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Optimization of pigment production by *Rhodotorula minuta* URM 5197 and *Rhodotorula mucilaginosa* URM 7409 using yellow passion fruit peel (*Passiflora edulis*)

Otimização da produção de pigmentos por *Rhodotorula minuta* URM 5197 e *Rhodotorula mucilaginosa* URM 7409 utilizando casca de maracujá - amarelo (*Passiflora edulis*)

Optimización de la producción de pigmento por *Rhodotorula minuta* URM 5197 y *Rhodotorula mucilaginosa* URM 7409 utilizando cáscara de maracuyá - amarillo (*Passiflora edulis*)

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Abstract
The objective of this work was to optimize the production of pigments by *Rhodotorula minuta* and *Rhodotorula mucilaginosa* through submerged fermentation, using yellow passion fruit peel (*Passiflora edulis*), as the only substrate. The independent variables evaluated to optimize were: yellow passion fruit peel (YPFP), in grams (g) as culture medium, pH and fermentation time, in days. The study of pigment production and its optimization was carried out using the Doehlert matrix, with fifteen experimental conditions, of which thirteen had different combinations and two repeated the central point. The fixed variables were 30ºC and 150 rpm. Data analysis was performed using the Statistica software version 10.0. The largest amount of total pigments and total carotenoids produced by *R. minuta* was 28±0.01 mg/L and 72.8±0.026 µg/g, respectively; while for *R. mucilaginosa* the production of total pigments was 37±0.002 mg/L and total carotenoids 236.8±0.013 µg/g. The optimum production point of total pigments for *R. minuta* was 2.3g of YPFP, pH 6.5 and 5 days and for *R. mucilaginosa* 2.5g of YPFP pH 6 and 5 days. In samples of total pigments, the presence of 0.29mg/L of β-carotene for *R. minuta* and 0.83 mg/L for *R. mucilaginosa* was identified. It is possible to conclude that yellow passion fruit peel can be used as a nutrient source for *Rhodotorula spp* growth and pigment production with total carotenoids and β-carotene in its composition.
Keywords: Bioprocesses; Carotenoids; Agro-industrial waste; Response surface.

Resumo
O objetivo desse trabalho foi otimizar a produção de pigmentos por *Rhodotorula minuta* e *Rhodotorula mucilaginosa* através de fermentação submersa, utilizando a casca de maracujá - amarelo (*Passiflora edulis*), como único substrato. As variáveis independentes avaliadas em relação a otimização foram: casca de maracujá-amarelo (CMA), em gramas (g) como meio de cultivo, pH e tempo de fermentação, em dias. O estudo da produção do pigmento e sua otimização

foi realizado através da matriz de Doehlert, com quinze condições experimentais, das quais treze apresentavam diferentes combinações e duas repetiam o ponto central. As variáveis fixas foram 30ºC e 150 rpm. A análise dos dados foi realizada a partir do Software Statistica versão 10.0. A maior quantidade de pigmentos totais e carotenoides totais produzidos pela *R. minuta* foi 28±0.01 mg/L e 72.8±0.026 µg/g, respectivamente; enquanto que para *R. mucilaginosa* a produção de pigmentos totais foi 37±0.002 mg/L e de carotenoides totais 236.8±0.013 µg/g. O ponto ótimo de produção de pigmentos totais para *R. minuta* foi 2.3g de CMA, pH 6.5 e 5 dias e para *R. mucilaginosa* 2.5g de CMA, pH 6 e 5 dias. Nas amostras de pigmentos totais foi identificada a presença 0.29mg/L de β-caroteno para *R. minuta* e 0.83 mg/L para *R. mucilaginosa*. É possível concluir que a casca de maracujá-amarelo pode ser utilizada como fonte de nutriente para crescimento das *Rhodotorula spp* e produção de pigmento com carotenoides totais e β-caroteno em sua composição.
Palavras-chave: Bioprocessos; Carotenoides; Resíduos agroindustriais; Superfície de resposta.

Resumen

El objetivo de este trabajo fue optimizar la producción de pigmentos de *Rhodotorula minuta* y *Rhodotorula mucilaginosa* mediante fermentación sumergida, utilizando como único sustrato la piel de maracuyá - amarillo (*Passiflora edulis*). Las variables independientes evaluadas en relación a la optimización fueron: piel de maracuyá amarilla (PMA), en gramos (g) como medio de cultivo, pH y tiempo de fermentación, en días. El estudio de la producción de pigmentos y su optimización se realizó utilizando la matriz de Doehlert, con quince condiciones experimentales, de las cuales trece tenían combinaciones diferentes y dos repetían el punto central. Las variables fijas fueron 30ºC y 150 rpm. El análisis de los datos se realizó con el software Statistica versión 10.0. La mayor cantidad de pigmentos totales y carotenoides totales producidos por *R. minuta* fue 28±0.01 mg/L y 72.8±0.026 µg /g, respectivamente; mientras que para *R. mucilaginosa* la producción de pigmentos totales fue 37±0.002 mg/L y carotenoides totales 236.8 ± 0.013 µg/g. El punto óptimo de producción de pigmentos totales para *R. minuta* fue 2.3 g de PMA, pH 6.5 y 5 días y para *R. mucilaginosa* 2.5 g de PMA, pH 6 y 5 días. En muestras de pigmentos totales se identificó la presencia de 0.29 mg/L de β-caroteno para R. minuta y 0.83 mg/L para *R. mucilaginosa*. Es posible concluir que la cáscara amarilla de maracuyá se puede utilizar como fuente de nutrientes para el crecimiento de *Rhodotorula spp* y la producción de pigmentos con carotenoides totales y β-caroteno en su composición.
Palabras clave: Bioprocesos; Carotenoides; Residuos agroindustriales; Superficie de respuesta.

