Available online at www.sciencedirect.com
ScienceDirect
Enzyme and Microbial Technology 40 (2007) 614–622
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Improvement of *Bacillus thuringiensis* delta-endotoxin production by overcome of carbon catabolite repression through adequate control of aeration

Dhouha Ghribi, Nabil Zouari *, Hassen Trabelsi, Samir Jaoua

Laboratoire des Biopesticides, Centre de Biotechnologie de Sfax, P.O. Box K, 3038 Sfax, Tunisia

Received 6 April 2006; received in revised form 11 May 2006; accepted 16 May 2006

Abstract

Over-production of delta-endotoxin by *Bacillus thuringiensis*, strain BNS3, entomopathogenic towards lepidoptera through overcome of catabolic repression of its synthesis, was investigated into full-controlled 3 l fermenter using simple and complex media. Equilibrium between density of the vegetative cells and their ability to synthesize toxins during sporulation was shown to be necessary to take into account. By application of various dissolved oxygen profiles during fermentation, it was clear that the level of dissolved oxygen saturation in the fermentation broth affected cell density as well as delta-endotoxin synthesis. Indeed, the adequate dissolved oxygen profiles to be followed throughout the fermentation for high production of bioinsecticides were determined using the two different media. It was found out that aeration rates corresponding to 60% and 70% oxygen saturation during the first 6 h of fermentation should be applied into 15 g l^{-1} glucose-based medium and 42 g l^{-1} gruel-based one, respectively. Then, 40% oxygen saturation should be ensured up to the end of fermentation, independently of the carbon source origin. With higher oxygen saturation values, cell densities were increased, but delta-endotoxin synthesis yields were strongly reduced. Cells produced with low aeration into the medium acquired higher capacities to synthesize toxins during sporulation. Interestingly, by following adequate oxygen profiles, it was possible to use increased initial glucose or gruel concentrations, without significant decrease of delta-endotoxin production yields. Consequently, the adequate control of dissolved oxygen in the culture media of *B. thuringiensis* allowed at least partial overcome of the carbon catabolite repression which seemed to be an apparent regulation, mostly due to the rate of use of energy generated during a first step of high growth of *B. thuringiensis* cells. These results are of great importance from practical point of view, since it could be possible to produce large quantities of insecticidal crystal proteins during large-scale fermentation, contributing to the reduction of *B. thuringiensis* insecticides production cost. Interestingly, in bioinsecticides produced with adequate aeration profiles, spores have lower yield, which is in favor of the dissemination of less spores into the environment.

Keywords: *B. thuringiensis*; Bioinsecticides; Catabolite repression; Cheap media; Dissolved oxygen; Fermentation

1. Introduction

Bioinsecticides, based on preparations of spores and parasporal crystal proteins (ICPs), named delta-endotoxin, produced by the bacterium *Bacillus thuringiensis* proved to be a high tool for fighting some agricultural pests and vectors of diseases. Delta-endotoxin exhibits larvicidal toxicity upon ingestion by susceptible insect larvae, dissolution and activation by larval gut-juices proteases [1]. In *B. thuringiensis*, regulation of sporulation-specific crystal protein gene expression and temporal role of specific sigma factors in this context were well reported [2]. However, there is virtually no information available on the environmental signals involved in regulating toxin gene expression in *B. thuringiensis*. Since accumulation of crystal proteins coincides with sporulation, it is not known if the environmental signals which induce sporulation trigger also toxin synthesis. Bacteria are endowed with systems that allow them to sense environmental and/or physiological signals, integrate them and transmit an output response [2]. Frequently, these responses are channeled through signal transduction pathways that ultimately allow the regulation of large set of genes. One of such global responses is the catabolic repression, corresponding to limitation of protein synthesis in presence of high concentrations of readily assimilated carbon sources. The car-

* Corresponding author. Tel.: +216 74274110; fax: +216 74275970.
E-mail address: Nabil.Zouari@cbs.rnrt.tn (N. Zouari).

0141-0229/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2006.05.015

bon catabolite repression was shown to be one of the major limitations of delta-endotoxin production by fermentation [3]. The use of *B. thuringiensis* as commercial insecticides would remain prohibitively, relatively expensive if this bacterium is not able to produce remarkable large quantities of insecticidal proteins during large-scale fermentation. Actually, more attention has been given to the regulation mechanisms that ensure the efficient production of the insecticidal proteins. Improvement of bioinsecticides production could be achieved by application of an adequate fermentation technology [4], essentially with use of appropriate media [5], overcome of metabolic limitations [6], improvement of *B. thuringiensis* strains through mutagenesis [7] and adaptation to abiotic stress conditions [8]. The aim of the present work was to study the overproduction of *B. thuringiensis* bioinsecticides by overcome of the carbon catabolite repression. The mechanism of the global response related to carbon catabolite repression was investigated. We determined the period of the culture during which the repressive effect of glucose occurred, and we showed that HPr kinase pathway, commonly found in Gram-positive bacteria, was not involved in the repressive regulation of delta-endotoxin synthesis. In contrast, by adequate control of dissolved-oxygen profiles into the medium, we evidenced that such global response was an apparent regulation, mostly due to the rate of use of energy generated during high growth of *B. thuringiensis* cells. Consequently, it was possible to overcome the repressive regulation of delta-endotoxin synthesis, by controlling oxygen in the culture media.

2. Materials and methods

2.1. B. thuringiensis strains

B. thuringiensis subsp. *kurstaki* strain BNS3 was used in the present work. It was isolated by Jaoua et al. according to the method described in [9]. This strain was used for large-scale bioinsecticides production studies because of the novelty of its delta-endotoxin encoding genes [10] and its toxicity to lepidopterean larvae was confirmed by bioassay on insect larvae [9]. The acrystalliferous strain, BNS3cry-, was obtained by plasmid curing from the wild strain BNS3 [10]. The amylases and proteases derepressed mutants Ma2, Ma10, Ma11, Ma15, Ma22 and Ma32 were isolated from the strain BNS3 through application of classical mutagenesis according to the method described in [7].

