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Production of natural astaxanthin by *Phaffia rhodozyma* and its potential application in textile dyeing

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ABSTRACT

Microbial astaxanthin are preferred over the synthetic equivalent, not only to impart color in a wide variety of products but also to provide bioactive antioxidants nutrients to human health. The yeast *Phaffia rhodozyma* is considered as one of the most important natural sources of astaxanthin. The present research work was designed to enhance the biosynthesis of astaxanthin by using synthetic substrates and moving from orbital shaker to 4 L stirred tank bioreactor (STB). Moreover, it was also evaluated the potential of astaxanthin-rich extracts in dyeing textile fabrics. Under optimal conditions in STB [carbon source (glucose/xylose) and aeration rate (1 vvm) with constant light irradiation], the process production achieved a 503.66 $\mu g/g_{DCW}$ (+ 48.99%) of astaxanthin compared to the orbital shakers (256.88 $\mu g/g_{DCW}$). The *in vitro* antioxidant activity and potential cytotoxicity assessment of astaxanthin-rich extracts was evaluated, indicating the potential of astaxanthin to be used as a natural dyeing agent for textiles, envisioning the replacement of synthetic colorants by natural counterpart.

1. Introduction

Currently, the dyes and pigments market demand are fulfilled by chemical synthesis using petroleum-based feedstocks [1–3]. However, the consumer chemophobia, environmental concerns, structural instability and racemic mixtures of artificial colorants have created the need for alternative pathways of natural colorants production [4,5]. In nature, colorants are extensively produced by several microorganisms, such as yeast, microalgae, bacteria, among others [6]. The heterobasidiomycetous yeast *Phaffia rhodozyma* sexual form, *Xanthophyllomyces dendrorhous*, has been widely investigated as a potential source of high-value added natural colorants also called biocolorants, *cf*., astaxanthin (the major carotenoid produced) and β-carotene [7], with great potential for textile dyeing due to their antibacterial and antioxidant functions [8,9]. In fact, the most textiles available in the market do not have these properties and due to that the materials are always contaminated by different microorganisms that can cause some allergic reactions, dermal infections or some bad odors [10]. The antioxidant properties of textiles have been less reported in the literature; however, there are some studies in the literature reporting [11] the ability of textiles incorporated with antioxidants to scavenge free radicals caused by skin degeneration, and protection of skin against oxidative stress. Nowadays, the textile production is among the most pollutant industrial activity, requiring new technologies to be applied throughout the textile production chain to minimize the environmental impact and increase the sustainability of the process [12]. Yeast-based biocolorants can replace synthetic ones in textile staining [13], however to address this challenge, previous improvement of upstream and downstream procedures is required.

The production of astaxanthin by *P. rhodozyma* (~300 $\mu g/g_{yeast}$) is lower compared to the microalgae counterpart (*Haematococcus pluvialis*) (~1100 $\mu g/g_{cell}$) [14,15], however despite the low production yields, the yeast has several advantages, *viz*., metabolic versatility, easy cultivation, no seasonal limitation, low nutritional requirements, shorter production time, among others [1]. Besides these relevant advantages, the commercial production of carotenoids, particularly astaxanthin by

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P. rhodozyma is still hampered by several factors, including the high production costs (expensive downstream processing), instability of strains (tendency to form mutants), and low biochemical conversion of β-carotene into astaxanthin (lack of metabolic studies) [16].

Therefore, the improvement of natural yeast-based astaxanthin production by *P. rhodozyma* are challenging to the industry. Several research activities were performed in order to reduce the production costs and increase the astaxanthin production yield using this microrganism ([16–20], and in most of the cases, the researchers have used the breeding high-yield strains (by mutation method), that can affect the physiology and metabolic disorder in the strains and genetic instability [21]. To overcome this drawback, the production of astaxanthin by *P. rhodozyma* can be stimulated optimizing the process parameters such as culture media, pH, temperature, agitation, among others. The implementation of microbial stress induction in the submerged cultivation also plays a pivotal role on cell growth and high astaxanthin accumulation in *P. rhodozyma,* that could reduce significantly the production cost [22,23]. As for example, Parajó et al., [24] evaluated the production of carotenoids by *P. rhodozyma* growing on culture media composed of hemicellulosic hydrolysates of Eucalyptus globulus wood (containing 16.6 g/L of xylose), reaching 4.6 mg/L of astaxanthin. Following this line, in another work [25], the same authors evaluated the fed-batch cultures of *P. rhodozyma* in xylose-containing media made from wood hydrolysates reaching 7.19 mg/L of astaxanthin. The higher volumetric astaxanthin yield (2.95 mg/L) obtained for *P. rhodozyma* NRRL Y-17268 was achieved by Vázquez et al., [26] in orbital shakers using xylose as the main carbon source.

In the light of the needs for the functional properties of textile fabrics with biological properties for health care application, in this study, the influence of cultivation conditions on enhancement of astaxanthin biosynthesis by *P. rhodozyma* strain NRRL Y-17268 was assessed, as well as the evaluation of its antioxidant and toxic properties. Finally, the industrial potential of astaxanthin-rich extracts to be used as a biocolorant agent in textiles fabrics were investigated as well as their washing durability. To the best of our knowledge this is the first study reporting the application of astaxanthin-rich extracts produced by *P. rhodozyma* in textile matrices.

2. Materials and methods

2.1. Materials

The carbon sources tested were glucose obtained from Synth (Diadema, SP, Brazil) and xylose from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride was obtained from Synth (Diadema, SP, Brazil), peptone (bacteriological), malt extract and yeast extract were obtained from Kasvi (Sao Jose dos Pinhais, PR, Brazil). β-carotene and astaxanthin standards were acquired from Sigma-Aldrich (St. Louis, MO, USA). All the other reagents were of analytical grade and purchased from Exodo Científica (Sumaré, SP, Brazil).

2.2. P. rhodozyma strain and carotenoids production

The red yeast *Phaffia rhodozyma* NRRL Y-17268 (Northern Regional Research Laboratory, Peoria, USA) was used in this study. The yeast pre-inoculum was prepared in 100 mL Erlenmeyer flasks containing 25 mL of Yeast Extract–Peptone–Dextrose (YPD) medium (g/L in ultrapure water): peptone bacteriological (20), yeast extract (10), glucose (20) for 48 h at 22 °C and 300 rpm in an orbital shaker (Amerex- Gyromax 737 R, Lafayette, CA, USA). For the improvement of astaxanthin production, three different strategies were evaluated:

(i) In the first screening, the influence of glucose:xylose ratio (g/L in ultrapure water): 10:10 (media 1:1), 10:0 (media 1:0), 0:10 (media 0:1), 20:10 (media 2:1), 10:20 (media 1:2), as primary carbon sources were assessed for the production of astaxanthin, maintaining the fixed variables (g/L): malt extract (3), yeast extract (3), and peptone (5) at 22 ºC, 300 rpm for 72 and 198 h. Note that in this first approach the kinetic parameters were also assessed. For the medias 1:1, 1:0, 0:1, 2:1 and 1:2, the C/N ratios were 2/1, 1/1, 1/1, 3/1 and 3/1, respectively.

(ii) Second, to determine the effect of osmotic stress (effect of salinity on the production of astaxanthin), different concentrations of NaCl (0.5, 1.0 and 2.0 M) were added to media 2:1 and inoculated at 22 ºC, 300 rpm for 96 h.

