World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.org/
Original Article

Microbial Diversity of Proteolytic Bacteria in the Gut of *Helicoverpa armigera*

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Received: 28-03-2015 / Revised: 21-04-2015 / Accepted: 25-04-2015

ABSTRACT

The present study describes the microbial diversity of proteolytic bacteria in the gut of *Helicoverpa armigera*. The insect was reared under laboratory conditions on chickpea (*Cicer* arietinum) and the life cycle was studied for two generations, from which healthy insects were selected for further study. The gut flora of *H.armigera* was isolated on Nutrient agar and MacConkeys agar, followed by incubation at room temperature and at 37ºC respectively for 24 to 48 h. The ability of these isolates to hydrolyse casein and gelatin was used as an indicator to screen out proteolytic organisms. Out of 24 isolates 10 isolates showed casein and gelatin hydrolysis activity. The isolates were identified by using 16 SrRNA sequencing. This study provides a basis for deciding the pest control strategies.

Keywords: Proteolytic, Chickpea, Nutrient agar, MacConkeys agar, Casein hydrolysis, 16SrRNA.

INTRODUCTION

Helicoverpa armigera (Hübner) of the Lepidoptera family is a very serious pest of many valuable crops. It is a polyphagous pest of a number of plant species, including **chickpea** (*Cicer arietinum*), pigeonpea (*Cajanus cajan*), tomato (*Lycopersicon esculentum*), okra (*Abelmoschus esculentus*), and cotton (*Gossypium* species), and is expected to become a fatal pest in other crops as well.For example sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*), maize (*Zea mays*), tobacco (*Nicotiana tabacum*) and groundnut (*Arachis hypogeal)*[1].As early instars larvae of *H. armigera* are greedy foliar feeders which later shift to the developing seeds, fruits, or bolls, leading to large reductions in yield [2].This way the pest in the country causes large financial loss per year [3].

In addition to feeding on high value crops it is an extremely dangerous pest because its production rate is extremely high; and it can migrate over a long distance [4].Therefore a thorough knowledge of life history of insect and its status as a pest can provide an important basis for developing efficient pest management strategies. In this study the ability of the isolates to carry out protein hydrolysis was established following which the organisms were identified by 16SrRNA sequencing.

MATERIALS AND METHODS

The life cycle study of *Helicoverpa armigera* was done on fresh and healthy insects obtained from the local farmers from areas adjacent of Aurangabad (MS).

Rearing of H.armigera: The insect was reared as per the procedure of [5] and vital statistics were recorded on a daily basis and continued till the death of the female.

Isolation of gut flora of Helicoverpa armigera: The healthy larva was selected from the reared organisms. The larva was cleaned with distilled water. The insect was cut open using a sterile blade. The mid-gut content was collected in 5 ml sterile saline under aseptic condition and then diluted in 1:10 under sterile conditions. The excreta was also taken and homogenized in saline. Both the samples were streaked on different media such as Nutrient Agar and MacConkeys Agar and incubated at room temperature and 37°C selectively for 24 to 48 h. Morphological and cultural characteristics of colonies that developed

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on the two mentioned media were studied. Isolates obtained on Nutrient and MacConkeys agar were purified by repeated sub culturing on fresh media. Twenty four pure cultures were selected for preliminary screening tests.

Screening for caseinase and gelatinase activity: The ability of the selected isolates to produce caseinase and gelatinase was investigated on Milk agar and Gelatin agar media respectively. The isolates were incubated at room temperature for 24 h. Diameter of casein and gelatin hydrolyzing zones produced by the test cultures were measured.

Identification of screened isolates by 16 SrRNA sequencing: Freshly cultured 24 isolates were sent to NCCS, Pune for 16SrRNA sequencing.

RESULTS

Rearing and study of Helicoverpa armigera larval lifecycle: It was found that the life cycle of first generation was completed in 61 days under laboratory conditions. Previous studies have shown that the insect complete the life cycle in 35- 45 days to maximum 56-65 days under field environmental conditions [3].

Isolation of gut flora of Helicoverpa armigera: Isolates that were obtained on Nutrient agar and MacConkeys agar were purified using standard microbiological procedures. Twenty four of such isolates were purified and selected for preliminary screening tests.

