Chapter 3. Isolation of 2,4,6-TCA-degrading microorganisms and characterization of the biodegradation pathway
3. Isolation of 2,4,6-TCA-degrading microorganisms and characterization of the biodegradation pathway

3.1.- Introduction.

Chlorophenols are pollutants of environmental significance (Czaplicka et al., 2001; and Czaplicka et al., 2003) due to their recalcitrance and high persistence in many of the ecosystems.

They have been industrially produced on a large scale: 2,4,6-TCP and PCP have been used extensively as preservatives of wood, leather and textile goods, and also as pesticides (Rappe, 1980). Besides, 2,4-dichlorophenol and 2,4,5-TCP are used as precursors in the synthesis of the herbicides 2,4-dichloro- and 2,4,5-trichlorophenoxyacetic acids (Rappe, 1980).

Also, 2,4,6-TCP is a precursor for the synthesis of PCP and prochloraz (Sitting, 1981).

In addition, 2,4,6-TCP is one of the main components in chlorine bleaching Kraft pulp mill effluents (Huynh et al., 1985).

For all these reasons, of the six isomers of TCP, 2,4,5-TCP and 2,4,6-TCP are considered as priority pollutants (Sitting, 1981) due to their toxicity, persistence and bioaccumulative character.
Consequently, many different studies have been conducted to isolate chlorophenols-degrading microorganisms and characterize the different biodegradation pathways in both aerobic (Xun y Orser, 1991; Wieser et al., 1997; Bhasker Reddy et al., 1998; Bhasker Reddy and Gold, 2000; Louie et al., 2002) and anaerobic conditions (Liu and Pacepavicius, 1990; Anmenante et al., 1992; Lacorte y Barceló, 1994; Chang et al., 1995; Masunaga et al., 1996; Dennie et al., 1998).

Irrespective of the presence or absence of oxygen in the ambient, dechlorination is the first critical step in the bacterial degradation of many chlorinated pollutants including chlorophenols. Nevertheless, we can clearly distinguish two completely different biodegradative mechanisms for chlorophenols:

- In anaerobic conditions they are biodegraded via reductive dechlorination.
- On the contrary, under aerobic conditions, the CPs are generally transformed via oxidative dechlorination.

Remarkably, in aerobic conditions chlorophenols exhibit a higher resistance to biodegradation. This is due to the fact that the chlorine atoms interfere with oxygenases enzymes, which normally initiate the attack of aromatic rings (Copley, 1997). So, pentachlorophenol is faster biodegraded in anaerobic than in aerobic conditions.

Next we will review briefly the biodegradation of chlorophenols in both anaerobic and aerobic conditions, focusing more attention to the aerobic condition since cork is an aerobic environment.

### 3.2.- Anaerobic biodegradation of chlorophenols.

As we previously indicated biodegradation of chlorophenols in anaerobic environments takes place by a reductive dehalogenation mechanism. In this process, chlorines are replaced with hydrogen, while degrading microorganisms use chlorinated chlorophenols as terminal electron acceptors in an anaerobic respiration. Therefore reductive dechlorination is partially (or sometimes completely) inhibited by the presence of other electron acceptors such as sulfate, nitrate, $O_2$ or $CO_2$.

As evident from the review of literature, only few microorganisms able to reductively dehalogenate chlorophenols have been isolated. This degradative charac-
teristic is well documented in the gram-positive *Desulfitobacterium* and also in the gram-negative sulfate-reducing bacteria *Desulfomonile tiedjei* (Mohn and Kennedy, 1992).

The genus *Desulfitobacterium* is very interesting since includes several species, like *D. frappieri*, *D. dehalogenans*, *D. chlororespirans*, *D. hafniensi* and *Desulfitobacterium* sp. Strain PCP-1 able to degrade many different chloroaromatic compounds (Dennie et al., 1998).

An in depth characterization of *D. frappieri* PCP-1 indicates that this microorganism is able to dehalogenate many of the chlorophenolic isomers in ortho-, meta-, or para- position (see table 3.1), and also other halogenated compounds including 2,4,5-tribromophenol, tetrachlorocatechol and tetrachloroguaiacol, pentachloroanisole and pentachloronitrobenzene among others (Dennie et al., 1998).

<table>
<thead>
<tr>
<th>Chorophenol</th>
<th>Chlorophenol dehalogenation rate (nmol/min/mg of cell protein)</th>
<th>Position dechlorinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCP</td>
<td>833</td>
<td>ortho</td>
</tr>
<tr>
<td>2,3,4-TCP</td>
<td>417</td>
<td>ortho</td>
</tr>
<tr>
<td>2,3,5-TCP</td>
<td>68</td>
<td>meta</td>
</tr>
<tr>
<td>2,3,6-TCP</td>
<td>25</td>
<td>meta</td>
</tr>
<tr>
<td>2,3,5-TCP</td>
<td>1158</td>
<td>ortho</td>
</tr>
<tr>
<td>2,3,6-TCP</td>
<td>567</td>
<td>ortho</td>
</tr>
<tr>
<td>2,4,6-TCP</td>
<td>500</td>
<td>ortho</td>
</tr>
<tr>
<td>3,4,5-TCP</td>
<td>400</td>
<td>para</td>
</tr>
<tr>
<td>2,4,-DCP</td>
<td>33</td>
<td>ortho</td>
</tr>
<tr>
<td>2,4-DCP</td>
<td>315</td>
<td>para</td>
</tr>
<tr>
<td>2,6-DCP</td>
<td>92</td>
<td>ortho</td>
</tr>
<tr>
<td>3,5,-DCP</td>
<td>667</td>
<td>meta</td>
</tr>
</tbody>
</table>

Table 3.1. Chlorophenol dehalogenation rates produced by *D. frappieri* in anaerobic conditions (Dennie et al., 1998)
As it can be seen in **table 3.1** dehalogenation of chlorophenols in anaerobic condition can occur at any position (*ortho-, meta- or para-* position. See **figure 3.1**). Also the same compound can undergo dehalogenation at different positions and rates, resulting in the production of different biodegradative intermediates.

