5. RESULTS

5.1. Isolation of bacteria from three sporadic tea pests

5.1.1. Characteristics and symptoms of three infected pest species

Since the gut is the initial organ affected in bacterial infections, the first signs of disease are related to feeding and assimilation. Loss of appetite, cessation of feeding, diarrhoea, gut paralysis and regurgitation are characteristic initial symptoms of bacterial infections. The bacteria infected larvae of *Arctornis submarginata*, *Andraca bipunctata* and *Orgyia postica* showed significant shrinkage of body due to cessation of eating and discolouration followed by blacking first around the gut region then the whole body and rapid decomposition. The healthy larvae and dead larvae due to bacterial infection are evident in the Fig. 5.1 A, B; Fig. 5.2 A, B and Fig. 5.3 A, B.

5.1.2. Quantitative estimation of bacteria

Total number of bacteria per larva was determined by counting the number of colonies on the plates, which were inoculated with diluted bacterial suspensions. The total number of bacteria recorded per advanced larva was $5.631X10^6 \pm 0.313$ for *A. submarginata*, $5.793X\ 10^6 \pm 0.240$ for *A. bipunctata* and $4.316X10^6 \pm 0.268$ for *O. postica*.

5.1.3. Isolation and screening of the entomopathogenic *Bacillus* strains from the cadaver of three sporadic tea pests

Naturally occurring entomopathogenic bacteria were isolated from the dead larvae (n=50) of *A. submarginata*, *A. bipunctata* and *O. postica* collected from different tea gardens of Darjeeling Foothills, Terai and the Dooars.

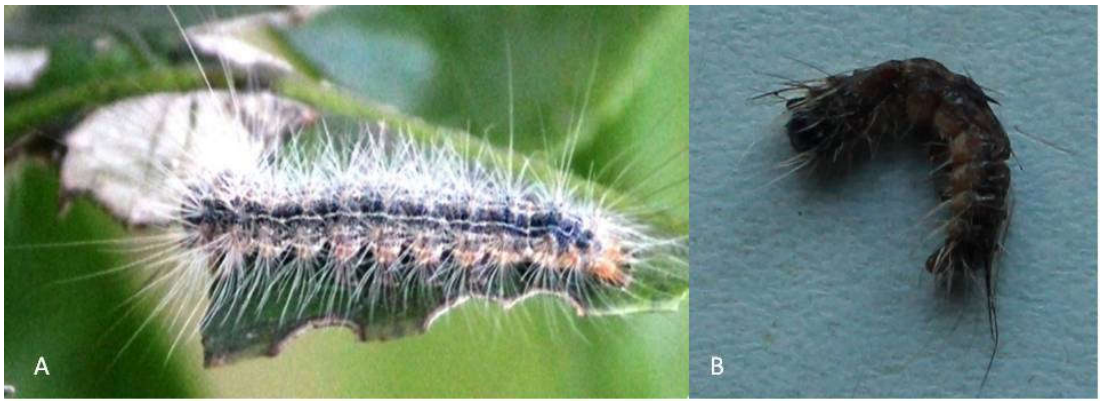

Fig. 5.1: *Arctornis submarginata* larva A) Healthy caterpillar; B) Bacterial infected cadaver.

Fig. 5.2: *Andraca bipunctata* larva A) Healthy caterpillar; B) Bacterial infected cadaver.

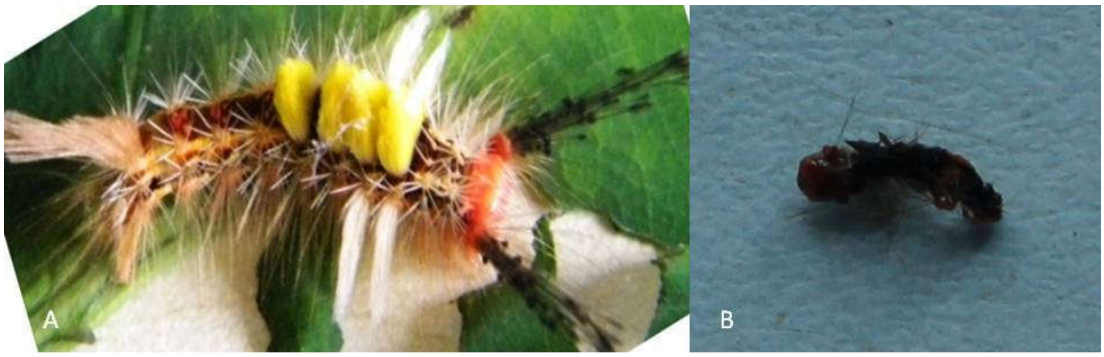

Fig. 5.3: *Orgyia postica* larva A) Healthy caterpillar; B) Bacterial infected cadaver.

I. *A. submarginata*: Out of 50 cadavers of *A. submarginata*, 21 seemed to harbour 23 *Bacillus* like colonies. Among these 23 *Bacillus* positive strains, seven (07) were picked according to the high propotion of bacteria in the cadavers. The same were tested for Koch's postulate. All the 07 bacterial strains were found to be positive for Koch's test. These were recorded as commonly occurring entomopathogens of *A. submarginata* caterpillar and were coded as Arc01-Arc07. Of these the most commonly occurring strains (Arc01, Arc02 and Arc03) were considered for detailed study (Fig. 5.4).

II. *A. bipunctata*: Among 50 cadavers of *A. bipunctata*, 25 cadavers showed 19 *Bacillus* like colonies (white and depressed with rough edge/ opaque and raised with smooth margins). Out of 19 *Bacillus* positive isolates, ten (10) were picked according to high proportion of bacteria in the cadavers. The same were tested for Koch's postulate. All the strains were positive to Koch's postulate and the most commonly occurring entomopathogens of *A. bipunctata* caterpillar were coded Ab01, Ab02, Ab03 and Ab04. They were considered for detailed study. The rest of the strains occurred in low proportion, as such only preliminary characterization has been done for them (Fig. 5.5).

III. *O. postica*: A total of 50 cadavers of *O. postica* were used for bacterial isolation, of which 22 cadavers had 18 *Bacillus* like colonies. Among these 18 bacterial isolates, six (06) were selected according to their occurrence in high proportion in the insect cadaver. They were tested for Koch's postulate and all fulfilled the test. The most commonly occurring entomopathogens against *O. postica* were coded Org 2A and Org 6A. These strains were considered for detailed study. The rest of the strains occurred in low proportion as such only preliminary characterization were done for them (Fig. 5.6).

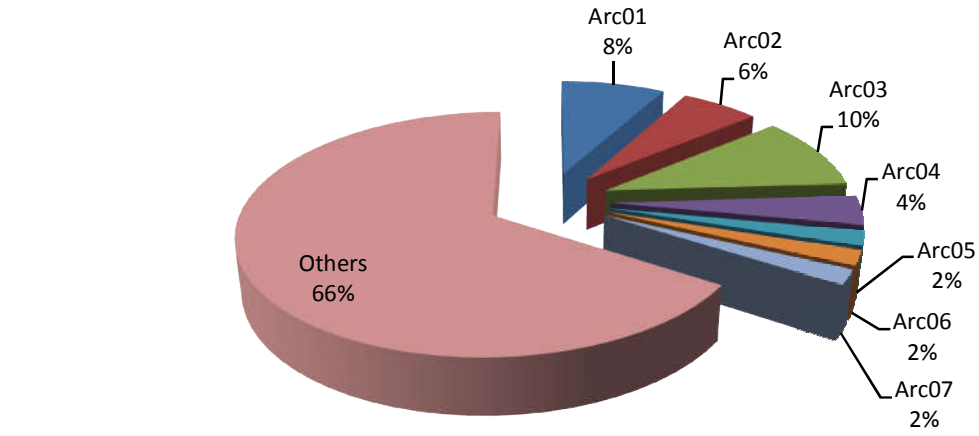

Fig. 5.4: Proportion of different *Bacillus* strains in *A. submarginata* cadavers.

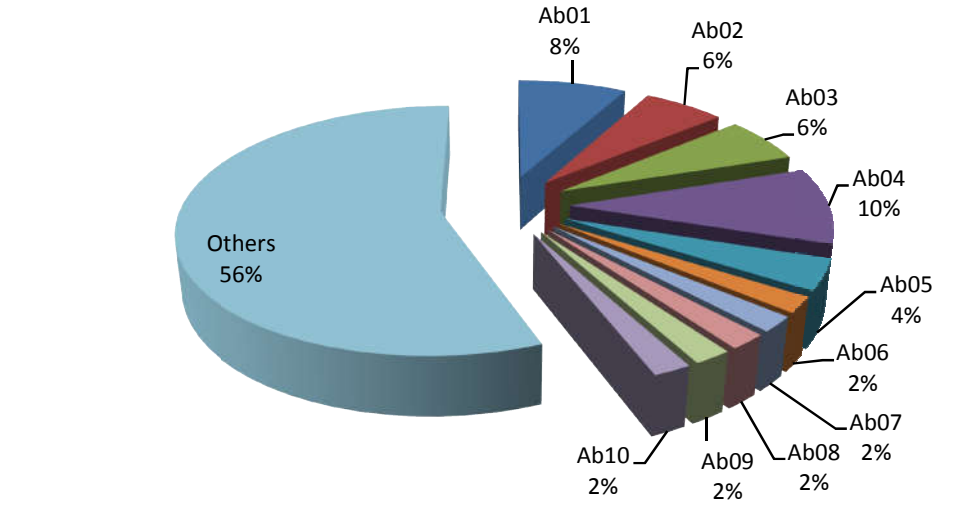

Fig. 5.5: Proportion of different *Bacillus* strains in *A. bipunctata* cadavers.

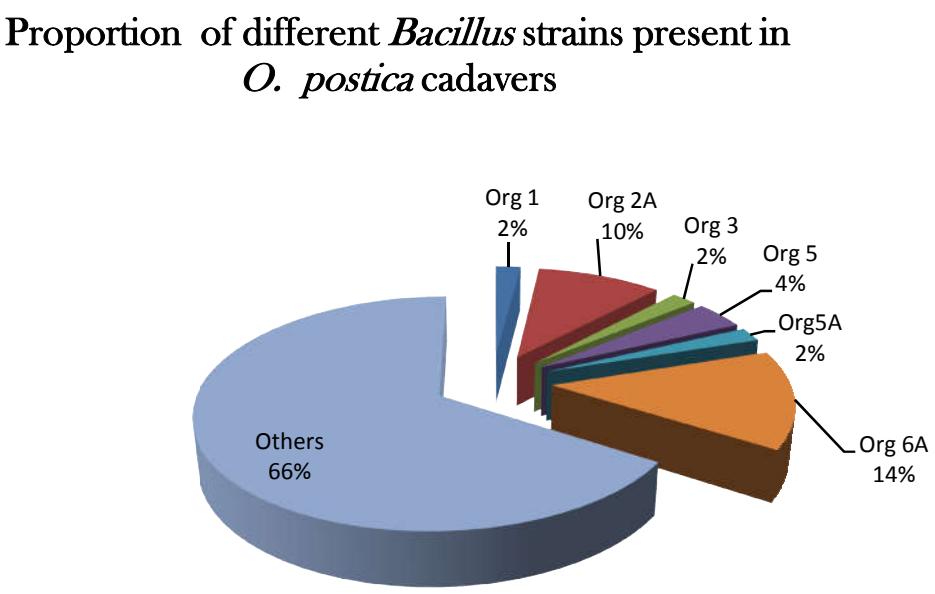

Fig. 5.6: Proportions of different *Bacillus* strains in *O. postica* cadavers.

After primary isolation, all the *Bacillus* strains from *A. submarginata* (07), *A. bipunctata* (10) and *O. postica* (06), respectively, were processed for further characterization (Table 5.1).

For convenience in addressing, describing and discussing these strains, in the forthcoming text of the thesis, designations along with coding of the strains have been done as mentioned in Table 5.2.

5.2. Characterization of the isolates

5.2.1. Physiological characteristics of the bacterial isolates

5.2.1.1. Determination of generation/ doubling time of the *Bacillus* strains

The generation/ doubling time of Arc01 isolated from *A. submarginata* was 84 min which was double of the generation time of *Btk* i.e. 42 min. The doubling time of Arc02and Arc03 was recorded as 20 min and 35 min, respectively. Further, it was found that the doubling time of other *Bacillus* isolates was i.e. Arc04, Arc05, Arc 06 and Arc07 were 72 min, 54 min, 51 min and 48 min, respectively (Table 5.3). All the isolates showed different generation time and Arc02 showed the least dobling time of 20 min followed by Arc03 which had a generation time of 35 min. Further, one way ANOVA of doubling times of different strains of *Bacillus* sp. isolated from

A. submarginata and reference strain *Btk* were found to be significantly different suggesting that all the isolates as well as *Btk* are different strains (Table 5.4).

Table 5.1: *Bacillus* sp. from dead larvae samples from tea gardens of Darjeeling foothills, Terai and Dooars.

Name of Tea Pests	Number of dead larvae samples	No. of cadavers harbouring *Bacillus* colonies	No. of *Bacillus* strains isolated	No. of Koch's postulate positive isolates
Arctornis submarginata	50	21	23	7
Andraca bipunctata	50	25	19	10
Orgyia postica	50	22	18	6

Table 5.2: Bacterial strains/isolates from *A. submarginata*, *A. bipunctata* and *O. postica* at a glance.

Name of Tea Pests	Code and Designations of the *Bacillus* strains isolated
Arctornis submarginata	Arc 01, Arc 02, Arc 03, Arc 04, Arc 05, Arc 06 and Arc 07
Andraca bipunctata	Ab 01, Ab 02, Ab 03, Ab 04, Ab05, Ab06, Ab07, Ab08, Ab09 and Ab10
Orgyia postica	Org 1, Org 2A, Org 3, Org 5, Org 5A and Org 6A

Table 5.3: Comparative account of doubling times of *Bacillus* sp. isolated from *A. submarginata* and reference strain *Btk.*

Name of Bacteria	**Doubling time (minutes)**
Btk	**42**
Arc 01	**84**
Arc 02	**20**
Arc 03	**35**
Arc04	**72**
Arc05	**54**
Arc06	**51**
Arc07	**48**

Table 5.4: ANOVA of generation/doubling times of different strains of *Bacillus* sp. isolated from *A. submarginata*.

ANOVA						
Source of Variation	SS§	df	MS¥	F	P-value	*F crit***
Between Groups	14227.5	7	203.25	203.25	1.64E-40	2.312741
Within Groups	32	32	1			
Total	14259.5	39				

§ Sum of squares

¥ Mean of squares

**Analysis showed that F> F crit (critical value). Therefore the null hypothesis was rejected and it was concluded that the variables (doubling time) are significantly different.

The generation/doubling time of four strains isolated from *A. bipunctata* were less than that of doubling time of *Btk.* Ab01, Ab02, Ab03 had doubling time of 36 min, 35 min and 33 min, respectively, whereas, Ab04 had the shortest doubling time of 24 min. Further, doubling time of rest of the six *Bacillus* strains of *A. bipunctata* were calculated as 62 min, 44 min, 53 min, 31 min, 38 min and 60 min for Ab05, Ab06, Ab07, Ab08, Ab09 and Ab10, respectively, (Table 5.5). All the isolates showed different generation time. Further, one way ANOVA of doubling times of different strains of *Bacillus* sp. isolated from *A. bipunctata* and reference strain *Btk* were found to be significantly different suggesting that all the isolates as well as *Btk* are different strains (Table 5.6).

The generation/doubling time of the isolates of *O. postica*, Org1, Org 2A, Org3, Org5, Org 5A and Org 6A were 69, 66, 74, 86, 82 and 30min respectively. Except Org 6A, all isolates took more time to double than *Btk*, which took 42 min to double (Table 5.7). One way ANOVA of doubling times of different strains of *Bacillus* sp. isolated from *O. postica* and reference strain *Btk* were found to be significantly different suggesting that all the isolates as well as *Btk* are different strains (Table 5.8).

5.2.1.2. Colony forming unit/ml of *Bacillus* strains

Microbial growth can be quantified by counting the total number of viable colonies formed by the inoculums of bacteria on the nutrient agar plate during one generation time. Assumption is that each cell in the aliquot can form one colony forming unit on the solid media. The cfu/ml was calculated to be $17.76x10^9$, $14.33x10^9$, $19.31x10^9$ for Arc01, Arc02 and Arc03, 8.15x109, $11.45x10^9$, $11.38x10^9$, $14.54x10^9$ for Ab01, Ab02, Ab03 and Ab04, while cfu/ml was found to be $10.54x10^9$ and $17.84x10^9$ for Org 2A and Org 6A, respectively. Reference strain *Btk* had the cfu/ml of $16.3x10^9$.

Table 5.5: Comparative account of doubling times of *Bacillus* sp. isolated from *A. bipunctata* and reference strain *Btk.*

Name of Bacteria	**Doubling time (minutes)**
Btk	**42**
Ab 01	**36**
Ab 02	**35**
Ab 03	**33**
Ab 04	**24**
Ab05	**62**
Ab06	**44**
Ab07	**53**
Ab08	**31**
Ab09	**38**
Ab10	**60**

Table 5.6: ANOVA of doubling times of different strains of *Bacillus* sp. isolated from *A. bipunctata*.

ANOVA

Source of Variation	SS§	df	MS¥	F	P-value	*F crit***
Between Groups	7372.727	10	737.27	737.27	1.5E-45	2.053901
Within Groups	44	44	1			
Total	7416.727	54				

§ Sum of squares

¥ Mean of squares

**Analysis showed that F> F crit (critical value). Therefore the null hypothesis was rejected and it was concluded that the variables (doubling time) are significantly different.

Table 5.7: Comparative account of doubling times of *Bacillus* sp. isolated from *O. postica* and reference strain *Btk.*

Name of Bacteria	**Doubling time (minutes)**
Btk	**42**
Org 2A	**66**
Org 6A	**30**
Org1	**69**
Org3	**82**
Org5	**74**
Org5A	**86**

Table 5.8: ANOVA of doubling times of different strains of *Bacillus* sp. isolated from *O. postica*.

ANOVA

Source of Variation	SS[§]	df	MS[¥]	F	P-value	*F crit***
Between Groups	8897.143	6	148.285	148.285	1.07E-33	2.445259
Within Groups	28	28	1			
Total	8925.143	34				

§ Sum of squares

¥ Mean of squares

**Analysis showed that F> F crit (critical value). Therefore the null hypothesis was rejected and it was concluded that the variables (doubling time) are significantly different.

