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Review

Mosquitocidal toxins of spore forming bacteria: recent advancement

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Mosquito borne diseases form a major component of vector borne diseases from all over the world. Several control strategies have been adopted to control diseases transmitted by mosquitoes. The discovery of highly potential bacteriocides like *Bacillus sphaericus* (*Bs*) and *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) have revolutionized over conventional insecticides in mosquito control programs. The potential genes in *Bs* for mosquitocidal actions have been cloned and expressed recently. Some mosquito species (*Culex pipiens pipiens, C. quinquefasciatus*) which had been susceptible to *Bs* toxin in the field have developed resistance to *Bs*. But, this was not possible to *Bti*. The molecular mode of action and mechanism of resistance involved in developing resistance in vector species have been recently explored. The current review paper explores the novelty of these mosquito pathogenic bacteria for the control of disease transmitting mosquitoes.

Key words: *Bacillus sphaericus*, *B. thuringiensis* serovar. *israelensis*, mode of action, binding assays, resistance, management, cost-effective culture media.

INTRODUCTION

Mosquito-borne diseases form a major group of communicable diseases such as malaria, filariasis, dengue and Japanese encephalitis in India as well as other developing nations. Every year about 300 million people are estimated to be affected by malaria, a major killer disease, which further threatens 2,400 million (about 40%) of the world's population (Sharma, 1999). Similarly, lymphatic filariasis caused by *Wuchereria bancrofti* which affects about 106 million people world wide and the closely related *Brugia malayi* and *B. timori* affect 12.5 million people in South East Asia. About 20 million people are infected every year by dengue virus transmitted by *Aedes* mosquitoes with about 24,000 deaths. The incidence of mosquito-borne diseases is increasing due to uncontrolled urbanisation creating mosquitogenic conditions for the vector mosquito populations. Therefore, mosquito control forms an essential component for the control of mosquito borne diseases. Several strategies have been adopted to control these diseases but perils of epidemics still loom large in most States in the country. Vector control as an in-built component of the nation-wide disease control strategy has been the main plank so far wherein synthetic insecticides have been effectively used during past several decades to control varied dipteran pests. However, the use of chemical insecticides has been greatly impeded due to development of physiological resistance in the vectors, environmental pollution resulting in bio-amplification of food chain contamination and harmful effects on beneficial non-target animals. Therefore, the need of alternate, more effective and environment-friendly control agents became urgent.

The last decade has evidenced an increased interest in biological control agents. More number of bio control agents was screened for their efficacy, mammalian safety and environmental impact. Many organisms have been investigated as potential agents for vector mosquito control, including viruses, fungi, bacteria, protozoa, nematodes, invertebrate predators and fish. However, most of these agents were shown to be of little operation-

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al use, largely because of the difficulty in multiplying them in large quantities. Only, a few spore forming bacteria, copepods and fish have reached operational use and are undergoing extensive field trials. The discovery of a bacteria-like *Bacillus sphaericus* Neide (*Bs*) and *B.thuringiensis* serovar. *israelensis* deBarjac (*Bti*) which are highly toxic to dipteran larvae have opened up the possibility of its use as potential biolarvicides in mosquito eradication programs the world over (Poopathi and Tyagi, 2002; Poopathi et al., 2002). These bacteria have some important advantages over conventional insecticides in mosquito control operations, besides being safe to non-target organisms including human being. Also, it is innocuous to the environment. Besides these bacteria, several other types of bacteria such as *B.t. jegathesan*, *B.t. morrisoni*, *B.t.* subsp. *medellin, B.t.* subsp. *malaysiensis*, *B.t.* subsp. *canadensis, Asticcacaulis excentricus, Clostridum bifermentans* subsp. *malaysia* and *Synechococcus* are being examined as an effective biological control agents. The *Bti* has been used operationally for the control of mosquitoes for over two decades and its formulations are highly effective against *Anopheles, Aedes* and *Culex* mosquitoes (Mahmood, 1998). No evidence has been found that *Bs* and *Bti* toxins harm aquatic organisms sharing the breeding sites of these vectors or have an adverse effect on the environment. Although *Bti* is effective, specific, bio-degradable and possesses a long shelf life, it does not recycle in the environment at levels high enough to provide significant residual activity. It has a short duration of toxic action, usually 24 to 48 hours and must, therefore, be applied at frequent intervals. Moreover, current spore forming *Bt* formulations sink in water and are consequently less efficient in controlling species of mosquito larvae that feed only near the water surface. The rate of killing with spores is slow compared with the chemical insecticides and the toxins have a narrower mosquito host range than the chemicals. *Bacillus sphaericus,* on the other hand, has been shown to recycle in the field conditions and exert larvicidal activity for a long period. However, the spores of *Bti* have the advantage over *Bs* that *Bti* has a wider spectrum of activities against *Anopheles*, *Culex* and *Aedes* spp, while *Bs* has its effect mainly on *Culex,* for a lesser extent to *Anopheles* and it is strongly species specific and act against only a few *Aedes* species. Field resistance has been only reported for *Bs,* while for *Bti,* it seems more difficult for mosquitoes to develop resistance even under intensive laboratory selection, which may be due to the multiple toxin complex of this bacterium. This review focuses on the recent advancement on research on the production of biopesticides (*Bs* and *Bti*) by using cost-effective technology in mosquito control operations.

