CHAPTER-3

MATERIALS AND METHODS

CHAPTER- 3
MATERIALS AND METHODS

3.1. Collection of *S. ricini* seeds

Out of the 26 eco-races of the *S. ricini* silkworm available in India (Directorate of Sericulture, Govt. of Assam), the Kokrajhar eco-race was chosen for the experiment (Sarmah *et al.*, 2012). Disease free layings (DFLs) of *S. ricini*, Kokrajhar eco race were collected from the Directorate of Sericulture, Kokrajhar, India, with their prior permission. The eggs were then brought and incubated at room temperature for hatching and further studies.

3.2. Selection of food plants

Three food plants, one each from primary, secondary, and tertiary namely Castor (*Ricinus communis*), Tapioca (*Manihot esculenta*) and Papaya (*Carica papaya*) respectively were selected for the experiment. The selection of food plants was solely done based on the availability of plants in the area and their easy accessibility (Plate 1).

3.3. Scientific Identification of host plants

For the identification of host plants used for rearing purposes, herbarium sheets were prepared from plant samples and submitted to the Taxonomy laboratory, Department of Botany, Bodoland University for valid identification and accession numbers were obtained (Plate 2).

3.4. Rearing of silkworm

Immediately after hatching, the newly hatched larvae of *S. ricini* were initially fed tender castor leaves until they reached the second instar stage. The larvae were then divided into three groups (n =100 per group), each fed on a different food plant i.e., castor, tapioca, and papaya, until they matured to the fifth instar stage (Plate 3). The groups were then designated as Sample C for those reared on castor, Sample T for those reared on tapioca, and Sample P for those reared on papaya. The entire rearing process of *S. ricini* was conducted under the climatic conditions of Kokrajhar district, with daily recordings of room temperature and relative humidity using a digital thermometer and hygrometer respectively throughout the rearing period.

3.5. Proximate analysis of Castor, Tapioca and Papaya leaves

3.5.1. Sample preparation

For proximate analysis, the leaves of all the three food plants were collected from the local areas of Kokrajhar district. After collection, leaves were washed properly to remove dust and dirt particles. Thereafter, the leaves were dried under hot air oven at a temperature of 40ºC until fully dried. Fully dried crispy leaves were then taken out of the hot air oven and ground into powders using an electronic grinder. The powdered leaves samples were then used as a sample for proximate analysis.

3.5.2. Proximate analysis of sampled food plant leaves

Moisture content, Crude fibre content, Ash content, Fat content, Crude protein content and Total carbohydrate content of plant samples were determined according to the protocol described in Association of Official Analytical Chemist (AOAC) methods (AOAC, 1995, 2006).

I. **Moisture content:** First, the weight of the fresh samples was taken. Then, the samples were dried in an incubator for 3 hours at 105°C. After that, the samples were cooled in desiccators and weighed again. The moisture content was obtained from the difference between wet weight and dry weight using the following formula:

$$\text{Moisture \%} = \frac{W1 - W2}{W1} \times 100$$

Where,

W1= Weight (g) of sample before drying

W2= Weight (g) of sample after drying.

II. **Ash content:** After moisture content analysis, the samples were placed on porcelain dishes and then placed in a furnace at 550°C for 4 h. The ash content was calculated using the following formula:

$$\text{Ash \%} = \frac{Weight\ of\ Ash}{Weight\ of\ dried\ sample} \times 100$$

III. **Crude fibre content:** The dried samples were digested with 1.25% sulphuric acid, filtered, and washed and then digested with 1.25% sodium hydroxide, again filtered, washed and dried. This dried sample was then ignited. The crude fibre content was calculated using the following formula:

$$\text{Crude fibre\%} = \frac{Weight\ of\ sample\ after\ ignition}{Weight\ of\ sample} \times 100$$

IV. **Fat content:** The samples were kept in diethyl ether for 2 h at 90°C. After extraction, the samples were then kept at 70°C in an incubator for 30 min, cooled in desiccators and weights were taken. The fat content was calculated using the following formula:

$$\text{Crude fat} = \frac{Original\ weight\ of\ the\ sample - Weight\ of\ the\ sample\ after\ extraction}{Original\ weight\ of\ the\ sample} \times 100$$

V. **Protein content:** The nitrogen content was evaluated by Kjeldahl method. The crude protein content was calculated by multiplying the crude nitrogen content by a factor of 6.25.

VI. **Carbohydrate content:** The total carbohydrate content was determined by the following formula.

Carbohydrate (%) = *100 − [moisture (%) + ash (%) + crude protein (%) + crude fat (%) + crude fibre (%)]*

3.6. Assessment of Larval parameters

The growth parameters such as larval duration, length, weight, percentage survivability, lentgth and weight of silk glands were recorded following the protocol described by Kumar and Elangovan (2010).

3.7. Harvesting of larvae

Fully matured fifth instar larvae reared on three sampled food plants were picked randomly from each group and then starved for 24 h prior to the extraction of gut. All the experiments done on sampled groups were performed in triplicates.

3.8. Sample sterilization and gut extraction

Larvae were taken on a clean sterile dissecting tray for experiment, and then the larvae were rinsed in distilled water for 30 sec followed by 75 % ethanol for 60 sec and again rinsed in distilled water for 30 sec to remove the surface disinfectant. Larvae were then kept at deep freezer -20ºC for 10 min to make them stable before dissection. The sterilized larvae from three sampled groups were dissected using sterile micro scissors under UV treated laminar air flow (LAF) cabinet to extract the gut (Plate 4 A to C).

3.9. Sample pre-processing

The extracted total guts from three sampled groups were then washed with 1 X Phosphate buffered saline (PBS) buffer and extracted guts from each separate groups were then pooled into three separate centrifuge tubes containing same volume of 1 X PBS buffer as preservative then samples were immediately stored in -20ºC until further use.

3.10. Gut digestive enzyme assay of *S. ricini*

Total gut digestive enzyme assay such as α-amylase, cellulase, proteinase and lipase activity of the larvae fed on three sampled food plants were assayed by following the protocols given below (Plate 4).