1. Introduction

Natural pigments can be obtained from plants, animals, and microorganisms (Heer & Sharma, 2017). This last group is highlighted when compared to the others, as it manages to make the pigment available immediately, does not depend on seasonality, allows for expansion of production, uses less physical space, and can be cultivated in alternative substrates, such as agro-industrial waste (Venil et al., 2013; Manimala & Murugesan, 2017; Sen et al., 2019).

These substrates, most often arise from the processing of fruits for the production of pulp and can consist of peel, seeds, and bagasse (Viana & Cruz, 2016). We know that degradation and environmental impacts arise in some situations from the inadequate disposal of waste generated by different sectors, necessary to find viable alternatives to reverse this scenario (Menezes et al., 2012).

Some studies report the possibility of reusing industrial waste for the growth of microorganisms and production of metabolites of interest, where at the same time this substrate acts as a source of carbon and nitrogen, it acts in the preservation and mitigation of the environment (Panesar et al., 2015; Kot et al., 2016). In this context, Brazil is one of the largest producers of passion fruit in the world and 95% of this product is destined for the juice industries that generate a lot of fruit peel wastes (Cavalcante & Melo, 2019).

Regarding the production of pigments by microorganisms, the genus *Rhodotorula* is known for producing natural pigments called carotenoids that can appear in yellow, orange, and red hues; and are used in the pharmaceutical, chemical, food, and feed industries, as in addition to coloring, they act as vitamin A precursors and may have biological activities (Guzman et al., 2010; Mata-Gómez et al., 2014; Mussagy et al., 2018; Aruldas et al., 2018; Manimala & Murugesan, 2018; Nabi et al., 2020).

In this context, carotenoids are considered isoprenoids, more specifically tetraterpenes with forty carbons, consisting of eight isoprene units (5C); with a system of conjugated double bonds, which follow a certain pattern of head-tail union

(Delgado-Vargas & Peredes-López, 2002). The order of the inverted double bond only in the central part of the molecule directly interferes with its chemical, physical and biochemical properties. The chains may present cyclic terminal groups, with the presence of oxygen (Gómez-Garcia & Ochoa-Alejo, 2013). The conjugated double bonds in the molecule are the chromophore that allows the absorption of light and, consequently, give color to the compound, which is easily visualized (Weedon & Moss, 1995).

Thus, this study evaluated the possibility of optimizing the production of total pigments by *R. minuta* and *R. mucilaginosa* through submerged fermentation, using yellow passion fruit peel (*Passiflora edulis*) as the only substrate.

2. Methodology

2.1 Waste Collection and Preparation

The raw material used was yellow passion fruit peel (*Passiflora edulis*), a waste generated during processing for the production of ice cream. The waste was obtained in the city of Itapetinga, BA, Latitude: 15° 15′ 23" South, Longitude: 40° 15′ 27" West. After cleaning, it was reduced to smaller parts and dried (72 hours, 50°C) in an oven (SOLAB – SL 102). Subsequently, it was crushed in a Willey knife mill (ACB LABOR), with a particle size of 2 mm.

2.2 Centesimal Composition of Yellow Passion Fruit Peel Waste - *(Passiflora edulis)*

The analysis of the proximate composition of the waste was carried out by the Forage Laboratory of the State University of Southwest Bahia, Campus de Itapetinga-BA. The methodology of Van Soest et al. (1991) and AOAC (2016).

2.3 Obtaining Microorganisms and Maintenance Techniques

The yeasts *R. minuta* (isolated from the ration of the parrot enclosure tray) and *R. mucilaginosa* (isolated from the soil) were acquired from the Microorganisms Culture Collection at the Federal University of Pernambuco - Micoteca URM – UFPE. To guarantee the viability of the species, four maintenance techniques were used: continuous subculture - (short term method), mineral oil - (medium-term method), sterilized water - (medium-term method), and common freezing - (medium-term method term) (Sola et al., 2012).

2.4 Experimental Planning: Doehlert Matrix

In the experimental design, the effects evaluated in the production of pigments by yeasts were: Yellow passion fruit peel (g) as culture medium, pH, and fermentation time, in days. The study of pigment production and its optimization was carried out using the Doehlert matrix, with fifteen experimental conditions, of which thirteen had different combinations and two repetitions in the central point. The fixed variables were 30ºC and 150 rpm. The fifteen experimental conditions were determined from preliminary tests and carried out in triplicate. Data analysis was performed using the Statistica software version 10.0.

2.5 Inoculum Preparation, Culture Medium, and pH Standardization

The inoculum was obtained by cultivating *R.minuta* and *R.mucilaginosa*, in Erlenmeyers (250 mL), with 100 mL of YM broth; under the agitation of 150 rpm, at 30°C, for 48 hours. The inoculum were transferred to the culture medium (100ml total) at a concentration of 10% (v/v) and estimated initial biomass of 10^8 CFU/ml (Silva et al., 2020). For the cultivation of yeasts, a culture medium composed only of yellow passion fruit peel (*Passiflora edulis*) was used, according to the combinations proposed by the Doehlert matrix. The pH of the culture medium for all assays was standardized with sulfuric acid (2M) or sodium hydroxide (2M).

