DIVERSITY OF CULTURABLE ENDOSYMBIONTS IN DIAMONDBACK MOTH (*Plutella xylostella*)

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DEPARTMENT OF AGRICULTURAL MICROBIOLOGY

UNIVERSITY OF AGRICULTURAL SCIENCES

BENGALURU – 560 065

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Thesis submitted to the

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In partial fulfilment of the requirements

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in

AGRICULTURAL MICROBIOLOGY

BENGALURU JULY, 2017

DEDICATED TO MY LOVING FAMILY AND FRIENDS

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BENGALURU – 560 065

CERTIFICATE

This is to certify that the thesis entitled "**DIVERSITY OF CULTURABLE ENDOSYMBIONTS IN DIAMONDBACK MOTH (*Plutella xylostella*)**" submitted by **Mr. VIJAYAKUMAR WAGHMARE ID. No. PALB 5168** for the award of the degree of **MASTER OF SCIENCE (Agriculture)** in **AGRICULTURAL MICROBIOLOGY** to the University of Agricultural Sciences, GKVK, Bengaluru-560 065. This is a *bona-fide* record of research work done by him during the period of his study in this University, under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associate-ship, fellowship or other similar titles.

Bengaluru
July, 2017

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Bengaluru
July, 2017 **(VIJAYKUMAR WAGHMARE)**

DIVERSITY OF CULTURABLE ENDOSYMBIONTS IN DIAMONDBACK MOTH (*Plutella xylostella*)

VIJAYKUMAR WAGHMARE

ABSTRACT

Diamondback moth, *Plutella xylostella* is a major pest of cruciferous crops worldwide and it has developed resistance to almost all synthetic and microbial insecticides. Diamondback moth was known to harbour microorganisms which play important role in growth and development of the host. In the present study bacterial strains were isolated from third instar larvae of *P. xylostella* collected from three different locations of the state Karnataka. Morphological characters of all isolated bacterial strains were studied. Biochemical characters including catalase and IMVIC reactions were done. Total bacterial genomic DNA was extracted from third instar larvae and amplified using PCR with 16S rRNA primers. Twenty different bacterial strains were identified in total. The partial 16S rRNA sequences from *P. xylostella* revealed the presence of *Serratia* spp. which were more predominant in Sugatur, Kolar population, *Bacillus* and *Enterococcus* spp. in Rattihalli, Haveri population and *Proteus* spp. in Malligere, Shivamogga population. The 16S rRNA sequence results also revealed the microflora varied from three different locations across the Karnataka. These Studies suggested that a combination of molecular and traditional culturing methods can be effectively used to analyze and to determine the diversity of gut microflora. These bacterial strains may play important roles in growth and development of *P. xylostella.*

July, 2017

Department of Agril. Microbiology
UAS, GKVK, Bengaluru.

Dr. R. MUTHURAJU
Major Advisor

ವಜ್ರಬೆನ್ನಿನ ಪತಂಗದಲ್ಲಿನ ಬೆಳಸಬಹುದಾದಂತ ಎಂಡೊಸಿಂಬೊಯೆಂಟ್ಸ್‌ಗಳ ವೈವಿಧ್ಯತೆಯ ಬಗ್ಗೆ ಅಧ್ಯಯನ

ವಿಜಯಕುಮಾರ ವಾಘಮಾರೆ

ಸಾರಾಂಶ

ವಜ್ರಬೆನ್ನಿನ ಪತಂಗ (ಪ್ಲುಟೆಲ್ಲಾ ಕ್ಸೈಲೊಸ್ಟೆಲ್ಲಾ)ವು ವಿಶ್ವದಾದ್ಯಂತ ಕೋಸು ಜಾತಿಯ ಬೆಳೆಗಳ ಪ್ರಮುಖ ಕೀಟವಾಗಿರುತ್ತದೆ. ಈ ಕೀಟವು ಎಲ್ಲಾ ಬಗೆಯ ರಾಸಾಯನಿಕ ಮತ್ತು ಸೂಕ್ಷ್ಮಜೀವಿಯ ಕೀಟನಾಶಕಗಳಲ್ಲಿ ನಿರೋಧಕ ಶಕ್ತಿಯನ್ನು ಹೊಂದಿದೆ. ವಜ್ರಬೆನ್ನಿನ ಪತಂಗದ ದೇಹದ ಒಳಭಾಗದಲ್ಲಿ ಸಾಕಷ್ಟು ಸೂಕ್ಷ್ಮಜೀವಿಗಳನ್ನು ಹೊಂದಿರುತ್ತವೆ, ಈ ಜೀವಿಗಳು ಕೀಟದ ಬೆಳವಣಿಗೆ ಮತ್ತು ಅಭಿವೃದ್ಧಿಯಲ್ಲಿ ಪಾತ್ರವನ್ನು ವಹಿಸಿವೆ. ಪ್ರಸ್ತುತ ಅಧ್ಯಯನದಲ್ಲಿ ಮೂರನೇ ಹಂತದ ಮರಿಹುಳುಗಳನ್ನು ರಾಜ್ಯದ ಆಯ್ದ ಮೂರು ಜಿಲ್ಲೆಗಳಿಂದ ಸಂಗ್ರಹಿಸಿ, ಅವುಗಳ ದೇಹದಲ್ಲಿರುವ ದುಂಡಾಣುಗಳನ್ನು ವಿವಿಧ ರಾಸಾಯನಿಕಗಳಿಂದ ತಯಾರಿಸಲಾದ ಮಾಧ್ಯಮಗಳ ಮೇಲೆ ಬಳಸಿ ನಂತರ ಅವುಗಳ ಮೇಲ್ಮೈ ಲಕ್ಷಣಗಳ ಬಗ್ಗೆ ಅಭ್ಯಸಿಸಲಾಯಿತು. ಜೀವರಾಸಾಯನಿಕ ಪರೀಕ್ಷೆಗಳಾದ ಕೆಟಲೆಸ್ ಮತ್ತು ಇಮಿವಿಕ್ ಪ್ರತಿಕ್ರಿಯೆಗಳನ್ನು ಕೈಗೊಳ್ಳಲಾಯಿತು. ದುಂಡಾಣುಗಳ ಜಿನೋಮಿಕ್ ಡಿಎನ್‌ಎ ಯನ್ನು ಮೂರನೇ ಹಂತದ ಮರಿಹುಳುವಿನಿಂದ ಪ್ರತ್ಯೇಕಿಸಿ ೧೬ಎಸ್ ಆರ್.ಆರ್.ಎನ್.ಎ ಪ್ರೈಮರ್‌ನ್ನು ಬಳಸಿ ಪಿಸಿಆರ್ ಯಂತ್ರ ಉಪಯೋಗಿಸಿಕೊಂಡು ಇಪ್ಪತ್ತು ದುಂಡಾಣುಗಳನ್ನು ಗುರುತಿಸಲಾಯಿತು. ಪ್ಲುಟೆಲ್ಲಾ ಕ್ಸೈಲೊಸ್ಟೆಲ್ಲಾದ ಭಾಗಶಃ ೧೬ಎಸ್ ಆರ್.ಆರ್.ಎನ್.ಎ ಅನುಕ್ರಮಗಳು ಸೆರೆಷಿಯಾ ಜಾತಿಯ ದುಂಡಾಣುಗಳು ಸುಗಟೂರು, ಕೋಲಾರ ಜಿಲ್ಲೆಯ ಹುಳುಗಳಲ್ಲಿ, ಬ್ಯಾಸಿಲಸ್ ಮತ್ತು ಎಂಟೆರೊಕೊಕಸ್ ಜಾತಿಯ ದುಂಡಾಣುಗಳು ರಟ್ಟಿಹಳ್ಳಿ, ಹಾವೇರಿ ಜಿಲ್ಲೆಯ ಹುಳುಗಳಲ್ಲಿ ಹಾಗೂ ಪ್ರೊಟಿಯಸ್ ಜಾತಿಯ ದುಂಡಾಣುಗಳು ಮಲ್ಲಿಗೆರೆ, ಶಿವಮೊಗ್ಗ ಜಿಲ್ಲೆಯ ಹುಳುಗಳಲ್ಲಿರುವುದನ್ನು ಕಂಡುಬಂದವು. ೧೬ಎಸ್ ಆರ್.ಆರ್.ಎನ್.ಎ ಅನುಕ್ರಮದ ಫಲಿತಾಂಶಗಳಲ್ಲಿ ಕಂಡುಬಂದ ಅಂಶವೇನೆಂದರೆ ಮೂರು ವಿಭಿನ್ನ ಸ್ಥಳಗಳ ಹುಳುಗಳಲ್ಲಿ ವಿಭಿನ್ನ ಜಾತಿಯ ಬೇರೆ ಬೇರೆ ದುಂಡಾಣುಗಳು ಕಂಡುಬಂದವು. ಮೇಲ್ಕಂಡ ದುಂಡಾಣುಗಳು ವಜ್ರಬೆನ್ನಿನ ಪತಂಗದ ಬೆಳವಣಿಗೆ ಮತ್ತು ಅಭಿವೃದ್ಧಿಯಲ್ಲಿ ಪ್ರಮುಖ ಪಾತ್ರ ವಹಿಸುತ್ತವೆ. ಆಣ್ವಿಕ ಮತ್ತು ಸಾಂಪ್ರದಾಯಿಕ ವಿಧಾನಗಳ ಸಂಯೋಜನೆಯ ಕೀಟದ ಕರುಳಿನ ಸೂಕ್ಷ್ಮಜೀವಿವರ್ಗದ ವೈವಿಧ್ಯತೆಯನ್ನು ವಿಶ್ಲೇಷಿಸಲು ಮತ್ತು ನಿರ್ಧರಿಸಲು ಪರಿಣಾಮಕಾರಿಯಾಗಿ ಬಳಸಬಹುದೆಂದು ಈ ಅಧ್ಯಯನದಿಂದ ಧೃಡಪಟ್ಟಿದೆ.

ಜುಲೈ, ೨೦೧೭
ಸೂಕ್ಷ್ಮ ಜೀವಿಶಾಸ್ತ್ರ ವಿಭಾಗ
ಗಾ.ಕೃ.ವಿ.ಕೇ., ಬೆಂಗಳೂರು–೬೫

ಡಾ. ಆರ್. ಮುತ್ತುರಾಜು
(ಮುಖ್ಯ ಸಲಹೆಗಾರರು)

STUDY OF CULTURABLE ENDOSYMBIONTS DIVERSITY IN DIAMONDBACK MOTH, *Plutella xylostella* (Lepidoptera; plutellidae)

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INTRODUCTION:

Diamondback moth, *Plutella xylostella* (L.) is one of the most destructive insect pest of cruciferous crops world wide, infesting cabbage, cauliflower and broccoli etc. It is the most important pest of cabbage and other brassicas worldwide costing upto US $1 billion per year in damage and control costs.

It has become more resistant to each new class of insecticide because of intensive and repeated use of insecticides. Insect endosymbionts can help to produce nutrients that do not exist in the food. Both herbivore insects and symbiotic microbes can secrete celluloytic enzymes for biomass destruction and hydrolysis.

Detoxification by symbionts as a mechanism of resistance depends on the ability of the microbes to degrade the compound and rapidly evolve. Microbes play in metabolism, vitamin synthesis, pheromone production, pathogen prevention and pesticide degradation, the symbiotic relationship within the gut is far from being fully understood

With the development of molecular biology, 16S rRNA gene sequence for microbial identification of close relationships at the genus and species level has been the most universal method.

OBJECTIVES:

1. Isolation of endosymbionts from Diamondback moth, ***Plutella xylostella***.
2. Characterization of isolated endosymbionts from Diamondback moth, ***Plutella xylostella***.

MATERIALS AND METHODS:

1. Isolation of endosymbionts from Diamondback moth, *Plutella xylostella*

Larvae of Diamondback moth (DBM) were collected from the field. The endosymbionts were isolated from starved third instar larvae of DBM by serial dilution and plating method using Nutrient agar (NA), Luria bertani (LB), Potato dextrose agar (PDA) and Yeast extract peptone dextrose agar (YEPDA). The pure culture was made and preserved for further studies.

2. Characterization of isolated endosymbionts from Diamondback moth, *Plutella xylostella*.

Isolated strains were examined for the colony morphology, cell shape and Gram reaction as per the standard methods.

The biochemical tests like Indole production test, Methyl red test, Voger - Proskauer test, Citrate utilization test and Catalase test were carried out by standard procedures.

The molecular characterization, DNA isolation, gel electrophoresis and PCR were done according to standard protocols and finally PCR products were sent for sequencing for the identification of unknown isolated strains.

RESULTS

First Population: COLLEGE OF SERICULTURE, CHINTAMANI. DISTRICT: KOLAR

Sl.no.	Isolates	Morphological and Biochemical characterization of isolated endosymbiont strains from larvae of Diamondback moth							
		Morphological characters			Biochemical test				
		Colour	Cell shape	Gram stain	1	2	3	4	5
	A) NA								
1	10^{-3} R_3, I_1	Dark yellow	Cocci	- ve	+	+	-	-	-
2	10^{-3}, R_3, I_3	Yellow	Cocci	+ ve	+	+	-	-	-
3	10^{-5}, R_1, I_2	White	Cocci	+ ve	-	-	+	+	-
4	10^{-3}, R_3, I_2	Light yellow	Cocci	- ve	-	-	-	-	-
5	10^{-3}, R_3, I_4	White	Coccobacilli	- ve	-	+	+	+	-
6	10^{-3}, R_2, I_5	White	Bacilli	+ ve	-	+	-	-	-
	B) LB								
7	10^{-3}, R_3, I_1	Milky white	Cocci	- ve	-	+	-	+	-
8	10^{-3}, R_3, I_2	Creamy dark yellow	Coccobacilli	- ve	+	-	-	-	-
	C) YEPDA								
9	10^{-3}, R_2, I_1	White	Rod	- ve	-	+	+	+	+
10	10^{-3}, R_2, I_2	Creamy, yellow	Coccobacilli	- ve	+	-	-	+	-

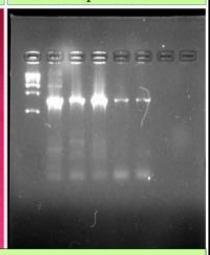
1. Indole production test, 2. Methyl red test, 3. Voges Proskauer test, 4. Citrate utilization test, 5. Catalase test, + : Positive, - : Negative.

Identification of isolated strains after sequencing

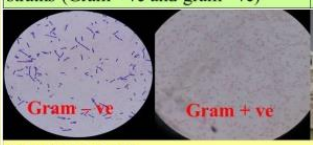
Sl.no.	Isolates	Organism
1	NA, 10^{-3}, R_3, I_1	*Serratia marcescens, Pseudomonos fluorescens*
2	NA, 10^{-3}, R_3, I_4	*Serratia marcescens, Gamma proteobacterium*
3	NA, 10^{-3}, R_2, I_5	*Bacillus cereus, B. thuringiensis, Bacillus* sp.
4	LB, 10^{-3}, R_3, I_1	*Serratia* sp., *Enterobacter* sp.
5	YEPDA, 10^{-3}, R_2, I_1	*Serratia marcescens, Enterobacter* sp.

Agar plate containing Colonies

Agarose gel electrophoresis



Morphological features of isolated strains (Gram +ve and gram –ve)



Biochemical tests

DISCUSSION:

There were 10 culturable strains which were isolated from third instar larvae of DBM collected from College of Sericulture, Chintamani.

Serratia marcescens, Bacillus cereus, Bacillus thuringiensis, Enterobacter sp. and *Pseudomonas* sp. were found as major bacterial isolates from third instar larvae of DBM.

Bacillus sp. and *Pseudomonas* sp. have been reported in gut of the *Discladispa armigera* (Olivier). Presence of *Serratia* sp., *Enterobacter* sp. and *Bacillus* sp. was reported in larval gut of *Helicoverpa armigera.* Therefore *Serratia* sp., and *Bacillus* sp. Were commonly found in various insects.

SUMMARY:

An attempt was made to identify different microbes from third instar larvae of Diamondback moth. 16s rRNA sequencing results revealed that the isolated strains belonged to the *Serratia marcescens, Bacillus cereus, Bacillus thuringiensis, Enterobacter* sp. and *Pseudomonas* sp. The microflora varied within species and also among different species. The identified microbes might be helpful as endosymbionts for providing defence against various biotic and abiotic stresses and act as antagonistic effect.

ADVISORY COMMITTEE

Chairperson:- Dr. R. Muthuraju
Members:- Dr. G. P. Brahmaprakash
Dr. K. M. Harinikumar
Dr. K. Muralimohan

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I INTRODUCTION

Diamondback moth, *Plutella xylostella* is the most important pest of the brassica, cabbage, cauliflower, radish, knol-khol, turnip, mustard and amaranthus in many parts of the world. DBM is globally distributed and found in Europe, Asia, Africa, America, Australia, New Zealand and the Hawaiian Islands. Geographically it might originate in Europe or Mediterranean region (Talekar and Shelton, 1993). *P. xylostella* was first observed in North America in 1854, in Illinois, but quickly spread across the continent. *P. xylostella* larvae feed only on cruciferous crops which contain mustard oil and glucosides (Hillyer and Thorsteinson, 1969).

In India, DBM is the major, ubiquitous and year round insect pest hindering the economic production of brassica crops (Sandur, 2004). The developing resistance and decline of insecticide efficiency against DBM become a limiting factor in cultivation of commercial crops like cabbage and cauliflower in India. An outbreak of DBM on cauliflower was reported in Aligarh during September to October 2006. The infestation increased gradually from first fortnight of August and led to total loss of the crop. Severity of this pest can increase in many regions of the country due to climatic changes (Dhaliwal *et al*., 2010).

The first report of DBM resistance to insecticides (DDT and parathion) in India was made by Varma and Sandhu (1968) in Ludhiana (Punjab) and it was confirmed by Deshmukh and Saramma (1973). Insecticide resistance and DBM control failures are common in states like Karnataka, Uttar Pradesh and Punjab. High level of DBM resistance to endosulfan was reported in Dharwad (81.75%), Belgaum (83.90%), Haveri (83.90%) and from Bidar (81.43%) in Karnataka by Vastrad *et al.* (2002). After the introduction of Bt in 1980s, it was used widely for control of DBM and the initial field resistance to Bt was reported in Philippines by Tabashnik *et al*. (1990).

Insect system harbors a wide range of microbial community. Microorganisms play an important and essential role in the growth and development of insects. Microbial flora of insect guts appears to be derived from diet upon which the insect feed (Hunt and Charnley, 1981). Inscets, like all other animals, live in a microbial world.

Microorganisms are everywhere, and insects are colonized by a wide diversity of microbes, resulting in many transient and some persistant relationships. Insect system also possesses permanent microorganisms which supply essential nutrients to their host and some possesses obligate microbial endosymbionts that benefit the insects (Bridges, 1981).

A largely untapped resource that may be used in pest management is the manipulation of microorganisms that live in symbiosis with insects (Rousk and bengtson, 2014). Microbial symbionts provide an diverse range of benefits in insect nutrition, e.g. by providing essential amino acids, digestive enzymes or vitamins (Brune, 2014).

The field that has received less attention is the roles that microbes play in protecting insects from toxic plant compounds and insecticides. This is despite the fact that it is known that many microorganisms contain enzymatic degradation mechanisms for a variety of plant secondary metabolites such as terpenes (Marrmulla and Harder, 2014), nicotine and cocaine (Brandsch, 2006) and even phosphorus- or sulfer-containing insecticides (Kertesz *et al.,* 1994). Oftentimes the interaction between microbe and insect are difficult to disentangle, and the relative contribution of insect versus microbial defence mechanism is not yet known. The molecular characterization and identification techniques have improved the analysis of diverse microbial populations (Muyzer *et al.*, 1993).

Studies on lepidopteron gut microbiota suggested that microorganisms provide essential nutrients and play a role in host digestion (Broderick *et al.*, 2004). Gayathri Priya *et al*. (2012) isolated and identified *Bacillus niabense, Paenibacillus jamilae, Cellulomonas variformis, Acinetobacter schidleri, Micrococcus yunnanesis, Enterobacter* sp and *Enterococcus cassilifavus* from *Helicoverpa armigera.* Ramesh *et al.* (2009) characterized gram negative microbes *Escherichia coli, Yersinia enterocolitica, Klebsiella, Pneumonia* sp. from the gut of silk worm. Madhusudan *et al.* (2011) isolated *Stenotrophomonas* sp., *Enterococcus casseliflavus, Enterococcus* sp., *Enterococcus gallinarum, Brevundimonas diminuta, Enterococcus faecium, Staphylococcus* sp., *Pseudomonas aeruginosa, Acinetobacter calcoaceticus, Bacillus subtilis, Rhodococcus*

sp. from the gut of field collected *H. armigera* larvae. The production of chitinase by gut bacteria from DBM appeared to contribute to host nutrition (Indiragandhi *et al.*, 2007).

Several molecular techniques have been developed to identify microbiota associated with the DBM. Methods based on the amplification of fragment coding for 16S rRNA have emerged as a powerful tool (Holben, 2002). The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria, but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level. Universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene and at either the 540-bp region or at the end of the whole sequence (about the 1550-bp region), and the sequence of the variable region in between is used for the comparative taxonomy (Chen *et al.,* 1989). Although 500 and 1500 bp is common lengths to sequence and compare, sequences in databases can be of various lengths. The 16S rRNA gene sequence has been determined for a large number of strains. GenBank, the largest databank of nucleotide sequences, has over 20 million deposited sequences, of which over 90,000 are of 16S rRNA gene to compare the sequence of an unknown strain.

The present study concentrates on the isolation of endosymbionts from *P. xylostella* that can be used to explore the link between host behavior and the microbiome by investigating microbial community variation within species and hypothesized that microbiomes within a DBM vary from location to location and farmer plant protection measured on endosymbionts.

The following objectives are framed to isolate, characterize, cultivate, preserve, phenological and genotypically identify endosybionts associated to *P. xylostella* in order to improve the understanding of this microbial environment.

Objectives:

1. Isolation of endosymbionts from Diamondback moth (*Plutella xylostella*)
2. Characterization of isolated endosymbionts from Diamondback moth (*Plutella xylostella*).

II REVIEW OF LITERATURE

The literature relevant to the present study pertaining to aspects of collection of Diamondback moth, *Plutella xylostella* from different locations of Karnataka, isolation, identification and characterization of endosymbionts isolated from Diamondback moth, are given under the following headings.

