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ORIGINAL PAPER

Biodegradation capabilities of filamentous fungi in high-concentration heavy crude oil environments

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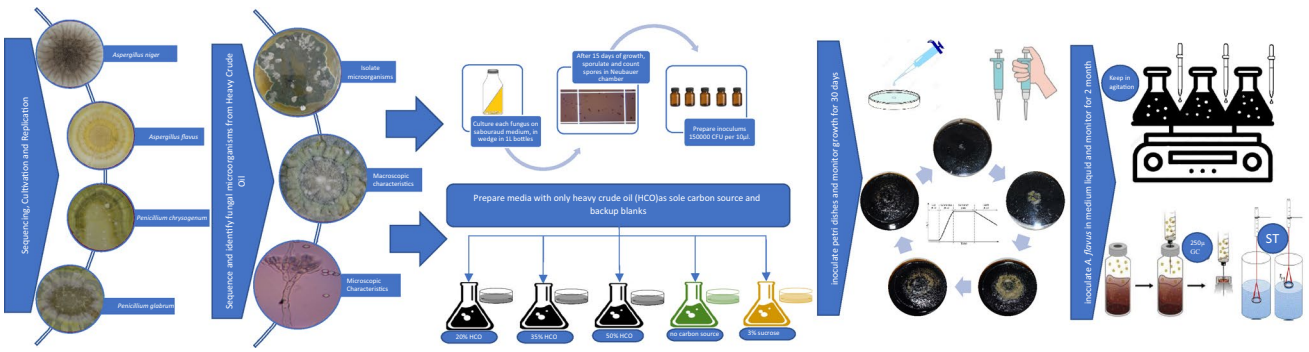
Abstract

In this comprehensive study, we delved into the capabilities of five fungal strains: *Aspergillus flavus*, *Aspergillus niger*, *Penicillium chrysogenum*, *Penicillium glabrum*, and *Penicillium rubens* (the latter isolated from heavy crude oil [HCO]) in metabolizing HCO as a carbon source. Employing a meticulously designed experimental approach, conducted at room temperature (25 °C), we systematically explored various culture media and incubation periods. The results unveiled the exceptional resilience of all these fungi to HCO, with *A. flavus* standing out as the top performer. Notably, *A. flavus* exhibited robust growth, achieving a remarkable 59.1% expansion across the medium's surface, accompanied by distinctive macroscopic traits, including a cottony appearance and vibrant coloration. In an effort to further scrutinize its biotransformation prowess, we conducted experiments in a liquid medium, quantifying CO_2 production through gas chromatography, which reached its zenith at day 30, signifying substantial bioconversion with a 38% increase in CO_2 production. Additionally, we monitored changes in surface tension using the Du Noüy ring method, revealing a reduction in aqueous phase tension from 72.3 to 47 mN/m. This compelling evidence confirms that *A. flavus* adeptly metabolizes HCO to fuel its growth, while concurrently generating valuable biosurfactants. These findings underscore the immense biotechnological potential of *A. flavus* in addressing challenges related to HCO, thereby offering promising prospects for bioremediation and crude oil bioupgrading endeavors.

Graphical abstract



Keywords Heavy crude oil metabolization · Biodegradation · Bioconversion · Biotensioactives · Filamentous fungi · Biotransformation capacity

Introduction

Petroleum, a natural hydrocarbon product formed through microbial biotransformation at elevated temperatures and pressures, alongside natural gas and coal, constitutes a

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significant portion, roughly 80–90%, of the world's total energy production (Graus et al. 2011; Strubinger et al. 2015). Despite ongoing efforts to develop alternative energy sources such as biofuels, solar, and wind energy, it is projected that fossil fuels will continue to meet at least 80% of the global energy demand over the next 20–30 years (IEA 2019; Mirchi et al. 2012; Shibulal et al. 2014).

However, global conventional oil production is on a downward trajectory due to the aging of existing oil fields and the challenges associated with discovering new ones to replace the depleted reserves. This predicament underscores the increasing significance of unconventional oil reserves, including heavy crude, extra-heavy crude, bitumen, tar sands, and shale oil, which constitute an estimated 50–70% of the world's recoverable oil resources (Head et al. 2003; IEA 2019; Demirbas et al. 2016; Central Intelligence Agency 2018; Singh and Choudhary 2021).

Unconventional or heavy crude oils (HCOs) are characterized by a molecular composition featuring a higher carbon content, elevated levels of heteroatoms (such as sulfur, nitrogen, and oxygen), and substantial quantities of heavy metals (including nickel, iron, copper, and vanadium). These unique physicochemical properties give rise to significant operational challenges throughout the heavy oil production chain, from extraction to transportation and, ultimately, refining. Consequently, the handling of HCOs involves lower recovery rates, increased waste generation, and notable environmental impacts (Castro and Vázquez 2009; Madden and Morawski 2011; Shibulal et al. 2014; He et al. 2015; Speight 2017).

In this context, the use of strategies based on hydrocarbon-degrading microorganisms as a biological treatment to reduce the viscosity and density of unconventional crudes emerges as a cost-effective and environmentally friendly alternative (Leon and Kumar 2005; Rana et al. 2007; Harner et al. 2011). These degrading microorganisms employ a range of mechanisms for hydrocarbon adsorption, and the remarkable versatility of microbial metabolism can be harnessed to address various challenges posed by unconventional crude oils. This includes the removal of heteroatoms from oil or modifying their chemical composition through diverse catabolic pathways capable of breaking down the complex mixture of compounds. As a result, these microorganisms excrete valuable substances such as biosurfactants, enzymes, and bioacids (Olivera et al. 2009; Ward 2010; Gargouri, et al. 2017).

The majority of research in this field has primarily focused on the degradation of specific petroleum fractions, such as asphaltenes, polycyclic aromatic hydrocarbons (PAHs), or the selective extraction of heteroatoms. These investigations are rooted in bioconversion principles encompassing various strategies, including microbial enhanced oil recovery (MEOR), biodesulfurization (BDS), biodenitrogenation (BDN), viscosity reduction/bioupgrading and/or extra-HCO bioconversion (BKN), and biodemetallization (BDM) (Borole and Ramirez-Corredores 2007; Speight 2014, 2017; Ayala et al. 2012; El-Gendy and Speight 2015; Speight and El-Gendy 2017).

In recent years, numerous bacterial strains, including those from *Bacillus* and *Petrotoga* species, have been isolated from oil reservoirs or oil-contaminated sites, exhibiting the remarkable ability to degrade the substantial alkyl chains present in various paraffinic blends and HCOs (Wang et al. 2006; Das and Mukherjee 2007; She et al. 2011; Gudiña et al. 2012, 2013a, b; Sugai et al. 2014). Microbial strains such as *Pseudomonas* spp., *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus cereus*, and *Bacillus firmus* have demonstrated their proficiency by effectivelydegrading 35% to 48% of asphaltenes after a 2-month incubation period at varying temperatures (ranging from 28 to 40 °C) using either pure or mixed cultures (Tavassoli et al. 2012; Gudiña and Teixeira 2017). Additionally, research has reported the valorization of Heavy Vacuum Gas Oil (HVGO) through the utilization of *Pseudomonas aeruginosa*, resulting in improved outcomes, albeit without significant alterations observed in the distillation profile of the biotreated crude oil (Ismail et al. 2017a, b). Furthermore, investigations into the crude oil biodegradation capacity of the *Stenotrophomonas maltophilia* (SS13) strain have revealed promising results. Following incubation with SS13, the crude oil exhibited substantial wax removal and viscosity reduction rates, reaching 74.36% and 47.83%, respectively (Wang et al. 2020). These findings underscore the potential of these bacterial strains in enhancing the bioprocessing of crude oil and provide valuable insights into their applications within the energy industry.

While a substantial body of research has predominantly focused on hydrocarbon biodegradation, particularly with bacteria, it is worth noting that the anaerobic hydrocarbon transformation pathway exhibits a comparatively slower rate when contrasted with the oxygen degradation pathway. Anaerobic bacterial degradation of crude oil primarily occurs under various conditions, including methanogenic, sulfate-reducing, and iron-reducing environments (Foght 2004; Fida et al. 2016; Chen et al. 2020). Fungi have advantages over bacteria, such as their adaptability to extreme conditions of temperature, pH, and salinity, which allows them to survive and maintain their activity in highly contaminated environments, including high-salinity seawater or hypersaline water (Varjani and Patel 2017; El-Shall et al. 2023), in addition to the formation of filamentous structures called mycelia, which allows them to spread and colonize larger areas in search of nutrients, which can be beneficial for the biodegradation of HCO (Amran et al. 2022), as well as the production of oxidative enzymes and secondary metabolites such as lipids that improve substrate access (El-Shall et al. 2023).

