# A simple method for the separation of *Bacillus thuringiensis* spores and crystals

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ABSTRACT

A simple new method, for separating *Bacillus thuringiensis* crystals from spores and cell debris, is described. The developed purification method uses hexane and low speed centrifugation and does not require any expensive material or reagents.

*Bacillus thuringiensis* (*Bt*) is a Gram-positive spore forming bacterium that is able to produce parasporal crystals made of proteins (Cry) during sporulation. The parasporal crystals of studied subspecies showed a wide range of toxicity against different insect orders (Diptera, Lepidoptera and Coleoptera) and other invertebrates (Feitelson, 1993; Schnepf et al., 1998). Assays of Cry proteins and the studying of the crystal biochemistry require pure crystals as standards. Moreover, assays of expression of the Cry transferred genes in *Bt* transgenic plants are based on a specific antibody reaction and they require pure biologically active crystals as antigens. A variety of techniques have been developed for the extraction of *B. thuringiensis* crystals. However, proper separation of crystals from bacterial spores' remains hampered by the proximity of their respective buoyant densities 1.25 and 1.30 $g \cdot cm^{-3}$, and by the tendency of spores to clump and entrap crystals. Reported techniques of *Bt* crystal purification include isopycnic centrifugation in caesium chloride (CsCl) (Fast, 1972), gradient centrifugation in Sodium bromide (NaBr) (Ang and Nickerson, 1978), density gradient centrifugation in Renografin (Sharpe et al., 1975), and centrifugation through step gradients of Ludox (Zhu et al., 1989). The most commonly adopted strategy is that described by Thomas and Ellar (1983) in which *Bt* spores and crystals are separated using discontinuous sucrose density gradients (67 to 72 to 79% [wt/vol] sucrose) with ultracentrifugation (Ito et al., 2004; Rolle, 2013). Foam flotation based extraction strategies (Sharpe et al., 1978) and methods using carboxymethyl cellulose column chromatography (Murty et al., 1994) have also been described. All the above-mentioned methods are partially successful and are time demanding or they require expensive equipment and reagents. Crystals of different strains differ by their shape, size and surface antigens whereas *Bt* spores are commonly hydrophobic (Doyle et al., 1984). We have taken the advantage of the latter characteristic to develop a simple and rapid method for preparation of highly pure crystals from crystal-spore mixtures of *Bt*. Protein crystals were purified from two subspecies of *B. thuringiensis* registered as $H_3^{MKA}$ and $Lip^{MKA}$, designated by "$H_3$" and "Lip", isolated from the Lebanese soil (El Khoury et al., 2014). Bacterial cultures were made in 3 L of Anderson medium (Anderson, 1990) at 30 °C in a 5 L bioreactor with a volumetric gas transfer coefficient (kla) of 13.32 $h^{-1}$. Cells were grown for about 48 h or until approximately complete autolysis had occurred releasing the spores and the toxin crystals in the culture medium. The developed purification procedure was first used in an attempt to isolate the spherical crystals of $H_3$. The liquid culture medium was centrifuged at 6000 rpm, 4 °C for 10 min and the obtained pellet was washed twice by suspending in 1 M NaCl containing 0.01% triton X-100. The pellet was then suspended in a 50 mL centrifuge tube with a saline solution, in order to enhance the hydrophobic interactions. An organic solvent

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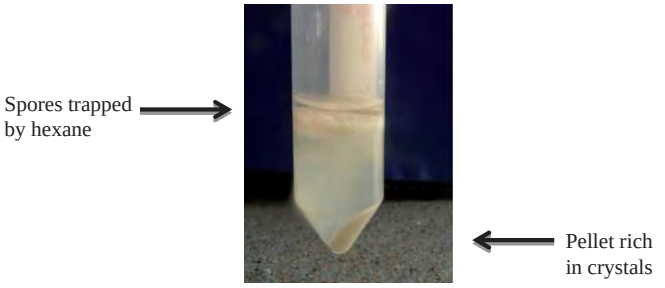


**Fig. 1.** Separation of spores, crystals and cell debris upon centrifugation (intended for color reproduction on the Web and in print).

