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"Phenomenological analysis of hydrocarbon degradation by a fungal electro-bio-catalyst in contaminated water"

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"PHENOMENOLOGICAL ANALYSIS OF HYDROCARBON DEGRADATION BY A FUNGAL ELECTRO-BIO-CATALYST IN CONTAMINATED WATER"

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Science is but a perversion of itself unless it has as its ultimate goal the betterment of humanity.

Nikola Tesla

Content

Abstract

Environmental pollution associated with petrochemical industry derivatives is a worldwide problem needing to be attended. In Mexico, 85% of environmental emergencies reported during 2015 were associated with hydrocarbons. Oil refinery wastewaters are the main source of hydrocarbon contaminated water bodies and soils. Then, it is necessary to design and implement efficient technologies for oil contaminated water treatment, before discharged into the environment.

The combination of biological and electrochemical techniques enhances the bioremediation efficiency of treating oil-contaminated environments. In this thesis, a method using two successive cultures, solid then liquid, for production and use of a non-growing fungal whole cell biocatalyst (*Aspergillus brasiliensis* attached to perlite) is proposed. A controlled electric field was applied during the fungal cell biocatalyst production in a solid state culture as a novel approach, denoted the electric field pretreatment, to enhance the hydrocarbon degrading activity. Afterward, the catalytic performance was determined in liquid culture using a 1 L airlift bioreactor.

In previous works the biocatalyst was evaluated as hexadecane (HXD) sorbent-degrader, demonstrating that biomass production on the electric field pretreated biocatalyst was only 20% of that on the untreated BC, but the maximum HXD sorption capacity was enhanced one order of magnitude by effect of the electric field pretreatment. The HXD degrading activity of the pretreated biocatalyst was 9-fold higher than that untreated.

To demonstrate applicability of the biocatalyst, it was used to degrade a hydrocarbon blend (hexadecane-phenanthrene-pyrene; 100:1:1 w/w) in liquid culture. During hydrocarbon degradation, all mass transfer resistances (internal and external) and sorption capacity were experimentally quantified. Internal mass transfer resistances were evaluated through the biocatalyst effectiveness factor analysis as a function of the Thiele modulus (using first order reaction kinetics, assuming a spherical biocatalyst where five particle diameters were assayed). External mass transfer resistances were evaluated by $k_L a$ determination, including each hydrocarbon molecule and oxygen. Electric field pretreatment during biocatalyst production promoted surface changes in biocatalyst and the production of an emulsifier protein in the airlift bioreactor was induced. The biocatalyst surface modifications enhanced

the affinity for hydrocarbons, improving hydrocarbon uptake by direct contact. The resulting emulsion was associated with decreased internal and external mass transfer resistances. Electric field pretreatment effects can be summarized as: a combined uptake mechanism (direct contact dominant followed by emulsified form dominant afterwards) diminishing mass transfer limitations, resulting in a non-specific hydrocarbon degradation in blend with an effectiveness factor close to unity.

Since the emulsifier protein played an essential role in the hydrocarbon degradation, it was partially characterized, measuring its emulsifying activity in the presence of different hydrophobic phases. A major emulsifying activity in the presence of polycyclic aromatic hydrocarbons in blend with aliphatic (1:1 w/w), 2.79 fold-higher than solely hexadecane, was observed. The environmental conditions were an important factor that affect the emulsifying activity; alkaline (pH 7-11) and thermal (25-92°C) environments significantly enhance the EA, but not the presence of salt (0-35 g L^{-1}). The emulsifying protein was 19.5 % of the total protein produced in the liquid culture, and purification enhanced 7 times its specific emulsifying activity.

The relevance of this thesis was, from the engineering point of view, the integration of microbiological and electrochemical inputs to solve an environmental problem. This piece of work considers three main topics for the enhancements of the global hydrophobic pollutants degradation rates: (i) a microorganism metabolically modified by an electric field, enhancing its pollutants degrading capabilities; (ii) the metabolic activity of an immobilized microorganism limited of nutrients mass transfer rates; (iii) the presence of an emulsifier agent that modifies the interfacial mass transfer resistances. The integration of these topics is expressed as kinetic parameters set, which can be a useful tool for design and scale-up of hydrocarbons biodegradation airlift bioreactors.

Resumen

La contaminación ambiental provocada por actividades de la industria petroquímica es un problema de gran interés a nivel mundial. El 85% de los incidentes ambientales registrados en México, durante el 2015, se relacionan con hidrocarburos. Una de las principales fuentes de contaminación asociada a hidrocarburos, tanto en suelos como en cuerpos acuíferos, son las descargas de aguas residuales de refinerías de petróleo. Por lo tanto, es necesario diseñar e implementar tecnologías eficientes para el tratamiento de aguas residuales de refinerías, antes de ser descargadas al ambiente.

Se ha demostrado que la combinación de métodos biológicos y electroquímicos aumenta la eficiencia de remediación de ambientes contaminados con hidrocarburos. El presente trabajo propone un método que consta de dos cultivos sucesivos: en el primer cultivo (sólido), se produce un biocatalizador constituido por biomasa de *Aspergillus brasiliensis* inmovilizada en agrolita. Durante la producción del biocatalizador se aplicó un campo eléctrico, de manera controlada, como un pretratamiento, con el propósito de aumentar la capacidad degradadora de hidrocarburos. Posteriormente, el biocatalizador se utilizó en la degradación de hidrocarburos en medio líquido usando un biorreactor airlift de 1L.

En trabajos previos se estudió el biocatalizador como un sistema sorbente-degradador de hexadecano (HXD). Se demostró que la producción de biomasa en el biocatalizador pretratado fue sólo el 20% de la que se produce sin pretratamiento; sin embargo, su capacidad de sorción de hidrocarburos aumentó en un orden de magnitud y la actividad degradadora fue 9 veces mayor por efecto del pretratamiento con el campo eléctrico.

Para demostrar la aplicación del biocatalizador, se utilizó con el objetivo de degradar una mezcla de hidrocarburos, compuesta por hexadecano, fenantreno y pireno, 100:1:1 p/p en cultivo líquido. Durante la degradación de hidrocarburos se evaluaron, experimentalmente, las resistencias a la transferencia de masa (internas y externas), y la capacidad de sorción de los hidrocarburos. Las resistencias internas a la transferencia de masa se evaluaron mediante el análisis del factor de efectividad, como una función del módulo de Thiele (usando una cinética de reacción de primer orden y suponiendo partículas catalíticas esféricas, utilizando cinco diámetros de partícula). Las resistencias externas a la transferencia de masa se evaluaron mediante la determinación de los coeficientes volumétricos de transferencia de

masa (k_La), considerando a cada hidrocarburo y al oxígeno. El pretratamiento con el campo eléctrico modificó la superficie del biocatalizador aumentando su afinidad por los hidrocarburos y, aumentando en consecuencia, el consumo por contacto directo; por otro lado, indujo la producción de una proteína emulsificante en el biorreactor airlift y, la emulsión formada mejoró los fenómenos de transferencia de masa.

Los efectos del pretratamiento con el campo eléctrico pueden resumirse en: un mecanismo combinado en el consumo de hidrocarburos (dominante por contacto directo en las primeras horas y, posteriormente, vía emulsificación de hidrocarburos) disminuyendo las resistencias a la transferencia de masa; dando como resultado la degradación de hidrocarburos de manera no específica y con un factor de efectividad cercano a uno, para los tres hidrocarburos utilizados.

Dado que la producción de la proteína emulsificante tuvo un papel muy importante en la degradación de hidrocarburos, se realizó una caracterización parcial sobre su actividad emulsificante con diferentes mezclas de compuestos hidrofóbicos y su estabilidad ante variaciones de pH, salinidad y aumento en la temperatura. Se observó que la actividad emulsificante fue 2.79 veces mayor en mezclas de hidrocarburos que contenían aromáticos policíclicos con respecto a la observada con el alifático hexadecano. Por otro lado, las condiciones ambientales fueron un factor importante en la actividad emulsificante, se observó que en ambientes alcalinos (pH 7-11) o precalentando las proteínas (25-92 °C) la actividad emulsificante aumentó significativamente y, la presencia de sal en el medio (0-3.5 g L-1) no tuvo un efecto significativo. Se demostró que la proteína emulsificante fue el 19.5% del total de proteínas producidas en el medio líquido y, su purificación aumentó 7 veces la actividad emulsificante.

La relevancia de esta tesis radica en la integración, desde el enfoque de la ingeniería, de un problema multidisciplinario que aborda la restauración de ambientes contaminados acoplando métodos microbiológicos y electroquímicos, considerando tres aspectos: (i) un microorganismo que puede ser modificado por la presencia de un campo eléctrico, aumentando su capacidad degradadora de contaminantes; (ii) la actividad metabólica de un microorganismo inmovilizado en un soporte poroso puede estar limitada por las resistencias a la transferencia de nutrientes y (iii) la producción de un agente emulsificante puede abatir

las resistencias a la transferencia de masa interfacial, aumentando la tasa de degradación de contaminantes hidrofóbicos. La integración de estos aspectos se puede expresar como un conjunto de parámetros cinéticos útiles para el diseño y escalamiento de biorreactores airlift con fines de remediación de aguas contaminadas con hidrocarburos.

1. Introduction

Hydrocarbon degradation in contaminated environments is a quite concern topic. According to Web of Science database, more than 6000 papers were published during 2016, in which 40% used electrochemical techniques, 30% was related to mass transfer approaches and 25% to the bioremediation assisted by surfactants; however, an integral study, including the role of biosurfactant or bioemulsifier in the hydrocarbon mass transfer resistances diminishment and microbial degradation rates in oil contaminated water, is not currently found.

This thesis proposed a novel method that consisted in two successive cultures: firstly, solid state culture, wherein the presence of a controlled electric field, as pretreatment, produced a fungal biocatalyst; subsequently, in a liquid airlift culture the biocatalyst was used for hydrocarbon degradation in an artificialy contaminated water, mimicking an oil refinery wastewater hydrocarbon blend. Therefore, this thesis was aimed to characterize the catalytic performance during the liquid phase hydrocarbon degradation. The evaluation of the biocatalyst mechanical resistance enhanced by the electric field pretreatment, its hydrocarbon sorption capability, as well as the hydrocarbon mass transfer resistances and degradation kinetics carried out. Besides, the elucidation of a role of an emulsifier protein produced during hydrocarbon degradation.

This thesis is divided into five chapters, including the Introduction section. In Chapter 2, a bibliographical overview, considering the hydrocarbon as pollution source from the oil refineries wastewaters, is presented. The possible methods for bioremediation approaching a heterogeneous catalytic process development are described. Chapter 3 presents preliminary results that gave origin to the catalytic performance characterization wherein the biocatalyst was evaluated as hexadecane sorbent-degrader. Chapter 4 shows the catalytic performance characterization and the phenomenological sequence here considered, this chapter includes: (i) fungal biomass attachment to the support, (ii) all mass transfer resistances and (iii) both hydrocarbons sorption and degradation improved by an emulsifier agent produced as a consequence of the electric field. Finally, Chapter 5 presents a functional characterization of the emulsifier protein, which was produced during hydrocarbon degradation in the airlift bioreactor.

2. Bibliographic review

2.1.Hydrocarbons as pollutants

Oil products are the worldwide highest energy sources, so the major environmental pollution problem. In Mexico, 85% of environmental emergencies reported during 2015 were associated to hydrocarbons activities [1]. The principal pollution sources are the oil refineries wastewaters [2, 3]; which can't generally be characterized due to their variable production and composition; however, a mean value of 3.5-5 m^3 per processed oil ton is estimated. The observed hydrocarbon concentration in oil refinery wastewaters may be up to 3000 mg L^{-1}, depending on the performed processes: distillation, catalytic reforming, catalytic cracking, hydrocracking or desulfurization [4]. During 2014, 1184 millions of barrels per day were processed in Mexico [5], the wastewater discharges could be about 4.6-6.6 x 10^5 m^3 per day.

When wastewaters are discharged without previous depuration, several negative consequences can be presented; in which, some of these could affect the human health due to most of the oil components are highly hazardous and carcinogenic; *e.g.* polycyclic aromatic hydrocarbons [3]. Then, it is necessary to design and implement efficient technologies for oil contaminated water treatment, before they are discharged in the environment.

2.2. Oil contaminated water treatment

Wastewater depuration process consists of three main treatments: (i) primary, physical separation; (ii) secondary, chemical or biological degradation and; (iii) third, usually chemically treatment [6].

Nowadays, several physical and chemical highly efficient oil contaminated water treatment methods have been developed, *e.g.* El-Naas *et al*. [7] proposed electrocoagulation using aluminium electrodes arrangement; in which, 63% of organic matter was removed. Yan *et al*. [8] used an electrochemical oxidation process, in which Fe particles and an airflow were introduced oxidizing 92.8 % of total hydrocarbon. On the other hand, Hami *et al*. [9] used a flotation method for removing 72-92 % of the organic matter. The physical and chemical methods for oil contaminated water could be highly effectives; however, they are expensive

and produce excessive amounts of sludges, which often content toxic compounds. The fate of excess sludges is normally dehydration and incineration [10].

The most common secondary treatment is performed in two steps: (i) a stirred and aerated tank with activated sludges and (ii) a clarifier where suspended biomass precipitates, then it is recirculated to the stirred tank [11,12,13]. Recently, several reactor configurations have been developed to enhance the hydrocarbon removal/degradation efficiency. Ishak *et al*. [11] compare some reactor configurations, concluding that sequential stirred tank bioreactors are easily operable. Another configuration is membrane bioreactors which are highly efficient, but operationally expensive than stirred tank. On the other hand, the use of fluidized bed, such as airlift bioreactors allows reaching similar removal and degradation efficiencies than stirred tank bioreactors; also, their design and operation is more accesible. Moreover, if an airlift bioreactor operates with immobilized microorganisms, highly hydrocarbon degrader active biomass concentration can be controlled, cancelling the biological sludges excess production [14]. For instance, Khondee *et al*. [15] used an internal loop airlift bioreactor inoculated with *Sphingobium* sp., immobilized in chitosan particles, for degradation of lubricants in wastewater, they observed 90% of total petroleum hydrocarbons degradation, operating in continues mode during 70 d. Immobilized biomass is a better alternative for wastewater treatment than free suspended aerobic microorganisms or sludges, due to the capability of biomass concentration control. The excess of biological sludge production becomes an operational problem in which, 40-60 % of operation costs could be intended for sludges disposal [16]. Another attractive option, although poorly studied, for biological oil contaminated water treatment is the use of filamentous fungi, which can produce extracellular enzymes able to oxidize the hydrocarbons. Thus, quickly absorb them into the biomass, also morphology (pellets or immobilized on inert support) allows separating the biomass from the effluent without an extra unit operation [16]. Since hydrocarbons are compounds water immiscible, their microbial degradation is a multiphasic process which could be limited by: (i) interfacial and diffusional mass transfer resistances and (ii) microbial capabilities for hydrocarbon uptake.

2.3. On the limitations and uptake mechanism of hydrocarbon biodegradation

During hydrocarbons degradation, the main weakness is their low bioavailability, which is result of their low solubility [17]. An alternative for enhancing the hydrocarbon bioavailability is the abatement of the interfacial tension that could be achieved by using surfactants, which are amphiphilic molecules able to improve the interfacial uptake of hydrophobic substrates. The use of synthetic surfactants could be self-defeating when they are in high concentrations because they could be inhibitors of hydrocarbon degradation; so, using microbial strains that produce biological surfactants (biosurfactants) is a better option than synthetics [18]. Biosurfactants, can enhance the solubility of the hydrophobic compounds forming micelles and increase the interfacial surface area by stable emulsions formation. They can also modify the cell surface hydrophobicity, allowing direct contact between microorganisms and the hydrophobic substrate [19] *e.g.* Bouchez-Naïtali *et al*. [20] discussed that long chain alkanes can be degraded by two main ways: (i) direct contact removal and (ii) surfactant mediated removal.

On the other hand, the filamentous fungi can produce small proteins, with high surface activity, denominated hydrophobins [21]. Hydrophobins have a defined biological role. They are produced and excreted to the culture medium to diminish the surface tension that way fungi can upload to the gas phase and form aerial hyphae, which are hydrophobic by a hydrophobin coating [22]. Another biological role of hydrophobins is acting as an anchor that facilitates the fungal biomass attachment on solid supports or hydrophobic substrates, such as hydrocarbons [23].

Another way for the interfacial tension abatement is the presence of an electric field, which modifies the electrical surface properties, altering the adsorption phenomena on the hydrophobic-hydrophilic interface [24]. The combined methods for hydrocarbon bioremediation (biological and electrochemical methods) has been demonstrated as bioremediation enhancers by two ways: (i) enhancing the microbial hydrocarbon degrader activity [25] and (ii) modifying surface interactions between hydrocarbons, aqueous phases and solids [26].

2.4.Combined biological-electrochemical hydrocarbon biodegradation

During the last two decades, combination of biological and electrochemical treatments has been of great interest for hydrocarbon degradation, arising from the electrokinetic approach for contaminated soils remediation; however, all secondary phenomena produced by the electric field, such as changes in temperature and relative humidity, make challenging to scale up and perform an electro-bioremediation in a real application [27]. So, along the time, the researchers have observed that the exposure of microbial communities to a low intensity electric field, modifies the hydrocarbon biodegradation rates and the composition of communities near to the electrodes [25, 28]. The electric field *in situ* application can modify the dehydrogenase activity in the soil modifying the microbial viability and growth kinetics [29], maintaining these modifications for a period [30]. All effects mentioned above of electric field applications suggest the use of electrochemical bioreactors, where the operational conditions can be controlled and the secondary effects diminished.

Currently, microbial metabolic changes promoted by an electric field are not completely understood; however, bacterial respiratory activity is directly related to a low intensity electric field [31]. In general, microbial metabolic changes have been studied in bacteria, but not in filamentous fungi; although, in our research group *Aspergillus brasiliensis* (formerly *A. niger*) was exposed to a controlled electric field during growth with hexadecane as carbon source. It was found that molecular oxygen consumption was increased by the electric field, promoting high lipoperoxidation levels and consequent alterations in membrane permeability [32] As a consequence, an enhanced mineralizing activity and low biomass production were observed [33]. In order to find the maintenance of the metabolic changes observed in *A. brasiliensis* exposed to an electric field, a two subsequent cultures strategy was performed *i.e.* the fungus, previously exposed to the electric field, used as a biocatalyst for hexadecane biodegradation in a subsequent solid and liquid culture. The hexadecane degradation was observed in both cultures without biomass production and an emulsifier production in the liquid culture [34]. Then, an interest of hydrocarbon biodegradation in the liquid culture aroused focused on the role of the emulsifier in the hydrocarbon degradation using the aforementioned fungal biocatalyst, previously exposed to an electric field as an activation procedure. So, in this work more complex hydrocarbons, such as polycyclic aromatics in a blend with an aliphatic were assayed in order to expand our knowledge on the applicability

of the biocatalyst. Hydrocarbon biodegradation was evaluated as a heterogeneous biocatalytic reaction.

2.5.Hydrocarbon biodegradation; a heterogeneous catalytic process

Bioremediation is a, natural or human handled, process in which biological catalysts are used for pollutants degradation, whether in water, wastewater, sludges, soils, aquifers or gaseous streams [35]. Hydrocarbon contaminated water treatment in multiphasic bioreactors is a heterogeneous catalytic reaction, carried out in seven sequential steps [36]:

1. Hydrocarbon mass transfer from contaminated matrix to the catalytic particle.
2. Hydrocarbon diffusion into the catalytic particle pores.
3. Hydrocarbon sorption onto the catalytic particle.
4. Hydrocarbon degradation reactions.
5. Excretion of intermediates and final hydrocarbon oxidation products.
6. Diffusion of hydrocarbon oxidation products.
7. Products transport to the matrix.

