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Organic and inorganic nitrogen source ratio effects on *Bacillus thuringiensis* var. *israelensis* delta-endotoxin production

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The influence of different organic and inorganic nitrogen source combinations and C:N ratios was studied in connection with growth and protein production of *Bacillus thuringiensis* var. *israelensis*. Protein production was assumed to be proportional to delta-endotoxin production. Delta-endotoxin concentration increased when media were supplemented with $(NH_4)_2SO_4$, but the delta-endotoxin: biomass dry weight ratio was unaffected by different C:N ratios. Organic nitrogen source, yeast extract, could be partially replaced by $(NH_4)_2SO_4$ with a significant increase in delta-endotoxin production.

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During sporulation, *Bacillus thuringiensis* var. *israelensis* produces a cytolytic protein (called delta-endotoxin) that is lethal to mosquito and blackfly larvae (de Barjac 1978; Armstrong *et al.* 1985; Hurley *et al.* 1987). This property has given rise not only to scientific inquiry but also commercial interest in *B. thuringiensis* var. *israelensis* since its discovery by Goldberg & Margalit (1977). One of the keys to successful commercialization of microbial bioinsecticides is the development of an adequate fermentation medium (Couch & Ross 1980). In this respect, it is very important to design optimal media for growth and delta-endotoxin production (Ertola 1987). This requires not only the selection of the suitable carbon and nitrogen source but also the search for the optimum C:N ratio. The influence of this parameter on the cellular composition and on the ability to utilize mixed carbon sources has been reported for *Hansenula polymorpha* (Egli & Quayle 1986). The effect of the different combinations of organic and inorganic sources has also been reported to affect the metabolism of *Clostridium acetobutylicum* (Welsh *et al.* 1987). Similar behaviour of the same microorganism was reported by Roos *et al.* (1985) when using different ammonium:glucose ratios. As far as the authors know, little or no information has been reported about the effect of these interactions on *B. thuringiensis* var. *israelensis* cultures.

This paper reports the results obtained when sequentially designed experiments were used to check up growth and delta-endotoxin production of *B. thuringiensis* var. *israelensis* under several carbon and organic and inorganic nitrogen combinations.

Materials and Methods

Microorganism and Growth

Bacillus thuringienis var. *israelensis* was grown in shake-flask cultures as previously described (Faloci *et al.* 1990) using a basal salts medium composed by (g/l):$MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.08; $MnSO_4.H_2O$, 0.05; KH_2PO_4, 3; K_2HPO_4, 3. This medium was supplemented with glucose, yeast extract (N

On a étudié l'influence de diverses sources d'azote organique et inorganique et des rapports C:N en relation avec la croissance et la production de protéine par *Bacillus thuringiensis* var. *israelensis*. On fait l'hypothèse que la production de protéines est proportionnelle à la production de delta-endotoxine. La concentration de delta-endotoxine croît quand on ajoute au milieu du $(NH_4)_2SO_4$, mais le rapport delta-endotoxine: poids sec de biomasse n'est pas affecté par différents rapports C:N. On peut remplacer partiellement la source organique d'azote, l'extrait de levure, par du $(NH_4)_2SO_4$ avec une augmentation significative de production de delta-endotoxine.

content 10% w/w) and $(NH_4)_2SO_4$ at various concentrations (see Results and Discussion). Media were identified taking into account the glucose and yeast extract concentrations, as follows: basal medium supplemented with 6 g glucose/l and 4 g yeast extract/l was referred to as medium 6C-4N, and so on. All media were sterilized at 121°C for 15 min with the pH adjusted to 7.0 before autoclaving. Six replicates were performed for each experiment. The results were subjected to statistical analyses by running the Statistics with Daisy, version 1.2.2. programme, in an Apple IIe computer. Analytical techniques were carried out as previously described (Faloci *et al.* 1990). Ammonium ion concentrations were determined with a Tecator Kjeltec Auto 1030 Analyzer and glucose concentrations by the DNS method (Miller 1959).

