

Improvement in biomass, lipid production and biodiesel properties of a euryhaline *Chlorella vulgaris* NIOCCV on mixotrophic cultivation in wastewater from a fish processing plant

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Abstract

In this study, the changes in biomass productivity, wastewater treatment, lipid production and biodiesel properties from mixotrophic cultivation of *Chlorella vulgaris* NIOCCV in untreated wastewater from a fish processing plant over F/2 medium was reported in this study. A significant 6-fold increase in biomass productivity ($257.87 \pm 1.27 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$) and 1.3-fold in lipid accumulation (48%) was recorded for *C. vulgaris* NIOCCV cultivated in wastewater enrichment of 15% (v/v) over F/2 medium. The biomass productivity was significantly higher in wastewater enrichment up to 50% (v/v) and salt concentration 5% (w/v) than in F/2 medium. The fatty acid methyl esters based theoretical calculation for biodiesel properties from the microalgal biomass cultivated in wastewater enriched medium were found superior to the same from the F/2 medium. The resultant biodiesel properties (CN value, oxidation stability, and kinematic viscosity) were in compliance to both the ASTM D6751 and EN 14214 fuel standards. The cultivation of *C. vulgaris* NIOCCV in wastewater resulted in significant decrease in nutrient levels is another advantage. Therefore, *C. vulgaris* NIOCCV can effectively be cultivated in untreated fish processing industry wastewater without addition of nutrients. Further, *C. vulgaris* NIOCCV can effectively be utilized as bioresource for biodiesel production.

Keywords: Biodiesel, Biomass productivity, *Chlorella*, Fish processing wastewater, Lipid, Wastewater treatment

1. Introduction

Over-dependency on non-renewable fossil fuels with the concomitant increase in greenhouse gases emission is the major 20th century scourge. Biodiesel has been considered as renewable and to a greater extent carbon-neutral fuel. Microalgae in this context have gained considerable global attention as sustainable bioresource to supplement the growing energy demand [1]. The microalgal biomass further has high bio-carbon sequestration potentials because of their higher photosynthesis efficiency, and growth rates than other terrestrial plants [2]. Despite concerted efforts, biofuel production from microalgae remained economically unfavorable mainly due to higher cultivation cost [3,4]. The culture medium compositions itself impose 30%-40% of the total cultivation cost [5]. The life cycle assessment of microalgal biodiesel production estimated input of about 3726 kg of water, 0.33 kg of nitrogen, 0.71 kg of phosphorous to produce 1 Kg of biodiesel [6]. This accounts to 32%–49 % of nitrogen and phosphorous world surplus values. The advancement in cultivation strategies as heterotrophic and mixotrophic cultivation showed promising potentials [7]. However, the cost of organic carbon substrate in such cultivation strategies is about 80% of the total cultivation medium cost [7]. Further, supplementation with fertilizer imposes a severe threat of eutrophication from runoff of fertilizer-enriched water to different aquatic streams [8]. Therefore, microalgae cultivation along with wastewater treatment may potentially reduce both the cultivation cost and environmental deteriorating impacts [9,10].

The untreated wastewater from seafood processing industry has high loads of organic nutrients inclusive of total organic carbon (TOC), ammonical nitrogen ($\text{NH}_3\text{-N}$), nitrate nitrogen ($\text{NO}_3\text{-N}$), and phosphorus ($\text{PO}_4\text{-P}$). Their outpaced growth in last one decade has been considered as one of the the principal source of coastal water pollution. The untreated discharge of wastewater from seafood processing industries can create hypereutrophication leading to eutrophication in the receiving water bodies. Therefore, utilization of such wastewater for microalgal cultivation may be interesting not only for the economic sustainability of cultivation step but also for environmental sustainability.

The feasibility study on microalgal cultivation in untreated fish processing industry wastewater was carried out using a strain, *C. vulgaris* NIOCCV isolated in the author's department. The *C. vulgaris* NIOCCV have been reported to grow and assimilate nutrients from wastewaters from different industries mainly the tannery and mixed industrial [11,12]. The effect of cultivation of *C. vulgaris* NIOCCV in untreated wastewater from fish processing industry in terms of biomass production and subsequent changes in lipid accumulation were investigated and compared with the cultivation in the F/2 medium. Further, the effects of different salt concentrations and light intensity regimes on

biomass productivity, lipid accumulation, and fatty acid composition were also evaluated. This study also determined the nutrient removal efficiency of the algal strain from the wastewater.

