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Fed-batch carotenoid production by Phaffia rhodozyma Y-17268 using agroindustrial substrates

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ABSTRACT

This work aim the carotenoid bioproduction by the yeast *Phaffia rhodozyma* Y-17268 in a fed-batch bioreactor with different low-cost agroindustrial substrates (crude glycerol, corn steep liquor, and rice parboiling water). The maximum concentration of total carotenoid and cell productivity were 4118 µg/L (835 µg/g) and 0.05 g/L. h, respectively, with a feed volume of 75 mL every 12 h. The medium were composed of 100 g/L crude glycerol, 100 g/L corn steep liquor, and 20 g/L rice parboiling water at 25ºC, $pH_{initial}$ 4.0, agitation rate of 250 rpm, aeration rate of 1.5 vvm and 96 h of bioproduction. 0.188 h^{-1} of maximum specific growth speeds (μ_{max}) was obtained for the major carotenoid - (all-E)-β-carotene (75.9%). Thus, the yeast *P. rhodozyma* produced in a fed-batch bioreactor demonstrated a great potential to produce the β-carotene.

Keywords: *β-carotene; bioreactor; substrates; low-cost; productivity.*

1. INTRODUCTION

The large demand for resources generated by industries and a growing consumer´s request for natural products. The natural pigments most studied are the carotenoids, responsible for the yellow, orange, and red colors of some fruits, vegetables, flowers, fish, crustaceous, bacteria, and fungi [1]. The carotenoids production via biotechnology can be obtained by a diversity of microorganisms, such as bacteria [2], yeasts [3–5], microalgal [6], and filamentous fungi [7].

The worldwide market for Natural Carotenoids presents an expectation of growth of roughly 3.2% according to the Compound Annual Growth Rate (CAGR) over the next 5 years, will reach 910 million US$ in 2024, from 750 million US$ in 2019 [8]. The most common consumed carotenoid by humans and animals is the β-Carotene, however, it still has a high production cost. The substrates (raw materials) used in the bioprocess represent around 30 - 70% of the production cost [9], that make the process expensive.

Therefore, an alternative to minimize the production cost is the biosynthesize from inexpensive nutrient sources as agroindustrial byproducts (substrates) containing raw materials with mono and disaccharides (some exemples are the rice parboiling water, corn steep liquor, molasses, whey, crude glycerol, among others) [3]. These agroindustrial byproducts mitigating the disposal problem of agricultural activities from agroindustry wastes, transforming into valuable material for biotechnological process. The agroindustrial byproducts can increase the process economic viability for being a source of nutrients like nitrogen, carbon, and minerals. Studies report the efficient use and viable economic point of view of substrates and their derivate such as glycerol [10,11], sugar cane [12–14], corn [15–17], among others. Fed-batch processes are reportedly effective and versatile in the most fermentation processes, including the carotenoid production. In such processes, especially those with high cell densities, the productivity is improved, due to the high viable cell counts. The fed-batch mode enhances the control of substrate concentration while minimizing substrate inhibition [18].

In a fed-batch fermentation procedure, a controlled substrate feeding profile can provide by the amount of glucose concentration in the mixture. Monitoring feeding of the substrate allows to overcome the inhibitory issues (mainly related to the osmotic stress), and sometimes, it can also restrict the cell's growth rate and so, avoid the limitations in oxygen and heat transfer [19].

The yield can be increased (carotenoid productivity) changing the physicochemical and nutritional parameters in a bioreactor. The change of the substrate concentrations presents an effect on cell biomass and metabolite production in fed-batch fermentation. Therefore, the optimization of carotenoid production is very important to reduce costs and maximize the productivity [3].

In this sense, this work aims to use the fed-batch strategy using corn steep liquor, crude glycerol, and rice parboiling water as substrates for enhanced carotenoid bioproduction by *P. rhodozyma* (Y-17268) in a bioreactor fermentation.

2. MATERIALS AND METHODS

2.1. Conditions of cultivation and carotenoid bioproduction.

Y-17268 was provided by the Bioprocess Engineering Laboratory, (FURG, RS/Brazil), certified as GRAS (Generally Recognized as Safe). The conditions of inoculum preparation were described previously [5]. This yeast was used for the carotenoid bioproduction in a bioreactor.

