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Biodegradation of crude oil by *Aspergillus tubingensis*: biosurfactant-producing strain

Biodegradación de petróleo crudo por *Aspergillus tubingensis*: cepa productora de biosurfactantes

D.L. Peraza-Liñan[1], B.A. Ayil-Gutiérrez[2], A.A. Urías-Salazar[3], J.A. López-Santillán[1], B. Estrada-Drouaillet[1], M.T.J. Segura-Martínez[1], W.A. Poot-Poot[1]*

[1]División de Estudios de Posgrado e Investigación, Facultad de Ingeniería y Ciencias, Universidad Autónoma de Tamaulipas, Centro Universitario Adolfo López Mateos, Victoria, Tamaulipas, C.P. 87120, México.

[2]Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Boulevard del Maestro s/n, Esquina Elías Piña, Reynosa, Tamaulipas, C.P. 88710, México.

[3]Universidad del Valle de México, Campus Victoria, Calle Juan B. Tijerina 306, Zona Centro, Victoria, Tamaulipas, C.P. 87000, México.

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Abstract

Crude oil is one of the most demanded fuels which causes an increase in environmental pollution due to spills. Bioremediation by hydrocarbonoclast microorganisms can be an alternative for hydrocarbon degradation through biochemical processes involving enzymes and biosurfactants. The objective of this work was to evaluate the capacity of oil biodegradation by a hydrocarbonoclast strain through growth kinetics and biosurfactant production. The strain was inoculated in flasks with mineral medium (MM) supplemented with oil (0, 20, 40, 60, and 80 %) and grown for 20 days. The variables evaluated were oil degradation by Soxhlet extraction, quantification of extracellular proteins, proteolytic activity, biosurfactant production by lysis in blood agar and emulsification capacity (E_{24}). The results allow concluding that the strain identified as *Aspergillus tubingensis* was able to biodegrade 78.38 % of the crude oil at the 80 % concentration and maintain a biomass production of 55.12 g/L, a low proteolytic activity of 0.0015 U/mL, as well as biosurfactant production and an E_{24} of 65.16 %. Therefore, *A. tubingensis* can be used in bioremediation works to recover oil contaminated sites.

Keywords: Bioremediation, Hydrocarbons, Filamentous Fungi, Biosurfactants, Growth Kinetics.

Resumen

El petróleo es uno de los combustibles de mayor demanda lo que ocasiona un aumento en la contaminación ambiental por derrames. La biorremediación por microorganismos hidrocarbonoclastas puede ser una alternativa para la degradación de hidrocarburos mediante procesos bioquímicos en donde participan enzimas y biosurfactantes. El objetivo de este trabajo fue evaluar la capacidad de biodegradación de petróleo por una cepa hidrocarbonoclasta mediante cinéticas de crecimiento y producción de biosurfactantes. La cepa se inoculó en matraces con medio mineral (MM) suplementado con petróleo (0, 20, 40, 60, y 80 %) y se cultivó durante 20 días. Las variables evaluadas fueron la degradación de petróleo por extracción Soxhlet, cuantificación de proteínas extracelulares, actividad proteolítica, producción de biosurfactantes por lisis en agar sangre y capacidad de emulsión (E_{24}). Los resultados permiten concluir que la cepa identificada como *Aspergillus tubingensis* pudo biodegradar el 78.38 % del crudo en la concentración del 80 % y mantener una producción de biomasa de 55.12 g/L, una baja actividad proteolítica de 0.0015 U/mL, así como la producción de biosurfactantes y un E_{24} de 65.16 %. Por lo tanto, *A. tubingensis* puede utilizarse en trabajos de biorremediación para recuperar lugares contaminados por petróleo.

Palabras clave: Biorremediación, Hidrocarburos, Hongos Filamentosos, Biosurfactantes, Cinética de Crecimiento.

*Corresponding author. *E-mail*: wpoot@docentes.uat.edu.mx;
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1 Introduction

Petroleum is a mixture of hydrocarbons, which are classified into aliphatic, aromatic, resins and asphaltenes, each of which has physicochemical characteristics that make them susceptible to degradation depending on their molecular weight (Mishra and Singh, 2012). It is used as a source of energy in the industry, however, its demand at a global and national level has increased extraction, refining, production and distribution activities, which in turn causes an increase in the number of spills in the environment due to leaks in storage tanks, damage to pipelines, accidents during transportation, among others (Binazadeh *et al*., 2020; Benguenab and Chibani, 2021).

In the environment, spills remain for a prolonged period of time due to slow degradation, which prevents the recovery of the site and reduces flora and fauna populations due to the mortality rate caused by the spill (Barrios, 2011; Farrington, 2014); in humans, can cause harmful effects derived from its toxic characteristics, such as cancer and DNA mutations (Mahjoubi *et al*., 2013; Zhang *et al*., 2019). Although there are techniques for oil removal, such as the use of containment booms, chemical dispersants, incineration and UV oxidation, they generally have low efficiency, require constant application and raise operating costs (Sayed *et al*., 2021). Bioremediation is a low-cost technique that operates under controlled conditions and with few inputs; it uses the metabolic capacity of microorganisms to degrade hydrocarbons present in oil to simpler molecules such as CO_2, H_2O, fatty acids and produce cell biomass (Perera *et al*., 2019; Czarny *et al*., 2020).

