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FACTORS AFFECTING FERMENTATIVE PRODUCTION *OF BACILLUS SPHAERICUS*

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ABSTRACT

If *Bacillus sphaericus* is to be successfully developed for use as a mosquito larvicide, it must be possible to produce the bacterium and its associated toxin inexpensively on a large scale. This will require the selection of a strain that is suitable to growth in large fermcntors using simple, rather crude substrates to produce a highly toxic cell mass. That cell mass should be readily recoverable, stable in storage, and amenable to formulation and dispersal. With respect to growth, *B. sphaericus* is not able to metabolize carbohydrates and this prevents the use of such common fermentation substrates as molasses (sucrose), whey (lactose), and com syrup and cellulose hydrolysates (glucose). We have found that some of the enzymes of the Embden-Meyerhof path and of other metabolic paths present in other bacteria are missing in *B. sphaericus.* Furthermore, *B. sphaericus* is unable to transport glucose or sucrose into the bacterial cells. It is unknown if the genes coding for the enzymes of these pathways and for the proteins used for transport are missing or if they are not being expressed. Thus, these bacteria must be grown on proteinaceous substrates. Of the rather small number of strains which have been examined in some detail, strain 2362 seems to be the most toxic and to possess certain other properties which make it suitable for development. For example, it produces toxin at a higher temperature and at a higher protein content in the medium than two other strains examined (1593, 2297). Since toxin synthesis requires the onset of sporulation, it is important that a high percentage of the bacteria] cells which are grown be induced to *initiate* sporulation. It is less important that sporulation actually be completed i.e. it is not necessary that final, mature, heat resistant spores be formed for a high level of toxicity to be achieved. However, it may be important to have a high number of spores in the larvicide to produce the extended larvicidal effect which has sometimes been reported for this bacterium. This question has not been answered experimentally. During growth of strain 2362 in proteinaceous media, a high pH develops (8.5-9.0). We found that control of the pH in the fermentor at 7.2 did not increase the final level of toxicity achieved. Since B. *sphaericus* is an aerobic bacterium, it is necessary to provide air to a fermentor during growth, however, increasing the level of dissolved oxygen by the use of pure oxygen instead of air (21% O_2) did not increase the final toxicity. At the completion of sporulation and toxin synthesis, the bacterial cells lyse and liberate the spore and the attached toxic parasporal body. Cell lysis results in a lower amount of cell solids being recoverable by centrifugation since cytoplasmic proteins and ribosomes arc lost. However, the solids which are recovered contain most of the toxin (the paraspore) and thus the toxicity of the recovered solids after cell lysis may be higher per unit weight than was the toxicity of the solids prior to cell lysis even though no new toxin was synthesized.

INTRODUCTION

The successful development of *Bacillus sphaericus* as a mosquito larvicide will depend upon the production of the bacterium and its associated toxin inexpensively on a large scale. This wiII require the selection of a bacterial strain that is suitable for growth in large fermentors using simple, rather crude substrates to produce a highly toxic cell mass. That cell mass should be readily recoverable,

stable in storage, and amenable to formulation and dispersal. This report describes experiments which provide information of use in the production of *B. sphaericus* larvicide.

MATERIALS AND METHODS

B. sphaericus 1593 and 2362 were grown in NYSM broth (nutrient broth supplemented with 0.05% yeast extract, 5×10^{-5} M $MnCl_2$, 7×10^{-4} M $CaCl_2$, 5×10^{-3} M $MgCl_2$). The effects of temperature and of varied medium protein concentration were carried out in cultures grown in 50 ml of NYSM broth in 500 ml flasks which were shaken at 175 rpm for 24 h. The incubation temperature in the protein level trials was 30°C. Fermentations were carried out in a New Brunswick Scientific Co. F-2000 Multigen fermentor operated at 30°C. Aeration was provided at 1 liter/l/min and agitation was set at 400 rpm. Inoculum for 1 liter of NYSM broth in the fermentor was prepared as 50 ml of NY broth (nutrient broth supplemented with 0.05% yeast extract) in a 500 ml flask shaken at 175 rpm for 4 h at 30°C.

