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Impact of liquid formulation based on ultrafiltration-recovered bioactive components on toxicity of *Bacillus thuringiensis* subsp. *kurstaki* strain BLB1 against *Ephestia kuehniella*

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ABSTRACT

A study of the recovery process of *Bacillus thuringiensis* (*Bt*) strain BLB1-based bioinsecticides using ultrafiltration showed that the highest recovery of BLB1 bioactive components was achieved by using a 5 kDa MWCO ultrafiltration membrane to concentrate the supernatant. A bioassay against *Ephestia kuehniella* showed that the retentate was approximately 49% more toxic than the supernatant, with a lethal concentration (LC_{50}) of 194.00 $\mu g\,g^{-1}$ in the retentate compared to 380.00 $\mu g\,g^{-1}$ in the supernatant. Furthermore, compared to the centrifugate (spore-crystal mixture) mixed with supernatant or saline water, the centrifugate retentate showed 42.7% and 56% higher toxicity against *E. kuehniella*, respectively. The involvement of proteases, chitinases and Vip3 proteins restored to the retentate suggests synergetic activity with delta-endotoxins and spores, and this activity was confirmed by enzymatic activity and western blot analysis. The formulated centrifugate retentate maintained stability and toxicity after 1 year of storage at 4 °C. These data suggest that the ultrafiltration process enabled significant virulence factors recovery from BLB1 supernatant grown in a complex medium and resulted in a highly toxic and stable formulation.

1. Introduction

Bacillus thuringiensis var. *kurstaki* (*Bt*) has been used exclusively as a biological control agent against many lepidopteran larvae, including the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lep.: Pyralidae). Its use has affected stored products, such as corn, wheat, rice, and flour [1]. An interesting characteristic of this bacterium is its production during sporulation of crystalline protein inclusions called delta-endotoxins that have pronounced insecticidal activity. Upon ingestion by the larvae of a susceptible species, these proteins (130–140 kDa) are activated by proteolysis into 60–70 kDa toxins that interact with specific receptors on the surface of midgut epithelial cells, leading to cell lysis [2]. In addition to the delta-endotoxins and spores responsible for the insecticidal activity, *Bt* also secretes large amounts of other insecticidal proteins into the culture medium, including vegetative insecticidal proteins (Vip3s) (90 kDa), which are active against lepidopteran species, and Cry1I proteins (70–81 kDa), which are toxic toward lepidopteran and coleopteran pests [3]. The secreted proteases and chitinase enzymes (74 kDa) also represent an important class of secreted virulence factors that are involved in the enhancement of *Bt* insecticidal activity against several lepidopteran and dipteran species [4,5].

Numerous processes, including adsorption, evaporation and precipitation, have been used in efforts to isolate soluble *Bt* bioactive components such as Vip3 proteins, Cry1I proteins and various enzymes that are typically lost in the supernatants of centrifuged fermented broths [6,7]. However, none of these methods have efficiently recovered the target virulence factors, resulting in the loss of large yields in the supernatant. Recent studies have attempted to recover active components from the supernatant by using membrane filtration, which is commonly used in biotechnology. Ultrafiltration (UF) is an effective technique that uses a pressure gradient and results in high yield and purity. It can be used for the separation and purification of various products, including enzymes and other proteins [8,9], or to recover microbial products (cells and spores) that are present in the culture medium [10,11]. The process of UF is affected by many physical and chemical factors, including pH, temperature, enzyme concentration and the composition of the fermented broth, as well as the type and size of the membrane that is used, which is typically selected based on the molecular weight of the desired proteins [12]. However, the use of UF has some

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drawbacks, including membrane blockage by the precipitation of particles onto the membrane. To minimize this effect, tangential flow filtration can be used. Unlike normal flow filtration, the feed flow is tangential to the membrane surface and perpendicular to the permeation flux. Consequently, the accumulation of solid particles can be reduced by employing the action of the flow [13]. The UF process has been successfully utilized to recover bioactive components from *Bt* cultured from wastewater and wastewater sludge [11]. By using the UF process, a feed stream can be separated into a retentate with a significant level of bioactive components from the *Bt* fermented broth and a permeate that is nearly 100% free of these components, improving the *Bt* entomotoxicity of the retentate [14].

In addition to the potency of the fermented broth, the use of biopesticides may also be restricted by their limited shelf life [15]. The development of *Bt* formulations from a synthetic medium has been thoroughly investigated [16]. However, to the best of our knowledge this is the first study to investigate the formulation of *Bt* produced in a complex medium [17] and examine its stability at different temperatures. The purposes of this study were to investigate the efficiency of UF use for the recovery of *Bt* bioactive components when cultured in a complex medium and to choose an appropriate membrane based on the increase in spore counts and toxin levels against *E. kuehniella* larvae. We also examined the involvement of Cry1I, Vip3 proteins and enzymes working synergistically with delta-endotoxins, as well as the stability of the new formulation after 1 year of storage at different temperatures.

