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THE DEVELOPMENT OF *BACILLUS THURINGIENSIS* AND *BACILLUS SPHAERICUS* AS BIOCONTROL AGENTS: FROM RESEARCH TO INDUSTRIAL PRODUCTION

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For many years the control of vectors of human diseases such as mosquitoes and black flies was achieved by the extensive use of highly toxic chemical larvicides. As a result of the insect resistance developed against chemical pesticides used as well as the negative environmental impact of these materials, there is an increase in the search for safer and more efficient alternatives. In the past 15 years emphasis has been placed on the development of biological agents as possible alternatives to chemicals for the control of vectors of human diseases.

Among the most promising biological control agents for mosquito and black fly are *Bacillus thuringiensis* var. *israeliensis* (Bti) (Goldberg & Margalit, 1977; De Barjac, 1978; Undeen & Berl, 1979) and several strains of *Bacillus sphaericus* (Bs) (Singer, 1980; Davidson et al., 1984; Davidson, 1985).

Various formulations of Bti have already been commercialized, but still a number of problems have restricted its use to very specific habitats. One of the main limitations of Bti is its short residual activity especially in polluted water and lack of persistence in the environment (Davidson et al., 1981). Alternatively, Bs-based products have become important in mosquito control of standing and highly polluted water reservoirs. Bs shows high level of activity against different species of *Culex* and experimental formulations were shown to have long-term activity in polluted habitats which appears to be the result of spore persistence or alternatively its capability to recycle in the environment (Nicolas et al., 1987). Due to these intrinsic properties, Bs can certainly complement Bti in the control of *Culex* and *Anopheles* species of mosquitoes over a wide range of habitats.

For commercial purposes it seems that both Bti and Bs formulations will be needed for the comprehensive control of insect vectors of diseases. Eventually the ideal product for the control of these vectors might be a genetically engineered microorganism containing the characteristics of both Bti and Bs.

With the objective of developing commercial Bti and Bs-based products we have concentrated our efforts on establishing cost efficient fermentation processes and formulations for the industrial production of Bti and Bs-strains. We have also developed a recombinant Bs which express the Bti toxin gene products.

Fermentation process — The results described are for Bs-strain 2362. A study of fermentation conditions of Bs strain 2362 has recently been published (Yousten & Wallis, 1987). In comparison with other Bs strains, this strain produces higher quantities of toxin, sporulates at higher temperatures and is not inhibited by protein enriched media. Still there is no enough information concerning the industrial production of Bs in general and of strain 2362 in particular. Some general rules though seem to be common to the growth of several Bs strains: a) these bacilli do not utilize sugars as carbon source and b) amino acids are the best source of both nitrogen and carbon while pyruvate, lactate, acetate or some Krebs cycle intermediates may serve as carbon source supplements (Lacey, 1984; Singer et al., 1966; de Barjac, 1980).

For the purpose of industrial production we have grown Bs 2362 on media supplemented with proteinaceous digests in the presence of various carbon sources. Industrial proteins for the preparation of media (designated P1-P7) were collected from various producers in Israel and abroad. Most of these

proteins were waste products of industry which have been subjected to autolysis or treated with proteases. Their protein content varied between 50 and 70% of dry weight. The rest was made up of carbohydrates and inorganic material. All media contained about 5 g/l of an industrial protein in 50 mM phosphate buffer, (pH = 7.0). Following fermentation the insoluble fraction was recovered by centrifugation, washed once with distilled water and freeze-dried.

The industrial proteinaceous digest solutions had to be supplemented with 2 g/l yeast extract as a source of vitamines and other growth factors in order to achieve optimal fermentation (Lacey, 1984; Singer et al., 1966).

The best results were obtained in protein media designated P5 and P7 while the lowest was the one designated as P4 (Table I). In general, media containing the most digested proteins (average peptide chain length of 3.4 to 5.7) resulted in higher toxin production than undigested protein media (average peptide chain length between 35.4 to 55.6).

