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Raw Glycerol and Parboiled Rice Effluent for Carotenoid Production: Effect of the Composition of Culture Medium and Initial pH

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Summary

Search for naturally grown food has stimulated the biotechnological production of carotenoids. Therefore, the use of the yeast *Xanthophyllomonas dendrorhous* has been researched due to its abilities to assimilate different sources as substrates and to produce high amounts of carotenoids. Furthermore, alternative sources have been used as the culture medium to reduce costs and environmental impact. A potent carotenoid is astaxanthin in view of its health-promoting and antioxidative properties. It consists of different geometrical isomers with *trans* and *cis* configuration. In *X. dendrorhous* this carotenoid is mostly found in the *trans* form, but *cis* isomers can also be found. Carotenoid production was investigated in culture medium containing by-products such as raw glycerol (from biodiesel) and parboiled rice effluent. The effects of the culture medium components on biomass concentration and specific and volumetric productions of carotenoids were verified by the Plackett-Burman design. Cultivations were carried out with yeast *Xanthophyllomonas dendrorhous* NRRL Y-17268 at 25 °C and 150 rpm for 168 h. In this study, maximum production of carotenoids was obtained under the following conditions (in g/L): raw glycerol 10, glucose 10, yeast extract 10, malt extract 10 and peptone 1 at pH=6. Resulting specific and volumetric productions of carotenoids were 326.8 and 4.1 μg/g, respectively.

Key words: natural pigments, by-products, Plackett-Burman design, *Xanthophyllomonas dendrorhous*

Introduction

Carotenoids are some of the most important compounds found in food. These natural colorants are responsible for yellow, orange and red colors in many kinds of food and play a decisive role in their acceptability (*1*). Besides, they have several other benefits to health since they protect humans against certain forms of cancer, cardiovascular disease, cataracts and macular degeneration (*2–4*).

Rise in consumers' health awareness has made them seek functional foods, a fact that may trigger an increase in the demand for carotenoids in the food industry (*5*). The global market demand for carotenoids has grown 2.9 % per year (*6*) and is expected to reach almost 10 million tons by 2017 (*7*). However, most of the commercial carotenoids derive from chemical synthesis and cannot meet consumers' demands for natural carotenoids (*5*). Due to concerns over the use of chemical additives in food, there has been an increasing interest in carotenoids that are naturally obtained by biotechnological processes.

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A large variety of substrates and microorganisms has been tested with special emphasis on the ever-increasing interest in the production of microbial carotenoids. Therefore, the yeast *Phaffia rhodozyma* (also referred to as *Xanthophyllomonas dendrorhous*) stands out as a natural source of carotenoids (*8–12*) due to its ability to assimilate different carbon sources as substrates, heterotrophic metabolism, relatively fast growth rate, ability to reach high cell density and nutrient quality and safety as a food additive. Besides, it has been certified as a GRAS (Generally Recognized as Safe) microorganism.

The production of bioproducts with alternative substrates, such as a source of carbon and/or nitrogen, is very important. Therefore, Brazil has the advantage of having various industrial by-products that can be used to add value, decrease the risk of having them discarded into the environment and reduce costs of medium production.

The effect of the experimental design methodology on the conditions of the process that involves the production of carotenoids has been studied (*6*,*13*,*14*). In a previous study carried out by our research group, the production of carotenoids by the wild yeast *Sporidiobolus pararoseus* with the use of a complex medium containing by-products such as raw glycerol, corn steep liquor and sugar cane molasses was investigated (*15*). The production of carotenoids from *Phaffia rhodozyma* NRRL Y-17268 was investigated in a culture medium containing pure glycerol and raw glycerol as additional carbon sources (*8*) and parboiled rice effluent as nitrogen and phosphorus sources (*16*). Moreover, carotenoids produced by this yeast strain had antioxidant activity (expressed as Trolox equivalent) of 4.00 and 3.66 mM/μg in ABTS radical cation discoloration and ferric reducing antioxidant power assays, respectively (*17*). However, the combined use of both by-products as substrates in the culture medium to maximize the production of carotenoids by an experimental design has not been studied yet.

