

CO₂ fixation and production of biodiesel by *Chlorella vulgaris* NIOCCV under mixotrophic cultivation

Deepti Jain^a, Supriya S Ghonse^{ab}, Tanmay Trivedi^{ab}, Genevieve L Fernandes^a, Larissa D Menezes^a, Samir R Damare^a, Mamatha S S^a, Sanjay Kumar^c, Vishal Gupta^{a*}

^a Biological Oceanography Division, CSIR-National Institute of Oceanography, Goa-403004, India

^b both the authors contributed equally

^c Department of Chemistry, University of Petroleum and Energy Studies, Dehradun-248007, India

* **Correspondence:** * vishalg@nio.org

Abstract

In this study, *Chlorella vulgaris* NIOCCV was cultivated in seafood processing industry wastewater with continuous supply of 5%, 10%, and 20% CO₂. The optimum CO₂ fixation efficiency (R_{CO_2}), biomass productivity, specific growth rate (SGR), and lipid content recorded were 0.43 mg L⁻¹ d⁻¹, 264.58±8.8 mg L⁻¹ d⁻¹, 0.46 d⁻¹, and 38±2.6% on dry weight basis, respectively at CO₂ supply of 10%. The fatty acid methyl ester-derived biodiesel properties determined at same condition were in compliance with national and international fuel standards. The higher calorific value (HHV) of the resultant biomass was 11.14, 16.41 and 12.83 MJ Kg⁻¹ for CO₂ enrichment of 5%, 10%, and 20%, respectively. The synergistic environmental benefit of nutrients removal from wastewater is shown as an additional advantage of microalgal cultivation. Thus, integration of algae-based CO₂ fixation with wastewater treatment and biodiesel production may realize microalgal CO₂ capture technology as environmentally sustainable and economically more attractive.

Keywords: Biodiesel, *Chlorella vulgaris*, CO₂ Fixation, Microalgae, Photobioreactor, Wastewater treatment.

Introduction

Mitigation for the accelerated release of greenhouse gases, in particular CO₂, is a major global concern (Duarte et al., 2017, Prabakar et al., 2018). A cleaner source of fuel with efficient CO₂ capture and storage technologies can potentially reduce CO₂ release (Prabakar et al., 2018). Microalgae have been established as a renewable feedstock for clean fuel or other energy conversion processes coupled with absorption of atmospheric CO₂. Most microalgal species investigated showed tolerance to CO₂ concentrations in the range of 5%-10% (v/v) (Zhou et al., 2017). Zhou et al. (2017) in their review highlighted a concern around the limited availability of microalgal strains tolerating higher CO₂ concentrations. Furthermore, the need for microalgal nutrients supplementation (Tan et al., 2018) and freshwater for cultivation (Zhou et al., 2017) challenges the economic sustainability of microalgal-based energy production. Thus, the synergy of CO₂ sequestration, wastewater utilization, and biofuel production may improve the efficiency of microalgae-based CO₂ capture and energy production (Prabakar et al., 2018).

Untreated wastewater from the seafood processing industry has high loads of organic nutrients inclusive of total organic carbon (TOC), nitrogen (ammoniacal, nitrite, and nitrate), and phosphorus. The untreated discharge from seafood processing industries can create local eutrophic conditions, rendering the coastal ecosystem highly vulnerable.

In this context, the objective of this study was to evaluate the CO₂ utilization efficiency of a euryhaline *Chlorella vulgaris* NIOCCV cultivated mixotrophically in seafood processing industry wastewater with the continuous supply of different CO₂ concentrations. The effects of CO₂ supply on biomass characteristics such as lipid, fatty acid composition, and higher calorific value were also studied.

