Production of carotenoids by *Rhodotorula mucilaginosa* using sugarcane juice (*Saccharum officinarum*) in the fermentation.

Produção de carotenóides pela Rhodotorula mucilaginosa utilizando sumo de cana (Saccharum officinarum) na fermentação.

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ABSTRACT
This study aims to investigate the production of carotenoids from pigmented yeasts isolated from Bahia's semi-arid region, using sugarcane juice as an alternative fermentation medium. The 2^3 full factorial statistical design was used to test the following independent variables: concentration of sugarcane juice (%) and yeast extract (g L^{-1}). The Doehlert design was used to test the variables of initial pH and stirring speed. The maximum production obtained showed a concentration of 40% sugarcane juice, 6.0 g L^{-1} yeast extract, initial pH of 6.8 and stirring speed of 176 rpm, which enabled a carotenoid production of 1300 µg L^{-1}. As for the cell growth, the maximum was obtained at the concentration of 43% sugarcane juice, 9.2 g L^{-1} yeast extract, with an initial pH of 6.8 and stirring speed of 176 rpm, resulting in production of 14.7 g L^{-1}. The HPLC analysis showed the presence of β-carotene among the compounds produced by the yeast. The results showed the use sugarcane supplemented with yeast extract and by controlling environmental conditions such as initial pH and stirring speed, it is possible to promote an increase in the bioproduction of carotenoids and biomass of *R. mucilaginosa*.

Keywords: agro-industrial product, yeast, β- carotene

RESUMO
Este estudo visa investigar a produção de carotenóides a partir de leveduras pigmentadas isoladas da região semi-árida da Bahia, utilizando o caldo de cana como meio de fermentação alternativo. O desenho estatístico factorial completo foi utilizado para testar as seguintes variáveis independentes: concentração de caldo de cana (%) e extracto de levedura (g L-1). O desenho Doehlert foi utilizado para testar as variáveis de pH inicial e velocidade de agitação. A produção máxima obtida mostrou uma concentração de 40%

de sumo de cana de açúcar, 6,0 g de extracto de levedura L-1, pH inicial de 6,8 e velocidade de agitação de 176 rpm, o que permitiu uma produção de carotenóides de 1300 μg L-1. Quanto ao crescimento celular, o máximo foi obtido à concentração de 43% de sumo de cana, 9,2 g de extracto de levedura L-1, com um pH inicial de 6,8 e velocidade de agitação de 176 rpm, resultando numa produção de 14,7 g de L-1. A análise HPLC mostrou a presença de β-caroteno entre os compostos produzidos pela levedura. Os resultados mostraram a utilização de cana-de-açúcar suplementada com extracto de levedura e através do controlo das condições ambientais tais como o pH inicial e a velocidade de agitação, é possível promover um aumento na bioprodução de carotenóides e biomassa de R. mucilaginosa.

Palavras-chave: produto agro-industrial, levedura, β- caroteno

1 INTRODUCTION

Carotenoids are natural pigments responsible for providing the colors yellow, orange and red to many foods such as fruits and egg yolk, as well as to some insects and marine animals [1]. Its chemical composition is primarily constituted of carbon and hydrogen.

Although plants are the main sources of carotenoids, these can also be produced by photosynthetic microorganisms (algae and cyanobacteria) and non-photosynthetic ones (bacteria, yeasts, and molds) [2].

The carotenoids have a wide variety of functions, especially with respect to human health. They function as biological antioxidants, and some of them are precursors to vitamin A and are used to treat the deficiency of this vitamin. They are able to relieve chronic diseases, help preventing cardiovascular diseases and several types of cancers, besides being in high demand as nutritional supplements and food colorings [1].

One of the main ways of obtaining carotenoids is through chemical synthesis; however, the increasing search for natural products has led to research for new sources of this compound, to replace or reduce the use of artificial compounds by biosynthesized ones [3].

