



# Exploring the use of hexadecane by *Yarrowia lipolytica*: Effect of dissolved oxygen and medium supplementation

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## ABSTRACT

This work aimed to evaluate the effect of medium composition and volumetric oxygen transfer coefficient ( $k_{La}$ ) on *Y. lipolytica* growth and production of microbial lipids and enzymes from hexadecane. In the stirred tank bioreactor, increasing  $k_{La}$  from 11 h<sup>-1</sup> to 132 h<sup>-1</sup> improved the hexadecane assimilation rate, biomass concentration, and lipids synthesis (0.90 g·L<sup>-1</sup>). A cost-effective hexadecane-based medium supplemented with corn steep liquor and a low amount of ammonium sulfate boosted lipids production up to 2.1 g·L<sup>-1</sup>, composed of palmitic, palmitoleic, oleic, and linoleic acids. The unsaturated/saturated fraction was dependent on the C/N ratio. Lipids of *Y. lipolytica* CBS 2075 are promising feedstock for animal feed, food additives, or the biodiesel industry. Simultaneous synthesis of extracellular lipase and protease from hexadecane was observed, which is a new feature that was not previously reported. The highest enzyme activity was obtained at the highest C/N ratio conditions. These results open new perspectives on the application of *Y. lipolytica*-based cultures for the biotransformation of hexadecane-polluted streams into valuable compounds, fulfilling an interesting strategy towards the circular economy concept.

## 1. Introduction

Petroleum refineries and petrochemical industries produce high volumes of oily effluents containing hydrocarbons, water, solids, and heavy metals. The improper disposal or ineffective treatment of these petroleum-derived effluents has detrimental impacts on both the environment and human health (Varjani et al., 2020). The implementation of microbial-based bioprocesses is one of the most promising alternatives posing as a cost-effective and eco-friendly way toward the removal and biotransformation of petroleum hydrocarbons from wastewaters (Sowani et al., 2019), ubiquitously available as inexpensive feedstocks for bioprocesses in contrast to sugar substrates commonly used in industry (e.g., the price of glucose syrup is, approximately, 1.42 €/kg) (USDA, 2023). Hexadecane, an aliphatic hydrocarbon, is a major compound of several crude oil, diesel fuels, and petroleum-based effluents and is frequently used as a model compound for the study of hydrocarbon biodegradation by microorganisms (Varjani and Upasani, 2017).

*Yarrowia lipolytica* is an oleaginous and strictly aerobic yeast, commonly found in lipid-rich products and oil-contaminated environments (Hassanshahian et al., 2012). Several authors have demonstrated the potential of this yeast to use hydrophobic wastes/by-products

(wastewaters from vegetable oils refinement process, pork lard, or waste cooking oils), producing valuable compounds, such as extracellular enzymes, organic acids, and microbial lipids (Darvishi et al., 2019; Liu et al., 2015a; Lopes et al., 2019, 2018). These unique features of *Y. lipolytica*, together with the production of large amounts of oily effluents, owing to a high dependency on petroleum-based products, have triggered the interest in using this yeast in the bioconversion processes of hexadecane.

Like most environmental pollutants, hexadecane is a hydrophobic compound with very low solubility in water (or culture medium). Therefore, access to hexadecane as a carbon and energy source for microbial species is limited. Generally, in *Y. lipolytica* cultures, the uptake of hexadecane is facilitated by cell attachment to oil droplets, aided by hydrophobic cell surface, or is mediated by the production of biosurfactants (Ferreira et al., 2023). Inside the cell, hexadecane is oxidized to fatty alcohols and then converted to fatty acids by fatty-alcohol-oxidizing enzymes. Lately, fatty acids are activated to synthesize acyl-CoA, which undergoes the  $\beta$ -oxidation pathway and is metabolized into acetyl-CoA. This intermediate metabolite could undergo be incorporated in the glyoxylate, Krebs or methyl-citrate cycles. Alternatively, fatty acids synthesized after metabolization of long-chain

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alkanes (C14 – C18), could be transported and stored in lipid bodies as triacylglycerols or steryl esters (Fig. 1) (Fickers et al., 2005; Fukuda, 2013).

The first aim of this work was to assess the ability of six *Y. lipolytica* strains to assimilate hexadecane (as a hydrocarbon model) for biomass production and lipids accumulation. The effect of volumetric oxygen transfer coefficient ( $k_L a$ ), medium composition, and carbon/nitrogen (C/N) on the production of lipid-rich biomass by *Y. lipolytica* CBS 2075 (the selected strain) from hexadecane was studied in an STR lab-scale bioreactor. As side-products, extracellular lipase, and protease were also detected in the medium. It is important to highlight that, thus far, most of the studies developed with *Y. lipolytica* strains were focused on hydrocarbon assimilation and only three explored its bioconversion to added-value compounds, such as succinic acid (Kamzolova et al., 2012), microbial lipids from pentadecane or heptadecane (Matatkova et al., 2017), and 4-(2,4-dichlorophenoxy)-butyric acid from 1-alkoxy-2,4-dichlorobenzenes through oxidative metabolism (Lim et al., 2016).

