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Evaluation of the Growth Pattern of *Bacillus thuringiensis* Bt_{III} in Different Media Containing Low-Cost Agro-Industrial Based Carbon Sources

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Abstract: Synthetic pesticides have a number of ill effects on soil, water, human health, wildlife and ecological balance. These ill effects have led to the use of microbial insecticides against insect pests of order Lepidoptera, Coleoptera, Diptera and Hemiptera. *Bacillus thuringiensis* is a gram-positive endospore forming bacterium that produces a parasporal crystal protein inclusion during sporulation that is insecticidal to insect pests of various orders. An attempt has been made to develop low-cost *Bacillus thuringiensis* formulations for the management of *Helicoverpa armigera*, which is a major problem in cotton, gram, vegetables and other crops. Agro-industrial residues and their byproducts can be used for the large-scale production of *B. thuringiensis* biomass to reduce the cost of production of these bio-pesticides. Different low-cost agro-industrial based carbon sources such as potato extract, cornstarch, wheat flour, sugarcane molasses, barley flour and soluble starch were evaluated for *Bacillus thuringiensis* biomass production. The media containing different carbon sources were inoculated with *Bacillus thuringiensis* strain Bt_{III} and kept under submerged conditions. The growth pattern was observed by plate count and absorbance method. Among the different carbon sources evaluated, maximum biomass of *B. thuringiensis* Bt_{III} was achieved after 48h of growth in the medium containing 1.2% potato extract in basal medium. The biomass production in this medium was equivalent to that achieved in LB medium after 48h of growth.

Key Words: *Bacillus thuringiensis*, biomass, agricultural by-products

Introduction

The *Bacillus thuringiensis* bacteria is receiving increasing attention for use in integrated pest management programs for agricultural and forest insect pests and insect vectors of human and other mammalian transmissible diseases. Taxonomically, these entomopathogenic (causing disease in insects) bacteria belong to the family Bacillaceae and are members of the genus *Bacillus*. Typically, they are Gram-positive, rod-shaped, form a spore and are motile by flagellae (whip-like appendages). The unique characteristic to this species is that they form a protein crystal next to the spore at the time of sporulation. *B. thuringiensis* occurs naturally in numerous species of agricultural and forest insects and is a component of the soil microbiota worldwide (Martin and Travers 1989). The worldwide market for *Bt* var. *kurstaki*-based products for forestry and agriculture is estimated to be about US\$60 million-80 million per year. At present, the single largest market for these products is against forest insect pests in North America.

B. thuringiensis is commercially produced by liquid fermentation. Large vats (5,000- to 140,000-liter capacity) filled with water, proteins, carbohydrates (sugar) and other ingredients needed to sustain *Bt* growth, are seeded with a small amount of *Bt* and incubated under specific conditions. When cell replication is complete (i.e., 10 to 100 millionfold increase in 72-120 hours), a spore (or endospore, dormant stage of the bacteria), and a diamond-shaped protein inclusion referred to as the crystal (or parasporal body) are formed within the vegetative cell (sporangium). At the completion of spore formation, the wall of the sporangium breaks down releasing both the spore and crystal into the surrounding growth medium. The spores, crystals and other residual fermentation solids are then harvested, stabilized, standardized, and formulated into the commercial product. Therefore, commercial formulations of *Bt* contain both the spore and crystal as their entomopathogenic ingredients. In susceptible insects, the alkaline midgut environment (pH>8.0) and proteolytic enzymes, dissolve ingested crystals and release smaller delta-endotoxins. These proteins, also known as the insecticidal crystal proteins (ICP's), recognize and bind to specific receptors on the cellular lining of the midgut. Depending on the *Bt* strain used, one or several different types of ICP's may be released from the crystal matrix. Once bound to the receptors, ICP's penetrate through the cell membrane and form ion-selective channels. The selective permeability characteristic of the cell membrane is then disrupted, causing the cell to absorb water, swell, and burst. This results in a perforation of the gut and leakage of gut content, including spores, into the hemolymph. At this point, gut paralysis (and in some cases, paralysis of the mouthparts) occurs, the larva stops feeding, and dies in a few hours to a few days.

