

ORIGINAL ARTICLE

Evaluation of the process conditions for the production of microbial carotenoids by the recently isolated *Rhodotorula mucilaginosa* URM 7409

Avaliação das condições de processo para a produção de carotenoides microbianos pela recém-isolada Rhodotorula mucilaginosa URM 7409

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Abstract

This study aimed to improve the physical and nutritional process conditions for the production of carotenoids by the newly isolated *Rhodotorula mucilaginosa*, a red basidiomycete yeast. The carotenoid bioproduction was improved using an experimental design technique, changing the process characteristics of agitation (130 rpm to 230 rpm) and temperature (25 °C to 35 °C) using seven experiments, followed by a 2⁵⁻¹ fractional design to determine the relevant factors that constitute the culture medium (glucose, malt extract, yeast extract, peptone and initial pH). A complete second order experimental design was then carried out to optimize the composition of the culture medium, the variables being yeast extract (0.5 to 3.5 g/L), peptone (1 to 5 g/L) and the initial pH (5.5 to 7.5), with 17 experiments. The maximum carotenoid production was 4164.45 µg/L (252.99 µg/g), obtained in 144 h in YM (yeast malt) medium with 30 g/L glucose, 10 g/L malt extract, 2 g/L yeast extract, 3 g/L peptone, an initial pH 6, 130 rpm and 25 °C, demonstrating the potential of this yeast as a source of bio-pigments. In this work, the nitrogen sources were the factors that most influenced the intracellular accumulation of carotenoids. The yeast *R. mucilaginosa* presented high production at a bench level and may be promising for commercial production.

Keywords: Basidiomycota; Pigmented; Submerged culture; β-Carotene; Experimental design; Optimization; *Rhodotorula*.

Resumo

Este estudo teve como objetivo melhorar as condições físicas e nutricionais do processo de produção de carotenoides pela recém-isolada *Rhodotorula mucilaginosa*, uma levedura basidiomiceta vermelha. A melhoria da bioprodução de carotenoides foi obtida pela técnica de delineamento experimental, alterando as



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características dos processos [agitação (130 rpm a 230 rpm) e temperatura (25 °C a 35 °C)] com sete experimentos, e após um planejamento fracionário 2^{5-1} para a classificação dos fatores relevantes que constituem o meio de cultura (glicose, extrato de malte, extrato de levedura, peptona e pH inicial). Um delineamento experimental de segunda ordem completo foi realizado para otimizar a composição do meio de cultura e as variáveis foram: extrato de levedura (0,5 a 3,5 g/L), peptona (1 a 5 g/L) e pH inicial (5,5 a 7,5), com 17 experimentos. A produção máxima de carotenoide foi de 4.164,45 µg/L (252,99 µg/g) em 144 h, em meio YM com 30 g/L de glicose, 10 g/L de extrato de malte, 2 g/L de extrato de levedura e 3g/L de peptona, e pH inicial 6, 130 rpm a 25 °C. Neste trabalho, as fontes de nitrogênio foram os fatores que mais influenciaram o acúmulo de carotenoides intracelular. A levedura *R. mucilaginosa* apresentou alta produção em nível de bancada, podendo ser promissora para a produção comercial.

Palavras-chave: Basidiomicota; Pigmento; Cultivo submerso; β-Caroteno; Delineamento experimental; Otimização; *Rhodotorula*.

1 Introduction

Biomolecules produced by the secondary metabolism of microorganisms are receiving much attention by researchers. Amongst these biomolecules, carotenoids stand out for exhibiting several important biological properties such as provitamin A, antioxidant and anticancer activities, besides conferring coloration to the industrialized foods (Gómez et al., 2014; Ünlü & Takaç, 2011; Wang et al., 2014).

The industrial production of these microorganisms (bacteria, fungi, microalgae and yeast) has several advantages, such as a small area for cultivation, rapid cell growth, easy scale up and adaptation to various sugar and nitrogen sources (Venil et al., 2013). On the other hand, not all microorganisms have the desired production capacity. The use of prospecting techniques allows for the discovery of new microorganisms with this potential (Chang et al., 2016; Singh et al., 2014).

Of the different sources of carotenoids, yeasts of the genus *Rhodotorula* have been reported as carotenogenic yeasts or pink yeasts due to their production of carotenoids. They are unicellular, their morphology has been described as soft, smooth and moist, and sometimes the presence of mucoid provides a high growth rate. They do not compete with food production and produce a wide variety of secondary metabolites (saponifiable lipids for the production of biodiesel, antibactericides, organic acids, B vitamins and enzymes), that can be obtained simultaneously in the biorefinery. In addition, they are easily adaptable to different nutritional sources, making them yeasts of industrial interest (Mao et al., 2012; Venil et al., 2013; Yoo et al., 2016).

Currently, there is a great interest in carotenoids due to their use in dietary supplements, as food coloring and as additives for pharmaceuticals and cosmetics. The demand for carotenoids has increased by 3.9% per year on the global market (Saini et al., 2018), but much of this market is dominated by chemical synthesis. These synthetic molecules have an impact on human health (toxicity, hypersensitivity and carcinogenicity) (Amchova et al., 2015) which has a negative effect on processed foods. On the other hand, the use of microbial sources could improve this negative characteristic for consumers, in addition to enriching the product with antioxidants and antimicrobial agents and being able to label it as containing natural dyes, causing no health malfunctions (Yolmeh & Khomeiri, 2017).

