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Optimization of canthaxanthin production by *Dietzia natronolimnaea* HS-1 from cheese whey using statistical experimental methods

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Abstract

Sequential methodology combining a screening test by fractional factorial design and an optimization test by central composite design was applied to enhance canthaxanthin production by *Dietzia natronolimnaea* HS-1 from cheese whey in shake flask cultures. Six variables (pH, luminous intensity, inoculum percent and concentrations of whey lactose, yeast extract and KH_2PO_4) were studied with the screening test. The results revealed that three factors of pH, concentrations of whey lactose and yeast extract had greater influence on the canthaxanthin production ($P < 0.01$). The central composite design was then used to determine the maximum canthaxanthin concentration. The optimum conditions for the highest canthaxanthin production (2871 ± 76 μg/l) were pH 7.66, whey lactose concentration 55.54 g/l and yeast extract concentration 7.36 g/l.

Keywords: Canthaxanthin; *Dietzia natronolimnaea*; Growth kinetic; Whey lactose; Optimization; Response surface methodology

1. Introduction

Carotenoids are yellow to red pigments that occur widely in nature. More than 700 different carotenoids are synthesized by animal, plant and microbial species [1]. Among them, canthaxanthin (4,4′-diketo-β-carotene) is a ketocarotenoid that is responsible for the orange-red color of egg yolks and the flesh of many marine animals [2]. The properties of canthaxanthin as anti-oxidant [3], anti-cancer agents [4], anti-tumor or anti-dermatosis [5], immune response stimulants [6] and coloring agents [7] are well known. Because of these properties, canthaxanthin is widely used in medical, pharmaceutical, cosmetic, chemical and food industries [8].

Commercial demand for canthaxanthin is mainly fulfilled by chemical synthesis. However, in food, cosmetic and pharmaceutical industries, the application of synthetic compounds is restricted due to their possible toxic effects [9]. Therefore, production of canthaxanthin using carotenoid-produced microorganisms has received increasing attention [10]. At present, canthaxanthin has been found in several microorganisms, including the bacteria *Micrococcus roseus* [8], *Rhodococcus maris*, *Brevibacterium* KY-4313 [2], *Bradyrhizobium* strains [11], *Gordonia jacobaea* [12], and *Dietzia natronolimnaea* HS-1 [13]; various algae including *Dictyococcus cinnabarinus* [14], *Chlorella emersonii* [15], and *Chlorella zofingiensis* [16]; and the halophilic archaeon *Haloferax alexandrinus* [10]. Among them, *Brevibacterium* KY-4313 and *D. natronolimnaea* HS-1 bacteria are reported as the most promising sources for biological production of canthaxanthin [2,13]. Canthaxanthin production by microbial sources can become industrially feasible if the cost of production is minimized by use of agro-industrial by-products such as carbohydrate sources [17]. A great variety of by-products has been used by microorganisms for carotenoid production such as grape must [18], beet molasses [19], sugarcane molasses [20], glycerol [21], peat extract [22] and cheese whey [23–25]. Whey is a liquid by-product of the cheese industry and its disposal without expensive sewage treatments represents a major source of water pollution [26,27]. Whey contains approximately 4.5% lactose, 0.8% protein, 1% salt, 0.1–0.8% lactic acid and some vitamins. Among these, whey lactose is a suitable carbon source for many microorganisms. Therefore, use of whey as an inexpensive medium for fermentation processes has long been of industrial interest. Nowadays, products such as ethanol, lactic acid, acetic acid, and single cell protein have been achieved through fermentation of whey lactose. Cheese whey may be a low-cost and favourable carbon source for carotenoid production by fermentation [25]; however, its

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utilization for the production of canthaxanthin has not been reported.

Optimization of the medium and environmental conditions is necessary in microbial fermentations to fully exploit the potential of selected microbial strains [28]. Statistical experiment design provides an efficient approach to optimization. Fractional factorial design is especially suitable to account for the interactions and identify the most significant components in the medium formula [29]. A combination of factors generating a certain optimum response can be identified through factorial design and the use of response surface methodology. The response surface methodology is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and searching optimum conditions of factors for desirable responses [30]. This method was successfully applied in many areas of biotechnology, including some recent studies on carotenoid production [31–33].

