3. MATERIALS AND METHODS

The research work of the present investigation entitled "**Biodiversity of fruit flies (Tephritidae: Diptera) and utilization of gut bacteria in their management"** was carried out in the Departments of Entomology, Microbiology and Plant Pathology (Molecular Plant Pathology laboratory), CSK Himachal Pradesh Krishi Vishvavidyalaya, and Division of Natural Plant Products, Institute of Himalayan Bioresource Technology (IHBT), Palampur (H.P.), during 2008 to 2010. Geographically, the experimental site is situated at $32^{o}6'$ N latitude and $76^{o}3'$ E longitude and at an elevation of 1290.8 m above mean sea level in North Western Himalayas.

The details of materials used and methods employed during the present investigations are described in this chapter.

3.1 Survey and identification of fruit flies infesting cucurbits

An extensive survey was undertaken to know the prevalence and diversity of fruit fly species in Himachal Pradesh and other states of India (Table 3.1) with the help of insect collecting net, fruit fly para-pheromones and collection of infested fruit and flower samples comprising minimum of ten fruit fly infested fruits during the peak activity of fruit flies in the area. The fruit fly infested samples from each location were kept in separate rearing cages (20 x 15 x 18 cm^3) under laboratory conditions at Palampur. The emerging fruit fly adults were identified on the basis of morphological descriptions given by Kapoor *et al.* (1980), Agarwal and Kapoor (1988), White and Elson-Harris (1992), Hardy and Drew (1996), Drew *et al.* (1998), Hancock and Drew (1999), and Drew and Raghu (2002). Identified fruit flies were kept in separate vials and stored under refrigerator at $-20^{o}C$ for DNA extraction.

Table 3.1: Surveyed locations for sample collection

Sr. No.	State (s)	District (s)	Place (s)	Latitude* DM	Longitude* DM	Elevation* m (amsl)	Sample collected/ Method
India							
1	Himachal Pradesh	Bilaspur	Chandpur	31°21' N	76°47' E	1020	Infested fruits
2			Ghumarwin	31°25' N	76°43' E	625	Infested fruits
3			Nihari	31°25' N	76°39' E	681	Trapping
4		Chamba	Banikhet	32°33' N	75°57' E	1538	Infested fruits
5		Hamirpur	Bhota	31°37' N	76°33' E	889	Infested fruits
6			Nadaun	31°46' N	76°20' E	460	Infested fruits
7		Kangra	Indora	32°7' N	75°40' E	329	Infested fruits
8			Jawalamukhi	31°53' N	76°17' E	470	Infested fruits
9			Kangra	32°4' N	76°16' E	792	Infested fruits
10			Palampur	32°6' N	76°32' E	1290	Infested fruits and flowers, trapping, with insect collection net
11			Shahpur	32°13' N	76°11' E	912	Infested fruits
12			Paragpur	31°48' N	76°14' E	606	Trapping
13		Kullu	Naggar	32°5' N	77°9' E	2067	Infested fruits
14		Mandi	Barot	32°02' N	76°50' E	2690	Infested fruits and flowers
15			Mandi	31°42' N	76°55' E	806	Infested fruits

Sr. No.	State (s)	District (s)	Place (s)	Latitude* DM	Longitude* DM	Elevation* m (amsl)	Sample collected/ Method
16			Nagwain	31°49' N	77°10' E	1116	Infested fruits
17			Sundernagar	31°31' N	76°54' E	1120	Infested fruits
18		Solan	Nauni	30°56' N	77°2' E	1546	Infested fruits
19			Saproon	30°56' N	77°31' E	2386	Infested fruits
20		Una	Haroli	31°33' N	75°59' E	593	Infested fruits
21	Haryana	Karnal	Karnal	29°41' N	76°59' E	71	Infested fruits
22	Karnataka	Bengaluru	Bengaluru	12°58' N	77°38' E	280	Trapping
23	Delhi	Delhi	IARI	28°37' N	77°9' E	229	Infested fruits
24	Bihar	Patna	Patna	25°37' N	85°12' E	60	Infested fruits
25		Nalanda	Bihar Sharif	25°11' N	85°31' E	65	Infested fruits
26		Samstipur	RAU, Pusa	25°58' N	85°40' E	58	Trapping
27	Uttar Pradesh	Ghaziabad	Ghaziabad	28°46' N	77°30' E	217	Infested fruits
28	Maharashtra	Solapur	Solapur	17°40' N	75°55' E	460	Infested fruits
Nepal							
29			Dhankuta	26°58' N	87°20' E	1445	Trapping

***Lat_lon (DM) and elevation in meter (above mean sea level) were provided by CGIRT, CSKHPKV, Palampur, Himachal Pradesh-176 062**

