Biofertilizers & Biopesticides

Rashad et al., J Biofertil Biopestici 2013, 4:1
http://dx.doi.org/10.4172/2155-6202.1000134

Research article Open Access

Optimization of Bio-Mosquitocidal Production using Local Bacterial Strains of *Bacillus Sphaericus*

Ferial M Rashad*, Waleed D Saleh and Hayam M Fathy

Department of Microbiology, Faculty of Agriculture, Cairo Univ. Giza, Egypt

Abstract

Highly active local strains of *B. sphaericus* were used along with the reference strains 1593 and 2297 to optimize the biocide production. The optimum conditions were as follow: 20% of total flask volume, pH 7.0, inoculum size 3-5×10^4 cfu / ml, 30° C and 200 rpm/5 days. Glycerol was the best C source at 0.5%; a great suppression up to >80% with propionic acid. All tested strains failed to synthesize biocide in the presence of inorganic N as a sole source; beef extract gave the supreme yield. Mg^{2+} was necessary for maximal productivity; however, Ca^{2+} damaged the fermentation. The uppermost productivity was achieved from 25 g/l fodder yeast, 500 ml/l corn steep liquor and 0.3 g/l $MgSO_4$ as a medium. Chicken feather at 30 g/l proved to be an excellent mono component; a superlative improvement was achieved when feather (3.0%) supplemented with 0.03% of $MgSO_4$.

Keywords: *Bacillus Sphaericus*; Low cost-effective media; $MgSO_4$; Chicken feather; Fooder yeast; Corn steep liquor; Toxicity bioassay

Introduction

Various kinds of direct and indirect harm to public health worldwide are transmitted during the blood meals of mosquitoes, resulting in periodic outbreaks in the populations of various countries. Controlling insect populations with chemical insecticides has proven useful. Over time, the use of chemical insecticides has resulted in two major impacts including environmental disruption and mosquitoes developed resistance which undermined this control strategy's value. This quandary has driven the search for alternative control agents/ methods. Within this condition, bacterial insecticides have proven effective in controlling insect vectors [1]. In this concern, *B. sphaericus* and *B. thuringiensis* subsp. *israelensis* have been recommended by World Health Organization to be used as an environmentally friendly mosquito control agent.

Compared to *B. thuringiensis* subsp. *israelensis*, which is the other major bacterium used in the biological control of mosquitoes, *B. sphaericus* offers a distinct advantage, having higher levels of efficacy and environmental persistence. Highly toxic strains of *B. sphaericus* produce two groups of proteinaceous toxins, binary toxin and mosquitocidal toxins (Mtx1, Mtx2, Mtx3). Binary toxin consisting of BinA and BinB that act together to kill mosquito larvae is produced in crystal form during sporulation. Mosquitocidal toxins are produced during the vegetative phase of growth and can act independently without the requirement for any other proteins [2,3].

In view of the fact that the use of locally available effective strains is always advisable in insect control programs, local facilities should be directed for biocide production in a cost effectual manner. In Egypt massive quantities of organic wastes are disburdened from food processing industries; however disposal of these wastes using traditional methods might be detrimental to the environment. The exploitation of such waste through biotechnology seems to meet the objectives of the growing interests for acquiring additional benefits. In one hand the manufacturing a cost-effective biocides; on the other hand diminish the environmental pollution.

In fermentation, *B. sphaericus* does not require a complex composition medium for growth. It has several important phenotypic properties, including those of being incapable of polysaccharide utilization and having exclusive metabolic pathways for a wide variety of organic compounds and amino acids [4,5].

Development of cheaper media for biocide production is a most important factor to be considered. Most of studies cited aimed to obtain microbial growth and toxic protein yield similar to or higher than that attained by reference laboratory medium, in such comparative studies researchers always used one random reference medium [6-11]. In our previous study [12], we evaluated five conventional laboratory media named: poly medium, nutrient yeast extract salt, glucose-glutamate-salts-EDTA, Luria Bertani and acetate yeast extract medium, for the growth and biocide production by *B. sphaericus* strains EMCC1931, EMCC1932 along with the reference strains. The results proved that medium composition has a great effect on the growth, sporulation and biocide production. Poly medium was found to be the most propitious medium. In the present study poly medium used to optimize nutritional requirements as well as cultural conditions and to develop low cost effective media for maximal production of mosquitocidal bacterial toxins.

Materials and Methods

Microorganisms

Two actively marked toxic strains of *Bacillus sphaericus* EMCC 1931 and *B. sphaericus* EMCC 1932, previously isolated from the soil of north Sinai in Egypt [12], were used in the present study along with the reference strains of *B. sphaericus* 1593 and 2297 as highly toxic strains [2]. The reference strains, *B. sphaericus*1593 and *B. sphaericus* 2297, were kindly provided by Prof. Dr. Y. A. Osman, Mansoura University and Prof. Dr. M. S. Foda, National Research Center, respectively.

B. sphaericus strains were grown on nutrient agar slants at 30°C for 72 hr. Seed cultures were carried out following the technique of Obeta and Okafor [13]. The slant cultures were washed with 5.0 ml sterile distilled water, which were then added to 250 ml flasks containing 50

***Corresponding author:** Ferial M Rashad, Department of Microbiology, Faculty of Agriculture, Cairo University Giza, Egypt. E-mail: ferialrashad@yahoo.com

Received May 16, 2013; **Accepted** June 27, 2013; **Published** July 01, 2013

Citation: Rashad FM, Saleh WD, Fathy HM (2013) Optimization of Bio-Mosquitocidal Production using Local Bacterial Strains of *Bacillus Sphaericus*. J Biofertil Biopestici 4: 134. doi:10.4172/2155-6202.1000134

ml nutrient broth. The flasks were placed on a rotary shaker at 200 rpm and incubated for 24 hr at 30°C. From these first- passage seed cultures, 5.0 ml were used to inoculate similar seed flasks and treated as above for 18 h.

Optimization of Fermentation Conditions

Series of experiments were carried out using the poly medium [14] that showed the best productivity [12] to optimize fermentation conditions for maximum growth, sporulation, toxic proteins synthesis and their activity against 3rd instar *Culex pipiens* larvae.

Effect of fermentation time

Flasks in triplicate containing medium (20% of total flask volume, Pyrex Erlenmeyer flask, Cat No: #4980-250) were inoculated from a second passage seed cultures of the four strains and incubated on rotary shaker following the same previous conditions. Samples from each culture were taken at zero time, 3, 6, 9, 12 h, then at daily intervals until 7 days to follow up the fermentation time. pH values of culture samples were directly measured using a digital pH meter (3020, Jenway, UK).

Effect of inoculum size

Flasks were loaded with poly medium, at 20% of total flask volume, inoculated in triplicate with different volumes of a second passage seed culture of each *B. sphaericus* strain to give initial counts of 5-7×10^3 to 1-2 ×10^5 cfu / ml and allowed to grow following the same previous conditions.

Effect of working volume

Different working volumes of poly medium were loaded to the flasks, in triplicate, at levels of 10, 20, 40 and 60% of total flask volume. The loaded flasks were inoculated with the selected best inoculum from a second passage seed cultures and incubated following the same previous conditions.

Effect of shaker speed (rpm)

Flasks were loaded, in triplicate, with medium at the selected best level of 20% of total flask volume, inoculated with the selected best inoculum from a second passage seed culture of each *B. sphaericus* strain and incubated for 5 days on a rotary shaker at 150, 200, 250 and 300 rpm at 30°C ± 1.

Effect of temperature

Flasks were loaded, in triplicate, with medium at level of 20% of total flask volume, inoculated with the selected best inoculum from a second passage seed culture of each *B. sphaericus* strain and incubated at 20, 25, 30, 35 and 40°C for 5 days on a rotary shaker at 200 rpm as the selected best speed.

