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Study of diversity of culturable gut bacteria in diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae)

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Abstract
The diamondback moth (DBM) *Plutella xylostella* (Lepidoptera: Yponomeutidae), a well-known destructive insect pest that infests Bracicaceous (=Cruciferae) crops, like broccoli, brussel sprouts, cauliflower, collards, mustard, rapeseed, radish, and turnip worldwide, has developed resistance to synthetic insecticides. Microbial symbionts provide an diverse range of benefits in insect nutrition, e.g. by providing essential amino acids, digestive enzymes or vitamins and responsible for insecticide resistance. The population of DBM was collected from field of Malligere village, Shivamogga district of the state Karnataka, India. The different bacterial strains were isolated from third instar larvae of DBM and characterized each strain based on morphology and biochemical characterization. Most of the isolates were gram negative bacteria. All the isolates were shown positive results for citrate utilization test and catalase test. The genomic DNA, 16Sr RNA sequencing, PCR were done for identification of isolates at genus level. Six different bacterial isolates were identified such as *Enterobacter cloacae, Enterococcus gallinarum, Proteus mirabilis, Providencia* sp., *Enterobacter hormaechei and Proteus mirabilis.* This study aimed to determine the diversity and characterization of gut bacteria of diamondback moth.

Keywords: Diamondback moth (DBM), gut bacteria, morphology, biochemical tests, 16S rRNA

Introduction
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) [5]. 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) [1]. The bacterial association with insects plays a significant role in the host insect morphogenesis, food digestion, nutrition, antifungal toxin production, pheromone production, regulation of pH, synthesis of vitamins, temperature tolerance, resistance against parasitoid development, and detoxification of noxious compounds (Dillon and Dillon 2004) [4].

In India, DBM is the major, ubiquitous and year round insect pest hindering the economic production of brassica crops (Sandur, 2004) [11]. 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) [2].

The first report of DBM resistance to insecticides (DDT and parathion) in India was made by Varma and Sandhu (1968) [13] in Ludhiana (Punjab) and it was confirmed by Deshmukh and Saramma (1973) [3]. 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) [14]. 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) [12].

The 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 *Brach bacterium* sp. PSGB10, *Pseudomonas* sp. PRGB06, and *Serratia marcescens* FLGB16 strains were found to cross-utilize the siderophores of various entomopathogens, including *Bacillus thuringiensis* (Indiragandhi *et al*,. 2008) [6]. The different life stages (fourth instar larvae, pupae and adults) of the Diamondback moth were collected to find out different microbial diversity of culturable endosymbionts in Diamondback moth (*Plutella xylostella*). 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 (Lin *et al.,* 2014) [8].

Ramya *et al.* (2016) [9] 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.

Material and Methods

Isolation of culturable gut bacteria

The different life stages of DBM such as larvae, pupae and adults were collected from the field of Malligere, Shivamogga district (13.92° N, 75.56° E) of the state Karnataka, India. The larvae of DBM were kept starvation for 24 hours before killing. The starved third instar larvae were selected, surface sterilized with 70 per cent ethanol for 1 minute followed by 0.1 per cent sodium hypochlorite for 1 minute, then rinsed with sterile distilled water for 2 to 3 times to remove the external microflora.

Serial dilution and plating

The surface sterilized larvae were crushed 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 was made up to 10^{-6} dilutions. The aliquot of 100 µl of all the dilutions were plated on Nutrient Agar (NA) and Luria bertani (LB) agar for the isolation of bacteria. 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. Colonies of different morphological characters were picked up and pure cultured on the same media and then preserved. The quadrant streak method was used primarily to make pure cultures from specimen containing mixed microflora.

Characterization of isolated bacteria

The preliminary identification of bacterial isolates was based on morphological characteristics, gram staining and biochemical analysis. Bacterial isolates were selected based on morphology like size, shape and colour. Gram staining was done based on standard protocol (Fig 2).

Biochemical characterization of isolated bacteria

The basic biochemical characterizations such as IMVIC and catalase test were done for all selected bacterial isolates. IMViC reactions consist of Indole production test, Methyl red and Voges proskauer test, Citrate test and Catalase test.

