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Overproduction of mosquitocidal toxins of *Bacillus thuringiensis* serotype 14 on cost effective media

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ABSTRACT

Bacillus thuringiensis serotype 14 (*Bti*) is being widely used in mosquito control programs. Large scale production of this subspecies is expensive because of the high cost of the production medium ingredients. In this study, we attempted to develop two cost effective media based on inexpensive locally available raw materials namely fodder yeast, a byproduct produced in plenty from ethanol industry in Egypt and grinded seeds of black eyed pea (cowpea; *Vigna unguiculata* subspecies *unguiculata)* a highly protein rich legume seed. Various nutritional and cultural parameters influencing diptera-specific delta-endotoxin synthesis by *Bti* serotype 14 were investigated. Optimum concentration of both fodder yeast and black eye pea was about 9%(w/v) after which a notable decrease in mortality percentage was evident. Adequate concentration of inorganic phosphate (50 mM K_2HPO_4) was required for an effective synthesis of mosquitocidal toxin. High aeration level corresponding to 20 : 1 air : medium ratio favored toxin production. Within tested sizes of inocula, 26×10^6 CFU and 13×10^5 /mL were suitable for attainment of highest mortality percentage against second instar larvae of *Culex pipiens* for fodder yeast(65.4% at 5ppm) and black eyed pea (97.5% at 1ppm)based media after 72 hour and 48 hour incubation period respectively. Addition of 0.1% Tween 80 to fodder yeast based medium nearly doubled toxicity percentage, while Ca inhibited toxin biosynthesis. LC_{50} value was determined for toxin produced on the two media were 1.5 ppm and 0.37 ppm for fodder yeast and black eyed pea based media respectively.The obtained results were discussed in the light of lowering the poduction cots this important biological control bacterium .

Key words: *Bacillus thuringiensis*, *Culex pipiens*, fodder yeast, black eyed pea.

INTRODUCTION

Use of entomopathogenic bacteria as biological control agents constitutes one of the main strategies for control of insect disease vectors. *Bacillus thuringiensis* var. *israelensis* (*Bti*) is considered ideal for mosquito control because of its high mosquito larvicidal activity and cost effectiveness (Balaraman and Hoti, 1987), ease of production (Prabakarn and Balaraman, 2006; Balaraman, 1980), amenability to a variety of formulations (Lacey *et al* 1984; Su and Mulla, 1999), safety to non – target organisms (Merritt *et al* ., 2005 ; Brown *et al* , 2004) and mammals (Thanabalu *et al* , 1993 ; Pauchet *et al* , 2005). Therefore , *Bacillus thuringiensis var. israelensis* has long been employed as a vector control agent (Manonmani and Hoti, 1995). Furthermore, the major advantage of this biocide is that risk of resistance to mosquitoes to *Bacillus thuringiensis* var *israelensis* (*Bti*) based products is very low, due to its multi toxin complex (Wirth *et al* 1998). During sporulation, *Bti* produces crystals which contain protoxins. The mode of action of these proteins on larvae involves the ingestion of crystals and spores. Inside the mid-gut lumen, under the action of alkaline pH and proteinases, protoxins in the crystals are solubilized and activated leadning to death of larvae.

Bacillus thuringiensis serotype 14 is the most effective microbial control agent active against disease – transmitting mosquitoes that is available to date (Goldberg and Margalit , 1977 ; de Barjac , 1978; Tyrell *et al* ., 1979 ; de Barjac and Larget – Thiery, 1984; Federici *et al*., 1990 ; Mahammod, 1998; Su and Mulla, 1999). It synthesizes intracellular crystal inclusions containing multiple protein components with molecular weights of 134 , 125 , 67 and 27 KDa (Sekar , 1986 ; Hofte and whiteley, 1989 ; Wirth *et al* ., 1998 ; Poopathi and Tyagi 2006). However, the combination of these proteins seems to exhibit much higher toxicity than any of the individual components.

