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EFFICACY OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AGAINST THE LARVAE OF *AEDES AEGYPTI*

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Abstract

Efficacy of aqueous suspension of *Bacillus thuringiensis var. israelesis* (Bti) was studied against the laboratory reared 3rd and 4th instar larvae of *Aedes aegypti*. Mortality rates as well as histological changes in the larval midgut due to toxic effect of Bti were investigated. Several microscopic techniques were also used to identify the toxic effect on the mosquito larvae. The highest mortality rate (96.66%) was found in case of 3rd instar larvae at 1.0µl/ml dose where LC_{50} value was 0.0097. Larval mortalities increased significantly to *A. aegypti* as doses increased ($p< 0.05$). Histological study revealed that cellular layers of the midgut epithelium were intact in control sample, but in case of the Bti treated larvae none of these were found in the midgut, only bacterial spores were seen. Results of the microscopic studies indicated that, among the six different colonies found in bacterial cultures, Bti spore, sporangium and vegetative cells were confirmed from one colony by phase transition and fluorescent microscopy. The Cry (crystals) endotoxins and Bti spore were confirmed by the SEM.

Key words: *Aedes aegypti*, *Bacillus thuringiensis* var. *israelensis,* Midgut*,* Phase transition microscopy, LC_{50}, Scanning electron microscopy

Introduction

Bacillus thuringiensis var *israelensis* is a ubiquitous gram positive spore forming bacterium, which produces proteinaceous insecticidal crystal during sporulation that is widely used as an alternative to synthetic chemical pestcides (Mendoza *et al*. 2012 and Ferre and Van Rie 2002).The Bti-crystals are β-sheet aggregates of a ca. 130 KDa protein that is actually a protoxin and must be activated by certain mid gut proteases to become a 60 to 65 KDa active toxin, which is highly insoluble in normal pH condition (Hofte and Whitely 1989, Schnepf *et al.* 1998 and Knowles and Dow 1993). *In vitro* studies of the cytolytic activity of the number of Cry toxin have revealed that the toxicity affects the Lepidopteran and Dipteran cell lines (Knowles and Ellar 1987 and Thomas and Ellar 1983).The molecular mechanisms of Cry-toxins have been discussed intensively, with several hypothesis based cascades of multi-step activities (de Maaged *et al.* 2001 and Knowles and Dow 1993). During these steps of route, the active toxin passes through the

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peritrophic membrane (PM) and binds to specific receptor of the brush border membrane vescicles (BBMVs) of the midgut epithelial cell (Gill *et al.* 1992, Hofmann *et al.*1988 and Van Rie *et al.* 1989).

Therefore, the structure and functional properties of the PM for Bt-toxicity are of vital importance. However, the interaction of Cry toxins with the PM, a major component of the mid-gut lumen, is not well understood (Granados *et al.* 2001). Biotechnologists and entomologists agree that mosquito control efficiency should be with selectivity for a specific target organism. New control methodologies aim at reducing mosquito breeding sites and biting activity. Combination of chemical-biological control methods for decreasing the population of mosquito and to regulate the man-vector contact has also required (Service 2008). *B. thuringiensis* var. *israelensis* has been proved to be successful weapon for fighting against mosquito. The present study was carried out to investigate the effect on toxicity and microbial activity of Bti against *A. aegypti.*

Materials and Methods

The selected strain of *Bacillus* used throughout the experiment was *Bacillus thuringiensis* var. *israelensis* (Bti). The aqueous suspension of Bti containing 1200 International unit/ml with 1.2% active ingredient and 98.8% inert ingredient was collected from the laboratory for Plant and Food Science, School of Agriculture, Food and Wine, University of Adelaide, Australia. One ml of Bti was dissolved in one liter of distilled water to prepare 1 µl/ml solution. This solution was diluted 10 times to formulate 0.1 µl/ml dose and 0.01 and 0.001 µl/ml Bti doses were prepared accordingly.

