Saccharomyces exiguus Uses Kerosene as a Source of Carbon and Energy

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Kerosene is a fuel derived from petroleum, a mixture of aliphatic and aromatic hydrocarbons, which eventually cause environmental pollution. In nature, there are genera and species of aerobic heterotrophic microorganisms, native to all environments, that have the potential capacity to degrade kerosene, such as some genera and species of yeast, to synthesize protein of unicellular origin or to bioaugment the negative environmental impact of kerosene. for the above. The objective of this work was to analyze the ability of Saccharomyces exiguus to use kerosene as a carbon and energy source. For this, S. exiguus was isolated from oil wells, it was grown in 5% kerosene with 1.2% NH_4Cl and 50 ppm yeast extract. The growth of S. exiguus in kerosene was analyzed using the response variables: dry weight, protein quantification, and gas chromatography showed the use of kerosene components as a carbon and energy source. The results showed that S. exiguus can use the aliphatic and aromatic hydrocarbons of kerosene as the only source of carbon and energy, this potential is applicable to synthesize unicellular protein or in the recovery of environments impacted by kerosene.

Keywords: soil, hydrocarbon mixtures, biodegradation, non-filamentous fungi, β oxidation

INTRODUCTION

In Mexico and around the world, kerosene, an insoluble mixture of aliphatic and aromatic hydrocarbons, is a petroleum derivative toxic to the environment[1], such as benzene, n-hexane, toluene, xylene, as well as other polycyclic aromatics like naphthalene and n-propyl benzene. These compounds are susceptible to microbial attack depending on the metabolic characteristics of the microorganism, as well as the environmental conditions that exist in nature or in the laboratory[1-3]. When kerosene impacts natural environments such as soil, it induces the selection of a wide diversity of microorganisms, which may be prokaryotes including genera and species of bacteria common in that environment, in addition to heterotrophic, aerobic eukaryotes such as filamentous fungi and some genera of yeasts that have the ability to biodegrade kerosene by using it as their sole source of carbon and energy[4-6] According to Prince et al.[7] over 150 genera and species of kerosene-degrading bacteria are known, such as: *Rhodococcus aetherivorans* and *R. wratislaviensis*[8], *Streptomyces spp*.[9], *Pseudomonas aeruginosa*[10,11], *Vibrio*, *Brevibacterium*, *Achromobacter*, *Mycobacterium* and *Bacillus spp.*[12-14]. Conversely, a lower diversity of filamentous fungi have been reported to degrade aromatic hydrocarbons[15, 16], the most representative examples of which are: *Aspergillus sp*., *Penicillum sp*, and *Trichoderma asperellum*[17-19]. However, there is interesting information related to genera and species of environmental yeasts that have been less well investigated for the elimination of aliphatic and aromatic hydrocarbons, which they use as the sole source of carbon and energy[20, 21]. There is also information regarding the synthesis of protein of unicellular origin as food for humans and animals[22, 23]. Examples in nature include the genera *Candida*[24, 25], *Cryptococcus*, *Pichia* and *Yarrowia*[26-28], as well as: *Meyerozyma*, *Rhodotorula*, *Wickerhamia* and *Rhodosporidium*[6]. Therefore, there is evidence that the yeast genus *Saccharomyces* has the potential to be exploited for the synthesis of protein of unicellular origin to produce low-cost food of excellent nutritional quality[22] as well as for the recovery of environments impacted by hydrocarbon mixtures, such as kerosene, by means of bioaugmentation, a process in which *Saccharomyces* would be fundamental for it to succeed[27.] In this sense, it has been reported that *Saccharomyces* species have the biochemical capacity to utilize 12- to 16-carbon hydrocarbon fractions analogous to kerosene to mineralize them to CO_2 and water[28]. Therefore, the objective of this work was to analyze the ability of *Saccharomyces exiguus* to use kerosene as a sole source of carbon and energy.