The basal amino acid medium can be supplemented with alternative carbon and nitrogen sources. We have observed that in our media the addition of urea, uric acid or citric acid had no effect either on the toxin or biomass yields. These compounds were probably not taken up by the strain 2362. On the other hand, acetate and lactate induced a 2 to 3 fold increase in biomass production but did not increase the toxicity of the preparation. The only improvement in toxin production was observed with the addition of glycerol (5 g/l), reaching a 10 fold increase in medium P3 (Table I). Consumption of glycerol was well correlated with increase in larvicide yield.

Amino acid consumption averaged at about 60% in all media. However, the consumption seems to be selective since several amino acids like L-proline were depleted from the media more than average, while L-arginine was almost not utilized and L-histidine accumulated in the broth above its initial concentration. Average figures on utilization of amino acids in the media were similar with or without glycerol, however they were depleted to a larger extent in glycerol enriched media.

Further improvement of toxin yield can be obtained by supplementing the media with corn steep liquor (CSL), a waste-product in the production of maize starch. It contains a variety of biologically active compounds and in our experiments has a syngergistic effect with glycerol. The fermentation process developed for Bs-2362 was later scaled up to 250 l pilot fermentation with a toxin yield of 25,000 IU/ml. Most importantly, we have observed that similar proteinaceous media can also be used for the efficient production of Bti in pilot-scale.

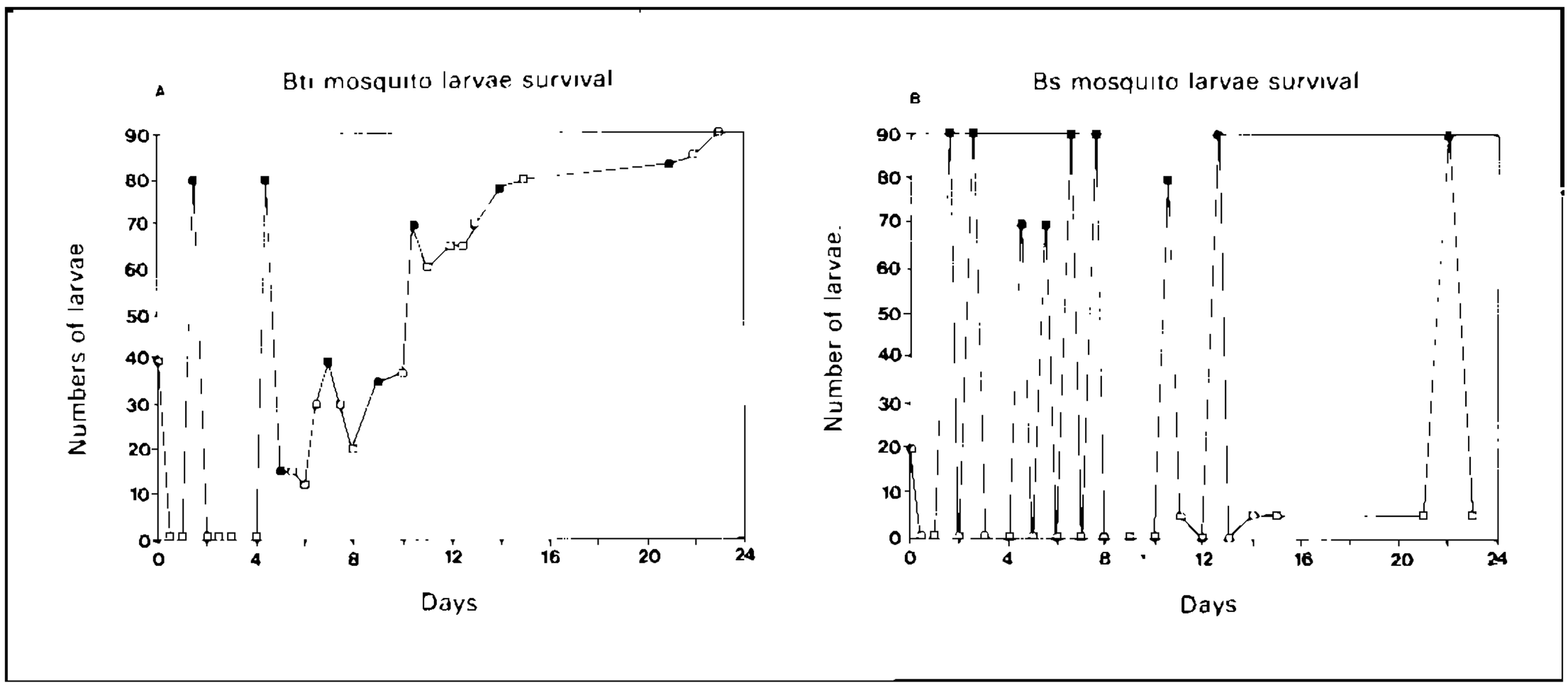
TABLE I

Production of *Bacillus sphaericus* toxin on protein media supplemented with minerals and carbon sources