BACTERIAL TOXINS

B. sphaericus is an aerobic, rod-shaped, endospore forming Gram positive soil bacterium. The first discovery of *Bs* strain toxic to mosquito larvae was reported by Kellen et al. (1965). Thereafter, more than 300 strains have been isolated and identified from all over the world (Singer, 1997; de Barjac et al., 1988; Thiery and Frachon, 1997). More than 180 *Bs* strains (belonging to six H serotypes) have been assayed on a wide variety of mosquito species and it has been found that the most potent strain was the H5a5b serotype. Sporulation of these *Bs* strains in a liquid culture medium was studied under the electron microscopy. Crystal-like inclusions first appeared (7 hours after lag phase) and reached their final size in 72 hours. The release of the spore/crystal inclusion complex occurred at 22 hours after incubation. Careful choice of culture medium and bacterial serotype is needed for high spore yield and high larvicidal activity. There are two kinds of insecticidal toxins (crystals and Mtx toxins), which differ in composition and time of synthesis. The crystal toxins are the main toxic factors in highly larvicidal strains. It contains two polypeptides of molecular weight 51 and 42 kDa (BinB and BinA respectively) which act as a binary toxin (Charles et al., 1997). The genes encoding for BinB and BinA are located on the chromosome in the strains of *B. sphaericus* (*Bs* 2362, *Bs*1593, *Bs* 2297). The amino acid sequence of these two polypeptides differs marketedly from those of other bacterial or larvicidal toxins, including *Bti*. However, the BinB and BinA share four segments of sequence similarity. The 42 and 51 kDa protein genes of *Bs* have been sub-cloned independently downstream of the CytA gene promotor of the toxin gene in *Bti* and introduced into a non-mosquitocidal strain of *Bt*. Each protein was overproduced and accumulated as inclusion bodies which were purified. The 42 kDa protein inclusions were found to be toxic to *Culex* larvae in contrast to the 51 kDa protein inclusions which were not toxic on their own but a synergistic effect between these two components was observed. Studies conducted with recombinant bacteria expressing these polypeptides individually have revealed that BinA could be toxic at high dosage in the absence of BinB, but this was not in the case for the BinB alone. However, presence of both BinB and BinA in equimolar amounts showed highest toxicity in larvae, since they seem to act in synergy. In addition to the binary toxin, another mosquitocidal protein with molecular weight of 100 kDa, appears to be synthesized in low-toxicity strains (Nielsen -LeRoux and Charles, 1992) as well as in some of the highly toxic strains and this polypeptide is expressed during the vegetative phase and is not homologous with the 51 kDa and 42 kDa proteins. The efficient expression of this 100 kDa mosquitocidal toxin in protease deficient recombinant *Bs* was thoroughly studied and It was concluded that protease negative *Bs* strains expressing Mtx and other toxins may form the basis of an alternative to the natural highly toxic strains for mosquito control. The location of the binary toxin (btx) and mosquitocidal toxin (mtx) genes

in *Bs* strains were determined by hybridization of specific gene probes to chromosomal DNA in Southern blots. The identification and introduction into *Bs* of the *Bt* subsp. *medellin* Cyt1 Abt gene results in higher susceptibility of which are otherwise resistant mosquito larval populations to *Bs*. Apart from *Bs* and *Bti*, the cloning and expression of other mosquitocidal strains such as *Bt* subsp. *medellin, Bt* subsp. *jegathesan* and *Clostridium bifermentans* have been reported (Delecluse et al., 1995).

