Chapter - III

Materials and Methods
CHAPTER - III
MATERIALS AND METHODS

EXPERIMENTAL DESIGN

SOIL COLLECTION, EVALUATION, BACTERIAL ISOLATION AND CHARACTERIZATION
1) Sample Collection
2) Analysis of the Physico-Chemical Properties of Soil
3) Bacterial Culture and Isolation
4) Molecular Identification of Bacterial Isolates using 16S Ribosomal DNA Sequencing

PLANKTONIC CELLS
5) Susceptibility to Antimicrobials
6) Assay of Tolerance to Organic Solvents
7) Efflux Pumps
8) Cell Surface Hydrophobicity - MATH and SAT assay
9) Cell Surface Charges - MATS assay
10) Assaying for Auto-aggregation and Co-aggregation
11) Exopolysaccharides and Bioemulsifier Secretion
12) Floating behaviour
13) Chemotaxis
14) Culture-dependent Screening of Integron and Gene Cassettes in Genomic DNA.

BIOFILM
15) Biofilm Formation
16) Confocal Laser Scanning Microscopy Analysis of Biofilm
17) Biofilm’s Resistance to Antimicrobials
18) Effect of Degrading Enzymes on Biofilm.
19) Efflux Pump Inhibitor on Biofilm Formation
20) Isolation and Characterization of Exopolymeric Substances
21) Extracellular DNA - Isolation, Characterization and Secretion
22) Effect of Exogenous DNA on Biofilm: Surface Hydrophobicity, Surface Charges and Aggregation
23) Culture-Dependent Screening of Class I Integron in eDNA.
Table 5: Media, Chemicals and Kits

<table>
<thead>
<tr>
<th>Test</th>
<th>Reagents / Chemicals</th>
<th>Sourced from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil analysis</td>
<td>Aqua Check™ kits for testing Iron, Total hardness, Fluoride, Chlorine, Hydrazine, and Arsenic</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Biochemical tests</td>
<td>KB003 Hi25™ <em>Enterobacteriaceae</em> identification kit, HiCarbo™ kit</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Grams staining kit</td>
<td>Identification of Gram positive and Gram negative as well as rods &amp; cocci</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>Catalase test</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Mac conkey agar medium</td>
<td>Identification of <em>Enterobacteriaceae</em> bacteria</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>MIC</td>
<td>Microfuge tubes, pipette tips, Microtiter plates (96 well flat bottom)</td>
<td>Tarsons, India</td>
</tr>
<tr>
<td>MIC, Culture of bacteria</td>
<td>Luria Bertani and Mueller Hinton broth</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Antibiotic resistance</td>
<td>Chloramphenicol, ampicillin, geneticin, bacitracin, hygromycin and rifampicin</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Heavy metals resistance</td>
<td>Heavy metal salts</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Detergent tolerance</td>
<td>Sodium dodecyl sulphate, Sodium lauryl sulphate and CTAB</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Dye resistance &amp; Visualization of DNA</td>
<td>Ethidium bromide and Acridine orange</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Efflux pump inhibitor</td>
<td>CCCP and 2,4 DNP</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Organic solvent tolerance</td>
<td>Cyclohexane, Hexane, Toluene, Xylene, Petroleum ether</td>
<td>Merck, India</td>
</tr>
<tr>
<td>PCR</td>
<td>λDNA, DNA Ladders, dNTPs, MgCl₂, Taq DNA Polymerase, 6X gel loading dye</td>
<td>Fermentas, USA</td>
</tr>
<tr>
<td>PCR</td>
<td>Primers</td>
<td>Sigma Aldrich, Bangalore</td>
</tr>
<tr>
<td>Guanidine thiocyanate, MES, phenol red</td>
<td>Gel extraction of amplicons</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Spin column</td>
<td>DNA extraction from agarose gel</td>
<td>Bangalore Genei</td>
</tr>
<tr>
<td>Exopolysaccharides detection</td>
<td>Congo red</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>MATH Assay</td>
<td>Salts</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>MATS assay</td>
<td>KNO₃</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Floating test</td>
<td>Salts</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Effect of enzymes activity on biofilm</td>
<td>Diastase &amp; Lysozyme</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Effect of enzymes activity on biofilm</td>
<td>DNase, Proteinase K, Trypsin, EcoRI, Hind III, Taq 1, Bam H1, RNase A, RNase T1</td>
<td>Fermentas, USA</td>
</tr>
<tr>
<td>Extracellular DNA secretion</td>
<td>Propidium iodide</td>
<td>Himedia, Mumbai</td>
</tr>
<tr>
<td>Isolation of DNA from biofilm</td>
<td>MO BIO™ Soil DNA isolation kit</td>
<td>MO BIO, USA</td>
</tr>
</tbody>
</table>
Table 6: Instruments used in the study

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Purpose</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shaker</td>
<td>Cultivation of the bacterial cells</td>
<td>Scigenics, Bangalore, India</td>
</tr>
<tr>
<td>Refrigerated centrifuge</td>
<td>Pelleting of bacterial cells and EPS</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>Flasks and glassware</td>
<td>Microbial and Molecular studies</td>
<td>Borosil, India</td>
</tr>
<tr>
<td>PCR</td>
<td>Amplification of desired genes</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>pH measurement</td>
<td>Measurement of hydrogen ion concentration</td>
<td>Elico, India</td>
</tr>
<tr>
<td>Milli-Q Water Purification System</td>
<td>Molecular Biology grade water</td>
<td>Millipore, Germany</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>Visualization of PCR amplicons and genomic DNA</td>
<td>Hoefer Scientific Instruments, USA</td>
</tr>
<tr>
<td>Incubator (4°C - 30°C)</td>
<td>Bacterial Growth and cultivation</td>
<td>Scigenics, India</td>
</tr>
<tr>
<td>Microscope</td>
<td>Grams staining</td>
<td>Olympus, India</td>
</tr>
<tr>
<td>FTIR</td>
<td>Identification of functional groups</td>
<td>Perkin Elmer, USA</td>
</tr>
<tr>
<td>DNA sequencing</td>
<td>Sequencing of amplicons</td>
<td>ABI Prism DNA Sequencer, USA</td>
</tr>
<tr>
<td>Micro quant Spectrophotometer</td>
<td>Biofilm and planktonic cell studies</td>
<td>Biotek Instruments, USA</td>
</tr>
<tr>
<td>Micropipettes</td>
<td>PCR, Planktonic cells and biofilm studies</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>CLSM</td>
<td>Biofilm images</td>
<td>Leica Microsystems, Germany</td>
</tr>
<tr>
<td>Gel Documentation</td>
<td>DNA analysis</td>
<td>Total Lab, England</td>
</tr>
</tbody>
</table>

3 SOIL COLLECTION AND EVALUATION, BACTERIAL ISOLATION AND CHARACTERIZATION

3.1 Sample Collection (Ojo, 2006)

Soil samples were collected from three petroleum contaminated sites, all in the State of Tamilnadu: Korukkupettai Railway station, Chennai; Ponmalai Railway workshop, Tiruchirappalli; and Petroleum storage facility at Ponmalai in Tiruchirappalli. Soil samples were collected randomly 5-10 cm beneath the surface using spatula and packed in sterile centrifuge tubes. Once transferred to the laboratory, they were stored at 4°C for further studies.
3.2 Physico-Chemical Properties of the Sampled Soil

3.2.1 Soil pH

Five grams of soil was dissolved in MilliQ water and the pH was checked with pH meter against standard buffer solutions.

3.2.2 Free carbonates were evaluated by treating the soil with acetic acid solution. Formation of effervescence is indicative of the presence of carbonates.

3.2.3 Preparation of filtrate

The physico-chemical properties of the soils were analyzed by mixing 30g soil in 150 ml MilliQ water and kept in a shaker incubator for 24 hrs at 37°C. The extract was filtered using Whatman filter paper and finally centrifuged at 11,000 rpm for 20 min. The filtrate was tested for total hardness, iron, fluoride, chloride, hydrazine and arsenic in the filtrate using standardised kits as per manufacturers’ instructions. The tests are describes hereunder.

3.2.3.1 Aqua Check™ iron testing

5 ml filtrate was mixed with a spoonful of reagent Fe. The contents were swirled for 5min and allowed to stand. The colour was matched with the standard colour chart.

3.2.3.2 Aqua Check™ total hardness

The aqua check test jar was filled with the filtrate upto the 10ml mark. A spoonful of powder reagent ID-1 was mixed with the filtrate. The powder was dissolved completely. 4-5 drops of ID-2 reagent were added and mixed well. The change in colour was taken note of. If the soluton turns blue, then the water sample is soft. Blue indicated soft; and red indicated high concentration of salts. Up on the solution turning red, reagent ID-3 was added by counting the number of droplets while mixing, until the colour changed from red to blue. Total hardness formula was calculated as follows (as ppm CaCO₃): 2 x (Number of Drops of ID3).
Materials and Methods

3.2.3.3 Aqua Check™ fluoride testing

10ml of filtrate was added with 2 drops of reagent 12A. The contents were mixed well. Then 5 drops of reagent 12B were added. The contents were mixed and allowed to stand for 5 minutes. The developing colour was matched with standard chart provided in the kit.