The tryptone broth for Indole production test, MR-VP broth for Methyl Red and Voges Proskauer tests, Simmons Citrate Agar for Citrate utilization test and trypticase soy agar for Catalase test were prepared in test tubes in the form of slants and broth. The pure cultures of selected bacteria were added in all the test tubes and incubated for 24-48 hours. In Indole production test, cheery red ring on the top layer of the broth was formed after adding kovac's reagent, which indicates positive for the test. In Methyl Red test, methyl red indicator were added in test tubes containing MR-VP broth, the production of red colour indicates the positive result and having ability to oxidize glucose. In Voges Proskauer test, pinkish red color of the broth was appeared after adding VP reagent 1 and 2, which indicates the positive result. For Citrate utilization test, changes in color of the media from green to blue, indicates positive reaction and for catalase test, formation of bubbles on the surface after adding hydrogen peroxide indicate positive for this test as shown in Fig 3 and Table 3.

Molecular identification

Isolation of bacterial genomic DNA by CTAB method

Bacterial cultures were grown in Luria Bertani broth (LB). The pellet was obtained by centrifugation at 10000 rpm for 1 minute and resuspended in 1X TE buffer, then 10% SDS, 5 µl of RNase, 4 µl of Proteinase K and 4 µl of lysozyme were added. The tubes were kept in hot water bath for 30 minutes at 65°C. The 5M NaCl and 80 µl of CTAB buffer was added. The tubes were incubated in hot water bath for 30 minutes at 65°C. The equal volume of Chloroform: Isoamyl alcohol (24:1) was added and centrifuged for 5 minutes at 10000 rpm. The equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was prepared and added in to the supernatant, centrifuged for 5 minutes in 10000 rpm. Again supernatant was transferred to a fresh tube and 1 volume of chilled isopropanol was added to precipitate the nucleic acid. Tubes were incubated at room temperature and centrifuged at 10000 rpm for 5 minutes. The 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.

Agarose gel electrophoresis

1% agarose gel was prepared by using 1X TAE buffer and added 2 µl of ethidium bromide. Comb was placed in boat, agar solution was poured slowly and kept for solidification. The comb was removed carefully and the gel was immersed with buffer in electrophoresis tank. The DNA samples were mixed with gel loading dye and then loaded into the wells. The gel was run at 60 volts for approximately 30 minutes. Gel was viewed under gel documentation unit and was photographed.

Polymerase Chain Reaction (PCR)

The 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, Fp forward primer (GAGTTTGATCCTGGTCA) and Rp reverse primer (ACGGCTACCTTGTTACGACTT). The 16S rRNA fragment was amplified in thermocycler. All the ingradients ie Master mix, Fp, Rp, sterile water and DNA templates (samples) were added and kept on ice. To ascertain the specificity of the PCR amplification, negative control (PCR mixture without DNA template) was also prepared. The tubes

were loaded into PCR and selected the appropriate conditions (94˚C for 3 minute, 94˚C for 30 seconds, 60˚C for 1 minute, 72˚C for 1 minute and 72˚C for 2.5 minutes) for the region being amplified.

Phylogenetics analysis

The purified PCR products were sent for sequencing. The 16S rRNA gene sequences was blast searched in NCBI database (http://www.ncbi.nlm.nih.gov), which were used to construct a phylogenetic tree by the character-based maximum-likelihood method with molecular evolutionary genetic analysis (MEGA7) software after multiple alignments of the data by CLUSTAL W. The phylogenetic tree was visualized by using tree view. Based on maximum query coverage the bacterial species was identified.

Results

Isolation and characterization of bacterial isolates

The totally six bacterial isolates were obtained based on their morphology among them four bacteria from nutrient agar media and two bacteria from LB media. The bacterial isolates were predominantly circular, small, raised, smooth, pasty looking, white in color. Some colonies were slightly dry texture, raised, irregular, concave, yellow color and others were smooth, circular, creamy white color. The five isolates were gram negative and remaining one was gram negative bacteria. Among six isolates, five isolates were rod shaped and one was cocci shaped bacteria (Table 1).

