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Effect of Dissolved Oxygen on the Growth, Sporulation and Larvicidal Activity of *Bacillus sphaericus* 2362

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ABSTRAK

B. sphaericus telah dikenalpasti sebagai agen biologikal yang menghasilkan protein toksik di sporanya dan berupaya membunuh larva nyamuk yang terlibat dalam penyebaran penyakit manusia seperti filariasis, encephalitis dan malaria. Dalam kultura sesekelumpuk terdapat hasilan bilangan spora maksima 2.9×10^9/ml media selepas 48 jam eraman. Dalam kultura selanjar, kadar dilusi 0.10 jam^{-1} didapati menghasilkan bilangan spora yang tinggi berbanding dengan kadar dilusi 0.15 jam^{-1} dan 0.2 jam^{-1}. Kajian kinetik ke atas tahap oksigen larut (DO) menunjukkan 20%, 50% dan 100% DO menghasilkan ketinggian spora bebas, sebaliknya 5% DO memberi keputusan yang tidak baik. Sel-sel yang tumbuh pada tahap 5%, 20% dan 50% DO memberi nilai bandingan aktiviti larvisid nyamuk tetapi pada 100% DO nilai larvisid adalah rendah.

ABSTRACT

Bacillus sphaericus produces bacterial protein toxin capable of killing larvae of mosquitoes which spread diseases such as filariasis, encephalitis and malaria. In batch culture, a maximum spore count of 2.9×10^9/ml media was obtained after 48 h cultivation. In continuous culture, a dilution rate of 0.10 hr^{-1} was found to give a higher spore count than 0.15 h^{-1} and 0.2 h^{-1} dilution rates. Kinetic studies on dissolved oxygen (DO) levels showed that 20, 50 and 100% gave rapid formation of free spores, whilst 5% DO gave poor results. Cells grown at 5, 20 and 50% DO levels gave comparable values of mosquito-larvicidal activity while those grown at 100% DO were significantly less toxic.

Keywords: *B. sphaericus* 2362, larvicidal activity, dissolved oxygen, growth, sporulation

INTRODUCTION

The use of bacteria belonging to the genus *Bacillus* as biological control agents for mosquitoes has been studied by many researchers. Strains of *B. sphaericus* 2362 are known to produce parasporal crystals consisting of two proteins with molecular weights of 42 and 51 kDa which synergistically

exhibit toxicity to mosquito larvae (Baumann *et al.* 1988). This strain is highly toxic towards the mosquito species *Culex*, has moderate toxicity toward *Anopheles*, and is relatively ineffective towards *Aedes*. *B. sphaericus* is a highly aerobic bacterium and the critical effects to the availability of oxygen towards growth, sporulation and toxicity formation need to be investigated. The economic choice of batch culture, fed-batch or the continuous system for the production of bacterial spores which contain the protein toxin depends upon determining which system gives maximum spore production. This investigation reports the growth and sporulation kinetics and toxicity of *B. sphaericus* 2362 in batch fermentation culture using various levels of dissolved oxygen (DO). It also compares batch and continuous systems for bacterial spore production.

MATERIALS AND METHODS

The strain of *B. sphaericus* 2362 used in the study was kindly provided by Dr. S. Singer, Western Illinois University, Macomb, Illinois, USA.

Culture of the bacteria strain were maintained on NYSM (nutrient yeast salt medium) agar with the nutrient composition (g/l) of nutrient agar 28, yeast extract 0.5, minerals – $MnSO_4 \cdot 4H_2O$ 0.111, Ca $Cl_2 \cdot 2H_2O$ 0.103, Mg $Cl2 \cdot 6H_2O$ 0.203). All fermentation experiments were carried out in NYSM broth. Batch and continuous experiments were carried out in a 2-litre LH fermenter Model 502D (LH Fermenter Ltd, UK) which was equipped with pH, temperature, agitation, antifoam and dissolved oxygen control units.