The aim of this study was optimization of canthaxanthin production by *D. natronolimnaea* HS-1 from cheese whey using statistical experimental methods. The effect of variables (pH, luminous intensity, inoculum percent and concentrations of whey lactose, yeast extract and KH_2PO_4) on the production of canthaxanthin was investigation by combining fractional factorial design and response surface methodology.

2. Materials and methods

2.1. Materials

The glucose, all nitrogen sources, KH_2PO_4 salt and agar were all obtained from the Sigma–Aldrich Chemical Company (USA). The pure ethanol (99.9%) was purchased from the Bidestan Company (Iran), the canthaxanthin standard supplied by Dr. Ehrenstorfer GmbH (Germany), and the acetonitrile and methanol were HPLC grade from Merck (Germany).

2.2. Microorganism

The strain *D. natronolimnaea* HS-1 (DSM 44860) was used and maintained on yeast/malt (YM) agar plates containing: 10 g/l glucose, 5 g/l peptone, 3 g/l malt extract, 3 g/l yeast extract and 15 g/l agar. Single colonies were transferred to a fresh plate every month, incubated for 4 days, and thereafter kept under refrigeration at 4 °C.

2.3. Preparation of inoculum

A pure culture of *D. natronolimnaea* HS-1 from the YM agar was transferred into 500-ml Erlenmeyer flasks containing 100 ml cheese whey (45 g/l lactose; supplemented with 6 g/l peptone and 6 g/l yeast extract), incubated in a rotary shaker (200 rpm) at 30 ± 1 °C, and after 5 days used as the inoculum.

2.4. Culture conditions

Calculated amounts of inoculum (according to the experimental designs) were transferred into 500-ml Erlenmeyer flasks containing 100 ml cheese whey supplemented with yeast extract and KH_2PO_4 salt. The concentration of whey lactose, yeast extract and KH_2PO_4 were considered according to the experimental designs. The flasks were incubated in a rotary shaker (200 rpm) at 30 ± 1 °C for 8 days. The culture conditions varied according to the experimental designs. To study the effect of light on the canthaxanthin production, light was provided by white fluorescent tubes. Some of Erlenmeyer flasks were covered with aluminium foils making them impermeable to light.

2.5. Cheese whey preparation

Cheese whey was obtained from Sahar Company (Ghazvin, Iran). Deproteinization was carried out by heat treatment (121 °C, 15 min) of the acidified (pH 5) cheese whey. The precipitates were removed by centrifugation at 4 °C and 8000 × *g* for 10 min. The clear liquid was diluted or concentrated as necessary and used.

2.6. Dry weight and whey lactose measurement

Biomass dry weight was determined by harvesting 5-ml culture samples, filtering the cells through 0.2-μm filter (Sigma–Aldrich Co., USA) (dried at 65 °C for 4 h), washing the cells with distilled water, and drying them at 105 °C to a constant weight (48 h). Meanwhile, the whey lactose was measured using an HPLC method.

2.7. Extraction and analysis of carotenoids

10-ml aliquots were centrifuged at 5000 × *g* for 10 min at 4 °C. The pellets were then washed twice with a solution of 9 g/l NaCl and centrifuged again. Next, the supernatant was resuspended in 3 ml of pure ethanol by vortexing for 5 min, and the pellets centrifuged again to extract the pigment. This was repeated three times. Thereafter, the pigments were completely extracted using a water bath (45 °C), and the carotenoid extracts subsequently filtered through a 0.2 μm hydrophobic fluorophore membrane (Sigma–Aldrich Co., USA) and analyzed by scanning the absorbance of the wavelength spectra of 300–600 nm using a spectrophotometer (UV-Visible, Cary 300, Varians Co., Germany). The maximum absorbance was determined at a wavelength of 474 nm, which conformed to standard canthaxanthin λ_{max}. The total carotenoid concentration was calculated following the formula provided by An et al. [34], and the concentrations of individual carotenoids determined according to the modified method of Razavi et al. [35], using an HPLC (Knauer, Germany) equipped with a UV–visible detector (K-2600, Knauer, Germany) and pump (K-1001, Knauer, Germany). The chromatographic separation was performed on a Nucleosil 100 C18, 5.0 μm (125 × 4.0 mm), where the temperature of the column was maintained at room temperature and the mobile phase was acetonitrile: methanol (80:20, v/v) at a flow rate of 1 ml/min. The eluant was monitored at 480 nm. To protect the column, a pre-column (5 × 4.0 mm) of the same material was also used. The volume of the injected solutions was 100 μl.