High Efficiency Production of Mosquitocidal Toxin by a novel*Bacillus sphaericus*isolate from Egyptian Soils on Local Agroindustrial Byproducts

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Abstract: Eighty six cultures were isolated from soil of different Egyptian Governorates including Quina, El-Menofeya, El-Gharbia, El-Sharkia, El-Behera and Kafr EL-Sheikh Governorates. Investigations of the mosquitocidal Egyptian isolates have revealed that isolate No.1 have the ability to form more toxin than the international reference strain *Bs*2362.The selected isolate No.1 exhibited a lower LC_{50} and LC_{90}values than the International strain *B. sphaericus* 2362 uponbioassay against secondinstars' larvae of *Culexpipiens.* The Egyptian isolate No.1was identified morphologically and biochemically as *Bacillussphaericus.* Physiological factors affecting growth and toxin formation in *B. sphaericus*No 1 in comparison to *B.s* 2362 were carried out. THE organism grown on modified Nutrient broth medium yielded the highest larval toxicity against the second instars' of *Culexpipiens*for both *Bacillus sphaericus*isolate No 1 and the international strain *Bacillus sphaericus.* The Optimum air : medium ratio were 9:1 and 4:1 of the flask volume for 4 and 3 days incubation periods using 2%and 3% sizes of inocula for *B. sphaericus* 2362 and the Egyptian isolate *B. sphaericus*No.1, respectively. Sodium acetate was the suitable carbon source for the isolate *B. sphaericus* No.1, while *B. sphaericus* 2362 was capable to utilize both sodium acetate and sodium succinate as carbon sources.The Egyptian isolate *B. sphaericus* No.1exhibited the highest mosquitocidal activity upon growth on kidney beans seeds and sesame meal as nutrient substrates at 3% final concentration, while *B. sphaericus*2362 exhibited the highest mosquitocidal activity by utilizing soy beans, lentils and sesame meal as complete media for growth and mosquitocidal toxin production.

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Key words: *Bacillus sphaericus,* isolation, characterization, mosquitocidal toxin, physiology, agroindustrial byproducts.

Introduction

The mosquito acts as a vector for many of the world´s most serious diseases, both parasitic e.g. malaria (*Anopheles*), lymphatic filariasis (*Anopheles*, *Aedes*, *Culex* and *Mansonia*) and viral e.g. yellow fever, dengue (both *Aedes*) and encephalitis (*Culex*).**(Baumann *et al*., 1991).**

*Bacillus sphaericus*has been successfully used for the the biological control of numerous of disease -transmitting mosquitoesmosquito and black fly species **(Lacey and Undeen, 1986)**. The prime advantage of the *B. sphaericus* strain lies in their ability to persist for longer periods in the environment than *Bacillus thuringiensis*var. *israelensis*. This may be due to recycling and amplification of spores in larval cadavers under certain aquatic situations or may be simply due to the long–term persistence of sufficient and accessible toxin in the environment or a combination of both of the above **(Singer, 1990; Correa &Yousten, 1995)**. Major advantage of these bacterial insecticides are thrir safety, biodegradability, and low environmental impact **(Maramorosch,1987)**

Opota*et al.* (2008) reported that the binary toxin (Bin) from *Bacillus sphaericus* exhibits a high insecticidal activity against *Culex* and *Anopheles* mosquitoes. The cytotoxicity of Bin requires an interaction with a specific receptor present on the membrane of midgut epithelial cells in larvae, a direct correlation exists between binding affinity and toxicity. The toxin binds with high affinity to its receptor in its primary target namely, *Culexpipiens.*

The present work paper aims for isolation of new *Bacillus sphaericus*strain with mosquitocidal activity that exceeds the existing international strains e.g.*B. sphaericus*2362. In the hope to reduce Production costs of mosquitocidal toxin used for biological control of disease- transmitting mosquitoes in the developing countries production physiology of the bacterial toxin was studied on synthetic andagroindustrial byproducts.

MATERIALS AND METHODS

Microorganisms

The International strain *Bacillus sphaericus*2362 was kindly obtained from prof. F.G. Priest, school of life sciences, Heriot watt university, UK

A new *Bacillus sphaericus* isolate namely No.1 was isolated from soils of QuinaGovernorate ,Egypt.