Effect of medium pH

Poly medium was adjusted to different pHs: 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. Three flasks of each pH level were loaded at 20% (of total flask volume), inoculated with the selected best inoculum from a second passage seed culture of each *B. sphaericus* strain and incubated at 30°C ± 1 as the selected best temperature for 5 days on a rotary shaker at 200 rpm.

Optimization of Nutritional Requirements

Series of experiments were designed to optimize the nutritional requirements in the growth medium of *B. sphaericus*, using different carbon and nitrogen sources based on the same C/N ratio of the poly medium. The ratio of carbon to nitrogen was determined as 3.5:1.0 and calculated to give final concentrations of carbon and nitrogen as 8.43 and 2.40 g/l [12]. In addition, the effect of inorganic mineral salts was also studied.

Three flasks were loaded from each prepared medium (pH=7.0) at 20% (of total flask volume), inoculated with selected best inoculum from a second passage seed culture of each *B. sphaericus* strain and incubated at 30°C ± 1 for 5 days on a rotary shaker at 200 rpm.

Carbon source

An equimolar C concentration of acetate, citrate, pyruvate and propionic acid were added instead of glycerol (sodium acetate, 13.3; sodium citrate, 11.6; sodium pyruvate, 11.9 g/l and propionic acid, 8.0 ml /l), depending on the same final concentrations of C and N of the poly medium. Another experiment was carried out to detect the best concentration of glycerol depending on different C\N ratios as the most obtained favorable carbon source (Table 1).

Nitrogen source

Six sources of inorganic and organic nitrogen were used singly and/or combined together to prepare new media having the same concentrations of C and N as 8.43, 2.40 g/l, respectively, of the selected poly medium as presented in Table 2.

Minerals

To study the effect of mineral salts, the selected poly medium was supplemented with the following salts: $MgSO_4$ (0.3 g/l), $MnSO_4$ (0.02 g/l), $CaCl_2$ (0.1 g/l), NaCl (3 g/l), KH_2PO_4 (0.5 g/l) and K_2HPO_4 (0.5 g/l), singly or in combination as illustrated in Table 3. KH_2PO_4 and K_2HPO_4 were added as phosphorous and potassium sources alone and/or with all minerals mixture. The prepared media with different substitutions were loaded, inoculated and incubated as previously mentioned.

Development of a Cost Effective Fermentation Medium

Solid and liquid proteinaceous organic by-product wastes of the food processing sector including fodder yeast, corn steep liquor and chicken feathers were used to develop a cost effective fermentation medium. These by-products were prepared as follows:

Corn steep liquor (CSL)

Corn steep liquor was obtained from Egyptian Company of Starch and Glucose, Torah, Cairo. It was boiled for 10 min, cooled to room temperature and kept refrigerated at 4°C overnight; then centrifuged to remove the precipitates. Total nitrogen was estimated according to micro-Kjeldahl metod, and carbon content according to the method of Walklay and Black's [15].

Fodder yeast

Fodder yeast was obtained from Sugar and Integrated Industries

Treatments	Glycerol (ml/l)	C (g/l)	N (g/l)	C/N ratio
1	5	6.48	2.40	2.70/1
2	10	8.43	2.40	3.51/1
3	15	10.38	2.40	4.33/1
4	20	12.33	2.40	5.14/1
5	25	14.28	2.40	5.95/1

* Poly medium as control

Table 1: Treatments for determination the best glycerol concentration.

Treatment	N-Sources* Inorganic N- sources (g/l)			Organic N- sources (g/l)			Glycerol (ml/l)
	$(NH_4)_2SO_4$	$NaNO_3$	NH_4Cl	Beef extract	Peptone	Yeast Extract	
1	22.6	-	-	-	-	-	21.50
2	-	14.6	-	-	-	-	21.50
3	-	-	9.2	-	-	-	21.50
4	-	-	-	19.4	-	-	12.82
5	-	-	-	-	15	-	9.76
6	-	-	-	-	-	24.5	8.41
7	-	-	-	7.5	9.2	-	10.95
8	-	-	-	7.5	-	15	10.12
9	-	-	-	-	5.8	15	8.95
Control	-	-	-	5	5	10	10.00

*At C/N ratio of 3.51/1

Table 2: Treatments for determination of the best nitrogen source.

Treatments	Minerals g /l $MgSO_4$ 0.3	$MnSO_4$ 0.02	$CaCl_2$ 0.1	NaCl 3	K_2HPO_4, 0.5 KH_2PO_4, 0.5
1	+	-	-	-	-
2	-	+	-	-	-
3	-	-	+	-	-
4	+	-	-	+	-
5	+	+	-	-	-
6	+	-	+	-	-
7	-	+	-	+	-
8	-	+	+	-	-
9	-	-	+	+	-
10	-	-	-	-	+
11	+	+	-	+	-
12	+	-	+	+	-
13	+	+	+	-	-
14	-	+	+	+	-
15	+	+	+	+	-
16	+	+	+	+	+
Control	-	-	-	+	-

Table 3: Treatments for determination the mineral requirements.

Company, El-Hawamdia, Giza. It is the biomass of *Saccharomyces cereviciae* yielded as a by-product from ethanol production. Total nitrogen and carbon contents were determined, before using in fermentation media.

Chicken feathers

Chicken feathers as poultry industry waste were obtained from chicken shop, Giza. The feathers were washed, air dried; then stored at room temperature to be used as a whole or crushed to fine powder using grinding mill and stored in packed condition at room temperature [9,10]. Total nitrogen and carbon were estimated.

Series of experiments were designed to formulate fermentation media using the above mentioned by-products wastes under the optimized conditions. Poly medium was used in all experiments to compare the results.

In the first set: The basic medium (poly medium) was modified by replacing yeast extract with fodder yeast and supplemented with $MgSO_4$ (Table 5). The prepared media were loaded, inoculated and incubated as previously mentioned.

In the second set: Seven media were formulated using fodder yeast and /or corn steep liquor as carbon and nitrogen sources. The final concentrations of C and N were calculated as g/l (Table 5).

In the third set: A weight of 100 g of the air dried whole feathers was boiled in one liter of tap water for 15 min. After cooling, the feather extract was filtered and the pH of the filtrate was adjusted (pH 7.0 ± 0.1); then dispensed in each of the three flasks at 20% (v/v) for culturing different strains of *B. sphaericus*, sterilized at 121°C for 15 min. After cooling, flasks were inoculated and incubated as previously mentioned.

In the fourth set: Different concentrations of air dried powdered feathers 5, 10, 20, 30 and 40 g/l were mixed with tap water and the pH was adjusted to 7 ± 0.1. Different feather media were dispensed, sterilized, inoculated, and incubated similarly as previously mentioned.

In the fifth set: Feather medium (30% feather) that gave the best yield of cells, spores and toxin proteins was supplemented with 0.3 g/l of $MgSO_4$.

In all fermentation runs, the yield of cells, spores, and toxic proteins were estimated and the final whole cultures were biologically assayed to determine the LC_{50} values against the 3rd instar larvae of *C. pipiens*. Starting and final pH of the fermentation cultures also admeasured.

Total Viable and Spore Counts

Serial decimal dilutions of culture samples were prepared; 1 ml of each dilution (in triplicates) was added to Petri dish, followed by addition of nutrient agar medium. For spore counts, the serial dilutions of culture samples were pasteurized at 80°C for 15 min before plating. Plates were incubated at 30°C for 48 h and the developing B. sphaericus colonies were counted and expressed as cfu/ml and/or spores/ml.

Biochemical studies and toxicity bioassay

Whole culture samples for each strain on different media were centrifuged at 6000 rpm and 4°C for 10 min and washed twice with distilled water. The pellets resuspended in distilled water and used for protein determination and toxicity bioassay.