1. Materials and methods

1.1. Algal species and its cultivation in photobioreactor (PBR) in the presence of CO₂

Chlorella vulgaris NIOCCV maintained as an axenic culture in the author's laboratory at CSIR-National Institute of Oceanography, Goa, India was employed in this study. Before cultivation with CO₂, the microalga was acclimatized to the aquaculture wastewater used. The characteristics of the wastewater are summarized in Table S1. The biomass productivity was optimized for wastewater enrichment (15% v/v), salinity (0.5-3.5%), light conditions (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$), and time period (4 days) (unpublished). The *C. vulgaris* NIOCCV was grown in a 13 L tubular PBR (Eppendorf, Germany) with a working volume of 4 L. The PBR culture was illuminated with a light intensity of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ using LED lights fixed inside the PBR, with a photoperiod of light: dark of 12: 12 h

and maintained at a temperature of 28 °C. A commercial grade CO₂ cylinder was used for purging the growth medium. The CO₂ gas mixed with air at concentrations of 5%, 10% and 20% (v/v) was purged continuously from the bottom of the PBR through silicon tubing during both the light and dark period. Air was supplied from a compressor with a sintered sparger. The desired concentrations of CO₂ in the mixture were maintained by rotameters. The gas flow was set to 0.5 vvm. Before entering the PBR, gases were passed through 0.22 μm filters.

1.2. Cell harvest and microalgal growth determination

The microalgal biomass was harvested every alternate day during the cultivation period of six days to monitor the growth. Microalgal cells were collected by centrifugation at 7,000 × g for 5 min. The cell pellet was washed with distilled water and freeze-dried. The hitherto separated liquid was collected for the determination of changes in nutrient levels after cultivation. To avoid any alteration in cell division from the change in diurnal rhythm, the cultures were sampled at approximately the same time of the day, 11:00 h.

The biomass productivity per day (mg L⁻¹ d⁻¹) and specific growth rate (SGR) were measured using the equations (Duarte et al., 2017): $(DW_T - DW_0)/(T_F - T_0)$ and $\ln(DW_T / DW_0)/(T_F - T_0)$ respectively, where DW_T and DW₀ are the dry biomass weight at final (T_T) and initial (T₀) time.

1.3. Elemental composition and higher calorific value (HHV) analysis

The elemental composition of freeze-dried biomass from each experimental condition was determined using a CHNS/Perkin Elmer 2400 Series Elemental Analyzer. The oxygen content of the biomass was calculated from the difference method on an ash-free basis. The H/C, O/C ratio and HHV values were calculated using the Van Krevelen diagram and according to Demirbas (1997) respectively.

1.4. CO₂ fixation rate

The CO₂ fixation rate (R_{CO_2} mg L⁻¹ d⁻¹) was determined following the equation given by Duarte et al., (2017): $P_X \times X_{cbm} \times (M_{CO_2}/M_C)$ where P_X is biomass productivity (mg L⁻¹ d⁻¹), X_{cbm} is carbon (%) determined from elemental analysis, M_{CO₂} is the molecular weight of carbon dioxide and M_C represents the molecular weight of carbon.

1.5. Total lipid estimation, fatty acid profiling, and biodiesel properties

The total lipid content was determined gravimetrically according to Bligh and Dyer (1959). Fatty acid components were determined by the transesterification of the lipid following the method described by Kumari et al. (2013) using a gas chromatography-mass spectrometer (GC-MS)

equipped with a DB-23 capillary column (60 m × 0.25 µm ID, 0.25 µm film thickness). Helium was used as a carrier gas at a flow rate of 1 mL min⁻¹. The mass selective detector was operated by electronic impact (70 eV). Injector and interface temperatures were 250 °C. The column oven temperature was programmed as follows: 60 °C for a 1 min hold and then raising to 150 °C at a rate of 20 °C min⁻¹ and then raising to 245 °C at a rate of 5 °C min⁻¹ and holding for 10 min at 245 °C. The contribution of each fatty acid was calculated from the total identified fatty acids and values are presented as percentage (%). The biodiesel properties were analyzed theoretically by incorporating the determined percentages of fatty acid methyl esters into the Biodiesel analyzer version 2.2.