Interestingly, reports indicate that fungi, including *Amorphoteca*, *Talaromycetes*, *Fusarium*, *Penicillium*, *Trichoderma*, *Aspergillus*, *Neosartorya*, *Pseudallescheria*, *Cladosporium*, *Pestalotiopsis*, *Phoma*, and *Paecillomyces*, synthesize extracellular oxidative enzymes, particularly those associated with lignin-degrading enzyme systems, under aerobic conditions. Additionally, they produce monooxygenase enzymes that effectively degrade a wide range of aliphatic compounds through terminal or subterminal oxidation, while aromatic compounds are broken down via dioxygenase activity (Naranjo et al. 2007; Al-Hawash et al. 2018; Singh and Choudhary 2021). Notably, a strain of *Neosartorya fischeri* has demonstrated the ability to grow using asphaltenes as its sole carbon and energy source, resulting in the degradation of up to 13% of the asphaltenes. Laccase activity was detected in the medium, indicating its potential involvement in the degradation process (Uribe-Alvarez et al. 2011; Hernández-López et al. 2016). Moreover, a co-culture of the fungi *Pestalotiopsis* spp. NG007 and *Polyporus* spp. S133 exhibited remarkable efficiency in the degradation of the asphaltic fraction of crude oil. After a 30-day incubation period at 25 °C, 81% of the resins and 79% of the asphaltenes were degraded. This synergy was attributed to the increased production of degradative enzymes, including catechol dioxygenases, laccase, manganese peroxidase (MNPp), and lignin peroxidase (LIPp), compared to pure cultures. Furthermore, biodegradation tests utilizing crude enzymes extracted from the fungal cultures revealed that the enzyme mixture produced by both fungi was more effective in degrading the asphalt fraction compared to the crude enzymes produced by each fungus individually (Yanto and Tachibana 2014).

Despite numerous reports showcasing the ability of various microorganisms to metabolize crude oil or its fractions, there remains a critical knowledge gap in this field. This gap primarily stems from the fact that the substrate percentages employed in previous research rarely exceed 4% v/v. While such concentrations may suffice for applications like bioremediation, they are often inadequate and unscalable for bioupgrading or resource utilization purposes.

In light of these limitations, the objectives of this study are as follows:

(i) To isolate and identify fungal microorganisms sourced from a 17°API HCO, specifically from the Napo region in Ecuador.
(ii) To quantitatively assess the growth of pre-selected and isolated fungal strains when cultivated in culture media containing 20%, 35%, and 50% v/v of HCO as the sole carbon source.
(iii) To demonstrate the assimilation capacity of the fungal strain that exhibits the most robust growth.

With these objectives in mind, this research endeavors to bridge the knowledge gap and provide valuable insights into the potential of fungal microorganisms in utilizing substantial concentrations of HCO as a carbon source, thereby contributing to advancements in bioupgrading and resource utilization technologies.

Materials and methods

The HCO used in this study was generously supplied by "Empresa Nacional del Petróleo de Chile" (ENAP). This crude oil originated from Ecuador and possesses specific characteristics, including an API gravity of 17.1°, a viscosity of 348.48 cST at 50 °C, and a sulfur content of 2.411%. The non-isolated microorganisms employed in this research were sourced from the Chilean Culture Collection of Type Strains (CCCT) at the BIOREN-UFRO Scientific and Technological Bioresource Nucleus, affiliated with the "Universidad de La Frontera."

Isolation of microorganisms

A HCO sample was introduced into Petri dishes utilizing a metal clamp and Sabouraud solid medium (Emmons): consisting of peptone at a concentration of 10 g/L, glucose or dextrose at 20 g/L, and agar at 15 g/L, as per (Hare 2013).

Identification of microorganisms

DNA was extracted from fresh mycelia obtained from plate cultures of the collected fungi and the isolated strain. Subsequently, the ITS region was amplified via PCR using DNA from pure cultures, and Sanger sequencing was carried out utilizing primers ITS1 and ITS4. These primers produce two sequences, approximately 500 bp each, on both the forward and reverse strands. The resulting sequences were later assembled, and a blast analysis was conducted, as outlined by Jasalavich et al. (2000). The sequencing process was executed using a 3500 Genetic Analyzer instrument.

Preparation of inoculums

The fungi were initially cultured on Sabouraud's agar in Petri dishes. Subsequently, to expand the fungal growth, an increased quantity of this medium was prepared to accommodate fungal replication, but in a slanting manner within 1-L bottles. This approach provided a larger surface area conducive to microbial development. The fungal cultures were maintained at 25 °C for an initial incubation period of 15 days, following the protocol outlined by Araujo et al. (2016). Following incubation, spores were harvested using 10 mL of sterile distilled water and sterile metal tweezers

to gently remove the fungus from the surface. The collected spores were then deposited into an amber bottle, employing sterile gauze as a filtration medium. Subsequently, spore quantification was promptly carried out using a Neubauer chamber Barbedo (2013).

Solid culture medium

To assess the growth capacity of fungi when metabolizing HCO, an experimental design employing a 3 × 3 × 3 matrix was conducted. This design encompassed variations in the culture medium, incubation time, and fungal strain type, with the response variable being the radial biomass growth observed in Petri dishes.

For this purpose, Czapeck base culture media were meticulously prepared with yeast extract (CzY), which consisted of the following components: Dipotassium phosphate at 1 g/L, a concentrated solution of salts at 10 mL/L, yeast extract at 5 g/L, sucrose at 30 g/L, and agar at 15 g/L. These media were sterilized using an autoclave at 120 °C for a duration of 15 min. The concentrated solution of salts, an essential component, comprised of distilled water at 100 mL, sodium nitrate at 30 g, potassium chloride at 5 g, magnesium sulfate heptahydrate at 5 g, and ferrous sulfate heptahydrate at 5 g. This solution was stored at room temperature in a sealed flask, following the procedure detailed by Carrillo (2003) and Carrillo et al. (2007). To replace the carbon source (sucrose) with HCO, concentrations of 20% v/v, 35% v/v, and 50% v/v were utilized. Emulsification of these culture media was achieved using Triton X-100, as (Martínez-Martín et al. 2016).

In the experimental setup, the original culture medium (CzY) containing sucrose served as the positive control, while the CzY0 medium, devoid of sucrose and, thus, lacking a carbon source, was prepared as the negative control. Each of these media was prepared in triplicate for all five fungal strains, resulting in 75 Petri dishes for experimentation.

Seeding of microorganisms

In the Petri dishes containing solid media, 10 µL of fungal inoculum was carefully deposited at the center of each dish. Specifically, for each fungus, five plates with different media were inoculated in triplicate. This process was carried out within a UV hood, and burners were employed to prevent contamination. Subsequently, these plates were incubated for a period of 30 days at room temperature, which ranged between 25 and 30 °C, in accordance with the methodology established by Hildebrand (1938).

Biotransformation test

Three Czapeck liquid nutrient media, devoid of agar, were meticulously prepared, each incorporating yeast extract. These media were formulated by substituting the carbon source (sucrose) with HCO at concentrations of 20% v/v, 35% v/v, and 50% v/v. Notably, no emulsification was performed. Each of these nutrient solutions was dispensed into 300 mL bottles at a volume of 200 mL.

In addition to the experimental media, a positive control, consisting of Czapeck liquid nutrient medium with the original yeast extract, was prepared and inoculated with a 10 mL fungal inoculum. Furthermore, a negative control was established, comprising a culture medium containing 35% uninoculated HCO. These media were prepared in triplicate, resulting in a total of 15 bottles.

All bottles were stored at ambient temperature, maintained between 25 and 30 °C, and subjected to orbital shaking at 200 rpm for 10 weeks. This experimental protocol adhered to the methods outlined by Yanto and Tachibana (2013) and Ismail et al. (2017).

Production de CO_2

Headspace analysis was conducted daily for the first week, followed by measurements every other day for the subsequent 2 weeks. Subsequently, weekly measurements were continued until a total of 10 weeks had been covered. For each analysis, 250 µL of the sample was extracted and subjected to analysis using an Agilent Technologies 7820A gas chromatograph. This instrument was equipped with an Agilent 250361-01 Carboxen 1010 Plot column and a Thermal Conductivity Detector (TCD). The oven temperature was maintained at 200 °C, while the detector temperature was set at 230 °C. The operational temperature range spanned from − 60 °C to 250 °C, and the column dimensions were 30 m × 530 µm × 30 µm.

To quantify the results, a standard calibration curve was previously established to correlate CO_2 concentrations with peak area. The data obtained from the analysis are expressed as a percentage of the total volume generated, in accordance with the methodology outlined by Uribe-Alvarez et al. (2011).

Changes in surface tension

Sample measurements were conducted at the same frequency as the headspace analysis using a Kruss Tensiometer, Model Easy Dine K20, manufactured by Kruss in Germany. This equipment was outfitted with a platinum ring and operated at room temperature, which was maintained within the range

of 25–30 °C. The measurements were carried out utilizing the Du Noüy ring method, following the protocol established by Walter et al. (2010).

To ensure accuracy, the instrument was meticulously calibrated by adjusting the measurements to achieve a water surface tension reading of 72 mN/m, as outlined in the procedure described by Ismail et al. (2014). Subsequently, the culture media were subjected to centrifugation at 5000 rpm for 10 min.

Results and discussion

Microorganisms identification

A total of five active fungi, consisting of four purchased fungi and one fungus isolated from crude oil, were subjected to sequencing for identification purposes. Photographic documentation was captured of the prominent growth, highlighting the most pertinent observations (refer to Table 1).