2. Materials and methods

2.1. Algal species and culture

The *Chlorella vulgaris* NIOCCV isolated from the mangrove regions of Goa, India and maintained as an axenic culture in the author's laboratory was employed in this study. The *C. vulgaris* was cultivated in 500 mL flask containing 200 mL modified F/2 medium with composition as NaNO₃ (75 g L⁻¹), NaH₂PO₄.H₂O (5 g L⁻¹), trace metal solution containing FeCl₃.6H₂O (3.15 g L⁻¹), Na₂EDTA.2H₂O (4.36 g L⁻¹), and 1 mL each of CuSO₄.5H₂O (9.8 g L⁻¹), Na₂MoO₄.2H₂O (6.3 g L⁻¹), ZnSO₄.7H₂O (22 g L⁻¹), CoCl₂.6H₂O (10 g L⁻¹), MnCl₂.4H₂O (180 g L⁻¹). The medium was enriched with vitamins as thiamine HCl (0.2 g L⁻¹), biotin (1 mg L⁻¹), and cyanocobalamin (1 mg L⁻¹). The cultures were incubated at temperature 28±2 °C under cool white fluorescent lights of intensity 100 μmol m⁻² s⁻¹ with a photoperiod of 12: 12 h light: dark cycle.

2.2. Wastewater characteristics

The untreated wastewater was collected from local fish processing industry. The nitrate, nitrite, phosphate, and ammonia concentration were determined using standard protocols (APHA –AWWA-WPCF, 1992). The characteristics of the raw wastewater are given in Supplementary Table 1.

2.3. Experimental design

Since the investigated microalgal species *C. vulgaris* NIOCCV was isolated from low salinity waters [11,12], initial culture experiments were performed in batch mode in 500 mL flask with 200 mL medium consisting of different strengths of wastewater (5%, 10%, 15%, 30%, 50%, 75% and 100% v/v) in artificial seawater (ASW). The ASW was prepared from 0.5% (w/v) artificial sea salt dissolved in distilled water. The wastewater was not enriched with additional nutrients. The *C. vulgaris* NIOCCV was also cultivated in the modified F/2 medium and treated as control culture. Both the culture medium were sterilized before microalgal inoculation. The inoculum added in the experimental and control condition was 10% (v/v) culture of exponentially growing alga. Both the experimental and control cultures were incubated at temperature 28±2 °C under cool white fluorescent lights of intensity 100 μmol m⁻² s⁻¹ with a photoperiod of 12 h: 12 h light: dark. The microalgal growth was monitored at different time intervals (4, 8, 12, 16 and 20 days).

2.4. Cell harvest and drying

Microalgal cells from both control and experimental conditions were collected by centrifugation at $7,000 \times g$ (10,000 rpm) for 5 minutes. The cell pellet thus collected from different experimental conditions were washed with distilled water and oven dried at $60\text{ }^{\circ}\text{C}$ until constant weight. To avoid any alteration in cell division from the change in diurnal rhythm, the cultures were sampled approximately at the same time of the day at 11:00 h.

2.5. Determination of microalgal growth

The microalgal growth was determined by measuring the optical density at a wavelength of 600 (OD₆₀₀) nm using spectrophotometer (UV-1800, Shimadzu, Japan). A linear regression between the OD₆₀₀ and the dried biomass weight was determined following the method described by Zhu et al. [8]. The regression equation determined was:

$$\text{Dry weight L}^{-1} = (\text{OD}_{600} - 0.1989) \div 0.0043, R^2 = 0.9826$$

The biomass productivity per day ($\text{mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$) was measured using the following equation:

$$\text{Productivity (mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}) = (\text{DW}_T - \text{DW}_0) / (\text{T}_T - \text{T}_0)$$

Where DW_T and DW₀ are the dry biomass weight at time T and 0 respectively.

The specific growth rate (SGR) was determined by the following equation [13,14]:

$$\text{Specific growth rate (day}^{-1}) = \ln(\text{N}_F / \text{N}_0) / (\text{T}_F - \text{T}_0)$$

Where N_F and N₀ are the initial and final dry biomass weights generated at final time T_F and initial time T₀ respectively.

2.6. Effect of salt concentration on microalgal growth

The effect of salt concentration on microalgal growth was determined after optimization of the wastewater concentration in the cultivation medium. The different salt concentrations investigated were 0.5%, 1.5%, 3.5%, 5% and 10% (w/v) which were achieved from the appropriate dissolution of artificial sea salt in distilled water. The culture inoculation, subsequent incubation, biomass productivity, and specific growth rates were determined as mentioned above.

2.7. Effect of light intensity on microalgal growth

The biomass productivity was then optimized for different light intensities. The microalgal culture with optimized wastewater and salt concentrations were incubated under the cool white light of

intensities of 50, 100, 200 and 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with 12 h: 12h light: dark photoperiod. The biomass productivity was determined after 8 days of incubation.

After optimization of the aforementioned cultivation conditions, the changes in water quality parameters were also determined in the residual water. The *C. vulgaris* NIOCCV was cultivated under optimized conditions for 6 days. The microalgal cells were harvested by centrifugation and water was collected for residual nutrient analysis.