5.2.2. Morphological Characteristics of the *Bacillus* strains

5.2.2.1. *Bacillus* strains of *A. submarginata* Arc01, Arc02, Arc03, Arc04, Arc05, Arc06 and Arc07

All the isolates of *Bacillus* strains (Arc01-Arc07) from *A. submarginata* had rod shaped vegetative body, they were found to be gram positive, facultatively anaerobic, endospore forming, catalase positive and could produce acid from glucose and were highly motile. All these characteristics were similar to the characteristics of the members of genus *Bacillus* (Sneath, 1986). Further, all the morphological characteristics of the bacterial isolates (Arc01, Arc02, Arc03) such as colony morphology (Fig.5.7A, B, C & D), vegetative body structure, spore-shape (Fig. 5.8A, B, C & D), presence of parasporal crystals (Fig. 5.9A, B, C & D) were found to be similar to *Bacillus thuringiensis* (*Bt*) (Assaeedi et al., 2011). A comparison of morphological characteristics of the isolated strains with reference strain *Bacillus thuringiensis kurstaki* (*Btk*) is given in Table 5.7.

5.2.2.2. *Bacillus* strains of *A. bipunctata*: Ab01, Ab02, Ab03, Ab04, Ab05, Ab06, Ab07, Ab08, Ab09 and Ab10

All the morphological characteristics of *Bacillus* strains (Ab01, Ab02, Ab03 and Ab04) of *A. bipunctata* such as colony morphology (Fig. 5.10 A, B, C & D) vegetative body structure, spore-shape (Fig. 5.11 A, B, C & D), presence of parasporal crystals (Fig. 5.12 A, B, C & D) were found to be similar to *Bt* and the strains were comparable to the reference strain *Btk* (Table 5.10).

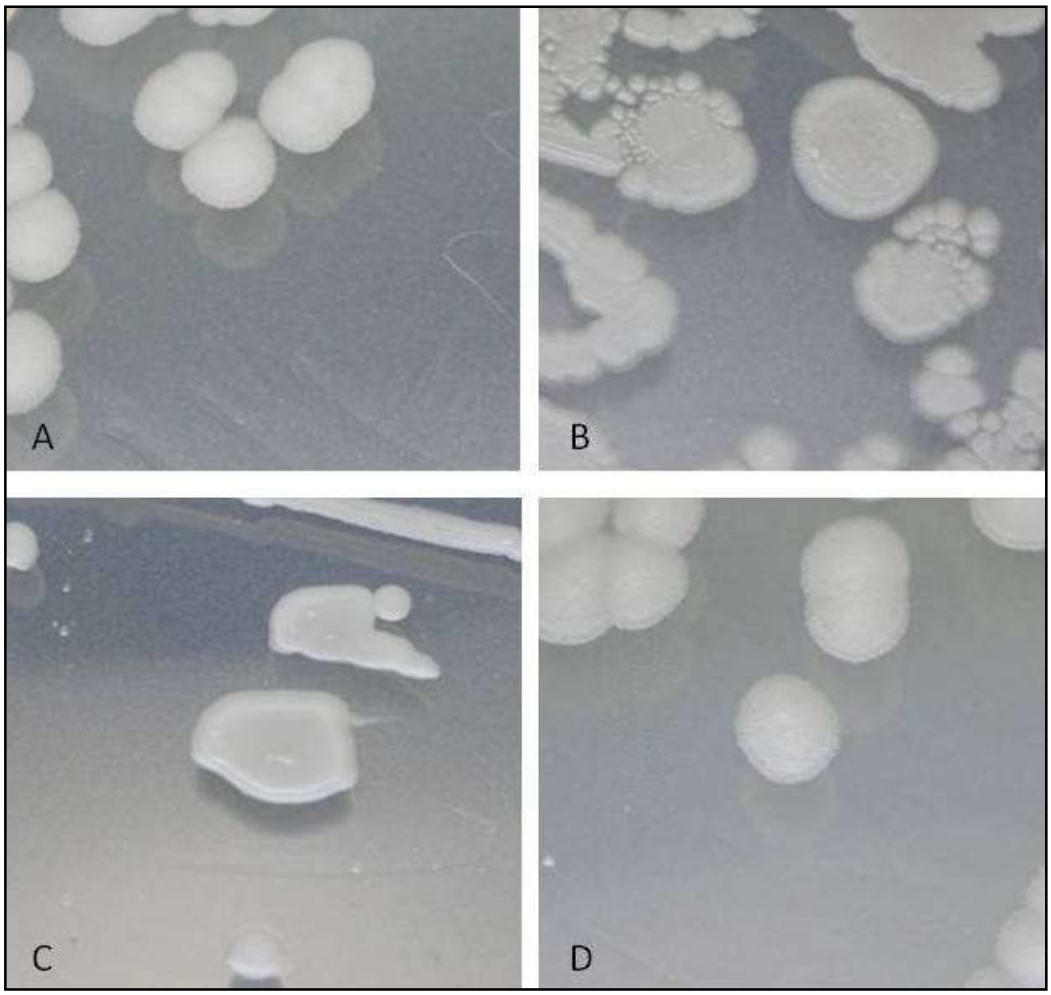

Fig. 5.7: Colony morphology of strains of *Bacillus*:- A) *Btk* (reference); B) Arc01; C) Arc02; D) Arc03.

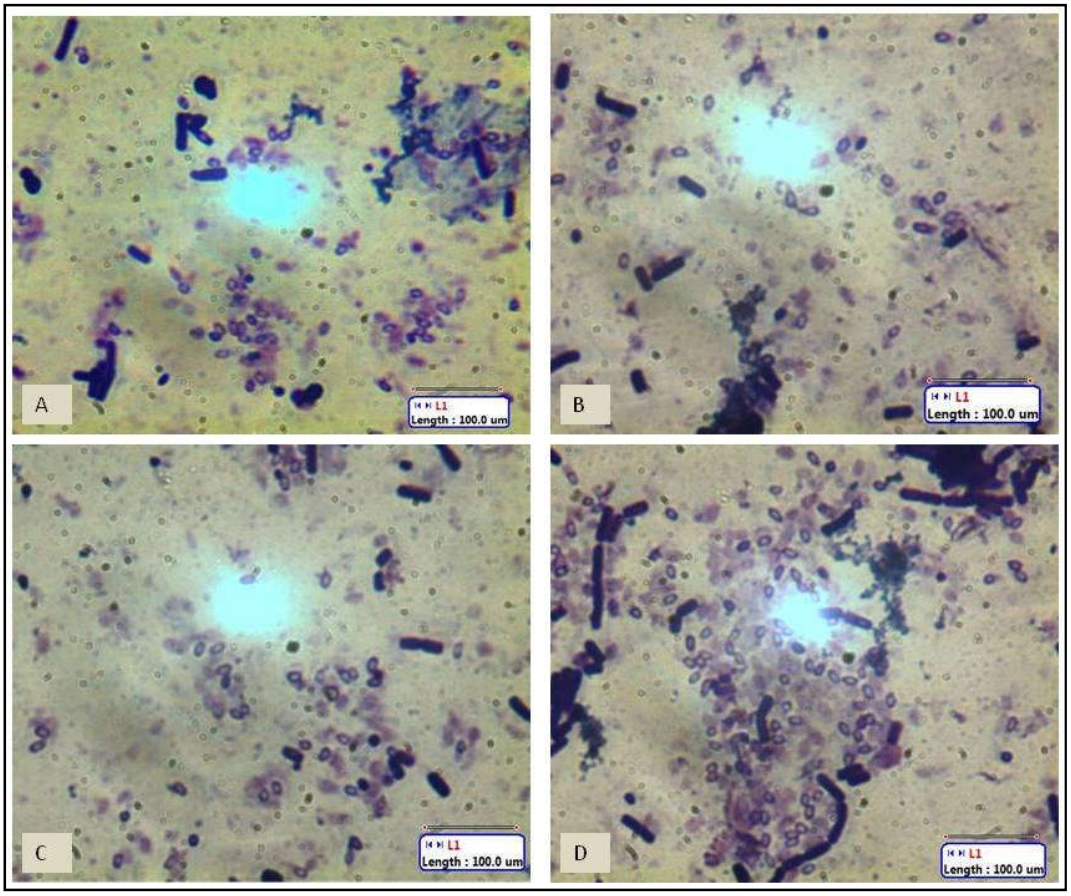

Fig. 5.8: Gram stained microphotographs (Vegetative cell, spore and crystal) of *Bacillus* strains:- A) *Btk* (reference); B) Arc01; C) Arc02; D) Arc03.

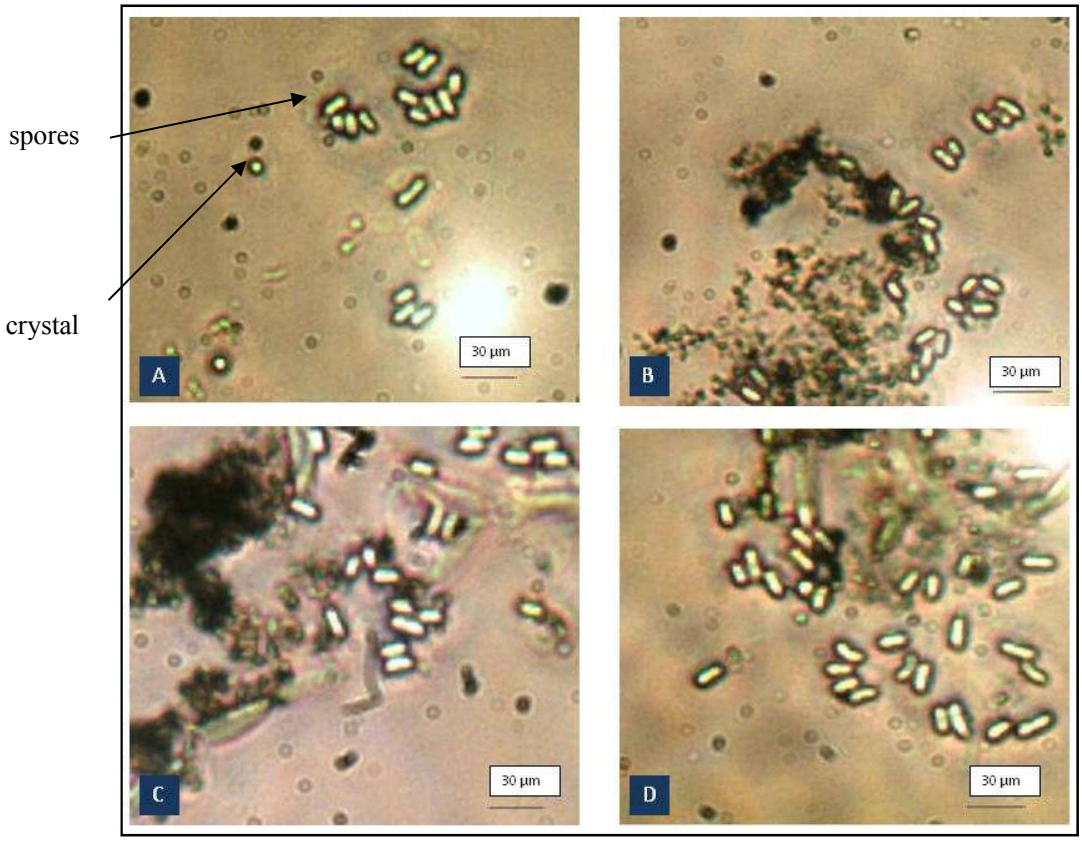

Fig. 5.9: Phase contrast microphotographs (Spore and crystal) of *Bacillus* Strains:- A) *Btk* (reference); B) Arc01; C) Arc02; D) Arc03.

Table 5.9: Morphological characteristics of the *Bacillus* strains of *A. submarginata* (Arc01-Arc07) and reference strain *Btk*.

Morphological Characteristics	*Bacillus thuringiensis kurstaki* (*Btk*)	Arc01	Arc02	Arc03	Arc 04	Arc 05	Arc 06	Arc 07
Shape of Vegetative cell	R	R	R	R	R	R	R	R
Chains of cells	+	+	+	+	+	+	+	+
Motility	HM	HM	HM	HM	HM	HM	HM	HM
Cell length > 3µm	+	+	+	+	+	+	+	+
Spore position and shape	VX	VX	VX	VX	VX	VX	VX	VX
Swelling of cell body by spore	-	-	-	-	-	-	-	-
Crystal protein structure	Bipyramidal	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical
Gram staining	+	+	+	+	+	+	+	+
Growth at 50°C	-	-	-	-	-	-	-	-
Growth at 10% NaCl	-	-	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+	+	+
Colony shape and configuration	Circular	Irregular	Circular	Circular	Rhizoid	Irregular	Circular	Fried egg
Colony texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Margin and elevation	Entire	Undulate	Entire	Entire	Entire	Undulate	Entire	Entire
Density/opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Pigmentation	White	White	White	White	White	White	White	White

R -Rod shaped; HM- Highly motile; V- spore central/subterminal; X- spore oval/ellipsoidal

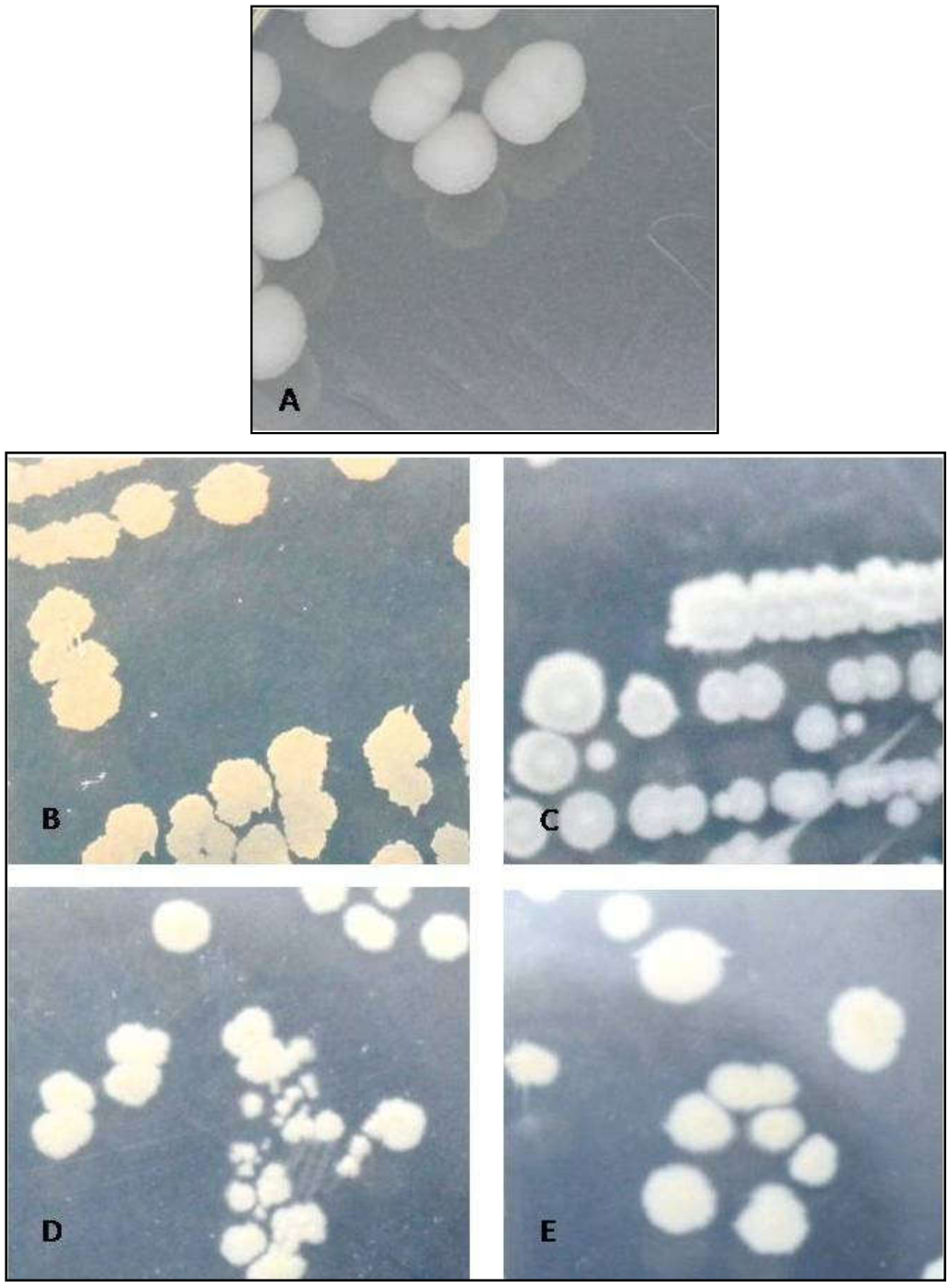

Fig. 5.10: Colony morphology of strains of *Bacillus* :- A) *Btk* (reference); B) Ab01; C) Ab02; D) Ab03; E) Ab04.