Since hydrocarbons remain in an organic phase which is non-water soluble, the first step could be limiting the hydrocarbon degradation rate. The first three steps, which refer to hydrocarbon removal from the contaminated matrix, can be enhanced by adding a surfactant. The enhancement could be related to hydrocarbon pseudo-solubilisation or emulsification as previously reported [37].

Some reports about hydrocarbon remediation suggest that the transport phenomena, associated to the first three steps, could be the main limitations. Then several studies have been committed to mass transfer resistances diminishment, *e.g.* Vieira *et al*. [38] proposed an intermittent aeration strategy for improve the depuration of gasoline and diesel contaminated water. The approach allowed to enhance the hydrocarbon degradation from 70% to 90 % in 22 d. Both oxygen and hydrocarbons are immiscible with water; however, the hydrocarbon solubility and mass transfer to the aqueous phase are slower than oxygen. Lizardi-Jiménez *et al*. [39] found that hexadecane mass transfer rate was stoichiometrically four times lower than oxygen transfer rate to the aqueous phase in an airlift bioreactor during hexadecane degradation. The three-phase system was observe, where the microbial cells were in suspension; but, if the microorganisms were immobilized on a porous support, an

additional internal mass transfer resistance would be present as diffusion into the pores, which would be more critical than interfacial mass transfer, having a great influence on the kinetics of hydrocarbon degradation.

Even though the transport phenomena could be the limitations during hydrocarbon degradation, it is necessary to discern amongst all steps involved into the hydrocarbon degradation. One way to achieve this is through the regimen analysis, in order to find the slowest step, which is the limiting for all degradation process [40].

2.6. Scope of the thesis

Hydrocarbon biodegradation in contaminated water is a heterogeneous process in which the limiting step could be on the mass transfer resistances or in the intrinsic kinetics of degradation reactions. *Aspergillus brasiliensis* formerly (*A. niger*) was proposed as a porous attached fungal biocatalyst with hydrocarbon degradation activity, produced in solid state culture which is a possible oil contaminated water remediator. The biocatalyst after an electric field pretreatment enhanced its hydrocarbon degrader activity acquiring a bioemulsifier production capability in a subsequent liquid culture. The bioemulsifier could have an enhancing hydrocarbon degradation role, either in the transport phenomena or in the reaction rates. So, it is necessary to evaluate each step related to the global degradation phenomenologically. A regimen analysis is a useful tool for finding the limiting step, then the obtained information could be a starting point to an efficient bioreactor design and operation.

Since the previously observed biocatalytic hexadecane biodegradation activity, in this thesis, an interest for more complexes hydrocarbons (such as polycyclic aromatic) degradation in an airlift bioreactor arise. The central hypothesis that supports this thesis is that the bioemulsifier produced after an electric field application diminish all the mass transfer resistances, enhancing the hydrocarbon blend degradation. So that, this thesis aimed to evaluate the hydrocarbon degradation using the fungal biocatalyst in an airlift bioreactor, visualized as heterogeneous biocatalytic process. All mass transfer limitations and the role of the bioemulsifier on the hydrocarbon uptake mechanism and the mass transfer resistances diminishment were evaluated as a consequence of an electric field pretreatment.

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3. Electric field as pretreatment to enhance the activity of a whole-cell biocatalyst for hydrocarbon degradation in contaminated water

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Abstract

In this paper, a method using two successive cultures, solid then liquid, for the production and use of a hydrocarbon degrading biocatalyst (BC) is proposed. Hexadecane (HXD) was used as a model hydrocarbon. An electric field was applied during the solid state culture (SSC) as a novel approach, denoted the electric field pretreatment, to enhance the HXD degrading activity. Afterward, the catalytic activity (CA) was determined in liquid culture. The CA of the biocatalyst, pretreated and untreated, was evaluated discriminating between sorption capacity and HXD degradation rate; biomass production on the EF pretreated BC was only 20% of that on the untreated BC, but the maximum BC sorption capacity was enhanced from 119 $\pm$ 41 mg (g BC)$^{-1}$ to 207 $\pm$ 23 mg (g BC)$^{-1}$ by the effect of the EF pretreatment. The activity of the biocatalyst was mainly associated with the pretreated fungal biomass; its activity was 9-fold higher than that of the untreated biomass. Linear model was used to obtain the affinity constant and Langmuir to adjust maximum sorption capacity. This enhancement in sorption capacity was associated with the high HXD degradation rate observed here; 86% of the initial HXD was eliminated in 42 h by the pretreated BC, whilst 53% was eliminated in 48 h by the untreated BC.

Keywords

Electric field, catalytic activity, sorption capacity, air-lift bioreactor, hydrocarbon degradation.

3.1.Introduction

Effluents from the oil industry are an important source of air, water and soil pollution. Oil extraction produces large volumes of wastewater discharge, approximately 10 times higher than the volume of oil extraction [1]; the chemical composition depends on the nature of the geological formation [2]. Moreover, oil refineries can produce an average flow of wastewater from 3.5 to 5 m^3 per ton of processed crude; reusing water cooling [3].Their composition varies according to the process to be carried out at each refinery [4]. A total of 1.184 million barrels per day of crude refining [5] was processed in Mexico during 2010. This could generate an average discharge of wastewater ranging from 460 000 to 660 000 m^3 d^{-1}. Biological methods are often suggested and even used to treat the effluent generated by the oil industry [4]. Regarding the configuration of bioreactors used to treat hydrocarbon-contaminated water, fluidized bed reactors achieve higher degradation rates over stirred tank or fixed bed bioreactors. Cell immobilization in the bioreactor can also improve biomass growth control [6]. A clear advantage of using immobilized cells is the savings in subsequent treatments generated by excessive biomass production, eliminating operational problems such as the stagnation caused by solid particles [7]. Sokól [8], using the sludge from the wastewater treatment plant of a refinery, immobilized cells on polypropylene particles and reported a 90% reduction in the organic matter. Air-lift bioreactors are one type of fluidized bed that can be used for the purpose of remediating contaminated water; for example, Khondee *et al*. [9] used an internal loop air-lift bioreactor with *Sphingobium sp.* immobilized on chitosan, observing 80–90% removal of total petroleum hydrocarbons (TPH) from initial concentration of 200 mg L^{-1} operating with a hydraulic retention time of 2 h.

Some research on microbial hydrocarbon degradation has visualized microbial cells as BCs able to remove environmental pollutants, and evaluated their metabolic activity; interest has focused primarily on understanding the relationships among the bioavailability of contaminants, transport mechanisms into the cells and the ability of microorganisms to remediate polluted environments [10]. More recent studies suggested a mechanism of removing contaminants in two stages, consisting of simultaneous sorption and degradation [9. 11]. However, in all cases strains endowed with a large capacity for hydrocarbon degradation were used. One way to increase the efficiency of microorganisms to degrade hydrocarbons is through the application of electric fields. There have been several studies on

the effects caused by exposing microbial communities to an electric field. Changes in the soil dehydrogenase activity and composition of microbial communities near to the anode have been reported [12, 13]. Ye *et al*. [14] observed a 20.3% increase in the HXD degradation rate by bacteria isolated from a contaminated site on the edge of Daqing Oil Field by applying a constant voltage gradient of 1.3 V cm^{-1} for 42 days. Previously, our group showed that exposure of the mycelium of *A. niger* to an electric field during HXD degradation changed the microbial metabolism, orienting it towards catabolic pathways. Apparently, important changes occur at the membrane level that favour the passage of HXD into the cells and, consequently, its mineralization [15]. This paper proposes the use of A. niger immobilized on perlite, growing in solid state culture as follows: (i) spores germination during 4.5 d, (ii) electric field treatment (1.0 d), (iii) growth after electric field treatment (6.5 d); resulting in a supported BC (total 12 d), as a novel approach to increase its activity in degrading HXD in a subsequent culture cycle (in liquid culture) under the assumption that the changes promoted by the electric field are maintained. BC activity was evaluated according to the Langmuir-Hinshelwood mechanism for a heterogeneous catalytic reaction, always distinguishing between surface activity (sorption capacity at equilibrium) and the HXD degradation rate.

3.2.Materials and methods

The experimental strategy proposed here consists of two stages (Fig. 1). First, a previously reported technique [15] was used to produce a fungal whole-cell BC (*A. niger* supported on perlite) on SSC. At this stage, an electric field was applied in a controlled manner as a pretreatment to modify the physiology of the fungus. In the second stage, the BC was used in serological bottles to determine surface activity (HXD sorption capacity) and, finally, an air-lift bioreactor containing the BC in suspension was prepared in which HXD-degrading activity was also evaluated. During the second stage a comparison between the pretreated and untreated BC was also performed.

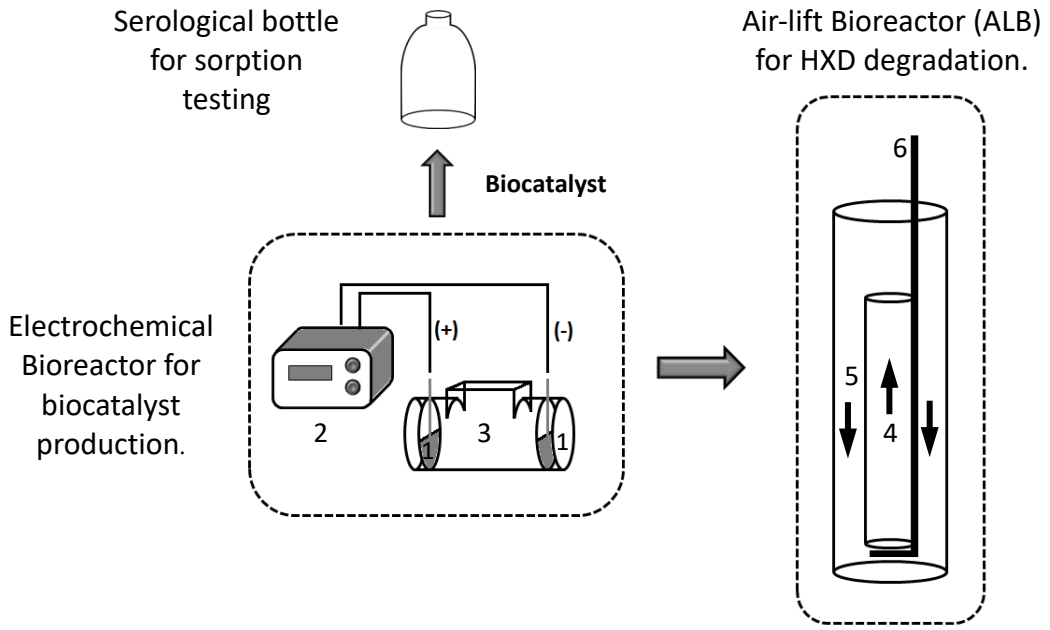

Figure 1. Scheme of production-use of the biocatalyst, (1) electrodes and electrolyte compartments, (2) potentiostat, (3) solid state culture bioreactor, (4) rising zone into air-lift bioreactor, (5) down comer zone into air-lift bioreactor, (6) air distributor.

3.2.1. Microorganism

The strain was *Aspergillus niger* ATCC 9642, propagated in flasks with 50 mL of potato dextrose agar (PDA). It was seeded by groove and held for 7 days at 30°C. Spores were harvested using 0.05% Tween 80 and the spore suspension was used as an inoculum to produce the BC. The strain was stored on PDA slants at 4°C, reseeded every 2 months.

3.2.2. Biocatalyst production

The BC was produced by SSC using a horizontal cylindrical bioreactor 450 mL in volume (Fig. 1) packed with 15 g of dry inert support (DIS). During production, an electric field was imposed (see below). Hereafter this will be referred to as the electric field pretreatment. Perlite (Dicalite, México, particle size 1.19–1.68 mm) was used as an inert support [15]. It was impregnated with HXD (SIGMA-ALDRICH, 99%) (180 mg (g DIS)$^{-1}$) dissolved in hexane (20% v/v); afterwards, it was spread in trays to evaporate hexane at room temperature. To the impregnated and dried perlite was then added 2.33 mL (g DIS)$^{-1}$ of mineral medium. The mineral medium was composed (in g L^{-1}) of: $NaNO_3$, 21.230; KH_2PO_4, 3.004; $MgSO_4$, 0.882; KCl, 3.050; trace element solution, 6 mL L^{-1}. The trace element solution was composed (in g L^{-1}) of: $FeSO_4 \cdot 7H_2O$, 0.001; $CuSO_4 \cdot 5H_2O$, 0.015; $ZnSO_4 \cdot 7H_2O$, 0.161;

$MnSO_4 \cdot 7H_2O$, 0.008. The medium was sterilized at 15 psi for 15 min; the initial moisture content was 70%. Biomass and residual HXD content in the BC was determined at the end of the SSC (12 d).

3.2.3. Electric field pretreatment

The bioreactor used to produce the BC was provided with two 12 cm separated electrodes (built in titanium coated with ruthenium oxide with a contact surface of 14 cm^2) both completely submerged into the solid medium, and two reservoirs for electrolyte (0.1 M KH_2PO_4) one for each bioreactor end (Fig. 1). The bioreactor was packed with perlite impregnated with HXD, mineral medium and inoculum. After spore germination (4.5 d) a 6 mA electric current was applied (resulting in 0.75 V cm^{-1} across the wet culture porous media) for only 24 h, after which growth was allowed to continue up to 12 d. Afterwards the catalytic activity was evaluated for HXD degradation in a subsequent liquid culture cycle, using an air-lift bioreactor with model contaminated water.

3.2.4. Sorption isotherms

The BC produced was sterilized at 15 psig for 15 min to eliminate metabolic activity; afterwards it was used for sorption experiments. To analyse the kinetics of sorption, serological bottles (150 mL) were used, containing 50 mL of MM to which were added 2.6 g L^{-1} HXD and 3 g L^{-1} of BC or perlite (inert support) as a control. All treatments were agitated at 150 rpm (Incubator shaker I26, New Brunswick, USA). In order to measure sorbed HXD, all serological bottles were filtered (Whatman paper 42), thoroughly water-washed and dried (60°C). The HXD concentration was measured every 6 h until a steady state was achieved. All measurements were performed in both the BC and perlite alone under the same conditions, both pretreated and untreated. To generate isotherms the initial HXD concentration in the MM was modified as follows: 0, 0.325, 0.65, 1.3, 2.6 and 5.2 g L^{-1}. After 48 h, the HXD dispersed in the aqueous phase and that sorbed on the solid phase were measured.

3.2.5. Sorption-degradation test

Sorption-degradation tests were performed at the same conditions as sorption isotherms, using BC sterile and non-sterile. HXD concentration was measured in both liquid and solid phases. To quantify degraded HXD, a mass balance was performed. Total HXD mass was

quantified (solid plus liquid) and then normalized based on maximum initial HXD mass assayed (260 mg per serological bottle in the case of 5.2 g L^{-1}). Final quantified HXD mass was graphically compared to initial HXD mass. If the resulting slope (measured HXD (theoretical HXD)$^{-1}$) is 1.0, then the total initial HXD mass is detected; whilst a slope lower than unity represents non-degraded HXD and the difference to the unity is the degraded HXD fraction.

3.2.6. Catalytic activity for HXD degradation

Biocatalytic activity was determined via HXD-specific consumption. Batch liquid cultures were set up using a cylindrical glass air-lift bioreactor (7 cm diameter, 50 cm length) provided with a concentric tube (4.2 cm diameter, 20 cm length) located 2.8 cm above the bottom with model contaminated water, i.e. 1 L of mineral medium (MM) supplemented with 1.3 g L^{-1} of HXD and 5 g L^{-1} of BC. Pneumatic agitation was provided by air-sparged into the concentric tube at 6 cm s^{-1} (5 VVM). The HXD concentration was measured every 6 h during 48 h. The mineral medium (MM) was composed (in g L^{-1}) of: $NaNO_3$, 0.3575; KH_2PO_4, 0.0059; $MgSO_4$, 0.0303; KCl, 0.0514; trace element solution, 2 mL L^{-1}. To distinguish the surface activity of the catalyst, sorption isotherms were carried out.

3.2.7. Analytical methods

3.2.7.1.Biomass in the biocatalyst

Biomass was quantified through soluble protein determination using the Lowry method (1951). Dry samples (0.5 g) were milled with a mortar and pestle, suspended in 10 mL of 0.5 N NaOH and boiled in a water bath for 10 min. Soluble protein was quantified in the hydrolysed samples with a commercial kit (Bio-Rad Dc protein Assay Kit). A calibration curve was obtained from the biomass of *A. niger* previously grown in 250-mL shaken flasks (50-mL of liquid medium; 72 h). The biomass was vacuum-filtered through Whatman 42 paper and milled. Soluble protein was determined spectrophotometrically with a Varian Cary 50, Australia.

3.2.7.2.HXD quantification

HXD content in the BC was obtained by solid-liquid microwave-assisted extraction using a MARS 5 unit, CEM, USA. Dry samples (1.0 g) were suspended in 10 mL each of a hexane-

acetone mixture (1:1) and extraction was performed for 15 min at 150 °C; the resulting extract (1.5 mL) was filtered and HXD was quantified by gas chromatography. For quantification of HXD dispersed in the liquid medium, liquid-liquid extraction was used. Five millilitres of each liquid sample was taken and mixed with 5 mL of a hexane-acetone mixture (1:1). The mixture was stirred and allowed to stand for 30 min. Then HXD was quantified in the organic phase by gas chromatography.

The HXD extracted was quantified by gas chromatography using a Varian 3900, USA, with a flame ionization detector (290°C). An Alltech 16367 capillary column was used (15 m x 0.25 mm x 0.1 µm) with helium as carrier (30 mL min^{-1}, 40 psi); the column temperature was 120°C initially then increased up to 200°C at 30°C/min. External standards were used.

3.2.8. Mathematical model

The results for the sorption isotherms were adjusted to the Langmuir model and the parameters defining the isotherms for the support (perlite sole), untreated BC and pretreated BC, were compared. The Langmuir model describes the amount of HXD sorbed on the solid phase as a function of HXD dispersed in the liquid phase, both variables measured at equilibrium, and is defined by two parameters indicating the ability of the sorbent to retain a compound and the affinity between the sorbate and sorbent. The model is described according to the following equation.

$$q_A = \frac{q_A^* C_A}{K_{Af} + C_A}$$

Where q_A is the concentration of HXD in the solid phase (expressed in g (g DM)$^{-1}$), C_A is the concentration of HXD dispersed in the liquid phase (expressed in g L^{-1}), q_A^* is the maximum sorption capacity (expressed in g (g DM)$^{-1}$) and K_{af} is the affinity constant between HXD and solid (expressed in g L^{-1}), defined as the equilibrium desorption constant for HXD. If the assayed concentrations are less than saturation concentration, a linear isotherm can describe the experimental data, and then, the model is according to the following equation:

$$q_A = KC_A$$

Where K is an apparent affinity constant, expressed in L (g DM)$^{-1}$.

3.2.9. Statistical analysis

All measurements were performed in triplicate and are presented as average value and standard deviation. The obtained data for the sorption isotherms were analysed by nonlinear regression using the Marquardt-Levenberg (1944) algorithm and the thermodynamic parameters of the Langmuir model were estimated. In order to compare Langmuir and linear model, the obtained data also were analysed by linear regression; these are presented as the estimated values and standard errors. The estimated parameters were compared using ANOVA and Tuckey test (α=0.05). For comparisons between the activity of the support and the pretreated and untreated BCs, pair-wise curves using the t-Student test (α=0.05) were contrasted. The statistical package IBM SPSS 18 was used.

