Mesa redonda: Toxinas de *Bacillus thuringiensis* e suas aplicações.

PRODUCTION OF PROTEIC PROTOXIN BY BACILLUS THURINGIENSIS BY SEMI-SOLID FERMENTATION

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Recently it has been recognized that the chemical insecticides are by no means, the ideal solution for the control of insects that inflict the heaviest losses on agricultural crops. Their toxicity to non-target species, particularly to men and animals, in addition to their steady loss in potency due to development of resistance among target species, were among the factors that urged the search for new means of insect control. Thus, the introduction of highly specific biological means for insect control, has offered a sound alternative to chemical insecticides.

In a rapid review of literature, invertebrates appear to have more fungal than bacterial diseases, probably because fungi are usually more obvious than bacteria, and they are usually more easily identified as a primary pathogen. Although, a wide variety of rod-shaped, aerobic, spore-forming bacteria are associated with disease. Some of these produce one or more toxins which are of primary importance in their pathology. All these pathogens have in common the failure to produce hemocoelic septicemia until near or after death of the host. Some of these bacilli carry their toxins with them, when ingested, having produced them elsewhere ; others produce the toxins while multiplying in the host gut. Among the best studied species is the commercially important Bacillus thuringiensis.

B. thuringiensis, as the other bacteria that to the genus Bacillus, forms endospores following the vegetative growth phase. These endospores are resistant against extreme environmental conditions, and are able to preserve viability over many years. The B. thuringiensis produces secondary metabolites exhibiting biological activities. Such compounds include toxins as well as enzymes, and their production occur either during vegetative growth phase or during sporulation phase. The crystalline protein inclusion which is formed during sporulation phase, is a gut poison for larvae of more than one hundred insect species belonging to the order Lepidoptera and Diptera.

In addition to the crystalline inclusion (also known as parasporal body, crystal, or as delta-endotoxin, in its actived state), some varietes release a nucleotide during the vegetative growth phase, which is called beta-exotoxin. This exotoxin has a much broader spectrum of activity, covering invertebrates and some vertebrates. Other insecticidal metabolites of B. thuringiensis strains, the alfa exotoxin and the gama-exotoxin, as well as some enzymes, are of only secondary importance for insect control.

Angus (1954) succeeded in proving that the crystalline inclusion formed during sporulation, was responsible for the insecticidal action. The unusual method of biosynthesis and deposition in a crystalline form, must have been the reason that more than 50 years elapsed between the isolation of the microorganisms (in 1902, in Japan), and the discovery of the toxic inclusion. The practical use of entomopathogenic microorganisms for crop protection, is only possible if a large scale industrial production of these organisms is developed. This principle was understood even by the early workers who attempted to industrialize their cultivation process for B. thuringiensis at the middle of the twenty century (Megna, 1963; Mechalas, 1963). Techniques developed by these authors were replaced by liquid culture techniques, and then later, by more efficient fermentation processes.

The feasibility of B. thuringiensis production depends, to a large degree, on the cost at which this product can be produced. This in turn, depends on the efficiency at which the organism produces the product (choice of the proper strain), the fermentation conditions (temperature, aeration, etc.), the means by which the product is recovered from the beer's produced in the fermentation process, and finally, by the cost of the medium used in the fermentation. Although in many countries, B. thuringiensis formulations are economical to be used in the control of several insects, in Brazil the wider use of this formulation has been restricted by economic reasons. So, in our country, fermentation technology has to aim the production of these endotoxins at reduced costs. However, the composition of new fermentation media and newer fermentation processes has received very little atention.

Dulmage (1971, 1973, 1981) successfuly used agroindustrial by-products in submerged fermentation for producing active delta-endotoxins. Salama (1983a, 1983b, 1983c, 1984) also developed practical liquid media by means of using several agroindustrial by-products. In the same line, attempts were made by Foda et al (1984, cited by Salama, 1984) to employ new methods of fermentation in the hope to arrive at a less expensive and more feasible method for B. thuringiensis production. They employed a semi-solid type of fermentation where the bacillus strains were grown in rectangular trays containing thin layers of the semi-solid medium with agar. Heavy inocula were spread over the surface of the medium. This technique gave promising results in endotoxin yields, and potential biological activity.

In our search for new fermentation process, and based on the few works in this area, we studied the production of B. thuringiensis spores and toxins, in a low cost medium - using agroindustrial by-products- by the semi-solid fermentation process in flasks.

FERMENTATION PROCESS

By semi-solid fermentation, we mean the growth of microorganisms on solid materials, not in a liquid phase (Hesseltine, 1977; Thieman, 1985). The substrate may be put on a tray or in a flask, and inoculated; the microorganism then, develops on the substrate as much they do in nature; and the substrate can be occasionally shaked, so that part of the bottom is moved to the top, and the medium becomes more uniform.

