Chapter-3. Material and Methods

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All the chemicals used during this study were procured from HI-Media Mumbai, Sigma Aldrich, USA. All the chemicals used were of highest purity and of analytical grade. All glassware’s used for practical work were used after sterilization. All the experiments were carried out in aseptic conditions.

3.1 Experimental site:

The experiments on “bioremediation of textile effluent by using microorganisms” were carried out in the department of Microbiology, Sau. K.S.K. College of Food technology, Beed, Maharashtra (India).

MATERIALS

3.2.1 Textile dyes:

1. Reactive blue 59
2. Reactive golden Yellow 84
3. Reactive orange 96

3.2.2 Culture Media:

All media was prepared using distilled water. All media recipes are per liter of distilled water. pH was adjusted to 7.0 with 0.1 N dilute hydrochloric acid and 0.1 N sodium hydroxide. Media was sterilized by autoclaving at 121°C for 15 min.

1. Mineral Salts Medium (MSM) :

\[
\begin{align*}
\text{K}_2\text{HPO}_4 & \quad - \quad 1.6 \text{g} \\
\text{KH}_2\text{PO}_4 & \quad - \quad 0.2 \text{ g} \\
\text{(NH}_4\text{)}_2\text{SO}_4 & \quad - \quad 1.0 \text{ g}
\end{align*}
\]
Chapter 3. Material and Methods

\[
\begin{align*}
\text{Mg SO}_4 & \quad - \quad 0.2 \text{ g} \\
\text{FeSO}_4 & \quad - \quad 0.01 \text{ g} \\
\text{NaCl} & \quad - \quad 0.1 \text{ g} \\
\text{CaCl}_2 & \quad - \quad 0.02 \text{ g}
\end{align*}
\]

The mineral salts medium was supplemented with 3 g/l of glucose and 1 g/l of yeast extract.

2. **Nutrient Agar:**

\[
\begin{align*}
\text{Peptone} & \quad - \quad 10 \text{ g} \\
\text{Yeast extract} & \quad - \quad 3.0 \text{ g} \\
\text{NaCl} & \quad - \quad 5.0 \text{ g} \\
\text{pH} & \quad - \quad 7.2 \text{ g} \\
\text{Agar} & \quad - \quad 20 \text{ g}
\end{align*}
\]

3.2.3 **Metals:**

1. Magnesium sulphate
2. Copper Sulphate
3. Zinc Sulphate
4. Potassium Chloride
5. Silver Chloride
6. Mercuric Chloride

3.2.4 **Inducers:**

1. Calcium Chloride
2. Calcium Carbonate
3.2.5 **BOD Reagents:**

1. **Sodium sulphite 0.025 N:** Dissolve 1.575 gms of Na$_2$SO$_3$ in distilled water and make the volume to 1 litter.

2. **Ferric chloride:** Dissolve 0.25 gm FeCl$_3$·6H$_2$O in distilled water and make the volume one liter.

3. **Calcium chloride:** Dissolve 27.5 gms of anhydrous CaCl$_2$ in distilled water to prepare one liter with water.

4. **Magnesium sulphate:** Dissolve 22.5 gms of MgSO$_4$·7H$_2$O in distilled water and make the volume to one liter with distilled water.

5. **Phosphate buffer:** Dissolve 8.5 gms of KH$_2$PO$_4$, 21.75 gms of Na$_2$HPO$_4$ and 1.7 gms of NH$_4$Cl and make volume one liter with distilled water.

6. **Sodium thiosulphate:** Dissolved 24.82 gms of Na$_2$S$_2$O$_3$·5H$_2$O in distilled water and make the volume to one liter with distilled water. This is 0.1 N Na$_2$S$_2$O$_3$ solution. Now 250 ml of this is diluted to one liter with distilled water.

7. **Alkaline azide solution:** 50 gm of NaOH + 13.5 gms NaI and 1.0 gm NaN$_2$ diluted to one liter.

8. **Manganese sulphate solution:** Dissolve 80 gms of MnSO$_4$ in 200 ml of distilled water and filter.

9. **Starch indicator:** Dissolve 1 gram of starch in 100 ml warm distilled water and add few drops of formaldehyde solution as preservative.

10. **Conc. H$_2$SO$_4$.**

3.2.6 **COD Reagents:**

1. **K$_2$Cr$_2$O$_7$ solution 0.25 N:** Dissolve 12.300 gms of A.R. K$_2$Cr$_2$O$_7$ in distilled water and make the volume one liter.
Chapter-3. Material and Methods

2. **K$_2$Cr$_2$O$_7$, 0.025N:** Dilute 0.25 N K$_2$Cr$_2$O$_7$ 10 times (100 to 1000 ml).

