Chapter 3

MATERIALS AND METHODS
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Oils used
Crude oil used for the experiments was obtained from the Navagam field of the Oil and Natural Gas Commission (ONGC), in Gujarat. API gravity of this oil was 31.

Servo 2T lubricating engine oil and pharma grade castor oil were also used as sole carbon source for some experiments.

Media
Bushnell-Haas media which was supplied by Hi-Media was used as minimal media.

Soil for isolation of micro-organisms capable of degrading crude oil
Crude oil containing soil was collected from various locations on the Navagam oil field of ONGC.

Media for:

a) Enrichment and growth of microorganisms capable of degrading crude oil
Bushnell and Haas media obtained from Hi-Media labs, supplemented with crude oil was used for growth of hydrocarbon degrading micro-organisms.

b) Growth Curve
Bushnell-Haas media was supplemented with crude oil, castor oil and lubricating oil as and when required, for the experiment.
Enumeration of heterotrophs and hydrocarbon degraders

Determination of frequency of heterotrophs and hydrocarbon degrading organisms was carried out by the MPN method using nutrient broth for heterotrophs and Bushnell-Haas broth supplemented with crude oil for hydrocarbon degrading bacteria.

Soil for Phytotoxicity studies
A mixture of soil: sand in the proportion of 1:1 was prepared. It was supplemented with 5% crude oil, mixed and sterilized. Soil used for control was not supplemented with crude oil. Unsterilised soil was also used as one of the controls. Ammonium nitrate and dipotassium hydrogen phosphate were used for fertilization. 10 μM/g of N as ammonium nitrate and proportionate concentration of P as di-potassium hydrogen phosphate was used.

Column Chromatography
Silica gel of 60-120 mesh for fractionation of crude oil was obtained from Qualigens. Hexane, Benzene, Chloroform (S.D. Fine Chem) and Methanol (Qualigens) were used for the fractionation of crude oil into saturates, aromatics, asphaltenes and resins.

Chemicals Used For Extraction Of Plasmid DNA
Sodium Chloride, Tris hydroxymethylaminomethane, Glacial acetic acid, Ethylene diaminetetra acetate (di-sodium salt), Sodium Acetate, Glucose, Sodium hydroxide and Chloroform were obtained from Qualigens.

Phenol and 8-hydroxyquinoline obtained from S.D. Fine Chem.

Bromophenol Blue and Ethidium Bromide were obtained from Sigma.

Agarose and sodium dodecyl sulfate from Hi-Media
Lysozyme was obtained from SRL
Alcohol was obtained from Alembic chemicals, Vadodara
Protein estimation
Copper sulfate, NaOH, Na$_2$CO$_3$, Na K tartarate, standard bovine albumin and Folin-ciocalteau reagent were obtained from M/S Qualigens, India.

Methods

Isolation of crude oil degrading bacteria
Soil from the crude oil contaminated site was used to screen for crude oil degrading microorganisms by enrichment technique. Soil (0.5 g) collected from various locations of the Navagam field area was added to 50 ml of sterile Bushnell-Haas medium (Hi-Media) in 250ml Erlenmeyer flasks. The BHM was supplemented with 1% crude oil as sole carbon source and incubated at 37± 2°C on a rotary shaker at 120 rpm for 7 days. Crude oil samples collected from the same site were also used for screening of hydrocarbon degrading bacteria. Isolation and maintenance of bacteria was carried out on Nutrient Agar (Hi-Media).

Enumeration of hydrocarbon degrading organisms
A five tube MPN technique was used to estimate the number of hydrocarbon utilizing organisms. The media used was crude oil supplemented Bushnell Haas media (Hi-Media). Three sets of five tubes each containing 5 ml of autoclaved medium containing 1% crude oil was inoculated with 1, 0.1 and 0.01 ml of appropriately diluted soil suspension. These tubes were incubated at 37± 2°C on static conditions. The MPN values were obtained from the MPN index for the five tubes. The same procedure was followed for enumerating heterotrophs from the same soil sample. The media used was nutrient broth.

