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Bacillus thuringiensis growth, sporulation and δ-endotoxin production in oxygen limited and non-limited cultures

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The production of crystals and spores of *Bacillus thuringiensis* var. *israelensis* was studied under different aeration conditions. The results with 4 l batch cultures showed that for O_2 non-limited cultures cell yield, toxin production and spore count were constant for all oxygen transfer rates (OTR). Under O_2 limitation, δ-endotoxin concentrations and spore counts were lower than those obtained in non-limited cultures. In addition, δ-endotoxin yields diminished under O_2 limitation, suggesting that the toxin synthesis mechanism could have been affected.

Key words: Bacillus thuringiensis, cell yield, δ-endotoxin, oxygen, oxygen-limited growth

Oxygen plays an important role in fermentation processes and usually overall yields are closely related to its supply. In relation to this, it has been demonstrated that, for several species of the genus *Bacillus*, sporulation is highly related to O_2 supply (Hanson *et al.* 1963; Murrell 1967; St Julian & Bulla 1971; Dingman & Stahly 1983). Yousten & Wallis (1987) demonstrated that, for *B. sphaericus*, toxin synthesis and spore formation are highly dependent on O_2. The necessity of high aeration rates has been reported for *Bacillus thuringiensis* (Holmberg *et al.* 1980; Foda *et al.* 1985; Pearson & Ward 1988), but only Moraes *et al.* (1980) studied the effect of dissolved O_2 on the respiration rate and growth of *Bacillus thuringiensis* NCIB 9207 and they did not report on the relationship between δ-endotoxin synthesis and O_2 supply.

The production of crystals and spores of *Bacillus thuringiensis* var. *israelensis*, as affected by oxygen supply, and some stoichiometric coefficients and parameters of growth were made the subjects of the present study, so that the characteristics of cultures of this microorganism can be elucidated.

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Materials and Methods

Microorganism and Inocula

The microorganism employed was *Bacillus thuringiensis* var. *israelensis* (serotype H-14), supplied by Dr. H. de Barjac (Institut Pasteur, Paris). Inocula were prepared by transferring cells from nutritive agar slants into 1-l Erlenmeyer flasks, containing 100 ml of culture medium. After 8 h of incubation (30°C, 200 rev/min) these cultures were employed to inoculate the bioreactor.

Medium and Culture Conditions

The composition of the culture medium was (g/l): glucose, 7; yeast extract, 3; $(NH_4)_2SO_4$, 0.47; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.08; $MnSO_4.H_2O$, 0.05; KH_2PO_4, 1.5; K_2HPO_4, 1.5. The medium was autoclaved at 121°C for 15 min. The pH was adjusted to 7 before sterilization.

Fermentation Procedure

The experiments were performed in 6-l bioreactors (LKB 1601D Ultroferm), at 30°C. The working volume was 4 l, and the aeration rate was 0.5 vol/vol/min. Batches lasted 48 h in order to ensure total lysis of the cells. The maximum oxygen transfer rate ranged from 3.1 to 48.4 mmol O_2/l/h, by varying the agitation speed between 150 and 650 rev/min.

Analytical Procedures

Biomass and δ-endotoxin concentrations were determined as described by Faloci *et al.* (1990). Glucose was measured as described by Miller (1959). Overall cell yields based on glucose ($Y_{x/s}$) were calculated as g of biomass produced per g glucose consumed up to the moment when maximum biomass was reached. The same criterion was employed to calculate overall cell yields based on

oxygen ($Y_{x/o}$). $Y_{p/x}$ was defined as the ratio of δ-endotoxin concentration achieved to the maximum dry weight (g/g). Spore count was evaluated by determining the number of heat resistant colony forming units (c.f.u.) at the end of each experiment. Dissolved O_2 concentration was continuously monitored by means of a sterilizable dissolved O_2 electrode. O_2 and CO_2 concentrations in the outcoming gases were measured employing a Beckman OM-14 O_2 analyser and a Horiba PIR-2000 infrared CO_2 analyser. O_2 uptake rate and CO_2 production rates were calculated according to Cooney *et al.* (1977). Total O_2 consumption was calculated by an integrating method.