Therefore, the effect of the concentration of culture medium containing two agro-industrial by-products, raw glycerol and parboiled rice effluent, as sources of carbon and nitrogen, respectively, on carotenoid bioproduction by *Xanthophyllomonas dendrorhous* was studied to add value to these by-products, thus possibly reducing the cost of the culture medium.

Materials and Methods

Alternative substrates

Raw glycerol and parboiled rice effluent, which were used in this study as alternative sources of carbon and nitrogen, were supplied by industries located in the south of Brazil and previously characterized (*8*).

Culture conditions and production of carotenoids

The yeast *Xanthophyllomonas dendrorhous* NRRL Y-17268 used in this research was kept in 10 mL of yeast malt (YM) agar (*18*) with the addition of 10 % (98 % by mass) analytical grade glycerol (Synth, Diadema, Brazil) and stored at –18 °C.

The inoculum was prepared by transferring 10 mL of the previously thawed microorganism suspension kept in analytical grade glycerol (–18 °C) to 90 mL of the modified YM medium (in g/L: glucose 10, yeast extract 3, peptone 5, malt extract 3 and KNO_3 0.2) (*19*). It was incubated at 25 °C and 150 rpm for 48 h or for the time required for the cell count to be at least 10^8 cells/mL, counted by a Neubauer chamber (Optik Labor, Lancing, UK) (*9*).

Bioproduction tests were conducted on an orbital shaker (model TE 425; Tecnal, Piracicaba, Brazil) with controlled stirring rate and temperature. Erlenmeyer flasks (500 mL) with 153 mL of culture medium were previously sterilized in an autoclave (model 103; Fabbe, São Paulo, Brazil) at 121 °C for 15 min. Each flask was inoculated with 17 mL of the previously prepared yeast suspension, which corresponds to 10^7 cells/mL (*20*), and incubated at 25 °C and 150 rpm for 168 h. Every 24 h 10-mL samples were collected and centrifuged at 1745×*g* for 10 min for the analysis of the supernatant (pH) and of the pellet (biomass and carotenoids).

Experimental design

In the experimental design, the effects of the concentration of the medium components and initial pH on carotenoid production were evaluated using the Plackett-Burman design, assuming that there are no interactions among the different variables (x_i) in the studied range. A linear approach is considered to be sufficient for screening, according to the following equation:

$$y=\beta_0+\Sigma\beta_i x_i (i=1,...,n) \qquad /1/$$

where y is the estimated target function, β_i are the regression coefficients and n is number of variables. The Plackett-Burman design is a fractional factorial design whose main effects may be simply calculated as the difference between the average value of the measurements made at the low level (–1) of the factor and the average value of the measurements at the high level (+1) (*21*).

The independent variables (factors) under study were raw glycerol, parboiled rice effluent, glucose, yeast extract, malt extract, peptone and initial pH (Table 1). In this study, seven variables were screened in 12 assays with three central points (Table 2). The responses or dependent variables during 168 h of study were total carotenoids (in μg/mL or μg/g) and biomass (in g/L).

Table 1. Levels of factors, concentrations of variables and pH values studied by Plackett-Burman design

Variable	γ/(g/L)		
	Level –1	Level 0	Level +1
Raw glycerol	10	25	40
Effluent	0	25	50
Glucose	1	5.5	10
Yeast extract	1	5.5	10
Malt extract	1	5.5	10
Peptone	1	5.5	10
pH	4	5	6

pH determination

The pH of the sample was determined by a potentiometer (model MB-10; Marte, São Paulo, Brazil), in accordance with the AOAC method no. 972.44 (*22*).

