SOLID AND LIQUID WASTE UTILIZATION IN FERMENTATION PROCESSES TO GET BACTERIAL INSECTICIDE

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ABSTRACT

Economic reasons have delayed the commercial production of *Bacillus thuringiensis* insecticide, by fermentation, in Brazil, using conventional substrates. The development of this type of insecticide is very important in our country, where almost 40% of food and stored grains, "in natura" or processed, are lost by insect pests attack. Based on this fact, this work was conducted, since 1972 at the School of Food Engineering. It was studied the feasibility of using solid or liquid wastes, as substrates, employing semi-solid or submerged fermentation to produce *B. thuringiensis* endotoxins. Many agroindustrial by-products, including solid residues from paper industry, residual fermented malt from beer industry, wastewater from coconut processing, from alcohol fermentation (molasses), from corn industry (cornsteep liquor), and a meal from cookies and biscuits residues of the bakery industry, were used to verify their abilities to support growth and sporulation of *B. thuringiensis*. Growth and sporulation in the semi-solid process took 168 hours, and produce a spore count between 10 and 100 billions spores per gram of culture medium, but in the submerged fermentation, spore count reached the same range in a shorter time of 48 - 72 hours. The fermentative process was followed by determining the growth and sporulation kinetics, the sugar consumption, dipicolinic acid evolution and pH behaviour. Scale up of the submerged fermentation process was done until 200 liters. In this process, aeration is very important, as well as the agitation rate, and were used 0.8 vvm and 250 rpm, respectively. The use of solid and liquid wastes from food industry is advantageous because this minimized pollution problems of the environment, offering, through fermentation of *B. thuringiensis*, a safe product to vertebrates, fishes and the eco-system at leastcost. Bioassays with the product are being performed to establish rate dosages against insect-tests, and to determine the effective potency of the product (conclusive test), aside the spore count, that is a presumptive test to standardize the product.

INTRODUCTION

The toxicity of chemical insecticides to non-target species, particularly to man, animals and beneficial insects, in addition to the steady loss of effectiveness were some of the factors that urged the search for new means of insect control.

Pathogens, inhibitors and competitors are generally accepted as desirable agents as alternatives for pest control. Promising candidate agents and agents already registered for use in agriculture have presented outstanding safety records [1].

Biological control has been widely studied and used in many countries throughout the word and to a large extent also in Brazil. Although in a sporadic manner and in a few localities in the beginning, the study of this pest control method was initiated in Brazil in 1970 [2]. Nowadays state institutions, universities and the Brazilian Agricultural Research Corporation (EMBRAPA) of the Ministry of Agriculture, are carrying out experiments in this field [3].

The National Research Center for Agriculture Defense (CNPDA) has been giving priority to biocontrol since 1985, and one of the studies that are going on, at CNPDA, is about massive production of *Bacillus thuringiensis* (*Bt*) by fermentation means, that is at present, one of the most studied and commercially available biocontrol agent. Its use is permitted up to harvest time on several crops used for fresh consumption, and, more recently on stored grain [4].

The toxic activity associated with *Bt* takes place primarily in the parasporal crystal formed within the mother cell during sporulation, either *in vivo* or *in vitro*. This crystal is composed of a glycoprotein subunit that is believed to be a protoxin which is converted to a toxin by proteolytic activity. This specific protein is a feeding poison which destroy the gut epithelia of most Lepidoptera and some Diptera. Among the Lepidoptera there are numerous pest species important in agriculture, pasture and forestry [5, 6].

In addition to the crystalline inclusion, some *Bt* varieties release a nucleotide-like particle, called beta exotoxin, during the vegetative growth phase. Other insecticide metabolites produced by some strains of *Bt* - alfa and gama exotoxins - as well as some enzymes are of only secondary importance for insect control.

A mixture of spores and crystals of *Bt* are commercially produced and economic marketed in many countries as specific insecticide. However, in Brazil economic reasons have restricted the wider use of it. As it is produced by fermentation means, fermentation technology in Brazil has to aim the production of these endotoxins at reduced costs.

The media for industrial production of *Bt* are basically composed of complex carbon and nitrogen sources. In a rapid review one can find that starch and molasses are suitable carbon sources, and protein-rich material of plant and animal origin such as soybean, corn steep liquor or casein hydrolisates provide good and cheap nitrogen sources for *Bt* growth and sporulation.

