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Oxygen transfer rate and pH as major operating parameters of citric acid production from glycerol by *Yarrowia lipolytica* W29 and CBS 2073

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The amount of citric acid (CA) produced by *Yarrowia lipolytica* is dependent on the yeast strain and growth conditions such as pH, oxygen availability and medium composition. In this work, an experimental design based on the Taguchi method was applied to evaluate the effect of parameters: pH, carbon/nitrogen (C/N) ratio in the medium, oxygen mass transfer rate (OTR) and salts concentration, on the CA production by two *Y. lipolytica* strains, W29 (ATCC 20460) and CBS 2073. OTR and pH showed higher influence on the CA production for both strains. The increase of OTR from air to the culture medium led to a two- and three-fold improvement of the CA production by *Y. lipolytica* CBS 2073 and W29, respectively. Besides the individual effects of the parameters, a significant influence of the interaction between these parameters was observed, mainly between OTR and salts. Different values of the parameters were found at the optimum conditions for each strain, but the theoretically predicted and experimentally obtained citric acid concentrations (c_{CA}) were approximately 10 g L^{-1} for both strains. The optimal conditions were also validated employing crude glycerol from biodiesel industry as a substrate, and similar behavior of the strains was observed.

Keywords: *Yarrowia lipolytica*, citric acid, pH, oxygen mass transfer rate, salts concentration, glycerol

Introduction

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid, CA), an organic acid intermediate of the tricarboxylic acids cycle, is largely used in food, pharmaceutical and detergent industries (Kamzolova et al., 2008). With a global annual production exceeding 1.6 million tons (Morgunov et al., 2013), this organic acid is mainly produced by submerged fermentation with *Aspergillus niger* from glucose syrup and molasses (cane and beet) (Förster et al., 2007). This process is well established but it has a few disadvantages (Förster et al., 2007), together with the constant increase of CA consumption which leads to the need of searching for new microorganisms, e.g. yeasts species, for CA production.

Yarrowia lipolytica, a strictly aerobic yeast, is known for its ability to use a wide range of carbon sources (Fickers et al., 2005; Rymowicz et al., 2010; Lazar et al., 2011) and it was already described as a CA producer from ethanol (Arzumanov et al., 2000), oils (Venter et al., 2004; Kamzolova et al., 2008), sucrose (Förster et al., 2007), glucose (Antonucci et al., 2001; Kamzolova et al., 2008), *n*-paraffins (Crolla & Kennedy, 2004) and glycerol (Imandi et al., 2007; Papanikolaou & Aggelis, 2009; Rymowicz et al., 2010). Additionally, also low cost by-products, such as olive mill wastewater (Gonçalves et al., 2009) and crude glycerol from biodiesel industry (Papanikolaou & Aggelis, 2003; Chatzifragkou & Papanikolaou, 2012), can be used as substrates.

The production and accumulation of CA by

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Y. lipolytica occurs under nitrogen-limited conditions and an excess of carbon source (Papanikolaou et al., 2002); other parameters, such as pH (Papanikolaou et al., 2002) and carbon/nitrogen (C/N) ratio (Levinson et al., 2007), also influence the amount of CA produced. Since *Y. lipolytica* is a strictly aerobic microorganism, oxygenation of the culture is also a factor to be considered. Studies have shown that higher CA production is obtained at high oxygen concentrations in the culture medium (Rywińska et al., 2012). Besides the operational and media conditions, the amount of CA produced is also dependent on the microbial strain (Kamzolova et al., 2003).

In this work, the effect of selected parameters on the CA production by two strains of *Y. lipolytica* was studied applying the Taguchi method. This fractional factorial design method allows identifying the effect of each parameter on the CA production, and predicting the optimum values of the parameters. Thus, growth conditions such as the C/N ratio, salts concentration, pH and oxygen mass transfer rate (OTR) were optimized to maximize the CA production by the two *Y. lipolytica* strains employed. Although several authors have mentioned the individual effect of each parameter on the CA production, few reports on the combined effect of these parameters are available.

Experimental validation of the optimum culture conditions was done using pure and crude glycerol from biodiesel industry.

Experimental

Yeast strains

Two strains of *Yarrowia lipolytica*, W29 (ATCC 20460) and CBS 2073, which have never been tested for CA production from glycerol, were used in this study. The strains were maintained on yeast extract peptone dextrose (YPD) agar medium at 4 °C to a maximum of two weeks. The YPD agar medium was composed of peptone (20 g L^{-1}), glucose (20 g L^{-1}), yeast extract (10 g L^{-1}) and agar (20 g L^{-1}).