A. aegypti was reared in the laboratory of The Department of Zoology, University of Dhaka in an ambient environmental condition at 28±6°C and 70-80% RH. The adult mosquitoes were kept in a rearing cage made of steel frame, covered with mesh net (30×30×30 cm in size). The larvae were kept in a water plastic bowl (7cm in diameter) covered with a piece of fine mesh net. The larvae were fed with cereals and adult female mosquitoes were fed with pigeon blood meal. Adult males were supplied with sugar solution soaked in wads of cotton wool.

A batch of 20 healthy, laboratory reared 3rd and 4th instar larvae of *A. aegypti* were placed separately in beakers containing 200 ml Bti solution each containing 1.0, 0.1, 0.01 and 0.001µl/ml concentration of bacterial doses to determine the mortality of this mosquito. One ml of Bti was dissolved in 999 ml of distilled water to make 1.0 µl/ml dose. Other doses of Bti were prepared likewise. *A. aegypti* larval mortalities after 24 hours were recorded. Besides the bacterial doses, whether other factors were involved in the mortality or not, a control replicate was also used. All bioassay experiments were repeated three times.

The mean percent of mortalities of different doses of Bti was statistically analyzed using ANOVA. Multiple comparisons were done by Tukey's honest significance test. A probit analysis was also done by following EPA program, version 1.5 for calculating LC values of Bti against 3rd and 4th instar larvae at 95% confidence limit. Mean percent of mortality and LC values of 3rd and 4th instar larvae were compared by t–tests in each dose.

Histological slides of the Bti treated 3rd instar larvae of *A. aegypti* in control and in 0.1µl/ml were prepared by longitudinal sectioning the tissues of the larval midgut region. Ethanol, Myer's albumin and Xyline were used as fixatives. Serial Longitudinal sections of the tissues were cut at 0.5 µm thickness with the help of a rotary microtome machine (model 08–260–02, ERMA INC, Japan). The tissue sections were stained with eosin and Heidenhein's haematoxylene in the laboratory condition.

Bti treated larval midgut samples were streaked onto a Petri - dish containing nutrient agar medium with a sterile platinum loop to find specific bacterial cultures. After overnight incubation at 37˚C, six different bacterial colonies were found in the nutrient agar plate. Subcultures were also done for the six colonies distinguished as sample 1, 2, 3, 4, 5 and 6 separately in the same culture media using the same procedure. Each of the above samples was stained following simple staining procedure with Acridine orange and Crystal violet dye. Bacterial spore staining was done by malachite green dye to observe whether Bt spore available or not. All the stained samples were observed under a Nikon Microphot microscope in phase contrast condition. A Nikon optiphot florescent microscope was also used to observe the sample under UV light in bright field condition. Photographs were taken with a Nikon UFX – II camera in all cases. Scanning electron microscopic (SEM) studies were done from the six pre fixed bacterial sample slides. The samples were sputter coated by platinum and processed to observe under a Jeol JSM 6490 LA scanning electron microscope. This technique is necessary for the identification of bacterial spores and crystal (Cry) toxin.

Results and Discussion

Percent of deceased 3rd and 4th instars larvae of *A. aegypti* at different doses of Bti are presented in Table 1. The highest mortality was 96.66% and the lowest mortality was 21.66% at 1.0 µl/ml and 0.001µl/ml doses, respectively. No mortality was observed in control for both larval instars. Susceptibilities of laboratory reared 3rd and 4th instars larval *A. aegypti* to an aqueous suspension of Bti are presented in Table 2. LC_{50} values for the 3rd instar larvae was 0.0097µl/ml and for the 4th instar larvae was 0.0087µl/ml. There was no significant difference at 5% level between the 3rd and 4th instar larvae in terms of mortality and LC values. However, significant difference was observed among the doses (Tables 1 and 2) ($F=8, 12$; $SE = 2.31$; $p< 0.05$). The aqueous suspension of Bti was highly effective against the 3rd and 4th instar larvae of *A. aegypti* (Tables 1 and 2). The findings of the present study showed that increasing in the doses of Bti increased the mortality of the mosquito larvae significantly (Table1). These results are in agreement

