

Development of a Cheap Media for Bacillus Thuringiensis Growth

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Abstract:

Bacillus thuringiensis (Bt) is being widely used in mosquito control programs. However, large-scale production of this bacillus is expensive due to the high cost of the production medium. In this study, we attempted to develop a cost-effective medium, based on locally available raw materials which is available in plenty as waste product from several industries. The results of the optimization studies showed that the optimum pH is at (7.2) and the optimum incubation temperature at (30°C) using standard nutrient broth (NB) media. Raw materials (wheat bran, rabbit feed, cabbage leaves, potato tubercles, and cactus extracts as carbon and protein sources were tested. In addition, seawater and stock solution as mineral sources were also prepared to select the most appropriate growth media. 35 trials (single or in combination) from those materials were experimented. The growth was determined by spectrophotometer at 600 nm. Medium composed of cactus extract and seawater was shown to be superior to other media for the growth of B. thuringiensis.

In conclusion, the finding of this cheap formulation should contribute to the reduction of the cost of B. thuringiensis bioinsecticide production. This would encourage local large scale production

Keywords: B. Thuringiensis, Mosquito Control, Cost-Effective, Pesticides, Raw Materials

Introduction

Overview

Global use of insecticides for mosquito control in recent decades has caused environmental pollution of aqueous ecosystems and has resulted in the development of insecticide resistance in many mosquito species [1]. Production and use of bioinsecticides have been drawing increasing attention to environmentally hazardous chemicals. Bacillus thuringiensis is one of the most commonly used products of such microbial insecticides. B. thuringiensis bioinsecticides are composed of mixture of spores and crystals harvested from the production media [2].

More than 400 insect species have developed the ability to resist chemical insecticides and this reduces their field efficacy [3]. Many chemical pesticides are difficult-to-degrade, can accumulate in the environment and hence might pollute surface and underground waters, soils and foodstuffs [4].

The high cost of B. thuringiensis products is due to production being located in the developed countries where production costs are higher, and also due to expenses paid in transportation to the op-

erational sites. Thus, local production should significantly reduce costs of pest control and could help the development of local fermentation industries and their improvement, besides the utilization of agro-industrial byproducts. However, BT is considered ideal for mosquito control because of its high specificity, lack of toxicity to humans [5], and to non target aquatic organisms found in association with mosquito larvae [6]. Furthermore, the major advantage of this biocide is that risk of development of resistance of mosquitoes to BT. based products is very low, due to its multi-toxin complex [7].

A less expensive medium for culturing of BT. will facilitate the production of biopesticides in a cost-effective manner. Bacillus thuringiensis was produced in different media using the seeds of legumes, dried cow blood, and mineral salts. The resulting insecticidal toxins were effective against Aedes, Anopheles, and Culex species [8]. Defatted groundnut cake as the Worst nitrogen source and gram Xour, soybean, and defatted milk powder as the second nitrogen source for the bulk production of BT have also been tested [9]. Like other microorganisms, BT grows in a culture medi-

um containing sources of carbon and nitrogen as well as mineral salts. Production of BT can be described by three phases: vegetative growth (exponential phase), transition phase and sporulation phase. Use of some of these raw materials resulted in biopesticides having good entomotoxicity [10]. In the conventional processes, the production of T and BS (*Bacillus sphericus*) based biopesticides requires a synthetic medium containing soybean [1], glucose [11], corn extract [12], common sugar [13]. More ingredients are added to enhance the sporulation process. Poopathi and co-workers have also reported cost-effective culture media, using potatoes, for the growth of mosquitocidal bacterial toxins [13]. Recently, a cost effective culture medium was successfully developed from poultry industry waste, i.e., chicken feathers, enabling the growth of BS and BT [13]. Wheat bran has been widely used for production of entomopathogenic fungi such as *Cordyceps* spp. and *Beauveria bassiana* [14], and the antagonistic fungus *Trichoderma* spp. [15]. The primary composition of wheat bran is 71% carbohydrate, 16% fat, and 13% protein. Wheat bran is a good source of carbohydrates, vitamins, and minerals with a low level of saturated fat.

