Israel Journal of Entomology Vol. XXXH (1998) pp. 37-44

PRODUCTION OF 5-ENDOTOXIN BY *BACILLUS THURINGIENSIS* SUBSP. *ISRAELENSIS* H-14 BASED ON AGRO-INDUSTRIAL BYPRODUCTS IN NORTHEASTERN MEXICO

MARIA GUADALUPE MALDONADO-BLANCO, LUIS JESUS GALAN-WONG, KATIUSHKA AREVALO-NINO, HIRAM MEDRANO-ROLDAN, REYES TAMEZ-GUERRA AND CRISTINA RODRfGUEZ-PADILLA *Departamento de Microbiologia e Inmunologia, Facultad de Ciencias Biologicas (FCB), UniversidcidAutonoma de Nuevo Leon (UANL), A.P. 414, C.P. 66450, Monterrey, N.L., Mexico*

ABSTRACT

Five media were designed using agroindustrial byproducts with locally available ingredients, suitable for the production of *B. thuringiensis* subsp. *israelensis,* a mosquito larvicide. The final whole cultures of seven bacterial strains propagated on these media were evaluated against *Aedes aegypti* larvae. The highest mortalities were recorded with cultures of strain 225, propagated on a medium containing corn steep liquor, soup paste powder, and molasses, which was formulated in six different combinations; the combinations differed in concentrations of the latter two constituents for the purpose of increasing yields and insecticidal activity. The most toxic extract was obtained with the combination medium containing 1% molasses, 3% soup paste powder, and 1 % corn steep liquor, which yielded a mean of 13.05 g of extract/L, a LC_{50} of 0.0317 mg/L and potency of 4,400 ITU/mg. This extract, formulated later with inert ingredients such as talc and diatomaceous earth, resulted in potencies of 105 and 73 ITU/mg, respectively. In comparison with four commercial bioinsecticides it was more potent than Vectobac®, and less potent than Bactimos®, Teknar® and ABG®-6168.

KEY WORDS: *Bacillus thuringiensis* subsp. *israelensis,* bioinsecticide production, *Aedes aegypti,* fermentation media.

INTRODUCTION

Mosquitoes are common insects in tropical and subtropical regions of the world, in which species of the genera *Aedes, Culex* and *Anopheles* act as vectors of important diseases such as yellow fever, dengue, lymphatic human filariasis and malaria (Faust et al., **1974).** Biological control emerges as an alternative strategy, because of the damages to the environment and nontarget organisms caused by the abuse of chemical insecticides, in addition to the resistance that insects have developed towards them. Two species of entomopathogenic bacteria, *Bacillus thuringiensis* subsp. *israelensis* **H-14** *(Bti)* and *Bacillus sphaericus,* which produce toxic crystals during sporulation (Goldberg and Margalit, **1977;** De Barjac, **1978;** Singer **1980),** are employed successfully in the control of larvae of mosquitoes and black flies **(WHO, 1982).** In the production of bioinsecticides by these strains, interest has developed in the use of the most economic substrates, such as molasses, soya flour, cotton seed flour, and dried and ground leguminous seeds (Smith, 1982; Vandekar and Dulmage, 1983; Obeta and Okafor, 1984),

leguminous seeds (Smith, 1982; Vandekar and Dulmage, 1983; Obeta and Okafor, 1984), to try to increase the insecticidal activity. In Mexico, the use of microbial insecticides is limited by high import costs; furthermore, little information is available relating to the biotechnology of production of this bacterium. The objective of the present work was the design of economic media for the production of the δ-endotoxin of *Bacillus thuringiensis* subsp. *israelensis*, using different agroindustrial byproducts available in Northeastern Mexico; and, furthermore, the evaluation of the insecticidal extracts obtained and their formulations, with some inert components, against *Aedes aegypti*, as well as of several commercial bioinsecticides.