3.2.3.4 Aqua Check™ chlorine testing

The aqua check test jar was filled with the filtrate up to the 10ml mark. One tiny spoonful of reagent 04A-1 and 2 drops of reagent 04A-2 were added and mixed well. The reagent 04A-3 was added drop by drop until the colour changed to bluish violet. Chloride mg/L (ppm) as Cl⁻ = 10 x (Number of Drops of 04A-3)

3.2.3.5 Aqua Check™ hydrazine testing

The aqua test jar was filled with the filtrate up to the 10ml mark. A spoonful of reagent 011 was added and mixed well to dissolve the powder completely. The jar was incubated for 5 mins at the room temperature. The colour developed was matched with the colour chart provided in the kit.

3.2.3.6 Aqua Check™ arsenic testing

5 ml of filtrate was added into the reaction vessel. One spoonful of reagent AS/01 was added and swirled gently for 1 min. Then one spoonful of reagent AS/2 was added. The test strip consists of Mercury (II) bromide was inserted with test field 2cm into reaction vessel and clamped in with lid. The vessel was incubated for 12 minutes by gentle swirl. Care must be taken that the test field must not touch the sample. After 12 min incubation, the test strip was removed from reaction vessel and dipped it for 2 sec with the test field into water. The colour developed in the test field was compared with the colour scale provided in the kit.

3.3 Culture and Isolation of Soil Bacteria

The soil sample was made into slurry, overlaid with petrol and kept still for two days to concentrate the bacterial population in the overlaid water. The sample was serially diluted and plated on LB agar plates and incubated at 37°C. Colonies appeared after 2
Materials and Methods

days. Nine bacterial populations were isolated, pure cultured and stored at -20°C. The isolates were inoculated as stab culture in tubes and utilized for further studies.

**Preservation of bacterial isolates**

For long term preservation, stock culture from each purified isolate was stored in 10% glycerol and kept at –80°C for further experiments.

**Biochemical tests on the isolates**

**3.3.1 Gram staining**

Gram staining was performed on the isolates using standard protocol. The bacteria were smeared on clean glass slides. Few drops of crystal violet were added to individual slides and allowed to stand for 60 seconds. Excess crystal violet solutions were drained by repeated washing with distilled water. Next the slides were flooded with grams iodine and incubated for 60 seconds. After incubation, the slides were washed with distilled water. Then the bacterial smears were decolorized by 95% ethanol, followed by washing the slides with distilled water. Finally the slides were flooded with grams saffranin and incubated by 60 seconds. The slides were repeatedly washed with distilled water and examined under microscope. The isolates were grouped into Gram positive and Gram negative as well as rods and cocci.

**3.3.2 Catalase test**

Few drops of 3% hydrogen peroxide were overlaid on the bacterial smear and the changes were noted.

**3.3.3 Mac conkey agar medium**

The bacteria were streaked on Mac conkey agar medium, incubated at 37°C for 48 hrs and the change of medium colour was observed.

**3.3.4 Preliminary characterization**

Morphological features of bacterial colonies were studied by streaking the bacteria on LB agar media. The biochemical tests of bacteria were carried out according to the instructions given in the KB003 Hi25™ *Enterobacteriaceae* identification kit consisted of 25 tests and HiCarbo™ kit which consisted of 35 carbohydrates and one
control test. Freshly prepared single colony of bacteria was inoculated in each well and incubated at 37°C for 24 hrs and the colour changes of the medium were noted.

3.4 Molecular Identification of Bacterial Isolates Using 16S Ribosomal DNA Sequencing

3.4.1 Extraction of bacterial genomic DNA (Pitcher et al., 1989)

DNA extraction was performed by guanidine thiocyanate method with certain modifications to standardize. 250µl of cell lysis solution was added to the cell pellet and incubated for 5 min. Then sample was homogenized by vortexing gently and centrifuged at 10,000 rpm for 5 min. 20µl lysozyme (10 mg/ml), 1µl RNase (5mg/mL) was added to the pellet and incubated for 1 hr at 45°C. 3µl Proteinase K (20 mg/ml) was added and mixed gently for 10 min, followed by the addition of 100 µl of protein precipitate solution and subsequent heating at 60°C for 10 min. With gentle vortex, equal volume of phenol: chloroform: isoamyl alcohol (24:24:1) was added and centrifuged at 12000 rpm for 5 minutes. To the aqueous layer, 90 µl of 5M sodium acetate, double the volume of 100% ethanol was added & mixture was centrifuged at 12000 rpm for 5 minutes. To air dried DNA pellet, 100µl of 10 mM Tris-Cl (pH 8.0) solution was added & stored at –20°C.

3.4.2 Purification of DNA

Further purification of the isolated DNA was accomplished by adding equal volume of (600µl) of phenol: chloroform (1:1). Resulting solution was vortexed and centrifuged 6000 x g for 10mins. Phenol: chloroform extraction ensures removal of protein impurities. Supernatant was subjected to another round of Phenol: chloroform extraction. The supernatant was collected and equal volume of chloroform was added and centrifuged at 6000 x g for 10 mins. The upper aqueous layer was harvested and 1/10 volume of 3M Sodium acetate and 2.5 volumes of chilled 95% ethanol were added to it and incubated at -20°C for 1 hr. The solution was centrifuged at 5000 x g for 15mins. Supernatant was discarded and the pellet was washed in 70% ethanol. A short spin was given and supernatant was discarded by inverting the tubes. Another short spin was given and the little soup left was removed with a micropipette. Finally the pellet was air dried and dissolved in sterile double distilled water and kept in room temperature for about 24 hrs before quantification of the DNA.
3.4.3 Quantification of genomic DNA

The extracted genomic DNA was quantified on 0.8% agarose gel. Aliquots of 50, 100, 150 and 200 ng λ-DNA were used as standards. DNA was quantified on the UV transilluminator as compared to the standard λ DNA loaded.

3.4.4 Primer dilution

The primers were dissolved in Sterile Milli Q Water and gently tapped and spinned for few seconds to ensure proper dissolution. The stock solutions were prepared as 1ng/1µl. 2µl of the stock solution was diluted in 198µl of Sterile Milli Q water to have a 10pm/µl dilution.

3.4.5 Preparation of Master Mix

The Master Mix contained the components necessary to make new strands of DNA in the PCR process. The Master Mix reagents included buffer, deoxyribonucleotides in equal amounts (dATP, dTTP, dCTP, dGTP), primers – both forward and reverse primers and Taq DNA polymerase

3.4.6 PCR optimization of 16S ribosomal RNA gene

The PCR parameters for the amplification of 16S rRNA genes were optimized (Pidiyar et al., 2004) except Dietzia sp. and Gordonia sp. 50 µl of PCR master mix contained 25pm each of 16F27 (5´-CCA GAG TTT GAT CMT GGC TCA G-3´) forward and 16R1525 (5´-TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC-3´) reverse primers, 10 mM of dNTPs, 1X PCR Buffer, 1U Taq polymerase, 2mM Mg++ , 1 µl (100-200 ng) template DNA, sterile milli Q water.

For the 16S rRNA amplification of Dietzia sp., and Gordonia sp., separate primers were used. 50 µl of PCR master mix contained 25pm each of 16F (5’-AGATRTGATCATYGCATYGC-3’) forward and 16R (5’-CAYTAMCTTWTAAACGRGT-3’) reverse primers, 10 mM of dNTPs, 1X PCR Buffer, 1U Taq polymerase, 2mM Mg++, 1 µl (100-200 ng) template DNA, sterile Milli Q water.
Table 7: PCR Mix for 16S rRNA amplification

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Taq Buffer</td>
<td>5 µl</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2 µl (1.5 mM)</td>
</tr>
<tr>
<td>Primer 1</td>
<td>5 µl (50 pM)</td>
</tr>
<tr>
<td>Primer 2</td>
<td>5 µl (50 pM)</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>1 unit (0.33 µl)</td>
</tr>
<tr>
<td>Template DNA</td>
<td>5 µl (50 ng)</td>
</tr>
<tr>
<td>Sterile MilliQ water</td>
<td>26.67 µl</td>
</tr>
<tr>
<td>Total</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

Agarose gel electrophoresis

The DNA samples were prior checked in agarose gels for PCR analysis. Agarose gels was prepared by dissolving 1gm agarose in 100ml of 1X TAE-running buffer containing ethidium bromide for visualization of the DNA in the gel matrix. Ten µl of each amplified sample was loaded into each well, after mixing with 1 µl of 6X gel loading dye (Fermentas, USA). DNA ladders of uniform sizes ranging in size from 100bp to 1000bp were used to determine the size of the PCR amplicons. Gels were run at 50V for 1 hr on a Hoefer Scientific Instruments, HE 33 Submarine agarose gel unit. Gels were visualized under UV transilluminator and photographed.