Results and Discussion

Growth of *Bacillus thuringiensis* var. *israelensis* and yield of delta-endotoxin with the different media are shown in Tables 1 and 2. The higher the yeast extract concentration, the higher were the delta-endotoxin and biomass dry weight values. The same effect was obtained by keeping the nitrogen

Table 1. Biomass dry weight and delta-endotoxin obtained in basal medium with different combinations of glucose and yeast extract.

Glucose (g/l)		Yeast extract (g/l)				
		2	4	6	8	10
2	p	0.164	0.164	0.204	0.298	0.317
	x	0.78	0.95	1.14	1.48	1.63
4	p	0.154	0.296	0.230	0.347	0.470
	x	0.88	1.28	1.38	1.85	2.17
6	p	0.180	0.321	0.347	0.360	0.559
	x	1.03	1.67	1.70	1.99	2.52
8	p	0.221	0.336	0.451	0.407	0.466
	x	1.14	1.99	2.04	2.21	2.41
10	p	0.202	0.308	0.464	0.529	0.506
	x	1.16	2.03	2.43	2.53	2.71

p: delta-endotoxin (g/l)
x: biomass dry weight (g/l)

Table 2. Biomass dry weight and delta-endotoxin obtained in basal medium with different combinations of glucose and yeast extract supplemented with 1 g $(NH_4)_2SO_4$/l.

Glucose (g/l)		Yeast extract (g/l)				
		2	4	6	8	10
2	p	0.184	0.215	0.303	0.357	0.395
	x	0.72	0.95	1.18	1.54	1.64
4	p	0.179	0.309	0.395	0.414	0.529
	x	1.06	1.27	1.46	1.75	1.89
6	p	0.292	0.364	0.454	0.588	0.734
	x	1.39	1.62	1.72	1.77	2.28
8	p	0.258	0.702	0.454	0.609	0.867
	x	1.39	2.08	1.95	2.32	2.64
10	p	0.280	0.606	0.609	0.587	0.824
	x	1.37	2.53	2.80	2.25	3.05

p: delta-endotoxin (g/l)
x: biomass dry weight (g/l)

concentration constant and increasing the initial glucose concentration. As the method employed for delta-endotoxin evaluation measures total protein (i.e. the Lowry method) it is assumed that the proportion of delta-endotoxin to the total protein is constant at the end of each experiment.

Mean values for the delta-endotoxin:biomass dry weight ratio ($Y_{p/x}$) obtained in the different experiments were 0.19 (standard error of the mean ± 0.004) and 0.25 (SEM ± 0.009) for the results shown in Tables 1 and 2, respectively. The difference between these mean values is highly significant ($P<0.01$). The similarity of different $Y_{p/x}$ values indicates that, whatever the C:N ratios used these did not affect the metabolic pathways of the organism. Large differences were only observed when yeast extract was supplemented with 1 g $(NH_4)_2SO_4$/l.

Although no data related to glucose consumption is shown, glucose was completely metabolized in most, but not all, media. Exceptions were media 6C-2N, 8C-2N, 10C-2N and 10C-4N, either with or without $(NH_4)_2SO_4$.

In the assays carried out with 2 g glucose/l, a high biomass dry weight to consumed glucose ratio was achieved, with a maximum value of 0.82 (using 10 g yeast extract/l). These high values reveal that once glucose had been completely metabolized, *B. thuringiensis* var. *israelensis* synthesized new cellular material from the carbon compounds supplied by yeast extract. This happens mainly when the media employed have C:N ratios between 0.66 and 1.0, which are the lowest used in these experiments.

Values of delta-endotoxin and biomass dry weight, shown in Tables 1 and 2, were compared by using a mean difference analysis; the differences in biomass dry weight were not significant ($t = 0.76$ for 24 degrees of freedom), while the differences in delta-endotoxin concentrations were highly significant, at the 0.1% level ($t = 5.46$ for 24 degrees of freedom).