Optimization of growth condition – experimental design

CA production was optimized using the Taguchi method, a fractional factorial experimental design. This method uses orthogonal arrays for the optimization of different parameters and studies some parameter combinations instead of all possible combinations, which reduces the time and resources needed for the optimization. Orthogonal arrays selection is decided according to the number of parameters (P) and the variation of levels (L) of each parameter. The number of experiments (N) is calculated as: $N = (L - 1)P + 1$ (Sathish Kumar et al., 2015).

The experimental design was performed using an L9 orthogonal array employing the Qualiteck-4 software (Nutek, Bloomfield Hills, USA). Four parameters (C/N ratio, pH, salts concentration and OTR) were combined and varied in three experimental setups (levels). From the Qualiteck-4 software, a total of nine experiments were planned. The experiments were performed in 500 mL flasks filled with 200 mL of the production medium. Yeast cells were pre-grown in YPG medium (20 g L^{-1} of glycerol, 20 g L^{-1} of peptone, 10 g L^{-1} of yeast extract), centrifuged and resuspended in the production medium (glycerol as the carbon source, yeast extract as the nitrogen source, 1.5 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 6 g L^{-1} of KH_2PO_4 and 0.5 g L^{-1} of Na_2HPO_4). These experiments were performed for both strains.

pH control was carried out by adding a 5 M KOH solution. The C/N ratio (g g^{-1}) was obtained varying the glycerol and yeast extract concentrations: 156 (20 g L^{-1} glycerol/0.5 g L^{-1} yeast extract), 391 (50 g L^{-1} glycerol/0.5 g L^{-1} yeast extract) and 1956 (50 g L^{-1} glycerol/0.1 g L^{-1} yeast extract). These experiments were performed at different OTR values (in mg L^{-1} h^{-1}): 48, 192 and 576, achieved at 140 min^{-1} using flasks without baffles, at 140 min^{-1} using flasks with baffles, and at 200 min^{-1} using flasks with baffles, respectively. OTR was estimated in blank assays by the sulfite oxidation method as described by Lopes et al. (2013). Salts concentration was: 0.15 g L^{-1} $CaCl_2$, 0.15 g L^{-1} $FeCl_3 \cdot 6H_2O$, 0.06 g L^{-1} $MnSO_4 \cdot H_2O$, 0.02 g L^{-1} $ZnSO_4 \cdot 7H_2O$ (level 3), half of these concentrations (level 2) and no salts solution (level 1).

The response of c_{CA} obtained in the experimental design was processed in the Qualiteck-4 software applying "bigger is better" quality characteristics to evaluate the optimum culture conditions for the CA production maximization. These optimal conditions were assessed for both strains using pure and crude glycerol (provided by Prio Energy – Prio Biocombustíveis, Portugal) with the following composition (mass %): 90.4 % of glycerol, 9 % of water, 4.9 % of NaCl, less than 0.001 % of methanol and less than 0.5 % of organic matter (non-glycerol).

Analytical methods

Samples were collected to analyze the cell concentration (optical density of 600 nm and converted to dry cell mass per liter), glycerol concentration (c_{GL}) and c_{CA}. The c_{GL} was determined by high-performance liquid chromatography (HPLC) using a Metacarb 67H (Varian) column (300 mm × 7.7 mm) coupled to a Jasco RI-1530 detector. The column was eluted with 5 mM H_2SO_4 at 0.5 mL min^{-1} and the column temperature was 60 °C. The value of c_{CA} was measured by HPLC using an YMC ODS-Aq (250 mm × 4.6 mm) reverse phase column coupled to a DAD detector at 214 nm. The mobile phase was 20 mM KH_2PO_4 (pH

Table 1. Parameters and levels used in the experimental design for each assay performed and c_{CA} obtained in the experiments designed using the Taguchi L9 orthogonal array in batch cultures of *Y. lipolytica* W29 (ATCC 20460) and CBS 2073; data are the average and standard deviation of two independent replicates