Gel purification of 16S rRNA amplicons (Kim et al., 2009)

After the fractionation of PCR amplicons by agarose gel electrophoresis, the DNA band was cut from the gel, placed in an eppendorf tube, weighed and 3 volumes of gel solubilization buffer was added and gently heated. After solubilization, 1 volume of isopropyl alcohol was added to the equal volume of gel fragment. The mixture was directly added to the spin column (Bangalore Genei, India) and the solution was allowed to flow through the column by centrifugation at 10,000rpm for 2 mins. After washing with 500µl of wash buffer, the spin column was dried, eluted with 500 µl of TE buffer (pH 8.0). The eluent was mixed with 300 µl of sodium acetate (pH 5.1) and 600 µl of ice-cold isopropyl alcohol. The mixture was filtered through a spin column; the column washed with 800 µl of 70% ethanol and bound DNA eluted with 100 µl of TE buffer (pH 8.0).
3.4.7 DNA Sequencing

The sequence of the PCR amplicons was determined by the method developed by Sanger and colleagues. The principle was based on the generation of DNA fragments using a DNA polymerase in the presence of deoxynucleotide-triphosphates and fluorescent-labeled dideoxynucleotide-TP which terminate the synthesis at nucleotide specific points along the target strand. The fragments which vary in size were separated by electrophoresis on an acrylamide gel and the nucleotide order can be determined by scanning up the gel and analyzing the bands.

The sequencing reaction was performed with ABI big dye cycle sequencing terminator reactions (Applied Biosystems) in a total volume of 20 µl. The reaction contained 4µl of purified PCR product, 4 µl of sequencing buffer and 4 µl of either forward or reverse primer at a concentration of 10mM. The sequencing PCR program was run in 25 cycles for 3 min at 96°C, 15 sec at 96°C, 10 sec at 55°C, and 4 min at 60°C. The ABI genetic analyzer (Applied Biosystems) was used to read the sequences and the obtained sequence was analyzed and manually edited by the Sequencher program (GeneCodes, USA).

3.4.8 Phylogenetic analysis of bacterial isolates

The isolates were identified by partial sequencing of the PCR amplified 16S rDNA. Detection of closely related species with our isolates (Gordonia sp. PCSB4 (GenBank Accession Number: HM449700.1), Dietzia sp. PCSB5 (GenBank Accession Number: HM449701.1), Micrococcus luteus PCSB6 (GenBank Accession Number: HM449702.1), Bacillus cereus PCSB8 (GenBank Accession Number: HM449698.1), Microbacterium testaceum strain PCSB7 (GenBank Accession Number: HM449703.1), Micrococcus sp. PCSB3 (GenBank Accession Number: HM449699.1), Micrococcus luteus PCSB9 (GenBank Accession Number: HM749895.1), Bacillus oleronius PCSB2 (GenBank Accession Number: HM012706.1), Brevibacterium casei PCSB1 (GenBank Accession Number: HM012705.1) is essential for the construction phylogenetic tree. The 16S rRNA of our bacterial sequences was compared with the previously published DNA sequences by BLAST that are available at the NCBI website (http://www.ncbi.nlm.nih.gov). The sequences that reveal the closest
Materials and Methods

Similarity to our bacterial isolates were retrieved from Genbank and used for the phylogenetic tree construction: *Brevibacterium massiliense* 7400794 (GenBank Accession Number: GQ222264.1), *Brevibacterium celere* KMM 3637 (GenBank Accession Number: AY228463.1), *Brevibacterium sanguinis* CF63T (GenBank Accession Number: AJ564859.1), *Brevibacterium oceani* BBH5 (GenBank Accession Number: AM158905.2), *Brevibacterium picturae* LMG 22061T (GenBank Accession Number: AJ620364.1), *Brevibacterium antiquum* VKM Ac-2118 (GenBank Accession Number: AY243344.1), *Bacillus sporothermodurans* (GenBank Accession Number: BSU49079), *B. acidocola* strain TCCCC27037 (GenBank Accession Number: EU231617.1), *B. shackletonii* LMG 18435 (GenBank Accession Number: NR_025373.1), *B. ginsengihumi* Gsoil 114 (GenBank Accession Number: NR_041378.1), *B. aquimarum* strain DS1 (GenBank Accession Number: EU835729.1), *B. pichinotyi* RS2 (GenBank Accession Number: AF519464.1), *Cloacibacterium normanense* tu33 (GenBank Accession Number: FJ544402.1), *Micrococcus indicus* IARI-CS-31 (GenBank Accession Number: JF343132.1), *M. alkanovora* (GenBank Accession Number: AJ702663.1), *M. endophyticus* cp1 (GenBank Accession Number: JN082275.1), *M. yunnanensis* B177 (GenBank Accession Number: HQ439904.1), *Actinobacterium* C19 gene (GenBank Accession Number: AB302333.1), *Micrococcus sp.* BD-15 (GenBank Accession Number: GU085223.1), *Micrococcus sp.* B-D-TSA2 (GenBank Accession Number: HM629400.1), Glacial ice bacterium SB12K-6-3 (GenBank Accession Number: AF479366.1), *Microbacterium trichothecenolyticum* 1CK48 (GenBank Accession Number: JQ229809.1), *M. resistens* KNUC2105 (GenBank Accession Number: JN084033.1), *M. hominis* 1P10AE (GenBank Accession Number: EU977655.1), *M. testaceum* DSM 20166 (GenBank Accession Number: NR_026163.1), *Bacillus anthracis* strain BVC19 (GenBank Accession Number: JQ407793.1), *B. mycoides* F4 (GenBank Accession Number: JQ579627.1), *B. thuringiensis* DSS7 (GenBank Accession Number: HM217124.1), *B. lentus* NCIMB8773 (GenBank Accession Number: NR_040792.1), *Dietzia psychralcaliphila* KNUC9014 (GenBank Accession Number: JF505948.1), *D. timorensis* CA155 (GenBank Accession Number: GQ870424.1), *D. natronolimnaea* 15LN1 (GenBank Accession Number: X92157.1), *Tsukamurella paurometabola* DSM20162T (GenBank Accession Number: X80628.1), *Dietzia kunjamensis* K30-10
(GenBank Accession Number: AY972480.1), *D. maris* C6115 (GenBank Accession Number: FJ588198.1), *Gordonia rubripertinctus* AGP1-3 (GenBank Accession Number: AY277554.1), *G. namibiensis* NAM-BN063B (GenBank Accession Number: AF380931.1), *G. terrae* IFM 0412 (GenBank Accession Number: FJ536292.1), *G. lacunae* A4-20 (GenBank Accession Number: JN627169.1), *G. amicalis* T3 (GenBank Accession Number: EU427321.1), *Microbacterium testaceum* DSM 20166 (GenBank Accession Number: NR_026163.1), *Bacillus lentus* NCIMB8773 (GenBank Accession Number: NR_040792.1), *B. shackletonii* LMG 18435 (GenBank Accession Number: NR_025373.1), *B. ginsengihumi* Gsoil 114 (GenBank Accession Number: NR_041378.1).

3.4.9 Nucleotide sequence accession numbers

Nucleotide sequences of partial 16S rRNA genes were deposited with GenBank (http://www.ncbi.nlm.nih.gov) using sequin submission tool.

3.4.10 Phylogenetic tree of 16S ribosomal RNA of bacterial isolates

Multiple sequence alignment of our 16S rDNA sequences with other bacterial sequences has done with ClustalW which determine the details of the phylogenetic distance between the isolates and its closest relatives. The aligned data set was analyzed in Data Analysis in Molecular Biology and Evolution (DAMBE) (Xia and Xie, 2001) and was converted into Molecular Evolutionary Genetics Analysis (MEGA) format. Creating the phylogenetic tree, the parameters used were: complete deletion of gaps/missing data, distance model set to applying the nucleotide kimura-2-parameter, homogeneous pattern among lineages and uniform rates among sites and using the maximum composite likelihood model. The scale unit is number of substitutions per site. Distances and clustering with the neighbour-joining, maximum-parsimony and maximum-likelihood methods were performed using software package MEGA version 5.0 (Tamura et al., 2011). Kimura-2 parameter was used as the nucleotide substitution model. The bootstrap values (%) presented at the branches was calculated from 1000 replications. Scale bar indicates 0.02 substitutions per nucleotide position.
A. PLANKTONIC CELLS

3.5 Susceptibility to Antimicrobials

All the metal salts, antibiotics, dyes, efflux pump inhibitors and detergents was dissolved in Milli Q water, filter sterilized, prepared as a stock solution (10mg/ml) and stored at –20°C till use. MICs of heavy metals, antibiotics, dyes, efflux pump inhibitors and detergents were determined by the broth micro-dilution method using cation-adjusted Mueller–Hinton broth (MHB). The range of concentrations of antimicrobials (10µg/ml, 20µg/ml, 40µg/ml, 80 µg/ml, 160 µg/ml, 320 µg/ml, 640 µg/ml and 1280µg/ml) was selected. The antimicrobials were diluted at 1/10 in Mueller Hinton broth. Finally 200µL of MH broth supplemented with the antimicrobials were added to the wells of microtitre plates and inoculated with 10µL of bacterial suspensions. Controls were included along with the tested samples. All samples were run in quadruplicate. The results were recorded after 24 h of incubation at 37°C by reading the planktonic cells at Optical density (OD\textsubscript{630}) using a plate reader (Biotek Instruments, USA). MIC was determined as a lower concentration of antimicrobials that prevent the visible turbidity of bacterial culture.