Furthermore, a microscopic image was acquired for the isolated fungus, and based on its distinctive characteristics, it was identified as belonging to the *Penicillium* genus (see Fig. 1). The spore count in the inocula was determined to be 150,000 colony-forming units (CFU) per 10 µL.

Plate growth analysis

Images of the growth plates were captured daily over a 30-day period, and the irregular areas were quantified using the ImageJ software. Growth patterns were compared across different culture media, as depicted in Fig. 2a–e. The standard deviation among the triplicate measurements was less than 1, indicating consistency close to the mean. Consequently, the calculated growth areas were averaged among the triplicates, facilitating a comparison of growth trends, as summarized in Table 2.

Fungal growth became evident on the 3rd day across all grow media. Notably, in media with a white base (+*y*-), the peak growth occurred between the 8th and 10th days for all fungi. In contrast, media containing HCO as the sole carbon source exhibited peak growth between the 14th and 16th days for all fungal strains. Beyond this point, growth ceased, and a stationary state was observed (refer to Table 2 and Fig. 2a–e). Among the fungal strains, *A. flavus* consistently exhibited the highest growth area across all media. Notably, in the presence of HCO, the greatest growth was recorded in the medium containing 35%v/v HCO as the carbon source, with a diameter of 5.32 cm out of the 9 cm total Petri dish diameter. This corresponded to an impressive coverage of 59.1% of the plate's growth area. Figures 3 and 4 illustrate the initial growth and growth at the peak day (15 days), offering a visual comparison of the fungal growth across media.

These results affirm the tolerance of these five fungal microorganisms to HCO, aligning with previous findings regarding the aerobic degradation abilities of fungal species such as *Aspergillus, Penicillium, and Cunninghamella*. These fungi are known to activate monooxygenase, which reduces molecular oxygen and promotes degradation. Moreover, the activity of lignin-degrading enzymes like laccase (LACp), LIPp, MNPp, versatile peroxidase (VEPp), and non-lignolytic enzymes like cytochrome P450 further facilitates the degradation of polyaromatic hydrocarbons, n-alkanes, and crude oil (Naranjo et al. 2007; Elshafie et al. 2007; Aydin et al. 2017; Singh and Choudhary 2021).

Among the fungal strains, *A. flavus* demonstrated the highest growth on the plates, followed by *Aspergillus niger*, *Penicillium chrysogenum*, *Penicillium rubens* (isolated strain), and *Penicillium glabrum*. It is worth noting that previous studies reported favorable results for hydrocarbon and crude oil degradation by *A. flavus*; however, these experiments involved low substrate concentrations ranging from 0.1% to 1% v/v crude oil in the culture medium. These studies revealed that *A. flavus* degraded 82.7% of total PAH compounds after 15 days of incubation (Al-Dossary et al. 2020).

While these percentages are acceptable for bioremediation studies involving small hydrocarbon quantities, research related to oil spills and heavy oil bioupgrading necessitates investigations with substantially higher substrate percentages. Such studies provide valuable insights for biotechnological applications and reduce water consumption. Hence, this research employed culture media with elevated HCO concentrations by volume (20%, 35%, and 50%).

To comprehensively assess *A. flavus*'s proficiency in metabolizing HCO as a carbon source, detailed observations of its macroscopic characteristics were conducted across different culture media with varying HCO percentages. Comparisons were made with the original culture medium (containing sucrose) and the medium devoid of a carbon source, enabling the qualitative identification of evolutionary differences.

Figure 5 offers a detailed view of the macroscopic characteristics of *A. flavus* growth. In the medium containing sucrose (A), the fungal growth is remarkable, exhibiting a cottony appearance with white hues toward the outer region and vibrant yellow circles toward the center. The coloration is vivid, and spores are distinctly visible. Notably, growth is observed radiating outward from the center, giving it a three-dimensional aspect.

In contrast, the negative control without a carbon source (only containing 0.5% yeast extract) (B) displays growth extending across the plate but with weaker and less clustered spores. This variant lacks the cotton-like appearance, the

Table 1 Identification of microorganisms (own source)

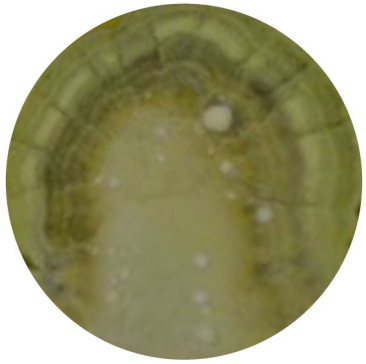
Image	Characteristics	Sequence	Name
	Cottony appearance, white at first darkening to black, has a fast growth rate	GGTCATTAGAGGAAGTAAAAGTCGTAACAAGGTTTC CGTAGGTGAACCTDNABasNrAssNmbNNrdNmNvN-rsNNnNNNrchasNNNcNnsNtNrNmNvNthNsadTTCGGC GGGCCCGCCGCTTGTCGGCCGCCGGGGGGGCGC CTCTGCCCCCCGGGCCCGTGCCCGCCGGARACC CCAACACGAACACTGTCTGAAAGCGTGCAGTCT GAGTTGATTGAATGCAATCAGTTAAAACTTTCA ACAATGGATCTCTTGGTTCCGGCATCGWKGAA RAA	*Aspergillus niger*
	Cottony appearance, white at the beginning, as time goes by it replaces the white color with a bright yellow and as it ages the yellow darkens to a green color, it has a fast growth rate	AATCAACTCAGACTTCACTAGATCGACAGAGTM-MAGYGGYCGTCTCCGGCGGGCGCGGGCCCGGGGC TGAGAGCCCCCGGCGGCCATGAATGGCGGGCCC GCCGAAGCAACTAAGGTACAGTAAACACGGGTG GGAGGTTGGGCTCGCTAGGAACCCTACACTCGG TAATGATCCTTCCGCAGGTTCACCTACGGAAAC CTTGTTACGACTTTTACTTCCTCCTAAATGACCA	*Aspergillus flavus*
	Cottony appearance, yellow with white at the beginning, with the passage of time replaces the yellow to kiwi green color, has a medium growth rate	TTARGRGGAAGTAAAAGTCGTAACAAGGTTTCCGTA GGTGAACCTGCGGDNABasNrAssNmbNNrdNmNvN-rsNNnNNNrchasNNNcNnsNtNrNmNvNthNsadGGCGGG CCCGCCTTAACTGGCCGCCGGGGGGCTTACGCC CCCGGGCCCGCGCCCGCCGAAGACACCCTCGAA CTCTGTCTGAAGATTGTAGTCTGAGTGAAAATA TAAATTATTTAAAACTTTCAACAACGGATCTCTTGG TTCCGGCATCGATGAARAACGCA	*Penicillium chrysogenum*

Table 1 (continued)

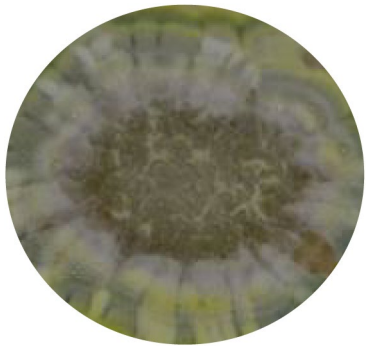
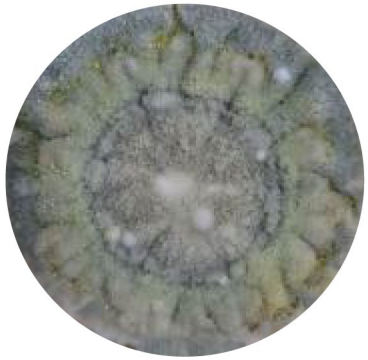
Image	Characteristics	Sequence	Name
	Cottony appearance, it starts with pink, white and yellow tones, with the passage of time it turns green, it has a medium growth rate	TTTTACTTATTTAGTTTATGCTCAGACTGCAATCTT CAGACAGAGTTCAATAGTGTCTCCGGTGCGCGC GGACCCGGGGGCAGAAGCCCCCCGGCGGCCGTG AGGCGGGCGCACCGAAGCAACAAGGTACAATAA ACACGGGTGGGAGGTTGGACCCAGAGGGCCCTC ACTCAGTAATGATCCTTCCGCAGGTTCACCTAC GGAAACCTTGTTACGACTTTTACTTCYCCTAAA TGACC	*Penicillium glabrum*
	Cottony appearance, starts with white, pink and green tones, with the passage of time the green is intensified to a turquoise green, is of average growth	TGCGGAAGGATCATTACCGAGTGAGGGCCCTCTGGG TCCAACCTCCCACCCGTGTTTATTTTACCTTGTTGC TTCGGCGGGCCCGCCTTAACTGGCCGCCGGGGG GCTTACGCCCCCGGGCCCGCGCCCGCCGAAGAC ACCCTCGAACTCTGTCTGAAGATTGTAGTCTGA GTGAAAATATAAATTATTTAAAACTTTCAACAA CGGATCTCTTGGTTCCGGCATCGATGAAGAACG CAGCGAAATGCGATACGTAATGTGAATTGCAAA TTCAGTGAATCATCGAGTCTTTGAACGCACATT GCGCCCCCTGGTATTCCGGGGGGCATGCCTGTC CGAGCGTCATTTCTGCCCTCAAGCACGGCTTGT GTGTTGGGCCCCGTCCTCCGATCCCGGGGGACG GGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGT CCTCGAGCGTATGGGGCTTTGTCACCCGCTCTG TAGGCCCGGCCGGCGCTTGCCGATCAACCCAAA TTTTTATCCAGGTTGACCTCGGATCAGGTAGGGATA CCCGCTGAACTTAAGCATAT	*Penicillium rubens* (Isolated)

Fig. 1 Image taken with a 2MP microscope camera, from a model 902N microscope at 40× objective, showing 1:conidiophores, 2:metula, 3:phialides, 4:ascospore of the fungus isolated from the heavy crude, indicating that it belongs to the genus *Penicillium*

yellow coloration, and the outward radial growth. Instead, growth is confined to the agar surface.