3. **Ferrous ammonium sulphae 0.1N:** Dissolve 39.2 gms of ferrous ammonium sulphate in little amount of distilled water. Then add 20 ml of conc. H$_2$SO$_4$ to increase the solubility and make the volume one liter.

4. **Ferrous ammonium sulphate 0.01 N:** Dilute 0.1N ferrous ammonium sulphate 10 times (100 to 1000ml).

5. **Ferroin indicator:** 1.485 gm of 1.10 phenonthroline + 0.695 gms of ferrous sulphate and dilute to make 100 ml solution.

6. **18 N H$_2$SO$_4$ i.e. 50% H$_2$SO$_4$**

7. **Silver sulphate (Ag$_2$SO$_4$ solid)**

8. **Mercuric Chloride (HgSO$_4$ Solid)**

METHODS

3.3 **Collection of soil samples:**

Soil samples were collected from various sites contaminated with textile industry effluent, Ichalkaranji, India. All the samples were placed in sterilized polythene bags and the soil samples were immediately transported to laboratory at 4°C.

3.4 **Enrichment and isolation of dye degrading bacterial cultures:**

Isolation of the bacterial cultures was carried out from the soil contaminated with effluent discharge of textile processing unit, Ichalkaranji, India by enrichment culture technique. 1 gm of soil was inoculated into 3 different Erlenmeyer flask (250 ml) containing 100 ml of Mineral salts Medium(MSM), amended separately with 50 mg/l of textile dyes reactive blue 59, reactive golden yellow 84 and reactive orange 96 and incubated in orbital shaker at 30°C for 24 hrs. Repeated transfers were carried out in the dye containing media till stable dye decolorizing and degrading bacterial cultures were obtained which showing consistent growth and decolorization.
during successive transfer. A loopfull of culture from decolorized broth was streaked on MSM agar plates containing 50 mg/l of textile dyes. The plates were incubated at 30°C for 24 hours. After incubation colonies showing decolorization zones were selected for screening for dye decolorization in liquid medium (Junnarkar et al., 2006; Jadhav et al., 2010).

### 3.5 Screening of dye degrading bacterial cultures:

0.1 ml aliquot of the decolorized broth was spread on MSM agar plates each supplemented with 50 mg/l of reactive blue 59, reactive golden yellow 84 and reactive orange 96 and plates were incubated at 30°C 24 hours. After incubation bacterial colonies showing maximum zone of dye degradation against all tested dyes were selected and the most efficient dye degrading isolates were used for the further studies. The bacterial isolates were then further screened for their dye degradation ability in liquid medium to select the most efficient isolates.

### 3.6 Preservation of isolated cultures:

All the isolated dye degrading bacterial cultures were maintained on Nutrient agar slants. These slants were then preserved in refrigerator at 4°C for further study.

### 3.7 Dye degradation assay:

To measure the percentage of degradation 5 ml samples of the cultures were withdrawn after different time intervals and centrifuged at 5000 rpm for 10 minutes. The degradation of dye was monitored spectrophotometrically by measuring the absorbance of the culture supernatant at maximum absorbance wavelength at 588 nm for reactive blue 59, 416 nm for reactive golden yellow 84 and 460 nm for reactive orange 96. An UV-Visible spectrophotometer (Elico-171) was used for absorbance measurement. Degradation activity was expressed in terms of the percentage degradation. The percentage degradation was calculated as follows (Ren et al., 2006; Telke et al., 2010).

\[
\% \text{ Degradation} = \frac{\text{Initial absorbance} - \text{observed absorbance}}{\text{Initial absorbance}} \times 100
\]
3.8. Determination of growth of bacterial strain F4:

The experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml Mineral salts medium, under aseptic conditions. In each of these flasks, 50 mg/l of each of reactive blue 59, reactive golden yellow 84 and reactive orange 96 were added separately. These flasks of mineral salts medium containing textile dyes 50 mg/l were inoculated with 10 ml of bacterial strain F4 having optical density of 0.5 at 600 nm. The flasks were incubated at 30°C in orbital shaker for 24 hours. After 24 hours of incubation growth of bacterial isolate F4 was monitored spectrophotometrically. The cell pellet obtained upon centrifugation (5000 rpm for 15 min) of 5 ml culture was resuspended in 5 ml distilled water and its absorbance was studied at 660 nm (Moosvi et al., 2007).