The production of bio-colorants, in relatively pure and concentrated forms, is the main technological challenge for this industry. The strategy to improve the process conditions and change the nutritional sources may positively reflect on the production of carotenoids by microorganisms (Machado & Burkert, 2015), and the use of statistical tools to study these factors and improve the process is essential.

Statistical methodologies such as factorial designs and central composite rotational designs (CCRD) may provide a comprehension of the factors involved in bioproduction based on experimental trials. These tools make it possible to reduce the number of tests without losing the response, as well as making an evaluation

in the range of the factors studied, selecting only the determinant and significant factors to be evaluated in another design, aiming at higher production (Dhaliwal & Chandra, 2015). The non-significant elements in the study can be omitted in the new experiment and hence these statistical tools can be adopted as a strategy to increase the production of carotenoids (Machado & Burkert, 2015; Valduga et al., 2007). Thus, the objective of this work was to carry out a screening of yeasts with the potential for carotenoid production using an experimental design technique, studying the effects of the process operating conditions (agitation and temperature), as well as the composition of the culture medium in order to isolate yeasts with the best potential for this application (*Rhodotorula mucilaginosa*).

2 Material and methods

2.1 Screening and isolation of the yeasts

Samples were collected from soil, leaves and flowers in a region of environmental biodiversity (latitude – 20.785218 and longitude – 49.359897) and stored in pre-sterilized high-density polyethylene thermoplastic containers until the analyses were carried out. Samples were randomly collected from soil, tree bark, flowers and grass in an area of environmental biodiversity near the research laboratory. The samples obtained were transferred to 250 mL conical flasks containing 50 mL of yeast and malt extract medium (YM: 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 10 g/L glucose) and incubated at 25 °C and 150 rpm for 48 h for microbial enrichment. They were then inoculated in Petri dishes with YM agar medium and incubated at 25 °C for 120 h (Cabral et al., 2011).

Yeast colonies that showed yellow to red coloring were transferred to test tubes with YM agar medium and incubated at 25 °C until satisfactory growth. Isolation of the yeasts was confirmed on YM agar Petri dishes at 25 °C for 120 h (Valduga et al., 2007) and the purity of the microorganism was checked by microscopic examination. Confirmation of the yeast was carried out in PCA (23.5 g/L), a selective medium for yeasts, with 50 mg/L chloramphenicol (Neo Química®) (Cabral et al., 2011; Pereira et al., 2014).

2.2 Microorganism maintenance and reactivation

The microorganisms were maintained in slant culture tubes with GYMP agar (2 g/L glucose, 1 g/L malt extract, 0.5 g/L yeast extract, 0.2 g/L NaH₂PO₄ and 1.8 g/L agar) and mineral oil, under refrigeration at 4 °C, and replicated every three months (Lopes et al., 2017). For reactivation, replication from stock cultures was carried out in test tubes with YM medium, incubating at 25 °C for 48 h.

2.3 Inoculum

One milliliter of sterile peptone water was added to the medium containing the activated microorganism (previous step), to obtain a cell suspension by scraping the surface of the medium. This was transferred to test tubes containing 9 mL YM broth and maintained at 25 °C for 48 h. The cultures were then stirred on a vortex and transferred to 250 mL conical flasks containing 50 mL YM broth, previously sterilized at 121 °C for 15 min. These cultures were incubated at 150 rpm and 25 °C, either for 48 h or the time required for the cell count to reach 10⁷ cells/mL, counting in a Neubauer chamber (Cipolatti et al., 2015).

2.4 Carotenoid bioproduction and yeast identification

Carotenoid bioproduction was carried out in an orbital shaker (Tecnal, TE 424, Brazil) in 250 mL conical flasks containing 112.5 mL YM medium at an initial pH 6.0. A ten percent inoculum was added and the cultivation started with 1x10⁷ cells/mL, incubating at 25 °C and 180 rpm for 144 h (Machado & Burkert, 2015).

Samples were collected at the end of cultivation to determine the biomass by a gravimetric method and also the pH value and total carotenoid concentration.

Based on the bioproduction results, the yeast strain with the highest yield was selected for DNA analysis (PCR). It was identified, stored and made available to all researchers by the Micoteca URM at the Federal University of Pernambuco, Brazil.

2.5 Experimental design

The design of the study consisted of three steps: (i) analysis of the operational conditions of the process; (ii) preliminary analysis of the factors influencing the behavior of *Rhodotorula mucilaginosa* in YM medium and; (iii) determination of the effects of these factors on carotenoid production for optimization and subsequent validation of the process operating conditions.

A central composite design (CCD) with four experiments and three replicates at the central point was used first. The independent variables (factors) were agitation (130 to 230 rpm) and temperature (25 °C to 35 °C).

After the statistical analysis, a second factorial design was carried out (2^{5-1}). To determine the effects of the five factors on carotenoid production, it was proposed to use the effect of the graph together with an alpha (α) of 5%, using Statistica® software version 10.0. The factors investigated were the components of the YM medium (yeast extract (1 to 5 g/L), peptone (1 to 10 g/L), glucose (10 to 30 g/L), malt extract (1 to 10 g/L) and the initial pH (4 to 6)). The results found for each factor were considered for the next experiment in sequence.