Table 1: Morphological features of bacterial strains of DBM

S. 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
Isolate O	$10^{-3,}$ R_2, I_1	White (slightly dry)	Rod	Negative

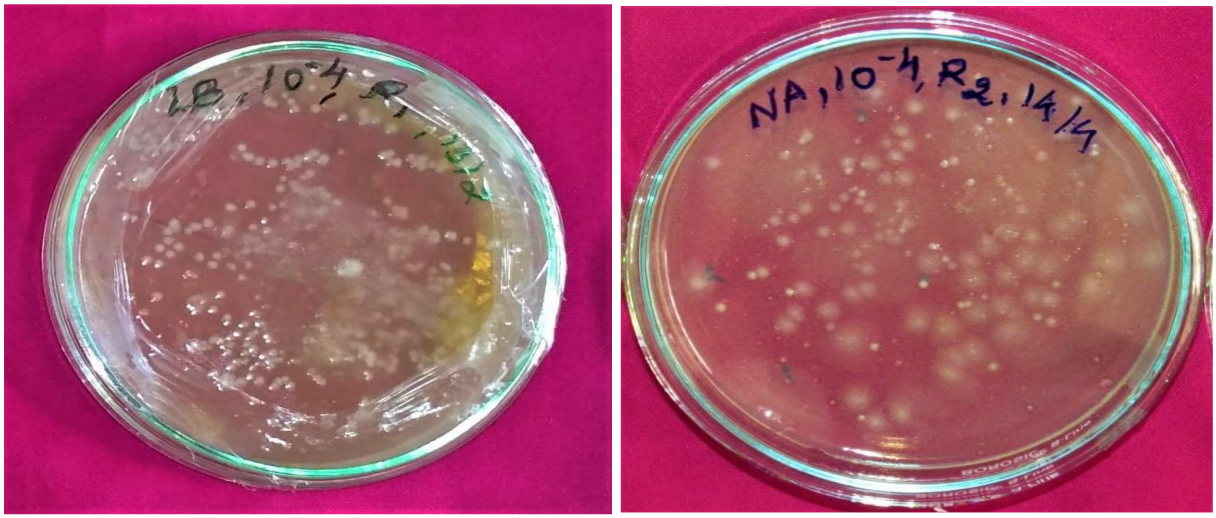


Fig 1: Bacterial colonies of Diamondback moth (*Plutella xylostella*)

