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Phenotypic and biochemical characterization of microorganisms associated with the entomopathogenicity against *Helicoverpa armigera* and selection of best potent bacteria having insecticidal activity

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Abstract
There was a sum of four strains which were found to be entomopathogenic against *Helicoverpa armigera*. All of these bacterial strains were gram positive endospore forming strains. In the present study, the phenotypic and biochemical nature of all larvicidal strains was studied followed by a leaf based bioassay to obtain the best potent strain. As a result of this study, 4c culture was found to be best potent as 100% mortality was observed.

Keywords: *Helicoverpa armigera*, gram pod borer, bio-insecticide, bio-control of insect, microbes based insecticide

Introduction
Helicoverpa armigera is n economic important insect pest which is also known as gram pod borer. It plays a detrimental role and damage the complete chickpea crop. Chickpea is the world's third most important pulse crop. This pod borer, *Helicoverpa armigera* is highly poly phagous in nature and attacks over 182 plants species as cotton, maize, tobacco, pigeon pea, chickpea and tomato etc. [1]. So it is a harmful pest for both, widely grown and economically important crops. The yield loss in chickpea due to pod borer was reported as 10-60 per cent in normal weather conditions [2]. It attacks on the pod of the chickpea which is consumed by a huge number of public and agriculturalist face this problem as it is one of the main food crop of developing nation including India. As this damage is caused during flowering and pod formation stages so it results in substantial yield loss [3]. There are a number of chemical pesticides available to control this insect pest but chemicals are harmful for complete eco-system so there biological control agents got introduced in market among which *Bacillus thuringiensis* is the most popular insecticidal bacteria which causes toxicity due to the presence of crystal proteins. But now a day, these pod borer have obtained resistance against *Bt.* based bio-control. This work was done to study the other bacteria which have the ability to act as a bio-control and all the biochemical tests were done.

Materials and Methods
Bacterial culture showing insecticidal activity: The entomopathogenic bacteria were isolated and analysed using a castor leaves based test coated with the bacterial cultures. These leaves were fed by larvae of borer and insecticidal activity was observed. Only positive cultures were selected for future studies [4].

Media to study colony morphology and phenotypic characteristic: Nutrient agar media was used. The selected strains were subcultured on Nutrient agar plated and their morphology was observed as shown in Table 1 and fig 1.

Biochemical Analysis
Motility test, Methyl red and Voges-proskaeur tests were performed following Olutiola *et al.*, 2000 [5] and Catalase test, Indole test and Oxidase tests were performed following Cheesbrough, M., 2006 [6].

Comparative study of all e*ntomopathogenic* strains

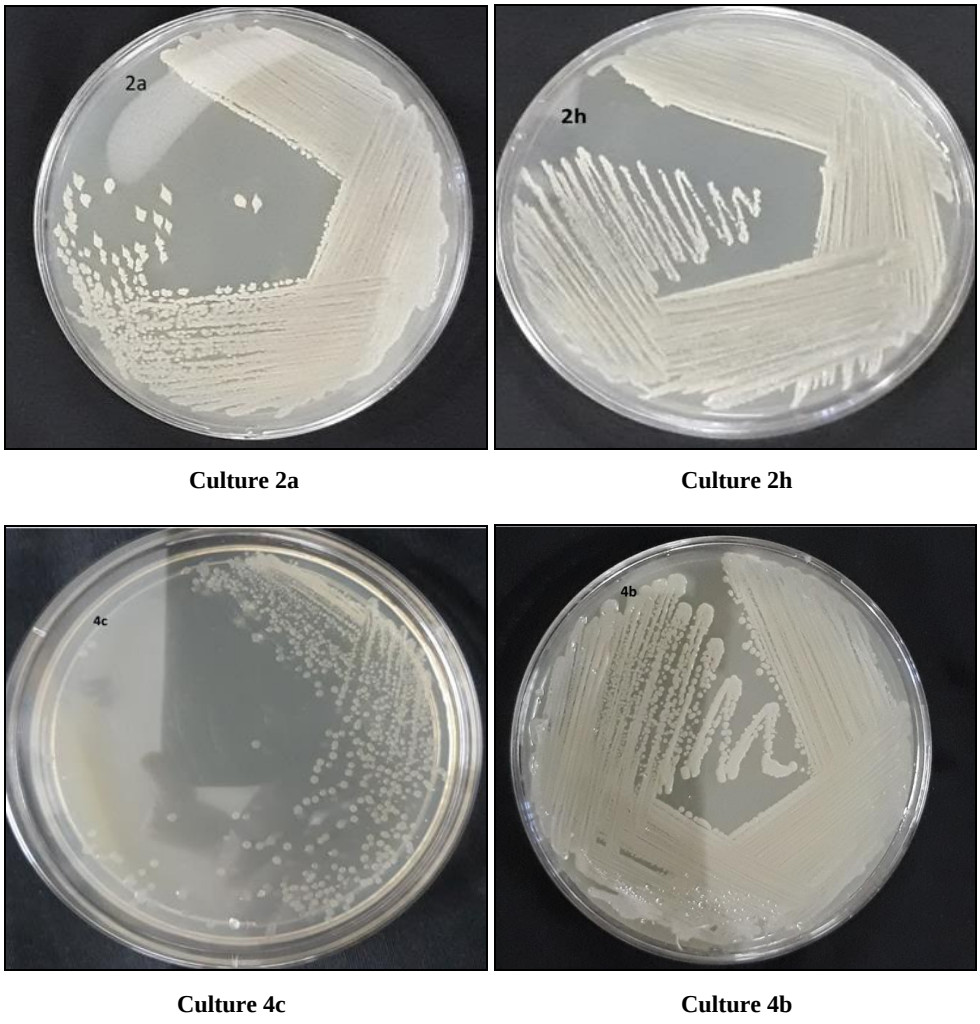
There were a total of four strains 2a (D1), 2h (D2), 4b (D3) and 4c (D4) and one known insecticidal strain of *Bacillus thuringiensis* (D5). The insecticidal test was repeated to get the most effective isolate and 4c was the most potent one as shown in figure 2. In this study water was used as a negative control (C) and showed the lowest mortality in larvae.