2.8. Scale-up cultivation

After optimization of cultivation parameters such as wastewater enrichment, salt concentration, and light intensity, the cultivation was scaled up to 60 L working volume capacity. The scale-up experiment was carried out in a horizontal flat panel glass tank. The exponentially growing culture of microalgae was inoculated at 10% (v/v) density. The culture was continuously aerated. Biomass was harvested after 4 days, centrifuged, washed with distilled water and lyophilized. Biomass productivity and SGR was determined as mentioned above.

2.9. Lipid estimation

For lipid estimation, 100 mg dried algal biomass was extracted with chloroform: methanol (2: 1 v/v) [15]. The mixture was bead beaten at 6.5 m s^{-1} for 30 min (3 min cycle with 5 min rest) with intermittent incubation on an ice bath. The extract was separated from cell mass by centrifugation. The extract was then washed thrice with distilled water. The organic layer was collected and transferred to a pre-weighed rotary evaporator flask and dried completely. The total lipid content was determined gravimetrically as the difference of weight between the flask with dried organic layer and the empty flask.

2.10. Transesterification and fatty acid profiling

Transesterification of the fatty acid components of the total lipid was carried out following the method described by Kumari et al. [16]. Fatty acid methyl esters were analyzed on Gas Chromatography mass spectrometer (GCMS) system (QP-2010, Shimadzu, Japan) equipped with a DB-23 capillary column (60 m \times 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 temperature was 250 $^{\circ}\text{C}$. The column oven temperature programmed was as follows: 60 $^{\circ}\text{C}$ for 1 min hold and then raised to 150 $^{\circ}\text{C}$ at the rate of 20 $^{\circ}\text{C min}^{-1}$ and then raised to 245 $^{\circ}\text{C}$ at the rate of 5 $^{\circ}\text{C min}^{-1}$ and held for 10 min at 245 $^{\circ}\text{C}$. The contribution of each fatty acid

was calculated from the total identified fatty acids and values are presented as a percentage (%). Further, the effect of salinity and light on fatty acid profile were also determined.

2.11. Biodiesel properties

The biodiesel properties were analyzed theoretically by incorporating the fatty acid percentages into the Biodiesel analyzer version 2.2 [17].

2.12. Statistical analysis

All the experiments were performed in the biological replicate of three. The results presented were the average value and expressed as the mean value (\pm SD). The effect of different experimental conditions were analysed using one-way analysis of variance (ANOVA) at the confidence level of 95% ($p < 0.05$) with statistical software package Minitab (USA).

3. Results and discussion

3.1. Effect of wastewater enrichment on biomass productivity

The comparative assessment of microalgal biomass productivity in F/2 and wastewater enriched medium is shown in Fig. 1. The biomass productivity of *C. vulgaris* NIOCCV cultivated in F/2 medium for different cultivation durations followed the order, $42.82 \pm 0.36 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1} > 45.99 \pm 0.33 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1} > 40.68 \pm 0.77 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1} > 34.82 \pm 1.12 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ after 4, 8, 12 and 16 days respectively. The biomass productivity of *C. vulgaris* NIOCCV in wastewater was found to be dependent upon both the enrichment concentration and incubation duration (Fig. 1). The microalgal growth curve in wastewater exhibited exponential phase followed by a stationary and senescent phase for all the dilutions $< 50\%$ (w/v). A prolonged lag phase of 8 days was recorded only for the cultivation condition wherein wastewater enrichment was 50% (v/v). The lack of lag phase in other enrichment concentrations is attributed to the fact that the microalgal cells inoculated were already in exponential phase while in case of 50% (w/v) enrichment, the turbidity of the medium would have limited the light availability for photosynthesis. Similar, results of growth with exponential followed by stationary phase has been reported for different microalgal species cultivated in concentrated municipal wastewater [18]. The optimum biomass productivity of *C. vulgaris* NIOCCV as $257.87 \pm 1.27 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ in shortest culture duration of 4 days was recorded for wastewater enrichment concentration of 15% (v/v). The culture duration for attaining the highest biomass productivity was found to be directly proportional to the wastewater enrichment. The biomass productivity similar to optimum ($258.30 \pm 1.04 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ and $209.68 \pm 1.46 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$) were determined after incubation duration of 8 and 20 days for enrichment concentrations of 30% and 50% (v/v), respectively (Fig 1). The optimum biomass productivities for lower wastewater enrichment concentrations of 5% and

10% were $67.07 \pm 0.22 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$, $184.97 \pm 0.55 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$, respectively after 8 days of cultivation. Relatively lower biomass productivity recorded at these wastewater enrichment concentrations of 5% and 10% may be attributed to limited nutrient availability due to higher dilutions. Nevertheless, remarkably lower biomass productivity at wastewater enrichment of 75% and 100% (v/v) may reasonably be associated with higher turbidity levels retarding the photosynthetic rates as well as the toxicity of ammonium ions. These trends are in accordance to an earlier study on *Nannochloropsis gaditana* wherein concentrate concentration lower than 20% and higher than 80% resulted in lower biomass productivity due to the insufficient concentration of nutrients and toxicity of ammonium, respectively [19]. Therefore, appropriate dilution of wastewater would be required for attainment of the optimum biomass productivity in a shorter duration of time.

