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Production of carotenoids by *Rhodotorula toruloides* isolated from Brazilian tropical savannah

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Abstract

Several artificial colouring agents are used as food additives to improve the foods' visual appearance. A recent increase in the use of natural colouring with bioactive properties (antioxidants) as a substitute for additives used in the food industry has led to the search for novel sources to produce such substances with functional colouring. The present work was aimed to isolate and select yeasts from the Cerrado (Savannah) biome (Central region, Brazil) to produce carotenoids. Sixty-nine of the 470 colonies, selected after screening, presented colours from yellow to red. These yeasts were grouped into three colours: yellow, pink and orange. Yeast belonging to the pink group, identified as *Rhodotorula toruloides*, was chosen for improvement of the factors (physical and nutritional) involved in submerged cultivation. Carotenoid bioproduction was improved by using an experimental design which evaluated the characteristics of the physical processes (agitation and temperature), followed by 2^{5-1} factorial experimental design to select the relevant factors for the culture medium. Following statistical analysis, a complete second-order experimental design was employed to optimise the composition of the culture medium. The maximum carotenoid production obtained was 1,333.11 $\mu g.L^{-1}$ (106.92 $\mu g.g^{-1}$) after 144 h at 25°C and 130 rpm in yeast malt (YM) medium containing 45.95 $g.L^{-1}$ glucose, 1 $g.L^{-1}$ malt extract, 0.7 $g.L^{-1}$ yeast extract, and 0.4 $g.L^{-1}$ peptone, with an initial pH value of 6. This result showed the potential of this yeast as a viable source of biopigments.

Introduction

In higher organisms such as plants, carotenoids play a key role in protecting the photosynthetic apparatus against photo-oxidative damage (Sun *et al.*, 2018). Carotenes can also be synthesised by some non-photosynthetic bacteria and fungi (Sowmya and Sachindra, 2015). However, since animals (including humans) are incapable of generating these biomolecules, they must be ingested via foods. Carotenoids exhibit several biological activities in human beings, such as anti-cancerous activity, besides the prevention of cardiovascular diseases, cataracts and other chronic diseases (Mata-Gómez *et al.*, 2014; Saini and Keum, 2018). This broad range of activities is due to the stimulation of the immune system by carotenoids. Therefore, carotenoids are becoming increasingly important to both pharmaceutical and food industries.

The food colouring market generated revenues of $1.5 billion in 2014 and is expected to yield $1.8 billion after carotenoid commercialisation in 2019, representing an increase of 3.9% per year (BCC Research, 2018). However, only 37.9% of this market is constituted of β–carotene originating from a natural source, i.e., the greater part of this market is dominated by synthetic colorants. Since synthetised compounds are considered noxious and have been banned from the pharmaceutical and food markets in several countries, the search for natural colorants is still in progress (Venil *et al.*, 2013; Grand View Research, 2016).

Pigment extraction from higher plants is onerous, since it involves numerous sample pre-treatment steps, difficulty in simultaneous extraction due to the complexity of several carotenoids in the matrix, and the use of mixtures of organic solvents to extract both polar and non-polar carotenes, besides its dependence on raw-materials and their availability throughout the year (Saini and Keum, 2018). Although the

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cultivation of microorganisms is promising, the use of microscopic beings, such as bacteria and fungi, leads to low biomass production and these microorganisms may be resistant to antibiotics (Ferrer *et al.*, 2017). Thus, the cultivation of microalgae is promising and economically viable when cultivated in open tanks, but the development of bacteria and other microorganisms may pose health risks, which must be considered (Singh and Sharma, 2012; Pankratz *et al.*, 2017). Yeasts, such as those belonging to the genus *Rhodotorula*, stand out in terms of cell growth and the assimilation of different carbon and nitrogen sources when compared with heterotrophic microalgae (Petrik *et al.*, 2014; Qin *et al.*, 2017).

