Materials & Methods
2. Materials and Methods

2.1. CHEMICALS

Media components - proteose peptone, tryptone, yeast extract, beef extract, agar agar and chemicals such as sodium chloride, ammonium chloride, disodium hydrogen phosphate, dipotassium hydrogen phosphate, glycerol and magnesium sulphate were purchased from Hi-Media, India and Fischer Scientific, South Korea. Streptavidin coated Dynal beads and Dynal – MPC magnetic rack were purchased from Invitrogen, USA. PCR reagents namely, Taq DNA polymerase, magnesium chloride, reaction buffer (10X) and deoxyribonucleotide triphosphates (dNTPs) were purchased from Invitrogen, USA. PCR Primers for 16S rRNA and 18S rRNA were purchased from Cosmo Genetech, South Korea and the biotinylated primers for MCH - PCR were obtained from Cosmo Genetech, Japan. DNA extraction kits, Ultra Clean™ (MoBio) kit and ChargeSwitch® gDNA Mini bacterial kit were purchased from MoBio Scientific, South Korea and Invitrogen, USA. MEGA – spin™ agarose gel extraction kit for DNA purification was purchased from MoBio Scientific, South Korea. All chemicals, solvents and reagents used were of analytical reagent grade.

2.2. MICROBIAL STRAINS

Pseudomonas LB400 strain was used for the optimization of 16S rRNA PCR and for 16S rRNA MCH-PCR, Micrococcus luteus ATCC-4698 and Micrococcus nishinomiyaensis DSMZ-2909 were used. Fungal 18S rRNA PCR optimization was done with Fusarium oxysporum and for 18S rRNA MCH-PCR Aspergillus niger van Tieghem, anamorph ATCC-9142 and Aspergillus niger
van Tieghem, anamorph DSMZ-16620 were used. The microbial strains were purchased from American Type Culture Collection (ATCC), USA and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), German collection of microorganism and cell cultures, Germany. All cultures used in the study are maintained at Molecular Bioremediation Division, Department of Environmental Biotechnology, Bharathidasan University, Tiruchirappalli, India.

2.3. MEDIA

2.3.1 Nutrient Broth (NB) (Atlas, 1993)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.5 g</td>
</tr>
</tbody>
</table>

pH was adjusted to 7.2 ± 0.2 and the volume was made to 1000 ml using distilled water.

2.3.2 Nutrient Agar (NA) (Atlas, 1993)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
</tbody>
</table>

pH was adjusted to 7.2 ± 0.2 and the volume was made to 1000 ml using distilled water.

2.3.3 Luria Bertani broth (LB) (Bertani, 1951)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
</tr>
</tbody>
</table>

pH was adjusted to 7.2 ± 0.2 and the volume was made to 1000 ml using distilled water.
2.3.4 **Luria Bertani agar (LBA) (Bertani, 1951)**

- Tryptone 10 g
- Yeast extract 5 g
- NaCl 10 g
- Agar 15 g

pH was adjusted to 7.2 ± 0.2 and the volume was made to 1000 ml using distilled water.

2.3.5 **Potato dextrose broth (PDB) (Atlas, 1993)**

- Potato (peeled) 200 g
- Dextrose 20 g

Potatoes were peeled off, cut into small pieces and boiled (200 g) in 500 ml of water, till they were easily penetrated by a glass rod and filtered through cheese cloth. Dextrose was added to the filtrate. pH was adjusted to 5.1 ± 0.2 and the final volume was made to 1000 ml using distilled water.

2.3.6 **Potato dextrose agar (PDA) (Atlas, 1993)**

- Potato (peeled) 200 g
- Dextrose 20 g
- Agar 15 g

Peeled off the potato skin, cut into small pieces and boiled (200 g) in 500 ml of water, till they were easily penetrated by a glass rod and then filtered through cheese cloth. Dextrose was added to the filtrate. Agar was dissolved in water and pH was adjusted to 5.1. The final volume was made to 1000 ml with distilled water.
**2.3.7 Super optimal catabolite medium (SOC)**

Tryptone 20 g  
Yeast extract 5 g  
NaCl 0.5 g  
KCl (1M) 2.5 ml  

pH was adjusted to 7.2 ± 0.2 and the volume was made to 1000 ml using distilled water. After sterilization, 20ml of glucose (1M) was added.

**2.4. BUFFERS AND SOLUTIONS (Sambrook et al., 1989)**

**2.4.1 Phosphate buffered saline (PBS)**

Sodium dihydrogen phosphate 780 mg  
Disodium hydrogen phosphate 709.8 mg  
Sodium chloride 2.34 g  

pH was adjusted to 7.2 and made up to 100 ml with distilled water

**2.4.2 Bradford’s reagent**

Coomassie Blue G250 0.025 g  
Ethanol (95%) 12.5 ml  
Ortho Phosphoric acid (85%) 25 ml  

The final volume was made up to 250 ml with distilled water

**2.4.3 Sodium dodecyl sulphate (10%)**

SDS 1 g  
Distilled water 10 ml

**2.4.4 Acrylamide solution (30%)**

Acrylamide 14.6 g  
Bis-acrylamide 0.4 g  
Distilled water 50 ml
2.4.5 Upper Tris
Tris buffer 3.025 g
SDS (10%) 2 ml
pH was adjusted to 6.8 and made up to 50 ml with distilled water

2.4.6 Lower Tris
Tris buffer 9.085 g
SDS (10%) 2 ml
pH was adjusted to 8.8 and made up to 50 ml with distilled water

2.4.7 Ammonium per sulfate (10%)
Ammonium per sulfate 1 g
Distilled water 10 ml

2.4.8 Tank buffer
SDS 1 g
Glycine 14.4 g
Tris buffer 3 g
Distilled water 1000 ml

2.4.9 Sample loading dye
Upper Tris 1.25 ml
Glycerol 1 ml
β-mercaptoethanol 0.5 ml
SDS 150 mg
Bromophenol blue 0.001 g
The final volume was made up to 10 ml with distilled water
2.4.10 Coomassie blue staining solution
Coomassie Blue R 250 1 g
Ethanol 250 ml
Glacial acetic acid 35 ml
The final volume was made upto 500 ml with distilled water

2.4.11 Destaining solution
Acetic acid 35 ml
Ethanol 150 ml
The final volume was made upto 500 ml with distilled water

2.4.12 Tris EDTA buffer
Tris base 242 g
Glacial acetic acid 57.1 ml
EDTA (pH 8.0) (0.5 M) 100 ml
pH was adjusted to 7.2 and made up to 1000 ml using distilled water.