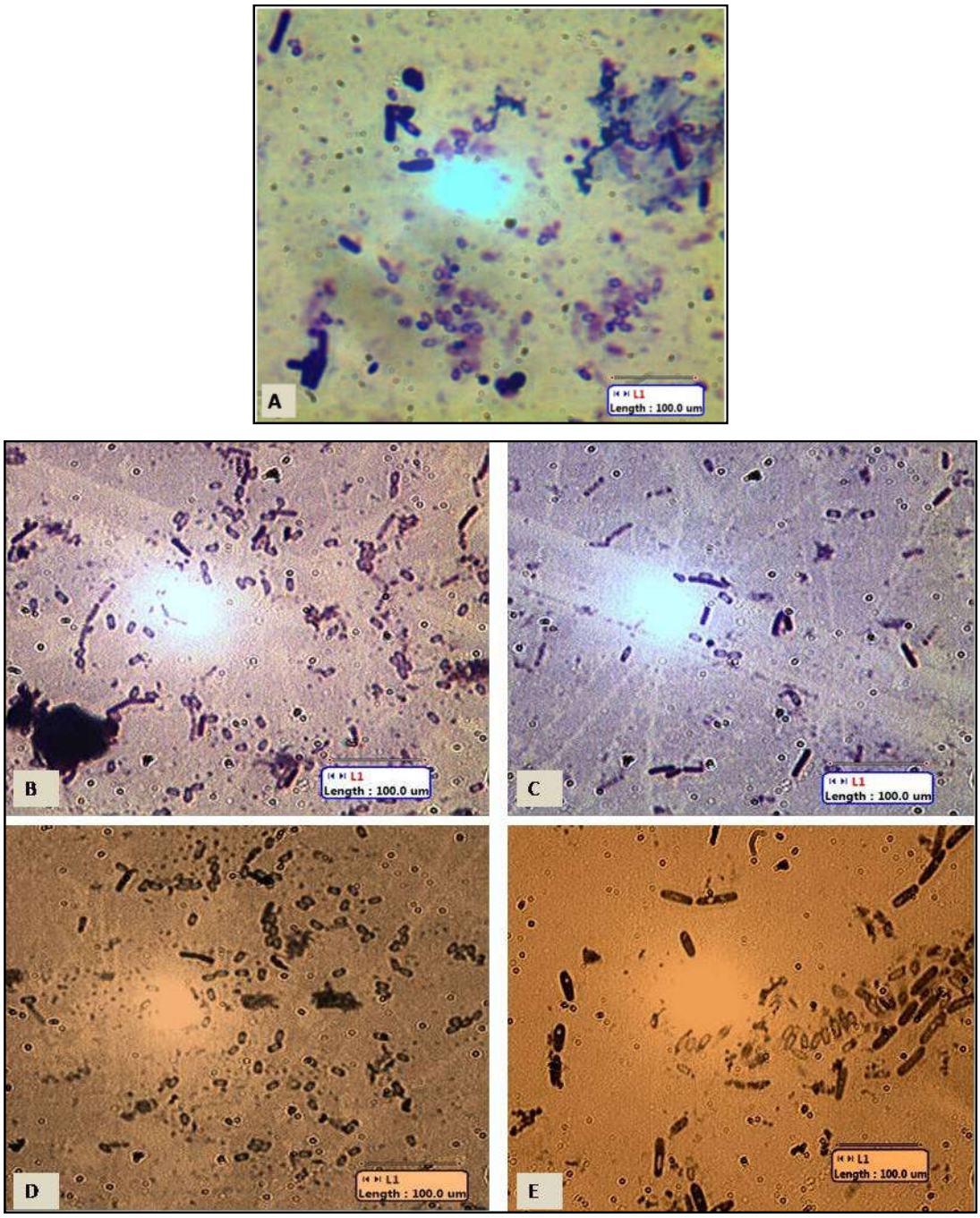

Fig. 5.11: Gram stained microphotographs (Vegetative cell, spore and crystal) of *Bacillus* strains:- A) of *Btk* (reference); B) Ab01; C) Ab02; D) Ab03 and D) Ab04.

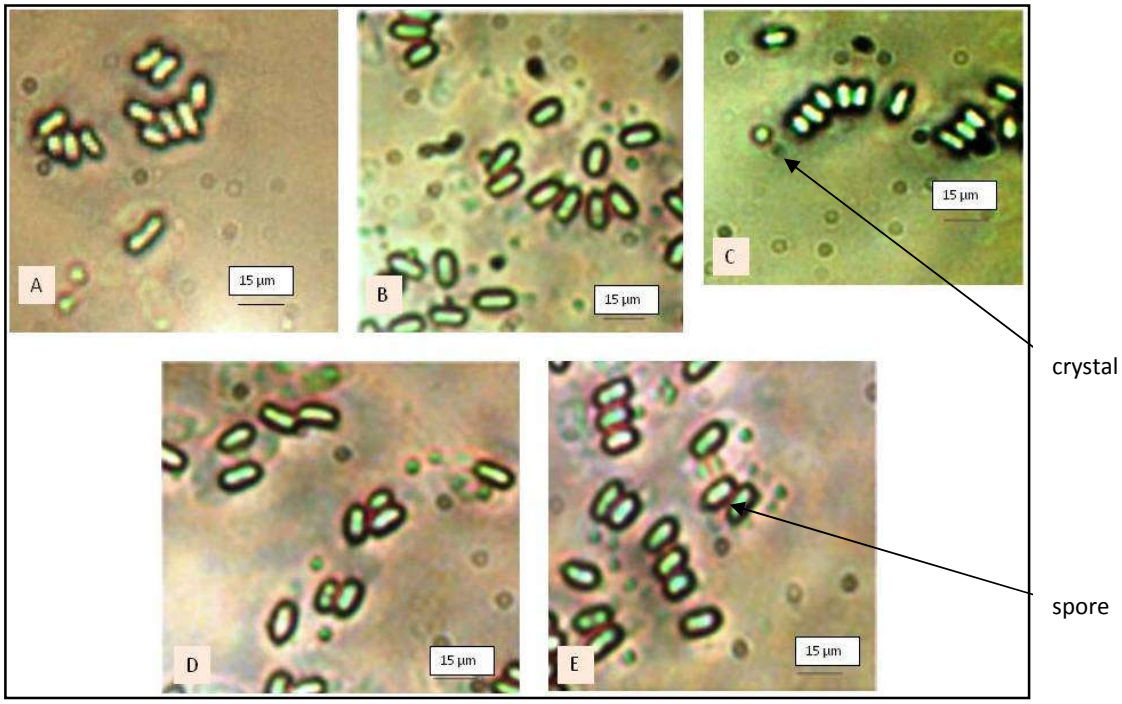

Fig. 5.12: Phase contrast microphotograph (Spore and crystal) of *Bacillus* strains:- A) *Btk* (reference); B) Ab01; C) Ab02; D) Ab03 and D) Ab04.

Table 5.10: Morphological characteristics of the bacterial isolates of *A. bipunctata* (Ab01-Ab10) and reference strain *Btk*.

Morphological Characteristics	*Bacillus thuringiensis kurstaki* (*Btk*)	Ab01	Ab02	Ab03	Ab04	Ab05	Ab06	Ab07	Ab08	Ab09	Ab10
Shape of Vegetative cell	R	R	R	R	R	R	R	R	R	R	R
Chains of cells	+	+	+	+	+	+	+	+	+	+	+
Motility	HM	HM	HM	HM	HM	HM	HM	HM	HM	HM	HM
Cell length > 3µm	+	+	+	+	+	+	+	+	+	+	+
Spore position and shape	VX	VX	VX	VX	VX	VX	VX	VX	VX	VX	VX
Swelling of cell body by spore	-	-	-	-	-	-	-	-	-	-	-
Crystal protein structure	Bipyramidal	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical
Gram staining	+	+	+	+	+	+	+	+	+	+	+
Growth at 50°C	-	-	-	-	-	-	-	-	-	-	-
Growth at 10% NaCl	-	-	-	-	-	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+
Colony shape and configuration	Circular	Circular	Irregular	Circular	Circular	Circular	Irregular	Circular	Circular	Fried egg	Circular
Colony texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Margin and elevation	Entire	Entire	Undulate	Entire	Entire	Entire	Undulate	Entire	Entire	Entire	Entire
Density/opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Pigmentation	White	Cream	White	White	White	Cream	White	White	White	White	White

R -Rod shaped; HM- Highly motile; V- spore central/subterminal; X- spore oval/ellipsoid

5.2.2.3. *Bacillus* strains of *O. postica:* Org2A, Org6A, Org1, Org3, Org5 and Org5A

All the morphological characteristics of *Bacillus* strains (Org2A and Org6A) such as colony morphology (Fig. 5.13 A, B, C & D) vegetative body structure, spore-shape (Fig. 5.14 A, B, C & D), presence of parasporal crystals (Fig. 5.15 A, B, C & D) were found to be similar to *Bt* and were comparable to reference strain *Btk* (Table 5.11).

5.2.3. Biochemical characteristics of the *Bacillus* strains

5.2.3.1. *Bacillus* strains of *A. submarginata*: Arc01, Arc02, Arc03, Arc04, Arc05, Arc06 and Arc07

Biochemical characteristics of Arc01 showed positive reactions for lysine decarboxylase, ornithin decarboxylase, Voges-Proskaur, citrate utilization, nitrate reduction and utilization of trehalose and glucose. It showed differences with *Btk* in ONPG test, and in utilization of citrate, arabinose, xylose, cellobios, melibiose and saccharose. Arc02 strain showed positive reactions for lysine utilization, ornithin utilization, citrate utilization, malonate utilization, esculin hydrolysis, rhamnose, cellobiose, raffinose and glucose utilization, while differences with *Btk* were observed in ONPG test, V-P test, nitrate reduction, esculin hydrolysis and in utilization of citrate, malonate, arabinose, xylose, rhamnose, melibiose, saccharose, raffinose and trehalose. Likewise, Arc03 strain showed positive reaction in nitrate reduction, H_2S production, V-P test, esculin hydrolysis and utilization of citrate, saccharose, trehalose and glucose. It showed differences with *Btk* in ONPG test, H_2S production, esculin hydrolysis and utilization of lysine, ornithin, citrate, malonate, arabinose, xylose, rhamnose and melibios. When tested for startch hydrolysis, casein hydrolysis and catalase test all found to be positive except Arc02 which was negetive for casein

hydrolysis. The strain Arc04 showed positive reaction in lysine decarboxylase, ornithin decarboxylase, urease, Voges-Proskaur and in utilization of trehalose and glucose, but differed from *Btk* in ONPG, urease and nitrate test. Further, it showed differences in utilization of arabinose, xylose, cellobiose, melibiose, saccharose and lactose. Strain Arc05 showed positive reaction in lysine decarboxylase, ornithin decarboxylase, nitrate reduction,Voges-Proskaur, and urease tests, and in utilization of citrate, saccharose, trehalose and glucose, however, showed differences with *Btk* in ONPG, and urease tests, and in utilization of citrate, arabinose, xylose, cellobiose, melibiose and lactose. On the other hand, Arc06 strain was positive for ONPG, lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, esculin hydrolysis and Voges-Proskaur tests, and in utilization of citrate, malonate, xylose, cellobiose, melibiose, saccharose, raffinose, trehalose and glucose. It showed differences with *Btk* in urease and esculin hydrolysis tests, and in utilization of citrate, malonate, arabinose, raffinose and lactose. The strain Arc07, was positive for ONPG, lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, esculin hydrolysis and Voges-Proskaur tests and in utilization of citrate, malonate, xylose, arabinose, melibiose, saccharose, raffinose and trehalose. It showed differences with *Btk* in urease and esculin hydrolysis tests and in utilization of citrate, malonate, cellobiose, raffinose, glucose and lactose. When tested for starch hydrolysis, casein hydrolysis and catalase tests, all the strains were found to be positive except Arc02 which was negative to casein hydrolysis In comparision to *Btk*, *Bacillus* strains Arc01-07 of *A. submarginata* showed either positive (+) or negative (-) reactions for different tests used for Biochemical characterization of bacteria (Table 5.12).

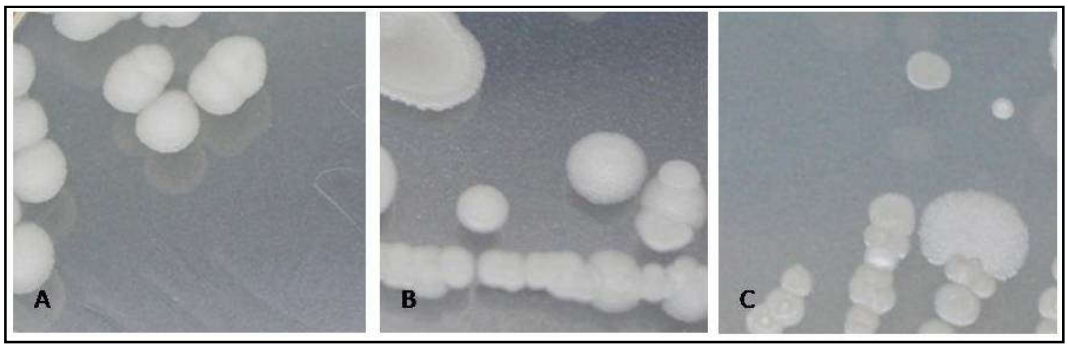

Fig. 5.13: Colony morphology of strains of *Bacillus*:- A) *Btk* (reference); B) Org 2A; C) Org 6A.

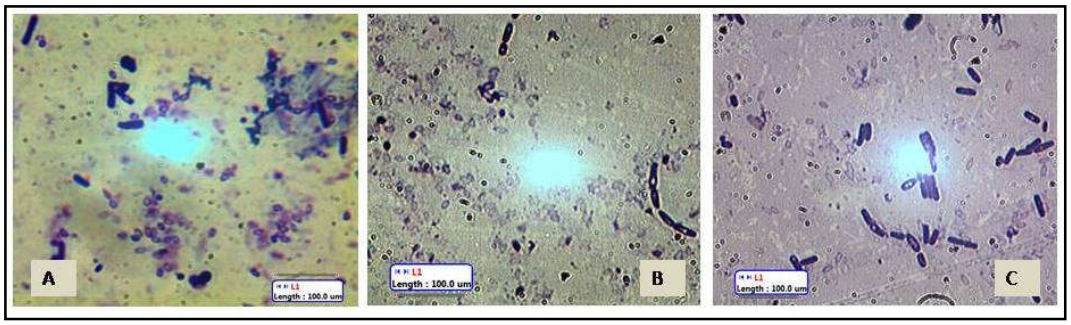

Fig. 5.14: Gram stained microphotographs (Vegetative cell, spore and crystal) :- A) *Btk* (reference); B) Org 2A; C) Org 6A.

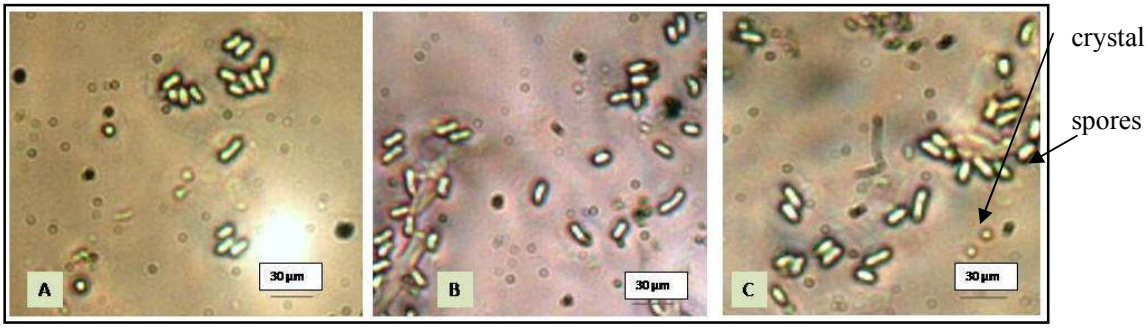

Fig. 5.15: Phase contrast microphotograph (Spore and crystal):- A) of *Btk* (reference); B) Org 2A; C) Org 6A.

Table 5.11: Morphological characteristics of the bacterial isolates of *O. postica* (Org 2A- Org5A) and reference strain *Btk*.

Morphological Characteristics	*Bacillus thuringiensis kurstaki* (*Btk*)	Org 2A	Org 6A	Org 1	Org 3	Org 5	Org 5A
Shape of Vegetative cell	R	R	R	R	R	R	R
Chains of cells	+	+	+	+	+	+	+
Motility	HM	HM	HM	HM	HM	HM	HM
Cell length > 3µm	+	+	+	+	+	+	+
Spore position and shape	VX	VX	VX	VX	VX	VX	VX
Swelling of cell body by spore	-	-	-	-	-	-	-
Crystal protein structure	Bipyramidal	Oval	Oval	Cubic	Spherical	Spherical	Spherical
Gram staining	+	+	+	+	+	+	+
Growth at 50°C	-	-	-	-	-	-	-
Growth at 10% NaCl	-	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+	+
Colony shape and configuration	Circular	Circular	Circular	Irregular	Circular	Rhizoid	Circular
Colony texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Margin and elevation	Entire	Entire	Entire	Undulate	Entire	Entire	Entire
Density/opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Pigmentation	White	White	White	Cream	White	Cream	White

R -Rod shaped; HM- Highly motile; V- spore central/subterminal; X- spore oval/ellipsoidal

lysine decarboxylase, ornithin decarboxylase, urease, Voges-Proskaur and in utilization of trehalose and glucose, but differed from *Btk* in ONPG, urease and nitrate test. Further, it showed differences in utilization of arabinose, xylose, cellobiose, melibiose, saccharose and lactose. Strain Arc05 showed positive reaction in lysine decarboxylase, ornithin decarboxylase, nitrate reduction,

Voges-Proskaur, and urease tests, and in utilization of citrate, saccharose, trehalose and glucose, however, showed differences with *Btk* in ONPG, and urease tests, and in utilization of citrate, arabinose, xylose, cellobiose, melibiose and lactose. On the other hand, Arc06 strain was positive for ONPG, lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, esculin hydrolysis and Voges-Proskaur tests, and in utilization of citrate, malonate, xylose, cellobiose, melibiose, saccharose, raffinose, trehalose and glucose. It showed differences with *Btk* in urease and esculin hydrolysis tests, and in utilization of citrate, malonate, arabinose, raffinose and lactose. The strain Arc07, was positive for ONPG, lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, esculin hydrolysis and Voges-Proskaur tests and in utilization of citrate, malonate, xylose, arabinose, melibiose, saccharose, raffinose and trehalose. It showed differences with *Btk* in urease and esculin hydrolysis tests and in utilization of citrate, malonate, cellobiose, raffinose, glucose and lactose. When tested for starch hydrolysis, casein hydrolysis and catalase tests, all the strains were found to be positive except Arc02 which was negative to casein hydrolysis In comparision to *Btk*, *Bacillus* strains Arc01-07 of *A. submarginata* showed either positive (+) or negative (-) reactions for different tests used for Biochemical characterization of bacteria (Table 5.12). The similarity coefficient among the bacterial isolates of *A. submarginata* (Arc01-Arc07) varied from 0.50 (50%) to 0.958 (95.8%). The lowest level of similarity 0.50 (50%) was recorded

between *Btk* and Arc02, whereas highest similarity of 0.958 (95.8%) was found between Arc01 and Arc05 (Table 5.13).