2.2. Agro-industrial byproducts.

The agroindustrial byproducts (substrates) used for the carotenoid bioproduction in a bioreactor were corn steep liquor donated by Corn Products, Mogi Guaçu/SP/Brazil, parboiled rice water acquired from Industrial Nelson Wendt - Pelotas/RS/Brazil, and crude glycerol (by-product of the conversion of oils into biodiesel) acquired from industry (Olfar – Erechim/RS/Brazil).

The corn steep liquor was prepared according to described by Valduga et al. [12] at a concentration of 100 g/L, and pH 3.0 (adjusted with 1 mol/L phosphoric acid). The agro-industrial byproducts were centrifuged (Eppendorf 5403) at 5000 rpm for 15 min at 24°C, and the pH value adjusted to 5.5 (2 mol/L sodium hydroxide).

2.3. Carotenoid bioproduction in fed-batch bioreactor.

In the fed-batch bioproduction was employed optimized medium [5] composed of crude glycerol (100 g/L), corn steep liquor (100 g/L), and rice parboiling water (20 g/L). An initial volume of 500 mL of the medium was autoclaved in the Biostat bioreactor (Braun Biotech International) before star the fermentation process. The feed rate for the carotenoid bioproduction employed varied from 50, 75, 112.5 and 150 mL of medium, each 12 h. The operational conditions used were based on our previous work [5]: 1.5 vvm of aeration rate-air with 180 rpm stirring rate, 25°C, and 4.0 $pH_{initial}$ for 96 h bioproduction.

2.4. Recovery of total carotenoids.

Recovery of total carotenoids followed the steps of lyophilization, cell disruption with dimethyl sulfoxide (DMSO, Quimex, Brazil), successive extractions with propanol (Quimex, Brazil), and centrifugation (MPW-351R refrigerated Laboratory Centrifuge) until cells were colorless. Next, for phase separation, DMSO removal and excess water were employed sodium chloride solution and petroleum ether.

2.5. Kinetics of the carotenoid bioproduction.

The kinetics for total cell mass, carotenoid production, pH evolution, substrate consumption (glycerol, total nitrogen – TN, and total organic carbon - TOC in the medium) was followed by periodic sampling of the medium (12 in 12 h for a total period of 120 h). The experiments were performed in triplicate (n = 3).

The conversion factor for the substrate in the product were determined in the different feed rate in the bioreactor at fed-batch mode as: $Y_{p/s}$ (μg carotenoids/g substrate), substrate in the biomass, $Y_{x/s}$ (g cells/g substrate), and the specific carotenoid production, $Y_{p/x}$ (μg carotenoids/g cells). The rates of microbial growth (r_x), product formation (r_p) and substrate consumption (r_s) were determined by the mass balance for each component at a given time.

The specific rate for growth (μ_x), product formation (μ_p) and substrate consumption (μ_s) were obtained dividing the instantaneous rate by the cell concentration.

2.6. Analytical methods.

The total carotenoids were estimated by the maximum absorbance at 474 nm in a spectrophotometer using the extinction coefficient (β-carotene $A^{1\%}_{1cm}$=2592, for petroleum ether). The concentration of carotenoids was expressed in specific carotenoids (μg/g) and total carotenoid bioproduction (μg/L). The total carotenoid bioproduction of represents the production of lyophilized cells (g) in relation to a fermented medium (1 L).

After extraction, the carotenoid cells were washed several times with distilled water, next dried at 105°C until a constant mass. The cell mass was determined by weight difference.

In the carotenoid bioproduction, the pH evolution was determined using a pH meter, and the total organic carbon (TOC) and total nitrogen (TN) in the medium by the catalytic combustion method, using a total organic carbon/total nitrogen analyzer (Shimadzu model TOC-VCSH, China). The determination of glycerol followed the standard method UNE-EN 14105 [20].

The separation, identification, and quantification of the compounds were performed by high-performance liquid chromatography with diode array detector coupled with Chemical Ionization at Atmospheric Pressure with a mass spectrometer (HPLC -DAD/APCI-MS). For the determination of the carotenoids a Shimadzu model 20-AD liquid chromatograph with an automatic injector, mobile phase degasser DGU-20A3 heating furnace CTO-20A and diode array detector SPD-M-20A coupled to a mass spectrometer (Bruker model Esquire 6000) with Ionization at Atmospheric Pressure source (APCI-MS).

