3. Materials and Methods

3.1 Reagents

3.1.1 Chemicals

Amersham Pharmacia (USA): Low melting agarose and Restriction enzymes.

B. genei Bangalore (INDIA): Taq polymerase, dNTP mix, DNA ladders, Restriction enzymes.

HiMedia, Pvt. limited Mumbai (INDIA): casein enzyme hydrolysate, agar agar, glucose, glycerol, NaNO₃, NaOH, potassium acetate, sodium acetate, sodium chloride, sodium hydroxide, sodium dodecyl sulphate, Tween-20, Tween-80, yeast extract, Bromophenol blue, Proteinase K, EDTA, Dimethylformamide, Potassium iodide, Iodine, Agarose and Tween 20.

MBI Fermentas (USA): T4 DNA ligase and Calf intestinal alkaline phosphatase. 3 kb DNA Ladder, 10 kb DNA Ladder, ATP, dNTPs, HindIII digested λ DNA ladder, MgCl₂, restriction enzymes.

Qiagen: QIAEX II gel extraction kit, Qiagen plasmid isolation kit

Mobio (USA): Soil DNA isolation kit, PCR purification kit.

Sigma chemical company (USA): X-Gal, IPTG, Tris base, SDS, Ethidium bromide, Ampicillin, Streptomycin, Tetracycline, Acrylamide, Bisacrylamide, TEMED, Ammonium per sulphate.

Molecular BioProducts (San Diego, CA, USA)
0.2-cm gap electroporation cuvette.

Rankem (ND, India)
Benzyl alcohol, chloroform, glacial acetic acid, hydrochloric acid, isoamyl alcohol, isopropanol, methanol and phenol.
Materials and Methods

Sigma Chemicals Co. (St. Louis, MO)
agarose, bovine serum albumin, ampicillin, tetracycline, DMSO, DNA gel loading
dye (5X), ethidium bromide, ethylene diamine tetra acetic acid, low melting point
agarose, lysozyme, sodium bicarbonate and sodium carbonate,

Tarson (Kolkata, INDIA)
1.5 ml microcentrifuge tubes, 50 ml centrifuge tubes, ELISA plates, glass petri plates,
microtips, PCR tubes and sterilized disposable petri plates.

3.1.2 Strains, plasmids and primers.

Table 3.1: Lists all the strains, plasmids and primers used in this study

<table>
<thead>
<tr>
<th>Strains/vectors/primers</th>
<th>Relevant characteristics</th>
<th>Sources /References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM110</td>
<td>damdcm supE44 hsdl7 thi leu rpsL lacY galK galT aratonA thr tsx Δ(lac-proAB) F' [traD36 proAB lacZ ΔM15]</td>
<td>STRATAGENE</td>
</tr>
<tr>
<td>SURE</td>
<td>endA1 glnV44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5 uvrC c14- Δ(mcrCB-hsdSMR-mrr)171 F' [proAB lacZ ΔM15 Tn10] uncertain status of TraD36 in F plasmid increased stability for inverted repeats and Z-DNA nalidixic acid resistant kanamycin resistant tetracycline resistant.</td>
<td>STRATAGENE</td>
</tr>
<tr>
<td>Vectors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>E.coli plasmid, 2686 bp in length. The pMB1 replicon rep responsible for the replication of plasmid high copy number bla gene, coding for beta-lactamase that confers resistance to ampicillin region of E.coli operon lac containing CAP protein binding site, promoter P lac, lac repressor binding site and 5'-terminal part of the lacZ gene encoding the N-terminal fragment of beta-galactosidase.</td>
<td>FERMENTAS</td>
</tr>
<tr>
<td>pGEMT-easy</td>
<td>Size 3015 bp, T7 promoter, SP6 promoter, Ampr, lac operator, LacZ start codon, phage fl region, pUC M13 priming sites, 3' – T overhangs.</td>
<td>PROMEGA</td>
</tr>
<tr>
<td>SuperCos1</td>
<td>Size 7.9 kb, T3 promoter, BamH I cloning site T7 promoter, cos recognition sequence 1, cos site 1, Xba I</td>
<td>STRATAGENE</td>
</tr>
</tbody>
</table>
Materials and Methods

### 3.2 Culture Media

#### 3.2.1 LB broth (per litre)

- **Casein enzyme hydrolysate**: 10 gm
- **Yeast extract**: 5 gm
- **Sodium chloride**: 5 gm

pH was adjusted to 7.4 by using NaOH and autoclaved at 15 lbs/sq.inch at 120°C for 15 min.

#### 3.2.2 LB agar (per litre)

- **Casein enzyme hydrolysate**: 10 gm
- **Yeast extract**: 5 gm
- **Sodium chloride**: 5 gm
- **Agar**: 15 gm

pH was adjusted to 7.4 by using NaOH and autoclaved at 15 lbs/sq.inch at 120°C for 15 min.

#### 3.2.3 LB agar maltose-magnesium sulphate (per litre)

- **Tryptone**: 10 gm
- **Yeast extract**: 5 gm
- **Sodium chloride**: 5 gm
- **MgSO₄**: 2.46 gm
- **Maltose**: 2.0 gm

pH was adjusted to 7.4 by using NaOH and autoclaved at 15 lbs/sq.inch at 120°C for 15 min.

---

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>530F</td>
<td>5’TGACTGACTGAGTGCCAGCMGCCGCGG ‘3</td>
<td>SIGMA</td>
</tr>
<tr>
<td>1494R</td>
<td>5’TGACTGACTGGYTACCTTGTTACGACTT’3</td>
<td>SIGMA</td>
</tr>
<tr>
<td>T7</td>
<td>5’TAAATACGACTCACTATAGG’3</td>
<td>SIGMA</td>
</tr>
<tr>
<td>SP6</td>
<td>5’ATTTAGGTTGACACTATAG’3</td>
<td>SIGMA</td>
</tr>
<tr>
<td>M13F</td>
<td>5’GTAAAACGACGGCCAGT’3</td>
<td>SIGMA</td>
</tr>
<tr>
<td>M13R</td>
<td>5’CAGGGAAACAGCTATGAC’3</td>
<td>SIGMA</td>
</tr>
<tr>
<td>S-D-Arch-0344-a-S-20</td>
<td>5’ACGGGGCGCCAGCAGGCGGCA’3</td>
<td>SIGMA</td>
</tr>
<tr>
<td>Univ-1517-a-A-21</td>
<td>5’ACGGCTACCTTGTTACGACTT’3</td>
<td>SIGMA</td>
</tr>
</tbody>
</table>

Restriction site, cos recognition sequence 2, cos site 2, SV40 promoter, neomycin resistance ORF, pUC origin, ampicillin resistance (*bla*) ORF.
3.2.4 Media for lipase activity (per litre)
Casein enzyme hydrolysate       10 gm
Yeast extract                       5 gm
Sodium chloride                 5 gm
Agar                               15 gm

pH was adjusted to 7.4 by using NaOH and autoclaved at 15 lbs/sq.inch at 120°C for 15 min. After autoclaving 20 μl of sterile tween 20 and 200 μl of filtered tributyrin were added before pouring of the plates.

