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Semi-continuous Carotenoid Production in Bioreactor from *Phaffia rhodozyma* Using Agro-industrial Residues

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Abstract: This work has studied the semi-continuous process for carotenoids production in the bioreactor by *Phaffia rhodozyma* (Y-17268) using agro-industrial residues as substrate. The kinetics process was evaluated using different cuts (25, 50, and 75%) every 96 h, until 288 h, to achieve a high level of productivity in total carotenoids. In a period of 192 h was obtained the maximum total carotenoids concentration (477 µg/g) with agro-industrial residues (100 g/L corn maceration water, 100 g/L crude glycerol, and 20 g/L rice parboiling water), under the following conditions: 250 rpm agitation rate, 1.5 vvm aeration rate, 25ºC, pH initial 4.0, with 50% working volume cut. It was obtained 42.51 µg/L .h of maximum productivity on total carotenoids, 0.063 g/L. h the maximum productivity on cells, and 0.067 h^{-1} specific maximum growth speeds. In this way, it was possible to achieve high production of carotenoids in a semi-continuous process and is a sustainable option to obtain this colorant on an industrial scale.

Keywords: Bioreactor; substrates; glycerol; corn maceration water; rice parboiling water; productivity; agro-industrial residues.

1. Introduction

Carotenoids have been widely used as additives in food and feed, nutraceuticals, and cosmetics due to biological activities [1]. The global market for carotenoids is reported to reach $ 1.5 billion in 2018. It will increase to an estimated value of $ 2.0 billion in 2022 [2]. The industrial demand for carotenoids such as β-carotene and astaxanthin has been growing due to the large variety of its applications in foods, cosmetics, and pharmaceutical industries. As well as their use as colorants, such compounds are vitamin A precursors (pro-vitamin A), and they have antioxidant properties, allowing the use of carotenoids in preventive cancer treatments, heart diseases, and macular degeneration related to aging [3,4].

Although the demand for carotenoids is rapidly increasing, their supply is limited due to inefficient production methods [1]. Many of the structurally more complex carotenoids cannot be chemically synthesized via economically viable methods [5]. Most carotenoids are extracted from natural sources with low yields due to complicated multi-step processes and low concentrations in the raw materials, which can also be further affected by unfavorable environmental conditions [6]. Therefore, the carotenoids production via microbial provides an attractive alternative, as it does not require direct land use or the time invested in growing crops for harvest and extraction.

That has contributed to researchers interest on discovering new sources, processes, and techniques that could be employed on the intensification of these pigments by microorganisms, considering that carotenoids obtained by chemical synthesis involve a great number of complex reactions. In contrast, these same carotenoids are naturally found in microalgae, bacteria, yeasts and fungi [7].

Alternative agro-industrial residues such as molasses, sugarcane bagasse, whey, cornmeal, corn liquor, rice parboiling water, glycerol, and others, have been used for carotenoids production [8-13] to reduce production costs.

Bioprocesses are generally applied on large scale production of natural colorants due to the high cellular density and a greater microorganism growth rate [14]. In semi-continuous fermentation, a portion of the culture is removed as output, and a fraction of a culture medium is replaced with fresh media at determinate intervals of time. This fermentation has been applied to obtain carotenoid as a simple means of approximating continuous fermentation in shake flasks. Thus, it makes the semi-continuous fermentation similar to the continuous fermentation process, once the volume is kept constant. However, the difference from the fed-batch is that the volume will increase with time. The semi-continuous fermentation showed as advantages a simplified operation and reduced bioreactor size, with extremely small feed rates in relation to continuous fermentation [15,16]. In this process, the feed solution with agro-industrial byproducts was fed at constant intervals while the effluent is removed discontinuously. So, some problems can be avoided by intermittent feeding of the substrate as the inhibition and catabolite repression [17].

