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# THE USE OF AZOTOBACTER STRAINS FOR THE BIODEGRADATION OF RECALCITRANT COMPOUNDS

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## General aspects of biodegradation of recalcitrant compounds.

One of the factors which affects the ecosystem is the existence of non-biodegradable compounds called persistents or recalcitrants. Although these terms are used in some cases as synonims, Bull (1980) makes a clear distinction between them. Recalcitrants are those compounds which resist whatever kind of biodegradation, whereas the persistent compounds can be degraded under certain circunstances. Biodegradation means the biological transformation of an organic compound to another form but it is commonly used to denote mineralization of organic matter. Recalcitrant compounds can be natural or xenobiotic. The latter expression is applied to a novel compound to which microorganisms have not been exposed in their evolution.

Before the industrial development the concentration of organic compounds in nature remained more or less constant through the activity of living organisms.

The environmental balance is being disturbed by those activities which are the result of the industrial development. Thus, recalcitrants compounds-natural or xenobiotic-enter the environment and usually are accumulated. It is important to point out that in either case the compounds would appear to be recalcitrants simply because it is concentrated (Leisinger, 1983).

The appearence of recalcitrant compounds in the environment is produced by different ways as is shown in Table 1.

Table 1: Entry of pollutants into the environment.

1.- Industrial Effluents
2.- Domestic Effluents
3.- Biocides
4.- Fertilizers
5.- Accidents (Seveso, Bopal, etc)

Table 2 shows a selection of priority pollutants evaluated in U.S. Environmental Protection Agency (EPA) (Callahan et al., 1979).

Table 2: Pollutants groups according to U.S. Environmental Protection Agency (EPA) (EPA Selection).

1.- Metals and inorganics
2.- Pesticides
3.- Polychlorinated biphenils ($PCB_S$) and related compounds
4.- Halogenated aliphatic hydrocarbons
5.- Halogenated ethers
6.- Monocyclic aromatics
7.- Phtalate esthers
8.- Policyclic aromatic hydrocarbons
9.- Nitrosamines

The behaviour of recalcitrant compounds in the environment depends on coexisting inorganic and organic compounds and on a variety of chemical processes (such as hydrolysis, photolysis, oxidation and reduction) or on biological processes (Alexander, 1981). The biological processes are, in general, a consequence of the macro and micro flora activity which may cause mineralization, cometabolism or accumulation of the recalcitrant compounds.

Through mineralization, carbon and energy sources are generated for microbial growth and this leads to the disappearance of compounds. The term cometabolism has been used in a number of ways (Alexander, 1973; Horvath,

1972) but has been recently defined as "the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compounds (Dalton et al., 1982). Cometabolism may lead to the accumulation of transformed products which may contain an increased or decreased toxicity in relation to the original compounds (Alexander, 1981).

Through accumulation, microorganisms can uptake recalcitrant compounds. This phenomenon may lead to bioconcentration of hazardous compounds and to their entry into the food chain (Baughman et al., 1981; Lal et al., 1982).

Since complete biodegradation of organic chemicals in nature is primarily due to microorganisms (Alexander, 1981) a major factor determining their susceptibility to microbial attack is the lengh of time it has been on earth.

In the case of chemical compounds wich have been present for millions of years there are organisms somewhere in the nature (biosphere) which can initiate their mineralization. Many of the recalcitrant compounds which have been produced by the modern industry are structuraly closely related to natural compounds. For this reason its biodegradation is possible because the same enzymatic systems are involved.

On the other hand, many other xenobiotic compounds have only recently introduced into the environment. In this case the microorganisms are unable to degrade these compounds. The challenge to the evolutionary potential of microorganisms produced by xenobiotic compounds has stimulated basic research on experimental enzyme evolution (Clarke, 1980; Mortlock, 1982; Wu, 1975).

