Sugar-stimulated CO_2 sequestration by the green microalga *Chlorella vulgaris*

Weiqi Fu [a,b,*], Steinn Gudmundsson [a], Kristine Wichuk [a], Sirus Palsson [a], Bernhard O. Palsson [a,c], Kourosh Salehi-Ashtiani [b], Sigurður Brynjólfsson [a]

[a] Center for Systems Biology and Faculty of Industrial Engineering, Mechanical Engineering and Computer Science, School of Engineering and Natural Sciences, University of Iceland, 101 Reykjavík, Iceland
[b] Division of Science and Math, Center for Genomics and Systems Biology (CGSB), New York University Abu Dhabi, Abu Dhabi, United Arab Emirates
[c] Department of Bioengineering, University of California, San Diego, La Jolla, CA 92093–0412, USA

HIGHLIGHTS

- Fine-tuning mixotrophic culture of microalgae enhances CO_2 bio-fixation.
- CO_2 capture in sugar-stimulated algal culture enhanced with increased light supply.
- Sugar-stimulated algae have as much pigments as its photoautotrophic counterpart.
- A techno-economic model evaluates the feasibility of large-scale algae cultivation.

GRAPHICAL ABSTRACT
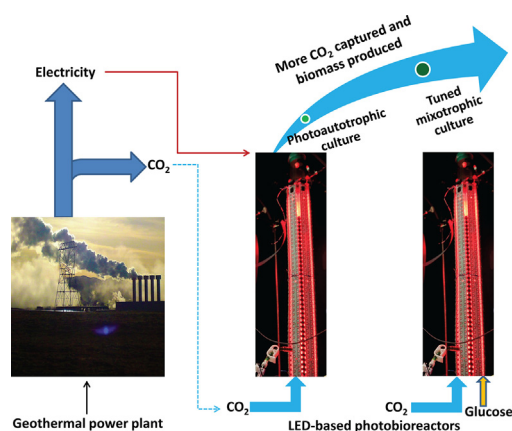
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ABSTRACT

To convert waste CO_2 from flue gases of power plants into value-added products, bio-mitigation technologies show promise. In this study, we cultivated a fast-growing species of green microalgae, *Chlorella vulgaris*, in different sizes of photobioreactors (PBRs) and developed a strategy using small doses of sugars for enhancing CO_2 sequestration under light-emitting diode illumination. Glucose supplementation at low levels resulted in an increase of photoautotrophic growth-driven biomass generation as well as CO_2 capture by 10% and its enhancement corresponded to an increase of supplied photon flux. The utilization of urea instead of nitrate as the sole nitrogen source increased photoautotrophic growth by 14%, but change of nitrogen source didn't compromise glucose-induced enhancement of photoautotrophic growth. The optimized biomass productivity achieved was 30.4% higher than the initial productivity of purely photoautotrophic culture. The major pigments in the obtained algal biomass were found comparable to its photoautotrophic counterpart and a high neutral lipids productivity of 516.6 mg/(L·day) was achieved after optimization. A techno-economic model was also developed, indicating that LED-based PBRs represent a feasible strategy for converting CO_2 into value-added algal biomass.

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* Corresponding author at: Center for Systems Biology and Faculty of Industrial Engineering, Mechanical Engineering and Computer Science, School of Engineering and Natural Sciences, University of Iceland, 101 Reykjavík, Iceland.
E-mail address: weiqi@hi.is (W. Fu).

1. Introduction

To reform the global energy production with a low carbon footprint, numerous efforts have been made to search for alternative fuels and sustainable feedstocks. Geothermal energy is found almost everywhere in the world and can be used as an energy source in several ways. In 2010, direct uses of geothermal energy were deployed in 78 nations, amounting to ~50 $GW_{thermal}$ and global installed capacity of geothermal base-load power was ~11 $GW_{electric}$ (Goldstein et al., 2011). However, geothermal power plants extract fluids for energy/electricity production while at the same time releasing gaseous contaminants into the atmosphere in the form of non-condensable gases (NCGs). The exact composition of the gas pollutants depends on the temperature in the wells and the chemical composition of the ground where the boreholes are drilled, and varies greatly from one field to another. However, the principal component is always carbon dioxide (CO_2), which comprises up to 95% (v/v) of NCGs from geothermal plants in Iceland (Ármannsson et al., 2005) and in the U.S.A. (Bloomfield and Moore, 1999). For instance, the NCGs produced at the Icelandic power plants are released directly into the air, mostly without any treatment, resulting into a CO_2 emission rate >100 g per kWh. This negatively impacts the utilization of geothermal resources as a sustainable energy source in the future. For example, geothermal plants in Iceland release up to 30 million tonnes of CO_2 per year into the atmosphere (Ármannsson et al., 2005). Currently, geothermal energy accounts a minute fraction of the world's energy output and only a few countries such as Iceland, Philippines and New Zealand generate >10% of their electricity from geothermal resources. As the geothermal energy emerges as a sustainable technology for future energy solutions, the overall global release rate of CO_2 from geothermal power plants is likely to increase in the coming years as more such plants are developed and built (Goldstein et al., 2011). Several strategies for reducing CO_2 emissions have been investigated. These include carbon capture and storage, a viable but expensive technology for removing CO_2 from the flue gases of power plants (Chen et al., 2012; Markewitz et al., 2012). Bio-mitigation of CO_2 is a promising strategy for reducing CO_2 emissions, which also derives added benefits from the biomass from the fixed CO_2, which can be used for other purposes such as food additives and cosmetics (Chen et al., 2012; Fu et al., 2016; Kassim and Meng, 2017). The current bio-mitigation technologies are currently hampered by low efficiencies and low capacity of CO_2 capture (Chen et al., 2012). Further biotechnological development is needed to make bio-mitigation a feasible alternative for offsetting the CO_2 emissions from geothermal power plants.