## 2. Materials and methods

### 2.1. Yeasts strain preservation

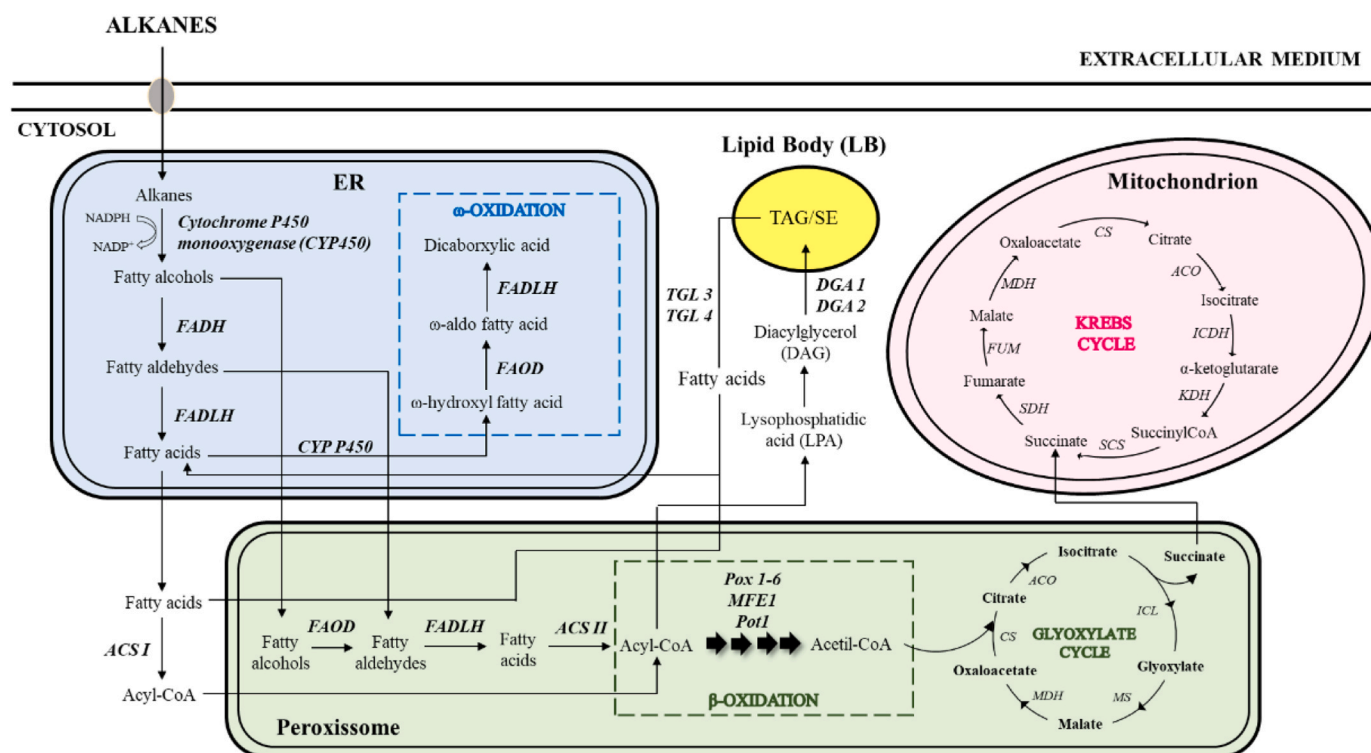
Cells of *Y. lipolytica* strains were grown overnight in a YPD medium composed of 20 g·L<sup>-1</sup> glucose, 20 g·L<sup>-1</sup> peptone, and 10 g·L<sup>-1</sup> yeast extract to prepare cryo-stocks in sterile microtubes (800 µL of yeast culture and 200 µL of glycerol 99.5%). All cryo-stocks were preserved at -80 °C until used to inoculate sub-cultures. These were inoculated with one microtube and placed in an orbital incubator at 27 °C and 200 rpm for around 16 h.

### 2.2. Screening of *Y. lipolytica* strains for hexadecane assimilation

In this work, six *Y. lipolytica* strains were studied for their ability to assimilate hexadecane as the sole carbon source: NCYC 2904, CBS 2073, CBS 2075, IMUFRJ 50682 - isolated from Guanabara Bay in Rio de Janeiro, Brazil (Hagler and Mendonça-Hagler, 1981), and strains ch 1/5 and ch 3/4 - isolated from cheese (Nagy, 2014). Batch cultures were carried out in 250-mL Erlenmeyer flasks containing 100 mL of culture medium composed of 5 g·L<sup>-1</sup> hexadecane and 6.7 g·L<sup>-1</sup> yeast nitrogen base without amino acids and with ammonium sulfate (YNB). Cells of each *Y. lipolytica* strain, pre-cultured in a YPD medium (prepared as described in Section 2.1), were harvested by centrifugation and used to inoculate the culture media at an initial cell density of 0.5 g·L<sup>-1</sup>. All experiments were conducted at 27 °C and 200 rpm in an orbital incubator for 72 h. The biomass concentration was monitored through time and the concentration of hexadecane in the medium was measured at the beginning and the end of the experiments.

### 2.3. Bioreactor batch experiments

Bioreactor batch experiments were carried out in a 5-L stirred tank bioreactor (STR) (BIOSTAT® B Plus, Sartorius) with 2-L of culture medium, at 27 °C and pH 5.5 automatically controlled by the addition of NaOH 2 M or HCl 2 M. Dissolved oxygen concentration was measured with an optical probe and the respective meter. Samples were collected at specific interval times for quantification of biomass and hexadecane concentration, and extracellular enzymes' activity. At the end of the experiments, yeast cells were harvested by centrifugation and stored at -20 °C for further quantification of intracellular lipids and long-chain fatty acids composition.



**Fig. 1.** An overview of metabolic pathways and respective cellular compartments involved in alkanes assimilation by *Yarrowia lipolytica*. Main metabolic flux during alkanes oxidation is shown with black arrows and respective enzymes are written, in italic, next to the arrows. Alternative metabolic fluxes, diterminal alkane oxidation or  $\omega$ -oxidation pathway and  $\beta$ -oxidation, are highlighted in the boxes with dashed blue and green lines, respectively. Abbreviations: ACO: aconitate; ACS I and II: fatty acyl-CoA synthetase; CS: citrate synthase; DAG 1 and 2: diacylglycerol acyltransferase; ER: endoplasmic reticulum; FADH: fatty alcohol dehydrogenase; FALDH: fatty aldehyde dehydrogenase; FAOD: fatty alcohol oxidase; FUM: fumarase; ICDH: isocitrate dehydrogenase; ICL: isocitrate lyase; KDH:  $\alpha$ -ketoglutarate dehydrogenase; MDH: malate dehydrogenase; MFE1: peroxisomal multifunctional enzyme; MS: malate synthase; Pot1: peroxisomal 3-oxoacyl-CoA thiolase; Pox 1–6: acyl-CoA oxidases; TAG: triacylglycerol; TGL 3 and 4: triacylglycerol lipase 3 and 4; SE: steryl esters.

To evaluate the effect of volumetric oxygen transfer coefficient ( $k_La$ ) on the lipid-rich biomass and hexadecane assimilation by *Y. lipolytica* CSB 2075, four agitation rates from 200 rpm to 600 rpm were tested at a specific airflow rate of 1 vvm (volume of air per volume of medium per minute) for 30 h. Yeast cells from a YPD pre-inoculum were centrifuged and resuspended (to obtain an initial cell density of  $0.5 \text{ g}\cdot\text{L}^{-1}$ ) in a 2-L medium composed of  $5 \text{ g}\cdot\text{L}^{-1}$  hexadecane and  $6.7 \text{ g}\cdot\text{L}^{-1}$  YNB.

The best condition of  $k_La$  was validated using a more economical medium, replacing YNB with corn steep liquor (CSL, C4648–500 G, Sigma-Aldrich®), tested at two concentrations of  $1.7 \text{ g}\cdot\text{L}^{-1}$  or  $3.4 \text{ g}\cdot\text{L}^{-1}$  and added to the cultivation medium composed of  $5 \text{ g}\cdot\text{L}^{-1}$  hexadecane and  $5 \text{ g}\cdot\text{L}^{-1}$  ammonium sulfate. Additionally, experiments with  $3.4 \text{ g}\cdot\text{L}^{-1}$  CSL (total organic carbon:  $19.5\% \pm 0.9\%$  (w/w), total nitrogen:  $3.2\% \pm 0.1\%$  (w/w), soluble protein:  $0.33\% \pm 0.03\%$  (w/w), glucose:  $2.5\% \pm 0.1\%$  (w/w), fructose:  $3.22\% \pm 0.01\%$  (w/w), and lactic acid:  $16.9\% \pm 0.1\%$  (w/w)) and  $0.5 \text{ g}\cdot\text{L}^{-1}$  ammonium sulfate were also performed to study the effect of ammonium sulfate concentration and, consequently, of the C/N ratio, on hexadecane utilization by *Y. lipolytica* CBS 2075.