According to Leisly Grady (1985) a fortuitous or gratuitous metabolism (Slater, 1982; Knackmus, 1981) is the major mechanism involved when a bacteria attack xenobiotic compounds. The success of gratuitous metabolism depend on a number of factors, not the least of which is the ability of xenobiotic compounds to induce the synthesis of the requisite enzyme. This will depend to a large degree on the similarity in structure between xenobiotic compounds and the natural substrate. Appart from the observation that biodegradability decreases as the number of substitutions increases, it is

difficult to generalize about the effect of the type or positions of the xenobiotic moiety because they are both influenced by the nature of the parent structure (Alexander, 1973).

It is apparent, however, that if the xenobiotic compound is unable to cause induction of the requisite enzyme, its biodegradation will only occur in the presence of a natural inducer. This could severely limit the usefullness of the enzymatic capability. Another important factor is the nature of the resulting product from the enzymatically catalized reaction. Sometimes the reaction product could be more toxic than the original compound or it could be less susceptible to further microbial attack, thereby making it more likely to persist or it could be more susceptible to be bioaccumalated, causing it to be more of a hazard to the higher organisms in the food chain (Alexander, 1980).

Luckly there are many examples in which the product of the fortuitous reaction was able to serve as a substrate for another enzymatically catalyzed step (Slater et al., 1982).

Enzymes are often induced coordinately i.e. one inducer may cause the synthesis of several enzymes.

When a single microbial species carried out gratuitous metabolism on a xenobiotic substrate as sole carbon and energy source they must ultimately be able to extract energy and reducing power from the reaction. If not, they will be unable to mantain their cellular integrity against the natural forces leading toward disorder and the culture will eventually die. In this case biodegradation of the xenobiotic compound will takes place through the use of additional carbon and energy sources supplied from the environment or from the action of other organisms in a mixed microbial community.

If a xenobiotic compound has been exposed to a pure culture of microorganisms two possibilities may occur: a) The xenobiotic has been transformed by gratuitous metabolism. If the tranformation product is more toxic than the original substrate it is accumulated. If the transformation product is benign

it will be futher transformed and the process may continue until has been converted into a biodegradable compound. b) A xenobiotic compound has been cometabolized. In this case the transformation products will always accumulate.

The production of a more toxic product than the original one by gratuitous metabolism or by cometabolic attack of a xenobiotic compound may will not result in their accumulation in the environment if the organism performing this transformation is growing in a mixed microbial community (Bull, 1980). Microbial communities are able to use carbon sources that cannot be degraded by any single organism alone (Slater, 1982; Harder, 1981; Jones, 1972).

Biodegradative capacity of a community is much greater (both quantitatively and qualitatively) particularly where xenobiotic compounds are involved. (Harder, 1981; Slater, 1981; Meers, 1973).

Several works have shown that adaptation of microorganisms may play a major role in determining biodegradation (Felset et al., 1981; Fournier et al., 1981; Senior et al., 1976; Kilbane et al., 1982; Stanlake et al., 1982; Briton et al., 1981; Kellogg et al., 1981; Chatterfee, 1981; Kulla, 1981; Spain et al., 1983).

Adapted microorganisms for degrading non-degradable compounds can be selected by using continuous cultures techniques but these adaptations require extended periods of cultivation. Two types of adaptation are involved. Firstly, the adaptation of existing catabolic systems to the degradation of novel compounds and secondly the attaintment of novel metabolic pathways through selection/mutation or by a chromosomal or plasmid encoded catabolic pathway (Chaterjee et al., 1981; Knacknuss, 1981; Slater et al., 1979). In Pseudomonas putida naphtalene degradation is encoded by a plasmid (Cane et al., 1982; Yen et al., 1982; Haas, 1983), but In Pseudomonas PMD-1, the biodegration of naphtalene depend on chromosomal genes (Zuniga et al., 1981). Some examples of plasmid-encoded pathways include a diverse group of aliphatic compunds - octane (Fennewald, 1979); aromatic an polynuclear aromatic hydrocarbons:-xylene, naphtalene (Yen, 1982), phenanthrene (Kiyohara et al.,1980), toluene (Franklin, 1981); chlorinated hydrocarbons such as 2,4-diclhorophenoxyacetate (2,4-D) (Don et al., 1981), chlorobiphenil (Furukawa

et al., 1982), 3-chlorobenzoate (Chatterjee, 1983; Reineke, 1982), chlorotoluene (Pierce, 1982).