The binary toxin of *Bs* strains is generally very toxic to *Anopheles* and *Culex* species but poorly or non-toxic to most *Aedes* species. However, susceptibility appears to depend on the species of mosquito and can thus vary within a genus, a serotype or even within the same serotype. The susceptibility also appears to depend on the stability of bacterial strains, appropriate methodology etc. Since these bacteria are safe for animals, environment and cause no health risk to humans, several formulations in the form of wettable powder (WP), water dispersable concentrate (WDC), emulsifiable concentrate (EC), flowable concentrate (FC), granules (G) and dust (D) have been produced to control many species of mosquitoes. These products have been tested extensively in USA, France, Brazil, Zaire, India and in Bangladesh. Like *B. sphaericus*, *B. thuringiensis* serovar *israelensis* (*Bti*) is also a spore forming Gram-positive soil bacterium since its discovery two decades ago (Goldberg and Margalit, 1977). More than 50,000 isolates have been screened and tested in insect control. This bacterium synthesizes proteins during sporulation that assemble into crystals which are toxic to mosquitoes. Crystal development during sporulation of *Bt* strains has been studied extensively. The crystals are composed of four polypeptides (M.wt. 125, 135, 68 and 28 kDa proteins) referred to as CryIVA, CryIVB, CryIVD and CytA . These genes encoding this Cry toxins are located on a 72 kDa resident plasmid and they have been cloned and expressed in various hosts. Chromosomal Cry genes have also been reported in some *Bt* strains and the role, structure and molecular organization of genes coding for the parasporal ∂-endotoxin of *Bt*. A review of the biochemical mechanisms of resistance of insects to *Bt* indicates that altered proteolytic processing of *Bt* crystal proteins may be involved in one case of resistance in mosquitoes. The presence of IS240 elements responsible for mosquitocidal action was investigated in sixty nine *Bt* strains. A PCR-based approach for detection of Cry genes in *Bt* has been reported. Since the toxins of this bacterium are highly potent for mosquito control, evaluation of the activity of *Bt* preparations is currently carried out by bioassay with a target insect and compared to a defined standard.

Resistance to bacterial toxins

Though *B. sphaericus* spore/crystal toxins are powerful tools to control mosquito vectors, the recent development of resistance in *Culex* species has impeded progress in mosquito control operations. The magnitude of *Bs* resistance and cross-resistance to different strains of *Bs* and *Bti* in filarial vector of *Culex quinquefasciatus* have been reported (Poopathi et al., 1999a,b,c, 2000a,b; Wirth et al., 2000) (Table 1). The resistance ratio recorded between *Bs* resistant and susceptible larvae were several thousand fold at the LC_{50}, and LC_{95} levels. These results indicated a need for judicious use of appropriate strains of *Bs* and *Bti* in the event of biopesticide resistance for mosquito control.

MODE OF ACTION

Crystal toxins from *Bs* are ingested by mosquito larvae, and after solubilization and proteolytic cleavage, the activated toxin interacts with the midgut epithelium leading to death of larvae. In mosquito larvae, the sequence of events follow in the manner given below, (i) ingestion of spore/crystal toxin (ii) toxin solubilization in the midgut (iii) activation of the protoxin by protease into active toxin i.e 42 and 52 kDa of *Bs* to 39 and 43 kDa proteins (iv) binding of active toxin to specific receptors present in the midgut brush border membrane, and (v) putative internalization of toxin and cell lysis. However, the eventual intracellular action of binary toxin in the cells is not completely clarified except for a few reports on cytopathological effects caused by the action of the toxin (Singh and Gill, 1988; Poopathi et al., 1999d, e).

