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RESEARCH ARTICLE

ISOLATION, CHARACTERIZATION & OPTIMIZATION OF CULTURAL CONDITION FOR *BACILLUS THURINGINSIS* BT FROM SOILS OF GAZA STRIP

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ABSTRACT

Bacillus thuringiensis (Bt) is a ubiquitous Gram-positive, rod-shaped and sporulating bacterium that has been isolated worldwide from a great diversity of ecosystems including soil, water, dead insects, dust from silos. It makes proteins that are toxic to immature insects (larvae). In current study *Bt* was isolated from different agricultural and non-agricultural soils for different locations in Gaza strip. The effect of different parameters on biomass yield production such as pH, temperature and incubation time discuss. Present study show the optimum conditions of pH, temperature and incubation time, for growth as well as biomass production, and was obtained at pH 7.0, 30°C, 24 hours respectively. Morevore, compare between the Btg (isolated from Gaza soil) and Bti (refferance strain) the growth and yield of biomass production was similar.

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INTRODUCTION

In the mid-1970s, the World Health Organization (WHO) and other international institutions initiated studies on the development of existing and new biological control agents (Boisvert and Boisvert, 2000), the organism that suppresses the pest or pathogen is referred to as the biological control agent (BCA) (Krishna *et al.,* 2005). Moreover, in 1975-76 under a World Health Organization sponsored project, Goldberg and Margalit discovered a new Bt strain in the Negev desert in occupied Palestine. The strain was isolated from Culex sp. dead larvae mosquito. Later was identified as Bacillus thuringiensis israelensis, serotype H14 according to its flagellar antigenicity by de Barjac (Mario, 2012).

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Bt strain, a naturally occurring in the soil, plants (Geetha, 2010), dust from stored grain, leaves of conifemus and deciduous trees (Ohba *et al.,* 2002; Yasutake *et al.,* 2007; Geetha, 2010; Leopoldo Palma *et al.,* 2014). Moreover, Bt is a facultative anaerobic bacteria (WHO, 2009), Gram-positive, rod-shaped and sporulation bacterium. In the other hand, the toxic components of Bt are a range of endotoxins bound up in stable protoxin molecules in the parasporal inclusion (Boisvert and Boisvert, 2000). The selective process for isolation of *Bacillus* spp. involves inhibition of germination of *Bacillus* spores by sodium acetate (0.25 M), while allowing all other organisms to grow in a liquid medium. Subsequent heat treatment of the culture at 80°C for 3 min eliminates all of the non-sporulated microbes. The surviving spores are then plated on an agar plate to grow (Xavier *et al.*, 2007).

In order, Bt activity and biomass yield is affected by many environmental factors including temperature, rainfall, pH, and sunlight. (Acharya and Chaudhary, 2012; Immanuel *et al.,* 2006) reported that the pH, temperature is the most important parameters essential for the success of a fermentation reaction. Current study was conducted to isolates a local larvicidal Bacillus strain from Gaza soils and examine the influence of cultural conditions such as pH, T°C and incubation time for production Bt.

MATERIAL AND METHODS

Soil Sampling and Site Selection

Eight soil samples were collected from different positions soil from Gaza strip (Rafah, wadi Gaza, Khanyunes, abbasan, alshikh ejleen, Islamic university, chicken farm soil, mint farm soil, Citrus farm soil). About 25 grams of top soil (after removed 2 cm of soil surface) were collected in a sterile cup, labeled with date and source of collection. Transported to the laboratory and processed within 2 hours of collection. The sources of these samples had not been sprayed with any of the *Bt*-biopesticide before. Table 1 shows the Global positioning system (GPS) different sampling location:

The broth was buffered with 0.25M sodium acetate. The mixture was shaken for 4hrs on a rotary shaker Model Sunior-Orbital having speed of 150 rpm at 37°±2°C. At the end of this time 0.5 ml of the soil suspension was taken and heated in a water bath at 80°C for 10 minutes. The mixture was then serially diluted with sterile distilled water. A volume of 0.1 ml of each dilution was streaked on agar medium.

The same aforementioned broth medium was solidified by the addition of agar and poured in Petri dishes. Plates were incubated at 30°C overnight. The cultures were incubated at 30°C for 24hrs. Colonies were then examined microscopically for sporulation and crystal formation. Isolates suspected to be Bt were kept in slant agar in tubes at 4°C for further characterization and study.