Don and Pemberton (1981) reported the isolation of six plasmids that encode for degradation of 2,4-D and monochlorophenoxy acetic acid and which could be transfer from Alcaligenes into Escherichia coli, Pseudomonas putida, Ps. fluorescens, Rhizobium Sp., Rhodopseudomonas sphaeroides and Acinetobacter calcoaceticus. Plasmid transfer is an important component of the evolution of degradative pathway in nature and in waste water treatment system (Leslie Grady, 1985; Simon-Sylvestre et al., 1979; Spain et al., 1980).

The knowledge of the degradation mechanisms which is another basic aspect of the subject is important for improving effluent treatment. Examples of mechanisms involved in the degradation of some recalcitrant compounds are cited below.

Furukawa et al. (1978) have studied the degradation of various isomers of PCB by Alcaligenes and Acinetobacter strains. The degradative pathway is shown in Fig. 1.

The bacterial degradation of herbicide 2,4-D has been studied with Flavobacterium and Arthrobacter strains. The degradative pathway is shown in the Fig. 2 (Motosugi et al., 1983).

Enzymological aspect of chloroaromatics compounds have been performed by Dorn et al. (1978). They worked with Pseudomonas Sp. B13 and found two forms of catechol dioxygenase:Pyrocatechase I and Pirocatechase II. An enzyme which catalyzes the chlorine eliminating cycloisomerization of 2- and 3-chloro-cis and cis-muconic acid has been isolated from cells of Pseudomonas sp B13 and was designated muconate cycloisomerase II (Schmidt et al., 1980).

Monomethyl sulfate (MS) which is frequently found in effluents produced by agrochemical and dyestuff industries is formed as a by-product in stechiometric quantities when methylations in organic syntheses are carried out with dimethylsulfate.

Fig. 3 shows the pathway proposed for the biodegradation and utilization of MS by Hyphomicrobium M5 strains. (Ghisalba et al., 1983).

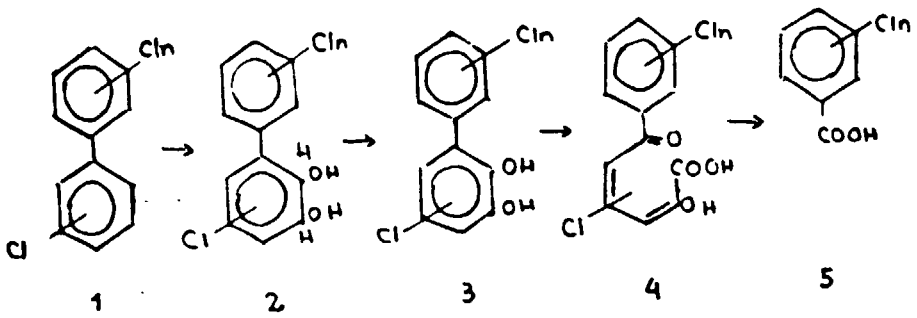


Fig. 1: Degradative pathway of $PCB_S$ (From Motosugi et al., 1983)
1. $PCB_S$ (n = 1-4)
2. cis-dihydroil compound
3. 2'3' dihydroxy compounds
5. derivates chlorinated benzoic acid.

