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STUDIES ON THE PRODUCTION OF SPORE CRYSTAL BY *BACILLUS THURINGIENSIS* CAMB 3-023 IN THE STIRRED FERMENTOR

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The insect pathogen *Bacillus thuringiensis* CAMB 3-023 was cultured using CSL-salt medium, in the stirred fermentor of 14 l capacity by batch process. The rates of aeration and agitation were kept at l/l/min and 200-300 rpm respectively. The sporulation hence in crystal protein synthesis was faster and much improved in comparison with shake flasks. It reached maximum ($4.2x10^9$/ml) in 48 h after inoculation with vegetative inoculum (10% v/v) developed in shake flasks. The bacterium was grown in the presence of different amounts of CSL (2.0-4.0% w/v). The optimum level of CSL was found to be 2.0% w/v. The addition of $MnSO_4$ and $CaCl_2$ during the course of fermentation enhanced sporulation.

Key words**:** *Bacillus thuringiensis*, Spore crystal protein, Fermentation.

Introduction

The bioinsecticide preparations of *Bacillus thuringiensis* are of great economic importance owing to their lethal effect against a wide variety of Lepidoptera, Diptera and Coleoptera larvae (Jamnback 1973; Scherrer *et al* 1977; Aronson *et al* 1986; Hofte and Whiteley 1989; Karim *et al* 1999a). The production of bioinsecticide is basically a cell biomass production process, provided the nutritional requirements of the bacterium for sporulation and endotoxin formation is fulfilled. Extensive studies have been carried out in connection with the nutritional requirements for growth and sporulation by submerged fermentation in shake flasks (Kang *et al* 1992; Zafar and Riazuddin 2002) and stirred fermentors (Goldberg *et al* 1980; Mohd-Salleh and Beegle 1980; Smith 1982, Kang *et al* 1992). The use of carbon sources such as glucose, a nitrogen source like yeast extract and the presence of potasium salts, manganese and some micronutrients are essential. Yeast extract is a source of amino acids, vitamins and minerals and the strain employed does not require vitamins as reported by the workers (Nickerson and Bulla 1974; Arcas and Yantorno 1987, Zafar *et al* 2001). Production of crystal protein in the bacterial cell follows the end of exponential growth phase and involves the assembly of proteins synthesized *de novo* at the beginning of sporulation (Scherrer *et al* 1973; Goldberg and Margalit 1977). Fed-Batch culture method has also been employed as a means of increasing cell mass or product concentration (Kang *et al* 1992). One reasonable way to increase spore production is to achieve high cell biomass and subsequently allow sporulation to occur. The spore concentrations obtained during different studies were $6.5x10^7$ (Pearson and Ward 1988), $1.8x10^8$ (Zafar and Riazuddin 2002a), $2.1x10^9$ (Arcas *et al* 1984) and $1.25x10^{10}$ per ml (Kang *et al* 1992) by using fed-batch culture method.

In continuation of our studies in shake flasks (Zafar and Riazuddin 2002a) the present work describes the propagation of a local isolate of *Bacillus thuringiensis* CAMB 3-023 (Karim *et al* 1999a) in the stirred fermentor for sporulation by batch process. Corn steep liqour (CSL) was used as a basal medium. The parameters such as concentration of CSL, agitation rate, size of inoculum, fermentation time for sporulation, dry pellet mass and crystal protein yield during fermentation were optimized.

Materials and Methods

Sources of raw materials/chemicals: Corn steep liquor (C.P.C. Rafhan Industries, Faisalabad, Pakistan), silicon based antifoam 30% (Sigma, Cat.No.A5758), Acetone (Merk, Cat.No.822251), $MnSO_4$ (Riedel-De Haenag,Cat.No.OE406-24) and $CaCl_2$ (Merk, Cat.No.102820).

Organism: The bacterium used was locally isolated *Bacillus thuringiensis* CAMB 3-023, carrying CryIA(a)/ IA(c) genes, previously characterized through molecular analysis according to the nomenclature of Bt. pesticidal crystal protein genes. It was maintained on Luria agar slants consisting of (g/l). Tryptone 10.0; Yeast extract, 5.0, NaCl, 5.0 and Difco Agar 15.0, pH 7.5. The cultures were incubated at 30˚C for 72 h and then stored at 4˚C. All the media, otherwise stated, were sterilized at 121˚C for 15 min.

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Inoculum preparation: The vegetative inoculum for fermentor studies was developed step wise in two stages: (i) First inoculum stage: 50ml LB medium, contained in 250ml cotton wool plugged conical flask, was inoculated by transferring a loopful of cells from LB agar slants. The flasks were rotated at 200 rpm using New Brunswick Incubator Shaker at 30°C for 24 h. (ii) Second inoculum stage: 500ml of 2.5% CSL medium in 2 liter flask was inoculated by transferring Ist inoculum and incubated for 24 h as mentioned above. Vegetative growth/sporulation by using Gram's staining and spore staining reactions were observed throughout the process of fermentation.

