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Oxygen mass transfer impact on citric acid production by *Yarrowia lipolytica* from crude glycerol

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ABSTRACT

Production of citric acid from crude glycerol from biodiesel industry, in batch cultures of *Yarrowia lipolytica* W29 was performed in a lab-scale stirred tank bioreactor in order to assess the effect of oxygen mass transfer rate in this bioprocess.

An empirical correlation was proposed to describe oxygen volumetric mass transfer coefficient (k_La) as a function of operating conditions (stirring speed and specific air flow rate) and cellular density. k_La increased according with a power function with specific power input and superficial gas velocity, and slightly decreased with cellular density.

The increase of initial k_La from 7 h^{-1} to 55 h^{-1} led to 7.8-fold increase of citric acid final concentration. Experiments were also performed at controlled dissolved oxygen (DO) and citric acid concentration increased with DO up to 60% of saturation. Thus, due to the simpler operation setting an optimal k_La than at controlled DO, it can be concluded that k_La is an adequate parameter for the optimization of citric acid production from crude glycerol by *Y. lipolytica* and to be considered in bioprocess scale-up. Our empirical correlation, considering the operating conditions and cellular density, will be a valid tool for this purpose.

1. Introduction

Yarrowia lipolytica is one of the most extensively studied "non-conventional" yeast, and is capable of using a wide range of carbon sources, such as sugars, alcohols, organic acids and hydrophobic compounds, like lipids and hydrocarbons [1–4], and convert them into several value-added products, such as lactones, enzymes and organic acids [5–8]. Moreover, this species has been proved to be very efficient using carbon sources from agro-industrial wastes [9–11].

Citric acid is traditionally produced by *Aspergillus niger* from molasse, a by-product of sugar industry (cane and beet), although other agro-industrial wastes have been successfully studied as alternative low-cost carbon source, such as apple pomace [12], corn cobs [13], carob pod [14], kiwi fruit peel [15], pineapple waste [16], orange waste [17], banana extract [18] and jackfruit wastes [19]. The production of citric acid by *Y. lipolytica* from low-cost carbon sources has also been reported, namely from glucose hydrol [20], pineapple waste [21], olive mill wastewater [22], cooking oil [23] and crude glycerol [24].

Biodiesel, a renewable energy, is an important alternative to the fossil fuels and its production increased considerably in the past few years. One problem associated with biodiesel industry is still the production costs and the accumulation into the market of high quantities of crude glycerol [25]. The purification of biodiesel-derived crude glycerol is not economically viable for the biodiesel plants. Therefore, the development of high-value compounds production from crude glycerol is critically important for the long-term sustainability of biodiesel industries. Some authors have reported the use of crude glycerol in microbial processes for production of, for example, 1,3-propanediol [26], poly(3-hydroxybutyrate) (PHB) [27], erythritol, mannitol [28] and citric acid [24]. In fact, under specific growth conditions, crude glycerol can be used by *Y. lipolytica* to produce citric acid [29].

Y. lipolytica is strictly aerobic and some studies have already showed the effect of oxygen mass transfer rate on yeast metabolism and products formation. The raise of oxygen transfer from gas phase to the culture medium resulted in an increase of cellular growth [30], lipase production [31] and γ-decalactone secretion [32].

Oxygen availability is a key parameter in *Y. lipolytica* cultivation, substrate uptake rate and citric acid accumulation [33]. Moreover, dissolved oxygen concentration in the medium could directly influence the amount and type of organic compounds produced by the yeast [34–37]. Workman et al. [33] demonstrated that in *Y. lipolytica*

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Nomenclature

List of abbreviations

a	Specific Interfacial area (m^{-1})
c	Constant dependent on the impeller
C	Dissolved oxygen concentration in the liquid ($mg\,L^{-1}$)
C_i	Dissolved oxygen concentration in the beginning ($mg\,L^{-1}$)
C_0	Dissolved oxygen concentration when aeration is restarted ($mg\,L^{-1}$)
C^*	Solubility of oxygen in the liquid ($mg\,L^{-1}$)
D_i	Impeller diameter (m)
DO	Dissolved oxygen concentration
F_g	Volumetric gas flow rate ($m^3\,s^{-1}$)
ICA/CA	Isocitric/citric acids ratio ($g\,g^{-1}$)
k_L	Liquid side mass transfer coefficient ($m^3\,s^{-1}$)
k_La	Oxygen volumetric mass transfer coefficient (h^{-1})
K_T	Constant dependent on the impeller
N	Stirring speed (rps)
N_p	Power number
OUR	Oxygen uptake rate ($mg\,g^{-1}\,h^{-1}$)
OTR	Oxygen mass transfer rate ($mg\,L^{-1}\,h^{-1}$)
p_g	Power input to the aerated system (W)
P_{max}	Maximum productivity ($g\,L^{-1}\,h^{-1}$)
q_S	Specific substrate consumption rate ($g\,g^{-1}\,h^{-1}$)
Re	Reynolds number
rpm	Rotation per minute
STR	Stirred tank reactor
t	Time (h)
t_0	Time when aeration is restarted (h)
V	Bioreactor working volume (m^3)
ν	Liquid viscosity ($kg\,m^{-1}\,s^{-1}$)
ν_s	Superficial gas velocity ($m\,s^{-1}$)
vvm	Volume of air per volume of medium per minute
X	Cellular concentration ($g\,L^{-1}$)
YPDA	Yeast extract, peptone, dextrose, agar medium
$Y_{CA/S}$	Citric acid yield per substrate mass consumed ($g\,g^{-1}$)
$Y_{X/S}$	Cell mass yield per substrate mass consumed ($g\,g^{-1}$)