Examining the fungus's growth in media containing crude oil (C and D), while the radial expansion may not be as extensive, it still exhibits significant macroscopic characteristics. Notably, spores appear denser, imparting a three-dimensional cotton-like appearance.

Similar to the sucrose-containing medium, there are white regions toward the outer periphery and yellow hues at the center. This indicates that the fungus effectively metabolizes HCO, utilizing it as a substrate for growth while harnessing other nutrients from the culture medium.

Considering the structural complexity of HCO, characterized by its high content of heavy fractions such as asphaltenes and resins, as well as heteroatoms, it can be confidently stated that the fungus activates its enzymatic arsenal. This activation process aligns with previous research (Speight 2014, 2017; Hernández-López et al. 2015; Naranjo et al. 2007) and potentially involves secondary metabolites like biosurfactants or cometabolites.

These substances facilitate substrate availability and contribute to the degradation of HCO into simpler compounds. Notably, the depolymerization of asphaltenes and resins likely occurs through cleavage at active sites containing heteroatoms, liberating trapped small molecules (Gudiña and Teixeira 2017; Heimann et al. 2017; Speight 2019; Nikolova and Gutierrez 2020).

The tests conducted in the solid medium provided valuable qualitative insights into the growth of fungal microorganisms and served as an initial indicator of HCO metabolization. However, to precisely quantify the utilization of HCO as a carbon source by *A. flavus*, a series of tests were replicated, this time in a liquid medium. The liquid medium encompassed the same range of HCO percentages (20%, 35%, and 50%) v/v and extended over a longer duration (10 weeks). Continuous agitation at 200 rpm was maintained at room temperature (25–30 °C).

CO_2 production directly correlates with substrate (HCO) degradation, particularly in a hydrocarbon mixture rich in heavy fractions such as asphaltenes and resins. In a prior study, the focus was solely on the mineralization of asphaltenes from Mayan crude oil. This research employed a strain, *N. fischeri*, isolated from a natural asphalt lake. The study observed CO_2 evolution over 9 weeks, considering

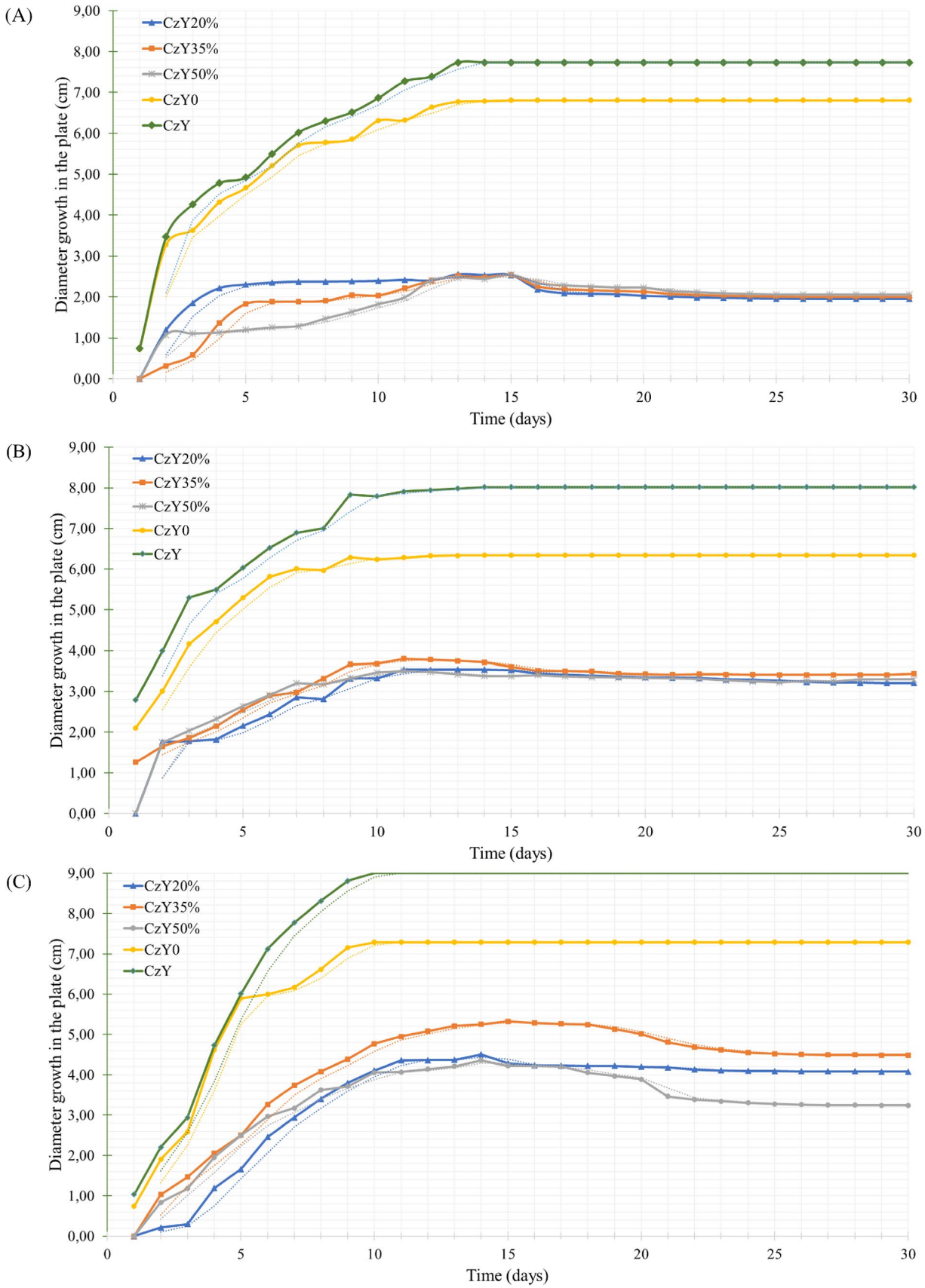


Fig. 2 **A** Diameter growth on the plate (cm) of *P. glabrum*. **B** Diameter growth on the plate (cm) of *P. chrysogenum*. **C** Diameter growth on the plate (cm) of *A. flavus*. **D** Diameter growth on the plate (cm) of *A. niger*. **E** Diameter growth on the plate (cm) of *P. rubens*