with the studies conducted by Ignoffe *et al.* (1981), Lacey and Lacey (1981), Farghal and Temerak (1981), Ahmed *et al.* (1986) and Begum *et al.* (2012), who have showed that *A. aegypti* was very susceptible to Bti. Van Essen and Hembree (1980) determined the LC_{50} of Bti exposed with the larvae of *A. aegypti* for 72 hours as 0.36 ppm. Results of the present experiments are in close agreement to the results of Van Essen and Hembree (1980).

Table 1. Mortality of the 3rd and 4th instar larval *Aedes aegygti* at different doses of *Bacillus thuringiensis* var. *israelensis* after 24 hours exposure (n=60).

Doses (µl/ml)	Percent of larval mortality (Mean±SD)	
	3rd instar	4th instar
0.001	26.66±2.885	25.0±5.0
0.01	46.66±5.77c	45.0±5.0c
0.1	85.0±5.0b	83.33±5.0b
1.0	96.66±2.885a	93.33±5.0a

* Values of 3 replications of each of which comprised of 20 larvae. Different letters indicates significant difference from the mortality of 0.001 µl/ml dose of Bti ($p<0.05$).

Table 2. Susceptibility of laboratory reared *Aedes aegypti* larvae (3rd and 4th instars) to an aqueous suspension of *Bacillus thuringiensis* var. *israelensis* (n=60).

Larval instars		LC values(µl/ml)	95% confidence limit		Slope values ±SE
			Lower	Upper	
3rd instar	LC_{50}	0.0097	0.002	0.023	0.99±0.25
	LC_{90}	0.2753	0.087	2.996	
	LC_{95}	0.753	0.189	15.85	
4th instar	LC_{50}	0.0087	0.003	0.022	0.83±0.17
	LC_{90}	0.2413	0.079	1.990	
	LC_{95}	0.591	0.161	8.859	

* Values of 3 replications of each of which comprised of 20 larvae.

Histological study revealed that the cellular layers and tissues of the midgut of the larvae of *A. aegypti* in control were intact (Plate1), but in the Bti treated larvae, no cellular parts were observed, and only bacterial spores were found (Plate 2). The pathological effects of Bti Cry endotoxins (δ-endotoxin) on susceptible insect larvae are responsible for extensive damage on midgut epithelial cells and biocide characters (Ferre and Van Rie 2002). The most important feature of spore forming Bti is to production of Cr–toxin during sporulation. During proteolytic activation, the active endotoxin of Bti binds to specific receptors in the BBMVs of midgut epithelial cells through PM (Gill *et al.* 1992, Hofmann *et al.*, 1988 and Van Rie *et al.* 1989). The PM may involve in several functions

of critical importance for the insect survival, including defense barrier against ingested parasites, pathogens and toxins (Binnington *et al*. 1998, Wang and Granados 1997 and Wang and Granados 2000). It has also been reported that midgut epithelial cells of the host are affected and eventually destroyed, probably because of the permeability of the cell membrane is affected (Aronson *et al.* 1986, Aronson and Wu 1989 and Chilcott *et al.* 1990). Cry-toxins activated in the cell membrane, form pores and channels in the gut membrane which is followed by the destruction of epithelial cells and paralysis of the digestive system and causes spore germination and septicemia and larval death occurs (Balaraman *et al.* 1981). The present findings of the histological works are in the agreement with the above mentioned investigators.

