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EVALUATION OF CONVENTIONAL LABORATORY MEDIA FOR PRODUCING ACTIVELY TOXIC STRAINS OF *Bacillus sphaericus*

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ABSTRACT

Two actively marked toxic strains of *Bacillus sphaericus* NSE1 and NSE2, previously isolated from the soil of North Sinai in Egypt, identified morphologically, biochemically and assayed biologically against mosquito larvae, were used in the present study along with the reference strains of *Bacillus sphaericus* 1593 and 2297. The PCR product profiles indicated the presence of genes encoding *Bin A*, *Bin B* and *Mtx1* in all analyzed strains; they are consistent with protein profiles. PM achieved the highest significant yield during the first 72 hr of fermentation course, but after 120 hr NYSM attained the highest significant yield of 1.1 - 1.3 × 10^9 CFU/ml and stand with PM without significant differences. AYEM, LBM and GGSEM, in that order, came after. The maximum yield of spores was reached at the end of the fermentation time in both PM and NYSM followed by GGSEM. However, the highest significant sporulation rate (88.5 - 100%) was obtained in NYSM followed by PM and GGSEM (71.4 – 93.3%), (40.0 – 80%), respectively. The synthesis of protein was observed only from 6 hr (NSE1, 2297), 9 hr (1593) and 12 hr (NSE2) onwards and reached the maximum level after 120 hr. The significant highest quantities was attained in PM. Local strains, NSE1 and NSE2, respectively, always produced significantly higher protein amounts in fermentation media than the standard strains 2297and 1593. The maximum toxicity against 3rd instar of *Culex pipiens* (LC_{50} 5 x 10^3 and 8 x 10^3 CFU/ml) was noticed with local strains NSE1 and NSE2, respectively in PM by 5 days without significant differences. In all of the fermentation runs, changes in pH showed a similar pattern, rising to reach about 8.3 - 9.0 at the end of fermentation.
Keywords: *Bacillus sphaericus, Culex pipiens,* Larvicide, Conventional media.

INTRODUCTION

Bacillus sphaericus has been successfully used for the biological control of mosquito species which are vectors of important human diseases. In general, the bacterium is more active against the larvae of *Culex and Anopheles* spp. and are poorly or not toxic to larvae of *Aedes* spp. The highly toxic mosquitocidal strains of *B. sphaericus* produce three kinds of toxins 42-, 51- and 100-kDa proteins. Production of crystal toxins (42- and 51- kDa) is usually closely related to spore formation, however, 100-kDa (Mtx1) is usually produced during vegetative stage (Yousten, 1984; Lacey and Undeen, 1986; Berry *et al.*, 1993; Ostuki *et al.*, 1997; Monnerat *et al.*, 2004).

Bacillus sphaericus has different nutritional requirements; it does not utilize sugars (Fridlender *et al.*, 1989) and does not grow well with starch and several other carbohydrates as carbon sources (Lacey, 1984). Apparently, amino acids are the best source of both nitrogen and carbon, while pyruvate, lactate, acetate or some Krebs cycle intermediates may serve as carbon source supplements (Fridlender *et al.*, 1989).

Many conventional laboratory media have been used by many searchers for the biocidal production. Early work of Knight and Proom (1950) revealed that the *Bacillus* required a fairly complex medium including seven amino acids in addition to thiamine and biotin. This medium was simplified considerably by Power and Pelczar (1964) with the supplementation of only glutamic acid and the vitamins. Afterward, it has been proved that the simplified basal medium of Power and Pelczar (1964) supported only a limited growth of *B. sphaericus,* NCA Hoop 1-A-2 as documented by Chan *et al.*, 1973. Therefore, they formulated the basal glucose-glutamate-salt (GGS) as a simple chemically defined medium for the vegetative growth of that strain. In the presence of 25-50 µg/ml of disodium ethyleneaminetetra-acetate (EDTA), abundant growth and sporulation to varying degrees were obtained.

Bourgouin *et al.* (1984) used Poly medium for growing *B. sphaericus* 1593 and 1881 strains. They illustrated that in spite of the absence of mineral supplements, the sporulation rate reached about 40% after 55 hr of incubation.

Hoti and Balaraman (1986) studied the growth cycle of *B. sphaericus* B.64 using nutrient yeast salt medium (NYSM) and found that the organism entered exponential phase after lag of two hr and the toxin synthesis was initiated around the 7th hr of incubation and reached maximum when the cells completed the exponential phase and entered the stationary phase. However, *B. sphaericus* (VCRC B-42) entered the exponential growth phase after 6 hour in the same medium (NYSM) and the toxin was observed only from 9 hr onwards and reached maximum level at stationary phase (Prabakaran *et al.*, 2007).

Good growth and sporulation were obtained when acetate yeast extract medium (AYEM) was used for the production of *B. sphaericus* 2362 by Sasaki *et al.* (1998). Spore initiation was started after 23hr, maximal yield of cells (13.0 g/l) and spores (4×10^9/ml) were obtained.

Luria Bertani (LBM) medium has been used as a reference medium in the work of Poopathi and his co-workers in 2002 for the production of *B. sphaericus* IAB-59. They found that increasing incubation time resulted in parallel increase in culture density, spores and crystals.

A program on biological control of mosquitoes, virulence prospecting and evaluation of new isolates around the world is one of the most important steps taken to determine their effect on target populations and thereby selecting the most promising strains for producing biological insecticides (Litaiff *et al.*, 2008).

In the present study, we screened these conventional media to select the best one that support growth, sporulation and toxin production of the local isolated strains for further studies.

MATERIALS AND METHODS

Microorganisms:

Two actively marked toxic strains of *Bacillus sphaericus* (Fathy, 2002), previously isolated from the soil of North Sinai in Egypt, identified

morphologically, biochemically and assayed biologically against mosquito larvae, were used in the present study along with the reference strains of *Bacillus sphaericus* 1593 and 2297. The reference strains were kindly provided by Prof. Dr. Y. A. Osman, Mansoura University and Prof. Dr. M. S. Foda, National Research Center, respectively.