3.9. Effects of different environmental factors on degradation of textile dyes by bacterial isolate F4:

3.9.1 Effect of different concentrations of dyes:

The experiment was carried out in two sets of 250 ml Erlenmeyer flasks containing 100 ml mineral salts medium, under aseptic condition. In each 100 ml mineral salts medium, different concentrations of reactive blue 59 (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mg/l) was added separately. In each 100 ml mineral salts medium, different concentrations of reactive golden yellow 84 (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mg/l) was added separately. In each 100 ml mineral salts medium, different concentrations of reactive orange 96 (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mg/l) was added separately. These flasks of mineral salts medium containing textile dyes were inoculated with 10 ml of bacterial isolate F4 having optical density of 0.5 at 600 nm. These flasks were incubated at 30°C in orbital shaker for 24 hours.

3.9.2 Effect of different carbon sources:

The experiment was carried out in two sets of 250 ml Erlenmeyer flasks containing 100 ml mineral salts medium amended separately with 50 mg/l of each of reactive blue 59, reactive golden yellow 84 and reactive orange 96. This mineral salts medium was supplemented with
different concentrations (0.5, 1, 1.5 and 2% (w/v)) of carbon sources i.e. glucose, fructose, starch, sucrose, maltose, lactose, dextrose and sodium acetate. This mineral salts medium containing different carbon sources and textile dyes were inoculated separately with 10 ml of bacterial isolate F4. These flasks were incubated at 30°C in orbital shaker at 150 rpm for 24 hours. Control set was prepared similarly without addition of carbon sources.

3.9.3 Effect of different nitrogen sources:

The effect of different nitrogen sources on degradation of reactive blue 59, reactive golden yellow 84 and reactive orange 96 was carried out in 250 ml Erlenmeyer flasks containing 100 ml mineral salts medium. The mineral salts medium containing 50 mg/l of each of textile dyes was supplemented with different concentrations (0.5, 1, 1.5 and 2% (w/v)) of different inorganic nitrogen sources such as ammonium acetate, ammonium nitrate, ammonium chloride, ammonium sulphate, sodium nitrate and organic nitrogen sources such as peptone, yeast extract, beef extract and urea. These flasks of mineral salts medium containing different nitrogen sources were inoculated separately with 10 ml of bacterial isolate F4. These flasks were incubated at 30°C in orbital shaker at 150 rpm for 24 hours. Similarly, control set was prepared without addition of nitrogen sources.

3.9.4 Effect of inoculum size:

The experiment was carried out in two sets of 250 ml Erlenmeyer flasks containing 100 ml mineral salts medium was amended separately with 50 mg/l of each of reactive blue 59, reactive golden yellow 84 and reactive orange 96. This mineral salts medium was inoculated separately with 5, 10, 15, 20, 25, and 30% (v/v) of inoculum of bacterial isolate F4 having optical density of 0.5 at 600 nm. These flasks of mineral salts medium were incubated at 30°C in orbital shaker at 150 rpm for 24 hours.

3.9.5 Effect of pH:

The experiment was carried out in two sets of 250 ml Erlenmeyer flasks amended separately with 50 mg/l of each of reactive blue 59, reactive golden yellow 84 and reactive orange 96. This mineral salts medium before addition of textile dyes was distributed in three sets of seven
different flasks. The pH of these mineral salt medium were adjusted by 0.1N HCL and 0.1N NaOH from 2, 3, 4, 5, 6, 7, 8, 9, 10, 12 and 14. Then these flasks of mineral salts medium after addition of textile dyes (50 mg/l) were inoculated with 10 ml of bacterial isolate F4 having optical density of 0.5 at 600 nm and incubated at 30°C in orbital shaker at 150 rpm for 24 hours.

3.9.6 Effect of temperature:

The experiment was carried out in two sets of 250 ml Erlenmeyer flasks containing 100 ml mineral salts medium amended separately with 50 mg/l of each of reactive blue 59, reactive golden yellow 84 and reactive orange 96. Each of these two sets of flasks containing mineral salts medium and textile dyes (50 mg/l) were inoculated with 10 ml of bacterial isolate F4 having optical density of 0.5 at 600 nm. These flasks were incubated at different temperatures ranging from 30, 35, 37, 40, 45 and 50°C in orbital shakers for 24 hrs. These flasks were also incubated at room temperatuures.

