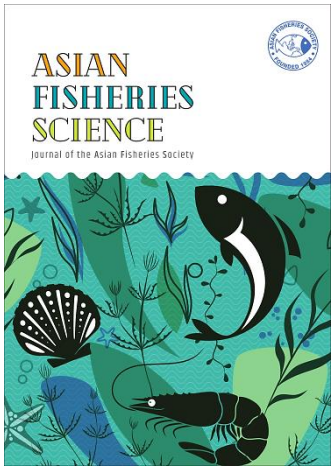

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Production and Purity of Phycobiliproteins from Selected Marine and Freshwater Cyanobacteria Subjected to Different Drying Methods

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Abstract

Phycobiliproteins, light-harvesting pigments found in cyanobacteria, red algae and cryptomonad are gaining importance in food, nutraceutical and pharmaceutical industries. Thus, a sustainable source of phycobiliproteins production is an essential consideration in meeting the increasing demand for these natural pigments. The present work aimed to compare the concentration and purity of phycobiliproteins from marine (*Geitlerinema* sp. and *Synechococcus* sp.) and freshwater (*Oscillatoria* sp. and *Spirulina* sp.) cyanobacteria, when subjected to different drying methods viz. sun-drying, oven-drying and freeze-drying. Results showed that the three different drying methods influenced the concentration and purity of phycobiliproteins from the different cyanobacteria. Under oven drying condition, phycocyanin concentration ($mg.mL^{-1}$) was significantly higher ($P < 0.05$) in marine *Geitlerinema* sp. followed by *Oscillatoria* sp. *Synechococcus* sp. and *Spirulina* sp., respectively, compared to sun-drying and freeze-drying methods. Phycoerythrin and allophycocyanin concentrations were also significantly higher ($P < 0.05$) in marine periphytic *Geitlerinema* sp. when compared to other cyanobacteria subjected to oven drying. In addition, results from oven-dried marine periphytic *Geitlerinema* sp. showed that total phycobiliproteins production and purity ratio of phycocyanin were significantly higher ($P < 0.05$) in comparison to sun-drying or freeze-drying *Spirulina* sp., *Synechococcus* sp. and freshwater *Oscillatoria* sp.

Keywords: cyanobacteria, phycobiliproteins, phycocyanin, phycoerythrin, drying conditions

Introduction

Cyanobacteria and algae contain valuable pigments such as carotenoids and phycobiliproteins, in addition, they are the immense sources of several metabolites such as alkaloids, carbohydrates, flavonoids, pigments, phenols, saponins, steroids, tannins, terpenes, and vitamins which can be utilised in biotechnology and industrial fields (Guihéneuf et al., 2016). The potential use of cyanobacteria in agriculture, aquaculture, nutraceuticals, bioenergy and pollution control (bioremediation) is well recognised (Abed et al., 2009). The unique spectral features such as strong absorbance and fluorescence, proteinaceous nature and, some imperative properties like hepato-protective, antioxidants, anti-inflammatory and anti-ageing activity of phycobiliproteins enable their use in foods, cosmetics, pharmaceutical and biomedical industries (Sonani et al., 2016). Moreover, phycobiliproteins are used as colouring agents in cosmetics, dairy products, ice creams, jellies, diagnostics, biomedical research and oxidative stress-induced diseases as they are proteinaceous and possess unique colour, fluorescence and antioxidant properties (Pandey, 2013). Phycocyanin from *Spirulina* plays an important role in inducing apoptosis on HeLa cells (Li et al., 2009), enhancing wound healing (Madhyastha et al., 2008), retardation of platelet aggregation (Chiu et al., 2006) and eradication of cancer cells *in vitro* (Li et al., 2010).

Phycobiliproteins in cyanobacteria contribute 50 % of the total cellular proteins, and these include phycocyanin (blue pigment), phycoerythrin (red pigment) and allophycocyanin (Madhyastha et al., 2008) based on inherent colour and absorbance properties. Phycocyanin is an accessory pigment of cyanobacteria, but not all cyanobacteria are bluish due to the presence of phycoerythrin which gives them a red or pink colouration. Phycocyanin (blue) and phycoerythrin (red) are the two major natural pigments commercially used from cyanobacteria.

