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PROPAGATION OF LOCALLY ISOLATED *BACILLUS THURINGIENSIS* (CAMB 3-023) FOR THE PRODUCTION OF SPORE-CRYSTAL PROTEIN IN SHAKE FLASK

A U Zafar* and S Riazuddin

National Centre of Excellence in Molecular Biology, University of the Punjab, Canal Bank Road, Lahore-53700, Pakistan

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The shake flask cultural conditions were optimized for the production of spore/crystal protein by locally isolated *Bacillus thuringiensis* strain CAMB 3-023. Of all the culture media tested, Corn Steep liquor medium with the addition of $MnSO_4$ and $CaCl_2$ gave highly encouraging results of spore counts ($1.8X10^8$/ml), crystal protein yield (42 mg g^{-1} dry mass) and also maximum dry cell mass (3.9 g l^{-1}). The final pH of the fermented mash was alkaline (9.0), 72 h after inoculation with vegetative cells.

Key words: *Bacillus thuringiensis,* Spore protein, Propagation.

Introduction

Extensive studies have been carried out on the propagation of *Bacillus thuringiensis* for the commercial production of microbial insecticides. (Goldberg *et al* 1980; Pearson and Ward 1988; Feitelson *et al* 1992; Kang *et al* 1992; Karim *et al* 1999b) The bacterium is an insect pathogen which produces a proteinaceous crystaline toxin that causes paralysis of the larval gut on ingestion. Moreover, large quantities of spores with high insecticidal activity are required for wide spread field use (Stockdale 1985). It is generally observed that high yielding plant varieties show more susceptibility to insect attack than wild type. Indiscriminate use of chemical insecticides has posed serious problems of environmental contamination through interference in the food chain. Apart from this, the resistance of pests to these chemical insecticides increases over the course of time (Burges and Hussey 1971). Thus there is a need for safe ecologically tenable pesticides. Insect pests are susceptible to a wide variety of microbial pathogens such as viruses, bacteria, fungi etc. that have been exploited as biopesticides (Aizawa 1982). Insect pathogens have no effect on humans and higher animals, which makes them more desirable over chemical insecticides. The potential of microbes in pest control, therefore, lies in directing their lethal or sterilizing effect to unwanted host insects and minimizing the same in domesticated insects. The biological control of pests should be scientifically exploited in accordance with economic or social needs. To achieve these objectives, a survey was conducted in isolating local strains of the bacterium *Bacillus thuringiensis* from different local habitats including dead or diseased insects (Khan *et al* 1995 a & b; Karim *et al* 1999a). The culture of *Bacillus thuringiensis* (CAMB 3-023) with maximum sporulation and toxicity to various Lepidoptera was selected for the development of bioinsecticide. The present work is, therefore, concerned with the optimization of cultural conditions in shake flasks for the propagation of the locally isolated strain.

* Author for correspondence

Materials and Methods

Organism: Bacillus thuringiensis CAMB 3-023 carrying CryIA(a)/1A(c) gene was used in the present study. The culture was maintained on Luria Agar medium consisting of (g l^{-1}):- Tryptone, 10.0; yeast extract, 5.0, NaCl, 5.0 and agar 15.0, pH 7.5. The culture was incubated at 30°C for 72 h afterwords the cultures were stored at 4°C. All the culture media used in the present study, unless otherwise stated, were sterilized at 121°C for 15 min.

Fermentation technique: The vegetative inoculum was developed in 250ml shake flask containing 50ml Luria broth described earlier. The medium was inoculated by transfering small amount of growth from 5 to 7 days old agar slant. Shake flask cultures were incubated at 30°C for 24 h. The incubator shaker (New Brunswick series 25), was rotated at 200 rpm. For fermentation studies 200ml of basal medium contained in IL cotton wool plugged conical flask was inoculated by 2.0% (v v^{-1}) vegetative inoculum. The flasks were shaken on rotary shaker. Following culture media were used in the present investigation:

Table 1
Selection of culture media for the propagation of *Bacillus thuringiensis* CAMB 3-023 in shake flasks

Medium	PH final	Sporulation spore/ml	Dry cell mass g l^{-1}	Crystal protein mg g^{-1} dry mass
M 1	8.2	6.90X105	1.44	24.0
M 2	7.8	1.30X106	5.40	10.5
M 3	8.6	2.00X106	6.30	11.0

Table 2
Effect of the concentration of CSL on the production of cell biomass, sporulation and crystal protein by *Bacillus thuringiensis* CAMB 3-023 in shake flasks

CSL g l^{-1}	PH final	Sporulation spore/ml	Dry cell mass g l^{-1}	Crystal Protein mg g^{-1} dry mass
10	8.89	2.35X104	1.2	19.2
20	8.90	2.10X107	2.4	22.4
30	8.72	4.37X107	3.2	26.8
40	8.49	2.80X106	4.4	11.6
50	8.45	1.28X106	6.4	10.4

M1 (g l^{-1}): Yeast extract, 0.2; glucose, 1; casaminoaicd, 0.4,$MgSO_4$, 0.2; K_2HPO_4, 0.6; KH_2PO_4, 0.4; $MnSO_4$, 0.5; $ZnSO_4$, 0.4; $CuSO_4$, 0.5; $FeSO_4$, 0.02;

M2 (g l^{-1}): Cane molasses, 50.0

M3 (g l^{-1}): Corn Steep liquor (CSL), 50.0

Analysis: Spore counts of test samples were determined by standard plate count method. Ten fold dilutions of samples were prepared and heat shocked at 80°C for 15 min to kill the vegetative cells. 100 microliter samples were spreaded on each agar plate. The number of colonies appeared on each plate, were multiplied with there dilution factor which represent the number of spores in the propagated cultures. The fermented mash was centrifuged at 7000 rpm for 15 min to collect the cell mass. The cell mass pellet was washed with acetone for the removal of water. The pellet was dried at 50°C in a hot air oven.

