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CAROTENOID-PRODUCING YEASTS IN THE BRAZILIAN BIODIVERSITY: ISOLATION, IDENTIFICATION AND CULTIVATION IN AGROINDUSTRIAL WASTE

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Abstract - Different yeast strains from forests located in southern Brazil, with potential to produce carotenoids, were isolated. Three microorganisms were selected as potential carotenoid producers. *Sporiodiobolus pararoseus*, *Rhodotorula mucilaginosa* and *Pichia fermentans* were grown in Yeast Malt (YM) medium and the carotenoids produced identified as cryptoxanthin and β-carotene. In order to reduce production costs, agroindustrial residues were used in the formulation of medium A (parboiled rice water and crude glycerol) and medium B (parboiled rice water and sugar cane molasses). The highest carotenoid production was obtained with *S. pararoseus*. It reached 905.30 μ gL^{-1} (122.82 μg g^{-1}) in YM medium, 820 μg L^{-1} (68.04 μg g^{-1}) in medium B and 710 μg L^{-1} (86.46 μg g^{-1}) in medium A. *R. mucilaginosa* exhibited the best performance in medium B (360 μg L^{-1} and 30.16 μg g^{-1}) and a new microorganism - *P. fermentans* - reached 48% (medium A) and 78% (medium B) of the value found in YM medium. Therefore, the agroindustrial residues under evaluation, which replaced the commonly used nitrogen and carbon sources in culture media, enabled the isolated yeasts to yield carotenoids.
Keywords: Agroindustrial byproducts; β-carotene; Yeast screening; Natural pigments.

INTRODUCTION

Carotenoids, which are the most widespread class of pigments in nature, are usually tetraterpenoids with 40 carbon atoms. These molecules have a system of double bonds that constitute the chromophore group responsible for the colors - red, orange and yellow - they exhibit. Widely found in plants, animals and microorganisms (Maldonade et al., 2008) and applied in food, pharmaceutical, cosmetic and feed industries (Zeni et al., 2011), they have higher economic and technological value than other microbial pigments (Tuli et al., 2015).

Importance has recently been given to carotenoids in the scientific field, not only due to their wide use as pigment in foods and precursors of vitamin A, but also because of their beneficial effects on human health. Some carotenoids, such as β-carotene and β-cryptoxanthin, have been highlighted due to their antioxidant action and protection against cardiovascular diseases and some types of cancer (Rao and Rao, 2007), besides the fact that they inhibit lipoprotein oxidation processes. Moreover, zeaxanthin exerts protective action against macular degeneration and cataracts (Ma and Lin, 2010), strengthens the

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immune system (Fusco et al., 2007) and has anti-inflammatory and anticarcinogenic activity (Preetha et al., 2008).

Habits of health-conscious people and their concern for the use of chemical additives in food have intensified researches on carotenoid production by biotechnological processes with algae such as *Dunaliella salina, Tetraselmis suecica, Isochrysis galbana, Pavlova salina* (Ahmed et al., 2014) and *Haematococcus pluvialis* (Machado Jr. et al., 2016); bacteria such as *Rhodopseudomonas palustris* (Kuo et al., 2012), *Halobacterium salinarum*, *Halorubrum sodomense*, *Haloarcula valismortis* and *Salinibacter ruber* (Jehlička et al., 2013); and yeasts such as *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous* (Pollmann et al., 2017; Silva et al., 2016; Cipolatti et al., 2015; Rios et al., 2015), *Rhodotorula mucilaginosa* (Manimala and Murugesan, 2017; Maldonade et al. 2012) and *Sporidiobolus pararoseus* (Machado and Burkert, 2015; Wei et al., 2014; Manowattana et al., 2018). Carotenoids, such as trans-violaxanthin, antheraxanthin, astaxanthin, lutein epoxide, lutein, zeaxanthin, α- and β-carotene were produced by algae (Ahmed et al., 2014). Bacterioruberin and salinixanthin were obtained with bacteria (Jehlička et al., 2013), while yeasts synthesized carotenoids such as β-carotene, lutein, torulene, torularhodin and astaxanthin (Cipolatti et al., 2015; Wei et al., 2014). Other multi-oxygenated carotenoids were produced by genetically modified microorganisms (Pollmann et al., 2017).

Yeasts are notable for their growth capacity and production using different agroindustrial wastes as substrate. *Phaffia rhodozyma*, with the use of crude glycerol, a byproduct of biodiesel production, and parboiled rice water (Silva et al., 2012), reached 1,700 μg L^{-1} (275.5 μg g^{-1}). With the same yeast, the optimization of the culture medium with parboiled rice water led to a production of 5,300 μg L^{-1} (628.8 μg g^{-1}) (Rios et al., 2015). Banzatto et al. (2013), in the cultivation of *R. rubra*, reached 274 μg L^{-1} (33 μg g^{-1}) with sugar cane juice, sugar cane molasses and syrup and produced torulene, torularhodin and β-carotene. *S. pararoseus*, with corn steep liquor and sugar cane molasses, reached a production of 521 μg L^{-1} (73 μg g^{-1}), while the use of corn steep liquor and crude glycerol led to a production of 780 μg L^{-1} (66 μg g^{-1}) (Machado and Burkert, 2015). Thus, they are promising alternatives for carotenoid industrial bioproduction, since they minimize costs of the cultivation medium and add value to the agroindustrial waste used as substrate.

Brazil has the greatest biodiversity on the planet, even though its microbiota is still largely unknown. Yeasts represent a part of the microflora of natural ecosystems, which may be desirable sources of various compounds, such as carotenoids. However, isolation and selection of potential carotenoid-producing yeasts have been poorly explored in Brazil (Zeni et al., 2011; Maldonade et al., 2008).

Therefore, the main goal of this study was the isolation, selection and identification of new yeast strains from forests located in southern Brazil as potential carotenoid producers. Besides, the potential of agroindustrial waste as substrate for the production of high value-added bioproducts was also evaluated.

MATERIAL AND METHODS

Pigmented yeast screening

For yeast isolation, samples of soil, bark, flowers, leaves and fruits were collected in different regions (the Sul-Riograndense Shield and the Eastern Coast) in southern Brazil. These samples were aseptically packaged. Strains that showed coloration were isolated and codified to facilitate further identification. Identified yeasts were then deposited in the André Toselo Tropical Culture Collection (Campinas, Brazil).

Yeast isolation and selection

Samples were placed in Erlenmeyer flasks with 20 mL YM broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1.0% glucose) and incubated at 30°C for 48 h. They were then inoculated in Petri dishes with YM agar and incubated at 30°C for 120 h. Yellow to red colonies were transferred to slant tubes with YM agar and incubated at 30°C until satisfactory growth. These colored cultures were re-isolated in Petri dishes with YM agar (incubated at 30°C for 72 h), transferred to slant tubes with GYMP agar (2.0% glucose, 0.5% yeast extract, 0.3% malt, 0.5% peptone, 0.2% monobasic sodium phosphate and 2.0% agar) and stored at 4°C after having grown (Maldonade et al., 2007).