5.2.3.2. *Bacillus* strains of *A. bipunctata*: Ab01, Ab02, Ab03, Ab04, Ab05, Ab06, Ab07, Ab08, Ab09

Biochemical characteristics of Ab01 strain showed positive reactions for lysine decarboxylase, ornithin decarboxylase, oxidase tests, esculin hydorlysis and in utilization of citrate, arabinose, xylose, adonitol, cellobios, melibios, sacchrose, raffinose and lactose. It showed differences with *Btk* in ONPG, nitrate reduction and esculin hydrolysis tests and utilization of citrate, adonitol, raffinose, trehalose and glucose. Strain Ab02 showed positive reactions for nitrate reduction, esculin hydrolysis and in utilization of xylose, adonitol, rhamnose, cellobios, melibios, raffinose and lactose. It showed differences with *Btk* in ONPG, esculin and nitrate reduction tests and in utilization of adonitol, rhamnose, saccharose, raffinose, trehalose and glucose. The strain Ab03 was found to be positive for urease, nitrate reduction, esculin hydrolysis and in utilization of citrate, arabinose, xylose, adonitol, rhamnose, cellobios, melibios raffinose and lactose, while differences with *Btk* were observed in ONPG, lysine and ornithin decarboxylation, urease, esculin hydrolysis tests and in utilization of citrate, adonitol, rhamnose, saccharose, raffinose, trehalose and glucose. The strain Ab04 was positive for nitrate reduction, V-P test and in utilization of arabinose, xylose, adonitol, rhamnose, and lactose. It showed differences with *Btk* in ONPG, lysine and ornithin decarboxylation, urease, and in utilization of adonitol, rhamnose, cellobios, melibios, saccharose, trehalose and glucose.

Table 5.12: Biochemical characteristics of *Btk* (reference), Arc01, Arc02, Arc03, Arc04, Arc05, Arc06 and Arc07.

Biochemical tests	*Btk*	Arc 01	Arc 02	Arc 03	Arc04	Arc05	Arc06	Arc07
ONPG	+	-	-	-	-	-	+	+
Lysine decarboxylase	+	+	+	-	+	+	-	+
Ornithin decarboxylase	+	+	+	-	+	+	+	+
Urease	-	-	-	-	+	+	+	+
Phenylalanine deamination	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	-	+	-	+	+	+
H_2S production	-	-	-	+	-	-	-	-
Citrate utilization	-	+	+	+	-	+	+	+
V-P Test	+	+	-	+	+	+	+	+
Methyl red	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-
Malonate	-	-	+	+	-	-	+	+
Esculin hydrolysis	-	-	+	+	-	-	+	+
Arabinose	+	-	-	-	-	-	-	+
Xylose	+	-	-	-	-	-	+	+
Adonitol	-	-	-	-	-	-	-	-
Rhamnose	-	-	+	-	-	-	-	-
Cellobiose	+	-	+	-	-	-	+	-
Melibiose	+	-	-	-	-	-	+	+
Saccharose	+	-	-	+	-	+	+	+
Raffinose	-	-	+	-	-	-	+	+
Trehalose	+	+	-	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	-
Lactose	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	-	+	+
Casein hydrolysis	+	+	-	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+

+ denotes positive test result; - denotes negative test result.

Table 5.13: Similarity coefficient among the bacterial isolates of *A. submarginata* (Arc01-Arc07) and *Btk* (reference).

	Btk	Arc01	Arc02	Arc03	Arc04	Arc05	Arc06	Arc07
Btk	1.00							
Arc01	0.75	1.00						
Arc02	0.50▲	0.708	1.00					
Arc03	0.583	0.73	0.625	1.00				
Arc04	0.666	0.916	0.666	0.666	1.00			
Arc05	0.708	0.958▪	0.625	0.791	0.708	1.00		
Arc06	0.75	0.666	0.666	0.666	0.625	0.791	1.00	
Arc07	0.708	0.625	0.541	0.708	0.583	0.708	0.916	1.00

▲ indicates lowest similarity between two strains

▪ indicates highest similarity between two strains

Ab05 showed positive reaction for ornithin decarboxylase, urease, phenylalanine deamination, nitrate reduction, H_2S production and esculin hydrolysis tests, and in utilization of citrate, malonate, arabinose, xylose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose and lactose, but differed with *Btk* in ONPG, lysine decarboxylase, urease, phenylalanine deamination, H_2S, V-P and esculin hydrolysis tests, and in utilization of citrate, malonate and raffinose. Ab06 showed positive reactions for urease, nitrate, methyl red and esculin hydrolysis tests, and in utilization of malonate, arabinose, xylose, cellobiose, melibiose, trehalose, glucose, raffinose and saccharose. It showed difference with *Btk* in ONPG, lysine decarboxylase, ornitin decarboxylase, urease, V-P, methyl red and esculin hydrolysis tests and in utilization of malonate, raffinose and lactose. Ab07 was found to be positive for ONPG, lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, H_2S production, methyl red and esculin hydrolysis tests and in utilization of malonate, arabinose, xylose, adonitol, rhamnose, trehalose, cellobiose, melibiose, glucose and lactose. It showed differences with *Btk* in urease, H_2S, V-P, methyl red and esculin hydrolysis tests and in utilization of malonate, adonitol, rhamnose, saccharose and raffinose. Ab08 showed positive reactions for ONPG, lysine decarboxylase, ornithin decarboxylase, urease, nitrate and esculin hydrolysis tests and in utilization of citrate, malonate, arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose and lactose. It showed differences with *Btk* in urease, V-P and esculin hydrolysis tests and in utilization of citrate, malonate, adonitol, rhamnose and raffinose. The strain Ab09 was positive for nitrate, methyl red and esculin hydrolysis tests and for utilization of citrate, malonate, arabinose, xylose, cellobiose, melibiose, saccharose, trehalose, raffinose and glucose. It showed differences with *Btk* in ONPG, lysine decarboxylase, ornithin decarboxylase, V-P,

methyl red and esculin hydrolysis tests and in utilization of citrate, malonate, raffinose and lactose. Ab10 showed positive reaction for ornithin decarboxylase, urease, nitrate, methyl red and esculin hydrolysis tests and in utilization of citrate, malonate, arabinose, xylose, cellobiose, melibiose, saccharose, raffinose and glucose. It showed differences with *Btk* in ONPG, lysine decarboxylase, urease, V-P, methyl red and esculin hydrolysis tests and in utilization of citrate, malonate, raffinose, trehalose and lactose.

When tested for starch hydrolysis, casein hydrolysis and catalase tes,t all the strains were found to be positive except Ab02 and Ab10, which were negative to casein hydrolysis (Table 5.14). The similarity coefficient among the bacterial isolates of *A. bipunctata* (Ab01-Arc10), ranged from 0.52 (52%) between *Btk* and Ab02 to 0.88 (88%) between the pairs Ab02 & Ab04, Ab06 & Ab09, Ab06 & Ab10 (Table 5.15).

5.2.3.3. *Bacillus* strains of *O. postica*: Org01, Org2A, Org03, Org 05, Org5A and Org6A

In biochemical tests, Org 2A showed positive reaction for lysine carboxylase, urease, H_2S productin, V-P test, esculin hydrolysis and utilization of citrate, malonate, rhamnose, sccharose, trehalose, glucose and lactose, while it differed from *Btk* in ONPG test, ornithin decarboxylation, urease, nitrate reduction, H_2S production, esculin hydrolysis and utilization of lysine, citrate, malonate, arabinose, xylose, rhamnose and melibios. The strain Org 6A was found to be positive for lysine carboxylase, ornithin decarboxylase, urease, H_2S production, V-P test and utilization of citrate, malonate, arabinose, cellobios, melibiose, raffinose, and glucose. Org 6A differed from *Btk* in ONPG test, nitrate reduction, H_2S production and in utilization of citrate, malonate, saccharose, raffinose and trehalose.

Table 5.14: Biochemical characteristics of *Btk* (reference), Ab01, Ab02, Ab03, Ab04, Ab05, Ab06, Ab07, Ab08, Ab09 and Ab10.

Biochemical tests	*Btk*	Ab01	Ab02	Ab03	Ab04	Ab05	Ab06	Ab07	Ab08	Ab09	Ab10
ONPG	+	-	-	-	-	-	-	+	+	-	-
Lysine decarboxylase	+	+	-	-	-	-	-	+	+	-	-
Ornithin decarboxylase	+	+	-	-	-	+	-	+	+	-	+
Urease	-	-	-	+	-	+	+	+	+	-	+
Phenylalanine deamination	-	-	-	-	-	+	-	-	-	-	-
Nitrate reduction	+	-	+	+	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	+	-	+	-	-	-
Citrate utilization	-	+	-	+	-	+	-	-	+	+	+
V-P Test	+	+	+	+	+	+	+	+	+	+	+
Methyl red	-	-	-	-	-	-	+	+	-	+	+
Indole	-	-	-	-	-	-	-	-	-	-	-
Malonate	-	-	-	-	-	+	+	+	+	-	+
Esculin hydrolysis	-	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	-	+	+	+	+	-	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+
Adonitol	-	+	+	+	+	-	-	+	+	-	-
Rhamnose	-	-	+	+	+	-	-	+	+	-	-
Cellobiose	+	+	+	+	-	+	+	+	+	+	+
Melibiose	+	+	+	+	-	+	+	+	+	+	+
Saccharose	+	+	-	+	-	+	+	-	+	+	+
Raffinose	-	+	+	+	-	+	+	+	+	+	+
Trehalose	+	-	-	-	-	+	+	+	+	+	-
Glucose	+	-	-	-	-	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	-	+	+	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	-	+	+	+	+
Casein hydrolysis	+	+	-	+	+	+	+	+	+	+	-
Catalase test	+	+	+	+	+	+	+	-	-	+	+

+ denotes positive test result; - denotes negative test result.

Table 5.15: Similarity coefficient among the bacterial isolates of *A. bipunctata* (Ab01-Ab10) and *Btk* (reference).

	Btk	Ab01	Ab02	Ab03	Ab04	Ab05	Ab06	Ab07	Ab08	Ab09	Ab10
Btk	1.00										
Ab01	0.68	1.00									
Ab02	0.52▲	0.72	1.00								
Ab03	0.56	0.80	0.84	1.00							
Ab04	0.56	0.64	0.88▪	0.76	1.00						
Ab05	0.64	0.666	0.541	0.708	0.458	1.00					
Ab06	0.6	0.583	0.625	0.708	0.541	0.76	1.00				
Ab07	0.6	0.541	0.625	0.583	0.50	0.64	0.64	1.00			
Ab08	0.72	0.75	0.625	0.791	0.541	0.76	0.68	0.80	1.00		
Ab09	0.68	0.708	0.666	0.75	0.541	0.72	0.88▪	0.52	0.72	1.00	
Ab10	0.6	0.708	0.583	0.75	0.50	0.72	0.88▪	0.60	0.72	0.84	1.00

▲ indicates lowest similarity between two strains

▪ indicates highest similarity between two strains

Similarly, Org01 showed positive reaction for ONPG, lysine decarboxylase, ornithin decarboxylase, nitrate reduction, H_2S production, V-P test, esculine hydrolysis and utilization of arabinoe, cellobios, melibios, sccharose, trehalose, glucose and lactose and showed differences with *Btk* in H_2S production, esculine hydrolysis and utilization of xylose and lactose. Org03 showed positive reaction for nitrate reduction, V-P test, esculine hydrolysis and utilization of citrate, arabinose, sccharose, trehalose, glucose and lactose. It showed diffrences with *Btk* in ONPG, lysine decarboxylase, esculine hydrolysis and utilization of citrate, xylose, cellobiose, mellibiose and lactose. The strain Org05 showed positive reaction for ONPG, phenylalanine deamination, V-P test, esculine hydrolysis and utilization of citrate, malonate, arabinose, xylose, cellobios, sccharose, raffinose, trehalose, glucose and lactose differing from *Btk* in lysine decarboxylase, ornithin decaboxylase, phenylalanine deamination, nitrate reduction, esculine hydrolysis and utilization of citrate, malonate, melibiose, raffinose and lactose tests. Org5A showed positive reactions for ONPG, lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, H_2S production, V-P test, esculine hydrolysis and utilization of citrate, arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, trehalose, glucose and lactose. It showed difference with *Btk* for urease, H_2S production, esculine hydrolysis and utilization of citrate, adonitol, rhamnose, sccharose and lactose. When tested for startch hydrolysis, casein hydrolysis and catalase test all the strains were positive to the above tests. (Table 5.16). The similarity coefficient among the bacterial isolates of *O. postica* (Org01-Org6A), varied from a lowest 0.44 (44%) between *Btk* and Org2A to a highest value of 0.84 (84%) between *Btk* and Org01 (Table 5.17).

Table 5.16: Biochemical characteristics of *Btk* (reference), Org 2A, Org 6A, Org04, Org03, Org05 and Org5A.

Biochemical tests	*Btk*	Org2A	Org6A	Org1	Org3	Org5	Org5A
ONPG	+	-	-	+	-	+	+
Lysine decarboxylase	+	+	+	+	-	-	+
Ornithin decarboxylase	+	-	+	+	-	-	+
Urease	-	+	+	-	-	-	+
Phenylalanine deamination	-	-	-	-	-	+	-
Nitrate reduction	+	-	-	+	+	-	+
H_2S production	-	+	+	+	-	-	+
Citrate utilization	-	+	+	-	+	+	+
V-P Test	+	+	+	+	+	+	+
Methyl red	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-
Malonate	-	+	+	-	-	+	-
Esculin hydrolysis	-	+	-	+	+	+	+
Arabinose	+	-	+	+	+	+	+
Xylose	+	-	+	-	-	+	+
Adonitol	-	-	-	-	-	-	+
Rhamnose	-	+	-	-	-	-	+
Cellobiose	+	-	+	+	-	+	+
Melibiose	+	-	+	+	-	-	+
Saccharose	+	+	-	+	+	+	-
Raffinose	-	-	+	-	-	+	-
Trehalose	+	+	-	+	+	+	+
Glucose	+	+	+	+	+	+	+
Lactose	-	+	-	+	+	+	+
Oxidase	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	+
Casein hydrolysis	+	+	+	+	-	+	+
Catalase test	+	+	+	+	+	+	-

+ denotes positive test result; - denotes negative test result.

Table 5.17: Similarity coefficient among the bacterial isolates of *O. postica* (Org01-Org6A) and *Btk* (reference).

	Btk	Org2A	Org6A	Org01	Org03	Org05	Org5A
Btk	1.00						
Org2A	0.44▲	1.00					
Org6A	0.64	0.56	1.00				
Org01	0.84▪	0.58	0.708	1.00			
Org03	0.64	0.83	0.541	0.60	1.00		
Org05	0.60	0.58	0.708	0.60	0.72	1.00	
Org5A	0.68	0.58	0.708	0.76	0.56	0.52	1.00

▲ indicates lowest similarity between two strains

▪ indicates highest similarity between two strains

5.3 Sodium-dodecyl sulphate (SDS) polyacralamide gel electrophoresis (PAGE) of bacterial proteins

5.3.1 SDS-PAGE of Crystal protein

- ***Bacillus* strains: Arc01 and *Btk* (reference).**

When crystal protein (cry) composition was analysed by SDS-PAGE, difference in molecular weight was found between *Bacillus* strain Arc 01 and the *Btk* (Fig. 5.16). The strain Arc01 and *Btk* both showed four bands of cry proteins of 128, 81, 64 and 55.6 kDa were present in Arc01 and 76, 67, 56.6, 44.4 and 29.5 kDa were observed in *Btk*, which suggests variation in crystal protein profile of *Bacillus* strain Arc01 and the reference strain *Btk*.

- ***Bacillus* strains: Arc02, Arc03 and *Btk* (reference).**

When composition of crystal protein (cry) was analyzed by SDS-PAGE, Arc 02 showed five protein bands having the molecular weight 117.3, 75.1, 55.9, 39.1 and 29 kDa, which in case of *Btk* were 115.8, 75.1, 56.6, 44.4 and 29.5 kDa. Arc 03 also revealed five protein bands of 122.7, 85.8, 56.3, 46.8 and 29 kDa but they were different from Arc 02 and *Btk*. So, a sharp difference in banding pattern was found between Arc 02, Arc03 and *Btk*. Again variability in the crystal protein profile was seen in the *Bacillus* strains Arc02, Arc03 and reference strain *Btk*. Presence of more than one crystal protein band also suggests that these isolates may have newer crystal toxins different from one another and also with reference *Btk* (Fig. 5.17).

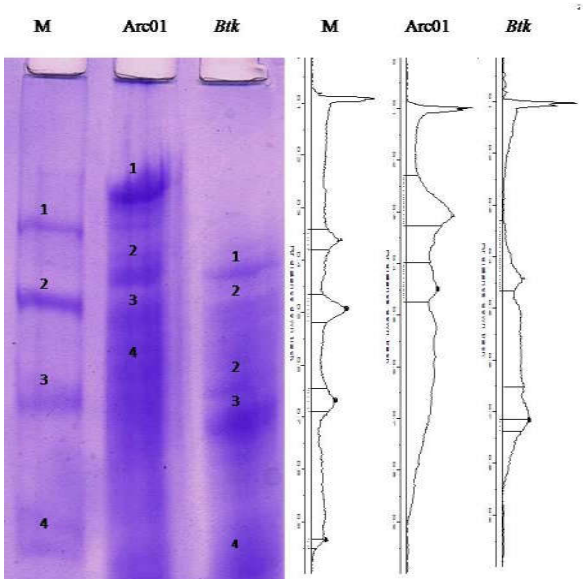

Fig. 5.16: SDS PAGE analysis of Whole body protein of Arc 01 and *Btk* (reference) [M: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa; *Btk*: 76 kDa, 67 kDa, 56.6 kDa, 44.4 kDa, 29.5 kDa; Arc01: 128 kDa, 81 kDa, 64 kDa, 55.6 kDa] with corresponding pixel graph.

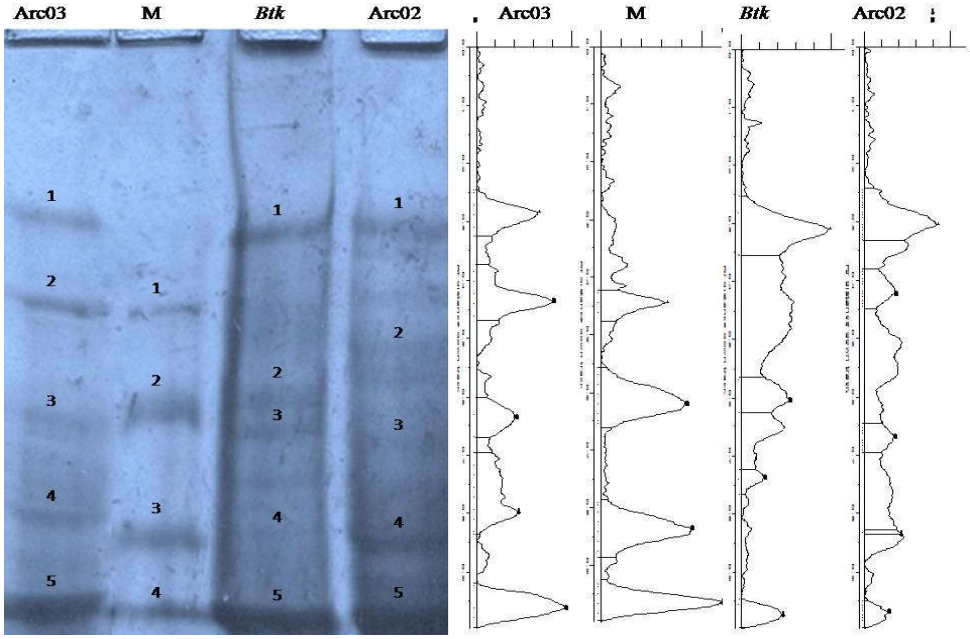

Fig. 5.17: SDS-PAGE of crystal protein of Arc02, Arc03 and *Btk* (reference) [Arc02: 117.3 kDa, 75.1 kDa, 55.9 kDa, 39.1 kDa, 29 kDa ; Arc03: 122.7 kDa, 85.8 kDa, 56.3 kDa, 46.8 kDa, 29 kDa; *Btk*: 115.8 kDa, 75.1 kDa, 56.6 kDa, 44.4 kDa, 29.5 kDa and Marker: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa] with corresponding pixel graph.

