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Supercritical Carbon Dioxide Micronization of Zeaxanthin from Moderately Thermophilic Bacteria *Muricauda lutaonensis* CC-HSB-11^T

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ABSTRACT: Moderately thermophilic bacterial strain CC-HSB-11^T (*Muricauda lutaonensis*), which was described recently from a coastal hot spring of Green Island, Taiwan, has been identified to produce zeaxanthin as a predominant xanthophyll by liquid chromatography−tandem mass spectrometry (LC−MS/MS). Cell culture in bioreactor produced 3.12 ± 0.18 mg zeaxanthin L^{-1} of culture. Micronization of zeaxanthin was achieved through supercritical carbon dioxide antisolvent precipitation method. Yield of zeaxanthin after the process was 53.4%. Dynamic light scattering assay determined the polydisperse existence of micronized particles of size 3 nm to 2 μm. Field emission scanning electron microscopy revealed distinct morphology and size distribution heterogeneity of particles. Integrity of zeaxanthin after the antisolvent process was assessed by LC−MS/MS. The technique capitalizes on the inherent ability of CC-HSB-11^T to synthesize zeaxanthin and the work demonstrated feasibility of antisolvent precipitation method to produce microparticles exploiting a bacterial strain.

KEYWORDS: *Muricauda lutaonensis*, microparticles, zeaxanthin, supercritical carbon dioxide, dynamic light scattering

INTRODUCTION

Xanthophylls are oxygenated carotenoids contributing to the maintenance of human health.[1] Zeaxanthin (3,3′-dihydroxy-β-carotene) is a dominant xanthophyll localized in the central macular region of human retina improving visual performance.[2,3] Like other xanthophylls, zeaxanthin is not produced by the human body and must be consumed through diet.[1−3] The dietary intake of zeaxanthin is believed to reduce the risk of age related macular degeneration, cataract, several heart related diseases and cancer.[4] Due to the outstanding photoprotective and antioxidant properties, zeaxanthin is widely used in the pharmaceutical industry and also as food and feed additives.[1−4] Since the optimum dietary source of zeaxanthin is scarce, studies have been carried out to upgrade the zeaxanthin content in agricultural products.[5,6] Commercial demand of zeaxanthin is mainly accomplished by chemical synthesis; however, multiple reaction steps, poor yield and undesirable byproducts have been major drawbacks.[4]

Microbes are attractive alternatives for the production of xanthophylls.[1] Extraction of xanthophylls such as astaxanthin, zeaxanthin and lutein from algal biomass has been documented.[7−9] Bacterial strains were also characterized to synthesize canthaxanthin[1,10] astaxanthin[11] and zeaxanthin.[4,12−15] Recently, biosynthesis of zeaxanthin was tested using a recombinant bacterial strain[16] showing its commercial interest from microbial sources. The large-scale production of potential carotenoids such as β-carotene and astaxanthin from microbial sources involve sophisticated technologies comprising patented processes; however, among xanthophylls only astaxanthin is being marketed as a microbial product.

Supercritical carbon dioxide (SC-CO_2) tends to improve the product quality with minimal residual toxicity and hence widely used in the precipitation of various bioactive compounds.[17−19] The SC-CO_2 antisolvent precipitation (SC-CO_2 ASP) is reasonably distinct from the SC-CO_2 extraction technology since the former often tend to precipitate the compounds during the process. The SC-CO_2 ASP technique has been employed in the precipitation of carotenoids from various sources.[20−23] Micronization of precipitated compound is an important consequence of antisolvent process besides the enhancement of product quality.[18−23] In fact, the compounds in micronized forms attain much importance in the pharmaceutical industry due to their enhanced efficacy, and tendency to accumulate at the target site.[24]

In the present work, a moderate thermophile of the family *Flavobacteriaceae* was characterized for the biosynthesis of zeaxanthin. Cells cultured under integrated bench size bioreactor were used as a source of zeaxanthin to test the feasibility of SC-CO_2 ASP to produce microparticles. The dynamic light scattering (DLS) technique was adopted to assess accurately the size distribution of particles. Morphology of particles was studied by scanning electron microscopy. Xanthophyll composition and integrity of zeaxanthin in the particles were determined by HPLC and mass spectrometry.