Among the microorganisms that degrade petroleum hydrocarbons are fungi, which stand out as the main degraders of organic matter, capable of degrading compounds with structures similar to polycyclic aromatic hydrocarbons (PAHs), where species of the genera *Trichoderma* (Al-Zaban *et al*., 2021)*, Penicillium* (Benguenab and Chibani, 2021), *Alternaria* (Balaji *et al*., 2014) and *Aspergillus* (Al-Hawash *et al*., 2018a; Al-Hawash *et al.,* 2018b) have been reported in degradation, the latter being the most abundant in most contaminated sites, suggesting a potential for use in the degradation of a wide range of hydrocarbons (Balaji *et al*., 2014; El-Hanafy *et al*., 2016; Al-Hawash *et al*., 2018a; Benguenab and Chibani, 2021). Within the species of the genera *Aspergillus* are found *A. niger, A. ustus, A. fumigatus, A. ochraceus, A. parasitics, A. tamarii, A. versicolor* (Balaji *et al.,* 2014). In addition to biodegrading hydrocarbons and petroleum derivatives such as polyester polyurethane (Khan *et al*., 2017), *Aspergillus tubingensis* is capable to remove heavy metals such as Pb^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} in soils contaminated with these ions, which it does through mechanisms of adsorption on the cell surface, intracellular uptake, as well as biomineralization (Nadumane *et al*., 2016; Jha *et al*., 2024). However, an excess of heavy metals in contaminated soils can inhibit the biodegradation of the hydrocarbons present in the crude oil (Hkiri *et al*., 2023).

Hydrocarbonoclast fungi isolated from oil contaminated sites have the potential to degrade hydrocarbons through the production of biosurfactants, which are amphipathic molecules that aid in the solubilization of hydrocarbons allowing their bioavailability to the microorganism, which ensures a reduction of the surface tension between the culture medium interface and the hydrocarbon (Lu *et al*., 2009; Al-Hawash *et al*., 2018a). This degradation capacity is also associated with the production of extracellular enzymes with low substrate specificity that catalyze degradation reactions of organic compounds to less hazardous forms (Yuniati, 2018; Arregui *et al*., 2019; Espinosa-Ortiz *et al*., 2021). Therefore, the objective of this work was to evaluate the capacity of oil biodegradation by a hydrocarbonoclast strain through growth kinetics and biosurfactant production.

2 Materials and methods

2.1 Isolation and selection of the fungal strain

The strains were isolated from water samples contaminated with crude oil from the Dos Bocas refinery, located in Tamalin, Veracruz, Mexico (21°31'27.3" N and 97°38'01.7" W). Isolation was performed by inoculating 100 μL of the serial dilutions (10^{-1}-10^{-5}) in Petri dishes with 20 mL of sterile potato dextrose agar (PDA) (39 g/L, autoclaved at 15 psi, 121 °C for 15 min). Petri dishes were incubated at 28 °C for 7 days and strains were purified by hyphal tip technique. Strain selection was performed by inoculating 50 mm mycelial discs in Petri dishes with PDA and crude oil (20-80 %), incubated under the same conditions and the strain with the highest growth in the crude oil culture medium was selected for the experiment.

2.2 Genomic DNA extraction and molecular identification of the strain

Genomic DNA extraction was performed from colonies grown in Petri dishes with potato dextrose agar (PDA) of the strain for 7 days. Molecular identification was carried out by PCR amplifying the ITS region (Internal Transcribed Spacer) with

primers ITS1 (5'-TCCGTAGGTGAACCTTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR reactions were carried out in volumes of 25 μL containing 2 μL of DNA, 2.5 μL of Taq buffer (10 X), 2 μL of $MgCl_2$ (50 mM), 2.5 μL of dNTPs (10 mM), 3 μL of each primer (20 mM) and 1 μL of Taq DNA polymerase (0.5 U/μL, Meridian Bioscience). Amplification was carried out in a thermal cycler (Cleaver Scientific) at 94 °C for 2 min and 40 amplification cycles at 94 °C for 90 s, at 54 °C for 30 s and a final extension at 72 °C for 1 min. PCR products were visualized in a 2 % agarose gel (BIOLINE®) at 80 v for 1 h, purified and sequenced at the Institute of Biotechnology of the National Autonomous University of Mexico. For molecular identification, the BLAST database of the NCBI (National Center of Biotechnology Information) was used.

2.3 Obtaining inoculum

The strain preserved in 10 % glycerol was activated in Petri dishes with PDA (39 g/L, autoclaved at 15 psi, 121 °C for 15 min) and incubated at 28 °C for 7 days, then three 50 mm diameter agar plug as inoculum were taken from the periphery of the mycelium and transferred to 125 mL flasks with 50 mL of mycological broth (50 g/L), added with 1.5 g of yeast extract (MB+YE) then incubated at 28 °C in an orbital shaker at 140 rpm for 3 days. The inoculum was obtained by taking 2 mL of the biomass and washed three times with sterile distilled water until the residue of the MB+YE culture medium was removed and then stored at 4 °C until use in the crude oil bioassays.

