



## Metal leaching from a spent catalyst by *Alternaria alternata*

### Lixiviación de metales a partir de un catalizador agotado por *Alternaria alternata*

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#### ABSTRACT

The study evaluated the leaching ability of the extracellular medium produced by a fungus, which was identified as *Alternaria alternata*. The study included the selection of a culture medium that favored the organic acid and siderophore production; sucrose and Czapek media were evaluated. Once the culture media was selected, bioleaching study of the catalyst at 1 and 5% continued, using only the extracellular media incubating at 30° C, 150 rpm during 7 days. Results indicated that the extracellular media produced by *A. alternata* in sucrose media decreases pH from 6 to 4.8; in this media siderophores were also produced. The study of metal bioleaching, using the extracellular media, showed that metals were leached at different extent, and it was observed that catalyst concentration had an important effect on the metal removal and on the order of the removal. It was found that at 1% catalyst and after 48 h of treatment, the following metals were removed, in descending order, V> Al> Si> Mo > Mg> Fe> Ni> As> Cr. This is the first report on the metal removal from the catalyst by the extracellular media of *A. alternata* which opens new research alternatives for the treatment of hazardous wastes with high metallic content.

Keywords: Bioleaching, Fungi, Leaching agent, Siderophore, Spent catalyst.

#### RESUMEN

El estudio evaluó la habilidad de lixiviación del medio extracelular producido por un hongo, el cual fue identificado como *Alternaria alternata*. El estudio comprendió la selección de un medio de cultivo que favorece la producción de ácidos orgánicos y de sideróforos, los medios de cultivo evaluados fueron medio de sacarosa y Czapek. Una vez

seleccionado el medio de cultivo, se continuó con el estudio de biolixiviación de metales de un catalizador al 1 y 5%, utilizando el medio extracelular en medio de sacarosa e incubando a 30°C, 150 rpm durante 7 días. Los resultados indicaron que el medio extracelular disminuyó su pH de 6 a 4.8; en este medio se producen sideróforos. El estudio de lixiviación de metales en presencia del catalizador demostró que el extracto del hongo es capaz de lixiviar metales; la concentración del catalizador tiene un efecto importante sobre la remoción de los metales y sobre la especificidad hacia los metales; a una concentración de catalizador del 1%, se removieron los siguientes metales, en orden descendente, V > Al > Si > Mo > Mg > Fe > Ni > As > Cr después de 48 h. Este es el primer reporte sobre la remoción de metales a partir de un catalizador por el medio extracelular de *A. alternata* lo cual abre nuevas alternativas de investigación para el tratamiento de residuos peligrosos con alto contenido metálico.

Palabras clave: Agente lixiviante, Biolixiviación, Catalizador agotado, Hongos, Sideróforos.

## 1. INTRODUCTION

The oil refining process use great amounts of catalysts, however due to their extensive use they become inactivated due to poisoning by foreign materials and impurities; then they are discharged and disposed representing an environment concern since spent catalysts are considered as hazardous wastes due to their high metals content. Catalysts contain a wide variety of metals such as Al, Mo, Ti, V, C, Ni among others; they facilitate different oil transformations. Actually, there are different metallurgical processes for their treatment; the most used are the hydro-pyrometallurgical ones. These methods dissolve the valuable metals efficiently, however significant amounts of pollutants are released to the environment, and that is the reason why they are not considered ecological methods.

The use of biological processes such as bioleaching, which is based on the ability of microorganisms or their byproducts to transform solid compounds into soluble and extractable elements represent a good alternative that can be considered as a clean technology due to their low requirements in cost and energy, if these are compared to the traditional methods.