Treat-Ment	Yeast ext.	Fodder** yeast	Beef ext.	Peptone	Glycerol	C	N	C/N
PM	10	-	5	5	10	8.43	2.40	3.5/1
1	-	5.0	5	5	10	7.38	1.70	4.3/1
2	-	7.5	5	5	10	7.90	1.83	4.3/1
3	-	10	5	5	10	8.43	1.97	4.3/1
4	-	20	5	5	10	10.53	2.52	4.2/1
5*	-	20	5	5	10	10.53	2.52	4.2/1

*plus of $MgSO_4$ (0.3 g/l)
**Fodder yeast contains 21% C and 5.5% N.

Table 4: Modification of the basic fermentation medium (g/l).

Treat-ment	Fodder yeast (g /l)	Corn steep** liquor (ml /l)	C g/l	N g/l	C/N ratios
1*	40	-	8.40	2.20	3.82/1
2	40	100	9.45	2.37	3.99/1
3	30	200	8.40	1.99	4.22/1
4	25	300	8.40	1.89	4.44/1
5	20	400	8.40	1.78	4.72/1
6	25	500	10.50	2.23	4.71/1
7*	25	500	10.50	2.23	4.71/1

* plus 0.3 g/l of $MgSO_4$
** Corn steep contains 1.05% C and 0.17% N.

Table 5: Formulated media containing different concentrations of fodder yeast and corn steep liquor.

Protein determination

Protein extracts were prepared by adding 25 µl of 2 M NaOH solution to each ml suspension followed by incubation at 37°C for 3 hr [16]. After centrifugation and extraction as mentioned above, protein concentrations in the clarified supernatant were determined using the technique of Bradford (1976) with bovine serum albumin (BSA, Sigma) as standard.

Bioassay against *Culex pipiens* larvae

The *Culex pipiens* 3rd instar larvae were obtained from mosquito rearing laboratory in Research Institute of Medical Entomology, Ministry of Health. Serial dilutions of the previously resuspended pellets were prepared in distilled water, and then one ml of each dilution was added to 100 ml distilled water in 200 ml plastic cups. Twenty, 3 rd instar larvae of *C. pipiens* were placed in each cup and suitable amount of larval food was added (ground dried bread: dried Brewer's yeast as 2:1). Experi- ments were conducted at room temperature of 28°C ± 2. Each experiment included 3 concen-trations in triplicates, as well as appropriate control. Larval mortality was scored after 48 h and corrected (if needed) for control mortality using Abbott's formula [17].

Statistical Analysis

Differences were determined with Analysis of Variance (ANOVA) using MSTAT-C statistical package (Michigan State University). Whenever significant differences were detected, means were separated using least significant differences (LSD) at 1% level of significance (MSTAT-C Version 4, 1987).

Results and Discussion

Bio-mosquitocidal production depends on the presence of active entomopathogenic strains, optimization of the fermentation conditions and the composition of fermentation medium. The variation in the composition of the tested media in their contents of carbon and nitrogen sources as well as minerals found to affect wholly both yield and toxicity of biocide [12]. Although, *B. sphaericus* is considered an undemanding bacterial species, neither requiring differential or selective culture media, nor it depends on special conditions to develop. But, vigilance should be exerted in application of conditions developed for one strain of *B. sphaericus* to the fermentation of another.

Optimization of Fermentation Conditions

After a lag phase of 0-6 h, there was a gradual increase in the yield of cells of all tested strains to reach a plateau in the range of 2.5 - 3.1×10^9 cfu /ml after 48 h (Figure 1a). The maximum growth was completed after 96 h and the highest sporulation rate was observed after 120 h and increased with extending the incubation time up to 144 h. The starting time at which the protein synthesized was varied among strains from 6-12 h (Figure 1b). The bioassay showed that 6h old cells of 2297 and EMCC1931 showed larvicidal activity against 3rd instar larvae of *Culex pipiens*, the LC_{50} values were 223 and 466 ng/ ml, respectively. After 12 h the toxicity increased and the LC_{50} values decreased to record 70, 117, 119 and 136 ng/ ml for EMCC1932, EMCC1931, 2297 and 1593 *B. sphaericus* strains, respectively. Significant increase in potency and yield of protein was observed with prolonging fermentation time up to 120 h, whereas the lowest LC_{50} values were 2.8, 3.3, 13.3 and 13.8 ng/ ml for EMCC1931, EMCC1932, 2297 and 1593, respectively. It is evident that highly toxic stains of *B. sphaericus* have the capacity to synthesize mosquitocidal (Mtx1, Mtx2, Mtx3) and binary toxins. Mosquitocidal toxins found to be expressed predominantly in the vegetative phase of

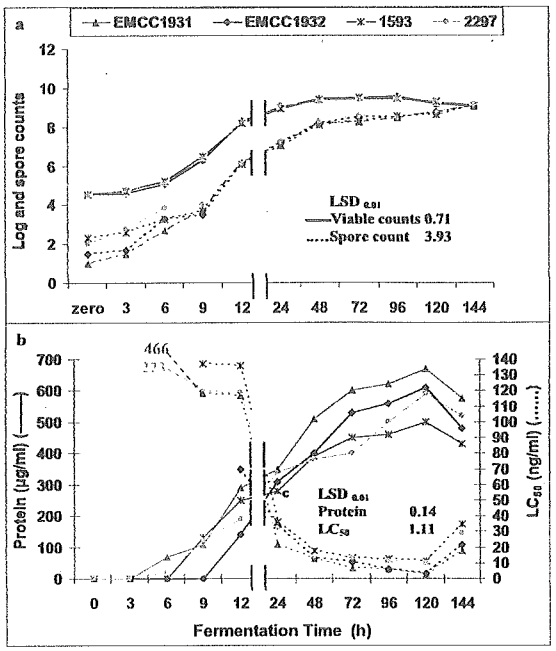

Figure 1: a) Growth cycle and sporulation of local and reference strains of *Bacillus sphaericus in poly medium, b) relation between the protein synthesis* and mosquitocidal activities during growth of local and reference strains of *B. sphaericus*.

growth. Binary toxins as more potent proteins are produced at the onset of sporulation and reached the peak at the completion of sporulation, at which, the bacterial cells lyse and liberate the spore and the attached toxic parasporal body [2,13,18].

Although the rate of sporulation reached the maximum with prolonging fermentation time to 144 h, both the synthesized protein and larvicidal activity showed an influential decrease. Similarly Foda et al. [7] observed progressive increase in toxin production by *B. sphaericus* 14N, 2362 strains followed by decline in toxin level upon prolonged incubation.

The initial density of *B. sphaericus* cells in the fermentation medium had great effect on cell growth, protein synthesis and its mosquitocidal activity. The highest protein yield accomplished by highest toxicity was achieved at 3-5×10^4 cfu / ml as indicated by the lowest values of LC_{50} were 4.2, 8.2, 10 and 13 ng/ ml for EMCC1931, EMCC1932, 2297 and 1593 strains of *B. sphaericus*, respectively (Figure 2a). However, both protein synthesis and potency were in decreasing rate with diminishing or increasing initial concentration of cells. In this concern El-Bendary (1999), obtained the highest toxicities of both reference strain 2362 and Egyptian strain 69 at initial count of 3.7×10^5 cfu / ml, however, an increase of the initial inoculum by at least 10 folds lowered the toxicity under the condition of batch fermentation. In contrary, Foda et al. [7] observed no remarkable variations in growth yields and sporulation titers of the cultures that were initiated with different sizes of inocula in semi solid fermentation.

Working volume and shaker speed (aeration) affected both productivity and lethality of protein by all *B. sphaericus* strains, it was altered by varying loaded volume / flask and the speed rate of shaker.

