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Utilising light-emitting diodes of specific narrow wavelengths for the optimization and co-production of multiple high-value compounds in *Porphyridium purpureum*

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Abstract

The effect of specific narrow light-emitting diode (LED) wavelengths (red, green, blue) and a combination of LED wavelengths (red, green and blue - RGB) on biomass composition produced by *Porphyridium purpureum* is studied. Phycobiliprotein, fatty acids, exopolysaccharides, pigment content, and the main macromolecules composition were analysed to determine the effect of wavelength on multiple compounds of

commercial interest. The results demonstrate that green light plays a significant role in the growth of rhodophyta, due to phycobiliproteins being able to harvest green wavelengths where chlorophyll pigments absorb poorly. However, under multi-chromatic LED wavelengths, *P. purpureum* biomass accumulated the highest yield of valuable products such as eicosapentaenoic acid (~2.9 %DW), zeaxanthin (~586 μg g^{-1} DW), *β*-carotene (397 μg g^{-1} DW), exopolysaccharides (2.05 g/L^{-1}), and phycobiliproteins (~ 4.8 % DW). This increased accumulation is likely to be the combination of both photo-adaption and photo-protection, under the combined specific wavelengths employed.

Keywords: *Porphyridium*, Phycobiliproteins, Fatty acids, Pigments, FTIR, Exopolysaccharides

1. Introduction

There is significant and increasing pressure within the cosmetic, pharmaceutical, biopharmaceutical and nutraceutical sectors to deliver so called “green pharmaceuticals”, i.e. those derived from a more environmentally friendly and sustainable production process (Ottman, 2011). This demand is the result of an increased consumer awareness and demand for personal care products, cosmetics, and pharmaceuticals to be derived from natural products rather than synthetic origin (Gernaey et al., 2012).

Porphyridium purpureum has the potential to fill a number of niche areas within these industrial sectors by possessing the ability to produce a broad range of commercially valuable chemicals such as phycobiliproteins (PBP) (Guihéneuf & Stengel, 2015), sulphated exopolysaccharides (EPS) (Fuentes-Grünewald et al., 2015), and polyunsaturated fatty acids (PUFAs) (Durmaz et al., 2007). Phycobiliproteins are a group of coloured water-soluble, αβ heterodimeric proteins that constitute the major

complex light-harvesting pigments of cyanobacteria, red algae, glaucocystophytes, and cryptophytes (Bermejo Román et al., 2002; Roy et al., 2011). PBPs can be applied as a colourant in the food and drink industry, as a pharmaceutical agent, and as a fluorescent agent (Bermejo Román et al., 2002; Spolaore et al., 2006). Due to the wide range of applications of PBPs, the total market value is estimated to be >US$ 60 million (Borowitzka, 2013). EPS are complex sulphated polysaccharides composed of different sugar monomers such as xylose, glucose, and galactose (Fuentes-Grünewald et al., 2015; Patel et al., 2013; Sun et al., 2008). *Porphyridium spp.* has been shown to synthesize and secrete EPS into the culture medium, which can be easily extracted from the culture medium (Fuentes-Grünewald et al., 2015; Sun et al., 2012; Velea et al., 2011). A number of studies have demonstrated antiviral, anti-radiation and antioxidant activities, antitumor and immunomodulatory activities properties of this compound (Patel et al., 2013; Raposo et al., 2013; Sun et al., 2012). Polyunsaturated fatty acids (PUFA) have been shown to be the predominant fatty acids detected in *Phorphyridium spp*., reaching 43.7% of total fatty acids (Durmaz et al., 2007), PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are known to have a variety of health benefits such as hypotriglyceridemic and anti-inflammatory properties (Bergé et al., 2002; Ryan et al., 2009; Spolaore et al., 2006).

P. purpureum possess several mass production advantages such as ease of culturing, the ability to grow in a range of latitudes, and a broad range of useful products enabling the potential for a bio-refinery approach to be adopted; which focuses on the co-production of multiple value added product streams, improving process economics, and therefore market viability (Guihéneuf & Stengel, 2015; Vanthoor-Koopmans et al., 2013). However, in order to realise the value of such molecules, algal

production facilities must adopt control systems which guarantee quality, purity, potency, consistency, efficacy, efficiency, and safety. Photobioreactors (PBRs) allow for the common biotic (e.g. pathogen contamination and competition with other microorganisms) and abiotic (e.g. temperature, gases, pH, and nutrients) bottlenecks in microalgal growth to be greatly reduced or even removed in a managed environment. Therefore, in autotrophic cultivation, light becomes the most critical processing parameter (Carvalho et al., 2011). Although sunlight offers a zero-cost source of illumination, the diurnal fluctuations of light can significantly decrease the total biomass concentration and consistency of product formation (Pérez-López et al., 2014). Algal cells are only able to utilise light energy in the photosynthetically active radiation (PAR) range (400-700nm), which equates to ~48% of total solar energy (Park et al., 2011). Furthermore, calculations have shown that only 10–14.4% of solar energy in the PAR range can in theory be converted into algal biomass (Benemann, 2008; Park et al., 2011; Williams & Laurens, 2010). However, typical yields gained by outdoor cultures have a photosynthetic conversion efficiency of only 1.3–2.4% of total solar radiation (Benemann, 2008; Park et al., 2011). Functionally, photons are harvested by the phycoerythrin -chlorophyll complex in rhodophytes, or peridinin-chlorophyll complex in dinoflagellates, which increase the portion of the spectrum that can be used for photosynthesis (Williams & Laurens, 2010). The pigment composition of algae defines the PAR utilisation range and differs according to the pigments acquired or lost during the organism's evolutionary history (Schulze et al., 2014). Light-emitting diodes (LEDs) are a relatively cheap, highly versatile, rapidly advancing modern lighting technology that can provide narrow spectral output (Glemser et al., 2016) and colour blending capability using red, green, blue (RGB) lights. A number of studies have

demonstrated that single wavelength LEDs at various light intensities can be used in the culturing process to adjust the biochemical composition of the biomass produced by microalgae (Atta et al., 2013; Das et al., 2011; Wang et al., 2007). Therefore, due to the unique spectral properties of LEDs when compared to other light sources, the specific light responses of microalgae can be analysed (Glemser et al., 2016). The cyanobacteria *Nostoc sp.* has been shown to exhibit significant changes in the levels of PBP when grown under specific LED wavelengths. Red wavelengths resulted in an overexpression of phycocyanin, whereas green wavelengths resulted in an overexpression of phycoerythrin (Johnson et al., 2014). The chromatic adaptation of *P.purpureum,* especially under green and RGB wavelengths, positively influences PBP production due to its specific role in adaptive photosynthetic activity (Chen et al., 2010).

