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Optimization of agroindustrial medium for the production of carotenoids by wild yeast *Sporidiobolus pararoseus*

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This study addresses the optimization of agroindustrial medium used to obtain carotenoids with the wild yeast, *Sporidiobolus pararoseus*. The optimal condition for the production of total carotenoids was achieved in 168 h obtaining 779.60 µg/L (65.64 µg/g) with 30 g/L of raw glycerol (from the synthesis of biodiesel) and 52.9 g/L of corn steep liquor at 25°C and 180 rpm. The formulation of another culture medium was also optimized by combining 40 g/L of sugar cane molasses and 6.5 g/L corn steep liquor, achieving a total carotenoid of 520.94 µg/L (73.19 µg/g) under the same process conditions, demonstrating the potential of this yeast as a biopigment source.

Key words: Agroindustrial, by-products, culture medium, optimization.

INTRODUCTION

Carotenoids are hydrocarbons found abundantly in nature with over 600 characterized structures. They vary in color, ranging from yellow to red, and are present in fruits, vegetables, flowers and microorganisms. These pigments play an important role in human health, as precursors of vitamin A, antioxidants and stimulants of the production of antibodies (Cabral et al., 2011; Amar et al., 2012).

Carotenogenic pigments are used in the preparation of cosmetics and pharmaceuticals, in textile industries, in the food industry as a food additive, colorants and functional ingredients (Venil and Ahmad, 2013). In recent decades, there has been a change in consumer behavior due to discoveries of the use of chemical additives that may

have allergenic and carcinogenic potential. Therefore, consumers now seek foods with functional properties and without synthetic additives (Machado et al., 2014). The global market demand for carotenoids for the year 2017 is forecast at 10 billion tonnes, with a compound annual growth rate of 2.9%/year (Venil and Ahmad, 2013). However, most of these pigments are derived from chemical synthesis and do not satisfy the desire of consumers seeking natural colorants.

The negative aspects of using carotenoids obtained through chemical synthesis have prompted growing interest in obtaining them through biotechnological processes using different yeasts (Michelon et al., 2012; Silva et al., 2012; Cipolatti, 2012; Fonseca et al., 2011;

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Otero, 2011), bacteria (Sutthiwong et al., 2014) and microalgae (Machado et al., 2014; Rodrigues et al., 2014; Ahmed et al., 2014). Other advantages to be gained, such as the microbial production of pigments, are obtainable quickly and at any time of year, in a small space, using low cost substrates, with control of the culture conditions and receiving the designation of natural colorants, as cited in literature (Valduga et al., 2009).

Among the microorganisms capable of producing carotenoids, the wild yeast *Sporidiobolus pararoseus,* isolated from the ecosystem of Rio Grande do Sul, in the Escudo Sul-riograndense (Shield of Rio Grande do Sul) region stands out (Otero, 2011). It is a producer of β-cryptoxanthin, lutein and β-carotene mostly obtained from the use of different co-products and agro-industrial residues, with antioxidant potential (Otero, 2011; Cipolatti, 2012).

However, microbial obtaining of carotenoids is mainly limited by the high cost of production. This cost can be minimized by improving/optimizing the use of industrial byproducts as sources of nutrients because they are widely available, and combined with experimental design technique can obtain high production of carotenoids (Taskin et al., 2011). To this strain of *S. pararoseus* has been found an adaptation to different medium complex using alternative sources of carbon and nitrogen, such as parboiled rice wastewater, corn steep liquor, raw glycerol (from the synthesis of biodiesel) and cane molasses sugar for the production of biopigments (Otero, 2011; Cipolatti, 2012).

On the other hand, the effect of the influence of process conditions involving the production and recovery of carotenoids using experimental design methodology has been applied. The recovery of carotenoids produced by *Sporidiobolus salmonicolor* involved studying the temperature and concentration of the enzyme complex to cell disruption (Monks et al., 2013), while Michelon et al. (2012) also assessed the enzymatic method for the recovery of carotenoids produced by *Phaffia rhodozyma* checking the influence of the pH of the reaction medium, temperature, initial activity of β-1,3-glucanase and reaction time. The composition of the production medium using *S. salmonicolor* (Valduga et al., 2009, 2014) and for *S. pararoseus* (Cabral et al., 2011). This methodology is also applied to obtain other bioproducts, such as biomass (Santos et al., 2012), galacto-oligosaccharides (Lisboa et al., 2012a; Lisboa et al., 2012b), dairy drinks (Martins et al., 2011; Martins et al., 2012; Burkert et al., 2012), enzymes (Maldonado et al., 2012; Campello et al., 2012; Alves et al., 2010), synthesis of natural flavors (Anschau et al., 2011; Aragão et al., 2011), phycocyanin (Moraes et al., 2010) and rhamnolipids (Rosa et al., 2010).

Thus, the aim of this study was to optimize concentrations of the substrates of two types of agroindustrial production medium using corn steep liquor combined with raw glycerol (derived from the synthesis of biodiesel) or sugar cane molasses, via response surface analysis,

using wild yeast *S. pararoseus* for carotenoid production.

MATERIALS AND METHODS

Microorganism

The *S. pararoseus* yeast used in this work was previously isolated (Otero, 2011) from environmental samples from the ecosystem of the Escudo Sul-Rio-Grandense region (Rio Grande do Sul - Brazil), identified and deposited in the André Toselo Tropical Culture Collection (CCT 7689).