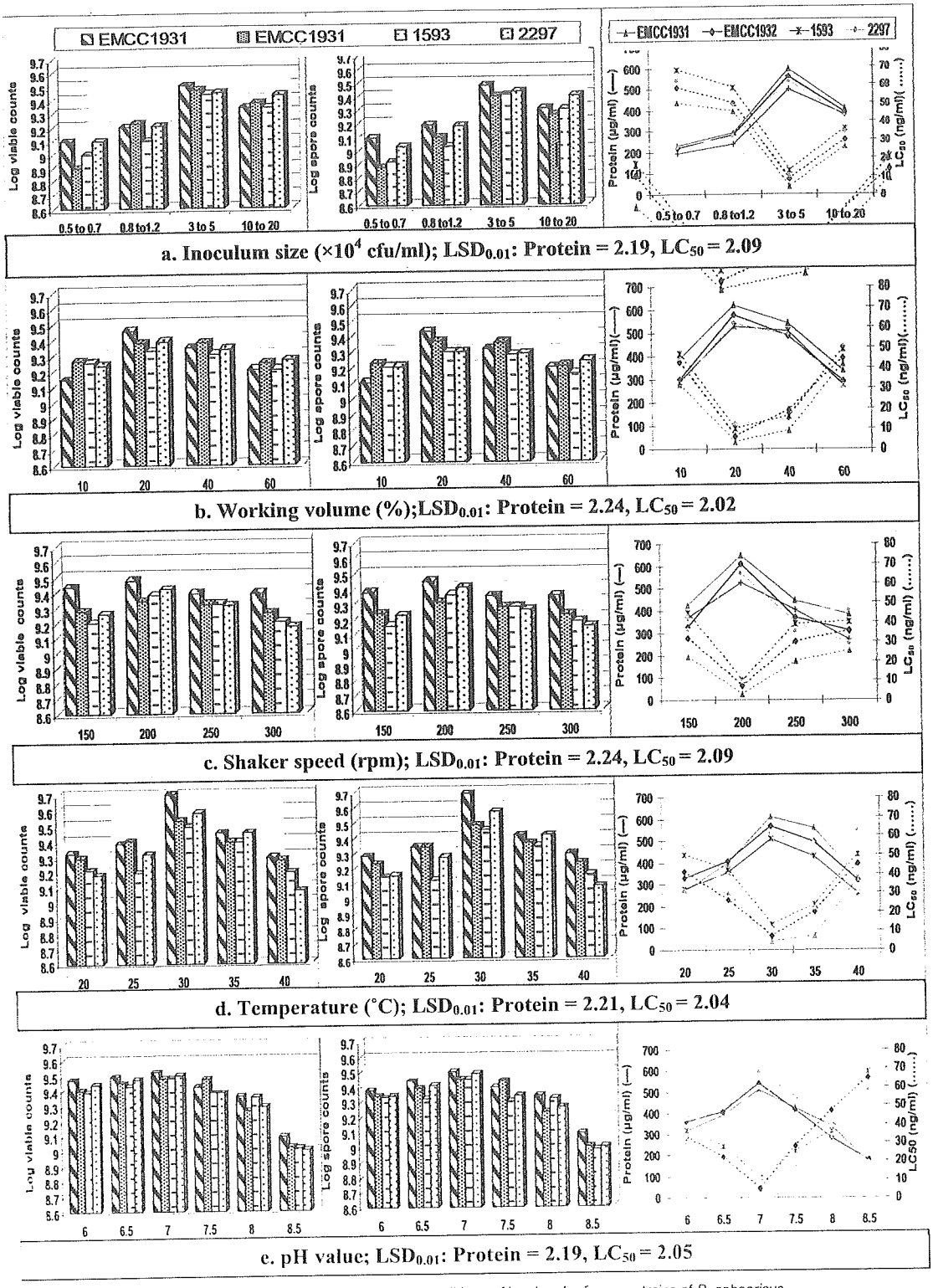

Figure 2: Optimization the fermentation conditions of local and reference strains of *B. sphaericus*.

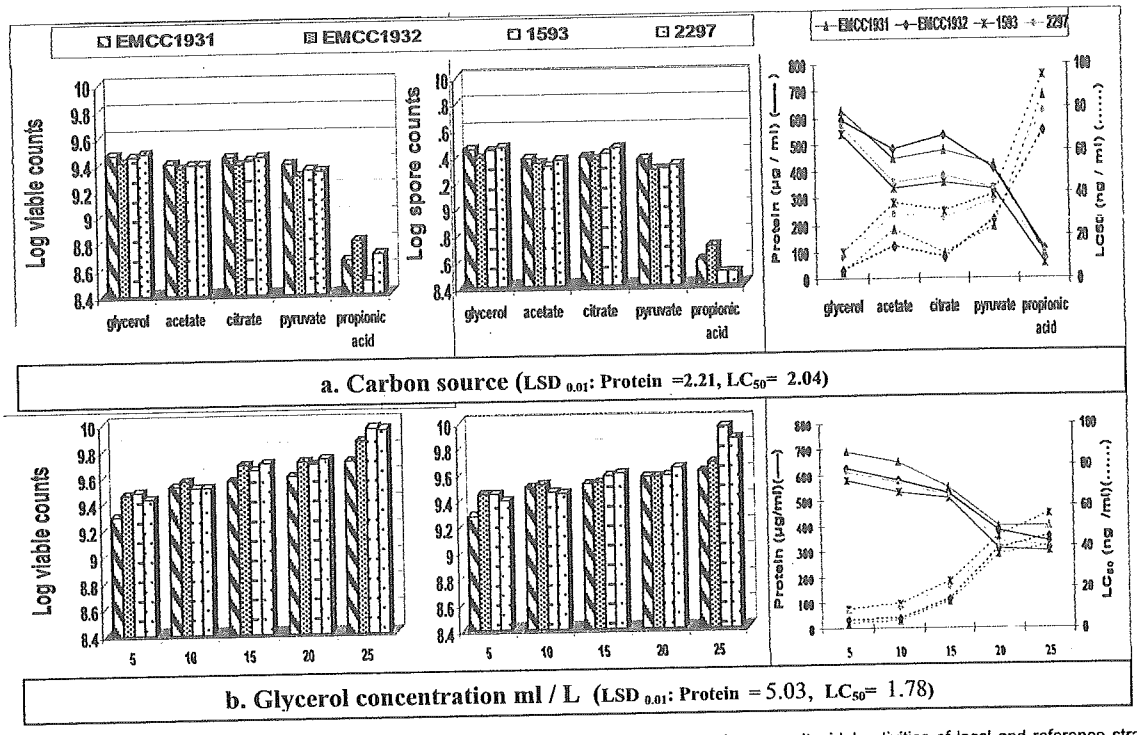

Figure 3: Effect of different: a) carbon sources, b) glycerol concentration on protein synthesis and the mosquitocidal activities of local and reference strains of *B. sphaericus*.

The obtained results indicated that loading volume at 20% of total flask volume and speed rate of 200 rpm being the most optimum at which the utmost yield of toxic proteins and growth was attained by all tested strains. Highest significant synthesis of protein was in parallel with the highest toxicity against the 3rd instar larvae of *C. pipiens* by all the tested strains of *B. sphaericus*. (Figure 2b and 2c). Great inhibition in protein potency by about 50% was observed at 10, 60% loaded volume and shaker speed 150, 300 rpm. These results corroborate the finding of El-Bendary (1999) who found that, the sporulation and toxin activity gave the highest values when the medium volume occupied 20% of the flask for both 2362 and 69 *B. sphaericus* strains. However, some authors reported that sporulation but not toxin production, is inhibited when pure oxygen is substituted for air [13,18,19]. Some others found that, larvicidal activity was greatly affected by the aeration rate [19,20].

