Natural ecosystems harbour microorganisms that are capable of degrading aromatics in freshwater, marine and soil ecosystems. In unpolluted ecosystems, hydrocarbon-degrading organisms represent less than 0.1% of the culturable heterotrophic microbial community, whereas, in contaminated environments they constitute up to 100% of the viable microbial population (Atlas 1981, Ridgeway et al 1990).

Bacteria and fungi capable of utilizing phenolic compounds which are found in soil and water environments (Kumaran and Paruchuri, 1997). Phenol degraders have been isolated both from contaminated and uncontaminated environments (Bastos et al 2002b, Vojta et al 2002, Santos and Linardi 2004). However, this present study focuses on isolation of phenol degrading bacterial consortium from saline environment, which had the potential to degrade selected phenolic compounds under saline conditions. Microorganisms from contaminated environments are cultured under laboratory conditions for a number of reasons. These include isolation and characterization of microorganisms that are able to degrade specific pollutant, for production of large-scale inoculum for bioaugmentation to accelerate remediation of contaminated environments (Watanabe 2001). An understanding of microorganisms as pure cultures or consortia may assist the development of bioremediation technology and bioremediation monitoring systems (Head 1998). Attempts to isolate these specific pollutant-degraders
and adapt them to biological wastewater treatment processes have been made by many researchers (Masqué et al 1987, Hinteregger et al 1992, Woolard and Irvine 1995).

This chapter describes the enrichment of phenol degrading bacterial consortium from saline environment. Isolation, biochemical characterization and molecular identification of the bacterial strains present in the consortium. Further, growth of the bacterial consortium on selected phenolic compounds at different concentrations of the substrate. Detection of dioxygenase enzyme activity produced during the degradation of phenol and 4-CP by the bacterial consortium. Finally, bioreactor study on the treatment of phenol containing saline wastewater by the acclimatized bacterial consortium.

4.1 SELECTION AND ENRICHMENT OF PHENOL DEGRADING BACTERIAL CONSORTIUM UNDER SALINE CONDITIONS

This chapter describes the enrichment of bacterial strains from soil samples from phenol contaminated sites and from soils with proximity to saline environment in Chennai.

In present study soil samples were collected from six different habitats in Chennai, were mixed and enriched with phenol under saline conditions to isolate halophilic phenol degrading bacterial consortium. Phenol-degrading microorganisms are usually isolated from environments exposed to this organic pollutant (Hofrichter et al 1993, Zaitsev et al 1995). During the isolation period, several bacterial strains co-existed in the consortium which could degrade phenol (50 mg/L) under saline conditions. After successive transfers, during the enrichment period the bacterial consortium contained 6 strains, which survived and degraded phenol as the
sole carbon source. This condition could be due to selection within the population for specific bacterial strains, which are more tolerant to phenol, probably due to changes in the regulatory system, achieved by substrate specificity or over expression of a pre-existing system for degradation of related substrates (Arai et al. 1998). Another reason could be when the enrichment culture is exposed to higher concentrations of the pollutant, there is further selection of microorganisms that are highly tolerant to the compound. Acquisition of degradative abilities by selective enrichment has been seen in laboratory ecosystems for many organic compounds and heavy metals (Alexander 1994, Van der Meer et al. 1992).

4.1.1 Growth of Bacterial Consortium on Different Phenolic Compounds

The bacterial consortium was examined for utilization of different phenolic compounds to be used as a sole source of carbon source and energy in the presence of 30 g/L NaCl concentration. A quantitative assessment of growth of the bacterial consortium on different phenolic compounds is given in Table 4.1. As the consortium was isolated from phenol contaminated sites and acclimatized to grow on phenol during the enrichment period, the consortium was able to grow on all the selected phenolic compounds which is indicated by increase in viable on different phenolic compounds (Figure 4.1). The growth of the bacterial consortium was comparatively less on chlorinated compounds like 2,4,6-TCP and PCP. This may be due to the substituted chlorine atom attached to the substrate.
Table 4.1 Growth of the bacterial consortium on different phenolic compounds

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>+++</td>
</tr>
<tr>
<td>Catechol</td>
<td>+++</td>
</tr>
<tr>
<td>o-Cresol</td>
<td>+++</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>+++</td>
</tr>
<tr>
<td>m-Cresol</td>
<td>+++</td>
</tr>
<tr>
<td>2-CP</td>
<td>++</td>
</tr>
<tr>
<td>4-CP</td>
<td>++</td>
</tr>
<tr>
<td>2,4-DCP</td>
<td>++</td>
</tr>
<tr>
<td>2,4,6-TCP</td>
<td>++</td>
</tr>
<tr>
<td>PCP</td>
<td>+</td>
</tr>
</tbody>
</table>

(*** - good, ++ - moderate, + - poor)

Figure 4.1 Growth of the bacterial consortium on different phenolic compounds
The isolated bacterial consortium was able to grow on phenols (Phenol, Catechol, o-Cresol, m-Cresol, p-Cresol) and on substituted chlorophenols (2-CP, 4-CP, 2,4-DCP and 2,4,6-TCP) but the growth was very less on PCP. The growth of bacterial consortium on PCP was very slow, probably because of its recalcitrant structure with five chlorine atoms substitutes attached to the aromatic ring structure.

The toxicity of phenolic compounds tends to increase with relative degree of chlorination (Reineke and Knackmuss 1988). This view was also supported by Saber and Crawford (1985), where they reported that PCP was resistant to degradation because of its stable aromatic ring system and high number of chlorine substitution. In present study, it was found that the growth of the consortium on PCP was inhibited thereby a reduction in the viable cell count. Gonzalez (1995) reported a decrease in the viability of the mixed culture during the degradation of PCP under non-saline conditions.

The isolated bacterial consortium utilized phenol as the sole carbon source, were the cell count increased to a maximum of $9 \times 10^8$ cfu/mL at the end of 3rd day. Growth of the bacterial consortium on catechol showed that the viable cell count increased to $4 \times 10^4$ to $6 \times 10^7$ cfu/mL in 3 days.

In the case of derivatives of phenol, o-cresol, m-cresol, and p-cresol the viable cell count increased to $6 \times 10^6$, $6 \times 10^6$ and $5 \times 10^6$ cfu/mL on the 3rd day respectively. When compared with phenols, chlorophenols gave less colony forming units, this may be due to the toxicity of chlorine atom present in the compounds. During the utilization of 2-CP and 4-CP the viable cell counts increased to $7 \times 10^6$ and $4 \times 10^6$ cfu/mL respectively. The maximum cell count of the bacterial consortium with 2,4-DCP increased to $6 \times 10^6$ cfu/mL, the viable cell count for 2,4,6-TCP was comparatively less, where the count was $5 \times 10^5$ cfu/mL on 3rd day. The cell count was very less
when the bacterial consortium was grown on PCP \((4 \times 10^5 \text{ cfu/mL})\) (Figure 4.1). This preliminary study on the growth of bacterial consortium on selected phenolic compounds showed that the consortium was able to utilize most of the phenolic compounds under saline conditions.

Garcia et al (2005) studied the degradation of low-molecular-weight aromatic compounds (benzoic acid, \(p\)-hydroxy benzoic acid, cinnamic acid, phenylacetic acid, \(p\)-coumaric acid, ferulic acid, salicylic acid) by a group of halophilic bacteria. When the isolates were enriched on phenol, they were able to utilize a greater number of aromatic compounds than the rest of the isolates enriched on other aromatic compounds other than phenol, showing their wider substrate specificity. But the mixed isolates were unable to utilize \(p\)-Cresol.

Hence it may be concluded that the bacterial consortium enriched on phenol was able to utilize all the selected phenolic compounds including substituted chlorophenols under saline conditions.

4.2 OPTIMIZATION OF GROWTH CONDITIONS IN THE BIODEGRADATION OF PHENOL

4.2.1 Effect of NaCl Concentrations on the Degradation of Phenol

To study the influence of salt on the degradation of selected phenolic compounds, phenol was used as the model compound in the preliminary study. This section describes the degradation of phenol at different salt concentrations \(((1-15 \%)\) i.e 10, 30, 50, 70, 100 and 150 g/L). (Figure 4.2).
At 3% NaCl, the phenol utilization was up to 80% with a maximum protein yield of 34 mg/L and the degradation reached a maximum of 85% at 5% NaCl, where the protein concentration reached a maximum of 37.5 mg/L. When the NaCl concentration was increased to 7% and 10% the degradation markedly reduced to 64% and 54% respectively with a reduction in the protein content to 30 and 23.4 mg/L respectively. At 15% NaCl, the
growth of the bacterial consortium was inhibited, which is represented with a very less protein content (12.5 mg/L) and the degradation dropped to 9 %. The bacterial consortium at 1% NaCl showed a lowest degradation of 7.5 %. Finally, this experiment showed that the bacterial consortium utilised phenol as the carbon source at wide range of NaCl concentrations from 30 g/L to 70 g/L, where the optimum growth was achieved at 5 % NaCl. When the concentration of sodium chloride was increased to above 5 % the growth and degradation reduced and at 15 % NaCl the growth was inhibited.

Overall in this experiment, the maximum degradation of phenol was 85 % at 5 % NaCl. Increase in the salinity would have inhibited the growth of the bacterial consortium and reduced the degradation. This was in view with the earlier reports, where they proved that increase in salinity decreased the degradation of the substrate (Mille et al 1991, Oren et al 1992, Bertrand et al 1993).

4.2.2 Effect of NaCl Concentrations on the Degradation of Phenol with Yeast Extract

It was observed from the previous experiment that the phenol degradation was only 85 % at an optimum NaCl concentration of 5 %. The experiment also showed that the protein content reduced at 1 % NaCl. When the NaCl concentration was increased to 3 %, the growth of the bacterial consortium was enhanced, which showed that the consortium is moderately halophilic in nature. It was suggested that halophiles have more demanding nutritional requirements at high salt concentrations, and hence, complex media containing growth promoting factors may help to stimulate growth of halophilic bacteria at high salt concentrations (Oren et al 1992). Yeast extract has water soluble portion of autolyzed yeast containing vitamins, nitrogen, amino acids, and carbon for bacterial growth. To enhance the degradation ability of the bacterial consortium above 3 % NaCl concentration, yeast
extract of 0.01 % was added to the mineral salts medium. Experiments were conducted with phenol (50 mg/L) at different salt concentrations in the presence of 0.01 % yeast extract is represented in the Figure 4.3.

Figure 4.3 Degradation of Phenol by the bacterial consortium and protein yield at different NaCl concentrations with yeast extract
At 3 % NaCl, the degradation of phenol was 95 % with a maximum protein yield of 37.6 mg/L and the degradation reached maximum of 99 % at 5 % NaCl in 4 days. The total protein concentration reached a maximum of 43.2 mg/L on the 2nd day. When the NaCl concentration was increased to 7 %, 10 % and 15 %, the degradation decreased to 93 %, 89 % and 14 % with a decrease in protein yield to 15, 7.6 and 5.2 mg/L respectively. The reduction in the degradation of phenol above 7 % NaCl may be due to higher salt concentrations, which affected the metabolic rate of bacterial cells; this was also supported by few studies (Ventosa et al 1998, Diaz et al 2000, Diaz et al 2002). Similar observations were also made by few researchers on polyaromatic hydrocarbons (Rambeloarisoa et al 1984, Mille et al 1991, Oren et al 1992, Bertrand et al 1993).

The utilization of phenol by the bacterial consortium was as low as 10 % at 1% NaCl, this was also indicated by the very less protein yield of 7.4 mg/L which emphasizes that the bacterial consortium needed salinity for utilizing phenol as the substrate; this proved that the bacterial consortium is moderately halophilic in nature. The optimum growth of the bacterial consortium in utilizing phenol was at 5 % NaCl. Previous literature on the degradation of phenol under saline conditions by individual strains showed that optimum degradation was achieved at 5 % NaCl (Woolard and Irvine 1995, and Hinteregger and Streichsbier 1997). From this experiment it is proved that the bacterial consortium was able to degrade phenol (50 mg/L) up to 99 % at optimum salinity of 5 % in the presence of yeast extract in 4 days. Hence for further experiments 0.01% of yeast extract was used in the mineral salts medium.

In earlier investigations, phenol was utilized by single halophilic bacterial strains, in which the incubation period for the growth on the substrate was more than 5 days (Bastos et al 2000b, Alva and Peyton 2003,
Garcia et al 2004). To our knowledge there are no reports on degradation of phenol using bacterial consortium under saline conditions. Even the studies which investigated the degradation of phenol with individual strains, the degradation took more than 4 days (Bastos et al 2000b, Alva and Peyton 2003). In the present investigation the isolated bacterial consortium was able to completely mineralize the substrate within an incubation period of 4 days at different NaCl concentrations with a optimum degradation at 5 % NaCl.

4.2.3 Effect of pH on the Degradation of Phenol

For optimum microbial activity in the environment, the preferred range of pH is between pH 6 to 8 (McLelland 1996). Therefore, it is not surprising to find that most microorganisms have evolved pH tolerances within this range (Suthersen 1999). Most heterotrophic bacteria favour a pH near neutrality (Atlas 1988). Nevertheless there are bacterial strains which can thrive outside this limit which belongs to the group ‘acidophiles’ (grows at lower pH) or ‘alkaliphiles’ (grows at higher pH).