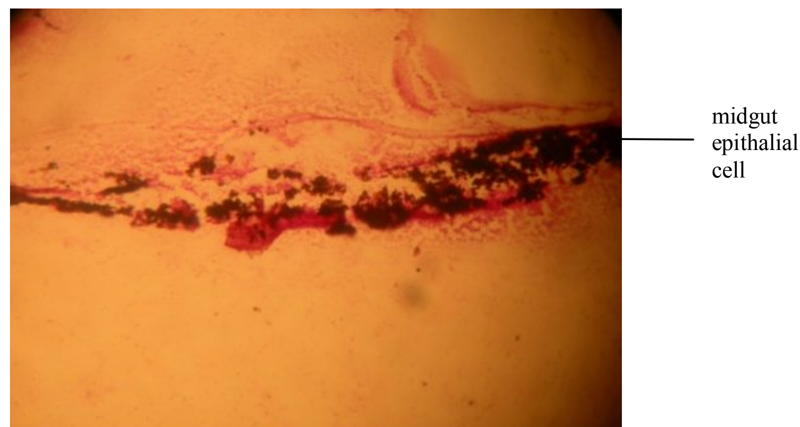


Plate 1. L.S. of the midgut of the 3rd instar larvae of the *Ae. aegypti* (40X).

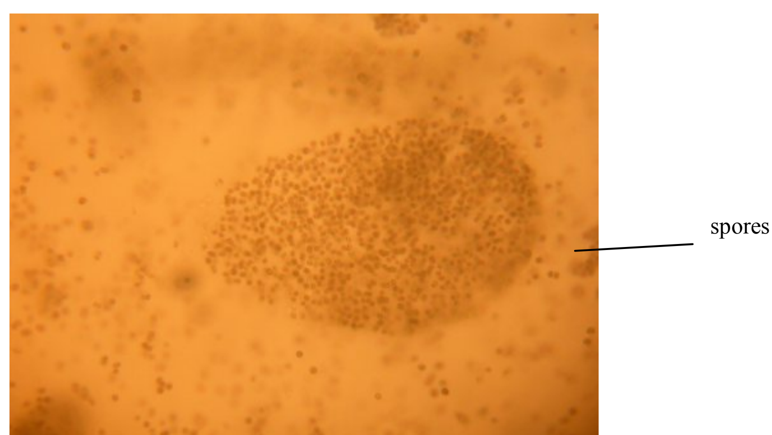


Plate 2. L.S. of the midgut of Bti treated 3rd instar larvae of *Ae. aegypti* showing only Bti spores; no tissue structures were found(40X).

The results of histological examination have been made attention on microbial study of the treated larval midgut element by several microscopic techniques, whether Bti Cry-toxin identification could be possible or not. Different types of dyes were used in several microscopic studies. Six different colonies were found from the subculture of the mid gut sample. Phase contrast and fluorescent microscopic study showed that among the six different stained with acridine orange, only samples 3 and 6 were rod-shaped and contained spores which were thought to be Bti (Plates 3A and B). The spores of the sample 3 were situated in the middle portion of the bacterium and some parts of this bacterium were darker which might be the Cry-endotoxin (Plate 3A). But, in case of the sample 6, the spores were rounded and located in the anterior terminal portion (Plate 3B). Bacterial vegetative cells and spores were also identified in phase contrast microscopic study of the sample 3 stained with malachite green (Plate 4). Vegetative cell, spore and sporangium were also recognized fluorescent microscopic study of sample 3 stained with acridine orange in bright field ailment (Plate 5A). The same bacterial stuffs were also documented under ultraviolet and bright field state of fluorescent microscopic study (Plates 5B and C). Stained with crystal violet, the sample 3 also showed vegetative cells, spore and sporangium under fluorescent microscope in bright field condition (Plate 6). Scanning electron microscopic images of 12000X from the sample 3 showed spore and possible crystals of Bti (Plate 7).