MATERIAL AND METHODS

Bacterial strains

Seven strains of *Bacillus thuringiensis* subsp. *israelensis* H-14 were used, namely strains 1, 7, 196, 197, 210, 223 and 225, obtained from the International Entomopathogen Bacilli Collection of the Department of Microbiology and Inmunology of the FCB-UANL. Subcultures on nutrient agar slants were incubated at 30°C for 72 h and stored at 4°C.

Bioinsecticide production media

Five different media were formulated, according to Vandekar and Dulmage (1983) and Sikdar et al. (1991), constituted by a basal medium containing, in g/L: $MgSO_4.7H_20$, 0.3; $CaCO_3$, 1.0; $MnSO_4.H_2O$, $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$, 0.02 each; corn steep liquor, 10; cane molasses, 20. The following byproducts were added, in g/L, for differentiating each one of the five media: (A) Harinolina, (Purina, S.A.), a waste product of cotton seed, 20; (B) soybean \paste (Purina, S.A.), 20; (C) fish meal (Purina, S.A.), 10; (D) soup paste powder (Gamesa, S.A.), waste of fabrication of soup paste, 70; and, (E) corn gluten, (Almidones Mexicanos, S.A.), 20. Another medium was included, (F), which contained, in g/L, molasses, 20; corn steep liquor, 10; soya flour, 20; $CaCO_3$, 1.0; it has been employed in fermentation of other varieties of *B. thuringiensis*, active against Lepidoptera (Galán-Wong et al., 1994). The pH of the media was adjusted to 6.8–7.0 with 1 N NaOH. After that 10 mL were dispensed individually into 50-mL Erlenmeyer flasks, and sterilized at 121°C for 15 min.

Propagation of the strains

The strains were grown on nutrient agar slants at 30°C for 24 h, and a loopful of slant culture was used to inoculate the seed cultures, for the six media, respectively. The flasks were placed on an orbital shaker (New Brunswick Scientific Co.) at 200 rpm and incubated at 30°C (Abdel-Hameed et al., 1991) for 12 h. From these seed cultures, 2% v/v was taken to inoculate similar flasks containing the six media, which were incubated as above, for 72–96 h, until 90–100% sporulation, detected through light microscopy, was obtained. The flasks were kept frozen until the bioassay test.

Tests with final whole cultures

These were made with third-instar larvae of *Aedes aegypti* (Linnaeus), native of Northeastern Mexico, according to the method recommended by H. De Barjac, from the Institute Pasteur, Paris, France (personal communication). Twenty-five mosquito larvae were placed in 250-mL plastic cups, containing 150 mL of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions from final whole cultures.

Triplicates were run per dilution, and four cups with 150 mL distilled water, were used as control; both tests and controls were maintained at 25 ± 2°C and 70–80% relative humity, during 24 h.

The most toxic culture was selected, according to the mortality data, by analysis of variance (ANOVA), and least significant differences (LSD) (p = 0.05) were computed to compare means.

Optimization of the fermentation media

In order to increase the production and activity of the insecticidal extract, six different combinations of the selected medium were prepared, changing the molasses concentration (10 and 20 g/L) and that of the soup paste powder, (30, 50 and 70 g/L), while the remaining ingredients stayed unchanged, as in the original medium. Each one of six combinations was prepared in triplicate, in volumes of 100 mL in 500-mL Erlenmeyer flasks. Inoculum of 2% v/v of a 12-h-old culture of *Bti* 225 grown in broth-triptose-phosphate (Difco), was used to inoculate the six combinations of the fermentation medium, which were incubated at the conditions already mentioned (Abdel-Hameed et al., 1991). During the fermentation the values of the bacterial population were determined through total viable count, made in plates with nutrient agar, in triplicate and expressed in CFU/mL of beer. The pH and the microscopic observation of spores and crystals determined the end of the fermentation. The recovery of the spore-crystal complex was done according to Dulmage et al. (1970).