Shake flask cultivation in YM medium

For yeast selection, an inoculum was prepared in 250 mL Erlenmeyer flasks with 100 mL YM medium at initial pH 6.0, previously sterilized at 121°C for 15 min. Flasks were inoculated with a loopful of suspended cells from the slant tubes and incubated at 25°C and 180 rpm for 48 h.

Cultures for carotenoid bioproduction were prepared in triplicate in 500 mL Erlenmeyer flasks with 250 mL culture medium at initial pH 6.0. Ten percent inoculum was added to each flask (to reach an initial cell concentration of $1x10^7$ cells mL^{-1}), then they were disposed in an orbital shaker (Tecnal model TE 424, Brazil) at 25ºC and 180 rpm without light for 168 h (Michelon et al., 2012). At this time, biomass concentration and total carotenoids were determined.

Yeast identification

Selected yeasts were characterized by PCR fingerprinting, i.e., the mini/microsatellite-primed PCR technique (MSP-PCR) (Libkind et al., 2003).

DNA extraction

For DNA extraction, yeast colonies were grown on modified Sabouraud agar (2% glucose, 1% peptone, 0.5% yeast extract and 2% agar) at 25°C overnight, transferred to 2 mL sterile tubes (Eppendorf) with 100 mL extraction buffer solution (50 mmol Tris, 250 mmol NaCl, 50 mmol EDTA, 0.3% wv^{-1} SDS, pH 8) and incubated at 65°C for 30 min. After incubation, 100 mL phenol/chloroform/isoamyl alcohol (25:24:1) was added. The mixture was vigorously vortexed, incubated for 3 min and centrifuged at 7558 x g for 3 min. DNA was dried at room temperature overnight, suspended in 100 mL TE buffer (10 mM Tris, 10 mM Na-EDTA, pH 8.0) and stored in a refrigerator.

PCR fingerprinting

Synthetic oligonucleotide (GTG) 5 and the core sequence of phage M13 (GAGGGTGGCGGTTCT) were used in MSP-PCR experiments, respectively. PCR reactions were performed in agreement with Libkind et al. (2003). Twenty yeast strains with identical DNA banding patterns were grouped and putatively considered to belong to the same species (Gadanho and Sampaio, 2002). At least one representative strain of each MSP-PCR group was subjected to the sequence analysis of D1/D2 domains of the large subunit of the rRNA gene, as described below. Physiologically distinct strains with unique MSP-PCR banding patterns were also selected for direct identification by sequencing the D1/D2 region of the rRNA gene. Whenever necessary, internal transcribed spacer (ITS) domains of the rRNA gene were also sequenced.

Sequence analysis

Total DNA was extracted and the D1/D2 variable domains of the large subunit of the rRNA gene were amplified (Lachance et al., 1999) with primers NL-1 (50-GCATATCAATAAGCGGAGGAAAAG-30) and NL-4 (50-GGTCCGTGTTTCAAGACGG-30). ITS regions of rRNA genes were amplified with universal primers ITS1 (50-TCCGTAGGTGAACCTGCGG-30) and ITS4 (50-TCCTCCGCTTATTGATATGC-30) (White et al., 1990). Primers used by this study have high accuracy for yeast identification and have also been used by most researches on yeast taxonomy (Kurtzman et al., 2011)

Sequencing of the D1/D2 region and ITS domains was directly performed with purified PCR products by a MegaBaceTM 1000 automated sequencing system (Amersham Biosciences, USA). Sequences were compared with those found in the GenBank database by the BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST at http://www.ncbi.nlm.nih.gov). In general, strains of a species show no more than 0 to 3 nucleotide differences (0-0.5% identity) in D1/D2 domains, while strains that show 6 or more noncontiguous substitutions (1% identity) are separate species (Kurtzman and Robnett, 1998). According to Daniel et al. (2009), the most commonly detected intraspecific sequence variability is 0-4 differences in the ITS region. Species with more nucleotide differences are not considered generally conspecific.

Shake flask cultivation in agroindustrial medium

Agroindustrial waste

Crude glycerol (83.08% w w^{-1} glycerol) derived from the synthesis of biodiesel, sugar cane molasses and parboiled rice water were kindly provided by industries located in the south of Brazil. Parboiled rice water was maintained under refrigeration (4 ± 2°C) and centrifuged at 1,745 x g for 10 min in order to enable particle removal before use.

Partial characterization of substrates

Partial characterization of substrates of the culture media (yeast extract, malt extract, peptone, parboiled rice water, sugar cane molasses and crude glycerol) was performed by determining carbon, nitrogen and hydrogen concentrations by a CHNS/O analyzer (Perkin Elmer, 2400, Germany). For equipment calibration, acetanilide was used as the certified reference material.

Except for carbon determinations in sugar cane molasses, a total organic carbon analyzer (TOC-V CPH / CPN, Shimadzu, Japan) was used with the NPOC (non-purgeable organic carbon) method. Nitrogen concentrations of parboiled rice water and sugar cane molasses were determined by Micro Kjeldahl (AOAC, 2000).

Shake flask cultivation

A similar procedure to the one described aboce for YM medium was used, but the volume of the culture medium was adjusted to 306 mL.

In order to obtain the same Carbon/Nitrogen (C/N) ratio found in YM medium (6.20), the Solver tool (Excel 2010 software, Microsoft Inc., USA) was used for calculating component concentrations by linear programming, considering both nitrogen and carbon contents of agroindustrial waste. Culture media for carotenoid production (C/N ratio of 6.20) were: medium A, which consisted of parboiled rice water (39.91 g L^{-1}) and crude glycerol (17.31 g L^{-1}); medium B, which was composed of parboiled rice water (44.01 g L^{-1}) and sugar cane molasses (23.6 g L^{-1}); and YM medium (3 g L^{-1} malt extract, 3 g L^{-1} yeast extract, 5 g L^{-1} peptone, 10 g L^{-1} glucose).

Analytical methods

Total carotenoid determination

For total carotenoid recovery, samples were centrifuged at 1800 x g at 25°C for 10 min to separate biomass, which was submitted to drying (at 35°C for 48 h) and successive maceration in a mortar with a pestle. Then, it was frozen (at -18°C for 48 h). Afterwards, 0.05 g cell was added to 2 mL DMSO (dimethylsulphoxide) (Nuclear, Brazil), previously heated at 55°C (Fanem 102, Brazil) for 1 h, with periodic vortex homogenization (Biomixer, QL-901, Brazil). In agreement with Fonseca et al. (2011), 6 mL of acetone (Quimex, Brazil) was then added, followed by centrifugation at 1800 × g at 25°C for 10 min. The supernatant was separated and successive extractions were carried out until cells were colorless. Ten mL of 20% NaCl (w v^{-1}) solution and 10 mL of petroleum ether were added. After stirring and phase separation, the excess of water was removed with Na_2SO_4 in order to obtain carotenogenic extracts (Rodriguez-Amaya et al., 2008).

Total carotenoid concentration was determined by spectrophotometric (Biospectro, SP 220, China) reading at 474 nm (Rodriguez-Amaya et al., 2008). Values were obtained by Eq.1 with the specific absorptivity coefficient in petroleum ether, i. e., 2100 mol L^{-1} cm^{-1} (Domínguez-Bocanegra and Torres-Munoz, 2004).

$$SPC = \frac{100A_{474}V}{21m} \quad (1)$$

where SPC is the specific total carotenoid concentration (μg.g^{-1}), A_{474} is the absorbance at 474 nm, V is the filtrate volume (mL), and m is the dry cell mass (g).