3.2.5 Media for amylase activity (per litre)
Casein enzyme hydrolysate           10 gm
Yeast extract                               5 gm
Sodium chloride                         5 gm
Starch                                        1 gm
Agar                                              15 gm

pH was adjusted to 7.4 by using NaOH and autoclaved at 15 lbs/sq.inch at 120°C for 15 min.

3.2.6 Media for cellulase activity (per litre)
Casein enzyme hydrolysate             10 gm
Yeast extract                                5 gm
Sodium chloride                             5 gm
Carboxyl methyl cellulose (CMC)  0.5 gm
Agar                                               15 gm

pH was adjusted to 7.4 by using NaOH and autoclaved at 15 lbs/sq.inch at 120°C for 15 min.

3.2.7 Media for protease activity (per litre)
Casein enzyme hydrolysate         10 gm
Yeast extract                               5 gm
Sodium chloride 5 gm  
Commercial non fat skimmed milk 1 gm  
Agar 15 gm  

pH was adjusted to 7.4 by using NaOH and autoclaved at 15 lbs/sq.inch at 120°C for 15 min.

3.3 Antibiotics

3.3.1 Ampicillin (50 mg/ml)  
Ampicillin powder 500 mg  
Millipore water 10 ml  
Solution is filter sterilized and stored at −20°C. Ampicillin was used at the concentration of 75 µg/ml.

3.3.2 Streptomycin (34 mg/ml)  
Streptomycin powder 340 mg  
Millipore water 10 ml  
Solution is stored at −20°C. Streptomycin was used at the concentration of 50 µg/ml.

3.3.3 Tetracycline (50 mg/ml)  
Tetracycline powder 500 mg  
Millipore water 10 ml  
Solution is filter sterilized and stored at −20°C away from light. Tetracycline was used at the concentration of 50 µg/ml.

3.4 Solutions and Buffers

SM Buffer (1L)  
NaCl 5.8 gm  
MgSO_{4} 2 gm  
Tris-Cl (1M, pH 7.5) 50 ml  
2% gelatin 5 ml  
Millipore water 1L  
Solution was autoclaved at 15 lbs/sq.inch at 120°C for 15 min and stored at room temperature.
**Materials and Methods**

**10mM MgSO\(_4\)**

MgSO\(_4\) 2.46 gm  
Millipore water 1L  
Solution was autoclaved at 15 lbs/sq.inch at 120°C for 15 min and stored at room temperature.

**EDTA (0.5 M solution)**

EDTA 18.6 gm  
Distilled water 80.0 ml  
Vigorously stirred on a magnetic stirrer, pH was adjusted to 8.0 by using NaOH pellets and volume was made up to 100 ml. Solution was autoclaved at 15 lbs/sq.inch at 120°C for 15 min and stored at room temperature.

**Tris-Hydrochloride (1 M)**

Tris-HCl 12.11 gm  
Distilled water 80.0 ml  
Vigorously stirred on a magnetic stirrer, pH was adjusted to 8.0 by using HCl and volume was made up to 100 ml. Solution was autoclaved at 15 lbs/sq.inch at 120°C for 15 min and stored at room temperature.

**Tris-HCl buffer (10mM solution)**

1 M Tris HCl (pH 8.0) 1.0 ml  
Distilled water 80.0 ml  
Mixed properly and volume was made upto 100 ml. Solution was autoclaved at 15 lbs/sq.inch at 120°C for 15 min and stored at room temperature.

**3M Sodium acetate (pH 4.6)**

Sodium acetate 246.09 gm  
Distilled water 1L  
pH was adjusted to 4.6 by using glacial acetic acid and volume was made up to 100 ml. Solution was autoclaved at 15 lbs/sq.inch at 120°C for 15 min and stored at room temperature.
Tris EDTA (TE) buffer
1 M Tris HCl (pH 8.0) 1.0 ml
0.5 M EDTA (pH 8.0) 0.2 ml
Volume was made up to 100 ml. Solution was autoclaved at 15 lbs/sq.inch at 120°C for 15 min and stored at room temperature.

Lysozyme solution (10 mg/ml)
Lysozyme 10 mg
TE 1 ml
Solution was kept at −20°C.

10% SDS (sodium dodecyl sulfate) solution
Sodium dodecyl sulfate 50 gm
Deionized water 400 ml
Dissolved by heating at 55°C, pH was adjusted to pH 7.2 using HCl and volume was made up to 500 ml. The solution was stored at room temperature.

Proteinase K solution
Proteinase K powder 100 mg
Dissolved in 10 ml of deionized water and kept at −20°C.

Sodium chloride (5 M solution)
Sodium chloride 29.22 gm
Dissolved in 80 ml of deionized water and vigorously stirred on a magnetic stirrer and volume was made up to 100 ml. Solution was autoclaved at 15 lbs/sq.inch at 120°C for 15 min and stored at room temperature.

Tris acetate EDTA (TAE) buffer, 50X (100 ml)
Tris-Base 24.2 gm
0.5M EDTA 10.0 ml
Acetic acid 5.7 ml
Volume was made up to 100 ml. Solution was autoclaved at 15 lbs/sq.inch at 120°C for 15 min and stored at room temperature. The working solution was 1X.
**Phenol:Chloroform:Isoamyl alcohol (50 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris saturated phenol (pH 8.0)</td>
<td>25 ml</td>
</tr>
<tr>
<td>Chloroform</td>
<td>24 ml</td>
</tr>
<tr>
<td>Isoamyl alcohol</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

The solution was stored at 4°C.

**Chloroform:Isoamyl alcohol (50 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>24 ml</td>
</tr>
<tr>
<td>Isoamyl alcohol</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

The solution was stored at 4°C.

**Solution I (100 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris-HCl (pH 8.0)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>0.5M EDTA (pH 8.0)</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>1M Glucose</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

Volume was made up to 100 ml. Solution was autoclaved at 10 lbs/sq. inch for 15 min and stored at 4°C.

**Lysozyme (1 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme powder</td>
<td>20 mg</td>
</tr>
<tr>
<td>TE</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Stored at −20°C.

**Solution II (2 ml)**

**NaOH (100 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10N NaOH pallets</td>
<td>40.0 gm</td>
</tr>
</tbody>
</table>

Volume was made up to 100 ml and stored at room temperature.

**SDS solution (100 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% SDS</td>
<td>10.0 gm</td>
</tr>
</tbody>
</table>

Volume was made up to 100 ml dissolved by heating at 65°C and stored at room temperature.

Solution II was prepared by adding 10N NaOH 0.04 ml, 10% SDS 0.20 ml and Millipore Water 17.60 ml. The solution is always freshly prepared, just before use.
### Solution III (100 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5M Potassium acetate</td>
<td>60.0 ml</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>11.5 ml</td>
</tr>
</tbody>
</table>

Volume was made upto 100 ml. Solution was autoclaved at 15 lbs/sq.inch at 120°C for 15 min and stored at 4°C.

