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ORIGINAL ARTICLE

High lipids accumulation in *Rhodosporidium toruloides* by applying single and multiple nutrients limitation in a simple chemically defined medium

Yolanda González-García[1] · L. Melisa Rábago-Panduro[2] · Todd French[3] · David Isidoro Camacho-Córdova[2] · Porfirio Gutiérrez-González[4] · Jesús Córdova[2]

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Abstract Microbial oil produced by the oleaginous yeast *Rhodosporidium toruloides* ATCC 204091 (formerly referred to as *Rhodotorula glutinis*) has a similar fatty acid composition to the vegetable oils and represents a potential alternative for biodiesel production. Finding strategies to improve the oil production by this yeast is desirable, as it is one of this nutrient's limitations during the accumulation phase, as well as one of the main factors influencing the process. Therefore, the effect of single or combined nutrient limitation on lipid accumulation by *R. toruloides* was investigated. Biomass production and lipid accumulation by *R. toruloides* was improved using experimental designs in a two-step batch culture on a chemically-defined culture medium with high initial glucose concentration. For the first culture step, a Box–Behnken design was applied to optimize the main medium components' concentrations, while maintaining a high biomass production. A biomass concentration of 44.3 g/L was reached with a medium composed of (g/L): glucose, 100; KH_2PO_4, 4.6; $NaNO_3$, 13.4; $MgSO_4 \cdot 7H_2O$, 0.2; and $CaCl_2 \cdot 2H_2O$, 0.11. For the second culture step, the biomass was transferred to lipid accumulation media. A 2^3 factorial experimental design was conducted to investigate the effect of N, P and S limitations (individually or jointly) on lipid production from glucose (100 g/L). Lipid accumulation on dry cell mass was 77.04, 65.42, 70.13 and 69.84% for N, P, S and simultaneous nutrients' limitations, respectively.

Keywords Microbial oil · Nutrient limited medium · Oleaginous yeast · Rhodosporidium

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✉ Yolanda González-García
yolacea@yahoo.com

L. Melisa Rábago-Panduro
meli_rplm@hotmail.com

Todd French
french@che.msstate.edu

David Isidoro Camacho-Córdova
davidicaco@yahoo.com.mx

Porfirio Gutiérrez-González
pgutierrezglez@gmail.com

Jesús Córdova
jesuscordovaudg@yahoo.com.mx

[1] Departamento de Madera, Celulosa y Papel, Universidad de Guadalajara, Km 15.5 Carretera Guadalajara-Nogales, 45010 Zapopan, Jalisco, Mexico

[2] Departamento de Química, Universidad de Guadalajara, Blvd. Marcelino García Barragán 1421, 44430 Guadalajara, Jalisco, Mexico

[3] Dave C. Swalm School of Chemical Engineering, Mississippi State University, Starkville, MS 39762, USA

[4] Departamento de Matemáticas, Universidad de Guadalajara, Blvd. Marcelino García Barragán 1421, 44430 Guadalajara, Jalisco, Mexico

Introduction

Incentives for biofuel production have driven the search for environmentally friendly practices and methods to convert non-food sources into combustibles. One of the main biofuels produced is biodiesel; however, its demand has been traditionally satisfied by oils derived from vegetable seeds or animal fats. Recently, microorganisms are being viewed as a source of oils, due to their capacity to store lipids, their fast growth rate and their ability to utilize waste materials. Microorganisms (mostly bacteria, algae, molds and yeast) storing more than 20% w/w of lipids in their dry cell mass are termed oleaginous (Meng et al. 2009). Oleaginous yeasts are particularly useful for the renewable and sustainable production of biodegradable oils for fuels and chemicals (Subramaniam et al. 2010). Recently, studies such as the discovery of yeast species accumulating very high oil concentration, the improvement of culture conditions that promote lipid accumulation, the implementation of metabolic engineering strategies, the conversion of waste products to yeast oil, and microbial oil production at pilot-scale level have been carried out intensively (Poli et al. 2014; Yen and Liu 2014). *Rhodosporidium toruloides* is an oleaginous yeast able to grow from several carbon sources (Easterling et al. 2009) including waste waters (Gonzalez-Garcia et al. 2013). It also produces carotenoids, which is an advantage since it might have the potential to be used for the production of both metabolites in the same process. Due the interest in this particular strain, its genome was recently sequenced (Paul et al. 2014) to explore genes encoding useful products. Furthermore, among oleaginous yeasts, this strain stands out due to its high oil accumulation capacity, mainly under nitrogen-limited conditions (Sitepu et al. 2014). However, the information regarding its ability to accumulate lipids under other nutrient (e.g., phosphorus and sulfur)-limited conditions is scarce.

