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Use of low-cost agro products as substrate in semi-continuous process to obtain carotenoids by *Sporidiobolus salmonicolor*

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ABSTRACT

This work has studied the semi-continuous carotenoids production by *Sporidiobolus salmonicolor* (CBS 2636) comparing the use of complex substrates and agroindustrial residues. The maximum total carotenoids concentration was 4376 μg L^{-1} in a 264 h period with complex substrates (80 g L^{-1} glycerol, 5 g L^{-1} malt extract, and 15 g L^{-1} peptone) and 7388 μg L^{-1} in a 288 h period with agroindustrial residues (80 g L^{-1} crude glycerol, 80 g L^{-1} corn maceration water, and 20 g L^{-1} rice parboiling water), showing an of approximate 54% increase, under the following conditions: 25 °C, $pH_{initial}$ of 4.0, 180 rpm agitation rate, 1.5 vvm aeration rate, with 50% working volume cut. The maximum productivity on total carotenoids was 34.8 and 41.4 g L^{-1} h^{-1}, the maximum productivity on cells was 0.058 and 0.104 g L^{-1} h^{-1}, with specific maximum growth speeds of 0.05 and 0.08 h^{-1}, obtaining the major carotenoid all-trans-β-carotene (83% and 81%), respectively.

1. Introduction

The appearance of natural or processed food is of extreme importance for its acceptance, and that is the reason why the colour is one of the most important sensorial properties (Botella-Pavía and Rodríguez-Concepción, 2006). Carotenoids are natural pigments responsible for the red, orange and yellow colors of foods, which has seen an increase in global marketplace demand at a rate of 2.9% per year, and it might reach nearly 10 million tons in 2017 (Venil et al., 2013). The industrial demand for carotenoids such as β-carotene and astaxanthin has been growing due to the large variety of its application in foods, cosmetics, and pharmaceutical industries. As well as their use as colorants, such compounds are vitamin A precursors (pro-vitamin A) and they have antioxidant properties, allowing the use of carotenoids in preventive cancer treatments, heart diseases and macular degeneration related to ageing (Venil et al., 2013; Vílchez et al., 2011; Krinsky and Johnson, 2005).

That has contributed to researchers interest on discovering new sources, processes and techniques that could be employed on the intensification of these pigments by micro-organisms, considering that carotenoids obtained by chemical synthesis involve a great number of complex reactions, while these same carotenoids are naturally found in microalgae, bacteria, yeasts and fungi (Hui et al., 2007; Bhosale et al., 2004; Fábregas et al., 2001).

A large number of substrates such as: molasses, sugarcane bagasse, whey, corn meal, corn liquor, glycerol and others, are considered potential carbon sources for the biotechnological production of carotenoids (Silveira et al., 2013; Bhatt et al., 2013; Saenge et al., 2011; Valduga et al., 2011, 2009b, 2009c, 2009d, 2009e, 2008). The study by Tinoi et al. (2005) demonstrates the effectiveness of the use of an agroindustrial residue widely available as substrate and the importance of the sequential method optimization to obtain high carotenoids yielding. Significant amounts of residues are generated on parboiled rice production, approximately 0.83 L per kg of paddy rice (Faria et al., 2006). Glycerol is the main sub-product derived from vegetable oils converted into biodiesel, corresponding to around 10% in mass of the oils fed for the process (Dasari et al., 2005). The amount of glycerol from this process exceeds the current market needs, and the use of crude glycerol by thermal, chemical and biological conversions on added value products, such as 1.3-propanediol, citric acid, carotenoids and others is an alternative (Bhatt et al., 2013; García et al., 2013; Kachrimanidou et al., 2013; Valduga et al. 2014; Cavalheiro et al., 2012; Tang et al., 2009; Zheng et al., 2008; Mu et al., 2007; Cerrate et al., 2006).

Bioprocesses are generally applied on large scale production of natural colorants due to the high cellular density and a greater

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microorganism growth rate (Gharibzahedi et al., 2012). The semi-continuous process consists of a series of sequential operations, where a part of the fermented medium is collected (fermentation cut) in sequential periods adding to the reactor a volume from the fermentation medium equal to the volume of the removed one, where the reactor's remaining fermented medium works as an inoculum to the added medium (Schimidell et al., 2007). The semicontinuous mode is often employed because, according to Ho et al. (2012), it can prevents a low rate of cell division during the early process stages, and it reduces the limitations in relation to the nutrients and light penetration during the later stages.

In the literature there are no reports of the bioproduction of carotenoids by yeasts using a semi-continuous system with agroindustrial residues, it has been reported for commercial production (Caldeira, 2015) and other products: microalgae cells (Radmann et al., 2007; Henrard et al., 2011, Da Rosa et al., 2015, Ayre et al., 2017) and orange vinegar (Ferreyra et al., 2014). In our previous work, we evaluated the production of carotenoids by *Sporidiobolus salmonicolor* (CBS 2636) in bioreactor and fed-batch using synthetic media and agroindustrial residues (Colet et al., 2015; Valduga et al., 2014, 2011, 2009b, 2009c, 2009d, 2009e). As in the literature there are few works related to semi-continuous for carotenoids production, the aim of the present study was to evaluate the kinetic and stoichiometric parameters on carotenoids bioproduction by *S. salmonicolor* (CBS 2636) in a semi-continuous system, comparing the use of complex substrate based medium and agroindustrial residues.