3.6 Assay of Tolerance to Organic Solvents

Five organic solvents (Cyclohexane, Hexane, Toluene, Xylene and Petroleum ether) were filter sterilized and utilized for tolerant assay.

Solid medium overlay assay (Asako et al., 1997)

The bacterial isolates were grown to late logarithmic phase and diluted to a concentration of approximately10^7 cfu/mL. 5µL aliquot of the bacterial suspension was spotted onto LB agar plates and air dried. n-Hexane, cyclohexane, xylene, petroleum ether and toluene was added to the plates at a depth of 2 to 3 mm. Plates were sealed with parafilm to prevent evaporation of solvents and incubated at 30°C for up to 48 h. Plating was done in triplicates and solvent tolerance was measured as a function of bacterial growth. Growth was recorded as confluent growth (++), visible growth (+) or no growth (-) after 24 h.
3.7 Efflux Pumps

**Preparation of efflux pump inhibitors**

Efflux pump inhibitors (CCCP and 2, 4 – DNP) was utilized for efflux pump studies.

3.7.1 Presence of efflux pumps in bacterial strains

The presence of efflux pumps in bacterial isolates was studied by treating the individual bacteria with 80µg/ml of CCCP and DNP and plated on LB agar media with the inclusion of organic solvents at a depth of 2-3cm. Growth was recorded as confluent growth (C), single colony (SC), or no growth (NG) after 24 h.

3.7.2 Effect of DNP on cell surface hydrophobicity and cell surface charges

The effect of inhibiting efflux pumps and its effect on cell surface hydrophobicity and cell surface charges were evaluated according to the protocol of Jain *et al.*, 2007. For assessing the effect of DNP on bacterial cell surface hydrophobicity and surface charges, each culture was inoculated in 150 ml LB medium in 250 ml conical flasks, with the concentration of 80µg/ml of 2,4 DNP and incubated for 24 hours at 37°C. Each culture broth was centrifuged at 5000 rpm for 20 minutes. The bacterial pellet was washed twice with PBS and later resuspended in the same buffer (pH 5, 7 & 9). The MATH and MATS assay were done according to the below mentioned protocol.

3.7.3 Screening for *qacEΔ1* efflux pump gene *(Rosser and Young, 1999)*

Planktonic cells genomic DNA was screened for the presence of *qacEΔ1* class efflux pump gene, reported to be involved in the extrusion of organic solvents. The primers such as CASSF (5’-TGATCCGCATGCCCCTCCATA-3’) and CASSR (5’-GGCAAGCTTAGTAAAGCCCTCGCTAG-3’) were utilized for PCR studies. Varying concentrations of template DNA, primers, Mg⁺⁺ ions and different annealing temperatures were set for the PCR optimization. PCR mixtures were prepared with 1µl (100ng) of genomic DNA, 5 µl of PCR buffer 10X, 1.5 mM of MgCl₂, 200 µmol of each deoxynucleoside triphosphate, 25 pmol each primer, and 1U *Taq* polymerase (Fermentas, USA) and sterile Milli Q water to a final volume of 50 µl. PCR includes initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min., annealing at 54°C for 1 min., elongation at 72°C for 1 min. and final extension at
Materials and Methods

72°C for 3 min. Finally the amplicons were checked in 1 % agarose gel. Size of fragments was estimated by using DNA marker (100 base-pair ladder).

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Taq Buffer</td>
<td>5 µl</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2 µl (1.5 mM)</td>
</tr>
<tr>
<td>Primer 1 (forward)</td>
<td>1 µl (10 pM)</td>
</tr>
<tr>
<td>Primer 2 (reverse)</td>
<td>1 µl (10 pM)</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>1 unit (0.33 µl)</td>
</tr>
<tr>
<td>Template DNA</td>
<td>5 µl (50 ng)</td>
</tr>
<tr>
<td>Sterile MilliQ water</td>
<td>34.67 µl</td>
</tr>
<tr>
<td>Total</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

Table 8: PCR mix for the screening of qacE△l efflux pumps

3.8 Cell Surface Hydrophobicity

The cell surface hydrophobicity of bacterial isolates was performed by inoculating the individual isolates in 100ml of LB broth in a 250ml Erlenmeyer flask at 37°C for 24 hours. Bacterial cells were immediately centrifuged (10000 × g, 15 min), washed twice, suspended in PUM Buffer & PBS buffer (pH 5, 7 & 9) for MATH assay and PBS (pH 5, 7 & 9) for SAT assay respectively. The Initial OD₆₀₀ (A₀) of cell suspensions in fresh PUM and PBS buffers was adjusted to 1.0 for microbial adhesion to hydrocarbon (MATH) test, and to 1.2 for the salt aggregation test (SAT). Hydrophobicity assays were carried out in triplicate.

MATH assay (Rosenberg et al., 1980)

Microbial surface hydrophobicity of bacterial isolates was assessed with five different solvents such as cyclohexane, hexane, toluene, petroleum ether and xylene with slight modifications. 500 µl of respective organic solvents was added to 2 ml of microbial suspension, vortexted for 1 min and stood for 10 minutes to ensure the complete separation of solvent phase from aqueous phase. After 10 min, the final OD of the aqueous-phase was measured (A₁) at 600 nm. The degree of hydrophobicity was calculated as 1 – (A₁/A₀) x100.
Salt aggregation test (Krepsky et al., 2003)

Salt aggregation test was performed with varying concentration of ammonium sulfate solutions (10mM, 25mM, 50mM, 100mM, 250mM, 500mM, 1M, 2M, 3M, 4M)) in 0.2 M phosphate buffer (pH 5, 7 & 9). 50 µl of the bacterial suspension was mixed with equal volume of varying ammonium sulfate solution in V – shaped microtitre plates, incubated for 10 minutes at room temperature and then the results were scored. The bacteria were considered to hydrophobic if it aggregates at lower ammonium sulfate concentrations.

3.9 Cell Surface Charges: MATS assay (Bellon-Fontaine et al., 1996; Kos et al., 2003)

MATS were measured using three different solvents xylene, an apolar solvent; chloroform, a monopolar and acidic solvent; and ethyl acetate, a monopolar and basic solvent. The bacterial isolates were grown in 100ml of LB broth in a 250ml Erlenmeyer flask at 37°C for 24 hours. Bacterial cells were centrifuged (10000×g, 15 min), washed twice and resuspended in 0.1M KNO₃ (pH 5, 7, 9) at an initial OD of about 1.0 (A₀) at 600 nm. 500 µl of solvent was added to 2 ml of bacterial suspension (pH 5, 7 & 9), vortexed for 1min and kept stand for 10 minutes. The aqueous phase was removed and its final OD at 600 nm (A₁) was measured. The percentage of bacterial adhesion to solvent was calculated as 1 – (A₁/A₀) x 100. Adhesion of bacteria to xylene represents the cell surface hydrophobicity or hydrophilicity with other two solvents values chloroform and ethyl acetate, were regarded as a measure of electron donor (basic) and electron acceptor (acidic) characteristics of bacteria, respectively.

3.10 Assaying for Auto-aggregation and Co-aggregation

Auto-aggregation of bacterial isolates were done according to the protocol of Del Re et al., 2000. Individual bacteria were inoculated into LB broth and incubated overnight. The cells were centrifuged at 5000g for 15 min, washed twice with phosphate buffered saline and resuspended in the same buffer (pH 5, 7 & 9). 2ml of bacterial suspensions were taken in test tubes, vortexed for 20 s and checked for auto-aggregation during 5 h incubation at room temperature. Initial OD was checked at
600 nm by taking 200 µl of upper aqueous suspension. During one hour interval, 200 µl of upper suspension were taken and its final OD at 600 nm was checked. The auto-aggregation percentage is expressed as: \( 1 - \frac{A_t}{A_0} \times 100 \), where \( A_t \) represents the absorbance at time \( t \): 2 h and \( A_0 \) the absorbance at \( t = 0 \).

Co-aggregation of bacteria was done according to the protocol of Del Re et al., 2000 by mixing equal volumes of each cell suspension, vortexed for 20 s and incubated at room temperature for 10 min. Initial OD was taken from the aqueous phase. During one hour interval, 100 µl of upper aqueous suspension was taken and its final OD at 600 nm was taken. The percentage of co-aggregation was calculated by the formula (Handley et al., 1987):

\[
\text{Co-aggregation} \, (\%) = \frac{(Ax + Ay / 2) - A(x + y)}{(Ax + Ay / 2)} \times 100
\]

Where, \( x \) and \( y \) represent each of the two strains in the control tubes, and \( (x + y) \) the mixture.