Cytopathological effects by bacterial toxins

Transmission electron microscopic (TEM) studies showed that the midgut epithelial cells of a *Bs* susceptible and resistant strains of *C. quinquefasciatus* had well defined microvilli in a parallel line on the outer boundary. Each microvillus contained a microfibrillar core and it extended below the plasma membrane to form a terminal web. It has been reported that *Bs* and *Bti* treatments bring about some changes in the midgut structure of the mosquitoes (Poopathi et al., 1999a,b, 2000c). Before *Bs* treatment, the nuclei of midgut epithelial cells were packed with nucleolar granules inside the nucleoplasm. The nucleolemma was well defined on the outer boundary. The mitochondria, rough endoplasmic reticulam, lysosome and Golgi body were also visible in the cytoplasm. The binary toxin from *Bs* and the multiple toxin from *Bti* after being absorbed into the gut, cells exert their effects on the midgut epithelium by causing disruption, separation and ploughing of columnar epithelial cells into the gut lumen. It has been argued that disruption and swelling of the midgut causes the death of the insect following *Bs* or *Bti* poisoning. *Bacillus sphaericus* toxin is a slow acting larvicide that does not paralyze mosquito larvae until 24 to 48 hours after treatment. However, pathological lesions in the midgut

Table 1. Cross-resistance to *Bacillus sphaericus* strains in *Culex quinquefasciatus* selected for resistance to *B. sphaericus* 1593M.

Strains	Intercept	Slope ± SE	LC_{50} (mg/l)	LC_{90} (mg/l)	LC_{95} (mg/l)	X^2 (df)	RR (at LC_{50}) [f]	RR (at LC_{90}) [f]	RR (at LC_{95}) [f]
B.sphaericus 1593M	6.86 [a]	1.64 ± 0.4	0.073(0.122 - 0.044) [c]	0.442(1.157 - 0.169) [d]	0.736(2.39 6 - 0.226) [e]	19.65 (4)	9.6(11.0 - 8.7)	29.5(27.5 - 33.8)	39.4 (45.2 -37.7)
	13.99 [b]	4.25 ± 1.8	0.0076(0.0 1-0.004)	0.01(0.02 - 0.005)	0.0187(0.0 53 0.006)	16.78 (2)			
B. sphaericus 2397	6.631	1.64 ± 0.5	0.101(0.25 2 - 0.04)	0.612(1.7 26 -0.065)	1.02 (2.29 -0.068)	44.18 (4)	27.3(29 .3 - 2.5)	19.7(38.7 - 10.8)	18.2 (38.3 - 9.7)
	8.430	1.402 ±0.3	0.0037(0.008 6 - 0.0016)	0.031(0.14 - 0.006)	0.056(0.39 9 - 0.007)	38.01 (4)			
B. sphaericus 2362	6.808	1.66 ± 0.7	0.213(0.30 9 - 0.075)	1.267(1.96 - 0.073)	2.099(3.16 9 - 0.063)	30.81 (3)	18.4(23.1 7.5)	23.0(27.2 - 1.7)	24.4(26.6 - 1.26)
	8.65	1.89 ± 0.3	0.0116(0.0 13 - 0.01)	0.055(0.0 720.043)	0.086(0.1 19 - 0.05)	6.85 (4)			
B. sphaericus IAB 59	6.808	3.9 ± 1.8	0.344(0.80 4 - 0.147)	0.733(3.9 620.136)	0.909(6.7 140.123)	24.48 (2)	9.6 (12.4 - 7.7)	4.7(9.6 - 2.3)	3.9(8.7 - 1.7)
	7.908	2.01 ± 0.4	0.036(0.06 5 - 0.019)	0.156(0.41 - 0.058)	0.236(0.79 - 0.072)	35.24 (4)			
B. thuringiensis var*israeloensis* PG 14	z9.65	1.66 ± 0.5	0.0016(0.0 04 -0.0007)	0.0095(0.0 61 –0.002)	0.016(0.5 7 - 0.002)	30.74 (3)	0.8(1.6 - 0.4)	1.5 (3.4 - 0.3)	1.8 (3.9 - 0.23)
	12.34	2.77 ± 0.5	0.002(0.00 25 -0.0019)	0.0065(0.01 8 - 0.005)	0.0088(0.0 4 - 0.0067)	5.15 (3)			
B. thuringiensis var *israelensis* 426	9.50	2.37 ± 0.6	0.013(0.01 9 - 0.008)	0.044(0.08 8 - 0.022)	0.063(0.15 - 0.027)	12.22(3)	1.7(2.0-1.2)	1.8(2.5 - 0.4)	1.8 (2.9 - 0.5)
	10.39	2.56 ± 0.7	0.0078(0.0 15-0.004)	0.025(0.094 - 0.0065)	0.034(0.17 - 0.0069)	9.68 (2)			

[a] Gandhinagar resistant strain (GR); [b] Madurai susceptible strain (MS); [c d e] 95% Fiducial limits of upper and lower at LC_{50}, LC_{90} and LC_{95} levels ;
[f] Resistance ratio = Experimental values (GR) ÷ Control values (MS).