## 2.4. Analytical methods

For the quantification of biomass, the optical density of cultures was measured at 600 nm and converted to dry cell weight ( $\text{g}\cdot\text{L}^{-1}$ ) using a calibration curve.

Extracellular lipase activity was quantified in the cultures' supernatant using *p*-nitrophenyl butyrate 1 mM as substrate dissolved in 250  $\mu\text{L}$  of reaction mixture (phosphate buffer 50 mM pH 7.3, acetone 4% v/v, and Triton-X 4% v/v) [10]. One unit of activity was expressed as the amount of enzyme that produces 1  $\mu\text{mol}$  of *p*-nitrophenol per minute under the assay conditions. Protease activity was quantified according to Lopes et al. (2019) in cultures supernatant using 0.5% (w/v) azocasein dissolved in sodium acetate buffer 50 mM (pH 5) as substrate. One unit of enzyme activity was defined as the amount of enzyme that causes an increase of 0.01 of absorbance comparatively to the blank (supernatant' cultures were replaced by sodium acetate buffer 50 mM) per minute under assay conditions.

Microbial lipids content was quantified in lyophilized cells by the phospho-vanillin colorimetric method as described by Lopes et al. (2019). Fatty acids composition was analyzed by gas chromatography after extraction from lyophilized cells with chloroform and further methylation with methanol and sulfuric acid (85:15, v/v). Pentadecanoic acid was used as the internal standard. The relative amount of each fatty acid (% w/w) was determined as the ratio between its concentration ( $\text{g}\cdot\text{L}^{-1}$ ) and the sum of the concentrations of all fatty acids identified in the sample.

Hexadecane was extracted from the culture medium samples by liquid-liquid extraction, using hexane as solvent (1:6, v/v) and undecane (C11) as the internal standard. Hexadecane concentration was quantified using a PerkinElmer® Clarus® 580 gas chromatograph, equipped with an FID detector and a BR-1 ms column (15 m x 0.25 mm, Bruker, USA). The injector and detector temperatures were set at 285 °C and 300 °C, respectively. The initial oven temperature was 60 °C, maintained for 1 minute, followed by 8 °C  $\text{min}^{-1}$  ramp up to 300 °C. The isothermal conditions were maintained at 300 °C for 5 minutes and then programmed down to 60 °C at a rate of 40 °C  $\text{min}^{-1}$ . Helium was used as carrier gas at 1  $\text{mL}\cdot\text{min}^{-1}$ .

## 2.5. Volumetric oxygen transfer coefficient ( $k_La$ ) measurement

The experimental  $k_La$  at different agitation rates was measured by a static gassing-out technique (Ferreira et al., 2016). Briefly, in blank assays (culture medium without cells), the concentration of dissolved oxygen in the liquid was followed until its saturation at specific conditions of agitation rate, after a prior gassing-out with nitrogen to remove the oxygen.

## 2.6. Statistical analysis

All data represent the mean of two independent replicates. Statistical analysis was performed with Statgraphics Centurion XVI Version 16.2.04 (StatPoint Technologies Inc., USA), using one-way analysis of variance (ANOVA) and Tukey's multi-range test to identify statistically significant differences in mean values of the different measured parameters (95% level of confidence).

## 3. Results and discussion

### 3.1. Screening of *Y. lipolytica* strains for hexadecane assimilation

The yeast *Y. lipolytica* has unique physiological features and distinctive metabolic pathways (which include several multi-gene families) that mediate the efficient assimilation of hydrocarbons such as alkanes and aromatic compounds (Madzak, 2021). All *Y. lipolytica* strains tested were able to grow in medium with the addition of  $5 \text{ g}\cdot\text{L}^{-1}$  of hexadecane (Fig. 2A). Particularly, ch 1/5 and ch 3/4 strains grew at a higher initial rate but reach a final biomass concentration similar to that attained with CBS 2073 and CBS 2075 strains after 48 h and 72 h of cultivation. Furthermore, these four yeast strains consumed all hexadecane in the culture medium within that period (Fig. 2B). By contrast, in the experiments with NCYC 2904 and IMUFRJ 50682 strains, approximately  $1 \text{ g}\cdot\text{L}^{-1}$  and  $2 \text{ g}\cdot\text{L}^{-1}$ , respectively, of unconsumed hexadecane remained in the medium at the end of the selected cultivation time.