3.2 Raising of parental stock cultures

A parental stock culture of fruit flies in the laboratory was raised from field collected infested fruits of cucumber at room temperature in specially designed rearing cages (38 x 38 x 45 cm^3). A polyethylene sheet was fitted over the base of the cage and filled with mixture of sterile fine sand and saw dust upto 5 cm height for pupation. The adults were provided with their natural host as well as a mixture of dry glucose and protein hydrolyzate (Protinex® Dumex Sciences, New Delhi) in the ratio of 1:1 in a Petri plate which was replaced at weekly intervals. Flies were also provided with water soaked cotton in a 50 ml beaker *ad libitum*. To prevent access of predatory ants to the cages, these were placed on water filled plastic plates in which water was changed daily.

3.3 Molecular characterization of fruit flies

3.3.1 DNA extraction

Total genomic DNA of each isolate was extracted following the procedure of Sharma *et al*. (2005) with minor modifications. Details of different isolates of fruit flies used for molecular characterization are presented in Tables 3.2a and 3.2b. For extracting total genomic DNA, the individual fruit fly was immersed in liquid nitrogen container for one min. and ground to fine powder using micro pestle. To each tube 700 µl of CTAB extraction buffer (Table 3.3) was added. All tubes were incubated at 65^oC for 1h in a water bath (YORK Scientific Industries, Delhi).

To each tube equal volume (700 µl) of chloroform: isoamyl alcohol (24:1) was added. The contents were mixed thoroughly and tubes were spun at 10,000 rpm for 12 min. in high speed refrigerated centrifuge (REMI India) at 4^oC. Aqueous phase was transferred to new tubes and 450 µl prechilled isopropanol was added and kept at -20^oC for 20-30 min. to precipitate the DNA. Tubes were then spun at 10,000 rpm for 12 min. and supernatant was decanted. The DNA pellet was washed thrice with 70 per cent ethanol, dried

Table 3.2a: *Bactrocera cucurbitae* (Coquillett) isolates used for molecular characterization

Sr. No.	Isolate number	Host/ Trap	Host scientific name/ lure name	Location	District
1	P101	Bitter gourd	*Momordica charantia* Linnaeus	Nadaun	Hamirpur
2	P102	Cucumber	*Cucumis sativus* Linnaeus	Bhota	Hamirpur
3	P103	Cucumber	*Cucumis sativus* Linnaeus	Sundernagar	Mandi
4	P104	Cucumber	*Cucumis sativus* Linnaeus	Mandi	Mandi
5	P106	Cucumber	*Cucumis sativus* Linnaeus	Patna	Patna (Bihar)
6	P107	Bottle gourd	*Lagenaria siceraria* (Molina)	Bihar Sharif	Nalanda (Bihar)
7	P108	Bottle gourd	*Lagenaria siceraria* (Molina)	Ghaziabad	Ghaziabad (U.P)
8	P109	Bottle gourd	*Lagenaria siceraria* (Molina)	IARI	Delhi
9	P110	Cucumber	*Cucumis sativus* Linnaeus	Ghumarwin	Bilaspur
10	P111	Cucumber	*Cucumis sativus* Linnaeus	Solapur	Solapur (MH)
11	P112	Bitter gourd	*Momordica charantia* Linnaeus	Indora	Kangra
12	P113	Bottle gourd	*Lagenaria siceraria* (Molina)	Indora	Kangra
13	P114	Bitter gourd	*Momordica charantia* Linnaeus	Nagwain	Mandi
14	P115	Cucumber	*Cucumis sativus* Linnaeus	Jawalamukhi	Kangra
15	P117	Cucumber	*Cucumis sativus* Linnaeus	Haroli	Una
16	P119	Cucumber	*Cucumis sativus* Linnaeus	Karnal	Karnal (Haryana)
17	P120	Trap	*Cue lure*	Bengaluru	Bengaluru (Karnataka)
18	P121	Trap	*Cue lure*	RAU Pusa	Samastipur (Bihar)
19	P122	Trap	*Cue lure*	Nihari	Bilaspur
20	P123	Trap	*Cue lure*	Dhankuta	Dhankuta (Nepal)