The aim of the present study was to improve the biomass production of *R. toruloides* by designing and optimizing a mineral culture medium, as well as to investigate the effect of single or combined nutrient limitations (N, S and P sources) on lipid accumulation.

Materials and methods

Microorganism

Rhodosporidium toruloides ATCC 204091 (formerly referred to as *Rhodotorula glutinis*) was used. The strain was propagated on YPD agar (yeast extract, 10 g/L; peptone, 20 g/L; glucose, 20 g/L, agar, 16 g/L), incubated at 30 °C for 5 days, and then used to inoculate slant tubes with YPD agar, which were incubated at 30 °C for 3 days and stored at 4 °C for strain preservation.

Inoculum

Cells from a slant tube were suspended in 5 mL of YPD broth and transferred to a 250-mL Erlenmeyer flask containing 45 mL of the same medium. This was incubated in a rotatory shaker at 30 °C and 150 rpm, until an optical density (OD_{600nm}) between 0.6 and 0.8 was achieved (applying a 1:10 dilution).

Experimental phases and general culture conditions

Three experiments were conducted in order to improve *R. toruloides* biomass production and lipid accumulation. First, the design of a simple chemically-defined medium for *R. toruloides* growth. This experiment was focused on studying the highest amount of initial glucose concentration that the yeast is able to use to produce biomass efficiently in only one step (batch culture). This was based on the fact that carbon is the main component of biomass, and a high amount of biomass mostly depends on the amount of initial carbon source used. Second, optimization of the culture medium for improving biomass production. After fixing the amount of initial glucose concentration (as a result of the previous experiment), other important components of microbial cells were studied, specifically the amount of N, P, and S. Three variables is an ideal number to apply a response surface design, and therefore a Box–Behnken design was chosen since it offers the advantage of requiring a smaller number of runs. And third, evaluation of N, P and S limitations (singularly or in combinations) on lipids accumulation by *R. toruloides*. For this experiment, a full factorial design 2^3 was applied, since such a design can estimate all the main effects (each nutrient limitation independently) and higher-order interactions (combined nutrient limitations).

All experiments were carried out in 500-mL Erlenmeyer flasks with 100 mL of culture medium, according to the corresponding experimental design. The cultures were inoculated with 10% (v/v) and incubated at 30 °C and 150 rpm until reaching a stationary phase (120–320 h, according to the culture medium composition). Initial pH was 5.5 and was not controlled during the cultures. Samples (5 mL) were withdrawn every 20–24 h for analyzing the biomass, glucose and lipids concentration.

Designing a suitable mineral culture medium for *R. toruloides* growth

R. toruloides was cultured in a chemically defined medium containing (g/L): glucose, 20; $MgSO_4 \cdot 7H_2O$, 0.41; KH_2PO_4, 1.4; and $CaCl_2 \cdot 2H_2O$, 0.15, whereas two mineral nitrogen

sources were evaluated: $(NH_4)_2SO_4$, 21 g/L (Medium A), or $NaNO_3$, 26.7 g/L (Medium B). A reference complex medium (YPD broth) was also used as a control.

After selecting the more suitable mineral nitrogen source, the medium was redesigned to reach a higher biomass concentration. The components concentrations in the medium were balanced, fixing a glucose concentration of 100 g/L, and considering a biomass yield ($Y_{X/S}$) of 0.5 and the elemental composition for yeasts (Stanbury et al. 2013), resulting in the following concentrations (g/L): glucose, 100; $NaNO_3$, 26.7; KH_2PO_4, 5.7; $MgSO_4 \cdot 7H_2O$, 1.7; and $CaCl_2 \cdot 2H_2O$, 0.11. The experiment was conducted with two replicates, as well as for biomass and glucose quantification in each sample taken.

Optimizing the culture medium for improving biomass production

In order to increase biomass production in batch culture (first step), the initial glucose concentration in the previously redesigned medium was varied from 20 to 300 g/L, while the concentration of the other components (N, P, S, Ca) remained constant. Furthermore, a Box–Behnken experimental design with three variables ($NaNO_3$, KH_2PO_4 and $MgSO_4 \cdot 7H_2O$ concentrations) at three levels (Supplementary Table 1) was developed, resulting in 15 treatments (media B.1– B.15). The response variable was the biomass production (g/L). Concentrations of other medium components remained constant for all cultures (with glucose at 100 g/L). Two replicates for each experimental treatment as well as for biomass and glucose determination were used.