Recovery and determination of total carotenoids

Recovery of total carotenoids was carried out in agreement with the methodology adapted from Fonseca *et al.* (*10*). Cells were centrifuged at 1745×*g* for 10 min, dried at 35 °C for 48 h in an oven (Eletrolab, São Paulo, Brazil) and frozen (at –18 °C for 48 h) in a refrigerator (Consul, Joinville, Brazil). Afterwards, at room temperature, 2 mL of dimethylsulphoxide (DMSO; Synth, Diadema, Brazil) were added (previously heated at 55 °C for 1 h, in a water bath (model 102; Fanem, São Paulo, Brazil) to 0.05 g of dried and frozen cells and shaken on a vortex agitator (model QL-901; Biomixer, Ningbo, PR China) and left to rest for 30 min.

After cell disruption, carotenoids were extracted by adding 10 mL of acetone (Synth). Then, centrifugation was conducted at 1745×*g* for 10 min. The supernatant was separated and successive extractions were carried out until the solvent and cells were colorless. A volume of 20 mL of NaCl solution (20 % by mass per volume) and 20 mL of petroleum ether (Synth) were added. After agitation and phase separation, excess water was removed with anhydrous sodium sulfate (Synth) and total carotenoids were determined in petroleum ether as astaxanthin by spectrophotometric reading (model SP-220; Biospectro, Zhejiang, PR China) at λ=474 nm (*23*) with the molar absorption coefficient of 2100 L/(mol·cm) (*24*,*25*), according to the following equation:

$$\mathrm{SPC}=\frac{A_{474\,\mathrm{nm}}\cdot V\cdot 100}{21\cdot m} \qquad /2/$$

where SPC is the specific production of total carotenoids (μg/g), A is the absorbance, V is the filtrate volume (mL), and m is the dry cell mass (g).

Volumetric production of carotenoids (VPC/(μg/mL)) was calculated from SPC (μg/g) and biomass concentration (g/mL).

Determination of biomass

Cell count was estimated by absorbance readings at λ=620 nm (*26*). The conversion of absorbance into biomass concentration was performed using a previously determined standard curve.

Statistical analysis

A statistical analysis of the estimated effects of each variable was performed. All analyses were done at 90 % confidence level ($p<0.1$) by the STATISTICA v. 5.0 (StatSoft Inc., Tulsa, OK, USA) software.

Results and Discussion

Carotenoid production kinetics

Total nitrogen (15.2 mg/L) and phosphorus (30.7 mg/L) concentrations in parboiled rice effluent are in agreement with data found in the literature regarding this agro-industrial by-product (*27*). The carbon mass fraction (44.4 %) in raw glycerol used in this study was higher than the one (39 %) used by Chatzifragkou *et al.* (*28*) in the study of the ability of eukaryotic microorganisms to assimilate raw glycerol derived from biodiesel and convert it into products with high added value. Therefore, the concentrations of nitrogen and phosphorus essential for the cell growth of the parboiled rice effluent and the carbon mass fraction in raw glycerol influence the transformation of these materials into potential sources of such nutrients in the culture medium used in this study.

The determination of the C:N ratio is a way to explain the cell growth and carotenoid production. Results in Table 2 show that reduction of the nitrogen content stimulated the production of carotenoids (and increased the value of the C:N ratio). Vustin *et al*. (*29*) found that a C:N ratio above 5 improves the biosynthesis of carotenoids. However, this ratio, by itself, cannot explain cell growth and carotenoid production. Other nutrients, such as vitamins, amino acids and minerals, and the conditions of the process (temperature, pH, oxygen content) exert strong influence on the metabolism of yeasts (*29*,*30*). On the other hand, the trials with C:N ratio under 5 had the worst performance (trials 7, 9 and 11). Similar results were observed by Rios *et al.* (*16*) with the same microorganism in the medium containing alternative carbon and nitrogen sources, but without raw glycerol.

Table 2 and Fig. 1 show a variation in the maximum biomass concentration obtained in the different trials using the Plackett-Burman design, from 2.4 (trial 11) to 17.2 g/L (trial 10). Regarding the maximum production of carotenoids, SPC ranged from 113.5 (trial 11) to 326.8 μg/g (trial 8), whereas VPC ranged from 0.3 (trial 11) to 4.1 μg/mL (trial 8).