1- Detection of toxin genes by Polymerase chain reaction (PCR)

a- Isolation of genomic DNA

The tested strains were inoculated in 50 ml LB medium and incubated at 30C for 18h, then centrifuged at 6000 rpm for 10 min. The pellets were resuspended in 50µl of TE buffer. Phenol/chloroform mixture was added at 500µl and all components were vortexed strongly and centrifuged at 6000 rpm for 30 min. The supernatant was transferred in new tube then 1ml ethanol 95% was added for half an hour. The mixture was centrifuged at 6000 rpm for 15 min. the pellets were washed with 500µl ethanol 70% then centrifuged for 5 min. and dried at room temperature (Marmur, 1961).

b- Primers

As shown in Table (1), three pairs of specific primers (Biobasic, Canada) were used to identify genes *Bin A*, *Bin B* that encode binary toxin (41.9 and 51.4 kDa) and gene *Mtx1* that encode mosquitocidal toxin, 100 kDa, (Shanmugavelu *et al.*, 1995).

Table (1): The sequence of primers and the expected size of the amplified products

Primer	Sequence	Length meres	Standard	Product size
BinA	5'ATGAGAAATTTGGATTTTATT 3'	21	*B.s.*1593M	1.3kb
	5'TTAGTTTTGATCATCTGTAAT 3'	21	*B.s.*1593M	
BinB	5'ATGTGCGATTCAAAAGACAAT3'	21	*B.s.*1593M	1.1kb
	5'TCACTGGTTAATTTTAGGTA 3'	20	*B.s.*1593M	
Mtx1	5'ATGGCTATAAAAAAAGTATTA 3'	21	*B.s.*1593M	2.6kb
	5'TACTATCTAGGTTCTACACC 3'	20	*B.s.*1593M	

c- PCR reaction

The genomic DNAs were transferred to 25 µl of PCR-reaction mixture containing 0.5µM of each primer, 0.2mM of each dNTP, 1x of *Taq* polymerase buffer, 1.5 mM $MgCl_2$ and 2.5U of *Taq* polymerase (Red Hot). The PCR amplifications were performed as follows: initial denaturation of DNA at 94°C for 5 min, 35 cycles comprised of 1 min denaturation at 94°C, 1 min annealing at 55°C, 2 min elongation step at 72°C followed by a final extension step at 72°C for seven min. Amplicons were visualized by electrophoresis on 1% agarose gel stained with ethidium bromide. The banding was visualized at short UV light (Carozzi *et al.*, 1991).

2- Analysis of protein profiles

Protein analyses were made for both 18 and 120 hr nutrient broth cultures of *Bacillus sphaericus* strains. Samples were prepared and separated on sodium dodecyle sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970).

Seed cultures preparation:

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, 1983. The slant cultures were washed with 5.0 ml sterile distilled water, which were then added to 250 ml flasks containing 50 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 18h.

Production media:

Five conventional laboratory media that have been recommended as reference media by many authors were used for *B. sphaericus* production as follows: Glucose-Glutamate-Salts- EDTA (GGSEM), Chan *et al.*, 1973, g/l, glucose 5, monosodium glutamate 10, K_2HPO_4 0.5, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.2, $FeSO_4.7H_2O$ 0.01, $MnSO_4.4H_2O$ 0.01, $ZnSO_4.7H_2O$ 0.013, $CaCl_2$ 0.025, thiamine 0.0005, biotin 1μg, EDTA 25 μg/ml; Nutrient Yeast Extract Salt Medium (NYSM) without glucose, Yousten and Davidson, 1982, g/l, peptone 5, beef extract 3, yeast extract 0.5, $MnCl_2$ 0.01, $CaCl_2$ 0.1, $MgCl_2$ 0.2 ; Poly Medium (PM), Bourgouin *et al.*, 1984, g/l, peptone 5, beef extract 5, yeast extract 10, glycerol 10, NaCl 3; Acetate Yeast Extract Medium (AYEM), Sasaki *et al.*, 1998, g/l ,sodium acetate 5.45, yeast extract 10, $MnCl_2$. $4H_2O$ 0.02, $CaCl_2$. $2H_2O$ 0.2, $MgCl_2$. $6H_2O$, 1.02, KH_2PO_4 0.5 and Luria Bertani (LBM), Poopathi *et al.*, 2002, g/l, peptone 10, yeast extract 5, NaCl 10. The pH of all media was adjusted to 7.1 ± 0.1 with 1N NaOH, and the media were dispensed in flasks as 20% v/v and sterilized at 121°C for 20 min.

Fermentation procedure:

Production flasks of each medium were inoculated in triplicate with ($\times 10^4$ CFU/ml) of a second passage seed culture of each *B. sphaericus* strain and allowed to grow at 30°C for 5 days on a rotary shaker at 200 rpm (Cole Parmer, 51604). Culture samples were drawn from each culture medium at 0, 1, 3 and 5 days intervals.

Growth cycle:

The medium showed the best result was chosen to study the growth cycle of *Bacillus sphaericus*. Flasks containing medium (20% v/v) were inoculated from 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 hr, then at daily intervals until 5 days.

In each sample, total viable count, spores count, pH, protein estimation and larvicidal activity against 3rd instar larvae of *Culex pipiens* were performed.

Total viable and spore counts:

Serial decimal dilutions of culture samples were prepared where 1ml 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 48h and the developing *B. sphaericus* colonies were counted and expressed as CFU/ml and/or spores/ml. The pH of culture samples was estimated using a digital pH meter (JEN WAY, 3305)

Biochemical studies and toxicity bioassay:

Whole culture samples for each strain on different media were centrifuged at 6000 rpm for 10 min, then the pellets were harvested and washed twice with distilled water (6,000 rpm/10 min/ 4 C). Thereafter, pellets were resuspended in distilled water.

Protein estimation:

Protein extracts were prepared by adding 25 µl of 2M NaOH solution to each ml suspension followed by incubation at 37°C for 3hr (Sasaki *et al.*, 1998). 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 and Population. The toxicity of the whole culture samples was determined. Serial dilutions from the pellet suspension of culture samples were prepared, then one ml of each dilution was added to 100 ml distilled water in 200 ml plastic cups. Twenty, 3rd 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). Each experiment included 3 concentrations in triplicates, along with the appropriate control and conducted at room temperature 28 $\pm$ 2 C. Larval mortality was scored after 48 hr and corrected (if needed) for control mortality using Abbott's formula (Abbott, 1925).

Statistical analysis:

Factorial ANOVA test was used to compare the mean maximum amount of crystal protein and low LC_{50} value among different treatments (M-statc).

