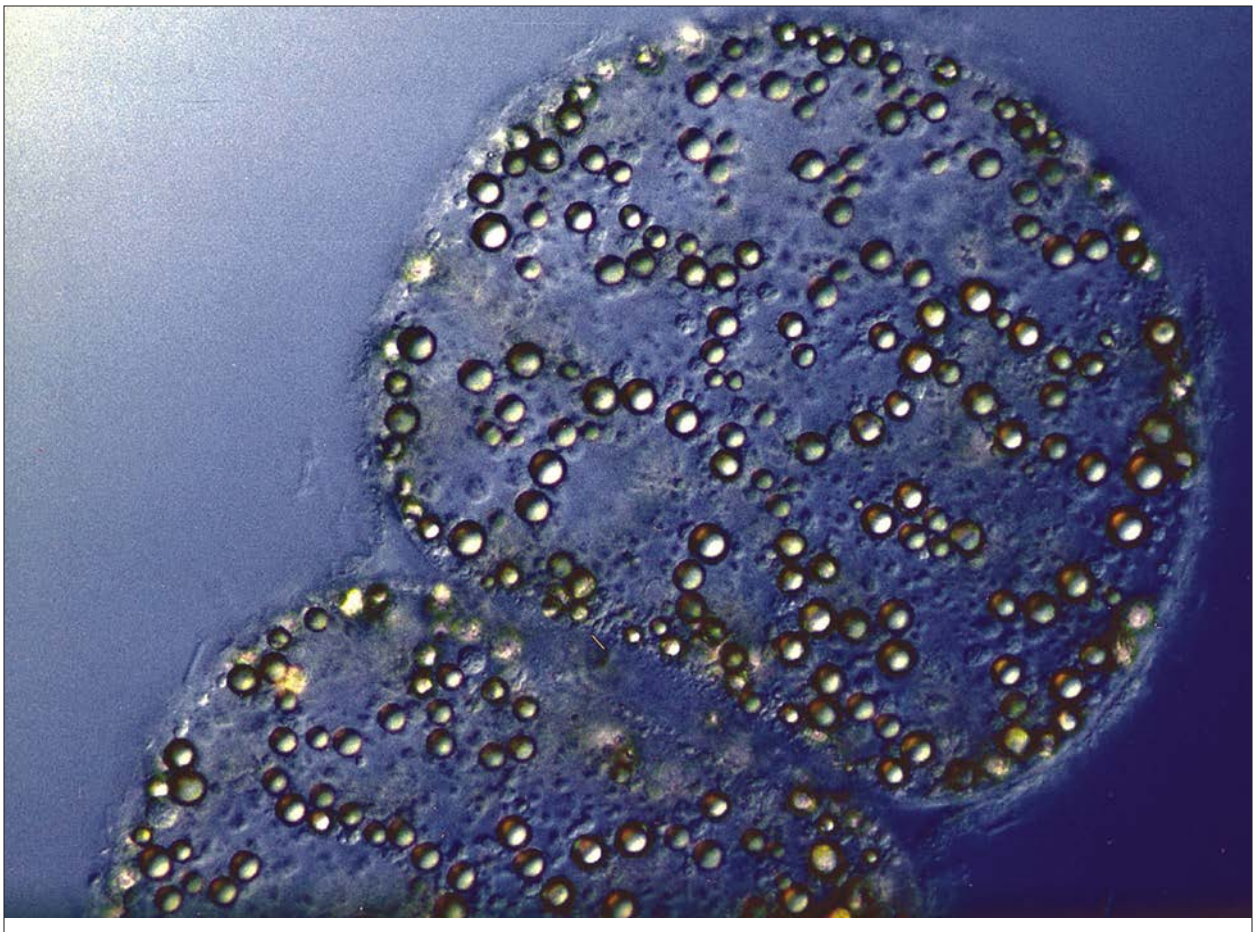

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Development of Cost Effective Medium for Production of *Bacillus sphaericus* Strain Isolated from Assam, India

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ABSTRACT

The commercially available nutrient yeast salt medium (NYSM) was compared with the low cost and locally available media for the production of mosquito larvicidal bacteria *Bacillus sphaericus* strain GC IV in the laboratory. *B. sphaericus* strain GC IV was produced using egg yolk, soybean flour, coconut water and banana pulp as growth media and their mosquito larvicide efficacy was compared with the same bacteria produced in NYSM as reference medium. *B. sphaericus* GC-IV cultured in egg yolk medium produced 100% *Culex quinquefasciatus* larval mortality ($p = 0.0035$, $df = 1$, $\chi^2 = 8.53$) at LC_{90} dose of reference NYSM medium, whereas the total bacterial biomass production was high in soybean flour medium ($p<0.0001$, $df = 4$, $F= 66.83$). The overall cost ratio obtained by considering medium cost and mosquito mortality obtained in all the media used favoured the egg yolk medium. The egg yolk provided a low cost yet efficient medium for culturing the *B. sphaericus* GC IV strain, therefore, it can be used for the large scale production of the mosquitocidal bacterial strain. The present study emphasizes the use of potential and cost effective growth media for mosquitocidal bacteria production in integrated vector control programmes.