2.4 Bioassays with crude oil

Bioassays were performed in 125 mL Erlenmeyer flasks with 50 mL of mineral medium (MM). The medium contained (in g/L), 1 $K_2HPO_4{\cdot}3H_2O$, 0.4 KH_2PO_4, 0.5 NaCl, 0.2 $(NH_4)_2SO_4$, 0.1 $NaNO_3$, 0.025 $MgSO_4{\cdot}7H_2O$ and 10 glucose, and the pH was adjusted to 7.0 with HCl and autoclaved at 15 psi, 121 °C for 15 min. Crude oil concentrations (0, 20, 40, 60 and 80 %) were added to the MM medium. The flasks were inoculated with 1 g of biomass (section 2.3) and incubated at 28 °C in orbital shaker at 140 rpm and the samples were taken every two days until 20 days of culture were completed.

2.5 Biomass production and growth kinetics

Biomass production was determined for each combination of crude oil and sampling time. This was obtained by filtering (0.25 μm) the culture media in a Kitasato flask adapted to vacuum pump (BIOBASE®) and then the biomass was dried in a heating oven (BOEKEL®) at 50 °C for 24 h. The kinetics and specific growth rate were obtained from the changes in dry weight (g/L) [X=X(t)] by using the Verhulst-Pearl equation (Equation 1), which has been used in biodegradation works (Ahuactzin-Pérez *et al*., 2018; Canul-Chan *et al*., 2023).

$$\frac{dX}{dt} = \mu\left(1 - \frac{X}{X_{\max}}\right)X, \text{ or } X\left(\frac{X_{\max}}{1} + Ce^{-\mu t}\right) \quad (1)$$

Where $X = X_0$ is the initial biomass value (g/L), $C = (X_{max} - X_0) / X_0$, X_{max} is the maximum or equilibrium value of biomass (g/L) and μt represents the specific growth rate at a given time. The μ parameter of the model was evaluated by a nonlinear least squares fitting program using the Microsoft Excel Solver function. The exponential growth phase was calculated by transforming the values obtained from the Verhulst-Pearl equation to base logarithm *e*.

2.6 Crude oil extraction and degradation

Samples were analyzed for each of the following oil combinations (20, 40, 60 and 80 %) and in two sampling times, 10 and 20 days. This was performed using the Soxhlet extraction method where the samples were mixed with anhydrous sodium sulfate (1:1), was loaded on a cartridge (filter paper 0.22 μm) and extracted for 7 h with dichloromethane (CTR Scientific®) at 39 °C. The results were analyzed by gravimetry by EPA method 9071 (1998) and the oil degradation was obtained by difference with the extraction yield.

$$\text{Extraction yields (\%)} = \frac{WFS - WF}{S} \times 100 \quad (2)$$

Where WFS is the weight (g) of the flask with sample, WF is the weight (g) of the flask and S is the weight (g) of the initial sample.

2.7 pH determination

The pH was determined with a potentiometer (ThermoScientific®) for each of the crude oil combinations (0, 20, 40, 60 and 80 %), this measurement was carried out every two days until 20 experimental days were completed.

2.8 Quantification of extracellular protein

For the quantification of extracellular protein, the extracellular protein quantification method was used Lowry *et al*. (1951), where the samples of each combination of the bioassays (0, 20, 40, 60 and 80 %) and sampling times, were filtered with a PTFE membrane (polytetrafluoroethylene terephthalate, 0.22 μm, Labfil®) for the elimination and obtaining the cell-free supernatant. The samples processed with the Lowry method were read in a spectrophotometer UV-VIS (UNICO®) at 590 nm and quantification was

performed using a calibration curve (0-500 μg/mL) and bovine serum albumin as standard (1 mg/mL, Sigma-Aldrich®).

2.9 Quantification of proteolytic activity

The quantification of the proteolytic activity of each of the crude oil combinations (0, 20, 40, 60 and 80 %) and sampling times, was performed using the Kunitz method *et al*. (1946) which consists of casein hydrolysis. For this, cell-free supernatants were inoculated in a mixture of 2 % (w/v) casein in phosphate buffer (200 mM and 8.7 % NaCl, pH 7.6). The mixture was incubated at 30 °C for 30 min and the reaction was stopped by adding 150 μL of 10 % (w/v) trichloroacetic acid, then centrifuged at 12 000 rpm for 10 min, the supernatant was read at 590 nm in a spectrophotometer UV-VIS (UNICO®) and the quantification was performed using a calibration curve (0-500 μg/mL) with the tyrosine standard (Sigma-Aldrich®). One unit of enzyme activity (U) was defined as the catalytic activity responsible for the transformation of 1 μmol of substrate per minute under defined conditions.

2.10 Biosurfactant production and emulsification index determination (E_{24})

Biosurfactant production was determined for each of the following oil blends (0, 20, 40, 60 and 80 %) and sampling times (2-20 days), using the blood agar lysis method as reported by Youssef *et al*. (2004). Samples of the cell-free supernatants were inoculated onto 0.5 cm diameter filter paper discs, then the discs were seeded in plates with 5 % (v/v) blood agar and incubated at 37 °C for 24 h. Biosurfactant production was determined by visual inspection of clear halos indicative of the presence of biosurfactants.