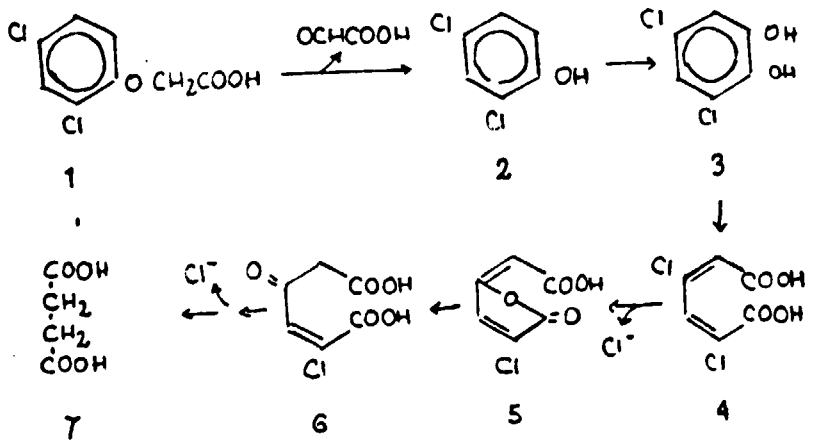


Fig. 2: Degradative pathway of 2,4-D (from Motosugi et al., 1983)
1. 2,4-D
2. 2,4 dichlorophenol
3. 3.5 dichlorocatechol
4. cis-cis 2.4 dichloromuconic acid
5. 2-chloro- 4-carboxymethylen but-2-enolide
6. chloromaleyacetic acid
7. succinic acid

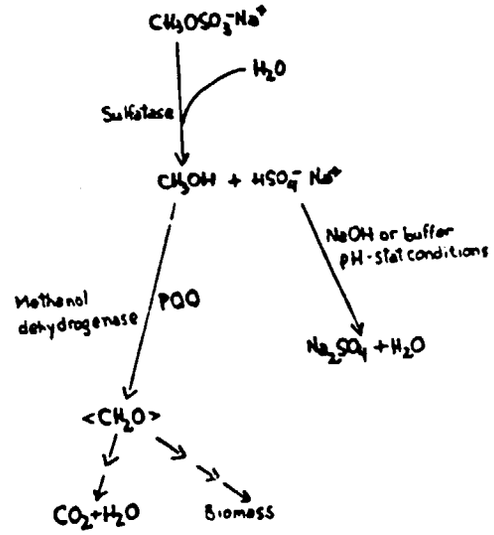


Fig. 3: Pathway for the biodegradation and utilization of MS (from Ghisalba et al., 1983).

Microbial treatment of industrial effluents containing recalcitrant compounds by Azotobacter strains

The production of industrial substances such as dyestuffs, vinyl chloride, drugs, agrochemicals etc is generally associated with the formation of large amount of toxic compounds which are finally disposed in the waste waters (Stuki et al., 1983; Ghisalba, 1983; Ghisalba et al., 1983).

According with the 80's EPA statistics based on more than 3100 samples from a total of 35 industrial categories, those pollutants observed with highest frecuency are: chloroform (37%), methylene chloride (36%), benzene (26%), toluene (28%), phenol (24%), ethylbenzene (15%), naphthalene (10%), phenanthrene/anthracene (10%) and trichloromethane (11%) (Patterson et al. 1981).

The use of conventional biological treatments is inadequate for treating industrial effluents containing recalcitrant compounds, and the efficiency of its removal depend on the kind of mixed community and also on the complexity of the present organisms.

In aerobic and anaerobic biological waste treatment processes, the most importants pollutants can give different types of response (Ghisalba, 1983): 1) inhibition and deterioration of the treatment efficiency, 2) formation of non-biodegable compounds 3) passing unchanged through the system 4) biochemica conversion with formation of different compounds nor necessarily simples ones 5) biodegradation (mineralization) with formation of $CO_2$, $H_2O$, $NO_3^-$, $SO_4^=$ and biomass 6) acclimatization of the population and degradation of the recalcitrant compounds by means of population shifts and microbial adaptation 7) absorption into soil particles and into primary slugde and 8) volatilization of the recalcitrant compounds.

In some cases, some compounds tested were concentrated on sludge by factors ranging between 5 to 170 (Tabak et al., 1983; Leisinger, 1983).

In spite of the abundance of biochemical information on catabolism, little data is available for the design and operation of waste treatment facilities containing recalcitrant compounds. It is important to have kinetic data on growth and degradation rates as well as on other parameters of growth

like the saturation constant (Finn, 1983; Cook et al., 1981).

Finn (1983) point out the neccesity of using starter cultures and treating pills for improving the effluents treatment. Sometimes this is achieved by using active cultures obtained from an efficient plant. In other cases is convenient to inoculate pure or mixed cultures (Senior, 1976), but it is important that the microorganism which is seeded succeedto be dominant (Stanlake et al., 1982; Edgehill et al., 1982). In the Azotopure process, Finn (1973) recommends the use of Azotobacter sp. for treating nitrogen deficient effluents.