Fermentation temperature is a transaction between microorganism stability and the time it takes for the completion of fermentation course. Our results proved that 30°C is the optimum temperature for both local and reference strains illustrated in Figure 2d. At elevated temperatures growth and sporulation did not influence, but toxicity was decreased to drastic levels at both 20 and 40°C. It has been found that *B. sphaericus* strain 1593 grew well at incubation temperature between 25°C and 40°C, but sporulation and toxin production could be inhibited. However, strain 2362 sporulated better than 1593 at all temperatures and the development of toxicity was not suppressed by growth at 35°C [21].

In almost all previous fermentation runs, changes in pH showed a similar pattern, rising from 7.0 ± 0.1 to reach about 8.1-9.1 at the end. However, at the highest loading volumes (60%) or when the fermentation broths incubated at 20°C, the relatively low values of pH (7.1-7.3), (7.2-7.8) were recorded, respectively. Since the members of *B. sphaericus* do not use sugars as a source of carbon, acids are not formed. Rather, ammonia accumulates in the proteinaceous fermentation broth, possibly due to deamination of amino acids; thus, the final pH values of all fermentation broths were arisen. However, lower pH values that were attained at high loading volume of 60% v/v may result from the accumulation of CO_2 in the fermentation broths [19]. As a consequence, both nitrogen and oxygen can have a profound influence on the pH during the fermentations.

Growth of *B. sphaericus* strains in adjusted poly medium to different pH levels revealed that maximum production of lethal protein yield occurred in the medium adjusted at pH 7.0, but it diminished as the pH drifted from neutrality and drastically affected at pH 8.5 (Figure 2e). Local strains, EMCC1931, EMCC1932 were more potent than reference strains 2297 and 1593, respectively, as indicated by LC_{50} values of 3.2, 5.1, 10.2 and 11 ng/ ml. It should be considered that a particular medium pH that may keep up maximum toxic protein production by one strain may be less satisfactory for another. Toxin synthesis in *B. sphaericus* B. 64 remained unaffected in the pH range of 5.5 to 10, *B. sphaericus* 2362 gave relatively the highest toxicity when grown in media buffered at pH 6.0 and 7.0; but gave the lowest values at pH 8.0. Moreover, maintenance of pH around 7.0 resulted in an increase in the toxicity of *B. sphaericus* 1953 [17,22,23].

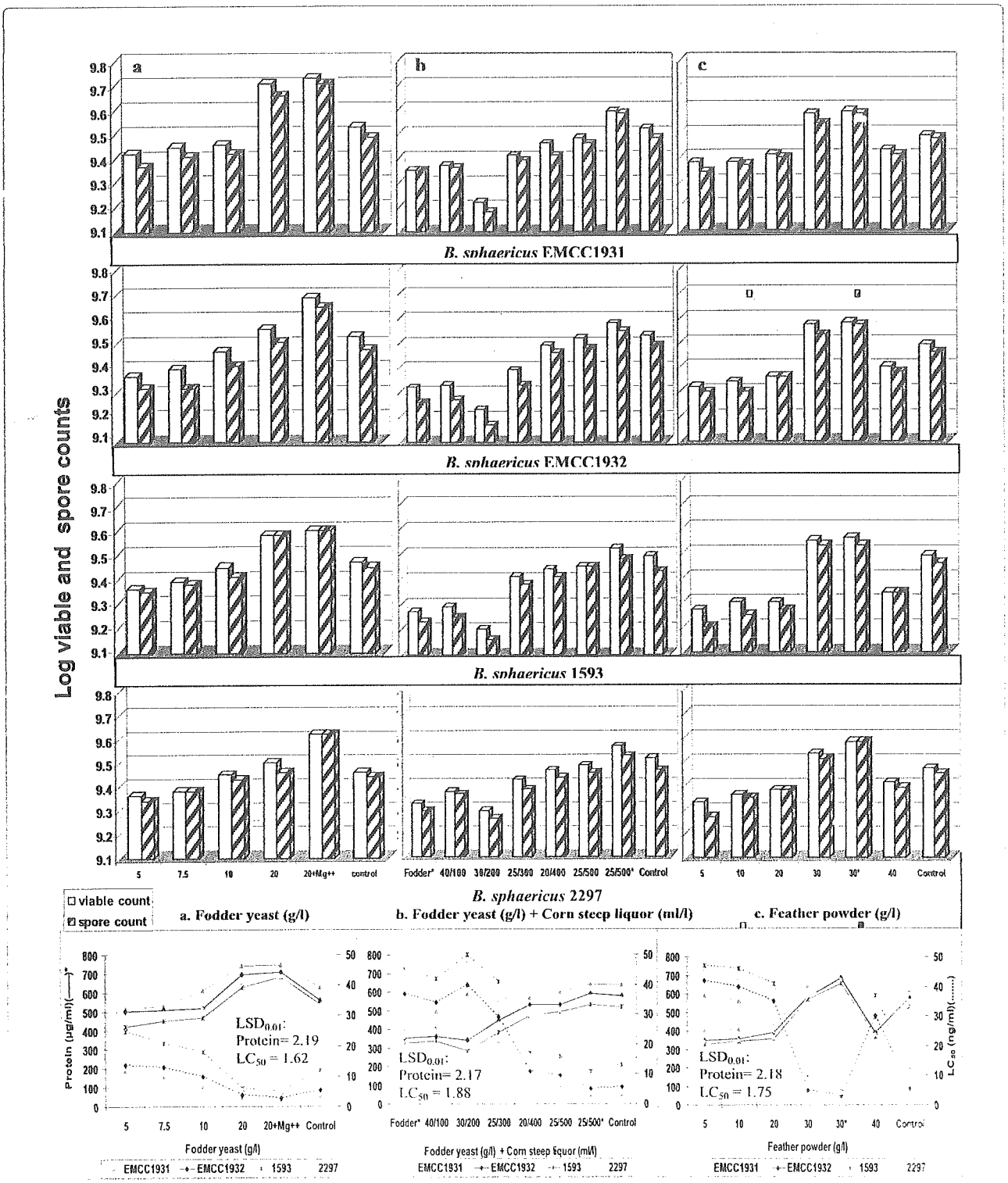

Figure 4: Effect of: a) fodder yeast, b) fodder yeast and corn steep liquor and c) feather powder (g/l) on the growth, sporulation, toxic protein yields and mosquitocidal activities of local and reference strains of *Bacillus sphaericus*. * = + Mg

Optimization the Nutritional Requirements of *B. sphaericus*

In the present study, adding equimolar C concentration of acetate, citrate or pyruvate in lieu of glycerol (1%) in poly medium (C/N ratio, 3.5/1) supported the good growth and sporulation but, unlike glycerol, toxic protein yield was lessened and a great suppression up to >80% in all growth parameters was observed with propionic acid as confirmed with bioassay (Figure 3a). The aforementioned results proved that glycerol at 1.0% (10 ml/l) as a normal component in poly medium is the most suitable carbon source for obtaining the utmost significant yields of potent protein. Therefore, an experiment was designed to study the effect of glycerol at different concentrations and subsequently different C / N ratios (Table 1) on the growth, protein quantity and lethality against the 3rd instar larvae of *Culex pipiens*. At 0.5% glycerol (C/N ratio, 2.7 /1) a considerable toxin yield was obtained. The highest significant yield of potent protein was achieved by local strains followed by reference ones (Figure 3b). Increasing the C/N ratios was interrelated positively with both viable counts and spores yield but linked negatively with protein potency and quantity. Therefore, it could be suggested that considerably lower concentrations of glycerol in the poly medium might be enough for achievement a significant larvicide yield.