2.4.13 Sample loading dye for SDS-PAGE
Glycerol (50%) 5 ml
Bromophenol blue (0.05%) 0.005 g
Distilled water 10 ml

2.4.14 Sample loading dye for AGE
Ethisdium bromide 10 mg
Distilled water 1 ml

2.4.15 Dynal beads 2X binding wash buffer
Tris-HCl, pH 7.5(10mM) 0.1576 g
EDTA (1mM) 0.0372 g
2.4.16 Dynalbeads MYOne™ Streptavidin C1

10mg (~7-12x10^9) Dynal beads per mL dissolved in phosphate buffered saline with a pH 7.4 containing 0.01% Tween 20 and 0.09% NaN₃.

2.5. MAINTENANCE OF MICROORGANISMs

Bacterial cultures were maintained on LB agar plates at 4°C for routine use. For long term storage, cells were suspended in sterile water and stored at 4°C or preserved in 40% glycerol at -70°C. Fungal cultures were maintained on PDA plates at 4°C for routine use and for long duration, the fungal cultures were maintained on PDA slants in a sterile test tube at 4°C.

2.6. STERILIZATION

All the media, buffers, eppendorff tubes, tips and reagents etc., used in this study were sterilized at (15 lbs/inch²) for 20 min unless otherwise specified. Heat labile chemicals were filter sterilized using 0.22 μm filter (Millipore, Molsheim, France).

2.7. SOIL SAMPLING

Soil samples were collected from the epicenter of a 30 year old small scale electroplating industry at Sipcot, Ranipet, Vellore district, Tamil Nadu, India. Ranipet is the third largest contaminated site in the world (http://www.tehelka.com/story_main13.asp?filename=Ne071605Tanneries_pollute.asp; http://www.pollutedplaces.org/region/south_asia/india/ranipet.shtml; http://www.adb.org/Projects/PEP/ind.asp.). Soil samples were collected from about 3 feet depth. The samples were stored at -80°C (Desjardin et al., 2002).
2.8. ANALYSIS OF SOIL SAMPLE

2.8.1 Measurement of pH

Soil was dried to remove the moisture content. Approximately 10 g of soil was mixed with 50 ml of sterile distilled water. The electrode was immersed into the soil suspension and the pH was measured.

2.8.2 Estimation of chromium and nickel by atomic absorption spectrophotometer

Soil samples were dried at 95°C at 24 h, ground, sieved (mesh size 63 μm) and dried at 100°C for 24 h. Approximately 10 g of soil was taken in 100 ml beaker and 10 ml of 1:1 conc. HNO₃ was added and heated at 95±5°C for about 20 min. The mixture was cooled and 5 ml of conc. HNO₃ was added and refluxed for 30 min (This step was repeated until known brown fumes were given off). The sample was heated at 95±5°C without boiling until the volume was reduced to approximately 5 ml. The mixture was cooled and 2 ml of deionized water and 3 ml of 30% hydrogen peroxide was added and heated until the effervescence subsides. The digestate was cooled and 30% hydrogen was added to it in 1ml aliquots with warming until the effervescence was minimum (not more than 10 ml). It was heated at 95°C until the volume reduced to approximately 5 ml. To this, 10 ml of conc. HCl was added to it and refluxed at 95±5°C for about 15 min. The digestate was cooled and filtered using Whatmann filter paper No.41 (63μm). The filtrate was collected and diluted to 100 ml in a volumetric flask and then analysed for chromium and nickel using Atomic Absorption Spectrophotometer (Perkin Elmer 30303B model) (APHA-AWA-WEF, 1998).
2.9. ENUMERATION AND SCREENING HEAVY METAL RESISTANT BACTERIAL SPECIES

Chromium and nickel resistant bacterial strains were isolated from the soil samples using bacterial medium. Approximately 10 g of soil sample was serially diluted with peptone water and 100 μl of suspension from 10^{-7} dilutions from the broth was spreaded on to the nutrient agar plates (standard spread plate technique) amended with different concentration of chromium (50, 100, 150, 200 and 250 mgL^{-1}) and nickel (25, 50, 75 and 100 mgL^{-1}) to isolate chromium and nickel resistant organisms. The plates were incubated at room temperature (30–35 °C) for 2-3 days. After incubation, the number of colonies grown in the media was counted on a Quebec colony counter.

Chromium and nickel resistant bacteria were screened by selecting the metal tolerant identical colonies from metal amended nutrient agar plate. The isolated colonies were grown in LB broth and these colonies were further characterized and employed for heavy metal tolerance studies.

2.10. IDENTIFICATION OF THE ISOLATED HEAVY METAL RESISTANT BACTERIAL STRAINS

Morphological, physiological, and biochemical characteristics of the isolated chromium and nickel tolerant bacterial species were performed according to Bergey’s Manual of Systematic Bacteriology (Holt et al., 2000).
2.11. ENUMERATION AND SCREENING OF HEAVY METAL RESISTANT FUNGAL SPECIES

Chromium and nickel resistant fungal strains were isolated from the soil samples using fungal medium potato dextrose broth (PDB). Approximately 10 g of soil sample was serially diluted water and 100 µl of suspension from 10^{-7} dilutions was spread on to the potato dextrose agar plates (standard spread plate technique). These medium were amended with different concentration of chromium (50, 100, 150, 200 and 250 mgL^{-1}) and nickel (25, 50, 75 and 100 mgL^{-1}) to isolate chromium and nickel resistant fungal species. The plates were incubated at room temperature (25 °C) for 4-6 days.

Metal resistant fungi were screened by selecting the tolerant fungi from metal amended potato dextrose agar plate. The isolated colonies were grown in PDB broth and these colonies were further characterized and employed for heavy metal tolerance studies.

2.12. IDENTIFICATION OF ISOLATED HEAVY METAL RESISTANT FUNGAL STRAINS

Based on the morphological characteristics, the isolated chromium and nickel tolerant fungal species were identified.

