Journal Pre-proof

Improvement of carotenoids production from *Rhodotorula glutinis* CCT-2186

Cassamo Ussemane Mussagy (Conceptualization) (Investigation) (Formal analysis) (Data curation)<ce:contributor-role>Writing-Original draft preparation), Ana Alice Carizia Guimarães (Investigation), Lara Vicente Ferreira Rocha (Investigation), James Winterburn (Conceptualization) (Methodology)<ce:contributor-role>Writing-Review-editing), Valéria de Carvalho Santos Ebinuma (Conceptualization)<ce:contributor-role>Writing-Review-editing) (Visualization) (Supervision) (Project administration) (Funding acquisition), Jorge Fernando Brandão Pereira (Conceptualization)<ce:contributor-role>Writing-Review-editing) (Visualization) (Supervision) (Project administration) (Funding acquisition)

PII: S1369-703X(20)30381-8

DOI: https://doi.org/10.1016/j.bej.2020.107827

Reference: BEJ 107827

To appear in: *Biochemical Engineering Journal*

Received Date: 21 July 2020

Revised Date: 8 October 2020

Accepted Date: 10 October 2020

Please cite this article as: Mussagy CU, Guimarães AAC, Rocha LVF, Winterburn J, Ebinuma VdCS, Pereira JFB, Improvement of carotenoids production from *Rhodotorula glutinis* CCT-2186, *Biochemical Engineering Journal* (2020), doi: https://doi.org/10.1016/j.bej.2020.107827

Improvement of carotenoids production from *Rhodotorula glutinis* CCT-2186

Cassamo Ussemane Mussagy[1,2], Ana Alice Carizia Guimarães[1], Lara Vicente Ferreira Rocha[1], James Winterburn[3], Valéria de Carvalho Santos Ebinuma[1*], Jorge Fernando Brandão Pereira[1,4*]

[1]Department of Engineering Bioprocesses and Biotechnology, School of Pharmaceutical Sciences, Sao Paulo State University (UNESP), Araraquara,14800-903, SP, Brazil

[2]CICECO-Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193, Aveiro, Portugal

[3]Department of Chemical Engineering and Analytical Science, The University of Manchester, Manchester, United Kingdom

[4]Univ Coimbra, CIEPQPF, Department of Chemical Engineering, Rua Sílvio Lima, Pólo II – Pinhal de Marrocos, 3030-790 Coimbra, Portugal

---

*Corresponding Authors:

Valéria Carvalho Santos-Ebinuma – Email: valeria.ebinuma@unesp.br

Jorge Fernando Brandão Pereira – Email: jfbpereira@eq.uc.pt

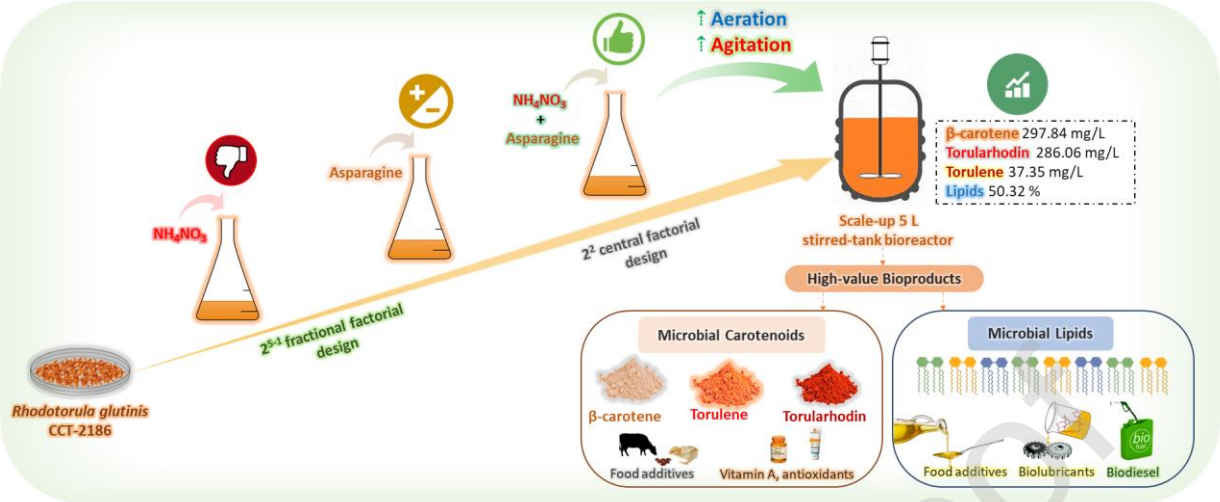
Department of Engineering Bioprocesses and Biotechnology, School of Pharmaceutical Sciences, Sao Paulo State University (UNESP), Araraquara,14800-903, SP, Brazil

Rodovia Araraquara-Jaú/ Km 01, Campos Ville - Araraquara/SP

14800-903 - Araraquara - SP/Brazil

Phone: +55-16-3301-4647

Graphical abstract



Highlights

- The production of carotenoids by *Rhodotorula glutinis* were optimized applying two statistical designs.
- *R. glutinis* produced simultaneously carotenoids (β-carotene, torularhodin and torulene) and lipids.
- Combined supplementation with organic and inorganic nitrogen sources have a key role in the production of carotenoids.
- The production yield of biomass and carotenoids were increased in the stirred-tank bioreactor.

Abstract

Rhodotorula strains can produce industrial valuable bioproducts. In this work, the production of carotenoids, and lipids by *Rhodotorula glutinis* using different nitrogen sources was evaluated. Two statistical experimental designs were applied to improve carotenoid production: a first 2^{5-1} fractional factorial design evaluating the influence of independent variables pH, nitrogen source, glucose, KH_2PO_4 and $MgSO_4$ concentrations; a second 2^2 central factorial design to optimize the effect of

pH and nitrogen sources. After the optimization using two statistical designs, a culture media composed of (in g/L) glucose (10), asparagine (10), NH_4NO_3 (4), KH_2PO_4 (0.52), $MgSO_4$ (0.52) was found as the best for the production of carotenoids at a pH 5 and 30 ºC. The best bioprocess was scaled-up to a 5 L stirred-tank bioreactor. The change to a bioreactor allowed to improve aeration and agitation conditions, and consequently, increasing the production yields (m/v) in, approximately, 25.83%, 11.88%, 24.50% and 10.32% for β-carotene, torularhodin, torulene and lipids, respectively. The combined supplementation of the culture media with both organic (asparagine) and inorganic nitrogen (ammonium nitrate) sources was primordial for enhancing the carotenogenesis. *R. glutinis* are very efficient in the production of valuable carotenoids and lipids, presenting high potential of yeast for the industrial production of more sustainable cosmetic, pharmaceutical, and food products.

Keywords: *Rhodotorula glutinis*; production; carotenoids; lipids; yeast.

1. Introduction

Carotenoids are lipid-soluble pigments responsible for the yellow, orange and red colors [1]. These are widespread substances with important physiological roles, for example, as high-quality food color and feed additives, antioxidants, as well as an alleged role in the prevention of breast, cervical, ovarian, colorectal cancers and cardiovascular and eye diseases [2,3]. The global market for carotenoids reached $1.5 billion in 2017 with an expected market of $2.0 billion by 2022 [4]. The growing consumer interest in “more natural products”, due to the concern with synthetic pigments, has made the microbial production of carotenoids more favorable and sustainable alternative [5,6]. Several studies have been demonstrating the biotechnological potential of Rhodotorula yeast, suggesting it as one of the most promising microorganisms for industrial food, feed and pharmaceutical processes [7–10].

The yeast-based processes for obtaining carotenoids and lipids have been extensively studied and, as demonstrated for other microorganisms, with a strict dependence on production yields and growing conditions (e.g., nutritional content, pH, and temperature) [11]. The efficiency of carotenoids’ biosynthesis using R. glutinis is achieved by adjusting the composition of the cultivation medium [12], commonly, through the evaluation of individual effect of different carbon (e.g., such as sucrose and glucose) [13,14] or inorganic nitrogen sources [15,16]. However, the number of studies distinguishing the effect of inorganic and organic nitrogen sources on yeast carotenogenesis are scarce, particularly, when used cultivation medium with reduced carbon supplementation.

In any case, if the development of industrial processes for the production of microbial carotenoids is foreseen, compositional cultivation conditions are a minor part and other operational costs must also be considered for commercial viability [17]. For that purpose, scale-up studies using bench-scale bioreactors bring further advantages, such as simple operation and low energy consumption, appearing as a low-expensive solution to validate the best conditions for the production of microbial carotenoids using *R. glutinis* [12,17], as previously demonstrated [17–19].

Considering that the optimization of cultivation conditions is of upmost importance to maximize the biomass, carotenoids and lipids production yields, in this work, two-statistical optimization designs were applied to improve the production of carotenoids (β-carotene, torularhodin, and torulene) using *R. glutinis* CCT 2186 yeast, namely: a first 2^{5-1} fractional factorial design - to evaluate the influence of independent variables pH, nitrogen source (organic or inorganic), glucose, KH_2PO_4 and $MgSO_4$; and a second 2^2 central factorial design - to optimize the pH and nitrogen sources. As a final test to validate the industrial potential of *R. glutinis* in producing carotenoids and lipids, the condition optimized after the second statistical design was scaled-up in a 5 L stirred-tank bioreactor.