Comparative assessment of two cultivation conditions in this study revealed a 6-fold increase in biomass productivity in wastewater enriched medium than the F/2 medium. The availability of high nutrient concentrations, particularly, the organic carbon, nitrogen and phosphorous in wastewater enriched medium may result in higher microalgal growth over F/2 medium in this study. This increase in biomass productivity may be attributed to the mixotrophic mode of cultivation in wastewater enriched medium. Mixotrophy induced improvements in biomass growth has been reported by various authors [20,21]. Similar results of the increase in microalgal biomass productivity in different wastewaters compared to standard cultivation medium have widely been reported [8,22,23,24]. The cultivation of *C. vulgaris* NIOCCV in the presence of light and fish processing industry wastewater containing organic carbon (Supplementary Table 1) might have increased the rate of photoautotrophic and heterotrophic metabolisms in alga because of simultaneous proceedings of both photosynthesis and oxidative phosphorylation. This would have resulted in a significant increase in both the respiration rate and light compensation irradiance, and the same may be attributed to high biomass production in microalgae [25]. The presence of high organic carbon in the cultivation medium is well known to induce biomass productivity [26,27]. The biomass productivity recorded in this study was similar to *Chlorella* spp grown in livestock waste compost [8], anaerobically digested activated sludge [28], and grown in either autotrophic, heterotrophic or mixotrophic manner [21]. The biomass productivity recorded in this study was significantly higher than that of the same reported for *Chlorella* sp. ($56.25 - 58.35 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$) cultivated in secondary treated domestic wastewater and also supplemented with fertilizer [22]. Furthermore, the biomass productivity achieved in this study was even higher than the same reported for *Chlorella* sp. grown in the commercial medium under highly efficient photosynthetic light conditions [29,30], and with $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ as the carbon source [31].

The determined senescence growth phase after 8-10 days of cultivation duration for different wastewater enrichments may be attributed to various factors mainly to the significant decline in nutrients relative to microalgal metabolic needs (Supplementary Table 1). Also, the cell density-dependent shading effect retarding the photosynthesis rate may also be another factor. Interestingly, the biomass productivities recorded at higher wastewater enrichments, mimicking the light-limiting conditions, were still found superior to F/2 medium. This may be attributed to the mixotrophic cultivation mode utilizing organic carbon present in wastewater.

The recorded improvement in biomass productivity of *C. vulgaris* NIOCCV grown in wastewater enriched medium highlights the potential of fish processing industry wastewater as a suitable nutrient resource. The improved biomass productivity in the wastewater used in this study dispenses the need for nutrient and organic carbon supply to the cultivation medium. The results also indicate the possibility of developing an integrated model for wastewater treatment coupled with biomass generation.

3.2. Effect of salt concentrations on biomass productivity

The effect of different salt concentrations on biomass productivity of *C. vulgaris* NIOCCV under optimized wastewater enrichment is shown in Fig. 2. The results revealed optimum biomass productivity of $252.33 \pm 1.38 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ in salt concentration of 0.5% (w/v) after 4 days of culture duration. The biomass productivity declined to 211.54 ± 1.44 , 178.48 ± 0.9 , $100.60 \pm 0.87 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ for salt concentration of 1.5%, 3.5% and 5% (w/v) after 4 days, respectively. A significant decline in biomass productivity was determined at a salt concentration of 10% (w/v). Further, the time required for attaining the maximum biomass productivity was found to be dependent upon the salt concentration. The microalgal growth curve showed stationary phase after 4 days when cultivated under salt concentration of 0.5% and 1.5% (w/v). However, the same for the salt concentration of 3.5% and 5% (w/v) was attained after 8 days. Further increase in the culture duration, a decline in biomass productivity was recorded at all the salt concentrations investigated except for 10% (w/v). The determined biomass productivity after 4 days of culture duration in this study was substantially higher than the same reported by Church et al. [32] after 8 days at similar salt concentrations. The tolerance of *C. vulgaris* NIOCCV to different salt concentrations was found to be significantly higher than that of the same reported for different *Chlorella* sp. isolated from hypersaline Bardawil lagoon [33], and different low salinity waters [14]. Further, earlier studies have shown limited growth of microalgae in wastewater if salt concentration was above 2% [34]. This study, therefore, presented a unique metabolic feature of *C. vulgaris* NIOCCV to grow at wider salinity regimes confirming the euryhaline physiology of this strain.