Maintenance and reactivation of the microorganism

The microorganism was maintained in test tubes containing GYMP agar inclined (2.0 g/L glucose, 1.0 g/L of malt extract, 0.5 g/L yeast extract, 0.2 g/L NaH_2PO_4 and 1.8 g/L agar) with mineral oil under refrigeration at 4°C (Fonseca et al., 2011) by 3 months. For reactivation, samplings were carried out from stock cultures to other test tubes with the same medium and incubated for 25°C at 48 h. A cell resuspension (pre-inoculum) was performed on 1.0 mL of peptone water (0.1%) and added to 9 mL of modified YM medium (3.0 g/L of yeast extract, 3.0 g/L of malt extract, 5.0 g/L peptone, 10.0 g/L of glucose, added to 0.2 g/L KNO_3) (Parajó and Vázquez, 1998) and incubated under the same conditions described above.

Agroindustrial substrates

The agroindustrial substrates that were used were kindly provided by industry in the region. The carbon sources were raw glycerol derived from the synthesis of biodiesel (BSBIOS Indústria e Comércio de Biodiesel Sul Brasil S/A - Passo Fundo – RS) and sugar cane molasses (Guimarães Indústria e Comércio Ltda. - RS), and corn steep liquor (Corn Products Balsa Nova - PR) from the wet corn milling, used as a nitrogen source, previously characterized (Cipolatti, 2012; Silva et al., 2012; Otero, 2011). For the formulation of the culture medium, the agroindustrial substrates were weighed according to the assay of the experimental design (Tables 1 and 5), centrifuged (3439 x *g* for 10 min) separately, transferred to the same Erlenmeyer flask to adjust the initial pH of the culture medium at 6.0 and kept standing until the sterilization at 121°C for 15 min.

Inoculum preparation

One milliliter cell suspension was made in sterile peptone water (0.1%) from the tubes containing the *S. pararoseus* in inclined YM agar and added to 9.0 mL of modified YM broth and incubated at 25°C for 48 h. The inoculum was grown in 250 mL Erlenmeyer flasks containing 90 mL of YM medium, previously sterilized at 121°C for 15 min, the cell suspension was added and incubated at 25°C, 150 rpm for 48 h or the time necessary to achieve 1×10^8 cells/mL, enumerated in a Neubauer chamber (Michelon et al., 2012).

Cultures in shake flasks

The cultures for the bioproduction of carotenoids were prepared in 500 mL Erlenmeyer flasks with 250 mL culture medium, an initial pH of 6.0 and the addition of 10% inoculum (starting cultivation with 1×10^7 cells/mL), under operating conditions of 25°C, 180 rpm (orbital shaker, Tecnal model TE 424), without lighting for 168 h (Fonseca et al., 2011; Michelon et al., 2012). Samples were taken every 24 h to monitor biomass concentration, pH, sugars and total

Table 1. Real values and coded levels (in parentheses) for the central composite design (CCD) and central composite rotational design (CCRD) using raw glycerol and corn steep liquor, and maximum concentration response of total carotenoids and biomass.

Assay	CCD				CCRD			
	X_1	X_2	Y_1	Y_2	X_1	X_2	Y_1	Y_2
1	5 (-1)	20 (-1)	154.86	4.42	15.8 (-1)	44.5 (-1)	450	8.32
2	10 (+1)	20 (-1)	283.19	6.05	44.2 (+1)	44.5 (-1)	599.5	9.02
3	5 (-1)	30 (+1)	163.03	4.55	15.8 (-1)	65.5 (+1)	591.72	7.79
4	10 (+1)	30 (+1)	640.54	6.47	44.2 (+1)	65.5 (+1)	338.03	6.78
5	7.5 (0)	25 (0)	348.95	5.83	10 (-1.41)	55 (0)	637.64	9.49
6	7.5 (0)	25 (0)	349.63	5.97	50 (+1.41)	55 (0)	707.78	9.23
7	7.5 (0)	25 (0)	345.83	5.99	30 (0)	30 (-1.41)	656.2	10.26
8	-	-	-	-	30 (0)	80 (+1.41)	575.32	5.91
9	-	-	-	-	30 (0)	55 (0)	822.57	9.90
10	-	-	-	-	30 (0)	55 (0)	820.79	9.98
11	-	-	-	-	30 (0)	55 (0)	822.03	9.95

X_1 = Raw glycerol concentration (g/L), X_2 = corn steep liquor concentration (g/L), Y_1 = total carotenoids concentration (µg/L) and Y2 = biomass concentration (g/L). - Assays that do not exist in the CCD.

carotenoids.

Experimental designs for bioproduction of carotenoids

The composition of the agroindustrial production medium using the corn steep liquor and raw glycerol byproducts was studied using a central composite design (CCD) followed by a 2² central composite rotational design (CCRD) (Table 1). The other carotenoid producing medium used the sugar cane molasses and corn steep liquor through a 2² CCRD (Table 5). The responses or dependent variables studied were maximum concentration of total carotenoids (µg/L), with the respective amount of biomass concentration (g/L) obtained for each experimental assay.

In the validation of each optimal carotenoid production condition using defined agroindustrial substrates, cultures were performed in triplicate under the same process conditions as described above.

Recovery of total carotenoids

The recovery of total carotenoids began with the centrifugation of the biomass at 3439 x g for 10 min, which was then transferred to a Petri dish and placed in a circulating air oven (35°C for 48 h) (Fonseca et al., 2011), and subsequently macerated in a degree, standardized in a mesh 115 sieve and frozen at -18°C for 48 h (Cipolatti, 2012). Once frozen, the biomass was lysed by a rupture agent dimethylsulfoxide - DMSO ((CH$_3$)$_2$SO), followed by vortexing for 1 min in 15 min intervals, a total of 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 performed until total bleaching of the cell was achieved.