2.1 Diamondback moth, *Plutella xylostella*

Diamondback moth, *Plutella xylostella* is distributed in all parts of the world where crucifers are grown. *P. xylostella* feeds upon all cruciferous plants, cole crops and several greenhouse plants and causing 50-80 % annual loss in marketable yield (Devjani and Singh, 1999). In India, estimated loss is about $16 million annually in a cultivated area of 0.5 million ha (Mohan and Gujar, 2003). It thrives under extremely varied agro climatic conditions prevailing in India and it reproduces year round and completes 13-14 generations. The DBM has the ability to multiply rapidly in the favorable tropical climates due to its high reproductive capacity and wide host range and to develop resistance against an array of insecticides (Shelton *et al*., 2000).

Outbreaks of *P. xylostella* have occurred frequently in various parts of the world that resulted in severe losses and the cost of control is about $ 1 billion (Talekar and Shelton, 1993). In September, 2006 outbreak of DBM has occurred on cauliflower in peri urban area of Aligarh district (U.P.), India, where the farmers plough down their field without harvest.

2.2 Endosymbionts in insects

Endosymbionts play a major role in various aspects within its host. Symbiotic relation of microorganisms with its host are one of those adoptive methods to overcome various problems such as poor nutrition, lack of amino acids, poor immunity and also plant secondary metabolites (Douglas, 2011). There are lot of scientific studies conducted on various sucking pests, confirm the involvement of symbiotic bacteria enhancing biotic fitness of their host insects (Brownlie and Johnson, 2009). Indigenous gut microbes of lepidopteran and other insects have been found to detoxify harmful secondary

metabolites (Morrison *et al.,* 2009) and to protect the host against the colonization of pathogens (Dillon and Dillon, 2004). Recent study by Kikuchi *et al.* (2012) showed the involvement of secondary microbial symbionts in the insecticide resistance attributes. Among various secondary endosymbionts, bacteria are main microbes, which are generally soil bacteria and able to establish a symbiotic relation with insects hosts by providing some benefits to their hosts and in return get the protection out of their hosts.

Xiang *et al*. (2006) compared the bacterial communities in the larval midgut of field and laboratory populations of cotton bollworm (*Helicoverpa armigera*), using denaturing gradient gel electrophoresis (DGGE) of amplified 16S rDNA sequences and 16S library sequence analysis. They observed that more diverse microbial community in caterpillars obtained from field and for the field population, phylotypes belonging to the *Enterococcus* (28%), *Acenitobacter* (19%), *Lactococcus* (11%), *Flavobacterium* (10%) and *Stenotrophomonas* (10%) were dominant members. They suggested that the environment and food supply might influence the diversity of gut bacterial community.

Jennifer and Marjorie (2008) conducted a microbial survey of fruit piercing and blood feeding moth *Calyptra thalictri* borkhausen (lepidoptera: noctuidae) by using polymerase chain reaction (PCR) primers for 16S rRNA sequences for eubacteria, and primers for archaea, fungi including yeast-like organisms, *microsporidia*, and *wolbachia.* High-fidelity PCR and subsequent DNA analyses indicated that at least five microorganisms belonging to the α-, β-, and γ-proteobacteria were present. No archaea, fungi including yeast-like organisms, *microsporidia,* or *wolbachia* were detected.

Anand *et al.* (2009) obtained eleven bacterial isolates from the digestive tract of *B. mori*, including the Gram positive *Bacillus circulans* and Gram negative *Proteus vulgaris, Klebsiella pneumoniae, Escherichia coli, Citrobacter freundii, Serratia liquefaciens, Enterobacter* sp., *Pseudomonas fluorescens, P. aeruginosa, Aeromonas* sp. and *Erwinia* sp., *Aeromonas* sp. was able to utilize the CMcellulose and xylan. *S. liquefaciens* was able to utilize three polysaccharides including CMcellulose, xylan and pectin. *B. circulans* was able to utilize all four polysaccharides with different efficacy. The gut of *B. mori* has an alkaline pH and all of the isolated bacterial strains were found

to grow and degrade polysaccharides at alkaline pH. The number of cellulolytic bacteria increases with each instar

Kikuchi (2009) focused on diversity of bacterial endosymbionts in insects and several model system with culturable endosymbionts, which provide a new perspective towards understanding how intimate symbiotic associations may have evolved and how they are maintained insects. They observed endosymbiotic associations *in vivo* through culture- independent molecular techniques, such as quatitative PCR, molecular phylogeny and *in situ* hybridization, as well as genomic and metagenomic analysis.

Visotto *et al.* (2009) obtained twelve aerobic and anaerobic proteolytic bacteria from the gut of velvetbean catterpilller, *Anticarsia gemmatalis* in calcium caseinate agar. The isolated bacteria were divided into five distinct groups, according to their polymerase chain reaction-restriction fragment-length polymorphism profiles and that bacteria were identified as *Bacillus subtilis, Bacillus cereus, Enterococcus gallinarum, Enterococcus mundtii,* and *Staphylococcus xylosus.* They assessed bacterial proteolytic activity through in vitro colorimetric assays for (general) proteases, serine proteases, and cysteine proteases and they observed that the isolated bacteria were able of hydrolyzing all tested substrates, except *Staphylococcus xylosus*, which did not exhibit serine protease activity. They concluded that gut proteases from velvetbean caterpillar are not exclusively secreted by the insect cells but also by their symbiotic gut bacteria.

Shi *et al.* (2010) applied various molecular biology techniques to insect gut symbiont analysis. This aimed to serve as an informative resource for research strategy development in the field and covered traditional molecular techniques like DGGE, RFLP and FISH with respect to how they are applied to study the composition, diversity and dynamics of insect gut symbiotic microbiota, then focused on the various 'omics' techniques. They analysed metagenomic sequencing together with recent adavancement in next generation sequencing that provide enormous sequencing information, allowing in-depth microbial diversity analysis and modeling of pathways for biological processes such as biomass degradation. The integration of different 'omics' level data allow to understand how insect gut works as a system to carry out its function.

Madhusudhan *et al.* (2011) identified culturable bacterial species isolated from gut of wild and laboratory larvae of *Helicoverpa armigera* by sequence analysis of 16S rRNA gene. Eleven bacterial species of different genera were identified from wild populations, in which *Enterococcus* were found to be predominant and six bacterial species of different genera from laboratory populations. The nucleotide sequences of 11 isolates from wild and 6 isolates from laboratory populations were submitted to NCBI-GenBank and finally showed variation in the bacterial sp. in laboratory and wild populations.

Murthy *et al.* (2011) collected *Cotesia plutellae* populations, a parasitoid of the diamond back moth *Plutella xylostella* from cauliflower fields and were assessed for the prevalence of the bacterial endosymbionts. They detected Bacterial endosymbionts in the genus *Wolbachia* in the populations obtained from Hoskote (Karnataka) and Thirupathi (Andhra Pradesh). After PCR amplification, Sequencing of the *Wolbachia* surface protein, wsp, revealed *Wolbachia* infection to be related to *Wolbachia* endosymbiont of *Cotesia glomerata* outer surface protein, wsp gene. they concluded that the detection of *Wolbachia* in the parasitoid signifying its role in biological manipulations of the parasitoid for enhanced efficiency.

Secil *et al*. (2012) studied Isolation, characterization and virulence of the culturable bacteria from entire tissues of larval *Ostrinia nubilalis* (Lepidoptera: Pyralidae) to obtain new microbes for biological control. They isolated total 16 bacteria from living and dead larvae and were identified as *Pseudomonas aeruginosa* (On1), *Brevundimonas aurantiaca* (On2), *Chryseobacterium formosense* (On3), *Acinetobacter* sp. (On4), *Microbacterium thalassium (*On5), *Bacillus megaterium* (On6), *Serratia* sp. (On7), *Ochrobactrum* sp. (On8), *Variovorax paradoxus* (On9), *Corynebacterium glutamicum* (On10), *Paenibacillus* sp. (On11), *Alcaligenes faecalis* (On12), *Microbacterium testaceum* (On13), *Leucobacter* sp. (On14), *Leucobacter* sp. (On15) and *Serratia marcescens* (On16) based on their morphological and biochemical characteristics. They determined partial sequence of the 16S rRNA gene to confirm strain identification. They obtained highest insecticidal activities from *P. aeruginosa* On1 (80%), *Serratia* sp. On7 (60%), *V. paradoxus* On9 (50%) and *S. marcescens* On16 (50%)

against larvae 14 days after treatment ($p < 0.05$). The results indicate that *P. aeruginosa* On1, *Serratia* sp. On7, *V. paradoxus* On9, *S. marcescens* On16 and *B. thuringiensis* subsp. tenebrionis Xd3 show potential for biocontrol of *O. nubilalis.*

Tang *et al.* (2012) determined the composition of the gut microbiota of two lepidopteran pests, *Spodoptera littoralis* and *Helicoverpa armigera* and applied cultivation-independent techniques based on 16S rRNA gene sequencing and microarray. They revealed that core community, consisting of *Enterococci, Lactobacilli, Clostridia,* etc. in the insect larvae and these bacteria are constantly present in the digestion tract at relatively high frequency despite that developmental stage and diet had a great impact on shaping the bacterial communities. They demonstrated that a core microbial community exists in the insect gut, which may contribute to the host physiology. The gut microbiota might also serve as a reservoir of microorganisms for ever-changing environments. Understanding these interactions might pave the way for developing novel pest control strategies.

Mason and Raffa (2014) used gypsy moth (*Lymantria dispar* L.) as a model to investigate interactions between egg mass and environmental sources of bacteria on larval midgut communities. They collected egg masses from several wild and laboratory populations, and evaluated the effects of diet, initial egg mass community, and internal host environment using 454 16S-rRNA gene pyrosequencing. They revealed that while midgut membership was more similar to bacteria associated with diet than with egg mass-associated bacteria, we were unable to detect distinct, persistent differences attributable to specific host plants. They finally conclude that gypsy moth has a relatively characteristic midgut bacterial community that is reflective of, but ultimately distinct from, it's foliar diet. This work demonstrates that environmental acquisition of diverse microbes can lead to similar midgut bacterial assemblages, underscoring the importance of host physiological environment in structuring bacterial communities.

Kotilingam *et al.* (2015) reported that the gut bacteria (endosymbionts) of *Cotesia vestalis* were isolated and identified. Predominant among them, *Enterobacter cancerogenus* was evaluated for its role in pesticide degradation of the widely used

organophosphorus insecticide, Acephate. Growth of the bacterium on minimal salt media utilizing the pesticide as carbon source and reduced peaks of the degradative product in Liquid Chromotagraphy Mass Spectrometry (LCMS) analysis confirmed the role of the bacterium in the degradation of the pesticide. The association of the symbiont contributed to pesticide resistance in the parasitoid. They discussed effective utilization of the symbiont in pest management programme.

Raina *et al.* (2015) used Polymerase chain reaction (PCR) and Flourescence in situ Hybridisation (FISH) for identification and localization of bacterial endosymbionts in *B. tabaci* as it harbors one of the highest numbers of endosymbionts which have helped it in becoming a successful global invasive agricultural pest. They identified seven different bacterial endosymbionts and These bacterial endosymbionts are known to provide various nutritional, physiological, environmental and evolutionary benefits to its insect host. Analysis of results obtained by these two techniques revealed the advantages of FISH over PCR. On a short note, performing FISH, using LNA probes proved to be more sensitive and informative for identification as well as localization of bacterial endosymbionts in *B. tabaci* than relying on PCR. This study would help in designing more efficient experiments based on much reliable detection procedure and studying the role of endosymbionts in insects

Chen *et al.* (2016) investigated biodiversity and activity of gut microbiota across the holometabolous life cycle of Spodoptera littoralis using ribosomal tag pyrosequencing of DNA and RNA. They reported that *Enterococcus, Pantoea* and *Citrobacter* were abundant in early-instar, while *Clostridia* increased in late instar. Comparative functional analysis with PICRUSt indicated that early instar larval microbiome was more enriched for genes involved in cell motility and carbohydrate metabolism, whereas in late-instar amino acid, cofactor and vitamin metabolism increased. Understanding the metabolic activity of these herbivore associated microbial symbionts may assist the development of novel pest management strategies.

Gracy *et al.* (2016) analysed the possibility of the involvement of gut symbiotic bacteria isolated from *Helicoverpa armigera* in insecticide resistance. Insecticide

resistance and Insecticide susceptible populations of *H. armigera* were collected from crops in India resulted in 6 different species of bacteria isolated from resistant populations and only one from the susceptible laboratory-reared population. Gut microbiota of the resistant populations had the highest diversity index, followed by the moderately resistant and susceptible populations. Six bacterial species were isolated from *H. armigera* larvae by culture. Four bacterial species had a long-term and mutual association with the *H. armigera* populations and some of the microflora associated with insecticide resistant populations differ quantitatively from insecticide-susceptible populations.

Ranjith *et al.* (2016) analysed the composition and diversity of gut bacteria of tomato fruit borer *H. armigera* using Illumina Next-Generation Sequencing of 16S ribosomal RNA amplicons. They identified total of 17 bacterial phyla, 34 classes, 84 orders, 173 families, 334 genera, and 707 species from sequence analysis. *Actinobacteria* was the most dominant groups, followed by *Proteobacteria* and *Firmicutes.* They revealed that function of different gut inhabiting bacteria of *H. armigera,* their role in nutrition, detoxification of lethal insecticide molecules, and defensive action against pathogens. They also found Insecticidal toxin producing bacterial species in associated with the *H. armigera* gut.

2.3 Endosymbionts in insect physiology and insecticide resistance

Mohan and Gujar (2003) collected five different field populations of the Diamondback moth larvae within 15 km radius of Ottanchathiram and studied IARI 17-65 laboratory strain to Biobitr (*Bacillus thuringiensis* var. kurstaki HD-1), cartap, Cry1Ab, fenvalerate, fipronil, flufenoxuron and RH 2485 using cabbage leaf disc feeding bioassays. All field populations were highly tolerant to fenvalerate and flufenoxuron compared with the IARI 17-65 strain. Wide variation in susceptibility of the field populations to insecticides appeared to be due to differential usage of these insecticides. Biobitr; Cry1Ab and RH 2485 were highly toxic to field populations of diamondback moth and could be used as alternate insecticides. An increased rate of detoxifying enzymes-*hydrolases, transferases* and *oxygenases* was responsible for imparting

resistance in *Plutella xylostella* larvae to insecticides. These results emphasize a need for developing a location-specific insecticide resistance management.

Verma *et al*. (2006) isolated bacterial strain *Rhodococcus* MTCC 6716 from the gut microflora of an Indian earthworm (*Metaphire posthuma*). This bacterial strain used Endosulfan as a carbon source and degraded it up to 92.58% within 15 days. The isolated strain of the bacterium did not produce the persistent form of the toxic metabolite endosulfan sulfate. This strain exhibits luxury growth in minimal medium with high concentrations of endosulfan (80 mg mL^{-1}). Degradation of the endosulfan occurred simultaneously with bacterial growth and an increase in chloride ion (87.1%) in the growth medium, suggesting nearly complete degradation of the insecticide. Since endosulfan is used worldwide for pest control and its residues have been retained for long periods in soil, water, and agricultural products, the isolated bacterial strain is valuable for bioremediation of endosulfan-contaminated soil and water.

Rajagopal (2009) studied beneficiary roles that bacteria play in the success and establishment of insects. After 16S rRNA sequencing, it has been shown that some of these bacteria, like *Wolbachia* and *Cardinium* are involved in manipulating insect populations and distorting their sex ratio. Attempts have been made to culture these bacteria in insect cell lines, as they are recalcitrant to culture under normal microbiological conditions. Finally discussed the diversity of bacteria associated with insects and the functional role played by them in the insect.

Kikuchi *et al.* (2012) reported unknown mechanism of insecticide resistance: Infection with an insecticide-degrading bacterial symbiont immediately establishes insecticide resistance in pest insects. They demonstrated that the fenitrothion-degrading *Burkholderia* strains establish a specific and beneficial symbiosis with the stinkbugs and confer a resistance of the host insects against fenitrothion. They identified a stinkbug population live on sugarcane in a Japanese island where fenitrothion has been constantly applied to sugarcane fields and 8% of them host fenitrothion-degrading *Burkholderia.* Their finding suggests that the symbiont-mediated insecticide resistance may develop

even in the absence of pest insects, quickly establish within a single insect generation and potentially move around horizontally between different pest insects and other organisms.

Eleftherianos *et al.* (2013) provided an overview of the effects of endosymbiotic bacteria on the insect immune system as well as on the immune response of insects to pathogenic infections. They discussed potential mechanisms through which endosymbionts can affect the ability of their host to resist an infection. They finally point out unresolved questions for future research and speculate how the current knowledge can be employed to design and implement measures for the effective control of agricultural insect pests and vectors of diseases.

Engel and moran (2013) studied diversity in structure and function of gut bacteria in insects. Insect guts present distinctive environments for microbial colonization, and bacteria in the gut potentially provide many beneficial services to their hosts. . Insect digestive tracts vary extensively in morphology and physicochemical properties, factors that greatly influence microbial community structure. Social insects, such as termites, ants, and bees provide opportunities for transfer of gut bacteria, and some of the most distinctive and consistent gut communities, with specialized beneficial functions in nutrition and protection. Gut bacteria of other insects have also been shown to contribute to nutrition, protection from parasites and pathogens, modulation of immune responses, and communication.

Ramya *et al.* (2016) isolated Acephate degrading bacterial isolates from the larval gut of Diamondback moth, *Plutella xylostella,* a notorious pest of cruciferous crops worldwide that has developed resistance to insecticides. They identified the isolates as *Bacillus cereus* (PXB.C.Or), *Enterobacter asburiae* (PXE), *Pantoae agglomerans* (PX-Pt.ag.Jor). They grew all isolates on minimal media (MM) in the presence of acephate at 100 and 200 ppm, with maximum growth at 200 ppm. All three isolates used acephate as a source of carbon and energy for growth; however, *Pantoae agglomerans* used it also as source of sulphur. Strong evidence revealed that the bacterial communities present in the gut of diamondback moth might aid in acephate degradation and play a role in the development of insecticide resistance.

Sivakumar *et al.* (2016) collected Field populations of cotton leafhopper, *Amrasca biguttula biguttula* (Ishida) exposed to heavy applications of imidacloprid, acephate, acetamiprid and dimethoate from the seven cotton (*Gossypium* spp) growing areas of the country. They islolated thirty culturable bacteria from the guts of 16 populations of leafhoppers and were characterized through morphological and molecular methods. There was more number of gut microflora associated with the leafhoppers collected from Dharwad where the insecticide usage pattern and the number of sprays were very high as compared to other locations. Various *Bacillus* spp. were reported in the Dharwad population. They identified *Serratia, Bacillus, Enterococcus, Enterobacter, Pantoea, Methylobacterium, Stenotrophomonas, Pseudomonas* and *Paenibacillus* in *A. biguttula biguttula.*

WenHong *et al.* (2016) investigated that the phenotypic characteristics of bacterium *Erwinia persicina* from larval gut of the Diamondback moth, *Plutella xylostella.* They analyzed phenotype of *E. persicina* with BIOLOG Phenotype MicroArray (PM) and tested totally 950 different metabolic phenotypes using the PM plates 1-10. The result showed that *E. persicina* was able to metabolize 39.47% of the tested carbon sources, 89.74% of nitrogen sources, 100% of sulfur sources, and 100% of phosphorus sources. Most informative utilization patterns for carbon sources of *E. persicina* were organic acids and carbohydrates, and for nitrogen were various amino acids. The gut bacterium showed both decarboxylase and deaminase activities in the presence of various amino acids. They concluded that the phenotypic characterization of *E. persicina* increased our knowledge of the bacterium, in particular its interactions with insect hosts and the adaptability in gut environments, and showed some possible approaches to controlling diamondback moth through decreasing *E. persicina* density.

Bosch and Welte (2017) studied defensive symbiosis between the pest insect and different types of microorganisms *i.e.* detoxifying symbioses. The insects have to withstand toxic molecules that are inherent to plant defences, as well as those that are produced and introduced by humans in the form of insecticides and leads to economical losses on crops worldwide. They recognized that microbial symbionts may play a role protecting against these toxins and insecticides. They highlighted well-characterized and

emerging insect model systems of detoxifying symbioses and assess how the microorganisms influence the host's success.

Li *et al.* (2017) evaluated detail difference of midgut bacteria in deltamethrin-resistant, deltamethrin-susceptible and field-caught populations of Diamondback moth, and studied phenomics of the predominant midgut bacterium *Enterococcus mundtii*. They revealed that *E. mundtii* and *Carnobacterium maltaromaticum* dominated the bacterial populations from deltamethrin-resistant and deltamethrin-susceptible larval midguts, whereas *E. mundtii* was predominant in field-caught population. Illumina sequencing analysis indicated that 97% of the midgut bacteria were from the phyla Firmicutes, Proteobacteria and Cyanobacteria. They analysed different populations of DBM midgut bacteria using both high-throughput DNA sequencing and cultivation methods and also first report concerning the phenomics of *E. mundtii*. The phenomics of *E. mundtii* provide a basis for the future study of gut bacteria functions.

2.4 Endosymbionts in Diamondback moth, *plutella xylostella*

Gonzalez-cabrera *et al.* (2001) conducted experiment on populations of diamondback moth for resistence to *Bacillus thuringiensis* Toxins. Demonstrated the occurrence in a laboratory colony of Diamondback moth of two different genes (either allelic or nonallelic) that confer resistance to Cry1Ab. At the concentration tested, resistance was dominant in one selection line and partially recessive in the other. The Diamondback moth colony was derived from a field population from the Philippines, which originally showed a different resistance phenotype. This is the first time that an insect population has been directly shown to carry more than one gene conferring resistance to the same Cry toxin.

Indiragandhi *et al.* (2008) isolated culturable gut bacteria from three different populations (insecticide-resistant, insecticide susceptible and field-caught populations) of lepidopteran insect pest Diamondback moth, *Plutella xylostella (*L.*)* to find out their role in host protection and nutrition and were found to be significantly different, irrespective of the developmental stage.

Indiragandhi *et al.* (2008) revealed that after 16S rRNA gene sequence analysis, the bacterial population from the prothiofos-resistant larval gut was more diversified with *Pseudomonas* sp., *Stenotrophomonas* sp., *Acinetobacter* sp. and *Serratia marcescens*. Meanwhile, the susceptible larvae were associated with *Brachybacterium* sp., *Acinetobacter* sp. and *S. marcescens* and the field caught population harboured a rather simple gut microflora belonging to *Serratia*.