### 3.11 Exopolysaccharides and Bioemulsifier Secretion

**Congo red binding** (Solano et al., 2002)

Detection of exopolysaccharides secretion was carried out by qualitatively assessing the level of Congo red binding of colonies grown for 48 h on LB agar supplemented with 40 µg/ml congo red. Bacterial colony colour was used to judge Congo red binding.

**Drop collapse test** (Bodour and Maier, 1998)

2 µl of diesel and petrol oil was added to the surface of glass plates respectively. To the surface of oil suspension, 5 µl of culture supernatant was added and observed after 1 min. The culture supernatant that make the oil drop collapsed was indicated as positive result and that drops remain beaded were scored as negative which was examined with distilled water as control.


**Materials and Methods**

**Oil displacement test** (Rodrigues et al., 2006)

The oil displacement test is a method employed to evaluate the surface tension reduction efficiency of cell free supernatant by measuring the diameter of the clear zone, which occurs after a drop of cell free extract was added to an oil-water interphase. 50 ml of distilled water was added to a petri dish (diameter of 15 cm), followed by the addition of 20 µl of diesel and petrol respectively onto the surface of the water, then by dropping of 10 µl of cell free supernatant. The diameter of clear halo was immediately measured.

**Emulsification test** (Cooper and Goldenberg, 1987)

The emulsification index was estimated by treating the equal volume of cell free supernatant and bacterial cells to diesel and petrol respectively, briefly vortexed for 2 minutes and left stand for 24 hrs. The emulsification index was determined as the ratio of the height of the emulsion layer versus the total height of the mixture.

**3.12 Floating Test** (Young et al., 2005)

The floating and non-floating nature of isolates was performed with slight modifications. The bacterial isolates were inoculated into a 78-ml test tube (220mm in length and 24mm in diameter) containing 40 ml of liquid mineral Bushnell Haas medium, added with 3% v/v of diesel as the carbon source. The culture was maintained at 30°C with 160 rev/min agitation for 72 hrs and was then kept still for 24 hrs to observe the spatial distribution of cells in the culture.

**3.13 Chemotaxis** (Grim and Harwood, 1997)

The attractant nature of bacterial isolates towards diesel and petrol was performed by a drop assay with slight modifications. 100 µl of individual overnight culture was inoculated into 40 ml of LB broth. The cells were harvested in the logarithmic phase and resuspended in 12 ml of chemotaxis buffer. The cell suspension was then layered on the bottoms of 60-mm-diameter petri dishes to a depth of about 3 mm. 50 µl drops of diesel was added to the center of a dish and analyzed. A chemotactic response of cells to the added compound results in the formation of a ring of turbidity near the center of the petri dish after about an hour.
3.14 Culture-dependent Screening of Integron and Gene Cassettes in Genomic DNA (Mazel et al., 2000)

The genomic DNA of all planktonic cells was screened for Class I, II and III Integrons. PCR mixtures were prepared with 1µl (100ng) of genomic DNA, 5 µl of PCR buffer 10X, 1.5 mM of MgCl₂, 200 µmol of each deoxynucleoside triphosphate, 25pmol each primer, and 1U Taq polymerase (Fermentas, USA) and sterile Milli Q water to a final volume of 50 µl. PCR includes initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min., annealing at 55°C for 1 min., elongation at 72°C for 1 min. and final extension at 72°C for 3 min. Finally the amplicons were checked in 1 % agarose gel. Size of fragments was estimated by using DNA marker (100 base-pair ladder). PCR products were separated on 1.5% agarose gel and were photographed by using Total Lab Gel Documentation System.

<table>
<thead>
<tr>
<th>Integrons / Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I intI1F (5’-GGGTCAAGGATCTGGATTTCG-3’)</td>
<td>intI1R(5’-CATGGGTGTAATCATTGTC-3’)</td>
</tr>
<tr>
<td>Class II intI2.F(5’-CACGGATATGCGACAAAAGGT-3’)</td>
<td>intI2.R(5’-GTAGCAAAACGAGTGCAGAAATG-3’)</td>
</tr>
<tr>
<td>Class III intI3.F(5’-GCCTCCTGCGACGCAGCCTTTCAG-3’)</td>
<td>intI3.R(5’-ACGGATCTGCCCAACCTGACT-3’)</td>
</tr>
</tbody>
</table>

Table 10: PCR Master Mix for the screening of Class I, II & III Integrons

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Taq Buffer</td>
<td>5 µl</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2 µl (1.5 mM)</td>
</tr>
<tr>
<td>Primer 1 (forward)</td>
<td>1 µl (10 pM)</td>
</tr>
<tr>
<td>Primer 2 (reverse)</td>
<td>1 µl (10 pM)</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>1 unit (0.33 µl)</td>
</tr>
<tr>
<td>Template DNA</td>
<td>10 µl (100 ng)</td>
</tr>
<tr>
<td>Sterile MilliQ water</td>
<td>29.67 µl</td>
</tr>
<tr>
<td>Total</td>
<td>50 µl</td>
</tr>
</tbody>
</table>
3.14.1 Screening for Class I integron-associated gene cassettes (Elsaied et al., 2007)

The PCR parameters for the amplification of gene cassettes were optimized. Forward primer (GCF1-5′-GCSGCTKANCTCVRRCGTTRRRY-3′) and Reverse primer (GCR1-5′-TCSGCTKARGAMTTGTTRRRY-3′) were utilized for the amplification of gene cassettes associated with the class I Integron. PCR mixtures were prepared with 5µl (50ng) of genomic DNA, 5 µl of 10X PCR buffer, 1.5 mM of MgCl₂, 200 µmol of each deoxynucleoside triphosphate, 25 pmol each primer, and 1 U Taq polymerase (Fermentas, USA) and sterile Milli Q water to a final volume of 50 µl. PCR includes initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min., annealing at 54°C for 1 min., elongation at 72°C for 1 min. and final extension at 72°C for 3 min. Amplicons of varying sizes were generated for all isolates. Size of fragments was estimated by using DNA marker (100 base-pair ladder). Finally the amplicons were checked in 1 % agarose gel and gel purified using Chromous Gel Extraction Kit (Chromous biotech, Bangalore, India). The protocols were followed as per the instructions of manufacturer: PCR amplicons were sliced from the agarose gel and weighed in a 2ml eppendorf tube. 3 volumes of gel extraction buffer II was added to 1 volume of gel (100 mg -100 µl) and the tube was incubated at 55°C for 10 minutes in dry bath. To the dissolved gel, one volume of isopropanol was added and mixed well. Total solution was poured into 2ml spin column and centrifuged at 13,000 rpm for 1 min. at room temperature. The eluent was discarded and again the spin column was kept in the same collection tube. 500 µl of wash buffer was added to the spin column and centrifuged at 13,000 rpm for 1 min. The eluent was discarded. The spin column was again kept in the same collection tube and the washing step was repeated once. The empty spin column along with collection tube was spun at 13,000 rpm for 3 min. to remove residual wash buffer droplets. The spin column was placed in a fresh 1.5 ml eppendorf tube and 20 µl of elution buffer was added to the spin column, incubated for 20 mins and centrifuged at 13,000 rpm for 1 min. at room temperature. The above step was repeated once. Finally the eluent was collected and the purified PCR amplicons were sequenced commercially using ABI genetic analyzer (Applied Biosystems) DNA sequencer.
Materials and Methods

Table 11: PCR Master Mix for the screening of Class I Integron associated gene cassettes

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Taq Buffer</td>
<td>5 µl</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2 µl (1.5 mM)</td>
</tr>
<tr>
<td>Primer 1 (forward)</td>
<td>1 µl (10 pM)</td>
</tr>
<tr>
<td>Primer 2 (reverse)</td>
<td>1 µl (10 pM)</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>1 unit (0.33 µl)</td>
</tr>
<tr>
<td>Template DNA</td>
<td>5 µl (50 ng)</td>
</tr>
<tr>
<td>Sterile MilliQ water</td>
<td>34.67 µl</td>
</tr>
<tr>
<td>Total</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

3.14.2 Phylogenetic analysis of gene cassettes

Partial DNA sequences of gene cassettes were compared with the deposited sequences in Genbank by BLASTN. The sequences that reveal the closest similarity to gene cassettes were retrieved and multiple sequence alignment was done with ClustalW which determine the details of the phylogenetic distance between the gene cassettes and its closest relatives (Thompson et al., 1997).

DNA sequences of gene cassettes that do not match any similar sequences in Genbank will be subjected to theoretical translations of all possible reading frames as query (BLASTX). Related protein sequences that show similarity to our gene cassettes protein sequences were retrieved and protein alignments of translated cassette-encoded genes were generated using ClustalW.

Nucleotide sequence deposition and accession

Nucleotide sequences of partial gene cassettes were deposited with GenBank using BankIT submission tool (http://www.ncbi.nlm.nih.gov).