2. Materials and methods

2.1 Materials

β-carotene and torularhodin standards were acquired from Carbosynth (San Diego, CA, U.S.A). Dimethyl sulfoxide (DMSO) (P.A) was acquired from Exodo Científica (Sumaré, SP, Brazil); peptone (bacteriological) and yeast extract were obtained from Kasvi (Sao Jose dos Pinhais, PR, Brazil), glucose (P.A) from Synth (Diadema, SP, Brazil); 3,5-dinitrosalicylic acid (DNS), potassium dihydrogen phosphate (KH_2PO_4) from Vetec (Rio de Janeiro, RJ, Brazil), magnesium sulfate ($MgSO_4$) and ammonium nitrate (NH_4NO_3) from Dinâmica (Indaiatuba, SP, Brazil) and asparagine (P.A) from Inlab (Sao Paulo, SP, Brazil). All the other reagents were of analytical grade and purchased from Exodo Científica (Sumaré, SP, Brazil).

2.2 Microorganism and growth conditions

Rhodotorula glutinis CCT 2186 yeast was acquired from the Tropical Culture Collection André Tosello (Campinas, SP, Brazil), isolated from the leaves of a kaki fruit (Diospyros). The inoculum was prepared by the activation of yeast *R. glutinis* CCT-2186 in Yeast Extract-Peptone-Dextrose (YPD) medium, with the following composition (g/L in deionized water): peptone (bacteriological) (20); yeast extract

(10); glucose (20). The inoculum culture was prepared in Erlenmeyer® type (100 mL) flasks containing 25 mL of the YPD medium. Cells were then grown for 48 h at 30 °C and 150 rpm using a temperature-controlled orbital shaker (Tecnal, model TE- 421, Piracicaba, SP, Brazil).

For the production of carotenoids and lipids, it was used a basal medium composed of (g/L in deionized water): glucose (10); KH_2PO_4 (0.52); $MgSO_4$ (0.52); NH_4NO_3 (4); and asparagine (10). The initial pH of the medium was adjusted to 5.0 by adding 2 mol/L of HCl or NaOH solution, before autoclaving. Erlenmeyer® type flasks (500 mL) containing 100 mL of the basal medium were sterilized at 121 ºC for 15 min in an autoclave (Tecnal®, model AV 30 (Piracicaba, SP, Brazil)). Afterwards, the flasks were inoculated with 5% v/v of a preculture (yeast in YPD medium) and incubated at 30 °C, 170 rpm for 72 h. Since carotenoids are intracellular, after cellular growth, the cells were then separated from the supernatant by centrifugation at 2500x*g* for 10 min at 4º C using a Hitachi CR-22N (Tokio, Japan) centrifuge. The supernatant was used to determine the residual glucose content, while cell pellets were used to quantify biomass content, *i.e.* cellular growth, carotenoid and lipid production. All the production assays using shake flasks were performed in duplicate. To optimize the production of carotenoids, the culture nutritional conditions were changed according the experimental design described in section 2.3.

2.3 Experimental design to optimize the production of carotenoids

A statistical design tool was used to improve the production of carotenoids by employing two statistical design approaches. In the first step, two 2^{5-1} fractional factorial (16 experimental runs plus 4 central points) designs were used to determine the influence of (A) glucose, (B) K_2HPO_4, (C) $MgSO_4$, (D) nitrogen source and (E) pH in the production of carotenoids, *i.e.* considering as response variables β-carotene, torularhodin and torulene. Although the amount of biomass and lipid produced by *R. glutinis* were determined for all experimental conditions, these were not considered as target-compounds and were not considered as response variables in the statistical design (*i.e.* this work has the aim to optimize *R.*

glutinis carotenogenesis). In the first fractional factorial design, the effect of NH_4NO_3 as inorganic nitrogen source was evaluated, whilst in the second experimental design the effect of an organic nitrogen source was considered, namely asparagine as a nitrogen source. The range and levels of the components used are detailed in **Table 1**.

From the results of the 2^{5-1} statistical design a second statistical design was performed, where the effects of pH and asparagine (most significant parameters in the first experimental design) for the production of β-carotene, torularhodin and torulene (response variables) were optimized in a 2^2 central composite design (the range and levels of the components detailed in **Table 2**). In these experiments NH_4NO_3 (at 4 g/L) was added and maintained constant in the cultivation media. All the experiments were performed in an orbital shaker at 30 °C and 170 rpm for 72 h, as detailed in the section 2.2.

Table 1. Variables and factor levels employed in the two 2^{5-1} fractional factorial designs for studying β-carotene, torularhodin and torulene production by *R. glutinis,* at 30 °C, 170 rpm for 72 h.

Factor	**Symbol**	**Range and levels**		
		-	0	+
Glucose (g/L)	A	5.0	12.5	20.0
K_2HPO_4 (g/L)	B	0.5	1.0	1.5
$MgSO_4$ (g/L)	C	0.5	1.0	1.5
Nitrogen source* (g/L)	D	2.0	4.0	6.0
pH	E	4	5.5	7.0

* In the first fractional factorial design the effect of NH_4NO_3 was evaluated, whilst in the second experimental design the effect of asparagine was studied.

Table 2. Variables and factor levels used in the 2^2 central composite design for studying β-carotene, torularhodin and torulene production by *R. glutinis*, at 30 °C, 170 rpm for 72 h.

Factor	**Range and levels**				
	Axial (-1,41)	Lower (-1)	Center (0)	Higher (+1)	Axial (+1,41)
pH	2.2	3.0	7.0	5.0	7.8
Asparagine (g/L)	0.0	5.0	10.0	20.0	20.6

* Fixed variables (g/L): Glucose (10), KH_2PO_4 (0.52), $MgSO_4$ (0.52), NH_4NO_3 (4)

Statistica® software Version 10.0 (StatSoft, Tulsa, OK, USA) was used for the regression analysis of experimental data and to estimate the corresponding regression coefficients. The quality of the fit of the polynomial model was

expressed by determination of the correlation coefficient, R^2, and statistical significance was validated by an F-test at a significance level of (p) ≤ 0.05 (in this work a confidence level of 95% was considered, considering the independent variables with p values lower than 0.05 as significant).

2.4 Production of carotenoids using a 5 L stirred-tank bioreactor

After the two optimizations of the production in orbital shaker, a 5 L bioreactor fermentation was performed to scale-up carotenoid production. The inoculum was prepared in Erlenmeyer® type (500 mL) flasks containing 100 mL of the YPD medium at 30 °C, 170 rpm for 48 h. Afterwards, an inoculum with a cell concentration of 0.2 mg/mL was transferred to a 5 L stirred-tank bioreactor (Tecnal®, model Tec-Bio-Flex (Piracicaba, SP, Brazil), equipped with a disc impeller, oxygen and pH electrodes) containing 4 L of the optimized cultivation medium from the 2^2 central factorial design, which was composed of (g/L in deionized water): glucose (10); asparagine (10); KH_2PO_4 (0.52); $MgSO_4$ (0.52); NH_4NO_3 (4). The cultivation medium was previously sterilized at 121 ºC for 15 min. The pH of the medium was adjusted to 5.0 at beginning of the fermentation, remaining constant throughout all the process. The fermentation was then conducted at 30 °C, 300 rpm and 1 vvm (air volume/medium volume/min) for 72 h. Antifoam was added as necessary. Samples were collected every 6 h for determination of glucose concentration, dry cell weight, lipids, β-carotene, torularhodin and torulene content.

2.5 Analytical Methods

2.5.1 Determination of residual sugars and dry cell weight

Total reducing sugars were determined spectrophotometrically with 3,5-dinitrosalicylic acid (DNS) method, as described by Miller [20], using a standard glucose calibration curve. Dry cell weight (DCW) of each sample was determined according to the method described by Buzzini and Martini [13]. Briefly, each sample of fermented broth was centrifuged at 2500 x*g* for 5 min, washed twice with

the same volume of distilled water, dried in petri dishes at 50 °C for 48 h, and then weighed in an analytical balance (Shimadzu, model AUY220, Sao Paulo, SP, Brazil) ($\pm 10^{-4}$ g) for determination of the DCW.

2.5.2 Extraction and determination of lipid content

To determine the lipid content of the yeast cells, lipids were extracted, dried and weighed by using a method adapted from Bligh and Dyer [21]. Briefly, the cells were harvested by centrifugation (2500 *xg* for 10 min at 10 °C), washed and dried to obtain constant weight. The weight of the dry pellet was measured in an analytical balance. The dry pellet was then resuspended in 30 mL of a chloroform/methanol (2:1 v/v) mixture and vortexed for 15 min at room temperature. Further, 10 mL of a NaCl aqueous solution (2 mol/L) was added, and the mixture centrifuged at 2500 x*g* for 15 min to separate the aqueous and organic phase. The organic phase was transferred to rotary evaporator flasks (previously weighed, w_1), which were dried under vacuum (at 300 mbar for 30 min) until the organic phase was fully evaporated, and the final weight of the flask measured (w_2). Lipid content, expressed as % dry cell weight *per* initial weight of the pellet (w) was determined according to Equation 1:

$$Lipid\ content\ (\%) = \frac{W_2 - W_1}{W} \times\ 100 \qquad \text{Equation 1}$$

In 5 L stirred-tank bioreactor, for comparison purposes the lipid concentration was evaluated in g/L.

2.5.3 Isolation, characterization, and quantification of carotenoids

Carotenoids from *R. glutinis* yeast were first isolated by using a conventional method described by Mussagy et al [22]. Briefly, *R. glutinis* wet biomass was subjected to chemical treatment with successive solvent extractions using DMSO. After the extraction, the samples were centrifuged at 2500 *xg* at 4 °C for 10 min and the supernatants containing carotenoids carefully recovered and lyophilized. Lyophilized carotenoids-rich extracts were solubilized in acetone (1 mL) and separated by liquid chromatography in a column filled with silica gel 60 (Merck,

Pinheiros, SP, Brazil) and a mixture of hexane/ethyl ether/acetic acid (70:29:1 v/v/v) as mobile phase. A qualitative analysis of the extracts by thin-layer chromatography (TLC) using pre-coated TLC sheets ALUGRAM (silica gel 60, Macherey-Nagel, Germany) was also carried out (a mixture of hexane/ethyl ether/acetic acid (70:29:1 v/v/v) used as mobile phase). The three fractions of the carotenoid lyophilized extracts separated in the column liquid chromatography were collected in different glass test tubes and then identified by reversed-phase high-performance liquid chromatography, RP-HPLC (using column chromatograph Shimadzu Shim-pack C_{18} (Japan), 4.6 mm x 250 mm and methanol/acetonitrile/dichloromethane (60:10:30, v/v/v) as the mobile phase). The chemical structures and purities of the three fractions of carotenoids were confirmed by proton nuclear magnetic resonance (^{1}H NMR) using a Bruker Avance III HD 600 (14.1T) (Massachusetts, USA).