Indiragandhi *et al.* (2008) examined gut bacterial strains of Diamondback moth (DBM) for siderophore production, plus the cross-utilization of these siderophores and expression of outer membrane receptor proteins (OMRPs). The *Brachybacterium* sp. PSGB10, *Pseudomonas* sp. PRGB06, and *Serratia marcescens* FLGB16 strains were found to cross-utilize the siderophores of various entomopathogens, including *Bacillus thuringiensis*. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed the presence of the OMRPs responsible for the siderophore cross-utilization. They concluded that siderophore cross-utilization and OMRP expression by the DBM gut bacterial strains support the potential for microbial populations in the insect gut to evolve efficient mechanisms to overcome any iron limitation imposed by the host insect and eventually contribute to the defense mechanism of the host insect.

Xia *et al*. (2013) conducted experiment on a Diamondback moth, DNA sequencing was used to examine midgut microbiota of diamondback moth, *Plutella xylostella* (L.), a destructive pest that attacks cruciferous crops worldwide. They examined Bacteria of the DBM larval midgut in a susceptible and two insecticide (chlorpyrifos and fipronil) resistant lines by Illumina sequencing sampled from an insect generation that was not exposed to insecticide and was revealed that more than 97% of the bacteria were from three orders: Enterobacteriales, Vibrionales and Lactobacillales. They also revealed that the difference related to inter and intra generational exposure to insecticides. Differences in the midgut microbiota among susceptible and insecticide resistant lines are independent of insecticide exposure in the sampled generations.

Lin *et al.* (2014) collected different life stages (fourth instar larvae, pupae and adults) of the Diamondback moth, *Plutella xylostella L.,* to find out different microbial

abundance and diversity of gut bacteria. This was investigated using bacteria culture-dependent method and PCR-DGGE analysis based on the sequence of bacteria 16S rRNA V3 region. A large quantity of bacteria was found in all life stages, out of which higher quantity of bacteria was found in larval gut. Firmicutes bacteria, *Bacillus* sp., were the most dominant species in every life stage. Phylogenetic analysis showed the sequences of the bacteria belonged to the Actinobacteria, Proteobacteria and Firmicutes. *Serratia* sp. in proteobacteria was abundant in the larval gut. Their study also suggested that a combination of molecular and traditional culturing methods can be effectively used to analyze and to determine the diversity of gut microflora.

Lin *et al.* (2014) screened gut bacteria from larvae of DBM and evaluated the effect of antibiotics by treating the larvae with 5 different antibiotics (rifampicin, ampicillin, tetracycline, streptomycin sulfate and chloramphenicol) on the gut bacterial diversity of *Plutella xylostella* larvae. All antibiotics reduced the larval growth, out of which tetracycline was found to be more toxic and streptomycin sulphate was relatively mild one. Species diversity was significantly reduced in the artificial diet and antibiotics treatment and they also showed that the larval gut bacteria could not be removed completely.

Li *et al.* (2015) isolated bacterial symbionts from third instar larvae of DBM and identified using 16S rRNA analysis to test antibiotic susceptibilities of the gut bacteria from feces of DBM by Kirby Bauer disk diffusion method. There were six bacterial species of five genera in the gut of the third instar larvae of *Plutella xylostella*. This entire bacterial microflora had the characteristics of diversity and these bacteria had natural resistance to some antibiotics. This study provided foundation for further study on functional bacterial microflora in the gut of larval *Plutella xylostella.*

Ramya *et al.* (2016) screened 11 geographic populations of DBM in India and analyzed them for bacterial diversity to determine the diversity of gut microflora in DBM, quantify esterase activity and elucidate their possible role in degradation of indoxacarb. They also concluded that apart from the insect esterases, bacterial carboxylesterase aid in the degradation of insecticides in DBM.

Ramya *et al.* (2016) isolated culturable gut bacterial flora from both larvae and adults of Diamondback moth and underwent molecular characterization with 16S rRNA. They obtained 25 bacterial isolates from larvae (n = 13) and adults (n = 12) of DBM. In larval gut isolates, gammaproteobacteria was the most abundant (76%), followed by bacilli (15.4%). Molecular characterization placed adult gut bacterial strains into three major classes based on abundance: gammaproteobacteria (66%), bacilli (16.7%) and flavobacteria (16.7%).

III MATERIAL AND METHODS

The present study on "Isolation and characterization of culturable endosymbionts in Diamondback moth, *Plutella xylostella* (Plutellidae : Lepidoptera)" was carried out in the Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, Bengaluru in collaboration with Department of Agricultural Entomology, UAS, GKVK, Bengaluru. The details of the material and methods employed during the course of study are presented here under.

3.1 Collection of Diamondback moth larval population

The DBM larvae were collected from the cabbage field in different locations by using plastic boxes with same host and brought to the laboratory for experiment purpose. These population were reared on rearing cage for first generation. From first generation, third instar larvae were selected for experimental purpose.

Table 1: Collection of Diamondback moth larval population in different locations

SI. No.	LOCATION	LATITUDE	LONGITUDE
01	Sugutur, Kolar	13.13801°N	78.13696 °E
02	Rattihalli, Haveri	14.42368 °N	75.51329 °E
03	Malligere, Shivamogga	13.92992 °N	75.56810 °E

3.2 Isolation of endosymbionts from larvae of Diamondback moth

3.2.1 Killing and surface sterilization

The third instar larvae of DBM were selected, starved for 24 hours and surface sterilized with 70% ethanol for 1 minute followed by 0.1% sodium hypochloride for 1 minute, then rinsed with sterile distilled water for 2 to 3 times to remove the external microflora.

3.2.2 Serial dilution and plating

The homogenized larvae were crushed using pestle and mortar with 1 ml Phosphate Buffer Saline (PBS) solution (pH 7.4). The homogenized samples were centrifuged at 2000 rpm for 10 minutes. Serial dilution of samples were made up to 10^{-6} dilutions. The aliquot of 1 ml of 4-6 fold dilutions were plated on four different media *i,e* Nutrient Agar (NA) and Luria Bertani (LB) agar for bacteria, Potato Dextrose Agar (PDA) and Yeast extract peptone dextrose agar (YEPDA) for fungi and yeast respectively. 10 µg of the suspension was inoculated on plates containing media. Aliquot was spread using sterilized spreader. The plating was done by spread plate technique. The plates were incubated at 28°C for 48 hours. After every 24 hours, plates were observed for microbial growth. based on morphology, selected the colonies and made pure culture. Purified colonies were grouped according to their colony morphology.

3.2.3 Purification of colonies

Purification of colonies was done by picking isolated colonies and streaking loopfull of culture on four new different media plates by following quadrant streak plate method. The quadrant streak method is used primarily to isolate pure cultures from specimen containing mixed microflora.

3.2.4 Storage of microbes

The isolated pure colonies were streaked on NA and LB agar slants for bacteria. The tubes were incubated till the bacteria showed growth in agar slants and later they were stored in refrigerator for further studies.

3.3 Identification and characterization of microflora

3.3.1 Colony morphology

Colonies that differed in appearance were typically different bacterial strains, species, or genera. The term “Colony morphology” refers to the visible characteristics of the colony. However, colony morphology was not reliable way to identify bacteria, as many different types of bacteria had similar colony morphology. Based on size, shape,

colour and margins of the isolates colonies differentiated and subjected for further studies.

3.3.2 Staining technique to study morphological features under microscope

3.3.2.1 Bacteria

Gram staining technique was used to differentiate Gram positive and Gram negative bacteria. The selected microbes from the pure culture plates were tested whether they are Gram positive or Gram negative.

3.3.2.2 Gram staining

- The 24 hours old cultures were subjected to Gram reaction. Thin smear was prepared, air dried and heat fixed. They were stained with crystal violet for 60 seconds and washed with water.
- Gram's iodine (mordent) was added for 60 seconds, washed with water and dried.
- The slide was then dipped in 70% ethyl alcohol for 45 seconds. Ethyl alcohol is used to decolorize Gram negative bacteria.
- The slide was counterstained with safranine for 60 seconds, washed with water, dried and observed under microscope (oil immersion 100X).

3.3.3 Biochemical characterization of isolated bacteria

The bacterial isolates were taken from streaked four different media plates and subjected to basic biochemical characterization, including catalase and IMVIC reaction. IMVIC reactions consist of Indole production test in tryptone broth, Methyl Red and Voges Proskauer tests in an MR-VP broth and citrate utilization test in Simmons Citrate Agar.

3.3.3.1 IMVIC Procedure

- **Indole test**

Procedure

Using selective technique each experimental organisms were inoculated into labelled test tube containing tryptone broth. The last test tube served as control. The test tubes were incubated for 48 hours at 37°C. On adding kovac's reagent, along the edge of the tube, cherry red ring on the top layer of the broth indicates the production of indole.

- **Methyl red test**

Procedure

MRVP broth was prepared and 5-10 ml of broth was taken in each test tube and was sterilized by autoclaving at 15 lbs pressure for 15 minutes. The test organisms were inoculated into the broth tubes using sterile inoculation loop. One uninoculated MRVP broth was maintained as control. Test tubes were incubated at 36°C for 48 hours. For each test tube 5 drops of methyl red was added and observed for colour change. Production of red colour indicates that the culture in the tubes is positive and having ability to oxidize glucose.

- **Voges proskauer test**

Procedure

MRVP broth was prepared and 5-10 ml of broth was taken in each test tube and sterilized by autoclaving at 15 lbs pressure for 15 minutes. The test organisms were inoculated into the broth tubes using sterile inoculation loop. One un inoculated MRVP broth was maintained as control. Test tubes were incubated at 35°C for 48 hours. 15 drops of VP- reagent 1 and 2-3 drops of VP-reagent 2 was added to all the culture tubes. Tubes were gently shaken for 30 seconds with the caps off to expose the media to oxygen. The appearance of pinkish red colour indicates the positive result of the test.

- **Citrate utilization test**

Procedure

Simmons citrate agar slants were prepared. Slants were inoculated with the cultures and control was kept uninoculated. Tubes were incubated at 37°C for 48 hours. Tubes were observed for growth of organisms and changes in colour as an indicator in the media, which is from green to blue, indicates that the positive citrate utilization test.

- **Catalase test:**

Procedure:

Trypticase soy agar slants were prepared and inoculated with cultures. One uninoculated slant was maintained as control. Slants were incubated at 35°C for 48 hours. The slants were observed for formation of bubbles after adding 3-4 drops of hydrogen peroxide. Formation of gas bubbles indicates positive result of test.

3.4 Molecular characterization

3.4.1 Isolation of Bacterial genomic DNA

3.4.1.1 DNA isolation procedure

- Isolated bacteria were multiplied in Luria Bertani broth (LB).
- Required amount of bacterial pellet was obtained by centrifugation at 10000 rpm for 1 minute in centrifuge until the compact pellet forms.
- Supernatant was removed and pellets were resuspended in 567 µl of TE (1X) buffer, 20 µl of 10% SDS, 5 µl of RNase, 4 µl of Proteinase K (10 mg/ml) and 4 µl of lysozyme (100 mg/ml) was added.
- Vortex thoroughly for 2-3 minutes and then kept the tubes in hot water bath for 30 minutes at 65°C.
- 100 µl of 5M NaCl was added and mixed thoroughly.

- Then 80 µl of CTAB buffer was added (Before adding CTAB solution was heated in water bath for 10 minutes at 65°C) and mixed thoroughly then incubated in hot water bath for 30 minutes at 65°C.
- Equal volume of Chloroform: Isoamyl alcohol (24:1) was prepared and added, centrifuged for 5 minutes at 10000 rpm. Aqueous, viscous supernatant was transferred to a fresh tube.
- Equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was prepared and added, centrifuged for 5 minutes in 10000 rpm.
- Aqueous, viscous supernatant was transferred to a fresh tube and 1 volume of chilled isopropanol was added to precipitate the nucleic acid.
- Incubated for 10 minutes at room temperature.
- Shaking the solution for a while and centrifuged at 10000 rpm for 5 minutes
- Supernatant was removed then small pellet was formed at the bottom of the test tube.
- 1000 µl of 70% chilled ethanol was added to pellet, centrifuged for 1 minute at 10000 rpm.
- Air dried and then dissolved in 80 µl of TE buffer or molecular grade water.
- Stored in freeze.

3.4.2 Agarose gel electrophoresis

3.4.2.1 Verification of DNA levels at 1% agarose gel

1. 1% agarose gel was prepared by using 1X TAE buffer (Tris Acetate EDTA buffer) and heated in microwave oven until it dissolved completely.
2. The clear solution was cooled to 45-50°C and 2 µl of ethidium bromide was added.
3. Comb was placed in boat, clear agar solution was poured slowly to avoid bubbles and kept undisturbed till gel get solidified.
4. After solidification, comb was removed carefully. The gel was immersed with buffer (1X TAE) in horizontal electrophoresis tank. 2 µl DNA samples were mixed with 1

µl gel loading dye (gel loading dye is helpful in visualization of bands on gel) were loaded into the wells.

5. Then the gel was run at 60 volts for approximately 30 minutes. Gel was viewed under gel documentation unit and was photographed.

3.4.3 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) has become a tool, used almost universally by molecular geneticists as one can use it to quickly amplify or create millions of copies of specific regions of a DNA strands without resorting to labour intensive cloning procedures.

3.4.3.1 Principle

PCR is based on the features of semi conservative DNA carried out by DNA polymerase in prokaryotic and eukaryotic cells. A specific region which is flanked by two oligonucleotide primers that share identity to the opposite DNA strands is enzymatically amplified. Amplification of selected region from a complex DNA mixture is carried out in vitro by the DNA polymerase from *Thermus aquaticus*, a bacterium that live in hot springs.

In this study, 16S rRNA based approach was used to determine and identify bacterial populations. Nearly full length bacterial 16S r RNA fragments were amplified by PCR from each representative isolate using primers, Fd1 forward primer (GAGTTTGATCCTGGTCA) and Rp2 reverse primer (ACGGCTACCTTGTTACGACTT). The 16S rRNA fragment was amplified in thermocycler.

Master mix includes all the ingredients except the template DNA (samples) was prepared. Ingredients were added in the following order and kept on ice. Table 2 shows ingredients per reaction mixture.

Table 2: Preparation of PCR mixture (50µl)

Master mix	25 µl
Forward primer	2.5 µl
Reverse primer	2.5 µl
Sterile water	16.0 µl
DNA template	4.0 µl

- To ascertain the specificity of the PCR amplification, negative control (PCR mixture without DNA template) was also prepared.

- 46 µl of master mix divided into individual reaction tubes and 4 µl of template DNA (amount dependent upon the strength of DNA from agarose gel) was added to each tube. No DNA was added to the negative control.

- Load tubes into PCR machine and select the appropriate program for the region being amplified.

3.4.3.2 Steps in Polymerase Chain Reaction (PCR)

- **Initial denaturation:** This step consists of heating the reaction to a temperature of 94°C which is held for 3 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

- **Second denaturation:** This step is the first regular cycling event and consists of heating the reaction to 94°C for 30 seconds which breaks apart the hydrogen bonds that connect the two DNA strands. Prior to the first cycle, the DNA is often denatured for extended time to ensure that both the template DNA and the primers have completely separated and is now single-strand only.

- **Annealing:** The reaction temperature is lowered to 60°C for 1 minute allowing annealing of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches

these template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.

- **Initial elongation:** The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 72°C for 1 minute. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute.

- **Final elongation:** This single step is occasionally performed at a temperature of 72°C for 2.5 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Table 3: Conditions maintained in PCR

Initial denaturation	94°C for 3 minute
Second denaturation	94°C for 30 seconds
Annealing	60°C for 1 minute
Initial elongation	72°C for 1 minute
Final elongation	72°C for 2.5 minutes

Levels of PCR products has been verified by running on an agarose gel, in the same way as when total DNA.

3.4.4 Agarose gel electrophoresis

Agarose gel electrophoresis is a method used in molecular biology to separate DNA strands by size, and to estimate the size of the separated strands by comparison to known fragments (DNA ladder). This is achieved by pulling negatively charged DNA molecules through an agarose matrix with an electric field. Shorter molecules move faster than longer ones.

3.4.4.1 Principle

Electrophoresis possess mechanism similar to sieving objects through a sieve. Here an electric field is used to push negatively charged DNA molecules through a gel matrix. Shorter DNA molecules move faster than long ones, since they are able to slip through the matrix more easily. It can be used for separation of DNA fragments of 50 base pairs up to several mega bases (millions of bases).

3.5 Homology

3.5.1 16S rRNA Sequencing analysis

The unpurified PCR products were sent for sequencing. The nucleotide sequencing of the PCR fragments was performed at Agri genome Pvt. Ltd. (Kerala, India). The DNA sequences corresponding to 16S rRNA gene, obtained from individual bacteria were reverse complemented using software Bio edit. Further DNA sequences aligned using the MEGA 7 software.

The aligned DNA sequences were compared using NCBI data base for identification of bacteria homology searches with 16S rRNA sequences in GenBank are performed with the BLAST program. This program gives DNA query, returns the most similar DNA sequences from the DNA database that the user specifies. The reference sequences required for comparison are downloaded from the EMBL database using the site http://www.ncbi.nlm.nih.gov/Genbank. Based on maximum query coverage the bacterial species was identified.

IV EXPERIMENTAL RESULTS

The results of the experiment on Isolation, Characterization and Diversity analysis of endosymbionts associated with Diamondback moth (*Plutella xylostella*) collected from three different locations of Karnataka. That was undertaken during 2016-17 are presented here under.

4.1 Collection of Diamondback moth(DBM)

The DBM population were collected from different locations of the state Karnataka *viz.,* Sugutur, Kolar; Rattihalli, Haveri; Malligere, Shivamogga. Collected larvae, pupae and adult of DBM. These populations were used for further studies.

4.2 First Population: Sugutur, Kolar District

The larvae, pupae and adult of DBM were collected from Sugutur, Kolar. The population brought in to the laboratory and reared in rearing cage for one generation (Plate 1). From first generation, the third instar larvae were selected and used for further study.

4.2.1 Isolation of culturable endosymbionts

The isolation of culturable endosymbionts was carried out by conventional method and identification by both conventional as well as molecular methods. The isolation of culturable endosymbionts from third instar larvae were carried out by quadrant plate method.

4.2.1.1 Colony characterization

Colony morphology includes its shape, the margins or edges of the colony, its colour, and surface features (Plate 2). These colony plates were used for the obtaining pure cultures of bacterial strains (endosymbionts). Some colonies were round and smooth; others had wavy edges and a wrinkled appearance.

4.2.1.2 Bacterial strains isolated from larvae of Diamondback moth (*Plutella xyostella*)

Well-developed different colonies were picked from inoculated plates and streaked on specific medium. The isolated bacterial strains were observed after 24 hours of inoculation. Totally 10 bacterial strains were isolated from the starved third instar larvae of DBM. Among them, 6 isolates from Nutrient Agar (NA), 2 isolates from Luria Bertani (LB) media, 2 isolates from Potato Dextrose Agar (PDA) media and 2 isolates from Yeast Extract Peptone Dextrose Agar (YEPDA) media. The fungi and yeasts were not found in any plates containing PDA and YEPDA media respectively.

4.2.1.3 Differentiation of bacteria using staining technique

The isolated bacterial strains were observed after 24 hours of inoculation. Different colonies were picked from inoculated plates and gram's staining was done for each isolate. Out of 10 bacterial isolates, Isolate 2, 3 and 6 were gram positive bacteria. Isolate 1, 4, 5, 7, 9, 10 and 8 were gram negative bacteria. Among 10 larval isolates, 2 were yellow rods, 3 were yellow cocci, 4 were white rod and remaining 1 was white cocci as shown in Table 4 and Plate 3.

4.2.2 Biochemical characterization of isolated bacterial strains

All the isolates were subjected to biochemical tests, after 48 hours. Observations were recorded and presented in Table 5 and Plate 4.

- **Indole production test**

After adding kovac's reagent to each tubes including unioculated tube, we observed that the Isolate 1, 2, 6, 8 and 10 strains were developed cherry red colour in the tubes (positive test) and Isolate 3, 4, 5, 7 and 9 strains were not found any cherry red colour in the tubes (negative test).

- **Methyl-red test**

Methyl red indicator was added to each tubes including uninoculated tube and observed that the Isolate 1, 2, 5, 6, 7 and 9 strains were developed red colour throughout

Plate 1: Diamondback moth (*Plutella xylostella*) reared in cage: First population

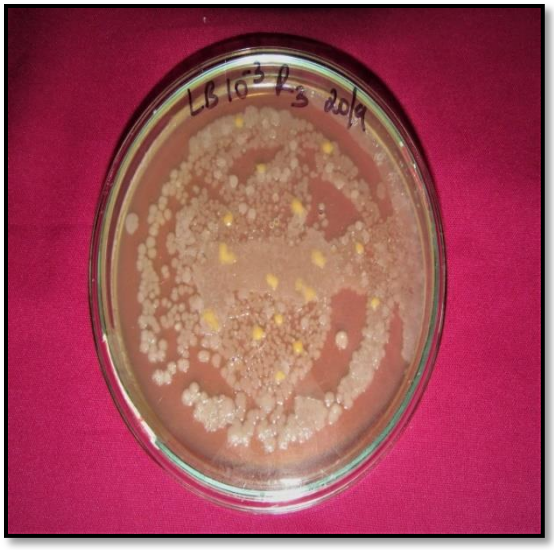
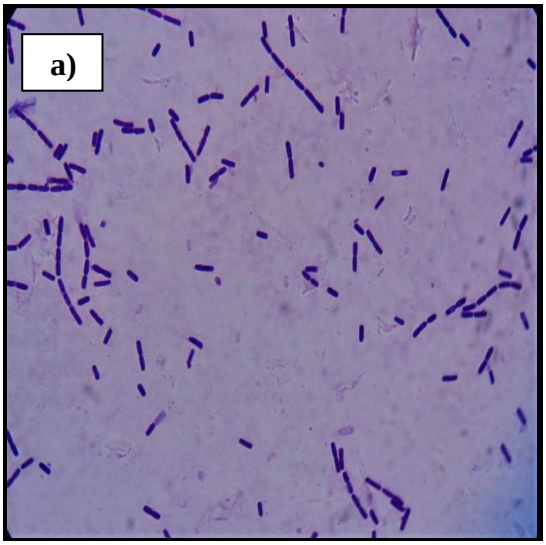


Plate 2: Bacterial colonies of Diamondback moth (*Plutella xylostella*): First population



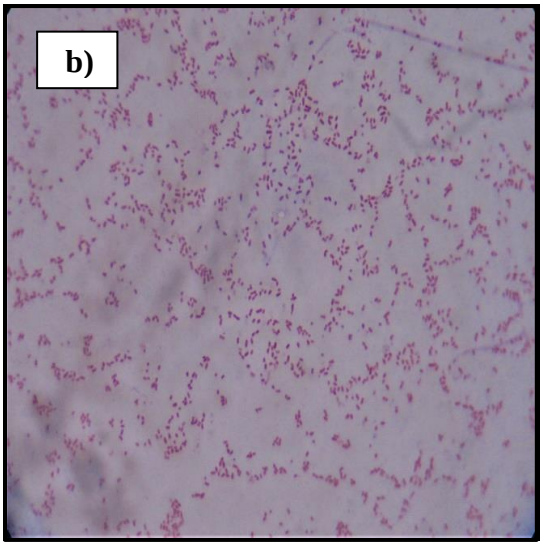


Plate 3: Gram's staining pictures of bacterial samples of Diamondback moth, *Plutella xylostella*: First population

a) GRAM NEGATIVE BACTERIA b) GRAM POSITIVE BACTERIA

the tubes indicated positive for the test whereas, Isolate 3, 4, 8 and 10 strains were not found any red colour throughout the tubes indicated negative for the test.