The use of yeasts in carotenoid bioproduction by the semi-continuous system with agro-industrial byproducts is still little explored. In this sense, this work aims to study the parameters (kinetic and stoichiometric) for carotenoids bioproduction using *Phaffia rhodozyma* (Y-17268) in a semi-continuous system with agro-industrial residues

2. Materials and Methods

2.1. Conditions of cultivation and carotenoids bioproduction.

Phaffia rhodozyma (Y-17268) certified as GRAS (Generally Recognized as Safe) was obtained from the Bioprocess Engineering Laboratory (FURG, Brazil). The strain was maintained at 4ºC on YMA medium (Yeast Malt Extract Agar) and supplemented with 0.2 g/L of KNO_3 (Vetec, Brazil). The medium was composed of yeast extract (3 g/L), malt extract (3 g/L), peptone (5 g/L), glucose (10 g/L), and agar (20 g/L). 100 mL of yeast malt extract (YM) medium was used for the inoculum preparation. A suspension of cells (10% v/v) from the stock slants was added to a sterilized Erlenmeyer flask to obtain the inoculum. The strain was incubated for 48 h at 25ºC, 180 rpm (New Brunswick Edison, NJ, USA) [18].

Carotenoid bioproduction was carried out for 288 h in a room without illumination. A Biostat bioreactor (Braun Biotech International, Holland) was utilized for experiments with a total volume of 1 L. During the bioproduction experiments, the temperature was controlled with a water bath (Tecnal, Brazil). The pH monitored using a digital pH meter (Digimed DMPH- 2, Brazil).

2.2. Agro-industrial substrates.

The agro-industrial residues used were corn maceration water (CMW), parboiled rice water (PRW), and crude glycerol all provide and acquired from the Industrial production process. The residues were supplied frozen and stored at -20°C until use. The CMW was previous chemically pre-treated, according to Valduga *et al*. [18] using phosphoric acid, and PRW and glycerol were used without previous treatment.

2.3. Bioproduction in a semi-continuous process.

Carotenoid bioproduction was performed at 25°C, $pH_{initial}$ 4.0, with a medium composed of agro-industrial residues (100 g/L CMW, 20 g/L PRW, and 100 g/L glycerol). These conditions were based on the previous study of the group [10]. Prior to inoculation, the bioreactor and its contents (1 L of the medium bioproduction) were sterilized by autoclaving at 121°C for 20 min. 10% (v/v) of inoculum was inoculated in 1 L medium, and cultivated for 96 h, aeration rate (1.5 vvm), stirring rate (250 rpm), $pH_{initial}$ 4.0, maintaining the temperature at 25ºC. Next, were taken 25, 50, and 75% of the working volume of the bioreactor to perform the carotenoid extraction. For the semi-continuous process, a fresh volume of sterilized medium was added to the bioreactor and cropped at the same condition of the inoculum, performing two cuts every 96 h of bioproduction, totalizing 288 h. In this way, were carried out 3 cycles in the semi-continuous process. These experiments were performed in triplicate.

2.4. Recovery and quantification.

The extraction and quantification of total carotenoids were performed according to a previous study [10]. The cells obtained from the carotenoid production were lyophilized for 36 h. 0.05 g dry cells were added to the test tubes along with 2 mL of $(CH_3)_2SO$ (Quimex, Brazil). Test tubes were homogenized in a shaker (New Brunswick) at 150 rpm, 35ºC, for 60 min for the cell disruption. The samples were extracted with 4 mL of C_3H_6O (Quimex, Brazil) and centrifuged (MPW-351R refrigerated Laboratory Centrifuge, Poland) 4,478 x g at 5ºC for 10 min. The supernatant obtained in the test tubers was separated, and the carotenoid extractions were carried out until both the solvent and cells were colorless. Following, 10 mL NaCl solution (20% w/v) and 10 mL petroleum ether were added to the samples, stirred for phase separation, and remove the $(CH_3)_2SO$. Then, the solution was filtered with the addition of 2 g Na_2SO_4 (Merck, USA), and the supernatant phase was collected.

The absorbance was read at 448 nm in a UV/Vis spectrophotometer (Agilent UV-8553, USA). The concentration of total carotenoid (µg/L) was estimated by Davies [19] equation using an absorbance coefficient of $E1_{\%1\ cm} = 2592$ (referent to β-carotene in petroleum ether). Also, the carotenoids were expressed in the specific production of carotenoid (µg/g), which characterizes the total carotenoid concentration (µg) by the cell mass of dried yeast (1 L of fermented medium).

