

## **ENZYMATIC REMEDIATION METHODS FOR POLLUTED AREAS BY MINING OPERATIONS**

Environmental pollution is growing more and more due to the indiscriminate and frequently deliberate release of hazardous, harmful substances. Research efforts have been devoted to develop new, low-cost, low-technology, eco-friendly treatments capable of reducing and even eliminating pollution in the atmosphere, the hydrosphere and soil environments. Among biological agents, enzymes have a great potentiality to effectively transform and detoxify polluting substances because they have been recognized to be able to transform pollutants at a detectable rate and are potentially suitable to restore polluted environments.

Several substances with high polluting potential are present in the environment and affect soil, sediments, water, air, microbial organisms, plants, animals, and humans. They may be distributed in one or all environmental compartments. The origins and sources of pollution are different: industrial activities such as mining and metal processing, petrochemical and industrial complexes, industry effluents, chemical weapons production, pulp and paper industries, dye industries and industrial manufacturing; and anthropogenic activities such as traffic, agricultural practices, and others. Several methodologies have been applied for the remediation of polluted systems and many of them, when implemented in the target sites, have led to successful results.

Two basic strategies have been utilized: engineering and biological ones. Engineering strategies are basically founded on physical and chemical methods, whereas biological strategies require the involvement of biological agents. Composting, land farming, bioreactors, bioremediation, and phytoremediation are the main biological methods applicable to soil and groundwater. Land farming is not suitable for the latter. Regardless the adopted method, the decontamination of polluted sites may be carried out by in-situ (if soils and water are treated directly on site) or ex-situ (if they are excavated, transported to another site and, then treated) treatments. In situ techniques are physical treatments, whereas ex situ treatments require higher costs and an increased environmental disturbance. Bioremediation and phytoremediation appear now as appealing technologies being based on the use of living organisms, microorganisms, plants, and their enzymatic set. (Rao, Scelza, Scotti, & Gianfreda, 2010)

### **ENZYMES AS DECONTAMINATING AGENTS**

Enzymes have several beneficial characteristics. They are the main effectors of all the transformations occurring in the biota. They are catalysts with either narrow (chemo-, region-



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and stereo-selectivity) or broad specificity and, therefore, they can be applied to a large range of different compounds in mixture, as well. They may produce extensive transformations of structural and toxicological properties of contaminants, and even their complete conversion into innocuous inorganic end products. They may perform processes for which no efficient chemical transformations have been devised.

Moreover, enzymes may present advantages over traditional technologies, and also over microbial remediation. Indeed, enzymes are not inhibited by inhibitors of microbial metabolism. They can be used under extreme conditions limiting microbial activity. They are effective at low pollutant concentrations and are active in the presence of microbial predators or antagonists. They act against a given substrate (microorganisms may prefer more easily degradable compounds than the pollutant), and are more mobile than microorganisms because of their smaller size. All these characteristics render enzymes eco-friendly catalysts as well as enzymatic techniques environmentally friendly processes. These latter may have the capability of remediation of many compounds that are unfriendly to the environment by the present ecological standards of our societies. As claimed Alcade et al., (2006), biocatalysis by enzymes (very often known as white biotechnology) “fully participates in the “green chemistry” concept introduced in the 90s by Sheldon and van Rantwijk, (2004), and its effect on sustainability is now established beyond question”.

Enzymes may act intracellularly, i.e. in the presence of or inside their originating cells; extracellularly, i.e. both in the presence or absence of their originating cells; free, i.e. soluble in solution and the catalysis will be homogenous; or immobilized, i.e. linked through different links to a solid matrix and the catalysis will be heterogeneous.

The most representative enzymatic classes in the remediation of polluted environments are: hydrolases, dehalogenases, transferases and oxidoreductases. Their main producers are bacteria, fungi, mainly white-rot fungi, plants and microbe-plant associations. For many of these enzymes the transformation of different xenobiotic substances has been tested mainly under laboratory conditions. Reagents and activity assay conditions are available for many of these enzymes, and they are described in detail to allow their easy detection and application.

