CHAPTER - II

Isolation, Identification and screening of Carbon-tetrachloride degrading microorganisms
Successful bioremediation depends on the availability of appropriate microorganisms. Microorganisms thrive in a particular habitat, mainly because they are metabolically able to exploit the resources of that habitat. Microorganisms, able to either metabolise xenobiotics or survive in the presence of such pollutant chemicals, can establish themselves in those habitats where these pollutants are present. The soil microflora in the vicinity of industries discharging anthropogenic chemicals will thus comprise of microorganisms capable of growth in the presence of them and be capable of transforming them by processes like induction evolved with time. Microorganisms of interest can hence be isolated from the appropriate natural environments and niches.

The search for useful biological agents to destroy hazardous wastes consists of an assortment of methodologies ranging from attempts to isolate pure strains from real-life contaminated situations to sophisticated genetic-engineering applications to produce a tailor-made organism capable of degrading a specific compound. The present chapter outlines the isolation of Carbon-tetrachloride degrading microorganisms from different contaminated sites and their identification, and screening from among them the most suitable culture, degrading Carbontetrachloride.

Collection of Samples

Soil samples from different ecosystems like soil from petrol pumps, and soil near the effluent discharge outlets
of chemical and pharmaceutical industries were collected. A total of 100 samples were collected. The soil samples were transported to the laboratory as early as possible in order to minimize any biochemical changes in the soil samples. The samples were refrigerated at 4°C and marked by numbering, and taken up serially for isolation of microorganisms degrading Carbontetrachloride.

Isolation of Carbontetrachloride degrading microorganisms

An enrichment culture technique, as modified from the method used by Criddle et al (1990a) and glucose mineral salts medium as described in Appendix I was used to isolate Carbontetrachloride degrading microorganisms. Glucose mineral salts medium was prepared in bulk and 50 ml aliquots of this medium was distributed into each 250 ml Erlenmeyer flask and sterilised separately at 121°C for 15 minutes. 0.5mM of Carbontetrachloride was added to each flask. 1 gm of the different soil samples collected from different ecosystems was added to the flasks. The flasks were incubated at 30°C, on a rotary incubator shaker,(Model E-25, EMENVEE Engineering, Pune). After 8 days of incubation, 2.0 ml of the enriched broth was added to 50 ml of fresh sterile glucose mineral salts medium. Four such successive transfers from the previous glucose mineral salts medium to fresh glucose mineral salts medium, were carried out with increasing concentrations of Carbontetrachloride at each successive transfer. The concentration of Carbontetrachloride added was 0.5mM for first week followed by 1mM in the second enrichment transfer and
2.5 mM in third and 5.0 mM in successive transfers thereafter. Glucose was excluded from the mineral salts medium after one month of acclimation. The enrichment flasks were regularly tested for the presence of microorganisms like bacteria, fungi and yeasts.

Isolation was carried out by streaking a loopful of growing culture from each of the last enrichment flasks on to mineral salts agar containing 5 mM Carbontetrachloride (Appendix I). The colony characteristics of the isolates were recorded and each of the isolated colonies were examined microscopically after staining. The same process was repeated twice to obtain pure culture and these isolated pure cultures were maintained on mineral salts medium containing Carbontetrachloride for further study.

4. Identification of Carbontetrachloride degrading microorganisms

A total number of 50 isolates belonging to the different groups of microorganisms namely yeast, fungi and bacteria (Table 5) were found to exhibit the ability to grow on Carbontetrachloride.

The isolated yeasts, belonged to the genera Candida sp, Rhodotorula sp and Saccharomyces sp, the isolated fungi belonged to the genera Penicillium sp, Fusarium sp and Aspergillus sp, the isolated bacteria belonged to Pseudomonas sp, Staphylococcus sp and Nocardia sp (Table 6).

The isolated colonies were identified according to the standard procedures of identification (Harrigan and McCance 1966, Hendrie and Shewan 1966, Buchanan and Gibbons 1974,
Table: 5 Microorganisms growing on Carbontetrachloride

<table>
<thead>
<tr>
<th>Types of microorganisms</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast</td>
<td>25</td>
</tr>
<tr>
<td>Fungi</td>
<td>17</td>
</tr>
<tr>
<td>Bacteria</td>
<td>8</td>
</tr>
</tbody>
</table>

