LIQUID CULTURE FOR ISOLATING MICROORGANISMS WITH POTENTIAL TO DEGRADE METHYL PARATHION FROM AGRICULTURAL SOILS AND ORGANIC WASTE*

L. R. Botero**; N. Nagles***; E. Barriuso****; G. A. Peñuela*****

Recibido: 18/04/2012
Aceptado: 05/10/2012

ABSTRACT

The search for microorganisms with high capacity for pesticide degradation is a very interesting attempt to approach bioremediation strategies in order to prevent contamination. This study evaluates the potential of agricultural soils and solid organic waste cultures microbial isolation as a strategy for screening microorganisms with potential for Methyl parathion (MP) degradation. For the study, microbial consortia were recovered from the liquid fraction of suspension cultures of agricultural soils and solid organic waste. Then, the MP degradation ability and its toxic effect on microbial activity were determined during microbial incubations under laboratory controlled conditions. The results showed that it is not possible to recover the active microorganisms that have potential to degrade the MP from the agricultural soils. However, an active consortium that would degrade both MP and its degradation products was isolated from the organic solid waste.

Key words: Methyl parathion, organophosphate, bioremediation, liquid culture, microbial isolation

* Article product of the research project of PhD thesis of the first author.

** Liliana Rocío Botero: Biologist, M.Sc in Biology, Research Professor. Coordinator of the Biodiversity, Biotechnology and Bioengineering Research Group (GRINBIO). Universidad de Medellín, Carrera 87 No. 30 - 65, Laboratories Center, Bloque 3-202, Medellín - Colombia. Tel: 057- 3405675, lbotero@udem.edu.co.

*** Nelson Nagles: Environmental Engineer, M.Sc. Group of Pollution Diagnosis and Control (GDCON). University Research Headquarters, Universidad de Antioquia, Calle 62 No. 52 - 59, Laboratorio 232, Medellín - Colombia, Tel: 057- 2196570. nnagles@udea.edu.co (Colombia).

**** Enrique Barriuso: Chemist, Ph.D. in Earth and Soil Sciences. Research Director of the National Institute of Agronomic Research I.N.R.A - AgroParisTech, Unité "Environnement et Grandes Cultures". 78850 Thiverval-Grignon. barriuso@grignon.inra.fr (France).

***** Gustavo Antonio Peñuela: Chemist, Ph.D. in Chemistry, Research Professor. Coordinator of the Group of Pollution Diagnosis and Control (GDCON). University Research Head quarters, Universidad de Antioquia, Calle 62 No. 52 - 59, Laboratory 232, Medellín - Colombia, Tel: 057- 2196570. gpenuela@udea.edu.co

CULTIVO LÍQUIDO PARA AISLAR MICROORGANISMOS CON POTENCIAL PARA DEGRADAR METIL PARATIÓN A PARTIR DE SUELOS AGRÍCOLAS Y RESIDUOS ORGÁNICOS

RESUMEN

La búsqueda de microorganismos con alta capacidad de degradación de los plaguicidas es un intento muy interesante de acercarse a las estrategias de biorremediación con el fin de evitar la contaminación. Este estudio evalúa el potencial del cultivo de microorganismos de suelos agrícolas y de residuos sólidos orgánicos como una estrategia para la detección de microorganismos con potencial para el metil paratión (MP) la degradación. Para el estudio, consorcios microbianos fueron recuperados de la fracción líquida de los cultivos en suspensión de los suelos agrícolas y los residuos sólidos orgánicos. La capacidad de degradación de la MP y su efecto tóxico sobre la actividad microbiana se determinó durante las incubaciones de los microorganismos en condiciones controladas de laboratorio. Los resultados mostraron que no es posible recuperar los microorganismos activos que tienen potencial para degradar el MP de los suelos agrícolas. Sin embargo, un consorcio activo que degradaría tanto MP y como sus productos de degradación se aisló a partir de los residuos sólidos orgánicos.

Palabras clave: Metil paratión, organofósforo, biorremediation, cultivo líquido, aislamiento microbiano.

