08-1	**Micro encapsulation of proteins and spores of *Bacillus thruringiensis* HD-1**	

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INTRODUCTION AND OBJECTIVES

The importance of bio-insecticides is its specific toxicity for certain insects in their larval phase. However, its marketing is still limited due to problems of stability and persistence of the protoxins in the environment by the effect of several factors from which radiation has been considered the most critical. In the spectrum of protective measures, microencapsulation seems to be a reliable, safe and feasible alternative to be carried out at an industrial level (Poncelet et al., 1992).

In the field of biological control most reported encapsulation methods are based on the extrusion of suspensions of biological material in sodium alginate solutions. The drawback of this methodology could be the particle diameter (Modifi et al, 2000). Biological control using entomopathogenic microorganisms only applies to the insect's larval stage, therefore, microcapsule size is a crucial factor.

This article presents a test of the microencapsulation process of a lyophilized spore protein aggregate (SPA) produced from *Bacillus thuringiensis var. kurstaki* HD-1 (Bt-HD-1). The methodology is based on the emulsification/internal gelation method (Poncelet et al., 1992), and was focused to produce microcapsules of short diameter (< 10 µm) and enable to protect the SPA when exposed to UV radiations of short (UV-C) and medium (UV-B) wavelength.

MATERIALS AND METHODS

Microorganisms

Bt-HD1 was obtained from the strain collection of CINVESTAV. It was grown in a soy flour medium (SF) at 30 °C and 200 rpm until 90% sporulation (1×10^8 spores/mL). The SPA was separated from cellular debris by repeated washing with acidulated (pH 2.5), saline (0.85%) and distilled water. The recovered SPA was lyophilized (VirTis, Benchtop K) and kept under refrigeration for later use.

Microencapsulation

An emulsion-gelling method in stirred tank was applied (Rodrígues et al. 2006). This method allows to adjust the microcapsule diameter by changing stirring speed (*v*, rpm), concentration of the emulsifier reagent (C_{SALG}; SALG = sodium alginate) and concentration of the gelling reagent (C_{CaCl2}, $CaCl_2$ = calcium chloride). To obtain the desired microcapsule diameter (D_{MC}), an experimental design of three factors at two levels (2^3) was implemented: C_{SALG}, 2% w / w and 3% w / w; *v*, 1500 rpm and 2000 rpm; C_{CaCl2}, 0.1M and 0.3M.

The analysis of the microencapsulation process comprehend: SPA microencapsulation efficiency and microcapsule characterization by morphology and size distribution. The microcapsule morphology was evaluated by scanning electron microscopy (JEOL JSM-35C) (SEM) and its diameter measured with the ImageJ program.

UV radiation

Samples of a microencapsulated SPA of known concentration were put per triplicate into glass dishes of 5 cm diameter and exposed to UV-C (254 nm) and UV-B (365 nm) wavelengths (Entela lamp model UVGL-25) over 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 min. The energy density on the sample surface was recorded with a radiation meter (Ophir). Free and microencapsulated SPA, irradiated or non irradiated, were analyzed for spore viability (UFC/mL), which was carried out by serial dilution. Degrading proteins was analyzed by polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

Diameter of microcapsules

The ANOVA of the effect of stirring speed, $CaCl_2$ concentration and alginate concentration on the microcapsules diameter had a significant effect with a p(F) <0.0001, being in the range of 3.1±0.2 - 6.8±0.4 µm. According to the results shown in Figure 1, for a fixed C_{SALG}, an increase of *v* from 1500 to 2000 rpm diminished the D_{MC} by 14.6 ± 0.1% and 47.2 ± 7.5%, when the $CaCl_2$ was added at concentrations of 0.1% and 0.3% respectively. When *v* was fixed to 1500 rpm, C_{CaCl2} increase from 0.1% to 0.3% increased the D_{MC} in 40.5% and 31.2% when the alginate was added at concentrations of 2 and 3%, respectively.

The interaction between calcium cations and carboxyl groups of alginates impacts the porosity of microcapsules. In this study, the C_{CaCl2} = 0.1M was assumed the most suitable because it produces less dense matrices that allowed the SPA release; $CaCl_2$ 0.3M prevented the release of the SPA, regardless of soaking time.