3.3.Results and discussion

3.3.1. Electrochemical pretreatment

When the electric field was applied during growth of *A. niger* in SSC, the consumption of HXD was enhanced, increasing from 84.7$\pm$1.2% to 94.6$\pm$1.2%, and biomass production was decreased from 168.0$\pm$12.3 mg (g BC)$^{-1}$ to 35.9$\pm$1.9 mg (g BC)$^{-1}$ by the untreated and pretreated BC, respectively. Similar results (carbon mineralization increased from 24% to 74% when the electric field was applied) were obtained by Velasco-Alvarez *et al*. [15], who first demonstrated that the application of an electric field modifies *A. niger* metabolism, reorienting it towards catabolic pathways. Podolska *et al*. [16] showed that the application of low intensity electric pulses (20 V) to *Pseudomonas fluorescens* can modify respiratory activity and the changes were reversible. Based on the hypothetical assumption that the time required to reverse the effects of the electric field on *A. niger* is sufficiently long, the electric field in our work was applied as an electrochemical pretreatment in order to evaluate the catalytic activity of the BC by means of the surface activity (HXD sorption) and the HXD transforming capacity.

3.3.2. HXD sorption kinetics

The HXD sorption kinetic runs were analysed for perlite and BC (both pretreated and untreated) in order to determine the steady state time. The HXD concentration related to one gram of the dry perlite reached was 5.3 $\pm$ 0.1 mg (g DM)$^{-1}$ (Fig 2). This concentration is low compared to sorbents used for hydrocarbon removal in polluted water; for example, activated

coal or hydrophobic clays, among others [17]. After applying the electric field, the perlite's sorption capacity decreased, achieving a HXD concentration of 1.2 ± 0.1 mg (g DM)$^{-1}$ in the steady state. This might be because of a change in surface charge distribution provoked by the electric field. The resulting redistribution of surface charges may change the electrokinetic properties of the solid-liquid interface [18]; therefore, the affinity between perlite and HXD could be negatively affected. In both cases a steady state was observed after 30 h.

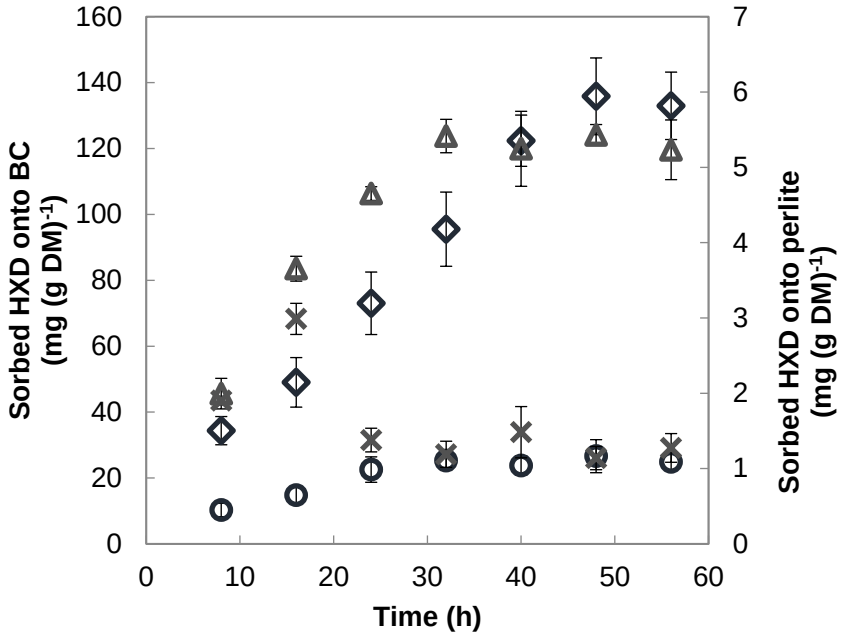

Figure 1. HXD sorption kinetics of pretreated perlite (✖), untreated perlite (▲), pretreated biocatalyst (◆) and untreated biocatalyst (○).

In the case of the untreated BC a steady state was reached after 24 h, with a sorbed HXD concentration of 25.1 ± 1.1 mg (g BC)$^{-1}$. With the pretreated BC, the steady state was not reached until 40 h, showing a concentration of 134.4 ± 2.0 mg (g BC)$^{-1}$. It was clear that EFPT increases the sorption capacity, but only in the presence of fungal biomass; even more, the change in sorbed HXD on the biomass was an order of magnitude greater than that observed on perlite. Based on these results, we decided to run experiments up to 48 h in order to construct sorption isotherms.

3.3.3. HXD sorption isotherms

Sorption isotherms (shown in Figure 3) were performed with three different sorbents: (i) perlite, (ii) the untreated BC and (iii) the pretreated BC. The pretreated perlite was not taking

into account because its sorption capacity totally decreased after pretreatment. The highest concentration of HXD observed in perlite was 9.7±1.7 mg (g DM)$^{-1}$ and in the untreated BC it was 34.0±6.8 mg (g DM)$^{-1}$ (Fig. 3). The difference may be because the biomass covering the support was probably hydrophobic and could increase the affinity between the BC and HXD. Teas *et al*. [19] suggest that oil sorption capacity could be increased by increasing the hydrophobicity of the perlite. In some reports, it is argued that an important property of a good hydrocarbon sorbent is surface hydrophobicity, such as activated coal from lignocellulosic biomass [20]. For example, Chen *et al*. [21] attribute the high sorption capacity of polycyclic aromatic hydrocarbons (80–90% in a week) by a consortium of white rot fungi to the surface hydrophobicity of the biomass.

When analysing the pretreated BC, it was observed that the sorption capacity increased by an order of magnitude compared to untreated BC up to 147.8±14.2 mg (g BC)$^{-1}$ (see Figure 3). To our point, this is the first work that discusses the sorption capacity of a microorganism previously modified with an electric field. Some researchers have suggested that application of a low intensity electric field may affect the permeability of cell membranes; however, the detailed effects of applying a low intensity electric field on the surface properties of microbial cells are unknown [16, 22].

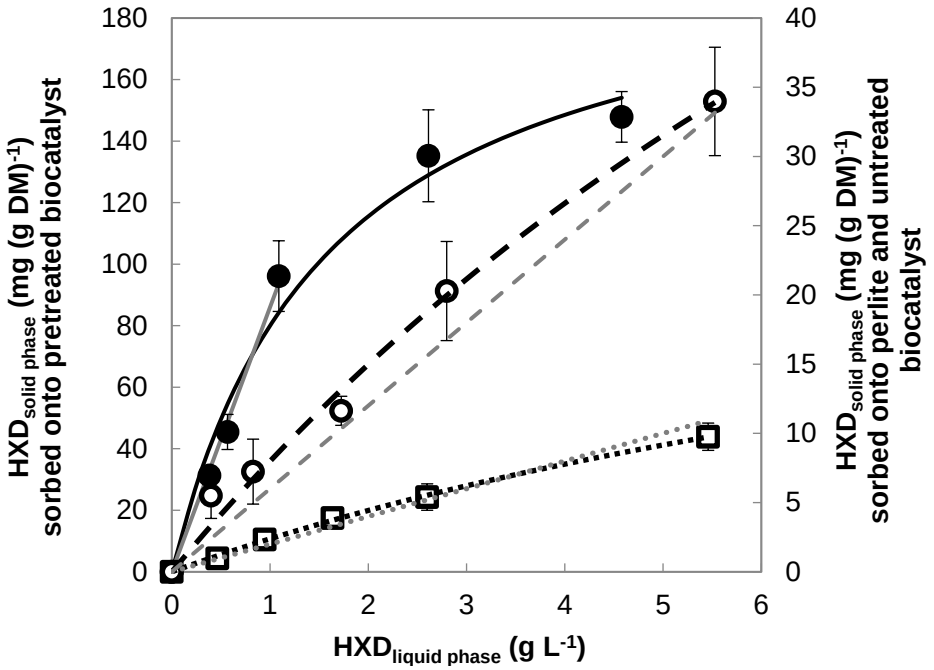

Figure 2. Sorption isotherms of HXD onto perlite (▫), untreated biocatalyst (○) and pretreated biocatalyst (●). Langmuir (black) and linear (grey) model fitting.

In order to perform a quantitative comparison of HXD sorption among three of the studied sorbents, the experimental results were fitted to the Langmuir and linear models and characteristic parameters defining the isotherms were compared. Table 1 shows the values of the parameters generated by two models for each sorbent.

Table 1. Estimated Langmuir and Linear Isotherms parameters for the three different sorbents.

Sorbent	**Langmuir R^2**	**K_A (10^4) (mg L^{-1})**	**q^*_{HXD} (mg (g DM)$^{-1}$)**	**Q^*_{HXD} (mg (mg BM)$^{-1}$)**	**Linear R^2**	**K (10^{-3}) (L (g DM)$^{-1}$)**
Perlite (support)	0.999	1.23 ± 0.18^{a}	32 ± 3^{A}	---	0.99	$2 \pm 0.1^{\#}$
Untreated Biocatalyst	0.99	1.39 ± 0.62^{a}	119 ± 41^{B}	0.7	0.98	$6 \pm 0.3^{\dagger}$
Pretreated Biocatalyst	0.98	0.15 ± 0.04^{b}	207 ± 23^{C}	5.7	0.99	$86 \pm 2^{\ddagger}$

Langmuir Isotherm parameters: K_A, affinity constant; q^*_{HXD}, maximum sorption capacity; Q^*_{HXD}, maximum sorption capacity related to biomass (BM). Linear Isotherm parameter: K, affinity constant. The average value and standard error are expressed. Different letters or symbols represent significant difference.

The affinity constant values for the support and the untreated BC are not actually well explained by the Langmuir model, because the large standard error found (approximately 40% of the average) was probably due to the absence of a concentration range where the amount of sorbed HXD was independent of the HXD concentration in the liquid phase, i.e., the concentration related to saturation was not clearly observed, in this case it was observed a linear isotherm. Linear model shows that the slope was highest for preteated BC (0.086 L $(g\ DM)^{-1}$), lower for untreated BC (0.006 L $(g\ DM)^{-1}$) and lowest for untreated perlite (0.002 L $(g\ DM)^{-1}$). Pretreated BC was analysed considering only the linear range (from 0 to 1.3 g L^{-1} of initial HXD). The maximum sorption capacity (q_A*) was obtained from Langmuir model. The highest q_A* value was observed for pretreated BC (207 mg $(g\ DM)^{-1}$), lower for untreated BC (119 mg $(g\ DM)^{-1}$) and lowest for untreated perlite (32 mg $(g\ DM)^{-1}$). The estimated values of the Langmuir parameters for the pretreated BC were similar to those reported by El-Naas *et al*. [23], where date-pit activated coal was used to adsorb organic matter. Those values were 252 mg g^{-1} for maximum sorption capacity, respectively. Activated coal is classified as a good sorbent of organic matter. Considering that the active phase of the BC is the biomass of *A. niger* and biomass production in the pretreated BC was only 21% compared to the untreated BC, we observed that the pretreated biomass sorbs 9-fold higher than the untreated biomass (Table 1). Carberry [24] mentions that the activity of a catalyst is related to the surface concentration of affinity sites for the reactants; thus, knowing the sorption capacity helps to understand and correctly interpret the catalytic activity. In order to discriminate between the possible effects of autoclaving on the sorption capacity and that induced by the electric field pretreatment, we decided to conduct a similar experiment to generate sorption isotherms but without sterilizing the BC. In this experiment, sorption phenomena and simultaneous degradation were expected.

3.3.4. Sorption-degradation test

In order to exclude the effect of autoclaving on the sorption capacity of the pretreated BC, non-sterile BC was used and a similar sorption isotherm experiment was performed; the results are shown in Figure 4. The resulting HXD concentrations were similar to those observed in the sorption isotherms with the pretreated BC (Fig. 3); however, the dispersed HXD concentrations in the liquid phase in our sorption-degradation test were lower than those obtained in the autoclaved assays, suggesting that a portion of the initial HXD was metabolized by the living fungus.

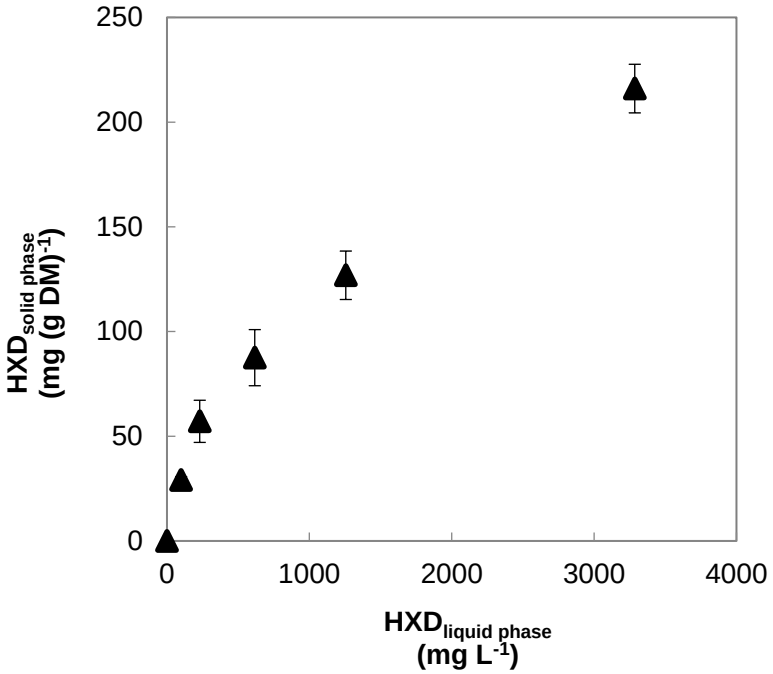

Figure 4. HXD concentration onto non-sterile pretreated biocatalyst as a function of the dispersed HXD concentration at T = 48 h.

In order to quantify the HXD that was probably metabolized, we performed a mass balance for HXD at the end of the experiment, identifying only 73 % of the initial HXD (Fig. 5), whilst in the autoclaved BC 100 % of the HXD was identified. Our results suggest that the sorption capacity was not affected by sterilization and the pretreated BC maintained its acquired surface activity when pretreated. In order to confirm the HXD consumption capacity, we evaluated the catalytic activity of the BC in liquid medium using an air-lift bioreactor.

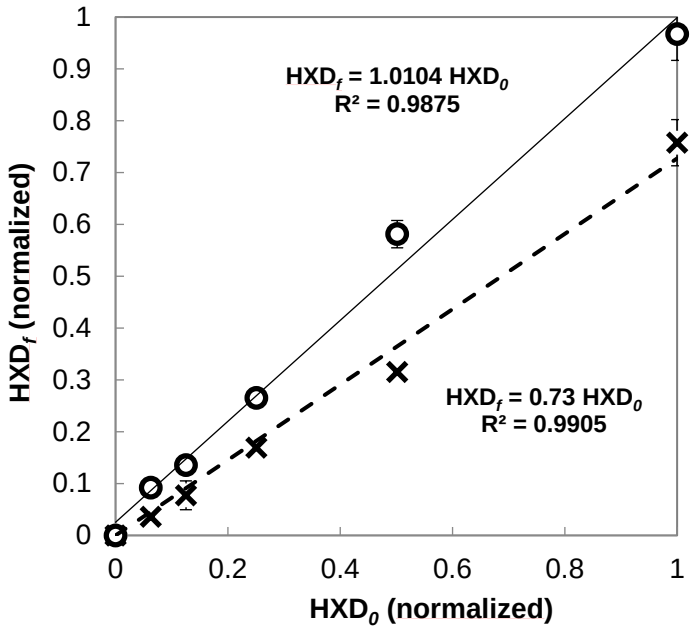

Figure 5. HXD balance at the end of the sorption-degradation test with HXD at T = 48 h using sterile (⚬) and non-sterile biocatalyst (✖). Normalized values are expressed based on the maximum initial HXD mass.

3.3.5. Hexadecane degradation in liquid medium

As far as we could determine, the electrochemical pretreatment affected the surface activity of the BC; therefore, we evaluated the catalytic activity in the liquid medium, again expecting an increase in the HXD degradation rate. The results are shown in Figure 6. The untreated BC consumed 53% of the initial HXD (0.81 mg HXD (mg BM)$^{-1}$) at 48 h, with an average consumption rate of 0.34 g (L d)$^{-1}$. Volke-Sepulveda *et al*. [25] observed lower degradation rates of HXD, from 0.09 g (L d)$^{-1}$ to 0.2 g (L d)$^{-1}$, using initial HXD concentrations from 20 to 80 g L^{-1} in 31 d cultures in serological bottles with orbital shaking. The difference may be better dispersion of the organic phase due to the hydrodynamics of the bioreactor; another possibility could be that the fungus previously grown on solid media and then transferred to liquid medium acclimated to the presence of HXD.

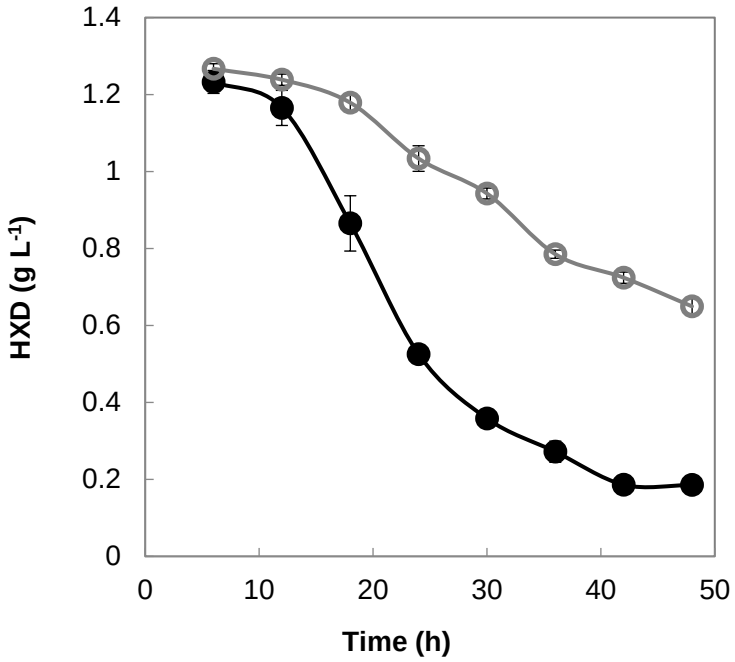

Figure 6. HXD degradation kinetics with pretreated (—●—) and untreated (—○—) biocatalyst.

Cuhna *et al*. [26] mention that a solid-liquid culture sequence, where a fungus is grown in a solid culture and then transferred to a liquid culture to produce protein, performs better than starting a conventional liquid culture inoculated with spores. In this case a similar phenomenon would be observed, where the BC is more efficient compared to HXD degradation by *A.niger* using spores as inoculum in liquid cultures as observed by Volke-Sepulveda *et al*. [25]. In the case of the pretreated BC, at 42 h of culture, up to 86% of the initial HXD (6.23 mg HXD (mg BM)$^{-1}$) was degraded, with an average consumption rate of 0.55 g HXD (L d)$^{-1}$. This result demonstrates that the increase in the BC's activity caused by the pretreatment is present in at least one subsequent culture.

3.4.Conclusions

Electric field pretreatment increased the BC's activity and this was maintained in a subsequent liquid culture. Sorption kinetics showed that electric field pretreatment decreased the sorption ability of perlite, but increased the fungal whole-cell BC sorption capacity. Linear isotherm fits experimental results better than Langmuir for affinity constant and Langmuir resulting in a useful tool in order to obtain the maximum sorption capacity. We demonstrate with both mathematical criteria that BC sorption capacity increased after electric field pretreatment and this may be the cause of the increased catalytic activity during HXD degradation. However, the relationship between electric field pretreatment and functional microbial activity needs to be investigated further. To our knowledge, this is the first work that proposes a method of both production and utilisation of a fungal whole-cell BC that was exposed to an electric field during fungal growth.

Acknowledgments

We acknowledge Consejo Nacional de Ciencia y Tecnología (CONACYT) for a scholarship to V. S-V.; and PEMEX-Refinación for partial support.

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Partial contribution

In the Chapter 3, two subsequent cultures (solid then liquid) were proposed for HXD degradation in the airlift bioreactor using the fungal whole cell biocatalyst. The biocatalyst was pretreated with an electric field in a solid state culture, then it was evaluated as HXD sorbent-degrader. It was demonstrated that the electric field pretreatment enhanced the biocatalyst's properties as a good applicant to remediate hydrocarbon contaminated water. At this point it was necessary to evaluate the biocatalytic activity approaching two topics: (i) to characterize the catalytic performance (mass transfer limitations, sorption capacity and reaction kinetics) regarding hydrocarbon degradation in the airlift bioreactor and (ii) the biocatalytic degradation of more complex hydrocarbons such as polycyclic aromatics.