Determining the effect of N, P and S limitation on lipids accumulation by *R. toruloides*

For the second culture step (lipid accumulation in batch culture system), the effect of the three main culture medium components concentrations ($NaNO_3$, KH_2PO_4 and $MgSO_4 \cdot 7H_2O$) on *R. toruloides* lipid accumulation (% of dry cell weight) was evaluated using a factorial 2^3 experimental design with four central points (Supplementary Table 2). It is worth noting that the concentrations of the other media components (for lipids accumulation) were kept constant (glucose at 100 g/L and $CaCl_2.2H_2O$ at 0.11 g/L).

Initially, *R. toruloides* was cultured in the biomass production optimized medium to reach 20 g/L of biomass. The cells were then aseptically recovered by centrifugation and resuspended in a fresh medium (L.1–L.12) suitable for lipids accumulation (limiting N, P and S sources individually or jointly) according to the experimental design (assays were done in duplicate). Statgraphics Centurion XVI.II software was used to carry out the experimental designs and statistical analysis of the data.

Lipid production by *R. toruloides* in optimized culture medium

A final experiment was performed in order to validate the information obtained from the previous experimental design, where the optimized culture medium for lipid production (Medium L.7) was used (g/L): glucose, 100; $NaNO_3$, 0.1; KH_2PO_4, 4.5; $MgSO_4 \cdot 7H_2O$, 0.1; and $CaCl_2 \cdot 2H_2O$, 0.11. *R. toruloides* was first cultured in the biomass production optimized medium (Medium B.3), in g/L: glucose, 100; KH_2PO_4, 4.6; $NaNO_3$, 13.4; $MgSO_4 \cdot 7H_2O$, 0.2; and $CaCl_2 \cdot 2H_2O$, 0.11, to reach about 20 g/L of biomass. Then, the biomass were aseptically recovered by centrifugation and resuspended in fresh L.7 medium. A 1-L shake flask (with 250 mL of medium) was used, and incubated at 30 °C and 150 rpm. Control of pH was not required due to this parameter presenting minimal variation when using nitrate as a nitrogen source. Samples were withdrawn every 24 h and analyzed for biomass production, lipid content, and glucose concentration (assays were carried out in duplicate).

Glucose, biomass and lipids assays

Culture samples (5 mL) were centrifuged at 4000 rpm for 15 min. The supernatant was recovered for glucose analysis by DNS assay (Gong et al. 2016) while the pellets were washed twice with distilled water and used for biomass and lipids quantification.

For biomass quantification, the washed pellets were dried in pre-weighted tubes to a constant weight in an oven set at 100 °C for 24 h. A standard curve of biomass was established by plotting optical density at 600 nm as a function of dry cell weight.

For total lipids quantification, a lipidic extraction of biomass was performed by adding 0.5 mL of 0.1 N HCl in glass tubes and incubating at 100 °C in a water bath for 30 min. After cooling, 5 mL of isopropanol:hexane:water mixture (55:20:25) was added. Tubes were tightly closed and incubated at 60 °C and 200 rpm for 15 min. Tubes were then centrifuged at 4000 rpm for 15 min, and the upper layer (containing the lipids) was transferred to clean and dry glass tubes. This extraction procedure was repeated three times (from one single time course), the solvents were evaporated and the lipidic extract redissolved in 5 mL of hexane. Total lipids were quantified by the Sulfo-phospho-vanillin method (Yamada et al. 2017). A calibration curve was prepared using *R. toruloides* lipidic extracts (dissolved in hexane) in concentrations from 0 to 2.2 g/L.

Results

Designing a suitable mineral culture medium for *R. toruloides* growth

Growth, glucose consumption and pH were evaluated by the kinetics of *R. toruloides* using two mineral media containing the same N concentration (4.4 g/L), but supplemented in different N-sources: 21 g/L of $(NH_4)_2SO_4$ (Medium A) or 26.7 g/L of $NaNO_3$ (Medium B). Both media contained 20 g/L of glucose, and they were also compared to that kinetic using YPD, also containing 20 g/L of glucose (Fig. 1). Maximal biomass productions of 3.68 and 10.27 g/L were reached at 72 and 90 h of culture, using Medium A and Medium B, respectively (Fig. 1a). When *R. toruloides* stopped growing, glucose consumption was around 50 and 90%, using Medium A and Medium B, respectively (Fig. 1b). Biomass yields were also higher using Medium B than Medium A ($Y_{X/S}$ = 0.53 and 0.37, respectively). It is important to note that a faster growth rate (μ_{MAX}) was obtained using Medium A (0.135 h^{-1}), rather than Medium B (0.065 h^{-1}), suggesting that *R. toruloides* has preference in consuming ammonium instead of nitrate. However, using ammonium as a nitrogen source, growth and glucose consumption were prematurely stopped. This phenomenon could be explained by the pH changes observed during *R. toruloides* cultures (Fig. 1c). In fact, the pH of Medium A gradually fell from 5.5 to 3.1 in 96 h of culture. This medium acidity could have inhibited *R. toruloides* growth. In contrast, the pH of Medium B increased from 5.5 to 6.4 in 66 h of culture and maintained this level until the cultivation ended (120 h). Therefore, nitrate was selected as the nitrogen source for further studies and YPD was discarded because of the null feasibility of using it in a large-scale lipid production process.