- ***Bacillus* strains: Ab01, Ab02, Ab03, Ab04 and *Btk* (reference).**

When composition of cry protein was analyzed by SDS-PAGE for the *Bacillus* strain isolated from *A. bipunctata*, Ab01and Ab02 revealed four bands each of 88, 56.7 and 43.3, 28 kDa and 88.9, 54.5, 43.8 and 27.5 kDa, respectively. Therefore, these two strains had crystal protein bands of comparable molecular weight. Each of the Ab03, Ab04 and reference stain *Btk* strains showed five distinct bands, Ab03 had bands of 56.2, 43, 38.2, 31 and 28 kDa, whereas 129.8, 97, 56.5, 44 and 29.4 kDa bands were present in Ab04 and *Btk* revealed 128.4, 97, 56.6, 44.4 and 29.5kDa bands. All the *Bacillus* strains Ab01-04 differed in crystal protein composition from the reference strain *Btk,* suggesting variability in crystal protein composition present among them and with reference strain *Btk* (Fig. 5.18).

- ***Bacillus* strains: Org2A, Org6A and *Btk* (reference).**

Similarly, SDS-PAGE analysis of cry protein Org2A and Org 6A was also carried out. Both the strains, Org2A and Org6A showed one major protein band having the molecular weight 36.5 kDa and 57.7 kDa, respectively. In contrast, *Btk* had five bands of 110.7, 97, 56.6, 44.4 and 29.5 kDa (Fig. 5.19). Unlike other strains mentioned above these two strains possessed only one bands suggesting they have only specific crystal toxin. All the band profiles showed difference within and between the strains.

5.3.2 Qualitative and Quantitative assay of whole cellular proteins of the isolates

Comparison of the whole cell proteins of different *Bacillus* strains of *Bt* isolated from *A. submarginata*, *A. bipunctata* and *O. postica* showed that the protein amount varied from 1.094-2.356 mg/ml in overnight grown bacterial culture.

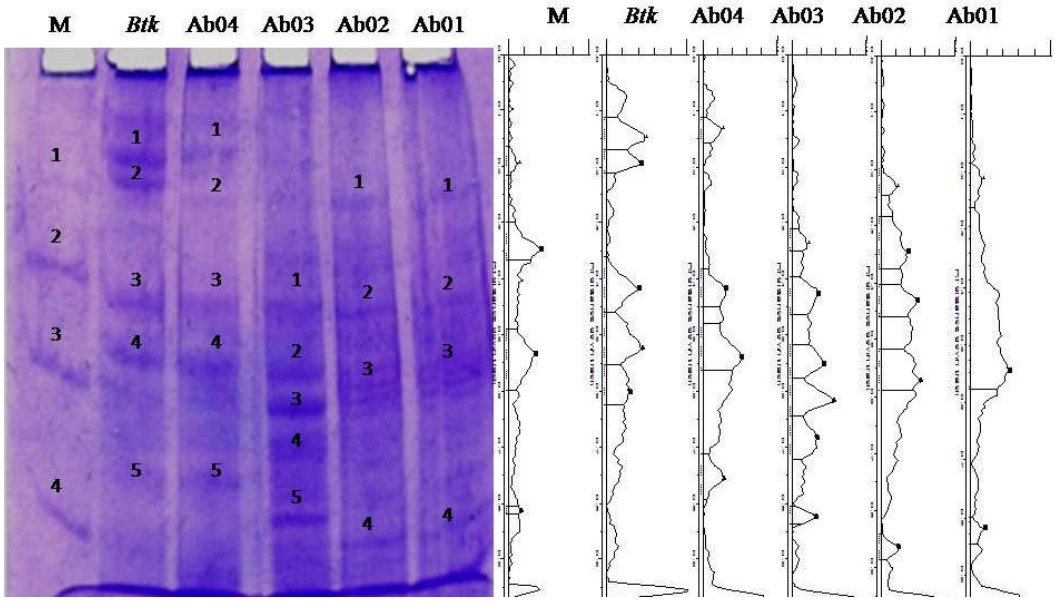

Fig. 5.18: SDS-PAGE of crystal proteins of *Bacillus* Ab01, Ab02, Ab03, Ab04 and *Btk* (reference) [Ab01: 88 kDa, 56.7 kDa,43.3kDa, 28 kDa; Ab02: 88.9 kDa, 54.5 kDa, 43.8 kDa, 27.5 kDa; Ab03: 56.2 kDa, 43 kDa, 38.2 kDa, 31 kDa, 28 kDa, Ab04: 129.8 kDa, 97 kDa, 56.5 kDa, 44 kDa, 29.5 kDa; *Btk*: 128.4 kDa, 97 kDa, 56.6 kDa, 44.4 kDa, 29.5 kDa; Marker: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa] with corresponding pixel graph.

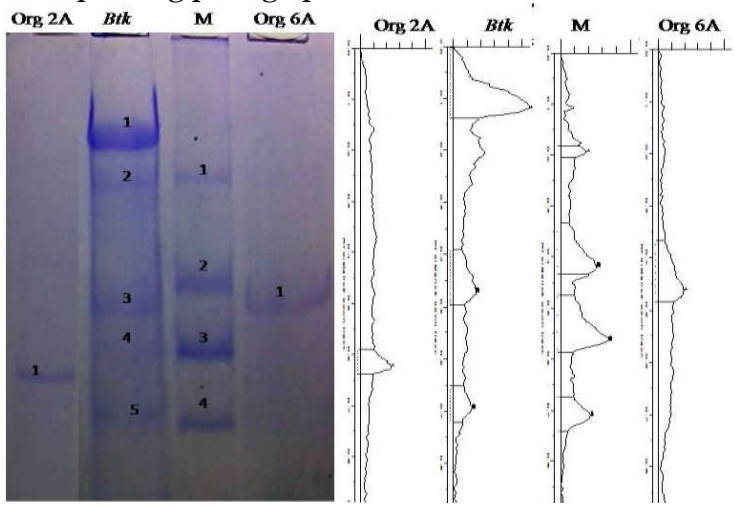

Fig. 5.19: SDS-PAGE of crystal protein of *Bacillus* sp Org 2A, Org 6A and *Btk* (reference) [Org2A: 36.5 kDa; Org6A: 57.7 kDa; *Btk*: 110.7 kDa, 97kDa, 56.6 kDa, 29.5 kDa; Marker: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa] with corresponding pixel graph.

The amount of proteins were 1.346, 1.420, 2.356, 1.094, 1.175, 1.244, 2.103, 1.994, 1.920 mg/ml for Arc01, Arc02, Arc03, Ab01, Ab02, Ab03, Ab04, Org2A, Org6A, respectively, compared to 2.128 mg/ml in *Btk* (Table 5.18). It indicated that different strains varied in whole cellular protein concentration.

In SDS PAGE analysis, the total cellular proteins of ten *Bacillus* strains as well as reference strain (Fig. 5.20, 5.21, 5.22, 5.23) were separated into several protein bands ranging from 10 to 126 KDa (Tables 5.19). The electrophoregram (Scans of protein profiles) revealed that the protein profiles can be distinguished into different groups on the basis of the molecular mass, which were in the range of 31 to 97 kDa (Arc01), 27 to 126 kDa (Arc02), 10 to 124 kDa (Arc03), 41 to 54 kDa (Ab01), 40 to 85 kDa (Ab02), 40 to 98 kDa (Ab03), 29 to 110 kDa (Ab04), 31 to 78 kDa (Org2A) and 31 to 77 kDa (Org6A). In *Btk* (reference strain) molecular masses were in the range of 25- 110 kDa combining four gels (Table 5.18). The protein profiles of isolated *Bacillus* strains could be distinguished into three protein groups, i.e. i) the proteins of low molecular masses of 25 to 59 kDa ii) the proteins of moderate molecular masses of 60 to 85 kDa and iii) the proteins of high molecular masses of 110 to 128 kDa (Table 5.19).

Table 5.18: Quantification of whole cellular proteins (Arc01, Arc02, Arc03, Ab01, Ab02, Ab03, Ab04, Org2A, Org6A) and *Btk* (reference).

Quantitative (Spectrophotometer)				
Bacillus strains	Optical density (OD)(at 545 nm)	Protein amount(mg/ml)	No. of bands	Molecular Weight Range (kDa)
Arc01	0.267	1.346	4	31-97
Arc02	0.280	1.420	3	25.8-126.6
Arc03	0.442	2.356	8	10-124
Ab01	0.224	1.094	2	40.8-54.1
Ab02	0.238	1.175	3	40.5-85
Ab03	0.250	1.244	4	40.5-98
Ab04	0.398	2.103	5	29-110
Org 2A	0.379	1.994	4	31.2- 78.5
Org 6A	0.367	1.920	3	30.8- 76.8
Btk	0.403	2.128	8	25-110

Table 5.19: Protein groups of nine *Bacillus* strains (Arc01, Arc02, Arc03, Ab01, Ab02, Ab03, Ab04, Org2A, Org6A) and *Btk* (reference).

Molecular masses of protein groups			
Bt strains	Group I (25 -59 kDa)	Group II (60 -80 kDa)	Group III (81 -135 kDa)
Arc01	31, 42.7	65.5	97
Arc02	27.7, 43	-	126.6
Arc03	10, 18, 26.8, 42.9, 50.3,	60.4	85.5, 124
Ab01	40.8, 54.1	-	-
Ab02	40.5, 58.1	-	85
Ab03	40.4, 55.8	-	84.8, 98
Ab04	29, 40.8	60.4	81.4, 110
Org2A	31.2, 42.6, 51	78.5	-
Org6A	30.8, 50	76.8	-
Btk	25, 31, 41, 55, 58	67, 76	110

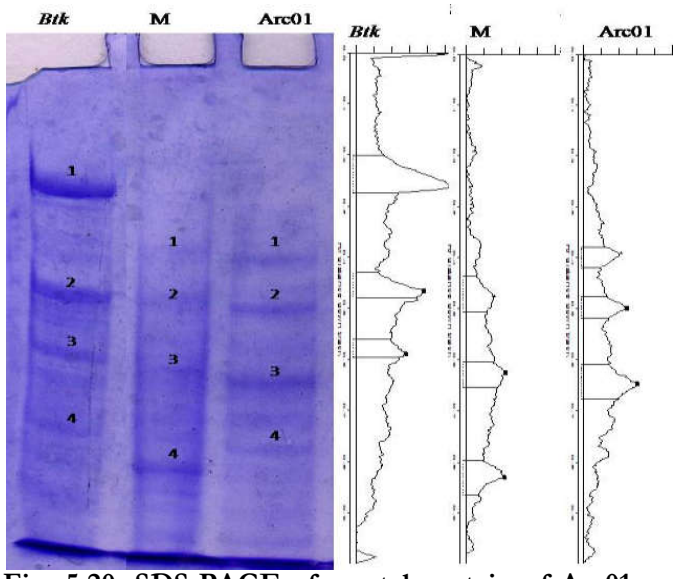

Fig. 5.20: SDS-PAGE of crystal protein of Arc01 and *Btk* (reference) [*Btk*: 115.8 kDa, 67 kDa, 45 kDa, 32 kDa; Arc01: 97 kDa, 65.5 kDa, 42.7 kDa, 31.2 kDa; M: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa;] with corresponding pixel graph.

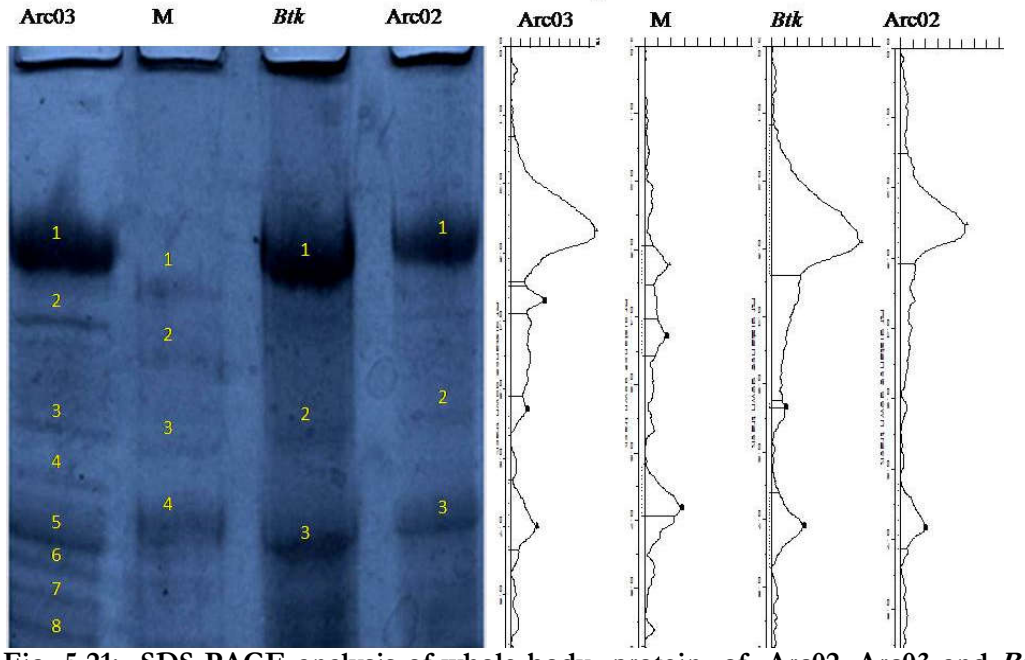

Fig. 5.21: SDS-PAGE analysis of whole body protein of Arc02, Arc03 and *Btk* (reference) [Arc02: 126.6 kDa, 43 kDa, 27.7 kDa; Arc03: 124 kDa, 85.8 kDa, 60.4 kDa, 50.3 kDa, 42.9 kDa, 26.8 kDa, 18 kDa, 10 kDa; *Btk*: 110.5 kDa, 55.3 kDa, 25.8 kDa and Marker: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa.] with corresponding pixel graph.

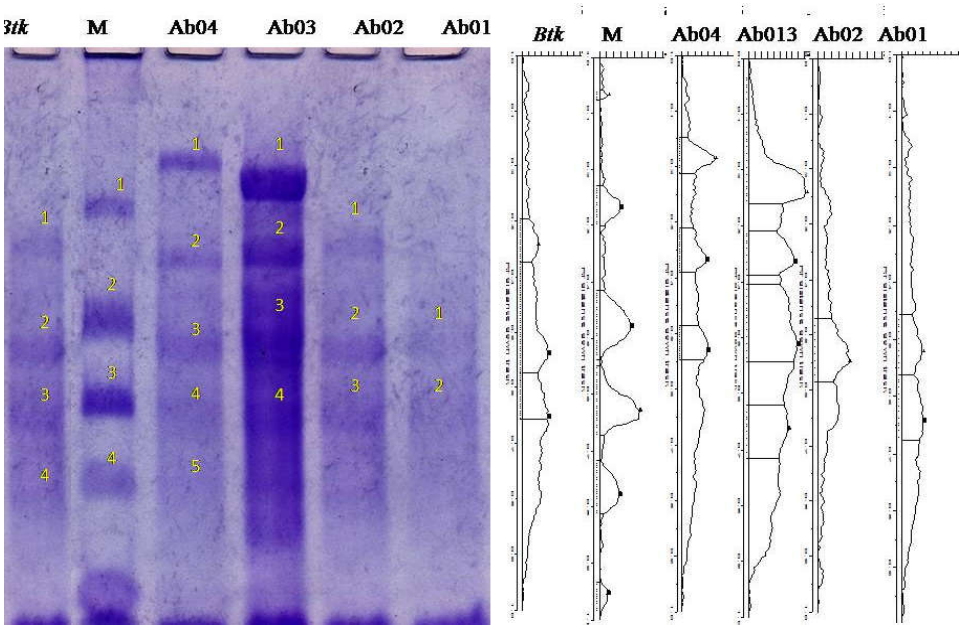

Fig. 5.22: SDS-PAGE of whole body protein of Ab01, Ab02, Ab03, Ab04 and *Btk* (reference) [Ab04: 110 kDa, 81.4 kDa, 60.4 kDa, 40.8kDa, 29 kDa; Ab03: 98 kDa, 84.8 kDa, 55.8 kDa, 40.5 kDa; Ab02: 85 kDa, 58.1 kDa, 40.5 kDa; Ab01: 54.1kDa, 40.8 kDa; *Btk*: 76.3 kDa, 58 kDa, 40.5 kDa, 25.8 kDa and Marker: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa] with corresponding pixel graph.

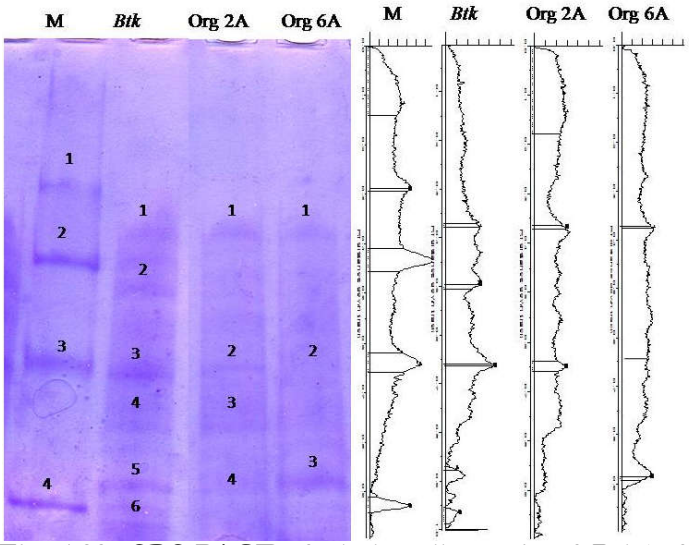

Fig. 5.23: SDS-PAGE of whole-cell protein of *Btk* (reference), Org 2A and Org 6A [Marker: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa; *Btk*: 76.3 kDa, 58 kDa, 51 kDa, 41.9 kDa, 25.5 kDa; Org 2A: 78.5 kDa, 51 kDa, 42.6 kDa, 31.2 kDa; Org 6A: 76.8 kDa, 50 kDa, 30.8 kDa] with corresponding pixel graph.