3.10.1. α-amylase assay

The α-amylase activity of gut samples were assayed according to the protocol described by Bernfeld (1955) using 1% starch as a substrate and 3,5, Dinitrosalicylic acid (DNSA) as a stopper. The absorbance of the sample mixtures were recorded on UV-VIS spectrophotometer at 540 nm. Enzyme activity was expressed as one unit of enzyme activity equivalent to the enzyme amount required for producing 1µM of maltose per min under the assay conditions.

3.10.2. Cellulase assay

Cellulase activity of gut samples were assayed using 1% Carboxymethylcellulose (CMC) as substrate. In the mixture solution, DNSA was added to terminate the enzyme reaction. The absorbance was measured at 540 nm. One enzyme unit was defined as the enzyme amount which releases 1 µM of glucose equivalent from substrate per min (Anand *et al.,* 2010).

3.10.3. Proteinase assay

Proteinase enzyme activity was assayed according to the protocol described by Cupp-Enyard (2008) by using Casein as a substrate and Trichloroacetic acid (TCA) as stopper of the enzyme activity. Absorbance of the samples was recorded using UV-VIS spectrophotometer at 660 nm. Proteinase enzyme activity of samples were calculated from the tyrosine standard curve and one unit of proteinase activity was defined as the quantity that is required to produce 1 µM of tyrosine equivalents per ml of TCA filtrate under the assay conditions.

3.10.4. Lipase Assay

Lipase enzyme activities of isolates were assayed using p-nitrophenylpalmitate (pNPP; Sigma Aldrich) as substrate (Winkler & Stuckmann, 1979) following the protocol described by Sarate *et al.* (2012). Absorbance of the samples was measured using UV-VIS spectrophotometer at 410 nm against a substrate free blank. One unit of enzyme activity was defined as 1 µM of p-nitrophenol released per min under the assay conditions (Massadeh, and Sabra, 2011).

3.11. To study the diversity of bacterial communities in the larval gut of silkworm *Samia ricini* feeding on different food plants using a culture independent method

3.11.1. Bacterial DNA isolation

Gut homogenates were prepared following the above mentioned steps (3.7 to 3.9). DNA was isolated from the samples using Sodium Dodecyl Sulfate (SDS) DNA isolation method according to the protocol described by Han *et al*. (2018). In each samples, 750 µl of TE Buffer (10mM Tris HCL, 1 mM EDTA, pH 8.0) and 50 µl of Lysosome (20mg/ml) were added and then incubated at 60ºC for 30 min. Subsequently,

10 µl of RNase (20 mg/ml) were added to the centrifuge tube and then incubated the suspension at 30ºC for 30 min. The tubes were then incubated at 65°C for 60 min with inversion in every 20 min after adding 100 µl of 10% SDS (pH 7.4) and 30 µl of proteinase K (20 mg/ml). After that, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) were added, and the sample was mixed by inversion. The samples were centrifuged at 21,500 x g for 2 min, and then supernatant were discarded from the centrifuge tubes. In each tube, an equal volume of chloroform: isoamyl alcohol (24:1) was added, and then the suspension was mixed gently and centrifuged again at 21,500 x g for 2 min. The upper aqueous layer were transferred to another 2 ml sterile centrifuge tubes, and the DNA were precipitated using a 1/10 volume of NaCl (3 M, pH 5.2) and 2 volumes of ice-cold (−20°C) 95% ethanol, followed by centrifugation at 21,500 x g for 5 min at 4°C. The isolated DNA pellets were washed twice using 1 ml of 70% ethanol before being air dried and finally resuspended in 100 µl of TE buffer (preheated to 50°C) and the DNA concentration were estimated using Qubit Fluorimeter (V.3.0).

3.11.2. PCR amplification of 16S rRNA gene

The specific V3 forward primer (CCTACGGGNBGCASCAG) and V4 reverse primer (GACTACNVGGGTATCTAATCC) were utilized to amplify the V3-V4 region of the 16S rRNA gene. The reaction mixture (25 µl) was prepared by using components [dH_2O (13 µl), 5X Phusion ™ HFBuffer (5µl), 10mM dNTPs (0.5µl), forward primer (1.25 µl), reverse primer (1.25 µl), template DNA (2 µl), DMSO (1.5 µl), Phusion ™ high fidelity DNA polymerase (0.5µl). PCR reactions were performed in Eppendorf Master cycler nexus by using Thermal Cycler (C1000TM, BIORAD) under 30 cycle PCR reaction conditions. The quality of the amplicons was checked on agarose gel electrophoresis before the preparation of library.

3.11.3. Preparation of libraries and sequencing of the 16S rRNA gene

The purified amplicon product (5ng) was utilized to generate a library through the NEBNext Ultra DNA library preparation kit. Subsequently, the library's quantity and quality were assessed using the Agilent 2200 TapeStation. Following this evaluation, the prepared library underwent sequencing on the Illumina HiSeq 2500

platform, employing a 250x2 pair end cycle (Figure 3.1), conducted at AgriGenome Labs Pvt Ltd., Kerela, India.

