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Chlorella vulgaris cultivation in photobioreactor using municipal wastewater for biofuel

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Microalgae, with their potential applications, particularly in food industry and Livestock feed as well as biofuel, has gained considerable attention in recent decades. As their culture using commercial media is expensive, an integrated approach using municipal wastewater is considered for simultaneous biomass production. Therefore, in this study, we evaluated the growth and biochemical composition of the green microalgae *Chlorella vulgaris* grown in municipal wastewater. Cultures in vertical column photobioreactor in outdoor/indoor conditions were also compared. *C. vulgaris* showed significantly higher (P <0.05) cell density, biomass, specific growth rate, lipid and carbohydrate in indoor culture condition than outdoor comparatively. Saturated fatty acids and polyunsaturated fatty acids containing 14-18 carbon molecules were significantly higher (P <0.05) in indoor culture. The result suggests that wastewater could be used as a low cost medium to grow *C. vulgaris* to obtain higher biomass and lipids.

Keywords: Biomass, Biofuel, Dietary supplement, Microalgae, Sewage

Microalgae represent a varied set of autotrophic, mainly photosynthetic, unicellular organisms. Different types of microalgae have been investigated in recent decades to identify their potential as alternate sources of feed supplements, medicinal products and foods[1]. The depletion of fossil fuel reserves, concerns over global warming and the high demand for natural functional foods and products have valorized microalgae as an alternative renewable feedstock for productive biodiesel. Algae-based biofuels can surmount the inadequacies presented by conventional fuels, thereby reducing the 'food *vs*. fuel' debate[2,3]. Although the production of biodiesel from microalgae is a newly emerging field, numerous reports described advantages of using microalgae in comparison with other available feedstock or terrestrial plants[4].

Chlorella has long been used as health food and additive for human consumption, as well as animal feed. Chlorella microalgae have attracted considerable attention due to their great potential as feedstock for production of biofuels and high-value products[5]. Its distribution is broad in fresh, marine and terrestrial habitats[6]. *Chlorella* is one of the most interesting algae for biofuel production as they produce more lipid under stress condition, and yield starch under best growth conditions. During stress condition, stored starch are gradually converted into lipid[7]. Hence, *Chlorella vulgaris* is considered a promising candidate for commercial lipid production due to its fast growth and easy cultivation[8]. However, *C. vulgaris* has high cell growth but low lipid content[9].

It is well known that wastewater is a good nutrient source for microalgae[10]. At present, the utilization of wastewater as a source of nutrients has become an interesting alternative to explore when the aim is biofuels. Wastewater treatment by cultivating microalgae for biomass production has been reported as a cost effective strategy[11]. Domestic wastewater can be used for microalgal biomass production and act as a cost-effective substrate for microalgal growth[12]. In addition, Kumar *et al.*[12] have also reported microalgae cultivation using organic wastewater like kitchen wastewater (KWW) and sewage wastewater (SWW). When microalgae start to

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grow and multiply, nitrogen and phosphorus content in wastewater medium decrease significantly as they are converted into useful nutrients[13]. Using wastewater to cultivate microalgae not only makes the process cost-effective but also the decreases the need for nutrients supplementation significantly.

One of the major operational expenses of algal biomass production in closed reactors is temperature control (cooling)[14]. According to Carneiro *et al.*[15] temperature has shown to have effects on the biochemical composition of microalgae. There is limited research detailing the effects of temperature on the lipid content of microalgal production and the type of fatty acids formed using wastewater as cultivation medium. Mata *et al*.[16] analyzed temperature as one of the important limiting factors that influence microalgal growth in both closed and open outdoor systems. They reported that a lot of microalgae can tolerate fluctuation of temperatures up to 15°C or lower, but exceeding the optimum temperature by only 2-4°C may result in the total culture loss.

There is an increasing demand of microalgal biomass for commercial purposes and cultivation is presently carried out mainly in open systems. Outdoor ponds culture suffers several limitations due to bacterial contamination, predation by protozoa and competition from other microalgae. Inexpensive plastic bags commonly used to cultivate microalgae suffer from low cell density and biomass productivity. To overcome the problems of low productivity and contamination, vertical photobioreactors (VPBR) have become an attractive alternative for microalgal cultivation. Microalgae grown in VPBR stimulate a closed controlled condition for maximum production of microalgae. Closed controlled photobioreactor is able to provide unialgal culture at the optimum condition. In addition, there are several advantages such as good mixing, well-defined fluid flow pattern and relatively high gas-liquid mass transfer rate. Therefore, in the present investigation, we have tried to elucidate the productivity and biochemical composition of the green microalgae *Chlorella vulgaris* cultivated in vertical column photobioreactor under different environmental conditions.