Vegetative cells, spores, Crystal toxin and paraposal Crystal formation of *B. thuringiensis* species was identified by phase contrast microscopy, fluorescent microscopy, scanning electron microscopy and electron microscopy (Mendoza *et al.* 2012 and Noguera and Ibarra 2010). Crystal toxin and spores of *B. thuringiensis var. thompsoni* and *B. sphaericus* was identified by phase contrast microscopic study (Surendran and vennison 2011). Results of the present investigations are in close agreement with the upshots of research done by above authors. Thus, it may be inferred that the Bti is highly susceptible to the larvae of *A. aegypti*; it causes tissue disruption in the midgut of the larvae of this insect.

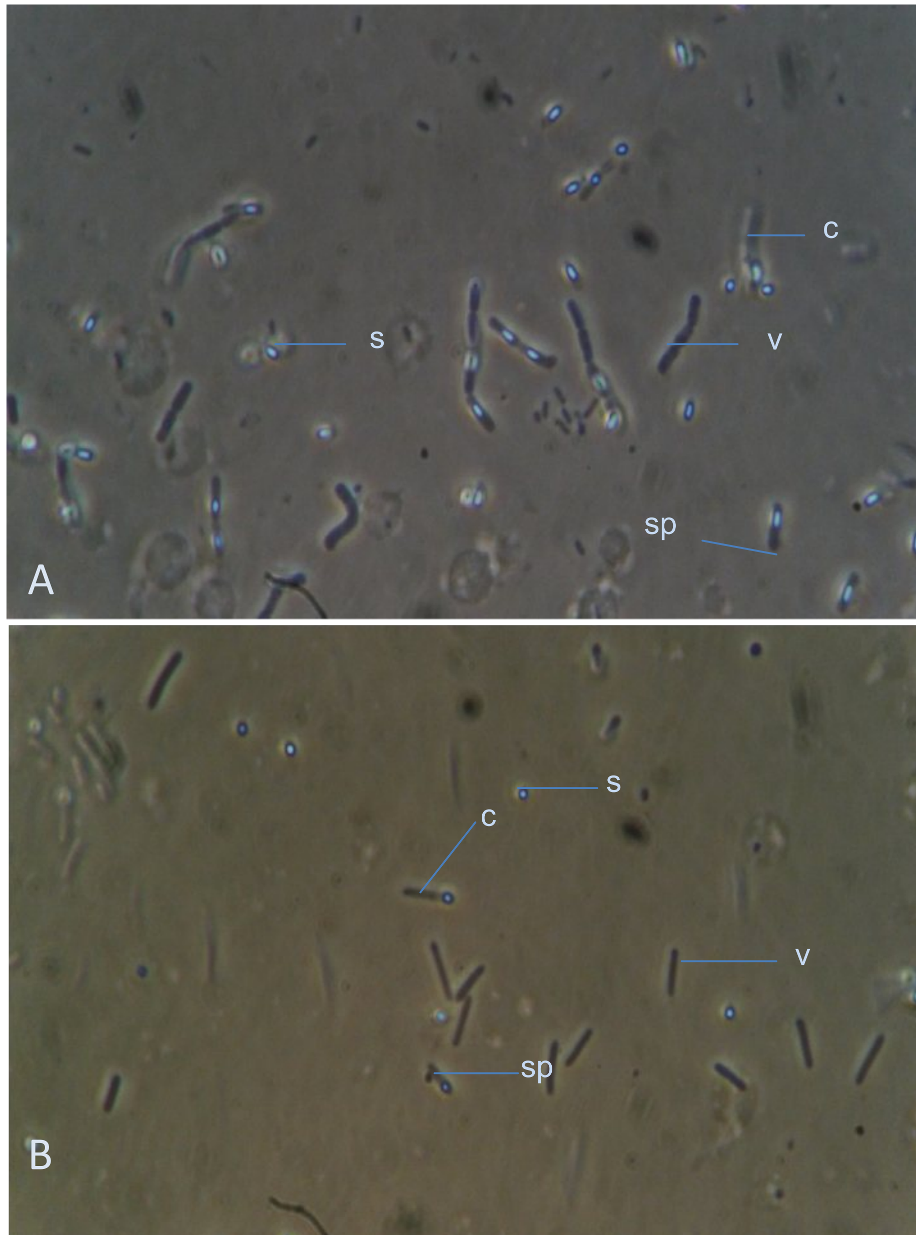


Plate 3. Photomicrograph viewed with phase contrast microscopy under an oil–immersion objective(100X) showing vegetative cells (v), spores(s), sporangia(sp) and crystals(c) of cultured bacterial sample no.3(Plate 3A) and 6(Plate 3B).

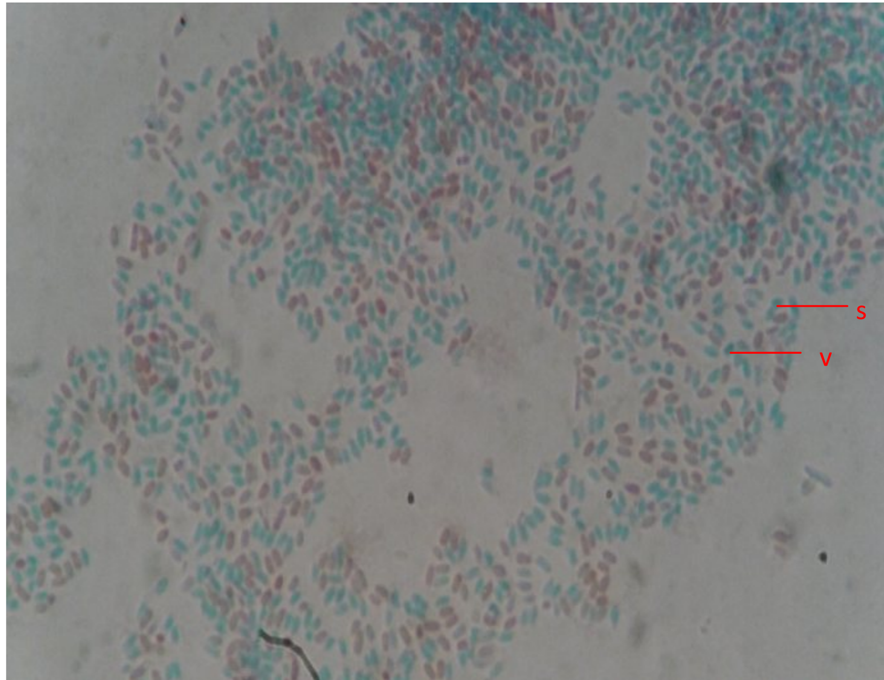


Plate 4. Photomicrograph of sample no. 3 stained with malachite green showing vegetative cells (v) and spores(s).