INTRODUCTION

Methyl parathion (MP) is an organophosphate pesticide widely used in agriculture to control insects [1], but its use is now restricted. Organophosphate pesticides represent 38% of the pesticides used in the world [2]. These compounds have an inhibitory effect of acetyl cholinesterase that is required for the normal operation of the nervous system [1, 3]. The hydrolysis of the phosphodiester bond drastically affects the toxicity of organophosphate pesticides, thus being an important step for their detoxification [4, 5]. In the case of MP, this transformation leads to the generation of p-nitrophenol (PN) [6]. This hydrolysis, and most of the degradation process of the organophosphate pesticides, has a microbiological origin. The microbial ability to degrade organophosphate pesticides has proven to be a genetically preserved character [7] that facilitates the pesticide degradation, solving problems of soil contamination [8-11].

The capacity of agricultural soils to degrade organophosphate pesticides such as the MP has been widely reported [7, 12, 13], and it allows the implementation of bio-augmentation plans for bio-remediation of contaminated soils [14]. This strategy assumes that the frequent application of the same pesticides induces an increase on the native fungi and bacteria that are adapted to the pesticide, thus, being suitable to be used as a carbon and energy source. These microorganisms naturally do the degradation of soil contaminants [9, 10, 15]. Moreover, the organic pollutant degradation of microorganisms coming from the transformation of organic solid waste has been also reported [16]. Thus, the process of organic material degradation from municipal waste promotes the development of microorganisms with a variety of enzymatic capabilities, which promotes degradation of recalcitrant compounds [14].

Under laboratory conditions, the use of pesticides as a source of carbon and energy aims to select populations capable of degrading recalcitrant compounds [17, 18]; this strategy has been widely reported in the case of organophosphate pesticides [18, 19]. The laboratory enrichments induce the proliferation of degrading microorganisms; therefore, allowing their isolation [14]. Water extraction and liquid cultures have been the most common strategies to extract microorganisms from the solid samples and to conduct enrichment experiments [19].

This study evaluated the potential of the liquid culture enriched with MP as a strategy for preselecting microorganisms and consortiums with MP degrading potential. Two sources of microorganisms were studied: agricultural soils with MP degrading potential, due to the fact that they were periodically treated with organophosphate pesticides and an organic solid residue from fruit wastes.

1 METHODOLOGY

Chemical Products: Microbial culture media were made with NaCl, KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4$, $ZnSO_4 \cdot 7H_2O$, $MgCl_2 \cdot 4H_2O$,boric acid; $CoCl_2 \cdot 6H_2O$, $CuSO_4$, $NiCl_2$,$FeSO_4$, EDTA, glucose and analytical grade yeast extract (Merck). For the MP degradation studies, a commercial concentrated emulsifiable liquid of MP (480 g L^{-1}; Methion 48 EC; from the company Agroser SA) was used. MP and PN with analytical standards (99.5% purity; Chem Service SA company), and acetonitrile (HPLC grade; Mallenckrodt) and water MilliQ (as solvents) were used for the high performance liquid chromatography (HPLC) analysis.

Microbial sources: three agricultural soil samples that were in presence of organophosphate pesticides were selected due to the presence of microorganisms with MP degradation capacities of over 80% after 5 days of incubation at 28°C (unpublished results). Two soil samples were collected in the state of Antioquia, Colombia (one obtained from a strawberry plantation and the other from a export-quality flower field both routinely sprayed with malathion and chlorpyrifos organophosphorated pesticides). An additional soil sample frequently fumigated with MP was obtained from an extensive cotton plantation (state of Córdoba,

Colombia). The samples were collected at 10 cm of soil depth, and were later put through a 2-mm sieve, mixed in equal proportion and preserved at 4°C until the time of testing. The solid organic waste consisted of a mixture of fruit waste (mango, papaya, pineapple and bananas) in equal fresh weight proportions.

To determine the total microorganism content, the samples (agricultural soil mixture and organic solid waste) were serially diluted in a buffered saline solution prepared with 8 g L^{-1}NaCl, 1.21 g L^{-1} of K_2HPO_4 and 0.34 g L^{-1} KH_2PO_4, and cultured in an agar dilution plate count. The cultures were maintained in the darkness at 28°C for 7 days; then, the number of colony forming units per gram of soil (CFU g^{-1}) was determined. The Walkley-Black wet oxidation method (OM) [20] was used to determine the organic matter of the agricultural soil mixture and the organic solid waste of the essays.