Optimizing the culture medium for improving biomass production

The kinetics of biomass production and glucose consumption of *R. toruloides* were performed for the different initial glucose concentrations (from 20 to 300 g/L) in mineral medium with $NaNO_3$ (26.7 g/L), and the results are shown in Table 1. For initial glucose concentrations less than or equal to 100 g/L, biomass production increased as glucose concentration increased. For these cultures, glucose was almost exhausted in less than 160 h, which suggested that *R. toruloides* growth was only limited by carbon source (glucose) and not by other media components. In contrast, for initial glucose concentrations of 200 and 300 g/L, total biomass production decreased and glucose was consumed in a lower percentage compared to the initial glucose concentration of 100 g/L.

The kinetic parameters (specific growth rates, biomass yield and biomass production) were calculated (based on the logistic growth model) from *R. toruloides* cultures for the media supplemented with different glucose concentrations. A maximum biomass production of 39.28 g/L was obtained using a glucose concentration of 100 g/L, while maximal values of biomass yield ($Y_{X/S}$ = 0.53) and specific growth rate (μ = 0.065 h^{-1}) were found at 20 g/L of glucose (Table 1). The high value of biomass production obtained in this work was superior to other reports for batch cultures in shake flasks, and similar to other fed batch cultures (Yen and Yang 2012; Liu et al. 2015). In Table 1, it can also be seen that, as glucose concentration increased, μ decreased, and that for glucose concentrations between 20 and 80 g/L, $Y_{X/S}$ varied from 0.48 to 0.53, while for concentrations between 100 to 300 g/L, $Y_{X/S}$ varied from 0.41 to 0.44.

The pH profiles (not shown) were similar to those presented in Fig. 1c, reaching at the culture end (314 h) an average pH of 6.4 ± 0.3, for cultures performed at 20, 40, 60, 80 and 100 g/L of glucose, and pH of 5.4 ± 0.1 at 200 and 300 g/L of glucose (Table 1). These results confirmed a slight medium alkalization of *R. toruloides* cultures for initial glucose concentrations less than or equal to 100 g/L, while pH was relatively constant for cultures performed at 200 and 300 g/L of glucose (the initial pH was 5.5). An initial glucose concentration of 100 g/L was chosen for further experiments, due to the highest biomass production obtained.

Table 2 shows the results of *R. toruloides* growth obtained by modifying $NaNO_3$, KH_2PO_4 and $MgSO_4 \cdot 7H_2O$ concentrations in the culture media, according to the Box–Behnken

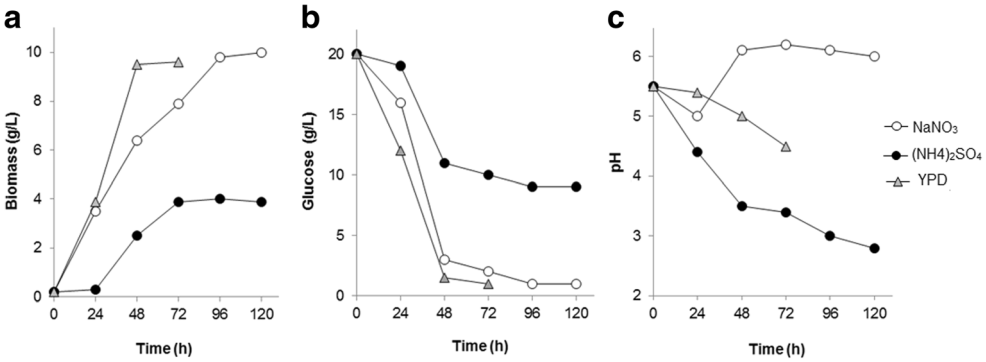

Fig. 1 Kinetics of biomass production (**a**), glucose consumption (**b**) and pH (**c**) of *R. glutinis* batch culture (30 °C, 150 rpm), using different nitrogen sources and glucose as carbon source (20 g/L)