Medium	Toxin yield, IU x 10^{-3} per ml broth				
	NA	Min	Glycerol, 5 g/l	Acetic acid, 5 g/l	D,L-lactic acid, 5 g/l
P1	0.73	0.94	4.34	0.94	1.30
P2	0.84	ND	1.60	ND	ND
P3	0.35	0.36	3.96	0.36	0.50
P4	0.09	ND	0.36	ND	ND
P5	1.65	0.95	1.40	2.61	ND
P6	0.52	0.40	0.87	0.07	2.50
P7	1.50	ND	2.90	0.60	1.70

NA – No additions.
Min – Mineral mixture at final concentrations:
$MgSO_4$ 12 mM; $CaCl_2$ 0.15 mM; $FeSO_4$ 0.1 mM; $MnSO_4$ 2.5 μM; $ZnSO_4$ 15 μM; $CoCl_2$ 20 μM; Na_2MoO_4; $CuCl_2$ and H_3BO_3 8 μM.

The toxicity of the powder was determined according to the standard method as recommended by Dr de Barjac (Pasteur Institute and WHO). Toxicity was expressed as International Units (IU) calculated by comparing LC_{50} values of the samples to the LC_{50} value of a standard Pasteur Institute powder (RB-80, 1000 IU/mg).



Survival of mosquito larvae *(Culex pipiens)* under simulation of water ponds (i. e. 80l-barrels in the open-air).
A. Pretreatment with liquid formulation of Bti
B. Pretreatment with liquid formulation of Bs-2362.
Rates are equivalent to 0.5 liter/acre. Filled squares (■) indicate the time and number of larvae added into the barrels; age of larvae varied in the range of first to third instar.

Using the material obtained from the pilot fermentation, liquid formulations of Bti and Bs were prepared with specific preservatives and other additives commonly used in the food industry. The activity of the products in the presence of such additives has been preserved at 4 °C for one and a half years.

Simulated field studies – The liquid formulations of Bti and Bs were tested by simulated field trials. The experimental systems were such that reflected environmental conditions of water reservoirs and sewage conditions. Tests were conducted in 80L-metal barrels filled up to two thirds with tap water. Two kg of sandy loam and 200 gr of poultry manur was added to each barrel. Vegetation of water ponds was represented by the addition of determined quantities of weeds. The barrels were positioned in the open environment and exposed to all the changes occurring in the natural habitat. A fixed number of *Culex pipiens* larvae was added to barrels containing either Bti or Bs formulations while non-treated barrels were used as control. Fresh larvae were added at different times and mortality was monitored daily. The activity of Bti persisted for about 6 days while Bs was still active after 23 days, which was the limit of reliable monitoring in the simulation system (Figure 1). From laboratory experiments we observed that the rapid sinking of crystals as well as photoinactivation were among the main reasons for the decay in Bti activity.

In trying to obtain a more persistent formulation in the field we addressed ourselves firstly to the question of photoinactivation of the microbial preparations using Bti as a model system.

Exposure of Bti (liquid formulations) to sunlight for 4 days under laboratory conditions resulted in over 90% inactivation of the active material (Table II). This inactivation could be prevented when the preparations were stored in the dark. A number of sun-screens were tested for their potential to prevent photoinactivation of the toxin. The best results were obtained using charcoal in the presence of adjuvants. Under these conditions most of the toxin remains active even after four days of exposure to sunlight. It proves that the inactivation observed by sun exposure is due to photoinactivation and not temperature or other factors that can destroy the toxin activity.