Liquid pre-culture: A suspension pre-culture was prepared for the liquid microbial growing activation from the soil and solid organic waste, the soil pre-culture was prepared using 200g of the agricultural soil mixture and adjusting the final volume to 1 L with a salt solution enriched with 1.5 g L^{-1} de K_2HPO_4, 0.5 g L^{-1} de KH_2PO_4,1 mg L^{-1} de $(NH_4)_2SO_4$, 0.21 g $L^{-1}$of $MgSO_4{\cdot}7H_2O$ and 0.5 g L^{-1} of yeast extract. For the solid organic waste suspension pre-culture preparation, 200 g of fruit waste was subjected to a liquid pre-culture process adjusting the final volume to 1L. The volume was adjusted with a salt solution similar to the one used for the soil pre-culture but without yeast extract. Both pre-cultures were placed in 10L glass containers and maintained under horizontal stirring (100 rpm) under laboratory conditions 25±2°C for 4 days.

In order to obtain the microbial consortia present in the liquid fraction of the pre-cultures after the incubation time, the agitation was stopped for 16 hours and the supernatant fluids were transferred to sterile containers glass by suction.

Media and culture conditions: microbial culture media were prepared with NaCl, KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4$, $ZnSO_4{\cdot}7H_2O$, $MgCl_2{\cdot}4H_2O$, boric acid; $CoCl_2{\cdot}6H_2O$, $CuSO_4$, $NiCl_2$,$FeSO_4$, EDTA, glucose and analytical grade yeast extract (Merck). 250 mL of this microbial suspension were placed in 750 mL glass containers of sealed amber screw cap. For the contaminated cultures, suspension was enriched with 20 mg L^{-1} of MP (Methion 48 EC). Uncontaminated microbial suspensions were used as controls. The cultures were maintained on horizontal shakers at 100 rpm under laboratory conditions at 25±2°C for 6 days. The tests were performed in triplicate.

Determination of toxic effect: the total respiration estimated through the kinetics of mineralization was used to determine the effect of the MP on the microbiological activity. The carbon dioxide (CO_2) generated was measured by depositing a 25 mL glass jar with 10mL of 0.8N NaOH solution in the interior of each culture vessel. The cultures were subjected to processes of aeration and the NaOH solution was replaced daily. The determination of $CO_2$was carried out by titration of NaOH with HCl (0.4N) after the addition of $BaCl_2$ to saturate, and 1% phenolphthalein as a pH indicator. The milligrams of CO_2 produced per mL of liquid culture of agricultural soil mixture or soil organic waste were calculated daily by means of the following equation.

$$mgCO_2 = \frac{(Vb - Vm) \times N_{HCl} \times 22.005}{M} \qquad (1)$$

Where: Vbis the volume of HCl used for the titration of the blank (mL);Vm is the volume of HCl used for sample titration (mL);NHC lis the normality of HCl; and M is the sample volume (mL).

Methyl-parathion degradation assessment: aliquots of10 mL of the culture were sampled on days 0, 1, 2, 3, 5 and 6 and stored at-4°C to monitor the MP degradation process. For performing the analysis, samples were thawed and subjected to

centrifugation procedures (1000 rpm, 30min) to remove the cell pellet. Finally, the liquid fraction obtained was filtered through a nylon membrane (45µm). The analysis of the samples was performed through a high performance liquid chromatography (HPLC), using a liquid high-resolution chromatograph (Agilent Technologies, model 1100, equipped with a G1311A quaternary pump), a diode array detector G1315B DAD, an automatic injector equipped with an injection loop of 20µl, and a SB-C-18 reversed phase column Zorbax (150 X 4.6 min diameter and 5µm particle size). The mobile phase used was a mixture of acetonitrile and water with the following gradient change times: 3, 5, 9, 15 and 16 minutes, and the following proportions of acetonitrile 0.5, 70, 70, 80 and 0.5. The flow rate was 1mL min^{-1}. The analysis time was 16 minutes. Standard curves for chromatographic analysis were done with concentrations of 0.1, 5, 10, 20 and 50 mg L^{-1} of MP (274nm), and 0.5, 1, 2, 2.5, 5 mg L^{-1} for the NP (320nm). The chromatographic data were processed using the ChemStation software, version A.10.02.