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of toxin treated larvae are observed as early as 7 to 10 hours after treatment. This causes a delayed paralysis and death of *Bs* exposed larvae was a certainty (Poopathi et al., 2000c). *Bacillus thuringiensis* subsp. *israelensis* toxin destroys the structure of cells in the midgut epithelium, whereas *Bs* toxin does not and takes a longer time to disintegrate (Singh and Gill, 1988; Poopathi et al., 1999d). The difference in toxin effect is probably due to variation in the size of active toxins from the two bacteria. Ultrastructural variations were also found to be similar in both *Bs* resistant and susceptible larval strains (Poopathi et al., 1999e).

BINDING KINETICS

From studies of binding kinetics (direct binding and homologous competition assays) of *Bs* binary toxin to the midgut brush border membrane fractions (BBMFs) of *Anopheles* and *Culex* mosquito larvae, it was reported that the radiolabeled toxin bound specifically to a single class of receptors. The BBMF of *An. gambiae* had the highest binding affinity for the toxin among the species examined, with a dissociation constant K_d of 30 ± 15 nM and a maximum receptor concentration of 5 ± 1 p mole.mg^{-1}. Toxin binding to *An. gambiae* BBMF was compared with that to BBMF from *Bs* susceptible and *Bs* resistant *C. pipiens* populations (Silva-Filha et al., 1997). Brush border membrane fractions (BBMF) toxin binding was slower in *An. gambiae* than in the *C. pipiens* populations. The BBMF of the *Bs*- resistant population of *C. pipiens* had an association profile that was similar to the susceptible population, despite of the lack of susceptibility *in-vivo*. No relationship between toxicity and irreversibility of toxin binding was detected. On the contrary, toxin dissociation was fast and almost complete in BBMF of all species studied. Similarly, the crystal toxins (BinB and BinA) of *Bs* were also used for *in-vitro* binding competition assays with BBMF from *C. pipiens* and *An. gambiae* and the results were the same (Charles et al., 1997). Identification of the receptor for the *Bs* crystal toxin in the BBMF of *C. pipiens* showed that a single 60 kDa midgut membrane protein is identified as the binding protein. This protein is anchored in the

Table 2. Direct-binding assay of 125 I labeled Bacillus sphaericus (Bs) binary toxin to Culex quinquefasciatus larval BBMF from Bs-susceptible, resistant and their back- crosses.

Mosquito strains	Specific binding (p mole toxin / mg BBMF protein)		
	8 nM	50 nM	150 nM
Madurai (MS)	1.14 (1.19 – 0.022)[a]	1.48 (1.58 – 1.39)[a]	1.74 (1.84 – 1.65)[a]
Gandhinagar (GR)	0.065 (0.13 – 0.004)	0.48 (0.65 – 0.31)	0.67 (1.10 –0.24)
MS ♂ x GR ♀	0.06 (0.08 – 0.038)	0.39 (0.58 – 0.21)	0.37 (0.62 – 0.11)
MS ♀ x F_3 ♂	0.95 (1.20 – 0.82)	1.18 (1.33 – 0.91)	1.44 (1.63 – 1.32)
GR ♀ x F_3 ♂	0.116 (0.13 – 0.096)	0.25 (0.27 – 0.24)	0.097 (0.21 – 0.02)

[a] 95 % fiducial limits of upper and lower at different concentrations.