Media used for growth, sporulation and mosquitocidal toxin production in shake cultures.

a-Media based on Agroindustrial by-products:

These media included offal's meal, feather meal and cotton seed meal. Most of these agroindustrialby-products are currently used in animals feed and available in Egyptian market.

b-Media based on cheap, locally available plant proteins: Certain legume seeds that are locally available in Egypt were examined as protein sources for growth, sporogenesis and mosquitocidaltoxin production. These legumes seeds such as soy beans, kidney bean, black eyed bean, yellow splite pea, and lentils were finely grinded and used in conjunction with the standard mineral salt solution at appropriate concentration.

Bioassay of bacterial toxins against Mosquitoes larvae.

Bioassay of locally isolated *Bacillus* cultures including *B. thuringiensis*and *B. sphaericus* were carried out as described by **Priest and Youstin (1991)**.Toxicity was determined with laboratory reared *Culexpipiens.*Serial dilutions in distilled water were tested in a preliminary toxicity screen. The range of concentration of full grown whole culture (FWC) which killed 50% and 90% of the larvae were identified. Then further toxicity tests were done in the range recorded to evaluate precisely the LC_{50} and LC_{90} values for each highly promising bacterial culture.

The corrected mortality was then plotted against culture dilution of cells/ml on log paper to determine LC_{50} andLC_{90} values for each highly promising bacterial cultures.

The bacterial dilutions were placed in small cups in duplicates along with 10 second instar larvae. Appropriate controls were run simultaneously using distilled water instead of cultures. The cups kept at room temperature 27±2°C. The mortality percentage was recorded by counting the number of living larvae and corrected by using appropriate control and applying Abbott's formula **(Abbott, 1925)**. The medium lethal concentrations LC_{50} of potent isolates was computed through probit analysis within 95% confidence limits using propan program.

Abbott's formula:

Corrected mortality % =

$$\frac{\text{Observed mortality \% - Corrected mortality\%}}{\text{100-Control mortality \%}} \times 100$$

RESULTS

3.1. Isolation, Identification and Mosquitocidal Toxin Production by *Bacilli* isolated from the Egyptian environments

Eighty six isolates were obtained from soils and mud samples ofsix different Egyptian Governorates including Quina, El-Menofeya, El-Gharbia, El-Sharkia, El-Behera and Kafr EL-Sheikh. Among these isolates, isolate No.1 obtained from QuinaGovernorate was the only isolate giving 100% mortality up to culture dilution 10^{-5}. Accordingly this isolate obtained from Quina Governorate was selected for further investigation.

3.2. Determination of LC_{50} and LC_{90} values of the Egyptian isolate No.1 obtained from Quina Governorate soils.

LC_{50} and LC_{90} of isolate No.1 and *B. sphaericus*2362 bioassayed against second instar larvae of *Culex. pipiens* revealed that the Egyptian isolate No.1 is more toxic than the reference strain 2362**. Table (1).**

3.3. Identification of the Egyptian isolate No.1 isolated from Quina Governorate.

The colonies exhibited beige color with medium size colonies, The texture is smooth semi-glistening

with round margin; The appearance of colonies is shiny with little elevation and flat.

Examination of the cells with the electron microscope revealed the rod-shaped of the vegetative cells as shown in Fig (1); sporulated cells (sporangia) with subterminal spores that are round in shape giving the sporangia club shaped appearance as shown in Fig (2). Also it was observed that isolate No.1 produced a spherical spore and round crystals when examined under the electron microscope as shown in Fig (3).

Some biochemical tests for the identification of the Egyptiaisolate No.1 obtained from QuinaGovernoratewere carried out Table (2).

3.4. Comparative Physiological studies on factors affecting growth, sporulation, and toxin production of the Egyptian isolate*B. sphaericus*No.1 and the International *B. sphaericus* 2362 strain under submerged fermentation conditions

3.4.1. Effect of types of media on growth parameters, sporulation titer and mosquitocidal toxicity under submerged conditions.