The decrease in biomass productivity with an increase in NaCl concentration have been reported in different studies on *C. vulgaris* [14,32]. The salinity induced energy expense diversification for the accumulation of osmoprotectants, regulation of ion exchange and maintaining the turgor pressure has been reported essential for microalgal sustainability in the medium [32]. The determined decline in growth of *C. vulgaris* NIOCCV with an increase in salinity may best be explained by the same fact that the energy expense for maintaining the osmotic balance under the hypersaline condition is higher than the energy required for cell division and growth.

Earlier studies investigating the effect of salinity on biomass growth for *Chlorella* spp were mainly restricted to the use of single salt either of Na or K [14,32,35]. On the contrary, this study highlights another potential of *C. vulgaris* NIOCCV to tolerate the synergistic effect of multiple metal salts present in ASW. Based on aforementioned results, the optimum biomass productivity can be achieved at a salt concentration of 0.5% or 1.5% (w/v) ASW after 4 days of cultivation in wastewater enrichment of 15% (v/v). Though wastewater could form excellent nutrient source for microalgal cultivation, higher salinity of wastewaters may limits the microalgal growth. Further, the recurring cultivation cost because of the nutrients supplementation and larger quantities of freshwater has been argued as economically unviable for developing microalgal-based industry [6,7]. Thus, dispensing the need for fresh water along with nutrient supplementation will be of direct support in cultivation cost reduction. The results of this study substantiate the potential of *C. vulgaris* NIOCCV as an efficient bioresource for cultivation in saline wastewaters dispensing the need of fresh water.

3.3. Effect of light intensity on biomass productivity

The biomass productivity of *C. vulgaris* NIOCCV cultivated under different light intensities (50, 100, 200 and 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at optimum wastewater enrichment, and salt concentration is shown in Fig. 3. The biomass productivity recorded at light intensities of 50, 100, 200 and 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively were 163.39 ± 1.59 , 246.27 ± 3.7 , 103.43 ± 0.97 and $38.39 \pm 0.77 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$. This confirms that the optimum biomass productivity can be realized at a light intensity of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The results of this study were concurrent with most of the previous reports wherein high light intensity above 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ imparts a negative effect to microalgal growth [36]. Similar results were also reported by Cheirsilp and Torpee [20] for *Chlorella* sp. showing highest biomass productivity at a light intensity of 8000 lux ($\sim 112 \mu\text{mol m}^{-2} \text{s}^{-1}$). A two-fold and six-fold decrease in biomass productivity at higher light intensities of 200 and 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively was determined (Fig. 3). A sudden exposure to higher light intensity may pose a severe stress to microalgae leading to the significant retardation in growth. Further, the variability in light saturation level of photosystem may also result change in biomass productivity for *C. vulgaris* NIOCCV. The

light intensity below and/or above the saturation level retard the microalgal growth because of the photo-limited condition and photo-inhibition, respectively [37,38]. The decline in biomass productivity at higher light intensity may be attributed to the inhibition of photosystem, in particular, the PS II causing subsequent inactivation of oxygen-evolving systems, electron carriers and the associated D1/D2 proteins [37]. For optimal biomass productivity, the photosynthetic unit turnover time needs to be determined which eventually depends upon both the light intensity and the photoperiod [38]. Intermittent exposure of higher light intensities to *C. vulgaris* NIOCCV may improve its adaptability and enhance the biomass productivity. Nevertheless, microalgal cultivation under controlled light conditions in photobioreactor may be possible as optimized in this study. However, utilization of solar radiations under open cultivation may significantly reduce the operational costs. Thus, the impact of open cultivation on biomass productivity of *C. vulgaris* NIOCCV needs further investigation.

3.4. Scal- up experiment

After optimization of different cultivation conditions, the microalgal biomass production was scaled up to 60 L in horizontal flat panel glass tanks (Supplementary Figure 1). The recorded biomass productivity was $287.52 \pm 3.20 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$. The biomass productivity obtained from scale-up cultivation was slightly higher than that of the same determined from small-scale experiments. This increase in biomass productivity in scale-up cultivation may be attributed to continuous aeration avoiding both the microalgal cell settlement at the bottom of the tank and cell aggregates formation. The assessment of SGR is an important parameter while scaling up the cultivation in order to avoid the discrepancies associated with initial stocking density. Further, SGR determines a proper hydraulic retention time (HRT) in continuous culture and therefore is an important parameter to assess the microalgae growth. The SGR for scale-up experiment was 1.5 d^{-1} which was similar to the SGR from small-scale experiment (1.43 d^{-1}). No significant change in SGR from scale up cultivation indicates the possibilities for outdoor cultivation of *C. vulgaris* NIOCCV in seafood processing industry wastewater.