In this investigation a study was carried out on the effect of pH (5.5, 6, 6.5, 7, 7.5, 8 and 8.5) on phenol (50 mg/L) degradation by the bacterial consortium at the optimum salinity of 50 g/L. Figure 4.4 shows the effect of pH on the growth and degradation of phenol (50 mg/L) at 5 % NaCl. The consortium was able to grow on a wide range of pH from 5.5 to 8.5. A maximum degradation of 99 % was achieved at pH 7. However, when the pH was lower than 7 and higher than 8, the degradation of phenol by the consortium reduced significantly. This shows that the bacterial consortium was able to utilize phenol from pH 7.0-8.0. This pH range serves to be the optimum for the growth of microorganisms (Gaudy and Gaudy 1981, Metcalf and Eddy 2003).
The growth of the bacterial consortium at pH 7, 7.5 and 8, the degradation were 99 %, 95 % and 74 % respectively. At pH 8.5 the degradation of phenol reduced to 42 %. At lower pH i.e., 6.5 and 6 the degradation was to 24 % and 9 %. The degradation was least at pH 5.5 (5 %). Among the different pH examined, pH 7.0 is optimum and recorded the highest degradation (99 %).
The optimum pH conditions for the growth of the bacterial consortium was comparable to phenol-degrading mixed cultures such as *Pseudomonas* sp., *Arthrobacter* sp., *Bacillus cereus*, *Citrobacter freundii*, *Micrococcus agilis* and *Pseudomonas putida* biovar B, *Nocardiodes* sp. and *Alcaligenes faecalis* which degraded phenol at a pH range of 7.0 to 10 (Sarnaik and Kanekar 1995, Kanekar et al 1999, Bastos et al 2000b).

In the present study, the isolated bacterial consortium utilized phenol at neutral pH 7. This was in accord with the results of Woolard and Irvine 1995, which showed a mixed culture degrading phenol (99% degradation) in 140 g/L NaCl at neutral pH. Hinteregger and Streichsbier (1997) reported an unidentified *Halomonas* sp. that was able to completely degrade phenol at pH 7 in a bubble reactor, where the concentration of phenol was 100 mg/L at 140 g/L of NaCl. Alva and Peyton (2003) reported the degradation of phenol by a haloalkaliphilic bacteria *Halomonas campisalis*, which was able to degrade the substrate at an alkaline pH range of 8-11, where the maximum degradation was at pH 9.5.

### 4.3 IDENTIFICATION OF BACTERIAL STRAINS USING BIOCHEMICAL AND MOLECULAR TECHNIQUES

Microbial isolation and screening is an important stage for evaluating the potential bio-degraders of different pollutants in the environment that can be used in environmental biotechnology, which relies on the pollutant-degrading capacities of naturally occurring microorganisms (Liu and Suflita 1993). Phenolic compounds are widely distributed in the environment both from natural and industrial sources. Several aerobic microorganisms that degrade phenol have been enriched and isolated under

The bacterial consortium was isolated from the mixed samples collected from different phenol contaminated sites and saline habitats. After successive transfers from the adaptation phase, the bacterial consortium had only 6 strains which degraded phenol as the sole carbon source in the mineral salts medium. In the present study, enrichment method was not used for selective isolation of individual microorganisms but rather to get isolates which can completely degrade phenol and other substituted phenolic compounds as the sole carbon source.

The isolated bacterial strains were subjected to biochemical and molecular characterisation (Cloning). There were six bacterial strains isolated and identified from the bacterial consortium and the results are presented below.

4.3.1 Morphological and Biochemical Characteristics

Consortium consisted of six strains of which four strains were gram positive and two strains were gram negative. The classification of bacteria was carried out entirely based on phenotypic characteristics, such as morphology, motility (hanging drop technique) and nutritional requirements. Morphology of the bacterial strains present in the consortium was studied using scanning electron microscope (SEM) shown in Figure 4.5. Table 4.2 summarizes the results on biochemical tests, and morphological characteristics of the isolated strains.
Figure 4.5 SEM photographs of the six bacterial strains

Table 4.2 Morphological and Biochemical characteristics of the isolated Bacterial strains

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Bacillus sp. KVGNV1</th>
<th>Arthrobacter sp. KVGNV2</th>
<th>Bacillus sp. KVGNV3</th>
<th>Halomonas sp. KVGNV4</th>
<th>Bacillus sp. KVGNV5</th>
<th>Pseudomonas sp. KVGNV6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram reaction</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Motility</td>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Catalase</td>
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<td>+</td>
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</tr>
<tr>
<td>Oxidase</td>
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<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Indole</td>
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<td>–</td>
<td>–</td>
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<td>+</td>
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<tr>
<td>Methyl red</td>
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</tr>
<tr>
<td>Voges-proskauer</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>Citrate</td>
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<td>+</td>
<td>–</td>
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<tr>
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<td>Lysine</td>
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<td>Phenylalanine</td>
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Table 4.2 (Continued)

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<th>Characteristics</th>
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<th>Arthrobacter sp. KVGNV2</th>
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<th>Halomonas sp. KVGNV4</th>
<th>Bacillus sp. KVGNV5</th>
<th>Pseudomonas sp. KVGNV6</th>
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<td>Glucose</td>
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</tr>
<tr>
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<td>–</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>Rhamnose</td>
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<td>Saccharose</td>
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</tr>
<tr>
<td>Tween 80</td>
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<tr>
<td>Starch</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Tributyrin</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Esculin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

All isolates grown on mineral salts medium with 50 g/L NaCl with phenol were observed as creamy and white gelatinous colonies. All the six isolates were round in shape with either smooth or irregular edges. Based on the biochemical characterisation, the bacterial isolates were identified as Bacillus sp. (KVGNV1), Arthrobacter sp. (KVGNV2), Bacillus sp. (KVGNV3), Halomonas sp. (KVGNV4), Bacillus sp. (KVGNV5), and Pseudomonas sp. (KVGNV6). Figure 4.6 and Figure 4.7 depict the photographical view of various biochemical tests for Bacillus sp. Genus level identification of the bacterial isolates was done with the use of Bergey’s Manual of Systematic Bacteriology (2005).

The biochemical characteristics of the six bacterial strains showed that they belong to three main phyla namely Firmicutes, Actinobacteria, γ-Proteobacteria. These groups of bacteria are found to be commonly present in the contaminated soil, water, and wastewater.

**Figure 4.6 Biochemical characteristics of *Bacillus sp.* (KVGNV1)**

(a) Tributylin agar (b) Starch agar

**Figure 4.7 Hydrolysis of Tributylin and Starch by *Bacillus sp.* (KVGNV1)**
4.3.2 Molecular Characterisation

4.3.2.1 Cloning of the mixed DNA

Approximately 900 base pairs of bacterial 16S rDNA fragments were amplified from the 5’-terminus (nucleotide positions 530-1490 and 21-958 region of *E.coli*). These sections contain the variable and conserved regions which accurately reflect the phylogenetic position of the corresponding 16S rDNA sequences and the total degree of diversity in a clone library (Lane et al 1985). The detail results obtained from each step during the cloning procedure is shown in the Figure 4.8.

4.3.2.2 Analysis of the 16S rRNA gene clone library

From the bacterial 16s rDNA clone library, 200 clones were randomly selected and each clone was subjected to colony-PCR. DNA isolated from a single representative clone was PCR-amplified and was analyzed for 16S rRNA sequencing. The sequences (with an average length of 900 base pairs) obtained from the above study were subjected to phylogenetic analysis.

A total of 175 clones were found positive for the insert and were partially sequenced, and 58 of which were found to contain the amplified 16S rRNA gene. On the basis of sequence similarity to the existing GenBank database entries, the clones were clustered together to form three major groups: the Firmicutes group, the Gamma proteobacteria group, and the Actinobacteria. Firmicutes contributed the major phyla (32 clones, 55%) \(\gamma\)-Proteobacteria (20 clones, 34%), and Actinobacteria group (6 clones, 10%). Most of the phylotypes were related to pollutant degrading bacteria.
Detailed phylogenetic affiliations of 16S rRNA gene phenotypes from the bacterial consortium are presented in Table 4.3.

(1) Mixed DNA Extraction  (2) PCR  (3) PCR Amplified DNA  (4) PCR purified Product  
(5) DNA Concentration, Ligation, Transformed DNA  (6) Colony PCR  (7) Colony PCR Product  
(8) Checked for positive clones, purified, sequencing  (9) Sequenced 16srRNA Gene  
(10) Phylogenetic Analysis

Figure 4.8 Cloning and sequencing of 16sr DNA of bacterial consortium
Table 4.3 Bacterial strains identified by 16S rRNA Sequence

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>No. of Clones</th>
<th>Phylum</th>
<th>Nearest Phylogenetic neighbour (Accession No.)</th>
<th>Affiliation</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KVGNV1 EU780459</td>
<td>14</td>
<td>Firmicutes</td>
<td><em>Bacillus cereus</em> ATCC 10987, complete genome (AE017194.1)</td>
<td><em>Bacillus cereus</em></td>
<td>99%</td>
</tr>
<tr>
<td>KVGNV2 EU780460</td>
<td>6</td>
<td>Actinobacteria</td>
<td><em>Arthrobacter</em> sp. TCCC23001(EU231606.1)</td>
<td><em>Arthrobacter</em> sp.</td>
<td>99%</td>
</tr>
<tr>
<td>KVGNV3 EU780461</td>
<td>10</td>
<td>Firmicutes</td>
<td><em>Bacillus licheniformis</em> 16S ribosomal RNA gene, partial sequence (EF059752.1)</td>
<td><em>Bacillus licheniformis</em></td>
<td>99%</td>
</tr>
<tr>
<td>KVGNV4 EU780462</td>
<td>7</td>
<td>Gamma Proteobacteria</td>
<td><em>Halomonas salina</em> 16S rRNA gene, strain F8-11 T (AJ295145.1)</td>
<td><em>Halomonas salina</em></td>
<td>99%</td>
</tr>
<tr>
<td>KVGNV5 EU780463</td>
<td>8</td>
<td>Firmicutes</td>
<td><em>Bacillus pumilus</em> strain BSH-4 16S ribosomal RNA gene, partial sequence (EF488975.1)</td>
<td><em>Bacillus pumilus</em></td>
<td>98%</td>
</tr>
<tr>
<td>KVGNV6 EU780464</td>
<td>13</td>
<td>Gamma Proteobacteria</td>
<td><em>Pseudomonas aeruginosa</em> strain MM1 16S ribosomal RNA gene, partial sequence. (EU583722.1)</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>99%</td>
</tr>
</tbody>
</table>

The relative abundance of bacteria identified in clone libraries is depicted in Figure 4.9. It could be seen that the predominate bacterial species present in the consortium belongs to *Bacillus cereus* (14 clones, 24 %), followed by *Pseudomonas aeruginosa* (13 clones 22 %), then *Bacillus licheniformis* (10 clones, 17 %) other group of bacteria showed clones less than 10 as *Bacillus pumilus* (8 clones, 13 %) *Halomonas salina* (7 clones, 12 %) and *Arthrobacter* sp. (6 clones, 10 %). The nucleotide sequences of the
identified six strains were submitted to GenBank. The Genbank accession EU780459, EU780460, EU780461, EU780462, EU780463, and EU780464 for the six bacterial strains is available in http://www.ncbi.nlm.nih.gov/Genbank.

Figure 4.9 Comparison of relative abundance of bacteria identified in clone library

4.3.2.3 Phylum description

The phylum that was most represented was Firmicutes, that includes, *Bacillus cereus*, *Bacillus licheniformis* and *Bacillus pumilus*. The second most represented phylum was Proteobacteria that includes subclass of γ-proteobacteria, β-proteobacteria and α-proteobacteria wherein, γ-proteobacteria was only represented by *Halomonas salina* and *Pseudomonas aeruginosa*. The strains identified in this study were in correlation with the work of Kanekar et al (1999), where they isolated mixed strains of phenol-degrading microorganisms of bacterial origin such as *Pseudomonas* sp., *Arthrobacter* sp. *Bacillus cereus*, *Citrobacter freundii*, *Micrococcus agilis*, *Pseudomonas putida* biovar B, *Nocardioides* sp. and
Alcaligenes faecalis, where the strains were slightly halophilic, and were able to grow at 2.6 g/L of NaCl and were alkaliphilic in nature.

(i) Description of Strains in Firmicutes

The major group of organisms in the consortium belonged to the Firmicutes. The Firmicutes are a group of bacteria, most of which have Gram-positive strains. Bacillus is a genus of rod-shaped bacteria and a member of the division Firmicutes. The 3 major strains in the consortium belonging to this group are Bacillus cereus, Bacillus licheniformis and Bacillus pumilus. Bacillus is a major group of bacteria degrading phenolic compounds, there are several reports on the degradation of phenol by single and mixed cultures of Bacillus sp. (Duffener et al 2000, Ali et al 2000, Tallur et al 2006, Arutchelvam et al 2006).

(ii) Description of strains in Gamma proteobacteria

The Proteobacteria are a major group (phylum) of bacteria. Gamma proteobacteria includes heterotrophs like Pseudomonas aeruginosa and Halomonas salina. Proteobacteria group reported to show high degradation capacity, assimilating acetate, and other organic acids under oxic and anoxic conditions (Bramucci et al 2003). The bacterial sequences assigned to Proteobacteria and Bacilli also possess hydrocarbon degrading activity.

Pseudomonas strains are dominant in degradation of these aromatic compounds (Watanabe et al 1998, Whiteley et al 2001, Watanabe et al 2002, El Fantroussi and Agathos 2005). The key components of microbial communities responsible for degradation of phenolic wastes are Pseudomonads. Their physiological and genetic basis of phenol degradation has been described by many researchers (Puhakka et al 1995, Srivastava et al

*Pseudomonas halodurans* was a marine bacterial isolate, which was able to tolerate 1.7 % NaCl to 15 % in synthetic sea water, during the utilization of benzoate (Oren et al 1992). Different species of *Halomonas* are reported in the degradation of phenol under saline conditions *Halomonas sp*. Hintregger and Streichsbier (1997), *Halomonas campisalis* Alva and Peyton (2003), *Halomonas* sp. EF11 (Maskow and Kleinsteuber 2004), *Halomonas organivorans* (Garcia et al 2005).

(iii) **Description of strains in Actinobacteria**

The phylum *Actinobacteria* is made up of gram-positive organisms. This includes *Arthrobacter sp.* which are motile rods and appear as rod-coccoid organisms in microscopy, it is a common genus of soil bacteria. This genus is metabolically versatile, producing many different enzymes allowing it to grow on a wide range of substrates (Prescott et al 2002).