Four types of media were used in this study namely Nutrient yeast salt medium, Luria –Bertani medium, Nutrient broth (NB), and modified Nutrient broth (NB+ 0.5% yeast extract)The obtained results showed that modified Nutrient broth medium yielded the highest larval toxicity against the second instars' larvae of *Culexpipiens*for both *Bacillus sphaericus*isolate No 1and the international strain *Bacillus sphaericus*2362(Data not shown).

3.4.2 Effect of aeration level on growth and toxicity of *B. sphaericus*

In this experiment the extent of aeration was altered by varying the air : medium ratio (amount of medium in the culture flask). The effect of aeration extent on growth parameters and toxicity of the mosquitocidal agent produced by the organisms under study are shown in Figures (4,5). It was noted that the viable count and toxicity increased with increasing the air : medium ratio. Furthermore, The sporulation and toxin production gave the highest titers when the medium volume occupied 10% and 20%, i.e. corresponding to air: medium ratio 9:1 and 4:1 of the flask volume for *B. sphaericus* 2362 and the Egyptian isolate *B. sphaericus*No.1, respectively.

3.4.3. Effect of different carbon sources utilized by *B.sphaericus*on growth parameters and mosquitocidal toxin formation.

It is known that *B. sphaericus*can not utilize carbohydrates for growth due to the lack of many of the enzymes of Embden Meyerhof and hexose monophosphate path ways **(Russel*et al.*, 1989).**In this experiment different carbon sources were used for testing the ability of the tested organisms*B. sphaericus* No.1 and *B. sphaericus* 2362 to utilize this carbon sources. The results revealed that sod. acetate was utilized by the isolate *B. sphaericus* No.1, at which the sporulation and toxin production yielded the highest titers. On the other hand, *B. sphaericus* 2362 was capable to utilize sod. acetate and sod. succinate, as shown in Figures (6,7).

3.4.5.. Effect of inoculum size on growth parameters and mosquitocidal toxin formation of *B. sphaericus*.

Different volumes of overnight growing culture were used as inocula for a set of 250 ml conical flasks each containing 25ml of modified nutrient liquid medium . The results of growth parameters and toxin production of tested organisms are illustrated by Figures (8,9).

The increase of inoculum size has led to the increase of sporulation titer and toxin production up to 3% inoculum size, and then decreased with the increasing of inoculums size in case of the Egyptian *B. sphaericus* No.1. However the sporulation and toxin production of *B. sphaericus* 2362 showed a little effect by changing the inoculum size. The highest toxicity were achieved using 3% inoculums size and 2% by isolate *B. sphaericus* No.1 and *B. sphaericus* 2362, respectively.

3.4.6. Effect of incubation period on growth parameters and mosquitocidal toxin formation of *B. sphaericus*.

This experiment was carried out under standard conditions by using a set of 250 ml conical flasks containing 25ml of modified nutrient liquid medium, then the extent of growth, sporulation titer and toxin production were followed and determined by harvesting after 2, 3, 4 and 7 days of incubation at 28 ± 2°C on a rotary shaker.

The results are shown in Figures (10,11). The mortality increased with increasing the incubation period until 3 days incubation period in case of *B. sphaericus* No.1 and 4 days for*B. sphaericus* 2362.

Table(1): Values of LC_{50} and LC_{90} for mosquitocidal toxins of the Egyptian isolate No.1in comparison with those of the International strain of *Bacillus sphaericus* 2362 at confidence limits(95%). The bioassay were carried out against second instar larvae of *Culexpipiens.*

Isolate	LC_{50} (Confidence limits at 95 %) by µl	LC_{90} (Confidence limits at 95 %) by µl
The Egyptian isolate *B. sphaericus* No.1 from Quina. Egypt	264.4 (155.3-365.8)	725.9 (517.3-1351.7)
The Internationalstrain *B. sphaericus*2362	359.2 (228.5-479.3)	932.4 (674.3-1818.9)

3.4.7. Effect of different by-products and grinded legumes seeds used as complete media on growth, sporulation and toxin production of *Bacillus sphaericus.*

Ten agroindustrialbyproducts that are available in Egypt were examined as a complete cost effective media for toxin production. The datain Figures(12,13) illustrated that the Egyptian isolate *B. sphaericus* No.1exhibited the highest mosquitocidal activity by utilizing kidney beans and sesame meal as nutrient substrate at 3% final concentration, while *B. sphaericus*2362 exhibited the highest mosquitocidal activity by utilizing soy beans, lentils and sesame meal as growth media for growth and mosquitocidal toxin production.