After confirming the significance of the effects of the factors, the third stage was carried out, a central composite rotatable design (CCRD) with 14 experiments and three replicates at the central points, studying the significant variables of yeast extract (0 to 2 g/L), peptone (0 to 2 g/L) and glucose (30 to 50 g/L). The three variables were studied at three levels: low (−1), medium (0) and high (+1). The CCRD was designed using Statistica® software version 10.0 and the other non-significant process variables were fixed as follows: malt extract 1 g/L, initial pH 6.0, with agitation and temperature at the highest condition obtained, namely 130 rpm and 25 °C. Table 1 shows the treatments carried out with the levels tested. The dependent variables or responses of the experimental design were the volumetric ($\mu\text{g/L}$) and specific ($\mu\text{g/g}$) concentrations of carotenoids and the biomass (g/L).

Table 1. Variables used in the growth of *R. mucilaginosa* to obtain carotenoids.

Variables	Levels				
	−1.68	−1	0	+1	+1.68
Yeast extract (g/L)	0	0.4	1	1.60	2
Peptone (g/L)	0	0.4	1	1.60	2
Glucose (g/L)	30	34	40	46	50

The results were evaluated by linear regression testing the complete quadratic model with interactions. The non-significant parameters were eliminated from the model by the Student t-test at 5% of significance, resulting in a polynomial empirical model for these three process variables (Equation 1):

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{12}AB + \beta_{13}AC + \beta_{23}BC \quad (1)$$

where Y is the response measured; β_0 is the model intercept; β_1 , β_2 and β_3 are linear coefficients; β_{11} , β_{22} and β_{33} are the quadratic coefficients; β_{12} , β_{13} and β_{23} are interaction coefficients and A, B and C are independent coded variables.

The quality of the models developed was evaluated by two types of value: coefficient of determination – R^2 (capacity to explain the variance) and p -value, obtained from ANOVA. The adjusted empirical polynomial equations were expressed in contour form and three-dimensional plots, to illustrate the relationship between

the responses as a function of the combination of the two factors to be optimized, keeping the other variables in central positions. The interaction of any two variables of the study can be examined from the prototype of the contour plots. Furthermore, the optimized numerical method was used to obtain optimal regions (optimal solutions).

2.6 Validation of the model

To confirm the theoretical results obtained from the adjusted polynomial equations, the culture was carried out in triplicate under the conditions described as optimal, to confirm the legitimacy of the models generated by the software. The results obtained for the volumetric and specific concentrations of the carotenoids were compared with the values predicted by the mathematical model.

2.7 Recovery of total carotenoids

Total carotenoid recovery was carried out according to the methodology described by Michelon et al. (2012) and Cipolatti et al. (2015). The biomass was centrifuged at $3439 \times g$ for 10 min, transferred to a Petri dish, dried in a freeze-dryer for 48 h, macerated in a mortar and standardized using a Tyler n° 115 sieve. After the standardization step, 0.05 g of biomass were submitted to lysis with 2 mL of rupture agent: dimethyl sulfoxide or DMSO ((CH₃)₂SO) and then agitated by vortex for 1 min every 15 minutes for 1 h. After rupture, 8 mL of acetone were added and the mixture centrifuged ($3439 \times g$ for 10 min). The supernatant was then separated from the precipitate and several successive extractions carried out to total cell whitening.

The supernatants were combined and 10 mL of 20% NaCl (p/v) and 10 mL of petroleum ether added in order to form two phases. The polar phase was collected and the excess of water removed using sodium sulphate (Na₂SO₄), originating the extracts containing the carotenoids (Michelon et al., 2012).

2.8 Determination of total carotenoids

The total carotenoid concentration in the extracts was determined in a spectrophotometer at 448 nm (Cabral et al., 2011), expressed as its major carotenoid (β -carotene in petroleum ether, whose specific absorptivity is $A_{cm}^{1\%} = 2592$), according to Equation 2 (David, 1976).

$$TC = \frac{A * V * 10^6}{A_{cm}^{1\%} * 100 * m_{sample}} \quad (2)$$

where TC is the total carotenoid concentration ($\mu\text{g/g}$); A is the absorbance; V is the volume (mL); m_{sample} is the dry cell mass (g) and $A_{cm}^{1\%}$ is the specific absorptivity. To calculate the volumetric concentration of total carotenoids ($\mu\text{g/L}$) using the result obtained for the concentration of total carotenoids ($\mu\text{g/g}$) and the biomass concentration (g/L), a unit conversion was carried out.

2.9 Determination of biomass

The cells were centrifuged ($3439 \times g$) at 25 °C for 10 min to separate the supernatant, washed with distilled water and centrifuged again. The cell mass was quantified after drying at 105 °C (Fanem SE-320) to constant mass, according to Association of Official Analytical Chemists (2012).

3 Results and discussion

Thirty colonies were isolated from the environmental samples, and eight presented orange and pink pigmentation, with emphasis on the genus *Rhodotorula*. This group of yeasts are abundant in various regions, such as Patagonia and near Cheon city in South Korea (Libkind & Van Broock, 2006; Yoo et al., 2016). According to Chang et al. (2016), 64.5% of all yeasts isolated from the Taiwanese sea coast contain *Rhodotorula*.

Table 2 shows the bioproduction in terms of biomass and carotenoid concentrations (specific and volumetric) for eight selected yeasts. The yeast with the highest bio-pigment yield (635.94 µg/L) and cell growth (6.30 g/L) was UYG9. Identification (PCR) by the URM Fungal Collection verified that this yeast was *Rhodotorula mucilaginosa*, obtained from soil samples. This yeast was deposited in the same institution, identified with the access code of URM 7409.

