Journal of Food Composition and Analysis 70 (2018) 78–88

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Optimization of phycocyanin extraction from *Spirulina platensis* using different techniques

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ARTICLE INFO

Keywords:
Food colorants
Food composition
Phycocyanin
ABTS scavenging activity
Ultrasound extraction
Microwave extraction
Classical homogenisation

ABSTRACT

Phycocyanin is an important commercially available blue food colorant. Herein we report an optimization study of various phycocyanin extraction methods from *Spirulina platensis* cyanobacterium biomass (dry, frozen and wet). Three different solvents i.e. distilled water, Na-Phosphate pH: 7.4 suspension and 1.5% $CaCl_2$ (w/v) water solution were applied as the extraction medium. The highest total phycocyanin content (55.33 mg/g) was extracted from frozen biomass using 1.5% $CaCl_2$ (w/v aq.) solution. Process variables of classical, ultrasound and microwave extraction methods (biomass/solvent ratio, extraction time, vibration, speed, and power) were optimized considering the CCRD experimental design to enrich phycocyanin. The optimum conditions of extraction methods; classical, ultrasound and microwave were determined as: 1.71% biomass/solvent ratio, 6237.66 homogenization rate and 15 min extraction time; 1% biomass/solvent ratio, 60% amplitude and 16.23 min extraction time; 2.34% biomass/solvent ratio, 133.29 W and 165.96 s extraction time. Classical extraction method provided vivid blue color, a higher amount of phycocyanin, and maximum antioxidant activity as compared to other extraction methods.

1. Introduction

Spirulina platensis is a non-toxic cyanobacterium characterized by high levels of carbonate and bicarbonate in an alkaline environment. *S. platensis* has been utilized as a food supplement as it contains polysaccharides, vitamins, minerals, unsaturated fatty acids, carotenoids, and phycobiliproteins (Su et al., 2014; Wu et al., 2016). Biomass of *S. platensis* consists 55–70% proteins, 3–9% fats, and 15–30% carbohydrates in addition to fibers and pigments (Kay and Barton, 1991).

Phycobiliproteins are accessory photosynthetic pigments that are aggregated in the cell as phycobilisomes, which are attached to the thylakoid membrane of chloroplast (Arad and Yaron, 1992). Cyanobacterial phycobiliproteins can be divided into three main classes; phycoerythrin (PE –bright pink, red), phycocyanin (PC –dark blue), and allophycocyanin (AP –brighter blue) (Ghosh et al., 2015; Kumar et al., 2014; Singh et al., 2015). These phycobiliproteins are widely used in medicines, foods, cosmetics and fluorescent materials (Eriksen, 2008; Kannaujiya and Sinha, 2016).

Phycocyanin is a water soluble natural pigment that is widely used as color additive in foods (chewing gums, dairy products, gellies etc.), cosmetics (lipstick and eye liners), fluorescent reagent, and as probe and tracer in clinical diagnostic instruments as well as in immunology (Eriksen, 2008; Kumar et al., 2014). It has significant antioxidant, anti-inflammatory, hepatoprotective, and radical scavenging properties (Martelli et al., 2014; Wu et al., 2016).

There are various techniques reported to extract phycocyanin (Sekar and Chandramohan, 2008) from *S. platensis* biomass in various physical forms (dry, wet and frozen). Its extraction is difficult as the cell wall of the cyanobacteria is quite resistant (Wyman, 1992), which is made of four layers i.e. fibril, peptidoglycan, proteins, and analogous to gram-negative bacteria (Van Eykelenburg, 1977). There are several methods reported to disrupt the cell wall including homogenization, sonication, microwave, supercritical fluid extraction and lysozyme disintegration (Deniz et al., 2016; Duangsee et al., 2009; Martinez et al., 2016).

The extraction process should be effective in terms of high extraction yield and must be environmental friendly. Ultrasonic cleaning baths or probe systems are mainly used to breakdown the cell wall of the bacteria (Vinatoru, 2001). Laboratory scale ultrasonic baths provides higher extraction yields as compared to the probe system (Vinatoru et al., 1999). Microwave extraction utilizes microwaves

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https://doi.org/10.1016/j.jfca.2018.04.007
Received 2 August 2017; Received in revised form 11 April 2018; Accepted 16 April 2018
Available online 20 April 2018
0889-1575/

(300 MHz–300 GHz) to heat the extraction medium (usually solvent) to extract most of the useful chemical contents out of bacterial biomass (Jain et al., 2009). Classical homogenization is generally applied using Ultra Turrax equipment to efficiently bring targeted compounds and solvent into contact; without extracting unwanted components from biomass. However, all of these cell disruption methods lack specificity as cell debris and other unwanted impurities are also released. Nevertheless, phycocyanin is sensitive to light, oxygen, moisture, and temperature. Therefore, it is able to readily degrade under certain physicochemical conditions (Kannaujiya and Sinha, 2016).

This study was primarily aimed to determine the most effective form of biomass (frozen, dried and fresh) and most suitable extraction medium (distilled water, Na-phosphate buffer pH: 7.4 solution and 1.5% $CaCl_2$ (w/v)) to obtain higher amounts of phycocyanin. The second aim of this study was to compare classical, ultrasound and microwave extraction methods and their conditions targeting vivid blue color, maximum phycocyanin concentration, and acceptable antioxidant activity.