Promising results of carotenoid production above 3.0 μg/mL (200 μg/g) in 168 h, with the combination of the by-products raw glycerol and parboiled rice effluent at different concentrations, added to other medium components, as shown in trial 2 (in g/L: raw glycerol 40, parboiled rice effluent 50, glucose 1, yeast extract 10, malt extract 1, peptone 1, initial pH=4), trial 8 (in g/L: raw glycerol 10, glucose 10, yeast extract 10, malt extract 10, peptone 1, initial pH=6), trial 10 (in g/L: raw glycerol 40, glucose 1, yeast extract 1, malt extract 10, peptone 10, initial pH=6) and central points 13, 14 and 15 (in g/L: raw glycerol 25, parboiled rice effluent 25, glucose 5.5, yeast extract 5.5, malt extract 5.5, peptone 5.5, initial pH=5) were achieved in this study.

In trial 8 (in g/L: raw glycerol 10, glucose 10, yeast extract 10, malt extract 10, peptone 1, initial pH=6), SPC was the highest, equal to 326.8 μg/g (Fig. 1c), whereas VPC was 4.1 μg/mL (Fig. 1e). The exponential growth phase occurred up to 120 h, followed by the stationary phase up to 168 h (Fig. 1a), when maximum production of carotenoids was achieved, an evidence of the fact that carotenoid synthesis is not directly associated with cell growth of this yeast. Likewise, Chávez-Cabrera *et al*. (*11*) found that the concentration of total proteins and pigments shows opposing trends, implying that protein synthesis restriction, necessary for cell growth, stimulates carotenoid synthesis by *X. dendrorhous*. Liu and Wu (*30*) also observed that carotenoids are synthesized as secondary metabolites

Table 2. Plackett-Burman design with real values, C:N ratio and responses for biomass concentration, specific production of carotenoids (SPC) and volumetric production of carotenoids (VPC) in 168 h

Assay	X_1	X_2	X_3	X_4	X_5	X_6	X_7	C:N	Y_1	Y_2	Y_3
1	40	0	10	1	1	1	6	30.2	8.4	190.7	1.6
2	40	50	1	10	1	1	4	9.2	16.3	204.1	3.3
3	10	50	10	1	10	1	4	6.0	9.0	213.4	1.9
4	40	0	10	10	1	10	4	10.0	5.7	201.3	1.1
5	40	50	1	10	10	1	6	7.6	16.1	119.5	1.9
6	40	50	10	1	10	10	4	8.0	14.7	171.0	2.5
7	10	50	10	10	1	10	6	4.9	3.2	175.2	0.6
8	10	0	10	10	10	1	6	6.9	12.7	326.8	4.1
9	10	0	1	10	10	10	4	4.6	4.9	163.8	0.8
10	40	0	1	1	10	10	6	8.8	17.2	221.9	3.8
11	10	50	1	1	1	10	6	4.0	2.4	113.5	0.3
12	10	0	1	1	1	1	4	13.1	5.9	187.3	1.1
13	25	25	5.5	5.5	5.5	5.5	5	7.7	15.1	210.1	3.2
14	25	25	5.5	5.5	5.5	5.5	5	7.7	16.0	224.4	3.6
15	25	25	5.5	5.5	5.5	5.5	5	7.7	15.5	201.0	3.1

X_1=raw glycerol (g/L), X_2=effluent (g/L), X_3=glucose (g/L), X_4=yeast extract (g/L), X_5=malt extract (g/L), X_6=peptone (g/L), X_7=initial pH, Y_1=biomass (g/L), Y_2=SPC (μg/g) and Y_3=VPC (μg/mL)

by *X. dendrorhous*, especially when cells are under stress (nutrient limitation) and when cell growth (primary metabolism) is suppressed.

VPC (Figs. 1e and f) trend was similar to biomass production (Figs. 1a and b), since it presents a relationship between SPC and cell count.

