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ORIGINAL PAPER

Influence of amino nitrogen in the culture medium enhances the production of δ-endotoxin and biomass of *Bacillus thuringiensis* var. *israelensis* for the large-scale production of the mosquito control agent

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Abstract We reported here the role of amino nitrogen in the commercial production of *Bacillus thuringiensis* var. *israelensis* media design. The insect pathogen *B. thuringiensis* var. *israelensis* was cultured in different media containing varying initial levels of amino nitrogen sources obtained from three different commercial venders. The biomass, mosquito larval toxicity and spore count produced were measured during the fermentation process. The results showed that the higher level of initial amino nitrogen concentrations in the medium led to higher yield of biomass (dry weight 4.78 g l^{-1}), larvicidal activity (LC_{50} 18.52 ng ml^{-1}) and spore count (3.24×10^{11} CFU ml^{-1}). Similarly decreasing the initial amino nitrogen concentration in the medium led to a decreased biomass (dry weight 1.64 g l^{-1}), larvicidal activity (LC_{50} 27.01 ng ml^{-1}) and spore count (3.7×10^{10} CFUml^{-1}).

Keywords Amino nitrogen content · *Bacillus thuringiensis* var. *israelensis* · *Aedes aegypti* larvae · Toxicity · Bioassay · Spore count

Introduction

Biological means of mosquito control based on the entomopathogenic bacteria, *Bacillus thuringiensis* var. *israelensis* [7] is being advocated for more than 20 years. The insecticidal activity of *B. thuringiensis* var *israelensis* is primarily due to its crystalline inclusion bodies and spores play only a secondary role [14]. The δ-endotoxin embodied in the parasporal bodies cause destruction and breakdown of the gut epithelium in susceptible insects. Presently *B. thuringiensis* var. *israelensis* is considered ideal for mosquito control because of high mosquito larvicidal activity and its cost-effectiveness [2], ease of production [3, 17], amenability to a variety of formulations [10, 20], safety to non-target organisms [5, 13] and mammals [16, 21]. These attributes of *B. thuringiensis* var. *israelensis* were merited it as an ideal tool and hence has long been employed in vector control agent [12]. Furthermore, the major advantage of this biocide is that risk of development of resistance by mosquitoes to it products based on it is very low, due to its multitoxin complex [22].

Amino nitrogen is a measure of the concentration of individual amino acids and small peptides (one to three units) which can be utilized by bacteria for cell growth and proliferation. For vigorous fermentation, a sufficient quantity of amino nitrogen must be present in the media. Depending upon the quantity of amino nitrogen in the growth medium biomass will increase or decrease. The media for industrial production of *B. thuringiensis* var. *israelensis* are basically composed of complex carbon and nitrogen sources. Starch and molasses are suitable carbon sources and protein-rich material of plant and animal origin such as soybean, corn steep liquor, or casein hydrolysates provide good and cheap nitrogen sources [9, 14, 17]. Since the insecticidal activity of *B. thuringiensis* is primarily based on the parasporal bodies, high yields of δ-endotoxin are desirable. The production of δ-endotoxin can often be improved by strain selection and by changing the culture parameters and have so far received little attention. In this paper we report the influence of amino nitrogen concentration of the culture medium in the commercial production of

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B. thuringiensis var. *israelensis* and its role in the media design and raw material quality control.

Materials and methods

Organism and materials

Bacillus thuringiensis var. *israelensis* (VCRC B-17) an indigenous isolate [4] obtained from the culture collection of Vector Control Research Center (Indian Council of Medical Research, Puducherry, India) was used in the present study. The strains were maintained on the nutrient yeast salt medium agar slants (NYSM) [15]. The composition of the medium is as follows: glucose 1% (W/V), peptone (0.5%), sodium chloride (0.5%), beef extract (0.3%), yeast extract (0.05%), $MgCl_2$ (0.0203%), $CaCl_2$ (0.0102%) $MnCl_2$ (0.001%) (Himedia, India), pH 7.0).