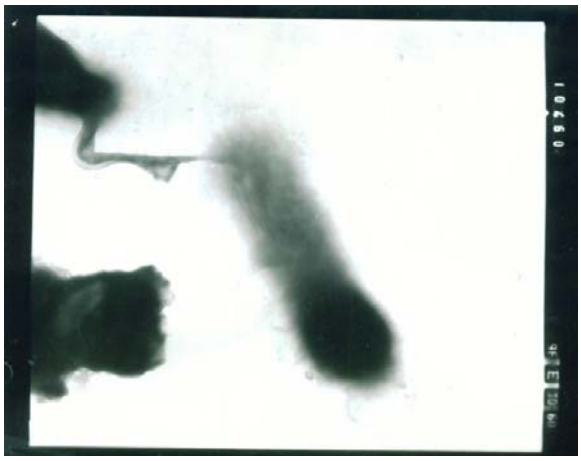


Fig (1) E.M. showing chain of vegetative cells of the Egyptian isolates *B. sphaericus* No.1 isolated from Quina Governorate (X 10.000).

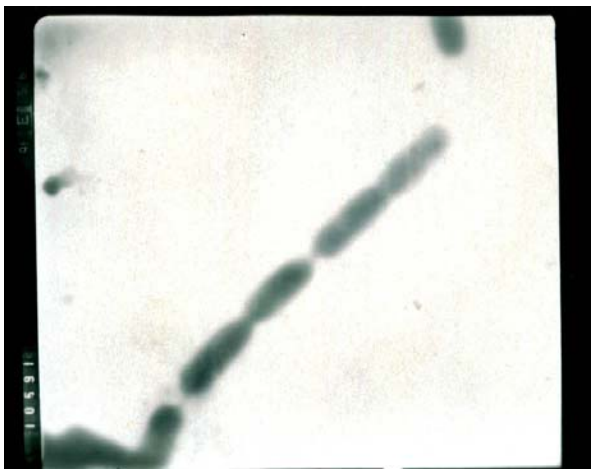


Fig (2) E.M. the Egyptian isolates No.1 isolated from Quina Governorate grown on nutrient liquid medium showing the club-shaped cells (X 20.000).

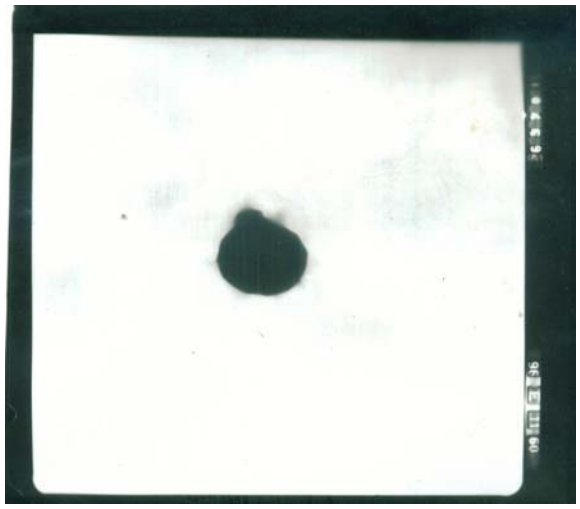
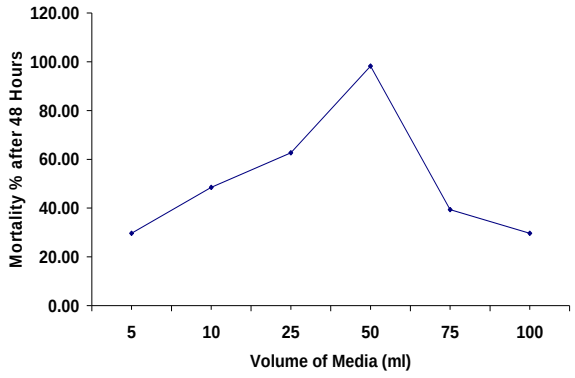


Fig (3) Electron Micrograph (E.M.) showing spherical spore and crystal of the Egyptian *B. sphaericus* isolates No.(1) after 3 days of incubation (X 20.000).