Greek letters

α, β, δ	Non-linear models parameters to be fitted
ρ	Liquid density ($kg\,m^{-3}$)
μ	Maximum specific growth rate (h^{-1})
τ	Probe response time (s)

cultures mannitol and arabitol were produced from glycerol when oxygen limitation occurred. Additionally, it was reported that the increase of dissolved oxygen in glycerol media led to an improvement of citric acid production and to a reduction of isocitric/citric acid ratio [36,37], but no reference of oxygen mass transfer rate was reported.

Oxygen is a key substrate in any aerobic bioprocess since it is an important nutrient for microbial growth, maintenance and metabolites production. Thus, a continuous supply of oxygen to the culture broth is needed due to its low solubility in aqueous medium [38]. It is very important to know and, if possible, to predict the oxygen mass transfer rate (OTR) and volumetric oxygen mass transfer coefficient (k_La) for different operating conditions to ensure sufficient oxygen transfer from the gas phase to the culture medium. OTR can be affected by several factors, such as geometrical characteristics of the bioreactor, operating conditions, physical properties of gas and liquid phases and by the presence of cells [38,39]. OTR and k_La can be related by:

$$OTR = k_La(C^* - C) \quad (1)$$

where k_La is the mathematical product of mass transfer coefficient (k_L) and specific interfacial area (a), C^* is the solubility of oxygen and C is the dissolved oxygen concentration in the liquid phase.

Although several works regarding the production of citric acid from crude glycerol have been published, data on oxygen volumetric mass transfer rate and dissolved oxygen concentration required to support the production in this medium is still limited. Moreover, there is a lack of correlations to predict k_La in production medium and the effect of active cells on oxygen mass transfer. The correct prediction of k_La is a crucial step to achieve an optimum design operation and scale-up of bioreactors. Thus, experimental values of k_La were obtained in a lab-scale stirred tank bioreactor (STR), by varying the stirring speed and the specific air flow rate, and its effect on citric acid production was evaluated. Data fitting to an empirical correlation for the prediction of k_La as a function of superficial gas velocity and specific power input of the aerated bioreactor, based on Eq. (2), was attempted with a correction in order to predict the effect of cells in k_La.

Finally, the behavior of yeast growth, substrate consumption and citric acid production under constant dissolved oxygen concentration in the medium were analyzed in order to find which intrinsic parameter of oxygenation is more important for citric acid production, initial k_La or controlled dissolved oxygen (DO). Thus, several batch experiments were performed at 20%, 40% and 60% of dissolved oxygen saturation.

2. Materials and methods

2.1. Strain and medium

Y. lipolytica W29 (ATCC 20460) was maintained in YPDA medium (glucose 20 g L^{-1}, peptone 20 g L^{-1}, yeast extract 10 g L^{-1} and agar 20 g L^{-1}) at 4 °C for a maximum of two weeks.

2.2. Bioreactor assay

Yeast cells were pre-grown for 18 h in 500 mL Erlenmeyer flask filled with 200 mL of pure glycerol 20 g L^{-1}, peptone 20 g L^{-1} and yeast extract 10 g L^{-1} medium, at 27 °C and at 200 rpm.

Cells were centrifuged and resuspended in the production medium composed by (g L^{-1}): crude glycerol 50; yeast extract 0.5; $MgSO_4 \cdot H_20$ 1.5; KH_2PO_4 6; Na_2HPO_4 0.5; $CaCl_2$ 0.75; $FeCl_3 \cdot 6H_2O$ 0.75; $ZnSO_4 \cdot 7H_2O$ 0.1; $MnSO_4 \cdot H_20$ 0.3. Crude glycerol was provided by Prio Energy–Prio Biocombustíveis, SA and has the following composition (w/w): 90.4% glycerol, 9% water, 4.9% NaCl and less than 0.001% methanol and 0.5% of organic matter (non-glycerol).

Batch assays were carried out in a 3.7 L bioreactor (RALF PLUS SOLO, Bioengineering, Switzerland) with 31 cm height and 17 cm diameter, and with Rushton impeller, 6-blade, 6 cm outside diameter. The medium pH was maintain at 5 by addition of potassium hydroxide (2 M) or orthophosphoric acid 21% (v/v), through Peripex peristaltic pumps (Bioengineering, Switzerland). Dissolved oxygen concentration was measured with a polarographic-membrane probe (InPro 6000, Mettler Toledo, USA) using the BioScadaLab software.

The bioreactor, filled with 1.7 L of production medium, was inoculated at an initial cell density of 0.5 g L^{-1} *Y. lipolytica* cells and the assays were performed at 27 °C.

In order to evaluate the effect of initial k_La on citric acid production, several experiments were carried out varying the specific air flow rate from 1 vvm to 3 vvm and changing the stirring speed from 200 rpm to 600 rpm.