Screening test of organism to produce gelatinase and caseinase and its likely role as an inhibitor: Out of twenty four isolates ten of these were given a positive gelatinase and caseinase activity. Figure 1, 2, 3 shows the zone of clearance. The results are same for gelatinase activity. The diameter of zone of clearance was as per table 1 and 2.

DISCUSSION

The cotton, maize, chick peas, tomatoes etc are most affected plants due to the *H.armigera.* In cotton crops, blooms that have been attacked may open prematurely and stay fruitless. When the bolls are damaged, either some fall off and others will fail to produce fur or product is of poor quality. Secondary infections by fungi and bacteria are common and may lead to putrid fruits [6-7].Therefore in order to develop proper pest control strategies and to understand the host related interactions a thorough knowledge of life history of *H.armigera* was studied first under laboratory conditions. The results obtained are similar to those of [3].

The previous studies have shown that the gut micro flora plays a vital role in overall growth and development of insects [8].They also control host population which may help in controlling the pest. Similarly in plant species the proteinase inhibitors have a role in defense against pests [9-10-11].Therefore the beneficial and harmful role played by the gut flora and their possible role as inhibitor was investigated in this study.

In present study the isolation of caseinase and gelatinase producing bacteria in the gut flora of this insect is an early pointer of a secondary activity of these enzymes, i.e. these may act as inhibitors which further inactivate the protein utilizing ability of the insect and lead to its death, So the gut flora might contribute to pest control and thus prevent the proliferation of the insect.

CONCLUSION

From the present life cycle study we found that the pest developed the ability to adjust itself in the maintained laboratory conditions. When the organisms were checked for caseinase activity, then in ten cultures area surrounding the colonies showed clearing zones indicating that utilization of casein implies the gut flora contains proteolytic bacteria, which shows capacity to produce protease enzymes which result into digestion of protein (casein) in the media. From the16SrRNA sequencing we found that *Enterobacter* and *Enterococcus* the *Acinetobactor, Stenotrophomonas, B.cereus, Planococous, Arthrobactor, Bacillaceae bacterium etc,* were dominant members. This shows that microbial diversity of the midgut bacteria and their proteolytic nature was confirmed by caseinase and gelatinase activity. This study will be helpful for developing efficient pest management strategies in future.

Acknowledgements: Author is thankful to Dr. Aditi Bhattacharya, Head of Department of Microbiology and Mrs. Madhuri Sahastrabudhe, Maulana Azad College, Aurangabad for their valuable guidance and support. Author also owes her thanks to Principal, Shri Bhagwan College of Pharmacy, Aurangabad (MS), India, for providing laboratory and library facilities to carry out the research work. Author would also like to thank NCCS, Pune for providing 16SrRNA sequencing.

TABLE 1: DIAMETER OF ZONES OF CLEARANCE ON MILK AGAR MEDIUM

Sr.no	Isolate	Diameter of zone of clearance (cm)
1	1	0.6
2	3	1.0
3	5	0.9
4	7	0.4
5	10	0.8
6	11	0.5
7	12	0.3
8	16	0.6
9	17	0.7

TABLE 2: DIAMETER OF ZONES OF CLEARANCE ON GELATIN AGAR MEDIUM

Sr.no	Isolate	Diameter of zone of clearance (cm)
1	1	0.7
2	3	1.2
3	5	0.9
4	7	0.5
5	10	0.9
6	11	0.6
7	12	0.4
8	16	0.6
9	17	0.8
10	18	0.4