The yeasts XPS2 and DRG3 showed high volumetric concentrations of carotenoids and no significant difference from UYG9, but the strain UYG9 presented higher values for volumetric and biomass concentrations.

Table 2. Bioproduction of carotenoids by the isolated yeasts.

Strains	Biomass (g/L)	SCC (µg/g)	VCC (µg/L)
XPS2	5.42 ± 0.12b	107.90 ± 7.14ab	584.48 ± 38.70ab
DRG3	5.27 ± 0.03bc	108.51 ± 3.64ab	571.51 ± 19.16ab
EGE5	3.88 ± 0.13e	122.46 ± 3.53a	474.55 ± 18.03c
RTH6	4.98 ± 0.15c	56.84 ± 4.10d	283.35 ± 20.42e
UHJ7	4.60 ± 0.17d	111.69 ± 3.35ab	513.78 ± 21.76bc
ODK8	6.18 ± 0.08a	88.29 ± 7.27c	545.94 ± 44.97bc
UYG9	6.30 ± 0.03a	100.94 ± 9.21bc	635.94 ± 58.01a
UG10	3.27 ± 0.17f	104.03 ± 3.21b	339.85 ± 10.48d

Mean ± deviation. Lowercase letters in the same column mean the samples differ statistically from each other ($p < 0.05$). SCC = Specific concentration of carotenoids. VCC = Volumetric concentration of carotenoids.

After the yeast URM 7409 was selected, an experimental design (CCD) was carried out, evaluating the operational cultivation factors as presented in Table 3. The results showed that lower agitation (130 rpm) and temperature (25 °C) favoured the production of carotenoids by this microorganism (91.78 µg/g and 679.15 µg/L, respectively). Thus, the operational conditions of agitation and temperature were standardized according to this plan and a new 2^{5-1} experimental design used to optimize the culture medium.

Table 3. 2^2 Central composite design (real and coded values) to maximize the process conditions (agitation and temperature).

Assay	X ₁	X ₂	Y ₁	Y ₂	Y ₃
1	-1 (130)	-1 (25)	7.4	91.78	679.15
2	+1 (230)	-1 (25)	6.55	66.69	436.83
3	-1 (130)	+1 (35)	2.7	48.85	131.89
4	+1 (230)	+1 (35)	3.65	17.49	63.85
5	0 (180)	0 (30)	6.8	55.94	385.95
6	0 (180)	0 (30)	6.8	54.52	376.29
7	0 (180)	0 (30)	7.1	55.61	383.74

X₁ = Agitation (rpm). X₂ = Temperature (°C). Y₁ = Biomass (g/L). Y₂ = Specific concentration of carotenoids (µg/g). Y₃ = Volumetric concentration of carotenoids (µg/L).

Table 4 shows the results obtained for the 2^{5-1} factorial design. The results showed that the total carotenoid concentration decreased when the yeast extract and peptone concentrations increased in most of the experiments, and that the assays at pH 6 may favour bioproduction. On the other hand, when one of the nitrogen sources was low (yeast extract or peptone) and the pH was 4, bioproduction was low in most experiments.

Table 4. 2^{5-1} Factorial design (real and coded values) for the optimization of the culture medium for the bioproduction of carotenoids.

Assay	X ₁	X ₂	X ₃	X ₄	X ₅	Y ₁	Y ₂	Y ₃
1	-1 (1)	-1 (1)	-1 (1)	-1 (10)	+1 (6)	7.25	252.53	1831.41
2	+1 (5)	-1 (1)	-1 (1)	-1 (10)	-1 (4)	10.05	261.41	2627.77
3	-1 (1)	+1 (10)	-1 (1)	-1 (10)	-1 (4)	13.50	225.30	3042.46
4	+1 (5)	+1 (10)	-1 (1)	-1 (10)	+1 (6)	12.93	237.52	3070.13
5	-1 (1)	-1 (1)	+1 (10)	-1 (10)	-1 (4)	10.33	281.94	2912.77
6	+1 (5)	-1 (1)	+1 (10)	-1 (10)	+1 (6)	8.53	239.41	2041.86
7	-1 (1)	+1 (10)	+1 (10)	-1 (10)	+1 (6)	13.67	249.08	3404.39
8	+1 (5)	+1 (10)	+1 (10)	-1 (10)	-1 (4)	13.47	172.62	2325.51
9	-1 (1)	-1 (1)	-1 (1)	+1 (30)	-1 (4)	17.51	262.52	4891.13
10	+1 (5)	-1 (1)	-1 (1)	+1 (30)	+1 (6)	17.79	198.80	3535.81
11	-1 (1)	+1 (10)	-1 (1)	+1 (30)	+1 (6)	18.81	261.96	5099.55
12	+1 (5)	+1 (10)	-1 (1)	+1 (30)	-1 (4)	16.66	36.59	609.57
13	-1 (1)	-1 (1)	+1 (10)	+1 (30)	+1 (6)	13.60	178.57	2428.92
14	+1 (5)	-1 (1)	+1 (10)	+1 (30)	-1 (4)	17.87	30.51	545.10
15	-1 (1)	+1 (10)	+1 (10)	+1 (30)	-1 (4)	16.36	42.60	696.97
16	+1 (5)	+1 (10)	+1 (10)	+1 (30)	+1 (6)	20.05	66.43	1332.22
17	0 (3)	0 (5.5)	0 (5.5)	0 (20)	0 (5)	16.14	190.52	3081.57
18	0 (3)	0 (5.5)	0 (5.5)	0 (20)	0 (5)	16.21	186.79	3021.27
19	0 (3)	0 (5.5)	0 (5.5)	0 (20)	0 (5)	16.17	194.25	3141.88

X₁ = Yeast extract (g/L). X₂ = Malt Extract (g/L). X₃ = Peptone (g/L). X₄ = Glucose (g/L). X₅ = pH. Y₁ = Biomass (g/L). Y₂ = Specific concentration of carotenoids (µg/g). Y₃ = Volumetric concentration of carotenoids (µg/L).