### RNase solution (1 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase powder</td>
<td>10.0 mg</td>
</tr>
</tbody>
</table>

Dissolved in 1 ml of sterile Millipore water. The solution was boiled for 5 min and stored at −20°C.

### 70% Ethanol (20 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol absolute</td>
<td>14 ml</td>
</tr>
<tr>
<td>MQH₂O (autoclaved)</td>
<td>6 ml</td>
</tr>
</tbody>
</table>

Stored at 4°C

### IPTG (1M)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPTG</td>
<td>1190.0 mg</td>
</tr>
<tr>
<td>Millipore water</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

Solution is filter sterilized and stored at −20°C.

### X-gal (5 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-gal</td>
<td>350.0 ml</td>
</tr>
<tr>
<td>Dimethyl formamide</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

The solution is stored at −20°C.

### 20 mM Sodium Phosphate Buffer.

(a) 0.2 M monobasic Sodium phosphate (1 litre)

27.6 gm of monobasic Sodium phosphate dissolved in 1 litre of Millipore water. Solution was autoclaved at 15 lbs/sq.inch at 120°C for 15 min and store at room temperature.

(b) 0.2 M dibasic sodium phosphate. (1 litre)
Materials and Methods

35.61 gm of dibasic Sodium phosphate dissolved in 1 litre of Millipore water. Solution was autoclaved at 15 lbs/sq.inch at 120°C for 15 min and store at room temperature.

To make 0.1M of Sodium Phosphate Buffer (100 ml). Mix 25.5 ml of monobasic Sodium phosphate buffer and 24.5 ml of dibasic Sodium phosphate buffer. Adjust pH 6.9 with 1 M NaOH. For 20 mM Sodium Phosphate Buffer, dilute the 0.1M of Sodium Phosphate Buffer 1:5 times.

DNA loading dye
8 gm of sucrose and 0.05 gm of Bromophenol blue dissolved in 20ml distilled water and stored at room temperature.

3.5 Soil samples
Seven different soil samples were collected from Kargil, a high altitude region of North-western Himalayas (Figure 3.1) and one soil sample was from salt pan Mumbai in India Unique niches selected for sampling were like high salt lakes, high altitude, radiations exposed. Plant debris and stones were removed from soil and sieved through a sterile 2 mm brass sieve. Soil samples was transported on dry ice and stored at -70 °C prior to DNA extraction.

3.6 Total community DNA Isolation, Purification and Quantification.
3.6.1 DNA Extraction with Chemical Method

Community DNA extractions were performed according to the modified Zhou protocol (Stach et al. 2001). Aliquots of soils (5 g) were weighed out into sterile 50 ml Tarson centrifuge tubes followed by the addition of 6.75 ml soil extraction buffer [1% CTAB (w/v); 100 mM Tris, pH 8.00; 100 mM NaH$_2$PO$_4$(pH 8.0); 100 mM EDTA; 1.5 M NaCl; 0.02% Protease K (w/v)]. The tubes were incubated horizontally at 37°C for 30 min with shaking. 750 μl 20% (w/v) SDS was added to each tube followed by further 2 h incubation at 65°C with gentle inversions every 20 min. Following incubation, the tubes were centrifuged at 3000 × g for 10 min at room temperature and the supernatant pooled into a sterile Tarson 50 ml centrifuge tube. An equal volume Phenol/Chloroform/Isoamyl was added and mixed gently followed by centrifugation at 12000 rpm for 10 min. Supernatants were again transferred to sterile 50 ml centrifuge tubes with the addition of an equal volume of chloroform. After careful mixing, the tubes were centrifuged at 12000 rpm for 10 min at room temperature and supernatants were recovered. 0.6 volumes of isopropanol was added
Figure 3.1: Site of sample collection from Jammu and Kashmir.
to the supernatants and DNA precipitation allowed to take place overnight at room temperature. DNA was pelleted by centrifugation at 15,000 rpm for 10 min, washed with 70% ethanol, recentrifuged at 15,000 rpm for 5 min, and air dried in a sterile hood. Sterile TE buffer was used to resuspend the DNA pellet. All samples were pooled and a small fraction was analysed by agarose gel electrophoresis.

3.6 Total community DNA Isolation, Purification and Quantification.

3.6.1 DNA Extraction with Chemical Method

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3.6.2 DNA Extraction with MoBio DNA extraction kit

DNA extraction was done by UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA, USA) according to manufacturer’s instruction. For DNA isolation, 5-10 gm of soil samples were taken and lysis of the microorganisms
Materials and Methods

in the soil carried out by a combination of heat, detergent and mechanical force against specialized beads. The released DNA was then bound to a spin filter. The filter was washed and then PCR quality DNA released into a buffer. DNA was analyzed by gel electrophoresis.

3.6.3 Agarose gel electrophoresis
Analysis of DNA was performed by agarose gel electrophoresis (Sambrook et al. 1982) in 0.7% agarose gel at 100 V in 1X TAE To allow visualization of the DNA on a UV transilluminator, the gels were supplemented with 0.5 μg/ml ethidium bromide.

3.6.4 Purification of Metagenomic DNA through low melting agarose
Agarose in which hydroxyethyl groups have been introduced into the polysaccharide chain was used. This substitution causes the agarose to gel at approximately 30°C and to melt at approximately 65°C below the melting temperature of most double-stranded DNA. These properties have been exploited to develop a simple technique for the recovery of DNA from gels.
0.5% (w/v) low-melting agarose was poured in a cassette in cold room to ensure that it solidifies completely. Samples of DNA were mixed with gel loading dye and loaded into the wells. Electrophoresis was carried out at 40–50 V. Using a sharp scalpel or razor blade, a slice of agarose containing the DNA was cut out and transferred to a sterile eppendorf tube. DNA was eluted from the gel slice using Qiagen gel extraction kit and checked on 0.7% (w/v) agarose gel.

3.6.5 Quantification of DNA
The spectrophotometric quantification of DNA was carried out at OD at 260 nm and 280 nm in a double bean UV spectrophotometer (Shimadzu UV 160A). The instrument was pre-set in such a way so that it could measure the absorbance at two wavelengths (WL1= 260 nm and WL2= 280 nm) simultaneously. The WL1 measures the DNA concentration whereas the WL2 reflects the concentration of proteins in solution. The ratio of absorbance at WL1 to WL2 around 1.8 is an indicator for good quality DNA. Sterile water was used to set the “blank”. The OD of community DNA was measured at both wavelengths. DNA concentration from the OD value at WL1 (260 nm) was calculated as per following formula.
1OD at WL1 for dsDNA = 50 ng/μl of dsDNA.
The original concentration of the DNA sample was calculated from the equation below:
Original concentration = OD at WL1 x 50 ng/μl × dilution factor.

3.7 Soil analysis
3.7.1 Dry weight assessment and water content
Dry weights were determined by placing 10 gm samples of soil, in duplicate in pre-weighed glass petri dishes. The samples were incubated at 100°C and weighed every 24 h for a period of 2 days. Water content was determined as the total difference in soil sample weight, expressed as a percentage.

3.7.2 pH measurement
1 gm of soil sample was mixed with 10ml of sterile MQ water and slurry was prepared. pH was measured using pH meter.

