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Batch and continuous culture production of the mosquito larval toxin of *Bacillus sphaericus* 2362

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SUMMARY

Batch and continuous culture were used to investigate the production of *Bacillus sphaericus* mosquito larvicide. In batch culture, control of the pH at 7.2–7.3 rather than allowing the normal rise to about 8.6 decreased the toxicity of the cells. Oxygen was required for toxin formation but increasing the level of dissolved oxygen in the medium by use of pure oxygen in the gas stream lowered toxin production. Sporulation and toxin production occurred in continuous culture and were greater at lower dilution rates. However, toxin yield in continuous culture was too low to be a likely alternative to batch culture.

INTRODUCTION

The control of mosquito larvae by use of toxins produced by bacteria has been shown to be a practical supplement or alternative to the use of organic chemicals. Products containing the toxin of *Bacillus thuringiensis* var. *israelensis* are already on the market. Another bacterium which holds some promise for development as a mosquito larvicide is *Bacillus sphaericus* [5,12,16]. This aerobic, sporeforming bacterium has a somewhat different spectrum of insecticidal activity than *B. thuringiensis* [16] and has been reported to persist somewhat longer in the aquatic environment [7,12,13]. The toxin produced by *B. sphaericus* accumulates inside the bacterial cell as a parasporal inclusion body at the time of sporulation [6,11,14,17]. The toxin may be synthesized as a 125-kDa protein which is converted in some strains (e.g., 2362 and 2297) to a non-toxic 63-kDa moiety and a toxic 43-kDa moiety during the process of sporulation [1,3]. The latter is later converted to an even more toxic form in the gut of mosquito larvae [4].

Although small quantities of *B. sphaericus* have been produced for use in field trials, there are few published reports concerning the effects of growth conditions on the toxicity of the bacteria. Dharmsthiti et al. [9] reported the use of byproduct from a monosodium glutamate factory as an ingredient in the fermentation broth. The effect of

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oxygen on sporulation and toxin synthesis by strain 1593 has been studied [19]. *B. sphaericus* strain 2362, which was isolated by Weiser [15], is now generally considered to be the most promising strain available for development. This report describes studies using batch culture to analyze the effects of pH control and varied oxygen levels on sporulation and toxin synthesis by this strain as well as a comparison of batch and continuous culture as possible means of production.

MATERIALS AND METHODS

Bacteria and growth conditions

B. sphaericus 2362 was maintained on slants of NYSM agar (nutrient agar supplemented with 0.05% yeast extract, 5×10^{-5} M $MnCl_2$, 7×10^{-4} M $CaCl_2$, 1×10^{-3} M $MgCl_2$). Both batch fermentation and continuous culture experiments were performed in 1 liter of NYSM broth in a New Brunswick Scientific Co. (NBS) F-2000 Multigen fermentor operated at 30°C. Aeration was provided at 1 liter/l/min and agitation was set at 400 rpm unless otherwise indicated. The pH was monitored continuously with an Ingold, steam-sterilized electrode and was regulated automatically by an NBS pH 40 control unit adding 5% H_2SO_4. The dissolved oxygen (D.O.) was monitored with an Instrumentation Laboratories polarographic, steam-sterilized electrode. Inoculum for the fermentor (5% v/v) was prepared as 50 ml of NY broth (nutrient broth supplemented with 0.05% yeast extract) in a 500 ml flask grown at 30°C with shaking for 4 h at 175 rpm. During continuous culture, medium was added at a constant rate by a peristaltic pump and was removed from the vessel by an overflow siphon. Samples for spore count and for bioassay of toxicity were removed from the chemostat after the culture had remained at one set of growth conditions for 48 h.

Growth was determined by measurement of the A_{660}. Spore counts were made by heating 1.5 ml of broth at 80°C for 12 min. The heated sample was sonicated for 1 min with the small probe of a Fisher model 300 sonic dismembrator to unclump spores. Diluted samples were plated in NY agar and incubated at 30°C for 48 h. Total cell counts were made microscopically using a Petroff-Hauser counting chamber. Dry weights were determined in triplicate after drying aliquots for 48 h at 110°C.