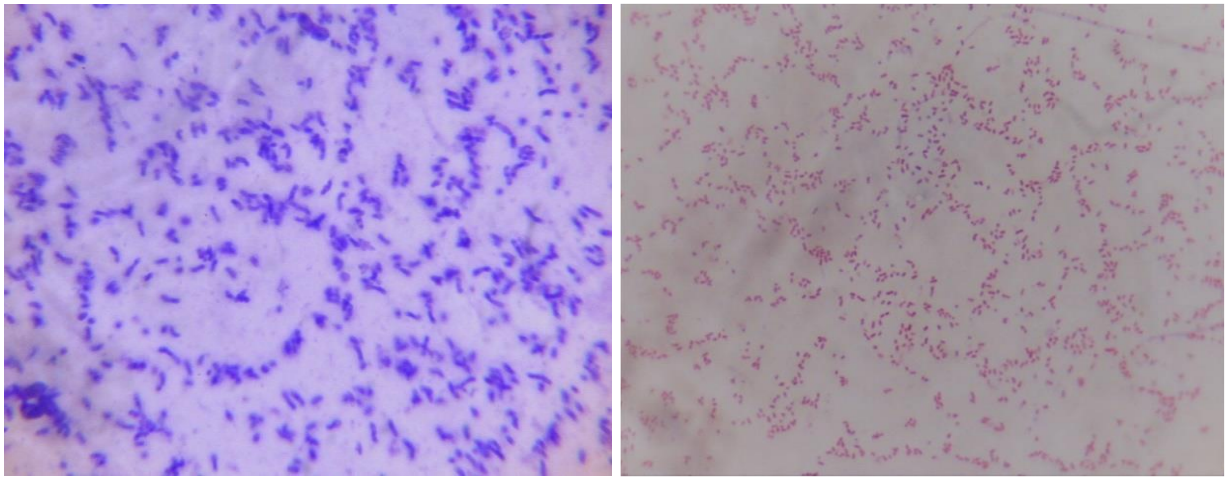
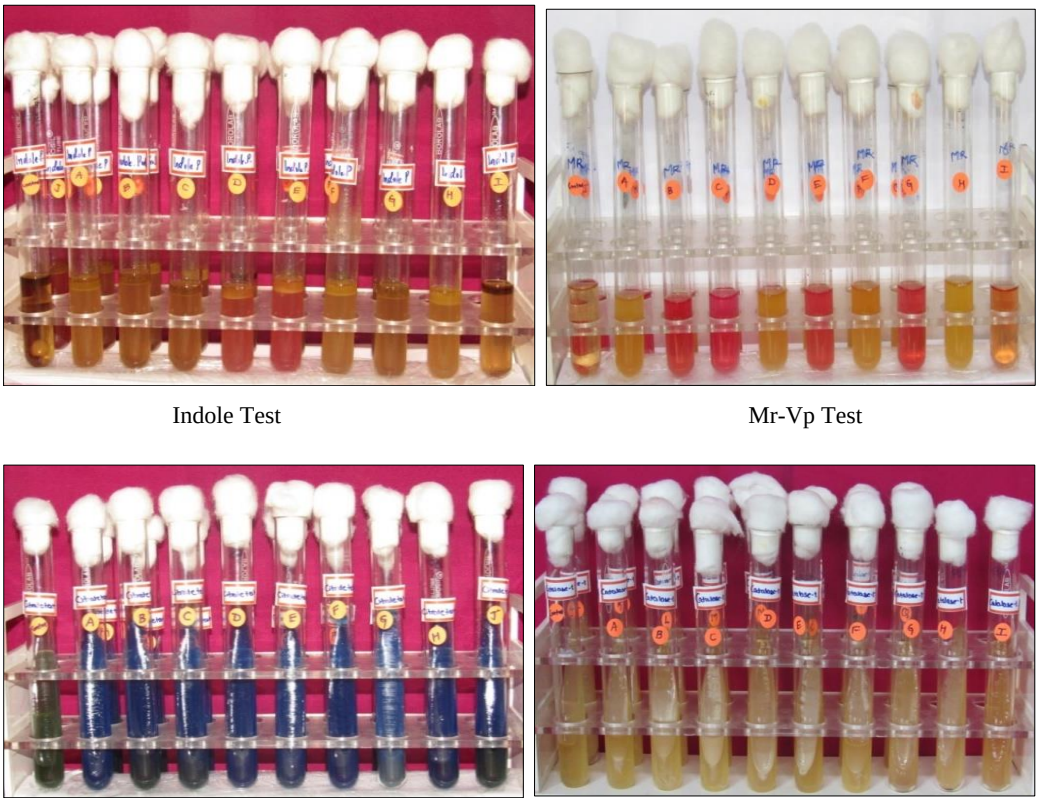


Fig 2: Gram's staining of bacteria. a) Gram negative bacteria b) Gram positive bacteria

Biochemical characterization of isolated bacteria

Through the morphology almost same type of bacterial colonies were analysed therefore the bacterial isolates were subjected for biochemical characterization. Among six isolates, all the bacterial isolates were shown positive results for Citrate utilization test and catalase test. Isolate D shown positive result for all the tests except methyl red test which shown negative result. Isolate E shown positive result for all the tests except Voges Proskauer test which shown negative result as shown in Table 2 and figure 3. For further confirmation and identification of isolates, molecular identification was performed.



Indole Test | Mr-Vp Test

Citrate Utilization Test | Catalase Test

Fig 3: Biochemical characterization of isolated bacteria of Diamondback moth (*Plutella xylostella*)

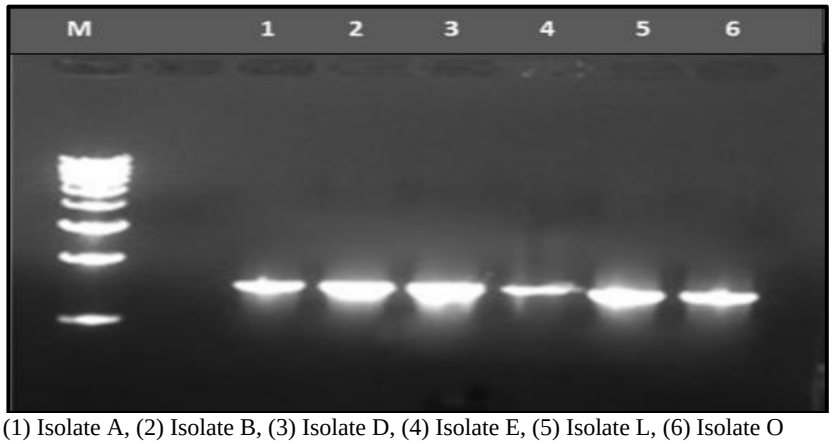
Table 2: Biochemical features of bacterial strains of DBM