The bacterial strain, *Arthrobacter citreus* isolated from a petroleum contamination site is able to metabolize different phenolic compounds under non-saline conditions. Immobilization of this strain with inert support was able to promote phenol degradation to higher concentration of 22 mM (Karigari et al 2006).

*Arthrobacter chlorophenolicus* A6 was isolated and enriched with phenol, which was able to grow on very high concentrations of 4-Chlorophenol (175 µg/g) within 10 days, it was used in bioremediation of phenol in soil (Westerberg et al 2000).

The six nucleotide sequences of strains obtained from the bacterial consortium were processed by a Blast search of the GenBank database
(www.ncbi.nlm.nih/BLAST) for the identification of the bacterial strains. All the sequences showed >98% similarity to the 16S rRNA sequences available in Gene Bank.

The evolutionary history was inferred using the Maximum Parsimony method (Eck and Dayhoff 1966). The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates that were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was shown next to the branches (Felsenstein 1985). The Maximum Parsimony (MP) tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar 2000) with search level 3 (Felsenstein 1985, Nei and Kumar 2000) in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). There were a total of 1063 positions in the final dataset, out of which 352 were parsimony informative. The phylogenetic tree constructed with the MEGA 4 software of the bacterial domain showing the distribution of major sequence styles is shown in Figure 4.10.

From the identification results it was inferred that, the bacterial consortium consisted of three different groups of bacteria. Firmicutes contributed three different strains of *Bacillus* sp. (*Bacillus cereus, Bacillus licheniformis, and Bacillus pumilus*), two Gamma proteobacterial groups (*Halomonas salina and Pseudomonas aeruginosa*) and Actinobacteria represented by only single strain of *Arthrobacter* sp. Most of these strains have been reported to degrade phenolic compounds under non-saline conditions. It is interesting to note that *Halomonas salina and Bacillus licheniformis* which belong to halophilic group of organisms were able to
coexist with other strains in the consortium to degrade phenolic compounds. The reason for such a co-existence might be due to the selection of enrichment of bacterial strains from six different habitats.

![Phylogenetic tree showing evolutionary relationships of 14 taxa including bacterial 16S rRNA sequences of the bacterial consortium](image)

(Numbers at the nodes indicate the percentage of bootstrap samplings)

**4.4 BIODEGRADATION OF PHENOL AT OPTIMUM CONCENTRATION OF NaCl (50 g/L)**

**4.4.1 Degradation of Phenol at Different Concentrations by the Bacterial Consortium**

From the previous study it was observed that the phenol degradation was optimum at 50 g/L NaCl concentration, and hence further study on the degradation of different concentrations of phenol was conducted at 50 g/L NaCl. The ability of the consortium to use phenol, as sole source of carbon and energy, was studied at 100, 150, 200, 250 and 300 mg/L of
phenol. There were few previous reports, where they used phenol as the model compound for degradation studies under saline conditions (Woolard and Irvine 1995, Hinteregger and Streichsbier 1997, Bastos et al 2000b, Peyton et al 2002, Alva and Peyton 2003, Garcia et al 2005).

Phenol at 100 mg/L, 150 mg/L, 200 mg/L concentrations was almost completely degraded (> 93 %) by the bacterial consortium in 4 days. At optimum salinity of 5 %, the bacterial consortium utilized 100 mg/L with an increase in growth in terms of protein from 3.5 mg/L on zero day to 45.2 mg/L in 2 days. The degradation reached a maximum of 99 % in 4 days. At 150 mg/L of phenol, the degradation was 96 % with a maximum protein yield of 37.6 mg/L. When the concentration of phenol was increased to 200 mg/L, the degradation percentage reduced to 93 % in four days. There was a corresponding decrease in the protein yield to 34.5 mg/L on the 3rd day. Further phenol degradation reduced to 90 % and 55 % at 250 mg/L and 300 mg/L of phenol respectively with a protein yield of 29.5 and 24.3 mg/L respectively (Figure 4.11). Optimum degradation was achieved at 100 mg/L concentration of phenol. Similar results were reported (Hinteregger and Streichsbier 1997), wherein the isolated *Halomonas* sp. from the brine wastewater could degrade phenol (100 mg/L) as sole source of carbon and energy at NaCl concentrations varying between 1 % and 14 % (w/v), where the maximum degradation of phenol at 100 mg/L was reported at 5 % NaCl. Woolard and Irvine (1995) also showed that halophilic mixed cultures degraded only up to 100 mg/L of phenol at 140 g/L NaCl with 99 % degradation in 150 h.

The bacterial consortium used in the present study, could utilize up to 300 mg/L of phenol with optimum degradation at 100 mg/L, in 4 days after
which there was no further degradation of phenol. The gas chromatographic profile of phenol degradation is represented in the Figure 4.12.

Figure 4.11 Degradation of Phenol at different concentrations by the bacterial consortium and protein yield at 50 g/L NaCl
Figure 4.12 GC profile of phenol degradation by the bacterial consortium at 50 g/L NaCl
From the above experiments it is proved that the bacterial consortium was able to degrade phenol almost completely (>93%) up to 200 mg/L concentration in 4 days. Above 250 mg/L, the degradation percentage reduced significantly. This may be due to substrate inhibition or product inhibition. Thus, when the concentration of phenol was increased to 300 mg/L, it affected the growth of the bacterial consortium and the degradation of the substrate. This behaviour is characteristic of toxic substrate metabolism as suggested by Hill and Robinson (1975), who reported that, as concentration of toxic substance increases, the more detrimental it becomes to the organism to degrade the substrate. Longer lag phase corresponding to increased phenol concentrations have been reported in non-halophilic bacteria. Wayman and Tseng (1976) suggested that microbial growth is inhibited firstly due to increase in the concentration of substrate to a level such that the growth decreases and ultimately ceases (substrate inhibition) and secondly by allowing the metabolite concentration to a toxic level, such as alcohol fermentation (product inhibition). Woolard and Irvine (1994) reported phenol inhibition, at concentrations above 30 mg/L, for a culture procured from the Salt Lake region.

Peyton et al (2002) reported the biodegradation of phenol by halophilic mixed cultures, in which one of the cultures was able to degrade up to 300 mg/L phenol at 100 g/L NaCl in 70 h. Hinteregger and Streichsbier (1997) showed that an unidentified Halomonas sp. degraded phenol up to 100 mg/L concentration at 140 g/L of NaCl in 13 h. Kanekar et al (1999) isolated alkaliphilic strains of Arthrobacter sp., Bacillus cereus, Citrobacter freundii, Micrococcus agilis and Pseudomonas putida biovar B which were capable of removing phenol from wastewaters arising from industries manufacturing methyl violet, where the bacterial strains were able to degrade
100 % of phenol. Alva and Peyton (2003) have shown the degradation of phenol (130 mg/L) by *Halomonas campisalis* in 100 h.

From the previous reports it may be concluded that most of the reports show that either the concentration of phenol used was less or the time taken for degradation of phenol was more than 4 days. The present study shows that the bacterial consortium was able to grow up to 300 mg/L of phenol, where optimum degradation of phenol was achieved at 100 mg/L in 4 days.

### 4.4.2 Degradation of phenol by individual bacterial strains

Preliminary studies have shown that the bacterial consortium is capable of utilizing selected phenolic compounds as sole carbon source. The degradation of phenol by the bacterial consortium was tested at various concentrations of phenol from 50 mg/L to 300 mg/L at optimum salinity of 50 g/L. It was observed from the previous experimental results that the bacterial consortium utilized phenol at an optimum concentration of 100 mg/L, which showed a degradation of 99 % in 4 days. Thus to further analyse the ability of the individual strains present in the bacterial consortium to degrade phenol, studies were conducted at optimum concentration of 100 mg/L of phenol at 50 g/L NaCl. To compare the degradation of the individual isolates, each strain was grown separately on phenol and their extent of degradation is presented in the Figure 4.13.

Among the six strains present in the bacterial consortium, *Arthrobacter sp.* was able to degrade 94 % of phenol with a maximum protein yield of 45.6 mg/L. *Pseudomonas aeruginosa* showed about 90 % degradation with 42.3 mg/L of protein yield. *Bacillus cereus* and *Bacillus licheniformis* showed 86 % and 83 % degradation, with the maximum protein yield of 35.2
and 33 mg/L respectively. At phenol concentration of 100 mg/L the growth was less with *Halomonas salina* and *Bacillus pumilus* where the protein content in the log phase was 30 and 27 mg/L with a degradation of 64 % and 61 % phenol respectively.

![Graph showing phenol degradation](image)

**Figure 4.13** Degradation of phenol by the bacterial strains and protein yield at 50 g/L of NaCl
Peyton et al (2002) showed the degradation of phenol by five different halophilic bacterial strains which utilized 50 mg/L of phenol in 17 h at 100 g/L NaCl. The protein yield was in a range of 30-40 mg/L by the individual cultures. In the present study the individual strains showed higher protein yield in the range of 30-47 mg/L and was able to utilize 100 mg/L of phenol at 50 g/L NaCl.

The most prevalent bacterial hydrocarbon degraders belong to the genera *Pseudomonas, Achromobacter, Flavobacterium, Nocardia* and *Arthrobacter* (Harayama and Timmis 1992). Kanekar et al (1999) isolated alkaliphilic strains of *Pseudomonas* sp., *Arthrobacter* sp. *Bacillus cereus*, *Citrobacter freundii*, *Micrococcus agilis* and *Pseudomonas putida* biovar B, *Nocardiodes* sp. and *Alcaligenes faecalis* from Lonar lake India, which were able to grow on 2.6 g/L of NaCl and was able to remove about 368 mg/L of phenol in the wastewaters from industries manufacturing methyl violet (using phenol as one of the major raw materials) and cumene-phenol.

Karigari et al (2006) isolated *Arthrobacter citreus* from hydrocarbon contaminated site, which was able to tolerate up to 22 mM of phenol under non-saline conditions. above that concentration was inhibitory to the growth of the bacterium. The organism had additional ability to degrade catechol, cresols and naphthol. Unell et al (2008) reported the degradation of mixture of phenols (4-chlorophenol, 4-nitrophenol and phenol) by *Arthrobacter chlorophenolicus* A6 under non-saline conditions.

*Pseudomonas* group of organisms predominate in degradation of phenolic wastes as previously shown in Table 2.4 of Chapter 2. But under saline conditions there are no reports on the degradation of phenol by individual strains of *Pseudomonas sp.*
In present work with bacterial consortium, the genus *Bacillus* is predominant with three different species. Next to *Pseudomonas* sp. many studies reported the degradation of phenols by *Bacillus* sp. under non-saline conditions (Duffener et al 2000, Ali et al 2000, Tallur et al 2006, Arutchelvan et al 2006)

A significant work has been done on the degradation of phenolic compounds by *Halomonas* sp. under saline conditions (Hinteregger and Streichbier 1997, Garcia et al 2004, 2005, Alva and Peyton 2003). As the genus *Halomonas* comprises of great number of species and they commonly inhabit saline habitats. They include species with few nutritional requirements and have the ability to grow in a wider range of salinities as they are very easy to culture, they play important part in the biotechnological processes at different salinities (Ventosa et al 1996).

On comparison with earlier reports it may be inferred that, the bacterial strains isolated in the present study were able to degrade phenol individually and as well as a consortium with a degradation of more than 90 % at 5 % NaCl. This suggests that the bacterial strains can also be used as pure cultures for degradation of phenolic compounds under saline conditions.

**4.4.3 Comparison of Degradation of Phenol by the Bacterial Consortium and the Bacterial Strains**

The degradation of phenol (100 mg/L) at 5 % salinity by the isolated bacterial consortium and the individual bacterial strains were compared and illustrated in the Figure 4.14. It is clear from the result that the consortium was able to completely degrade phenol (100 mg/L) with degradation of 99 %. *Arthrobacter sp.* gave 94 % and *Pseudomonas aeruginosa* showed 90 % degradation. While the degradation reduced with
*Bacillus cereus* and *Bacillus licheniformis* to 86 % and 84 % respectively. The degradation of phenol relatively reduced to 64 % and 61 % with *Halomonas salina* and *Bacillus pumilus*. Figure 4.14 shows the comparison of degradation of phenol (100 mg/L) by bacterial strains and consortium. Based on the results it was inferred that using a bacterial consortium in the degradation of phenol proved to be more efficient in completely degrading phenol (99 %) in 4 days. The reason might be due to bacterial strains present in the consortium co-exists and takes part in the degradation of phenol, while as individual strains tolerance to the toxic substrate was limited. Hence, for the further degradation studies on phenolic compounds and in the treatment of phenol containing saline wastewater the bacterial consortium was used as the inoculum. It is interesting to note that bacterial strains belonging to three different phyla could coexist as a consortium to completely degrade phenol under saline conditions.

![Figure 4.14 Comparison of degradation of phenol by the bacterial strains and consortium at 50 g/L of NaCl](image)

Figure 4.14 Comparison of degradation of phenol by the bacterial strains and consortium at 50 g/L of NaCl
4.4.4 Effect of nitrogen sources on the degradation of phenol by the bacterial consortium

To examine the influence of alternative nitrogen sources, tryptone and urea were used in place of yeast extract in the medium. Tryptone is composed of an assortment of peptides formed from digestion of casein. Urea has two amino groups, which act as a good source of nitrogen.

The effect of alternate nitrogen source on the degradation of phenol at 50 g/L of NaCl was determined by replacing 0.01 % of yeast extract with 0.01 % tryptone or 0.01 % urea. The degradation of phenol (100 mg/L) in the presence of tryptone and urea is given in the Figure 4.15. The results showed that addition of tryptone in the place of yeast extract gave only 94 % degradation in 4 days, with a maximum protein yield of 39.3 mg/L. When urea was substituted for yeast extract, the degradation still reduced to 91 % with a maximum protein yield of 32 mg/L.

![Figure 4.15 Effect of nitrogen sources on the protein yield and degradation phenol by the bacterial consortium](image)

Figure 4.15 Effect of nitrogen sources on the protein yield and degradation phenol by the bacterial consortium
From the experimental results of previous section, it may be concluded that addition of yeast extract in the medium showed highest phenol degradation of 99% and it also enhanced the growth of the consortium. Altering the nitrogen source with tryptone or urea didn’t play an important role in improving the degradation of the substrate; this might be due to the composition of yeast extract as it is readily available as aminoacids.