Statistical Analysis: Data normality verification was performed, regression analysis and calibrations were done to explain the variation of the compound and the CO_2 generated by the microbial activity, the intercept of the models was removed in cases where the p value of the intercept was greater than 0.05. Multivariate analyses were performed using the Pearson correlation test to determine the correlation between the change of the detected compounds and their dependence of the time culture. Multivariate analyses were performed using the Pearson correlations tests to determine the correlation between the evolved concentrations (mgL^{-1}) of the compounds (MP and PN) found by HPLC versus the culture time. Some unidentified degradation product (UDP) analyses were included in this study; for them, the variation of the maximum absorbance (muA) was used. In order to graphically visualize the evolution of the different compounds during the culture, the concentrations were normalized using the *C/ Cmax* relationship, where C is the concentration of a compound at a given time and *Cmax* is the maximal concentration found during the experiment. Finally, multiple regression analyses were performed to obtain the general model equations that predicted MP and PN time variations.

2 RESULTS AND DISCUSSION

Soil and solid organic waste initial evaluation: big differences of organic matter (4.4% and 29%) and microorganisms (4.40 $x10^5$ and 5.60 x 10^{11} CFU g^1) for the soil agricultural mixture and solid organic waste were found for the suspension pre-cultures. As it was expected, the differences affected the forecasted microbial activity and development in the liquid fraction of the suspension pre-culture.

Total biological activity and toxicity effects of methyl-parathion: the implementation of the regression analysis allowed the generation of highly reliable models (R^2>97%; p=0.0001) to determine and predict the activity of microbial consortia that were recovered from the liquid fraction of the pre-cultures in the suspension of organic waste residues and agricultural soils (Table 1). Additionally, these models allowed comparing the activity of the cultures exposed to MP with the ones that were

Table 1. Models of CO_2 variation according to the time (t), R2 and p values for the different essays: soil control (SC), solid organic waste (SOW), Solid organic waste/ contaminated with MP (SOW+MP), Soil contaminated with MP (S+MP)

Essay	*SC*	*SOW*	*SOW+MP*	*S+MP*
R2	97%	97%	97%	97%
p	0.0001	0.0001	0.0001	0.0001
Model	CO_2(mg)=18.9*sqrt(t)	CO_2(mg)=10.1*t	CO_2(mg)= 9.2*t	CO_2(mg)=7.2*t

Source: the authors

used as control without exposure to MP, in order to determine the toxic effect of this compound on consortia for both types of cultures.

The total respiration of the consortium in liquid cultures expressed as the cumulated mineralization (Figure 1) showed an exponential mineralization rate starting at 18.9 mgd^{-1} of CO_2.while for the consortia from soil cultures that were exposed to MP, the mineralization kinetics is linear, with a slope of 7.2 $mgd^{-1}$of CO_2. The mineralization kinetics of the consortia extracted from the organic waste was also linear, with mineralization rates of 10.14 and 9.2 mgd^{-1} of CO_2 for the control and MP exposed cultures, accordingly.

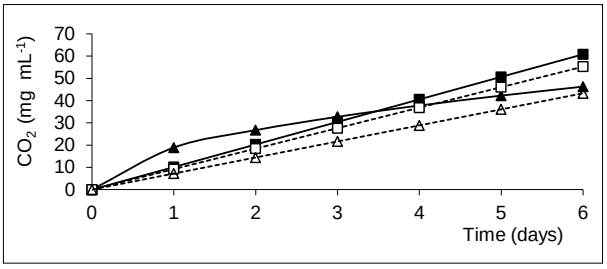

Figure 1. Kinetics of mineralization for microbial consortia extracted from the liquid fractions of the pre-cultures of solid organic waste and agricultural soils cultured under liquid conditions. Consortia fromsoil control not exposed to MP (▲), soil exposed to MP (Δ), solid organic waste not exposed to MP (■), solid organic waste exposed to MP (□).

