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Bioremediation of Herbicide Velpar K® In Vitro in Aqueous Solution with Application of EM-4 (Effective Microorganisms)

Márcio Antônio Gomes Ramos* **and Sergio Akinobu Yoshioka**
[1]*Instituto de Química de São Carlos; Universidade de São Paulo; Av Trab. São-Carlense, 400; C. P.: 780; 13560-970; São Carlos - SP - Brasil*

ABSTRACT

This work assessed the bioremediation of herbicide Velpar K®, in vitro in aqueous solution, used against weeds in sugar cane in São Paulo state. The herbicide contained Hexazinone and Diuron. It was used the microbial inoculant denominated Effective Microorganisms (EM-4), pool of microorganisms from soil that contained lactic and photosynthetic bacteria, fungi, yeasts and actinomycetes for bioremediation. Results for the depth of cultivation on agar-agar inoculated with EM-4 showed the microorganisms growth in the concentrations between 0.2% and 1.0% of the Velpar K® in the gel. The analysis of high performance liquid chromatography (HPLC) showed that the EM-4 was effective for the bioremediation of the herbicide, which reached the values of 80% for diuron and 70% for hexazinone after 21 days in solution of 2:1 of Velpar K®/EM-4 ratio. These results could be useful for planning the bioremediation of contaminated areas with Velpar K®.

Key words: Velpar K®, herbicide, bioremediation, effective microorganisms

INTRODUCTION

Fertilizers and pesticides are used with the main objective of increasing the agricultural productivity, although most of them are substances that cause environmental and public health damages. The natural degradation process of such substances in the environment and their elimination is called bioremediation. This method has mainly applications in contaminated environments such as water, soil, sediments from the rivers, lakes and oceans. During the last decades, there has been considerable developments on the use of microorganisms to promote biodegradation and bioremediation (Boopathy 2001).

The natural environment can be modified or benefitted with different objectives, including the application for microbial bioremediation of impacted areas (Silva et al. 1999). With the complexity of the environment where the contamination occurs as well as bioremediation, which is dependent on interdisciplinary approaches (involving the areas of chemistry, microbiology, biochemistry, engineering, ecology, geology and others), the bioremediation technology can be applied as a treatment *in situ* or *ex situ* of contamination (Melo et al. 1999). Several studies have been performed to minimize, reduce or even eliminate the presence of toxic materials into the environment, such as the biodegradation of organochlorine, carbamates and organophosphates. The bioremediation process of

*Author for correspondence: marcioagramos@hotmail.com

organic matter is based on the metabolic activity of heterotrophic aerobic and anaerobic microorganisms, and is affected by a large number of physical and chemical factors, which include energy sources (electron donors), electron recipients, nutrients, pH, temperature and metabolic inhibitors (Seabra 2008). Bioremediation is a strategy or process that uses microorganisms or their enzymes to detoxify the contaminants to acceptable levels of pollutants in the environment through the transformation of the contaminant into harmless forms (Tapajós 2008). Therefore, biodegradation is based on the microbial degradation processes and chemical processes combined with engineering, which is able to maximize the transformation of organic contaminants from the environment (Moreira et al. 2002). Between the bacteria and fungi described as efficient decomposers, there are those belonging to the genera: *Azospirillum, Pseudomonas, Alcaligenes, Enterobacter, Proteus, Klebsiella, Serratia. Bacillus, Arthrobacter, Nocardia, Streptomyces, Mucor, Fusarium, Chaetomium, and Phanerochaete Trametes*.

Several chemicals can be biologically treated successfully, such as crude oil, oil derived from oil refining and cracking as gasoline, benzene, xylene, toluene, ethylbenzene, diesel oil, kerosene, wood preservatives, solvents, various urban sewage sludge or industrial, and other biogenic or xenobiotic compounds. Among the xenobiotics, the insecticides and herbicides are substances of widespread use in agriculture, and are classified according to chemical structure, but the most common method is that one implemented according to action and biological species reached.

In Brazil, large amounts of Velpar K® herbicide is used in sugar cane cultures. This herbicide is selective in controlling the pre- and post-germination of weeds that have broad leaves. The product operates in the photosynthesis inhibition, and it is commercialized in the form of granules which are soluble in water. Its active ingredients belong to the groups of substituted urea and triazinones, whose IUPAC names are: 3 - (3,4 - (dichlorophenyl) -1,1-dimethylurea or diuron, containing 53.3% and 3-cyclohexyl-6(dimethylamine)-1methyl-1,3,5, triazine-2, 4 (1H, 3H) dione or hexazinone, containing 6.7% and the remaining 40% of inert ingredients (DU PONT BRASIL 1989). Because of high solubilization, the diuron and hexazinone present great risks since they are easily leached and offer greater risks for contamination of groundwater (Gomes et al. 2001). For this reason, there is interest in monitoring of contamination in groundwater by these herbicides. Hexazinone present in this herbicide should be removed after performing its role when present in excess. This is done often through the microbial route, which depends on the dynamic equilibrium of microbial population. This can be altered by the changes in environmental conditions such as the pesticides addition or other biologically active substances (Silva 1996).