What is more, good growth and sporulation were achievable when the bacteria were grown in defined media containing glycerol as sole carbon source at equimolar carbon concentration of 8.43 g/l and inorganic nitrogen 2.40 g/l, however, no biocide was synthesized by all tested strains (Table 2 and Table 6) [17] stated that growth of *B. sphaericus* 2362 was probably depending on amino acids when glycerol present as supplement to a complex, proteinaceous medium rather than as carbon source. However, they obtained very poor growth when glycerol was present as the sole carbon source in defined medium.

Adding an equimolar N concentration (2.40 g/l) of beef extract, yeast extract and peptone as an organic source individually or in combination supported the growth of all tested strains as illustrated in Table 6, nevertheless, the protein yield and potency was significantly varied. On the whole, it seems that beef extract alone had the superiority in invigorating the synthesis of potent protein comparing to the other compounds when presented individually or in combinations; which indicates that beef extract containing essential nutrients required to enhance the synthesis of biocide by all the tested strains.

Based on the present results, it is palpable that spore count was not always proportional to protein production or toxicity, and the larvicidal toxicity of a strain is the most trustworthy indicator. Thus, it could be concluded that biocide yield is the genuine parameter for a successful fermentation.

Using poly medium as a basis, different mineral salts were added singly or in combination (Table 3) to assess its effect on the growth, sporulation, protein yield and larvicidal activity of *B. sphaericus* strains. Our results evinced that Mg^{2+} is the most necessary metallic ion required for the outmost yield of potent proteins by all tested strains when Mg^{2+} added lonely to the fermentation medium followed by its combination with Na^+ and potassium phosphate salts, respectively (Tables 7).

Minerals are essential in the growth, sporulation and toxin production by the bacilli. They serve primarily as cofactors, or non protein components of a variety of enzymes termed metalloenzymes. K^+ activates enzymes involved in the formation of peptide bonds during protein synthesis. Mg^{2+} functions as a cofactor for all phosphorylating enzymes, including the enzyme that catalyzes the conversion of ADP to ATP; it is also a component of ribosomes. Ca^{2+} is a constituent of several enzymes and bacterial endospores and is essential for their heat resistance. Potassium, magnesium and calcium also function as cations to neutralize or buffer electrical charges within the cell.

Concerning iron, it is a key component of heme-containing respiratory enzymes. Mn^{2+} as a microelement functions as a cofactor for phosphorylating enzymes in a fashion similar to Mg^{2+}. RNA, DNA

	Parameter				
N-source	Total count x10⁹	Total Spore X10⁹	Protein (µg/ml)	LC_{50} (ng/ml)	Final Ph
			Strain EMCC1931		
$(NH_4)_2SO_4$	1.40	1.3	0	0	6.6
$NaNO_3$	1.90	1.80	0	0	6.5
NH_4Cl	2.30	1.99	0	0	6.4
Beef extract	3.97	2.80	653	4.2	8.5
Peptone	2.56	2.30	448	30	8.5
Yeast extract	2.78	2.5	499	15	8.3
Beef / peptone	2.70	2.40	467	22	7.9
Beef / yeast extract	2.81	2.6	508	10	7.9
Peptone / yeast extract	2.17	2.13	340	36	8.3
Control	2.92	2.69	630	5.0	8.5
			Strain EMCC1932		
$(NH_4)_2SO_4$	1.40	1.32	0	0	7.0
$NaNO_3$	1.80	1.70	0	0	7.2
NH_4Cl	2.70	2.50	0	0	6.9
Beef extract	3.92	3.81	600	5.0	8.6
Peptone	2.42	2.24	332	33	8.5
Yeast extract	2.57	2.6	438	25	8.3
Beef / peptone	2.43	2.46	392	29	8.4
Beef / yeast extract	2.88	2.79	452	21	9.0
Peptone / yeast extract	1.95	1.79	298	45	8.9
			Strain 1593		
$(NH_4)_2SO_4$	2.50	2.30	0	0	6.9
$NaNO_3$	3.31	3.20	0	0	6.8
NH_4Cl	3.70	3.60	0	0	6.4
Beef extract	3.62	3.40	576	10	8.6
Peptone	2.02	2.00	427	34	8.5
Yeast extract	2.50	2.30	439	21	8.3
Beef / peptone	2.15	2.71	438	26	8.1
Beef / yeast extract	2.80	2.10	510	17	9.0
Peptone / yeast extract	1.69	1.67	276	49	8.9
Control	2.90	2.80	540	13	8.6
			Strain 2297		
$(NH_4)_2SO_4$	2.1	1.9	0	0	6.9
$NaNO_3$	2.5	2.3	0	0	6.8
NH_4Cl	2.7	2.6	0	0	6.9
Beef extract	2.8	2.7	588	9.2	8.6
Peptone	2.4	2.2	394	39	8.5
Yeast extract	2.5	2.4	410	30	8.2
Beef / peptone	2.4	2.3	401	35	8.2
Beef / yeast extract	2.7	2.61	447	24	8.8
Peptone / yeast extract	1.87	1.85	315	42	8.2
Control	2.79	2.69	570	11	8.6
$LSD_{0.01}$		2.18	1.89		

Table 6: Effect of nitrogen sources on the growth, sporulation, protein yields and mosquitocidal activities of local and reference strains of *B. sphaericus*.

polymerase are zinc-containing enzymes [24-27]. It was surprising that supplementation of the fermentation medium with Ca^{2+} alone or in mishmash with Na^{+} and/or Mn^{2+}, Mg^{2+} inhibited the synthesis of lethal protein by at least 60% as confirmed by bioassay against the 3rd instar larvae of *C. pipiens* (Table 7). This might be due to the undefined poly medium used in the fermentation contains yeast extract, peptone and beef extract in a sufficient concentrations, most of these minerals are normally present at sufficient levels and there may be no need to add most of these ions to the fermentation media. Consequently an extra supplementation of calcium and manganese led to inauspicious effect.

Replacement of the yeast extract by 2.0% fodder yeast (21% C and 5.5% N) in the basal medium with or without Mg^{2+} gave supreme augmentation in growth, sporulation and synthesis and lethality of protein as indicated by lowering the LC_{50} values (Table 4 and Figure 4a). When it used alone plus $MgSO_4$ at the concentrations of 40 and 0.3 g/l, respectively, it proved to be a complete medium for providing *B. sphaericus* cells with their nutritional requirements as indicated by growth and sporulation, however, low toxic protein yield was produced. A complementary effect have been proven as indicated by the uppermost productivity of all the tested strains when the fermentation medium was formulated using corn steep liquor at concentration of 500 ml/l, 25 g/l fodder yeast and 0.3 g/l of $MgSO_4$ (Table 5 and Figure 4b). However, corn steep liquor as a waste by product contains 0.17% N, and 1.05% C as indicated by chemical analysis, it has been found to contain a variety of necessary biologically active compounds, vitamins and growth stimulants [20,28]. The current study evinced that, the yield of synthesized protein is the virtual consideration for successful fermentation. Yet, toxicity must be confirmed by bioassay against *Culex pipiens* larvae to be quite exact.