Cyanobacteria are a potential source for the commercial production of phycocyanin. In cyanobacteria, a significant proportion of the total protein is contributed by the phycobiliproteins, which are located in granules attached to the photosynthetic membrane (Santiago-Santos et al., 2004). They can use light spectra between absorption peaks of chlorophyll *a* and carotenoids with the help of accessory pigments (phycocyanin, phycoerythrin and allophycocyanin) (Santiago-Santos et al., 2004). The contents of pigments depend on the species and cultivation conditions (Begum et al., 2016). Currently, phycocyanin and phycoerythrin have been extracted from *Spirulina* sp. (Hemlata, 2009), *Calothrix* 7601 (Prasanna et al., 2004) and marine cyanobacterium *Porphyridium cruentum* (Roman et al., 2002).

The drying of cyanobacterial biomass before extraction of phycobiliproteins is an important step as bacterial degradation of the wet biomass could affect the pigment production. In general, drying methods can reduce the quantity and purity of phycocyanin. Thus, a method that could preserve the original material, but minimise thermal damages such as freeze-drying is preferred. During the freeze-drying process, water is removed via vacuum pumping from the material while it is frozen (Nireesha et al., 2013).

Processing of cyanobacterial biomass by sun-drying is perhaps the cheapest drying method engaged (Prakash et al., 1997). However, sun-drying technique is a lengthy process and requires large drying surface. In addition, there is a risk of loss of some bio-reactive products. Low-pressure shelf drying is another low-cost drying technology that has been investigated (Prakash et al., 1997) but has low efficiency. Sarada et al. (1999) reported 50 % loss of phycocyanin when *Spirulina* was dried using spray or convective dryers. Drum-drying (Prakash et al., 1997), spray-drying (Desmorieux and Decaen, 2006), fluidised bed drying (Leach et al., 1998), freeze-drying (Millamena et al., 1990), and refractance window dehydration technologies are commonly used as drying methods which are efficient but costly.

There is great market demand for phycobiliproteins at present, but the scarcity of information in terms of species selection for high-quality pigment and drying methods of algal biomass hampers its commercial production. Therefore, this study aimed to compare the production and purity of phycobiliproteins from selected marine and freshwater cyanobacteria using different drying methods.

Materials and Methods

Cyanobacteria culture conditions

Two marine (*Geitlerinema* sp., *Synechococcus* sp.) and two freshwater (*Oscillatoria* sp., *Spirulina* sp.) cyanobacteria were used in this study to determine whether there are differences between marine and freshwater algae in terms of phycobiliproteins contents. All the cyanobacteria were isolated from Malaysian water bodies, and pure cultures were maintained in Microalgae Culture Unit, Universiti Putra Malaysia. Marine periphytic *Geitlerinema* sp. and *Synechococcus* sp. were cultured in Conway medium (Tompkins et al., 1995), whereas freshwater *Oscillatoria* sp. was cultured in Bold's basal medium (Nichols and Bold, 1965) and *Spirulina* sp. in Zarrouk's medium (Aiba and Ogawa, 1977). All the species were cultured in 20 L glass tanks, aerated and kept under a shade with an average light intensity of 140 µmol photons m^{-2} s^{-1}. All treatments were carried out in triplicates. The biomass of cultured species was calculated according to Lavens and Sorgeloos (1996). In addition, the specific growth rate (µ day^{-1}) of the cultured species were calculated according to Clesceri et al. (1989).

Identification of cyanobacteria

All four isolates of marine and freshwater cyanobacteria were identified using the conventional method. Isolates were identified to generic level (Bellinger, 1992). The isolate which showed the highest production and purity was confirmed by molecular identification. Molecular identification was done according to Nübel et al. (1997) with minor modifications.

Harvesting

The cultures were harvested at their stationary phase by centrifugation at 6,000 ×g for 15 min. The cell pellets were washed three times with distilled water.