Algae are an attractive source of biomass, lipids, and valuable bioactive compounds as they can grow on marginal lands where their cultivation does not compete directly with food crops (Fu et al., 2017; Raheem et al., 2018). Furthermore, algae can have higher yields per unit area than land crops (Clarens et al., 2010; Trentacoste et al., 2013). The green microalga *Chlorella vulgaris* is one of the fastest growing algae (Anjos et al., 2013; Najm et al., 2017). It is widely cultivated, and is used in food and feed production, as well as in the treatment of industrial wastewater and flue gases (Heredia-Arroyo et al., 2011; Olguín, 2012). *Chlorella* is able to grow under autotrophic, heterotrophic, and mixotrophic conditions (Zuniga et al., 2016) and also has the potential to be an excellent candidate to produce commercially interesting pigments (Cordero et al., 2011). Its robustness, high growth rate and high content of neutral lipids make this species a promising candidate for bioenergy production (Liang et al., 2009). In order to reduce processing costs and to make algal biomass production more economical, indoor photobioreactor (PBR) systems have been developed for high density culture (Choi et al., 2013; Fu et al., 2012a). Light-emitting diode (LED)-based PBRs have also been developed for algae cultivation and CO_2 capture based on the extensive use of geothermal resources, which includes the use of geothermally produced electricity to generate lights for photosynthesis and the use of waste CO_2 as the carbon source for algal biomass and high value products (Fu et al., 2012a).

Light supply and delivery are limiting factors when scaling up PBRs for photoautotrophic cultivation. The extra light/dark cycles due to mixing effects in large PBRs are unfavorable to algal growth (Wichuk et al., 2014). They largely decrease the biomass productivity and the biomass concentration and this greatly increases the operating and downstream processing costs (Liang et al., 2009). In this regard, a strategy using a mixotrophic culture mode (Juntila et al., 2015) may be beneficial for biomass generation in PBRs. Liang et al. (2009) studied the effects of acetate, glucose and glycerol on the growth of *C. vulgaris* and obtained the highest growth rate with glucose under mixotrophic conditions. Mixotrophic cultivation of algae can produce more cellular biomass than either the autotrophic or heterotrophic cultures individually, and the choice of substrate and its concentration may greatly influence the final biomass yield of the mixotrophic culture (Heredia-Arroyo et al., 2011).

Although the target will be to reduce CO_2 emissions from geothermal plants, pure CO_2 balanced in air was utilized instead of waste CO_2 to cultivate algae, as the experimental conditions are more controllable and reproducible for algal growth. In this study, we assessed the scalability of the PBRs based on the key parameters, i.e. biomass productivity and biomass yield on light energy. In order to make the system both cost-effective and efficient, we explored mixotrophic growth condition of *C. vulgaris* where sugar is only used at minimized levels to stimulate light-driven photosynthetic growth in biomass production. We examined the effects of glucose and other monosaccharides on *Chlorella* growth and found that the appropriate addition of sugars could not only provide additional biomass generation, but also stimulate photoautotrophic growth with enhanced photosynthetically derived biomass generation and CO_2 sequestration. We further developed a mixotrophic culture of *C. vulgaris* in LED-based PBRs in order to improve the economic feasibility of PBR-based algal production systems a techno-economic analysis was also performed to provide an in depth understanding of the PBR system for algae cultivation and CO_2 sequestration.

2. Materials and methods

2.1. Algal strain and culture conditions

The algal strain *C. vulgaris* (UTEX 26, UTEX Culture Collection of Algae) was cultivated using 2.5% (v/v) CO_2 - air mixture at 25 ± 2 °C in LED-based PBRs according to methods described in our previous study (Fu et al., 2012a). The initial biomass concentration for batch and semi-continuous culture was 0.84 g dry cell weight per litre (gDCW/L), unless otherwise stated, with an optical density OD_{660nm} ≥2 and transmittance ≤1%. It was assumed that all light provided on the inner surface of the PBRs would be absorbed by the high-density algal culture from the start (Fu et al., 2012a). A cultivation regime of three days was used which enabled achieving high density cultures and a high level of average growth rate (Fu et al., 2012a). The mixotrophic culture experiments were conducted in bubble column LED-based PBRs that had working volumes of 500 ± 5 mL (height = 60 cm and diameter = 4.0 cm). For scalability experiments, PBRs with a fixed height of 60 cm and diameters of 4.0, 6.0, 8.0 and 10.0 cm were used. The average incident light intensity was 300 μE/(m²·s) at a 25% duty cycle, while the input gas had a superficial velocity of 0.24 cm/s and a CO_2 level of 2.5% (v/v). The working volume of the PBRs was 500 mL, 1200 mL, 2400 mL and 3600 mL for diameters of 4.0 cm, 6.0 cm, 8.0 cm and 10.0 cm, respectively. Student's *t*-test was used to determine the significance of variance between groups.

2.2. Growth media

The growth media used in this study were K-8 and K-9, in which the former was used in scalability experiments and most mixotrophic experiments and the latter only to study the effects of glucose on algal growth. The K-8 medium was prepared according to the methods used in our previous study (Fu et al., 2012a). The K-9 medium was prepared by modifying the K-8 medium such that 15.6 mM urea was used as a nitrogen source instead of potassium nitrate. All compounds required for the

media were obtained from Sigma–Aldrich Co. (St. Louis, U. S. A.). The pH of cultures grown on both K-8 and K-9 media was kept stable for a short-term cultivation of three days, at levels between 6.0 and 6.5; this was accomplished mainly by the phosphate and bicarbonate buffer systems in the media.

2.3. Photobioreactor setup

The light supply and photobioreactor setups were as follows: blue and red LED arrays with narrow output spectra of 470 $\pm$ 20 nm and 660 $\pm$ 20 nm, respectively, were purchased from LUMEX Inc. (Carol Stream, Illinois, U.S.A.). Different average photon fluxes were supplied by using flashing light at a frequency of 10 kHz, but with different duty cycles for mixotrophic experiments. The photon flux of the light supplied to the PBRs was measured on the inner surface of each PBR using a quantum sensor (SR. NO. Q40526 of QUANTUM, Model LI-1400, LI-COR biosciences, Lincoln, Nebraska, U.S.A.). More details of the photobioreactor setup can be found in Fu et al. (2012a).

2.4. Addition of monosaccharides to mixotrophic cultures

The timing and amount of glucose and other monosaccharides added in different mixotrophic experiments are described and summarized in Table S1 (Supplementary Data 1). We evaluated the effects of D-mannose, D-galactose, D-fructose and L-arabinose on the growth rate in a three day mixotrophic batch culture, with the addition strategy that gave highest yields on D-glucose, i.e., 1.0 mM at 0 h and 2.0 mM at 48 h. L-arabinose which is not consumed by Chlorella (Rodriguez-Lopez, 1966) was used as a negative control in the study. Student's t-test was used to determine the significance of variance between groups.

