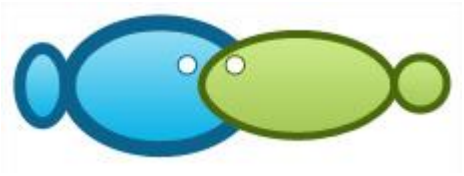

Chromatographic isolation of ß-phycoerythrin from *Porphyridium cruentum* using ceramic hydroxyapatite

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Abstract. The pigment ß-phycoerythrin is useful as a natural pigment in food and cosmetics, and also serves as a fluoroprobe for laboratory analyses. This study evaluated the effectiveness of ceramic hydroxyapatite as a stationary phase to purify ß-phycoerythrin using medium-pressure liquid chromatography. The microalga, *Porphyridium cruentum*, was cultivated in artificial sea water consisting of 20% effluent biogas of tapioca waste water, as well as in modified F2 nutrient medium consisting of NaH_2PO_4 $2H_2O$ and $NaNO_3$, stock solution of trace elements (Na_2EDTA, $NaMoO_4$ $2H_2O$, $CoCl_2$ $6H_2O$, $FeCl_3$ $6H_2O$, $CuSO_4$ $5H_2O$, $ZnSO_4$ $7H_2O$, $MnCl_2$ $4H_2O$), and stock solution of vitamins (biotin, cyanocobalamin, and thiamine HCl). *P. cruentum* biomass was harvested after 8 days by centrifuging at 8000 g for 10 min at 4°C. Fresh biomass was resuspended in buffer solution and sonicated for 30 min. Then the slurry was centrifuged at 12000 g for 4 min. ß-phycoerythrin-rich supernatants were pooled and treated as a crude extract. The crude extract was then passed over a ceramic hydroxyapatite column at medium pressure. Ceramic hydroxyapatite was prepared using a precipitation method and sintering at 1200°C. The composition and microstructures of the ceramic were characterized with a scanning electron microscope equipped with an energy dispersive X-ray spectrometer. The purity of ß-phycoerythrin was confirmed using UV-Vis spectroscopy. Ceramic hydroxyapetite offers a scalable method for isolation of ß-phycoerythrin. Moreover, effluent biogas of tapioca waste water should be considered as an alternative cultivation medium for sustainable production of microalgae. This study succeeded in cultivating *P. cruentum* in modified F2 media with 20% biogas waste and utilizing ceramic hydroxyapatite to recover 60% of ß-phycoerythrin with an A546/A280 ratio of 3.995.
Key Words: ion chromatography, microalgae, phycobiliprotein, waste water.

Introduction. *Porphyridium cruentum*, a unicellular, marine red alga, belongs to the phylum *Rhodophyta*. This microalga has received considerable attention in recent years because of its production of ß-phycoerythrin (BPE) which is widely used as a colorant in food, nutraceuticals, and pharmaceuticals (Gujar et al 2019). However, to date, commercial cultivation of *Porphyridium* spp. has not been achieved due to high production costs. Many factors can influence production costs, such as the microalga species, cultivation condition, and isolation techniques. Due to its diverse applications in various fields, it seemed worthwhile to develop a simple, cost effective method to recover BPE from *P. cruentum* to meet the increasing demand.

Researchers have made many attempts to cultivate *Porphyridium* species and to devise simple methods to extract and purify BPE from them. Several studies showed that waste water can be used as media to cultivate *Porphyridium* sp. (Widiyaningsih et al 2013; Ulusoy Erol et al 2020). Application of agricultural waste for mass cultivation of *Porphyridium* sp. is a first step to reduce cost and to ensure sustainability of biomass production. *Porphyridium* sp. productivity depends on various parameters, such as temperature, pH, and carbon and nitrogen sources (Su et al 2016). However, information about using effluent biogas from tapioca waste water as a carbon source is still limited.

The second crucial step concerns optimized recovery of BPE from microalgal biomass. Some methods have been reported for extracting and purifying BPE from *Porphyridium* biomass (Gallego et al 2019; Ardiles et al 2020). Extraction and purification are essential steps to optimize BPE recovery. Microwave-assisted extraction, ammonium sulfate precipitation (Cai et al 2012), coupled with ion-exchange chromatography (Bermejo Roman et al 2002) have been employed to purify BPE from *Porphyridium* sp.