Table (2): Some biochemical tests for the identification of the Egyptian isolate No.1 obtained from Quina Governorate as compared with *B. sphaericus* 2362.

Biochemical tests	*Standard strain B. sphaericus* 2362	The Egyptian isolate No.1
Tolerance to NaCl 2%	+	+
5%	+	+
7%	–	–
10%	–	–
Degradation of adenine	+	+
Decomposition of urea	+	+
Hydrolysis of casein	+	+
Hydrolysis of Starch	+	+
Hydrolysis of gelatin	+	+
Utilization of citrate	–	–
Methyl red test	+	–
Vogesproskauer test	–	–
Catalase test	–	–
Nitrate reduction test		



Fig(4): Effect of volume of media on toxin production by the Egyptian isolate *B. sphaericus* No.1

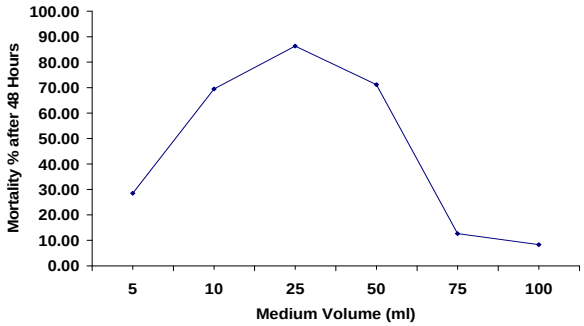


Fig (5): Effect of volume of media on toxin production by *B.sphaericus* 2362.

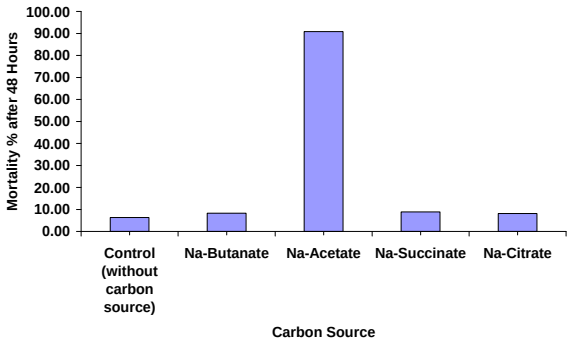


Fig (6): Effect of different carbon sources (salts of organic acids)on toxin production of the Egyptian isolate *B. sphaericus*No.1.

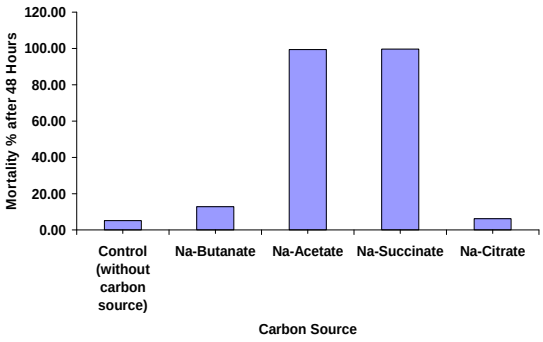


Fig (7): Effect of different carbon sources (salts of organic acids) on toxin production by *B.spharricus* 2362.

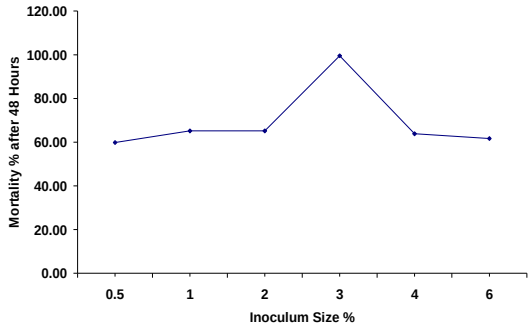


Fig (8): Effect of inoculum size on toxin production of the Egyptian isolate *B. sphaericus* No.1.

