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Biochemical and Physical Requirements of *Bacillus thuringiensis* subsp. *kurstaki* For High Biomass Yield Production

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ABSTRACT

The effects of total carbohydrate, total protein, carbohydrate/protein ratio, amino acid contents, initial pH and aeration on biomass yield product of *Bacillus thuringiensis* subsp. *kurstaki* was investigated. The bacteria were cultured in economical media comprising agricultural products and by-products including fishmeal, cottonseed meal, defatted soybean meal, cornsteep liquor, yeast extract, scotafeme, sorghum and peptone as protein sources and glucose and beet molasses as carbohydrate sources. The results indicated the presence of a direct correlation between yield biomass and carbohydrate/protein (C/N) ratio as well as the glutamic acid content of the media. The highest biomass was produced in the media providing 0.4 – 0.5 C/N ratio and 13.9% glutamic acid. A pH range of 7.0 to 8.0 was needed for high yield production. The optimal ratio for culture volume to flask size and shaking speed were 1/5 and 250 rpm, respectively. The biochemical factors described can be considered as the minimal criteria to evaluate culture media for biomass production from *Bacillus thuringiensis* subsp. *kurstaki*.

Keywords: *Bacillus thuringiensis*, Biomass, Aeration.

INTRODUCTION

Bacillus thuringiensis (*B. thuringiensis*), a gram positive spore-forming bacterium, is the most widely used biological agent for controlling insect pests of agricultural, forestry, medical and veterinary importance. An important feature of the bacterium is the production of large, proteinaceous crystalline inclusions during sporulation. These crystals consist of one or more proteins (Cry and Cyt proteins with molecular masses of 60-130 and 20 kD, respectively) which are responsible for the insecticidal properties of many *B. thuringiensis* strains. Upon ingestion by insect larva, crystalline toxins are dissolved by alkaline proteases in the midgut and are converted to the active form by trimming with the gut proteases. The activated toxin then binds to insect-specific receptors exposed on the surface of the plasma membrane of midgut epithelial cells and then inserts itself into the membrane to create transmembrane leakage pores that cause cell swelling and lysis and eventual death of the insect.

Because of the high efficiency of *B. thuringiensis* endotoxins against pest larvae, these toxins are of high commercial importance and strain selection and varying cultural conditions have been widely investigated to improve *B. thuringiensis* endotoxin production (Dulmage and Rhodes,

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1982; Holmberg and Sievanen, 1980). The media used for the industrial production of *B. thuringiensis* are composed of complex carbon and nitrogen sources. Starch and molasses are considered as suitable carbon sources and protein-rich materials such as soybean flour, cornsteep liquor and casein are among the cheap sources of nitrogen used. The addition of macronutrients such as Mg^{2+} and Ca^{2+} has been reported to increase the yield and insecticidal activities of *B. thuringiensis* (Goldberg *et al*., 1980).

In the present study, a number of different culture media were used to produce *B. thuringiensis* subsp. *kurstaki* spore-crystal mixtures. The main objective was to determine a relationship between the amounts of spore-crystal mixture produced and the biochemical constituents, including carbohydrate and protein, of the culture media. The effect of aeration and initial pH on biomass yield were also investigated.

MATERIALS AND METHODS

Bacterial Strain and Growth Condition

Lepidopteran-active *B. thuringiensis* subsp. *kurstaki* was obtained from the commercial product Dipel®. To obtain the spore-crystal mixtures, single colonies from overnight LB plates were inoculated into 100 ml T3 sporulation medium (per liter: 3 g of tryptone, 2 g of tryptose, 1.5 g of yeast extract, 0.05 M sodium phosphate [pH 6.8] and 0.005 g MnCl) and cultured for 60 hours in an orbitary shaker incubator at 30° C and 200 rpm. Spores and crystals were harvested by centrifugation at 7000 g for 10 min and, after two washes with sterilized distilled water, the pellet was resuspended in water and stored at –20° C until required. For long-term storage, the slurry was mixed with glycerol to make a final 20% glycerol suspension and stored at –80° C.