Brazilian work with *Bacillus thuringiensis*

Studies were initiated in 1972, at the Department of Engineering at the Faculty of Food Engineering of the State University of Campinas (UNICAMP), to explore the feasibility of producing endotoxin preparations of *Bt* using submerged fermentation with cheap liquid by-products as components of the fermentation medium. That Department owned some submerged batch mini

and pilot scale fermentors and its facilities, so they explore the problem of the medium composition, its price and their influence on the final cost of the product. The results obtained, generated two industrial patents, (one in 1976 and another in 1985) [7, 8], about the fermentation process by means of submerged culture using sugar cane molasse and corn steep liquor as nutrient medium.

Based on the promising results obtained in laboratory and pilot scale production of *Bt*, the CNPDA with the cooperation of UNICAMP, initiated in 1986 studies to employ different methods of fermentation to spare the need of large aerated fermentors.

Besides these pioneer works in Brazil, other institutions are developing studies on *Bt* genetics, its side effects, safety, formulation techniques, improvement of its stability in the field, and so on.

THE SEMI-SOLID FERMENTATION

Semi-solid fermentation means the growth process of microorganisms on solid materials, not in a liquid phase [9, 10]. In this type of fermentation the substrate may be put on a tray or in a flask; after the inoculation the microorganism develops on the substrate. The substrate can be ocasionally shaked, so that part of the bottom moves to the top providing the necessary aeration for the growth of the microorganism, and promoting the uniformity of the medium.

The culture medium

First of all, to obtain an economical production, it requires that the fermentation medium has to be as cheap as possible, as well as able to support conveniently the endotoxin production. Many workers have already studied the influence of the composition media over the sporulation and endotoxin formation [9, 11, 12, 13, 14, 15, 16], but did it for the submerged fermentation process.

To iniciate the study of the semi-solid fermentation process it was necessary to select a suitable substrate. The selection was made based primarily on the following guidelines:
agroindustrial solid by-products, available in the region where the work was going to be done; the by-product must have a stable and nearly constant composition; and, it must be really a by-product or residue of low cost.

Many contacts were made with different industries to get information about their liquid and solid residues and by-products generated, their mean chemical composition, the cost and quantities available. From these data, some by-products were selected to be used in the experiments: solid residue from pulp and paper industry (A); residual fermented malt from beer industry (B); a special kind of meal obtained from residual cookies and biscuits of bakery industry (C); two kinds of meal from chicken slaughter house (D and E).

The process

Bacillus thuringiensis var. *thuringiensis* was used throughout the study. Its culture was maintained on slants of nutrient agar medium, in refrigerator. Each month it was transferred to a new slant.

Erlenmeyers of 250 ml of nominal capacity, containing 10 g of each residue separately, were used for the semi-solid fermentation experiments. The solid medium stayed between 1.5 and 2.0 cm (height), to facilitate the

TABLE 1
Production of *Bacillus thuringiensis* spores by semi-solid fermentation process

Residue used as fermentation medium	Time of fermentation (h)	pH	Maximum number of viable spores/g of fermented medium at harvest time
A solid residue from pulp and paper industry	0 169	7.2 5.5	- - - 3.0×10^{13}
B residual fermented malt from beer industry	0 144 223	5.7 8.0 8.1	- - - 2.2×10^{14} 1.0×10^{17}
C meal from residual cookies / biscuits of bakery industry	0 169	6.0 6.0	- - - 7.8×10^{8}
D meal from chicken slaughter house	0 169	5.9 7.9	- - - 1.0×10^{10}
E meal from chicken slaughter house	0 168	6.2 7.8	- - - 1.2×10^{10}
C + A (1:1)	0 168 192	6.4 6.1 6.4	- - - 3.2×10^{13} 3.0×10^{18}
C + A (1:1) with addition of mineral salts	0 168 264	6.9 6.8 6.8	- - - 7.8×10^{14} 3.0×10^{23}
C + B	0 168	6.0 7.5	- - - 1.6×10^{8}
C + B (1:1) with addition of mineral salts	0 168	6.0 7.3	- - - 1.2×10^{15}

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oxigen transfer for the growing microorganisms.

Each flask was previously sterilized at 121ºC for 20 minutes, and then humidified with 3 ml of a 22.5% sterilized glucose solution and inoculated with 7 ml of the submerged pre-fermentation of Bt (nearly 10^8 bacterial cells/ml, obtained in 15 h).

The incubation time ranged between 0 and 7 days. The flasks were maintained in the incubator, with only one hand-shake each day to avoid formation of clumps, and to promote a good aeration.

For analysis purposes, two flasks were taken at each harvest time, and 80 ml of sterilized water was added to each one. They were agitated in a rotary-shaker for 20 minutes at 200 rpm and then the supernatant of both was used to count the viable spores and to do the pH analysis.