4. Fungal biocatalyst activated by an electric field: improved mass transfer and non-specificity for hydrocarbon degradation in an airlift bioreactor

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Abstract

The combination of biological and electrochemical techniques enhances the bioremediation efficiency of treating oil-contaminated water. In this study a non-growing fungal whole cell biocatalyst (BC; *Aspergillus brasiliensis* attached to perlite) pretreated with an electric field (EF), was used to degrade a hydrocarbon blend (hexadecane-phenanthrene-pyrene; 100:1:1 w/w) in an airlift bioreactor (ALB). During hydrocarbon degradation, all mass transfer resistances (internal and external) and sorption capacity were experimentally quantified. Internal mass transfer resistances were evaluated through BC effectiveness factor analysis as a function of the Thiele modulus (using first order reaction kinetics, assuming a spherical BC, five particle diameters). External (interfacial) mass transfer resistances were evaluated by k_La determination. EF pretreatment during BC production promoted surface changes in BC and production of an emulsifier protein in the ALB. The BC surface modifications enhanced the affinity for hydrocarbons, improving hydrocarbon uptake by direct contact. The resulting emulsion was associated with decreased internal and external mass transfer resistances. EF pretreatment effects can be summarized as: a combined uptake mechanism (direct contact dominant followed by emulsified form dominant) diminishing mass transfer limitations, resulting in a non-specific hydrocarbon degradation in blend. The pretreated BC is a good applicant for oil-contaminated water remediation.

Keywords: *Aspergillus brasiliensis* (*niger*), Thiele modulus, effectiveness factor, aliphatic and polycyclic aromatic hydrocarbon degradation, airlift bioreactor.

4.1. Introduction

Bioremediation is the most common technique for oil contaminated water treatment; however, an important non-easily biodegradable fraction, *e.g.* polycyclic aromatic hydrocarbons, such as phenanthrene (PHE) and pyrene (PYR), requires new strategies to enhance remediation efficiency [1]. The combination of microbial and electrochemical techniques, referred to as microbial bioelectrochemical systems (BES), have been shown to enhance bioremediation efficiency, for example a microbial electrochemical cell (MEC) that uses an external electric energy source to drive the intended reactions for pollutant degradation [2]. *Aspergillus brasiliensis* ATCC 9642, formerly *Aspergillus niger* ATCC 9642 [3], grown on perlite in solid state culture has been used as a MEC, associated with a high hexadecane (HXD) degradation capability and low biomass production [4]. The degradation rate of hydrocarbons is limited due to their poor solubility and bioavailability. Lizardi-Jiménez *et al*. [5] found that HXD consumption by an oil-degrading consortium was limited by the hydrocarbon transfer rate rather than the oxygen transfer rate.

As with immobilized enzymes, if a microorganism is attached to a porous support, internal and external mass transfer resistances could have a great influence on the kinetics. Internal mass transfer barriers can be studied through particle size and the evaluation of effectiveness [6].The relationship between particle size and mass transfer resistance can be represented by the Thiele modulus, which reflects the combined effects of particle diameter, the maximum reaction rate and the diffusivity of the reactants into the pores of the catalyst [7]. The effectiveness factor, defined as the ratio between the observed reaction rate (with mass transfer resistances) and the maximum reaction rate (without mass transfer resistances) [8], as a function of the Thiele modulus is currently the preferred tool for chemical engineers to simulate complex heterogeneous reaction kinetics [9]. However, for heterogeneous reaction systems, it is necessary to experimentally evaluate the mass transfer resistances in catalytic particles, especially in biological catalysts, due to their dependency on complex biochemical reaction mechanisms. A particle size might exist at which the effectiveness factor is maximal and, therefore, the immobilized biocatalyst performance is optimal [10].

Hydrocarbon uptake in multiphasic bioreactors can be carried out by two mechanisms: (i) direct contact between microorganisms and hydrocarbon drops and (ii) by emulsifying oil in water phases. It was found that predominant bacterial hydrocarbon uptake mechanism is by

direct contact [11]; however, when an immobilized microorganism is used as hydrocarbon degrader, the external cells present high metabolic activity in contrast to starved cells into the pores *i.e.* low effectiveness biodegradation [12]. Then two possible alternatives able to enhance effectiveness: (i) the particle size diminishment and (ii) the hydrocarbon degradation assisted by a bioemulsifier agent.

Previously, *A. brasiliensis* attached to perlite and pretreated with an electric field, visualized as a fungal whole cell biocatalyst (BC), was proposed as a promising method to remediate contaminated water, due to its HXD sorbent-degrader capability [13]; however, it is necessary to expand our knowledge on the degradation capabilities of more complex and hazardous hydrocarbons, *e.g.* PAH, and the related kinetics and mass transfer limitations. The aim of this study was to characterize BC performance (mass transfer limitations, sorption capacity and reaction kinetics) regarding hydrocarbon blend degradation in an airlift bioreactor. Internal mass transfer resistances and biodegradation kinetics were experimentally evaluated through the effectiveness factor, as a function of the Thiele modulus. Additionally, the specificity for the three different hydrocarbons assayed in the blend was assessed through independent sorption isotherms and effectiveness factor analysis.

4.2.Materials and Methods

4.2.1. Microorganism

The strain was *Aspergillus brasiliensis* ATCC 9642, propagated in flasks with 50 mL of potato dextrose agar (PDA). It was seeded by groove and maintained for 7 days at 30°C. Spores were harvested using 0.1% Tween 80 and the spore suspension was used as an inoculum to produce the BC. The strain was stored on PDA slants at 4°C, reseeded every 2 months.

4.2.2. BC production

The BC was produced in solid state culture according to the previously reported method [13]. During the BC production, the electrochemical pretreatment (activation by an electric current, 6 mA during 24 h after spore germination) was performed. Afterwards, growth was allowed to continue up to the end of culture. The end of culture was determined before sporulation began (see section 3.1).

4.2.3. Drying and disrupting the biocatalyst

The produced BC was dried for 8 h in a desiccator (initial relative humidity ~5%, 30°C) until a 20% moisture content in the BC was achieved. The dried BC was crushed using a mortar and pistil, disrupted into different diameters and sized passing through five different meshes (<0.105, 0.105-0.42, 0.42-0.59, 0.59-1.19 and 1.19-1.68 mm). Dried and sized BC was evaluated for biomass detachment and metabolic activity. Biomass detachment was evaluated by biomass quantification for each BC diameter. The results are presented as normalized biomass content in the BC based on the biomass content in non-crushed BC samples. Viability was determined through radial growth on PDA in Petri dishes after 48 h.

4.2.4. External Mass transfer resistances evaluation

4.2.4.1. Interfacial oxygen mass transfer

Dissolved oxygen was measured with a Clark type electrode (ADI dO_2, Applisens, Netherlands). The dynamic numerical method proposed by Fujio *et al*. [14] was used to determine the volumetric mass transfer coefficient (k_La).

4.2.4.2. Interfacial hydrocarbon mass transfer

The ALB was started and allowed to stabilize hydrodynamic conditions, a pipet (4.42×10^{-3} cm^2 transversal area) with a defined hydrocarbon volume was introduced (see Figure 1) as early reported [15]. An aliquot was taken each 0.5 h during 3 h, and hydrocarbon concentration was measured.

Figure 1. Experimental arrangement for interfacial hydrocarbon mass transfer quantification, pipet contains rhodophile stained hydrocarbon blend.

4.2.5. Aliphatic-PAH sorption isotherms

The produced BC was thermally sterilized (15 psig, 15 min) in order to eliminate metabolic activity; afterwards it was used for sorption experiments. All treatments were agitated at 150 rpm (Incubator shaker I26, New Brunswick, USA). To generate isotherms (30 °C) the initial hydrocarbon blend concentration in the MM was: 0, 0.32, 0.65, 1.3, 2.6, 5.2 and 10.4 g L^{-1}. After 48 h, the hydrocarbon dispersed in the aqueous phase and that sorbed on the BC were measured according to the methods previously reported [13].

4.2.6. Aliphatic-PAH degradation

The degradation of an aliphatic-PAH blend (100:1:1; HXD-PHE-PYR; mimicking the aliphatic-PAH proportion in diesel) was carried out in batch liquid cultures using a previously reported airlift bioreactor [13]. Hydrocarbon degradation assays were carried out using all previously established particle diameters. In all cases, fluidization and instantaneous mixing was visually assured. Hydrocarbon (sole carbon source) concentration was measured every 6 h, during 48 h of culture time. In order to visualize the specificity during hydrocarbon blend degradation, each hydrocarbon concentration was reported as normalized value, based on the initial concentration (1.287, 0.013 and 0.013 g L^{-1} for HXD, PHE and PYR, respectively). The biomass content in the BC was measured at the initial and final time.

4.2.7. Analytical methods

Biomass quantification

Biomass was quantified through total protein determination using the Lowry method [16]. Dry samples (0.5 g) were prepared and analyzed as early reported [4]. Soluble protein was determined spectrophotometrically with a Varian Cary 50 (Australia).

4.2.7.1.Spore quantification

The solid culture was homogenized and suspended (1 g based on dry matter) in 10 mL of 0.1% Tween 80, then vortexed for 2 min. Next, it was filtered through Miracloth (Merck Millipore). The filtrate was successively diluted. The number of spores in each dilution were counted using a Neubauer chamber.

4.2.7.2.CO_2 quantification

The equipment to quantify respiratory activity was designed and constructed in our laboratory (Figure 2). An intermittent air flow controlled with a solenoid valve was passed through. When the CO_2 level reached 1%, the air flow was opened to change the culture head space, then the produced CO_2 was quantified. One cycle (open-close of the solenoid valve) was considered one sample. The sampling frequency was adjusted according to the respiration rate of each culture. Operation parameters (flow rate and sampling frequency) were measured and controlled with LabView software (2011, National Instruments; USA). A Vernier relative humidity sensor, as well as CO_2 and O_2 gas sensors (Vernier Software and Technology; USA) were connected to an NI CompactDAQ data acquisition system (National Instruments; USA). When the air flow was opened, the flow rate and concentration of CO_2 and O_2 were measured. CO_2 concentrations were integrated with respect to time, resulting in milligrams of CO_2 produced during the established sampling period. The results during BC production are expressed as mg CO_2 per g of dry inert support (DIS).

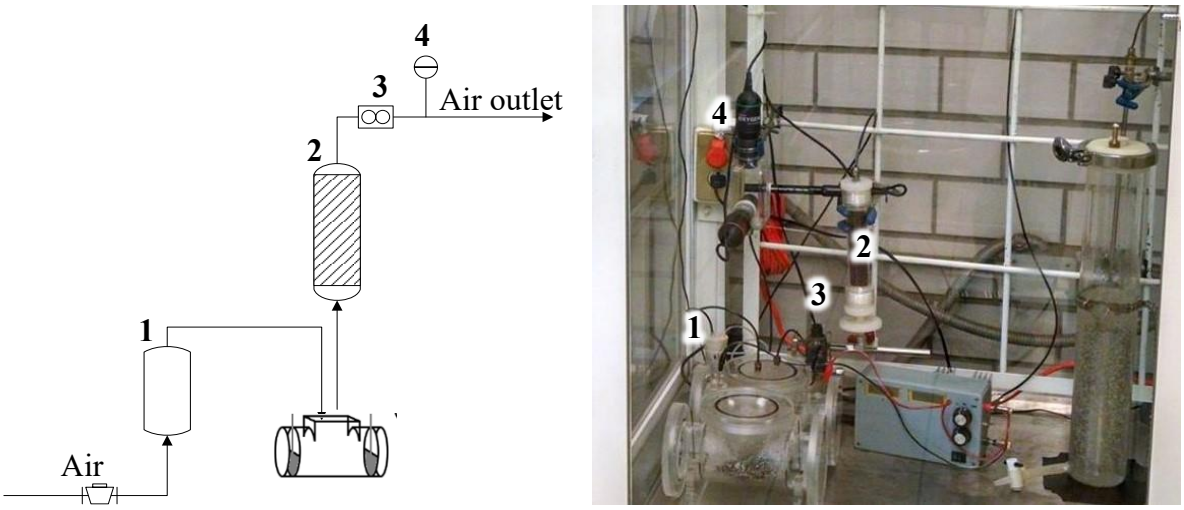

Figure 2. Respirometer for solid state culture analysis. Humidifier on the inlet to the bioreactor (1), drier column on the outlet of the bioreactor (2), flow meter (3) and sensor chamber to measure CO_2 and O_2 concentrations and relative humidity in the air flow (4).

4.2.7.3.Hydrocarbon quantification

The hydrocarbon content in the BC was obtained by solid-liquid microwave-assisted extraction using a MARS Xpress unit, CEM, USA. Dry samples (1.0 g) were suspended in 10 mL of a hexane-acetone blend (1:1) and extraction was performed for 15 min at 150°C; the resulting extract was filtered and each hydrocarbon was quantified by gas chromatography. For the quantification of dispersed hydrocarbons in the liquid medium, liquid-liquid extraction was used. Five milliliters of each liquid sample was taken and mixed with 5 mL of a hexane-acetone (1:1). The mixture was stirred and allowed to stand for 30 min. Then, each hydrocarbon was quantified in the organic phase by gas chromatography using a Shimadzu GC-2010 Plus (Japan) apparatus with a flame ionization detector (290°C). An Alltech 16367 capillary column was employed (15 m x 0.25 mm x 0.1 µm) with N_2 as the carrier gas (30 mL min^{-1}, 40 psi); the column temperature was initially set at 120°C and thereafter increased up to 200°C at 30°C min^{-1}. External standards were used.

4.2.7.4.Soluble protein quantification

Protein production (at 24 h) in the ALB was determined using the Bradford method [17]. One milliliter of liquid media was extracted from the ALB and centrifuged (6000 *g*; 10 min); then, 200 µL of Bradford reagent (Sigma-Aldrich) was mixed with 800 µL of the sample. The mixture was allowed to stand for 10 min and absorbance was measured (550 nm) using a Cary 50, apparatus (Varian, Australia). Bovine serum albumin (Sigma-Aldrich, ≥98%) was

used as the standard. The soluble protein concentration was reported as normalized values dividing protein production for each particle diameter assayed by the maximum protein production.

4.2.7.5.Emulsifying activity determination

The activity of bioemulsifier produced in the ALB during hydrocarbon degradation was determined by measuring the stable emulsified droplets, using a Mastersizer 2000 (Malvern, UK). At 24 of liquid culture, an aliquot was taken and droplets Sauter mean diameter (D_{32}) was measured. Stable emulsion was considered as micrometric droplets without significant diameter changes within 24 h.

4.2.8. Statistical analysis

All cultures were carried out in duplicate. The hydrocarbon degradation kinetics of the liquid cultures were evaluated by linear regression, fitted to a first order reaction model. All measurements were carried out in triplicate samples and compared using ANOVA and the Tukey test ($\alpha \leq 0.05$). Correlation analysis was carried out between the each hydrocarbon degradation kinetic constant and the soluble protein production at 24 h of ALB culture. In order to standardize the scales; both, kinetic constants and protein production were normalized based on the estimation for the smallest BC diameter.

4.3.Results and discussion

4.3.1. Biocatalyst production

The BC was produced in SSC; after spore germination (4.5 d), an electric current of 6 mA (1 V cm^{-1}) was applied for 24 h. Afterwards, growth was allowed to continue up to the end of the culture period. During BC production, mineralization and sporulation were measured. Figure 3 shows that CO_2 production was near to 380 mg CO_2 per g of dry inert support, with 4.5 d lag time, similar to previously observed [4]. CO_2 production rate was enhanced by the electric current (i-ii, Figure 3). Podolska *et al*. [18] discussed that a low intensity electric field acts as an electron pump, thus accelerating respiratory activity. The maximum CO_2 production rate was reached at 6.5 d; the CO_2 production rate was not zero after 12 d, possibly due to some intermediate metabolites such as carboxylic acids that accumulated in the cell and gradually oxidized [19]. The CO_2 production rate and sporulation, which started after 8

d, were considered for the improvement of BC characteristics, *i.e.* high hydrocarbon oxidation capability and the absence of spores that could detach from the BC and form independent mycelial structures. Based on these characteristics, the selected time for BC production was 8 d. The next step was the establishment of conditions for drying and disrupting the BC, followed by the evaluation of its hydrocarbon degradation capability using several BC diameters.

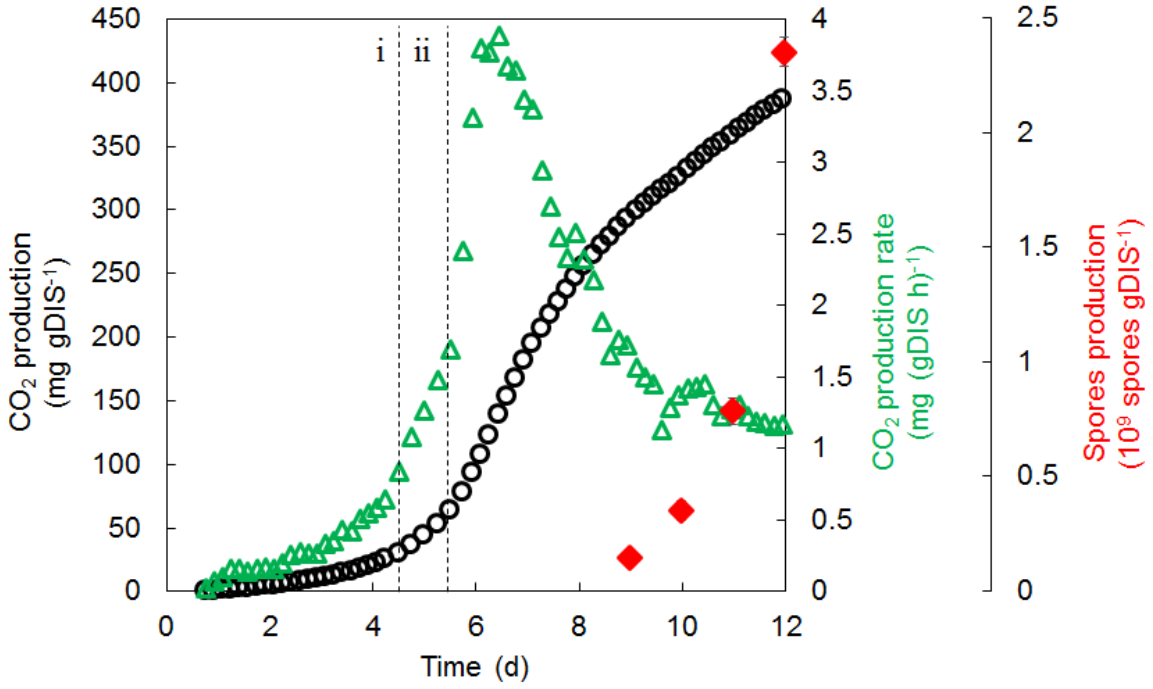

Figure 3. Hexadecane mineralization (open symbols), accumulated production of CO_2 (○), CO_2 production rate (Δ) and spore production (♦) during biocatalyst production with a controlled (6 mA) electric field applied between 4.5 d (i) and 5.5 d (ii).