Although there are several microorganisms that produce carotenoids, only a few are of biotechnological interest. Hence, the isolation and screening of microorganisms, which are potential producers of carotenoids such as astaxanthin, torulene, lutein and β–carotene must be encouraged (Cabral *et al.*, 2011; Valduga *et al.*, 2014). Screening is a valuable tool which enables the search for new microorganisms with biotechnological potentials. The Brazilian savannah (known as the Cerrado in Brazil) is one of the several biomes in the country, and comprises an area that stretches over 2 million km^2, that is, 25% of the country's territory, and is estimated to harbour close to 70 to 100 thousand species of moulds/yeasts (Monteiro Moreira *et al.*, 2016). However, to date, its biotechnological and commercial potential has been poorly explored.

The production of commercial colorants is expensive, but the costs could be decreased by using cell stress techniques that cause changes either in the process conditions (agitation and temperature) or in the nutrition sources. As a result, this may enable the production of natural colorants on an industrial scale (Cabral *et al.*, 2011). Individual studies on the factors involved in submerged cultivation are not only difficult and expensive but may lead to wrong conclusions due to the lack of analyses that include all the associated factors. The use of statistical tools, such as experimental design, may help to clarify the interactions amongst different parameters at different levels, and calculate the optimal level for each parameter to reach a determined target (Saenge *et al.*, 2011).

Therefore, the present work was aimed to screen yeasts using an experimental model in a less explored biome that has the potential to produce carotenoids. The effects of the operational process conditions (agitation and temperature) and the composition of the culture medium used to grow the isolated yeast (*Rhodotorula toruloides*), which showed the highest potential for this application, were both studied.

Materials and methods

Yeast screening and isolation from the Savannah biome

Samples were collected from the soil, leaves, and flowers found in the savannah biome (latitude -15.926710 and longitude -52.234504) and stored in pre-sterilised thermoplastic containers made of high-density polyethylene until further analysis. They were then transferred to 250 mL conical flasks containing 50 mL yeast malt extract (YM: 3 $g.L^{-1}$ yeast extract, 3 $g.L^{-1}$ malt extract, 5 $g.L^{-1}$ peptone, and 10 $g.L^{-1}$ glucose) and incubated at 25°C and 150 rpm for 48 h to promote microbial enrichment. They were then inoculated onto YM agar in Petri dishes and incubated at 25°C for 120 h (Cabral *et al.*, 2011).

Yeast colonies that varied from yellow to red were transferred onto YM agar slants and incubated at 25°C to reach satisfactory growth. The isolation of the yeasts was confirmed on YM agar in Petri dishes incubated at 25°C for 120 h (Valduga *et al.*, 2007) and microbial purity was checked by microscopic examination. Yeast confirmation was carried out using Plate Count Agar - PCA (23.5 $g.L^{-1}$) supplemented with 50 $mg.L^{-1}$ chloramphenicol (Neo Química®), a selective medium for yeasts (Cabral *et al.*, 2011; de Melo Pereira *et al.*, 2014).

Microbial maintenance and reactivation

The yeasts were maintained under refrigeration at 4°C in test tubes on GYMP agar (glucose, yeast malt, and peptone) slants (2 $g.L^{-1}$ glucose, 1 $g.L^{-1}$ malt extract, 0.5 $g.L^{-1}$ yeast extract, 0.2 $g.L^{-1}$ NaH_2PO_4, and 1.8 $g.L^{-1}$ agar), covered with a mineral oil layer over the agar, and replicated every three months (da Fonseca *et al.*, 2011). Reactivation was carried out by replication from stock cultures into other test tubes containing YM medium and incubated at 25°C for 48 h.

Inoculum

Sterile peptone water (1 mL) was added to the activated yeasts (from the previous step) and the surface of the medium was scraped to obtain a cell suspension. The suspension was then transferred to test tubes containing 9 mL YM broth and incubated at 25°C for 48 h. The cell cultures obtained were homogenised, transferred to 250 mL conical flasks containing 50 mL YM broth previously sterilised at 121°C for 15 min, and incubated at 25°C and 150 rpm for either 48 h or the time required for the cell culture to reach a density of 10^7 $cells.mL^{-1}$, as counted in a Neubauer chamber (Valduga *et al.*, 2007).