2.2. Gruel and fish meal sources

Gruel was obtained from a local semolina factory processing durum wheat producing mainly semolina (65%), flours (10%), gruel (14%) and other by-products. Gruel contains starch (60%), other carbohydrates (5%), cellulose (1.5%), gluten (12%) and total nitrogen (2.1%). Fish meal was obtained from "Office National de la Pêche" (Mahdia, Tunisia). It contains 43% proteins and 7.23% total nitrogen.

2.3. Inocula preparation

Inocula were prepared by transferring cells from nutrient agar slants into 3 ml of Luria broth (LB) medium and incubated overnight at 30 °C. Aliquots (0.2 ml) were used to inoculate 250 ml Erlenmeyer flasks containing 50 ml LB medium [11]. After 6 h incubation at 30 °C in a shaker set at 200 rpm, the OD_{600} was determined. The culture broth was used to inoculate the studied media to start with an initial OD_{600} of 0.15 corresponding to almost 2×10^8 CFU ml^{-1} and 0.05 $g\,l^{-1}$ dry biomass.

2.4. Bioinsecticides production, in shake flask, using glucose-based medium

The *B. thuringiensis* strains were grown in a liquid medium at the optimized conditions for delta-endotoxin production [4]. The carbon source was from glucose (15 $g\,l^{-1}$) and glycerol (5 $g\,l^{-1}$). The medium also contained yeast extract (5 $g\,l^{-1}$) and ammonium sulfate (5.4 $g\,l^{-1}$) to get a C/N ratio of 7.0. The following minerals were used ($g\,l^{-1}$): KH_2PO_4, 1; K_2HPO_4, 1; $MgSO_4$, 0.3; $MnSO_4$, 0.01; and $FeSO_4$, 0.01. The pH was adjusted to 7.0 before sterilization at 121 °C for 20 min. In each shake flask, $CaCO_3$ was added at a concentration of 20 $g\,l^{-1}$ for the improvement of pH stability. Carbon source and $CaCO_3$ were sterilized separately. The 1000 ml flasks with four baffles containing 50 ml of culture medium were incubated for 72 h at 30 °C in a rotary shaker set at 200 rpm.

2.5. Bioinsecticides production, in shake flask, using gruel- and fish meal-based medium

In the complex medium, glucose, glycerol and yeast extract were substituted by 42 $g\,l^{-1}$ crude gruel and 20 $g\,l^{-1}$ fish meal powder. Ammonium sulfate was used at a concentration of 6.6 $g\,l^{-1}$ to get a C/N ratio of 7.0. The minerals were added at same concentrations as in glucose medium, sodium chloride (0.5 $g\,l^{-1}$) and Tween-80 (Fluka AG, CH-9470 Buchs, Switzerland) (1 $g\,l^{-1}$) were added to the medium. The pH was adjusted to 7.0 before sterilization at 121 °C for 20 min. In each shake flask, 1 g of $CaCO_3$ was added for maintaining of pH stability. Carbon source and $CaCO_3$ were sterilized separately. The preparation and the incubation of the 1000 ml and the 250 ml flasks were done as described for glucose medium.

2.6. Bioinsecticides production into 3 l fermenter

Production experiments were carried out at 30 °C into 3 l Labfors (Infors, Switzerland) full automatically controlled fermenter containing 2 l of culture medium with continuous regulation of pH using 2N HCl and 2N NaOH. Aeration of the medium was automatically controlled by control of agitation and air flow as described in Section 3. Dissolved oxygen was continuously monitored by an oxygen sensor (InPro 6000 Oxygen sensor, Mettler Toledo, Switzerland). Foaming was controlled by the use of an antifoam (Struktol SB2020, Schill seilacher, Hamburg, Germany), through the fermentation.

2.7. Biomass and delta-endotoxin determination

After 72 h of growth, the *B. thuringiensis* cultures are composed of a mixture of spores, crystals and minor cell debris. The number of spores was estimated by counting colony forming units (CFU) at the end of the experiments. Hence, culture samples were heated at 80 °C for 10 min, appropriate dilutions were plated on solid LB medium and incubated at 30 °C overnight. Delta-endotoxin concentration was determined in the solubilized crystal preparation from each culture medium as described in [3]. In summary, 1 ml of culture medium was centrifuged for 10 min at 10,000 × *g* and the pellet was washed twice with 1 M NaCl and twice with distilled water. The pellet was suspended in 1 ml of 50 mM NaOH (pH 12.5) in order to solubilize delta-endotoxin crystals. After 2 h incubation at 30 °C, total solubilized proteins in the supernatant were measured by using Bio-Rad reagent (Bio-Rad Protein assay, cat. 500-0006, München, Germany) according to the method of Bradford [12]. The acrystalliferous strain, BNS3 cry-, was used as a negative control for lepidoptera-specific strains, to take into account possible contribution of dissolved proteins from spore coat, cell debris and particulate or insoluble materials. The negative control was included in each experiment and each cultural condition. Indeed, we considered the effect of growth conditions on the content of insoluble materials in the culture media and, thus, their contribution to the protein levels measured after treating the pellets by NaOH. Toxin contents were calculated as the result of subtracting the total proteins measured with the BNS3 cry- strain from the total proteins measured with the toxin producing strains. The values presented are the average of the results of three determinations of two separate experiments for each cultural condition. All the results related to determination of delta-endotoxin production and CFU counts were statistically analysed by SPSS software (Version 100) using Duncan test performed after analysis of variance (ANOVA). The yield of