- **Voges-Proskauer test**

After adding V-P reagent I (napthol solution) and V-P reagent II (40% potassium hydroxide) to tubes including uninoculated tube, we found that the ruby pink (red) colour developed in Isolate 3, 5 and 9 tubes indicated positive for this test where as in Isolate 1, 2, 4, 6, 7 and 8 tubes were not developed ruby pink colour indicated negative for the test.

Table 4: Morphological features of bacterial strains isolated from larvae of Diamondback moth: First population

SI. No.	Isolates	Colony morphology	Cell shape	Gram reaction
	NA			
Isolate 1	10^{-3}, R_3, I_1	Round, regular, dark yellow	Straight Rod	Negative
Isolate 2	10^{-3}, R_3, I_3	Yellow	Cocci	Positive
Isolate 3	10^{-5}, R_1, I_2	White	Cocci	Positive
Isolate 4	10^{-3}, R_3, I_2	Light yellow	Cocci	Negative
Isolate 5	10^{-3}, R_3, I_4	Opaque, irregular, White	Rod	Negative
Isolate 6	10^{-3}, R_2, I_5	Large, irregular, convex, White	Rod	Positive
	LB			
Isolate 7	10^{-3}, R_3, I_1	Large, concave, White	Rod	Negative
Isolate 8	10^{-3}, R_3, I_2	Filamentous, Creamy dark yellow	Rod	Negative
	YEPDA			
Isolate 9	$10^{-3,}$ R_2, I_1	Small, round, mucoid White	Rod	Negative
Isolate 10	$10^{-3,}$ R_2, I_2	Creamy yellow	Cocci	Negative

Table 5: Biochemical features of bacterial strains isolated from larvae of Diamondback moth (*Plutella xylostella*): First population

SI. No.	1	2	3	4	5
Isolate 1	+	+	-	-	-
Isolate 2	+	+	-	-	-
Isolate 3	-	-	+	+	-
Isolate 4	-	-	-	-	-
Isolate 5	-	+	+	+	-
Isolate 6	+	+	-	-	-
Isolate 7	-	+	-	+	-
Isolate 8	+	-	-	-	-
Isolate 9	-	+	+	+	+
Isolate 10	+	-	-	+	-

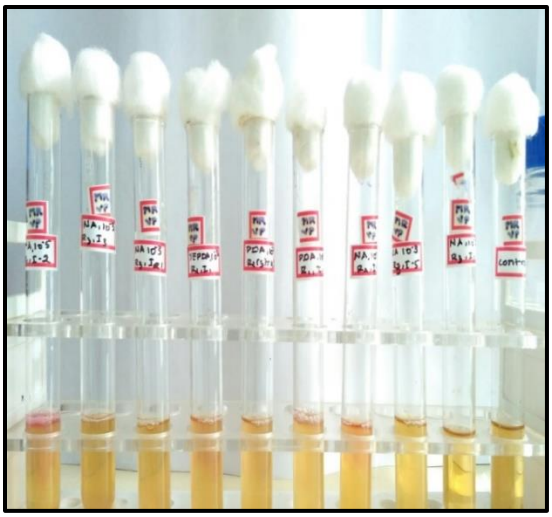
1. Indole production test, 2. Methyl red test, 3. Voges proskauer test, 4. Citrate utilization test, 5. Catalase test. + - Positive, - - Negative.

- **Citrate utilization test**

After 48 hours incubation, observed that the Isolate 3, 5, 7, 9 and 10 tubes were changed the colour from green to blue indicated positive for this test whereas Isolate 1, 2, 4, 6, and 8 tubes were negative for this test which remains green in colour.

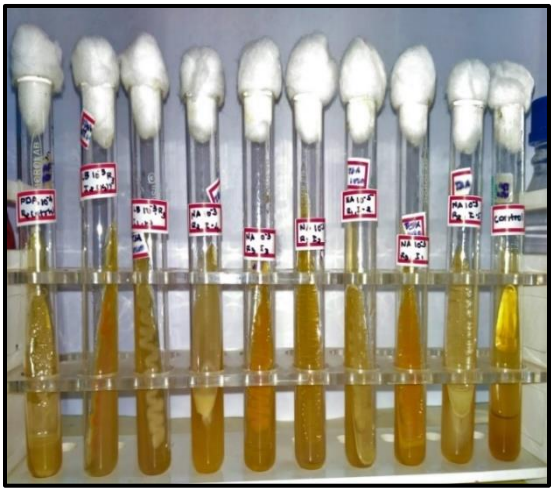
- **Catalase test**

Hydrogen peroxide at 4-5 drops was added to each tubes including uninoculated tube and observed that the Isolate 1, 2, 3, 4, 5, 6, 7, 8 and 10 tubes were not produced gas bubbles indicated negative for this test. Isolate 9 tube produced gas bubble indicated positive for catalase test.



MR-VP TEST

INDOLE TEST



CATALASE TEST

CITRATE UTILIZATION

Plate 4: Biochemical characterization of isolated bacteria of Diamondback moth (*Plutella xylostella*): First population

4.2.3 Molecular characterization for the identification of isolated bacterial strains

4.2.3.1 DNA bands visualization on Agarose gel

The six bacterial strains were isolated from third instar larvae of DBM were subjected to DNA isolation under conditions described in Section 3.4.1 and DNA bands were observed on agarose gel as thick bands under UV transmission and were used for further studies. It was found that the sharp bands of genomic DNA under Gel documentation photograph were clear (Plate 5). These sharp bands represented the presence of DNA and which was subjected to PCR as template DNA for the amplification by using the 16s rRNA specific forward and reverse primers.

4.2.3.2 16S rRNA analysis and gel electrophoresis

The DNA was amplified using PCR under conditions described in Section 3.4.4.3. In this study, 16S rRNA–based approach was used to determine bacterial populations associated with larvae of DBM. Nearly full-length bacterial 16S rRNA fragments were amplified by PCR from each representative isolates using the 16S rRNA primer sets, Fd1 forward primer (GAGTTTGATCCTGGTCA) and (ACGGCTACCTTGTTACGACTT) Rp2 reverse primer. The 16S rRNA fragment was amplified in thermocycler. Amplification products were of the expected 1000bp size and were run on agarose gel. All six bacterial isolates were amplified with Fd1 and Rp2 primers.

4.2.4 Diversity analysis

The bacterial microflora isolated from DBM were identified as *Serratia marcescens, Bacillus cereus* and *Myroides odoratus.* The Isolate 1 and 9 were identified as *Serratia marcescens,* Isolate 5 was identified as *Serratia nematodiphila*, Isolate 7 was identified as *Serratia* sp., Isolate 6 was identified as *Bacillus cereus* and Isolate 8 was identified as *Myroides odoratus.* The bacteria identified differed from location to location (Table 7 and Fig. 1).

The bacterial strains isolated from starved third instar larvae of DBM were placed together to generate the phylogenetic tree by neighbor joining method and in this similarity probabilities were shown. Bacterial species with maximum query coverage

Table 6: Fasta aligned sequences of different isolated bacterial endosymbionts: First population

SI. No.	ISOLATE	FASTA SEQUENCES OF ISOLATES BACTERIAL ENDOSYMBIONTS
	A) NA	
ISOLATE 1	10^{-3}, R_3, I_1	TCACCTCATGCGCAGCTTACACATGCAAGTCGAGCGGTAGCACAGGGGAGCTTGCTCCCCG GGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAAC TACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGC CTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAG GCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCA GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCA TGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGT GAGCTTAATACGCTCATCAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCC AGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCAC GCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAA ACTGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTA GAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGT GCGAAAGCGTGGGGGAGCAAACAGGATTAGA
ISOLATE 5	10^{-3}, R_3, I_4	CCCCCAGCTCCCCATCATTCCCCGTCATGCAGGTCGAGCTAGTAGCACTGGGGAGCTTGCTC CCCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGA TAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTC GGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCAC CTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGG TCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCA GCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGG TGGTGAGCTTAATACGCTCATCAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGT GCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG CACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTT GAAACTGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGC GTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGA

SI. No.	ISOLATE	FASTA SEQUENCES OF ISOLATES BACTERIAL ENDOSYMBIONTS
ISOLATE 6	10^{-3}, R_3, I_5	GAATTATTCGGGGGGGACGAGGGGCCCCTTAGGGGGGGGCTCCAAAAGGTACCCACCGACT TCGGGTGTTACCACTCTCGGGGGGGGAGGGGGGGTGTGTACAAGGCCCGGGAACGTATTCC CGCCGGCTTGCTGATCCGCAATAACAACCAATTCCACCTTCATGTATGCAAGTTGCGACCGA CAATCCGAACTGAGAAGGGGTTGTGGGATTAGCTTCACCTCGCGGTCTTGCAGCTTTTTGTC CGCCCATTGTAGCACGTGTGTACCAAGGTGTAGGGACATGATGATTGGACGTCATCCCCAC CTTCTCCGCTTTGTCGACGGCGGTCTCTTGAGAGTGCCCCACTTAAGGATGGGTCTAAGATC AAGGGGTGGCGTTTTGAGGGATTAACCCAAAATCTCACGGGCGAGCTGGAAAACCATGCAC CACCTGTGGTTCTGGTTCCGAAAGAAACCCCTATTTTTTGGGTTTTCAAATGATGGCCAAGG CCGGGAAGGTTCCTCTCCGTTCTTTCAAATAAAACAATTTGTGCACCGGTTGTGGGGCCCCC CTCATTTTCTTTTAGATTTTACTTGCGGCCGTACCCCCCAGGGGGAATGGTAAAGGGTGAAT CTCGCATAAAAATGGGAGAGCGTCTAAACTTAGTAGAGATCTTTTACGGGGGGGCTTCAAG GTATTAATCGTGTTTGGCTTCTGCGCTATTGACCTCCTTGTGGTATCAGACAAAGAAACCCC TTCTCGCTCAGTGTTGGGTGCTTTAT
	B) LB	
ISOLATE 7	10^{-3}, R_3, I_1	CGACGACTTGCCGGAGCTTACCATGCAGTCGAGCGGTAGCACAGGGGAGCTTGCTCCCCGG GTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGATAACTA CTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCT CTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGC GACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGA CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATG CCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGA GCTTAATACGCTCATCAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAG CAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGC AGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAAC TGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGA GATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGC GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGT

SI. No.	ISOLATE	FASTA SEQUENCES OF ISOLATES BACTERIAL ENDOSYMBIONTS
ISOLATE 8	10^{-3}, R_3, I_2	GGGCATCGACAGCTACCATGCAGTCGAGGGGTATAGAGAGCTTGCTTTCTAGAGACCGGCG GATGGGTGAGTAACGCGTATGCAACCTACCTTTTACAGGGGAATAGCCCGGAGAAATTCGG ATTAATGCTCCATGGTTTATATGAATGGCATCGTTTGTATAATAAAGATTTATCGGTAAAAG ATGGGCATGCGTATCATTAGCTAGTTGGTGTGGTAACGGCATACCAAGGCTACGATGATTA GGGGTCCTGAGAGGGAGATCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGA GGCAGCAGTGAGGAATATTGGTCAATGGAGGCAACTCTGAACCAGCCATGCCGCGTGCAGG ATGACGGTCCTATGGATTGTAAACTGCTTTTGTACGGGAAGAAATGTAATTACGTGTAATTA TTTGACGGTACCGTAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG AGGATCCGAGCGTTATCCGGAATTATTGGGTTTAAAGGGTTCGTAGGCGGTTGAGTAAGTC AGTGGTGAAATCTTATAGCTTAACTATAAAATTGCCGTTGATACTGCTTGACTTGAATAGTA TGGAAGTAATTAGAATATGTAGTGTAGCGGTGAAATGCTTAGATATTACATGGAATACCAA TTGCGAAGGCAGATTACTACGTACTTATTGACGCTGATGAACGAAAGCGTGGGTAGCGAAC AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTCGGTTTTCGGA CTGAGTGGCTAAGCGAAAGTGATAAGTATCCCACCTGGGGAGTACGTTCGCAAGA
	C) YEPDA	
ISOLATE 9	10^{-3}, R_2, I_1	GGGCTATGGGCGGCAGCTTACACATGCAAGTCGAGCGGTAGCACAGGGGAGCTTGCTCCCC GGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAA CTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGG CCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAG GCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCA GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCA TGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGT GAGCTTAATACGCTCATCAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCC AGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCAC GCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAA ACTGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTA GAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGT GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG

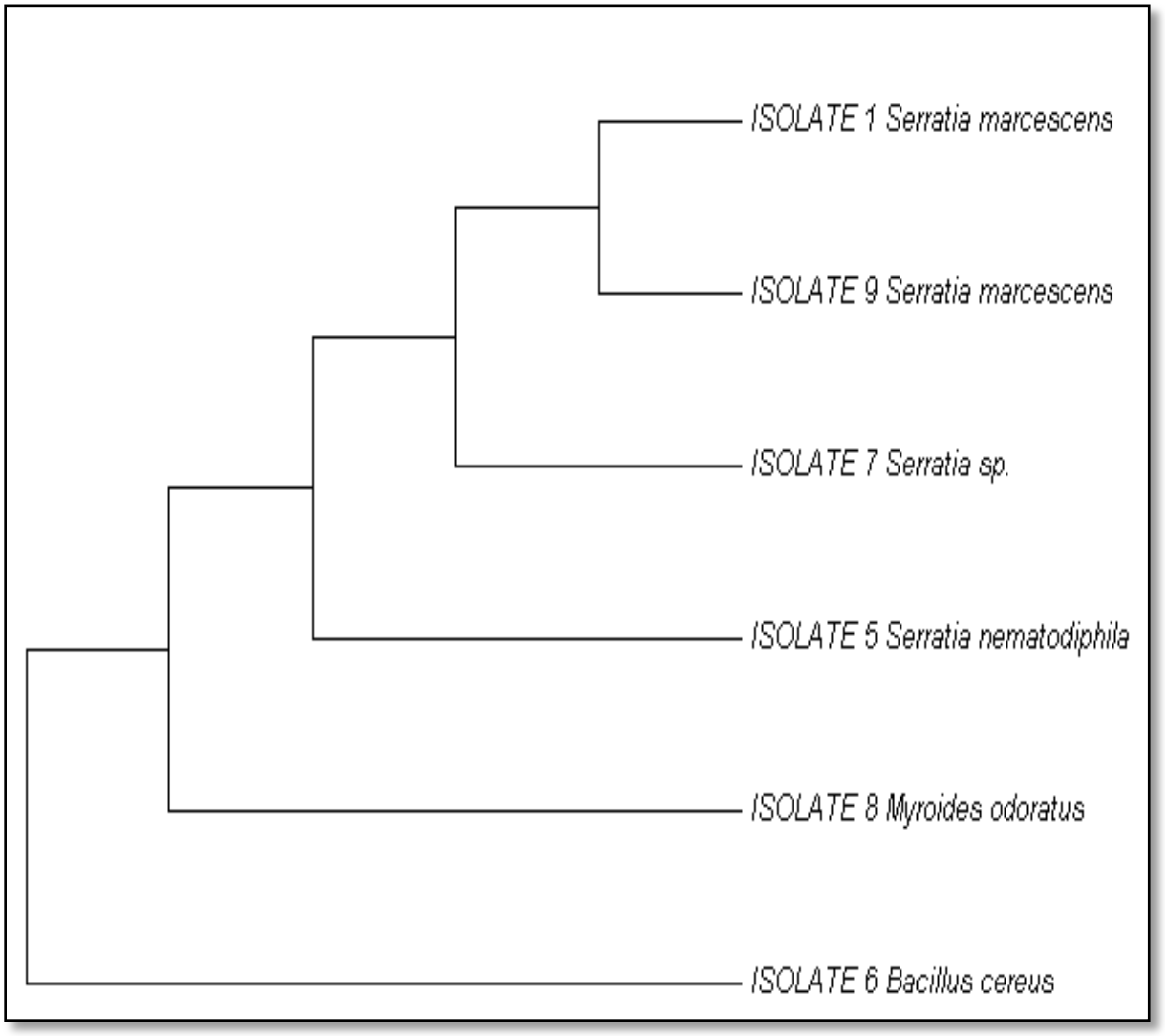


Fig. 1. Phylogenetic tree showing bacterial diversity in Diamondback moth (*Plutella xylostella*): first population

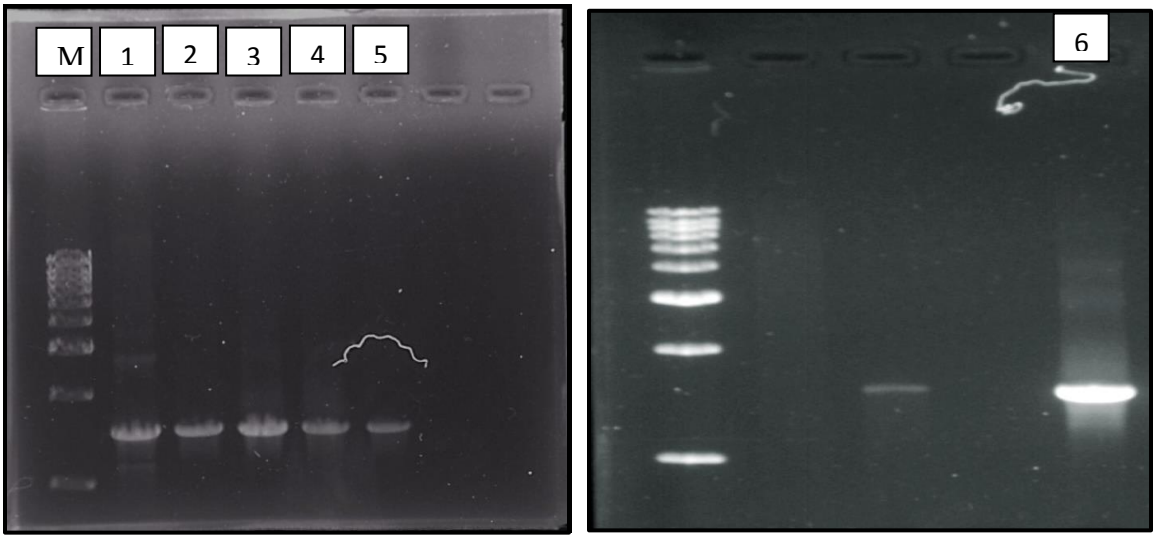


Plate 5: Agarose gel showing amplification of 1000 bp gene corresponding to 16S rRNA, M-Marker DNA-1000bp: First population

(1) ISOLATE 1, (2) ISOLATE 5, (3) ISOLATE 6, (4) ISOLATE 7,

(5) ISOLATE 8, (6) ISOLATE 9.

were identified and those sequences are submitted to GenBank for the accession numbers to know the diversity in gut microflora by constructing phylogenetic tree in future (Table 6).

Table 7: Bacterial endosymbionts identified in larvae of Diamondback moth (*Plutella xylostella*): First population

Isolate	Identified bacterial endosymbionts
Isolate 1	*Serratia marcescens*
Isolate 5	*Serratia nematodiphila*
Isolate 6	*Bacillus cereus*
Isolate 7	*Serratia* sp.
Isolate 8	*Myroides odoratus*
Isolate 9	*Serratia marcescens*

4.2.4.1 Identification of bacterial endosymbionts after sequencing

The identification of bacterial endosymbionts from the larvae of DBM by 16S rRNA sequence analysis was tabulated above. The above results revealed that *Serratia marcescens* exclusively, or predominant in third instar larvae of DBM (Table 7).

4.3 Second Population: Rattihalli, Haveri

4.3.1 Isolation of endosymbionts from larvae of Diamondback moth

Third instar larvae of DBM were collected from Rattihalli, Haveri District. The field collected larvae were brought to the laboratory, reared and used for further study.

4.3.1.1 Colony characterization

The colony characters were recorded and enumerated (Plate 6). The bacterial colonies isolated from starved third instar larvae of DBM were predominantly slightly dry texture, raised, pasty looking and white in colour. Some colonies were irregular, concave, dry, dry yellow and others were smooth, opaque, circular, creamy white colour.

4.3.1.2 Bacterial strains isolated from larvae of Diamondback moth (*Plutella xyostella*)

Different colonies were picked from inoculated plates and streaked for purification. plates were observed after 24 hours of inoculation. Totally eight bacterial strains were isolated from third instar larvae of DBM.

4.3.1.3 Differentiation of bacteria using staining technique

The isolated bacterial strains were observed after 24 hours of inoculation. Gram's staining was done for each isolate which were picked from different inoculated colony plates. Among the eight isolated bacterial strains, Isolate 1, 2, 5 and 6 were found to be gram negative bacteria. Isolate 3, 4, 9, and 10 were gram positive bacteria. Most of the strains were predominantly rod shaped bacteria (Table 8 and Plate 7).

4.3.2 Biochemical characterization of isolated bacterial strains

All the isolates were subjected to biochemical tests. After 48 hours, Observations were recorded and presented in Table 9 and Plate 8.

- **Indole production test**

After adding kovac's reagent to each tubes including unioculated tube, Isolate 3, 4, 5, and 6 strains were developed cherry red colour in the tubes (positive test) and Isolate 1, 2, 9, and 10 strains were not developed any cherry red colour in the tubes (negative test).