Growth was determined by measurement of the culture turbidity at 660 nm. 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. This procedure often doubled the spore count compared to unsonicated samples. Diluted samples were plated in NY agar and incubated at 30°C for 48 h. Dry weights were determined by recovering cells from fermentation samples by centrifugation, resuspending the cells in distilled water, and drying aliquots (in triplicate) for 48 h at 110°C.

Toxicity of washed bacterial cells was determined by bioassay against second instar larvae of *Culex quinquefasciatus* as previously described (Yousten and Wallis, 1987). Toxicity units were determined by comparing the LC_{50} of the assayed sample to the LC_{50} produced by a standard *B. sphaericus* 1593 powder, designated RB-80, which was assigned a value of 1000 toxin units/mg (Bourgouin et al., 1984).

Enzymes were assayed by growing *B. sphaericus* 2362 to mid-exponential phase ($A_{660} = 0.2$) in NYSM broth (supplemented with 0.1% glucose) shaken at 30°C. Cells were harvested by centrifugation, resuspended in buffer appropriate to the assay, and broken by sonication. The broken cells were removed by centrifugation and the cell-free extract was centrifuged at 120,000 g for 1 h. All extracts were kept cold and were assayed within a few hours of preparation. The assays either directly involved or were coupled so as to involve oxidation or reduction of NAD(P) or NAD(P)H which was monitored spectrophotometrically at 340 nm. Amylase activity was tested by observing zones of clearing around colonies grown on NYSM agar supplemented with 2% Argo brand starch. *B. subtilis* 168 served as a positive control. *B. sphaericus* 2362 grown in NYSM broth supplemented with 0.1% lactose was assayed for beta galactosidase by measuring hydrolysis of o-nitrophenyl-beta-D-galactopyranoside spectrophotometrically at 420 nm. *E. coli* K-12 served as a positive control.

RESULTS AND DISCUSSION

Carbohydrate metabolism by *B.sphaericus*

Carbohydrates are a common source of carbon for the growth of microorganisms. They are frequently provided in fermentation systems in the form of molasses (sucrose), starch or cellulose hydrolysate (glucose), or whey (lactose). These fermentation carbon sources are readily available and inexpensive in many parts of the world. However, *B.sphaericus* is unusual among microorganisms in that it is unable to utilize carbohydrate as a source of carbon for growth. This requires the use of more expensive and sometimes less available protein material for fermentation substrate. Dharmsthiti et al. (1985) suggested the use of by-product from a monosodium glutamate factory as

an ingredient in media to grow *B. sphaericus*. If the inability to metabolize carbohydrate is due to a single metabolic lesion e.g. a missing enzyme, it might be possible to remedy this problem by transfer of the gene for the missing enzyme into *B. sphaericus*. Analysis of the usual pathways of carbohydrate metabolism revealed that the following enzymes are missing or below the level of detection in *B. sphaericus* 2362: glucokinase, hexokinase, phosphoglucose isomerase, 6-phosphofructokinase, glucose-6-phosphate dehydrogenase, glucose dehydrogenase, and phosphogluconate dehydratase/KDPG aldolase (assayed as a pair). In each case the enzyme was detected in an appropriate control bacterium to verify the effectiveness of the assay itself. The lack of these enzymes prevents the functioning of the Embden–Meyerhof–Parnas (EMP) pathway, the hexose monophosphate pathway, and the Entner–Doudoroff pathway. Enzymes of the lower portion of the EMP pathway were shown to be present by the ability of the bacteria to convert glycerol to acetic acid when glycerol was added as a supplement to NYSM broth. In addition, strain 2362 grew with acetate as sole carbon source. To do this, the bacteria must possess the lower portion of the EMP pathway to synthesize fructose-6-phosphate (for peptidoglycan synthesis) and ribose (for nucleic acid synthesis). In addition to the enzymes already listed, the cells lacked amylase to degrade starch and beta galactosidase to hydrolyze lactose. Slow growth took place when gluconate was the sole carbon source. This is permitted by the presence of gluconokinase and 6-phosphogluconic acid dehydrogenase.