After the identification and characterization of the chemical structures of carotenoids produced by *R. glutinis* yeast, the respective visible-light absorption spectra were determined using a Thermo Scientific (Genesis10S) UV−Vis spectrophotometer (accuracy: ± 0.005 AU at 1.0 AU) (China). The visible-light spectra from 380 to 600 nm were obtained, and the respective carotenoids calibration curves established at 450 nm (β-carotene), 480 nm (torulene), and 500 nm (torularhodin). The carotenoid concentrations (mg/L) were determined according to the pre-established calibration curves obtained from pure β-carotene and torularhodin standards, and from the purified fraction of torulene.

2.5.4 Extraction and determination of carotenoids

Carotenoid extraction was carried out according to the modified method of Mussagy et al [22]. After lipid extraction, the cell pellets were mixed with 5 mL of DMSO and disrupted by maceration (5 min ON/15 min OFF for a total 1 h). After the procedure the supernatant was recovered, and the procedure repeated until the cells become fully bleached. The supernatant recovered from the cell disruption was mixed with 10 mL of 20 % (w/v) of NaCl aqueous solution and 10 mL of petroleum ether. After the formation of both phases, the nonpolar phase was

collected and excess water was removed with sodium sulfate (Na_2SO_4), the carotenoid-rich extracts were then dissolved in acetone (1 mL), filtered with Polytetrafluoroethylene (PTFE) membrane of 0.22 μm pore size, and quantified using external calibration curves in a Thermo Scientific® UV-Vis spectrophotometer (model Genesis 10S, China). Since carotenoids are light-sensitive, all assays were performed by using sealed vials covered with aluminum foil to protect carotenoids from oxidation, photodegradation and isomerization.

3. Results and discussion

3.1 Optimization of the production of carotenoids by statistical design

The production of a microbial metabolite is largely influenced by the components of the growth medium, particularly, carbon, nitrogen and mineral sources, pH and temperature [23–25]. The use of experimental design methodology allows a reduction of the number of experiments and assess the interaction among the independent variables [26]. In the present work, two 2^{5-1} fractional factorial designs and one 2^2 central factorial design were applied to improve the production of three intracellular carotenoids (β-carotene, torularhodin and torulene) by *R. glutinis* CCT 2186.

In the first set of experiments, a 2^{5-1} design was applied to assess the significance of the following independent variables on carotenoid production, *i.e.*: glucose (A); K_2HPO_4 (B); $MgSO_4$ (C); NH_4NO_3 (D); pH (E). The 2^{5-1} design strategy is adequate for initial screenings, since it decreases the number of experiments (in comparison to the full factorial design), as well as it elucidates the main effects in the microbial processes, and allows the adjustment of the next statistical optimization strategy [27]. The matrix of the first 2^{5-1} fractional factorial design and corresponding experimental results of biomass, lipid and carotenoid production by *R. glutinis* are shown in **Table 3**.

Table 3. Matrix of 2^{5-1} fractional factorial design for study of β-carotene, torularhodin and torulene production by *R. glutinis* using NH_4NO_3 as nitrogen source, at 30 °C, 170 rpm for 72 h.*

Run	Design matrix					Experimental results				
	A	B	C	D	E	DCW (g/L)	Lipid content (% w/w)	**β- carotene (mg/L)**	**Torularhodin (mg/L)**	**Torulene (mg/L)**
1	-	-	-	-	+	2.71	42.04	**106.30**	**135.84**	**11.55**
2	-	-	-	+	-	6.06	21.75	**111.55**	**141.98**	**11.74**
3	-	-	+	-	-	3.96	4.95	**80.30**	**101.79**	**8.41**
4	-	+	-	-	-	5.58	13.66	**157.05**	**187.07**	**15.60**
5	+	-	-	-	-	2.67	29.78	**73.80**	**92.59**	**7.64**
6	-	-	+	+	+	3.13	18.05	**123.30**	**153.94**	**12.81**
7	-	+	-	+	+	3.06	24.08	**119.30**	**157.62**	**13.50**
8	-	+	+	-	+	5.02	20.33	**140.80**	**178.79**	**15.07**
9	-	+	+	+	-	2.33	26.45	**117.55**	**143.20**	**11.12**
10	+	+	+	-	-	4.84	3.46	**88.55**	**113.45**	**9.40**
11	+	+	-	+	-	4.32	21.13	**103.05**	**134.31**	**11.32**
12	+	+	-	-	+	5.30	10.06	**90.80**	**112.83**	**9.40**
13	+	-	+	-	+	3.70	4.87	**106.05**	**131.24**	**10.17**
14	+	-	+	+	-	3.50	48.99	**74.05**	**92.59**	**7.64**
15	+	-	-	+	+	3.71	37.23	**142.30**	**176.33**	**14.11**
16	+	+	+	+	+	3.60	38.53	**88.05**	**107.62**	**8.90**
17	0	0	0	0	0	4.65	9.40	**63.55**	**79.09**	**5.96**
18	0	0	0	0	0	4.68	6.60	**67.05**	**81.85**	**6.57**
19	0	0	0	0	0	4.57	7.24	**75.05**	**90.75**	**6.84**
20	0	0	0	0	0	4.74	8.61	**59.80**	**73.88**	**5.65**

*Glucose (A); K_2HPO_4 (B); $MgSO_4$ (C); NH_4NO_3 (D); pH (E)

From **Table 3**, among the 20 runs performed, it was observed that the DCW varied from 2.33 to 6.06 g/L, the lipid content from 3.46 to 48.99 %, whilst the production of carotenoids varied from 59.80 to 157.05 mg/L for β-carotene, 73.88 to 187.07 mg/L for torularhodin and 5.65 to 15.60 mg/L for torulene. The highest DCW concentration (6.06 g/L) was achieved in run 2 [(glucose (5.0 g/L), K_2HPO_4 concentration (1.5 g/L), $MgSO_4$ concentration (0.5 g/L), NH_4NO_3 concentration (0.5 g/L) and pH (4.0)], while the highest production of carotenoids and content of lipids occurred at run 4 [(glucose (5.0 g/L), K_2HPO_4 (1.5 g/L), $MgSO_4$ (0.5) NH_4NO_3 (2.0 g/L), and pH (4.0)] and run 14 [(glucose (20.0 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4$ (1.5 g/L), NH_4NO_3 (6.0 g/L), and pH (4.0)], respectively.

R. glutinis accumulate lipids and carotenoids, in which their biosynthesis share acetyl-CoA as common precursor, although these compounds have distinct metabolic pathways [28]. This observation is in line with our results, since the experimental conditions for obtaining the highest lipid content were different from those where an increase of carotenoid production yields was observed. In fact,

while lipids are produced and accumulated during the exponential growth of the microorganism, in which the consumption of carbon source is rapid, the carotenoids are secondary metabolites produced from the beginning of the yeast's stationary phase to the death phase [29]. Carbon/nitrogen (C/N) ratios of the growth medium influence the production of lipids, specially, affecting the activity of adenosine monophosphate (AMP) dependent isocitrate dehydrogenase in the tricarboxylic acid cycle (TCA) [30], and thus, channelizing the acetyl CoA for lipid biosynthesis. With the yeast growing, the C/N ratio is altered, with higher levels of nitrogen than carbon, which will activate the precursors for the production of carotenoids, usually, in the deceleration phase of the yeast (*i.e.* between the end of the exponential phase and the beginning of the stationary phase) [31]. A medium with a high C/N ratio tends to produce lipids rather than carotenoids, whilst the increase of Nitrogen tends to privilege the carotenogenesis [31].

Although the highest content of carotenoids was achieved in run 4, it is important to note that, among them, torularhodin (187.07 mg/L) was the main carotenoid produced, followed by β-carotene (157.05 mg/L) and with lower torulene content (15.60 mg/L). The low amount of torulene can be explained by a preferential production of torularhodin by the *R. glutinis*, which according to the their biosynthesis pathway, torularhodin is always obtained after transformation of the torulene, including, hydroxylation and oxidation [32,33].

As the main objective of this work is to evaluate carotenoid production by *R. glutinis,* the analysis of variance (ANOVA) of production of the three carotenoids production as variables response was performed. First order models were fitted to the experimental data in order to evaluate the significance of five factors (pH, NH_4NO_3, glucose, K_2HPO_4 and $MgSO_4$), as detailed in **Table 4**.

Table 4. Analysis of variance (ANOVA) applied to the regression models according to 2^{5-1} fractional factorial design to evaluate the influence of pH, NH_4NO_3, glucose, K_2HPO_4 and $MgSO_4$ on carotenoid production by *R. glutinis,* at 30 °C, 170 rpm for 72 h.