Shake flask Production in Experimental Media

The agricultural raw materials used in this study are listed in Table 1. The biochemical components chosen to characterize these products were total carbohydrate, total protein and essential amino acids for spore and crystal production (Fast, 1981; Nickerson and Bulla, 1974). The raw materials were ground in a laboratory grinder (if necessary) and sifted through a 180 µm mesh sieve before use. The initial concentrations for carbohydrate sources including glucose and beet molasses were 4.0, 7.0 and 15.0 g/lit and for protein sources was 30.0 g/lit. Usually five different media, in triplicates, were simultaneously cultured in 500 ml Erlenmeyer flasks. This was done to minimize expected variations in biomass production between batches. The pH of all cultures was adjusted to 7.0 at the beginning of the shake culture using 1.0 M sodium hydroxide or concentrated hydrogen chloride. After 60 hours of incubation, the spore-crystal slurries were harvested by centrifugation at 7000 rpm for 10 minutes.

Cell Mass Analysis

Colony-forming units (CFUs) were determined by heating 1.0 ml of freshly homogenized aliquots of the broth at 65° C for 10 minutes in a water bath. Serial dilutions were then prepared in sterile saline and 0.1 ml aliquots of the appropriate dilution were spread on T3 plates in triplicate and each count was repeated once. The plates were incubated at 30° C for 24 h and only those plates containing 15-200 colonies were used for enumeration.

Effect of Aeration and pH on Biomass

The effect of aeration on biomass yield of *B. Thuringiensis* subsp. *kurstaki* was determined by growing bacteria in varying ratios of the culture volume to flask size (500 ml) and different shaking speeds ranging from 150 to 300 rpm. The ratio of culture to flask volumes was 0.2, 0.3 and 0.4. To investigate the effect of the initial

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Table 1. Agricultural products and by-products used as protein and carbohydrate sources for *Bacillus thuringiensis* subsp. *kurstaki* production.

	Composition[a]								
				Amino acid (% of protein)					
Product	Protein (%)	Carbohydrate (%)	C/N ratio	Glutamic acid	Arginine	Glycine	Leucine	Histidine	Methionine
Defatted soybean meal	54.5	28.2	0.5	-	-	-	-	-	-
Cornsteep liquor	42.8	12.8	0.4	13.9	4.4	1.1	8.2	2.8	2.1
Yeast extract	43.8	8.0	0.2	6.5	0.8	2.4	0.8	3.6	0.8
Cottonseed meal	61.0	23.2	0.4	16.0	7.2	2.8	6.5	2.0	1.4
Sorghum	10.0	72.0	7.2	2.8	0.4	0.4	1.4	0.2	0.2
Dried milk	13.1	54.8	4.18	5.3	0.8	-	2.49	0.69	0.64
Peptone	13.2	-	-	17.3	0.4	1.3	7.2	2.0	2.7
Scatefome	-	-	-	-	-	-	-	-	-
Fishmeal	-	-	-	-	-	-	-	-	-
Beet molasses	6.7	65.1	9.7	-	-	-	-	-	-

[a] Data from Paul and Southgate (1978), Solomons (1969), Anonymous (1971), Miller and Churchill (1986) ; -, no available dat

pH on biomass yield, the initial pH of soybean medium was adjusted to 5.0, 6.0, 7.0, and 8.0 and the bacterial CFU/ml was determined at 60 hours after incubation.

RESULTS AND DISCUSSION

Biochemical analyses of different media relative to *B. thuringiensis* biomass production were mainly based on the C/N ratio and amino acid content. *B. thuringiensis* produces large parasporal crystals composed of 95% protein and 5% carbohydrate and the media rich in protein could efficiently support high biomass production. The amount of biomass yield produced in different media as determined by viable spore count method is summarized in Table 2.