These independent factors can best be observed individually in Figure 1, where the Pareto graph is presented. In this type of graph, it is possible to verify which are the main factors of greater occurrence in a process, allowing one to concentrate the efforts on them.

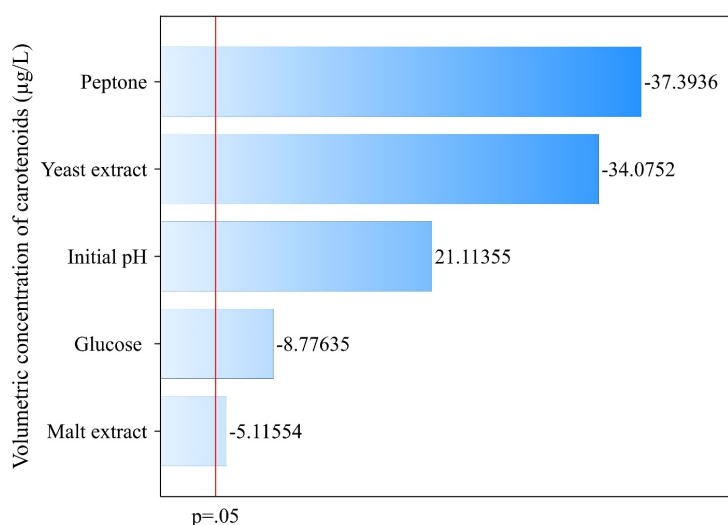


Figure 1. Effect of the variables on the volumetric concentration of carotenoids in the 2^{5-1} factorial design at 25 °C and 130 rpm.

Yeast extract and peptone were shown to be the variables with the greatest influence, with a negative effect on carotenoid production, followed by pH (positive effect). This inhibitory effect of the yeast extract and peptone can be explained by the excess of nitrogen source or a low C:N ratio, which limits the accumulation of carotenoids in oleaginous microorganisms, including the *Rhodotorula* strains (Taskin et al., 2016). The microorganism-producing carotenoids require minimal amounts of nitrogen, serving as maintenance for their development, allowing the excess carbon in the culture medium to be used for the synthesis of pigments (Spier et al., 2015), a fact observed in this work, for *R. mucilaginosa*, which requires smaller amounts of nitrogen.

For bio-pigment-producing yeasts, organic acids are produced at the start of cultivation, acidifying the medium, and then these acids are assimilated, increasing the pH value. The use of acidified culture media (initial pH 4) causes disarray in the metabolic pathway, impairing the formation of acetic acid, alcohol or the citric acid cycle intermediates during the adaptation phase (Machado & Burkert, 2015). Aksu & Eren (2007) studied the pH range from 3 to 8, for the production of carotenoids by *R. glutinis*, and found that pH 6.5 was more favourable for pigment production, because it induced cell growth and high carotenoid production rates.

Therefore, to maximize carotenoid bioproduction, the yeast extract concentrations were modified to lower values, lowering the value from 0.5 to 3.5 g/L and increasing peptone from 1 to 5 g/L, a region close to the central point of Table 4. As the pH had a significant positive effect ($p < 0.05$), its levels were shifted to higher values (5.5 to 7.5). Considering that the other variables were significant, such as malt extract (10 g/L) and glucose (30 g/L) and exerted little influence on carotenoid production, they were fixed at their highest level (+1), for a high C/N ratio. In short, the strategy adopted was to reduce the nitrogen sources to better absorb the excess of available carbon sources in the culture medium, as observed in other studies (Spier et al., 2015; Taskin et al., 2016).

In this fractional design (2^{5-1}), it was found that the significant variables adopting a 5% alpha were the nitrogen sources (peptone and yeast extract), followed by the initial pH value. In this type of experimental design, the interactions between the factors cannot be obtained, due to the low number of degrees of freedom in relation to the number of experiments. Fractional designs are used for screening and for the evaluation of important factors that influence the response. In addition, a new design with significant factors was carried out and a new concentration interval was organized to obtain an optimized region.

Table 5 presents the real and coded values for the 2^3 central compound rotational design (CCRD) and the responses in terms of cell concentration, and the specific and volumetric carotenoid concentrations. Table 4 shows that the highest volumetric carotenoid concentrations were obtained in the central point region, while the cell concentration remained practically the same in all the assays.

Table 5. 2^3 Central composite design (real and coded values) for the optimization of the culture medium.