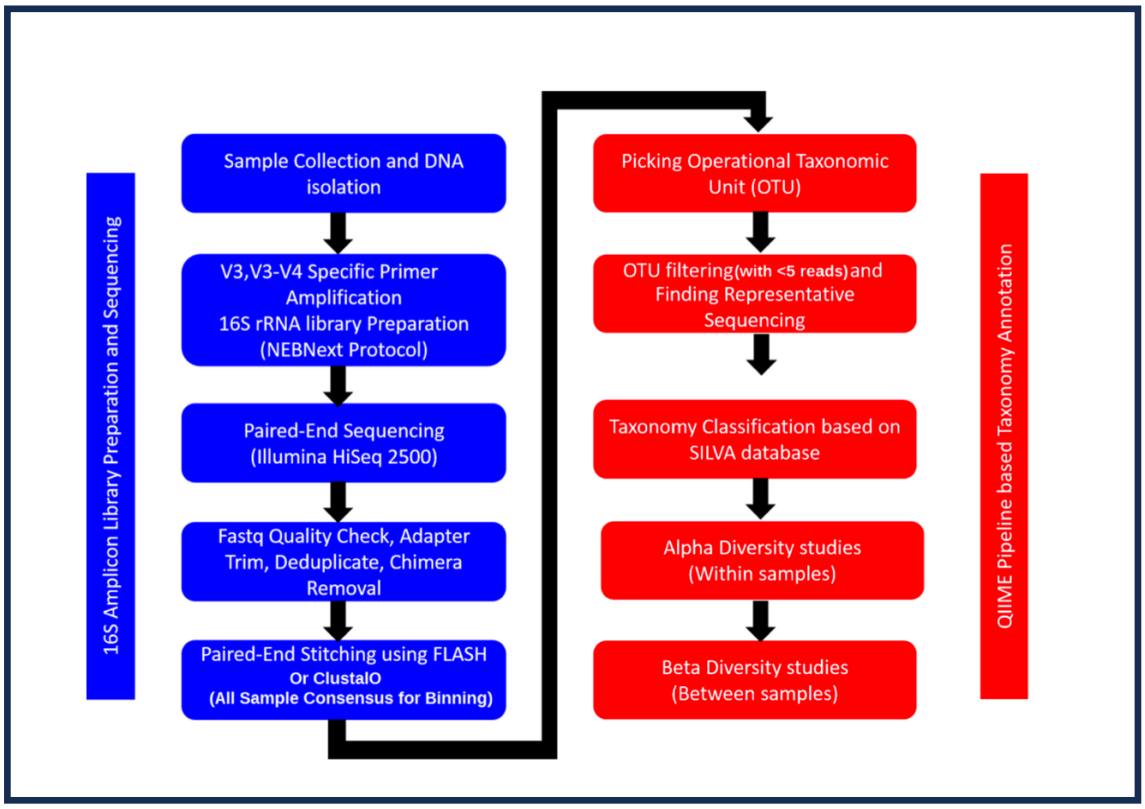


Figure 3.1. Metagenomic flowchart for the wet lab and bioinformatics protocols

3.11.4. Bioinformatics data analysis

I. Sequence data Fastq quality checking

The raw reads obtained from Illumina sequencing platform after demultiplexing was subjected to FastQC program (latest version.0.11.8) to check the quality of the reads with default parameters. The base quality (PhredScore; Q), base composition, GC content, ambiguous bases (other than A, T, G, C) and adapter dimers were thoroughly checked prior to the bioinformatics analysis.

II. Data filtering, consensus sequence generation and OTU clustering

The specific forward primer for the V3 region and reverse primers for the V4 region were trimmed utilizing a PERL script. The pair end reads with a Phred score quality (Q>20) were selected for the generation of consensus sequences for the V3-V4

region. After trimming the primers, the high-quality paired-end reads were pairwise combined to create consensus FASTA sequences for the V3-V4 amplicon. This merging of reads was accomplished using the FLASH program (version 1.2.11), with a minimum overlap of 10bp and a maximum overlap of 240 bp, with zero mismatches permitted. Any chimeric sequences were eliminated through the de novo chimera removal approach utilizing UCHIME (version 11), integrated into the VSEARCH tool.

III. OTUs picking and taxonomic classification

The process of selecting Operational Taxonomic Units (OTUs) and taxonomic classification was carried out using the pre-processed consensus sequences derived from the V3-V4 region. Pre-processed reads from all samples were pooled and grouped into OTUs according to the similarity of sequences employing the Uclust program (similarity cut off = 0.97) within the Quantitative Insights into Microbial Ecology (QIIME) software suite. The entire downstream analysis was conducted using QIIME1 program (Version: 1.9.1) (Caporaso *et al*., 2010). Representative sequences from each clustered OTU were chosen and aligned with the SILVA core set of sequences using the Python Nearest Alignment Space Termination (PyNAST) program. Subsequently, taxonomy classification and the generation of relative abundance plots at various taxonomic levels (phylum, class, order, family, genus, and species) based on OTUs were performed using the Ribosomal Database Project (RDP) classifier (Cole *et al.*, 2014), by matching each representative sequence with the SILVA OTUs database (Yilmaz *et al*., 2014).

IV. Construction of Krona plot

Krona Tools (Version: 2.8.1) was used to create the krona plot of the bacterial communities in the samples applying tab-delimited text file listing quantities (abundances) and taxonomy lineages. Result was obtained in the form of html file.

IV. Alpha diversity analysis of samples

The microbial diversity within the samples was calculated by using alpha diversity indices including Shannon, Chao1 and observed species metrics. The metric calculation was performed using QIIME software.

V. Venn diagram

A venn diagram was constructed using bioinformatics and evolutionary genomics tool. OTU Id and sample count reads were used as input for this analysis. Furthermore, Sorenson's similarity index was computed based on the Venn diagram data, revealing shared and unshared bacterial OTUs between samples C, T, and P, to get insights into the similarity among these samples.

VI. Co-occurrence analysis of gut bacterial genera

SCNIC (Version: 0.6.2) was used for co-occurrence analysis of bacterial genera using genus level biom formatted file applying correlation method sparcc (distance metric) and the output consisted of a directory containing both a 'correlation.txt' file and a 'correlation_network.gml' file. Then, a network plot was generated using Python script, with the 'correlation_network.gml' file as input. The result representing the network plot derived from the correlation analysis.

VII. Principal coordinate analysis (PCoA)

PCoA was performed by following two main steps. In the first step, analysis was conducted using the QIIME 1 script `principal_coordinates.py` (Version: 1.9.1). The input for the step was a distance matrix file, and the output yielded a file containing the principal coordinate (PC) axes presented as columns corresponding to each sample (rows). Subsequently, in the second step, a 2D PCoA plot was generated using an in-house Python script, with the input being the previously obtained principal coordinates.txt file. The plotting process involved setting ellipsoid parameters, specifically opacity of 0.5 and a method based on the Interquartile Range (IQR). The outcome of the step was a .png file visually representing the 2D PCoA plot with the specified ellipsoid characteristics.

