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A new combined approach to improved lipid production using a strictly aerobic and oleaginous yeast

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Practical application

The oleaginous yeast *Rhodosporidium toruloides* represents a promising candidate for the successful production of microbial lipids. This microorganism could be used to produce a variety of metabolites useful for industrial biotechnology. Microbial lipids have potential applications in energy and food industry, since most of those lipids are triacylglycerol with long-chain fatty-acids.

This paper describes the conditions for the production and the factors that could improve the production of microbial lipids, focusing in aeration factors correlated with oxygen mass transfer in broth culture. The combined approach, high aeration combined with an oxygen-vector, improved the lipid accumulation and reduced the cultivation time. These two factors are key factors for the success of scale-up and profitability of a bioprocess.

Abstract

Microbial lipids have potential applications in energy, and food industry, because most of those lipids are triacylglycerol with long-chain fatty-acids that are comparable to conventional vegetable oils and can be obtained without arable land requirement. *Rhodosporidium toruloides* is a strictly aerobic strain, where oxygen plays a crucial role in growth, maintenance and metabolite production, such as lipids and carotenoids. Dissolved oxygen concentration is one of the major factors affecting yeast physiological and biochemical characteristics. In this context, different approaches have been developed to increase available oxygen by the increasing the aeration and the addition of an oxygen-vector. The grown of *R. toruloides* in 2-L mechanical stirred tank reactor equipped with 1 or 2 porous sparger and a 70 C/N ratio, revealed a lipid content of 0.47 and 0.52 g/g and a lipidic productivity of 0.16 and 0.17 g/L.day, respectively. The oxygen-vector addition, increased the lipidic productivity for 0.20 g/L.day and a lipid contend of 0.51 g of lipids/g of biomass. The combined approach, combining high aeration (AA) and 1% of n-dodecane addition (DA), produced a significant improvement in the lipid accumulation (62%, w/w), when compared with the DA (51%, w/w) and the AA (52%, w/w) approaches. The increasing of lipids accumulation and smaller culture time are key factors for the success of scale-up and profitability of a bioprocess.

1. Introduction

Single cell oil, namely microbial oil, is the lipid accumulated by oleaginous microorganisms in their cell body. Because of its important function in energy, and food industry, it has been the focus of many researches. Biodiesel production has been the subject of considerable attention from the scientific community due to the intense discussion on renewable energy, particularly with respect to the limited supply and increasing cost of conventional fossil fuels [1]. Biodiesel is a proven biofuel that can be added directly in the established infrastructure and blended legally in any percentages with petroleum diesel [2]. Currently, the majority of biodiesel produced worldwide is considered 1st generation, since its production is based on plant oils, animal fats and algal oils [3, 4]. However, microbial oils can be a considerable alternative, resulting in the production of 2nd generation biodiesel, which doesn´t compete with the food industry nor gives rise to ethical or social issues. Microbial lipids have potential applications in biodiesel production because the majority of those lipids are triacylglycerol with long-chain fatty acids that are comparable to conventional vegetable oils and can be obtained without an extensive arable land requirement [5–7].

Oleaginous microorganisms can accumulate lipids more than 20% of their dry biomass [8], for lipid accumulation, oleaginous species should be cultivated with at least one limited nutrients such nitrogen, phosphorus, sulphur, zinc, iron or magnesium and with excess carbon [9–11]. Among these cases, nitrogen deprivation is the most easy-controlling and low-cost cultivation regime [12]. Thus, when the limiting nutrient becomes exhausted the cell growth is inhibited and remaining carbon source is channelled to lipid accumulation. The accumulation capability is extremely conditioned by the carbon-to-nitrogen (C/N) ratio and other factors in particular, aeration, pH, inorganic salt, etc. [9–11, 13].

In particularly, yeast are potentially useful for lipid production compared to other microorganisms because of their rapid growth, high cell biomass, high content of lipid, no endotoxin and easily large scale growth [14]. Among them, the yeast *Rhodosporidium toruloides* has been found to be an exceptional storage lipid producer due to high cellular lipid content of over 70% and high cell density of 100 g/L [15, 16].

The major limiting factor for aerobic microorganism's growth is the oxygen supply into the broths, due to the poor solubility of oxygen in aqueous media. Oxygen limitation can be surpassed by increasing the stirrer speed and aeration rate, or by changing the reactor geometry/dimension. However, this may causes uncontrolled foam formation in the reactor, excessive power consumption and stress to the microorganism, which is neither an economical process nor effective [17–20]. An effective and effortless approach may be the addition of oxygen-vectors, which are capable of enhancing the oxygen transfer into microorganisms. Oxygen-vectors are organic solvents with high oxygen solubility, wherein its solubility is 15 to 20 times higher than in water [21–23]. The most common oxygen vectors utilized in biotechnology are perfluorocarbons [21, 24, 25] and hydrocarbons [18, 21, 26, 27].

The presence of oxygen-vectors originates another phase, the liquid organic phase, leading to the formation of new interfacial areas between the gas and liquid phases. This new phase can enhance an extensive amount of oxygen from the air bubbles. In literature, several oxygen transfer mechanisms were reported, however the most plausible mechanism assumes that the

hydrocarbons are adsorbed into the bubble surface, followed by the formation of a continuous film, and subsequent oxygen diffusion from the air bubble to microorganisms in the aqueous phase through oxygen-vector [20, 28, 29].

The *Rhodosporidium toruloides* is a strictly aerobic strain, oxygen plays a crucial role in growth, maintenance and metabolite production, such as lipids and carotenoids. Due to the high oxygen requirement, it is crucial that sufficient amounts of oxygen be present in the liquid phase. The oxygen transfer rate (OTR) is dependent upon the volumetric mass transfer coefficient, K_La, and the driving force between gas-liquid phase, C_L^*-C_L, where C_L is the dissolved oxygen concentration and C_L^* is the oxygen saturation concentration in the liquid phase at the gas-liquid interface [30, 31].

Dissolved oxygen concentration is one of the major factors affecting yeast physiological and biochemical characteristics, in this context, different approaches have been developed to increase available oxygen in the yeast culture of *R. toruloides* CECT 1499 by the increasing the aeration and the addition of an oxygen-vector. The *R. toruloides* yeast culture were grown in a batch mechanical stirred-tank reactor (STR) in a limited nitrogen medium, with a molar C/N ratio of 70.

The fatty acid compositional profile was also analysed and compared with the existing standards (European Standard EN 14214) for biodiesel production. The properties of biodiesel produced have been estimated using equations from simulations models of the properties estimated based on the fatty acid composition of vegetable oils.

2. Materials and methods

2.1 Microorganism and maintenance medium

This study used the yeast *R. toruloides* CECT 1499, from Spain Culture Collection Centre (University of Valencia). Stock cultures were maintained on solid PDA (HIMEDIA, India) agar plates at 30 °C, during 2 days and afterwards, maintained at 4 °C and subcultured twice a month.

2.2 Pre-culture conditions and culture media

R. toruloides CECT 1499 yeast cells were grown in 250 mL shake flasks containing medium YPD (20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose) on an orbital shaker (NeifoPentlab, Portugal), at 150 rpm and 30 °C for 48h. Then, cultures were initiated with 10 % (v/v) of pre-culture to a 250 mL shake flask with fresh lipid production medium (pH 5-6) contained: glucose 13 g/L, yeast extract 0.75 g/L, $(NH_4)_2SO_4$ 0.1 g/L, $MgSO_4 \cdot 7H_2O$ 1.5 g/L and KH_2PO_4 0.4 g/L. These cultures were incubated in an orbital shaker at 150 rpm and 30 °C, and used as inoculum to get an initial cell concentration of 1 x 10^7 cell/mL.