The aim of this study was to determine the effect of specific LED wavelengths on the formation of multiple high-value products derived from the microalgae *P. purpureum,* and to assess if particular wavelengths trigger specific metabolite induction. All wavelengths tested were assessed for biomass yields and value-added product formation which would allow for bio-refinery processing to be adopted. To the best of the authors knowledge this is the first study to investigate the production of multiple high-value products by *P. purpureum* in response to specific LED wavelengths.

2. Materials and Methods

2.1 Strain, medium and pre-cultivation conditions

The Rhodophyta microalgae *P. purpureum* (CCAP 1380/3) was conditioned in indoor cultures at the Centre for Sustainable Aquatic Research (CSAR), Swansea University, UK. Non-axenic cultures of this species were scaled up using natural

autoclaved seawater (20 min at 121°C) from 250 mL to 1 L flasks using standard F/2 commercial media (Cell-hi F2P, Varicon). The flask cultures were grown in triplicate for seven days, illuminated with approximately 60 μmol photons m^{-2} s^{-1} using cool white fluorescent tubes perpendicular to the culture, with a light/dark cycle of 18/6 h. The flask cultures where then pre-adapted using the relevant LED wavelength for a minimum of 3 days prior to inoculation into the 10 L^{-1} bubble columns.

2.2 Culture conditions

The flask cultures were used to inoculate 3, 10 L bubble columns (acrylic plastic, 0.1 m diameter/light path, 1.2 m height) to an initial cell density of $1.30x10^5$ cells mL^{-1} using a standard F/2 commercial media (Cell-hi F2P, Varicon). The columns were maintained in a controlled temperature room at 19–21 °C, aerated with filtered (0.2 μm) ambient air (0.039% CO_2) at a rate of 0.1 min^{-1} (v/v) into the base of the tube through a 1 mm plastic capillary tube as described by Mayers et al. (2013). Each column was illuminated by a 1 meter length flexible cable of 60 tri-coloured LED chips (red, green, blue LED) (Figure 1), coiled externally around the column. The LED light strips were purchased from Taotronics, and the LEDs were linked to an Aleph 1 CV driver (Enttec Melbourne, Australia). The wavelength and photoperiod (light/dark cycle of 18/6 h) was controlled via a laptop using the lighting software myDMX 2.0 (ADJ Products, Los Angeles, USA). All columns were provided with the same LED wavelength and light intensity for a 10 day growth-period to gain a triplicate of data. The wavelengths used were red (620-625nm), green (520-525nm), blue (465-468nm), and the combination of red, green and blue (RGB) (620-625, 520-525 and 465-468 nm). The light intensity was measured using a cosine-corrected light meter (WALZ Model US-SQS/L) and due to light attenuation within column the light intensity was measured

1 cm in front of bulb (Table 3, supplementary material). The spectral distributions of the different irradiance fields were measured using an AvaSpec ULS-2048 spectrophotometer (Avantes, UK) (Figure 5, supplementary material). The bubble columns were located in a shared temperature-controlled room, that was illuminated with approximately 60 µmol photons m^{-2} s^{-1} using cool white fluorescent tubes. To ensure that no external light sources influenced the algal growth and metabolite formation, the bioreactors were surrounded on three sides with black plastic sheeting and one side with sliver reflective sheeting. The light intensity of the external light source able to penetrate the covered reactors was measured as <5 µmol photons m^{-2} s^{-1}, using a cosine-corrected light meter (WALZ Model US-SQS/L). The columns were sampled daily from day 0 to 3, and then on days 6, 8, and 10.

2.3 Microalgal analysis

Cell number, total cellular volume, and cell size were recorded using a Coulter counter (C4 Beckman Coulter GmBH, Drefield, Germany). Each sample was counted in duplicate; therefore 6 samples were gained for the average of each light condition. During sampling days a minimum of 50 mL of culture was taken from each tube and centrifuged (Beckman Coulter Centrifuge, Avanti J-20XP) for 20 minutes at 8000 rpm. The biomass was washed twice with deionized (DI) water, centrifuged for 20 minutes at 8000 rpm, then collected and freeze dried (ScanVac Cool Safe, LaboGene, Lynge, Denmark) for 24 h prior to further analysis. The supernatant was collected and frozen until required for EPS extraction.

The specific growth rate (µ) was determined for all LED light treatments using equation (1):

$$\mu = \frac{lnN_1 - lnN_0}{t_1 - t_0} \qquad (1)$$

where N_0 and N_1 are the cell concentrations (cells per mL^{-1}) at times t_0 and t_1.

The duplication time or doubling time (DT) was calculated using equation (2):

$$DT = \frac{ln\,(2)}{\mu} \qquad (2)$$

2.4 Exopolysaccharides (EPS) extraction

An alcohol precipitation method was used to extract the EPS from the collected supernatant. The carbohydrates present in the sample were extracted following the method of Patel et al. (2013) using 99% ethanol in the ratio 1:1 EtOH:Supernatant in a 15 mL tube as described by Fuentes-Grünewald et al. (2015).

2.5 Fourier Transformed Infa-Red (FTIR) analysis

FTIR attenuated total reflectance (ATR) spectra were collected using a PerkinElmer Model Spectrum Two instrument equipped with a diamond crystal ATR reflectance cell with a DTGS detector scanning over the wavenumber range of 4000–450 cm^{-1} at a resolution of 4 cm^{-1} as described by Fuentes-Grünewald et al. (2015) and Mayers et al. (2013). Briefly, ethanol (70%) was used to clean the diamond ATR before the first use and between samples. Approximately 3–5 mg of finely powdered freeze-dried *P. purpureum* biomass or EPS extract was applied to the surface of the crystal and then pressed onto the crystal head. A duplicate (each consisting of an average of 12 scans) of each bioreactor sample was conducted for each light type, therefore results of 6 ATR spectra were gained and results averaged. Background correction scans of ambient air were made prior to each sample scan. Scans were recorded using the spectroscopic software Spectrum (version 10. PerkinElmer, Germany). The content of lipid, protein and carbohydrate in biomass samples was determined using FTIR, which had previously been calibrated using D-Glucose for carbohydrates, palmitic acid for

lipids and BSA for proteins at different concentrations, the carrier powder for the FTIR calibration was potassium bromide (KBr).