Media preparation

Peptone and yeast extract obtained from three different commercial venders were used for the experiment. All the peptones (vegetable) were obtained by acid hydrolysate and yeast extract (brewer's yeast) obtained by autolysate. Production medium "A" was procured from (Hi-pure chemicals, Chennai, India). Peptone it contained (total nitrogen 7.2%, protein 46%, amino nitrogen 2%, ash 15%, moisture 5% and sodium chloride 5% pH 6.8) and yeast extract (total nitrogen 7.2%, protein 46%, sodium chloride 5%, moisture 5%, ash 15%, pH 6.8). Production medium "B" was obtained from (Romali chemicals, Chennai, India). Peptone and it contained (total nitrogen 9.5%, protein 61%, amino nitrogen 3%, ash 15%, moisture 5% and sodium chloride 5% pH 6.9) and yeast extract (total nitrogen 10.7%, protein 68%, amino nitrogen 3%, sodium chloride 5%, moisture max 7%, ash 15%, pH 6.8). And, production medium "C" was obtained from (Hi-media Laboratories limited, Mumbai, India). Peptone, which comprised (total nitrogen 11.5%, protein 73%, amino nitrogen 5.0%, ash 9.0%, moisture 5% and sodium chloride 5%, pH 6.8) and yeast extract (total nitrogen 11.0%, protein 70%, amino nitrogen 5.0%, sodium chloride 5%, moisture 3.3%, ash 9.0%, pH 6.9). Peptone and yeast extract obtained from these companies had different initial amino nitrogen level in the medium after preparation. Medium "A" contained 0.17 mg ml^{-1}, medium "B" contained 0.33 mg ml^{-1} and medium "C" contained 0.66 mg ml^{-1} amino nitrogen level. All the media had the same quantity of the ingredients but only difference was the different amino nitrogen level. Production medium "A" consist of 2% each peptone and yeast extract obtained from company A, production medium "B" consists of 2% each peptone and yeast extract obtained from company B and production medium "C" consist of 2% each peptone and yeast extract obtained from company C. To each medium was added 0.5% glucose and 10 ml of stock salt solution per liter medium. [Stock salt solution: $MgCl_2$ (20.3 g), $CaCl_2$ (10.2 g) and $MnCl_2$ (1.0 g) in 1 l of distilled water]. The pH of the medium was adjusted to 7.0 prior to inoculation.

Seed culture preparation

The seed culture was produced using shake flasks. First stage seed culture was prepared by inoculating 10 ml of NYSM broth in a test tube with one loopful of culture and incubating the tube on a rotary shaker at 30 °C, 250 rpm for a period of 6 h. Second stage seed culture was prepared by transferring 10 ml of first stage seed culture into 600 ml × 2 of the appropriate medium in a 2-l Erlenmeyer flask and incubating on a rotary shaker at 30 °C, 250 rpm for a period of 6 h. The seed culture was maintained in the log phase.

Fermentation procedure

Actively growing second stage seed culture was used to inoculate the bioreactor at 2% V/V. The optical density (OD) of the inoculums was 0.5 when measured at 600 nm. The bioreactor used in this study (100 l capacity Bioengineering fermentor, Wald, Switzerland) had a working volume of 60 l and was set to control the fermentation variables automatically. The appropriate production medium (58.8 l) was sterilized in situ and the fermentation was started in batch mode with the following conditions: temperature 30 °C, stirrer speed 200 rpm, pH controller was connected only with 2 N NaOH and the pH was never allowed to drop below 7.0. However, rise in pH above 7.0 was not controlled. Dissolved oxygen (DO) was maintained between 30 and 40% saturation by controlling the air flow that was set at 1 $l\,l^{-1}$ min^{-1}. Silicone M30 (Dow-Corning Co, Midland, MI, USA) at 10% in mineral oil was used as an antifoam agent. Fermentation was terminated after the completion of spore crystal complex (24 h). Samples were withdrawn at 4 h intervals and used for the determination of the cell mass, spore count and the toxicity test. For each medium the fermentation was repeated three times on separate days.

Biomass

Culture samples (100 ml) drawn from the fermentor were centrifuged at 15,000×*g* for 20 min, the supernatant was discarded and the wet biomass was lyophilized and the dry weight expressed in g l^{-1}. This lyophilized powder was used for the determination of the mosquito larvicidal toxicity test.

Spore count

Culture samples were heat treated at 80 °C for 15 min, serially diluted and plated on NYSM agar plates. Plates were incubated at 30 °C for 48 h and the developing *B. thuringiensis* var. *israelensis* colonies were counted and expressed in CFU ml^{-1}.