Analysis of Crude oil
Crude oil analysis was done by gravimetric measurement. The amount of oil was determined by measuring the weight of the sample, the oil degrading capacity being evaluated by the decrease in the weight compared to that of the control sample. Uninoculated flasks containing Bushnell-Haas medium supplemented with crude oil were used to estimate the loss due to weathering. Chloroform (Qualigens) was used as a solvent for this experiment.
**Growth of Bacterial isolates**

Bacteria was grown in Bushnell-Haas media supplemented with 2% crude oil on a rotary shaker at 120 rpm, 37± 2° C. Growth of bacteria was measured spectrophotometrically (Beckman DU-64) by reading at 600 nm at an interval of 24 hours till a near constant reading was obtained. Bushnell-Haas media was used as a blank. Cultures were also grown in Castor oil (growth was measured at $A_{600\text{nm}}$ at an interval of 6 or 12 hours) and 2T oil (growth was measured at $A_{600\text{nm}}$ at an interval of 24 hours).

**Effect of Sodium Chloride concentration**

Bacterial isolates were inoculated in Bushnell-Haas medium supplemented with crude oil. Sodium chloride concentration was varied from 0 to 2M and bacterial growth was measured spectrophotometrically. Media devoid of externally added NaCl was also used.

**Effect of Hydrocarbon concentration**

Bushnell-Haas medium containing varying concentrations of crude oil was inoculated with the bacterial isolate and $A_{600}$ was measured. Concentration of crude oil used for this purpose was 1% - 20%.

**Studies on phytotoxicity of crude oil and bioremediation**

These experiments were carried out to study the efficacy of crude oil degradation by cultures in supporting seed germination and plant growth. Soil weighing 150 grams (mixture of garden soil and sand in the ratio 1:1) was mixed with 5% crude oil and sterilized by autoclaving at 15lbs, 121°C for 15 minutes. It was then put in small plastic pots (2.4" x 2.4" x 2.6") which were UV sterilized. The soil was then inoculated with approximately $3.3 - 4 \times 10^7$ cfu/g of soil. It was incubated at 37± 2° C for 10 days. Controls used for this purpose were garden soil devoid of oil, soil containing crude oil which was uninoculated and soil inoculated with the microorganisms but not containing oil. 25 seeds of *Vigna radiata* (Linn.), 20 *Oryza sativa* L. and 20 seeds of *Brassica juncea* (L.) Coss. were sown in separate pots after incubation period. Germination percentage, root and shoot length and wet weight of seedlings was measured.
For studies on effect of fertilization and bioaugmentation, different experimental pots were set up. To check the efficacy of indigenous soil microorganisms, garden soil was artificially contaminated as described above and was left unsterilized so that the microorganisms present in the soil could degrade the oil. To other pots in this experiment, N: P in the different proportions like 1:1, 1:2, 1:5 and 1:10 was added.

To check whether addition of an organism known to degrade crude oil enhances rate of seed germination, *Pseudomonas* XRF15, an organism isolated by us from the Navagam oil field was added to the unsterilized contaminated garden soil. Fertilization in the above mentioned proportion was also carried out.

To check for the effect of seeding of crude oil contaminated soil with *Pseudomonas* spp. (XRF15) (seeding) and fertilization treatment, the soil preparation was autoclaved after addition of crude oil thus leaving the soil free of indigenous soil organisms and then seeding and fertilization was carried out.

**Protein estimation**

Estimation of protein was done by Lowry’s method (1951).

**Chlorophyll content**

Chlorophyll content of the plants was estimated using Harborne’s method (1973). Plant material was powdered in a mortar for a few seconds to break the tissues. 25 ml of acetone was added and the tissues were macerated further. A pinch of calcium carbonate was added to this mixture to prevent the degradation of chlorophylls to pheophytin. The mixture was decanted to a filter leading to a 50 ml volumetric flask. The residue in the mortar was mashed further with 5 ml of acetone and the mixture was transferred to the filter. The residue remaining in the filter funnel was washed with 2 x 5ml quantities of acetone so that the acetone dripping down to the flask became colourless. 10 ml of distilled water was added to the flask to make the volume upto the 50 ml mark. The extract was shaken well and the absorbance was measured using Beckman DU 530 spectrophotometer at 663 nm and 645 nm in 1 ml cells. The concentrations of the various chlorophylls was calculated using the given formulae:
Chlorophyll \( a (mg/l) = 12.7 A_{663} - 2.69 A_{645} \)

Chlorophyll \( b (mg/l) = 22.9 A_{645} - 4.66 A_{663} \)

Total chlorophyll \( mg/l = 20.2 A_{645} + 8.02 A_{663} \)