There are several studies on the use of actinomycetes, fungi, bacteria and yeast in an individual or consortium for the bioremediation of pesticides in the soil for the purpose of reducing or eliminating their dangerous products (Gaylarde 2005). The use of EM-4 promoted the practice of natural agriculture (Okuda et al. 1999). The aim of this work was to assess the bioremediation of Velpar K in aqueous solution using EM-4, and to analyze by HPLC its active principles without biodegradation.

MATERIAL AND METHODS

Inoculant EM-4 is a mixed culture of many species of bacteria and leavenings, whose development is carried out preferably in molasses from diluted sugar cane. The diuron and hexazinone standards were purchased from Aldrich. Velpar K® (Du Pont Brazil 1989) was obtained from a local agricultural store.

Growth of EM-4 in Velpar K® as the only carbon source

A sample of 1.0mL of the EM-4 ($5x10^2$ cells/mL), previously activated with molasses was plated using the inoculation technique by depth (pour plate) in triplicate. Then 15-20 mL of solid agar Velpar-K® were poured into sterile Petri dishes, previously melted at 45°C, containing 2% agar (Merck®) and variable concentrations of Velpar K® (0.0, 0.2, 0.8 and 1.0% ,v/v) and cooled. The Petri dishes were maintained at 30 ± 1°C for seven days in order to check the microorganisms growth by counting the colony forming units (CFU) with a colony counter (Phoenix - model EC 589).

Quantification by HPLC of Velpar K®

For this, Shimadzu C-R4A chromatograph, coupled to LC 9A pump and UV detector (SPD - 6

HS) at 254 nm, SCC-6B controller, with column HP - C 18 to 20 cm in length and internal diameter of 4.6 mm and a particle size of 10 µm was used. The flow rate was 1mL/min, using 55% (v/v) acetonitrile diluted in Milli-Q water as mobile phase.

Standard curve of hexazinone and diuron

A standard curve was built up using 20 µL of the hexazinone and diuron standards solutions at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/mL concentrations using HPLC.

Quantification of bioremediation

The *in vitro* bioremediation in aqueous solution was performed in 500 mL Erlenmeyer flasks containing 200 mL of solution, in which EM-4 (500 cells/mL) was added in the solution of Velpar K® (6.25g/L) as the protocol of Table 1 under agitation of 100 rpm. During 21 days, samples of 10 mL were taken as shown in Table 1, which were analyzed by HPLC after dilution between 25 and 100 times with Milli-Q water.

Table 1 - Proportions and dilution factor of aqueous solutions of K Velpar®(6.25g/L):EM-4(1:1000), for quantification by HPLC of the bioremediation of compounds diuron and hexazinone. (Volume total =200mL).

Ratio	A	B	C	D	E
Velpar K ®/EM-4	1/0	1/ 1	2/ 1	9/ 1	0/1
Dilution factor	1.0	2.0	1.5	1.1	1.0

RESULTS AND DISCUSSION

Biodegradation of Velpar K® by EM-4

The counting of colony forming unit per milliliter (CFU/mL) in plates A, B, C and D after the 5th day of cultivation showed significant increase in the number of cells present for all concentrations used with Velpar K®, compared to the control (agar:EM-4) of the plate (a). This indicated that Velpar K® was used as the only source of energy and carbon by the EM-4 microorganisms, as shown in Table 2.

Standardization curve of the HPLC of diuron and hexazinone.

A calibration curve was made analysing 20 µL of solutions of the standards of diuron and hexazinone at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/mL concentrations by (HPLC. Results are shown in Table 3.

Table 2 - Quantification of colony forming units per milliliter (CFU/mL) the EM-4 growth with or without the Velpar K® presence.

Sample	CFU/mL
Agar : EM-4	$2.3 \times 10^3 \pm 0.4 \times 10^3$
Agar:EM-4:Velpar ®	$1.4 \times 10^5 \pm 0.2 \times 10^5$

Table 3 - Areas of the diuron and hexazinone chromatograms, which indicated the concentrations curve of the standards by HPLC.