MATERIALS AND METHODS

Microorganism and Media. The bacterial strain CC-HSB-11^T (*Muricauda lutaonensis;* KCTC 22339^T) was the isolate[25] which was grown by using commercial marine broth (Difco 2216).

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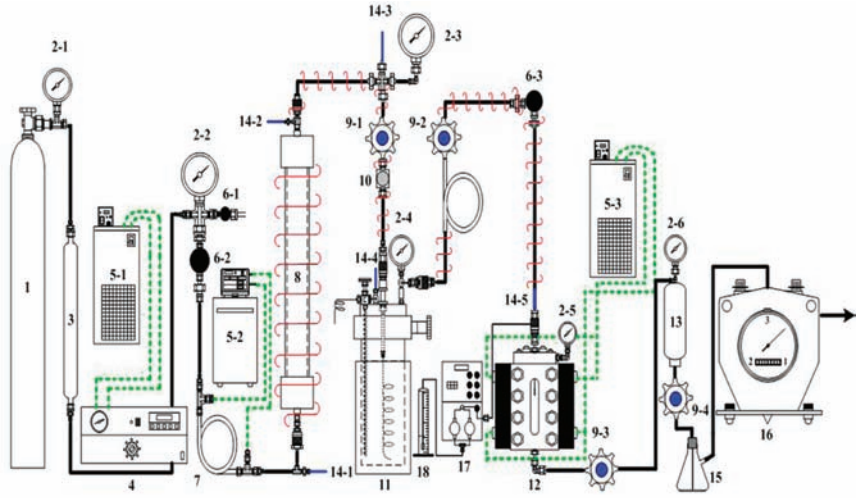

Figure 1. Schematic diagram of supercritical carbon dioxide antisolvent precipitation unit used to produce microparticles of zeaxanthin from CC-HSB-11^T.

Reagents and Chemicals. Authentic xanthophyll standards such as lutein and zeaxanthin (all-*trans*) were purchased from Sigma-Aldrich chemical company, USA and Fluka, Switzerland, respectively. HPLC grade solvents were used for chromatographic analysis. Carbon dioxide (99.95%, Toyo gas, Taiwan) was used as an antisolvent agent during the precipitation process.

Bacterial Cell Fermentation in Bioreactor. Integrated bench size bioreactor (Biostat *B plus*, $W \times H \times D$, 590 × 730 × 565 mm, 2 L capacity) was employed for fermentation by inoculating 100 mL (~0.1 g freeze-dried biomass) of seed culture aseptically into the reactor as to initiate the process. Culture media pH was adjusted automatically to 7.4 ± 0.2 by using 1 N NaOH/HCl with a constant temperature of 40 °C. The stirrer speed was 150 rpm during the process, and sterile air supply was initiated with a constant flow rate of 1 VVM to maintain minimum (>15%) dissolved oxygen to accomplish the requirement by strain. Culture conditions were maintained stable during fermentation by using integrated digital control of the system and monitored through the screen. Cells harvested at log phase by high speed centrifugation (13,000 rpm, 10 min, 4 °C; Himac CR 21G, Hitachi) were subjected to freeze-drying (Eyela FDU 1200) for 8 h. The resultant freeze-dried biomass was quantified and transferred into a brown bottle under N_2 stream and preserved at −20 °C for further analysis.