4.3.2. Drying and disrupting the biocatalyst

Drying the BC was necessary in order to avoid the formation of a paste, which could be hard to handle when the BC was disrupted. The drying and disrupting steps could promote both hydric and mechanical stresses and thus affect the capability of BC to degrade hydrocarbons. The attached biomass in the BC, after being dried and disrupted, was another essential characteristic evaluated and associated with mechanical stability. Figure 4 shows the relative biomass content for all BC diameters for both EF untreated and pretreated BC. In untreated

BC, significant differences in the relative biomass content were observed for smallest diameter. In pretreated BC, the biomass content was not significantly affected by the disruption procedures. This result shows that surface BC properties were affected by EF pretreatment, resulting in stronger biomass sorption (attachment) onto the support than with untreated BC. The biomass sorption onto the support is related to the interfacial tension and its dependence on the electrical state of the interface [20]. Barzyk and Pomianowski [21] proposed that the change in interfacial tension as a function of an applied potential is equal to the surface polarizable charge. According to this, the presence of an EF can modify the support surface properties and its sorption capacity, *e.g.* biomass attachment, as shown in this work. An important characteristic observed in the BC is the preservation of biomass attachment strength, even in the ALB. This behavior is not observed in abiotic experiments, similar to those reported by Qin *et al.* [22]. They found that PHE adsorption on graphite was enhanced by an EF, but this enhancement was observed only while applying the EF. Since attached biomass is the active phase in the BC, it is necessary to consider the relative biomass content in the degradation of a specific hydrocarbon per milligram of biomass. In the case of EF pretreated BC, the relative biomass content was independent of the particle diameter (Figure 4); therefore, it can be assumed with reasonable reliability that the concentration of the reactive species (biomass content in the BC as active phase and aliphatic-PAH blend) is the same for all BC diameters assayed for hydrocarbon degradation.

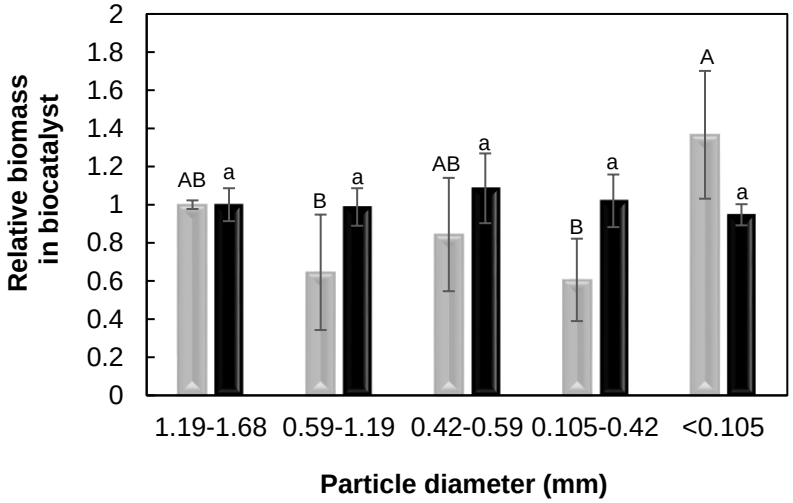

Figure 4. Relative biomass content in electric field pretreated (black) and untreated (grey) biocatalyst dried and disrupted into different diameters. Histograms with different letters indicate significant differences ($\alpha \leq 0.05$) according to the Tukey multiple comparison test.

Figure 5 shows all assayed BC diameters (a), lowest (disrupted; b) and highest (non-disrupted; c). Disrupted BC showed mycelial fragments which were assumed without any diffusional mass transfer limitations. When non-disrupted BC, with diffusional mass transfer limitations, was stained with lactophenol blue, mycelial structures were clearly attached to the perlite. Both disrupted and non-disrupted BC were able to grow in PDA Petri dishes (Figure 5d and e). The relative radial growth of *A. brasiliensis* was not significantly different for any dried and disrupted particle diameter. Figure 5e shows as an example for the highest particle diameter (2.87 ± 0.09 cm d^{-1}); therefore, the fungus was assumed to be metabolically active (this assumption was later tested; see section 3.4).

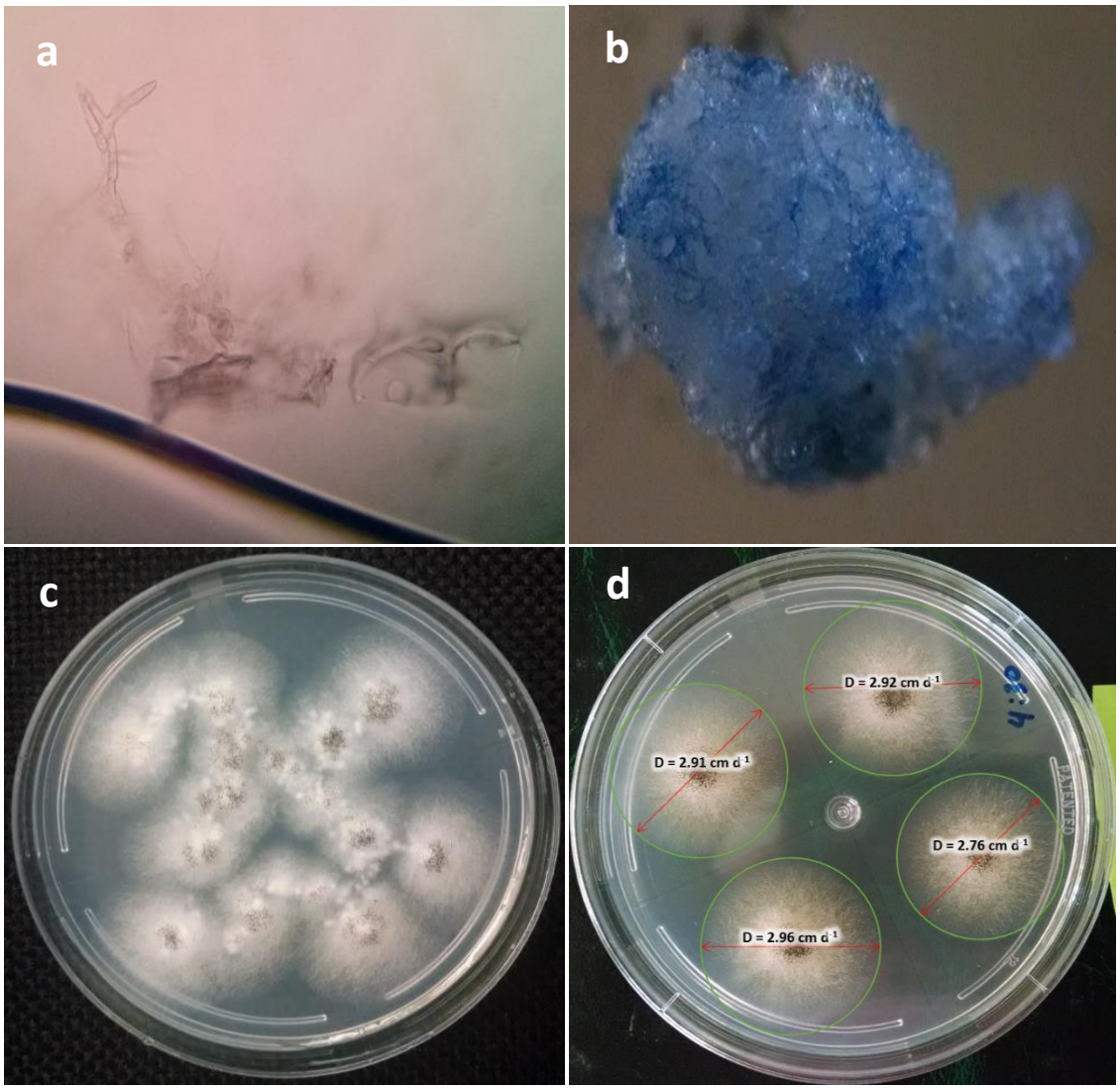

Figure 5. Pictures of assayed biocatalyst diameters (a), lowest (b) and highest (c) diameter. Viability test; (d) and (e) for "b" and "c", respectively.

4.3.3. Aliphatic-PAH sorption

Based on the previous section, BC acquired new sorption properties that improve the biomass attachment. The acquired sorption properties are apparently related to surface modifications promoted by the EF pretreatment. In order to establish the role of the aforementioned surface modifications in the BC hydrocarbon sorption capacity, sorption isotherms were performed (see Figure 6). Same as in our previous report, the EF pretreatment enhanced (an order of magnitude) the BC maximum sorption capacity [13], Figure 6a shows that the untreated BC was not saturated with hydrocarbon (neither HXD, PHE and PYR) under assayed conditions. Contrary to the pretreated BC, in which the saturation was clearly reached for all three hydrocarbons (Figure 6b).

The multicomponent Langmuir model was used to determine the maximum sorption capacity and affinity (by equilibrium constant) of BC for hydrocarbon. Table 1 shows that equilibrium constant was enhanced after the pretreatment *i.e.* the BC affinity for hydrocarbon was enhanced, as we previously found for HXD sorption [13]. However, the maximum sorption capacity was 35% lower using the hydrocarbon blend than only HXD. On the other hand, PAH equilibrium constants were 18 and 28 fold-higher than HXD for PHE and PYR, respectively. *i.e.* the hydrocarbon sorption is preferable to PYR, followed by PHE and, finally by HXD.

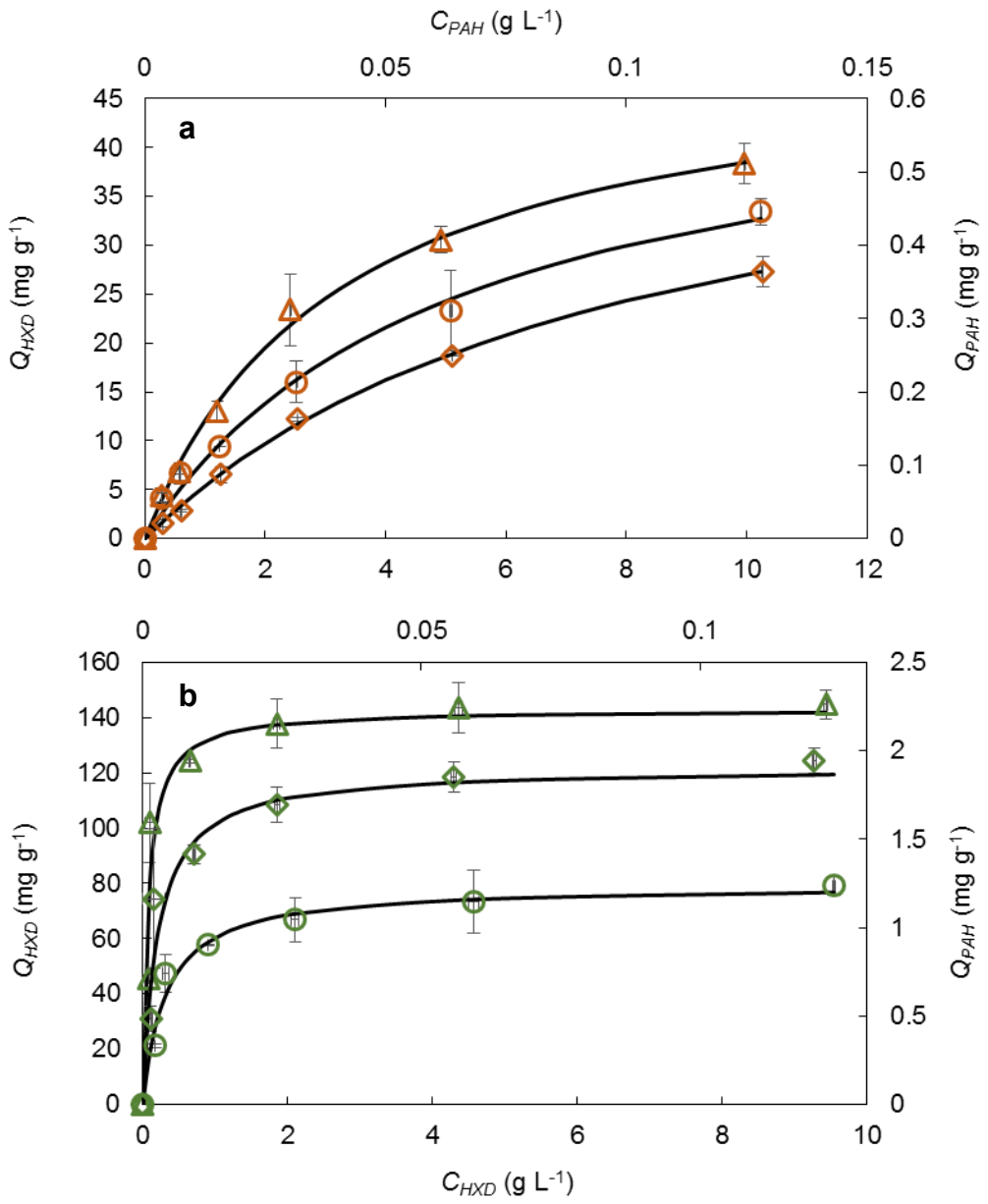

Figure 6. Sorption isotherms of hydrocarbons (hexadecane, triangles; phenanthrene, circles; and pyrene, diamonds) in blend, onto untreated (a) and pretreated (b) biocatalyst. Continues curves represent the multicomponent Langmuir adsorption model fitting.

Table 1. Estimated multicomponent Langmuir isotherms parameters for all three hydrocarbon sorption.

BC	hydrocarbon	q_{Mi} (mg g^{-1})	K (L g^{-1})	R^2
EF Untreated	HXD	50.90 ± 3.56	0.31 ± 0.02	0.99
	PHE	0.65 ± 0.05	15.81 ± 1.16	0.98
	PYR	0.65 ± 0.07	9.94 ± 0.78	0.99
EF Pretreated	HXD	143.03 ± 15.03	13.39 ± 0.94	0.87
	PHE	1.24 ± 0.08	244.73 ± 17.12	0.95
	PYR	1.91 ± 0.22	381.87 ± 25.85	0.82

4.3.4. Aliphatic-PAH degradation

The BC was separated into five diameters, and their hydrocarbon degradation capabilities were evaluated. Figure 7 shows hydrocarbon degradation using the highest and lowest BC diameters. The untreated BC (highest diameter; ii filled data, Figure 7) was capable of degrading all three hydrocarbons; however, only hexadecane and PHE were initially degraded (57.6% and 62.1% at 48 h, respectively), whilst PYR consumption began after 24 h (41.6% degraded at 48 h). The PAH have unusual chemical stability due to electronic resonance, and their chemical stability is greater with a higher number of aromatic rings, which could be related to the slower biodegradation of PYR [23]. When the lowest diameter untreated BC was used, the hydrocarbon degradation rate was enhanced by 50% for all three hydrocarbons (ii unfilled data, Figure 7).

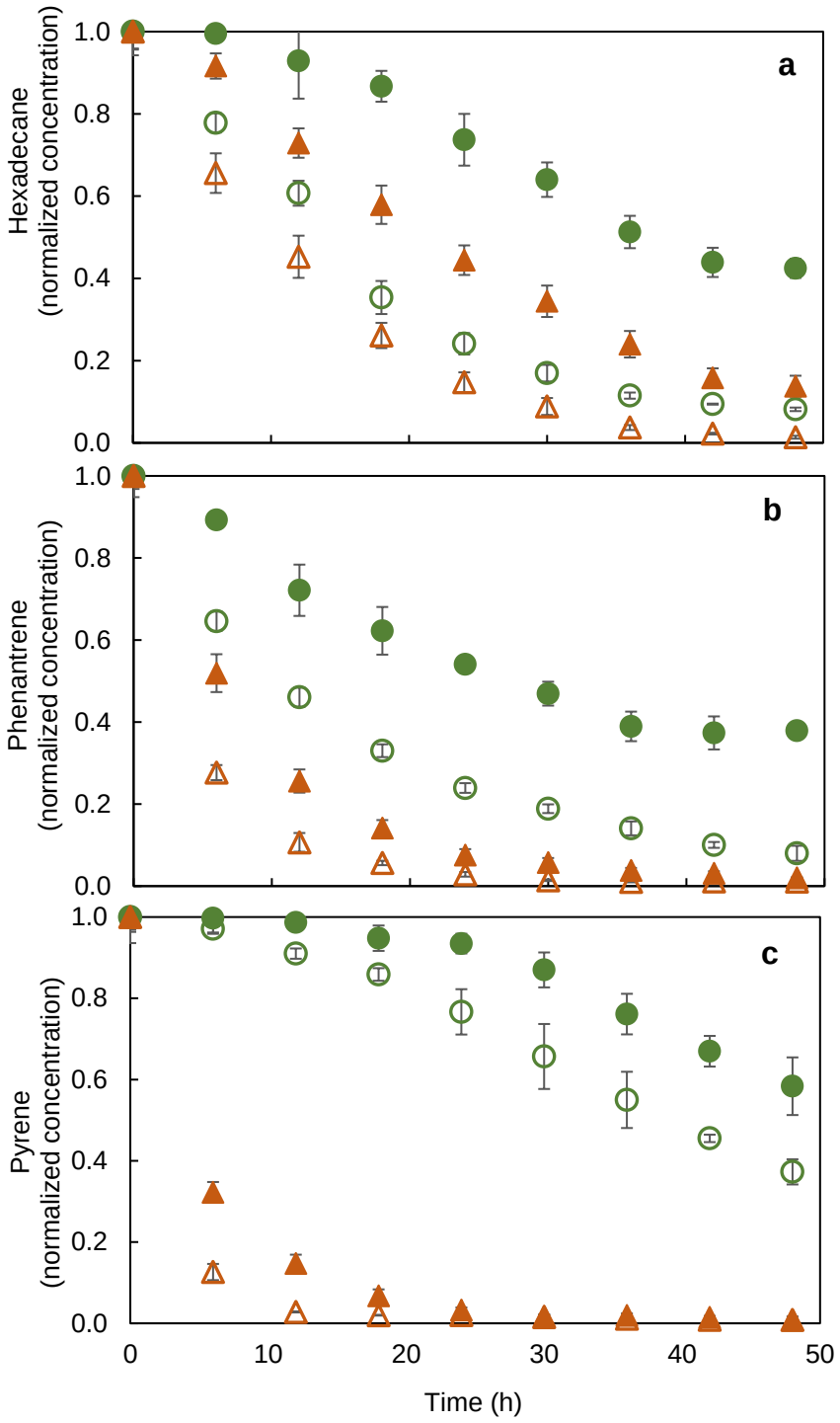

Figure 7. Normalized concentration of hexadecane (a), phenanthrene (b) and pyrene (c), using EF pretreated (i, triangles) and untreated (ii, circles) biocatalyst. Only the largest (filled symbols) and smallest (unfilled symbols) diameter particles are shown.

On the other hand; when the EF pretreated BC was used, PHE and PYR disappeared within 24 h, and the hydrocarbon degradation rate was also enhanced by decreasing the BC diameter. HXD degradation increased from 87% to 99% within 48 h of culture, using the highest and lowest particle diameters, respectively. The results depicted in Figure 7a show that decreasing the particle size provided an enhanced degradation rate compared to EF pretreatment only for HXD degradation. For PAH, the degradation rate was enhanced by EF pretreatment rather than by decreasing the particle size (Figures 7b, 7c). This significant result suggests that untreated BC degrades hydrocarbons with clear specificity for HXD and PHE while EF pretreated BC was able to degrade all three hydrocarbons in a non-specific way.

Ercan *et al*. [24] identified the physiology of microorganisms in zero-order growth being as cell factories for metabolite or enzyme production. In this context, BC seemed to act as a non-specific hydrocarbon degrading cell factory, since biomass production during liquid culture was negligible (the initial biomass content in the BC added to the ALB was 37.6 ± 2.4 mg g BC^{-1}, while the final biomass content was 34.2 ± 4.7 mg g BC^{-1}); however, the fungus in the BC was able to grow on PDA Petri dishes.