Chlorophyll content on a fresh weight basis was calculated by the following formula:

\[
\text{Chlorophyll a (mg/g)} = \frac{12.3 A_{663} - 0.86 A_{645} \times V}{1000 \times W}
\]

\[
\text{Chlorophyll b (mg/g)} = \frac{19.3 A_{645} - 3.6 A_{663} \times V}{1000 \times W}
\]

\[V = \text{volume} = 50 \text{ ml}\]
\[W = \text{fresh weight in grams i.e 10 gms.}\]

**Extraction of plasmid DNA**

Plasmid DNA was extracted using alkaline lysis method (Birnboim and Dolly, 1979)

- Bushnell-Haas media supplemented with 2% crude oil was inoculated with 1% inoculum.

- It was incubated on shaking conditions for 72 hrs at 37±2°C at 120 rpm.

- 1.5 ml culture was taken into a microfuge tube centrifuged at 12000g for 5 minutes at 4°C.

- Supernatant was removed and pellet was resuspended in 100µl of ice cold Solution I (Glucose 50mM, Tris-Chloride (pH8) 25mM, EDTA (pH8) 2mM by rigorous vortexing.

- 200µl of freshly prepared Solution II (1% SDS in 0.2 N NaOH) was added and mixed by inverting the tube five times. (Do not vortex). The tube was stored on ice.
• 150μl of ice cold solution III (5M Na acetate, glacial acetic acid and water) was added, the tube closed and vortexed gently in inverted position. The tube was stored in ice for 5 minutes.

• It was then centrifuged at 12000g for 5 minutes at 4°C. The supernatant was transferred to a fresh tube.

• An equal volume of 1:1 Phenol :Chloroform was added, the solution was mixed and centrifuged at 12000g for 2 minutes at 4°C.

• The aqueous layer was transferred to a fresh tube and extracted with equal volume of Chloroform. It was then mixed and centrifuged as above.

• The aqueous layer was carefully removed to a fresh microfuge tube and precipitated with 2 volumes of ethanol. It was stored for 5 minutes at -20°C and centrifuged at 12000g for 5 minutes at 4°C.

• The absolute alcohol was decanted and the pellet was rinsed with 1 ml of chilled 70% ethanol.

• It was stored at -20°C for 24 hours and centrifuged at 12000g, 5 minutes at 4°C.

• Alcohol was decanted, the pellet air-dried and dissolved in 30μl TE(pH=8).

• It was loaded onto 0.8% agarose gel + Ethidium Bromide (1μl/ml of gel from a 10mg/ml stock) after addition of bromophenol blue loading buffer (0.25% bromophenol blue in 40% sucrose).

**Fractionation of Crude Oil**

• A column of 60cm x 10mm was packed with silica gel (60-120 mesh).
• 10ml of residual oil was extracted with CHCl₃.
The oil was then loaded onto the silica column on the top and sequentially eluted with:

A) Hexane : 100 ml for saturate fraction
B) Benzene : 45 ml for aromatic fraction
C) Methanol : 50 ml for resin fraction
D) Chloroform : 35 ml for asphaltene fraction

- Fractions were dried in the oven and gravimetrically estimated.
- An equal weight of each fraction was taken and Bushnell-Haas media was added to it and sterilized.
- One flask was left uninoculated whereas the other flask was inoculated with a loopful of culture and incubated on a rotary shaker for 120 hrs at 110 rpm, 37± 2°C.
- Percentage degradation of each fraction was calculated gravimetrically after extracting each fraction with its respective solvent.

IDENTIFICATION OF MICROORGANISMS

In order to identify the isolates, morphology, pigmentation and detailed colony characteristics were noted. Characteristic of gram staining were recorded, gram-positive, gram-negative or gram variable. Morphology of the cell was also studied carefully to ascertain the purity of the culture.

After preliminary identification, final characterization was done by detection of biochemical or metabolic activity, following the procedures as described in the Bergey's Manual of Systematic Bacteriology (1984). All the media and reagents used for the identification procedure have been described in the Annexure.

STUDY OF BIOCHEMICAL AND METABOLIC ACTIVITY

Certain characteristics are unique to each species and help in the characterization of unknown bacterial isolates. For these purposes bacteria have to be first isolated
under strict sterilized conditions for obtaining pure cultures. These methods include standard methods of isolation like streak plate technique, pour plate technique, serial dilution method etc. Thereafter preliminary identification like colony characteristics and gram’s staining are performed which groups the bacteria of interest.