Biopesticide application in mosquito control operations has gained much importance, during the last two decades, in view of environmental protection (Poopathi and Abidha, 2007). Yet, an important advantage of biopesticide application in cost effective manner. In this paper we report over production of mosquitocidal toxins by *Bacillus thuringiensis* serotype 14 on fodder yeast and grinded seeds of black eyed pea, *Vigna unguiculata*.

MATERIAL AND METHODS

Bacteria

Bacillus thuringiensis var *israelensis (Bti*) was obtained from a commercial bioinsecticide based on *Bacillus thuringiensis israelensis*. The method of the microorganism isolation of *Bacillus thuringiensis (Bti)* was implemented as described by Travers *et al* (1987) and organism identification *(Bti)* was further confirmed according to the method of Rabinovich *et al* (1999).The isolated *Bti* culture was maintained on nutrient agar slants at 4°c.

Bacterial culture media

The conventional laboratory culture broth (Nutrient broth) was used for inoculum preparation by mixing 5g peptone and 3g beef extract / 1 L dist water. 25 ml of sterile medium was inoculated with one loopful of bacteria slant and incubated for 24 hour on an orbital rotary shaker at 30° C. Finely grinded legumes seeds and agroindusterial byproducts were incorporated at 3% final concentration in 25 ml 0.05 M dipotassium hydrogen phosphate containing 0.3%, glucose that was added for growth initiation. The flasks were inoculated and incubated on a rotary shaker (150 rpm) for 72 h at 30° C.

Toxicity test

Full grown culture on different substrates were assayed against 2nd instar laboratory reared Larvae *Culex pipiens*. Desired dilutions were placed into 100 ml beakers in duplicates along with 10 second instar *Culex pipiens* larvae. About 100 mg of ground fish meal was added to each cup. The beakers were covered with muslin and kept at 26 ± 2 ° C with 10 h / 14 h dark cycle. The mortality percentage was recorded by counting the number of dead larvae. (Priest and Yousten, 1991).

Statistical analysis

LC_{50} of *Bti* grown on fodder yeast and black eyed pea were determined according to Probit regression analysis (Finney 1971).

RESULTS AND DISCUSSION

Potentials of some agricultural byproducts and legume seeds as base media for toxin production

Four different byproducts namely offals meal, feather meal , cotton seed meal and fodder yeast in addition to four grinded legume seeds namely soy beans, kidney beans, black eyed pea and horse beans were screened as complete media for larvicidal toxin production. Table (1) . Fodder yeast and black eyed pea gave the highest biotoxin yields at high dilution of 15 ppm. Thus those two substrates were selected for further investigations as their toxin productivity exceeded that of both the standard complete commercial media (Nutrisoy and Proflo) as standard control substrates.

Poopathi and abidha (2007) reported that the use of chicken feather waste as culture medium is highly economical for the industrial production of these mosquito pathogenic bacilli. Soy bean flour, ground nut cake powder and wheat bran extract were reported for large scale production of *Bti* by Prabakaran and Balaraman (2006).

Effect of different concentrations of fodder yeast and black eye pea on toxin production

A range of 1.5% to 12% of the selected

substrates, fodder yeast and black eyed pea were tested for mosquitocidal toxin production, Table (2). The obtained results revealed that the mortality % is significantly affected by the concentration of the nutrient used. Although 9% (w/v) fodder yeast and black eye pea represented the most suitable concentration for toxin productivity at 5 ppm of culture dilution against 2nd instar larvae of *Culex pipiens*, yet high mortality was obtained by black eyed pea reaching 97.9% while, only 62.6% was gained by fodder yeast.

With increasing the concentration to 12% (w/v), the mortality was notably decrease to 27.9%. On the other hand , at the same conc. black eyed pea medium supported the formation of higher toxicity yielding 78.0% mosquitocidal activity. Gangurde and Shethna (1995) used 2% (w/ v) defatted mustard seed meal as a growth medium for *Bti*. Under these conditions , 46% mortality was achieved. At 4% (w / v) mustard seed meal, *Bti* grew better but had undetectable larvicidal activity.