Source: the authors

In the initial stage, the rate of mineralization of consortia of soil cultures was even higher than these obtained from organic solid waste cultures under non contaminated control conditions (Figure 1). The mineralization rate of consortia of soil cultures decreased on the second day of incubation, probably because the depletion of the nutrients, which had been quickly consumed. Mineralization rates showed that microbial activity caused a decrease of the MP consortia derived from agricultural soils to the value of 7.2 mgd^{-1}, the lowest CO_2 obtained in all trials, which was in accordance to the expectations since the toxic effect of MP on soil microorganisms has been widely reported [6, 21-24]. It is possible that nutrients were utilized more slowly in cultures enriched with MP, allowing a constant utilization and thus, obtaining constant mineralization rates without evidence of depletion of nutrients 7 days after starting the culture, as it was presented in uncontaminated control cultures.

MP toxicity was also evident on the slopes that showed a decrease from 10.14 to 9.2 mg d^{-1} of CO_2 for the microorganisms recovered from the liquid fraction of solid organic waste. It is likely that the nutrient content available is enough for the maintenance and development of microbial activities for the associations of organic solid residues. As it was expected, the final value obtained for soil consortia showed a lower mineralization than the one for waste consortia, as soil nutrients are more limited and quickly exhausted (4.4% compared with 29%, respectively, as it was detected in the raw materials).

Degradation: the HPLC analysis allowed the identification of the PN and another 2 possible unidentified degradation products (UDP) of MP: they were named according to their time retention: UDP_3 (5.2 min; 320 nm) and UDP_4 (6.3 min, 274 nm).

The *C/Cmax* analysis (Figures 2 and 3)considered *Cmax* to be the maximum muA values detected for the compounds when they were at their maximum level during the culture time: MP (1420 muA; 0 days of culture), PN (776 muA, 0 days of culture, detected to be as degradation product of the commercial product). The *Cmax* of UDP_3 (259 muA; 5 days of culture) and UDP_4 (352 muA; 6 days of culture) were included only after microbial consortia activity for organic solid waste because they were not detected for solid organic waste. It can be observed that the most important activity of degradation in liquid cultures inoculated with microorganisms recovered from the liquid fraction of solid organic waste occurred in day 1. MP and PN are present in the culture for 3-5 days until they begin to degrade. UDP_3 and UDP_4 appear as a result of the advanced degradation of the MP.

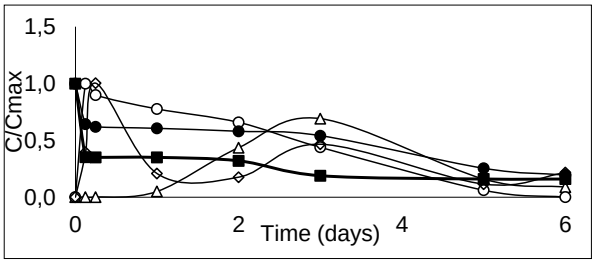

Figure 2. Variation in *C/Cmax* of the different compounds detected in the liquid culture obtained from the microbial consortia solid organic waste. Symbols: (■) MP, (●) PN, (Δ) UDP_3, (O) UDP_4, (◊) UDP_5.

Source: the authors

For the liquid culture obtained from the microbial consortia of agricultural soils mixture (Figure 3) *C/Cmax* analysis showed that MP remained almost unchanged during the incubation; however, the PN that was initially present in the commercial product of MP was degraded after three days. It clearly shows the simultaneous degradation of PN, and how the MP remains unchanged during the time of cultivation.