3.9.7 Effect of incubation period :

The experiment was carried out in two sets of 250 ml Erlenmeyer flasks containing 100 ml mineral salts medium under aseptic conditions. In each of these flasks 50 mg/l of each of reactive blue 59, reactive golden yellow 84 and reactive orange 96 were added separately. In each of these flasks of mineral salts medium containing textile dyes (50 mg/l) were inoculated with 10 ml of bacterial isolate F4 having optical density 0.5 at 600 nm. These flasks were incubated at 30°C in orbital shaker at 150 rpm for different incubation period of time. Percentage degradation was recorded at different time intervals i.e. 24, 48, 72, and 120 hrs.

3.9.8 Effect of shaking and static conditions:

The experiment was carried out in two sets of 250 ml Erlenmeyer flasks containing 100 ml mineral salts medium of pH 7.0, under aseptic conditions. In each of these flasks 50 mg/l of reactive blues 59, reactive golden yellow 84 and reactive orange 96 were added separately. Each of these flasks of mineral salts medium containing textile dyes (50 mg/l) were inoculated with 10 ml of bacterial isolate F4 having optical density 0.5 at 600 nm. These flasks were incubated at 30°C in orbital shaker (with shaking) at 150 rpm for 24 hours.
Another experiment was carried out in two sets of 250 ml Erlenmeyer flasks containing 100 ml mineral salts medium of pH 7.0, under aseptic conditions. In each of these flasks 50 mg/l of reactive blues 59, reactive golden yellow 84 and reactive orange 96 were added separately. Each of these flasks of mineral salts medium containing textile dyes (50 mg/l) were inoculated with 10 ml of bacterial isolate F4 having optical density 0.5 at 600 nm. These flasks were incubated at 30°C under static condition (without shaking) for 24 hours.

3.10 Effect of inducers on degradation of textile dyes by bacterial isolate F4:

3.10.1 Effect of calcium chloride (CaCl$_2$):

Effect of calcium chloride (CaCl$_2$) on degradation of textile dyes was carried out in two sets of 250 ml Erlenmeyer flasks containing mineral salts medium 100 ml. In each of these flasks 50 mg/l of each of reactive blue 59, reactive golden yellow 84 and reactive orange 96 were added separately. These flasks of mineral salts medium containing textile dyes were added separately with different concentrations of calcium chloride (0.01, 0.02, 0.03, 0.04 and 0.05 g/l). Each of these two sets of flasks were inoculated with 10 ml of bacterial isolate F4 having optical density of 0.5 at 600 nm. These flasks were incubated at 30°C in orbital shaker at 150 rpm for 24 hours.

3.10.2 Effect of calcium carbonate (CaCO$_3$):

The effect of calcium carbonate (CaCO$_3$) on degradation of textile dyes was carried out in two sets of 250 ml Erlenmeyer flasks containing 100 ml mineral salts medium. In each of these flasks 50 mg/l of each of reactive blue 59, reactive golden yellow 84 and reactive orange 96 were added separately. These mineral salts medium flasks containing textile dyes were then added separately with different concentrations of calcium carbonate (0.01, 0.02, 0.03, 0.04 and 0.05 mg/l). Each of these two sets of flasks were inoculated with 10 ml of bacterial isolate F4 having optical density of 0.05 at 600 nm. These flasks were incubated at 30°C in orbital shaker at 150 rpm for 24 hours.

3.11 Effect of metals:

The experiment was carried out in two sets of 250 ml Erlenmeyer flasks containing 100 ml mineral salts medium amended separately with 50 mg/l of each of reactive blue 59, reactive
golden yellow 84 and reactive orange 96 and supplemented with different concentrations of metals (0.01, 0.02, 0.03, 0.04 and 0.05 mg/l) such as magnesium sulphate, copper sulphate, zinc sulphate, potassium chloride, silver chloride and mercuric chloride. These flasks of minerals salts medium containing textile dyes and different concentrations of metals were inoculated with 10 ml of bacterial isolate F4 having optical density of 0.5 at 600 nm. These flasks were incubated at 30°C in orbital shaker at 150 rpm for 24 hours. Control set was prepared similarly without addition of metals.

3.12 Effect of different concentrations of sodium chloride:

The experiment was carried out in two sets of 250 ml Erlenmeyer flasks containing 100 ml of mineral salts medium and 50 mg/l of each of reactive blue 59, reactive golden yellow 84 and reactive orange 96. Each of these two sets of flasks were added separately with different concentrations of sodium chloride (0.5, 1, 1.5, 2, 2.5, and 3% (w/v)). These two sets of flasks were inoculated with 10 ml of bacterial isolate F4 having optical density of 0.5 at 600 nm. These flasks were incubated 30°C in orbital shaker at 150 rpm for 24 hours.