- **Methyl-red test**

Methyl red indicator was added to each tubes including uninoculated tube and observed that the Isolate 2, 3, 5, 6, and 9 strains were developed red colour throughout the tubes indicated positive for the test. Isolate 1, 4 and 10 strains were not found any red colour throughout the tubes indicated negative for the test.

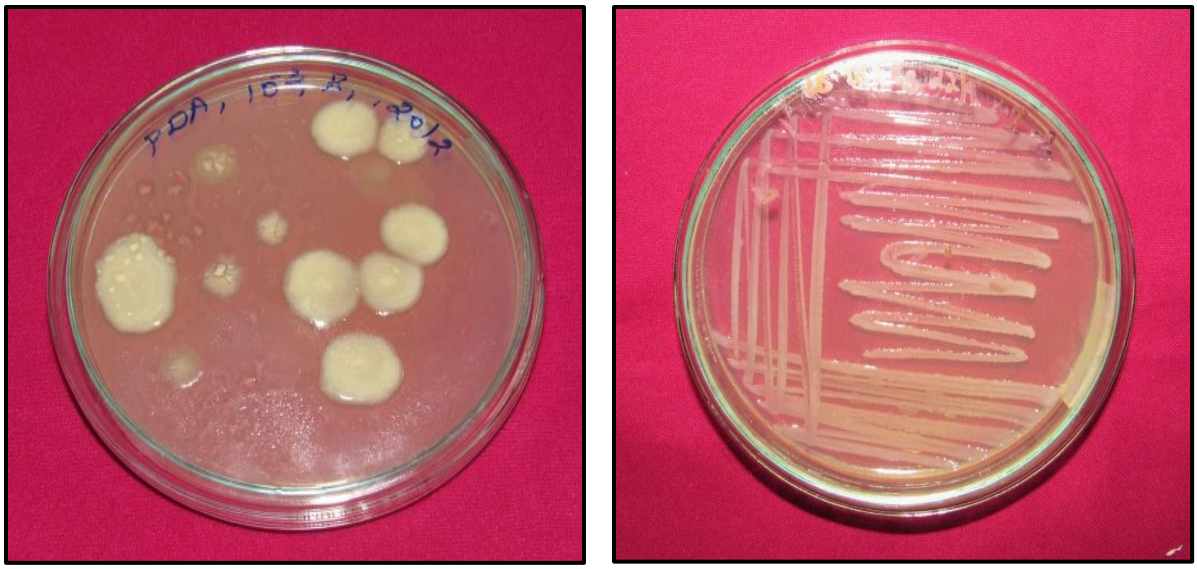


Plate 6: Bacterial colonies of Diamondback moth (*Plutella xylostella*): Second population

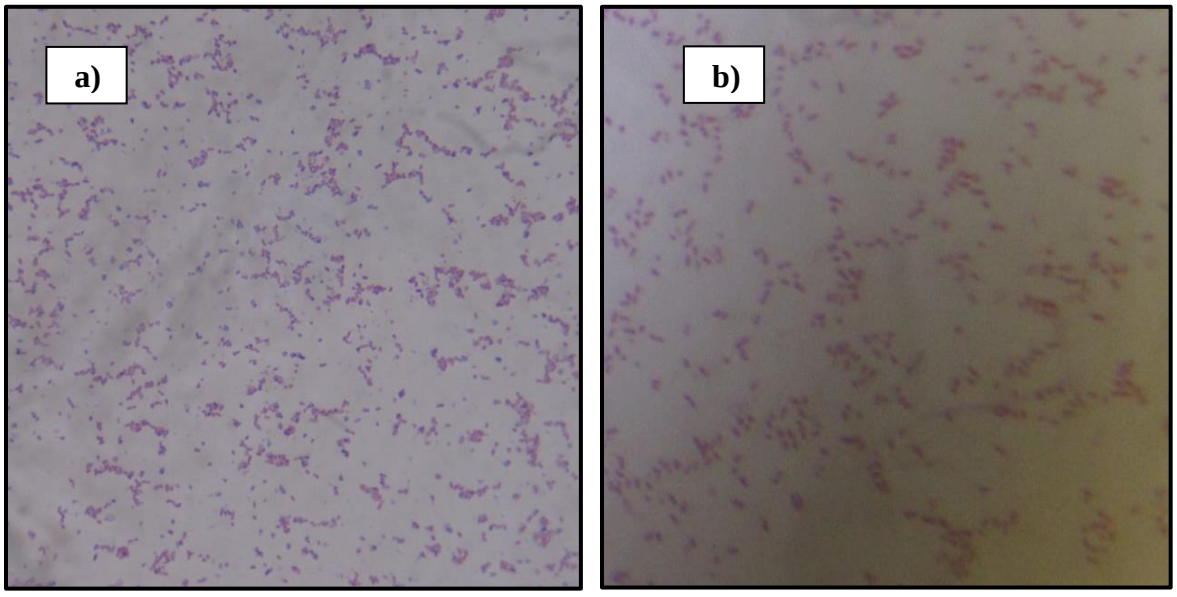


Plate 7: Gram's staining pictures of bacterial isolates of Diamondback moth (*Plutella xylostella*): Second population

a) GRAM NEGATIVE BACTERIA b) GRAM POSITIVE BACTERIA

Table 8: Morphological features of bacterial strains: Second population

SI. No.	Isolates	Colony morphology	Cell shape	Gram reaction
	NA			
Isolate 1	10^{-3}, R_2, I_1	White, circular, concave	Rod	Negative
Isolate 2	10^{-4}, R_1, I_1	Dark yellow	Rod	Negative
Isolate 3	10^{-4}, R_2, I_1	Dry, dull white	Rod	Positive
Isolate 4	10^{-6}, R_1, I_1	Raised, irregular, creamy	Rod	Positive
Isolate 9	10^{-6}, R_1, I_2	Circular, smooth, yellow	Cocci/rod	Positive
Isolate 10	10^{-5}, R_2, I_1	Smooth, shiny, circular, convex, pinkish	Cocci	Positive
	LB			
Isolate 5	10^{-4}, R_1, I_1	Large fluidal white	Rod	Negative
	PDA			
Isolate 6	10^{-3}, R_1, I_2	Dense dark white	Rod	Negative

Table 9: Biochemical features of bacterial strains isolated from larvae of Diamondback moth (*Plutella xylostella*): Second population

SI. No.	1	2	3	4	5
Isolate 1	-	-	+	+	-
Isolate 2	-	+	-	+	+
Isolate 3	+	+	+	+	+
Isolate 4	+	-	-	-	+
Isolate 5	+	+	+	-	-
Isolate 6	+	+	+	+	-
Isolate 9	-	+	-	+	-
Isolate 10	-	-	-	-	-

1. Indole production test, 2. Methyl red test, 3. Voges proskauer test, 4. Citrate utilization test, 5. Catalase test. + - Positive, - - Negative

- **Voges-Proskauer test**

After adding V-P reagent I (napthol solution) and V-P reagent II (40% potassium hydroxide) to each tubes including uninoculated tube, ruby pink (red) colour developed in Isolate 1, 3, 5 and 6 tubes indicated positive result for this test. Isolate 2, 4, 9, and 10 tubes were not developed ruby pink colour indicated negative for this test.

- **Citrate utilization test**

After 48 hours incubation, observed that the Isolate 1, 2, 3, 6, and 9 tubes were changed the colour from green to blue indicated positive result for this test whereas Isolate 4, 5, and 10 tubes were negative for this test which remains green in colour.

- **Catalase test**

Hydrogen peroxide at 4-5 drops was added to each tubes including uninoculated tube and observed that the Isolate 2, 3, and 4 tubes were produced gas bubbles indicated positive for this test. Isolate 1, 5, 6, 9, and 10 tubes were not produced gas bubbles indicated negative for catalase test.

4.3.3 Molecular characterization of isolated endosymbionts

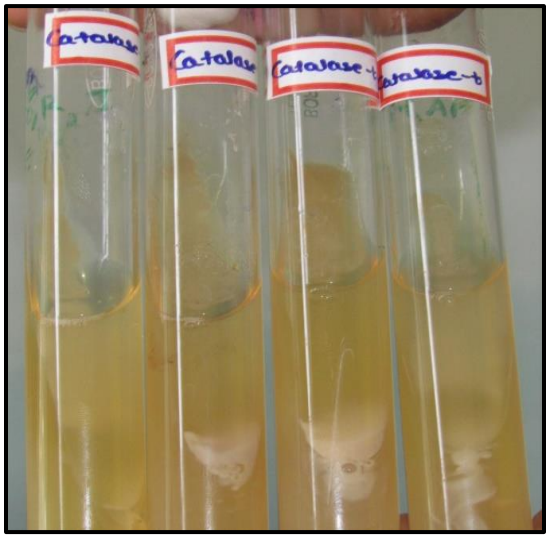
4.3.3.1 DNA bands Visualization from larvae of Diamondback moth (*Plutella xylostella*) on agarose gel

The eight bacterial strains isolated from starved third instar larvae of DBM were subjected to DNA isolation under conditions described in Section 3.4.1 and DNA bands were observed on agarose gel as thick bands under UV transmission and were used for further studies. The sharp bands of genomic DNA were found under Gel documentation photograph (Plate 9). This visualance of sharp bands represented the presence of DNA and which was subjected to PCR as template DNA for the amplification by using the 16S rRNA specific forward and reverse primers.

INDOLE TEST

MR-VP TEST

CITRATE UTILIZATION TEST



CATALASE TEST

Plate 8: Biochemical characterization of isolated bacteria of Diamondback moth (*Plutella xylostella*): Second population

4.3.3.2 16s rRNA analysis and gel electrophoresis

The DNA was amplified using PCR under conditions described in Section 3.4.4.3. In this study, 16S rRNA–based approach was used to determine bacterial population. Nearly full-length bacterial 16S rRNA fragments were amplified by PCR from each representative isolate using the 16S rRNA primer sets, Fd1 forward primer (GAGTTTGATCCTGGTCA) and Rp2 reverse primer (ACGGCTACCTTGTTACGACTT). The 16S rRNA fragment was amplified in thermocycler. Amplification products were of the expected 1000bp size and were run on agarose gel. All eight bacterial isolates were amplified with Fd1 and Rp2 primers.

4.3.4 Diversity analysis

The bacterial microflora isolated from DBM were identified as *Pseudomonas otitidis, Dyella japonica, Bacillus* sp., *Aneurinibacillus aneurinilyticus, Ralstonia solanacearum, Ralstonia picketti, Brachybacteria* sp. and *Kocuria turfanensis.* The Isolate 1 was identified as *Pseudomonas otitidis,* Isolate 2 was identified as *Dyella japonica,* Isolate 3 was identified as *Bacillus* sp.*,* Isolate 4 was identified as *Aneurinibacillus aneurinilyticus,* Isolate 5 was identified as *Ralstonia solanacearum,* Isolate 6 was identified as *Ralstonia picketti,* Isolate 9 was identified as *Brachybacteria* sp. And Isolate 10 was identified as *Kocuria turfanensis* (Table 11 and Fig. 2).

The bacteria isolated from larvae of Diamondback moth (*Plutella xylostella*) were placed together to construct the phylogenetic tree by neighbor joining method and in this similarity probabilities were shown. Whereas bacterial species with maximum query coverage were identified and those sequences are submitted to GenBank for the accession numbers to know the diversity in gut microflora by constructing phylogenetic tree in future (Table 10).

Table 10: Fasta aligned sequences of different isolated bacterial endosymbionts: Second population

SI. No.	ISOLATE	FASTA SEQUENCES OF ISOLATES BACTERIAL ENDOSYMBIONTS
	NA	
ISOLATE 1	10^{-3}, R_2, I_1	CCCAAATGCCGGCTGCCTCACCATGATAGTCGAGCGGATGAGTGGAGCTTGCTCCATGATT CAGCGGCGGACGGGTGAGTAAAGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTTCG AAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCAC GCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCCCACCAAGGCGAC GATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTC CTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCC GCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGAGCAGTAA GTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGC AGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGT AGGTGGTTCAGCAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATCCAAAA CTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGTGAAATGCGTAGA TATAGGAAGGAACACCAGTGGCGAAGCGACCACCTGGACTGATACTGACACTGAGTGCGA AAGCGTGGAGCAAAACAGGATTAGATACCTG
ISOLATE 2	10^{-4}, R_1, I_1	GGGTAGGCTCATTGATTCGACTTACCCCAGTCATGAACCACTCCGTGGTCGTCGTCCCCCTT GCGGTTAGACTAACGGCTTCTGGAGCAACTCACTCCCATGGTGTGACGGGCGGTGTGTACA AGGCCCGGGAACGTATTCACCGCAGCATAGCTGATCTGCGATTACTAGCGATTCCGACTTC ATGGAGTCGAGTTGCAGACTCCAATCCGGACTGGGATCGGCTTTCTGGGATTGGCTCCACC TCGCGGTATTGCAACCCTCTGTACCGACCATTGTAGTACGTGTGTAGCCCTGGCCGTAAGG GCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAG TTCCCACCATTACGTGCTGGCAACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCC AACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTTCTGATTCCCGAAGGC ACTCCCGCATCTCTGCAGGATTCCAGACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCA TCGAATTAAACCACATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGT CTTGCGACCGTACTCCCCAGGCGGCGAACTTAACGCGTTAGCTTCGACACTGATCTCCGAG TTGAGACCAACATCCAGTTCGCATCGTTTAGGGCGTGCACTACCAGGGTATCTAATCCTGTT TGCTCCCCACGCTTTCGTGCCTCAGCGTCAG

SI. No.	ISOLATE	FASTA SEQUENCES OF ISOLATES BACTERIAL ENDOSYMBIONTS
ISOLATE 3	10^{-4}, R_2, I_1	GCCCTAAGGACTCATATTACGACTTCCCCCAATCATCTGTCCCACCTTAGGCGGCTGGCTCC AAAAGGTTACCCCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTA CAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTT CATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAATGGTTTTATGGGATTCGCTTAAC CTCGCGGTCTTGCAGCCCTTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGG GGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAG TGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAA CATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACCCTGTCCCCCGAAGGGG AACGCCCTATCTCTAGGGTTGTCAGGGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTT CGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCC TTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTTTGCTGCAGCACTAAAGGGCGGAA ACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGACTACCAGGGTATCTAATCCTGTTTG CTCCCCACGCTTT
ISOLATE 4	10^{-6} , R_1, I_1	GTCCCAATGACATGCATCTAAGACTTCCCCCAATCATCTGCCCCACCTTCGGCGGCTGGCTC CTTGCGGTTACCTCACCGACTTCGGGTGTTGCAAACTCTCGTGGTGTGACGGGCGGTGTGT ACAAGACCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCT TCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAATGGTTTTAAGGGATTGGCGCAC TCTCGCGAGTTGGCTGCCCGTTGTTCCATCCATTGTAGCACGTGTGTAGCCCAGGACATAA GGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGTCTTGTCGACGGCAGTCTCCCTAG AGTGCCCAACTGAATGCTGGCAACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCC AACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACCGCTGCTCCGAAGAG AGCTCCTATCTCTAGGAGGGTCAGCGGGATGTCAAGCCCTGGTAAGGTTCTTCGCGTTGCT TCGAATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAGC CTTGCGGCCGTACTCCCCAGGCGGAGTGCTTATTGCGTTAGCTGCGGCACTGAGGATTGGA GTCCCCAACACCTAGCACTCAACGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTC GCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGGCCAGAGAGCCGCCTTCGCCACGGG TGTT

SI. No.	ISOLATE	FASTA SEQUENCES OF ISOLATES BACTERIAL ENDOSYMBIONTS
ISOLATE 9	10^{-6} , R_1, I_2	TGCTCTCCTGGCTAGTGGCTACTTAATCCCATCACTATCACCACACTTACGACAGCTCCC TCACGAGGATGGGCCACTGGCTTCGCGGTGTGACCGACTTTCAGGACTTGACGGGCGGTGT GTACAAGGCCCGGGAACGTATTCACCGCAGCGTTGCTGATCTGCGATTGCAACCATTTCCT ACTTCATGGGGTCAAGTTGCAGACCCCAATCCGAACTGAGGCCGGTTTTTTGGGATTCGCT CCACCTCACAGTCTTGCAACTTTTGTACCGACCATTGTAGCATGGGTGAAGCCAAAGACAA AAGGGGCATGATGATTTGACGTCGTCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCT ATGAGTCCCCACCATGACGGGCGGAAAAAAAAAAACGGGGGTTGCGCTCGTGGGGGGACT TAACCAAACATCTCACGACACGAGGTGACAAAAACCATGCACCACCTGTGCACCAGTCCG AAGAAAACCCCATCTCTGGAGGCGTCGGGAGAATGTCAAGCCTTGGGAAAGTTCTTCGCGT TGCATCGAAATAAACAGCATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTT TAGCCTTGCGGGCGTACTCCCCAGGGGGGGAATTTATTGCGTTATATATGCCGCGGAAAAA GGTGGAAAGCCCCCCACACCTAGTGCCCAACGTTTACGGGATGGACTACCAGGGTATCTAA TCCTGTTCGCTACCC
ISOLATE 10	10^{-5} , R_2, I_1	CCAGCGAACCCCGTCCACGAGTGAGCGTCACGTTGATCTATCAGCGGTATGCTCGCTTGCG ACTTAATTAGTGAAAAAAGGATGAGTAATAGGTGAGTACCTGCCCTTGACTCTGGGATAAG CCTGGGAAACCGGGTCTAATACTGGATACGACCCCCTGTCGCATGGTGGGGGGTGGAAAG GGTTTGACTGGTTTTGGATGGGCTCACGGCCTATCAGCTTGTTGGTGGGGTAATGGCTCACC AAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGG CCCAGACTCCTACGGAAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCTGATGCA GCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAG CCACAAGTGACGGTACCTGCAGAAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGT AATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGGCGTAAAGAGCTCGTAGGCGGGT TTGTCGCGTCTGCTGGTGAAACCCCGGGGCCTCACCCCCGGGGTCTGCAGGGGGGTACGGG CAGACTAAAGTGCAGTAGGGAAGACTGGAATTCCTGGTGTAGCGGTTAATATGCGCAGAT ATCAGGGAGGAAACTCCGATGGCGAAGCAGGTCTCTGGGCTGTTACTGACGCTGAGGAGC GAAAGCATG

SI. No.	ISOLATE	FASTA SEQUENCES OF ISOLATES BACTERIAL ENDOSYMBIONTS
	LB	
ISOLATE 5	10^{-4}, R_1, I_1	CCTTAGGCAACCTCACACAGACTTGACCCCAGTCATGAACCCTACCGTGGTAATCGCACAC TCCTTGCGGTTAGGCTAACTACTTCTGGTAAAGCCCACTCCCATGGTGTGACGGGCGGTGT GTACAAGACCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAG CTTCACGTAGTCGAGTTGCAGACTACGATCCGGACTACGATGCATTTTCTGGGATTAGCTCC ACCTCGCGGCTTGGCAACCCTCTGTATGCACCATTGTATGACGTGTGAAGCCCTACCCATA AGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCTCTA GAGTGCCCTTTCGTAGCAACTAGAGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACA TCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCCACTTTCTCTTTCGAGCAC CTAATGCATCTCTGCTTCGTTAGTGGCATGTCAAGGGTAGGTAAGGTTTTTCGCGTTGCATC GAATTAATCCACATCATCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTTAATCT TGCGACCGTACTCCCCAGGCGGTCAACTTCACGCGTTAGCTACGTTACTAAGGAAATGAAT CCCCAACAACTAGTTGACATCGTTTAGGGCGTG
	PDA	
ISOLATE 6	10^{-3}, R_1, I_2	TTGCTAAGAAACTCGTCGCGACTTCACCCCAGTCATGTACTCTACACGTGGTAATCGCCCTC CTTGCGGTTAGGCTAACTACTTCTGGTAAAGCCCACTCCCATGGTGTGACGGGCGGTGTGT ACAAGACCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCT TCACGTAGTCGAGTTGCAGACTACGATCCGGACTACGATGCATTTTCTGGGATTAGCTCCA CCTCGCGGCTTGGCAACCCTCTGTATGCACCATTGTATGACGTGTGAAGCCCTACCCATAA GGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCTCTAG AGTGCCCTTTCGTAGCAACTAGAGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACAT CTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCCACTTTCTCTTTCGAGCACC TAATGCATCTCTGCTTCGTTAGTGGCATGTCAAGGGTAGGTAAGGTTTTTCGCGTTGCATCG AATTAATCCACATCATCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTTAATCTT GCGACCGTACTCCCCAGGCGGTCAACTTCACGCGTTAGCTACGTTACTAAGGAAATGAATC CCCAACAACTAGTTGACATCGTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTC CCCACGCTTT

Table 11: Bacterial endosymbionts identified in larvae of Diamondback moth (*Plutella xylostella*): Second population

Isolate	Identified bacterial endosymbionts
Isolate 1	*Pseudomonas otitidis*
Isolate 2	*Dyella japonica*
Isolate 3	*Bacillus* sp.
Isolate 4	*Aneurinibacillus aneurinilyticus*
Isolate 5	*Ralstonia solanacearum*
Isolate 6	*Ralstonia picketti*
Isolate 9	*Brachybacteria* sp.
Isolate 10	*Kocuria turfanensis*

4.3.4.1 Identification of bacterial endosymbionts after sequencing

The identification of bacterial endosymbionts from the larvae of DBM by 16S rRNA sequence analysis was tabulated. The results revealed that *Enterobacter* sp., and *Proteus mirabilis* exclusively, or predominant in third instar larvae DBM (Table 11).

4.4 THIRD POPULATION: MALLIGERE, SHIVAMOGGA

4.4.1 Isolation of endosymbionts from larvae of Diamondback moth (*Plutella xylostella*)

Third instar larvae of DBM were collected from Malligere, Shivamogga District. The larvae were brought to the laboratory and used for further study.

