Chapter 6

Results and Discussion

The experimental results obtained in the present study are described in detail in this chapter. In particular, the results obtained for (a) phytotoxicity of perchlorate on plant systems, (b) batch study of perchlorate degradation, (c) isolation and characterization of Perchlorate Respiring Bacteria (PRB), (d) effect of environmental parameters on perchlorate degradation, (e) growth curve and kinetics of PRB in batch reactor and (f) microbial degradation of perchlorate in STBR system using real effluent and synthetic effluents are presented in different sections and discussed.

The first part of the section 6.1 discussed about the phytotoxicity of perchlorate on *Lycopersicum esculentum*, *Vigna mungo*, *Vigna radiate*, *Zea mays* seeds. The effect of perchlorate on seed germination and root elongation was examined. The growth inhibitory effect against plant systems has been studied. Further, the presence of perchlorate in seeds were qualitatively determined by FTIR analysis and summarized. Finally, the experimental uncertainty is discussed.

The second part of this chapter (section 6.2) discussed about the results of perchlorate degradation by mixed microbial consortium in batch bio-reactor. In particular, sulphidogenesis and perchlorate reduction was examined. The ORP, pH and salinity profile inside the bio-reactor has been studied. The MLSS behaviour during the reactor operation has also been reported.

Subsequent section 6.3 of this chapter discussed about the results obtained for isolation and characterization of pure isolates of PRB. In particular, cellular morphology, metabolic characteristics, gram staining and scanning electron microscopic (SEM) analysis has been described. Further, bio-chemical characterization and molecular characterization of pure isolates was examined. The 16S rRNA gene sequence data of the isolates published in NCBI- Gene Bank is given in Appendix A. The phylogenetic tree constructed for strains ARJR SMBS, LMN and LMN SMBS is shown in Appendix B. The restriction site analysis of 16S rRNA gene sequence data obtained in the present study is reported in Appendix C. Finally, the
secondary structure obtained for gene sequence data in the present study is presented in Appendix D.

The section 6.4 of this chapter discussed about the results obtained for effect of environmental parameters on perchlorate degradation. In particular, acetate as an electron donor in perchlorate biodegradation was examined. The inhibitory effect of nitrate on perchlorate removal has been analysed. The effect of pH and saline tolerance of mixed cultures have been examined and summarized at the end of this section.

The following section of this chapter discussed about the results obtained for growth curve and kinetics of PRB in batch reactor. In particular, the growth curve study, dry weight cell measurement of PRB has been studied. The Colony Forming Unit (CFU) analysis of pure isolates was examined. Finally, the results of perchlorate degradation measurement and bacterial growth kinetic study have been summarized with a closure.

At the end of this chapter (section 6.6) the results obtained for microbial degradation of perchlorate in STBR system for the synthetic effluent and real effluent, by the strain ARJR SMBS has been reported. The bioaccumulation of chloride and the biomass profile of strain ARJR SMBS in STBR were analyzed. The mass balance for perchlorate degradation was examined and then, the effect of pH, salinity and ORP on STBR system performance was evaluated. Finally, the results obtained for microbial growth kinetics of strain ARJR SMBS in STBR system has been presented with a closure.

6.1 PHYTOTOXICITY OF PERCHLORATE

Phytotoxicity of ClO$_4^-$ was carried out according to US EPA protocol (US EPA, 1996). Seed germination and root elongation is a rapid and widely used acute phytotoxicity test that have several advantages like sensitivity, simplicity, low cost and suitability for unstable chemicals or samples (Munzuroglu and Geckil, 2002; Wang et al, 2001). Toxicity studies of ClO$_4^-$ were done on four seeds namely, *L.*
*esculentum*, *V. mungo*, *V. radiate* and *Z. mays* to find out the effects of ClO$_4^-$ on seed germination and root elongation activity. The results of the formulated ClO$_4^-$ doses (10, 25, 50, 75 and 100 mg L$^{-1}$) on treated plant systems are presented in Table 6.1 and explained as below.

### 6.1.1 EFFECT OF PERCHLORATE ON SEED GERMINATION

Kordan have reported that germination is normally known as a physiological process which begins with water inhibition by seeds and culmination in the emergence of the rootlet (Kordan, 1992). The germination (%) for the sample treated with 100 mg L$^{-1}$ of perchlorate in case of *L. esculentum* and *Z. mays* were 83.33 % and 66.67 % respectively where as control seeds showed 100 % germination on both the cases. The summary of effect of perchlorate on seed germination of *L. esculentum* (A), *V. mungo* (B), *V. radiate* (C) and *Z. mays* (D) are showed in Figure 6.1. The control treated seeds has taken as 100 % germination in all the test systems. Results reveal that *Z. mays* showed the lowest germination percentage compared to other test plant seeds.

![Figure 6.1: Effect of perchlorate on germination (%) of *L. esculentum* (A), *V. mungo* (B), *V. radiate* (C) and *Z. mays* (D). Data are presented as mean ± SD of three independent experiments with 95% confidence interval.](image-url)
Table 6.1: Root elongation and germination index test results obtained for *L. esculentum*, *V. mungo*, *V. radiate* and *Z. mays* after treatment with varying initial perchlorate concentrations (Control=0, A=10, B=25, C=50, D=75 and E=100 mg L⁻¹ perchlorate) in this study.

<table>
<thead>
<tr>
<th>TO with varying ClO₄⁻ (mg L⁻¹)</th>
<th>Root Elongation (cm)</th>
<th>Germination Index</th>
<th>RSG*</th>
<th>RRG**</th>
<th>Germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L. esculentum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (0 mg L⁻¹)</td>
<td>4.1 ± 0.21</td>
<td>100 ± 0.0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>A (10 mg L⁻¹)</td>
<td>3.48 ± 0.62</td>
<td>80.22 ± 7.29</td>
<td>93.33</td>
<td>84.79</td>
<td>93.33</td>
</tr>
<tr>
<td>B (25 mg L⁻¹)</td>
<td>3.33 ± 0.52</td>
<td>76.7 ± 2.86</td>
<td>93.33</td>
<td>81.3</td>
<td>93.33</td>
</tr>
<tr>
<td>C (50 mg L⁻¹)</td>
<td>3.35 ± 0.46</td>
<td>76.09 ± 1.3</td>
<td>93.33</td>
<td>81.6</td>
<td>93.33</td>
</tr>
<tr>
<td>D (75 mg L⁻¹)</td>
<td>2.86 ± 0.45</td>
<td>62.77 ± 1.81</td>
<td>90</td>
<td>69.75</td>
<td>90</td>
</tr>
<tr>
<td>E (100 mg L⁻¹)</td>
<td>2.59 ± 0.28</td>
<td>53.19 ± 1.18</td>
<td>83.33</td>
<td>63.25</td>
<td>83.33</td>
</tr>
<tr>
<td><strong>V. mungo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (0 mg L⁻¹)</td>
<td>4.52 ± 0.19</td>
<td>100 ± 0.0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>A (10 mg L⁻¹)</td>
<td>3.43 ± 0.33</td>
<td>73.07 ± 2.66</td>
<td>96.67</td>
<td>75.88</td>
<td>96.67</td>
</tr>
<tr>
<td>B (25 mg L⁻¹)</td>
<td>2.9 ± 0.19</td>
<td>64.15 ± 4.42</td>
<td>100</td>
<td>64.15</td>
<td>100</td>
</tr>
<tr>
<td>C (50 mg L⁻¹)</td>
<td>2.69 ± 0.81</td>
<td>59.58 ± 6.81</td>
<td>100</td>
<td>59.58</td>
<td>100</td>
</tr>
<tr>
<td>D (75 mg L⁻¹)</td>
<td>2.55 ± 0.18</td>
<td>56.41 ± 4.09</td>
<td>100</td>
<td>56.41</td>
<td>100</td>
</tr>
<tr>
<td>E (100 mg L⁻¹)</td>
<td>1.99 ± 0.18</td>
<td>39.95 ± 8.2</td>
<td>90</td>
<td>44.09</td>
<td>90</td>
</tr>
</tbody>
</table>

TO indicates Test Organism; *RSG-Relative Seed Germination rate; **RRG- Relative Root Growth; Data are presented as mean ± standard deviation of three independent experiments with 95 % confidence interval.
Table 6.1: Continued

<table>
<thead>
<tr>
<th>TO with varying ClO$_4^-$ (mg L$^{-1}$)</th>
<th>Root Elongation (cm)</th>
<th>Germination Index</th>
<th>RSG*</th>
<th>RRG**</th>
<th>Germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>V. radiate</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control (0 mg L$^{-1}$)</td>
<td>4.02 ± 0.42</td>
<td>100 ± 0.0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>A (10 mg L$^{-1}$)</td>
<td>3.41 ± 0.69</td>
<td>82.70 ± 8.76</td>
<td>96.67</td>
<td>84.91</td>
<td>96.67</td>
</tr>
<tr>
<td>B (25 mg L$^{-1}$)</td>
<td>3.23 ± 0.56</td>
<td>78.29 ± 5.3</td>
<td>96.67</td>
<td>80.51</td>
<td>96.67</td>
</tr>
<tr>
<td>C (50 mg L$^{-1}$)</td>
<td>3.13 ± 0.43</td>
<td>74.95 ± 6.3</td>
<td>96.67</td>
<td>77.93</td>
<td>96.67</td>
</tr>
<tr>
<td>D (75 mg L$^{-1}$)</td>
<td>2.62 ± 0.17</td>
<td>65.08 ± 4.32</td>
<td>100</td>
<td>65.08</td>
<td>100</td>
</tr>
<tr>
<td>E (100 mg L$^{-1}$)</td>
<td>2.27 ± 0.68</td>
<td>54.52 ± 9.13</td>
<td>96.67</td>
<td>56.63</td>
<td>96.67</td>
</tr>
<tr>
<td><strong>Z. mays</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (0 mg L$^{-1}$)</td>
<td>2.14 ± 0.03</td>
<td>100 ± 0.0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>A (10 mg L$^{-1}$)</td>
<td>2.13 ± 0.14</td>
<td>89.55 ± 5.49</td>
<td>90</td>
<td>99.35</td>
<td>90</td>
</tr>
<tr>
<td>B (25 mg L$^{-1}$)</td>
<td>1.38 ± 0.13</td>
<td>53.49 ± 2.01</td>
<td>83.33</td>
<td>64.48</td>
<td>83.33</td>
</tr>
<tr>
<td>C (50 mg L$^{-1}$)</td>
<td>1.26 ± 0.2</td>
<td>50.9 ± 6.31</td>
<td>86.67</td>
<td>59.03</td>
<td>86.67</td>
</tr>
<tr>
<td>D (75 mg L$^{-1}$)</td>
<td>1.01 ± 0.11</td>
<td>35.19 ± 3.18</td>
<td>73.33</td>
<td>47.34</td>
<td>73.33</td>
</tr>
<tr>
<td>E (100 mg L$^{-1}$)</td>
<td>0.72 ± 0.08</td>
<td>22.56 ± 4.25</td>
<td>66.67</td>
<td>33.64</td>
<td>66.67</td>
</tr>
</tbody>
</table>

TO indicates Test Organism; *RSG-Relative Seed Germination rate; **RRG- Relative Root Growth; Data are presented as mean ± standard deviation of three independent experiments with 95 % confidence interval.
6.1.2 EFFECT OF PERCHLORATE ON ROOT ELONGATION

The results of the root elongation for the sample treated with 100 mg L\(^{-1}\) of perchlorate in case of *V. mungo* and *Z. mays* were 1.99 cm and 0.72 cm respectively where as control seeds showed a visible growth difference (4.1 cm and 2.14 cm) in both the test systems. The summary of effect of perchlorate on root length of *L. esculentum* (A), *V. mungo* (B), *V. radiate* (C) and *Z. mays* (D) are showed in Figure 6.2. The measurement of root elongation study also reveals that the toxicity effect of perchlorate was predominant in *Z. mays* compared to other test seeds.

![Figure 6.2: Effect of perchlorate on root length](image)

Figure 6.2: Effect of perchlorate on root length of *L. esculentum* (A), *V. mungo* (B), *V. radiate* (C) and *Z. mays* (D). Data are presented as mean ± SD of three independent experiments with 95% confidence interval.

6.1.3 GROWTH INHIBITORY EFFECT AGAINST PLANT SYSTEMS

Perchlorate of greater than 50 mg L\(^{-1}\) showed considerable level of phytotoxicity action against all the four tested seeds and the effect was more in *Z. mays* compared to other test seeds analyzed for this study. Perchlorate of less than 25 mg L\(^{-1}\), showed negligible inhibitory effect on seed germination and root elongation in all the tested plant seeds. Control treated seeds showed 100 % root germination in all cases and this suggests that as perchlorate concentration increases, the toxicity effect towards test plant systems also increases. It is evident from Figure 6.3 that,
exposure to perchlorate, at 100 mg L\(^{-1}\), did affect seed germination and there was visible or textural difference in the 100 mg L\(^{-1}\) plant seeds and the control plant seed samples of \(Z\). \textit{mays}. Germination of \(Z\). \textit{mays} was shown, since it was more sensitive to perchlorate.

Figure 6.3: The visible growth difference observed with perchlorate exposure in \(Z\). \textit{mays} (A) control seeds (0 mg L\(^{-1}\) perchlorate) and, (B) Seeds treated with 100 mg L\(^{-1}\) perchlorate.

Seyfferth and Parker showed that, the uptake rate of perchlorate in different genotypes of lettuce exhibited a linear relationship of perchlorate accumulation in tissues with increasing perchlorate concentration in culture medium (Seyfferth and Parker, 2007). They also predicted that the perchlorate accumulation had a larger influence on geological location rather than the genotype. Many crops like lettuce, cucumbers, tomatoes, carrots, spinach, alfalfa, fruits grains and feed plants concentrate perchlorate in their tissues, when grown with contaminated water. Our results suggests that a linear relationship between ClO\(_4^-\) concentration and bioaccumulation rate which causes the inhibitory effect on seed germination and root elongation processes. In 1999 from a study conducted by the US EPA, perchlorate was accumulated in the leaves of lettuce seedlings by factors of 100 times or more (US EPA, 2002). Therefore, vegetables irrigated by water with even low levels of perchlorate could deliver large doses of the toxin to consumers. Further, the present study also emphasis that ClO\(_4^-\) of higher concentration has been found to have growth inhibitory effect towards plant community in the environment.
6.1.4 FTIR ANALYSIS

Perchlorate is a polyatomic anion with tetrahedral symmetry which exhibit four vibrational modes at the following frequencies: 935 (Cl-O), 460 (O-Cl-O), 1050-1170 (Cl-O) and 630 (O-Cl-O) cm\(^{-1}\). Previous reports shows that Raman spectroscopy was used to detect perchlorate in lettuce extracts (Williams et al, 2001) and the band intensity at 1092 and 1331 cm\(^{-1}\) are assigned to be for perchlorate and nitrate respectively (Mosier-Boss, 2006). Present study FTIR spectra (sodium perchlorate + KBr pellets) showed the band width maximum at 1090.55 cm\(^{-1}\) for perchlorate (control) and experimental ones (seed powdered sample) obtained the peaks with in 1090.55 ± 31 cm\(^{-1}\). In all the test samples, we have analyzed the FTIR spectra for the highest (100 mg L\(^{-1}\)) and lowest (10 mg L\(^{-1}\)) concentration of perchlorate treated in plant seeds. The band width observed for \(L.\) esculentum was 1059.69 cm\(^{-1}\) and 1060.66 cm\(^{-1}\) at 10 and 100 mg L\(^{-1}\) of perchlorate concentration respectively are showed in Figure 6.4. The band width observed for \(V.\) mungo was 1062.59 cm\(^{-1}\) and 1076.08 cm\(^{-1}\) at 10 and 100 mg L\(^{-1}\) of perchlorate concentration respectively (Figure 6.5). The band width observed for \(V.\) radiate was 1077.04 cm\(^{-1}\) and 1077.04 cm\(^{-1}\) at 10 and 100 mg L\(^{-1}\) of perchlorate concentration respectively (Figure 6.6). The band width observed for \(Zea\) mays was 1078.97 cm\(^{-1}\) and 1075.12 cm\(^{-1}\) at 10 and 100 mg L\(^{-1}\) of perchlorate concentration respectively (Figure 6.7). The small changes in peak position may be due to the effect of various environmental conditions like weathering, mechanical treatment such as grinding, pressure and temperature changes (Brindley et al, 1986).
Figure 6.4: FTIR spectra of *Lycopersicum esculentum* at 10 and 100 mg L\(^{-1}\) of perchlorate concentration: A– 10 mg L\(^{-1}\) spectra, B– 100 mg L\(^{-1}\) spectra, C– control spectra (perchlorate + KBr).