B. thuringiensis (*Bt*) is highly effective against the American boll worm, *Helicoverpa armigera*, at the larval stage which cause great damage to various commercially important crop plants worldwide. Bioinsecticides containing *B. thuringiensis* are target-specific and can minimize the possibility of development of resistance in insects. The media for the production of *Bt* biomass contain nitrogenous sources and energy producing carbohydrate sources which are easily

assimilated by bacteria. For optimal growth, *B. thuringiensis* also require a variety of inorganic sources which function physiologically as protein, nucleic acid and co-enzyme constituent and as cofactor of numerous enzyme reactions and bacterial cytochromes (Morris *et al.* 1997). But media ingredients are too expensive to be used in the large-scale production of *B. thuringiensis*. Therefore, there is a need to substitute less expensive agricultural products or agro-industrial based by-products. The cost of fermentation ingredients is especially important since they will determine the cost-benefit ratio of the final product. Therefore, the present investigation was undertaken to investigate growth pattern analysis of *Bacillus thuringiensis* Bt_{III} in different media containing low cost agro-industrial based carbon sources.

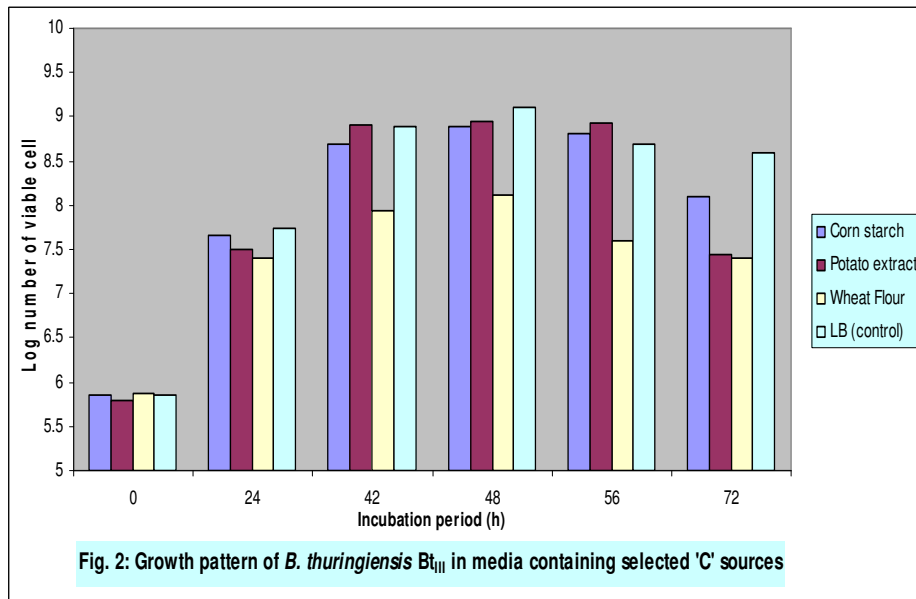
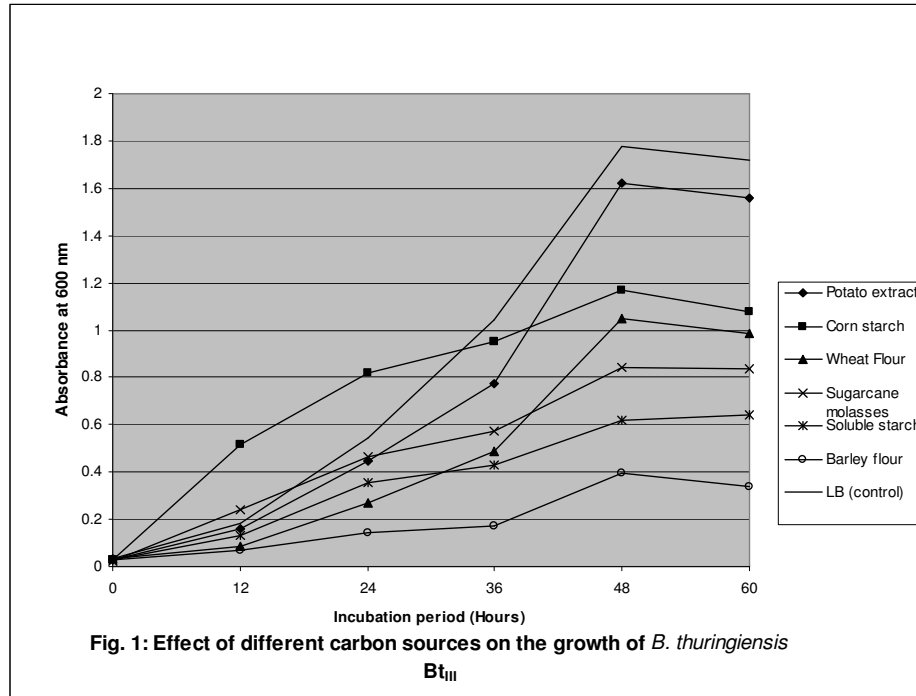
Materials and Methods