Total isolates = 50.
<table>
<thead>
<tr>
<th>Genus</th>
<th>Number of Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I Yeasts</strong></td>
<td></td>
</tr>
<tr>
<td>Candida sp</td>
<td>8</td>
</tr>
<tr>
<td>Rhodotorula sp</td>
<td>12</td>
</tr>
<tr>
<td>Saccharomyces sp</td>
<td>5</td>
</tr>
<tr>
<td><strong>II Fungi</strong></td>
<td></td>
</tr>
<tr>
<td>Penicillium sp</td>
<td>9</td>
</tr>
<tr>
<td>Fusarium sp</td>
<td>8</td>
</tr>
<tr>
<td>Aspergillus sp</td>
<td>3</td>
</tr>
<tr>
<td><strong>III Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas sp</td>
<td>5</td>
</tr>
<tr>
<td>Staphylococcus sp</td>
<td>3</td>
</tr>
<tr>
<td>Nocardia sp</td>
<td>2</td>
</tr>
</tbody>
</table>

The key formulated for the identification of the isolates as per above references are shown in the Fig 4 to 8. The characteristic features of the isolates are given in Tables 7 to 15.

Even though a good number of fungal cultures were isolated having the ability to degrade Carbontetrachloride, for further studies we have selected yeast and bacteria because of ease of cultivation, and shorter generation periods.

**Screening of Carbontetrachloride degrading strains**

All the bacterial and yeast strains isolated were inoculated into 50 ml of mineral salts medium containing 5mM of Carbontetrachloride and checked for the growth and dehalogenation. All these sets were incubated at 30°C on rotary incubator shaker for eight days taking care to seal the flasks with Teflon. After 8 days growth as optical density at 600nm and dehalogenation as the chloride ions released (Appendix II) was measured for yeast and bacterial strains. The results are as shown in Table 16 & 17.

Among all the different strains tested for growth and dehalogenation on 5mM Carbontetrachloride, the yeast strains *Rhodotorula* R3 and *Candida* C4 exhibited maximum growth and dehalogenation activity. From the results obtained (Tables 16 & 17) it was observed that yeast *Rhodotorula* R3 and *Candida* C4 showed the highest dehalogenation activity as compared to bacteria. Hence from among all the isolates *Rhodotorula* R3 and *Candida* C4 were selected for Carbontetrachloride tolerance studies.
Figure: 4  KEY FOR IDENTIFICATION OF YEASTS

Spores

- Ascospores
  - Endomycetaceae
    - True mycelium
    - Polar budding: glucose not fermented
      - Debaryomyces
  - Endomycopsis
    - Salt concentration high
      - Use ethanol as carbon source
      - Debaromyces

- Ballistospores
  - Sporobolomyctaceae
    - Pseudomycelium
    - Multilateral: glucose fermented
      - Saccharomyces
  - Ballistosporales
    - Neither ascospores nor Ballistospores
      - Cryptococcaceae
      - No sexual spores
        - Multilateral budding
        - Rhodotorula
      - Off white
        - NO3-
        - Innositol
      - Red/Pink
        - Orange pigment

- False mycelium
  - Cryptococcus

TABLE: 7 CHARACTERISTICS OF GENUS SACCHAROMYCES

They are unicellular, spherical, ovoid or longer cells with rounded ends.

Pseudomycelium is formed sometimes.

Vegetative reproduction by budding on solid media.

Colonies are usually white or cream, domed, smooth, semi-mat and shiny.

Ascospores (1-4/ascus) are usually spherical or ovoid.
Carbohydrates are fermented vigorously.
TABLE 8 CHARACTERISTICS OF GENUS CANDIDA

Budding unicellular organism. Sometimes forms true mycelium.

Blastospores are frequently formed on the mycelia.

Reproduction is by budding or fission.

On solid media colonies are off white, cream or yellow.
TABLE 9  CHARACTERISTICS OF GENUS RHODOTORULA

Usually budding cells but occasionally form rudimentary pseudomycelium.

The colonies on solid media are brightly coloured red, pink, orange, or yellow.

They don't ferment carbohydrates.

They do not reduce nitrate.

They do not grow on inositol.
Figure 5  KEY FOR IDENTIFICATION OF FUNGI

Deuteromycetes (Fungi imperfecti)
Lack sexual phase. Reproduction by means of conidia/Pseudomycelium/true mycelium.

- Moniliaceae
  - produce conidia on unorganised hyaline conidiophores or directly from hyaline hyphae

- Tuberculariaceae
  - produce sporodochia.conidia are produced from phialides produce macroconidia and microconidia long multisep-tate crescent shaped.