To calculate the volumetric concentration of total carotenoids (VPC) (μg L^{-1}), both total carotenoid concentration (μg g^{-1}) and biomass concentration (g L^{-1}) were used. Units were converted.

Carotenoid identification

Identification of carotenoids produced with YM medium (168 h) was carried out by extract concentration in a rotary evaporator (Fisaton model 802, Brazil) and re-dissolution in 5 mL methanol:acetonitrile 30:70 (v:v). A 20 μL aliquot was then injected into the liquid chromatography. An HPLC system (Shimadzu, Japan), consisting of an automatic sampler (SIL-10AF), a solvent mixing module (LC-10 ALvp), an automatic degasser (FCV-10ALvp), a quaternary pump (DGU-14A), a column oven compartment (CTO-10ASvp) set at 25°C, a UV-Vis spectrophotometric detector (SPD-10Avp) set at 450 nm and a control system (SCL-10avp), were used. A Shim-Pak reverse phase CLC-ODS column (4.6 cm x 150 mm x 5 μm particle size, Shimadzu) with an octadecyl stationary phase and a guard column CLC- GODS (5 μm, 2 cm x 4 mm, Supelco) were also used.

Elution was carried out at flow rate of 1 $mLmin^{-1}$ with an initial mobile phase of 30% methanol (A), 70% acetonitrile (B) and 0% acetyl acetate (C) for 10 min. Percentages remained 10% (A), 80% (B) and 10% (C) up to 35 min. Afterwards, they were 5% (A), 80% (B) and 15% (C) up to 40 min. Finally, the initial mobile phase was maintained up to 45 min, and then kept for 3 min (Marinova and Ribarova, 2007).

Carotenoids were identified by comparing their retention times, with the help of a calibration curve obtained with the following standards: β-carotene, which was provided by Fluka (Saint Louis, USA) and β- cryptoxanthin, zeaxanthin and lycopene, which were supplied by Chromadex (Irvine, CA, USA). All had 97% purity.

Biomass determination

For yeast selection, a 10 mL sample was centrifuged at 1800 × g and 25°C for 10 min. Then, cells were washed with distilled water and centrifuged again. Cell mass was quantified by drying at 105°C until constant weight (AOAC, 2000). In the case of the selected yeasts, biomass concentration was estimated by absorbance reading at 620 nm and the value was converted to g L^{-1} with the use of a previously constructed standard curve (Kusdyantini et al., 1998).

pH determination

pH was determined by a potentiometer (Marte, MB-10, Brazil), in agreement with the AOAC (2000).

Determination of total reducing sugar concentration

Total reducing sugar (TRS) concentrations were determined in cell free supernatant previously centrifuged at 3439 x *g* for 10 min for YM medium and B medium (parboiled rice water and sugar cane molasses). One mL culture medium with the agroindustrial byproducts was submitted to hydrolysis with 2 mL of 2.0 moL L^{-1} HCl in a water bath at 55°C for30 min, followed by the addition of 2 mL of 2.0 mol L^{-1} NaOH for acid neutralization (Liu et al., 2012). Subsequently, TRS was determined by the spectrophotometric method of 3,5-dinitrosalicylic (DNS), in accordance with Miller (1959), with the use of a standard glucose curve (between 0.1 and 1.0 g L^{-1}).

Kinetic parameters of cultivation

The substrate-to-product conversion factor ($Y_{P/S}$, microgram carotenoids per gram glucose), the substrate-to-biomass conversion factor ($Y_{X/S}$, gram cells per gram substrate) and the biomass to product conversion factor ($Y_{P/X}$, microgram carotenoids per gram cells) were calculated (Bailey and Ollis, 1986).

Cell productivity (P_x, g L^{-1} h^{-1}) and total carotenoid productivity (P_c, µg g^{-1} h^{-1}) were determined by cell growth curves and carotenoid production (Bailey and Ollis, 1986).

Maximum specific growth rate ($\mu_{máx}$) was calculated by Eq. 2, by using the Microcal Origin® 5.0 software.

$$\mu_{max} = \frac{1}{X}\frac{dX}{dt} \quad (2)$$

where $\mu_{máx}$ is the maximum specific growth rate (h^{-1}) and X is the biomass concentration (g L^{-1}).

Statistical analysis

All experiments were performed in triplicate (n=3) and results were treated by the analysis of variance followed by the Tukey's post-hoc test ($p<0.05$), using Statistica 5.0 software (StatSoft Inc., USA).

RESULTS AND DISCUSSION

Yeast diversity

In this study, 147 environmental samples were collected in two ecosystems: 42, in flowers; 19, in fruits; 35, in bark; 14, in the soil; and 37, in leaves. At the end, 683 yeast colonies were isolated. However, since only 64 yeasts had some coloration (30 yellow, 10 orange and 24 pink), they were selected and re-isolated. Results are shown in Tables 1, 2 and 3.

Table 1. Biomass (g L^{-1}) and total carotenoids (µg L^{-1} or µg g^{-1}) of yellow yeasts.

Code	Biomass (g L^{-1})	Carotenoids (µg g^{-1})	Carotenoids (µg L^{-1})
A1	7.43± 0.15^{g,h,i,j}	17.52±2.96^{d}	140±37.40^{d}
A2	1.28± 0.24^{n,o}	2.76±0.27^{k,l,m,n}	3.53±08^{j}
A10	7.00±0.10^{i,j,k}	2.67±0.33^{l,m,n}	18.33± 2.05^{h,i,j}
A12	9.00±0.10^{d,e,f,g,h}	4.60±0.15^{h,i,j,k,l,m}	40.00± 1.25^{f,g,h,i,j}
A14	7.13±0.86^{h,i,j,k}	2.88±0.11^{k,l,m,n}	20.33±2.49^{h,i,j}
A15	8.37±0.83^{f,g,h,i,j}	3.52±0.30^{j,k,l,m,n}	29.00±2.16^{g,hi,j}
A16	4.46±0.217^{l,m}	6.37±0.08^{g,h,i,j,k,l}	20.00±1.411^{g,h,i,j}
A20	11.67±1.42^{a}	11.15±0.15^{e,f}	126.67±12.47^{d}
A21	8.57±1.10^{i,j,k}	16.02±0.02^{e,f}	133.33±17^{d}
A23	7.13±0.85^{h,i,j,k}	7.26±0.06^{f,g,h,i,j}	20.33±2.49^{f,g,h,i,j}
A24	8.10±0.26^{f,g,h,i,j}	14.31±0.27^{d,e}	113.33±4.71^{d,e}
A27	2.27±0.47^{m,o}	10.19±0.12^{f,g}	19.67±0.47^{h,i,j}
A28	9.20±0.45^{c,d,e,f,g}	6.74±0.12^{g,h,i,j,k}	61.6±3.30^{f,g,h,i}
B2	10.80±0.61^{a,b,c,d}	8.47±1.11^{f,g,h}	88.33±8.50^{d,e,f}
B7	8.07±0.40^{f,g,h,i,j}	0.00±<0.01^{n}	0.00±<0.01^{j}
B8	0.80±0.30^{o}	0.00±<0.01^{n}	0.00±<0.01^{j}
B9	9.53±0.25^{b,c,d,e,f}	1.28±0.14^{m,n}	11.67±0.94^{i,j}
B10	9.07±0.21^{d,e,f,g,h}	4.50±0.13^{h,i,j,k,l,m}	3.70±0.6^{f,g,h,i,j}
B11	9.07±0.21^{d,e,f,g,h}	7.40±0.16^{f,g,h,i,j}	76.70±18.90^{e,f,g,h}
B12	11.37±1.06^{a,b}	34.29± 0.17^{b}	383.73±3.3^{b}
B13	10.43±1.10^{a,b,c,d,e}	4.20±0.03^{i,j,k,l,m}	44.33±3.30^{f,g,h,i,j}
C3	11.07± 0.63^{a,b,c}	24.56±1.89^{c}	271.03±14.20^{c}
C6	8.73±0.25^{e,f,g,h,i}	2.01±0.07^{m,n}	17.50±0.22^{h,i,j}
D2	9.07±0.81^{d,e,f,g,h}	9.04±2.21^{f,g}	66.70±2.49^{e,f,g}
D3	7.87±0.28^{f,g,h,i,j}	122.82±5.27^{a}	905.30±41.35^{a}
D4	8.77±0.32^{e,f,g,h,i}	4.53±0.17^{h,i,j,k,l,m}	38.67±0.47^{f,g,h,i,j}
D5	2.60±0.40^{m,o}	7.63±0.38^{f,g,h,i}	19.00±2.94^{h,i,j}
D6	5.32±0.32^{k,l}	1.35±0.09^{m,n}	7.00±<0.01^{j}
D7	2.96±0.09^{m,n}	7.70±0.25^{f,g,h,i}	22.67±0.94^{h,i,j}
D12	6.53±0.70^{j,k}	7.54±0.10^{f,g,h,i,j}	44.33±3.30^{f,g,h,i,j}

