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Sustainable approach in phlorotannin recovery from macroalgae

Shir Reen Chia,[1,2] Pau Loke Show,[2] Siew-Moi Phang,[3] Tau Chuan Ling,[4] and Hwai Chyuan Ong[1,*]

Department of Mechanical Engineering, Faculty of Engineering, University of Malaya, 50603 Kuala Lumpur, Malaysia,[1] Bioseparation Research Group, Department of Chemical and Environmental Engineering, Faculty of Engineering, University of Nottingham Malaysia Campus, Jalan Broga, 43500 Semenyih, Selangor Darul Ehsan, Malaysia,[2] Institute Ocean and Earth Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia,[3] and Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia[4]

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In this present study, alcohol/salt liquid biphasic system was used to extract phlorotannin from brown macroalgae. Liquid biphasic system is a new green technology that integrated with various processes into one-step, by concentrating, separating and purifying the bioproduct in a unit operation. The solvent used is non-toxic and there is potential for solvent recovery which is beneficial to the environment. Phlorotannin is a bioactive compound that has gained much attention due to its health beneficial effect. Therefore, the isolation of phlorotannin is lucrative as it contains various biological activities that are capable to be utilised into food and pharmaceutical application. By using 2-propanol/ammonium sulphate system, the highest recovery of phlorotannin was 76.1% and 91.67% with purification factor of 2.49 and 1.59 from *Padina australis* and *Sargassum binderi*, respectively. A recycling study was performed and the salt phase of system was recycled where maximum salt recovery of 41.04% and 72.39% could be obtained from systems containing *P. australis* and *S. binderi*, respectively. Similar recovery of phlorotannin was observed after performing two cycles of the system, this concludes that the system has good recyclability and eco-friendly.

[**Keywords:** Liquid biphasic system; Phlorotannin recovery; Macroalgae; *Padina australis*; *Sargassum binderi*]

Phlorotannin is a type of polyphenolic compounds exists in macroalgae (commonly known as seaweed), especially brown macroalgae (Phaeophyta). It was synthesized to defence against stress conditions, for example grazing, UV radiation and bacterial infection (1,2) or acts as the secondary defence metabolites during the development of algal cell walls (3). This type of bioactive compound has attracted huge interest from community due to the variation of their bioactivities, such as antidiabetic, antioxidant, anti-bacterial, and anti-inflammatory (4). Stern et al. (5) have stated that the precipitation of protein could be occurred through the interaction of phlorotannin and protein. Protein precipitation is significant in downstream processing to isolate the protein from all possible contaminants in blood. Phlorotannins are made up of oligomers and polymers of phloroglucinols (1,3,5-trihydroxybenzene). Different structure of phlorotannins may formed via various polymerization degrees (6). Natural bioactive compounds or metabolites in algae are often extracted due to their non-toxicity properties and potential health benefits. The conventional methods are solid–liquid extraction, supercritical fluid extraction, pressurised liquid extraction and centrifugal partition extraction (3). Conventional methods are time-consuming and often required high volume of toxic organic solvent during the extraction process. Therefore, liquid biphasic system (LBS) is introduced in extracting and purifying the components or metabolites from plant and aquaculture at the same time.

LBS is a better alternative compared to conventional methods as higher yield and purified product could be obtained through this method. There are several types of LBS, for example polymer/salt, alcohol/salt or polymer/polymer as the phase forming components (7,8). Over decades, this technique has been studied intensively as a separation technology in separating and purifying biological products such as enzyme, proteins and metabolites from biological sources without denaturing the targeted product. It was reported that LBS is capable to extract biomolecules in a short mass transfer period with high efficiency and selectivity using lower cost (9,10). However, parameters such as types of phase forming components, pH, temperature or addition of inorganic salt may affect the extraction and purification process accordingly depending on the targeted product. Apart from that, the LBS formed by alcohol/salt has been proved to extract out and purify high value product (11,12) like laccase from processed *Hericium erinaceus* (Bull.:Fr) Pers. Fruiting bodies (13), γ-cyclodextrin by *Bacillus cereus* cyclodextrin glycosyltransferase (14) and fucoxanthin from *Isochrysis galbana* and *Phaeodactylum tricornutum* (15) successfully.