2.13. QUANTIFICATION OF CHROMIUM, NICKEL AND MICROBIAL BIOMASS

2.13.1 Estimation of chromium (APHA-AWWA-WEF, 1998)

Chromium concentrations were determined by 1,5 diphenylcarbazide method (APHA-AWWA-WEF, 1998) with potassium dichromate as standard. Different aliquots of standard was taken in the concentration range of 0.2 – 2 mg/ml. To this 3.5 ml of 6N H₂SO₄ and 1 ml of diphenyl carbazide reagent was
added to the solution and mixed thoroughly and allowed to stand for 10 min at room temperature. To 1 ml of the sample, the above reagents were added. The absorbance was read at 540 nm in a spectrophotometer. A standard graph was plotted against the concentration of chromium and absorbance. From the standard graph, the concentration of the sample can be calculated. The linear regression was found to be 0.998. The applicable concentration limit of chromium is 100–1000 μgL⁻¹ using diphenylcarbazide method. The reaction is very sensitive with the molar absorptivity based on chromium being about 40,000 L g⁻¹ cm⁻¹ at 540 nm.

2.13.2 Estimation of nickel

Nickel concentration was estimated using dimethylglyoxime (DMG) (Armit and Harden, 1906) with nickel sulfate as standard. Aliquots of standards were taken in the concentration range of 0.1 – 1 mg/ml. About 2 ml of dimethyl glyoxime reagent was added to the solution and mixed thoroughly. Reddish-pink color appeared upon addition of DMG to which, 1 ml of 1% KCL solution was added after 1 min. The solution was then transferred to separating funnel and 10 ml of chloroform was added. The solution was shaken thoroughly and the chloroform layer separated out. The absorbance was taken at 366 nm against a blank. To 1 ml of the sample, the above reagents were added. The absorbance was read at 366 nm using spectrophotometer. A standard graph was plotted against the concentration of nickel and absorbance. From the standard graph, the concentration of the nickel present in the sample was calculated. The linear regression was found to be 0.965. Estimation of nickel using DMG is a very sensitive method and it can estimate as low as 1/1000 mg.
2.13.3 Biomass quantification

Fungal and bacterial biomass was quantified by drawing 2.5 ml of broth culture from the fungal and bacterial medium and the absorbance was measured using spectrophotometer at 405 and 595 nm, respectively. Uninoculated growth medium was used as blank (Prasenjit and Sumathi, 2005).

2.14. BIOSORPTION STUDIES FOR CHROMIUM AND NICKEL REMOVAL USING THE ISOLATED METAL RESISTANT MICROORGANISMS

2.14.1 Optimization of pH for chromium removal by isolated bacterial species

The bacterial isolates were inoculated into a series of 250 ml conical flasks containing nutrient broth amended with 100 mgL\(^{-1}\) of chromium. The pH was varied from 3 to 11 (3, 5, 5.2, 7, 7.5, 9, and 11) using dilute HCl or NaOH. The cultures were shaken (120 rpm) at 35°C for 24 h. After 24 h incubation, the adsorbate were separated by centrifugation at 3,000 rpm for 15 min and the chromium concentration was determined by a spectrophotometrically (APHA, 1980). The initial and the final concentration of chromium used in batch mode studies were calculated by estimating the concentration of chromium spectrophotometrically. From the difference in concentration the removal efficiencies of the microorganism was calculated. Based upon the chromium removal and biomass data, the optimum pH was determined.

2.14.2 Optimization of pH for nickel removal by isolated bacterial species

The bacterial isolates were inoculated into a series of 250 ml conical flasks containing nutrient broth amended with 50 mgL\(^{-1}\) of nickel. The pH was varied from 3 to 11 using dilute HCl or NaOH. The experiment was carried as given in section 2.14.1. The nickel concentration were determined by a spectrophotometrically (Armit, and Harden, 1906). The initial and the final
concentration of nickel were estimated. From the difference in concentration the removal efficiencies of the microorganism was calculated. Based upon the nickel removal and biomass data, the optimum pH was determined.

2.14.3 Optimization of pH for chromium removal by isolated fungal species

The fungal isolates were inoculated into a series of 250 ml conical flasks containing potato dextrose broth amended with 100 mgL⁻¹ of chromium. The pH was varied from 3 to 11 using dilute HCl or NaOH. The experiment was carried out as given in section 2.14.1. Based upon the chromium removal and biomass data, the optimum pH was determined.

2.14.4 Optimization of pH for nickel removal by isolated fungal species

The fungal isolates were inoculated into a series of 250 ml conical flasks containing potato dextrose broth amended with 50 mgL⁻¹ of nickel. The pH was varied from 3 to 11 using dilute HCl or NaOH. The experiment was carried out as given in section 2.14.1. Based upon the nickel removal and biomass data, the optimum pH was determined.

2.14.5 Optimization of temperature for chromium removal by isolated bacterial species

The bacterial isolates were inoculated into a series of 250 ml conical flasks containing nutrient broth amended with 100 mgL⁻¹ of chromium. The temperature was varied from 29 to 36°C (29, 30, 31, 32, 33, 34, 35, and 36°C). The experiment was carried out as given in section 2.14.1. The chromium concentration was determined spectrophotometrically (APHA-AWWA-WEF, 1998). Based upon the chromium removal and biomass data, the optimum temperature was determined.
2.14.6 Optimization of temperature for nickel removal by isolated bacterial species

The bacterial isolates were inoculated into a series of 250 ml conical flasks containing nutrient broth amended with 50 mgL\(^{-1}\) of nickel. The temperature was varied from 29 to 36°C (29, 30, 31, 32, 33, 34, 35, and 36°C). The experiment was carried as given in section 2.14.1. The nickel concentration was determined spectrophotometrically (Armit and Harden, 1906). Based upon the nickel removal and biomass data, the optimum temperature was determined.

2.14.7 Optimization of temperature for chromium removal by isolated fungal species

The fungal isolates were inoculated into a series of 250 ml conical flasks containing potato dextrose broth amended with 100 mgL\(^{-1}\) of chromium. Temperature was varied from 29 to 36°C (29, 30, 31, 32, 33, 34, 35, and 36°C). The experiment was carried out as given in section 2.14.1. The chromium concentration was determined spectrophotometrically (APHA-AWWA-WEF, 1998). Based upon the nickel removal and biomass data, the optimum temperature was determined.

2.14.8 Optimization of temperature for nickel removal by isolated fungal species

The fungal isolates were inoculated into a series of 250 ml conical flasks containing potato dextrose broth amended with 50 mgL\(^{-1}\) of nickel. The temperature was varied from 29 to 36°C (29, 30, 31, 32, 33, 34, 35, and 36°C). The experiment was carried as given in section 2.14.1. Chromium concentrations was determined spectrophotometrically (Armit and Harden, 1906). Based upon the nickel removal and biomass data, the optimum temperature was determined.
2.14.9 Kinetics of chromium removal and cellular growth by isolated bacterial species

The bacterial isolates were inoculated into a series of 250 ml conical flasks containing nutrient broth amended with 100 mgL$^{-1}$ of chromium. The cultures were shaken in a rotary shaker (120 rpm) at pH 7 and 35°C. During the incubation period, chromium concentration and biomass were monitored for every two hours interval until chromium removal attains a saturation level. The chromium concentration was estimated as before (APHA-AWWA-WEF, 1998).

