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CAROTENOID PRODUCTION BY *SPORIDIOBOLUS PARAROSEUS* IN AGROINDUSTRIAL MEDIUM: OPTIMIZATION OF CULTURE CONDITIONS IN SHAKE FLASKS AND SCALE-UP IN A STIRRED TANK FERMENTER

Carina Molins Borba[1*], Millene das Neves Tavares[1], Caroline Costa Moraes[2] and Janaína Fernandes de Medeiros Burkert[1]

[1]Laboratório de Engenharia de Bioprocessos, Universidade Federal do Rio Grande, Avenida Itália, km 08, Campus Carreiros, Escola de Química e Alimentos, Mailbox: 474, Zip code: 96203900 - Rio Grande, RS - Brazil.
[2]Laboratório de Microbiologia e Toxicologia de Alimentos, Universidade Federal do Pampa, Maria Anunciação Gomes de Godoy, n°1650, Malafaia, Zip Code: 96413-170-Bagé, RS- Brazil.

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Abstract – Biotechnological production of carotenoids can be affected by cultivation conditions, such as temperature, pH and agitation. The aim of this study was to maximize carotenoid production by *Sporidiobolus pararoseus* in shake flasks with agroindustrial by-products as substrates. The best conditions were used in a stirred tank fermenter. The medium consisted of corn steep liquor and sugar cane molasses pretreated with sulfuric acid. In order to evaluate the effects of the variables, a central composite rotatable design was used. The highest values of total carotenoids (565 μg L^{-1}), biomass (13.6 g L^{-1}) and $Y_{p/s}$ (10.9 μg g^{-1}) were obtained in Assay 13 at 27.5ºC, 150 rpm and pH=4, in Erlenmeyer flasks. For the best conditions defined for carotenoid productivity and 1.2 vvm in the stirred tank fermenter, the maximum value was 1969.3 μg L^{-1} of total carotenoids. It was 3.5-fold higher than the value obtained when shake flasks were used.

Keywords: yeast, molasses, experimental design.

INTRODUCTION

Biotechnology has great importance in various areas, such as pharmaceutical, food, agricultural and environmental (Fossi et al., 2016; Pleszczynska et al., 2016; Riaño et al., 2016; Kavino et al., 2007). The use of yeast has been highlighted in the production of a wide range of compounds, such as lipids (Gong et al., 2016), enzymes (Otero et al., 2015) and carotenoids (Cipolatti et al., 2015; Damodaran et al., 2007).

Red yeasts belonging to the genus *Sporidiobolus* can grow and produce carotenoids (the major one is β-carotene) efficiently in an alternative medium, such as corn steep liquor combined with sugar cane molasses (Machado and Burkert, 2015; Cipolatti, 2012).

More than 15 million tons of corn were produced in Brazil in 2015 (IBGE, 2016). In order to reduce losses, corn is subjected to humidification during its processing, and the residual water is called corn steep liquor (Liggett and Koffler, 1948). The composition of the water, which is quite variable depending on the corn which is used, may include carbon, hydrogen

**Corresponding author.* carinamolinsborba@yahoo.com.br

and nitrogen (Cipolatti, 2012). More than 10 million tons of sugar cane were also produced in Brazil in 2015 (IBGE, 2016). The industrial processing of sugar cane results in the by-product known as sugar cane molasses, whose composition has more than 30% of carbon (Valduga et al., 2007; Cipolatti, 2012).

This ability makes them promising for the industrial bioproduction of carotenoids, not only to minimize costs with the cultivation medium, but also to add value to the agroindustrial waste. Cultivation conditions can interfere in carotenoid production by red yeasts. Temperature is one of the most important environmental factors for the development of organisms, since it entails changes in biosynthetic pathways, including carotenoid biosynthesis (Bhosale, 2004). The pH level can affect cell growth and product formation. In general, the resulting effects depend on the microorganisms, medium composition and operational conditions (Saenge et al., 2011).

In aerobic microorganisms, aeration plays the major role in the growth rate and carotenoid production, since oxygen transfer becomes a limiting factor when cells grow and the viscosity of the broth increases (Mantzouridou et al., 2002). In shake flasks, oxygen transport is a consequence of the incubator shaking, while in stirred tank fermenters, oxygen is provided by a compressed air line. Stirring blades improve medium mixing and the distribution of air bubbles; reduction in the size of the bubbles increases their surface areas (Garcia-Ochoa et al., 2010).

Data collected by BCC Research (2015) show that the global carotenoid market value was $1.5 billion in 2014 and it is expected to reach nearly $1.8 billion in 2019. The favorable scenario, along with increasing concerns for the risks of consuming synthetic dyes (Batada and Jacobson, 2016; Dafallah et al., 2015), makes of great interest studies whose objectives are to increase productivity and reduce production costs of natural dyes, such as carotenoids. Therefore, the aim of this study was to maximize carotenoid production by the new strain *Sporidiobolus pararoseus* in shake flasks with agroindustrial by-products as substrates. The best conditions were used in a stirred tank fermenter.

MATERIALS AND METHODS

Microorganism

The new yeast strain *Sporidiobolus pararoseus,* which was used in this study, had been previously isolated in Caçapava do Sul, a city located in the Escudo Sul-riograndense ecosystem (Rio Grande do Sul state, Brazil) from environmental samples (Otero, 2011), identified and deposited at the André Tosello Tropical Culture Collection (CCT 7689).