Key words: *Bacillus sphaericus*, bioinsecticide, vector control, nutrient yeast salt medium, egg yolk medium

INTRODUCTION

Mosquitoes cause various kinds of direct and indirect harm to public health worldwide. For many years, campaigns to combat mosquitoes were based on spraying chemical insecticides to kill larvae and adults. The initial result of this strategy proved quite satisfactory, leading to the control of various mosquito borne epidemics, especially in early 20th century (Melo *et al*., 2009). However, after several decades, the consequences of this approach proved quite harmful 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 (Poopathi and Tyagi, 2004).

Since the last few decades, the bacilli based mosquito larvicides popularly known as biocides or biolarvicides have become popular in vector control (Porter *et al*., 1993). Mosquito vector control, based on entomopathogenic bacteria *Bacillus thuringiensis* and *B. sphaericus* has been studied for more than 20 years. These biolarvicides have been proved very efficient in controlling various mosquito vectors under laboratory and field conditions. The larvicidal materials of these bacterial

formulations are endotoxin proteins produced by bacteria during sporulation. The major advantages of these biolarvicides are, low application cost and safety to environment, human, animals and other non-target organisms (Yadav *et al*., 2010).

At present, high cost of the nutrient medium for bacterial agents is a major factor to be considered when it comes to large scale production and use of biopesticides (Prabakaran and Balaraman, 2006). Hence, attempts are being made to develop nutrient medium using cheaper raw materials for mass production of these bacteria. The growth, sporulation and enzyme production in bacteria depends upon various conditions and compositions of the culture medium (Utong *et al*., 2006). Various media have been tested earlier for the growth of *B. sphaericus* using raw materials like potato, common sugar and chickpea (Poopathi *et al*., 2002). The *B. sphaericus* strains grown on corn steep liquor produced yield and toxicity comparable to that of strain grown on peptone yeast extract using a laboratory bioreactor (Kuppusamy and Balaraman, 1991). Five different media including seeds of legumes, dried cow blood mineral salts were used to produce the bacteria which were effective against *Aedes*, *Anopheles* and *Culex* mosquito species (Obeta and Okafor, 1983). A simple chemical based defined medium containing sodium acetate (37 or 74 mM) as only source of carbon has been used for the growth of *B. sphaericus* (Massie *et al*., 1985). Egg yolk and white soybean meal are easily available and have been used for the production of a *Bacillus* based mosquito control agent (Prabakaran and Hoti, 2008).

With an aim to develop a low cost medium, the present study was carried out by testing the locally available sources like soybean flour, coconut water, banana pulp and egg yolk as nutrient materials for growing *Bs* strain GC Subgroup IV isolated from Assam, India. The product performance was assessed in term of biomass production, larvicidal efficacy and cost effectiveness as compared to NYSM (Myers and Yousten, 1980) as reference medium.

MATERIALS AND METHODS

Organisms: *B. sphaericus* GC subgroup IV has been isolated previously from the soil samples in Assam, identified at Institute of Microbial Technology (IMTECH) Chandigarh, India and deposited in Microbial Type Culture Collection and Gene Bank (MTCC, AC. No. 3910) (Baruah *et al*., 2008). The strain is maintained on NYSM agar slants in the Defence Research Laboratory, Tezpur itself. The *B. sphaericus* strain isolate was inoculated in NYSM (Hi-Media ingredients) broth and incubated at 37°C for 48 h for complete sporulation before harvesting by centrifugation at 15,000x g for 20 min. Supernatant was discarded and the cell pellet was lyophilized. The larvae of *Cx. quinquefasciatus* were taken from the rearing facility of Defence Research Laboratory, where mosquitoes are reared for research purpose. The present study was carried out during 2009-10.