  - Fusarium
    - conidiophores noseptate and turn to vesicle.
    - conidia in unbranched chain

  - Aspergillus

  - Penicillium
    - conidiophores branched to form a brush like conidial head.
    - Conidia are present as bottle shaped sterigmata (Finger shape arrangement)
The black aspergilli or *Aspergillus niger* group are the fungi commonly called black mold.

Conidiophores are non septate and arise from specialised, thick walled hyphal cells (Foot cells). Each conidiophore ends in a terminal enlarged ellipsoidal hemispherical or spherical swelling which ears phialides.

Conidia are unicellular, vary in their colour and shape. The conidia form unbranched chains.

Colonies are of different colours such as green, brown, grey, orange, yellow and off white.
TABLE: 11 CHARACTERISTICS OF GENUS *Penicillium*

They are generally called as green molds and blue molds. The mycelium produces simple, long, erect conidiophores that branch about two thirds of the way to the tip, in characteristic symmetrical or asymmetrical broom like fashion.

The conidiophores commonly referred to as the brush.

The multiple branching of the conidiophores ends in a group of phialides that bear the long conidial chains.

The conidia are globose to ovoid.

With the production of conidia colonies usually become green, grey-green, blue green or yellow-green.
<table>
<thead>
<tr>
<th>Table 12: Characteristics of Genus <em>Fusarium</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>A number of <em>Fusarium</em> sp are parasitic.</td>
</tr>
<tr>
<td>Mycelium of the fungus invades vascular tissue.</td>
</tr>
<tr>
<td>The macroconidia produced by members of this genus have many characteristics, being multicellular and spindle shaped or sickle shaped. One cell with ovoid microconidia is also formed.</td>
</tr>
<tr>
<td>Colonies on suitable media may be fluffy and spreading, and often coloured rose-pink, salmon-pink, brown-purple or yellow.</td>
</tr>
<tr>
<td>Chlamydosporas are frequently formed in the mycelium.</td>
</tr>
</tbody>
</table>
FIGURE 6 KEY FOR IDENTIFICATION OF *PSEUDOMONAS* SP.

Gram negative rods
Motile with polar flagella

- **Aerobic**
  - Very poor or no growth in the presence of animal protein;
growth at pH 4.5
-Rapid production of acetic acid from ethanol.
  - *(Acetomonas)*

- **Anaerobic** *(Symbomonas)*
  - Growth on media containing meat extract and/or peptone.
  - Oxidase positive/weak positive *(Kovac’s)*

- **Non fermentative in Hugh and Leifson’s medium** *(Glucose)*
- Fermentations in Hugh and Leifson’s medium *(Glucose)*
  - *(Aeromonas, Vibrio)*
  - Green fluorescent *(Pseudomonas)*
  - No fluorescent pigment produced *(Xanthomonas)*
A] MORPHOLOGY: Cell single, straight or curved rods but not helical. Gram negative, non sporulating motile rods. Do not produce sheaths or prosthecae.

B] BIOCHEMICAL CHARACTERS:

1] Catalase positive.
2] Oxidase positive.
3] Chemoorganotroph.
4] Metabolism oxidative never fermentative
5] Denitrifying
6] Produces acid and gas from glucose, galactose and mannose.
Fig: 7 Key for identification of Staphylococcus sp

- Gram positive cocci in clusters
  - Glucose fermentation and growth in presence of erythromycin
    - Positive
      - Motility
        - Motile (Planococcus)
        - Non motile (Micrococcus)
    - Negative
      - Motility
        - Motile (Aerococcus)
        - Non motile (Staphylococcus)
### Table: 14  Characters of the genus *Staphylococcus*

**A) MORPHOLOGY:** Cells spherical, occurring in clusters and characteristically dividing in more than one than one plane. Gram positive.

**B) BIOCHEMICAL CHARACTERS:**

1} Glucose fermentation is anaerobic.

2} Catalase positive.

3} Sensitive to lysozyme.

4} Oxidase positive.
Fig: 8 Key for identification of *Nocardia* sp.

Gram positive hyphae and rod shaped fragments

- Mycelium rudimentary or absent (*Mycobacterium*)
- Substrate mycelium, fragments into bacillary and coccoid elements
- Substrate mycelium, stable not fragmenting (*Streptomyces*)

Metabolic type

- Oxidative, partially acidfast, cell wall type IV
  - Hypoxanthine degraded (*Nocardia*)
  - Hypoxanthine not degraded (*Rhodococcus*)
- Fermentative
  - Actinomyces
Table: 15  Characters of genus Nocardia.

A) Morphology: Gram positive, substrate mycelium fragments into bacillary and coccoid elements. Non acid fast, non motile and non spore forming.