Examples of hydrolases are phosphotriesterases, amidases, proteases, carbohydrases (cellulases and amylases), depolymerase. Mono- or di-oxygenases, reductases, dehalogenases, cytochrome P450 monooxygenases, phenol oxidases (laccases, tyrosinases) and peroxidases (lignin and manganese peroxidases) are the main classes of oxidoreductases.

The breakdown of esteric, amidic and peptidic bonds by esterases, amidases and proteases may lead to products with little or no toxicity. For instance, bacterial hydrolases such as carbamate or parathion hydrolases from *Achromobacter*, *Pseudomonas*, *Flavobacterium*, *Nocardia*, and *Bacillus cereus* have been successfully used in the transformation of pollutants such as carbofuran and carbaryl or parathion, diazinon and coumaphos. Similarly, carbohydrases,



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depolymerases, proteases and phosphatases, produced by several bacteria and fungi, can be suitable for the transformation of insoluble materials such as carbohydrates, plastics and proteins.

Sornyotha et al. (2010) showed that the combination of xylanase and cellulose (two hydrolases acting as plant cell wall degrading enzymes) exerted a synergistic action on the removal of linamarin (a cyanogenic glycoside found in the leaves and roots of plants such as cassava, lime beans, and flax) from cell walls of cassava roots, a high-cyanogen variety, and on the release of linamarase. Linamarin liberation from parenchyma was enhanced by 90% with the combined enzyme treatment. In addition, when the combined enzymes were applied for detoxification during cassava starch production, a low-cyanide product was obtained with a decreased linamarin concentration (96%) compared to the non-enzyme treated tissues. The author concluded that this method is more effective than the traditional detoxification method of adding linamarase directly and/or the treatment with some microorganisms. Moreover, this method could be used in the low-cyanide-cassava starch production and is suitable for detoxification of cassava products during processing. enzymes involved in the transformation of nitrile compounds. Nitrile is any organic compound which has a  $-C\equiv N$  functional group. The  $-C\equiv N$  functional group is called a nitrile group in which the carbon atom and the nitrogen atom are triple bonded together. The prefix cyano is used in chemical nomenclature to indicate the presence of a nitrile group in a molecule. A cyanide ion is a negative ion with the formula  $CN^-$ . The  $-CN$  group is sometimes, less properly, referred to as a cyanide group or cyano group, and compounds with it are sometimes referred to as cyanides. Many cyanide-containing compounds are highly toxic and deadly poisonous while some nitriles (which do not release cyanide ions) may have low toxicities. Nitrile compounds are synthesized by plants, fungi, bacteria, algae, insects and sponges.

There are two different enzymatic pathways for the degradation of nitrile compounds (Figure 3). One is a two step degradation involving nitrile hydratase and amidase via an amide as an intermediate. The other is the direct hydrolysis of nitriles to the corresponding acids and ammonia, catalyzed by nitrilase. Nitrilases (EC 3.5.5.1) constitute branch 1 of the nitrilase superfamily, which comprises enzymes acting on nonpeptide  $C\equiv N$  bonds. They are produced by both bacteria, such as *Nocardia* sp. and *Rhodococcus* sp., and fungi, as *Fusarium solani* or *Aspergillus niger*. Some of the nitrilases are capable of hydrolyzing nitriles stereospecifically. While much information is available on the structure and function of bacterial nitrilases, a lesser amount of findings is available for nitrilases from filamentous fungi. Martínková et al. (2009) reviewed the current knowledge of these enzymes by examining findings on enzyme screening, production, purification and immobilization and prospective applications in the field of biocatalysis. In particular, they investigated the potentiality of fungal nitrilase and compared their performance with some from bacterial origins. Their studies established that the nitrilases of