Additionally, several assays with constant dissolved oxygen (20%, 40% and 60%) were performed. The DO concentration in the culture medium was controlled by manipulating the stirring speed and specific air flow rate, through a cascade control mode. In the cascade mode, the stirring speed and specific air flow rate automatically varied between the values studied in k_La modeling (200–600 rpm of stirring speed and 1–3 vvm of specific air flow rate).

Each experiment was replicated twice to ensure the repeatability and the reproducibility of the results.

2.3. k_La calculation

Numerous empirical correlations have been proposed to calculate k_La, depending on the bioreactor configuration [38]. For a stirred tank bioreactor (STR) the most common function is given by Eq. (2) [40]:

$$k_La = \alpha\left(\frac{P_g}{V}\right)^{\beta} v_s^{\gamma} \quad (2)$$

where P_g is the power input to the aerated system, V is the working volume, v_s represents the superficial gas velocity and α, β and γ are non-linear models parameters to be fitted.

In order to estimate the power input to the aerated system (P_g), the Reynolds number (Re) is determined by Eq. (3) and the power number (N_p) by Eq. (4):

$$Re = \frac{D_i^2 N\rho}{\nu} \quad (3)$$

$$N_p = \frac{P_g}{\rho N^3 D_i^5} \quad (4)$$

where D_i represents the impeller diameter, N the stirring speed, ρ the liquid density and ν the liquid viscosity. As the amount of glycerol added to the medium was small (73 mL of glycerol in 1700 mL of medium, it was diluted 23-fold) and no other component of the medium or product would affect the viscosity of the medium, it was assumed the viscosity of water. Since the cell density was low and the metabolites produced did not significantly altered the viscosity, in k_La determination in the fermentation medium with cells it was also assumed the viscosity of water.

If the Reynolds number is between 19,070 and 38,141 the flow regime inside the system is considered turbulent and N_p is not a function of Re [41]. Thus, P_g without aeration (P'_g) can be calculated by Eq. (5):

$$P'_g = K_T D_i^5 N^3 \rho \quad (5)$$

where K_T represents a constant dependent on the impeller.

Finally, P_g in the aeration system is determined by Eq. (6):

$$P_g = c\left(\frac{P'_g N D_i^3}{F_g^{0.56}}\right)^{0.45} \quad (6)$$

where c represents a constant that depends on the impeller used and F_g is the volumetric gas flow rate.

2.3.1. Static gassing-out technique

For experimental k_La determination in blank assays (without cells), the static gassing-out technique was used. This method allows evaluating the effect of operational parameters, such as stirring speed and specific air flow rate, in the oxygen transfer efficiency [42]. After a preliminary gassing-out with compressed nitrogen to remove the oxygen in the medium, the aeration was switched on at specific conditions of specific air flow rate and stirring speed until saturation.

The technique is based in the oxygen mass balance equation (Eq. (7)) which, in the absence of cells and in batch mode, is simplified to the equality between the time variation of the dissolved oxygen concentration $\left(dC/dt\right)$ and the oxygen transfer rate from the gas to the liquid.

$$\frac{dC}{dt} = k_La(C^* - C) \quad (7)$$

Integrating this equation, the value of k_La was obtained, which is equal to the symmetrical slope of the plot of $\ln(C^* - C)$ *vs.* time [43].

The probe response time (τ) was estimated according to [44], and a value of 7 s was obtained. k_La values were corrected according to Eq. (8)

$$\frac{1}{k_La'} = \frac{1}{k_La} + \tau \quad (8)$$

where k_La' is the oxygen volumetric mass transfer coefficient determined experimentally.

2.3.2. Dynamic gassing-out technique

During citric acid production by *Y. lipolytica* cells, k_La was determined using the dynamic gassing-out technique. The method is based on following the dissolved oxygen concentration in cultivation medium during a short interruption of the aeration [45]. In the presence of active cells and in the absence of aeration, the respiratory activity of yeast cells leads to the removal of oxygen of the liquid medium.

The procedure involves two steps: one to stop aeration and another to restart aeration at the operating conditions. Thus, in the first step, monitoring the decrease of dissolved oxygen concentration will allow to determine the specific oxygen uptake rate (OUR):

$$\frac{dC}{dt} = -\text{OUR} \quad (9)$$

Aeration is restarted before reaching the critical dissolved oxygen concentration value [44]. After the resumption of aeration, the oxygen mass balance in the liquid phase is expressed by Eq. (10):

$$\frac{dC}{dt} = k_La(C^* - C) - \text{OUR} \quad (10)$$

2.4. k_La modeling

To take into account the effect of cellular concentration, X, on k_La, a correction of Eq. 2 was made (Eq. 11).

$$k_La = \alpha\left(\frac{P_g}{V}\right)^{\beta} v_s^{\gamma}(1+X)^{\delta} \quad (11)$$

The power input to the aerated system (P_g) and the superficial gas velocity (v_s) were calculated using the equations presented in the Section 2, converting the specific air flow rate to real volumetric gas flow rate (F_g). According to Michel and Miller [46], the parameters of these empirical equations depend of system geometry and are only valid for superficial gas velocity between 0.042 m s^{-1} and 0.180 m s^{-1} and stirring speed between 180 rpm and 960 rpm, that cover the conditions used in this work.