Figure 6.5: FTIR spectra of *Vigna mungo* at 10 and 100 mg L\(^{-1}\) of perchlorate concentration: A– 10 mg L\(^{-1}\) spectra, B– 100 mg L\(^{-1}\) spectra, C– control spectra (perchlorate + KBr).
Figure 6.6: FTIR spectra of *Vigna radiate* at 10 and 100 mg L\(^{-1}\) of perchlorate concentration: A– 10 mg L\(^{-1}\) spectra, B– 100 mg L\(^{-1}\) spectra, C– control spectra (perchlorate + KBr).

Figure 6.7: FTIR spectra of *Zea mays* at 10 and 100 mg L\(^{-1}\) of perchlorate concentration: A– 10 mg L\(^{-1}\) spectra, B– 100 mg L\(^{-1}\) spectra, C– control spectra (perchlorate + KBr).
All the 10 mg L⁻¹ perchlorate treated seeds (10 mg L⁻¹ perchlorate + seed + KBr) FTIR spectra showed lesser peak height than the respective seeds treated with perchlorate of higher concentration (100 mg L⁻¹ perchlorate + seed + KBr). The spectral analysis of samples are made on the basis of the magnitude and relative intensities of the recorded spectra and in the analogy with the assignments made by other researchers on the similar type of perchlorate compounds. The accuracy of chlorate evaluation increases using the control derivative spectra. Our initial observation related to FTIR spectra revealed that all the solid seed samples are dominated by the –OH stretch which exhibit absorption bands at 3250-3350 cm⁻¹, 2940-2920 cm⁻¹ dominated by methylene group (–CH₂–), 1660-1620 cm⁻¹ dominated by nitrates group (–ONO) and 1420-1400 cm⁻¹ dominated by primary amides group (–CONH₂), thus indicating the similarity of the chemical composition and qualitative identity. The C–H bands associated with methyl and methylene groups that usually occur at 2920 cm⁻¹ (CH asymmetric stretch) and at 2860 cm⁻¹ (CH symmetric stretch) are superimposed as a shoulder of the broad O–H band. Other bands characteristic to humic substances appear at 1633.41, 1640.16, 1643.05, 1630 and 1411.64 cm⁻¹. Also at 1630-1640 cm⁻¹ appear the vibration band for absorbed water, C=C, C=O from amide and benzophenones from humic substances. Each seed sample spectrum appears to have distinctive spectra in the 400-4000 cm⁻¹ region with maximum intensive peak at 3289-3434.59 cm⁻¹, most probably due to the presence of various organic constituents present in test seed (Cox et al, 2000).

The summary of common observed absorption frequencies (cm⁻¹) and its tentative assignment of bond vibrations for *L. esculentum*, *V. mungo*, *V. radiate* and *Z. mays* after treatment with 10 and 100 mg L⁻¹ perchlorate are summarized in Table 6.2. In the control sample (sodium perchlorate + KBr) spectra, water has strong IR absorbance at three prominent bands around 3349.75 (O-H stretching), 2025.85 (water association), and 1617.02 cm⁻¹ (H-O-H bending). Its broadness is generally attributed to hydrogen bonding. If the sample particle size is minimum, then the sample (seed in powder form) in most disordered state and it produces better FTIR spectra. The absence of some peaks from sample to sample, suggesting that perchlorate interacted with the seeds and created a change in its composition or due to the differenece in particle size which is used for FTIR characterization.
Table 6.2: Summary of the common observed absorption frequencies (cm$^{-1}$) in FTIR spectra and its tentative assignment of bond vibrations for Control, *L. esculentum*, *V. mungo*, *V. radiate* and *Z. mays* after treatment with 10 mg L$^{-1}$ and 100 mg L$^{-1}$ of perchlorate.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Common Observed Frequency (cm$^{-1}$)</th>
<th>Mode of Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Sodium perchlorate + KBr)</td>
<td>640.25, 1617.02, 1090.55, 2025.85</td>
<td>O-Cl-O vibration, C-C stretch, Cl-O vibration, H-O-H bending, water association</td>
</tr>
<tr>
<td><em>L. esculentum</em></td>
<td>1059, 1640, 2925.48</td>
<td>Cl-O vibration, -ONO- stretch, -CH$_2$- vibration</td>
</tr>
<tr>
<td><em>V. mungo</em></td>
<td>1415, 1633.41</td>
<td>C-S stretch, Ring Vibration (boat)</td>
</tr>
<tr>
<td><em>V. radiate</em></td>
<td>1077.05</td>
<td>Cl-O vibration mode</td>
</tr>
<tr>
<td><em>Z. mays</em></td>
<td>1412, 1637.27, 3398.92</td>
<td>C-N-C bend, -ONO- stretch, NH$_2$ stretch</td>
</tr>
</tbody>
</table>
6.1.5 CLOSURE

The fate of pollutants uptake and bioaccumulation mechanism are not yet well understood which effects the seed germination and root elongation processes. This is because of the synergistic or antagonistic effects of complex physico-chemical or biochemical processes, such as adsorption, binding to components present in seeds; reaction with other compounds, particle size distribution, biodegradation etc which can modify specific pollutants properties. Present study showed the toxicity effect of perchlorate in seed germination and root elongation is a concentration-dependent process. From the obtained results, greater than 50 mg L$^{-1}$ of perchlorate was found to be toxic against plant systems. Among the four test systems (*L. esculentum*, *V. mungo*, *V. radiate* and *Z. mays*), *Z. mays* was more sensitive to perchlorate than other plants. The perchlorate compound can be qualitatively identified in the FTIR absorption spectra by the characteristic band 1090.55 ± 30 cm$^{-1}$. The limitation of study is that the detection of trace amount of perchlorate present in aqueous matrices is not possible due to the intense bands which may occur due to OH stretching and bending modes of water that dominate large regions of the infrared spectrum. The results also provide evidence that a linear relationship between perchlorate concentration and bioaccumulation rate was observed in plant systems and leads to a growth inhibitory effect. Nevertheless, it act as an effective remedies for removing perchlorate from the contaminated sources such as soil and water present in the environment.
6.2 BATCH STUDY OF PERCHLORATE DEGRADATION

The raw sludge sample was obtained from waste water treatment plants is used as the seed for development of active potential perchlorate degrading microorganisms through slow adaptation and acclimatization process. After the preliminary treatment, the raw sludge was introduced into the BC (total volume 3L, working volume 2L) for acclimatization study. The fed batch reactor for perchlorate reduction was operated for 156 days. The start up period was nearly one month during which the system attained more or less stable condition. Reduction of the initial influent perchlorate concentration of 100 mg L⁻¹ achieved within 10 days. The first one month of low perchlorate removal rate could be attributed to acclimatizing of the sludge as well as for development of an active PRBs consortium. After domestication, the perchlorate-reducing ability of the sludge was greatly improved and the influent perchlorate concentration was gradually increased to 600 mg L⁻¹ and subsequently the inlet concentration maintained the same for rest of the period. The initial perchlorate, pH, ORP, salinity, conductivity, TDS and MLSS level of the raw sludge was found to be: 0.07-0.65 mg L⁻¹, 7.2, 5.9 mV, 2.34 ppt, 4.18 mS, 1.27 ppt and 1.35 g L⁻¹ respectively. Perchlorate degradation was monitored daily by estimating the residual perchlorate in the reactor liquid.

Figure 6.8: Profile of influent & effluent perchlorate and percentage of reduction in the batch reactor. The data represent the mean values of three independent experiments.
Figure 6.8 shows the profile of influent and effluent perchlorate concentrations and percentage removal in the batch container during the period of study. Batch experiments were conducted to study the effect of various parameters using the prior acclimatized effluent sludge. The gradual acclimation to increased perchlorate/salinity resulted in better performance of biological treatment processes and a higher perchlorate reduction rate, as reported by Kincannon and Gaudy 1966; Burnett, 1974 and Park and Marchand, 2002. The acclimatization of reactor sludge to a maximum level of $\text{ClO}_4^-$ (600 mg L$^{-1}$) was achieved on 40$^{\text{th}}$ day of the system start up and the same concentration was able to maintain continuously till the end of reactor analysis.

6.2.1 SULPHIDOGENESIS AND PERCHLORATE REDUCTION

Sulphidogenesis had a positive attribute to the perchlorate reduction in the batch container. Being a strong oxygen scavenger, it remove even traces of oxygen in the reactor there by promoting the facultative anaerobic PRBs to use $\text{ClO}_4^-$ as terminal electron sink. The sulphate present in the mineral medium ((NH$_4$)$_2$SO$_4$, MgSO$_4$.7H$_2$O, FeSO$_4$.7H$_2$O) was found to be decreasing under anaerobic conditions by Sulphate Reducing Bacteria (SRB) which results in the formation of hydrogen sulphide. The anaerobic effluent had a typical smell of H$_2$S during the period of study. The dissolved sulphide imparted black colour to the container sludge and the sludge floc with sulphide precipitation observed inside the reactor as shown in Figure 6.9. Studies show that several PRB have been shown to use inorganic electron donors such as H$_2$ (Giblin et al, 2000; Logan and LaPoint, 2002; Zhang et al, 2002), sulphide and Fe (II) (Bruce et al, 1999; Achenbach et al, 2001).

![Image](Figure 6.9: The gas production profile and the sludge floc with sulphide precipitation (arrow) observed inside the batch reactor during acclimatization process.)
Mass transfer was expected to be an important rate limiting factor in the overall process. The impact of increasing $S^0$ concentrations on ClO$_4^-$ reduction kinetics was dependent on particle size. The increase in ClO$_4^-$ reduction rate is limited with increase in powder $S^0$ concentrations, whereas the rates increased dramatically with granules $S^0$ concentration. The enrichment culture was observed to have the capacity to ferment $S^0$ in a reaction known as sulphur disproportionation which is shown in equation (6.1) below:

$$4 \text{S}^0 + 4\text{H}_2\text{O} \rightarrow 3\text{H}_2\text{S} + \text{SO}_4^{2-} + 2\text{H}^+ \quad (6.1)$$

Studies show that $S^0$ can be used as an effective electron donor for ClO$_4^-$ reduction in a chemolithotrophic enrichment culture derived from aerobic activated sludge (Sholeh et al, 1997). In 1999 Bruce observed that sulphide (H$_2$S) oxidation linked to perchlorate reduction in a PRB isolate, strain CKB (Bruce et al, 1999). A sulphide oxidizing PRB, *Dechloromonas agitata* was isolated that reduced perchlorate to chloride but only partially oxidize H$_2$S to $S^0$ (Achenbach et al, 2001).

6.2.2 ORP, PH AND SALINITY PROFILE

The perchlorate degradation mechanism is predominant under oxygen free environment, an attempt is made to study the effect of oxidation reduction state during perchlorate reduction process inside the batch reactor. The ORP measurement was taken into account throughout the reactor at regular intervals. Removal rates were found higher when dissolved oxygen level is low as shown in Figure 6.10. ORP was taken as an indicator of the extent of anoxic environment status within the system. It is used as an effective method of monitoring and an important control parameter in wastewater treatment processes. ORP measurement showed the ability of water samples observed to be either accept or donate electrons and it is expressed in millivolt. ORP rates were calculated from the slope of the dissolved oxygen concentration change over time. The initial ORP of the raw sludge was found to be 5.9 mV.
Figure 6.10: Profile of ORP and percentage of perchlorate reduction in the batch bioreactor during the period of study. The data represent the mean values of three independent samples.

Song and Logan (2004) showed that, a perchlorate-degrading culture *Dechloromonas* sp. KJ which degraded 5 mg L\(^{-1}\) of ClO\(_4^-\) was returned to reducing conditions under anaerobic environment after a limited period of 8 h of aeration. Previous reports showed that high concentrations of dissolved oxygen (DO) repress perchlorate degradation (van Ginkel et al, 1996; Wallace et al, 1998) and that all perchlorate reducing bacteria isolates are either facultative or microaerophiles (Bruce et al, 1999; Achenbach et al, 2001; Wallace et al, 1998). Present study suggests that, during the reactor operation, the optimum range of ORP values should be in the range of -75 mV to -205 mV and which leads to removal of 5 to 600 mg L\(^{-1}\) of ClO\(_4^-\) present in the influent water. This leads to a biotransformation mechanism such as methanogenesis which is well known to occur at low redox potentials. Lovley and Phillips (1988) reported that, methanogenesis is generally occurs at -200mV or lower redox potentials. In fact, the oxygen produced during perchlorate degradation does not accumulate in pure cultures; it is consumed by facultative microbes faster than it is produced (Chaudhuri et al, 2002). Because of this reason, during the reactor operation, the oxygen which is generated would be consumed by the facultative microbes present in the ClO\(_4^-\) acclimatized effluent sludge.
Figure 6.11: Profile of pH, Salinity variation and percentage reduction observed in the perchlorate reducing reactor during the period of study. The data represent the mean values of three independent samples.

The batch reactor pH was maintained in the neutral range (6.5 - 7.5) during the reactor usage. The initial pH of the raw sludge sample was found to be 7.2. The variation from neutral pH showed that no significant effect on the perchlorate reduction. Figure 6.11 shows that the optimal pH for perchlorate reduction occurs around neutral pH. The Dechloromonas and Azospira species generally grow optimally at pH values near neutrality in fresh water environments (Bruce et al, 1999). Achenbach et al, (2001) reported that, some species comes under the genera Dechloromonas and Azospira were capable of growth and perchlorate respiration at pH values as low as 5.

The initial salinity of the raw sludge was found to be 2.34 g L\(^{-1}\). During the period of study, the salinity of the reactor varies from 1.45-4.46 g L\(^{-1}\) and this is mainly dependence upon the perchlorate concentration in the influent (Figure 6.11). There is no significant variation in perchlorate removal even at high salinity on the reaction medium. The mixed microbial consortium would have been acclimatized to higher salinity (4 g L\(^{-1}\) NaCl) level through slow adaptation mechanism. Further studies have been done to isolate and characterize saline tolerant microbial culture/consortium for in situ bioremediation under real field conditions. Many research has shown that gradual acclimation to increased salinity resulted in better
performance of microbial treatment processes and a higher perchlorate reduction rate (Kincannon and Gaudy 1966; Burnett 1974; Park and Marchand 2002).

