CHAPTER 8

STUDY THE SURVIVAL OF BACTERIA IN THE EFFLUENT DURING TREATMENT PROCESS

8.1. Introduction

Survivability is the ability to remain alive or continue to exist. Survival of bacterial consortium in the effluent during the treatment process played an important role in the reduction of pollution. It is necessary to check that the bacteria of our selected consortium is surviving or not in the effluent during treatment process. Morphologically it is difficult to identify the bacteria; therefore a molecular typing for identification of bacteria was used in the study.

Traditional methods used for the typing of bacteria are often based on phenotypic characteristics; such methods include biotyping (Raien et al., 1994), antibiogram analysis (Modi et al., 1987), O serotyping (Gastone et al., 1983), and phage typing (Gaston, 1987). Most of these techniques are not sufficiently sensitive to distinguish different strains or affected by physiological factors. Later on, ribotyping (Bingen et al., 1992; Garaizaret al., 1991; Grattard et al., 1994) and pulsed-field gel electrophoresis (PFGE) (Haertl and Bandlow, 1993) have been used for typing of E. cloacaee, with high discriminatory potential and good reproducibility. Unfortunately, Pulsed-field gel electrophoresis (PFGE) is expensive, time consuming, and labour intensive and can suffer DNA degradation during electrophoresis. Polymerase chain reaction (PCR)-based methods are cheaper, faster, and easier to perform.

ERIC PCR is polymerase chain reaction based method used to confirm the survival of bacteria of selected consortium in the current study. ERIC stands for Enterobacterial Repetitive Intergenic Consensus sequences which are 127-bp imperfect palindromes that occur in multiple copies in the genomes of enteric bacteria.
8.2. Materials and Methods

8.2.1. Equipments, Plastic Ware and Glassware

(i) Equipments

The equipment used in the present study includes water purifier (Millipore), weighing balance (Sartorius), centrifuge (Heraeus Multifuge), horizontal gel electrophoresis (Bangalore GENEI), PCR (Bio-Rad), incubator shaker (New Brunswick – Innova 4300), water bath (Poly science), UV Transilluminator (G-Biosciences), vortex (Spinix), incubator (Hicow), hot air oven (Metrex), autoclave (Hicow), hot plate (Tarson), gel documentation system (Bio digital), laminar air flow (Atlantis), micro pipettes {2.5µl, 10µl, 20µl, 100µl, 200µl, 1000µl, 5000 µl} (Accupipet and Eppendorf).

(ii) Plastic Ware

Plastic ware such as micro centrifuge tubes (0.6 µl, 1.5 µl and 2µl), low retention micro pipette tips (0.2-10µl, 20-200µl, 200-1000µl), cryo vials, micro tip boxes, storage and cryo boxes, -20°C mini cooler and micro centrifuge racks, ice bucket, floating rack, sterile petridishes, PCR tubes (0.2 ml), micro magnetic stirrer bar, purple nitrile gloves, parafilm, cryo tags, tough spot tags and tissue paper (kimwipes) were procured from Tarsons.

(iii) Glassware

Glassware such as measuring cylinders (1000ml, 500 ml, 250 ml, 100ml, 50ml, 25 ml, and 10ml), beakers (50 ml, 100 ml), conical flasks (50ml, 150 ml, 250 ml, and 500 ml,) and bottles (50 ml, 250ml, 500 ml and 1000 ml) were purchased from Borosil.