2.6 Fatty acid analysis

Fatty acid composition was determined for all light treatments at the end of the cell culture (day 10) using a DB-5 column in an Agilent Technologies GC Model 789083 (Agilent Technologies, Germany). Total lipid was gained from 10-15 mg of the freeze dried cells grown under all light treatments via a modified method according to Folch et al. (1957). Heptadecanoic acid (C17:0) was added to the recovered total lipid as an internal standard. Fatty acid methyl esters (FAMEs) were extracted by adding 1 mL trans-esterification mix (methanol:toluene:2,2 dimethoxypropane (DMP):sulphuric acid; 39:20:5:2, by volume) (Garcés & Mancha, 1993) to the vial. Samples were heated to 90°C for 90 minutes and then allowed to cool to room temperature. 2 mL of hexane was then added to each tube, vortexed, and then left for 1 hour to allow the layers to partition. The hexane (top layer) was pipetted into a pre-weighed glass vial. An additional 2 mL of hexane is then added to the lipid sample to repeat the extraction, and both fractions are then combined in the pre-weighed glass vial evaporated to dryness under a nitrogen stream. Prior to GC analysis 300 µL of GC-grade hexane was added to re-suspend the FAME and the sample transferred to a GC vial. A Xevo G2S (Waters Corporation, Massachusetts, USA) connected to a 7890B gas chromatograph (Agilent Technologies, Yarnton, UK) was used for the FAME analysis. An injection volume of 1 μL was loaded onto a column type J&W DB5-MS capillary column (Agilent Technologies, Yarnton, UK) (30 m length ×0.25 mm ID, 0.25 μm film) using Helium as carrier gas. The gas flow rate was 1.2ml/min and the injector temperature

was 280 °C. The oven programme was as follows: Initial temp 50 °C for 1 min, then ramped at 25 °C per min to 280 °C which was maintained for 4 minutes (total run time 14.2 mins). Fatty acids were identified by comparing the obtained retention times with that of known standards (37 component FAME mix, Supelco™) using the software MassLynx (version 4.1) (Waters Corporation, Massachusetts, USA).

2.7 PBP extraction and determination

PBPs were extracted using a modified version of the method developed by Lawrenz et al. (2011). The freeze-dried biomass of each bubble column sample taken on day 0-3, day 6-8 and day 10 were pooled and weighed to a known weight on a semi-micro and analytical balance (MSE 124S-100-DU, Sartorius balance, Germany), the sample weight was noted to the nearest 0.1 mg, all PBP extractions were conducted in triplicate. The samples were transferred into 15 mL falcon tubes, and subjected to a minimum of five freeze-thaw cycles; this being, the samples were immersed in 5 mL of 0.1 mol L^{-1} phosphate buffer (pH=6) and stored at -20 °C until frozen (~2 hrs), they were then thawed and subjected to 10 minutes sonication on ice (Bermejo Román et al., 2002), the samples were then vortexed for 5 minutes and then placed back into -20 °C, and the process repeated. After the final freeze-thaw cycle the cell debris was removed via centrifugation at 8,000 rpm for 5 minutes. The supernatant was recovered and used for PBP measurements. Absorbance of the extracts was measured at 455, 564, 592, 618 and 645 nm using a UNICAM UV 300 spectrophotometer. The concentrations of the PBPs phycoerythrin (PE) and phycocyanin (PC) were determined using the equations of Beer and Eshel (1985):

$$\mathrm{PE(mg\,L^{-1}} = [(\mathrm{OD_{564\,nm}} - \mathrm{OD_{592\,nm}}) - (\mathrm{OD_{455\,nm}} - \mathrm{OD_{592\,nm}})\ \mathrm{x}\ 0.2]\ \mathrm{x}\ 0.12$$

(3)

$$\mathrm{PC(mg\,L^{-1}} = [(\mathrm{OD_{618\ nm}} - \mathrm{OD_{645\ nm}}) - (\mathrm{OD_{592\ nm}} - \mathrm{OD_{645\ nm}})\ \mathrm{x}\ 0.51]\ \mathrm{x}\ 0.15$$

(4)

Where OD is the optical density of the pigment at the particular wavelength. The total PB content was calculated by adding values from Equations (3) and (4).

2.8 Pigment analysis

Pigments were extracted from a known volume of *P. purpureum* biomass using HPLC grade dimethyl sulfoxide (DMSO). The extracted pigments were analysed and quantified using a HPLC (Agilent 1200 Series, Agilent Technologies, Waldbronn Germany) equipped with an UV-VIS diode array detector (DAD). The separation was performed on a Hypersil reversed phase C8 column (100mm x 4.6mm, 5µm) using the combined methods of Zapata et al. (2000) and Barlow et al. (1998). A flow rate of 1 ml min^{-1}, by a linear gradient follows (minutes; % Solvent A; % solvent B): (0;75:25), (1; 50:50), (20;30:70), (25;0:100), (32;0:100). Solvent A consisted of 70:30 (v/v) methanol: 1 M ammonium acetate and solvent B was 100% methanol. Chromatographic data was processed using UV-Vis ChemStation B.04.02 software (Agilent Technologies, Yarnton, UK). Pigments were detected by absorbance of 440 nm and identified by comparison of retention time and spectra with standards purchased from Sigma –Aldrich Co. USA.

2.10 Statistical data analysis

All experiments were carried out with a minimum of three replicates. The results are expressed as averages ± standard deviation (SD). The effects of the LED wavelength on the different variables or parameters investigated were analysed using a two-way

analysis of variance (ANOVA) with level of significance: P <0.05). All statistics were performed using Minitab software (release 15, Minitab Inc., State College, PA).

3. Results and discussion

The Effect of different LED wavelengths on *P. purpureum* on the growth and product formation was investigated. This involved inoculating 10L bubble columns with pre-adapted flask grown cultures, and culturing over a 10 day growth period under various LED lighting regimes. Four different LED wavelengths were used to illuminate the cultures: red, green, blue, and the combination of red, green and blue (RGB). After 10 days of cultivation, growth performance and major cellular components such as cellular PBP, excreted EPS, dry biomass biochemical composition, pigments, and fatty acids was examined and a comparison between the different lighting conditions was made to determine the effect of specific wavelengths, and to assess if one specific wavelength gained beneficial biomass yield and co-product formation.