In this study, wheat bran and potato were tested as carbon sources, while, rabbit feed as protein source, seawater and or stock solution for mineral source, and we investigated *B. thuringiensis* growth in cabbage and cactus extract based on the fact that BT are usually isolated from soils planted with cactus and cabbage. Optimization of pH and temperature was also performed.

Objective

1. To develop a cost effective media, based on a locally available raw material.
2. To develop a biological pesticide instead of harmful chemical pesticide.
3. To recycle waste product media and used it in useful applied.

significant

Today, the world tend to use biological insecticides as safe and economic alternative of chemical insecticides which may be toxic and causes diseases for humans and animals., In addition, pollution with insecticides has long term hazardous effects. Therefore, the development of a cheap media based on raw and locally available materials to maximize the growth of BT which is considered as safe insecticide would encourage local production and use of biological control methods in Gaza strip.

Our research will provide chance for use that bacteria which is friendly to the environment contributing to reducing of chemical pollution resulting from the traditional methods of mosquitoes control. It is also well documented the mosquitoes transmit several diseases. Reducing the number of mosquitoes acting as vectors will also reduce incidence of those diseases.

Literature review

Definition of bacteria

Bacillus is a gram positive, aerobic, spore forming soil bacterium that has been used in recent years as a microbial larvicide, to control mosquito vector populations [16]. It shows major toxic effect against larvae of filariasis (*Culex*) and malaria (*Anopheles*) vectors

[13]. This bacterium is characterized by its ability to produce crystalline inclusions proteins or crystals called endotoxin during sporulation and/or stationary phase. The crystalline inclusions along with the spores have a great potential to control a great number of pest insects belonging to the order Lepidoptera, Diptera and Coleoptera [28]. The Bt biopesticide preparations are based on endotoxin proteins accumulated as parasol crystals produced by the bacterial cells. Various agricultural and industrial by-products may be used as raw materials in biopesticide industry, such as maize glucose, soybean flour, peanuts, sugarcane molasses, which also contain N, and liquid swine manure [29]. Also mentioned less expensive complex sources of organic nitrogen such as grains, beans, oilseeds, fish and meat meals. Moreover, the low cost of by-products as nutrients sources in fermentation media of Bt biopesticide production has received little attention. However, a higher level of entomotoxicity is desired to reduce biopesticide production costs.

Bacterial delta-toxin

Bt toxins are a potential alternative to broad-spectrum insecticides. The toxicity of each Bt type is limited to one or two insect orders; it is nontoxic to vertebrates and many beneficial arthropods, because Bt works by binding to the appropriate receptor on the surface of mid gut epithelial cells. Any organism that lacks the appropriate receptors in its gut cannot be affected by Bt. A 2007 study funded by the European arm of Greenpeace suggested the possibility of a slight but statistically meaningful risk of liver damage in rats [17]. The observed changes have been found to be of no biological significance by the European Food Safety Authority. Furthermore, the major advantage of this biocide is that risk of development of resistance of mosquitoes to *B. t. i.* based products is very low, due to its multi-toxin complex [7]. The main criterion for *B. thuringiensis* differentiation from other soil-forming bacteria was crystal production by sporulation cultures [30].

Bacterial culturing media

In spite of many advantages of Bt based biopesticides, their application is limited due to high production costs [18]. In conventional processes, Bt insecticide is commercially produced on synthetic medium comprising soybean meal, fishmeal, glucose, yeast extract, peptone and trace elements and sometimes more ingredients are added to enhance the sporulation process. The cost of Bt production depends on many factors; however, the raw material cost is one of the most important factors and may comprise 30–40% of the overall production cost [19]. Therefore, selection of growth medium or raw material is critical for commercial production of this biopesticide. Several raw materials (industrial and agriculture byproducts) have been tested as alternative culture media for the production of Bt biopesticides. Wastewater sludge can be a very good source of carbon, nitrogen, phosphorus, and other nutrients for many microbial processes that could add value to sludge by producing certain valuable metabolic products e.g. endotoxins. Recently, the expensive soya based synthetic media for Bt production have been successfully substituted with agro-industrial wastes (maize starch, wheat bran, rice straw, corn steep liquor, etc.) and wastewater sludge [20]. Solid-state fermentation