Results and Discussion

The phenotypic and biochemical characters of isolated strains are shown in table 1 and 2. The strains were tentatively recognized as different *Bacillus spp.* The 4c strain showed the highest mortality as given in fig. 2. *Bacillus thuringiensis* and 4c strain showed 100% mortality (fig 3 and 4).

Table 1: Phenotypic characterization of *Entomopathogenic strains*

Isolate number	Colour	Appearance	Size	Edge
D1	Cream	Dry	Small	Irregular
D2	Cream	Shiny	Small	Smooth
D3	Cream	Shiny	Small	Irregular
D4	Cream	Slimy & Shiny	Big	Smooth
D5	Cream	Slimy & Shiny	Big	Smooth



Culture 2a **Culture 2h**

Culture 4c **Culture 4b**

Fig 1: Pure cultures of selected Entomopathogenic strains

Table 2: Biochemical characterization of *Entomopathogenic strains*

Sample	Colony Morphology	Motility	Indole	Oxidase	citrate utilization	Catalase	VP test	Methyl red test
D1	S,	+ve	-ve	-ve	-ve	+ve	+ve	+ve
D2	S,	+ve	-ve	-ve	-ve	+ve	+ve	+ve
D3	S,	+ve	-ve	-ve	-ve	+ve	+ve	+ve
D4	R	+ve	-ve	-ve	-ve	+ve	+ve	+ve
D5	R	+ve	-ve	-ve	-ve	+ve	+ve	+ve

S-smooth; R-rough; +ve= positive; -ve=negative.

Four isolates were selected to be insecticidal strains. These strains were used for the next insecticidal test to select the most potent strain among all including known insecticidal strain of *Bacillus thuringiensis* and water as control. Here the test was performed on ten larvae for each strain and mortality rate was calculated at different time intervals 0 hour, 0.5 hour, 3 hours, 9 hours, 12 hours, 18 hours, 24 hours, 32 hours and 48 hours. This study is represented in table 3 (3-8). In these tables the number of larvae were represented with "L" and number of dead larvae were represented with "X". The percent mortality was calculated as follows:

% mortality = [(Number of total larvae-number of dead larvae)/Number of total larvae] X 100

1. Mortality percentage caused by 2(a) strain: The per cent mortality caused by 2a isolate is given in table 3. The initial number of larvae were 10, at time 0 the number of dead larvae was 0. As the time increased from 0 hour to 48 hours, the mortality per cent was observed from 0 to 90%. A graphical representation of per cent mortality is shown in fig 1. The graph showed mortality by 2a strain, known *Bacillus thuringiensis* strain and water. It was seen that known *Bacillus thuringiensis* was potent than 2a but after 48 hours both showed 90% mortality.