3.1 Effect of P. purpureum growth rate under different LED light wavelengths

Figure 2A. shows *P. purpureum* growth curves when subjected to different lighting conditions during the 10 day cultivation period. Generally, cells cultivated under RGB or green LED wavelengths had a similar growth rates, gaining significantly higher cell densities ($6.24x10^5$ and $6.59x10^5$ respectively) when compared to cells grown under red or blue wavelengths (P=<0.001). As there was no significant difference in the cell densities achieved under RGB or green wavelengths from day 8-10 (P=0.928), the inference was made that the increase in light intensity for cells grown under RGB wavelengths (Table 3, supplementary material) did not have a significant

effect on cell growth. The maximum average doubling time (RGB= 2.4 days and green=2.3 days) and fastest average growth rate (div/day) (RGB= 0.283, green= 0.306) for these cultures were similar to that gained by Fuentes-Grünewald et al. (2015) in outdoor semi-continuous and batch cultures of *P. purpureum*, however the cell densities gained during these trials were significantly lower due to the lower illumination levels and lower cell density inoculum. There was no significant difference (P=0.996) between day 0-10 in the cell densities for cultures illuminated with blue LED wavelengths. The cell density of cultures illuminated with red wavelengths significantly declined from day 0-3 (P=<0.001) and then remained stable from day 6-10 with no significant difference in the cell density (P=0.698). This data would suggest that cultures grown under RGB wavelengths are only able to harvest the green LED light wavelengths, as would be expected as PBP are the major light-harvesting pigments in red algae (Roy et al., 2011) such as *P. purpureum.* The PBP content of *P. purpureum* has been reported to reach 25% of dry weight (DW) (Velea et al., 2011). PBP are able to harvest the 'green gap' where the chlorophyll pigments absorb poorly (Roy et al., 2011). PBP in *P. purpureum* has been reported to mainly consist of PE (48-95%), and smaller quantities of PC (40-20%), and allophycocyanin (APC) (~10%) (Guihéneuf & Stengel, 2015; Kathiresan et al., 2007; Velea et al., 2011). The absorption and fluorescence features of PE are in the spectral range of 450–600 nm (Roy et al., 2011). The green LED wavelengths (520-525nm) fall centrally in the photosynthetically usable radiation (PUR) for PE, while blue LED wavelengths (465-468 nm) are on the edge of PE PUR, and red is outside the PUR region (Figure 5, supplementary material). For cell growth LED wavelengths need to fall in the PUR.

3.2. Influence of different wavelengths on the excreted EPS

Light quality has been demonstrated to be a key factor for controlling the polysaccharide production of *Porphyridium spp.* (Sun et al., 2008). EPS extracted over the 10 days of culture from the *P. purpureum* culture supernatant from cells cultured under red, green, and blue wavelengths showed no significant difference the yield (P=0.875) (average g/L^{-1} 1.73,1.75, and 1.74 respectively), whereas cells cultured under RBG wavelengths excreted significantly higher EPS yields of 2.05 g/L^{-1} on day 10 (P=0.033) (Figure 2B). The yields for all light wavelengths were higher than those gained by Sun et al. (2008), who gained 0.99 g/L^{-1} under green wavelengths after 14 days of culture of *Porphyridium cruentum* (Sun et al., 2008). FTIR has been proposed as a good tool for assessing the quality of the products obtained microalgae cultures such as EPS, due to the strong peak at 1087 cm^{-1} corresponding to polysaccharides (Fuentes-Grünewald et al., 2015). Therefore, the EPS from each LED wavelength was pooled to assess the quality via the percentage of carbohydrate. EPS excreted from cells grown under green LED illumination resulted in significantly higher EPS purity (38.36 % carbohydrates), when compared to the three other wavelengths (P=<0.001) (Figure 2C). Fuentes-Grünewald et al. (2015) measured the carbohydrate percentage of EPS excreted by *P. purpureum* during batch and semi-continuous outdoor culture, in this study the highest percentage of carbohydrate gained in the EPS content was ~31%, gained after 20 days of semi-continuous culture.

3.3 Biomass composition under different LED wavelengths (FTIR analysis)

FTIR has been shown to be a rapid and reliable tool for the analysis of microalgae composition evolution (Fuentes-Grünewald et al., 2015; Mayers et al., 2013) and has the additional benefit of only requiring a small quantity of biomass for analysis (3-5 mg) (Mayers et al., 2013). The concentration of the main macromolecules

(proteins, carbohydrates and lipids) was followed for *P. purpureum* cultures illuminated under different LED wavelengths. The protein fraction generally increased in percentage from day 0 to day 8 for cultures grown under RGB, green and blue LED wavelengths. From day 6 to day 10 there is no significant difference in the percentage of proteins in the cell biomass for cultures grown under RGB, green, and blue light (P=0.257). *P. purpureum* has been shown to produce large amounts of proteins, as much as 39% DW (Becker, 1994). The highest average protein content of 36.5% DW, was produced in cells illuminated by blue LED wavelengths. Cells grown under red LED wavelengths had significantly lower protein yields (P=<0.001), with an average protein content of 20.6±2.3% DW. PBP are the only antenna pigments that lack protective carotenoids. Instead, the phycobilisome-associated carotenoid-protein, *Synechocystis* PCC 6803, provides photoprotection and regulates energy from the PBP to the reaction centre (Roy et al., 2011; Wilson et al., 2008). The high protein content of cells cultivated under RGB, green and blue wavelengths are most likely due to an increase in *Synechocystis* PCC 6803 in response to the higher energy of the blue and green light when compared to red light (Figure 3A). Light is known to produce stress that can impact lipid accumulation in many microalgae species (Atta et al., 2013; Hu et al., 2008). However, very little change occurs in the total lipid, with each sample containing approximately 17 (% DW) total lipids under all wavelengths over the 10 day growth period, however there were significant differences in the FAME content (see section 3.4). It is likely that the stable lipid content is in response to the adequate nutrient availability; this is also reflected in the cellular carbohydrate content. In general, Figure 3C illustrates that that cellular carbohydrates either slightly decrease or remain stable (total average DW content: 19.8±2.4, 19.6±1.0, 21.72±1.39, 21.78±2.30 for RGB, red,

green and blue respectively). A rise in lipids and carbohydrate cellular content in microalgae is linked with carbon storage products and is generally triggered by nutrient depletion (Fuentes-Grünewald et al., 2015; Guihéneuf & Stengel, 2015). *Porphyridium spp.*, has been shown to increase cellular carbohydrate content under reduced nitrogen availability (Fuentes-Grünewald et al., 2015; Guihéneuf & Stengel, 2015).

3.4 Effect of LED wavelength on fatty acid content and composition

The total FAME content ranged from 3.4± 0.7 % DW in cells grown under red light to 6.6± 2.2 % DW in cells grown under RGB wavelengths (Table 1). All light treatments gained higher total FAME yields when compared to the total FAME of between 1.1–2.8% DW gained by Guihéneuf et al. (2015) when growing *P. purpureum under* nitrogen-replete and deplete culture conditions.