The emulsion index was determined by mixing 2 mL of cell-free supernatant with 2 mL of oil in polypropylene tubes (CORNING®). The mixture was left to stand for 24 h and then vortexed for 2 min. Photographs were taken for each of the tubes and analyzed with the ConfocalUniovi ImageJ version 1.49 program and to calculate the percentage of emulsion (E_{24}), the equation of Menezes *et al*. (2005).

$$E_{24} = \frac{HE_L}{TH_S} \times 100 \quad (3)$$

Where E_{24} is the emulsion index (%) at 24 h, HE_L is the height of the emulsified layer (cm), TH_S is the total height of the suspension (cm).

2.11 Statistical analysis

The experiment was established under a completely randomized design with factorial arrangement where the factors were crude oil concentration (%) and time of sampling (days). Growth kinetics was modeled using the Verhulst-Pearl equation and the exponential growth phase was calculated by transforming the values obtained from the Verhulst-Pearl equation to base logarithm *e*. Factor and interaction effects were analyzed with a multifactor ANOVA ($p \leq 0.05$); for response variables with significant interaction ($p \leq 0.05$) a Poisson regression was performed to estimate variances, and 95 % confidence intervals were calculated using the GENMOD procedure, variables with significant factor effect ($p \leq 0.05$) were analyzed by means of a comparison of means with Tukey's test (P=0.05), using the statistical program SAS version 9.0 (Statistical Analysis System, Institute Inc. 2002).

3 Results and discussion

3.1 Molecular identification of the strain

The fungus isolated from crude oil-contaminated water from the Dos Bocas oil field in Tamalin, Veracruz, Mexico was molecularly identified as *Aspergillus tubingensis* with a 99.56 % identity in NCBI GenBank and accession number MT609909.1. Fungi isolated and identified from contaminated sites include species of the genera *Trichoderma* (Al-Zaban *et al*., 2021), *Aspergillus* (Al-Hawash *et al*., 2018a; Al-Hawash *et al.,* 2018b), *Penicillium* (Benguenab and Chibani, 2021) and *Alternaria* (Balaji *et al*., 2014), nevertheless, *Aspergillus* is one of the most abundant genera in crude oil contaminated sites (Zhang *et al*., 2015; El-Hanafy *et al*., 2016). Among the reported species of *Aspegillus* are found *A. niger, A. ustus, A. fumigatus, A. ochraceus, A. parasitics, A. tamarii, A. versicolor* (Balaji *et al.,* 2014). *Aspergillus tubingensis* has demonstrated the ability to eliminate Pb^{2+} (98 %), Ni^{2+} (96.45 %), Cu^{2+} (92.19 %) and Zn^{2+} (93.99 %) (Nadumane *et al*., 2016), as well as potential for biodegrading polyester polyurethane (Khan *et al*., 2017), while in our case, the capacity to degrade crude oil.

3.2 Growth kinetics

The Verhulst-Pearl model made it possible to determine the dynamics and modeling of biomass production and thus obtain optimal knowledge of the growth kinetics of the strain at crude oil concentrations. With the logarithmic transformation (*e*) of the Verhulst-Pearl values obtained, it could be observed that in the control medium, exponential growth occurred from day 1 to 3 with 48.71 g/L of biomass, while the stationary phase was between days 8 and 20 with biomass of 48.02 and 48.71 g/L,

respectively. The maximum growth of the strain was 48.71±1.29 g/L on day 4 with a specific growth rate of 0.62 d^{-1} (Figure 1a). Our results differ from those reported by Pérez-Montiel *et al*. (2021) who evaluated the growth of *Pleurotus ostreatus* in mineral medium supplemented with glucose, registering a maximum biomass value of 3.45±0.10 g/L at 20 days, while in our work it was 48.71±1.29 g/L; suggesting that this growth is related to glucose consumption and that once the microorganism reaches its maximum biomass value, the amount of glucose decreases.

The growth kinetics of *A. tubingensis* in the presence of crude oil was different at each concentration according to the Verhulst-Pearl model. The kinetics of *A. tubingensis* in 20 % crude oil presented an exponential growth from day 1 to 3 (47.99 g/L) and a stationary phase from day 10 onwards with biomass values from 52.02 to 52.75 g/L. Maximum strain growth was 52.75±1.50 g/L on day 14 with a specific growth rate of 0.51 d^{-1} (Figure 1b). At the 40 % oil concentration *A. tubingensis* presented an exponential biomass growth from day 1 to 2 (44.49 g/L) and a stationary phase from day 12 to 20 of 48.20 to 48.36 g/L, respectively. The maximum growth value was 48.37±1.92 g/L on day 8 with a specific growth rate of 0.53 d^{-1}.