2.2 Culture conditions in aerated STR

All cultures were performed in a 2-L (1.6-L working volume) stirred tank reactor, STR (Bio Controller ADI 1030, Thermo Circulator ADI 1018, Apllikon, Holland), a jacketed vessel, equipped with a Rushton turbine (radial flow) and operated at 30 °C, 200 rpm and aerated at 0.5 and 0.75 vvm. STR was equipped with one or two microporous spargers, depending on the

assay. The STR experiments were performed with lipid production medium supplemented with antifoam B (Sigma A-5757) at 0.1 mL/L. The medium pH was adjusted to 5.0 before inoculation. The culture monitoring was followed through the BioXpert program, version 2.1. The measurement of temperature, pH and dissolved oxygen was made with specific probes, polarographic DO_2 sensor, Pt-100 sensor in thermowell in topplate and classic pH sensor respectively, and the values were constantly registered. Samples were taken for 144 h at regular intervals to determine the biomass dry weight, sugar consumption, neutral lipid accumulation and fatty acid composition, as described in ''Analytical methods''.

In the assays with oxygen-vector, n-dodecane (BDH Prolabo, France; oxygen solubility 54.9 x 10^{-3} g/L at 35 °C and atmospheric air pressure) was added to the culture medium at different volumetric fractions of 1 and 2 % (v/v).

2.3 Analytical methods

Dry cell weight (DCW) was determined by centrifuging (Hettrich Zentrifugen Universal 320, Germany) 5 mL of culture, in a pre-weighed dried tube, at 2 370 g for 20 min and dried at 60 °C to a constant weight, and weighed on a precision balance (Precisa XB 120A, Switzerland).

The glucose concentration was determined using 3,5-dinitrosalycylic acid (DNS) method [32].

Inorganic nitrogen (NH_4^+ form) was quantified using the Phenol-hypochlorite method [33].

2.4 K_La measurements

Volumetric mass transfer coefficient (K_La) was measured using the dynamic gassing-out method [30]. For the K_La determination, was utilized the polarography oxygen probe of the bioreactor. Oxygen probe calibration was realized at atmospheric pressure by setting zero and 100 % saturation under nitrogen and air sparging, respectively. The K_La determinations were performed in the medium culture before inoculation, in the middle of the cell growth, and at the final growth stage. The inlet of air flow to the culture broth was interrupted and the decrease of the dissolved oxygen concentration due to cellular respiration was observed and recorded by a polarographic oxygen probe. The K_La value was calculated by the integrated form of the equation proposed by Stanbury *et al.* [30], where the slope of the resulting straight line representing the ln ($C^* - C_L$) versus time is equal to the value of $-K_L$a.

K_La determination was performed without and with 1 and 2 % (v/v) of oxygen-vector, n-dodecane, added to the culture medium.

2.5 Lipid analysis and fatty acid composition

Lipid concentration was estimated by fluorescence spectrometry. *R. toruloides* CECT 1499 cells were stained with Nile Red according to the protocol describe by Kimura *et al.* [34], which allows monitoring neutral lipids content during the accumulation process. A calibration curve between fluorescence intensity and Triolein concentration was developed. This standard curve was performed according to Bertozzini *et al.* [35].

Fatty acid analyses were performed by high performance liquid chromatography (HPLC) using samples previously extracted from yeast cells following the modified method proposed by

Folsh *et al.* [36] and then hydrolyzed according to Hein *et al.* [37]. Fatty acid composition was analyzed in a high-performance liquid chromatography system (Hitachi LaChrom Elite HPLC, Japan) equipped with a refractive index detector (Hitachi L-2490, Japan) and an Alltima Alltech Hi-Load C18 column (Grace, USA) was used with an isocratic elution of acetonitrile: water (99:1) at 30 °C.

2.6 Determination of culture yields and kinetics parameters

The specific growth rates (h^{-1}) were calculated using the DMFIT modelling tool (http://modelling.combase.cc).

The lipid content ($Y_{L/X}$) was calculated according to the following formula:

$$Y_{L/X} = \frac{L_i - L_0}{X_i - X_0}$$

where, $X_i$and L_i are the dry cell weight and lipid concentration on day t_i, respectively and X_0 and L_0 are the dry cell weight and lipid concentration on the first day (t_0), respectively.

The lipid yield ($Y_{L/S}$) was calculated according to the following formula:

$$Y_{L/S} = \frac{L_i - L_0}{S_0 - S_i}$$

where, $S_i$and S_0 are the glucose concentration on day t_i and on the first day (t_0), respectively. L_i and L_0 are the lipid concentration on day t_i and on the first day (t_0), respectively.

The lipid productivity (P_{lipid}) was calculated by the equation:

$$P_{lipid}(g\ L^{-1}\ day^{-1}) = \frac{L_{maxi}}{t_i}$$

where, L_{maxi} is the maximum lipid concentration on day t_i.

The Biomass productivity (P_{Biom}) was calculated by the equation:

$$P_{Biom}(g\ L^{-1}\ day^{-1}) = \frac{(X_i - X_0)}{t_i}$$

where, X_0 and X_i is the biomass concentration on day t_0 and t_i, respectively.

2.7 Determination of biodiesel properties

Different physicochemical fuel properties namely Cetane Number (CN), Long Chain saturated factor B (LCSF(B)), Cold Filter Plugging Point (CFPP), Saponification Number (SN), Iodine Value (IV), Higher Heating Value (HHV) and Flash Point (FP) were determined using predictive models and mathematical equations [38-41].

Cetane Number was obtained using the multiple linear regression equation:

$$CN = 1.068\sum(CN_i \times W_i) - 6.747$$

where, CN_i represent reported CN of pure fatty acid methyl ester available in reference [38], collected from the literature and W_i is the mass fraction of each fatty acid methyl ester detected and quantified by HPLC [38].

Long Chain Saturated Factor (B) and Cold Filter Plugging Point were calculated by the following empirical equations [39]:

$$LCSF\ (B) = 0.1 \times C16(wt.\%) + 0.5 \times C18(wt.\%) + 1 \times C20(wt.\%) + 1.5 \times C22(wt.\%) + 2 \times C24(wt.\%)$$

$$CFPP = 3.1417 \times LCSF(B) - 16.477$$

where, C16, C18, C20, C22 and C24 are the composition of saturated fatty acids with a long chain.

Iodine Value and Saponification Number were calculated by the following empirical equations:

$$IV = \sum(254 \times D \times A_i/MW_i)$$

$$SN = \sum(560 \times A_i/MW_i)$$

where, A_i is the percentage, D is the number of double bonds and MW_i is the molecular mass of each fatty acid methyl ester[40].

The Higher Heating Value is related with IV and SN, for calculation of HHV has been used the following equation[40]:

$$HHV = 49.43 - ((0.015 \times IV) + (0.041 \times SN))$$

Flash Point was calculated according to the following formula [41]:

$$FP = (47.62 \times HHV) - 1802.7$$

3. Results and Discussion

3.1 Effect of oxygen availability in lipid accumulation (High Aeration Approach)

Previous studies in our laboratory, indicates that C/N ratio of 70 enhanced lipid accumulation by the yeast culture *R. toruloides* CECT 1499 [42, 43]. These assays was performed in erlenmeyer, where the maximum lipid content was 0.22 g/g (Table 1), a low value for oleaginous microorganisms [10, 44–48] . This low lipid content might had been associated with low oxygen availability in the shake flask, where the K_La is approximately 5 h^{-1} [49], since the accumulation of lipids is an aerobic process in which oxygen dissolved in the culture should be a crucial factor [50]. To better understand the effect of oxygen on growth kinetics and lipid accumulation of *R. toruloides* CECT 1499 yeast cultures, different experiments were conducted in a STR bioreactor where the K_La was suitably changed.