Drying and extraction methods

The harvested biomass of different cyanobacteria was subjected to different drying methods such as oven-drying (40 °C), freeze-drying and sun-drying. Harvested biomass was dried overnight in the oven according to Sarada et al., (1999). Samples were dried in freeze dryer until it was fully dry. In case of sun-drying, biomass was kept outside under shade until dry. The dried powders were then ground using mortar and pestle and sieved (120 µ mesh). Dried powder (40 mg) was then soaked in 10 mL phosphate buffer (0.1 M; pH 7.0) and vortexed to mix and stored at 4 °C for 24 h for phycobiliproteins extraction. Then

the phycobiliproteins containing supernatant was centrifuged at 6000 rpm for 10 min. The supernatant was collected and absorbance was read at different wavelengths (562, 615 and 652 nm) using phosphate buffer as blank. All experiments of drying and extraction were carried out in triplicates.

Spectrophotometric estimation of phycobiliproteins

The amount of phycocyanin (PC), phycoerythrin (PE) and allophycocyanin (APC) in the sample was calculated using simultaneous equations (Bennett and Bogorad, 1973) and the extinction coefficients (Siegelman and Kycia, 1978) as follows:

$$\text{Phycocyanin (PC) mg.mL}^{-1} = \{A615 - (0.474 \times A652)\} / 5.34$$

$$\text{Allophycocyanin (APC) mg.mL}^{-1} = \{A652 - (0.208 \times A615)\} / 5.09$$

$$\text{Phycoerythrin (PE) mg.mL}^{-1} = \{A562 - (2.41 \times PC) - (0.849 \times APC)\} / 9.62$$

Total phycocyanin, phycoerythrin and allophycocyanin (mg.g^{-1}) were calculated according to Silveira et al. (2007):

$$\text{Pigment concentration} \times V / DB$$

where, V = solvent volume and DB = dried biomass.

Purification factor

Phycocyanin, phycoerythrin and allophycocyanin were extracted and the purity was monitored spectrophotometrically by the A620/A280, A565/A280 and A650/A280 ratio (Bennett and Bogorad, 1973).

Statistical analysis

Data were analysed using both one way and two-way ANOVA. Duncan multiple range test at $P < 0.05$ level of probability was used to determine significant differences among different cyanobacteria and drying methods. All the data which were expressed in percentages were arcsine-transformed to satisfy the condition of homogeneity of variance (Zar, 1984). Statistical analyses were accomplished using the statistical analysis system (SAS, 2002) computer software.

Results

All the four isolates were identified according to their morphological feature and confirmed as *Geitlerinema* sp., *Synechococcus* sp., *Oscillatoria* sp. and *Spirulina* sp. *Geitlerinema* sp., which showed maximum production and purity of phycobiliproteins and were further confirmed by molecular identification (Figs. 1, 2). Based on BLAST comparison, the sequence from the isolated marine periphytic cyanobacteria possessed 99 % sequence identity to reference *Geitlerinema* sp. from NCBI (National Center for Biotechnology Information) GenBank (Accession number: HQ197684).

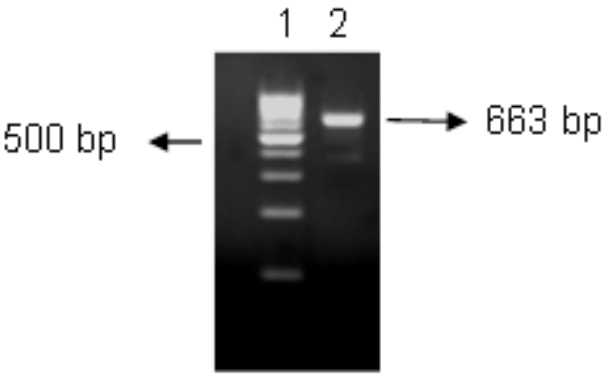

Fig. 1. Gel electrophoresis of PCR product amplified using 16S rRNA forward and reverse primers. Lane 1 represents the 100bp DNA ladder (Fermentas) and Lane 2 PCR product of marine periphytic cyanobacteria.