The microbial consortium recovered in the liquid fraction of the organic solid waste showed a degradation of the MP activity that was higher than the ones obtained from the preselected agricultural soils mixture. The degradation of both the MP and the PN that was observed in the cultures inoculated with the consortium obtained from organic solid waste (Figure2) suggests that the microbial consortia that was extracted from the aqueous phase of pre-cultivation of solid organic waste has a wide enzymatic range, which allows to efficiently degrade both compounds. There are several alternative explanations for the observed degradation capacity in the consortium that was recovered from solid organic waste. Microbial diversity enables co-metabolic degradation processes [4]. However, the presence of microorganisms capable of carrying out simultaneous degradation of both compounds is also possible, as it has been reported by other authors [11].

The accumulation and subsequent degradation of the UDP_3 was possibly submitted, as a result of the activation of enzymatic systems [10]. These results indicate that microbial consortia in the aqueous fraction that was obtained from the pre-culture liquid of the solid organic waste have the ability to degrade not only the MP and NP but also other MP degradation products, increasing the process efficiency and reducing the risk of accumulation of other toxic sub-products [25]. These results support the possibility of implementing this type of pre-cultures as an alternative to increase MP degradation in contaminated soils. This proposal is similar to the one reported to degrade PN using the sludge treatment plant waste water [26].

A direct correlation was found between the change in the concentration of MP and PN (constant 1.83688 mg L^{-1} and 1.83684 mg L^{-1}, respectively) in the statistical analyses of MP degradation as a result of microbial activity of the consortia obtained from solid organic waste; however, no correlation was detected regarding the culture time for the MP or for the PN.

In the case of the consortia degradation activity recovered from the liquid fraction of agricultural soils, the most decisive factor was the lack of activity of the microorganisms on the MP (Figure 3), as well as the fact that its concentration remained unchanged during the 7 days of cultivation. The findings of these studies contrast the findings reported by several authors who implemented the liquid culture of solid substrates (such as agricultural soils) as a strategy for screening microorganisms with potential to degrade the pesticide [7, 12, 13]. These results show that this strategy is not always feasible, and that the active microorganisms in the degradation process can grow only if they are attached to the solid fraction, which consequently leads to the fact that they cannot be separated and isolated by means of liquid culture conventional techniques or direct isolation techniques using serial dilutions and subsequent dumped plating [7, 12, 13]. This study also showed that the microorganisms responsible for the degradation of the NP, the main known product of degradation of

the MP and other organophosphate [2, 18] are not necessarily the same microorganisms responsible for the MP degradation on the agricultural soils that remained attached to the solid matrix and failed to be available in the liquid fraction. These results show that the PN degradation capacity is not always associated to the MP as it has been reported by some authors who found a strong genetic linkage between *opd* genes (associated with the degradation of MP) and *orf* genes (associated with the degradation of PN) [27]. The results support the fact that recalcitrant organophosphate pesticides such as MP, mainly by phosphorus-ester bond and the aromatic ring structure, make the biodegradation process more difficult [2, 3, 10].

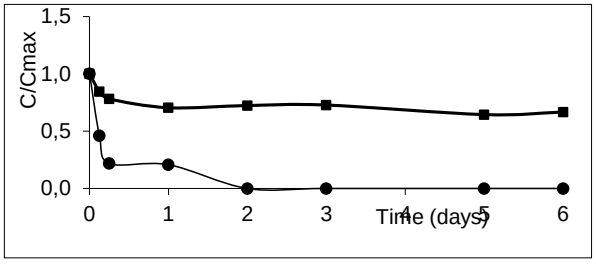

Figure 3. Variation in *C/Cmax* of the different compounds detected in the liquid culture obtained from the microbial consortia of agricultural soils exposed to MP. Symbols: (▪) MP; (•) PN.

Source: the authors

The comparative analysis of the compounds variation performed in liquid cultures enriched with MP (Figure 4a) showed that the microorganisms that have a potential to degrade the MP could only be recovered in the liquid fraction of solid organic waste and not in the agricultural soils. The results of NP degradation (Figure 4b),as a result of microbial activity in the liquid fraction obtained from both types of culture, showed a similar pattern, being slightly more efficient for the degradation in the initial stage PN consortia that were obtained from soil samples extracted from solid organic waste. According to the results, although the time for NP degradation is two times the amount time for MP degradation, the conversion of MP to NP is the crucial step to achieve detoxification.