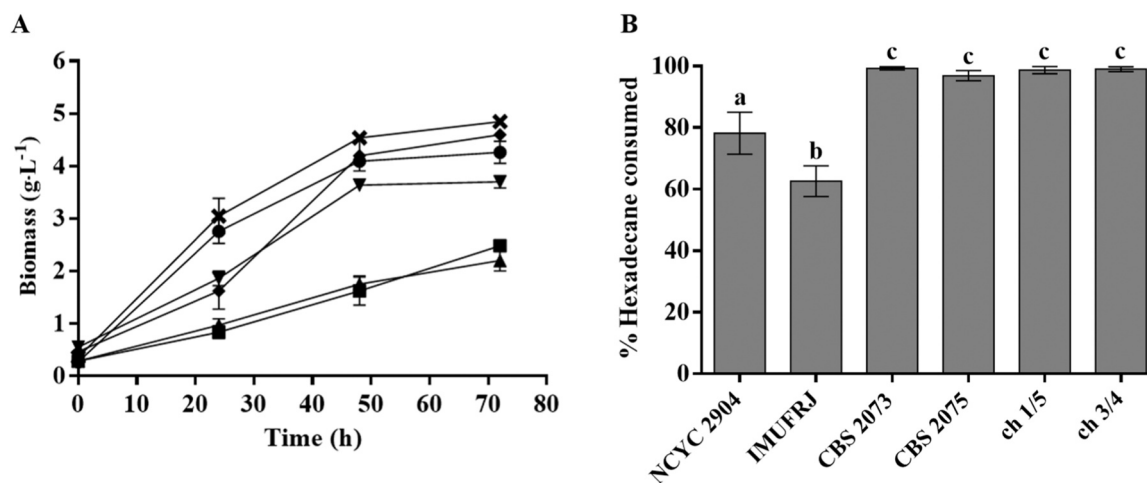
At the end of the selected cultivation time. The highest biomass yield (mass of cells per mass of hexadecane consumed after 72 h) was achieved in cultures of *Y. lipolytica* CBS 2075 ( $0.83 \text{ g}\cdot\text{g}^{-1} \pm 0.01 \text{ g}\cdot\text{g}^{-1}$ ) and ch 3/4 ( $0.87 \text{ g}\cdot\text{g}^{-1} \pm 0.03 \text{ g}\cdot\text{g}^{-1}$ ) strains, which were approximately 1.6-fold higher than those obtained in *Y. lipolytica* NCYC 2904 ( $0.51 \text{ g}\cdot\text{g}^{-1} \pm 0.03 \text{ g}\cdot\text{g}^{-1}$ ) and IMUFRJ 50682 ( $0.55 \text{ g}\cdot\text{g}^{-1} \pm 0.13 \text{ g}\cdot\text{g}^{-1}$ ) cultures. The biomass yields of *Y. lipolytica* CBS 2073 ( $0.63 \text{ g}\cdot\text{g}^{-1} \pm 0.02 \text{ g}\cdot\text{g}^{-1}$ ) and ch 1/5 ( $0.75 \text{ g}\cdot\text{g}^{-1} \pm 0.05 \text{ g}\cdot\text{g}^{-1}$ ) strains were statistically equal. These results demonstrated the ability of different *Y. lipolytica* strains to consume hexadecane at  $5 \text{ g}\cdot\text{L}^{-1}$  of concentration in 72 h and use it as a carbon source for biomass proliferation. *Yarrowia lipolytica* strains developed specialized mechanisms to overcome the low solubility of hydrocarbons in water and to facilitate its uptake. In general, the ability of *Y. lipolytica* to synthesize extracellular biosurfactants and/or the direct attachment between cells and oil droplets, owing to the high hydrophobicity of cell surface, are described as fundamental features of *Y. lipolytica* to grow in hydrocarbons (Amaral et al., 2006; Kim et al., 2000). Albeit some authors described the capacity of *Y. lipolytica* strains to assimilate hexadecane at flask-scale, the initial hexadecane concentration used and substrate consumption rates obtained in this study are higher than those described in the literature. Hassanshahian et al. (2012) reported that two *Y. lipolytica* strains, isolated from a marine polluted environment, were able to grow in 1% (v/v) of crude oil, but the total assimilation of medium chain-length alkanes, such as hexadecane, was observed after one week of yeast cultivation. *Y. lipolytica* RM7/11, a cold-adapted strain, grew in a medium with  $1 \text{ g}\cdot\text{L}^{-1}$  of hexadecane, but only 50% were consumed after 5 days at 15 °C (Margesin et al., 2003).

Considering the similarity of results among the two best strains, *Y. lipolytica* 3/4 and CBS 2075, this last one was selected for further experiments conducted in the STR lab-scale bioreactor.

### 3.2. Bioreactor experiments

#### 3.2.1. Effect of volumetric oxygen transfer coefficient ( $k_La$ )

The microbial conversion of hydrophobic substrates into biomass, microbial lipids, and enzymes is affected by the oxygen transfer rate (OTR) and the amount of dissolved oxygen concentration (DOC) in the culture medium (Lopes et al., 2008, 2018). The increase in agitation rate



**Fig. 2.** Biomass profile (A) and hexadecane consumption (B) obtained in batch cultures of *Y. lipolytica* strains carried out in Erlenmeyer flasks with the addition of hexadecane (5 g·L<sup>-1</sup>): NCYC 2904 (■), IMUFRJ 50682 (▲), CBS 2073 (▼), CBS 2075 (◆), ch 1/5 (●) and ch 3/4 (x). The error bars represent the standard deviation of two independent replicates. Bars followed by the same letter (Figure B) do not present statistically significant differences (95% confidence level).

is one strategy that enables the enhancement of the volumetric oxygen transfer coefficient ( $k_{La}$ ) and, consequently, OTR. Therefore, this study aimed to evaluate the effect of  $k_{La}$ , by varying the agitation rate in an STR lab-scale bioreactor, on *Y. lipolytica* CBS 2075 growth, hexadecane consumption, and metabolites production.

As expected, the increase in agitation rate enhanced  $k_{La}$ , and the value at 600 rpm was 12-fold higher than that at 200 rpm (Table 1). This increase in  $k_{La}$  had an impact on DOC in the culture medium and different oxygen profiles were obtained depending on the  $k_{La}$  condition (Fig. 3C). In the experiments carried out at  $k_{La}$  of 11 h<sup>-1</sup> and 50 h<sup>-1</sup>, DOC dropped to zero in the first 10 h and remained at this value until the end of the cultivation. At the  $k_{La}$  condition of 84 h<sup>-1</sup>, DOC was equal to zero during all exponential growth phases, increasing progressively after this period. By contrast, in the experiments carried out at the highest  $k_{La}$  (132 h<sup>-1</sup>), DOC gradually fell to approximately 36% of saturation after 22 h of cultivation and stabilized above 90% of saturation by the end of cultivation.

The non-limiting oxygen conditions with a  $k_{La}$  of 132 h<sup>-1</sup> had a remarkable positive effect on *Y. lipolytica* growth. A 2.3-, 1.6- and 3.7-fold improvement in final biomass concentration (Fig. 3A), biomass yield, and maximum growth rate (Table 1), respectively, were obtained by increasing  $k_{La}$  from 11 h<sup>-1</sup> to 132 h<sup>-1</sup>.

**Table 1**

Experimental values of volumetric oxygen transfer coefficient ( $k_{La}$ ), specific growth rate ( $\mu$ ) calculated at the exponential phase, biomass yield ( $Y_{x/s}$ ), specific lipid content, lipids concentration and maximum protease (Prot<sub>max</sub>) activity obtained after 30 h of *Y. lipolytica* CBS 2075 batch cultures carried out in an STR bioreactor. Data are average  $\pm$  standard deviation of two independent replicates. Values followed by the same letter in each column do not present statistically significant differences (95% confidence level).

Agitation rate (rpm)	$k_{La}$ (h <sup>-1</sup> )	$\mu$ (h <sup>-1</sup> )	$Y_{x/s}$ (g·g <sup>-1</sup> )	Specific lipid content (% w/w)	Lipids (g·L <sup>-1</sup> )	Prot <sub>max</sub> (U·L <sup>-1</sup> )
200	11 $\pm$ 1 <sup>a</sup>	0.04 $\pm$ 0.01 <sup>a</sup>	0.9 $\pm$ 0.1 <sup>a</sup>	14 $\pm$ 3 <sup>a</sup>	0.35 $\pm$ 0.08 <sup>a</sup>	197 $\pm$ 12 <sup>a</sup>
	300	50 $\pm$ 2 <sup>b</sup>	0.06 $\pm$ 0.01 <sup>ab</sup>	1.05 $\pm$ 0.12 <sup>ab</sup>	11 $\pm$ 2 <sup>a</sup>	0.41 $\pm$ 0.09 <sup>a</sup>
400	84 $\pm$ 2 <sup>c</sup>	0.10 $\pm$ 0.01 <sup>b</sup>	1.01 $\pm$ 0.05 <sup>ab</sup>	11 $\pm$ 1 <sup>a</sup>	0.50 $\pm$ 0.02 <sup>a</sup>	193 $\pm$ 9 <sup>a</sup>
	600	132 $\pm$ 2 <sup>d</sup>	0.149 $\pm$ 0.01 <sup>c</sup>	1.4 $\pm$ 0.1 <sup>b</sup>	16 $\pm$ 1 <sup>a</sup>	0.90 $\pm$ 0.05 <sup>b</sup>

$Y_{x/s}$  was expressed as mass of cells per mass of total hexadecane consumed after 30 h. Specific lipid content was expressed as mass of lipids per mass of cells.