VIII. Functional annotation of Gut bacterial genome

Functional roles of bacterial genes involved in metabolism were analysed by taking KEGG pathway using bioinformatics software Picrust2 (Version 1.1.3). Heatmap was constructed to know the dominant role of bacteria.

3.11.5. Statistical Analysis of Metagenomic Profiles (STAMP)

STAMP analysis of functional profile of gut bacterial data was carried out using the STAMP software (Version 2.1.3) to identify and compare functional profile of the gut bacterial sequences using profile file of predicted metagenome and group metadata file generated from the gut bacterial 16S rRNA gene sequencing data (Parks and Beiko, 2010).

3.12. To isolate and characterize some beneficial gut bacteria using a culture dependent method

3.12.1. Isolation of gut bacteria

Fifth instar *S. ricini* larvae from three sampled groups were picked randomly from each group and were starved for 24 h prior to their gut extraction. The larvae were then brought under LAF cabinet and surface sterilized using 70% ethanol followed by thoroughly rinsing with distilled water to remove any impurities and to avoid contamination. The guts were then extracted from larvae by piercing the abdomen from the head. The extracted guts were then taken carefully into sterile eppendorf tubes for their homogenization process. The gut homogenates were centrifuged at 14,000 rpm for 10 min for purification. Clear supernatant (1ml) with bacterial cells were taken for serial dilution process (10^{-1} -10^{-8}). From each tube, 0.1 ml aliquot was taken and inoculated by using spread plate method into individual nutrient agar media plates for gut bacterial culture. After inoculation, the plates were incubated under BOD incubator at 37ºC for 24-48 h and plates were observed regularly to check bacterial growth (Plate 4).

3.12.2. Isolation of pure culture

After 36 h incubation, all the plates of different dilution factor were observed. The plates where all the bacterial colonies were distinct and non-overlapping to each other were selected and taken as standard dilution (Plate 5). Same process was carried out in all 3 groups. Bacterial colonies were then picked from standard dilution plates for purification. The isolates were purified many times until contamination free colonies were found. All the isolates were purified on nutrient agar plates and were kept under 37ºC condition in BOD. The isolates from *S. ricini* larvae fed on Castor was referred as

Isolate C, on Tapioca was referred as Isolate T and Papaya was referred as Isolate P (Plate 8).

3.12.3. Qualitative screening of digestive enzyme producing gut bacterial isolates

Pure bacterial isolates from all the sampled groups were then subjected into different substrate medium to check their ability to produce extracellular digestive enzymes.

I. α-amylase

To evaluate α-amylase activity, bacterial isolates were cultured individually on starch-agar medium plates (1% soluble starch, tryptone, yeast extract, and agar, pH 7) for 48 hours at 37°C. Subsequently, the plates were flooded with Lugol's iodine solution. The appearance of transparent zones surrounding the colonies upon addition of the solution indicates the production of amylase (Amoozegar *et al*., 2003).

II. Cellulase

To assess cellulase activity, the isolates were cultured on CMC-agar media plates containing specific components (CMC, tryptone, KH_2PO_4, Na_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2$, $FeSO_4.7H_2O$, agar, at pH 7). Following 48 h incubation, at 37°C, the plates underwent staining with Congo red solution (2 g/L) for 10 min, followed by destaining with 1 M NaCl for 15 min. The presence of halo zones surrounding the colonies indicated the production of cellulase (Meddeb-Mouelhi *et al*., 2014).

III. Proteinase

Skim milk agar medium was utilized to screen for bacterial isolates capable of producing proteinase. The medium composition included 15g/L agar, 5g/L casein, 1g/L dextrose, 28g/L skim milk powder, and 2.5g/L yeast extract, with a pH of 7. After incubation at 37°C for 48 hours, the formation of a halo or clear zone surrounding the colonies indicates the presence of enzyme activity (Manorma *et al.,* 2017).

IV. Lipase

Rhodamine B agar medium supplemented with tween 80 was used to screen for lipase activity in the isolates. The bacterial isolates were cultured on Rhodamine B agar media plates [0.5% beef extract, 0.5% peptone, 0.5% $(NH_4)2SO_4$, 0.05% $MgSO_4$, 0.4% K_2HPO_4, 0.02% $CaCl_2$, 0.2% $NaNO_3$, 12.0% olive oil emulsion, 2.0% Rhodamine B (0.1mg/ml), 1.8% agar, and tween 80, with a pH range of 9.2-9.8]. These plates were then incubated at 37°C for 12-24 hours before inoculation. Subsequently, utilizing a sterilized inoculation loop, the bacterial isolates were inoculated onto the Rhodamine B plates and incubated at 37°C for 48 h. Lipase production was identified by the appearance of a pinkish to white precipitation zone surrounding the bacterial colonies (Ilesanmi *et al*., 2020).

3.12.4. Phenotypic characterization of enzyme producing gut bacterial isolates

Phenotypic characterization was performed by using two methods viz. Morphological characterization and Physiological characterization.

3.12.4.1. Morphological characterization

For the morphological characterization, the individual isolates were subjected to microscopic observation for their colony morphology viz. shape, size, colour, consistency, margin, elevation, and opacity and the result was recorded by following the manual of Cappuccino and Sherman, (2013).

3.12.4.2. Physiological characterization

I. Gram Staining

For the Gram staining, firstly a bacterial smear was prepared by taking a clean glass slides, and then a drop of water was placed on its surface. Then aseptically, small sample of bacterial cells was collected from an isolated colony using a loop and spread onto the slide to create a smear. The smear was then air-dried by passing it over a flame, ensuring it's heat-fixed without overheating. Subsequently, the smear was flooded with crystal violet solution for 60 sec and excess stains were washed off with distilled water. Gram's iodine solution was applied for another 60 sec to fix the stain, followed by a gentle wash with distilled water. Decolorization is achieved by adding ethyl alcohol

(75%) drop by drop until the stain no longer appears in the washes, usually took around 10 sec. After quickly washing off the decolorizer, the slides were blotted dry. The smear was then flooded with safranin as a counterstain for 30 sec, washed gently, and excess water was drained. Air drying was performed carefully, and the finished slide is observed under a microscope light microscope (Labomed-LX- 300) at 100X using the oil immersion objective for detailed examination, facilitating the differentiation of bacteria based on their cell wall properties (Cappuccino and Sherman, 2013) (Plate 9).