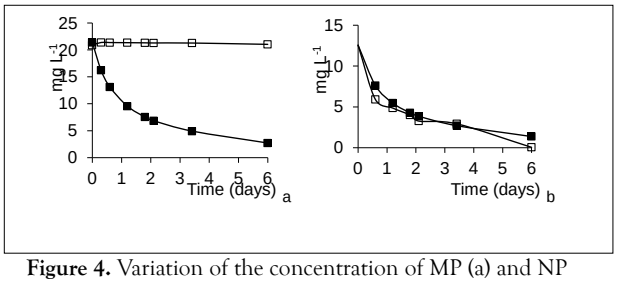

Figure 4. Variation of the concentration of MP (a) and NP (b) in liquid cultures inoculated with microbial consortia recovered from the liquid fraction from suspension pre-cultures of soil agricultural (•) or solid organic waste (□).

Source: the authors

3 CONCLUSIONS

It is feasible to recover microorganisms that have a potential for degrading MP in the liquid phase of the pre-cultures of solid organic waste. The application of the liquid fractions obtained after a suspension culture of solid organic waste on the soils that have been contaminated with MP could be evaluated under farm conditions. It could be a good inexpensive alternative of bio-stimulation and bio-augmentation to increase the efficiency of native soil microorganisms and accelerate MP degradation. It is not feasible for agricultural soils under the pre-culture liquid conditions described here, which precludes this approach for screening and isolation.

4 ACKNOWLEDGEMENTS

The authors would like to thank Universidad de Antioquia and Universidad de Medellín for their support regarding personnel, facilities and equipment. The authors would also like to express their gratitude to the Alfa-Caribiotec Project (European Community), COLCIENCIAS, Universidad de Medellín and Universidad de Antioquia for their financial support.

REFERENCES

[1] F. L. Edwards and P. B. Tchounwou, "Environmental Toxicology and Health Effects Associated with Methyl Parathion Exposure - A Scientific Review " *International Journal of Environmental. Research in Public Health*, vol. 2, pp. 430-441, 2005.

[2] B. K. Singh and A. Walker, "Microbial degradation of organophosphorus compounds. ," *FEMS Microbiology Reviews,* vol. 30, pp. 428-471 2006.

[3] F. Worek, *et al.*, "Inhibition, reactivation and aging kinetics of highly toxic organophosphorus compounds: Pig versus minipig acetylcholinesterase," *Toxicology,* vol. 244, pp. 35-41, 2008.

[4] D. G. Karpouzas and B. K. Singh, "Microbial Degradation of Organophosphorus Xenobiotics: Metabolic Pathways and Molecular Basis," in *Advances in Microbial Physiology*. vol. Volume 51, K. P. Robert, Ed., ed: Academic Press, 2006, pp. 119-225.

[5] I. Horne, *et al.*, "dentification of an opd (organophosphate degradation) gene in an Agrobacterium isolate," *Applied Environmental Microbiology* vol. 68, pp. 3371-3376., 2002.

[6] X. Qiu, *et al.*, ""Biodegradation of p-nitrophenol by methyl parathion-degrading Ochrobactrum sp. B2."," *International Biodeterioration and Biodegradation,* vol. 59, pp. 297-301, 2007.

[7] R. Zhang, *et al.*, "Diversity of organophosphorus pesticide-degrading bacteria in a polluted soil and conservation of their organophosphorus hydrolase genes.," *Canadian Journal of Microbiology,* vol. 51, pp. 337-343, 2005.

[8] K. Ohshiro, *et al.*, "Biodegradation of organophosphorus insecticides by bacteria isolated from turf green soil," *Journal of Fermentation and Bioengineering,* vol. 82, pp. 299-305, 1996.

[9] B. K. Singh, *et al.*, "Cross-enhancement of accelerated biodegradation of organophosphorus compounds in soils: Dependence on structural similarity of compounds," *Soil Biology and Biochemistry,* vol. 37, pp. 1675-1682, 2005.

[10] D. G. Karpouzas, *et al.*, "Non-specific biodegradation of the organophosphorus pesticides, cadusafos and ethoprophos, by two bacterial isolates," *FEMS Microbiology Ecology,* vol. 53, pp. 369-378, 2005.