Table 3.2b: Fruit fly species used for molecular characterization

Sr. No.	Isolate(s) number	Fruit fly species name	Host plant/ trap	Host scientific name/ lure name	Location	District
1	P1	*Bactrocera tau* (Walker)	Bottle gourd	*Lagenaria siceraria* (Molina)	Nadaun	Hamirpur
2	P2	*Bactrocera tau* (Walker)	Bitter gourd	*Momordica charantia* Linnaeus	Nadaun	Hamirpur
3	P4	*Bactrocera tau* (Walker)	Cucumber	*Cucumis sativus* Linnaeus	Palampur	Kangra
4	P5	*Bactrocera tau* (Walker)	Summer squash	*Cucurbita pepo* Linnaeus	Nauni	Solan
5	P7	*Bactrocera tau* (Walker)	Cucumber	*Cucumis sativus* Linnaeus	Banikhet	Chamba
6	P8	*Bactrocera tau* (Walker)	Cucumber	*Cucumis sativus* Linnaeus	Nadaun	Hamirpur
7	P9	*Bactrocera tau* (Walker)	Cucumber	*Cucumis sativus* Linnaeus	Mandi	Mandi
8	P10	*Bactrocera tau* (Walker)	Cucumber	*Cucumis sativus* Linnaeus	Ghumarwin	Bilaspur
9	P11	*Bactrocera tau* (Walker)	Bitter gourd	*Momordica charantia* Linnaeus	Nagwain	Mandi
10	P12	*Bactrocera tau* (Walker)	Bitter gourd	*Momordica charantia* Linnaeus	Palampur	Kangra
11	P13	*Bactrocera tau* (Walker)	Pumpkin	*Cucurbita maxima* Duchesne	Barot	Mandi
12	P14	*Bactrocera tau* (Walker)	Cucumber	*Cucumis sativus* Linnaeus	Jawalamukhi	Kangra
13	P15	*Bactrocera tau* (Walker)	Bitter gourd	*Momordica charantia* Linnaeus	Jawalamukhi	Kangra

14	P16	*Bactrocera tau* (Walker)	Cucumber	*Cucumis sativus* Linnaeus	Chandpur	Bilaspur
15	P18	*Bactrocera tau* (Walker)	Bitter gourd	*Momordica charantia* Linnaeus	Shahpur	Kangra
16	P20	*Bactrocera tau* (Walker)	Trap	Cue lure	Nihari	Bilaspur
17	P302	*Bactrocera scutellaris* (Bezzi)	Trap	Cue lure	Palampur	Kangra
18	P401	*Bactrocera zonata* (Saunders)	Trap	Methyl eugenol	Palampur	Kangra
19	P501	*Bactrocera dorsalis* (Hendel)	Litchi	*Litchi chinensis* Sonnerat	Palampur	Kangra
20	P502	*Bactrocera dorsalis* (Hendel)	Mango	*Mangifera indica* Linnaeus	Palampur	Kangra
21	P503	*Bactrocera dorsalis* (Hendel)	Trap	Methyl eugenol	Palampur	Kangra
22	P504	*Bactrocera dorsalis* (Hendel)	Guava	*Psidium guajava* Linnaeus	Palampur	Kangra
23	P508	*Bactrocera dorsalis* (Hendel)	Trap	Methyl eugenol	Paragpur	Kangra
24	P601	*Bactrocera nigrofemoralis* White & Tsuruta	Trap	Cue lure	Palampur	Kangra
25	P701	*Dacus longicornis* Wiedemann	Trap	Cue lure	Palampur	Kangra
26	P1601	*Dacus sphaeroidalis* (Bezzi)	Trap	Cue lure	Palampur	Kangra
27	P1602	*Dacus sphaeroidalis* (Bezzi)	Trap	Cue lure	Palampur	Kangra

and dissolved in 100 µl of Tris EDTA (10mM Tris HCl and 1mM EDTA pH 8.0). RNAase @ 10 µl/ ml (MBI Fermentas) was added and emulsion was incubated for half an hour at 37^{o}C. The amount of DNA was quantified by recording the absorbance at 260 nm wavelength using UV/VIS spectrophotometer (Bio Rad, SmartSpec 3000). DNA was stored at -20^{o}C for further use.

Table 3.3 : Reagent and concentration of DNA extraction buffer

Reagent	Stock concentration	Working concentration	Working solution (100 ml)
Tris HCl (pH- 8.0), 100 mM	1 M	100 mM	10 ml
NaCl 1.4 M	5 M	1.4 M	28 ml
EDTA (pH- 8.0)	0.5 M	20 mM	4 ml
CTAB (2%)			2 g
PVP (1 %)			1 g
Water (RNAase and DNAase free)			55 ml
Total			100 ml

3.3.2 Primers used

A 700 bp long fragment of mitochondrial cytochrome oxidase subunit I gene (*mtCOI* gene) was amplified using the forward primer UEA 7 and reverse primer UEA 10, developed by Lunt *et al.* (1996). The base sequences of primers (Table 3.4) were custom synthesized (Life Technologies (India) Pvt. Ltd.).