Bioassay

Toxicity of the bacterial cells was determined by bioassay against second instar larvae of *Culex quinquefasciatus*. The cells were recovered from the broth by centrifugation, washed twice with sterile distilled water, and resuspended in sterile distilled water for dry weight determination and bioassay. Dilutions of the bacteria were made in dechlorinated, sterile tap water, and 5 ml of the diluted suspension was placed into plastic cups with 45 ml of sterile tap water, 1 drop of 10% w/v debittered brewers yeast, and 10 larvae. Three cups at each dilution and 10 cups of untreated control larvae were held at 25°C for 48 h when mortality was recorded. Data were corrected for control mortality with Abbott's formula and LC_{50} values were determined by probit analysis. Toxicity units were determined by comparing the LC_{50} of a fermentor sample with the LC_{50} produced by a standard *B. sphaericus* 1593 powder, designated RB-80, which was assigned a value of 1000 toxin units/mg [2]. The RB-80 powder was obtained from H. de Barjac (Institut Pasteur, Paris).

RESULTS

Batch fermentations

B. sphaericus 2362 grew rapidly in NYSM broth and achieved a final cell population of (1–2) $\times$ 10^9/ml. Upon the initiation of sporulation, the swollen cells containing forespores clumped, whereas the vegetative cells remained unclumped. Although the fermentations were continued for 24 h, sporulation was completed several hours prior to that time, and at 24 h many of the sporangia had lysed, liberating free spores with attached parasporal bodies. The pH of the broth, which was about 6.7 at the time of inoculation, increased steadily to a final pH of about 8.5–8.6 (Fig. 1). In

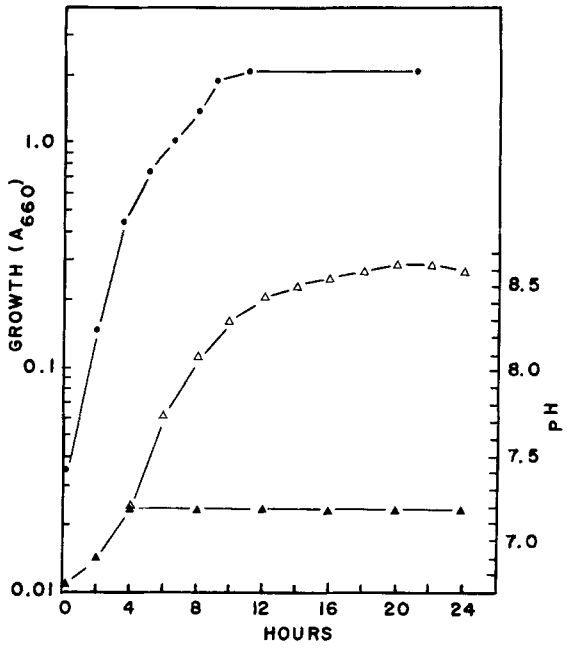

Fig. 1. Change in pH during growth of *B. sphaericus* 2362 in batch culture. ●, growth profile (absorbance) of bacteria grown without pH control; △, pH profile in batch culture; ▲, pH profile in batch culture with pH controlled at 7.2. The growth curve with pH control was very similar to that for cells grown without pH control.

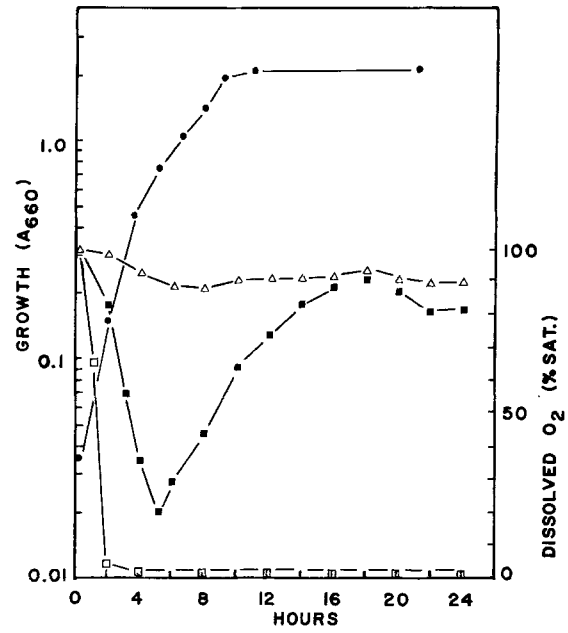

Fig. 2. Changes in the level of dissolved oxygen during growth of *B. sphaericus* 2362 in batch culture. ●, growth profile of bacteria grown with aeration at 1 liter of air/l/min. The growth curve was very similar for growth under the other aeration conditions shown here. ■, dissolved oxygen at 1 liter air/l/min and 400 rpm; □, dissolved oxygen at 0.5 liter air/l/min and 200 rpm; △, dissolved oxygen at 1 liter pure oxygen/l/min and 400 rpm.