RESULTS

The PCR product profiles of 2 local (NSE1, NSE2) and 2 standard strains (1593, 2297) are shown in Fig.1 (a, b & c). Analysis of these profiles proved that both the standard and local strains harbour the *Bin A, Bin B* and *Mtx1* genes encoding Bin A 42-, Bin B 51- and Mtx1 100-kDa proteins. These results were consistent with the protein profiles for these strains where the presence of 51-, 42- and 100 kDa bands, corresponding to the sizes of Bin B, Bin A and Mtx1, respectively, were observed (Fig. 2).

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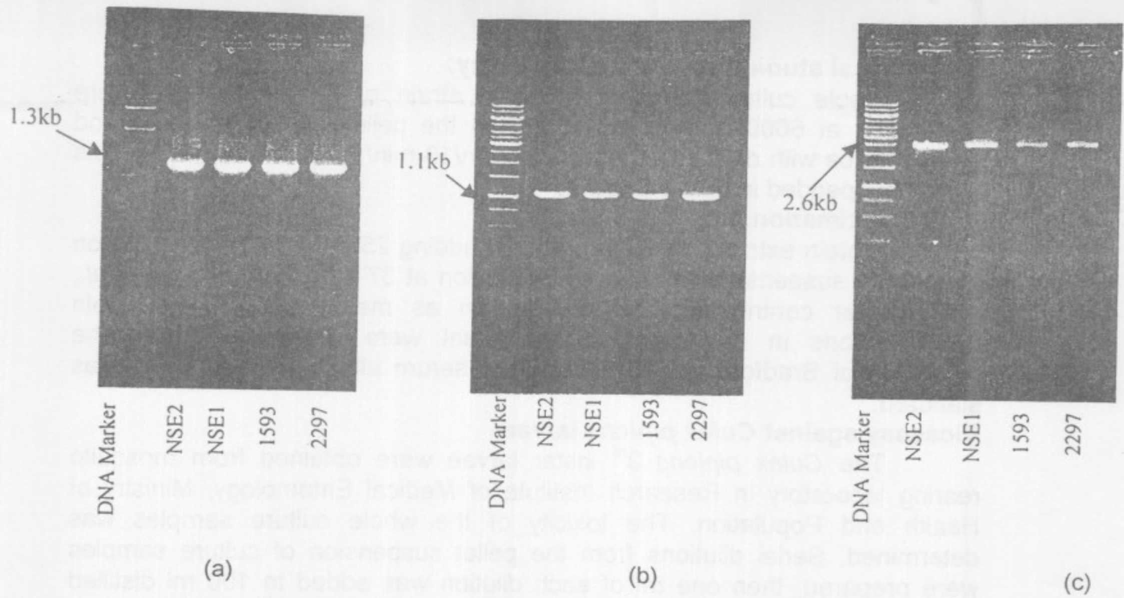

Fig.1. The PCR product profiles of *B. sphaericus* isolates.

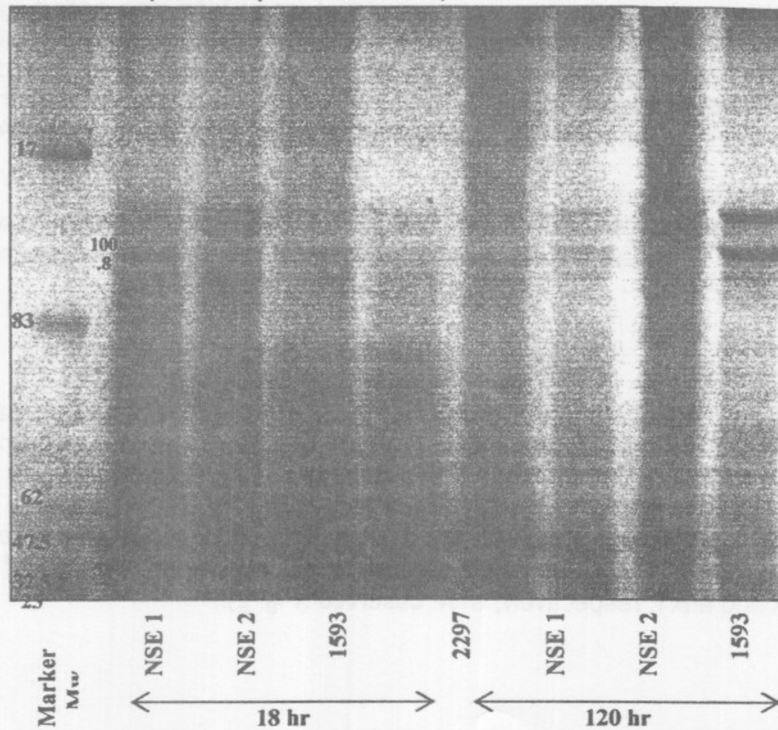

Fig. 2. Gel electrophoresis (SDS-PAGE) of crude toxin extracts from *Bacillus sphaericus* strains.Molecular weight standards in kDa; 175, 83, 62, 47.5, 32.5 and 25.

Regarding the overall growth and toxin production in all conventional laboratory media, it is palpable that the viable counts of *Bacillus sphaericus* strains varied according to growing medium, PM had the highest viable counts for all strains throughout the fermentation time (Fig. 3). After 24 hr cultivation, PM achieved the highest significant counts (8.7×10^8 -1.2×10^9 CFU/ml), however no significant differences could be observed between the other tested media. In addition, there was no significant differences between local and reference strain 2297. Increasing fermentation time up to 72 hr resulted in increasing growth yield; PM was still the best medium supporting the growth of different *B. sphaericus* strains (about 43- 300 fold over the other tested media). After 5 days and at the end of the fermentation course, the viable counts of different strains decreased in PM, but they increased in the other tested media with multiplication rate ranged from 1.25 to 58.3 fold. NYSM attained the highest significant yield of 1.1 - 1.3×10^9 CFU/ml and stand with PM without significant differences. AYEM (1 – 1.8×10^8 CFU/ml), LBM (2×10^7 – 2.7×10^8 CFU/ml) and GGSEM (2×10^7 – 1×10^8 CFU/ml) came after (Fig. 3).

