CPQ Microbiology (2018) 1:6
Research Article

CIENT PERIODIQUE

Easy and Non-Expensive Method to Increase the Production, Enhance the Stability and Improve the Purification of Cry1Ab Crystals

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Received: 30 October 2018

Published: 27 November 2018

Keywords: *Bacillus Thuringiensis; Cry1Ab; PH Stability; Filtration; Purification*

Abstract

We were able to increase *Bacillus thuringiensis* Cry1Ab crystals production and enhance its stability by using C3.5:N4.5 nutrients ratio. Crystal size was conveniently reduced from ≥1.0 to ≤ 0.5 µm by increasing culture aeration conditions, in order to facilitate their purification by a simple filtration procedure The recombinant Cry1Ab protein was produced 10 times more using the C3.5:N4.5 ratio, with respect to the amount obtained using the C7:N1 and C1:N7 proportions.

No gradients neither solvent treatments were needed to get a highly purified crystals with insecticidal activity. No spores were observed under the microscope using malachite green stain.

Introduction

Bacillus thuringiensis (Bt) is a Gram-positive spore forming bacterium with great importance to agriculture because the insecticidal activity produced by its Cry proteins; also known as δ-endotoxins [1,2]. Insecticidal activity is principally induced by Cry proteins specific interaction with the membrane receptors of midgut epithelium cells of insects [3]. In this sense, increasing Cry proteins production, stability and purification changing culture conditions, will facilitate the assays development on insect and human cell lines [4]. Cry proteins are produced as crystal inclusion bodies (CIB) through the Bt sporulation process. CIB production levels depends on Bt strain selected, culture medium ingredients utilized and growth conditions established [5]. Its purification has been extensively reported, however most methods are expensive and time consuming, because the physical and chemical similarities between CIB and spores [6,7]. Hence, to improve CIB production, stability and purification without affecting their insecticidal activity; medium ingredients, growth conditions and a simple filtration procedure were evaluated. Thus, several carbon:nitrogen (C7:N1, C3.5:N4.5, C1:N7) nutrient ratios were assayed to evaluate their influence on the Cry proteins production and stability. However, it is reported that a batch fermentation using Bt and high carbon concentrations (C7:N1) may decrease lower than five units the pH, affecting the bacterium development [8]. On the other hand, high nitrogen concentrations as those found in C1:N7 ratio; could increase the pH over nine units decreasing the bacterial development and the crystal stability [1,9].

Material and Method

Culture Mediums and Bt Growth

In this work 600ml of C3.5:N4.5 medium containing; 2.0gr/L of sucrose, 1.5gr/L of corn syrup, 4.0gr/L of peptone and 0.5gr/L of yeast extract, were used to growth a Bt recombinant strain (BtRS) harboring the pHT315-1Ab plasmid, which contains the Cry1Ab gene [10]. The BtRS growth and its Cry1Ab crystal production in C3.5:N4.5 medium was compared with C7:N1 medium containing 7gr/lt of sucrose and 1 gr/l of peptone and with C1:N7 medium, which contains 1gr/lt of sucrose and 7gr/lt of peptone. In addition, 0.250gr/L of $MgSO_4$ and 1g/L of KCl were added to all mediums, pH was adjusted to 7.0 and mediums were sterilized. Subsequently, 2ml/L at 10mM of $MnCl_2$, 1ml/L at 0.5M of $CaCl_2$, 1ml/L at 0.15M of $FeSO_4$ and 25μg/ml of erythromycin were supplemented. Growth conditions were settled at 200rpm and 30°C using Erlenmeyer flasks of 1000ml. BtRS strain was grown 72 h in 600 ml of each medium, samples were taken each hour during exponential growth and every 12 h through the sporulation phase.

