Chapter 3
1. Experimental animal

The silkworm is the larva or caterpillar of the domesticated silkworm *Bombyx mori*. It is an economically important insect, being a primary producer of silk. Due to its miniature size and ease of culture, the silkworm has become a model organism. It is found to be an important laboratory tool in the study of Lepidopteran and arthropod biology. It is estimated that more than 3000 silkworm strains are available all over the world due to various ongoing breeding programmes (Nagaraju, 2002; Thangavelu et al., 2003). These silkworm varieties include univoltines, bivoltine and polyvoltines. Univoltines and bivoltines are qualitatively and quantitatively superior races whereas polyvoltines are relatively inferior in both the traits but superior in their survival and hardiness. But in tropical countries like India, polyvoltine silkworm strains play important role in the production of silk, since they are well accustomed to the tropical climatic conditions.

Plate-1a : Showing the mulberry silkworm *Bombyx mori* (L)

The model organisms selected for this study was the mulberry silkworm, *Bombyx mori* belonging to the order Lepidoptera, class Insecta and Family Bombicidae.
The race chosen was a hybrid of LXNB4D2 (L - Local multi voltine variety, NB4D2 - New bivoltine with dumbbell shaped cocoon) which feeds, mainly on mulberry leaves (PLATE 1a). It is a holometabolous insect, whose life cycle has four distinct stages namely the egg, larva, pupa and adult.

1.1. Rearing of silkworm

Eggs of *Bombyx mori* (LXNB4D2) was obtained from the grainage of Regional Sericulture Centre of V.M. Chathram, Tirunelveli, Tamilnadu, India. The V instar larvae were reared by the tray rearing method by adapting Krishnaswami *et al.* (1970). In this method tray were lined with newspaper and care was taken to maintain the required temperature and humidity. MR2 variety of mulberry was used to feed the worms. Which belong to the family Moraceae and *Morus sp*. Fresh healthy leaves were collected during the cool hours of the day and stored in wet gunny bags. The leaves were chopped and fed to the early larval instars. The larvae were fed 5 times a day. The beds were cleaned every day and sufficient spacing was adopted during rearing.

2. Methoxyfenozide

**RH - 2485: A Pure Synthetic Non-Steroidal Ecdysone Agonist**

Physical and Chemical Properties of Methoxyfenozide

- **Code Number**: RH - 2485, RH - 112, 485
- **Molecular formula**: C22H28N2O3
- **Molecular weight**: 368 Dalton
Structural Formula

Common Name : Intrepid 2F and Intrepid 80 WSP
Chemical Name : Benzoic acid, 3-methoxy-2-methyl-2-(3,5-dimethylbenzoyl)-2-(1, 1-dimethylethyl) hydrazide
(IUPUC) : N-tert-butyl-N'-(3-methoxy-o-toluoyl)-3, 5-xylohydrazide
ISO Name : Methoxyfenozide
Physical State : Light brown liquid
Melting Point : 204 – 205°C
Odour : None
Vapour Pressure : <1 X 10⁻⁷ mmHg (25°C)
Stability : Stable at 54°C, stable to hydrolysis at pH5.7 And 9
Solubility : 3.3 ppm (20°C)
DMSO : 11%
Cyclohexane : 9.9%
Acetone : 9%
Formulation : 250mg/ [AI]/L

2.1. Preparation of Methoxyfenozide

100µl of methoxyfenozide was taken from 250 mg / [AI]/ liter, dissolved in 900µl of acetone and considered as stock from that and 1 µg ([AI]/litre) concentrations were applied topically to the V instar larva on day one.
3. Study Design

Bombyx mori

V instar larva

Control

Experimental (acetone 1µg/larva) (Methoxyfenozide 1µg/larva / [AI]/ liter)

12, 24, 48, 72 and 96 hours after treatment

Extraction

Haemolymph Fat Body Silk gland Mid gut

Haemogram

THC

DHC

Cytomorphology

- Protein
- Carbohydrate
- Lipid

Biochemical analysis

Histology

Enzymes

Microbial fauna

Protease I, II

Proteolysis

Amylase

Acid phosphatase

Lactate dehydrogenase
4. Haemogram and cytomorphology of Haemocytes

The experiment consisted of 100 V instar larva. A group of 50 larvae served as control and other 50 as experimental. The control groups were applied with acetone mid dorsally. The experimental groups were topically applied with 1µg/larva of RH-2485 in acetone on day 1 after molting. Care was taken to ensure that control and treated groups were kept in separate places. THC and DHC and cytomorphology were studied every day by collecting the haemolymph after 12, 24, 48, 72 and 96h through cutting the pro legs.