3.13 Effect of repeated addition of dyes:

The intention of this study was to check the ability of bacterial isolate F4 for the repeated degradation of textile dyes. The experiment was carried out in two sets of 250 ml Erlenmeyer flasks containing 100 ml of mineral salts medium. In each of these two sets of flasks 50 mg/l of each of reactive blue 59, reactive golden yellow 84 and reactive orange 96 were added separately. Each of these two sets of flasks were inoculated with 10 ml of bacterial isolate F4 having optical density of 0.5 at 600 nm. These flasks were incubated at 30°C in orbital shaker at 150 rpm for 24 hours. After degradation of textile dyes 50 mg/l of reactive blue 59, reactive golden yellow 84 and reactive orange 96 were added separately again in each of two sets of flasks without supplementation of any extra nutrients. Dyes were added continuously without further inoculation until the culture lost its dye degradation ability to the significant extent.

3.14 Collection of textile industry effluent:

Highly colored textile effluent was collected from Radhamohan textile industry, Ichalkaranji, India. The effluent was collected in plastic containers. The effluent was black colored due to the
presence of dyes. After the collection, the effluent was immediately transported to laboratory and stored in refrigerator at 4°C.

3.15 Analysis of the textile industry effluent:

The physico-chemical parameters such as pH, temperature, total solids, total suspended solids, biological oxygen demand (BOD) and Chemical oxygen demand were analyzed by method of APHA (2005). The pH and temperature were determined at the sampling site.

3.15.1 Determination of total solids (TS):

A clean dish of suitable size was dried at 103-105°C in an oven to a constant weight. 100-150 ml of thoroughly mixed textile effluent sample was accurately added into a dish. The dish was weighed and evaporated to dryness on a water bath. The residue was dried in an oven for about 1 hour at 103-105°C and reweighed after cooling to room temperature. The cooling was done until the weight of the dish plus residue was constant to within 0.05 mg. The weight of the dish was subtracted to obtain the weight of the total solids (APHA, 2005).

3.15.2 Determination of suspended solids (SS):

100 ml of the sample of textile effluent was withdrawn into a conical flask with a pipette. It was filtered in Gooch funnel fitted with glass fiber filter paper which has been pre-dried at 103-105°C. The glass fiber was carefully removed from the Gooch and dried to a constant weight at 103-105°C and the weight subtracted from the weight of the filter paper to obtain the weight of the suspended solids (APHA, 2005).

3.15.3 Determination of chemical oxygen demand (COD):

The untreated sample of the effluent was first analyzed for COD immediately after collection. The biologically treated sample was also analyzed for COD. The sample is refluxed in strongly acid solution with a known excess of K₂Cr₂O₇. Oxygen consumed is measured against standards at 600 nm with a spectrophotometer (Elico-171) (APHA, 2005).
3.15.4 Determination of biological oxygen demand (BOD):

The untreated sample of the effluent was first analyzed for BOD immediately after collection. The biologically treated sample was also analyzed for BOD. The 5-day BOD at 20°C was determined by the standard dilution technique. The method consists of filling an airtight bottle of the specified temperature for 5 days. Dissolved oxygen concentration (DO) is measured initially and after incubation. Then the BOD is computed from the difference between initial and final DO (APHA, 2005).

3.16 Decolorization of textile effluent:

The experiment was carried out in two sets of 250 ml Erlenmeyer flasks. The original textile effluent was filtered by using the filter paper. 100 ml of the filtered effluent was taken in two different 250 Erlenmeyer flasks. These flasks were sterilized at 121°C for 20 min. Thereafter, these flasks were inoculated with 10 ml of bacterial isolate F4 having optical density 0.5 at 600 nm. These flasks were incubated at 30°C in orbital shaker for different time periods i.e. 24, 48, and 72 hours. Samples were withdrawn after regular intervals and analyzed for color removal.

The intensity of color was measured at its maximum absorbance wavelength at 490 nm. Percentage decolorization was calculated as follows (Telke et al., 2010).

\[
\text{Decolorization (\%)} = \frac{(\text{Initial absorbance}) - (\text{observed absorbance})}{(\text{Initial absorbance})} \times 100
\]

Abiotic controls (without microorganisms) were also included.