3.8 PCR amplification using 16S rDNA eubacterial and archaeal primers
3.8.1 PCR assay using eubacterial primers
High molecular weight community DNA was used as a template for 16S rDNA amplification using primers 530F and 1494R (Borneman et al. 1996). The sequences representing the 3’ end are hypervariable while the 5’ end sequences are more conserved. Positive control reaction contained 60 ng DNA of E.coli JM110; however negative reaction contained no exogenous template. PCR products were examined by gel electrophoresis on 1% agarose gel in 1X TAE buffer as described in Section 3.6.3. Amplified DNA fragment was recovered with PCR purification kit (Mo Bio Laboratories, Solana Beach, CA, USA)

1M Tris : 1.0 µl
25 m M MgCl$_2$ : 2.5 µl
BSA : 5.0 µl
10 m M dNTPs : 2.0 µl
Primer (F) : 1.5 µl
Primer (R) : 1.5 µl
DNA : 1.0 µl
Materials and Methods

Isolation of metagenomic DNA from soil

Sequencing of recombinants

ARDRA analysis

16S gene amplification

Transformation and spreading on AXI plates

Selection of positive recombinants

Ligation with vector

Operon in E.coli host cells

Alignment of sequences with database

Phylogenetic Relationship between the sequences

Figure 3.2: Schematic representation of 16S library construction and analysis of sequences.
Figure 3.3: pGEM®-T Easy Vector circle map and sequence reference points. Figure was adapted from www.promega.com/tbs/tm042/tm042.pdf
**Materials and Methods**

MQ H\(_2\)O : 4.5 µl  
Taq Polymerase (1.5 U) : 1.5 µl  
**Total** : 20.0 µl

**PCR PROGRAMME:**

1. Initial Denaturation 94°C for 1 min.  
2. Denaturation 94°C for 30 sec.  
3. Annealing 50°C for 30 sec.  
4. Extension 72°C for 1 min.  
5. Final Extension 72°C for 5 min.  
**Total Cycles : 40**

### 3.8.2 PCR assay sing 16S archaeal primers

Amplification of 16S rRNA genes was done by PCR using a primer set specific to Archaea (Costantino *et al.* 1998). S-D-Arch-0344-a-S-20 and Univ-1517-a-A-21 were the primers used in this study. The positive control reaction contained 10 ng DNA of *Halobacterium salinarium* (MTCC 1626) however, the negative reaction contained no exogenous template.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>25 m M MgCl(_2)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>BSA</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>10 m M dNTPs</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Primer (F)</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>Primer (R)</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>DNA</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>MQ H(_2)O</td>
<td>4.5 µl</td>
</tr>
<tr>
<td>Taq Polymerase (1.5 U)</td>
<td>1.5 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20.0 µl</td>
</tr>
</tbody>
</table>

**PCR PROGRAMME :**

1. Initial Denaturation 94°C for 3 min.  
2. Denaturation 94°C for 30 sec.
3. Annealing 60°C for 30 sec.
4. Extension 72°C for 1 min.
5. Final Extension 72°C for 5 min.

Total Cycles : 30

3.9 Construction of 16S eubacterial and archael library in pGEMT-easy
The pGEM®-T and pGEM®-T Easy Vector Systems (a,b) are convenient systems for the cloning of PCR products. The vectors are prepared by cutting the pGEM®- 5Zf (+) and pGEM®-T Easy Vectors, respectively, with EcoRV and adding a 3´ terminal thymidine to both ends. These single 3´ T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. The high-copy-number pGEM®-T and pGEM®-T Easy Vectors contain T7 and SP6. RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by blue-white screening on indicator plates. Both the pGEM®-T and pGEM®-T Easy Vector contain multiple restriction sites within the multiple cloning regions. The pGEM®-T Easy Vector multiple cloning region is flanked by recognition sites for the restriction enzymes EcoRI, BstZI and NotI, thus providing three single-enzyme digestions for release of the insert. The pGEM®-T and pGEM®-T Easy Vectors also contain the origin of replication of the filamentous phage f1 for the preparation of single-stranded DNA.

3.9.1 Preparation of host cells for electroporation
Cloning host *E. coli* JM110 cells were used for the construction of 16S libraries. The competent host cells were prepared according to the protocol given by Sambrook and Russell (2001). The host cells were grown overnight in 20 ml LB broth in the presence of antibiotic at 37°C and at 200 rpm. 300 ml of fresh LB broth in a 1000 ml flask was inoculated with 3 ml of overnight grown culture and incubated at 37°C in 200 rpm shaker until they reached mid-log phase of growth (0.5-0.6 OD at 260 nm). The host cells were harvested in 50 ml prechilled centrifuge tubes (Tarson) by
centrifugation at 6,000 × g for 10 min at 4°C. The supernatant was decanted aseptically and cells were washed twice with an equal volume of chilled sterile water followed by centrifugation at same speed, time and temperature. Upon centrifugation the water was decanted and cells pellet was washed again with 1/10 volume of prechilled 10% glycerol solution and centrifuged at 6,000 × g for 10 min. The supernatant was discarded and cells pellet were resuspended in 1 ml of 10% glycerol and the cell density at OD$_{600}$ adjusted to between $2 \times 10^{10}$ to $3 \times 10^{10}$ cells ml$^{-1}$. The cells were aliquotted into 50 µl volumes, and stored at -70°C immediately until required. Transformation efficiency of electrocompetent cells was measured by transforming an aliquot with 1 µl of pUC19 vector containing 20 ng of DNA. The transforming efficiency of _E. coli_ JM110 was 3 to $2.0 \times 10^7$ CFU/µg DNA.

### 3.9.2 Ligation

Purified PCR product was ligated with pGEMT-easy vector according to manufacturer’s instructions (Promega corporation, USA) using the recommended ratio of the insert DNA and vector from 1:3 to 3:1. The ligation reaction was set up at 4°C for overnight.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product of 16S eubacteria</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>2X buffer</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>pGEMT vector</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Ligase</td>
<td>1.0 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>10.0 µl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product of 16S archael</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>2X buffer</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>pGEMT vector</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Ligase</td>
<td>1.0 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>10.0 µl</td>
</tr>
</tbody>
</table>
3.9.3 Transformation of E. coli cells by Electroporation.

An Eppendorf tube containing 50 μl of electrocompetent cells (E.coli JM110) was removed from -70°C and allowed to thaw on ice. 3.5 μl of ligation mix was added to the thawed cells and gently mixed. The mixture was returned to ice for ~ 1 min then pipetted into a pre-cooled 0.1 cm sterile electroporation cuvette (Bio-Rad Laboratories, Hercules, CA, USA). Electroporation was performed using the following conditions: 2.5 kV, 25 μF, 200 Ω. Immediately following electroporation, 1000 μl LB broth, was added to the cuvette, the cells transferred to a 20 ml test tube and incubated at 37°C for 1 h with agitation. The cells were plated in aliquots of 100 to 200 μl onto LB-agar plates supplemented with100 µg/ml ampicilin, 80 μg/ml X-Gal (5-bromo- 4 chloro 3-indolyl-β-D-galactopyranose) and 0.5 mM IPTG (isopropyl-b-D-thiogalactoside) and incubated overnight at 37°C. After the incubation period the plates were observed for growth of clones. Recombinant transformants were selected by blue/white color selection based on insertional inactivation of the lacZ gene.

