Process of extraction and isolation of canthaxanthin from saline bacteria biomass Paracoccus carotinifaciens VTP20181 isolated in Vietnam

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Abstract
Canthaxanthin is a natural carotenoid, which can be biosynthesized from several different bacterial strains and exhibits a wide range of biological benefits such as antioxidant, immune booster, and vascular stabilization. Canthaxanthin is widely used in the food and pharmaceutical industries as a natural colourant, and its production from natural sources has been the topic of growing interest. In this study, we established the process of extracting, enriching and isolating canthaxanthin from the post-fermentation biomass of the saline bacteria Paracoccus carotinifaciens VTP20181 isolated in Vietnam at laboratory scale. This process consists of 5 steps: Dry biomass generation, dry biomass extraction, removal of saturated fat, fast column chromatography and slow column chromatography, canthaxanthin purification. Canthaxanthin compound is obtained with 98% purity. Canthaxanthin extraction and purification efficiency of the whole process reached 55.5%.

Keywords: Canthaxanthin, Paracoccus carotinifaciens VTP20181, extraction process.

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INTRODUCTION

Special attention is being paid to natural pigments that replace beta-carotene derived synthetic pigments such as canthaxanthin, astaxanthin, lycopene, lutein with many biological activities such as antioxidant, colouring, and resistance enhancement both in research and commerce [1, 2]. Experimental studies and clinical evaluation of beta carotene and its derivatives such as canthaxanthin, astaxanthin, lutein have demonstrated the effects of this group on customer health. Carotenoids act as antioxidants, remove free radicals, strengthen cell walls as well as protect cells, thereby enhancing immunity, anti-tumour, support in treating cardiovascular disease and obesity [3–6]. Many surveys show that the most popular and most economically valuable fields of application for these pigments are food, pharmaceutical, cosmetic and livestock industries. The total value of carotene derived products worldwide in 2015 reached 1.21 billion USD, increasing gradually 3–5% each year. By 2025, the commercial value of these products is estimated at USD 2 billion [7].

Canthaxanthin (4,4'-diketo-β-carotene) is a carotenoid whose polyene backbone structure is more stable in the cell than active compounds with a single polyene backbone structure, thus providing a better antioxidant effect [8–10]. This compound is found in several plants, crayfish, salmon, birds, fungi, marine algae and bacteria. Canthaxanthin was discovered for the first time in an edible fungus called Paracoccus carotinifaciens [11, 12]. Canthaxanthin can be obtained from biosynthesis or chemical synthesis. In recent years, canthaxanthin isolated from microbiological sources has developed dramatically due to restrictions on the use of chemically synthesized carotenoids in the food and pharmaceutical industries. Compared with chemically synthesized canthaxanthin, microorganism-extracted canthaxanthin is of importance due to its safety and does not depend on factors such as geography, season.

The group of microorganisms that contain important canthaxanthin are bacteria (e.g: Dietzia natronolimnaea HS-1, Micrococcus roseus and Rhodococcus maris) and green microalgae (Clorococcum sp. MA-1, Chlorella emersonii, Chlorella zoofingiensis and Dictyococcus cinnabarinus). In which, canthaxanthin synthesized from D. natronolimnaea HS-1 takes more than 90% of total obtained carotenoid [13]. According to Razavi et al., canthaxanthin is the dominant pigment in this bacterium. When batch cultured on fermentation equipment, canthaxanthin content reached 2.87 mg/l and when batch fermented with additional medium, canthaxanthin content increased to 13.17 mg/l [14]. Currently, in Vietnam, several strains capable of producing high content of canthaxanthin have also been isolated such as Staphylococcus CNTP 4191, Staphylococcus CNTP 4192, Haloferax alexandrinus NBRC 16590 and Paracoccus carotinifaciens VTP20181. Based on the information above, the important role of the extraction and isolation of canthaxanthin from microorganisms and their biomass in the food and pharmaceutical industries is proven. In this study, we present the process of extraction and isolation of canthaxanthin at laboratory scale from biomass of bacteria Paracoccus carotinifaciens VTP20181 isolated in Vietnam.

TECHNOLOGICAL PROCESS OF EXTRACTING AND ISOLATING CANTHAXANTHIN FROM BIOMASS OF BACTERIA Paracoccus carotinifaciens VTP20181

Material

Biomass of the bacterium Paracoccus carotinifaciens VTP20181 obtained after fermentation was provided by the Food Industry Research Institute. Biomass was refrigerated (−40°C) to prepare for extraction. The biomass solution is red brown, the moisture content is 80% and the canthaxanthin content is 0.3 mg/g of wet biomass.

Equipment, solvents and chemical reagents

High-pressure liquid chromatograph system, analysis column C18 (4.6 mm × 250 mm, particle size 5 µm), detector PDA or UV-Vis, wavelength 475 nm, pump LC-20AD, solvent channel B: methanol (0.1% acetic acid), solvent channel A: acetonitrile.
Process of extraction and isolation of canthaxanthin

Column chamber temperature 30°C, flow rate 1 ml/min, injection volume 20 µl, column pressure 176 kgf/cm².

Rotary evaporator EYELA N-1200 A, magnetic stirrer, Hettich centrifuge, chromatographic column, vacuum pump, Buchner filter hopper, UP2000Ht probe ultrasound.

Solvents, chemical reagents: liquid nitrogen, argon gas, distilled water, acetone, n-hexane, methanol, acetonitril, glycerol monostearate (GMS), urea, 2 times distilled water.