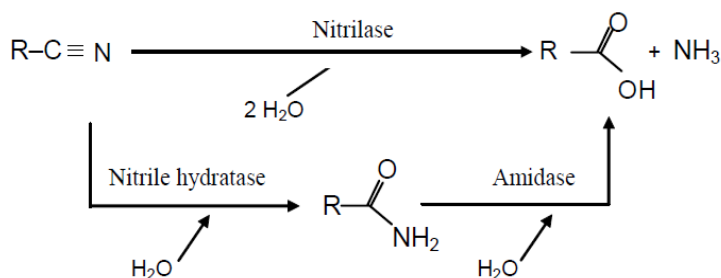


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filamentous fungi have high relative activities toward (hetero) aromatic nitriles, and accept a wide range of aliphatic and alicyclic nitriles.



**Figure 3.** Enzymatic pathways for hydrolysis of nitriles.

One of the benefits of fungal nitrilases is their high specific activity toward substances such as benzonitrile and analogues, 3- and 4-cyanopyridine and also some medium chain length aliphatic nitriles, considered their preferential substrates.

For instance, *A. niger* nitrilase was able to transform a large range of different substrates at high rate as compared with bacterial nitrilase. The biotechnological impact of nitrilases lies in their potential to accept a wide range of aliphatic and acyclic nitriles; to hydrolyze nitriles under mild conditions, with excellent regio- and enantioselectivities in some cases; to exhibit high activity, stability and thermostability. This makes these enzymes good candidates for biodegraders of nitrile contaminants.

Hydrogen cyanide (HCN) is a major environmental pollutant of the chemical and metallurgical industries. It is produced on large scale worldwide to satisfy major industrial countries needs. Cyanide is extremely toxic to aerobic forms of life since it inhibits respiration by strongly binding to cytochrome oxidase. Although extremely toxic, cyanide can enzymatically be converted to the less toxic thiocyanate by rhodanases (thiosulfate:cyanide sulfurtransferases, EC 2.8.1.1), highly conserved and widespread enzymes now considered as one of the mechanisms evolved for cyanide detoxification. A genetic system was engineered to express high levels of recombinant *Pseudomonas aeruginosa* rhodanese (r-RhdA) in *E. coli*, and this organism was used to test the role of r-RhdA in cyanide detoxification. Active r-RhdA was obtained over a 4-h period, and the enzyme distributed between the cytoplasm (95%) and the periplasm (5%), as it was assessed by Western blot analyses and enzymatic assays. The accessibility of thiosulfate to r-RhdA limited the sulfur transfer reaction in the cellular system, but permeabilization of the bacterial membrane increased the cyanide conversion into thiocyanate. Overall results indicated that engineered *E. coli* was able to perform cyanide detoxification even under laboratory conditions, and suggested that microbial rhodanases may contribute to cyanide transformation in natural environments. An important group of enzymes is

constituted by oxidative enzymes. They have a predominant role in the environment being involved in several processes including the detoxification of polluted environments.

Indeed, they are involved in the formation of humus material in soil, in the exchanges between plants and soil through degradation and synthetic mechanisms. Moreover, they can be involved in the reactions between humus constituents and xenobiotic molecules and form bound residues in water systems and humus materials in soil. (Rao, Scelza, Scotti, & Gianfreda, 2010)

## **MICROBIAL ENZYMES IN BIOREMEDIATION**

### **Monooxygenases.**

Monooxygenases incorporate one atom of the oxygen molecule into the substrate. Monooxygenases are classified into two subclasses based on the presence cofactor: flavin-dependent monooxygenases and P450 monooxygenases.