Carotenoid bioproduction and yeast identification

Carotenoid bioproduction was carried out in an orbital shaker (Tecnal, TE 424, Brazil) using 250 mL conical flasks containing 112.5 mL YM medium at an initial pH of 6.0. Ten percent of inoculum was added to reach a density of 10^7 cells.mL^{-1}, and incubated at 25°C and 180 rpm for 144 h (Machado and Burkert, 2015).

Samples were collected at the end of the cultivation period to determine their biomass using a gravimetric method. Additionally, the pH value of the culture and its total carotenoid concentration were also determined. Based on the bioproduction results, the yeast strains showing the highest yield was selected for DNA analysis (polymerase chain reaction, PCR). It was identified, stored, and made available to all researchers from the Micoteca URM at the Universidade Federal de Pernambuco, Brazil.

DNA extraction, PCR, and sequencing

For genomic DNA extraction, all the endophytic fungi isolated were cultured on Potato Dextrose Agar (PDA), and the Wizard® Genomic DNA Purification Kit (Promega, USA) was used to extract the DNA, according to the manufacturer's instructions. The primer set, NL1 and NL4 (O'Donnell, 1993), was used to amplify part of the ITS region (first and second internal transcribed spacer regions and intervening 5.8S nrDNA). PCR, sequencing, and sequence analyses were performed as described by Wang *et al.* (2015). The GenBank (http://www.Ncbi.nlm.nih.gov/BLAST/) was referred to analyse the ribosomal DNA sequences obtained.

Experimental design

The effects of both the operational conditions of the process and the composition of the culture medium were defined using an experimental design. In the first step, a central compound design (CCD) with three repetitions at the central point was used. The independent variables (factors) under investigation were agitation (from 130 to 230 rpm) and temperature (from 25°C to 35°C).

After the completion of the first step, a second factorial design (2^{5-1}) was employed to select the independent variables of the culture medium composition [yeast extract (from 1 to 5 g.L^{-1}), malt extract (from 1 to 10 g.L^{-1}), peptone (from 1 to 10 g.L^{-1}), glucose (from 10 to 30 g.L^{-1}), and initial pH (from 4 to 6)]. Agitation was set at 130 rpm while the temperature was set at 25°C. After the completion of this step, the third step was carried out, which consisted of a rotational central compound design (RCCD) with three central points to finalise the study of the independent variables, yeast extract (from 0 to 2 g.L^{-1}), peptone (from 0 to 2 g.L^{-1}) and glucose (from 30 to 50 g.L^{-1}). Malt extract was set at 1 g.L^{-1}, initial pH at 6.0, agitation at 130 rpm and the temperature at 25°C.

The dependent or variable responses under study were the biomass (g.L^{-1}), volumetric concentrations (μg.L^{-1}) and specific concentrations (μg.g^{-1}) of the carotenoids.

Total carotenoid recovery

The total carotenoid recovery was determined based on the method described by da Fonseca *et al.* (2011) and Michelon *et al.* (2012) and adapted by Cipolatti *et al.* (2015). The biomass was centrifuged at 3,439 g (5000 rpm) for 10 min, transferred to a Petri dish, placed in a freeze-dryer for 48 h, macerated in a mortar, and standardised using a Tyler 115 sieve (Machado and Burkert, 2015; Cipolatti *et al.*, 2015). Following standardisation, the biomass was submitted to lysis using the rupture agent dimethyl sulfoxide (DMSO, $(CH_3)_2SO$), and then agitated for 1 min in a vortex after every 15 min for 1 h (da Fonseca *et al.*, 2011). After rupture, acetone was added so that the mixture could again be centrifuged (3,439 g for 10 min). The supernatant was separated from the precipitate and several successive extractions were conducted until total cell whitening was achieved.

The supernatants were pooled together and 20% NaCl (w.v^{-1}) and petroleum ether was added to form two phases. The polar phase was collected and excess water was removed using Na_2SO_4, thus giving rise to the extracts containing carotenoids (Michelon *et al.*, 2012).