The major fatty acids gained under all wavelengths were EPA (EPA, C20:5n3), followed by palmitic acid (PA, C16:0), and smaller amounts of linoleic acid (LA, C18:2n6), and oleic acid (OA, 18:1n9). C20 PUFA's (especially EPA) have been shown to be the dominant fraction of *Porphyridium spp.* (Guil-Guerrero et al., 2000; Rebolloso Fuentes et al., 2000), and have several pharmaceutical properties due to their important role in modulating human metabolism (Bergé et al., 2002; Ryan et al., 2009; Spolaore et al., 2006). In this study, biomass grown under RGB wavelengths had significantly higher EPA content (43.1 % of total FAME) when compared to cells grown under monochromatic (blue, red, green) LED wavelengths, there was no significant difference in the EPA content of cells grown under red, green or blue LED illumination (P=0.184). Das et al. (2011) (Das et al., 2011) also gained higher yields of EPA in *Nannochloropsis* multichromatic white light LED, when compared to monochromatic (blue, red, green)

LED illumination. From a processing view point biomass grown under RGB wavelengths gained beneficial protein: FAME: EPA ratios of 1:0.21:0.08 when compared to cells grown under red light which gained ratios of 1:0.16:0.04.

Significantly higher levels of PUFAs were also gained when the cells were grown under the RGB wavelength, when compared to red, green and blue light treatments (P=<0.001), therefore multichromatic "white light" (RGB) are clearly beneficial for the accumulation of high value FAMEs when compared with narrow wavelengths such as red, green or blue. Despite very little change in the total lipid levels under specific wavelength treatments (Figure 3B), the FAME data demonstrates the significant impact of light wavelength on the FAME composition profile in *P. purpureum* cells.

3.6 Effect of LED wavelength on phycobiliproteins synthesis

The phycobilisome is an adaptive structure which can alter the light harvesting capabilities of the cell depending upon the by light intensity, quality, and nutrient supply available (Chen et al., 2010; Johnson et al., 2014; Roy et al., 2011). Many species of microalgae are able to regulate the synthesis of PE and PC in response to the spectral quality of their environment (Ley & Butler, 1980). In the present study, RGB LED wavelengths were found to be most suitable for total PBP yield (4.8 ± 1.1%DW) as well as maximum synthesis of PE (Figure 4). The PBP yield gained under RGB LED wavelengths was significantly higher than the PBP yield gained under red, green and blue LED wavelengths (P=0.001). The PBP yield gained under all wavelengths was found to be comparable with other studies reported for *Porphyridium spp.* (Guihéneuf & Stengel, 2015; Kathiresan et al., 2007; Rebolloso Fuentes et al., 2000). Cells grown under RGB wavelengths also gained the highest ratio of PBP per gram of protein DW

(gPBP/gPDW) of 0.151 gPBP/gPDW on day 10, when compared to cells exposed to red (0.089 gPBP/gPDW), green (0.080 gPBP/gPDW), and blue (0.0224 gPBP/gPDW) wavelengths after 10 days of culture. The higher PBP: protein ratios indicate greater ease of the downstream separation processing, therefore reducing costs associated to downstream processing. It is likely that cells grown under the multichromatic RGB LED wavelengths gained highest yield of PBP due to the combined effect of the blue and the green wavelengths which provide an increased the portion of spectrum that can be harvested by the PBP (Figure 5, supplementary material). The rapid and ongoing development of LED technology has created several wide spectrum white lights, tuneable at different emission peaks such as warm white and cool white LEDs (Massa et al., 2008). The data suggests higher productivity of biomass and PBP is generally seen with additional wavelengths, therefore similar affects may be achieved by broadening of the spectrum. Research into the effect of broad spectrum white LEDs is required. However, currently white LEDs, which are produced using blue LEDs with phosphor coatings, are less efficient than the single-wave-peak LEDs (Massa et al., 2008), therefore an economic assessment must be considered for the viable production of marketable products.

Interestingly there is a significant (P=<0.001) increase in total PBP yield under blue LED wavelengths at day 6-8, when compared to day 0-3 and day 10. During this time there is also a significant decrease in chlorophyll a, combined with a high increase in zeaxanthin (Table 2). As previously stated, the blue LED wavelengths (465-468 nm) are on the edge of PBP PUR (Figure 5, supplementary material), and blue light also has high energy photons. This would suggest the cells grown under blue LED wavelengths were under photo-adaption whilst trying to provide photo-protection in the form of

zeaxanthin, which reduces photo-oxidative damage. However, on day 10 cell density (Figure 2A), percentage of PBP in the dry biomass (Figure 2B), and the amount of zeaxanthin (Table 2) have all decreased suggesting only providing blue LED wavelengths caused photo-inhibition within the cell, due to the energy of blue photons being higher than that required for photosynthesis (Das et al., 2011; Schulze et al., 2014).

This data suggests light could be used as a trigger for product formation. Tailored light regimes may be developed for consistent and reliable *P. purpureum* culture yield and product formation. Cell growth could be conducted under broad green wavelengths; the cells could then be switched to narrow blue wavelengths to induce high carotenoid and PBP yields within a short time period.

3.5 Pigment analysis

The influence of LED wavelengths on the major pigments content of *P. purpureum* was determined for day 0, 6 and 10 of the growth period, the results are presented in table 2. Multiple studies have focussed on the effect of light, temperature, nutrients (Algarra & Rudiger, 1993; Cunningham et al., 1989; Guihéneuf & Stengel, 2015; Merchuk et al., 1998), and wavelength (Brody & Emerson, 1959; Ley & Butler, 1980) on the pigment composition of *Porphyridium spp*. Prior to LEDs, the effect of wavelength was conducted using glass filters or green and blue mercury lines to produce the desired wavelengths. However, the wavelengths produced using these methods had a wide spectral distribution. LEDs are the first light source to provide a narrow spectral output allowing the study of specific wavelengths of pigment production. To the authors best knowledge this is the first study to measure the effect of specific narrow LED wavelengths on the pigment composition of *P. purpureum*.

Table 2 illustrates that there is a distinct difference in the pigment composition due to the specific LED wavelength treatments. This was also noted by Ley & Butler (1980), when *P. cruentum* was grown under different coloured filters at roughly the same levels of total irradiance.

Chromatic adaptation has a strong influence on the accessory pigments (carotenoids and PBP) to help to absorb the light in wavelengths that chlorophyll is inefficient at absorbing, such as the blue to green wavelengths, transferring the light energy to chlorophyll *a* (Chl *a*), the core pigment of photosynthetic activity. Another role of accessory pigments is to prevent photo-oxidative damage (due to reactive oxygen species (ROS)) to cells. Zeaxanthin is specifically involved in de-epoxidation, as this pigment contains double carbon bonds and can accept an excess of energy from excited chlorophyll and dissipate this as a heat (Sujatha, 2015). This adaptation is reflected by Chl *a* concentration. The highest yield of Chl *a* was obtained under blue LED wavelengths at day 0 (1764 $\mu g\ g^{-1}\ DW$), however these levels significantly decline by day 10 (785 $\mu g\ g^{-1}\ DW$). The energy of blue photons is higher than that required for photosynthesis (Schulze et al., 2014), The initial high levels of Chl *a* compared to zeaxanthin, followed by the continual drop in Chl *a* blue LED light may have caused nonphotosynthetic quenching (NPQ), generating reactive oxygen species (ROS) (Schulze et al., 2014). In the early stages of culture cells had not adapted to have high enough levels of zeaxanthin to protect against ROS and dissipate the excess photon energy.