II. Catalase test

A small amount of growth culture was placed onto a clean microscope slide. Then added a few drops of H_2O_2 (3%) onto the smear then observed the slide for the result. A positive result was the rapid evolution of O_2, as evidenced by bubbling. A negative result recorded no bubbles or only a few scattered bubbles (Cappuccino and Sherman, 2013).

III. Oxidase test (OX)

A good-sized amount of inoculum (already incubated and grown) from a culture plate was picked and spread on an oxidase disc (Himedia), then was observed for the colour change. A positive reaction usually occurs within 5-10 sec, and culture will become purple whereas an oxidase negative results in no colour change (Shields and Cathcart, 2010).

IV. Substrate utilization using Analytical Profile Index (API) 20E strip.

Substrate utilization tests such as β-galactosidase, Arginine dihydrolase, Lysine decarboxylase, Ornithine decarboxylase, Citrate utilization, H_2S production, Urease, Tryptophan deaminase, Indole production, Acetone production (Voges-Proskauer), Gelatinase, Fermentation/Oxidation of Glucose, Mannitol, Inositol, Sorbitol, Rhamnose, Saccharose, Melibiose, Amygdalin, and Arabinose activity of bacterial isolates were analyzed using the API 20E strip (bioMerieux™). Firstly, a humid atmosphere was created in an incubation box by adding about 5 ml of sterile distilled water to the tray's honeycombed wells. The strain reference is recorded on the tray's elongated flap. In the next, the strips were taken out of their packaging and placed inside the incubation box.

A 5 ml ampule of API suspension medium was opened and used without additives. Then, a single, well-isolated colony from an 18-24 h old culture was removed using a pipette and emulsified to produce a uniform bacterial suspension, which was then immediately used for inoculation of the API 20E strip following the manufacturer's guidelines. The incubation box was closed, and incubation was carried out for 18-24 h at 36°C ± 2°C. Following the incubation period, the strips were read and interpreted based on the manufacturer's provided instructions (Table 3.1).

V. Tolerance to pH, Salt and Temperature:

The pH, salt and temperature (5-45 ºC) tolerance were studied using nutrient agar for 96 h. The pH optimization was determined on nutrient agar plates with pH 7-11 with an increment of 0.5 pH unit by KH_2PO_4/K_2HPO_4 or $Na_2CO_3/NaHCO_3$ buffer system at 37ºC. Salt tolerance was examined at 0-10 % (w/v) of NaCl concentration, with an increment of 2 % at 37°C on nutrient agar plates.

3.13. To study some nutritionally important cultivable gut bacteria through quantitative enzymatic assay

3.13.1. Bacterial sample preparation

Newly grown 24 h old bacterial samples were collected and inoculated into the nutrient broth medium (Peptone 5g/L, Beef extract 1.5g/L, Sodium Chloride 5g/L, Yeast extract 1.5g/L) containing 1% substrates for individual enzyme activities at pH 7.2 ± 0.2 (Suvarna *et al*., 2013) and then incubated inside BOD incubator at 37ºC for 24 h. The 24 h old bacterial isolates in culture broths were centrifuged at 14,000 rpm for 5 min at 4ºC. The clear bacterial supernatant generated were collected and used as an enzyme solution for various enzyme assays.

3.13.2. Digestive enzyme assays

I. α-amylase assay

The α-amylase activity of bacterial samples was assayed according to the protocol described by Bernfeld (1955) using 1% starch as a substrate and DNSA as stopper. The absorbance of the sample mixtures was recorded at 540 nm. Final enzyme activity was

expressed as one unit of enzyme activity corresponding to the enzyme amount required for producing 1µM of maltose per min under the assay conditions.

II. Cellulase assay

Cellulase activity of isolates was assayed using 1% CMC as substrate. In the mixture solution, DNSA was added to terminate the enzyme reaction. The absorbance was measured at 540 nm. One enzyme unit was defined as the enzyme amount which releases 1 µM of glucose equivalent from substrate per minute (Anand *et al.,* 2010).

III. Proteinase assay

Proteinase enzyme activity was assayed according to the protocol described by Cupp-Enyard (2008) by using Casein as a substrate and TCA as stopper of the enzyme activity. Absorbance of the samples was recorded at 660 nm. Proteinase enzyme activity of samples was calculated from the tyrosine standard curve and one unit of proteinase activity was defined as the enzyme quantity that is required to produce 1 µM of tyrosine equivalents per ml of TCA filtrate under the assay conditions.

IV. Lipase assay

Lipase enzyme activities of isolates were assayed using p-nitrophenylpalmitate (pNPP; Sigma Aldrich) substrate (Winkler & Stuckmann, 1979) following the protocol described by (Sarate *et al*., 2012). Absorbance of the samples was measured using UV-VIZ spectrophothometer at 410 nm against a substrate free blank. One unit of enzyme activity was defined as 1 µM of p-nitrophenol released per min under the assay conditions (Massadeh, and Sabra, 2011).

3.13.3. Molecular identification of bacterial isolates

3.13.3.1. DNA isolation

The cultures are sub-cultured by inoculating in Lysogeny Broth (LB) medium for overnight growth at 37°C. The genomic DNA was extracted by using DNA isolation kit (Himedia).

