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Efficient extraction of canthaxanthin from *Escherichia coli* by a 2-step process with organic solvents

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ABSTRACT

Canthaxanthin has a substantial commercial market in aquaculture, poultry production, and cosmetic and nutraceutical industries. Commercial production is dominated by chemical synthesis; however, changing consumer demands fuel research into the development of biotechnology processes. Highly productive microbial systems to produce carotenoids can be limited by the efficiency of extraction methods. Extraction with hexane, acetone, methanol, 2-propanol, ethanol, 1-butanol, tetrahydrofuran and ethyl acetate was carried out with each solvent separately, and subsequently the most efficient solvents were tested in combination, both as mixtures and sequentially. Sequential application of methanol followed by acetone proved most efficient. Extraction efficiency remained stable over a solvent to biomass range of 100:1 to 55:1, but declined significantly at a ratio of 25:1. Application of this method to a canthaxanthin-producing *Escherichia coli* production system enabled efficient canthaxanthin extraction of up to 8.5 mg g^{-1} dry biomass.

1. Introduction

Carotenoids consist of a large and structurally diverse group of natural products represented by >600 known compounds. Canthaxanthin (β,β-carotene 4,4′-dione), a direct product of β-carotene diketolation, is synthesized by many organisms, including microalgae, fungi and bacteria (Asker et al., 2002; Asker and Ohta, 1999; Hannibal et al., 2000; Hua-Bin et al., 2006; Khodaiyan et al., 2007; Krupa et al., 2010; Lorquin et al., 1997; Nasri Nasrabadi and Razavi, 2010; Nelis and De Leenheer, 1989; Takaichi et al., 2009; Tao and Cheng, 2004; Veiga-Crespo et al., 2005). Canthaxanthin is approved for application in both animal feed and food for human consumption, and as a result is used widely as a feed supplement in poultry and aquaculture farms, as well as in cosmetics, nutraceuticals and food products for human consumption (Aguilar et al., 2010; Beardsworth and Hernandez, 2003).

When consumed by laying hens, broilers, salmon and trout, canthaxanthin deposition results in highly desirable pigmentation of flesh and/or eggs. The cultivation of farmed salmon and trout employs canthaxanthin at 25 mg per kg^{-1} feed (maximally), while both broiler and laying hen feed is composed of up to 8 mg per kg^{-1} (Beardsworth and Hernandez, 2003). Canthaxanthin's estimated global market for 2010 was approximately US$154 million, which represents approximately 6% of the world market of carotenoids (Global Industry Analysts, 2006). Although the application of pelletized biomass containing canthaxanthin eliminates the requirement for extraction, the production of canthaxanthin for cosmetic, nutraceutical, pharmaceutical and food products (destined for human consumption) require production and extraction of high purity canthaxanthin.

Commercial production of canthaxanthin is currently dominated by chemical synthesis (Bohsale and Bernstein, 2005); however, changing consumer demands and the desire for enantiopure products is driving investigations into biological production of canthaxanthin. Several microorganisms that naturally synthesize canthaxanthin have been evaluated for their potential as commercial production hosts, such as *Gordonia jacobaea*, *Sporidiobolus salmonicolor*, *Dietzia natronolimnaea* and *Bradyrhizobium* sp. (Lorquin et al., 1997; Valduga et al., 2009; Veiga-Crespo et al., 2005). Since low yields have limited the commercial appeal of such strains, metabolic engineering studies have been completed, targeting increased canthaxanthin biosynthesis (Das et al., 2007; Zhu et al., 2009). However, commercial feasibility of a biological production system is still inhibited by a paucity of efficient extraction methods. Such methods must achieve nearly complete canthaxanthin extraction in minimal time with minimal solvent volume.

Efficient carotenoid extraction is dependent on the nature of the biomass containing the target carotenoid, the carotenoid compound itself, and the specific extraction conditions such as solvent type, solvent to biomass ratio, extraction temperature and particle

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size (Gu et al., 2008; Krupa et al., 2010). Solvent based extraction methods typically employ acetone, hexane, ethanol, tetrahydrofuran, ethyl acetate, individually or in combination (Papaioannou et al., 2008; Roukas and Mantzouridou, 2001; Schiedt and Liaaen-Jensen, 1995). Ultrasonic (Macias-Sanchez et al., 2009), acid-based (Ni et al., 2008) and supercritical CO_2 extraction (de Moraes et al., 2006; Jaime et al., 2007; Krichnavaruk et al., 2008; Macias-Sanchez et al., 2009; Ota et al., 2009) methods have also been developed, as have biomass pre-treatments (Papaioannou et al., 2008), including acid/alkali and enzyme treatments, and exposure to heat and mechanical disruption forces (Li et al., 2006; Mendes-Pinto et al., 2001; Ni et al., 2008). In spite of extensive research, protocols remain inefficient, requiring prolonged contact between biomass and solvent, or repeat exposure of the carotenoid containing biomass to the extraction solvent to achieve efficient extraction. This inefficiency represents a significant limitation to the commercial application of such methods.

Our research goal was the development of a suitable process for the potential commercial production of canthaxanthin from genetically engineered *Escherichia coli*. Presented here are steps taken in the development of a rapid protocol for the complete extraction of canthaxanthin from *E. coli* biomass.