Phylogenetic tree

The gene cassettes were identified by partial sequencing of the PCR amplicons. Detection of closely related gene/protein sequences with our gene cassette sequences:
Materials and Methods

JQ923487 Unknown gene cassettes PCSGC1 [B. oleronius PCSB2], JQ923488 Putative alanine transaminase Transcriptional regulator (Lrp/AsnC family) PCSGC2 Gene cassettes [B. cereus PCSB8], JQ923489 Putative acyl-[acyl-carrier protein] desaturase Gene cassette PCSGC3, [B. cereus PCSB8] JQ923490 unknown Gene cassette PCSGC4 [B. cereus PCSB8], JQ923491 Unknown Gene cassette PCSGC5 [B. cereus PCSB8], JQ923492 Unknown gene cassette PCSGC6 [B. casei PCSB1], JQ923493 Unknown Gene cassette PCSGC7 [M. luteus PCSB6], JQ923494 Putative SSS Family solute/sodium (Na+) symporter Gene cassette PCSGC8 [Dietzia sp. PCSB5] is essential for the construction of phylogenetic tree. Since closely related DNA sequences to our gene cassette sequences are limited, an attempt was done to perform the six frame translation of nucleotide sequences of gene cassette and comparing the theoretical protein sequences of gene cassettes with the previously published protein sequences by BLASTX that are available at the NCBI website (http://www.ncbi.nlm.nih.gov). The protein sequences that reveal the closest similarity to our theoretical protein sequences of gene cassettes were retrieved from Genbank and used for the phylogenetic tree construction: ZP_06073317.1 glutamate-5-semialdehyde dehydrogenase [Acinetobacter radioresistens SH164], ZP_05362316.1 glutamate-5-semialdehyde dehydrogenase [A. radioresistens SK82], ZP_06066724.1 glutamate-5-semialdehyde dehydrogenase [A. junii SH205], ZP_06692586.1 conserved hypothetical protein [Acinetobacter sp. SH024], ZP_03824418.1 gamma-glutamyl phosphate reductase [Acinetobacter sp. ATCC 27244], YP_045295.1 gamma-glutamyl phosphate reductase [Acinetobacter sp. ADP1], YP_004997670.1 proA gene product [A. calcoaceticus PHEA-2], YP_003733655.1 gamma-glutamyl phosphate reductase [A. oleivorans DR1], ZP_06058697.1 glutamate-5-semialdehyde dehydrogenase [A. calcoaceticus RUH2202], ZP_04660087.1 gamma-glutamyl phosphate reductase [A. baumannii AB900], YP_001708152.1 gamma-glutamyl phosphate reductase [A. baumannii SDF], YP_080426.1 AsnC family transcriptional regulator [Bacillus licheniformis ATCC 14580], YP_005131607.1 alaR gene product [B. amyloliquefaciens subsp. plantarum CAU B946], YP_003921537.1 Lrp/AsnC family transcriptional regulator [B. amyloliquefaciens DSM 7], YP_001422413.1 AlaR [B. amyloliquefaciens FZB42], YP_001488023.1 unnamed protein product [Bacillus pumilus SAFR-032], YP_003974563.1 unnamed protein product.
[B. atrophaeus 1942], YP_004878639.1 ArsR family transcriptional regulator [B. subtilis subsp. spizizenii TU-B-10], AEP92148.1 transcriptional regulator [B. subtilis subsp. subtilis RO-NN-1], ZP_06873272.1 putative transcriptional regulator (Lrp/AsnC family) protein [B. subtilis subsp. spizizenii ATCC 6633], gZP_08004930.1 acyl-[acyl-carrier protein] desaturase [Bacillus sp. 2_A_57_CT2], ZP_09601233.1 fatty acid desaturase type 2 [Bacillus sp. 1NLA3E], ZP_04197551.1 acyl-[acyl-carrier protein] desaturase [B. cereus AH603], ZP_04105904.1 acyl-[acyl-carrier protein] desaturase [B. thuringiensis serovar berliner ATCC 10792], ZP_04087574.1 acyl-[acyl-carrier protein] desaturase [B. thuringiensis serovar huazhongensis BGSC 4BD1], ZP_04318405.1 acyl-[acyl-carrier protein] desaturase [B. cereus ATCC 10876], ZP_00738702.1 Acyl-[ACYL-carrier protein] desaturase [B. thuringiensis serovar israelensis ATCC 35646], ZP_04293860.1 acyl-[acyl-carrier protein] desaturase [B. cereus AH621], YP_001643941.1 fatty acid desaturase type 2 [B. weihenstephanensis KBAB4], ZP_01171821.1 delta-aminolevulinic acid dehydratase [Bacillus sp. NRRL B-14911], ZP_07709530.1 delta-aminolevulinic acid dehydratase [Bacillus sp. m3-13], BAK15800.1 delta-aminolevulinic acid dehydratase [Solibacillus silvestris StLB046], YP_004860575.1 delta-aminolevulinic acid dehydratase [B. coagulans 36D1], ZP_08095183.1 delta-aminolevulinic acid dehydratase [Planococcus donghaensis MPA1U2], ZP_09600354.1 Uroporphyrinogen III synthase HEM4 [Bacillus sp. 1NLA3E], ZP_01171820.1 uroporphyrinogen-III synthetase [Bacillus sp. NRRL B-14911], ZP_08005862.1 uroporphyrinogen-III synthetase [Bacillus sp. 2_A_57_CT2], ZP_09354283.1 delta-aminolevulinic acid dehydratase [B. smithii 7_3_47FAA], ZP_08005861.1 delta-aminolevulinic acid dehydratase [Bacillus sp. 2_A_57_CT2], ZP_04291369.1 Delta-aminolevulinic acid dehydratase [B. cereus R309803], YP_002369269.1 hemB gene product [B. cereus B4264], ZP_01172822.1 hypothetical Sugar diacid utilization regulator [Bacillus sp. NRRL B-14911], ZP_01172822.1 hypothetical Sugar diacid utilization regulator [B. megaterium WSH-002], YP_003597940.1 putative sugar diacid recognition [B. megaterium DSM 319], YP_003563219.1 putative sugar diacid recognition [B. megaterium QM B1551], ZP_02159188.1 hypothetical Sugar diacid utilization regulator [Shewanella benthica KT99], YP_004496553.1 transcriptional regulator CdaR [Desulfotomaculum carboxydivorans CO-1-SRB], ZP_08115633.1 transcriptional
Materials and Methods

regulator, CdaR [D. nigrificans DSM 574], YP_001094653.1 unnamed protein product [Shewanella loihica PV-4], YP_001473257.1 unnamed protein product [S. sediminis HAW-EB3], ZP_04818076.1 patatin [Staphylococcus epidermidis M23864:W1], ZP_07842391.1 putative phospholipase, patatin family [S. caprae C87], ZP_03613522.1 patatin [S. capitis SK14], EGS40975.1 phospholipase, patatin family [S. capitis VCU116], YP_002633198.1 putative esterase of the alpha-beta hydrolase superfamily [S. carnosus subsp. carnosus TM300], ZP_09203572.1 hypothetical protein CDLVIII_1472 [Clostridium sp. DL-VIII], ZP_03055338.1 SSS family solute:sodium [B. pumilus ATCC 7061], YP_005130664.1 yodF gene product [B. amyloliquefaciens subsp. plantarum CAU B946], EHM04782.1 YodF [B. amyloliquefaciens IT-45], YP_003920626.1 Na+/metabolite permease [B. amyloliquefaciens DSM 7], YP_001421527.1 hypothetical protein RBAM_019340 [B. amyloliquefaciens FZB42], YP_003973561.1 unnamed protein product [B. atrophaeus 1942], ZP_06872925.1 putative Na+/metabolite permease [B. subtilis subsp. spizizenii ATCC 6633], BA185631.1 hypothetical protein BSNT_03192 [B. subtilis subsp. natto BEST195], AEP91156.1 YolC [B. subtilis subsp. subtilis RO-NN-1],YP_004203891.1 unnamed protein product [B. subtilis BSn5], NP_389839.1 Na+/metabolite permease [B. subtilis subsp. subtilis str. 168].

Distances and clustering with the neighbour-joining, maximum-parsimony and maximum-likelihood methods were performed using software package MEGA version 5.0 (Tamura et al., 2011). The bootstrap values (%) presented at the branches was calculated from 1000 replications.
**Materials and Methods**

**B. BIOFILM**

### 3.15 Biofilm Formation (Rossi et al., 2007)

Biofilm formation by the bacterial isolates was assayed by microtitre plate assays. 10µL of overnight culture was added to 190µL of sterile LB broth taken into the wells of 96 flat well polystyrene microtitre plates and allowed to form biofilm by incubating the microtitre plates at 37°C statically. The planktonic cells OD were read at 630nm. The wells were thoroughly washed with the sterile PBS to remove the planktonic cells. After washing, the biofilm formation was stained by adding 200 µL of 0.01% (w/v) crystal violet and incubated for 30 min. After incubation, the excessive stains were removed by washing the wells with PBS and extracted with ethanol 95% (v/v). The amount of biofilm was quantified by measuring the optical density at 546 nm (OD546) of dissolved CV using a plate reader (Biotek Instruments, USA). Each isolate was assayed eight times. Non-inoculated medium controls (ODc) were included. The biofilm formation by isolates was classified as weak, moderate and strong biofilm formers based on the ODs of biofilms (Stepanovic et al., 2004). Cut–off OD (ODc) was deducted as four standard deviations above the mean OD of the negative control. Isolates were classified into:

1. OD < ODc, no biofilm producer,
2. ODc < OD < (2 x ODc) weak biofilm producer,
3. (2 x ODc) < OD < (4 x ODc) moderate biofilm producer and
4. (4 x ODc) < OD strong biofilm producer.

