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Original Research Paper

Carotenoids production from a newly isolated *Sporidiobolus pararoseus* strain using agroindustrial substrates

Eunice Valduga*, Arthur Henrique Rausch Ribeiro, Karine Cence, Rosicler Colet, Lídia Tiggemann, Jamile Zeni, Geciane Toniazzo

Department of Food Engineering, URI – Campus de Erechim, Av. Sete de Setembro 1621, Erechim – RS 99700-000, Brazil

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ABSTRACT

This work aimed at evaluating the carotenoids production by a newly isolated *Sporidiobolus pararoseus* using agroindustrial substrates (glycerol, corn steep liquor and parboiled rice water). Bio-production was carried out in an orbital shaker, using 10% (w/v) of inoculum (25 °C, 180 rpm for 35 h), incubated for 120 h in a dark room. Liquid N_2 and dimethylsulfoxide (DMSO) were used for cell rupture and carotenoids were extracted with a solution of acetone/methanol (7:3, v/v). Optimization of carotenoids bio-production was achieved by experimental design technique. Maximum concentration of 843 μg L^{-1} (β-carotene of 396 μg L^{-1}) of carotenoids was obtained in a medium containing 40 g L^{-1} of glycerol, 40 g L^{-1} of corn steep liquor and 20 g L^{-1} of parboiled rice water, 25 °C, initial pH 4.0 and 180 rpm. The kinetic evaluation showed that the maximum concentration of total carotenoids was reached after 96 h of bio-production and that carotenoids production was associated with cell growth. The substrate consumption showed that at 96 h of bio-production consumption of 76% of total organic carbon (54% of glycerol) and 76% of nitrogen occurred.

1. Introduction

Carotenoids are pigments responsible for the yellow, orange, red and purple colors in a wide variety of plants, animals and microorganisms (Botella-Pavía and Rodríguez-Concepción, 2006). Naturally occurring carotenoids are tetraterpenoids consisting of highly unsaturated isoprene derivatives, include both hydroxylated xanthophylls (lutein, zeaxanthin, astaxanthin, α-crytoxanthin, and others) and hydrocarbon (α-, β-carotene, lycopene, and others) species. The industrial demand for carotenoid pigments, such as b-carotene and astaxanthin, is increasing due to the wide variety of applications as food coloring agents, e.g., margarine, soft drinks, and baked goods, as precursors of Vitamin A (pro-Vitamin A) in food and animal feed, as additives to cosmetics and multivitamin preparations, and in the last decade as antioxidants to reduce cellular or tissue damage (Frengova and Beshkova, 2009; Frengova et al., 2006; Venil et al., 2013). It has been found that carotenoids can inhibit various types of cancer and guard one from other important "lifestyle-related" diseases, such as cardiovascular disease and age-related macular degeneration due to their antioxidant activity and provitamin A function (El-Agamey et al., 2004; Andrew and Young, 2001; Sotirios and Vassiliki, 2006).

Increasing health-consciousness among consumers is now an incentive for the search for functional foods, and it is likely to trigger an increase in the demand for carotenoids in the food industry. The global market demand for carotenoids has been growing at a rate of 2.9%/year, and is expected to reach almost 10 million tons by 2017 (Venil et al., 2013). However, most of the commercial carotenoids are derived from chemical synthesis and cannot meet consumers' desire for natural carotenoids.

Thus, the attention has shifted from chemical synthesis to the isolation of carotenoids from biological sources, including microbial production, using *Rhodobacter sphaeroides* (Gu et al., 2008), *Dunaliella salina* (Aguilar et al., 2004), *Dunaliella tertiolecta* (Fazeli et al., 2006), *Chlorella zofingiensis* (Po-Fung and Feng, 2005), *Haematococcus pluvialis* (García-González et al., 2005; Dufossé et al., 2005), *Blakeslea trispora* (Johnson and Schroeder, 1995), *Rhodospirillum rubrum* (Goodwin, 1980), *Xanthophyllomyces dendrorhous* (Hu et al., 2006), *Rhodotorula glutinis* (Aksu and Eren, 2007; Schneider et al., in press), *Rhodotorula mucilaginosa* (Aksu and Eren, 2005; Irazusta et al., 2013), *Sporobolomyces roseus* (Davoli and Mierau, 2004), *Sporobolomyces ruberrimus* (Razavi and March, 2006), *Sporobolomyces* sp. (Maldonado et al., 2008), *Sporidiobolus salmonicolor* (Valduga et al., 2008, 2009a,b,d, 2011) and *Phaffia rhodozyma* (Lim et al., 2002; Liu et al., 2006; Bhatt et al., 2013).

Numerous substrates (such as molasses, sugarcane bagasse, whey, corn bran, corn steep liquor, glycerol and others) have been considered as potential carbon sources for biotechnological

* Corresponding author. Tel.: +55 54 35209000; fax: +55 54 35209090.
E-mail address: veunice@uricer.edu.br (E. Valduga).

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production of carotenoids (Valduga et al., 2008, 2009a,b,c,d, 2011; Bhatt et al., 2013; Saenge et al., 2011; Silveira et al., 2013). Work of Tinoi et al. (2005) demonstrates the effectiveness of using a widely available agroindustrial waste product as substrate and the importance of the sequential simplex optimization method in obtaining high carotenoid yields. In parboiled rice are generated significant amounts of waste, approximately 0.83 L kg^{-1} of paddy rice (Faria et al., 2006). Glycerol is the main by-product of the conversion of oils into biodiesel, comprising approximately 10% by mass of the oils fed to the process (Dasari et al., 2005). The increased production of biodiesel has caused a sudden increase in the production of glycerol creating a glut in the glycerol market (Johnson and Taconi, 2007). Researchers around the world (Saenge et al., 2011; Cerrate et al., 2006; Zheng et al., 2008; Tang et al., 2009; Mu et al., 2007) are currently looking at the thermal, chemical, and biological conversion of crude glycerol to a variety of value-added products such (1,3-propanediol, citric acid, carotenoids and others).