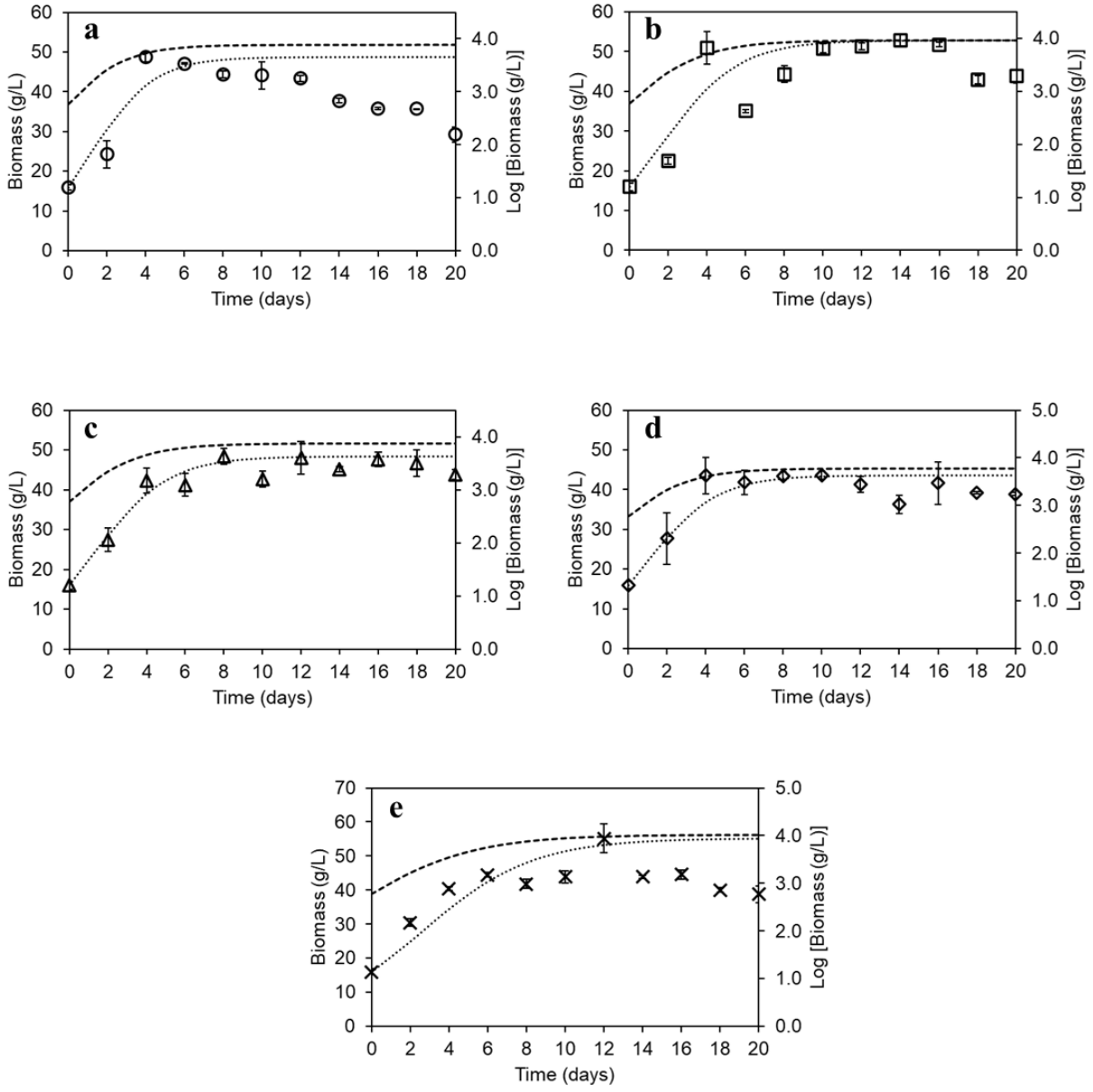

Figure 1. Growth kinetics of *Aspergillus tubingensis* biomass production in the presence of crude oil at different concentrations (%); a: control (0 %), b: 20 %, c: 40 %, d: 60 % and e: 80 %, (---) modelled with Verhulst-Pearl and (---) with the logarithm base (*e*).

With respect to the higher concentrations of 60 and 80 % crude oil, the model estimated changes in biomass production during the growth kinetics, where the specific growth rate was 0.56 d^{-1} 60 % of crude oil and 0.35 d^{-1} by 80 %. The exponential phase of *A. tubingensis* at the 60 % concentration occurred from day 1 to 2 (43.57 g/L), a stationary phase between days 10 to 20 with values from 43.32 to 43.59 g/L, as well as a maximum growth of 43.60 ± 1.10 g/L on day 10 (Figure 1d). While at the highest crude oil concentration of 80 %, the exponential phase occurred from day 1 to 4 (49.00 g/L) and the stationary phase between days 14 to 20 with values from 54.13 to 55.00 g/L, where the maximum growth was 55.12±4.20 g/L of biomass on day 12 (Figure 1e).

Our results differ from Benguenab and Chibani (2021) who reported that *Aspergillus ustus* presented a maximum biomass growth of 0.516 g/L using 6 % diesel as a carbon source, while in our work a value of 55.12 g/L of biomass was obtained with *A. tubingensis* in 80 % crude oil. For their part, Ahuactzin-Pérez *et al*. (2016) reported that *Fusarium culmorum* presented a maximum biomass value of 5.1 g/L using bis(2-ethylhexyl) phthalate at a concentration of 1000 mg/L, as well as a specific growth rate of 1.92 d^{-1}, while in our case the rate was 0.35 d^{-1} at 80 % crude oil concentration. The metabolic behavior of each microorganism depends on the complexity of the carbon source used, the exposure time and the composition of the culture medium, which is reflected in changes in biomass and growth rate, therefore, the kinetic characterization of the microorganism is important for a better efficiency in bioremediation processes (Kebria *et al*., 2009; Al-Hawash *et al*. , 2018b).