Toxicity test

The dried cell biomass produced by lyophilization was assayed against early fourth instars laboratory-reared *Aedes aegypti* larvae. Lyophilized powders of *B. thuringiensis* var. *israelensis* was prepared in sterile distilled water and added to disposable cups, each containing 50 larvae in 250 ml of chlorine free tap water. All tests were conducted at 28 ± 2 °C. Each experiment included six concentrations of four replicates each, along with the appropriate control. Larval mortality was scored after 24 h and corrected for control mortality, using Abbott's formula [1]. The experiment was done three times on different days. The results are expressed in ng ml^{-1}. Probit regression analysis [6] was carried out to calculate LC_{50}.

Amino nitrogen estimation

The amino nitrogen content of the fermentation broth was determined by the method of Sorenson's Formol Titration method [11] and is as follows: a aliquot of 20 ml of culture supernatant taken into a clean conical flask was added with 5 ml of HCHO and kept for 2 min. A few drops of phenolphthalein indicator were then added and titrate against NaOH. The end point was the appearance of the permanent pale pink color. The titration was repeated for concordant values and the results are expressed in mg ml^{-1}.

Statistical analysis

A one-way ANOVA test was used to compare the mean maximum spore count, biomass, mosquito larvicidal toxicity and amino nitrogen level among different media. Pairwise comparison of the media was done using the Post-hoc multiple comparison test based on least significant difference (LSD).

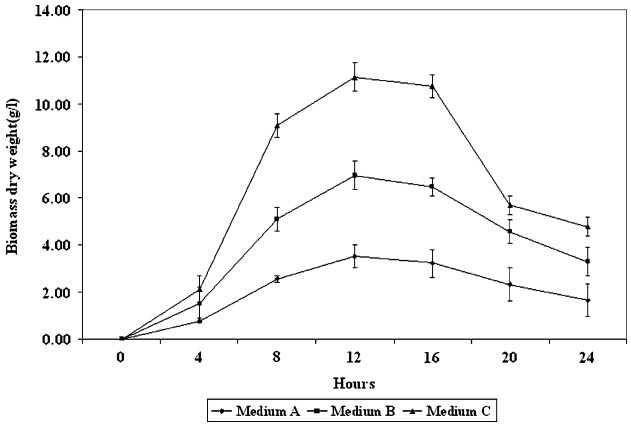

Fig. 1 Dry biomass of different hourly cultures of *B. thuringiensis* var. *israelensis* grown in medium A, B and C

Results

The biomass produced by different media containing different level of amino nitrogen varied significantly. The biomass (dry weight) obtained was 1.64 g l^{-1} (SE ± 0.4), 3.29 g l^{-1} (SE ± 0.34) and 4.78 g l^{-1} (SE ± 0.23) when grown in medium A, B and C, respectively (Fig. 1). The mean maximum biomass differed significantly between the three media [($F = 21.98$, $df = (2,6)$, $P = < 0.002$]. The mean biomass yield from medium "C" was significantly higher than that from medium "B" and "A" (Post-hoc multiple comparison test, $P < 0.0076$).

A maximum spore count of 3.24×10^{11} CFU ml^{-1} was obtained in medium "C" followed by medium "B" 8.5×10^{10} CFU ml^{-1} while medium "A" 3.7×10^{10} CFU ml^{-1} (Fig. 1). The mean maximum spore counts differed significantly between media [$F = 514.990$, $df = (2,6)$, $P = < 0.0001$]. Thus, the mean spore count from medium "C" was significantly higher than that from medium "B" and "A" (Post-hoc multiple comparison test, $P < 0.0001$).