Until now, most of the reported studies are related to solvent extraction and enzyme-assisted extraction to recover phlorotannin. This is the first report on phlorotannin recovery using LBS, which no study has done before. In this present study, we were focussing on the feasibility of alcohol/salt LBS technique in extraction/purification of phlorotannin from *Padina australis* and *Sargassum binderi*. Ammonium sulphate has been chosen to be the type of salt phase due to the versatility of ammonium sulphate forming two phases with other alcohols. Besides, it is a cheaper choice compared to the usage of polymer and copolymer as the bottom phase in the system. Ammonium sulphate is also known for its functional usage in

* Corresponding author. Tel.: +603 7967 5247; fax: +603 7967 5317.
E-mail address: onghc@um.edu.my (H.C. Ong).

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industrial especially as soil fertilizer where it can easily be obtained. The type of alcohol was conducted to investigate a better combination of alcohol with ammonium sulphate before optimising the system. Thus, in this study, several parameters have been investigated to determine the capability of LBS in partitioning phlorotannin from brown macroalgae, such as effect of tie-line length, volume ratio, pH study and sample concentration.

MATERIALS AND METHODS

Materials The marine algae, *P. australis* and *S. binderi* were collected from Cape Rachado, Port Dickson, Malaysia on September 2016. The samples were washed thoroughly three times with tap water to remove the sediments and marine salts attached on the surface of samples. The samples were rinsed with distilled water before drying in the oven. The cleaned samples were oven-dried at 50°C for 3 to 5 days. The dried samples were cut into small fragments prior to further analysis. Besides, phloroglucinol (1,3,5-trihydroxybenzene) for analysis, ammonium sulphate ($(NH_4)_2SO_4$), Folin-Ciocalteu reagent and sodium carbonate (Na_2CO_3) were purchased from Merck (Darmstadt, Germany). Ethanol, methanol, 1-propanol and 2-propanol were provided by R&M chemicals (Malaysia). All chemicals and reagents used in this paper were analytical grade.

Partitioning of phlorotannin in LBS The composition of LBS was prepared on weight percentage (%, w/w) basis according to the alcohol/ammonium sulphate phase diagram reported by Khayati and Shahriari (16) and Wang et al. (17). A concentrated stock solution of ammonium sulphate (40%, w/w) was prepared by mixing appropriate quantities of chemicals and distilled water at an appropriate ratio. The alcohol stock solutions were prepared at a weight basis of 80% prior for usage. The LBS was prepared in a 1.5 mL centrifuge tube by adding the appropriate quantity of 80% (w/w) alcohol stock solution, 40% (w/w) $(NH_4)_2SO_4$ stock solution, distilled water and 5% (w/w) of samples to a final mass of 1.5 g. The components were mixed homogenous by gentle agitation and subjected to centrifugation. The mixture was centrifuged using a Sigma 1–14 microcentrifuge (Sartorius, Germany) at 4000 $\times g$ for 10 min to ensure a complete separation. After phase separation, the top and bottom phases were collected and the concentration of phlorotannin within the system were analysed. All the experiments were performed triplicate at room temperature.

Determination of phlorotannin content The total phlorotannin content in brown macroalgae was examined by a modified version of Folin-Ciocalteu method described in the work by Ahmad et al. (18) with some modifications. A standard stock solution of phloroglucinol (20,000 mg L^{-1}) was prepared by dissolving 0.2 g of phloroglucinol into distilled water and made up to 10 mL. Then, the stock solution was diluted into a range of 20–600 mg L^{-1} as working solutions, in order to produce a calibration curve for measuring the phlorotannin concentration. The working solutions were prepared in a measurable range of the Epoch microplate reader (BioTek, USA). 0.2 mL of sample extract were extracted out from top and bottom phases, was mixed with 1.0 mL of 10% (v/v) Folin-Ciocalteu reagent (which the reagent diluted with distilled water) into test tube. The mixture was allowed to stand for 5 min and followed by the addition of 0.8 mL of 7.5% (w/v) Na_2CO_3 to the mixture. Each test tubes were capscrewed and vortexed for 20 s. The mixture was incubated at room temperature to react for 2 h in dark and was measured at wavelength of 740 nm using microplate reader in homogenous condition. The absorbance of the sample extract was measured against a blank sample which contained similar mixture without the sample extract. The concentration of the total phlorotannin content in sample extract was examined by comparing the value obtained from the calibration curve of phloroglucinol.