mosquito midgut membrane via a glycosyl phosphatidylinositol (GPI) anchor, and is partially released by phosphatidylinositol specific phospholipase. Further an enzymatic investigation showed that the receptor of the Bin toxin in *C. pipiens* midgut may be an α-glucosidase (Danboux et al., 2002). Binding experiments with the field population of *C. quinquefasciatus* that had been selected in the laboratory showing 100,000 fold resistance to *Bs* binary toxin failed to reveal the presence of any specific binding (Nielsen-LeRoux et al., 1995). The authors concluded that the resistant strain had lost the functional receptor for the *Bs* toxin. The binding characteristics of BBMF from the F_1 larval progeny (susceptible female x resistant males) were very close to those of the parental susceptible strain which is consistant with the resistance being recessive. Because, the resistance is encoded by a recessive gene-linked to the sex locus on chromosomal and it is not associated with any loss of binding affinity between BBMF and *Bs* radiolabeled toxin. It was reported in toxin binding assays that the sugar molecules had no detectable inhibitory effect on toxin binding to *C. pipiens* BBMF (Nielsen-LeRoux and Charles 1992). The role of gut proteinase in the mechanism of action and the specificity of *Bs* toxin reflects the fact that the susceptibility of mosquito cell culture differ from the specificity of the toxin. Immunological localization of *Bti* toxins in midgut cells of toxin treated *An. gambiae* showed that the midgut cells are the primary target for the toxins and that there is binding to specific receptors on the apical microvilli. Though a preliminary study of an *in vitro* binding assay using *Bti* toxin midgut cell of *An. gambiae* was reported through immuno light microscopy, the exact mode of action of *Bti* toxin in the mosquito is still not clear. We are exploring the possible *Bti* mode of action and eventual intracellular action (*in vitro*) in the cells.

Binding affinity of the *Bs* binary toxin to a specific receptor on the midgut brush border membrane from geographically different mosquito species of *C. quinquefasciatus* (Indian strain) of resistant, susceptible, F_1 progeny and back-crosses to susceptible and resistant strains have been studied recently (Poopathi et al., 2004). Toxicity assays in the larvae of these strains confirmed that the resistance was inherited by partially recessive gene. The similarities in susceptibility of *Bs* susceptible and the progeny from back-crosses strain with F_5 may be expected which may reflect lack of any susceptibility variations between these two strains. Whereas, the susceptibility of F_1 offspring was higher than that of susceptible parent but lower than that of resistant parent, indicating that resistance being controlled by partially recessive gene. This study was justified by *in vitro* binding assays in the larval strains developed from cross breeding experiments (Table 2). A new polypeptide (MW: 80 kDa) visualized in *Bs*-resistant strains, through SDS-PAGE has further substantiated the observation.

BINDING ASSAYS: A NEW APPROACH

In order to test the sensitivity of vectors such as *C. quinquefasciatus* for the *Bs* toxin binding mechanism in the BBMF, the normal practice has been to extract BBMF from frozen specimens of the vectors. This preservation of frozen specimens needs elaborate arrangements using liquid N_2 or dry ice. In fact, frozen samples cannot be transported as such under dry ice for studies between laboratories or countries. Alternate procedures to simplify preservation and transportation of samples between laboratories would be very useful. We reported a novel alternative method for preservation of mosquitoes for studying *Bs* binding mechanism in mosquitoes (Poopathi et al., 2002). Larvae that being kept air-dried or lyophilized were tested for their sensitivity to *Bs* toxins. Dried and lyophilized larvae of samples of French strains were studied in comparison with frozen larval samples. *C. pipiens pipiens* larvae were air-dried (at 30^{o} C for 24 hour) and lyophilized (at -50^{o}C, vacuum pressure = 0.1 m bar) individually for BBMF preparation by a differential centrifugation process and binding assays. Results showed good specific binding in all three larval samples, including the reference sample. It was also observed that there were no statistically significant differences of the toxin binding affinity between frozen larvae (standard), air-dried and lyophilized larval samples by our homologous competition experiments (figure not shown).

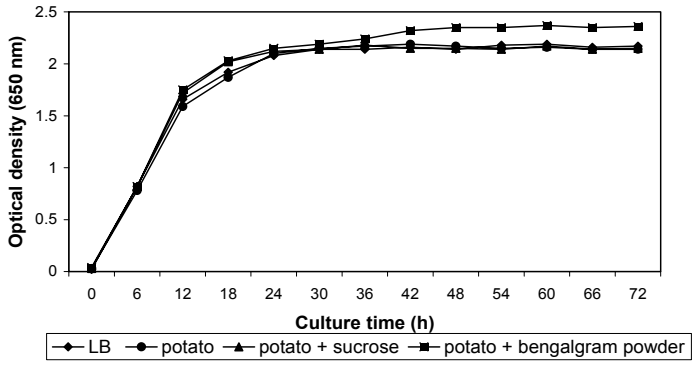

Figure 1. Growth pattern of *Bacillus thuringiensis* serovar *israelensis* in different culture media.