2.5. Sample collection and biomass determination

Samples of cell suspension (typically 2 mL) were collected every 24 h over three days and at the end of the experiments. The optical density at 600 nm was measured to determine the dry biomass as described in our previous study (Fu et al., 2012a).

2.6. Biomass yield on light energy

The biomass yield on light energy (the quantum yield), Ψ_E, is defined as the amount of biomass generated per unit of radiance absorbed by the algal culture during batch culture. More details of the calculations can be found in Fu et al. (2012a).

2.7. Biomass yield on sugars

The biomass yield on sugars, such as glucose and mannose is defined as the increase or decrease in biomass (g) produced due to the addition of sugars in comparison to that produced under photoautotrophic culture conditions per unit of sugars (g) consumed by algae during batch culture over three days.

2.8. Extraction and quantification of neutral lipids

Procedures for the extraction of neutral lipids (Bligh and Dyer, 1959) were as follows: Each 50-mL sample (OD$_{600}$ = 6.0) of C. vulgaris after batch culture over three days was centrifuged (2000g, 10 min) and re-suspended in 8.0 mL deionized water. The cells were disrupted using an ultrasonic disintegrator (MSE Ltd. London, United Kingdom) for 15 min in an ice/water bath. The sample was then homogenized in a 250-mL flask for 2 min with a mixture of 10.0 mL chloroform and 20.0 mL methanol. A further 10.0 mL chloroform was added to the mixture and, after blending for 30 s, 10.0 mL deionized water was added and blending continued for another 30 s. The mixture was then transferred to a 100-mL graduated cylinder and, after storing for 10 h or overnight at −20 °C for

complete separation and clarification, the volume of the chloroform layer, which contained the purified neutral lipids, was recorded and collected. A portion of the lipid extract containing 10–20 mg lipid was evaporated to dryness in a tared tube with a miVac QUATTRO concentrator (Genevac Inc., New York, USA). The weight of the lipid residue was determined. After weighing, a small volume of chloroform was added to each tube to detect the presence of non-lipid (insoluble) material. If non-lipids were present, the chloroform was carefully decanted and the tube was rinsed three times with chloroform. The dry weight of the residue was determined and subtracted from the initial weight.

2.9. Detection of monosaccharides

The presence of the reducing carbohydrates D-fructose, D-galactose, D-mannose and L-arabinose was detected using freshly prepared Tollens' reagent. Specifically, 2 mL of algal sample was collected at the end of each batch culture. After centrifugation (10,000g, 5 min), the liquid supernatant was transferred to a glass tube, then 10 mL Tollens' reagent was added to check the presence of stated carbohydrates. The presence of D-glucose was detected with a Glucose Assay Kit from Sigma–Aldrich Co. (St. Louis, U. S. A.). At the end of each batch culture, sugar determination was performed to confirm that all sugars except L-arabinose were completely consumed while the concentration of L-arabinose remained unchanged during batch culture.

2.10. Determination of carotenoids and chlorophylls

Cell pellets were collected from 500 μL of samples at the end of each batch culture over three days by centrifugation (1000g for 10 min) and then extracted with 3 mL of a 2:1 ethanol:hexane (v/v) containing 0.1% (w/v) butylated hydroxytoluene until colorless (García-González et al., 2005). Then 2 mL water and 4 mL hexane were added and the mixture was vigorously shaken and centrifuged again at 1000g for 5 min (Fu et al., 2013). Then 4 mL of the upper hexane layer were evaporated with a miVac QUATTRO concentrator at 25 $\pm$ 2 °C, reconstituted in a mixture of methyl tertiary butyl ether: acetonitrile (MTBE: ACN) (50:50 v/v) and analyzed by ultra-performance liquid chromatography, UV and mass spectrometry (UPLC-UV-MS) according to the procedures described in Fu et al. (2012b). Student's t-test was used to determine the significance of variance between groups.

2.11. Techno-economic analysis and cost model

The energy/electricity and local employment costs are quoted from the market price in Iceland. The main facilities as well as the LED modules are calculated from the setup we developed for our previous photobioreactor systems (Fu et al., 2012a) in collaboration with the Controlant company (Reykjavik, Iceland). The costs of consumables including materials and reagents are based on prices from Sigma-Aldrich/Merck (St. Louis, USA) and bulk prices are obtained from Amazon (Seattle, USA) and Alibaba (Hangzhou, China). The breakdown of total costs is shown in detail in Supplementary Data 2.
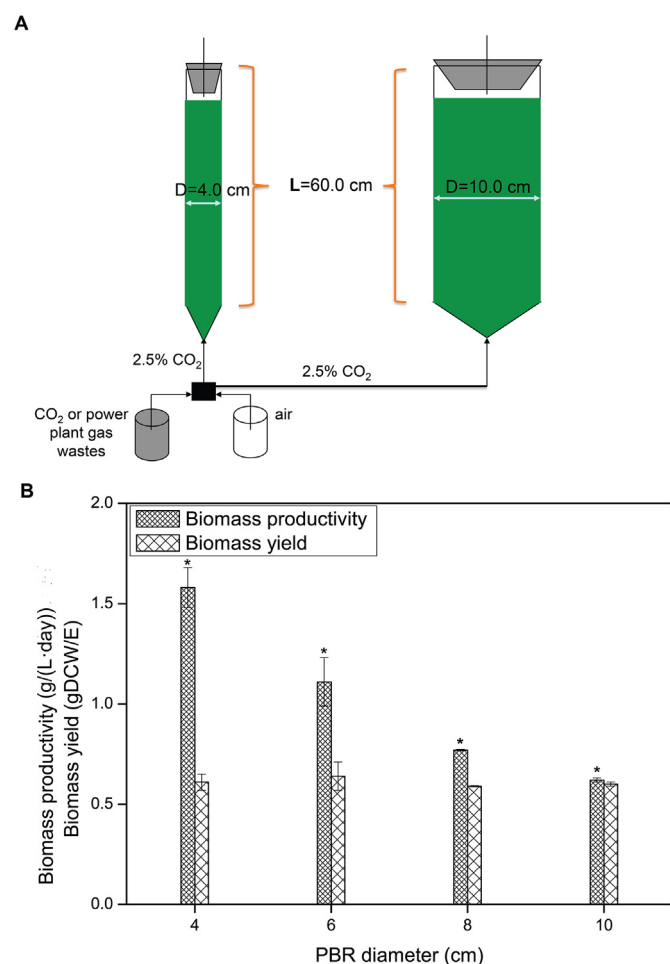
3. Results and discussion