**Figure 3.1.** Reductive dehalogenation of PCP (up) and 2,4,6-TCP (down) carried out by *D. frappieri* in anaerobic conditions.
3.3.- Aerobic biodegradation of chlorophenols.

Biodegradation of chlorophenols in aerobic conditions is mainly carried out by two different classes of microorganisms:

- Many aerobic bacteria can degrade chlorophenols by using oxygenases that initiate the attack on the aromatic ring.

- On the contrary, white-rot fungi used the enzymes of the ligninolytic complex to degrade chlorophenols.

Next we will briefly review this topic.

3.3.1.- Biodegradation of chlorophenols by aerobic bacteria: a general view.

Bacteria that catalyze the aerobic degradation of chlorophenols are divided into two classes (Tomasi et al., 1995):

✓ strains that degrade mono-, and dichlorophenols but not attack more highly halogenated phenols. These chlorophenols are metabolized through the chlorocatechols pathway.

✓ strains that exclusively degrade tri-, tetra- and pentachlorophenol. These highly chlorinated chlorophenols are metabolized through the hydroquinones pathway.

We will centre our attention onto this last group considering the high significance of TCPs and PCP as environmental pollutants, and as precursors of the 2,4,6-TCA and PCA that contaminate wines.

The biodegradation of chlorophenols by aerobic bacteria, as many others chloroaromatic compounds, is normally initiated by the removal of chlorine substituents from the benzene ring as a previous step to the cleavage of the aromatic rings. Many different bacteria can degrade highly chlorinated phenols:

✓ Burkholderia (Pseudomonas) cepacia AC1100 is able to degrade the herbicide 2,4,5-trichlorophenoxyacetate (2,4,5-T). The first intermediate in the degradation pathway is 2,4,5-TCP (Tomasi et al., 1995; Xun, 1996). Then the 2,4,5-TCP is dehalogenated to 2,5-dichlorohydroquinone (see figure 3.2) by the enzyme chlorophenol-4-monoxygenase (Xun, 1996).
The enzyme requires O\(_2\) and NADH as substrates. FAD acts as cofactor stimulating the reaction, although it is not essential for the catalysis. Interestingly, this enzyme also can dehalogenate other chlorophenols like 2,3,5,6-TCP and 2,4,6-TCP.

✓ The initial step of PCP biodegradation has been characterized in *Flavobacterium* sp. ATCC 39723 (Xun and Orser, 1991a). In this strain a pentachlorophenol hydroxylase catalyzes the dehalogenation of PCP into 2,3,5,6-tetrachlorohydroquinone in the presence of O\(_2\) and NADPH (figure 3.3).

A similar activity has been detected in cell-free extracts of *Arthrobacter* sp. strain ATCC 33790 (Schenk et al., 1989).

This strain can also degrade triiodophenol (TIP) and tribromophenol (TBP) (Xun and Orser, 1991b).

✓ The 2,4,6-TCP biodegradation pathway has been also characterized in *Ralstonia eutropha* JMP134 (Louie et al., 2002). The initial step of the biodegradation pathway is achieved by a FADH\(_2\)-dependent monooxygenase that converts 2,4,6-TCP into 2,6-dichlorohydroquinone (figure 3.4), and then into 6-chlorohydroxyquinol.
Figure 3.3. Initial reaction catalyzed by the enzyme pentachlorophenol hydroxylase for the biodegradation of pentachlorophenol by *Flavobacterium* sp. ATCC 39723

Figure 3.4. The initial step in the biodegradation of 2,4,6-TCP by *Ralstonia eutropha* JMP134 consist on the dehalogenation of 2,4,6-TCP into 2,6-dichlorohydroquinone in a reaction carried out by a 2,4,6-TCP monooxygenase activity.
The dehalogenation of 2,4,6-TCP has also been detected in many other aerobic bacteria like Rhodococcus chlorophenolicus (Uotila et al., 1992a), Mycobacterium fortuitum (Uotila et al., 1992b), Streptomyces rochei (Golovleva et al., 1992), and Pseudomonas picketti (Kiyohara et al., 1992).

From the review of all these data we can assume that the biodegradation of chlorophenols containing 3 or more chlorine atoms always begin with the attack of monooxygenase-type enzymes to form quinones. Therefore, we can conclude that the degradation always happens through the biodegradative quinone pathway.

3.3.2.- Biodegradation of chlorophenols by white-rot fungi.

Wood is formed when the protoplasts of plant cells, initially separated by the middle lamella and the primary wall, lay down a thick secondary wall and eventually dye (Anke and Weber, 2006). The main components of the primary wall and the middle lamella are cellulose and pectin respectively which consist of chains of repeated sugars units assembled in a regular fashion by means of specific enzymes. In both structures we can also find hemicelluloses: these polymers are formed by different sugar moieties.

On the contrary the secondary cell wall is formed by lignin, which consists of modified phenolic substances linked in a random fashion by way of free radical reactions to render a very complex tridimensional structure which is very difficult to degrade (see the structure of lignin in figure 1.5). Therefore, lignin has a non-repetitive structure very recalcitrant to biodegradation. In fact, the only microorganisms capable of mineralising (the conversion into water and carbon dioxide) lignin are a selected group of basidiomycetes fungi called white-rot fungi. However, even these fungi can not live on lignin alone but require other, more easily utilizable carbon sources to sustain lignin degradation, which is thus said to be co-metabolic (Anke and Weber, 2006).

Lignin biodegradation happens by way of free radical intermediates similar to those involve in its synthesis. The concerted and simultaneous action of several oxidative enzymes is required to break down lignin, which constitute the so-called ligninolytic complex.

✓ The initial attack is mediated by lignin peroxidases (LiP) and/or manganese-peroxidases (MnP).
• LiP appears to be the key enzyme in the oxidation of non-phenolic phenylpropanoid units which leads to polymer fragmentation. This enzyme also oxidizes aromatic nuclei to aryl cations radicals.