For separation, a C_{30} YMC column (5 μm, 4.6 × 250 mm, Waters, USA) was used. The conditions of the chromatographic method were flow rate of 0.9 mL/min and oven temperature 29°C. The linear gradient used was methanol/methyl tert-butyl ether went from 95:5 to 70:30 in 30 min, to 50:50 in 20 min, maintained at 50:50 for 10 min and finally modified to 95:5 in 2 min. Mass spectra were acquired with a scan range from 100 to 1500 m/z, and also by monitoring the characteristic ions of the studied compounds. The MS parameters were as follows: APCI source in positive mode; 4000 nA, source temperature: 450°C, dry gas: N_2 with a temperature of 350°C and a flow of 5 L/min, nebulizer: 60 psi. The compounds were identified on the basis retention time (chromatographic data, the UV–visible spectra features, such as spectral fine structure (% III/II) and intensity of cis peak (% A_B/A_{II}). The tentative identification of the compounds was possible by comparing the generated mass spectra (protonated molecule ($[M+H]^+$) and its MS/MS fragments)and the order of elution of the compounds in the system with data from the literature. The percentage of each carotenoid was calculated considering the total area of all identified compounds.

2.7. Statistical analysis.

The results were obtained in triplicate and treated by analysis of variance (ANOVA). The mean values were compared using the Tukey's test, by Statistica® software (Statsoft, Tulsa, USA, version 5.0).

3. RESULTS

Figures 1 and 2 show the kinetics of cell growth (biomass), total and specific carotenoid bioproduction, pH evolution and the consumption of substrates in bioreactor fed-batch with different feed volumes (50, 75, 112.5, and 150 mL) every 12 h.

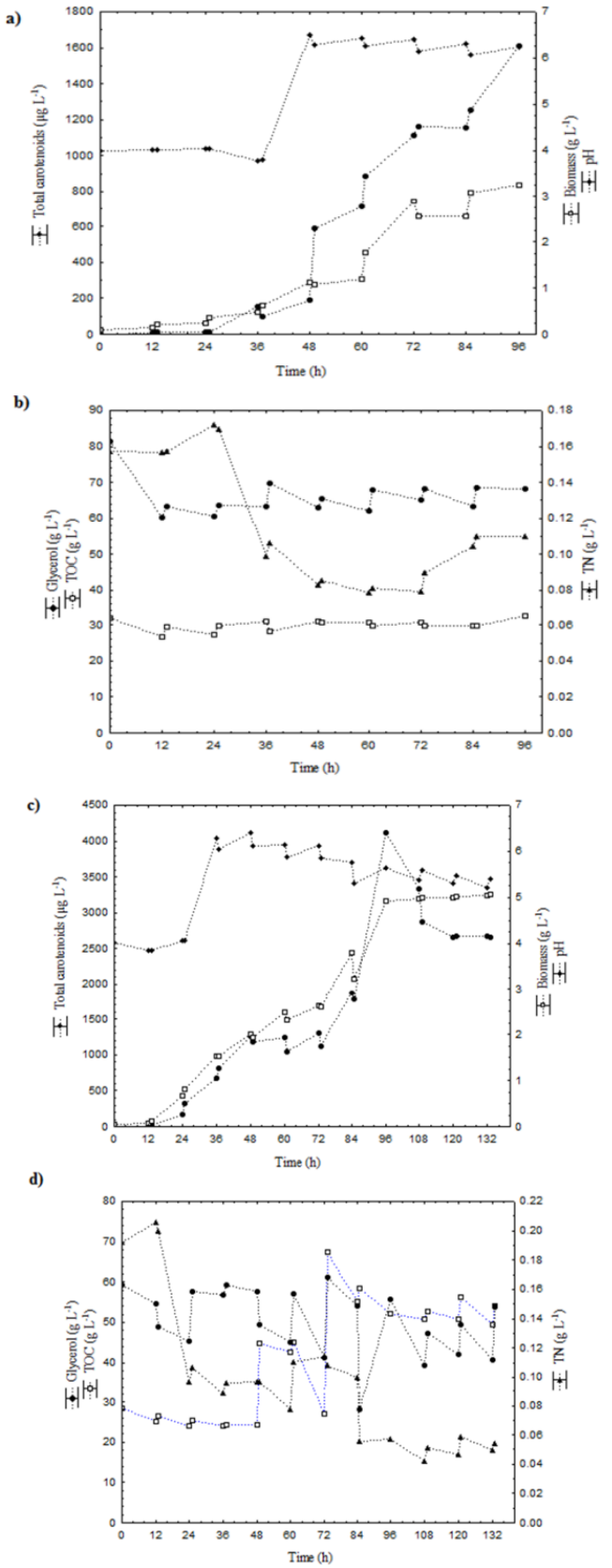
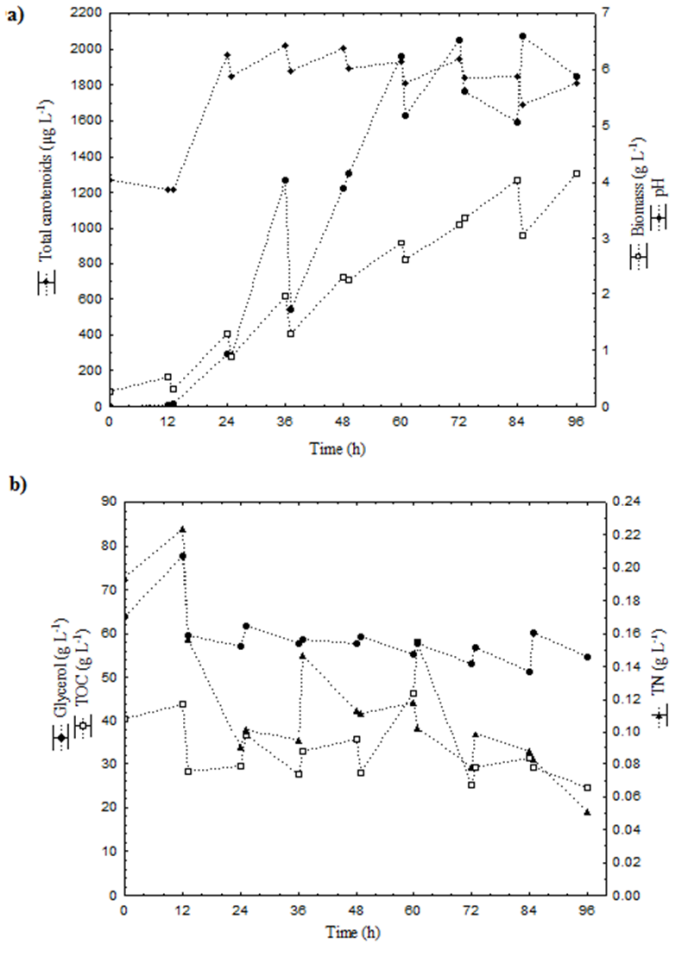