Not only were most of the enzymes which are normally present in the early part of carbohydrate metabolism missing in *B. sphaericus* 2362, but experiments using [^{14}C]glucose and [^{14}C]sucrose revealed that this bacterium is unable to transport these sugars into the bacterial cell (Russell et al., 1989).

It is unclear if the genes coding for the many missing enzymes are missing in this bacterium, if the proteins are synthesized at undetectable levels, if they are synthesized in an inactive form (perhaps due to separate mutations), or if the genes are present but are not expressed. The latter could be due to some regulatory defect. If the genes are all absent, then the possibility of replacement by genetic engineering may be an impractical task. However, if they are present but not expressed for some reason, perhaps the lack of a regulatory protein, then it might be possible to remedy this problem by the insertion of a gene. At the present time too little is known about regulation of the synthesis of these enzymes to suggest a simple remedy. It seems that it will continue to be necessary to grow *B. sphaericus* on proteinaceous carbon sources.

Properties of *B. sphaericus* strains

Of the limited number of strains of *B. sphaericus* which have been examined for their level of toxicity, those of bacteriophage group 3 serotype H5a5b (for example strains 2362,1593, 1881) and 4 (strain 2297) have proven to be the most toxic. A description of bacteriophage and serotype groups has been given (Yousten, 1984). Beyond the level of toxicity, other factors must be considered when selecting a strain for large scale fermentation. For example, to obtain a large cell mass, it will be necessary to grow the bacteria in a medium having a high protein content. When strains 1593 and 2362 were compared in media having increasing amounts of protein (tryptone i.e. hydrolyzed casein), toxin production by 1593 decreased as protein concentration increased. Toxin production by 2362 remained constant (Table 1). Another factor involved in fermentative production is the temperature at which toxin can be synthesized. This would be particularly important in tropical countries where the supply of fermentor cooling water may be limited and where growth of the bacteria at a high temperature may be necessary. A comparison of strains 1593 and 2362 at 3 temperatures indicated that 2362 could produce high toxicity at 35°C whereas 1593 was unable to do so (Table 2). Neither strain produced a high level of toxicity at 40°C. The comparisons were only made for these two strains (Yousten et al., 1984) but such manufacturing-related studies should be made for all strains which are being considered for production. Such studies must be an integral part of selecting the best strain for large scale production.

TABLE 1
Effect of increasing protein concentration on sporulation and toxin synthesis by *B. sphaericus* 1593 and 2362[a]

NYSM supplement[b]	Strain 1593		Strain 2362	
	Spores/ml	LC_{50}(ng/ml)	Spores/ml	LC_{50}(ng/ml)
None	3.5×10^8	1.1	1.3×10^9	0.01
2% tryptone	4.6×10^5	41.1	9.5×10^8	0.03
4% tryptone	9.9×10^5	33.5	3.6×10^8	0.04
6% tryptone	1.1×10^5	136.0	6.4×10^8	0.04

[a]From Yousten et al. (1984).
[b]NYSM broth contained 3 mg/ml protein; following supplementation with 6% tryptone, the broth contained 32 mg/ml protein.

TABLE 2
Effect of growth temperature on sporulation and toxin synthesis by *B. sphaericus* 1593 and 2362[a]

Temperature	Strain 1593		Strain 2362	
	Spores/ml	LC_{50}(ng/ml)	Spores/ml	LC_{50}(ng/ml)
25	3.2×10^8	0.1	9.7×10^8	0.07
29	2.2×10^8	0.6	1.0×10^9	0.05
35	3.5×10^6	9.6	6.9×10^8	0.04

[a]From Yousten et al. (1984).