4.1. THC (Total Haemocytes Count (Jones, 1962))

The *B.mori* larvae were taken from the container and the prolegs were cut to collect the haemolymph. The counting of the free haemocytes was done by using haemocytometer with improved double Naubauer ruling. The haemolymph was collected in the white blood cell pipette, which has calibrated up to 0.05 ml. The WBC pipette rinsed earlier with distilled water. Then 0.05ml of haemolymph was taken and immediately it was diluted by 0.8% stained saline solution (Gentian Violet) up to the mark of 1ml and shaken well. The mixed fluid was drawn in to the chamber and the cells were counted under the light microscope.

**Calculation:**

Haemocytes in the four corners (1mm square) were counted and THC/mm$^3$ was determined by the following formula.
THC  ==  Total number of cells counted x dilution factor x depth factor

Where,

\[
\text{Dilution factor} = 0.05\text{ml} = 20 \text{ (i.e., 0.5 ml = 20)}
\]

\[
\text{Depth factor} = 10
\]

\[
\text{No of squares counted} = 4
\]

The THC had been determined in at least five individuals and were recorded in relation to control and treated groups (1µg/larva of methoxyfenozide) taken after day by day.

### 4.2. DHC (Differential Haemocytes Count (Jones, 1962))

The hemolymph was collected by cutting proleg of experimental animal. The haemolymph was drawn on a glass slide which was already washed with 100% alcohol chocked cotton to remove impurities. The rectangular thin cover glass was taken and a thin blood smear was prepared. 25% Giemsa stain (which was prepared in methanol or 70% alcohol) was allowed to spread on the smear and air dried for 1 or 2 minutes. Then it was gently washed by adding distilled water and dried. Thus prepared slide was then observed under high power (45X) light microscope. Totally 100 cells were counted from each and five insects as prohaemocytes, plasmatocytes, oenocytoids, sperule cells and granular cells.

### 4.3. Cytomorphology

The haemocytes of both control and the treated V larva cytomorphology was studied by measuring the length and width of prohaemocytes, plasmatocytes, oenocytoids, sperule cells and granular cells. The morphometry were measured using
stage and ocular micrometer fitted to the light microscope at 100X. The cellular components were observed through 100X oil immersion and were photographed.

5. Biochemical analysis

Biochemical analysis like protein, carbohydrate and lipids were estimated from the collected samples of haemolymph, fat body tissue, silk gland and the mid gut.

5.1. Sample collection

The samples were extracted by following the methods given below.

5.1.1. Haemolymph

Collection of haemolymph protein was done by cutting the abdominal legs of *B. mori* larva after 12, 24, 48, 72 and 96h after treatment with methoxyfenozide and haemolymph was bled from the wound directly into the eppendorf tubes coated with phenylthiourea, an antioxidative agent and equal amount of extraction buffer with pH7.2 (50mM Tris Hcl : 607 mg, 400mM Nacl:2.338 gm, 1mM Phenyl methyl sulfonyl fluoride:17.4mg, GDW to 100ml adjust the pH with 0.1N NAOH) was added then centrifuged at low speed to remove haemocytes the supernatant was stored in a refrigerator until further analysis.

5.1.2. Fat body

Dissect the whole fat body of the *B.mori* larva and rinse with double distilled (DD) water after 12, 24, 48, 72 and 96h after treatment with methoxyfenozide. Grind with Extraction buffer (1-2ml) using tissue homogenizer. And centrifuge at 12,000 rpm for 10 minutes at 4°C. Collect the supernatant and store at deep freezer for further analysis.
5.1.3. Silk gland

Dissect the whole silk gland of the V instar *B. mori* larva and rinse with double distilled (DD) water after 12, 24, 48, 72 and 96h after treatment with methoxyfenozide. Grind with Extraction buffer (1-2ml) using tissue homogenizer. And centrifuge at 12,000 rpm for 10 minutes at 4°C. Collect the supernatant and store at deep freezer for further analysis.