**Biofilm formation in tubes** (Rossi et al., 2007)

Biofilm formation by isolates in polypropylene and borosilicate tubes was performed with slight modifications. 100 µL of overnight bacterial culture was inoculated into 10 ml of LB broth (pH 5, 7 & 9) and incubated statically in borosilicate (BS) or polypropylene (PP) tubes for 24hrs. The cultures were aspirated, washed with distilled water and stained with 2ml of Crystal violet (0.01%) for 30 min. The excess CV was washed with sterile distilled water and dissolved with 2 ml of ethanol. The biofilm formation was estimated by reading the OD at 546 nm and classified as strong, moderate, weak or negative. Biofilms formed at the air liquid interface were stained purple. All the strains were tested in triplicate.
3.16 Confocal Laser Scanning Microscopy Images of Biofilm Development

Biofilms were formed on pre-sterilized microscopic glass slides. Aliquots (200 µl) of overnight cultures were inoculated in sterile petri plates containing 20 ml of appropriate growth media. Pre-sterilized microscopic glass slides were immersed in the medium. The petriplates were incubated at room temperature for 24 h on a rocker. After incubation, the slides were removed and rinsed twice with PBS (0.1 M, pH 7.0) and stained with Live/Dead BacLight viability stains (Molecular Probes, Eugene). BacLight stains differentiate live cells from the dead ones on the basis of loss of membrane integrity (Jin et al., 2005). The Live/Dead stain was prepared by mixing 3 µl each of Syto 9 and propidium iodide (PI) in 1 ml of PBS. The biofilms were stained by incubating with the dyes for 10 min at room temperature and the unbound stain was removed by washing in PBS for 15 min. The cell viability was assessed by using a confocal laser scanning microscope (CLSM).

A confocal laser scanning microscope (TCS SP2 AOB5) equipped with DM IRE 2-inverted microscope (Leica Microsystems, Germany) was used to image bacterial biofilms. A 63 × 1.2 NA water immersion lens was used for obtaining the images. The confocal microscope was equipped with the 488 nm and 543 nm Ar lasers used for excitation and emission that were collected in the bandwidth 515-540 nm and 560 nm, respectively. SYTO 9 was excited by a 488 nm laser and the emitting light was collected by the filter 515-540, while PI was excited by a 543 nm laser and the filter 560 nm was used to collect emission light. About 20 biofilm stacks measuring an area of 238.1 × 238.1 µm were scanned and the images were acquired at 2 µm z-intervals, from the base of the biofilm to its top. Line averaging was applied and percentage biofilm disruption was determined by using the digital image analysis freeware ImageJ, downloadable from the site http://rsb.info.nih.gov/ij. Three dimensional projections of the biofilms were performed with IMARIS software (Bitplane, Zurich, Switzerland).

3.17 Biofilm’s Resistance to Antimicrobials (Cernohorska and Votava, 2004)

For determining the MIC-b to biofilm cells, we used the MIC for planktonic cells as the initial concentration in the tests and increased from that. 10µL of overnight culture was inoculated into 190µL of sterile LB broth added into 96 wells of microtitre plate
and incubated at 37°C for 24 h. After incubation, the medium was decanted and washed with PBS to remove the planktonic cells. The MIC-b for biofilm was determined by increasing the 10 – 75 times MIC of planktonic cells (640µg/ml to 24,000µg/ml). 200µL of respective heavy metals, dyes, detergents, efflux pump inhibitors and antibiotics at two-fold dilutions prepared in MHB was added to the established biofilms. The MIC-b for biofilm was determined according to the method given by Ceri et al., 1999 in which the lowest concentration of antimicrobials which reduce or prevent the visible turbidity of planktonic cells sheds from the biofilm. Controls were included along with the tested samples. All samples were run in quadruplicate. The viability of the biofilm was also determined by crystal violet method.

3.18 Effect of Degrading Enzymes on Preformed Biofilm

In general, the matrix of bacterial biofilms is enclosed in a matrix of exopolymeric substances, clothed with proteins, lipids, RNA and DNA. All these macromolecules helps in protecting the three dimensional structure of biofilms. An effort was taken to study the importance of these macromolecules in protecting the biofilm structure and function. Biofilm was allowed to form in the 96 well microtiter plates by adding 190 µl of LB broth and 10 µl of one day culture. After 2 days, 11 enzymes namely Trypsin (100µg/ml), Lysozyme (100µg/ml), BamHI (10U/ml), EcoRI (10U/ml), Hind III (10U/ml), Taq I (10U/ml), RNAse A (10U/ml), RNAse T1 (10U/ml), DNAse (10U/ml), Proteinase K (10U/ml) and Diastase (100µg/ml) were added to preformed biofilm. The contents were mixed well and the release of bacterial cells from the biofilm matrix was noted at OD$_{600}$ at different time interval (0 min, 30min and 60 min). The medium was discarded and the plates was washed with distilled water, which was followed by the crystal violet staining and decolourization by adding 100% ethanol. The effect of enzymes on preformed biofilm was spectrophotometrically analyzed at 546nm.

3.19 Efflux Pump Inhibitors on Biofilm Formation

Efflux pump inhibitors are the class of chemicals that inhibit the siphon action of efflux pumps. Varying concentrations (12.5mM, 25mM, 50mM, 100mM and 150mM)
Materials and Methods

of CCCP and DNP were utilized for the study on the effect of EPIs on biofilm formation. 190µl of respective concentration of EPIs in LB broth and 10µl of bacterial culture were inoculated into the wells of microtitre plates (96-well flat-bottom) respectively and incubated at 37°C for 24hrs. The effect of EPIs on biofilm formation was monitored by crystal violet staining.

3.20 Isolation and characterization of Exopolymeric Substances

EPS produced by each of the selected isolates was extracted by the method of Matjan et al., 2007. The bacteria were grown in LB broth at 37°C for 24 hrs. After incubation, the bacterial suspension was centrifuged at 10,000rpm for 30 minutes at 4°C. The biomass was discarded and two volumes of ice-cold isopropanol were added to the supernatant and allowed to be at -20°C for 24 hrs. Then it was centrifuged under the same conditions to obtain the EPS. The protein contamination in the EPS were precipitated with 25% (w/v) trichloroacetic acid (Himedia, India) on ice for 2 h and removed by centrifugation. Again the EPS was re-precipitated by the addition of two volumes of ice cold isopropanol, centrifuged and dialyzed against the distilled water. The pellet was air dried for analytical work.

Analytical procedures on EPS

The carbohydrate content of the EPS sample was determined according to the method proposed by Dubois et al., (1956) using glucose as a standard. 1mg of freeze dried EPS was added to 1ml of water, 50 µl of 80% (w/v) phenol/water, 5 ml of concentrated H₂SO₄ and heated at 100°C for 5 min, and then cooled to 20°C. An aliquot of the sample was removed, diluted 20 times in concentrated sulphuric acid mixed, and its absorbance at 490 nm for pentoses was read using a plate reader (Biotek Instruments, USA).

FTIR analysis of EPS

A pellet for infrared (IR) analysis was obtained by carefully grinding 2 mg of EPS with 200 mg of dry potassium bromide, ground well in mortar under an IR lamp for 30 minutes and then pressing in a mold. The IR spectrum of EPS from 400 to 4000 cm⁻¹ was obtained using a Perkin-Elmer Spectrum GX (Silverstein et al., 1991).
3.21 | EXTRACELLULAR DNA – ISOLATION, CHARACTERIZATION AND SECRETION

3.21.1 | Comparative evaluation of genomic and eDNA from biofilm

**Isolation of genomic DNA from biofilm**

The DNA was isolated from the four biofilm forming bacteria, using the MO BIO power soil™ DNA isolation kit.

1) 2 ml of bacterial culture was taken in microfuge tube and centrifuged at 10,000 rpm for 5 mins. The supernatant was discarded and the power beads were added to the bacterial pellet and gently vortexed.

2) 60 µl of solution C1 was added and the tubes were inverted several times.

3) Powerbead tubes were secured horizontally on a flat-bed vortex pad with tape. The tubes were vortexed at maximum speed for 10 minutes.

4) Then the tubes were centrifuged at 10,000 x g for 30 seconds at room temperature.

5) The supernatant was transferred to a clean 2ml collection tube.