Scaling up of photobioreactors is key to commercial utilization of algae-based systems for biomass production and CO$_2$ capture. In the context of LED illuminated reactors, one of the key parameters is the diameter of the cultivation vessels. To test what is the optimal diameter, a semi-continuous cultivation mode was used to evaluate the effects of PBR size on biomass productivity. The average biomass productivity decreased from 1.58 to 0.62 g dry cell weight per litre per day (g/(L·day)) as the diameter of the PBRs increased from 4.0 to 10.0 cm, while the biomass yield on light energy was nearly constant, approximately 0.60 gDCW/E (Fig. 1). The relatively constant values of biomass yield confirmed that the light

supply was the limiting factor during the scale up, which is in agreement with a previous study (Fu et al., 2012a). Although the biomass yield on light energy was maintained at a relatively high level during the PBR scale-up, the biomass productivity did decline significantly, resulting in a lower biomass concentration in larger-diameter reactors. A high-density culture upon harvest, along with high biomass productivity is desirable for downstream processing and would reduce the daily operating costs. Previous study had shown that biomass production of *C. vulgaris* was increased under a mixotrophic growth condition (Zuniga et al., 2016) and it would be interesting to see how different sugars can affect biomass productivity and if a more effective concentration of sugar can be found for high-dense cultures.

3.1. The effects of D-glucose addition on the growth of C. vulgaris under mixotrophic growth conditions

Glucose is one of the preferred organic carbon sources for *Chlorella* (Liang et al., 2009). Liang et al. (2009) found that levels of glucose addition had a significant impact on algal growth and a glucose concentration of 1% (55.6 mM) increased the generation of biomass while higher concentrations had an inhibitory effect on growth under

mixotrophic conditions. To avoid inhibition effects and to gain high biomass yield on glucose, we studied the effects of D-glucose addition at different levels below 55.6 mM on the growth of *C. vulgaris* in batch culture over three days. To activate the glucose transport system as well as the catabolic pathways such as glycolytic and oxidative pentose phosphate pathways during the transition from photoautotrophic growth to mixotrophic growth, glucose was added to the medium in small amounts, at most 1.0 mM, at the beginning of the experiment. This addition was intended to stimulate the activation, rather than to act as a major carbon source. In order to exhaust all the supplemented glucose before the end of batch culture, no glucose was added after 48 h. Additionally, each treatment was <50 mM, based on the estimation of the daily uptake rates (data not shown), in order to avoid inhibitory effects. The addition of glucose resulted in increased biomass at both high and low addition levels (Table 1). The yields of *C. vulgaris* were higher in comparison to those of other microorganisms; the maximum biomass yield on glucose was found to be 0.51 gDCW/gGlucose for *Saccharomyces cerevisiae* (Verduyn et al., 1991) and 0.52 gDCW/gGlucose for *Escherichia coli* in carbon limited cultures (Varma et al., 1993). For calculation of photoautotrophic growth in a mixotrophic culture, a conversion efficiency of 0.51 gDCW/gGlucose (Verduyn et al., 1991) was used to maximize glucose conversion capacity of *C. vulgaris* to the upper bound. Including glucose at a high level (>10 mmol/(L·day)), i.e., 12.2 and 13.7 mmol/(L·day) on average, significantly increased the biomass productivity of the batch culture (Table 1). Interestingly, adding glucose at low levels, i.e., between 1.0 and 2.8 mmol/(L·day), gave much higher yields on photoautotrophic growth, up to 1.75 g/(L·day) on average in comparison with photoautotrophic growth without glucose (Table 1). For the model cyanobacteria species *Synechocystis* sp. PCC 6803 cultivated in a medium with a glucose concentration of 5.0 mM, the heterotrophic biomass yield was equivalent to 0.50 gDCW/gGlucose (Yang et al., 2002). It has been suggested that heterotrophic cultures of microalgae like *Chlorella* cannot achieve higher biomass yields on glucose than purely heterotrophic microorganisms such as *E. coli* and *S. cerevisiae* since part of the energy derived from glucose is used for the maintenance of the photosynthetic apparatus, which doesn't contribute to biomass generation (Morales-Sánchez et al., 2013). The results of our study indicate that more CO_2 was converted into biomass photosynthetically under specific mixotrophic regime than under photoautotrophic conditions (Table 1). This enhanced conversion of CO_2 into biomass could be a consequence of potentially activated metabolic pathways due to glucose addition as well as glucose-derived metabolites. In the meantime, CO_2 concentrating mechanism

Fig. 1. Evaluation of effects of photobioreactor size on algal growth. (A) A schematic diagram of bubble column photobioreactors. (B) The effect of PBR diameter on the growth of *C. vulgaris* in batch cultures. The biomass productivity represents an average growth rate in batch culture over three days, starting from an initial density of 0.84 gDCW/L. The presented results are average values from two independent experiments. Error bars indicate standard error. The asterisk indicates significant difference between groups (p < 0.05). Biomass productivity is the volumetric daily biomass generation. Calculation of biomass yield on light energy is shown in the Materials and methods section. DCW, dry cell weight; E, Einstein, a unit for one mole of photons.

Table 1
The effect of two different levels of glucose addition on the growth of *C. vulgaris* in batch culture over three days.

Average glucose supplementation per day (mM)		Timing and levels of glucose addition (mM)			Growth performance[1]	
		0 h	24 h	48 h	Biomass productivity (g/(L·day))	Photoautotrophic production of biomass[2] (g/(L·day))
High level	13.7	1.0	10.0	30.0	2.58 ± 0.04	1.32 ± 0.04
	12.2	0.33	1.0	35.3	2.54 ± 0.01	1.42 ± 0.04
Low level	2.8	0.33	1.0	7.0	1.86 ± 0.001	1.60 ± 0.001
	1.7	0.50	4.5	0	1.91 ± 0.08	1.76 ± 0.08[ab]
	1.1	0.33	1.0	2.0	1.74 ± 0.02	1.64 ± 0.02
	1.0	1.0	0	2.0	1.84 ± 0.05	1.75 ± 0.05[ac]
Autotrophic	0	0	0	0	1.58 ± 0.05	1.58 ± 0.05[aa]

[1] The results presented are average values ± standard error from two independent experiments.
[2] Assuming all glucose added was consumed and converted into biomass based on a theoretical maximal conversion efficiency of 0.51 gDCW/gGlucose, the photoautotrophic production of biomass was calculated from the total biomass productivity by deducting the glucose-derived biomass generation during batch culture. The symbols (ab, ac, ad, and ae) indicate significant difference between mixotrophic growth and photoautotrophic growth (aa).