Figure 1. Kinetics of total carotenoid, biomass, pH and substrates consumption: glycerol, TOC and total nitrogen in fed-bath system with different feed volumes 50 mL (a - b), and 75 mL (c –d), every 12 h.

The maximum carotenoid concentration was 4118.18 µg/L in 96 h of bioproduction, with 75 mL feed volume, every 12 h (Figure 1c, Table 1). However, increasing the feed volume to 150 mL, every 12 h, in 96 h of bioproduction (Figure 2c), resulted in a carotenoid concentration of 2985.42 µg/L. This production was ~ 1.4 times lower than obtained with 75 mL feed volume, every 12 h. This may be related to inhibition by the excess of the substrate. Excess nutrients added stabilized the bioproduction, being observed by the high amount of residual TOC (Figure 2d).

Correlating the fed-batch yield with the optimized single batch obtained by Urnau et al. [5] was verified an increase of approximately 140%. Thus, demonstrating that the controlled addition of nutrients (corn steep liquor, rice parboiling water, and crude glycerol) and consequent substrate assimilation resulted in high production of biomass and total carotenoids. Therefore, the low-cost and nutrient-rich raw materials and substrates were better used in the bioproduction and it is a viable alternative to industrial by-products reuse. In another work of the group using semi-continuous process to carotenoid production using different yeast the *Sporidiobolus salmonicolor* it was verified that the corn steep liquor substrate presented the highest levels of nitrogen and minerals (sodium, magnesium, iron, zinc, and copper), and the glycerol has the highest concentration of TOC. Which is important nutrient for the cell metabolism and cofactors for various enzymatic reactions [3,21].