Maintenance and reactivation of the microorganism

The microorganism was maintained in inclined tubes with YM agar (3.0 g L^{-1} yeast extract, 3.0 g L^{-1} malt extract, 5.0 g L^{-1} peptone, 10.0 g L^{-1} glucose and 20 g L^{-1} agar). For reactivation, samples were transferred from stock cultures to other tubes with the same medium and incubated at 25ºC for 48 h. Cell resuspension (pre-inoculum) was performed in 1.0 mL peptone water (0.1%), added to 9 mL of previously optimized medium - composed of 40.0 g L^{-1} sugar cane molasses and 6.5 g L^{-1} corn steep liquor, pretreated with sulfuric acid (Machado and Burkert, 2015) - and incubated in the same conditions previously described.

Pretreatment of substrates

For the pretreatment of the medium, both substrates, molasses and corn steep liquor, were separately acidified to pH = 3.0 by the addition of 1 N sulfuric acid and allowed to rest for 24 h at room temperature. After this period, they were centrifuged at 3439 xg for 10 min and the pH was adjusted in agreement with the experimental design, before the sterilization process, with the addition of a NaOH or sulfuric acid solution (Machado and Burkert, 2015).

Inoculum preparation

The pre-inoculum was added to 250 mL Erlenmeyer flasks with 90 mL of medium (40.0 g L^{-1} sugar cane molasses and 6.5 g L^{-1} corn steep liquor, pretreated with sulfuric acid) which had been previously sterilized at 121ºC for 15 min (Machado and Burkert, 2015). The inoculum was incubated at 25ºC, at 150 rpm for 48 h or the time necessary to achieve $1x10^7$ cells mL^{-1}, counted with a Neubauer chamber (Michelon et al, 2012).

Influence of the volume of the production medium in relation to the volume of the reactor in shake flasks

In order to evaluate the influence of the volume of the production medium in relation to the volume of the reactor, experiments were carried out in three ratios: 30% (500 mL Erlenmeyer with 150 mL medium), 50% (500 mL Erlenmeyer with 250mL medium) and 70% (500 mL Erlenmeyer with 350 mL medium).

Previously optimized culture medium (Machado and Burkert, 2015) - composed of 40.0 g L^{-1} sugar cane molasses and 6.5 g L^{-1} corn steep liquor, pretreated with sulfuric acid at pH 5 (Rios et al., 2015) - and 10% inoculum were used.

Optimization of the conditions of carotenoid production in shake flasks with agroindustrial medium

For the carotenoid bioproduction, 500 mL Erlenmeyer flasks with 225 mL culture medium (40.0 g L^{-1} sugar cane molasses and 6.5 g L^{-1} corn steep liquor, pretreated with sulfuric acid) and 25 mL (10%) inoculum were used. To evaluate the effects of the variables temperature (20-35ºC), agitation (100-200 rpm) and initial pH (4-8) on carotenoid production by *S. pararoseus*, an experimental design was used (Table 1), with pretreated substrates. Samples were taken every 24 h to monitor biomass concentration, pH, total reducing sugars and total carotenoids. Total cultivation time was 240 h.

Culture in stirred tank fermenters

Production was carried out with the stirred tank fermenter Biostat 2 L (1.5 L working volume), using medium with 40.0 g L^{-1} sugar cane molasses and 6.5 g L^{-1} corn steep liquor pretreated with sulfuric acid, temperature control at 25ºC, initial pH = 6.0, at 158 rpm and 1.2 vvm (Hui et al., 2007) for 168 h. Biomass, pH, total reducing sugars and total carotenoids were monitored in this period.

Extraction and determination of total carotenoids

Recovery of total carotenoids began with biomass centrifugation at 3439 xg for 10 min. It was then transferred to a Petri dish, placed in a circulating air oven (35ºC for 48 h) (Fonseca et al., 2011), macerated to a standardized degree by a 115 mesh sieve and frozen at -18ºC for 48 h (Cipolatti et al., 2015). Once frozen, the biomass was lysed with the rupture agent dimethyl sufoxide (DMSO), followed by vortexing for 1 min at 15 min intervals for 1 h (Fonseca et al., 2011). After rupture, acetone was added, followed by centrifugation (3439 xg for 10 min). The supernatant was separated and successive extractions were performed until total bleaching of the cell.

To the solvent phases obtained by centrifugation, 20% NaCl solution (w v^{-1}) and petroleum ether were added. After the formation of both phases, the polar phase was collected and excess water was removed by sodium sulfate (Michelon et al., 2012), thus, forming the carotenogenic extracts.

Concentrations of specific carotenoids in the extracts were determined by a spectrophotometer (Biospectro SP-220, China). Maximum absorbance average was 448 nm, expressed in terms of its major carotenoid (β-carotene), using Equation 1 (Davies, 1976):

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

where TC is the total carotenoid concentration (μg g^{-1}), A is the absorbance, V is the volume (mL), m_{sample} is the

Table 1. Coded values and real values (in parentheses) for the central composite rotational design (CCRD) and the response variables.