Table 1 Kinetic parameters calculated from *R. glutinis* cultures in a chemically defined medium ($NaNO_3$ as nitrogen source), containing different glucose concentrations

S_0	X_{MAX}	μ_{MAX}	$Y_{X/S}$	Final pH
20	10.23 ± 0.44	0.065 ± 0.015	0.53 ± 0.05	6.8 ± 0.2
40	20.37 ± 0.8	0.035 ± 0.009	0.48 ± 0.09	6.4 ± 0.2
60	31.36 ± 0.95	0.030 ± 0.010	0.53 ± 0.11	6.4 ± 0.2
80	36.48 ± 1.41	0.018 ± 0.005	0.49 ± 0.04	6.3 ± 0.3
100	39.28 ± 0.48	0.025 ± 0.002	0.43 ± 0.01	6.1 ± 0.1
200	33.22 ± 0.37	0.014 ± 0.003	0.41 ± 0.01	5.5 ± 0.1
300	19.07 ± 0.87	0.014 ± 0.002	0.44 ± 0.03	5.4 ± 0.1

Final pH was reported at the culture end (314 h). Initial pH was 5.5

S_0 Initial glucose concentration (g/L), X_{MAX} maximum biomass concentration (g/L), μ_{MAX} maximum growth rate (h^{-1}), $Y_{X/S}$ biomass yield (g biomass produced/g glucose consumed)

design. The exponential growth of *R. toruloides* stopped at 192 h for all cultures, reaching maximal biomass concentrations fluctuating between 30.9 and 44.3 g/L for the evaluated $NaNO_3$, KH_2PO_4 and $MgSO_4{\cdot}7H_2O$ concentrations.

The maximal biomass produced in each culture medium (B.1–B.15) is shown in Table 2. It was observed that biomass production increased as KH_2PO_4 concentration increased and $NaNO_3$ concentration decreased. On the other hand, at the $MgSO_4{\cdot}7H_2O$ concentrations studied, this compound was not significant. Maximal experimental and predicted biomass productions (44.3 and 43.24 g/L, respectively) were obtained at 13.4 g/L $NaNO_3$, 4.6 g/L KH_2PO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$ and 0.11 g/L $CaCl_2{\cdot}H_2O$ (Medium B.3 in Table 2). This biomass production value was 12% superior to that obtained under no optimized conditions (39.28 g/L at S_0 = 100 g/L in Table 1). The maximum predicted biomass concentration can be easily seen in the response surface plot from the Box–Behnken design (Fig. 2); at that point, the calculated values of μ_{MAX} and $Y_{X/S}$ were 0.44 h^{-1} and 0.03, respectively. The significance of each variable and their interactions was additionally evaluated by analysis of variance (ANOVA). The simple effect of $NaNO_3$, the interaction effect of $NaNO_3{\cdot}KH_2PO_4$ and the quadratic effects of $NaNO_3$, KH_2PO_4 and $MgSO_4{\cdot}7H_2O$ were significant (P value <0.05) on the biomass production of *R. toruloides* (Supplementary Table 3).

Table 2 *R. glutinis* biomass production in culture media with 100 g/L of glucose and different concentrations (g/L) of $NaNO_3$, KH_2PO_4, and $MgSO_4{\cdot}7H_2O$, according to the Box–Behnken experimental design

Medium	$NaNO_3$	KH_2PO_4	$MgSO_4{\cdot}7H_2O$	X_{MAX}	μ_{MAX}	$Y_{X/S}$
B.1	13.40	1.4	0.2	38.6	0.028	0.49
B.2	40.10	1.4	0.2	35.6	0.027	0.44
B.3	13.40	4.6	0.2	44.3	0.030	0.44
B.4	40.10	4.6	0.2	33.3	0.024	0.33
B.5	13.40	3.0	0.1	37.0	0.028	0.53
B.6	40.10	3.0	0.1	30.9	0.030	0.54
B.7	13.40	3.0	0.3	37.1	0.018	0.51
B.8	40.10	3.0	0.3	30.9	0.022	0.54
B.9	26.75	1.4	0.1	33.3	0.029	0.52
B.10	26.75	4.6	0.1	32.1	0.031	0.37
B.11	26.75	1.4	0.3	31.7	0.025	0.43
B.12	26.75	4.6	0.3	32.4	0.022	0.39
B.13	26.75	3.0	0.2	34.0	0.024	0.38
B.14	26.75	3.0	0.2	33.0	0.022	0.50
B.15	26.75	3.0	0.2	32.3	0.023	0.51