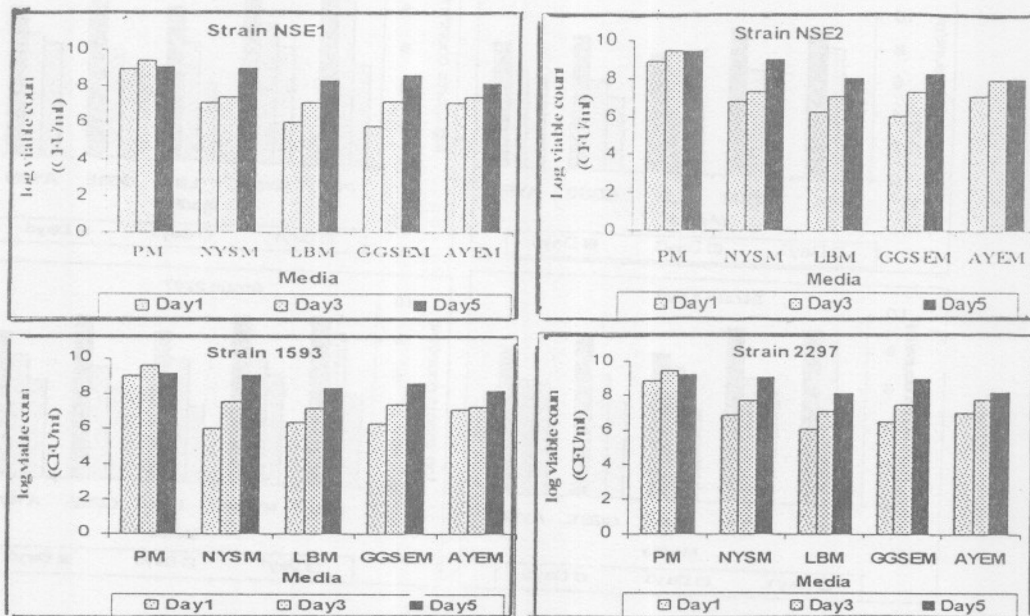

Fig.3. Total viable counts of local and standard strains of *Bacillus sphaericus* in different conventional laboratory media (LSD, 0.01 is 21.5)

Sporulation rate was generally at low level after 24 hr in all production media, PM recorded the lowest significant rate (1.3 – 1.7%), although it attained the higher viable spore counts. After 72 hr, sporulation rate was still low (Figs.4, 5) and significant differences were observed within the tested strains in PM and between PM and the other tested media. The lowest figure was found in GGSEM (3.7×10^5 – 2.4×10^6 spores/ml). Consequently, the maximum yield of spores was reached at the end of the fermentation time in both PM and NYSM ($1.0 – 1.4 \times 10^9$ spore/ml) followed by GGSEM . ($1.1- \quad 8.0 \times 10^8$ spores/ml). The highest significant sporulation rate (88.5 - 100%) was obtained in NYSM followed by PM and GGSEM (71.4 – 93.3%), (40.0 – 80%) in that order. There were no significant differences between NSE1 and the reference strain 2297 but they significantly varied with the other tested strains (Fig. 5).

Protein was produced after 24 hr in the fermentation media except in GGSEM and AYEM, whereas it was produced lately.

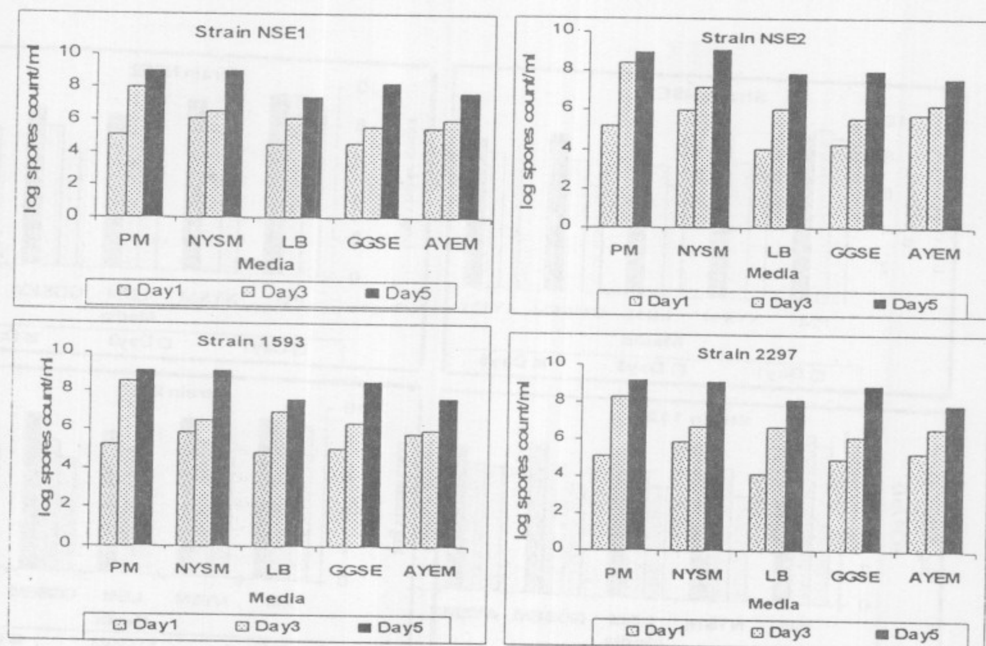

Fig.4. Total viable spores count of local and standard strains of *Bacillus sphaericus* in different conventional laboratory media (LSD, 0.01 is 0.95)

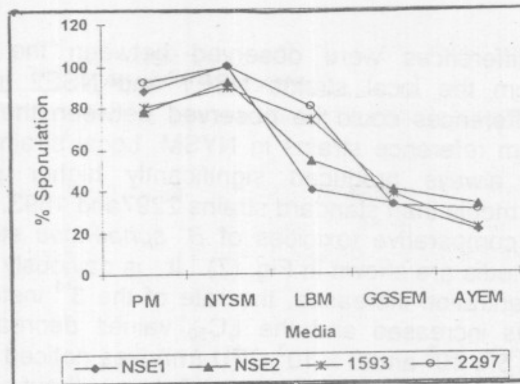

Fig.5. Sporulation rate of local and standard strains of *B. sphaericus* after 5 days of incubation in the different fermentation media (LSD, 0.01 is 0.47)

Protein was in increasing order along the fermentation course. The significant highest quantities were attained in PM by the local strains NSE1, NSE2 and the standards 2297 and 1593, in that order by 5 days (Fig. 6). Generally, the quantities of produced protein differed significantly among the tested media and strains along the fermentation time.