Table 3.4: Base sequences of UEA 7 and UEA 10 primer

Name of the Primer	Sequence (5’ to 3’)
UEA 7 (Forward)	5’ TACAGTTGGAATAGACGTTGATAC 3’
UEA 10 (Reverse)	5’ TCCAATGCACTAATCTGCCATATTA 3’

3.3.3 PCR amplification

The PCR amplification was carried out in 0.2ml PCR tubes with 20 µl reaction volume consisting following reaction mixture:

Reaction Mixture	Quantity (µl)
Buffer 10 X	2.0
$MgCl_2$ (25 mM)	2.0
dNTPs mix (10 mM each) (Fermentas)	0.5
Taq DNA polymerase (5U/µl), (Life Technologies (India) Pvt. Ltd)	0.2
Primer forward (10 µM) 20 pmol	1.0
Primer reverse (10 µM) 20 pmol	1.0
Water (SDW)	11.3
DNA (20ng)	2.0
Total Volume	20.0

Reaction mixture was vortexed and centrifuged in a microfuge (Bangalore Genei, India). Amplifications were performed using thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, USA) with following temperature transitions:

Steps	Temperature (^oC)	Time (minute)
1. Initial denaturation	94	3.00
2. Denaturation	94	1.00
3. Annealing	50	1.00
4. Elongation	72	1.00

The thermal cycler was programmed for 35 cycles with one cycle of initial denaturation and steps 2-4 were repeated 35 times and a final extension at 72^{o}C for 30 min. using fastest ramp time between the temperature transitions.

3.3.4 Agarose gel electrophoresis of PCR product

The PCR products were resolved by electrophoresis using 3 per cent agarose gel in 0.5X Tris borate EDTA buffer. DNA ladders of 100 bp and Lambda DNA / *EcoR* I – *Hind* III double digest were used as markers. The gels were run at 80V for 2 h using Bangalore Genei power pac system, stained with ethidium bromide (0.5 µg / ml) for 10 min after electrophoresis, viewed and images were captured using Alphalmager 2200 (Alpha Infotech Corporation, San Leandro, CA) gel documentation system.

3.3.5 Sequencing and Data Analysis

PCR products of *mtCOI* gene of different fruit fly isolates obtained through amplification with specific primer were freeze dried (CHRIST *ALPHA I-2LD*) and sent for custom sequencing using same upstream and downstream primers to Xcelris labs limited, Ahmadabad, India.

3.3.6 Nucleotide sequence analysis of *B. cucurbitae* isolates

The sequences of different fruit fly isolates were blasted using on-line NCBI Blastn program http://www.ncbi.nih.gov/blast (Altschul *et al.* 1997) and twenty three sequences of *mtCOI* of *B. cucurbitae* isolates available in the GenBank Nucleotide Database, NCBI were selected for sequence comparison (Table 3.5). The selected sequences along with thirty three submitted sequences were aligned by ClustalW program (http://www.ebi.ac.uk/clustalw/) (Higgins *et al.* 1994).

Analysis of genetic and phylogenetic relationships was performed using MEGA 4.1 Software (Tamura *et al*. 2007). Genetic distances among every isolate of *B. cucurbitae* and outgroups were calculated based on the pairwise matrix of sequence divergences using the Kimura two-parameter method

(Kimura 1980). The UPGMA (Sneath and Sokal 1973) or/and distance/neighbor-joining (Saitou and Nei 1987) method was/ were used for phylogeny reconstruction. Confidence levels (Felsenstein 1985) for UPGMA an NJ tree were assessed by bootstrap (500 replications). Population structure and minimum spanning tree (MST) among *B. cucurbitae* haplotypes was constructed using Arlequin 3.1 (Excoffier *et al.* 2005) and program TREEVIEW (Page 1996) was used to draw the minimum spanning tree (MST). A minimum spanning network was constructed using TCS1.21 (Clement *et al.* 2000)

Table 3.5: GenBank sequences of *mtCOI* gene of *Bactrocera cucurbitae* used in phylogenetic analysis

Sr. No.	Country name	GenBank accession number
1	China	EU599634
2	China	EU048559
3	China	EU048560
4	China	EU048563
5	China	EU048561
6	China	EU048564
7	China	EU048565
8	China	EU048566
9	China	EU048567
10	China	AY398758
11	Japan	AY530900
12	Japan	AB192449
13	Malaysia	FJ903497
14	Sri Lanka	AB192451
15	Thailand	AF423110
16	Thailand	AB192452
17	USA	AY945039
18	USA	AY945040
19	USA	AY945041
20	USA	AY945052
21	USA	AY945051
22	USA	AY945050
23	USA	AY945049

3.3.7 Nucleotide sequence analysis of *B. tau* isolates

The detailed procedures of nucleotide sequence analysis are given in the section 3.3.6.