6.2.3 MLSS ANALYSIS

MLSS is used as a measure of the concentration of microbial consortium or biomass present in the batch container. The concentration of suspended solids (both organic and inorganic) in the mixed liquor of an aeration tank is denoted by MLSS. The increase in MLSS value is an indirect indication of growth of PRBs present in the acclimatized sludge. The initial MLSS of the raw sludge was found to be 1.35 g L\(^{-1}\). After domestication, the perchlorate-reducing ability of the sludge was greatly improved and Mixed Liquor Suspension Solid (MLSS) of the reactor gradually increased from 1.35 to 6.48 g L\(^{-1}\) during the operated period of 160 days as shown in Figure 6.12. The gradual acclimation to increased perchlorate level (max. 600 mg L\(^{-1}\)) resulted in better performance of the batch bio-reactor and higher biomass formation. The biomass solids in a biological waste water reactor are usually indicated as Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS). The solids comprised of biomass, non biodegradable volatile suspended solids (nbVSS) and inert inorganic total suspended solids (iTSS). In fact, the biomass formation is an indirect measurement of higher perchlorate reduction rate in biological treatment processes.

![MLSS graph](image)

Figure 6.12: MLSS status of the batch reactor during the period of study. The data represent the mean values of three independent experiments.
6.2.4 CLOSURE

Present study has attempted to emphasize on microbial degradation of \( \text{ClO}_4^- \) and various environmental factors which affects on biodegradation. Present batch reactor results suggest that the \( \text{ClO}_4^- \) degradation depends on the influent \( \text{ClO}_4^- \) concentrations, influent acetate concentrations and concentration of alternate electron acceptors such as oxygen, presence of oxygen scavengers like sulphide, pH, salinity and the amount of active PRBs present in the reaction medium. Moreover, compared with individual isolates the enriched consortium developed in the batch reactor reduced \( \text{ClO}_4^- \) at faster rate.
6.3 ISOLATION AND CHARACTERIZATION OF PERCHLORATE RESPIRING BACTERIA (PRB)

The isolation and characterization of six pure PRBs, isolated from mixed waste water sludge through real effluent (RE) and synthetic effluent (SE) where waste water is mixed with mineral media components is the subject of discussion in this section. In particular, isolation of PRBs from effluents, phenotypic characterization, biochemical characterization and molecular characterization of PRB is described in detail.

6.3.1 ISOLATION OF PRB FROM SYNTHETIC AND INDUSTRIAL EFFLUENT

Six perchlorate reducing microorganisms namely: Proteobacterium ARJR SMBS, Methylophaga sp. LMN, Pseudomonas aeruginosa strain LMN SMBS, Exiguobacterium sp. LMNARJR, Proteus sp. LMNCRE and Bacillus sp. ARJR were isolated from SE and RE. The visible colonies are formed on petri dish after 24-48 h of incubation of six PRBs, has been shown in Figure 6.13. The pure isolates, Proteobacterium ARJR SMBS, Pseudomonas aeruginosa strain LMN SMBS and Methylophaga sp. LMN (represented as A, B and C in Figure 6.13) were isolated from SE sludge enriched with perchlorate (600 mg L⁻¹) and rest three isolates, Bacillus sp. ARJR, Proteus sp. LMNCRE and Exiguobacterium sp. LMNARJR (represented as D, E and F given in Figure 6.13) were isolated from a source of mixture of industrial effluent and soil samples collected from a contaminated place at Vellore.

Interestingly, most of the PRBs were found to be facultative in nature and perchlorate is used as a terminal electron acceptor during the oxidation of acetate and that would act as a substrate and carbon source in their growth medium (Xu et al, 2003; Wallace et al, 1996; Herman and Frankenberger, 1998; Coates et al, 1999; Logan et al, 2001a). However, in the reduction sequence of perchlorate none of the intermediate accumulates in the solution. The chlorite disproportionation to chloride and oxygen is a non-energy yielding step, catalyzed by chlorite dismutase, but both chlorate and oxygen are used as electron acceptors by perchlorate-respiring microbes.
(Rikken et al, 1996). Coates et al, (1999) and Logan (1998) reported that chlorate-degrading bacteria may also degrade perchlorate. Nevertheless, the degradation capacity of pure isolates differs and it dependence upon the effect of various environmental factors and the amount of enzymes (perchlorate reductase, chlorate reductase and chlorite dismutase) produced by the bacteria. Miller and Logan (2000) reported that *Dechloromonas* sp. strain JM, isolated from a perchlorate-degrading reactor was found to reduce perchlorate with hydrogen, but it required an organic substrate (acetate) for growth and could not fix CO₂. Zhang isolated chemolithoautotrophic, perchlorate-reducing, hydrogen oxidizing bacterium *Dechloromonas* sp. strain HZ showed optimum ClO₄⁻ reduction activity at pH 6.8 (Zhang et al, 2002).

### 6.3.2 PHENOTYPIC CHARACTERIZATION OF PRB

Colony morphology can cover further aspect of the species identity. The *proteobacterium* ARJR SMBS isolated in agar petri dish produced circular, raised, cream and curled colonies as shown in Figure 6.13 (indicated as A). The *pseudomonas aeruginosa* strain LMN SMBS colonies grown on agar medium observed to be in the range of 2 to 2.25 mm size, raised, filamentous and with time they tend to rapidly spread on the surface of the agar with green fluorescent pigment (Figure 6.13- indicated as B). Howard and Inglis, (2003) and Park, (2000) reported that *pseudomonas stutzeri* produces highly wrinkled, convex, non-circular, light yellow colonies on agar. The *Methylphaga* sp. LMN colonies grown on agar were observed to be in the range of 1.4 to 1.9 mm size, raised, circular, appeared moist in nature and with time they tend to spread on the surface of the agar with orange fluorescent pigment (Figure 6.13- indicated as C). The morphological characteristics of *Bacillus* sp. ARJR colonies grown on agar medium portrayed curled, circular, dense cream color and moist in nature (Figure 6.13- indicated as D). Further observed that strain ARJR belongs to Gram-positive, rod-shaped bacteria and comes under the division *Firmicutes*. *Proteus* sp. LMNCRE found to be in the Family *Enterobacteriaceae* within the *Proteobacteria*. Garrity et al, (2005) reported that *Proteus* genus includes five species, *P. vulgaris*, *P. mirabilis*, *P. penneri*, *P. hauseri* and *P. myxofaciens*, exist in manure, soil, polluted water etc.
Figure 6.13: Inorganic mineral medium with 100 mg L$^{-1}$ perchlorate and 200 mg L$^{-1}$ acetate showing growth of PRBs. A- *Proteobacterium* ARJR SMBS, B- *Pseudomonas aeruginosa* strain LMN SMBS, C- *Methylophaga* sp. LMN, D- *Bacillus* sp. ARJR, E- *Proteus* sp. LMNCRE and F- *Exiguobacterium* sp. LMNARJR.
The strain LMNCRE colonies grown on petri dish within the range of 1.2 to 1.6 mm size, filamentous, lobate and with time they tend to rapidly spread on the surface of the agar with pale green color (Figure 6.13- indicated as E). The *Exiguobacterium* sp. LMNARJR was identified as a Gram-positive, anaerobic short rod strain, formed round orange colonies on the solid medium containing 100 mg L\(^{-1}\) perchlorate and 200 mg L\(^{-1}\) acetate, which was smooth on the surface and irregular on the edge. The LMNARJR colonies grown on agar were 1.3 to 1.8 mm size, raised, circular and with time they tend to spread on the surface of the agar with dense orange fluorescent pigment (Figure 6.13- indicated as F). All the six strains were grown in the presence of perchlorate as a respiratory electron acceptor.

**GRAM STAINING:** The Gram stain procedure distinguishes between Gram-positive and Gram-negative groups by coloring the bacterial cell red or violet. It is based on the chemical and physical properties of bacterial cell wall. Primarily, it detects peptidoglycan, present in a thick layer in Gram-positive bacteria, which retains the crystal violet (Bergey et al, 1994). After staining, Gram-positive results in a purple/blue color while Gram-negative results in a pink/red color (Harrigan and McCance, 1990; Norris et al, 1981). The majority of the reported PRB have been found to be Gram-negative, rod-shape, motile bacteria that possess a multilayered cell wall structure (Coates and Achenbach, 2004).

The present work depicted that *Bacillus* sp. ARJR and *Exiguobacterium* sp. LMNARJR found to be Gram-positive, rod-shape in nature (Figure 6.14-indicated as D and F) and other strains observed to be Gram-negative (Figure 6.14- marked as A, B, C and E). Hence for ARJR and LMNARJR, crystal violet (CV) dissociates in aqueous solution into CV\(^+\) and chloride (Cl\(^-\)) ions. Further these ions penetrate through the cell wall and cell membrane. Subsequently the CV\(^+\) ion interact with negatively charged component of bacterial cell and stain the cell purple.

Gram negative bacteria stain red, which is attributed to a thin peptidoglycan wall, which does not retain the crystal violet during the decoloring process. Iodine is often referred to as a mordant, but act as a trapping agent that prevents the removal of the CV and iodine complex and, therefore, colour the cell (Llewellyn, 2005). The alcohol act as a decolourizer, however, if the alcohol remains on the sample for too
long, it may leads to erroneous results. If the bacteria is Gram positive, it will retain
the primary stain (CV) and not take the secondary stain (safranin), causing it to look
violet/purple under microscope. If the bacterium is Gram negative, it will lose the
primary stain and take the secondary stain, causing it to appear red under microscope.
Figure 6.14 shows the Gram staining results obtained for six strains used in the study.

Figure 6.14: Gram staining results obtained for strain ARJR SMBS (A), LMN (B),
LMN SMBS (C), ARJR (D), LMNCRE (E) and LMNARJR (F) in this study.
SEM ANALYSIS: The cell size and shape of bacteria were determined by SEM analysis as shown in Figure 6.15. Strain ARJR SMBS was observed to be straight-rod shape cell with 0.31 μm wide and 0.94 μm long in size (Figure 6.15- indicated as A). Strain LMN SMBS was identified as short-rod shape cell with 0.25 μm wide and 0.61 μm long in size (Figure 6.15- indicated as B). Further, strain LMN was found to be cocci shape of 0.1 μm wide and 0.18 μm long in size (Figure 6.15- indicated as C). The strain ARJR was detected to be rod-shape cell of 0.15 μm wide and 0.44 μm long in size (Figure 6.15- indicated as D). Strain LMNCRE detected to be elongated rod-shape cell with 0.18 μm wide and 0.61 μm long in size (Figure 6.15- indicated as E). Finally, strain LMNARJR identified as short rod-shaped cell that is 0.2 μm wide and 0.58 μm long in size (Figure 6.15- indicated as F). The phenotypic characterization of six isolates was performed and summarized in Table 6.3.
Figure 6.15: Scanning electron micrograph of *Proteobacterium* ARJR SMBS (A), *Pseudomonas aerogenosa* strain LMN SMBS (B) at scale bar 1μm, *Methylophaga* sp. LMN (C) at scale bar 2μm, *Bacillus* sp. ARJR (D), *Proteus* sp. LMNCRE (E) and *Exiguobacterium* sp. LMNARJR (F) at scale bar 1μm.
Table 6.3: Phenotypic characteristics results obtained for *Proteobacterium* ARJR SMBS (A), *Pseudomonas aeruginosa* strain LMN SMBS (B), *Methylophaga* sp. LMN (C), *Bacillus* sp. ARJR (D), *Proteus* sp. LMNCRE (E) and *Exiguobacterium* sp. LMNARJR (F).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gram staining</th>
<th>Shape</th>
<th>Color</th>
<th>CS* (mm)</th>
<th>Form</th>
<th>Elevation</th>
<th>Margin</th>
<th>G-NA*</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARJRSMBS(A)</td>
<td>Gram Negative</td>
<td>Elongated Rod</td>
<td>Cream</td>
<td>1.8 – 2.5</td>
<td>Circular</td>
<td>Raised</td>
<td>Curled</td>
<td>Good</td>
<td>Moist</td>
</tr>
<tr>
<td>LMN SMBS(B)</td>
<td>Gram Negative</td>
<td>Short rod</td>
<td>Green</td>
<td>2 – 2.25</td>
<td>Filamentous</td>
<td>Raised</td>
<td>Lobate</td>
<td>Good</td>
<td>Moist</td>
</tr>
<tr>
<td>LMN (C)</td>
<td>Gram Negative</td>
<td>Cocci</td>
<td>Orange</td>
<td>1.4 – 1.9</td>
<td>Circular</td>
<td>Raised</td>
<td>Curled</td>
<td>Good</td>
<td>Moist</td>
</tr>
<tr>
<td>ARJR (D)</td>
<td>Gram Positive</td>
<td>Rod</td>
<td>Cream</td>
<td>1.7 – 2.6</td>
<td>Circular</td>
<td>Raised</td>
<td>Curled</td>
<td>Good</td>
<td>Moist</td>
</tr>
<tr>
<td>LMNCRE (E)</td>
<td>Gram Negative</td>
<td>Elongated rod</td>
<td>Pale Green</td>
<td>1.2 – 1.6</td>
<td>Filamentous</td>
<td>Plane</td>
<td>Lobate</td>
<td>Moderate</td>
<td>Moist</td>
</tr>
<tr>
<td>LMNARJR (F)</td>
<td>Gram Positive</td>
<td>Short rod</td>
<td>Orange</td>
<td>1.3 – 1.8</td>
<td>Circular</td>
<td>Raised</td>
<td>Irregular</td>
<td>Good</td>
<td>Moist</td>
</tr>
</tbody>
</table>

CS* indicates Colony Size; G-NA* indicates Growth in Nutrient Agar; Data are presented the mean values of three independent experiments.
6.3.3 BIOCHEMICAL CHARACTERIZATION OF PRB

The bio-chemical test result is obtained for all the pure isolates and is discussed in this section. Standard methods were followed for characterization of PRBs as discussed in the chapter 4. The comparative analysis suggests that isolate ARJR SMBS identified as *Proteiobacterium*, isolate LMN SMBS as *Pseudomonas aeurogensoa* and isolate LMN as *Methylophaga* sp. Harrigan and McCance, (1990) reported in their research on *Methylophaga* sp. The isolates LMNARJR, ARJR and LMNCRE were subjected to 9 different biochemical tests; the citrate, indole, nitrate reduction and urease test were carried out using a rapid identification kit. Specific colour development after incubation served as indicator of reaction type. Results obtained were based on colour change which depends upon principle of pH change and substrate utilization. The test results of the biochemical reactions were compared with standard ready reckoner chart provided along with the biochemical kit for generic identification of the bacteria. Comparative analysis suggests that isolate LMNARJR identified as *Exiguobacterium* sp., isolate LMNCRE identified as *Proteus* sp. and isolate ARJR identified as *Bacillus* sp. The detailed description of biochemical tests and serological typing must be performed for definite identification and confirmation of organisms as described below.