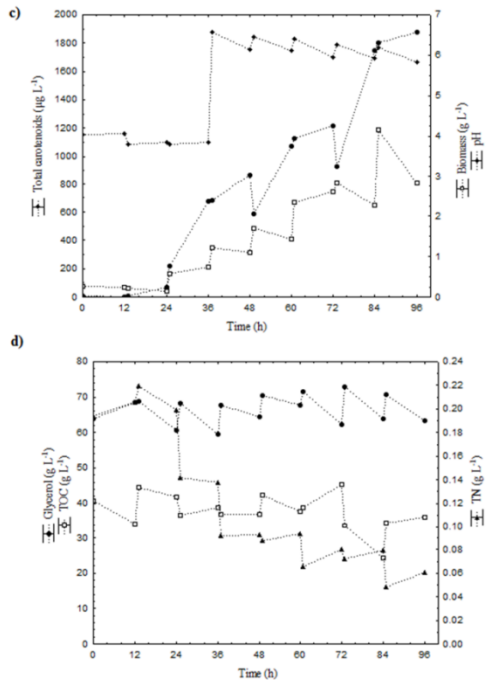


Figure 2. Kinetics of total carotenoid, biomass, pH, and substrates consumption: glycerol, TOC and total nitrogen in fed-bath system with different feed volumes 112.5 mL (a - b), and 150 mL (c –d), every 12 h.

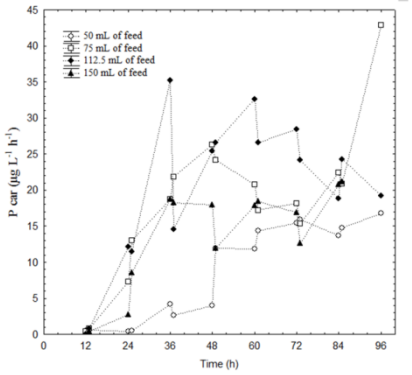


Figure 3. Global total carotenoid bioproduction (Pcar) (a) cell carotenoid conversion factor (Yp/x), and (b) bioproduction on the fed-batch process with different feed volumes (50, 75, 112.5, and 150 mL).

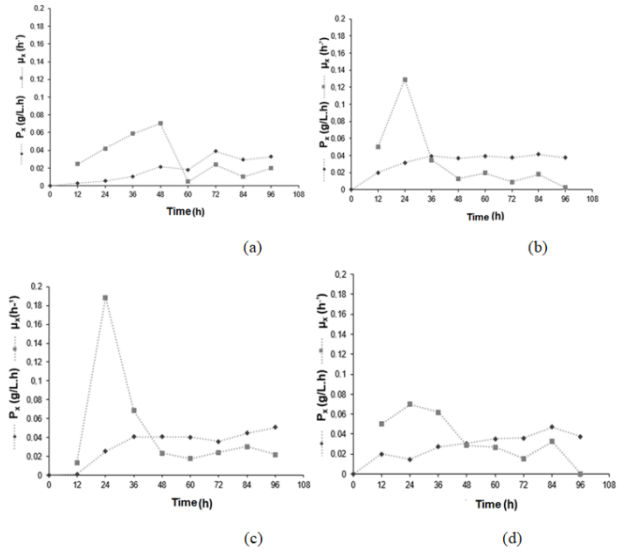


Figure 4. Specific growth rate (μx) and cell productivity (Px) during bioproduction on the fed-batch process with different feed volumes (a) 50 mL), (b) 75 mL, (c) 112.5 mL, and (d) 150 mL.

Carotenoids concentration can be related to the culture medium composition as well as to the bioproduction system. Saenge et al. [10] in carotenoids production using *Rhodotorula glutinis* (TISTR 5159) observed an increase of 13% bioconversion of crude glycerol to lipids and carotenoids in fed-batch system when compared to the simple-batch. Colet et al. [3] verified an increased of 52% in fed-bath when compared to simple-batch system in the carotenoid production with 112 mL feed volume, every 12 h.

Figure 1 (b and d) and Figure 2 (b and d) were observed a gradual TN, TOC, and glycerol consumption during the bioproduction process. This result suggested that the yeast cells consume glycerol as a carbon source. The higher nitrogen (76%), and TOC (42%) consumption was observed in experiments using 75 mL feeding volume, every 12 h (Figure 1d) for 96 h bioproduction. Colet et al. (2019) using *S. salmonicolor* also noted higher TN (72%) and TOC (42%) consumption in the bioproduction process with 112.5 mL feeding volume for 120 h.