To improve the oxygen mass transfer in the liquid phase and to improve access to oxygen by cells, the culture aeration system was changed, through the addition of one more air sparger in the vessel. Volumetric oxygen mass transfer coefficient was determined at different operational conditions, with one and two sparger's and different aeration rates (0.5, 0.75 and

1 vvm). Figure 1 shows the highest K_La was obtained with two porous sparger's and 1 vvm (61.41 h^{-1}), wherever this K_La is not statistically different to that obtained with two porous sparger's and 0.75 vvm (60.59 h^{-1}). In energetic and economic perspective, is more advantageous operate at 0.75 vvm than at 1 vvm, energy input is smaller and less costly. Therefore, the K_La of 60.59 h^{-1} was selected and designed as high oxygen availability and the K_La of 33.93 h^{-1} was selected as control conditions in STR-2L. The K_La of 5 h^{-1}, characteristic of 250 mL Erlenmeyer, was designed as low oxygen availability [42].

Despite of the different K_La, all three cultures, with identical molar ratio C/N of 70, became nitrogen limited within 24 hours (data not shown). Thus, we believed that, the performance among the three cultures on biomass concentration and lipid content were different not due to nitrogen exhaustion but presumably to the oxygen availability. Table 1 shows the kinetic and efficiency parameters of the cultures grown with low, high and control oxygenation. When scaled-up from 250 mL Erlenmeyer (low oxygen availability) to 2L-STR (control) the lipid content increased from 22 to 47% (w/w) and the K_La increased 6.8 times. This improvement in lipid content appears to be related with oxygen availability in the growth medium. Similar results were obtained with the yeast *R. toruloides* Y4, when operating in fed-batch mode, which accumulated 68% (w/w) of lipids with a dissolved oxygen of 40-50 % saturation. While, the shake flask fed-batch culture only reached 48% (w/w), with the same yeast strain [13]. In an aerobic culture, particularly for *R. toruloides* CECT 1499 yeast cultures, several biochemical reactions including carbon consumption, lipid production, and cell proliferation required large amounts of oxygen. So, oxygen has a great importance in the *R. toruloides* CECT 1499 yeast cultures and high oxygen promotes lipogenesis and consequently lipid accumulation, as a result of the up-regulation of enzymes involved in lipid biosynthesis such as malic enzyme and ATP-citrate lyase [51–53].

In systems with forced aeration such as STR, in addition to better dispersion and accessibility of dissolved oxygen, this importance is heightened due to the possibility of controlling aeration, agitation and vessel design, relatively to the shake flask.

It was also observed that K_La almost doubled with the increase of the number of spargers, from one to two porous spargers. The porous sparger promotes the formation of microbubbles with a high retention time in the liquid phase, thus enabling a greater mass transfer of oxygen and consequently a greater accessibility of this gas to the cells. Studies by Raposo *et al.* [54] showed that this type of sparger is more efficient with high K_La values than those obtained with the L-shaped sparger. High oxygen availability culture had improvements in all parameters, particular in lipid content increase to 52% (w/w) and achieved a biomass of 2.49 g/L (Table 1).

The yeast *R. toruloides* CECT 1499 shown an increase of lipid yield ($Y_{L/S}$) and glucose consumption with the increase of K_La, high oxygenation of culture medium almost certainly leads to a metabolically more active culture, not being so limited by oxygen availability. Although, the $Y_{L/S}$ obtained are consistent with the values referenced by other authors, with a similar initial glucose concentration, these values are low relative to the theoretical maximum. Andrade *et al.* [55] obtained a low lipid yield of 0.13 g/g when used the yeast *R. toruloides* NCYC 921 in a batch bioreactor, for lipid production with an initial glucose concentration of 10

g/L. The theoretical maximum lipid yield is 0.31 g/g [8, 56], i.e., the production of 1 mole of trioleoylglycerol requires 16 mole of glucose. For *R. toruloides*, the $Y_{L/S}$ frequently documented for oleaginous microorganisms is around 0.20 g/g [10, 44, 48, 57]. In this study, the culture with high oxygen availability, 60.59 h^{-1}, has a lipid yield two times lower than the maximum theoretical lipid yield. We believed that the cell culture can still be nutritionally limited by the oxygen, not being enough for the growth and lipid metabolism. It is observed that, although, the consumption of carbon source increased with increasing the K_LA, there is no depletion of the glucose, being limited by the other factor or nutrient, no by the substrate.

These results suggested that oxygen supply was limiting, and that oxygen-transfer should be intensified to further improve lipid productivity. For this purpose, we tested the addition of an oxygen vector in culture, in order to increase the oxygen solubility in the culture and promote greater accessibility of this gas to the cells.

3.2 Effect of n-dodecane in lipid accumulation (n-Dodecane Approach)

As showed before, oxygen availability promotes a higher lipid accumulation. The K_La was increased through aeration increment, high aeration lead to high power consumptions, foam formation and could adversely affect the microbial growth. Has been reported that the K_La value can decrease, remain unaffected or increase upon addition of oxygen-vector [58]. n-dodecane was added as oxygen-vector with the intent of increase K_La and reduce power consumption. Unexpectedly, K_La slightly decreased in presence of 1 and 2% (v/v) of n-dodecane from 33.93 to 28.47 and 28.02 h^{-1}, respectively (Table 2). This decline implies longer aeration times to saturate. But, on the other hand, n-dodecane has a large affinity for oxygen, allowing to increase the amounts of oxygen transferred from the gas phase to the cell. Therefore, the OTR maximum may increase due to the enhancement of saturation concentration in the liquid phase [30, 31]. Oxygen vector can also act as surface-active agent to lower the surface tension of water and increase the gaseous specific interfacial area and thus increase the OTR [58].

The glucose consumption increased when n-dodecane was added (Table 2). At the same initial glucose concentration, the additions of 1 and 2% (v/v) of n-dodecane, resulted in glucose consumption of 81.63 and 99.46 %, respectively, while a glucose consumption of 70.33 % was achieved by the control. Glucokinase is one of the first enzyme involved in the conversion of glucose into other metabolites, this enzyme increases in the presence of n-dodecane, by the work reported by Xu et al. [53]. Biomass production of culture control also differed from the cultures with the addition of n-dodecane. Relatively to the control, the addition of the oxygen-vector, increase the biomass production in 15% and 32 % with 1% and 2% of n-dodecane, respectively. The presence of n-dodecane promotes better glucose consumption and higher biomass production even with a minor decrease in K_La. During aerobic bioprocess, the oxygen is transferred from a rising gas bubble into a liquid phase and ultimately to the site of oxidative phosphorylation inside the cell. The liquid film resistances around bubbles usually control the overall transfer rate [59], the presence of n-dodecane could lower the surface tension of the broth, reducing the liquid film resistances around air bubbles fostering the overall transfer rate and increase the OTR.