Selective Isolation

One gram of soil sample placed in 9 ml of sterile saline, vortexes for one minute, and heated at 80 ºC for five minutes to eliminate all vegetative bacterial and fungal spores. A loopful from the heated vortexes soil is streaked on control diet of R & F *Bacillus cereus* / *Bacillus thuringiensis* Chromogenic Plating Medium. Plates are incubated at 37 °C for 48 hours.

Table 1. GPS locations of the collected soil samples from different locations of Gaza strip

Soil number	Location	GPS
1	Rafah	
2	Wadi Gaza	
3	Absaan	
5	Chicken Farm	
6	Farm fresh mint	
7	Citrus farm	
8	Islamic University of Gaza	
9	Alshikhejleen	

Bacillus Thuringiensis (Bt) Reference Strains

Reference strains used in this study were obtained from *B. Thuringiensis* subsp. is raelensis

ISOLATION OF LARVICIDAL BACILLUS THURINGIENSIS

Enrichment

Acetate selection method described by (Travers *et al.,* 1987) was used for the soil samples taken from the different sites. Half a gram of each sample was added to 10 ml of broth medium.

Suspected colonies characterized by pale blue colored on chromogenic media are then subcultured for testing their larvicidal activity before an identification process is initiated.

ISOLATE IDENTIFICATION

Microscopic examination

Gram staining

A smear was prepared and air-dried, fixed and stained with gram staining reagents. In short, smears were flooded with crystal violet for one minutes and rinsed with water.

Gram`s Iodine was added for one minutes and rinsed with water. Ethanol was used to decolorize smears for not more than 20 seconds and washed with water. Finally, safrannin was added for 30 seconds. Slides were rinsed and plotted onto absorbent tissue and examined under the high and oil immersion objectives.

Spore Staining (Schaeffer & Fulton`s)

A smear was prepared as previously described. The entire slide was Flooded with Schaeffer & Fulton`s Spore Stain A solution (malachite green). The slide was steamed for 5 minutes and rinsed under running tap water. The slide was counterstained with Schaeffer & Fulton`s Spore Stain B solution (safranin) for 30 seconds. Slides were rinsed and plotted onto absorbent tissue and examined under the oil immersion objectives.

Parasporal crystal toxin detection

At different growth stages of the cultured *Bacillus* isolate, samples were examined under a phase contrast microscope for parasporal crystal toxin.

Cultural characteristics

The selected isolate was plated onto the surface of *B. thuringiensis* chromogenic agar and incubated for 24 hours at 30 °C. Plates were inspected for growth and colony morphology (size, color and texture) were noted.

Biochemical identification

Lecithinase production

A loopful from a 24 hours old *Bacillus* isolate was inoculated onto the surface of Baired-Parker agar media (HiMedia) overnight at 30°C to examine for lecithinase production. Opacity around the colonies is considered positive.

Motility testing

An inoculating needle was used to inoculate (by stabbing into) sulfide-indole-motility (SIM) media (Himedia). The tubes are incubated overnight at 30oC. A diffusion of the growth away from the stab line was considered as positive results for motility. Positive and negative controls were used for comparison.

Penicillin susceptibility (According to the CLSI reference)

Three to five well-isolated colonies of the same morphological type from an agar plate culture were selected. A loop was used to transfer the growth into a tube containing 5 mL of a Brain Heart Infusion Broth. The broth culture was incubated at 30 °C until it achieves or exceeds the turbidity of the 0.5 McFarland standard. The suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard. Adequate light was used to visually compare the inoculum tube and the 0.5 McFarland standards against a card with a white background and contrasting black lines.

A sterile cotton swab was dipped into the adjusted inoculum tube and the excess fluid was drained by pressing the swab against the walls of the test tube. The dried surface of an MHA plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed.

A penicillin disk was applied onto the center of the inoculated MHA, left for 15 minutes at 4°C and incubated for 24 hours at 30°C. The zone of inhibition was measured and recorded in mm. A known Bacillus thurangensis control strain was tested as a control.

Culture Media preparation

Nutrient agar plates (NA), broth agar (NB), selected control diet of R & F Bacillus cereus / Bacillus thuringiensis Chromogenic Plating Medium and media of locally available carbon sources (HI media, india).

Growth Curves preparation

An initial experiment to determine the growth curve of the test strain will be carried out using shake flask culture technique to set a growth comparison point. A 500 µL of an overnight culture will be used to inoculate 50 mL of media of locally available carbon sources in a 250 mL Erlenmayer flask. The culture will be incubated at 30°C by shaking for 72 hrs at 200 rpm. Inoculation time will be considered as time zero. Samples will be taken from the culture with 2 h intervals and used for quantitative determination of growth which will be measured spectrophotometrically at 600 nm. Viable counts will be determined as “colony forming units/mL” simultaneously. A growth curve will be constructed by plotting the absorbance at 600 nm against sampling time. This curve will be used as a reference for comparison with subsequent experiments.