Assay	X ₁	X ₂	X ₃	Y ₁	Y ₂	Y ₃
1	-1 (1.11)	-1 (1.81)	-1 (5.90)	24.50	103.06	2715.46
2	+1 (2.89)	-1 (1.81)	-1 (5.90)	25.51	129.01	3290.67
3	-1 (1.11)	+1 (4.19)	-1 (5.90)	25.68	124.01	3184.53
4	+1 (2.89)	+1 (4.19)	-1 (5.90)	25.71	96.07	2469.73
5	-1 (1.11)	-1 (1.81)	+1 (7.10)	23.70	123.75	2932.41
6	+1 (2.89)	-1 (1.81)	+1 (7.10)	24.28	147.44	3580.17
7	-1 (1.11)	+1 (4.19)	+1 (7.10)	23.20	119.84	2780.57
8	+1 (2.89)	+1 (4.19)	+1 (7.10)	13.47	98.63	1328.73
9	-1.68 (0.5)	0 (3)	0 (6.5)	25.03	148.63	3719.66
10	+1.68 (3.5)	0 (3)	0 (6.5)	24.04	109.46	2971.32
11	0 (2)	-1.68 (1)	0 (6.5)	24.53	171.29	4201.30
12	0 (2)	+1.68 (5)	0 (6.5)	25.82	156.83	4050.11
13	0 (2)	0 (3)	-1.68 (5.5)	25.82	111.97	3532.97
14	0 (2)	0 (3)	+1.68 (7.5)	23.56	175.68	4139.86
15	0 (2)	0 (3)	0 (6.5)	24.36	179.75	4379.50
16	0 (2)	0 (3)	0 (6.5)	26.29	175.25	4269.78
17	0 (2)	0 (3)	0 (6.5)	24.15	169.70	4134.65

X₁ = Yeast extract (g/L). X₂ = Peptone (g/L). X₃ = Initial pH value. Glucose 30 g/L and malt extract 10 g/L. Y₁ = biomass concentration (g/L). Y₂ = Specific concentration of carotenoids (μg/g). Y₃ = carotenoid production (μg/L).

In the CCRD (Table 5), the maximum carotenoid production varied from 1328.73 µg/L (assay 8) to 4379.50 µg/L (assay 15) and the biomass concentration from 13.47 g/L (assay 8) to 26.29 g/L (assay 16) in 144 h. Using the design, the maximum carotenoid concentration increased more than six times and the biomass more than three times, when compared to the maximization of the process conditions (Table 3). The carotenoid values obtained in the present study with *R. mucilaginosa* were higher than those found for other *Rhodotorula* strains (Lopes et al., 2017; Maldonado et al., 2012).

The statistical analyses were carried out using the Statistica® software version 10.0. The quality of the model fit was expressed by the coefficient of determination R^2 and by the statistical significance of the regression. Equations 3 and 4 present the second-order coded model, which describes the volumetric and specific carotenoid concentrations as a function of the independent variables (factors analysed), which were the nitrogen sources (yeast extract and peptone) and the initial pH within the range studied. The model was validated by the variance analysis (Table 6). The factors and their interactions were very significant, less than 0.04 for the p -value.

The importance of the p -value is that it demonstrates how significant the terms making up the equation are. The non-significant variables of the model were added to the lack of fit.

Table 6. Variance analysis of the 2^3 central composite design for the yeast *R. mucilaginosa*.

Volumetric concentration of carotenoids (µg/L)						Specific concentration of carotenoids (µg/g)					
Variation source	Sum of squares	Degrees of Freedom	Mean sum of squares	p -value		Variation source	Sum of squares	Degrees of Freedom	Mean sum of squares	p -value	
x_1	35998	1	35998	0.040		x_1^2	5473.49	1	5473.49	0.005	
x_1^2	3243249	1	3243249	0.004		x_2	580.55	1	580.55	0.041	
x_2	663621	1	663621	0.022		x_2^2	1048.84	1	1048.84	0.023	
x_2^2	763910	1	763910	0.010		x_3	1531.18	1	1531.18	0.016	
x_3^2	1482772	1	1482772	0.010		x_3^2	3183.74	1	3183.74	0.008	
x_1x_2	1436182	1	1436182	0.027		x_1x_2	1219.93	1	1219.93	0.020	
x_2x_3	526035	1	526035	0.028		Regression	1012810	6			
Regression	6782347	7				Residual	392064	10			
Residual	3705554	9				Lack of fit	3869.95	8			
Lack of fit	3675471	7				Pure error	50.69	2			
Pure error	30083	2				Total	14048.74	16			
Total	10487901	16									

($p < 0.05$); X_1 = Yeast extract (g/L). X_2 = Peptone (g/L). X_3 = Initial pH (g/L). Volumetric concentration of carotenoids (R^2 : 65.0, R : 0.80). Specific concentration of carotenoids (R^2 : 0.72, R : 0.85).

Another parameter analysed was the coefficients of determination (R^2) for the volumetric (65%) and specific (72%) responses, which were both higher than 50%, indicating they describe a good part of the experiments. According to Anindyajati et al. (2018), a model having a R^2 value greater than 62% is acceptable. The R values showed a strong correlation between the variables (yeast extract, peptone and initial pH value), indicating good significance of the mathematical model in predicting the behaviour of *R. mucilaginosa*. Thus, with a coefficient of determination (R^2) greater than 50% and almost all the factors significant, it was possible to construct the contour curve presented in Figure 2a and b.

$$\text{Volumetric concentration of carotenoids}(\mu\text{g/L}) = 4324.84 - 161.30x_1 - 537.11x_1^2 - 220.54x_2 - 260.67x_2^2 - 363.17x_3 - 423.70x_1x_2 - 256.43x_2x_3 \quad (3)$$

$$\text{Specific concentration of carotenoids } (\mu\text{g/g}) = 176.64 - 22.07x_1^2 - 6.52x_2 - 9.66x_2^2 + 10.59x_3 - 16.83x_3^2 - 12.35x_1x_2 \quad (4)$$

The 3D contour curves were constructed using the mathematical models obtained, to better explain the interactions between the two factors on the response evaluated. Strong interactions are indicated by darker areas, and thus it was possible to obtain the optimum condition for each response. On the other hand, by overlapping the two figures, it was possible to obtain a region that satisfied both responses, thus reaching the maximum value provided by the two Equations 3 and 4.