Table 1 Toxicity of different hourly cultures of *B. thuringiensis* var. *israelensis* grown in Medium A, B and C against early fourth instar *Aedes aegypti* larvae

Hours	LC50 (ng ml^{-1}) (95% FL)		
	Medium A	Medium B	Medium C
4	0	0	0
8	0	0	0
12	458.04 (414.58–506.06)	395.29 (357.10–437.57)	351.92 (318.05–389.39)
16	79.89 (72.72–87.77)	67.84 (61.84–74.42)	59.51 (53.97–65.61)
20	38.41 (34.92–42.26)	31.47 (28.50–34.66)	22.62 (20.51–24.95)
24	27.01 (24.40–29.89)	22.56 (20.49–24.84)	18.52 (16.76–20.47)

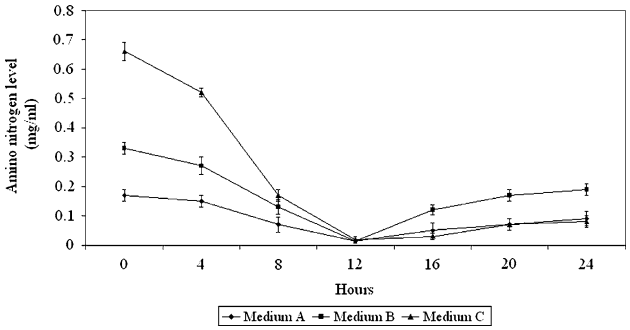

Fig. 2 Amino nitrogen level of different hourly cultures of *B. thuringiensis* var. *israelensis* grown in medium A, B and C

Similarly medium "C" was superior when the larvicidal activity of the biomass was taken into account, as the biomass grown in this medium exhibited maximum toxicity LC_{50} 18.52 ng ml^{-1} (Table 1). It is followed by medium "B" LC_{50} 22.52 ng ml^{-1} (Table 1) and medium "A" (LC_{50} 27.01 ng ml^{-1}). There is a significant difference between the LC_{50} values of biomass from different media [F = 217.048, df = (2,6), P = < 0.0001]. The larvicidal tests showed the mean LC_{50} of medium "C" is significantly higher than that of medium "B" and "A" (Post-hoc multiple comparison test, $P < 0.0012$).

The initial amino nitrogen level of each media varied significantly [F = 330.529, df = (2,6), P = < 0.0001]. The maximum amino nitrogen level in medium "C" was found to be 0.66 mg ml^{-1} (SE ± 0.22) while that of medium "B" 0.33 mg ml^{-1} (SE ± 0.01) and medium "A" 0.17 mg ml^{-1} (SE ± 0.01) (Fig. 2). Thus, the mean amino nitrogen level of medium "C" is significantly higher than that of medium "B" and "A" (Post-hoc multiple comparison test, $P <$ 0.0001).

Discussion

The present investigation indicated the important of the prior knowledge of amino nitrogen level in the fermentation medium at which the biomass, δ-endotoxin, spore count yield can be obtained maximally. In order to select the best and less expensive sources of nitrogen for the commercial production of *B. thuringiensis* var. *israelensis* different nitrogen sources like peptone and yeast extract from various commercial vendors was assayed amino nitrogen level in the raw materials procured and the one selected on the basis of higher level of amino nitrogen level. Thus, the data found to be appropriate from these experiments showed that better results can be obtained when nitrogen source containing high level of amino nitrogen. Peptone has been reported to be the better organic nitrogen source, supporting optimum production and sporulation as well as high cell density [8]. Shake flask culture of *B. thuringiensis* H-14 strains showed varied ability to produce toxins and δ-endotoxin lethal to mosquito larvae dependent upon the particular strain and growth medium used [19]. Highest sporulation titers were obtained at 2% fodder yeast concentration with endotoxin yields ranging between 7 and 9 g l^{-1} of medium [18]. The results obtained in the present study demonstrated the importance of amino nitrogen for the production of δ-endotoxin. The fact that the yield of δ-endotoxin per sporulated cell is strongly influenced by the selection of the medium rich in amino nitrogen and of the culture conditions is more important for the commercial production of *B. thuringiensis* var. *israelensis*. Attention should not only be directed towards high spore yields, but at the same time the components of the medium have to be balanced to obtain the best toxic activity per volume of medium. The additional amino nitrogen is present in the medium could increase the δ-endotoxin production and biomass. In summary, the results showed that increased initial amino nitrogen in the culture medium increases the biomass, spore count and toxicity. Similarly decreased amino nitrogen level in the culture medium decreases the biomass, spore count and toxicity is evidenced by this experiments.

In conclusion for the commercial production of *B. thuringiensis* var. *israelensis* measuring the amino nitrogen level is more important and can serve as a useful measure for assessing the quality of raw material in the production facilities for *B. thuringiensis* var. *israelensis*.

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