Table 3.1. Reading Table for API 20E strip analysis

TESTS	ACTIVE INGREDIENTS	QTY (mg/cup)	REACTIONS/ENZYMES	RESULTS	
				NEGATIVE	POSITIVE
ONPG	2-nitrophenyl-βD-galactopyranoside	0.223	β-galactosidase (Ortho Nitro Phenyl-βD-Galactopyranosidase)	Colourless	yellow
ADS	L- arginine	1.9	Arginine Dihydrolase	Yellow	Red/orange
LDC	L-lysine	1.9	Lysine Dearboxylase	Yellow	Red/orange
ODC	L-ornithine	1.9	Ornithine Decarboxylase	Yellow	Red/orange
CIT	Trisodium citrate	0.756	Citrate Utilization	Pale green/ yellow	Blue-green/yellow
H_2S	Sodium thiosulfate	0.075	H_2S production	Colourless/ greyish	Black deposit /thin line
URE	Urea	0.76	Urease	Yellow	Red/ orange
TDA	L-tryptophane	0.38	Tryptophane Deaminase	Yellow	Reddish brown
IND	L- tryptophane	0.19	Indole production	Colourless pale green/ yellow	Pink
VP	Sodium pyruvate	1.9	Acetone production (Voges Proskauer)	Colourless / pale pink	Pink/ red
GEL	Gelatin (bovine origin)	0.6	Gelatinase	No diffusion	Diffusion of black pigment
GLU	D-glucose	1.9	Fermentation/ oxidation (Glucose) (4)	Blue/ blue- green	Yellow / greyish yellow
MSN	D-mannitol	1.9	Fermentation/ Oxidation (Mannitol) (4)	Blue / blue-green	yellow
INO	Inositol	1.9	Fermentation/ Oxidation (Inositol) (4)	Blue / blue-green	yellow
SOR	D- sorbitol	1.9	Fermentation/ Oxidation (Sorbitol) (4)	Blue / blue-green	yellow
RHA	L- rhamnose	1.9	Fermentation/ Oxidation (Rhamnose) (4)	Blue / blue-green	yellow
SAC	D-sucrose	1.9	Fermentation/ Oxidation (Saccharose)	Blue / blue-green	yellow
MEL	D-melibiose	1.9	Fermentation/ Oxidation (Melibiose) (4)	Blue / blue-green	yellow
AMY	Amygdalin	0.57	Fermentation/ Oxidation (Amygdalin) (4)	Blue / blue-green	yellow
ARA	L- arabinose	1.9	Fermentation/ Oxidation (Arabinose) (4)	Blue / blue-green	yellow

3.13.3.2. DNA Quality checking

Extracted DNA was checked for quality in 1.8 % agarose gel using voltage of 140V for 36 min (Figure 3.2) and the concentration of DNA was checked on Nanodrop spectrophotometer (Table 3.2).

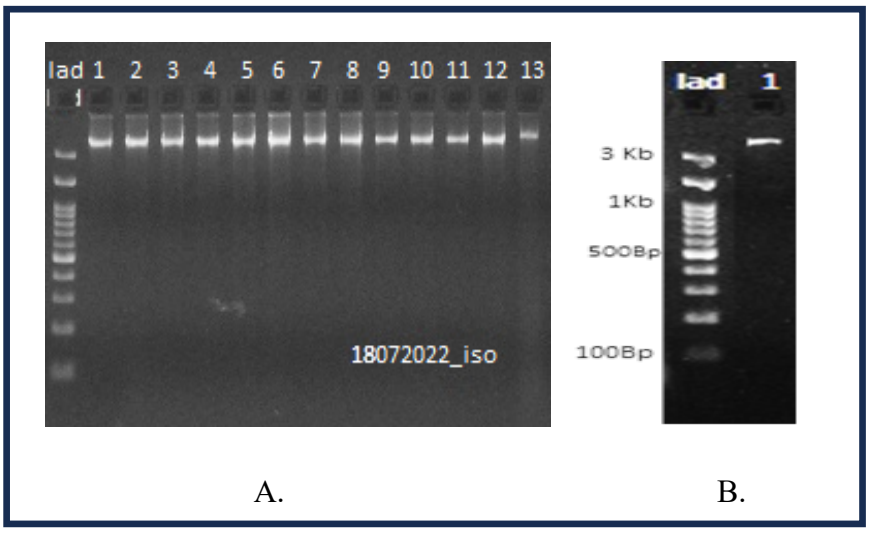


Figure 3.2. Agarose gel electrophoresis of crude DNA isolated from bacterial isolates: A1-C1; A2- C2; A3-C3; A4-C4; A5-C5; A6-C6; A7 T1; A8-T2; A9-T3; A10-P1 A11-P2; A12-P3; A13-P4; B1-T4.

3.13.3.3. PCR amplification

All the samples were amplified using 16S rRNA universal primers viz. 27F and 1492R as forward and reverse primers respectively. Primer 1 (907R) and Primer 2(785F) were used as sequencing primers in Thermocycler PCR. The PCR reaction mixture and reaction cycle were as follows:

PCR reaction mixture (20 µl)

HPLC water	11.4 µl
Primer 10 pmol Forward	1µl
Primer 10 pmol Reverse	1µl
PCR mix	4.6 µl
DNA template 20 ng/µl	2 µl

Following PCR steps were followed to amplify the target gene.

Step 1: Initial Denaturation: 95° C for 3 min.

Step 2: followed by 35 cycles of the following steps:

Denaturation : 95°C for 30sec

Annealing : 55°C for 30sec

Extension : 72°C for 1 min 30sec

Step 3: Final Extension: 72°C for 5 min.

Table 3.2. Genomic DNA concentrations of bacterial isolates

Sl. no.	Well no.	Sample ID	ng/µl	A260/A280	A260/A230
01	A1	ISOLATE C1	20.1	1.59	1.42
02	A2	ISOLATE C2	22.1	1.58	1.43
03	A3	ISOLATE C3	19	1.6	1.44
04	A4	ISOLATE C4	22	1.58	1.56
05	A5	ISOLATE C5	19.1	1.6	1.43
06	A6	ISOLATE C6	23.4	171	1.47
07	A7	ISOLATE T1	22.5	1.6	1.48
08	A8	ISOLATE T2	24.1	1.64	1.59
09	A9	ISOLATE T3	28.1	1.55	1.64
10	A10	ISOLATE P1	26.1	1.77	1.4
11	A11	ISOLATE P2	59.1	1.73	1.32
12	A12	ISOLATE P3	28.1	1.59	1.48
13	A13	ISOLATE P4	27.2	1.67	1.22
14	B1	ISOLATE T4	39.2	1.78	1.75

3.13.3.4. Purification of amplicons

After PCR amplification, the amplified PCR products were subjected to gel electrophoresis (Figure 3.3) for evaluation. Thereafter, the amplicons were purified with Qiagen gel extraction kit before being sent for sequencing.