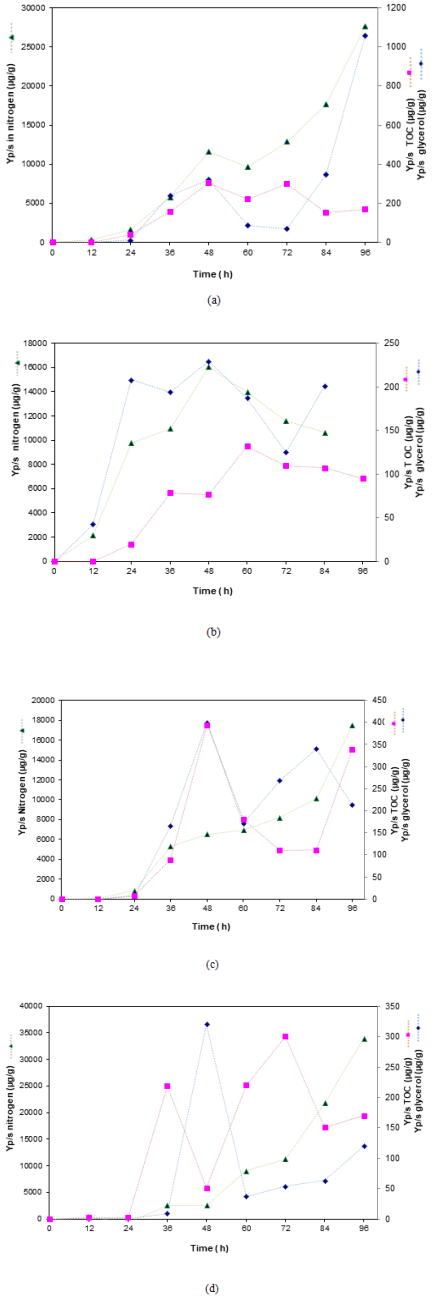


Figure 5. Glycerol, TOC, and TN conversion factors in carotenoid during the bioproduction on the fed-batch process with different feed volumes (a) 50 mL, (b) 75 mL, (c) 112.5 mL, and (d) 150 mL.

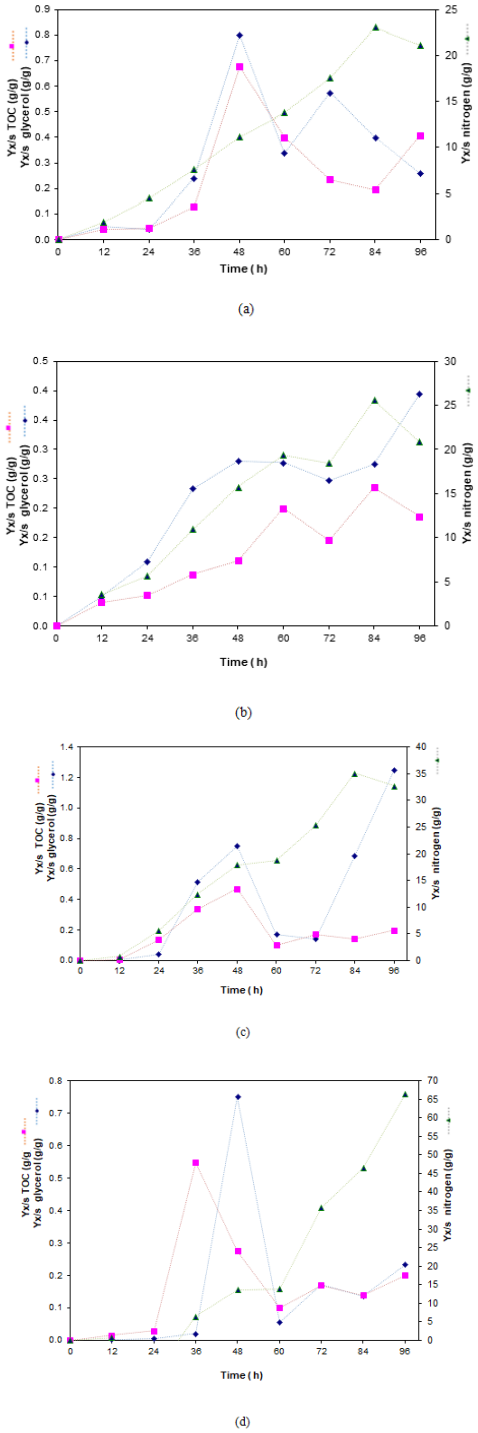


Figure 6. Glycerol, TOC, and TN conversion factors in carotenoid during the bioproduction on the fed-batch process with different feed volumes (a) 50 mL, (b) 75 mL, (c) 112.5 mL, and (d) 150 mL.