2.5. Kinetics of the carotenoid bioproduction.

The kinetics (n = 3) were evaluated periodically, collecting the medium every 24 h during 288 h of bioproduction. The parameters evaluated were the carotenoid production, pH evolution, cell mass, and substrate consumption in the medium, being the latter evaluated in terms of glycerol, total nitrogen (TN), and total organic carbon (TOC).

The conversion factor for the substrate in the product, $Y_{P/S}$ (µg carotenoids/g substrate), the substrate in the biomass, $Y_{X/S}$ (g cells/g substrate), and the specific carotenoid production, $Y_{P/X}$ (µg carotenoids/g cells), were calculated according to Bailey and Ollis [20].

The instantaneous productivity in cells (r_x) and carotenoids (r_p) in a semi-continuous process at constant volume also was determined according to Bailey and Ollis [20] and Colet *et al*. [21]. It was taken into account the rates of microbial growth (r_x), product formation (r_p), and substrate consumption (r_s). These rates were evaluated through the mass balance of each component at a specific time.

2.6. Analytical methods.

2.6.1. Cell Mass.

The cells obtained in the semi-continuous bioproduction process were centrifuged (MPW-351R refrigerated Laboratory Centrifuge, Poland) at 3,000 x g, at a constant temperature of 5ºC for 10 min. Next, were washed with distilled water several times and dried in an oven (Fanem SE-320, Brazil) at 105ºC until a constant mass.

2.6.2. Total Organic Carbon (TOC) and Total Nitrogen (TN).

The TOC and TN in the medium were evaluated using the catalytic combustion method at 680ºC and 720ºC, respectively. The analyses with infrared detection were performed on Shimadzu equipment with TOC/TN analyzer (TOC-VCSH, China). The samples were diluted in ultrapure water before reading.

2.6.3. pH.

The pH of the medium in the semi-continuous process using agro-industrial residues was determined using a digital pH meter (Digimed DMPH-2, Brazil).

2.6.4. Glycerol.

The glycerol of the medium in the semi-continuous process was determined according to the standard method UNE-EN 14105 [22]. The procedure steps for the determination of glycerol concentration were described previously by Colet *et al*. [21].

3. Results and Discussion

Figures 1, 2, and 3 show the kinetic curves of total carotenoid production by *P. rhodozyma* for the semi-continuous system. It is observed that from the second cycle, the high concentration of total carotenoids (5346 µg/L) was obtained in the system with a cut of 50% (Figure 2) with 192 h, when compared to bioproducts with cuts of 25 (4087 µg/L) and 75% (4127 µg/L). The maximum concentration of biomass obtained in each cut was higher in the process that used a cut of 50% (3.4 and 9.0 g/L for the first and second cycles) than in the process that was performed cut of 75% (3.4 and 4.9 g/L for the first and second cycles) and 25% (3.4 and 6.9 g/L for the first and second cycles, respectively).

According to Figure 2 (a) in a semi-continuous fermentation process with a cut of 50% every 96 h, it is observed after the second cut, a decrease in the concentration of carotenoids up to 288 h. Thus, it is convenient to take the semi-continuous bioproduction until

approximately 192 h. After this time which there is a decrease in the production of carotenoids and the production is no longer associated with cell concentration.