Materials and Methods

Wastewater collection

Wastewater was collected from oxidation pond of municipal wastewater treatment plant. Before use, firstly wastewater was filtered by through a filter bag than by glass microfiber filter (GFC, Whatman) and UV-treated as contamination with bacteria, protozoa or another species of microalgae is a serious problem for mono-specific cultures of microalgae. Finally, wastewater was stored in at 3 ± 2°C for *C. vulgaris* growth. The pH was measured using a pH meter (Orion, USA) and by using a ion chromatography (882 Compact IC Plus, Metrohm, Switzerland) anions and cations were analyzed from wastewater.

Stock culture of *Chlorella vulgaris*

Tropical *Chlorella vulgaris* was obtained from the Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, that was isolated from a fish farm located at the Kuala Terengganu, Malaysia. The culture was grown under artificial light condition (50 µmol/m^2/s) at 25°C using Bold's Basal Medium (BBM)[17] and later inoculated in flask containing filtered and UV treated wastewater and maintained at 25°C and 50 µmol/m^2/s light intensity. The wastewater grown *C. vulgaris* was used as the stock culture for the experiment.

Experimental design

In this experiment, *C. vulgaris* was grown in outdoors under shade and indoors under laboratory conditions in vertical column photobioreactor (VPBR) using pre-treated wastewater. Both treatments had three replicates. *C. Vulgaris* was gradually scaled up from an initial culture volume of 20 mL to 3 L. It was then transferred to VPBR containing 7 L wastewater as the medium (Total culture volume in each bioreactor was 10 L). The cultures were transferred in their log-phase of growth as determined by biomass, cell count and optical density (OD). Indoor VPBR was provided with artificial illumination by six 30 W fluorescent tubes (Philips, Indonesia) placed at the back of the VPBR while outdoor VPBR was exposed to natural daylight. Aeration was provided from the bottom of the VPBR to keep the microalgal cells in suspension and provide oxygen. The culture was harvested using a continuous centrifuge (Hitachi* High-speed Refrigerated Centrifuge, himac CR 21g-II) when it reached in the stationary phase for protein, carbohydrate and fatty acid analyses. Lipid was analyzed every alternate day.

Analysis of physical and growth parameters

During the culture of *C. vulgaris* in VPBR, physical parameters such as temperature and light intensity were measured using thermometer and light meter. Microalgal growth was measured using duplicate

aliquots removed daily from the culture for estimation of cell density, optical density and biomass. The cell density was determined using Neubauer haemocytometer (Assistant, Germany) and calculated according to Lavens & Sorgeloos[18]. Optical density (OD) was determined at a wavelength of 540 nm using a spectrophotometer (UV-VIS 1601, Shimadzu, Japan). Biomass was estimated by following the method of Ratha *et al*.[19] and specific growth rate (SGR) of microalga was calculated according to Daniel & Srivastava[20].

Proximate and biochemical composition

Protein was analyzed according to Lowry *et al*.[21]. Lipid and carbohydrate analysis were conducted according to the methods of Bligh & Dyer[22] and Dubois *et al*.[23]. Fatty acids were analyzed by "Two steps transesterification (2TE)" method after a little modification from[24].

Statistical analysis

All of the collected data were analyzed using one-way analysis of variance (ANOVA) and significant differences among the treatments were analyzed by the Duncan multiple range test at 95% confidence interval level. Protein, lipid and carbohydrate percentages were arcsine transformed before statistical analysis. The statistical analyses were done using the Statistical Analysis System (SAS) computer software.

Results

Physicochemical analysis of wastewater

The physicochemical characteristics of the wastewater used in this study for *C. Vulgaris* culture were shown in Table 1. It holds significant amount of anions and cations required for microalgae culture and confirms the suitability of wastewater for the *C. vulgaris* culture. Moreover, the most dominated microalgae genera in untreated wastewater were *Oscillatoria* (Cyanophyta), *Spirulina* and *Ankistrodesmus* (Chlorophyta).