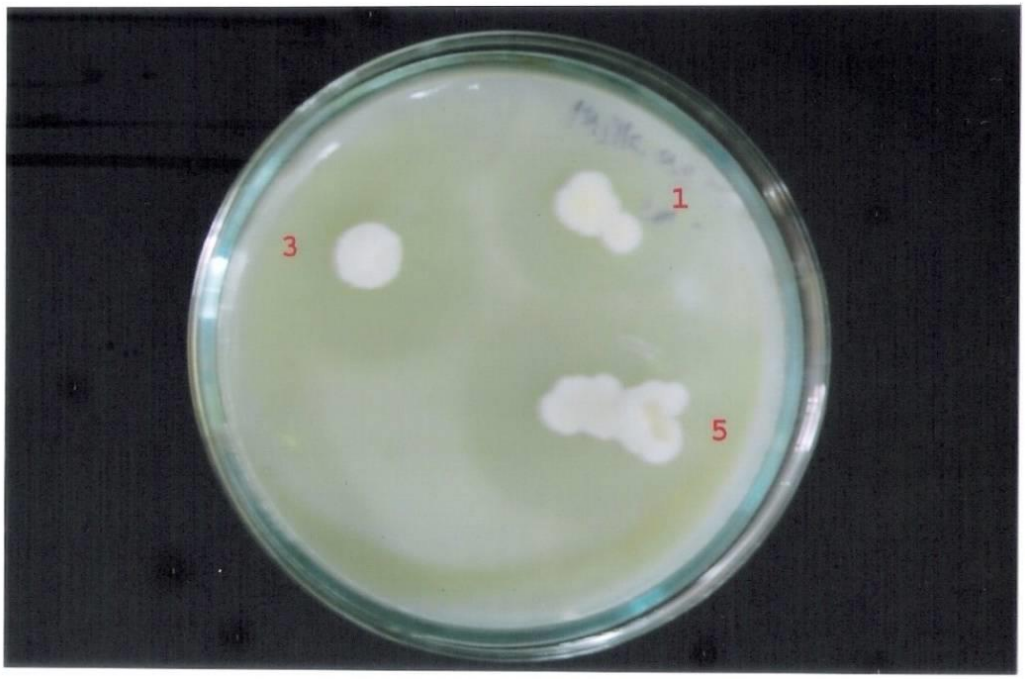

Figure 1: Caseinase activity for sample no-1, 3, and 5.