• MnP oxidizes Mn$^{+2}$ to Mn$^{+3}$, which in turn attacks phenol structures in lignin (Glen et al., 1986).

• Also a versatile peroxidase (VP), sharing LiP and MnP catalytic properties has been reported in some fungi like Pleurotus eryngii (Martínez et al., 1996; Ruiz-Dueñas et al., 1999) or Bjerkandera species (Mester and Field, 1998).

Remarkably, the efficiency of the attack of these enzymes requires an H$_2$O$_2$-producing system.

✓ Several enzymes are involved in the production of H$_2$O$_2$ like glucose oxidase, glyoxal oxidase or aryl alcohol oxidase. This last enzyme is very important because it uses chlorinated anisyl alcohols as substrates, and these compounds are potential environmental pollutants.

✓ A third group of enzymes capable of attacking lignin are laccases (benzene-diol:oxygen oxidoreductase). They are a group of enzymes characterized by their copper content and their oxidative capacity using phenolic compounds as substrates and oxygen as a terminal electron acceptor, with the concomitant reduction of O$_2$ to H$_2$O.

Remarkably, white-rot fungi have an immense potential, by means of the enzymes of the ligninolytic complex, to degrade many hazardous and contaminant substances, including phenolic compounds (Bollag et al., 1988), chloroaromatics, including chlorophenols (Anke and Weber, 2006), and other xenobiotics, like dioxins (Takada et al., 1996; Sato et al., 2002).

Next we will review the available information regarding biodegradation by 2,4,6-TCP and PCP by white-rot fungi.

### 3.3.2.a.- Biodegradation of 2,4,6-TCP.

The biodegradation of 2,4,6-TCP by several white-rot fungi has been extensively analyzed.
The biodegradation of 2,4,6-TCP by Phanerochaete chrysosporium (showed in Figure 3.5) is initiated by a lignin peroxidase (LiP) or manganese peroxidase (MnP) catalyzed oxidative dechlorination reaction to produce 2,6-dichloro-1,4-benzoquinone (Bhasker Reddy et al., 1998). This quinone is later reduced to 2,6-dichloro-1,4-dihydroxybenzene, which is reductively dechlorinated to yield 2-chloro-1,4-dihydroxybenzene. The latter is degraded further by one of two parallels pathways: it either undergoes hydroxylation to yield 5-chloro-1,2,4-trihydroxybenzene, which is reductively dechlorinated to produce the common key substrate 1,2,4-trihydroxybenzene; or alternatively it experiments reductive dechlorination to yield 1,4-hydroquinone. This compound is then ortho-hydroxylated to yield 1,2,4-trihydroxybenzene.

The biodegradation pathway of 2,4,6-TCP by Panus tigrinus and Coriolus versicolor (Leontievsky et al., 2000) shows some differences. The initial step in the pathway is the same: 2,6-dichloro-1,4-hydroxybenzene and 2,6-dichloro-1,4-benzoquinone were found as products for primary oxidation of 2,4,6-TCP (Figure 3.6). However, whereas the primary attack of 2,4,6-TCP by P. tigrinus was conducted mainly by MnP, in C. versicolor it was catalyzed by laccase, suggesting a different mode of regulation of this degradative process. Interestingly, also an oligomeric product (probably containing less than two chlorine atoms on the aromatic nucleus) was detected as product of these reactions.

Formation of 2,6-dichloro-methoxyphenol from 2,6-dichloro-1,4-hydroxyquinone was detected, whereas 2,5-dichloro-1,4-benzoquinone was a precursor for the formation of 2,6-dichloro-4-methoxy-1,3-dihydroxibenzene. The formation of methoxyl groups is probably a result of a side reaction catalyzed by cell-associated methyltransferases.

3.3.2.b.- Biodegradation of PCP by Phanerochaete chrysosporium.

Several intermediates originated during the biodegradation of PCP by P. chrysosporium have been detected and accordingly a putative biodegradation pathway (Figure 3.7) has been proposed (Bhasker Reddy and Gold, 2000).

The biodegradative process in initiated by a LiP- or MnP-catalyzed oxidative dechlorination to produce tetrachloro-1,4-benzoquinone. Then, depending of the metabolic conditions, this quinone is further metabolized by two parallel pathways with some cross-links among them.
Figure 3.5. Biodegradation pathway of 2,4,6-TCP by *Phanerochaete chrysosporium* according to Bhasker Reddy *et al.* (1998).
Figure 3.6. Biodegradation pathway of 2,4,6-TCP by Panus tigrinus and Coriolus versicolor according to the data of Leontievsky et al. (2000).
Figure 3.7. Hypothetical pathways for the biodegradation of pentachlorophenol by *Phanerochaete chrysosporium* (Bhasker Reddy and Gold, 2000).
In one of the branches, the tetrachloro-1,4-benzoquinone (compound I in figure 3.7) is reduced to tetrachloro-1,4-dihydroquinone (II). Next, this compound is reductively dehalogenated to trichlorohydroquinone (III). This reaction is catalyzed by a two-component tetrachlorohydroquinone reductive dehalogenase system (Bhasker Reddy and Gold, 1999). In the presence of glutathion (GSH), a membrane-bound enzyme converts tetrachlorohydroquinone into its glutathionyl conjugate. In a second step, a soluble cytoplasmic enzyme transforms the glutathionyl conjugate to trichlorohydroquinone. Later, and after other two successive reductive dehalogenation steps, trichlorohydroquinone is converted into 1,4-hydroquinone (V).

In the other branch, tetrachloro-1,4-benzoquinone is dehalogenated to 2,3,5-trichlorotrihydroxybenzene (IV), which by the means of three reductive dehalogenation steps generates 1,2,4-trihydroxybenzene (VI).

From the different data reviewed in the previous pages, four important conclusions can be deduced:

1). The initial attack of chlorophenols is always achieved by a LiP, MnP or laccase activity producing the corresponding benzoquinone or hydroquinone.