2.6 Pigment Extraction

Pigment extraction was performed based on an adaptation of the proposal by Moliné et al. (2012) and Silva et al. (2020), in four stages. In the first stage, there is centrifugation (refrigerated centrifuge - CIENTEC 6000R) of the fermented after filtration, at 3000xg, for 5 minutes. The culture medium was removed and the pellet was separated with sterile distilled water and the supernatant was discarded. In the second stage, 1mL of DMSO (Dimethyl sulfoxide) was used, associated with the "pellet" and vortexed (AP 56 - Phoenix Luferco) for 1 minute. The mixture was incubated in a water bath (CT-266-CIENTEC) for 1 hour at 55°C. Then centrifuged at 3000xg for 5 minutes. The supernatant was removed and stored at -20°C, in Falcon tubes, protected from light. For the third stage, 1mL of acetone was added to the pellet, followed by vortexing for 1 minute and subsequent centrifugation 3000xg for 5 minutes. The second and third steps were repeated until exhaustive extraction. The last step pooled the acetone and DMSO fractions. Then 2 ml of petroleum ether (35°C - 65°C) and 0.5 ml of saturated NaCl solution at 5°C were added. The mixture was vortexed for 15 seconds and subjected to centrifugation 3000xg for 10 minutes; at a temperature of 5°C. The petroleum ether phase was collected with the pigments and stored in Falcon tubes, wrapped in aluminum foil. The Falcon tubes were kept at room temperature until the ether had completely evaporated and the extract was obtained.

2.7 Analytical Methods

2.7.1 Determination of Total Carotenoids

After obtaining the extract of total pigments, the determination of the concentration of total carotenoids was performed in a spectrophotometer, at an absorbance of 448 nm. The results of the concentration of total carotenoids were obtained through Equation (1), expressed in terms of its main carotenoid, β-carotene (specific absorptivity in petroleum ether of 2592, obtained experimentally) (Davies, 1976; Moriel et al., 2005).

$$TC = \frac{A \times V \times 10^6}{A_{1cm}^{1\%} \times 100 \times m_{amostra}}$$

Where: TC = total carotenoids ($\mu g.g^{-1}$); A = absorbance; V = volume (mL); S_{sample} = dry cell mass (g); $A_{1cm}^{1\%}$ = specific absorptivity. To calculate the total carotenoids ($\mu g.L^{-1}$) with the results of the specific concentration and the biomass concentration, the unit conversion was performed.

2.7.2 Identification and Quantification of β - carotene by High-Performance Liquid Chromatography (HPLC)

β-carotene was identified and quantified by HPLC according to the adaptation of the methodology used by Silva et al. (2020). The extracts obtained were suspended in 1mL of methanol and HPLC acetone (9:1) and transferred to 2mL vials with lids and wrapped in aluminum foil to limit access to light and minimize carotenoid degradation. A 20µL aliquot of each sample was manually injected into the Shimadzu analytical HPLC, with a binary pump. The column used was a C18 Supelco column (25cm x 4.6mm x 5µm) and the mobile phase was acetonitrile/ethyl acetate/methanol, pre-sonified, in a ratio of 10:40:50 (v/v/v), maintained until the end of the 25-minute run/sample, under a flow of 0.6mL/min, with a wavelength of 460nm, at room temperature. The calibration curve for quantification of β-carotene was constructed with different concentrations of the standard submitted to HPLC through this methodology.

2.8 Statistical Analysis

The values found for the production of pigments were analyzed using the Statistica 10 program, through analysis of variance (ANOVA), Pareto diagram, and response surface graph.

3. Results and Discussion

The proximate composition of the chosen waste indicates that the yellow passion fruit peel can be used as a source of nitrogen and carbohydrate for the growth of the microorganism, as it has 12.93% crude protein, 1% ether extract, 7.35% mineral material, 49.09% neutral detergent fiber - FDN (8.94% lignin + 29.5% cellulose + 10.65% hemicellulose), 38.44% acid detergent fiber - FDA (8.94% lignin + 29.50% cellulose) and 0.36% ash.

With this composition, the yellow passion fruit peel was effective as a substrate for the growth of the two studied yeast species. The waste with a greater number of lignocellulosic compounds (lignin, cellulose, and hemicellulose) has a greater potential to generate fermentable sugars, functioning as a substrate for yeast growth and pigment production (Silva et al., 2020).

Previous studies demonstrate that the genus *Rhodotorula* is capable of growing and producing pigments in different alternative culture media, including fermented radish brine (Malisorn & Suntornsuk, 2008); chicken feather peptone (Taskin et al., 2011); glycerol wastes from the production of biodiesel with glucose (Petrik et al., 2013); coconut water or rice (Yadav & Prabha, 2014); crude glycerin and used brewer's yeast (Rodrigues et al., 2019), orange bagasse (*Citrus sinensis*) with sugarcane molasses (Machado et al., 2019) and potato wastewater and glycerol fraction (Kot et al., 2020). This demonstrates that in the presence of alternative substrates, whose composition includes proteins and carbohydrates, there is a possibility that the yeast of the genus *Rhodotorula* can grow and produce the pigment.

Table 1 shows the results obtained for the production of pigments (g/L) and total carotenoids by *R. minuta* and *R. mucilaginosa*, according to the conditions proposed in the Doehlert matrix. The response values of pigments and total carotenoids are the means of the triplicate value performed in the tests.

Table 1. Production of total pigments and total carotenoids according to Doehlert matrix for *R. minuta* and *R. mucilaginosa*.