Results and Discussion

Seven different conditions of O_2 supply were studied. Time courses of biomass, dissolved O_2 evolution, O_2 consumption rate (rO_2) and CO_2 production rate (rCO_2) are shown for three of them. Figure 1a shows linear growth after dissolved O_2 reached a value of 0%. Figure 1b shows a culture in which the maximum O_2 transfer rate (OTR) just meets the maximum O_2 demand, and the dissolved O_2 reaches a value of 0% and rises immediately after. This curve corresponds to a maximum OTR of 14.9 mmol O_2/l/h. Figure 1c corresponds to the higher OTR employed (48.4 mmol-O_2/l/h). In this case, the value of dissolved O_2 was always higher than 70%. Figures 1b and 1c show that the time courses of biomass, rO_2 and rCO_2 are similar, thus indicating that an OTR of 14.9 mmol O_2/l/h is enough to support growth in the experimental conditions employed. Maximum biomass, final spore count, δ-endotoxin concentration and the yields achieved are presented in Table 1.

In cultures performed at OTRs ranging from 14.9 to 48.4 mmol O_2/l/h (Table 1A), where no O_2 limitation was observed, the maximum biomass concentration was reached after 12 h of cultivation, whereas sporulation had scarcely begun at that time. Cell yields based on glucose, ($Y_{x/s}$), showed no significant differences, giving an average value of 0.66. This value is significantly higher than that obtained under limited conditions (0.40) (Table 1B). Cell yields obtained in non-limited cultures were rather high for glucose as the carbon source. This can be attributed to the assimilation of carbon compounds from the yeast extract.

The evaluation of total consumed O_2 from the beginning of the fermentation up to the moment when maximum biomass was reached, allowed the calculation of cell yields based on O_2 ($Y_{x/o}$). Again, significant differences were observed between cultures with and without O_2 limitation. For O_2 non-limited cultures the average $Y_{x/o}$ was 1.45 g biomass/g O_2 (Table 1A), while a value of 2.45 was reached for O_2-limited cultures (Table 1B). These results suggest a higher efficiency for O_2 consumption when cultures were under conditions of O_2 limitation. This conclusion agrees with reports given by other authors (Harrison & Pirt 1967; Rizzi *et al.* 1989). In spite of this, Holmberg *et al.* (1980) did not find differences in growth of *Bacillus thuringiensis*

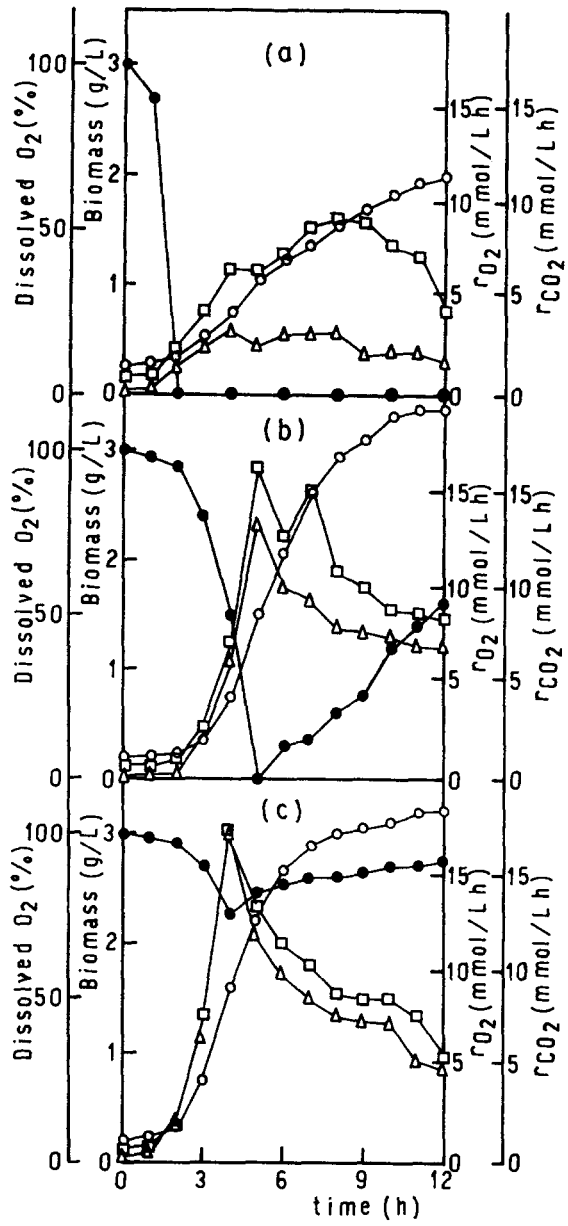

Figure 1. Time course of biomass (○), dissolved oxygen (●), oxygen consumption rate, rO_2 (△) and carbon dioxide production rate, rCO_2 (□) under three different oxygen supply conditions with oxygen transfer rates (mmol O_2/l/h) of: (a) 3.1; (b) 14.9; (c) 48.4.

