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CONSULTATION ON THE DEVELOPMENT OF BACILLUS SPHAERICUS AS A MICROBIAL LARVICIDE

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OPTIMIZATION OF MEDIA AND SELECTION OF RAW MATERIAL FOR MASS PRODUCTION

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The development of fermentation media and procedures for the production of B. sphaericus is not unlike the development of many other fermentations -- with one important restriction -- the media used in the fermentations must be adaptable to the recovery and formulation processes that the fermentation products must undergo. Aside from their nutritive qualities, these nutrients must be of a fine enough mesh to pass through whatever equipment is used in the application of the product in the field. This is essential if the costs of recovery are to be kept to a minimum: the separation of residues of coarsly-ground nutrients can be expensive. Leaving this to one side as a general principle, I would like to preface this discussion with a general summary of just what is needed to develop any fermentation and then to see how these procedures are applied to the development of the B. sphaericus fermentations.

1) Selection of a suitable bioassay procedure

In the initial search for a useful entomopathogen, it is enough to find out whether the microbe or its products kills or does not kill a desired target insect. However, once a culture has been selected as a proposed microbial control agent, it becomes essential to develop a suitable assay procedure. Most fermentation studies result in modest gains in yields -- 20 to 30% being a common increment. It is the summation of these modest gains that enable us to make major progress in increasing yields and decreasing costs, so it is desirable that a fermentation assay be accurate to within ± 30% so as to be able to detect such gains. This is an attainable goal, but attaining it in the case of B. sphaericus bioassays (or in any bioassay of a microbial insecticide) entails the use of many insects, testing them against many concentrations of the proposed product, and incubating the test animals for a significant period of time. All this makes the insect bioassay slow and costly of time and manpower and limits the number of assays that can be run.

2) Selection of a suitable fermentation procedure

The initial screen should tell us whether a new isolate is pathogenic to the test insect species. Development of a bioassay should have begun Now we need to study as many isolates of this new pathogen as are available, and to do this, we have to develop a screening program to select the most promising isolates for further study. At this stage, fermentation studies are begun. It is now necessary to design and select a medium on which to grow these isolates so that we can compare their ability to produce the entomopathogen in submerged fermentation. The medium we use must support the growth at at least most isolates of the candidate microbe and also support the production of reasonable amounts of the insecticidal products. There is no way to lay down a general rule as to the makeup of this screening fermentation medium. We can develop it only by applying our own backgrounds of experience, coupled with a considerable amount of trial and error. Let me emphasize that at this stage of our program, economics plays very little part in the selection of a medium: We are evaluating the product, not the fermentation. What we do hope, of course, is that we will be able to develop fermentations that are practical and economical for the production of whatever insecticidal agent that we choose. Fortunately, in the case of the entomopathogenic bacilli, this is not too difficult to achieve.

a) Media ingredients

In considering the development of a screening medium, we must keep in mind that we are not trying to optimize production of the microbial insecticide at this stage. The medium will probably not be optimum for all strains of the microbe. This is all right, so long as the medium will allow us to produce enough of the insecticide to evaluate it. As fermentation studies proceed, new ingredients will be added, concentrations of nutrients will be changed, and the fermentation media will become more complex and specialized. These new media are essential to the development of economical production of the microbial insecticide, but they are not necessarily good screening medium.

As examples of this, I'd like to draw from our experience with Bacillus thuringiensis. We have developed a screening medium, which we call medium B-4, for use in screening isolates of this bacillus. The medium has been basic to our fermentation research. This medium contains only low concentrations of nutrients and contains/liter of medium, 10 g degossypolized cottonseed flour and 15 g dextrose as the sole carbon and nitrogen sources. This medium is highly satisfactory for laboratory studies in shaken flasks, but it is much too light for studies in fermentors where improved agitation and aeration permit the microbe to utilize the media ingredients more efficiently. On the other hand, shake flask fermentations can be harmed by the high concentrations of solids used in some fermentor media, probably because the presence of excess nutrient in shake flasks (which are much less efficiently aerated than are fermentors) lead to abnormal fermentation sequences.