(SSF) technology offers an alternative fermentation method that possesses several biotechnological advantages over the conventional submerged fermentation [21]. They are: low medium cost and energy consumption, low wastewater output, high stability of the products [22], and some spore-forming microorganisms only sporulate when grown on a solid substrate. Though SSF technology has recently been practiced to produce Bt and other microbial control agents using soybean seed or agricultural wastes as raw materials [23]. Several locally available waste materials such as corn steep liquor, coconut waste, rice bran and molasses have been used for the production of B. t. i. [24].

Bacillus thuringiensis was produced in different media using the seeds of legumes, dried cow blood, fishmeal and corn steep liquor [25], powders of edible leguminous seeds and cane sugar molasses [25], corn extract and corn steep liquor [11,12] or potato starch Bengal gram (Cicer arietinum) [26]. In the conventional processes, the production of Bti- and Bsbased biolarvicides requires a synthetic medium containing soybean [1], glucose [11], corn extract [12], common sugar [13], etc. and sometimes more ingredients are added to enhance the sporulation process. The expensive cost of raw materials highly constrains their commercial production.

Recently, we have successfully developed a cost-effective culture medium from poultry industry waste, i.e., chicken feathers, enabling the growth of Bs and Bti [13], but in other research they used locally available raw materials such as soybean flour (Gly-

cine max), groundnut cake powder (Arachis hypogea) and wheat bran extract (Triticumaestivum) for the large-scale production of B. t. i. [1].

Control of Mosquitoes

Mosquitoes cause great nuisance to human beings and pose threats to public health as vectors of diseases like malaria, filariasis, dengue, Japanese-encephalitis, West Nile fever. Annually 300 million people are estimated to be affected by malaria transmitted by Anopheles mosquitoes with more than one million death [31]. Several strategies have been adopted to control these dipteran pests and to reduce vector-borne diseases. Synthetic insecticide have been effectively used during the past several decades for mosquitoes control operations. But the chemical approach has demerits such as the development of insecticide resistance, environmental pollution bioamplification of contamination of food chains and harmful effects of beneficial insects. Hence, there has been an increased interest in recent years in the use of biological control agents for mosquitoes control [32]. The discovery of bacteria like Bacillus thuringiensis subsp. Which are highly toxic to dipteran larvae opened up the possibility of the use of these biolarvicide in mosquito eradication programs [33]. Mosquito pathogenic bacilli have some advantages over conventional insecticide in mosquito control operations because they have a broader host spectrum, are safer for non-target organisms (including human) and are more environmental friendly.

Materials and Methods

Materials

Equipments

Centrifuge	Hanil S500 4B
pH meter	PinPoint
Autoclave	Delama
Shaker Incubator	n-biotek.inc
Incubator	Memmert
Automatic pipettes	Dragon-Med
Digital camera	S4
Glass tube, elementary flask, beaker	Simax, Germany
Spectrophotometer	Lange DR5000

Disposals

Cotton, Filter paper, Glassware, Inoculating plastic loops, Parfilm, Plastic droppers, Plastic Petri plates, Tips, cuvettes.

Methods

Optimization of Ph and Temperature

Ph optimization

Five different Ph media (6.5, 6.7, 6.9, 7.2, 7.4) PH were prepared by using NB (Nutrient Broth) glass tube, each one repeated three-times, then inoculated with isolated Bacillus thuringiensis and incubated 24hr at 37°C. The Ph was controlled with Ph meter by adding tiny drops of NaOH or HCl with constant stirring.

Temperature optimization

Five NB glass tubes (7.2 Ph) were incubated at different temperatures (25, 27, 30, 33, 36)°C for 24hr. The growth were indicated by using spectrophotometer (600nm), and NB media as blank.