Some genera of carotenogenic yeasts such as *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces* and *Phaffia* are currently known. The biotechnological use of microorganisms for industrial applications has attracted the interest of many researchers due to several advantages, such as: the use of low-cost substrates, natural origin, occupation of small spaces, independence regarding the environmental conditions, and the easy control of culture conditions [4 -7]. Thus, studies have been conducted to obtain new fermentation media for these carotenogenic microorganisms, with the aim of

reducing the cost of the process. In this context, studies were carried out to obtain new means of fermentation for these carotenogenic microorganisms, to reduce the cost of the process [8 - 12].

The preference for alternative means, such as cane juice, is due to the ease of obtaining sugar on a large scale, which contributes to the reduction of production costs [10, 13]. The high nutritional value of sugarcane juice and the considerable interest in its use in fermentation processes have generated many studies on this topic, which has resulted in significant advances [13, 14].

The innovative aspect of this experiment consists of proposing a viable alternative to produce microbiological carotenoids, in addition to highlighting the predictive character of the microorganism in question, which was isolated in the semi-arid region of Bahia. The region that, despite the hot and dry character of the climate, reveals a high degree of biodiversity and points to the possibility of microorganisms from this biome, to produce substances with peculiar characteristics, and great resistance in relation to environmental conditions, including the possibility of withstanding lower temperatures, high, often used in industrial processes [15].

Thus, this study aims to investigate the production of carotenoids from pigmented yeasts isolated from the semi-arid region of Bahia, using sugarcane juice as an alternative means of fermentation

2 MATERIAL AND METHODS

2.1 MICROORGANISM AND CULTURE CONDITIONS

The *Rhodotorula mucilaginosa* cultures used in this study belong to the collection of the Laboratory of Enzymology and Fermentation Technology at the Health Department of the State University of Feira de Santana. They were preserved (g L^{-1}) in YM (dextrose 10, peptone 5, malt extract 3, yeast extract 3, agar 15) at 4ºC. Yeasts were identified according to the standard methodology [16].

The strain was subjected to fermentation in different media according to the following formulations: 1 (25% sugarcane juice; 2 g L^{-1} yeast extract); 2 (25% sugarcane juice; 2 g L^{-1} yeast extract; 10 g L^{-1} peptone); 3 (25% sugarcane juice; Ammonium sulphate ($(NH_4)_2$ SO_4 – 5.3g L^{-1}); Monopotassium phosphate (KH_2PO_4 – 5.5g L^{-1}); Sodium phosphate dibasic (Na_2HPO_4 – 3.7g L^{-1}); Magnesium sulfate ($MgSO_4$.$7H_20$) – 0.5 g L^{-1}; 2.0 g L^{-1} yeast extract); 4 (25% sugarcane juice; 1.5 g L^{-1} peptone; Ammonium phosphate monobasic ((NH_4) H_2PO_4 -0.45g L^{-1}); 5 (25% sugarcane juice (control). The

formulation showing the best result was used as the basis for the implementation of the other statistical plannings.

3 EXPERIMENTAL DESIGNS

Two (2) experimental designs were used to optimize pigment production. The first 2^3 full factorial statistical design was used in order to test the following independent variables: concentration of sugarcane juice (10% - 30% - 50%), and yeast extract at the concentrations of (0 - 5 g L^{-1} - 10 g L^{-1}).

The optimum value obtained in the first experimental design was set and used during the second one, was performed by using the Doelhert design, in which the following variables were assessed: stirring speed (130 - 180 - 230 rpm) and initial pH (5.0 – 6.0 – 7.0 – 8.0 – 9.0). The parameters used were based on data taken from the literature for related research.

3.1 PREPARATION OF THE CULTURE MEDIUM

Sugarcane juice was diluted in distilled water according to the desired dilutions. The pH was adjusted to 2.0 with 5 mol L^{-1} hydrochloric acid (HCl) and it was held in boiling water bath for 40 min for sucrose hydrolysis. After hydrolysis, the solution was cooled to room temperature and its pH was adjusted to 6.0 with 1 mol L^{-1} sodium hydroxide (NaOH). The precipitate was removed by centrifugation. Total reducing sugar (TRS) was assayed spectrophotometrically using dinitrosalicylic acid (DNS). The supernatant was sterilized for 30 min at 121ºC and used as nutrient source [13].