X_{MAX} Maximum biomass concentration (g/L), μ_{MAX} maximum growth rate (h^{-1}), $Y_{X/S}$ biomass yield (g biomass produced/g glucose consumed)

The equation fitting the biomass production was established as follows:

$$Y_1 = 33.12 - 3.29a + 3.22a^2 - 2ab + 1.62b^2 - 2.36c^2$$

where Y_1, a, b and c are the biomass, $NaNO_3$, KH_2PO_4 and $MgSO_4{\cdot}7H_2O$ concentration (in g/L), respectively. For this model, the calculated (predicted) coefficient of determination (R^2 = 0.97) guaranteed that the fit to the data was accurate. This mathematical model was used to generate the response surface plot (Fig. 2). Remarkably, the biomass production of *R. toruloides* reached in this work by the Box–Behnken design (44.3 g/L) was higher than other batch cultures reported elsewhere for this yeast in shake flasks (Braunwald et al. 2013; Kolouchová et al. 2016a, b).

Determining the effect of N, P and S limitation on lipids accumulation by *R. toruloides*

Lipid accumulation in nutrient-limited culture media (L.1-L.12) is shown in Table 3. The combination of factors (components concentration in the culture medium) had a significant effect on lipids accumulation, since values ranged from 50.52

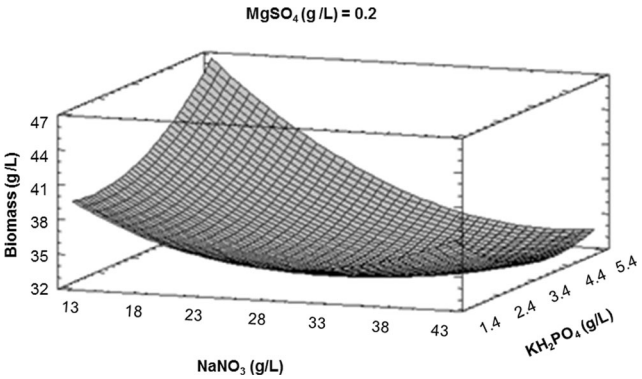

Fig. 2 Response surface plot from the Box–Behnken design, showing the predicted effects of $NaNO_3$ and KH_2PO_4 concentration (g/L) on *R. glutinis* biomass production, with $MgSO_4{\cdot}7H_2O$ concentration kept constant (at 0.2 g/L)

Table 3 *R. glutinis* lipid accumulation (% of dry cell biomass) in nutrient-limited culture media, prepared with 100 g/L of glucose and different concentrations (g/L) of $NaNO_3$, KH_2PO_4 and $MgSO_4{\cdot}7H_2O$, according to a 2^3 experimental design with four central points

Medium	$NaNO_3$	KH_2PO_4	$MgSO_4{\cdot}7H_2O$	Lipid accumulation
L.1	0.1	0.005	0.005	69.84
L.2	13.5	0.005	0.005	60.86
L.3	0.1	4.5	0.005	61.83
L.4	13.5	4.5	0.005	70.13
L.5	0.1	0.005	0.2	50.52
L.6	13.5	0.005	0.2	65.42
L.7	0.1	4.5	0.2	77.04
L.8	13.5	4.5	0.2	58.85
L.9	6.8	2.3	0.097	59.69
L.10	6.8	2.3	0.097	59.40
L.11	6.8	2.3	0.097	60.38
L.12	6.8	2.3	0.097	61.97

to 77.04%. As far as we are concerned, 77.04% is the highest value reported for lipid accumulation in *R. toruloides*. This maximum lipids accumulation was obtained using the medium with the following concentrations (g/L): glucose, 100; $NaNO_3$, 0.1; KH_2PO_4, 4.5; $MgSO_4{\cdot}7H_2O$, 0.2; and $CaCl_2{\cdot}2H_2O$, 0.11, in which the limiting nutrient was the nitrogen source.

According to the ANOVA for the results from the 2^3 factorial design (Supplementary Table 4), the simple effects of KH_2PO_4 and $MgSO_4{\cdot}7H_2O$, the interaction effects of $NaNO_3{\cdot}KH_2PO_4$ and $KH_2PO_4{\cdot}MgSO_4{\cdot}7H_2O$; and the triple effect $KH_2PO_4{\cdot}NaNO_3{\cdot}MgSO_4{\cdot}7H_2O$ were significant (P value <0.05) on the lipids accumulation of *R. toruloides*.