Good reproducibility of the carotenoid bioproduction can be verified in trials 13, 14 and 15 (central points) of the Plackett-Burman design (Table 2 and Fig. 1).

The pH curves are shown in Figs. 1g and h. Depending on the composition of the medium culture and process conditions during its growth, the yeast *X. dendrorhous* can excrete carbonic intermediaries, such as acetic acid, an alcohol or an intermediate of the citric acid cycle that is subsequently reabsorbed, thus stimulating carotenogenesis (*31*) and influencing pH decrease in the first 24 h of cultivation. This behavior was better represented in trial 8 (Fig. 1g), according to the SPC peak observed (Fig. 1c) at 168 h.

Effect of the culture medium composition on carotenoid production

Fig. 2 shows the analysis of the main effects of the variables on the biomass concentration, SPC and VPC during 168 h of cultivation using the Plackett-Burman design, which was performed at a 90 % confidence level ($p<0.1$). Increasing the raw glycerol concentration from 10 to 40 g/L had significant effect on the biomass concentration and VPC, leading to an average increase of 6.7 g/L and 0.9 μg/mL, respectively. Glucose had negative effect when the medium concentration was increased from 1 to 10 g/L. It decreased biomass concentration to 1.5 g/L, but increased SPC to 44.7 μg/g.

It is likely that these effects happened because glucose and raw glycerol are metabolized quickly. Glycerol passes through the cell membrane by facilitated diffusion (*32*). Inside the cell and under the conditions without nitrogen, it causes stress to the red yeast, thus stimulating carotenoid and other secondary (as well as primary) metabolite production (*33*,*34*). Therefore, the energy for efficient acetyl-CoA and NADPH synthesis required for increased carotenoid production may derive from the catabolism of glycerol *via* glycolysis (*34*).

Bellou *et al.* (*35*) found that the use of glycerol by *Mortierella ramanniana* did not alter the amounts of biomass in comparison with other strains of *Mortierella* cultivated on multiple carbon sources. However, these fungi accumulated more interesting compounds (lipids) whose production was associated with primary metabolism during mycelial growth. In the yeast *Yarrowia lipolytica*, higher assimilation rates of glycerol than of glucose were also observed (*36*). However, they were not significantly affected by the increase in dissolved oxygen concentration (*37*).

Under aerobic conditions and easily metabolizable high levels of sugars, Deken (*38*) found that the Crabtree effect occurs and results not only in the decrease of biomass production but also in ethanol and acetic acid formation. Rivaldi *et al*. (*39*) showed that the route of glycerol assimilation is similar to the glycolysis, since the oxidation of glycerol by yeast consists in the phosphorylation of glycerol by the glycerol kinase enzyme to form glycerol-3-phosphate, which is reduced to dihydroxyacetone phosphate and is considered an important intermediate to a gluconeogenesis molecule (hexose synthesis), and to obtain a number of compounds by oxidative pathways.

Reynders *et al*. (*40*) also observed the Crabtree effect in fed-batch fermentation by *Phaffia rhodozyma*. In fed-batch culture at feed concentrations of 27 and 55 g/L of glucose, there was a good agreement between the result-