MATERIALS AND METHODS

Origin of *Saccharomyces exiguus*. This research was based on the premise that *S. exiguus* as well as other genera and species of yeasts are part of the microbiota that exists in the soil of PEMEX oil wells in Altamira, Tamaulipas, Mexico, and around the world[26]. Soil contaminated by petroleum derivatives was analyzed at the Industrial Microbiology and Soil Laboratory of the Department of Biological Sciences (FCB, in Spanish), Universidad Autónoma de Nuevo León (UANL) and at the Environmental Microbiology Laboratory of the Chemical Biological Research Institute (IIQB, in Spanish) of the Universidad Michoacana de San Nicolás de Hidalgo (UMSNH). For this purpose 1.0 g of soil was seeded in 50 mL of a mineral medium with the following chemical composition ($g \cdot L^{-1}$): 5% (v/v) kerosene sterilized by filtration with a Millipore 0.2 µm membrane, NH_4Cl 2.0, NaCl 4.0, $MgSO_4$ 0.5, K_2HPO_4 0.5, KH_2PO_4 1.0, with pH adjusted to 5.5, which was incubated at 30 °C/15 days under agitation at 200 rpm, which upon appearance of turbidity was seeded on 5% kerosene agar which was incubated at 30 °C/15 days with growth of *S. exiguus* colonies, which were reseeded on Sabouraud agar (AS) with the following chemical composition ($g \cdot L^{-1}$): 10.0 polypeptone, 40.0 glucose, 18.0 agar, with pH adjusted to 5.6, incubated at 30 °C/40 h, then these colonies were suspended in saline solution (NaCl 0.85 %) mixed with La CoronaMR 0.01 % detergent (DSS): the yeast was agitated at 200 rpm/2 h, then centrifuged, suspended in 0.85 % SSD and inoculated in nephelometric flasks: absolute control (AC) a flask filled only with 0.85 % DSS adjusted to 0 at an absorbance of 440 Klett units (KU) in a Klett Summerson photocolorimeter with red filter (650 nm). Finally, 5 mL of the suspension of *S. exiguus* were taken and inoculated in 50 mL of 5 % liquid mineral kerosene, with 1.2 % NH_4Cl and 50 ppm of yeast extract at pH 5.5[6].

Growth kinetics of *S. exiguus* in kerosene. *S. exiguus* yeast reproduced in 5 % kerosene with 1.2 % NH_4Cl and 0.025 % corn steeping liquid (CSL) (Maize product, Guadalajara, Jalisco, Mexico), or with 50 ppm malt extract (Merck), and/or 50 ppm yeast extract (Merck). *S. exiguus* was then incubated at 30 °C/4 days/200 rpm in triplicate. In this phase, different variables were used to measure the growth of *S. exiguus* in kerosene: i) optical density in a Klett Sumerson photocolorimeter at 650 nm every 24 h, with 5% mineral kerosene without *S. exiguus* as reference, ii) dry weight with 0.2 µm and 13 mm diameter Millipore membrane (Millipore Corporation, USA), which were tared in an oven at 110 °C, to filter 0.4 mL of the cellular suspension of *S. exiguus*(Millipore Corporation, USA), tared again and the difference in weight of the membrane with cells and weight of the membrane without cells was expressed in g/L to plot in grams (g) of *S. exiguus* in relation to time, iii) protein concentration: calculated by the Lowry method as an indirect measurement of the growth of *S. exiguus* with the Folin-Ciocalteu reagent, for which 0.4 mL of supernatant of kerosene 5% mineral liquid was taken at intervals for 24 h, centrifuged at 2500 rpm/15 min, and measured in a Coleman Junior II Model/620 spectrophotometer (Beckman Instruments, Inc.). The experimental data were validated with the statistical program ANOVA/Tukey HSD $P<0.05$ % with Statgraphics Centurion[29], iv) the identification of *S. exiguus* and other genus analogues was made according to the manual of Lodder[30] and Phaff et al.[31], based on the morphological and physiological characteristics listed in Table 2 using the following culture media, malt extract agar (MEA) (g/L): malt extract 3.0, yeast extract 3.0, peptone 5.0, glucose 10.0, agar 18.0, at pH 5.0, malt yeast agar (MYA) (g/L): malt extract 3.0, yeast extract 3.0, peptone 5.0, glucose 10.0 and pH at 5.0, Gorodkowa agar (g/L): glucose 2.5, NaCl 5.0, meat extract 10.0 and pH of 6.5, Fowell's acetate agar (g/L): sodium acetate 0.5, agar 2.0 at pH 6.5, Starkey's modified ethanol (g/L): ethanol 0.05, K_2HPO_4 0.025, $MgSO_4*H_2O$ 0.025 at pH 6.5, Wickerham* (g/L): yeast extract 4. 5, peptone 7.5 and pH at 6.5, and Wickerham** (g/L): KH_2PO_4 0.15, $MgSO_4*H_2O$ 0.5, NaCl 0.1, $CaCl_2$ 0.1, dextrose 10.0, NH_4Cl 0.5 and pH of 6.5. As reference for identification of yeast isolates, commercially obtained yeast strains were used[32].