(iii) Lastly, the effect of light on the production was tested using white light irradiation Empalux IB 14027 (40 W) (São José dos Pinhais, PR, Brazil). Tests were performed using two-different culture media: the first containing Media 2:1 + light and the second with Media 2:1 + NaCl (0.5 M) + light, at 22 ºC, 300 rpm for 96 h.

In all cases the culture media were inoculated with 10% (v/v) of yeast inoculum, and the cultures were grown in 250 mL Erlenmeyer flasks (contained 50 mL of medium) on an orbital shaker (Amerex-Gyromax 737 R, Lafayette, CA, USA) at different conditions according to the experiment. After cellular growth, the culture media containing colored cells were centrifuged at 2500 x*g* for 5 min at 10 ºC using a Hitachi CR-22 N (Tokio, Japan) the supernatant was used to determine the residual xylose and glucose content and pH, while colored cells were used to quantify dry cell weight, astaxanthin and other carotenoids content. At this stage, carotenoids were recovered from yeast biomass according to the procedure previously described by Mussagy et al. [27]. Briefly, the dry biomass was mixed with 5 mL of acetone and macerated. After that, the carotenoids-rich supernatant was recovered, and the procedure repeated until the yeast biomass become bleached. The obtained colored fractions were filtered with a Millipore® Polytetrafluoroethylene Polymer (PTFE) membrane (0.22 μm pore size), the solvents evaporated at 60 °C and 100 mbar using a Heidolph (Hei-VAP) rotaevaporator (Schwabach, Germany) and the 'total astaxanthin-rich extracts' were concentrated in 1 mL of acetone for quantification. For the antioxidant and cytotoxicity assays, the acetone was evaporated and the extracts were solubilized in the solvent used in each procedure. All the astaxanthin-rich extracts production strategies were performed in triplicate.

2.3. Carotenoid's production in stirred-tank bioreactor

The bioreactor cultivations of *P. rhodozyma* were performed on Minifors II bioreactor (Infors, New Jersey/USA) under a useful volume of 4 L equipped with two-rushton impeller, oxygen and pH electrodes, coupled with white light irradiation using illumination equipment, consisted of AVANT Led-reflector SLIM 50-BR6500K (Sao Paulo, SP, Brazil) (37.5 W ~ 50 W, a beam of light of 75 lm/W and color temperature 6500 K). The inoculum was prepared in 500 mL Erlenmeyer flasks (containing 100 mL of the YPD medium) at 25 °C, 300 rpm for 48 h. Afterwards, an 10% (v/v) of pre-inoculum was transferred to the Minifors II bioreactor containing 4 L of the cultivation medium with high astaxanthin production yields, which was composed of culture media Media 2:1 at 22 °C, 300 rpm and 1 vvm (air volume/medium volume/min) for 168 h. Before the production, the cultivation media was previously sterilized in an autoclave Tecnal®, model AV 30 (Piracicaba, SP, Brazil) at 121 ºC for 15 min. The pH (5.5) of the medium was monitored throughout all the process and 2 g of antifoam was added in the first 24 h. Samples (50 mL) were collected every 24 h for the determination of astaxanthin and other carotenoids content as presented previously (Section 2.2), xylose and glucose concentration and dry cell weight.

2.4. Antioxidant activity

From stock solution (200 μg/mL in methanol) of carotenoids extracts rich in astaxanthin, different concentrations (from 6.1 to 97.2 μg/mL)

were prepared and added to the equivalent 1.8 mL of DPPH•, then mixed and standing in dark room for 30 min at 25 °C. The absorbance of solutions was measured by Thermo Scientific® (Genesis 10 S) UV–vis spectrophotometer at 518 nm. The evaluation was performed in triplicate and the percentage reduction of the DPPH• was calculated by the following Eq. (1):

$$A_{DPPH\bullet}(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (1)$$

where $A_{control}$ is the absorbance at 518 nm of the initial DPPH• solution and A_{sample} corresponds to the absorbance after the addition of carotenoids extracts.

2.5. Cytotoxicity assay using Resazurin test

NIH/3T3 cells were seeded into a 96-wells plate (5 $\times 10^3$ cells/well) and incubated for 24 h in DMEM/FBS at 10% (v/v). Carotenoids extracts rich in astaxanthin in range concentration of 0.5–15 μg/mL using mean DMEM/FBS at 3% v/v for dilutions and incubated for 3 h. After 24 h of treatment, 20 μL of Resazurin solution (25 μg/mL in PBS) and 180 mL of DMEM phenol-red free were added and incubated for 4 h. The fluorescence analyses were carried out in the microplate reader EnSpire® (PerkinElmer, USA) with excitation wavelength at 540 nm and emission at 590 nm. The percentage of viable cells was calculated following the Eq. (2):

$$Viable\ \ cells\ \ (\%) = \frac{OD_{sample}}{OD_{control}} \times 100 \quad (2)$$

2.6. Dyeing potential of astaxanthin

To evaluate the feasibility of *Phaffia*-based astaxanthin in dyeing, three-different textile fabrics (cotton, 100%) purchased in the local market at Araraquara, São Paulo (Brazil) was selected: *i)* Textile fabric 1 (TF1) with a thickness of 0.16 mm; *ii)* Textile fabric 2 (TF2) with a thickness of 0.08 mm; and *iii)* Textile fabric 3 (TF3) with a thickness of 0.07 mm. The 3 samples of white cottons fabrics (7 ×5 cm) were prepared by washing in NaCl solution (1% w/v) for 30 min at 25 °C and drying at 50 °C. The 200 μL of astaxanthin-rich extracts were dissolved in 20 mL of ethyl acetate and used as stock solution (47.6 μg/mL). The textile fabrics were carefully dipped in 20 mL of colorant solution at room temperature (25 °C) for 48 h in dark environment. Following, the textile fabrics were removed from the colorant solution and washed with distilled water to remove the excess of colorant, and it was dipped in $CuSO_4$ solution (0.1% w/v) used as a mordent for 15 min at 25 °C [28]. To evaluate the performance of colorants incorporation in textile fabrics (with good fixation), the samples were washed for 10 min in the presence of 1% (v/v) liquid detergent Triex (Sertãozinho, SP, Brazil) and cleaned with tap water to remove released astaxanthin and left to dry at 50 °C (this procedure was repeated 6 times). The white uncolored cotton textile fabrics (TFC) was used as control.

2.7. Analytical methods

2.7.1. Dry cell weight and quantification of sugars

Dry cell weight of each sample was determined after all carotenoid's extraction procedures described below to avoid the degradation of the biomolecules. Briefly, after the carotenoid's extraction using acetone, the colorless biomass was quantitatively transferred into falcon tubes and dried in an oven at 50 °C for 48 h, and then weighed in an analytical balance (Shimadzu, model AUY220, Kyoto, Japan) [29]. The concentrations of glucose and xylose were determined using a High-Performance Liquid Chromatography (HPLC) system equipped with a refractive index RID detector (Shimadzu model RID - 20 A) and Aminex HPX-87 H column (300 × 7.8 mm) (Bio-Rad, Hercules, California, USA) at a flow rate of 0.6 mL/min and column temperature of 60 °C, with 0.005 M of H_2SO_4 as the mobile phase [30].

