Bioremediation of domestic water polluted by benzene of waste motor oil with crude extract of *Pleurotus Florida* and mineral solution

Bioremedição de água doméstica poluída por benzeno de óleo residual de motor com extrato bruto de *Pleurotus Florida* e solução mineral

Bio-mediación de agua doméstica contaminada con benceno de aceite residual automotriz con extracto crudo de *Pleurotus Florida* y solución mineral

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ABSTRACT
In Mexico and in the world, a problem of contamination in domestic water is due to petroleum derivatives such as waste motor oil (WMO), whose chemical composition is aliphatic and aromatic hydrocarbons. An ecological solution is to biostimulation with extracellular enzymes of *Pleurotus florida* and mineral solution to eliminate both by the aerobic native heterotrophic microbiota. The objective of this research was to analyze the bioremediation of domestic water polluted by benzene of WMO with an extract of *P. florida* and mineral solution. In that sense, *P. florida* was grown in straw with lignin content, that induces enzymes to hydrolyze benzene for 5 days, for obtaining crude extract of *P. florida* (*ePf*). This *ePf* used for the biostimulation of domestic water polluted by benzene from WMO and a mineral solution: to oxidate the benzene that was measured by CO$_2$ generated from its mineralization and mass-coupled gas chromatography to demonstrate its elimination. The results indicate that in biostimulation of domestic water polluted by benzene from WMO with *ePf*, caused its hydrolysis followed by the mineral solution that eliminated it. Compared to domestic water polluted by benzene from WMO non biostimulated by *ePf* neither mineral solution where benzene did not disappear. This data supports biostimulation domestic water polluted by benzene from WMO with *ePf* and mineral solution for its rapid reutilization.

Keywords: water, hydrocarbons, environmental contamination, aromatic extracellular enzymes, mineralization.
**Palavras-chave**: água, hidrocarbonetos, contaminação ambiental, enzimas extracelulares aromáticas, mineralização.

**RESUMEN**
En México y el mundo, un problema de contaminación en aguas domésticas se debe a derivados del petróleo como el aceite residual de motor (ORM), cuya composición química está compuesta por hidrocarburos alifáticos y aromáticos. Una solución ecológica es la bioestimulación con enzimas extracelulares de Pleurotus florida y una solución mineral para su eliminación por microbiota heterótrofa nativa aeróbica. El objetivo de esta investigación fue analizar la biorremediación de aguas domésticas contaminadas con benceno ORM con un extracto de P. florida y una solución mineral. En este sentido, P. florida se cultivó en paja con contenido de lignina, la cual induce a las enzimas a hidrolizar benceno durante 5 días, para obtener extracto crudo de P. florida (ePf). Este ePf se utiliza para la bioestimulación de agua doméstica contaminada por ORM benceno y una solución mineral: para oxidar el benceno que fue medido por el CO2 generado a partir de su mineralización y cromatografía de gases acoplada a masa para demostrar su eliminación. Los resultados indican que, en la bioestimulación del agua doméstica contaminada por el benceno de la ORM con Fe, hubo hidrólisis seguida de la solución mineral que la eliminó. En comparación con el agua doméstica contaminada por benceno de ORM no bioestimulada por FEP, ni con la solución mineral donde el benceno no ha desaparecido. Estos datos apoyan la bioestimulación del agua doméstica contaminada con benceno ORM con ePf y solución mineral para su reutilización rápida.