6) To the supernatant, 250 µl of solution C2 was added and vortexed for 5 seconds and the tubes were incubated at 4°C for 5 minutes.

7) The tubes were centrifuged at room temperature for 1 minute at 10,000 x g.

8) 600 µl of supernatant was transferred to a clean 2ml collection tube. To that, 200 µl solution C3 was added and vortexed briefly. The tubes were incubated at 4°C for 5 min.

9) The tubes were centrifuged at room temperature for 1 minute at 10,000 x g.

10) 750 µl of supernatant was transferred to a clean 2ml collection tube. To that solution, 1.2ml of solution C4 was added and vortexed for 5 seconds.

11) 675 µl of solution was loaded approximately onto a spin filter and centrifuged at 10,000 x g for 1 minute at room temperature. Flow through was discarded and additional 675 µl of supernatant to the spin filter was added and centrifuged at 10,000 x g for 1 minute at room temperature. The remaining supernatant was loaded onto the spin filter and centrifuged at 10,000 x g for 1 minute at room temperature.

12) 500 µl of solution C5 was added and centrifuged at room temperature for 30 seconds at 10,000 x g.
Materials and Methods

13) The flow through was discarded from the 2ml collection tube and the tubes were centrifuged at 10,000 x g for 1 minute at room temperature.

14) The spin filter was carefully placed in a clean 2ml collection tube. Splashing of any solution C5 on to the spin filter was avoided.

15) 100 µl of solution C6 was added to the centre of the white filter membrane. The content was centrifuged at room temperature for 30 seconds at 10,000 x g.

16) The spin filter was discarded and the DNA solution in the tube was used for downstream application. The DNA solution was stored at -20° to -80°C.

Isolation of eDNA from biofilm matrix

1) The bacterial cells were grown in 6 well flat bottom plates.

2) The supernatant was discarded and unwashed biofilm were harvested by resuspending in eDNA isolation buffer and transferred into chilled tubes.

3) After centrifugation for 5 minutes at 4°C and 18,000 g, 100 µl of each supernatant was transferred to a tube containing 300 µl of TE buffer and extracted once with an equal volume of chloroform/isoamylalcohol (24:1).

4) The aqueous phase of each sample was then mixed with 3 volume of ice cold 100% ethanol and 1/10 volume of 3 M Na acetate (pH 5.2) and stored at -20°C.

5) Next day, the ethanol precipitated DNA was collected by centrifugation for 20 minutes at 4°C and 18,000g, washed with ice cold 70% ethanol, air dried and dissolved in 20 µl of TE buffer.

3.21.2 RAPD analysis of genomic DNA and eDNA (Venieri et al., 2004)

In order to detect differences between the extracellular and the genomic DNA of biofilm forming isolates, RAPD PCR was done. Four different primers were used: primer 1247 (5′-AAGAGCCCGT-3′), 1254 (5′-CCGCAGCCAA-3′), 1290 (5′-GTGGATGCCGA-3′) and RP15 (5′-AATGCGGCAG-3′). 50 µL of PCR master mix reaction volume contained 50 ng DNA (Genomic DNA or eDNA), 1.5 mM MgCl₂, 5 µL 10 × PCR buffer, 100 pmol of primer, 1 U of Taq DNA Polymerase and 10mM dNTP mix. PCR conditions were as follows: cycling program of 45 cycles (94°C for 5min, 94°C for 1 min; 31°C for 3 min; 72°C, 2 min). Subsequently, 20 µL aliquots of the products were electrophoresed in 1.5% agarose gels run for 2 h at 90 V. Gels were
Materials and Methods

stained for 20 min with ethidium bromide and photographed under UV light. A molecular size marker (Mass Ruler DNA Ladder High Range, Fermentas, Germany) was used as a reference. Comparison was made between both the amplified DNA sample and difference was observed.

Table 12: PCR Mix for RAPD analysis of genomic DNA and eDNA

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Taq Buffer</td>
<td>5 µl</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2 µl (1.5 mM)</td>
</tr>
<tr>
<td>Primers</td>
<td>10 µl (100 pM)</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>1 unit (0.33 µl)</td>
</tr>
<tr>
<td>Template DNA (Genomic DNA or eDNA)</td>
<td>5 µl (50 ng)</td>
</tr>
<tr>
<td>Sterile MilliQ water</td>
<td>26.67 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50 µl</strong></td>
</tr>
</tbody>
</table>

3.21.3 Extracellular DNA released by Planktonic Cells and Biofilm (Allesen-Holm et al., 2006)

Release of eDNA by planktonic cells and biofilm forming isolates of B. casei, M. testaceum and B. cereus was studied in 96 wells microtitre plates using 50µM propidium iodide. For the measurement of eDNA release by biofilm, 10 µl of individual bacterial cultures and 50µM (7µl from stock solution) of propidium iodide was added to 83µl LB Broth and incubated for 48 hours in an unshaken condition and the OD was checked at 480nm for propidium iodide absorbance and 600nm for bacterial density. For assessing the release of eDNA by planktonic cells, 10 µl of bacterial culture and 50µM (7µl from stock solution) of propidium iodide was added to 83µl LB broth and incubated for 48 hours in the shaken condition and the OD was checked at 480nm for propidium iodide absorbance and 600nm for bacterial density.

3.22 Effect of Exogenous DNA on Biofilm
3.22.1 Exogenous DNA and cell surface hydrophobicity

MATH assay was done to assess the effect of λ DNA on the cell surface hydrophobicity of B. cereus, M. testaceum, B. oleronius and M. luteus (Y2). PBS
(pH7) was used as an aqueous phase. Initial OD of the culture is taken before the addition of organic solvents. The procedure can be carried out by mixing the bacterial suspension (2 ml) with 0.5 ml of Cyclohexane, 5µl (5µg) & 10µl (10µg) of λ DNA to each tubes respectively and vortexed briefly for 60s. The two phases (Organic & Aqueous) were allowed to separate by incubating the test tubes for 20 min. The aqueous phase was taken and Final OD was noted at 600nm. The difference between the initial OD and final OD indicates the hydrophobicity of the bacterial cultures.

3.22.2 Exogenous DNA and cell surface charges

The effect of external DNA on the surface charges of bacterial isolates were measured by Microbial adhesion to solvents (MATS) assay with slight modifications. The assay were done using three different solvents xylene, an apolar solvent; chloroform, a monopolar and acidic solvent; and ethyl acetate, a monopolar and basic solvent. The bacterial isolates were grown in 100ml of LB broth in a 250ml Erlenmeyer flask at 37°C for 24 hours. Bacterial cells were centrifuged (10000×g, 15 min), washed twice and resuspended in 0.1M KNO₃ (pH 5, 7, 9) at an initial optical density (OD) of about 1.0 (A₀) at 600 nm. 500 µl of solvent, 5 µl (5µg) of λ-DNA was added to 2 ml of bacterial suspension (pH 7), vortexed for 1min and kept stand for 10 minutes. The aqueous phase was removed and its final OD at 600 nm (A₁) was measured. The percentage of bacterial adhesion to solvent was calculated as 1 – (A₁/A₀) x 100. Adhesion of bacteria to xylene represents the cell surface hydrophobicity or hydrophilicity with other two solvents values chloroform and ethyl acetate, were regarded as a measure of electron donor (basic) and electron acceptor (acidic) characteristics of bacteria, respectively (Bellon-Fontaine et al., 1996; Kos et al., 2003).

3.22.3 Exogenous DNA on cell aggregation

The effect of external DNA on the aggregation pattern of bacterial isolates was done according to the protocol of Del Re et al., 2000 with slight modifications. Individual bacteria were inoculated into LB broth and incubated overnight. The cells were centrifuged at 5000g for 15 min, washed twice with phosphate buffered saline and resuspended in the phosphate buffer (pH 7). Required volume of exogenous λ-DNA
Materials and Methods

[5μl (5μg) and 10μl (10μg)] was added to 2ml of bacterial suspensions in respective test tubes, vortexed for 20s and checked for aggregation during 5 h incubation at room temperature. Initial OD was checked at 600nm by taking 200 μl of upper aqueous suspension. During one hour interval, 200 μl of upper suspension were taken and its final OD at 600nm was checked. The aggregation percentage is expressed as: 

1 – (A_t/A_0) X 100, where A_t represents the absorbance at time (t): 5 h and A_0 the absorbance at t = 0.

3.23 Culture-Dependent Screening for Class I Integrons on genomic DNA and eDNA of biofilm (Mazel et al., 2000)

Both genomic DNA and eDNA of biofilm were screened for the presence of Class I integrons by PCR. The PCR parameters were same as that of utilized for the screening of Class I Integron in planktonic cells. The amplified PCR products were electrophoreosed on 0.8% agarose gel at 50V using TBE buffer and the bands were observed under UV transilluminator.

3.24 Statistical analysis

Statistical analysis was performed using SPSS software (version 11.5). Statistical analysis included mean ± SD, ANOVA and Student's t test. Results were considered significant at P < 0.05.