Factors	β- carotene			Torularhodin			Torulene		
	SS	*F*-ratio	*p*-value	SS	*F*-ratio	*p*-value	SS	*F*-ratio	*p*-value
Glucose	40.64	0.96	0.40	14.73	0.30	0.62	0.47	1.57	0.30
K_2HPO_4	907.52	21.45	0.02*	1435.08	28.79	0.01*	12.67	42.50	0.01*
$MgSO_4$	9.77	0.23	0.66	25.63	0.51	0.53	0.11	0.35	0.59
NH_4NO_3	650.25	15.36	0.03*	1191.11	23.90	0.02*	12.71	42.62	0.01*
pH	228.77	5.40	0.10	409.96	8.23	0.03*	4.12	13.82	0.03*
Pure Error	126.92			149.51			0.89		

*Significant at $p < 0.05$

The ANOVA statistical analysis of the results presented in **Table 4** shows that the production of β-carotene was significantly ($p < 0.05$) influenced by the concentration of K_2HPO_4 and NH_4NO_3, while the production of torulene and torularhodin, in addition to the concentration of salts, also were affect by the pH of the cultivation medium (*cf.* Pareto chart from **Fig. 1-A**). It is important to note that the inorganic source of nitrogen (NH_4NO_3) revealed a significant effect ($p < 0.05$) on the production of the three carotenoids within all the ranges explored in our experiments.

Interestingly, the yields of carotenoid production obtained were higher than those reported in previous studies, using inorganic nitrogen source in the culture medium [16,34]. Saenge et al., [16] used different inorganic nitrogen sources to produce carotenoids in a growth medium containing palm oil mill effluent (POME), producing 115.76 mg/L and 105.85 mg/L of carotenoid, with using ammonium sulfate and ammonium nitrate, respectively. Bhosale and Grade [34] also evaluate the influence of different ammonium salts as source of inorganic nitrogen in cultivation media containing molasses to produce carotenoids with *R. glutinis* mutant 32; however, regardless the ammonium-salt added, the concentration of total carotenoid produced were significant lower (*i.e.*, ammonium sulfate ≈ 14.14 mg/L, ammonium nitrate ≈ 8.8 mg/L and ammonium chloride ≈ 17.8 mg/L).

In order to increase the production of carotenoids, and considering that NH_4NO_3 had a significant effect (at a confidence level $p < 0.05$) on the production

of the three carotenoids, the next step was to determine whether changing from a source of inorganic nitrogen for an organic (in this case asparagine) has significant influence on the production yields. Asparagine was chosen as organic nitrogen source, based on previous work by our group [22], where the ability of this amino acid to stimulate the carotenogenesis in *R. glutinis* was observed. Except for the nitrogen source, all other growth conditions were the same as in the first 2^{5-1} factorial design. The main results of the production of biomass, lipids and carotenoids by *R. glutinis* obtained with the second 2^{5-1} fractional factorial design are detailed in **Table 5**.

Table 5. Matrix of 2^{5-1} fractional factorial design for study of biomass, lipid content, β-carotene, torularhodin and torulene production by *R. glutinis* using asparagine as nitrogen source, at 30 °C, 170 rpm for 72 h.

Run	**Design matrix**					**Experimental results**				
	A	B	C	D	E	DCW (g/L)	Lipid content (% w/w)	**β-carotene (mg/L)**	**Torularhodin (mg/L)**	**Torulene (mg/L)**
1	-	-	-	-	+	2.65	26.81	**121.91**	**124.87**	**14.21**
2	-	-	-	+	-	3.57	13.14	**190.52**	**203.31**	**23.50**
3	-	-	+	-	-	2.58	2.62	**140.37**	**161.87**	**18.42**
4	-	+	-	-	-	4.20	17.85	**166.54**	**171.74**	**19.14**
5	+	-	-	-	-	0.27	61.60	**124.16**	**129.87**	**14.65**
6	-	-	+	+	+	4.95	23.04	**72.84**	**71.11**	**6.03**
7	-	+	-	+	+	2.62	27.62	**110.87**	**115.99**	**13.09**
8	-	+	+	-	+	4.90	38.02	**130.17**	**136.25**	**15.29**
9	-	+	+	+	-	2.50	18.03	**100.04**	**104.87**	**11.65**
10	+	+	+	-	-	4.07	15.13	**140.17**	**147.37**	**16.83**
11	+	+	-	+	-	2.80	28.20	**132.04**	**137.00**	**16.17**
12	+	+	-	-	+	3.66	13.69	**172.04**	**176.99**	**20.30**
13	+	-	+	-	+	1.92	13.62	**114.04**	**120.12**	**14.06**
14	+	-	+	+	-	3.32	14.56	**177.27**	**188.37**	**21.40**
15	+	-	-	+	+	3.17	43.19	**112.54**	**121.12**	**13.81**
16	+	+	+	+	+	4.86	6.36	**201.92**	**155.46**	**9.38**
17	0	0	0	0	0	2.22	27.39	**137.92**	**137.37**	**14.06**
18	0	0	0	0	0	2.22	26.83	**117.67**	**118.62**	**12.90**
19	0	0	0	0	0	2.19	26.76	**123.42**	**129.37**	**14.10**
20	0	0	0	0	0	2.36	22.86	**126.54**	**129.49**	**12.56**

*Glucose (A); K_2HPO_4 (B); $MgSO_4$ (C); Asparagine (D); pH (E)

As can be seen in **Table 5**, the presence of asparagine (D) increased the production of the three intracellular carotenoids compared to the first fractional factorial design. Interestingly, the highest production rates were not achieved in the

same run, *i.e.*, the highest concentration of β-carotene (201.92 mg/L) was obtained in the run 16, while both highest concentration of torularhodin and torulene were obtained in run 2, with 203.31 mg/L and 23.50 mg/L, respectively. Comparing the carotenoid production rates using the source of organic nitrogen (**Table 5**) with those using an inorganic one (**Table 3**), there was an increase of 22.23%, 7.98% and 31.62% in the production of β-carotene, torularhodin and torulene, respectively. Despite the focus on the production of carotenoid, it is important to note that supplementation with asparagine also favored the production and accumulation of lipids by *R. glutinis*, reaching, in run 5, a production of approximately 61.60% of lipids, which corresponds to an increase of about 20% than in the absence of a source of organic nitrogen (first factorial design).

The experimental results of carotenoid production were subjected to analysis of variance (ANOVA) (**Table 6**), in which first order models were used to adjust the experimental data, evaluating the main effects of the five factors (pH, asparagine, glucose, K_2HPO_4 and $MgSO_4$).

Table 6. Analysis of variance (ANOVA) applied to the regression models according to 2^{5-1} fractional factorial design that evaluated the influence of pH, asparagine, glucose, K_2HPO_4 and $MgSO_4$ on carotenoid production by *R. glutinis*, at 30 °C, 170 rpm for 72 h.

Factors	β- carotene			Torularhodin			Torulene		
	SS	*F*-ratio	*p*-value	SS	*F*-ratio	*p*-value	SS	*F*-ratio	*p*-value
Glucose	5457.52	75.16	0.00*	3448.33	58.17	0.00*	15.62	24.91	0.02*
K_2HPO_4	985.02	13.57	0.03*	467.97	7.89	0.07	0.67	1.07	0.38
$MgSO_4$	897.30	12.36	0.03*	2249.84	37.95	0.01	66.06	105.35	0.00*
Asparagine	536.85	7.39	0.07	82.31	1.38	0.32	0.03	0.05	0.83
pH	470.67	6.48	0.08	2002.34	33.78	0.01*	56.66	90.37	0.00*
Pure Error	217.82			177.847			1.881		

*Significant at $p < 0.05$

The ANOVA analysis of **Table 6** and Pareto chart of **Fig. 1-B** show that, in the study range, K_2HPO_4, $MgSO_4$ and glucose concentrations had a significant effect (at 95% of confidence level) on the variable response β-carotene. Regarding torularhodin as a variable response, glucose concentrations and pH have significant effect, while for torulene, glucose, $MgSO_4$ concentrations and pH exhibited a significant effect with 95% of confidence level.

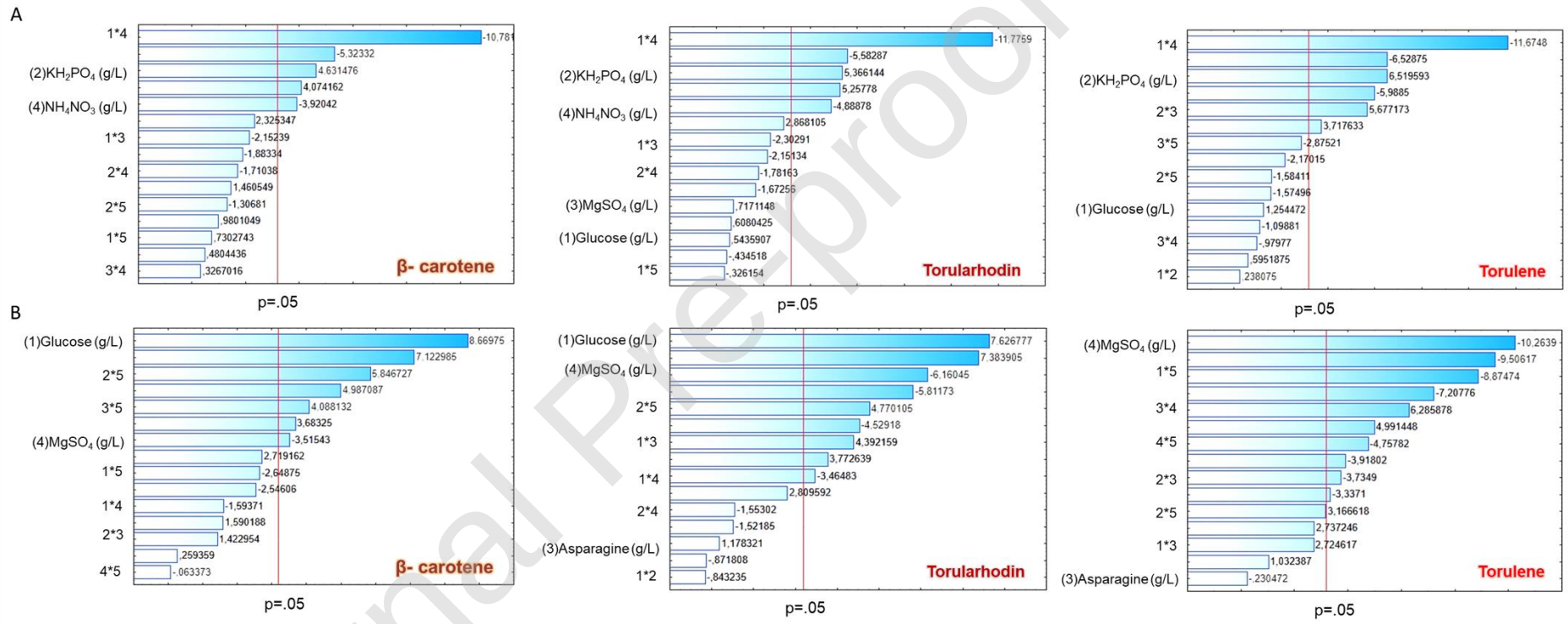


Fig. 1 Pareto chart for the effects of variables: (1) glucose, (2) K_2HPO_4, (3) $MgSO_4$, (4) nitrogen source (A: NH_4NO_3 and B: asparagine) and (5) pH under β- carotene, torularhodin and torulene production by *R. glutinis*, at 30 °C, 170 rpm for 72 h, according to the 2^{5-1} fractional factorial design.