In the solvent phases, obtained from the centrifugation, 20% NaCl solution (w/v) and petroleum ether were added. After the formation of the two phases, the polar phase was collected and excess water was removed with sodium sulfate (Na$_2$SO$_4$), forming carotenogenic extracts (Michelon et al., 2012).

Determination of total carotenoids

The concentration of total carotenoids in the extracts was deter-

mined using a spectrophotometer (Biospectro SP-220, China) through the average maximum absorbance at 448 nm[1] expressed in terms of its major carotenoid (β-carotene in petroleum ether with specific absorptivity of $A_{1cm}^{1\%}$ = 2592), using equation 1 (Davies, 1976).

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

Where, specific TC is the total concentration of carotenoids (µg/g), A is absorbance, V is the volume (mL), m_{sample} is dried cell mass (g) and $A_{1cm}^{1\%}$ is specific absorptivity. To calculate the volumetric concentration of total carotenoids (µg/L) using the result of the concentration of total carotenoids (µg/g) and biomass concentration (g/L) a unit conversion was performed.

Determination of pH

The pH was determined by reading the sample in a potentiometer, according to AOAC (1995).

Determination of biomass concentration

The cell concentration throughout the bioproduction of carotenoids was estimated by reading the absorbance at 620 nm, through a previously constructed standard curve (Kusdiyantini et al., 1998).

Determination of the concentration of total reducing sugars

The concentration of total reducing sugars (ART) was determined in cell free supernatant previously centrifuged at 3439 x g for 10 min. One milliliter of culture medium with the agroindustrial byproducts were submitted to a hydrolysis with 2 mL of HCl 2.0 moL/L in a water bath at 55°C for 30 min, followed by the addition of 2 mL of NaOH 2.0 moL/L for acid neutralization (Liu et al., 2012). Subsequently, the total reducing sugars (ART) were determined
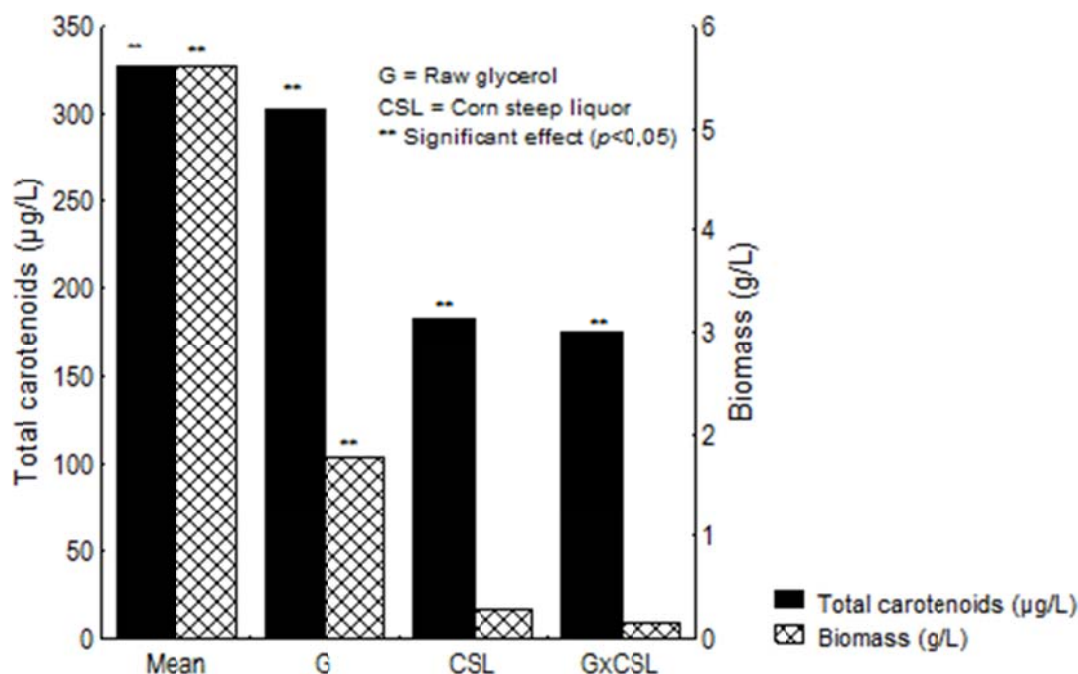
Figure 1. Effects of variables on the responses total carotenoids concentrations and biomass concentrations for experimental design CCD using raw glycerol and corn steep liquor.

using the spectrophotometric method of 3-5 dinitrosalicylic (DNS) in accordance with Miller (1959), using standard glucose curve whose concentration vary between 0.1 and 1.0 g/L.

Statistical analysis

The data were treated with the aid of Statistica 5.0 (StatSoft Inc., Tulsa, OK, USA). All the analyses were performed considering a 95% of confidence level ($p<0.05$). The analysis of variance (ANOVA) was used to estimate the statistical parameters. The response surfaces and contour diagrams were drawn in accordance with Box et al. (1978).

RESULTS AND DISCUSSION

Optimization of the composition of the production medium containing raw glycerol and corn steep liquor

The prior partial characterization of the industrial substrates used in this work, such as the amount of carbon in the raw glycerol 44.4% (Silva et al., 2012), the sugar cane molasses 36.5% (Otero, 2011) and the nitrogen in corn steep liquor approximately 4% (Cipolatti, 2012), showed that these agroindustrial substrates are sources of nutrients in the culture medium and can minimize the cost of obtaining these biopigments.