Analysis of genetic and phylogenetic relationships was performed using MEGA 4.1 Software (Tamura *et al*. 2007). Genetic distances among every isolates of *B. tau* and outgroups were calculated based on the pairwise matrix of sequence divergences using the Kimura two-parameter method (Kimura 1980). The UPGMA (Sneath and Sokal 1973) method was used for phylogeny reconstruction. Confidence levels (Felsenstein 1985) for UPGMA tree were assessed by bootstrap (500 replications).

Table 3.6: GenBank sequences of *mtCOI* gene of *Bactrocera tau* used in phylogenetic analysis

Sr. No.	Country name	GenBank accession number
1	China	AY398753
2	China	EU048569
3	Japan	AY530901
4	Malaysia	FJ903496
5	Thailand	AF400067
6	Thailand	AY151138
7	Thailand	AF400073
8	Thailand	AF400072
9	Thailand	AF400071
10	Thailand	AF400070
11	Thailand	AF400069
12	Thailand	AF400068

3.3.8 Nucleotide sequence analysis of other fruit flies species

The same procedures were employed for the analysis of all fruit fly sequences as described in the section 3.3.6. Analysis of genetic and phylogenetic relationships was performed using MEGA 4.1 Software (Tamura *et al*. 2007). Genetic distances among every isolates of fruit flies and outgroups were calculated based on the pairwise matrix of sequence divergences using the Kimura two parameter method (Kimura 1980). The UPGMA (Sneath and Sokal 1973) method was used for phylogeny reconstruction. Confidence levels (Felsenstein 1985) for UPGMA tree were assessed by bootstrap (500 replications).

3.4 Isolation and characterization of gut bacteria from *B. tau*

Brain heart infusion agar (BHIA) (Hi-media) and peptone yeast extract agar (PYEA) were used for isolating bacteria from gut of *B. tau*. Peptone yeast extract broth (PYEB) and peptone yeast extract agar (PYEA) were then used throughout the experiment for culturing bacteria outside the host tissues.

PYEB: Peptone-10 g, Yeast extract- 5 g, NaCl- 5 g, Distilled Water- 1000 ml, pH- 7.2

PYEA: Peptone-10 g, Yeast extract- 5 g, NaCl- 5 g, Agar- 15 g, Distilled Water - 1000 ml, pH- 7.2

3.4.1 Isolation of bacteria from fruit fly gut

The adult flies after surface sterilization with alcohol (70%) for 30 sec. followed by sodium hypochloride (0.25%) for one min. and then washed three times with sterilized distilled water (SDW), were dissected open with the help of sterilized needles, forceps and scissors. Gut of the fruit fly was removed under aseptic conditions (in the laminar flow).

The gut of individual fly was rinsed with alcohol (70%) for 30 sec. followed by sodium hypochloride (0.25%) for one min. and then washed three times with sterilized distilled water (SDW). A loopful content from each was streaked separately on PYEA and BHIA plates. The plates were incubated at 30^{o}C for 48-72 h and were examined for bacterial growth. The whole set of experiment was repeated three times at weekly intervals, using randomly trapped flies from the stock culture.

3.4.2 Screening of gut bacterial isolate for fruit fly attractancy

Thirty gut bacterial isolates were used to study the attractiveness to *B. tau*. Pure culture (72 hrs old) of different bacterial isolates was grown on PYE broth medium. The bacterial isolates were taken in separate Petri plates and kept inside the cage (45 x 45 x 55 cm^{3}) with un-inoculated PYE broth as control. Twenty five pairs of 5 days old fruit flies were released in the cage and flies visiting each treatment were recorded for 30 min. The experiment was repeated six times for *B. tau* and data obtained were analyzed statistically. On the basis of screening, five most promising gut bacteria of *B. tau* were selected for their characterization, attractiveness to fruit flies and GCMS analysis for identification of volatile chemicals.

3.4.3 Identification of bacterial isolates

The pure cultures of five promising gut bacteria *viz.* PIB, P3A, P10A, B4A and B10B of *B. tau* were maintained on PYEA slants and PYEA plates at refrigerated temperature (4-8^{o}C).

Morphological, cultural, biochemical and molecular characteristics were studied and an attempt was made to identify the bacterial isolates following the techniques given in the manual of Kanwar *et al.* (1997) and these results were compared with *Bergey's Manual of Determinative Bacteriology* (Holt *et al.* 2000).

For studying the morphological, cultural and biochemical characteristics, 48 h old culture of the test bacterium on PYEA was employed. All tests were carried out in duplicate along with a control set.

3.4.3.1 Morphological characterization

Cell shape and Gram's reaction were studied, following the standard procedures and staining reagents as described by Kanwar *et al*. (1997). Colony morphology, growth pattern and pigment production were studied by following the standard procedures. The cultures were grown on PYEA plates to observe the colony morphology.