CITRATE TEST: The citrate test is an IMViC tests that distinguish between members of the *Enterobacteriaceae* family based on their metabolic by-products (Baron and Finegold, 1990; Davis, 1980; Harley, 2005). It is often used to identify gram-negative pathogens and environmental isolates (Tang et al, 1998). The citrate test screens a bacterial isolate for the ability to utilize citrate as its carbon and energy source (Difco, 1998; MacFaddin, 2000). Present results reveal that apart from strains LMN and LMNARJR, other isolates were able to utilize citrate as carbon and energy source (Table 6.4). Citrate tests results obtained for strains ARJR SMBS, LMN and LMN SMBS has been showed in Figure 6.16 (indicated as A). The visible presence of growth on the medium and the change in color due to the increased pH were the signs that an organism can import citrate in the medium and such organisms considered to be citrate positive. Citrate is an intermediate in Krebs cycle, is generated by many bacteria. Upon uptake by the cell, citrate is cleaved by citrate lyase to oxaloacetate and acetate. The oxaloacetate is then metabolized to pyruvate and CO₂. When citrate
is used as an energy source, alkaline carbonates and bicarbonates were produced as by-products raise the pH of the medium to above 7.6, causing the bromothymol blue to change from the original green color to blue (Koser, 1923; MacFaddin, 2000). However, only bacteria that can utilize citrate as the sole carbon and energy source will be able to grow on the Simmons citrate medium, thus a citrate-negative test culture will be virtually indistinguishable from an uninoculated slant.

**INDOLE TEST**: Indole test results obtained for strains ARJR SMBS, LMN and LMN SMBS is showed in Figure 6.16 (indicated as B). The indole production for the bacterial isolates is showed in equations 6.2 and 6.3 and summarized in Table 6.4.

\[
\text{Tryptophan} \xrightarrow{\text{Tryptophanase}} \text{Indole} + \text{pyruvic acid} + \text{NH}_3 \quad (6.2)
\]

\[
\text{Indole} + \text{Kovac's reagent} \xrightarrow{\text{Butanol}} \text{Rosindole} + \text{H}_2\text{O} \quad (6.3)
\]

Tryptophan, an essential amino acid, is oxidized by some bacteria by the enzyme tryptophanase resulting in the formation of indole, pyruvic acid and ammonia. The formation of a cherry red color represents a positive test. In the present study infers that, all the PRBs isolates were not able to oxidize tryptophan present in the medium and showed negative results for all.

**MANNITOL-MOTILITY TEST**: Motility has long been recognized as an important taxonomic tool and biological characteristic of microorganisms (Jordan et al, 1934; Leifson, 1951, 1960; Stanier and Van Neil, 1941). Motility test medium is used to determine the motility of microorganisms and it was determined by the diffuse spread of growth beyond the stab line of inoculation. Figure 6.16 (indicated as C) shows the manitol-motility test results obtained for strains ARJR SMBS, LMN and LMN SMBS and summarized in Table 6.4. Results infers that, strains ARJR SMBS, LMN, LMNARJR and ARJR were able to grow in manitol salts medium and observed to be non-motile in nature. In contrast to these isolates, strains LMN and LMNCRE are motile in nature and they were able to grow in the high salt differential manitol media (Table 6.4). In general, as organisms grow, creates a diffuse red color which is much easier to visualize. Mannitol salts agar is a selective and differential media due to the high salt concentration which prevents all bacterial sp. from growing. The media is differential due to the presence of mannitol and the indicator phenol red which turns yellow in low pH environments.
MR-VP TEST: The methyl-red (MR) and the Voges-Proskauer (V-P) tests are used to differentiate two major types of facultatively anaerobic enteric bacteria that produce large amounts of acid and those that produce the neutral product acetoin as end product. Figure 6.17 (indicated as A) shows the MR-VP test results obtained for strains ARJR SMBS, LMN and LMN SMBS and other isolates summarized in Table 6.4. Both these are performed simultaneously because they are physiologically related and are performed on the same medium MR-VP broth. Opposite results are usually obtained for the methyl-red and Voges-Proskauer tests, i.e. MR+, VP- or MR-, VP+. In these if an organism produces large amounts of organic acids: formic, acetic, lactic and succinic (end products) from glucose, the medium will remain red (a positive test) after the addition of methyl red a pH indicator (i.e. pH remaining below 4.4). In the present study results reveals that, strains ARJR SMBS and ARJR showed positive test for MR and negative for VP. Strains LMNARJR and LMNCRE showed negative test for MR and positive for VP (Table 6.4). In the strains LMNARJR and LMNCRE, methyl red will turn yellow (a negative test) due to the elevation of the PH above 6.0 because of the enzymatic conversion of the organic acids (produced during the glucose fermentation) to non-acidic end products such as ethanol and acetone. In contrast to the usual process, the strains LMN SMBS and LMN showed negative for both MR and VP as summarized in Table 6.4.
TSI TEST: The TSI tests results obtained for strains ARJR SMBS, LMN and LMN SMBS in this study is shown in Figure 6.17 (indicated as B). Results infer that, strain ARJR SMBS has the ability to ferment glucose, lactose and/or sucrose (slant/butt turn yellow represented as A/A in Table 6.4. In further, strain ARJR SMBS test tubes showed with excellent bubbles due to the formation of gas and no black precipitate observed on the slant. It indicates that there is no hydrogen sulfide (H$_2$S) production by strain ARJR SMBS. H$_2$S producing organisms on TSI Agar may produce so much of black precipitate, ferrous sulfide, that the acidity produced in the butt is completely masked. In contrast to strain ARJR SMBS, strain LMN SMBS does not have ability to ferment sugars, no gas production with dense black precipitate, which is an indication of good H$_2$S production and represented as K/K, G$, H_2$S$^{++}$ in Table 6.4. Strain LMN observed to be capable of glucose fermentation, peptone catabolised and there is no gas and H$_2$S production in the TSI agar medium (represented as A/K, G$, H_2$S$^-$ in Table 6.4). Further, strains LMN ARJR, LMN CRE and ARJR have the following characteristics as described above and represented as: A/K, G$, H_2$S$^-$; K/K, G$, H_2$S$^+$; A/A, G$, H_2$S$^-$ respectively (Table 6.4). However, the following uncertainties need to be considered while interpreting TSI test for microorganisms. Some organism may demonstrate H$_2$S production on Kliger Iron Agar, but not on TSI agar because utilization of sucrose in TSI agar suppresses the enzyme mechanism that results in the production of H$_2$S. Specifically, H$_2$S producing Salmonella and some members of Enterobacteriaceae may not be H$_2$S positive on TSI agar.

Figure 6.17: MR-VP (A) and TSI (B) tests results obtained for strain ARJR SMBS, LMN and LMN SMBS in this study.
NITRATE TEST: Nitrate reduction property of *Proteobacterium* ARJR SMBS and *Methylphaga* sp. LMN were confirmed by the addition of sulfanilic acid (0.8%) and N, N-dimethyl-alpha-naphthylamine (0.6%) in the reaction mixture which detects the presence of catabolic end product as depicted in Figure 6.18 (indicated as A). It infers that, test organism has reduced the NO$_3^-$ to NO$_2^-$ and a red color appeared within 2 min which indicates the presence of NO$_2^-$ in the tube. The nitrate reduced to nitrite as shown in equation (6.4).

\[
2e^- + 2H^+ + NO_3^- \rightarrow NO_2^- + H_2O \quad (6.4)
\]

If no color change is observed within 2 min, there are several possible reasons. Either the organism was unable to reduce NO$_3^-$ at all, or capable of reducing NO$_2^-$ as showed in equation (6.5), or reduced NO$_3^-$ directly to molecular nitrogen as depicted in equation (6.6). Equation (6.5) infers that the strain ARJR SMBS and LMN could reduce nitrate to nitrite then to nitric oxide or further to nitrous oxide or further to nitrogen gas.

\[
NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (6.5)
\]

\[
2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O \quad (6.6)
\]

Figure 6.18: Nitrate reduction test results obtained for strain ARJR SMBS, LMN and LMN SMBS (A- before zinc addition, B- after zinc addition) in the study.

In contrast, strain LMN SMBS nitrate reduction property was confirmed by the addition of zinc dust to the nitrate reduction medium which detects the amount of nitrate remained in the culture medium (Figure 6.18 -indicated as B). Zinc is a powerful reducing agent and if there is any NO$_3^-$ remaining in the tube, a small amount of zinc dust will rapidly reduce it to NO$_2^-$. Therefore the appearance of a red color after the addition of zinc dust to a colorless reaction tube indicates a negative reaction, i.e., the organism has failed to reduce NO$_3^-$. Further, if the broth remains
colorless after the addition of zinc, the organism has also reduced the NO$_2^-$ intermediate product to N$_2$ gas or some other nitrogenous product. Therefore, it concludes that strain LMN SMBS has reduced the NO$_2^-$ intermediate product to N$_2$ gas or some other nitrogenous product. Further, the nitrate reduction property of ARJR, LMNARJR and LMNCRE were conformed using biochemical test kit as described in section 4.5.2 and summarized in Table 6.4.

OXIDASE TEST: The oxidase test is a test used in microbiology to determine if a bacterium produces certain cytochrome c oxidases and it is used as a taxonomic tool for many microorganisms. Figure 6.19 (indicated as A) shows that the oxidase test results obtained for ARJR SMBS, LMN and LMN SMBS and for other isolates, test results were summarized in Table 6.4. Previous report shows that, the oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase, an enzyme sometimes called indophenol oxidase (Gaby and Free, 1958; MacFaddin, 1972; Oser, 1965). Present study proved that, apart from strain LMN and LMNARJR, the other isolates produces cytochrome c oxidases in cell. In the presence of an organism that contains the cytochrome oxidase enzyme, the reduced colorless reagent becomes an oxidized colored product (Gaby and Free, 1958; Gerhardt et al, 1981; Lui and Jurtshuk, 1986). When the reagent is oxidized by cytochrome c, it changes from colorless to a dark blue or purple compound, indophenol blue (Figure 6.19) (Gordon and McLeod, 1928; Lui and Jurtshuk, 1986).

UREASE TEST: The urease test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide (Day et al, 1930). Urea agar has reduced buffer content and contains peptones and glucose. This medium supports the growth of many enterobacteria allowing for the observation of urease activity. If the organism produces ammonia, it leads to an increase in pH in the medium and color change from yellow (pH 6.8) to bright pink (pH 8.2). Present study results shows that, all the isolates were not able to hydrolyze urea present in the Christensen’s urea agar as shown in Figure 6.19 (indicated as B) and infer urease test negative for all the isolates.
Figure 6.19: Oxidase (A) and urease (B) tests results obtained for strain ARJR SMBS, LMN and LMN SMBS in this study.

CATALASE TEST: Some bacteria can reduce diatomic oxygen to hydrogen peroxide or super oxide. Both of these molecules are toxic to bacteria. In fact, some resistant bacteria use two enzymes to catalyze the conversion of hydrogen peroxide and super oxide back into diatomic oxygen and water. One of these enzymes is catalase and its presence can be detected by adding hydrogen peroxide to the microbial culture and oxygen gas will be evolved (positive test). Catalase test results obtained for all strains were summarized in Table 6.4 and found to be positive test for all the six isolates. Hence all strains contains catalase enzyme and catalase contains four porphyrin heme (iron) groups which allow the enzyme to react with the hydrogen peroxide and convert to water and oxygen. This reaction can be summarized by the following equation 6.7.

\[ 2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \]  

(6.7)

Catalase is a hemoprotein and a tetramer of four polypeptide chains, each over 500 amino acids long. The optimum pH for catalase is approximately neutral (7.0). Bacteria cannot protect themselves from the lethal effect of hydrogen peroxide, which is accumulated as a product of carbohydrate metabolism. Catalytic decomposition of hydrogen peroxide involves the reduction of trivalent iron (Fe³⁺) to (Fe²⁺) and the reoxidation of the latter by oxygen as depicted in equation 6.7.
Table 6.4: Bio-chemical characteristics results obtained for *Proteobacterium* ARJR SMBS (A), *Pseudomonas aeruginosa* LMN SMBS (B), *Methylophaga* sp. LMN (C), *Bacillus* sp. ARJR (D), *Proteus* sp. LMNCRE (E) and *Exiguobacterium* sp. LMNARJR (F) in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Citrate</th>
<th>Indole</th>
<th>M-M**</th>
<th>MR-VP**</th>
<th>TSI</th>
<th>NR**</th>
<th>Oxidase</th>
<th>Urease</th>
<th>Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARJRSMB(S) (A)</td>
<td>Positive</td>
<td>Negative</td>
<td>M+M−</td>
<td>MR’VP’</td>
<td>A/A, G⁺⁺, H₂S⁻</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>LMN SMBS (B)</td>
<td>Positive</td>
<td>Negative</td>
<td>M-M+</td>
<td>MR’VP’</td>
<td>K/K, G⁺, H₂S⁺⁺</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>LMN (C)</td>
<td>Negative</td>
<td>Negative</td>
<td>M+M−</td>
<td>MR’VP’</td>
<td>A/K, G⁺, H₂S⁻</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>ARJR (D)</td>
<td>Positive*</td>
<td>Negative*</td>
<td>M+M−</td>
<td>MR’VP’</td>
<td>A/A, G⁺, H₂S⁻</td>
<td>Positive*</td>
<td>Negative*</td>
<td>Positive*</td>
<td>Positive</td>
</tr>
<tr>
<td>LMNCRE (E)</td>
<td>Positive*</td>
<td>Negative*</td>
<td>M-M+</td>
<td>MR’VP⁺</td>
<td>K/K, G⁺, H₂S⁻</td>
<td>Positive*</td>
<td>Positive*</td>
<td>Negative*</td>
<td>Positive</td>
</tr>
<tr>
<td>LMNARJR (F)</td>
<td>Negative</td>
<td>Negative*</td>
<td>M+M−</td>
<td>MR’VP⁺</td>
<td>A/K, G⁺, H₂S⁻</td>
<td>Positive*</td>
<td>Negative*</td>
<td>Positive</td>
<td></td>
</tr>
</tbody>
</table>

*Tests results obtained by using rapid identification kit; M-M** indicates Manitol-Motility; M+M- represents mannitol utilization and non-motile and vice versa; MR’VP’ indicates Methyl-Red utilization and Voges-Proskauer non-utilization and vice versa; A/A indicates glucose and lactose and/or sucrose fermentation; K/K indicates no fermentation of sugars and peptone catabolized; A/K indicates glucose fermentation only; G’ indicates gas produced; H₂S’ indicates hydrogen sulphide produced; NR’’ indicates Nitrate Reduction.
6.3.4 MOLECULAR CHARACTERIZATION OF PRB

Followed by phenotypic and bio-chemical characterization of isolates, molecular level characterization studies were carried out and the results obtained in the present study is the subject of discussion in this section. In particular, results obtained for 16S rRNA gene partial sequence analysis, phylogenetic comparison for the description of isolated strains, restriction site analysis for the description of isolated strains and RNA secondary structure analysis of isolated strains is described in detail.

