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Bioelectroremediation of hexadecane in electrical cells containing *Aspergillus niger* immobilized in alginate

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ABSTRACT

Bioelectroremediation (BER) uses electrical current to stimulate catabolism of environmental pollutants, such as petroleum spills. However, applying current to soil may have adverse effects on the microorganisms involved in petroleum degradation. We identify and evaluate the BER capabilities of a strain of *Aspergillus niger* obtained from petroleum-contaminated soil. Spores of this strain immobilized in alginate spheres (2 g) were mixed with 100 g of hexadecane-contaminated sandy loam soil and exposed to 5, 10, or 15 mA direct current in a 200 cm^3 cell with copper electrodes. Soil hexadecane concentration was measured by gas chromatography. More than 94% of hexadecane was removed from the soil within 12 days for the currents tested, and the *A. niger* grew to 6 x10^6 CFU g^{-1} in 15 days at 10 mA current. The maximum hexadecane degradation was achieved using a 10-mA current for 20 days, but more than 99% of the hexadecane was removed by the fifth day. These results suggest that the use of spore-containing alginate beads promotes growth and petroleum biodegradation of *A. niger* exposed to electrical currents.

Keywords: *Aspergillus*, bioelectroremediation, hexadecane, petroleum, soil.

Bioeletrorremediação de hexadecano em células elétricas contendo *Aspergillus niger* imobilizado em alginato

RESUMO

A bioeletrorremediação (BER) usa corrente elétrica para estimular o catabolismo de poluentes ambientais, como derramamentos de petróleo. No entanto, a aplicação de corrente ao solo pode ter efeitos adversos sobre os microrganismos envolvidos na degradação do petróleo.

Identificamos e avaliamos as capacidades de BER de uma cepa de *Aspergillus niger* obtida de solo contaminado com petróleo. Os esporos desta cepa foram imobilizados em esferas de alginato (2 g) foram misturados com 100 g de solo franco-arenoso contaminado com hexadecano e expostos a 5, 10 ou 15 mA de corrente contínua em uma célula de 200 cm^3 com eletrodos de cobre. A concentração de hexadecano no solo foi medida por cromatografia gasosa. Mais de 94% do hexadecano foi removido do solo em 12 dias para as correntes testadas, e o A. niger cresceu para 6 x 10^6 UFC g^{-1} em 15 dias na corrente de 10 mA. A degradação máxima do hexadecano foi alcançada usando uma corrente de 10 mA por 20 dias, mas mais de 99% do hexadecano foi removido no quinto dia. Esses resultados sugerem que o uso de grânulos de alginato contendo esporos promove o crescimento e a biodegradação do petróleo de A. niger exposto a correntes elétricas.

Palavras-chave: *Aspergillus*, bioeletrorremediação, hexadecano, petróleo, solo.

1. INTRODUCTION

Given the long-lasting environmental effects of petroleum spills, developing procedures for rapid remediation of contaminated soils has attracted considerable attention. (Kumar *et al.*, 2021). These remediation procedures are divided into two broad classes - bioremediation and physicochemical technologies. Bioremediation may be the less expensive alternative, but it requires more time (Raj *et al.*, 2018). Thus, ways to improve the efficiency of bioremediation will likely make it more competitive and widely applied.

One promising advance in bioremediation is bioelectroremediation (BER), which involves stimulating biological degradation of contaminants with low-amperage direct current (Gidudu and Chirwa, 2019). It has been shown that the application of a current modifies the metabolic responses in microorganisms, causing acceleration of biodegradation (Sarankumar *et al.*, 2020). Furthermore, when used for petroleum-contaminated soil, BER increases bioavailability of petroleum-derived pollutants, thus making it less recalcitrant to microbial degradation (Fan *et al.*, 2017).