(CCM) that plays a vital role in carbon fixation in microalgae may be affected due to the mass balances among different CO_2 forms as well as the balance between CO_2 and O_2 (Morales et al., 2015).

Mixotrophic growth of *Chlorella* has previously been widely reported (Juntila et al., 2015; Morales-Sánchez et al., 2013; Zuniga et al., 2016). However, using glucose for photosynthetically enhancing biomass production as well as for CO_2 incorporation has not yet been reported in green algae and its mechanism remains largely unknown. To investigate this interesting phenomenon, we started by studying the mixotrophic growth on D-glucose with the same addition strategy under different LED illumination conditions, i.e., red light with blue light present and red light alone. Combined blue and red LED lights (50%/50%) increased the biomass yield only slightly compared to red light only at the same photon flux level, indicating that light quality is not the key factor affecting mixotrophic growth on glucose (Table S2 in Supplementary Data 1). However, when the photon flux was increased from 85 to 170 $\mu E/(m^2 \cdot s)$, both the biomass yield on glucose and biomass productivity increased considerably under both light conditions (Table S2 in Supplementary Data 1). This is in agreement with results reported elsewhere that the yield of biomass (g biomass per mol C in glucose) increases as the amount of light energy provided increased for mixotrophic metabolism (Shastri and Morgan, 2005). As the light supply is the limiting factor in LED-based PBRs, we hypothesize that the enhancement of biomass productivity due to the synergistic interactions between the heterotrophic and photoautotrophic growth was related to adenosine triphosphate (ATP) generation, given that the ATP created by the light dependent photosynthetic reactions drives the transportation and phosphorylation of glucose (Muthuraj et al., 2013).

3.2. The effects of different monosaccharides on the mixotrophic growth of Chlorella

Glucose is the favored organic carbon source for many microorganisms, e.g. *E. coli*, *S. cerevisiae* and *C. vulgaris* (Martínez and Orús, 1991). It has been reported that *Chlorella* can utilize various hexoses for heterotrophic growth, e.g. glucose, fructose, galactose and mannose, but not pentoses like arabinose (Rodriguez-Lopez, 1966). We therefore tested these sugars as stimuli for the mixotrophic growth of *Chlorella*. The results were that low levels of D-galactose, D-fructose or L-arabinose had no obvious growth promoting effects on *C. vulgaris* (Fig. 2).

On the other hand, the addition of D-mannose at low levels, 1.0 mM per day on average in batch culture, was found to inhibit the growth of *C. vulgaris* significantly (Fig. 2). To investigate this observation further, we then tested how the addition of varying amounts of D-mannose affected *Chlorella* growth. It was found that increasing the initial level of D-mannose from 0.2 mM to 1.0 mM resulted in a dramatic decrease in growth rates. The addition of 1.0, 2.0 and 4.0 mM D-mannose showed similar inhibition effects on the growth (Fig. 2B). It has been reported that many plants cannot metabolize D-mannose 6-phosphate which results from the phosphorylation of D-mannose by hexokinase. The reason is that the gene encoding phosphomannose isomerase is either missing or its expression levels are low. This enzyme isomerizes D-mannose 6-phosphate, converting it into D-fructose-6-phosphate, which can be metabolized further in plants (Rosellini, 2011). The mannose added to *Chlorella* cultures under light is transported across the plasma membrane with the inducible hexose/H^+ symporter (hexose uptake protein 1) and phosphorylated by the hexokinase (Doebbe et al., 2007; Tanner, 2000). It is likely that the generation of D-mannose 6-phosphate which consumes ATP from the pool cannot be further metabolized to D-fructose-6-phosphate and is therefore unable to enter the glycolysis pathway, resulting in a decrease of ATP level for carbon fixation during Calvin cycle (Herold and Lewis, 1977; Maruta et al., 2008).
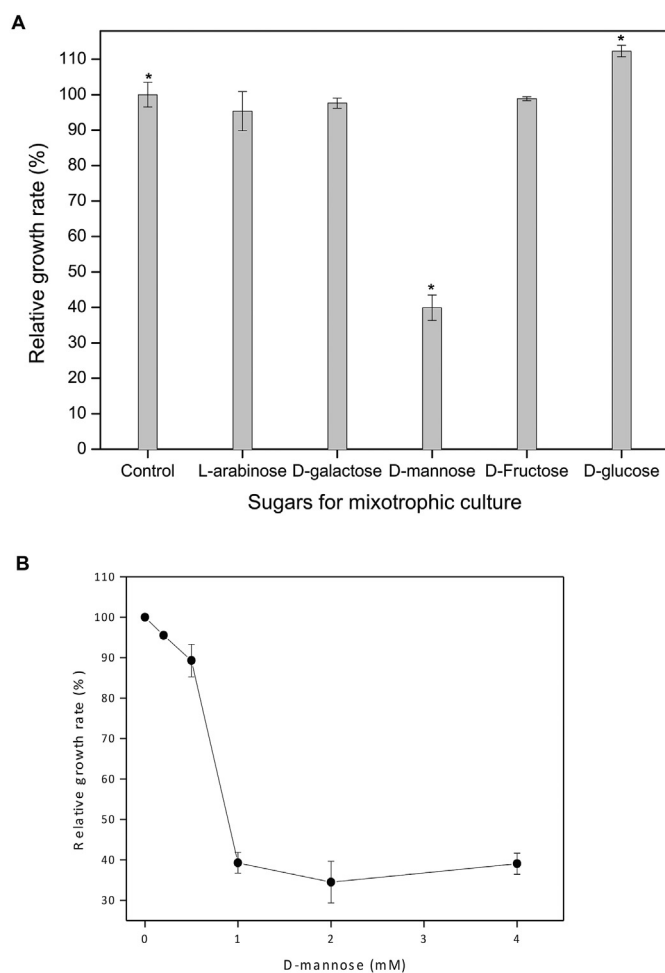
Fig. 2. The effects of monosaccharides on the mixotrophic growth of *C. vulgaris* using K-8 medium in batch culture. (A) Addition of different monosaccharides. Cells were cultivated for three days, starting from an initial density of 0.84 gDCW/L. The addition of monosaccharides was 1.0 mM at 0 h and 2.0 mM at 48 h, respectively. Relative growth rate (%) was defined as the ratio of growth rate in a specific mixotrophic growth condition to a photoautotrophic growth rate (control, 100%). (B) Different levels of D-mannose addition. In all the experiments, D-mannose was added to the culture (initial density, 0.84 gDCW/L) at the starting point (0 h). The average growth rate was measured at 24 h and normalized to the photoautotrophic growth rate (no addition of D-mannose). Error bars indicate standard error from two independent experiments. The asterisk indicates significant difference between the control and experimental group ($p < 0.05$).
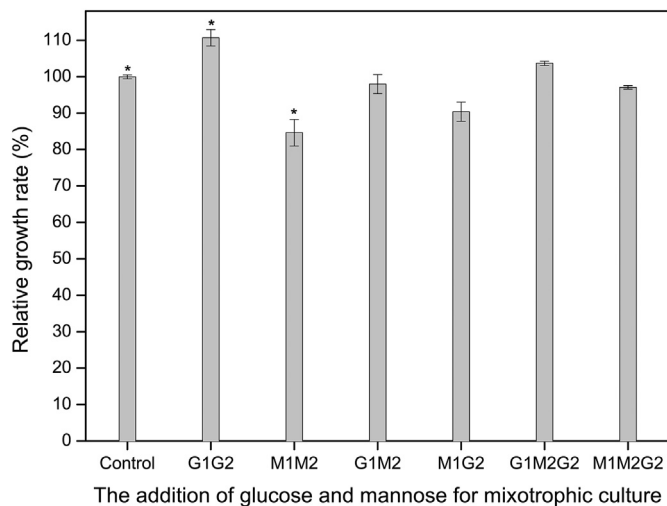
3.3. The effects of nitrogen source on growth