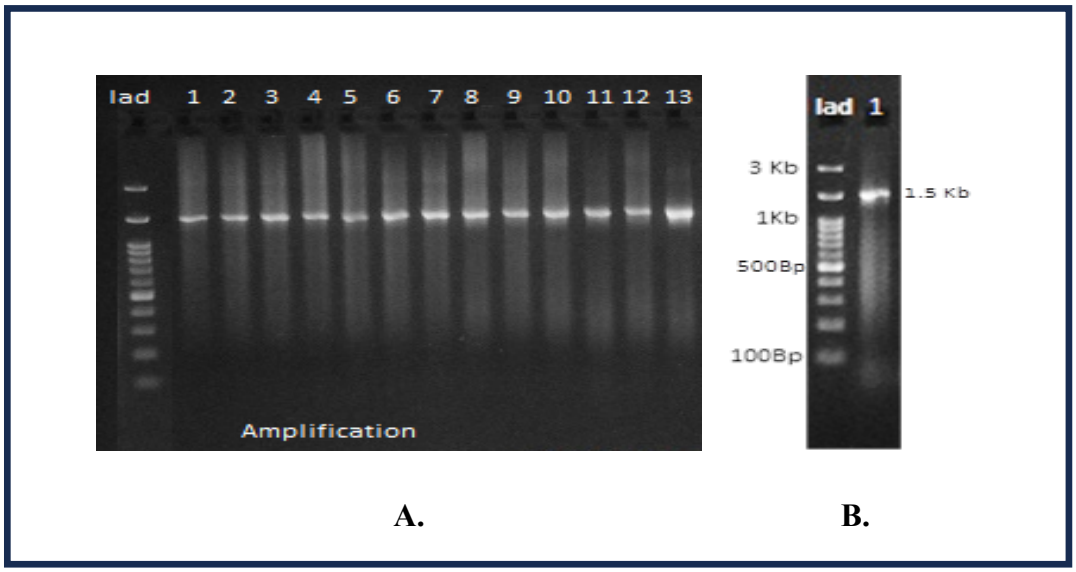


Figure 3.3. Agarose gel electrophoresis of 16S rRNA PCR products from bacterial isolates using universal primers: A1-C1; A2- C2; A3-C3; A4-C4; A5-C5; A6-C6; A7 T1; A8-T2; A9-T3; A10-P1; A11-P2; A12-P3; A13-P4; B1- T4

3.13.3.5. DNA sequencing

After the purification, 2 µl volumes of samples were taken for the sequencing per reaction using BDT V3.1 chemistry. After sequencing, data were generated in the form of FASTA file which were taken further for their quality checking, primer trimming, removal of ambiguous bases and further bioinformatics analysis.

3.13.3.6. Data quality control, trimming and sequence alignment

Poor contaminated bases of sequencing data were trimmed from both the end of forward and reverse sequencing data. Then the trimmed forward and reverse bases were aligned together using bioinformatics software (Bioedit) and consensus sequences were generated for each of the DNA samples. Processed concensus sequences were subjected to blast analysis (National Centre for Biotechnology Information, NCBI) for finding probable sequence similarity, E-value, query coverage and highest percentage identity, etc. The identified DNA sequences were then submitted to gene bank database and accession IDs were generated.

3.13.3.7. Construction of Phylogenetic Tree

The FASTA sequences of identified bacterial taxa isolated in present study were subjected to phylogenetic tree construction. Top five BLAST sequences for each bacterial isolate from NCBI database were used to allign and construct the phylogenetic tree to refer their evolutionary history. The FASTA sequences were aligned by ClustalW programme using MEGA11 (version 11.0.13) (Tamura *et al*., 2021). Phylogenetic tree was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). A bootstrap analysis of 1000 replicates was performed for clustering taxa (Felsenstein, 1985).

3.14. Statistical data analysis

All the statistical analysis was conducted in Paleontological Statistics (PAST) (v. 4.03) software, and all experiments were performed in triplicate, and results were represented as Mean ± SE, Significant variations in the data were calculated using one-way Analysis of Variance (ANOVA) at 95% level of significance ($P<0.05$).

PLATE: 1

Photograph of food plants used for rearing of *S. ricini*: A. Castor (*Ricinus communis*); B. Tapioca (*Manihot esculenta*); C. Papaya (*Carica papaya*)

PLATE: 2

Photograph of submitted herbarium sheets of sampled food plants: A. *R. communis* (BUBH0000827); B. *M. esculenta* (BUBH0000828); C. *C. papaya* (BUBH0000829).