Table 1

Sporulation and toxin formation by *B. sphaericus* 2362 grown by batch fermentation

Trial	pH[a] condition	O_2[b] condition	Spores/ml	Toxin units/mg ± S.D.[c]
1	uncontrolled	1	6.5×10^8	9359 ± 906
2	uncontrolled	1	6.0×10^8	7728 ± 539
3	uncontrolled	1	7.7×10^8	9190 ± 56
4	uncontrolled	1	8.6×10^8	14144 ± 59
5	7.3 ± 0.1	1	7.6×10^8	4309 ± 235
6	7.3 ± 0.1	1	7.8×10^8	2439 ± 13
7	7.3 ± 0.1	1	9.8×10^8	4125 ± 176
8	uncontrolled	2	2.1×10^8	4641 ± 450
9	uncontrolled	3	6.3×10^8	2716 ± 278
10	uncontrolled	3	6.1×10^8	2932 ± 96

[a] Typical pH profile of a fermentation without control is shown in Fig. 1.

[b] Oxygen conditions were as follows: 1 = 400 rpm, 1.0 l air/l/min; 2 = 200 rpm, 0.5 l air/l/min; 3 = 400 rpm, 1.0 l 100% oxygen/l/min.

[c] Toxin units derived from a comparison of the LC_{50} of the distilled water washed cell mass compared to the LC_{50} of the standard RB80 powder which is assigned a value of 1000 units/mg. Each value is the mean of two assays ± S.D.

four trials in which the pH was uncontrolled, there were (6.0–8.6) × 10^8 spores formed per ml and 7728–14 144 toxin units/mg dry weight of cell mass (Table 1). In three trials the fermentation was initiated at pH 6.7 and the pH was allowed to rise to 7.2, at which time it was controlled by the addition of acid. This did not significantly change the final spore count from that found without pH control. However, the toxicity of the cells produced with pH control was lower than that produced by cells grown in media in which the pH was allowed to rise (Table 1).

B. sphaericus is a strictly aerobic bacterium and it could be anticipated that the level of D.O. would influence sporulation and perhaps toxin synthesis. With air flow at 1 liter/l/min and 400 rpm agitation (trials 1–4), the D.O. dropped rapidly to about 20% saturation during exponential growth and then rose again as the cells entered stationary phase (Fig. 2). In fermentor trial 8, the air flow rate and the agitation rate were both reduced by one-half compared to that in trials 1–4. The level of D.O. dropped rapidly during the period of exponential growth and remained close to 0% saturation throughout the course of the fermentation (Fig. 2). This drop in the level of D.O. caused a decrease in the number of heat-resistant spores by about 71% and a decrease in the toxicity by about 54% (based upon the average toxicity of trials 1–4). When the inlet gas stream was changed from air to pure oxygen (1 liter/l/min and 400 rpm), a high level of D.O. was maintained throughout the fermentation (Fig. 2) and growth and sporulation were comparable to that found using air. However, the toxicity of the cell mass produced under these conditions was markedly reduced compared to that produced when the cells were grown in air (Table 1).