Analysis of variance shown in Table 3 indicates that the effect caused by the nitrogen source is significant at the 1% level ($P<0.01$). Taking into account this result, and the conclusions raised from the mean difference analysis, it is evident that delta-endotoxin production can be improved by supplementing culture media with $(NH_4)_2SO_4$. This suggests that, if the production medium is to be optimized, it is necessary to adjust the initial NH_4^+ concentration. It is interesting to point out that in all the media tested, NH_4^+ was completely consumed. However, when $(NH_4)_2SO_4$ was used as the sole nitrogen source, little or no growth was observed. This result is in good agreement with those reported by Nickerson & Bulla (1974) for other *B. thuringiensis* varieties.

Table 3. Analysis of variance for delta-endotoxin production

Source of variation	Sum of squares	DF	Mean of squares	$F_{calc.}$
Main effects				
Carbon source				
lineal	5.780×10^{-4}	1	5.780×10^{-4}	0.48
quadratic	1.750×10^{-3}	1	1.750×10^{-3}	1.44
cubic	1.625×10^{-3}	1	1.625×10^{-3}	1.34
residuals	1.827×10^{-4}	1	1.827×10^{-4}	0.15
Nitrogen source				
lineal	0.014	1	0.014	11.57**
quadratic	3.842×10^{-4}	1	3.842×10^{-4}	0.32
cubic	3.264×10^{-3}	1	3.264×10^{-3}	2.67
Residuals	0.02067	17	1.216×10^{-3}	
Total	0.04245	24		

** Significant at 1% ($P<0.01$)
DF: degrees of freedom

Table 4. Delta-endotoxin production and $Y_{p/x}$ yields as affected by different organic to inorganic nitrogen ratios, using basal medium supplemented with 6 g glucose/l.

Yeast extract (g/l)	$(NH_4)_2SO_4$ (g/l)	delta-endotoxin (g/l)	$Y_{p/x}$
1.84	1.0	0.433 ± 0.017	0.227 ± 0.019
2.6	0.66	0.433 ± 0.011	0.244 ± 0.019
3.0	0.47	0.387 ± 0.012	0.219 ± 0.004
4.0	0	0.307 ± 0.010	0.192 ± 0.004

(Total nitrogen concentration was kept constant at 0.4 g/l.)

The results obtained with the different combinations of yeast extract and $(NH_4)_2SO_4$ are given in Table 4. Yeast extract, as sole nitrogen source, was not enough to ensure a good production of delta-endotoxin. This was statistically confirmed by comparing the protein concentration reached in the medium with 4 g yeast extract/l with those attained with the other combinations (the differences were highly significant, $P<0.005$). The results observed with the other media showed significant differences ($P<0.01$), except between media with 1.84 and 2.6 g yeast extract/l. As little is known about the factors affecting *B. thuringiensis* var. *israelensis* physiology in relation to growth, sporulation and delta-endotoxin synthesis (Foda *et al.* 1985), it is difficult to find any explanation for the low values of delta-endotoxin concentration reached in the medium that was not supplemented with $(NH_4)_2SO_4$. This result could not be ascribed to an unfavourable effect caused by a possible inhibitor agent present in the yeast extract because neither growth nor delta-endotoxin synthesis was affected when other *B. thuringiensis* varieties were grown using higher yeast extract concentrations than those employed in this work (Goldberg *et al.* 1980); Dharmstiti *et al.* 1985; Arcas *et al.* 1987).

We can conclude that combinations of organic and inorganic nitrogen sources affect *B. thuringiensis* var. *israelensis* delta-endotoxin production more than does the C:N ratio. Consequently, as most of media described in the literature report effects of different concentrations of $(NH_4)_2SO_4$ (Dubois 1968; Yousten & Rogoff 1969; Shulz *et al.* 1985), further research will be necessary to acquire a better knowledge on the role played by NH_4^+ on *B. thuringiensis* var. *israelensis* physiology. This would allow a rational design of industrial culture media to maximize delta-endotoxin production at lower costs. Moreover, it would be necessary to study the interactions produced by other inorganic nitrogen sources and their effects on *B. thuringiensis* var. *israelensis* performance.

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