The native isolates of *Bacillus thuringiensis* were obtained from Microbial Biotechnology lab of BMB department, CCS HAU, Hisar. Agro-industrial residues and their byproducts can be used for the large-scale production of *B. thuringiensis* biomass to reduce the cost of production of these bio-pesticides. Different low-cost agro-industrial based carbon sources such as potato extract, cornstarch, wheat flour, sugarcane molasses, barley flour and soluble starch were used for *Bacillus thuringiensis* biomass production. For preparation of potato extract, 250g of potatoes were boiled in one liter of water and extract was used in the growth medium. The basal medium used for the growth of *B. thuringiensis* isolates was Morris *et al.* (1997) medium with modification which contained Peptone 2.0g/l, Dextrose 1.5g/l, Yeast extract 2.0g/l, MgSO₄·7H₂O 0.03g/l, FeSO₄·7H₂O 0.02g/l, ZnSO₄·7H₂O 0.02g/l, NaCl 5.0g/l, Tween 60 1.0ml/l along with agro-industrial based carbon source. Tween 60 was used as a surfactant to increase bacterial cell membrane permeability and consequent uptake of nutrients from the fermentation medium. All media were sterilized at 15 lb/sq inch for 20 min. *B. thuringiensis* isolate Bt_{III} was inoculated into these production media and incubated at 30°C on a rotary shaker. Samples were withdrawn at an interval of 12h, serially diluted and plated on the LB (Luria broth containing 10g/l tryptone, 10g/l yeast extract, 5g/l NaCl) agar plates. The plates were incubated for 24h at 30°C and then total numbers of colony forming units (cfu) were calculated. For optimizing the amount of carbon source in the media, different concentrations of carbon source were used in the production medium.

Results and Discussion

Different carbon sources were evaluated for the production of Bt biomass. Different media were inoculated with *B. thuringiensis* isolate Bt_{III} and growth was monitored at an interval of 12h for 60 hours. It was observed that *B. thuringiensis* isolate attained the maximum growth after 48 hours of inoculation in all the media evaluated. Among the different media evaluated, the medium containing potato extract showed maximum growth followed by the medium containing corn starch

(Figure 1 and Figure 2). The biomass production was the lowest in the medium containing barley floor as the carbon source.



To optimize the concentration of potato extract, its different concentrations were used in production medium. It was observed that maximum biomass was produced when PE was used at a concentration of 12ml/l (1.24×10^{10} cfu/ml in case of *B. thuringiensis* isolate Bt_{III}). By increasing the concentration of PE, there was a decrease in total cell number (Table 1). This suggested that the potato extract at a concentration of 12ml/l was found to be optimum for biomass production.

Tab. 1. Effect of potato extract concentration on production of *B. thuringiensis* Bt_{III} biomass

Potato extract conc.(ml/L)	Biomass production*					
	Incubation period (hours)					
	0	12	24	36	48	60
2	4.255	4.832	5.146	8.881	9.934	9.695
4	4.278	4.839	5.857	8.892	9.949	9.732
6	4.342	4.857	6.079	8.913	10.100	9.778
8	4.204	4.863	6.113	9.880	10.000	9.832
10	4.342	4.869	7.113	9.903	10.086	9.845
12	4.278	4.880	7.117	9.924	10.086	9.806
14	4.278	4.880	7.120	9.924	10.093	9.812
LB(control)	4.278	4.869	5.982	9.934	10.107	9.845
S.E.(±)	2.229	3.502	1.291	1.798	1.586	3.502
C.D.(p≤0.05)	3.381	3.599	4.275	4.955	3.254	3.599