The first experimental design was done to evaluate the effect of the concentrations of raw glycerol and corn steep liquor substrates in the production of carotenoids

by *S. pararoseus*. Table 1 shows the real and coded values of the 2^2 central composite design with the respective responses of maximum carotenoid concentration and its respective biomass concentration.

In Table 1 (CCD), the maximum concentration of carotenoids ranged from 154.86 (assay 1) to 640.54 µg/L (assay 4) and biomass concentration of 4.42 (assay 1) to 6.47 g/L (assay 4), in 168 h for all the experimental conditions except assay 2 where the greatest carotenoid concentration occurred at 144 h.

The use of experimental design enables the study on 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). Through the analysis of the main effects presented in Figure 1, it can be seen that the increase in the concentration of raw glycerol from 5 to 10 g/L and corn steep liquor from 20 to 30 g/L significantly increased ($p<0.05$) the concentration of total carotenoids by 302.92 and 182.76 µg/L, respectively. However, for cell growth, only the effect of increasing the raw glycerol was significant ($p<0.05$), increasing the biomass by 1.78 g/L.

Therefore, according to the analysis of the effects, the independent variables under study had a significant positive influence in the responses. The assay 4 (10 g/L of raw glycerol and 30 g/L corn steep liquor, carbon/ nitrogen ratio C/N = 8.26), formulated with the maximum

Table 2. Results of the regression coefficients (RC), standard error (SE), *t* and *p* from the central composite rotational design (CCRD) for agroindustrial substrates, and maximum concentration response of total carotenoids and biomass concentration.

CCRD	Volumetric carotenoids (µg/L)				Biomass (g/L)				
	RC	SE	*t* (2)	*p*		RC	SE	*t* (2)	*p*
Raw glycerol and corn steep liquor									
Mean**	822.39	0.53	1560.76	<0.01	Mean**	9.95	0.02	426.28	<0.01
X_1(L)	-0.66	0.65	-2.05	0.20	X_1(L)**	-0.08	0.01	-5.93	0.03
X_1 (Q)**	-112.59	0.77	-292.0	<0.01	X_1 (Q)**	-0.48	0.02	-28.13	<0.01
X_2 (Q)**	-29.31	0.65	-90.70	<0.01	X_2 (Q)**	-1.12	0.01	-78.01	<0.01
X_2 (Q)**	-141.24	0.77	-366.29	<0.01	X_2 (Q)**	-1.12	0.02	-65.69	<0.01
$X_1 x X_2$**	-100.80	0.91	-220.89	<0.01	$X_1 x X_2$**	-0.43	0.02	-21.16	<0.01
Sugar cane molasses and corn steep liquor									
Mean**	483.41	6.37	75.82	0.01	Mean**	8.02	0.09	84.44	0.01
X_3 (L)**	261.93	6.385	41.03	0.02	X_3 (L)**	3.23	0.1	33.93	0.02
X_3 (Q)**	-206.27	8.47	-24.36	0.03	X_3 (Q)**	-3.38	0.13	-26.81	<0.01
X_2 (L)	-23.42	6.38	-3.67	0.17	X_2 (L)*	0.87	0.1	9.15	0.07
X_2 (Q)**	-143.69	8.47	-16.97	0.04	X_2 (Q)**	-2.30	0.13	-18.24	0.04
$X_3 x X_2$*	62.96	9.02	6.98	0.09	$X_3 x X_2$	0.28	0.13	2.05	0.29

X_1 = Raw glycerol concentration (g/L), X_2 = corn steep liquor concentration (g/L), X_3 = sugar cane molasses concentration (g/L) *(*p*<0.1), **(*p*<0.05).

levels (+1) of agroindustrial substrates, corroborates the analysis of effects resulting in higher responses for the bioproduction of carotenoids (640.54 µg/L) and biomass concentration (6.47 g/L). Thus, for the sequence of optimization of this production medium, the levels of agroindustrial substrates were expanded through a second experimental design (2^2 CCRD) with concentration ranges of the raw glycerol from 10 to 50 g/L and corn steep liquor from 30 to 80 g/L (Table 1).

The practice of wholly or partly replacing a complex commercial medium with byproducts is interesting, since the presence of microelements can enhance the desired bioproduction. The presence of vitamin B and several minerals (K, P, Mg, Na, Ca, Fe, Zn, Mn and Cu) was also found (Gao and Yuan, 2011) in the corn steep liquor; just as the presence of minerals Ca, K, Mg, Na and P in raw glycerol was determined by Quispe et al. (2013).

The potential of the increase in the concentration of the raw glycerol has also been observed previously (Silva et al., 2012), in the production of carotenoids by the yeast *Phaffia rhodozyma*, in which the use of up to 40 g/L of this substrate did not differ significantly (*p*>0.05) from the pure glycerol. The composition of raw glycerol from biodiesel synthesis can be quite variable, however, the nutrients with higher content (K, Na and P) up to an optimal concentration, can positively influence the biosynthesis of carotenoids and cell growth (Santos et al., 2011).

In the CCRD (Table 1), the maximum concentration of carotenoids ranged from 338.03 (assay 4) to 822.57 µg/L (assay 9) and biomass concentration from 5.91 (assay 8) to 10.26 g/L (assay 7) in 168 h, except for assays 1 (144

h), assay 7 (144 h) and assay 4 (120 h). With the CCRD, the maximum concentration of carotenoids and biomass were increased by approximately 28 and 59% respectively as compared to CCD.