**Palabras clave**: agua, hidrocarburos, contaminación ambiental, enzimas extracelulares aromáticas, mineralización.

**1 INTRODUCTION**

Environmental pollution related to petrochemical products is recognized as one of the most serious problems in domestic water, groundwater and superficial and other water bodies. In México, the annual production of waste motor oil (WMO) is approximately 325 million liters (1-3). It is estimated that only 20% of the generated volume under goes proper final treatment. The composition of WMO is aliphatic with chain lengths ranging from C15 to C50, a broad range of aromatic compounds, such as benzene (2,3), higher percentages of polycyclic aromatic hydrocarbons (PAHs), WMO may also contains more additives compared to fresh oil and the concentration of PAHs may range from 34 to 190 times higher than those in fresh motor oil (4-6) minor quantities of gasoline, additives, detergents, viscosity improvers, oxidation and/or rust inhibitors, etc. Nitrogen (N) and sulfur (S) compounds, and metals as lead (Pb), zinc (Zn), barium (Ba) and magnesium (Mg). All these contaminants arise from normal wear of engine components and from heating and oxidation of lubricating oil during engine operation. Therefore, WMO as a mixture of aliphatic and aromatic hydrocarbons involves a risk to the human health and
the environment, particularly domestic water (7-9). In WMO on the most common aromatic is benzene is particularly has relatively high-water solubility at 1.8 g/l, 15 ºC, it is easily transferred from spills and leaking storages in domestic water, groundwater and drinking water supplies (8). Benzene from WMO is difficult to remove in those sites because it lacks an activating (O2) oxygen or N substituent group, making the oxidation of the ring not energetically feasible. Long term health effects of benzene exposure include harmful effects on bone marrow and cancer in humans (9-12).

An ecological alternative of solution es bioremediation schemes is investigated for the treatment of domestic water and industrial effluents containing WMO with aliphatic and aromatic hydrocarbons as benzene (13,14). Bioremediation is the most widely used for aliphatic hydrocarbons mineralization by biostimulation of the aerobic native heterotrophic microbial consortium with a mineral solution having in balance basic inorganic compounds such as N, phosphorous (P), potassium (K) and others important minerals for microbial metabolism (2,5,8). However, biostimulation methods are limited by the toxicity of WMO due to benzene and other aromatics and the corresponding low concentrations of the source organic carbon for coometabolism of these hydrocarbons in order to clean polluted environment (3-5). However, most of the studies have focused on microorganisms able to do so, however little is known on the contribution of fungi or its metabolic product to bioremediation of environment polluted by benzene from WMO. Bioremediation due fungal mediated mineralization of soil pollutants as aromatics from WMO are testing with white-rot fungi (16,17). Since it’s demonstrated that a lot of species of fungy belonging to the group of white-rot fungi are capable to degrade lignin, that is a naturally occurring polymer in plants (18,19). Thus, an ecological solution to induce the bioremediation environmental polluted by WMO (8,9) as could be in domestic water is to apply the biostimulation of the native microbiota with essential macronutrients (6,7). The elimination of the aromatic fraction could be with an extracellular enzymatic extract of P. florida (ePf), a basidiomycete that synthetizes: a Laccase, Manganese peroxidase or MnP, and Lignin peroxidase or LiP (17-19). This is an enzymatic complex has not substrate chemical specific to hydrolyze aromatic rings, similar to those in the chemical composition of WMO. The aim of this work was biostimulation of domestic water polluted by benzene of WMO with ePf and mineral solution.
2 MATERIAL AND METHODS

2.1 PLEUROTUS FLORIDA CULTIVATION AND OBTAINING OF ENZYMATIC CRUDE EXTRACT

The fungi *P. florida* was donated by Kamuro Inc., based in Morelia, Michoacán, México. It was cultivated preparing malt extract and incubated at 28 °C for seven days. Thereupon, the fungi were inoculated in a flask containing distilled water and 7.5 g of sterile wheat straw as only source of carbon and energy. The flask was incubated at 28 °C for 14 days according to techniques reported (16-17). At the end of the period, the flask content was centrifuged at 1000 rpm/10 min, the supernatant was filtered using a Millipore membrane, 0.2 µ. The protein concentration was measured using a curve of bovine albumin as standard. The *ePf* was conserved in glycerol at -20 °C until use. 2.2. Biostimulation of crude extract of *P. florida* on benzene hydrolysis from WMO diluted (1:100) in concentration of 11,000 ppm of WMO in distilled water. Immediately, a sample of 10 ml was transferred to a Bartha flask 500 ml capacity, with H$_2$O$_2$ at 2 ppm; MnSO$_4$: 2 mM 1.0 ml and *ePf*: 1mg/ml. All Bartha flasks with 10,000 were incubated at 30 °C (±2°C), 100 rpm for a three-week period. A relative control was 100 ml of domestic water polluted by WMO and not biostimulated with *ePf*. An absolute control was 100 ml of domestic water polluted by 10 ml of diluted WMO, then biostimulated with sterilized *ePf* and sodium azide to inhibit any biological activity (19). All assays were carried out as a triplicate.