2.7.2. Quantification of carotenoids

After the extraction and characterization procedures, the colored acetone-rich carotenoids were quantified using reversed-phase high-performance liquid chromatography (RP-HPLC) Shimadzu® (Kyoto, Japan) with Shim-pack C_{18} (Japan), 4.6 mm × 250 mm column chromatograph using methanol: acetonitrile: dichloromethane (40:50:10, v/v/v) as the mobile phase eluting isocratically for 12 min. The flow rate was 1 mL/min at 30 °C. Astaxanthin and other carotenoids were detected using UV-Vis detector at λ_{max} 450 and 480 nm [31]. The carotenoid concentrations were obtained using pre-established calibration curves of pure astaxanthin and β-carotene as standards, providing a carotenoids concentration in μg/mL. The carotenoid concentrations in μg/g were determined based on total carotenoid production *per* gram of dry cell weight.

2.8. Colorimetric analysis

Colored textile fabrics with yeast-based astaxanthin, were subjected to the quantitative analysis of the color, evaluated by a chromameter with the CIELAB color system (Hunterlab ColorQuest XE). The color was measured considering the CIE color coordinates used to calculate chroma (C^*) and hue angle (H°): L^* [lightness from black (0) to white (100)], a^* [positive a represent red while negative is green], and b^* [positive a represent yellow while negative is blue]. The C^* value (Eq. 3) represents the purity of the color and saturation while H° value (Eq. 4) is directly related to the redness (0), yellowness (90), greenness (180) and blueness (270) [30,32]. The total color difference (ΔE) was also calculated to the samples with astaxanthin incorporated and control and after the washing procedure according Eq. 5.

$$C = \sqrt{a^2/b^2} \quad (3)$$

$$H^\circ = arctg \left(\frac{b^*}{a^*}\right) \quad (4)$$

$$\Delta E = \sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2} \quad (5)$$

2.9. Statistical analysis

All trials were performed in triplicate (n = 3) and the results compared using analysis of variance (ANOVA) with Tukey's HSD to verify significant differences of astaxanthin and others carotenoids at 95% confidence level ($p \leq 0.05$). The analysis was performed using Origin Software version 10.0 (Northampton, Massachusetts, USA).

3. Results and discussion

3.1. Improvement on astaxanthin production

As widely described in the literature, the red yeast *P. rhodozyma* is an important source of high-value added carotenoids, *viz*, natural astaxanthin produced by the biotechnological route. In this work, a preliminary study was carried out in order to establish the influence of carbon source and the profile of carotenoids produced on culture media containing the selected carbon sources. Since the ability of *P. rhodozyma* strains to metabolize several carbon sources is well described in the literature [33], two-different sugars were considered for this work: (*i*) glucose (that can be fully obtained by hydrolysis of cellulosic raw materials) and (*ii*) xylose (produced by lignocellulosic substrates) [34]. First, *P. rhodozyma* was grown at different glucose:xylose ratio media (0:1, 1:0, 0:1, 2:1 and 1:2), maintaining the fixed variables (g/L): malt extract (3), yeast extract (3) and peptone (5) at 22 °C, 300 rpm for 72 and 198 h. At this point, taking into consideration that the conversion of

β-carotene into astaxanthin requires a single enzyme (astaxanthin synthase) to introduce the oxygenated groups of astaxanthin, in which several factors including the carbon source can affect the accumulation of the target carotenoid (i.e., astaxanthin), in this work the strategy to evaluate the production of astaxanthin and β-carotene to comprehend the most efficient conversion under the selected culture media was used (Fig. 1**A-B**).

As depicted in Fig. 1**A-B**, the pH remained relatively constant during cultivations in orbital shaker (varied from 6.64 to 8.06). In the condition [glucose:xylose ratio (1:2)], the biomass yield (DCW) and carotenoids production increased at 72 and 198 h, achieving the maximum yield of astaxanthin and β-carotene: 218.71 – 225.24 μg/g_{DCW} at 72 h and 231.41 – 212.99 μg/g_{DCW} at 198 h. However, as also depicted in Fig. 1, the production of astaxanthin was observed in culture media containing glucose:xylose at ratio 2:1 at 72 h, achieving 245.59 μg/g_{DCW} of astaxanthin and 173.87 μg/g_{DCW} of β-carotene. At this point, the DCW was 9.42 g/L and pH ~7.25. The carotenoids biosynthetic pathway in *P. rhodozyma*, revealed that β-carotene is the main precursor of astaxanthin [1], thus in order to evaluate the astaxanthin ratio production, we first compared the conversion efficiency of β-carotene into astaxanthin. At 20 g/L glucose and 10 g/L xylose, the red yeast *P. rhodozyma* had astaxanthin level strike greater than others conditions, but lower β-carotene content (Fig. 1**A-B**). Note that, carotenoids are secondary metabolites synthesized during the stationary phase of yeast growth, and high content of carbons source are always desired to improve the biomass production and carotenoids metabolic pathway. Lower carbon source content in the medium (1:0 and 0:1) matched with decrease in biomass, and consequently in the low production of astaxanthin. The culture media containing a sole carbon source (glucose or xylose), in concentrations up to 10 g/L, produced low amount of both carotenoids. Cultivation of *P. rhodozyma* in xylose (10 g/L) only produces a slightly amount of astaxanthin and high β-carotene content (160.33 and 221.73 μg/g_{DCW}, respectively) at 72 h, which can be attributed to the low DCW concentration of 6.0 g/L. Different behavior was observed using only glucose as carbon source (10 g/L) at 72 h, achieving 200.04 and 142.40 μg/g_{DCW} of astaxanthin and β-carotene, respectively with 6.83 g/L of DCW.

In summary, from the results obtained in Fig. 1**A-B**, *P. rhodozyma* appears to perform better when cultivated in a mixture of carbon sources at glucose:xylose mass ratio (2:1), indicate the important role of carbon source metabolism during the microorganism cultivation. To confirm our results concerning the simultaneous consumption of glucose and xylose, the microorganism was cultivated in orbital shaker on a mixture of 20 g/L glucose and 10 g/L xylose (media 2:1) to evaluate the kinetics of carotenoids production. The samples were collected at different time intervals (24 h) and analyzed. After 48 h, the results showed glucose depletion and then xylose consumption starts (which was not unexpected), the carbon source was completely consumed after 72 h (Fig. 1-**C**). The DCW and carotenoids content increased gradually with final values of 10.08 g/L and 240.00 and 173.00 μg/g_{DCW} of astaxanthin and β-carotene respectively.