Diverse microorganisms have been reported for the bioleaching of heavy metals contained in solid wastes including *Aspergillus niger*, *Penicillium simplicissimum*, *Penicillium purpurogenum*, *Rhodotorula rubra*, *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans* (Deng *et al.*, 2013). Most studies have been performed with bacteria although fungi appear as promising but studies are scarce. Some species of heterotrophic fungus (e.g., *Aspergillus* and *Penicillium*) have shown potential for metal bioleaching of various waste materials, such as fly ash, spent catalysts and electrical wastes. The most important mechanism of metal leaching by heterotrophic fungus is an indirect process, that is, by the microbial production of metabolites such as amino acids, organic acids or siderophores (Yang and Bin, 2013; Deng *et al.*, 2013). The aim of the present study was to determine the role of fungal metabolites, contained in extracellular media, such as organic acids and siderophores in the bioleaching of metals contained in a spent catalyst.

## **2. MATERIALS AND METHODS**

### **2.1. Fungus strain**

Isolate MVSS-AH-5 was obtained from a site with high metallic content located in Guanajuato, México; it was identified by molecular techniques in the Biotechnology Genomic Center of the National Polytechnic Institute.

### **2.2. Fungus Identification**

A collection of taxonomically related sequences was obtained from the NCBI Taxonomy Homepage and used to perform a multiple alignment analysis with CLUSTAL W (Larkin *et al.*, 2007) and T-Coffee (Magis *et al.*, 2014). Only common 16Sr and ITS genes regions were included in the phylogenetic tree, and similarity analyses using the Jukes-Cantor model were performed with the MEGA 5.2 (Tamura *et al.*, 2011). The phylogenetic tree was constructed using the UPGMA method, and 1000 bootstrap replications were assessed to support internal branches (Hillis and Bull, 1993). Sequences with identity scores to well-characterized 16S and ITS sequences greater than 97% identity (<3% divergence) were resolved at the specie level, between 95% and 97% at the genus level (Rosello-Mora and Amann, 2001).

### **2.3. Hydrotreating catalyst**

The spent catalyst used in the present study was coded as Ecat-Ni/V-H-oil, which was prepared from three different spent catalysts. The Mexican Petroleum Institute provided catalyst. Metallic content was determined by ICP-EOS following the methodology explained later. The particle size was lower than 500  $\mu\text{m}$ . The catalyst was stored at room temperature.

### **2.4. Effect of culture media on fungal metabolites production**

Isolate MNSS-AH-5 was cultivated on potato dextrose agar plates and was kept in an incubator at 30°C until sporulation. The conidia suspension of fungus was prepared at  $1 \times 10^7$  conidia/mL, which was determined in a Newbauer chamber. To obtain fungal metabolites, two media were evaluated: sucrose (Aung and Ting, 2005) and Czapek media (Deng *et al.*, 2013) as follows: 10% (3 mL) of conidia solution were inoculated in 50 mL Erlenmeyer flasks containing 30 mL of media, then systems were incubated at 30°C, 150 rpm during 7 days. Every 24 h, organic acid production was indirectly determined by pH in supernatant according to NMX-AA-008-SCFI-2011 method, using a digital potentiometer (PerpHecT LogR meter 310) and siderophores production was determined by CAS method as follows.

### **2.5. Evaluation of siderophore production**

The siderophore production was determined by chrome azurol S (CAS) method (Milagres *et al.*, 1999; Santos *et al.*, 2014). Briefly, the CAS method consist on mixing 0.5 mL of the cell-free filtrate with 0.5 mL of CAS assay solution and 1 mL of distilled water. The

mixture was allowed to equilibrate during 2 h in the dark at room temperature; later the absorbance was measured at 630 nm. Adjustment to zero absorbance was done with a mixture containing the same volume of CAS; the volume of cell-free filtrate was replaced by culture medium. Positive reactions were recorded by a change in the color of the CAS reagent from blue to yellow, light orange, orange or purple (Silva-Stenico *et al.*, 2005; Perez-Miranda *et al.*, 2007). Siderophore content in aliquot was calculated by using following formula (Afreen *et al.*, 2014):

$$\% \text{ Siderophore unit (\% SU)} = (A_r - A_s / A_r) \times 100$$

Where:

$A_r$  = absorbance of reference (non inoculated SM + CAS reagent)

$A_s$  = absorbance of sample (supernatant of production medium + CAS reagent) at 630 nm

For bioleaching studies, the extracellular media obtained at those previously selected conditions (pH reduction and maximal siderophore production) was produced and then used. However, to obtain a higher amount of media, in this case, extracellular media was produced in 1 L Erlenmeyer flasks containing 250 mL of sucrose medium as mentioned above; then at the appropriate time, fungus biomass was harvested and then extracellular media was vacuum filtered through sterile fine pore filter paper; the media containing the leaching agent was passed through 0.22  $\mu\text{m}$  membrane (Whatman) to obtain the cell-free leaching media which was used for further studies.