3.9.4 Plasmid DNA extraction using Alkaline lysis Method.

White colonies were picked from the agar plates, inoculated into 5 ml of LB broth supplemented with the appropriate antibiotic and incubated overnight at 37°C with agitation. Plasmid DNA was isolated from the cultures by the alkaline lysis method (Birnbiom and Doly 1979). The cells were harvested at 4,000 rpm for 10 min at 4°C and washed with 100 μl of Solution I. The supernatant was discarded. 100 μl of solution I along with lysozyme (4 mg/ml) was added to each vial, which was thereafter vortexed and incubated at 37°C for 1 hour. 200 μl of Solution II was added and the tubes were incubated on ice for 10 min. Following the addition of 150 μl of Solution III, the tubes were incubated on ice for 10 min, and then centrifuged at 15,000 for 15 min at 4°C. Supernatant was collected in 1.5 ml eppendorf followed by addition of RNase 4 μl (10 mg/ml) to the supernatant and incubated at 42°C for 1 hour. Equal volume of Phenol: Chloroform: Isoamyl alcohol in ratio of 25:24:1 was added to supernatant and centrifuged at 12000 rpm for 10 min at 4°C followed by washing with choloroform: isoamyl alcohol in ratio of 24:1. To the aqueous layer 10% volumeof 3M Na-acetate and 70% volume of isopropanol were added and kept at 20°C for overnight. The plasmid DNA was pelleted down at 15,000 rpm for 20
minutes at 4°C and washed with 70% ethanol and air dried. The pellet was finally dissolved in 30 µl of TE.

3.9.5 Restriction analysis of plasmids for presence of insert

Plasmids isolated from randomly picked colonies were subjected to restriction analysis with EcoRI. The size of insert was approximately 1kb.

\[
\begin{align*}
\text{DNA} & : 3.0 \mu l \\
10x \text{ Buffer} & : 2.0 \mu l \\
\text{MQH}_2\text{O} & : 13 \mu l \\
\text{Eco RI} & : 2.0 \mu l \\
\hline
\text{Total} & : 20.0 \mu l
\end{align*}
\]

Restricted products were resolved in 0.7% agarose as described in section 3.6.3.

3.10 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

rDNA inserts from recombinant clones were reamplified by Specific PCR primers. The cycle parameters applied were the same as for the initial amplification of the rDNA as already mentioned in 3.8. Aliquots of crude reamplified rDNA PCR products were digested with of the tetra cutter base specific restriction endonucleases enzyme \textit{MspI} and \textit{AluI} (New England Bio labs, Beverly, Mass), for 3hrs at 37°C. Digested products were separated by agarose (2 %) gel electrophoresis as described in 3.6.3. The reaction was as following.

\[
\begin{align*}
\text{Amplified product} & : 15 \mu l \\
10X \text{ buffer} & : 2 \mu l \\
aluI/\textit{MspI} & : 1 \mu l \\
\text{MQH}_2\text{O} & : 2 \mu l \\
\hline
\text{Total} & : 20 \mu l
\end{align*}
\]

3.11 Template preparation and Nucleic acid sequencing

Plasmid DNA isolated from positive clones was used as template for PCR amplification for the generation of sequencing templates.
Materials and Methods

1M Tris : 1.0 µl
25 mM MgCl₂ : 2.5 µl
10 mM dNTPs : 2.0 µl
T7Primer : 1.0 µl
DNA : 1.0 µl
MQH₂O : 11.5 µl
Taq Polymerase : 1.0 µl (1 U)

Total : 20.0 µl

The cycle parameters applied were the same as for the initial amplification of the rDNA as already mentioned in section 3.8.

Sequencing PCR

The Big Dye Terminator sequencing Kit (ver.3) was used in the sequencing reaction.

The sequencing PCR assay as follows

Ready reaction mix : 8.0 µl
T7 Primer : 1.0 µl
DNA : 5 µl
MQ : 6 µl

Total : 20 µl

PCR PROGRAMME:

1. Denaturation 96 °C for 10 sec.
2. Annealing 50°C for 5 sec.
3. Extension 60°C for 4 min.

Total : 25 cycles

PCR products were purified by increasing the volume upto 100 µl with sterile water and precipitating with equal volume of ice cold ethanol in the presence of 0.3 M sodium acetate. The tubes were incubated in ice for 10 min and centrifuged at 13000 × g for 20 min. The supernatant was removed and the pellet was washed with 250 µl of 70% ethanol and centrifuged again at 13000 × g for 10 min at 4°C. The supernatant
was discarded and the pellet was air dried. The dried DNA pellet dissolved in 15 μl of TSR (template suppressor reagent) and heated at 95°C for 5 min before loading onto the automated ABI Prism 377 automatic DNA sequencer (Perkin-Elmer, Applied Bioystems).

3.12 Phylogenetic Analysis
All the 16S rDNA eubacterial and archael gene sequences were compared to closest databased-relatives available in the National Centre for Biotechnology Information (NCBI) using BLAST analysis. MegAlign program of Lasergene 5.0 (DNASTAR Inc., USA) was used for multiple sequence alignment. Sequence regions containing gaps and ambiguous nucleotides were removed from the multiple alignments before analysis. CHECK_CHIMERA programme was used for inspection of putative secondary structures. Phylogenetic affiliation among these sequences were performed by using Clustal W1.81 and the tree was created by the programme MEGA version 3.1 (Tamura et al. 2007).

All 16S rRNA eubacterial sequences were deposited in the GenBank database under the following accession numbers: DQ659419 to DQ659428, DQ866063 to DQ866079 and EF577248 to EF577256.

The sequences of 16S archael have been deposited in the GenBank database under accession numbers EF619315- EF619320, EF619323- EF619323, EF619324, EF619326- EF619332, EF619334, EF619342, EF990132-EF990139, EU145969-EU145974 and DQ866137.

3.13 Preparation of metagenomic library
3.13.1 Preparation of metagenomic DNA
3.13.1.1 Partial Digestion of Metagenomic DNA
In cosmid library construction, it is essential to have DNA fragments of 30-42 kb, therefore the chromosomal DNA should be partially digested with Sau3AI or MboI to make it compatible to BamHI site of Supercos vector. Approximately 2 μg of community DNA was used for restriction enzyme digestion using Sau3AI. Digestions were performed using Sau3AI. The reactions were stopped at different time interval of 2, 5, 10, 15 and 30 min. To stop the reaction, 4 μl of 50 mM EDTA was added to each reaction and incubated the tubes at 68°C for 10 min.
Figure 3.4: Schematic representation of metagenic library construction screening and subcloning of amylolytic clone.
A large scale (10X) digestion of metagenomic DNA was performed for the optimized time.

Following digestion, reaction mixtures and λ-HindIII molecular weight marker were loaded into separate wells of a 0.8% agarose gel and electrophoresed as described in Section 3.6.3.