Effect of phosphate concentration

Four concentrations of K_2HPO_4 as an inorganic phosphate namely 0.05 , 0.075 , 0.1 and 0.15 M were added to 9% fodder yeast and black eye pea based media, Table (3). The results obtained showed that increasing phosphate concentrations inhibited mosquitocidal toxin production by *Bti* grown on the two media. This is because additions of phosphate may affect the initial pH value of media. Ozkan *et al* (2003) studied the effects of various nutritional and cultural factors on the biosynthesis of Cry 4 Ba and Cry 11 Aa toxins in *Bacillus thuringiensis* subsp *israelensis* HD 500. They reported that the highest yields of both Cry 4 Ba and Cry 11 Aa , and the highest sporulation frequency were obtained when the cells were grown on 50 – 100 m M K_2HPO_4.

Effect of aeration level

Aeration level was changed by varying the volume of the fermentation media per 250 ml conical flasks. Four air: medium ratios were tested, Table (4). Highest mortality percentage was achieved at 20 : l air: medium ratio. As air space decrease, a notable decrease in the mortality percentage was evident reaching 7.5% and 3.8% at 1.5 : 1 ratio. No studies could be found in the literature concerning the effect of aeration level on toxin production by *Bti* sero type 14. Ghribi *et al* (2006) reported improvement of *Bacillus thuringiensis* delta – endotoxin production by overcome of carbon catabolite repression through adequate control of aeration. They found that aeration rates corresponding to 60% and 70% oxygen saturation during the first 6 h of fermentation should be applied into 15 gl^{-1} glucose based medium and 42 gl^{-1} gruel based media; then 40% oxygen saturation should

Table 1: Potential of some agricultural by-products and legumes seeds as growth media for production of mosquitocidal toxins of *Bti*

Tested substrate	Final pH	Mortality % after at 15 ppm after (h)	
		24	48
Offals meal	8.8	40	55
Feather meal	8.8	60	70
Cotton seed meal	8.6	70	90
Fodder yeast	8.8	85	100
Soy beans	8.7	60	70
Kidney beans	7.3	45	65
Black eyed pea	8.2	90	100
Horse beans	8.0	55	70
Nutrisoy (standard medium)	8.0	60	70
Proflo (standard medium)	8.6	50	75

be ensured upto the end of fermentation. With higher oxygen saturation values, cell densities were increased, but delta- endotoxin synthesis yields were strongly reduced.

Effect of inoculum size on toxicity of *Bti*

Seven inoculum sizes in term of colony forming units (CFU) were applied to inoculate both types of media. As shown in Table (5), the effect of inoculum size differs by the type of media used. In case of the agroindustrial byproduct , fodder yeast, the larval toxicity increased with increase of the size of inoculum up to 26 × 10 ^{6} CFU/mL reaching 65.4% mortality at 5 ppm after which clear decrease in mortality was evident. In case of using black eyed pea, the pattern was different as upon using only 1.3 × 10 ^{6} CFU/mL, larval toxicity reached 97.5% at only 1 ppm. This indicates that this medium based

Table 2: Effect of different concentrations of fodder yeast and black eyed pea on toxicity of *Bti* against larvae of *Cx. pipiens*

Concentration (%)	Mortality* % after 48h at 5 ppm of *Bti* grown in	
	Fodder yeast	Black eyed pea
1.5	4.0 ± 0.4 f	7.1 ± 0.7 f
3.0	14.6 ± 1.1 e	24.2 ± 1.5 e
4.5	23.6 ± 1.3 d	38.8 ± 1.4 d
6.0	38.3 ± 1.3 b	61.3 ± 2.0 c
7.5	60.8 ± 1.2 a	77.1 ± 1.9 b
9.0	62.9 ± 1.6 a	97.9 ± 0.7 a
12	27.9 ± 1.7 c	78.0 ± 1.0 b

*Mortality % is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at $P = 0.05$.