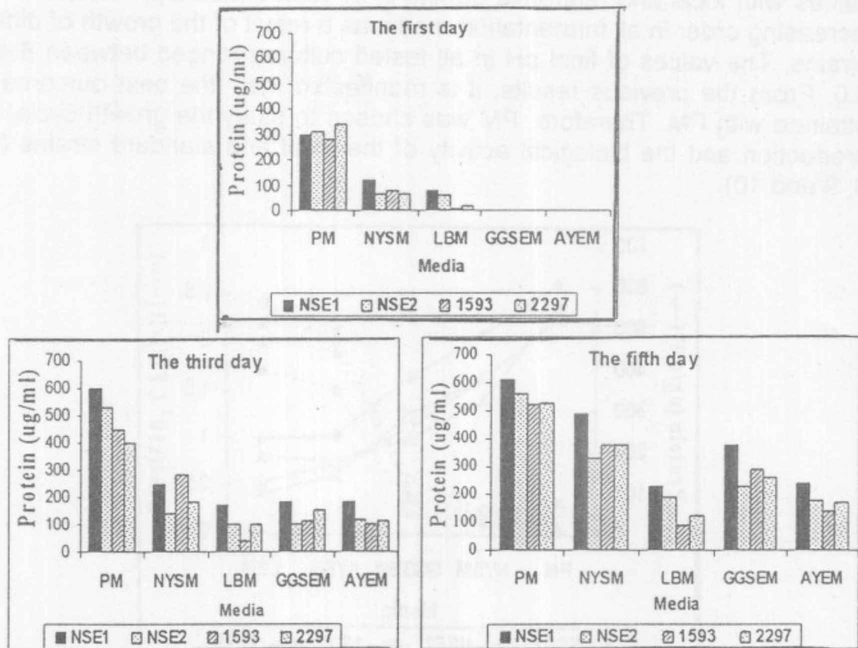

Fig.6. Protein synthesis by local and standard strains of *Bacillus sphaericus* in the conventional media during fermentation course (LSD, 0.01 is 0.09).

After a lag phase of 0-6 hr, 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 hr (Fig. 8). The maximum growth was completed after 96 hr and the highest sporulation rate was observed after 120 hr in a range of 78-92%. The highest percentage was obtained with 2297, NSE1 (92, 88), however, the lowest one was observed with NSE2 strains, in that order (Fig. 9). Statistical analysis revealed that all strains were significantly varied.

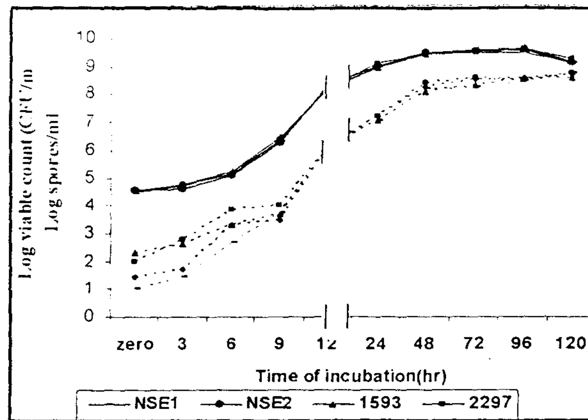

Fig. 8. Growth cycle and sporulation of local and standard strains of *Bacillus sphaericus* in PM (____)Total viable count, LSD,0.01 is 0.71),(---)Total spores count,(LSD, 0.01 is 393.4).

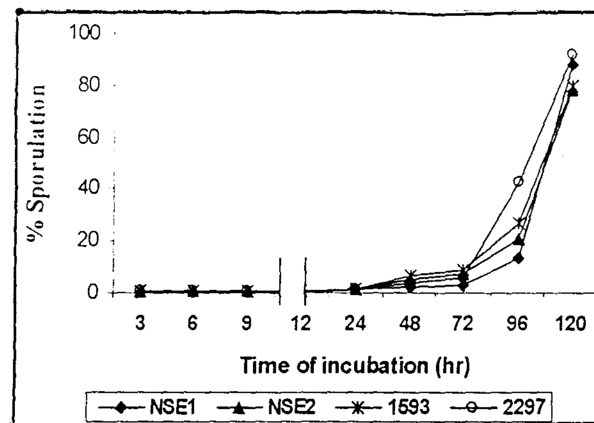

Fig. 9. Sporulation rate during the growth of local and standard strains of *B. sphaericus* in PM (LSD, 0.01 is 0.29).

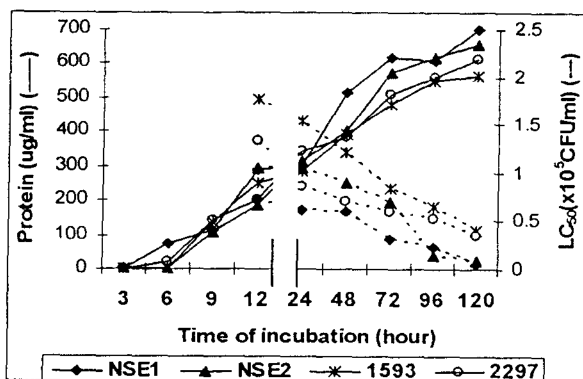

Fig.10. Relation between the protein synthesis and larvicidal activity during growth of local and standard strains in PM.

DISCUSSION

Polymerase chain reaction (PCR) analysis proved that both the standard 1593, 2297 and local NSE1, NSE2 are highly toxic strains. They harbor the *BinA, BinB* and *Mtx1* genes encoding Bin A 42-, Bin B 51- and Mtx1 100-kDa proteins. The well known, highly toxic mosquitocidal strains harbor the 42-, 51- and 100-kDa genes (Shanmugavelu *et al.,* 1995; Otsuki *et al.*, 1997). The presence of 100-kDa corresponding to the Mtx1 toxin observed on SDS-PAGE in 18 hr and 120 hr cultures was symmetrical with the PCR product profile obtained in the present work. However, it was not previously observed on SDS-PAGE, according to the fact that this is a vegetative protein; it has not been observed in spores crystal mixture (Thanabalu *et al.*, 1992; Thanabalu and Porter, 1995; Monnerat *et al.*, 2004).