Concentration (mg/L)	Area Unit (cm^2)	
	Diuron	Hexazinone
0.5	46235	24791
1.0	87684	46663
1.5	132988	70571
2.0	185304	99040
2.5	219082	117082
3.0	267390	142817

The method of linear regression was applied for the equation of the straight calibration standards of diuron and hexazinone, indicating a correlation between the concentrations injected, as shown in Figure 1. The values of the factor of linear regression (R-diuron = 0.99903, and R-hexazinone = 0.99895) allowed to quantify the diuron and hexazinone in the aqueous solution of Velpar K®. Figures 2 and 3 show the bioremediation dynamics with EM-4 (500 cells/mL) of the Velpar K® active principles during 21 days. The bioremediation for the ratio of the Velpar K®: EM-4 solution from 2:1 in the first three days reached 42% for diuron and 29% for hexazinone.

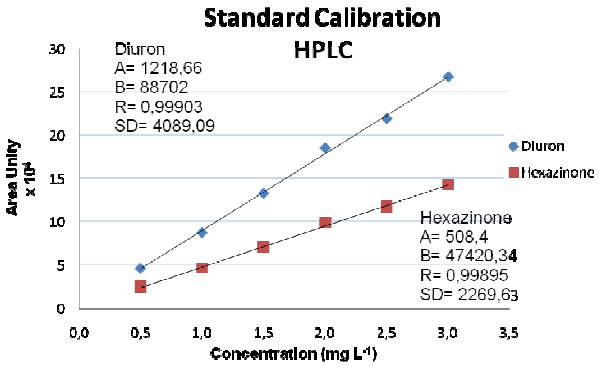

Figure 1 - HPLC calibration curve of standard solutions of diuron and hexazinone.

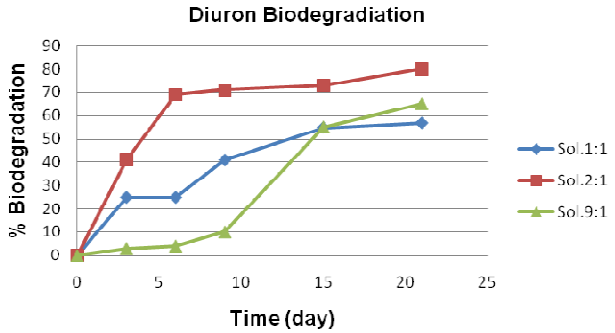

Figure 2 - Bioremediation of diuron by EM-4 during 21 days at 25°C.

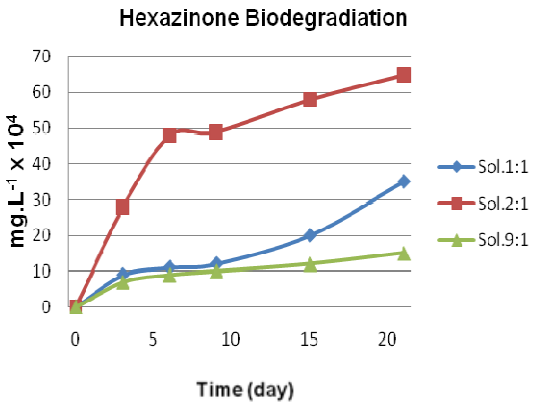

Figure 3 - Bioremediation of hexazinone by EM-4 during 21 days at 25°C.

Figures 2 and 3 showed that diuron and hexazinone could be bioremediated, and that the proportion of the EM-4 had an influence on the kinetics of bioremediation. The application of EM-4 in water contaminated with diuron and hexazinone, highlighted the possibility that the mixed culture of EM-4, was able to degrade diuron and hexazinone.

Dellamatrice et al (2001), studying the degradation of ^{14}C-diuron, showed that *Actinobacter baumannii*, D12 and 12 and other soil microorganisms degraded the diuron. The degradation was more efficient when the organism had historical presence of the herbicide to reach these conditions up to 27% of mineralization, with about $2x10^6$ CFU/g soil. The authors found that in soils with a history of application, the average life of diuron was 72 days, while in soils with no history of application it was 444 days. Considering the results in Figures 4 and 5, it could be concluded that EM-4 supported the degradation of the herbicide, reaching the values of 80% for diuron 70% for hexazinone after 21 days. These results could be useful for planning the bioremediation of contaminated areas, or when applying this herbicide on the crops.

CONCLUSIONS

The results obtained in this work showed that it was possible to use the EM-4 for the bioremediation of contaminated areas (water or soil), using Velpar K® herbicide for sugar cane culture.

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