For k_La modeling, the data fitting to Eq. (11) was performed by least-squares non-linear regression using the Solver tool of Microsoft Excel 2010 software.

2.5. Analytical methods

Samples were collected at appropriate intervals for measurement of cell, glycerol and citric acid concentration. For quantification of cell concentration, optical density of cultures was

measured at 600 nm and converted to dry cell mass per liter by a calibration curve. Glycerol concentration was quantified by high-performance liquid chromatography (HPLC) using a Metacarb 87H column (300 mm × 7.7 mm) coupled to a RI detector. The eluent was H_2SO_4 5 mM at 0.5 mL min^{-1} and the column temperature was 60 °C, maintained with a column thermostat. Citric acid concentration was measured by HPLC using aYMC ODS-Aq (250 × 4.6 mm) reverse phase column coupled to an UV detector at 214 nm. The mobile phase was KH_2PO_4 20 mM, pH 2.8 at room temperature and a flow rate of 0.7 mL min^{-1}.

3. Results and discussion

3.1. k_La modeling in STR bioreactor

To evaluate the effect of specific power input in the aerated system, the superficial gas velocity and cell density on k_La values, several experiments were carried out in a 3.7-L stirred tank bioreactor, by changing simultaneously stirring speed and specific air flow rate. The experimental results of k_La obtained in the different experimental conditions are presented in Table 1. As expected for a STR bioreactor, the increment of stirring speed and specific air flow rate led to an enhancement of k_La value. A 18-fold improvement in k_La values were obtained by increasing the specific air flow rate from 1 vvm to 3 vvm and the stirring speed from 200 rpm to 600 rpm. The k_La experimental value for the assay at 200 rpm of stirring speed and 1 vvm of specific air flow rate with cells was not possible to calculate, since a total depletion of oxygen through time of production process was recorded.

Table 1
Experimental k_La values under different experimental conditions. Data are presented as the average and standard deviation of two independent experiments.

Experimental conditions		k_La (h^{-1})	
Specific air flow rate (vvm)	Stirring speed (rpm)	Without cells	With cells
1	200	7 ± 1	–
1.5	300	30 ± 1	18 ± 2
2	400	55 ± 3	48 ± 3
2.5	500	84 ± 3	81 ± 1
3	600	125 ± 15	89 ± 3

Several parameters, from fluid properties to bioreactor geometry, can influence the k_La estimation in the system. The determination of k_La is a crucial step in bioreactor design, allowing to quantify the effect of the operating variables on the provision of oxygen and to establish the aeration capacity of the bioreactor system. Using the experimental data obtained in the assays with increased stirring speed and specific air flow rate (Table 1), in citric acid medium with and without yeast cells, the values of α, β, γ and δ parameters from Eq. (11) were estimated as indicated in Eq. (12):

$$k_La = 86\left(\frac{P_g}{V}\right)^{0.51} v_s^{0.46}(1+X)^{-0.12} \quad (12)$$

For the empirical equation, P_g/V values ranged from 7.9 W m^{-3} to 191.0 W m^{-3}, v_s from 2.3×10^{-3} m s^{-1} to 6.9×10^{-3} m s^{-1} and X from 0 g L^{-1} to 6.23 g L^{-1}. For aqueous systems, a wide range for the parameters values were proposed in the literature, in which exponent of P_g/V varied between 0.3 and 0.8 and exponent of v_s varied from 0.4 to 1 [38,47]. These variations resulted from differences in physicochemical properties (ionic strength, viscosity and surface tension) of the culture broths used by the authors. The results obtained in the present work, in a citric acid production medium, show that the k_La dependence is slightly higher on the specific power input than on the superficial gas velocity, once the exponent of v_s is lower than the exponent of $\left(P_g/V\right)$. In previous work [32], in the same bioreactor but with a biphasic culture medium (oil-in-water emulsion), a same influence of the power input (β of 0.5) was obtained, but a lower influence of superficial gas velocity (α of 0.2) was found.

The presence of cells in the system resulted in a slight negative effect on k_La. Also other authors reported a decrease in the k_La values with the increase in cell concentration [48,49]. This effect may be explained by the effect of cells as solid particles that may block the transfer of oxygen from air bubbles to the liquid phase [38].

In Fig. 1, predicted *vs.* experimental k_La results are represented with a line slope close to 1, which indicates a good approximation between real k_La values and the values calculated by the correlation.

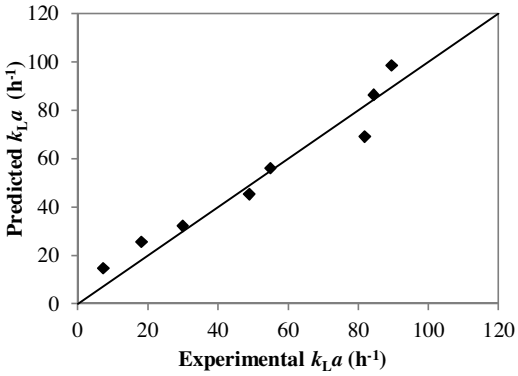

Fig. 1. Correlation between the experimental and predicted k_La values using Eq. 12.