Among hydrocarbons, hexadecane (HXD), a common soil pollutant, is used frequently as a petroleum contamination proxy in bioremediation experiments (Dehghani *et al.*, 2013) because, like other hydrocarbons, it has low water solubility (0.9 µg L^{-1}) but is biologically labile (Okoye *et al.*, 2020). Using the HXD model to test traditional bioremediation has yielded some successful results using different microorganisms, such as *Pseudomonas aeruginosa* (Cruz *et al.*, 2021). Additionally, Yuan *et al.* (2013) achieved an HXD biodegradation of 53.7% using a bacterial consortium with a constant voltage gradient of 1.3 V cm^{-1} for 42 days in pristine soil; and Wang *et al.* (2016) used alternative bioremediation and electrokinetic technologies with soil bacteria to obtain 78.5% HXD degradation after 45 days of treatment. Using the fungus *Aspergillus brasiliensis*, Velasco-Alvarez *et al.* (2011) achieved a biodegradation of 96% with a current of 0.42 mA cm^{-2}.

While it is true BER has been successful in some laboratory-scale experiments, several parameters need to be explored further to create a coherent set of recommendations for successful BER use in the field. Therefore, evaluating ecological parameters, different microorganisms, pH, voltage gradient, electrochemical cell design, microorganism resistance to electric current, design and configuration of electrodes, costs, and energy efficiency are important factors for improving efficient elimination of pollutants and BER recommendations (Li, *et al.*, 2020; Annamalai and Sundaram, 2020).

To this end, we characterized a strain of *Aspergillus niger* isolated from petroleum-contaminated soil and evaluated its BER potential by exposing spores immobilized in alginate to different currents in HXD-containing soil.

2. MATERIAL AND METHODS

2.1. Isolation of *Aspergillus niger* strain from contaminated soil

Contaminated soil (1 kg) was collected from near Huamacucho, Peru (7°48'40.3" S 78°03'41.8" W, Figure 1A) where an oil spill occurred 3 days earlier. The soil (10 g) was combined with 100 ml of sterile saline solution (0.85% NaCl), homogenized, and allowed to settle at room temperature for 15 min. A 100 µL aliquot of the supernatant was spread on the surface of a petri dish containing potato dextrose agar (PDA, glucose 15 g L^{-1}, potato infusion 200 g L^{-1} and agar agar 15 g L^{-1}; pH 6) and incubated at 30ºC for 5 days. A colony with *Aspergillus*-like density and morphology with black conidia was selected and replicated once in tubes with PDA agar to obtain pure cultures (Al-Dossary *et al.*, 2019).

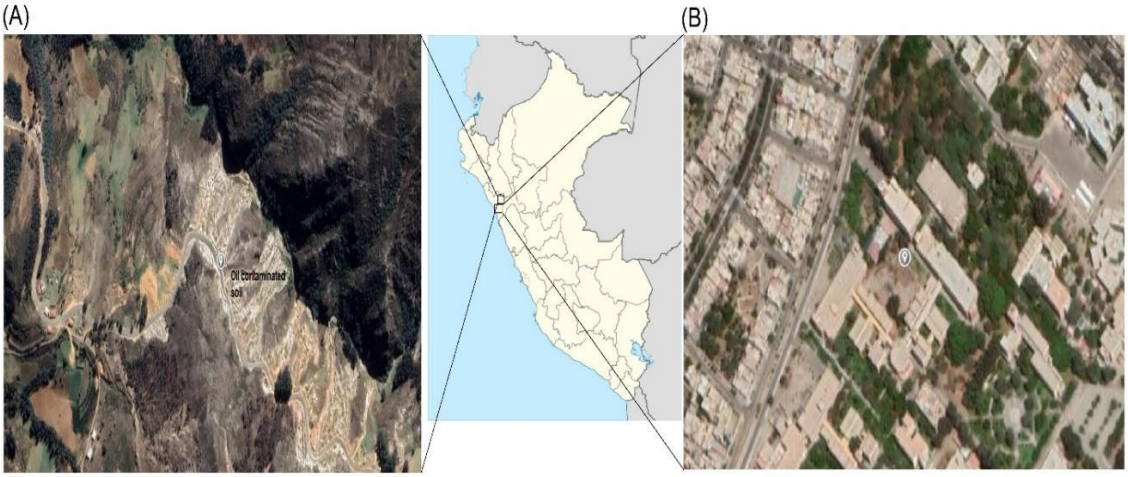

Figure 1. (A) Sampling site of oil-contaminated soil used in the isolation of *A. niger* near Huamachuco, Peru. (B) Location of the oil-free sampling site for the experimental trials, in Trujillo, Peru.
Source: Google Maps.