Growth and toxin production by *B. sphaericus* 2362

When *B. sphaericus* 2362 was grown in NYSM medium, the pattern of growth, sporulation, and toxin synthesis presented in Fig.1 was found. The pH of the medium, which was initially about 6.7, increased to about 8.8 in the stationary phase. This was caused at least in part by the accumulation of ammonia in the medium. The ammonia is probably derived from the deamination of amino acids as the bacteria metabolize these compounds for carbon and energy for growth. Bacterial cells displayed swollen ends containing forespores at about 6 h after inoculation. This preceded the development of heat resistant spores by several hours (Fig.1). Toxicity had already reached a high level before the appearance of heat-resistant spores. Between the 14th and 24th hours, the cells lysed and set free the spores and toxic paraspores. As the cells lysed, cytoplasmic materials which could no longer be recovered by centrifugation were released into the medium. This lysis was reflected in a decrease in the cell solids which were recoverable by centrifugation (refer to mg/ml dry weight data in Fig. 1). The toxin is associated primarily with a parasporal body which along with the spore is still sedimented when the culture is centrifuged. Thus, the solids which are recovered by centrifugation late in the fermentation period, are enriched in toxin. This accounts for the almost doubling of toxicity (expressed as units/mg dry weight) between 14 and 24 hours. It might appear that a considerable amount of toxin had been synthesized during this period, but in reality about the same total amount of toxin was present in the culture at 14 and at 24 hours. When analyzing the results of fermentation studies, it is critical to know how the samples were prepared for bioassay. For example, was it possible to wash off excess medium ingredients from the cells prior to assay or

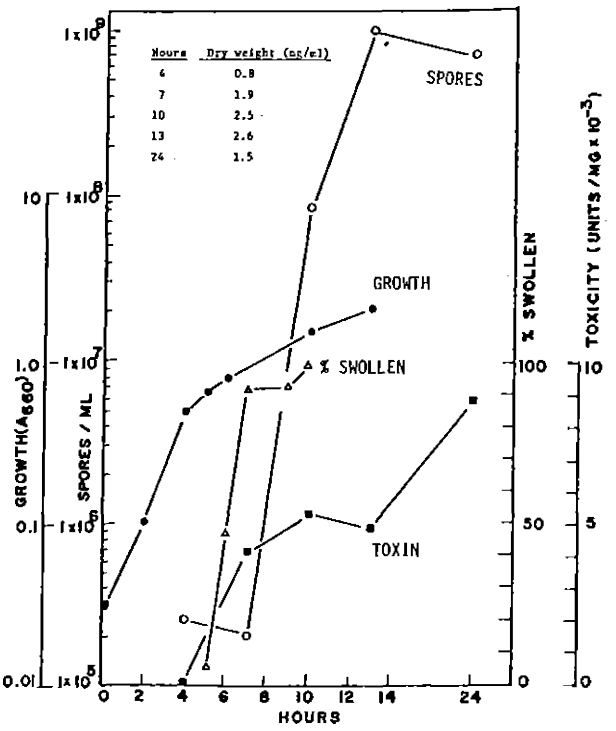

Fig. 1. Growth, sporulation and toxin synthesis by *B. sphaericus* 2362. Symbols: (●) growth; (o) heat resistant spores; (Δ)cells with swollen ends indicating the beginning of sporulation; (■) toxin.

was this extra (inert) weight included in the LC_{50} calculation? Was the dry weight of the cell mass in a preparation actually determined or was the weight of 1 ml of a liquid cell suspension simply set to 1 g in calculating toxicity? Very different levels of toxicity are obtained in each case and this can be misleading when comparing results from different laboratories.

Since the pH of *B. sphaericus* fermentations increased dramatically during growth of the bacteria, pH control during growth was tested as a means of possibly increasing toxicity. When the pH was held at 7.2 by the automatic addition of sterile H_2SO_4, the toxicity of strain 2362 was not increased (Yousten and Wallis, 1987). However, the toxicity of the usually less toxic strain 1593 was improved by this technique (Yousten et al., 1984).

B. sphaericus is an aerobic bacterium and oxygen is rapidly removed from the medium during growth. An adequate level of dissolved oxygen is necessary to assure good growth and the initiation of sporulation. However, increasing the level of dissolved oxygen by changing from air (21% oxygen) to pure oxygen in the gas stream to a fermentor containing strain 2362 did not increase the toxicity of the bacterial cells even though it did increase the level of dissolved oxygen throughout the fermentation (Yousten and Wallis, 1987).

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