3.5. Wastewater treatment

A comparative assessment of the quality of untreated fish processing plant wastewater prior and post microalgal cultivation was made through measurement of key water quality parameters. Fish processing industry wastewater was characterized to have essential nutrients for microalgal growth (Supplementary Table 1). The nutrient analysis after 6 days of cultivation revealed a significant decline in the nutrient load. The percentage reduction in concentrations of total organic carbon

(23.46%), total N (72.73%), total ammoniacal nitrogen (79.87%), and total phosphorous (53.24%) was noticeable after *C. vulgaris* NIOCCV cultivation (Supplementary Table 1). The wastewater nutrient profile revealed that ammonium form ($\text{NH}_4^+\text{-N}$) accounts as major nitrogen content (>90%). A significant decrease in the concentration of $\text{NH}_4^+\text{-N}$ than total nitrogen (nitrate+nitrite) indicates the preferential utilization of *C. vulgaris* NIOCCV. Similar source preference have been reported by Deng et al. [39,40] for *C. vulgaris* grown in different wastewaters. The wastewater with higher ammonium content were not supportive in microalgal growth because of its toxicity [41]. The preferential utilization of ammonia as N-content resulted in higher remains of nitrate in the wastewater after microalgal cultivation [27,42]. A significant decline in both the ammonia and nitrate and nitrite concentrations in this study, therefore, represent the efficient metabolic ability of the strain to sequentially utilize these components. Further, assimilation of ammonium-N is considered to be an energy efficient metabolic adaptation [41], thus, represents another unique feature of *C. vulgaris* NIOCCV. This physiological adaptation of *C. vulgaris* NIOCCV might have resulted an improvement in biomass productivity in wastewater enriched medium over the F/2 medium.

In addition to N-utilization, the presence of organic carbon (TOC) in cultivation medium affect the microalgal growth through mixotrophic mode. A significant decline in TOC by 23% after cultivation confirms the mixotrophic growth of *C. vulgaris* NIOCCV. This reduction in TOC is independent of interferences by associated biotic factors as cultivation was carried out under axenic conditions. This mixotrophic mode could further be correlated with higher growth in fish processing wastewater than in the F/2 medium. The nutrient removal efficiency (%) for nitrate and phosphate by *C. vulgaris* NIOCCV in this study was relatively higher than those of the same reported by Fernández-Linares et al. [22], and Deng et al. [40]. Further, the highest nutrient removal efficiency of *C. sorokoniana* reported was 48% and 40% for nitrogen and phosphorus under heterotrophic cultivation than autotrophic and mixotrophic cultivation [21]. The significant reduction in nutrient loads from the wastewater by *C. vulgaris* NIOCCV may, therefore, be attributed to its mixotrophic cultivation.

The results of the present study showed significant nutrient removal efficiencies of *C. vulgaris* NIOCCV from untreated fish processing industry wastewater. Since cultivation medium in this study was enriched with 15% (v/v) of wastewater which could be discharged after microalgae cultivation. However, at higher enrichment concentrations decline in biomass productivity indicated sub-optimal nutrient removal efficiency because of lower metabolism. Thus, nutrient removal efficiency may be enhanced after either primary and/or secondary treatment of wastewater. Abiotic and biotic factors have been reported to influence the removal efficiencies of key wastewater

characteristics [18,39,40]. Further, the effect of various other factors mainly the biotic factors on nutrient removal efficiency of microalgae needs to be investigated in order to develop open pond cultivation.

3.6. Total lipid content and FAME components

A significant increase in the lipid production was recorded for the alga cultivated in wastewater enriched medium ($48\pm 2.75\%$) over the same in F/2 medium ($36\pm 1.86\%$). Wastewater-induced increase in lipid accumulation have been reported for different *Chlorella* spp. [8,22,24,32]. In contrast, Ji et al., [43] reported a decline in lipid accumulation for *Chlorella* sp. grown in wastewater over the same in BG 11 medium. In general, the lipid accumulation is directly related to the biomass productivity. The recorded increase in biomass productivity of *C. vulgaris* NIOCCV after cultivation in wastewater enriched medium might have resulted in higher lipid accumulation. The lipid content recorded in this study was found higher than those reported for different *Chlorella* spp. cultivated under different culture conditions. For example, the maximum lipid content reported was 25.4% after cultivation in monosodium glutamate supplemented wastewater [43], 16% in water supplemented with different salt types and concentrations [32], 25.7% in treated wastewater supplemented with fertilizer [22], 30.3% in nutrient rich piggery wastewater [9]. Further, the recorded lipid content in this study was significantly higher than the same reported after heterotrophic cultivation of different *Chlorella* spp using sucrose [7] and starch digested wastewater [41] as carbon source. Zhu et al. [8] reported the lipid content as high as 44.30%, similar to this study, for *Chlorella* sp. grown in livestock waste. The cultivation medium composition with availability of organic carbon induced higher microalgal growth, hence, assimilate more nitrogen resulting in higher C/N ratio [44]. This condition mimics the state of N-deprivation and results in an increase in lipid accumulation. Thus, high organic carbon content of the fish processing industry wastewater potentially increases the biomass productivity which in turn result in N-depletion in shorter cultivation period, thus, pushes the carbon assimilation towards higher lipid biosynthesis.