Assay	X_1	X_2	X_3	R_1*	R_2*	R_3	R_4	R_5	R_6	R_7	R_8
1	-1.00 (23)	-1.00 (120)	-1.00 (5.2)	324 (192 h)	11.1 (240 h)	0.036	6.5	0.20	28.3	0.04	1.5
2	1.00 (32)	-1.00 (120)	-1.00 (5.2)	164 (216 h)	3.6 (216 h)	0.002	2.9	0.07	42.9	0.01	0.5
3	-1.00 (23)	1.00 (180)	-1.00 (5.2)	412 (168 h)	12.5 (192 h)	0.039	7.8	0.22	33.6	0.06	2.3
4	1.00 (32)	1.00 (180)	-1.00 (5.2)	172 (240 h)	7.7 (240 h)	0.009	3.0	0.14	17.0	0.03	0.5
5	-1.00 (23)	-1.00 (120)	1.00 (7.2)	185 (120 h)	5.8 (240 h)	0.030	5.2	0.11	28.5	0.02	1.2
6	1.00 (32)	-1.00 (120)	1.00 (7.2)	89 (240 h)	2.6 (240 h)	0.024	1.6	0.04	40.8	0.01	0.3
7	-1.00 (23)	1.00 (180)	1.00 (7.2)	396 (144 h)	9.7 (216 h)	0.039	7.2	0.23	28.7	0.04	1.7
8	1.00 (32)	1.00 (180)	1.00 (7.2)	222 (216 h)	5.3 (240 h)	0.010	4.6	0.10	44.7	0.02	0.9
9	-1.68 (20)	0 (150)	0 (6)	287 (168 h)	10.7 (216 h)	0.039	4.9	0.27	17.0	0.04	1.0
10	1.68 (35)	0 (150)	0 (6)	137 (24 h)	3.4 (24 h)	0.001	0.0	0.44	0.0	0.10	0.0
11	0 (27.5)	-1.68 (100)	0 (6)	133 (48 h)	3.2 (48 h)	0,025	1.5	0.07	22.3	0.05	1.1
12	0 (27.5)	1.68 (200)	0 (6)	232 (192 h)	6.6 (216 h)	0.025	4.6	0.19	22.9	0.03	0.7
13	0 (27.5)	0 (150)	-1.68 (4)	565 (240 h)	13.6 (192 h)	0.025	10.9	0.26	43.0	0.07	2.3
14	0 (27.5)	0 (150)	1.68 (8)	203 (144 h)	4.1 (240 h)	0.025	6.1	0.07	57.7	0.01	1.3
15	0 (27.5)	0 (150)	0 (6)	503 (168 h)	6.7 (168 h)	0.030	9.5	0.11	83.7	0.03	2.9
16	0 (27.5)	0 (150)	0 (6)	520 (168 h)	6.6 (216 h)	0.030	10.2	0.12	87.9	0.03	3.0

X_1: Temperature (ºC); X_2: Agitation (rpm); X_3: initial pH ; R_1: Total Carotenoids (μg L^{-1}); R_2: Biomass (g L^{-1}); R_3: $\mu_{máx}$ (h^{-1}); R_4: $Y_{p/s}$ (μg g^{-1}); R_5: $Y_{x/s}$ (g g^{-1}); R_6: $Y_{p/x}$(μg g^{-1}); R_7: P_x (g L^{-1} h^{-1}); R_8: P_c (μg L^{-1} h^{-1}); *In parentheses is the time at which this variable was at the maximum value.

dry cell mass (g) and $A_{1cm}^{1\%}$ is the specific absorptivity (β-carotene in petroleum ether = 2592). To calculate total carotenoids (μg L^{-1}) with the results of the specific concentration and biomass concentration, unit conversion was performed.

Determination of pH

Culture pH was determined by a pHmeter (Marte, MB-10, Brazil), in agreement with AOAC (2000).

Biomass concentration

Cell concentration was estimated throughout the process of carotenoid bioproduction by reading the absorbance at 620 nm with a previously constructed standard curve (Choi and Park, 2003).

Determination of total reducing sugars

Total reducing sugar (TRS) concentrations were determined in cell-free supernatant. Two mL of culture medium were submitted to hydrolysis with 2 mL HCl 2.0 mol L^{-1} in boiling water for 10 min, followed by the addition of 2 mL of NaOH 2.0 mol L^{-1} for acid neutralization (Maldonade et al., 2013). Subsequently, total reducing sugars were determined by the spectrophotometric method of 3,5-dinitrosalicylic acid (DNS), according to Miller (1959), with a standard glucose curve.

Kinetic parameters

For kinetic parameters, data were obtained by analytical methodologies and calculated according to Hiss (2001). Maximum specific growth rate - μ_{max} (h^{-1}) was calculated with the help of the Software Grapher 8 by linear regression of the exponential phase, using Equation 2:

$$\mu_{\max} = \frac{1}{X}\frac{dX}{dt} \tag{2}$$

TRS in product conversion factors ($Y_{p/s}$ (μg g^{-1})), TRS in biomass ($Y_{x/s}$ (g g^{-1})) and biomass in product ($Y_{p/x}$ (μg g^{-1})) were calculated by Equations 3, 4 and 5, respectively:

$$Y_{p/s} = \frac{P_m - P_0}{S_0 - S} \tag{3}$$

$$Y_{x/s} = \frac{X_m - X_0}{S_0 - S} \tag{4}$$

$$Y_{p/x} = \frac{P_m - P_0}{X_m - X_0} \tag{5}$$

where X_m is the maximum biomass concentration (g L^{-1}), X_0 is the initial biomass concentration (g L^{-1}), P_m is the maximum carotenoid concentration (μg L^{-1}), P_0 is the initial carotenoid concentration (μg L^{-1}), S_0 is the initial TRS concentration (g L^{-1}) and S is the final TRS concentration (g L^{-1}).