Detection of certain enzyme systems that are unique to each species can serve as identification markers that usually does the final characterization of unknown bacteria into genus and species. These enzyme systems are detected by the study of biochemical or metabolic activity of bacteria through a series of differential tests depending on the substrates utilized: proteins, lipids and carbohydrates. Of the several characteristic tests available to us we have to choose the tests depending on the preliminary identification such as Gram reaction, morphology studies and colony characteristics.

Ingredients for final characterization ingredients were dissolved in distilled water and pH was adjusted to 7.4, media was sterilized at 121°C for 20 minutes and dispensed in autoclaved petridishes.

GRAM'S STAINING METHOD

This was a differential staining technique used to demonstrate the properties of bacteria.

Principle
Crystal Violet (Gentian Violet) serves as a primary stain, which binds to the bacterial cell wall after treatment with iodine solution which serves as a mordant. Some bacterial species which have a higher content of peptidoglycan retain the crystal violet even after treatment with alcohol which serves as a decolorizer. Such bacteria are gram-positive. The gram-negative bacteria contain higher lipid content in their cell wall and lose the crystal violet primary stain on treatment with alcohol. When counter stained with basic fuschin or safranine they appear pink in colour.
Procedure

- A thin smear of bacterial suspension was made on the slide and allowed to air dry.
- The smear was fixed by passing it quickly through the flame 3-4 times.
- It was stained with crystal violet for 30 seconds.
- The stain was drained off and the smear washed with water.
- Lugol's iodine was applied for 30 seconds.
- After draining the iodine solution, it was decolorized with absolute alcohol till violet colour washed off.
- After washing the smear with running tap water it was counter stained with 0.5% safranin for 30 seconds.
- Stain was drained, smear was washed with water and air-dried.
- A drop of cedar wood oil was placed and the slide was examined under oil immersion objective (100X) of the microscope.

Interpretation

Gram – Positive bacteria: stained purple
Gram Negative bacteria: stained pink

CITRATE UTILIZATION TEST

Sodium citrate is a simple organic compound found as one of the metabolites in the tricarboxylic acid (TCA/Kreb's) cycle. Some bacteria can obtain energy in a way other than the fermentation of carbohydrates by utilizing citrate as a sole source of carbon. Any medium used to detect citrate utilization must be devoid of protein and carbohydrates as sources of carbon.

Principle

The utilization of citrate as a sole source of carbon by the test organism is detected by the presence of alkaline by-products in the citrate medium, which contain sodium citrate, an anion, as the sole carbon source and ammonium phosphate as a sole nitrogen source. The production of ammonia (NH₃) from the ammonium salts leads to the alkalization of the medium from conversion of ammonia to ammonium hydroxide (NH₄OH). Bromothymol blue indicator used in the medium is yellow in acidic pH and blue at pH above 7.6.
Procedure
Simmon’s Citrate agar slant was streaked with a loopful of pure culture suspension and incubated at 37°C for 24-48 hours.

Interpretation
The development of blue colour on the slant indicated the capacity of the organism to utilize citrate and the test was considered as positive.

CYTOCHROME OXIDASE TEST
The cytochromes are iron-containing hemoproteins serving in the respiratory chain of aerobic organisms by transferring electrons (hydrogen) to oxygen with the formation of water.

Principle
The cytochrome oxidase test utilizes the dye phenylenediamine dihydrochloride, that substitutes oxygen as an artificial electron acceptor. In the reduced state the dye is colourless. However in the presence of cytochrome oxidase and atmospheric oxygen, P-phenylenediamine is oxidized, forming indophenol blue.

Procedure
Two to three drops of the reagent were added onto the isolated bacterial colonies on a nutrient agar plate.

Interpretation
Bacterial colonies having cytochrome oxidase activity developed a deep blue colour at the inoculation site within a few seconds.

FLUORESCENCE–DENITRIFICATION TEST
The ability to produce fluorescein pigment and to reduce nitrates and/or nitrites completely to nitrogen gas are the two important characteristics in the identification of the *Pseudomonads* and other non-fermentative bacilli. The medium can also be modified by addition of lactose and phenol-red to detect acid from lactose fermentation which is useful in identification of strong lactose fermenters from non-fermenters.
Principle
Fluorescein is an organic luminescent dye which upon excitation with ultraviolet light emits a green yellow fluorescence. Fluorescence of colonies can be detected on media containing cationic salts such as magnesium sulfate which behaves as activators or coactivators to intensify luminescence.