3.13.1.2 Recovery of restriction digested metagenomic DNA from agarose gel

Partially digested DNA sample was mixed with 5X DNA loading dye solution and electrophoresed at 1 v/cm in a horizontal slab gel electrophoresis unit (8 × 10 cm; model B.GENEI). A low melting 0.7% agarose gel was prepared in 1X TAE buffer containing 0.5 μl/ml of ethidium bromide. λ-HindIII (Fermentas) was taken as molecular weight marker. After electrophoresis, the digested DNA was visualized under shortwave UV light and photographed. Genomic DNA fragments ranging in size from the 30-40 kb were eluted from the agarose gel and purified by using the
QIAEX II gel extraction kit (Qiagen Valencia, CA, USA) as per manufacturer’s instructions and kept at –20°C until used.

### 3.13.1.3 Dephosphorylation of partially digested metagenomic DNA
Dephosphorylation of 5’ ends of partially digested-purified genomic DNA was performed using Calf Intestinal Alkaline Phosphatase (CIAP).

<table>
<thead>
<tr>
<th>Partially Digested DNA</th>
<th>: 60.0 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X dephosphorylation buffer</td>
<td>: 10.0 µl</td>
</tr>
<tr>
<td>MQH₂O</td>
<td>: 15.0 µl</td>
</tr>
<tr>
<td>CIAP</td>
<td>: 15.0 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>: 100.0 µl</td>
</tr>
</tbody>
</table>

The reaction was kept at 37°C for 1 h. The DNA was extracted once with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamylalcohol (24:1) at 12,000 × g for 10 min at 4°C. The supernatant containing DNA was collected in another micro-centrifuge tube and precipitated with 2 volume of cold ethanol in the presence of 0.3 M sodium acetate (pH 4.6) and kept at –20°C for overnight. DNA was pelleted down, washed twice with 70% ethanol and air dried. The DNA was dissolved in 20 µl of sterile water and kept at –20°C until used.

### 3.13.2 Preparation of Vector
The metagenomic library of soil was constructed in SuperCos 1(Stratgene), is a novel, 7.9 kb cosmid vector that contains bacteriophage promoter sequences flanking a unique cloning site.

### 3.13.2.1 Digestion of Vector DNA with XbaI
About 25 µg of the Supercos cosmid vector was digested with XbaI in a reaction volume of 20 µl containing 1U of XbaI, Fermentas with 10X buffer supplied with enzyme. The reaction was incubated at 37°C for 3 hrs.
**Figure 3.5:** Circular map and features of the Super Cos 1 cosmid vector. Figure was adapted from www.stratagene.com/manuals/251301.pdf.
Super Cos 1 cosmid vector : 2.5 μl

10x Buffer : 2.0.0 μl
MQH₂O : 11.0 μl
BSA (1%) : 2.0 μl
XbaI : 2.5 μl

Total 20.0 μl

A large scale (10X) digestion of vector DNA was performed for the optimized time.

Super Cos 1 cosmid vector : 25.0 μl
10x Buffer : 20.0 μl
MQH₂O : 110.0 μl
BSA (1%) : 20.0 μl
XbaI : 25.0 μl

Total 200.0 μl

The linearized vector was observed in agarose gel electrophoresis as described in 3.6.3 The linearized vector was extracted once with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1) at 12,000 × g for 10 min at 4°C. The supernatant containing DNA was collected in another micro-centrifuge tube and precipitated with 2 volume of cold ethanol in the presence of 0.3 M sodium acetate (pH 4.6) and kept at −20°C for overnight. DNA was pelleted down, washed twice with 70% ethanol and air dried. The DNA was dissolved in 200 μl of sterile TE buffer.

### 3.13.2.2. Dephosphorylation of Xba I -Digested vector DNA

Dephosphorylation of XbaI digested-purified vector DNA was performed using Calf Intestinal Alkaline Phosphatase (CIAP).

XbaI digested vector DNA : 200.0 μl
10X dephosphorylation buffer : 30.0 μl
The reaction was followed by incubation at 37°C for 1 h. The DNA was extracted once with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1) at 12,000 × g for 10 min at 4°C. The supernatant containing DNA was collected in another micro-centrifuge tube and precipitated with 2 volume of cold ethanol in the presence of 0.3 M sodium acetate (pH 4.6) and kept at –20°C for overnight. DNA was pelleted down, washed twice with 70% ethanol and air dried. The DNA was dissolved in 100 µl of sterile TE buffer.

3.13.2.3 Digestion of Xba I digested and CIAP treated vector DNA with Bam HI

Xba I digested and CIAP treated 100 µl of vector DNA was restricted with 10U of BamHI in total volume of 200 µl at standard buffer conditions for 3 hours at 37°C for 3 hours.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xba I digested and CIAP treated Vector DNA</td>
<td>100.0 µl</td>
</tr>
<tr>
<td>10xBuffer</td>
<td>20.0 µl</td>
</tr>
<tr>
<td>MQH₂O</td>
<td>60.0 µl</td>
</tr>
<tr>
<td>BamHI</td>
<td>20.0 µl</td>
</tr>
<tr>
<td>Total</td>
<td>200.0 µl</td>
</tr>
</tbody>
</table>

The DNA was extracted once with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1) at 12,000 × g for 10 min at 4°C. The supernatant containing DNA was collected in another micro-centrifuge tube and precipitated with 2 volume of cold ethanol in the presence of 0.3 M sodium acetate (pH 4.6) and kept at –20°C for overnight. DNA was pelleted down, washed twice with 70% ethanol and air dried. The DNA was dissolved in 50 µl of sterile TE buffer.
3.13.3. Ligation
The reaction of 20 µl was prepared in PCR tube. The reaction was incubated at 4°C for overnight.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (~2.5 µg)</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>Vector (~1.0 µg)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>MQ H₂O</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>2X ligation buffer</td>
<td>10.0 µl</td>
</tr>
<tr>
<td>Ligase (5U)</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>ATP (10mM)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20.0 µl</td>
</tr>
</tbody>
</table>

3.13.4. Packaging of DNA
Packaging of the ligated DNA was carried out by using Gigapack III gold packaging extract (Stratagene) and as per the instructions of the manufacturers. The packaging mix (25 µl) was thawed and immediately ligated mix (20 µl) was added to it and mixed gently by pipetting. After a short spin tubes were incubated at RT (20-22 °C) for 2-3 hrs. 200 µl of SM buffer was added and then 20 µl of chloroform was added and mixed. The tubes were spinned briefly and the supernatant containing the phage was ready for titrating.

3.13.5 Preparation of host cells
The bacterial glycerol stock of SURE (E.coli) host cells were streaked on fresh antibiotic containing plates (Tetracycline, 15 µg/ml), incubated for overnight at 37°C. Single colony from the plate was transferred to 250 ml LBMM. The culture was incubated at 30 °C at 220 rpm till the OD of cells reached 1.0 at 600 nm. Cells were pelleted at 500 X g for 10 min. and resuspended in half the original volume of sterile 10 mM MgSO₄. Host cells were diluted to an OD₆₀₀ of 0.5 with sterile 10 mM MgSO₄.