Media preparation

- Coconut water (CW) broth was prepared by adding 10 mL L^{-1} of stock salt solution (stock salt solution: $MgCl_2$ 20.3 g; $CaCl_2$ 10.2 g; $MnCl_2$ 1.0 g) to 100 mL of coconut water. The pH of the medium was adjusted to 7.2
- Soybean flour medium (SF) was prepared by dissolving 2.5% (w/v) soybean flour in distilled water and filtered through muslin cloth. Then, 10 mL L^{-1} of stock salt solution was added in the soybean solution and pH was adjusted to 7.2
- Ripe Banana Pulp (BP) medium was prepared by blending the ripe banana in a mixer and reduced to puree. Distilled water was added in puree at ratio of 3:1 to make a final puree which

could be poured. After puree preparation 10 mL L^{-1} of stock salt solution was added and pH was adjusted to 7.2

- Egg Yolk (EY) medium was prepared in distilled water by adding 2% (w/v) egg yolk powder (produced by boiling chicken's egg until egg yolk and egg white were solid. Boiled eggs were cooled for 5 min in cold water and egg yolks were separated from the white albumin layer, lyophilized, powdered and stored at 4°C). Ten mililiter per litre of stock salt solution was added and pH of medium was adjusted to 7.2 prior to inoculation

Growth conditions: First stage seed was prepared by inoculating 10 mL of NYSM broth with one loopful of *Bs* cells from a slant culture and incubating on an orbital shaker at 37°C, 180 rpm for 6 h. The seed thus prepared was added to 100 mL of various medium in a 500 mL flask at 2% level (v/v) and the flasks were incubated on an orbital shaker at 37°C, 250 rpm for a period of 36 h. This sample was used for assessment of cell mass and larvicidal activity.

Cell mass: Each culture was centrifuged at 15,000 x g for 20 min (Prabakaran and Hoti, 2008), supernatant was discarded and the cell pellet was lyophilized. Dry weight was calculated (g/100 mL) and noted in g L^{-1}. The dried samples were used to determine the toxicity against third instar *Cx. quinquefasciatus* larvae.

Toxicity test: A stock solution of 100 ppm was prepared from *Bs* grown in each medium and desired concentrations were made from the stock solution. Bioassays were conducted in 500 mL glass beakers (n = 6) and required concentration of spore crystal complex formed in each medium was added in 250 mL of water. 25 healthy third instar larvae of *Cx. quinquefasciatus* were introduced in each test concentration. The mortality was scored 24 h post treatment and corrected to control mortality, if any. Larvae exposed to water only were treated as control.

Data analysis: Mortality was corrected using Abbot's formula (Abbott, 1925). The LC_{50} and LC_{90} values were calculated by probit regression analysis (Finney, 1971). Mortality and bacterial biomass production in different media were compared using one way analysis of variance (ANOVA). Mortality in NYSM and egg yolk media were compared using chi-square test. Costs of media were obtained by adding the individual cost of each ingredient and overall cost comparison was made by factoring in the cost involved, biomass produced and percentage mortality achieved.

RESULTS AND DISCUSSION

Dry biomass produced (Table 1) in soybean flour medium (5.20±0.12 g L^{-1}) was significantly greater than other media used ($p<0.0001$, degrees of freedom (df) = 4, $F= 66.83$). The LC_{50} (at 0.02 mg L^{-1}) and LC_{90} (at 0.05 mg L^{-1}) value of indigenous *Bs* strain grown in NYSM media was determined earlier (2,19) and were taken as reference dosages for evaluation of other media sources in the present study. Maximum toxicity in terms of percentage mortality observed in *Bs* produced in egg yolk medium was 76 and 100% at reference LC_{50} and LC_{90} doses after 24 h of treatment (Table 2). Whereas in coconut water 31 and 41.8%, soybean flour 23.2 and 36.4%, banana pulp 13 and 19% LC_{50} and LC_{90} mortality were recorded, respectively. The LC_{50} dose mortality in egg yolk medium was higher than those in other media ($p<0.0001$, df = 3, $F = 89.99$). Similarly LC_{90} dose

Table 1: Comparative cost analysis for production of 1 L conventional (NYSM) and other indigenous media

Culture medium	Media components	Total cost (INR) for 1 L of media	Dry weight of cell mass (g L^{-1}±SEM)	Production cost comparison	Overall cost comparison
NYSM (Conventional medium)	Peptone + beef extract + yeast extract + sodium chloride + salt solution ($MgCl_2$, $CaCl_2$ and $MnCl_2$)	92.67	2.70±0.14	16.5	18.0
Soybean flour (SF)	Soybean flour + salt solution	21.08	5.20±0.12	0.9	2.6
Coconut water (CW)	Coconut water + salt solution	50.08	2.72±0.19	8.3	19.9
Banana pulp (BP)	Banana pulp+ salt solution	10.00	3.02±0.10	1.6	8.4
Egg yolk (EY)	Egg yolk + salt solution	5.00	2.54±0.12	1.0	1.0

L: litre; SEM: standard error mean

Table 2: Toxicity of *Bs* grown in different media against 3rd larval instar of *Culex quinquefasciatus*

	Larval mortality (%)	
Nutrient medium	0.02 mg L^{-1} *	0.05 mg L^{-1} *
Soybean flour	23.2±1.9	36.4±3.1
(95% CI)	(18.05- 28.35)	(27.68-45.12)
Coconut water	31.0±2.9	41.8±2.42
(95% CI)	(22.95-39.03)	(35.09-48.51)
Banana pulp	13.0±2.0	19.0±1.9
(95% CI)	(7.45-18.55)	(13.81- 24.19)
Egg yolk	76.0±4.3	100±0.0
(95% CI)	(64.06-87.94)	(100-100)