Medium ± standard deviation (n=3). Different letters indicate significant difference among means in the same column ($p<0.05$).

Table 2. Biomass (g L^{-1}) and total carotenoids (µg L^{-1} or µg g^{-1}) of orange yeasts.

Code	Biomass (g L^{-1})	Carotenoids (µg g^{-1})	Carotenoids (µg L^{-1})
A6	1.63±0.25^{b}	2.62±0.33^{d}	4.23±0.58^{d}
A9	3.90±0.20^{f}	3.85±0.73^{d,e}	14.33±1.89^{d}
A13	3.81±0.31^{f}	8.69±0.18^{c}	33.66±0.89^{e}
A17	8.20±0.30^{e}	<0.01±<0.01^{f}	1.00±<0.01^{d}
A18	11.53±0.47^{c}	5.00±0.10^{e}	57.33±0.94^{c}
A25	8.33±0.58^{e}	9.71±0.18^{c}	78.67±0.94^{b}
A26	9.83±0.45^{d}	4.40±0.15^{e}	42.67±2.36^{c,e}
B1	11.23±0.06^{c}	25.33±1.60^{a}	280.00±14.1^{a}
D1	9.40±0.53^{d}	3.70±0.30^{d,e}	34.33±4.19^{e}
D11	7.13±0.15^{a}	7.00±0.05^{b}	46.67±4.71^{c,e}

Medium ± standard deviation (n=3). Different letters indicate significant difference among means in the same column ($p<0.05$).

Table 3. Biomass (g L^{-1}) and total carotenoids (µg L^{-1} or µg g^{-1}) of pink yeasts.

Code	Biomass (g L^{-1})	Carotenoids (µg g^{-1})	Carotenoids (µg L^{-1})
A3	1.27± 0.21^{i}	4.13±0.60^{g}	5.17±0.65^{g}
A4	1.73 ±0.08^{i}	2.55±0.50^{g,h,i}	4.40±0.78^{g,h,i}
A5	1.78±0.19^{i}	26.55±1.75^{c}	46.67±4.11^{c}
A7	1.17±0.31^{i}	2.67±0.73^{g,h,i}	3.13±0.99^{g,h,i}
A8	0.55±0.15^{i}	<0.01±<0.01^{i}	1.00±<0.01^{i}
A11	6.63±0.18^{g,h}	5.47±0.21^{f,g}	35.67±1.89^{f,g}
A19	11.97±0.25^{a,b}	54.07±0.15^{a}	640.00±8.18^{a}
A22	8.13±1.25^{d,e,f,g}	13.26±0.13^{d}	103.00±9.43^{d}
B3	2.41±0.30^{i}	3.23±1.80^{g,h,i}	7.57±3.59^{g,h,i}
B4	1.70±0.03^{i}	2.52±0.10^{g,h,i}	4.23±0.21^{g,h,i}
B5	1.36±0.26^{i}	42.27±3.16^{b}	57.67±11.81^{b}
B14	10.67±0.73^{a,b,c}	10.03±0.05^{d,e}	103.33±4.71^{d,e}
C1	7.33±0.85^{f,g}	<0.01±<0.01^{i}	1.00±<0.01^{g,h,i}
C2	6.30±1.08^{g,h}	0.52±0.11^{h,i}	3.47±0.97^{g,h,i}
C4	2.31±0.49^{i}	28.17±0.52^{f,g}	12.33±4.01^{f,g}
C5	9.97±1.51^{b,c,d,e}	5.51±0.14^{f,g}	54.33±6.34^{f,g}
D8	8.30±1.24^{d,e,f,g}	2.40±0.23^{g,h,i}	20.00±1.41^{g,h,i}
D9	12.67±060^{a}	2.32±0.06^{g,h,i}	26.33±4.50^{g,h,i}
D10	4.77±0.85^{h}	5.66±0.06^{f,g}	23.33±4.71^{f,g}
D13	7.57±1.12^{f,g}	8.16±0.04^{e,f}	60.67±7.04^{e,f}
D14	1.53±0.15^{i}	3.60±0.08^{g,h}	1.00±<0.01^{g,h,i}
E1	9.07±1.00^{c,d,e,f}	0.40±0.05^{h,i}	3.70±0.6^{g,h,i}
E2	7.97±0.91^{e,f,g}	5.26±1.81^{g}	34.00±25.7^{g}
E3	10.33±0.55^{b,c,d}	9.88±1.11^{d,e}	378.30±39.1^{d,e}

Medium ± standard deviation (n=3). Different letters indicate significant difference among means in the same column ($p<0.05$).

Yeast screening for carotenoid production

Isolated yeasts were grown to determine biomass and total carotenoid concentration (168 h cultivation). In this growth period, a statistical evaluation ($p<0.05$) was carried out to select the most promising yeasts for yellow, orange and pink carotenoid production.

Strains D3, B12 and C3 produced yellow carotenoids: 905.30 µg L^{-1} (122.82 µg g^{-1}) with 7.87

g L^{-1} biomass, 383.73 µg L^{-1} (34.29 µg g^{-1}) with 11.37 g L^{-1} and 271.03 µg L^{-1} (24.56 µg g^{-1}) with 11.07 g L^{-1}, respectively. Carotenoid production by strain D3 was higher and statistically different ($p<0.05$) from the others. These yeasts provided the highest carotenoid production in this study.