Activity of the insecticidal extract

The toxicity of the obtained extracts was evaluated against third-instar larvae of *Aedes aegypti*, according to the methodology of Hall and Arakawa (1980), starting from a stock solution of 50 mg/L; 8–10 concentrations of the spore-crystal complex were used in a range of 0.005 to 10 mg/L, with 3 repetitions of 25 larvae per concentration and four for the control, the toxicity being compared with the International Standard IPS-82 obtained from the Institute Pasteur of Paris, France. The mortality data presented at the end of the 24-h exposure were processed through a Probit Analysis program (Finney, 1971). Each bioassay was repeated 3 times. On base of the mean lethal concentration of the standard and of the ready extracts, the unknown titre of the latter was determined by the formula:

$$\frac{15{,}000 \times LC_{50}\ \text{IPS-82 standard}}{LC_{50}\ \text{of the extract}} = \text{Unknown titre (International Toxic Units/mg)}$$

Formulation of the insecticidal extracts obtained

These were formulated at 10% w/w active ingredient (insecticidal extract); furthermore, the following inert ingredients were added: 1) talc, and 2) diatomaceous earth (Sigma Chem. Co.) and also, as coadjuvant, 1% Triton X-114 (Angus and Lüthy, 1971; Rodríguez-Tovar, 1994).

Potency of formulations and commercial bioinsecticides

The commercial bioinsecticides evaluated were Teknar® (Sandoz), Vectobac® G, ABG® 6168 (Abbott) and Bactimos® (Biochem) and the two formulations prepared with the active

ingredient from *Bti* 225, bioassayed at laboratory level. The method of Mulla et al. (1982) was employed: from a 1% stock solution, seven serial dilutions were prepared, of which 150 mL were placed in plastic cups with 25 larvae/cup, with three repetitions for each concentration and four for the control. All formulations were assayed against the same insect and under the same conditions as in the previous bioassays.

RESULTS AND DISCUSSION

The greatest dilution of the final whole culture to which there was response was 10^{-6}, as shown by the mortality used to make the differentiation. In Fig. 1 the mortality results of *Aedes aegypti* larvae with 42 final whole cultures of *Bti* are shown. Variable percentages of mortality were noted; the highest was registered with the cultures propagated in medium D (soup paste powder), up to 42%, while media C (fish meal) and F (soya flour) brought about mortalities of 20% and below; in media A (waste of cotton seed), B (soybean paste) and E (corn gluten) low percentages of mortality were obtained. Such differences in the larvicidal activities should be due to the composition of the medium, the strain and the fermentation conditions (Dulmage, 1971; Smith, 1982); Abdel-Hameed et al. (1991) and Pearson and Ward (1988) obtained highly potent extracts in media with molasses (10 g/L) and soybean flour (15–30 g/L), while other investigators such as Obeta and Okafor (1984) obtained effective products in media with blood meal (10 g/L) in addition to seeds of leguminosae (7.5 g/L).

ANOVA for two factors (strain and medium), with a 0% error, established significant