3.2 ANALYTICAL PROCEDURES

To standardize the inoculum, isolated colonies were transferred to a Petri dish containing YM agar and incubated in a greenhouse at 28 °C for 48h. After this period, the colonies were diluted in 0.45% saline solution, and then held with a spectrophotometer at 600 nm to standardize the inoculum in 10^7 number of microorganisms. Subsequently, 10 mL of inoculum were inoculated in 100 mL of medium and they were incubated in an orbital shaker (Tecnal, model 420) at 28 °C for 120h at 200 rpm.

After fermentation, samples were taken from each culture for calculating the biomass and reducing sugars and to produce carotenoids. The cells in 1.5 mL of culture were collected and washed twice by centrifugation weight was constant.

The residual sugar concentration was measured in the supernatant according to DNS method [17].

Carotenoid pigment extraction. Wet cells were collected by centrifugation (4.000 g of 5 min), washed twice with distilled water, and ruptured twice with 500 µL of preheated (55 ºC) dimethyl sulfoxide (Merck). After centrifugation, the supernatants were pooled. Final pigment extraction from the pellet was carried out by successively removing and centrifuging 1.5 mL acetone (Merck) aliquots. All supernatants were pooled and 0.5 mL of a saturated solution of sodium chloride (NaCl) was added. Full pigment extraction was achieved with a series of 3 mL petroleum ether volumes until exhaustion. Ether aliquots were collected, washed in distilled water to remove acetone traces, evaporated under nitrogen (N_2), and freeze-dried for water removal when required. Samples were kept on ice and protected from light during the procedures [18].

3.3 ANALYTICAL METHODS

Freeze-dried pigment samples were dissolved in 2–4 mL diethyl ether and total carotenoids were assayed spectrophotometrically at 485 nm. Data were expressed in µg of β-carotene/ L^{-1} of culture broth or in µg of β-carotene g^{-1} of the dry biomass, according to the eq. 1 and 2:

Carotenoids (µg g^{-1}) $= [A_{485} \times V(mL) \times 10^4] / [A_{1cm}^{1\%} \times P]$ /1/

Carotenoids (µg L^{-1}): $[A_{485} \times V(mL) \times 10^4] / [A_{1cm}^{1\%} \times v]$ /2/

Where: A = absorbance (485nm) V = extract volume (ml), $= A_{1cm}^{1\%}$ absorption coefficient of torulene or β-carotene in petroleum ether (2.680 or 2.592), P = weight of sample (g). v =volume of sample [19].

3.4 CHROMATOGRAPHIC ANALYSIS

For chromatographic analysis, extracts belonging to the maximum pigment production were dried out under nitrogen stream, re-dissolved in acetone and individually injected into the chromatographer. It was used a high performance liquid chromatographer controlled by Elite EZcrom and equipped with quaternary pump, degassing system, automatic injector and diode array detector. All analyses were performed by using a reverse phase column kept at 20 °C. The mobile phase was composed of acetone and ethyl water in a linear gradient of 95:5 for 20 minutes [20].

3.5 STATISTICAL ANALYSIS OF THE DATA

Statistical analysis of biomass, carotenoid and residual sugar production data was performed by means of ANOVA, using the *Statistic* statistical package [21].

The responses of the experiments were given by assessing biomass growth in liquid medium (g L^{-1}), and carotenoid (µg g^{-1} e µg L^{-1}) and residual sugar (µg L^{-1}).

4 RESULTS

4.1 EFFECT OF THE CONCENTRATION OF SUGARCANE JUICE AND YEAST EXTRACT

The results obtained from the optimization of the concentration of sugarcane juice (%) and yeast extract (g L) are shown in Table 1.