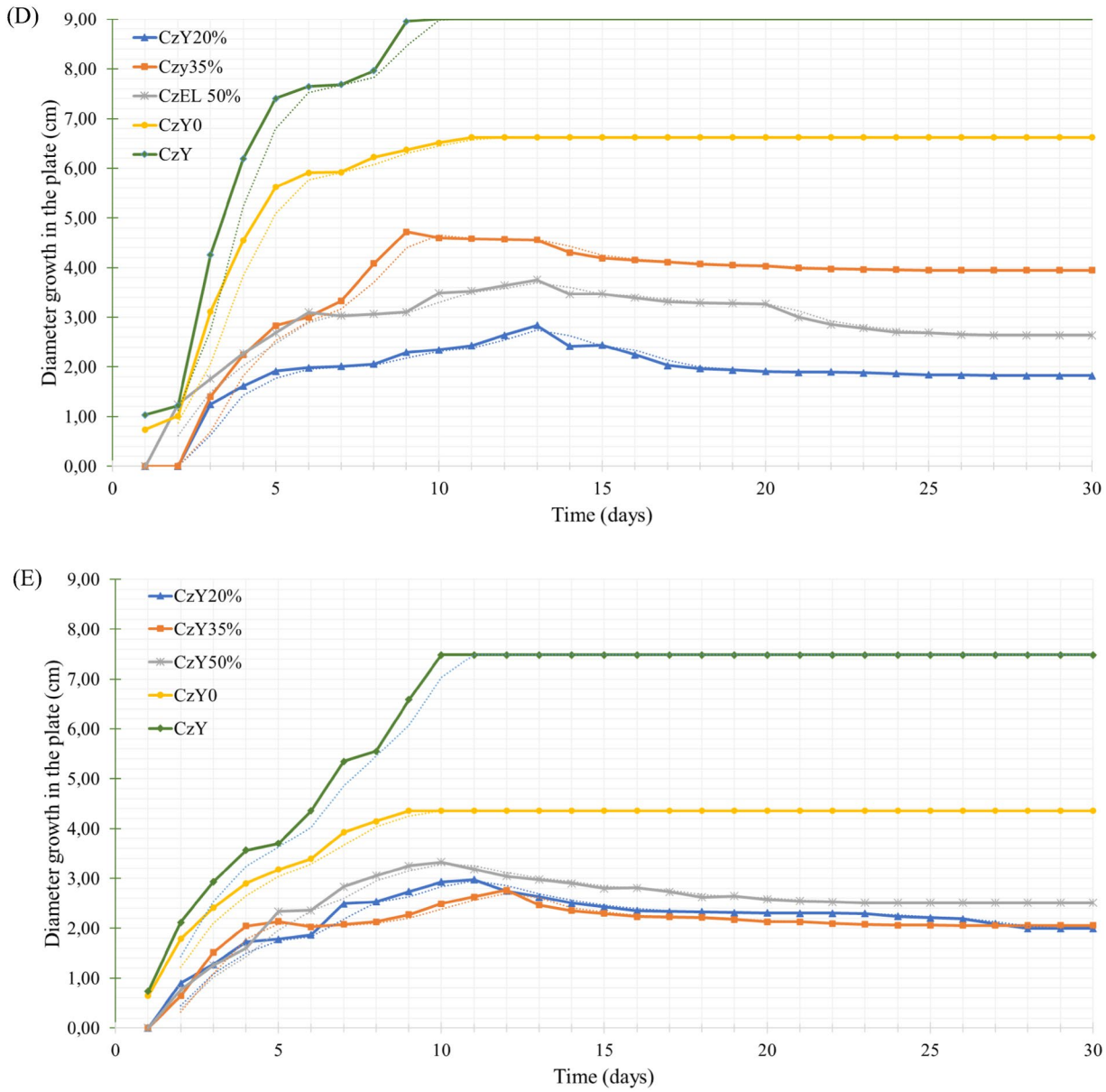


Fig. 2 (continued)

initial and final weights, as well as the weight of the inoculum. Additionally, an elemental analysis of carbon revealed that carbon represented 83.5% of the total weight of asphaltenes in the crude oil. The balance indicated a mineralization of at least 15.5% of the asphaltenes (Durand et al. 2010; Uribe-Alvarez et al. 2011).

In our current research, CO_2 production was meticulously monitored through headspace analysis employing gas chromatography, as displayed in Table 3 and Fig. 6. The percentage by volume of CO_2 gas produced exhibited a consistent increase until day 30. The positive control (with sucrose) demonstrated a 58% production of CO_2, indicating proper inoculation of the media. In contrast, the control medium or negative blank (without inoculum) did not exhibit any significant CO_2 production. Concerning the media containing HCO as a carbon source, higher gas production was observed in the media with 20% and 35% substrate, registering production rates of 33% and 35%, respectively. This finding aligns with the outcomes of the solid media experiments. Notably, the medium containing 50% HCO displayed lower gas production, suggesting substrate supersaturation in the medium, rendering it impossible to achieve correct mineralization.

Furthermore, the production of biosurfactants plays a pivotal role in enhancing the availability of water-insoluble or immiscible hydrocarbons, facilitating improved

Table 2 Fungal growth diameter according to medium and days (own source)

		Time (days)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
		Culture mediums																														
Type of fungal strain	*P. glabrum*	CzY20%	0.00	1.19	1.86	2.22	2.30	2.36	2.38	2.38	2.38	2.39	2.42	2.41	2.55	**2.54**	2.54	2.19	2.09	2.08	2.07	2.03	2.01	1.99	1.98	1.97	1.96	1.95	1.95	1.95	1.95	1.95
		CzY35%	0.00	0.32	0.59	1.36	1.84	1.89	1.89	1.91	2.05	2.04	2.22	2.41	2.52	2.49	**2.54**	2.27	2.19	2.17	2.15	2.13	2.08	2.05	2.03	2.02	2.01	2.01	2.01	2.01	2.01	2.01
		CzY50%	0.00	1.08	1.10	1.13	1.19	1.25	1.29	1.47	1.64	1.83	1.99	2.41	2.48	2.45	**2.54**	2.34	2.28	2.26	2.23	2.23	2.14	2.11	2.09	2.07	2.06	2.06	2.06	2.06	2.06	2.06
		CzY0	0.73	3.28	3.64	4.32	4.67	5.21	5.71	5.79	5.86	6.31	6.33	6.64	6.78	6.79	**6.81**	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81
		CzY	0.74	3.48	4.27	4.78	4.92	5.50	6.02	6.30	6.51	6.86	7.27	7.39	**7.73**	7.73	7.73	7.73	7.73	7.73	7.73	7.73	7.73	7.73	7.73	7.73	7.73	7.73	7.73	7.73	7.73	7.73
	P. chrysogenum	CzY20%	0.00	1.76	1.78	1.82	2.15	2.44	2.86	2.81	3.32	3.32	3.54	3.53	**3.54**	3.54	3.52	3.43	3.41	3.39	3.36	3.35	3.34	3.33	3.30	3.28	3.26	3.24	3.22	3.22	3.21	3.21
		Czy35%	1.26	1.65	1.86	2.14	2.54	2.89	2.98	3.32	3.66	3.68	**3.80**	3.78	3.75	3.72	3.60	3.51	3.50	3.49	3.44	3.43	3.41	3.43	3.42	3.41	3.41	3.41	3.41	3.41	3.41	3.44
		CzY50%	0.00	1.75	2.04	2.33	2.63	2.91	3.20	3.17	3.32	3.47	**3.50**	3.48	3.42	3.38	3.37	3.40	3.37	3.35	3.34	3.33	3.32	3.31	3.27	3.25	3.23	3.26	3.24	3.29	3.29	3.29
		CzY0	2.10	3.01	4.16	4.71	5.29	5.82	6.01	5.97	6.29	6.24	6.28	6.33	6.33	**6.34**	6.34	6.34	6.34	6.34	6.34	6.34	6.34	6.34	6.34	6.34	6.34	6.34	6.34	6.34	6.34	6.34
		CzY	2.79	3.99	5.30	5.50	6.03	6.52	6.89	7.00	7.83	7.79	7.91	7.94	7.98	**8.02**	8.02	8.02	8.02	8.02	8.02	8.02	8.02	8.02	8.02	8.02	8.02	8.02	8.02	8.02	8.02	8.02
	A. flavus	CzY20%	0.00	0.21	0.30	1.19	1.66	2.46	2.94	3.40	3.80	4.11	4.36	4.37	4.37	**4.50**	4.28	4.23	4.23	4.22	4.22	4.20	4.18	4.13	4.11	4.10	4.09	4.09	4.09	4.08	4.08	4.08
		CzY35%	0.00	1.04	1.47	2.05	2.51	3.27	3.74	4.08	4.39	4.77	4.95	5.08	5.21	5.25	**5.32**	5.28	5.27	5.25	5.13	5.01	4.81	4.68	4.62	4.55	4.52	4.50	4.50	4.49	4.49	4.49
		CzY50%	0.00	0.84	1.18	1.96	2.50	2.97	3.18	3.62	3.71	4.06	4.07	4.14	4.21	**4.36**	4.22	4.21	4.21	4.05	3.97	3.89	3.46	3.39	3.35	3.31	3.28	3.26	3.25	3.25	3.24	3.24
		CzY0	0.74	1.90	2.59	4.62	5.90	6.00	6.17	6.61	7.16	**7.29**	7.29	7.29	7.29	7.29	7.29	7.29	7.29	7.29	7.29	7.29	7.29	7.29	7.29	7.29	7.29	7.29	7.29	7.29	7.29	7.29
		CzY	1.03	2.20	2.94	4.73	6.01	7.12	7.77	8.31	8.81	**9.00**	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	A. niger	CzY20%	0.00	0.00	1.25	1.62	1.92	1.99	2.01	2.06	2.30	2.34	2.43	2.64	**2.84**	2.42	2.44	2.24	2.03	1.97	1.94	1.91	1.90	1.90	1.88	1.86	1.84	1.83	1.83	1.83	1.83	1.83
		CzY35%	0.00	0.00	1.40	2.24	2.83	3.01	3.33	4.08	4.72	4.60	4.58	**4.57**	4.56	4.30	4.19	4.15	4.11	4.07	4.05	4.03	3.99	3.98	3.97	3.96	3.95	3.95	3.95	3.95	3.95	3.95
		CzY50%	0.00	1.25	1.76	2.27	2.69	3.10	3.03	3.07	3.10	3.49	3.53	3.64	**3.75**	3.47	3.47	3.39	3.31	3.29	3.28	3.27	3.00	2.86	2.78	2.70	2.69	2.65	2.64	2.64	2.64	2.64
		CzY0	0.74	1.01	3.12	4.54	5.62	5.91	5.92	6.22	6.37	6.51	**6.62**	6.62	6.62	6.62	6.62	6.62	6.62	6.62	6.62	6.62	6.62	6.62	6.62	6.62	6.62	6.62	6.62	6.62	6.62	6.62
		CzY	1.03	1.22	4.26	6.19	7.41	7.65	7.68	7.96	8.96	**9.00**	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	P. rubens	CzY20%	0.00	0.90	1.27	1.73	1.78	1.87	2.50	2.53	2.74	2.93	**2.98**	2.75	2.63	2.50	2.43	2.35	2.34	2.33	2.32	2.31	2.30	2.30	2.29	2.24	2.21	2.19	2.09	2.00	2.00	2.00
		Czy35%	0.00	0.64	1.52	2.05	2.14	2.03	2.08	2.13	2.27	2.49	2.63	**2.76**	2.47	2.35	2.30	2.24	2.23	2.22	2.18	2.13	2.13	2.10	2.08	2.07	2.06	2.06	2.06	2.06	2.06	2.06
		CzY50%	0.00	0.77	1.26	1.60	2.33	2.36	2.84	3.05	3.26	3.33	**3.18**	3.05	2.98	2.90	2.80	2.81	2.73	2.63	2.65	2.58	2.55	2.53	2.51	2.51	2.51	2.51	2.51	2.51	2.51	2.51
		CzY0	0.64	1.79	2.41	2.90	3.18	3.39	3.93	4.15	**4.36**	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36
		CzY	0.74	2.12	2.93	3.56	3.70	4.35	5.34	5.55	6.59	**7.48**	7.48	7.48	7.48	7.48	7.48	7.48	7.48	7.48	7.48	7.48	7.48	7.48	7.48	7.48	7.48	7.48	7.48	7.48	7.48	7.48