4.4.1.1 Colony characterization

The colony character recorded and enumerated (Plate 10). The most of the bacterial colonies isolated from starved third instar larvae of DBM were predominantly slightly dry texture, raised, pasty looking and white in colour. Some colonies were

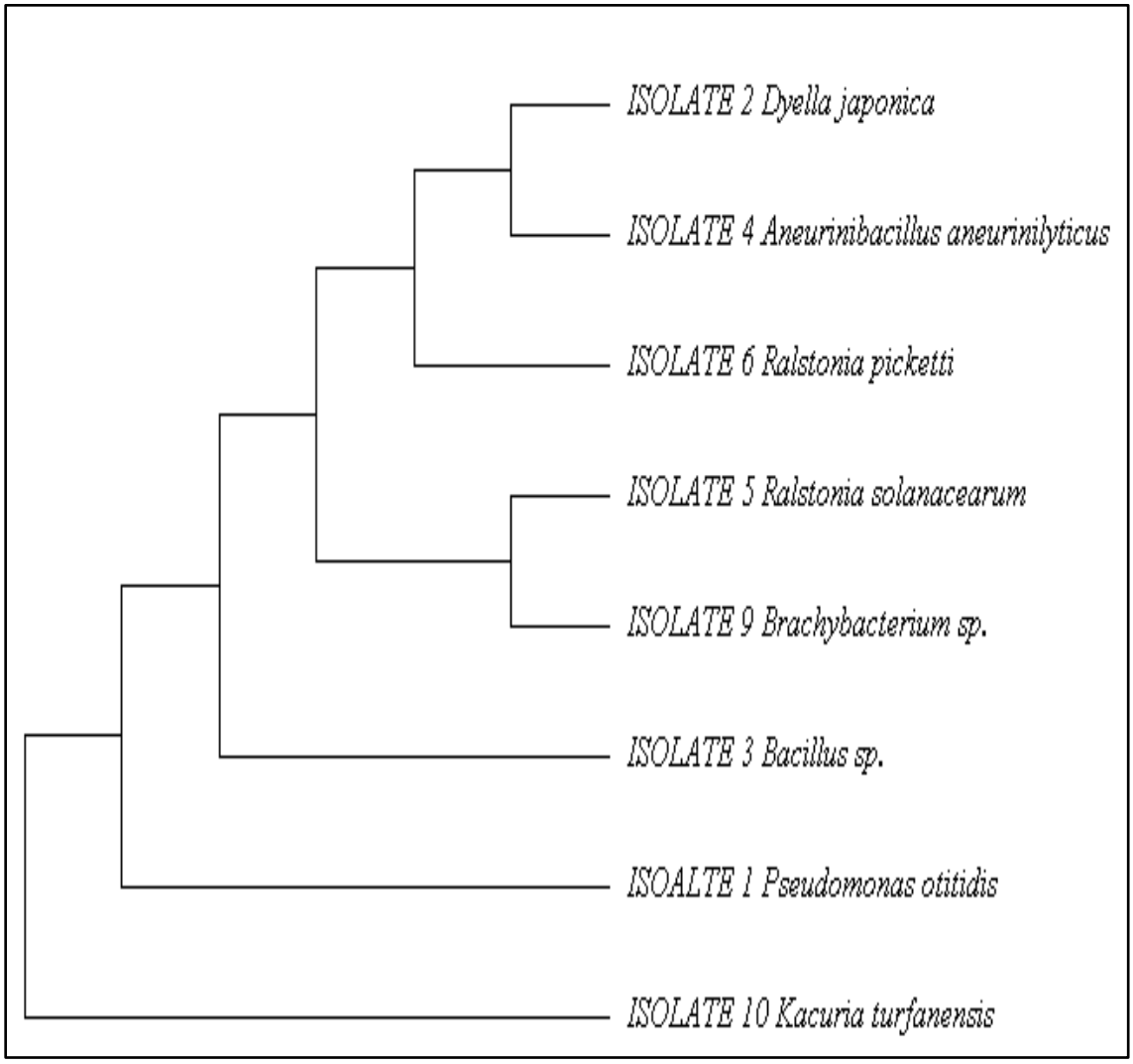


Fig. 2. Phylogenetic tree showing bacterial diversity in Diamondback moth (*Plutella xylostella*): Second population

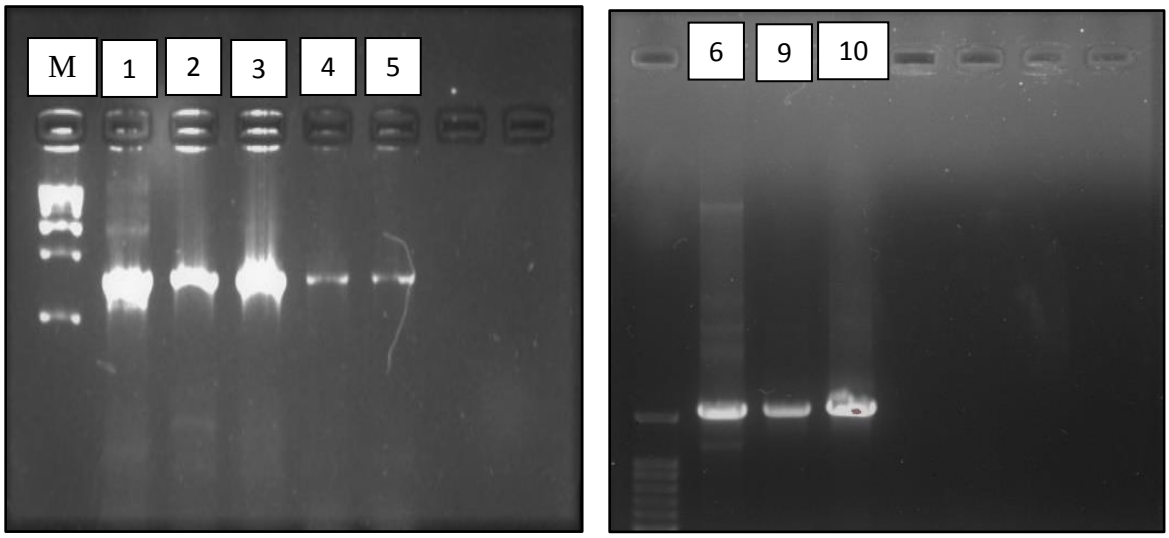


Plate 9: Agarose gel showing amplification of 1000 bp gene corresponding to 16S rRNA, M-Marker DNA-1000bp: second population

(1) ISOLATE 1, (2) ISOLATE 2, (3) ISOLATE 3, (4) ISOLATE 4, (5) ISOLATE 5, (6) ISOLATE 6, (7) ISOLATE 9 (8) ISOLATE10

smooth, small, convex, creamy white coloured and others were rough, large, concave, yellow coloured (Table 12).

4.4.1.2 Bacterial strains isolated from larvae of Diamondback moth (*Plutella xyostella*)

Different colonies were picked from inoculated plates and streaked for purification plates were observed after 24 hours of inoculation. Totally six bacterial strains were isolated from third instar larvae of DBM as shown in Table 12.

4.4.1.3 Differentiation of bacteria using staining technique

The isolated bacterial strains were observed after 24 hours of inoculation. Gram's staining was done for each isolate which were picked from different inoculated colony plates. Among the six isolated bacterial strains, five strains were found to be gram negative rods and remaining one was gram positive ovoid shaped bacteria (Table 12 and Plate 11).

Table 12: Morphological features of bacterial strains: Third population

SI. No.	Isolates	Colony morphology	Cell shape	Gram reaction
	NA			
Isolate A	10^{-4}, R_2, I_1	Shiny, small, round, convex, Yellow	Rod	Negative
Isolate B	10^{-4}, R_1, I_1	Smooth, Creamy white with entire edges	Cocci (Ovoid)	Positive
Isolate D	10^{-4}, R_1, I_3	Mucoid, small round, tan, waves across plate	Rod	Negative
Isolate E	10^{-5}, R_2, I_1	Large, Dull gray, non-swarming	Rod	Negative
	LB			
Isolate L	10^{-4}, R_1, I_1	Convex, small, White	Rod	Negative
	PDA			
Isolate O	$10^{-3,}$ R_2, I_1	White (slightly dry)	Rod	Negative

4.4.2 Biochemical characterization of isolated bacterial strains

All the isolates were subjected to biochemical tests, after 48 hours. Observations were recorded and presented in Table 13 and Plate 12.

- **Indole production test**

After adding kovac's reagent to each tubes including unioculated tube, Isolate D and E strains were developed cherry red colour in the tubes (positive test) and Isolate A, B, L and O strains were not developed any cherry red colour in the tubes (negative test).

- **Methyl-red test**

Methyl red indicator was added to each tubes including uninoculated tube and observed that the Isolate B and E strains were developed red colour throughout the tubes indicated positive for the test whereas, Isolate A, D, L, and O strains were not found any red colour throughout the tubes indicated negative for the test.

- **Voges-Proskauer test**

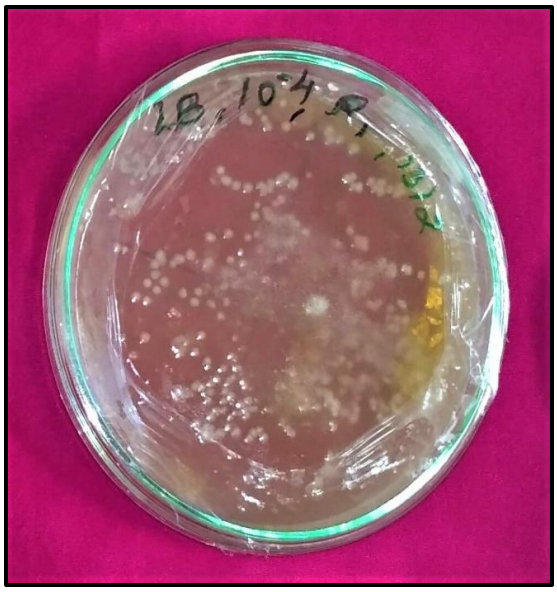
After adding V-P reagent I (napthol solution) and V-P reagent II (40% potassium hydroxide) to each tubes including uninoculated tube, ruby pink (red) colour developed in Isolate A, B, E and O tubes were indicated positive for this test whereas Isolate D and L tubes were not developed ruby pink colour in tubes indicated negative for the test.

- **Citrate utilization test**

After 48 hours incubation, observed that the Isolate A, B, D, E, L and O tubes were changed the colour from green to blue indicated positive for this test. No negative result observed for this test.

- **Catalase test**

Hydrogen peroxide at 4-5 drops was added to each tubes including uninoculated tube and observed that the Isolate A, B, D, E, L and O tubes were produced gas bubbles indicated positive for this test. No negative result observed for this test.



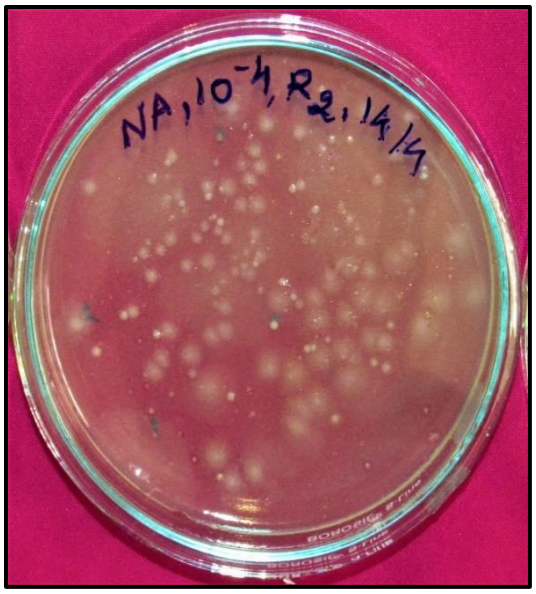
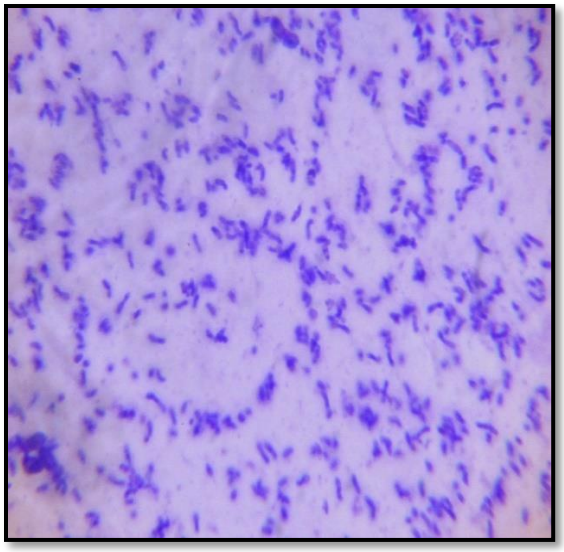


Plate 10: Bacterial colonies of Diamondback moth (*Plutella xylostella*): Third population



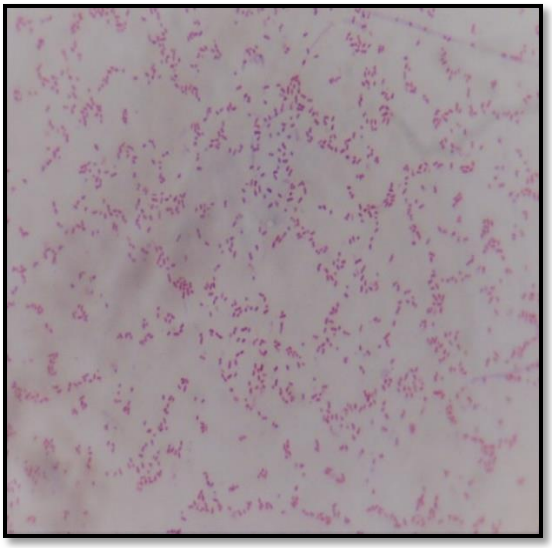
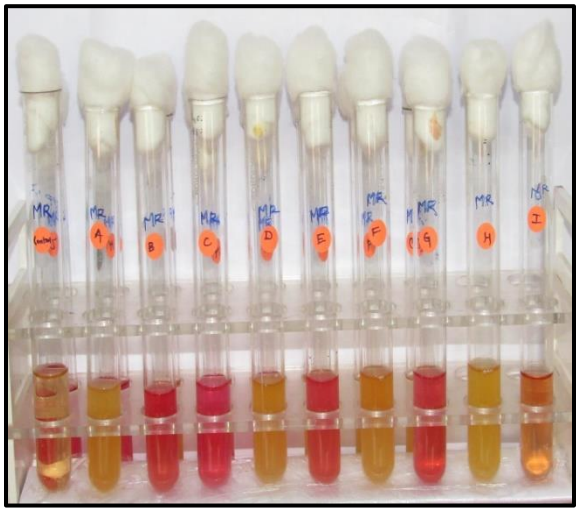


Plate 11: Gram's staining pictures of bacterial samples of Diamondback moth (*Plutella xylostella*): Third population

a) Gram negative bacteria **b) Gram positive bacteria**

INDOLE TEST



MR-VP TEST

CITRATE UTILIZATION TEST

CATALASE TEST

Plate 12: Biochemical characterization of isolated bacteria of Diamondback moth (*Plutella xylostella*): Third population

Table 13: Biochemical features of bacterial strains isolated from larvae of diamondback moth: Third population

SI. No.	1	2	3	4	5
Isolate A	-	-	-	+	+
Isolate B	-	+	-	+	+
Isolate D	+	-	+	+	+
Isolate E	+	+	-	+	+
Isolate L	-	-	+	+	+
Isolate O	-	-	-	+	+

1. Indole production test, 2. Methyl red test, 3. Voges proskauer test, 4. Citrate utilization test, 5. Catalase test. + - Positive, - - Negative

4.4.3 Molecular characterization of isolated endosymbionts

4.4.3.1 DNA bands Visualization from larvae of Diamondback moth (*Plutella xylostella*) on agarose gel

The six bacterial strains were isolated from starved third instar larvae of DBM were subjected to DNA isolation under conditions described in Section 3.4.1 and DNA bands were observed on agarose gel as thick bands under UV transmission and were used for further studies. The sharp bands of genomic DNA were found under Gel documentation photograph (Plate 13). This visualance of sharp bands represented the presence of DNA and which was subjected to PCR as template DNA for the amplification by using the 16s rRNA specific forward and reverse primers.

4.4.3.2 16S rRNA analysis and gel electrophoresis

The DNA was amplified using PCR under conditions described in Section 3.4.4.3. In this study, 16S rRNA–based approach was used to determine bacterial population. Nearly full-length bacterial 16S rRNA fragments were amplified by PCR from each representative isolate using the 16S rRNA primer sets, Fd1 forward primer (GAGTTTGATCCTGGTCA) and Rp2 reverse primer

(ACGGCTACCTTGTTACGACTT). The 16S rRNA fragment was amplified in thermocycler. Amplification products were of the expected 1000bp size and were run on agarose gel. All 6 bacterial isolates were amplified with Fd1 and Rp2 primers.

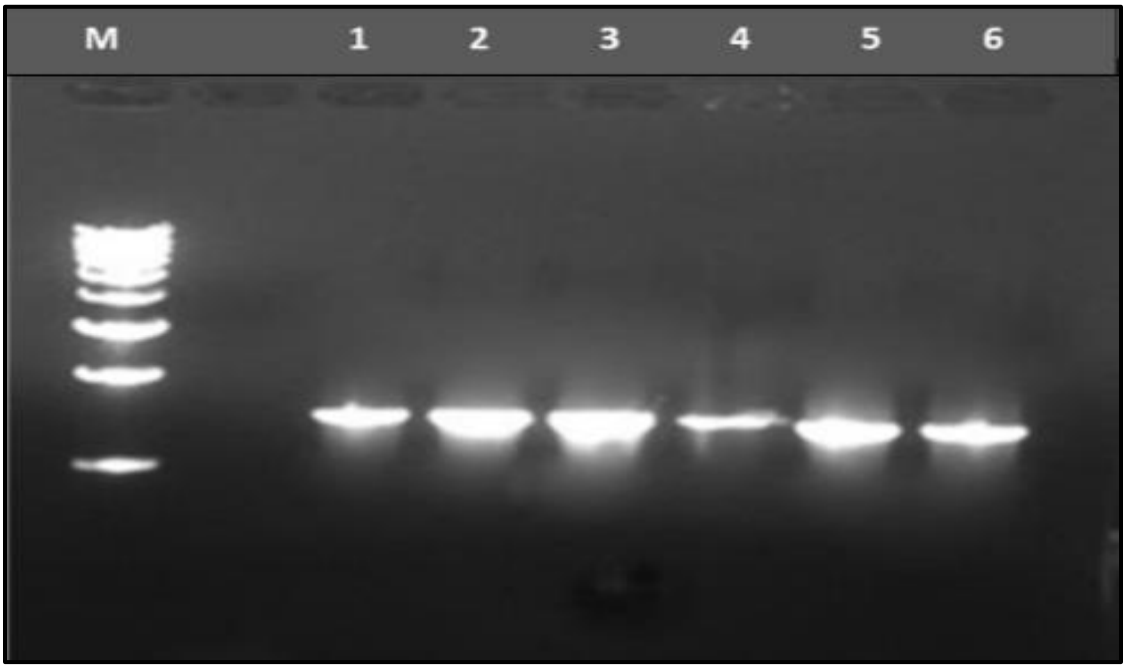
4.4.4 Diversity analysis

The bacterial microflora isolated from DBM larvae were identified as *Enterobacter cloacae, Enterococcus gallinarum, Proteus mirabilis, Providencia vermicola* and *Enterobacter ludwigii*. The Isolate A was identified as *Enterobacter hormaechei,* Isolate B was identified as *Enterococcus gallinarum,* Isolate E was identified as *Providencia vermicola,* Isolate O was identified as *Enterobacter ludwigii.* The Isolate D and Isolate L were identified as *Proteus mirabilis* (Fig. 3).

The bacteria isolated from larvae of DBM were placed together to construct the phylogenetic tree by neighbor joining method and in this similarity probabilities were shown. Whereas bacterial species with maximum query coverage were identified and those sequences were submitted to GenBank for the accession numbers to know the diversity in gut microflora by constructing phylogenetic tree in future (Table 14).

4.4.4.1 Identification of bacterial endosymbionts after sequencing

The identification of bacterial endosymbionts from the larvae of DBM by 16S rRNA sequence analysis was tabulated. The results revealed that *Enterobacter* spp., and *Proteus mirabilis* exclusively, or predominant in third instar larvae DBM (Table 15).



(1) ISOLATE A, (2) ISOLATE B, (3) ISOLATE D, (4) ISOLATE E, (5) ISOLATE L, (6) ISOLATE O.