Chromatographic analysis of *S. exiguus* growth in kerosene. The remaining kerosene that *S. exiguus* did not use as a carbon and energy source was separated from the mineral medium by centrifugation and then analyzed in a Beckman GC 72-5 gas chromatograph. To demonstrate and quantify the aliphatic and aromatic hydrocarbons in kerosene that were consumed by S. exiguus, a mixture of standards was used: C_{10}, C_{12}, C_{13}, C_{14}, C_{16} (in varying configurations) were analyzed by elution of the gas chromatographic peaks before and after growth of *S. exiguus* in kerosene[33].

RESULTS

FIGURE 1
INDIRECT MEASUREMENT OF THE ABILITY OF *S. exiguus* TO UTILIZE 5% KEROSENE AS A CARBON AND ENERGY SOURCE AND 1.2% NH_4CL AS A NITROGEN SOURCE WITH DIFFERENT GROWTH FACTORS

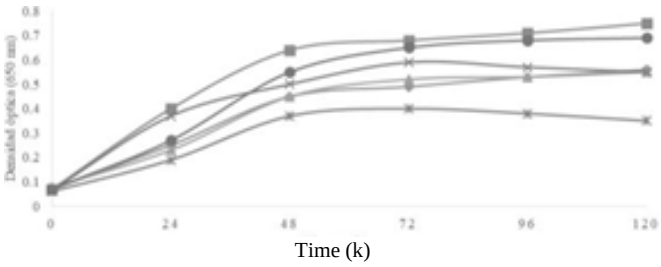

Figure 1 shows the growth of *S. exiguus* in 5 % kerosene as the sole source of carbon and energy enriched with 50 ppm yeast extract: it indicates that the adaptation phase started before 8 h, while the logarithmic phase started within the first 12 h to conclude at 48 h, followed by the stationary phase up to

120 h. This is analogous to when *S. exiguus* grew in 5 % kerosene with 1.2 % NH_4Cl as a mineral nitrogen source, enriched with 50 ppm yeast extract.

Figure 2, shows the growth of *S. exiguus* in 5 % kerosene, without 1.2 % NH_4Cl, enriched with 50 ppm yeast extract, in this case, the highest amount of cellular protein of 39 µg/mL was recorded in the first 24 h, with an increase up to 190 µg/mL at 120 h. When *S. exiguus* was given a source of mineral nitrogen in the form of 1.2 % NH_4Cl, enriched with 50 ppm of yeast extract, a measurement of 36 µg/mL of protein was recorded at 24 h and 153 µg/mL at 120 h, which constitutes indirect evidence that *S. exiguus* utilized kerosene as a carbon and energy source.

FIGURE 2
PROTEIN CONCENTRATION OF *S. EXIGUUS* WHEN USING 5% KEROSENE AS A CARBON AND ENERGY SOURCE AND 1.2% NH_4CL WITH DIFFERENT GROWTH FACTORS

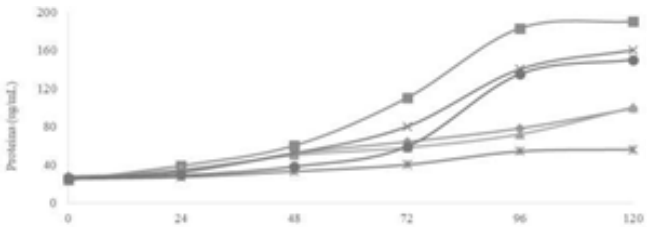

FIGURE 3
DRY WEIGHT OF *S. EXIGUUS* USING 5% KEROSENE AS A CARBON AND ENERGY SOURCE WITH 1.2% NH_4CL AND DIFFERENT GROWTH FACTORS

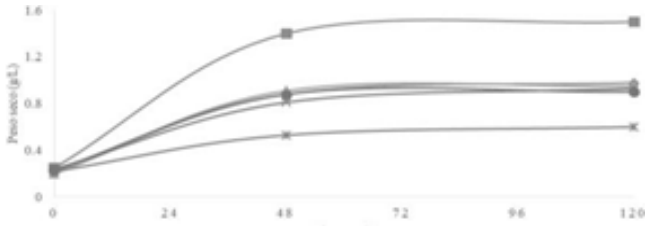

Figure 3 shows the indirect measurement of growth by dry weight of *S. exiguus* in 5% kerosene when used as a carbon and energy source, without the 1.2% NH4Cl but enriched with 50 ppm yeast extract, the measurement indicated 1.35 g/L in the first 48 h with an increase up to 1.45 g/L at 120 h, indicating the yeast extract is also a source of organic nitrogen, limited in availability for *S. exiguus* because of the low concentration added to the mineral medium.