Yeast B1 was the best producer of orange pigments with 280 µg L^{-1} (25.33 µg g^{-1}) total carotenoid concentration and 11.23 g L^{-1} biomass concentration. Strains A19 and E3 were the best producers of pink carotenoids. They produced 640 µg L^{-1} (54.07 µg g^{-1}), with 11.97 g L^{-1} biomass concentration, and 378.3 µg L^{-1} (9.88 µg g^{-1}), with 10.33 g L^{-1} biomass concentration, respectively.

Yeasts with the highest potential for carotenoid production came from the following sources: bark (D3 - Sul-Riograndense Shield), flowers (B12 - Eastern Coast; B1 - Sul-Riograndense Shield), soil (C3 - Sul-Riograndense Shield), leaves (A19 - Eastern Coast) and fruit (E3 - Sul-Riograndense Shield). Thus, three yeasts with highest potential as carotenoid producers were selected: D3 (yellow), B1 (orange) and A19 (pink). These microorganisms underwent genus and species identification, carotenoid bioproduction kinetics and identification of the major carotenoid producers.

Yeast identification

After genetic sequencing of the three isolated yeasts, results obtained from GenBank information were compared and led to the following identification: D3 was identified as *Sporidiobolus pararoseus* (Fig. 1a); B1, as *Rhodotorula mucilaginosa* (Fig. 1b); and A19, as *Pichia fermentans* (Fig. 1c). These species showed 100% of sequence identities in the ITS region and D1/D2 domains with the sequences of reference strains of these species deposited in the GenBank. Among yeasts isolated and selected as carotenoid producers in this study, only *Rhodotorula* (Libkind et al., 2004; Aksu et al., 2005 and Maldonade et al., 2008) and *Sporodiobolus* (Cabral et al, 2011; Valduga et al., 2014) have been known as yeasts which produce these pigments. To date, no reports of carotenoid production by *Pichia fermentans* have been found in the literature.

Partial characterization of substrates

Partial characterization of yeast extract, malt extract, peptone, crude glycerol, parboiled rice water and sugar cane molasses is described in Table 4. Total nitrogen concentration of parboiled rice water complies with the one found in the literature (Queiroz et al., 2007). Crude glycerol used in this study showed higher carbon concentration (49.3%) than that used by Silva et al. (2012), when they studied carotenoid production by *Phaffia rhodozyma*. Therefore, amounts of nitrogen and carbon found in parboiled rice water and crude glycerol, respectively, are essential for cell growth. Thus, these substrates are potential sources of nutrients in the culture medium.

Bioproduction kinetics of selected yeasts

Sporidiobolus pararoseus

The behavior of *S. pararoseus* can be observed by growth kinetics, pH, TRS concentration and carotenoid production found in YM medium (Fig. 2a). It started after 24 h of culture (5.69 µg g^{-1}) with 50% TRS (6.54 g L^{-1}), which had already been previously consumed, at pH 5.39 and biomass of 3.17 g L^{-1}. It increased up to 60 h and then remained almost constant until 100 h (TRS depletion). There was an increase in total carotenoids, which reached the maximum of 905.30 µg L^{-1} (122.82 µg g^{-1}) in 168 h, at final pH 7.6 and biomass of 7.32 g L^{-1}. Such a pH change may have happened because a carbon intermediate was excreted by the yeast during growth as acetic acid, alcohol or intermediate of the citric acid cycle, thus causing a decrease in pH. Since this intermediate is subsequently reassimilated, it stimulates strong carotenogenesis and causes an increase in pH (Chociai et al., 2002). Thereafter, the pH remains constant and indicates the end of the culture.

Carotenoid production in medium A (parboiled rice water and crude glycerol) (Fig. 2b) started after 32 h (3.82 µg g^{-1}) at pH around 6 and biomass of 4 g L^{-1}. It increased after 160 h (12.06 g L^{-1}) and decreased slightly at the end of the cultivation. There was an increase in total carotenoids, whose maximum was 820 µg L^{-1} (68.04 µg g^{-1}) after 168 h, at final pH 8.5.

Carotenoid production in medium B (parboiled rice water and sugar cane molasses) (Fig. 2c) started after 40 h (1.37 µg g^{-1}) with approximately 70% TRS consumption, at pH 5 and biomass of 4 g L^{-1}, which increased up to 168 h (10.69 g L^{-1}). There was an increase in total carotenoids, whose maximum concentration was 710 µg L^{-1} (86.46 µg g^{-1}) after168 h at final pH 8.2.

Biomass concentration of this yeast achieved lower results in YM medium than in agroindustrial substrates; however, most carotenoid production occurred in YM medium. But in media A and B, volumetric production was promising because it reached about 90 and 78%, respectively, by comparison with YM medium, considering the same processing time (168 h).

Rhodotorula mucilaginosa

Fig. 3a shows that *R. mucilaginosa* consumed less TRS in YM medium than in other media. However, TRS consumption was similar in medium B for *S. pararoseus* (Fig. 2c) and *R. mucilaginosa* (Fig 3c). Cultures reached similar maximum biomass concentrations after 168 h, i. e., the agroindustrial medium A reached 12.49 g L^{-1} (Fig. 3b) at final pH