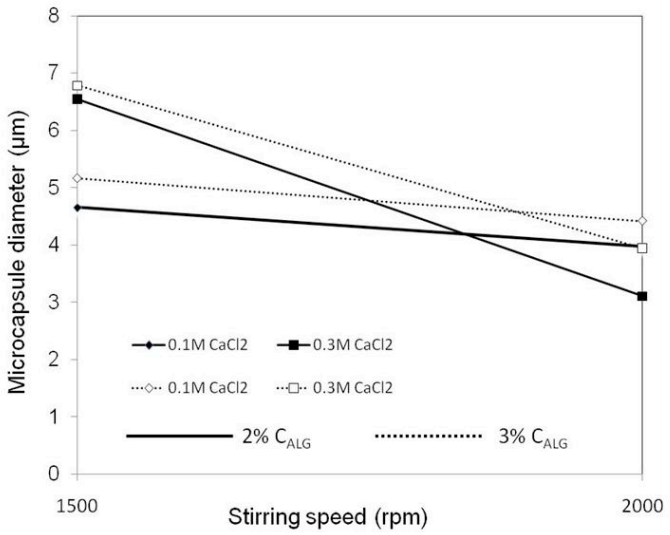

Figure 1: ANOVA of the effect of the stirring speed and $CaCl_2$ concentration on the microcapsule diameter at fixed alginate concentration.

The efficiency of the SPA microencapsulation was 90%, with a final spore and protoxin concentration of 0.9×10^8 spores/mL and 300 µg/mL, respectively. The microcapsules showed an irregular and ovoid form with a smooth surface that became corrugated after subjection to a lyophilization process (Figure 2). The SEM analysis allowed the identification of the spore-crystal aggregate fully cover by the alginate matrix.

UV radiation of the spore-crystal aggregate

The viability of free and microencapsulated spores after their exposure to UV-C and UV-B radiation is presented in Figure 3. Under UV-C radiation, free spores viability fell to zero in 14 minutes. However, this was remained when UV-B radiation was applied. These results agree with Myasnik, et al. (2001) who reported viabilities of 85% in Bt spores exposed to UV-B. Spore sensitivity to UV radiation has been associated to factors such as: number of plasmids, spore nature, protein nature and low molecular weight of some protoxins. These reports corroborate the results obtained and presented in this work.

CONCLUSIONS

The spore-protoxin aggregate was microencapsulated and the diameter was in the range 3.1±0.2 - 6.8±0.4 µm. Microencapsulated spores kept their viability when they were exposed to short and medium UV radiation in the range of 0 – 0.5 J and 0-0.2 J, respectively. Free spores exposed to the same UV radiation intensities lost their viability within the first 14 minutes. Verification of the spore and protein immobilization into the microencapsulate assumes the effectiveness of *Bt*-HD-1 in insect combat.

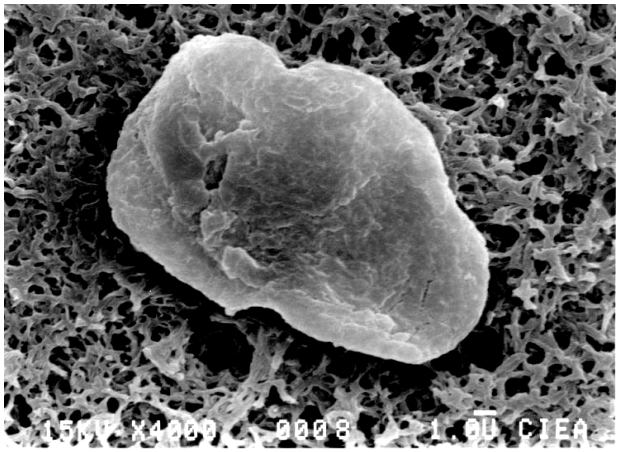

Figure 2. Dried alginate microcapsule

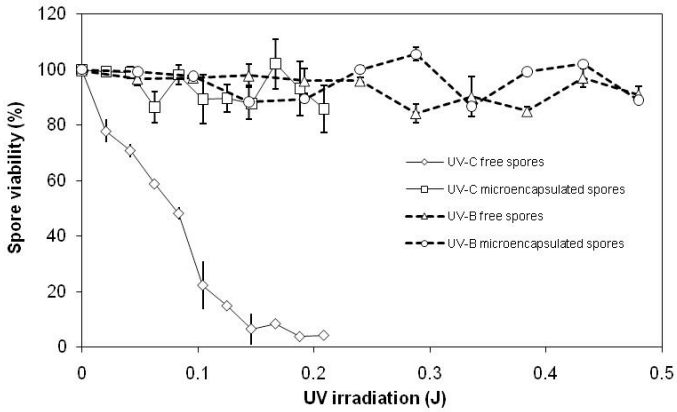

Figure 3. Viability of free and microencapsulated spores (Bt-HD-1) exposed to UV radiation of 254 nm and 365 nm during 20 minutes.

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