2). In *P. chrysosporium* these compounds latter undergo reductive dehalogenation to finally yield the key intermediate 1,2,4-trihydroxybenzene. This is an unexpected result because reductive dehalogenation of chloroaromatics is a typical biodegradation mechanism carried out by bacteria in anaerobic conditions. However, a two-component enzymatic system has been identified in *P. chrysosporium*, responsible of the reductive dehalogenation of tetrachlorohydroquinone to trichlorohydroquinone (Bhasker Reddy and Gold, 1999).

3). In other species like *C. versicolor* or *P. tigrinus* the quinones can be biomethylated by some cell-associated methyltransferase activity.

4). Therefore, the degradation pathways for chlorophenols in white-rot fungi involved different mechanisms depending on the species analyzed by combining extracellular oxidative and intracellular reductive dechlorination reactions (Bhasker Reddy and Gold, 2000).
3.3.3.- Significance of biodegradation of chloroanisoles.

On the contrary to the high number of studies focussed on chorophenols biodegradation, almost no literature and data available can be found on chloroanisoles degradation.

✓ Lamar and coworkers (1990) described the formation of pentachloroanisole (PCA) in soils inoculated with the white-rot fungus *Phanerochaete chrysosporium* and *P. sordida* in the presence of PCP. Depletion of PCP by these fungi occurred in a two-stage process. The first stage was characterized by a rapid depletion of PCP that coincided with an accumulation of PCA. In the second stage, the levels of PCP and PCA were reduced. These microorganisms were able to mineralize PCA in liquid medium, so it was supposed that the depletion of PCA in contaminated soils was due to the involvement of some fungal enzymatic activity.

✓ In anaerobic conditions it has been reported the *para*-dehalogenation of pentachloroanisole to produce 2,3,5,6-tetrachloroanisole (**figure 3.8**) by *Desulfitobacterium frappieri* PCP-1 (Dennie *et al.*, 1998).

Remarkably, we have not been able to find any reference regarding biodegradation of chloroanisoles by bacteria in aerobic conditions. Several reasons could be pointed out as the cause of this situation:

• Perhaps, this is due to the absence of toxicity of chloroanisoles as compared to the high toxicity of chlorophenols (see section 1.5 in chapter 1).

• Also we must also consider that chloroanisoles are much more stable chemical compounds than chlorophenols, and obviously they could be much more difficult to be degraded. We can speculate that the methylation of the hydroxyl group of chlorophenols produce some steric or spatial impediment for the action of the monooxygenase that degrades chlorophenols.

However, we think that the isolation of 2,4,6-TCA-degrading microorganisms is of great interest for the cork industry. Indeed, these microorganisms would be a potential source of enzymes involved in the biodegradation of chloroanisoles, that could be applied for the development of cleaning treatments for the corks.
Therefore, one of the main objectives of this research program would be the isolation of microorganisms able to degrade chloroanisoles, the identification and characterization of degradative enzymes and the development of strategies to clean the corks, diminishing the contamination level for this off-odor compounds.
3.3.- Isolation of 2,4,6-TCA-degrading microorganisms from biological samples.

In order to isolate from soil samples microorganisms that could potentially degrade 2,4,6-TCA we developed the strategy summarized in figure 3.9:

✓ Two different soil samples were analysed: a sample of PCP-contaminated soil taken from a wood industry, and also normal garden soil obtained from the campus of the University of León (Spain).

✓ Several dilutions of each sample were used to inoculate flasks containing minimal medium with glucose (0.1%) as the only carbon source. The cultures were inoculated at 28ºC for three days until a good growth was evident, and hypothetically many of the microbial subpopulations of the soil sample had been amplified.

✓ Next, we used this culture to inoculate flasks containing the same minimal medium with glucose, but now supplemented with the pesticide 2,4,6-TCP (10 µg/ml). We assumed that using this pesticide we could select 2,4,6-TCP-degrading microorganisms, and giving the high structural similarity with 2,4,6-TCA, some of these microorganisms could have the capability to degrade 2,4,6-TCA. After four days of incubation at 28ºC a evident growth was detected.

✓ We used this culture to inoculate flask containing two different liquid cultures:
  • a minimal medium (MM) containing 2,4,6-TCA (200 g/ml) as the sole carbon source (TCAMM).
  • Composition of 2,4,6-TCA minimal medium (TCAMM)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂HPO₄</td>
<td>2.61 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.70 g</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>0.80 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.25 g</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>0.02 g</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.0002 g</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.00018 g</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>0.00002 g</td>
</tr>
<tr>
<td>2,4,6-TCA</td>
<td>0.20 g</td>
</tr>
<tr>
<td>pH 7.2</td>
<td></td>
</tr>
</tbody>
</table>
• The same TCAMM but supplemented with the pesticide 2,4,6-TCP (25 \( \mu \text{g/ml} \)).

The flasks were incubated at 25ºC until a clear growth was detected.

As it can be seen in figure 3.9 (down to the left) a dense growth was detected in the flasks containing TCAMM supplemented with the pesticide, whereas a slightly lower growth was detected for the cultures developed in plane TCAMM.

✓ Finally, we isolate several microorganisms from this last media, which were further analyzed to check their capability to degrade 2,4,6-TCA.

3.4.- Analysis of 2,4,6-TCA biodegradation by microorganisms isolated from soil samples.

The isolation protocol described above led us to isolate 8 phenotypically different bacteria from PCP-contaminated soil and 6 bacteria from standard garden soil.

Once isolated, we tested the capability of all these bacteria to degrade 2,4,6-TCA in TCAMM, and also in TCAMM supplemented with benzoic acid (1 mM).