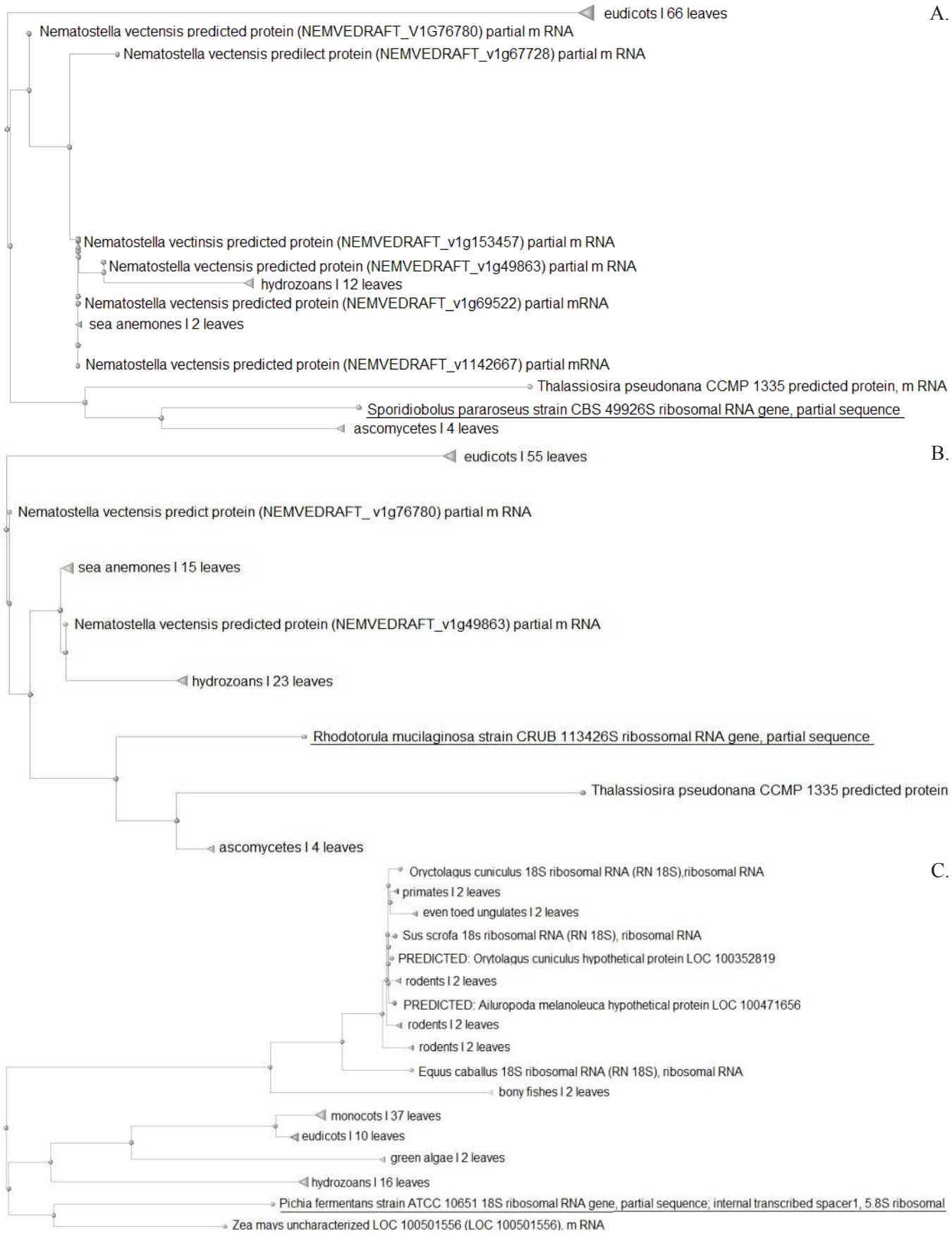

Figure 1. Dendrogram of identification of the yeast for *Sporidiobolus pararoseus* (a), *Rhodotorula mucilaginosa* (b) and *Pichia fermentans* (c).

Table 4. Partial characterization of substrates found in culture media.

Substrates	% C	% H	% N
Yeast extract	38.40	5.65	10.67
Malt extract	39.24	6.54	10.93
Peptone	43.47	6.71	14.47
Parboiled rice water	0.16	2.25	3.02
Sugar cane molasses	36.50	ND*	0.30
Crude glycerol	49.30	ND*	1.05
Standard			
Sample 1	100.24	99.55	99.90
Sample 2	100.29	102.83	99.81
% recovery	100.63	106.90	100.87

*ND- not determined.

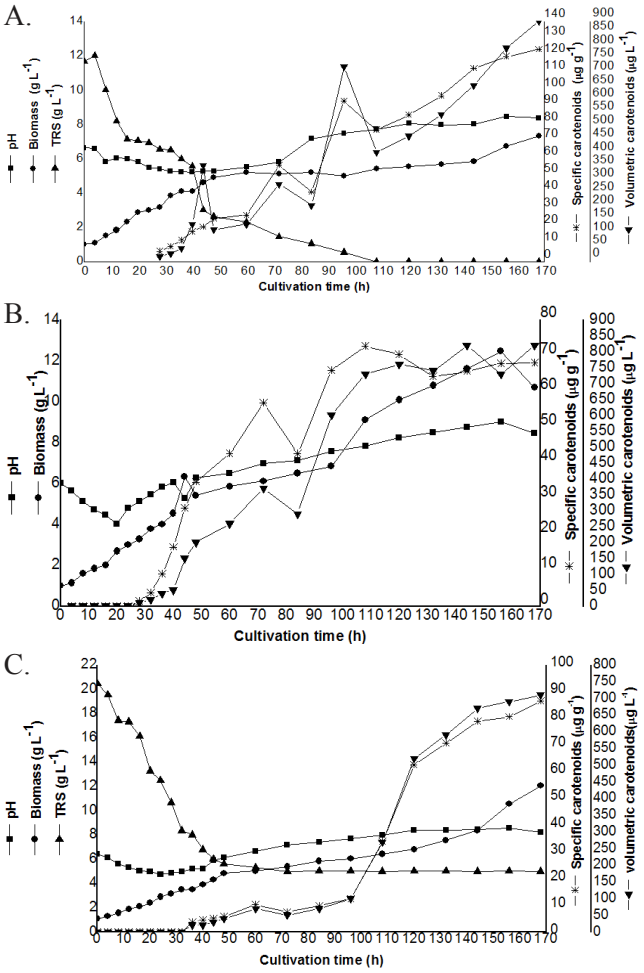

Figure 2. Growth kinetics, consumption of TRS, pH and production of carotenoids in YM medium (a), medium A parboilized rice water and raw glycerol (b) and medium B parboilized rice water and sugar cane molasses (c), at 25ºC and 180 rpm for 168 h for *Sporidiobolus pararoseus*.

8.20 whereas medium B attained 12.02 g L^{-1} (Fig. 3c) at final pH 8.50. YM medium reached 11.18 g L^{-1} (Fig. 3a) at final pH 8.10. The highest carotenoid production began at 40 h and reached 360 µg L^{-1} (30.16 µg g^{-1}) after 168 h in medium B. Carotenoid production started later (72 h) and reached 250 µg L^{-1} (28.06 µg

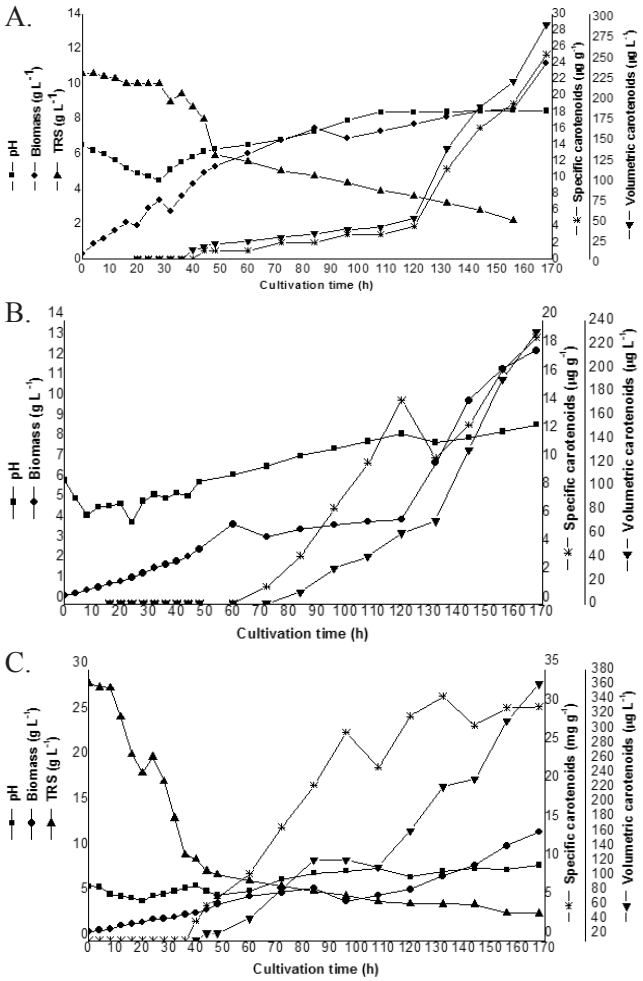

Figure 3. Growth kinetics, consumption of TRS, pH and production of carotenoids in YM medium (a), medium A parboilized rice water and raw glycerol (b) and medium B parboilized rice water and sugar cane molasses (c), at 25ºC and 180 rpm for 168 h for *Rhodotorula mucilaginosa.*

g^{-1}) and 230.00 µg L^{-1} (18.76 µg g^{-1}), respectively, in YM medium and medium A after 168 h. Therefore, the medium with sugar cane molasses produced 44% more than YM medium.