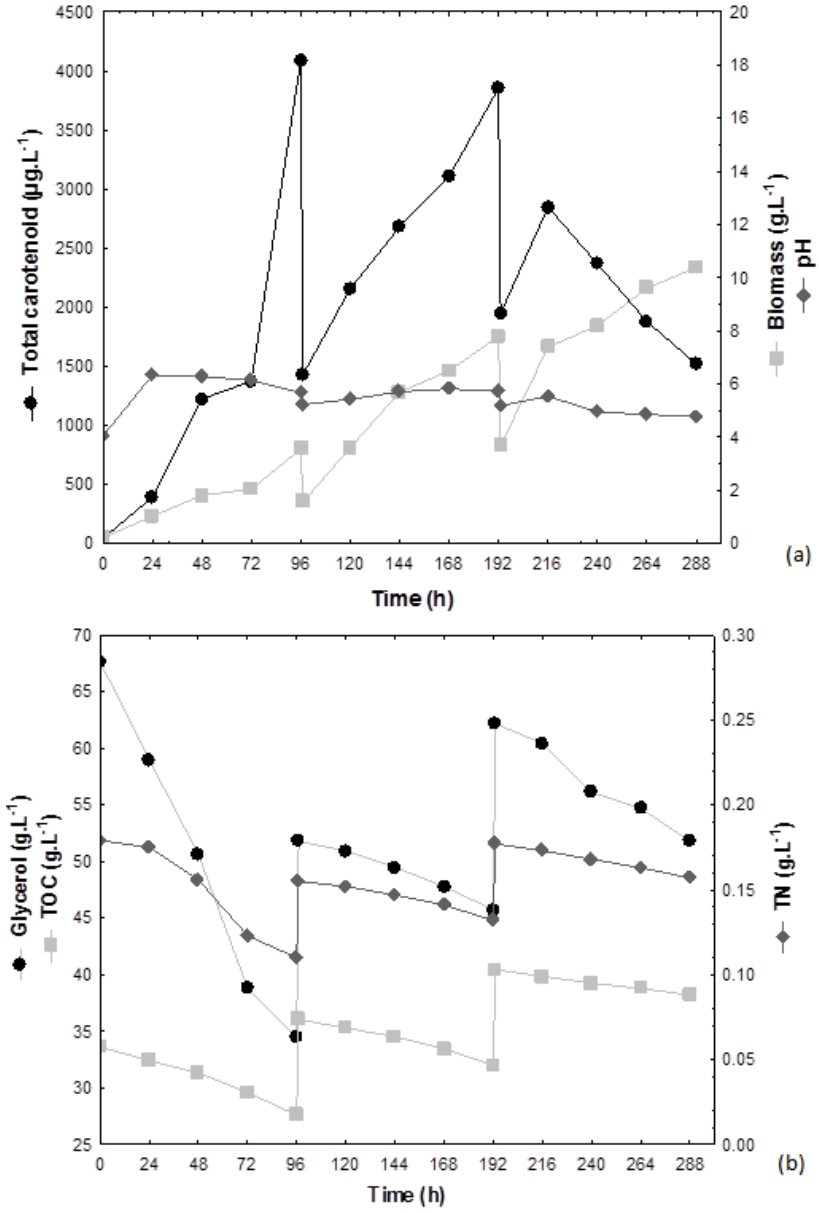

Figure 1. Kinetics of carotenoid production, biomass, and pH (**a**); substrates consumption: glycerol, TOC and TN (**b**) semi-continuous process using agro-industrial residues with a cut of 25%, every 96 h, until 288 h.

Urnau *et al*. [9] observed in a semi-continuous bioproduction process, an increase of ~55% in the total carotenoid when compared to the simple batch system, which was 2380 µg/L. Colet *et al*. [21] obtained the maximum carotenoids production by *S. salmonicolor* in a semi-continuous system of 7388 µg/L at 288 h, with a cut of 50%, every 96 h, using an optimized medium (80 g/L CMW, 80 g/L glycerol and 20 g/L RPW), showing an increase around 55% in relation to the fed-batch process [8], and 90% in relation to simple batch [24].

pH is one of the main environmental parameters that influence cell growth and product formation. In general, it increases in the first hours of fermentation (Figure 1, 2, and 3 (a)). At the beginning of the fermentation, there was an increase in pH, possibly related to proteolysis of the microorganism. This is a natural phenomenon, which occurs after high consumption of the substrate, especially when the strain does not use another carbon source. So, result in the

degradation of amino acids, the formation of ammonia responsible for raising the pH. The pH in the agro-industrial medium decreased after the second cut, reaching the higher carotenoids production at a pH of 5.67.