2. Materials and methods

2.1. Conditions of Cultivation and the Bioproduction of Carotenoids

Sporidiobolus salmonicolor (CBS 2636) (Centraalbureau voor Schimmelcultures, Utrecht, The Nederlands) was used in the bioproduction of carotenoids in a bioreactor. Initially, the lyophilized culture was hydrated in a YM medium (Yeast Malt Extract), at 25 °C for 72 h. Then, a culture was transferred to slant tubes, containing YMA medium (Yeast Malt Extract Agar), and incubated at 25 °C for 48 h. After growth, the slants were kept at 4 °C and were subcultured for 2 months each. The inoculum was prepared in Erlenmeyer flasks with 100 mL of YM medium. After sterilization, these flasks were inoculated with a suspension of cells from the stock slants and incubated at 25 °C, 180 rpm for 48 h (Valduga et al., 2008).

Carotenoid bioproduction was carried out for 480 h in a room without illumination, in a Biostat bioreactor (Braun Biotech International) with 1 L of working volume. Temperature was controlled using a water bath and pH was monitored during the bioproduction.

2.2. Agroindustrial substrates

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

The corn steep liquor was chemically pre-treated used phosphoric acid according to the methodology described by Valduga et al. (2008).

2.3. Production of Carotenoids in semi-continuous process

Based on the preliminary tests in experimental design (Colet et al., 2015), the production medium was defined for the runs in bioreactor, with agroindustrial residues comprising crude glycerol 80 g L^{-1}, corn steep liquor 80 g L^{-1} and parboiled rice water 20 g L^{-1}; with complex substrates comprising peptone 15 g L^{-1}, malt extract 5 g L^{-1} and 80 g L^{-1} glycerol, based study by Colet et al. (2015). An initial volume of 1 L of the medium bioproduction was autoclaved in the bioreactor. Crops were by 96 h, after were collected 25%, 50% and 75% of the working volume of bioreactor, this was carried out extraction of carotenoids. Then, the same amount of fresh volume medium sterilized was added to the bioreactor, maintaining the fixed settings for aeration rate (1.5 vvm), stirring rate (180 rpm), temperature (25 °C), $pH_{initial}$ 4.0, and 480 h carrying four cutting after every 96 h of bioproduction, totaling 5 cycles.

2.4. Recovery of total carotenoids

The recovery of total carotenoids was carried out according to the method described by (Valduga et al., 2009b). Cells were were subjected to successive macerationswith liquid nitrogen. Dimethylsulphoxide - DMSO (Nuclear) was subsequently added in a ratio of 2:1, and the mixture heated at 55 °C/30 min (Fanem 102) with periodic vortex homogenization (Phoenix AP-56). A solution of acetone (Quimex) and methanol (7:3 v/v) was then added, followed by centrifugation (3,000 × g) at 5 °C for 10 min. The supernatant was separated, was added 10 mL of NaCl solution at 20% (w/v) and 10 mL of petroleum ether (Dinâmica). After stirring and phase separation was carried out filtering with addition of sodium sulfate (Na_2SO_4, Merck) and a supernatant phase with withdrawal pipette aid, evaporated off in a rotary evaporator (Tecnal TE-210) at 35 °C, and the pigments dissolved in methanol (Merck).

2.5. Kinetics of the carotenoid bioproduction

The kinetics of substrate consumption (glycerol, total nitrogen and total organic carbon -TOC in the medium), cell mass, pH evolution and carotenoid production were followed by periodic sampling of the medium (24 in 24 h).

The conversion factors ($Y_{P/S}$, $Y_{X/S}$ and $Y_{P/X}$), productivity in cells and carotenoids, instantaneous and specific rates (r_x, r_p, r_{sg}, r_{sN}, μ_x, μ_p e μ_s) were determined in the different runs performed in the bioreactor at semi-continuous mode.

The conversion factor for the substrate in the product, $Y_{P/S}$ (μg carotenoids/g substrate), substrate in the biomass, $Y_{X/S}$ (g cells/g substrate), and the specific carotenoid production, $Y_{P/X}$ (μg carotenoids/g cells), were calculated by Eqs. (1)–(3), respectively (Bailey and Ollis, 1986).

$$Y_{P/S} = r_X/r_S \quad (1)$$

$$Y_{X/S} = r_X/r_S \quad (2)$$

$$Y_{P/X} = r_P/r_X \quad (3)$$

where r_x = the cell growth rate (g L^{-1} h^{-1}); r_{sg}; r_{sN}; r_{sC} = the glycerol, nitrogen and TOC consumption rate (g L^{-1} h^{-1}) respectively; r_p = the carotenoid production rate (μg L^{-1} h^{-1}).

The instantaneous productivity in cells and carotenoids in a semi-continuous system at constant volume are defined as the r_x or r_p rate, respectively. The rates of microbial growth (rx), product formation (rp) and substrate consumption (rs) can be determined by the mass balance for each component at a given time, as presented in Eqs. (4)–(6) (Bailey and Ollis, 1986).

$$r_X = d_X/dt \quad (4)$$

$$r_P = d_P/dt \quad (5)$$

$$r_S = -d_S/dt \quad (6)$$

The specific rate for growth (μ_x), product formation (μ_p) and substrate consumption (μ_s) can be obtained dividing the instantaneous rate by the cell concentration, as expressed by Eqs. (7)–(9) (Bailey and Ollis, 1986).

$$\mu_x = r_X/X \quad (7)$$