While the conventional microbiological methods have been useful, they are time-consuming, error-prone and involve tedious procedures. These methods lack specificity unless the microorganisms are endowed with well identified genetic markers such as mosquito larvicidal genes (MLGs) coding for the mosquito larvicidal protein (Baumann *et al.*, 1991; Porter *et al.*, 1993). When any of the primers were tested with genomic DNA of other *Bacillus* species, absolute specificity was seen only for *B. sphaericus* DNA and no products were amplified from purified genomic DNA of *B. amyloliquefaciens or B. thuringiensis var israelensis* (Shanmugavelu *et al.*, 1995). Since the use of locally available effective bacterial strains is always advisable in biological control programs, it should be genetically analyzed to screen the presence of newer or perhaps more toxic strains.

From the present results, it is clearly evidenced that media, incubation time and *B. sphaericus* strains play a key role in growth, sporulation, protein synthesis and potency. A marked difference in growth, sporulation, time at which toxin was produced and the level of toxin production during fermentation was observed for both local and standard strains. The same observations were reported early by many workers

(Singer, 1979 & 1980; Bourgouin and *de* Barjac, 1980; Meyers and Yousten, 1981; Lacey, 1984).

Prolonging fermentation time up to 5 days actualized the maximum lethal activity and sporulation rate in all media tested. Antipodal, Klein *et al.* (1989) found that prolonged fermentation times up to 48 hr have never resulted in consistent increase of spore counts. The present results revealed a steady increase in spore counts in all the tested media along the fermentation time. Growth cycle of both local and standard strains insinuated that growth and sporulation in PM were almost completed after 96 and 120 hr, respectively. The time at which the protein synthesized in PM was varied with *B. sphaericus* strains and ranged from 6 to 12 hr. In this regard, it has been found that, after a lag phase of 0-6 hr, toxin synthesis by *B sphaericus* B.64 in NYSM was initiated around 7 hr after incubation and became at maximum when the cells completed the exponential phase and entered the stationary phase (Hoti and Balaraman, 1986). With strain 1593, Meyers *et al.* (1979) reported that the toxicity increased markedly as the cells entered the early stage of sporulation which corresponded to the stationary phase. Also, Prabakaran *et al.* (2007) found that *B. sphaericus* (*VCRC B-42*) did not produce toxin during the lag phase of 0-6 hr in NYSM. The toxin was observed after 9 hr, however it was observed after 6 hr in medium contained 2 % peanut cake powder.

In Egypt, Foda *et al.* (2003) noted progressive increase in toxin production by *B. sphaericus* 14N, 2362 strains in semi-solid fermentation with extending incubation time; the maximum level was attained after 9 days. Recently, Poopathi and *Abidha*. (2007 and 2008) found that in NYSM both *B. sphaericus* strains, IAB-59 and 2362 showed a rapid multiplication after a lag phase of about an hour to reach the plateau after 48 hr as measured by optical density at 650 nm, while the maximum growth, sporulation and endotoxin release was completed after 72 hr.

The PM and NYSM respectively supported good growth and spore production of *B. sphaericus* strains without significant differences in the growth profile especially at the end of the incubation time. However, marked differences of larvicidal activities were observed between local and standard strains. Toxins produced by the NSE1, NSE2 strains in PM, NYSM media, respectively, were significantly more lethal than the standard strains 2297 and 1593 of *B. sphaericus.*

The PM was found to be the most auspicious medium. This might be attributed to the medium contents of yeast extract and glycerol. It is well known that yeast extract is mainly used as an excellent source of B-complex and also rich in amino acids which support both growth and sporulation. A considerable improvement of larvicide production and almost complete sporulation in the presence of glycerol (5 g/l) have been proved by Fridlender *et al.* (1989) and Klein *et al.* (1989).

At the end of the fermentation course, complete sporulation never obtained in PM but attained by NSE1 and 2297 strains in NYSM, although an equal numbers of viable spores/ml was obtained in both media. It is worthy mentioning that NYSM supplemented with minerals such as Mn^{++}, Mg^{++} and Ca^{++} supported sporulation and toxin protein production as indicated by

Dharmsthiti *et al.,* 1985. Likewise, Rady and el-Deen (1991) stated that the concentrations of the extracellular proteins were always higher in the absence of the inorganic minerals, though the toxicity of the proteins against 3rd instar *Culex pipiens* L. larvae increased in the presence of inorganic elements. Our results denoted that the toxicity of proteins released by reference strains in NYSM at the end of fermentation course was significantly more lethal than that obtained in PM; however the toxicity of proteins released in PM by local strains were significantly more lethal than standard strains and equally toxic to that obtained in NYSM. Meyers and Yousten (1978) found that excluding Mn^{2+} from the complex medium resulted in culture with few spores but was of equal toxicity to a culture containing many spores.

Unsatisfied results either in growth, spores and/or toxin yields were obtained when GGSEM was used for growing *B. sphaericus* strains, although, it contains the vitamins biotin and thiamine, minerals including Ca^{2+} and Mn^{2+} as well as glutamate which recommended for the bacterium growth and sporulation (Singer *et al.*, 1966; Meyers and Yousten, 1978). Sporulation had been found to be varied widely from about 10% to near total sporulation from experiment to another even though all conditions were kept constant (Chan *et al.*, 1973)

It has been reported that although *B. sphaericus* may be grown with glutamic acid as the sole carbon source, growth and toxin yield were improved considerably with the addition of lysine, methionine, isoleucine and valine in synthetic media (Singer, 1981; Singer, 1982). In 1989, Klein and his coworkers, obtained high larvicide yield equally to that attained in media supplemented with glycerol when peptone was substituted with L- glutamate in fermentation medium for growing *B. sphaericus* 2362.

Although, AYEM is containing acetate, minerals required for sporulation and yeast extract at 10 g/l, it gave significantly low yield of spores and toxins. This result was in congruence with those obtained by Fridlender *et al.*, 1989 and Sasaki *et al.,* 1998.

A relatively meager larvicide yield was formed by all tested strains in LBM, this may be due to the shortage of the nutrients necessary for toxin synthesis comparing to the other tested media.

The present results gave an idea about the most effective medium that will be used in further study to optimize the biocide production and to develop a low cost effective production medium.

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REFERENCES

Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. Journal of Economic Entomology, 18: 265-267.