Motility: Motility was studied by hanging drop technique. A small drop of liquid bacterial culture was placed in the center of a cover slip (No. 1) with the help of an inoculating needle. A concavity slide with a central depression was used for this technique. Vaseline was applied around the depression of the slide and it was inverted over the drop of culture by keeping the drop in the centre of depression (well). The slide was turned quickly the right side up so that the hanging drop was suspended in the well. The motility was observed by focusing the edge of the drop under the microscope.

3.4.3.2 Biochemical characterization

Catalase activity: A loopful of 24 h old bacterial culture was placed on a clean glass slide. A drop of 20 per cent hydrogen peroxide was added over it, mixed with an inoculating needle and observed for production of gas bubbles which indicated positive catalase activity.

Oxidase test (Kovacs 1956): For the oxidase test, a 24 h old culture of the test bacterium was rubbed with a sterilized glass rod on a filter paper, impregnated with freshly prepared 1 per cent (w/v) aqueous tetramethyl-p-phenylene diamine dihydrochloride solution. The test was oxidase positive if a purple colour developed within 10-60 sec. and negative if no colour developed within 60 sec.

Carbohydrate metabolism test: Five ml peptone water (double strength) with 1 to 2 drops of phenol red indicator was added to test tubes with Durham's fermentation tube in each test tube. The tubes were autoclaved at 121^{o}C for 15 min. Five ml of 2 per cent sterilized sugar solution was then added to make the final concentration of 1 per cent in the medium. All the

test tubes were inoculated with loopful of 24 h old culture of test bacterium. After 24 h, observations were taken for fermentation and gas production. Change in medium colour from light red to yellow and gas bubbles in Durham tubes indicated positive reaction for fermentation and gas production, respectively.

Medium**:** Peptone water (Hi-Media)

Sugar used for test**:** D-Glucose

Utilization of citrate (Simmons 1926): Simmon's citrate agar medium was prepared, dispensed in test tubes, autoclaved at 121^{o}C for 20 min. and slants were prepared. After inoculation with the test bacterium, the slants were incubated at 37^{o}C for 24 h and observations were recorded. Growth and conversion of original green colour to blue colour indicated citrate utilization.

Citrate medium**:** Simmon's citrate media (Hi-Media)

Indole test: Five ml medium in test tubes was inoculated with pure culture of bacterial isolates and incubated at 37^{o}C for 24 h. Kovac's reagent (0.2 to 0.3 ml) was added and shaken vigorously. The tubes were kept standing for about 10 min. and observed. Red colour in the alcohol surface layer indicated a positive indole test whereas, the original colour indicated negative test.

Medium**:** Peptone water (Hi-Media)

Methyl red (MR) test (Clark and Lubs 1915): Five ml each of the MR-VP (Voges-Proskauer) broth was dispensed into test tubes and sterilized in autoclave at 121^{o}C for 20 min. The tubes were inoculated with the test bacterium and incubated at 37^{o}C for 24 h. After incubation, 5-6 drops of methyl-red reagent were added to each tube and shaken well. Formation of bright red colour indicated positive reaction.

Media**:** MR-VP broth media (Hi-Media)

Methyl red reagent**:** 25 mg of methyl red was dissolved in 75 ml of 95% ethanol + 50 ml of distilled water.

Voges-Proskauer (VP) test: MR-VP broth was inoculated with the test bacterium and incubated at 37^{o}C for 24 h. After incubation, 0.6 ml of 5 per cent α-naphthol (in 95% ethanol) and 0.2 ml of 40 per cent aqueous solution of KOH were added to 1 ml of broth culture and shaken well. Observations were recorded after 5 min. Development of red colour indicated positive VP-test.

Triple Sugar Iron (TSI) test: Commercially available TSI medium (Hi-Media) was dispensed into test tubes and sterilized in autoclave at 121^{o}C for 20 min. and slants were prepared. TSI slants were inoculated by the test bacteria and incubated at 37^{o}C for 24 h.

3.4.3.3 Molecular characterization of bacterial isolates

Molecular characterization of five promising gut bacteria *viz.* PIB, P3A, P10A, B4A and B10B associated with *B. tau* was done by sequencing *16S rRNA* gene.

3.4.3.3.1 Extraction of genomic DNA

Total genomic DNA of each isolate was extracted following the procedure of Prabhakar *et al*. (2009b) with minor modifications. For extracting total genomic DNA, the individual bacterial isolate was grown in peptone yeast extract broth (PYEB) for 72 h at 37^{o}C. Each bacterial culture was transferred to 1.5 ml eppendorf tube and spun at 10,000 rpm for 12 min. The supernatant was discarded and eppendorf tubes containing bacterial pellets were immersed in liquid nitrogen container for one min. and the pellet was ground to fine powder using micro pestle. Rest of the procedure for DNA extraction was same as given in section 3.3.1.