The fermentations were developed at 25ºC and 200 r.p.m in a orbital shaker for 108 hours. Briefly, samples (1 ml) were collected at different times, acidified by adding 50 \( \mu \text{l} \) of HCl 1N and then extracted twice with 1 ml of ethyl acetate. The organic phases were mixed, evaporated, and the residue resuspended in 50 \( \mu \text{l} \) of acetonitrile:water (70:30) and analyzed by HPLC by using a reverse phase Lichrospher RP-C18 column with acetonitrile:water (70:30) as a mobile phase at a flow rate of 0.7 ml/min. Eluted peaks were detected at 285 nm.

To confirm the results obtained by the HPLC analyses we also titrated at 48 hours of fermentation the release of chloride ions in the culture media. An increase in the chloride ion concentration would indicate the dehalogenation of 2,4,6-TCA, since the only putative origin of this ion is the 2,4,6-TCA molecule. The chloride ion detection was carried out by using a chloride ion electrode (Cole-Parmer), calibrated against NaCl solutions of known concentration, and coupled to a bench pH/Ion/mV meter (Oakton).
Figure 3.9. Protocol for the isolation of 2,4,6-TCA degrading microorganisms from soil simples (PCP-contaminated soil from a wood industry and soil from a garden). Note the high growth in the flask to the left containing 2,4,6-TCP and 2,4,6-TCA as carbon sources and the slight growth (flask to the right) in the minimal medium containing 2,4,6-TCA as the only carbon source.
The results obtained are summarized in table 3.2 and table 3.3. From these data we can derive the following conclusions:

✓ Three out of the 8 bacterial strains isolated from PCP-contaminated soil (table 3.3) showed a high capability to degrade 2,4,6-TCA. They were identified at the species level by partial sequencing of the 16S rDNA gene by using the primers 16SA and 16SB and the conditions described by Álvarez-Rodríguez et al. (2003b).

*Xanthomonas retroflexus* INB/B2 and *X. retroflexus* INB/B4 degraded after 48 hours of growth the 88% and 100% respectively of the 2,4,6-TCA initially presented in the culture media, with a concomitant 2,8 and 3,8 mM chloride release.

Another strain identified as *Pseudomonas putida* INB/P1 also totally degraded 2,4,6-TCA at 48 hours, producing the releasing of 3,2 mM chloride to the culture media. In all the cases the chloride release, around the 3 mM value indicated that during the 2,4,6-TCA degradation the three chlorine atoms were released from its structure.

The time course of the biodegradation process is shown in figure 3.10. As it can be seen the biodegradation process is faster and more effective in TCAMM than when benzoic acid was added to the culture media, indicating that the cometabolism of this product slowed down the 2,4,6-TCA biodegradation. A small amount of 2,4,6-TCA (about 10%) dissapeared in the *C. albicans* negative control, probably due to evaporation.

✓ Also two of the strains isolated from garden soil (see table 3.3), identified as *Acinetobacter radioresistans* INB/S1 and *X. retroflexus* INB/S3 were able to completely remove 2,4,6-TCA from the culture. Like with the strains isolated from PCP-polluted soil a chloride release around 3 mM was detected, indicating the total dehalogenation of 2,4,6-TCA.

The biodegradation kinetics is summarized in figure 3.11: note like again the metabolism of 2,4,6-TCA is less efficient in the presence of benzoic acid.
<table>
<thead>
<tr>
<th>Strain</th>
<th>2,4,6-TCA degradation rate (%) at 48 h of growth in TCAMM</th>
<th>Chloride release at 48 hours (mM)</th>
<th>Chloride release at 108 hours (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthomonas retroflexus INB/B2</td>
<td>93%</td>
<td>3.5</td>
<td>2.8</td>
</tr>
<tr>
<td>Xanthomonas retroflexus INB/B4</td>
<td>38%</td>
<td>1.6</td>
<td>3.8</td>
</tr>
<tr>
<td>Pseudomonas putida INB/P1</td>
<td>46%</td>
<td>1.9</td>
<td>3.2</td>
</tr>
<tr>
<td>P2</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>P4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>P5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>P6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>P8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 3.2. 2,4,6-TCA biodegradation rate (%) and release of chloride ions (mM) for bacteria isolated from PCP-contaminated soil. *ND: not detected.
<table>
<thead>
<tr>
<th>Strain</th>
<th>2,4,6-TCA degradation rate (%) at 48 h of growth in TCAMM</th>
<th>Chloride release at 108 hours (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter radioresistans S1</td>
<td>94.7% 93.2% 100%</td>
<td>3.0 3.1 3.1</td>
</tr>
<tr>
<td>S2</td>
<td>ND* ND ND* 100%</td>
<td>ND ND ND</td>
</tr>
<tr>
<td>Xanthomonas retroflexus INB/S3</td>
<td>100% ND ND</td>
<td>ND ND ND</td>
</tr>
<tr>
<td>S4</td>
<td>ND ND ND ND 100%</td>
<td>ND ND ND</td>
</tr>
<tr>
<td>S5</td>
<td>ND ND ND ND ND 100%</td>
<td>ND ND ND</td>
</tr>
<tr>
<td>S6</td>
<td>ND ND ND ND ND ND</td>
<td>ND ND ND</td>
</tr>
</tbody>
</table>

Table 3.3. 2,4,6-TCA biodegradation rate (%) and release of chloride ions (mM) for bacteria isolated from a sample of garden collected at the campus of the University of León (Spain). *ND: not detected.
Figure 3.10. Time course biodegradation of 2,4,6-TCA in TCAMM supplemented with benzoic acid (A) and in TCAMM (B) by three bacterial strains isolated from PCP contaminated soil. [◆ C. albicans negative control; ◆ X. retroflexus INB/B2; X. retroflexus INB/B4; and ▲ P. putida INB/P1].

Figure 3.11. Time course biodegradation of 2,4,6-TCA in TCAMM supplemented with benzoic acid (A) and in TCAMM (B) by two bacterial strains isolated from garden soil and a phenol-degrading strain of Rhodococcus erythropolis M1 (Goswami et al., 2005). [◆ C. albicans negative control; ◆ R. erythropolis M1; A. radioresistans INB/S1; and ▲ X. retroflexus INB/S3].
3.5.- Prediction of putative 2,4,6-TCA biodegradation pathways: identification of biodegradative intermediates.