Test	YPFP (g)	pH	T (days)	TP RMI (mg/L)	TP RMU (mg/L)	TC RMI (µg/g)	TC RMU (µg/g)
1 (C)*	3	6	5	26.80±0.010	31.50±0.005	69.68±0.026	201.60±0.032
2	3	4	5	21.00±0.020	15.45±0.025	54.60±0.052	98.88±0.159
3	5	6	5	13.10±0.005	11.75±0.022	34.06±0.013	75.20±0.140
4	3	8	5	26.00±0.001	27.25±0.012	67.60±0.002	174.40±0.076
5	1	6	5	23.20±0.005	24.10±0.003	60.32±0.013	154.24±0.019
6	2	5	6	23.85±0.040	24.20±0.025	62.01±0.103	154.88±0.159
7	4	5	6	12.25±0.005	6.75±0.030	31.08±0.013	43.20±0.191
8	4	7	6	7.50±0.002	4.50±0.020	19.50±0.005	28.80±0.127
9	2	7	6	25.40±0.056	26.25±0.005	66.04±0.144	168.00±0.032
10	2	5	4	22.20±0.325	17.50±0.030	57.72±0.838	112.00±0.191
11	4	5	4	19.90±0.010	14.25±0.060	51.74±0.026	91.20±0.384
12	4	7	4	20.15±0.060	15.05±0.002	52.39±0.155	96.32±0.013
13	2	7	4	24.95±0.001	2.55±0.001	64.87±0.003	16.32±0.006
14 (C)*	3	6	5	27.15±0.023	35.50±0.001	70.59±0.059	227.2±0.006
15 (C)*	3	6	5	28.00±0.010	37.00±0.002	72.80±0.026	236.8±0.013

(C)*: Central point; YPFP: Yellow passion fruit peel (*Passiflora edulis*); T: Time; TP: Total pigments; RMI: *R.minuta*; RMU: *R.mucilaginosa*; TC: Total carotenoids. Source: Authors.

According to the results presented in the Doehlert matrix, there was a difference in the number of pigments and total carotenoids produced between the different species. *R. minuta* produced 28±0.01 mg/L of total pigments using only the passion fruit peel (*Passiflora edulis*) as substrate, while *R. mucilaginosa* presented better total pigment production, obtaining 37±0.002 mg/L when compared to *R. minuta*. Regarding the production of total pigments, Buzzini e Martini (2000) used grape must as substrate for pigment production by *R. glutinis* DBVPG 6439 and obtained 5.95mg/L. While Squina et al. (2002) worked with *R. rubra* and *R. glutinis* using sugarcane juice as substrate, yeast extract, and peptone found a pigment production of 4.4 mg/L for *R. rubra* and 6.7 mg/L for *R. glutinis*.

In this same context, Tinoi et al. (2005) used hydrolyzed bean waste flour and sweet potato extract as a substrate for *R. glutinis* and obtained 3.48±0.02 mg/L of total pigments. Cheng e Yang (2016) used *R. mucilaginosa* F-1 and as a substrate for their growth wasted food including ketchup, sugarcane molasses, and healthy drinks; the authors found a pigment concentration of 2.23 mg/L when using ketchup, 2.61 mg/L with sugarcane molasses, and 1.10 mg/L with the healthy drink.

Although the species differ among the studies cited, they belong to the same genus. The values found are very close to each other and lower than those found in this study. Among the authors studied, only Taskin et al. (2011) when using the mutant species *R. glutinis* MT-5 found a value higher than this study, 92mg/L of total pigment when using 8g/L of chicken feather peptone as substrate.

Thus, the result found for the production of total pigments by the two species stands out for being a higher value than that found in studies that used yeast without mutations and agro-industrial waste as the only substrate.

As for the production of total carotenoids, *R.minuta* produced 72.8±0.026 µg/g, a value lower than that produced by *R.mucilaginosa*, which was 236.8±0.013 µg/g. When comparing this result of the two species obtained in this study with the study of Machado et al. (2019), similar values are identified, as the authors also used *R. mucilaginosa* for pigment production and found a total carotenoid production of 252.99 µg/g, varying the amount of yeast extract, peptone, and initial fermentation pH.

Also, on the production of total carotenoids, Silva et al. (2020) obtained a total carotenoid production of 179.66 µg/g produced by *R. mucilaginosa*, with sisal bagasse as the main substrate, varying the supplementation of the culture medium, pH, and temperature. In another study, when using *R. mucilaginosa* F-1, the authors varied the culture media and found a production of 315.9 µg/g when using the YM medium (10g/L glucose, 5g/L peptone, 3g/L of yeast extract, 3g/L of yeast extract), 376.3 µg/g with ketchup, 268.6 µg/g with molasses, and 245 µg/g with a healthy drink (Cheng & Yang, 2016).

When using another species of the same genus, in this case, *R.glutinis* R12 with different culture media, the total carotenoid production found was 283.71 µg/g. However, the three-culture media used in the study were not constituted by agro-industrial waste, were formed by compounds that are normally present in standard yeast culture media (Gerelmaa et al., 2018).

Thus, when comparing the different studies and the production of total carotenoids, we can observe that the values found are very similar, regardless of whether the culture medium consists of agro-industrial or standard waste, as in both the yeast can access nutrients that allow its growth and produce pigments; which consolidates the possibility of reducing the production cost and also allows an application for agro-industrial waste.

According to Silva et al. (2020) pH is a variable that can influence the production of total pigments and carotenoids, which allows inferring the presence of an adaptive response in *Rhodothorula ssp.* Studies carried out by Machado et al. (2019), highlight that the initial pH of the fermentation positively influences the volumetric production of carotenoids; which corroborates other studies (Nahas et al., 1982; Peñalva & Arst, 2004; Freitas et al., 2007) that demonstrated the role of pH in microorganisms, emphasizing its physiological versatility and the existence of extracellular monitoring that does not it only

regulates the homeostatic pH as well as the synthesis/secretion of biomolecules occurring only at pH values that function effectively (Caddick et al., 1986; Maccheroni et al., 1991).

The experimental values found for the total pigments were used to make statistical adjustments to generate significant models, as shown in Table 2.

Table 2. Analysis of Variance (ANOVA) for total pigment production by *R. minuta* and *R. mucilaginosa* by the Doehlert matrix.