TABLE 1
GROWTH OF *S. exiguus* USING 5% KEROSENE AS A CARBON AND ENERGY SOURCE AND NH_4CL WITH AND WITHOUT DIFFERENT GROWTH FACTOR

Saccharomyces exiguus in 5% kerosene.	X (g/L)	µexp (h-1)	t.d (h)	pH final
1.2% NH_4Cl without growth factor	0.73c**	0.049b	14c	4.3a
1.2% NH_4Cl + yeast extract 50 ppm	1.15a	0.081a	8.5d	3.5b
NH_4Cl at 1.2 % + malt extract 50 ppm	0.87b	0.047b	14.5c	4.0a
NH_4Cl at 1.2 % + corn soaking liquid at 0.025 %	0.72c	0.031c	22.0b	3.8a
Without 1.2% NH_4Cl, no growth factor	0.28e	0.022d	30a	3.9a

Without 1.2% NH_4Cl with yeast extract 50 ppm 0.63d 0.038c 18c 3.5b

Conditions: Inoculum 10% (v/v), agitation 200 rpm, 30°C, initial pH 5.5, X = cell yield, μexp = growth rate, t.d. = doubling time, *n= 3. **values with different letter indicate statistical difference according to ANOVA/Tukey ($P \leq 0.05$).

FIGURE 4
ANALYSIS OF *S. exiguus* UTILIZATION OF 5% KEROSENE HYDROCARBONS AS A CARBON AND ENERGY SOURCE, 1.2% NH_4CL AND YEAST EXTRACT AT 30°C/200 RPM

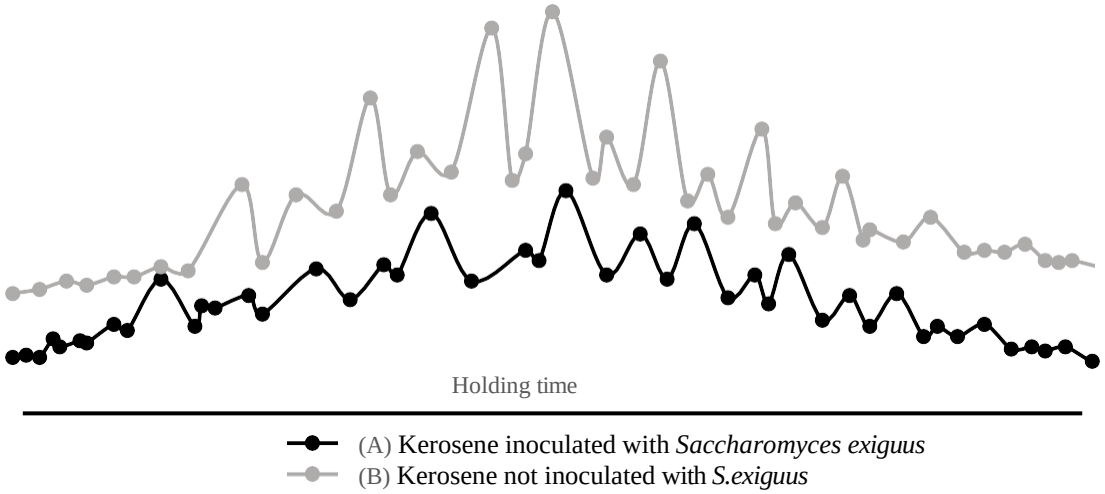

Table 1 shows the growth of *S. exiguus* in 5% kerosene when used as a carbon and energy source with 1.2% NH_4Cl, enriched with yeast extract, where the highest yield or cell production of up to 1.15 g/L was recorded with a maximum growth rate of 0.081 h^{-1}, with a lower doubling time of 8.5 h, which caused a decrease in pH to 3.5. This showed that the chemical composition of the mineral medium with 5% kerosene, NH_4Cl and yeast extract as a growth factor was the best nutritional condition for the biodegradation of kerosene.