Identification of Carotenoids. For the analysis of carotenoids, 10 mg of finely powdered freeze-dried biomass was added to 1 mL of ethanol, mixed thoroughly and agitated overnight in the dark at 40 °C. The mixture was centrifuged (13,000 rpm, 10 min, 4 °C), and the supernatant was filtered through Millipore filter paper (PVDF; 13 mm, 0.22 μm). The yellow-colored crude ethanol extract was further diluted with ethanol and subjected to full wavelength scan (250−700 nm) using UV−visible spectrophotometer (U-3010, Hitachi) operated at room temperature for preliminary identification of carotenoids. Subsequently, chromatographic separation of polar and nonpolar carotenoids was achieved according to the previously published methods.[11] For liquid chromatography, a HPLC pump (L-2130, Hitachi) equipped with an autosampler (AS-4000) and diode array detector (L-2455, Hitachi) was used. A reverse-phase column (CAPCELL PAK C18 MG S-5, 35 × 4.6 mm, 5 μm particle size; Shiseido, Tokyo, Japan) connected through a C18 guard column (Phenomenex) maintained at 35 °C was applied for carotenoid separation. For the confirmation of carotenoids, mass spectrometry was performed in a Thermo Finnigan LTQ linear ion trap mass spectrometer (Thermo LTQ XL, San Jose, CA, USA) connected to Thermo Scientific Surveyor LC plus system equipped with a Surveyor MS pump plus and a Surveyor autosampler (Thermo Scientific, San Jose, CA, USA). An APCI source operated in the positive ion mode during analysis under the following conditions: sheath gas flow (N_2), 50 arbitrary units; auxiliary gas flow (N_2), 10 arbitrary units; source voltage, 6 kV; capillary temperature, 300 °C. For the data-dependent MS/MS experiments, precursor ion detected in the full MS scan was selected with an isolation width of 2 m/z unit for collision-induced dissociation with the collision energy of 25 eV. Carotenoids were identified based on its absorption spectrum, retention time (t_R) and m/z values with reference to authentic standards.

Preparation of Microparticles by SC-CO_2 Antisolvent Precipitation. Extractability of zeaxanthin from 10 mg freeze-dried biomass was tested by following the method published elsewhere[15] using 1 mL of solvents such as ethanol, methanol, acetone, hexane and tetrahydrofuran. Subsequently, for primary extraction, 1 g of powdered freeze-dried bacterial biomass was introduced into 10 mL of ethanol and mixed thoroughly for 10 min. Sample was placed in a 40 °C rotary oven overnight (~16 h) at 100 rpm to bleach the cells in the dark. The bleached mixture was centrifuged (13,000 rpm, 10 min, 4 °C), and supernatant was collected. The pellet was resuspended in 20 mL of ethanol followed by bleaching and centrifugation. Supernatants were pooled together and concentrated (~10 mL) by using rotary vacuum evaporator (EYELA, Japan). The resultant yellow-colored oily concentrate containing zeaxanthin was subjected to SC-CO_2 ASP as feed solution.

A schematic diagram of the unit used for SC-CO_2 ASP is depicted in Figure 1. The operating parameters for SC-CO_2 ASP were selected on

the basis of previously published work with minor modifications.[21] As an initiation step, liquid CO_2 charged from a CO_2 cylinder (1) was passed through a gas-dryer (3) and compressed by a high-pressure double-piston pump (*Spe-ed* SFE, Applied Separations, USA) (4) into a 75 mL high-pressure surge tank (8) and a 750 mL middle-pressure surge tank (11) at a constant flow rate after preheating it in a double-pipe heat exchanger (7). Temperature in pump and heat exchanger was maintained at 25 and 40 °C, respectively using two circulators (5-1, 5-2). Then, CO_2 expanded through two back-pressure regulators (9-1, 9-2) and a metering valve (6-3) was plumbed into a 200 mL visible precipitator equipped with two pieces of safety glasses (TST, Taiwan) (12). For each SC-CO_2 ASP, after the release of CO_2 into the precipitator under a selected supercritical condition, a solution of 1 g of freeze-dried biomass extract concentrated to 10 mL of solvent was delivered through the feeding buret (18) into the precipitator through a coaxial nozzle at a constant flow rate of 1 mL min^{-1} using a HPLC pump (CM-3200, Thermo Separation Products, USA) (17). Meanwhile, the SC-CO_2 was continuously introduced into the precipitator at a flow rate of 4.5 L min^{-1}. A nylon filter paper (Nylaflo, 0.45 μm) was tightly packed over a stainless sintered frit filter (37 μm) and fixed at the bottom of the precipitator to prevent an entrainment of particles.