16S rRNA GENE SEQUENCE ANALYSIS: 16S rDNA sequencing is a powerful tool for elucidating phylogenetic relationships among prokaryotes (Stackebrandt et al, 1997; Woese, 1987) and for differential identification of bacterial species and genera (Goto et al, 2000; Joung and Cote, 2002). PCR amplification of the 16S rRNA genes allowed us to determine the sequence of 1,431 nucleotide bases for strain ARJR SMBS, 1,440 nucleotide bases for strain LMN SMBS, 1,284 nucleotide bases for strain LMN, 1,409 nucleotide bases for strain ARJR, 1,432 nucleotide bases for strain LMNCRE and 1,451 nucleotide bases for strain LMNARJR respectively. Coates and Achenbach reported that, up to now, more than 45 (per) chlorate-reducing bacteria have been isolated from a broad diversity of environments (Coates and Achenbach, 2004). Phylogenetic affiliations have found that (per) chlorate-reducing bacteria belong to members in the α, β, γ, and ε subclasses of the *Proteobacteria*. Bacterial genera that contain microbes capable of reducing (per) chlorate include *Azospirillum*, *Dechlorospsirillum*, *Ideonella*, *Dechloromonas*, *Azospira*, *Dechlorormarinus*, *Pseudomonas*, and *Wolinella*. Species of *Dechloromonas* and *Azospira* in the β- *Proteobacteria* are known to be the most predominant (per) chlorate reducers in the *Proteobacteria* (Coates and Achenbach, 2004).

BLAST analysis for comparing the 16S rRNA sequence of strain ARJR SMBS to the 16S rRNA sequence of *Klebsiella* sp SRC DSB23 (GenBank accession number, GU374056) and *Klebsiella* sp SRC_DSB8 (GenBank accession number, GU374027) indicates that these two sequences were closely related (99% query coverage) to strain ARJR SMBS. Phylogenetic affiliations have found that strain ARJR SMBS belong to members of γ subclasses of the *Proteobacteria*. Therefore, it
reveals that, strain ARJR SMBS is belonging to the members of both perchlorate-reducing bacteria and in γ-subdivision of the Proteobacteria. BLAST analysis for comparing the 16S rRNA sequence of strain LMN SMBS to the 16S rRNA sequence of Pseudomonas aeruginosa strain R7-539 (GenBank accession number, JQ659890) and Pseudomonas aeruginosa strain RI-1 (GenBank accession number, JQ773431) indicated that, sequences are closely related to strain LMN SMBS with 98% query coverage. Phylogenetic affiliations have found that strain LMN SMBS belong to members of γ subclasses of the Proteobacteria. Therefore, it reveals that, strain LMN SMBS is belongs to the members of both perchlorate-reducing bacteria and in γ-subdivision of the Proteobacteria.

Logan et al. (2001a) reported that, Pseudomonas sp. strain PM1 and PM2 is related to Pseudomonas sp. PDA at the species level, but the two Pseudomonas species appear to be different with respect to their metabolic properties because chlorate-respiring Pseudomonas sp. PDA could not reduce perchlorate as the terminal electron acceptor. Xu and Logan also reported that, Pseudomonas aeruginosa belonged to a non-perchlorate reducer due to the absence of the chlorite dismutase for perchlorate reduction (Xu and Logan, 2003). In contrast, we found here that the isolate Pseudomonas aeruginosa LMN SMBS could reduce perchlorate as the terminal electron acceptor, but the perchlorate degrading efficiency of isolate was negligible as compared with other reported PRBs in literature. Moreover, Park showed that, Pseudomonas sp. strain PM2 showed 100% similarity to the 16S rRNA sequence of P. aeruginosa ATCC BAA-1006 and the isolate PM2 could reduce perchlorate as the terminal electron acceptor (Park, 2005). According to these criteria, we designated the strain LMN SMBS as Pseudomonas aeruginosa strain LMN SMBS. The 16S rRNA gene sequence data of Proteobacterium ARJR SMBS and Pseudomonas aeruginosa LMN SMBS published in NCBI (GenBank accession numbers: HQ148164, HQ148165 respectively) as shown in Appendix A.

BLAST analysis for comparing the 16S rRNA sequence of strain LMN to the 16S rRNA sequence of Methylophaga sp. CRR1-17 (GenBank accession number, HQ222610) and Methylophaga sp. DMS048 (GenBank accession number, DQ660930) indicates that, sequences are closely related (96 and 98% query coverage) to strain LMN. Phylogenetic affiliations have found that strain LMN belong to
members of γ subclasses of the *Proteobacteria*. Therefore, strain LMN is belongs to the members of both perchlorate-reducing bacteria and in γ-subdivision of the *Proteobacteria*. BLAST analysis for comparing the 16S rRNA sequence of strain ARJR to the 16S rRNA sequence of *Bacillus* sp. S2-3 (GenBank accession number, FJ217159) and *Bacillus* sp. SA-3 (GenBank accession number, AY695834) depicted that these sequences were closely related (97% query coverage) to strain ARJR. In contrast to other isolates, the phylogenetic affiliations have found that strain ARJR belong to members of Bacilli class of the *Firmicutes*. Therefore, strains ARJR is the first reported organism that belongs to the members of perchlorate-reducing bacteria and outside the subdivision of the *Proteobacteria*. The 16S rRNA gene sequence data of *Methylophaga* sp. LMN and *Bacillus* sp. ARJR published in NCBI (GenBank accession numbers: HQ268014 and JQ695939 respectively) as shown in Appendix A.

BLAST analysis for comparing the 16S rRNA sequence of strain LMNCRE to the 16S rRNA sequence of *Proteus mirabilis* strain FFL2 (GenBank accession number, JN092590) and *Proteus mirabilis* strain FFL1 (GenBank accession number, JN092589) indicated that these two sequences are closely related (98% query coverage) to strain LMNCRE. Phylogenetic affiliations have found that strain LMNCRE belong to members of γ subclasses of *Proteobacteria*. Therefore, it reveals that strain LMNCRE belongs to the members of both perchlorate-reducing bacteria and in γ-subdivision of the *Proteobacteria*. BLAST analysis for comparing the 16S rRNA sequence of strain LMNARJR to the 16S rRNA sequence of *Exiguobacterium* sp. BTAH1 (GenBank accession number, AY205564) and *Exiguobacterium* sp. CmLB12 (GenBank accession number, HM352336) indicated that these two sequences are closely related (97% query coverage) to strain LMNARJR. In contrast to other isolates, the phylogenetic affiliations have found that strain LMNARJR belong to members of Bacilli class of the *Firmicutes*. Therefore, strain LMNARJR is identified to be belongs to the members of perchlorate-reducing bacteria and outside the subdivision of the *Proteobacteria*. The 16S rRNA gene sequence data of *Proteus* sp. LMNCRE and *Exiguobacterium* sp. LMNARJR published in NCBI with GenBank accession numbers JQ695940 and JQ695941 respectively as shown in Appendix A.
Like other reported PRBs, all the strains used in this study are metabolically versatile and can utilize acetate as electron donor and perchlorate as the electron acceptor in the medium. Phylogenetic analysis of the 16S rRNA gene sequence analysis as described above infers that, except strain ARJR and LMNARJR, all the strains belong to γ subclasses of the *Proteobacteria*. However, since high 16S rRNA sequence similarity (>97.5%) does not assure species identity, the metabolic and morphological characterization of all the strains was assayed to support the further description of newly identified PRBs (Stackebrandt and Goebel, 1994). Regarding this metabolism, it is generally accepted that all PRBs are capable of denitrification and the same found with the present bio-chemical characterization results in this study (Table 6.4). However, previous reports infers that *Pseudomonas stutzeri* is capable of denitrification, but *Pseudomonas* sp. PDA and strain PM1 which showed 100% similarity to the 16S rRNA sequence of *P. stutzeri* CCUG 11256 could not reduce nitrate as the terminal electron acceptor (Logan et al, 2001a; Park, 2000; Zumft, 1997).

**PHYLOGENETIC COMPARISON FOR THE DESCRIPTION OF ISOLATED STRAINS:** Woese reported that, the evolutionary conservation of 16S rRNA sequences makes it difficult to define phylogenetic relationships among closely related species (Woese, 1987). In this regard, the phylogenetic trees were constructed by the neighbor-joining method (Kumar et al, 2004) and plotted for *Proteobacterium* ARJR SMBS, *Pseudomonas aeruginosa* LMN SMBS and *Methylophaga* sp. LMN as depicted in Appendix B. For neighbor-joining (NJ) analysis, the distance between the sequences was calculated using Kimura's two-parameter model. The robustness of the trees was statistically evaluated by bootstrap analysis with 1,000 bootstrap samples and the obtained bootstrap values are represented at the nodes. Treeview software is used for phylogenetic tree construction and to determine the taxonomic position of ARJR SMBS, LMN SMBS and LMN. Phylogenetic analyses of the 16S rRNA gene sequences of known PRBs infers that they are members of *Proteobacteria*, with majority of them (≥70%) belongs to the Beta subclass, such as members of the *Dechloromonas* and *Azospira* sp. in the β- *Proteobacteria* and members of the *Pseudomonas* sp. in the γ-*Proteobacteria*, respectively (Rikken et al, 1996; Wallace et al, 1996; Coates et al, 1999; Achenbach et al, 2001; Zhang et al, 2002; Coates and Achenbach, 2004).
Upon comparison of strain ARJR SMBS with those of its phylogenetic
neighbours, it was found that closely related to *Klebsiella* sp. enrichment culture clone
SRC_DSB23 (GU374056), *Klebsiella* sp. enrichment culture clone SRC_DSB8
(GU374027) and so on. Therefore, phylogenetic tree analysis placed the strain ARJR
SMBS in the gamma subgroup of *Proteobacteria* and within the genus *Klebsiella*.
Further, strain LMN SMBS clustered within the family of *Pseudomonas aeruginosa*
and the gamma subgroup of *Proteobacteria*. The phylogenetic study reveals that, the
close relatives of strain LMN SMBS include *Pseudomonas aeruginosa* strain SU8
(HQ283487), *Pseudomonas* sp. OU-67 (FN663622), *Pseudomonas aeruginosa* JCM
2776 (AB594757) and so on as summarized in Appendix B. Hence, phylogenetic tree
analysis placed the strain LMN SMBS in the gamma subgroup of *Proteobacteria* and
within the genus *Pseudomonas*. Similarly, strain LMN clustered within the family of
*Methylophaga* sp. and the gamma subgroup of *Proteobacteria*. The close relatives of
strain LMN includes *Methylophaga* sp. CRR-17 (HQ222610), *Methylophaga*
sulfidovorans strain RB-1 (NR_026313) as depicted in Appendix B. Hence, phylogenetic tree analysis placed the strain LMN in the gamma subgroup of
*Proteobacteria* and within the genus *Methylophaga*. It is known that (per)chlorate-
reducing bacteria in *Proteobacteria* are metabolically versatile in terms of utilizing
various electron acceptors and donors, while halo-respiring organisms in the low G+C
Gram positive category, and *Chlororflexi* (*Dehalobacter* sp., *Dehalococcoides* sp.)
are metabolically limited and strictly depend on halo-respiration for growth (Coates and
Achenbach, 2004; Smidt and de Vos, 2004). Overall, perchlorate-respiring organisms
are being found in all subclasses of *Proteobacteria* and indicating that they can utilize
chlorinated compounds as an alternative electron acceptor.

RESTRICTION SITE AND RNA SECONDARY STRUCTURE ANALYSIS:
Restriction site analysis is generally used in molecular studies to determine the
orientation of an insert in a cloning vector. The restriction sites analysis of the 16S
rRNA gene sequences of all the six isolates is summarized in Appendix C.
Approximately 47 restriction sites for various restriction enzymes such as EcoRI,
BmrI, NcoI, AvrII etc. were found in *Proteobacterium* ARJR SMBS with 55 mol% 
G+C content. Subsequently, the RNA secondary structure was predicted for 16S
rRNA gene sequence data and depicted in Appendix D. In general, the stability of the
structure is based on the number of loops and stems present in the RNA molecule.

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Approximately 33 loops and 54 stems were observed in strain ARJR SMBS as shown in Appendix D. The restriction site analysis for strain LMN SMBS reveals that 51 restriction sites (EcoI, Apol, BanI, BsrGI etc) were found in the molecule with G+C content 54 mol%. The RNA secondary structure analysis of strain LMN SMBS infers that, around 34 loops and 47 stems were found in the structure as depicted in Appendix D. Further, the free energy of the structure for strain ARJR SMBS and LMN SMBS was found to be -338.9 kcal/mol and -323.5 kcal/mol respectively. Similarly, the restriction site analysis of strain LMN SMBS and strain LMN showed 51 and 54 restriction sites respectively with G+C content 54 mol%. For strain LMNARJR and LMNCRE depicted 47 restriction sites with G+C content of 56 mol% and 60 restriction sites with G+C content of 53 mol% respectively. In strain ARJR, the free energy of structure was observed to be -324.4 kcal/mol as shown in Appendix D.

6.3.5 CLOSURE

Bioremediation is proven to be a suitable option for removing low level of perchlorate from contaminated water and other sources because of its high efficiency and minimal impact on water quality. During our present study, we found that the six new microbial isolates (strain ARJR SMBS, LMN SMBS, LMN, ARJR, LMNCRE and LMNARJR) could reduce perchlorate as the terminal electron acceptor. All are observed to facultative anaerobes. The growth pattern and perchlorate degradation mechanism varies for each isolates. Therefore, we speculate that the isolated facultative anaerobes appear to have different energy-generating mechanism for perchlorate respiration in the growth medium. The biochemical characterization results proved that all the cultures have nitrate reduction property. The range of metabolic diversity can be extended when these facultative anaerobes use multiple electron acceptors (NO₃⁻) instead of O₂ for respiration in their energy metabolism. However, O₂ or NO₃⁻ is essential to the growth of other microorganisms. All the six strains isolated here have a better advantage than other applicants in an attempt to utilize alternative electron acceptors for the continuous operation of the electron transport pathways in their respiratory systems. Perchlorate is one of the preferential alternatives for isolates in reaction medium. Under suitable conditions, the electrons can be derived from various electron donors (acetate), and transferred to the electron
acceptor, perchlorate. The presence of nitrate in the culture medium that would leads to act as a protonophore in the medium results in uncertainty of the experiment. Xu et al, (2004) reported that, *Pseudomonas* sp. PDA was unable to degrade perchlorate or grow using nitrate, and the induction of enzymes necessary for chlorate respiration differed for strains like KJ and PDA. Considerable molecular stability was found for the strains ARJR SMBS and L MNARJR with free energy of the RNA molecule as -338.9 kkal/mol as compared to other isolated PRBs.

After an extensive characterization studies of six strains suggests that these microbes isolated from the perchlorate-reducing enrichment culture may play simultaneously promising role in perchlorate with a minimal effect on the other reactions. However, additional research on the reduction of perchlorate could be performed using molecular techniques (e.g., fluorescent in situ hybridization, FISH, DNA-DNA hybridization). This would allow analysis of the spatial relationship between PRB and halo-respiring organisms in the perchlorate-acclimated enrichment culture. Specifically, these *in situ* molecular techniques will be useful to track specific microbes or to design new gene probes or groups of microorganisms believed to play an important role in simultaneous reductions of perchlorate and other toxic pollutants present in the contaminated water. In particular, new probes may also help to detect previously unknown microorganisms present in the perchlorate-acclimated enrichment culture. Moreover, a combination of up-to-date cultivation and molecular techniques should be used to understand the ecological roles of both PRB and other halo-respiring organisms in a dynamic enrichment culture in real-field conditions.
6.4 EFFECT OF ENVIRONMENTAL PARAMETERS ON PERCHLORATE DEGRADATION

Batch experiments were performed in batch reactor (100 ml volume), to study the effect of various environmental parameters on bio-degradation of perchlorate and the results obtained were summarized below. In particular, acetate as an electron donor, inhibitory effect of nitrate, effect of pH and saline tolerance of mixed microbial consortium is described in detail.