Among the products where both carbohydrate and protein contents were known, the C/N ratio of 0.5 provided optimal conditions for biomass production. Spore counts on defatted soybean meal and cornsteep liquor with C/N ratios of 0.5 and 0.4, respectively, were the highest. Desai and Shethna (1991) reported that the toxicity of *B. thuringiensis* subsp. *israelisiensis* was tripled at a C/N ratio of 0.5 compared with one at 0.2. The optimal C/N ratio for *B. thuringiensis* subsp. *aizawai* was 0.3-0.5 (Morris *et al*., 1997). Dried milk with a C/N ratio of 4.18 could efficiently support bacterial multiplication which is possibly due to the highly neutrified and optimized nature of this product which can provide almost all nutritional requirements of dividing and developing cells (Table 1).

The optimal media, in terms of protein composition, contained high levels of glutamic acid, the most abundant amino acid constituent of the crystal protein (Fast, 1981). Cornsteep liquor had large amounts of glutamic acid (13.9%) as well as arginine and leucine, both of which are among the major constituents of *B. thuringiensis* parasporal crystal (Fast, 1981). Glutamic acid, glycine, arginine and methionine stimulated growth of *B. thuringiensis* subsp. *thuringiensis* (Rajalakshmi and Shethna, 1977) and glutamic and aspartic acids are known to be essential for the growth of *B. thuringiensis* subsp. *Israeliensis* (Dharmsthiti, 1985).

Cottonseed meal has an optimal C/N ratio as well as high levels of glutamic acid but could not support biomass production. This was possibly because of the nature of this nutrient source, with its constituents

Table 2. Laboratory production of spore-crystal mixture of *Bacillus thuringiensis* subsp. *kurstaki* from different protein and carbohydrate sources.

Protein source (30.0 g/lit)	CFU/ml[a] in varying concentrations of carbohydrate sources[b]			
	Glucose (4.0 g/lit)	Glucose (7.0 g/lit)	Molasses (7.0 g/lit)	Molasses (15.0 g/lit)
Defatted soybean meal	1.3×10^9	1.7×10^9	0.4×10^7	0.6×10^7
Cornsteep liquor	1.0×10^8	1.4×10^9	1.0×10^5	1.0×10^6
Yeast extract	0.4×10^8	0.7×10^8	0.3×10^6	0.8×10^6
Sorghum	0.1×10^7	0.3×10^7	1.0×10^6	1.2×10^6
Fishmeal	1.0×10^6	1.0×10^6	1.0×10^6	1.0×10^6
Cottonseed meal	1.1×10^7	1.2×10^7	1.0×10^7	1.0×10^7
Dried milk	0.2×10^8	0.8×10^8	0.8×10^7	0.1×10^7
Peptone	1.2×10^7	2.5×10^7	1.0×10^6	0.5×10^7
Scatefome [c]	0.4×10^7	0.7×10^7	0.6×10^6	0.4×10^6

[a] 1×10^8 heat-resistant spores/ml corresponds to 1×10^9 spores/ml (Goldberg *et al*., 1980).
[b] Data are average number of triplicates repeated at least once at different times. Bacteria were cultured in shaking flasks at 30° C , 250 rpm, medium volume/flask volume at 1/5 and at initial pH of 7.0. Data are obtained at 60 h after incubation.
[c] 30 ml/lit was used.

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Table 3. Effect of the culture volume/flask size, shaking speed and initial pH on biomass product of *Bacillus thuringiensis* subsp. *kurstaki*.