2. Mortality percentage caused by 2(h) strain: The per cent mortality caused by 2h isolate is given in table 4. The initial number of larvae were 10, at time 0 the number of dead larvae was 0. As the time increased from 0 hour to 48 hours the mortality per cent was observed from 0 to 80%. A graphical representation of per cent mortality was shown in fig 1. The graph represented mortality by 2h strain, known *Bt.* strain and water. It was seen that known *Bt.* was potent than 2h as it continuously showed more number of dead larvae as compared to 2h strain. After 48 hours *Bt.* showed 90% mortality while 2h showed 80% mortality and 30% mortality was also observed in control.

3. Mortality percentage caused by 4(b) strain: The per cent mortality caused by 4b isolate is given in table 5. The initial number of larvae were 10, at time 0 the number of dead larvae was 0. As the time increased from 0 hour to 48 hours the mortality per cent was observed from 0 to 80%. A graphical representation of per cent mortality was shown in fig 1. The graph represented mortality by 4b strain, known *Bt* strain and water. It was seen that known *Bt* was more potent than 4b as it continuously showed more number of dead larvae as compared to 4b strain. After 48 hours *Bt.* showed 90% mortality while 4b showed 80% mortality and 30% mortality was also observed in control.

4. Mortality percentage caused by 4(c) strain: The per cent mortality caused by 4b isolate is given in table 6. The initial number of larvae were 10, at time 0 the number of dead larvae was 0. As the time increased from 0 hour to 48 hours the mortality per cent was observed to be increased from 0 to 80% shown in fig 1. The graph represented mortality by 4c strain, known *Bt.* strain and water. It was seen that known *Bt.* was less potent than 4c. The isolate 4c was observed as the most potent insecticidal strain among all including known insecticidal strain of Bt. After 48 hours *Bt.* showed 90% mortality while 4b showed 100% mortality and 30% mortality was also observed in control.

A comparative graph for all four isolates, known strain of *Bacillus thuringiensis* and control has represented in fig 1. The isolate 4c strain was found as the most potent strain and showed 100% mortality at 48 hours.

Table 3: Larvicidal activity of 2a strains on larvae of *H. armigera* at different time intervals

Time (hrs)	0	0.5	3	9	12	18	24	32	48
L	10	10	9	9	7	5	4	2	1
X	0	0	1	1	3	5	6	8	9
Mortality (%)	0	0	10	10	30	50	60	80	90

Table 4: Larvicidal activity of 2h strains on larvae of *H. armigera* different time intervals

Time (hrs)	0	0.5	3	9	12	18	24	32	48
L	10	10	8	6	6	6	6	3	0
X	0	0	2	4	4	4	4	7	8
Mortality (%)	0	0	20	40	40	40	40	70	80

Table 5: Larvicidal activity of 4b strains on larvae of *H. armigera* at different time intervals

Time (hrs)	0	0.5	3	9	12	18	24	32	48
L	10	8	7	4	4	3	3	2	2
X	0	2	3	6	6	7	7	8	8
Mortality (%)	0	20	30	60	60	70	70	80	80

Table 6: Larvicidal activity of 4c strains on larvae of *H. armigera* at different time intervals

Time (hrs)	0	0.5	3	9	12	18	24	32	48
L	10	10	9	7	4	3	0	0	0
X	0	0	1	3	6	7	10	10	10
Mortality (%)	0	0	10	30	60	70	100	100	100

Table 7: Larvicidal activity of Reference strains (*Bacillus thuringiensis* MTCC strain) on larvae of *H. armigera* at different time intervals

Time (hrs)	0	0.5	3	9	12	18	24	32	48
L	10	9	9	7	7	5	4	4	1
X	0	1	1	3	3	5	6	6	9
Mortality (%)	0	0	0	30	30	50	60	60	90

Table 8: Larvicidal activity of Control (sterilized distilled water) on larvae of *H. armigera* at different time intervals

Control experiment									
Time (hrs)	0	0.5	3	9	12	18	24	32	48
L	10	10	10	10	8	8	8	7	7
X	0	0	0	0	2	2	2	3	3
Mortality (%)	0	0	0	20	20	20	20	30	30