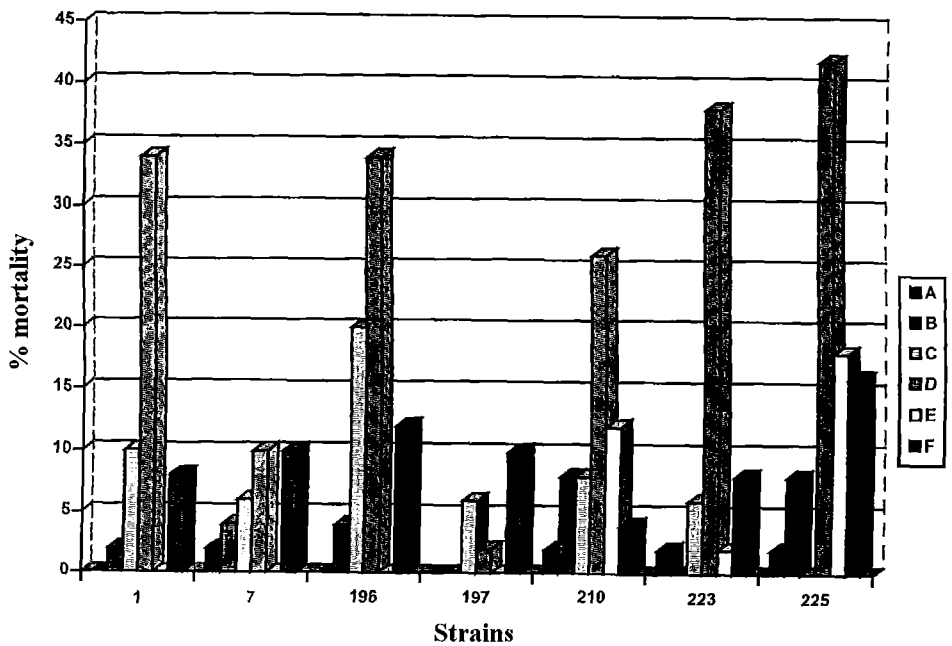

Fig. 1. Percentage mortality of *Aedes aegypti* larvae brought about by 10^{-6} final whole cultures of *Bti* propagated in six media.

TABLE 1
Comparison of mean mortality of *Aedes aegypti* larvae by *Bti* final whole cultures at 10^{-6} dilution — the influence of the factors strain and medium

Strain		Medium		
Bti strain	Mean no. of larvae/25 killed by each *Bti* strain grown in the six media*	Variable ingredient	Medium	Mean no. of larvae/25 killed by seven *Bti* strains grown in each medium**
225	3.6667 a	Soup paste powder	D	6.8095 a
196	2.8889 b	Soybean flour	F	2.4762 b
210	2.5 b	Fish meal	C	2.0476 b
223	2.4444 b	Corn gluten	E	1.1905 c
1	2.3333 b	Soybean paste	B	0.9524 c
7	1.4444 c	Waste of cotton seed	A	0.3333 d
197	0.8883 c			

Means from 18 values* (left); and from 21 values** (right). Numbers within a column not followed by the same letter are significantly different, ANOVA, LSD ($p \leq 0.05$).

differences of toxicity between the strains and the media; in Table 1 the results of the comparison of the mean mortality are shown. The factor strain gave origin to three groups. In the first, strain 225 was the most effective, the second group consisted of strains 196, 210, 223 and 1, and the third consisted of strains 7 and 197. In comparison of the factor medium, four groups resulted, with medium D being significantly superior to the other five media.

In the stage of optimization of the fermentation medium, accomplished with the strain *Bti* 225 indigenous from Kenya and propagated in six combinations from the selected medium D, similar fermentation patterns were obtained, with bacterial growth levels of about 10^9 cells/mL of beer. These values are comparable to those obtained by others investigators (Abdel-Hameed et al., 1991; Obeta and Okafor, 1984; Pearson and Ward, 1988), where sporulation percentages of 80–90% were reached during 72–96 h fermentation; the production values of the extracts recovered at the end of the fermentation varied widely, from 8.6 to 42.5 g/L, with the result that when a greater weight of extract was obtained, the presented activity was smaller. This must have been due to the insoluble solids of residual substrate, that remained at the end of the fermentation and that would precipitate together with the extract in the recovery process of the bioinsecticide, such as indicated by some authors (Vandekar and Dulmage, 1983; Abdel-Hameed et al., 1991) and thus interfere with the activity of the extract.

In Table 2 the mean lethal concentrations from the insecticidal extracts obtained in the six combinations of medium D are shown, within a range of 0.0317 to 5.55 mg/L. Also, the potencies obtained against *Aedes aegypti* larvae are presented. It was observed that the most potent extract was obtained with combination 1, constituted of 1% molasses and 3% soup paste powder. The standard IPS-82 showed a potency 3.4 times higher than our most active extract.