Media Selection

Material preparation

A 30g of wheat bran or rabbit feed were added to 1L distilled water (DW) and boiled for 10 min, then centrifuged at 3000 rpm for 10 min to have less turbid liquid. A 200g cabbage leave, potato, or cactus were placed in 200 ml boiling DW for 20 min, then centrifuged (3000rpm, 10min). A 1.0g MnCl₂, 20.3g MgCl₂, 10.2g CaCl₂ were put to 100 ml DW to make 1% stock solution (7.2 pH).



Figure3.1.: Wheat bran extract.

Media preparation

A Collected mixtures of the previous liquid were tested as a whole combinations of all trials, as shown in Table3.1..



Figure3.3.combinations of media.

Sterilization and Cultivation

All media combination for the proper media selection were steril-



Figure3.5.:Media after cultivation



Figure3.2.: Centrifuge with 50 ml tube capacity.

All the selected media had a constant pH and so temperature as it determined in the first place, which is 7.2 pH and 30°C. The pH was controlled with the same procedure (NaOH, HCl, pH meter), and the temperature was set on the shaker incubator.



Figure3.4.:Shaker Incubator

ized at 121°C for 30 min, then when cooled were inoculated with 200 µl of *B. thuringiensis* in normal saline and cultivated for 24 hr at 30°C using shaker incubator.



Figure3.6.: Spectrophotometer

Data analysis

Data generated from the experiments were tabulated as Microsoft Excel sheets. Table and graphs were generated.

Media combination	Fractional Volumes (ml)		
1-wheat bran	100	17-cabbage+rabbitfeed+ wheatbran+ seawater+ stock	25+25+25+25+10
2-wheatbran+ seawater	50+50	18-potato	100
3-wheatbran+ stock solution	100+10	19-potato+ seawater	50+50
4- rabbit feed	100	20-potato+ wheatbran	50+50
5-wheatbran+ rabbit feed	50+50	21-potao+rabbitfeed	50+50
6-wheat bran+ rabbit feed+ stock	50+50+10	22-potato+ seawater+ stock	50+50+10
7-wheatbran+ rabbitfeed+ seawater	25+25+50	23-cactus	100
8-rabbitfeed +seawater	50+50	24-cactus+ seawater	50+50
9-rabbitfeed+ stock	100+10	25-cactus+ wheatbran	50+50
10-cabbage water	100	26-cactus+ rabbitfeed	50+50
11-cabbage water+ seawater	50+50	27-cabbage+rabbitfeed +wheatbran+potato+ seawater	25+25+25+25+10
12-cabbage+ stock	100+10	28-cabbage+rabbitfeed + wheatbran+ potato+ seawater+ stock	25+25+25+25+5+5
13-cabbage+ wheat bran	50+50	29-potato +wheatbran + rabbitfeed+seawater + stock	25+25+25+25+10
14cabbage+rabbit feed	50+50	30-Potato+ stock	100+10
15cabbage+rabbit feed+ wheatbran	50+25+25	31-Cactus+ stock	100+10
16cabbage+rabbit feed+ wheatbran + seawater	25+25+25+25	32-Cactus+ seawater + stock	50+50+10
		33-cactus+ potato	50+50
		34-wheatbran+cactus + rabbitfeed	25+50+25
		35- seawater+ stock	100+10

Table3.1.: Media preparation and their respective volumes.

Optimization of pH and Temperature

PH Optimization

The highest absorbance at(600nm) for the growth of the test

organism at a temperature of 30°C was at pH7.2, as show in table (4.1).

pH	Absorbance (600nm)
6.5	0.313
6.7	0.489
6.9	1.122
7.2	1.125
7.4	0.445

Table 4.1.: Bacterial growth at different pH .

Temperature optimization

Different absorbance values (600nm) for bacteria growth resulted for predetermined different temperature, and it was the 30°C

temperature that showed significantly highest one as it is shown in table 4.2..

Temperature(°C)	Absorbance (600nm)
25	0.458
27	0.712
30	2.935
33	0.774
37	0.594

Combinations of the media

Thirty five media combinations showed greatly varied bacterial

growth, which was elevated in some and decreased in others. The results of these combination is listed in table 4.3..