S. 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.

Molecular identification of bacterial isolates

The genomic DNA was isolated from six bacterial isolates. The thick DNA bands (Fig. 4) were visualized on agarose gel under gel documentation photograph represents the presence of DNA and which was subjected to PCR amplification in thermocycler with 16S rRNA primers. The amplified products were expected 1000 bp in size. The bacterial isolates were identified as *Enterobacter cloacae* strain BR6-B, *Enterococcus gallinarum* strain 99B (BR46), *Proteus mirabilis* strain ALK418, *Providencia* sp. Strain LJ20, *Proteus mirabilis* strain ALKO44, *Enterobacter hormaechei* strain EAF52 in Table 3.



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

Fig 4: Agarose gel showing amplification of 1000 bp gene corresponding to 16S rRNA gene of Diamondback moth, M-Marker

Table 3: Molecular detection and identification of bacterial isolates of Diamondback moth (*Plutella xylostella*) with percent homology

S. No.	Nucleotide sequence identification	Isolates name	% homology	Accession no.
1.	*Enterobacter cloacae* strain BR6-B	Isolate A	99.46%	MF498504.1
2.	*Enterococcus gallinarum* strain 99B (BR46)	Isolate B	99.87%	KF254559.1
3.	*Proteus mirabilis* strain ALK418	Isolate C	99.77%	KC456548.1
4.	*Providencia* sp. Strain LJ20	Isolate E	99.87%	MG049767.1
5.	*Proteus mirabilis* strain ALKO44	Isolate L	100%	KC456539.1
6.	*Enterobacter hormaechei* strain EAF52	Isolate O	99.88%	MG594001.1

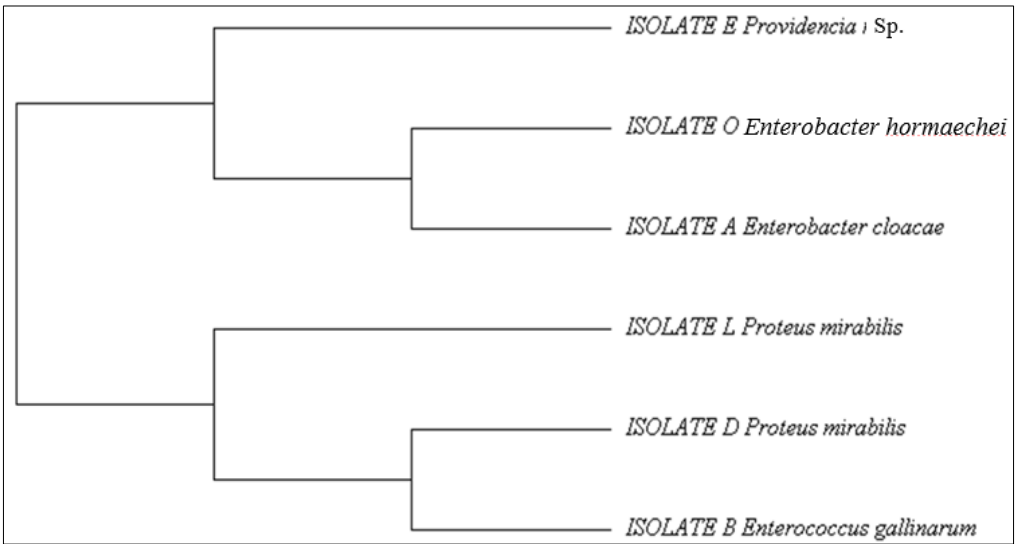


Fig 5: Maximum likelihood tree constructed for partial 16S rRNA gene of bacterial isolates of Diamondback moth (*Plutella xylostella*)

Diversity analysis

From the six bacterial sequences, 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. The 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 (Kikuchi, 2009) [7].

Discussion

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 1). 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 indicates positive result for that particular test (Table 2).

The isolated six bacteria identified after sequencing were *Enterobacter cloacae, Proteus mirabilis Enterococcus gallinarum, Providencia* sp., *Enterobacter hormaechei* and *Proteus mirabilis* (Table 3). The 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) [15].

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