AGRO-INDUSTRIAL RESIDUES AS A MEDIUM FOR GROWTH AND SPORULATION OF BACILLUS THURINGIENSIS

I.O. MORAES
Faculty of Food Engineering
Universidade Estadual de Campinas

D.M.F.CAPALBO
Brazilian Enterprise for Agriculture Research
EMBRAPA / CNPDA

SUMMARY

Spore-forming bacilli having larvicidal activity against lepidopterous can be grown in liquid raw material. For this reason, agro-industrial residues were studied as culture medium for growing bacilli in submerged culture. Sugar cane molasses,fresh coconut water and corn steep liquor were utilized in this research. The best growing medium was attained with a combination of sugar cane molasses and corn steep liquor, rich in carbohydrates and nitrogen, respectively.

INTRODUCTION

One of the most important reasons for the current interest in entomo pathogens lies in the fact that they will be sufficiently specific not to affect beneficial insects. At present the most studied and commercialy produced is Bacillus thuringiensis.

Bacillus thuringiensis, a gram-positive, aerobic,sporogenic bacteria produces several toxins, two of then are considered the most important for agriculture: delta-endotoxin (or crystalline toxin or just crystal) and the spore. The spectrum activity of delta-endotoxin is limited to certain Lepidoptera , mosquitoes and blackflies. This toxin is atoxic for the insects until dissolved by alkali or alkaline solutions in their guts.

The mixture of the delta-endotoxin and spores is generally more active than the crystal or the spores alone. Experimental results indicate a sinergistic effect of the spores and the toxin,so the end result is an

infection higher than the simple summation of their effects. Thus, the commercial products are mixtures of the spores and delta-endotoxin.

The production of the insecticide based on spores and endotoxins, requires a proliferation of the initial organism, besides growing the cells; sporulation needs to occur in order to produce endotoxins. Bacillus thuringiensis is very effective when produced "in vitro", by a fermentation process. It requires carbon for byosinthesis, nitrogen, mineral elements and other growth factors (Dulmage, 1970; Dubois, 1968; Goldberg, 1980).

Sources of carbon, usually cited in literature are carbohydrates like starch, molasses, raw grain mashes or grain processing by-products. Nitrogen can be supplied by ammonium salts, amino acids and protein-rich materials like soybean meals, corn steep liquor, yeast extracts and other hydrolysates. Inorganic salts are essential for growth, sporulation and heat stability. The growth factors, like vitamins, are essential as components of enzyme systems of cells.

The economics of the production require the fermentation medium to be as cheap as possible, and more research is needed in this area. Salama et al (1983a, 1983b, 1984) investigated several agro-industrial by-products for their abilities to support toxin production. These products were described as low-price and universally available. They indicated fish meal, bovine blood and fodder yeast as the principal by-products for a high production of active toxins for Spodoptera littoralis and S. exigua, and other egyptian pests. Arcas (1984) proposed a medium based on malt sprouts producing a concentration of nearly $2x10^9$ spores.ml^{-1}, achiving high mortality against Galleria mellonella. Fernandez (1975) included coconut water at 0%, 50% and 100% to replace distilled water in nutrient broth and in other simple media, observing low levels of spore counts possibly due to a nutrient imbalance. Moraes (1976) studied the composition of the culture medium in the growth of B. thuringiensis, utilizing corn steep liquor, sugar cane molasses and urea. She concluded that the combination of 10g of sugar cane molasses and 30g of corn steep liquor was effective in producing spores and crystalls active against Ascia monuste orseis.

The present work was undertaken in order to study the formulation of media considering the influence of its components in the yields of spore-crystal and also to study a simple and cheaper medium for commercial production. Corn steep liquor, sugar cane molasses and coconut water were studied as a cheaper and convenient agro-industrial by-products in our region.

MATERIAL AND METHODS

Microorganism and inocula

The microorganism used was a strain of B.thuringiensis, serotype 1, isolated from commercial product Bactospeine (Rhône-Poulenc).

Inocula were prepared from a 48h agar nutritive slants cultures with no more than 72h in refrigerator (+4°C), after the incubation.

The medium was prepared according to Moraes(1976) in erlenmeyers mecanically agitated for 17 hours at 30°C.

Media

Corn steep liquor, sugar cane molasses and fresh filtered coconut water (liquids agro-industrial by-products) were investigated for their potential to support the production of spore-delta-endotoxin complexes (Table 1). No other nutrient was added to the media. Each one was used to give a final concentration of 8.3 g/l of total sugar, determined by the anthrone method (Scott et al, 1953).