1.6. Statistical analysis

All the experiments were performed in as three biological replicates. The results presented were the average values and expressed as mean values (±SD). The effects of different experimental conditions were analyzed using one-way analysis of variance (ANOVA) at the confidence level of 95% (p<0.05) with the statistical software package Minitab (USA).

3. Results and discussion

3.1. CO₂ utilization efficiency of *C. vulgaris* NIOCCV

Determination of CO₂ utilization efficiency (R_{CO_2}) is essential for defining a microalgal strain suitable for CO₂ capture, storage, and mitigation. In this study, microalgal tolerance towards moderate to very high concentrations of CO₂ in continuous supply was investigated. The CO₂ concentrations injected for the cultivation medium of *C. vulgaris* NIOCCV in this study were similar to the concentrations released (12% to 20%) from fossil fuel burning (Saravanan et al., 2018). A significant change in R_{CO_2} with the change in CO₂ supply was determined in this study. The highest R_{CO_2} (430 mg L⁻¹ d⁻¹) was recorded for the microalgae cultivated with a CO₂ concentration of 10%. The rate of R_{CO_2} declined to 149 mg L⁻¹ d⁻¹ and 236 mg L⁻¹ d⁻¹ at CO₂ concentrations of 5% and 20%, respectively. The determined R_{CO_2} in this study was found to be significantly higher than that of the same reported in other studies. For instance, the optimum R_{CO_2} was 247.98 mg L⁻¹ d⁻¹ for *C. minutissima* cultivated with a CO₂ supply of 10% and in medium supplemented with xylose, arabinose and nitrogen (Freitas et al., 2017b), 260 mg L⁻¹ d⁻¹ for *C. pyrenoidosa* with 10% CO₂ supply (Tang et al., 2011), 252 mg L⁻¹ d⁻¹ for *C. pyrenoidosa* with 5% CO₂ supply (Sydney et al., 2010), 255-300 mg L⁻¹ d⁻¹ for *C. fusca* with intermittent CO₂ supply (Duarte et al., 2017), 95.30 mg L⁻¹ d⁻¹ for *Chlorella* sp. with atmospheric level CO₂ supply (Ansari et al., 2017), 287 mg L⁻¹ d⁻¹ for *Chlorella* sp. with supply of CO₂ at a concentration of 5% (Yadav et al., 2015). The reported R_{CO_2}

greater than in this study were 510 mg L⁻¹ d⁻¹ for *Chlorella* sp. supplied with 20% CO₂ at 0.2 vvm flow rate (Zhao and Su, 2014), 674 mg L⁻¹ d⁻¹ for mutant *Chlorella* sp. MTF-7 with 25% CO₂ supply (Chiu et al., 2011). A comparison of R_{CO_2} for different *Chlorella* spp. from previous studies is shown in Table S2. The results clearly highlight the efficiency of *C. vulgaris* NIOCCV as a resource for industrial level CO₂ capture and mitigation.

3.2. Effect of different CO₂ concentrations on microalgal growth

In addition to the efficient CO₂ fixation, the effect of biomass productivity at different CO₂ supply was determined at different cultivation time. The time series results of the effect of CO₂ concentrations on biomass production is shown in Fig. 1a. The highest growth recorded was at the CO₂ concentration of 10%, with biomass productivity of 264.58±8.8 mg L⁻¹ d⁻¹, and specific growth rate of 0.46 d⁻¹. The same growth parameters for 20% and 5% CO₂ supply were 163.12±3.3 mg L⁻¹ d⁻¹, and 0.36 d⁻¹; 110.41±7.83 mg L⁻¹ d⁻¹, and 0.32 d⁻¹, respectively. The continuous supply of CO₂ at concentrations above 5% have been reported toxic for most microalgal species (Duarte et al., 2017, Tang et al., 2011). However, a CO₂ supply with a gradual increase in concentration and intermittent supply for a shorter duration of time have been shown to improve the biomass productivity at CO₂ concentrations >15% (Duarte et al., 2017, Kuo et al., 2017). Freitas et al. (2017a) reported highest biomass productivities of 140±10 mg L⁻¹ d⁻¹, 150±10 mg L⁻¹ d⁻¹ for *C. minutissima* cultivated under CO₂ concentrations of 10% and 20%, respectively with the supplementation of an organic carbon source. In comparison, the biomass productivities reported in this study were significantly higher at 10% CO₂ concentration and similar to 20% without nutrient supplementation. The biomass productivities in this study were higher than the same reported by Freitas et al. (2017b) as 100 mg L⁻¹ d⁻¹ during raceway cultivation using atmospheric CO₂ concentration. Kuo et al. (2016) reported biomass productivity as high as 641 mg L⁻¹ d⁻¹ for *Chlorella* sp. after 3 days of cultivation in aquaculture effluent with CO₂ supply at a concentration of 2%.