The number of studies evaluating the effect of specific amino acids on the production of carotenoid using *R. glutinis* yeast are scarce [34–36], and among them with inconclusive results on the effects of amino acids on carotenogenesis. For example, Bhosale and Gadre [34] observed a decrease in the production of carotenoids by *R. glutinis* (mutant 32) with the supplementation of the growth media with amino acids (namely, threonine, glycine, aspartic acid, histidine, lysine, serine, tryptophan and tyrosine), while Voaides and Dima [36] achieved the highest carotenoid rates by cultivating *R. glutinis* strain Rd3 in amino acid-supplemented media (*i.e.*, using 0.1% (m/v) of glutamic acid ≈ 87.20 µg/g cell dry mass or 0.1% (m/v) of threonine ≈ 63.81 µg/g cell dry mass). Mihalcea et al., [35] also obtained a greater accumulation of carotenoids (≈ 1500 µg/L) when *Rhodotorula sp.* yeast were grown in a medium supplemented with threonine (0.2% (m/v)) and alanine (0.1% (m/v)).

Although the asparagine concentration had no significant effect (at 95% confidence level), compared to the first fractional factorial design (which used only NH_4NO_3 as nitrogen source), it was evident that the addition of a source of organic was positive for the carotenoid production, specially torularhodin and torulene, in which asparagine has significance as interaction parameter with the other variables. This is in line with the findings of Alcantara and Sanchez [37], who stated that the production of zeaxanthin by *Flavobacterium sp.* can be significantly improved (up to 4-fold increase) by combining organic nitrogen and carbon sources, in particular, supplementing the defined culture medium (NaCl; K_2HPO_4; $MgSO_4$ and inorganic salts) with L-asparagine and glucose.

The two initial 2^{5-1} statistical designs demonstrated that both inorganic and organic sources influence the production of carotenoids, but not exclusively, since the other experimental parameters exhibited significance in the three carotenoids as response variables. It is expected because the microbial production of carotenoids is very complex and depends on several factors, *e.g.*, nitrogen and carbon source, presence of microelements in the medium, temperature and pH [38].

Based on the results of the two-fractional factorial design, and considering that the use of sources of organic and inorganic nitrogen could allow an improvement in the carotenoids production rates, a 2^2 central factorial design was employed to evaluate the influence of pH and the simultaneous use of inorganic and organic nitrogen sources, as independent variables, in the production of carotenoids (β-carotene, torularhodin and torulene, as response variables). Industrially, high production titers are desired for most microbial-based processes, usually achieved by using high concentration of carbon source (*e.g.*, glucose) in the growth medium. The first optimization, like the previous literature [34], demonstrated that high concentrations of glucose allow high biomass production, but do not necessarily reflect high yields of yeast carotenoid biosynthesis. Therefore, in the second design, low concentrations of glucose (10 g/L) were used, first to reduce some negative effect on carotenogenesis that can occur at high carbon concentration and, second, to clearly elucidate the role of nitrogen in carotenoid production.

For the 2^2 central factorial design, a basal medium composed of (g/L in deionized water) glucose (10), KH_2PO_4 (0.52) and $MgSO_4$ (0.52) was used. All other cultivation parameters were maintained as the previous designs (*i.e.*, temperature at 30 °C and aeration at 170 rpm). This design evaluated the combination level of organic and inorganic nitrogen source, changing the concentration of asparagine (from 5.0 to 20.6 g/L) to a fixed NH_4NO_3 concentration (4 g/L), while the level of pH ranged from 2.2 to 7.8. All the details about the design matrix and corresponding experimental results *i.e.* DCW, lipid and carotenoids content, are detailed in **Table 7**.

Table 7. Experimental design matrix and experimental results for the 2^2 central factorial design, on carotenoid production by *R. glutinis*, at 30 °C, 170 rpm for 72 h.

Run	Design matrix		Experimental results				
	pH	Nitrogen source (g/L)	DCW (g/L)	Lipid content (% w/w)	**β- carotene (mg/L)**	**Torularhodin (mg/L)**	**Torulene (mg/L)**
1	3.0 (-1)	5.0 (-1)	3.67	38.73	**247.63**	**190.25**	**28.08**
2	3.0 (-1)	20.0 (1)	5.01	44.08	**193.15**	**183.4**	**21.78**
3	7.0(1)	5.0 (-1)	4.63	15.54	**223.98**	**208.49**	**25.78**
4	7.0 (1)	20.0 (1)	5.18	43.46	**163.40**	**156.83**	**14.47**
5	2.2 (-1.41)	10.0 (0)	5.16	41.06	**165.05**	**121.53**	**18.54**
6	7.8 (1.41)	10.0 (0)	5.48	33.92	**126.42**	**118.78**	**14.53**
7	5.0 (0)	0.0 (-1.41)	3.26	25.13	**128.55**	**117.23**	**14.96**
8	5.0 (0)	20.6 (1.41)	5.67	39.18	**190.91**	**183.31**	**20.95**
9 (C)	5.0 (0)	10.0 (0)	4.28	39.75	**248.25**	**216.61**	**28.26**
10	5.0 (0)	10.0 (0)	4.15	34.46	**253.33**	**218.48**	**28.45**
11	5.0 (0)	10.0 (0)	4.08	32.08	**249.12**	**216.09**	**28.26**
12	5.0 (0)	10.0 (0)	4.05	37.53	**252.01**	**220.93**	**28.20**

*Fixed variables (m/v): glucose (10 g/L), KH_2PO_4 (0.52 g/L), $MgSO_4$ (0.52 g/L), NH_4NO_3 (4 g/L). *C- Central point.

As can be seen in **Table 7**, regardless of the run, all conditions of the 2^2 experimental design allowed a significant increase in the production of the three carotenoids by *R. glutinis*. The maximum values of β-carotene (from 216.61 to 220.93 mg/L), torularhodin (from 248.25 to 253.33 mg/L) and torulene (from 28.20 to 28.26) concentration were obtained in the center point runs (9, 10, 11 and 12), corresponding to cultures supplemented with 10 g/L of asparagine, 4 g/L of NH_4NO_3 and pH 5. The combination of nitrogen source and pH also influenced the biomass production, especially the first variable, since the largest DCW (5.67 g/L) was achieved with the highest concentration of asparagine (20.6 g/L) - run 8.

Regarding the lipid content, the nitrogen combination (organic and inorganic) increased the lipid production (run 5), which, under same processual conditions, allowed a two-fold increase compared to cultivation with a single inorganic nitrogen source (run 2 from **Table 3**), and three-fold compared to the cultivation with an organic source (run 2 from **Table 5**). The same positive effect on production of carotenoids was observed for the other equivalent processual conditions, namely, in run 2 (**Table 7**) which exhibited an increase of 15.6% and 2.01% compared to cultivations using a single inorganic (run 15 of **Table 3**) and organic nitrogen source (run 15 of **Table 5**), respectively. Interestingly, the highest

production of lipids was obtained by cultivating *R. glutinis* in a medium rich in nitrogen sources and with minimal concentrations of carbon (10 g/L of glucose).

To obtain further information about the influence of each variable (organic and inorganic nitrogen supplementation and pH), as well as the interaction between both variables in the production of β-carotene, torularhodin and torulene (response variables), the Pareto charts and response surfaces ($p < 0.05$) were prepared, as depicted in **Figs. 2 A**, **B** and **C**, respectively.