Immediately after shaking, a sample of the liquid was transferred to a sterilized flask and heat stimulated. After this heat shock, the sample was serially diluted and the count of viable spores was done by means of the pour plate count colony method, on nutrient agar.

The liquid phase previously obtained was left resting for 10 minutes. This pH, measured with a potenciometer, expressed the pH value of the semi-solid fermented sample, as suggested by A.O.A.C. (1984) methods for meals.

The preliminary fermentation tests described, showed some interesting results summarized on TABLE 1.

The best results were attained using residues A and B or its combination with residue C.

Many authors [17, 18] affirm that the presence of ions calcium and magnesium improves the sporulation of most of the spore-former bacteria. So, it was added to the culture medium a product usually recommended for meal animal supplementation. This meal is rich in mineral salts and it is not expensive. Its addition to the residues into the media (see TABLE 1) increased the sporulation yields as expected.

As the fermentation went on, it was observed that the moisture content of the medium decreased too much. The evaluation of its content showed nearly 30% of moisture in culture media on the 7th day of fermentation. Under very dry conditions, the viable cells became unviable, and no increase in spores was attained.

To overcome this problem, some experiments were done to attain the appropriated humidification needed by these growing bacteria. Periodically it was added one or two milliliters of sterilized water to each fermentation flask. The results attained are summarized in TABLE 2.

TABLE 2
Influence of periodic humidification on the production of *Bacillus thuringiensis* spores, by semi-solid fermentation process (typical results)

Harvest time (h)	Moisture content (%)			Maximum number of viable spores/g of fermented medium at harvest time		
	Water added / flask			Water added / flask		
	0 ml	1 ml	2 ml	0 ml	1 ml	2 ml
0	50.17	50.17	50.17	4.5×10^4	4.5×10^4	4.5×10^4
96	47.50	50.85	51.54	9.9×10^{10}	8.9×10^{14}	9.2×10^{12}
198	43.16	52.90	57.71	10^{10}	1.1×10^{17}	1.0×10^{23}
288	40.49	53.36	59.98	1.4×10^9	5.3×10^{13}	1.0×10^{32}
336	38.00	54.15	62.10	1.2×10^9	1.8×10^{12}	4.0×10^{32}

Those data showed that the addition of 2 ml of water each 48 hours was the best quantity. In the first two or three days of fermentation, the erlenmeyers became humid inside. Although the temperature was not measured, that humidity certainly indicated that the temperature of the fermentation medium reached higher values than the observed outside the flasks.

In a small scale work volume, the increased temperature was not a real problem, because with only one handshake of the flask, the medium brought good mixture and the heat excess was dissipated. But it will be a real problem to be solved for the next step of scaling up the process.

The bioassay

In order to standardize the product, the spore count is not sufficient. So, it was necessary to establish bioassays with insect-tests, pests of the agriculture, mainly Lepidoptera. *Plodia interpunctella* (stored grains pest) and *Asciamonuste orseis* (foliar damage) had been chosen as insect-tests and the bioassays demonstrated the toxicity of the product obtained by submerged fermentation. The products resulted from semi-solid fermentation, were not bioassayed yet, but to do these assays the Laboratory of Insect--tests/CNPDA is also doing massive production of *Anticarsia gemmatalis* (soybean pest insect).

DISCUSSION AND CONCLUSION

Based on the results showed previously, it was concluded that the semi--solid fermentation process could be used for *Bacillus thuringiensis* spores production.

The fermented malt from beer industry and the solid residue from pulp and paper industry could both be used as a complete medium for growth and sporulation of Bt. Furthermore, the supllementation of these media with a cheap mineral salt source could improve the production of spores.

The semi-solid fermentation process showed some advantages over the conventional stirred or aerated liquid fermentations, and they can be described mainly because:
the medium is relatively simple since only meal plus water is needed. Cheap mineral salt may be added just to enrich it; the required space occupied by the fermentation equipment is relatively small if compared to the yield of the product; the conditions under which the microorganism grows are more like the conditions under which it grows in nature; aeration is easyly 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 fermentation become obvious when one works with it:
during the fermentation of small scale batches, no problem occurs, however with large amount of material the heat becomes a problem that must be controlled; monitoring devices to determine moisture, pH and product yield, become a necessity in this type of fermentation. These parameters can not be measured in the fermentation flask, and the removal of the samples represents a risk of contamination; the substrate treatment must be considered; in some cases it must be cracked lightly, but the formation of flour should be avoided.

Brazil is a big country, so as the semi-solid fermentation process demonstrated to be economically interesting for Bt endotoxins production,

it could be adopted in the future for small scale local production. Although promising results had been achieved, further studies will be required before final evaluation of the suggested approaches.

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