A model fitting was accomplished for the second experimental design CCRD (Table 1). The independent variables (raw glycerol concentration and corn steep liquor concentration) and responses (total carotenoid concentration and biomass concentration) fitted the second-order model equations and examined in terms of the goodness of fit. The ANOVA were used to evaluate the adequacy of the fitted model. The R-squared value provided a measure of how much of the variability in the observed response values could be explained by the experimental variables and their interactions. A good model (values above 0.85 are considered good) explains most of the variation in the response. The closer the R-squared value is to 1.00, the stronger the model and the better the response predictions (Haaland, 1989).

Table 2 shows the regression coefficients, standard deviation, *p* and *t* values used in the construction of the models (Equations 2 and 3). On the basis of the analysis of variance (ANOVA), as shown in Table 3, a second order models Equations 2 and 3 were established, describing the total carotenoids concentration and biomass concentration, respectively, as a function of raw glycerol and corn steep liquor concentrations. The pure error was very low, indicating a good reproducibility of the experimental data. Based on the F test, the models are predictive, since its calculated F value is higher than the critical F value (1.29 and 1.41 times for carotenoid and biomass concentrations, respectively) and the regression

Table 3. ANOVA for the CCRD for agroindustrial substrates, and maximum concentration response of total carotenoids and biomass concentration.

Source of variation	Quadratic Sum		Degrees of freedom		Quadratic Mean		F calculated	
	C_{total}	Bio	C_{total}	Bio	C_{total}	Bio	C_{total}	Bio
Raw glycerol and corn steep liquor								
Regression	190487.6	17.92	4	47621.90	47621.90	3.58	5.84	7.16
Pure error	48936	2.53	6	8156	8156	0.50		
Total	239423.60	20.45	10					
Sugar cane molasses and corn steep liquor								
Regression	1933420	36.29	4	4	48335.50	9.07	9.29	8.48
Pure error	2609.20	5.34	5	5	5201.84	1.07		
Total	219351.20	41.63	9	9				

C_{total} = Total carotenoids concentration, Bio = biomass concentration, R = coefficient of correlation. Raw glycerol and corn steep liquor concentrations C_{total} (R: 0.89, $F_{tab,\ 95\%}$ = 4.53) and Bio. (R: 0.94 $F_{tab,\ 95\%}$ = 5.05). Sugar cane molasses and corn steep liquor concentrations C_{total} (R: 0.88, $F_{tab,\ 90\%}$ = 3.52) and Bio. (R: 0.87, $F_{tab,\ 90\%}$ = 2.41).
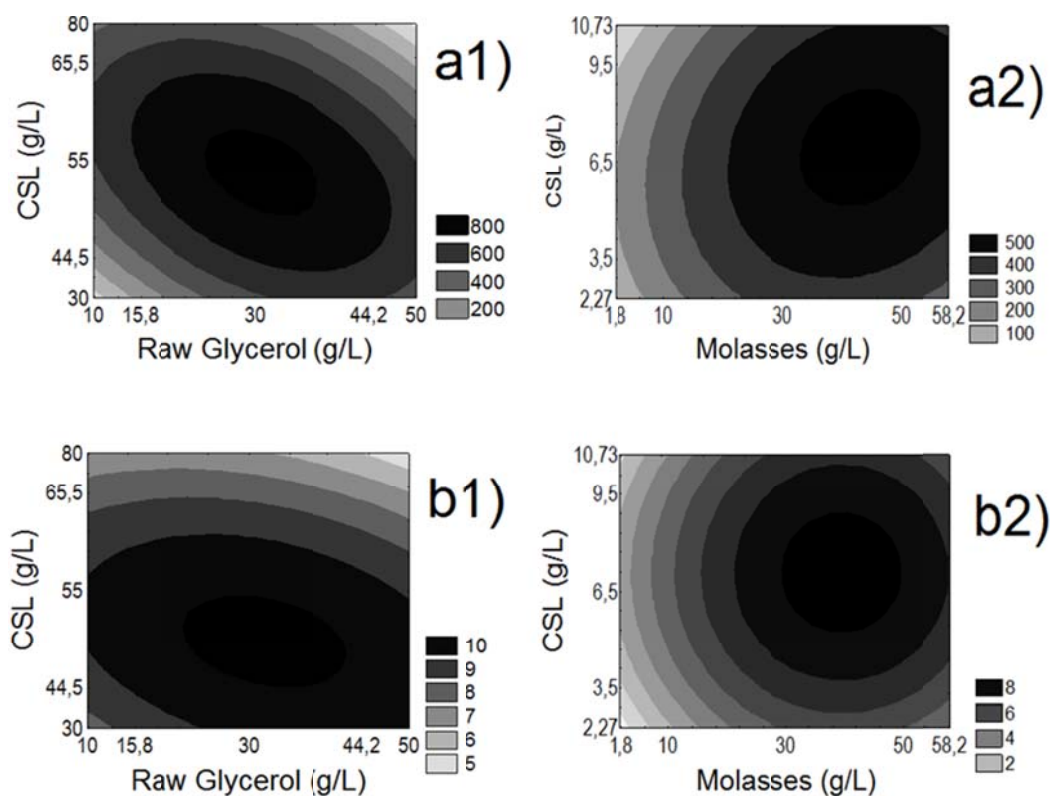

Figure 2. Contour curves for the total carotenoids concentrations (a1 and a2) and biomass concentrations (b1 and b2) as a function of the concentration of raw glycerol and CSL-corn steep liquor (1) and sugar cane molasses and CSL-corn steep liquor (2), at 25°C, 180 rpm, initial pH of 6.0 at 168 h.

coefficient (0.89 and 0.94 for carotenoid and biomass concentrations, respectively). The parameters that were not significant were added to lack of fit in the analysis of variance. The coded models were used to generate response surfaces (Figure 2) for the analysis of the variable effects on bioproduction carotenoid.