R. toruloides CECT 1499 achieved a lipid contend of 0.52 and 0.51 g/g under 2 and 1% (v/v) of n-dodecane, respectively. With no n-dodecane addition lipid content was 0.47 g/g. n-Dodecane clearly affect lipid biosynthesis and accumulation. However, how n-dodecane affect lipid accumulation remains unknown. Possibly, the yeast is metabolically more active in presence of oxygen-vector, due to a greater availability and accessibility of oxygen, not being the culture limited nutritionally. As previously shown, the addition of one more air sparger in the vessel and an aeration of 0.75 vvm has promoted a K_La of 60.59 h^{-1} and achieved a lipid content of 0.52 g/g, the same lipid content was obtained with the addition of n-dodecane. However, the K_La is around half in presence of 1 and 2% of n-dodecane. These are two different approaches, which result in the increase of lipid content.

n-Dodecane stimulated the aerobic metabolism of *R. toruloides* CECT 1499, resulting in a faster synthesis of lipids and cell growth, lipid productivity relates the quantity of lipid synthesized per time. In presence of n-dodecane lipid productivity was stimulated and reached 0.20 and 0.21 g/L.day, with 1 and 2% of n-dodecane, respectively, while in culture without addiction of n-dodecane, lipid productivity dropped to 0.16 g/L.day. Overall, there are two hypothetical possibilities for the enhancement triggered by the addition of n-dodecane. Firstly, n-dodecane increment OTR as already mentioned. The secondary possibility lays on the fact that previous studies demonstrate that n-dodecane addition increases the transcription level of several genes involved in mechanisms that supply metabolites to fatty acid biosynthesis. That genes encoded enzymes like glucokinase, citrate synthase, malate dehydrogenase and glucose-6-phosphate dehydrogenase [52, 53]. According to C. Ratledge [60] the ability of an oleaginous microorganism to accumulate large quantities of lipid lays on two key factors, the ability to produce a continuous supply of acetyl-CoA directly in the cytosol of the cell as an indispensable precursor for fatty acid biosynthesis, and, the ability to produce a sufficient supply of NADPH as the essential reductant used in fatty acid biosynthesis. Citrate synthase and malate dehydrogenase are involved in the citrate/malate cycle and in the cytosolic "transhydrogenase" cycle that provide precursors of acetyl-CoA and NADPH [60]. Glucose-6-phosphate dehydrogenase overexpression also contributes for NADPH generation, through the pentose phosphate pathway. It is worth to point out that when the fatty acid accumulation process is active, down-shift of the TCA cycle would be a rational requirement. As mentioned before, nitrogen starvation significantly inhibits isocitrate dehydrogenase [8, 61]. However, how n-dodecane affects growth kinetic and lipid production remains unclarified.

3.3 Combination of high aeration and 1% of n-dodecane in lipid accumulation

The availability of oxygen to the *R. toruloides* CECT 1499 yeast cells is extremely important, especially for lipid accumulation, which is strictly an aerobic process. Two approaches were studied, one by increasing the K_La using two air dispersers and the other by adding an oxygen vector, the n-dodecane. High values of K_La (60.59 h^{-1}), were obtained by the utilization of two porous sparger´s and 0.75 vvm (high aeration approach, AA), and the addition of n-dodecane (n-dodecane approach, DA), improved growth kinetics and lipid production of *R. toruloides* CECT 1499. We expect that the combination of these two approaches could result in a significant improvement in lipid production and growth of *R. toruloides* yeast. Thus, the effect

of combined approaches (CA) will be studied. From an economical perspective, was selected 1% of n-dodecane to perform this study, the cost of adding 1 or 2 % of n-dodecane have a marginally impact on laboratory scale, however in comparison with industrial scale the impact would be tremendous. Another important parameter was the time at which it reached the maximum lipid concentration, being achieved at 108 hours of culture with 1% of n-dodecane. For the culture with 2% (v/v) n-dodecane, were needed more 38 hours to achieve maximum lipid concentration. (data not shown).

The increase of biomass productivity occurs during the exponential phase and decreased at 30 h of culture with the drastic reduction of the nitrogen concentration in the medium. Up to 30 h the decrease of the substrate concentration is noticeable, reflected in the increase of biomass productivity (Figure 2). After the exhaustion of the nitrogen source, it was observed the increase in lipid content indicates that nutrient stress occurred. Nitrogen exhaustion leads to an increase of AMP deaminase that releases ammonium ions for compensating for nitrogen exhaustion in the cells. This compensation, leads to a decrease of AMP concentration. NAD^+-isocitrate hydrogenase (NAD-ICDH) is dependent on AMP, low levels of NAD-ICDH results in the accumulation of isocitrate and thence citrate in the mitochondrion [60]. At the end of the exponential phase the pH dropped from 5.5 to 3.27 (data not shown), probably, this parameter is related with citrate secretion to the extracellular medium due to nitrogen exhaustion. Higher lipid content is attained during the stationary phase, as it observed after the 30 h of culture and remains constant throughout the cultivation, which trigged by a down-shift of TCA cycle due to low levels of NAD-ICDH.

The combined approach had a positively effect on lipid accumulation (62%, w/w), with a clearly increase when compared with the DA (51%, w/w) and the AA (52%, w/w) approaches. Nambou *et al.* [62] found the same competence in lipid accumulation with *Y. lipolytica* DSM3286 STR culture, with a lipid content of 65% (w/w) and a glucose consumption of 15 g/L.The lipid content achieved by CA is among the highest [10, 13, 44, 45, 48, 63, 64].

Figure 2 shown that after 30 h, glucose continues to be consumed, with a decrease in biomass productivity, however it was observed an increase of the lipid accumulation. The lipid yield, glucose consumption and lipid content showed that in CA approach, the consumed glucose was actively channelled for lipid biosynthesis. In terms of lipid productivity, the CA increase from 0.17 (AA) and 0.20 g/L.day (DA) to 0.30 g/L.day (Figure 3), being these values within the range referenced by other authors [46].

The CA approach, which resulted by the combination of high aeration with the addition of 1% of n-dodecane, produced a significant improvement in the culture of *R. toruloides* CECT 1499, reflected in the increasing of lipids accumulation and in decreasing of the culture time. Key factors for the success of scale-up and profitability of a bioprocess.

3.4 Fatty acid compositional profile and estimated properties of biodiesel from the lipid products

Lipid samples from the cells of *R. toruloides* CECT 1499 grown in the CA approach were transmethylated and the resulting fatty acid methyl esters (FAMEs) were analysed by HPLC, to

verify whether it complies the existing standards (European Standard EN 14214). The fatty acid profile of *R. toruloides* CECT 1499 yeast is shown in Table 3 also is displayed profile of other authors work and the different raw materials. The predominant fatty acids in lipid extracted from *R. toruloides* yeasts, this work, were palmitic (C16:0), oleic (C18:1) and linoleic (C18:2), with over 90% of detected fatty acids. The fatty acid composition of yeast species are very similar [13, 65–67], except for *R. toruloides* NCYC 921 [68], that present a high value of stearic acid, 71.3 % (w/w). In our case, the composition of fatty acids seems to be promising for biodiesel production, since the most suitable fatty acids for biodiesel production are palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3). In particular oleic acid because of their properties as chain length, cetane number, oxidative stability and meting point [67, 69]. Other relevant specification is the percentages of polyunsaturated methyl esters with more than three double bonds and linolenic methyl ester (C18:3), must be lower than 1% and 12% (w/w), respectively.

The potential quality of biodiesel was tested according to the European Standard EN 14214. Table 3 shows physicochemical biodiesel properties determined through prediction models and mathematical equations [38–40, 70]. Cetane number is extensively used as diesel fuel quality parameter related to a measurement of how well it combusts, however should be taken into consideration that high CN is an advantage in terms of engine performance and emissions, on the other hand, will make the fuel ignite in a short distance to the injector and the cooked fuel particles inside the injector may plug the injector nozzle [71]. Most diesels for standard vehicles have a CN between 45 and 60, the minimum CN for biodiesel is 51 (European Standard EN 14214). It is possible to assume that values ranging from 51 to 60 are suitable for most diesel engines. All the FAMEs of oils produced by microorganisms (Table 3), including *R. toruloides* CECT 1499, had CN values of 51 – 61, which were higher than minimum requirement and close to the maximum for standard diesel vehicles.

The Iodine value (IV) measure the degree of unsaturation of an oil and must be lower than 120 g I_2/100g due to the fact that heating fuels with high IV tend to polymerise and form deposits on engine nozzles, piston rings and piston ring grooves [72]. The predicted IV of *R. toruloides* CECT 1499 oils meet the specification of the European Standard EN 14214.