(diethyl ether, dichloromethane or hexane) was added to a ratio less or equal to 10% (50, 75, or 100 μL/mL of aqueous suspension) to minimize the risk of altering crystals. The suspension was sonicated at 100 W for 10 min to dispel clumping then centrifuged at 6000 rpm for 10 min. The obtained pellet was resuspended in a saline solution, the organic solvent was added again and the same procedure was repeated three to four times. Finally the pellet was washed twice with cold distilled water. Among the hydrophobic organic solvents tested (diethylether, dichloromethane and hexane), only the hexane could trap the spores under the conditions described. The compositions of the different phases of the centrifuged solution were verified by observation at 100× using a light microscope after staining with Coomassie blue solution (0.13% (w/v) Coomassie blue reagent; 50% (v/v) acetic acid). Spores were trapped by the hexane on the top layer; cell debris remained in the aqueous phase while the crystals accumulated to form the pellet (Fig. 1).

The highest crystal purity was attained when the hexane was added at a concentration of 10% which is the closest to the ratio found by Doyle et al. (1984) (approximately 133 μL hydrocarbon/mL cell suspension) for maximal adherence of spores to hydrocarbons. The purified crystals were air dried (away from light) and weighed. About 10 mg of 99% pure crystals was obtained from 35 mL of culture. After alkaline dissolution of the toxin proteins obtained in the culture medium (Rivera, 1998), the toxin protein concentration was estimated by the Bradford method, using the bovine serum albumin as a standard (Bradford, 1976). The crystal's recovery yield was calculated according to the following equation:

$$\text{Recovery yield} = \frac{\text{Crystals dry weight} \times 100}{\text{Mass of toxin proteins in the culture}}.$$

The recovery yield of $H_3$ crystals was 44%. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2) indicated that the proteins

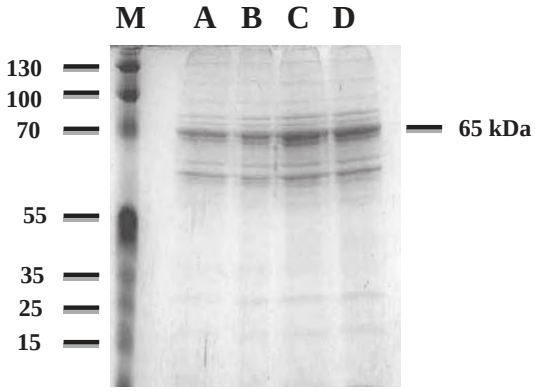


**Fig. 2.** Stained sodium dodecyl sulfate 10% polyacrylamide gel after electrophoresis of $H_3$ crystals before (lanes A, B) and after purification (lanes C, D), lane M, molecular weight standards.

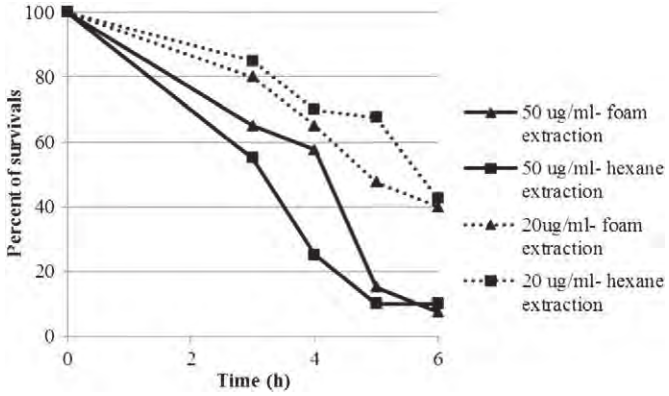


**Fig. 3.** Survival of *Anopheles gambiae* four instar larvae infected with $H_3$ purified toxins. This result is representative of two independent experiments. Same results have been obtained on third instar Larvae.

composing the crystals of $H_3$ were unaffected and that proteolysis did not occur during the purification.

The toxicity of the purified $H_3$ crystals was compared to that of traditionally extracted crystals using the foam separation technique (Sharpe et al., 1978). As shown in Fig. 3 the new extraction protocol does not affect the efficiency of crystal toxicity against *Anopheles gambiae* larvae.

The purification method was also used to separate the crystals of another *B. thuringiensis* strain "Lip" which produces bipyramidal crystals and few cuboidal crystals. About 67 mg of 99% pure crystals was obtained from 35 mL of culture medium which corresponds to a recovery yield of 92%. The toxin protein concentration determined in "Lip" culture medium was 3-fold greater than that obtained in $H_3$ culture medium. Lip crystals are bigger and have higher content of proteins than $H_3$ crystals which could explain the higher recovery yield of these crystals.

The purification procedure described herein was shown efficient at separating spores and crystals of two different *B. thuringiensis* strains having different crystals shapes. It is an easy and fast separation method that does not require ultracentrifugation or any expensive material. Moreover larger quantities of purified crystals could be prepared using this method than are conveniently available from density gradient centrifugation.

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