3.13.6 Titerating the cosmid library:
Phage mix was diluted to 1:10 with SM buffer. In microfuge tubes, 25 μl of each of dilution was mixed with 25 μl of host cells as prepared in section 3.11.5 and incubated at RT for an hour. 200 μl of LB was added and incubated for another hour at 37 °C with in between 2-3 gentle inversions. Microfuge tubes were spinned for 2 min at 1200 rpm and pellet was resuspended in 50 μl of fresh LB. The cells were diluted appropriately and plated on LB agar plates containing ampicillin and tetracycline then incubated at 37°C for overnight.

### 3.13.7 Checking clones for the presence of Insert

A few clones were randomly picked up and cosmids were extracted using alkaline lysis method as described in section 3.9.4. These cosmids were digested in reaction volume of 20 μl using 1U of *EcoRI*

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>10.0 μl</td>
</tr>
<tr>
<td>10xBuffer</td>
<td>3.0 μl</td>
</tr>
<tr>
<td>MQH2O</td>
<td>15.0 μl</td>
</tr>
<tr>
<td><em>EcoRI</em></td>
<td>2.0 μl</td>
</tr>
</tbody>
</table>

Total : 30.0 μl

The reactions were incubated for 3 hrs at 37°C. The restriction of cosmid clones was visualized on 0.7% agarose gel as described in section 3.6.3

### 3.13.8 Amplification and Cryopreervation of cosmid library

The individual cosmid clones were replicated in 96 well microtiter plates containing 100 μl of LB with ampicillin 50 mg/ml in each well and incubated at 37°C for overnight.100 μl of autoclaved glycerol was added to each well and individual copies of the library were stored at -70 °C.

### 3.14 Activity-based screening of the metagenomic library

The clones from the library were replicated in microtiter plates containing 200 μl of Luria-Bertani broth + 50 μg/ml Ampicillin using a 96 pin array individual. For detecting Lipase, Amylase, Cellulase and Protease activities the library was replicated
Materials and Methods

to agar plates containing appropriate substrate given in table 3.2. Plates were incubated for 28 hrs at 37°C before scoring phenotype.

3.14.1 Amylase activity

For observing the amylase activity, the plate was flooded with Gram’s Iodine. Clones showing amylase activity produce clear halo around the colony.

3.14.2 Protease activity

For protease activity, the plates were observed for clear halo zone around the colony.

3.14.3 Lipase activity

For lipase activity, the plates were observed for clear halo zone around the colony.

Table 3.2: Different substrates used for activity based screening of various hydrolytic enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipase</td>
<td>Tributyrin</td>
</tr>
<tr>
<td>Amylase</td>
<td>Soluble starch</td>
</tr>
<tr>
<td>Protease</td>
<td>Commercial non fat skimmed milk</td>
</tr>
<tr>
<td>Cellulase</td>
<td>Carboxyl methyl cellulose sodium</td>
</tr>
</tbody>
</table>

3.14.4 Cellulase activity

The cellulase activity was observed by staining the plates with congo red dye for half an hour followed by destaining with 1M NaCl. The clear zone around the colony shows cellulase activity.

3.15 Subcloning of amylase positive cosmid clone (cAMY)

3.15.1. Vector DNA preparation

pUC19 strain was used for vector preparation. Plasmid was extracted by alkaline lysis method as described in section 3.9.4. pUC19 vector is small, high copy number, E.coli plasmids, 2686 bp in length. Insertion of DNA into the MCS located within the
Figure 3.6: Circular map of vector pUC19. Figure was adapted from www.fermentas.com/en/products/all/molecular-cloning
lacZ gene inactivates the N-terminal fragment of beta-galactosidase and abolishes alpha-complementation. Bacteria carrying recombinant plasmids therefore give rise to white colonies.

### 3.15.1.1 Digestion of pUC19 Vector with BamHI

About 5 μg of vector DNA was digested with BamHI in a reaction volume of 200 μl using 10U of BamHI. The sample was mixed by centrifugation for 5 sec and incubated at 37°C for overnight. The linearized vector was gel purified by using the QIAEX II gel extraction kit (Qiagen) as per manufacturer’s instructions and stored at –20°C until used.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>MQH₂O</td>
<td>14.5 μl</td>
</tr>
<tr>
<td>10X Buffer</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>BamHI</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>2.0 μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20.0 μl</strong></td>
</tr>
</tbody>
</table>

A large scale (10X) digestion of vector DNA was performed for the optimized time.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>MQH₂O</td>
<td>145 μl</td>
</tr>
<tr>
<td>10X Buffer</td>
<td>20 μl</td>
</tr>
<tr>
<td>BamHI</td>
<td>15 μl</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>20 μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>200 μl</strong></td>
</tr>
</tbody>
</table>

### 3.15.1.2 Dephosphorylation of BamHI digested pUC19 Vector

BamHI digested pUC19 vector DNA was dephosphorylated by treating with shrimp alkaline phosphatase (SAP; Fermentas) in a reaction volume of 100 μl. The reaction was mixed well and incubated at 37°C waterbath for 30 min. After the incubation period, 2 μl of SAP was again added to the reaction and reincubated at same
temperature for another 30 minutes. The reaction was terminated by incubating at 68°C for 10 min.

\[ \text{BamH1 digested vector DNA} : 120 \mu\text{l} \]
\[ \text{10X dephosphorylation buffer} : 20.0 \mu\text{l} \]
\[ \text{MQH}_2\text{O} : 50.0 \mu\text{l} \]
\[ \text{SAP} : 10 \ (8+2) \mu\text{l} \]
\[ \text{Total} \quad 200 \mu\text{l} \]

The vector DNA was extracted once with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1) at 12,000 x g for 10 min at 4°C. The supernatant containing vector DNA was collected in another micro-centrifuge tube and precipitated with 2 volume of cold ethanol in the presence of 0.3 M sodium acetate (pH 4.6) and kept at –20°C for overnight. DNA was pelleted down, washed twice with 70% ethanol and air dried. The DNA was dissolved in 20 μl of sterile water and kept at –20°C until used.

3.15.2 Preparation of DNA
Cosmid DNA was extracted from cAMY. Cosmid DNA was extracted by alkaline lysis method as described in section 3.9.4.