Increase in salinity and depletion of nutrients inhibit the growth of the bacterial cells. In general, to support the growth of bacterial cells, nutrients such as glucose, yeast extract and acetate were added as additional carbon source (Yuan et al 2000). Carla and Babu (2004) reported the degradation of BTEX compounds by a halophilic and halotolerant microcosms, where benzene was completely degraded in 8 days after the addition of yeast extract, vitamins or trace elements, but in the absence of the stimulants it took 15 days. An archaea (strain EH4) isolated from a salt marsh was able to degrade a higher amount of eicosane (C\textsubscript{20}H\textsubscript{42}) in the presence of yeast extract, peptone, and casaminoacids (Bertrand et al 1990). Woolard and Irvine (1995) also reported that addition of yeast extract in the medium enhanced the degradation of phenol.

4.4.5 Effect of Additional Salts on the Degradation of Phenol by the Bacterial Consortium

Degradation of phenolic compounds by microorganisms is also affected by some external factors such as salt ions, pH and temperature of the wastewater. Especially, high salt concentration is the most important factor which reduces microbial activity. Biodegradation of phenol in the presence of high salt concentration, particularly sodium chloride, has been reported by many researchers. Saline wastewater contains phenolic compounds, along with different salts other than NaCl. To study the effect of other salts under
saline conditions (50 g/L NaCl); experiments were conducted with additional salts such as K\(_2\)SO\(_4\), KCl, Na\(_2\)SO\(_4\), and NaNO\(_3\) (Figure 4.16).

![Figure 4.16 Effect of additional salts on the protein yield and degradation of phenol by the bacterial consortium](image)

From the results it was observed that the bacterial consortium utilized phenol in the presence of additional salts (K\(_2\)SO\(_4\), KCl, Na\(_2\)SO\(_4\) and NaNO\(_3\)) under saline conditions. In the presence of KCl and K\(_2\)SO\(_4\) the degradation of phenol was 90 % and 84 %. Least degradation was observed
with NaNO$_3$ (79 %). Growth of the consortium in terms of protein yield during the degradation of phenol in the presence of additional salts is given in the Figure 4.16. The maximum protein yield for sodium salts was in the range of 32.5 to 46.4 mg/L on 3$^{rd}$ day with NaNO$_3$ and Na$_2$SO$_4$ respectively. In the case of potassium salts, the protein yield was 38.2 and 42.3 mg/L for K$_2$SO$_4$ and KCl on 3$^{rd}$ day.

4.4.6 Identification of Metabolites Formed during the Degradation of Phenol

Intermediates formed during the degradation of phenol (100 mg/L) was analysed by Gas Chromatograph-Mass Spectral (GC-MS) analysis. Analyses of the 72 h supernatant, from the growth of bacterial consortium on phenol, showed the presence of the metabolites. A comparison of the mass spectra of extracted compounds with the standards (phenol and catechol) showed that the peaks in Figure 4.17 are phenol (peak 1), catechol (peak 2) and cis, cis–muconic acid (peak 3), indicating that the phenol degradation by the bacterial consortium followed via ortho- cleavage.

The GC-MS chromatogram showed four peaks, first being the parent compound phenol at retention time of 8.35 min; corresponding mass analyses yielded m/z (25,39,55,66,74,94), followed by peak 2 with a retention time of 9.863 min represented catechol, intermediate compound of phenol, with the masses m/z (40,53,64,81,92,110,112,136,151,166,207). At the retention time of 15.925 min peak 3 was observed which represented the ortho- cleavage product cis-cis muconic acid with m/z (97,137,163), the peak 4 was an unidentified product (Figure 4.17).
Figure 4.17 GC-MS Spectrum showing metabolites formed during the degradation of phenol

Hinteregger and Streichsbier (1997) studied the degradation of phenol at optimum salinity of 50 g/L where they reported that the disappearance of phenol was accompanied by accumulation of cis-cis muconic acid, which is a dead end product of ortho-cleavage pathway. Oren et al (1992) reported the same ortho-pathway in benzoate degradation by a marine bacterial isolate *Pseudomonas halodurans*, which exhibited a similar behaviour of tolerating increased salt concentrations from 17 g/L NaCl to 150 g/L in synthetic sea water, but there was no further intermediate products in the ortho-cleavage pathway.
Many researchers have reported the production of catechol and cis-cis muconic acid as intermediates of ortho pathway during the degradation of phenol (Müller and Babel 1996, Bastos et al 2000b, Wuster et al 2003, Tsai et al 2005, Alva and Peyton 2003).

In the present study formation of cis-cis muconic acid as the metabolite during the degradation suggested that the degradation of phenol was by ortho-cleavage pathway by the bacterial consortium.

4.5 DEGRADATION OF DIFFERENT CONCENTRATIONS OF SUBSTITUTED PHENOLS BY THE BACTERIAL CONSORTIUM AT 50 g/L NaCl

Next to phenols, many substituted phenols including chloro/nitrophenols and Cresols are listed as priority pollutants by the U.S. Environmental Protection Agency (1989). Cresols are present in crude oil and coal tar. Cresols are isomeric substituted phenols with a methyl substituent at either the ortho, meta or para positions relative to the hydroxyl group. These compounds are most commonly used in the production of fragrances, antioxidants, dyes, pesticides and resins. Ortho- and para-cresols are used in the production of lubricating oils, motor fuels and rubber polymers, while meta-cresol is used in the manufacture of explosives. Concentrations as high as 2100 µg/L for o-cresol and 1200 µg/L for mixed m- and p-cresols have been detected in wastewaters.

This section presents the degradation of different concentrations of Cresol derivatives (o-Cresol, m-Cresol, p-Cresol) and dual substrates (Phenol and o-Cresol) by the bacterial consortium.
4.5.1 Degradation of Different Concentrations of Cresols

Degradation of different concentrations of o-cresol with the protein yield is depicted in the Figure 4.18. o-Cresol at 50 mg/L, 75 mg/L and 100 mg/L concentrations were almost completely (93 %) degraded by the bacterial consortium in 4 days. At 50 mg/L and 75 mg/L of o-cresol, the bacterial growth in terms of protein yield increased to 42.3 mg/L and 35.6 mg/L respectively in 2 days. At the end of 4 days the degradation of o-cresol 50 mg/L and 75 mg/L was 99 % and 95 % respectively. When the concentration of o-cresol was increased to 100 mg/L, the degradation reduced to 93 % and with a decrease in the protein yield of 32 mg/L in the log phase (2 days). This shows that the growth of the consortium was inhibited with an increase in o-cresol concentration to 100 mg/L. The result of gas chromatographic analysis of o-cresol is shown in the Figure 4.19.

![Figure 4.18 Degradation of o-Cresol at different concentrations and protein yield by the bacterial consortium](image-url)
Figure 4.19 GC profile of o-Cresol degradation by the bacterial consortium at 50 g/L NaCl
Degradation of \( m \)-cresol at concentrations such as 50 mg/L, 75 mg/L and 100 mg/L with the yield of protein is shown in the Figure 4.20. \( m \)-cresol was comparatively degraded less than \( o \)-cresol, where the degradation reduced to 94% at 50 mg/L of \( m \)-cresol with a maximum protein yield of 37.6 mg/L. When the \( m \)-cresol concentration was increased to 75 mg/L there was a drop in the degradation (92%) with a corresponding protein yield of 30 mg/L. At highest concentration of \( m \)-cresol (100 mg/L) the bacterial consortium gave a lowest degradation of 82% with the increase in protein yield to 26.3 mg/L. Such decrease in the degradation might be due to the position of the functional group of \( m \)-cresol. The gas chromatographic profile of degradation of 50 mg/L of \( m \)-cresol is given in the Figure 4.21.

![Figure 4.20 Degradation of \( m \)-Cresol at different concentrations and protein yield by the bacterial consortium](image)
Figure 4.21 GC profile of \( m \)-Cresol degradation by the bacterial consortium at 50 g/L NaCl
Figure 4.22 depicts the degradation of \( p \)-cresol at different concentrations of (50 mg/L, 75 mg/L and 100 mg/L). When compared to \( o \)- and \( m \)-cresols, the degradation of \( p \)-cresol reduced considerably, which is observed from the results. At 50 mg/L, the degradation was 93% with a maximum protein yield of 33.2 mg/L. The degradation decreased to 90% and 79% at 75 mg/L and 100 mg/L with the maximum protein yield of 33.2 mg/L and 25.6 mg/L respectively.

![Degradation of \( p \)-Cresol at different concentrations and protein yield by the bacterial consortium](image)

**Figure 4.22 Degradation of \( p \)-Cresol at different concentrations and protein yield by the bacterial consortium**

A decrease in the degradation of \( m \)-cresol and \( p \)-cresol compared with \( o \)-Cresol could be due to the function of the methylation, which reduces the solubility of the substrate; as the methyl position increases from \textit{ortho} through \textit{meta} to \textit{para} positions. Thus \( o \)-cresol is rendered more available to the bacterial consortium during the biodegradation process. Among the cresols, \( p \)-cresol proved to be more recalcitrant than the other cresols. The gas chromatographic profile of degradation of 50 mg/L of \( p \)-cresol is given in the Figure 4.23.
Figure 4.23 GC profile of p-Cresol degradation by the bacterial consortium at 50 g/L NaCl
The bacterial consortium in the present study was able to degrade > 92 % of o-Cresol under saline conditions which was achieved after 4 days of incubation. This is higher than the degradation (87 %) by the biofilm of *Arthrobacter viscosus* (Quintelas et al 2006) and also with the fungal population (84 % degradation) previously reported by (Atagana 2004) under non-saline conditions.

Liu and Pacepavicius (1990) showed the biodegradation of the three isomeric cresols under non-saline conditions increased in the following order, *p*- > *m*- > *o*- cresol. This was reflected in the present study showing that the degradation of cresols in the order of *o>*-*m>*-*p*-cresols under saline conditions. Another reason for lesser degradation of *p*-cresol with other cresols is that, they are reported to degrade slower than that of other substituent.

Garcia et al (2005) studied the catabolic versatility of many low molecular weight-aromatic compounds including phenols and *p*-cresols. Most of the isolates belonging to the genus *Halomonas* were able to grow on most of the aromatic compounds except for *p*-cresol. The isolates enriched on phenol were able to utilize a great number of aromatic compounds than the rest of the isolates enriched on other aromatics. There are several reports on the degradation of phenol under saline conditions (Hinteregger and Striechbier 1997, Alva and Peyton 2003), but there are no reports on the degradation of cresols by individual strains or by a bacterial consortium under saline conditions. In present study, the bacterial consortium enriched with phenol had the ability to grow on all the cresol substrates with more than 90 % degradation efficiency.
4.5.2 Degradation of Dual Substrates (Phenol with $o$-Cresol)

Phenolic compounds are important constituents of petrochemical wastewaters, arising from the transformation processes. The refinery wastewater contains phenol together with substituted alkyl phenols as the main constituents. The phenolic compounds in the spent caustic liquors are in the range of 2-50 g/L, where 75 % is phenol and 10 % are alkyl-substituted compounds like cresols, xylenols and ethylphenols (Berné and Cordonnier 1995). Heinaru et al (2000) reported the occurrence of phenolic compounds mainly phenol (70 mg/L) and $p$-cresol (40 mg/L) from the leachate of oil shale water. As industrial wastewater contains complex mixtures of phenolic compounds and salts, it is particularly desirable to use the microorganisms, which are previously acclimatized on phenolic compounds under saline conditions to degrade such substrates.

From the results of the previous study, it was proved that the optimum concentration of phenol was 100 mg/L and $o$-cresol was 50 mg/L, where almost complete degradation of the individual substrate was achieved in 4 days. For the degradation of dual substrates by the bacterial consortium, Phenol (100 mg/L) and $o$-cresol (50 mg/L), were used for studying the simultaneous degradation of the substrates. The results from the experiments showed that phenol was degraded 95 % and $o$-Cresol was degraded 91 % with the maximum protein yield of about 29.5 mg/L on 2nd day (Figure 4.24).
Figure 4.24 Degradation of Dual substrates and protein yield by the bacterial consortium

The gas chromatographic profile of degradation of the dual substrates is presented in Figure 4.25. From the figure it was observed that when the consortium degraded phenol and \( o \)-cresol together. There are previous reports on the degradation of mixture of substrates by pure cultures and mixed cultures. The simultaneous metabolism of phenol and cresol has been reported in aerobic and anaerobic conditions (Hutchinson and Robinson (1988), Tawfiki et al (1999), Heider and Fuchs (1997), Fang and Zhou (2000) under non- saline conditions, where the degradation of phenol was inhibited by the presence of alkylphenols \( o-, m-, \) or \( p- \) cresols).
Figure 4.25  GC profile of degradation of dual substrates by the bacterial consortium at 50 g/L NaCl
Chang et al (1992) have reported the degradation of toluene and benzene by *Pseudomonas fragi*, where they observed rate of degradation of either benzene or toluene in the presence of the other was slower than the degradation of either of the substrate alone; a competitive type of inhibition between toluene and xylene.

Kar et al (1997) showed a competitive inhibition between Phenol and *p*-cresol by *Arthrobacter* sp., where the inhibition constants showed that the phenol degradation was strongly inhibited by the presence of *p*-cresol. While o-cresol enhanced the degradation of phenol marginally but *o*-cresol degradation was not affected by the presence of phenol as the co-substrate.

Paraskevi and Polymenakou (2005) reported that the addition of *o*-cresol strongly inhibited phenol transformation during the co-metabolism of phenol and *o*-cresol.