In addition of containing recalcitrant compounds, many effluents from chemical and petrochemical industries are deficient in nitrogen and phosphorous sources which are essencial for microbial growth (Rozich, 1984).

When waste waters are deficient in nitrogen compounds three alternative process could be applied: a) assimilation treatment, 2) enzymatic method 3) azotopure process (Finn, 1973).

The microbial uptake of organic compounds via oxidative assimilation and the conversion of these compounds into non-nitrogenous cellular components takes place when cell replication is not possible.

A lack or an insufficient amount of nitrogen will result in non-proliferating conditions and the microbes will be prompted to remove organics via an oxidative assimilation mechanism. The advantage of the oxidative assimilation process over a conventional activated slugde process, which is usually employed for treating nitrogen-deficient wastes, is that a substantial saving in chemical costs (ammonia).

The enzymatic method has been mentioned for the removal of phenol (Atlow et al., 1984) $PCB_S$ (Klivanor, 1983) and pesticides (Johnson et al, 1983) from industrial effluents.

In the Azotopure process Finn recommends the use of Azotobacter sp. for treating nitrogen-deficient effluents. In pilot plants ($3m^3$/day) tests with Azotobacter, a water stream containing mostly ethanol (2000 mg/l COD) was treated in 5 hours detention time at 30°C to remove 80% of organic matter

even without cell separation (Finn, 1983).

The use of Azotobacter strains is an alternative microbial process that may be applied for the treatment of nitrogen deficient wastes which contain recalcitrant compounds. In the literature very little information is available on the degradation of recalcitrants by Azotobacter strains (Barabarz, 1982; Ghisalba, 1983).

The main biochemical characteristics of the members of the genus Azotobacter is the non-symbiotic nitrogen fixation. The nitrogen fixation is accomplished by the operation of the nitrogenase system, a very specialized enzymatic system which is composed of two metaloproteins highly sensitive to oxygen. The culture conditions, nutritional requirements and biochemical properties of Azotobacter have been described by Burton (1973).

The importance of using Azotobacter cultures systems is mainly due to the wide range of possible applications of this microorganisms such as alginate production (Horan et al., 1981), vegetables growth promoting substances like auxines and gibberellines, solubilization of potasium from minerals (Giulietti et al., 1982); inoculants for non-symbiotic $N_2$ fixation (Burton, 1973); treatment of wastes with low nitrogen content (Finn et al., 1973; Waehner et al., 1985) and production of ammonia by fermentation (Narula et al., 1981).

The most important advantages of the use of the Azotobacter genus in connexion with effluent treatment are as follows: 1) non-symbiotic nitrogen fixation, 2) Azotobacter cultures are able to be mantained as the dominant population in a quasi-pure culture in a nitrogen deficient medium, 3) Azotobacter strains are able to grow using different organic compounds such as alcohols, acids, sugars, aromatic compounds like benzoate, salicilate, phenol, etc as a sole carbon source. 4) considerable chemical oxygen demand (COD) reduction is attained 5) limited sludge produced, 6) Low yield of biomass, the organic matter being mainly converted into carbon dioxide and water, 7) production of polimers (alginate) which may enhance the removal or hydrocarbon and oils from waste waster (Henkel, 1982).

The main disadvantage of Azotobacter strains is the inability to hydrolize esthers, but is has been recently mentioned the possibility of introducing

this property by genetic manipulation.

In our laboratory we have been working on the use of Azotobacter strains for treating recalcitrant compounds (Molina et al., 1985). The strains used were as follows: Azotobacter SL, Azotobacter BA1 and Azotobacter BA2 all isolated from Argentina soils. We studied the influence of temperature and pH on growth rate using a mineral base medium with glucose as sole carbon source by using Azotobacter SL strain. The activation energy (E) for growth from Arrhenius plot obtained was 10.929 cal. $mol^{-1}$ between 15-30°C and 18.970 $cal.mol^{-1}$ between 30-35°C. The optimum pH was 6.5-7.0.