## 2.6. Bioleaching of metals from spent catalyst

To evaluate the leaching of metals from spent catalyst by the extracellular media from fungus, two experimental sets were prepared in 50 mL Erlenmeyer flasks containing 15 mL of fungus leaching media; a) catalyst was used at 1% pulp density and b) catalyst was added at 5 % (w/v) pulp density and then incubated at 30°C, 150 rpm during 48 h. All experiments were done in duplicate. Controls containing sucrose media plus spent catalyst at 1 % and 5 % (w/v) pulp density were also included in order to evaluate the abiotic leaching. At the end of experimentation, total content of flasks was separated by filtration then the catalyst was dried at room temperature; filtrate was collected in 50 mL glass vials and stored at 4°C until be analyzed by ICP-EOS (Gómez-Ramírez *et al.*, 2015). Metal removal from catalysts due to extracellular media was reported as the difference between the metal concentrations in controls and those concentrations in treatments.

## 2.7. Digestion and analysis of metals

Metal concentrations (bioleached) in liquid media and those remaining in catalysts were determined at the beginning and at the end of treatment. Samples of 1 mL of liquid samples or 100 mg of spent catalyst were subjected to metal analyses using an ICP-OES, Varian Model 710-ES after acid digestion. Catalyst treated was placed in cylindrical vial of silicon carbide, 6 mL of HNO<sub>3</sub> and 2 mL of HCl were added; samples were digested in a Microwave Reactions System: Multiwave PRO (Anton Paar), using a rotor HF100. Digestion conditions were: power 800 W for eight vessels, 40 Bar, temperature 210-240°C, with pRate of 0.3 bar seg-1, ramp 15 min, hold 20 min and cooling 15 min. Afterwards, 20 mL of deionized water

was added to cylindrical vial and the supernatant was collected in a 100 mL flask and set with deionized water. Metal analysis was performed using different wavelengths (nm) according with the metal. Metal concentrations in spent catalyst were calculated based on calibration curve of 0.1 - 10 ppm using a commercial standard (High-Purity) Cat. # ICP-200.7-6. The data were statistically analyzed using the Minitab 17 software with Tukey's HSD pairwise comparisons.

### 3. RESULTS

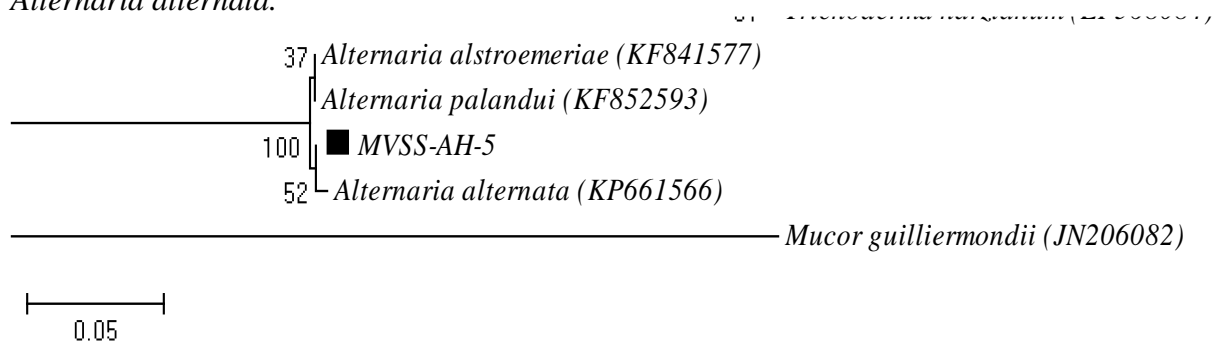
As can be observed in Table 1, ICP analyses showed that catalyst was composed mainly by aluminum; other elements were also presented however, although their concentration in catalyst is important when catalyst was used at 1 and 5% (w/v) the concentration was low.