6.4.1 ACETATE AS AN ELECTRON DONOR

Since acetate was found to be the best carbon source for perchlorate reduction (Wu et al, 2008), studies were carried out to find out the optimum molar ratio of acetate to perchlorate. Batch experiments were performed with an initial concentration of 150 mg L\(^{-1}\) perchlorate injected in BBRs. The acetate concentration is varied such that the ratio of acetate and perchlorate are 0, 0.5, 1.0, 2.0, 3.0 and 4.0 respectively. The level of perchlorate kept constant at 150 mg L\(^{-1}\). The pH of the reaction medium was adjusted to 7.5 using 2N NaOH and salinity at 0.25% w/v NaCl. The reactor operation was initiated by means of 750 mg L\(^{-1}\) of biomass and operated in fed-batch mode for 3.5 days to optimize the acetate concentration. After periodic incubation (12 h) sample was decanted, centrifuged and residual perchlorate level measured by using ORION ion analyzer. Results obtained for effect of acetate on perchlorate bio-degradation mechanism by mixed microbial consortium in batch reactor is shown in Figure 6.20.

The perchlorate reducing culture substantially reduced the perchlorate, when the acetate concentration is at 300, 450 and 600 mg L\(^{-1}\). Previous report shows that, acetate is used as a single substrate for heterotrophic Cl\(^{O_4}^-\) reduction (Coates et al, 1999; Logan et al, 2001b). The reduction reaction was considerably slower at acetate concentration lesser than 150 mg L\(^{-1}\) as depicted in Figure 6.20. However, the optimum reduction of Cl\(^{O_4}^-\) was observed at molar ratio of perchlorate to acetate at 1:2. Experiments conducted by Bardiya and Bae (2005) suggested that no growth of PRB was observed when acetate was removed from the medium. Interestingly, found
here that even without acetate, ClO$_4^-$ reduction by mixed microbial consortium was observed in batch reactor, however, the rate of perchlorate reduction mechanism was considerably low in the medium. The possible reason may be the mixed microbial inoculum used for batch reactor study has been pre-acclimatized to perchlorate to a considerable level (600 mg L$^{-1}$) in the reaction medium.

Figure 6.20: Results obtained for effect of acetate on perchlorate bio-degradation mechanism by mixed microbial consortium in batch reactor. Data are presented as mean ± standard deviation of three independent experiments with 95% confidence interval.

6.4.2 INHIBITORY EFFECT OF NITRATE

Since in most waste waters, perchlorate is present along with other anions such as nitrate. Hence, it is important to understand microbial ClO$_4^-$ reduction in the presence of other anions. In fact, ammonium perchlorate is widely used in rocket fuels and the oxidation mechanism leads to the release of nitrate along with the excess ClO$_4^-$ used in fuels. Therefore, the co-existing nature of ClO$_4^-$ and nitrate used as oxidizers in rocket fuels (Logan and LaPoint, 2002), experiments were carried out to estimate the effect of nitrate on ClO$_4^-$ bio-degradation mechanism. An initial concentration of 100 mg L$^{-1}$ perchlorate was injected in BBRs and nitrate varied from 0, 10, 20, 50, 100 and 200 mg L$^{-1}$. The reactor was operated at pH 7.5 and salinity 0.25% w/v NaCl. The reactor operation was initiated by means of 750 mg L$^{-1}$ of
biomass and operated in fed-batch mode for 2.5 days. After periodic incubation (12 h) sample was decanted, centrifuged and sent for analysis. Results obtained for effect of nitrate on perchlorate bio-degradation mechanism by mixed microbial consortium in batch reactor is shown in Figure 6.21. Generally, nitrate biologically reduced to nitrogen gas in four sequential steps and it has been reported that membrane bound nitrate reductase (NO$_3^-$ to NO) is involved in denitrification and the same known to reduce chlorate (Xu et al, 2004). Results reveal that as nitrate concentration increases, the rate of ClO$_4^-$ degradation decreases continuously. This shows that nitrate act as an inhibitor on perchlorate degradation mechanism and the same supported in the literature (Nerenberg et al, 2002; Rikken et al, 1996). It reveals that nitrate act as an electron acceptor competes with available ClO$_4^-$ in the medium.

![Residual ClO$_4^-$ Conc. (mg/L) vs. Time (h)](image)

Figure 6.21: Results obtained for effect of nitrate on perchlorate bio-degradation mechanism by mixed microbial consortium in batch reactor. Data presented as mean ± standard deviation of three independent experiments with 95% confidence interval.

Chaudhuri reported that, if a nitrate grown culture of *D. Stuillum* was used to inoculate medium containing equimolar amounts of ClO$_4^-$ and nitrate, the growth and respiration were inhibited for an extended lag period of 40 h, after which nitrate reduction occurred prior to ClO$_4^-$ reduction (Chaudhuri et al, 2002). When the initial concentration of nitrate (200 mg L$^{-1}$) is twice as that of ClO$_4^-$ (100 mg L$^{-1}$), nearly 31.3% decline in ClO$_4^-$ reduction has been observed compared to those batch experiments performed without nitrate (0 mg L$^{-1}$) and 100 mg L$^{-1}$ ClO$_4^-$ (Figure 6.21).
Xu and his workers looked at reduction of ClO$_4^-$ and nitrate occurs when the bacteria are acclimated to only one of the two electron acceptors (Xu et al, 2004). They found that the enzymes for perchlorate and nitrate reduction are separately expressed in bacteria, however, also observed that bacteria that were grown in the presence of both nitrate and ClO$_4^-$ had better reduction when compared to bacteria grown only on ClO$_4^-$ . In fact, bacteria grown on only nitrate did not show any ClO$_4^-$ reduction. Studies conducted by Herman and Frankenberger reported that PRB isolate Perclace can also reduce nitrate along with ClO$_4^-$ present in the medium (Herman and Frankenberger, 1999). It is noteworthy that many sites contaminated with perchlorate also contain high nitrate levels and low concentrations of chlorinated aliphatics. Usually nitrate is removed along with ClO$_4^-$ in bioreactors because most PRBs are denitrifiers. The effect of a perchlorate-reducing environment along with nitrate degradation and on downstream processes, such as those using fluidized bed columns, also need to be investigated, due to there appear to be technical roadblocks for process optimization in bioreactor systems. These results are important with regards to enzymatic reactions in the presence of other anions and to develop more efficient strains for microbial ClO$_4^-$ reduction in bioreactor systems.

6.4.3 EFFECT OF PH

Since most of the industrial effluent and contaminated sites have more than one pollutant makes a long variation in the pH of the waste water and soil sediments. Hence to evaluate the growth ability of the perchlorate-enriched consortium at different pH was carried out in batch reactor (100 ml volume). The reactor operation was initiated by means of 100 mg L$^{-1}$ perchlorate, 200 mg L$^{-1}$ acetate and pH varied from 4, 5, 6, 7, 7.5, 8, 9 and 10. The pH of the medium was adjusted with 1N HCl or NaOH. An initial biomass of 750 mg L$^{-1}$ was used and operated in fed-batch mode for 6 days to optimize the pH. After periodic incubation (12 h), sample was decanted, centrifuged and perchlorate quantity measured by using ion analyzer. Results show that the maximum ClO$_4^-$ degradation was found to be at pH 8.0 as depicted in Figure 6.22. The optimum pH at which active ClO$_4^-$ reduction observed under alkaline range (7.5-8.5) and there was a slight decrease in reduction when pH moves towards more acidic side. Compared to control pH (7.5), nearly 12.8% decline in ClO$_4^-$ reduction
was observed under acidic pH. The responses of different batch bottles to different pH suggest that the efficiency of ClO$_4^-$ reduction is sensitive to the change in pH.

![Graph showing ClO$_4^-$ concentration over time for different pH levels.](image)

**Figure 6.22:** Results obtained for effect of pH on perchlorate degradation mechanism by mixed microbial consortium in batch reactor. Data are presented as mean ± standard deviation of three independent experiments with 95% confidence interval.

Wu et al. (2008) reported that after an initial adaptation period, successive ClO$_4^-$ reduction cycles were monitored at initial pH 7, 8 and faster reduction observed at pH 7 (0.059 h$^{-1}$, $r^2=0.99$). The present results found good match with previous report by Zhang and they found optimum ClO$_4^-$ reduction activity of *Dechloromonas* sp. HZ at pH 6.8. When the initial pH is at 8, the reduction rate was about one-half of that at pH 7 (0.033 h$^{-1}$, $r^2=0.81$). Subsequently at initial pH of 6 and 9, little reduction of ClO$_4^-$ was observed in the solution (Zhang et al, 2002). Overall perchlorate reduction occurred throughout the pH from 5.0 to 9.0 (Wang et al, 2008) and the same supported from our findings that the microbial ClO$_4^-$ reduction by a unit mass of mixed and heterotrophic bacteria observed throughout the pH from 4.0 to 10.0 and the maximum reduction obtained at pH 8.0.
6.4.4 SALINE TOLERANCE OF MIXED CULTURES

Reverse osmosis/nanofiltration (RO/NF) are promising technology alternatives, ion exchange systems have received more attention for perchlorate treatment in drinking water applications. However, these water treatment systems can generate high concentration of perchlorate (200–500 mg L\(^{-1}\) Cl\(_{O4}^-\)) and saline waste streams, which require further treatment technologies before disposal (Gu et al, 2001; Gingras and Batista, 2002; Batista et al, 2002). In general, due to the high affinity of perchlorate for anion exchange sites of the ion exchange resins, high concentration of regenerant brines with 6–12% NaCl would be generated and required further treatment. Biological treatment offers an attractive option for removing high concentrations of Cl\(_{O4}^-\) from these waste streams, and can eliminate the need for Cl\(_{O4}^-\) disposal of treatment system residuals. In this regard, to study the effect of varying salinity on Cl\(_{O4}^-\) reduction and to analyze the saline tolerant capacity of mixed microbial consortium obtained from the batch reactor, batch studies were carried out in BBR (100 ml volume). An initial concentration of 100 mg L\(^{-1}\) perchlorate, 200 mg L\(^{-1}\) acetate was used and salinity varied from 0.1, 0.25, 0.5, 1, 2, 4, 6 and 8% w/v NaCl. The reactor operation was initiated by means of 750 mg L\(^{-1}\) of inoculum and operated in fed-batch mode for 7.5 days. After periodic incubation (12 h) sample was decanted, centrifuged and sent for analysis.

![Figure 6.23: Results obtained for effect of salinity on perchlorate bio-degradation mechanism by mixed microbial consortium in batch reactor. Data are presented as mean ± standard deviation of three independent experiments with 95% confidence interval.](image-url)
Results show that the ability of perchlorate respiring microorganisms to grow under high salinity (> 4% w/v NaCl) conditions appears to be limited as depicted in Figure 6.23. There was a considerable decrease in rate of ClO₄⁻ reduction when the salinity increases continuously. The bacteria responsible for reductive respiration were active below 2% w/v NaCl salinity and saline tolerance would be a limiting factor in the biodegradation process. At 6% and 8% salinity, microbial community could not withstand to follow the degradation action. Bruce and his workers reported that, a ClO₄⁻ degrading isolate had an optimal salinity of 1% (Bruce et al, 1999) and Ideonella dechloratans failed to grow at 3% NaCl (Malmqvist et al, 1994). This suggests that the anaerobic mixed microbial consortia may reach to high salt tolerant limit through slow adaptation and acclimatization process. This was supported by studies conducted by Coppola and McDonald, reported that W. Succinogenes HAP-1 degrade ClO₄⁻ in brines derived from regeneration of ion exchange resins containing 7% NaCl and 180 mg L⁻¹ of ClO₄⁻ (Coppola and McDonald, 2000). Further studies conducted by Batista proved that, a small increase of 1% NaCl reduces the ClO₄⁻ reduction rate by half (Batista et al, 2002). Another PRB Dechloromonas agititates strain CKB isolated from paper mill wastewater grew well in environments with less than 2% NaCl and optimal growth at 1% NaCl, but no growth observed at 4% NaCl (Coates et al, 2000). Therefore, exploitation of the salt-tolerant bacteria would be an alternative to the conventional biological treatment systems for perchlorate removal from contaminated wastewater.

6.4.5 CLOSURE

Based on data obtained in a series of batch tests, we determined that nitrate acts as an inhibitor in the perchlorate degradation mechanism. Perchlorate reduction under denitrifying conditions has shown that efficiency of perchlorate reduction is sensitive to the influent concentration of nitrate and accumulating nitrite in the medium. Higher concentration of nitrate and nitrite has got an inhibitory effect on ClO₄⁻ degradation and matched with many previous reports. It is suggested that inhibitory effect of nitrate in ClO₄⁻ reduction is due the toxic effect of accumulating nitrite (as a protonophore) in the medium rather than competition with NO₃⁻ for electron donor. The result shows that acetate is a very effective carbon source and electron donor for ClO₄⁻ reduction. It was observed that the efficiency of perchlorate
reduction is not very sensitive to the influent concentration of acetate, the supplied acetate should exceed the stoichiometric demand of the perchlorate (ClO₄⁻/CH₃COO⁻ = 1:2). Perchlorate reduction under high saline conditions shows that salinity has a detrimental effect on ClO₄⁻ degradation, as salinity increases the ClO₄⁻ degradation decreases. Research in progress for exploitation of the salt-tolerant bacteria would be an alternative to the conventional biological treatment systems for effective perchlorate removal from contaminated sources. Finally, to conclude this section, present experimental findings observed in batch reactor studies would help us in understanding the successful removal of perchlorate and its related problems present the environment.
6.5 GROWTH CURVE AND KINETICS OF PRB IN BATCH REACTOR

Individual batch cycle experiments were performed in batch reactor (250 ml volume), to find out the best perchlorate degrading bacterium from the six pure strains and the results obtained in the present study is discussed below. In particular, growth curve and growth kinetics of pure isolates, dry weight cell measurement of strains: ARJR SMBS, LMN and LMN SMBS, Colony Forming Unit (CFU) analysis and perchlorate degradation study of pure isolates is described in detail.

6.5.1 GROWTH CURVE AND GROWTH KINETICS STUDY

Subsequent on the microbial screening and isolation studies, the growth curve analysis of all the pure isolates (ARJR SMBS, LMN SMBS, LMN, ARJR, LMNARJR and LMNCRE) is performed in BR of 250 ml volume as shown in Figure 5.1 and the results are discussed below. All the strains were enriched in the dark at 37°C under anoxic condition with acetate as electron donor and perchlorate as the electron acceptor. The results obtained for microbial growth kinetics of all the bacterial isolates at different perchlorate levels of 0, 10, 25, 30, 50, 75 and 100 mg L\(^{-1}\) is described in detail. The BBR operation was initiated by means of 1ml of a 24 h-old mother culture which was estimated to be in the log phase of growth and the same procedure was continued for each perchlorate concentration study. Initial pH and salinity of the medium adjusted at 7.5±0.5 and 0.25% w/v NaCl respectively. Batch study was performed with a positive control and a negative control of experiments. The positive control indicates reactor’s containing perchlorate, nutrient medium and without inoculum. The negative control represent reactor’s containing nutrient medium, inoculum and without perchlorate. The negative control indicates the microbial growth profile in the absence of perchlorate (0 mg L\(^{-1}\)). Samples were withdrawn periodically at regular intervals (3 h) and sent for growth analysis. The bacterial growth was measured by using a UV-Visible spectrophotometer at 600 nm. The absorbance values for positive control were subtracted from the experimental BBR containing nutrient medium, inoculum and respective perchlorate (0, 10, 25, 30,
50, 75 and 100 mg L\(^{-1}\)) concentration. The experiment was carried out in triplicates and the mean value reported.