Selected gas pressure was 10 MPa (100 bar) and kept constant manually using a back-pressure regulator (9-3). System temperature was 40 °C and maintained constant throughout the dynamic run time by using a water bath circulator (5-3). Following the SC-CO_2 ASP, a vapor−liquid stainless steel separator (13) was connected to the precipitator maintained at 5 MPa using a back-pressure regulator (9-4) to stabilize the expanded mixture. A 250 mL flask separator (15) was placed therein to collect the mixture of CO_2 and solvent under ambient conditions. Consumption of CO_2 was estimated using a wet gas meter (TG3, Ritter, Germany) (16). Temperature and pressure in the system was monitored using several K-type thermocouples (14-1−14-5) and several Bourdon-type pressure gauges (2-1−2-6) respectively. Experiments were repeated at least three times with the same set of conditions.

Quantification of Zeaxanthin. A standard curve was plotted for the commercial zeaxanthin using HPLC. Carotenoids present in crude extracts were completely separated and peak area of target compound was integrated for quantification with reference to standard curve. Similarly, quantity of precipitated zeaxanthin after SC-CO_2 ASP process was also estimated by the HPLC method. Percentage yield of zeaxanthin, Y_Z, was calculated by using the following formula.

$$Y_Z\ (\%) = \frac{\text{quantity of zeaxanthin in microparticles}}{\text{quantity of zeaxanthin in ethanol extract}} \times 100$$

Characterization of Microparticles. Morphology of microparticles of zeaxanthin was analyzed using JEOL-7401 F field emission scanning electron microscope (FESEM). Microparticles of zeaxanthin collected over the nylon filter paper after dynamic run time of SC-CO_2 antisolvent precipitation were suspended in high purity Milli-Q water and mixed briefly (Vortex Genie 2) for 2 min to get the primary suspension. Particle size distribution analysis was carried out subsequently by diluting the suspension suitably with Milli-Q water and loaded into N5 submicron particle size analyzer (Beckman-Coulter, High Wycombe, U.K.) maintained at 25 °C. Data acquisition was 200 s long, and at least three runs were made in each case with a detection angle of 90°. Data were recorded in terms of unimodal and size distribution processor (SDP) format and analyzed using photon correlation spectroscopy (PCS) control software (Version 2.02, Non Compliant).

RESULTS AND DISCUSSION

Biomass Production and Identification of Carotenoids. Preliminary growth experiments were conducted before starting the fermentation to optimize the culture conditions. Periodical monitoring of optical density (600 nm) values of culture media showed that the cell growth continued up to 56 h, reaching its maximum at 72 h. Subsequent studies were carried out using a bench size stirred tank bioreactor with a working volume of 1 L marine broth. Log phase harvest of bacterial cells fermented under bioreactor yielded 1.17 ± 0.13 g freeze-dried biomass L^{-1} culture.