3.2. Effect of initial k_La on citric acid production

In order to evaluate the effect of initial k_La on citric acid production, several experiments were performed varying simultaneously the stirring speed and specific air flow rate. The raise of initial k_La from 7 h^{-1} to 125 h^{-1}, due to an increase of specific air flow rate and stirring speed, had a positive impact on citric acid production (Fig. 2c). At lower initial k_La value (7 h^{-1}) the cells presented longer lag phase than in the higher values, and no significant differences were observed for cellular growth between them (Fig. 2a). Additionally, the crude glycerol consumption profile was similar for all the experiments, with exception for the assay at lower initial k_La value (Fig. 2b).

As expected, according with initial k_La conditions, different dissolved oxygen profiles were observed in batch cultures of *Y. lipolytica* (Fig. 3). During the first hours of yeast cultivation (corresponding to the exponential growth phase) a decrease on oxygen concentration in the medium was observed, particularly in the experiments with lower values of initial k_La (7 h^{-1} and 30 h^{-1}). In fact, for an initial k_La value of 7 h^{-1}, a full depletion of oxygen from the medium was observed through all the process, which can justify the lower biomass and citric acid concentrations obtained in this condition. In the phase of citric acid production (after the nitrogen source had been completely consumed), the oxygen demand is lower and a raise of oxygen concentration in the medium has been reported [36,50,51]. In the experiments with an initial k_La value equal to 30 h^{-1}, the dissolved oxygen concentration dropped to zero in the first hours but stabilized around 20% during the citric acid production. For the other initial k_La conditions, the oxygen concentration in the medium never fell to zero and stabilized around 55%, 70% and 85% for k_La values of 55 h^{-1}, 84 h^{-1} and 125 h^{-1}, respectively.

The raise of initial k_La from 7 h^{-1} to 55 h^{-1} led to an increase of citric acid concentration and maximum productivity (Fig. 4).

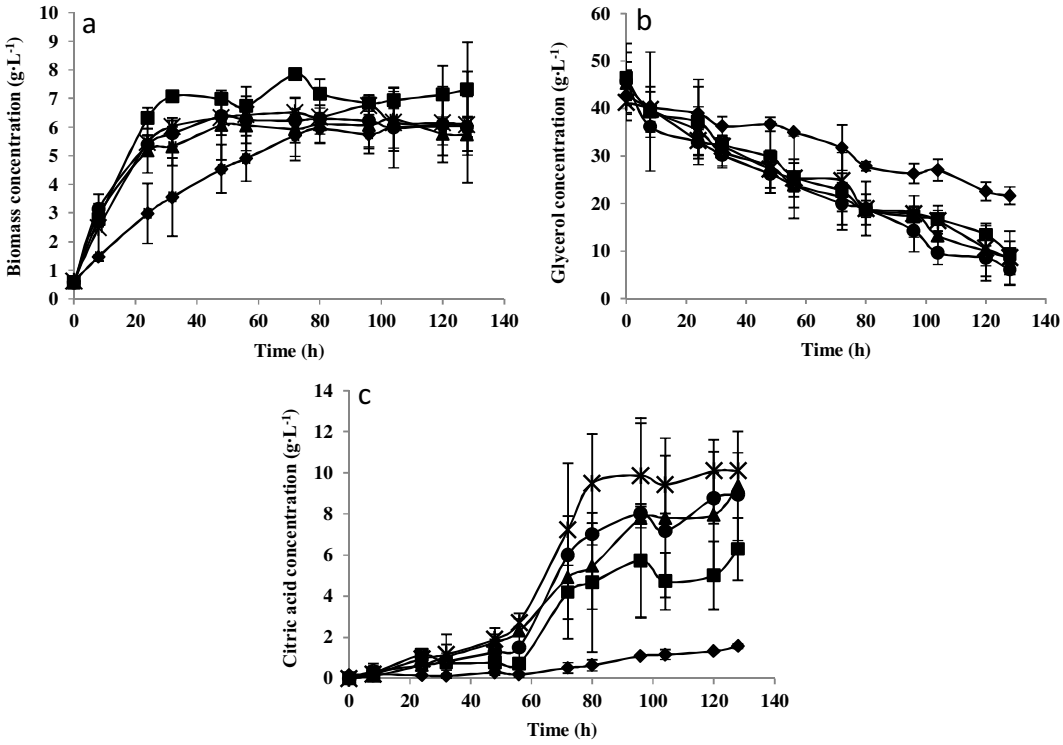

Fig. 2. Biomass production (a), crude glycerol consumption (b) and citric acid production (c) in batch cultures of *Y. lipolytica* W29 at different k_La values (h^{-1}): 7 (♦), 30 (■), 55 (▲), 84 (●) and 125 (×). The error bars represent the standard deviation of two independent replicates.