Through the cultivation of *P. rhodozyma* on the carbon source mixture (media 2:1), both sugars were assimilated sequentially (first glucose and then xylose). As depicted in Fig. 1-**C**, the microorganism growth did not present a lag phase and β-carotene is produced since the inoculation while the astaxanthin has a more pronounced production from 24 h of cultivation. Besides that, the sugar-consumption for glucose and xylose exhibited different pattern, *viz.*, the glucose and xylose were completely depleted, at different times, indicating that glucose is still the first sugar to be fully exhausted by the yeast *P. rhodozyma* followed by xylose. Similar sugar uptake behavior for *P. rhodozyma* using glucose and xylose as carbon source, has been reported by Montanti et al., [35]

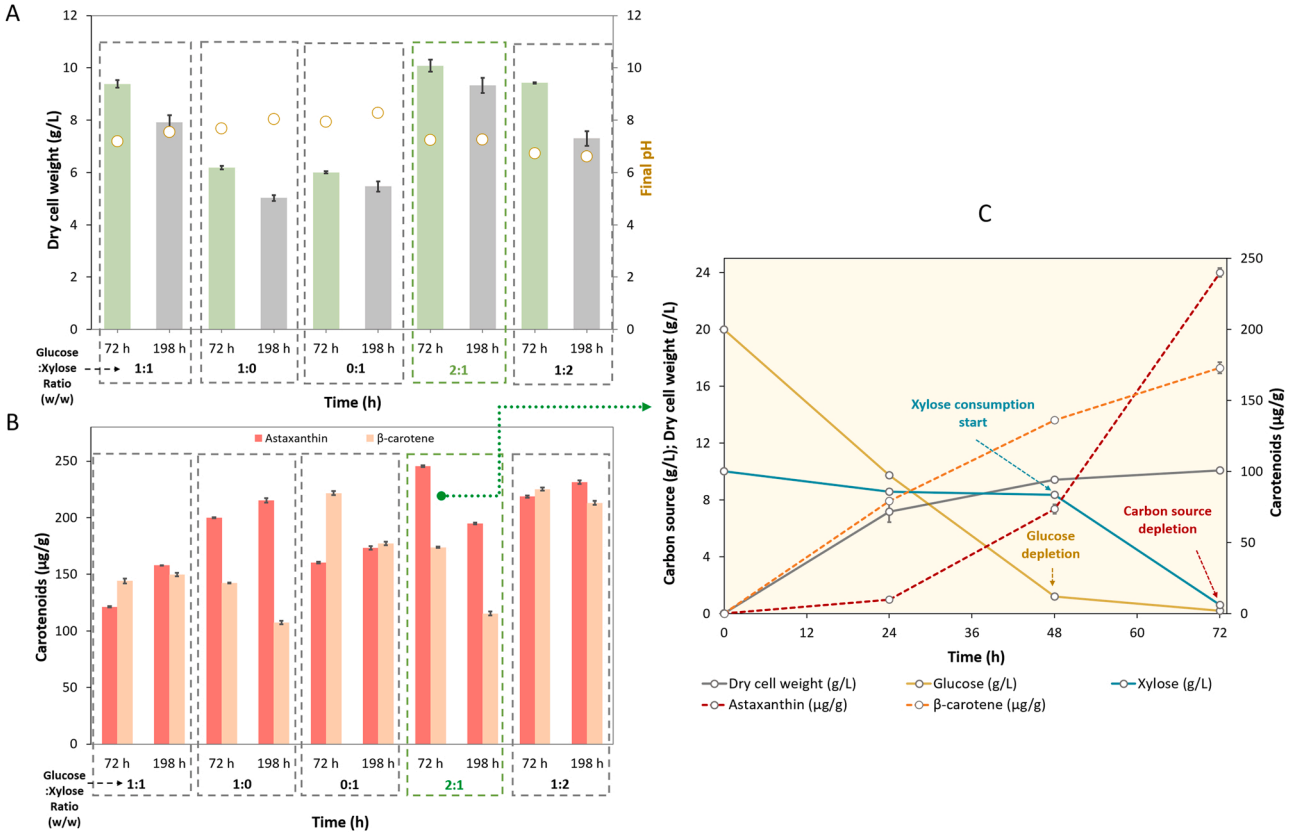

Fig. 1. A-B - Effect of carbon source (glucose:xylose ratio) on growth and carotenoid production by *P. rhodozyma* NRRL Y-17268 after incubation for 72 and 198 h at 22 °C, 300 rpm in orbital shaker. **C-** Kinetics of carotenoids production by *P. rhodozyma* NRRL Y-17268 cultivated in orbital shaker on a mixture of glucose (20 g/L) and xylose (10 g/L) at 22 °C, 300 rpm for 72 h. The data represent the means ± standard deviations of triplicate (n = 3).

using the strain JTM 185. In this particular work, the yeast used glucose preferentially, and xylose was only consumed once glucose was depleted, followed by arabinose. Stoklosa et al., [36], also indicated the preferential consumption of glucose compared to xylose by *P. rhodozyma* ATCC 74219. The results of this first approach, indicate that this strain grows more favorably in the presence of hexose sugars (i.e., glucose). It has been frequently reported in the literature that many red yeasts, including *Phaffia* strains prefer glucose as the primary carbon source over other sugars (xylose, arabinose, among others), and glucose metabolism often represses the consumption of other sugars [35]. These results clearly demonstrate that *P. rhodozyma*, including the strain NRRL Y-17268 can switch its metabolism to the other pentose sugars once glucose is exhausted, enhancing the production of microbial carotenoids, such as β-carotene which is subsequent converted into astaxanthin. The results from Fig. 1**-C,** also indicate that the DCW increase and then tends to stabilize until the full consumption of the carbon sources, and the conversion of β-carotene into astaxanthin was slower during the first 48 h. The high accumulation was observed from 48 to 72 h when glucose depletes, suggesting that the synthesis of both carotenoids is among other factors influenced by the glucose level in the culture media, i.e., the astaxanthin synthesis is improved when glucose is exhausted. In this particular case, and having in mind that carotenoids are produced in stationary phase of yeast growth, from the kinetics obtained in Fig. 1**-C,** it is clear that up to 72 h (equivalent to 24 h of the stationary phase), the maximum production of astaxanthin was not reached.

As widely reported in the literature, the stress induced by salts in microorganisms, stimulates the cation toxicity, oxidative and osmotic stress, forcing the yeast to regulate their intracellular biochemical processes (regulation of ROS, osmotic and ion homeostasis) to control or repair of damage [37,38]. In this line, in the next design, the effect of salinity on the production of astaxanthin were evaluated, first to understand the effect of osmotic stress in the production of astaxanthin, i.e., addition of NaCl at different concentrations (0.5, 1.0 and 2.0 M) in culture media (media 2:1), and second to evaluate the conversion of β-carotene into astaxanthin (Fig. 2**A**).

As shown in Fig. 2**A**, the results showed that an increase in NaCl concentration in the media significantly inhibits the growth of *P. rhodozyma*, and consequently the accumulation of low amounts of astaxanthin. Moreover, supplementation of media with 0.5 and 1.0 M of NaCl had almost no impact on the growth of *P. rhodozyma* compared to the optimized media 2:1 (Fig. S1**-A** from Supplementary material). Remarkably, the high salt conditions (2.0 M) resulted in significant growth inhibition and consequently low accumulation of both carotenoids (Fig. S1**-A** from Supplementary material). The astaxanthin yield in the sample treated with 0.5 and 1.0 M of NaCl increased by about 3 times compared to the samples containing 2.0 M after 96 h of incubation. The production yields remained in 171.81 and 173.89 μg/g_{DCW} (β-carotene) and 152.61 and 117.73 μg/g_{DCW} (astaxanthin) at 0.5 and 1.0 M of NaCl respectively, revealing that the treatment containing 0.5 M of NaCl achieved the best conversion of β-carotene into astaxanthin. Furthermore, the increase of astaxanthin accumulation on samples containing 0.5 M of NaCl in the yeast was not because the salt promoted the *P. rhodozyma* growth, but was directly related to the low concentration of salt that favored the astaxanthin biosynthesis, as previously aforementioned. The preliminary results depicted in Fig. 2**A**, suggested that the synthesis of intracellular β-carotene and astaxanthin assists *P. rhodozyma* to alleviate the stress caused by salt treatment (NaCl at 0.5 M). The synthesis of several carotenoids by microorganisms, proved to be an adaptative mechanism against several biotic and abiotic factors, including salt stress [38]. For example, the oxidative stress tolerance of modified *Saccharomyces cerevisiae* was significantly enhanced after the introduction of the *P. rhodozyma* crtS gene (to produce astaxanthin), these metabolic engineering revealed the potential for overproduction of ketocarotenoids (i.e., astaxanthin) and also breeding new oxidative stress-tolerant yeast strains [39]. In another work, the authors revealed that some oxidative stress substances (i.e., TiO_2), produced stress response in *P. rhodozyma*, leading to the accumulation of 2-fold more astaxanthin than the control group; and also proved that TiO_2 had no effect on biomass and apoptosis of the cells [16].