2.14.10 Kinetics of nickel removal and cellular growth by isolated bacterial species

The bacterial isolates were inoculated into a series of 250 ml conical flasks containing nutrient broth amended with 50 mgL$^{-1}$ of nickel. The cultures were shaken in a rotary shaker (120 rpm) at pH 7 and 35°C. During the incubation period, nickel concentration and biomass were monitored for every two hours interval until nickel removal attains a saturation level. The nickel concentration was determined spectrophotometrically (Armit and Harden, 1906).

2.14.11 Kinetics of chromium removal and cellular growth by isolated fungal species

The fungal isolates were inoculated into a series of 250 ml conical flasks containing potato dextrose broth amended with 100 mgL$^{-1}$ of chromium. The cultures were shaken in a rotary shaker (120 rpm) at pH 5 and 35°C. During the incubation period, chromium concentration and biomass were monitored for every two hours interval until chromium removal attains a saturation level. The chromium concentration was determined by a spectrophotometrically (APHA-AWWA-WEF, 1998).
2.14.12 Kinetics of nickel removal and cellular growth by isolated fungal species

The fungal isolates were inoculated into a series of 250 ml conical flasks containing potato dextrose broth amended with 50 mgL$^{-1}$ of nickel. The cultures were shaken in a rotary shaker (120 rpm) at pH 5 and 35°C. During the incubation period, nickel concentration and biomass were monitored for every two hours interval until nickel removal attains a saturation level. The nickel concentration was determined spectrophotometrically (Armit and Harden, 1906).

2.15. TOLERANCE IN RESPONSE TO WIDELY VARYING HEAVY METAL CONCENTRATION

2.15.1 Chromium tolerance response by isolated bacterial and fungal species

Fungal and bacterial isolates were added into a 250 ml flask containing potato dextrose broth and nutrient broth was amended with chromium using potassium dichromate salt (chromium concentrations ranging from 100 to 10,000 mgL$^{-1}$). The cultures were shaken in a rotary shaker (120 rpm) in a temperature controlled water bath at pH 7 and 35°C for 24 h. After 24 h incubation, the biomass was measured. The extent of tolerance was compared and the “normalized” biomass was calculated, i.e., biomass at each heavy metal concentration per biomass using a control. Bacterial and fungal biomasses were quantified by spectrophotometer at 595 and 405 nm, respectively.
2.15.2 Nickel tolerance response by isolated bacterial and fungal species

Fungal and bacterial isolates were added into a 250 ml flask containing potato dextrose broth and nutrient broth was amended with nickel using nickel sulphate (nickel concentrations between 50 and 500 mgL\(^{-1}\)). The cultures were shaken in a rotary shaker (120 rpm) at pH 5 and 35°C for 24 h. After 24 h incubation, the biomass was measured. The extent of tolerance was compared and the “normalized” biomass was calculated, i.e., biomass at each heavy metal concentration per biomass using a control. Bacterial and fungal biomasses were quantified by spectrophotometer at 595 and 405 nm.

2.16. PROTEOMIC EXPRESSION ASSAYS IN HEAVY METAL-RESISTANT MICROORGANISMS

Proteomes from the bacterial and fungal isolates were extracted and purified using microbial lyses method. Cell samples were taken from mid-log phases of cellular growth (at optical density of 0.3–0.4) under the conditions of the experiments to measure the kinetics of cellular growth and heavy metal removal. The isolated proteins were quantified by Bradford’s method with bovine serum albumin as standard (Bradford, 1976). To 10 \(\mu\)l of the protein extract, distilled water was added to make up the volume to 100 \(\mu\)l. About 2.5ml of Bradford’s reagent was added and mixed thoroughly and allowed to stand for 5 min. The absorbance was read at 595nm in a spectrophotometer.

Proteomic expression was performed by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) for the samples at room temperature (Laemmli, 1970). A 10% linear resolving gel and 2% stacking gel were used to separate the proteins. A sandwich was made with two glass plates separated by spacer strips (1.5 mm thickness). The resolving gel mix was poured into the space between the glass plates. A stream of distilled water was layered
Materials and Methods

on to the gel to exclude oxygen from inhibiting polymerization and to ensure a uniform flat gel surface. Presence of a sharp interface between the polymerized gel and the overlay was an indication of complete polymerization. After decanting the water layered on the surface, 2% stacking gel was prepared and poured over the separating gel and Teflon comb (1.5 mm thickness) was inserted to form wells. Care was taken not to trap any air bubbles while casting the gel. Teflon comb was removed, after polymerization. The basal strip was removed and the glass plates with polymerized gel were fixed to the electrophoretic apparatus. Protein samples were mixed with equal volumes of sample loading buffer and kept in boiling water bath for a few seconds and then loaded on to each well.

The resolving gel was prepared as follows:

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Resolving gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide solution (30%)</td>
<td>2.1 ml</td>
</tr>
<tr>
<td>Lower Tris (pH 8.8)</td>
<td>1.6 ml</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>2.6 ml</td>
</tr>
<tr>
<td>APS</td>
<td>40 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

The stacking gel was prepared as follows

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Resolving gel (3%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide solution (30%)</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>Lower Tris (pH 8.8)</td>
<td>0.45 ml</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>0.72 ml</td>
</tr>
<tr>
<td>APS</td>
<td>14 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>4 μl</td>
</tr>
</tbody>
</table>

Electrophoresis was carried out in tank buffer for 3 h at a constant voltage of 30V in the region of stacking gel and 90V in the region of resolving gel. The
Materials and Methods

gels were stained with coomassie blue R-250 for over night. The gels were then
destained with destaining solution for 3 h.