Media combination	Abs 600nm
1-wheat bran	0.618
2wheatbran+seawater	0.453
3-wheat bran+ stock	0.009
4- rabbit feed	0.312
5-wheatbran+ rabbitfeed	0.785
6-wheatbran+ rabbit feed+ stock	0.004
7-wheat bran+ rabbit feed+ seawater	0.332
8rabbitfeed+seawater	0.235
9-rabbit feed+ stock	0.002
10-cabbage water	0.193
11-cabbage+seawater	0.006
12-cabbage+ stock	0.241
13cabbage+wheatbrn	0.308
14-cabbage+ rabbitfeed	0.689
15-cabbage+ rabbit feed+ wheatbran	0.698
16-cabbage+ rabbit feed+ wheatbran+ seawater	0.080
17-cabbage+ rabbitfeed+ wheatbran+ seawater+ stock	0.110
18-potato	0.424
19-potato+ seawater	0.160
20-potato+ wheatbran	0.052
21-potao+rabbitfeed	0.059
22-potato+ seawater+ stock	0.756
23-cactus	0.259
24-cactus+ seawater	1.043
25-cactus+ wheatbran	0.294
26-cactus+ rabbitfeed	0.819
27-cabbage+ rabbitfeed+ wheatbran+ potato+ seawater	0.549
29-potato+wheatbran +rabbitfeed+seawater+stock	0.390
30-Potato+ stock	0.038
31-Cactus+ stock	0.113
32-Cactus+seawater+ stock	0.119
33-cactus+ potato	0.462
34-wheatbran+cactus+ rabbitfeed	0.332
35- seawater+ stock	0.756

Evaluation of media selection according to B. thuringiensis growth

From the 28 media combination trials, there are 8 suggestions for the media selection, which is considered to have the most highest

values of absorption for bacterial growth (≥ 0.500 at 600 nm). In table4.4, cactus and seawater media seemed to have the biggest value for suggestion as B. thuringiensis media which is cheap enough for toxin production.

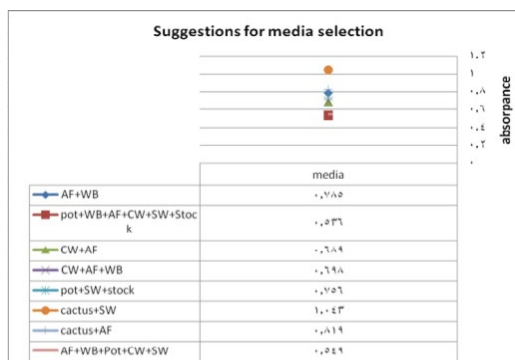


Table4.4.: Suggestions for media selection

Discussion

Mosquito borne diseases remain a serious global, public health problem. Mosquito control is an essential component of disease control and relies on the use of chemical insecticides, though they are expensive and toxic to non target organisms. The discovery of biopesticides (Bs and Bti) has revolutionized over conventional insecticides in mosquito eradication programs. The high cost of conventional media components, to produce these biopesticides on a large scale, make it necessary to utilize cheap and commonly available biological waste materials through simple fermentation technology. During development of cost effective media, we had variable results for several media combinations, some of them consider suitable for optimum bacterial growth, while others weren't ideal for reasonable production.

In our study, wheat bran, rabbit fed, seawater and others consider to locally cheap and available waste product, in other research they used some material such as coconut cake, feather, rice water, and sludge waste that is relatively economically cost effective and suitable for media optimization.

pH and Temperature Optimization

In this study 7.2 Ph and 30°C had the best reading for *B. thuringiensis* growth, which had significant results in comparison to other Ph and temperature intervals. While in other studies, The pH of the medium was adjusted to 7.2. NYSM broth was used for comparison, The flasks were incubated on a rotary shaker at 30 °C and at 250 rpm for a period of 36 h [1]. The pH of the medium was adjusted to 7.2 prior to Inoculation, and the Second stage, seed was prepared by transferring 10ml of first stage seed into 600 ml x2 of the appropriate medium in a 2 L Erlenmeyer flask and incubating on a rotary shaker at 30 °C, 180 rpm for a period of 6 h [1].