A single colony culture maintained on NA plate was transferred into 100 ml NY medium (NYSM medium without minerals salts) in a 500-ml Erlenmeyer flask and incubated at 30°C in a rotary shaker (250 rpm) for 8 h. Five ml of this culture was then transferred into 70 ml of fresh NY medium and incubated for a further 12 h. The culture was then inoculated aseptically into 1.5 l of NYSM broth in a fermenter and run at 30°C, agitation speed 350 rpm, aeration rate 0.5 l/min and with saturation values of DO concentrations 5, 20, 50, 100% using saturated air. Higher levels of DO were achieved by the addition of pure oxygen. Fermentation was carried out for 72 h and samples were taken at intervals for analysis of dry cell wt, vegetative and spore count, and larvicidal activity.

Dry cell wt was determined by the standard methods (AOAC 1980). Ten-ml culture samples were centrifuged twice with distilled water. The pellets of cells and spores were oven dried at 80°C overnight and the constant dry weights were recorded. Vegetative and spore counts were done microscopically using a Weber Thoma bacterial counting chamber (Weber Scientific International Ltd., England)

Larvicidal activity was estimated using the third instar *Culex quinquefasciatus* mosquito. Ten mosquito larvae placed in 100 ml deionised

H_2O fed with 5% baker's yeast were tested for mortality against the culture. After 48 h the response was converted to an LD_{50} by probit analysis (Finney 1971). The samples of *B. sphaericus*, including a control, were tested against mortality of the mosquito larvae. Larvicidal activities (LD_{50}) were tested using the standard (SP88-Pasteur Institute, Paris, France) sample provided by Pasteur Institute, France.

For both batch and continuous culture systems, culture preparation and inoculum were carried out as described above, except for the continuous system. The feeding medium was fed to cells grown in batch culture after 8 h. Feeding of the medium was done using a peristaltic pump (Watson & Marlow, Model 402, UK) and the feeding rates were set at 0.10, 0.15, and 0.20 h^{-1}. A fixed DO concentration of 20% air saturation was used. Samples were taken for analysis at specific intervals.

RESULTS AND DISCUSSION

B. sphaericus grew rapidly in all treatments and gave a final concentration of $2.9 - 3.2 \times 10^9$ cells/ml at the end of 72-h batch fermentation. In each case the initial pH was between 6.8 and 6.9, and rose steadily throughout the fermentation process to a final pH of 8.8 – 9.0.

The growth and sporulation of *B. sphaericus* 2362 are summarized in *Fig. 1.* The growth curve shown is for cells grown with low set DO