Biostimulation of domestic water polluted by benzene from WMO with *ePf* and mineral solution

Six Bartha flasks were used, containing 100 ml of domestic water, WMO diluted 1:100 was biostimulated by 1 and 1 mg/ml of the *ePf* and mineral solution with the following chemical composition (g/l): K$_2$HPO$_4$: 4; MgSO$_4$: 3; NH$_4$NO$_3$: 10; CaCO$_3$: 1; KCl: 2; ZnSO$_4$: 0.5; CuSO$_4$: 0.5; FeSO$_4$: 0.2; EDTA 8.0; tween 20 0.01%; H$_2$O$_2$: 2 ppm; MnSO$_4$: 2 mM. All Bartha flasks were incubated at 30 °C (±2°C), 100 rpm for another three-week period (20-22). The Bartha flasks were incubated under shaking at 100 rpm at room temperature for 18 days. To quantify the generation of CO$_2$ derived from the mineralization of the WMO, due to biostimulation by mineral solution, were taken 10 mL of 0.1 N KOH was added to one of the arms of the Bartha flask, every 24 h/18 day. The remaining alkali was determined by titration with 0.1 N HCl (2,4,7). All experimental data were analyzed by ANOVA and Tukey (P ≤ 0.05), to establish the minimum significant difference. The experiment was carried out as a triplicate.
Analysis of aliphatic hydrocarbons.

Analysis of benzene concentration was carried out using a Gas Chromatograph (Perkin Elmer Autosystem Series) coupled to FID, using an Elite 5 capillary column coated with a 5% diphenyl/95% Dimethyl Polysiloxane stationary phase, 30m length, 0.25 diameter, 0.25 mm film thickness in a split injection mode. The carrier gas was Helium; the column oven temperature was 40º C for 8 min and was increased from 40-180º C at 6º C min-1. The injector temperature was 250º C (19,21).

Experimental design

Five treatments were employed, as shown in Table 1 in order to analyze biostimulation of domestic water polluted by benzene from WMO with ePf and mineral solution. All experimental data were analyzed by ANOVA and Tukey (P ≤ 0.05), to establish the minimum significant difference.

Table 1. Experimental essay to biostimulation of domestic water polluting by benzene from waste motor oil with crude extract of *Pleurotus florida* followed by biostimulation with mineral solution.

<table>
<thead>
<tr>
<th>Treatments (T)</th>
<th>Domestic Water</th>
<th><em>P. florida</em> extract</th>
<th>WMO</th>
<th>Na Azide</th>
<th>Tween 80</th>
<th><em>H_2O_2</em></th>
<th><em>MnSO_4</em></th>
<th>Mineral solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (relative control)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>*</td>
<td>+</td>
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<tr>
<td>2 (control absolute)</td>
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<td>3</td>
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</tbody>
</table>

*4 repetitions for each treatment; *Sterilized, (+) = apply; (-) non apply.
Source: self-authored

3 RESULTS

In the Figure 1 shows the biostimulation of domestic contaminated by benzene from WMO with ePf supporting that a fungal extracellular enzyme from ePf involved in lignin degradation of plants. The analysis of benzene from WMO yielded an initial concentration of 34.2 µmol in the Bartha flasks. The biostimulation of domestic water polluted by benzene from WMO with ePf induced the depletion of benzene concentration in only four days; due to high activity of Laccase, main extracellular enzyme of ePf, however in the same period, there was also an abiotic loss of benzene, detected concentration on day three was 19.2 µmol; benzene from WMO was depleted after four days, in the Bartha flask control treatment non biostimulated with ePf; this loss of benzene was effected by evaporation was up to 30% according to the figure 1, since it has been reported abiotic elimination of benzene from WMO increased by incubation time because
it’s high solubility in water, volatilization and adsorption on the walls of the Bartha flasks (2,6,8).