*log number of total viable cells

Designing a cheap, industrial fermentation medium is one of the most important aspects of process development. An important aspect of reducing fermentation costs can be substitution of high-cost media ingredients by agro-industrial residues and by-products. *B. thuringiensis* strains are known to utilize a large number of different carbon and nitrogen sources for growth and delta-endotoxin production (Schisler *et al.* 2004). Various agricultural nitrogen sources like deoiled and expeller cakes of mustard, cottonseed, groundnut, corn steep liquor have been used to design low-cost fermentation media in batch culture process (Johnson *et al.* 1994). High spore yield was reported with a medium containing cheese whey, soya bean milk and molasses (Alves *et al.* 1997). *B. thuringiensis* also require, for optimal growth, a variety of macro and microelements which function as co-factors of numerous enzyme reactions (Sarrafzadeh *et al.* 2005). In minimal medium, various ingredients are too expensive for large-scale production of *B. thuringiensis*. Therefore, agricultural products and by-products were evaluated as sources of nutrients for production of *B. thuringiensis*. Cotton seed meal, defatted soya flour and corn gluten meal were found to be the most efficient substrate for the production of *B. thuringiensis* biomass (Morris *et al.* 1997). Bengal gram (Poopathi and Kumar 2003), defatted groundnut cake, gram flour and soy bean (Desai and Shethna 1991) have been used as different nitrogen sources for the production of *B. thuringiensis*.

Conclusions

In the present study, six different cost effective carbon sources such as potato extract, cornstarch, wheat flour, sugarcane molasses, barley flour and soluble starch were evaluated for biomass production. The results in this study indicated that the potato extract supported the highest biomass production of *Bt* followed by the cornstarch-containing medium and that 1.2% concentration of potato extract produced the optimum biomass after 48h of incubation i.e. 1.24×10^{10} cfu/ml which was comparable to growth in LB medium (1.1×10^{10} cfu/ml). Therefore, LB medium could be easily replaced for industrial scale production of *B. thuringiensis* because of its high cost in comparison to the medium containing potato extract as carbon source.

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ISPITIVANJE RASTA *Bacillus thuringiensis* Bt_{III} NA RAZLIČITIM SUPSTRATIMA KOJI SADRŽE JEFTINE IZVORE UGLJENIKA POREKLOM IZ POLJOPRIVREDE I INDUSTRIJE

- originalni naučni rad -

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Rezime

Sintetički pesticidi ispoljavaju brojna štetna dejstva na zemljište, vodu, ljudsko zdravlje, divlje životinje i ekološku ravnotežu uopšte. Zbog ovakvih efekata za suzbijanje insekatskih štetočina iz redova Lepidoptera, Coleoptera, Diptera i Hemiptera, sve više se počinju primenjivati mikrobiološki insekticidi. *Bacillus thuringiensis* je gram-pozitivna bakterija koja formira endospore i stvara kristalni protein uz samu sporu tokom sporulacije koji ima insekticidno dejstvo na različite redove insekatskih štetočina. Cilj rada je bio da se proizvedu jeftini preparati na bazi *Bacillus thuringiensis* koji bi se primenili u suzbijanju *Helicoverpa armigera*, jednog od najglavnijih insekatskih štetočina useva pamuka, indijskog pasulja, povrća i drugih kultura. Ostaci iz poljoprivrede i industrije i njihovi sporedni proizvodi mogu se upotrebiti u masovnoj proizvodnji biomase *B. thuringiensis* u cilju smanjenja troškova proizvodnje ovih biopesticida. Za tu svrhu izvršeno je ispitivanje različitih jeftinih izvora ugljenika poreklom iz poljoprivrede i industrije – ekstrakt krompira, kukuruzni skrob, pšenično brašna, melasa iz šećerne trske, ječmeno brašno i rastvorljivi skroba. Hranljive podloge sa različitim izvorima ugljenika inokulisane su sojem Bt_{III} bakterije *Bacillus thuringiensis*. Rast bakterija praćen je izračunavanjem ukupnog broja bakterija, primenom apsorpcione metode. Maksimalna biomasa *B. thuringiensis* Bt_{III} postignuta je nakon 48-časovnog rasta na hranljivoj podlozi sa 1,2% krompirovog ekstrakta u baznoj sredini. Količina biomase dobijena na ovoj podlozi bila je istovetna količini proizvedenoj na LB podlozi nakon 48-časovnog rasta.