5.4 Plasmid profiling of the bacterial strains:

- ***Bacillus* strains: Arc01, Arc02, Arc03 and *Btk* (reference).**

Plasmid profiling of *Bacillus* strains of *A. submarginata* (Arc01, Arc02 and Arc03) showed one major plasmid band with approximate molecular weight 20.7 kbp, 18.5 kbp and 18.1 kbp respectively, which were comparable to the reference strain *Btk* which had 19 kbp band but slightly differed in plasmid size among themselves and also with the *Btk* (Fig. 5.24).

- ***Bacillus* strains: Ab01, Ab02, Ab03, Ab04 and *Btk* (reference).**

Plasmid profiling of *Bacillus* of *A. bipunctata* (Ab01, Ab02, Ab03 and Ab04) and reference strain *Btk* showed one major plasmid band ranging from 16 to 19 kbp. Ab01 had a band of 16 kbp followed by 17 kbp band of Ab02, while Ab03 and Ab04 had 18.2 kbp and 19.4 kbp bands, respectively. *Btk* yielded a major band of 19 kbp. All the isolates differed among themselves and also with the *Btk* in their plasmid profile (Fig. 5.25).

- ***Bacillus* strains: Org 2A, org 6A and *Btk* (reference).**

Plasmid profiling of *Bacillus* of *O. postica* (Org2A and Org6A) and *Btk* showed one major plasmid band for each with molecular weight 20.5 kbp, 19.8 kbp and 19 kbp, respectively which differed among themselves and with the *Btk* as well (Fig 5.26).

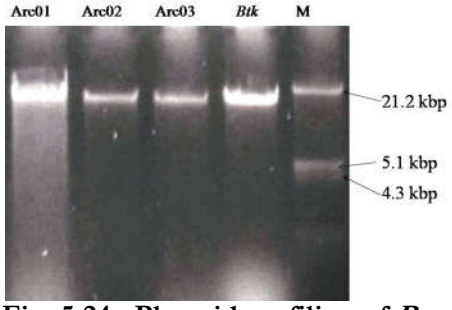

Fig. 5.24: Plasmid profiling of *Bacillus* sp. Arc01 [20.7 kbp], Arc02[18.5 kbp], Arc03[18.1 kbp] and *Btk* (reference)[19 kbp]with *Hind* III and *Eco* RI double digested λ DNA as Marker.

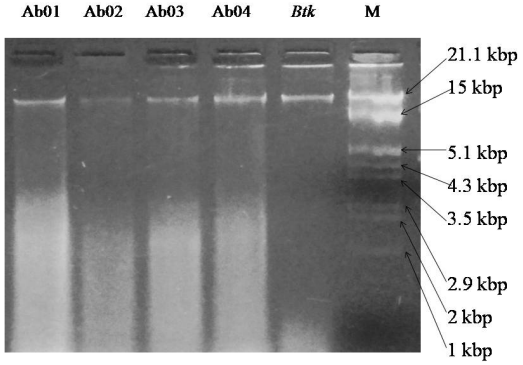

Fig. 5.25: Plasmid profiling of *Bacillus* sp. Ab01 [16 kbp], Ab02 [17 kbp], Ab03 [18.2 kbp], Ab04 [19.4 kbp] and *Btk* (reference)[19 kbp] with *Hind* III and *Eco* RI double digested λ DNA as Marker.

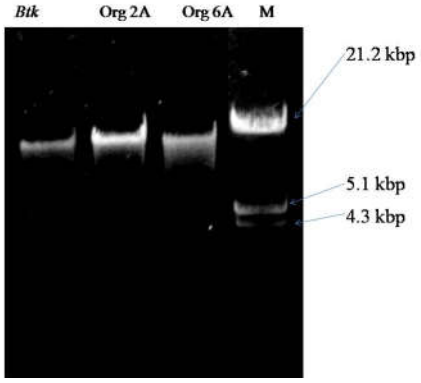

Fig. 5.26: Plasmid profiling of *Bacillus* sp. Org2A [20.5 kbp], Org6A [19.8 kbp] and *Btk* (reference)[19 kbp] with *Hind* III and *Eco* RI double digested λ DNA as Marker.

5.5 Bioassay

5.5.1 Rearing of the test insects in laboratory

5.5.1.1 Rearing of sporadic pests in laboratory

(i) *Arctornis submarginata* (Walker)

Systematic position

Phylum: Arthropoda

Class: Insecta

Order: Lepidoptera

Family: Lymantriidae

Genus: *Arctornis*

Species: *A. submarginata*

Moths of *A. submarginata* were collected from the tea plantations of Darjeeling Terai and the Dooars. They were then allowed to mate and lay eggs in sterilized plastic container, towelled with tissue paper. Fertilized eggs were then transferred in fresh containers for hatching. After hatching, the 1st instar caterpillars were transferred to small transparent plastic buckets and thoroughly washed fresh tea leaves (with petioles of young leaves submerged in water filled 2ml micro centrifuge tube) were provided as food to the neonates. The bucket was sanitized and food was changed regularly. At 3rd instar stage the caterpillars were transferred to bigger transparent buckets provided with mature tea leaves as food. Leaves were changed every day.

Eggs were round biconcave, olive green laid in clusters of 260-280 on the dorsal surface of tea leaves. They hatched in 6-7 days with 75% success. The post-embryonic development was for 39-46 days with six larval instars; first instars were light yellow, 2.81 mm in length, which grew 17-18 folds to reach brownish-black 6th instar stage, covered profusely with hair. Pictorial presentation of Life cycle of the insect is given in Fig. 5.27 with details in tabular form (Table 5.20).

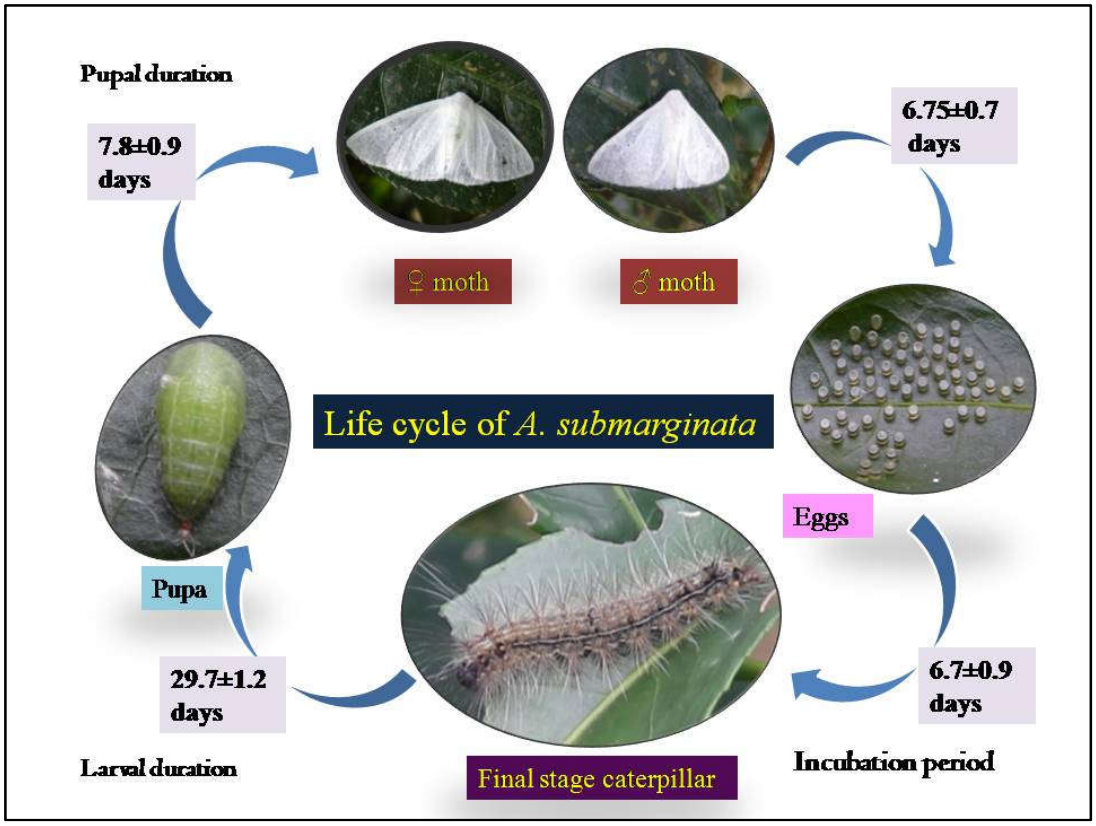

Fig. 5.27: Life cycle stages of *A.submarginata* reared in laboratory.

Table 5.20: Biological parameters of Life cycle of *A. submarginata* in laboratory.

	Observations *	
Biological parameters	Duration (days)	Average length (mm)
Egg Incubation	6.7±0.94 (n=30)	
1st larval Instar	8.5±0.57	2.81±0.31
2nd larval Instar	6.75±0.5	3.73±0.25
3rd larval Instar	5.5±0.57	9.6±0.96
4th larval Instar	5.75±0.5	17.7±0.82
5th larval Instar	3.5±0.57	34.8±0.77
6th larval Instar	2.8±0.78	39.1±0.99
Pupa	7.8±0.92	15.5±0.51
Adult longevity	6.75±0.73	
Secondary sex ratio (M:F)	1:0.5	
Fecundity	260-280	
Hatchability	75%	

*values are mean±S.D.

-experiments were conducted during November to February (average temp min: 13°C; max: 24°C; RH- 70±5%)

(ii) *Andraca bipunctata* Walker

Systematic position

Phylum: Arthropoda

Class: Insecta

Order: Lepidoptera

Family: Bombycidae

Genus: *Andraca*

Species: *A. bipunctata*

Moths of *A. bipunctata* were collected from the tea plantations of Darjeeling Terai and the Dooars. They were then allowed to mate and lay eggs in sterilized plastic container, towelled with tissue paper. Fertilized eggs were then transferred in fresh containers for hatching. After hatching the 1st instar caterpillars were transferred to small transparent plastic buckets. Thoroughly washed fresh tea leaves (with petioles of young leaves submerged in water filled 2ml micro centrifuge tube) were provided as food to the caterpillars. The bucket was sanitized and food was changed regularly. At 3rd instar stage the caterpillars were transferred to bigger transparent buckets provided with young and mature tea leaves as food. Leaves were changed as and when required. The eggs were light yellow, hard, leathery and oval with hatchability of 70%. The 1st instars were pale yellow, with black head capsule and distinct body segmentation, which later on grows up to 12-14 times when they reach maturity. Pictorial presentation of Life cycle of the insect is given in Fig. 5.28 with details in tabular form (Table 5.21).

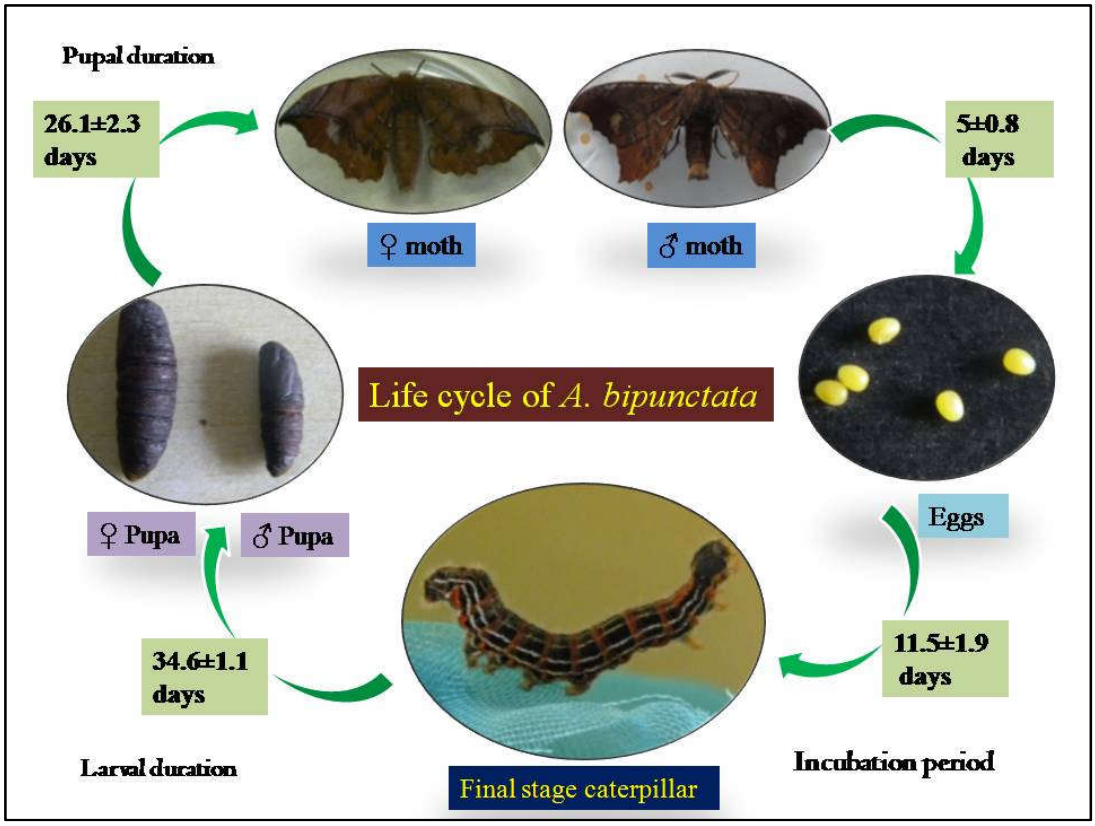

Fig. 5.28: Life cycle stages of *A.bipunctata* reared in laboratory.

Table 5.21: Biological parameters of Life cycle of *A. bipunctata* in laboratory.

	Observations *	
Biological parameters	Duration (days)	Average length (mm)
Egg Incubation	11.5±1.96	
1st larval Instar	8.2±0.95	4.12±0.88
2nd larval Instar	7.5±0.57	8.6±0.14
3rd larval Instar	6.5±0.57	14.6±0.15
4th larval Instar	7±0.81	21±0.10
5th larval Instar	6.7±0.5	48.1±0.32
Pupa(male)	26.1±2.37	18.8±0.1
Pupa(female)		25.4±0.11
Adult longevity♀	5.2±0.78	
Adult longevity♂	2.4±0.51	
Secondry sex ratio (M:F)	1:1	
Fecundity	360-400	
Hatchability	70%	

*values are mean±S.D.

-experiments were conducted during April to July (average temp min: 25°C; max: 32°C; RH-85±5%)

(iii) *Orgyia postica* Walker

Systematic position

Phylum: Arthropoda

Class: Insecta

Order: Lepidoptera

Family: Lymantriidae

Genus: *Orgyia*

Species: *O. postica*

Moths of *O.postica* were collected from the tea plantations of Darjeeling Terai and the Dooars. They were then allowed to mate and lay eggs in sterilized plastic container, towelled with tissue paper. Fertilized eggs were then transferred in fresh containers for hatching. After hatching the 1st instar caterpillars were transferred to small transparent plastic buckets. Thoroughly washed fresh tea leaves (with petioles of tea leaves submerged in water filled 2ml micro centrifuge tube) were provided as food to the caterpillars. The bucket was sanitized and food was changed regularly. At 3rd instar stage the caterpillars were transferred to bigger transparent buckets provided with mature tea leaves as food. Leaves were changed every day. The eggs were round, dull white in colour with hatchability of 73%. The larvae passed through six larval instars with a total post embryonic development period of 25-30 days. The 1st instar was about 2.3 mm, yellow in colour having patches of black bristles only on 4th segment. Post embryonic development lasted for 59-61 days with six larval instars. Pictorial presentation of Life cycle of the insect is given in Fig. 5.29 with details in tabular form (Table 5.22).

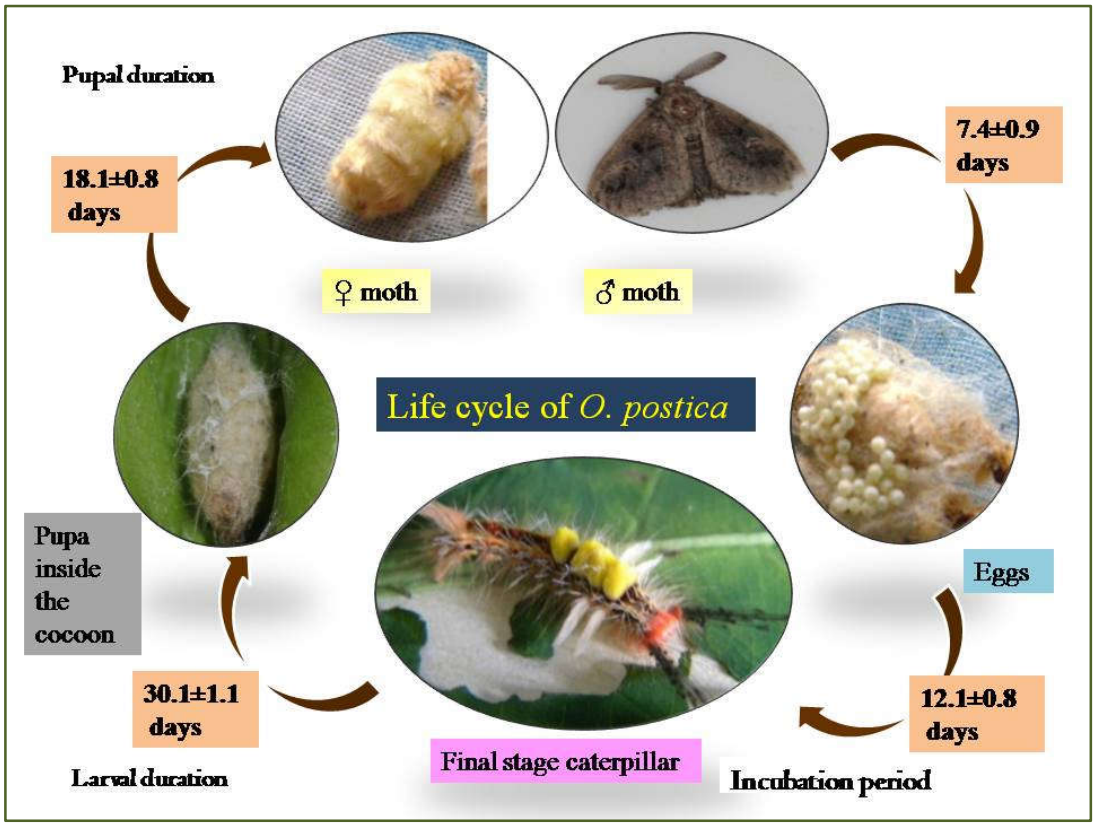

Fig. 5.29: Life cycle stages of *O.postica* reared in laboratory.