CIB Purification

The BtRS strain was grown 72 h in 200ml of C3.5:N4.5 medium, this volume was centrifuged at 8000 x g by 10 min and pellet was resuspended in 200ml of TBE buffer pH 8.5 to maintain CIB stability. Resuspended CIB were passed through a 0.6µm filter (ULTA Prime PP 0.6 µm from GE Healthcare Life Sciences, CA, USA), to retain cells and spores. 50ml samples were concentrated until 2ml using an Amicon Ultra-15 filter of 100KDa and CIB were stored at -20°C.

Results and Discussion

Figure 1 shows that BtRS strain did not grow in C7: N1 medium, as was expected by the assays developed by Rivera in 1998. However, small differences in growing were obtained between C3.5:N4.5 and C1:N7 mediums (Fig. 1). Nonetheless, CIB size and concentration were greater in C3.5:N4.5 than in C1:N7, indicating carbon and nitrogen sources as well as its concentration are very important for crystal production and stability (Fig. 2a, b).

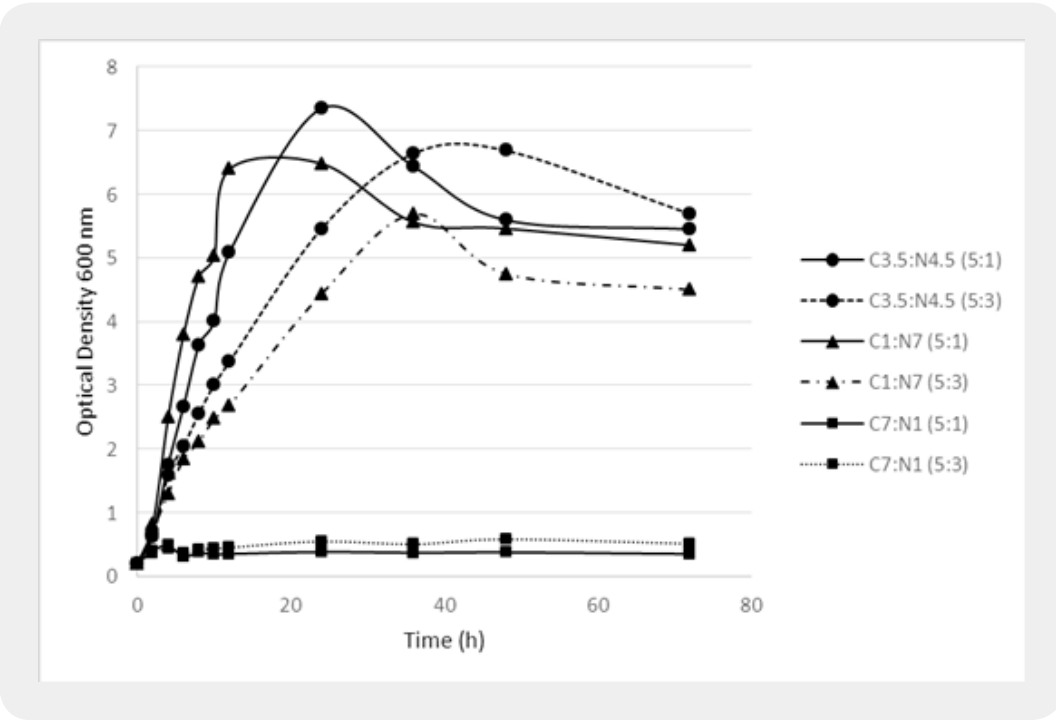

Figure 1: *Bt growth at different C:N ratios and air:medium proportions.*

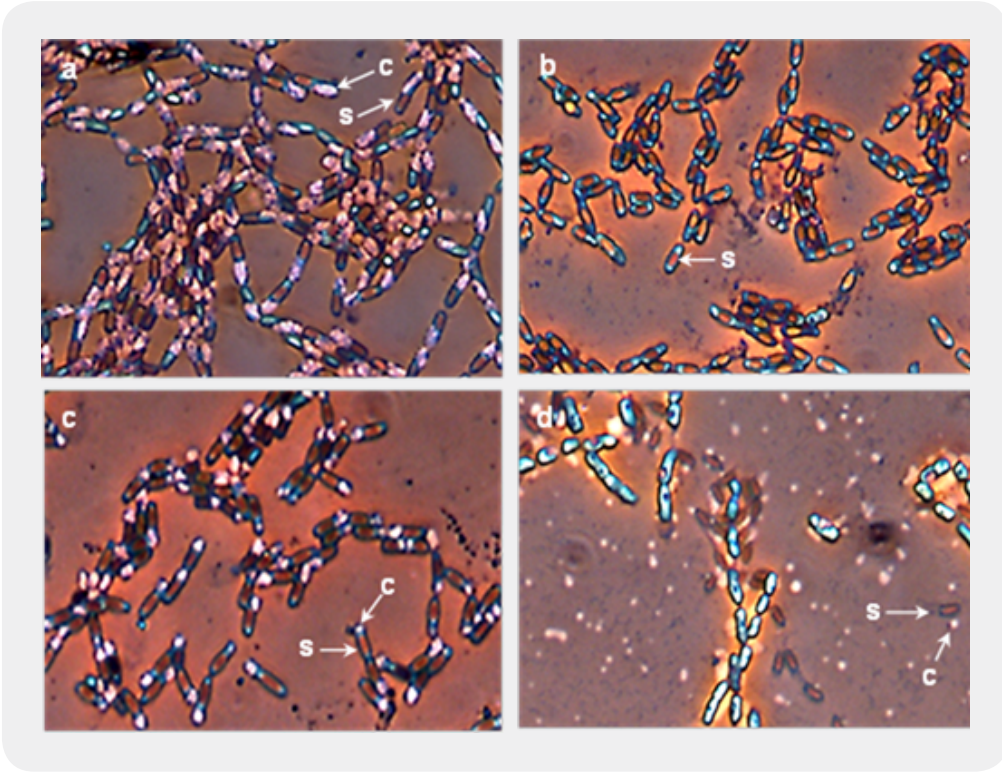

Figure 2: *Bt Cry1Ab crystal production in; a) C3.5:N4.5 medium and 5:3 air:medium proportion at 36 h, b) C1:N7 medium and 5:3 air:medium proportion at 36 h, c) C3.5:N4.5 medium and 5:1 air:medium proportion at 36 h, d) C3.5:N4.5 medium and 5:1 air:medium proportion at 48 h. Spores (s), CIB (c).*