The higher heating value (HHV) is one of the most important properties of a fuel [73]. This value represents the amount of heat released during the combustion of a specified amount of it and is a characteristic for each oil/fuel. The HHV of biodiesels produced from rape and corn had values near 40 Mj/kg, being this parameter quite similar among all the cultures present in table 3. It would be expected that there no discrepancy, considering that carbon number and carbon/hydrogen ratio in fatty acid molecules are similar. The flash point (FP) must have a minimum value of 101 °C. The FP doesn´t affect the combustion directly, but it is used in shipping and safety regulations that define flammable and combustible materials. Essentially FP is the temperature at which the fuel will start to burn when it comes to contact with fire [71]. The FP predicted in this study from *R. toruloides* cells, had a value of 132°C, which is above the required minimum, thus ensuring that presents no problem when stored in the fuel tank.

Another important norm for choosing FAMEs is the cold filter plugging point (CFPP). Biodiesel fuels typically have operability problems such as plugging of filters and fuel lines when temperatures approach -10 °C [39]. The Portuguese legislation (NP EN 14214) establishes maximum limits for CFPP, depend on the season. The CFPP maximum limit in summer, winter and spring is 0, -10 and -5 °C, respectively. *R. toruloides* CECT 1499 biodiesel (prediction) have one of the lowest CFPP (-6 °C), despite this cannot be used in winter. This parameter is the most difficult to fulfil (in winter). *R. toruloides* NCYC 921 [68] have the worst CFPP (174 °C) due to high content of stearic and lignoceric acid. However, it is possible to lower the CFPP through additions of different sources of oils and/or through the addition of cold flow improvers.

The exploration of these properties showed that fatty acid compositional profile of *R. toruloides* CECT 1499 respect the conditions of Portuguese legislation (NP EN 14214) and has great potential as raw material for biodiesel production.

4.Concluding remarks

In order to improve the lipid production on oleaginous yeast, such as *R. toruloides,* several culture factors are important. In present work are proposed combinate approach, combining an already optimized C/N ratio with high aeration in an optimized design STR bioreactor and oxygen vector addiction. The optimized conditions were, C/N ratio 70, two porous spargers at 0,75 vvm and 1% n-dodecane.

Lipid samples from CA approach were transmethylated and the resulting fatty acid methyl esters was verifying whether it complies the existing standards (European Standard EN 14214) and has great potential as raw material for biodiesel production.

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References

[1] Concas, A., Steriti, A., Pisu, M., Cao, G., Comprehensive modeling and investigation of the effect of iron on the growth rate and lipid accumulation of Chlorella vulgaris cultured in batch photobioreactors. *Bioresour. Technol.* 2014, *153*, 340–50.

[2] Röttig, A., Wenning, L., Bröker, D., Steinbüchel, A., Fatty acid alkyl esters: Perspectives for production of alternative biofuels. *Appl. Microbiol. Biotechnol.* 2010, *85*, 1713–1733.

[3] Vasudevan, P.T., Briggs, M., Biodiesel production-current state of the art and challenges. *J. Ind. Microbiol. Biotechnol.* 2008, *35*, 421–30.

[4] Kosa, M., Ragauskas, A.J., Lipids from heterotrophic microbes: advances in metabolism research. *Trends Biotechnol.* 2011, *29*, 53–61.

[5] Li, Q., Du, W., Liu, D., Perspectives of microbial oils for biodiesel production. *Appl. Microbiol. Biotechnol.* 2008, *80*, 749–56.

[6] Adamczak, M., Bornscheuer, U.T., Bednarski, W., The application of biotechnological methods for the synthesis of biodiesel. *Eur. J. Lipid Sci. Technol.* 2009, *111*, 800–813.

[7] Vicente, G., Bautista, L.F., Gutiérrez, F.J., Rodríguez, R., et al., Direct Transformation of Fungal Biomass from Submerged Cultures into Biodiesel. *Energy & Fuels* 2010, *24*, 3173–3178.

[8] Ratledge, C., Wynn, J.., The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv. Appl. Microbiol.* 2002, *51*, 1–51.

[9] Gill, C.O., Hall, M.J., Ratledge, C., Lipid accumulation in an oleaginous yeast (Candida 107) growing on glucose in single-stage continuous culture. *Appl. Environ. Microbiol.* 1977, *33*, 231–239.

[10] Wu, S., Hu, C., Jin, G., Zhao, X., et al., Phosphate-limitation mediated lipid production by Rhodosporidium toruloides. *Bioresour. Technol.* 2010, *101*, 6124–6129.

[11] Wu, S., Zhao, X., Shen, H., Wang, Q., et al., Microbial lipid production by Rhodosporidium toruloides under sulfate-limited conditions. *Bioresour. Technol.* 2011, *102*, 1803–1807.

[12] Liu, B., Benning, C., Lipid metabolism in microalgae distinguishes itself. *Curr. Opin. Biotechnol.* 2013, *24*, 300–9.

[13] Li, Y., Zhao, Z. (Kent), Bai, F., High-density cultivation of oleaginous yeast Rhodosporidium toruloides Y4 in fed-batch culture. *Enzyme Microb. Technol.* 2007, *41*, 312–317.

[14] Wang, G.-Y., Chi, Z., Song, B., Wang, Z.-P., et al., High level lipid production by a novel inulinase-producing yeast Pichia guilliermondii Pcla22. *Bioresour. Technol.* 2012, *124*, 77–82.

[15] Freitas, C., Parreira, T.M., Roseiro, J., Reis, A., et al., Selecting low-cost carbon sources for carotenoid and lipid production by the pink yeast Rhodosporidium toruloides NCYC 921 using flow cytometry. *Bioresour. Technol.* 2014, *158*, 355–9.

[16] Zhao, X., Wu, S., Hu, C., Wang, Q., et al., Lipid production from Jerusalem artichoke by Rhodosporidium toruloides Y4. *J. Ind. Microbiol. Biotechnol.* 2010, *37*, 581–5.

[17] Cascaval, D., Galaction, a.-I., Folescu, E., Turnea, M., Comparative study on the effects of n-dodecane addition on oxygen transfer in stirred bioreactors for simulated, bacterial and yeasts broths. *Biochem. Eng. J.* 2006, *31*, 56–66.

[18] Lai, L.-S.T., Tsai, T.-H., Wang, T.C., Application of oxygen vectors to Aspergillus terreus cultivation. *J. Biosci. Bioeng.* 2002, *94*, 453–9.

[19] Leung, R., Poncelet, D., Neufeld, R.J., Enhancement of oxygen transfer rate using microencapsulated silicone oils as oxygen carriers. *J. Chem. Technol. Biotechnol.* 1997, *68*, 37–46.

[20] Rols, J.L., Goma, G., Enhancement of oxygen transfer rates in fermentation using oxygen-vectors. *Biotechnol. Adv.* 1989, *7*, 1–14.

[21] Rols, J.L., Condoret, J.S., Fonade, C., Goma, G., Mechanism of enhanced oxygen transfer in fermentation using emulsified oxygen-vectors. *Biotechnol. Bioeng.* 1990, *35*, 427–35.

[22] Liu, Y.S., Wu, J.Y., Use of n-hexadecane as an oxygen vector to improve Phaffia rhodozyma growth and carotenoid production in shake-flask cultures. *J. Appl. Microbiol.* 2006, *101*, 1033–8.

[23] Battino, R., Rettich, T.R., Tominaga, T., The Solubility of Oxygen and Ozone in Liquids. *J. Phys. Chem. Ref. Data* 1983, *12*, 163.

[24] Elibol, M., Mavituna, F., Effect of perfluorodecalin as an oxygen carrier on actinorhodin production by Streptomyces coelicolor A3(2). *Appl. Microbiol. Biotechnol.* 1995, *43*, 206–210.

[25] Castro, C.I., Briceno, J.C., Perfluorocarbon-based oxygen carriers: review of products and trials. *Artif. Organs* 2010, *34*, 622–34.