The Chl *a* content of the cells grown under both RGB and green LED wavelength gradually increases over the growth period. However, RGB cells gain significantly higher yields of Chl *a* by day 10 ($P=<0.001$), most likely due to the

combined effect of the blue and green wavelengths. The combination of blue and green light may also improve the ability of the light to penetrate into cultures as the density of cells increases during growth, due to the higher energy blue photons. The highest zeaxanthin : Chl *a* and PBP: Chl *a* ratios on day 10 of the culture period was observed in cells grown under RGB (32.9 and 0.40 respectively) and green (18.8 and 0.7 respectively) LED wavelengths when compared to red (13.7 and 0.01), while blue gained high PBP: Chl *a* ratios (0.1) and low zeaxanthin : Chl *a* (7.29), demonstrating that the cell is able to apply differing adaption mechanisms for the higher energy (blue and green) photons (Table 2). Therefore, it seems that that NPQ of the blue light is reduced when applying multichromatic RGB LED wavelengths.

Cells grown under red light treatments were shown to gain the lowest pigments concentrations when compared to the other light treatments over the same period (Table 2). Ley & Butler (1980) used absorption and fluorescence emission spectra to measure the ratio of photosystem II (PSII) to photosystem I (PSI) as a function of excitation wavelength in *P. cruentum*. The work demonstrated that cells grown in light absorbed primarily by the phycobilisomes (green) packaged a larger fraction of their chlorophyll into PSI, whereas cells grown in light absorbed by chlorophyll distribute their chlorophyll more equally. However as stated previously, this work was conducted using filters with broad spectral distributions (red = ~625-700 nm). *P. purpureum* has no chlorophyll b and no accessory light-harvesting chlorophyll a/b protein complexes (Kopecký et al., 2004), therefore at a wavelength of 620nm algae grown under red LED wavelengths could have effectively been in the dark as they are unable to utilize the wavelength with Chl *a*, PBP, or carotenoids (Figure 5, supplementary material). Future work using absorption and fluorescence emission spectra to measure the ratio of

photosystem II (PSII) to photosystem I (PSI) as a function of excitation wavelength from algal cells grown under LED wavelengths needs to be carried out.

Guihéneuf et al. (2015) have demonstrated that *P. purpureum* gained the highest productivities of Chl *a*, zeaxanthin, and β-carotene under low light intensities (30 μmol m^{-2} s^{-1}). This study was also conducted under low light intensities, however in order to gain a significant amount of biomass for large scale production, investigation into LED light intensity and scale of production need to be studied. It is likely that at larger scales and higher light intensities the productivity of Chl *a*, zeaxanthin, β-carotene, PBP will be lower than reached in this study. Again, there is potential to produce a tailored LED lighting programme that switches colour and intensities to improve both biomass yield and product formation.

4. Conclusion

Results indicate that specific wavelengths have impact on metabolite production in microalgae. Green light improved the growth of the rhodophyta as the phycobiliproteins were able to harvest this wavelength, whereas chlorophyll pigments absorbed poorly. However, under multichromatic RGB LED wavelengths the *P. purpureum* biomass was able to accumulate the highest yields amounts of valuable products such as eicosapentaenoic acid, zeaxanthin, β-carotene, exopolysaccharides, and phycobiliproteins. This is due to a combination of photo-adaption and photo-protection, under combined specific wavelengths. Blue LED wavelengths resulted in poor growth, but an increase in phycobiliproteins, pigments, and protein over the growth period.

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Figure and Table Captions

Figure 1: The experimental set- up of the 10 L^{-1} bubble columns. Each column was wrapped with 1 m of LED strips containing 60 LEDs. The photoperiod and wavelength was controlled via the laptop and drivers.

Figure 2: **A)** Growth curve (cell count) of *P. purpureum* under different LED wavelengths during the 10 day cultivation period. **B)** EPS extracted over the 10 days of culture from the *P. purpureum* culture supernatant from cells cultured under different LED wavelengths. **C)** The quality (% carbohydrate) of EPS extracts gained each LED wavelength, analysis by FTIR

Figure 3: *P. purpureum* biomass composition with time analysed by FTIR in cultures grown under different LED wavelengths. A) Protein (%DW); B) Lipid (%DW); C) Carbohydrate (%DW)

Figure 4: Changes of PBP content (% DW) and composition (% total PE and PC) in cultures of *P. purpureum* cultivated under different LED wavelengths treatments. All data represent the average ± SD of three individual replicates

Table 1: Fatty acid composition (% of total FAME) of *P. purpureum* cultivated under different LED light wavelengths (day 10). All data represent the average ± SD.

Table 2: The pigment content ($\mu g\ g^{-1}\ DW$) of *P. purpureum* under different LED light wavelengths. All data represent the average ± SD.