3.15.3 Digestion of Cosmid DNA with \textit{BamH1}
About 10 μg of Cosmid DNA was digested with \textit{BamH1} in a reaction volume of 200 μl using 10U of BamH1. The sample was mixed by centrifugation for 5 sec and incubated at 37°C for overnight. The restricted Cosmid DNA was gel purified by using the QIAEX II gel extraction kit (Qiagen) as per manufacturer’s instructions and stored at –20°C until used.

\[ \text{DNA} : 20.0 \mu\text{l} \]
\[ \text{10xBuffer} : 20 \mu\text{l} \]
\[ \text{MQH}_2\text{O} : 90.0 \mu\text{l} \]
\[ \text{BamH1} : 10 \mu\text{l} \]
\[ \text{Total} : 200 \mu\text{l} \]
3.15.4 **Ligation:**

For the subcloning of Cosmid clone, it is essential to have DNA fragments and vector DNA in a ratio around 10:1. Vector and cosmid DNA fragments were ligated. The reaction of 15 μl was prepared in PCR tube. The reaction was incubated at 4°C for overnight.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>3 μl</td>
</tr>
<tr>
<td>Vector (pUC19)</td>
<td>1 μl</td>
</tr>
<tr>
<td>MQH₂O</td>
<td>7 μl</td>
</tr>
<tr>
<td>2X ligation buffer</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>1 μl</td>
</tr>
<tr>
<td>ATP</td>
<td>1.5 μl (10 mM)</td>
</tr>
</tbody>
</table>

Total 15 μl

3.15.5 **Transformation**

The transformation of ligated product into competent *E.coli* JM110 was done as described in section 3.9.3.

3.15.6 **Screening of plasmid recombinant clone for amylase activity**

Plasmid recombinants were grown in LB media for overnight at 37°C and then plated into well on starch agar plate and incubated at 37°C for 28 hrs. After 28 hrs, overlaid the plate with iodine solution and scored for clear halo zone around the well.

3.15.7 **Check for the size of insert**

Plasmid was extracted by Qiagen plasmid isolation kit and amplified with M13 sequencing primers.

**Amplification of plasmid isolated from pAMY**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>MQH₂O</td>
<td>13 μl</td>
</tr>
<tr>
<td>taq buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>dNTPs (200 μM)</td>
<td>1 μl</td>
</tr>
</tbody>
</table>
M13 F Primer           :    1 μl
M13 R Primer        :    1 μl
DNA                          : 1 μl
Taq                             : 1 μl

Total                           20 μl

**PCR Programme :**

1. Initial Denaturation 94°C for 3 min.
2. Denaturation 94°C for 1 min
3. Annealing 60°C for 30 sec
4. Extension 72°C for 1 min.
5. Final Extension 72°C for 5 min.

Total cycles : 30

The PCR product was resolved in a 1% (w/v) agarose gel. Amplified DNA fragment was recovered with PCR purification kit (Mo Bio Laboratories, Solana Beach, CA, USA)

### 3.16 Sequencing of PCR product

The amplicon was subjected to sequence analysis as described in section 3.11 for the identification of putative amylase gene.

### 3.17 Preparation of crude enzyme extract

The pellet of 50 g wet weight of cells was suspended in 150 ml of phosphate buffer (20 mM, pH 6.0) and disrupted by Ultrasonication (MSE Manor Roya Crawley RH 10 2QQ) at 16 Hz (30 s for five times with intermittent cooling for 5 min) and then centrifuged at 20,124 × g for 10 min at 4°C. The supernatant was then taken in a tube and stored at -20°C for further analysis.

### 3.18 Partial purification of enzyme

The supernatant obtained by centrifugation was used as crude enzyme and then fractionated with 10% to 60% saturation of ammonium sulphate. The precipitate was recovered by centrifugation (20,124 g× 10 min at 4°C), dissolved in phosphate buffer
(20 mM, pH 6.0) and dialyzed overnight against the same buffer. The dialyzed enzyme solution was used for further studies.

3.19 Characterization of pAMY.

3.19.1 Quantification of enzyme activity in pAMY

Enzyme assay was carried out by using the dinitrosalicylic acid method for the determination of reducing sugar (Miller 1959). The enzyme reaction mixture was composed of substrate and an appropriate quantity of enzyme in 20 mM sodium phosphate buffer (pH 6.5); 500 μl of the enzyme mixture was incubated at 30°C for 10 min. The reaction was stopped by adding 500 μl of dinitrosalicylic acid solution (10.6 g of 3,5- dinitrosalicylic acid, 19.8 g of NaOH, 306 g of potassium sodium tartrate, 7.6 ml of phenol, 8.3 g of sodium metabisulfate and 1,416 ml of distilled water). The reaction mixture was boiled for 5 min and cooled by placing the tubes on ice. Absorbance was measured at 575 nm in a 1 cm polystyrene cuvette by using a Perkin Elmer spectrophotometer. Standard curve relating maltose concentration was plotted using 0.1 to 1.0 mg/ml. Amount of reducing sugar liberated by the enzyme was determined with respect to concentration of standard maltose. One unit of hydrolyzing activity was defined as the amount of enzyme required to produce 1 μmol of maltose in 1 min.

3.19.2 SDS –PAGE analysis of pAMY.

Equal amounts of protein (in μg) was taken from each fraction and mixed with 1.5 volumes of 1X gel loading buffer. Heat shock was given minutes at 100°C for three min. 10% SDS-Gel was prepared by mixing solutions in appropriate proportions. Samples were loaded and gel was run at 100 volts electric supply. When the front indicator dye Bromophenol Blue reached the bottom, electrophoresis was stopped and the gel slab was removed from electrophoresis apparatus by separating the spacers and glass plates.

The gel was stained for protein with Coomassie brilliant blue solution for 2 hr and the repeatedly destained in the destaining solution. Destaining was continued till the gel turned transparent and distinct blue bands were visible

Formulations of SDS-PAGE resolving and stacking gel

Resolving gel (10%)
MQH₂O : 11.9 ml
Materials and Methods

1.5 M Tris Cl pH 8.8 : 7.5 ml
10% SDS : 0.3 ml
Acrylamide/ Bis (30%) : 10 ml
10% ammonium per sulphate : 0.3 ml
TEMED : 0.012 ml

Stacking gel (10%)
MQ H₂O : 1.4 ml
1.5 M Tris Cl pH 6.8 : 0.25 ml
10% SDS : 0.02 ml
Acrylamide/ Bis (30%) : 0.33 ml
10% ammonium per sulphate : 0.02 ml
TEMED : 0.002 ml

3.19.3 Effect of pH on amylase activity.
The pH profile of pAMY activity was determined using the following buffers for the different pH ranges: pH 4.5 to 5.5, 50 mM sodium citrate; pH 6.5 to 7.5, 50 mM sodium phosphate; pH 8.5 to 9.5, 50 mM Tris-HCl at 40°C.

3.19.4 Effect of temperature on amylase activity
The optimum temperature for amylase activity of pAMY was measured by determining its hydrolytic activity at different temperatures (10-60°C) for 10 min at pH 6.5. The thermostability of amylase was determined by pre-incubating the enzyme for up to 30 min at temperature ranging between 10-60°C, followed by residual activity determination with added substrate at 40°C. The substrate specificity of the amylase (pAMY) was also evaluated on soluble starch, amylose, glycogen and maltose under optimal conditions of temperature and pH.

3.19.5 Effect on enzyme activity using metal ions and chemicals
Effect of metal ions and chemicals on the activity of recombinant pAMY was determined at 40°C for 10 min with appropriate metal salts and chemical agents at final concentrations of 1mM and 0.5% w/v, respectively. The extent of inhibition and activation of enzyme activity was described as percentage of the ratio of residual
activity to complete enzyme activity in the control sample (without addition of metal ions and chemical agents).