During hydrocarbon degradation by EF pretreated BC, a biosurfactant protein (probably a hydrophobin; results not shown) was detected, which emulsified the hydrocarbon blend by transforming hydophobic droplets into hydrophilic micelles. A bimodal micelle distribution diameter (22.9 and 2.9 μm in proportion 4:1) was found. These values were smaller than the perlite pore diameter (average 100 μm) [25], which facilitated transport into the BC pores, resulting in enhanced interfacial mass transfer; this was likely the reason for improved hydrocarbon degradation. Several studies have focused on the role of surfactants in the remediation of water polluted with hydrocarbons, concluding that they can enhance remediation efficiency due to improved interfacial transport, resulting in major pseudo-solubility and the bioavailability of hydrophobic molecules [26]. At this point, the two possible known hydrocarbon uptake mechanisms in multiphasic bioreactors (direct contact and oil phase emulsification) were defined by interfacial interaction, as previously reported [27]. To our point, in case of pretreated BC a combined uptake mechanism probably takes place. During first culture hours, direct contact uptake was dominant. As emulsifier protein was released to the liquid medium, emulsified hydrocarbon uptake became dominant. In

contrast, dominant uptake mechanism by untreated BC was direct contact, limited by internal and external mass transfer resistances.

The global degradation rate could also be limited by hydrocarbon diffusion to the cell surface [28]. This is an important issue because the effects of a low intensity electric field on microbial metabolism are not completely understood [18], even though it enhances microbial processes like hydrocarbon degradation [4]. At this point, it is important to know the magnitude of the mass transfer resistances from the bulk to the BC, in order to discriminate between transport phenomena and the intrinsic kinetics of hydrocarbon biodegradation.

4.3.5. Mass transfer resistances evaluation

In order to determine the principal limitation between hydrocarbon and oxygen external (interfacial) mass transfer, volumetric coefficients (k_La) were evaluated (see Table 2).

Table 2.Volumetric mass transfer coefficients determination by using numerical dynamic method. Mean estimated parameter and standard error is presented.

	k_La (10^{-4} s^{-1})			
	O_2	HXD	PHE	PYR
In the absence of bioemulsifier	267 ± 17	1.692 ± 0.34	0.047 ± 0.001	0.026 ± 0.004
In the presence of bioemulsifier	351 ± 21	44.288 ± 1.24	1.181 ± 0.022	1.23 ± 0.16

According to the k_La orders of magnitude, the principal limitations are in the PAH mass transfer to the aqueous phase (PYR > PHE > HXD >> O_2); however, when the bioemulsifier was produced, all k_La values were enhanced: 1.3 for O_2 and 27, 25 and 47 times for HXD, PHE and PYR, respectively. The presence of bioemulsifier improve the interfacial mass transfer; however, PAH mass transfer were the slowest, similarly to recently reported [5].

The understanding of mass transfer effects on reaction rates and specificity is suitable for use in reactor modelling studies [29]. Table 3 shows the effective diffusivity parameters, which are in two orders of magnitude lower than kLa for PAH. *i.e.* the main limiting step is in the hydrocarbon mass transfer into the BC. The kinetic data for hydrocarbon degradation were fitted to a first order reaction model and employed to obtain the Thiele modulus. The estimated kinetic parameters are shown in Table 3 for EF untreated and pretreated BC. In the

case of untreated BC, the estimated PYR degradation kinetic constant was in the same order of magnitude than those early reported [30], where a bacterial strain immobilized in diatomite was used, evaluating sorption and degradation of PYR. However, the kinetic constant values for pretreated BC were 3, 5 and 7-fold higher than untreated for HXD, PHE and PYR, respectively.

Table 3. Kinetic constants estimated for hydrocarbon degradation and parameters for the Thiele modulus calculation.

BC	D_P (cm)	D_{eff} ($*10^{-6}$ cm^2 s^{-1})	k_{HXD} ($*10^{-3}$ s^{-1})	k_{PHE} ($*10^{-5}$ s^{-1})	k_{PYR} ($*10^{-6}$ s^{-1})
EF untreated	0.168	1.36	4.40 ± 0.45	0.93 ± 0.08	4.14 ± 0.33
	0.119	1.76	4.90 ± 0.53	1.15 ± 0.10	3.99 ± 0.31
	0.059	1.50	4.90 ± 0.47	1.18 ± 0.11	4.42 ± 0.35
	0.042	2.14	5.07 ± 0.55	1.46 ± 0.13	6.35 ± 0.51
	0.0105	2.25	6.13 ± 0.67	2.02 ± 0.17	8.20 ± 0.65
EF pretreated	0.168	1.85	14.51 ± 1.59	5.97 ± 0.54	36.10 ± 2.88
	0.119	1.87	14.78 ± 1.63	5.94 ± 0.53	28.85 ± 2.30
	0.059	2.04	14.93 ± 1.62	5.80 ± 0.50	38.77 ± 3.10
	0.042	2.31	15.34 ± 1.71	6.13 ± 0.55	42.03 ± 3.36
	0.0105	2.48	15.57 ± 1.72	9.32 ± 0.84	42.49 ± 3.39

The resulting effectiveness factors of BC (untreated and pretreated) for the three hydrocarbons tested are depicted in Figure 8. The effectiveness factor for HXD using EF untreated BC was similar to the theoretical values for a first order reaction without external mass transfer resistances. When the Thiele modulus is greater than unity, the global degradation rate might be limited by diffusional resistances into BC pores. This result was according to reported by Liu *et al*. [7] for the evaluation of mass transfer resistances into aerobic microbial granules and the model proposed by Gonzo *et al*. [9]. For PAHs, the effectiveness factor decreased when the Thiele modulus values were 2 or 3 orders of

magnitude lower than HXD (Figure 8a). This is an important result as it suggests that PAH degradation is limited by both internal and external (interfacial) mass transfer resistances [31]. Delidovich *et al*. [32] evaluated the influence of mass transfer in the catalytic oxidation of glucose, concluding that the interfacial mass transfer of oxygen is able to essentially influence the overall kinetics; a similar result was observed in this work.

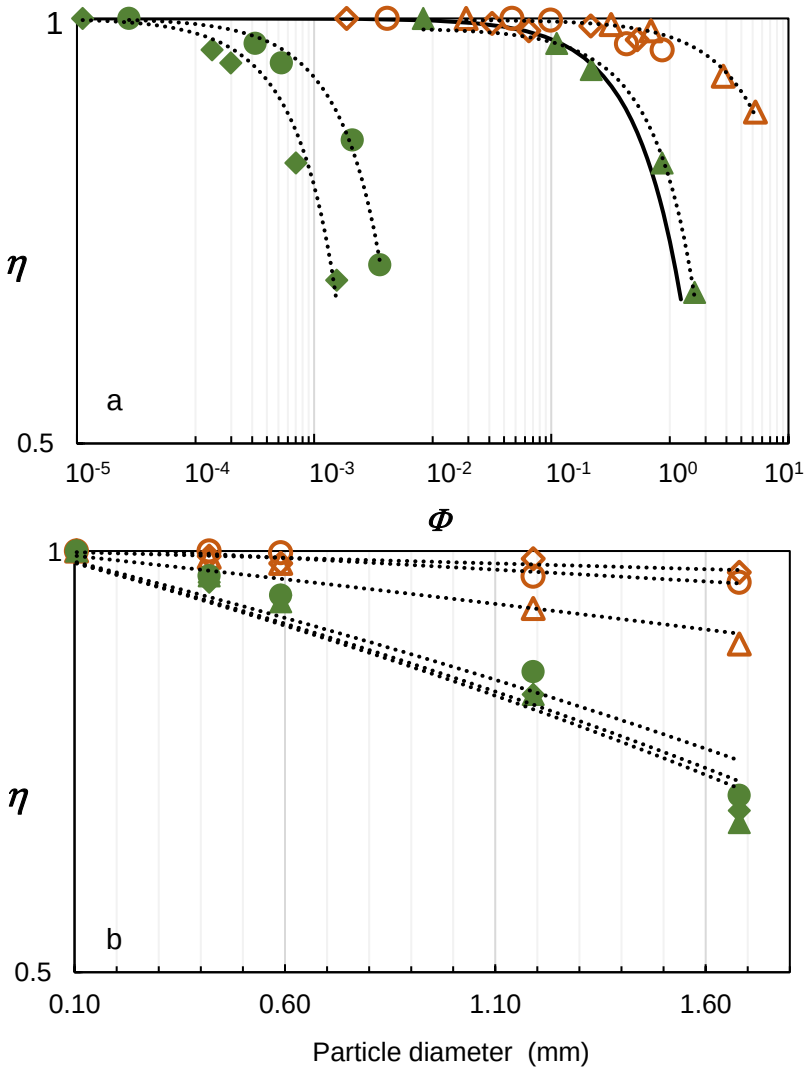

Figure 8. Effectiveness factor as a function of the observable Thiele modulus for hexadecane (triangles), phenanthrene (circles) and pyrene (diamonds) using EF pretreated (open symbols) and untreated (filled symbols) biocatalyst. The continuous line represents a theoretical case with a first order reaction for a spherical catalyst with negligible external mass transfer resistances.

Figure 8b shows that the effectiveness factor was approximately 0.63 when larger particles of untreated BC were used, *i.e.* in order to enhance the effectiveness factor, untreated BC requires to be less than 0.6 mm, in contrast to pretreated BC, which can be 1.19-1.68 mm.

Surprisingly, EF pretreated BC was not susceptible to mass transfer resistances. This was consistently observed with the effectiveness factors for the three hydrocarbons (Figure 8b), which were close to unity for all assayed BC diameters. The highest effectiveness observed with EF pretreated BC could be due to the surface changes promoted by EF on BC, thereby increasing its affinity for hydrocarbons, as has been described in a previous work [13]. However, the surfactant produced in the ALB may have contributed to the enhanced degradation rate by forming an emulsion, which increased the specific surface area of the organic phase and thus the hydrocarbon concentration in the bulk [33], resulting in an enhanced mass transfer rate to the BC. Our results agree with those reported by Vital-Jacome *et al*. [34]; they experimentally demonstrated that the effectiveness factor was approximately to unity when the substrate bulk concentration was increased.

In order to identify the role of the surfactant in the hydrocarbon degradation kinetics, the kinetic constant for each hydrocarbon degradation was correlated with protein production at 24 h (Figure 9). For HXD and PHE, the correlation coefficients were close to 0.8, *i.e.* the protein, as an emulsifier, was directly related to hydrocarbon degradation. For PYR, the correlation coefficient was lower (0.27), which could be explained by two-fold higher PYR sorption compared to PHE (see Fig. 6), *i.e.* the protein was indirectly associated with PYR degradation, since an important fraction of PYR was not detected due to simultaneous sorption onto the BC and degradation. Interfacial transport of PAH is one of the major limitations. This limitation was diminished when using EF pretreated BC, resulting in an effectiveness factor close to unity. Our results show that all mass transfer resistances implicated in hydrocarbon degradation were diminished using EF pretreated BC, supporting its application in the remediation of water contaminated with PAHs. In fact, this method could be as effective as chemical methods such as the ozonation of PAH in oil/water emulsions studied by Kornmuller and Wiesmann [35].

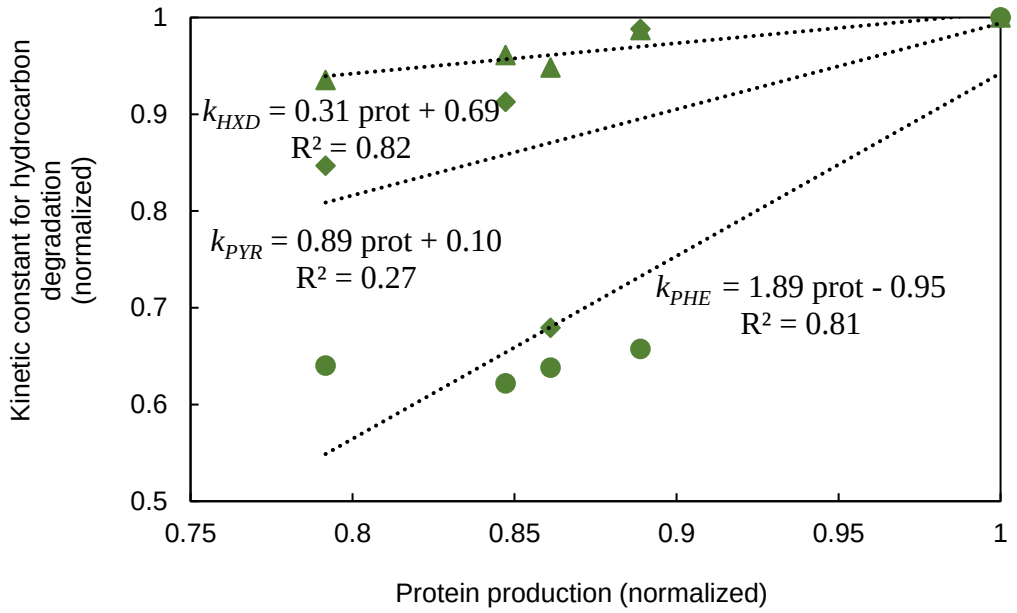

Figure 9. Correlation analysis between kinetic constant for hydrocarbons (hexadecane, ▲; phenanthrene, ●; and pyrene, ♦) and protein production in the airlift bioreactor. Kinetic constant and protein production data were normalized based on the lowest particle diameter.

4.4.Conclusions

The BC was capable of degrading a hydrocarbon blend composed of HXD, PHE and PYR. EF pretreatment during BC production promoted surface changes in BC and production of an emulsifier protein in the ALB. Surface changes enhanced the affinity between BC and hydrocarbons, improving hydrocarbon uptake by direct contact. The resulting emulsion was associated with decreased internal and external mass transfer resistances. We demonstrated that the hydrocarbon degradation rate was also increased as compared with the untreated BC: (*i*) the kinetic constant values were 3, 5 and 7-fold higher than those with the untreated BC for HXD, PHE and PYR, respectively and (*ii*) hydrocarbon degradation was non-specific with a combined uptake mechanism (direct contact and emulsified form) whilst for the untreated BC degradation was only by direct contact. Finally, the effectiveness factor was close to unity for all three hydrocarbons, even for larger BC diameters.

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Appendix A. Theoretical considerations

A.1. Multicomponent sorption

Langmuir model is one of the most used model to describe the amount of a compound sorbed on any surface, Langmuir model is as a function of two parameters: (i) a maximum sorption capacity and (ii) sorption equilibrium constant; these constants allow us to determine the saturation concentration and the affinity between sorbate and sorbent. The Langmuir model is expressed as shown in equation A.1.1.

$$q = \frac{q_M K_e C}{1 + K_e C} \quad \text{(A.1.1)}$$

Where q represents de adsorbed amount, C represents the dispersed sorbate in a fluid phase, q_M and K_e are two parameters that describe the maximum sorption capacity and the sorption equilibrium constant, respectively. When sorbate is a blend in which all components can competitively be adsorbed, eq. A.1.1 is expressed as a generalizable mode [36, 37] as shown in equation A.1.2.

$$q_i = \frac{q_{M,i} K_{e,i} C_i}{1 + \sum_{j=1}^{N} K_{e,j} C_j} \quad \text{(A.1.2)}$$

Where i represents the component (in the blend) to be described, j represents each component and N is the total number of components in the blend.

A.2. Internal mass transfer resistances in the BC

Diffusional mass transfer resistance was quantified in terms of an effectiveness factor as a function of the Thiele modulus, defined as shown in equation A.2.1, assuming first order reaction kinetics and spherical BC.

$$\phi = \frac{R_P}{3} \sqrt{\frac{k}{D_{eff}}} \quad \text{(A.2.1)}$$

where R_P is the BC radius, k is a kinetics reaction constant and D_{eff} is the effective diffusivity, which can be expressed as a function of porosity (ε) and molecular diffusivity (D), according to the random model, as shown in equation A.2.2 [8]:

$$D_{eff} = \varepsilon^2 D \quad \text{(A.2.2)}$$

A generalized form of the Thiele modulus (in terms of the observable variables) proposed by Weisz and Prater [38] is shown in equation A.2.3.

$$\Phi = \frac{R_P^2}{D_{eff} C_{HC}} \frac{dC_{HC}}{dt} \quad \text{(A.2.3)}$$

Where $\frac{dC_{HC}}{dt}$ is the global average reaction rate and C_{HC} is the bulk hydrocarbon concentration. If the reaction rate occurs according to first order reaction kinetics, the observable Thiele modulus can be written as equation A.2.4.

$$\Phi = \eta\phi^2 \quad \text{(A.2.4)}$$

The effectiveness factor (η) was evaluated as a function of R_P and Φ, as discussed by Carberry [31].

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Partial contribution

In the Chapter 4, the fungal biocatalyst was used to degrade a hydrocarbon blend (hexadecane-phenanthrene-pyrene, 100:1:1 w/w/w) in the airlift bioreactor, this chapter was focused on the catalytic performance characterization. All mass transfer resistances, biocatalyst's hydrocarbon sorption capacity and degradation kinetics were phenomenologically evaluated. The effectiveness factor was experimentally evaluated, as a function of the Thiele modulus, in order to determine the diffusional mass transfer resistances into the biocatalyst pores (Figure 8). Concomitant an emulsifier protein was produced during the hydrocarbon degradation as a consequence of the electric field pretreatment. The emulsifier protein was related to the mass transfer diminishment and modified hydrocarbon uptake mechanisms. So that, a functional characterization was necessary to know the applicability of the emulsifier protein into environmental engineering topics.

5. Stable protein able to emulsify hydrocarbons, produced by *Aspergillus brasiliensis* (*niger*) in an airlift bioreactor, after an electrochemical pretreatment

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Abstract

Bioemulsifier compounds can enhance the bioremediation efficiency in contaminated environments. An emulsifier protein was produced and easily separated from oil-contaminated water as cheap substrate when *A. brasiliensis* (formerly *Aspergillus niger*), pretreated with an electric field, was used in an airlift bioreactor. The hydrocarbons-emulsifier protein was 19.5 % of the total protein produced in the liquid culture, and its purification enhanced 7 times the specific emulsifying activity, and the interfacial surface area was increased from 1047 to 8400 m^{-1}. Its emulsifying activity (EA) was measured using polycyclic aromatic hydrocarbons in a blend with hexadecane (1:1 w/w), which was 2.79-fold higher than sole hexadecane. The influence of operational conditions such as pH and salt concentration on the EA were assessed to characterize the emulsions stability. Alkaline environments (pH 7-11) enhanced 19% the EA that was not affected by the presence of salt (0-35 g L^{-1}). On the other hand, preheating the emulsifier protein samples (60°C) enhanced 2.5 times the EA. Based on EA, this emulsifier protein can be applied as bioremediation enhancer in contaminated soils.

Keywords: *Aspergillus brasiliensis* (*niger*), emulsifier protein, electrochemical pretreatment, airlift bioreactor, stability of emulsifying activity.

5.1. Introduction

Emulsions are multiphasic systems in which oil phase droplets are suspended in continuum water phase (oil in water); or vice versa, water in oil [1]. Since emulsions are thermodynamically unstable systems, adsorption of surface active compounds (emulsifiers) on the liquid-liquid interface is necessary to stabilize it [2]. Emulsifiers could be produced by chemical synthesis or microbial metabolism (Bioemulsifiers). Bioemulsifiers exhibit some advantages on the chemical synthetized, such as lower toxicity and higher biodegradability [3]. Bioemulsifiers are considered versatile chemical compounds, which can be used in different industrial applications, they are most often proposed in the petroleum industry as extraction process enhancers [4] and environmental such as bioremediation of contaminated soil or water, due to their ability to counter the high viscosity and low solubility of hydrocarbons [5,6]. However, surrounding conditions such as pH, temperature or salt concentration can diminish the bioemulsifier surface activity [1]; thus, they would be evaluated before any application proposal.

Bioemulsifiers production and application for environmental proposes are limited by high costs of biosynthesis and downstream separation and purification [7]. The appropriate approach for downstream depends on the nature of substrates and secreted biosurfactants [8]. A good option to easily recover a bioemulsifier or biosurfactant could be a foam recovery technique, such as previously reported [9].