In the present study, we report a new method for efficient BPE production from *P. cruentum* cultivated using effluent biogas derived from industrial tapioca waste water. This microalgal strain grows normally in a 20% mixture of the effluent from biogas process and artificial sea water. BPE was recovered from biomass *P. cruentum* extract using a single medium-pressure ion-exchange chromatography step with ceramic hydroxyapatite (HAp) as the stationary phase.

Material and Method

Synthesis and characterization of HAp. HAp powder was prepared using a wet chemical precipitation method from $CaCl_2$ $2H_2O$ and Na_2HPO_4 (Ramesh et al 2013). An aqueous solution of 0.3 M Na_2HPO_4 was added dropwise to a 1.66 M solution of $CaCl_2$ at a rate of 1 mL min^{-1}. During this process, the pH of the solution was adjusted to 10 using NaOH solution. The reaction was carried out at 32°C with continuous stirring (800 rpm). The resulting precipitate was aged for 15 h and then repeatedly centrifuge-washed with distilled water until the pH became neutral. Then it was dried at 80°C for 24 h. For sintering, HAp was heated to 1200°C for 1 h using a furnace Lenton type UAF 16/10. The HAp surface was characterized using scanning electron microscopy/dispersive X-ray spectroscopy (SEM-EDX) Zeiss EVO MA 10 at Technical Service Unit, Integrated Laboratory of Innovation and Technology Center Lampung University, Indonesia.

Algal strain and cultivation conditions. This study was performed using the microalga *P. cruentum*. Microalgae were obtained from the culture collection of the Biochemistry Laboratory Institute of Technology at Bandung, Indonesia. A stock culture was maintained in a 250-mL flask containing modified artificial sea water (ASW). The starter culture was grown in a 6×60 cm glass column containing ASW (g L^{-1}) composed of: 27 g NaCl, 5.6 g $MgCl_2.6H_2O$, 1.5 g $CaCl_2.2H_2O$, 1 g KNO_3, 0.07 g K_2HPO_4, 6.6 g $MgSO_4.7H_2O$, and 0.04 g $NaHCO_3$ while media were adjusted to a pH between 7.4-8.2. Illumination was provided by fluorescent lamps producing 350 µmol photons m^{-2} s^{-1}. The photoperiod was 12:12 h (light to dark), and the growth temperature was maintained at 28±2°C. An additional carbon source of media culture and agitation were supplied with an aerator. Microalgal cells were harvested via centrifugation (8000 g for 10 min at 4°C).

Preparation of a crude extract. *P. cruentum* was cultivated in modified F2 mixed with 20% effluent biogas. The modified F2 nutrient medium consisted of the following (pH 8): NaH_2PO_4 $2H_2O$ 0.0056 g; $NaNO_3$ 0.0752 g; stock solution of trace elements - 1 mL L^{-1} (Na_2EDTA 4.15 g, $NaMoO_4$ $2H_2O$ 0.20 g, $CoCl_2$ $6H_2O$ 0.011 g, $FeCl_3$ $6H_2O$ 3.17 g, $CuSO_4$ $5H_2O$ 0.012 g, ZnSO4 $7H_2O$ 0.024 g, $MnCl_2$ $4H_2O$ 0.19 g); and stock solution of vitamin mix - 1 mL L^{-1} (cyanocobalamin 0.0004 g, thiamine HCl 0.13 g, and biotin 0.0005 g) (Hawrot-Paw et al 2020). Microalgae were harvested after eight days of cultivation, and biomass was collected using a HITACHI CF 16 RXII centrifuge at 8000 g for 10 min at 4°C. After that, pelleted *P. cruentum* were mixed with 0.1 M buffer phosphate (pH 6.7, 0.05 M K_2HPO_4, 0.05 M KH_2PO_4) and sonicated to homogeneity. Then the cell lysate was centrifuged again. The supernatant was gradually saturated with 60% ammonium sulfate. The resulting solution was kept for 2 h at 4°C and centrifuged at 12000 g for 15 min at 4°C, using a TOMY CAX-370 centrifuge. The precipitate was kept at 4°C until use.