5.1.4. Mid gut

Dissect out the mid gut of the *B. mori* larva and rinse with double distilled (DD) water after 24, 48, 72 and 96h after treatment with methoxyfenozide. Grind with Extraction buffer (1-2ml) using tissue homogenizer. And centrifuge at 12,000 rpm for 10 minutes at 4°C. Collect the supernatant and store at deep freezer for further analysis.

5.2. Estimation of protein (*Lowry et al.*, 1951)

The protein content were analyzed from the collected samples by following *Lowry et al.* (1951) method and BSA was used as standard.

Lowry’s reagent C (sol A: 2% NaCo3 10g; and 0.1 N NaOH 2g in 500ml H2O, sol B:0.5% CuSO4 in 2% potassium sodium tartarate and make upto 100ml, Sol C: 50 ml of sol A and 1ml of sol B). Follin’s Reagent: (1N strength) 1:1 ratio approximately before use.

To 1 ml of the standard protein solutions containing 10-100 µg of protein (or) appropriately diluted unknown protein sample solution add 4ml of reagent D and mix the contents. After 10 minutes of incubation at room temperature, add 0.4 ml of Follin’s reagent and vortex the content immediately. Run a reagent blank with 1 ml of distilled
H₂O along with the standards and sample. Incubate this for 30 minutes at room temperature, read the O.D at 720nm, in a photometer and record the absorbance.

**Calculation**

\[
\text{Amount of protein in sample} = \frac{\text{OD of the sample} \times \text{Concentration of the standard (µg / µl)}}{\text{OD of the standard}}
\]

5.3. **Estimation of carbohydrate** *(Carrol et al., 1956)*

The weighed (10mg) samples were put into mortar and 2 ml of 80% ethanol was added and ground well. This homogenate was centrifuged for 10 minutes at 3000 rpm. To the supernatant 1ml of 10% TCA was added. The precipitated protein is removed by centrifuging at 3000rpm for another 10 minutes. 1ml of supernatant was taken and 5ml of 0.2% Anthrone reagent was added. Test tubes are covered with cotton on the top and kept in a boiling water bath for 10 minutes. They were cooled at room temperature and OD is measured at 620 nm using spectrophotometer. A respective volume of reagent is added to 1ml of distilled H₂O served as blank. Glucose served as Standard. The OD is compared with standard graph to estimate the carbohydrate.

5.4. **Estimation of lipid** *(Zak et al., 1954)*

A gram of tissue was homogenized using 0.1 M phosphate buffer and filtered, centrifuged at 3000 rpm for 5 minutes. The supernatant were collected from which 2.5 ml was taken and 2.5 ml of FeCl₃, diluting reagent 4 ml of H₂SO₄ was added and mixed thoroughly. Standard was prepared by taking 0.1, 0.2, 0.3, 0.4, and 0.5 ml of standard cholesterol and dilute at 5 ml with FeCl₃ diluting reagent and 4 ml of H₂SO₄ and blank was also prepared and read OD at 560 nm.
5.5. Protein separation using SDS PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis)

5.5.1. Sample collection

For preparing samples for SDS-PAGE collection of haemolymph proteins were collected as given in 5.1.1. Protein content of the sample was estimated by adopting the method of Lowry et al. (1951) and BSA was used as standard.

7% SDS-Polyacrylamide gel electrophoresis was carried out following the method of Laemmli (1970). The cathode migrating proteins according to their molecular weight started migrating and separated according to charge. 7% resolving gel and 4% stacking gel were used to separate various proteins. The resolving gel was prepared by adding the following.

5.5.2. Preparation of Gel

The gel was prepared by mixing stock solution as per the following schedule.