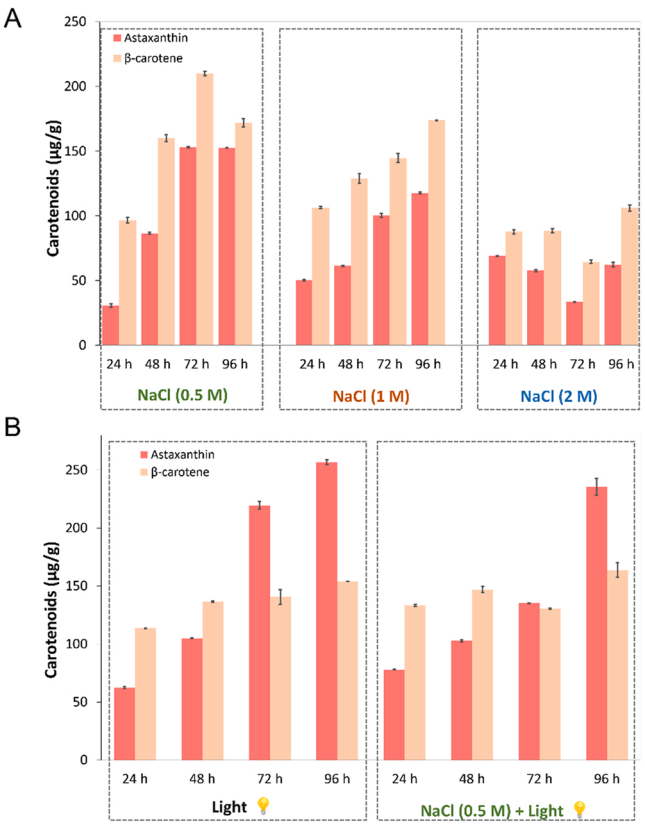

Fig. 2. Influence of NaCl (0.5, 1.0, and 2.0 M) (**A**) and light (**B**) on the production of carotenoids by *P. rhodozyma* NRRL Y-17268 in a media containing glucose:xylose (ratio of 2:1), incubated at 22 °C, 300 rpm in orbital shaker. The data represent the means ± standard deviations of triplicate experiments (n = 3).

Furthermore, associating the carotenoids production yields obtained in the initial cultivation (media 2:1) with those obtained in the presence of NaCl (0.5 M) at 72 h, no significant changes was observed in the biomass production, however an increase in the β-carotene was observed (from 171.81 to 173.89 μg/g_{DCW}), a significant reduction in the accumulation of astaxanthin was also detected (from 240.00 to 152.61 μg/g_{DCW}). Despite the benefits of osmotic stress caused by the salts on the biosynthesis of carotenoids, for this work the addition of NaCl was not very effective in relation to the conversion of β-carotene into astaxanthin. The mechanism behind the biosynthesis of carotenoids produced by *P. rhodozyma* in response to oxidative stress substances (i.e., salts) are less clear, and further research is still needed.

In order to improve the production of astaxanthin by *P. rhodozyma*, the influence of higher intensities of light on grow and carotenogenesis was also examined. Two-different culture media were evaluated: the first containing media 2:1 + light and the second with media 2:1 + NaCl (0.5 M) + light, at 22 ºC, 300 rpm for 96 h. The content of carotenoids biosynthesized by *P. rhodozyma* yeast strains was determined under the conditions of exogenous stress (Fig. 2**B**).

As depicted in Fig. 2**B**, after 96 h of cultivation under osmotic stress and white light irradiation [media 2:1 + NaCl (0.5 M) + light], the amount of carotenoid biosynthesized in the yeast biomass (β-carotene= 154.11 μg/g_{DCW} and astaxanthin= 256.88 μg/g_{DCW}) was higher compared to the previous conditions evaluated (Figs. 1 to 2**A**). The same relationship was found in the culture media composed of [media 2:1 + light], after 96 h at 22 °C. As clearly observed, the process of

astaxanthin production was more efficient under white light irradiation without addition of salt (β-carotene= 163.80 μg/g_{DCW} and astaxanthin= 235.76 μg/g_{DCW}), revealing that white light irradiation effectively stimulated the biosynthesis and conversion of β-carotene into astaxanthin by *P. rhodozyma*. A significant difference in the DCW content across both cultivations was observed, but in general the values ranged from 9 to 10 g/L at 96 h.

The use of exogenous stress factor (i.e., light irradiation) have been widely reported in the literature [40–42]. The importance of light irradiation on carotenogenesis, in particular on the production of astaxanthin is directly associated with the mechanism of photoprotection, and protection of cells against reactive oxygen species (ROS) caused by light stress [41]. Jacobson et al., [42], exposed the astaxanthin over-producing strain of *P. rhodozyma* to a continuous low-intensity light source, during the yeast maturation phase, enhancing the astaxanthin concentration by at least 2-fold or 3-fold. Stachowiak [41] evaluated the influence of illumination for *Xanthophyllomyces dendrorhous* DSM 5626 and its mutants for astaxanthin production. In this particular work, the author revealed that the application of adequate values of light close to natural (within the range of 670–718 lux), improve astaxanthin production by approximately 5–15%, depending on the strain. In the same line, Vázquez and Santos [40] related that astaxanthin produced by *P. rhodozyma,* was the main carotenoid (83.4%) produced under constant illumination (500 lux).

In summary, from the evaluated conditions of this study, it was observed that light is the most significant factor governing the process of astaxanthin production by *P. rhodozyma* as it can bring significant improvements in the conversion of β-carotene into astaxanthin. These findings suggested that *Phaffia* cells could be produced by tuning the light and color irradiation. However, there's no universal model to use light to produce carotenoids; the light color and intensity must be tailored for each strain. Furthermore, from the results of this section, the culture media composed of media 2:1 + light was the best condition for the production and conversion of β-carotene (154.11 μg/g_{DCW}) into astaxanthin (256.88 μg/g_{DCW}). Comparing the results from media 2:1 + light (96 h) (Fig. 2**B**) with those from the media 2:1 without light irradiation (at 72 h) (Fig. 1**A**), increases of 6.57% for the production of astaxanthin and decrease of 10.91% for β-carotene, respectively were achieved. It is evident that the supplementation of the cultivation medium with NaCl favors the carotenegenesis, but the production yields was very low compared to other tested conditions. The results also revealed that NaCl (2.0 M) decrease the ability of yeast to synthesize carotenoids resulted in the low production yields probably due to the inhibition of yeast growth.