2.2. Morphological and molecular characterization of *A. niger*

A microculture of the fungus was used to observe microscopic morphological structures (conidiophore, vesicle and phialid) by the Riddell technique (Ordóñez-Valencia *et al.*, 2018). For genomic characterization, DNA was extracted from a pure 5-day old culture (Yeast DNA Extraction Kit, Thermo Scientific, Part No. 78870). As previously described, 18S rRNA sequences were amplified by PCR, using primers ITS-1 and ITS-4 and the amplified rDNA was characterized on a 1.5% agarose gel (Oduro-Mensah *et al.*, 2018). The purified PCR product was sequenced (Macrogen USA) and aligned to related known sequences using BLAST (National Centre for Biotechnology Information) (Buehler *et al.*, 2017). A phylogenetic tree was built from related rDNA sequences with MEGA-X (Kumar *et al.*, 2018).

2.3. Immobilization of fungal spores

A. niger spores were immobilized in calcium alginate spheres, according to the method described by Buehler *et al.* (2017). A spore suspension of the culture of *A. niger* containing $4x10^8$ CFU per mL in 1.5% Tween 80 (Sigma Aldrich, USA) was mixed 1:1 with sterile 3% sodium alginate solution (w v^{-1}). This mixture was added dropwise to sterile 0.2 M $CaCl_2$ using a 10 mL syringe. The resulting 3-5 mm spheres were rinsed with sterile water to discard excess calcium ions. The calcium alginate spheres contained $2x10^7$ UFC per gram and were preserved in a sterile solution of 0.1% NaCl at 4°C.

2.4. Addition of HXD to oil-free soil

Oil-free soil (4 kg) was obtained from the campus of National University of Trujillo, Peru (8°06′45.7″ S 79°02′19.1″ W) (Figure 1B), dried at room temperature, and homogenized with a mortar and sieved to a grain size of 1.15 µm (Table 1). The sieved soil was sterilized in an oven at 180°C for 1 h. The pH and conductivity of the soil was analyzed using a Consort 5010, while elemental composition was determined by atomic absorbance. A solution containing 180 mg of HXD (>98%, Merck, USA) dissolved in 1.9 mL n-heptane (≥ 99%, Merck, USA) was mixed thoroughly with 1 kg of soil. The soil was then spread on trays to let the n-heptane evaporate at room temperature for 24 hours (Velasco-Alvarez *et al.*, 2011).

2.5. Electrochemical chamber

The electrochemical chamber followed the specifications of Sumbarda-Ramos *et al.* (2010) with some modifications. The reaction chamber was a glass cylinder (external diameter of 5.0 cm, height of 9.8 cm). On either end of the reaction chamber was an electrolytic cell (diameter of 6.5 cm, height of 3.3 cm) containing a copper electrode with a contact area of 37.37 cm^2 (Figure 2). Microporous cellulose discs were used to facilitate ion exchange and current between the electrolytic cells and the reaction chamber. The chamber was sterilized with UV-A radiation before use.