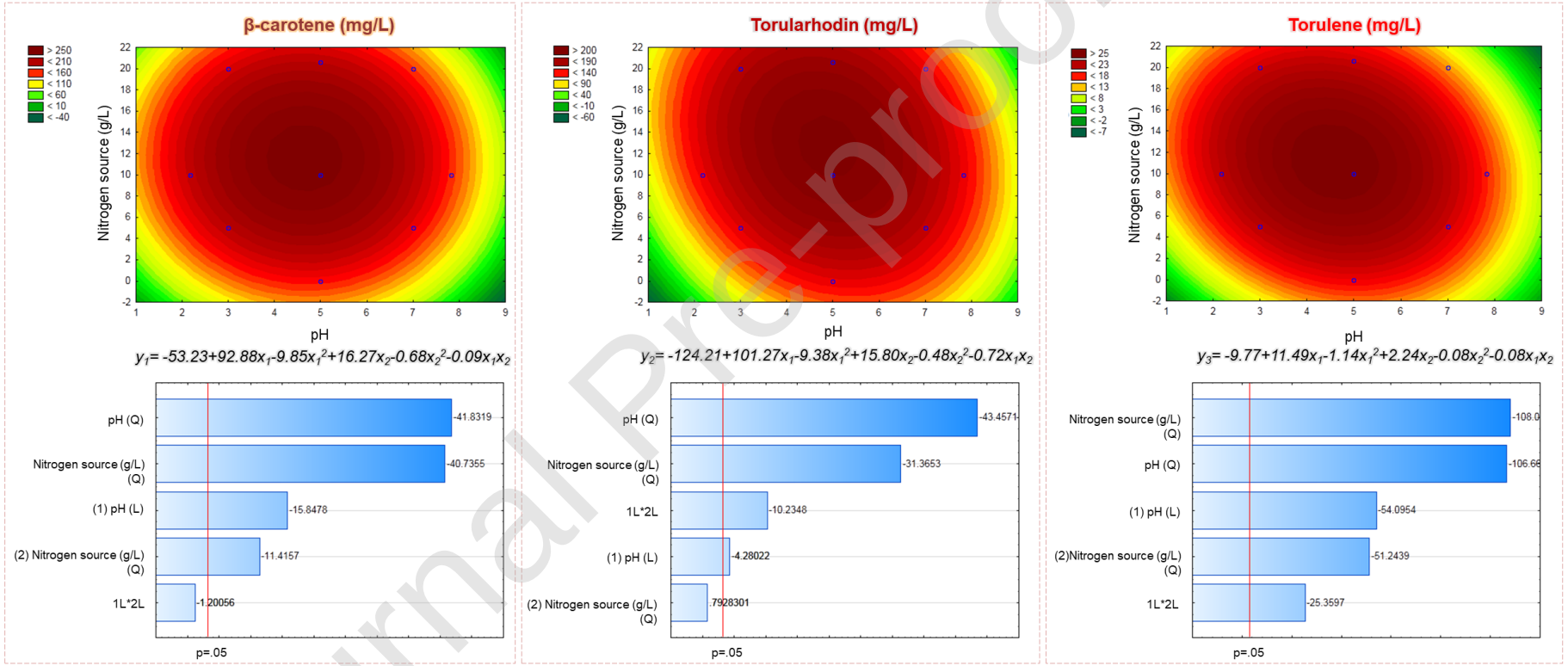


Fig. 2 Response surface and Pareto Chart for studies of the effects of independent variables x_1: pH and x_2: nitrogen source on y_1: β-carotene (A), y_2: torularhodin (B) and y_3: torulene (C) production by *R. glutinis* CCT-2186 in 72 h in an orbital shaker at 30 °C and 170 rpm.

As shown in **Figs. 2A**, **B** and **C**, for all the response variables, both pH and combined nitrogen source, in linear and quadratic terms, exhibited significant negative effects ($p < 0.05$), *i.e.*, in the study range, the simultaneous increase of organic and inorganic nitrogen concentration and pH caused a reduction in the production of carotenoids. **Table S1** in the **Electronic supplementary material** shows the probability values (*p*) for the β-carotene, torularhodin and torulene. Based on the results of the complete regressions, the models were determined to pH (x_1) and nitrogen combination (x_2) under the production of β-carotene (y_1), torularhodin (y_2) and torulene (y_3), as described by the equations shown in **Fig. 3**.

The production of secondary metabolites by *R. glutinis* is dependent on both pH and concentrations of nitrogen and carbon sources, which will have a direct impact on microbial carotenogenesis, namely on the type and amount of carotenoids synthesized by yeast cells [39]. So, as needed, different conditions can be usefully exploited to maximize the production of carotenoids.

In summary, from the results of **Fig. 2,** the central runs (*i.e.*, pH 5, 10 g/L of asparagine and 4 g/L of NH_4NO_3) were the best conditions for the simultaneous production of β-carotene (220.93 mg/L), torularhodin (253.33 mg/L) and torulene (28.26 mg/L). Comparing the optimized results from the 2^2 central factorial design (**Table 7**) with those from the second 2^{5-1} fractional design (**Table 4 -** run 16,), increases of 8.61%, 19.74% and 17.37%, respectively, for the production of β-carotene, torularhodin and torulene were achieved. It is evident that a combined supplementation of the cultivation medium with asparagine and NH_4NO_3 favors the carotenegenesis as well as lipid accumulation by *R. glutinis*. However, together, the optimized results also revealed that at higher pH values the yeast's ability to synthesize lipids is decreased, probably due to the inhibition of yeast growth.

3.2 Carotenoid production using a 5 L stirred-tank bioreactor

As shown in the previous section, the use of adequate statistical design approaches allows to find the optimal conditions for the microbial production of biomolecules, in this case intracellular carotenoids. However, mostly of the experiment designs are operated at lab scale, which are far from representative of the bioreactor-based processes at industrial scale. Thus, in order to provide

further information about the ability of *R. glutinis* to produce carotenoids at a larger scale, as final state the optimized culture media was tested in a 5 L stirred-tank bioreactor. After the three experimental designs for the carotenoid production using the orbital shaker, a significance of both independent variables (pH and nitrogen combination) for the biosynthesis of the three carotenoids was demonstrated. Thus, it was found as the optimal conditions for the production of carotenoids, the use of a culture medium composed of 10 g/L of glucose (as carbon source), 10 g/L of asparagine (as organic nitrogen source), 4 g/L of NH_4NO_3 (as inorganic nitrogen source), 0.52 g/L of KH_2PO_4, 0.52 g/L $MgSO_4$, as well as its growth at pH 5. This condition was then used in the cultivation of *R. glutinis* in the 5 L stirred-tank bioreactor. The bioreactor was operated in a batch mode for 72 h, monitoring at each 6 h of cultivation, the pH, DCW, glucose consumption, as well as the lipid and carotenoid (β-carotene, torularhodin and torulene) concentrations, as depicted in **Fig. 3** (all values are detailed in **Table S2** in the **Electronic supplementary material**).

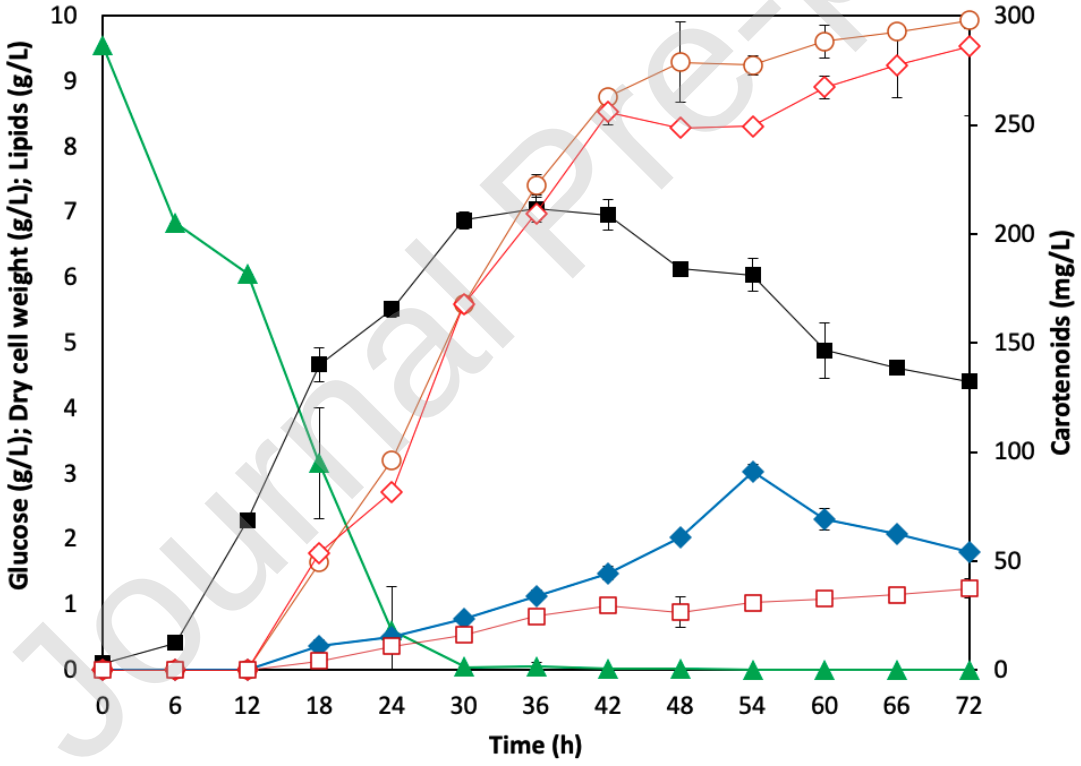


Fig. 3 Dry cell weight (g/L) (-■-), glucose (g/L) (-▲-), lipids (g/L) (-♦-) and production of β-carotene (-○-), torularhodin (-◇-) and torulene (-□-) by *R. glutinis* CCT-2186 in 5 L stirred-tank bioreactor at 30 ºC, 1 vvm, 300 rpm for 72 h. The error bars in some cases, are smaller than the markers.

The cellular biomass is the primary bioproduct of a yeast biorefinery, acting as a key performance indicator in the production lipids and carotenoids by yeast (produced intracellularly). As shown in **Fig. 3**, the fermentative process started with low cell concentration, with an initial DCW in the bioreactor of 0.09 g/L. After 6 h, exponential cellular growth started, reaching the maximum DCW of 7.14 g/L after 36 h of cultivation. After 42 h, a decrease in DCW occurred, with a final DCW of 4.42 g/L after 72 h of cultivation, a value equivalent to that obtained for the cultivations in the orbital shaker (*cf.* **Table 7**-center runs of 2^2 factorial design results). Regarding the DCW decrease, it is a result of both glucose depletion and lipids turnover (as noted by the decrease of lipid content). Maintenance of cell viability and function is highly dependent of the carbon source (in this case glucose). The results from **Figure 3** indicate that the DCW decrease occurs in few hours after the full consumption of the glucose. These results are in accordance with those obtained by: Dai et al [43], using *Rhodotorula glutinis* supplemented with xylose, which also demonstrated a decrease in biomass after the depletion of the carbon source; Yen et al [44], which grown *R. glutinis* in an internal-loop airlift bioreactor by using mixture substrates of rice straw hydrolysate and crude glycerol and found that when the carbon source is depleted, biomass tends to stabilize and then decrease. It is noteworthy that after 30 h, where the maximum DCW concentration was reached, the glucose concentration decreased to 0.04 g/L, corresponding to a consumption of approximately 99.5% of the carbon source, *i.e.*, the glucose was fully consumed during the exponential cell growth phase.