As observed from the Figure 4.24, irrespective of the concentration levels of the compounds, Phenol was degraded higher than that of *o*-Cresol. The disparity observed in the growth of the bacterial consortium on phenol and *o*-cresol may be due to the presence of methyl group on the compound, which affects some of their physical properties, such as solubility. This may be due to the fact that phenol, compared to *o*-cresol, is a much simpler carbon source and therefore is easily metabolized by the bacterial consortium. Another reason would be that the bacterial consortium was primarily enriched on phenol as the carbon source.

Saravanan et al (2008) reported the degradation of dual substrates Phenol and *m*-cresol in an internal loop airlift bioreactor study, where the Phenol has been preferentially degraded by the microbial culture rather than *m*-cresol probably owing to the toxic effect of the later.
Wang et al (2009) indicated that there was almost no effect on the growth of *Arthrobacter* sp. and the degradation of mixtures of phenol (200 mg/L) and *p*-cresol (100 mg/L) with less than 5 % NaCl; and concentration above 5 % NaCl, the growth was inhibited at late lag phase and complete degradation was achieved in 88 h. Whereas in the present study, it could be seen that the degradation of mixture of substrates were degraded more than 90 % within 4 days in the presence of 5 % NaCl without any lag phase.

In the present study the bacterial consortium could degrade the dual substrates with greater than 90 % degradation in 4 days without any lag phase. The presence of dual substrates did not affect the degradation of individual compounds as a mixture, which shows that the bacterial consortium could be used in the degradation of different combination of substrates.

### 4.5.2.1 Effect of nitrogen sources on the degradation of dual substrates

To study the effect of alternate nitrogen sources to yeast extract, tryptone and urea were used during the degradation of dual substrates. Figure 4.26 shows the degradation of dual substrates phenol (100 mg/L) and *o*-cresol (50 mg/L) in the presence of tryptone. It was observed from the Figure 4.26 that phenol was degraded up to 93 % and *o*-cresol was degraded up to 82 % with maximum protein yield of 32.4 mg/L in 2 days.

When urea was used as the nitrogen source, phenol degradation reduced to 90 % and *o*-cresol was degraded up to 70 % with a maximum protein yield of 27.2 mg/L in 2 days (Figure 4.27). The nitrogen sources like tryptone and urea were not able to enhance the degradation of dual substrates. During the degradation of dual substrates in the presence of yeast extract, the degradation was more than 90 % for both the substrates in mixture. This may be because of the composition of yeast extract, as it is rich in vitamins,
minerals, and digested nucleic acids and readily available as aminoacids for growth of the bacterial consortium.

Figure 4.26 Effect of tryptone on the protein yield and degradation of dual substrates by the bacterial consortium

Figure 4.27 Effect of urea on the protein yield and degradation of dual substrates by the bacterial consortium
4.5.2.2 Effect of additional salts on the degradation of dual substrates

As industrial wastewater from pharmaceutical industries contains mixtures of phenolic compounds and different salts, it is very difficult for the conventional microorganisms to treat such saline wastewater containing the phenolics. A bacterial consortium of utilizing the mixer of substrates and tolerating saline conditions is essential for the treatment of such saline wastewaters. Effect of salts on the biodegradation of dual phenolic compounds were determined with additional salts such as KCl or Na$_2$SO$_4$ or K$_2$SO$_4$ or NaNO$_3$. These studies were carried out in the medium containing 100 mg/L of Phenol and 50 mg/L of o-Cresol. The results are shown in the Table 4.4. The bacterial consortium was able to degrade the mixed phenolic compounds in the presence of the all the additional salts. The dual substrates (i.e) 100 mg/L and o-cresol 50 mg/L was degraded in 4 days in the presence of 50 g/L of NaCl.

Table 4.4 Effect of additional salts on the degradation dual substrates and protein yield by the consortium

<table>
<thead>
<tr>
<th>Salts (50g/L)</th>
<th>Protein Yield in log phase (2 days) (mg/L)</th>
<th>Degradation efficiency of phenol (%)</th>
<th>Degradation efficiency of o-Cresol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>32.4</td>
<td>87</td>
<td>78</td>
</tr>
<tr>
<td>K$_2$SO$_4$</td>
<td>26.7</td>
<td>80</td>
<td>65</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>35.6</td>
<td>90</td>
<td>85</td>
</tr>
<tr>
<td>NaNO$_3$</td>
<td>28.7</td>
<td>75</td>
<td>72</td>
</tr>
</tbody>
</table>

In the presence of Na$_2$SO$_4$ the degradation of phenol was 90 % and o-Cresol was degraded simultaneously to 85 % at the end of 4 days. In the case of KCl the degradation was 87 % and 78 % for Phenol and o-Cresol respectively. It was shown that in the presence of K$_2$SO$_4$ the degradation still
reduced to 80 % and 65 % for Phenol and o-Cresol. NaNO₃ showed the least degradation of 75 % and 72 % for Phenol and o-Cresol. Overall this experiment showed that the bacterial consortium grown on dual substrates in the presence Na₂SO₄ and KCl gave higher degradation of not less than 78 % compared with K₂SO₄ and NaNO₃ were the degradation was not less than 72 %.

Wang et al (2009) reported the degradation of mixtures of substrates (Phenol (100 mg/L) and p-Cresol (100 mg/L)) in the presence of additional salts, where the degradation percentage with Na₂SO₄ and KCl was not less than 77 % and in the presence of K₂SO₄ and NaNO₃ degradation was not less than 59 %. On comparison with the results of the present study with the isolate used by Wang et al (2009), it inferred that bacterial consortium could degrade considerably higher amount of phenol and o-cresol than that evidenced with isolate *Arthrobacter* W1 strain.

### 4.5.2.3 Identification of metabolites during the degradation of dual substrates

The analyses of the 72 h culture extracts of the bacterial consortium grown on mixtures of phenol and o-cresol, produced intermediate metabolites as shown by GC-MS analyses (Figure 4.28). The chromatogram showed three peaks, on comparison of the mass spectrum from extracted samples with the standards it proved that the first peak was catechol an intermediate compound of phenol at retention time of 9.88 min; corresponding mass analyses yielded m/z (40,53,63,81,95,110,112), followed by peak 2-Hydroquinone with a retention time of 11.525 min, where the m/z (25,27,39,55,63,81,92,110,112). At the retention time of 18.35 peak 3 was observed which represented the metabolized product of o-cresol, 3-methyl catechol with masses
(33,42,52,67,85,110,124,134). The structures of the compounds are represented in the top of the peak in Figure 4.28.

Figure 4.28 GC-MS Spectrum of the metabolites formed during degradation of dual substrates

Masunaga et al (1983) reported four different metabolites dihydroxytoluenes, 3-methylcatechol, 4-methylresorcinol and methyl hydroquinone, which were produced during the degradation of mixture of phenol and o-cresol.

Ahamed et al (2001) reported the presence of 3-methylcatechol and 2-ketohe-xcis-4-enoate during o-cresol degradation. Saravanan et al (2008) showed that hydroquinone, benzoquinone, catechol, oxalic acid, maleic acid,
acetic acid, formic acid and tartaric acid were formed as intermediates during the degradation of phenol and m-cresol as mixed substrates under non-saline conditions.

Mesophilic bacteria have been widely reported to catabolize phenol and the isomeric cresols by converting them before aromatic-ring fission into catechol and methylcatechols, respectively. Based on the literature survey on halophilic degradation of aromatics, only few works have been reported on the degradation of aromatics by the pure cultures, but there are no reports on degradation of phenolic compounds by bacterial consortium. The present study results indicated that the bacterial consortium was able to convert the dual substrates to their intermediates such as catechol and 3-methyl catechol, in 4 days under saline conditions.

4.6 DEGRADATION OF DIFFERENT CONCENTRATIONS OF SUBSTITUTED CHLOROPHENOLS BY THE BACTERIAL CONSORTIUM AT 50 g/L NaCl

Chlorophenols are carcinogenic and toxic environmental pollutants which are massively discharged to the environment from uncontrolled industrial activities. The annual global demand for chlorophenols is ~100 kilo tonnes (Fetzner 1998), and the global production of higher and lower chlorinated phenols is ~25-30 kilo tonnes and ~60 kilo tonnes, respectively (Ullmann’s 1998). Chlorophenols enter into the environment from the industrial waste discharges and leachates from landfills and places where they are used as pesticides. Mono and di-chlorophenols also enter into the atmosphere by volatilization. Chlorinated phenolics have been found to accumulate in freshwater and marine environments. The annual industrial
production of chlorophenols was estimated at $0.2 \times 10^6$ metric tonnes in 1989 (WHO 1989, Xun et al 1992a, b, Stuart and Woods 1998, Tiirola et al 2002).

To determine the ability of the bacterial consortium to degrade chlorophenols, experiments on degradation were performed with selected chlorophenols, such as mono-hydroxy group -4-chlorophenol (4-CP), dihydroxy group -2,4-dichlorophenol (2,4-DCP), trihydroxy group- 2,4,6 trichlorophenol (2,4,6- TCP).

### 4.6.1 Degradation of Different Concentrations of 4-CP

To study the ability of the bacterial consortium to degrade different concentrations of 4-CP at optimum salinity of 5 % NaCl, the growth and degradation were studied with the consortium on different concentrations of 4-CP ranging from 25 to 100 mg/L. Initially, the degradation increased at 25 mg/L and up to 75 mg/L of 4-CP, with a steady increase in the protein yield. When the concentration of the substrate was increased to 100 mg/L, the growth of the consortium reduced, with a decrease in degradation of 4-CP as shown in Figure 4.29.

During the batch study at 25 mg/L of 4-CP, the maximum degradation was 93 %, with a protein yield of 37.5 mg/L in two days. When the concentration of 4-CP was increased to 50 and 75 mg/L the degradation was 90 % and 88 % respectively with a decrease in the protein yield on the 2nd day 35.4 mg/L and 32.1 mg/L respectively. At 100 mg/L, the degradation of 4-CP reduced to 84 % with a respective decrease in the protein yield on the second day to 27.5 mg/L. This indicated that the growth of the bacterial consortium reduced with the increase in substrate concentration and there was no further degradation. Figure 4.30 shows the gas chromatographic profile of degradation of 4-CP (25 mg/L) at 50 g/L NaCl. An increase in the 4-CP
concentration to 100 mg/L decreased the degradation, which might be due to the toxic effects of the compound at its higher concentration.

Figure 4.29 Degradation of different concentrations of 4-CP by the bacterial consortium and protein yield at 50 g/L NaCl
Figure 4.30  GC profile of 4-CP degradation by the bacterial consortium at 50 g/L NaCl
Hollender et al (1997) reported the degradation of 4-CP by *Comamonas testosteroni* JH5 under non-saline conditions, where above 1.24 mM (158 mg/L) concentration the degradation was inhibited.

Biodegradation of chlorophenols have been studied under non-saline conditions by pure cultures and by mixed cultures, which were initially grown on various growth substrates, including phenol, toluene and other more common carbon sources (Schmidt et al 1983, Spain and Gibson 1988, Topp et al 1988). Farrell and Quilty (1999) reported 4-CP degradation by mixed cultures which proved to be more efficient than pure species. Further they reported that pure species may suffer from contamination problems and thus may be impossible to maintain in the field. *Pseudomonas putida* CP1 completely degraded monochlorophenols (2- and 3- Chlorophenols) via an ortho-cleavage pathway up to 1.56 mM and 4-CP at 2.34 mM (Farrell and Quilty 2002).

Bae et al (1996) reported that the strain *Comamonas testosteroni* CPW301 was able to degrade 4-CP at 0.75 mM (96 mg/L) in 340 h and 0.9 mM (115 mg/L) was inhibitory. *Arthrobacter chlorophenolicus* could degrade 100 mg/L 4-CP within 24 h (Westerberg et al 2000).

Yang et al (2008) reported *Rhizobium* sp. 4-CP-20 could degrade 100 mg/L of 4-CP in 4 days under non-saline conditions, concentrations above 240 mg/L was inhibitory. They also proved that mixed culture had better capability to degrade about 200 mg/L of 4-CP in 7 days.

In present study, the bacterial consortium could degrade 4-CP up to 100 mg/L under saline conditions at 50 g/L NaCl, this shows the ability of the consortium to grow and tolerate 100 mg/L of 4-CP as the sole carbon source equivalent to non-halophilic strains.
Results from the previous literature are on degradation of 4-CP by pure and mixed cultures under non-saline conditions. These non-halophilic strains have degraded 4-CP either in the presence additional carbon sources or the time taken for degradation of the substrate was more than 5 days. In the present study the bacterial consortium was able to degrade not less than 84% up to 100 mg/L of 4-CP under saline conditions in 4 days. The growth of the bacterial consortium did not show any lag phase and was able to degrade 4-CP up to 75 mg/L with complete degradation of the substrate. Above 75 mg/L the degradation reduced which may be attributed to the cytotoxicity of chlorophenols at elevated concentrations (Herrera et al 2008).

4.6.1.1 Effect of Nitrogen Sources on the degradation of 4-CP

Increase in salinity and depletion of nutrients inhibit the growth of the bacterial cells. To support the growth of bacterial cells, nutrients such as yeast extract and carbon sources glucose, acetate are added to the mineral salts medium (Yuan et al 2000). In the present study (0.01%) tryptone and urea were used as nitrogen sources instead of yeast extract, to study the effect on the degradation of 4-CP.

At 25 mg/L of 4-CP the bacterial consortium was able to show a maximum degradation of 93%, hence this optimum concentration was taken to further evaluate the degradation efficiency of alternate nitrogen sources in the mineral salts medium. The effect of the alternate nitrogen sources in the degradation of 4-CP is given the Figure 4.31. The degradation of 4-CP was influenced by other nitrogen sources. In the presence of tryptone as the nitrogen source, the degradation reduced to 89% with a maximum protein yield of 27.5 mg/L. In the presence of urea, the degradation still reduced to 83% with corresponding protein yield of 24.3 mg/L in 2 days. The results showed that altering the yeast extract with other nitrogen sources like tryptone or urea reduced the degradation of 4-CP.
Figure 4.31 Effect of nitrogen sources on the protein yield and degradation of 4-CP at 50 g/L NaCl

Fakhruddin and Alamgir (2007) reported that degradation of 4-CP under non-saline conditions by *Pseudomonas putida* CP1, in the presence of yeast extract the rate of removal of chlorophenol (200 mg/L) was increased up to a value of 0.5 % (w/v).