**Table 1.** Elemental composition of the spent catalyst analyzed by ICP-EOS

Metal	mg/kg	Metal	mg/kg
Al	195482.7	Mg	1615.5
Si	28324.5	As	1349.8
V	17185.8	Zn	190.9
Fe	11742.8	Cr	153.6
Mo	6224.8	Sn	146.4
Ni	2765.8	Pb	62.2
P	2111.4	Cd	47.6
Sr	25.3	Cu	23

#### 3.1. Fungus identification

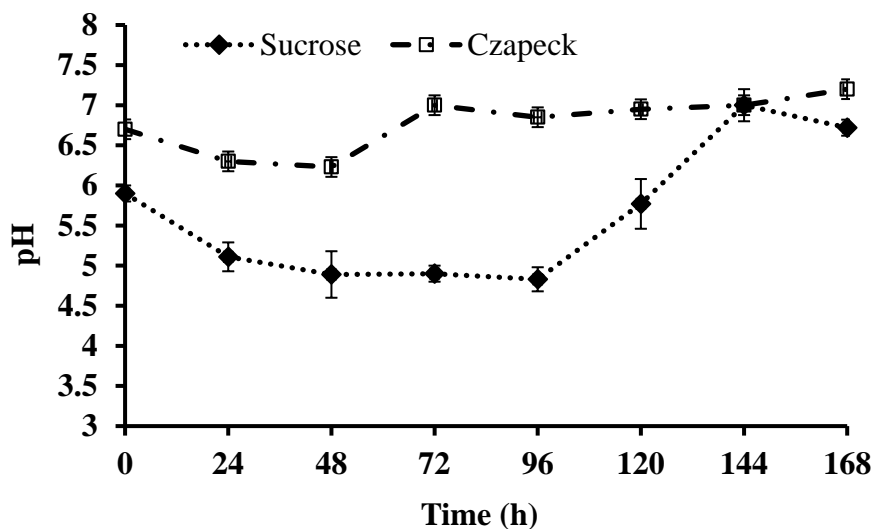
As it was mentioned previously, isolate coded MVSS-AH-5 was identified by molecular techniques. Fig. 1 shows the cladogram were isolate MVSS-AH-5 was identified as *Alternaria alternata*.



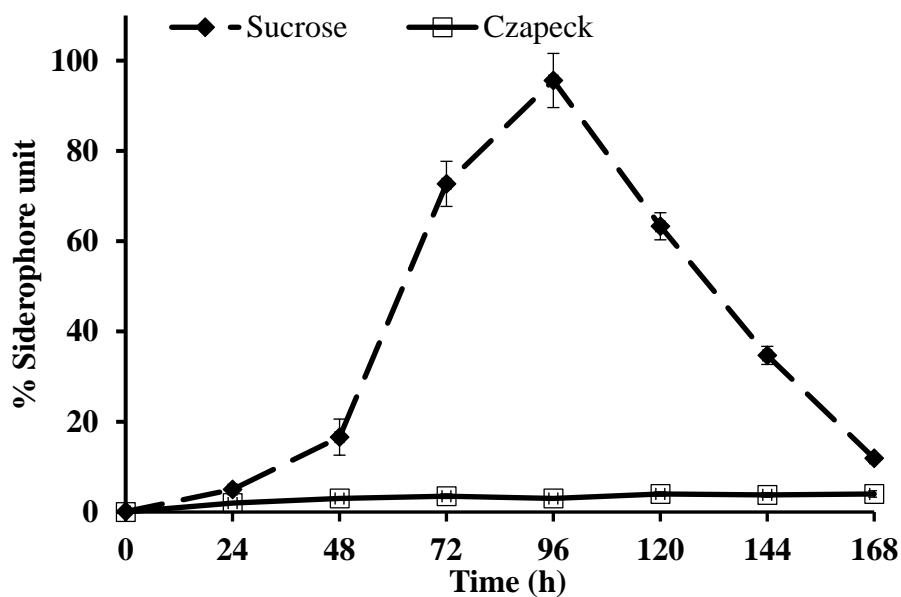
**Fig. 1.** The cladogram was constructed by using MEGA 6 software by Tamura *et al.*, (2011). Evolutionary relationships were estimated by UPGMA method (Sneath and Sokal, 1973) with 1000 bootstrap value to obtain the consensus tree (Felsenstein, 1985). Evolutionary distances were calculated using the Jukes-Cantor method (Jukes, 1969). *Mucor guilliermondii* was used as the outgroup. Bar 0.05 substitutions per nucleotide position.