Determination of total carotenoid

The total carotenoid (TC) concentrations in the extracts were determined by evaluation of absorbance in a spectrophotometer at 448 nm (Cabral *et al.*, 2011), and expressed as the major carotenoid (β–carotene in petroleum ether, whose specific absorptivity is $A_{cm}^{1\%}$ = 2592), with the use of Eq. 1 (David, 1976).

$$TC = \frac{A * V * 10^6}{A_{1cm}^{1\%} * 100 * m_{sample}} \qquad \text{(Eq.1)}$$

where TC = total carotenoid concentration (μg.g^{-1}), A = absorbance, V = volume (mL), m_{sample} = dry cell mass (g), and $A_{cm}^{1\%}$ = specific absorptivity. To calculate the volumetric concentration of the carotenoids (μg.L^{-1}), a unit conversion was applied (direct multiplication) using the results obtained for the specific concentration of carotenoids (μg.g^{-1}) and the biomass concentration (g.L^{-1}).

Determination of biomass

The cells were centrifuged at 3,439 g for 10 min to separate the supernatant. The pellet was then washed with distilled water and centrifuged again. The cell mass was quantified after drying at 105°C to constant mass according to AOAC (2012).

Determination of pH

The pH was determined by analysing the sample in a potentiometer, according to AOAC (2012).

Determination of reducing sugars

The concentrations of reducing sugars were determined using the spectrophotometric method of 3-5 dinitrosalicylic (DNS) in accordance with the method previously described by Miller (1959), using a standard glucose curve in which standard concentration varies between 0.1 and 1.0 $g.L^{-1}$.

Statistical analysis

The data were analysed using Statistica 10.0 (StatSoft Inc., Tulsa, OK, USA) at a 95% confidence level ($p < 0.05$), and the analysis of variance (ANOVA) was used to estimate the statistical parameters. Response surfaces and contour diagrams were constructed in accordance with the method described previously by Box (1978).

Results and discussion

The screening of yeasts from the Savannah biome that produce microbial carotenoids was conducted for the first time. Sixty-nine of the 470 colonies selected from the environmental samples obtained from the Brazilian biome, were either orange, pink, or yellow. The yeasts were grouped into three colour classes (yellow, orange, and pink); however, in the present work, only the selection of pink yeasts was investigated (34 yeasts harboured this pigmentation).

In the search for carotenoid-producing yeasts, the yeast showing the highest biopigment production was strain 5 (639.05 $\mu g.L^{-1}$), followed by strain 8 (609.80 $\mu g.L^{-1}$). The other strains, such as strain 7 (519.44 $\mu g.L^{-1}$) and strain 34 (461.59 $\mu g.L^{-1}$), showed inferior production. The yeast strains 5 and 8 were identified by genomic sequencing using GenBank, by the Culture Collection at the Micoteca URM (Recife, Brazil), as *Rhodotorula toruloides* (ID: MK532267, shows 99.66% similarity with ID: KY109177) and *Rhodotorula* sp. (ID: MK532268), respectively. These were both found in flower samples deposited in the institution, and were identified by the access codes of URM 7406 and URM 7407, respectively.

Both yeasts strains 5 (URM 7406) and 8 (URM 7407) produced high levels of carotenoids, with no significant difference in yield. However, the former showed a higher average volumetric concentration of carotenoids than the latter.

The results hereafter presented are based on the analysis of strain URM 7406. An experimental design (CCD) was used to evaluate the operational cultivation factors. The results showed that the lowest agitation (130 rpm) and temperature (25°C) favoured both the specific and volumetric carotenoid production by this strain (92.52 $\mu g.g^{-1}$ and 669.54 $\mu g.L^{-1}$, respectively). On the other hand, when the temperature was above 25°C and agitation at 130 rpm, the production was low (145.08 $\mu g.L^{-1}$). Similarly, when the agitation was above 130 rpm and temperature at 25°C, the carotenoid production was also low (480.51 $\mu g.L^{-1}$).

This step led to standardisation of both operational conditions (agitation and temperature) and a new 2^{5-1} factorial experimental design was then used to optimise the culture medium.