$$C_{total} = 822.39 - 112.60.GLY^2 - 29.31.CSL - 141.24.CSL^2 - 100.80.GLY.CSL \quad (2)$$

$$Bio. = 9.95 - 0.08.GLY - 0.48.GLY^2 - 1.12.CSL - 1.12.CSL^2 - 0.43 CSL.GLY \quad (3)$$

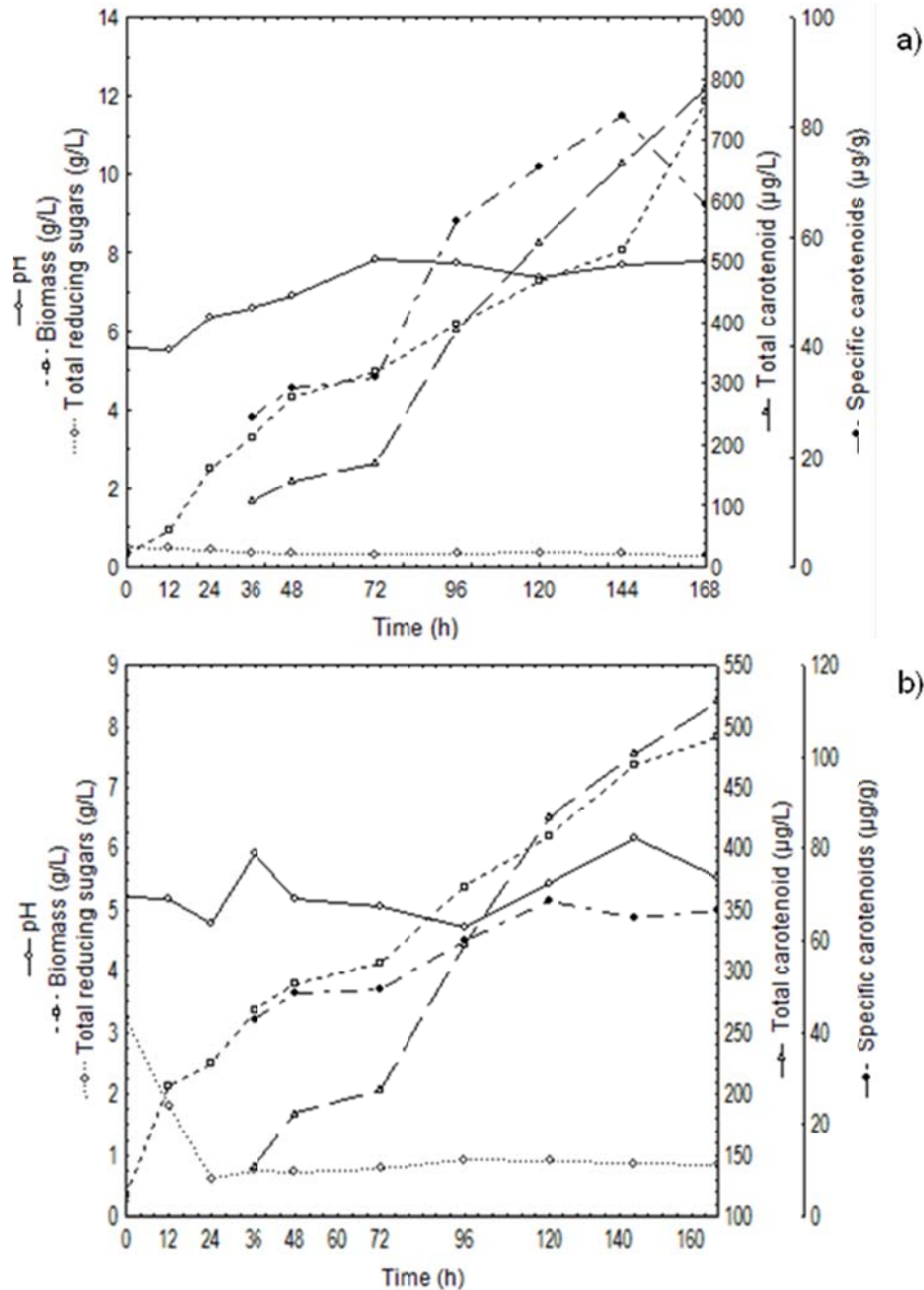Where, C_{total} is the total carotenoids concentration (μg/L),

Figure 3. Kinetics of the production carotenoid by *S. pararoseus* in the validation of empirical models using raw glycerol and corn steep liquor (a) and sugar cane molasses and corn steep liquor (b), at 25°C, 180 rpm, initial pH of 6.0 at 168 h.

Bio is the biomass concentration (g/L), GLY is raw glycerol concentration and CSL is corn steep liquor concentration.

As can be seen in Figure 2 a1, the culture medium may be within the ranges of concentrations of industrial substrates of 24.32 to 35.68 g/L raw glycerol and 50.8 to 59.2 g/L corn steep liquor. For optimal biomass concentration (Figure 2 b1), the culture medium may contain from 24.32 to 41.36 g/L of raw glycerol and 44.5 to 55 g/L corn steep liquor.

Therefore, the optimum culture medium for the production of carotenoids with agroindustrial byproducts by *S. pararoseus* was set in 30 g/L raw glycerol and 52.9 g/L corn steep liquor, as used in the validation of models.

Figure 3a shows the average results of the kinetic for pH, biomass concentration, and the volumetric and specific concentration of carotenoids during the validation of the models (Equations 2 and 3) in the agroindustrial production medium containing raw glycerol and corn steep liquor (C/N ratio = 7.93).