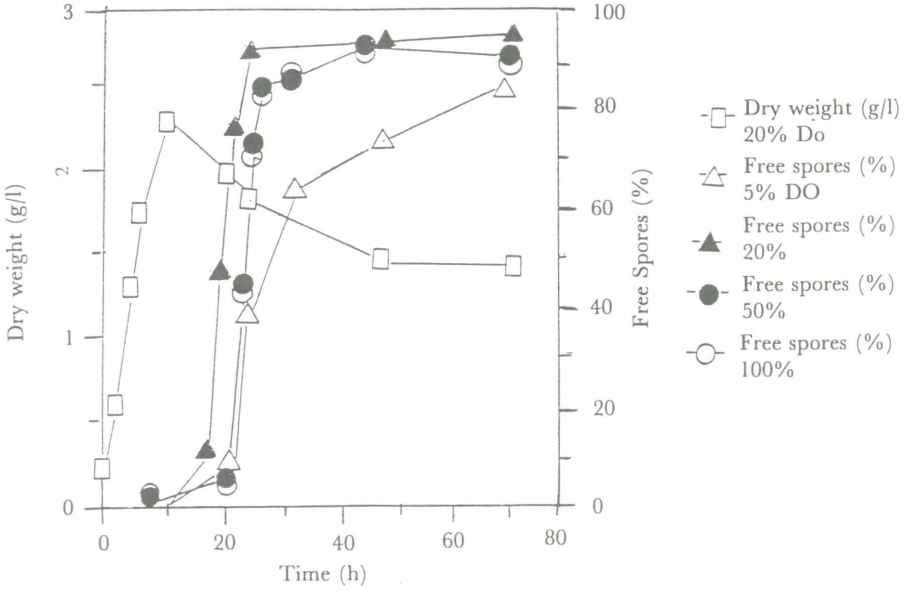

Fig. 1: Growth and sporulation of B. sphaericus *grown using various levels of DO (Average of 5 replicates)*

concentration of 20%. Cells grown under the other conditions were very similar to those shown for 20%. This result is in agreement with that reported by Youstern and Wallis (1987). The percentage of total cells which formed free spores rose from 87 to 94% within 72 h of batch fermentation. Spore numbers increased rapidly at DO values of 20, 50 and 100% (from approximately 10 to 85% of the total cell count) in about 20 h fermentation. At 5% DO the formation of free spores proceeded more slowly, taking around 60 h to increase from 10 to 80% free spores. This trend in the rates of sporulation at the lowest DO levels is significant when considering commercial production of the culture as it would incur higher costs. Thus, 20% DO can be considered a suitable level for the formation of free spores from *B. sphaericus* 2362. A spore preparation is expected to have a far longer shelf-life than preparation of either the vegetative or incompletely sporulated cells. Toxin formation is associated with sporulation and a spore preparation would therefore have an immediate impact on the target mosquito population.

Toxicity studies via larval assays were performed on 48-h culture grown at DO levels of 20, 50 and 100%, and on a 72-h sample from the 5% DO fermentation. Results of the bioassays are shown in *Fig. 2.* Yousten and Wallis (1987) reported toxin production by *B. sphaericus* 2362 grown at 0, 20 and 90% DO, as the minimum required by the culture. They reported that the level of toxicity from cells grown at 0% DO was intermediate. A high level of toxicity was observed from those grown at 20% DO and the lowest level of toxicity was from cells grown at 90% DO. Our results for 100 and 20% DO were found to be comparable to those reported by Yousten and Wallis (1987) for 90 and 20% DO values. However, our cells grown at 5% DO showed significant toxicity. It is possible that at 0% DO, cell growth is hampered and toxin production by the culture may be incomplete, thus resulting in low toxicity level. The results also showed that significant toxicity levels for samples grown at DO of 5, 20 and 50%. However, those