Procedure
The medium was inoculated by stabbing with a heavy suspension of the culture and then the slant area was streaked. It was incubated at 37°C for 24-48 hrs.

Interpretation
Examination of the tube for fluorescence was done under a UV source. A bright yellow-green glow indicated a positive test. The presence of gas bubbles in the butt of the medium indicated that nitrogen gas was produced from denitrification and a yellow slant with FLN medium indicator acid was produced from the utilization of lactose by the test organisms.

GLUCONATE TEST
Gluconic acid is one of the oxidation products formed from glucose by aerobic microbes that utilize carbohydrates via Enter-Douderoff pathway. Gluconate medium contains potassium gluconate to serve as the substrate for those bacterial species which can oxidize it to 2-ketogluconic acid and other metabolites.

Principle
A gluconate from potassium gluconate is degraded by test organism into 2-ketogluconic acid and other intermediate metabolites which then reduce the cupric hydroxide (Benedict's reagent) to cuprous oxide and can be detected visually as a yellow orange precipitation by addition of Benedict's reagent into the medium.

Procedure
Gluconate test medium was inoculated with heavy suspension of the test organism from an over night broth culture and incubated at 37°C for 24-48 hours. After incubation 1 ml of Benedict's reagent was added to the tube of the culture medium.
**Interpretation**
The development of a yellow-orange precipitation indicated the presence of reducing substances and the test was positive. If the colour of the medium after addition of Benedict's reagent remained bluish-green, the test was negative.

**INDOLE TEST**
Indole (benzylpyrole) is one of the metabolic degradation products of the amino acid tryptophan. Bacteria that possess the enzyme tryptophanase are capable of hydrolyzing and deaminating tryptophan to pyruvic acid and ammonia. It is important in separation of *E. coli* which generally yields a positive test from members of the *Klebsiella-Enterobacter* group (mostly negative).

**Principle**
When indole reacts with the aldehyde group of p-dimethylamino benzaldehyde it forms a red coloured complex.

**Procedure**
Tryptophan broth was inoculated with overnight grown culture inoculum of the test organism and incubated at 37°C for 18-24 hours. After incubation five drops of reagent were added down the inner wall of the tube.

**Interpretation**
The development of a bright red colour at the interphase of the reagent and the broth within a few seconds after adding the reagent was indicative of the presence of indole positive test.

**METHYL RED TEST**
Methyl red is a pH indicator with a range between 6.0 (yellow) and 4.4 (red). In order to produce a colour change, the test organisms must produce a large quantity of acid from the carbohydrate being used.

**Principle**
This is a quantitative test for acid production from glucose via the mixed acid fermentation pathway. For positive methyl red test, test organisms should produce strong acids to overcome the buffering system of the medium.
Procedure
Glucose-phosphate broth was inoculated with a loopful of the organisms and incubated at 37°C for 24-48 hours. At the end of incubation 5 drops of methyl red reagent were added directly to the broth.

Interpretation
The development of a stable red colour on the surface of the medium was indicative of a positive test. Since some organisms produced lesser quantity of acid from the substrate, an intermediate orange colour developed indicating a negative test.

NITRATE REDUCTION TEST

The capability of an organism to reduce nitrate to nitrites is an important characteristic used in the identification and species differentiation of many groups of micro-organisms.

Principle
Organisms demonstrating nitrate reduction have the capability of deriving oxygen from nitrate to form nitrites and other reduction products. Presence of nitrites in the test medium is detected by the addition of α-naphthylamine and sulfanilic acid with the formation of a red diazonium dye, P-sulphobenzene-azo-α-naphthylamine.

Procedure
The nitrate broth was inoculated with a loopful of the test organisms from an overnight grown pure culture broth and incubated at 37 °C for 24 hours. After incubation reagents A and B were added to the test medium in the respective order.

Interpretation
The development of red colour within 30 secs after adding the test reagents, was indicative of the presence of nitrites and represents a positive reaction for the reduction of nitrates. Development of no colour after the adding of test reagents it indicated either that the nitrates have not been reduced (a true negative reaction) or that they have been reduced to products other than nitrites, such as ammonia, molecular nitrogen(denitrification), nitric oxide or nitrous oxide. Since the test
reagents detect only nitrites, it was necessary to add a small quantity of zinc dust to all negative reactions to prevent false negative reading. Zinc ions reduced nitrates to nitrites and the development of red colour after the addition of Zinc dust indicated the presence of residual nitrates and confirmed a true negative reaction.