Table 1: Planning 2^3 factorial design used to optimize the production of carotenoid *Rhodotorula mucilaginosa.*

Essay	Sugarcane juice (%)	Yeast Extract (g L^{-1})	Carotenoids (µg g^{-1})	Carotenoids (µg L^{-1})	Biomass (g L^{-1})	Residual Sugar (g L^{-1})
1	50.0	0.0	18.2	26.7	4.1	107.8
2	50.0	5.0	94.2	828.0	12.5	89.4
3	50.0	10.0	58.5	605.8	13.6	81.3
4	30.0	0.0	26.2	118.8	6.1	51.1
5	30.0	5.0	93.0	855.2	12.4	22.6
6	30.0	5.0	94.9	942.7	13.2	20.8
7	30.0	5.0	97.4	868.9	12.6	22.6
8	30.0	10.0	44.0	433.5	13.0	27.6
9	10.0	0.0	20.7	128.6	3.4	14.7
10	10.0	5.0	22.5	383.8	3.7	3.8
11	10.0	10.0	38.3	166.1	3.9	4.2

According to the data obtained, the increase in specific and volumetric production of carotenoids was on average 95.1 µg g^{-1} and 888.9 µg L^{-1} (test 5, 6 and 7). The highest production of biomass 13.6 g L^{-1} occurred in test 3 (Table 1).

The results obtained were submitted to analysis of variance (ANOVA), which showed the correlation coefficient ($R^2 = 0.94$) to produce carotenoids indicating that there is a fit between the model and the experimental data. The corresponding regression is significant Fcal.> Ftab. The lack-of-fit test was not significant Fcal. <Ftab., with an $R^2 = 0.96$. Thus, from the analysis of variance it is noticed that the model is significant and that the proposed equations are valid for both production of carotenoids (µg L^{-1}) and biomass (g L^{-1}).

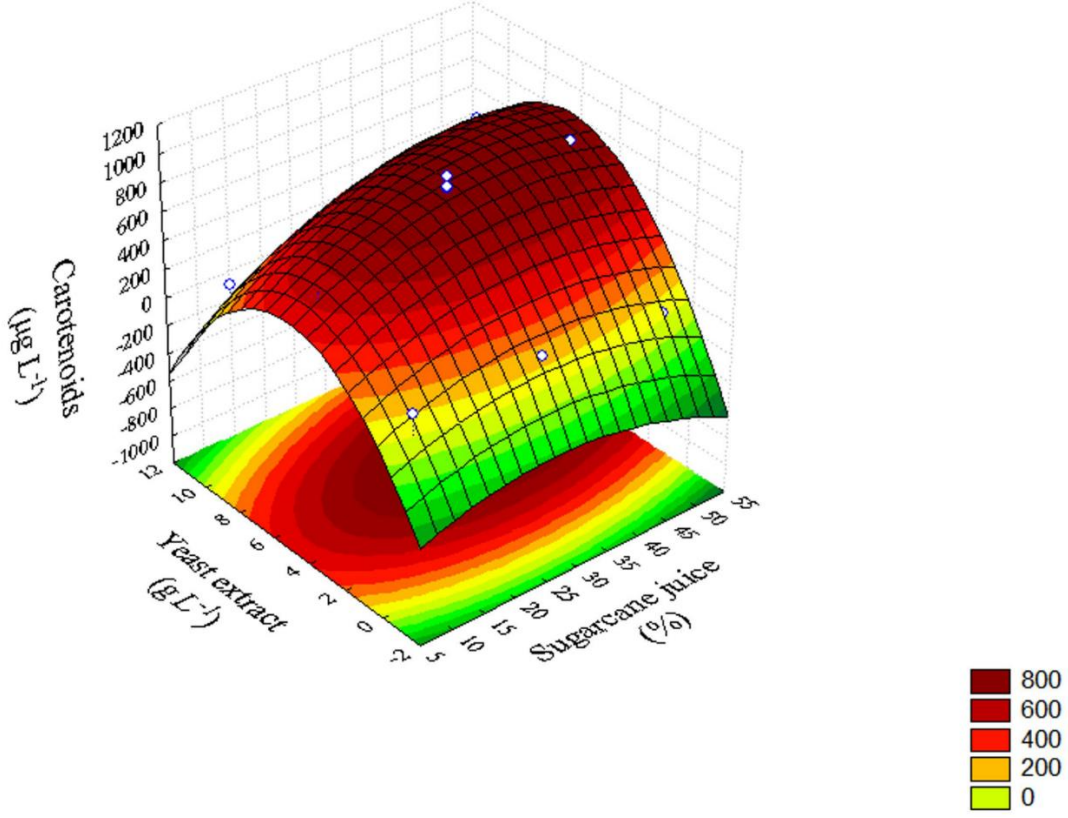
The model described for the volume production of carotenoids and cell growth, depending on the variables of sugarcane juice concentration and yeast extract, follow the eq. 3 and 4 below.