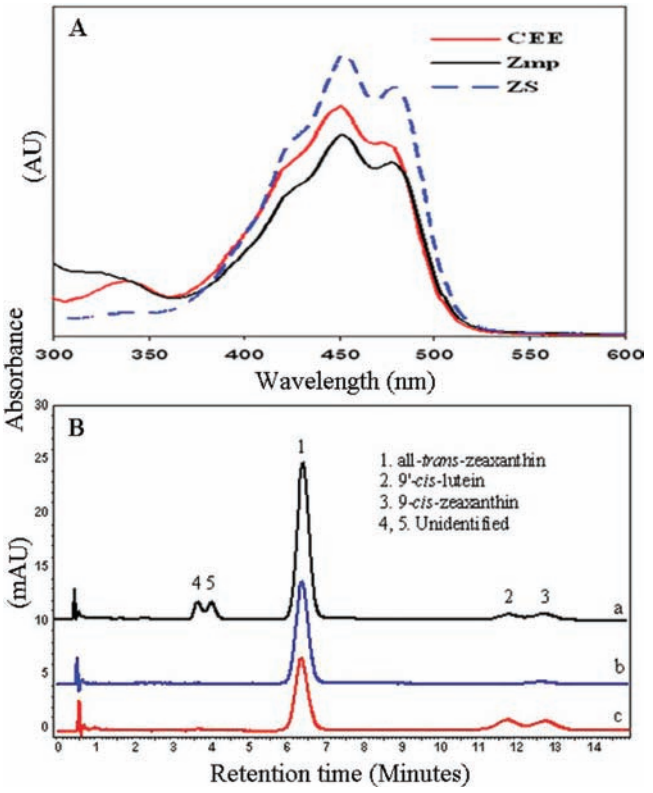

Figure 2. Panel A: UV−visible absorption spectrum of crude ethanol extract (CEE), microparticles of zeaxanthin (Zmp) and zeaxanthin standard (ZS) solubilized in ethanol. Panel B: Comparison of chromatograms representing the separation or elution of zeaxanthin in microparticles produced by supercritical carbon dioxide antisolvent precipitation method (a), the standard all-*trans*-zeaxanthin (b) and the crude ethanol extract comprising carotenoids isolated from strain CC-HSB-11^T (c) as observed under 450 nm detection wavelength of HPLC.

The crude ethanol extract comprising carotenoids isolated from the strain CC-HSB-11^T displayed typical absorption spectrum identical to zeaxanthin during preliminary carotenoid analysis performed using UV−visible spectrophotometer as shown in Figure 2. Polar carotenoids present in the crude ethanol extract were completely separated through HPLC. Comparison of the chromatograms representing separation and elution of carotenoids present in crude ethanol extract (trace c) and standard all-*trans*-zeaxanthin (trace b) is displayed in Figure 2. Chromatogram of crude ethanol extract (trace c) showed the presence of three distinct peaks (peaks 1−3). The t_R of predominant peak 1 (65.5 ± 2.37% of total carotenoids) was similar to that of all-*trans*-zeaxanthin standard (trace b). In addition, the UV−visible absorption spectrum of peak 1 was identical to that of standard all-*trans*-zeaxanthin as monitored through diode array detector which exhibits characteristic vibronic spectra with λ_{max} of 450 nm consisting of adjacent typical shoulder peaks. The mass spectrum of peak 1 gave parent ion $[M + H]^+$ at m/z 569 and

collision-induced dissociation fragments of m/z 561 and 475 identifying the compound as all-*trans*-zeaxanthin. Subsequent chromatographic quantification revealed that the strain CC-HSB-11^T produced 2.67 ± 0.15 mg zeaxanthin (all-*trans*) g^{-1} freeze-dried biomass. The compounds representing peak 2 (15.6 ± 0.26% of total carotenoids) and 3 (14.8 ± 0.34% of total carotenoids) were identified to be 9′-*cis*-lutein and 9-*cis*-zeaxanthin respectively based on mass spectroscopic data, t_R, UV−visible absorption spectra and with reference to standards as well as information available in the literature.[26] Compounds corresponding to peaks 4 (2.51 ± 1.61% of total carotenoids) and 5 (1.60 ± 0.83% of total carotenoids) were present in negligible amounts and remain unidentified.

Microbes are attractive alternative sources of carotenoid production due to their small population doubling time and relatively simple extraction steps. Zeaxanthin biosynthesis was previously reported in algal species such as *Neospongiococcum*, *Dunaliella salina*, *Microcystis aeruginosa, Spirulina* and *Nannochloropsis oculata,* yeast species such as *Phaffia rhodozyma* and nonphotosynthetic bacterial strains such as *Erwinia herbicola*.[1,4,20] However, low production efficiency was a major obstacle for large-scale applications. Although several members of the family *Flavobacteriaceae* have been characterized to produce zeaxanthin, to date, only *Flavobacterium multivorum* is studied substantially for enhanced zeaxanthin production.[27,28] The quantity of zeaxanthin synthesized by CC-HSB-11^T was 3.12 ± 0.18 mg L^{-1} of culture, which is comparatively higher than the quantity reported from *Flavobacterium multivorum* (1.77 ± 0.09 mg L^{-1} media,[27]) indicating its high zeaxanthin biosynthesis efficiency. The inherent ability of CC-HSB-11^T to synthesize a large amount of zeaxanthin offers an attractive alternative for industrial and pharmaceutical applications. In addition, postoptimization scaleup can determine the competitive future of CC-HSB-11^T as a potential source of zeaxanthin.