It is reported that crystal stability was maintained below pH 9 [1]; therefore, in the C3.5:N4.5 medium, ingredients and its concentrations were carefully selected to get pH values between 8 and 8.5 throughout the crystal production phase. In this sense, after 72 h BtRS strain initiated its growth in this medium the pH remained around 8.5 units, while in C1:N7 medium pH overpassed the 9.5 units. Therefore, CIB low production and its low stability found in the C1:N7 medium (Fig. 2b), must be directly correlated with nutrients (C1:N7) and pH values obtained, as a consequence of the high Nitrogen concentration utilized. Furthermore, extracellular proteases analysis developed from BtRS strain grown in C1:N7 medium shows its maximum enzyme activity between pH 9 to 10 (data not shown). In this sense, these enzymes could be carrying out soluble Cry1Ab protein degradation on the C1:N7 medium (Fig. 2b). On the other hand, Cry1Ab higher levels found in C3.5:N4.5 medium seems to be a consequence of the nutrients concentration, the lower pH and the higher crystal stability obtained (Fig. 2a, d).

Once CIB production was increased and its stability was enhanced with C3.5:N4.5 medium its purification was carried out using a simple filtration procedure, to improve the whole process. However, in this medium the BtRS strain produced bigger crystals than spores (Fig. 2a); therefore, to reduce crystal size the culture aeration was increased at 5:1 (1000/200ml) instead of the 5:3 (1000/600ml) that was used in the first assay [5].