### 3.2. Effect of culture media on fungal metabolites production

In Fig. 2, it is observed that the pH reduction was observed only in sucrose media from 6 to 4.8 at 96 h whereas in Czapek medium no important changes in pH were detected. In the case of siderophores production, these were evidenced (Fig. 3) because changes in color from blue to orange-yellow were observed within 72-120 h with a maximal production at 96 h. No siderophore production was observed in Czapeck media. Based on these results, further studies were done using sucrose media to produce the extracellular media at 96 h.



**Fig. 2.** Monitoring of pH during the fungus growth in two different media at 30°C, 150 rpm.

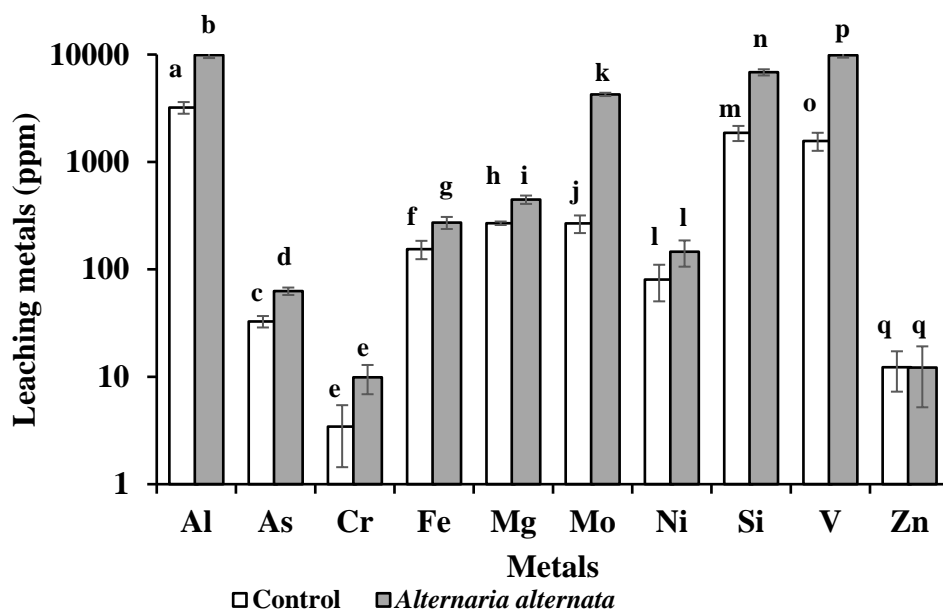


**Fig. 3.** Monitoring of siderophore production during the fungus growth in two different media at 30°C, 150 rpm.

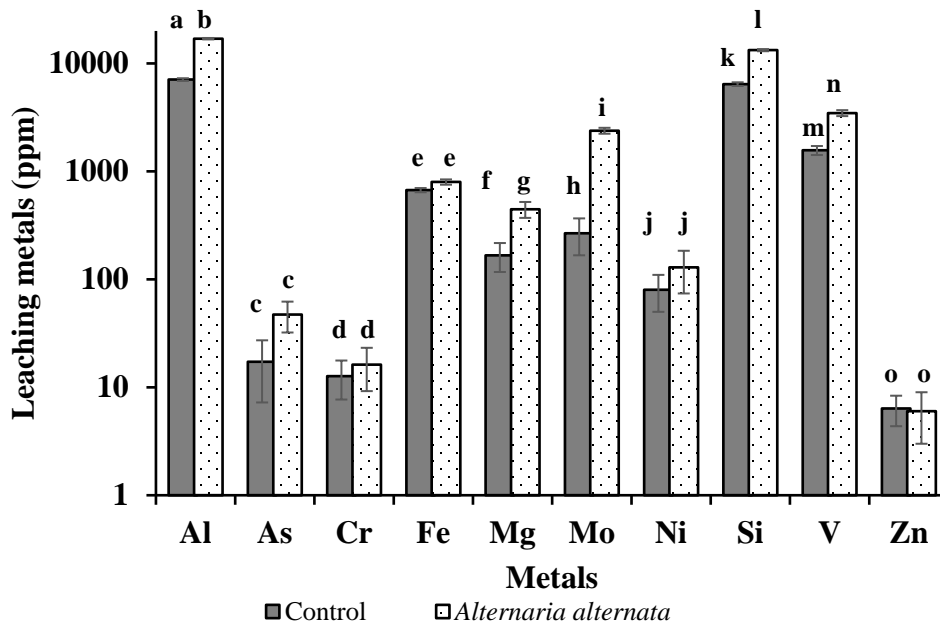
### 3.3. Metal removal from spent catalyst

According with results, extracellular media produced by *A. alternata* in sucrose media was able to remove V (8285.3 mg/kg) > Al (6662.0 mg/kg) > Si (4973.8 mg/kg) > Mo (3990.2 mg/kg) > Mg (177.7 mg/kg) > Fe (118.2 mg/kg) > As (29.9 mg/kg) from the spent catalyst at 1 % (w/v). Fig. 4 shows the leaching metals detected in liquid media, as can be observed the extracellular media of *A. alternata* removed more metals than control, except for Cr, Ni and Zn, when catalysts was used at 1%. Then, difference in concentrations between treatment and control corresponded to metal removed from catalyst.