Comparing the production yields obtained in the cultivation in the bioreactor with those obtained with the orbital shaker, a significant increase in the production of the three carotenoids was observed, namely, from 220.93 to 297.84 mg/L for β-carotene (+ 25.83%), from 252.01 to 286.06 mg/L for torularhodin (+ 11.88%) and from 28.20 to 37.35 mg/L for torulene (+ 24.50%). Although the cultivation medium, pH and temperature are the same, in the stirred-tank bioreactor, the agitation rate was increased (from 150 rpm to 350 rpm) and a further aeration source (1 vvm of air) was added. The improvement of cultivation agitation and aeration conditions is beneficial in microbial biosynthesis, mainly because it favors the mass transfer of substrate, product

and oxygen, and consequent, all specific metabolic pathways. The influence of aeration on yeast carotenogenesis was previously assessed by Davoli et al. [40], observing that *R. glutinis* yeast produce higher concentrations of carotenoids at higher aeration rates (an increase of approx. 45% in the carotenoids content). Malisorn and Suntornsuk [12] also demonstrated a positive influence of aeration on carotenoid production by *R. glutinis* (DM28) in an 3 L stirred-tank bioreactor, specifically, achieving a maximum of β-carotene (0.21 mg/L after 24 h fermentation) by increasing the oxygen dissolved in the medium from 60% to 80%. Yamane et al. [41] also obtained high astaxanthin production rates by *P. rhodozyma* at high oxygen concentrations.

It has been shown that these microorganisms (*R. glutinis*) do not have a hyperactive fatty acid biosynthesis system, but they are capable of producing in significant quantities, acetyl-CoA, the basic unit of fatty acid biosynthesis [30,42]. *R. glutinis* is one of the oleaginous microorganisms with the greatest potential to synthesize and accumulate lipids intracellularly, for example, in this work it accumulated up to 61.60% (run 5, **Table 5**) of its total biomass in lipids (interestingly, obtained under normal, *i.e.,* "stress-free", cultivation conditions). Lipids are preferentially produced (and accumulated) during exponential yeast growth, when the increase in biomass is proportional with the carbon consumption [29]. In the bioreactor, as shown in **Fig. 3**, *R. glutinis* achieved a maximum of lipid concentration of 3.04 g/L (50.32% w/w) after 54 h of cultivation, which corresponds to DCW of 6.04 g/L. The lipid content remained above 2.30 g/L until 60 h, but then, decreased to 1.80 g/L after 72 h (end of the cultivation), because of the cell death in consequence of the yeast lipid depletion.

Together with the results of the orbital shaker, the cultivation in the 5 L stirred-tank bioreactor cultivation confirmed that a simple adjustment of the nitrogen sources and pH can significantly increase the production of the three carotenoids by *R. glutinis*, as well as to obtain other valuable by-products, e.g., lipids. The performance of the optimal cultivation medium was validated in a 5 L stirred-tank bioreactor, demonstrating that the combined use asparagine and NH_4NO_3 as complex nitrogen source is beneficial for the production of carotenoids (β-carotene, torularhodin and torulene) and lipid accumulation.

This study provides valuable information about the importance of using complex nitrogen source for the production of yeast biomass, from which more than one commercial valuable products can be simultaneously obtained, *i.e.,* i) carotenoids from yeast biomass can be used as supplement in functional foods; additive in cosmetics and animal feeds; ii) lipids can be used as food additives, diet supplements, substitutions for precious fats, and can also be used as substrates in the third-generation biodiesel production; and iii) carotenoids and lipids-free biomass (after the extraction of both added-value compounds) can be even used as protein and carbohydrate (after carotenoid and lipid extraction) to animal feed or agricultural fertilizers.

4. Conclusions

From sequential optimization studies using fractional factorial designs followed by central composite design, a clear effect was found between supplementation with organic and inorganic nitrogen sources and the production of carotenoids (β-carotene, torularhodin and torulene) and lipids from *Rhodotorula glutinis* CCT-2186. The use of combined sources of organic and inorganic nitrogen is recommended to supplement simple synthetic nutritional media for the cultivation of *R. glutinis*. The performance of the yeast was validated in a 5 L stirred-tank bioreactor, in which, as demonstrated by the improvement in carotenoids production yields, the importance of performing the initial optimization of the nutritional content of cultivation media was confirmed. Despite the promising results using combined sources of inorganic and organic nitrogen sources, additional studies focused in the optimization of C/N balance are recommended, particularly, envisaging the improvement of the *R. glutinis* biomass content and guaranteeing, at same time, the maintenance of high productivity yields for carotenoids and lipids.

Credit authorship contribution statement

Conceptualization: CUM, VCSE and JFBP. Literature review: CUM. Investigation: CUM, AACG and LVFR. Writing original draft: CUM, VCSE, and JFBP. Figures: CUM. Funding: CUM, VCSE, and JFBP. Validation: CUM, JW, VCSE, and JFBP. Writing-Review and Editing: CUM, JW, VCSE, and JFBP. All authors read and approved the final manuscript.

Acknowledgments

This research was supported by grants from the Ministry of Science and Technology, High Education and Technical Vocational Training of Mozambique (MCTESTP, Maputo, Mozambique) through the HEST Project-World Bank and the FAPESP (São Paulo Research Foundation, Brazil) through the projects: 2014/19793-3; 2014/16424-7; 2014/01580-3; 2017/50303-0 and 2019/09618-3. The authors also acknowledge the support from the CAPES-PRINT (Coordination of Superior Level Staff Improvement, Brazil) - Finance Code 88887.367977/2019-00 and CAPES-PROEX- Finance code 001.

Credit author statement
Cassamo Ussemane Mussagy: Conceptualization, Investigation, Formal analysis, Data curation, Writing- Original draft preparation.
Ana Alice Carizia Guimarães: Investigation,
Lara Vicente Ferreira Rocha: Investigation
James Winterburn: Conceptualization, Methodology, Writing-Review-editing
Jorge Fernando Brandão Pereira: Conceptualization, Writing-Review-editing, Visualization, Supervision, Project administration, Funding acquisition.
Valéria de Carvalho Santos Ebinuma: Conceptualization, Writing-Review-editing, Visualization, Supervision, Project administration, Funding acquisition.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that can constitute potential conflict of interest.

References

[1] M.M.S. Cabral, K. Cence, J. Zeni, S.M. Tsai, A. Durrer, L.L. Foltran, G. Toniazzo, E. Valduga, H. Treichel, Carotenoids production from a newly isolated *Sporidiobolus pararoseus* strain by submerged fermentation, Eur. Food Res. Technol. (2011), 233, 159–166. doi:10.1007/s00217-011-1510-0.

[2] M. del C. Cerón García, C.V. González López, J.M. Fernández Sevilla, E. Molina Grima, Preparative recovery of carotenoids from microalgal biomass, in: Methods Mol. Biol., 2018, 1852, 107-115. doi: 10.1007/978-1-4939-8742-9_6.

[3] A. Milani, M. Basirnejad, S. Shahbazi, A. Bolhassani, Carotenoids: biochemistry, pharmacology and treatment, Br. J. Pharmacol. (2017), 174 (11), 1290-1324. doi: 10.1111/bph.13625.

[4] C.U. Mussagy, J. Winterburn, V.C. Santos-Ebinuma, J.F.B. Pereira, Production and extraction of carotenoids produced by microorganisms, Appl. Microbiol. Biotechnol. (2018). 103(3), 1095-1114. doi:10.1007/s00253-018-9557-5.

[5] A. Das, S.H. Yoon, S.H. Lee, J.Y. Kim, D.K. Oh, S.W. Kim, An update on microbial carotenoid production: Application of recent metabolic engineering tools, Appl. Microbiol. Biotechnol. (2007). 77, 505–512. doi:10.1007/s00253-007-1206-3.

[6] V.M. Ye, S.K. Bhatia, Pathway engineering strategies for production of beneficial carotenoids in microbial hosts, Biotechnol. Lett. (2012). 34, 1405–1414. doi:10.1007/s10529-012-0921-8.

[7] A.M. Kot, S. Błażejak, A. Kurcz, I. Gientka, M. Kieliszek, *Rhodotorula glutinis*—potential source of lipids, carotenoids, and enzymes for use in industries, Appl. Microbiol. Biotechnol. (2016), 100, 6103–6117. doi:10.1007/s00253-016-7611-8.

[8] F.M. Squina, A.Z. Mercadante, Influence of nicotine and diphenylamine on the carotenoid composition of *Rhodotorula* strains, J. Food Biochem. (2005), 29 (6), 638-652. doi:10.1111/j.1745-4514.2005.00030.x.

[9] J. Tinoi, N. Rakariyatham, R.L. Deming, Simplex optimization of carotenoid production by *Rhodotorula glutinis* using hydrolyzed mung bean waste flour as substrate, Process Biochem. (2005), 40 (7), 2551-2557. doi:10.1016/j.procbio.2004.11.005.

[10] K. Jeevaratnam, B. V Latha, Purification and Characterization of the Pigments from *Rhodotorula glutinis* DFR-PDY Isolated from Natural Source, Glob. J. Biotechnol. Biochem. (2010), 5 (3), 166-174.