8.2.2. Study of Bacterial Survival in effluent of pulp and paper mill

8.2.2.1. ERIC PCR

The survival of the bacteria, of selected consortium i.e. PNP3, PNP6 and PNP8 were checked in sludge sample by using ERIC-PCR. For this the sludge samples (100 ml) were collected from bioreactor after two months of initial inoculation. The collected sample (1 ml) was suspended in 9 ml of saline water (0.85% NaCl). To obtain the isolated colonies, the prepared suspension was serially diluted up to 10^-10 dilutions and then spreaded on nutrient agar plates. The plates were then incubated at 35°C for two
days. The bacterial colonies that appeared after two days of incubation enabled us to
determine the total population of bacteria surviving during the treatment time. To create
the DNA fingerprint profile, the obtained bacterial colonies were subjected to ERIC-
PCR. To preserve the obtained bacterial colonies, they were simultaneously streaked on
nutrient agar plates. The primers used for ERIC-PCR were adapted from Singh et al,
(2010) and were procured from TCGA, India. The sequence of the primers used for
ERIC-PCR were ERIC-1 5′-ATG TAA GCT CCT GGG GA T TCA C-3′ and ERIC-2 5′-
AAG TAA GTG ACT GGG GTG AGC G-3′. For colony PCR, each bacterial colony
was picked up and suspended in 25 μl of MiliQ water in sterile centrifuge tube.
Bacterial suspension was incubated at 95°C for 3 min on boiling water bath and
centrifuged at 8,601.6×g for 2 min. Following centrifugation, supernatant was used as
the template for amplifying ERIC sequences. A reaction mixture of 30 μl was prepared
by adding 2.5 μl of 1.5 mM of PCR buffer, 4 μl of template, 0.5 μl of 400 μM of dNTPs
Mix, 1 μl of 20 mM of each primer, 0.5 unit of Taq polymerase and 20 μl of distilled
water. The prepared reaction mixture was incubated in a thermal cycler (BIORAD) and
amplification was carried out at set program. The thermal cycler program starts with an
initial denaturation at 95°C for 2 min and followed by denaturation, annealing and
elongation step for 35 cycles at 92°C for 30 s, 55°C for 80 s and 70°C for 3 min 20 s.
The thermal cycler program ends with the final extension at 70°C for 8 min. The
amplified ERIC PCR products were separated electrophoretically on 2% agarose gel
containing with 1x TAE buffer and EtBr (0.5 μg/ml). The separated ERIC-PCR
products were seen in UV transilluminator and their image was saved using a gel
documentation system. The genomic fingerprints thus obtained were compared with the
fingerprint of the used bacterial consortium, which was considered as a standard for this
analysis. To generate the genomic fingerprint of the bacteria constituting consortium,
their DNA was subjected to ERIC-PCR under similar reaction conditions.

8.2.2.2. Verification Experiment

Verification of survival of bacteria in effluent was done by performing identification of
bacteria which having similarly in DNA fingerprint with our selected bacteria in
consortium 9. Identification of bacteria was done by 16S r RNA gene sequence
analysis. Procedure for identification was used as in chapter 7. Three different bacteria
which having similar DNA band pattern in ERIC-PCR with PNP3, PNP6 and PNP8 were identified and their phylogenetic analysis was done.

8.3. Results

8.3.1. ERIC PCR

The selected consortium i.e. 9 (as mentioned in chapter 5) was further used for the study of bioremediation of pulp and paper mill effluent. Since the effluent are toxic in nature and there is quite a possibility that the bacteria cannot survive in this toxic environment for longer period. So to confirm the survivability of these bacterial strains during the treatment process, ERIC-PCR was performed.

Total 22 bacterial colonies were obtained from the NB agar plates, which was plated with 10⁻⁹ dilution. All 22 bacterial colonies, along with the bacteria of selected consortium were subjected to colony ERIC-PCR. The ERIC-PCR result (Figure 8.1 and 8.2) shows that the three bacterial strains of the selected consortium were serving in the effluent. Lane 1 and 16 of figure 8.1 & 8.2 having DNA ladder (100bp New Englands biolabs), whereas lanes 2, 3 and 4 of figure 8.1 & 8.2 shows the banding pattern obtained from bacterium PNP3, PNP6 and PNP8.

![Figure 8.1: ERIC PCR](image)

Figure 8.1: ERIC PCR: Lane 1 & 16 (100bp New England biolabs), Lane 2 (PP1), Lane 3 (PP 2), Lane 4 (PP 3), Lane 6 & 13 showing presence of another bacteria in the sample. Rest of the lanes is similar to our isolates.
Lane 5-15 shows banding pattern obtained from all 22 bacterial isolates. In fig 8.1 the banding pattern in lane 5, 10, 11 and 14 are similar with that of lane 2, so it is inferred that the bacteria representing the banding pattern in lane 5, 10, 11 and 14 are similar to PNP 3. Similarly lane 7, 8, 9, 12 and 15 are showing similarity with lane 3 i.e., PNP 6 and likewise the banding pattern in lane 8 is showing similarity with the banding pattern of lane 4 i.e., bacteria PNP 8. DNA fingerprint of two bacterial of lane 6 and 13 are different and do not have similarity with any one of our consortium.

Figure 8.2: Lane 1 & 16 (100bp New England biolabs), Lane 2 (PNP 3), Lane 3 (PNP 6), Lane 4 (PNP 8), Lane 6 & 13 showing presence of another bacteria in the sample. Rest of the lanes is similar to our isolates.