Nitrates (NO_3^-) are key nutrients as well as signaling molecules that impact both the metabolism and the development of plants, and that its

Table 2
Effect of glucose and mannose on the growth of *C. vulgaris* using urea as the sole nitrogen source (K-9 medium).

Organic carbon source	Sugar addition (g/L)			Growth performance [a]	
	0 h	24 h	48 h	Biomass productivity (g/(L·day))	Average yield (gDCW/gSugar)
D-Glucose	0.18	0	0.36	2.06 ± 0.04	1.11 ± 0.16
	0	0	0	1.86 ± 0.01	Not applicable
D-Mannose	0.18	0	0.36	1.59 ± 0.01	−1.59 ± 0.06
	0	0	0	1.88 ± 0.09	Not applicable

[a] The results presented are average values ± standard error from two independent experiments.

Fig. 3. The effects of D-glucose and D-mannose addition on the mixotrophic growth of *C. vulgaris* in K-9 medium (urea as the sole nitrogen source) in batch culture. Cells were cultivated for three days, starting from an initial density of 0.84 gDCW/L. G1G2: adding glucose at 0 h and 48 h; M1 M2: adding mannose at 0 h and 48 h; G1 M2: adding glucose and mannose at 0 h and 48 h, respectively; M1G2: adding mannose and glucose at 0 h and 48 h, respectively; G1M2G2: adding glucose at 0 h and both mannose and glucose at 48 h; M1M2G2: adding mannose at 0 h and both mannose and glucose at 48 h. All the doses of sugar supplementation were in low levels at 1.0 mM or 2.0 mM. Details of adding strategies were summarized in Table S1 (Supplementary Data 1). The presented results are average values from two independent experiments. Error bars indicate standard error. The asterisk indicates significant difference between the control and experimental group (p < 0.05).

assimilation is regulated through a complex network of transporters, reductases and synthetases (Krouk et al., 2010). It is, however, unknown whether the substitutions of nitrates with other nitrogen-containing compounds will affect the photoautotrophic and mixotrophic growth of

Chlorella. Because urea has the highest nitrogen content among all the nitrogen sources in algal cultivation, we tested the utilization of urea (K-9 medium) instead of potassium nitrate (K-8 medium) as the sole nitrogen source for the photoautotrophic growth of *C. vulgaris*. We found that the K-9 medium increased the biomass productivity by 14% compared to the K-8 medium (Table S3 in Supplementary Data 1). According to the elemental analysis of biomass composition, carbon is the major component of *Chlorella*, accounting for 51.4–72.6% of the biomass (by weight) while nitrogen accounts for 6.2–7.7% (Mandalam and Palsson, 1998), so carbon fixation through the Calvin cycle can significantly affect cell growth as well as its central metabolism. The enzymatic hydrolysis of one mole of urea to carbamate by urease and the subsequent non-enzymatic decay of carbamate leads to the release of one mole of carbon dioxide and two moles of ammonia (Witte, 2011). Presumably due to the fact that ammonium ions do not require further reduction and are thus metabolically more affordable than nitrate, urea as sole nitrogen source enhanced biomass production of *Chlorella* (Table S3 in Supplementary Data 1). However, ammonia at high concentrations could be toxic to many green microalgae, including *Chlorella*, due to pH and ionic imbalance (Scherholz and Curtis, 2013). It is likely that the balance between the intracellular pool of ammonium and its supply also affects nitrogen metabolism as well as biomass production (Zuniga et al., 2018).