Lastly, in order to provide additional information about the ability of *P. rhodozyma* to produce astaxanthin, further improvement in the production process was achieved by cultivating cells in lab scale stirred-tank bioreactor (STB). After the screening of several factors to improve the production of carotenoids using rotatory shakers, the simple culture media (media 2:1) composed of (g/L): glucose (20), xylose (10), malt extract (3), yeast extract (3) and peptone (5) subjected to a continuous white light intensity was found as the optimal conditions for the production and conversion of β-carotene into astaxanthin. The aforementioned condition was used in the cultivation of *P. rhodozyma* in the STB working volume of 4 L. The STB was operated in a batch mode for 144 h, at 22 °C, 300 rpm, air flow rate (1 vvm). Fig. 3 provides the glucose consumption, pH and DCW as well as the overall product titers for β-carotene and astaxanthin, monitored at each 24 h of cultivation.

As depicted in Fig. 3, the yeast growth started at 24 h (DCW= 3.82 g/L), reaching the maximum DCW value of 9.88 g/L after 96 h of cultivation, a value equivalent to that obtained for the media 2:1 + light (Fig. 2**B**). After 96 h, a slight decrease in DCW was observed, reaching a final DCW of 8.80 g/L after 144 h of cultivation. The decrease of DCW is expected and it is a result of the carbon source depletion. The process in STB was much faster than in the cultures using orbital shaker (Fig. 1**C**), as indicated by the total glucose-consumption rate (0.73 g/L/h), which

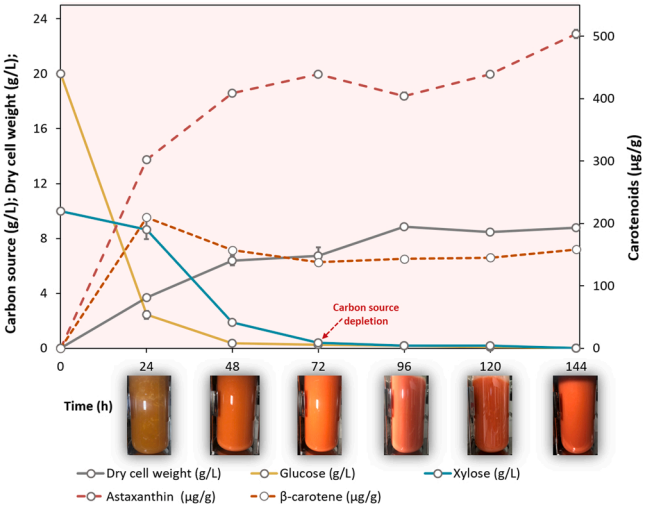

Fig. 3. Production of astaxanthin rich extracts (astaxanthin and β-carotene) by *P. rhodozyma* NRRL Y-17268, in stirred-tank bioreactor incubated for 144 h at 22 °C, 300 rpm, air flow rate (1 vvm), exposed to white light irradiation. The data represent the means ± standard deviations of quantification procedures. The error bars in some cases, are smaller than the markers.

might be the result of better control of pH, suitable homogenization and oxygen transfer rates. The cells viability is usually dependent on the carbon source, and after the carbon source depletion the DCW decrease few hours after as observed in Fig. 3. The increase of DCW content from 72 to 96 h upon depletion of the carbon sources, is related to the growth on overflow metabolites that leads to additional biomass formation. Similar results were reported by Stoklosa et al. [36], using yeast *P. rhodozyma* cultivated in sweet sorghum juice supplemented with ammonium sulfate, which revealed the decreased of biomass after the depletion of glucose, sucrose and fructose. Kusdiyantini et al. [43], also found that when the carbon source (glycerol) is depleted the biomass tends to decrease (from 3.2 to 1.2 g/L) during *P. rhodozyma* PR 190 batch fermentation. It is noteworthy that during batch cultivation of *P. rhodozyma* on the optimal defined medium, pH slightly decreases during the exponential growth phase, indicating the possible production of acid(s), *viz.*, varied from 6.54 (time zero) to 5.85 at the end of cultivation (144 h) (data not shown) but with no significant changes in the carotenoids astaxanthin content. A similar result was reported by Meyer et al. [44] using the astaxanthin overproducing *P. rhodozyma* mutant J4–3. This result can also explain the decrease in biomass during the cultivation.

Relating the production yields obtained in the cultivation in the STB with those obtained with the orbital shaker (Fig. 2**B**), a significant increase in the production of the astaxanthin was observed, *viz.,* from 256.88 to 503.66 μg/g_{DCW} (+ 48.99%) while for β-carotene, no significant difference was observed, i.e., from 154.11 to 158.46 μg/g_{DCW}. This results clearly demonstrated that the combination of carbon source (glucose/xylose), aeration rate (1 vvm) with constant light irradiation, is favorable for the biosynthesis and conversion of β-carotene into astaxanthin compared to orbital shakers, mainly because it favors the mass transfer of substrate, improving the specific metabolic pathways. Several stress factors including the lack of carbon source induce changes in the metabolic activities of a cell such as accumulation of carotenoids to protect the cells under oxidative stress, *viz.,* the synthesis of carotenoids in *P. rhodozyma* is induced when glucose depleted and under aerobic metabolism could be related to the antioxidant properties of carotenoids, especially astaxanthin [45]. As previous reported by Tsai et al. [46], under oxygen-limiting conditions, the microorganisms accumulate NADH mainly due to the reduction of oxidative phosphorylation. To preserve the NADH equilibrium on cell during the mevalonate pathway, the reduction of excess NADH molecules is required, and

it is implemented by repressing a carotenoids biosynthetic pathway which produces large amounts of NADH. Evidently, large amount of NADH is generated as a side product of the conversion of β-carotene into astaxanthin, demonstrating the suppression of astaxanthin production by an increase in NADH accumulation (in oxygen-limiting conditions) [47]. On contrary, when an adequate amount of oxygen molecule is provided to the yeast cell, the accumulated NADH is re-oxidized to NAD^+ allowing the high production titers of carotenoids. In addition, since an oxygen molecule is introduced into the astaxanthin molecule during the STB [48], the suitable aeration in the culture media is crucial prerequisite for astaxanthin biosynthesis. Probably, this is the main reason that the conversion of β-carotene into astaxanthin was enhanced with the aeration (i.e., 1 vvm) in the STB and consequently, a better oxygen transfer. As shown in the present section, which is in accordance with previous reports, adequate balance of carbon source, light and aeration, were found to have a positive effect on the conversion of β-carotene into astaxanthin. The performance of the optimal cultivation medium obtained in STB cultivation are in agreement with those obtained by the orbital shaker, validating that simple adjustment of the glucose and xylose ratio, light irradiation and controlled aeration can significantly increase the production of carotenoids by *P. rhodozyma*. The results of astaxanthin production (503.66 μg/g_{DCW}) obtained in this work, are in accordance with those obtained by Parajó et al. [49] that reported up to 571 mg of astaxanthin *per* kg biomass (571 μg/g_{DCW}) for the same *P. rhodozyma* strain NRRL Y-17268 growing on synthetic media supplemented with xylose as a carbon source and with inorganic nitrogen sources (ammonium phosphate, ammonium sulphate, ammonium nitrate or potassium nitrate). Vázquez et al. [25] have reported the high intracellular astaxanthin concentrations (up to 996 μg/g_{DCW}), in fed-batch cultures of *P. rhodozyma* in xylose-containing media made from wood hydrolysates. In this particular work, the authors improved the air flow rate (3 L/min) and agitation speed (400 rpm) and constant illumination (300 lux) provided by cool white fluorescent lamps. In this section valuable information about the use of *P. rhodozyma* to produce astaxanthin-rich extracts was discussed, revealing that this microorganism is not only able to ferment glucose and xylose but also produces carotenoids of commercial interest, mainly astaxanthin. This results also opens new opportunities to explores the possibility of using a cheap and abundantly available raw materials from industrial and agricultural origin rich in glucose and xylose to increase the conversion of β-carotene into astaxanthin. The exposure of yeast to white light, play a significant role in the induction of the carotenogenesis, confirming the antioxidative response of *Phaffia* to protect the cell integrity against the ROS generated by illumination stress.