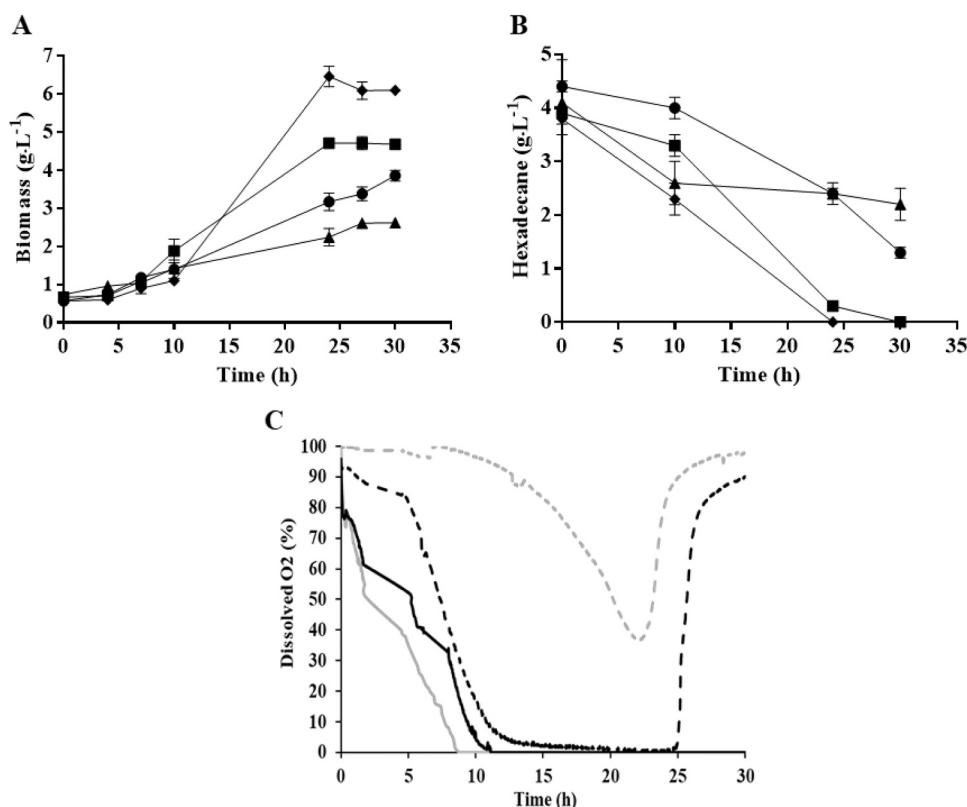
Moreover, regardless  $k_{La}$  conditions, the growth rate of *Y. lipolytica* CBS 2075 was higher than in the Erlenmeyer flask (Fig. 2A). After 24 h of cultivation, a 4-fold improvement in biomass concentration was obtained in the experiments at  $k_{La}$  of 132 h<sup>-1</sup> comparatively to the flasks, demonstrating the importance of DOC, controlled pH, and mechanical agitation in cultures of *Y. lipolytica* growing in hexadecane. As a strictly aerobic yeast, the enhancement in biomass concentration under conditions of high DOC was also reported for different *Y. lipolytica* strains growing in crude oil (Martins et al., 2012), waste cooking oils (Lopes et al., 2019), volatile fatty acids (Pereira et al., 2021), and crude glycerol (Ferreira et al., 2022).

The assimilation of hexadecane by *Y. lipolytica* CBS 2075 cells was also highly dependent on the DOC. Whereas a complete assimilation of hexadecane was observed in the experiments at  $k_{La}$  of 84 h<sup>-1</sup> and 132 h<sup>-1</sup>, in the experiments conducted at the lowest  $k_{La}$  conditions, hexadecane was not completely consumed, and approximately 2.2 g·L<sup>-1</sup> and 1.3 g·L<sup>-1</sup> of unconsumed hexadecane remained in the medium (Fig. 3B). According to Fickers et al. (2005) and Fukuda (2013), the metabolization of *n*-alkanes, such as hexadecane, occurs through a metabolic route highly dependent on oxygen ( $\beta$ -oxidation pathway). Moreover, besides the effect of high stirring rates on  $k_{La}$  values, there is also a positive impact on the dispersion of hexadecane droplets in the aqueous medium, which favors the contact between yeast cells and hexadecane, then will facilitate substrate assimilation by *Y. lipolytica* cells.

Despite the significant effect of  $k_{La}$  and DOC on *Y. lipolytica* growth and hexadecane assimilation, no enhancement was attained in specific lipid content by increasing the  $k_{La}$  value from 11 h<sup>-1</sup> to 132 h<sup>-1</sup> (Table 1). However, since higher biomass was achieved in experiments carried out at the highest  $k_{La}$  conditions, a 2.6-fold improvement in lipids concentration was obtained. Although no studies regarding the effect of  $k_{La}$  or DOC on lipids synthesis from hexadecane were found in the literature, Matatkova et al. (2017) observed that *Y. lipolytica* CCY 30–26–36 was able to accumulate between 12% (w/w) and 18% (w/w) of lipids from pentadecane and heptadecane in Erlenmeyer flask experiments. Other authors demonstrated that lower quantities of microbial lipids were produced by *Y. lipolytica* strains from stearin (Papanikolaou et al., 2007), waste cooking oils (Lopes et al., 2019), pork lard (Lopes et al., 2018), volatile fatty acids (Pereira et al., 2021) and glucose (Bellou et al., 2014) when high oxygenation conditions were employed. According to Papanikolaou et al. (2007), under high oxygenation conditions, the activity of acyl-CoA oxidases increases, which will hinder the synthesis of lipids by *Y. lipolytica*, since the carbon source is directed towards the production of lipid-free biomass.

Besides the production of microbial lipids, simultaneous synthesis of





**Fig. 3.** Time course of cellular growth (A), hexadecane assimilation (B) and dissolved oxygen concentration (% of saturation) (C) obtained in batch cultures of *Y. lipolytica* CBS 2075 carried out in an STR bioreactor at different  $k_{La}$  ( $\text{h}^{-1}$ ): 11 (▲, grey line), 50 (●, black line), 84 (■, black dashed line) and 132 (◆, grey dashed line). The error bars represent the standard deviation of two independent replicates.