[11] E. Valduga, A. Valério, P.O. Tatsch, H. Treichel, A. Furigo, M. Di Luccio, Assessment of Cell Disruption and Carotenoids Extraction from *Sporidiobolus salmonicolo*r (CBS 2636), Food Bioprocess Technol. (2009). https://doi.org/10.1007/s11947-008-0133-3.

[12] C. Malisorn, W. Suntornsuk, Improved β-carotene production of *Rhodotorula glutinis* in fermented radish brine by continuous cultivation, Biochem. Eng. J. (2009), 43 (1), 27-32. doi:10.1016/j.bej.2008.08.005.

[13] P. Buzzini, A. Martini, Production of carotenoids by strains of *Rhodotorula glutinis* cultured in raw materials of agro-industrial origin, Bioresour. Technol. (2000), 71 (1), 41-44. doi:10.1016/S0960-8524(99)00056-5.

[14] I.R. Maldonade, A.R.P. Scamparini, D.B. Rodriguez-Amaya, Selection and characterization of carotenoid-producing yeasts from Campinas region, Brazil, Brazilian J. Microbiol. (2007), 38, 65-70 doi:10.1016/0370-2693(74)90070-7.

[15] Z. Aksu, A.T. Eren, Production of carotenoids by the isolated yeast of *Rhodotorula glutinis*, Biochem. Eng. J. (2007), 35, 107-113. doi:10.1016/j.bej.2007.01.004.

[16] C. Saenge, B. Cheirsilp, T.T. Suksaroge, T. Bourtoom, Efficient concomitant production of lipids and carotenoids by oleaginous red yeast *Rhodotorula glutinis* cultured in palm oil mill effluent and application of lipids for biodiesel production, Biotechnol. Bioprocess Eng. (2011), 16(1), 23-33. doi:10.1007/s12257-010-0083-2.

[17] H.W. Yen, Y.X. Liu, J.S. Chang, The effects of feeding criteria on the growth of oleaginous yeast- *Rhodotorula glutinis* in a pilot-scale airlift bioreactor, J. Taiwan Inst. Chem. Eng. (2015), 49, 67-71. doi:10.1016/j.jtice.2014.11.019.

[18] Z.T. Harith, M. de A. Lima, D. Charalampopoulos, A. Chatzifragkou, Optimised production and extraction of astaxanthin from the yeast *Xanthophyllomyces dendrorhous*, Microorganisms. (2020). 8. 430.doi:10.3390/microorganisms8030430.

[19] A.H. Mondala, R. Hernandez, T. French, L. McFarland, J.W. Santo Domingo, M. Meckes, H. Ryu, B. Iker, Enhanced lipid and biodiesel production from glucose-fed activated sludge: Kinetics and microbial community analysis, AIChE J. (2012). 58 (4), 1279-1290. doi:10.1002/aic.12655.

[20] G.L. Miller, Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar, Anal. Chem. (1959), 31(3), 426-428. doi:10.1021/ac60147a030.

[21] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification., Can. J. Biochem. Physiol. (1959), 37(8), 911-917. doi:10.1139/o59-099.

[22] C.U. Mussagy, V. de C. Santos Ebinuma, M. Gonzalez-Miquel, J.A.P. Coutinho, J.F.B. Pereira, Protic ionic liquids as cell disrupting agents for the recovery of intracellular carotenoids from yeast *Rhodotorula glutinis* CCT-2186, ACS Sustain. Chem. Eng. (2019), 7 (19), 16765-16776. doi:10.1021/acssuschemeng.9b04247.

[23] F. de Oliveira, D.B. Pedrolli, M.F.S. Teixeira, V. de Carvalho Santos-Ebinuma, Water-soluble fluorescent red colorant production by *Talaromyces amestolkiae*, Appl. Microbiol. Biotechnol. (2019), 103(16):6529-6541. doi: 10.1007/s00253-019-09972-z.

[24] V.C. Santos-Ebinuma, I.C. Roberto, M.F.S. Teixeira, A. Pessoa, Improvement of submerged culture conditions to produce colorants by *Penicillium purpurogenum*, Brazilian J. Microbiol. (2014), 45(2), 731-42. doi:10.1590/S1517-83822014000200049.

[25] F. de Oliveira, L.C. Ferreira, Á.B. Neto, M.F. Simas Teixeira, V. de Carvalho Santos Ebinuma, Biosynthesis of natural colorant by *Talaromyces amestolkiae*: mycelium accumulation and colorant formation in incubator shaker and in bioreactor, Biochem. Eng. J. (2020), 161 (15), 107694. doi: 10.1016/j.bej.2020.107694.

[26] P.K. Park, E.Y. Kim, K.H. Chu, Chemical disruption of yeast cells for the isolation of carotenoid pigments, Sep. Purif. Technol. (2007), 53(2), 148-152. doi:10.1016/j.seppur.2006.06.026.

[27] M.I. Rodrigues, A.F. Iemma, Experimental design and process optimization, 2014. doi:10.1201/b17848.

[28] V. Perrier, E. Dubreucq, P. Galzy, Fatty acid and carotenoid composition of *Rhodotorula* strains, Arch. Microbiol. (1995), 164 (3), 173-179. doi:10.1007/BF02529968.

[29] G. Singh, A. Jawed, D. Paul, K.K. Bandyopadhyay, A. Kumari, S. Haque, Concomitant production of lipids and carotenoids in *Rhodosporidium toruloide*s

under osmotic stress using response surface methodology, Front. Microbiol. (2016), 7, 1686. doi: 10.3389/fmicb.2016.01686.

[30] C. Ratledge, Single cell oils - have they a biotechnological future?, Trends Biotechnol. (1993), 51, 1-51. doi:10.1016/S0065-2164(02)51000-5.

[31] D. Somashekar, R. Joseph, Inverse relationship between carotenoid and lipid formation in *Rhodotorula gracilis* according to the C/N ratio of the growth medium, World J. Microbiol. Biotechnol. (2000). 16(5), 491-493. doi:10.1023/A:1008917612616.

[32] O. Gribanovski-Sassu, F.H. Foppen, Light and temperature effect on *Epicoccum nigrum*, Phytochemistry. (1968), 7 (9), 1605-1612. doi:10.1016/S0031-9422(00)88613-6.

[33] A.M. Kot, S. Błazejak, I. Gientka, M. Kieliszek, J. Bryś, Torulene and torularhodin: "New" fungal carotenoids for industry?, Microb. Cell Fact. (2018), 17 (49). doi: 10.1186/s12934-018-0893-z.

[34] P.B. Bhosale, R. V. Gadre, Production of β-carotene by a mutant of *Rhodotorula glutinis*, Appl. Microbiol. Biotechnol. (2001). 55(4), 423-427. doi:10.1007/s002530000570.

[35] A. Mihalcea, C. Ungureanu, M. Ferdes, A.A. Chirvase, C. Tanase, The influence of operating conditions on the growth of the yeast *Rhodotorula rubra* ICCF 209 and on torularhodin formation, Rev. Chim. (2011), 62 (6), 659-556.

[36] C. Voaides, R. Dima, The effect of nitrogen source on carotenoids production by *Rhodotorula* sp., Rom. Biotechnol. Lett. (2012), 17(5):7570-7576.

[37] S. Alcantara, S. Sanchez, Influence of carbon and nitrogen sources on *Flavobacterium* growth and zeaxanthin biosynthesis, J. Ind. Microbiol. Biotechnol. (1999), 23(1), 697-700. doi:10.1038/sj.jim.2900688.

[38] A.M. Kot, S. Błażejak, M. Kieliszek, I. Gientka, J. Bryś, L. Reczek, K. Pobiega, Effect of exogenous stress factors on the biosynthesis of carotenoids and lipids by *Rhodotorula* yeast strains in media containing agro-industrial waste, World J. Microbiol. Biotechnol. (2019), 35 (157). doi: 10.1007/s11274-019-2732-8.

[39] F. Ramel, S. Birtic, S. Cuine, C. Triantaphylides, J.-L. Ravanat, M. Havaux, Chemical Quenching of Singlet Oxygen by Carotenoids in Plants, PLANT Physiol. (2012), 158(3), 1267-78. doi:10.1104/pp.111.182394.

[40] P. Davoli, V. Mierau, R.W.S. Weber, Carotenoids and fatty acids in red yeasts *Sporobolomyces roseus* and *Rhodotorula glutinis*, Appl. Biochem. Microbiol. (2004), 40(4), 392-397. doi:10.1023/B:ABIM.0000033917.57177.f2.

[41] Y.I. Yamane, K. Higashida, Y. Nakashimada, T. Kakizono, N. Nishio, Influence of oxygen and glucose on primary metabolism and astaxanthin production by *Phaffia rhodozyma* in batch and fed-batch cultures: Kinetic and stoichiometric analysis, Appl. Environ. Microbiol. (1997), 63(11), 4471-8.

[42] S. Papanikolaou, G. Aggelis, Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production, Eur. J. Lipid Sci. Technol. (2011), 113(8), 1031-1051. doi:10.1002/ejlt.201100014.

[43] C. Dai, J. Tao, F. Xie, Y. Dai, and M. Zhao, Biodiesel generation from oleaginous yeast *Rhodotorula glutinis* with xylose assimilating capacity." Afr. J. Biotechnol (2007), 6, 2130-2134. doi: 10.5897/AJB2007.000-2331.

[44] H.W. Yen, J.T. Chang, J.S. Chang, The growth of oleaginous *Rhodotorula glutinis* in an internal-loop airlift bioreactor by using mixture substrates of rice straw hydrolysate and crude glycerol, Biomass and Bioenergy, (2015), 80, 38-43. doi: 10.1016/j.biombioe.2015.04.026.