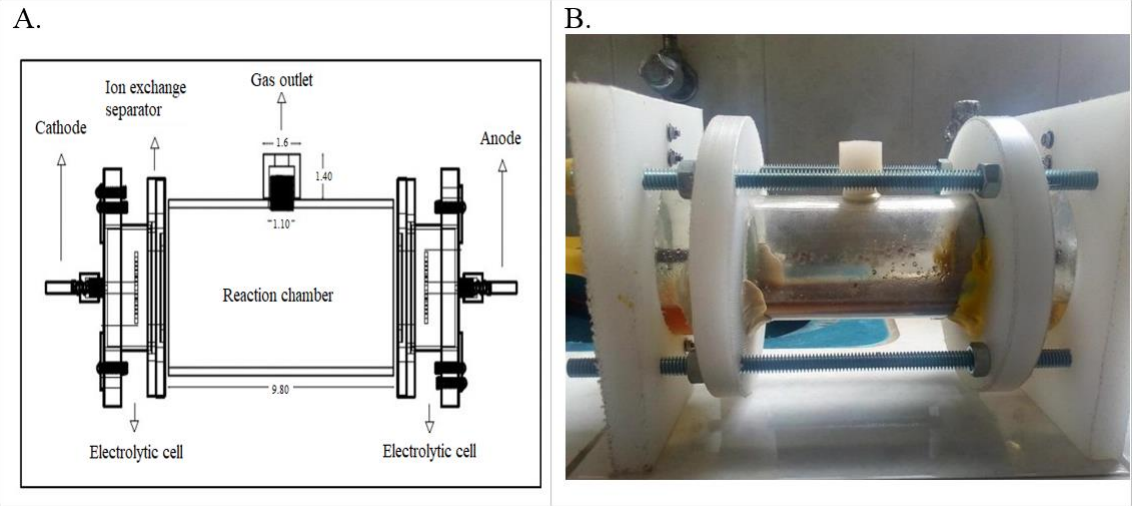

Figure 2. (A) Design and (B) photo of electrochemical chamber in use.

2.6. Bioelectrodegradation experiments

In each experimental run, 100 g of HXD-contaminated soil, 2 g of inoculated spheres, 43.5 mL of aqueous medium (250 mM $NaNO_3$, 22 mM KH_2PO_4, 7.5 mM $MgSO_4 \cdot 7H_2O$, 40 mM KCl, 2.1 µM $FeSO_4 \cdot 7H_2O$, 0.36 µM $CuSO_4 \cdot 5H_2O$, 3.3 µM $ZnSO_4 \cdot 7H_2O$, 0.17 µM $MnSO_4 \cdot 7H_2O$, pH 5) was added to the reaction chamber (Volke-Sepúlveda *et al.*, 2006). Phosphate buffer (0.1 M, pH 7, 30 mL) was added to the electrolytic cells. The electrochemical chamber was hermetically secured with teflon and steel screws. Constant direct current (5, 10, or 15 mA) was applied to the electrodes for 6 or 12 days at room temperature (23±2ºC); current was monitored with a multimeter (Prasek, PR-301C). Controls omitted both current and inoculum but were otherwise treated the same as the experimental runs. The resulting soil samples (6 experimental and 2 control) were then analyzed for HXD content.

2.7. Determination of residual HXD

Soil samples were analyzed for HXD by EPA Method 8015C at an accredited laboratory (Corlan SAC, Lima, Perú) using a gas chromatograph (Perkin Elmer CLARUS 690 GC) with

a capillary-type injector and a flame ionization detector with a sensitivity greater than 0.0131 C g^{-1}. Soil samples were tested in triplicate. The chromatographic data were analyzed by TotalChrom software (Lima *et al.*, 2020).

2.8. Statistical analysis

The results from different experimental conditions were compared using ANOVA and Tukey HSD tests by Minitab statistical software 18, with a level of significance $p < 0.05$ (Mena *et al.*, 2015).

2.9. Confirmation of optimized results

After completing the first set of experiments, a second series of confirmatory tests were completed using the same methods at a current of 10 mA and incubation times of 5, 10, 15, and 20 days with a similar control. In addition to HXD analysis of soil samples, 1 g of each soil sample was analyzed for *A. niger* colony-forming units (CFU) by serial dilution on Sabourand agar plates which were incubated at 30ºC for 5 days post-inoculation.

3. RESULTS AND DISCUSSION

3.1. Isolation of an *A. niger* strain

Soil contaminated with crude oil near Huamachuco, Peru was selected for isolation of an *A. niger* strain that has an active capacity to degrade hydrocarbons using standard purification techniques (Okoye *et al.*, 2020). This strain had macroscopic and microscopic morphology consistent with *A. niger*: a black globose aspergillar head, smooth brown conidiophores and globose spores or conidia. PCR amplification of the ITS-1 region of the 18S rRNA gene produced 612-617 bp oligonucleotides; sequences were compared to the Genbank database (BLAST). Phylogenetic analysis revealed that the isolated strain was 99% similar to *Aspergillus niger* (KY702576) (Figure 3). The 18S rRNA nucleotide sequence of the isolated strain was deposited in the GenBank database under the accession number MT180482 with designation QH27.