2.17. EXTRACTION OF GENOMIC DNA FROM THE SOIL AND METAL RESISTANT MICROORGANISMS

2.17.1 Extraction of genomic DNA from the contaminated soil

Isolation of genomic DNA from soil was carried out by Ultra Clean™ (MoBio) Kit. The soil samples were dried to remove the moisture content and it was sieved to form a uniform structure. Approximately 1 g of soil sample was weighed and added to the 2 ml bead solution and vortexed for 30 sec in horizontal position and 75 μl of S1 solution (supplied in the kit) and 200 μl of IRS solution were added. The tubes containing the mixture were secured horizontally on a flat bed vortex pad and made to vortex for 12 min at 5,000 rpm and centrifuged at 10000 × g for 30 sec. The supernatant was transferred to a clean microcentrifuge tube and 250 μl of S2 solution (supplied in the kit) was added and vortexed for 10 sec followed by incubation at 4°C for 3 min. The tubes were centrifuged for 1 min at 10,000 x g and 450 μl of supernatant was transferred to a clean microcentrifuge tube containing 1300 μl of solution S3 (supplied in the kit) and vortexed for 5 sec. Approximately 700 μl of supernatant was transferred onto a spin filter and centrifuged at 10,000 x g for 1 min. Flow through was discarded and the remaining supernatant was added to the spin filter, and centrifuged at 10,000 x g for 1 min. It was repeated until all supernatant has passed through the spin filter (a total of 3 loads were performed). To this solution 300 μl of solution S4 (supplied in the kit) was added and centrifuged for 30 sec at 10,000 x g. The flow through was discarded and the tubes were air dried. Spin filter was placed on a new clean tube and approximately 30 μl of sterile water was added in the center of the membrane filter. The Spin filters were kept as such for 1 min, followed by centrifugation at
10,000 x g for 2 min. The spin filter was discarded and the DNA was collected and stored at – 20°C for further analysis.

2.17.2 Extraction of genomic DNA from chromium and nickel resistant bacterial species

Bacterial isolates were added into a 250 ml flasks containing LB broth amended with 100 mgL⁻¹ of chromium and 50 mgL⁻¹ of nickel. The cultures were shaken in a rotary shaker (120 rpm) at pH 7 and 35°C for 24 h. Bacterial genomic DNA was isolated using ChargeSwitch® gDNA Mini Bacterial Kit, California, U.S (Parrilli et al., 2008). Approximately, 0.75 ml of over night grown pure culture was taken and centrifuged to form pellet at 14,000 rpm, the supernatant was discarded and the pellet was retained. To the pellet, 100 µl of resuspension buffer (R4 - supplied in the kit) which contains RNase A followed by 5 µl of lysozyme solution with a concentration of 50 mg/ml was added. The samples were mixed by pipetting up and down gently several times to ensure that the cells are evenly distributed. The samples were incubated for 10 min at 37°C and 500 µl of lysis buffer (L14 - supplied in the kit) which contains 10 µl of premixed Proteinase K was added and mixed thoroughly. It was kept at room temperature for 2 min and centrifuged at 12,000 rpm for 15 min. The supernatant was discarded and the pellet was dissolved in 20 µl of TE buffer. Isolated DNA was stored at – 20°C for further analysis.
2.17.3 Extraction of genomic DNA from chromium and nickel resistant fungal species

Fungal isolates were plated into a potato dextrose agar amended with 100 mgL⁻¹ of chromium and 50 mgL⁻¹ of nickel. The plates were incubated at 35°C for 48 h. Fungal genomic DNA was isolated using the Glass bead procedure (De Maeseneire et al., 2007). To 500 μl of sterile milliQ water, freshly grown individual strains of fungal colonies were taken and centrifuged at ~10000 x g. To the pellet, 700 μl of buffer (200 mM Tris pH 7.5, 10 mM EDTA, 0.5 M NaCl and 1% SDS) was added and the pellets were dissolved. To this 40 mg glass beads was added followed by addition of 0.2 ml phenol, chloroform solution (1:1). Vortexed the tube for 6 min and centrifuged for 1 min at 10000 x g. The supernatant was transferred to a clean centrifuge tube and 300 μl of buffer solution and 300 μl phenol - chloroform solution was added. The mixture was centrifuged at 10000 x g for 30 sec. The upper liquid phase (~ 550 μL) was transferred to sterile centrifuge tube and 500 μl of 95% ethanol was added. The tubes were inverted and incubated at -20°C for 30 min and centrifuged for 10 min at 10000 x g. The supernatant was discarded without disturbing the pellet. The pellets were washed with 500 μl of 70% ethanol and air dried. The pellets were dissolved in 50 μl of sterile milli Q water and stored at – 20°C.

2.17.4 Extraction of genomic DNA for MCH-PCR

The samples were incubated for 10 min at 55°C. To the sample, 40 μl of evenly distributed ChargeSwith® magnetic beads (Figure 1) were added and mixed well and ensured that was no formation of air bubbles. Binding buffer (B8 - supplied in the kit) (300 μl) was added and vortexed for 2 to 3 seconds. The samples were incubated at room temperature for 1 min and placed on a MagnaRack™ (Magnetic rack) (Parrilli, et al., 2008) for 3 to 5 min. The supernatant was discarded without disturbing the pellets of beads. The
eppendorff tubes were removed from the magnetic rack and 1000 μl of wash buffer (W12 - supplied in the kit) was added to the tube and tapped up and down gently for 3 to 5 times to avoid the formation of air bubbles. The eppendorff tubes containing the mixed solutions were placed on the magnetic rack and beads were concentrated for 3 to 5 min and the supernatant was discarded without disturbing the magnetic bead cluster. The tubes were removed from the magnetic rack and 1000 μl of wash buffer (W12 - supplied in the kit) was added and mixed gently. The tubes were placed on the magnetic rack and the magnetic beads were concentrated and the supernatant was discarded. The tube containing the magnetic bead from the magnetic rack was removed and 200 μl of elution buffer (E5 - supplied in the kit) was added. The tubes were incubated for 5 min at 55 to 65°C and placed in the magnetic rack for 5 min or until the beads formed a tight pellet. The supernatant containing the extracted DNA was transferred to a sterile microcentrifuge tube. The purified DNA was stored at -20°C for further analysis.
2.18. AGAROSE GEL ELECTROPHORESIS

1% agarose gels were prepared in 1X TAE buffer containing 6 µl of ethidium bromide dye. The samples were run for 1 h at 100 V (Bio rad) and visualized for the presence of DNA under UV illumination. Along with sample 1kb ladder was used as marker.