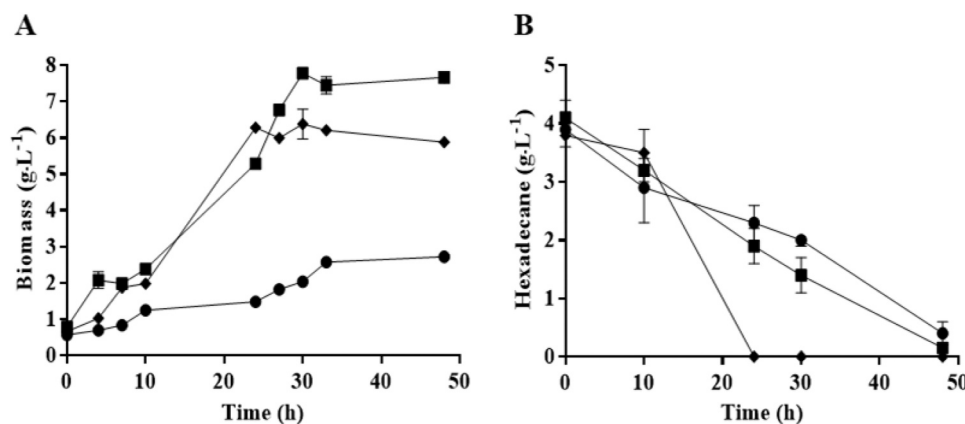
extracellular protease by *Y. lipolytica* CBS 2075 from hexadecane was observed (Table 1), a new feature that was not previously reported in hexadecane medium. The ability of *Y. lipolytica* strains to produce protease from oily substrates (Gomes et al., 2013; Lopes et al., 2019, 2009a; Najjar et al., 2011) or sugars (Lopes et al., 2008, 2009b) was already reported, but this is the first work describing its synthesis from hexadecane. Similar profiles of protease activity were obtained in all  $k_{La}$  conditions, remaining at low values during the first 10 h and increasing until 25 h of cultivation. Though the proteolytic activity was not affected by the increase in agitation rate, an increase of 88% in protease activity was achieved by raising  $k_{La}$  from 11  $\text{h}^{-1}$  to 50  $\text{h}^{-1}$ , but a further increase in  $k_{La}$  to 132  $\text{h}^{-1}$  did not improve the enzyme synthesis by *Y. lipolytica* CBS 2075. Albeit the maximum protease activity was attained at a  $k_{La}$  of 50  $\text{h}^{-1}$  and the further increase in  $k_{La}$  did not improve the enzyme synthesis (Table 1), Lopes et al. (2019) observed a 3.5-fold improvement in the proteolytic activity of *Y. lipolytica* W29 cells growing in waste cooking oils by rising the  $k_{La}$  from 9  $\text{h}^{-1}$  to 93  $\text{h}^{-1}$ . Similarly, the enhancement of DOC by increasing the values of total air pressure had a clear positive effect on protease production by *Y. lipolytica* W29 from glucose (Lopes et al., 2009b).

### 3.2.2. Effect of medium composition

The development of bioprocesses using low-cost media components has become a crucial step to reduce costs and increase market competitiveness. The composition of the culture media plays a key role in the biosynthesis of valuable compounds by *Y. lipolytica* strains (Lopes et al., 2022). Furthermore, 50% - 80% of the total production costs of added-value compounds are affected by the cost of feedstocks used for microbial cultivation (Koutinas et al., 2014). Corn steep liquor (CSL) is a by-product of corn starch processing, containing mostly proteins, reducing sugars, organic acids, minerals, and some free amino acids, commonly used as an alternative to more expensive nitrogen sources (e.

g. yeast extract, YNB) in microbial processes (Cavallo et al., 2020; Liu et al., 2017; Martínez-Arcos et al., 2021). In the current study, YNB was replaced by CSL in bioreactor batch cultures, at two different concentrations (1.7  $\text{g}\cdot\text{L}^{-1}$  or 3.4  $\text{g}\cdot\text{L}^{-1}$ ). Additionally, it was evaluated the effect of the C/N ratio (by adding different concentrations of ammonium sulfate, 0.5  $\text{g}\cdot\text{L}^{-1}$  or 5  $\text{g}\cdot\text{L}^{-1}$ ) on *Y. lipolytica* lipid-rich biomass. Regardless of medium composition, complete assimilation of hexadecane was observed in all experiments, but its consumption profile was dependent on medium supplementation (Fig. 4B). In addition, the consumption patterns were related to biomass production (Fig. 4A). In the experiment where CSL was used at the same concentration as YNB (1.7  $\text{g}\cdot\text{L}^{-1}$ ), the total assimilation of hexadecane occurred after 50 h of cultivation, with a consumption rate lower than in the other experiments, in which CSL concentration was doubled.

The lowest value of specific growth rate, final biomass concentration, and biomass yield (Table 2) was also obtained in this condition. The increase of CSL concentration from 1.7  $\text{g}\cdot\text{L}^{-1}$  to 3.4  $\text{g}\cdot\text{L}^{-1}$  at a C/N ratio of 4 led to faster consumption of hexadecane. Moreover, a 2.2-fold improvement was obtained in final biomass concentration and biomass yield. In these experiments, yeast cells reached the stationary growth phase after 24 h, at the same time as total hexadecane assimilation. By contrast, the stationary phase of cultures supplemented with 0.5  $\text{g}\cdot\text{L}^{-1}$  ammonium sulfate was reached later (30 h) owing to the presence of hexadecane until the end of cultivation. The final biomass concentration attained in the experiment carried out with the lowest ammonium sulfate concentration and 3.4  $\text{g}\cdot\text{L}^{-1}$  CSL (C/N ratio of 40) was 34% higher than that obtained in the experiments with YNB (Fig. 3A). Some authors also demonstrated the potential of CSL as a nitrogen source in *Y. lipolytica* cultures, being a low-cost alternative to yeast extract (Cavallo et al., 2020), ammonium chloride (Liu et al., 2017) or urea (Santos et al., 2013). Moreover, CSL was already used as an efficient nitrogen source in the production of lipase (de Freitas et al., 2021;



**Fig. 4.** Time course of cellular growth (A) and hexadecane assimilation (B) obtained in batch cultures of *Y. lipolytica* CBS 2075 carried out in an STR bioreactor supplemented with ammonium sulfate  $5 \text{ g}\cdot\text{L}^{-1}$  + CSL  $1.7 \text{ g}\cdot\text{L}^{-1}$  (C/N ratio 4, ●), ammonium sulfate  $5 \text{ g}\cdot\text{L}^{-1}$  + CSL  $3.4 \text{ g}\cdot\text{L}^{-1}$  (C/N ratio 4, ◆) and ammonium sulfate  $0.5 \text{ g}\cdot\text{L}^{-1}$  + CSL  $3.4 \text{ g}\cdot\text{L}^{-1}$  (C/N ratio 40, ■). The error bars represent the standard deviation of two independent replicates.