Thus, BtRS strain was grown in 200 ml of C3.5:N4.5 medium at conditions mentioned above and obtained results show that CIB size decreased from ≥ 1.0 to ≤ 0.5µm (Fig. 2a, c, d). In this sense, crystal size reduction could be a consequence of the different replication rates between pHT315-1Ab plasmid and BtRS strain; because the cells number found in 5:1 proportion was higher with respect to those found in 5:3 (Fig. 1). In this sense, more bacterial cells found at the same time means faster replication rate and consequently less Cry1Ab protein production by cell.

The 0.85 µm Bt spore size reported by Carrera and coworkers in 2007 [11] was not affected by using 5:1 proportion (Fig. 2a,c), therefore, a filtration procedure was carried out using a 0.6 µm cut-off filter to separate CIB (≤ 0.5µm) from the spores (~0.85µm) (materials and methods). Crystal's disaggregation was carried out at 37°C for 1h mixing 400µl of purified CIB with 100 µl of 0.5M Na_2CO_3/$NaHCO_3$ pH 10.5 solution and toxin activation was carried out for 30 min at 37°C using the same reaction tube plus the addition of 20 µg/ml of trypsin. PMSF at 1mM was used to stop the reaction and activated Cry1Ab toxin was analyzed by SDS-PAGE (Fig. 3). This figure shows both Cry1Ab tetrameric and monomeric bands of ~260KDa (lane 2) and ~65 KDa (lane 3), were visible at pH 8.5 and 10.5, respectively. Cry1Ab protein identification was corroborated by mass spectrometry analysis after bans were cut out from the gel (data not shown). The Cry1Ab activated protein was stored at -70°C until it was used in insect assays. Protein quantification was carried out using the Bradford methodology.

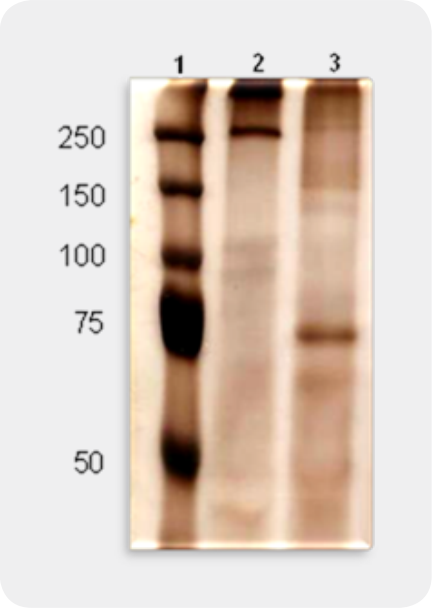

Figure 3: *PAGE of Cry1Ab protein from C3.5:N4.5 medium and 5:1 air:medium proportion at 72 h: lane 1, MWM; lane 2, Cry1Ab tetrameric conformation at pH 8.5; lane 3, Cry1Ab monomeric at pH 10.*

Cry1Ab toxicity was tested against *Manduca sexta larvae*; toxin was applied at 1, 0.5 and 0.05µg/cm^2 onto diet surface, incubated seven days at room temperature and insect survival was recorded. It is known that Cry1Ab toxin presents high specificity against Bt-R1 cadherin-like receptor from *Manduca sexta* [10]; in this sense, insecticidal assays developed in this work shown a LD_{50} of 0.05µg/ml that corresponds with results previously reported [2]. Furthermore, these results corroborate that Cry1Ab protein produced in C3.5:N4.5 medium not suffered any modifications after all treatments [12,13].

Conclusion

CIB production was increased and its stability was enhanced using the C3.5:N4.5 medium with respect to C7:N1and C1:N7 frequently used mediums. CIB purification using a simple filtration procedure instead of gradient or solvents utilization seems to be an easier and more convenient alternative. For this reason, we believe this methodology is a friendly and non-expensive alternative to improve the production, stability and purification of Cry toxins.

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