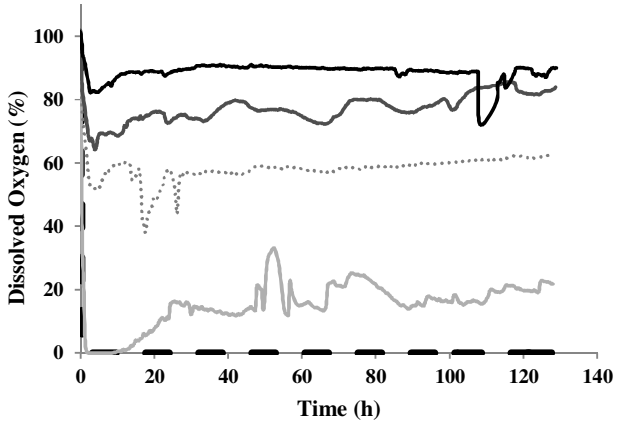

Fig. 3. Dissolved oxygen concentration profiles during citric acid production in batch cultures of *Y. lipolytica* W29 at different k_La values (h^{-1}): 7 (dashed line); 30 (light grey line); 55 (dotted line); 84 (dark grey line); 125 (black line).

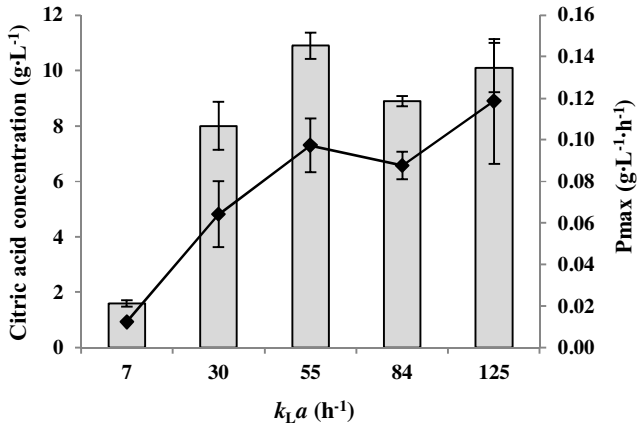

Fig. 4. Effect of k_La on citric acid concentration (bars) and maximum productivity (dots). Citric acid productivity was calculated by the ratio between the maximum citric acid concentration obtained in each experiment and respective time. The error bars represent the standard deviation of two independent replicates.

Approximately 8-fold improvement in citric acid concentration and maximum productivity was observed. The lower concentration was attained in the experiments where a full oxygen depletion from the medium was observed and the highest citric acid concentration was reached in the experiments where the dissolved oxygen remained above 55%.

Other authors have demonstrated the positive effect of increasing stirring speed from 400 rpm to 800 rpm [36] and to 1000 rpm [2] on citric acid production, but did not calculate or estimate k_La.

The small amount of citric acid produced at lower initial k_La value can be due to variations on the activity of important enzymes involved on citric acid production. Some authors observed a decrease in citric acid production under low aeration conditions, which was associated with a decrease in the activity of enzymes involved in tricarboxylic acid cycle and glyoxylate cycle [52,53].

At low aeration conditions, a reduced activity of citrate synthase, aconitate hydrate and NAD-dependent isocitrate dehydrogenase was observed. Additionally, an increase on the ATP-citrate lyase activity, responsible to the cleavage of citrate, was noticed. Moreover, the activity of isocitrate lyase and malate synthase, which are enzymes from glyoxylate cycle and are involved in the cleavage of isocitrate to succinate and malate, decreased under low oxygen concentration conditions. This pathway presents the major source of succinate and malate to mitochondria during intensive citric acid production. With the reduction of these enzymes activity from glyoxylate cycle, the increase of ATP-citrate lyase activity provides an alternative source for mitochondrial activity, not allowing an accumulation of citric acid [52].