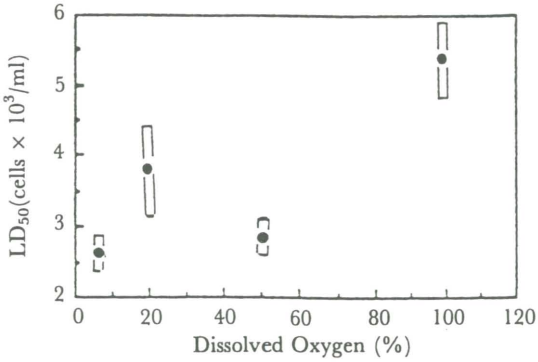

Fig. 2: Larvicidal activity (LD_{50}) of B. sphaericus *at various levels of DO*

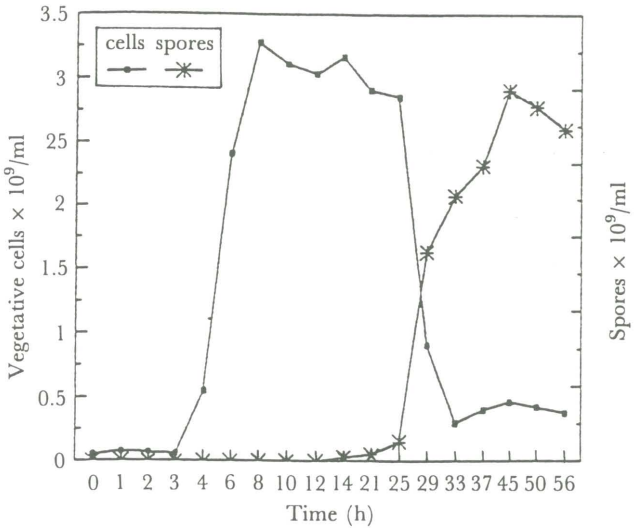

Fig. 3: Vegetative growth cells and spore count of Bacillus sphaericus *2362 in batch culture fermentation system grown at 20% DO (Average of 5 replicates)*

grown at 100% DO exhibited low toxicity level; this is probably due to the inhibitory effect of protein toxin formation at higher oxygen levels.

In batch culture fermentation, an initial incoculum of 4.9×10^6 vegetative cell/ml was used. During fermentation, maximum vegetative cells (3.28×10^9/ml) and spores (2.9×10^9) were obtained after 6 h and 45 h fermentation, respectively (*Fig. 3*). For the continuous culture system, steady-state culture was observed on samples grown at 35-h fermentation. Using different dilution flow rates it was found that a dilution rate of 0.10 hr^{-1} gave higher spore count than higher dilutions (*Fig. 4*). After 35 h fermen-tation constant spore production of around 1.3×10^8/ml, 0.75×10^8/ml and 0.3×10^8/ml were obtained on samples at dilution rates of 0.10, 0.15 and 0.01 hr^{-1}, respectively. Our results are in agreement with those reported by Yousten and Waills (1987) in which spore production was found to decrease with an increase in dilution rate. Thus, in the continuous system a lower dilution rate would give better spore production than a higher dilution rate. A higher dilution rate would decrease the availability of more nutrients for cell growth and spore formation.

The productivity of spores from batch culture was found to be higher than in the continuous culture system. However, economics and other fermentation considerations must be taken into account when dealing with biomass production using the batch and continuous systems.

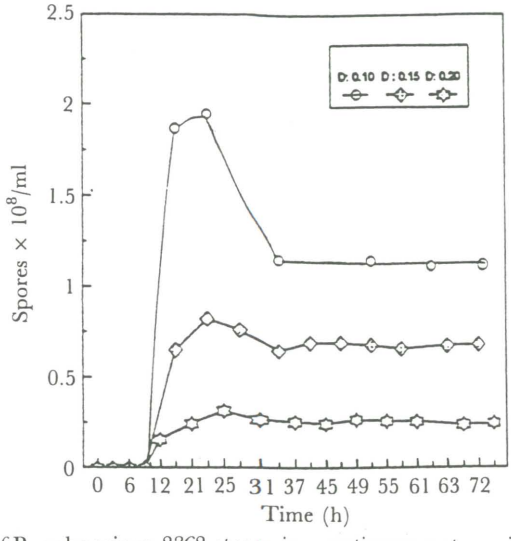

Fig. 4: Production of B. sphaericus *2362 spores in a continuous system with various dilution flow rates (0.10 hr^{-1}, 0.15^{-1}, 0.20^{-1}) (Average of 5 replicates)*

REFERENCES

AOAC. 1980. *Official Methods of Analysis*. Washington, DC.

BAUMANN, L., A.H. BROADWELL and P. BAUMANN. 1988. Sequence analysis of the mosquitocidal toxin genes encoding 51,4 – and 41.9 – kDa protein from *Bacillus sphaericus* 2362 and 2297. *J. Bacteriol.* **170**: 2045-2050.

FINNEY, D.J. 1971. *Probit Analysis*. 3rd. edn. Cambridge University Press.

YOUSTEN, A.A. and D.A. WALLIS. (1987). Batch and continuous culture production of the mosquito larval toxin of *Bacillus sphaericus* 2362. *J. Ind. Microbiol.* **2**: 277-283.