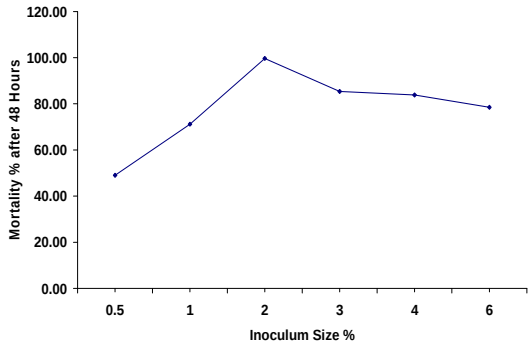


Fig (9): Effect of inoculum size on toxin production of *B. sphaericus* 2362.

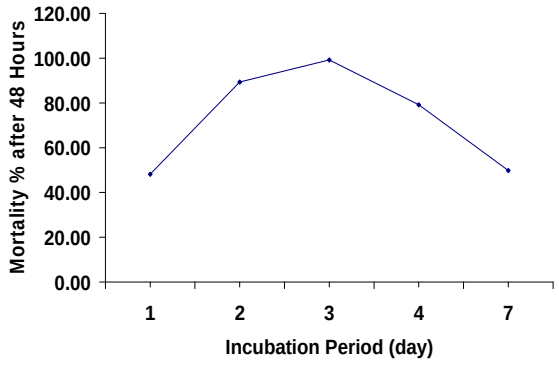


Fig (10): Effect of incubation period on toxin production of *B. sphaericus* No.1.

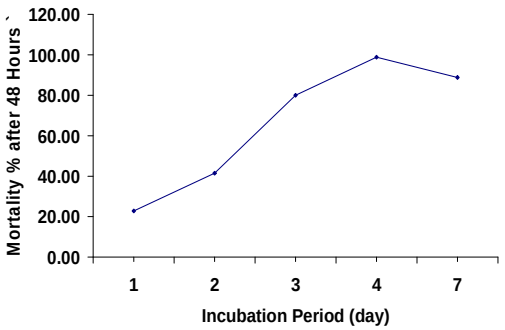


Fig (11): Effect of incubation period on toxin production of *B. sphaericus* 2362.

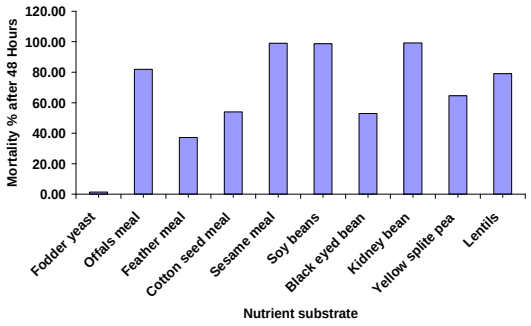


Fig (12): Effect of some agroindusterial by-products and grinded legumes seeds used as complete growth media on mosquitocidal toxicity of the Egyptian isolate *B. sphaericus* No.1 using substrate concentration 3%.

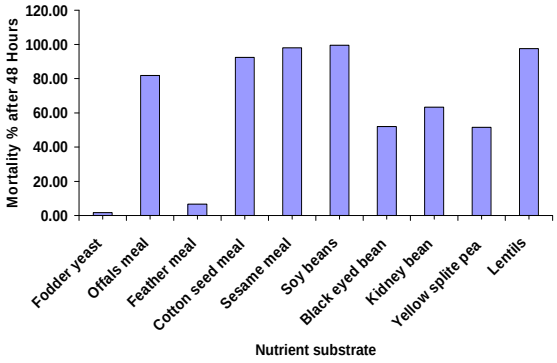


Fig (13): Effect of some agroindusterial by-products and grinded legumes seeds on mosquitocidal toxicity of *B. sphaericus*2362 using substrate concentration 3%.