The volumetric carotenoid concentration increased when the glucose concentration was increased, but in the case of the other carbon sources, such as malt extract, increasing the concentration of carbon source did not affect the bioproduction. In contrast, when the concentrations of yeast extract and peptone were increased, there was a decrease in the amounts of pigment obtained in most experiments. Although there was no difference in carotene production in the pH range of 5 to 6, experiments did not show good carotene production at pH 4. These independent factors are better observed in Figure 1, which shows the effects of increasing the concentrations of the variables on the range of responses under study.

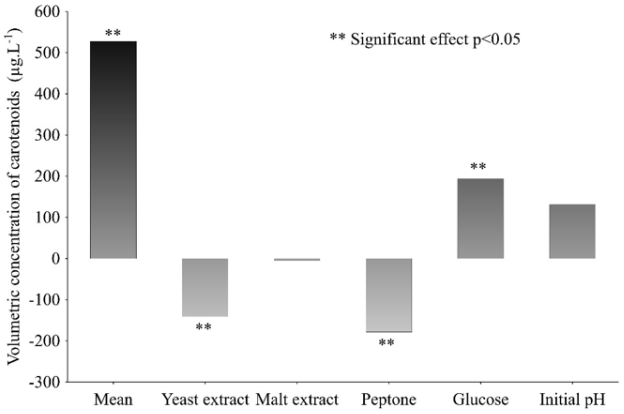

Figure 1: Effect of variables on responses of total carotenoids in 2^{5-1} factorial design at 25°C and 130 rpm.

Glucose was the only carbon source which exhibited a positive and significant effect on carotenoid production, followed by pH. The stimulating effect of glucose may be explained by the fact that it is easily

assimilated in the metabolic pathway of β–carotene biosynthesis. Glucose, when associated with pH, increased the bioproduction of carotenoids by up to 45% (Cabral *et al.*, 2011).

Although the pH value exhibited a positive, but insignificant effect, it has been described as a regulator of organic acid production at the beginning of cultivation (Machado and Burkert, 2015). The use of an acidified medium (initial pH 4) leads to a disturbance in the metabolic pathway and negatively affects the formation of acetic acid, alcohol, and the citric acid cycle intermediates in the adaptation phase. Studies on the production of carotenoids by *R. toruloides* in the pH range from 3 to 8, show that pH 6.5 is the most favourable pH to produce pigment, since it increases the cell growth rate and carotenoid production (Aksu and Eren, 2007).

The yeast extract and peptone concentrations exhibited negative effects on production. Even though the nitrogen source used in the present work was known to promote satisfactory cell growth, its high concentrations in the culture medium might have suppressed the biosynthesis of carotenoids and other secondary metabolites, such as lipids (Saenge *et al.*, 2011). Some researchers have stated that the C:N ratio influences the production of colorants by several microorganisms (Cheirsilp *et al.*, 2012; Braunwald *et al.*, 2013; Machado and Burkert, 2015; Tkáčová *et al.*, 2017). In the present work, it was observed that *R. toruloides* required only minimum quantities of nitrogen to maintain cell development, and utilised the excess of carbon in the culture medium to synthesise carotene. Therefore, to maximise carotenoid bioproduction, changes in the levels of concentration of carbon and nitrogen sources were made. The concentrations of yeast extract and peptone were decreased, i.e. moved from 0 to 2 $g.L^{-1}$ range, close to the central point in Figure 1. On the other hand, glucose exhibited a significant positive effect ($p < 0.05$) and therefore its level was increased (from 30 to 50 $g.L^{-1}$). Considering the fact that the pH exhibited a positive, but insignificant ($p > 0.05$) effect, it was set at 6 since good results were obtained at this value. The change in concentrations of malt extract exhibited little effect and was administered at a lower level (1 $g.L^{-1}$). In short, the strategy was to increase the C:N ratio by decreasing the nitrogen sources, so as to better absorb the excess of carbon sources available in the culture medium (Spier *et al.*, 2015; Taskin *et al.*, 2016).