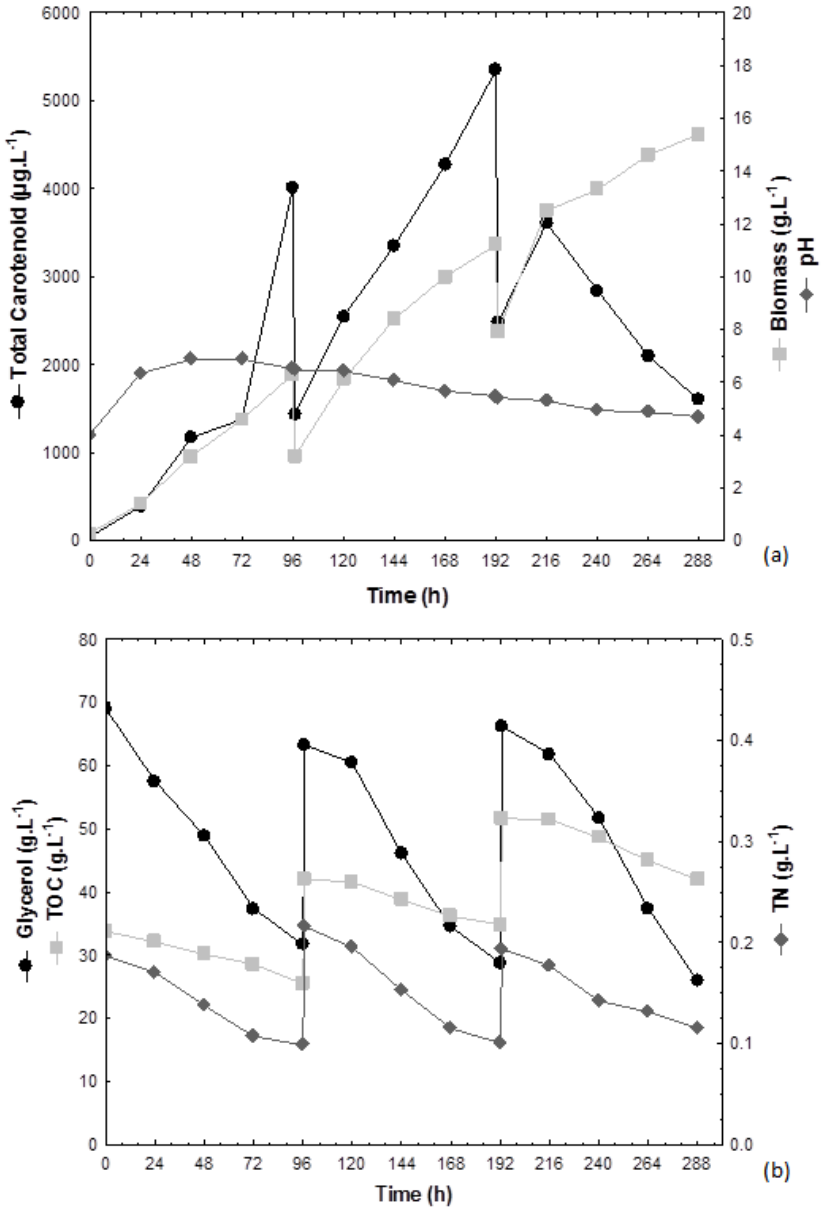

Figure 2. Kinetics of carotenoid production, biomass, and pH (**a**); substrates consumption: glycerol, TOC and TN (**b**) semi-continuous process using agro-industrial residues with a cut of 50%, every 96 h, until 288 h.

Colet *et al*. [21] verified the effect of pH on the bioreactor bioproduction in a semi-continuous system with agro-industrial residues using *S. salmonicolor* and observed that the pH control (4.0) throughout the bioproduction reduced the carotenoid bioproduction in relation to the process without pH control.

In Figure 2b, the cut of 50% showed higher consumption of glycerol (61%) after the second cut in 288 h, total organic carbon (25%) after 96 h of bioproduction, and of nitrogen (54%) after the first cut in 192 h, wherein this phase it was observed the high concentration of total carotenoid (5346 µg/L).

In the cut of 75% with agro-industrial residues (Figure 3) no increase in the total carotenoid production was observed when compared to the other cuts (25 and 50%). This

decrease may be related to the formation of secondary compounds, such as β-ionone that is formed by the carotenoid oxidation [18], and/or inhibition by the excess of nutrients.