This finding indicated that the binding kinetics of the *Bs* toxin studied from dried and lyophilized larval samples were comparable to frozen larval samples. This novel approach opens up a possibility of using dried or lyophilized vector specimens like larvae for test of binding kinetics against biolarvicides such as *Bs* and their derivatives. It appears that no such study was initiated in medical and agricultural research on the mode of action of bacterial toxins in insects. If proved on a large scale this approach would simplify preservation and transportation of vectors for testing at a later stage in the laboratory for their susceptibility to various insecticides and their derivatives.

RESISTANCE MANAGEMENT

Combined application of neem based biopesticides with microbial agents revealed that the neem biopesticide showed synergistic interaction with the *Bs* toxin against resistant larvae of *C. quinquefasciatus* (Poopathi et al., 1997). However, no synergistic action was reported against the *Bs* susceptible strain. The toxin alone yielded only 10 to 5% larval mortality. The observed LC_{50} and LC_{90} of the *Bs* and neem mixture were compared with the expected lethal concentrations and it was found that the combination of neem and *Bs* toxin exerted a synergistic effect in *C. quinquefasciatus* resistant strain. Tabashnik (1992) re-examined the data reported by Van-Frankenhuyzen (1991) and concluded that the 27 kDa (CytA) toxin and the 130 or 65 kDa (CryIV) toxin from *Bti* synergistically interact against *Ae. aegypti*. A very low concentration of CryIc toxin with endochitinase exerted a synergistic effect in *Spodoptera littoralis*. In *Musca domestica* also, synergism is observed only in resistant strains such that the synergist may be reducing the level of resistance. The efficacy of malathion and pyrethroids for killing *Bs* resistant *C. quinquefasciatus* was evaluated recently and the results were promising (Poopathi, 2001; Poopathi and Baskaran, 2001). Resistance is believed to be a complex, genetic, evolutionary and ecological phenomenon. Resistance management tactics are most likely to succeed if they are directed at reducing the single-factored selection pressure that occurs with conventional biocide or chemical control. Obvious counter measures include: (i) rotation or alternation of *Bs* or *Bti* toxins with other toxins, insecticides, or cultural or biological control strategies, (ii) reducing the frequency of biocide treatments, (iii) avoiding insecticides with prolonged environmental persistance and slow - release formulations, (iv) avoiding treatments that apply selection pressure, and (v) incorporating source reduction methods. The combination of these principle is essentially a blue print for integrated pest management (IPM) which will successfully delay or prevent the development of resistance in vector population. Theoretically, integrated pest management (IPM) helps delay resistance by providing multiple sources of pest mortality.

COST-EFFECTIVE CULTURE MEDIA FOR BIOPESTICIDE PRODUCTION

Although the potential of biopesticides (*Bs* and *Bti*) and its derived toxins has been demonstrated in mosquito control, the existing technology to grow and to produce *Bs* and *Bti* formulations is not cost-effective. Development of cheaper media would facilitate the culture and production of bacteria in a cost-effective manner. Obeta and Okafor (1984) formulated five media from the seeds of legumes, dried cow blood and mineral salts, and assessed growth and production of insecticidal toxins of *Bti* which were effective against *A. aegypti*, *C. quinquefasciatus* and *A. gambiae*. Similarly, other culture media containing fishmeal, soybean, cornsteep liquor for the production of *Bti* and *Bs* have also been reported to be most effective and compared well with the standard (Salma et al., 1983, Kuppusamy 1990, Kumar et al., 2000). In Peru, a field trial is currently under operation using *Bti* produced from the whole ripe coconut for the control of malarial vectors (P. Ventosilla, IMTAH, personal communicaton). An attempt has been made to evaluate the cost-effectiveness of *Bs* and *Bti* produced from potato based culture medium vis-à-vis that of conventional medium (Luria Bertani, LB) to deduce the level of commercial viability based on laboratory production (Figure 1). The amount of potato material required in this study to prepare 10 L of culture medium was 1.5 kg which has a cost of US $ 0.11. In comparison, preparation of 10 L of LB medium costs US $ 15.10. Thus, the use of potato based culture medium may be much more economical for the industrial production of these mosquito-pathogenic bacilli (Poopathi,\ et al., 2003a,b). In view of these facts, the application of potato based culture medium appears to be quite promising and feasible for the mosquito control program in the field, especially in developing countries.

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