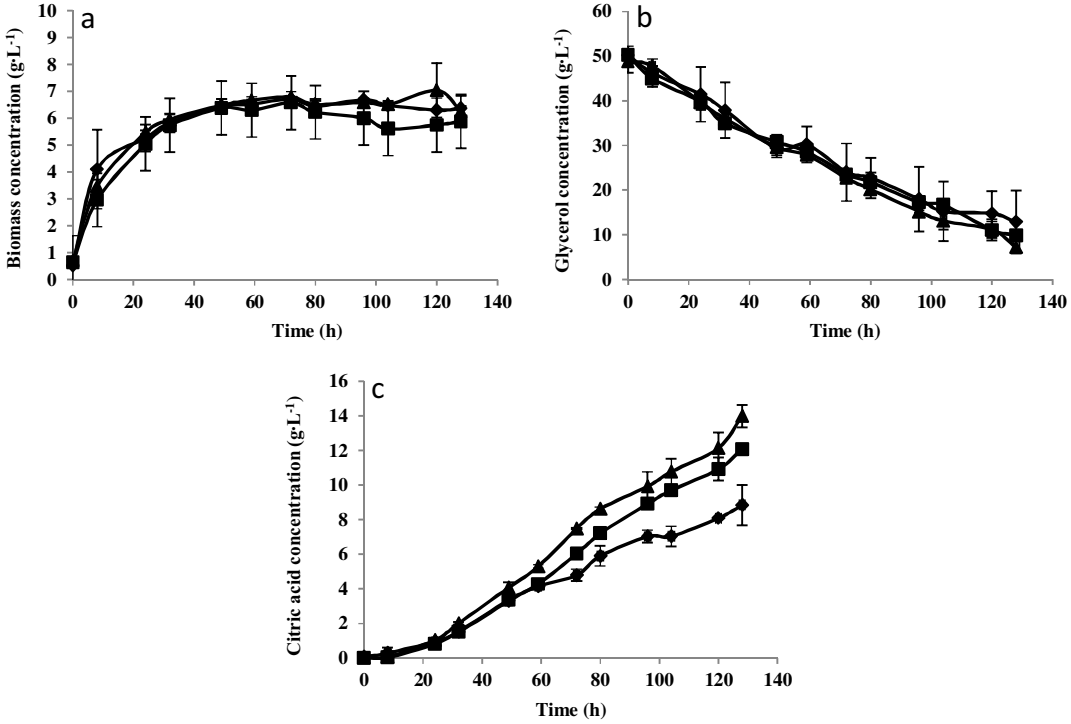

Fig. 5. Biomass concentration (a) crude glycerol consumption (b) and citric acid production (c) in batch cultures of *Y. lipolytica* W29 with different dissolved oxygen concentrations (%): 20 (♦), 40 (■), 60 (▲). The error bars represent the standard deviation of two independent replicates.

Above an initial k_La of 55 h^{-1}, no differences were observed in citric acid concentration and productivity. Probably, the highest stirring speed used in this study affected cell morphology and viability and, consequently, yeast metabolism. Changes in morphology of *Y. lipolytica* cells growing in similar range of stirring speed and specific air flow rate were reported by Braga et al. [32].

The increase of initial k_La from 7 h^{-1} to 30 h^{-1} led to a 48% increase in specific growth rate (Table 2). At initial k_La of 30 h^{-1}, 55 h^{-1}, 84 h^{-1} and 125 h^{-1}, no statistical differences in specific cellular growth rates were observed. Moreover, the highest biomass yield was attained with the lowest value of initial k_La. Also Crolla and Kennedy [2] demonstrated that increasing stirring speed from 400 rpm to 900 rpm (with constant specific air flow rate of 1 vvm) did not affect the *Candida lipolytica* cells concentration, but the lower stirring speed resulted in higher biomass yield. The highest specific glycerol consumption rate (q_s) and citric acid yield ($Y_{CA/S}$) were obtained in the experiments performed with an initial k_La equal to 55 h^{-1}. A 2.7-fold and 4.4-fold improvement in glycerol consumption rate and citric acid yield, respectively, was obtained increasing the initial k_La from 7 h^{-1} to 55 h^{-1}.

In addition to the influence on citric acid production, k_La also affected the isocitric/citric acid (ICA/CA) ratio. A considerably decrease (2.1-fold) of this parameter was attained raising the initial k_La from 7 h^{-1} to 125 h^{-1}. Other studies described that isocitric acid percentage decreased with an increase of stirring speed or specific air flow rate [36]. This result is particularly important for the downstream process, diminishing the global cost of citric acid purification.

3.3. Effect of controlled dissolved oxygen on citric acid production

Dissolved oxygen concentration in the production medium is an operational parameter that can influence the bioprocess overall performance. The maintenance of an adequate oxygen concentration through all the time is a challenge and a crucial step to maximize microbial growth and metabolites production.

Considering the oxygen profiles obtained in the previous assays and discussed above, several experiments were performed with constant dissolved oxygen concentrations and the values of 20%, 40% and 60% were chosen. These values of DO were controlled by manipulating the stirring speed and specific air flow rate, through a cascade control mode. The concentrations were selected taking into account the DO profiles obtained from the k_La assays: (a) 60% was, approximately, the value at which the dissolved oxygen stabilized in the initial k_La condition with higher concentration of citric acid; (b) 20% was the lowest dissolved oxygen concentration (different of 0%) obtained during citric acid production; and (c) 40% is an intermediate value.

Although *Y. lipolytica* is a strictly aerobic microorganism, no differences were found in the growth profiles and cell density reached with increased DO concentrations in the medium (Fig. 5a). Also, glycerol consumption had the same behavior for all conditions tested (Fig. 5b). In contrast, an enhancement of citric acid production was obtained with the raise of DO concentration (Fig. 5c). A 1.4- and 1.6-fold improvement in citric acid concentration was attained by increasing the DO concentration in the medium from 20% to 40% and to 60%, respectively (Fig. 6). Additionally, the maximum productivity was positively affected by the raise of DO concentration up to 60% and a 1.3-fold improvement was obtained compared to the assays carried out with 20% DO.

Independently the DO concentration in the medium, no differences were observed in specific growth rate, biomass yield and glycerol specific consumption rate (Table 3). However, a 1.6-fold improvement in citric acid yield was attained in the experiments with 60% of DO compared to the assays carried out at 20%.

These results are in accordance with previous works published by Kamzolova et al. [53] and Finogenova et al. [52], which reported the increase of citric acid production, in batch and continuous cultures of *Y. lipolytica* N1, when the DO concentration in the medium was equal to 60%. However, Anastassiadis and Rehm [54] observed that the maximum citric acid production by *Candida oleophila*, in continuous mode, was achieved with a DO of 20%.