3.2. In vitro antioxidant activity

The ability of astaxanthin-rich extracts (astaxanthin and β-carotene) to capture free radicals and reactive oxygen species is well known [8,50,51]. In this work, the DPPH• radical scavenging assay has been used to estimate the free radical scavenging activities of carotenoids extracts rich in astaxanthin produced by *P. rhodozyma* at different concentrations (from 6.2 to 100 μg/mL), and the results are depicted in Fig. 4.

The scavenging activities of DPPH• exerted by astaxanthin-rich extracts is depicted in Fig. 4. The concentration of 100 μg/mL exhibited 100% inhibition, indicating the hydrogen donating capability of astaxanthin (*i.e.,* hydroxyl and keto endings groups) presented in the extract. As expected, as lower the concentration of astaxanthin, lower the antioxidant power of the extract.

3.3. Cytotoxicity assay

The *in vitro* cytotoxicity assay of biosynthesized astaxanthin-rich extracts by *P. rhodozyma* was performed in the range of 0.5–15 μg/mL (Fig. 5). The viability of the cells treated with astaxanthin extracts (from 0.5 to 5 μg/mL) for 4 h showed a significant absence of cytotoxicity,

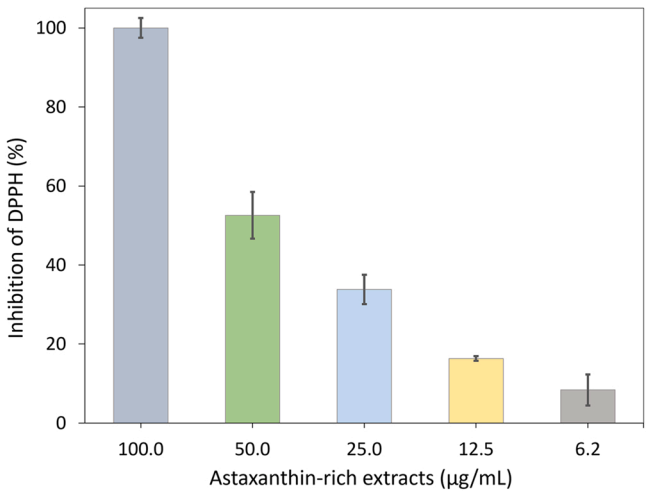

Fig. 4. DPPH• scavenging activity of astaxanthin-rich extracts produced by *P. rhodozyma* at different concentrations.

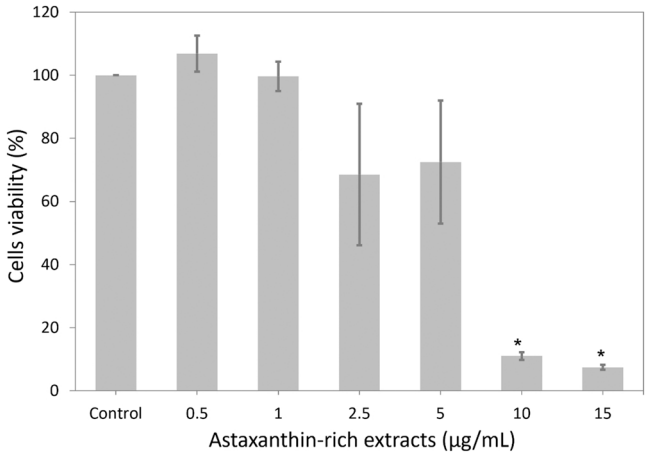

Fig. 5. Cytotoxicity of astaxanthin produced by submerged cultivation of *P. rhodozyma* on 3T3/NIH murine fibroblast cells. Cells were incubated with different concentrations (positive control, 0.5, 1, 2.5, 5 10 and 15 μg/mL) for 4 h at 38 °C and 5% CO_2 atmosphere. Data are presented as mean ± SEM of three independent experiments (*$p < 0.05$) by ANOVA statistical test and Tukey's post-test for multiple comparisons ($n = 3$).

therefore only concentrations of 10 and 15 μg/mL showed a high rate of cell death that was statistically significant. These results revealed that the astaxanthin-rich extracts (from 0.5 to 5 μg/mL) do not affect the viability of the studied NIH/3T3 cells. Therefore, its plausible that in a reasonable dosage range of the extract, the application of yeast-based astaxanthin is safe for textiles dyeing development in the range of concentration studied.

3.4. Astaxanthin-rich extracts as a textile colorant

The dyed cotton fabrics using astaxanthin-rich extracts exhibited a significant color difference compared to the undyed fabric upon visual inspection and colorimetric evaluation. The color characteristics of the undyed textile fabrics used as control (TF1-C, TF2-C and TF3-C), the luminosity (L^*) varied from 55 to 65, the parameter a^* from 1.41 to 2.44 and b^* from − 12 to − 10 (Fig. 6-**B** and Tables S1 from Supplementary material). Concerning to the purity of samples (C^* value), the undyed textiles varied from 10 to 12, indicating a low vivid color. As visual observed (Fig. 6-**A**) only sample TF1 allowed the good-looking incorporation of astaxanthin to the textile, with color characteristics ranged

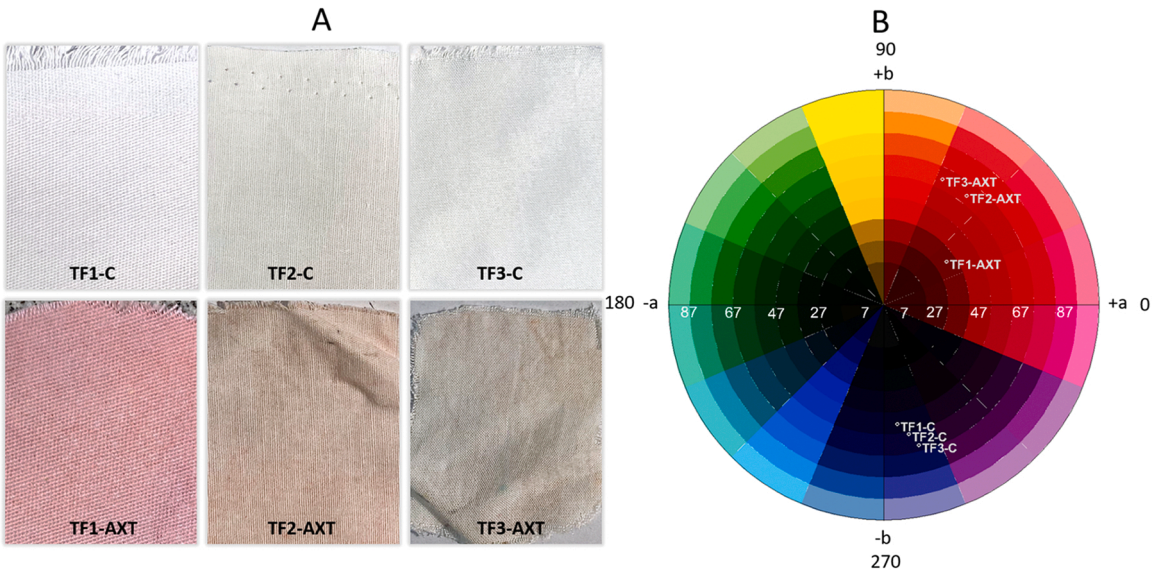

Fig. 6. A- Visual aspect of textile fabrics with astaxanthin-rich extracts, **B-** Qualitative color change in CIELAB color space for astaxanthin-rich extracts on textile fabrics: Undyed white textile fabrics used as control (TF1-C, TF2-C and TF3-C), dyed textile fabrics with astaxanthin (TF1-AXT, TF2-AXT and TF3-AXT).