According to Figure 2, in order to obtain maximum carotenoid production (volumetric and specific), the peptone concentration should be in the range from 1 to 3.5 g/L, yeast extract between 1.2 and 2.5 g/L and the initial pH should be adjusted between 6 and 7, maintaining malt extract at 10 g/L, glucose at 30 g/L, agitation at 130 rpm and the temperature at 25 °C.

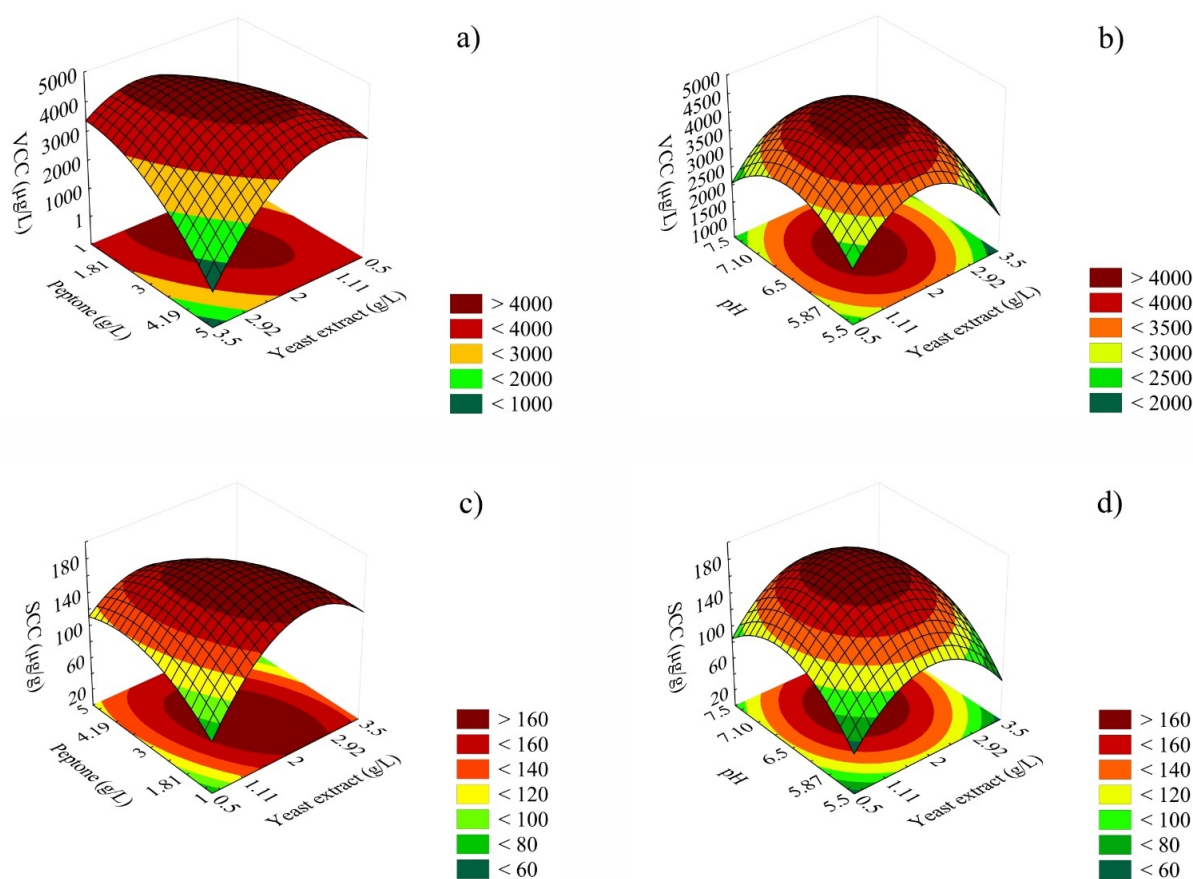


Figure 2. 3D response surface curves for carotenoid production (a, b, c and d) as a function of yeast extract, peptone and initial pH at 25 °C and 130 rpm for 144 h. SCC = Specific concentration of carotenoids (μg/g). VCC = Volumetric concentration of carotenoids.

3.1 Validation of the experiments

Based on the results of the statistical analyses, triplicate tests were carried out to confirm the predictions of the mathematical models. The optimal condition found for the yeast *R. mucilaginosa* was 3 g/L peptone (level 0), 2 g/L yeast extract (level 0), 10 g/L malt extract, 30 g/L glucose, an initial pH of 6.5 (level 0), agitation at 130 rpm and temperature at 25 °C (a moderate ratio between the carbon and nitrogen sources).

When the experiment was repeated, a production of 4261.31 $\mu\text{g/L}$ (252.99 $\mu\text{g/g}$) was obtained. The relative deviations obtained between the experimental results and those predicted by the model during validation were lower than 3.71% for the volumetric concentration of carotenoids, indicating good reliability of the model (Table 7). On the other hand, the specific concentration was higher than that of the model equation, making it possible to further increase the intracellular concentration of carotene. Therefore, both models were accepted for the prediction of the carotenoid concentrations.