R. minuta					
Source of variation	SQ	GL	QM	Calculated F	Tabulated F
Regression	489.3298	9	54.3699	10.50	3.31
Waste	25.8792	5	5.1758		
Lack of adjustment	25.1175	3	8.3725	21.98	9.16
Pure error	0.7617	2	0.3808		
Total SQ	515.2090	14			
R2	95%				
R. mucilaginosa					
Source of variation	SQ	DF	MS	Calculated F	Tabulated F
Regression	1.499,586	9	166.62	5.27	3.31
Waste	158,153	5	31.6306		
Lack of adjustment	141.986	3	47.3285	5.86	9.16
Pure error	16.167	2	8.0833		
Total SQ	1657.739	14			
R2	90%				

SQ = Sum of Squares, DF = Degree of freedom; MS = Medium square; F = Fisher Test; R^2 = Determination coefficient. Source: Authors.

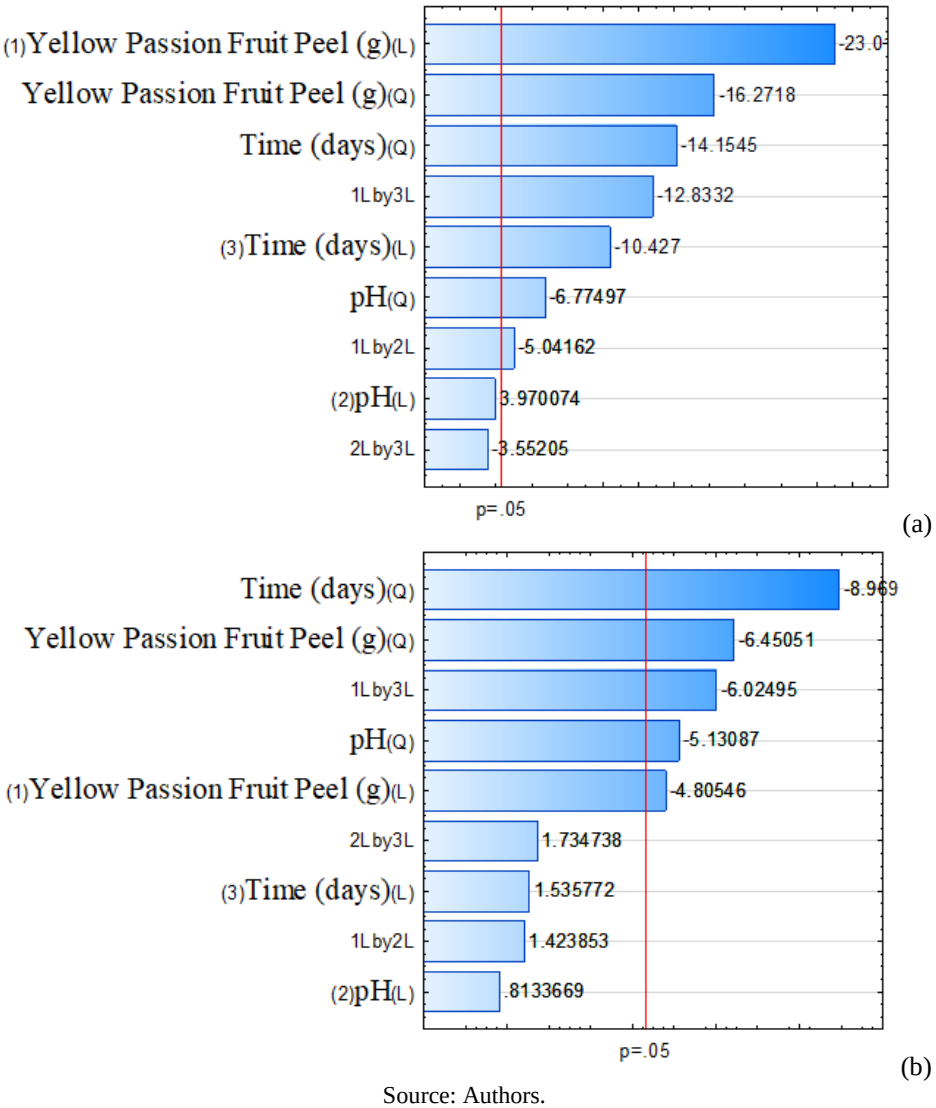
ANOVA assessed the quality of adjustment of the models generated using the Fisher test (F test), regression significance, lack of adjustment, and the coefficient of multiple determination. There is statistical significance for the effects when the p-value is less than 0.05.

Considering the Fisher test for the production of total pigments of *R. minuta*, the calculated F (10.50) is greater than the tabulated F (3.31) when considering the regression, this means that it is possible to trust the obtained data. When analyzing the lack of adjustment, the calculated F (21.98) was greater than the tabulated F (9.16), indicating that there was a lack of adjustment, the R2 was equal to 95%, demonstrating that 95% of the results found are explained by the experimental model.

For the production of total pigments by *R. mucilaginosa*, in the regression, the calculated F (5.27) is greater than the tabulated F (3.31), so it is possible to trust the data. As for the lack of adjustment, the calculated F (5.86) is lower than the tabulated F (9.16) indicating that there was no lack of adjustment, the results found are not random, the R2 was 90%, which means that 90 % of the results found are explained by the experimental model.

Figure 1 shows the Pareto diagram that demonstrates the statistical significance of the quadratic and linear terms and their interaction concerning the production of total pigments by *R. minuta* and *R. mucilaginosa*.

Figure 1. Pareto diagram for total pigment production (mg/L) for *R.minuta* (a) and *R. mucilaginosa* (b).