Table 1 shows the real and codified values of the 2^3 Rotational Central Compound Design (RCCD) and the responses in terms of cell concentration, besides the specific and volumetric carotenoid concentrations. The Table shows that the highest carotenoid concentrations were reached in the region with the highest levels of carbon sources and lowest levels of nitrogen sources, thus characterising a high C:N ratio.

Table 1. 2^3 rotational central compound design (real and codified values) to optimise the culture medium.

Runs	X_1	X_2	X_3	Y_1	Y_2	Y_3
1	-1 (0.4)	-1 (0.4)	-1 (34.05)	9.24	75.04	693.69
2	+1 (1.60)	-1 (0.4)	-1 (34.05)	10.68	105.86	1130.83
3	-1 (0.4)	+1 (1.60)	-1 (34.05)	10.03	104.7	1049.76
4	+1 (1.60)	+1 (1.60)	-1 (34.05)	9.54	98.53	939.81
5	-1 (0.4)	-1 (0.4)	+1 (45.95)	10.49	103.48	1085.19
6	+1 (1.60)	-1 (0.4)	+1 (45.95)	12.61	106.43	1342.54
7	-1 (0.4)	+1 (1.60)	+1 (45.95)	12.83	105.17	1349.54
8	+1 (1.60)	+1 (1.60)	+1 (45.95)	5.36	106.71	572.05
9	-1.68 (0)	0 (1)	0 (40)	9.24	108.39	1002.00
10	+1.68 (2)	0 (1)	0 (40)	7.59	73.61	559.03
11	0 (1)	-1,68 (0)	0 (40)	10.53	97.85	1029.97
12	0 (1)	+1,68 (2)	0 (40)	11.28	110.16	1242.48
13	0 (1)	0 (1)	-1,68 (30)	9.17	104.86	961.77
14	0 (1)	0 (1)	+1,68 (50)	11.89	96.15	1143.60
15	0 (1)	0 (1)	0 (40)	12.31	101.31	1215.82
16	0 (1)	0 (1)	0 (40)	10.68	110.67	1328.19
17	0 (1)	0 (1)	0 (40)	11.69	104.77	1257.39

X_1 = yeast extract ($g.L^{-1}$); X_2 = peptone ($g.L^{-1}$) and X_3 = glucose. Malt extract in 1 $g.L^{-1}$, initial pH 6. Y_1 = biomass ($g.L^{-1}$); Y_2 = specific concentration of carotenoids ($\mu g.g^{-1}$) and Y_3 = volumetric concentration of carotenoids ($\mu g.L^{-1}$).

In the RCCD (Table 1), the maximum carotenoid production ranged from 559.03 $\mu g.L^{-1}$ (assay 10) to 1,349.54 $\mu g.L^{-1}$ (assay 7), whereas the biomass concentration ranged between 5.36 $g.L^{-1}$ (assay 8) and 12.83 $g.L^{-1}$ (assay 7), at 144 h. After employing the experimental design, the maximum carotenoid and biomass concentrations increased by 121.31% and 45.45%, respectively, in comparison with the initial process conditions.

Eq. 2 shows the codified second-order model which describes the carotenoid concentration in

terms of the independent variables (yeast extract, peptone, and glucose), in the time interval under study. The model was validated by ANOVA (Table 2). The determination of the coefficient of variation (R^2) of 86% and the value for $F_{calculated}$ of 2.37–fold higher than that established for total carotenoids, enabled the contour plot to be constructed (Figure 2). For the specific carotenoid concentrations, no factor was significant and the R^2 value was only 42%, signifying that the model did not explain the experiments satisfactorily.

$$\text{Volumetric concentration of carotenoids } (\mu g.L^{-1}) = 1263.93 - 68.68x_1 - 138.74x_1^2 + 61.61x_3 - 54.76x_3^2 - 197.74x_1x_2 - 105.92x_1x_3 - 83.90x_1x_3 \quad \text{(Eq. 2)}$$

Where x_1 = yeast extract; x_2 = peptone, and x_3= glucose.

Table 2. Analysis of variance for the 2^3 central compound design.