Purification and characterization. Purification of a crude extract of BPE was accomplished using medium-pressure liquid chromatography (MPLC). Chromatography was performed on a Buchi type Sepacore X50 system connected with an inner diameter 2x12 cm plastic column of ceramic HAp (20 g). The mobile phase consisted of sodium

phosphate buffer (pH 6.8, 1 M NaH_2PO_4, 1 M Na_2HPO_4) with 0.1% NaCl using a 10 mL sample loop at a flow rate of 20 mL min. Samples (2 mL) were loaded onto the HAp column and eluted with a linear gradient of 0.1% NaCl (from 20% to 90%). The resulting eluent was monitored by UV absorbance at 220, 254, and 364 nm and 5 mL fractions were collected. The purity of isolated BPE was determined from the A_{565}/A_{280} ratio using a Cary 50 spectrophotometer (Bennet & Bogorad 1973).

Results and Discussion

Synthesis and characterization. The reaction of Na_2HPO_4 with $CaCl_2$ yielded 16.9 g of white, crystalline HAp. Crystalline HAp was sintered at 1200°C to obtain ceramic HAp (15.8 g). HAp synthesis was performed several times to obtain 60 g of ceramic HAp. Surface morphology of ceramic HAp was investigated with SEM, showing that HAp had formed agglomerated, rod-shaped structures (Figure 1).