Figure 4 shows the chromatographic profile of 5% kerosene uninoculated with *S. exiguus* used as absolute control, where the highest number of elution peaks indicating aliphatic hydrocarbons of the iso-alkane type, according to the retention time similar to the n-alkanes of the standards used as reference were recorded[34,35].

TABLE 2
MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF YEAST GENERA USING KEROSENE AS A CARBON SOURCE

Morphological and physiological characteristics	*Aureobasidium* sp	*Rhodotorula* sp	*Saccharomyces exiguus*
Type of gemination	Polar	Multilateral	Multilateral
Growth on malt extract-yeast agar (MEYA)	Abundant	Abundant	Abundant
Ascus formation	-	-	1 to 4 per ascus
Glucose fermentation	+	-	-
Assimilation of NO_3 (nitrates)	-	-	-
Pigment synthesis	Olive green to black	Orange	-
Film and/or pellet formation	+*	+	-

No growth (-), growth (+), *generates film or pellet.

Table 2 shows the morphological and physiological characteristics of the yeast genera and species isolated from different PEMEX oil wells in the city of Altamira, Tamaulipas, Mexico, which use kerosene as the only source of carbon and energy. Those that according to the morphological and physiological characteristics of the yeast genera in the cultivation methods used for the identification of the yeasts as: SA, malt yeast agar (MYA), Gorodkowa, Fowell's acetate agar, Starkey's ethanol, Wickerham and those described in the Manual of Lodder[30] and Phaff et al.[31], in relation to the genus *Aureobasidium*, with oval polar gemmation cells, without ascospores, with septate mycelium, blastospores and chlamydospores, which forms a type of pellet in liquid cultures and did not ferment glucose, although in anaerobiosis it reduced NO_3- to NO_2 -.

DISCUSSION

Figure 1 shows that *S. exiguus* grew without problems even though the kerosene concentration was 5%, without the 1.2% NH_4Cl, but enriched with the yeast extract containing B-complex vitamins in sufficient concentration for *S. exiguus* to utilize the aliphatic and aromatic hydrocarbons of kerosene as the sole source of carbon and energy by the aerobic, O_2-dependent pathway, with the enzymes of the respiratory chain which depend on the concentration of B vitamins in the yeast extract[32,36,37]. In this sense, it is reported that different genera and species of yeasts, such as *Candida albicans* have the ability to metabolize polycyclic aromatic hydrocarbons similar to those in kerosene. While *S. exiguus* had poor growth in 5% kerosene with 1.2% NH_4Cl as a nitrogen mineral source in the absence of the B-complex vitamins as a growth factor, in the form of yeast extract, thus preventing *S. exiguus* from activating enzymes to metabolize the aliphatic hydrocarbons in kerosene, *S. exiguus* had poor growth in 5% kerosene with 1.2% NH_4Cl as a source of nitrogen mineral. Figure 2 shows the increase in protein concentration produced by *S. exiguus* when using 5% kerosene as the sole source of carbon and energy in the culture medium, favored with an inorganic nitrogen source such as 1.2% NH_4Cl, and enriched with yeast extract with B-complex vitamins, which enabled the activation of the enzymes necessary for the oxidation of aliphatic hydrocarbons in kerosene by *S. exiguus* through the beta oxidation biochemical degradation pathway for kerosene[23,34]. In contrast to the growth of S. exiguus in 5% kerosene with 1.2% NH_4Cl, without the growth factor, there was a protein concentration with values ranging from 25-56 μg/mL at the end of the stationary phase at 120 h, indicating that the absence of the yeast extract slowed and inhibited the ability of *S. exiguus* to utilize the aliphatic hydrocarbons in kerosene as a carbon and energy source[33, 37]. Figure 3 shows the dry weight of *S. exiguus* when using the aliphatic and aromatic hydrocarbons of kerosene as the only source of carbon and energy, where the yeast extract enriched the mineral culture medium with B-complex vitamins, activating the enzymes required for the oxidation of kerosene hydrocarbons, as detected by the increase in the synthesis of the unicellular protein of *S. exiguus* as an indirect measure of the utilization of kerosene as a source of carbon and energy. When *S. exiguus* was grown in 5% kerosene, but now enriched with either malt extract or CSL as inducers of growth factors, a weight of 0.8 g/L was recorded at 24 h, indicating that the chemical composition of the malt extract or CSL did not contain the enzyme inducers necessary for *S. exiguus* to utilize the aliphatic hydrocarbons in the kerosene as the sole source of carbon and energy. Consequently, there was an evident inhibition of the growth of *S. exiguus*, since the dry weight detected at the end was similar to that recorded in the 5% kerosene used as AC without inoculation with *S. exiguus*[23,35,38]. Table 1 shows the growth of *S. exiguus* in 5% kerosene, using it as the sole source of carbon and energy, where it was shown that it requires 1.2% NH_4Cl for protein and nucleic acid synthesis, while the yeast extract provided B vitamins for the activation of enzymes associated with the biodegradation of aliphatic and aromatic hydrocarbons of kerosene[26,39,40]. The numerical values of the growth or yield of *S. exiguus* in kerosene when metabolized as the sole source of carbon and energy, with 1.2 % NH_4Cl plus yeast extract as the growth factor, were statistically different compared to the values of the cell growth or yield of 0.73 g/L, which reached the maximum growth rate at 0.049 h^{-1}, in contrast to the prolonged 14 h doubling time of *S. exiguus* in 5 % kerosene as carbon and energy source without NH_4Cl or any growth factor, which was used as the AC.