Source of variation	Quadratic sum	Degrees of freedom	Quadratic mean	*p*–value
x_1^*	64363.8	1	64363.8	0.047
x_1^{2*}	249848.5	1	249848.5	0.013
$x_1x_2^*$	312812.8	1	312812.8	0.010
$x_1x_3^*$	89746.0	1	89746.0	0.034
$x_2x_3^*$	56312.0	1	56312.0	0.052
Regression	861792.1	7	123113.16	
Residual	133896.5	9	14877.39	
Lack of fit	127440.6	7		
Pure error	6455.9	2		
Total	995688.6	16		

*($p < 0.1$); x_1: yeast extract ($g.L^{-1}$); x_2: peptone ($g.L^{-1}$) and x_3: glucose ($g.L^{-1}$). Volumetric concentration of carotenoids: (R^2: 86%, $F_{7;9;0.90\ (tabulated)} = 2.51$, $F_{calculated}$(model) = 8.28). Ratio $F_{calculated}/F_{tabulated} = 3.30$.

Contour plots were obtained from the second-order models. When both figures were overlapped, an optimal region favouring the parameters under study could be found. In order to obtain the maximum concentration of carotenoids (Figure 2), the culture medium should contain 0.2 to 1.2 $g.L^{-1}$ yeast extract, 0 to 0.4 $g.L^{-1}$ peptone, 45.95 to 50 $g.L^{-1}$ glucose, 1 $g.L^{-1}$ malt extract, and 0.2 $g.L^{-1}$ KNO_3. The initial pH should be 6.0, agitation at 130 rpm, and temperature at 25°C.

After analysing Figure 2, to validate the model optimised for the production of carotenoids by *R. toruloides* URM 7406, the optimal production medium, which satisfied the three parameters under study, was selected as follows: 0.4 $g.L^{-1}$ peptone (level -1), 0.7 $g.L^{-1}$ yeast extract (level -0.5), 45.95 $g.L^{-1}$ glucose (level +1), 1 $g.L^{-1}$ malt extract, and 0.2 $g.L^{-1}$ KNO_3, with an initial pH value of 6.0, agitation at 130 rpm, and temperature of 25°C in order to reach maximum pigment production.

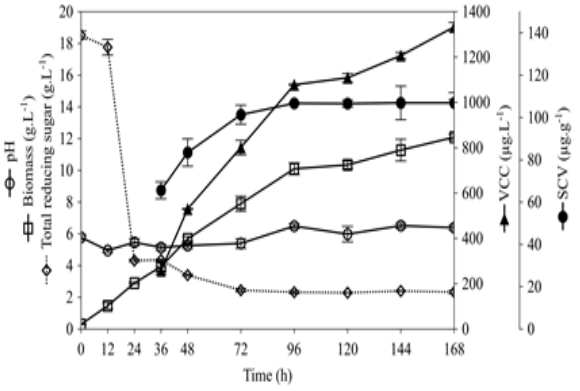

Figure 3: Production kinetics of total carotenoids by Rhodotorula toruloides (URM 7406) in the validation of the empirical models in yeast malt. VCC = volumetric concentration of carotenoids, SCV = specific concentration of carotenoids