Research diagram

The research process diagram is shown in figure 1 including 5 main steps: Dry biomass generation, total extract collection, fat removal using urea crystallization method, flash column chromatography to collect canthaxanthin-rich inoculants and column chromatography to obtain purified canthaxanthin.

Figure 1. Diagram of the research process for extracting and isolating canthaxanthin
**Experimental process**

*Dry biomass generation*

About 5,000 g of wet biomass was dissolved with water at the ratio (1/2, w/w), 5% maltodextrin carrier was added to the wet biomass weight and stirred to create a homogeneous mixture. Spray drying was carried out to collect dry biomass. The technology parameters of the spray drying process are as follows: inlet and outlet hot air temperature is 1,650°C/850°C; the refuelling rate is 2.5 litres/hour, the rotation speed is 20,000 rpm. 1,250 g of dry biomass flowing powder was obtained with moisture 6%.

*Extract of dry biomass*

1,000 g of dry biomass was extracted using ultrasound with mixture solvent (ethanol 96 + 0.5% glycerol monostearate (GMS)) with solvent/material ratio (v/w) of 5/1. The extraction was repeated 3 times. Ultrasonic wattage is 120 W, ultrasonic time is 90 minutes/extraction, extraction temperature is 30°C. The extracts were combined and purified using a Buchner funnel. Solvents were evaporated to obtain 45.5 g of total extract containing canthaxanthin.

*Removal of saturated fat*

40 g of total extract containing canthaxanthin and saturated fatty acids were crystallized with saturated urea in ethanol 96% according to the following technological parameters: the ratio of ethanol/total fatty acids 6/1 (v/w), urea/total fatty acids ratio 3.5/1 (w/w), crystallization temperature 0°C, crystallization time 12 h. Filtration was carried out and crystallization was removed to obtain an aqueous solution. Solvent was evaporated to obtain 38.2 g of fat removed total extract.

*Flash column chromatography*

Flash column chromatography was performed using diethyl ether solvent. 35 g of total extract was diluted with ethanol and mixed with silica gel. The mixture was subjected to column chromatography, eluted with diethyl ether acetone (6/1 → 3/1, v/v) to give 6 fractions C1 → C6. The presence of canthaxanthin in fractions was checked using thin layer chromatography (TLC). The fractions containing pure canthaxanthin were collected, evaporated to obtain 345 mg of pure canthaxanthin. The purity was evaluated using high performance liquid chromatography (HPLC) and its physicochemical data was compared. Canthaxanthin extraction and purification efficiency of the whole process reached 55.5%. Canthaxanthin purity reached over 98%.

*Column chromatography, canthaxanthin purification*

1.1 g of an extract was dissolved with analytical ethanol, then mixed with silica gel. The mixture was subjected to column chromatography, eluted with n-hexane/acetone (6/1 → 3/1, v/v) to give 6 fractions C1 → C6. The presence of canthaxanthin in fractions was checked using thin layer chromatography (TLC). The fractions containing pure canthaxanthin were collected, evaporated to obtain 345 mg of pure canthaxanthin. The purity was evaluated using high performance liquid chromatography (HPLC) and its physicochemical data was compared. Canthaxanthin extraction and purification efficiency of the whole process reached 55.5%. Canthaxanthin purity reached over 98%.

**The physicochemical parameters of canthaxanthin**

The physicochemical parameters of this compound were determined using different physical and chemical methods as follows:

- ESI-MS mass spectrometry: m/z = 565 [M + H]+;
- Molecular formula: C_{40}H_{52}O_2;
- State: red solid;
- Melting point: 211–213°C;
- Maximum absorption at the wavelength $\lambda_{\text{max}}$ 470 nm;
- Solubility: insoluble in water, well soluble in acetone, chloroform.

Nuclear magnetic resonance spectrum was determined on a 500 MHz Bruker nuclear magnetic resonator at the Institute of Chemistry - Vietnam Academy of Science and Technology.

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 1.86 (2H, m, H-2, 2’), 2.51 (2H, m, H-3, 3’), 6.25 (2H, m, H-7, 7’), 6.36 (2H, d, H-8, 8’), 6.27 (2H, m, H-10, 10’), 6.68 (2H, m, H-11, 11’), 6.40 (2H, d, H-12, 12’), 6.29 (2H, m, H-14, 14’), 6.65 (2H, m, H-15, 15’), 1.20 (6H, s, H-16, 16’), 1.20 (6H, s, H-17, 17’), 1.88 (6H, s, H-18, 18’), 2.00 (6H, s, H-19, 19’), 2.18 (6H, s, H-20, 20’).

$^{13}$C NMR: δ 198.7 (C=O), 160.9 (C-6, 6’), 141.1 (C-8, 8’), 139.3 (C-12, 12’), 136.6 (C-13, 13’), 134.8 (C-9, 9’), 134.3 (C-10, 10’), 133.6 (C-14, 14’), 130.5 (C-15, 15’), 129.9 (C-5, 5’), 124.7 (C-11, 11’), 124.2 (C-7, 7’), 160.9 (C-6,
Process of extraction and isolation of canthaxanthin

6'), 37.7 (C-2, 2'), 35.7 (C-1, 1'), 34.3 (C-3, 3'), 27.7 (C-16, 16'), 27.7 (C-17, 17'), 13.7 (C-

Figure 2. Structure of canthaxanthin

CONCLUSION
In this study, post-fermentation saline bacteria strain Paracoccus carotinifaciens VTP20181 was used in a process to extract and isolate canthaxanthin. Chromatography methods were employed to purify the canthaxanthin. Current results suggest new research direction in creating canthaxanthin products from microbiological sources for the food and pharmaceutical industries in Vietnam.

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