Baumann, P.; Clark, M.; Baumann, L. and Broadwell, A. (1991). *B. sphaericus* as mosquito pathogen: Properties of the organism and its toxins. Microbiology Review, 55: 425-436.

Berry, C.; Hindley, J.; Grounds, T.; Desouza, I. and Davidson, E. W. (1993). Genetic determinants of host ranges of *Bacillus sphaericus* mosquito larvicidal toxins. Journal Bacteriology, 175: 510-518.

Bourgouin, C. and *de* Barjac, H. (1980). Evaluation du potential de *Bacillus sphaericus* comme larvicide anti-moustiques. WHO mimeo. doc. WHO/VBC/80(792):24pp.

Bourgouin, C.; Larget, T.I. and *de* Barjac, H. (1984). Efficacy of dry powders from *B. sphaericus* RB80, a potent reference preparation for biological titration. Journal of Invertebrate Pathology, 44: 146-150.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254.

Carozzi, N.B.; Kramer, V.C.; Warren, G.W.; Evola, and Koziel , M.G. (1991). Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymierase chain reaction product profiles. Applied and Environmental Microbiology, 57(11): 3057-3061.

Chan, E.C.; Rutter, P.J. and Wills, A. (1973). Abundant growth and sporulation of *Bacillus sphaericus* NCA Hoop 1-A-2 in a chemically defined medium. Canadian Journal of Microbiology, 19: 151-154.

Dharmsthiti, S.C.; Pantuwatana, S. and 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.

Fathy, H. M. (2002): Studies on some biocide-producing microorganisms. M. Sc. Thesis, Faculty of Agriculture, Cairo University, 274.

Foda, M.S.; El-Bendary, M and Moharam, M. E. (2003). Salient parameters involved in mosquitocidal toxins production from *Bacillus sphaericus* by semi-solid substrate fermentation. Egyptian Journal of Microbiology, 38(3): 229-246.

Fridlender, B.; Keren-Zur, M.; Hofstein, R.; Bar, E.; Sandler, N.; Keynan, A. and Braun, S. (1989). The development of *Bacillus thuringiensis* and *Bacillus sphaericus* as biocontrol agents: from research to industrial production. Memorias do Instituto Oswaldo Cruz, 84(3): 123-127.

Hoti, S.L. and Balaraman, K. (1986). Parameters facilitating local production of *Bacillus sphaericus*. Indian Journal of Medical Research, 83: 166-170.

Klein, D.; Yanai, R.; Hofstein, R.; Fridlender, B. and Braun, S. (1989). Production of *Bacillus sphaericus* larvicide on industrial peptones. Applied Microbiology and Biotechnology, 30: 580- 584.

Knight, B. C. and Proom, H. (1950). A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus*. Journal of General Microbiology, 4: 508-538.

Lacey, L. A. (1984). Production and formulation of *Bacillus sphaericus*. Mosquito News, 44(2): 153-159.

Lacey, L. A. and Undeen, A. H. (1986). Microbial control of black flies and mosquitoes. Annual Review of Entomology, 31:265-296.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Litaiff, E. C.; Tadel, W.P.; Porto, J. I.R and Oliveira, I, M, A (2008). Analysis of toxicity on *Bacillus sphaericus* from Amazon soils to *Anopheles darlingi* and *Culex quinquefasciatus* larvae. Acta Amazonica: 38(2): 225- 262.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. Journal Molecular Biology, 3:208-218.

Monnerat, R.; da Silva, S.F.; Dias, D.S.; Martins, É.S.; Praça, L.B.; Jones, G.W.; Soares, C.M.; de Souza Dias, J.M.C. and Berry, C. (2004). Screening of Brazilian *Bacillus sphaericus* strains for high toxicity against *Culex quinquefasciatus* and *Aedes aegypti*. Jen, 128(7): 469-473.

Meyers, P. and Yousten, A. A. (1978). Toxic activity of *Bacillus sphaericus* SSII-1 for mosquito larvae. Infection and Immunity, 19: 1047-1053.

Meyers, P. and Yousten, A. A. (1981). Toxic activity of *Bacillus sphaericus* for mosquito larvae. Development of Index Microbiology, 22: 41-52.

Meyers, P.; Yousten, A. A. and Davidson, E. W. (1979). Comparative studies of the mosquito-larval toxin of *Bacillus sphaericus* SSII-1 and 1593. Canadian Journal of Microbiology, 25:1227-1231.

Obeta, J.A.N. and Okafor, N. (1983). Production of *Bacillus sphaericus* strain 1593 primary powder on media made from locally obtainable Nigerian agricultural products. Canadian Journal of Microbiology, 29: 704-709.

Otsuki, K; Guaycurus, T.V. and Vicente, C.P. (1997). *Bacillus sphaericus* entomocidal potential determined by polymerase chain reaction. Memorias do Instituto Oswaldo Cruz, 92(1): 107-108.

Poopathi, S. and Abidha, S. (2007). Use of feather-based culture media for the production of mosquitocidal bacteria. Biological Control, Cited in http://www.elsevier.com / locate/ibiod.

Poopathi, S. and Abidha, S. (2008). Biodegradation of poultry waste for the production of mosquitocidal toxins. International Biodeterioration and Biodegradation, Cited in http://www.elsevier.com / locate/ibiod.

Poopathi, S.; Anup Kumar, K.; Kabilan, L. and 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.

Porter, A. G.; Davidson, E. W. and Wei, Liu, J. (1993). Mosquitocidal toxins and their genetic manipulation for effective biological control of mosquitoes. Microbiolocical Review, 57: 838-861.

Power, D. A. and Pelczar, M. J. (1964). Microbial interactions: population and sporulation studies of *Bacillus sphaericus* grown in association with *Erwinia atroseptica*. Antonie van Leeuwenhoek Journal Microbiology Serology, 30: 97-108.

Prabakaran, G.; Balaraman, K.; Hoti, S. L. and Manonmani, A. M. (2007). A cost- effective medium for the large- scale production of *Bacillus sphaericus* H5a5b (VCRC B42) for mosquito control. Biological Control, 41: 379-383.

Rady, M.H. and el-Deen, A. F. (1991). Effect of nutritive elements on the extracellular protein of different *Bacillus* strains, toxic to mosquito larvae. Journal of Egypt Society and Parasitology, 21:575-583.