* Reference LC_{50} and LC_{90} doses from NYSM medium; CI: confidence interval

mortality was also significantly greater in egg yolk media ($p<0.0001$, df = 3, F = 257.73). Further LC_{50} ($p = 0.0003$, df = 1, chi-square (χ^2) = 13.41, relative risk (R^2) = 0.5873, 95% confidence interval [CI] = 0.4497- 0.7670) and LC_{90} ($p = 0.0035$, df = 1, $\chi^2 = 8.53$, $R^2 = 0.4737$, 95% CI = 0.4077- 0.5503) dose mortality were significantly greater in egg yolk medium than in NYSM medium.

Larval mortality rates clearly favored the *Bs* strain cultured in egg yolk medium, the comparison proved even better when the cost of the ingredients was factored in (Table 1). The amount of egg yolk powder required to prepare 1 L of culture medium was 2.0 g which costs INR 5.00 while the same amount of NYSM medium requires INR 92.67. Hence, egg yolk medium was found to be 18.5 times less expensive than the conventional medium. The overall cost comparison ratio (EY: SF: BP: NYSM: CW) comes out to be 1: 2.6: 8.4: 18.0: 19.9 in favor of egg yolk medium.

The production cost of *B. sphaericus* is high primarily due to its inability to utilize carbohydrate based substrate (Kuppusamy and Balaraman, 1990) and requirements for proteinaceous sources for growth, sporulation and toxin production (Jeff-Agboola *et al*., 2006). The difference in the cost of media largely depends upon the various components of a medium. The conventional NYSM medium contains high cost laboratory chemicals which are sold in very small amounts. On the other hand the egg yolk is available throughout the year at much cheaper cost. The overall cost comparison indicated that soybean medium was more than two times and NYSM medium was eighteen time costlier as compared to egg yolk medium. Many attempts have been made in the past to cultivate *Bacillus* to produce toxin at cheaper costs which has provided similar mosquito toxicity

as achieved in the present study (Prabakaran and Balaraman, 2006; Prabakaran *et al*., 2008). The egg yolk medium cultured bacteria have produced high activity comparable to that of commercially available medium against *Cx. quinquefasciatus* (Prabakaran and Hoti, 2008). Similar results have been obtained in the present study where mosquitocidal activity achieved using egg yolk cultured *B. sphaericus* was comparable to the NYSM medium.

The difference in the bacterial bioinsecticide efficiency indicates better *B. sphaericus* fermentation in egg yolk medium which may be due to higher production of endotoxin. *B. sphaericus*, unlike most microorganisms, does not use sugars in energy metabolism because the bacteria lack enzymes that are capable of metabolizing sugars as a source of carbon. The energy compounds, saccharides and polysaccharides are completely absent in the soybean flour medium and protein material are at large. Thus it becomes a favorable growth medium to *B. sphaericus* production. In egg yolk medium there is abundance of proteins and mineral salts which make it suitable for growth and endotoxin production. Therefore, the substrates present in the culture media play important role in the growth and endotoxin production. Martins *et al.* (2006) have found that yeast brewery residues and thrub (heavy fats, proteins and inactive yeast) brewery residue supported the growth and sporulation of *B. sphaericus* strain but only yeast brewery residues media could show better toxicity against *Culex* larvae. Further the different strains of same bacteria may show different growth and toxic activities, which may be due to difference in growth requirements of different strains (Boulenouar *et al*., 2006). In corn steep liquor medium *Bs* 1593 showed no significant changes in biomass yield and toxicity whereas, strain VCRC B-42 showed increased cell yield and toxicity in the same medium (Kuppusamy and Balaraman, 1991). Desai and Shethna (1991) have showed that bulk produced *B. sphaericus* 1593 in medium containing defatted peanut cake and milk powder was highly toxic for fourth instar larvae of *Cx. quinquefasciatus*. These results are similar as obtained in the present research and strengthen the view that cheap culture media with high protein and minerals salt are efficient in bulk production of entomopathogenic bacteria. Media containing dried cow blood, mineral salts and legumes seed has been used to cultivate *B. sphaericus* in Nigeria which were very effective against *Cx. quinquefasciatus* and *An. gambiae* (Obeta and Okafor, 1983).

CONCLUSION

In the present study we have found that egg yolk medium supports the maximum growth of indigenous *B. sphaericus* strain with higher larvicidal toxicity as compared to the other media sources used. Hence it may be very useful substrate for the mass production of *B. sphaericus* and other entomopathogenic bacteria at low cost.

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