Pichia fermentans

P. fermentans (Fig. 4a) was able to consume all TRS in YM medium (100 h) and up to 90% in medium B by the end of the process (Fig. 4c). The behavior of pH was similar to the one shown by the other yeasts, i. e., it decreased in the first 50 h (4.41) and reached 8.36 or 8.15 at the end of the process in YM medium (Fig. 4a). In media A and B, the pH decrease occurred until 24 h and 28 h, respectively, reaching 8.45 and 8.96 after 168 h in media A and B.

Biomass increased gradually and reached a maximum of 12.32 g L^{-1} in medium B (Fig. 4c), followed by YM medium, where *P. fermentans* produced 11.84 g L^{-1} (Fig. 4a). In medium A, it

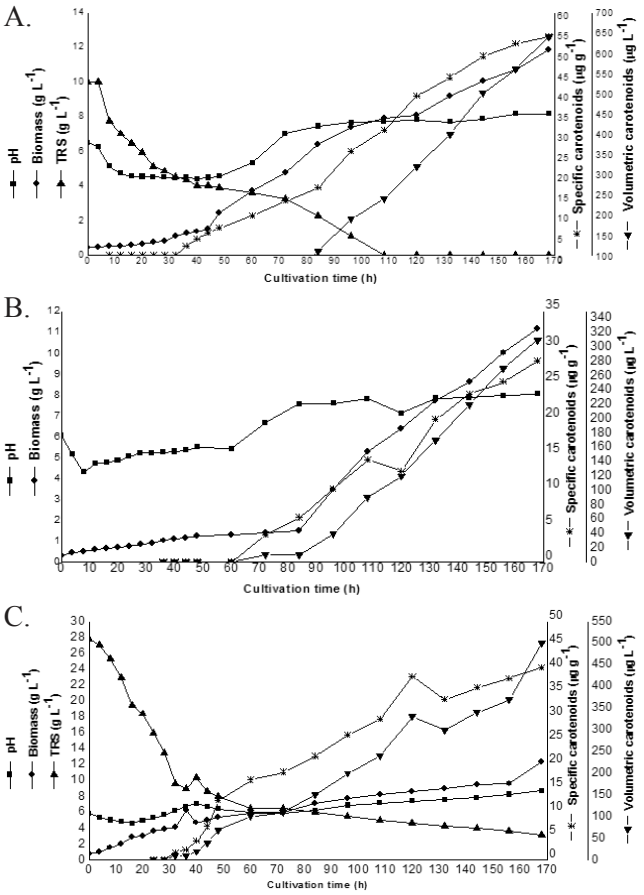

Figure 4. Growth kinetics, consumption of TRS, pH and production of carotenoids in YM medium (a), medium A parboilized rice water and raw glycerol (b) and medium B parboilized rice water and sugar cane molasses (c), at 25ºC and 180 rpm for 168 h for *Pichia fermentans*.

produced 9.09 g L^{-1} (Fig. 4b) in 168 h. In YM medium, maximum carotenoid production started after 36 h and reached 640 µg L^{-1} (54.07 µg g^{-1}) after 168 h. In the medium with sugar cane molasses (B), bioproduction started after 32 h, reached 500 µg L^{-1} (40.33 µg g^{-1}) and was higher than the one in medium A, whose value was 310 µg L^{-1} (27.83 µg g^{-1}) and started after 40 h. The highest carotenoid production occurred in YM medium, but both media A and B reached 48% and 78%, respectively, in the same processing period.

In this study, the agroindustrial medium with sugar cane molasses showed promising results for carotenoid production by the three selected microorganisms. Sugar cane molasses is an excellent culture medium for carotenoid production in microorganisms because its very complex composition contains nutrients, such as nitrogen, potassium, magnesium, manganese and iron, which, individually, can positively influence carotenoid bioproduction. Mevalonate, for instance, is a natural precursor of carotenoids (Valduga et al., 2008).

Some authors have carried out yeast isolation and identification with the objective of carotenoid production. In the agroindustrial media investigated in this study, carotenoid production by *S. pararoseus* (820 µg L^{-1} medium B and 710 µg L^{-1} medium A) was similar or higher than the ones reported in the literature by Maldonade et al. (2007), using *S. roseus* with YM medium (237 µg L^{-1}). Machado and Burkert (2015) reached 521 µg L^{-1} (40 g L^{-1} sugar cane molasses and 6.5 g L^{-1} corn steep liquor) and 780 µg L^{-1} (30 g L^{-1} crude glycerol and 52.9 g L^{-1} corn steep liquor) with the same microorganism of this study. Cabral et al. (2011), with another strain of *S. pararoseus*, using 60 g L^{-1} glucose, 15 g L^{-1} peptone and 15 g L^{-1} malt extract, reached a production of 856 µg L^{-1}. With the same strain, but with agroindustrial substrates (40 g L^{-1} glycerol, 40 g L^{-1} corn steep liquor and 20 g L^{-1} parboiled rice water), the carotenoid production was optimized and reached a maximum value of 843 µg L^{-1} (396 µg L^{-1} β-carotene) (Valduga et al., 2014).

R. mucilaginosa (360 µg L^{-1} with medium B and 230 µg L^{-1} with medium A) isolated in this study had better performance than *R. mucilaginosa* (100 µg L^{-1}) using glucose:glycerol:yeast extract (1:2), 0.5% Tween 20, 10 mM H_2O_2, according to Dhaliwal and Chandra (2015). Aksu et al. (2007), using a complex culture medium (15 g L^{-1} glucose, 2.5 g L^{-1} yeast extract, 2 g L^{-1} malt extract, 1 g L^{-1} $(NH_4)_2SO_4$, 1 g L^{-1} NaH_2PO_4 and 0.25 g L^{-1} $MgSO_4 \cdot 7H_2O$) in *R. glutinis* cultivation, reached 125 µg L^{-1}. Banzatto et al. (2013) produced 274 µg L^{-1} with *R. rubra* (40 g L^{-1} sugarcane juice, 40 g L^{-1} molasses, 40 g L^{-1} syrup, 2 g L^{-1} urea, 0.5 g Nitrofos KL/L). However, the performance of the strain of *Rhodotorula* isolated in this study showed lower carotenoid production (β-carotene and torulene) in YM medium than *R. glutinis* (881 µg L^{-1}), *R. graminis* (594 µg L^{-1}) and *R. mucilaginosa* (487-590 µg L^{-1}) (Maldonade et al., 2007). Zeni et al. (2011) isolated three yeasts, among other microorganisms, with great potential to produce red carotenoids, with total carotenoid production of 707-818 µg L^{-1} in a culture medium composed of 40 g L^{-1} glucose, 14 g L^{-1} bacto peptone and 10 g L^{-1} malt extract.