**Table 2**

Values of biomass yield ( $Y_{x/s}$ ), specific lipid content, lipids concentration, and maximum lipase ( $\text{Lip}_{\max}$ ) and protease ( $\text{Prot}_{\max}$ ) activities obtained in *Y. lipolytica* CBS 2075 batch cultures carried out in STR bioreactor. Data are average  $\pm$  standard deviation of two independent replicates. Values followed by the same letter in each column do not present statistically significant differences (95% confidence level).

Ammonium sulfate ( $\text{g}\cdot\text{L}^{-1}$ )	CSL ( $\text{g}\cdot\text{L}^{-1}$ )	$Y_{x/s}$ ( $\text{g}\cdot\text{g}^{-1}$ )	Specific lipid content (% w/w)	Lipids ( $\text{g}\cdot\text{L}^{-1}$ )	$\text{Lip}_{\max}$ ( $\text{U}\cdot\text{L}^{-1}$ )	$\text{Prot}_{\max}$ ( $\text{U}\cdot\text{L}^{-1}$ )
5	1.7	$0.63 \pm 0.01^a$	$12 \pm 1^a$	$0.38 \pm 0.07^a$	$34.4 \pm 0.2^a$	$355 \pm 49^a$
5	3.4	$1.40 \pm 0.02^b$	$16 \pm 2^a$	$1.0 \pm 0.1^b$	$56.7 \pm 0.2^a$	$464 \pm 17^a$
0.5	3.4	$1.7 \pm 0.2^b$	$27.3 \pm 0.2^b$	$2.06 \pm 0.01^c$	$201 \pm 8^b$	$526 \pm 23^a$

$Y_{x/s}$  was expressed as mass of cells per mass of total hexadecane consumed after 48 h. Specific lipid content was expressed as mass of lipids per mass of cells.

Duarte et al., 2021), citric acid (Liu et al., 2015b), and microbial lipids (Osman et al., 2022; Souza et al., 2016) from substrates other than hexadecane. According to the literature, nitrogen is essential during cellular growth since it is required for vital metabolic processes such as amino acids and nucleotide biosynthesis, and in nitrogen-limited conditions, occur a decrease in cell proliferation (Morin et al., 2011). However, in the current study, the decrease of ammonium sulfate concentration was not limiting for *Y. lipolytica* CBS 2075 growth, which represents a cost-saving in medium components.

As occurred in the yeast cultures supplemented with YNB, synthesis of extracellular protease was also observed in experiments supplemented with CSL. Though the proteolytic activity was not affected by CSL concentration or C/N ratio (Table 2), interestingly, an increase of 42% in protease activity was attained in the experiments with the addition of ammonium sulfate  $0.5 \text{ g}\cdot\text{L}^{-1}$  and CSL  $3.4 \text{ g}\cdot\text{L}^{-1}$  comparatively to the values obtained in the cultures supplemented with YNB. Additionally, the production of extracellular lipase was also observed in experiments supplemented with CSL. Microbial production of extracellular lipase from hexadecane by bacteria species such as *Burkholderia glumae* (Boekema et al., 2007), *Alcanivorax borkumensis* (Kadri et al., 2018) and *Pseudomonas aeruginosa* (Mishra and Singh, 2012) was already reported. However, it is important to highlight that this is the first work reporting the induction of lipase by *Y. lipolytica* in hexadecane-based cultures, which could be promising since lipase has a high commercial value owing to its several industrial applications, including as a biocatalyst for

biodiesel production. The increase of the C/N ratio from 4 to 40 had a positive effect on lipase synthesis by *Y. lipolytica* CBS 2075. Lipase activity attained at C/N ratio 40 was 3.5-fold higher than that achieved at C/N ratio 4 (Table 2). Likewise, lipase synthesis by *Y. lipolytica* W29 and IMUFRJ 50862 in olive mill wastewaters was favored at a high C/N ratio (Lopes et al., 2009a). By contrast, a low C/N ratio triggered lipase synthesis by *Y. lipolytica* M53 and ACA-DC 50109 growing in waste cooking oils (Liu et al., 2017) and stearin (Papanikolaou et al., 2007), respectively. In *Y. lipolytica* W29 cultures using pork lard (Lopes et al., 2018) or waste cooking oils (Lopes et al., 2019) as substrate, the C/N ratio had no significant effect on lipase production.

According to the literature, lipids synthesis by *Y. lipolytica* strains from oils and hydrocarbons occurs by the *ex novo* pathway, in which microbial lipids are accumulated simultaneously with the production of lipid-free biomass, independently of nitrogen concentration in the culture medium (Papanikolaou and Aggelis, 2011). Though the synthesis of lipids by *Y. lipolytica* strains from hydrophobic substrates was already studied by several authors, lipids accumulation by *Y. lipolytica* from hexadecane has still not been explored. In the current study, howbeit the increase in the concentration of CSL did not improve specific lipid content, a 2.6-fold improvement in lipids concentration was attained by increasing CSL concentration from  $1.7 \text{ g}\cdot\text{L}^{-1}$  to  $3.4 \text{ g}\cdot\text{L}^{-1}$  (Table 2), as result from high biomass concentration obtained under such conditions. On the contrary, specific lipid content and lipids concentration were, respectively, 41% and 51% higher in experiments conducted at a C/N ratio of 40 compared to the higher nitrogen concentration conditions. The results herein demonstrated that hexadecane-based cultures with a simple medium formulation, composed of CSL and ammonium sulfate in low concentration, were suitable to produce *Y. lipolytica* CBS 2075 biomass enriched with lipids. Similarly, the increase of the C/N ratio in the culture medium from 25 to 100 had a positive effect on lipids accumulation by *Y. lipolytica* MTCC 9520 growing in chicken tallow (Radha et al., 2020). According to the authors, under limited nitrogen conditions, a decrease in  $\text{NAD}^+$ -isocitrate dehydrogenase activity represses the Krebs cycle, and the carbon source is channeled toward intracellular lipids synthesis. By contrast, *Y. lipolytica* W29 was able to accumulate significant amounts of lipids from pork lard independently of the C/N ratio in the culture medium (Lopes et al., 2018).

Microbial lipids accumulated by *Y. lipolytica* CBS 2075 from hexadecane were composed of palmitic, palmitoleic, oleic, and linoleic acids, regardless of medium composition (Table 3). In cultures supplemented with pentadecane or heptadecane, *Y. lipolytica* CCY 30–26–36 accumulated lipids mostly composed of palmitic, oleic, and linoleic acids (Matatkova et al., 2017). Though lipids accumulated by *Y. lipolytica* CBS 2075 in experiments carried out with  $1.7 \text{ g}\cdot\text{L}^{-1}$  or  $3.4 \text{ g}\cdot\text{L}^{-1}$  of CSL have predominantly the same fatty acids profile, a

**Table 3**

Fatty acids composition of microbial lipids produced by *Y. lipolytica* CBS 2075 from hexadecane in STR-bioreactor batch cultures. Data are average  $\pm$  standard deviation for two independent replicates. Values followed by the same letter in each column do not present statistically significant differences (95% confidence level).