$$Y = -150.26 + 23.35x_1 - 0.39x_1^2 + 185.28x_2 - 19.44x_2^2 + 1.34x_1x_2 \quad /3/$$
$$Z = -3.08 + 0.63x_1 - 0.009x_1^2 + 0.85x_2 - 0.10x_2^2 + 0.02x_1x_2 \quad /4/$$

Where Y = production of carotenoids (µg L^{-1}), Z = total biomass (g L^{-1}), x_1 = sugarcane juice (%), x_2 = yeast extract (g L^{-1}).

The model proposed for the production of carotenoids was used to plot the presented graph response surface. The maximum yield of total carotenoids was obtained at the concentration of 40.0% sugarcane juice and 6.0 g L^{-1}yeast extract resulting in a production of 890 µg L^{-1} (Figure 1).

Figure 1. Surface response of carotenoids yield to sugar cane juice (%) versus yeast extract.



The model proposed for the production of biomass was used to plot the presented graph response surface one. The maximum biomass production occurred at the concentration of 43.2% sugarcane juice and 9.2 µg L^{-1} yeast extract, obtaining a yield of 14.7 µg L^{-1}.

4.2 EFFECT OF STIRRING SPEED AND INITIAL PH.

The specific carotenoid production value obtained from the pigment concentration per gram of dry mass (µg g^{-1}) and the volumetric production obtained from the pigment concentration per liter of fermented medium were evaluated (µg L^{-1}). The experimental results are presented in (Table 2).

Table 2: Doehlert matrix used to optimize the production of carotenoids from *Rhodotorula mucilaginosa*.

Assay	Stirring Speed (rpm)	initial pH	Carotenoids (µg g^{-1})	Carotenoids (µg L^{-1})	Biomass (g L^{-1})	Residual Sugar (g L^{-1})
1	230	6	68.9	382.9	7.2	29.8
2	230	8	69.5	400.3	7.6	25.3
3	180	5	148.2	847.5	7.0	34.2
4	180	7	149.1	1271,5	8.0	33.3
5	180	7	167.2	1207.3	8.1	32.6
6	180	7	170.5	1314.9	8.2	21.6
7	180	9	40.1	344.8	8.8	9.0
8	130	6	108.0	431.3	5.2	39.8
9	130	8	50.1	225.7	6.4	26.6

The highest specific and volumetric production of carotenoids 170.5 µg g^{-1} and 1314.9 µg L^{-1}, respectively occurred in assay 6. The highest biomass production obtained was 8.8 g L^{-1} (assay 7).

The results obtained were submitted to analysis of variance (ANOVA) which showed the correlation coefficient (R^2 = 0.98) for the carotenoids and biomass production indicating that there is a fit between the model and the experimental data. The corresponding regression is significant Fcal> Ftab. The lack-of-fit test was not significant Fcal. <Ftab. Thus, from the analysis of variance it is noticed that the model is significant and that the proposed equations are valid for the production of carotenoids (µg L^{-1}) as biomass (g L^{-1}).

The models describing the response surface for maximum yield of carotenoids and biomass are in eq. 5 and 6 below.

$$Y = -1347.13 + 93.7x_1 - 0.28037x_1^2 + 1869.2x_2 - 154.9x_2^2 + 1.1x_1x_2 \quad /5/$$