In order to evaluate the performance of the fermentation process, productivities of carotenoid - P_c (μg L^{-1} h^{-1}) and biomass - P_x (g L^{-1} h^{-1}) were calculated from Equations 6 and 7, respectively:

$$P_c = \frac{P_m - P_0}{t_f} \tag{6}$$

$$P_x = \frac{X_m - X_0}{t_f} \tag{7}$$

where X_m is the maximum biomass concentration (g L^{-1}), X_0 is the initial biomass concentration (g L^{-1}), P_m is the maximum carotenoid concentration (μg L^{-1}), P_0 is the initial carotenoid concentration (μg L^{-1}) and t_f is the fermentation time in which maximum biomass or product (h) was obtained.

Statistical analysis

Data were treated with the aid of Statistica 5.0 (StartSoft Inc., Tulsa, OK, USA). All analyses considered 90% confidence level ($p<0.1$). The analysis of variance (ANOVA) was used for estimating statistical parameters. Response surfaces were drawn in agreement with Box et al. (1978).

RESULTS AND DISCUSSION

Nitrogen, carbon, temperature, agitation and pH are some of the factors that can affect carotenoid production separately or synergistically (Aksu and Eren, 2007; Rios et al., 2015). In this study, temperature, pH and agitation were investigated in the search for the best conditions for carotenoid production by *Sporidiobolus pararoseus*.

Influence of the volume of the production medium in relation to the volume of the reactor

Figure 1 shows the results of total carotenoids obtained for the ratios medium volume: reactor volume of 30, 50 and 70%. The volume variation of the medium in the reactor can affect oxygen transfer. Liu et al., (2006) characterized the transfer of oxygen during the cultivation of *Phafiia rhodozyma* and, based on linear regression of experimental data, observed that the oxygen transfer coefficient was correlated

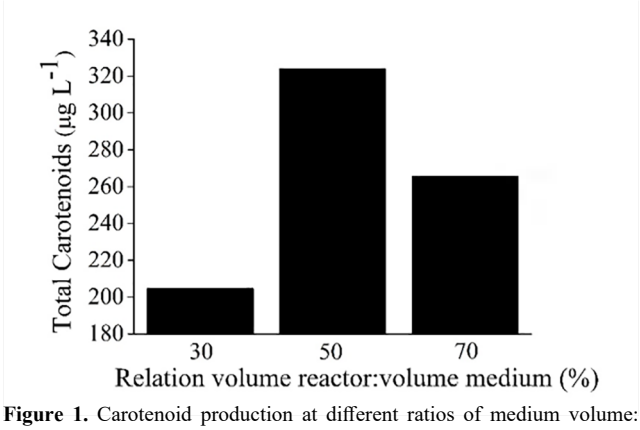

Figure 1. Carotenoid production at different ratios of medium volume: reactor volume

with shaker speed and liquid volume. In the same study, the authors report that the carotenoid yield showed a strong linear correlation with the oxygen transfer rate demonstrating that oxygen supply is crucial for carotenoid production in liquid cultures. The effect of oxygen transfer on the production of biomass and carotenoids was also demonstrated by other authors using *Rhodotorula rubra* (Cutzu et al., 2013) and *Blakeslea trispora* (Mantzouridou et al., 2005). Therefore, it is of great importance to define a ratio of medium volume: reactor volume that allows an adequate transfer of oxygen, since this parameter is directly dependent on the liquid side mass transfer coefficient, the total specific surface area available for mass transfer and the driving force in terms of concentrations (Garcia-Ochoa et al., 2010). In the present study the highest concentration of carotenoids was obtained with the 50% ratio, which was chosen for further work.

Optimization of the conditions of carotenoid production in shake flasks with agroindustrial medium

Table 1 shows the central composite rotatable design (CCRD) 2^3. Times in which the highest carotenoid and biomass concentrations were obtained are indicated between parentheses. It is worth mentioning that the smallest values of responses under study were obtained in conditions in which temperatures were maintained above 30ºC (Assays 2, 4, 6, 8 and 10). Low productions observed at high temperatures may have been the result of changes in the major carotenoid. According to Frengova and Beshkova (2009), the effect of temperature depends on the microorganism species; it often manifests itself in a number of variations in synthesized carotenoids. El-Banna et al. (2012) showed that temperature range from 15 to 35ºC in the cultivation of *Rhodotorula glutinis* led to changes in the percentage of produced β-carotene and torulene. When the temperature was raised to 30ºC, β-carotene was the major carotenoid produced by the yeast, representing up to 60% of the production. However, when the temperature reached 35ºC, this ratio was reversed and torulene represented 61% of total carotenoid production.

