems to be related to the *B. sphaericus* strain used. On the other hand, Yousten and Wallis *(17)*, in later studies, did not observe any significant difference for spore count and insecticidal activity for *B. sphaericus* 2362 when the pH was controlled between 7.2 and 7.3.

The trub-containing media (T and YT) showed a decrease in the pH values after fermentation by S1 and S2 strains, suggesting the formation of metabolic acids. The oxidative metabolism of the bacilli occurs through the Embden-Meyerhof-Parnas and/or Entner-Doudoroff pathway, yielding pyruvate, which is subsequently metabolized by the tricarboxylic acid

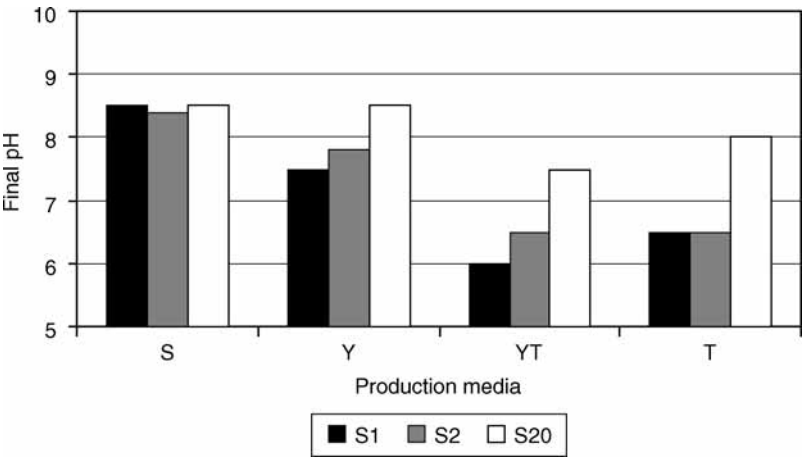

Fig. 3. Effect of growth of different strains (S1, S2, and S20) on different media (S, standard medium; Y, 100% residual yeast; YT, 50% residual yeast plus 50% trub; and T, 100% trub) after 48-h incubation in pH values.

(TCA) cycle. According to Russel et al. *(24)*, the pH decreases during vegetative growth, as the glucose consumption leads to the accumulation of acetate, until a depression of the TCA enzymes occurs; then, the acetate can be metabolized by this pathway, as observed for *B. cereus*. At the end of the exponential phase, which coincides with the transition of the cell from the vegetative to the sporulated form, an increase in the pH is observed as a consequence of the oxidation of organic acids, which are intermediate compounds of this metabolic pathway. However, owing to the incapacity of *B. sphaericus* to assimilate carbohydrates, it requires alternative carbon sources for its metabolism, such as protein material *(24,25)*. The presence of protein materials in the culture medium as carbon and nitrogen sources results in a greater assimilation of carbon in comparison with nitrogen, causing an accumulation of ammonia ions and a subsequent increase of the pH.

Concerning the organic material present in the alternative media, it can be observed that they contain a much higher quantity of organic carbon than the medium S (Fig. 4). After fermentation, a reduction in the organic material of both residues was verified, especially for the S20 culture in medium Y. The results obtained indicate the use of brewery residues for bioinsecticide production as another possible strategy in pollution treatment.

The protein uptake in the different media by the three *B. sphaericus* strains is shown in Fig. 5. The major consumptions are observed when strains S1 and S2 were cultivated in the medium T. The protein uptake by the S20 strain showed similar behaviors under all culture conditions tested.

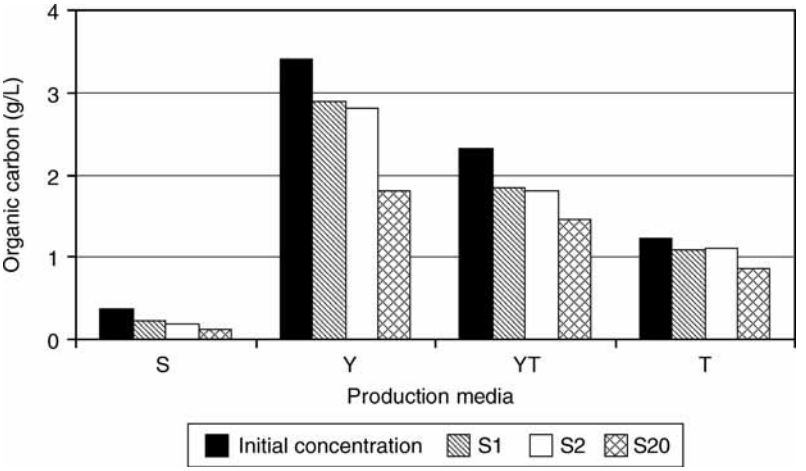

Fig. 4. Total organic carbon uptake by *Bacillus sphaericus* strains after 48-h incubation in different media (S, standard medium; Y, 100% residual yeast; YT, 50% residual yeast plus 50% trub; and T, 100% trub).

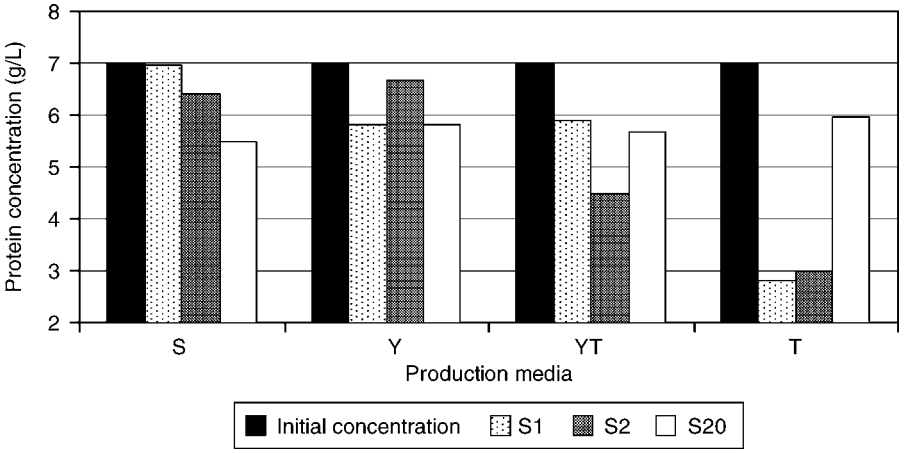

Fig. 5. Protein uptake by *Bacillus sphaericus* strains after 48-h incubation in different media (S, standard medium; Y, 100% residual yeast; YT, 50% residual yeast plus 50% trub; and T, 100% trub).

Conclusions

The Brazilian isolated strains of *B. sphaericus* S1, S2, and S20 were able to grow and sporulate when cultivated in brewery residues. Trub promoted the growth of the three strains, especially of S1. Maximum sporulation frequencies of most bacilli were achieved by cultivation in the brewery yeast residue. The spore forming of the bacilli in the alternative media was superior to that obtained in the medium S. However, different growth and sporulation patterns have been evidenced regarding the constituents of the industrial residues. The spore/crystals produced by *B. sphaericus*

strains on alternative media showed good entomotoxic activity against *C. quinquefasciatus*, especially S20. The results obtained recommend the use of brewery residues for *B. sphaericus* insecticidal toxin production because of their wide availability and low cost in Brazil and other countries. Considering the study, we can estimate a cost of 1 USD to prepare 100 L of medium according to data provided by the brewing industry. The organic matter was reduced after the fermentative process, mainly by the S20 strain cultivated in yeast brewery residue, thus reducing total carbon in the effluent stream and the cost owing to its treatment discharge.

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