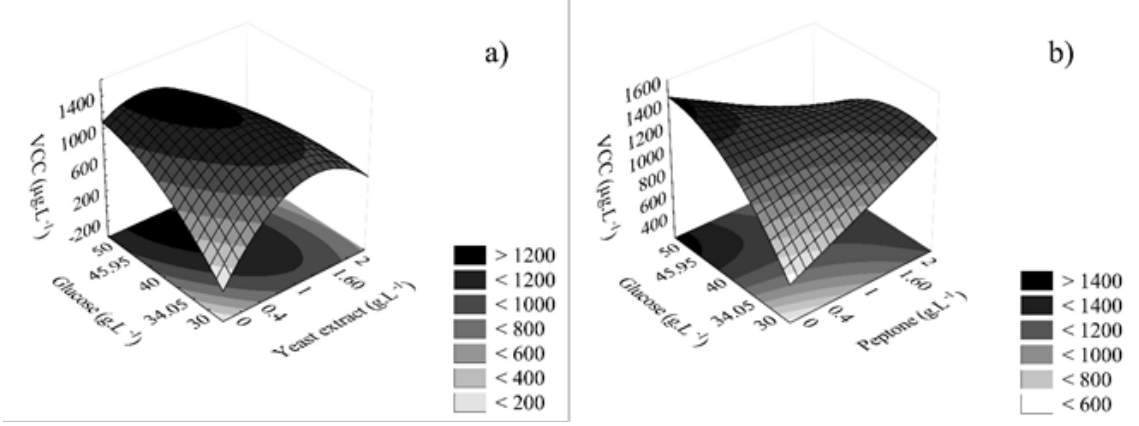

Figure 2: Contour plot for carotenoid production (a and b) regarding the yeast extract, peptone and glucose at 25°C, 130 rpm for 144 h. VCC = volumetric concentration of carotenoids.

Figure 3 shows the mean results for the cultivation kinetics with respect to pH, cell growth, and the specific and volumetric concentrations of carotenoids throughout validation of the model in Yeast Malt (YM) culture medium.

Figure 3 illustrates that there was a rapid sugar consumption during the first few hours of cultivation and elevation of cell growth up to the end of the process, i.e. cell multiplication progressed rapidly for 96 h. Unlike cell growth, pigment production depicted lower levels during the first 48 h and the highest volumetric concentration of carotenoids was detected after 96 h, due to a decrease in cell growth. On the other hand, the specific concentration of carotenoids increased during the first 96 h, and then, remained constant up to 168 h.

The pH value ranged from 5.8 to 5 during the first 12 h, and then it increased to 6.0, with some fluctuations. These changes in pH might be attributed to the capacity of the aerobic microorganism to convert sugars into organic acids. This natural phenomenon, which occurs during the first few hours of cultivation, has previously been reported by other studies (Machado and Burkert, 2015; Taskin *et al.*, 2016). After depletion of the carbon sources, peptide hydrolysis occurred, which increased the pH. This phenomenon was observed by Valduga *et al.* (2014) when they used agro-industrial substrates (glycerol, corn steep liquor, and parboiled rice water) to yield carotenoids by *Sporidiobolus pararoseus*. The effect of pH on *R. toruloides* has already been studied by Kot *et al.* (2017), who showed that change in pH influenced both cell growth and the carotenoid concentration. Carotene biosynthesis and cell growth elevation were induced by high pH values (from 4.0 to 7.0), which was confirmed in the present work (Figure 3), where maximum carotenogenesis was induced in 168 h at pH 6.4.

Validation of the ideal experimental condition

Since the model (Eq. 2) forecast the maximum response for carotenoid production of 1,308.43 $\mu g.L^{-1}$ when the ideal values of the significant independent variables (yeast extract, peptone, and glucose) were used, a real experiment had to be carried out using these values to make sure the forecast result was not polarised in relation to the practical value. Therefore, the experiment was repeated under the calculated ideal conditions and a product yield of 1,333.11 $\mu g.L^{-1}$ was obtained, thus validating the model. In fact, the production was 1.89% higher than that predicted by the model.

Conclusion

The present work showed that the Savannah biome, located in the central region of Brazil, was a good source of carotenoid-producing yeasts. The yeast, *R. toruloides*, isolated from flower samples, was identified as the largest carotenoid producer amongst 34 screened yeasts. The present work was performed in an orbital shaker with the culture medium composed of 45.95 $g.L^{-1}$ glucose, 0.4 $g.L^{-1}$ peptone, and 0.7 $g.L^{-1}$ yeast extract, at 25°C, 130 rpm, and initial pH 6. The combination of these factors led to the achievement of a total carotenoid production of 1,333.11 $\mu g.L^{-1}$ (106.92 $\mu g.g^{-1}$). The parameters that determine the rate of carotenoid production were the sources of nitrogen (peptone and yeast extract) and carbon (glucose). It is predicted in future studies that with the use of aerated reactors and fed-batch culture, even higher carotenoid yields could be obtained.

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