3.3 Crude oil degradation

Statistical analysis showed significant differences in crude oil degradation with the combinations of 20, 40, 60 and 80 %, as well as the exposure times of 10 and 20 days. At day 10 the oil degradation by *A. tubingensis* it was 66.63 % on the MM medium with 20 % crude oil, while on day 20 the degradation increased to 71.80 %, similarly degradation values were observed in the combination with 40 % crude oil, where the fungus degraded 50.36 % on day 10 and on day 20 it was 64.21 %. On day 10, at concentrations of 60 % and 80 % crude oil, the fungus degraded 50.36 % on day 10 and 64.21 % on day 20. *A. tubingensis* degraded 15.93 % and 12.67 %, while on day 20 the degradation was 32.91 % and 78.38 %, respectively (Figure 2). These results differ with those obtained by Benguenab and Chibani (2021), who evaluated the degradation of crude oil at 2 % with *Penicillium lilacinum* and *Aspergillus ustus* and reported a degradation of 44.55 % and 30.43 % at day 40, respectively, while in our work the degradation

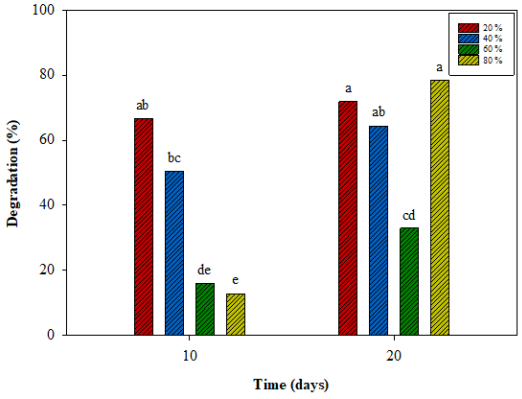

Figure 2. Evaluation of crude oil degradation by *Aspergillus tubingensis* at different concentrations at 10 and 20 days. Different literals (a, b, c, d, e) indicate significant differences (Tukey P=0.05).

was 78.38 % at 80 % oil concentration. The observed differences demonstrate that crude oil degradation depends on the metabolic capacity of the fungus, as well as on the complexity of the hydrocarbon, where the species of the genera *Aspergillus* have been reported for their high capacity to degrade crude oil, as well as aliphatic and aromatic hydrocarbons (Ye *et al*., 2011; Zhang *et al*., 2015; Al-Hawash *et al*., 2018b).

3.4 Culture pH

pH in crude oil combinations (0, 20, 40, 60 and 80 %) and exposure times showed significant interaction ($p \leq 0.05$). In all crude oil concentrations, the culture media presented a reduction in pH up to day 20 except for the media with 60 % and 80 % oil, where the strain presented values of 5.85 and 6.05 respectively at day 10, while in the medium with 40 % and in the control a similar behavior was observed with a decrease of 2.55 and 1.42 respectively. In the case of the culture medium with 20 % crude oil, a decrease in pH was observed during the evaluation time; however, an increase of 5.89 was detected on day 18. Similarly, the highest pH was recorded in the medium with 60 % on day 18 and was 6.84 (Figure 3). The results of our work differ from those reported by Al-Hawash *et al*. (2018a), who evaluated the degradation of 1% hexadecane by *Aspergillus* sp. RFC-1 and observed a reduction in pH from 7.1 to 5.1 at day 10, while in our work as the crude oil concentration increased from 60 % to 80 %, the pH changed from 6.05 to 5.85 and as it decreased from 40 % to 20 % the pH was maintained with minimal changes of 1.80 and 1.83 at day 10 of culture, respectively. These results suggest that, depending on the hydrocarbon complexity, the metabolic capacity of the fungus may vary. In addition, it has been reported that changes in the pH of the culture medium may be due to the production of organic acids or

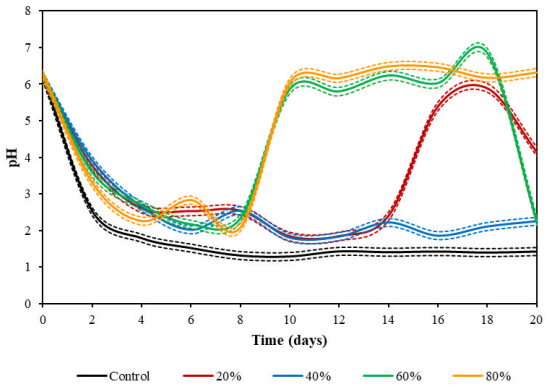

Figure 3. Estimated pH values in MM mineral medium with different crude oil concentrations (%) for 20 days; (---): 95 % confidence intervals.

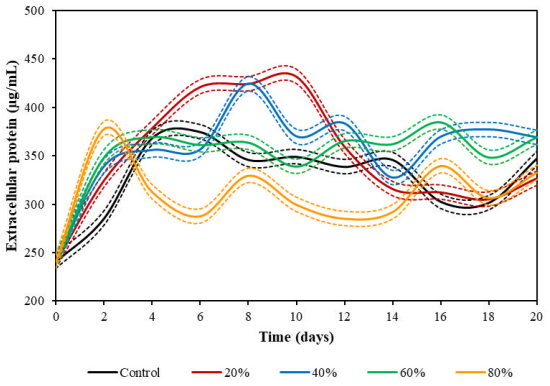

Figure 4. Estimated values of fungal extracellular protein of *Aspergillus tubingensis* in MM mineral medium with different crude oil concentrations (%) for 20 days; (---) 95 % confidence intervals.

hydrocarbon degradation intermediates and the release of hydronium ions (H_3O^+) that can affect the pH of the culture medium (Al-Hawash *et al*., 2018a).