Figure 1. Biostimulation of domestic water polluted by benzene of WMO with *Pleurotus florida* crude extract before biostimulation with mineral solution

On figure 2 showed biostimulation of domestic water polluted by benzene of WMO with ePf followed by mineral solution had different patterns on the CO$_2$ generated due to oxidation of benzene because there is a native wide heterotrophic diversity microbial capable to use benzene of WMO as only source of carbon and energy (16). The activity of extracellular enzyme to break benzene increased drastically in five days after biostimulation with mineral solution due to basic macronutrients that induced mineralization for the native microbiota, which was followed of constant decrease over the next 16 days of biostimulation with mineral solution according that chemical composition of domestic water (17,18).

On figure 2 the total mineralization of benzene from WMO at 32.77 μM (data showed on figure 1) was achieved in less than 5 days compared to domestic water non polluted by WMO used as absolute control, there was not CO$_2$ detected due non biostimulation by ePf and mineral solution. The high CO$_2$ production was observed on the biostimulation of domestic water polluted by benzene of WMO due to a wide diversity heterotrophic aerobic microbial population able to mineralize benzene (19, 20,21).
In Figure 3a the chromatogram demonstrated that in domestic water polluted by benzene was one the main aromatic hydrocarbons from WMO before biostimulation with ePf. While in 3b the chromatogram showed that biostimulation of domestic water polluted by benzene from WMO with ePf supporting that contain one common extracellular enzymes involved in breaking aromatic hydrocarbons of WMO as well as laccase (5,21).
Figure 3- a. Chromatogram showed the peak of benzene of waste motor oil polluted domestic water before biostimulation with crude extract of *Pleurotus florida*.

Figure b. Chromatogram showed benzene elimination from waste motor oil after biostimulation with crude extract of *Pleurotus florida* and mineral solution.

4 DISCUSSION

In figure 1 is showed that biostimulation of domestic water polluted by benzene of WMO with *ePf* which contains a complex group of fungal extracellular enzymes to degrade lignin, these enzymes are able to hydrolysis of aromatic compounds facilitated its mineralization by biostimulation with mineral solution for the heterotrophic aerobic native microbial population form domestic water (8,15). In contrast with the assay when
fungy is capable to mineralized aromatic compounds in soil or waters polluted by those chemicals in order to eliminate them (16-18), but limited to different environmental condition as well as the concentration of polluting compounds is regard low, since if those fungus are not able to tolerate and grow poor having not good results, in terms of destroying aromatic hydrocarbons (3,5,21). Benzene was only breaking by ePf due that its fungal extracellular enzimes are adapted to pollute environments where not living cell are able to show any activity, in that sense this kind of biostimulation tools is suitable to apply for destroying aromatic compounds as benzene (15,16). Those results support that the ePf involve enough concentration of Laccase to hydrolysis this toxic aromatic even the complex chemical and biological conditions found in domestic water, data obtained were consistent with the reports generated by other authors (17). That showed that benzene from WMO required the ePf in order to break it and then by biostimulation with mineral solution for the indigenous aerobic heterotrophic microbiota in domestic water to achieve its elimination (18). These results also confirm that benzene of WMO is more recalcitrant than its aliphatic hydrocarbons, in that sense ePf is a tool to apply when environment is impacted by aromatic compounds (16, 21).

At present, decontamination of domestic water polluted by benzene from WMO is carried out through in situ water for bioventing. With this technique, aromatic hydrocarbons are removed due combination of volatilization and because biostimulated induced its mineralization (20). Also, it has been reported that some fungi are able to grow on relative low concentration of volatile aromatic hydrocarbons have already been used successfully for the biofiltration of air containing volatile hydrocarbons (21,22).

This study to support that biostimulation of domestic water polluted benzene from WMO with ePf and mineral solution might be a viable technique to improve mineralization of benzene or other types of aromatic hydrocarbons (19,10). Further studies are still needed to assess the stability of crude ePf in domestic polluted with WMO on a large scale.

5 CONCLUSIONS

This research concluded that crude extract of P. florida has a potential value to apply in biorecovering domestic water polluted by benzene from WMO, supported by biostimulation with mineral solution cloud be led to reuse it in some agriculture soil or industrial process.
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