[11] Z. Arbeli and C. L. Fuentes, "Accelerated biodegradation of pesticides: An overview of the phenomenon, its basis and possible solutions; and a discussion on the tropical dimension," *Crop Protection,* vol. 26, pp. 1733-1746, 2007.

[12] C. Sreenivasulu and Y. Aparna, "Bioremediation of Methylparathion by Free and Immobilized Cells of Bacillus sp . Isolated from Soil " *Bulletin of Environmental Contamination and Toxicology,* vol. 67, pp. 98-105, 2001.

[13] P. S. Suresh, *et al.*, "An Insilco approach to bioremediation: Laccase as a case study," *Journal of Molecular Graphics and Modelling,* vol. 26, pp. 845-849, 2008.

[14] M. Megharaj, *et al.*, "Bioremediation approaches for organic pollutants: A critical perspective," *Environment International,* vol. 37, pp. 1362-1375, 2011.

[15] R. M. Atlas, *et al.*, "Response of microbial populations to environmental disturbance," *Microbial Ecology,* vol. 22, pp. 249-256, 1991.

[16] Q.-Y. Cai, *et al.*, "Bioremediation of polycyclic aromatic hydrocarbons (PAHs)-contaminated sewage sludge by different composting processes," *Journal of Hazardous Materials,* vol. 142, pp. 535-542, 2007.

[17] M. L. Ortiz , *et al.*, "Biodegradation of methyl-parathion by bacteria isolated of agricultural soil. ," *Revista Internacional de Contaminacion Ambiental* vol. 17, pp. 147-155, 2001.

[18] X. Qiua, *et al.*, "Biodegradation of p-nitrophenol by methyl parathion-degrading Ochrobactrum sp. B2," *International Biodeterioration and Biodegradation, vol. 59, pp. , 2007.*, vol. 59, pp. 297-301, 2007.

[19] M. Cycoń, *et al.*, "Biodegradation of the organophosphorus insecticide diazinon by Serratia sp. and Pseudomonas sp. and their use in bioremediation of contaminated soil," *Chemosphere,* vol. 76, pp. 494-501, 2009.

[20] D. Carreira, "Carbono oxidable. Una forma de medir la materia orgánica del suelo," in *Tecnologías en análisis de suelos*, M. S. R. asociación, Ed., ed: Asociación Argentina de la Ciencia del Suelo, 2005, pp. 91-102.

[21] M. Ortiz, *et al.*, "Biodegradation of methyl-parathion by bacteria isolated of agricultural soil," *Revista Internacional de Contaminacion Ambiental* vol. 17, pp. 147-155, 2001.

[22] I. Horne, *et al.*, "dentification of an opd (organophosphate degradation) gene in an Agrobacterium isolate. A," *Applied Environmental Microbiology* vol. 68, pp. 3371-3376., 2002.

[23] F. Edwards and P. Tchounwou, "Environmental Toxicology and Health Effects Associated with Methyl Parathion Exposure - A Scientific Review," *International Journal of Environmental. Research in Public Health* vol. 2, pp. 430-441, 2005.

[24] R. Zhang, *et al.*, "Diversity of organophosphorus pesticide-degrading bacteria in a polluted soil and conservation of their organophosphorus hydrolase genes," *Canadian Journal of Microbiology,* vol. 51, pp. 337-343, 2005.

[25] E. A. Lock, *et al.*, "Studies on the mechanism of 4-aminophenol-induced toxicity to renal proximal tubules," *Human and Experimental Toxicology*, vol. 12:, pp. 383-388., 1993.

[26] F. O. Sagban, "Impacts of wastewater sludge amendments in restoring nitrogen cycle in p-nitrophenol contaminated soil.," *Journal of Environmental Science (China)*, vol. 23, pp. 616-623., 2011.

[27] D. Siddavattam, *et al.*, "Transposon-Like Organization of the Plasmid-Borne Organophosphate Degradation (opd) Gene Cluster Found in Flavobacterium sp.," *Applied and Environmental Microbiology*, vol. 69, pp. 2533-2539, 2003.