Table 5.22: Biological parameters of Life cycle of *O.postica* in laboratory.

	Observations *	
Biological parameters	Duration (days)	Average length (mm)
Egg Incubation	12.1±0.87	
1st larval Instar	8.7±0.78	2.3±0.15
2nd larval Insatar	4.18±0.75	6.27±0.18
3rd larval Instar	3.72±0.78	7.6±0.13
4th larval Instar	3.72±0.64	12.4±0.33
5th larval Instar	3.7±0.94	15±0.41
6th larval Instar	10.8±0.9	31.5±0.26
Pupa	18.1±0.87	14.1±0.78
Adult longevity	7.4±0.96	
Secondary sex ratio (M:F)	1:1	
Fecundity	250-300	
Hatchability	73%	

*values are mean±S.D.

-experiments were conducted during March to April (average temp min: 17°C; max: 27°C; RH-80±5%)

5.5.5.2 Rearing of the control insect (silk worm) in laboratory for testing safety of entomopathogens

***Bombyx mori* Linnaeus**

Systematic position

Phylum: Arthropoda

Class: Insecta

Order: Lepidoptera

Family: Bombycidae

Genus: *Bombyx*

Species: *B. mori*

Disease free eggs of *Bombyx mori* nistari (DFL) collected from local Central Sericulture farm, Matigara (Dist. Darjeeling) were kept in a water soaked cotton bed inside Petri plates and placed in an incubator at 24° C and > 70% humidity for hatching of the neonates. After hatching the paper containing the first instar was transferred to paper box and covered with fresh sterilized mulberry leaves. The caterpillars ate voraciouly and on finishing the layer of mulberry leaf, crawl to the upper layer of mulberry leaves. This upward movement of the caterpillars helped in the cleaning of the wastes and faecal matters which lay at the bottom. The caterpillars are provided with the fresh leaves 3-4 times a day and the amount depended on the advancement of the caterpillar stage. In about 21 days they were ready for pupation. The fifth (final) instar stopped feeding before spinning the silk cocoon. Adult moths emerged from the cocoons in about a week. Pictorial presentation of Life cycle of the silk moth is given in Fig. 5.30 with details in tabular form (Table 5.23).

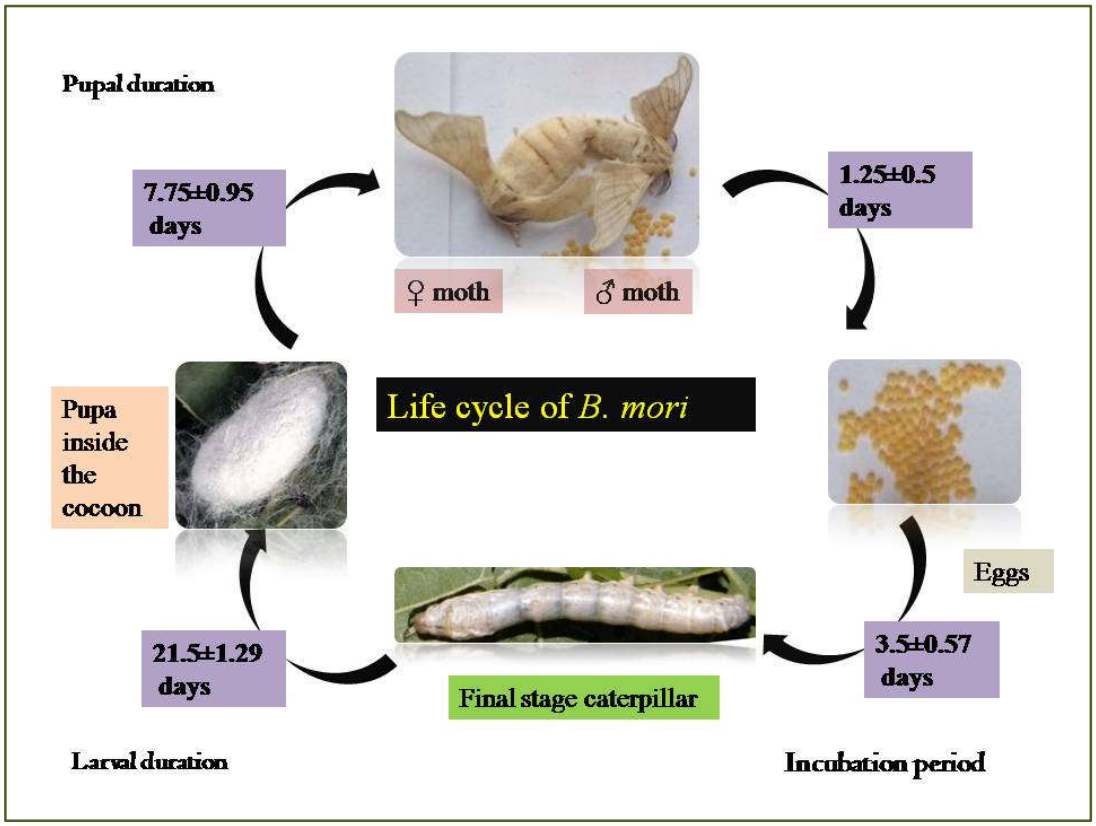

Fig. 5.30: Life cycle stages of silkworm (*B. mori* nistari) reared in laboratory.

Table 5.23: Biological parameters of Life cycle of *B. mori* in laboratory.

	*Observation
Biological parameters	Duration (days)
Egg Incubation	3.5±0.57
1st larval Instar	3.25±0.5
2nd larval Insatar	3.5±0.57
3rd larval Instar	3.75±0.5
4th larval Instar	5.5±1.2
5th larval Instar	7.25±0.95
Pupa	7.75±0.5
Adult longevity	1.25±0.5
Secondary sex ratio	1:1 (M:F)
Fecundity	350-400
Hatchability	95%

*values are mean±S.D.

-experiments were conducted in controlled temperature and relative humidity (24°C; RH-70%)

5.5.2 Bioassay (LT_{50} and LC_{50}) for *Bacillus* strains of *A. submarginata*

The percent mortality of second instar *A. submarginata* caterpillars when treated with *Bacillus* (Arc 01), ranged between 23% to 78% through 9 days observation. The LC_{50} value was recorded as 398.1 µg/ml with fiducial lower limit 353 µg/ml and upper limit 443.1 µg/ml. LT_{50} values were 7.28 days for 1000 µg/ml and 7.88 days for 750 µg/ml and 8.45 days for 500 µg/ml. In case of Arc02 strain of *Bacillus* the percent mortality of second instar larvae varied from 12% to 63% upto 9 days. The LC_{50} value was found to be 791.2 µg/ml with fiducial lower limit 662.5 µg/ml and upper limit 957µg/ml. The LT_{50} value was 5.5 days for 1000 µg/ml concentration. *Bacillus* sp. Arc03 showed a percent mortality between 26% to 79% upto 9 days. The LC_{50} was calculated as 342 µg/ml with fiducial lower and upper limit of 281.7 µg/ml and 414.9 µg/ml respectively. The LT_{50} of the same was found to be 5.28 days for 1000 µg/ml, 6.3 days for 750 µg/ml and 7.42 days for 500 µg/ml. *Btk* on the other hand showed the mortality ranging between 17% to 76% upto 9 days. LC_{50} value was 537 µg/ml with lower fiducial limit of 483.6 µg/ml and upper fiducial limit of 590.3 µg/ml. The LT_{50} values were 7.57 days and 8.5 days for 1000 µg/ml and 750 µg/ml, respectively (Fig. 5.31).

The data on dosage-mortality response of *A. submarginata* to Arc01, Arc02, Arc03 and *Btk* revealed that chi-square values were good fit of probit response. All the bioassays showed that there was no heterogeneity between observed and expected responses (Table 5.24).

Fig. 5.31: Bioassay of three entomopathogenic *Bacillus* strains: Arc01, Arc02, Arc03 and *Btk* (reference).

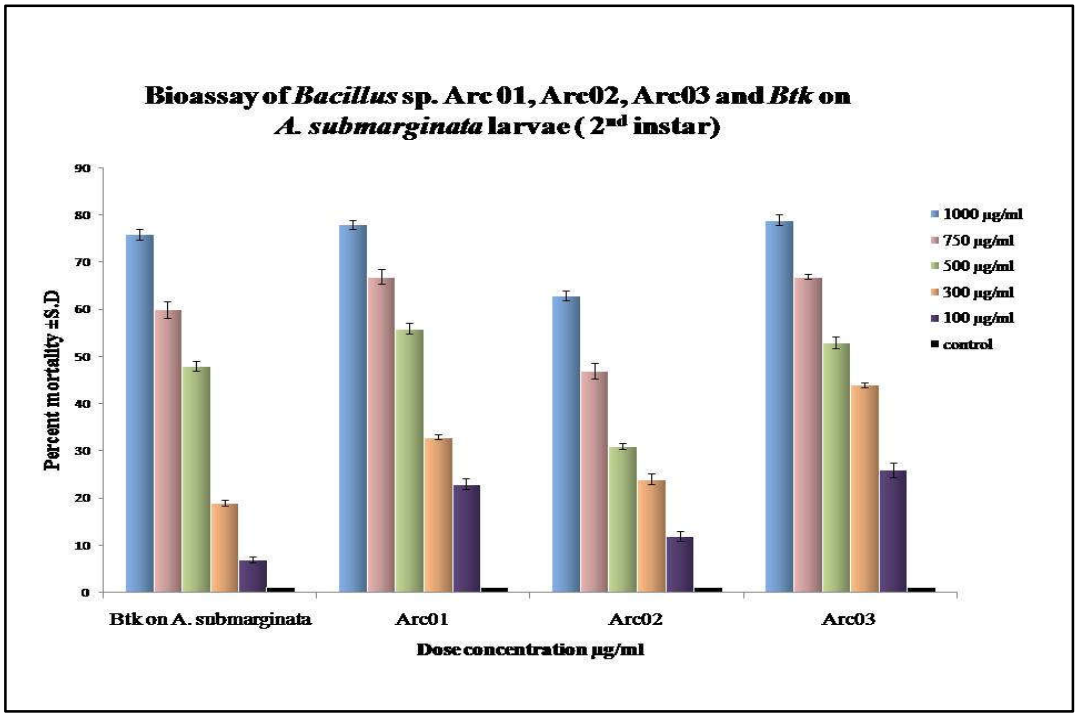

Table 5.24: Bioassay results of *Bacillus* strains: Arc01, Arc02, Arc03 and *Btk* (reference) on *A. submarginata* larvae (2nd instar; n=90).

						Fiducial limits		
Bacterial isolate	**#Concentration**	**% mortality**	**Heterogeneity (χ2)**	**Regression equation**	**LC_{50} (µg/ml)**	**Lower**	**Upper**	**LT_{50} (days)**
Arc 01	**1000** **750** **500** **300** **100**	**78** **67** **56** **33** **23**	**5.69112**	**Y=2.587569x+1.52924**	**398.1**	**353**	**443.1**	**7.28 for 1000** **7.88 for 750** **8.45 for 500**
Arc 02	**1000** **750** **500** **300** **100**	**63** **47** **31** **24** **12**	**4.81019**	**Y=2.901032x+1.47781**	**791.2**	**662.5**	**957**	**5.5 for 1000**
Arc 03	**1000** **750** **500** **300** **100**	**79** **67** **53** **44** **26**	**3.0022**	**Y=2.533x+ 1.365**	**342**	**281.7**	**414.9**	**5.28 for 1000** **6.3 for 750** **7.42 for 500**
Btk	**1000** **750** **500** **300** **100**	**76** **60** **48** **19** **17**	**5.07422**	**Y=1.520x+0.833**	**537**	**483.6**	**590.3**	**7.57 for 1000** **8.5 for 750**

 # Concentration in µg/ml

5.5.3 Bioassay (LT_{50} and LC_{50}) for *Bacillus* strains of *A. bipunctata*

The percent mortality of early 2nd instar *A. bipunctata* larvae increased from 20% to 55% within 9 days when treated with Ab01 strain of *Bacillus* in laboratory. The LC_{50} value for Ab01 was found to be 664 µg/ml with fiducial lower limit 519 µg/ml and upper limit 849.6 µg/ml. The LT_{50} values were found to be 7 days for 1000 µg/ml, 7.67 days for 750 µg/ml concentrations. In case of Ab02 strain of *Bacillus,* the percent mortality ranged from 13 % to 60% upto 9 days. The LC_{50} value was found to be 785.6 µg/ml with fiducial lower and upper limits 644.9 µg/ml and 956.9 µg/ml respectively. The LT_{50} value was 7.5 days for 1000 µg/ml. In Ab03 strain, the percent mortality of larvae varied from 13% to 57% upto 9 days in the laboratory condition. The LC_{50} value was found to be 783.2 µg/ml with fiducial lower limit 630.3 µg/ml and upper limit 973.3 µg/ml. The LT_{50} value was 7.5 days for 1000 µg/ml. The percent mortality of larvae in Ab04 strain of *Bacillus* varied from 27% to 87% upto 9 days. The LC_{50} value was found to be 385.8 µg/ml with fiducial lower limit 305.9 µg/ml and upper limit 486.6 µg/ml, while LT_{50} values were 5.83 days, 7.5 days and 7.67 days for 1000, 750 and 500 µg/ml concentrations, respectively. In case of *Btk*, the LC_{50} value was found to be 787.7 µg/ml with fiducial lower and upper limits 642.6 µg/ml and 965.5 µg/ml, respectively. The LT_{50} value recorded was 7.65 days for 1000 µg/ml (Fig. 5.32).

The data on dosage-mortality response of *A. bipunctata* against Ab01, Ab02, Ab03, Ab04 and *Btk* revealed that chi-square values were good fit of probit response. All the bioassays showed that there was no heterogeneity between observed and expected responses (Table 5.25).

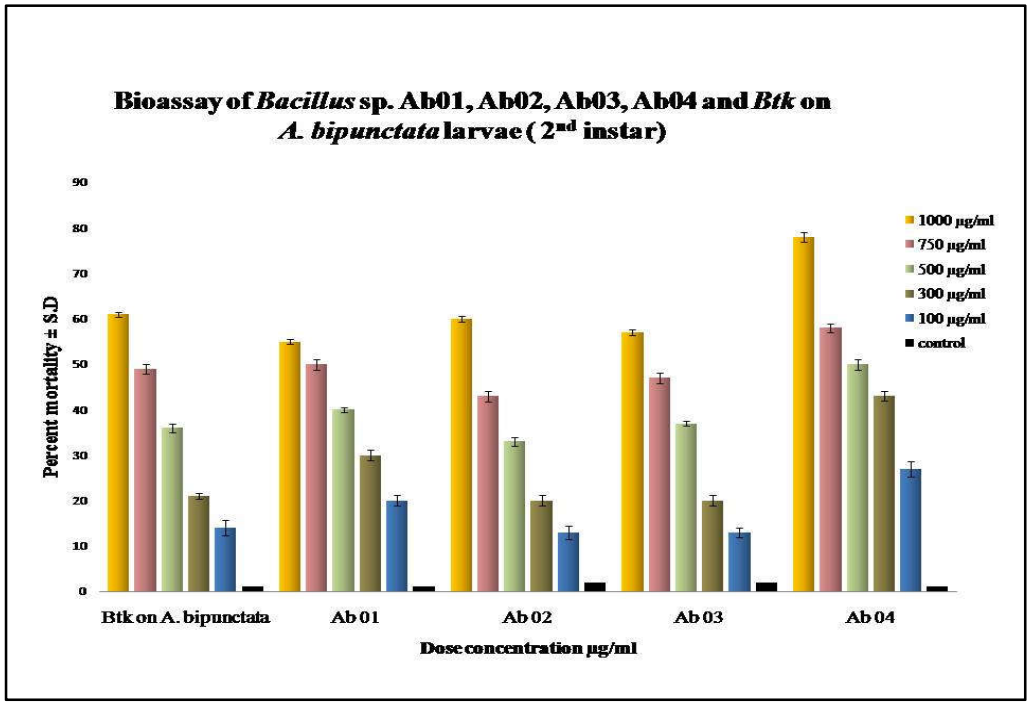

Fig. 5.32: Bioassay of four entomopathogenic *Bacillus* strains: Ab01, Ab02, Ab03, Ab04 and *Btk* (reference).

Table 5.25: Bioassay results of *Bacillus* strains: Ab01, Ab02, Ab03, Ab04 and *Btk* (reference) on *A. bipunctata* larvae (2nd instar; n=90).