The growth performance of experimental cyanobacteria species cultured in 20 L glass tanks showed that *Spirulina* sp. had the lowest biomass ($P < 0.05$) compared to the others (Table 1). Its specific growth rate was also the lowest, but not significantly different from *Synechococcus* sp.

Marine (*Geitlerinema* sp. and *Synechococcus* sp.) and freshwater cyanobacteria (*Oscillatoria* sp. and *Spirulina* sp.) were subjected to different drying methods (sun-drying, oven-drying and freeze-drying) and screened to compare the concentration and purity of phycobiliproteins (PC, PE and APC). Results showed that biomass dried in the oven under controlled temperature had significantly ($P < 0.05$) higher concentration (mg.mL^{-1} of culture volume) of PC, PE and APC in marine *Geitlerinema* sp. (0.55 ± 0.1; 0.08 ± 0.2; 0.20 ± 0.1) compared to sun-dried (0.34 ± 0.0; 0.04 ± 0.2; 0.14 ± 0.1) and freeze-dried (0.20 ± 0.1; 0.06 ± 0.1; 0.10 ± 0.2) biomass (Figs. 3a, 3b, 3c).

In case of total phycobiliproteins production under oven drying method, marine *Geitlerinema* sp. (208.1 ± 3.14 mg.g^{-1}) showed significantly higher content ($P < 0.05$) followed by freshwater *Oscillatoria* sp. (182.0 ± 1.0 mg.g^{-1}), *Spirulina* sp. (116.1 ± 1.3 mg.g^{-1}) and *Synechococcus* sp. (141.5 ± 2.2 mg.g^{-1}) (Table 2).

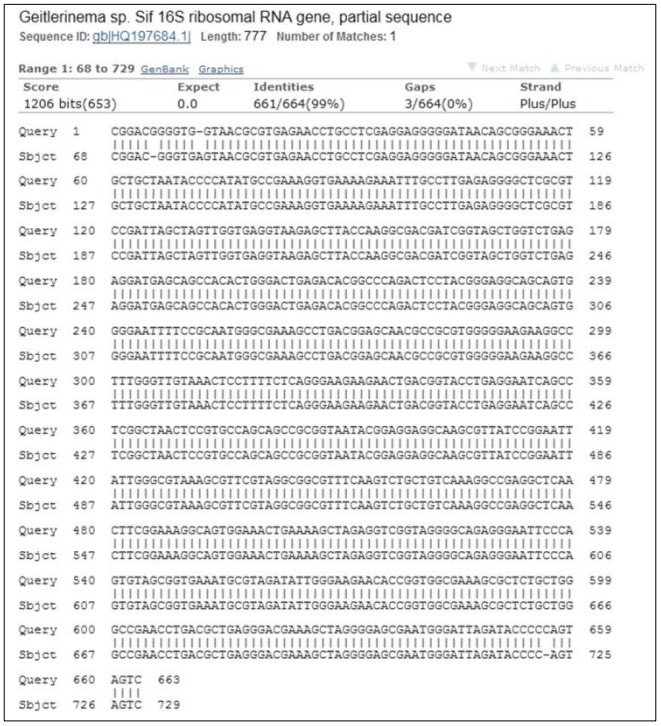

Fig. 2. Partial nucleotide sequence of the 16S rRNA gene from marine periphytic cyanobacteria aligned with *Geitlerinema* sp. (Accession number: HQ197684) partial sequence available in NCBI GenBank database.

Table 1. Mean values (± standard error of the means, n = 3) of biomass and specific growth rates (SGR) of experimental cyanobacteria species. Mean values in columns with different superscripts are significantly different at $P < 0.05$.