Run	pH	C/N ratio	OTR	Salts	c_{CA} /(g L^{-1})	
					Y. lipolytica	
					W29	CBS 2073
1	1	1	1	1	3.7 ± 0.2	3.5 ± 0.1
2	1	2	2	2	6.2 ± 0.8	7.0 ± 0.2
3	1	3	3	3	6.2 ± 0.1	4.7 ± 0.2
4	2	1	2	3	2.6 ± 0.2	8.1 ± 0.7
5	2	2	3	1	4.4 ± 0.3	4.5 ± 0.4
6	2	3	1	2	2.0 ± 0.2	2.8 ± 0.1
7	3	1	3	2	4.0 ± 0.4	2.0 ± 0.0
8	3	2	1	3	0.1 ± 0.1	2.2 ± 0.0
9	3	3	2	1	0.7 ± 0.1	1.7 ± 0.1

Level	pH	C/N ratio	OTR	Salts
1	5	156	48	0
2	6	391	192	1/2
3	7	1956	576	1

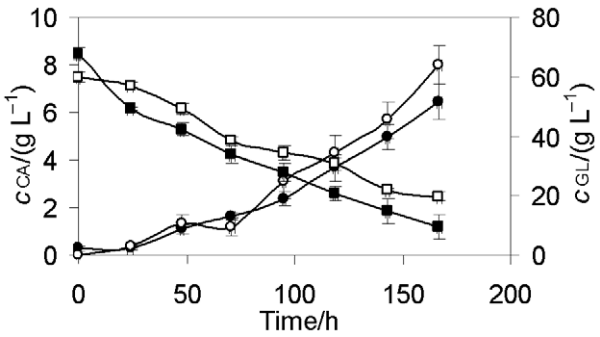

Fig. 1. Time profile of CA production (●, ○) and glycerol consumption (■, □) for *Y. lipolytica* W29 (black symbols) and *Y. lipolytica* CBS 2073 (white symbols) from run 2. pH = 5, C/N = 391, OTR = 192 and 1/2 salts concentration. Error bars represent the standard deviation for two independent replicates.

2.8) at ambient temperature and the flow rate of 0.7 mL min^{-1}.

Results and discussion

Y. lipolytica W29 has been successfully used for lipase (Lopes et al., 2008), γ-decalactone (Braga & Belo, 2015) and also for CA production (Sarris et al., 2011). Moreover, due to the availability of its complete genome sequence, *Y. lipolytica* W29 has been used as a cellular model for genetic modifications by several research groups (Nicaud et al., 2002). Also, *Y. lipolytica* CBS 2073 has been reported to grow efficiently and produce lipase in olive mill wastewater based media (Gonçalves et al., 2009). This work also intends to study the use of crude waste of the biodiesel industry (crude glycerol) for CA production, which justifies the selection of the strains.

The time profile of c_{CA} and c_{GL} is shown in Fig. 1 for run 2 as an example, since identical behavior was observed for all runs. Continuous CA increase and glycerol decrease in the medium with time was observed up to the end of the measurement (168 h). Thus, the response of interest was the final c_{CA} value shown for all runs in Table 1.

From the response obtained it is clear that CA production is strongly dependent on the combination of the various parameters studied. The c_{CA} value varied in the range of 0.1–8.1 g L^{-1}.

Individual effect of each parameter on the CA production is shown in Fig. 2. The increase of pH from 5 to 7 led to a decrease of c_{CA}, particularly for *Y. lipolytica* W29. In the experiments carried out at pH 5, c_{CA} was three times higher than that obtained at pH 7. However, no significant differences between pH 5 and 6 were observed for the CBS strain. The effect of the C/N ratio was similar for both strains; a small increase was observed at the ratio equal to 391. In the experiments with *Y. lipolytica* W29, the CA production increased proportionally to OTR and the increase of OTR from level 1 to 3 led to a three-fold improvement in c_{CA}. However, for *Y. lipolytica* CBS 2073, the best results were obtained in the intermediate OTR level, where a two-fold increase of the CA production was achieved compared to the lowest OTR value. The increase of salts concentration in the culture medium had a slightly positive effect on the CA production for both strains; however, for *Y. lipolytica* CBS 2073, the maximum CA production was obtained at the highest concentration of salts, while for *Y. lipolytica* W29, only half of the salts concentration was enough to pro-