The highest values of total carotenoids (565 μg L^{-1}), biomass (13.6 g L^{-1}) and substrate to product conversion factor ($Y_{p/s}$=10.9 μg g^{-1}) were obtained in Assay 13, at 27.5ºC, at 150 rpm (central points) and pH=4 (-1.68).

Valduga et al. (2008) found similar results to those obtained in this study regarding the increased production of carotenoids in acid pH with *S. salmonicolor* and medium composed of 10 g L^{-1} molasses, 5 g L^{-1} corn steep liquor and 5 g L^{-1} Prodex Lac®, pre-treated with sulfuric acid, at 25ºC, at 180 rpm, initial pH 4.0. Their production reached 541.5 μg L^{-1}.

TRS consumption (analyzed as glucose) during cultivation (data not shown) ranged from 68% (Assay 10) to 96% (Assay 3). Assays with the highest TRS consumption occurred at 180 rpm (Assays 3, 4 and 8) and at 150 rpm (Assays 13, 14, 15, 16 and 17). The lowest TRS consumption (Assays 11, 6 and 2) occurred at the lowest agitation (100-120 rpm) and caused the three lowest $Y_{p/s}$ (1.54; 1.56; 2.86 μg g^{-1}) and $Y_{x/s}$ (0.07; 0.04; 0.07 gg^{-1}) values. In Assay 10, despite the fact that agitation was 150 rpm, $Y_{p/s}$ was close to zero, probably because the temperature was 35ºC (level + 1.68). The effect of agitation on cell development was previously observed in *Rhodotorula glutinis* cultivation, from 100 to 150 rpm, when the yeast showed less cell growth, probably due to the decrease in nutrients available on the cell surface, whereas high stirring rates (more than 250 rpm) caused cell disruption (Tinoi et al., 2005).

The use of an experimental design enables the study of the influence of the levels of one variable on the responses. Table 2 shows regression coefficients, standard deviations and p and t values used in the construction of the models (Equations 8, 9 and 10).

On the basis of the analysis of variance (ANOVA), as shown in Table 3, second-order Equations 8, 9 and 10 were established to describe the total carotenoid concentration, biomass concentration and carotenoid productivity (P_c), respectively, as a function of temperature, agitation and pH. The pure error was very low, indicating good reproducibility of the experimental data. Based on the F test, the models are predictive, since the calculated F values are higher than the critical F values (3.6, 10 and 6.4-fold for carotenoid and biomass concentrations

Table 2. Regression coefficients (RC), standard errors (SE) and t from the central composite rotational design (CCRD) for the responses total carotenoids, biomass concentration and P_c.

Total Carotenoids (μg L^{-1})				Biomass (g L^{-1})				P_c(μg L^{-1} h^{-1})			
	RC	**SE**	**t(6)**		**RC**	**SE**	**t(6)**		**RC**	**SE**	**t(6)**
Mean	511.16*	50.80	10.06	**Mean**	6.61*	0.90	7.34	**Mean**	2.945*	0.29	10.04
X_1 (L)	-67.53*	39.02	-3.46	**X_1 (L)**	2.36*	0.69	-6.81	**X_1 (L)**	-0.447*	0.22	-3.97
X_1 (Q)	-105.59*	47.42	-4.45	**X_1 (Q)**	0.22	0.84	0.52	**X_1 (Q)**	-0.834*	0.27	-6.09
X_2 (L)	44.51*	39.02	2.28	**X_2 (L)**	1.30*	0.69	3.77	**X_2 (L)**	0.09	0.22	0.84
X_2 (Q)	-116.09*	47.42	-4.90	**X_2 (Q)**	-0.54	0.84	-1.29	**X_2 (Q)**	-0.07*	0.27	-5.11
X_3 (L)	-57.80*	39.02	-2.96	**X_3 (L)**	2.01*	0.69	-5.81	**X_3 (L)**	-0.170	0.22	-1.51
X_3 (Q)	-44.63	47.42	-1.88	**X_3(Q)**	0.85*	0.84	2.04	**X_3 (Q)**	-0.377*	0.27	-2.75
X_1X_2	-19.92	50.96	-0.78	**X_1X_2**	0.19	0.90	0.41	**X_1X_2**	-0.099	0.29	-0.67
X_1X_3	16.32	50.96	0.64	**X_1X_3**	0.59	0.90	1.30	**X_1X_3**	0.132	0.29	0.90
X_2X_3	30.90	50.96	0.21	**X_2X_3**	0.14	0.90	0.30	**X_2X_3**	0.052	0.29	0.35

*($p < 0.1$); X_1: temperature;X_2: agitation; X_3: pH.

Table 3. ANOVA for the CCRD responses total carotenoids, biomass concentration and productivity in carotenoids.