2. Methods

2.1. Biomass production

Unless otherwise stated, all chemicals, reagents and media components were purchased from Sigma–Aldrich (USA). Sufficient biomass to allow direct comparison of extraction protocols was generated via cultivation of a genetically modified *E. coli* MG1655. Briefly, chromosomal over-expression of limiting genes within the native non-mevalonate pathway facilitated the biosynthesis and accumulation of large quantities of carotenoids under fermentation conditions (unpublished data). Canthaxanthin biosynthesis was achieved through ligation of the *Anabaena variabilis* ATCC 29413 derived β-carotene ketolase into plasmid pAC-Beta, kindly supplied by Dr. F.X. Cunningham from the University of Maryland, College Park, MD (Cunningham et al., 1996) (unpublished data). Biomass production was achieved by the completion of two batch fermentations. Briefly, a seed culture was prepared by picking several individual colonies from a Luria–Bertani (miller) (LB) agar plate (Sigma–Aldrich, USA), supplemented with chloramphenicol (50 μg ml^{-1}). Biomass was used to inoculate 25 ml LB broth, containing chloramphenicol (50 μg ml^{-1}), within a 100-ml conical flask. The seed culture was incubated at 30 °C, 150 rpm, for 16 h. Ten milliliters of seed culture was used to inoculate each B-DCU 2 L bioreactor (Sartorius, USA), containing 1.5 L of LB (miller) broth (Sigma–Aldrich, USA), supplemented with glycerol (1.3, w/v) and chloramphenicol (50 μg ml^{-1}). Fermentation conditions were as follows: temperature was maintained at 37 °C for 2.5 h and subsequently reduced manually to 30 °C for further 22 h. Dissolved oxygen was maintained at 25% throughout via an agitation control loop. pH control and feed strategies were not employed. Biomass was harvested via the removal of 50 ml aliquots from the fermentor into sterile 50-ml tubes (pre-weighed). Biomass was pelleted via centrifugation, 900*g*, 20 min, at 4 °C. The supernatant was removed and cell pellets lyophilized. Biomass was stored at −80 °C until extraction.

2.2. Basic extraction protocol

All carotenoid extractions were completed in duplicate, unless otherwise stated. Carotenoid extraction proceeded via modification to established protocols (Armenta et al., 2006; Scaife et al., 2009). Briefly, solvents were pre-chilled to 4 °C and employed in predefined volumes (see Sections 2.4–2.7). Solvents were added to the weighed biomass samples, and biomass disrupted by mechanical grinding with a glass rod, combined with vigorous vortexing, to visual homogeneity. Samples were subsequently sonicated in an ice-water bath for 5 min, vortexed for an additional 30 s and centrifuged at 900*g* for 20 min at 4 °C. Extracts were transferred to chilled amber glass 30-ml screw-top vials, where applicable extracted carotenoids were dried under argon, and resuspended in 500 μl methanol. Two hundred and fifty microliters aliquots of samples destined for HPLC analysis were transferred to 1.7-ml microcentrifuge tubes, centrifuged at 900*g*, 5 min, and submitted to HPLC analysis. This procedure was employed for all subsequent extractions, with modification to biomass and solvents as described in the relevant sections.

2.3. Extraction 1

Two hundred milligrams lyophilized biomass was weighed into a sterile 50-ml centrifuge tube for each extraction and 10 ml of each solvent: hexane, acetone/methanol (7:3), 2-propanol, 95% ethanol, 1-butanol, tetrahydrofuran (THF), ethyl acetate or methanol, were added. Methanol was chosen over acetone due to methanol's relatively similar but still greater polarity (Kerton, 2009), which would be more effective for a targeted extraction of canthaxanthin, a xanthophyll with two ketonic groups that provide significant polarity to this carotenoid. Extraction was performed as described in Section 2.2, and repeated until total carotenoid extraction was achieved, based on visual examination of the biomass and extraction solvent, to a maximum of three repeats. Three milliliters of the total extract was dried under argon, and samples processed for HPLC analysis.

2.4. Extraction 2

Based upon results of Section 2.3, methanol and acetone were investigated further. Twenty-five milligrams biomass was weighed into a 15-ml falcon tube for each extraction. This assay employed methanol and acetone in sequential combinations. Following the addition of solvent 1 (Table 1), samples were processed as described in Section 2.2. Following the transfer of Extract 1 to a 30-ml screw top amber glass vial, solvent 2 (Table 1) was added to the biomass, and extraction repeated. Extracts were combined for each sample. A 250-μl aliquot was processed for HPLC analysis as described in Section 2.2.

2.5. Effect of solvent to biomass ratio

Briefly, 200 mg dry biomass were employed, extraction was achieved by the sequential addition of different volumes of methanol followed by acetone (Table 2). Extraction was completed as described in Section 2.2. Following extraction, carotenoid containing solvents were combined for each sample. A 250-μL aliquot was taken from each, and processed for HPLC analysis.

Table 1
Conditions for extraction via sequential or combined solvents.

Sample	Solvent 1	Solvent 2
1	5 ml Methanol	2 ml Acetone
2	3 ml Methanol	2 ml Acetone
3	5 ml Methanol mixed with 2 ml acetone	N/A
4	2 ml Acetone	3 ml Methanol