Optimization of Culture Conditions

Determination of optimum growth temperature

An inoculum will be introduced into a 500 ml of sterile broth nutrient media in 500 ml flasks. 5 flaks will be prepared (4 flasks containing *Bt* and one flask as a negative control sample) and incubated at the following temperatures (20, 25, 30, 35) and will be incubated for 24 hours. Inoculation time will be considered as time zero. Samples will be collected at (zero – 4 – 6 – 8 – 12 and 24 hour) of incubation to measure the optical density spectrophotometrically at 600 nm. Another experiment will be designed based on the results of the initial experiment to determine the exact optimum temperature.

Determination of optimum pH

A 500 ml of sterile nutrient broth medium will be prepared and distributed into 7 flasks (6 flasks containing *Bt* and one flask as a negative control sample). The pH of the medium will be adjusted using 1N HCl and 1N NaOH to obtain the following pH values (6, 6.5, 7.0, 7.5, 8.0) respectively. An equal volume

of the standardized inoculums will be added to each flask and incubated at the optimum temperature obtained from the previous experiment for 24 h. Samples will be collected at after 24 hour of incubation to measure the optical density spectrophotometrically at 600 nm. Another experiment will be designed based on the results of the initial experiment to determine the exact optimum pH using smaller intervals.

Determination of optimum incubation time

A standardized BT inoculum will be added into 3 separate flasks (triplicates) containing nutrient broth medium. A sample will collect at 4, 6, 8, 12, and 24 hours. Each sample will be tested for viable cell counts and the turbidity will be determined spectrophotometrically at OD 600 nm.

Data Analysis and presentations

All data generated was collected from the experiments and tabulated by Microsoft Excel for tables and graphs (Version 2010).

RESULTS

Isolation of *Bt* from Gaza Soil

Eight samples from different Gaza soils were collected. Sodium acetate selection methods were used for the isolation of Bt. Colonies on plates of nutrient agar that had similar colony morphology to Bt were selected as referances (Bti) and this colony as shown in (Figure 1).

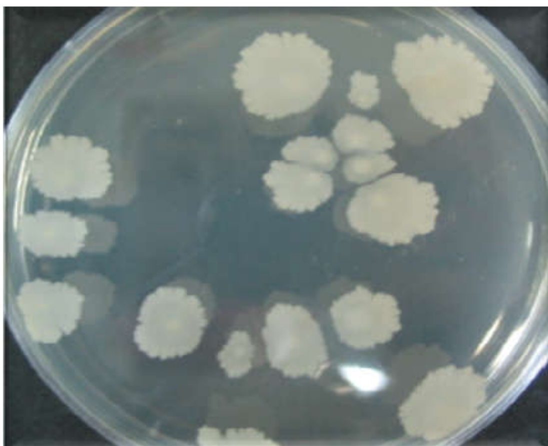

Figure 1. Bacillus colony forming in acetate nutrient agar as selective media

Isolates were named in the following order; sample number showing sampling site, Bt-like colony morphology with upper letter “Q” and a number to differentiate isolates obtained from the same sample. After observation by phase-contrast microscopy, three isolates from different sources were characterized as Bt based on the culture on the reference Bt selective media (sigma Aldrich), spore, gram negative staining, crystal protein and biochemical test. Bt isolates were found in 3 samples out of the 8 samples analyzed. The highest percentage of samples containing the bacterium was in mint soil.

Morphological and Cultural Properties

The cultural characteristics of the suspected *Bacillus thuringiensis* isolates were examined. Generally, colonies were white to cream in color, tend to have large frosted glass appearance, initially, but may become opaque. Some colonies were mucoid in nature, others brittle. The isolates are Gram-positive, spore formers and motile. The spore is found in the center of the cell. The shape of spores is ellipsoidal. All isolates produced crystal proteins with various forms and size.

Biochemical characterization

Lecithinase production

After the inoculation of the suspected colonies on Baired-Parker agar media, opacity around colonies appeared after an overnight incubation which considers as a positive result as shown in Figure 2.