Ejiofor and Okafor using different culture media, studied the influence of pH on the

growth and sporulation processes of Bt. When the pH was near 5, there was weak sporulation and crystal formation. These authors concluded that low pH could inhibit growth, sporulation and crystal formation of Bt [27]. Abdel-Hameed also stated that if the pH of a culture medium is not between the range 6.5–7.5, sporulation and d-endotoxin formation can be adversely affected [3].

Media selection

According to the result, cactus and seawater consider to have the major reading for bacterial growth (1.043 at 600nm), which may have suitable mineral and carbohydrates concentrations, while cactus and rabbit fed (0.819) relatively had good growth, in comparison with cactus and wheat bran (0.294), while cactus alone had a growth of (0.259), which may make its combination with seawater as critical source for salts. Wheat bran and rabbit fed had a growth of (0.785) which is higher than other wheat bran media, such as wheat bran (0.618), wheat bran and seawater (0.453), wheat bran, rabbit fed and seawater (0.332), wheat bran and cabbage (0.308), and with cactus (0.294), and the lowest one was with potato (0.052). Rabbit fed in combination with cactus

(0.819) had the highest growth in rabbit fed media combination, such as with wheat bran and cabbage (0.698), then with cabbage (0.689), and rabbit fed only had (0.312), and other combinations had much lower growth. Potato media had relatively intermediate growth (0.424), while with seawater had (0.160), and in different way from other media it raised with stock solution (0.756), which might be because potato extract had less mineral concentration. When combined overall material without stock solution, the growth was (0.549), but sharply decline when incubated with stock solution (0.110).

Earlier, many Bs and Bti formulations produced from conventional media (Luria Bertani and NYSM) have been tested in the field for mosquito control. Subsequently, cost effective formulations were utilized for biopesticide production. Obeta and Okafor cultured Bti on five formulated media from seeds of legumes (groundnut cake, cow pea of white and black varieties, soya bean, bambara beans), dried cow blood and mineral salts [8]. Poopathi, Poopathi and Anup kumar and Prabakaran also have used potatoes, coconuts, fishmeal, cornsteep liquor and soybean for the production of biopesticides [1,13,24]. A medium containing glucose solution, corn extract, sodium humate and mineral salts resulted in an increase in biomass titer by 45%, endotoxin by 220% as compared to the initial medium [12].

Chapter VI Conclusions and Recommendations

Conclusions

The selected media should contribute to a significant reduction of the cost of *B. thuringiensis* bioinsecticide production and utilization, which extremely expensive in the original media, that prevent them from use as economical and biological source of insecticide. And also played a significant role for elimination of disease incidence such as misquote that act as vector of Plasmodium which spread Malaria. In the other hand it consider as suitable alternative to the harmful chemical pesticide, that cause several side effects on the plants, animals and so human being.

In our study, we developed a cheap, safe, productive media for optimum production of *B. thuringiensis*, based on using of locally cost-effective raw materials, that easily to extract and prepare, and most of them consider to be waste product. We ended to select the "cactus and seawater" media to be the best media for bacterial growth.

Recommendations

In light of the result of this study and the above listed conclusions, we are recommended to:

- 1- Develop other media that based on utilizing of what might be more cost-effective and productive media.
- 2- Strategies to replace chemical pesticide of that biopesticide.
- 3- Strategies to recycle waste product to produce productive media.
- 4- Work on the results of this study, to develop it more.
- 5- Develop strategies to apply this study in the

Activity	2012				2013		
	Sep	Oct	Nov	Dec	Jan	Mar	May
Preparatory stage:							
-Literature survey and Proposal writing	<input type="checkbox"/>	<input type="checkbox"/>					
Lab. and Field work:							
-Collection of clinical and environmental material		<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			
-Optimization the cultural condition			<input type="checkbox"/>				
-Media preparation and cultivation				<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
-Data analysis				<input type="checkbox"/>			
-Research writing						<input type="checkbox"/>	<input type="checkbox"/>

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