from 33, 31, 21 of L^*, a^* and b^* (Fig. 6-**B**), respectively, demonstrating a clear red-yellow material with C^* value of 37 that suggests a more vivid color than the control (TF1-C). The total color difference (ΔE) among the undyed white textile fabrics and the dyed counterpart with astaxanthin was also calculated and the values determined were 49.84 to TF1-C/TF1-AXT, 13.38 to TF2-C/TF2-AXT and 8.77 to TF3-C/TF3-AXT (Tables S1 from Supplementary material) proving that the highest difference of color was achieved with the condition TF1-C/TF1-AXT.

The affinity of the astaxanthin-rich extracts to the textile fabrics depends on several parameters such as carotenoid/cotton interactions including the hydrophobicity and the nature of their functional groups as well as the concentration of the extract incorporated. Yeast-based astaxanthin extracts have the advantages to improve some shades of red color (depending on the concentration) that can become an alternative and sustainable choice for textile fabric dyeing. However, due to their structural nature, astaxanthin and other biomolecules such as β-carotene are easily hydrolyzed and degraded by several factors, such as washing, temperature, oxidation, among others [52]. In this work, to evaluate the wash fastness properties of astaxanthin-rich extracts in the textile fabrics, the samples (TF1) were washed and dried for approximately 6 times (Fig. 7).

As depicted in Fig. 7-**A** and Tables S2 from Supplementary material, the values of L^* (from 34.19 to 37.79) indicated that there is no pronounced variation in sample luminosity during each cycle of washing, however a slight loss of red-yellow color after in each cycle was observed [a^* (from 31 to 18) and b^* (from 21 to −0.9)]. It was found that washing fastness (6 cycles) of all dyed fabrics with astaxanthin showed considerably good, there is no significant deterioration in desirable properties (Fig. 7-**B**). According to the total color difference (ΔE*) (Tables S2 from Supplementary material) significant loss occurs of color after the first and second washing procedure, and behind the color difference keep close, there is no difference of color after the sixth washing, with a value of 1.11 to ΔE* , being ΔE* of 1.0 the smallest color difference that the human eye can see. The color of textile fabric slight changes after each cycle of washing (maximum 6 cycles), probably due to the oxidation of astaxanthin (non-polar molecule) [53], and also due to the high affinity of this molecule to the detergents in the reaction media, that also improves the color loss from the textile fabrics. To avoid the fast deterioration of astaxanthin-rich extracts in the textile fabrics, future studies should focus on the evaluation of the mordants effects on the color strength and fixation, dyeing time and temperature, among other

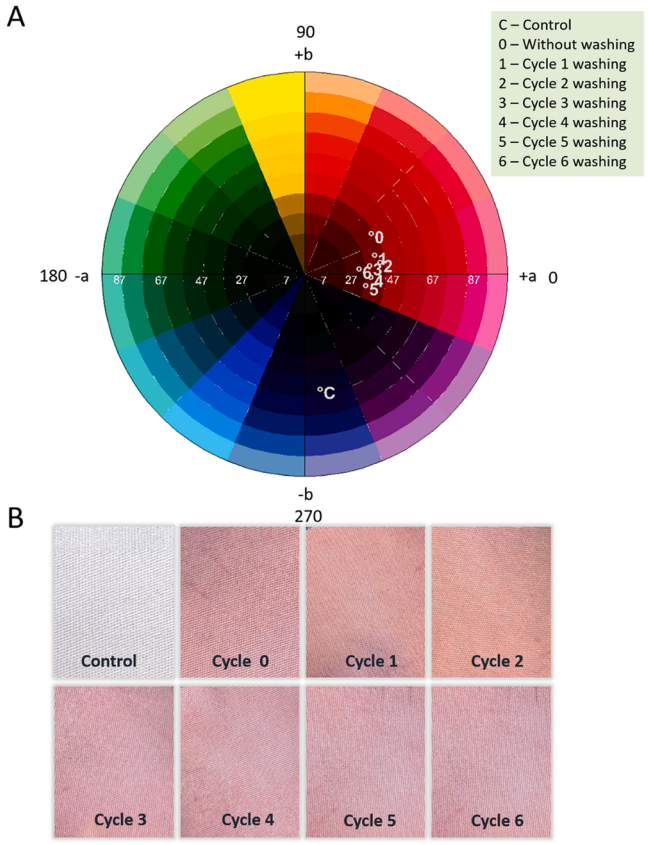

Fig. 7. A- Qualitative color change in CIELAB color space for astaxanthin-rich extracts on TF1-AXT and **B-** Visual aspect of washing cycles at 25 °C.

important parameters to improve product quality. However, these results show new insights into the application of astaxanthin produced by biotechnological route, thus taking into account that most of synthetic textile dyes are sold for approximately 100 USD/Kg and pure synthetic astaxanthin is usually sold by 2000 USD/Kg [1], we believe that the current experimental work is of paramount importance, not only as a support for our design and demonstration that *Phaffia*-based astaxanthin

can be used as dyeing agents, but also to help other researchers to follow similar or low-cost approaches and carry out important studies in this area.

4. Conclusions

In conclusion, the presence of light irradiation in the cultivation enhanced the production of astaxanthin by *P. rhodozyma* NRRLY-17268 growing on combined carbon source (glucose and xylose). Under selected conditions in STB at pH 5.8, incubation temperature of 22 °C with agitation of 300 rpm, aeration of 1 vvm for 144 h, astaxanthin production reached 503.66 μg/g_{DCW}. Furthermore, as demonstrated in this study, the best the astaxanthin-rich extracts showed antioxidant properties and no cytotoxicity until 5 μg/mL. *P. rhodozyma* is a novel candidate for the production of astaxanthin-rich extracts with biological activities to be effectively used as dyeing agent for textile materials as an alternative to the synthetic dyes. Further studies, some of which are currently underway in our laboratories, are needed to further evaluate the economic viability and environmental impact through techno-economic analysis (TEA) and life-cycle assessment (LCA) of the proposed application.

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CRediT authorship contribution statement

Cassamo U. Mussagy: Conceptualization, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Pedro G. P. Silva** and **Janaina F. M. Burkert**: Yeast acquisition, Writing – review & editing. **Camila F. Amantino** and **Fernando L. Primo**: Cytotoxicity evaluation, Writing – review & editing. **Adalberto Pessoa Jr:** Writing – review & editing, Supervision. **Valeria C. Santos-Ebinuma:** Conceptualization, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2022.108658.

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