It is clearly noticed that chicken feather could be sufficiently used

	Parameter									
Minerals	Total count x10⁹	Total Spore X10⁹	Protein (µg/ml)	LC_{50} (ng/ml)	Final pH	Total count x10⁹	Total Sporex10⁹	Protein (µg/ml)	LC_{50} (ng/ml)	Final pH
				Local strains						
		EMCC1931						EMCC1932		
Mg	4.34	4.30	724	1.5	7.3	3.29	3.20	680	3.1	7.8
Mn	2.90	2.00	523	9.5	6.5	2.44	2.30	444	21	7.5
Ca	1.09	1.08	193	64	8.4	0.84	0.83	151	66	8.4
Mg,Na	3.95	3.90	694	2.5	8.6	3.01	3.10	643	3.5	8.5
Mg,Mn	2.30	1.70	295	36	9.4	2.19	1.99	307	40	7.8
Mg,Ca	2.60	1.78	383	30	8.8	2.38	2.00	334	34	8.0
Mn,Na	2.80	2.30	549	7.1	7.1	2.45	2.31	481	17.7	7.1
Mn,Ca	1.54	1.37	261	44	8.8	1.65	1.55	270	45	8.9
Ca,Na	0.99	0.94	193	65	8.6	0.77	0.71	129	70	8.6
K,P	3.59	3.10	681	3.0	7.5	2.90	2.80	626	4.0	7.1
Mg,Mn,Na	2.22	1.48	279	40	8.3	1.84	1.79	290	42	8.3
Mg,Ca,Na	2.30	1.76	387	31	8.7	2.22	1.29	332	39	8.7
Mg,Mn,Ca	1.50	1.36	258	48	7.1	1.28	1.26	225	56	7.7
Mn,Ca,Na	1.5€	1.48	269	42	8.7	1.80	1.70	288	43	8.5
Mg,Mn,Ca,Na	1.41	1.30	252	54	8.5	0.95	0.83	167	64	8.4
Mg,Mn,Ca,Na,K,P	3.20	2.90	600	6.2	8.1	2.55	2.50	559	7.0	7.2
Control	3.25	3.07	640	3.9	8.8	2.85	2.71	577	4.4	8.8
$LSD_{0.01}$			9.59	1.75				9.59	1.75	
				Reference strains						
		1593						2297		
Mg	3.70	3.60	609	5.3	8.3	3.20	3.15	630	4.5	8.4
Mn	2.42	2.32	405	28.4	7.1	2.50	2.30	520	21.2	8.5
Ca	0.82	0.81	147	76	8.4	0.99	0.99	180	66	8.7
Mg,Na	3.48	3.30	571	6.2	8.1	3.15	3.10	591	7.5	7.2
Mg,Mn	1.59	1.39	272	52	7.9	1.78	1.67	308	42	8.1
Mg,Ca	1.80	1.65	310	48	8.6	1.99	1.85	323	35	8.5
Mn,Na	2.73	2.63	419	23	7.5	2.60	2.51	536	23	7.5
Mn,Ca	1.24	1.21	221	65	8.8	1.60	1.50	286	52	8.4
Ca,Na	0.53	0.52	98	85	8.6	0.77	0.76	140	76	8.4
K,P	3.30	2.70	544	10.2	7.1	3.15	3.00	578	10.7	8.0
Mg,Mn,Na	1.40	1.29	245	54	8.3	1.70	1.60	293	46	7.9
Mg,Ca,Na	1.60	1.54	283	50	8.7	1.89	1.74	320	39	8.2
Mg,Mn,Ca	1.19	1.15	218	68	7.7	1.36	1.31	228	58	8.7
Mn,Ca,Na	1.26	1.24	223	60	8.1	1.69	1.55	287	49	8.8
Mg,Mn,Ca,Na	0.94	0.83	171	70	8.5	1.29	120	225	60	7.6
Mg,Mn,Ca,Na,K,P	2.80	2.70	490	15.4	7.5	2.90	2.81	540	18.2	7.8
Control	3.13	2.90	540	13	8.5	3.10	2.90	560	12	8.4
$LSD_{0.01}$			9.59	1.75				9.59	1.75	

Table 7: Effect of different minerals on the growth, sporulation, toxic protein yields and mosquitocidal activities of local and reference strains of *B. sphaericus*.

as mono component medium for the growth and sporulation of *B. sphaericus*. As good as high counts were attained by all strains when feather extract (10%) was used as fermentation medium, although, lower quantities of toxic protein by Ca 22-32% were synthesized comparing to that obtained with control medium (Table 8). Bioassay of toxins produced by different strains of *B. sphaericus* against 3rd instar larvae of *Culex pipiens* showed relatively lower toxicity by 2.6-4.6 times than that obtained with control medium. The obtained low productivity in feather extract medium comparing to that obtained in the poly medium may be reflected the deficiency of the former fermentation medium in required nutrients. Wang and Parsons 1997 reported that the hydrothermal treatment resulted in yielding a product with poor digestibility and variable nutrient quality [29]. Our results are not in accordance with the findings of Poopathi and Abidha [10] perhaps due to the differences in reference media that used as a control. They used NYS medium that has lower productivity than poly medium.

Using air dried ground chicken feather, in different concentrations varied in the range between 5 to 40 g/l tap water, proved that chicken feather was a good mono component medium for the growth of different *B. sphaericus* strains even at the lowest concentration.. A progressive increase in synthesized lethal protein was recorded by augmenting the concentration of chicken feather powder up to 30 g/l to attain the highest significant productivity by all the tested strains and stand with or surpass the productivity with the control medium (Figure 4c). A supreme improvement in both protein yield and toxic activities of both local and reference strains were achieved at 30 g feather/l supplemented with 0.3 g/l of $MgSO_4$. The protein yield augmented by ca. 12-24%, the toxicity of all strains was at least two times greater than that obtained in the control medium. Conversely, increasing the feather concentration to 40 g/l led to an inhibition of the synthesized proteins by ca. 25-34% and subsequently decreased the toxicities of all strains by about 4-6 times less than those produced in control medium.

However, Poopathy and Abidha [10] found that the toxicity of *B. sphaericus* (2362 SPH-88) grown in medium contained chicken feather powder at 5 g/l was statically similar to that grown in NYSM. Our results indicated that chicken feather powder at concentrations up to 20 g/l is not sufficient for production of the maximal potent biocide comparing to poly medium. The chicken feather powder at 30 g/l proved to be an excellent mono component cost effective fermentation medium for the maximal productivity equally as or surpass the productivity of the poly medium; a superlative improvement was achieved when feather fermentation medium (3.0%) was supplemented with 0.03% of $MgSO_4$. The chemical analysis in the present study showed that chicken feather contains N, 13.1%, and 52.46% of C. Calculation the exact concentration of C and N present in the feather chicken powder medium at the optimum concentration (30 g/l) found to equal 15.72 and 3.93, comparing to 8.43 and 2.4%, respectively, present in the control poly medium. The aptitude of the *B. sphaericus* strains to utilize the feather protein, keratin, and obtain their all required nutrients for good growth and accordingly synthesis the biocide confirmed their capability like some other *Bacillus* sp. to produce enzymes needed for metabolizing and hydrolysis of keratin as indicated by increasing the pH of fermentation medium.

	Parameter				
Strains	Total count x10^9 cfu/ml	Total spores x10^9 / ml	Protein (µg/ml)	LC_{50} (ng/ml)	Final pH
EMCC1931	2.70	2.60	486	20	8.2
Control	3.10	3.00	620	4.8	8.5
EMCC1932	2.80	1.94	395	25	8.3
Control	3.10	2.91	580	5.5	8.5
1593	2.85	2.79	384	31	8.2
Control	3.10	2.90	530	12	8.5
2297	2.71	2.64	386	29	8.3
Control	2.97	2.80	560	11	8.7
$LSD_{0.01}$			2.39	2.07	

Table 8: Growth parameters of local and reference strains of *B. sphaericus* in media formulated from 10% extracted chicken feather waste.

It has been reported that bird's feathers represent over 90% proteins mainly keratins that are made up of long chains of various amino acids and two major types of alpha- and beta - keratin are known. It has been observed that neutral to alkaline pH from 6 to 9 supports keratinase production and feather degradation, alkaline pH possibly favors keratin degradation. Fermentation significantly increases the levels of essential amino acids. The higher alkalinity is attributed to deamination reactions leading to the release of ammonium and thus increase in pH and consequent increase in keratinolysis. In addition, the keratinases are generally stimulated in the presence of divalent metal ions like Mg^{2+} [30-34].