Micronization of Zeaxanthin by SC-CO_2 Antisolvent Precipitation. The overnight treatment of freeze-dried biomass with organic solvents such as methanol, ethanol, hexane, tetrahydrofuran and acetone extracted about 88.8%, 75.1%, 9.9%, 95% and 88% of zeaxanthin respectively. The extraction of zeaxanthin in ethanol was enhanced up to maxima (95%) by adding fresh solvent (ethanol) and repeating the extraction process. As compared to the extraction of xanthophylls from plant sources,[29,30] primary extraction from bacterial source is relatively simple and convenient since it has eliminated the multiple steps and minimized the use of hazardous solvents. Ethanol is generally regarded as safe compared to other conventional solvents and has a tendency to precipitate the compound during antisolvent processes.[20] Therefore it was chosen as a solvent for primary extraction of xanthophylls from freeze-dried biomass and also as feed solvent for subsequent antisolvent processes.

For the feasibility of the SC-CO_2 ASP process, operating parameters such as saturator pressure, temperature and composition are believed to play an important role.[19,20] These parameters, which cause reduction in the solubility and increase the supersaturation, are necessary to produce particles with smaller sizes.[23] During the antisolvent process, precipitation of zeaxanthin was observed on the inner walls of precipitator as a result of typical nucleation behavior in a disperse medium where the particles are spread throughout the whole volume of precipitator.[21] Despite these, micronized particles were deposited on the filter paper placed at the stainless steel sintered frit due to the accumulation of liquid phase carrying the precipitated zeaxanthin. The precipitation indicated the accomplishment of equilibrium solubility of solute which could determine the supersaturation.[23] In addition, the close contact between the sample solution delivered through coaxial nozzle and CO_2 in the apparatus (Figure 1) might have contributed to higher supersaturation and nucleation rates and hence formation of smaller particle sizes.[23] Increase in the liquid solution concentration (>10%) resulted in increased mean particle size and broad particle size distribution as consistent with previously published work.[18,20] After depressurizing, micronized particles were collected and analyzed for particle morphology and size distribution as well as for quantification of zeaxanthin. About 53.4% of zeaxanthin was recovered for the provided set of conditions as microparticles with a size of 3 nm to 2 μm.

Hydrophobic compounds, such as fats, hydrocarbons and essential oils, are selectively solubilized by SC-CO_2 due to its weak nonpolar solvent nature whereas polar hydrophilic molecules are weakly soluble.[31] Zeaxanthin is a polar carotenoid with two hydroxyl groups per molecule which might have contributed to its poor solubility in SC-CO_2 resulting in the precipitation. In addition, biocide property exhibited by SC-CO_2 results in the decontamination or sterilization of materials processed, improving the quality of particles produced.[31] The SC-CO_2 ASP of zeaxanthin from CC-HSB-11^T exhibits several advantages over the currently adopted conventional methods including minimum sample manipulation and ease of automation of various processes with reduced use of hazardous solvents. All these features outlined above illustrate that the technique is fairly reliable for the production of microparticles of zeaxanthin from bacteria.

Characterization of Microparticles of Zeaxanthin. Efficiency of water-dispersible beadlets and micelles as a vehicle for the delivery of carotenoids to the cells under culture has been documented.[32,33] In fact, the micron-meter and nanosized particles are rather effective in drug delivery due to their enhanced bioavailability.[24] Therefore, experiments were conducted to characterize microparticles of zeaxanthin generated by the SC-CO_2 ASP method. The particles collected were carefully recovered, solubilized in ethanol and analyzed through HPLC and mass spectrometry. The chromatogram (trace a) showed five distinct peaks as shown in Figure 2. The overall quantity of all-*trans*-zeaxanthin in microparticles was comparatively higher (peak 1, 75.0 ± 1.44% of the total carotenoids) than the amount present in crude ethanol extract. Interestingly, when compared to the crude ethanol extract (trace c), relative quantity of 9′-*cis*-lutein (peak 2, 5.21 ± 0.13% of the total carotenoids) and 9-*cis*-zeaxanthin (peak 3, 7.30 ± 0.46% of the total carotenoids) was decreased with concomitant increase in two unidentified carotenoid peaks (peak 4, 6.04 ± 0.39%; peak 5, 6.49 ± 0.46% of the total carotenoids) in microparticles (trace a). These additional peaks (peaks 4 and 5, trace a) indicate the possible transformation among isomeric forms during the antisolvent process.