Continuous fermentation

To determine the effect of the growth rate on sporulation and toxin formation, *B. sphaericus* 2362 was grown in continuous culture at dilution rates ranging from 0.15 to 0.46 h^{-1}. In Fig. 3 it is seen that sporulation decreased as the growth rate of the

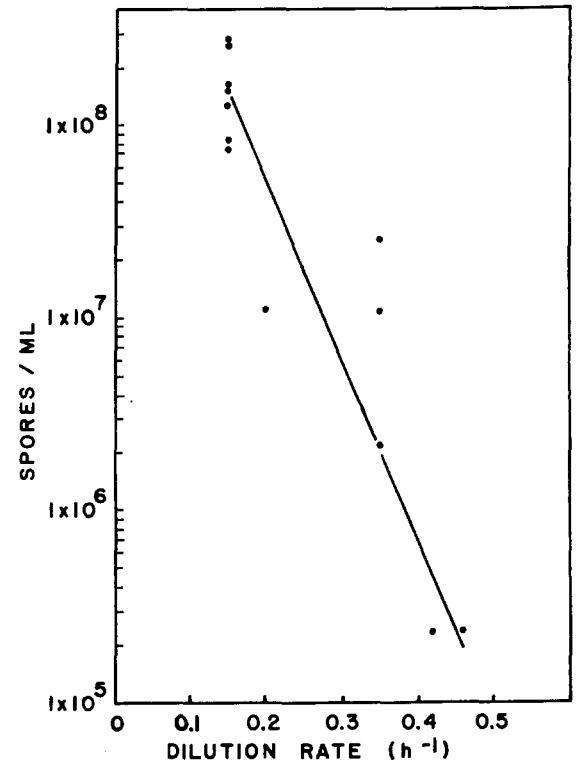

Fig. 3. Sporulation by *B. sphaericus* 2362 in response to varied dilution rates in a chemostat. Line drawn by linear regression.

culture was increased (increased dilution rate). At the lowest dilution rate tested, $D = 0.15$, only about 10% of the cells in the vessel were present as spores, in contrast to about 50% which were present as heat-resistant spores after 24 h of batch fermentation. Toxicity of the cell mass responded to variations in the dilution rate in the same manner as did sporulation; i.e., toxicity was highest at the lowest dilution rate (Fig. 4). However, even at the lowest dilution rate it was only about 12% of that produced by bacteria grown in the batch fermentation (comparing the mean of the seven continuous runs done at $D = 0.15$ to the mean of the four batch trials reported in Table 1). The pH in continuous culture remained in the range of 8.2–8.5.

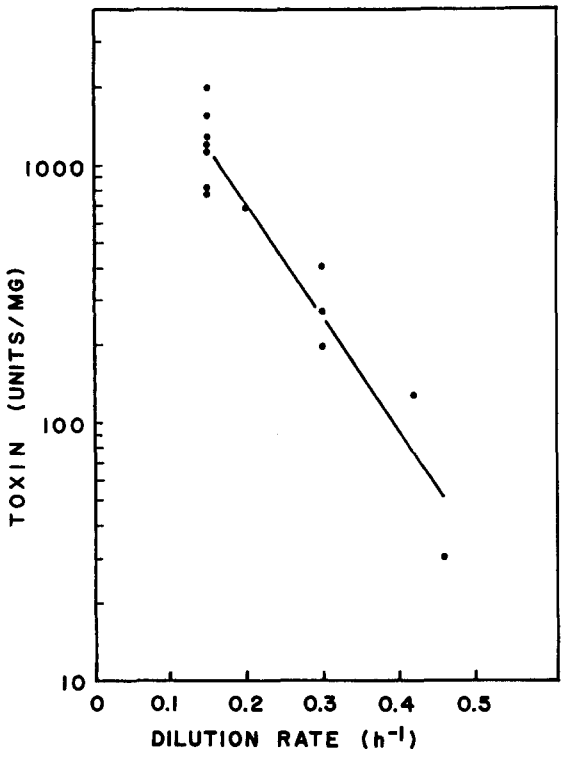

Fig. 4. Toxicity of *B. sphaericus* 2362 in response to varied dilution rates in a chemostat. Each data point is the mean of two bioassays. Line drawn by linear regression.

DISCUSSION

B. sphaericus 2362 grown in batch culture using a non-particulate, complex medium, washed free of medium ingredients following growth, and bioassayed soon after production without intervening spray drying or precipitation of the cells yielded a highly toxic cell mass. The pH rose from an initial value of 6.7 to a final value of about 8.6. Somewhat to our surprise, control of the pH at 7.2–7.3 not only did not increase toxicity, but caused it to decline. This is the reverse of what was found for strain 1593, a strain which without pH control produces a lower level of toxicity than strain 2362 [18]. It is unknown whether the higher level of toxicity of 2362 when the pH was uncontrolled resulted from more toxin synthesis or from increased toxin stability.