The lipid accumulation in microalgal cells has been known to vary with various biotic and abiotic factors. This study, therefore, determined the effect of different salt concentrations and light intensity regimes on lipid accumulation and subsequent change in fatty acid profile. The lipid production determined in this study was in the range of 30-48% at various treatments of salt concentration and light intensity (Table 1). The lipid accumulation was found to decline with increase in both the salt concentration (above 1.5%) and light intensity. There was no significant difference observed in lipid yield for salt concentrations of 0.5% and 1.5% (w/v). The lipid yields determined in this study for different salt concentrations follow the trends of biomass productivity. The decrease in lipid yields

with an increase in salt concentration has also been reported for *Botryococcus* spp. [45]. The significant decline in biomass productivity in the range of 1.4 to 2.5 fold for *C. vulgaris* NIOCCV with an increase in salt concentration relative to optimum biomass productivity indicates higher remains of N-content in the cultivation medium under former condition than later. The availability of N along with higher salts might be able to maintain the osmotic equilibrium without extra energy expense and lipid biosynthesis [32] therefore no increase in lipid was determined. Further, salinity induced oxidative damage dependent lipid accumulation has also been reported [14,32,35] which might not be true in this case during mixotrophic cultivation as both the photoautotrophic and heterotrophic metabolism continues during light and dark period. The presence of organic carbon in the cultivation medium was also reported to alter the microalgal metabolism [32]. This study, therefore, hypothesised that the continuation of carbon assimilation probably at a slower rate at higher salinities could be able to meet the energy expenses for maintaining the osmotic regulation without storing the energy in the form of lipid.

The effect of light intensity on lipid production is summarized in Table 1. The optimum lipid production ($48 \pm 2.75\%$) was recorded at light intensity of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$, with the salt concentration of 0.5% (w/v ASW) and, wastewater enrichment of 15% (v/v). Decline in lipid production as $36 \pm 1.95\%$, $32 \pm 2.45\%$, and $30 \pm 2.75\%$, but with no significant differences among each other, were determined for light intensities of 100, 200 and $300 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively. The optimum light intensity for achieving highest biomass productivity was found to be $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, but the same resulted in 25% decline in lipid production. This attributes to the fact that the light intensities for optimum biomass production and/or lipid accumulation are different. Similar trends of decline in lipid production with the increase of light intensity despite the increase in biomass productivity were also reported for marine *Chlorella* sp. and *Nannochloropsis* sp [20,36]. The determined increase in lipid yield with lower light intensity may be attributed to the photoautotrophic metabolic inactivation resulting in an increase in storage energy reserve mainly the lipids. Also, the light starved condition ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$), restrict the energy expense for cell division and biomass growth while diverting the same for the synthesis of storage materials such as carbohydrate and lipid. This fact is further justified by the recorded decline in biomass productivity at a light intensity of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$.

The fatty acid composition of the alga grown in modified F/2 medium and wastewater enriched medium under optimized cultivation conditions are given in Table 1. The dominant fatty acid methyl ester (FAMES) in both the conditions were palmitic acid (C16:0), Oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) along with myristic acid (C14:0), arachidic acid (C20:0), palmitoleic acid (C16:1 n9), hexadecadienoic acid (C16:2), hexadecatrienoic acid (C16:3). Along