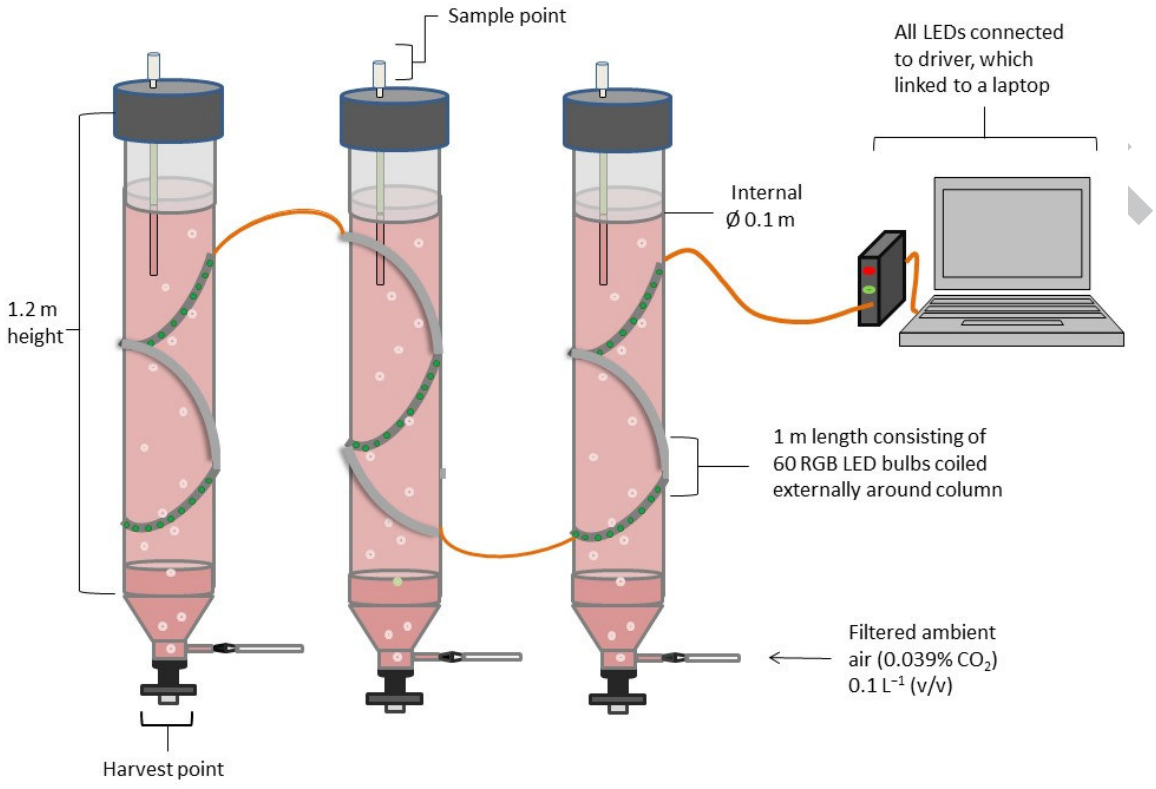


Figure 1

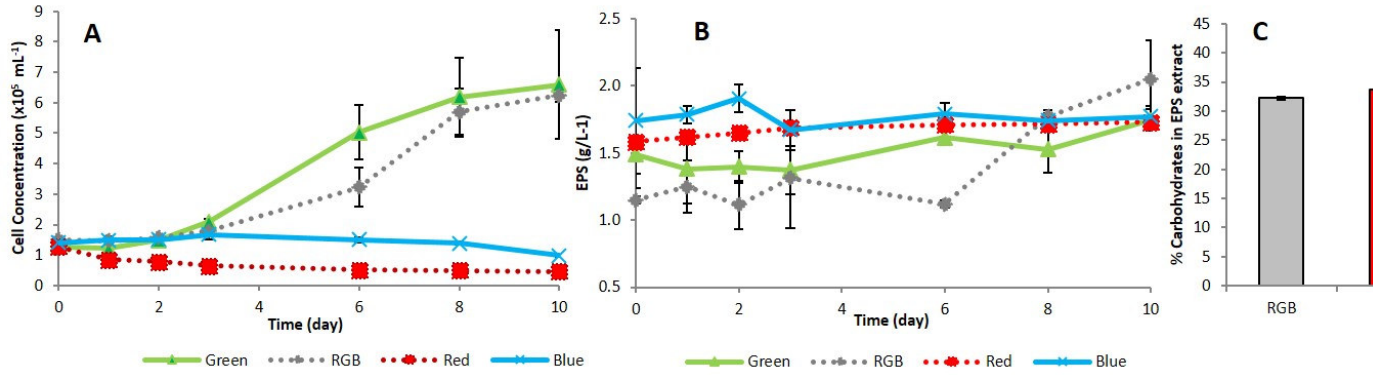


Figure 2

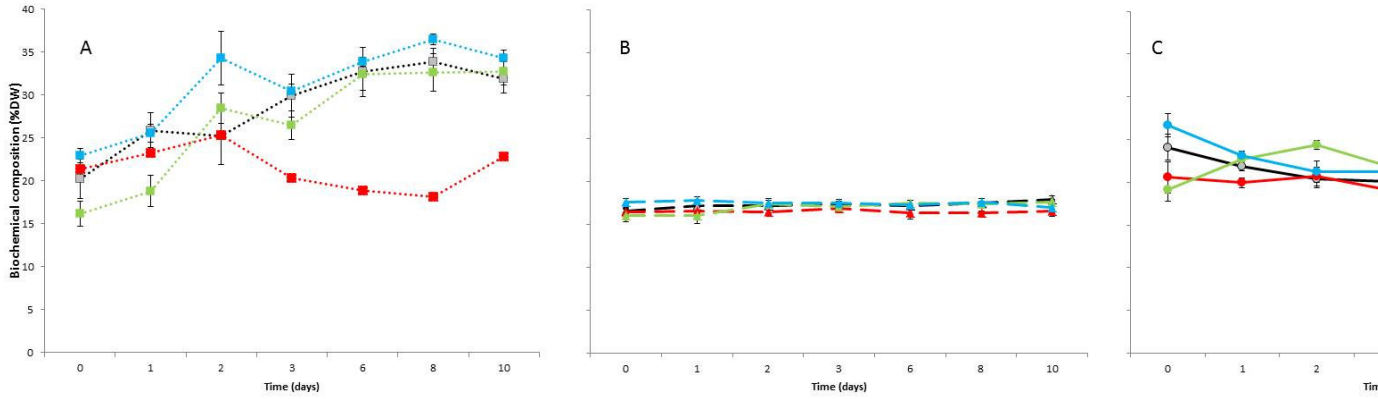
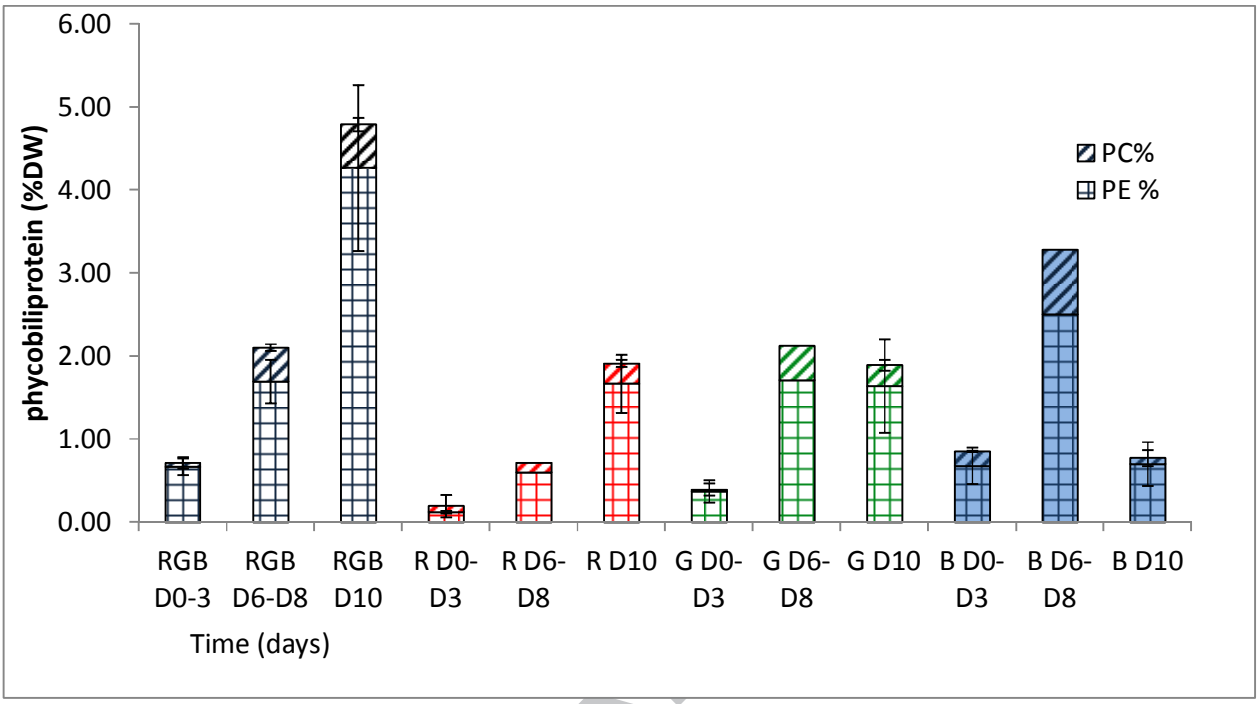