Total carotenoids					
Source of variation	**QS**	**DF**	**QM**	**F_{cal}**	**R^2**
Regression	282612.9	5	56522.6	9	0.82
Residue	62522.2	10	6252.2		
Total	345135.1	15			
Biomass					
Source of variation	**SQ**	**GL**	**MQ**	**F_{cal}**	**R^2**
Regression	165.5	4	41.4	25.3	0.90
Residue	17.97	11	1.6		
Total	183.5	15			
Productivity in carotenoids					
Source of variation	**QS**	**DF**	**QM**	**F_{cal}**	**R^2**
Regression	10.6	4	1.6	16.2	0.85
Residue	1.8	11	0.2		
Total	12.4	15			

QS: Quadratic Sum; DF: Degrees of freedom; QM: quadratic mean. Total carotenoids: $F_{tab} = 2.52$; Biomass and productivity in carotenoids: $F_{tab} = 2.54$.

and P_c, respectively). Regression coefficients (0.82, 0.90 and 0.85) for total carotenoids, biomass and P_c, respectively, are considered satisfactory. Parameters that were not significant ($p>0.1$) were added to lack of fit in the analysis of variance. Coded models were used to generate response surfaces (Figure 2).

$$\mathbf{TC\ (\mu g\ L^{-1})} = 441.1 - 67.5\ X_1 - 86.8\ X_1^2 + 44.5\ X_2 - 97.3\ X_2^2 - 57.8\ X_3 \quad (8)$$

$$\mathbf{Biomass\ (g\ L^{-1})} = 6.3 - 2.3\ X_1 + 1.30\ X_2 - 2.0\ X_3 + 0.9 X_3^2 \quad (9)$$

$$\mathbf{P_c\ (\mu g\ L^{-1} h^{-1})} = 2.9 - 0.4\ X_1 - 0.8\ X_1^2 - 0.7\ X_2^2 - 0.4\ X_3^2 \quad (10)$$

where TC is the total carotenoids, P_c is the carotenoid productivity, X_1 is the temperature, X_2 is the agitation and X_3 is the pH.

Figures 2a, 2b and 2c show that the maximum carotenoid production was optimized. Therefore, agitation should be maintained between 144 and 170 rpm, along with temperatures between 24 and 27.5ºC and pH between 4 and 4.2. In order to reach maximal production of biomass (Figure 2c, 2d and 2e), agitation should be maintained between 192 and 200 rpm, temperatures between 20 to 21ºC and initial pH from 4 to 4.3. Carotenoid productivity (Figure 2g, 2h and 2i) was also optimized; agitation had to be kept between 132 and 168 rpm, temperatures from 25 to 27.5ºC and pH from 5.4 to 6.6.

To find the highest content of carotenoids, models given by Equations 8, 9 and 10 were validated at 27.5ºC, at 150 rpm and initial pH = 4.0 (Figure 3a). In this assay, 591.4 μg L^{-1} was obtained in 240 h, approximately 9% more than the model had predicted (538.2 μg L^{-1}). In the same conditions, 12.8 g L^{-1}

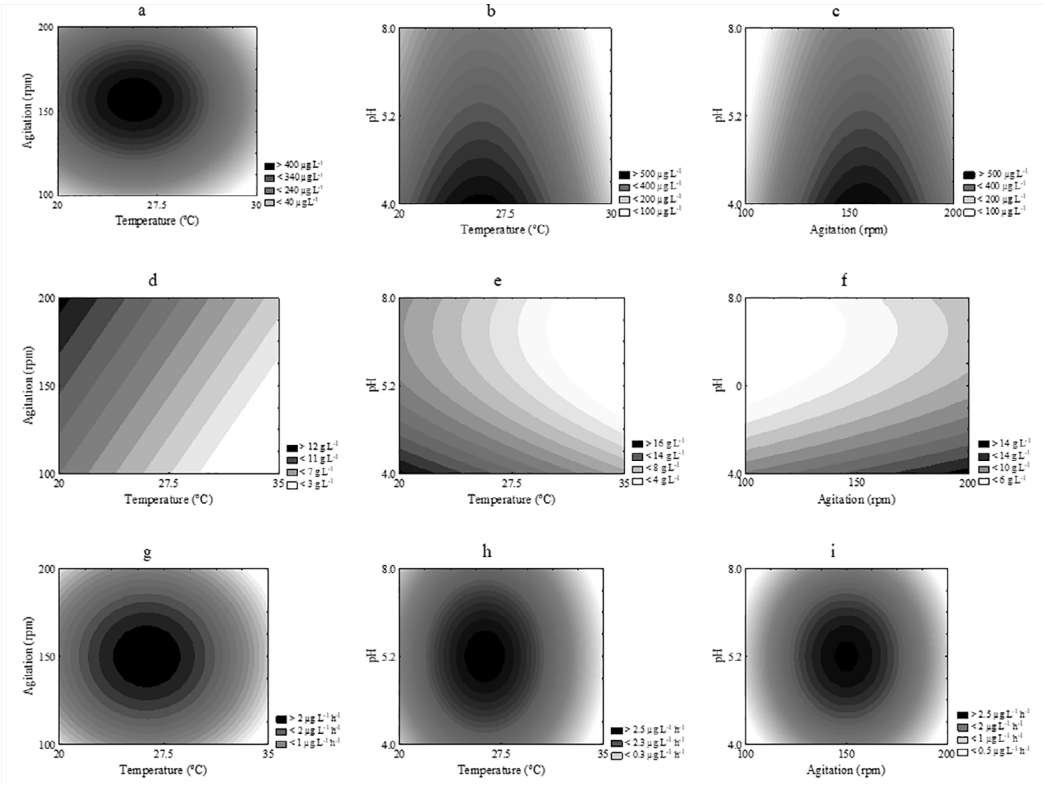

Figure 2. Contour curves of total carotenoids (a, b, c), biomass (d, e, f) and P_c (g,h,i).

biomass was obtained, approximately 5% more than the model had predicted (12.2 g L^{-1}), represented by Equation 9. Carotenoid productivity was 2.25 µg L^{-1} h^{-1}, 27% more than predicted by Equation 10 (1.77 µg L^{-1} h^{-1}). The pH rises until 72 h, whereas no changes were observed afterwards.