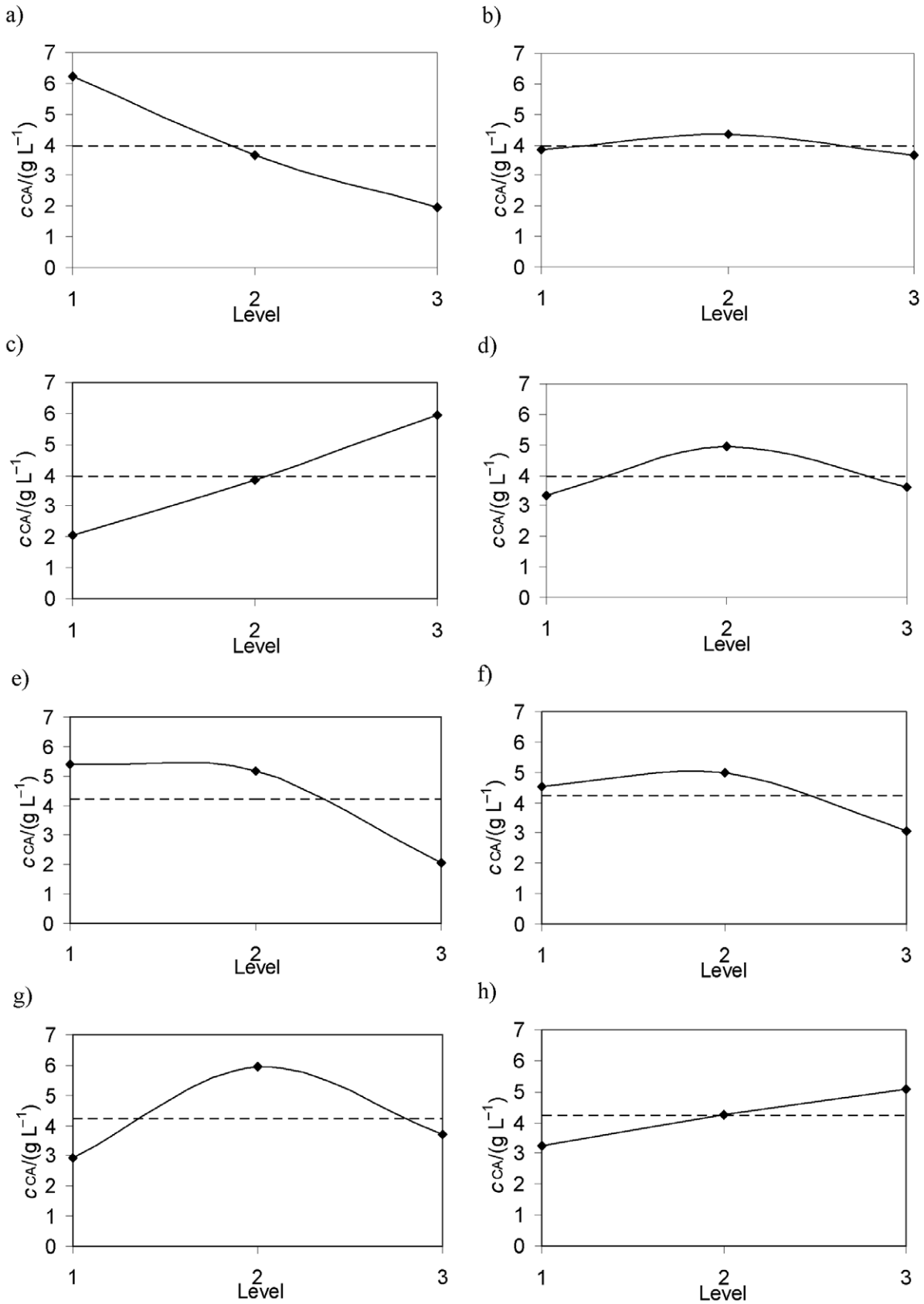

Fig. 2. Effect of individual parameters at different levels for *Y. lipolytica* W29 (a–d) and *Y. lipolytica* CBS 2073 (e–h): pH (a, e); C/N ratio (b, f); OTR (c, g); salts (d, h). Level description is shown in Table 1.

Table 2. Analysis of variance (ANOVA) for the Taguchi L9 orthogonal array

Y. lipolytica	Parameter	Sum of squares	Variance	*F*-ratio	*P*/%
W29	pH	55.86	27.93	106.48	48.13
	C/N ratio	1.62	0.81	3.08	0.95
	OTR	46.02	23.01	87.73	39.57
	Salts	9.12	4.56	17.38	7.47
	Error	2.36	0.26	–	3.88
CBS 2073	pH	51.00	25.50	37.31	45.82
	C/N ratio	9.52	4.76	6.96	7.53
	OTR	34.70	17.35	25.39	30.78
	Salts	6.94	3.47	5.08	5.15
	Error	6.15	0.68	–	10.73

vide the maximum CA production.