Analytical methods

The changes in total carbohydrates concentration (expressed as % of glucose) were determined by the anthrone method described by Scott el al(1953).

The spore concentration was measured by counting chamber method in a Neubauer chamber.

Biomass content was determined by optical density at 600 nm.

Protein content was expressed as total nitrogen determined by the micro-Kjeldahl method.

Conditions of growth

The study was carried out in a mini-fermentor M-1000 (1 liter) from Fermentation Design Inc. All fermentations were operated with 500 rpm of agitation (magnetic stirrer), aeration of 1.6 vvm (air volume/medium volume/minute), temperature controlled at 30°C ± 2°C. The initial pH of the media was adjusted to 7.0, before autoclaving. The pH of the fermentation liquor was not controlled during the fermentation,ended after 24 hours.

RESULTS

The composition of the agro-industrial by-products tested is given in Table 1.

By-product	Total sugar concentration (% glucose)	Total nitrogen concentration (% N)
filtered coconut water	4.5	0.02
sugar cane molasses	67	0.90
corn steep liquor	5.2	3.75

TABLE 1 . Composition of the agro-industrial by-products tested.

The kinectis of glucose utilization and pH variation in liquid fermentation of B. thuringiensis are important parameters to stablish the bacillus growth and sporulation. Sporulation occurs when the glucose of the medium is nearly exausted and the pH returns to neutrality (Figure 1) .

The results obtained with the three by-products in test, as a culture medium with or without combination are presented in Figures 2,3 and 4.

Results showed a tendency of pH to decrease in the first 8 hours of fermentation and then return to neutrality, indicating the occurence of sporulation, for all media tested. This effect was greater with the combination of media than when they were used alone. The same tendency occured with sugar variation.

The optical density, an indicator of biomass concentration, varied much more, indicating a high cell growth rate and low sporulation, during the same period.

When protein (corn steep liquor , Table 1), was added to the medium containing carbohydrates (sugar cane molasses, Table 1), the rate of comsumption of glucose and optical density increased. Also, the spore concentration increased, reaching the desired level (10^9 or more spores/ml)(Fig. 4).

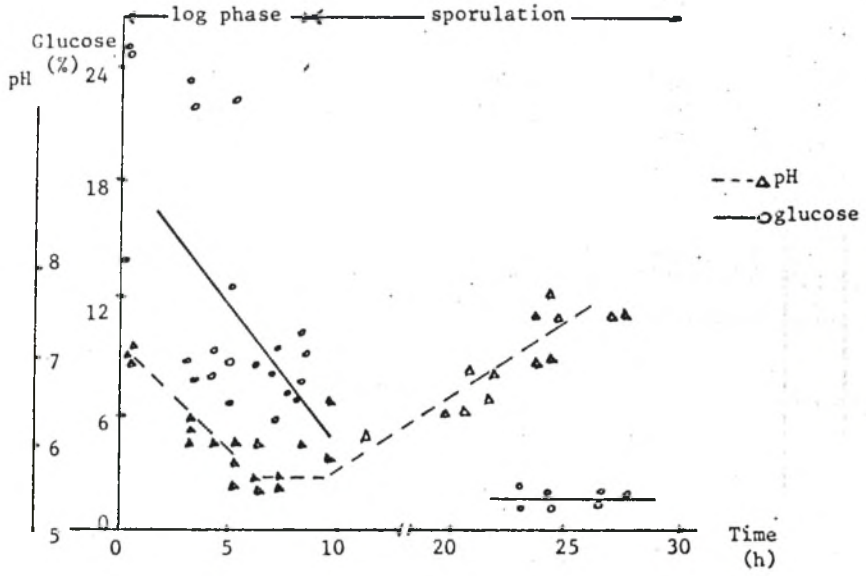

FIGURE 1 . Kinetics of glucose utilization and pH variation in batch fermentation of B. thuringiensis (Moraes et al. 1984)