The following equation predicting the lipids accumulation of *R. toruloides* was developed from the experimental design analysis, based on a first-degree model:

$$Y_2 = 69.59 - 0.76a - 2.2b - 210.61c + 0.33ab + 19.29ac + 83.59bc - .9.03abc$$

where Y_2 is the lipids accumulation (%), and a, b and c are $NaNO_3$, KH_2PO_4 and $MgSO_4{\cdot}7H_2O$ concentrations (in g/L), respectively. For this model, the calculated (predicted) coefficient of determination ($R^2 = 0.91$) guaranteed that the fit to the data was correct.

In the present study, the culture media deprived only of phosphorous (L.6 medium) or sulfur sources (L.4 medium) presented lipids accumulation of 65.42 and 70.13%, respectively (Table 3). And when the three nutrients are simultaneously limited (L.1 medium), lipids accumulation reached 69.84%, while the treatments limiting two of the medium components resulted in lower lipid accumulation values (from 50.52 to 61.97%) (Table 3). Limited media in nitrogen, phosphorous or sulfur sources (L.7, L.6 and L.4, respectively) as well as the altogether limited medium (L.1) resulted in 31, 11, 19 and 18% more lipids accumulation, respectively, in comparison with the lipid accumulation value obtained in the basal medium (L.8, 58.85%).

Lipid production by *R. toruloides* in optimized culture medium

In order to validate the results obtained from the previous lipid optimization experiment, medium L.7 (glucose, 100; $NaNO_3$, 0.1; KH_2PO_4, 4.5; $MgSO_4{\cdot}7H_2O$, 0.2; and $CaCl_2{\cdot}2H_2O$, 0.11) was inoculated with 21 g/L of *R. toruloides* biomass and the kinetics of lipid accumulation was followed. The results are depicted in Fig. 3. It was observed that, at the beginning of the accumulation process, biomass lipid content was around 10%, and after 120 h this value increased 5 times, reaching around 50%. From 120 to 260 h, the lipid accumulation was slower, increasing up to 72% ± 4%. This values correlate with the model prediction of around 77% of lipid accumulation. Almost 90% of the glucose was consumed at the end of the fermentation, and it was also observed that biomass (without lipids) remained almost constant during the culture, and therefore the increment in total biomass was due the accumulation of lipids.

Discussion

Numerous reports concerningthe biomass production of *R. toruloides* have been performed using ammonium sulfate as the nitrogen source; however, the media acidification during the culture (observed in this study) has rarely been addressed. On the other hand, reports using nitrate as the nitrogen source to produce biomass and lipids of oleagionous yeasts are very scarce (Latha et al. 2005; Easterling et al. 2009; Ferrao and Garg 2011; Zhu et al. 2012). The present study's results have shown that nitrate is a convenient nitrogen source favoring *R. toruloides* growth, since the maximum biomass production reached in Medium B (10.27 g/L) was similar to that obtained using YPD (9.71 g/L), a complex medium. Nevertheless, the growth rate was the sole difference observed when culturing *R. toruloides* in YPD and Medium B (Fig. 1). The rapid growth in YPD seemed to be related to the presence of growth factors contained in the yeast extract and peptone (i.e. amino acids and vitamins). The biomass production levels obtained with Medium B and YPD were similar to those obtained by Liu et al. (2015) for a mineral medium supplemented with ammonium sulfate (4 g/L) and corncob hydrolysate (containing 40.1 and 2.3 g/L of

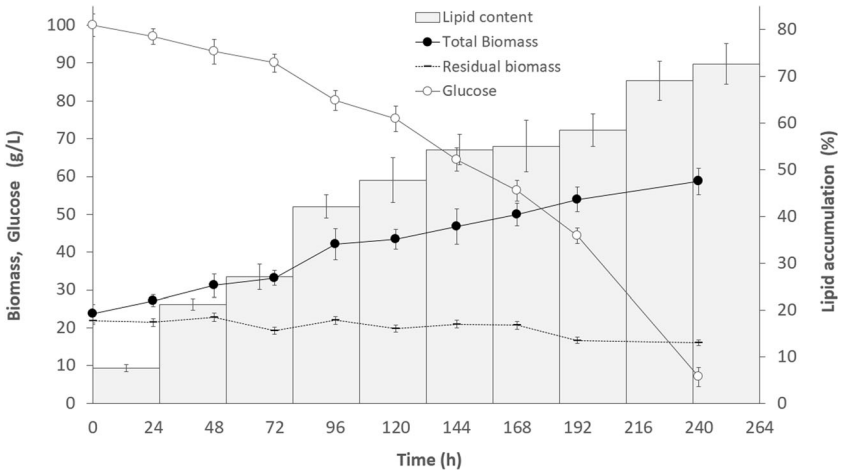

Fig. 3 Kinetics of lipids accumulation by *R. glutinis* in optimized culture medium (L.7) with 100 g/L of glucose as the carbon source

xylose and glucose, respectively) in *R. glutinis* cultures performed in shake flasks (11.7 ± 0.73 g/L of biomass).