However, the insecticidal activity presented by combination 1 (4,400 ITU/mg) was slightly higher than the one shown by an extract obtained as a recovered strain of the standard IPS-82 and produced locally in a medium with molasses and soybean flour (Rodríguez-Tovar et al., 1991).

TABLE 2
Comparative toxicity of six extracts of *Bti* 225 obtained with six combinations of medium D and with IPS-82 against *Aedes aegypti* larvae

Combination*	Variable ingredients** (%)	LC_{50} (mg/L)	ITU/mg
1	1M 3P	0.0317	4,400
2	1M 5P	0.1046	1,333
3	1M 7P	5.55	25
4	2M 3P	0.0382	3,651
5	2M 5P	0.7581	184
6	2M 7P	2.9535	47
IPS-82	–	0.0093	15,000

*From medium D. **Amounts of M = molasses, P = soup paste powder.

The potencies obtained with the commercial bioinsecticides tested, as well as for our formulations prepared with talc and diatomaceous earth as inert ingredients, are shown in Table 3. The latter formulations showed low toxicity, less than expected, since Rodríguez-Tovar (1994), in a similar work, reported an activity of 346 ITU/mg for her formulation with talc, at the same proportion as ours; meanwhile, the most potent commercial products were Bactimos® and Teknar®. The possible explanation for the low toxicity of our formulations, could be loss of toxin before or during the formulation stage, due to factors such as high temperature and dampness combined with the hygroscopic nature of the extract (Guillet et al., 1979; Vandekar and Dulmage, 1983). We may conclude that it is possible to produce bioinsecticides using byproducts wich are very cheap and available in our locality and which could substitute for soybean flour, a product of relatively higher cost. However, the formulation stage must be studied more in depth in order to obtain satisfactory results.

TABLE 3
Comparative activities of commercial bioinsecticides and two formulations prepared with the active ingredient of *Bti* 225 against *Aedes aegypti* larvae

Bioinsecticide	LC_{50} (mg/L)	95% C.L.	Potency (ITU/mg)
Bactimos®	0.0771	0.0304–0.1954	1,809
Teknar®	0.0925	0.0503–0.1702	1,508
ABG®-6168	0.516	0.4630–0.5750	270
Formulation 1*	1.3213	0.9978–1.7497	105
Formulation 2**	1.9073	1.1882–3.0615	73
Vectobac®	7.6332	5.9795–9.7443	18

*With talc and **with diatomaceous earth as inert ingredients.

REFERENCES

Abdel-Hameed, A., Carlberg, G. and El-Tayeb, O.M. 1991. Studies on *Bacillus thuringiensis* H-14 strains isolated in Egypt. IV. Characterization of fermentation conditions for δ-endotoxin production. *World Journal of Microbiology and Biotechnology* 7:231–236.

Angus, T.A. and Lüthy, P. 1971. Formulation of microbial insecticides. In: Microbial Control of Insects and Mites. Edit H.D. Burges and N.W. Hussey. Academic Press, New York, NY. pp. 623–638.

De Barjac, H. 1978. Une nouvelle varieté de *Bacillus thuringiensis* tres toxique pour les moustiques: *Bacillus thuringiensis* var. *israelensis* serotype 14. *Comptes Rendus Hebdomadaires des Seances de l'Académie des Sciences (Paris)* Série D 286:797–800.

Dulmage, H.T. 1971. Production of δ-endotoxin by eighteen isolates of *Bacillus thuringiensis* serotype 3, in 3 fermentation media. *Journal of Invertebrate Pathology* 18:353–358.

Dulmage, H.T., Correa, J.A. and Martínez, A.J. 1970. Coprecipitation with lactose as a means of recovering the spore-crystal complex of *Bacillus thuringiensis*. *Journal of Invertebrate Pathology* 15:15–20.

Faust, E.C., Rusell, P.F. and Jung, R.C. 1974. Parasitología Clínica, Reimpresión 1979. Salvat Editores S.A., Mallorca, España.