Table 3: Effect of phosphate concentration added to fodder yeast and black eyed pea on toxicity of Bti against larvae of *Cx. pipiens*

Concentration (%)	Mortality* % after 48h at 5 ppm of *Bti* grown in	
	Fodder yeast	Black eyed pea
0.05 (control)	58.8 ± 2.0 a	93.3 ± 1.1 a
0.075	39.0 ± 1.6 b	58.5 ± 1.5 b
0.10	15.0 ± 1.2 c	32.5 ± 1.6 c
0.15	3.8 ± 0.9 d	13.8 ±1.4 d

*Mortality % is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P = 0.05.

Table 4: Effect of aeration on toxicity of Bti against larvae of *Cx. pipiens* grown in fodder yeast or black eye pea media

Medium air ratio	Mortality* % after 48h at 5 ppm of *Bti* grown in	
	Fodder yeast (5 ppm)	Black eyed pea (3 ppm)
1:20	82.5 ± 2.0 a	90.0 ± 1.2 a
1:9	60.0 ± 1.8 b	78.8 ± 2.1 b
1: 4	34.4 ± 1.4 c	23.3 ± 1.3 c
1: 1.5	7.5 ± 1.2 d	3.8 ± 1.1 d

* Mortality % is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P = 0.05

Table 5: Effect of inoculum size on toxicity of Bti against larvae of *Cx. pipiens* grown in fodder yeast or black eye pea media

Inoculum size (CFU×10^{-6})	Mortality* % after 48h at 5 ppm of *Bti* grown in	
	Fodder yeast (5 ppm)	Black eyed pea (1 ppm)
1.3	32.5 ± 1.7 d	97.5 ± 1.0 a
3.2	40.0 ± 1.5 c	95.8 ± 1.2 a
6.5	51.3 ± 1.9 b	95.8 ± 1.5 a
13	60.8 ± 1.5 a	56.3 ± 2.2 b
26	65.4 ± 1.7 a	58.3 ± 1.1 b
52	36.7 ± 1.9 cd	58.8 ± 1.1 b
104	17.1 ± 1.8 e	24.3 ± 2.2 c

* Mortality % is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P = 0.05.

on legume seed powder, is an excellent medium for mosquitocidal toxin production.

Effect of Inoculum Type

A subsequent study was carried out to investigate the effect of the inoculum type on larvae toxicity of *Bti* on Fodder yeast and black eyed pea, Table (6). The obtained results showed that the type of inoculum clearly affects toxin productivity. The most suitable inoculum type for media based on fodder yeast was that grown on the same type of medium. Using nutrient broth for growth of *Bti* as an inoculum produced less significant mortality percentage, while a notable decrease in toxin production is evident upon using the inoculum grown on black eyed pea reaching 34.4% at 5 ppm. In case of media based on black eyed pea, larval toxicity was favored with either nutrient broth or black eyed pea inocula reaching 94.6 mortality percentage at only 1 ppm. In contrast inoculum grown on fodder yeast medium clearly inhibited toxin production yielding only 45% mortality against second instar larvae of *culex pipiens*. These results may be explained in light of induction effect initiated by the second transfer of the microorganism on the same medium towards the production of the mosquitocidal toxin

Table 6: Effect of inoculum type on toxicity of Bti against larvae of *Cx. pipiens* grown in fodder yeast or black eyed pea media

Inoculum period (h)	Mortality* % after 48h at 5 ppm of *Bti* grown in	
	Fodder yeast (5 ppm)	Black eyed pea (1 ppm)
NB	60.0± 1.6 b	94.2 ± 1.2 a
Fodder yeast	77.5 ± 1.4 a	45.0 ± 1.2 b
Black eye pea	35.4 ± 1.1 c	94.6 ± 1.4 a

* Mortality % is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P = 0.05.

Table 7: Effect of incubation period on toxicity of Bti against larvae of *Cx. pipiens* grown in fodder yeast or black eye pea media

Inoculum period (h)	Mortality* % after 48h at 5 ppm of *Bti* grown in	
	Fodder yeast (5 ppm)	Black eyed pea (1 ppm)
7	0.4 ± 0.4 d	15 ± 1.2 d
16	5.8 ± 1.0 c	40 ± 1.4 c
24	15.0 ± 1.1 b	45 ± 1.2 b
48	57.9 ± 1.7 a	96.7 ± 1.3 a
72	60.4 ± 1.1 a	95.8 ± 1.6 a
96	58.3 ± 1.7 a	95.0 ± 1.2 a

* Mortality % is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P = 0.05.