Models generated for the other responses were not predictive, so the effects of the variables were of no avail (Figure 4). The use of an experimental design enables the study of the influence of the levels of one variable on the response variable. Thus, the main effects of such a design may be simply calculated as the difference between the average of measurements made at the high level (+1) of the variable and the average of measurements at the low level (-1) (Rodrigues and Iemma, 2012). The variables temperature, agitation and pH, in the ranges under study, did not show significant effects (p>0.1) on the responses $Y_{x/s}$ and $Y_{p/x}$. But the change in the temperature level from -1 to +1 had negative effects on the values of $\mu_{máx}$ (-0.025 h^{-1}), $Y_{x/s}$ (-0.10 g g^{-1}) and P_x(-0.02 g L^{-1} h^{-1}). Concerning agitation, the effects were positive for $Y_{x/s}$ (0.07 g g^{-1}) and P_x(0.02 g L^{-1} h^{-1}). Regarding pH, the effect was negative for P_x (-0.01 g L^{-1} h^{-1}).

In the conditions of validation in shake flasks (27.5ºC, 150 rpm and initial pH = 4.0) 591.4 µg L^{-1} total carotenoids was obtained in 240 h. Biomass was 12.8 g L^{-1} whereas carotenoid productivity was 2.25 µg L^{-1} h^{-1}. Finally, μ_{max} was 0.042 h^{-1}, $Y_{p/s}$ was 18.35µg L^{-1}, $Y_{x/s}$ was 0.40 g g^{-1}, $Y_{p/x}$ was 45.33 µg g^{-1} and biomass productivity was 0.05 g L^{-1} h^{-1}.

Carotenoid production in a stirred tank fermenter

The principal aim of fermentation scale-up is to produce large quantities of product with high yields in the minimum time possible (Ju and Chase, 1992).

In the stirred tank fermenter, the conditions were the ranges considered to be the best in the experimental design previously introduced for carotenoid productivity: 25ºC, initial pH 6.0, 158 rpm. Aeration was maintained at 1.2 vvm (Hui et al., 2007) for 168 h.

In these conditions (Figure 3b), the maximum values were 1969.3 µg L^{-1} for total carotenoids, 18.05 g L^{-1} for biomass, 0.044 h^{-1} for $\mu_{máx,}$ 37.08 µg L^{-1} for $Y_{p/s}$, 0.33 g g^{-1} for $Y_{x/s}$, 112.48 µg g^{-1} for $Y_{p/x}$, 0.10 g L^{-1} h^{-1} for biomass productivity and 11.48 µg L^{-1} h^{-1} for carotenoid productivity. The pH ranged from 5.5 to 8.7, with the highest change in the first 72 h of cultivation. After 96 h of the process, 80% of TRS had been consumed.

Malisorn and Suntornsuk (2009) compared β-carotene production by *R. glutinis* DM28 in Erlenmeyer flasks and in a stirred tank fermenter. Carotenoid production in the stirred tank fermenter

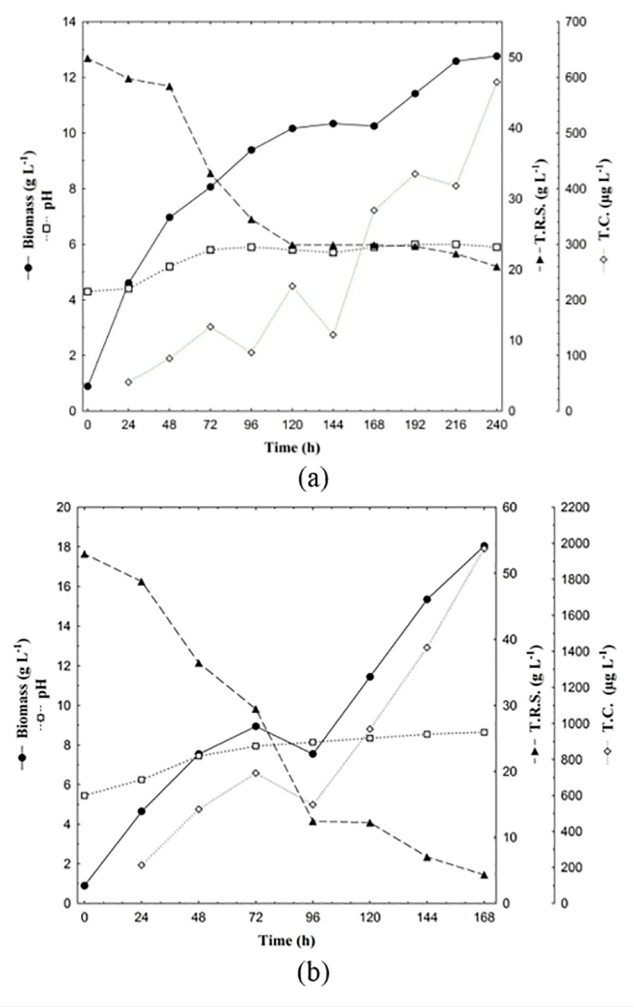

Figure 3. (a) Biomass, pH, total carotenoids and TRS consumption in shake flasks (27.5ºC, 150 rpm, initial pH = 4.0); (b) Biomass, pH, total carotenoids and TRS consumption in the stirred tank fermenter (25°C, 158 rpm, 1.2 vvm, initial pH = 6.0).