Finney, D.J. 1971. Probit Analysis. 3rd Edition. Cambridge University Press, Cambridge, England.

Galán-Wong, L.J., Sandoval-Coronado, C.F., Arévalo-Niño K., Rodríguez-Padilla, C. and Luna-Olvera, H.A. 1994. Evaluación de cepas nativas y de colección de *Bacillus thuringiensis* serotipo H8 contra larvas de lepidópteros. *Southwestern Entomologist* 19:301–307.

Goldberg, L.J. and Margalit, J. 1977. A bacterial spore demostrating rapid larvicidal activity against *Anopheles sergenti, Uranotaenia unguiculata, Culex univittatus, Aedes aegypti* and *Culex pipiens*. *Mosquito News* 37:355–358.

Guillet, P., Dempah, J. and Coz, J. 1979. Evaluation de *Bacillus thuringiensis* serotype H-14 de Barjac pour la lutte contre les larves de *Simulium damnosum* s.l. III. Données préliminaires sur la sédimentation del'endotoxine dans l'eau et sur stabilité en zone tropicale. Rapport *ORSTOM* No. 7:1–10.

Hall, I. and Arakawa, K. 1980. Development of a standard bioassay method for determining potency of commercial *Bacillus thuringiensis* subsp. *israelensis*, University of California, Mosquito Control Research, Annual Report: 63-65.

Mulla, M.S., Federici, B.A. and Darwaseh, H.A. 1982. Field evaluation of the microbial insecticide *Bacillus thuringiensis* serotype H-14 against flood water mosquitoes. *Applied and Environmental Microbiology* 43:1288–1293.

Obeta, J.A.N. and Okafor, N. 1984. Medium for the production of primary powder of *Bacillus thuringiensis* subsp. *israelensis*. *Applied and Environmental Microbiology* 47:863–867.

Pearson, D. and Ward, O.P. 1988. Effect of culture conditions on growth and sporulation of *Bacillus thuringiensis* var. *israelensis* and development of media for production of the protein crystal endotoxin. *Biotechnology Letters* 10:451–456.

Rodríguez-Tovar, M.L. 1994. Diseño y evaluación de formulados de *Bacillus thuringiensis* var. *israelensis* en laboratorio y campo en *Culex pipiens quinquefasciatus* (Say) y *Aedes aegypti* (Linnaeus). Tesis de Maestría. Facultad de Ciencias Biológicas, UANL, Monterrey, N.L., Mexico.

Rodríguez-Tovar, M.L., Morales-Ramos, L.H., Torres, R., Quiroz, H. and Culebro, M. 1991. Evaluación en laboratorio y campo de un formulado de *Bacillus thuringiensis* var. *israelensis* en larvas de *Culex* sp. *Southwestern Entomologist* 16:277–281.

Sikdar, D.P., Majumdar, M.K. and Majumdar, S.K. 1991. Effect of minerals on the production of the delta endotoxin by *Bacillus thuringiensis* subsp. *israelensis*. *Biotechnology Letters* 13:511–514.

Singer, C. 1980. *Bacillus sphaericus* for the control of mosquitoes. *Biotechnology and Bioengineering* 22:1335–1355.

Smith, R.A. 1982. Effect of strain and medium variation on mosquito toxin production by *Bacillus thuringiensis* var. *israelensis*. *Canadian Journal of Microbiology* 28:1089–1092.

Vandekar, M. and Dulmage, H.T. 1983. Guidelines for production of *B. thuringiensis* H-14. UNDP/ World Bank/WHO. Special Programme for Research and Training in Tropical Diseases. Geneva, Switzerland. pp. 38–110.

WHO 1982. Information document produced by the WHO Division of Vector Biology and Control. First revision of the data sheet which appeared as WHO/VBC/79.750 and VBC/BCDS/79.01. Geneva, Switzerland. pp. 1–44.