Individual contribution of each parameter is very important to define the parameters to be strictly controlled during the production process. The analysis of variance (ANOVA) for the selected response (Table 2) may contribute to the selection of these parameters considering their influence on the CA production by *Y. lipolytica* strains. The last column (*P*, %) in Table 2 indicates the contribution of each parameter; the higher percentage represents the parameter with more influence on the process. Considering these results, it was possible to select the parameters pH and OTR as the most significant for the CA production by both yeast strains. Although pH and OTR were by far the most significant parameters in the CA production for both strains, the best conditions of each process were slightly different, proving that optimal conditions are dependent on the yeast strain.

Papanikolaou et al. (2002) studied the production of CA by *Y. lipolytica* LGAM S(7)1 using a buffered and a non-buffered medium with the initial pH equal to 6. In the buffered medium (where pH dropped to 4.5), a ten-fold improvement in c_{CA} was reached compared to the non-buffered medium (where pH dropped to 2–3). Karasu-Yalcin et al. (2010) tested different initial pH values (in the range of 4.2–8.5) and observed that maximum CA production was obtained in the pH range of 5.2–7. Studies performed by Tomaszewska et al. (2014) showed that the maximum c_{CA} was obtained at pH 5.5 while lower pH values (3–4) favored the production of sugar alcohols. The negative effect of low pH can also be proved by its influence on the CA transport through the cell membrane. Anastassiadis and Rehm (2005) studied the effect of pH on the active CA transport and demonstrated that this transport system is pH-dependent.

Oxygen has also been reported as an important factor in the CA production. Rywińska et al. (2012) observed an improvement of the CA production when increasing the agitation rate from 400 min^{-1} to 900 min^{-1}, and the aeration rate from 0.18 vvm to 0.6 vvm (vvm = volume per volume per minute); however, no information on OTR was given.

In this work, the salts concentration in the production medium was a parameter with little influence on the CA production. However, some studies have shown that salts concentration can play an important role in the yeast growth and CA production. Finogenova et al. (2002) observed that ion zinc in limiting concentrations reduces the cellular growth and CA production. In this work, the effect of salts concentration was shown to be also dependent on the *Y. lipolytica* strain. Similarly, Karasu-Yalcin et al. (2010) reported that the supplementation of culture medium with zinc had distinct effects on the CA production by two *Y. lipolytica* strains: it decreased with *Y. lipolytica* NBRC 1658 and increased with *Y. lipolytica* NBRC 57.

The production of CA occurs in nitrogen-limited conditions and an excess of the carbon source. In this study, the C/N ratio was one of the parameters with lower influence on the CA production and the ratio equal to 391 led to higher c_{CA}. However, some studies showed that higher c_{CA} were reached at higher C/N ratios. Levinson et al. (2007) observed that C/N ratios between 343 and 686 led to higher c_{CA}. Also, André et al. (2009) reported that the increase of the amount of carbon source (glycerol), maintaining the nitrogen concentration, led to an improvement of the CA production.

Besides the individual effect of each p arameter, the interaction between the parameters contributes to the overall process. Estimated interaction severity index (SI) allows understanding the influence of the interaction of two parameters (Table 3). It is worth noticing that the highest SI is not associated with the most important parameters (individual effect) and the SI values for each parameter combination depend on the yeast strain studied. For *Y. lipolytica* W29, the interaction of the C/N ratio (parameter with little individual effect) with OTR (parameter with high individual effect) resulted in higher SI value (53.45 %), closely followed by the interaction of the salts concentration with OTR (52.23 %). The interaction between the parameters with higher individual influence (pH vs. OTR) provided lower SI values (24.71 %). For *Y. lipolytica* CBS 2073, interactions with higher SI

Table 3. Estimated interactions of studied parameters based on severity index (SI/%)

Interacting parameter pairs	*Y. lipolytica*	
	W29	CBS 2073
C/N ratio vs. OTR	53.15	9.86
OTR vs. salts	52.23	54.48
pH vs. salts	46.37	48.67
pH vs. OTR	24.71	6.51
pH vs. C/N ratio	10.8	62.67
C/N ratio vs. salts	5.7	38.8

values were those of the C/N ratio vs. pH (62.67 %) and of the salts concentration vs. OTR (54.48 %). It was observed that the oxygen requirement for CA production by *Y. lipolytica* are lower in the presence of higher concentrations of iron (Finogenova et al., 2002; Kamzolova et al., 2003), which is in accordance with the results reported here and demonstrates the importance of salts concentration vs. the OTR interaction. From the analysis of interactions it was also observed that the most important ones are those of a parameter with lower individual effect combined with a more significant parameter. These results suggest that the influence of a parameter depends on the other parameter in the CA production optimization.