Ammonium sulfate (g·L <sup>-1</sup> )	CSL (g·L <sup>-1</sup> )	Relative fatty acid content (%)					
		Palmitic	Palmitoleic	Oleic	Linoleic	TUFA (%)	TSFA (%)
5	1.7	28.5 $\pm$ 0.4 <sup>a</sup>	28 $\pm$ 1 <sup>a</sup>	15.4 $\pm$ 0.3 <sup>a</sup>	28 $\pm$ 1 <sup>a</sup>	71	29
5	3.4	17 $\pm$ 1 <sup>b</sup>	31 $\pm$ 1 <sup>ab</sup>	21.0 $\pm$ 0.4 <sup>b</sup>	31 $\pm$ 1 <sup>a</sup>	83	17
0.5	3.4	41 $\pm$ 1 <sup>c</sup>	34.0 $\pm$ 0.2 <sup>b</sup>	16.9 $\pm$ 0.3 <sup>c</sup>	7.8 $\pm$ 0.2 <sup>b</sup>	59	41

TUFA: total unsaturated fatty acids; TSFA: total saturated fatty acids.

1.4-fold improvement in the content of oleic acid was attained at the highest CSL concentration conditions. By contrast, a clear decrease (1.7-fold) in palmitic acid amount was attained by increasing CSL concentration from 1.7 g·L<sup>-1</sup> to 3.4 g·L<sup>-1</sup>. Thereby, lipids produced under these conditions were mostly composed of unsaturated fatty acids (83%). Notwithstanding, while the rise of CSL concentration from 1.7 g·L<sup>-1</sup> to 3.4 g·L<sup>-1</sup> increased the unsaturated fraction from 71% to 83%, microbial lipids produced in the experiments carried out at C/N ratio 40 has the lowest unsaturated fraction among the lipids produced in all conditions (Table 3). In experiments carried out at a C/N ratio of 40, a clear predominance of palmitic and palmitoleic acids (> 75%) and a low amount of linoleic acid (< 8%) were observed. The content of palmitic acid in this condition was 30% higher than that attained under higher nitrogen concentration conditions (C/N ratio of 4). Moreover, a 4-fold decrease in linoleic content was obtained by increasing the C/N ratio from 4 to 40. In cultures of *Rhodotorula glutinis* (Braunwald et al., 2013) and *Rhodospiridium toruloides* (Ye et al., 2021), the increase of the C/N ratio in glucose and sucrose media, respectively, also led to the accumulation of lipids rich in palmitic acid (> 20%) with a decrease in the content of linoleic acid. In this study, although lipids produced at high C/N ratio conditions had a low content of oleic and linoleic acids, high amounts of palmitoleic acid (> 34%) were accumulated, contrariwise to the fatty acids composition of microbial lipids obtained from waste cooking oils (Lopes et al., 2019) and pork lard (Lopes et al., 2018), predominantly composed of oleic, linoleic, palmitic and stearic acids. Albeit stearic acid was a common fatty acid of lipids accumulated by *Y. lipolytica* strains, even if in lower amounts (< 10%), herein lipids produced by *Y. lipolytica* CBS 2075 did not have stearic acid in their composition, regardless of medium composition.

From a commercial standpoint, these microbial lipids can be applied as a source for food and feed supplements or as feedstock for the biodiesel industry. The synthesis of palmitoleic acid, a monounsaturated fatty acid, by oleaginous yeasts has gained considerable attention due to its singularity in improving diet and human health (Kolouchová et al., 2015). Dried and killed *Y. lipolytica* biomass cultivated in wastes, such as industrial glycerol or biofuel waste, was already recognized as safe for food and feed products by the current food regulations (Jach and Malm, 2022). On the other side, lipids from *Y. lipolytica* CBS 2075 have the potential as feedstock for biodiesel production, due to their composition being similar to some vegetable oils (Folayan et al., 2019). Yet, since the fatty acids profile is one of the major parameters affecting biodiesel quality and properties, deepening studies are always needed to understand if lipids selected for biodiesel production meet the guidelines specified by international biodiesel standard requirements.

The combination of a by-product (CSL) with a low concentration of ammonium sulfate as a nitrogen source allows the formulation of a more economical hexadecane-based medium, which is of utmost interest for future bioprocesses implemented at an industrial scale. Besides biomass production, the simultaneous synthesis of enzymes (lipase and protease) and high-value lipids is an advantage for establishing an innovative and environment-friendly biotechnological approach under the scope of the biorefinery concept. This bioprocess opens new perspectives on the application of *Y. lipolytica*-based cultures for the biotransformation of pollutant compounds (hexadecane) in wastewaters into valuable metabolites, with economic (reducing costs) and environmental (reducing

environmental pollution) interest.

#### 4. Conclusions

The ability of different *Y. lipolytica* strains to efficiently grow on hexadecane-based medium was demonstrated in small-scale batch cultures. In lab-scale bioreactor experiments, dissolved oxygen concentration in the culture medium proved to be an important factor affecting *Y. lipolytica* CBS 2075 growth and microbial lipids accumulation from hexadecane. Additionally, the use of corn steep liquor (CSL) in the formulation of culture medium efficiently supported yeast growth, favored lipids biosynthesis, and concurrently induced lipase and protease production by *Y. lipolytica* CBS 2075. This demonstrated the feasibility of using CSL as an alternative low-cost source of nutrients in hexadecane-based media, which is a promising outcome for scale-up purposes. Moreover, in CSL-supplemented media, the increase in the C/N ratio boosted lipid-rich biomass production. Albeit lipids accumulated by *Y. lipolytica* CBS 2075 were composed of palmitic, palmitoleic, oleic, and linoleic acids in all media, the content of palmitic, oleic, and linoleic acids was highly dependent on the CSL concentration and C/N ratio. Hence, *Y. lipolytica* CBS 2075 biomass enriched in valuable lipids is promising as feedstock for animal feed or as human dietary supplements (owing to *Y. lipolytica* GRAS status), or for the production of lipids-derived biofuels. The possibility of using hexadecane as a carbon source by *Y. lipolytica* strains to produce lipid-rich biomass (and enzymes as a side-product) not only valorizes the waste management of hydrocarbons-polluted streams but also ensures the economic sustainability and viability of this bioprocess, fulfilling an interesting approach inserted in the circular economy concept.

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#### CRediT authorship contribution statement

**Miranda Sílvia M.:** Formal analysis, Investigation, Methodology, Writing – original draft. **Belo Isabel:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Lopes Marlene:** Supervision, Writing – original draft, Writing – review & editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.



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