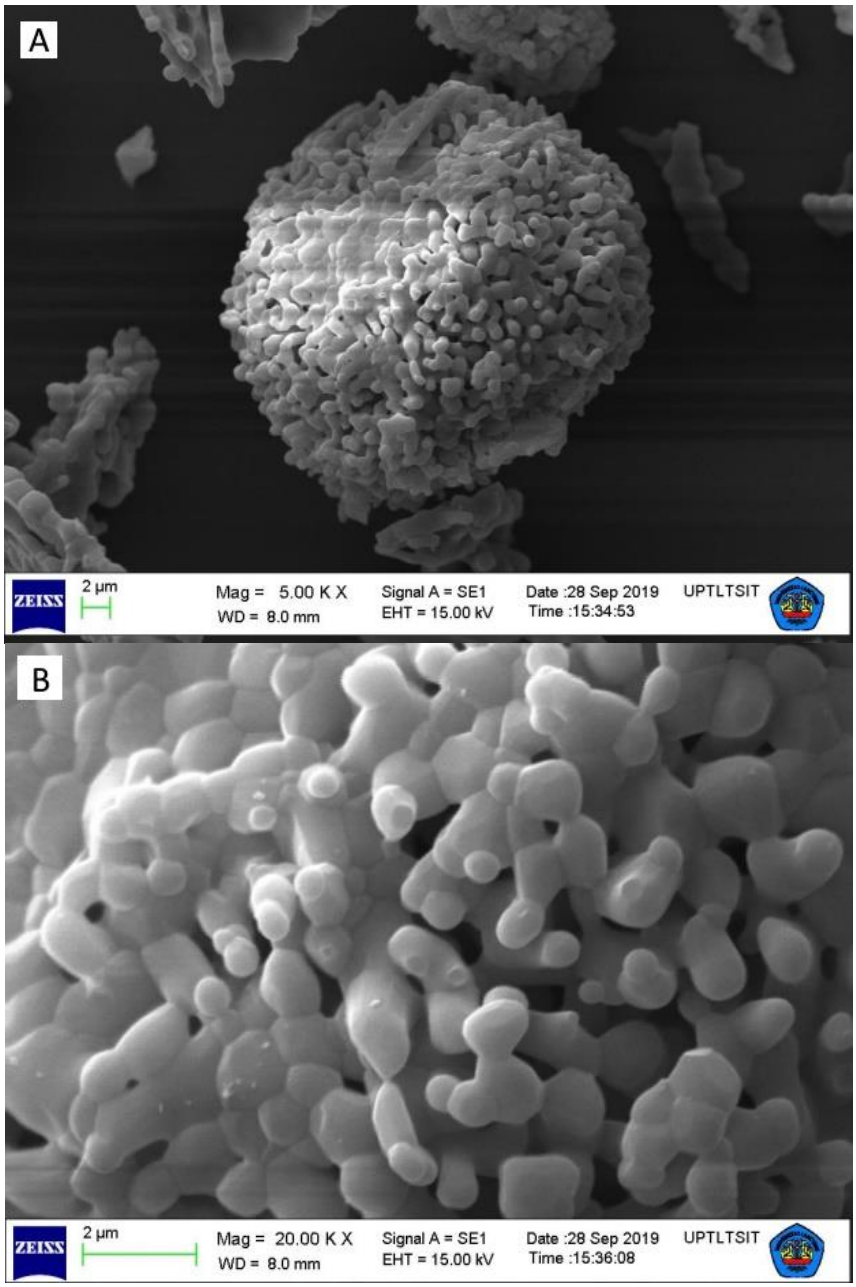

Figure 1. Visualization of sintered hydroxyapatite using a scanning electron microscope; A - magnification 500x; B - magnification 2000x.

As depicted in Figure 2, EDX spectra confirmed that ceramic HAp consists mainly of calcium, phosphorus, and oxygen, which together form calcium hydroxyl phosphate. Chemical analysis by EDX reveals a Ca/P ratio of 0.7, for the sample after heat-treatment at 1200°C (Table 1). Based on a chromatographic point of view, HAp with Ca/P ratio of 1.67 consists of positively charged pairs of calcium ions (C sites) and six negatively charged