	Ratio of medium volume /flask volume			Initial pH				Shaking speed (rpm)			
	0.2	0.3	0.4	5.0	6.0	7.0	8.0	150	220	250	300
CFU/ml $\times 10^{-9}$	1.6	0.95	0.07	0.01	0.02	1.5	1.6	0.3	1.2	1.6	0.9

Bacteria were grown in a broth medium composed of soybean meal and glucose at 30.0 g/lit and 7.0 g/lit, respectively. Data are average of triplicates at 60 h after incubation.

remaining insoluble even upon and following autoclaving. Morris *et al*. (1996) reported on the efficiency of cottonseed meal for *B. thuringiensis* biomass production. On the contrary, this could be due to differences in the quality or processes used for cottonseed meal production by different suppliers. It is suggested that additional treatments on cottonseed meal might be required before it could be used as a medium constituent.

Beet molasses did not support a high production of spore-crystal mixtures. Although the beet molasses contains nitrogen, inorganic constituents, vitamins and carbohydrates and is used widely as a low-cost carbon source in *B. thuringiensis* production, it is not as good a source of carbon as glucose. Furthermore, its nitrogen constituent is mostly in the form of betaine which is poorly assimilated by *Saccharomyces* spp. and other microorganisms (Zabriske *et al*., 1980; Morris *et al*., 1996).

Cultures grown at culture volume to flask volume ratios of 0.2 and 0.3 had higher growth rates than that at a ratio of 0.4 (Table 3). The high aeration needed to ferment *kurstaki* is in agreement with Moraes *et al.* (1981) who reported that the respiration rate of *B. thuringiensis* grown in a culture medium containing sugarcane molasses and cornsteep liquor increases when the oxygen concentration is increased from 2.5 to 10% of the saturation value. An optimal shaking speed was obtained at 250 rpm while speeds lower than 200 rpm failed to support bacterial aeration requirements (Table 3). In our experiments, starting pHs of 5.0 and 6.0 did not produce high biomass (Table 3). Biomass production was relatively high at pHs 7.0 and 8.0 which were equally appropriate for toxin production, indicating that the hydrogen requirements of *B. thuringiensis* subsp. *kurstaki* are typical of other *B. thuringiensis* strains (Morris *et al.,* 1996).

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نیازمندیهای بیوشیمیایی و فیزیکی در تولید انبوه باکتری *Bacillus thuringiensis* subsp. *Kurstaki*

م. کشاورزی، ح. سلیمی و ف. میرزانمادی

چکیده

تأثیر فاکتورهای بیوشیمیایی شامل کربوهیدرات کل، پروتئین کل، نسبت کربوهیدرات به پروتئین، محتوای اسیدهای آمینه و pH اولیه محیط کشت و همچنین میزان هوادهی بر محصول بیوماس باکتری *Bacillus thuringiensis* subsp. *kurstaki* بررسی گردید. باکتری در محیطهای کشت اقتصادی تهیه شده از محصولات کشاورزی و مواد جانبی فرآوری آنها از جمله پودر ماهی، کنجاله پنبه دانه، کنجاله سویا، آب پس مانده ذرت، عصاره مخمر، پس مانده الکل، دانه سورگوم و پپتون بعنوان منابع پروتئین و گلوکز و ملاس چغندر قند بعنوان منابع کربوهیدرات کشت داده شد. نتایج بیانگر وجود رابطه مستقیم بین میزان بیوماس و نسبت کربوهیدرات به پروتئین و همچنین محتوای اسید گلوتامیک محیط کشت داشت. بالاترین بیوماس در محیطهایی با نسبت کربوهیدرات به پروتئین ٤/. تا ٠/٥و اسید گلوتامیک ١٣/٩ درصد تولید شد. PH اولیه ٧ الی ٨ برای تولید بیوماس بالا لازم بود. نسبت بهینه حجم کشت به اندازه فلاسک و سرعت شیکر بترتیب ١ به ٥ و ٢٥٠ دور در دقیقه تعیین گردید. فاکتورهای بیوشیمیایی فوق می توانند در طراحی محیطهای کشت اقتصادی برای تولید انبوه باکتری *Bacillus thuringiensis* subsp. *kurstaki* در نظر گرفته شوند.

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