When spent catalyst was used at 5%, the metal removal was Al (9872.7 mg/kg) > Si (6839.0 mg/kg) > Mo (2115.8 mg/kg) > V (1903.0 mg/kg) > Mg (279.6 mg/kg), although not significant difference was found) after 48 h of treatment. Fig. 5 shows the leaching metals detected in liquid media, as can be observed the extracellular media of *A. alternata* removed more metals than control, except for As, Cr, Fe, Ni and Zn, when catalysts was used at 5%, a similar behavior than 1% although the order in metal removal was different. Then, difference in concentrations between treatment and control corresponded to metal removed from catalyst.



**Fig. 4.** Bioleaching of metals from spent catalyst at 1 % (w/v) pulp density after 48 of treatment at 30 °C, 150 rpm by the extracellular media produced by *Alternaria alternata*. Statistically significant differences (one-way ANOVA with Tukey's HSD (P < 0.05) are indicated by different letters.



**Fig. 5.** Bioleaching of metals in spent catalyst at 5 % (w/v) pulp density after 48 of treatment at 30 °C, 150 rpm by leaching agent produced by *Alternaria alternata*. Statistically significant differences (one-way ANOVA with Tukey's HSD ( $P < 0.05$ )) are indicated by different letters.

To the best knowledge of the authors, this is the first report where extracellular media obtained from *A. alternata* is used to remove metals from spent catalyst. The highest leaching of different metals was achieved using catalyst at 1% pulp density.