Species	Biomass ($g.L^{-1}$)	SGR ($\mu.day^{-1}$)
Geitlerinema sp.	0.88 ± 0.91^{a}	0.45 ± 0.01^{a}
Synechococcus sp.	0.78 ± 0.31^{a}	0.35 ± 0.03^{b}
Oscillatoria sp.	0.88 ± 0.11^{a}	0.45 ± 0.01^{a}
Spirulina sp.	0.48 ± 0.21^{a}	0.30 ± 0.02^{b}

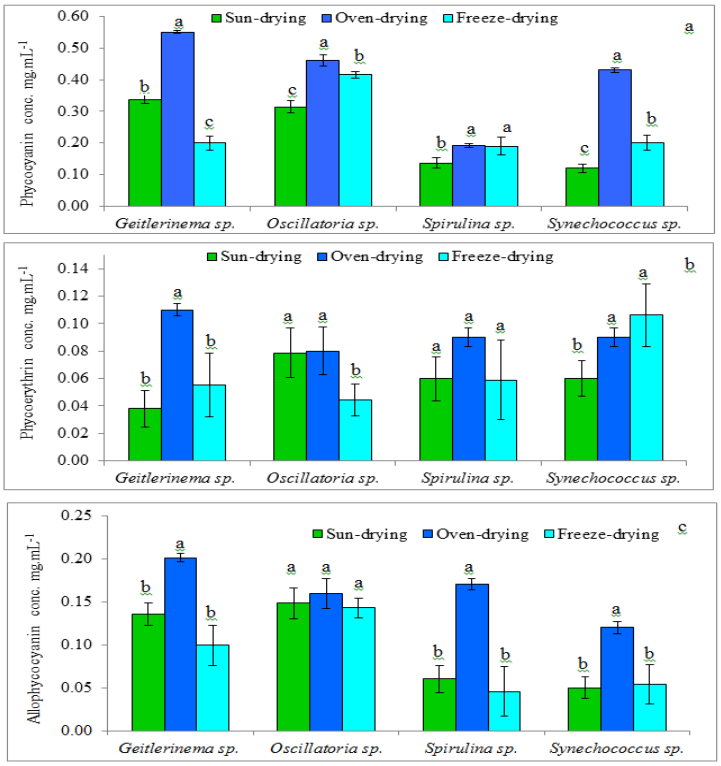

Fig. 3. Total phycocyanin concentration ($mg.mL^{-1}$)(a), phycoerythrin concentration ($mg.mL^{-1}$)(b) and allophycocyanin concentration ($mg.mL^{-1}$)(c) from *Geitlerinema* sp., freshwater *Oscillatoria* sp., *Spirulina* sp., and *Synechococcus* sp. under different drying methods.

In addition, the purity ratio of PC, PE and APC were also significantly higher ($P < 0.05$) under oven drying condition in marine *Geitlerinema* sp. (1.2 ± 0.1; 0.6 ± 0.1; 0.5 ± 0.2) compared to freshwater *Oscillatoria* sp. (0.7 ± 0.2; 0.4 ± 0.2; 0.5 ± 0.3); *Spirulina* sp. (0.5 ± 0.3; 0.3 ± 0.4; 0.3 ± 0.3) and *Synechococcus* sp. (0.4 ± 0.2; 0.3 ± 0.2; 0.3 ± 0.2), and also to other drying methods such as sun-drying and freeze-drying (Figs. 4a, 4b, 4c).

Discussion

Phycobiliproteins (PBPs) are a family of accessory light-harvesting macromolecules organised in supramolecular complexes, called phycobilisomes (PBSs) that function as components of the photosynthetic apparatus in cyanobacteria and some eukaryotic algae. Modern research and development in the synthesis and function of PBSs have expanded the potential applications of PBPs in biotechnology, diagnostic, food and medicine (Vinod et al., 2011). They are extensively commercialised for fluorescent application in clinical and immunological analysis.

Malaysia is a tropical country and it has an excessive diversity in its cyanobacterial resources that can be exploited commercially. Nevertheless, cyanobacterial phycobiliproteins are limited and need more attention to this aspect. In the United States, the price of phycobiliproteins products range from USD3 to USD25 mg.g^{-1} for native pigment but they can reach USD1,500 mg.g^{-1} for certain cross-linked pigments with antibodies or other fluorescent molecules. In the near future, the price is likely to increase by 20 % annually (Sekar and Chandramohan, 2008).