B) Biochemical characters:

- Reduction of nitrate: Positive
- Denitrification: Negative
- MR Test: Negative
- VP Test: Negative
- Indol production: Negative
- Citrate utilization: Positive
- O/F Test: Oxidative
- Hydrogen sulfide production: Negative
- Urease: Positive
- Hydrolysis of cellulose: Negative
- Heat resistance (in 10% skim milk at 72°C for 15 min): Negative
- Acid production from glucose: Positive
- Gas production from glucose: Negative
<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Growth O.D. 600nm</th>
<th>mM chlorine ions liberated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodotorula R1</td>
<td>0.040</td>
<td>0.02</td>
</tr>
<tr>
<td>Rhodotorula R2</td>
<td>0.046</td>
<td>0.022</td>
</tr>
<tr>
<td>Rhodotorula R3</td>
<td>0.072</td>
<td>0.038</td>
</tr>
<tr>
<td>Rhodotorula R4</td>
<td>0.034</td>
<td>0.016</td>
</tr>
<tr>
<td>Rhodotorula R5</td>
<td>0.036</td>
<td>0.018</td>
</tr>
<tr>
<td>Rhodotorula R6</td>
<td>0.043</td>
<td>0.022</td>
</tr>
<tr>
<td>Rhodotorula R7</td>
<td>0.038</td>
<td>0.018</td>
</tr>
<tr>
<td>Rhodotorula R8</td>
<td>0.041</td>
<td>0.020</td>
</tr>
<tr>
<td>Rhodotorula R9</td>
<td>0.052</td>
<td>0.024</td>
</tr>
<tr>
<td>Rhodotorula R10</td>
<td>0.061</td>
<td>0.028</td>
</tr>
<tr>
<td>Rhodotorula R11</td>
<td>0.055</td>
<td>0.026</td>
</tr>
<tr>
<td>Rhodotorula R12</td>
<td>0.047</td>
<td>0.022</td>
</tr>
<tr>
<td>Candida C1</td>
<td>0.054</td>
<td>0.014</td>
</tr>
<tr>
<td>Candida C2</td>
<td>0.035</td>
<td>0.01</td>
</tr>
<tr>
<td>Candida C3</td>
<td>0.034</td>
<td>0.01</td>
</tr>
<tr>
<td>Candida C4</td>
<td>0.061</td>
<td>0.016</td>
</tr>
<tr>
<td>Candida C5</td>
<td>0.052</td>
<td>0.012</td>
</tr>
<tr>
<td>Candida C6</td>
<td>0.059</td>
<td>0.016</td>
</tr>
<tr>
<td>Candida C7</td>
<td>0.036</td>
<td>0.01</td>
</tr>
<tr>
<td>Candida C8</td>
<td>0.056</td>
<td>0.014</td>
</tr>
<tr>
<td>Saccharomyces S1</td>
<td>0.069</td>
<td>ND</td>
</tr>
<tr>
<td>Saccharomyces S2</td>
<td>0.065</td>
<td>ND</td>
</tr>
<tr>
<td>Saccharomyces S3</td>
<td>0.067</td>
<td>ND</td>
</tr>
<tr>
<td>Saccharomyces S4</td>
<td>0.054</td>
<td>ND</td>
</tr>
<tr>
<td>Saccharomyces S5</td>
<td>0.049</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: No free chloride ions were detected in the medium.
Table: Screening of Carbontetrachloride degrading bacterial strains.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Growth O.D. 600nm</th>
<th>mM chlorine ions liberated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas P1</td>
<td>0.057</td>
<td>0.014</td>
</tr>
<tr>
<td>Pseudomonas P2</td>
<td>0.069</td>
<td>0.015</td>
</tr>
<tr>
<td>Pseudomonas P3</td>
<td>0.065</td>
<td>0.015</td>
</tr>
<tr>
<td>Pseudomonas P4</td>
<td>0.04</td>
<td>0.010</td>
</tr>
<tr>
<td>Pseudomonas P5</td>
<td>0.05</td>
<td>0.012</td>
</tr>
<tr>
<td>Staphylococcus ST1</td>
<td>0.059</td>
<td>0.015</td>
</tr>
<tr>
<td>Staphylococcus ST2</td>
<td>0.052</td>
<td>0.014</td>
</tr>
<tr>
<td>Staphylococcus ST3</td>
<td>0.049</td>
<td>0.012</td>
</tr>
<tr>
<td>Nocardia N1</td>
<td>0.05</td>
<td>0.010</td>
</tr>
<tr>
<td>Nocardia N2</td>
<td>0.054</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Since the yeast isolates Rhodotorula R3 and Candida C4 exhibited maximum dehalogenating activity they were tested for their ability to dehalogenate and grow at different concentrations of Carbontetrachloride in mineral salts medium. The concentrations of Carbontetrachloride used were 5mM, 10mM and 15mM. The flasks were sealed with Teflon and incubated at 30 °C on rotary incubator shaker for 8 days. After 8 days of incubation growth was measured as absorbance and dehalogenation by estimating chloride ions released turbidimetrically. The residual Carbontetrachloride in flasks of Rhodotorula R3 and Candida C4 was determined by gas chromatography (Appendix II).