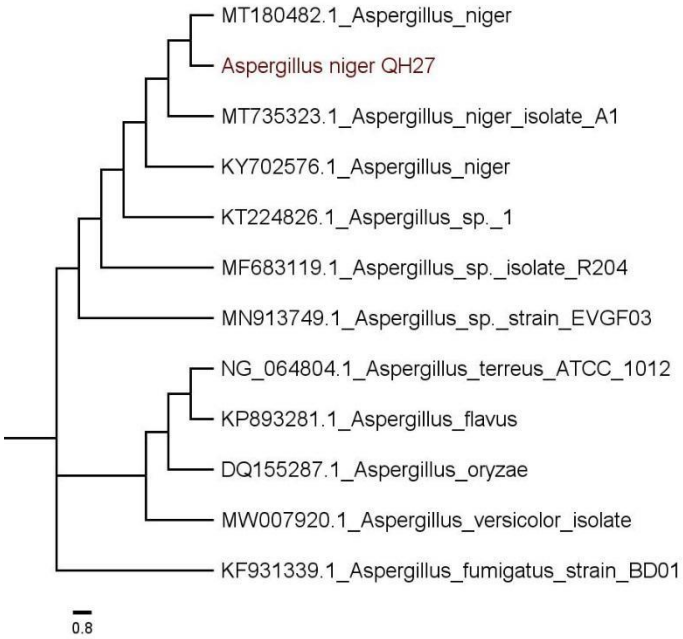

Figure 3. Phylogenetic analysis of 18S rRNA sequences from *Aspergillus niger* QH27.

3.2. Characterization of oil-free soil

In this investigation, a sandy loam soil with acceptable characteristics for BER was used (Table 1). Soil texture is acceptable for remediation experiments to evaluate organic compounds (Abdel-Moghny *et al.*, 2012). The soil sample had a total cation exchange capacity (CEC) of 11.59, which is appropriate for BER; but soils with CEC >15 experience physiochemical, hydrological, and mechanical changes when exposed to an electric field (Panjaitan and Andi, 2017).

Table 1. Physicochemical properties of the sandy loam soil used for BER studies.

Physical / chemical parameter	Value
pH (1:1)	8.0
C.E. (1:1) (dS/m)	0.8
CaCO3 (%)	8.4
M.O. (%)	1.1
P (ppm)	78
K (ppm)	427
Sand (%)	79
Silt (%)	10
Clay (%)	11
Textural class	Sandy loam
Ca^{+2} (cmol(+) kg^{-1})	7.42
Mg^{+2} (cmol(+) kg^{-1})	2.44
K^{+} (cmol(+) kg^{-1})	1.01
Na^{+} (cmol(+) kg^{-1})	0.72
$Al^{+3} + H^{+}$ (cmol(+) kg^{-1})	0.00
Total Cation Exchange Capacity	11.59

3.3. Degradation of HXD using BER with *A. niger* QH27

BER results in several physical processes in soil: electrolysis, electromigration, electroosmosis and electrophoresis, which drag water, ions, compounds, colloids and/or microorganisms through the pores of the soil. Thus, soil components are more thoroughly mixed, causing the degradation of pollutants on the surface of the soil particles, and/or on the electrodes by electrochemical oxidation. More importantly, these processes increase the bioavailability of pollutants to microorganisms, and may also promote the transfer of pollutants and nutrients into cells (Ramadan *et al.*, 2018).

The degradation of HXD by BER using *A. niger* was monitored under 3 different currents (5, 10, and 15 mM) at two incubation times (6 and 12 days). A control that omitted both *A. niger* spheres and current was also analyzed at both timepoints. Each soil sample was analyzed in triplicate (Table 2). All initial experimental conditions resulted in a degradation greater than 75%, with a maximum degradation of 99.836 ± 0.002% when 10 mA was applied for 12 days (Table 2). The control showed negligible HXD loss.

BER results obtained here using fungal biomass differ from other investigations with bacteria, such as Hassan *et al.* (2019), who achieved a 20-30% degradation of diesel hydrocarbons with an electric current of approximately 220 mA, using *Acinetobacter calcoaceticis, Sphingobacterium multivorum* and *Sinorhizobium.* Likewise Vaishnavi *et al.* (2021) obtained a remediation of 84% of the diesel in soil using *Staphylococcus epidermidis* EVR4 with 2 V. Taken together, these data point to more efficient BER with fungus when compared to bacteria.

Table 2. HXD elimination by BER under different currents and endpoints. The % HXD elimination is the mean ($\bar{x}$) with standard deviation (σ) of 3 analyses of a soil sample.