In view of the great demand for phycocyanin at commercial level, it is therefore important to develop a simple drying method to store maximum amount of phycocyanin in the biomass during the drying process. In the present study, different drying methods of selected cyanobacterial biomass were evaluated in order to minimise the loss of phycobiliproteins during the drying process. In addition, the purity ratio of phycobiliproteins

Table 2. Total production of phycobiliproteins (mg.g^{-1}) from *Geitlerinema* sp., freshwater *Oscillatoria* sp., *Spirulina* sp., and *Synechococcus* sp. under different drying methods.

Cyanobacterial species	Drying methods		
	Sun-drying	Oven-drying	Freeze-drying
Geitlerinema sp.	121.9 ± 1.1^{b**}	208.1 ± 3.1^{a*}	85.1 ± 1.5^{c**}
Oscillatoria sp.	135.3 ± 1.9^{c*}	182.0 ± 1.0^{a**}	148.1 ± 0.8^{b*}
Spirulina sp.	59.8 ± 2.1^{c***}	116.1 ± 1.3^{a****}	66.8 ± 1.2^{b****}
Synechococcus sp.	58.9 ± 0.7^{c***}	141.5 ± 2.2^{a***}	80.2 ± 2.5^{b***}

Mean values with superscripts $^{a, b, c}$ are significantly different from others in row ($P < 0.05$) and mean values with *, **, *** and **** are significantly different from others in column ($P < 0.05$).

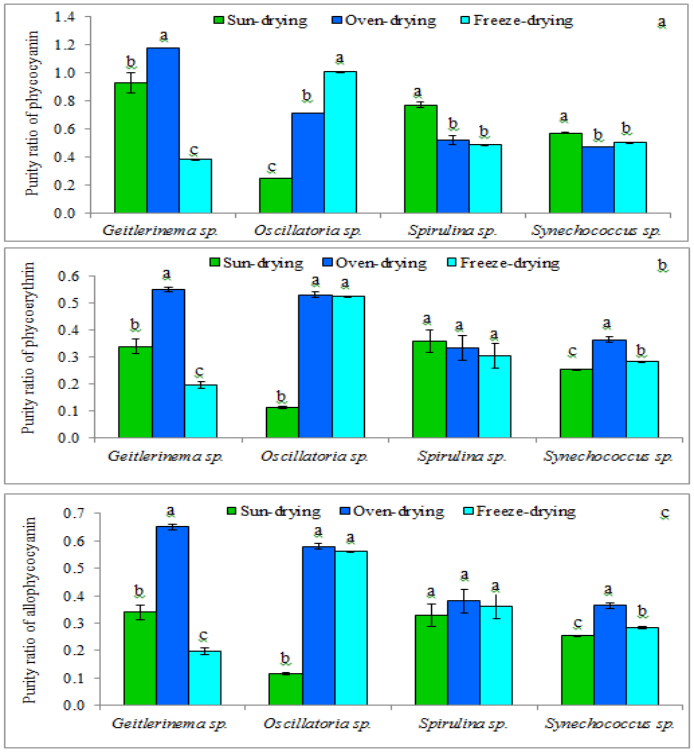

Fig. 4. Purity ratio of phycocyanin (a), phycoerythrin (b) and allophycocyanin (c) extract from *Geitlerinema* sp., freshwater *Oscillatoria* sp., *Spirulina* sp., and *Synechococcus* sp. under different drying methods.

extracted from the selected cyanobacterial biomass subjected to different drying methods was evaluated.