[26] Zhang, J.G., Wang, X.D., Zhang, J.N., Wei, D.Z., Oxygen vectors used for S-adenosylmethionine production in recombinant Pichia pastoris with sorbitol as supplemental carbon source. *J. Biosci. Bioeng.* 2008, *105*, 335–40.

[27] Galaction, A.-I., Cascaval, D., Oniscu, C., Turnea, M., Enhancement of oxygen mass transfer in stirred bioreactors using oxygen-vectors. 1. Simulated fermentation broths. *Bioprocess Biosyst. Eng.* 2004, *26*, 231–8.

[28] Galaction, A.-I., Cascaval, D., Turnea, M., Folescu, E., Enhancement of oxygen mass transfer in stirred bioreactors using oxygen-vectors 2. Propionibacterium shermanii broths. *Bioprocess Biosyst. Eng.* 2005, *27*, 263–71.

[29] Mizerovskii, L.N., Smirnova, K.P., Solubility of noble gases and nitrogen in n-alkanes at 298.15 K and atmospheric pressure. Correlation analysis. *Russ. Chem. Bull.* 2010, *58*, 1547–1561.

[30] Stanbury, P.F., Whitaker, A., Hall, S.J., Principles of Fermentation Technology, 2nd ed, Butterworth-Heinemann, Oxford 1995.

[31] Tribe, L.A., Briens, C.L., Margaritis, A., Determination of the volumetric mass transfer coefficient (k(L)a) using the dynamic “gas out-gas in” method: Analysis of errors caused by dissolved oxygen probes. *Biotechnol. Bioeng.* 1995, *46*, 388–392.

[32] Miller, G.L., Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* 1959, *31*, 426–428.

[33] Weatherburn, M.W., Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* 1967, *39*, 971–974.

[34] Kimura, K., Yamaoka, M., Kamisaka, Y., Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence. *J. Microbiol. Methods* 2004, *56*, 331–8.

[35] Bertozzini, E., Galluzzi, L., Penna, A., Magnani, M., Application of the standard addition

method for the absolute quantification of neutral lipids in microalgae using Nile red. *J. Microbiol. Methods* 2011, *87*, 17–23.

[36] Folch, J., Lees, M., Stanley, G.H., A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 1957, *226*, 497–509.

[37] Hein, M., Isengard, H.-D., Determination of underivated fatty acids by HPLC. *Zeitschrift Für Leb. Und -Forsch. A* 1997, *204*, 420–424.

[38] Tong, D., Hu, C., Jiang, K., Li, Y., Cetane number prediction of biodiesel from the composition of the fatty acid methyl esters. *JAOCS, J. Am. Oil Chem. Soc.* 2011, *88*, 415–423.

[39] Ramos, M.J., Fernández, C.M., Casas, A., Rodríguez, L., et al., Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresour. Technol.* 2009, *100*, 261–268.

[40] Khot, M., Kamat, S., Zinjarde, S., Pant, A., et al., Single cell oil of oleaginous fungi from the tropical mangrove wetlands as a potential feedstock for biodiesel. *Microb. Cell Fact.* 2012, *11*, 71.

[41] Demirbas, A., Relationships derived from physical properties of vegetable oil and biodiesel fuels. *Fuel* 2008, *87*, 1743–1748.

[42] Teresa, A.M., Leveduras oleaginosas para a produção de biodiesel, a partir de resíduos agroindustriais. Otimização das condições de crescimento e de produção lipídica, UAlg, 2012.

[43] Val, M., A influência das condições nutricionais e operacionais na produção de lípidos, a partir da levedura Rhodosporidium toruloides, UAlg, 2013.

[44] Zhao, X., Hu, C., Wu, S., Shen, H., et al., Lipid production by Rhodosporidium toruloides Y4 using different substrate feeding strategies. *J. Ind. Microbiol. Biotechnol.* 2011, *38*, 627–32.

[45] Dias, C., Sousa, S., Caldeira, J., Reis, A., et al., New dual-stage pH control fed-batch cultivation strategy for the improvement of lipids and carotenoids production by the red yeast Rhodosporidium toruloides NCYC 921. *Bioresour. Technol.* 2015, *189*, 309–318.

[46] Carvalho, A.K.F., Rivaldi, J.D., Barbosa, J.C., de Castro, H.F., Biosynthesis, characterization and enzymatic transesterification of single cell oil of Mucor circinelloides – A sustainable pathway for biofuel production. *Bioresour. Technol.* 2015, *181*, 47–53.

[47] Li, T., Gargouri, M., Feng, J., Park, J.J., et al., Regulation of starch and lipid accumulation in a microalga Chlorella sorokiniana. *Bioresour. Technol.* 2015, *180*, 250–257.

[48] Wu, S., Zhao, X., Shen, H., Wang, Q., et al., Microbial lipid production by Rhodosporidium toruloides under sulfate-limited conditions. *Bioresour. Technol.* 2011, *102*, 1803–1807.

[49] Eibl, R., Eibl, D., Disposable Bioreactors, Springer, London 2009.

[50] Yang, X., Jin, G., Gong, Z., Shen, H., et al., Recycling biodiesel-derived glycerol by the oleaginous yeast Rhodosporidium toruloides Y4 through the two-stage lipid production process. *Biochem. Eng. J.* 2014, *91*, 86–91.

[51] Bellou, S., Makri, A., Triantaphyllidou, I.E., Papanikolaou, S., et al., Morphological and

metabolic shifts of Yarrowia lipolytica induced by alteration of the dissolved oxygen concentration in the growth environment. *Microbiology* 2014, *160*, 807–817.

[52] Zhu, P., Dong, S., Li, S., Xu, X., et al., Improvement of welan gum biosynthesis and transcriptional analysis of the genes responding to enhanced oxygen transfer by oxygen vectors in Sphingomonas sp . *Biochem. Eng. J.* 2014, *90*, 264–271.

[53] Xu, Z., Bo, F., Xia, J., Sun, Z., et al., Effects of oxygen-vectors on the synthesis of epsilon-poly-lysine and the metabolic characterization of Streptomyces albulus PD-1. *Biochem. Eng. J.* 2015, *94*, 58–64.

[54] Raposo, S., Lima-Costa, M.E., Effects of the hydrodynamic environment and oxygen mass transfer on plant cell growth and milk-clotting protease production in a stirred-tank reactor. *Eng. Life Sci.* 2012, *12*, 441–449.

[55] Andrade, R., Leal, R., Roseiro, J., Reis, A., et al., Monitoring Rhodosporidium toruloides NCYC 921 batch fermentations growing under carbon and nitrogen limitation by flow cytometry. *World J. Microbiol. Biotechnol.* 2012, *28*, 1175–84.

[56] Papanikolaou, S., Aggelis, G., Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. *Eur. J. Lipid Sci. Technol.* 2011, *113*, 1031–1051.

[57] Wiebe, M.G., Koivuranta, K., Penttilä, M., Ruohonen, L., Lipid production in batch and fed-batch cultures of Rhodosporidium toruloides from 5 and 6 carbon carbohydrates. *BMC Biotechnol.* 2012, *12*, 26.

[58] Dumont, E., Delmas, H., Mass transfer enhancement of gas absorption in oil-in-water systems: a review. *Chem. Eng. Process. Process Intensif.* 2002, *42*, 419–438.

[59] Garcia-Ochoa, F., Gomez, E., Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. *Biotechnol. Adv.* 2009, *27*, 153–76.

[60] Ratledge, C., Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. *Biochimie* 2004, *86*, 807–15.

[61] Kolkman, A., Comparative Proteome Analysis of Saccharomyces cerevisiae Grown in Chemostat Cultures Limited for Glucose or Ethanol. *Mol. Cell. Proteomics* 2004, *4*, 1–11.