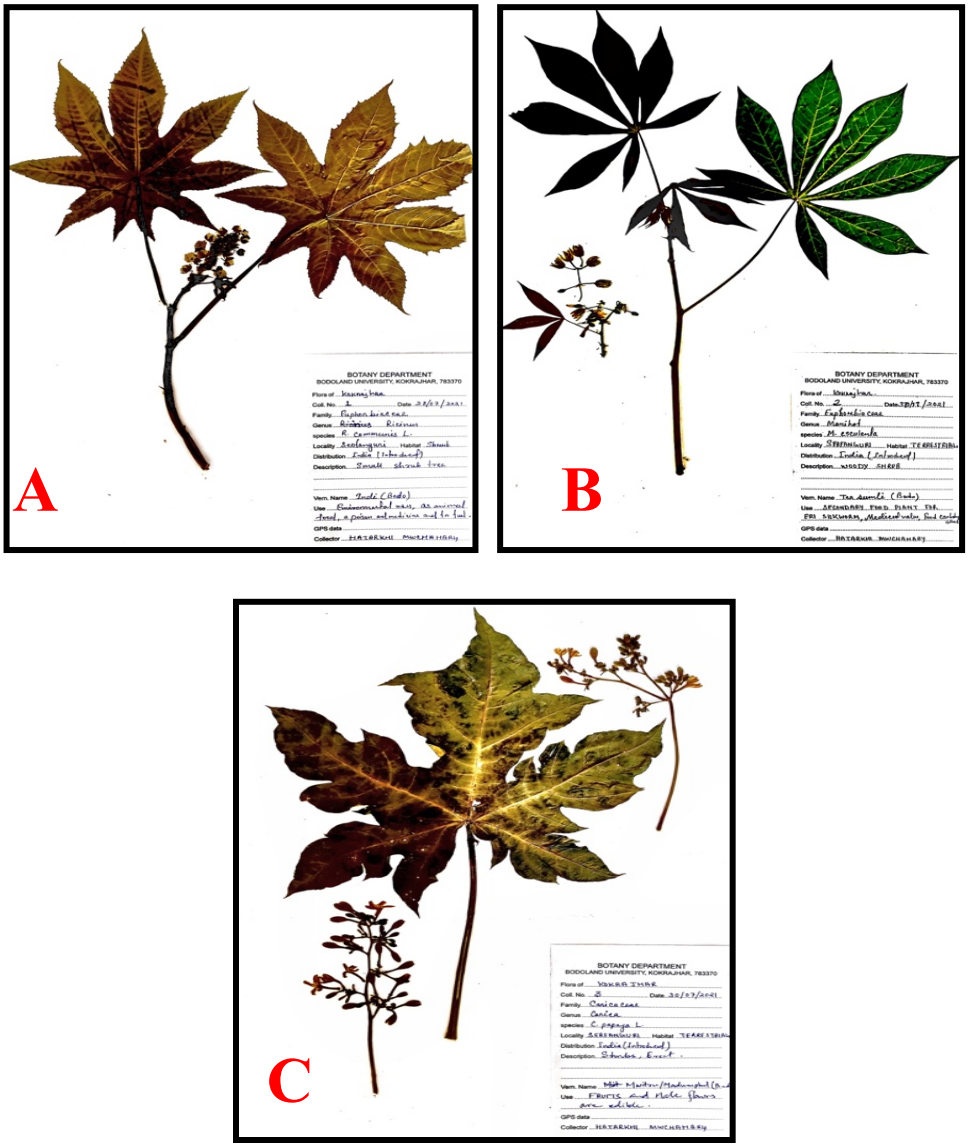
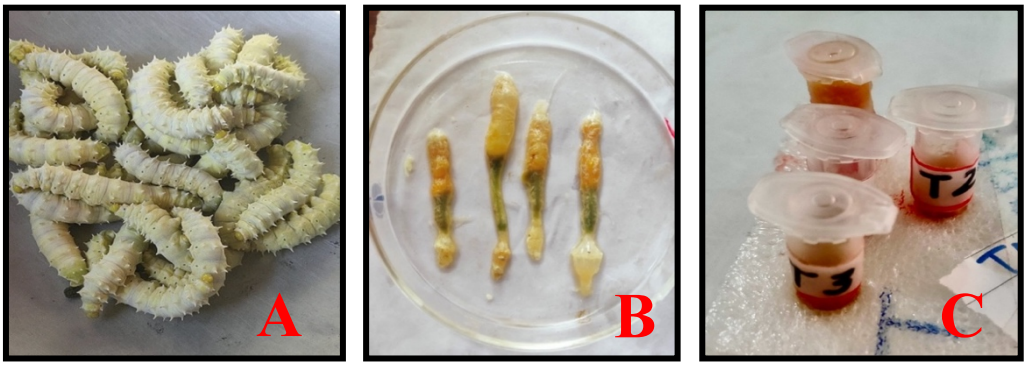


PLATE: 3

Photograph of Rearing of *S. ricini* on: A. *R. communis* leaves; B. *M. esculenta* leaves; C. *C. Papaya* leaves

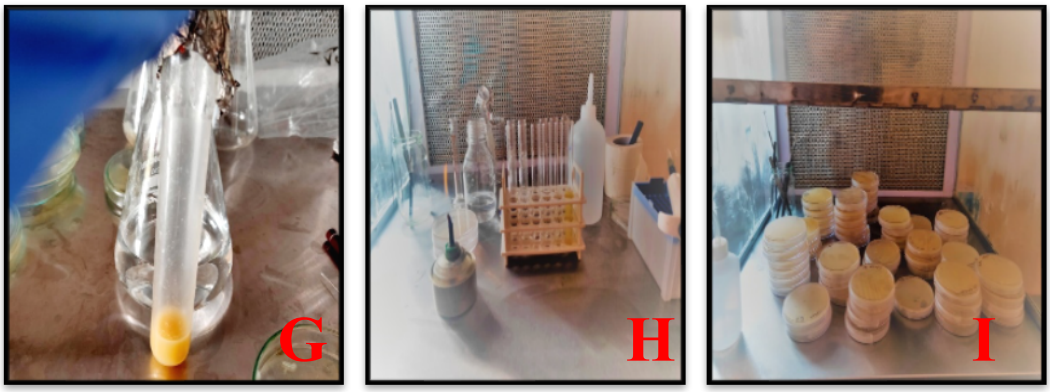
PLATE: 4

A to I: Photos of experimental procedures for isolation and culture of gut bacteria



A. Mature 5th instar *S. ricini* larvae; B. Gut samples extracted from the larvae; C. Collection of sample in 1.5ml eppendorf tube

D. Nutrient agar media preparation; E. Plating of media; F. Incubation of culture media under BOD under 37°C



G. Gut homogenate preparation; H. Serial dilution of gut homogenates; I. Inoculated plates of gut samples

PLATE: 5

Mixed culture of gut bacterial isolates: A. Mixed culture isolated from *R. communis* fed larvae (Isolate C); Isolated from *M.esculenta* (Isolate T); C. Isolated from *C.papaya* fed larvae (Isolate P)