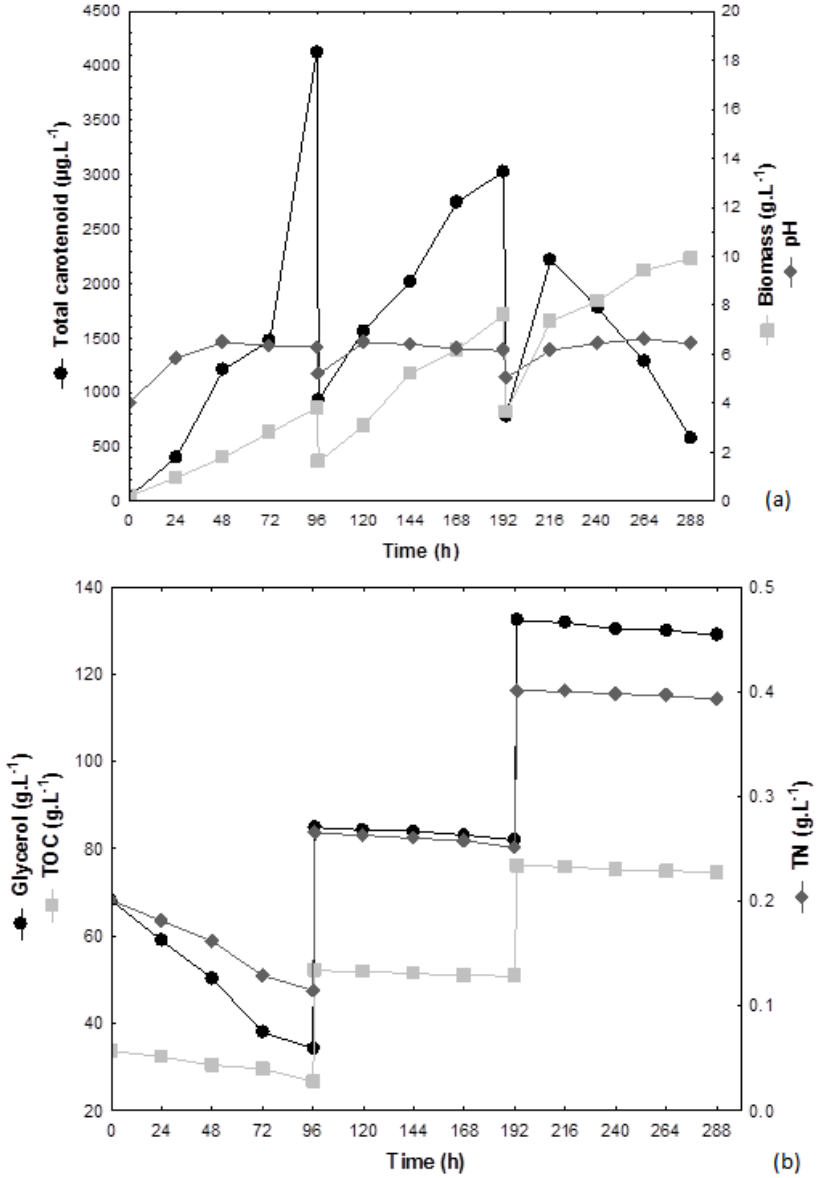

Figure 3. Kinetics of carotenoid production, biomass, and pH (a); substrates consumption: glycerol, TOC, and TN (b) semi-continuous process using agro-industrial residues with the cut of 75%, every 96 h, until 288 h.

In the cut of 75%, the volume of culture medium replaced at each beginning of the cycle was greater, that is, a greater amount of nutrients was available to the cells. However, it did not increment the cell growth (maximum 9.9 g/L). This can be explained by the time that the microorganism needed to adapt and grow in this new medium. Comparing the cuts of 25% and 50%, it is observed a lower amount of biomass by 25%. This may have occurred due to the small number of possible inhibitors and/or toxic metabolites removed from the fermented broth in the semi-continuous process. At the end of the bioproduction, there was a high concentration of total organic carbon (98.1%), glycerol (97.4%), and nitrogen (98%), indicating that there was no lack of nutrients. Microelements play an important role in cell metabolism, mainly due to their requirements as cofactors for various enzymes [25]. Deficiencies or high concentrations of minerals cause significant metabolic changes [26], increasing biomass production.