oxygen atoms associated with triplets of phosphates (P-sites). Functional groups of C-sites, P-sites, and hydroxyl groups are connected in a fixed pattern on the crystal surface (Kawasaki et al 1985). For a Ca/P ratio less than 1.67, this leaves phosphate residues as the dominant surface feature. HAp tends to repel negatively charged residues like carboxyl and phosphoryl groups (Jungbauer et al 2004).

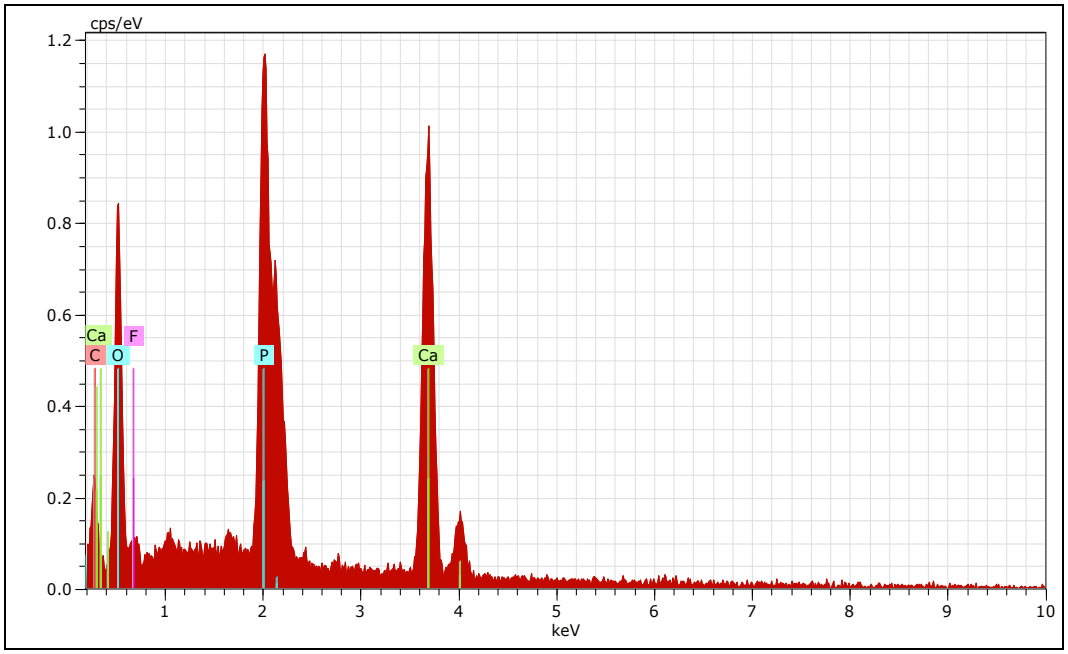

Figure 2. Energy-dispersive X-ray spectroscopy (EDX) characterization of ceramic hydroxyapatite used as a stationary phase in medium pressure liquid chromatography.