3.4.3.3.2 Primer used

16S rRNA (rss) gene amplification was done using universal bacteria specific primers. The base sequences of primers (Table 3.7) were custom synthesized (Life Technologies India, Pvt. Ltd.).

Table 3.7: Base sequences of *rss* gene primers

Name of the Primer		Sequence (5' to 3')
rrs gene	F	5' AGA GTT TGA TCA TGG CTC AG 3'
	R	5' TAC CTT GTT ACG ACT TCA CC 3'

3.4.3.3.3 PCR amplification for *rrs* gene

The PCR amplification was carried out in 0.2ml PCR tubes with 25 µl reaction volume consisting of following reaction mixture:

Reaction Mixture	Quantity(µl)
Buffer 10 X	2.5
$MgCl_2$ (25 mM)	1.5
dNTPs mix (10 mM each)	2.0
Taq DNA polymerase (5U/µl), (Life Technologies India, Pvt. Ltd)	0.2
Primer forward (10 µM) 20 pmol	0.8
Primer reverse (10 µM) 20 pmol	0.8
Water (SDW)	15.2
DNA (20ng)	2.0
Total Volume	25.0

Reaction mixture was vortexed and centrifuged in a microfuge (Bangalore Genei, India). Amplifications were performed using thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, USA) with following temperature transitions:

Steps	Temperature (^{o}C)	Time (minute)
1. Initial denaturation	94	5.00
2. Denaturation	94	0.75
3. Annealing	53	0.75
4. Elongation	72	0.50

The thermal cycler was programmed for 35 cycles with one cycle of initial denaturation and steps 2-4 were repeated 35 times and a final extension at 72^{o}C for 5 min. using fastest ramp time between the temperature transitions.

3.4.3.3.4 Agarose gel electrophoresis of PCR product

The digested PCR products were resolved by electrophoresis using 1.2 per cent agarose gel in 0.5X Tris borate EDTA buffer. DNA ladders of 100 bp and Lambda DNA / *EcoR* I – *Hind* III double digest were used as markers. The gels were run at 80V for 2 h using Bangalore Genei power pac system, stained with ethidium bromide (0.5 µg / ml) for 10 min after electrophoresis, viewed and images were captured using Alphalmager 2200 gel documentation system.

3.4.3.3.5 Sequencing and Data Analysis

PCR products of *rrs* gene of five gut bacteria obtained through amplification with specific primer (section 3.6.3.1) were freeze dried (CHRIST *ALPHA I-2LD*) and sent for custom sequencing using same upstream and downstream primers (Life Technologies India Pvt. Ltd.).

3.4.3.3.6 Nucleotide sequence analysis

The sequences of different bacterial isolates were blasted using on-line NCBI Blastn program http://www.ncbi.nih.gov/blast. For the purpose fourty two sequences of *16S rRNA* of different bacteria of high sequence similarity were selected for sequence comparison from GenBank Nucleotide Database, NCBI. The pair wise genetic distance between five bacterial isolates associated with *B. tau* and other selected bacterial sequences was determined (Table 3.8).

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal 1973). The percentage of replicated trees in which the associated taxa clustered together in the bootstrap test (500 replicates) with the Maximum Composite Likelihood method to compute evolutionary distances (Tamura *et al.* 2004) and in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 298 positions in the final dataset. Phylogenetic analysis was conducted in MEGA 4.1 Software programme (Tamura *et al.* 2007).

3.5 Gut bacteria as attractants to fruit flies

Five promising bacterial isolates constantly associated with fruit flies were evaluated for their attractiveness to fruit flies (*B. tau*) in presence of protein hydrolyzate (positive control) and sugar (negative control) under laboratory conditions. Pure culture (72 hrs old) of each bacterial isolate was grown in PYE broth was taken in Petri plate and kept inside the cage (45 x 45 x 55 cm^3). Twenty five pairs (5 days old) of fruit flies were released in the cage and flies visiting each treatment were recorded for 30 min. The experiment was repeated six times for *B. tau* and data obtained were analyzed statistically (Table 3.9).