During degradation process, the yeast extract is taken up from the medium by the cell and directly incorporated into proteins or transformed into other cellular nitrogenous constituents. By contrast, the cell spends more energy and time in synthesizing amino acids for protein synthesis from inorganic nitrogen sources (Mongi et al 2005). In the present study, addition of nitrogen sources like tryptone and urea reduced the degradation up to 80 % while in the presence of yeast extract the degradation of 4-CP (25 mg/L) was 93 %. The reason for greater degradation in the presence of yeast extract might be due to the easy availability of yeast extract in the form of aminoacids.
4.6.1.2  Effect of Additional Salts on the degradation of 4-CP

To study the effect of additional salts on the degradation of 4-CP, experiments were conducted with additional salts under saline conditions (50 g/L of NaCl). When Na$_2$SO$_4$ was used the degradation was 90 % with a maximum protein yield of 32.4 mg/L. In the case of K$_2$SO$_4$ and KCl the degradation reduced to 84 % and 75 % with the protein yield of 28.3 and 32.4 respectively (Figure 4.32).

![Figure 4.32 Effect of additional salts on the protein yield and degradation of 4-CP by the bacterial consortium](image)
The degradation was least with NaNO$_3$ (62%) with a maximum protein yield of 25.4 mg/L. The bacterial consortium was able to degrade 4-CP in the presence of additional salts where the presence of Na$_2$SO$_4$ didn’t have much influence the degradation compared to other salts (NaNO$_3$, KCl, K$_2$SO$_4$) where the degradation of 4-CP was reduced.

4.6.1.3 Identification of Metabolites during the degradation of 4-CP

To identify the metabolites from the degradation of 4–CP, 72 h culture supernatant was analysed in GC-MS. The mass spectra were compared with the standards (4-CP, 4-Chlorocatechol), which showed that the peaks as shown in Figure 4.33.

The chromatogram showed three peaks, first was the parent compound 4-CP at retention time of 8.92 min; corresponding mass analyses yielded m/z( 26,39,50,65,73,92 99.8,100,128). The second peak formed at the retention time of 14.131 represented the intermediate compound 4-Chlorocatechol (40,51,63,83,87,98,115,126,144,170,185,200,259,274) and peak three was the final degraded product of 4-CP, 5-chloro-2-hydroxymuconate at the retention time of 17.25 with the following masses (48,63,75,83,97,114,131,158,174,184,191,199). The structures of the compounds are represented on top of the peak in Figure 4.33. During the degradation of 4-CP, the degradation of 4-CP, the degradation pathway is initiated by the hydroxylation of 4-CP to the corresponding 4-chlorocatechol. The 4-chlorocatechol then undergoes a meta-ring cleavage by catechol 2,3 dioxygenase to produce 5-chloro-2-hydroxymuconic semialdehyde, which in turn is converted to 5-chloro-2-hydroxymuconate (El-Sayed et al 2009). In the present study, the GC-MS analysis of the degradation of 4-CP, showed that the key intermediate 5-chloro-2-hydroxymuconic semialdehyde was formed this suggest that the consortium
followed *meta*-cleavage pathway during the degradation. Activated aromatic compounds undergo ring cleavage reactions via lower pathway and are further processed to give molecules that can eventually enter the tricarboxylic acid cycle (Cafaro et al 2004).

Figure 4.33 GC-MS Spectrum of the metabolites formed during degradation of 4-CP

Sahinkaya and Dilek (2007) showed the degradation of 300 mg/L of 4-CP by acclimated and unacclimated activated sludge in aerobic batch reactors under non-saline conditions leading to the formation of 5-chloro-2-hydroxymuconic semialdehyde, which was further metabolized, indicating complete degradation of 4-CP via a *meta*-cleavage pathway.
Degradation of 4-CP by pure and mixed culture and the metabolites formed during the degradation was reported by many other researchers (Hollender et al 1997, Farrell and Quilty 1999, Yang and Lee 2008). Nordin et al (2005) reported *A. chlorophenolicus* A6 degraded 4-CP via hydroxyquinol. Moreover, hydroxyquinol was removed from cell extracts derived from 4-CP-grown cells but not from extracts of cells grown on succinate.

El Sayed et al (2009) reported that degradation of 4-CP by production of the 4-chlorocatechol and 5-chlorohydroxymuconic acid as intermediates of meta-cleavage pathway by *Bacillus subtilis* OS1, while the cell free extracts of *Alcaligenes* OS2 showed modified meta-cleavage pathway.

### 4.6.2 Degradation of Different Concentrations of 2,4-DCP

2,4–dichlorophenol (2,4-DCP) is a key intermediate in the synthesis of chloride-based herbicides, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 2- (2,4-dichlorophenoxy propionic acid (2,4 –DP). Due to their extreme toxicity and persistence in the soil, these compounds represent a serious ecological problem and public health risk (ATSDR 1999).

To our knowledge there are no reports on the degradation of 2,4-DCP by the bacterial consortium under saline conditions. Maltseva et al (1996) isolated three bacterial strains from alkaline lake in South Oregon, of which halophilic strain 1-18 strain was able to degrade up to 3000 mg/L of 2,4-dichlorophenoxyaxeticacid (2,4-D) in 3 days at pH 8.4-9.4.

In present study, the bacterial consortium was tested on 2,4- DCP, as sole source of carbon and energy source at 25 mg/L to 100 mg/L
concentrations at 50 g/L NaCl. Initially at 25 mg/L the degradation of 2,4-DCP was 92 % with a maximum protein yield of 32.5 mg/L on the 2\textsuperscript{nd} day. At substrate concentrations of 50 mg/L and 75 mg/L, the degradation decreased to 88 % and 86 % respectively with a maximum protein yield on the 3\textsuperscript{rd} day of 30 and 26.5 mg/L respectively. The degradation of 2,4-DCP reduced relatively to 82 % with a maximum protein yield of 25.6 mg/L at 100 mg/L of 2,4- DCP. The degradation of different concentrations of 2,4-DCP is presented in Figure 4.34. To our knowledge there are no reports on the degradation of 2,4-DCP under saline conditions. However, biodegradation of chlorophenols by a variety of microorganisms have been studied by many authors (Steinle et al 1998, Kim et al 2002, Kargi and Eker 2005) under non-saline conditions. According to these studies, the biodegradability of chlorophenols depends on the number and position of halogens in the aromatic ring. Furthermore, high chlorophenol concentrations are known to be inhibitory to microbial growth. There are contradicting reports regarding the advantages on the use of mixed cultures over pure cultures in the biodegradation of chlorophenols. Some reports (Caldeira et al 1999, Farrell and Quilty 1999) suggest that mixed microbial communities result in higher levels of biodegradation of chlorophenols. Farrell and Quilty (1999) reported that the mixed culture degraded 1.56 mM of 4-CP by meta cleavage pathway.

However, in present study from the preliminary experiments on degradation of phenol as sole carbon source by individual bacterial strains and the consortium proved that the degradation is most efficient with consortium compared to individual strains. Hence, mixed culture was used in the degradation of different phenolic compounds.
Koh et al (1997) have reported a 69 % dehalogenation of 2,4-DCP using *Alcaligenes eutrophus*, while Wang et al (2000) showed that the degradation of 2,4- DCP using *Bacillus insolitus* both as pure cultures and as immobilized cultures where concentrations above 200 mg/L the degradation was less than 50 %.
Gallizia et al (2003) reported that a pure culture of *Micrococcus* species was able to degrade phenol and 2,4-DCP under non-saline conditions, where 2,4-DCP (200 mg/L) was utilized in 10 days with higher degradation at alkaline pH (pH 10).

Goswami et al (2005) reported that degradation of 2-chlorophenol was efficient with a pure culture of *Rhodococcus erythropolis* M1 than with mixed culture. Matafonova et al (2006) reported that *Bacillus cereus* GN1 degraded 2,4-DCP up to 560 µM in submerged culture experiments in two days under non-saline conditions.

Herrera et al (2008) reported that a *Bacillus* consortium could degrade 2,4-DCP (2.5 mM) in the presence of NH₄Cl or KNO₂ as nitrogen sources, with a degradation of 85% in 21 days under non-saline conditions. Kiyohara et al (1992) reported, the concentration of the 2,4-DCP under non saline conditions, where inhibitory concentrations of the compound for individual cultures were for *Pseudomonas picketti* (15.6 mg/L), *Pseudomonas putida* (125 mg/L) and with *Pseudomonas aeruginosa* (62.5 mg/L).

Comparing the results of the present study with the previous reports, the concentration of 2,4 –DCP was not more than 125 mg/L under non –saline conditions. While the bacterial consortium in the present study was able to degrade up to 100 mg/L of 2,4-DCP without any lag period. Previous reports also showed that the period for degradation of the compound was more than 4 days and in most of the experiments they used additional nutrient supplements to enhance the degradation of 2,4-DCP even in non-saline conditions. However, in the present study the bacterial consortium was able to utilize different concentrations of 2,4- DCP up to 100 mg/L in 4 days under saline conditions with yeast extract (0.01 %). The gas chromatographic profile of degradation of 2,4-DCP (25 mg/L) is represented in the Figure 4.35.
Figure 4.35 GC profile of 2,4-DCP degradation by the bacterial consortium at 50 g/L NaCl
4.6.5.1 Effect of nitrogen sources on the degradation of 2,4-DCP

To study the effect of alternate nitrogen sources, tryptone and urea were used individually in the place of yeast extract in the mineral salts medium. Figure 4.36 shows the degradation of 2,4-DCP at its optimum concentration of 25 mg/L with protein yield by the bacterial consortium. The degradation of 2,4-DCP was reduced by altering the nitrogen source, where the degradation dropped in the absence of yeast extract.

In the presence of tryptone as the nitrogen source the degradation reduced to 86% with the maximum protein yield of 25.4 mg/L in 2 days. When yeast extract was alternated with urea, the degradation still dropped to 81%, with corresponding increase in the protein yield to 22.3 mg/L on the 2nd day. The results revealed that yeast extract proved to be a more efficient nitrogen source, when compared with tryptone and urea; this may be due to easy availability of aminoacids in yeast extract than the other sources.

![Figure 4.36 Effect of nitrogen sources on the protein yield and degradation of 2,4-DCP by the bacterial consortium](image)

Figure 4.36 Effect of nitrogen sources on the protein yield and degradation of 2,4-DCP by the bacterial consortium
4.6.2.2 Identification of metabolites during the degradation of 2,4-DCP

The intermediates formed during the degradation of 2,4-DCP was analysed by GC-MS and is presented in the Figure 4.37. The mass spectrum from the 72 h culture filtrate were compared with the standards (4-CP and 2,4-DCP). The chromatogram showed 3 peaks, first peak at a retention time of 9.314 represented the parent compound 2,4-DCP with masses m/z (40,49,63,73,97,98,125,126,145,162), peak 2 at retention time 9.717 represented 4-CP with masses m/z (14,18,39,46,65,73,93,100,128) followed by peak 3 representing 3,5-dichlorocatechol at retention time of 14.131 with masses m/z (40,51,63,83,87,98,115,126,144,170,185,200,259,274).

Figure 4.37 GC-MS Spectrum of the metabolites formed during degradation of 2,4-DCP
Herrera et al (2008) reported the degradation 2,4-DCP by a *Bacillus* consortium (CPHY1). Where GC-MS of the culture supernatant showed only 4-CP was produced as the intermediate, which indicated that 2,4-DCP was incompletely degraded.

In the present study the detection of the 3,5-dichlorocatechol in the supernatant showed that the substrate 2,4-DCP was degraded to form its intermediate metabolite. Dichlorocatechol is the central metabolite in the aerobic degradation of a wide range of chlorinated aromatic compounds (Farell and Quilty 1999). Degradation of 2,4-DCP by pure cultures and mixed cultures was reported by many other researchers with dichlorocatechol as the intermediate (Quan et al 2004, Matafonova et al 2006, Sahinkaya et al 2007).

### 4.6.3 Degradation of different concentrations of 2,4,6-TCP

2,4,6-Trichlorophenol (2,4,6-TCP) is widely used in industrial processes as a preservative of leather and textile goods, for wood and glue as an antimildew agent, and as a precursor for the synthesis of Prochloraz (fungicide) and some biocides such as 2,3,4,6-tetrachlorophenol and pentachlorophenol (Sittig 1981). 2,4,6-TCP is a toxic, persistent and bioaccumulable environment pollutant. Of the six isomers of trichlorophenols, 2,4,5- and 2,4,6-isomers have been placed on the U.S. Environmental Protection Agency’s priority pollutants list (EPA 1984a, b). The latter has been included in directive 76/464/EEC (European Economic Community) as a dangerous substance discharged into the aquatic environment (Vincent 1991).

From the six isomers of trichlorophenols, 2,4,6-TCP has been reported as highly toxic and frequently discharged into the aquatic environment. Hence batch studies were conducted with different concentrations of 2,4,6-TCP by the bacterial consortium at optimum salinity of 5% NaCl. The protein yield of the consortium and degradation was examined, with different concentrations of 2,4,6-TCP ranging from 25 to 100
mg/L. The protein yield of the consortium on 2,4,6-TCP was comparatively less than other phenolic compounds used in the study. This might be due to the recalcitrance of the compound. The degradation and protein yield of the 2,4,6-TCP is illustrated in the Figure 4.38.

![Figure 4.38 Degradation of 2,4,6-TCP at different concentrations and protein yield by the bacterial consortium at 50 g/L NaCl](image)

Figure 4.38 Degradation of 2,4,6-TCP at different concentrations and protein yield by the bacterial consortium at 50 g/L NaCl
Initially at 25 mg/L of 2,4,6-TCP the degradation was 89% with a maximum protein yield to 30.3 mg/L in 2 days. When the concentrations of 2,4,6-TCP was increased to 50 mg/L and 75 mg/L the degradation reduced to 84% and 80% with the corresponding decrease in the protein yield to 25.4 and 28.4 respectively. The degradation of 4-CP, dropped significantly with 100 mg/L of the substrate to 74% with protein yield increasing on the 2nd day to 21.5 mg/L. Figure 4.38 illustrates the degradation of 2,4,6-TCP and protein yield by the bacterial consortium at 50 g/L NaCl.