Proteins with high surface activity, as bioemulsifiers, have particular properties that distinguish them from other surfactant compounds; they can form a thin membrane that is resistant to rupture, stabilizing the droplets and avoiding their coalescence. The droplets size measurement is a useful tool to determine the extended interfacial surface area as well as the emulsifying activity, as early reported [10]. Filamentous fungi can produce a group of small proteins with highest observed surface activity (surfactant, emulsifier and solid wettability interchanger), denominated hydrophobins [1,11]. Hydrophobins, located on the cell surface, they have a defined biological role related to hydrophobic substrates uptake; although hydrophobins are good option for oil removal from contaminated water [12] their use, as bioemulsifier, is limited due to the lack of knowledge for the industrial application [13].

Aspergillus brasiliensis (formerly *niger*) acquired some physiological modifications after an electrochemical pretreatment on the fungal biomass, *e.g. A. brasiliensis* gets better hydrocarbon degrader capability producing an emulsifier protein (EP) when it is transferred to a subsequent liquid culture in an airlift bioreactor (ALB) [14]. The produced EP seems to be a good option to enhance the bioremediation efficiency, which could have higher surface activity than other bioemulsifier such as emulsan, alasan, biodispersan, protein complex, among others reported for hydrocarbon bioremediation [6]; due to the EP evaluated in this work is a subproduct directly synthetized from the hydrocarbons degradation using the wastewater as cheap substrate as recently reported [15]. However, surrounding aqueous solution composition (*e.g.* pH or salt concentration) and environmental stresses (*e.g.* temperature changes) can modify the emulsion stabilizing capabilities [1]; so that, a further EP surface activity characterization is needed in order to clarify on the possible petroleum mobilization applications. Thus, this work was aimed at the functional characterization of an unknown EP surface activity for a laboratory downstream separation and purification procedure. The EP was produced by *A. brasiliensis* in liquid culture with a hydrophobic substrate, after an electrochemical pretreatment in solid state culture. The characterization included surface activity measurement regarding (i) purity grades in a downstream procedure, (ii) several hydrophobic phases for oil in water emulsions formation and (iii) environmental conditions (pH, temperature, and salty media).

5.2. Materials and Methods

5.2.1. Microorganism

The strain was *Aspergillus brasiliensis* (formerly *niger*) ATCC 9642, propagated in flasks with 50 mL of potato dextrose agar (PDA). It was seeded by groove and maintained for 7 days at 30°C. Spores were harvested using 0.1% Tween 80 and the spore suspension was used as inoculum to fungal growth. The strain was stored on PDA slants at 4°C, reseeded every 2 months.

5.2.2. Fungal growth conditions

Aspergillus brasiliensis was produced by solid state culture (SSC) using a horizontal cilindrical electrochemical bioreactor with a working volume of 450 mL such as previously reported [16]. During fungal growth, a controlled electric field was imposed. The

electrochemical bioreactor was provided with two electrodes (built of titanium coated with ruthenium oxide with a contact surface of 14.13 cm2), and two reservoirs for the electrolyte (0.1 M KH2PO4) at the extremes. After spores germination (4.5 d) an electric current of 6 mA was applied for 24 h. Afterwards, growth was allowed to continue up to the end of culture (12 d). Hereafter, this will be referred to as the electrochemical pretreatment. At the end of SSC attached *A. brasiliensis* was transferred to a subsequent liquid culture using an airlift bioreactor.

5.2.3. Airlift bioreactor conditions

A cylindrical glass airlift bioreactor, such as early reported [14], was used with 5 g L^{-1} of attached *A. brasiliensis*. The total protein concentration in liquid culture was measured every 6 h during 48 h. The mineral medium (MM) was composed (in mg L^{-1}) of: $NaNO_3$, 357.5; KH_2PO_4, 5.9; $MgSO_4$, 30.3; KCl, 51.4; trace element solution, 2 mL L^{-1}.

5.2.4. Analytical methods

5.2.4.1.Emulsifier protein extraction and quantification

In order to quantify the total protein production, a foam breaker device was installed into the ALB as shown in Figure 1 (the total protein kept dissolved into the liquid medium). An aliquoted of culture (1 mL) was centrifuged (6000 x g; 10 min), protein was measured in the supernatant by Bradford method [17].

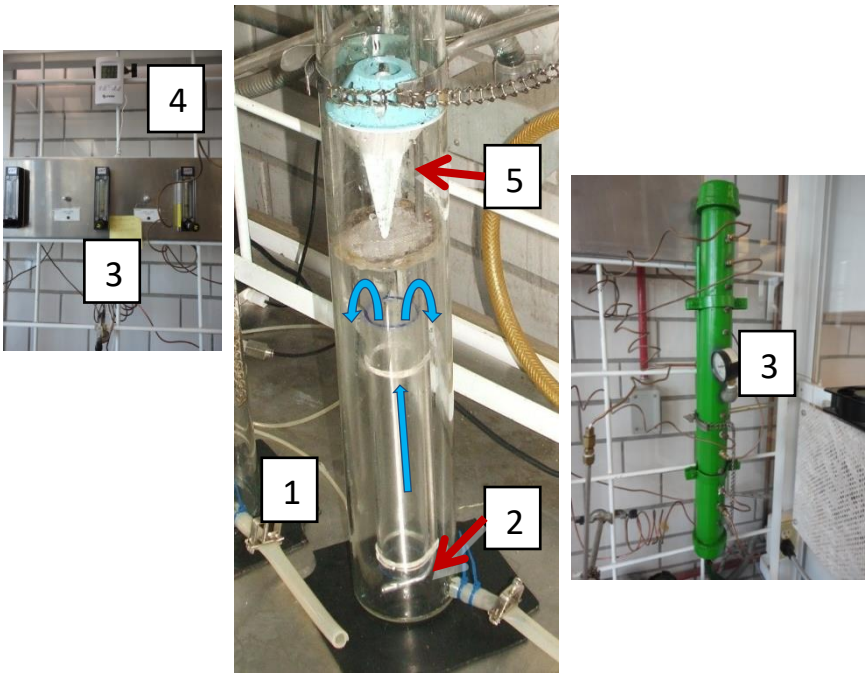


Figure 1. Airlift bioreactor arrangement. Image shows the bioreactor body (1), gas diffuser (2), pressure and gas flowrate control (3), temperature control (4) and foam breaker (5).

Emulsifier protein production was extracted according to the method used by Vigueras-Ramírez *et al*. [18] for extraction of hydrophobin-like proteins. The foam breaker was removed and the generated foam in the ALB was recovered and suspended in distilled water. An aliquoted of suspension was centrifuged (6000 x g, 10 min). Insoluble fraction was suspended in 2% SDS dissolved in Tris-HCL buffer (0.1 M, pH 9, 2 h); then the sample was centrifuged (7740 x g, 4 °C, 10 min), the soluble fraction was mixed with 2 M KCl (1:4) to precipitate SDS and discard after centrifugation (7500 x g, 4 °C, 10 min); the insoluble fraction was suspended in concentrated formic acid (FA) and sonicated three times during 30 s in cold water, the soluble fraction was separated by centrifugation (7500 x g, 4 °C, 10 min), then it was neutralized with 45% (w/v) NaOH to pH 7 and kept during 12 h at 4°C. All supernatants were stored (4°C) for further emulsifying activity determination.

5.2.4.2. Surface activity characterization

Two methods were used to characterize the protein surface activity: (i) emulsifying activity and (ii) extended interfacial surface area.

The emulsifying activity was determined by turbidimetry. The generated foam in the ALB was collected and suspended in distilled water. A method based on reported by Rosenberg *et*

al. [19] was used to measuring the emulsifying activity, in brief: 30 μL of suspended foam and 10 μL of oil phase were added to 2.96 mL of 0.1M Tris-HCl (pH 7); the mixture was vortexed (3000 rpm, 2 min) then, it was allowed to stand during 5 min and optical density was measured (600 nm) using a Cary 50, Varian, Australia. A difference of 0.1 absorbance unity in the mixture, with respect to a control (MM without EP) was considered one emulsifying activity unity (U). Specific emulsifying activity per milligram of protein was reported (U mg^{-1}). The emulsifying activity was determined using 5 hydrocarbon samples: I, HXD; II, diesel; III, 1% w/v pyrene and 1% phenanthrene dissolved in HXD; IV, a blend composed by HXD and 2-methyl naphthalene (1:1) and V, 1% phenanthrene and 1% pyrene dissolved in blend IV.

Extended interfacial surface area was determined measuring the droplets diameter in emulsion. A Mastersizer 2000, Malvern, UK, was used to measure the Sauter mean diameter (D_{32}) and the extended interfacial surface area was estimated as follows:

$$a_{HC} = \left(\frac{6}{D_{32}} \right) \phi$$

Where a_{HC} is the interfacial surface area (reported as m^{-1}) and, ϕ is the hydrocarbon dispersed phase fraction (dimensionless).

5.2.5. Statistical analysis

All cultures were carried out in duplicate and all measurements were carried out in triplicate. Experimental data were compared using ANOVA and the Tukey test ($\alpha \leq 0.05$).

5.3.Results and discussion

5.3.1. Bioemulsifier production

Figure 2a shows that *A. brasiliensis*, previously electrochemically treated, was able to produce the EP from early times, in the airlift bioreactor (ALB) during HXD consumption, reaching a maximal concentration at 18 h (0.5±0.02 mg mL^{-1}), which was maintained during subsequent 18 h; finally, protein was probably partway degraded. Otherwise, the negative control (*A. brasiliensis* without electrochemical pretreatment; Figure 2b) produced less protein reaching the maximum concentration (0.33±0.01 mg mL^{-1}) at 36 h, *i.e.* average

protein production rate using the pretreated *A. brasiliensis* was 3 fold-higher than the negative control. Emulsion and foam were only produced in the airlift bioreactor (ALB) during HXD consumption by *A. brasiliensis*, previously electrochemically treated. Since 52.9±0.3% of the foam dry weight was protein, it was considered to quantify the bioemulsifier production. A similar result was observed by Hosseini *et al*. [20], they observed a protein production enhanced two times by an electric current (5-10 mA) application during the *Fusarium oxisporum* growth in liquid culture; however, they applied the electric current into the same liquid culture that protein was produced. In contrast, in this work the electric current was applied in a solid state culture, which was independent to the liquid ALB where EP was produced.

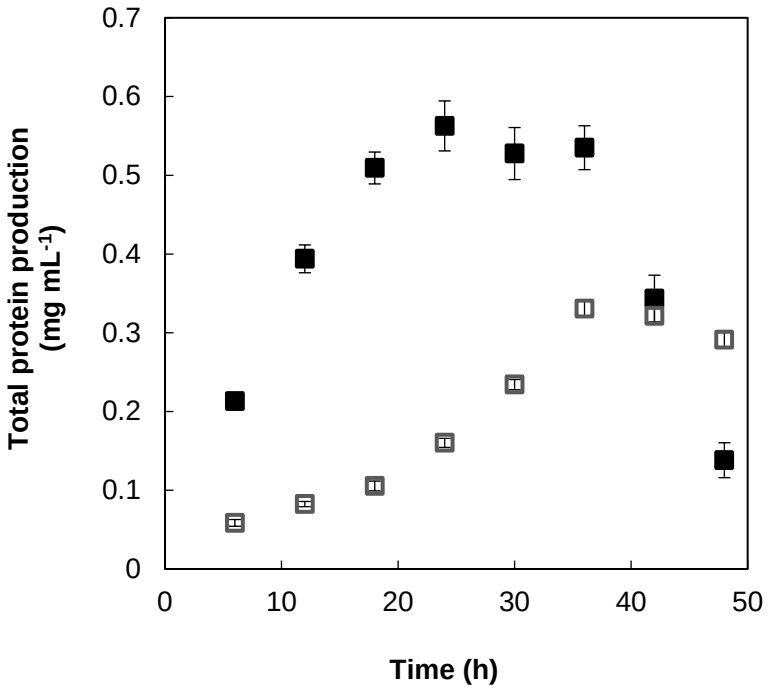


Figure 2. Total soluble protein production in the airlift bioreactor by the electric field pretreated (a, filled symbols) and untreated (b, open symbols) *Aspergillus brasiliensis*, using hexadecane as a carbon source.

The bioemulsifier could be, hypothetically: (i) a metabolic product when *A. brasiliensis* degraded hydrocarbons as reported for bioemulsifier producer bacterial consortium [21] or (ii) a product of the environmental change *i.e.* when the fungus was transferred from the electrochemical bioreactor (solid state culture) to the ALB (liquid culture), the cytoplasmatic proteins could be excreted due to enhance membrane permeability promoted by the electrical

pretreatment, as recently reported [22]; if so, the same EP production level should be observed independently of the supplied carbon source (hydrophobic or hydrophilic) in the ALB, suggesting damage in the fungal biomass. To reject damage in *A. brasiliensis*, two similar experiments (see Figure 3) were carried out as controls: (i) with glucose as carbon source and (ii) without carbon source; both were compared with and without the electrochemical pretreatment. When glucose was used as carbon source, the EP was not produced in the ALB using pretreated *A. brasiliensis*. When *A. brasiliensis* was used without electrochemical pretreatment, the total protein production was 0.04±0.01 mg mL^{-1} at 36 h, total protein production using glucose was an order of magnitude lower than using HXD. Also, neither emulsion nor foam was observed. When the experiment without carbon source was carried out, a basal protein concentration (0.02 ± 0.005 mg mL^{-1}) was observed during all culture time (48 h). This result demonstrates that the fungal biomass was maintained without damage after the electrochemical pretreatment; moreover, in a subsequent airlift liquid culture, protein production was probably regulated by the fungal physiological response to the presence of different carbon sources.

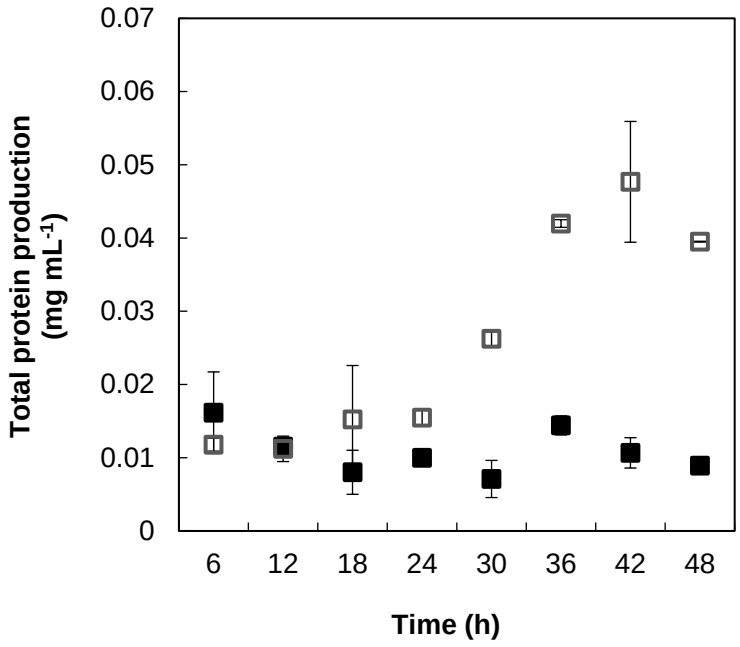


Figure 1. Total protein production in the airlift bioreactor by the electric field pretreated (filled symbols) and untreated (open symbols) *Aspergillus brasiliensis*, using glucose as carbon source.

So far, the pretreated *A. brasiliensis* can produce a bioemulsifier in an ALB, only in the presence of HXD as carbon source which was a hydrophobic substrate and the possible

inductor of the EP production. Filamentous fungi produce small proteins named hydrophobins with specific biological role [23], *e.g.*, up taking a hydrophobic substrate like gas oxygen or hydrocarbons, as in this work. Hydrophobins are the proteins which have the highest known surface activity, as either foam and emulsion stabilizers [1]. In this work, the EP seemed to be a good emulsion stabilizer. Then it was necessary to know its EA quantitatively.

An important characteristic in emulsion stabilization is the adsorption of emulsifier at the liquid-liquid interface [24,25] so that, interactions between the different phases (adsorbent) and the bioemulsifier (adsorbate) can be determined by their affinity. To evaluate the affinity between the EP and the organic phase, emulsifying activity was measured using five different hydrocarbons, pure HXD, and blends. Figure 4 shows that the specific activity was lowest when the organic phase is a sole aliphatic hydrocarbon such as HXD or a mixture rich in aliphatic such as diesel (I and II, Figure 4). Diesel is a blend of aliphatics with low HAPs concentration (up to 340 mg L^{-1}, mainly phenanthrene) [26]. When HXD was saturated with 1% w/v phenanthrene and pyrene the average emulsifying activity was enhanced 19% (not significantly) concerning the pure HXD. On the other hand, when bioemulsifier was in the presence of a blend with 50 %w/w 2-methylnaphthalene (VI and V. Figure 5), the emulsifying activity was 2.79 fold-higher than pure HXD; on the other hand, blend V was not significantly different to IV, but the measurements had lower variations. Similar results were observed by Rosenberg *et al.* [19] using a bacterial emulsifier. This result suggests that the affinity of EP is higher to HAP than aliphatic hydrocarbons. The next step was to evaluate the emulsifying activity as a function of the downstream procedure steps for separation and purification.

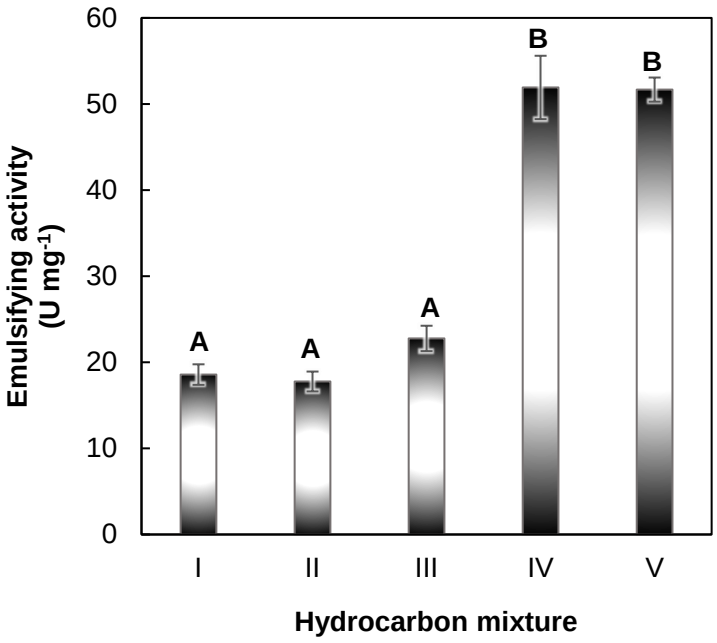


Figure 2. Emulsifying activity of emulsifier extract in front of different hydrocarbons blend: (I) hexadecane, (II) diesel, (III) 1% phenanthrene and 1% pyrene dissolved in hexadecane, (IV) hexadecane-2methyl naphthalene (1:1 v/v) and (V) 1% pyrene dissolved in mixture IV. Different letters on histograms indicate significant differences (ANOVA, Tukey; $p<0.05$).

5.3.2. Emulsifier extraction and purification

To assess the EP emulsifying activity, an adequate extraction/separation procedure is needed. Foam collecting seems to be an economic and simple downstream procedure to concentrate the bioemulsifier [27]. Protein concentration in the generated foam was 20 fold-higher than liquid phase into the ALB. The selected procedure for purification was based on a hydrophobin purification method [18]. The purification procedure used in this work has usually been focused on proteomic analysis and characterized regarding yields and specific surface activity obtained in each purification step [18,28]. In this work, it has been evaluated as a possible downstream process for emulsifier protein separation and purification, based on the EA. In that context the purification process needs to be characterized.