serotype 1 (ATCC 10792) under different aeration rates, but from their report there is no evidence that cultures were under O_2 limitation. From data reported by Moraes *et al.* (1980) a value of $Y_{x/o}$ of 1.28 can be derived for *Bacillus thuringiensis* NCIB 9207, a similar value to those obtained in the present study for non-limited cultures.

Regarding sporulation, it is important to note that when cultures were under O_2 limitation, spore count values were about 4×10^8 c.f.u./ml. In the experiments without oxygen

Table 1. δ-Endotoxin concentration, spore count, maximum dry weight and yields obtained under different oxygen supply conditions.

Oxygen supply	Agitation speed (rev/min)	OTR_{max} (mmol O_2/l/h)	δ-Endotoxin* (mg/l)	Spore count* (10^{-7} c.f.u/ml)	Dry weight (g/l)	$Y_{x/s}$	$Y_{x/0}$	$Y_{p/x}$
A. Non-limited	350	14.9	415	81	3.30	0.62	1.41	0.126
	450	23.4	410	91	3.12	0.68	1.47	0.131
	550	34.2	390	85	3.20	0.67	1.51	0.122
	650	48.4	390	86	3.20	0.67	1.41	0.122
B. Limited	150	3.1	239	39	2.78	0.39	2.48	0.086
	250	7.2	278	45	2.84	0.40	2.41	0.090
C. Interrupted	350	14.9	280	79	3.30	–	–	0.085

* Final values.

limitation, spore counts reached 9×10^8 c.f.u./ml. Similar behaviours were reported for *B. thuringiensis* var. *galleriae* (Foda *et al.* 1985). These authors noted that the lower the O_2 supply, the lower was the spore count. However, it is impossible to compare these results because Foda *et al.* (1985) gave no data about OTR.

In O_2-limited cultures, the δ-endotoxin concentrations reached were low compared with those obtained in non-limited cultures. From data in Tables 1A and 1B, it can be seen that in O_2-limited cultures the concentrations of δ-endotoxin achieved were as low as 58% of the maximum observed in the present study (415 mg/l). Abdel-Hameed *et al.* (1991) reported a decrease of almost 50% in toxin yields when low OTRs were employed for *B. thuringiensis* production. In O_2 non-limited cultures, an average value of $Y_{p/x}$ of 0.125 was achieved, higher than that in O_2-limited cultures. This result indicates that the mechanism of δ-endotoxin synthesis could have been affected. The same conclusion can be drawn in terms of number of spores formed: being 2.67×10^{11} c.f.u./g biomass, for non-limited cultures, and 1.46×10^{11} c.f.u./g biomass, for O_2-limited cultures.

To analyse if oxygen limitation affects both δ-endotoxin synthesis and spore formation during the first stages of growth, an experiment was performed in which aeration was interrupted immediately after maximum biomass concentration was reached (Table 1C). This experiment began under conditions of non-limitation (agitation speed of 350 rev/min; OTR of 14.9 mmol O_2/l/h), but aeration was interrupted after 12 h of cultivation (at a biomass concentration of 3.30 g/l). The δ-endotoxin concentration reached (280 mg/l) was not significantly different to those reached in O_2-limited cultures. However, the spore count was 79×10^7 c.f.u./ml, the same as obtained in non-limited cultures. So, the number of spores per g of maximum biomass was the same as that obtained for non-limited cultures. These results confirm that, although both sporulation and δ-endotoxin synthesis are greatly dependent on O_2 supply, once sporulation has been triggered it will be completed, even if O_2 supply is interrupted. However, δ-endotoxin synthesis is affected by such an interruption and only a fraction of the expected yield was achieved. Thus, O_2 must be continuously supplied if high δ-endotoxin concentrations are to be reached.

Since δ-endotoxin is responsible for the insecticidal activity of *Bacillus thuringiensis*, these conclusions must be considered when producing bioinsecticides based on this bacterium. Knowledge of stoichiometric coefficients and growth parameters should help to achieve optimum results when either laboratory or plant production of *Bacillus thuringiensis* is intended.

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