The requirement for oxygen for both sporulation and toxin synthesis was clearly demonstrated when availability was limited by reduced air flow rate and by reduced agitation. Despite the demonstrated oxygen requirement, increasing the percent saturation of the medium throughout the fermentation by supplying pure oxygen rather than air did not enhance either sporulation or toxicity. In fact, the use of pure oxygen caused about a 72% decrease in the level of toxicity compared to the use of air. It has not been determined whether this is an inhibition of toxin synthesis or whether it is due to destruction of the toxin protein by the high level of D.O. It should be noted that use of pure O_2 results in higher levels of D.O. than would be present at an equal percent saturation with air.

The toxic parasporal body of *B. sphaericus* appears in the cell after the initiation of sporulation [11,17], so that the onset of the morphogenic sequence of events which lead to formation of the spore is necessary for toxin synthesis. In a continuous culture system, the bacteria must continue to grow vegetatively or be washed out of the fermentor. Therefore, it was unclear whether sporulation and the associated toxin synthesis (and the cessation of vegetative growth for the sporulating cell) would be compatible with a continuous culture system. Dawes and Mandelstam [8] had shown that for *B. subtilis* grown in continuous culture at dilution rates of 0.05–0.45 h^{-1} there was a direct relationship between the dilution rate and the initiation of sporulation. That is, there was no threshold growth rate beneath which sporulation was massively initiated in all the cells. Rather, there was increasing probability that a cell would sporulate as the growth rate (dilution rate) decreased. Similarly, Frankena et al. [10] found that the production of exocellular proteinase by *B. licheniformis* in continuous culture increased as the specific growth rate (dilution rate) decreased. We observed that a similar situation holds for *B. sphaericus*; i.e., sporulation and toxin synthesis increased as the growth (dilution) rate decreased. The data obtained from batch and continuous fermentations can be used to make a comparison of the potential productivity of these two methods for production of *B. sphaericus* toxin. Assuming a yield of 10 100 units/mg and

1220 units/mg for batch and continuous systems respectively (averages obtained from trials 1–4 in Table 1 and from the seven continuous runs at $D = 0.15$), a cell yield of 1.5 mg/ml for both (obtained from the same runs as above), and that each batch run lasts 24 h and has a 10 h turnaround time for the fermentor to be prepared for another run, the figures in Table 2 can be derived. Despite the efficiency possible using continuous culture, a series of batch fermentations would allow greater toxin production over the projected time. Eventually, the declining productivity of batch culture and the constant productivity of continuous culture would allow the latter to be more productive. However, the much greater expenditure of media, the related downstream processing to recover and concentrate the toxin-containing bacterial cells produced by continuous culture, and the greater complexity of the continuous culture process may negate that advantage. The toxin yields using commercial types of media in large fermentors would likely be different than those reported here but the relationships would likely be similar. The efficiency of continuous culture is lost because of the large drop in toxin produced per unit of cell mass under these conditions. Unlike the production of single cell protein, which benefits from continuous culture, the *B. sphaericus* toxin is only produced by sporulating cells and these represent a small proportion of the total cells present in continuous culture. If the gene for the toxin could be placed under control of a promoter recognized in the vegetative cell and if the toxin could be produced and processed in an appropriate manner in such a cell, the use of continuous culture would be an appropriate means for production.

Table 2

Comparison of the productivity of batch and continuous fermentation

Hours	No. of runs	Units of toxin produced	Units of toxin · h^{-1}	Medium volume (l)
Batch[a]				
24	1	1.5×10^7	6.3×10^5	1
228	7	1.1×10^8	4.6×10^5	7
Continuous[b]				
24	–	6.6×10^6	2.7×10^5	3.6
228	–	6.2×10^7	2.7×10^5	34.2

[a] Calculated assuming a yield of 10 100 units/mg cell dry weight, 1.5 mg/ml dry weight of cells, 1.0 l medium in the fermentor, and a 10 h turnaround time between fermentor runs.

[b] Calculated assuming a yield of 1220 units/mg cell dry weight, 1.5 mg/ml dry weight of cells, a dilution rate of 0.15 h^{-1}, and 1.0 l of medium in the fermentor.

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