Table 2
Effect of k_La (h^{-1}) on maximum specific growth rate (μ), specific consumption rate (q_s), biomass yield ($Y_{X/S}$), citric acid yield ($Y_{CA/S}$) and isocitric/citric acid ratio (ICA/CA) during batch cultures of *Y. lipolytica* W29. Data are presented as average and standard deviation of two independent experiments.

	k_La (h^{-1})				
	7	30	55	84	125
μ (h^{-1})	0.062 ± 0.014	0.092 ± 0.004	0.086 ± 0.009	0.085 ± 0.005	0.085 ± 0.006
q_s ($g\,L^{-1}\,h^{-1}$)	0.24 ± 0.05	0.51 ± 0.02	0.65 ± 0.24	0.68 ± 0.32	0.61 ± 0.15
$Y_{X/S}$ ($g\,g^{-1}$)	0.26 ± 0.01	0.18 ± 0.01	0.14 ± 0.04	0.14 ± 0.08	0.14 ± 0.03
$Y_{CA/S}$ ($g\,g^{-1}$)	0.07 ± 0.00	0.17 ± 0.01	0.31 ± 0.11	0.23 ± 0.04	0.27 ± 0.06
ICA/CA ($g\,g^{-1}$)	0.21 ± 0.02	0.16 ± 0.03	0.14 ± 0.05	0.11 ± 0.01	0.10 ± 0.05

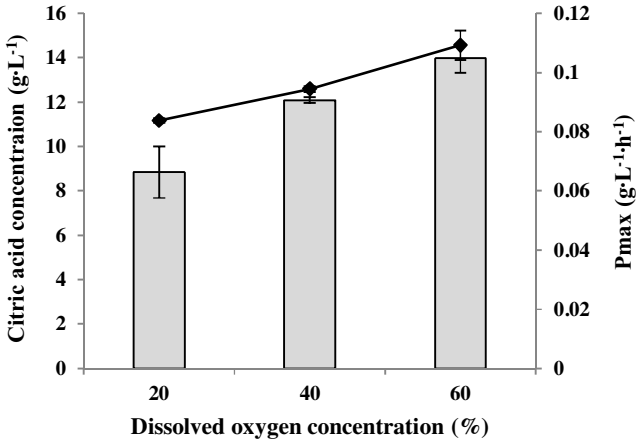

Fig. 6. Effect of dissolved oxygen (%) on citric acid concentration (bars) and maximum productivity (dots). Citric acid productivity was calculated by the ratio between the maximum citric acid concentration obtained in each experiment and respective time. The error bars represent the standard deviation of two independent replicates.

Table 3
Effect of dissolved oxygen concentration (%) on maximum specific growth rate (μ), specific consumption rate (q_s), biomass yield ($Y_{X/S}$) and citric acid yield ($Y_{CA/S}$) during bath culture of *Y. lipolytica* W29. Data are presented as average and standard deviation of two independent experiments.

	Dissolved oxygen concentration (%)		
	20	40	60
μ (h^{-1})	0.085 ± 0.002	0.079 ± 0.006	0.077 ± 0.000
$Y_{X/S}$ ($g\,g^{-1}$)	0.16 ± 0.04	0.13 ± 0.01	0.15 ± 0.02
q_s ($g\,g^{-1}\,h^{-1}$)	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
$Y_{CA/S}$ ($g\,g^{-1}$)	0.235 ± 0.006	0.299 ± 0.005	0.37 ± 0.04

Comparing the results obtained in the experiments carried out with initial k_La and controlled dissolved oxygen, it was observed that citric acid concentrations obtained using controlled DO (20% and 60%) was similar to those attained in experiments with initial k_La of 30 h^{-1} and 55 h^{-1}, respectively.

Considering the small differences in citric acid production obtained in the two operational approaches discussed above, and from an industrial perspective, operating at constant values of stirring speed and aeration rate will be easier and less technologically demanding. Moreover, in this work, when DO was controlled at 20% and 60%, the stirring speed needed to achieve these oxygen concentrations were higher than those used at initial k_La of 30 h^{-1} and 55 h^{-1}, respectively (data not shown), thus representing an increase of operating costs due to power consumption. For the reasons explained above, setting initial k_La was proven to be an adequate strategy of optimization of the bioprocess of citric acid production from glycerol by *Y. lipolytica* W29.

4. Conclusions

Citric acid production by *Y. lipolytica* W29, from crude glycerol, was evaluated considering the effect of oxygen using two different strategies: constant stirring speed and aeration to give a determined initial k_La and constant dissolved oxygen concentration.

An empirical correlation to predict k_La value as a function of operating conditions (superficial gas velocity and specific power input), as well of cellular density was established.

The increase of initial k_La resulted in an increase of citric acid production and a decrease of ICA/CA ratio. The maximum citric acid concentration was achieved with an intermediate value of initial k_La (55 h^{-1}). The raise of dissolved oxygen concentration led to an increase of citric acid yield and productivity, achieved either by controlling dissolved oxygen concentration at constant values or by increasing stirring speed and specific air flow rate to give adequate initial k_La values. This last strategy appears to be a more economically attractive approach, from an industrial implementation perspective.

This work demonstrated the importance of k_La in citric acid production by *Y. lipolytica* from crude glycerol and the correlation proposed will be very useful for further work on the development of strategies for the optimization and scale-up of this bioprocess.

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