3.17 Identification of metabolites formed after degradation of textile dyes by gas chromatography and mass spectra:

3.17.1 Extraction of textile dyes:

Under optimal conditions, degradation of textile dyes was carried out in two sets. In each 250 ml Erlenmeyer flasks of mineral salts medium (100ml) each containing 50mg/l of textile dyes...
reactive blue 59, reactive golden yellow 84 and reactive orange 96. Each flask of mineral salts medium containing textile dyes was inoculated with 10 ml of bacterial isolate F4 of optical density of 0.5 at 600 nm. These flasks were incubated at 30°C in orbital shaker for 24 hours.

After complete decolorization of textile dyes the culture broth was centrifuged at 7,000 rpm for 20 min to remove the cell mass. Culture supernatant containing the metabolites formed after degradation of textile dyes were extracted, using equal volume of ethyl acetate, dried over anhydrous Na₂SO₄ and concentrated in a rotary vacuum evaporator.

3.17.2 Analysis of the metabolites by GC/MS:

GC/MS analysis was carried out in Doctor’s analytical laboratory, Pune. Rotary vacuum concentrated sample was dissolved in methanol and GC/MS analysis of metabolites formed degradation of textile dyes was carried out using a QP 5000 mass spectrophotometer (Shimadzu). The ionization voltage was 70 eV. Gas chromatography was conducted in temperature programming mode with a Resteck column (0.25 mm × 30 mm; XTI 5). The initial column temperature was 40°C for 4 min, which was increased linearly at 10°C min⁻¹ to 270°C and held at 4 min. The temperature of injection port was 275°C and GC/MS interface was maintained at 300°C. The helium was used as carrier gas; flow rate was 1 ml min⁻¹ and 30 min run time. The compounds were identified on the basis of mass spectra and using the NIST library stored in the computer software (version 1.10 beta Shimadzu) of the GC/MS.

3.18 Identification of bacteria by morphological, cultural and biochemical characteristics.

The isolated efficient dye degrading bacterial isolate F4 was identified on the basis of morphological, cultural and biochemical tests according to the Bergey’s Manual of Determinative Bacteriology.

3.19 Identification of textile dye degrading bacteria:

3.19.1 Identification of bacteria by 16S rRNA sequence analysis:

DNA extraction:

Bacterial isolate was grown on LB Agar plates at 28°C for 48 hours. Single colonies were suspended in 200i PBS. Total DNA was extracted from the suspended bacterial cells using
geneO- Spin Bacterial DNA isolation kit. DNA was directly used for PCR amplification of 16S rRNA gene using geneOmbio Microbial Identification kit (geneOmbiotechnologies, Pune, India). After PCR is over, the PCR products were checked on 1% agarose by agarose gel electrophoresis and amplicon size was determined using reference Ladder.

1% agarose gel spiked with ethidium bromide at a final concentration of 0.5µg/ml was prepared using agarose (LE, Analytical Grade, Promega Corp., Madison, WI 53711 USA) in 0.5 X TBE buffer. 5.0 µl of PCR product was mixed with 1µl of 6X tracking dye. 5µl of gScale 1kb size standard (gene Ombiotechnologies, Pune; India) was loaded in one lane for confirmation of size of the amplicon using reference ladder. The DNA molecule were resolved at 5V/cm until the tracking dye is 2/3 distance away from the lane within the gel. Bands were detected under a UV Trans illuminator. Gel images were recorded using BIO-RAD GelDocXR gel documentation system.

**Purification of PCR products:**

Sequencing uses one primer, while PCR utilizes two. If we try to sequence with two primers present, we will get two sequences back, superimposed on each other and completely unreadable. Hence it is necessary to purify PCR products prior to sequencing. PCR products were purified using genePure PCR purification kit (geneOmbiotechnologies, Pune; India). The PCR products were eluted in final volume of 20µl.

**Agarose gel electrophoresis of purified PCR products:**

The protocol mentioned in previous was used for checking of purified PCR products on 1% agarose gel and determination of approximate concentration of DNA.

**DNA Sequencing:**

Using the geneOmbio Microbial Identification Kit sequencing primers and ABI BigDye® Terminator v3.1 Cycle Sequencing reaction kit (Applied Biosystems, USA), the purified PCR amplicons were sequenced.
Sequence analysis was performed using sequencing analysis V5.2 (ABI, USA). The sequence was assembles and used for further analysis. NCBI nucleotide database was used for BLASTn analysis and finding the closest five matches to the query sequence.

The NCBI sequence database hit that showed maximum match with the query sequence was identified as the closest organism of the organism analyzed. The phylogenetic tree was constructed using neighbor-joining method, Kimura-2 parameter test.