Determination of phase volume ratio, purification factor, partition coefficient and recovery Phase volume ratio is the volume ratio of alcohol to salt phase after the centrifugation which was calculated using the equation as shown below:

$$V_R = \frac{V_T}{V_B} \quad (1)$$

where V_T is the volume of the top phase and V_B is the volume of the bottom phase.

The purification factor (P_F) of the bottom phase was calculated using the formula below:

$$P_F = \frac{\text{SA of phase sample}}{\text{SA of crude feedstock}} \quad (2)$$

where SA is the specific activity and P_F is the purification factor.

The partition coefficient (K) of the phlorotannins between the phases was calculated as follows:

$$K = \frac{C_T}{C_B} \quad (3)$$

where C_T and C_B are equilibrium concentrations of the partitioned phlorotannin in the alcohol-rich top phase and the salt-rich bottom phase, respectively. The recovery of phlorotannin, R (%) in the bottom phase was examined using the formula as follows:

$$R(\%) = \frac{100}{1 + [1/(V_R \times K)]} \quad (4)$$

where K is the partition coefficient and V_R is the volume ratio.

Recycling study The recycling studies were performed in 50 ml test tubes, with known mass. A total mass of 20 g for both systems containing different macroalgae species were mixed, followed by centrifugation at 4000 $\times g$ for 10 min to separate the phases. The upper and bottom phase were separated and measured after extraction. Both phases were subjected to recycle process. The alcohol was recovered from the remaining water by using rotary evaporator. The volume of recovered alcohol was recorded and the refractive index value was measured. The yield of recovered 2-propanol (Y_A) was defined as followed:

$$Y_A = \frac{V_1}{V_0} \times 100\% \quad (5)$$

where V_1 and V_0 is the volume of 2-propanol recovered from the top phase and original volume of 2-propanol used in the system.

Recovery of salt was performed by adding methanol to the bottom phase (0.5–2 times the volume of bottom phase). The crystallized ammonium sulphate was recovered through filtration. The yield of ammonium sulphate recovered (Y_S) was defined as followed:

$$Y_S = \frac{M_1}{M_0} \times 100\% \quad (6)$$

where M_1 and M_0 is the mass of ammonium sulphate recovered from the bottom phase and original mass of ammonium sulphate used in the system.

RESULTS AND DISCUSSION

Selection of alcohol/salt LBS Four common types of alcohol, namely methanol, ethanol, 1-propanol and 2-propanol, were chosen to be examined the suitable phase forming components combined with ammonium sulphate in extracting phlorotannin from macroalgae. Several studies have shown phlorotannin activity remains stable after the extraction with these common alcohols (19–21). In general, both species have showed higher affinity towards the combination of 2-propanol/ammonium sulphate system compared to other systems. The higher the interaction of hydrogen bond between alcohol and water resulted in the lower solubility of targeted product in alcohol phase (22). Therefore, targeted product was preferentially partitioned to the salt phase. From Fig. 1, the highest recovery of phlorotannin obtained from *P. australis* is 59.3% which have comparable

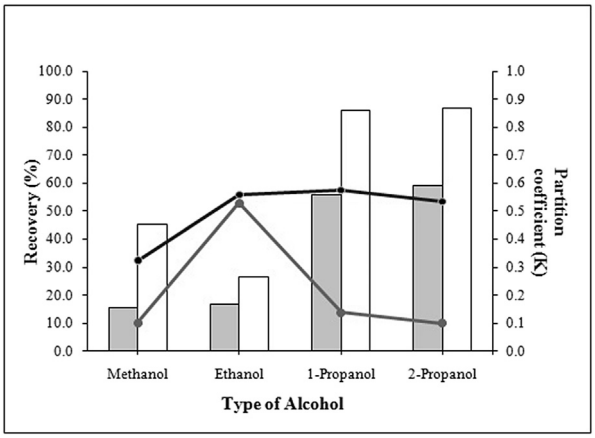

FIG. 1. Effect of alcohols on recovery and partition coefficient (K) of phlorotannin from *P. australis* and *S. binderi*. Shaded columns represent recovery of phlorotannin from *P. australis*; open columns represent recovery of phlorotannin from *S. binderi*; black line with marker represents partition coefficient of *P. australis*; shaded line with marker represents partition coefficient of *S. binderi*.

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