2. Materials and methods

2.1. Bacterial strains

The *Bt* strain BLB1 was isolated from a Tunisian soil sample. This strain was selected because of its novel delta-endotoxin encoding genes and its high toxicity against *E. kuehniella* larvae. Indeed, the BLB1 LC_{50} was nearly half that of the reference strain, HD1 [18]. Previous studies have demonstrated by real-time quantitative PCR assays that the BLB1 *cry1A* (*cry1Aa*, *cry1Ab*, *cry1Ac*) gene copy number is significantly higher than that of reference strain HD1 [18]. Moreover, the BLB1 Cry1Ac protein harbors three mutations involved in binding to insect receptors, which could be a major factor responsible for its hyper-toxicity against *E. kuehniella* larvae [19].

The *Bt* strains S1/4 and BUPM106 were used as positive and negative controls, respectively, for the detection of Vip3 proteins [20]. The *Bt* var. *kurstaki* H3a, 3b, 3c strain BNS3 and the non-crystalliferous strain BNS3cry- were used as positive and negative controls, respectively, for the detection of Cry1I proteins [21]. The *Bt* strain BUPM2 was used as a low-chitinase-producing strain for the detection of chitinase enzyme.

2.2. Bioinsecticides production

The bioinsecticide preparation was achieved by the fermentation of BLB1 in Erlenmeyer flasks. The complex culture medium used for BLB1 growth was composed of 30.00 g L^{-1} starch, 25.00 g L^{-1} soya bean and the following minerals (g L^{-1}): KH_2PO_4, 1.00; K_2HPO_4, 1.00; $MgSO_4$, 0.30; $MnSO_4$, 0.01; and $FeSO_4$, 0.01 [17]. The pH was adjusted to 7.00 before sterilization. In each shaken flask, $CaCO_3$ was added to a final concentration of 20.00 g L^{-1} to stabilize the pH. For the inoculant preparation, one isolated colony was inoculated in 3 mL of LB medium [22] and incubated overnight at 30 °C. Aliquots were used to inoculate 250 mL Erlenmeyer flasks containing 50 mL of LB medium. After 6 h of incubation at 30 °C in a rotary shaker set at 200 rpm, the absorbance at 600 nm was measured. The culture broth was then inoculated into the complex medium at an initial optical density of 0.15. The 1000 mL flasks with four quarrels containing 50 mL of complex medium were incubated for 72 h at 30 °C in a rotary shaker set at 200 rpm.

2.3. Recovery of entomotoxicity components from fermented broth

The fermented broth was centrifuged at 9000 × *g* for 30 min according to Brar et al. [23]. The resulting supernatant was used for UF studies with tangential flow filtration (Sartorious, Vivacell 250, Model N° 85030-519-49, Germany). Pressure was applied to force a portion of the fluid through the membrane to the permeate side. The permeability of the membrane was 49.2 mL m^{-2} h^{-1} atm^{-1} with a width of 62 mm and an effective surface area of 20 cm^2. The pressure difference across the membrane was 4 bars.

2.4. Selection of membrane size

During this study, polyethersulfone membranes with different molecular weight cut-offs (MWCO) of 100 (VS6041), 30 (VS6021), 10 (VS6001) and 5 kDa (VS6011) were used to identify the membrane with the best performance. Thus, the supernatant of the fermented broth was filtered at a fixed flux rate through the different membranes. The performance of each membrane was evaluated by determining the number of remaining spores by counting the colony forming units (CFU) and by toxicity bioassays against *E. kuehniella* larvae using the resulting retentates and permeates.

2.5. CFU determination

The number of spores was estimated by CFU after 72 h of growth in the complex medium. The culture samples, permeate, retentate and supernatant were heated to 80 °C for 10 min. Appropriate dilutions were plated onto solid LB medium and incubated at 30 °C for 24 h [24].

2.6. Proteolytic activity assay

Proteolytic activity was determined according to Kembhavi et al. [25] with 1% of casein substrate prepared in Tris–HCl buffer (0.1 mM) at pH 7.00. The reaction mixture contained 1 mL of casein solution and 1 mL of diluted supernatant or diluted retentate. The mixture was incubated for 20 min at 60 °C, and the reaction was terminated by the addition of 3 mL of 5% trichloroacetic acid (TCA). The absorbance of the supernatant was measured at 280 nm. Blank samples were prepared with inactivated enzyme heated at 100 °C for 5 min. Enzyme dilutions were performed accordingly to produce a linear reaction after 20 min incubation. One proteolytic activity unit was defined as the amount of enzyme required to liberate 1.00 μg of tyrosine from casein per minute under the experimental conditions.

2.7. Cry1I, vegetative insecticidal proteins and chitinase detection by western blot

Individual retentate samples were prepared by UF from the supernatant collected from the fermented broth of *Bt* strains BLB1, S1/4, BUPM106, BNS3, BNS3(−) and BUPM2. Collected retentates were precipitated with TCA [26]. Briefly, a 1/10 volume of 100% TCA was added to the retentate samples and incubated for 30 min on ice. The samples were centrifuged at 9000 × *g* for 20 min at 4 °C, and then the supernatant was carefully removed and the precipitated proteins re-suspended in one volume of Laemmli sample buffer (1×). The pH was adjusted during protein solubilization by adding