The results are as shown in Fig 9 and Table 18. Maximum growth and dehalogenation was observed at 10mM for Rhodotorula R3 and Candida C4. The % degradation was higher with Rhodotorula R3 than with Candida C4.

7. Maintenance of cultures

All the Carbontetrachloride degrading isolates were maintained on mineral salts medium slants containing 0.1% v/v Carbontetrachloride. The slants were routinely subcultured in order to maintain Carbontetrachloride degrading ability of the culture.
FIG.-9  Carbontetrachloride tolerance of
Rhodotorula  R 3  and  Candida  C 4
Table 18: Carbontetrachloride tolerance by *Rhodotorula R3* and *Candida C4.*

<table>
<thead>
<tr>
<th>Name of Yeast</th>
<th>Concentration of Carbontetra Chloride mM</th>
<th>Growth O.D. 600nm</th>
<th>mM Chloride released</th>
<th>% Degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodotorula R3</td>
<td>5</td>
<td>0.072</td>
<td>0.038</td>
<td>38.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.109</td>
<td>0.081</td>
<td>40.5</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.085</td>
<td>0.045</td>
<td>22.5</td>
</tr>
<tr>
<td>Candida C4</td>
<td>5</td>
<td>0.060</td>
<td>0.016</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.068</td>
<td>0.034</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.055</td>
<td>0.020</td>
<td>6.6</td>
</tr>
</tbody>
</table>
6. Discussion

Bioremediation of the environments containing xenobiotics can be achieved by the introduction of specialized microorganisms (bioaugmentation) with xenobiotic metabolising abilities or stimulating indigenous population (biostimulation). It seems very likely, that random mutation and environmental selection processes are responsible for the evolution of the ability to metabolize xenobiotics in bacteria. The ability of microorganisms to evolve new metabolic potential depends on their susceptibility to the alteration and exchange of genetic information. Specialized microorganisms can also be developed by techniques such as enrichment and genetic manipulation in the laboratory.

The catabolism of pollutants like halogenated hydrocarbons is generally initiated by dehalogenation and this process can be accomplished by indigenous microbes in the environment (Oldenhuis et al 1989). *Pseudomonas* sp KC isolated from ground water aquifer (Cridle et al 1990) was demonstrated to degrade Carbon tetrachloride. Degradation of Carbon tetrachloride by biostimulation has also been reported (Semprini et al 1992). Genetic engineering is one of the recent methods adopted, to clone genes, to produce microbial strains with xenobiotic degrading abilities. Yeasts are being subject to recombinant DNA procedures (Loper et al 1984) to produce strains which could be cultivated for field treatment of a hazardous-waste site or for use in an industrial waste treatment process. Due to the limited facilities in our laboratory, experiments on genetic engineering could not be carried out.
As many as 50 isolates capable of growth in the presence of Carbontetrachloride from different ecosystems were isolated. The isolates belonged to bacterial, fungal and yeast groups. From among the different isolates the maximum growth in the presence of 5mM Carbontetrachloride obtained and so also, the maximum dehalogenation activity observed by the free chloride ions liberated in the medium by the yeasts isolates, rendered it to be the most suitable microorganism for further degradation studies over bacterial isolates. From among the different yeast strains, Rhodotorula R3 and Candida C4 was found to exhibit maximum growth and dehalogenating activity in the presence of 5mM Carbontetrachloride. The Carbontetrachloride tolerance studies of Rhodotorula R3 and Candida C4 revealed that Rhodotorula R3 could tolerate and dehalogenate 10mM Carbontetrachloride better than Candida C4. Nearly 40% degradation of Carbontetrachloride by Rhodotorula R3 was observed. Mineralization of Carbontetrachloride to carbon dioxide ranging from 10 to 99% has been reported (Criddle 1989). Hence, it was decided to find out the optimal cultural conditions for the growth of Rhodotorula R3 on Carbontetrachloride and biodegradation of Carbontetrachloride.