[62] Nambou, K., Zhao, C., Wei, L., Chen, J., et al., Designing of a “cheap to run” fermentation platform for an enhanced production of single cell oil from Yarrowia lipolytica DSM3286 as a potential feedstock for biodiesel. *Bioresour. Technol.* 2014, *173*, 324–333.

[63] Li, M., Liu, G.-L., Chi, Z., Chi, Z.-M., Single cell oil production from hydrolysate of cassava starch by marine-derived yeast Rhodotorula mucilaginosa TJY15a. *Biomass and Bioenergy* 2010, *34*, 101–107.

[64] Pruvost, J., Van Vooren, G., Le Gouic, B., Couzinet-Mossion, a, et al., Systematic investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel application. *Bioresour. Technol.* 2011, *102*, 150–8.

[65] Yen, H.-W., Chen, P.-W., Chen, L.-J., The synergistic effects for the co-cultivation of oleaginous yeast-Rhodotorula glutinis and microalgae-Scenedesmus obliquus on the biomass and total lipids accumulation. *Bioresour. Technol.* 2015, *184*, 148–152.

[66] Wang, C.L., Li, Y., Xin, F.H., Liu, Y.Y., et al., Evaluation of single cell oil from Aureobasidium pullulans var. melanogenum P10 isolated from mangrove ecosystems for biodiesel production. *Process Biochem.* 2014, *49*, 725–731.

[67] Sitepu, I.R., Sestric, R., Ignatia, L., Levin, D., et al., Manipulation of culture conditions alters lipid content and fatty acid profiles of a wide variety of known and new oleaginous yeast species. *Bioresour. Technol.* 2013, *144*, 360–369.

[68] Freitas, C., Nobre, B., Gouveia, L., Roseiro, J., et al., New at-line flow cytometric protocols for determining carotenoid content and cell viability during Rhodosporidium toruloides NCYC 921 batch growth. *Process Biochem.* 2014, *49*, 554–562.

[69] Tanimura, A., Takashima, M., Sugita, T., Endoh, R., et al., Cryptococcus terricola is a promising oleaginous yeast for biodiesel production from starch through consolidated bioprocessing. *Sci. Rep.* 2014, *4*, 1–6.

[70] Bonturi, N., Matsakas, L., Nilsson, R., Christakopoulos, P., et al., Single Cell Oil Producing Yeasts Lipomyces starkeyi and Rhodosporidium toruloides: Selection of Extraction Strategies and Biodiesel Property Prediction. *Energies* 2015, *8*, 5040–5052.

[71] Wan Ghazali, W.N.M., Mamat, R., Masjuki, H.H., Najafi, G., Effects of biodiesel from different feedstocks on engine performance and emissions: A review. *Renew. Sustain. Energy Rev.* 2015, *51*, 585–602.

[72] Schober, S., Mittelbach, M., Iodine value and biodiesel: Is limitation still appropriate? *Lipid Technol.* 2007, *19*, 281–284.

[73] Demirbaş, A., Biodiesel fuels from vegetable oils via catalytic and non-catalytic supercritical alcohol transesterifications and other methods: a survey. *Energy Convers. Manag.* 2003, *44*, 2093–2109.

Table 1: Kinetics parameters on lipid production from different values of K_La by *R. toruloides* CECT 1499, grown with a molar ratio C/N of 70. Batch experiments were carried out in a 2L STR at 30 °C, 0.75 vvm and 200 rpm, equipped with a porous sparger, for 146 h. Shake flask culture was carried out on an orbital shaker at 150 rpm and 30 °C for 146 h.

Culture mode/Spargers	K_La (h^{-1})	Lipid content (g/g)	$Y_{L/S}$ (g/g)	$Gluc_{cons}$ (%)	$Prod_{Lip}$ (g/L.day)
Shake Flask	5^{c}	0.22 ± 0.02^{b}	0.03 ± 0.01^{c}	63.93	0.07 ± 0.01^{b}
Batch/one	33.93 ± 0.71^{b}	0.47 ± 0.04^{a}	0.11 ± 0.01^{b}	70.33	0.16 ± 0.04^{a}
Batch/two	60.59 ± 0.31^{a}	0.52 ± 0.03^{a}	0.14 ± 0.01^{a}	72.33	0.17 ± 0.00^{a}

K_La – Volumetric mass transfer coefficient; $Y_{L/S}$ – Lipid yield ; $Gluc_{cons}$ - Glucose Consumption; $Prod_{Lip}$ – Lipid Productivity. Values are expressed as means ± standard deviation, n=3. Different letters indicate statistically significant differences according to Student-Newman-Keuls ($p < 0.05$).

Table 2: Kinetics parameters on lipid production from different additions of n-dodecane by *R. toruloides* CECT 1499 in a 2-L stirred tank reactor at 30 °C, 200 rpm and aerated 0.50 vvm with one porous sparger's for 146 h.

n-Dodecane % (v/v)	K_La (h^{-1})	Lipid content (g/g)	$Y_{L/S}$ (g/g)	$Gluc_{cons}$ (%)	$Prod_{Lip}$ (g/L.day)
0	33.93±0.71[a]	0.47±0.02[a]	0.11±0.01[a]	70.33	0.16±0.04[a]
1	28.47±0.49[b]	0.51±0.03[a]	0.09±0.00[a]	81.63	0.20±0.01[a]
2	28.02±1.25[b]	0.52±0.06[a]	0.09±0.01[a]	99.46	0.21±0.02[a]

K_La – Volumetric mass transfer coefficient; $Y_{L/S}$ – Lipid yield; $Gluc_{cons}$ - Glucose Consumption; $Prod_{Lip}$ – Lipid Productivity. Values are expressed as means ± standard deviation, n=3. Different letters indicate statistically significant differences according to Student-Newman-Keuls ($p < 0.05$).

Table 3: Fatty acid compositions (wt. %) and properties of biodiesel from lipid extracts. *R. toruloides* CECT 1499 (this study), *R. toruloides* Y4 [13], *R. toruloides* NCYC 921 [68], *R. glutinis*/*S. obliquus* [65], *A. pullulans* [66] and *M. circinelloides* URM 4182 [46].

Property	This Study	[13]	[68]	[65]	[66]	[46]	Rape [39]	Corn [39]
Fatty acid (wt.%)								
C14:0	1.1	1.3	0.1	0.4	0.0	2.1	0.0	0.0
C16:0	21.1	20	6.8	12.9	26.7	22.2	4.9	6.5
C16:1	0.0	0.6	0.0	0.5	1.7	1.0	0.0	0.6
C18:0	2.7	14.6	71.1	2.8	6.1	7.7	1.6	1.4
C18:1	45.5	46.9	6.6	71.3	44.5	39.6	33.0	65.6
C18:2	25.0	13.1	2.7	10.8	21.0	9.7	20.4	25.2
C18:3	0.6	3.5	0.5	1.3	0.0	10.8	7.9	0.1
C20:0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.1
C20:1	0.0	0.0	0.0	0.0	0.0	0.7	9.3	0.1
C22:0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0
C22:1	0.0	0.0	0.0	0.0	0.0	0.0	23.0	0.1
C24:0	0.0	0.0	12.2	0.0	0.0	1.7	0.0	0.1
C24:1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Other's	4.6	0.0	0.0	0.0	0.0	3.6	0.0	0.0
Saturated	24.9	35.9	90.2	16.1	32.8	38.0	6.5	8.0
Monounsaturated	45.5	47.5	6.6	71.8	46.2	41,5	65.3	66.4
Polyunsaturated (2,3)	25.0	16.6	3.2	12.1	21.0	20.5	28.3	25.3
SN (mg KOH/g)	184.7	193.1	184.1	191.6	193.9	196.1	182.0	189.9
IV (g I_2/100g)	82.1	72.4	11.4	83.5	75.8	80.5	109.0	101.0
HHV (Mj/kg)	40.6	40.4	41.7	40.3	40.3	40.2	40.3	40.1
FP (°C)	132	122	184	117	118	111	170	170
CN	51	57	61	54	56	52	55	53
CFPP (°C)	-6	13	174	-8	1	17	10	-12