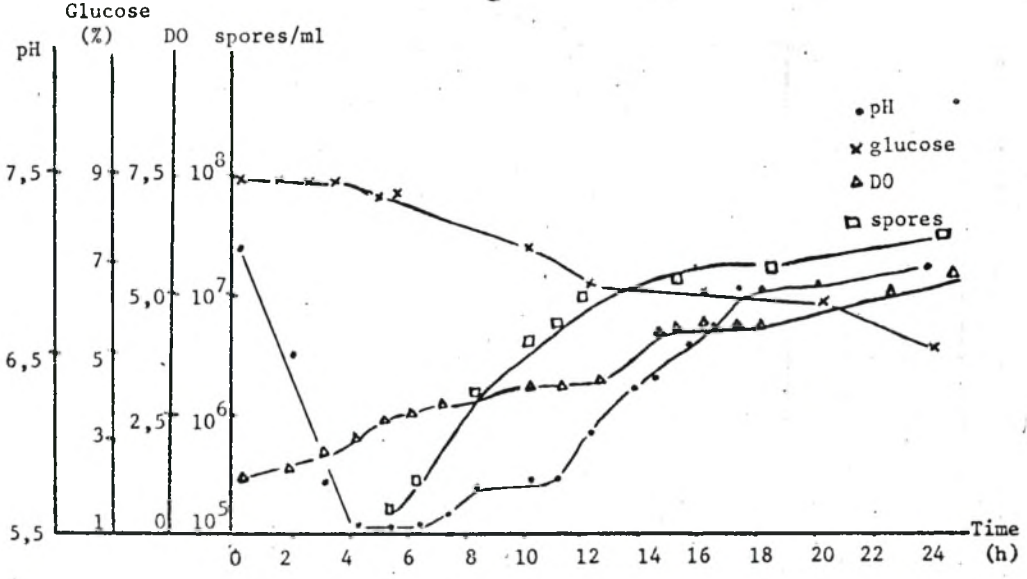

FIGURE 2 . Analysis of the pH, optical density(DO), glucose concentration and spores formed in the batch fermentation of fresh coconut water by B. thuringiensis.

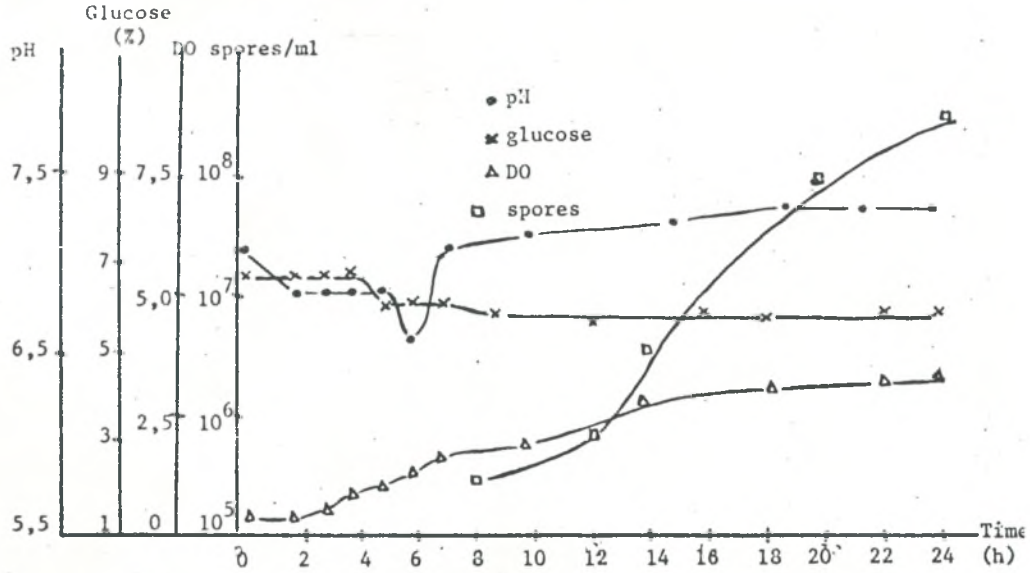

FIGURE 3 . Analysis of the pH, optical density (DO), glucose concentration and spores formed in the batch fermentation of sugar cane molasses by B. thuringiensis.