To study the possible action of perchlorate and metabolic activities of PRB, growth kinetic studies have been carried out within the range 0 to 100 mg L\(^{-1}\) perchlorate. From the growth curve of PRBs, the dynamic growth rate of a bacterial species in the logarithmic phase was identified and used for kinetic analysis. The specific growth rate (\(\mu\)) of PRB corresponding to various doses of perchlorate (\(x\)) was calculated. Subsequently a kinetic model equation was developed to predict the specific growth rate, cell density and perchlorate degradation level based on the experimental data. The model equation correlates the inter relationship between bacterial growth rate in BBR with respect to perchlorate level in the medium under defined environmental conditions.

GROWTH CURVE OF STRAIN ARJR SMBS: Since the isolate ARJR SMBS was isolated from a perchlorate pre-acclimatized effluent sludge, the perchlorate degrading property at 0 and 50 mg L\(^{-1}\) perchlorate in BR is determined. The initial colony forming unit per millilitre (CFU/ml) of bacteria was found to be 55 x 10\(^9\) CFU/ml. Samples were taken periodically (2 h) and measured the growth of microbial cells. Batch reactor studies on strain ARJR SMBS showed that, around 99.5% (\(\leq\)0.27 mg L\(^{-1}\)) of perchlorate was removed within 14 h of incubation as shown in Figure 6.24. The growth curve pattern observed for *Proteobacterium* ARJR SMBS at 0 and 50 mg L\(^{-1}\) perchlorate is depicted in Figure 6.24. Degradation results reveal that, at 50 mg L\(^{-1}\) perchlorate, growth retardation effect was observed on bacteria. In Figure 6.24 PD-ARJR SMBS corresponds to perchlorate degradation pattern observed at 50 mg L\(^{-1}\) perchlorate and GC-ARJR SMBS corresponds to growth curve pattern observed at 50 and 0 mg L\(^{-1}\) perchlorate. Growth curve profile observed at 0 mg L\(^{-1}\) perchlorate is taken as the negative control of the study. Further observed that, after an initial lag period of 6 h, the acclimated perchlorate enriched strain ARJR SMBS removed 38.4% of perchlorate and complete (100%) removal observed within 16 h of incubation.
In continuation of pervious study, experiments were carried out with different initial concentration of perchlorate (0, 10, 30 and 50 mg L⁻¹) in BR and results as shown in Figure 6.25. Samples were taken periodically (3 h) and measured growth of the cells. At a particular interval, reactor’s with higher concentration of perchlorate showed growth retardation on strain ARJR SMBS. The growth curve of bacteria was characterized as initial lag phase (0-6 h) (represented as ‘a’ in Figure 6.25), followed by exponential phase (6–24 h) (represented as ‘b’ in Figure 6.25) and finally reaches to stationary-death (≥ 27 h) phase (represented as ‘c’ in Figure 6.25). As infer from the results, after an initial lag period, the cell growth rate was exponential in nature. Further, observed that as perchlorate level increases the lag phase of the bacterium was extending towards to second phase of the growth curve and this was found to be maximum at 50 mg L⁻¹ perchlorate. This is due to the slow adaptation of strain ARJR SMBS to the new stressful environment or due to the inhibitory effect of perchlorate on growth of the bacteria. It is noteworthy that the above phenomena occurs, irrespective of the equal amount of inoculum (biomass) injected in all the BRs. It infers that, perchlorate show off a growth inhibitory effect on strain ARJR SMBS. In fact, each microbial species have specific resistance mechanism to toxic chemicals and pollutants present in the environment.
Figure 6.25: Growth curve of strain ARJR SMBS at varying perchlorate (0, 10, 30 and 50 mg L\(^{-1}\)) concentrations. a, b and c indicates lag phase (0-6 h), exponential phase (6-24 h) and stationary-death phase (\(\geq 24\) h) respectively. Error bar represent the means and standard deviation of three independent reactors with \(p < 0.05\).

GROWTH KINETICS OF STRAIN ARJR SMBS: The exponential phase of the isolate as shown in Figure 6.25 was used for kinetic study. Figure 6.26, depicting \(\text{Log (Cfu/ml)}\) vs. time has been used to determine the specific growth rate (\(\mu\)). The dynamic growth rate of a bacterial species in the logarithmic phase is represented by the following equation (6.8):

\[
\ln N = \ln N_0 + \mu t
\]

Where \(N_0\) is the initial cell count, \(N\) is the bacterial cell count at time \(t\) and \(\mu\) is the growth rate constant for the bacteria. From the growth curve of strain ARJR SMBS (Figure 6.25), the logarithmic phase was identified between 6 and 24 h of incubation at different concentrations of perchlorate. The growth data (\(\text{Log (Cfu/ml)}\)) in this time interval was plotted as shown in Figure 6.26 and the growth rate (\(\mu\)) corresponding to various doses of perchlorate (\(x\)) was calculated. The values of \(\mu\) for strain ARJR SMBS observed on the linear portion of exponential growth phase (6–24 h). As shown in Figure 6.26, strain ARJR SMBS exhibited different growth rate for the utilization of perchlorate which include \(\mu_1=0.0333\) h\(^{-1}\) for 10 mg L\(^{-1}\), \(\mu_2=0.0369\) h\(^{-1}\) for 30 mg L\(^{-1}\) and \(\mu_3=0.0393\) h\(^{-1}\) for 50 mg L\(^{-1}\) respectively. The strain ARJR SMBS exhibited higher rates of growth with respect to increasing concentration of perchlorate in the medium. This indicates that the growth of strain ARJR SMBS was slightly higher at 50 mg L\(^{-1}\) perchlorate than that of lower perchlorate concentrations.
(30, 10 mg L\(^{-1}\)) used in this study. However, culture exhibited lower rate of growth than perchlorate reducers that was reported in literature, such as strain KJ (0.14 h\(^{-1}\)) (Logan et al, 2001a) and strain PM1 and PM2 (0.072 h\(^{-1}\) and 0.063 h\(^{-1}\) respectively) (Park, 2005). Moreover, strain ARJR SMBS may be unique at practical level application for bioremediation of environments contaminated with perchlorate and nitrate.

![Graph showing cell growth rate of strain ARJR SMBS at varying concentration of perchlorate](image)

Figure 6.26: Cell growth rate of strain ARJR SMBS at varying concentration of perchlorate (50, 30 and 10 mg L\(^{-1}\)) with \( \mu \) values: \( \mu_1 = 0.0333 \) h\(^{-1}\) for 10 mg L\(^{-1}\), \( \mu_2 = 0.0369 \) h\(^{-1}\) for 30 mg L\(^{-1}\) and \( \mu_3 = 0.0393 \) h\(^{-1}\) for 50 mg L\(^{-1}\). Cfu- Colony forming units.

Further, the obtained \( \mu \) values (\( \mu_1 0.0333 \) h\(^{-1}\), \( \mu_2 0.0369 \) h\(^{-1}\) and \( \mu_3 0.0393 \) h\(^{-1}\)) were plotted against corresponding to various doses of perchlorate (\( x \)) and a linear relationship between \( \mu \) and \( x \) was calculated. The relationship has been represented by the following equation (6.9).

\[
\mu = ax + b
\]  
(6.9)

The above linear relationship obtained for strain ARJR SMBS is shown in Figure 6.27. The obtained relationship infers that value of \( a \) signifies the growth rate on the concentration of perchlorate. As we discussed previously, the growth rate at 50 mg L\(^{-1}\) was slightly higher as compared with at 30 and 10 mg L\(^{-1}\) perchlorate. This infers that, the \( \mu \) is directly related with concentration of perchlorate (\( x \)), hence, as perchlorate concentration increases the cell growth rate also increases. The relationship obtained for strain ARJR SMBS (Figure 6.27) with \( \pm 2.5\% \) deviation as shown below:

\[
\mu = 1.5 \times 10^{-4} x + 0.032
\]  
(6.10)
The observed relationship between growth rate ($\mu$) and perchlorate concentration ($x$) proves that perchlorate concentration and biomass level are the key factors in the sensitivity of bacteria (strain ARJR SMBS) during the biodegradation mechanism. It also reveals that the parameters like acetate concentration, pH, salinity and anoxic environment are also considered to be important factors that decide the rate of degradation process under steady state conditions. Even though growth retardation was observed in bacterium (Figure 6.25), the bacterial growth rate ($\mu$) showed a positive relationship (equation 6.10) with increasing concentration of perchlorate ($x$) (Figure 6.27) in the medium. This is may be due to inhibitory effect of perchlorate on growth of bacteria or may be due to the lack of enzyme production (perchlorate reductase and chlorite dismutase) in strain ARJR SMBS causes a negative impact on microbial population in the culture medium. The mechanism of action involves protons ($H^+$) from the aqueous solution combined with oxygen deducted from perchlorate anion ($\text{ClO}_4^-$) to form water with the help of perchlorate reductase and followed by the action of chlorate dismutase reduces and accumulated as chloride in the medium (O’Conor and Coates, 2002). The biochemical characterization infer that strain ARJR SMBS had nitrate reducing property. So this may be another reason if nitrate is present in the growth medium that could act as a protonophore and the inhibitory effect of nitrate is due to the toxic effect of
accumulating nitrite in the medium rather than it acts as an electron donor (Chaudhuri et al, 2002; Bender et al, 2002; Kengen et al, 1999).

GROWTH CURVE OF STRAIN LMN: The growth curve of Methylophaga sp. LMN at varying perchlorate concentrations (0, 25, 50, 75 and 100 mg L$^{-1}$) is shown in Figure 6.28. The growth curve consists of an initial lag phase (0 – 9 h), followed by an exponential phase (9 – 24 h) and finally reaches to a stationary-death ($\geq$ 24 h) phase. In fact strain ARJR SMBS had a lag phase period of 6 h (Figure 6.25) and infers that, strain ARJR SMBS has better and faster rate of adaptation in the growth medium as compared to strain LMN.

![Growth curve of strain LMN at varying perchlorate concentrations](image)

Figure 6.28: Growth curve of strain LMN at varying perchlorate (100, 75, 50, 25 and 0 mg L$^{-1}$) concentrations. a, b and c indicates lag phase (0-9 h), exponential phase (9-24 h) and stationary-death phase ($\geq$ 24 h) respectively. Error bar represent the means and standard deviation of three independent reactors with $p < 0.05$.

Result shows that during the lag phase of growth, microbial cells adapted to the new environment and at higher perchlorate concentration ($\geq$75 mg L$^{-1}$) growth inhibitory effect was observed in strain LMN. This may because the cell’s of strain LMN required more time for acclimatization as compared to strain ARJR SMBS. After an initial adaptation period, the cell count increased exponentially in the medium. Overall, strain ARJR SMBS had better efficiency for perchlorate removal.
and depicted that each microbial species follows different metabolic characteristics in the growth medium.

GROWTH KINETICS OF STRAIN LMN: As we discussed previously, kinetic studies will be carried out in the logarithmic phase (9 – 24 h) of the growth curve (Figure 6.28). The similar kinetic analysis have been performed and the growth data in the logarithmic phase at different initial perchlorate concentrations (100, 75, 50 and 25 mg L\(^{-1}\)) was plotted as shown in Figure 6.29. Subsequently the values of growth rate (\(\mu\)) corresponding to various doses of perchlorate (\(x\)) was calculated as follows: \(\mu_1=0.0714\) h\(^{-1}\) for 100 mg L\(^{-1}\), \(\mu_2=0.0895\) h\(^{-1}\) for 50 mg L\(^{-1}\) and \(\mu_3=0.0924\) for 25 mg L\(^{-1}\). Even though strain LMN exhibited higher growth rate in the reaction medium in contrast to strain ARJR SMBS, it depicted a decreasing growth rate with respect to increasing concentration of perchlorate in the medium (Figure 6.29). It infers that, perchlorate act as an inhibitor on bacterial growth of strain LMN, therefore strain ARJR SMBS may find useful for field applications. Strain LMN exhibited lower rates of growth compared to strain KJ (0.14 h\(^{-1}\)) (Logan et al, 2001) and observed comparable growth rate with strain PM1 (0.072 h\(^{-1}\)) and PM2 (0.063 h\(^{-1}\)) (Park, 2005).

![Figure 6.29: Cell growth rate of strain LMN at varying concentration of perchlorate (100, 75, 50, 25 and 0 mg L\(^{-1}\)) with \(\mu\) values \(\mu_1=0.0714\) h\(^{-1}\) for 100 mg L\(^{-1}\), \(\mu_2=0.0895\) h\(^{-1}\) for 50 mg L\(^{-1}\) and \(\mu_3=0.0924\) h\(^{-1}\) for 25 mg L\(^{-1}\).]
Logan reported that, *Pseudomonas aeruginosa* could not reduce perchlorate due to the absence of the chlorite dismutase enzyme (Xu and Logan, 2003). Interestingly, here we found that the isolate *Pseudomonas aeruginosa* strain LMN SMBS could reduce perchlorate present in the medium (Figure 6.31). Strain LMN SMBS was able to degrade around 52.6% perchlorate after an incubation period of 10 h from an initial concentration of 50 mg L\(^{-1}\). However, the above degradation efficiency was considerably lower than that of strain ARJR SMBS (81.2% within 10 h). Therefore, strain ARJR SMBS had nearly 30% higher degradation capacity than that of strain LMN SMBS. Overall, we speculate that strain ARJR SMBS showed better growth rate, cell adaptation and perchlorate degrading capacity than that of strain LMN and LMN SMBS.

![Graph showing perchlorate degradation and growth curve](image)

**Figure 6.31:** Growth and perchlorate degradation profile of *Pseudomonas aeruginosa* LMN SMBS on inorganic mineral medium. PD- perchlorate degradation; GC- growth curve. Error bar represent the means and standard deviation of three independent reactors with \(p < 0.05\).

Subsequently, experiments were conducted in BR at 0, 25, 50, 75 and 100 mg L\(^{-1}\) perchlorate and the growth curve pattern of strain LMN SMBS is shown in Figure 6.32. The growth curve consists of an initial lag phase (0–3 h), followed by an exponential phase (3–12 h) and a stationary-death (≥ 12 h) phase. The smaller period of lag phase (0-3 h) infers that, strain LMN SMBS had faster rate of cell adaptation in the growth medium as compared to strain ARJR SMBS and LMN. However, the growth curve pattern at 100 mg L\(^{-1}\) depicted that (Figure 6.32) microbial cells were
not able to grow actively as compared to lower perchlorate level in the medium. Hence, we speculate that the maximum perchlorate level at which strain LMN SMBS could withstand for active degradation would be within the limit of 50-75 mg L\(^{-1}\) and indicated as perchlorate degrading potential. This is may be due to the fact that each microbial species have different metabolic activity in the medium.