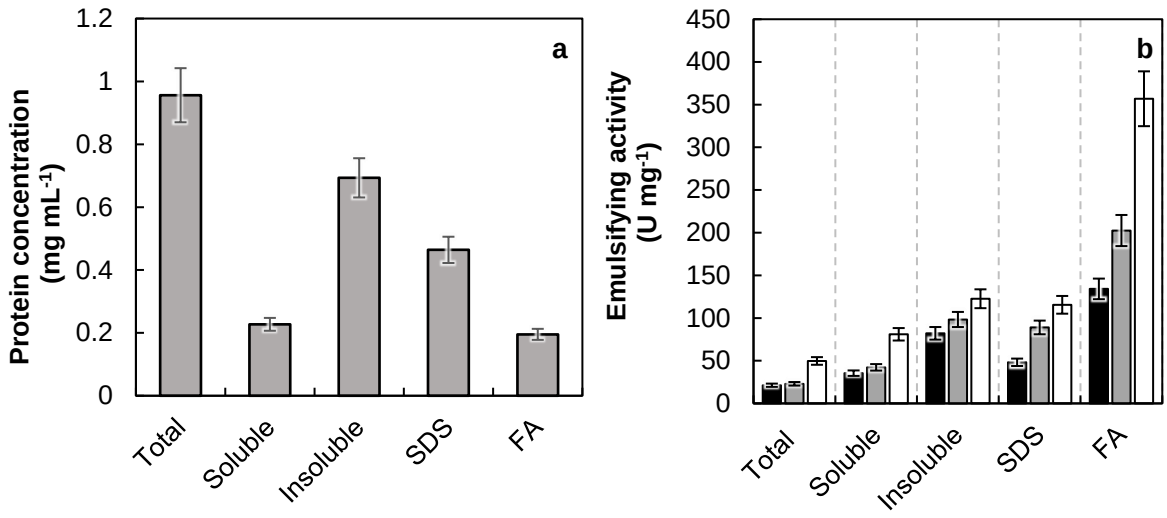


Figure 5. Recovery of emulsifier protein during each separation and purification steps (a) and emulsifying activity quantification (b) using hexadecane (black), 1% phenanthrene and 1% pyrene dissolved in hexadecane (grey) and hexadecane-2-methyl naphthalene (1:1 v/v, white).

The 96.2 % of total protein was recovered in the first purification step, observing a water insoluble fraction with activity 1.5 fold-higher than soluble (Figure 5 a). In the second purification step (2% SDS) 80% of insoluble protein was recovered. Finally, the FA soluble fraction was 19.5 % of total protein (Figure 5a). According to the purification procedure, the expected emulsifying activity should be enhanced in each purification step based on each hydrophobin class [28]. Figure 5b shows that the emulsifying activity in the FA soluble fraction was 7 fold-higher than emulsifying activity of the total protein, this is characteristic of class I hydrophobins [11]. Although several positive characteristics have been observed in laboratory scale, its use is very limited due to the lack of knowledge for industrial production [13]. Therefore, there is a need to evaluate the effectiveness of purification procedure based on the emulsifying activity on hydrocarbons and the evaluation of key factors that affect the EA, such as pH, temperature and salt concentration. The functionality of the emulsifier protein was easily measured as emulsifying activity resulting in an extended interfacial surface area.

5.3.3. Extended interfacial Surface area as emulsifying activity

The interfacial surface area a_{HC} in the emulsion is the result of EP adsorption on the liquid-liquid interface; its magnitude depends on the purity grade of the EP. The EP content in the collected foam stabilizes an emulsion with a bimodal distribution Sauter mean diameter

droplets (22.9-2.9 μm), resulting in an extended interfacial surface area of 1047 m^{-1}. On the other hand, the purified EP was able to stabilize an emulsion with a Sauter mean diameter of 1.2 μm, resulting in an extended interfacial surface area of 8400 m^{-1}. Here the question was: why purifying? Depending on the final customer or industrial application, a downstream process from separated foam, rich in EP, would be unnecessary *e.g.* hydrocarbon mobilization for remediation or enhanced oil recovery. Thus, this paper proposes a practical and staightforward method to produce, in bioreactors, a bioemulsifier able to mobilizing hydrocarbons in contaminated environments as a bioremediation agent similarly to early report [29]. To complete the characterization of EP, its stability under different operational conditions is needed to propose EP as remediation enhancers.

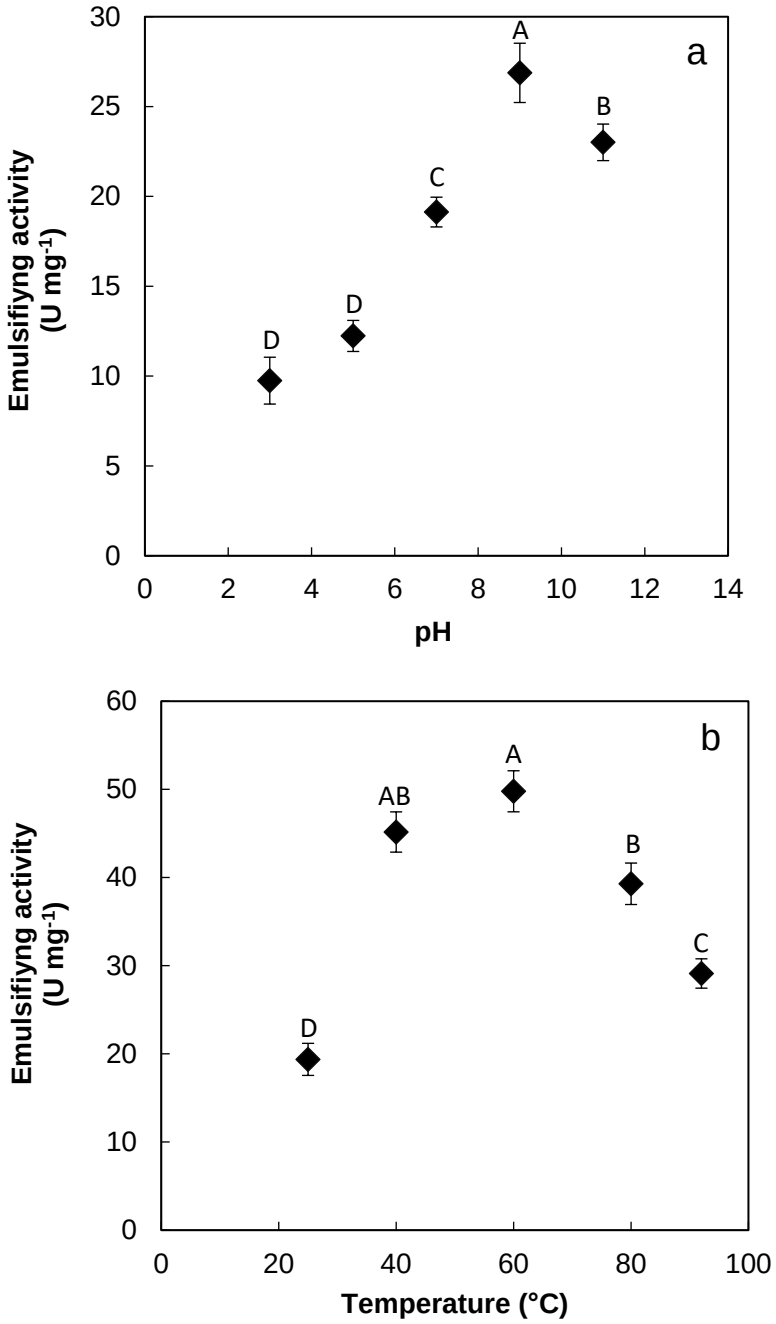
5.3.4. On the emulsifier protein stability

The stability of the EP and its emulsifying activity is a crucial parameter for elucidate the applicability on environmental remediation purposes. So, the EA was evaluated under extreme conditions of pH, temperature and salinity. Figure 6a shows that acidic pH was unfavourable for the emulsifying activity. At pH 9 the emulsifying activity was the highest observed. This could be related to the folding state of the EP as well as the protein net charge. Wierenga *et al.* [30] found that pH is thermodynamically related to the surface activity. Nearby the isoelectric point, there is a low energy barrier to interfacial adsorption, resulting in a high surface activity.

The temperature effect on the emulsifying activity was determined by sample preheating; results are in the Figure 6b. When bioemulsifier was preheated, up to 92 °C, its emulsifying activity was enhanced, 60 °C preheating was the highest assayed treatment (2.5 fold-higher than not preheated). Pan *et al.* [31] reported that adsorption of protein on hydrophobic surface involves a reversible folding-unfolding step. In our case, the preheating could reversibly unfold the protein facilitating its adsorption on hydrocarbon, enhancing its emulsifying activity. However, unfolding could be due to preheating and/or another surrounding chemical compounds such as pH or salt presence.

The stability of the emulsifying activity in salinity conditions is shown in the Figure 6c. An apparent diminishment of the emulsifying activity was found; however, in the assayed concentration range differences were not significant, this range was specified to mimic the

salinity in sea water. The salt concentration is important physicochemical parameter due to counter-ions can adsorb by ion-ion interactions altering the emulsifying activity [1]. EP obtained in separated foam showed an emulsifying activity with high versatility in different operational conditions; alkaline environments and preheating samples enhanced the emulsifying activity.



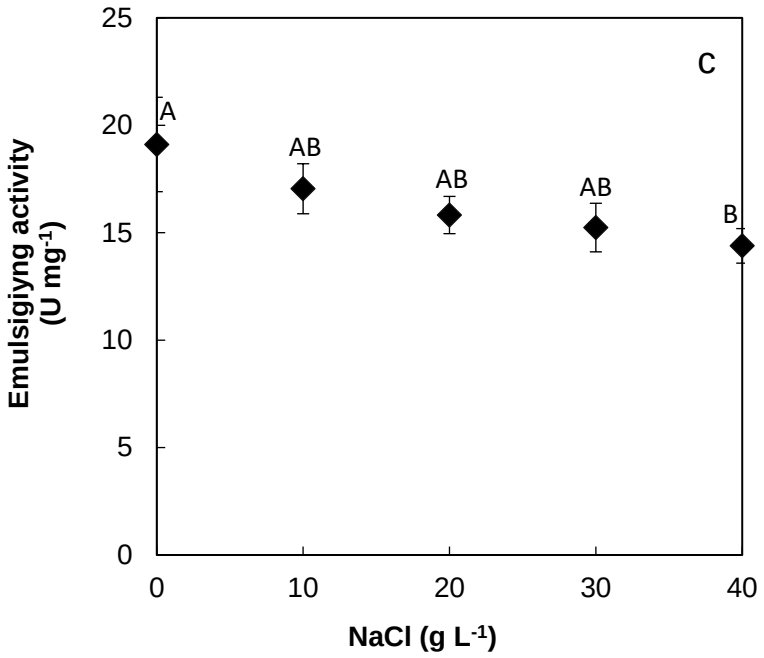


Figure 6. Variations of emulsifying activity in different environmental conditions of pH (a), temperature of preheating (b) and salinity (c). Different letters indicate significant differences (ANOVA, Tukey; $p<0.05$).

Conclusions

The method proposed in this work for emulsifier protein production is simple and practical for environmental bioremediation proposes. An electrochemical pretreatment on *Aspergillus brasiliensis* promoted the capability to produce an emulsifier protein in a subsequent liquid airlift culture. Emulsifier protein production was observed only in the presence of HXD as a hydrophobic substrate, being 19.5 % of the total protein produced in the liquid culture; after purification, its specific emulsifying activity enhanced 7 times. This emulsifier had higher affinity for polycyclic aromatic hydrocarbons than aliphatic. Emulsifying activity was enhanced in alkaline media and by preheating the emulsifier protein samples. The salinity in the liquid medium did not affect the emulsifying activity. The different results obtained in this work suggest that the emulsifier protein was a hydrophobin-like protein; however, a further proteomic and secretomic analysis of *A. brasiliensis* is needed to identify the bioemulsifier.

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Partial contribution

Chapter 4 shows the role of an emulsifier agent on the hydrocarbon degradation and makes evident the need to its functional characterization. The pretreated biocatalyst was able to produce an emulsifier protein in a liquid airlift culture. The emulsifier protein production was observed only after the electrochemical pretreatment, in the presence of a hydrophobic substrate such as hydrocarbons. The emulsifier protein was able to stabilize oil in water emulsions using different hydrocarbon blends, observing emulsifying activity values in the presence of polycyclic aromatic hydrocarbons a 2.79 fold-higher than solely aliphatic. Emulsifying activity was enhanced in alkaline environments and by preheating samples. The salinity in the liquid medium did not affect the emulsifying activity. The bioemulsifier protein was 19.5 % of the total protein produced in the liquid culture, and purification enhanced seven times its emulsifying activity. Our Results suggest that the emulsifier protein was a hydrophobin; however, a further analysis on proteomic and secretomic of *A. brasiliensis* is needed in order to protein identification.

General discussion as a run-down

In this work a fungal whole cell biocatalyst is proposed as hydrocarbon contaminated water remediation role. Biocatalyst is a good sorbent-degrader of aliphatic and polycyclic aromatics hydrocarbons, solely and in blend. With respect to the hydrocarbon degradation in blend, a phenomenological analysis in the multiphasic airlift bioreactor (liquid-liquid-gas-porous solid with attached fungal biomass) was performed. Results can be summarized using a regimen analysis technique, in which we can observe the characteristic times of each step during hydrocarbons degradation. Since Chapter 4 shows that hydrocarbons mass transfer into the biocatalyst are the main limitation, the characteristic times (t_c) were normalized based on the hydrocarbon effective diffusivity (see Table A).

Table A. Estimated kinetic parameters and characteristic times related to each step involved in hydrocarbon degradation in the airlift bioreactor.

		Parameter	**Characteristic times**
Untreated BC	$k_L a_{O_2}$	2.6 x 10^{-2} s^{-1}	37.0 s
	$k_L a_{HXD}$	1.6 x10^{-4} s^{-1}	1.6 h
	$k_L a_{PHE}$	4.7 x 10^{-6} s^{-1}	59.0 h
	$k_L a_{PYR}$	2.6 x 10^{-6} s^{-1}	107.0 h
	$D_{eff_{HC}}$	1.36 x 10^{-6} cm^2 s^{-1}	5.8 h
	k_{HXD}	6.13 x 10^{-3} s^{-1}	2.7 min
	k_{PHE}	2.02 x 10^{-5} s^{-1}	13.0 h
	k_{PYR}	8.2 x 10^{-6} s^{-1}	33.0 h
Pretreated BC	$k_L a_{o_2}$	3.5 x 10^{-2} s^{-1}	28.0 s
	$k_L a_{HXD}$	4.4 x 10^{-3} s^{-1}	3.8 min
	$k_L a_{PHE}$	1.2 x 10^{-4} s^{-1}	2.3 h
	$k_L a_{PYR}$	1.2 x 10^{-4} s^{-1}	2.2 h
	$D_{eff_{HC}}$	1.85 x 10^{-6} cm^2 s^{-1}	4.2 h
	k_{HXD}	1.4 x 10^{-2} s^{-1}	1.1 min
	k_{PHE}	5.9 x 10^{-5} s^{-1}	3.0 h
	k_{PYR}	3.6 x 10^{-5} s^{-1}	6.5 h

It can be observed that interfacial mass transfer characteristic times decreased one and two orders of magnitude for hydrocarbons, *i.e.* the formed emulsion improved the interfacial mass transfer, then the apparent concentration of hydrocarbon into the biocatalyst was enhanced, promoting a faster hydrocarbon degradation, with an effectiveness factor close to unity for all three hydrocarbons. The first order kinetic parameters for hydrocarbon degradation decreased to 40, 21 and 19% for HXD, PHE and PYR, respectively, when the emulsifier protein was produced. The effective diffusivity and these characteristic times values were not significantly modified by the emulsifier presence; however, the accuracy of the estimation method was not enough. In general, Chapter 4 provides a selected engineering parameters set for design and scale up of airlift bioreactors for oil contaminated water treatment.

On the other hand, hydrocarbons uptake mechanisms were modified after electric field pretreatment. Surface changes on BC improved the hydrocarbon degradation by direct contact, mainly for pyrene, during the first hours of culture time; then, dominant uptake mechanism became via emulsified hydrocarbons. Since the emulsifier protein have high emulsifier activity, which was stable on wide pH, temperature and salinity ranges, it could be applied into several environmental and industrial application.

Conclusions

Aspergillus brasiliensis attached to perlite acts as a fungal whole cell biocatalyst, able to degrade an aliphatic-polycyclic aromatic hydrocarbons blend. An electric field pretreatment on the biocatalyst as a method to enhance its hydrocarbon degrader activity was relevant for hexadecane, phenanthrene and pyrene, in blend.

The electric field pretreatment promoted surface changes on the biocatalyst improving both, biomass attachment strength and hydrocarbon sorption capacity. The biocatalyst affinity for hydrocarbon after the electric field pretreatment enhanced in an order of magnitude and, the enhancement was highest for pyrene followed by phenanthrene and lowest for hexadecane.

The biocatalyst was able to produce an emulsifier protein that was related with a modified hydrocarbon uptake mechanism. The emulsifier protein was produced only after the electric field pretreatment, in the presence of a hydrophobic substrate. The emulsifying activity was higher in the presence of polycyclic aromatic hydrocarbons in blend than solely aliphatic. The emulsifying activity was stable in salty medium and improved in alkaline media or by preheating sample up to 92 °C.

Surface changes on the biocatalyst and the produced emulsifier protein were related to a non-specific hydrocarbon degradation diminishing the mass transfer limitations, both internal and external *i.e.* the pretreated biocatalyst was able to degrade all three assayed hydrocarbons in blend with an effectiveness factor close to unity.

Perspectives and recommendations

In this thesis, a phenomenological analysis was developed for hydrocarbons degradation by a fungal whole cell biocatalyst. It was demonstrated that hydrocarbon degradation after an electric field pretreatment was non-specific, with lower mass transfer resistances. This would allow to achieve a kinetic study focused on performing a mechanistic intrinsic model. The intrinsic kinetics study could be a useful tool to identify the possible metabolic modifications associated to the electric field pretreatment. *i.e.* some enzymatic reactions accelerated by the pretreatment. Also, the intrinsic kinetics study can be experimentally accomplished by a proteomic and metabolomic analysis focused on bioreactor design and scale up.

The emulsifier protein, hypothetically a hydrophobin, observed in the airlift bioreactor was an essential element for hydrocarbon degradation. Furthermore, it is important to perform a chemical identification, based on proteomic tools. Hydrophobins of *Aspergillus brasiliensis* production from contaminated water could be an economically interesting process.

On the other hand, the solid state bioreactor is currently not optimized. A pH, and fungal growth gradient was observed during the electric field application, which could be an operational problem for scale up purposes. It is important to find another configuration based on scalable dimensionless parameters, diminishing the heterogeneity in the solid state culture associated to the electric field application.

Casa abierta al tiempo
UNIVERSIDAD AUTÓNOMA METROPOLITANA

ACTA DE DISERTACIÓN PÚBLICA

No. 00191
Matrícula: 2163804132

PHENOMENOLOGICAL ANALYSIS OF HYDROCARBON DEGRADATION BY FUNGAL ELECTRO-BIOCATALYST IN CONTAMINATED WATER

En la Ciudad de México, se presentaron a las 17:00 horas del día 17 del mes de enero del año 2018 en la Unidad Iztapalapa de la Universidad Autónoma Metropolitana, los suscritos miembros del jurado:

DRA. CONCEPCION KEIKO SHIRAI MATSUMOTO
DR. SERGIO ANTONIO GOMEZ TORRES
DR. GERMAN BUITRON MENDEZ
DR. CARLOS OMAR CASTILLO ARAIZA

Bajo la Presidencia de la primera y con carácter de Secretario el último, se reunieron a la presentación de la Disertación Pública cuya denominación aparece al margen, para la obtención del grado de:

DOCTOR EN BIOTECNOLOGIA

DE: VICTOR SANCHEZ VAZQUEZ

y de acuerdo con el artículo 78 fracción IV del Reglamento de Estudios Superiores de la Universidad Autónoma Metropolitana, los miembros del jurado resolvieron:

APROBAR

Acto continuo, la presidenta del jurado comunicó al interesado el resultado de la evaluación y, en caso aprobatorio, le fue tomada la protesta.

VICTOR SANCHEZ VAZQUEZ
ALUMNO

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