Once, it was obvious that five different bacterial strains isolated from soil samples were able to efficiently degrade 2,4,6-TCA in liquid cultures, our next goal was the elucidation of the first biodegradative reaction, and the prediction of a putative biodegradative pathway.

The identification of the first enzyme involved in 2,4,6-TCA biodegradation was specially interesting for us, because of its putative industrial application to develop some cleaning treatment for cork stoppers.

However, an extensive bibliographic search did not let us to detect any relevant data regarding the aerobic degradation of chloroanisoles (see section 3.3.3 in this chapter).

Therefore, we decided to use the Pathway Prediction System of the Biocatalysis/Biodegradation Database (UM-BBD) of the University of Minnesota (http://umbbd.msi.umn.edu). This database compiles all the available information regarding biodegradation, including biodegradative pathways, biodegradation rules, biodegradative enzymes and many other useful data.

The Pathway Prediction System employs all this theoretical information to predict hypothetical biodegradative pathways of new compounds, or like in the case of 2,4,6-TCA, pathways for compounds which biodegradative processes are unknown.

3.5.1.- Prediction of putative biodegradative reactions of 2,4,6-TCA in aerobic conditions.

By using that predictive tool we were able to predict five hypothetical different reactions for the first step of the 2,4,6-TCA biodegradation in aerobic conditions, as it can be seen in figure 3.12:
✓ The predicted reactions 1 and 2 are oxidative dehalogenations of the chlorine atoms in the positions 4 (para) or 6 (ortho), hypothetically catalyzed by and monooxygenase-type enzyme.

✓ Processes 3 and 4 are typical O-demethylation reactions, but while in reaction 3 the methyl group is release and transfer to some methyl-receptor compound (probably tetrahydrofolate), in the reaction 4 the released methyl group is oxidized to formaldehyde.

Figure 3.12. Prediction of initial reactions for the biodegradation of 2,4,6-TCA in aerobic conditions by using the pathway prediction system from the UM-BBD (University of Minnesota-Biocatalysis/Biodegradation Database)
Finally, it also would be possible the oxidation of the methyl group of 2,4,6-TCA to a primary alcohol, in a reaction presumably catalyzed by a hydroxylase- or monoxygenase-type enzymatic activity.

3.5.2. Identification in liquid cultures of degradative intermediates.

Once we had predicted the putative intermediates originated in the first biodegradative reaction of 2,4,6-TCA, our next goal would be the attempt to identify some of these molecules (or any other putative intermediate) in liquid cultures.

The study of metabolic intermediates of 2,4,6-TCA biodegradation was carried out for the isolated strains *X. retroflexus* INB/B4, *Pseudomonas putida* INB/P1 and *Acinetobacter radioresistance* INB/S1.

We boarded this task in two different ways:

**Identification of Metabolic intermediates of 2,4,6-TCA degradation by HPLC.**

The protocol followed was the next:

- The bacterial strains were grown in TCAMM without 2,4,6-TCA, but supplemented with methanol (200 ml/L of liquid medium) as carbon source, for 24 hr at 25°C.

- Then 2,4,6-TCA (dissolved in methanol) at a concentration of 200 µg/ml was injected into the media after 24 hr. A control flask was kept, as negative control, with 200 µl of methanol in 100 ml of minimal medium.

- Samples of supernatants were taken at an interval of 2hr.

- They were acidified with 50 µl 1M HCl to pH 2 and extracted twice with ethyl acetate. The organic fraction was evaporated to near dryness in a vacuum evaporator. The residue was dissolved in acetonitrile:water (70%:30%).

- The consumption of 2,4,6-TCA and concomitant production of metabolites were followed by analyzing the samples by Reverse Phase-HPLC by using the conditions previously described in section 3.4.
The results obtained are described next:

- **X. retroflexus INB/B4 strain.** The analysis of putative intermediates from 2,4,6 TCA degradation let us to detect the appearance in culture supernatants of a peak at 16hr of incubation (see panel B in **figure 3.13**), which was no present in a parallel control developed in the absence of 2,4,6-TCA (panel A in figure 3.13).

The retention time and absorption maxima of this peak were 7.2 minutes and 288.9 nm respectively, which are almost identical to one of a 2,4,6-TCP standard.

✓ **A. radioresistans INB/S1 strain.** On the contrary no peaks were detected in this case.

✓ **Pseudomonas putida INB/P1.** A similar result was detected: a clear peak of putative 2,4,6-TCP was detected in samples of 36, 40 and 44 hours of growth (data not shown).

**Figure 3.13.** HPLC analysis of 16 hours old culture supernatants from X. retroflexus INB/B4 strain developed in TCAMM (A), and the same minimal medium with methanol as carbon source (B). Peaks corresponding to 2,4,6-TCA and 2,4,6-TCP are indicated by arrows.
These results were confirmed by purification of the peaks and subsequent analysis by gas chromatography-mass spectrometry: the spectra of the compounds corresponding to putative 2,4,6-TCP was very similar to that of a authentic 2,4,6-TCP standard (data not shown).

✓ **Identification of Metabolic intermediates by Gas Chromatography-Mass Spectrometry (GC-MS).**

The bacterial cultures were developed as reported above, but in this case the supernatant samples were taken at an interval of 4hr up to 56 hr:

- The samples (1ml) were acidified with 1mM HCl to pH 4 and saturated with NaCl (1M).

- Next they were extracted twice with an equal volume of ethylacetate.

- The organic phase was dried in a vacuum evaporator and acetylated by dissolving in pyridine and acetic anhydride (1:3) vol/vol. In a parallel set of samples the organic fraction was dissolved in 50µl of hexane (without acetylation).

- Both kind of samples were analyzed by GC-MS.

This kind of analysis let us to detect in samples corresponding to the three bacterial strain analyzed a chlorinated compound, which mass spectrum was almost identical to that one of 2,4,6-TCA standard, as it can be seen in figure 3.14.