In the figure 8.2 lane 1 and lane 16 having DNA ladder (100bp New England biolabs), lane 2, 3 and 4 having PNP3, PNP6 and PNP8 as above. Band pattern of lane 6 and 11 are similar with lane 2 (PNP3). Lane 7 having similarity with lane 3 (PNP6). Lane 4 (PNP8) having similarity with lane 5, 10 and 14. Bacteria of lane 8, 9 and 15 showing negative result of ERIC PCR as don’t having amplification. DNA fingerprint of two bacteria of lane 10 and 12 are different and do not have similarity with any one of our consortium. In this we can say that out of 22 bacteria 6 are PNP3, 6 bacteria are PNP6 and 4 bacteria are similar as PNP8. Total 6 bacteria do not having similarity with any of three bacteria of selected bacterial consortium. These are may be of indigenous bacteria of pulp and paper industry effluent.
8.3.2. Verification Experiment

Total 22 bacterial colonies were obtained from the NB agar plates, which was plated with $10^9$ dilution. ERIC PCR was performed by using genomic DNA of all the 22 bacteria and their DNA band pattern are given in figure 8.1 and 8.2. In figure 8.1, DNA band pattern of bacteria of lane 5, lane 7 and lane 8 having similarity with PNP 3 PNP 6 and PNP 8. Bacterial of lane 5, 7 and 8 were identified by 16S rRNA gene sequencing. 16S rRNA gene sequences of these bacteria are given below in fasta formate and there blast results are in table 8.1. It was found that the bacterium of lane 5 and PNP3 shares 99% identity with *Klebsiella pneumoniae subsp. pneumoniae* MGH 78578 strain ATCC 700721; MGH 78578, accession number NR_074913.1. The bacterium of lane 7 and PNP6 showed 99% identity with the bacterium *Alcaligenes aquatilis* strain LMG 22996, accession number NR_104977.1. However, bacterium of lane 8 and PNP8 have shown, 99% identity with strain *Cronobacter helveticus* strain LMG 23732, accession number NR_104980.1. The 16S rDNA gene sequences of from the study along with the 16S rRNA gene of their closest relative were used to study their phylogeny via phylogenetic tree (figure 8.3). The phylogenetic tree was constructed using neighbour joining method via software MEGA. It was observed that PNP 3, lane 5 and *Klebsiella pneumoniae* (NR_074913.1) are in one clade. PNP 6, lane 7 and *Alcaligenes aquatilis* (NR_104977.1) are in same clade. Similarly PNP8, lane 8 and *Cronobacter helveticus* (NR_104980.1) shares similar clade. It can be conclude that bacterium of lane 5 and PNP 3 are similar i.e. *Klebsiella sp*. Bacterium of lane 7 and PNP 6 are similar i.e. *Alcaligenes sp*. and bacterium of lane 8 and PNP 8 are similar as *Cronobacter sp*. It can also be conclude that bacteria of selected consortium actively present in the effluent during treatment and survive after several round of treatment cycle in SBR.

>Lane5

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GTAGCGCCCTCCGGAAGTTAAGCTACCTACTTTTTTGCAACCCACTCCCATGGTGTAGCGGCGGTGTGTTACCAAGGCCGGGAAACGTATTACCGTAGCATTTCTGATCTA
CGATTACTAAGCGATTCTCGACTTCATGGAAGTCGAGTTCGAGACTCCAATCCGGACTCAGACTATTTATGAGGTCGCTTGCTCTCGGGAGGTCGCTTCTCTTTTGATATATGGCC
ATTGTAGACGTTGCTGTTAGCCCCTGGCTGTTAAGGCCATGATGCTGTAGCGACGCTGTGCTGGCAGTTTCTGACCCATTTCACAACATACCTCAACCACTGCGTCTTTGGGACG
CACCCTCCTTCAGTTTATCACAAGTTGCGCTTCCTGTCGCGGACTTTACCACATATTACAACACGATGAGACAGATGCATTGCTGCTCAGGTGACGGCGCACGAAGGCTGCGTCC
CTTACGGTCAAGGTGCTGCTGCTGAGTTGTTCAAGGAGTCAACGGCAACGAGTCAACGGCAACGAGTCAACGGCAACGAGTCAACGGCAACGAGTCAACGGCAACGAGTCAACGGC
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AACCTTGCGGCCGCTACTCCCAAAGGCGGTCGATTTACAGCGTTAGCTCGGAGAGCCA
CGGCTCAAGGGCCAACAATTCGAATCGTTTACAGGCTGTAACACTGAGGG
TACTTTAATCTGTTTGGCCTCCCCACCTCTTGCGGCTACTGAGTGTGCTGGCAGGG
GGGCGCCCTCCGGCCACCCGGTATTCTCCTCACAGTACTCTACCAGGCTACACCT
GAATTTCTACCCCCCTTACGCTCTGACCTGCTGACCCACTGACCTGCTTGGCAAG
CCGACTTTGCGATTTACAGCGTTAGCTTTGCTCCCCACGCTTTGCACCTGAGCGTCAGT
ATTATCCCAGGGGGCTGCCCTTCGCCACCGGTATTCCTCCACATCTACGCATTTCAC
GGAATTCTACCCCCCTCTACAAGACTCTAGCCTGCCARKTWCGAATGCAGTTCCCA
GGTATGACGGCGAGCCGCGGACAACTGCCACTGTTGCAAGAGGGCTGTAACACTGAG
GGTACAGACATGGCATGAGGCCGAAGGTCCCCAATTGGTCTTGCGACATTATGCA
GGTATTAGCTACGGTTTCCAGTATGTATTCACCCTCTCTCACTGAGCTAGTACAT
GCACTCACCAGCGCCTGCAGCTCAGTCAACCCCACACATGACATCTCGTACTGGCCTCA