Table 4. Validation responses of the empirical models for different agroindustrial substrates.

Validation responses	Medium 1		Medium 2	
	Y_1	Y_2	Y_1	Y_2
Response predicted by the model	755.44	9.95	492.07	7.52
Experimental response *	779.60	11.65	520.94	7.82
Deviation of the model	3.10	14.59	5.54	3.86

*Results are means of triplicate assays (n=3). Medium 1 = 30 g/L raw glycerol and 52.9 g/L corn steep liquor, medium 2 = 40 g/L sugarcane molasses and 6.5 g/L corn steep liquor, Y_1 = total carotenoids (µg/L) and Y_2 = biomass (g/L).

Table 5. Real values and coded levels (in parentheses) for the CCRD using sugar cane molasses and corn steep liquor, and maximum concentration response of total carotenoids and biomass concentration.

Assays	Independent variables		Responses	
	X_3	X_2	Total carotenoids (µg/L)	Biomass (g/L)
1	10 (-1)	3.5 (-1)	209.40	4.41
2	50 (+1)	3.5 (-1)	340.22	5.87
3	10 (-1)	9.5 (+1)	125.92	4.80
4	50 (+1)	9.5 (+1)	382.66	6.81
5	1.8 (-1.41)	6.5 (0)	89.52	1.03
6	58.2 (+1.41)	6.5 (0)	555.52	7.70
7	30 (0)	2.27 (-1.41)	403.29	4.68
8	30 (0)	58.2 (+1.41)	366.16	6.20
9	30 (0)	6.5 (0)	476.51	7.93
10	30 (0)	6.5 (0)	489.26	8.12

X_3 = Sugar cane molasses (g/L); X_2 = corn steep liquor (g/L).

The pH showed a decrease during the first 12 h of cultivation, increasing up to 72 h (7.0 - 8.0) and remained almost constant until the end of the process. Similar behavior had been previously observed for other micro-organisms (Silva et al., 2012; Frengova et al., 1994), where during the biosynthesis of carotenoids the changes in the pH of the culture medium occurred as a consequence of microbial growth and the release of compounds such as acetic acid, alcohol or intermediates of the citric acid cycle during the adaptation phase, causing a decrease in pH. At this point, this intermediate is then reassimilated and stimulates a strong caroteno-genesis, resulting in an increase in pH. Thereafter, the pH remains constant indicating the end of cultivation. The sugar was almost totally consumed by yeast at the end of the process (0.30 g/L), achieving a maximum concentration of carotenoids 779.60 µg/L (65.64 µg/g) at 168 h with a biomass concentration of 11.65 g/L

The relative deviations obtained during the validation of the experimental results and those predicted by the model (Table 4) were lower than 5 and 15% (Rodrigues and Iemma, 2012), respectively, for total carotenoids and biomass concentrations, considered good for these bioprocesses (Rodrigues and Iemma, 2012). Therefore, Equations 2 and 3 predict the behavior of the production of carotenoids.

Optimization of the composition of the production medium containing sugar cane molasses and corn steep liquor

A third experimental design was made using another combination of agroindustrial substrates such as sugar cane molasses and corn steep liquor for the production of carotenoids by *S. pararoseus*. Table 5 shows the real and coded values of the 2^2 CCRD with respective responses of maximum carotenoid concentration and its respective biomass concentration.

The maximum concentration of carotenoids ranged from 89.52 (assay 5) to 555.52 µg/L (assay 6) and biomass concentration of 1.03 (assay 5) to 8.12 g/L (assay 10), in 168 h for all experimental conditions (Table 5). The same sequence of adjustment and predictive model validation of the optimal condition of medium composition for the production of carotenoids by *S. pararoseus* was

followed with agroindustrial substrates sugar cane molasses and corn steep liquor.

Table 2 presented the regression coefficients, standard deviation, p and t values used in the construction of the models (Equations 4 and 5). On the basis of the analysis of variance (ANOVA), as shown in Table 3, a second order models Equations 4 and 5 were established, describing the total carotenoids concentration and biomass concentration, respectively, as a function of sugar cane molasses and corn steep liquor concentrations. The pure error was very low, indicating a good reproducibility of the experimental data. Based on the F test, the models are predictive, since their calculated F value is higher than the critical F value (2.05 and 2.41 times for carotenoid and biomass concen-trations, respectively) and the regression coefficient (0.88 and 0.87 for carotenoid and biomass concentrations, respectively). The coded models were used to generate response surfaces (Figure 2).

$$C_{total} = 483.41 + 130.97.MEL - 103.13.MEL^2 - 71.84.CSL^2 + 31.48.MEL.CSL \tag{4}$$
$$Bio. = 8.02 + 1.61.MEL - 1.69.MEL^2 + 0.44.CSL - 1.15.CSL^2 \tag{5}$$

Where, C_{total} is the concentration of total carotenoids concentration (µg/L), Bio. is the biomass concentration (g/L), MEL is sugar cane molasses concentration and CSL is corn steep liquor concentration.

As can be seen, the culture medium may have between 30 to 50 g/L of sugar cane molasses and 5.3 to 8.9 g/L for corn steep liquor to obtain a maximum concentration of carotenoids (Figure 2 a2) and biomass concentration (Figure 2 b2).

Therefore, to determine the optimum culture medium for the production of carotenoids with agroindustrial byproducts (sugar cane molasses and corn steep liquor) by *S. pararoseus*, the levels were set at 40 g/L sugar cane molasses and 6.5 g/L of corn steep liquor (C/N ratio = 42.94), composition used in the validation of models.