Since using urea as the nitrogen source in the K-9 medium enhanced the biomass productivity under photoautotrophic conditions (Table S3 in Supplementary Data 1), it was of interest to know whether the effects of glucose on mixotrophic growth are related to nitrogen/nitrate metabolism. As shown in Table 2, the addition of glucose to the K-9 medium had similar growth promoting effects as the glucose addition to the K-8 medium (Fig. 2A), with a 10.7% increase in biomass productivity and an average yield of 1.11 gDCW/gGlucose. The K-9 medium largely alleviated the inhibition effects of D-mannose observed in the K-8 medium (see Figs. 2B and 3), presumably due to enhanced biomass generation by urea-derived CO_2 in photosynthesis. The effects of D-glucose and D-mannose addition on growth were studied further in the K-9 medium.
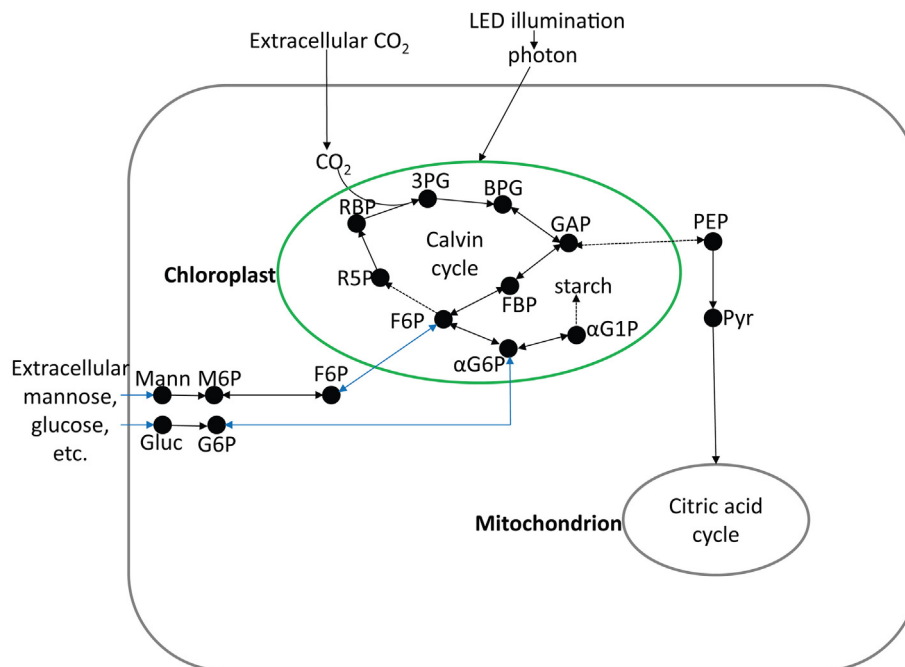
Fig. 4. A schematic diagram of the carbon fixation pathway in *C. vulgaris* under mixotrophic culture conditions based on KEGG database. Black arrows indicate the directions of reactions. Blue arrows indicate the possible membrane transport. Abbreviations: 3PG, 3-phospho glycerate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; BPG, 1,3 biphospho glycerate; CO_2, carbon dioxide; DHAP, dihydroxy acetone phosphate; F6P, fructose 6 phosphate; FBP, fructose 1,6 biphosphate; G1P, glucose 1 phosphate; G6P, glucose 6 phosphate; GAP, glyceraldehyde 3 phosphate; Gluc, glucose; Mann, mannose; NAD, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); NADP, nicotinamide adenine dinucleotide phosphate (oxidized form); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); O_2, oxygen photon, Photon (light); R5P, ribulose 5 phosphate; RBP, ribulose 1,5 bisphosphate. Descriptions of energy-related reactions are given in Table S4 (Supplementary Data 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Carbon capture efficiency of *C. vulgaris* under photoautotrophic and mixotrophic growth conditions and the associated neutral lipids.

Culture mode	Biomass productivity[a] (g/(L·day))	Neutral lipids content[b] (% of dry biomass)	Lipid productivity (mg/(L·day))	Bio-fixation of carbon dioxide (g/(L·day))
Photoautotrophic growth	1.86	25.0 ± 0.5	465.0 ± 9.3	3.51
Mixotrophic growth	2.06	25.1 ± 2.5	516.6 ± 5.3	3.71

[a] Biomass productivity data are from Table 2; all glucose added (0.18 g/(L·day)) is assumed to be consumed and converted into biomass with a maximal conversion efficiency of 0.51 gDCW/gGlucose (see Table 1); carbon content in the dry biomass is assumed as 51.4% (Mandalam and Palsson, 1998); for photoautotrophic culture, CO_2 fixation rate = biomass productivity ·51.4%/(12/44); for sugar-stimulated mixotrophic culture, CO_2 fixation rate = (biomass productivity − 0.18 · 0.51)·51.4%/(12/44).

[b] The results presented are average values ± standard error from two independent experiments.

It was found that the addition of D-glucose into algal cultures, together with or before D-mannose addition, reduced the growth inhibition effects of D-mannose, though the greatest growth rate was still observed for glucose addition alone (Fig. 3). As D-glucose is the preferred carbon source and a competitive substrate to D-mannose, we speculated that the presence of D-glucose in the medium mitigated the growth inhibition caused by D-mannose probably via hexose transport and catabolic pathways (Fig. 4).

To estimate the amount of carbon dioxide fixed into biomass, it was assumed that the biomass productivity in the mixotrophic culture corresponds to 2.06 g/(L·day) (Table 2), which is 30.4% higher than the initial photoautotrophic biomass productivity of 1.58 g/(L·day) (Table S3 in Supplementary Data 1) in a purely photoautotrophic culture. Further, assuming a carbon content of 51.4% (Mandalam and Palsson, 1998) in the dry biomass, approximately 3.71 g/(L·day) of carbon dioxide (Table 3) could be fixed into biomass. Therefore, a 200-tonne industrial scale photobioreactor unit could convert 270.9 t of CO_2 waste into 150.4 t of valuable dry biomass annually. For comparison, Landsvirkjun, the largest power company in Iceland, has put considerable effort into land reclamation and re-forestation, but the actual binding of CO_2 annually is <5 tonnes per hectare in the forest areas after >3000 seedlings per hectare are planted and allowed to grow for several years (Landsvirkjun, Environmental Report, 2012). Estimates of carbon capture efficiencies indicate that a higher efficiency is achieved under glucose-stimulated mixotrophic conditions than under photoautotrophic culture (Table 3). In addition, the CO_2 fixation rates obtained in this study were about 2.1 times better than the values reported for engineered cyanobacteria (Chen et al., 2012).

3.4. The effects of D-glucose addition on carotenoid and neutral lipids content of Chlorella

The mixotrophic culture strategy developed in this study improved both the biomass productivity and the biomass yield of *Chlorella*. It is important to determine whether the product quality of the algae, in terms of functional ingredients and biodiesel, is affected by the mixotrophic conditions. We therefore quantified selected carotenoids and neutral lipids in both photoautotrophic and mixotrophic cultures. There was no significant difference observed in the content of major carotenoids and chlorophylls, i.e., β-carotene, α-carotene, lycopene, lutein, zeaxanthin, and chlorophyll *a* and *b*, in the two culture modes (Fig. 5). We therefore conclude that key biomass nutraceuticals of the mixotrophic *Chlorella* developed in this study is as high as its photoautotrophic counterpart. In addition, it was found that the total content of neutral lipids in *Chlorella* was similar, i.e. 25% of the dry biomass under both photoautotrophic and mixotrophic culture conditions, which corresponds to an increase in volumetric lipid productivity from 465.0 to 516.6 mg/(L·day) (Table 3). These figures are higher than the neutral lipid levels that are typically 10% to 20% of dry biomass in the model diatom *Thalassiosira pseudonana*, which has recently been suggested as an attractive source of biofuels (Trentacoste et al., 2013). This study suggests that the sugar-stimulated mixotrophic cultivation of *Chlorella* is a promising approach for producing biofuels and bioactive products.