Flavin-dependent monooxygenases contain flavin as prosthetic group and require NADP or NADPH as coenzyme. P450 monooxygenases are hemecontaining oxygenases that exist in both eukaryotic and prokaryotic organisms. The monooxygenases comprise a versatile superfamily of enzymes that catalyzes oxidative reactions of substrates ranging from alkanes to complex endogenous molecules such as steroids and fatty acids. Monooxygenases act as biocatalysts in bioremediation process and synthetic chemistry due to their highly regionselectivity and stereoselectivity on wide range of substrates. Majority of mono-oxygenase studied previously are having cofactor, but there are certain monooxygenases which function independent of a cofactor. These enzymes require only molecular oxygen for their activities and utilize the substrate as reducing agent. The desulfurization, dehalogenation, denitrification, ammonification, hydroxylation, biotransformation, and biodegradation of various aromatic and aliphatic compounds are catalyzed by monooxygenases. These properties have been explored in recent years for important application in biodegradation and biotransformation of aromatic compounds. Methane mono-oxygenase enzyme is the best characterized one, among monooxygenases. This enzyme is involved in the degradation of hydrocarbon such as substituted methanes, alkanes, cycloalkanes, alkenes, haloalkenes, ethers, and aromatic and heterocyclic hydrocarbon. Under oxygen-rich conditions, mono-oxygenase catalyzes oxidative dehalogenation reactions, whereas under low oxygen levels, reductive dechlorination takes place. Oxidation of substrate can lead to de-halogenation as a result of the formation of labile products that undergo subsequent chemical decomposition.

### **Microbial Dioxygenases.**

Dioxygenases are multicomponent enzyme systems that introduce molecular oxygen into their substrate. Aromatic hydrocarbon dioxygenases, belong to a large family of Rieske nonheme iron oxygenases. These dioxygenases catalyze enantiospecifically the oxygenation of wide range of substrates. Dioxygenases primarily oxidize aromatic compounds and, therefore, have



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applications in environmental remediation. All members of this family have one or two electron transport proteins preceding their oxygenase components. The crystal structure of naphthalene dioxygenase has confirmed the presence of a Rieske (2Fe–2S) cluster and mononuclear iron in each alpha subunit. The catechol dioxygenases serve as part of nature's strategy for degrading aromatic molecules in the Environment. They are found in the soil bacteria and involved in the transformation of aromatic precursors into aliphatic products. The intradiol cleaving enzymes utilize Fe(III), while the extradiol cleaving enzymes utilize Fe(II) and Mn(II) in a few cases. (Rao, Scelza, Scotti, & Gianfreda, 2010)

## Conclusions

In conclusion, several are the fields in which enzymes can be applied (Figure 7). Enzymes present environmental advantages against chemicals and microorganisms. They are: the biotransformation does not generate toxic side products as is often the case with chemical and some microbiological processes; the enzymes are digested, in situ, by the indigenous microorganisms after the treatment; the requirement to enhance bio-availability by the introduction of organic co-solvents or surfactants is much more feasible from an enzymatic point of view than using whole cells; the production of enzymes at a higher scale, with enhanced stability and/or activity and at a lower cost is feasible by using recombinant-DNA technology.

However, the enzyme performance for in situ bioremediation of contaminated groundwater and soils may be affected by many factors.

They are:

- chemistry and toxicity of contamination,
- source and concentration of pollutants,
- solubility, transport, adsorption, dispersion and volatility of pollutant compounds,
- detection, determination and monitoring of pollutants,
- chemistry, physics and microbiology of groundwater and soil,
- chemistry and mechanics of soil at the contaminated site,
- hydrogeology and hydrology of the contaminated site,
- limitations of environmental standards for water and soil,
- environmental conditions, nutrient sources and presence of electron acceptors,
- and mainly the biodegradability of contaminants.

The scope of bioremediation is to decrease the concentration of organic pollutants at undetectable levels or, if measurable, lower than the limits established as safe or tolerable by regulatory agencies. Consequently several criteria concerning a) the selected agent to perform the bioremediation process, b) the toxicity of the end-products and c) the conditions at the site, favorable or not to the process, must be met to be bioremediation seriously considered as



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a practical method for treatment. In addition, the feasibility of the bioremediation program has to be evaluated by considering its real applicability, its possible limitations and drawbacks and its advantages.

Only when some or all these aspects will be addressed, an effective, successful, productive, and nondeleterious of environment quality and costly-convenient bioremediation process will be performed.

## Bibliography

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