The screening of the substrate have to be made, based on some guidelines. The mayor ones are:

- agroindustrial solid by-products has to be available in the region where the work is done;
- this product, has to have a stable, nearly constant, composition;
- it has to be a really by- product or a subproduct of low cost.

For the purpose of our work, developed in Campinas and in Jaguariúna, the selected by-products include solid residue from pulp and paper industry, residual fermented malt from beer industry, a special kind of meal obtained from residual cookies and biscuits of bakery industry, and two kinds of meal from chicken slaughter house.

They were air-dried, when necessary, to reach even 10% moisture, and were stored at ambient temperature until to be used.

For the fermentation process, 10g of each residue as such, or a combination of two, were dispensed in a 250 ml erlenmeyer, sterilized, humidified with a glucose solution and inoculated with a heavy inoculum of B. thuringiensis pre-fermented in a liquid medium. The principal results are summarized in Table I.

Table I - Production of Bacillus thuringiensis spores by semi-solid fermentation.

Residue used as fermentation medium	Time of fermentation (h)	Maximum number of viable spores/g of fermented medium at harvest time
A solid residue from pulp and paper industry	169	$3,0 \times 10^{13}$
B residual fermented malt from beer industry	144 223	$2,2 \times 10^{14}$ $1,0 \times 10^{17}$
C meal from residual cookies and biscuits of bakery industry	169	$7,8 \times 10^{8}$
D meal from chicken slaughter house (1)	169	$1,0 \times 10^{10}$
E meal from chicken slaughter house (2)	168	$1,2 \times 10^{10}$
C + A (1:1)	168 192	$3,2 \times 10^{13}$ $3,0 \times 10^{18}$
C + A (1:1) with addition of mineral salts	168 264	$7,8 \times 10^{14}$ $3,0 \times 10^{23}$
C + D	168	$1,6 \times 10^{8}$
C + B	168	$1,2 \times 10^{15}$

RESULTS

Based on the results showed on Table I, we concluded that the semi-solid fermentation process can be sucessfully used for B. thuringiensis spores production.

The fermented malt and the solid residue from pulp and paper industry, could both be used as a complete medium for growth and sporulation of B. thuringiensis. Furthermore, the supplementation of these media with a lowprice mineral salt source, could provide feasible enrichment sources yielding production of spores.

CONCLUSIONS

The semi-solid fermentation process demonstrated to be an interesting process that can be adopted in the future. Its advantages over the conventional stirred or aerated liquid media fermentations could be described:

- the medium is relatively simple since a single meal plus needed. Other nutrients, such as liquid agroindustrial residues, may be added to the meal;
- it is apparent from the works in the literature, that the process may be scaled up;
- the required space occupied by the fermentation equipment is relatively small if compared to the yield of product, because less water is used, and substrate is concentrated. The complexity of equipment is no greater than conventional fermentation equipment, and may still be smaller;
- specially important is the fact that the conditions under which the microorganisms grow, are more like the conditions under which they grow in nature;
- the substrate may be placed in plastic or paper bags, and frozen without having to store large volumes of liquid in breakble bottles or tie up large holding tanks;
- there are no enormous amount of liquid waste, as in liquid fermentations, to present a disposal treatment problem. In fact, there is no liquid to be disposed off;
- aeration is easily obtained since there are air spaces between each particle of the substrate;
- since the product is concentrated in the solid substrate, it may be dried and stored at less cost because less moisture must be removed.

Some problems with semi-solid fermentations, become obvious when one works with it:

- the variety of Bacillus thuringiensis has to be limited to those which do not produce exotoxins, since we are interested in the endotoxin;
- during the fermentation of small scale batches , no problem occurs; however, when one ferments large amount of material, heat becomes a problem that must be controlled;
- monitoring devices to determine moisture, pH and product yield, become a problem in this type of feremeentation. This will require considerable innovation by engineers and technicians;
- the substrate treatment must be considered. In some cases, the substrate must be cracked lightly, but the formation of flour should be avoided;
- the amount of inoculum required to inoculate large mass of substrate may be a problem in scalling up;
- the required addition of sterilized water or other liquids, during fermentation, offers the chance of introducing contaminants.

Due to its safety and specificity, the microbial insecticides as biological means of pest control, have found wide acceptance, particularly those produced by Bacillus thuringiensis. The present work we described above, using by - products from agroindustries, is favoured by the universality of these by-products, and also by the possible use of the semi-solid process, with such low price substrate. However, further studies will be required before final evaluation of the suggested approaches.

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