Figure 4 shows the global carotenoid production in the semi-continuous process with different cuts (25, 50, and 75%) during the cultivation. In 96 h, it is observed similar

productivity for both cuts (approximately 40 µg/L. h). In the bioproduction, it is observed that the cut of 50% maintained a superior behavior in relation to the other tests. However, after 96 h, carotenoid productivity showed a progressive decrease. Colet *et al*. [21] using the *S. salmonicolor* yeast in a semi-continuous process with a cut of 50%, obtained similar productivity (41.4 µg/L. h) in 48 h bioproduction.

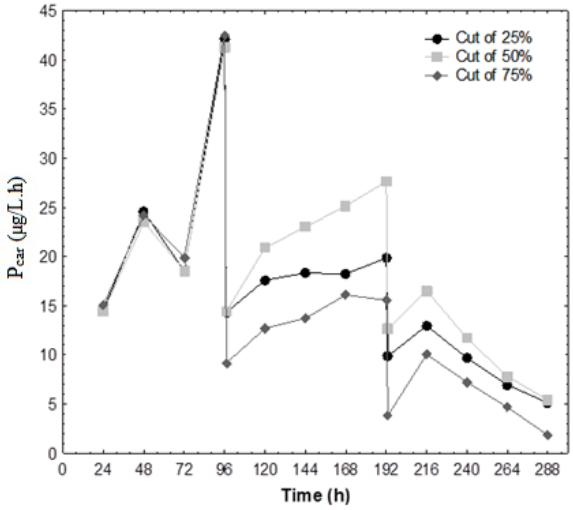

Figure 4. Global carotenoid production (P_{car}) on the semi-continuous process using agro-industrial residues, with a cut of 25%, 50%, and 75% at 1.5 vvm, 180 rpm, 25ºC, $pH_{initial}$ of 4.0, and 480 h.

Table 1 shows the kinetic and stoichiometric maximum parameters obtained for *P. rhodozyma* in the semi-continuous bioproduction process.

Table 1. Kinetic and stoichiometric maximum parameters of carotenoid production in a bioreactor from tests in the semi-continuous process using agro-industrial residues.

Parameters	Maximum obtained value	Cut (%)	Time (h)
$Y_{P/G}$ (µg/g) (Glycerol base)	847	50	193
$Y_{X/G}$ (g/g) (Glycerol base)	2.65	50	193
$Y_{P/C}$ (µg/g) (TOC base)	15343	25	168
$Y_{X/C}$ (g/g) (TOC base)	31.55	25	168
$Y_{P/N}$ (µg/g) (Nitrogen base)	362310	50	216
$Y_{X/N}$ (g/g) (Nitrogen base)	1740	25	193
$Y_{P/X}$ (µg/g)	1210	25	96
P_x (g/L.h)	0.063	50	96
μ_x (h^{-1})	0.067	50	24

It was observed that the maximum specific speed of growth (μ_{max}) of 0.067 h^{-1}, with maximum productivity in cells (P_x) of 0.063 g/L. h, and the productivity in total carotenoids of 42.51 µg/L. h, using the cut of 50% in 96 h of bioproduction. In this way, using the semi-continuous process was possible to improve the productivity of natural pigment using agro-industrial residues. The obtained carotenoid can be used by biotechnology and food industries as additives or supplements in foods, cosmetics, pharmaceutical, among other applications.

4. Conclusions

The kinetic bioproduction study in a semi-continuous system, with a cut of 50%, showed a high concentration of total carotenoids of 5346 µg/L (477 µg/g of specific carotenoids) in 192 h. The μ_{max} for *P. rhodozyma* Y-17268 in a semi-continuous system bioreactor was 0.067 h^{-1}, with maximum cell productivity of 0.063 g/L. h. By the results

obtained in the bioproduction of *P. rhodozyma* carotenoids, the semi-continuous process with a 50% cut and 192 h would be recommended for increasing the industrial scale, thus leading to an increase in production and consequent increase in yield. In this way, this fermentation process is very available using agro-industrial residues and is an important strategy for obtaining and recovery of pigments.

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Conflicts of Interest

The authors declare no conflict of interest.

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