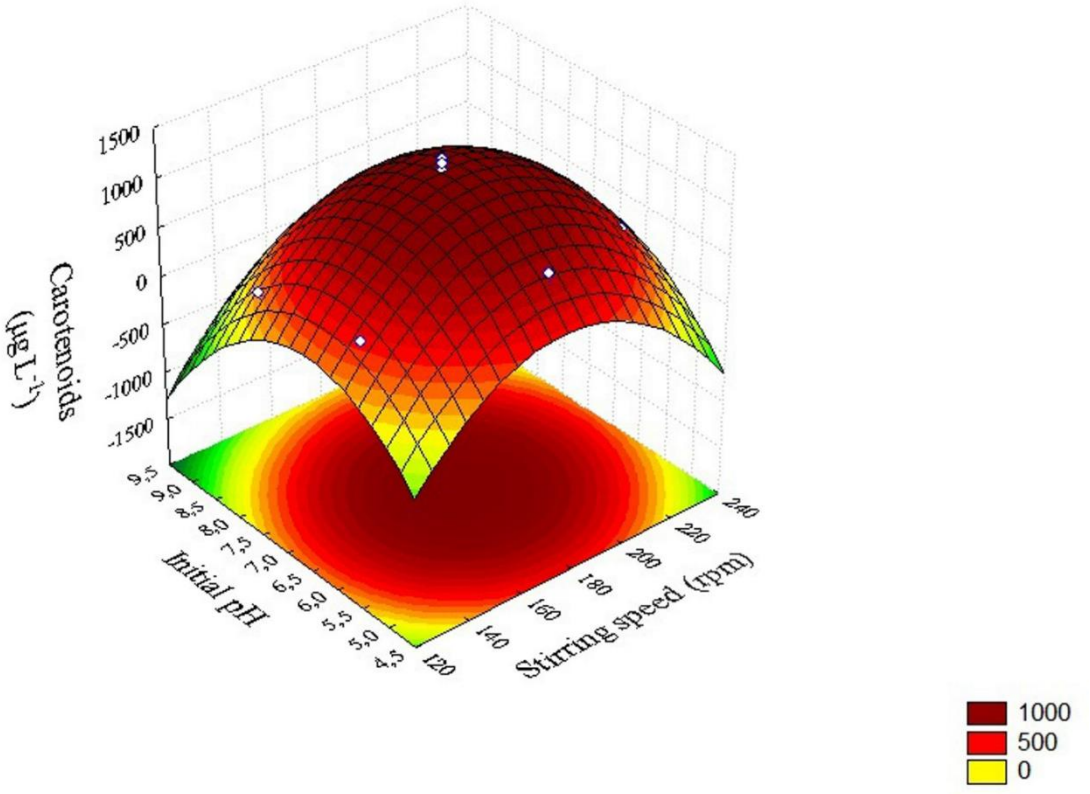
$$Z = -22.99 + 0.24x_1 - 0.0005x_1^2 + 1.75x_2 - 0.05x_2^2 - 0.003Zx_1x_2 \quad /6/$$

Where Y = production of carotenoids (µg L^{-1}), Z = Biomass production (g L^{-1}), x_1 = stirring speed (rpm), x_2 = initial pH.

The proposed equations were used to plot the response surface graphs for carotenoid production (Figure 2). Where we observed that the ideal region for maximum

carotenoid production is pH 6.7 and agitation speed of 180 rpm, resulting in a maximum yield of 1397.44 g L^{-1}. As the optimum region for biomass production and pH 9.0, stirring speed 180.6 rpm, resulting in a maximum yield of 9.0 g L^{-1}.

Figure 2. Response surface of the carotenoid yield for stirring speed (rpm) versus