Table 1 — Physicochemical characteristics of the wastewater assayed in the present study

Properties	Value
Physical properties	
pH	7.65±0.01
Temperature (°C)	25.84±0.02
Conductivity (ms/cm^2)	0.360±0.01
Dissolved oxygen (mg/L)	5.39±0.02
Chemical properties (mg/L)	
Calcium	13.60±0.12
Chloride	1128.21±01
Phosphate	6.70±0.02
Nitrate	0.79±0.01
Nitrite	3.90±0.02
Ammonium	3.32±0.01
Potassium	6.01±0.01
Sodium	12.19±0.01
Sulphate	38.04±0.01

[Values are means ± standard errors (n=3). Significant variations among the indoor and outdoor (P <0.05) are indicated by values in each series with a different letter]

Temperature and light intensity

In outdoor condition, the mean temperature was range from 26.5 to 30.3°C while in indoors it was from 22.9 to 23.9°C. The mean light intensity was ranged from 58.01 to 223.86 µmol/m^2/s in outdoors and from 23.15 to 24.2 µmol/m^2/s in indoors.

Growth of *C. vulgaris*

On growth, *C. vulgaris* showed a significant difference (P <0.05) from day 1 to day 3 in outdoor and indoor culture system in terms of cell density and biomass (Fig. 1 A and B). Highest growth in terms of cell density, optical density and biomass was found on day 5 for indoor culture. Along with this, *C. vulgaris* showed an earlier stationary phase and shorter exponential phase in outdoor culture condition than indoor culture condition. Moreover, specific growth rate of *C. vulgaris* was found to be significantly higher (P <0.05) in indoors (day 7; 0.28) than outdoors (day 5; 0.21).

Proximate and biochemical composition

Results showed that, lipid and carbohydrate content were significantly higher (P <0.05) in indoor condition (30% dry wt.) compared to outdoor condition (25% dry wt.) whereas protein was

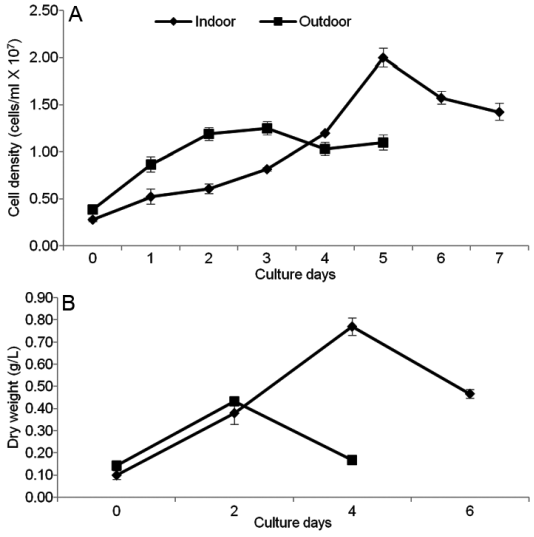

Fig. 1 — (A) Cell density; and (B) biomass of *Chlorella vulgaris* cultivated in vertical column photobioreactor under indoor and outdoor condition. [Values are mean ± standard error (n=3)]

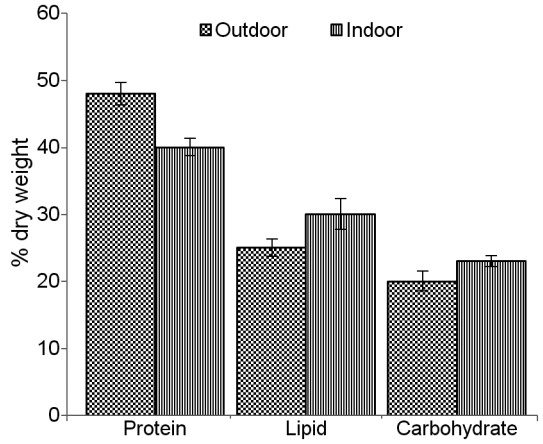

Fig. 2 — Protein, lipid and carbohydrate content (% dry wt.) of *Chlorella vulgaris* cultivated in vertical column photobioreactor under indoor and outdoor condition. [Values are mean ± standard error (n=3)]

Table 2 — Fatty acid composition (% of total fatty acids) of *Chlorella vulgaris* cultivated in vertical column photobioreactor (10 L) in indoor/outdoor culture condition