Culture mediums: *CzY20%* Czapeck medium with yeast extract and 20%v/v of heavy crude oil (HCO), *CzY35%* Czapeck medium with yeast extract and 30%v/v of HCO, *CzY50%* Czapeck medium with yeast extract and 50%v/v of HCO, *CzY0* Czapeck medium alone with yeast extract without carbon source (negative control −), *CzY* Czapeck medium with yeast extract and sucrose (positive control +)

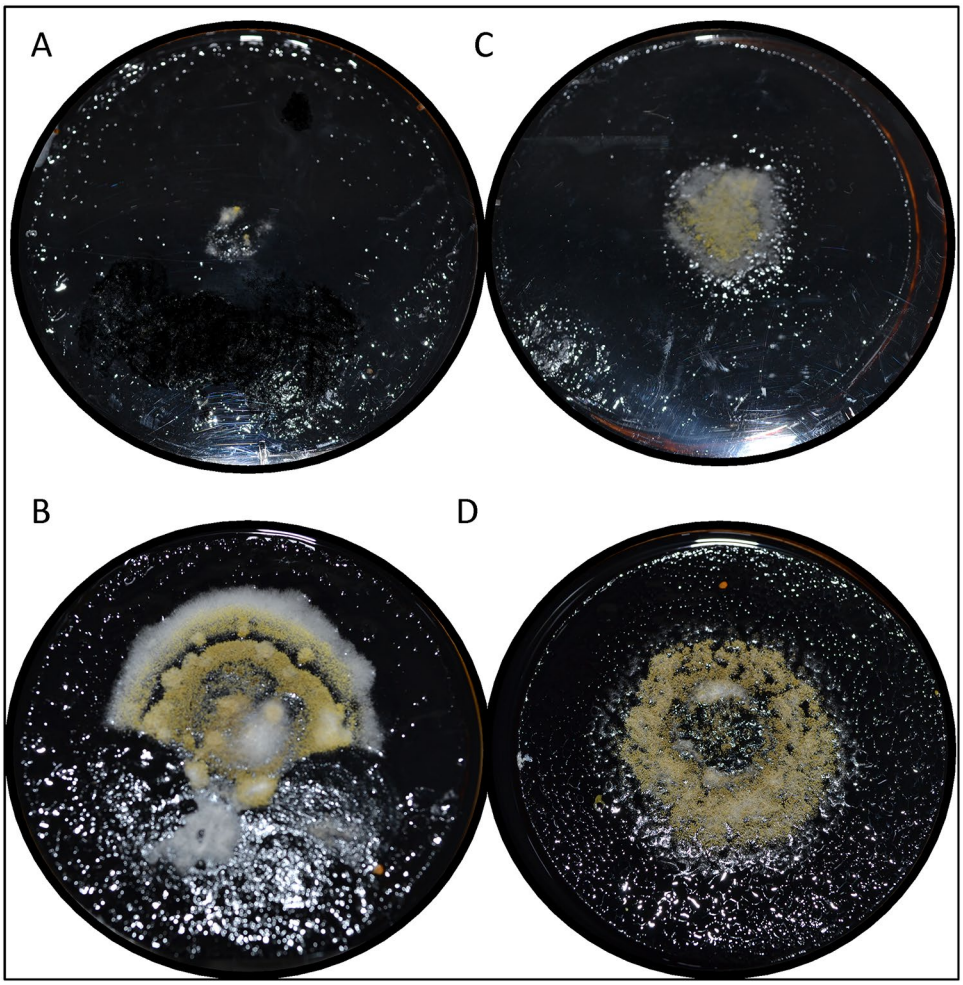


Fig. 3 Growth of *A. flavus* on 2 media: **A** CzY20 (Czapeck medium with yeast extract and 20% HCO as carbon source) at 3 days of growth; **B** CzY20 at 15 days of growth. **C** CzY35 (Czapeck medium with yeast extract and 35% HCO as carbon source) at 3 days of growth; **D** CzY35 at 15 days of growth

microorganism access to these substrates. Consequently, this promotes biodegradation efficiency. When faced with hydrophobic substrates like HCO, some microorganisms produce biosurfactants as secondary metabolites, a common physiological adaptation (Ward 2010; Mnif et al. 2011; Ismail et al. 2014, 2017a, b).

To provide direct evidence of biotensioactive production, we closely monitored the alteration in surface tension within the cultures over time, as depicted in Fig. 7. Several control measurements were incorporated into the study. First, the surface tension of Milli Q water, which typically registers at 72 mN/m, was employed as a reference, and used to calibrate the instrument during each measurement. Second, the surface tension of the heavy crude (HCO) devoid of culture medium was measured, yielding a value of 30 mN/m. Lastly, a negative blank consisting of a culture medium with 35% HCO as the sole carbon source, devoid of inoculum, resulted in a surface tension measurement of 30.80 mN/m, indicative of minimal emulsification due to the absence of microorganisms.

In contrast, when considering the culture media containing HCO as the carbon source with inoculum, we observed fluctuations in surface tension. In comparison to the surface tension of heavy crude and the negative blank, these media exhibited an increase in surface tension. Conversely, when compared to the surface tension of water, a decrease was observed, signifying biosurfactant production and emulsification of the media. Notably, the most substantial alteration occurred between days 9 and 11, representing the peak increase in media tension. Among these media, the most significant change was observed in the medium with 20% HCO, followed closely by the medium with 35% HCO.

At the culmination of the 10-week bioconversion process, all media underwent centrifugation at 5000 rpm for 10 min, separating the phases and allowing for the measurement of surface tension in the supernatants. Figure 8 illustrates that the surface tension of the aqueous phase experienced the most significant decrease in the media with 20% HCO, reaching a measurement of 47 mN/m.

This decline in surface tension serves as strong confirmation that the fungal microorganism *A. flavus* effectively utilized HCO as a carbon source for its own growth and the production of biotensioactives. Moreover, it demonstrates that the biosurfactants derived from HCO were subsequently