Table 1

Percent weight and atomic composition of ceramic hydroxyapatite

	Ceramic hydroxyapatite		
Element	*Weight %*	*Atomic %*	*Ca/P (atomic)*
O	47.85±3.05	57.45±3.05	
P	20.54±0.23	12.74±0.23	0.7±0.01
Ca	18.53±0.16	8.88±0.16	

Cultivation. Overall, *P. cruentum* required four days for adaptation in effluent biogas before entering an exponential growth phase (Figure 3a). The growth peak occurred on the 8th day of cultivation, for both growth media. After 10 days, the increase in biomass concentration in media F2 and effluent biogas was reduced. On the final day of culture, SEM was used to visualize the morphology of single cells of *P. cruentum* in effluent biogas (Figure 3b).

Single cells of *P. cruentum* grew rapidly in effluent biogas media, and displayed a characteristic red color during cultivation. The composition and physico-chemical properties of effluent biogas had no adverse effects on the morphology. After the 8th day of cultivation, the culture achieved 1.5 g of wet biomass per L, which compares favorably with cultures of other microalgae (Li et al 2019a).

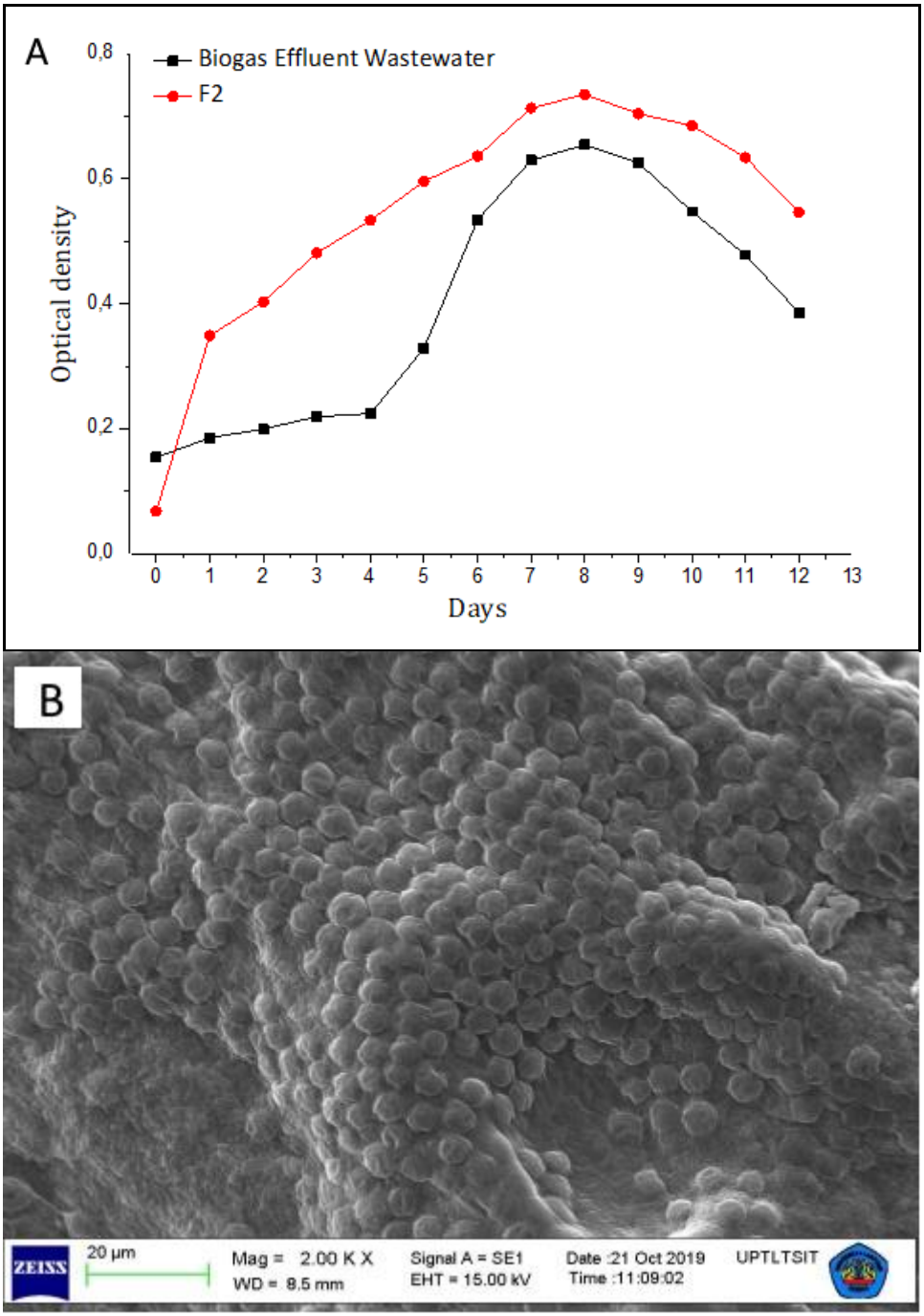

Figure 3. A - optical density growth curve of *Porphyridium cruentum* in F2 media (red) and media supplemented with tapioca waste water effluent biogas (black); B - morphology of *P. cruentum* under scanning electron microscopy (2000x magnification).

In general, microalgae can be grown in media mixed with 20% effluent biogas (Ichsan et al 2014). The BPE content in microalgal biomass depends on cultivation conditions and species. A study on *Porphyridium purpureum* (syn *P. cruentum*) showed that cells grown with sufficient nitrogen, were red, while nitrogen-limited cells were green (Li et al 2019b). The phycoerythrin content in the red microalga *P. purpureum* depends not only on nitrogen, but also on various macronutrients (Lu et al 2020).

Purification and characterization. The chromatogram showed a pronounced peak at a retention time of about 3 min after a delay of 1 min (Figure 4). Elution was accomplished at 20 mL min^{-1} with linear NaCl gradient. UV absorbance at 360 nm showed broad peak,

compared with those at 220 nm and 254 nm. Fraction 8 was collected and analyzed using a spectrophotometer.