We have previously observed that the presence of floating agents and adjuvants such as alginates prevented the fast sinking of the toxin crystals. The relative decrease in activity of the toxin in the presence of sun-screens but in the absence of floating agents is probably due to sinking of crystal toxin rather than to photoinactivation (Table II).

Cloning of Bti toxin gene into Bs – Product improvement by genetic manipulations have

TABLE II

Protection of Bti from photoinactivation

	LC_{50} (x 10^3)					
Sun-screen (units)	0		400		1600	
Adjuvant	–	+	–	+	–	+
Sun-exposure (4 days)	> 50	> 50	> 20	3	~ 10	1.5
Dark	3	3	2	1.5	2	2

Bti suspensions were mixed with the sun-screens and an adjuvant as indicated. The resulting formulations were diluted with water to 0.01 mg/ml (of the original Bti powder) and placed in polystyrene bottles.

To avoid local heating, the bottles were dipped in water in a shallow glass tray and placed on a table exposed to the sun. Similar bottles, containing the formulations, covered with aluminium foil, and dipped in the same water-bath served as "dark" control. Following four days of sun exposure, toxicity of the tested suspensions were determined by the standard bioassay.

TABLE III

Characterization of representative clones (of approximately 100 kanamycin-resistant transformants of *Bacillus sphaericus* 2362)

Strain	Kanamycin[a] 5 µg/ml	Immunological response[b]			LC_{50} µg/ml[c] (48 hrs) Aedes	Sporulation time (hrs)
		Bti-proteins		*B. sph. prot.*		
		28 kDa	65 kDa	110 kDa		
Bs 2362	–	–	–	+++	1.9	24
Clone 62	–	+++	+	+++	0.19	48
	+	++	+	+++	0.30	> 48
Clone 5	–	+	–	+++	0.57	> 48
	+	++	–	+++	1.16	> 48
Clone 13	–	++	–	+++	0.21	> 48
	+	++	–	+++	0.53	> 48
Clone 96	–	–	++	+++	1.7	24
	+	–	+	+++	2.1	24

a Initial stages of culture, up to the onset of sporulation, were always in the presence of kanamycin.
b Purification of proteins was carried out by gel filtration and antibodies were purified from sera of immunized rabbits.
c Bioassay with larvae of *Aedes aegyptii* was performed in comparison to the standard RB-80 of Bs (according to recommendations of Dr. de Barjac and WHO).

already proven to be useful for development of new biopesticides (Vaeck et al., 1987). There is no doubt that this technique can offer highly sophisticated solutions for the control of vectors of diseases.

As mentioned above, Bs has a narrower spectrum of activity against mosquito species while it is much more persistent than Bti in the field. Bs is toxic to *Culex* and *Anopheles* species of mosquitoes but is almost non active against *Aedes* species, Bti on the other hand is toxic to all three mosquito species and also to black flies, but it is much less persistent in polluted water. A recombinant microorganism containing the persistent characteristics of Bs and the spectrum of activity of Bti might therefore offer the ideal product for the biological control of mosquitoes and black flies.

In our experiments, fragments of the 112 kilobase plasmid containing the gene encoding the Bti toxin (Carlton & Gonzalez, 1985) was introduced into the *Bacillus subtilis* plasmid pPL603E (Mongkolsuk et al., 1984). This new plasmid construct was used for the transformation of Bs 2362.

Plasmid pPL603E codes for resistance to kanamycin and chloramphenicol, insertion of

Bti fragments resulted in inactivation of the gene coding for chloramphenicol resistance. Bs recombinants were selected by their resistance to kanamycin and loss of resistance to chloramphenicol. The new kan resistant clones were analysed by bioassays in which the toxicity against *Culex pipiens* and *Aedes egyptii* larvae was tested. In this system, only Bti and recombinant Bs are expected to control both types of larvae. Recombinant clones were significantly more active against *Aedes* larvae than the parental Bs 2362 (Table III). In the same system, Bti, Bs and the recombinant strains were equally active against *Culex* species (data not shown).

Based on the results described, we believe that Bti and Bs, as well as recombinant microorganisms will become useful biolarvicides which can be effectively implemented into mosquito control programs. Such liquid formulations of microbial pesticide could efficiently be used as replacements for toxic chemicals presently used.

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