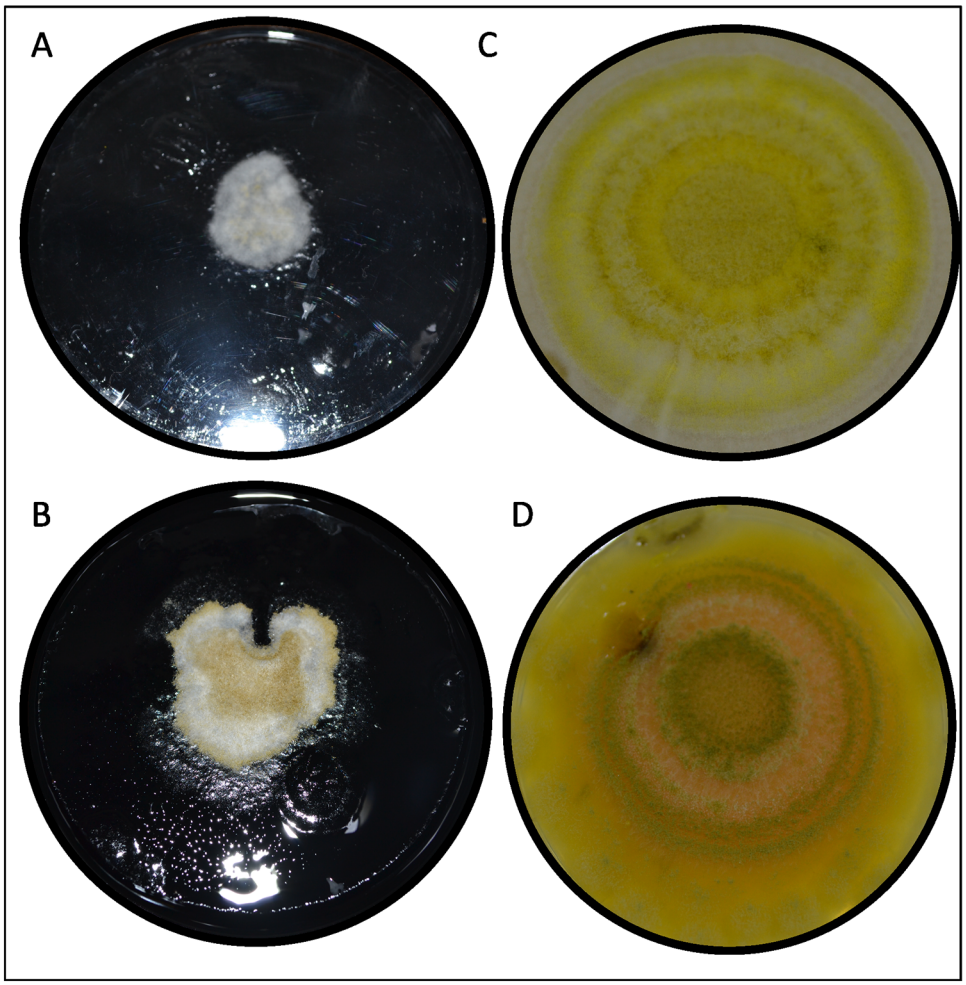


Fig. 4 Growth of *A. flavus* on 3 media: **A** CzY50 (Czapeck medium with yeast extract and 50% HCO as carbon source) at 3 days of growth; **B** CzY50 at 15 days of growth. **C** CzY (Czapeck medium with yeast extract and 3% sucrose as carbon source—positive blank) at 10 days of growth; **D** CzY0 (Czapeck medium with yeast extract and no carbon source—negative blank) at 10 days of growth

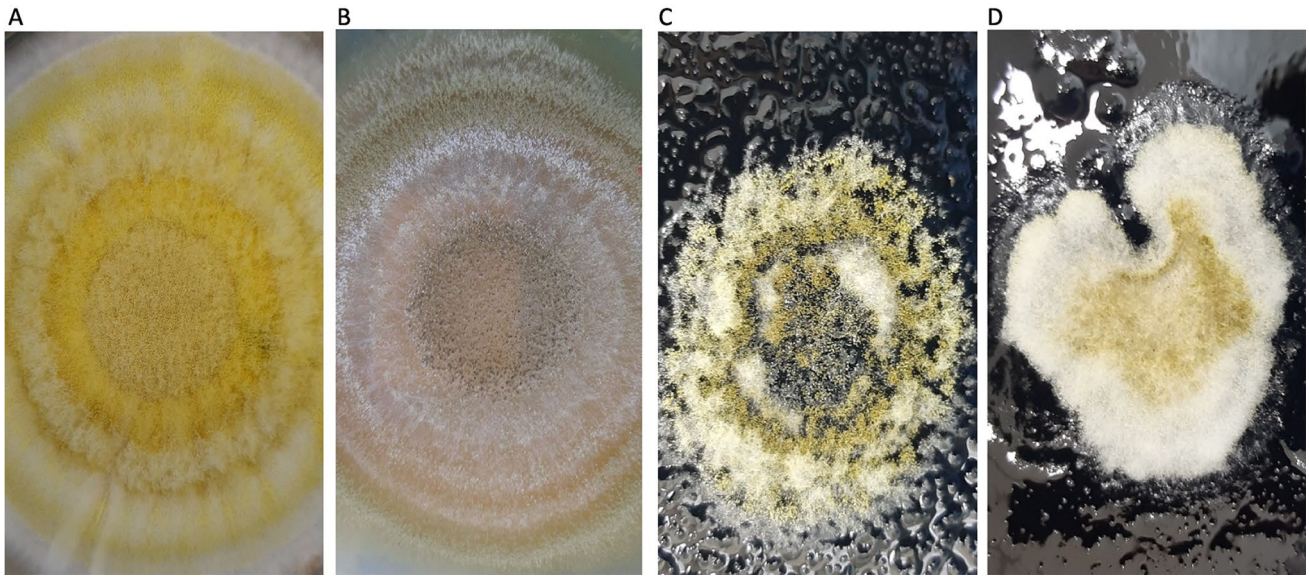


Fig. 5 Macroscopic characteristics of *A. flavus* according to different media. **A** CzY (Czapeck medium with yeast extract and 3% sucrose as carbon source-positive blank); **B** CzY0(Czapeck medium with yeast extract without carbon source—negative white); **C** CzY35 (Czapeck medium with yeast extract and 35% HCO as carbon source); **D** CzY50 (Czapeck medium with yeast extract and 50% HCO as carbon source)

Table 3 Production de CO_2

	CO_2				
time (days)	FCZ20 (%)	FCZ35 (%)	FCZ50 (%)	FCZ0W (%)	WCZ35 (%)
0	0	0	0	0	0
1	2	4	2	10	2
2	3	6	3	14	2
3	8	11	5	20	4
4	8	12	5	20	4
5	9	13	6	21	4
6	9	13	6	23	5
7	11	14	7	24	5
9	13	15	9	25	8
11	14	20	15	30	9
13	19	26	18	35	9
15	20	28	19	42	9
17	24	28	19	47	9
19	25	28	19	48	9
21	29	29	19	48	9
23	30	32	19	48	10
30	35	38	20	51	10
37	33	35	17	58	9
44	28	28	16	54	7
51	29	20	15	52	7
58	26	18	15	50	6
65	22	14	15	48	5

excreted into the extracellular medium (Soberón-Chávez and Maier 2011; Ismail et al. 2017a, b).

To ensure the reliability of the data, a statistical analysis of variance (ANOVA) was conducted, yielding a *p*-value of less than 0.05, further reinforcing the robustness of the findings.

Study limitations

While this study presents novel insights into the capacity of fungal microorganisms, particularly *A. flavus*, to metabolize HCO as a carbon source, some limitations and potential biases must be acknowledged. First, the study primarily relied on laboratory-based experiments conducted under controlled conditions, which may not fully replicate the complexities of real-world environments where HCO degradation occurs. The study's duration of 10 weeks may not capture long-term dynamics and variations in HCO biodegradation. Nevertheless, this research highlights the potential of *A. flavus* and other fungi in addressing challenges related to HCO bioupgrading. It offers valuable insights into sustainable and environmentally friendly solutions for unconventional oil resources, bridging the gap between laboratory findings and practical applications in the field.

Economic aspects motivating the research and use of bioupgrading systems

The use of HCO biodegradation systems prior to the refining process can have a significant economic impact, although the specific costs depend on several factors (operating costs, process efficiency, external factors, among others), but an effective biodegradation system can improve the quality of HCO, making it easier and more economical to refine. Reducing viscosity or the presence of impurities can reduce the costs associated with refining, such as energy consumption, the need for additives or more complex separation processes.

Considering the demand and use of this energy resource, any improvement, no matter how small, translates into a higher percentage of recovery, a greater amount of valuable product and less production of pollutants into the environment.

This research was challenging due to the high percentages of HCO (20%, 35%, 50%) v/v to which the fungal microorganisms were subjected, obtaining encouraging results that provide important information that can be complemented with rigorous analysis for future scale-up.