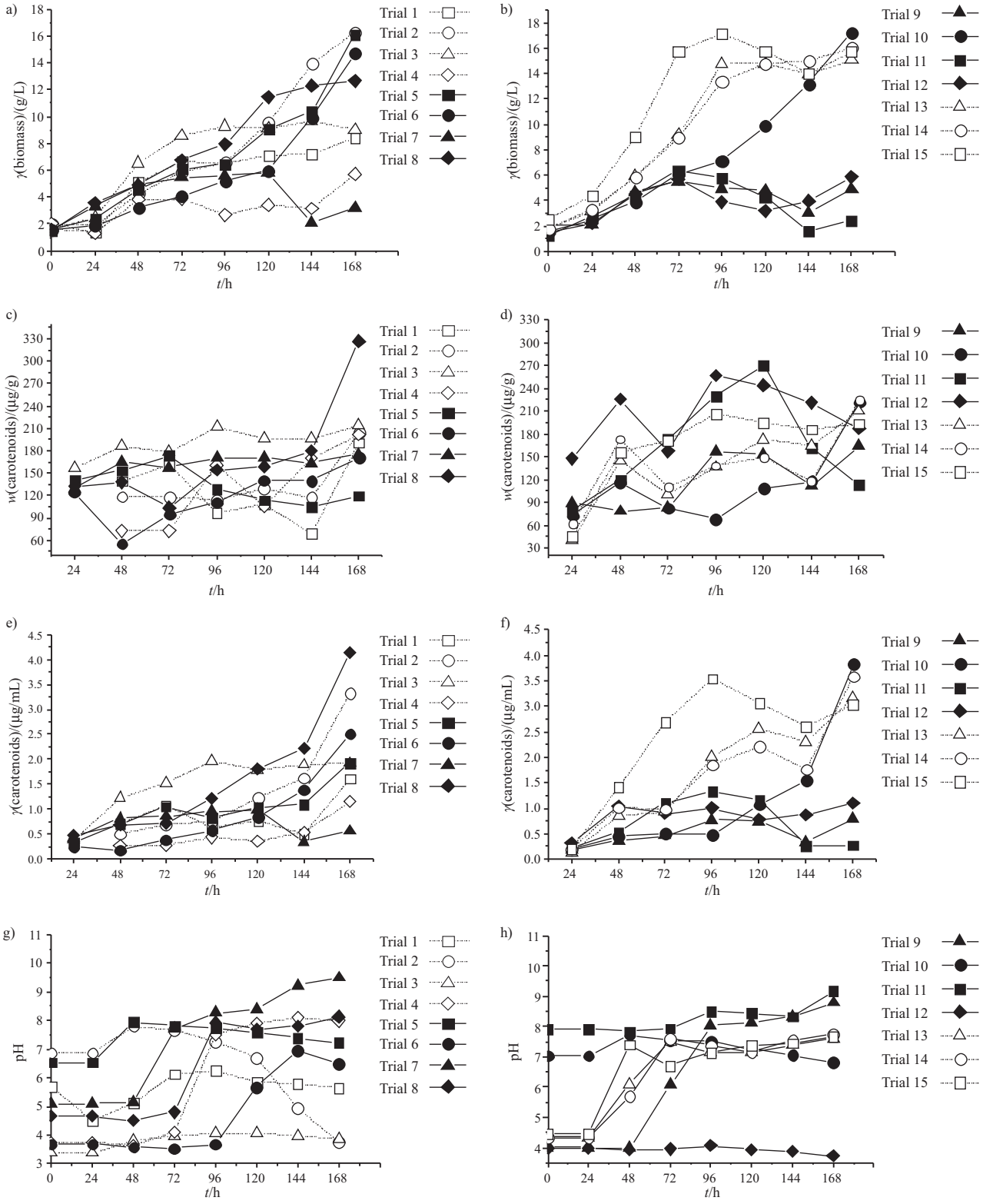

Fig. 1. Carotenoid production kinetics determined by the Plackett-Burman design at 25 °C and 150 rpm: a and b) biomass concentration, c and d) specific production of carotenoids (μg/g), e and f) volumetric production of carotenoids (μg/mL), and g and h) pH

ing biomass formation and the one expected by a mass balance model. At 125 g/L of glucose in the feed, the biomass formation was lower than the expected one and fermentation products, such as ethanol and acetic acid, accumulated in the culture medium.