The results showed that the increase in the concentration of the substrate reduced the growth of the consortium which was intern represented by the protein yield. When the concentration of the substrate increased to 100 mg/L the degradation reduced to 74%, this might be due to toxicity of the substrate or unavailability of 2,4,6-TCP to the consortium or accumulation of toxic metabolites or depletion of nutrients such as nitrogen and phosphate or all these factors together would have inhibited the growth of the bacterial consortium. Another possible reason would be other than the substrate no other carbon source like glucose was supplied in the medium.

Maltseva and Oriel (1997) reported bacterium *Nocardioides* sp Strain M6 was able to degrade 1600 mg/L of 2,4,6-TCP at pH 9.4 in 288 h. The mineral medium used in their study contained casminoacids and yeast extract (1 g each). In the present study, the bacterial consortium grew on mono-, di and trichlorophenols at 5% NaCl with trace amounts of 0.01% of yeast extract in the medium. This shows that the bacterial consortium is halotolerant and as the consortium had optimum pH of 7, increase in the concentration of 2,4,6-TCP (100 mg/L) reduced the degradation.
Kharoune et al (2002) reported the degradation of 2,4,6-TCP (200 mg/L) under non-saline conditions by a microbial consortium containing *Sphingomonas paucimobilis*, *Burkholderia cepacia*, *Chryseomonas luteola* and *Vibrio metschnikovii*, where only of about 51 mg of 2,4,6-TCP g/L cell protein/h was degraded in 160 h.

Pamukoglu and Kargi (2008) reported the degradation of 2,4,6-TCP under non-saline conditions by *Rhodococcus rhodochrous* (DSM 43241) where 150 mg/L of 2,4,6-TCP inhibited the growth and degradation, in the presence of glucose as the additional carbon source.

The bacterial consortium effectively degraded mono- and di-chlorophenols when these were used as the only sources of carbon and energy. The biodegradability of the compounds decreased in the order: 4-CP >2,4-DCP >2,4,6-TCP. In studies of chlorophenol biodegradation, it can be noted 4-CP, 2,4-DCP were degraded readily than 2,4,6-TCP.

Nutrients play a vital role in the degradation of aromatics under saline condition as the bacterial cells are under dual stress of both the organic pollutant and the salt concentration. Hence, addition of nutrients may enhance the degradation with an increase in cell count during the utilization of the 2,4,6-TCP as sole carbon source at its higher concentrations. The gas chromatographic profile of 2,4,6-TCP is given in the Figure 4.39.
Figure 4.39 GC profile of 2,4,6-TCP degradation by the bacterial consortium at 50 g/L NaCl
4.6.3.1 Effect of nitrogen sources on the degradation of 2,4,6- TCP

To enhance the degradation, a minimal quantity of yeast extract was added as a nitrogen source in the mineral salts medium. To study the influence of alternate nitrogen sources, at optimum concentration of 25 mg/L of 2,4,6-TCP, the mineral salts medium containing yeast extract was replaced with tryptone and urea. The results of the experiment are presented in the Figure 4.40. Addition of the nitrogen sources influenced the degradation of the substrate. In the presence of tryptone it was observed that degradation was 81 % with an increase in the protein content to 18.5 mg/L on the 2\textsuperscript{nd} day. When urea was used as the nitrogen source, it was found that the degradation reduced to 77 % with a protein yield to 16.5 mg/L.

![Figure 4.40](image)

**Figure 4.40 Effect of nitrogen sources on the protein yield and degradation of 2,4,6-TCP by the bacterial consortium**

Tryptone and urea reduced the degradation of 2,4,6-TCP, the bacterial consortium was able utilize yeast extract to enhance the degradation of the substrate, as it is readily available as aminoacids. Addition
of yeast extract enhanced the degradation of 2,4,6-TCP (Fava et al 1995 and Armenante et al 1999).

Leys et al (2005) reported that degradation of Polyaromatic hydrocarbons in the soil slurry was enhanced by addition of inorganic nutrients and nutrient ratio allows easy production of new cells at the expense of the poly aromatic hydrocarbons as sole source of carbon and energy.

4.6.3.2 Identification of metabolites during the degradation of 2,4,6-TCP

The formation of intermediates in the degradation of 2,4,6-TCP was analysed by Gas Chromatograph-Mass Spectral (GC-MS) analysis. The supernatant from the mineral medium grown with bacterial consortium on the substrate showed the presence of the metabolites during the degradation process. A comparison of the mass spectra from intermediates of sample-extracted compounds with the standards (2,4,6-TCP) showed that the peaks in Figure 4.41 are 2,4,6-TCP (peak 1) at retention time of 9.825 the corresponding mass yielded m/z (36,48,62,73,97,99,125,132,160,169,196), the peak 2 represented the intermediate 2,6-dichlorohydroquinone with m/z (53,60,88,120,141,158,176) at retention time of 12.025. The peak 3 and peak 4 were unidentified products. Peak 2 showing 2,6-dichlorohydroquinone indicated that 2,4,6 TCP was dechlorinated to form its intermediate. Figure 4.41 represents the GC-MS analysis results of 2,4,6-TCP, where the structure of the compounds are represented at the top of the peak. From the results it was obvious that 2,6-dichlorohydroquinone was the intermediate formed from 2,4,6-TCP degradation which may further undergo oxidation to the corresponding quinone compound and is only detectable when it is present in appreciable amounts. Matus et al 2003, Sanchez and Gonzalez (2007) reported that the 2,6-dichloroquinone was produced during the degradation of 2,4,6-TCP.
Xun and Webster (2003), studied the degradation of 2,4,6-TCP by Ralstonia eutropha JMP134 and described that 2,4,6-TCP monooxygenase catalyzes sequential dechlorination of 2,4,6-TCP to 6-chlorohydroxyquinol. The monooxygenase converts 2,4,6-TCP to 6-chlorohydroxyquinone by means of two different reactions first it oxidizes 2,4,6-TCP to 2,6-dichloroquinone, and then it hydrolyzes 2,6-dichloroquinone to 6-chlorohydroxyquinone.

Two main strategies are used to degrade chlorophenols by aerobic bacteria utilizing these compounds as a carbon and energy source (Solyanikova and Golovleva 2004). Lower chlorinated phenols mono – and dichlorophenols (1 to 2 chlorine substituents) are initially attacked by monooxygenases yielding chlorocatechols as the first intermediates
(chlorocatechol pathway), which are subject to ring cleavage prior to dechlorination. On the other hand, polychlorinated phenols (3 to 5 chlorines) are converted to chlorohydroquinones as the initial intermediates (hydroquinone pathway).

Subsequent reactions progressively remove chlorines from the ring prior to ring cleavage. The monooxygenase from *Ralstonia eutropha* strain JMP134 (pJP4) catalyzes successive dechlorination reactions of 2,4,6-TCP to 2,6-dichlorohydroquinone and then to 6-chlorohydroxyquinol prior to ring cleavage by a hydroxyquinol dioxygenase (Matus et al 2003).

The GC-MS analysis of the extracted sample proved that the bacterial consortium used for the degradation of 2,4,6-TCP was able to form intermediate 2,6-dihydroquinone which is the intermediate product of 2,4,6-TCP. This proved that the compound was dechlorinated by the monooxygenase enzyme which also occurs in the non-halophilic strains degrading 2,4,6-TCP. The results also suggest that the pathway followed by halophilic bacterial consortium was same as that of the conventional microorganisms. This was also supported by studies conducted by Oren et al (1992), Hintregger and Streichsbier (1997) and Alva and Peyton (2003).

4.7 DIOXYGENASE ACTIVITY DURING THE DEGRADATION OF PHENOL AND 4-CHLOROPHENOL BY THE BACTERIAL CONSORTIUM

Aerobic organisms degrade phenol to catechol followed by oxidative cleavage of the ring. This oxidative ring cleavage of catechol can occur in one of the two ways. (1) *Ortho* cleavage pathway to produce cis,cis-muconic acid and (2) *meta* cleavage pathway to produce 2-hydroxymuconic semialdehyde. The production and accumulation of these intermediates during phenol degradation has been commonly observed (Li and Humphrey 1989;
Morsen and Rehm 1990; Allsop et al 1993). For complete degradation of chlorinated aromatic compounds to occur, two steps are necessary, cleavage of the aromatic ring and the removal of the chloride atom (Haggblom, 1990). The initial step in the aerobic degradation of mono-chlorophenols is their transformation to chlorocatechols. Chlorocatechols are the central metabolites in the aerobic degradation of a wide range of chlorinated aromatic compounds. The identification of byproducts formed during the biodegradation process of phenol and chlorophenol is essential for a better understanding of the degradation mechanism.

Oxygenases catalyze reactions in which atoms of oxygen are incorporated into organic substrates. Such enzymes are widely distributed in nature and are involved in both biosynthesis and biodegradation. Dioxygenase enzyme is an intradiol enzyme that cleaves the aromatic ring by incorporation of both atoms of dioxygen into the substrates. The present study was centered mostly on ring cleaving dioxygenases. In this section the specific activity of dioxygenase assay performed during the degradation of phenol and 4-chlorophenol are reported.

For the detection of dioxygenase activity, phenol was used as the model compound for phenol derivatives and 4-chlorophenol was used as the model-compound in the case of chlorophenol derivatives.

### 4.7.1 Dioxygenase Activity on phenol

A batch study was conducted with an optimum phenol concentration of 100 mg/L to detect the presence of catechol 1,2 dioxygenase and catechol 2,3 dioxygenase produced by the bacterial consortium at 50 g/L of NaCl. The effect of substrate concentration was optimized for further studies. Enzyme assays conducted with phenol (100 mg/L) as the substrate at 50 g/L NaCl, suggested that the consortium produced only catechol 1,2 dioxygenase showing the ortho-cleavage pathway. There was no catechol 2,3...
dioxygenase activity detected when phenol was used as the substrate by the bacterial consortium. The experiment results of catechol 1,2 dioxygenase assay is presented in this section.

4.7.1.1 Effect of substrate (Catechol) concentration on catechol 1,2 dioxygenase activity

To study the effect of substrate on catechol 1,2 dioxygenase activity, experiments were conducted on degradation of phenol (100 mg/L) by the bacterial consortium on the 2nd day in their log phase at 50 g/L NaCl. The effect of substrate concentration on the activity of catechol 1,2 dioxygenase is shown in the Figure 4.42. Substrate concentrations were used from 1 µm – 10 µm. It was observed from the figure that the highest catechol 1,2 dioxygenase activity of 0.379 µm/min/mg of the protein was achieved at 5 µm of catechol, from 6 µm of the substrate concentration there was no increase in the activity of catechol1,2 dioxygenase. Hence, for the further 5 µm of catechol was used as the substrate.

![Figure 4.42 Effect of substrate concentration on catechol 1,2 dioxygenase activity](image-url)
Hinteregger and Streichsbier (1997) reported the degradation of phenol (100 mg/L) by *Halomonas* sp. at 50 g/L NaCl, where the catechol 1,2 dioxygenase activity was 760 nm/min/mg of protein, there was no catechol 2,3 dioxygenase activity and they showed that there was only ortho- pathway during the degradation of phenol.

The present study with the bacterial consortium showed that only catechol 1,2 dioxygenase activity during the degradation of phenol (100 mg/L) with optimum NaCl concentration of 50 g/L and there was no catechol 2,3 dioxygenase activity. This was also proved from the previous experiments with gas chromatography- mass spectral analysis. Use of the ortho- pathway is reported to be more efficient in carbon conversion to cell mass (growth yield) than the meta-pathway. The meta pathway utilizes phenol at a higher rate but results in lower cell yields (Kiesel and Muller 2002)

**4.7.1.2 Effect of pH on catechol 1,2 dioxygenase activity**

To determine the effect of pH on catechol 1,2 dioxygenase produced by the bacterial consortium, its activity was measured with 5 µm of catechol at different pH from 5 to 8. The specific activity was 0.085 µm/min/mg of protein at pH 5 during the log phase (on 2nd day) with total protein yield of 22.4 mg/L. However, activity increased to 0.268 µm/min/mg of protein, at pH 6 with the maximum protein yield at the end of 3rd day to 25.4 mg/L. The specific activity of catechol 1,2 dioxygenase was maximum at pH 7 (0.425 µm), with total protein of 30.3 mg/L. (Figure 4.43). Further increase in the pH resulted in the decrease in specific activity and finally it gave only 0.322 µm/min/mg of protein at pH 8 with total protein of 22.5 mg/L on 3rd day. The optimum pH for the enzymatic action of catechol 1,2 dioxygenase is pH 7. Previous experiments with growth also proved that the bacterial consortium showed highest growth and degradation of phenol at neutral pH of 7. This showed that the bacterial consortium was able to degrade the substrate at neutral pH with the production of catechol 1,2 dioxygenase.
Briganti et al (1997) reported phenol degradation by *Acinetobacter radioresistens*, where the purified catechol 1,2 dioxygenase showed that the optimum activity was in the range of pH 6.0 to 8.5.

Alva and Peyton (2003) reported degradation of phenol and catechol by the haloalkaliphilic *Halomonas campisalis*, where they showed the presence catechol 1,2 dioxygenase at pH 8.

**Figure 4.43** Effect of pH on catechol 1,2 dioxygenase activity by the bacterial consortium
Two *Halomonas* sp. have been reported to use the ortho-pathway for the biodegradation of aromatics under saline conditions and at neutral pH (Hinteregger and Streichsbier 1997, Rosenberg 1983).