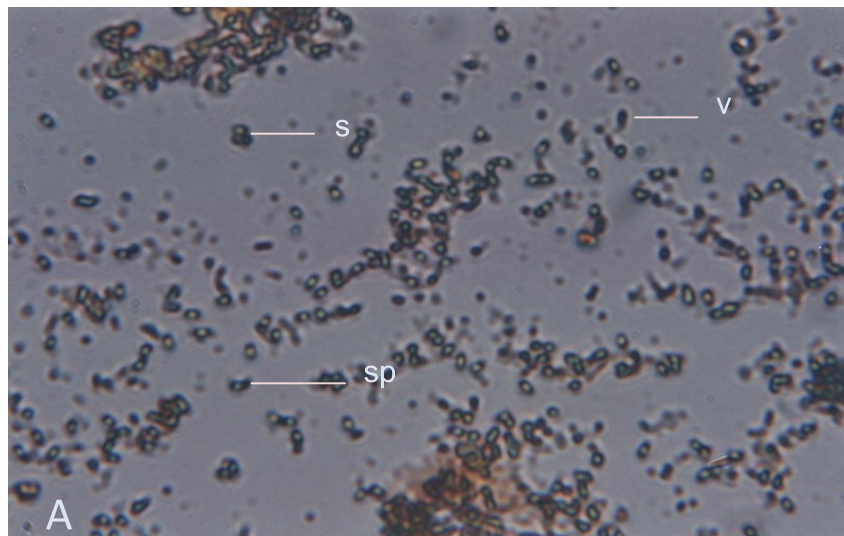
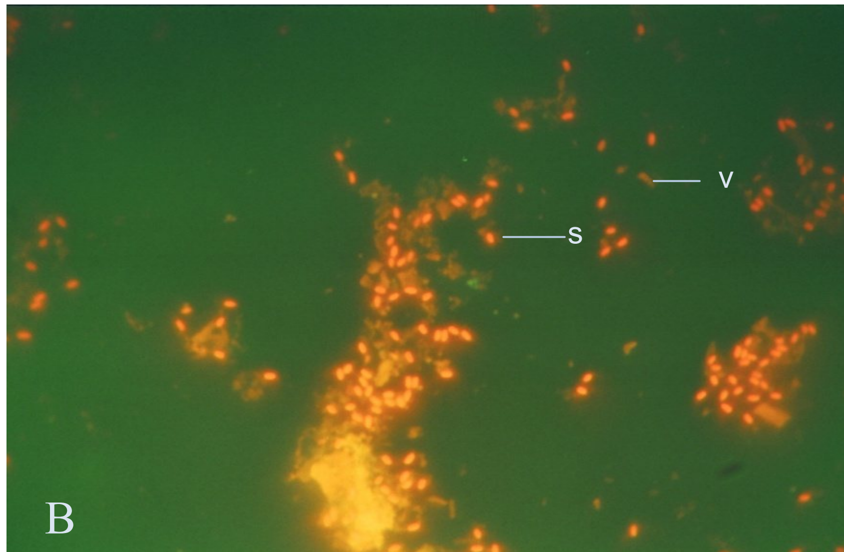
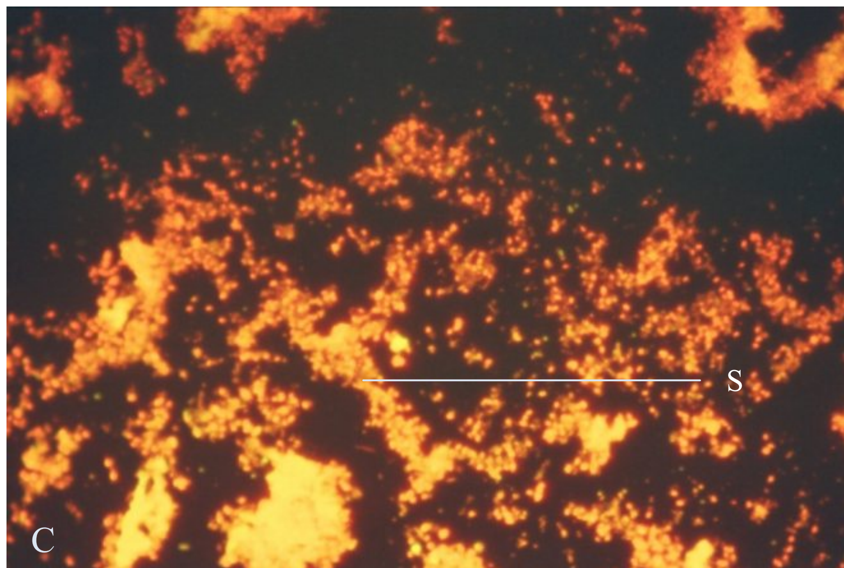


Plate 5A**.** Photomicrograph of bacterial sample no. 3 stained with acridine orange showing vegetative cell(v), spores(s), and sporangium under bright field microscopy(A) and under fluorescent microscopy(B and C)(100X).