3.5. Economic feasibility of LED-based PBRs

Techno-economic analysis of microalgae cultivation systems has been conducted mostly in regions with sufficient lights and mild temperature for high growth rates although there is also a need to investigate the efficiency of algal cultivation system in regions in high latitudes to assess whether there are effective solutions (Manninen et al., 2016). In this study, a comprehensive cost model (Fig. 6 and Supplementary Data 2) was made in order to evaluate the economic feasibility of the proposed strategy. In the modeling analysis, a single PBR with a diameter of 7.0 cm and a length of 600 cm was used and a total of 990 units was built and grouped in 30 modules in a plant. In the model, the costs for producing algal biomass can be roughly divided into three parts: direct costs; capital costs; and overhead costs. The energy cost for lighting accounts for >95% of the direct costs, while the cost of the LED boards is the largest part of the capital expenditure, accounting for 29% of the total. The labor cost is the major part of the overhead costs, at 34% of the total. This cost could be decreased by increased automation of the manufacturing process. Accordingly, the cost was estimated as 49.16 $/kgDCW for PBRs with a working volume of 22 L for each and 21.78 t for the whole suite. Our results indicate that LED-based PBRs may be economically feasible for the commercial production of value-added goods such as quality *Chlorella* powder, which retails for >100 $/kgDCW (Taiwan Chlorella Manufacturing Company). With continued development of the LED technology, the cost of LEDs is expected to decrease and the energy efficiency may still be improved significantly, reducing the capital cost of the LED boards and the direct energy
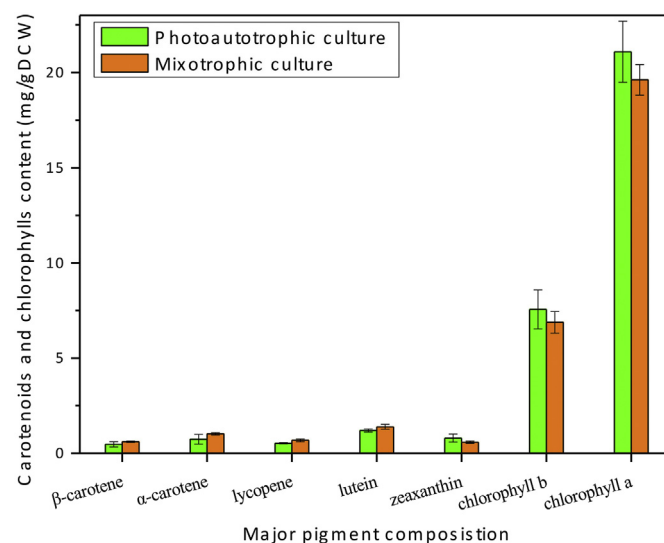
Fig. 5. The contents of major carotenoids and chlorophylls in *C. vulgaris*. K-9 medium was used in all experiments. For the mixotrophic culture, 1.0 mM and 2.0 mM D-glucose were added to the culture at the starting point (0 h) and at 48 h. The samples were collected at the end of batch culture over three days and pigments were extracted for LC/MS analysis. The presented results are average values from two independent experiments. Error bars indicate standard error. No significant difference was detected for major pigment accumulation between the mixotrophic culture and photoautotrophic culture (p > 0.05).

Total costs for biomass production: 49.16 $/kg
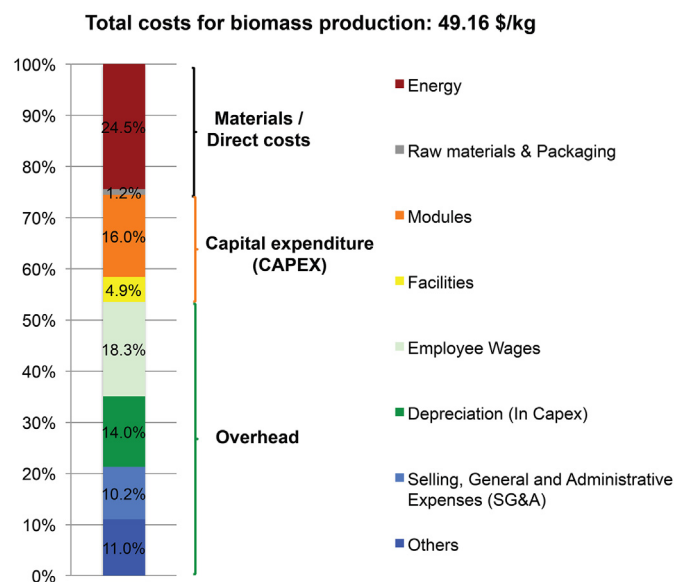
Fig. 6. A techno-economic analysis of total costs for algal biomass production. Detailed analysis is shown in Supplementary Data 2.

cost. Furthermore, algal strain development may improve the overall biomass productivity and consequently reduce the overhead costs. For instance, an increase of overall biomass productivity by 40% may lower the total cost to approximately 35 $/kgDCW. It is reasonable to expect that the total cost of the proposed process may be reduced significantly in the near future on the basis of improvements in biological performance of microalgae, as well as improvements in micro-electronics of LED illumination.

4. Conclusions

In this study, we have demonstrated how *Chlorella* culture can be developed using low dose of sugars at stimulating-levels for promoting photosynthetic efficiency and CO_2 sequestration. Using small doses of sugars together with LED illumination may be a promising strategy for producing algal biomass in an environmental-friendly and sustainable manner while fixing CO_2 from flue gas biologically. Further development of these processes with microalgae may contribute significantly to the mitigation of CO_2 emissions, particularly in locations such as Iceland, that LED-based PBRs are the main practical means for algal cultivation through the seasonal fluctuations of light and temperature.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.11.120.

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