DISCUSSION

The mosquito acts as a vector for many of the world's mostserious diseases, both parasitic e.g. malaria (*Anopheles*), lymphaticfilariasis (*Anopheles*, *Aedes*, *Culex* and *Mansonia*) and viral e.g. yellow fever, dengue (*Aedes*) and encephalitis (*Culex*). The present work aims to isolate some local isolates of *B. sphaericus*pathogenic to mosquito larvaefrom the Egyptian environment.Itwas also devoted to investigate the growth physiology and various factors that are affecting growth, sporulation and toxins formation. On the other hand, special attention was given to search for suitable media that are low-priced and locally available in Egypt for *B. sphaericus* production on a large scale. The goal stemmed from the fact that the feasibility of economic production of spores and toxin crystals of *B. sphaericus* is dependant to a large extent on production costs and availability of raw materials under the local conditions. Physiological factors affecting growth and toxin formation in *B. sphaericus* revealed that modified Nutrient broth medium yielded the highest larval toxicity against the second instars' of *Culexpipiens*for both *Bacillus sphaericus*isolate No 1 and the international strain *Bacillus sphaericus*2362. The requirements of individual bacterial strain for nutrients may vary for different strains and also of different isolates within the same strain within the same species. Thus optimal concentration of nutrients for one isolate may not necessarily be suitable for another. Therefore, it is impossible to recommend a fermentation medium that will be best for all isolates of the same species **(Foda*et al.* 2000).**

It is established that *B. sphaericus* is an obligate aerobe and adequate air supply is needed for growth, initiation of sporulation and toxin synthesis **(Yousten and Wallis, 1987)**.

In our studies, it was found that the maximum sporulation and toxicity were acheived when the medium volume to air ratio was 1:4 for the Egyptian isolate *B. sphaericus*No.1 and 1:9 for the International strain *B. sphaericus* 2362 that was used for comparative purposes. The increase in medium volume to air ratio has lead to the decrease in sporulation and toxicity. This result agrees with what reported by **Youysten and Wallis (1987)**. They found that oxygen was required for toxin production by *B. sphaericus* strain 2362. However, they reported that increasing the level of dissolved oxygen (DO) in the medium by use of pure oxygen in the gas stream lowered toxin production, while in case of strain 1593, (another *B. sphaericus*International strain), increased (DO) produced a block in sporulation, but toxin synthesis was normal **(Youstene*t al.*, 1984)**.

The result of growth parameters of tested organisms indicated that *B. sphaericus* isolate No.1 gave high sporulation titer and toxicity at inoculum size 3% and a decrease in toxicity was recorded by increasing the inoculum size, However the highest sporulation and toxin production levels of strain 2362 were achieved by inoculum size 2%. This result agrees with that reported by **Foda*et al.* (2000)**, they reported that the sporulation of the Egyptian isolate No. 69 increased by decrease in the inoculum size to reach 7.5×10^6/ml viable count whereas the sporulation of strain *B. sphaericus*2362 exhibited a little effect by changing the inoculum size.

*B. sphaericus*can not use carbohydrates for growth due to the lack of many of the enzymes of Embden Meyerhof and hexose monophosphate pathways**(Russel*et al.*, 1989).**

In the present study, it has been found that the Egyptian isolate *B. sphaericus* No.1 grew well using acetate as sole carbon source. On the other hand the International strain *B. sphaericus*2362 grew with acetate or succinate as sole carbon source. This result agrees with what reported by **(Gordon *et al.*, 1973; De BarJac*et al.*, 1980; Klein *et al.*, 1989;Widjaya*et al.*, 1992 and Ahmed *et al.*, 1993 and 1996).** They reported that numerous strains of *B. sphaericus*grew with acetate, Pyruvate, lactose, glutamate, succinate, histidine and arginine, as sole major carbon and energy sources.