The above demonstrated the utilization of kerosene as a sole source of carbon and energy by *S. exiguus* with NH_4Cl as a source of mineral nitrogen, and that growh was stimulated by yeast extract, a source of B-complex vitamins, which facilitated the reproduction of *S. exiguus*, indirectly measured by protein synthesis, or for biotreatment of kerosene-impacted environments known as bioaugmentation, a technique of targeted biological removal of some type of contaminant(s) by a specific microorganism such as *S. exiguus*, which in kerosene-impacted environments could remove it[33,34]. In addition, *Aureobasidium sp*. and *Rhodotorula sp*. were observed growing on kerosene as a sole source of carbon and energy, but without the ability to consume it as efficiently as *S. exiguus* does[23-25].

Figure 4 shows the chromatogram demonstrating that when *S. exiguus* used 5% kerosene as a source of organic carbon and energy it proved to have the ability to oxidize the aliphatic hydrocarbons in this mixture containing compounds of 12 to 16 carbon atoms, based on the disappearance of the elution peaks of these hydrocarbons[33-35,38]. In this sense, Okpokwasili & Amanchukwu (1988)[39] reported that the genus *Candida* possesses the genes to utilize hydrocarbon mixtures similar to those found in kerosene[40,41], therefore, these yeast genera and species are a suitable biological option for: i) the synthesis of single-cell protein of high nutritional value and/or ii) the bioaugmentation or "*ex situ*" removal of kerosene-impacted environments[42]. While Zinjarde & Pant (2002)[43] reported the effectiveness of *Candida* species to biodegrade it such as: *C. parapsilosis*, *C. guilliermondii* and *C. tropicalis* as well as *Yarrowia lipolytica* which showed the highest capacity to eliminate the aliphatic fraction of kerosene up to 78 %. Table 2 presents the main morphological and biochemical characteristics of the yeast genera that use kerosene hydrocarbons as a carbon and energy source, which were observed in the young 24-h yeast cultures that had bright convex colonies, with olivaceous pigments, and were characterized by a high degree of purity. Under the microscope, cells were observed dividing by polar budding, in 3-4 day cultures the colonies darkened as they formed a mycelium at the periphery[44]. The genus *Rhodotorula* was isolated from soil impacted by kerosene from Altamira, Tamaulipas, characterized by oval cells with multilateral gemmation observed by microscopy, without mycelium or pseudomycelium, the organism did not generate any film when grown in a liquid medium, in solid culture medium conical colonies were observed, with a white intracellular pigment of creamy consistency, convex in shape. *Rhodotorula* did not ferment glucose, although it assimilated NO_3^-[45], while the genus *Saccharomyces* species *exiguus* appeared as oval cells with multilateral gemmation under the microscope, possessing 1 to 4 ascospores, in liquid culture medium it did not generate any film on the surface, did not assimilate NO^-, but it did ferment glucose and galactose[44,45].

This research concludes that *S. exiguus* is capable of using up to 51% of the aliphatic hydrocarbons in kerosene as a carbon and energy source. Therefore, it is possible to grow it as a producer of protein of unicellular origin and remover of kerosene hydrocarbons by bioaugmentation for the recovery of water and soil impacted by petroleum derivatives similar or different from kerosene.

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ENDNOTES

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