When cultured in high glucose concentrations (100–300 g/L), it is worth noting that *R. toruloides* was able to grow, revealing its remarkable osmotolerance (Singh et al. 2016), though in general biomass yield decreased as initial glucose concentration increased, suggesting higher substrate utilization to synthetize compatible solutes to balance intra- and extracellular osmotic potential. Tchakouteu et al. (2017) studied *R. toruloides* for lipid production using high initial glucose concentration performing batch-bioreactor experiments and found that using up to 150 g/L of glucose resulted in satisfactory substrate assimilation by the yeast. Wiebe et al. 2012, reported 37 g/L as the highest *R. toruloides* biomass production with an initial glucose concentration of 100 g/L in batch culture, which is slightly less than we obtained in the present study (44.3 g/L).

Concerning oil accumulation, it is well known that lipids synthesis by oleaginous microorganisms is promoted by supplying an excess of the carbon source and limiting an essential nutrient for growth.

Most studies on oleaginous yeasts have been performed by limiting the nitrogen source in the culture media, reaching lipid contents up to 66% (Ageitos et al. 2011). Particularly for *R. toruloides* in batch culture under nitrogen limitation, and using glucose as carbon source (100 g/L), Wiebe et al. (2012) reported lipids accumulation around 50%. Shen et al. (2017) also studied this yeast under nitrogen limitation, but in continuous culture with different dilution rates, and found lipids accumulation up to 41.9%, while Tchakouteu et al. (2017) obtained 71.3% when combining nitrogen limitation with the addition of 4% (w/v) NaCl. On the other hand, a few studies have been performed in oleaginous yeasts by limiting other nutrients rather than the nitrogen source (Wu et al. 2010, 2011). In this regard, Granger et al. (in Patel et al. 2017) studied the individual effect of the limitation of the phosphorous source in the culture medium of *R. glutinis*, finding poor lipids accumulation (18.2%). In contrast, *R. toruloides* was studied for oil production under individual limitations of phosphorous or sulfur source cultures, and lipids accumulations of 58.3 and 62.2%, respectively, were reported (Wu et al., 2010, 2011), those values being lower than obtained in the present study: 65.42 and 70.13% for phosphorous or sulfur limitation, respectively.

Studies on the effect of the simultaneous limitation of nitrogen, phosphorous and sulfur sources on lipids biosynthesis by this strain have not previously been reported as far as we know, but in the present study it was observed that individual or multiple nutrient limitation could be an effective strategy to reach high oil production by this yeast. Such lipid accumulation enhancement was due the modifications of nutrient availability in the culture medium, which may cause metabolic deviations, while the carbon flux can be preferentially oriented to lipid synthesis in oleaginous microorganisms (Patel et al. 2017). On this subject, Xu et al. (2017) mentioned that the influence of some inorganic salts on lipid production by yeasts has been previously reported, and that, in particular, $MgSO_4$ and KH_2PO_4 have an influence on the lipid accumulation of *R. toruloides*. They mention that enzymes involved in lipid synthesis are affected by metal ions, such as the ATP-citrate lyase, malic enzyme, and isocitrate dehydrogenase.

Regarding the application of statistical experimental design methods for improving lipids accumulation in oleaginous yeasts, this has been used for *Lipomyces starkeyi* (Zhao et al. 2008), *R. glutinis* cultured in palm oil mill effluents (Saenge et al. 2011) and in *Trichosporon fermentans* (Huang et al. 2012). These studies resulted in optimized media, which, compared with initial media (before optimization), gave 1.59-, 1.67- and 1.33- fold improvement for lipids production of *L. starkeyi*, *R. glutinis* and *T. fermentans*, respectively. These improvement values were similar to that obtained in our study (1.31-fold).

Conclusion

Nitrate was revealed as a convenient nitrogen source for *R. glutinis* cultures without pH control, allowing the yeast to grow efficiently in a simple chemically defined medium at a high initial glucose concentration. Experimental design methodology showed this to be a successful tool for improving lipid production by this oleaginous yeast, demonstrating that, besides nitrogen limitation, phosphorous, sulfur, or combined nutrients limitation could also be used to promote high lipid accumulation in *R. glutinis*.

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