(186 µg L^{-1}) was approximately two times higher than in Erlenmeyer flasks (87 µg L^{-1}). According to the authors, this behavior indicates that better medium homogenization and oxygen distribution caused by cultivation in the stirred tank fermenter influence the accumulation of β-carotene in yeast cells significantly, possibly due to their involvement in the stimulation of enzymes, such as phytoene desaturase, β-carotene hydroxylase and lycopene cyclase, which act on the metabolism of this carotenoid. The comparison between the maximum amount of carotenoids found in the stirred tank fermenter and that found in the validation shows that this amount was 3.3-fold higher than the value obtained with the use of shake flasks.

Vigorous stirring is required to ensure homogeneity in the distribution of nutrients. In shake flasks, oxygen transport is a consequence of the incubator shaking,

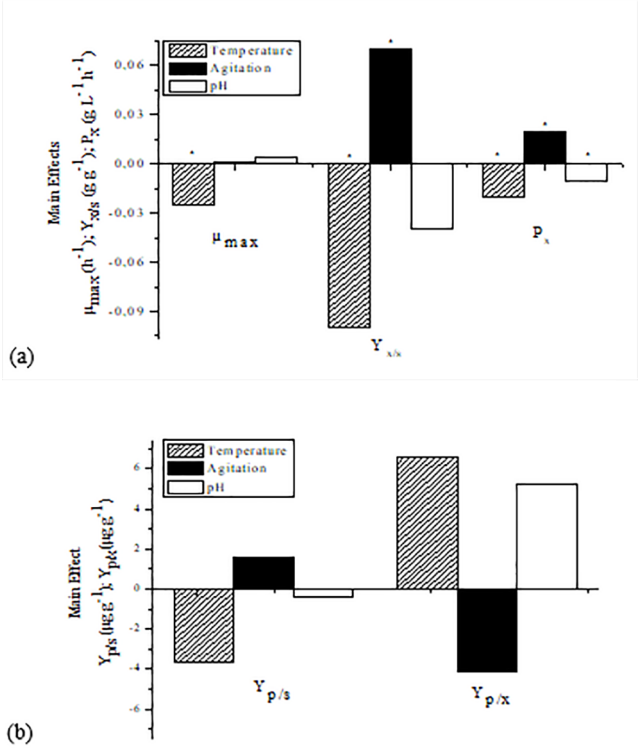

Figure 4: Effects of the variables temperature, agitation and pH on the responses $\mu_{máx}$ (a), $Y_{x/s}$ (a), P_x(a), $Y_{p/s}$ (b) and $Y_{p/x}$ (b) by the experimental design CCRD

while in stirred tanks fermenters, the oxygen is provided by a compressed air line and distributed by a diffuser. Besides, stirring blades improve the mixing of the medium and the distribution of air bubbles, reducing their size and increasing their surface area (Garcia-Ochoa et al., 2010).

CONCLUSIONS

Conditions of temperature, agitation and pH were optimized in order to evaluate total carotenoid and carotenoid productivity. In the 2^3 CCRD experimental design, carotenoid production showed its maximum value: 565.0 µg L^{-1}. Production in a stirred tank fermenter, in the best conditions observed in the experimental design for carotenoid productivity, reached 1969.3 µg L^{-1} for carotenoids and 11.48 µg L^{-1} h^{-1} for carotenoid productivity. Carotenoid production found in cultivation in the stirred tank fermenter was 3.5-fold higher than the value obtained with shake flasks. Therefore, this study not only demonstrates the influence of the parameters temperature, agitation and pH on carotenoid production by the yeast *S. pararoseus*, but also shows that its capacity can be increased by modifying the parameters aeration and agitation.

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NOMENCLATURE

TC	total concentration of carotenoids (μg g^{-1})
A	absorbance
V	volume (mL)
m_{sample}	dried cell mass (g)
$A_{1cm}^{1\%}$	specific absorptivity
TRS	total reducing sugars
DNS	3,5 dinitrosalicylic acid
$\mu_{máx}$	maximum specific growth rate (h^{-1})
$Y_{p/s}$	TRS to product conversion factor (μg g^{-1})
$Y_{x/s}$	TRS to biomass conversion factor (g g^{-1})
$Y_{p/x}$	biomass to product conversion factor (μg g^{-1})
X_m	maximum biomass concentration (g L^{-1})
X_0	initial biomass concentration (g L^{-1})
P_m	maximum concentration of carotenoids (μg L^{-1})
P_0	initial concentration of carotenoids (μg L^{-1})
S_0	initial concentration of TRS (g L^{-1})
S	final concentration of TRS (g L^{-1})
P_c	carotenoid productivity (μg L-1 h^{-1})
P_x	biomass productivity (g L^{-1} h^{-1})
t_f	time of fermentation where the maximum biomass or product was obtained (h)
CCRD	central composite rotatable design

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