2.19. PURIFICATION OF THE TARGET DNA

The genomic DNA was separated in agarose gel electrophoresis was sliced from the gel using a sharp individual blades with the help of ultra violet transilluminator and transformed into centrifuge tubes. Tubes were weighed and 3 volumes of agarose lysis buffer (supplied in the MEGA – spin™ agarose gel
extraction kit) (Sinigalliano et al., 1995) to 1 volume of the corresponding gel (≈ 300 μl per 100 mg of agarose gel) was added. The tubes were incubated for 10 min at 55°C until the gel slice has completely dissolved. To this, 1 volume of isopropanol was added and mixed well. About 800 μl of mixture was transferred to the collection tube and centrifuged for 1 min at 13,000 rpm and flow through was discarded. To the filter tube, 700 μl of washing buffer was added and centrifuged for 1 min at 13,000 rpm. The flow through was discarded and centrifuged at 13,000 rpm for 1 min to remove all the washing buffer solution. The filter tube was transferred to a sterile centrifuge tube and added 30 μl of sterile water at the center of the filter column and centrifuged at 13,000 rpm for 2 min.

2.20. QUANTIFICATION OF TARGET DNA BY NANODROP –UV METHOD

About 1 μl of the DNA sample was used for the estimation by using Nano drop (ND-1000) at 260 nm (De Maeseneire et al., 2007). Sterile milli Q water served as a blank.

2.21. PCR AMPLIFICATION FOR THE EXTRACTED DNA SAMPLES

2.21.1 PCR amplification for 16S rRNA

16S rRNA were amplified using universal 16S rRNA primers (Table 1) 11F, 27F and 1492R (Cosmo gene tech) (Soojeung et al., 2008). PCR reactions were performed in a 25 μl volume containing 0.1 ng of template DNA, 1X PCR buffer, 2 mM MgCl2, 200 μM concentration of each deoxy nucleoside triphosphates (dNTPs), 100 pmole concentration of each primer and 0.025 U of Taq DNA polymerase enzyme. The final volume was adjusted with sterile Milli-Q water. A DNA thermocycler (Bio-rad) was used to amplify the reactions through an initial denaturation step consisting of 94°C for 3 min and followed by
25 cycles at 94°C for 1 min, 50°C for 1 min and with an extension of 72°C for 1 min followed by an final extension temperature at 72°C for 2 min. This annealing temperature was selected as this consistency yielded a single PCR product of the expected (~ 1400bp) size. The amplified PCR products were stored at -20°C for further purification and down stream applications.

**TABLE 1**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Target group</th>
<th>Primer Sequence 5' – 3'</th>
<th>Amplification target</th>
<th>Purpose</th>
<th>mer (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>27F</td>
<td>16S rRNA</td>
<td>AGAGTTTGATCATGGGCTCAG</td>
<td>1492–1513</td>
<td>Cloning</td>
<td>20</td>
<td>Laemmli, 1970</td>
</tr>
<tr>
<td>1492R</td>
<td>16S rRNA</td>
<td>TACGGTTACCTTGTTACGACTT</td>
<td>1492–1513</td>
<td>Cloning/T-RFLP/MCR</td>
<td>22</td>
<td>Laemmli, 1970</td>
</tr>
<tr>
<td>11f</td>
<td>16S rRNA</td>
<td>GTTTGATCTGGCTCAG</td>
<td>1492–1513</td>
<td>Cloning</td>
<td>17</td>
<td>Kane et al., 1993</td>
</tr>
<tr>
<td>*27F- FAM</td>
<td>16S rRNA</td>
<td>AGAGTTTGATCATGGGCTCAG</td>
<td>1492–1513</td>
<td>T-RFLP</td>
<td>20</td>
<td>Laemmli, 1970</td>
</tr>
<tr>
<td>*27F- Biotin</td>
<td>16S rRNA</td>
<td>AGAGTTTGATCATGGGCTCAG</td>
<td>1492–1513</td>
<td>MCH/Cloning</td>
<td>20</td>
<td>Laemmli, 1970</td>
</tr>
<tr>
<td>ITS4R</td>
<td>ITS between 5S &amp; 18S</td>
<td>TCCTCCGCTTATTGATATGC</td>
<td>450–750</td>
<td>MCH/Cloning/T-RFLP</td>
<td>20</td>
<td>Anderson et al., 2003</td>
</tr>
<tr>
<td>*LROR-FAM</td>
<td>18S rRNA</td>
<td>ACCCGCTGAACCTAAGC</td>
<td>550</td>
<td>T-RFLP</td>
<td>17</td>
<td>Moriya et al., 2005</td>
</tr>
<tr>
<td>LR5</td>
<td>18S rRNA</td>
<td>TCCTGAGGGAAACTTCG</td>
<td>550</td>
<td>Cloning/T-RFLP/MCR</td>
<td>17</td>
<td>Moriya et al., 2005</td>
</tr>
</tbody>
</table>

**2.21.2 PCR amplification for 18S rRNA**

18S rRNA were PCR amplified using the universal fungal primers, EF3 and EF4 (Cosmo gene tech) (Soojeung et al., 2008). PCR reactions were performed in a 25 μl volume containing 0.3 ng template DNA, 10 X PCR buffer, 50 mM MgCl₂, 100 mM of each deoxy nucleoside triphosphates (dNTPs), 1000 pmole of each forward and reverse primers, 5U of Taq DNA polymerase and the final volume was made up to with sterile Milli-Q water. A DNA thermocycler (Bio-rad) was used to incubate the reaction mix using the
following thermocycle condition. The PCR amplification profile starts with an initial denaturation for 2 min at 94°C followed by 35 cycles of 94°C for 30 sec, annealing temperature at 45°C for 30 sec, 72°C for 2 min, followed by an final extension for 10 min at 72°C. The amplified products were stored at -20°C for further purification and down stream application.

2.22. PURIFICATION OF THE AMPLIFIED PCR PRODUCTS

Purification of amplified PCR products were done using PCR quick-spin™ PCR product purification kit. Column based method was used to purify the target DNA. To the amplified (25 μl) PCR product, 500 μl of binding buffer was mixed followed by addition of 150 μl of isopropanol. The mixture was mixed well by pipetting and allowed to stand for 1 min. The mixture was added to an spin column and centrifuged for 1 min at 13,000 rpm. The flow through was discarded and the centrifugation was repeated and the column was retained. To this 700 μl of washing buffer was added and centrifuged at 13,000 rpm for 1 min. The washing step was repeated with 500 μl of washing buffer to remove the remaining salts. The flow through was discarded and centrifuged for 1 min at 13,000 rpm to dry and remove the remaining ethanol in the washing buffer. The column was transferred to a sterile centrifuge tube and 30 μl of elution buffer was added in the center of the column and kept for 30 seconds. The column was spinned for 2 min at 13,000 rpm. The purified DNA was stored at -20°C.