3.5 Extracellular protein

The combinations of crude oil (0, 20, 40, 60 and 80 %) and exposure times showed significant interaction ($p \leq 0.05$) on extracellular protein. The values in all bioassays showed a trend in the first four days of culture, reaching values of 350 μg/mL of protein, except for the medium with 80 % crude oil. While extracellular protein showed changes from day 4 to 14 in all MM media with crude oil and the values ranged from 450 μg/mL (20 %) to 300 μg/mL (80 %). In the case of media with 20 %, 80 % oil and the control, the extracellular protein values were 335 μg/mL. Similarly, in the 40 % and 60 % oil media, the extracellular protein was 370 μg/mL. Despite these variations in the extracellular protein, the extracellular protein values were 335 μg/mL in the 40 % and 60 % oil media. *A. tubingensis* maximum values were obtained in the media supplemented with 20 % and 40 % crude oil, at 10 days (431.87 μg/mL) and at day 8 (424.37 μg/mL), for each concentration (Figure 4). These results are different with Khandelwal *et al*. (2021) who evaluated the degradation of crude oil (250 mg/L) with the fungi of *Trichoderma atroviridae*, *Aspergillus nidulans* and *Aspergillus sydowii* and reported values of 10.0 μg/mL, 9.8 μg/mL and 10.3 μg/mL of extracellular protein at 15 days. While Al-Hawash *et al*. (2018a) evaluated the degradation of hexadecane at 1 % by *Aspergillus* sp. RFC-1 and reported protein values of 510 μg/mL on day 8 of incubation, similar to those obtained in our work, where the highest amount of extracellular protein (431.87 μg/mL) was obtained in the presence of 20 % oil on day 10. These results suggest that crude oil is used as a carbon source by the fungus and that its degradation depends on extracellular enzymes, which may vary according to the complexity and concentration of the oil (Sharma *et al*., 2016).

3.6 Proteolytic activity

Proteolytic activity in MM culture media supplemented with oil (0, 20, 40, 60 and 80 %) showed significant interaction ($p \leq 0.05$) with the factors studied. Proteolytic activity tended to increase in the first four days in all bioassays with MM and crude oil, reaching values above 0.0016 U/mL. However, from day 6 to 18 a different behavior of the proteolytic activity was presented in all bioassays with oil, registering increases of 0.0013 U/mL on day 8 in 80 % oil as opposed to the control that had a decrease of 0.0013 U/mL; while in the 40 % concentration two peaks of 0. 0023 U/mL (day 12) and 0.0021 U/mL (day 16), in 60 % the peaks were less noticeable 0.0019 U/mL (day 8) and 0.0021 U/mL (day 16) and in the case of the culture medium with 20 % crude oil the proteolytic activity decreased reaching values of 0.0012 U/mL (day 14), while in the control a peak of 0.0023 U/mL was registered on day 18 (Figure 5). These results differ from those obtained by Balaji *et al*. (2014) who evaluated the enzymatic potential of fungi isolated from soil contaminated with crude oil and reported that in the species of *Aspergillus niger*, *A. fumigatus*, *A. terreus* and *A. versicolor* it was not possible to quantify the proteolytic activity, while in other species, such as *Mucor racemose* and *Colletotrichum falcatum* the proteolytic activity obtained was 3.2 U/mL and 0.1 U/mL respectively. In our case, the proteolytic activity presented by *Aspergillus tubingensis* was lower, registering values between 0.0013-0.0025 U/mL. The ability of fungi to degrade crude oil depends on their enzymatic potential and the complexity of the compound, mainly on extracellular enzymes, such as lipases, laccases, peroxidases and proteases that catalyze degradation reactions (Santos *et al*., 2008; Al-Zaban *et al*., 2021).

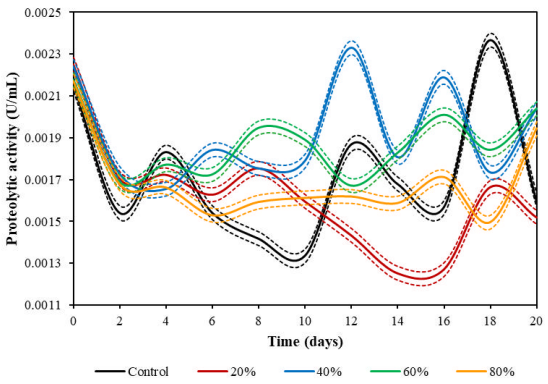

Figure 5. Estimated values of proteolytic activity of *Aspergillus tubingensis* in mineral medium with different oil concentrations (%) for 20 days; (---): 95 % confidence intervals.

However, high or low levels of proteolytic activity in oil-supplemented media may suggest degradation or turnover of enzymes related to oil use and/or adaptation of the fungus to its environment.