						Fiducial limits		
Bacterial isolate	**# Concentration**	**% mortality**	**Heterogeneity (χ2)**	**Regression equation**	**LC_{50} (µg/ml)**	**Lower**	**Upper**	**LT_{50} (days)**
Ab01	**1000 750 500 300 100**	**55 50 40 30 20**	**2.03714**	**Y= 2.82220x+1.13888**	**664**	**519**	**849.6**	**7 for 1000 µg/ml 7.67 for 750 µg/ml**
Ab02	**1000 750 500 300 100**	**60 43 33 20 13**	**4.93654**	**Y= 2.09497x+1.38113**	**890.7**	**723.2**	**1096.9**	**7.5 for 1000 µg/ml**
Ab03	**1000 750 500 300 100**	**57 47 37 20 13**	**3.02716**	**Y= 2.92713x+1.38015**	**845.5**	**687.1**	**1040.4**	**7.5 for 1000 µg/ml**
Ab04	**1000 750 500 300 100**	**78 58 50 43 27**	**5.97441**	**Y= 2.58642x+1.19189**	**385.8**	**305.9**	**486.6**	**5.83 for 1000 µg/ml 7.5 for 750 µg/ml 7.67 for 500 µg/ml**
Btk	**1000 750 500 300 100**	**61 49 36 21 14**	**4.86453**	**Y= 2.89636x+1.34546**	**787.7**	**642.6**	**965.5**	**7.65 days for 1000 µg/ml**

 # Concentration in µg/ml.

5.5.4 Bioassay (LT_{50} and LC_{50}) for *Bacillus* strains of *O. postica*

Bioassays have been carried out on the early 2nd instar *Orgyia postica* larvae using two of the *Bacillus* isolates Org 2A and Org 6A. The result showed that in case of Org 2A strain of *Bacillus,* the percent mortality of larvae varied from 17% to 67% upto 9 days. The LC_{50} value was found to be 543.3 µg/ml with fiducial lower limit 477.6 µg/ml and upper limit 659.6 µg/ml. The LT_{50} values were 6 days for 1000 µg/ml and 6.5 days for 750 µg/ml concentrations. In case of Org 6A the percent mortality of larvae varied from 21% to 78% upto 9 days. The LC_{50} value was found to be 354.8 µg/ml with fiducial lower and upper limits 299 µg/ml and 421.1 µg/ml, respectively. The LT_{50} values were 5.5 days for 1000 µg/ml, 6 days for 750 µg/ml and 6.19 days for 500 µg/ml concentrations. When *Btk* was applied to the *Orgyia* larvae the percent mortality varied from 20% to 76% upto 9 days. The LC_{50} value was found to be 386.8 µg/ml with fiducial lower limit 326.7 µg/ml and upper limit 457.9 µg/ml. The LT_{50} values were 6 days for 1000 µg/ml, 6.5 days for 750 µg/ml and 7.15 days for 500 µg/ml concentrations (Fig. 5.33).

The data on dosage-mortality response of *O. postica* against Org2A, Org6A and *Btk* revealed that chi-square values were good fit of probit response. All the bioassays showed that there was no heterogeneity between observed and expected responses (Table 5.26).

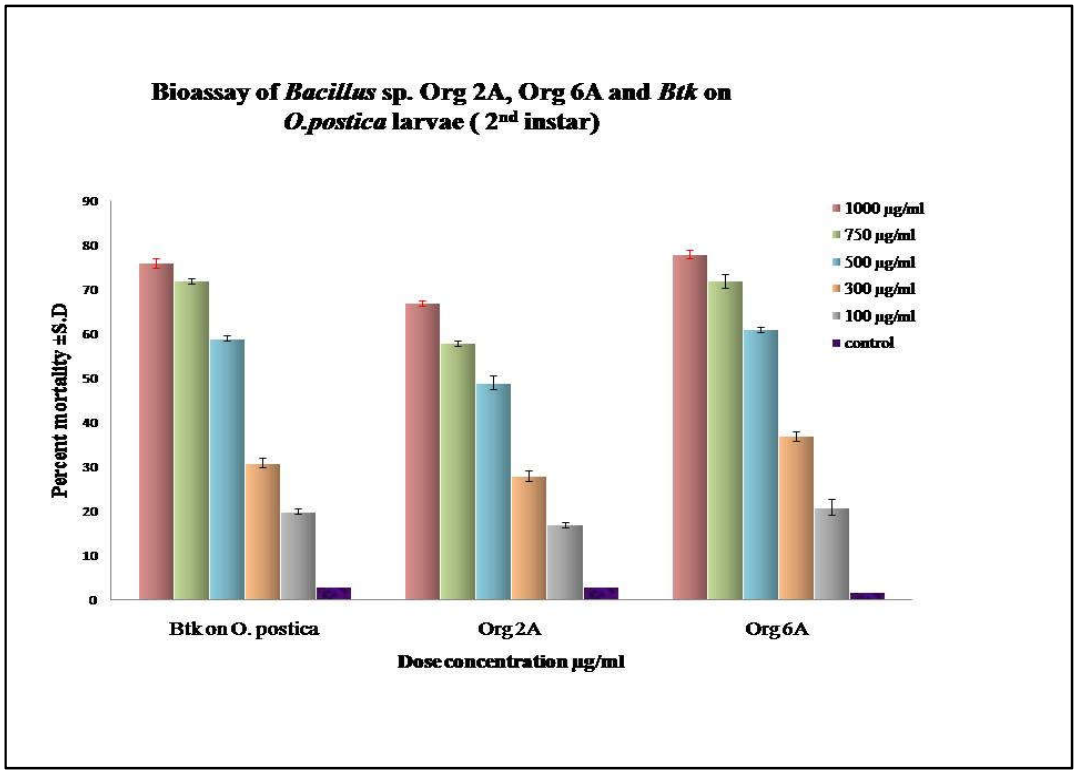

Fig. 5.33: Bioassay of two entomopathogenic *Bacillus* strains: Org2A, Org6A and *Btk* (reference).

Table 5.26: Bioassay results of *Bacillus* strains: Org 2A, Org 6A and *Btk* (reference) on *O. pstica* larvae (2nd instar; n=90).

						Fiducial limits		
Bacterial isolate	**# Concentration**	**% mortality**	**Heterogeneity (χ2)**	**Regression equation**	**LC_{50} (µg/ml)**	**Lower**	**Upper**	**LT_{50} (days)**
Org2A	**1000 750 500 300 100**	**67 58 49 28 17**	**2.72736**	**Y=2.735114x+1.4457**	**543.3**	**447.6**	**659.6**	**6 for 1000 µg/ml 6.5 for 750 µg/ml**
Org6A	**1000 750 500 300 100**	**78 72 61 37 21**	**2.88971**	**Y=2.55x+1.65871**	**354.8**	**299**	**421.1**	**5.5 for 1000 µg/ml 6 for 750 µg/ml 6.19 for 500 µg/ml**
Btk	**1000 750 500 300 100**	**76 72 59 31 20**	**6.50426**	**Y=2.58749x+1.68775**	**386.8**	**326.7**	**457.9**	**6 for 1000 µg/ml 6.5 for 750 µg/ml 7.15 for 500 µg/ml**

 # Concentration in µg/ml.

5.5.5 Cross-infection of the *Bacillus* isolates against silkworm larva

In the cross infectivity test using spore-crystal mixture of all of the nine isolates of *Bacillus* strains (Arc01, Arc02 and Arc03) isolated from *A. submarginata* cadavers, *Bacillus* strains Ab01, Ab02, Ab03 and Ab04) isolated from *A. bipunctata* cadavers, *Bacillus* strains (Org2A and Org6A) isolated from *O. postica* cadavers were prepared in five concentrations (1000, 750, 500, 300, 100 µg/ml) and the same were tested on *B. mori* (2^{nd} instar, n=90) by leaf dip method. Mortality were checked every 24 hrs upto 9 days. Bioassay setup showed that there was no significant mortality between inoculated and control set of silkworm larvae (Table 5.27, 5.29, 5.31) and there was no significant difference in the mortality when the mortality were compared through one way ANOVA (Table 5.28, 5.30, 5.32).

In general, no significant mortality could be observed in silkworms on exposure to the bacterial strains isolated from the sporadic lepidopteran tea pests under study. Suggesting these newly isolated *Bacillus* strains are safe for use in the tea gardens in North Bengal Terai area without harming the sericulture industry running parallaly in this area.

Table 5.27: Mortality in silkworm larvae when fed with bacteria isolated from *A. submarginata*.

		Arc01	Arc02	Arc03
Bacterial concentration (µg/ml)	Sample size x replicate =total	% mortality	% mortality	% mortality
1000	30x3=90	5.56	6.67	5.56
750	30x3=90	6.67	6.67	4.45
500	30x3=90	3.34	3.34	3.34
300	30x3=90	4.45	4.45	2.23
100	30x3=90	3.34	3.34	3.34
Control	30x3=90	3.34	3.34	3.34

Table 5.28: One way ANOVA comparing the effect of different *Bacillus* strains from *A. submarginata* (Arc01, Arc02 and Arc03) on *B. mori*.

Source	SS[§]	df	MS[¥]	
Between-treatments	0.345	2	0.1725	F =0.23596
Within-treatments	2.1931	3	0.731	
Total	2.5381	5		
F= 0.23596* P= 0.80321				

§ Sum of squares

¥ Mean of squares

*At 0.05 level, the means are NOT significantly different.

Table 5.29: Mortality in silkworm larvae when fed with bacteria isolated from *A. bipunctata*.

		Ab01	Ab02	Ab03	Ab04
Bacterial concentration (µg/ml)	Sample size x replicate =total	% mortality	% mortality	% mortality	% mortality
1000	30x3=90	11.12	10	7.78	10
750	30x3=90	10	8.89	10	11.12
500	30x3=90	7.78	8.89	10	10
300	30x3=90	8.89	7.78	8.89	8.89
100	30x3=90	6.67	6.67	7.78	8.89
Control	30x3=90	6.67	6.67	7.78	7.78

Table 5.30: One way ANOVA comparing the effect of different *Bacillus* strains from *A. bipunctata* (Ab01, Ab02, Ab03 and Ab04) on *B. mori*.

Source	SS[§]	df	MS[¥]	
Between-treatments	1.8248	3	0.6083	$F = 0.36524$
Within-treatments	6.6618	4	1.6654	
Total	8.4866	7		
F= 0.36524* P= 0.782887				

§ Sum of squares

¥ Mean of squares

*At 0.05 level, the means are NOT significantly different.

Table 5.31: Mortality in silkworm larvae when fed with bacteria isolated from *O. postica*.

		Org 2A	Org 6A
Bacterial concentration (µg/ml)	Sample size x replicate =total	% mortality	% mortality
1000	30x3=90	4.45	3.34
750	30x3=90	5.56	4.45
500	30x3=90	3.34	3.34
300	30x3=90	4.45	5.56
100	30x3=90	3.34	3.34
Control	30x3=90	4.45	5.56

Table 5.32: One way ANOVA comparing the effect of different *Bacillus* strains from *O. postica* (Org 2A and org 6A) on *B. mori*.

Source	SS[§]	df	MS[¥]	
Between-treatments	0.1971	1	0.1971	$F = 0.32$
Within-treatments	1.2321	2	0.6161	
Total	1.4292	3		
F= 0.32* P= 0.628609				

§ Sum of squares

¥ Mean of squares

*At 0.05 level, the means are NOT significantly different.

5.6 PCR amplification of 16S rRNA gene of most virulent strains

After isolation, purity of the total DNA of most virulent strains (Arc03, Ab04 and Org6A) was tested to make sure that the genomic DNA does not have any protein contaminations (Table 5.33). Next these pure DNA samples of most virulent strains isolated from three pest species was selected for PCR amplification of 16S rRNA gene using Universal Primer pair (Table 4.2). All the three strains under study and the reference *Btk* yielded a 1500 bp band after PCR (Fig. 5.34). All the PCR products were subjected to sequencing.

5.6.1 Characterization of the most virulent *Bacillus* strains using 16S rRNA

16S rRNA gene sequencing of the most virulent isolates, Arc03, Ab04 and Org6A yielded 1328, 1387 and 1386 nucleotides long amplicon with universal primer. The sequences were compared with the known sequences available in GeneBank of NCBI (National Centre for Biotechnology Information, http://www.ncbi.nih.gov/).

When sequences were analysed using BLAST search (Basic Local Alignment Search Tool) which provides a rapid comparison of related sequences, the 16S rDNA of Arc03, Ab04 and Org6A showed 99% sequence homology with *Bacillus thuringiensis* strain ATCC 10792 16S ribosomal RNA gene, partial sequence. [GenBank: NR_114581.1], *Bacillus thuringiensis* strain IAM 12077 16S ribosomal RNA gene, [GenBank: NR_043403.1] and *Bacillus thuringiensis* strain NBRC 101235 16S ribosomal RNA gene, [GenBank: NR_112780.1]. The partial sequences of these strains when aligned with related sequences (obtained from BLAST) using CLUSTAL W 2.0.12 program (multiple sequence alignment) resulted in the

generation of highest score of 99% among the sequences. These strongly supported that the isolated bacterial strains under study are indeed *B. thuringienseis.* The sequences of 16S ribosomal RNA genes were then submitted to NCBI and the same were given the Gene Bank Accession Numbers as KX245014, KX245015 and KX245016 for Arc03, Org6A and Ab04, respectively. Further, the sequence obtained for reference *Btk* showed difference with these virulent strains suggesting they are novel strains of *Bacillus thuringiensis* than the commercially available *Btk* which was used as reference for the study.

5.6.2 Cry gene amplifications of the most virulent strains

PCR analysis was carried out using the specific primers for the identification of *cry*1, *cry*2 and *cry*9 genes (toxic to lepidopterans) for most virulent *Bacillus* strains Arc03, Ab04 and Org6A and compared with reference *Btk* strain. Positive results were obtained for the *cry*1 (277 bp) and *cry*9 gene (354 bp) was obtained for Arc03, Ab04 and reference *Btk*. Further, Ab04 and reference *Btk* showed positive result for *cry*2 (1500 bp) gene whereas, Org6A only gave amplicon for *cry*2 gene (Fig. 5.35; 5.36; 5.37). The similar *cry* gene pattern obtained for the reference strain *Btk* indicates that these strains may belong to the serovar *kurstaki.*

Table 5.33: Quantification of genomic DNA of most virulent strains.

Strains	Wave length		Ratio[ж]
	$^{A}260$	$^{A}280$	
Arc03	0.024	0.12	2.0
Ab04	0.042	0.023	1.8
Org6A	0.017	0.009	1.9
Btk	0.019	0.01	1.9

[ж] The ratio lied between 1.7-2.0 indicating their purity.

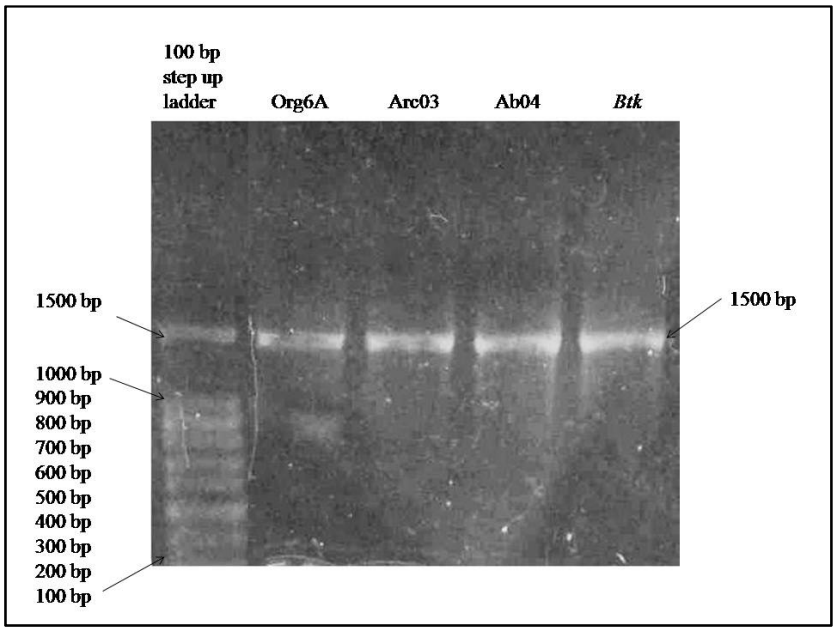

Fig. 5.34: 16S rRNA gene amplification of most virulent *Bacillus* strains (Arc03, Ab04 and Org6A) isolated from the pest species and reference strain *Btk*.

Nucleotide

GenBank

Bacillus thuringiensis strain ARC3 16S ribosomal RNA gene, partial sequence

GenBank: KX245014.1

FASTA Graphics

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  TITLE     16S rRNA partial sequence of Bacillus thuringiensis strain isolated
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GenBank

Bacillus thuringiensis strain ORG6A 16S ribosomal RNA gene, partial sequence

GenBank: KX245015.1

FASTA Graphics

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GenBank

Bacillus thuringiensis strain AB04 16S ribosomal RNA gene, partial sequence

GenBank: KX245016.1

FASTA Graphics

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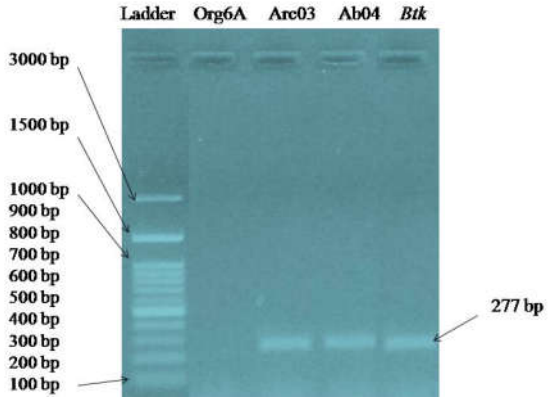

Fig. 5.35: Gene amplification of *cry* 1 of most virulent *Bacillus* strains (Arc03, Ab04, Org6A) isolated from the pest species and reference strain *Btk*.

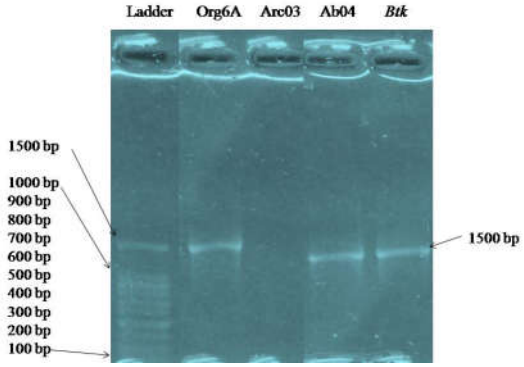

Fig. 5.36: Gene amplification of *cry* 2 of most virulent *Bacillus* strains (Arc03, Ab04, Org6A) isolated from the pest species and reference strain *Btk*.

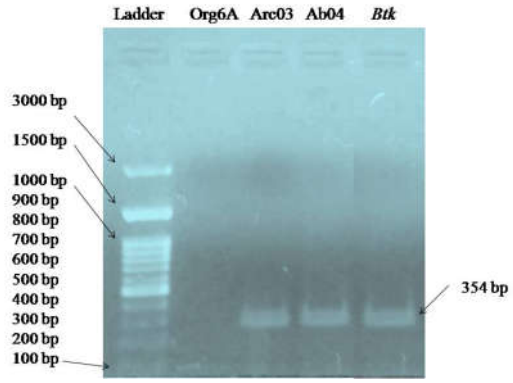

Fig. 5.37: Gene amplification of *cry* 9 of most virulent *Bacillus* strains (Arc03, Ab04, Org6A) isolated from the pest species and reference strain *Btk*.