In order to improve the yield of carotenoid pigments and subsequently decrease the cost of this biotechnological process, diverse studies have been performed by optimizing the culture conditions including nutritional and physical factors. Factors such as nature and concentration of carbon and nitrogen sources, minerals, vitamins, pH, aeration, temperature, light and stress have a major influence on cell growth and yield of carotenoids (Aksu and Eren, 2005; Hu et al., 2006; Razavi and March, 2006; Valduga et al., 2009d; Liu et al., 2006; Saenge et al., 2011; Tinoi et al., 2005; Buzzini et al., 2005). Thus, the development of optimization studies regarding the composition of the medium for the production of carotenoids is necessary to reduce costs and improve yields. In this context, this work aimed at evaluating the total carotenoids production by newly isolated *Sporidiobolus pararoseus* strain using agroindustrial substrates (glycerol, corn steep liquor and parboiled rice water).

2. Materials and methods

2.1. Conditions of cultivation and bio-production of carotenoids

The yeast used in the runs bio-production of carotenoids using agroindustrial residues (glycerol, corn steep liquor and parboiled rice water) was isolated from samples of orange and eucalyptus leaves and identified as *S. pararoseus* (Zeni et al., 2011; Cabral et al., 2011).

The inoculum was prepared in Erlenmeyer flasks of 250 mL with 100 mL of YM medium (Yeast Malt Extract). After sterilization, these flasks were inoculated with a suspension of cells from the stock slants and incubated at 25 °C, 180 rpm for 35 h (D.O.$\sim$1.0, $\sim 1 \times 10^6$ UFC mL^{-1}).

Bio-production tests were carried out in an orbital shaker (Nova Ética DRB-430) with stirring rate and temperature controlled. The 250 mL Erlenmeyer flasks contained 100 mL of fermentation medium and 10% (v/v) of inoculum ($\sim 1 \times 10^6$ UFC mL^{-1}) and were incubated for 12 h in the dark. Experimental design technique was used to study the effects of medium composition and the operational conditions.

2.1.1. 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 and crude glycerol were chemically pretreated used phosphoric acid according to the methodology described by Valduga et al. (2008). The corn steep liquor was used at concentrations of 100 g L^{-1}. The pH value of the corn steep liquor and crude glycerol were first adjusted to 3.0 using 1 mol L^{-1} phosphoric acid (Nuclear). The substrates were left for 24 h at 24 °C and centrifuged (Eppendorf 5403) at 5000 rpm for 15 min. Finally the pH value was adjusted to 5.5, using a 2 mol L^{-1} NaOH solution (Vetec).

2.1.2. Experimental design

The effects of the composition of the culture medium and the bioproduction conditions were assessed by a central composite rotatable design (CCRD) 2^3, and the independent variables investigated were glycerol (30–50 g L^{-1}), corn steep liquor (10.1–40 g L^{-1}) and parboiled rice water (8–32 g L^{-1}). Temperature (25 °C), initial pH (4.0) and stirring rate (180 rpm) were set at fixed levels, based study by Cabral et al. (2011). After statistical analysis, a second experimental design (full 2^2) was performed keeping constant pH (4.0), temperature (25 °C), stirring rate (180 rpm) and parboiled rice water (20 g L^{-1}). The independent variables (factors) studied were glycerol (0–80 g L^{-1}) and corn steep liquor (0–40 g L^{-1}).

The responses or dependent variables studied were: total carotenoids (μg L^{-1}), specific production of carotenoids (μg g^{-1}), biomass (g L^{-1}) and pH.

2.1.3. Recovery of total carotenoids

The recovery of total carotenoids was carried out according to the methodology described by Valduga et al. (2009c). The cells were centrifuged at (3000*g*), at 5 °C for 10 min (Eppendorf 5403), the residual glycerol in the cells was removed with diethyl ether and submitted to successive macerations in a pestle and mortar after freezing in liquid nitrogen. Dimethylsulfoxide – 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 (3000*g*) at 5 °C for 10 min. The supernatant was separated and successive extractions carried out until both solvent and cells were colorless. The solvent was evaporated off in a rotary evaporator (Tecnal TE-210) at 35 °C and the pigments dissolved in methanol (Merck).

2.1.4. Kinetics of the bio-production

Knowledge of the kinetics of a fermentative process is of extreme importance when transposing a laboratory experiment to an industrial scale, as well as when a quantitative comparison between different culture conditions is required (Hiss, 2001; Bailey and Ollis, 1986). 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.

2.2. Analytical methodology

2.2.1. Determination of total carotenoids

The absorbance of the sample after the extraction was measured in a spectrophotometer (Agilent UV-8553). The concentration of total carotenoids was estimated by the maximum absorbance at 448 nm, using the equation described by Davies (1976). The coefficient of absorbance used was that referent to β-carotene: $E^1\%$ 1 cm=2592, for petroleum ether and $E^1\%$ 1 cm=2550, for methanol.

Concentration of carotenoids was expressed in terms of total carotenoids (μg L^{-1}) and specific production of carotenoids (μg g^{-1}). The specific production of carotenoids represents the total concentration of carotenoids (μg) in relation to the biomass of dried yeast obtained in 1 L of a fermented medium.