Plates 5B and C. Photomicrograph of bacterial sample no. 3 stained with acridine orange showing vegetative cell(v), spores(s), and sporangium under bright field microscopy(A) and under fluorescent microscopy(B and C)(100X).

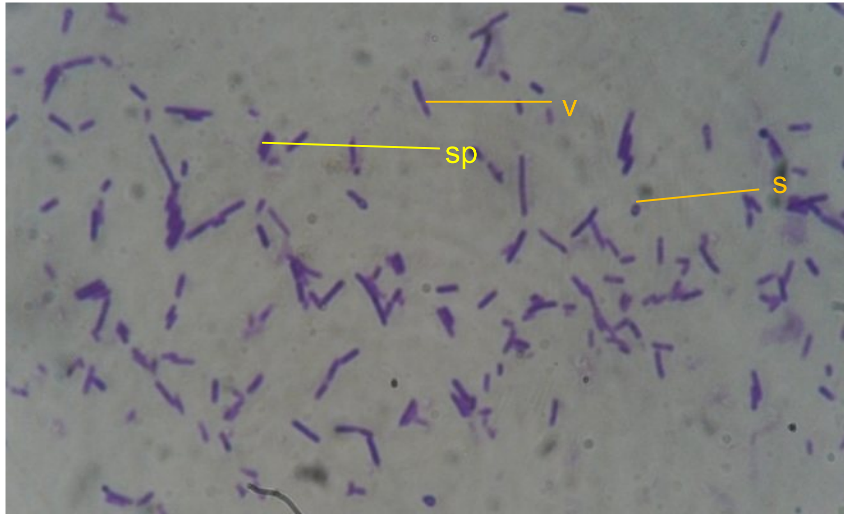


Plate 6. Photomicrograph of bacterial sample no.3 stained with crystal violet showing vegetative cells (v), spores (s) and sporangium (sp) (100X).

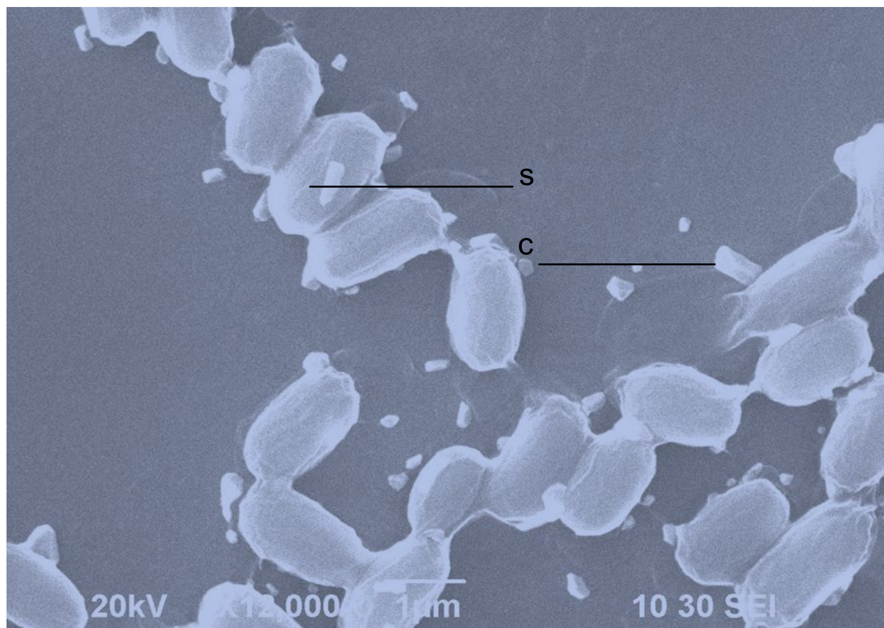


Plate 7. Scanning Electron Microscopic Image of Sample no.3 showing spores (s) and Crystals (c) (12000X).

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