<table>
<thead>
<tr>
<th></th>
<th>Monomer</th>
<th>Separating gel buffer</th>
<th>10.0% SDS</th>
<th>Initiator (APS)</th>
<th>GDW</th>
<th>Catalyst (TEMED)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a) Resolving gel (7%)</strong></td>
<td>-</td>
<td>0.65ml</td>
<td>-</td>
<td>60μl</td>
<td>3.39ml</td>
<td>3μl</td>
</tr>
<tr>
<td>Stocking gel (4%)</td>
<td>Monomer</td>
<td>- 0.25ml</td>
<td>Stocking gel buffer</td>
<td>0.5ml</td>
<td>Initiator (30%)</td>
<td>0.1ml</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>- 0.5ml</td>
<td>-</td>
<td>20μl</td>
<td>GDW</td>
<td>1.12ml</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>- 0.25ml</td>
<td>-</td>
<td>2μl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample Buffer</td>
<td></td>
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<tr>
<td>Tris Hcl (pH 6.8)</td>
<td>0.62ml</td>
<td></td>
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<tr>
<td>Glycerol</td>
<td>0.5ml</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>25ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>75mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>pinch</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Double distilled water</td>
<td>3.6ml</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Catalyst</td>
<td>N, N, N’, N’ - Tetramethylene diamine.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initiator</td>
<td>Ammonium per sulphate (APS) - 1.5g , GDW to 10.00 ml. This solution was prepared just before use.</td>
<td></td>
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</tr>
</tbody>
</table>

5.5.2. Casting of Gel

The resolving gel mixture were mixed gently but thoroughly and slowly poured into the glass plate sandwich. A fine mist of 0.1% SDS was layered on to the gel to exclude oxygen from inhibiting polymerization and to ensure a uniform flat gel surface. Presence of a sharp interface between the polymerized gel and the overlay was an indication of complete polymerization. At this time the overlaying SDS was decanted. 4% stacking gel was prepared by mixing the following solutions and poured over the separating gel.

A teflon comb of 1.5mm thickness was inserted to form the wells. After polymerisation, the comb was removed and the wells were rinsed with 0.1% SDS. Care was taken not to trap any air bubbles while casting the gel. Protein samples were mixed with equal volume of sample loading buffer of pH 6.8 and kept in boiling water bath for 30 seconds and then loaded on to each well. Individually, molecular weight marker proteins (phosphorylase b- 97kDa, bovine serum albumin- 66kDa, ovalbumin –43kDa and carbonic anhydrase-29kDa and Lysozyme-14kDa (Himedia, Mumbai, India) were loaded simultaneously for comparison.
Electrophoresis was carried at a constant voltage of 50V in the region of stacking gel and 100V in the region of resolving gel. The running buffer (electrode solution) of pH-8.3 (0.025M Tris, 0.815M glycine and 0.1%SDS) prepared by dissolving 3g of Tris, 14.4g of glycine and 1g of SDS in 1litre of distilled water. After electrophoresis, the gels were stained with staining solution for 30 minutes. Then, the gels were destained with destaining solutions. They were stored in 7% acetic acid for the purpose of photography.

<table>
<thead>
<tr>
<th>Staining Solution</th>
<th>Coomassie brilliant blue - 0.25g (R-250)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>- 50ml</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>- 7ml</td>
</tr>
<tr>
<td>GDW to 100ml</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Destaining Solution</th>
<th>Methanol - 30ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>- 7ml</td>
</tr>
<tr>
<td>GDW to 100ml</td>
<td></td>
</tr>
</tbody>
</table>

6. Enzyme activity


To 1ml of 1% casein solution (1g in 100 ml of distilled water) 0.5ml of extract was added and incubated at 35°C for 1h in water bath. Reaction was terminated by adding 5ml of 10% TCA. Centrifuged at 3000rpm for 10min and supernatant was collected. 1ml of supernatant was added with 5ml of Lowry’s reagent C. 0.5ml of three fold diluted Folin’s phenol reagent was added. The OD was measured at 670nm. BSA used as standard.
6.2. Estimation of Protease II (Eguchi and Iwamoto, 1976).

To 0.5ml of 1% casein add 2ml of 0.1M borate buffer and 0.5ml of extraction was added. Incubate at 30°C for 1h. And 2ml of 10% TCA was added to terminate the reaction and centrifuged. Collect the supernatant and add Folin’s reagent and read at 660nm.