P. fermentans (500 µg L^{-1} or 40.33 µg g^{-1} medium B and 310 µg L^{-1} or 27.83 µg g^{-1} medium A), a new isolated yeast, produced 10-fold the amount of carotenoids in agroindustrial medium (40.33 µg g^{-1}), by comparison with genetically modified yeast *P. pastoris* (4 µg g^{-1}), according to Araya-Garay et al. (2012).

Brazil has a broad microorganism diversity which has been inadequately investigated. However, major development in production processes is needed before these pigments can reach the consumer market (Mezzomo and Ferreira, 2016). Native microorganisms screened in this study showed that their potential is similar to or higher than the one of those described

in the literature as microbial sources for carotenoid production in submerse cultivation. Nevertheless, the performance of these yeasts can be improved by optimizing production conditions.

Table 5 shows kinetic parameters of the yeasts *S. pararoseus, R. mucilaginosa* and *P. fermentans* in three culture media. $\mu_{máx}$ was similar or higher in media with alternative substrates than in YM medium for all yeasts. *S. pararoseus* (YM medium and medium B) and *P. fermentans* (medium A) reached 0.28 h^{-1}, while *R. mucilaginosa* reached 0.24 h^{-1} (medium B). Similar behavior was observed in biomass productivity (P_x), which showed little variation in the conditions under study (0.04-0.07 g L^{-1} h^{-1}). Maximum P_x of 0.07 g L^{-1} h^{-1} was found for *S. pararoseus* (medium B), *P. fermentans* (YM medium and medium B) and *R. mucilaginous* (medium A).

Total carotenoid productivity (P_c) for *S. pararoseus* was higher in YM medium (0.70 µg L^{-1} h^{-1}), but with sugar cane molasses (medium B, 0.62 µg L^{-1} h^{-1}), it reached 88 % of this productivity. *P. fermentans* showed high P_c in YM medium (0.31 µg L^{-1} h^{-1}), whereas that of *R. mucilaginosa* was high in medium A (0.16 µg L^{-1} h^{-1}).

Regarding conversion factors, $Y_{X/S}$ was high in YM medium for *P. fermentans* (1.13 g g^{-1}) and *R. mucilaginosa* (1.30 g g^{-1}). However, *S. pararoseus* (0.70 g g^{-1}) showed high conversion in medium A while $Y_{P/S}$ was higher in YM medium, considering all yeasts. $Y_{P/X}$ was higher in YM medium for *S. pararoseus* (19.57 µg g^{-1}) and *P. fermentans* (4.75 µg g^{-1}), even though *R. mucilaginosa* showed higher values in medium B (2.74 µg g^{-1}).

Table 5. Kinetic parameters of *S. pararoseus, P. fermentans* and *R. mucilaginosa.*

	Kinetic parameters	YM	Medium A*	Medium B*
S. pararoseus	μ_{max} (h^{-1})	0.28	0.15	0.28
	$Y_{P/S}$ (µg g^{-1})	10.30	5.60	ND**
	$Y_{X/S}$ (g g^{-1})	0.53	0.70	ND**
	$Y_{P/X}$ (µg g^{-1})	19.57	7.90	10.94
	P_X (g L^{-1} h^{-1})	0.04	0.06	0.07
	P_C (µg L^{-1} h^{-1})	0.70	0.50	0.62
P. fermentans	μ_{max} (h^{-1})	0.17	0.28	0.20
	$Y_{P/S}$ (µg g^{-1})	5.40	1.35	ND**
	$Y_{X/S}$ (g g^{-1})	1.13	0.43	ND**
	$Y_{P/X}$ (µg g^{-1})	4.75	3.16	3.50
	P_X (g L^{-1} h^{-1})	0.07	0.05	0.07
	P_C (µg L^{-1} h^{-1})	0.31	0.05	0.07
R. mucilaginosa	μ_{max} (h^{-1})	0.16	0.14	0.24
	$Y_{P/S}$ (µg g^{-1})	3.36	0.94	ND**
	$Y_{X/S}$ (g g^{-1})	1.30	0.60	ND**
	$Y_{P/X}$ (µg g^{-1})	2.60	1.55	2.74
	P_X (g L^{-1} h^{-1})	0.06	0.07	0.06
	P_C (µg L^{-1} h^{-1})	0.14	0.10	0.16

*Medium A (parboiled rice water and glycerol) and medium B (parboiled rice water and sugar cane molasses).
**ND- not determined.

Parameters under evaluation, such as $\mu_{máx}$, P_x and P_c, obtained promising results with the use of agroindustrial substrates (medium A and medium B) for *S. pararoseus, R. mucilaginosa* and *P. fermentans*, while conversion factors showed different performances.

Identification of carotenoids produced by selected yeasts

Three carotenoids produced by the selected yeasts were identified. *S. pararoseus* and *P. fermentans* produced β-carotene and β-cryptoxanthin, respectively. However, only β-cryptoxanthin was identified as having been produced by *R. mucilaginosa*. Two peaks were not identified at elution times of 21.0 and 27.7 min. Maldonade et al. (2007, 2008) also reported β-carotene in *R. mucilaginosa, R. graminis, R. glutinis, R. minuta* and *Sporobolomyces* yeasts as one of the major carotenoids. However, except in *R. minuta*, these authors also found torulene at high concentrations.

This study offers an alternative for carotenoid production, i. e., β-carotene and β-cryptoxanthin, by a new yeast (*P. fermentans*), since, in the literature, this yeast is only described as producer of this biocompound by genetically modified strains (Araya-Garay et al., 2012). In addition, it demonstrated the potential of the biodiversity found in southern Brazil.

CONCLUSION

This study isolated 64 wild yeasts from environmental samples collected in ecosystems located in southern Brazil (the Sul-Riograndense Shield and the Eastern Coast). They are good sources of microbial diversity to obtain yeasts for carotenoid production with the use of alternative agroindustrial substrates so as to minimize future costs of biopigment production, add value to these byproducts and mitigate environmental impact. *S. pararoseus* is the most promising yeast for carotenoid production, since it produced β-cryptoxanthin and β-carotene, reaching 820 µg L^{-1} in medium B (parboiled rice water and sugar cane molasses) and 710 µg L^{-1} in medium A (parboiled rice water and crude glycerol). These values were 90 and 78%, respectively, of the production obtained in YM medium. The new yeast *P. fermentans* produced the same carotenoids, reaching 48% (medium A) and 78% (medium B), by comparison with the production in YM medium. The carotenoid production with *R. mucilaginosa* was lower than the one obtained with the other yeast, with 360 µg L^{-1} (medium B, 44% above the production in YM medium) and 230 µg L^{-1} (medium A, 92% above the production in YM medium). Parameters under evaluation, such as $\mu_{máx}$, P_x and P_c, gave promising results with the use of agroindustrial substrates (medium A and medium B), while

conversion factors showed different performances. Therefore, the use of agroindustrial residues in yeast cultivation aiming at carotenoid production is more efficient, more interesting and cheaper than complex media, such as YM.

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