#### 4. DISCUSSION

The genus *Alternaria* is a cosmopolitan widely distributing in soil and organic matter. At least 268 metabolites from *Alternaria* fungi have been reported. They include nitrogen-containing metabolites, steroids, terpenoids, pyranones, quinones, and phenolics. *Alternaria* metabolites exhibit a variety of biological activities such as phytotoxic, cytotoxic, and antimicrobial properties. Some reports mentioned the role of this genus to remove metals however; it has not been reported for the treatment of spent catalysts and less about the use of its extracellular media for metal bioleaching (Lou *et al.*, 2013).

Bioleaching processes of minerals, as can be considered the spent catalyst, require the use of organic acid-producing heterotrophic microorganisms or other chelating agents, most studied genera are *Aspergillus* and *Penicillium*. The organic acids could aid the solubilisation of hard and complex mineral materials as spent catalyst since it has been reported that organic acids are low-molecular weight carbon compounds capable to form complexes with various minerals. The actions of these organic acids can occur in two different ways: a) direct mineral attack by the metal-complexing organic acid anions as well as protons and b) there is a structural similarity between protons from organic acids and from monovalent elements so they can be substituted. Therefore, an important



screening process for bioleaching microbes involves the direct or indirect evaluation of the ability of the microorganisms to produce organic acids. Previous studies performed by our research group showed that *Penicillium sp* and *Acremonium sp* are able to remove metals from spent catalyst (Gómez-Ramírez *et al.*, 2015) however some drawbacks have been considered for field application, so in this study the first step was to evaluate the culture media where *A. alternata* produce organic acids. In addition, siderophore production was evaluated at the same conditions.

Siderophores have received much attention in recent years because of their potential roles and applications in various areas of environmental research. Their significance in these applications is because siderophores have the ability to bind a variety of metals in addition to iron, and they have a wide range of chemical structures and specific properties. The role of siderophores is primarily to scavenge Fe, but they also form complexes with other essential elements (i.e. Mo, Mn, Co and Ni) in the environment and make them available for microbial cells (Bellenger *et al.*, 2008). Results of the present study evidenced the production of organic acids by measuring pH decreasing (6.8 a 4.83) and siderophores (95%) by CAS method, respectively, in sucrose media by *A. alternata*. In Czapeck media, no organic acids (pH decreasing) or siderophores were produced. Therefore, more studies were done using the sucrose media. Although other chelating agents or compounds could be produced in liquid media of *A. alternata* only these mechanisms are exposed.

Regarding organic acids, it has been reported that organic acids leached more heavy metals than inorganic acids (Yixin Ya, 2014). The pathway of metal leaching is directly related to the coordinative ability of organic acids and metal ions. The more stable of the ligand formed, the more difficult to be adsorbed by the soil and sediment, and thus the metal leaching is much easier (Gao *et al.*, 2003). A number of studies of heavy metal pollution of soil have verified that the reactions between organic acid and heavy metal are related to the amount and place of the carboxyl and hydroxyl groups (Gao *et al.*, 2002). Generally, the structure with more carboxyl groups is beneficial to the heavy metal leaching.

Based on results and previous information, the study showed the ability of the extracellular media of *A. alternata* to leach or mobilize different metals from spent catalyst at 1 and 5% probably by means of siderophore or organic acids although more studies are needed to demonstrate it. The extracellular media of *A. alternata* mobilized metals at different extent when catalyst was used at 1 or 5%. The reason why this could occurs it is not completely understood due to the complex nature of the catalyst and due to the affinity of each metal to siderophores or organic acids.

Summarizing, this study report for the first time the use of the extracellular media of *A. alternata* containing organic acids and siderophores for the metal leaching from a spent catalyst. This media is able to leach metals although the mechanisms are unknown however, it is suggested that siderophores and organic acids production are involved. Nowadays, there are interest in the search of clean technologies to decrease the environmental impact of the chemicals industry, then the use of biological leaching using fungus or their metabolites could be a good alternative to treat non- sulfur materials with high metallic content. The leaching agent produced by *Alternaria alternata* could be suggested for the treatment of spent catalyst that came from oil industry.

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## CONFLICT OF INTEREST

All the researchers listed as authors of the current study declare that there is no conflict of interests regarding the publication of this manuscript.

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