Plate 13: Agarose gel showing amplification of 1000 bp gene corresponding to 16S rRNA gene of Diamondback moth, M-Marker DNA-1000bp

Table 14: Fasta aligned sequences of different isolated bacterial endosymbionts: Third population

SI. No.	ISOLATE	FASTA SEQUENCES OF ISOLATES BACTERIAL ENDOSYMBIONTS
	NA	
ISOLATE A	10^{-4}, R_2, I_1	GCCCCCTTAAGGGAGACTATTCATGCAATCGACGGTAACAGGAAGCAGCTTGCTGCTTCGCT GACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACT GGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCT TGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGA CGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACT CCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATAAGG TTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCA GCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAG GCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTG GCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGA TCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCACGTGCGAG AGCGTGTGGAGCAAACAGGATT
ISOLATE B	10^{-4}, R_1, I_1	GGCACTCGGCCTGCTATAATGCAAGTCGAACGCTTTTTCTTTCACCGGAGCTTGCTCCACCG AAAGAAAAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGA TAACACTTGGAAACAGGTGCTAATACCGTATAACACTATTTTCCGCATGGAAGAAAGTTGA AAGGCGCTTTTGCGTCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAAC GGCTCACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGA GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTC TGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAG AAGAACAAGGATGAGAGTAAAATGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGC TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGG CGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGA GGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCG GTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACT GACGCTGAGGCTCGAAAGCGTGGG

ISOLATE D	10^{-4}, R_1, I_3	GGGCATGCGCAGCTACCATGCAGTCGAGCGGTAACAGGAGAAGCTTGCTTTCTTGCTGACG AGCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGA AACGGTGGCTAATACCGCATAATGTCTACGGACCAAAGCAGGGGCTCTTCGGACCTTGCAC TATCGGATGAACCCATATGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGA TCTCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCT ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGC GTGTATGAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGATAAGGTT AATACCCTTGTCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGC CGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGC GGTCAATTAAGTCAGATGTGAAAGCCCCGAGCTTAACTTGGGAATTGCATCTGAAACTGGTT GGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGT GGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAG CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTAG AGGTTGTGGTCTTGAAGCGTGGCTTCTGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGT ACGGCCGCAAGGTTAAAAC
ISOLATE E	10^{-5} , R_2, I_1	CTCATACGCAGCTACCATGCAGTCGAGCGGTAACAGGGGAAGCTTGCTTCTCGCTGACGAG CGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAAAC GGTGGCTAATACCGCATAATCTCTTAGGAGCAAAGCAGGGGAACTTCGGTCCTTGCGCTATC GGATGAACCCATATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCC TAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG GAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTA TGAAGAAGGCCCTAGGGTTGTAAAGTACTTTCAGTCGGGAGGAAGGCGTTGATGCTAATAT CATCAACGATTGACGTTACCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG TAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTG ATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTGGGAATGGCATCTAAGACTGGTCAGCT AGAGTCTTGTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAG GAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTG GGGAGCAAACAGGATTAGATAC

	LB	
ISOLATE L	10^{-5}, R_1, I_1	CATTTCCCTAGCTTAAGTCTACCATGCAGTCGAGCGGTAACAGGAGAAAGCTTGCTTTCTTG CTGACGAGCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAGGGGGATAACTA CTGGAAACGGTGGCTAATACCGCATAATGTCTACGGACCAAAGCAGGGGCTCTTCGGACCT TGCACTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGC GACGATCTCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATG CCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGATAA GGTTAATACCCTTATCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGC AGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCA GGCGGTCAATTAAGTCAGATGTGAAAGCCCCGAGCTTAACTTGGGAATTGCATCTGAAACT GGTTGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAG ATGTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCG AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGAT TTAGAGGTTGTGGTCTTGAA
	PDA	
ISOLATE O	10^{-3}, R_2, I_1	GGCGATCCGTCTGACTACTATGACAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTTGCTG ACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTG GAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTT GCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGAC GATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTC CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCG CGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATAAGGT TAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG CCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG CGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGG CAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGAT CTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAA AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTT GGAGGTTGTGCCCTTGAGGCGTGGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGG AGTACGGCCGCAAGGTT

Table 15: Bacterial endosymbionts identified in larvae of Diamondback moth (*Plutella xylostella*): Third population

Isolate	Identified bacterial endosymbionts
Isolate A	*Enterobacter cloacae*
Isolate B	*Enterococcus gallinarum*
Isolate D	*Proteus mirabilis*
Isolate E	*Providencia rettgeri*
Isolate L	*Proteus mirabilis*
Isolate O	*Enterobacter ludwigii*

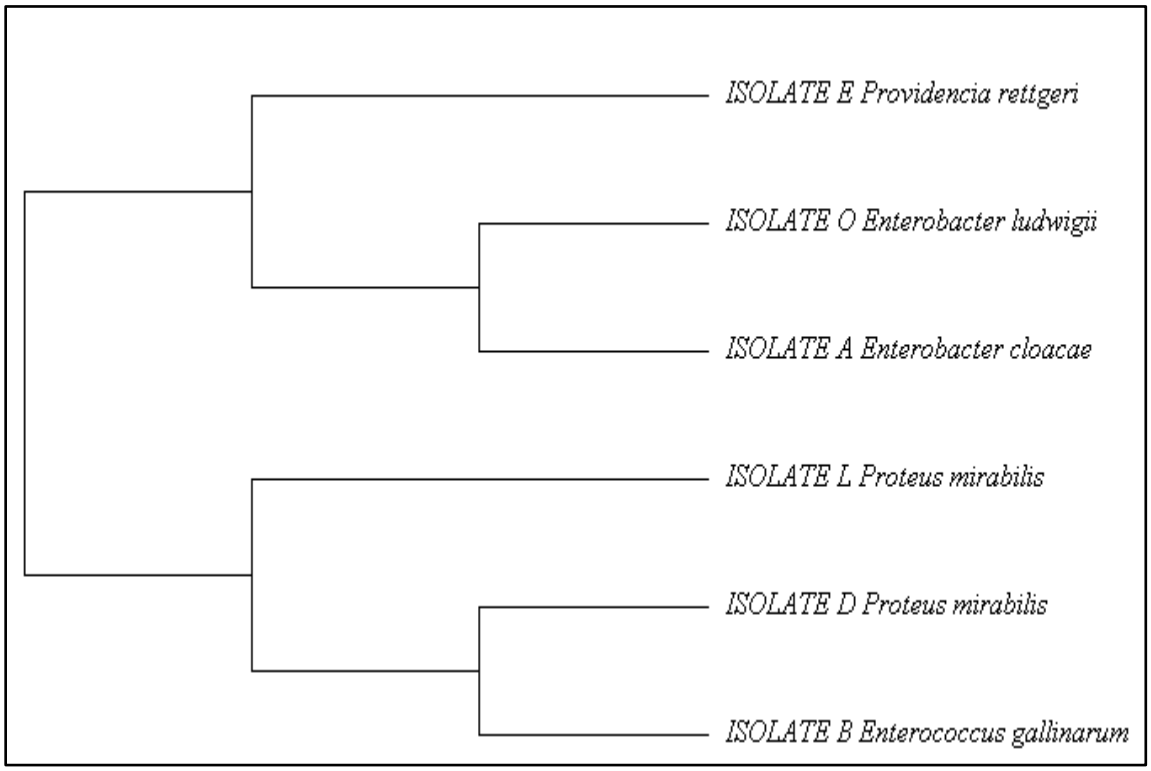


Fig. 3. Phylogenetic tree showing bacterial diversity in Diamondback moth (*Plutella xylostella*): Third population

V DISCUSSION

Diamondback moth, *Plutella xylostella* were found infesting Brassicaceae host plants throughout the year. A peak was recorded from September to October where the maximum population was observed during September and the minimum during May. Pupal population was greatest in September and lowest in July. The maximum number of adults was found in October (Kandoria and Singh, 1994). Devjani and Singh (1999) indicated that the maximum abundance of *P. xylostella* was observed during March. Hunt and Charnley (1981) and Idowu and Edema (2002) reported that the microorganisms in the guts of the insect usually indicate the types and numbers of microorganisms in the environment as microbes normally get into the gut of the insect along with the food.

The present study on "Isolation and characterization of culturable endosymbionts of Diamondback moth (*Plutella xylostella*)" was carried out in the Department of Agricultural microbiology, University of Agricultural Sciences, GKVK, Bengaluru in collaboration with Department of Entomology, UAS, GKVK, Bengaluru. The results discussed in details are presented in this chapter.

5.1 Collection of Diamondback moth (*Plutella xylostella*) populations

During this study, three populations of DBM were collected from three different locations of the state Karnataka: the collection of the larvae, pupae and adult of DBM were done from Sugutur, Kolar District: Rattihalli, Haveri District and from Malligere, Shivamogga District. DBM populations were reared on rearing cage. Indiragandhi *et al.* (2007) collected prothiofos- resistance, prothiofos- susceptible populations of Diamondback moth (*Plutella xylostella*) from the Korea Research Institute of Chemical Technology, Seoul National University, Republic of Korea and field caught population was collected from cabbage field in the Miwon region of Chungbuk province. 16S rRNA sequences revealed that the bacterial population from three DBM larvae population were most diversified and found to be significantly different, irrespective of developmental stage.

Lin *et al.* (2014) collected Different life stages of *P.xylostella* including the fourth instar larvae, pupae, adults, separately from cabbage fields in Yangling region, Shaanxi, China. They analyzed and determined diversity of gut microflora using molecular and traditional culturing methods.

5.2 First population: Sugutur, Kolar

5.2.1 Isolation of endosymbionts from Diamondback moth (*Plutella xylostella*) collected from Sugutur, kolar district

5.2.1.1 Colony characterization of isolated endosymbionts

Based on the bacterial colonies obtained after incubation, an effort was made to study Colony morphology which includes its shape, the margins or edges of the colony, its color, and surface features**.** The isolated bacterial colonies from third instar larvae of DBM were predominantly white rods followed by yellow cocci.

Ramya *et al.* (2016) studied on culturable gut microflora isolated from both larvae and adults of Diamondback moth (*Plutella xylostella*). They isolated 13 bacterial isolates from larvae of Diamondback moth (*Plutella xylostella*), 9 were Gram-negative rods, 2 were Gram positive rods and the remaining 2 were Gram-positive cocci. They also isolated bacterial isolates from adults of DBM, 9 were Gram-negative rods and 3 were Gram-positive rods.

5.2.1.2 Bacterial strains isolated from larvae of Diamondback moth (*Plutella xyostella*)

Totally ten bacterial strains were isolated from third instar larvae of DBM. Xia *et al.* (2013) examined bacteria of Diamondback moth (*Plutella xylostella*) larval midgut in a susceptible and two insecticide (chlorpyrifos and fipronil) resistant lines by Illumina sequencing and they revealed that more than 97% of the bacteria were from three orders: Enterobacteriales, Vibrionales and Lactobacillales. Both insecticide-resistant lines had more Lactobacillales and the much scarcer taxa Pseudomonadales and Xanthomonadales with fewer Enterobacteriales compared with the susceptible strain.

5.2.1.3 Staining technique to study morphological features under microscope

Different colonies were picked from inoculated plates and gram's staining was done for each bacterial isolates of Diamondback moth (*Plutella xyostella*) both gram positive and gram negative bacteria were isolated. Among the 10 bacterial isolates, Isolate 2, 3 and 6 were gram positive bacteria and Isolate 1, 4, 5, 7, 9, 10 and 8 were gram negative (Table 4).

5.2.2 Biochemical characterization of isolated bacterial strains

The pure culture of all isolated bacterial strains were used for biochemical tests including catalase and IMVIC reactions. All the isolates were inoculated into test tube containing particular media or broth and one test tube was kept uninoculated which served as control. The test tubes were incubated for 48 hours. Changes in colour of the media or broth after adding particular reagent for particular test which indicated positive for that particular test.

Anand *et al.* (2009) obtained eleven isolates from digestive tract of *Bombyx mori* and labelled as Isolate 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11. They characterized them morphologically and biochemically. Isolate 1, 2, 3, 4, 5, 6, 7, 9, 10 shown positive result for catalase test. Isolate 1, 2, 3, 4, 6, 7, 8 and 11 shown positive result for citrate utilization test. Isolate 3, 5, 8, 9, 10 and 11 shown positive result for Indole production test. Isolate 2, 3, 4, 5, 6, and 10 shown positive result for methyl red test and Isolate 4, 7, 8, 9 and 11 shown Positive for Voges- proskauer test.

5.2.3 Molecular characterization of isolated endosymbionts

5.2.3.1 DNA bands visualization from larvae of DBM on agarose gel

In this study, DNA was isolated from the six bacterial isolates from the third instar larvae of DBM and DNA bands were observed on agarose gel confirmed the presence of different bacterial DNA which was later sequenced. Ramya *et al.* (2016) isolated acephate degrading bacteria from larval gut of Diamondback moth (*Plutella xylostella*). They made partial 16S rRNA sequencing and all the isolates were identified as *Bacillus cereus* (PXB.C.Or), *Enterobacter asburriae* (PXE), and *Pantoae*

agglomerans (PX-Pt.ag.Jor). All three isolates used acephate as a source of carbon and energy for growth.

Xia *et al.* (2013) extracted total bacterial DNA from the third instar larvae of Diamondback moth (*Plutella xylostella*) using DNA isolation kit method and amplified using PCR with 16S rRNA primers. The amplified products were run on 1.0% agarose gels and purified. Products were sent to the Beijing Genomics Institute (BGI) in Shenzhen to construct the V6 library for sequencing. These 16S rRNA sequences revealed that more than 97% of the bacteria were from three orders: Enterobacteriales, Vibrionales and Lactobacillales. Both insecticide-resistant lines had more Lactobacillales and the much scarcer taxa Pseudomonadales and Xanthomonadales with fewer Enterobacteriales compared with the susceptible strain.

5.2.3.2 16S rRNA analysis and gel electrophoresis

In this study, 16S rRNA based approach was used to determine bacterial populations. Amplified products were of the expected 1000bp size and were run on agarose gel. All six bacterial isolates were amplified with Fd1 forward primer (GAGTTTGATCCTGGTCA) and Rp2 reverse primer (ACGGCTACCTTGTTACGACTT). As a result full-length bacterial 16S rRNA fragments were amplified by PCR.

5.2.4 Identification of bacterial isolates after sequencing

The bacteria identified after sequencing from the larvae of DBM were *Serratia marcescens, Serratia nematodiphila, Serratia* sp., *Bacillus cereus* and *Myroides odoratus.* Among these *serratia* sp. was most predominant (Table 6).

Pseudomonas sp., *Stenotrophomonas* sp., *Acinetobacter* sp., and *Serratia marcescens* strains were identified in prothiofos-resistence larval gut of Diamondback moth (*Plutella xylostella*), *Brachybacterium* sp., *Acinetobacter* sp. and *S. marcescens* were found in prothiofos-susceptible larval gut of Diamondback moth (*Plutella xylostella*) and the field caught population harboured a rather simple gut microflora of

phylotypes belonging to *Serratia.*They concluded that there was a significant variation in the gut bacteria from the three different populations of DBM (Indiragandhi *et al.,* 2007).

Xia *et al.* (2017) obtained gut microbiota of Diamondback moth (*Plutella xylostella*) by metagenomic sequencing. There were three bacterial isolates found more abundantly namely *Enterobacter cloacae, Enterobacter asburiae,* and *Carnobacterium maltaromaticum* and they revealed that these bacteria were responsible for breakdown of plant cell walls, detoxification of plant phenolics, and synthesis of amino acids. The presence of specific enzymes in the microbiota community, such as supporting amino acid synthesis, digestion and detoxification functions, demonstrates the beneficial interactions between *P. xylostella* and its gut microbiota.

Ramya *et al.* (2016) studied diversity of gut microflora in DBM. They screened 11 geographic populations of DBM in India and analyzed them for microbial diversity. They obtained 25 bacterial isolates from larvae (n=13) and adults (n=12) of DBM. They isolated culturable bacterial genomic DNA by SDS-lysis and quantity was checked with 1% agarose gel and amplified 16S rRNA gene with 16S rRNA primers. They made PCR and PCR products sent for sequencing. After 16S rRNA sequencing, they found that in larval gut isolates, gammaproteobacteria was the most abundant (76%), followed by bacilli (15.4%) and adult gut isolates, gammaproteobacteria (66%), bacilli (16.7%) and flavobacteria (16.7%).

Madhusudan *et al.* (2011) isolated gut microbial flora of the wild and laboratory larvae of *H. armigera* and identified culturable bacterial species by sequence analysis of 16S rRNA gene. They found eleven bacterial species of different genera from wild populations. Identified bacteria were *Stenotrophomonas* sp., *Enterococcus casseliflavus*, *Enterococcus* sp., *Enterococcus gallinarum, Enterococcus feacium*, *Bravundimonas diminuta, Staphylococcus sp., Pseudomonas aeruginosa, Acinetobacter calcoaceticus, Bacillus subtilis* and *Rhodococcus sp.* and bacterial species from laboratory populations were *Proteus vulgaris, Cellulosimicrobium cellulans, Klebsiella oxytoca, Bacillus subtillis, Stenotrophomonas maltophilla* and *Pseudomonas* sp.

5.2.4.1 Diversity analysis

From the six bacterial sequences isolated from larvae of DBM, a CLUSTAL W alignment was made and Phylogenetic tree was constructed using Neighbor-joining and Maximum likelihood methods. The several bacterial species isolated with maximum query coverage were identified and those sequences were submitted to GenBank for the accession numbers to know the diversity in gut microflora by constructing phylogenetic tree in future (Fig 1).

Sivakumar *et al.* (2016) isolated thirty culturable gut bacteria associated with sixteen field populations of cotton leafhopper *Ambrasca biguttula biguttula* and identified through 16S rDNA sequences with the available bacterial sequences in public database (GenBank, NCBI). The nucleotide sequences of the collected bacterial strains were subjected to homology searches in DNA databases, which revealed that the sequences of *Bacillus amyloliquefaciens, B. subtilis, Exiguobacterium* sp., *Proteus mirabilis, Staphylococcus pasteuri* of field caught population showed 100% similarity with the 16S rRNA gene sequences of the respective identified organism, while Agrobacterium sp., *Bacillus anthracis, B. atrophaeus, B. cereus, B. megaterium, Enterobacter hormaechei, Enterococcus silesiacus, Erwinia persicina, Klebsiella variicola, Lysinibacillus sphaericus, Microbacterium oxydans, Phenylobacterium sp., Serratia marcescens, Staphylococcus gallinarum, Xanthomonas* sp. showed 99% similarity. *Brevibacterium halotolerans, Hymenobacter gelipurpurascens, Massilia varians, Paenibacillus cineris, Ralstonia solanacearum, Stenotrophomonas maltophilia* showed 98% similarity and *Enterobacter asburiae, Methylobacterium komagatae, Pseudomonas geniculata, Ralstonia pickettii* showed 97% similarity.

5.3 Second population: Ratihalli, Haveri District

5.3.1 Isolation of endosymbionts from Diamondback moth (*Plutella xylostella*) collected from Rattihalli village, Haveri District

5.3.1.1 Colony characterization of isolated endosymbionts

Totally eight bacterial colonies were obtained from larvae of DBM based on their morphology (colour, shape, margin, size etc). Among eight bacterial colonies, most of the

colonies were found as white coloured, rod shaped bacteria, others were found as yellow coloured bacteria. Fungi and yeast was not found in any plates.

Priya *et al.* (2012) reported the bacterial community structure in the midgut of fifth instar larvae of *Helicoverpa armigera* collected from host plants grown in different parts of India. Using culturable techniques, They isolated and identified members *of Bacillus firmus, Bacillus niabense, Paenibacillus jamilae, Cellulomonas variformis, Acinetobacter schindleri, Micrococcus yunnanesis, Enterobacter* sp., and *Enterococcus cassiliflavus*. Besides these the presence of *Sphingomonas, Ralstonia, Delftia, Paracoccus* and *Bacteriodetes* was determined by culture independent molecular analysis.

5.3.1.2 Bacterial strains isolated from larvae of Diamondback moth (*Plutella xyostella*)

Totally eight bacterial strains were isolated from third instar larvae of DBM. Xia *et al.* (2013) examined bacteria of Diamondback moth (*Plutella xylostella*) larval midgut in a susceptible and two insecticide (chlorpyrifos and fipronil) resistant lines by Illumina sequencing and they revealed that more than 97% of the bacteria were from three orders: Enterobacteriales, Vibrionales and Lactobacillales. Both insecticide-resistant lines had more Lactobacillales and the much scarcer taxa Pseudomonadales and Xanthomonadales with fewer Enterobacteriales compared with the susceptible strain.

5.3.1.3 Staining technique to study morphological features under microscope

Totally eight bacterial strains were isolated from DBM and made pure culture. Pure cultures of all strains were used to differentiate between gram positive and gram negative organisms using Gram staining technique. Among the 8 bacterial isolates, Isolate 1, 2, 5, and 6 were found to be gram negative and Isolate 3, 4, 9, and10 were gram positive bacteria (Table 7).

Anand *et al.* (2009) *Bombyx mori* L. (Lepidoptera: Bombycidae) obtained eleven bacterial isolates from the digestive tract of *B. mori*, including the Gram positive *Bacillus circulans* and Gram negative *Proteus vulgaris, Klebsiella pneumoniae, Escherichia coli,*

Citrobacter freundii, Serratia liquefaciens, Enterobacter sp., *Pseudomonas fluorescens, P. aeruginosa, Aeromonas* sp., and *Erwinia* sp.

5.3.2 Biochemical characterization of isolated bacterial strains

The pure culture of all isolated bacterial strains were used for biochemical tests including catalase and IMVIC reactions. All the isolates were inoculated into test tube containing particular media or broth and one test tube was kept uninoculated which served as control. The test tubes were incubated for 48 hours. Changes in colour of the media or broth after adding particular reagent for particular test which indicated positive for that particular test (Table 8).

5.3.3 Molecular characterization of isolated endosymbionts

5.3.3.1 DNA bands visualization from larvae of Diamondback moth on agarose gel

DNA of eight bacterial isolates were extracted using standard protocol and were observed as bands under UV transmission and under Gel documentation photograph, sharp bands of genomic DNA were observed and that represents the presence of DNA and which was subjected to PCR as template DNA for the amplification by using the 16s rRNA specific forward and reverse primers.

Ramya *et al.* (2016) isolated acephate degrading bacteria from larval gut of Diamondback moth (*Plutella xylostella*). They made partial 16S rRNA sequencing and all the isolates were identified as *Bacillus cereus* (PXB.C.Or), *Enterobacter asburriae* (PXE), and *Pantoae agglomerans* (PX-Pt.ag.Jor). All three isolates used acephate as a source of carbon and energy for growth.

5.3.3.2 16S rRNA analysis and gel electrophoresis

In this study, 16S rRNA based approach was used to determine bacterial populations associated with larvae of DBM. Nearly full-length bacterial 16S rRNA fragments were amplified by PCR from each representative isolate using the Fd1 forward primer (GAGTTTGATCCTGGTCA) and Rp2 reverse primer (ACGGCTACCTTGTTACGACTT). The 16S rRNA fragment was amplified in

thermocycler. Amplification products were of the expected 1000bp size and were run on agarose gel.

Raina *et al.* (2015) used Polymerase chain reaction (PCR) and Flourescence in situ Hybridisation (FISH) for identification and localization of bacterial endosymbionts in *B. tabaci* as it harbors one of the highest numbers of endosymbionts which have helped it in becoming a successful global invasive agricultural pest. They identified seven different bacterial endosymbionts and These bacterial endosymbionts are known to provide various nutritional, physiological, environmental and evolutionary benefits to its insect host. Analysis of results obtained by these two techniques revealed the advantages of FISH over PCR. On a short note, performing FISH, using LNA probes proved to be more sensitive and informative for identification as well as localization of bacterial endosymbionts in *B. tabaci* than relying on PCR. This study would help in designing more efficient experiments based on much reliable detection procedure and studying the role of endosymbionts in insects.

5.3.4 Identification of bacterial isolates after sequencing

The bacteria identified after sequencing from the third instar larvae of DBM were *Pseudomonas otitidis, Kocuria turfanensis, Dyella japonica, Bacillus* sp., *Aneurinibacillus aneurinilyticus, Ralstonia solanacearum, Ralstonia picketti, Brachybacterium* sp. Among these *Ralstonia* sp. was most predominant (Table 9).

Madhusudhan *et al.* (2011) identified culturable bacterial species isolated from gut of wild and laboratory larvae of *Helicoverpa armigera* by sequence analysis of 16S rRNA gene. 11 bacterial species of different genera were identified from wild populations, in which *Enterococcus* were found to be predominant and six bacterial species of different genera from laboratory populations. The nucleotide sequences of 11 isolates from wild and 6 isolates from laboratory populations were submitted to NCBI-GenBank and finally showed variation in the bacterial sp., in laboratory and wild populations.