The gradual glycerol consumption during bioproduction was noted in all feed volumes studied (50, 75, 112.5, and 150 mL, every 12 h). An excess in the medium was observed in the experiments with higher fed volume (Figure 2 b and d), which can result in cellular inhibition due to substrate overload. However, excessive glycerol substrate can repress the synthesis of the carotenoids. In the highest bioproduction experiment (75 mL feed volume, every 12 h, in 96 h)had a high C/N ratio (916). The C/N ratio has a significant influence on cell growth and carotenoid biosynthesis. In other studies, was related that a high C/N ratio increased the carotenoid production yield due to the nitrogen limitation [22,23].

One of the parameters that influence cell growth and product formation is pH. Regardless of the feed volume, the pH remained constant during the first 24 h of bioproduction, when it showed an increase, and after this time small variations were observed (Figures 1 and 2, a and c). Carotenogenesis and cell growth accompanied the pH increase (4.0 to 6.5), and the maximum carotenoid production (4118.18 µg/L) was verified at pH 5.63 (Figure 1c). This may be related to the *P. rhodozyma* metabolism, a natural phenomenon that happens after high consumption of substrate. The increase in the pH level can be due to the ammonia formation as a consequence of amino acids degradation [21]. Colet et al. [18] observed in fed-batch system an increase of pH value, regardless of the fed volume. The authors observed an increase up to 7.68 in the first 24 h of bioproduction, after a progressive decrease occurred until to 4.0 at the end of the process.

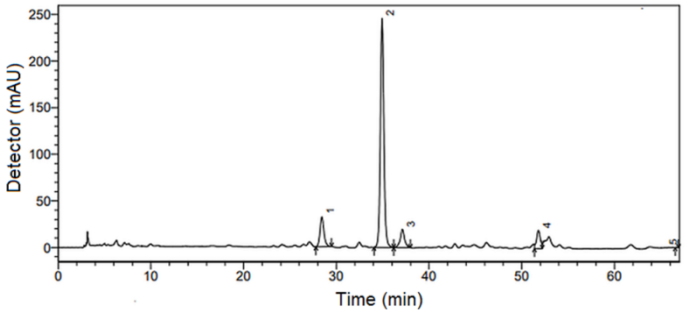


Figure 7. HPLC-DAD chromatogram of carotenoids obtained by fed-batch process.

Bioproduction by *P. rhodozyma* presents carotenoid formation partially associated with cell growth. In the experiment with 75 mL feed volume, every 12 h (Figure 1c), the highest cell concentration (4.93 g/L) was observed in 96 h of bioproduction. This increase in concentration when compared to the single batch (~15%) [5] is due to the periodically feeds. In this way, the cells can use larger sources of the substrate (carbon and nitrogen) and maintain its production of total and specific carotenoids.

The maximum yield was 42.87 µg/L.h at 96 h (Figure 3). The maximum $Y_{P/X}$ conversion factors (Figure 3b) were 888.82 µg/g at 36 h and 828.15 µg/g at 96 h of bioproduction, with feed volumes of 112.5 mL and 75 mL, every 12 h, respectively.

Maximum cell yield (Px) was 0.05 g/L.h at 9 h with 75 mL feed volume, every 12 h (Figure 4b, Table 1), with maximum specific growth rate (µmax) of 0.18 h^{-1}. With high substrate concentration, the microorganism grows at maximum velocity (µmax), but when it decreases without substrate inhibition, µ decreases to zero (substrate depleted), as shown in Figure 4.

Luna-Flores et al. [24] evaluated the *X. dendrorhous* carotenoids production in fed-batch and obtained 0.126 h^{-1}µmax, a similar value obtained in the present work with feed volume of 75 mL, every 12 h (Figure 4).

The highest conversion factors of glycerol, TOC and total nitrogen in carotenoids (Figure 5, Table 1) were 1058.1, 394.57, and 33938 µg/g with feed volumes of 75, 150 and 50 mL, every 12 h, respectively. The carbon source (TOC) influences the production of carotenoids, subsequently, it affects the production of acetyl-CoA. Once, the carotenoids biosynthesis starts with

conversion of acetyl-CoA in mevalonic acid, which is the first precursor for carotenoid production [25].