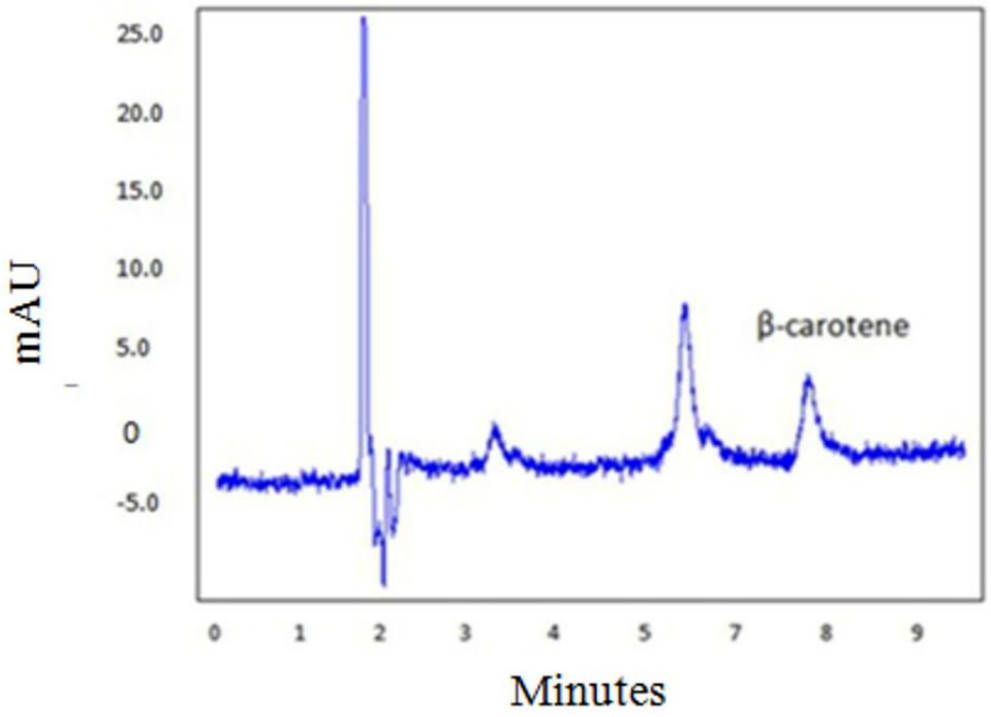
4.3 MODEL VALIDATION

Based on the results of the statistical analyses, triplicate tests were carried out to confirm the predictions of the mathematical models. The optimal condition found for the yeast *R. mucilaginosa* initial pH 6.7 and stirring speed of 180 rpm, concentration of 40.0% sugarcane juice and 6.0 g L^{-1} yeast extract. Thus the observed value proved to be very close to the predicted value (1300 µg L^{-1}), indicating that the proposed model can be used to predict future results.

4.4 CHROMATOGRAPHIC ANALYSIS

The HPLC analysis showed that the corresponding carotenoid spectrum showed a peak similar to that of β-carotene. A análise mostrou a presença de β-caroteno no espectro de UV (Figura 3).

Figure 3 - HPLC chromatogram of carotenoids obtained from *Rhodotorula mucilaginosa* yeast species.



5 DISCUSSION

5.1 EFFECT OF THE CONCENTRATION OF SUGARCANE JUICE AND YEAST EXTRACT.

The results obtained allow us to observe that cane juice 30% supplemented with yeast extract 5gL^{-1} resulted in the highest specific and volumetric production of carotenoids 95.1 μg.g^{-1} and 888.9 μg L^{-1}, respectively. For cell growth, it is noted that the concentration of sugarcane juice may promote an increase in biomass production when used in sugar 50.0% broth supplemented with concentrations of 10g L^{-1} yeast extract resulting in a production of 13.6 g L^{-1} (Table 1).

By analyzing the residual sugar, the concentration of sugarcane juice could have been more diluted, which would result in greater savings regarding industrial scale production (Table 1 and 2).

Sugarcane juice, despite its rather complex composition, acts as a source of glucose providing energy through its breakage and consequent production of ATP. It also acts as a source of carbon to be assimilated for molecules biosynthesis [24, 25].

On the other hand, yeast extract acts as an organic source of nitrogen. It is known that the presence of these two elements is an essential condition to promote the development of the primary metabolism of the microorganism, such as cell growth, as well as the secondary metabolism, including the production of pigments such as carotenoids. Results of Table 1 shows that if concentration of yeast extract was null, cell growth was visibly impaired.

In this study it can be seen that in the tests where there was a higher carbon / nitrogen (C / N) ratio, the volumetric production of carotenoids was significantly higher, in contrast, a lower C / N ratio led to a higher biomass production

The results obtained in this experiment were superior to those found with *Rhodotorula mucilaginosa* CCT3892 when grown in sugarcane molasses as a carbon source resulting in the production of 53.0 µg g-1 carotenoids [26].