Microparticles of zeaxanthin bound to the nylon filter after SC-CO_2 ASP were analyzed by FESEM (Figure 3). Superficial pores were not observed over the particles indicating the absence of liquid phase containing carbon dioxide entrapped in the particles formed.[21] The dimensions of the particles produced were varying with distinct morphology having well-defined boundaries as shown in Figure 3. Morphology of particles obtained during lower liquid solution concentration suggests its probable origin due to nucleation.[21,23] The image scale in Figure 3 partially depicts the presence of micron-sized meter-sized particles distributed independently without significant clumping, fusion or coalescence. The electron

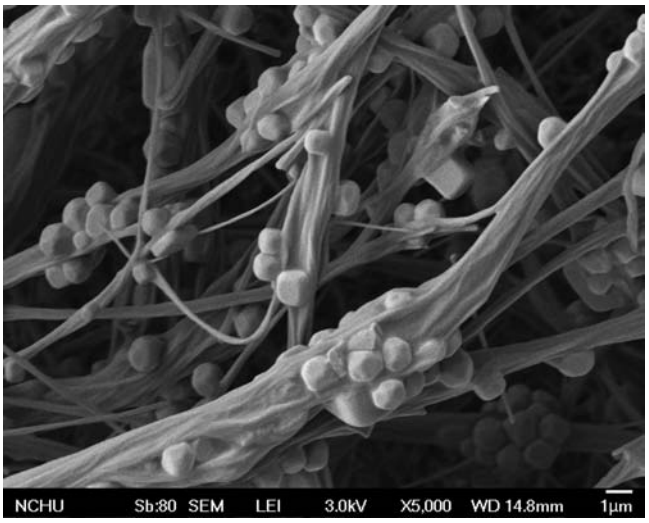

Figure 3. Field emission scanning electron micrograph representing microparticles of zeaxanthin produced by supercritical carbon dioxide antisolvent precipitation method.

microscopy and liquid chromatography evaluated morphology of micronized particles and feasibility of the precipitation process respectively.

Particle Size Distribution Analysis by Dynamic Light Scattering. To transform the qualitative observation into quantitative data, PCS was adopted, which is a technique based on the dynamic light scattering property of particles. The fingerprint reproducibility results of PCS provided a polydispersity index (PI) value of 1.44 ± 0.04, which is a measure of particle size distribution broadness. A PI value of 1 indicates large variations in particle size, a reported value of zero means that there is no variation in size.[34] In addition, the SDP analysis differentiated multimodal peak distribution and hence provided the actual distribution of particles in three different formats; volume, intensity and number distributions. In the present study, a histogram of intensity analysis obtained by autocorrelation function was selected to represent the percentage intensity of particles of each size in a sample. In this histogram, the magnitude of each bar is proportional to the percentage amount of total scattered intensity due to the particles. At 90° detection angle analysis, percentage intensity amount of 63, 20, 9 and 6 came from particles with a size of 1708 ± 352.2 nm, 706 ± 131.5 nm, 101 ± 12.4 nm and 3 ± 0.6 nm, respectively indicating particle size heterogeneity. Assessment of size of the particles generated by SC-CO_2 ASP through FESEM could be hindered by the presence of artifacts, and hence application of PCS was reasonable. The experimental data from the N5 analyzer using light scattering measured at 90° could detect the expected size distribution of microparticles of zeaxanthin (3 nm to 2 μm) indicating micronization (Figure 3). In conclusion, PCS revealed the presence of polydisperse and heterogeneous population of micronized particles produced by SC-CO_2 ASP method. The characterization of CC-HSB-11^T to produce zeaxanthin and antisolvent process-generated microparticles will be beneficial for the application of food and pharmaceutical industry.

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