The highest conversion factors of glycerol, TOC and total nitrogen in cells (Figure 6, Table 1) were 1.25, 0.67, and 66.28 g/g with feed volumes of 75, 150 and 50 mL, every 12 h, respectively. Carotenogenesis is regulated in many microorganisms by nutritional factors, as nitrogen availability (total nitrogen). The consumption rates (nitrogen and carbon) has an important role in the synthesis of secondary metabolites, that determine the production extension and the type of synthesized metabolites [26].

The conversion factors (carotenoids and cells) obtained in fed-batch were higher when compared to those found in single batch [5]. Thus, demonstrating that the fed-batch was effective in converting substrate into product and cells, with more production of carotenoids. This high concentration of carotenoids was due to additional media provide in fed-bath system, also the cells are able to use more nutrients available and maintain their metabolism. Thus, it contributes to the higher cell mass concentration that was converted into total carotenoids, as well as specific ones.

Figure 7 and Table 2 show the HPLC-DAD chromatogram of the carotenoids obtained from the fed batch-system (75 mL feed volume, every 12 h) and the identified compounds, respectively. It is observed separation in four carotenoids, which were identified based on elution order, retention time, absorption spectra, molecular ions, and generated fragments. The results of concentration (µg/g dry weight) and area quantification (%) were obtained from the β-carotene standard curve. The major compound found was (all-*E*) -β-carotene representing 76% of the total carotenoids (634 µg/g), followed by (13*Z*)-β-carotene (11.1%), (9*Z*)-β-carotene (7.4%), and γ-carotene (5.5%). The results obtained in the present study corroborate with Cipolatti et al. [27] in the evaluation of the carotenogenic profile of the extract obtained from *P. rhodozyma* that quantified the β-carotene as the major compound.

β-carotene due to the proven claim of antioxidant properties and can be used as food colorants, nutraceuticals, and cosmetics [28]. In this way, the β-carotene obtained in fed-batch bioreactor can be an alternative to industrial-scale production.

Table 1. Kinetic and stoichiometric maximum parameters of bioproduction in a bioreactor from tests in the fed-batch process with the agroindustrial byproduct.

Parameters	Maximum obtained value	Feed rate (mL)	Longest conversion time (h)
$Y_{P/S}$ (µg/g) (Glycerol base)	1058.1	75	96
$Y_{X/S}$ (g/g) (Glycerol base)	1.25	75	96
$Y_{P/S}$ (µg/g) (TOC base)	394.57	150	48
$Y_{X/S}$ (g/g) (TOC base)	0.67	150	48
$Y_{P/S}$ (µg/g) (Nitrogen base)	33938	50	96
$Y_{X/S}$ (g/g) (Nitrogen base)	66.28	50	96
$Y_{P/X}$ (µg/g)	888.82	112.5	36
P_x (g/L. h)	0.05	75	96
μ_x (h^{-1})	0.188	75	24

Table 2. Chromatographic UV–Vis mass spectroscopy characteristics and content of carotenoid bioproduction in a bioreactor from tests in the fed-batch process with the agroindustrial byproducts with feed rate 75 mL obtained by HPLC-DAD-APCI-MS/MS.

Peak	Carotenoid	Concentration (µg/g)	Retention time (min)	λmáx (nm)	Area (%)	$[M+H]^+$ (m/z)	Fragmentions (m/z)
1	(13*Z*)-β-carotene	92.93	28.4	338/443/469	11.13	537	444$[M-92]^+$
2	(all-*E*)-β-carotene	633.93	34.9	451/476	75.92	537	444$[M-92]^+$
3	(9*Z*)-β-carotene	62.04	37.1	345/447/471	7.43	537	n.d.
4	γ-carotene	46.09	51.8	460/491	5.52	537	n.d.

4. CONCLUSIONS

The maximum total carotenoids concentration and cell productivities were 4118 µg/L (835 µg/g) and 0.05 g/L. h, with a flow rate of 75 mL every 12 h. The μ_{max} was 0.188 h^{-1} with 93% the major carotenoid (all-E)-β-carotene. The use of glycerol, corn steep liquor, and rice parboiling water is a viable alternative to produce carotenoid, making the process less expensive. Also, the agro-industrial byproducts are important strategies to maximize the production and allows industrial scheduling.

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