2. Materials and methods

2.1. Materials and chemicals

Arthrospira (Spirulina) platensis EGEMACC 38, used to extract phycocyanin, was obtained from the Microalgae Culture Collection of Ege University. In the algal biotechnology laboratory of Ege University, *S. platensis* was grown in Zarrouk medium (Zarrouk, 1966) at 50 µmol photons $m^{-2} s^{-1}$ light intensity and aerated at an airflow rate of 2 L/min at 22 ± 2 °C.

Folin-Ciocalteu reagent (Merck, Darmstadt, Germany) and sodium carbonate (Fisher Science, UK) were employed to quantify total polyphenols, while gallic acid (Merck, Darmstadt, Germany) was used as a calibration standard. Trolox (Hoffman-La Roche) (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid; Aldrich Chemical Co., Gillingham, Dorset, UK) was used as an antioxidant standard. ABTS i.e. 2,2′-azinobis(3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt, and calcium chloride were obtained from Sigma-Aldrich (Poole, Dorset, UK).

2.2. Extraction procedures

2.2.1. Determination of biomass form and solvent type

Phycocyanin was extracted from frozen, dried and fresh biomass of *S. platensis*; in three different extraction mediums. Biomass was frozen at −20 ± 2 °C for 24 h while it was dried in the oven at 50 ± 2 °C with a target of about 8% moisture content. To determine the effect of pre-treatments (freezing and drying) applied to the biomass, dry matter content was kept constant. Classical phycocyanin extraction was performed under IKA-Turrax homogenizer rotating at 7000 rpm in distilled water with Na-phosphate buffer (pH: 7.4) and 1.5% $CaCl_2$ (w/v) as extraction medium at 25 °C for 10 min. The temperature was controlled with a circulator water bath (Daihan, WCR P8, Korean). After extraction, the cell residue was centrifuged at 4000 rpm for 10 min. Crude extracts were analyzed for phycocyanin content. The most appropriate form of biomass was chosen considering maximum phycocyanin concentration and vivid blue color.

Classical (7000 rpm, 10 min), ultrasound (50% amplitude, 15 min) and microwave (150 W, 180 s) extractions were performed at 1% biomass/solvent ratio to determine the most suitable extraction solvent. A mechanical homogenizer (Ultra Turrax, IKA, Model T25, Germany) was used during classical extraction. Ultrasound extraction was performed in an ultrasound bath (Daihan Wisd WUC-D06H, Korea, 290 × 150 × 150 mm) at 40 kHz. The intensity of sonication was amplitude controlled (independent variable). However, as reported earlier, the ultrasonic intensity distribution inside the ultrasonic bath is not homogeneous. Before starting extraction procedure in ultrasound bath, the most intense zone of sonication inside the bath (aluminum foil test) was determined for the extraction experiments.

A microwave device (Milestone, Start E, Italy, programmable, Teflon-coated built-in magnetic stirrer) was used at 2450 MHz in the microwave extraction method. The extractions were carried out in a 100 mL-sealed vessel made of high-purity TFM, surrounded by a safety shield (made of HTC, a new high-performance plastic) including a “vent-and-reseal” safety valve. Temperature was monitored and controlled with the aid of a shielded thermocouple, inserted directly into the vessel. The evolution of time, temperature, and power were continuously recorded during each experiment.

Selection of solvent to be used in the classical, ultrasound and microwave extractions have been determined considering the structural properties of the *S. platensis* (Jin et al., 2014; Silveira et al., 2007; Vali Aftari et al., 2017). Extraction phase in classical and ultrasound methods; the process temperature was kept constant at 25 ± 2 °C with the circulator water bath. In the microwave extraction method, the temperature didn't exceed 40 ± 2 °C in the extraction chamber. Three different solvents (distilled water, Na-phosphate buffer pH: 7.4 solution and 1.5% $CaCl_2$ (w/v)) were used as extraction medium. After extraction, the cell residue was removed and phycocyanin content was analyzed.

All experiments were performed in triplicate, and the presented results are means ± 95% confidence interval. The obtained data was statistically analyzed by analysis of variance using SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL) and mean analysis was performed using Duncan’s procedure. The differences were considered significant when $p \leqq 0.05$.

2.2.2. Process parameters optimization of the extraction methods

Effect of specific extraction process conditions (homogenization rate (rpm)/amplitude (%)/microwave power (W)), biomass/solvent ratio (%) and extraction time of classical, ultrasound and microwave extraction methods on total phycocyanin, total phenolic content, and $ABTS^{\cdot+}$ scavenging activity of extracts were investigated using Central Composite Rotatable Design (CCRD). While the range of biomass/solvent ratio (% w/w, A) was the same for all extraction methods, the extraction time (min, C) was variable for each procedure. Homogenization rate (rpm) (B) for classical extraction, amplitude (%, D) for ultrasound extraction and microwave power (W, E) for microwave extraction methods were selected as independent extraction method parameters. 1.5% $CaCl_2$ (w/v) was used as extraction medium for all the extraction methods. The boundary conditions created by the CCRD of the independent variables for each extraction method are given in Table 1.

Multiple regression analysis was conducted to fit the Eqs. (1)–(3) on the experimental data; significant terms of the model were determined by ANOVA. The CCRD and the corresponding data analysis were carried out using the Design-Expert 7.0.0 (Stat-Ease Inc., MN, USA).

$$PC = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} x_{ji} \quad (k = 1,2,3) \tag{1}$$

$$TPC = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} x_{ji} \quad (k = 1,2,3) \tag{2}$$

$$ABTS = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} x_{ji} \quad (k = 1,2,3) \tag{3}$$

2.3. Analysis

2.3.1. Phycocyanin content measurement

Prior to spectrophotometric measurement, the extract was