Medium B-4 would not be a satisfactory screening medium for use with B. sphaericus isolates. To begin with, the entomopathogenic strains of B. sphaericus do not utilize sugar, and there is no need for dextrose in our screening medium. Second, many isolates of B. sphaericus do not grow well on medium B-4 in shake flasks, although they will grow on this medium in fermentors. However, most isolates of B. sphaericus do grow well on a peptonized milk medium, designated in our laboratory as medium B-15a. This medium, like B-4, is quite "light" in nutrients and contains only 15.0 gm peptonized milk/liter of medium as the only carbon and nitrogen source. (Peptonized milk is a protein hydrolyxate of dehydrated skim milk and is available as a fermentation ingredient in the U.S. It is, however, rather expensive.) Yeast extract has played a useful role as a result, we have included 2 gm yeast extract per liter of medium. Yousten has also pointed out the value of minerals in B. sph. agar media, so we include small amounts of $MgSO_4$. MnSO4, $ZnSO_4$, and $FeSO_4$ in all our media for B. sph.

Medium B-15a is a satisfactory medium in which to screen the ability of a wide number of isolates of B. sph., but it is expensive and supports only moderate yields of B. sph. Therefore, we have tested the growth of B. sph. on a series of different media in small (14-liter) fermentors. To do this, we tested a wide series of fermentation nutrients that were available in the United States. Since our strains of B. sph. do not utilize carbohydrate, we made no attempt to try alternate carbohydrate sources, although we did test one or two molasses products. It is important to the development of these more advanced fermentations that we utilize cheap, locally-available nutrients. Thus most of the media developed at Brownsville revolve around such ingredients as corn steep liquor (essentially a waste product from the processing of corn), soybean flour, and cottonseed flour.

b. Fermentation conditions

The three key variables in fermentor studies with B. sph. are aeration, temperature of incubation, and pH. To study the effects of these variables on yields of B. sph. or its toxin, we need a very accurate bioassay. At Brownsville, we do not have the manpower to conduct such bioassays, although we can sometimes receive help from another laboratory. However, our limited data indicate that yields will increase dramatically when we can optimize these variables. At present, in Brownsville, we are using our B-16 medium (containing in gms/liter medium: 30 gm of soybean flour, 15 gm of a soy peptone, 10 gm of corn steep liquor, and 2 gm of yeast extract.) The pH of this medium is quite acid, so the medium is adjusted to pH 7.0 before sterilization. The fermentors are aerated with 0.67 volumes of air per volume of medium per minute. We incubate the fermentors at $30^{\circ}C$ for 45-48 hours, depending on how the culture sporulates. Because B. sph. does not utilize sugar, the pH of the fermentation rapidly becomes more alkaline reaching pH 9.5 or more during the incubation period.

c. Recovery procedures and evaluation

Recovery procedures must be simple and the number of steps in the recovery limited as far as possible if B. sph. is to become an economical tool for larva control. Centrifugation and spray drying have been the principle methods used in the recovery of the B.t.-delta-endotoxins from fermentation beers. We have assumed that the same methods will be used for B. sph. Much more work needs to be done to follow the yields from starting medium to final product.

It is frequently assumed that the potency of the final product is the only evaluation procedure needed in either B.t. or B. sph. fermentations. This is not correct. For example, if we grow B.t. on a medium such as tryptose broth, the powder recovered will be very high in potency. If we grow B.t. on a soybean-flour-based medium, the potency of the product may be only 1/5 as high. However, the yields, based on the amount of insecticidal activity present in a liter of fermentation medium will be the reverse, with the yields of B.t. activity being 4-5 times greater in the soybean medium. Superficially, the tryptose product looks better, but from an economic standpoint, the soybean product is far superior.