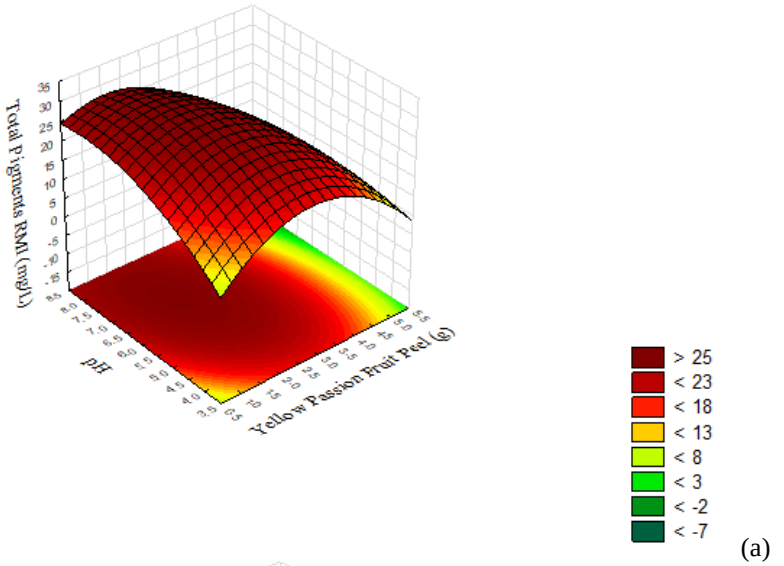


Source: Authors.

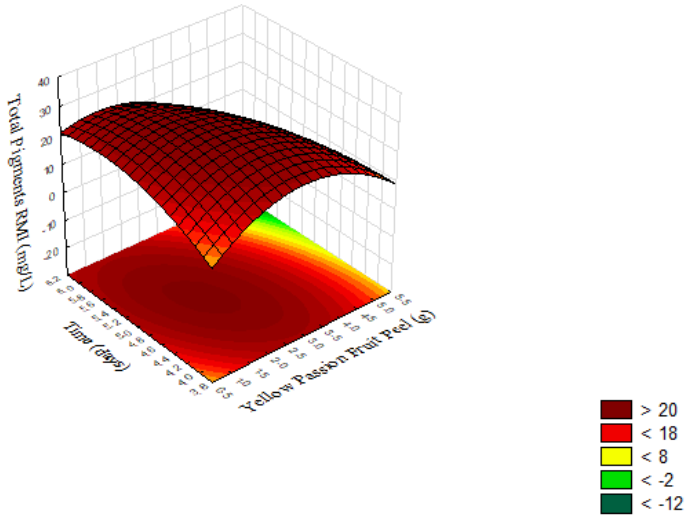
In the Pareto diagram for total pigment production, the three variables were statistically significant (p-value <0.05). When analyzing the correlation between the variables, for *R. minuta* there was statistical significance for pigment production, the relationship between yellow passion fruit peel and pH (p-value 0.037), and yellow passion fruit peel and time (p-value 0.006) (Figure 1a). While for *R. mucilaginosa*, when verifying the correlations between the variables, there was statistical significance only for yellow passion fruit peel and time (p-value 0.02) (Figure 1b). We can affirm that the yellow passion fruit peel influences pigment production. In both species, there is a correlation between yellow passion fruit peel and time (days) with statistical significance.

Figures 2 and 3 show the response surface indicating the optimal point for total pigment production by *R. minuta* and *R. mucilaginosa*.

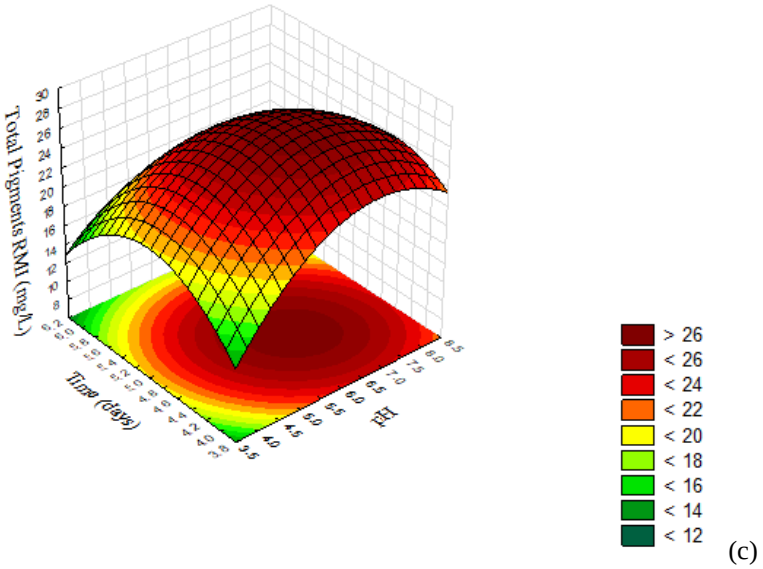
Figure 2. Response surface graphs for total pigment production by *R. minuta*, showing the effects of interactions of (a) pH and yellow passion fruit peel, (b) time (days) and yellow passion fruit peel, and (c) time (days) and pH.