In Figure 3b, during the validation of the models (Equations 4 and 5), the pH showed the same behavior as the previous culture medium, decreasing up to 24 h of cultivation, increasing up to 36 h (5.0 - 6.0) and remained almost constant until the end of the process, as well as having the total reducing sugar practically depleted (0.85 g/L in 168 h).

A maximum biomass concentration of 7.82 g/L was achieved, while the total carotenoid concentration was 522.62 µg/L. The agroindustrial mediums rich in molasses, which has greater complexity, containing nutrients (nitrogen, potassium, magnesium, manganese, iron, etc.), heavy metals, and salts, can cause changes in the pH as described by Valduga et al. (2009). The bioproduction of secondary compounds is also possible (Valduga et al., 2008).

The relative deviations obtained during the validation of

the experimental results and those predicted by the models (Table 4), using molasses and corn steep liquor, were less than 6% (Rodrigues and Iemma, 2012). Therefore, Equations 4 and 5 predict the behavior of the production of carotenoids and biomass concentration.

The advantages of optimizing the production medium using the Response Surface Methodology for obtaining maximum concentrations of microbial pigments observed in this study were also reported by Gharibzahedi et al. (2013), with a reduction in the number of experimental assays required to evaluate multiple variables and their interactions.

In literature, some studies have reported the use of different substrates and process conditions for the bioproduction of carotenoids using different strains of *Sporidiobolus pararoseus*. Maldonade et al. (2008) isolated and identified *Sporobolomyces roseus* achieving 237 (72 µg/g), and 3.3 g/L of biomass concentration in culture medium YM, at 25°C, 200 rpm, initial pH 6.0 and 120 h.

The effect of initial pH on the culture medium (YM) from 3.0 to 5.0 for the production of carotenoids by *S. salmonicolor* (CBS 2636) was evaluated, achieving 455.4 µg/L (111 µg/g) with 4.1 g/L of biomass concentration in 120 h at 25°C, 180 rpm and with an initial pH of 4.0 (Valduga et al., 2009).

In another study, Cabral et al. (2011) isolated and identified the yeast *S. pararoseus*, optimizing the production of carotenoids, through an experimental design that studied variables glucose (26.4 to 93.6 g/L), peptone (6.6 to 23.4 g/L) and malt extract (6.6 to 23.4 g/L). The maximum concentration was 856 µg/L (229.69 µg/g) with 3.69 g/L of biomass concentration for 120 h at 25°C, 180 rpm and an initial pH 4.0. Therefore, results are above those obtained in the present study, however with a formulation with 60 g/L glucose, 15 g/L peptone and 15 g/L of malt extract.

Valduga et al. (2014) evaluated the production potential of carotenoids by *S. salmonicolor* optimizing the culture medium through experimental design, achieving 843 µg/L (181.26 µg/g) with glycerol (40 g/L), corn steep liquor (40 g/L) and parboiled rice wastewater (20 g/L) for 96h at 25°C, 180 rpm and an initial pH of 4.0.

The supplementation of the production medium with raw glycerol derived from the synthesis of biodiesel (34 g/L of raw glycerol, 2.0 g/L of KH_2PO, 2.0 g/L of $(NH_4)_2SO_4$, 1.7 g/L of K_2HPO_4, 0.1 g/L of $MgSO_4 \cdot 7H_2O$, 0.1 g/L of $MnSO_4 \cdot H_2O$ and 0.1 g/L of NaCl, in 120 h at 25°C, 200 rpm and initial pH 6.0) as compared to two commercial mediums (YM and BMP) by *S. pararoseus* (TISTR5213) was higher, showing that the growth stimulation of red yeast is probably due to the presence of oligoelements in this substrate (Manowattana et al., 2012).

The strain of yeast used for this study, *S. pararoseus*, was previously isolated, selected and identified by Otero (2011) and it stood out among the others. With 39.91 g/L

of parboiled rice wastewater and 17.31 g/L of raw glycerol, and a second medium containing 44.01 g/L parboiled rice wastewater and 23.6 g/L of sugar cane molasses, a total carotenoid concentration was achieved which is 710 (86.46 µg/g) and 820 µg/L (106.20 µg/g), respectively, at 25°C, 180 rpm for 168 h, and initial pH 6.0.

From the previous work by Cipolatti (2012) on modifying the nitrogen source in a production medium with 4.8 g/L raw glycerol and 35.6 g/L of corn steep liquor (C/N ratio = 6.20) 634.5 µg/L (87.3 µg/g) of carotenoids concentration with 7.3 g/L of biomass in 120 h, under similar conditions was observed. Therefore, in this work with the new optimized medium proposed (30 g/L of raw glycerol and 52.9 g/L corn steep liquor), there was a gain of 22.86% in carotenoid concentration and 26.26% in biomass concentration, showing the potential application of this yeast enabling a reduction in the costs of the production medium.

Conclusions

The optimization of the composition of two agroindustrial culture mediums for the production of carotenoids by a wild strain of *S. pararoseus* in shake flasks using 30 g/L of raw glycerol and 52.9 g/L of corn steep liquor (C/N ratio = 10.42) and another with 40 g/L of sugar cane molasses, 6.5 g/L of corn steep liquor (C/N ratio = 42.94) achieved, respectively, 779.60 (65.64 µg/g) and 520.94 µg/L (73.19 µg/g) of carotenoids concentrations, at 25°C, 180 rpm, initial pH of 6.0 at 168 h. Therefore, this yeast strain is an interesting source of biopigment production for future studies on scale up.

Conflict of interest

The authors have not declared any conflict of interests.

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