Effect of incubation period

Different incubation periods were tested for maximum larval toxicity, Table (7). The obtained results revealed that on both media mosquitocidal activity was produced after 48 h of incubation at 150 rpm and 30º C, reaching maximum mortality percentage after 72 h for fodder yeast based medium and 48 h only for black eyed pea based medium. Nevertheless, the toxin was stable for 96 h of incubation under the same conditions. Poopathi and Abidha (2007) reported that the maximum

Table 8: Effect of supplementation of fodder yeast medium with some additives on toxicity of *Bti* against larvae of *Cx. pipiens*

Additives (concentration)	Mortality* % after 48h at 3 ppm
None (control)	42.1 ± 1.8 c
$CaCl_2$ (5.5×10^{-4})	56.3 ± 1.9 b
$MgCl_2$ (8×10^{-3})	29.6 ± 1.6 d
Tween 80 (0.1%)	80.4 ± 1.3 a

* Mortality % is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at *P* = 0.05.

Table 9: LC50 of *Bti* grown on fodder yeast and black eyed pea media

Substrate	LC50 (95%fiducial limits)
Fodder yeast	1.5 (1.3 -1.7)
Black eyed pea	0.37 (0.33 – 0.42)

growth and endotoxin release of *Bs* and *Bti* were completed after 72 h on both chicken feather waste medium and nutrient yeast extract salt medium.

Effect of additives to fodder yeast based medium

In this experiment calcium choride at 5.5 x 10 $^{-4}$ M , $MgCl_2$ at 8 x 10^{-3} M and Tween 80 at 0.1 % were supplemented to fodder yeast and black eyed pea aiming at the elucidation of their effect on toxin productivity. As shown at Table (8), $CaCl_2$ at the used concentration enhanced larval toxicity compared with the control medium at 3 ppm. In the same time, $MgCl_2$ inhibited the mortality percentage by about 30% The yields of bacterial toxin are known to be greatly influenced by trace metals and other minerals and in most cases it is not known whether the effect is a direct one or simply a manifestation of sporulation. Metals ranges selected in the present study were adapted from the previous documents to avoid insufficient or toxic concentrations, Weinberg (1970) and Weinberg (1977). Ozkan *et al* (2003) studied various nutritional and cultural parameters influencing delta endotoxin synthesis by *Bacillus thuringiensis* subsp *israelensis* HD 500. they reported that Mg and Ca favored toxin production when provided at 8 x 10^{-3} M and 5.5 x 10^{-4} M concentration respectively.

When the surface active agent Tween 80 was added at 0.1% to fodder yeast based medium, mortality percentage was significantly increased and nearly doubled at 3 ppm. Several surface active agents were screened is shake flask experiments to achieve higher entomotoxicity Zouari *et al* (1998). Other studies on semi – synthetic media have reported the use of Tween 80 as an additive to enhance substrate assimilation during *Bt* fermentation in shake hask experiments, Zouari *et al* ; (1999) and Liu *et al* (2003). There also has been extensive use of tween 80 as wetting and suspending agent in formulations of biopesticides and also as spary mixes to improve spreading on foliage, Lachhab *et al* (2001). Brar *et al* (2005) reported an increase of 49% in entomotoxicity upon addition of Tween 80 (0.2% v/v) to hydrolyzed sludge as a raw material for *Bacillus thuringiensis* based biopesticide.

When these additives were added to black eye pea based medium, no significant increase in mortality percentage was achieved (data not shown).

LC_{50} of *Bti* grown on fodder yeast and black eyed pea were determined as shown in Table (9). lC_{50} of *Bti* grown on fodder yeast based medium was 1.5 ppm and 0.37 ppm for black eyed pea .

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