Obviously, this data (and also the reported previously obtained by HPLC analysis) clearly indicated that in the three bacterial isolates the 2,4,6-TCA biodegradation is initiated with a O-demethylation reaction to produce 2,4,6-TCP.
Figure 3.14. Mass-spectra of a product detected in liquid cultures of the three bacterial strains analyzed (up) and of authentic 2,4,6-TCP (down).
3.6.- Detection of enzymatic activities involved in initial steps of the 2,4,6-tca biodegradation.

Once we had established the presence of 2,4,6-TCP in the supernatants of liquid cultures, we tried to confirm this finding by the detection of a putative O-demethylase enzymatic activity responsible for the formation of 2,4,6-TCP from 2,4,6-TCA biodegradation.

3.6.1.- The significance of O-demethylation in the biodegradation of aromatic compounds.

O-demethylation is a kind of reaction of environmental significance because many of natural phenylmethylethers, like vanillin and syringate, and also many xenobiotics undergo O-demethylation.

Vanillate and syringate are particularly interesting compounds because they can be produced in high amounts during lignin degradation, which mainly consist of two phases: firstly, white-rot fungi depolymeraze lignin, and secondly bacteria decompose the lignin-derived compounds to smaller molecules.

Several different kinds of demethylation reactions have been characterized:

✓ Anaerobic O-demethylation reactions.

In anaerobic conditions the C-O bound cleavage for phenylmethylethers proceeds mainly through a O-demethylation reaction, which requires tetrahydrofolate (THF) and ATP (Berman y Frazer, 1992; Stupperich and Konle, 1993).

Also very recently it has been characterized a three-component vanillate O-demethylase from *Moorella thermoacetica* (Naidu and Ragsdale, 2001). This enzymatic complex is composed of three different proteins: first, the protein MtvB catalyses methyl transfer from a phenylmethylether compound to the cobalt center of MtvC, a corrinoid protein, and next Mtva catalyses transmethylation from Mtvc to THF, forming methyltetrahydrofolate (MTHF).
Aerobic O-demethylation reactions.

In aerobic conditions two kind of mechanisms have been found involved in the cleavage of phenylmethylethers (Sonoki et al., 2000):

1). Oxygenase-type reaction. In this case a monoxygenases with two- or three-component enzyme system catalysed the O-demethylation. These enzymes systems contain terminal enzymes such as iron-sulfur proteins or cytochrome P450-like enzymes, and they require NADH/NADPH to carry out demethylation via electron transport. Some examples are cited next:

- a). In some microorganisms like *Nocardia* and *Moraxella* spp. the enzymes involved in the demethylation of isovanillate and guaiacols are cytochrome P450-monoxygenases (Dardas et al., 1985).

- Hibi and coworkers (2005) have recently described the vanillate-O-demethylase system of *Pseudomonas* sp., which reductively catalyses the demethylation of vanillic acid into protocatechuate, with the concomitant production of formaldehyde. It is a class IA oxygenase composed of two proteins: the terminal oxygenase VanA, and the reductase VanB, which use NADH/NADPH as electron donor. These proteins are component of the monooxygenase system, and VanB exhibit extensive homology to many members of the ferredoxin family (Brunel y Davison, 1988). This type of demethylase is involved in vanillate degradation in all the vanillate-utilizing bacterial reported so far, like *Pseudomonas* and *Acinetobacter*.

- In *Pseudomonas maltophilia* a three-component enzyme system catalyses the O-demethylation of the herbicide dicamba (Wang et al., 1997). The three proteins have been identified as a reductase, a oxygenase, and a ferredoxine-like protein.

2). In this case the O-demethylation is catalyzed by a THF-dependent O-demethylase. Both THF and ATP are essentials for the reaction. This kind of reactions resembles the anaerobic O-demethylation processes. For example, in *Sphingomonas paucimobilis* it has been cloned the ligM gene encoding a O-demethylase involved in the O-demethylation of vanillate and syringate (Abe et al., 2005). The enzyme LigM resembles some homology to THF-dependent transferases, and therefore this system is similar to anaerobic O-demethylases.
3.6.2.- Detection of 2,4,6-TCA \(\text{O}\)-demethylase activities in the bacterial strains isolated from soil samples.

In order to confirm that the initial step in the 2,4,6-TCA biodegradation was a \(\text{O}\)-demethylation step yielding 2,4,6-TCP we decided to perform enzymatic assays for the detection of putative 2,4,6-TCA \(\text{O}\)-demethylases in the strains *X. retroflexus* INB/B4, *P. putida* INB/P1 and *A. radioresistans* INB/S1.

✓ Firstly we obtained cell-free extracts for the bacterial strains indicated above as follows.

### Preparation of cell extracts for testing \(\text{O}\)-demehtylase activities

Cell-free extracts for each microbial strain (INB/B4, INB/S1 and INB/P1 were prepared as follows:

✗ Bacteria were grown in TSB (trypticase soy broth; Mac Faddin, 1985) medium for 24 hours at 30°C with strong agitation to obtain a good biomass.

✗ The cells were collected by centrifugation and the pellet washed with 1 volume of sterile NaCl 0.9% (weight/volume), and then transferred into TCAMM.

✗ The culture was incubated at 25°C and 200 r.p.m until the end of the experiment.

✗ Cells were harvested at different time intervals (12, 18, 24, 30, and 36 hours) by centrifugation and washed twice with sterile NaCl 0.9% (weight/volume).

✗ Cells were harvested by centrifugation and then resuspended in breaking buffer (1ml/100 ml culture) of the following composition: Tris 50 mM (pH 7.5); glycerol, 10%; EDTA, 1mM; DTT, 2mM; MgCl\(_2\), 2mM, and NaCl, 10mM.

✗ Lysozyme was added at a concentration of 2mg/ml and immediately cells were broken by sonication in an ice bath, but using 5-6 second burst pulses (total number of pulses applied being 20 with 1 min interval).
✓ The cell extract was centrifuged at 12,000 r.p.m for 20 min at 4°C to remove the cellular debris.