2.23. ETHANOL CONCENTRATION OF PURIFIED PCR PRODUCTS

To the 30 μl of purified DNA products, 2 volumes of 100% absolute ethanol was added and incubated at 4°C for 10 min. The tube was vortexed for 5 sec and centrifuged at 14,000 rpm for 10 min. Supernatant was discarded and air
dried. To this, 10 ~ 15 µl of sterile distilled water was added and stored at 4°C (Soojeung et al., 2008).

2.24. TERMINAL RESTRICTION LENGTH POLYMORPHISM (TRFLP) ANALYSIS

2.24.1 16S rRNA Polymerase chain reaction for TRFLP analysis

16S rRNA were amplified using universal 16S rRNA primers (Table 1) labeled forward FAM-27F and unlabelled reverse 1492R (Cosmogen) (Konstantinidis, et al, 2003). PCR reactions were performed in a 25 µl volume containing 0.1ng of template DNA, 1X PCR buffer, 2 mM MgCl₂, 200 µM concentration of each deoxy nucleoside triphosphates (dNTPs), 50 pmol concentration of each primer and 0.100 U of Taq enzyme. A DNA thermocycler (Bio-rad) was used to amplify as given in section 2.21.1.. The PCR products were purified using Qiagen spin columns (Qiagen Inc., Valencia, CA) as discussed in section 2.22. The purified PCR products were stored at -20°C for further purification and down stream applications.

2.24.2 18S rRNA Polymerase chain reaction for TRFLP analysis

18S rRNA were amplified using the universal primers (Table 1) FAM milliQ - EF3 and EF4 (Cosmo gene tech) (Borneman and Jack Hartin, 2000). PCR reactions were performed in a 25 µl volume containing 0.3 ng template DNA, 1X PCR buffer, 50 mM MgCl₂, 100 mM of each deoxy nucleoside triphosphates (dNTPs), 1000 pmole of each forward and reverse primers and 5U of Taq DNA polymerase. The final volume was adjusted with milli-Q water. A DNA thermocycler (Bio-rad) was used to amplify as given in reaction denaturation for 2 min at 94°C followed by 35 cycles at 94°C for 30 sec, annealing temperature at 45°C for 30 sec, 72°C for 2 min, followed by a final extension for 10 min at 72°C. The PCR products were purified using Qiagen spin
Materials and Methods

columns (Qiagen Inc., Valencia, CA) as discussed in section 20. The purified PCR products were stored at -20°C for further purification and downstream applications.

2.24.3 Enzyme digestion for T-RFLP analysis

Approximately ~ 40 ng of purified 16S rRNA and 18S rRNA PCR products were digested with Hha I for 12 h at 37°C (Konstantinidis et al., 2003) in a 20 µl reaction mixture which contained 2 µl of 10 x Tango enzyme buffer, 1 µl of restriction enzyme and 5 µl of amplified template DNA. The final volume was made up to 20 µl and using sterile milli-Q water. Mixture was kept at 65°C for 5 minutes and stored at -20°C for further analysis. The digested products were tested for completeness with a pure culture control (Micrococcus luteus ATCC-4698, Pseudomonas – LB 400 for 16S rRNA and Aspergillus niger van Tieghem, anamorph ATCC-9142, Fusarium oxysporum for 18S rRNA). The digested samples were sent to NICEM, Seoul National University (http://www.nicem.snu.ac.kr/) (Soojeung, et al., 2008) for sequence analysis. Terminal fragments smaller than 50 bases or larger than 600 bases were deleted from analysis, the former because of sizing inaccuracies for such large fragments. Finally, a level of 50 fluorescence units was imposed as a minimum threshold value for all peaks in the selected size range. Profiles were visually inspected and aligned based on relative peak distribution. For each enzyme digestion, duplicates were run as a means of confirming the reproducibility of the method.
2.25. PCR AMPLIFICATION USING MAGNETIC BEAD CAPTURE HYBRIDIZATION TECHNIQUE

2.25.1 Bead preparation and primer hybridization

The preparation of beads were performed under the 15 ~ 20°C room temperature (Langrell and Barbara, 2001). 2.5 μl of Dynal beads one™ streptavidin C1 (10mg/ml) were transferred from the stock to a sterile PCR centrifuge tube containing 22.5 μl of 2X binding and wash buffer placed in a Dynal magnetic rack. Care was taken to avoid the contact of magnetic beads to the tip of the pipette. Beads were washed twice with 2X binding and wash buffer. The magnetic beads were stored in 25 μl 2X binding and wash buffer (100 μg/μl). To this, 25 μl of single biotin labeled corresponding primer was added. The final primer concentration of 100 pmole in 50 μl was prepared. The mixture was incubated at room temperature in a table rocker with slow agitation for 15 ~ 30 min. The primer used for the bead preparation should be below 30 bp. The beads were washed twice with 1X binding and wash buffer. Resuspended the beads in 25 μl of sterile milli-Q water and final concentration of bead was 100 μg/μl and for biotin labeled primer was 100 pmole. The beads were stored at 4°C for downstream processing (Figure 2).
2.25.2 MCH-PCR for 16S rRNA

16S rRNA was amplified using universal 16S rRNA primers (Table 1), biotin labeled 27F and 1492R (Cosmo gene tech) (Jacobsen, 1995). The primers were hybridized with the streptavidin coated magnetic bead and used for PCR reaction. PCR reactions were performed in a 25 µl volume containing 0.1 ng of template DNA, 1X PCR buffer, 2 mM MgCl₂, 200 µM concentration of each deoxy nucleoside triphosphates (dNTPs), 100 pmol concentration of 1492 R primer, 5U of Taq DNA polymerase and 2.5 µl of magnetic bead containing the 27F primer. The final volume was adjusted with sterile milli-Q water. A DNA thermocycler (Bio-rad) was used to amplify the reaction mixture through an initial denaturation step consisting of 94°C for 3 min and followed by 25 cycles of 94°C for 1 min, 50°C for 1 min and with an extension of 72°C for 1 min.
followed by an final extension temperature at 72°C for 2 min. The PCR products were stored at -20°C for further purification and downstream applications.