Garcia et al (2005) reported the degradation of phenol by *Halomonas organivorans*, the specific activity achieved during the degradation was 0.061 µm/min/mg of the protein at 10% NaCl. In present study on phenol degradation by the bacterial consortium showed 0.425 µm/min/mg of the protein at the end of 2nd day, which proved that the consortium could produce a higher specific activity, this might be due to the existence of different bacterial strains in the consortium.

### 4.7.2 Dioxygenase activity on chlorophenol

To detect the presence of dioxygenase activity from the bacterial consortium during the utilization of 4-chlorophenol, experiment was performed at an optimum concentration of 25 mg/L of 4-CP under saline conditions (50 g/L of NaCl). Experiments conducted during the degradation of 4-CP, showed that there was only pronounced catechol 2,3 dioxygenase activity, which showed that the bacterial consortium utilized 4-chlorophenol by meta-cleavage pathway. Experiments were conducted to study the effect of substrate concentration on catechol 2,3 dioxygenase activity and the results are presented in this section.

#### 4.7.2.1 Effect of substrate (4-chlorocatechol) concentration on catechol 2,3 dioxygenase activity

To study the effect of substrate on catechol 2,3 dioxygenase activity, experiments were conducted with 4-chlorophenol 25 mg/L at 50 g/L NaCl. The effect of substrate concentration on the activity of catechol 2,3 dioxygenase is demonstrated in the Figure 4.44. Substrate concentrations were used from 10 mM – 100 mM of 4-chlorocatechol. It was observed from the figure that the highest catechol 2,3 dioxygenase activity of 0.425
µm/min/mg of the protein was achieved at 50 mM of 4-chlorocatechol, from 60 mM of the 4-chlorocatechol concentration there was no further increase in the activity of catechol 2,3 dioxygenase. Hence, 50 mM of 4-chlorocatechol was used as the substrate for dioxygenase assay.

Figure 4.44 Effect of substrate (4-chlorocatechol) concentration on catechol 2,3 dioxygenase activity

4.7.2.2 Effect of pH on catechol 2,3 dioxygenase activity

To study the effect of pH on catechol 2,3 dioxygenase the activity of the enzyme was measured with 50 mM of 4-chlorocatechol as optimum at different pH from 5 to 8. The enzyme activity was less (0.078 µm/min/mg of protein) at pH 5 at the on 2nd day with total protein of 21.5 mg/L. However it increased at pH 6 the activity was 0.223 with an increase in protein content to 25.4 mg/L. The specific activity of catechol 2,3 dioxygenase was maximum at pH 7 which showed highest activity of 0.412 µm/min/mg of protein with 30.3 mg/L of total protein (Figure 4.45). Further increase in the pH resulted in a decrease in enzyme activity and at pH 8.0 the enzyme activity was 0.321 µm/min/mg of protein with 22 mg/L of total protein on 3rd day. The optimum
pH for the enzymatic action of catechol 2,3 dioxygenase was similar to that of catechol 1,2 dioxygenase at neutral pH 7 and the activity was retarded at pH 8. This experiment further confirmed that the bacterial consortium was able to degrade the phenols with the intradiol enzymes at neutral pH. The levels of catechol 2,3-dioxygenase activity detected during the degradation of 4-Chlorophenol disappeared only after 4-chlorophenol was almost degraded by the mixed culture.

**Figure 4.45** Effect of pH on catechol 2,3 dioxygenase activity by the bacterial consortium
Hollender et al (1997) reported degradation of 1.8 mM 4-CP via the meta-cleavage pathway by Comamonas testosteroni JH5, where catechol 2,3 dioxygenase activity was 173 mU/mg of the protein, while catechol 1,2 dioxygenase activity was less than 1.

O’ Sullivan (1998) reported the degradation of 4-CP (1.56 mM) by mixed culture containing two Pseudomonas species were the degradation took place via the meta-cleavage pathway.

Farrell and Quilty (1999) reported the degradation of 4-CP via meta cleavage pathway, with catechol 2,3 dioxygenase activity of 0.096 µm/min/mg of the protein, by a mixed microbial community under non saline conditions.

As shown in the figure, the present study showed that the catechol 2,3 dioxygenase activity was 0.412 µm/min/mg of the protein in the log phase of 48 h by the bacterial consortium, which is comparatively higher than reported by earlier workers (Farrell and Quilty 1999, Sahinkaya and Dilek 2005).

According to the results on the dioxygenase enzyme activity, it can be concluded that the bacterial consortium followed ortho-cleavage pathway during the degradation of phenol and meta-cleavage pathway during the degradation of 4-CP.

4.8 TREATMENT OF SALINE PHARMACEUTICAL WASTEWATER BY THE BACTERIAL CONSORTIUM

The isolated bacterial consortium which was able to degrade phenol and substituted phenols under saline conditions was used in the treatment of
phenol containing saline wastewater in a lab-scale bioreactor. The wastewater was collected from a pharmaceutical industry near Chennai. In this section the efficiency of the bacterial consortium in the treatment of diluted and raw saline pharmaceutical wastewater (SPW).

### 4.8.1 Treatment of Diluted Saline Pharmaceutical Wastewater

The preliminary study was conducted on the treatment of phenol containing saline pharmaceutical wastewater prior to laboratory scale bioreactor study. In the preliminary study batch study was performed in shake flasks with combined pharmaceutical wastewater supplemented with mineral medium (80:20 v/v) along with the bacterial consortium. The results of the batch experiments in shake flasks are shown in Figure 4.46.

The initial COD and BOD concentration were 16,180 mg/L and 1961 mg/L, respectively after dilution with the mineral salts medium (80:20 v/v). The maximum COD and BOD removal efficiencies were 92 % and 90 % respectively with an effluent COD and BOD concentrations of 1,452 mg/L and 212 mg/L, at the end of 5 days. In the batch study the bacterial consortium removed about 92 % of total phenols with a maximum dry cell weight of 655 mg/L in the log phase on the 4th day. There was no significant increase in the biomass of the consortium after 5 days; this might be due to the depletion of nutrients and toxic intermediates produced during the degradation.
Figure 4.46 Reduction of Total Phenols, COD and BOD during the treatment of SPW
COD reduction was only 55% on the 3rd day and the COD removal gradually increased to 84% on the 5th day which reached a maximum of 92% at the end of 6 days. It is clear from the figure that the COD, BOD reduction starts during the logarithmic growth phase and the COD, BOD reduction reached maximum during the stationary phase. Phenolic compounds in the wastewater were rapidly degraded or transformed into their metabolites, which is also indicated by the COD removal.

Afzal et al (2007) reported the degradation of phenol with monocultures of *Pseudomonas aeruginosa* and *Pseudomonas pseudomallei*, where both the strains were able to degrade phenol (1500 mg/L) in 7 days. As individual cultures of *Pseudomonas aeruginosa* showed 96% removal of both COD and BOD, while *Pseudomonas pseudomallei* showed 86% and 80% of COD and BOD removal respectively.

In present study, the time taken by the bacterial consortium for the treatment of the saline wastewater was only 6 days and there was no lag period during the degradation of phenols and the consortium could effectively degrade the organics under saline conditions. The results obtained in the batch study on the treatment of phenol containing saline pharmaceutical wastewater by the bacterial consortium have shown the feasibility for high COD and BOD removal along with total phenols removal in the saline pharmaceutical wastewater. As the batch study proved to be efficient in the removal COD and BOD, the bacterial consortium acclimatized to the pharmaceutical wastewater was further employed in the lab-scale bioreactor studies.

4.8.2 Treatment of Raw Saline Pharmaceutical Wastewater

The characteristics of saline pharmaceutical wastewater used were: pH -7.8, colour- brown, salinity 3.5-3.6 %, a high COD 20,198-20,226 mg/L, BOD 3,822-3,952 mg/L, TKN - 980-995 mg/L, phosphate was 182-198
mg/L, and total phenols of 565-595 mg/L. The removal of different wastewater components such as pH, COD, BOD, MLSS, MLVSS, TKN, and Phosphate were observed at every 24 h time interval. The results obtained are presented and discussed in this section.

4.8.2.1 Efficiency of bacterial consortium in treating SPW

During the treatment process the pH in the bioreactor was between 7.4 and 7.8. Initially the pH was 7.8 in the raw effluent and at the end of 5 days, the pH was 7.4. This trend can be possibly attributed to the conversion of the recalcitrant compounds in the wastewater, and production of acidic intermediates.

Initial COD and BOD were 20,226 mg/L and 3,952 mg/L, which started to decrease from the second day of treatment and finally attained 2,225 mg/L and 625 mg/L, respectively on the 5th day of the treatment. The BOD and COD removal efficiencies were 84 % and 89 % respectively.

In present study, when the BOD of the saline wastewater decreased, the MLVSS increased from 585 mg/L to 1492 mg/L in 5 days. The MLSS concentration during the treatment study was between 585 mg/L to 1568 mg/L which is represented in the Figure 4.47. Woolard and Irvine (1995) reported the degradation of phenol with 99.5 % removal by moderately halophilic microorganisms during the treatment of synthetic hypersaline wastewater by sequential batch biofilm reactor, where the increase in MLVSS increased from 50 mg/L to 1020 mg/L in 180 h.

In the present work, when the MLVSS increased, the concentrations of nitrogen in the saline pharmaceutical wastewater decreased from 995 mg/L to 18 mg/L and phosphate decreased from 198 mg/L to 12 mg/L, which showed that they were utilized by the bacterial consortium for MLVSS production. The increase in MLSS and MLVSS during the treatment
process is represented in the Figure 4.47. The utilization of nitrogen and phosphate in the medium is shown in the Figure 4.48.

**Figure 4.47 Reduction of COD and BOD during the treatment SPW with concentrations of MLSS and MLVSS**

**Figure 4.48 Removal of TKN and Phosphate during the treatment of SPW**

The results of this experiment proved that the bacterial consortium is efficient in the treatment of SPW with a COD, BOD removal greater
than 80%. The reactor set up during the treatment of SPW is shown in the Figure 4.49.

![Figure 4.49 Reactor set up for the treatment of SPW](image)

**4.8.2.2 Degradation of phenolic compounds in SPW**

In the raw wastewater the total phenols contributed to 595 mg/L, which was utilized by the bacterial consortium. At the end of 5 days the total phenols concentration was 15 mg/L which amounted to degradation of 97% as represented in Figure 4.50. The growth of the bacterial consortium was shown as biomass (dry cell weight), which increased from 24.6 mg/L at the start to a maximum of 652 mg/L on 4\textsuperscript{th} day of treatment process. The phenol containing saline pharmaceutical wastewater was analysed for the presence of phenolic compounds by GC-MS which is represented in the Figure 4.51.
Figure 4.50 Removal of Total Phenols during the treatment of SPW

Figure 4.51 GC-MS Spectrum of the raw SPW
The GC-MS chromatogram showed five major peaks, the first peak representing 2,4-DCP with m/z (41,49,63,73,97,98,126,162), the 2nd peak at retention time of 9.52 represented Phenol with m/z (25,39,55,66,74,94) followed by the 3rd peak 2,4,6-TCP at retention time of 10.615 with masses m/z (40,48,62,73,97,99,125,132,143,160,169,196,198). At the retention time of 14.099, 4th peak was observed which represented 2,3,4,5-Tetrachlorophenol with masses m/z (40,44,61,83,97,98,131,168,196,232) and the 5th peak represented pentachlorophenol at the retention time 18.514 showing masses m/z (40,47,60,83,95,101,115,130,141,165,169,196,202,214, 230,239,266,268).

The bacterial consortium utilized the phenolic compounds in the wastewater as the sole source of carbon and energy. At the end of 5 days, the phenolic compounds in the wastewater were almost completely degraded with 97% degradation. The individual phenols and the intermediates at the end of 4 days was shown by GC-MS analysis as shown in the Figure 4.52.

It was found that phenolic compounds were metabolized almost completely, with formation of their intermediates at 9.717, peak 2 representing 4-Chlorophenol m/z (26,39,50,65,73,92,99.8,100,128), peak 3-Catechol at 9.863 min with the masses m/z (40,53,64,81,92,110,112,136, 151,166,207), peak 4- Hydroquinone retention time of 11.525 min with the m/z (25,27,39,55,63,81,92,110,112), and final intermediate at peak 5 being dichlorocatechol 14.131 with masses (40,51,63,83,87,98,115,126,144,170, 185,200,259,274). It was also found that few compounds were available in traces at peak 1- 2,4-DCP, peak 6 and peak 7 represented tetrachlorophenol and pentachlorophenol. Phenolic compounds such as 2,4-DCP and pentachlorophenol were found in trace amounts which would have contributed for the remaining 3% of total phenol at the end of 5 days treatment process.
Figure 4.52 GC-MS Spectrum of treated SPW

Woolard and Irvine (1995) used a sequencing batch biofilm reactor (SBBR) with moderately halophilic bacteria isolated from the Great Salt Lake, in order to treat a synthetic effluent containing 150 g/L of salt and here they showed a degradation efficiency of 99.5% of phenol (100 mg/L) concentration in 150 h.

Panswad and Anan (1999) showed 71% COD removal using an anaerobic/aerobic process and with a synthetic wastewater containing 3% salt, where the seeding material was acclimated to high salinity conditions.

In the present study the isolated bacterial consortium was able to degrade 97% of the total phenol present in the raw saline pharmaceutical
wastewater with initial concentration of phenol 595 mg/L within 6 days. The bacterial consortium has the ability to degrade higher concentration of phenols as well as different phenolic compounds which proved the feasibility in the treatment of raw saline wastewater.

Kubo et al (2001) reported the treatment of pickle plum saline wastewater by two salt–tolerant bacteria *Staphylococcus* sp. and *Bacillus cereus* using aerobic sequential batch reactor which showed 90% removal of COD during continuous operation at 15% NaCl in 7 days.

According to the results batch studies conducted with diluted SPW showed a COD removal of 92%, BOD removal of 90% and total phenols were degraded up to 92%. The treatment process with raw SPW showed that the COD removal was 89%, BOD removal was 84% and total phenol removal was 97% respectively. Thus, it can be concluded that the present study proves that the bacterial consortium is able to utilize the phenolic compounds present in the saline pharmaceutical wastewater.