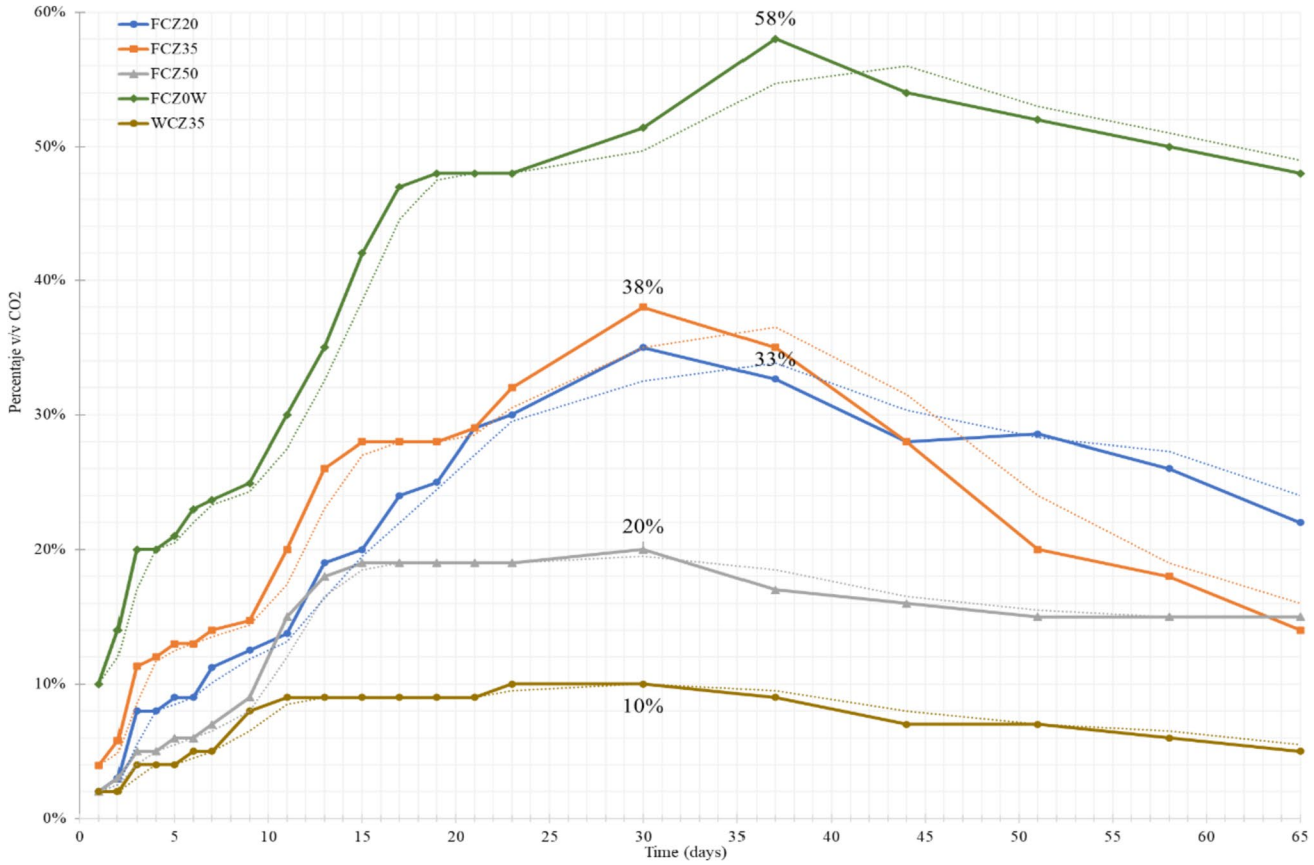


Fig. 6 CO_2 production according to culture media where: *FCZ20* Czapeck medium with yeast extract inoculated with fungus and 20% HCO, *FCZ35* Czapeck medium with yeast extract inoculated with fungus and 35% HCO, *FCZ50* Czapeck medium with yeast extract inoculated with fungus and 50% HCO, *FCZ0W* Czapeck medium with yeast extract, sucrose and inoculated with fungus (control), *WCZ35* Czapeck medium with yeast extract and 35% HCO without inoculum (control)

Conclusion

The five fungal microorganisms examined in this study exhibited remarkable tolerance to high-volume percentages (20%, 35%, and 50%) of HCO in the culture media. To assess their metabolic potential in degrading HCO, we closely monitored their growth and observed macroscopic characteristics over time. Among these fungi, *A. flavus* emerged as the most promising performer, achieving substantial growth at 25 °C, with 50% growth in plates containing 20% HCO, 59.1% in plates with 35% HCO, and 48.44% in plates with 50% HCO. It was followed by *A. niger*, *P. chrysogenum*, *P. rubens* (isolated from HCO samples), and *P. glabrum*. The analysis of CO_2 production further confirmed the superior performance of *A. flavus*, particularly in media containing 20% and 35% v/v HCO, with a 35% and 38% increase, respectively. Notably, the most significant reduction in the surface tension of the aqueous phase was observed in the culture medium with 20% v/v HCO, decreasing from 72.3 to 47 mN/m. These findings underscore *A. flavus*'s capability to metabolize HCO for growth and biosurfactant production, positioning it as a promising candidate for bioremediation of oil spills and bioupgrading of crude oil. Further research should focus on determining which fractions of crude oil are most susceptible to attack by this microorganism and elucidating the associated metabolic pathways, paving the way for viable biotechnological solutions.

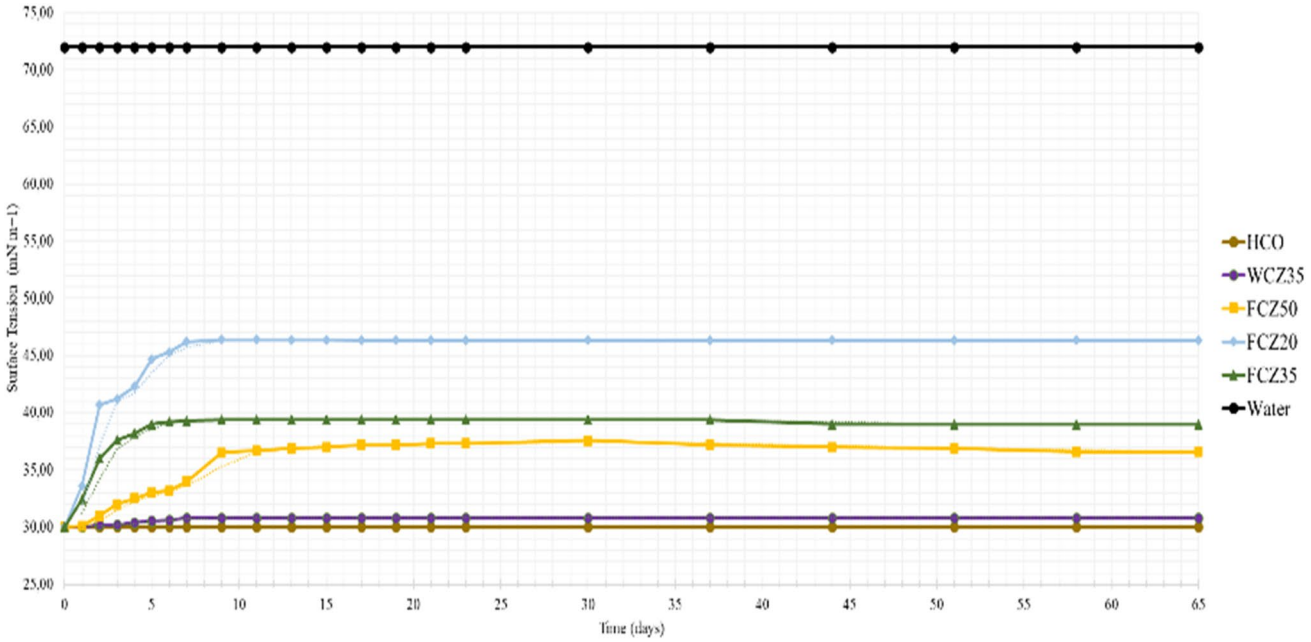


Fig. 7 Surface tension of the O/W emulsion according to culture media where: *HCO* heavy crude oil (control), *WCZ35* Czapeck medium with yeast extract and 35% HCO without inoculum (control), *FCZ20* Czapeck medium with yeast extract inoculated with fungus and 20% HCO, *FCZ35* Czapeck medium with yeast extract inoculated with fungus and 35% HCO, *FCZ50* Czapeck medium with yeast extract inoculated with fungus and 50% HCO

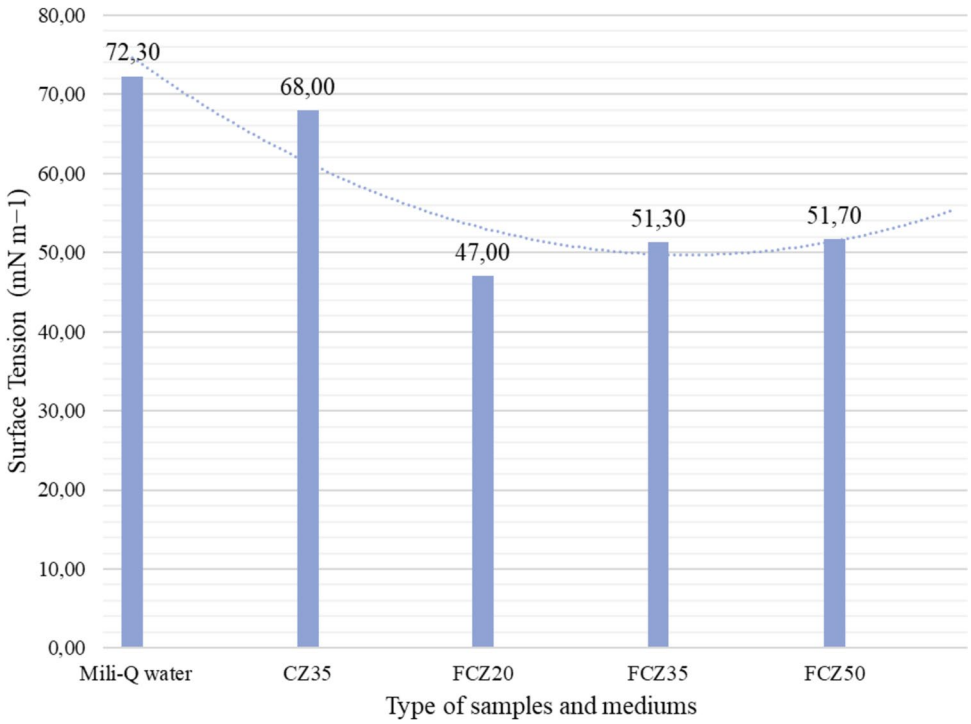


Fig. 8 Changes in the surface tension of the aqueous phase where: *Mili-Q water* control, *CZ35* aqueous phase of the medium with 35% of HCO without inoculum (control), *FCZ20* aqueous phase of the inoculated medium and 20% HCO, *FCZ35* aqueous phase of the inoculated medium and 35% HCO, *FCZ50* aqueous phase of the inoculated medium and 50% HCO

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Author contributions All authors contributed to the conception and design of the study. JZC-Z prepared the materials, collected and analyzed the data, performed the experiments, and wrote the first draft. LAR-C and CAS-N were responsible for the adaptation and improvement of the methodology, as well as the supervision of the inputs. YCR checked the data graphics and statistical analysis. All authors commented on earlier versions of the manuscript and read and approved the final manuscript.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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