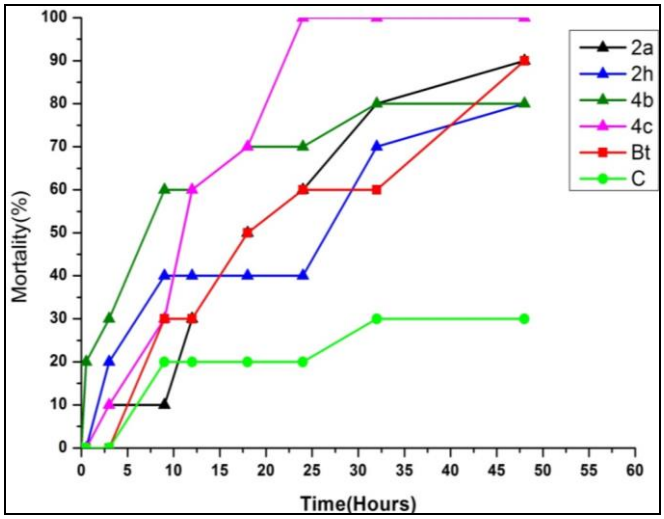


Fig 2: Comparative Graph of all isolate showing variation in mortality rate

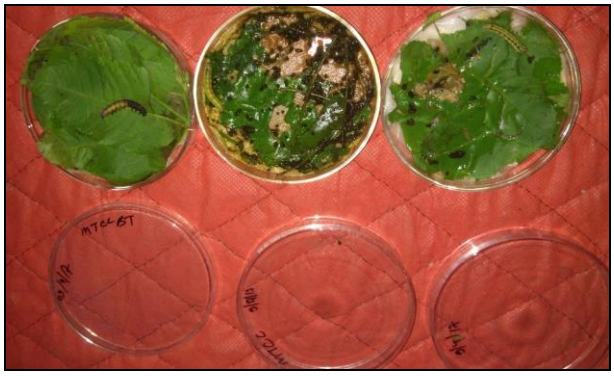


Fig 3: Mortality test by reference strain of *Bacillus thuringiensis*

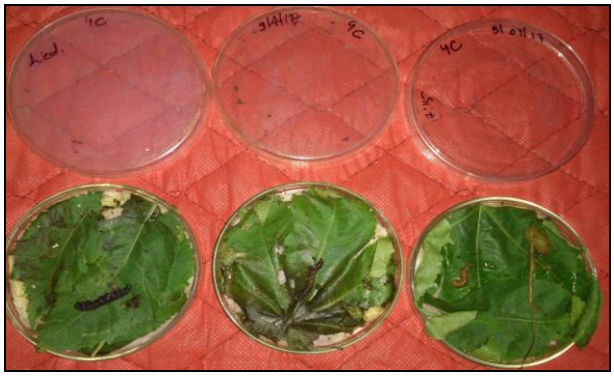


Fig 4: Mortality test by the most potent endospore forming insecticidal isolate 4c

Discussion

All the four insecticidal isolates were found gram positive, straight, thick, sporulating rods, occurring in long chains. The spores were ellipsoidal and sub terminal with round edges. The colonies were round, medium sized, elevated with irregular margins and the color was creamish white. The finding showed similar results to earlier finding by Chatterjee *et al.*, (2010), who found that the colony of the endospore forming bacterial isolate (SB1) was irregular, white, flat, undulate and gummy [7].

All the test isolates were studied for biochemical characters. In the present study, all the isolates were found negative for citrate utilization and Indole production test while production of catalase, oxidase, Voges Proskauer and Methyl red tests were positive in our study as shown in table 2. Similar findings have also been reported by Kaur *et al*. (2006) [8]. Mishra *et al*. (2016) showed that *B. thuringiensis* isolates failed to grow anaerobically and that all isolates were positive for gelatin, casein hydrolysis catalase, citrate and the VP reaction as the result of biochemical test [9].

This was a bacterial cells based mortality test and all selected insecticidal isolates showed different percentage mortality. Culture 2a showed 90 percent larval mortality of *H. armigera* as shown in table 3 and fig 2 and the known strain of *Bacillus thuringiensis* also showed 90% larval mortality (Table 7). The isolate 2a strain was as potent as known *Bacillus thuringiensis* strain while 80% mortality was observed by isolate 2h (Table 4 and Fig 2) as well as by isolate 4b (Table 5 and Fig. 2). The most potent culture 4c showed 100% mortality as the result of a leaf bioassay (Fig 2 and 4 and Table 6) and similar mortality percent was observed by Rehman *et al.*, (2002) who reported that laboratory evaluation of different *B. thuringiensis* subspecies showed on 10 [10] spore/ml concentration caused 100% mortality of larvae of the *H. armigera* during leaf bioassay [10].

Conclusion and Future Aspects

The work was to study the phenotypic and biochemical characteristics of microorganisms associated with the entomopathogenicity against *Helicoverpa armigera.* Different *Bacillus spp.* showed insecticidal activity. Following the finding, a number of insecticidal bacteria can be isolated from the environment to develop such natural biopesticides which may be new to the insect so there will not be a problem of resistance development as it was with known *Bacillus thuringiensis* based insecticides and it is safe for animals and environment so there will be no issues as it was with chemical pesticides. This type of endospore forming bacteria may lead to a new eco-friendly biopesticide.

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