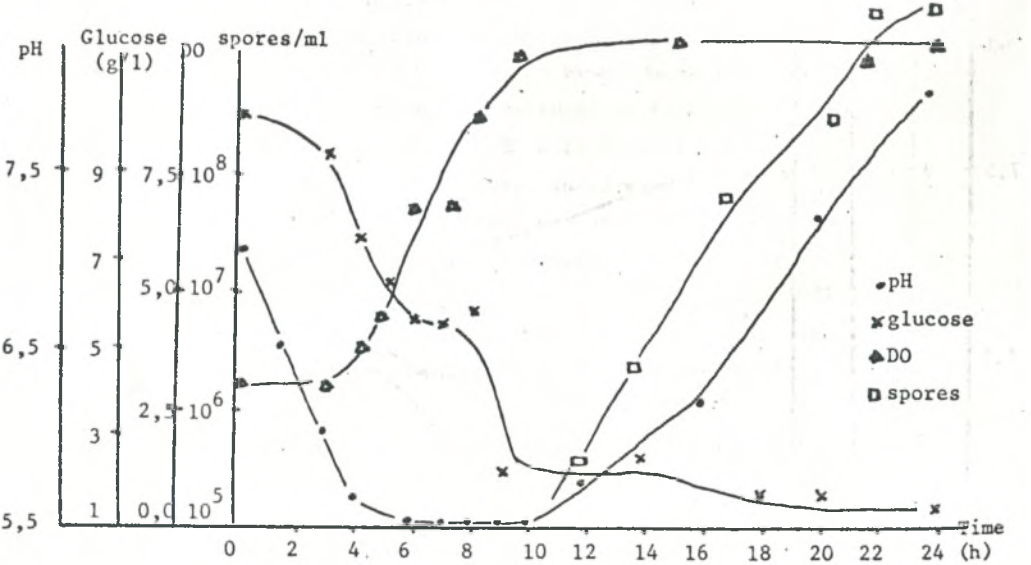

FIGURE 4 . Analysis of the pH,optical density (DO),glucose concentration and spores formed in the batch fermentation of sugar cane molasses (15 g/l) and corn steep liquor (60 g/l) by B. thuringiensis .

DISCUSSION

Although only three liquid agro-industrial by-products were tested, we observed that the combination of carbohydrate and nitrogen sources is necessary for a rapid and good yield in spores.

The combination of different levels of by-products, and the utilization of other available agro-industrial residues, are the steps to be studied in the future.

The utilization of waste coconut water, dispensed from coconut factories ,remains a good candidate for cultivation medium, despite the results obtained. It could be used not as a unique component but replacing the fresh water requirement as solvent for the ingredients of the medium. Also it would be a source of nutrients and reduce waterway pollution.

Brazil is a large country that possesses innumbered regionaly agro-industrial by-products, wich represent a potential source of nutrient for B. thurigiensis growth medium. The solid, semisolid and liquid by-products have to be examined for the fermentation process,searching for a cheaper medium and so, cheaper final products.

BIBLIOGRAPHY

ARCAS,J.; YANTORNO,O.; ARRARÁS,E. and ERTOLA,R. (1984) . A new medium for growth and delta-endotoxin production by Bacillus thuringiensis var. kurstaki . Biotechn.Let. 6 (8):495.

DUBOIS,N.R. (1968) Laboratory batch production of B.thuringiensis spores and crystals. Appl. Microbiol. 16 (7):1098.

DULMAGE,H.T. (1971). Production of delta-endotoxin by eighteen isolates of Bacillus thuringiensis serotype 3, in three fermentation media. J.Inv.Pathol.18: 353.

FERNANDEZ,W.L.;OCAMPO,T.A. and PEREZ,D.C. (1975). Coconut water in three media reduces cell yield of Bacillus thuringiensis var. thuringiensis Berliner. The Phillippine Agriculturist 58 (7/8): 273.

GOLDBERG,I.;SNEH,B.;BATTAT,E. and KLEIN,D. (1980). Optimization of a medium for a high yield production of spore-crystal preparation of Bacillus thuringiensis effective against the egyptian cotton leaf worm Spodoptera littoralis Boisd. Biotechn. Let. 2 (10): 419.

MORAES,I.O. (1973). Obtenção de inseticidas bacterianos por fermentação submersa. MSc. Thesis. State University of Campinas, UNICAMP,Brazil.

_____ (1976). Ensaios de fermentação submersa para produção de um inseticida bacteriano em mini-fermentador. PhD. Thesis. State University of Campinas,UNICAMP. Brazil

_____ and Capalbo,D.M.F. (1984). Study of continuous fermentation for obtaining bacterial insecticide to control agricultural pest. Proceedings of the IV Japan-Brazil Symposium on Science and Technology. Vol. II, p. 248-255.

SALAMA,H.S.; FODA,M.S.; DULMAGE,H.T. and EL-SHARABY,A. (1983a). Novel fermentation media for production of delta-endotoxins from Bacillus thuringiensis. J.Inv. Pathol. 41:8.

SALAMA,H.S.; FODA,M.S.; SELIM,M.H. and EL-SHARABY,A. (1983b). Utilization of fodder yeast and agro-industrial by-products in production of spores and biologically active endotoxins from Bacillus thuringiensis. Zbl. Mikrobiol.138: 553.

SALAMA,H.S. (1984).Bacillus thuringiensis Berliner and its role as a biological control agent in Egypt. Z.ang.Ent. 98: 206.

SCOTT,T.A. and MELVIN,E.H. (1953). Anal. Chem.:1656.