Gut microbiota of Diamondback moth (*Plutella xylostella*) obtained by metagenomic sequencing. There were three bacterial isolates found more abundantly namely *Enterobacter cloacae, Enterobacter asburiae,* and *Carnobacterium maltaromaticum* and they revealed that these bacteria were responsible for breakdown of plant cell walls, detoxification of plant phenolics, and synthesis of amino acids. The presence of specific enzymes in the microbiota community, such as supporting amino acid synthesis, digestion and detoxification functions, demonstrates the beneficial interactions between *P. xylostella* and its gut microbiota (Xia *et al.,* 2017).

Visotto *et al.* (2009) obtained twelve aerobic and anaerobic isolates of proteolytic bacteria from the gut of the velvetbean caterpillar (*Anticarsia gemmatalis*) in calcium caseinate agar. The isolated bacteria were divided into five distinct groups, according to their polymerase chain reaction-restriction fragment-length polymorphism profiles. After molecular analysis, the isolated bacteria were identified as *Bacillus subtilis*, *Bacillus cereus, Enterococcus gallinarum*, *Enterococcus mundtii,* and *Staphylococcus xylosus.*

5.3.4.1 Diversity analysis

From the eight bacterial sequences isolated from larvae of DBM, a CLUSTAL W alignment was made and Phylogenetic tree was constructed using Maximum Parsimony, Neighbor-joining and Maximum Likelihood methods. Several bacterial species isolated with maximum query coverage are identified and those sequences are submitted to GenBank for the accession numbers to know the diversity in gut microflora by constructing phylogenetic tree in future.

Chen *et al.* (2016) investigated biodiversity and activity of gut microbiota across the holometabolous life cycle of *Spodoptera littoralis* using ribosomal tag pyrosequencing of DNA and RNA. They reported that *Enterococcus, Pantoea* and *Citrobacter* were abundant in early-instar, while *Clostridia* increased in late instar. Comparative functional analysis with PICRUSt indicated that early instar larval microbiome was more enriched for genes involved in cell motility and carbohydrate metabolism, whereas in late-instar amino acid, cofactor and vitamin metabolism

increased. Understanding the metabolic activity of these herbivore associated microbial symbionts may assist the development of novel pest management strategies.

Ranjith *et al.* (2016) analysed the composition and diversity of gut bacteria of tomato fruit borer *H. armigera* using Illumina Next-Generation Sequencing of 16S ribosomal RNA amplicons. They identified total of 17 bacterial phyla, 34 classes, 84 orders, 173 families, 334 genera, and 707 species from sequence analysis. *Actinobacteria* was the most dominant groups, followed by *Proteobacteria* and *Firmicutes.* They revealed that function of different gut inhabiting bacteria of *H. armigera,* their role in nutrition, detoxification of lethal insecticide molecules, and defensive action against pathogens. They also found Insecticidal toxin producing bacterial species in associated with the *H. armigera* gut.

5.4 Third population: Malligere, Shivamogga

5.4.1 Isolation of endosymbionts from Diamondback moth (*Plutella xylostella*) collected from Shivamogga district

5.4.1.1 Colony characterization of isolated endosymbionts

Bacterial colonies (Pure culture) were used for observing the colony characters**.** single isolated colonies were picked up and observed there morphology. The isolated bacterial colonies from third instar larvae of Diamondback moth were predominantly white rods followed by yellow rod. Total six bacterial isolates were isolated and labeled as Isolate A, B, D, E, L and O. Isolate A was yellow, rod shaped bacteria. Isolate B was creamy white, cocci shaped bacteria. Isolate D was raised white, rod shaped bacteria. isolate E was dull gray straight rod shaped bacteria. Isolate L was white, rod and Isolate O was raised white slightly dry, rod shaped bacteria.

Iskender *et al.* (2017) collected larvae of chestnut gall wasp, *Dryocosmus kuriphilus* from chesnut field. They obtained four bacterial isolates and labelled as Dk1, Dk2, Dk3 and Dk4 and studied morphologically and biochemically. Among four bacterial isolates, two were spore forming (isolates Dk2 and Dk4) and two were non

spore forming bacteria (Dk1 and Dk3). They found that Dk1 was cream coloured, rod shaped bacteria and Dk2, Dk3, and Dk4 was cream coloured rod shaped bacteria.

5.4.1.2 Bacterial strains isolated from larvae of Diamondback moth (*Plutella xyostella*)

Totally six bacterial strains were isolated from third instar larvae of Diamondback moth (*Plutella xylostella*). Xia *et al.* (2013) examined bacteria of Diamondback moth (*Plutella xylostella*) larval midgut in a susceptible and two insecticide (chlorpyrifos and fipronil) resistant lines by Illumina sequencing and they revealed that more than 97% of the bacteria were from three orders: Enterobacteriales, Vibrionales and Lactobacillales. Both insecticide-resistant lines had more Lactobacillales and the much scarcer taxa Pseudomonadales and Xanthomonadales with fewer Enterobacteriales compared with the susceptible strain.

5.4.1.3 Staining technique to study morphological features under microscope

Different colonies were picked from inoculated plates and gram's staining was done for each bacterial isolates to study the gram character of the isolated bacterial strains. Among the six bacterial isolates, Isolate B was gram positive bacteria and Isolate A, Isolate D, Isolate E, Isolate L and Isolate O were gram negative bacteria (Table 10).

Iskender *et al.* (2017) isolated four bacterial isolates from the larvae of chestnut gall wasp, *Dryocosmus kuriphilus* and named as Dk1, Dk2, Dk3 and Dk4. They characterized all four isolates morphologically and biochemically, they made gram staining and result found that isolate Dk3 was gram negative bacteria and remaining Dk1, Dk2, and Dk4 were gram positive bacteria.

5.4.2 Biochemical characterization of isolated bacterial strains

The pure culture of all isolated bacterial strains were used for biochemical tests including catalase and IMVIC reactions. All the isolates were inoculated into test tube containing particular media or broth and one test tube was kept uninoculated which served as control. The test tubes were incubated for 48 hours. Changes in colour of the

media or broth after adding particular reagent for particular test which indicated positive for that particular test (Table 11).

5.4.3 Molecular characterization of isolated endosymbionts

5.4.3.1 DNA bands visualization from Apis cerana (worker, drone and Queen) guts on agarose gel

In this study, DNA was isolated from the six bacterial isolates from the third instar larvae of Diamondback moth (*Plutella xylostella*) and DNA bands were observed on agarose gel confirmed the presence of different bacterial DNA which was later sequenced. Ramya *et al* (2016) isolated acephate degrading bacteria from larval gut of Diamondback moth (*Plutella xylostella*). They made partial 16S rRNA sequencing and all the isolates were identified as *Bacillus cereus* (PXB.C.Or), *Enterobacter asburriae* (PXE), and *Pantoae agglomerans* (PX-Pt.ag.Jor). All three isolates used acephate as a source of carbon and energy for growth.

Xia *et al.* (2013) extracted total bacterial DNA from the third instar larvae of Diamondback moth (*Plutella xylostella*) using DNA isolation kit method and amplified using PCR with 16S rRNA primers. The amplified products were run on 1.0% agarose gels and purified. Products were sent to the Beijing Genomics Institute (BGI) in Shenzhen to construct the V6 library for sequencing. These 16S rRNA sequences revealed that more than 97% of the bacteria were from three orders: Enterobacteriales, Vibrionales and Lactobacillales. Both insecticide-resistant lines had more Lactobacillales and the much scarcer taxa Pseudomonadales and Xanthomonadales with fewer Enterobacteriales compared with the susceptible strain.

5.4.3.2 16s rDNA analysis and gel electrophoresis

In this study, 16S rRNA based approach was used to determine bacterial populations. Amplified products were of the expected 1000bp size and were run on agarose gel. All 6 bacterial isolates were amplified with Fd1 forward primer (GAGTTTGATCCTGGTCA) and Rp2 reverse primer

(ACGGCTACCTTGTTACGACTT). As a result full-length bacterial 16S rRNA fragments were amplified by PCR.

5.4.4 Identification of bacterial isolates after sequencing

The bacteria identified after sequencing from the third instar larvae of Diamondback moth (*Plutella xylostella*) were *Enterobacter cloacae, Enterococcus gallinarum, Proteus mirabilis, Providencia vermicola* and *Enterobacter ludwigii.* Among these *Proteus* sp. was most predominant (Table 12).

Gut microbiota of Diamondback moth (*Plutella xylostella*) obtained by metagenomic sequencing. There were three bacterial isolates found more abundantly namely *Enterobacter cloacae, Enterobacter asburiae,* and *Carnobacterium maltaromaticum* and they revealed that these bacteria were responsible for breakdown of plant cell walls, detoxification of plant phenolics, and synthesis of amino acids. The presence of specific enzymes in the microbiota community, such as supporting amino acid synthesis, digestion and detoxification functions, demonstrates the beneficial interactions between *P. xylostella* and its gut microbiota (Xia *et al.*, 2017).

Visotto *et al.* (2009) obtained twelve aerobic and anaerobic isolates of proteolytic bacteria from the gut of the velvetbean caterpillar (*Anticarsia gemmatalis*) in calcium caseinate agar. The isolated bacteria were divided into five distinct groups, according to their polymerase chain reaction-restriction fragment-length polymorphism profiles. After molecular analysis, the isolated bacteria were identified as *Bacillus subtilis, Bacillus cereus, Enterococcus gallinarum, Enterococcus mundtii,* and *Staphylococcus xylosus.*

Thirty culturable bacteria were isolated from the guts of 16 populations of cotton leafhoppers (*Ambrasca biguttula biguttula*) and were characterized through morphological and molecular methods. There was more number of gut microflora associated with the leafhoppers collected from Dharwad. Various *Bacillus* spp. were reported in the Dharwad population. The association of *Enterococcus asburiae, Enterobacter silesiacus* from the guts of leafhoppers of Guntur. *Enterococcus hormaechei* was isolated from the insects of Bangalore. The predominant bacterial genera

identified in *A.biguttula biguttula* were *Serratia*, *Bacillus*, *Enterococcus*, *Enterobacter*, *Pantoea*, *Methylobacterium*, *Stenotrophomonas*, *Pseudomonas* and *Paenibacillus* (Sivakumar *et al.*, 2016)

Xia *et al.* (2017) obtained gut microbiota of Diamondback moth (*Plutella xylostella*) by metagenomic sequencing. There were three bacterial isolates found more abundantly namely *Enterobacter cloacae*, *Enterobacter asburiae*, and *Carnobacterium maltaromaticum* and they revealed that these bacteria were responsible for breakdown of plant cell walls, detoxification of plant phenolics, and synthesis of amino acids. The presence of specific enzymes in the microbiota community, such as supporting amino acid synthesis, digestion and detoxification functions, demonstrates the beneficial interactions between *P. xylostella* and its gut microbiota.

Ramya *et al.* (2016) studied diversity of gut microflora in DBM. They screened 11 geographic populations of DBM in India and analyzed them for microbial diversity. They obtained 25 bacterial isolates from larvae (n=13) and adults (n=12) of DBM. They isolated culturable bacterial genomic DNA by SDS-lysis and quantity was checked with 1% agarose gel and amplified 16S rRNA gene with 16S rRNA primers. They made PCR and PCR products sent for sequencing. After 16S rRNA sequencing, they found that in larval gut isolates, gammaproteobacteria was the most abundant (76%), followed by bacilli (15.4%) and adult gut isolates, gammaproteobacteria (66%), bacilli (16.7%) and flavobacteria (16.7%).

Madhusudan *et al.* (2011) isolated gut microbial flora of the wild and laboratory larvae of *H. armigera* and identified culturable bacterial species by sequence analysis of 16S rRNA gene. They found eleven bacterial species of different genera from wild populations. Identified bacteria were *Stenotrophomonas* sp., *Enterococcus casseliflavus*, *Enterococcus* sp., *Enterococcus gallinarum*, *Enterococcus feacium*, *Bravundimonas diminuta*, *Staphylococcus sp.*, *Pseudomonas aeruginosa*, *Acinetobacter calcoaceticus*, *Bacillus subtilis* and *Rhodococcus sp.* and bacterial species from laboratory populations were *Proteus vulgaris*, *Cellulosimicrobium cellulans*, *Klebsiella oxytoca*, *Bacillus subtillis*, *Stenotrophomonas maltophilla* and *Pseudomonas* sp.

5.4.4.1 Diversity analysis

From the six bacterial sequences isolated from larvae of DBM, a CLUSTAL W alignment was made and Phylogenetic tree was constructed using Neighbor-joining and Maximum likelihood methods. The several bacterial species isolated with maximum query coverage are identified and those sequences are submitted to GenBank for the accession numbers to know the diversity in gut microflora by constructing phylogenetic tree in future.

Kikuchi., (2009) focused on diversity of bacterial endosymbionts in insects and several model system with culturable endosymbionts, which provide a new perspective towards understanding how intimate symbiotic associations may have evolved and how they are maintained insects. They observed endosymbiotic associations *in vivo* through culture- independent molecular techniques, such as quatitative PCR, molecular phylogeny and *in situ* hybridization, as well as genomic and metagenomic analysis.

Engel and moran, (2013) studied diversity in structure and function of gut bacteria in insects. Insect guts present distinctive environments for microbial colonization, and bacteria in the gut potentially provide many beneficial services to their hosts. . Insect digestive tracts vary extensively in morphology and physicochemical properties, factors that greatly influence microbial community structure. Social insects, such as termites, ants, and bees provide opportunities for transfer of gut bacteria, and some of the most distinctive and consistent gut communities, with specialized beneficial functions in nutrition and protection. gut bacteria of other insects have also been shown to contribute to nutrition, protection from parasites and pathogens, modulation of immune responses, and communication.

VI SUMMARY

Isolation of gut microflora is important to identify the microbial species by observing colony morphology and obtain pure cultures, by streaking a loop full of culture on nutrient agar medium by quadrant streaking to get well separate colonies. Isolated colonies were streaked on NA slants for bacteria. The tubes were incubated till the bacteria shown the growth in agar slant and later they were stored in refrigerator until further use. The investigation were undertaken on the Diamondback moth, *Plutella xylostella* collected from different locations of Karnataka state viz., Sugutur, Kolar district; Rattihalli, Haveri district; Malligere, Shivamogga district; to know the variation in microflora in the different locations and varied farmers plant protection.

Identification and characterization of microbes was done by gram staining technique for each isolated strain obtained from larvae of DBM to differentiate between gram positive and gram negative organisms, while simultaneously learning about the cellular morphology and arrangement.

In the present study, we isolated bacterial strains (endosymbionts) isolated from larvae of DBM collected from three different locations of the Karnataka state. Morphological feature studies of bacterial colonies isolated are as follows

- Morphological features of bacterial strains (endosymbionts) isolated from DBM collected from Sugutur, Kolar district

  Isolate 1- Round, regular, dark yellow, Straight Rod (gram negative). Isolate 2- Yellow, cocci (gram positive), Isolate 3- White, Cocci (gram positive), Isolate 4- Light yellow, cocci (gram negative). Isolate 5- Opaque, irregular, White, Rod (gram negative). Isolate 6- Large, irregular, convex, White, Rod (gram positive), Isolate 7- Large, concave, White, Rod (gram negative). Isolate 8- Filamentous, Creamy dark yellow, Rod (gram negative). Isolate 9- Small, round, mucoid White, Rod (gram negative). Isolate 10- Creamy yellow, Cocci (gram negative).

- Morphological features of bacterial strains (endosymbionts) isolated from DBM collected from Rattihalli, Haveri district

  Isolate 1- White, circular, concave, Rod (gram negative). Isolate 2- Dark yellow, Rod (gram negative). Isolate 3- Dry, dull white, Rod (gram positive). Isolate 4- Raised, irregular, creamy, Rod (gram positive). Isolate 9- Circular, smooth, yellow, cocci/rod (gram positive). Isolate 10- Smooth, shiny, circular, convex, pinkish, Cocci (gram positive). Isolate 5- Large fluidal white, Rod (gram negative). Isolate 6- Dense dark white, Rod (gram negative).

- Morphological features of bacterial strains (endosymbionts) from DBM collected from Mlligere, Shivamogga district

  Isolate A- Shiny, small, round, convex, Yellow, Rod (gram negative). Isolate B- Smooth, Creamy white with entire edges, Cocci (Ovoid), (gram positive). Isolate D- Mucoid, small round, tan, waves across plate, Rod (gram negative). Isolate E- Large, Dull gray, non-swarming, Rod (gram negative). Isolate L- Convex, small, White, Rod (gram negative). Isolate O- White (slightly dry), Rod (gram negative).

- Biochemical characterization of isolated bacterial strains- Sugutur, Kolar district

  Isolate 1, 2, 6, 8 and 10 indicated positive result for indole production test. Isolate 1, 2, 5, 6, 7 and 9 were observed positive result for methyl red test. Isolate 3, 5 and 9 were found positive result for Voges-Proskauer test. Isolate 3, 5, 7, 9, and 10 were found positive result for Citrate utilization test. Isolate 9 was observed positive result for catalase test.

- Biochemical characterization of isolated bacterial strains- Rattihalli, Haveri District

  Isolate 3, 4, 5 and 6 were indicated positive result for indole production test. Isolate 2, 3, 5, 6 and 9 were observed positive result for methyl red test. Isolate 1, 3, 5 and 6 were found positive result for Voges-Proskauer test. Isolate 1, 2, 3, 6, and 9 were found positive result for Citrate utilization test. Isolate 2, 3 and 4 were observed positive result for catalase test.

- Biochemical characterization of isolated bacterial strains- Malligere, Shivamogga District

  Isolate D and E indicated positive result for indole production test. Isolate B and E were observed positive result for methyl red test. Isolate A, B, E and O were found positive result for Voges-Proskauer test. Isolate A, B, D, E, L and O were found positive result for Citrate utilization test. Isolate A, B, D, E, L and O were observed positive result for catalase test.

Molecular characterization of bacterial isolates from larvae of DBM by extracting DNA from all the bacterial strains isolated from larvae of DBM and the sharp bands of genomic DNA under Gel documentation photograph was found. This visualance of sharp bands represented the presence of DNA and which was subjected to PCR as template DNA for the amplification by using the 16s rRNA specific Fd1 forward primer (GAGTTTGATCCTGGTCA) and Rp2 reverse primer (ACGGCTACCTTGTTACGACTT). Amplification products were of the expected 1000bp size and were run on agarose gel.

The 16S rRNA sequence analysis revealed the identification of bacteria isolated from the larvae of Diamondback moth as follows,

- The identified bacteria isolated from the larvae of DBM: Sugutur, Kolar

  Serratia marcescens, Serratia nematodiphila, Bacillus cereus, Serratia sp., *Myroides odoratus, Serratia marcescens.*

- The identified bacteria isolated from the larvae of DBM: Rattihalli, Haveri

  Pseudomonas otitidis, Dyella japonica, Bacillus sp., *Aneurinibacillus aneurinilyticus, Ralstonia solanacearum, Ralstonia picketti, Brachybacteria* sp., *Kocuria turfanensis.*

- The identified bacteria isolated from the larvae of DBM: Malligere, Shivamogga

  Enterobacter cloacae, Enterococcus gallinarum, Proteus mirabilis, Providencia rettgeri, Proteus mirabilis, Enterobacter ludwigii

The above result revealed that *Serratia* spp. exclusively and predominant in first population, *Ralstonia* spp. more predominant in second population and *Proteus spp.*in third population. The above 16S rRNA sequence results also revealed that, the microflora varied from three different locations across the Karnataka.

The bacteria identified in first population were *Serratia marcescens, Serratia nematodiphila, Bacillus cereus, Serratia* sp., *Myroides odoratus, Serratia marcescens* whereas in Second population *Pseudomonas otitidis, Dyella japonica, Bacillus* sp., *Aneurinibacillus aneurinilyticus, Ralstonia solanacearum, Ralstonia picketti, Brachybacteria* sp., *Kocuria turfanensis* were found and in third population *Enterobacter cloacae, Enterococcus gallinarum, Proteus mirabilis, Providencia rettgeri, Enterobacter ludwigii,* so there is no similar microbes found in three different populations which was collected from the state Karnataka which shows that the endosymbionts (microflora) were completely different from place to place and completely diversified.

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Annexure - I

Ingredients for preparation of symbionts culture media

Nutrient Agar (NA)

SI. No.	***Chemicals***	***Quantity***
01	Beef extract	3.0 gm
02	Peptone	5.0 gm
03	Nacl	5.0 gm
04	Agar	16.0 gm
05	Distilled water	1000 ml
06	pH	7.4

Luria Bertani Agar (LB)

SI. No.	***Chemicals***	***Quantity***
01	Tryptone	10.0 gm
02	Yeast extract	5.0 gm
03	Nacl	10.0 gm
04	Agar	16.0 gm
05	Distilled water	1000 ml
06	pH	7.0

Potato Dextrose Agar (PDA)

SI. No.	***Chemicals***	***Quantity***
01	Potato	200 gm / litre
02	Dextrsoe	20.0 gm
03	Agar	15.0 gm
04	Distilled water	1000 ml
05	pH	6.5

Yeast Extract Peptone Dextrose Agar (YEPDA)

SI. No.	***Chemicals***	***Quantity***
01	Yeast extract	10.0 gm
02	Peptone	20.0 gm
03	Dextrose	20.0 gm
04	Agar	16.0 gm
05	Distilled water	1000 ml
06	pH	6.5

1% Tryptone broth

SI. No.	*Chemicals*	*Quantity*
01	Peptone	10.0 gm
02	Distilled water	1000 ml

MRVP Broth

SI. No.	*Chemicals*	*Quantity*
01	Peptone	7.0 gm
02	Dextrose	5.0 gm
03	KH_2PO_4	5.0 gm
04	Distilled water	1000 ml
05	pH	6.9

Simmons Citrate Agar

SI. No.	*Chemicals*	*Quantity*
01	$NH_4H_2PO_4$	1.0 gm
02	K_2HPO_4	1.0 gm
03	NaCl	5.0 gm
04	Sodium Citrate	2.0 gm
05	$MgSO_4$	0.2 gm
06	Agar	16.0 gm
07	Bromothymol blue	0.8 gm
08	Distilled Water	1000 ml
09	pH	6.9

Trypticase Soy Agar

SI. No.	*Chemicals*	*Quantity*
01	Trypticase	15.0 gm
02	Phytone	5.0 gm
03	NaCl	5.0 gm
04	Agar	16.0 gm
05	Distilled Water	1000 ml
06	pH	7.3