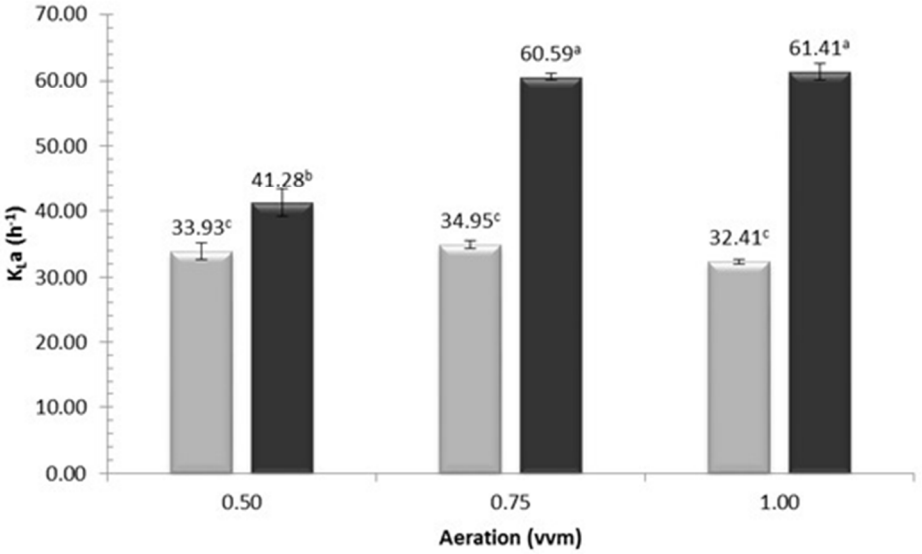
SN – Saponification Number; IV – Iodine value; HHV – higher heating value; FP – flash point; CN – Cetane number; CFPP - filter plugging point

Figure legends

Figure 1: Variation of K_La for different aeration rates determined in a STR-2L at 200 rpm and 30 °C, equipped with one (grey) and two porous sparger's (black). Values are expressed as means ± standard deviation, n=3. Different letters indicate statistically significant differences according to Student-Newman-Keuls ($p < 0.05$).

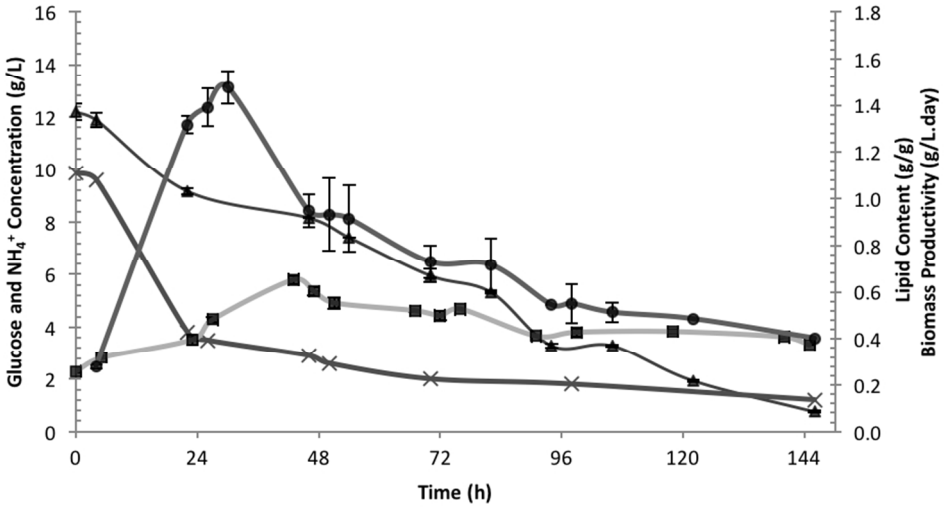
Figure 2: Substrate and nitrogen sources consumption, biomass productivity and lipid accumulation profile for *R. toruloides* CECT 1499 in a 2-L stirred tank reactor at 30 °C, 200 rpm, 1% of n-dodecane and aerated 0.75 vvm with two porous sparger´s. Biomass productivity (filled circles), glucose concentration (filled triangles), lipid content (filled squares) and 100 x NH_4^+ concentration (crosses). Values are expressed as means ± standard deviation, n=3. Different letters indicate statistically significant differences according to Student-Newman-Keuls ($p < 0.05$).

Figure 3: The comparison of lipid/DCW yield, lipid productivity, lipid/substrate yield in n-dodecane approach, DA (black bars), high aeration approach, AA (grey bars) and combined approaches, CA (black stripes bars). Values are expressed as means ± standard deviation, n=3. Different letters indicate statistically significant differences according to Student-Newman-Keuls ($p < 0.05$).

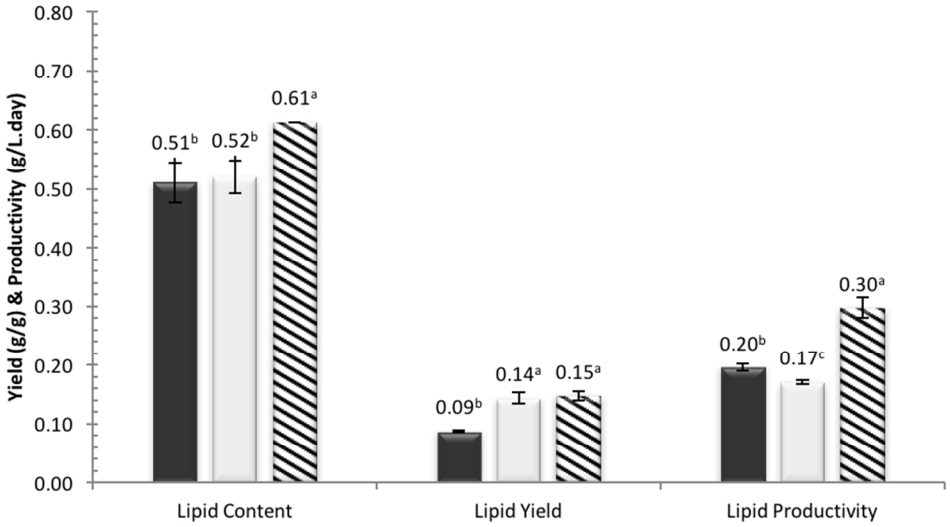


Variation of KLa for different aeration rates determined in a STR-2L at 200 rpm and 30 °C, equipped with one (grey) and two porous sparger's (black). Values are expressed as means ± standard deviation, n=3. Different letters indicate statistically significant differences according to Student-Newman-Keuls ($p < 0.05$).

155x92mm (96 x 96 DPI)



Substrate and nitrogen sources consumption, biomass productivity and lipid accumulation profile for R. toruloides CECT 1499 in a 2-L stirred tank reactor at 30 °C, 200 rpm, 1% of n-dodecane and aerated 0.75 vvm with two porous sparger´s. Biomass productivity (filled circles), glucose concentration (filled triangles), lipid content (filled squares) and 100 x NH4+ concentration (crosses). Values are expressed as means ± standard deviation, n=3. Different letters indicate statistically significant differences according to Student-Newman-Keuls ($p < 0.05$).



The comparison of lipid/DCW yield, lipid productivity, lipid/substrate yield in n-dodecane approach, DA (black bars), high aeration approach, AA (grey bars) and combined approaches, CA (black stripes bars). Values are expressed as means ± standard deviation, n=3. Different letters indicate statistically significant differences according to Student-Newman-Keuls ($p < 0.05$).