![Graph showing growth curve of strain LMN SMBS at varying perchlorate concentrations](image)

Figure 6.32: Growth curve of strain LMN SMBS at varying perchlorate (100, 75, 50, 25 and 0 mg L\(^{-1}\)) concentrations. a, b and c indicates lag phase (0-3 h), exponential phase (3-12 h) and stationary-death phase (\(\geq 12 \) h) respectively. Error bar represent the means and standard deviation of three independent reactors with \(p < 0.05\).

GROWTH KINETICS OF STRAIN LMN SMBS: The kinetic analysis has been performed and the growth data in the logarithmic phase (3 - 12 h) at different initial perchlorate concentrations (100, 75, 50 and 25 mg L\(^{-1}\)) was plotted as shown in Figure 6.33. The values of growth rate (\(\mu\)) corresponding to various doses of perchlorate (\(x\)) was calculated as follows: \(\mu_1 = 0.0333 \text{ h}^{-1}\) for 100 mg L\(^{-1}\), \(\mu_2 = 0.0667 \text{ h}^{-1}\) for 75 mg L\(^{-1}\), \(\mu_3 = 0.1433 \text{ h}^{-1}\) for 50 mg L\(^{-1}\) and \(\mu_4 = 0.1567 \text{ h}^{-1}\) for 25 mg L\(^{-1}\). The values of \(\mu\) for strain LMN SMBS was observed to be exhibited higher growth rate as compared to strain ARJR SMBS and comparable with strain LMN. However, the growth rate of strain LMN SMBS was decreasing continuously with respect to increase in level of perchlorate in the medium and it’s indirectly related with growth of the bacterium. Strain LMN SMBS exhibited lower rate of growth than strain KJ (0.14 h\(^{-1}\)) (Logan et al, 2001) and found good match with strain PM1 (0.072 h\(^{-1}\)) and PM2 (0.063 h\(^{-1}\))
(Park, 2005). Subsequently, the $\mu$ values of strain LMN SMBS were plotted against corresponding to various doses of perchlorate ($x$) (Figure 6.34) and a linear relationship between $\mu$ and $x$ was calculated as given below (6.12):

$$\mu = -1.8 \times 10^{-3} x + 0.2117$$  \hspace{1cm} (6.12)

The derived correlation infers that as perchlorate concentration increases the cell growth rate decreases continuously. Therefore, strain ARJR SMBS would be useful for field application.

Figure 6.33: Cell growth rate of strain LMN SMBS at varying concentration of perchlorate (100, 75, 50 and 25 mg L$^{-1}$) with $\mu$ values $\mu_i=0.0333$ h$^{-1}$ for 100 mg L$^{-1}$, $\mu_i=0.0667$ h$^{-1}$ for 75 mg L$^{-1}$, $\mu_i=0.1433$ h$^{-1}$ for 50 mg L$^{-1}$ and $\mu_i=0.1567$ h$^{-1}$ for 25 mg L$^{-1}$ obtained.

Figure 6.34: Dynamic cell growth rate ($\mu$) of Pseudomonas auerogena LMN SMBS at varying initial perchlorate ($x$) concentrations (100, 75, 50 and 25 mg L$^{-1}$).
GROWTH CURVE OF STRAIN ARJR: Experiments were conducted in BR at 0, 25, 50, 75 and 100 mg L\(^{-1}\) perchlorate and the growth curve pattern of strain ARJR as shown in Figure 6.35. The growth curve consists of an initial lag phase (0–3 h), followed by an exponential phase (3–21 h) and a stationary-death (≥ 21 h) phase. The smaller period of lag phase (0-3 h) infers that, strain ARJR had a faster rate of cell adaptation in the growth medium as compared to strain ARJR SMBS and LMN. However, the growth curve pattern at >75 mg L\(^{-1}\) depicted that (Figure 6.35) microbial cells were not able to grow in the medium. Hence, we speculate that the maximum perchlorate level at which strain ARJR could withstand for active degradation would be within the range of 25-50 mg L\(^{-1}\) and indicated as perchlorate degradation potential. It speculates that the lower degradation potential may be due to each microbial species exhibited different metabolic characteristics in the growth medium. Overall the degradation profile depicts that strain ARJR had comparatively lower perchlorate removal efficiency as compared to other isolates used in the study.

Figure 6.35: Growth curve of strain ARJR at varying perchlorate (100, 75, 50, 25 and 0 mg L\(^{-1}\)) concentrations. a, b and c indicates lag phase (0-3 h), exponential phase (3-21 h) and stationary-death phase (≥ 21 h) respectively. Error bar represent the means and standard deviation of three independent reactors with \(p < 0.05\).

GROWTH KINETICS OF STRAIN ARJR: The kinetic analysis has been undertaken and the growth data in the logarithmic phase (3 - 21 h) at different initial perchlorate concentrations (100, 75, 50 and 25 mg L\(^{-1}\)) was plotted as shown in Figure 6.36. The values of growth rate (\(\mu\)) corresponding to various doses of perchlorate (\(x\)) was
calculated as follows: $\mu_1 = 0.0103$ h$^{-1}$ for 100 mg L$^{-1}$, $\mu_2 = 0.0405$ h$^{-1}$ for 75 mg L$^{-1}$, $\mu_3 = 0.071$ h$^{-1}$ for 50 mg L$^{-1}$ and $\mu_4 = 0.0873$ h$^{-1}$ for 25 mg L$^{-1}$. The values of $\mu$ for strain ARJR was observed to be exhibited higher rate of growth as compared to strain ARJR SMBS and found good match with strain LMN and LMN SMBS.

![Graph 1](image1)

Figure 6.36: Cell growth rate of strain ARJR at varying concentration of perchlorate (100, 75, 50 and 25 mg L$^{-1}$) with $\mu$ values $\mu_1 = 0.0103$ h$^{-1}$ for 100 mg L$^{-1}$, $\mu_2 = 0.0405$ h$^{-1}$ for 75 mg L$^{-1}$, $\mu_3 = 0.071$ h$^{-1}$ for 50 mg L$^{-1}$ and $\mu_4 = 0.0873$ h$^{-1}$ for 25 mg L$^{-1}$ obtained.

![Graph 2](image2)

Figure 6.37: Dynamic cell growth rate ($\mu$) of *Bacillus* sp. ARJR at varying initial perchlorate (x) concentrations (100, 75, 50 and 25 mg L$^{-1}$).
However, the growth rate of strain ARJR was decreasing continuously with respect to increasing concentration of perchlorate in the medium and it’s indirectly related with growth of the bacterium. Strain ARJR exhibited lower rate of growth than strain KJ (0.14 h\(^{-1}\)) (Logan et al, 2001) and found considerably good match with strain PM1 (0.072 h\(^{-1}\)) and PM2 (0.063 h\(^{-1}\)) (Park, 2005). Subsequently, the \( \mu \) values of strain ARJR were plotted against corresponding to various doses of perchlorate (\( x \)) (Figure 6.37) and a linear relationship between \( \mu \) and \( x \) was calculated as given below (6.13):
\[
\mu = -1.0 \times 10^{-3} x + 0.1177
\]  
(6.13)
The derived correlation infers that as perchlorate concentration increases the cell growth rate decreases continuously. Therefore, strain ARJR SMBS would be useful for field application.

GROWTH CURVE OF STRAIN LMNCRE: Experiments were conducted in BR at 0, 25, 50, 75 and 100 mg L\(^{-1}\) perchlorate and the growth curve pattern of strain LMNCRE as shown in Figure 6.38. The growth curve consists of an initial lag phase (0–6 h), followed by an exponential phase (6–21 h) and a stationary-death (\( \geq 21 \) h) phase. Both cell production and perchlorate degradation decreases in the death phase of the growth curve. In general, usually samples were collected in the latter logarithmic growth phase and the early stationary phase of isolates because the microbial production of enzymes or degradation action would not increase after those stages and at the worst case it would decrease (Kurane and Nohata, 1991; Shih et al, 2001; Lu et al, 2005). The degradation activity of LMNCRE reached in its peak at the stationary phase (21 h), however, degradation rate was lower than strain ARJR SMBS, LMN and higher than strain ARJR, LMN SMBS, LMNARJR. It reveals that strain LMNCRE had a reasonable degradation activity in the growth medium. After 27 h, as the death rate of the strain began to exceed its birth rate, the strain entered the decline phase, and the degradation activity of the perchlorate decreased gradually. The growth pattern at 100 mg L\(^{-1}\) perchlorate after 30 h infers that (Figure 6.38), the strain entered the decline phase and the degradation activity of the perchlorate decreased gradually. Therefore, the optimum perchlorate level at which strain LMNCRE could do active degradation would be in between 75 ± 5 mg L\(^{-1}\).
Figure 6.38: Growth curve of strain LMNCRE at varying perchlorate (100, 75, 50, 25 and 0 mg L\(^{-1}\)) concentrations. a, b and c indicates lag phase lag phase (0-6 h), exponential phase (6-21 h) and stationary-death phase (\(\geq 21\) h) respectively. Error bar represent the means and standard deviation of three independent reactors with \(p < 0.05\).

GROWTH KINETICS OF STRAIN LMNCRE: The kinetic analysis has been undertaken and the growth data in the logarithmic phase (6 - 21 h) at different initial perchlorate concentrations (100, 75, 50 and 25 mg L\(^{-1}\)) was plotted as shown in Figure 6.39. The values of growth rate (\(\mu\)) corresponding to various doses of perchlorate (\(x\)) was calculated as follows: \(\mu_1 0.0505\) h\(^{-1}\) for 100 mg L\(^{-1}\), \(\mu_2 0.059\) h\(^{-1}\) for 75 mg L\(^{-1}\), \(\mu_3 0.0914\) h\(^{-1}\) for 50 mg L\(^{-1}\) and \(\mu_4 0.1352\) for h\(^{-1}\) 25 mg L\(^{-1}\). The values of \(\mu\) for strain LMNCRE exhibited higher growth rate as compared to strain ARJR SMBS and ARJR and found good match with strain LMN and LMN SMBS. However, strain LMNCRE depicted an indirect relationship with respect to increase in perchlorate in the medium. Further, the obtained \(\mu\) values were plotted against corresponding to various doses of perchlorate (\(x\)) (Figure 6.40) and a linear relationship between \(\mu\) and \(x\) was calculated as shown in equation (6.14).

\[
\mu = -1.1 \times 10^{-3} x + 0.1557
\]  

(6.14)

Therefore, the above relationship showed that \(\mu\) is indirectly related with the concentration of perchlorate in the medium.
Figure 6.39: Cell growth rate of strain LMNCRE at varying concentration of perchlorate (100, 75, 50 and 25 mg L$^{-1}$) with $\mu$ values $\mu_1=0.0505$ h$^{-1}$ for 100 mg L$^{-1}$, $\mu_2=0.059$ h$^{-1}$ for 75 mg L$^{-1}$, $\mu_3=0.0914$ h$^{-1}$ for 50 mg L$^{-1}$ and $\mu_4=0.1352$ h$^{-1}$ for 25 mg L$^{-1}$ obtained.

Figure 6.40: Dynamic cell growth rate ($\mu$) of *Proteus* sp. LMNCRE at varying initial perchlorate ($x$) concentrations (100, 75, 50 and 25 mg L$^{-1}$).

GROWTH CURVE OF STRAIN LMNARJR: The growth curve pattern of strain LMNARJR at different perchlorate level (0, 25, 50, 75 and 100 mg L$^{-1}$) as showed in Figure 6.41. The lag phase, logarithmic phase and stationary phase of bacteria was depicted in Figure 6.41. Result shows that $>25$ mg L$^{-1}$ perchlorate in the medium, inhibited the growth of the bacterium. Hence, the degradation potential of the
organism would be within the range of $25 \pm 2$ mg L$^{-1}$ and strain LMNARJR exhibited lowest degrading capacity as compared to other isolates used in the study.

![Graph](image)

Figure 6.41: Growth curve of strain LMNARJR at varying perchlorate (100, 75, 50, 25 and 0 mg L$^{-1}$) concentrations. a, b and c indicates lag phase (0-6 h), exponential phase (6-24 h) and stationary-death phase ($\geq 24$ h) respectively. Error bar represent the means and standard deviation of three independent reactors with $p < 0.05$.

GROWTH KINETICS OF STRAIN LMNARJR: The growth data in the logarithmic phase (6-24 h) at different initial perchlorate concentrations (100, 75, 50 and 25 mg L$^{-1}$) were plotted (Figure 6.42) to study the kinetic behaviour of strain LMNARJR. The values of growth rate ($\mu$) corresponding to various doses of perchlorate ($x$) were calculated as follows: $\mu_1$ 0.0071 h$^{-1}$ for 100 mg L$^{-1}$, $\mu_2$ 0.0238 h$^{-1}$ for 75 mg L$^{-1}$, $\mu_3$ 0.0369 h$^{-1}$ for 50 mg L$^{-1}$ and $\mu_4$ 0.0607 h$^{-1}$ for 25 mg L$^{-1}$. Overall, strain LMNARJR exhibited lowest growth rate as compared to other isolates used in the study. Hence, it is recommended that strain ARJR SMBS would be better for field applications. Subsequently, the obtained $\mu$ values of strain LMNARJR were plotted against corresponding to various doses of perchlorate ($x$) (Figure 6.43) and a linear relationship between $\mu$ and $x$ was calculated as shown in equation (6.15).

$$\mu = -0.7 \times 10^3 x + 0.0756$$  \hspace{1cm} (6.15)

This relationship infers that the value of $\alpha$ signifies the growth rate on the concentration of perchlorate and depicted that as perchlorate concentration increases the cell growth rate decreases continuously. It also supports previous findings
Subsequently, the obtained $\mu$ values were plotted against corresponding to various doses of perchlorate ($x$) and shown in Figure 6.30 a linear relationship was obtained as given in equation 6.11. The derived relation infers that as perchlorate concentration increases the cell growth rate decreases.

$$\mu = -3.0 \times 10^{-4} x + 0.1015$$  \hspace{1cm} (6.11)

The above relation shows that $\mu$ is negatively correlated with concentration of perchlorate and depicted that microbial growth retardation in the bacteria. The inhibitory effect of perchlorate on the growth of strain LMN was predominant at higher concentrations of perchlorate ($> 50$ mg L$^{-1}$) and therefore strain ARJR SMBS may be unique at a practical level for bioremediation of perchlorate.

![Graph showing growth rate against perchlorate concentration](image)

Figure 6.30: Dynamic cell growth rate ($\mu$) of *Methylophaga* sp. LMN at varying initial perchlorate ($x$) concentrations (100, 75, 50 and 25 mg L$^{-1}$).

GROWTH CURVE OF STRAIN LMN SMBS: Similar to previous batch experiments, growth curve study was done for strain LMN SMBS at 0 and 50 mg L$^{-1}$ perchlorate in batch reactor and results are shown in Figure 6.31. The initial CFU/ml was found to be $35 \times 10^9$ CFU/ml. In Figure 6.31 PD-LMN SMBS corresponds to perchlorate degradation pattern observed at 50 mg L$^{-1}$ perchlorate and GC-LMN SMBS corresponds to growth curve pattern observed at 50 and 0 mg L$^{-1}$ perchlorate. The perchlorate degradation profile showed that 99.2% removal efficiency was achieved within 24 h (Figure 6.31), however, this removal efficiency was lower than strain ARJR SMSB (99.5% removal within 14 h) for 50 mg L$^{-1}$ perchlorate. Xu and