(a)



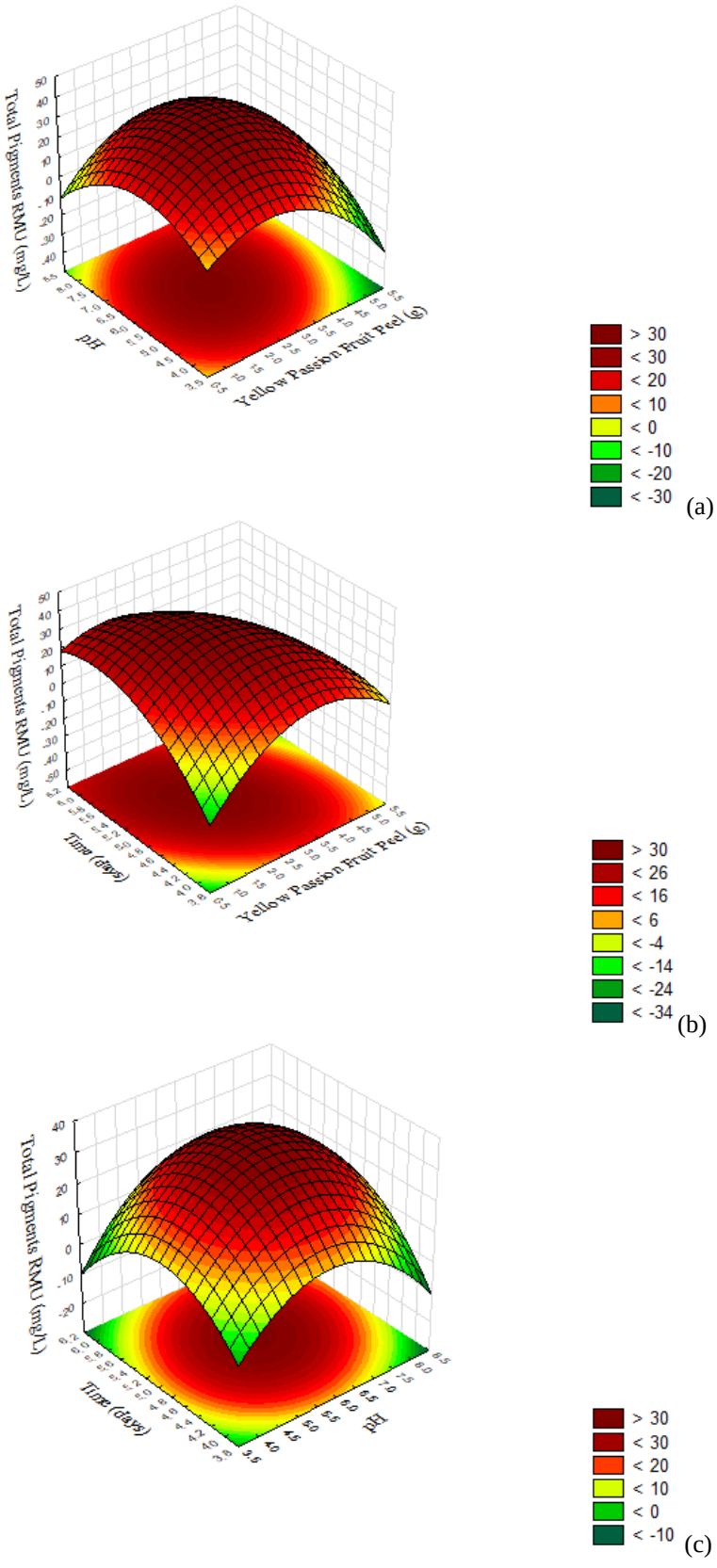
(b)



(c)

Source: Authors.

Figure 3. Response surface graphs for total pigment production by *R. mucilaginosa*, showing the effects of interactions of (a) pH and yellow passion fruit peel, (b) time (days) and yellow passion fruit peel, and (c) time (days) and pH.



Source: Authors.

From the results of Figures 2 and 3, through the response surface, an optimal point of total pigment production was found for *R. minuta* (2.3 g of YPFP, pH 6.5 and 5 days) and *R. mucilaginosa* (2.5g of YPFP, pH 6, and 5 days). The determination of an optimal production point is intended to indicate the best growing conditions to produce a greater quantity of pigment. Thus, we observed in the optimization of pigment production that the amount of substrate, pH, and time was similar for both species. The interaction of these variables, under these specific conditions, contributed to the access of nutrients by yeast, cell monitoring, production, and secretion of biomolecules. We also observed that acidic environments are not a premise for good pigment production by *Rhodotorula spp*. The neutral pH, around 6, demonstrated its functionality regarding an adaptive response of yeasts to cellular sensing, allowing an optimal point of production of total pigments, for both *R. minuta* and *R. mucilaginosa*.

The equation that describes the behavior of the response regarding the production of total pigments (mg/L) produced by *R. minuta* and *R. mucilaginosa* as a function of the variables, is described in Equations 2 and 3, respectively.

Total Pigment Production RMI (g/L) = - 208.00 + 30,800 (YPFP) - 2,292 (YPFP)2 + 19,238 (pH) - 0.954 (pH)2 +56,233 (T) - 4,546 (T)2 - 1,100 (YPFP) (pH) - 2,800 (YPFP)(T)