A research recently conducted with the production of carotenoids in *Rhodotorula* species was optimized using agro-industrial means, coconut water and rice grains as the culture medium and obtained as maximum yields: *R. minuta* (2.588 µg g^{-1}), *R. acheniorum* (1.091 µg g^{-1}) and *Rhodotorula sp* (2.160 µg g^{-1}) when using coconut water in fermentation at 30 ° C for 6 days [26]. For these researchers, the results obtained showed that the genus *Rhodotorula* will be a promising microorganism for the commercial production of carotenoids.

In another study, the optimization of carotenoid production in *Phaffia rhodozyma* the maximized conditions for carotenoid production (g L^{-1}) were: malt extract, 16.25; peptone, 15 and boiling water of rice, 87.5, with pH 5, at 25 ºC, 150 rpm for 144 h, reaching 5.3 µg mL^{-1} (628.8 µg g^{-1}) [27].

5.2 EFFECT OF STIRRING SPEED AND INITIAL PH.

The largest volumetric production of carotenoids occurs at 180 rpm and an initial pH of 7.0, resulting in of a specific and volumetric production of 170.5 µg g^{-1} and 1314.9 µg mL^{-1}, respectively. The highest biomass production, 8.8 g L-1, occurred at 180 rpm and initial pH 9 (table 2).

The data obtained in the experimental design shows that aerobic microorganisms such as *R. mucilaginosa* which primary metabolism requires the presence of oxygen; the change of stirring speed could contribute to a higher yield of cell growth.

Note also that the initial pH around 5.5 to 7.5 may provide the best conditions to produce the pigment and biomass. The pH is one of the most parameters in the production

of carotenoids of microbiological origin, due to the influence on the growth of the microorganism and product formation. Normally during the synthesis of carotenoids, a reduction in pH occurs due to other metabolites formed during the fermentation process, followed by a subsequent elevation of that pH during an intense phase of carotenogenesis [24]. Thus, highly acidic (2.0–4.0) and alkaline (10.0–13.0) media did not support pigment production [14].

In a recent study, the optimization of carotenoid production and cell growth of *Rhodotorula glutinis* yeasts in a medium containing cheese whey with different concentrations of NaCl resulted in the maximum growth of biomass (13.95 g L^{-1}) and carotenoids (6.544 mg L^{-1}) after incubation for 120 hours at 30 ° C and pH 6.6 in a medium containing 3% NaCl [28].

The results of the optimization of cell growth and carotenoid biosynthesis by *Phaffia rhodozyma* were tested by varying the pH and temperature in a medium containing sugarcane molasses as a carbon source. The pH and the optimum temperature were 5 and 25 ° C, a production of 317 µg g^{-1}. The growth of biomass was not influenced in the pH between 5 - 7, being around 7.68 g L^{-1} [27].

Studies like these demonstrate that the use of agro-industrial products and the use of appropriate environmental conditions can contribute to increase the productivity of the pigment, as well as reduce the costs of the process.

5.3 CHROMATOGRAPHIC ANALYSIS

The analysis showed the presence of β-carotene in the UV spectrum (Figure 3). The results of the retention times are in accordance with the data retention times in the literature [29, 30].

β-carotene is an organic red-orange pigment used in a variety of food items, ranging from red to yellow. In addition to being pharmacologically and biologically active, they can have a wide range of activities, including antioxidants, antimicrobials, anticancer compounds, immunoregulators and anti-inflammatories [31].

6 CONCLUSION

Thus, we could conclude that the juice of cane juice can be used as a culture medium for fermentation processes in yeasts. In addition, the combination of the use of low-cost raw materials, such as sugarcane, followed by supplementation with yeast extract and control of environmental conditions, such as initial pH and agitation speed, is

possible to promote an increase in bioproduction of carotenoids and biomass. With this, the performed experiment represents a viable option to produce β-carotene with the potential to be used in more diverse industrial sectors. Therefore, it is necessary to determine the best culture medium and the best environmental conditions for microbial fermentation, to explore the potential of the necessary strain

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