6.3. Estimation of Proteolytic activity (Briegel and Lea, 1975)

To 1ml of 1% casein in 0.1M phosphate buffer (pH 7.6), add 1ml of calcium chloride and 1ml of extract, incubate at 37°C for 1 hour. Centrifuge and use the supernatant. And one part of supernatant and another 5 parts of Lowry’s reagent C and add 3ml of diluted Folin’s phenol reagent (1:1 ratio). And read OD at 650nm.

6.4. Estimation of Amylase activity (Ishaaya and Swirski, 1970)

To 1ml of 0.2% soluble starch in borate buffer (pH 9.2) add 0.25 ml extract and incubate at 37°C for 30 min. Add 0.4 ml of DNS reagent (1% 100ml NaOH, 1g Dinitro Salicic acid, 200mg crystalline phenol, 50mg sodium sulphite, make upto 1 L with distilled H₂O and store at 4°C) and incubate it for 5 min at 100°C. Read the colour development at 575nm. Glucose used as standard.

6.5. Estimation of Acid phosphatase (Beaufay et al., 1954)

Incubate 1ml of 0.2M acetate buffer (pH 5.5) and 0.2 ml extract at 37°C for 10 min. Add 0.5 ml of substrate (1.49g of EDTA, 0.84g citric acid and 0.03g ρ–nitrophenol phosphate in 100 ml water and adjust to pH 5.3) and incubate at 37°C for 1h, then add 4.5 ml of 0.1 NaOH and read at 420 nm. ρ–nitrophenol used as standard.
6.6. Estimation of Lactate Dehydrogenase (King, 1965)

Incubate 0.1 ml of enzyme extract with 0.2 ml of NAD and 0.2 ml of distilled water at 37°C for 15 min. Add 1 ml of 2,4 Dinitro phenyl hydrazine and incubate at 37°C for 15 min and cool it to room temperature. Add 10 ml of 0.4N NaOH and read the O.D at 440 nm. NADH used as standard.

7. Gut histology

Haemocyte behavior and the gut pathologic conditions were studied using histological technique. Whole animal, gut treated with methoxyfenozide (1 µg/larva/ [AI]/litre) for 24h, 48h, 72h and 96h were fixed in Bouin’s fluid for 24h. Then the tissue were washed in running tap water and transferred to 70% ethyl alcohol. Tissue dehydration was accomplished by the use of graded alcohol series beginning with 70% ethyl alcohol. Xylene was used as a clearing agent. The tissues were impregnated with wax by keeping in three molten paraffin baths for 20 minutes each (melting point 56-60°C. Microtone sections were cut at 5-6µl thickness and stained in Harris haemotoxylin and eosin. The cellular changes in the gut epithelial cells were considered as the major criteria to assess the immunotoxic impact of growth regulator. These changes were observed under the light microscope (NIKON-IIIE-400Eclipse) and photographed at 10X, 40X and 100X (oil immersion).

8. Gut Microflora Studies

8.1. Enumeration of Total Heterotrophic Count (THC)

1 gram of each sample of control and methoxyfenozide treated silkworm gut were taken from the fifth instar (24h). The samples were homogenized with 10 ml sterile distilled water using mortar and pestle in the sterilized UV chamber. Then the
homogenized solution was centrifuged at 1000 rpm for 10 min. 1 ml of the supernatant was mixed with 9 ml of sterile distilled water and then it was serially diluted up to $10^{-7}$ dilution. Pour plate technique was carried out for the enumeration of total heterotrophic bacteria using sterile Nutrient agar (Himedia) for bacterial growth. 1 ml of diluted sample was pipetted out aseptically into sterile petri dishes. Sterilized Nutrient agar was poured into petridish containing the diluted samples. Following addition of the Nutrient agar the plate is gently rotated in a circular motion to achieve uniform distribution of microorganisms and immediately after solidification placed in an inverted position. The procedure was repeated for all dilutions to be plated. Dilutions were plated in triplicate. After 48 hours of incubation at room temperature, total colony forming units (CFU/gm) were determined.

The isolation and identification of different bacteria from control and treated samples were done based on the growth of bacteria on agar media such as nutrient Agar, EMB agar, Mac Conkey, *Pseudomonas* selective media and Mueller Hinton Agar. A representative number of the different colonial types were detected and collected from plates and streaked on Nutrient Agar for purity and to carry out identification. Isolates were stabbed on to Nutrient agar slants incubated for 24-48h maintained by overlying sterile liquid paraffin for further studies.