Fermentation: The fermentation studies were carried out in a 14 l New Brunswick Microferm Fermentor with working volume of 10 l. The fermentor containing CSL medium, with initial pH 7.0, adjusted by using NaOH solution (10.0%) was placed in the autoclave and sterilized at 121°C for 45 min. The size of the inoculum was kept at 10% v/v unless otherwise stated. The rates of agitation and aeration were 200-300 RPM and l/l/min, respectively. The foaming during fermentation was controlled manually by adding silicon based antifoam (30%) drop wise. The pH was recorded by pH probe and the sporulation of cells, however, was determined by microscopy during the course of fermentation. Finally, the number of spores/ml was estimated by colony forming unit assay. The solution containing $MnSO_4$ and $CaCl_2$ were added to the fermentor, 24 h after inoculation for the enhancement of sporulation. At the end of fermentation, the bacterial cell mass was harvested at room temperature by centrifugation (Beckman, Model J2-21) at 8000 rpm for 10-15 min. The wet pellet was treated with acetone to remove water and dried in a hot air oven at 50°C for 10 h. The dried cell mass was powdered using electric grinding jar mill to the mesh size 200 and formulation of bioinsecticide was developed for field studies (Karim *et al* 1999a; Zafar *et al* 2000; Karim *et al* 2000; Rehman *et al* 2002; Zafar *et al* 2002b).

Analysis: Colony forming units (CFU) of heat resistant spores were determined by making serial dilution of fermented culture, after giving heat shock at 80°C for 10 min. The samples were spreaded on LB agar plates and incubated at 30°C for overnight. The crystal protein concentration was estimated by dissolving known amount of dried cell mass in solublizing buffer (50mM sodium carbonate, 10mM Dithiothreitol, pH 10) and incubated at 37°C for 4-6 h with frequent shaking. The crystal protein in the suspension was solubilized at alkaline pH; insoluble fraction and cellular proteins were removed by centrifugation at 14,000 rpm for 10 min at room temperature. Protein concentration was measured by the method of Bradford (1976).

Results and Discussion

Corn steep liquor and byproduct of starch/glucose industries were found to be complete medium providing adequate amounts of all nutrients for the propagation of locally isolated *Bacillus thuringiensis* CAMB 3-023 instead of highly expensive raw materials such as yeast extract, peptone and casein hydrolysate.

Effect of the concentration of CSL (1.0-4.0% w/v) on the production of spore/ml, dry mass and crystal protein yield of *Bacillus thuringiensis* CAMB 3-023 was carried out in the stirred fermentor. The addition of $MnSO_4$ and $CaCl_2$ salts in CSL medium increased the yield substantially. For *Bacillus thuringiensis* propagation, it is necessary to use an adequate

Table 1

Effect of the concentration of CSL with and without salts on the production of spores, dry cell mass and crystal protein yeild of Camb Bt. 3-023 in the stirred fermentor*

CSL% (w/v)	Addition of salts**	Replicates	Sporulation spores/ml	Dry mass g/1	Crystal protein mg/g dry mass
1.0	–	2	$3.0x10^6$	1.5	100.0
	+	2	$3.0x10^6$	1.6	104.4
2.0	–	3	$3.5x10^7$	2.5	119.0
	+	4	$0.5x10^8$	3.0	159.0
2.5	–	3	$2.8x10^7$	2.6	105.3
	+	3	$4.3x10^7$	3.2	111.0
3.0	–	2	$5.0x10^6$	4.0	68.2
	+	3	$9.5x10^6$	4.4	72.0
4.0	–	3	$1.3x10^5$	4.9	47.4

* Agitation 200 RPM for 70 h cultivation ; ** $MnSO_4$ + $CaCl_2$

Table 2
Effect of agitaton on the production of spores, dry cell mass and crystal protein yield of Camb BT 3-023 in the stirred fermentor*

CSL** % (w/v)	Agitation RPM	Replicates	Sporulation spores/ml	Dry mass g/l	Crystal protein mg/g dry mass
2.0	200	2	$2.3x10^7$	2.7	106.4
	300	5	$1.4x10^9$	3.0	183.0
2.5	200	3	$0.6x10^7$	3.0	100.0
	300	3	$2.2x10^8$	3.4	154.7
3.0	200	2	$3.9x10^6$	3.4	54.4
	300	2	$0.2x10^8$	3.5	112.5

* Cultivation 48 h; ** CSL medium with salts

organic carbon and nitrogen source (Arcas and Yantorno 1987). Although CSL seems to be the most convenient source of carbon and nitrogen, the ratio of carbon and nitrogen source in the medium is very critical for the optimum sporulation. With the increase of CSL concentration in the medium, the rate of sporulation and crystal protein yield was decreased (Table 1). The maximum sporulation was observed in 2% CSL medium with $MnSO_4$ and $CaCl_2$ salts, at 30-32 h after inoculation. The cells were harvested for 68-70 h after inoculation. The maximum vegetative growth took place within first 32 hours and by the addition of salts at this point, the sporulation was $0.5x10^8$ per ml and crystal protein yield was 159 mg/g dry cell mass.

The adequate supply of dissolved oxygen to the culture is highly critical for maximum cell biomass formation. The supply of oxygen to the culture depends on both the rates of aeration and agitation during the course of fermentation. The rate of agitation was changed from 200 RPM to 300 RPM while the aeration was kept at l/l/min and it greatly enhanced both the sporulation and crystal protein yield. Maximum sporulation was observed 48 h after inoculation. The maximum vegetative cell growth reached within first 20-22 hrs and addition of salts was made at this point. In 2% CSL medium at 200 RPM, the rate of sporulation after 48 h fermentation is $2.3x10^7$ with the crystal protein yield of 106.4 mg/g dry mass. By increasing agitation upto 300 RPM, the maximum sporulation ($1.4x10^9$ spores/ml) was observed with the crystal protein yield of 183 mg/g dry mass (Table 2) whereas at 200 RPM the maximum sporulation/crystal protein was acheived in the same medium at 70 hrs fermentaiton after inoculation (Table 1).

Thus the CSL (2% w/v) medium containing salts was more effective in terms of spore/crystal protein yields, produced in 48 hrs fermentation at 300 RPM agitation rate with the aeration of l/l/min.

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