Figure 2. lecithinase production of the suspected colonies on Baired-parker agar media

Motility testing

From each isolate, 2-3 colonies inoculated into Sigma-Aldrich (SIM) media by stabbing to examine for motility, after an overnight incubation period, diffusion was seen away from the stab line to be considered as a positive result. For motility test as presented in Figure 3

Figure 3. Motility testing in SIM media

Penicillin susceptibility

The bacterial isolates from a sample was examined for penicillin susceptibility according to the CLSI reference. The result showed a resistance to Penicillin as shown in Figure 4.

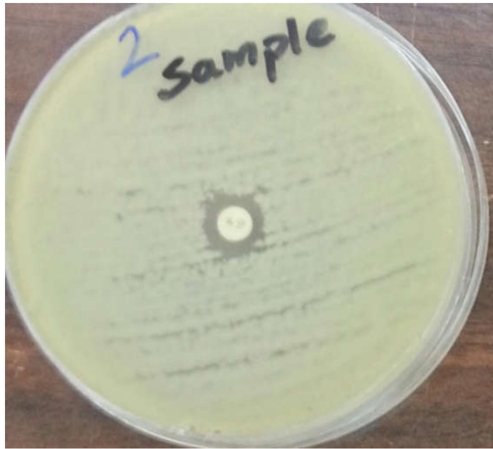

Figure 4. Penicillin susceptibility of the suspected colonies on Mueller Hinton agar media

Optimization of factors affecting on Bt growth

pH Optimization

Variation of pH values for incubated samples within 5.5 to 8 is high. These values varied from 0.469 OD to 0.713 OD. The lowest value of pH recorded was 0.469 at pH 5.5, while the highest was 0.713 at pH 7. Table 2 summarizes the Bt growth at different pH incubation at optimal temperature 30ºC

Table 2. *Bt* growth at different pH incubation at optimal temperature 30°C

Sample Trial	pH value					
	5.5	6	6.5	7	7.5	8
1	0.468	0.587	0.465	0.813	0.618	0.403
2	0.74	0.731	0.726	0.898	0.697	-
3	0.199	0.021	0.296	0.427	0.443	0.302
Mean value	0.469	0.446	0.496	0.713	0.586	0.353

Temperature and Incubation Time Optimization

Regarding temperature and time of incubation effect, (Table 3) summarizes the results pointed to the importance of incubation temperature which regulated the OD 600nm obtained from the test. At 30 °C, *Bt* recorded the highest growth of OD 0.89 and the lowest performing was observed at 20ºC OD 0.46 after 24 h incubation.

Table 3. Bacterial growth at different temperatures and incubation time

Incubation Temperature	Incubation Time (h)				
	T_4	T_6	T_8	T_{12}	T_{24}
20^0C	0.05	0.07	0.15	0.22	0.46
25^0C	0.07	0.175	0.28	0.35	0.6
30^0C	0.15	0.32	0.42	0.47	0.89
35^0C	0.17	0.4	0.415	0.418	0.66

DISCUSSION

The influence of temperature on the biomass production of the Bt strain is presented in Fig. 5. Present study showed the optimum growth temperature, as well as biomass production, was obtained at 30ºC. On other hand, a temperature is one of the critical parameter which has to control and maintain in an optimum condition for maximal biomass production. (Ozkan *et al.,* 2003), (Ghribi *et al.,* 2007) and (Jouzani *et al.,* 2015) reported similar results. Contrary to that, (Kumar *et al.*, 2014) reported that the optimum temperature for the growth from Bt sp. the Temperature range of 36 to 38, with maximum activity at 38°C. Moreover, (Chovatiya *et al.,* 2014) recorded maximum protease production at 37°C in medium. However, Bacillus sp. producing the Protease optimum activity of numerous protease from bacterial sources was between 30°C and 40°C (Dutta and Banerjee, 2006; Devi, 2014; Chovatiya *et al.,* 2014).

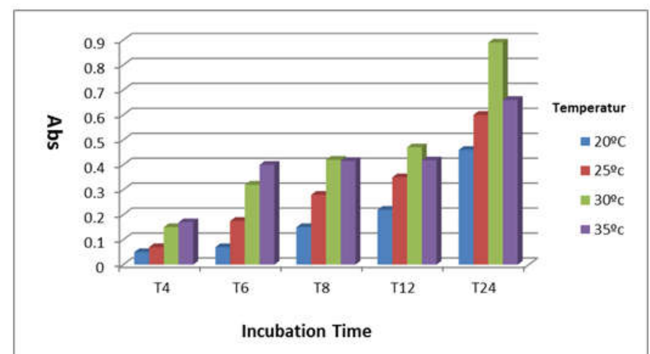

Figure 5. Effect of various Temperature and incubation time on biomass production