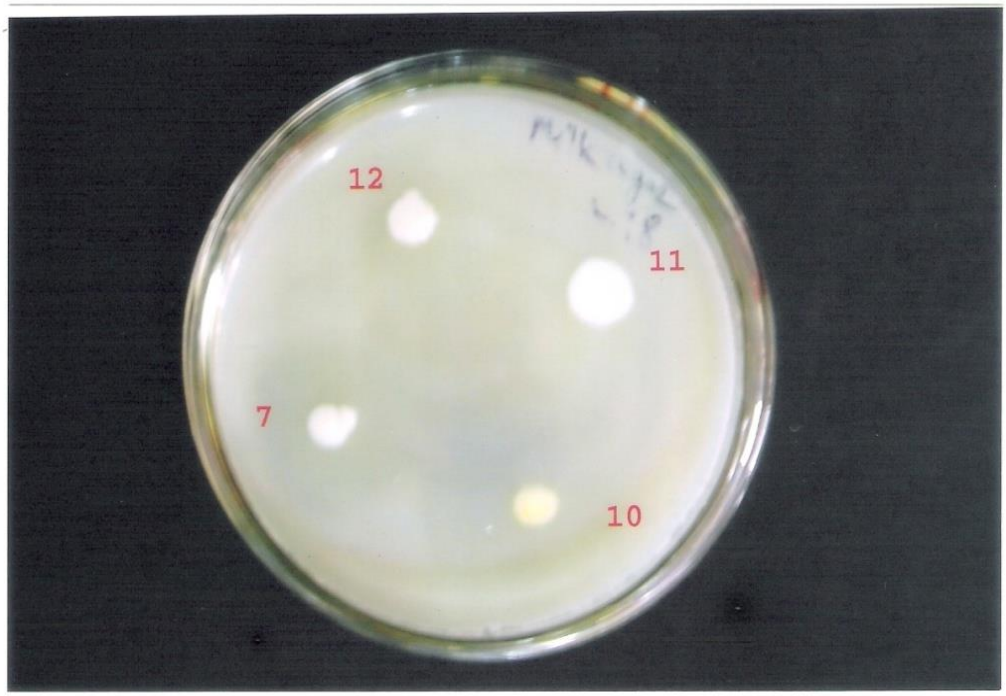

Figure 2: Caseinase activity for sample no 7, 10, 11 and 12

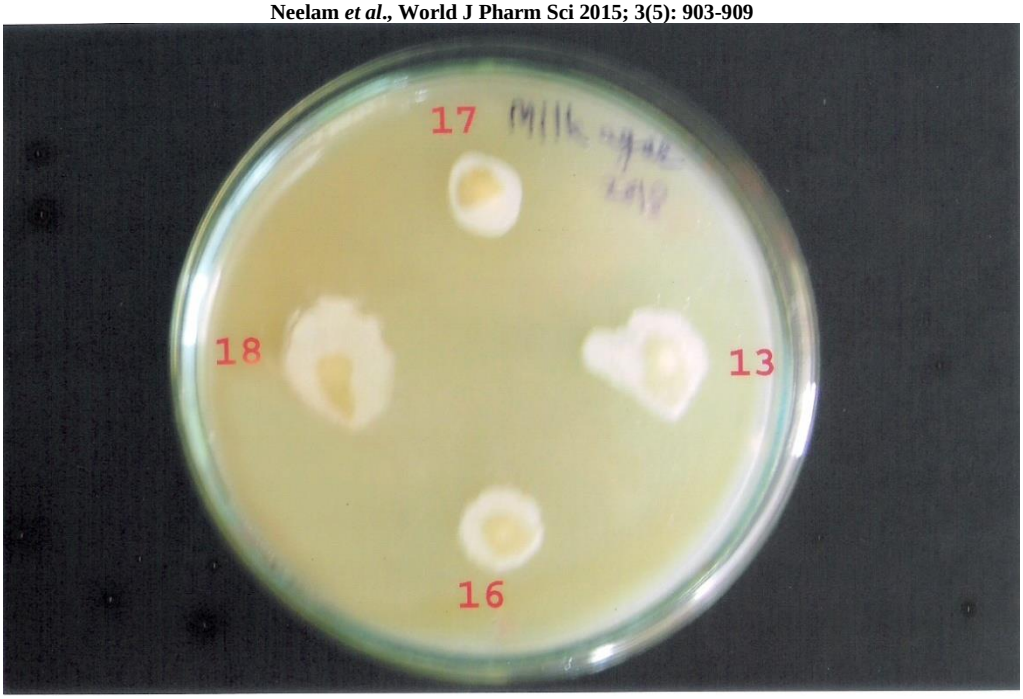

Figure 3: Caseinase activity for sample no-13, 16, 17 and 18.

16Sr RNA sequencing for isolate I:

>**Sample no. 1A_704F**

GCGAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTATTGC
TGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCAAAGCACCGCGAAAAACCTTACCAG
GTCTTGACTTCCTCTGACAACCCTAAAAATAGGGGTTCTCCTTCGGGAGCATAGTGACAGGTGGTGC
ATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACAAGCGCAACCCTGGATCT
TAGTTGCCATCATTT

Sequences producing significant alignments:

Sample no. 1

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU327888.1	Bacillus cereus strain SV1 16S ribosomal RNA gene, partial sequence	695	695	100%	0.0	97%

Sample no. 3

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF508128.1	Arthrobacter sp. XZ-3 16S ribosomal RNA gene, complete sequence	418	418	99%	1e-113	80%

Sample no. 5

Accession	**Description**	**Max score**	**Total score**	**Query coverage**	**E value**	**Max ident**
AM292318.1	Bacillus thuringiensis 16S rRNA gene, clone pAC	1402	1402	100%	0.0	98%

Sample no.7

Accession	**Description**	**Max score**	**Total score**	**Query coverage**	**E value**	**Max ident**
EU022688.1	Acinetobacter calcoaceticus strain YLZZ-1 16S ribosomal RNA gene, partial sequence	1389	1389	100%	0.0	99%

Sample no. 10

Accession	**Description**	**Max score**	**Total score**	**Query coverage**	**E value**	**Max ident**
AB194325.1	Stenotrophomonas maltophilia gene for 16S rRNA, partial sequence, strain:BL-15	939	939	100%	0.0	93%

Sample no. 11

Accession	**Description**	**Max score**	**Total score**	**Query coverage**	**E value**	**Max ident**
DQ520828.1	Bacillaceae bacterium NR184 16S ribosomal RNA gene, partial sequence	449	449	100%	4e-123	93%

Sample no 12

Accession	**Description**	**Max score**	**Total score**	**Query coverage**	**E value**	**Max ident**
EU339930.1	Arthrobacter sp. W1 16S ribosomal RNA gene, partial sequence	1386	1386	100%	0.0	100%

Sample no 16

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU221350.1	Acinetobacter baumannii strain N2S4 16S ribosomal RNA gene, partial sequence	1373	1373	100%	0.0	99%

Sample no. 17

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AY582938.1	Planococcus sp. D36 16S ribosomal RNA gene, partial sequence	1351	1351	100%	0.0	99%

Sample no. 18

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF522130.1	Acinetobacter sp. CU27 16S ribosomal RNA gene, partial sequence	1410	1410	99%	0.0	99%

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