>Lane7

AGCGGCTCTCCTTACGGTATTAGCTACCTACTTCTTGTTGAACCCCCACTCCCATGTTGT
GACGCGGCGGTTACGTTCAAGGACCCGGGAGACGAATCTCCGGAACATTTCTTCAACC
GATTACAGCGGATTCGCCATCAGCAGTAGTTGCAAGACTGAGCTCCGGAATACCT
GATCCGCGGTTAGTACCTGACTCCCCGCTGCCGCTGCCGCTACCCGCTTAACC
ATTGATAGCGTGTGACCCTACCAATGAGGGCTAGAAGTTGACTTCCATCCC
CACTCTTCTCCTCATTGAGTACCACGCTTACCTAGTGTTTCGATAGCTCACACTA
ATGCAACAGGGTGTGCGATCAGCATTCTGGTTGCTGTGCTGACCCACTGACCTGCTT
TTGGCGTACTTCTACGAGCTTGTTAGTACGTTCAAGAGGGCTGTAACACTGAG
GGTATTAGCTACGGTTTCCAGTATGTATTCACCCTCTCTCACTGAGCTAGTACAT
GCACTCACCAGCGCCTGCAGCTCAGTCAACCCCACACATGACATCTCGTACTGGCCTCA

>Lane8

AAAGTGTTAGCGCTCTCCGCAAGGTTAAGCTACCTACTTCTTGGTGAACCCCCACTCCCATGTTGT
GACGCGGCGGTTACGTTCAAGGACCCGGGAGACGAATCTCCGGAACATTTCTTCAACC
GATTACAGCGGATTCGCCATCAGCAGTAGTTGCAAGACTGAGCTCCGGAATACCT
GATCCGCGGTTAGTACCTGACTCCCCGCTGCCGCTGCCGCTACCCGCTTAACC
ATTGATAGCGTGTGACCCTACCAATGAGGGCTAGAAGTTGACTTCCATCCC
CACTCTTCTCCTCATTGAGTACCACGCTTACCTAGTGTTTCGATAGCTCACACTA
ATGCAACAGGGTGTGCGATCAGCATTCTGGTTGCTGTGCTGACCCACTGACCTGCTT
TTGGCGTACTTCTACGAGCTTGTTAGTACGTTCAAGAGGGCTGTAACACTGAG
GGTATTAGCTACGGTTTCCAGTATGTATTCACCCTCTCTCACTGAGCTAGTACAT
GCACTCACCAGCGCCTGCAGCTCAGTCAACCCCACACATGACATCTCGTACTGGCCTCA

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CAGGAGCTGACGACAGCCATCGACGACCTGTCTCAGATCGTTCCCAAGGCAAGCAACCAATCATCTCTGGAAAGTTCTCTGGATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCCTCACGGCGGCTTGCGGGGCCCCGTCACATCTATTGTAGTTTAGCTATCGACTCTCCAGTCAACTCTCAACCTCAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTA

Table 8.1: Blast Analysis

<table>
<thead>
<tr>
<th>Name of isolate</th>
<th>Closest relative of the isolate</th>
<th>Accession number</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNP3</td>
<td><em>Klebsiella pneumoniae</em> subsp. pneumoniae MGH 78578 strain ATCC 700721; MGH 78578</td>
<td>NR_074913.1</td>
<td>99%</td>
</tr>
<tr>
<td>Lane 5</td>
<td><em>Klebsiella pneumoniae</em> subsp. pneumoniae MGH 78578 strain ATCC 700721; MGH 78578</td>
<td>NR_074913.1</td>
<td>99%</td>
</tr>
<tr>
<td>PNP6</td>
<td><em>Alcaligenes aquatilis</em> strain LMG 22996</td>
<td>NR_104977.1</td>
<td>99%</td>
</tr>
<tr>
<td>Lane 7</td>
<td><em>Alcaligenes aquatilis</em> strain LMG 22996</td>
<td>NR_104977.1</td>
<td>99%</td>
</tr>
<tr>
<td>PNP8</td>
<td><em>Cronobacter helveticus</em> strain LMG 23732</td>
<td>NR_104980.1</td>
<td>99%</td>
</tr>
<tr>
<td>Lane 8</td>
<td><em>Cronobacter helveticus</em> strain LMG 23732</td>
<td>NR_104980.1</td>
<td>99%</td>
</tr>
</tbody>
</table>

Figure 8.3: Phylogenetic tree of the 16S r RNA gene sequences belonging to phylum *Klebsiella*, *Alcaligenes*, and *Cronobacter*. The bacteria from this study are denoted as PNP 3, PNP 6, PNP 8, Lane 5, Lane 7 and Lane 8.
8.4. Discussion

The genomic fingerprinting method employed is based on the use of DNA primers corresponding to naturally occurring repetitive elements in bacteria, and the polymerase chain reaction (rep-PCR). Bacterial genomes are generally considered to be streamlined, and yet numerous families of short (30–150 bp) interspersed repetitive sequences have been described (Lupski and Weinstock, 1992; Bachellier et al., 1996; Tobes and Ramos, 2005). Three families of repetitive sequences have been identified such as the 35-40bp repetitive extragenic palindromic (REP) sequence, the 124-127bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and 154bp BOX element. Little is known about the origins, evolution, mode of generation, and possible function of these elements. Some repetitive sequences have been reported to act as binding sites for a variety of proteins, including DNA polymerase and DNA gyrase (Gilson and Hofnung, 1990). Most short bacterial repetitive sequences are imperfect palindromes, with the potential to form secondary structures, which may enhance mRNA stability (Newbury et al., 1987). Alternatively, most repetitive elements may be non functional junk.

ERIC sequences also described as intergenic repetitive units differ from most other bacterial repeats in being distributed across a wider range of species. ERIC sequence repeat includes a conserved inverted repeat and is located in non-coding transcribed regions of the chromosome, in either orientation with respect to transcription. ERIC sequences were initially described in *Escherichia coli, Salmonella typhimurium* (now Salmonella enterica serovar Typhimurium), and other members of the Enterobacteriaceae, as well as *Vibrio cholera* (Sharples and Lloyd, 1990; Hultin et al., 1991). Initially the presence of ERIC sequences has been demonstrated only in gram-negative bacteria. Later on Belkum et al., used this fingerprinting technique to type *Staphylococcus aureus* strains. ERIC-PCR has subsequently been used in several studies of *E. coli* diversity (Lipman et al., 1995; Manges et al., 2004; Jeong et al., 2005; Ramchandani et al., 2005). Wei et al., (2004) determined the sequences of genomic fragments amplified using ERIC-PCR primers from unidentified microbial strains within human fecal samples. In 1992, de Bruijn, examined the distribution of dispersed repetitive DNA sequences in the genomes of a number of gram-negative soil bacteria using conserved primers corresponding to ERIC sequences by PCR.
ERIC sequences are highly conserved and their position in enterobacterial genomes varies between different species and has been used as a genetic marker to characterize isolates within a bacterial species (Versalovic et al., 1991). In ERIC-PCR, a banding pattern is obtained by amplification of genomic DNA located between ERIC elements or between ERIC elements and other repetitive DNA sequences. This technique uses consensus primers in the PCR to amplify DNA sequences located between successive repetitive elements (Hulton et al., 1991).

The results showed that out of 22 bacteria obtained from serial dilution, 6 were PNP3, 6 PNP6 and 4 bacteria were similar as PNP8. The rest of the 6 bacteria do not having similarity with any of three bacteria of selected bacterial consortium. They are might be of indigenous bacteria of pulp and paper industry effluent. So, it is concluded that the consortium bacteria have survived in the effluent long time after their inoculation.

8.5. Conclusion

The current study also emphasized the survival of bacterial isolates of selected consortium in the effluent after successful compilation of the bioremediation study. Results show the presence of microbial consortia in the sludge sample after treatment. Survival of bacterial consortium in the effluent during the treatment process played an important role in the reduction of pollution.