3.7 Biosurfactant production and emulsification capability

Biosurfactant production by *Aspergillus tubingensis* in mineral medium was dependent on the oil concentration (0, 20, 40, 60 and 80 %). This was detected by the formation of clear and visible halos in blood agar plates (β-hemolysis), with intensity and size of the halo according to the oil concentration (Figure 6). The results of our work are similar with those reported by Al-Hawash *et al*. (2018a) who evaluated the capacity of *Aspergillus* sp. RFC-1 to produce biosurfactants in the presence of 1 % hexadecane and observed the formation of visible halos in Petri dishes with blood agar, likewise in our work, in all combinations of oil we observed the formation of clear and visible halos. Carrillo *et al*. (1996) evaluated the production of biosurfactants by the *Pseudomonas* sp. and mentioned a relationship between hemolytic activity and the production of biosurfactants (surfactin and rhamnolipids) that lyse red blood cells, suggesting the use of blood agar as a preliminary and visual method to detect biosurfactant production. On the other hand, Cáceres-Zambrano *et al*. (2024) evaluated filamentous fungi (*Aspergillus flavus*, *Aspergillus niger*, *Penicillium chrysogenum*, *Penicillium glabrum* and *Penicillium rubens*) in environments of high heavy crude oil concentration and observed a reduction in the surface tension of the aqueous phase as a result of biosurfactant production.

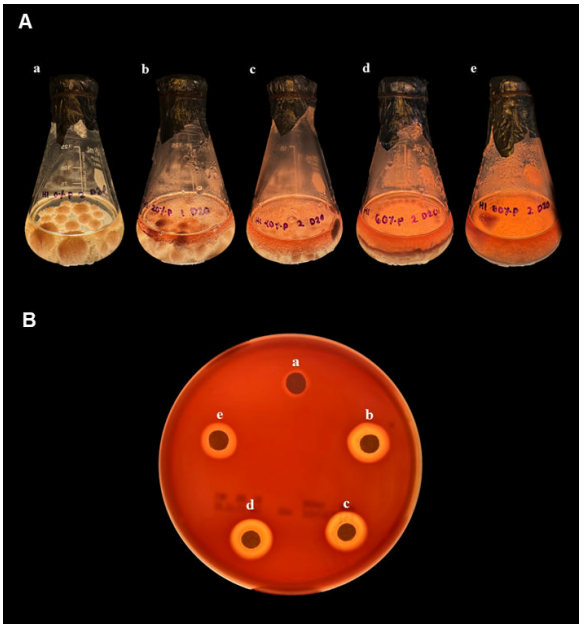

Figure 6. Growth of *Aspergillus tubingensis* on mineral medium (A) and biosurfactant production on blood agar (B) of different oil concentrations: a (0 % control), b (20 %), c (40 %), d (60 %) and e (80 %) for 20 days.

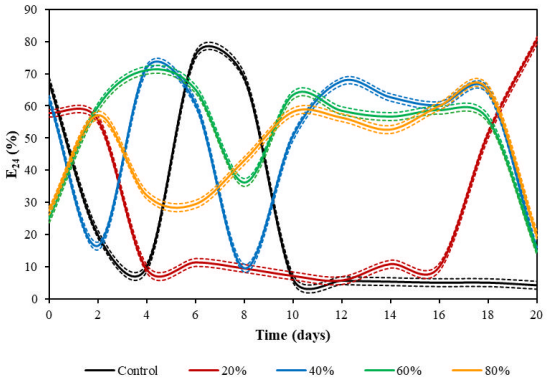

Figure 7. Estimated values of the emulsion index of *Aspergillus tubingensis* in mineral medium with different crude oil concentrations (%) for 20 days; (---): 95 % confidence intervals.

Another criterion used to evaluate biosurfactant production is the emulsification index (E_{24}). This was significant ($p \leq 0.05$) in all MM media supplemented with crude oil (0, 20, 40, 60 and 80 %). The highest values in the E_{24} were detected at day 20 (80.36 %), 6 (75.86 %) and 4 (71.7 %) at concentrations of 20 %, control and 40 % crude oil. While in the media with 60 %, 40 % and 80 % crude oil the rates were 71.70 %, 67.58 % and 64.60 % on days 4, 12, 18 and on days 2 (57.08 %), 10 (58.15 %) and 18 (65.16) respectively (Figure 7). These results differ from those reported by Al-Hawash *et al*. (2018b) who evaluated the degradation of crude oil (250 mg/L) by *Aspergillus* sp. RFC-1 and reported that the highest

emulsification capacity was 35.8 % on day 8 of culture, while in our work it was 80.36 % in the medium with 20 % crude oil on day 20. The reports suggest that the production of biosurfactants is related to the emulsification capacity of the microorganisms (fungus or bacteria) for the degradation of petroleum hydrocarbons (Atakpa *et al*., 2022).

Conclusion

According to the results, it can be concluded that *Aspergillus tubingensis* can maintain cell growth and biodegrade crude oil in the range studied. Furthermore, it was able to biodegrade 78.38 % of crude oil at the 80 % concentration and maintain a biomass production of 55.12 g/L, and a lower growth constant (0.35 d^{-1}), as well as a low proteolytic activity in all media. In addition, the production of biosurfactants was necessary to emulsify hydrocarbons and improve their biodegradation. Therefore, *Aspergillus tubingensis* can be used in bioremediation works to recover oil contaminated sites.

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