### 2.25.3 MCH-PCR for 18S rRNA

18S rRNA were amplified using universal 18S rRNA primers (Table 1), biotin labeled ITS1F and ITS4R (Cosmo gene tech) (Table 1) (Jacobsen, 1995). PCR reactions were performed in a 25 μl volume containing 0.3 ng template DNA, 10 X PCR buffer, 50 mM MgCl₂, 100 mM of each deoxy nucleoside triphosphates (dNTPs), 1000 pmol of EF4 reverse primers, 5U of Taq DNA polymerase and 2.5 μl of magnetic bead containing the EF3 forward primer. The final volume was adjusted with sterile milli-Q water. A DNA thermocycler (Bio-rad) was used to incubate the reaction mix using the following thermocycle conditions. The PCR amplification starts with an initial denaturation for 2 min at 94°C followed by 35 cycles of 94°C for 30 sec, annealing temperature at 45°C for 30 sec, 72°C for 2 min, followed by a final extension for 10 min at 72°C. The amplified products were stored at -20°C for further purification and downstream application.

### 2.25.4 Purification of MCH-PCR amplified products

The PCR products contained a biotin end coupled with streptavidin coated magnetic bead. The separation of the streptavidin and the biotin complex were carried. The amplified bead mixture was incubated at 65°C for 5 min using DNA thermocycler. The DNA and beads were separated using the Dynal magnetic rack. The extracted DNA was further purified using PCR quick-spin™ PCR product purification kit (column based method). To the amplified (25 μl) PCR product, 500 μl of binding buffer was added followed by addition of 150 μl of isopropanol and allowed to stand for 1 min. The mixture was added to spin
Materials and Methods

column and centrifuged for 1 min at 13,000 rpm. The flow through was
discarded and the column was retained. To the column, 700 μl of washing
buffer was added and centrifuged at 13,000 rpm for 1 min. The washing step was
repeated with 500 μl of washing buffer to remove the remaining salts. The flow
through was discarded and centrifuged for 1 min at 13,000 rpm and air dried.
The column was transferred to a sterile centrifuge tube and 30 μl of elution
buffer was added in the center of the column and kept for 30 sec. The purified
DNA was stored at -20°C for further use.

2.26. CLONING OF PCR PRODUCTS

The purified products of 16S rRNA and 18S rRNA were cloned into
TOPO – TA cloning vector (Figure 3). The cloning reaction for 16S rRNA and
18S rRNA were carried out in a separate reaction mixture and performed in 3
steps

a) Preparation of reaction mixture

b) Transformation of DNA into competent cell

c) Screening of transformed cells

FIGURE 3
Vector used in cloning

![Vector diagram](image-url)

pCR®4-TOPO®
3956 bp

49
a) **Preparation of reaction mixture**

In a sterile 100 µl PCR centrifuge tube, a total volume of 6 µl reaction mixture was prepared by adding 1 µl of salt solution, 2 µl of sterile milli Q water, 2 µl of target DNA and 1 µl of Topo Cloning vector supplied in the TOPO TA cloning kit. The reaction mixture was incubated at 22 ~ 23°C for 15 min and stored at 4°C.

b) **Transformation of DNA into competent cell**

To the competent cell supplied in the TOPO TA cloning kit, 3 µl of the reaction mixture was added. The competent cell containing the reaction mixture was incubated in ice for 15 min and heat shock treatment was given to the cells for 30 sec at 42°C with out shaking and kept in ice for 5 min. To this mixture, 250 µl of prewarmed (37°C) SOC medium was added. The tube was incubated at 37°C for 6 h at 200 rpm in a incubator shaker.

c) **Screening of transformed cells**

Kanamycin LB plates (50 µg /ml) were prepared and the plates were warmed at 37°C. To the antibiotic plates, 10 µl of the incubated transformed cells with 90 µl of SOC medium was spreaded evenly. The step was repeated with 20 µl and 30 µl of the transformed cells with 80 µl and 70 µl of SOC medium, respectively. The plates were incubated at 37°C for 24 h. The individual colonies were picked using a sterile toothpick and were transformed into a 96 well rack containing 800 µl of Kanamycin LB freezing buffer medium. The 96 well plate was incubated over night at 37°C at 200 rpm in a incubator shaker.
2.27. SEQUENCING ANALYSIS

The clones in the 96 well plates were sent to the Macrogen sequencing company. The plasmid DNA from each transformant was prepared by a modified alkaline lysis method. Nucleotide sequences of the clone inserts were determined by cycle sequencing using BigDye Terminator (Applied Biosystems) and 3.2 pM M13F (59-GTAAAACGACGGCCAG-39) and M13R (59-CAGGAAACAGCTATGAC-39) sequencing primers. Sequences were analysed on ABI 3730xl sequencers (Applied Biosystems). The vector sequences were removed and adjusted for quality values and the mean single-sequence read length was ~ 700 nt. Bidirectional sequence reads of clone inserts provided near full-length 16S bacterial and 18S fungal sequences, which were used for phylogenetic analysis.

2.28. PHYLOGENETIC ANALYSIS

Identification of 16S rRNA and 18S rRNA was done by phylogenetic analysis. Sequences with ≥97% similarity were assembled and the individual assemblies were analysed by comparison with known phylotypes via the Sequence Match Program of the Ribosomal Database Project II (RDP-II; version 3 release 9.38) (Cole et al., 2003). The relationships of the cloned assemblies to known phylotypes were expressed with S-ab scores in which the number of unique oligomers shared between the query sequence and the sequence in the RDP-II database, divided by the lowest number of unique oligomers in either of the two sequences. The top 20 hierarchical matches for each assembly consensus sequence were examined, and the sequence was designated the phylotype in the RDP-II database with the highest S-ab score. In the two cases in which the assembly sequences matched more than one phylotype with the same S-ab score, data are presented as the type species. Phylogenetic identification was verified to
the genus level using the RDP-II database classifier program, which uses a naive Bayesian rRNA classifier (version 1.0) to assign a sequence to a taxonomical hierarchy (Holt et al., 2000). The aligned sequences were then checked for gaps manually, arranged in a block of 250 bp in each row and saved as molecular evolutionary genetics analysis (MEGA) format in software MEGA V2.1. The pair wise evolutionary distances were computed using the Kimura 2-parameter model (Kimura, 1980). To obtain the confidence values the original data set was resampled 1000 times using the bootstrap analysis method. The bootstrapped data set was used directly for constructing the phylogenetic tree using the MEGA program for calculating the multiple distance matrixes. The multiple distance matrix obtained was then used to construct phylogenetic trees using neighbour-joining (NJ) method (Saitou and Nei, 1987). All these analysis were performed using the MEGA V 2.1 (Kumar et al., 2001).