Figure 3

Table 1

	Fatty acid composition (% of total FAME)			
Fatty acid	RGB	Red	Green	Blue
Myristic (C14:0)	0.0	0.2±0.0	0.0	0.0
Palmitoleic (C16:1)	0.3±0.1	0.5±0.2	0.3±0.2	0.5±0.4
Palmitic acid (C16:0)	23.3±2.3	26.6±2.1	26.3±2.3	26.4±3.2
Linoleic acid (C18:2n6)	3.3±0.8	3.1±0.4	3.1±0.9	2.5±0.2
Oleic acid (C181n9)	1.1±0.1	1.3±0.3	1.7±0.4	1.0±0.2
Eicosapentaenoic acid (C20:5n3)	43.1±3.3	23.9±1.5	29.1±1.2	29.1±7.1
Saturated fatty acids	23.3	26.9	26.3	26.4
Monounsaturated fatty acid	1.3	1.7	2.1	1.5
Polyunsaturated fatty acids	46.4	26.9	32.2	31.6


6.00
5.00
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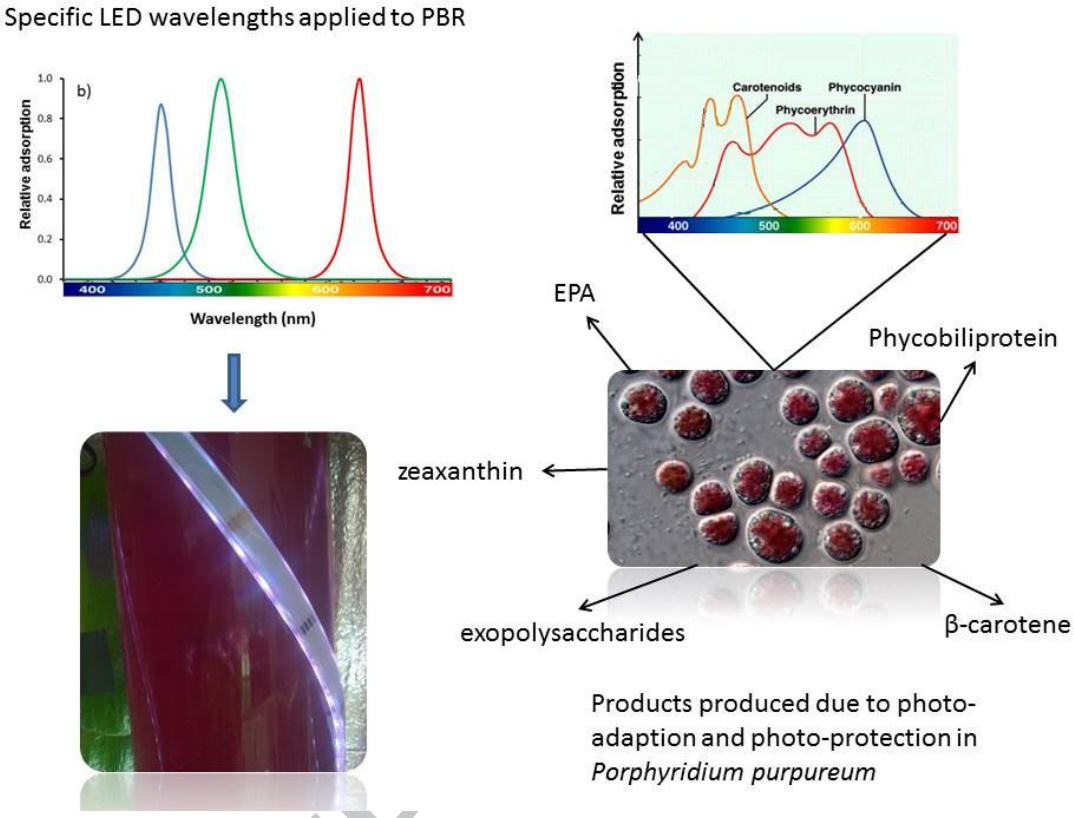
phycobiliprotein (%DW)
PC%
PE %
RGB D0-3
RGB D6-D8
RGB D10
R D0-D3
R D6-D8
R D10
G D0-D3
G D6-D8
G D10
B D0-D3
B D6-D8
B D10
Time (days)

Figure 4

Table 2

LED Light colour	Time (day)	Chlorophyll *a* *(μg g^{-1} DW)*	Zeaxanthin *(μg g^{-1} DW)*	β-carotene *(μg g^{-1} DW)*
RGB	0	506.7±11.1	275.0±3.2	136.2±0.7
	6	669.7±6.7	309.6±0.7	195.8±0.4
	10	1913.3±3.4	586.3±0.3	396.7±0.3
Red	0	378.7±1.5	180.8±0.2	108.0±1.3
	6	170.6±1.3	-	28.5±1.7
	10	256.8±2.1	18.8±2.3	56.3±1.1
Green	0	654.6±2.1	123.5±1.4	171.1±1.1
	6	1199.8±12.0	429.2±2.5	339.3±1.6
	10	1239.3±18.1	65.8±1.1	38.6±0.4
Blue	0	1763.8±17.0	129.4±0.6	229.7±4.4
	6	1092.1±1.8	204.9±4.2	213.9±1.8
	10	783.6±3.0	107.4±5.6	217.6±2.3

Graphical Abstract



Highlights

- Green light plays a significant role in the growth of *Porphyridium purpureum*
- Multi-chromatic LED wavelengths accumulated the highest yields of valuable products
- Photo-adaption and photo-protection are suspected to boost product yield
- Specific wavelengths can increase product ratios increasing biomass value