Equation 2

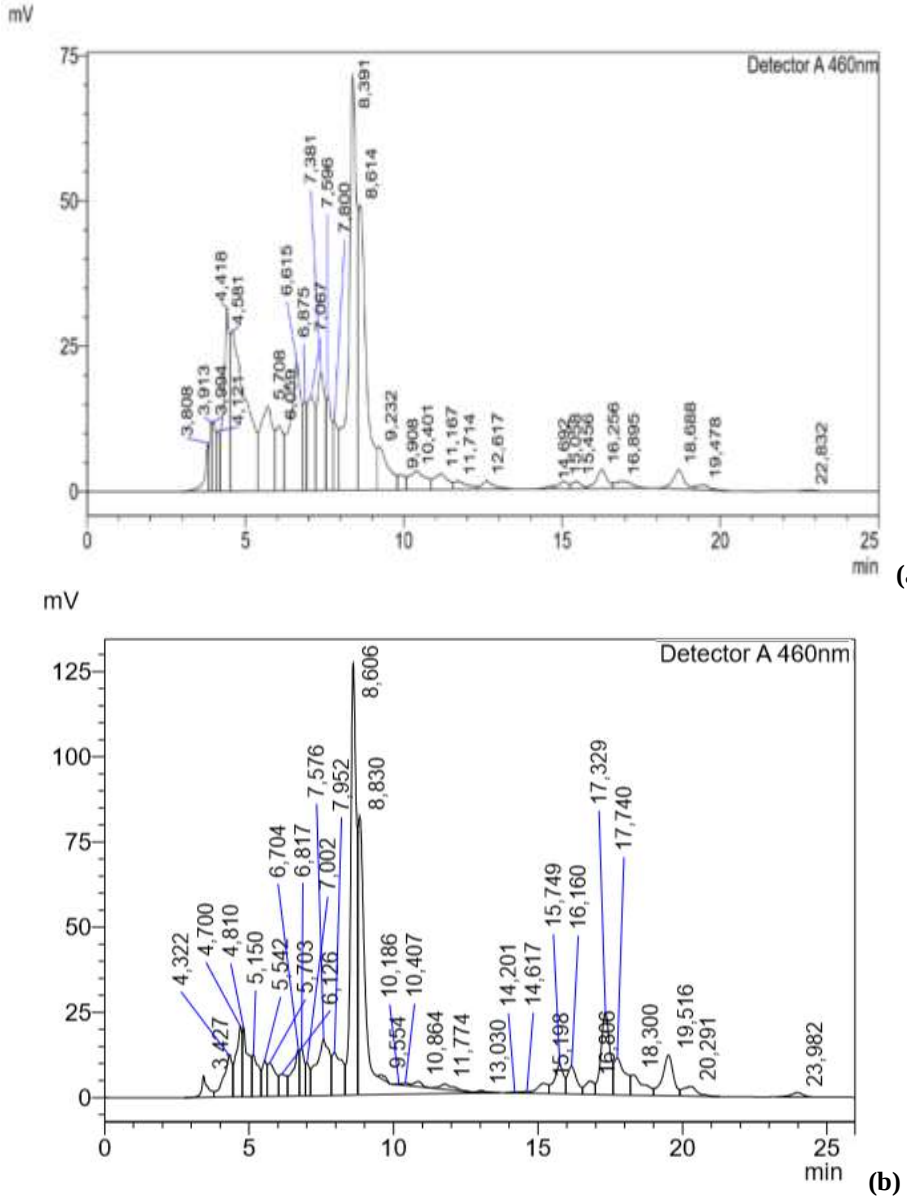
Total Pigment Production RMU (g/L) = - 468.33 + 43.39 (YPFP) - 4.18 (YPFP)2 + 27.52 (pH) - 3.33 (pH)2 + 141.96 (T) - 13.27 (T)2 + 1744 (pH))(T)

Equation 3

Where: YPFP: Yellow passion fruit peel, T: Time in days, RMI: *R. minuta*, RMU: *R. mucilaginosa*

Through the high-performance liquid chromatography (HPLC) represented by the chromatograms (Figure 4), we identified the amount of β-carotene present in samples of total pigments produced by *R. minuta* (Figure 4a) and *R. mucilaginosa* (Figure 4b).

Figure 4. Chromatograms obtained by HPLC considering the detector response (mV) x time (min), for β-carotene obtained by *R.minuta* (a) and *R.mucilaginosa* (b).



Source: Authors.

Regarding the chromatograms obtained by HPLC, we can state that the total pigments obtained by *R. minuta* (0.29mg of β-carotene per liter) and *R. mucilaginosa* (0.83 mg of β-carotene per liter) have β-carotene in its composition. The amount of β-carotene was determined by a straight-line equation obtained by the standard β-carotene curve at different known concentrations and the area corresponding to the β-carotene peak performed by HPLC, identified between 18 to 19 minutes for the pigment produced by *R.minuta* and 19 to 20 minutes for pigment produced by *R.mucilaginosa*.

Thus, the equation obtained was:

Y β-carotene = 648,868X-113,258. Where: Y= Area obtained at the peak of β-carotene by HPLC and X= Concentration in mg/L of β-carotene.

In the study published by Silva et al. (2020), when verifying the production of β-carotene by high-performance liquid chromatography by R.*glutinis* URM 6692, *R.minuta* URM 6693, and *R.glutinis* URM 6695 using residual glycerin as substrate, they identified production of 0.56mg/L, 1.02mg/L, and 0.13mg/L, respectively. These values demonstrate similarity with the results found in this study.

Because of the tests carried out, *R. mucilaginosa* presented a greater quantity of total carotenoids and β-carotene in total pigment samples, when compared to *R. minuta*. Thus, *R. mucilaginosa* is a promising species in terms of cellular adaptability against *R. minuta* for pigment production. Presenting and producing this type of substance in a different way is relevant, as carotenoids stand out in other pigments, as they have different purposes, concerning uses that meet the demands of different sectors of the industry; it can be defined in a moment of choice at the beginning of large-scale production.

4. Final Considerations

The results of this study indicate that yellow passion fruit peel can be used as a substrate for the growth of *Rhodotorula spp* and production of pigment with total carotenoids and β-carotene in its composition. By optimizing the cultivation conditions to which the species were subjected, we determined the optimal conditions for the production of total pigments. This demonstrates the versatility of yeasts that use wastes as substrates for the production of relevant biomolecules, such as pigments.

The presence of carotenoids in these pigments encourages and directs further studies for purification and verification of possible biological activities (antimicrobial, antioxidant, cytotoxic, toxicity, antifungal), to use the pigment not only to color some substance, but for use in the food, pharmaceutical, and cosmetic industry.

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