Table 7. Mathematical model validation responses.

	VCC ($\mu\text{g/L}$)	SCC ($\mu\text{g/g}$)
Response predicted by the model	4324.84	176.63
Experimental response*	4164.45	252.99
Model deviation	-3.71	43.23

*Results are means of triplicate assays. VCC = Volumetric concentration of carotenoids. SCC = Specific concentration of carotenoids.

Initially, the carotenoid production using the yeast selected was 635.94 $\mu\text{g/L}$, and reached 4.32 mg/L by improving the process conditions according to the mathematical model predicted by the design. The same occurred for the initial specific carotenoid production of 122.46 $\mu\text{g/g}$, obtaining a maximum concentration of 252.99 $\mu\text{g/g}$ with the improvements indicated by the design.

Similar behaviour was observed when comparing these results with those found in the current literature. According to Cutzu et al. (2013), of 18 yeasts found in different databases, *Rhodotorula glutinis* showed prominence in the assimilation of crude glycerol. In the culture medium optimization process, the variables studied were glycerol, yeast extract and peptone, reaching a production of 14.07 mg/L and increasing the capacity by 280%.

On the other hand, Cabral et al. (2011) used a Plackett-Burman design to improve both the process conditions (agitation and temperature) and the composition of the culture medium for yeast isolated (*Sporidiobolus pararoseus*), increasing the carotenoid production by up to 45%. It was found that high glucose (60 g/L) and malt extract (15 g/L) concentrations and low peptone (15 g/L) concentrations influenced the bioproduction. In the present work, it was verified that the yeast required large amounts of glucose and moderate amounts of the nitrogen source (yeast extract) for good performance.

In the screening process described by Aksu & Eren (2007), it was verified that the initial pH influenced the development of the yeast *Rhodotorula glutinis*, and that the range from 6 to 6.5 favoured its development, obtaining a final cell concentration of 4.9 g/L. In the present work, it was verified that the pH was a significant variable with a positive effect on pigment bioproduction.

Figure 3 shows the average results obtained for the culture kinetics of pH, biomass concentration and the volumetric and specific carotenoid productions during validation of the model in the yeast malt culture medium.

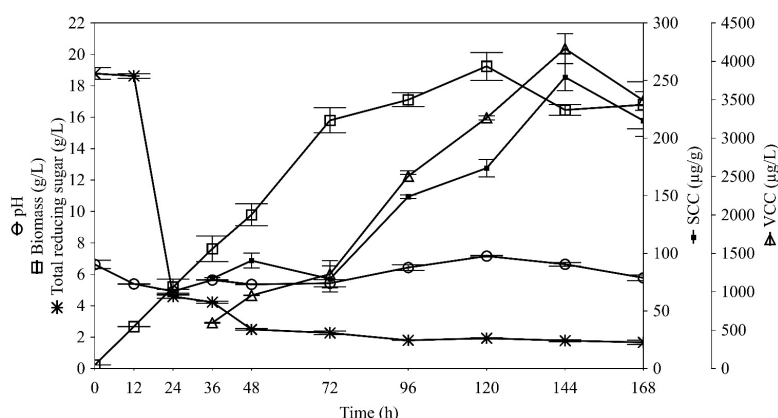


Figure 3. Kinetics of the total carotenoid production by *Rhodotorula mucilaginosa* according to the empirical models validated using the yeast malt culture medium. VCC = Volumetric concentration of carotenoids. SCC = Specific concentration of carotenoids.

Figure 3 shows rapid sugar consumption from 12 h to 24 h and rapid cell growth evolution up to 72 h of the process. In contrast to cell growth, pigment production was low during the first 72 hours and the largest increases in both specific and volumetric carotenoid production were detected after 72 h, at the same time as the values for reducing sugar stabilized.

Similar behaviour was observed for the cell growth of *Rhodotorula glutinis* in a medium such as beet molasses supplemented with ammonium sulphate (Taskin et al., 2016).

Maximum carotenoid production (4261.31 µg/L or 252.99 µg/g) occurred after 144 h with a low carbon source concentration (1.78 g/L), and from that time onwards, the production decreased. The pH of the culture showed little variation throughout the process. Similar behaviour was shown for the yeast *Sporidiobolus pararoseus* in agro-industrial coproducts (crude glycerol and corn steep water) where, after 24 hours of cultivation, the carbon source had been consumed and the carotenogenic phase started (Machado & Burkert, 2015).

4 Conclusion

From the screening carried out in a biodiverse environment (latitude – 20.785218 and longitude – 49.359897), it was possible to isolate the yeast strain *Rhodotorula mucilaginosa* URM 7409 on Brazilian territory. The study was carried out in an orbital shaker and the optimum rate of carotenoid production was obtained with a culture medium composed of 30 g/L glucose, 10 g/L malt extract, 2 g/L yeast extract and 3 g/L peptone, with a temperature of 25 °C, agitation at 130 rpm and an initial pH value of 6. This combination of factors achieved a total carotenoid production of 4261.31 µg/L (252.99 µg/g). The parameters that determined the rate of carotenoid production were the nitrogen sources followed by the initial pH value. It is to be expected that future studies in aerated reactors and the use of batch feed will surpass the values obtained here. This isolated yeast can be considered as a sustainable source of carotenoids from the perspective of a biorefinery, where diverse valuable components such as lipids, enzymes and antimicrobials are simultaneously obtained.

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