With the use of 50 g/L of parboiled rice effluent, there was a significant increase of biomass concentration of 1.1 g/L and decrease of SPC of 49.2 μg/g (Table 2). Therefore, phosphorus and nitrogen found in this substrate were probably used for cell growth. This result also shows that

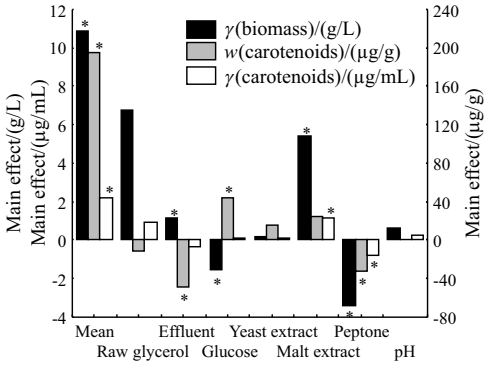

Fig. 2. Main effects of the variables on the biomass concentration (g/L), specific production of carotenoids (μg/g) and volumetric production (μg/mL) of carotenoids, *significant effect ($p<0.1$)

carotenogenesis can be stimulated by nitrogen limitation. Vustin *et al*. (*29*) suggested that nitrogen in excess in the medium culture can increase cell growth, but suppress the activity of enzymes in carotenoid conversion.

Likewise, increase in the malt extract concentration from 1 to 10 g/L resulted in the increase in the effects of all responses, namely biomass concentration, SPC and VPC of 5.4 g/L, 24.0 μg/g and 1.2 μg/mL, respectively. However, opposite effects were observed with the increase of peptone concentration, a variable that was studied at the same concentration range as the previous variable: there was a significant decrease in biomass concentration (3.4 g/L), SPC (32.5 μg/g) and VPC (0.8 μg/mL) responses. Furthermore, no significant effect on any response under study was observed when the concentration of the yeast extract increased from the –1 level (1 g/L) to the +1 level (10 g/L), as well as with the increase in the initial pH of the culture medium from 4 to 6.

Rios *et al*. (*16*) observed positive effects of the increase in malt extract concentration on carotenoid production but not of the yeast extract concentration when using *P. rhodozyma* in similar culture media without the use of raw glycerol. The yeast extract and other nutrients added to the culture medium did not influence the production of carotenoids by *Xanthophyllomyces dendrorhous* ENM 5 at 20 °C and 250 rpm. However, contrary results to the ones found in this study were obtained, with an increase in the initial pH of the culture medium from 4.5 to 6.5 (*30*). Studies show that the initial pH of the culture medium for cell growth and production of carotenoids depend on the microbial strain under study (*41*).

The production of carotenoids using agro-industrial by-products in this study was better than that obtained by Zeni *et al*. (*42*) using isolated microorganisms capable of producing carotenoids from soil, leaves, flowers, fruits, agro-industrial waste and decomposed processed products. The isolated microorganisms were cultured on an orbital shaker with 10 % (by mass per volume) inoculum (25 °C and 180 rpm for 48 h) for 120 h in the dark in YM medium. A total of 116 microorganisms were isolated (16 yeasts, 65 bacteria and 35 molds). Three yeasts showed great potential to produce red carotenoids in a concentration up to 707–818 μg/L (99–263 μg/g). One mold and two bacteria produced yellow pigments in a concentration ranging from 1063 to 2563 μg/L (239–2310 μg/g).

The Plackett-Burman design was adequate for screening medium culture variables in the production of carotenoids by *X. dendrorhous* NRRL Y-17268. Increases in raw glycerol and malt extract concentrations were the most important variables for the concomitant increase in cell growth and production of carotenoids. Increase in the peptone concentration influenced cell growth and production of carotenoids negatively. Glucose and parboiled rice effluent showed the opposite effect. The initial pH of the culture medium and the concentration of yeast extract were not significant ($p<0.1$) in the responses under investigation.

Conclusions

The use of the by-products raw glycerol and parboiled rice effluent in the culture medium for carotenoid bioproduction by *Xanthophyllomonas dendrorhous* NRRL Y-17268 under different conditions was observed. The effects of the concentration of culture medium components were verified in shake flasks by Plackett-Burman design. The maximum value was found at 168 h with volumetric production of carotenoids of 4.1 μg/mL (326.8 μg/g) in the culture medium containing (in g/L): raw glycerol 10, glucose 10, yeast extract 10, malt extract 10 and peptone 1, at initial pH=6.

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