Crystal protein concentrations were measured by Bradford (Bio-Rad) assay (1976) after alkaline solubilization of crystal protein from the dried cell mass pellet. Known amount of pellet was suspended in solubilizing buffer (50 mM sodium carbonate, 10mM Dithiothreitol, pH 10.5) and incuabted at 37°C for 4 h. The crystal protein in the suspension was solubilized and insoluble fraction was removed by centrifugation at 14,000 rpm for 10 min.

Results and Discussion

Selection of culture medium: The designing of fermentation medium is very critical for the optimum production of metabolites or cell mass. The sources of carbon, nitrogen and their ratios along with minerals and vitamines play an important role in fermentation process (Scherrer *et al* 1973). Different culture media (M1, M2 & M3) were examined for the production of dry cell mass, spores per ml and crystal protein by propagating *Bacillus thuringiensis* CAMB 3-023 in shake flasks (Table 1). Medium M1, consisting of yeast extract-casiene hydrolysate-salts produced both less number of spores ($6.9X10^5$/ml) and cell mass (1.44 g l^{-1}) but the amount of crystal protein (24mg g^{-1} dry mass) was higher in comparison with other culture media. The pH of the fermented mash became alkaline (8.2). In case of molasses medium (M2), the dry cell mass and number of spore per ml were higher than that obtained in medium M1. The final pH was 7.8. It could be due to higher level of sugar in molasses medium (M2). Of all the media, however, Corn Steep Liquor medium (M3) gave highly encouraging results of cell biomass, sporulation and crystal protein content. Though the number of spores were lesser as reported by other workers (Goldberg *et al* 1980, Arcus *et al* 1987; Pearson and Ward 1988;) but their studies were carried out in the stirred fermentor by both batch and fedbatch techniques. The final pH of the fermented medium was more alkaline (Stockdale 1985; Aizawa 1982). Corn Steep Liquor, byproduct of starch industry being a good source of all the nutrients and also due to economic reasons it was selected for further investigations.

Effect of the concentration of CSL: CSL is good source of both sugar and nitrogen and its optimum level was determined by adding different/concentrations (10.0 to 50.0 g l^{-1}) in the fermentation medium (Table 2). The bacterial cell mass hence sporulation was greatly affected when the amount of CSL in the medium was 10.0 g l^{-1}, i.e., $2.35X10^4$ spores/ml and 1.2 g l^{-1} dry cell mass. Further increase in the concentration of CSL (20.0 & 30.0 g l^{-1}) resulted in the enhancement of both the sporulation and cell mass. The sporulation, however, was maximum (4.37X107/ml), when the level of CSL was 30 g l^{-1}. At high level of CSL (4.0 to 5.0 g l^{-1}) the cell synthesis was increased but sporulation/crystal protein yield were both greatly affected. The optimum level of CSL, therefore, was found to be 30 g l^{-1} (w/v) in shake flask experiments.

The extract of cotton seed meal (CSM) was also added to CSL medium in order to study its effects on both cell forma-

Table 3

Effect of the addition of cotton seed meal extract of CSL medium on the sporulation and crystal protein by Bacillus thuringiensis CAMB 3-023 in shake flasks.

CSM* extract	Sporulation spore/ml	Dry Cell mass g l^{-1}	Crystal protein mg g^{-1} dry mass
0.00	4.37X107	3.2	26.8
0.25	1.00X107	3.8	18.4
0.50	9.00X106	4.0	17.9
1.00	1.20X16	4.2	14.2

* Cotton seed meal (CSM) extract was prepared by boiling 10% (w/v) cotton seed meal in distilled water for 15 minutes and used filterate in culture medium.

tion and sporulation (Table 3). The control cultures were also run in parallel. The addition of CSM extract resulted in the increase of cell synthesis but sporulation was greatly reduced. It shows that slight change in the Carbon-Nitrogen ratio greatly affected the sporulation. This has also been reported by Pearson and Ward (1988).

Effect of the addition of mineral salts: The presence of mineral salts in the basal medium is necessary for the enhancement of sporulation by the bacterial culture. Many workers have supplemented the fermentation media with different mineral salts (Goldberg *et al* 1980; Robert 1982).

The addition of different salts such as $MnSO_4$, $CaCl_2$, $MgSO_4$, $ZnSO_4$ or $FeSO_4$ was made to CSL medium (Table 4). The sporulation and cell synthesis were both affected in the presence of $MgSO_4$, $ZnSO_4$ or $FeSO_4$. The incorporation of $MnSO_4$ or $CaCl_2$, however, resulted in the enhancement of sporulation, but the combined effect of both these salts further improved the sporulation $1.8X10^8$ spores/ml and crystal protein yield of 42.0 mg g^{-1} dry mass.

Table 4

Effect of the addition of different salts to CSL medium on the cell biomass, sporulation and crystal protein by *Bacillus thuringiensis* CAMB 3-023 in shake Hasks

Salts	Conecn-ration g l^{-1}	Spor-ualtion spore/ml	Dry cell mass g l^{-1}	Crystal protein mg g^{-1} dry mass
$MnSO_4$	0.05	3.0X107	3.6	32.5
$CaC1_2$	0.10	1.2X107	3.5	33.2
$MnSO_4$+$CaC1_2$	0.05+0.10	1.8X108	3.9	42.0
$MgSO_4$	0.05	6.5X104	3.0	11.0
$ZnSO_4$	0.02	2.8X106	3.2	17.5
$FeSO_4$	0.02	4.0X105	3.2	14.0
Control	-	2.9X107	3.3	25.4

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