Table 3.8: Bacteria used for multiple sequence alignment

Sr. No.	GenBank accession no.	Name of Bacteria	Country
1	HQ113205	*Delftia acidovorans*	Canada
2	FR682935	*Delftia sp.*	Belgium
3	AF538930	*Delftia acidovorans*	Belgium
4	AF149849	*Delftia acidovorans*	Germany
5	FJ688376	*Delftia sp.*	France
6	AM910363	Uncultured *Delftia acidovorans*	Germany
7	EF692532	*Delftia sp.*	Uruguav
8	GQ466172	*Delftia acidovorans*	Turkey
9	AB517709	*Myroides odoratus*	Japan
10	GU350455	*Myroides sp.*	China
11	M58777	*Myroides odoratus*	-
12	D14019	*Flavobacterium odoratum*	Japan
13	GQ857652	*Myroides sp.*	Korea
14	AJ854059	*Myroides odoratimimus*	Germany
15	AM910365	*Uncultured Flavobacterium*	Germany
16	FJ965845	*Flavobacterium sp.*	India
17	EF125185	*Ochrobactrum guangzhouense*	China
18	FJ581024	*Pseudochrobactrum sp.*	India
19	EF071943	Brucellaceae bacterium	China
20	DQ334872	*Ochrobactrum sp.*	China
21	AM403218	*Ochrobactrum sp.*	Germany
22	AM041247	*Ochrobactrum oryzae*	India
23	EU543575	*Ochrobactrum sp.*	China
24	AJ920029	*Ochrobactrum shiyianus*	China
25	HM468098	*Pseudochrobactrum sp.*	China
26	GQ249219	Phyllobacteriaceae bacterium	China
27	AM884147	Phyllobacteriaceae bacterium	Germany
28	FJ542910	Uncultured *Defluvibacter sp.*	USA
29	EU870446	*Defluvibacter lusatiensis*	China

30	AM884144	Phyllobacteriaceae bacterium	Germany
31	FJ982919	*Defluvibacter lusatiensis*	Spain
32	AM884148	Phyllobacteriaceae bacterium	Germany
33	HM152635	Uncultured *Pseudomonas sp.*	France
34	AM910358	Uncultured *Pseudomonas sp.*	Germany
35	EU372964	*Pseudomonas sp.*	China
36	FJ472861	*Pseudomonas putida*	China
37	FJ472858	*Pseudomonas putida*	China
38	AM913888	*Pseudomonas sp.*	Germany
39	AM930519	*Pseudomonas putida*	China
40	DQ387441	*Pseudomonas putida*	Korea
41	AY741156	*Pseudomonas putida*	Korea
42	HM805109	*Pseudomonas geniculata*	India

Table 3.9: Attractancy of promising gut bacteria to fruit fly *B. tau* (Walker)

Treatment	Composition
T1	*Deftia acidovorans* (2 ml)
T2	*Pseudomonas putida* (2 ml)
T3	*Flavobacterium sp.* (2 ml)
T4	*Defluvibacter sp.* (2 ml)
T5	*Ochrobacter sp.* (2 ml)
T6 (Negative control)	Control (Sugar, 2 ml 10%)
T7 (Positive control)	Control (ProteinX ®, 2 ml 10%)

3.6 Volatile chemical compound identification of gut bacteria through Gas Chromatography Mass Spectrometry (GCMS)

Bacterial Preparations: The bacterial cultures were grown in peptone yeast extract broth (2 ml) for 72 hr at 30°C in GC-MS headspace tube (15 ml) with one un-inoculated control for qualitative analysis by GC-MS.

Chemical Identifications: GC-MS (70 eV) data were measured in MS-QP-2010 series (SHIMADZU CORPORATION, Tokyo, Japan) equipped with MS, AOC-20i auto sampler and BD-5 capillary column (SGC International, Ringwood, Australia) of 30 m length, 0.25 mm i.d. with film thickness 0.25 µm (Poly ethylene glycol) and helium as a carrier gas. The injector temperature was 250^{o}C with split ratio 1:50. Injection of volatiles for GC-MS analysis was by thermal desorption at 250°C in a split injector. The injector was operated in the split mode and the purge valve was opened after 1 min. Linear velocity of helium carrier gas was 40.80 cm/ sec. The GC column oven temperature was programmed to hold at 40^{o}C for 4 min and then increased up to 220^{o}C at increments of 4^{o}C/ min and finally holding at 220^{o}C for 15 min. Column flow rate was set at 1.28 ml/ min. Ion source temperature was 200^{o}C and the interface temperature was set at 250^{o}C. The MS was scanned at 70 eV over 40-600 a.m.u. at 2 scans/ sec.

GC-MS identifications were based on computer matching of unknown spectra with those in the Wiley 138K Mass Spectral Database (John Wiley & Sons, New York).

Calculation of Retention Index (I)

GCMS data were temperature programmed and then the Kovats index was calculated by the equation

$$I = \left[\frac{t_{r(unknown)} - t_{r(n)})}{t_{r(N)} - t_{r(n)}}\right] * (100 \times z) + (100 \times n)$$

Where;

I = Kovats retention index,

n = the number of carbon atoms in the smaller alkane,

N = the number of carbon atoms in the larger alkane,

z = the difference of the number of carbon atoms in the smaller and larger alkane,

t_r = the retention time.