In the present study, ten leguminous seeds and agroindustrial by-products were used as nutrient substances at concentration 3% and the result indicated that soy beans, kidney beans and sesame seed meal could be used efficiently as nutrientssources to support growth, sporulation and toxin production of the Egyptian isolate *B. sphaericus*No.1. High levels of toxicity were obtained even at low concentration of diluted culture (3×10^{-6}), as inocula. On the other hand, *B. sphaericus* 2362 grew well on a medium contained soy beans, lentils and sesame seed meal and the growth, sporulation and high levels of toxin production were achieved at the same culture dilution. Uses of such various by-products as well as legume seeds have shown that local production from inexpensive ingredients available in different regions is possible. Such studies may pave the way for mass production on industrial scales.**Dulmage*et al.* (1970)**culturd*B. sphaericus*1593 and 2362 separately in a fermentor on peptonized milk medium with yeast

extract and mineral supplements. The fermentor beer was centrifuged and then resuspended in lactose solution and precipitated with acetone. These powders were highly insecticidal to *Culexquinquifasciatus*larvae producing LC_{50} values in the range of 10^{-2}µg/ml. **Obeta and Okafor (1983)** formulated five media from dried cow blood, mineral salts and seeds from four species of legumes (ground nut cake, cowpea, mambara beans and soy beans) for production of *B. sphaericus* 1593. Good growth, sporulation and toxin activity of *B. sphaericus*1593 were obtained with all tested media. **Dharmsthiti*et al.* (1985)** grew *B. sphaericus* on a medium containing 7% hydrolyzed liquor by-product from a monosodium glutamate factory. **Klein *et al.* (1989)** used hydrolyzed industrial peptones (waste product of industry) for constructing seven media for production of *B. sphaericus*larvicides. These media contained 5 g/l industrial peptone in 50 mM phosphate buffer (pH 7.0) in combination with other carbon and nitrogen sources. Industrial peptone medium supplemented with glycerol was the most efficient medium for growth and larvicides production by *B. sphaericus* 2362.The local availability of proteinaceous materials is vitally important for *B.sphaericus* fermentation. For example, one of the most useful nitrogen sources is cotton seed flour **(Dulmage*et al.* 1990b)**.They reported that several nitrogen sources are used in *Bt* fermentation, including soybean flour, cotton seed flour and fish meal. The soy flour and cotton seed flour were both very good sources of nutrients for both*Bt*and *B. sphaericus*production.**Gangurde and Shethna (1995)** concluded that mustard seed meal (MSM) contains 40% protein, with glutamic acid and arginine as a major amino acids. Therefore, growth and larvicidal activity of *B.sphaericus* 2362 and 1593 produced in MSM can be attributed in part to the presence of these amino acids. **Ampofo (1995)** used some local row-materials for production of *Bs* insecticides in Ghana. He tested anchovy, spent grain form breweries, bambara beans and sprout maize as media for production of *B. sphaericus* IAB 881. He reported that larvicidal activity of *Bs* IAB 881 grown in anchovy, spent grain, bambara beans and sprout maize, was similar to that obtained in synthetic medium with LC_{50} ranging from 0.3×10^{-5} to 0.68×10^{-6} (dilution). Cell counts were in the range of 11×10^{8} – 36×10^{8} CFU/ml and spore counts were between 29×10^{7} and 61×10^{7} CFU/ml. **El-Bendary (1999)** used ground agroindustrial by-products and leguminous seeds at 2% final concentration as media for production of *B. sphaericus* in distilled water with or without addition of NYSM salts. The obtained results indicated that most of the tested substances supported formation of highly efficient media for *Bs* toxin production of appreciably high sporulation yield and toxicity. She also reported that the most efficient media for *B. sphaericus*toxin production were soy flour, cotton seed flour, corn steep solids and offals meal. Furthermore, it was observed that addition of NYSM salts to these substances incrased the *B. sphaericus* toxicity. Moreover, the toxicity of *B. sphaericus*increased about 1.5-4.5 times when these agroindustrial by-products were partially hydrolyzed by nuclease or alkalase enzymes before using as media. **El-Bendary*et al.* (2008),** used whey permeate (WP) for production of mosquitocidal toxin by *B. sphaericus* 2362 and thethe Egyptian isolate, *B.sphaericus* 14N1 under both submerged and solid state fermentation conditions. Under submerged fermentation, high mosquitocidal activity was produced by *B. sphaericus* 2362 and *B.sphaericus* 14N1 at 50% -100% and 25% -70% whey permeate, respectively.

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