The growth kinetics of *C. vulgaris* NIOCCV with different CO₂ concentrations showed an initial short lag phase for one day (Fig. 1a). A similar trend of a short slower phase was reported for *C. fusca* LEB 111 with flue gas (Duarte et al., 2017). A significant decline in the pH from 9.11 to 6.6 within a few hours of CO₂ injection was recorded in this study and may be attributed as a major factor for the observed time gap. With the increase in time and microalgal growth, the pH of the medium increased and stabilized at pH 8.8. The most likely reason for this might be the increased carboxylase activity of RuBisCo and another downstream enzyme carbonic anhydrase, which transports hydroxyl ions outside the cell into medium (Freitas et al., 2017b, Kumar et al., 2014). In this study, the biomass grown in one cycle of cultivation was used as an inoculum for subsequent

cycles of cultivation. The microalgal cells get adapted to CO₂ concentrations, therefore, no prolonged lag phase was observed with increased CO₂ concentrations in subsequent cultivation cycles. High-density microalgal inoculum exposed and adapted to an adequate CO₂ concentration has been reported as an effective strategy to overcome the growth inhibition by higher CO₂ concentrations (Duarte et al., 2017, Chiu et al., 2008).

3.3. Total lipid content and fatty acid profile

The total lipid content of *C. vulgaris* NIOCCV was found to be in the range of 18±2.8% to 38±2.6% on the dry weight basis with different concentrations of CO₂ supplied. The trends revealed an increase in total lipid content with an increase in CO₂ concentration from 5% to 10% as 28.5±2.1% and 38±2.8, respectively, while same declined to 18±2.8% at the higher CO₂ concentration of 20%. The growth of *C. vulgaris* NIOCCV at 10% CO₂ supply showed lower concentrations of total nitrogen content. This nutrient-deprived condition may be a key factor for obtaining higher lipid yield at CO₂ supply of 10%. The total lipid content determined in this study was significantly higher than that reported as 25% for *C. pyrenoidosa* (Tang et al., 2011), 34% for *C. fusca* (Chiu et al., 2008), 11.92% for *C. vulgaris* (Yoo et al., 2010), 25.2% for a mutant strain of *Chlorella* (Chiu et al., 2011), 21.1% for *Chlorella* sp. (Kumar et al., 2014), 12.72% for *Chlorella* sp. (Yadav et al., 2015), and 23.7% for *Chlorella* sp. GD (Kuo et al., 2016). Thus, the higher lipid yield obtained in this study, without additional nutrient supplementation, can significantly reduce the biodiesel production cost.