✗ Finally the supernatant was filtered through a PD-10 (Sephadex G-25) column to remove small molecules. The sample was eluted by adding 3.5 ml of breaking buffer.

✓ Once obtained the cell-free extracts we carried out two different types of enzymatic assays for O-demethylases:

| THF-dependent O-demethylase assay |
|-------------------------|----------------|
| ✗ Potassium phosphate buffer (pH 7.5) | 20 mM |
| ✗ 2,4,6 TCA | 1mM |
| ✗ Tetrahydrofolate | 1mM |
| ✗ ATP | 1mM |
| ✗ MgCl2 | 3.5mM |
| ✗ Cell extract | 350 µl |

The assays were performed in a final volume of 500 µl, in both anaerobic (due to the lability of THF in the presence of oxygen) and aerobic conditions, at 30°C and up to 2 hours. The reactions were stopped by adding 50 µl of 1M HCl.
The products of the enzymatic assays were analysed as indicated next: reactions were extracted twice with 0.5 ml of ethylacetate. The organic fractions were mixed and evaporated until near dryness. The residue was resuspended in 50 µl of acetonitrile and analysed by HPLC by using a reverse phase Lichrospher RP-C18 column with acetonitrile:water:acetic acid (70:30:0.1) as a mobile phase at a flow rate of 0.7 ml/min. Eluted peaks were detected at 285 nm.

The results obtained were the next:

**X. retroflexus INB/B4 strain.** Although 2,4,6-TCP was detected in culture supernatants by HPLC and GC-MS analyses, all the efforts carried out until now in order to detect a putative 2,4,6-TCA O-demethylase have been unsuccessful.

**X. radioresistans INB/S1 strain.** In this case we could detect in 18 and 24 old-hours cells a clear THF-dependent O-demethylase activity when the reactions were performed in aerobic conditions (see figure 3.15). No 2,4,6-TCP formation was detected in negative control reaction carried out in the absence of THF or ATP, neither when the reactions were developed in the absence of oxygen.

---

**Oxygenase-type O-demethylase assay**

- X Potassium phosphate buffer (pH 7.5) 20 mM
- X 2,4,6 TCA 1mM
- X NADPH 1mM
- X NADH 1mM
- X Glutathione reduced 1mM
- X Cell extract 350 µl

The assays were performed in a final volume of 500 µl, in both anaerobic (due to the lability of THF in the presence of oxygen) and aerobic conditions, at 30ºC and up to 2 hours. The reactions were stopped by adding 50 µl of 1M HCl.

✓ The products of the enzymatic assays were analysed as indicated next: reactions were extracted twice with 0.5 ml of ethylacetate. The organic fractions were mixed and evaporated until near dryness. The residue was resuspended in 50 µl of acetonitrile and analysed by HPLC by using a reverse phase Lichrospher RP-C18 column with acetonitrile:water:acetic acid (70:30:0.1) as a mobile phase at a flow rate of 0.7 ml/min. Eluted peaks were detected at 285 nm.
A clear oxygenase-type $O$-demethylase activity was detected in aerobic conditions in cell-free extracts corresponding to 24 hours of growth (data not shown). This activity was not observed when the reaction was developed in anaerobic conditions.

**Pseudomonas putida INB/P1.** A clear oxygenase-type $O$-demethylase activity was detected in aerobic conditions in cell-free extracts corresponding to 24 hours of growth (data not shown). This activity was not observed when the reaction was developed in anaerobic conditions.

**Figure 3.15.** THF-dependent $O$-demethylase activity detected in 18 hours old cultures from *A. radioresistans* INB/S1 strain developed in TCAMM. (A): Reaction developed without ATP (negative control), and (B) 1 hour $O$-demethylase reaction. 2,4,6-TCA and 2,4,6-TCP are indicated by arrows.

**3.7.- Conclusions and future Prospects.**

**3.7.1.- Conclusions.**

The main conclusions and results of this study are as follows:

1) We have isolated from soil samples five different bacterial strains able to efficiently degrade 2,4,6-TCA in liquid cultures: three of the strains belong to the species *X. retroflexus*, and the other two strains belong to *P. putida* and *A. radioresistans* species.
II).- The analysis, by both HPLC and GC-MS, of the culture supernatants of the three bacterial isolates detected the presence of 2,4,6-TCP, indicating that the first biodegradative step for 2,4,6-TCA is a O-demethylation.

III).- The O-demethylation of 2,4,6-TCA in A. radioresistans INB/S1 strain is carried out by a THF-dependent O-demethylase activity.

IV).- In Pseudomonas putida INB/P1 the O-demethylation of 2,4,6-TCA is performed by an oxygenase-type O-demethylase activity.

3.7.2.- Future prospects.

It is clear that the finding that in the bacterial strains analyzed the 2,4,6-TCA biodegradation is initiated by an O-demethylase step to yield 2,4,6-TCP is a negative finding in relation to their putative use to clean corks. Indeed, this kind of enzymes cannot be used to hypothetically clean cork samples since we would be generating 2,4,6-TCP, that could be again O-methylated by filamentous fungi to produce 2,4,6-TCA.

Therefore, our future efforts should try the isolation of another bacterial strains which biodegrade 2,4,6-TCA in a different way: maybe producing the dehalogenation of chlorine atoms in meta or para position or oxidating the methyl group to a hydroxyl group (see figure 3.12 in this chapter).

This future objective may be reached by changing the initial conditions of the isolation from the biological samples.
3.8.- Bibliography of the Chapter


132

Chapter 3 Isolation of 2,4,6-tca-degrading microorganisms and characterization...


Leontievsky, AA; Myasoedova, NM; Baskunov, BP; Evans, CS; and Golovleva, LA. (2000). “Transformation of 2,4,6-trichlorophenol by the white rot fungi *Panus tigrinus* and *Coriolus versicolor*”. *Biodegradation* **11**: 331-340.