Fatty acids	Indoor	Outdoor
C14:0	25.29^{a}±0.01	05.20^{b}±0.02
C16:0	33.72^{a}±0.20	31.72^{b}±0.10
C16:1	43.01^{a}±0.01	0.79^{b}±0.02
C17:1	0.10^{a}±0.02	0.19^{a}±0.02
C18:0	4.25^{a}±0.10	2.95^{b}±0.11
C18:1	10.70^{a}±0.10	7.48^{b}±0.01
C18:2	28.96^{a}±0.11	29.16^{a}±0.10
C18:3*n*-3	20.09^{a}±0.10	18.49^{b}±0.01
C20:1	1.2^{a}±0.13	0.4^{b}±0.10
C20:3*n*-3	2.03^{a}±0.21	0.20^{b}±0.10
C20:5*n*-3	0.6^{a}±0.18	0.5^{a}±0.10
C22:6*n*-3	1.53^{a}±0.13	0.29^{b}±0.10
Total saturated fatty acids	40.00^{a}±0.21	38.01^{b}±0.01
Total unsaturated fatty acids	58.39^{a}±0.21	52.01^{b}±0.11

[Values are means ± standard errors (n=3)]

significantly higher (P <0.05) in outdoor culture (48% dry wt.) compared to indoor culture (40% dry wt.) (Fig. 2). In addition, fatty acid profile showed that C14:0 - C18:0 was significantly higher (P <0.05) in indoor culture than outdoors (Table 2). The total saturated and unsaturated fatty acids were also significantly higher (P <0.05) in the indoor culture.

Discussion

Microalgae are reported to grow well in industrial, municipal and agricultural wastewaters. de Alva *et al.*[25] Found higher biomass productivity and lipid accumulation in *Scenedesmus acutus* cultured in previously treated wastewater than enriched medium. High nutrient content of wastewater makes it an efficient growth medium for microalgae culture to produce higher microalgal biomass and lipid for energy generation[26]. In the present experiment, *Chlorella vulgaris* was chosen because of its tolerance to organic pollution and its ability to rapidly colonize any environment that is rich in nitrogen, phosphorus and organic compound[27]. In the current study, comparatively higher cell growth, optical density and biomass productivity of *C. vulgaris* was obtained in indoor culture system than outdoor. In a previous study, Wild *et al*.[28] also reported similar result and claimed that higher growth rate and biomass productivity was obtained in control system than outdoor cultivation. In addition, in the present experiment nitrogen and phosphorus concentration in the treated wastewater was significant in comparison to BBM[17], and therefore supported good growth of *C. vulgaris*. Hence, cultivation of *Chlorella* sp. can be one of the potential methods to reduce nitrogen and phosphorus from wastewater to prevent eutrophication.

For proper cell growth rate, biomass production and lipid productivity *Chlorella vulgaris* requires some optimum culture environments such as, temperature, light intensity and quality, pH, salinity and culture condition[29]. According to Mata *et al*.[16], microalgae can easily tolerate the optimum temperature but if the optimum temperature is exceeding by only 2-4°C may result in loss of the culture. According to Soeder *et al*.[30], the optimal temperature measured under conditions of maximum microalgal growth rate varies between microalgal species and is often between 28-35°C. This explained why the outdoor culture had to be harvested earlier. Since cooling system was not equipped in the PBR, the temperature could not be decreased to improve the culture conditions.

Furthermore, temperature rise in outdoor culture was accompanied by high light intensity. Outdoor culture recorded mean light intensity ranging from 58.01 up to 223.86 μmol/m^2/s. High intensity of light may have led to the generation of oxygen by photosynthesis in culture medium, reaching values that in combination with intense light could have damaged the microalgal cells that finally led to senescence phase of the culture[31]. On the other hand, indoor temperature culture was not exposed to large temperature variation from day to day as the minimum temperature recorded was 22.5°C while the maximum was 25.6°C. The temperature variance was almost constant because the culture temperature in the PBR was regulated by the air-conditioner that acted as external cooling system. In this study, although the

temperature and light intensity of outdoor system are in line with the recommended ranges provided by previous researchers but the higher cell growth, optical density, biomass and lipid productivity was noticed in indoor cultivation system than outdoor. This could be due to the wide temperature and light intensity variation of outdoor system compared to indoor which has reduced cell growth, optical density and biomass of outdoor.