The first step in many extraction processes is the removal of water from the sample or drying. Drying minimises the microbial growth and deterioration by chemical reactions of the wet biomass and helps to conserve the desirable qualities. As reported by other studies, dried biomass is suitable and efficient for extraction of phycocyanin from *Spirulina* with high purity ratio. Temperature is an important factor and plays a significant role in the drying process of cyanobacterial biomass and extraction of phycocyanin. Considerable loss of phycocyanin concentration was observed by Sarada and co-researchers (Sarada et al., 1999) when wet biomass was dried between 60–150 °C. This could be due to phycocyanin's peripheral position in phycobilisomes on the thylakoid membrane and attributable to phycobilisomes sensitivity to temperature (Sarada et al., 1999). Phycocyanin is extremely sensitive to factors such as temperature, light, and pH. These factors can lead to excessive loss of phycocyanin (Martelli et al., 2014). High drying temperature (>60 °C) decreased the amount of the phycocyanin extractable from *Spirulina platensis* (Oliveira et al., 2008). Güroy et al. (2017) reported that biomass drying at 80 °C results in loss of phycocyanin. In the present study, biomass dried in oven under low temperature below 60 °C showed maximum production of phycobiliproteins with high purity ratio of phycocyanin. Drying method under shade by air circulation can be used for large-scale extraction of PC (Doke, 2005). In another study by Doke (2005), *Spirulina* biomass dried at 25 °C under shade by air circulation had maximum phycocyanin (80 $mg.g^{-1}$) production and relatively high purity ratio. Similarly, in the present study, *Geitlerinema* sp. biomass dried in oven under low temperature showed maximum production of phycobiliproteins with relatively high purity ratio of phycocyanin, phycoerythrin and allophycocynin in comparison to the other cyanobacterial species. In addition, phycocyanin extracted from oven-dried biomass also showed high purity ratio (1.14) than those reported in the literature for cell extract (0.97, 0.95) (Zhang and Chen, 1999).

The purity of phycocyanin plays a significant role in commercial applications. A purity of 0.7 is considered as food-grade, 3.9 as reactive grade and greater than 4.0 as analytical grade (Rito-Palmares et al., 2001). Different workers have applied different purification process in order to get high purity ratio. The purity of phycocyanin was reported as 1.8, 4.9 and 3.5 in *Spirulina platensis*, *Synechococcus* sp. I09201 and *Calothrix* sp. by using chitosan adsorption and aqueous two-phase extraction, hydrophobic and ion-exchange chromatography, Q-Sepharose and hydrophobic interaction chromatography, respectively (Abalde et al., 1998; Patil et. al., 2006). A study by Seo et al. (2013) demonstrated that phycocyanin from *Spirulina platensis* isolated using high-pressure process and hexane separation method had a purity of 0.909, which was better than that of the phycocyanin standard 0.904. In a subsequent study, they also reported that purity of 0.909 phycocyanin from *Spirulina platensis* was used in experiments to test for its anti-cancerous effect. However, in the present study phycocyanin purity of 1.2 was found in the crude extract of periphytic *Geitlerinema* sp. from oven-dried biomass which can be further used for different applications. In our study, we used this phycocyanin to test for anticancer activity on HepG2 cell line and found good inhibitory activity (Begum, 2014).

Production of phycobiliproteins from freshwater *Oscillatoria* sp., *Spirulina* sp., periphytic *Geitlerinema* sp. and blue-green *Synechococcus* sp. biomass dried under different conditions do greatly affect the pigment and purity ratio of phycocyanin. The present study showed that biomass dried in the oven at 40 °C had maximum production of phycocyanin, phycoerythrin and allophycocyanin. Hence, it can be summarised that to obtain maximum production and purity ratio of phycobiliproteins from the biomass, oven-drying method is the most suitable and efficient. The oven-drying method can be used commercially as it is simple, efficient and convenient.

Conclusion

The information from this study is important and valuable in selecting the potential cyanobacterial species and defining the most favourable drying conditions for maximum production of phycocyanin. This is because if a drying method works well for one species, it may not work well for other cyanobacterial species. During the screening of cyanobacteria for phycobiliproteins, *Geitlerinema* sp. produced maximum phycobiliproteins. Further research is required to determine how the spectral composition of light and extraction process controls pigments biosynthesis pathways.

Acknowledgements

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