We tested first the ability of the strain for reduction of COD of media containing glucose maltose, starch, sucrose and lactic acid as sole carbon source. The highest specific growth rate ($\mu = 0.20\ h^{-1}$) and highest COD removed, 85%, were obtained with lactic acid as sole carbon source. This behaviour is in agreement with Muñoz et al. (1981).

The strains were also used for treatment effluent from a pharmaceutical industry containing different organic compounds. Removal of 40-50% of COD were obtained.

The three strains were also used for treatment recalcitrant compounds such as: aromatic gas oil, hydrogenated gas oil, celulose acetate, DDT ; m-nitrotoluene; 2,4-D and benzene as sole carbon source, which were added to a mineral base medium. DDT was used insolubilized or previously dissolved in acetone.

Azotobacter SL, Azotobacter BA1 and Azotobacter BA2 grew in DDT and also were able to grow on aromatic and hydrogenated gas oil and benzene. However, all of them were unable to use cellulose and 2,4-D as carbon sources. Two (Azotobacter SL and Azotobacter BA2) strains were able to grow on m-nitrotoluene.

Table 3 shows the parameters of growth of the Azotobacter strains grown on mineral base medium supplemented with insolubilized DDT.

Table 3: Parameters of growth of the Azotobacter strains grown on mineral base medium supplemented with insolubilized DDT.

| Azotobacter strains | DDT (mg/l) | $L(h^{-1})$ | $\mu(h^{-1})$ |
|---|---|---|---|
| SL | 60 | 22 | 0.02 |
| | 80 | 28 | 0.02 |
| | 100 | 34 | 0.02 |
| BA 1 | 60 | 68 | 0.05 |
| | 80 | 57 | 0.05 |
| | 100 | 72 | 0.05 |
| BA 2 | 60 | 34 | 0.09 |
| | 80 | 32 | 0.08 |
| | 100 | 32 | 0.08 |

L: Lag period; $\mu$: specific growth rate.

These results clearly show that the strain Azotobacter BA2 is particularly suitable for using DDT as a sole carbon source. The specific growth rate ($\mu$) is remarkable.

Although phenol is easily degraded and for this reason not considered as a recalcitrant compound (Ghisaba, 1983; Yang et al., 1975), it is troublesome contaminant in surface waters owing to the fact that when phenol containing water is chlorinated, toxic polychlorinated phenols may result. For this reason it was also decided to test the strains for degrading phenol Table 4 shows the results of growth experiments carried out with the Azotobacter strains using mineral base medium supplemented with phenol.

Table 4: Parameters of growth and pehnol consumption of Azotobacter strains grown on mineral base medium supplemented with phenol.

| Azotobacter strains | Phenol# (mg/l) | L ($h^{-1}$) | μ ($h^{-1}$) | % Phenol consumption |
|---|---|---|---|---|
| SL | 60 | 19 | 0.07 | 95 |
| | 80 | 19 | 0.07 | 96 |
| | 100 | 20 | 0.08 | 95 |
| BA 1 | 60 | 11 | 0.02 | 93 |
| | 80 | 13 | 0.02 | 93 |
| | 100 | 12 | 0.02 | 92 |
| BA 2 | 60 | 13 | 0.2 | 95 |
| | 80 | 13 | 0.3 | 95 |
| | 100 | 13 | 0.2 | 95 |

# Concentrations higher than 1 g/l inhibited the growth of all the strains.

It is interesting to point out the ability of all the strains to degrade phenol in high percentage, and particularly that of Azotobacter BA 2, which specific growth rate is very high compared with the others strains.

It can be concluded that the recalcitrant compounds tested (with the exception of cellulose acetate and 2,4-D) were used as carbon source by Azotobacter strains in nitrogen deficient medium.

The treatment of recalcitrant compounds, by Azotobacter strains represents an interesting alternative which deserves further investigation, because conventional biological treatments are inadequate for the removal of these products from industrial effluents and other waste waters.

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