Taking into account the experimental data obtained, the Taguchi method established the optimum level of each factor for maximization of CA production and predicted a theoretical value in optimal conditions (Table 4). pH and C/N ratio are the same for both strains, despite the OTR and salt concentration needed to maximized c_{CA} for each strain being different. *Y. lipolytica* W29 needs a higher OTR value but lower salt concentration when compared with *Y. lipolytica* CBS 2073. The importance of OTR and salts interaction was reported by Finogenova et al. (2002) and Kamzolova et al. (2003). The authors observed that, in presence of high iron concentrations it was possible to achieve great amounts of CA with less quantity of oxygen.

In order to confirm the theoretical values and validate the experimental design, assays were carried out at optimal conditions predicted by the method for both strains (Table 4). For both strains, the experimental and predicted results were similar, validating the method and allowing to conclude which are the optimal conditions for CA production by these *Y. lipolytica* strains.

Besides the assay with pure glycerol, an experiment with crude glycerol was performed at optimal conditions for both strains. As the main by-product of biodiesel production, crude glycerol can now be found in abundance and at prices lower than those of pure glycerol, which makes crude glycerol a promising carbon source for bioprocesses with *Y. lipolytica*. No statistical differences ($p > 0.05$) in the CA production with pure and crude glycerol were observed (Table 4), which indicates that the impurities and additional nutrients present in crude glycerol do not affect the CA production by the *Y. lipolytica* strains used in this work. The current low cost of crude glycerol together with the present results show the possibility of using crude glycerol as a carbon source for CA production by *Y. lipolytica*. Similarly to pure glycerol, c_{CA} values obtained by the strains studied ($p > 0.05$) at the optimal culture conditions using crude glycerol are not statistically different.

Conclusions

The Taguchi experimental design for process optimization allowed to conclude that pH and OTR have higher influence on the CA production in batch cultures of two *Y. lipolytica* strains, W29 and CBS 2073, using glycerol as the substrate. Moreover, the interaction between OTR and the salts concentration showed also a significant effect for both strains. Thus, these parameters are very important for process scale-up.

Table 4. Optimum culture conditions, predicted and experimental c_{CA} obtained for batch cultures of *Y. lipolytica*. Data are the average and standard deviation of two independent replicates

Y. lipolytica	Parameter	Level	Values	$c_{CA}/(g\ L^{-1})$	
				Predicted	Experimental
W29	pH	1	5	9.6	9.5 ± 0.6 (pure) 10.3 ± 0.1 (crude)
	C/N ratio	2	391		
	OTR	3	576		
	Salts	2	1/2		
CBS 2073	pH	1	5	8.8	10.5 ± 0.3 (pure) 9.4 ± 0.8 (crude)
	C/N ratio	2	391		
	OTR	2	192		
	Salts	3	1		

Note: Current grand average performance: *Y. lipolytica* W29 – 3.9 g L^{-1}; *Y. lipolytica* CBS 2073 – 4.2 g L^{-1}.

Optimum conditions for c_{CA} maximization were determined as: pH 5 and C/N ratio of 391 g g^{-1} for both stains, OTR of 576 mg L^{-1} h^{-1} and half salts concentration for *Y. lipolytica* W29, and OTR of 192 mg L^{-1} h^{-1} and full concentration of salts for *Y. lipolytica* CBS 2073. The CA production was similar for both strains, but in a larger-scale production, the W29 strain requires smaller amounts of salts, provided that a good oxygen transfer into the medium is ensured. Good oxygenation of the medium is easily achieved with several bioreactors used for industrial CA production, e.g. stirred tank bioreactors.

Values of c_{CA} obtained for both stains using crude glycerol from biodiesel industry were similar to those obtained with pure glycerol, proving the possibility of using this by-product as a low cost carbon source for CA production by the *Y. lipolytica* strains used in this work.

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