8.2. Morphological characterization of selected bacteria

The morphological identification of all isolates studied were performed and compared to the description and keys as described in the Bergey’s Manual of systematic Bacteriology. The phenotypic features characterized are as follows: Cultural and Morphological characteristics: The colony morphology, cell morphology, and the motility of bacterial isolates from fresh cultures were evaluated.
8.3. Biochemical Identification of bacteria

Colonies obtained from nutritive agar selective plates were isolated and streaked on nutrient agar slants, and broth and incubated overnight at room temperature. The following biochemical tests were conducted to identify the bacteria following Bergey’s manual of bacteriological classification (Sneath et al., 1984; Simbert and Krieg, 1997; Hols et al., 1994)

Biochemical Tests (MacFadin, 1980)

8.3.1. Gram’s staining

A loopful of culture was placed on clean glass slide. Smear was made and subjected to Gram’s staining. Primarily, crystal violet was applied to the smear and kept for 60 sec and washed with distilled water. Secondly, Gram’s iodine was used as mordant and washed with water. Afterwards it was washed with 95% alcohol and then treated with saffranine and kept for 45 seconds. The gram positive organism appeared purple blue whereas gram negative organism appeared pink.

8.3.2. Motility (Hanging drop method) (Kannan, 1996)

Motility of organism was identified following hanging drop method. Vaseline was smeared in four edges of a cover slip and a very small drop of the culture was placed at the center of the cover slip. Then a concave slide was placed in inverted position carefully on the cover slip. The cover slip was turned up right and examined under the microscope.
8.3.3. **Indole production test**

One percent peptone broth was prepared, sterilized and inoculated with the isolates and incubated at room temperature for 48 hrs. After incubation, 1 ml of Kovac’s reagent was added and gently shaken. The results were observed after allowing the tubes to stand. A cherry red ring was taken as an indication of positive reaction.

8.3.4. **Kovac’s oxidase test**

The test culture was inoculated in the peptone broth and incubated at 37°C for 24 hr. After incubation, 0.2 ml of Kovac’s reagent was added and the colour change was noted. Appearance of cherry red colour in the alcohol layer indicated positive result.

8.3.5. **Catalase test**

Nutrient agar slant (or) broth with the test culture was inoculated and incubated at 37°C for 24 hr. Following incubation 1 ml of 3% hydrogen peroxidase was trickled down the slant (or) broth. They were examined immediately after 5 minutes (Kannan, 1996). Evolution of bubbles indicated a positive reaction.

8.3.6. **Methyl Red test**

The tubes of MR-VP broth was inoculated with the test culture and incubated at 37°C for 24h. Following incubation, 5–6 drops of methyl red solution was added. A bright red colour indicated positive result.

8.3.7. **Voges Proskeur test**

The test culture was inoculated in the MR-VP broth and incubated at 37°C for 48 hr. After 48 hrs of incubation, 1 ml of 40% potassium hydroxide (plus creatine) and
3 ml of a 5% solution of alphanaphthol were added to the test culture. A positive reaction was indicated by the development of a pink colour within 3 minutes, which became crimson red in 30 minutes. The tube was shaken at intervals to ensure maximum reaction.

8.3.8. Citrate test

Sterilized air dried Simmon’s citrate agar slant was prepared and a loopful of culture was streaked and incubated at $37^0$ C for 24 hrs. After incubation period the slants were observed for colour change. A positive test showed a blue colour on the streak growth.

8.3.9. Urease test

The test culture was inoculated heavily over the entire slope surface and incubated at $37^0$C. Reactions were recorded after 4, 8, 12, 24 and 48 hours of incubation. A positive urease reaction was indicated by change in the colour of the medium from yellow to purple.

8.3.10. Carbohydrate fermentation test

Peptone broth containing 1% of the glucose sugar and pH indicator was prepared. After inoculating bacterium, test tubes were incubated for 24-48 hrs, observed for growth, acid and gas production.

9. Statistical analysis

Statistical method was done using SPSS 16 (windows software). One way ANOVA comparison test was done using “Duncan Multiple Range Test”.

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