Furthermore, the derived fatty acid methyl esters (FAME) profile defines the quality of biodiesel. The quality of FAME derived biodiesel is mainly dependent upon the fatty acids C16:0, C18:0, C18:1, and C18:2 (Yoo et al., 2010), which were found to be in the range of 55% to 60% of the total FAMEs determined in this study. The FAME contribution of C21:0 and C24:0 in this study were found to be less than 0.5%, thus, ignored from calculation. The FAME profile of *C. vulgaris* NIOCCV cultivated under different CO₂ concentrations is given in Table S3. The quality parameters of FAME-based biodiesel mainly include cetane number (CN), oxidative stability (OS), cold flow properties (CFPP), saponification value (SV), iodine value (IV), degree of unsaturation (DU) and kinematic viscosity (K_v) (Knothe, 2012). The CN values determined in this study for all the tested conditions were found to be higher than those of the specified limits of fuel standards. This is an indicator of ignition delay time and the quality of combustion, offering advantages such as easier start-up of the engine, less knocking and low nitrous oxide emission (Arias-Peña et al., 2013). The other important parameters, K_v and CFPP (for adequate fuel supply to injector at different operating temperatures), OS (stability), and IV (tendency of oil polymerization and prevention of

their deposition in the engine) values, determined in this study were also found to be in the specified range for all the tested conditions. The lower IV (82-84 g I₂ 100 g⁻¹) values determined indicates the lesser tendency to oil polymerization thereby preventing the deposition of oil in the engine (Knothe, 2012). The values for biodiesel quality parameters in the present study were compared with different national and international fuel standards and are given in Table S4. Thus, the growth of *C. vulgaris* NIOCCV under the continuous supply of higher CO₂ concentration and the production of quality biodiesel, represents its potential as an efficient fuel resource.

3.4. Higher heating value (HHV) of *C. vulgaris* NIOCCV

According to the Van Krevelen diagram, H/C: O/C of the *C. vulgaris* NIOCCV was in the order of 2.48 > 1.88 > 1.66 for CO₂ concentration of 10% > 20% > 5%, respectively (Fig. 1b). The determined variations in HHV follow the trend of H/C: O/C with values of 11.14, 16.41 and 12.83 MJ Kg⁻¹ for CO₂ concentration of 5%, 10%, and 20%, respectively (Fig. 1c). These values showed a strong positive correlation with different biomasses e.g. straw, jute, cellulose, olive husk, corncob (Demirbas 1997), and *Saccharina japonica* (Kim et al., 2012).

3.5. Nutrient removal efficiency

The analysis of wastewater quality after cultivation of microalgae revealed a significant decline in the concentrations of total organic carbon, nitrate+nitrite-nitrogen, ammoniacal-nitrogen, and phosphate-phosphorous as 23.46%, 79.68%, 82.42%, and 63.64%, respectively. Furthermore, an 84% reduction in turbidity of wastewater was recorded. The nutrient removal efficiency was found to increase with the supply of CO₂. The results of this study showed a higher rate of ammonia removal than a similar study from Kuo et al. (2016), and higher nitrogen and phosphate removal than in Fernández-Linares et al. (2017). In general, high ammonium content in the growth medium is considered toxic to microalgal growth (Tan et al., 2018). A significant reduction in ammonium content from the cultivation of *C. vulgaris* NIOCCV indicates its metabolic adaptation, preferring direct assimilation of ammoniacal nitrogen (Tan et al., 2018). Thus, the results of this study advocate for an effective, economically favorable and environmentally sustainable strategy of microalgal cultivation using seafood industry wastewater.

4. Conclusion

The tolerance of *C. vulgaris* NIOCCV to the continuous supply of higher CO₂ concentrations represents the most striking feature of this strain. The results of this study advocated for an effective, economically favorable and environmentally sustainable strategy of cultivation of *C. vulgaris* NIOCCV in seafood processing industry wastewater without use of additional nutrients. The

integrated approach of wastewater treatment and quality biodiesel production may support a significant reduction in the CO₂ abatement cost. These merits support the potential of *C. vulgaris* NIOCCV as an effective bioresource for the development of sustainable biorefinery.

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Conflict of Interest

All the authors mutually agree and state that there is no conflict of interest.

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Figure captions

Fig. 1: Biomass productivity of *Chlorella vulgaris* NIOCCV at different cultivation duration and CO₂ concentrations (5%, 10% and, 20%).