OXIDATIVE-FERMENTATIVE TEST (O-F TESTS)

HUGH AND LEIFSON TEST

Principle
Sacchrolytic microorganisms degrade glucose either fermentatively or oxidatively. The end products of fermentation are relatively strong mixed acids that can be detected in a conventional fermentative test medium.

The Hugh and Leifson medium contains 0.25% protein and 1% carbohydrates. This lower protein to carbohydrates ratio reduce the formation of alkaline amine that can neutralize the small quantities of weak acid which may be formed from oxidative metabolism.

Procedure
Two tubes of the O-F medium were inoculated with overnight broth culture using stab technique. One of the tubes was layered with sterile liquid paraffin and both the tubes are incubated at 37°C for 48 hours or longer.

Acid production was detected in the medium by appearance of a yellow colour in the case of oxidative organisms. Colour formation was first noted near the surface of the medium

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<thead>
<tr>
<th>Type of Metabolism</th>
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<th>Closed tube</th>
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<tr>
<td>Oxidative</td>
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<td>Alkaline</td>
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<td>(Green)</td>
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<tr>
<td>Fermentative</td>
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<td>(Yellow)</td>
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<tr>
<td>Non-Sacchrolytic</td>
<td>Alkaline</td>
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</table>
PHENYLALANINE-DEAMINASE TEST

Phenyl alanine is an amino acid which upon deamination forms a keto acid, phenyl pyruvic acid.

**Principle**
This test depends upon the detection of phenyl pyruvic acid. The amino group of the amino acid is enzymatically removed. This results in production of ammonia and corresponding keto acid. This phenylpyruvic acid gives a green colour with a ferric salt.

**Procedure**
Phenylalanine agar slant was streaked with the test organism and incubated at 37°C for 24-48 hours. After incubation 4-5 drops of ferric chloride were added and the tube was rotated to dislodge the surface colonies.

**Interpretation**
The immediate appearance of an intense green colour indicated the presence of phenyl pyruvic acid and a positive test otherwise it was taken as negative.

VOGES-PROSKAUER (V-P) TEST

Voges-Proskauer is a double eponym named after two microbiologists. They first observed the red colour reaction produced by appropriate culture media after treatment of potassium hydroxide.

**Principle**
Pyruvic acid, the pivotal compound formed in the fermentative degradation of glucose is further metabolized via a number of metabolic pathways depending upon the enzyme systems possessed by different bacteria. One such pathway results in the production of acetoin (acetyl-methyl carbinol). In the presence of atmospheric oxygen and 40% potassium hydroxide, acetoin is converted to diacetyl and α-naphthol serves as a catalyst to bring out a red colour complex.

**Procedure**
Glucose-phosphate broth was inoculated with a loopful of test organism and incubated for 24 hours at 37°C. After incubation, 1 ml of the broth was taken in a
clean test - tube and added to 0.6 ml of 5% 1 - naphthol followed by 0.2% ml of 40% KOH.

The tube was gently shaken and exposed to atmospheric oxygen. The test tube was left undisturbed for a few minutes.

**Interpretation**
The development of a red colour after 15 minutes following the addition of reagent indicated a positive test and no development of colour was indicative of a negative test.

**CARBOHYDRATE FERMENTATION TEST**

The ability of bacteria to ferment carbohydrates and the types of products formed are very useful in bacterial identification. A given carbohydrate may be fermented to a number of different end products depending upon the microorganism involved. These end products are characteristic of the particular bacterium.

**Principle**
Anrade's added to 1% peptone media containing a particular sugar and inverted Durham’s tube is placed in the test tube. If acid is produced the colour changes to pink if gas is produced the medium inside the Durham tube will be displaced, entrapping the gas in the form of a bubble.

**Procedure**
6 ml media substituted with 1% sugar in a test tube was taken and a Durham tube was placed in an inverted position so that no bubble remained in the tube.

Media was inoculated with 0.1ml of an overnight growth culture of bacteria and incubated for 24-48 hours.

**Interpretation**
Pink colour in the media indicated acid production and the presence of bubble in the media indicated the production of gas.