Current (mA)	Time (days)	% HXD elimination ($\bar{x}\pm\sigma$)
5	6	89.972±0.002
	12	98.612±0.003
10	6	99.3335±0.0004
	12	99.836±0.002
15	6	79.964±0.007
	12	94.833±0.001
0 (control)	6	0.001±0.001
	12	0.001±0.002
10 (repeat)	5	99.278±0.0004
	10	99.745±0.003
	15	99.968±0.0003
	20	99.994±0.0002
0 (control repeat)	20	0.0004±0.0002

One concern with BER is that the current may alter the microorganisms involved in bioremediation. For example, it has been shown that electrical current may inhibit cell differentiation, minimize biomass production and promote the formation of spores or CFU. In order to overcome this potential difficulty, *A. niger* spores were immobilized in alginate, which likely shortens the latency time during degradation by promoting cellular growth (Velasco-Alvarez *et al.*, 2017).

In order to test *A. niger* growth in the presence of electrical current and to confirm the BER results of the first experiments, a second series of experiments was performed (Table 2, bottom). These experiments used a constant current of 10 mA for 5 to 20 days and also monitored fungal growth. Fungal concentration steadily increased to $6x10^6$ CFU g^{-1} after 15 days (Figure 4). Furthermore, HXD degradation was nearly complete by day 5, with the remaining <1% of HXD continuing to decrease (Table 2, bottom). These results confirm that the *A. niger* QH27 immobilized in alginate spheres both grows efficiently and uses HXD as a carbon source while exposed to electrical current (Habibul *et al.*, 2016).

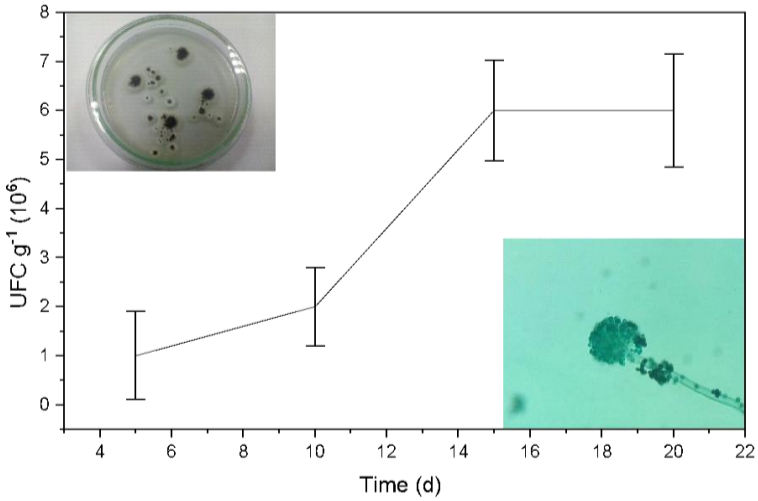

Figure 4. Growth curve of *A. niger* QH27 over 20 days exposed to 10 mA as measured by serial dilution (for example, Petri dish, top inset). *A. niger* was confirmed microscopically (bottom inset, 400x, Olympus CX31).

A one-way ANOVA ($F_{12,26}$=8.4x10^8, P=0) and Tukey's HSD test between runs and controls indicated statistically significant differences between all experimental runs tested, except for some control results. However, multiple repetitions of the entire experiment would likely increase error bars to roughly 10%. Therefore, nearly all treatments tested, except for 15 and 5 mA at 6 days, would almost completely remove HXD. Thus, an evaluation of energy consumption may be considered in choosing an appropriate current.

4. CONCLUSION

We identified a strain of *A. niger* that can rapidly grow and metabolize HXD as a carbon source while exposed to electrical current. Furthermore, we show that immobilizing spores in alginate spheres is one way to promote cellular growth and minimize latency. Under these conditions, *A. niger* QH27 can nearly eliminate HXD in loamy soils in as little as 5 days in an electrochemical chamber at 23ºC when exposed to 10 mA of direct current. This model opens the possibilities of testing additional strains and fungi for BER capacity exposed to a variety of pollutants, which may contribute to more efficient removal of contamination by hydrocarbons and their derivatives from soils.

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