Conclusion

The individual measure that governs maximal biocide production is type and concentration of carbon and nitrogen sources, Mg^{2+}, fermentation time, inoculum size, aeration, temperature and pH. A combination of these factors at optimum levels ultimately decides the biocide yield. Maximum growth and spore formation surely are a precondition for the maximum synthesis of lethal protein, however, both the parameters are not directly correlated. The current study evinced that, the yield of synthesized protein is the virtual consideration for successful fermentation. Yet, toxicity must be confirmed by bioassay against *Culex pipiens* larvae to be quite exact. Chicken feather plus $MgSO_4$ at concentrations of 30 and 0.3 g/l could be exploited as a cost-effective fermentation medium for local commercial production of value-added bio-mosquitocidal and diminished the environmental pollution.

References

1. Lacey LA (2007) Bacillus thuringiensis serovariety israelensis and Bacillus sphaericus for mosquito control. J Am Mosq Control Assoc 23: 133-163.
2. Jagtap SC, Jagtap CB, Kumar P, Srivastava RB (2009) Detection of Bacillus sphaericus mosquitocidal toxin genes by multiplex colony PCR. Can J Microbiol 55: 207-209.
3. Rungrod A, Tjahaja NK, Soonsanga S, Audtho M, Promdonkoy B (2009) Bacillus sphaericus Mtx1 and Mtx2 toxins co-expressed in Escherichia coli are synergistic against Aedes aegypti larvae. Biotechnol Lett 31: 551-555.
4. Yousten AA, Russell BL, Davidson EW, Faust RM, Margalit J, et al. (1989) Factors affecting fermentative production of Bacillus sphaericus. Israel Journal of Entomology 23: 233-238.
5. Han B, Liu H, Hu X, Cai Y, Zheng D, et al. (2007) Molecular characterization of a glucokinase with broad hexose specificity from Bacillus sphaericus strain C3-41. Appl Environ Microbiol 73: 3581-3586.
6. Poopathi S, Anup Kumar K, Kabilan L, Sekar V (2002). Development of low-cost media for the culture of mosquito larvicides, Bacillus sphaericus and Bacillus thuringiensis serovar. israelensis. World Journal of Microbiology & Biotechnology 18: 209-216.
7. Foda MS, El-Bendary M, Moharam ME (2003) Salient parameters involved in mosquitocidal toxins production from Bacillus sphaericus by semi-solid substrate fermentation. Egyptian Journal of Microbiology, 38(3): 229-246.

8. Prabakaran G, Balaraman K, Hotil SL, Manonmani AM (2007) A cost-effective medium for the large-scale production of *Bacillus sphaericus* H5a5b (VCRC B42) for mosquito control. Biological Control 41: 379-383.
9. Poopathi S, Abidha S (2007) Use of feather-based culture media for the production of mosquitocidal bacteria. Biological Control 43: 49-55
10. Poopathi S, Abidha S (2008) Biodegradation of poultry waste for the production of mosquitocidal toxins. Int Biodeter Biodegrad 62: 479-482.
11. Melo AL, Soccol CR, Thomaz-Soccol V, Nogueira M Jr (2009) Evaluation of Bacillus sphaericus bioinsecticide produced with white soybean meal as culture medium for the control of Culex (Culex) quinquefasciatus. Cad Saude Publica 25: 563-569.
12. Rashad FM, Saleh WD, Nasr M, Fathy HM (2012) Identification of mosquito larvicidal bacterial strains isolated from north Sinai in Egypt. AMB Express 2: 9.
13. Obeta JA, Okafor N (1983) Production of Bacillus sphaericus strain 1593 primary powder on media made from locally obtainable Nigerian agricultural products. Can J Microbiol 29: 704-709.
14. Bourgouin C, Larget-Thiery I, de Barjac H (1984) Efficacy of dry powders from Bacillus sphaericus: RB 80, a potent reference preparation for biological titration. J Invertebr Pathol 44: 146-150.
15. Cotteni A, Verloo M, Kiekens L, Velghe G, Camerlynck R (1982) Chemical analysis of plants and soils. Laboratory of Analytical and Agrochemistry State, University, Ghent, Belgium, p.60.
16. Abbott WS (1925) A method of computing the effectiveness of an insecticide. Journal of Economic Entomology 18: 265-267.
17. Russell BL, Jelley SA, Yousten AA (1989) Carbohydrate metabolism in the mosquito pathogen Bacillus sphaericus 2362. Appl Environ Microbiol 55: 294-297.
18. Klein D, Uspensky I, Braun S (2002) Tightly bound binary toxin in the cell wall of Bacillus sphaericus. Appl Environ Microbiol 68: 3300-3307.
19. Yousten AA (1984) Bacillus sphaericus: microbiological factors related to its potential as a mosquito larvicide. In Advances in Biotechnological Processes 3: 315-343.
20. Kuppusamy M, Balaraman K (1991) Effect of corn-steep liquor on growth and mosquito larvicidal activity of *Bacillus thuringiensis* var israelensis de Barjac 1978 and B. sphaericus Neide 1904. Indian J Exp Biol 29: 187-189.
21. Lacey LA (1984) Production and formulation of Bacillus sphaericus. Mosquito News 44: 153-159.
22. Hoti SL, Balaraman K (1986) Parameters facilitating local production of Bacillus sphaericus. Indian J Med Res 83: 166-170.
23. El-Bendary Magda A (1999) Growth physiology and production of mosquitocidal toxins from Bacillus sphaericus. Ph.D. Thesis, Faculty of Science, Ain-Shams University, Egypt.
24. Singer S, Goodman NS, Rogoff MH (1966) Defined media for the study of bacilli pathogenic to insects. Ann N Y Acad Sci 139: 16-23.
25. Myers P, Yousten AA (1978) Toxic activity of Bacillus sphaericus SSII-1 for mosquito larvae. Infect Immun 19: 1047-1053.
26. Dharmsthiti SC, Pantuwatana S, Bhumiratana A (1985) Production of B. thuringiensis subsp. israelensis and B. sphaericus strain 1593 on media using a byproduct from a monosodium glutamate factory. Journal of Invertebrate Pathology 40: 231- 238.
27. VanDemark PJ, Batzing BL (1987) The Microbes: An Introduction to their Nature and Importance.The Benjamin/Cummings publishing company, inc. pp. 136-137.
28. Fridlender B, Keren-Zur M, Hofstein R, Bar E, Sandler N, et al. (1989) The development of Bacillus thuringiensis and Bacillus sphaericus as biocontrol agents: from research to industrial production. Mem Inst Oswaldo Cruz 84 Suppl 3: 123-127.
29. Wang X, Parsons CM (1997) Effect of processing systems on protein quality of feather meals and hog hair meals. Poult Sci 76: 491-496.
30. Williams C. M, Lee CG, Garlich JD, Shih JCH (1991) Evaluation of a bacterial feather fermentation product, feather-lysate as a feed protein. Poultry Sci 70: 85–94.
31. Friedrich AB, Antranikian G (1996) Keratin Degradation by Fervidobacterium pennavorans, a Novel Thermophilic Anaerobic Species of the Order Thermotogales. Appl Environ Microbiol 62: 2875-2882.
32. Kim WK, Lorenz ES, Patterson PH (2002) Effect of enzymatic and chemical treatments on feather solubility and digestibility. Poult Sci 81: 95-98.
33. Riffel A, Lucas F, Heeb P, Brandelli A (2003) Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. Arch Microbiol 179: 258-265.
34. Gupta R, Ramnani P (2006) Microbial keratinases and their prospective applications: an overview. Appl Microbiol Biotechnol 70: 21-33.

Citation: Rashad FM, Saleh WD, Fathy HM (2013) Optimization of Bio-Mosquitocidal Production using Local Bacterial Strains of *Bacillus Sphaericus*. J Biofertil Biopestici 4: 134. doi:10.4172/2155-6202.1000134