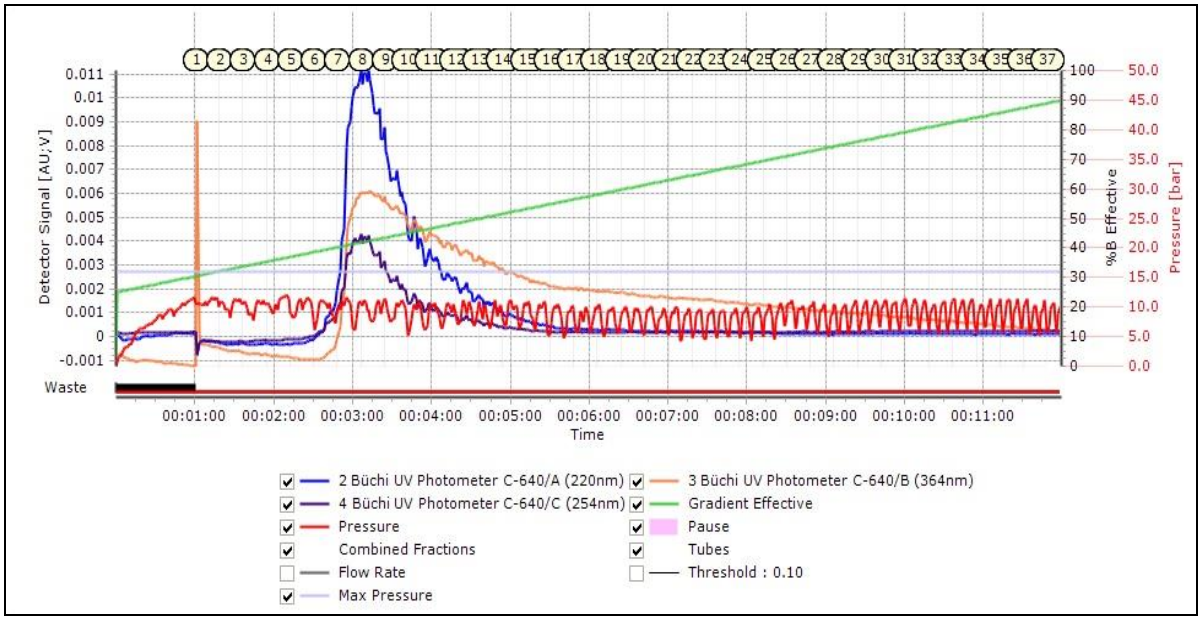

Figure 4. Elution of ß-phycoerythrin from a ceramic hydroxyapatite column (in a diameter of 2 cm x L 12 cm) at 20 mL min^{-1}, 0.1% NaCl (λ= 220, 254, and 360 nm).

The absorbance spectrum of crude *Porphyridium* extract showed absorbance peaks at 280, 545, 565, 620, and 652 nm, indicating a mixture of protein and phycobiliprotein. After separation using MPLC, the absorption spectrum of purified BPE showed absorption peaks at 545 and 565 nm. No absorbance peak was found at 620 or 652 nm, indicating the absence of phycourobilin, phycocyanin, and allo-phycocyanin in the purified BPE sample, while low absorption in the region of 280 nm suggested high purity of the BPE in fraction 8 (Figure 5). The A_{546}/A_{280} ratio of the purified BPE was 3.995, which was considered sufficient for food grade preparations (0.4-3.8) (Walter et al 2011).

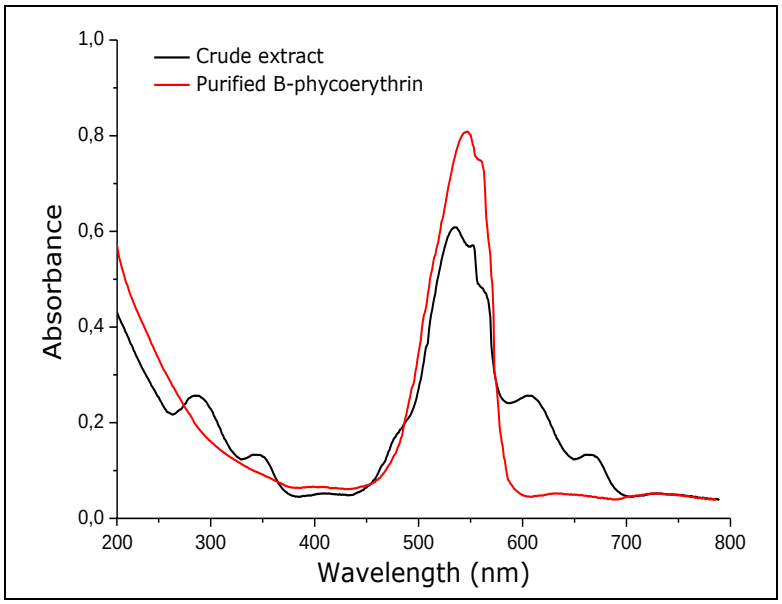

Figure 5. Absorbance spectrum of crude extract and purified ß-phycoerythrin.

Table 2

Purity ratio of ß-phycoerythrin

Sample	*A_{280}*	*A_{546}*	*Purity (A_{546}/A_{280})*	*% Recovery of BPE*
Crude Extract	0.237	0.608	2.565	60
Recover PE	0.202	0.807	3.995	

Note: BPE - ß-phycoerythrin.

Conclusions. This study successfully cultivated *P. cruentum* in F2 media with 20% effluent biogas and employed ceramic HAp to recover 60% of the BPE with an A546/A280 ratio of 3.995. We conclude that the above procedure has potential to improve the commercial production of BPE.

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Conflict of Interest. The authors declare that there is on conflict of interest.

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