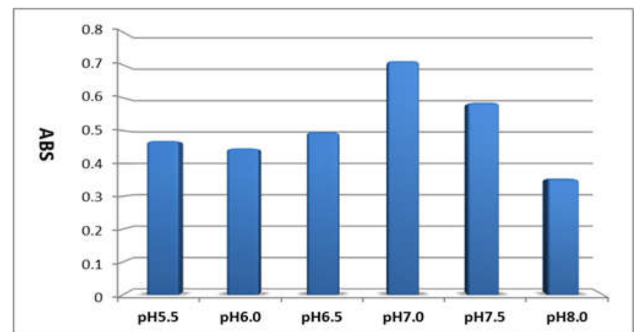

Figure 6. Effect of various pH on biomass production

The cultural conditions and environmental requirements for Bt growth and biomass production have been studied. The isolated strains of Bt were able to grow and produce biomass with a wide range of pH (5.5 to 8.0).Maximum growth, as well as biomass increased, were obtained at pH 7.0 (Fig.6), Similar results have been reported for several researcher (Rowe & Margaritis, 1987; Najafa, 2006; Hossain *et al.,* 2007; Ghribi *et al.,* 2007; Ernandes *et al.*, 2013; Anakwenze *et al.,* 2014; Lathwal *et al.,* 2015). This study suggested that the isolated strains of Bt are preferred the neutral pH in nature, According to our results, adjustment of pH for growth media as it strongly influences the biomass production. Most microorganisms grow optimally within a wide pH range.

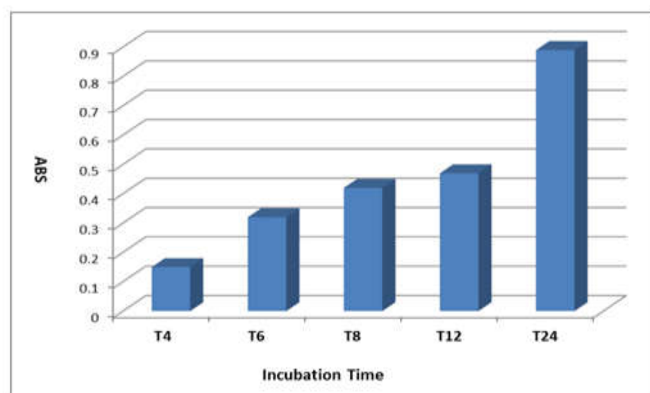

Figure 7. Relation between incubation time and biomass production

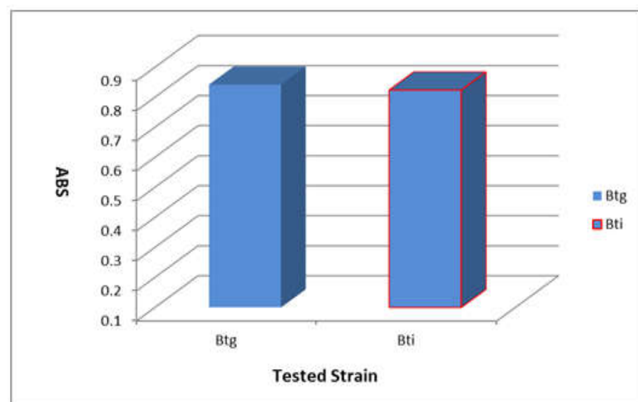

Figure 8. Growth pattern of Btg (isolated from gaza soil) and Bti (refferance strain) in growth medium (N.A)

(Immanuel *et al*. 2006) reported that the cellulolytic enzyme, endoglucanase from Bacillus sp. isolated from the estuarine coir netting effluents hydrolyzes substrate in the pH range of 4.0 to 9.0, with maximum activity at pH 7.0. Contrary to that, (Sivakumar *et al.* 2012) observed Maximum keratinase production at pH 10.0 by Bacillus thuringiensis TS2. In growth curve experiment, the effect of incubation time on biomass production was studied from different incubation time 4,6,8,12 and 24 h (Figure 7). The biomass production increased with increase in fermentation period. In this study maximum growth, was obtained at T24, Similar results were also reported by (kuberan *et al.,* 2010; saleem *et al.,* 2014; Awad *et al.*, 2015). Contrary to that, (valicente *et al.,* 2010) recorded maximum biomass production at incubation time 72h of fermentation. In the other hand, the growth pattern of Btg and Bti in growth medium was similar, corroborating the results of biomass production of isolated strain (Btg) in the present study (Figure 8) similar enhanced growth pattern of Bti.

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