Sasaki, K. Jiaviriyaboonya, S. and Rogers, P.L. (1998). Enhancement of sporulation and crystal toxin production by corn-steep liquor feeding during intermittent fed-batch culture of *Bacillus sphaericus* 2362. Biotechnology Letters, 20(2): 165-168.

Shanmugavelu, M.; Sritharan, V. and Jayaraman, K. (1995). Polymerase chain reaction and non- radioactive gene probe based identification of mosquito larvicidal strains of *Bacillus sphaericus* and monitoring of *B. sphaericus* 1593M, released in the environment. Journal of Biotechnology, 39(2): 99- 106.

Singer, S. (1979). Use of entomogenous bacteria against insects of public health importance. Development of Index Microbiology, 20: 117-122.

Singer, S. (1980). *Bacillus sphaericus* for the control of mosquitoes. Biotechnology and Bioengineer, 22: 1335-1355.

Singer, S. (1981). Mosquitoes: Time to look again at *B. sphaericus*. Practical Biotechnology, April 16- 20.

Singer, S. (1982). The biotechnology for strains of *Bacillus sphaericus* with vector control potential. Proc. III Int. Coll. Invertebrate Pathology, Sussex University, England, pp.485-489.

Singer, S.; Goodman, N. S. and Rogoff, M. H. (1966). Defined media for the study of bacilli pathogenic to insects. Annual N. Y. Academic Science, 139: 16-23.

Thanabalu, T. and Porter, A. (1995). Efficient expression of a 100-kDa mosquitocidal toxin in protease- deficient recombinant *Bacillus sphaericus*. Applied and Environmental Microbiology, 61:4031-4036.

Thanabalu, T.; Hindley, J. and Berry, C. (1992). Proteolytic processing of the mosquitocidal toxin from *Bacillus sphaericus*. SSII-1. Journal of Bacteriology, 174: 5051-5056

Yousten, A. A. (1984). *Bacillus sphaericus*: microbiological factors related to its potential as a mosquito larvicide. In "Advances in Biotechnological Processes". vol.4. (Ed., Mizrahi, A and Van Wezel, A.), New York, Alan R. Liss.

Yousten, A. A. and Davidson, E. W. (1982). Ultrastructural analysis of spores and parasporal crystals formed by *Bacillus sphaericus* 2297. Applied and Environmental Microbiology, 44(6):1449-1455.

تقييم البيئات المعملية التقليديـــة لإنتـــاج الـــسلالات الـــسامة النـــشطة لبكتريـــا *Bacillus sphaericus*

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أستخدم فى هذا البحث سلالتان محليتان SNE1, SNE2 لهما نشاط بيولـــوجى ضـــد يرقات بعوض الكيولكس (تم عزلهما سابقا من منطقة شمال سيناء بمصر وتعريفهما على المستوى المورفولوجى والكيميائى والبيولوجى) كما أستخدم معهما سلالتان من السلالات المرجعية ١٥٩٣، ٢٢٩٧ للمقارنة. وقد تم أولا التحليل بطريقة البلمرة الجزيئية PCR للكشف عن الجينات *BinA, BinB, Mtx1* المسؤلة عن التشفير للبروتينات السامة ذات الأوزان الجزيئيـــة التاليـــة ٤٢، ٥١، ١٠٠ كيلو دالتون- كذلك تم الفصل الكهربائي للبروتينات المنتجة في مـــزارع عمـــر ١٨ و ٢٤ ساعة باستخدام البولى أكريليميد جيل SDS-PAGE – ثانيا تنمية هذه السلالات علـــى خمـــس بيئات معملية تقليدية (PM, NYSM, GGSEM, AYEM, LBM) لتحديد أحسنهم إنتاجية من حيث النمو ، التجرثم وإنتاج البروتينات السامة وذلك لاستخدامها فى إجراء دراسات إضافية.**ثالثـــا** اختبار النشاط البيولوجي للسلالات المنماه علي البيئات المختلفة ضد الطور الثالث ليرقات بعوض الكيولكس. وقد أوضحت النتائج أن السلالات المحلية مثل السلالات المرجعية تحتوى على الجينات المسؤلة عن التشفير للبروتين ١٠٠ كيلودالتون أثناء النمو الخضرى وكذلك البروتينات ٤٢ ، ٥١ كيلودالتون أثناء مرحلة التجرثم وقد جاءت نتائج الفصل الكهربائى للبروتينات مطابقة وذلك بوجود البروتينات السابقة مما يؤكد أن السلالات المحلية على درجة عالية من الـــسمية مثـــل الـــسلالات المرجعية. أعطت البيئة PM أعلى محصول من الخلايا بعد ٧٢ ساعة وكانت الفروق معنوية بينها وبين البيئات الأخرى وبعد ١٢٠ ساعة تساوت معها البيئة NYSM لتعطـــى ١,١-١,٣ × ١٠ [٩] خلايا حية/ملى ، بينما جائت البيئات GGSEM, LBM, AYEM على التـــوالى. أيـــضا تـــم الحصول على أعلى محصول من الجراثيم على كل من بيئتى NYSM , PM (١-١,٤ × ١٠ [٩] جراثيم/ ملى) بعد نهاية مدة التخمر. كان أعلى معدل للتجرثم علـــى البيئـــة NYSM (٨٨,٥-١٠٠%) تلتها PM (٧١,٤- ٩٣,٣%) و GGSEM (٤٠ -٨٠%) علـــى التـــوالى و بفـــروق معنوية. لوحظ إنتاج البروتين بعد٦-٩ ساعات من بداية عملية التخمر وحتى النهايـــة ، وقـــد تـــم الحصول على أعلى إنتاج من PM بواسطة السلالات المحلية وكانت الفروق معنوية بينها وبـــين السلالات المرجعية. أيضا أعطت السلالات المحلية أعلى نشاط بيولوجى ضد الطور الثالث ليرقات بعوض الكيولكس حيث كان أقل تركيز من الخلايا ٥×١٠ [٣] ، ٨×١٠ [٣] خلايا حية / ملـــى مـــن المزرعة الكلية لكل من السلالتان NSE2, NSE1 على التوالى للحصول علـــى ٥٠% مـــوت لليرقات. إرتفعت درجة الـــ pH فى جميع البيئات أثناء فترة النمو لتصل ٨,٣ – ٩.