The total protein content of *Chlorella vulgaris* is 43-58% of its dry weight in terms of growing circumstances[32]. In the current study high amount of protein content was recorded in outdoor culture system compared to indoor culture system. Similar outcomes were recorded in the study of Wild *et al*.[28]. In this study, outdoor culture system of *Chlorella* provided the highest concentration (579 g/kg dry matter basis) of crude protein compared to control system. In the current study, the higher concentration of protein in outdoor culture system was may be due to the availability of nitrogen. When nitrogen is available in the culture system, the storage components can be accumulated by microalgae during the day time and then microalgae can do conversion of these accumulated components to protein at night. This activity changes the protein concentration resulting in higher protein concentration which is up to 70%[33]. In metabolic pathways, ATP dependent phases are included, as a result the storage components are degraded in the respiratory chain partially causing up to 10% biomass depletion. Depletion can be continuous if there remain unsuitable growth environment like, lower light intensity and temperature[34]. As a consequence, there lies a possibility of destructive metabolism of the accumulated storage components during the night time causing enhancement of the crude protein content of the outdoor sample in comparison to the indoor.

Higher carbohydrate and lipid percentages were obtained in indoor cultivation system than outdoor. It was evaluated by previous researchers that, when grown in poor conditions and with inadequate nitrogen, *Chlorella vulgaris* can have a total carbohydrate content of 12-55% (dry wt. basis)[35]. The carbohydrate percentages of indoor 23% (dry wt.) and outdoor 20% (dry wt.) are in the recommended ranges provided by previous researchers.

Lipid content is a key factor that should be considered when biofuel production is concerned. In *C. vulgaris* lipid percentages varies from (5-58)% (dry matter basis)[32]. In this study, the obtained total lipid percentages are within the range provided by Safi *et al*.[32]. In indoor cultivation, higher lipid percentage was achieved 30% (dry wt.) and comparatively lower percentage was found in outdoor 25% (dry wt.).

Microalgae contain significant fatty acids used for biodiesel production. In a previous study, Tokusoglu & Unal[36], found that among three different algae strain *Chlorella*, *Spirulina* and *Isochrisis*, the *Chlorella* showed highest content of PUFA 38.94% (DPA 3.11%, DHA 20.94%). Total n-3 fatty acid was also found higher in *Chlorella* 29.21% among the three cultivated strain. The EPA content was found 3.23% in *Chlorella* which is also higher than *Spirulina* microalgae.

Comparatively, high amount of oleic acid (C18:1) was obtained in current study in indoor culture system than outdoor. In the present experiment, the fatty acid composition of *C. vulgaris* grown in wastewater showed the presence of 14-18 carbon molecules. In addition, *C. vulgaris* cultured indoors contributed to higher unsaturated fatty acids under controlled conditions due to low temperature in comparison to outdoor cultures. Temperature has a significant role on the fatty acid composition of microalgae. *Chlorella ellipsoidea* also showed the similar results where the unsaturated fatty acids content was increased by 2-fold at lower temperature[37]. Unsaturated fatty acids increments at low temperature in *C. vulgaris* might be a response to maintain membrane fluidity and function, allowing the *C. vulgaris* to acclimatize to low temperature stress[38].

In the present experiment, continuous exposure to light indoors resulted in high content of unsaturated fatty acids whereas fluctuation of light outdoors led to a decrease. Different light intensities and wavelengths change the lipid metabolism in microalgae that affect the lipid content[39,40]. Light irradiation and temperature can only be controlled in closed system bioreactors. *C. vulgaris* biodiesel manufacturing is currently expensive, it might compensate the expense of integrating wastewater treatment facilities together with fuel production units. Microalgae consume municipal effluent nutrients, reduce pollution and aquatic eutrophication, and also generate biofuel and other value-added compounds at the same time. Therefore, the result of the present experiment implies that wastewater could be used as a medium to grow

C. vulgaris and may be done under controlled conditions of temperature and light in a VPBR to get quality lipid production aimed at biofuel feedstock.

Conclusion

Present findings revealed that wastewater can be used as a medium to cultivate *Chlorella vulgaris* under controlled conditions to obtain biomass and good quality lipids for biofuel production. The biomass cultivated from wastewater promises a wide range of applications. nutrients in the wastewater are utilized by algae otherwise, it would have been harmful to the environment and by growing algae in wastewater it reduces the medium costs and also saves the environment. The vertical photobioreactors (VPBR) would be used best under controlled operating conditions such as temperature for maximum productivity without contamination. More detailed studies should be done to utilize the wastewater for mass production of microalgae.

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Conflict of Interest

Authors declare no competing interests.

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