with these, longer chain FAME, behenic acid (C22:0) and tetracosanoic acid (C24:0) were also detected in this study. But, the contributions of these FAMEs were found to be less than 0.5%, thus, ignored from the calculations. The FAMEs profile determined in this study is in accordance with those reported in various earlier studies on *Chlorella* [1,4,32,33]. The *Chlorella* species has mostly been reported to contain shorter chain fatty acids of C16-C18 carbon length [33]. This carbon chain length of fatty acid is known to be suitable for biodiesel production. The fatty acids, C16:0 and C18:1, showed the increase of 2.2 and 4.1 fold, respectively for *C. vulgaris* NIOCCV grown in wastewater enriched medium compared to F/2 medium. However, C18:3 showed a significant decline of 2.5 fold in wastewater grown alga. The overall fatty acid profile results revealed 3.4 and 1.8 fold increase in monounsaturated fatty acids (MUFAs), and saturated fatty acids (SFAs) with a concomitant decrease of 1.2 fold in poly unsaturated fatty acids (PUFAs) for the biomass grown in wastewater over F/2 medium. The determined increase in SFAs is mainly attributed to the significant 2.24 fold increase in the content of C16:0. Fatty acid profile tends to change by the cultivation condition, the physiological growth of the alga, and other associated biotic and abiotic factors [18]. The increase in SFAs for microalgae grown in wastewater over standard medium have been reported for different *Chlorella* species [32,46,47]. This increase in SFAs for *C. vulgaris* NIOCCV grown in wastewater than in F/2 medium can be attributed to N-deprived state in the wastewater as determined after 6 days of cultivation period. The specific increase in C16:0, contributing largely to the resultant increase in SFAs, may be attributed to the high ammonium concentration in the culture medium [48]. Further, a relation of the N-deprived induced SFAs along with the proportional increase in MUFAs and a concomitant decrease in PUFAs have been recorded in earlier studies [46,49]. Similar trends of increase in SFAs and MUFAs with a decrease in PUFAs are determined in this study for *C. vulgaris* NIOCCV cultivation in wastewater over the same in the F/2 medium. This trend of increase in SFAs in the lipid from wastewater grown biomass is a clear indication for gaining quality biodiesel [50].

Sub-optimal growth conditions are known to shift the microalgal FAME profile and their percent composition. Upon increasing the salinity above 0.5% (w/v) for wastewater enriched medium, FAME profile revealed a decrease in SFAs with a concomitant increase in PUFAs. However, no significant change in MUFAs was recorded (Table 1). The determined increase in PUFA is mainly because of the increase in C18:2 with a relative decrease in C16:0. The similar trend were also recorded for increasing light intensity above $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ along with the decline in MUFA as well. Ghosh et al. [24] also reported similar fatty acid profiles for *C. vulgaris* grown in spent medium of thermophilic biohydrogen production. Achazi et al. [51] also reported the increased ratio of unsaturation to saturation in *D. salina* upon cultivation at higher salinities. The increase in PUFA

with the increase in salinity may be attributed to the adjustment of membrane fluidity to maintain the ion transport regulating the osmotic balance. The increase in PUFA over SFA were found significantly higher for *C. vulgaris* NIOCCV grown with increasing light intensities. However, this increase in PUFAs was accounted for increase chain length and unsaturation unlike one fatty acid (C18:2) in the salinity induced increase in PUFA. Light-induced increase in PUFAs are indicative of microalgal regulatory mechanism increasing its light-harvesting ability to protect itself from photooxidative damage and also to maintain the membrane fluidity [52,53].

3.7. Biodiesel properties

The biodiesel properties of fatty acid methyl esters (FAME) derived from the lipid extracted from *C. vulgaris* NIOCCV grown in both wastewater enriched and the F/2 medium is given in Table 2. The parameters mainly considered for determining the biodiesel quality are cetane number (CN), saponification value (SV), iodine value (IV), the degree of unsaturation (DU), oxidative stability (OS), and kinematic viscosity (Kv) [54]. The determined biodiesel properties of *C. vulgaris* grown in optimized wastewater enriched medium were CN (61.4), SV (162.94 mg g⁻¹), IV (121.31 g I₂ 100 g⁻¹), DU (104.81 wt%), OS (6.27 h) and Kv (2.68 mm² s⁻¹). Whereas the same for F/2 medium grown alga were CN (56.94), (141.61 mg g⁻¹), IV 123.99 g I₂ 100 g⁻¹, DU 103.12 wt%, OS 4.96 h, and Kv (2.23 mm² s⁻¹). The improvement in biodiesel properties for FAME derived from the lipid extracted from *C. vulgaris* NIOCCV grown in wastewater enriched medium was evident over those of same from F/2 medium (Table 2).

The minimum value of CN specified by EN 14214 and IS 15607 is 51 while the same specified by ASTM D6751-08 is 47. The CN values determined in this study was found higher than the specified limits of fuel standards at all the investigated cultivation conditions (Table 2). The CN is an indicator of ignition delay time and quality of combustion. This offers several advantages such as easier start-up of engine, less knocking and low nitrous oxide emission [55]. The CN value is dependent upon the content of SFA and MUFA. The significant increase in SFAs and decline in PUFAs content in *C. vulgaris* NIOCCV after cultivation in wastewater enriched medium at different salinity and light regimes compared to the same in F/2 medium resulted in superior CN values [56]. The other important parameter, Kv, for which the specified range is 1.9-6.0 mm² s⁻¹. The Kv values determined in the present study were in the specified range for all the tested conditions (Table 2). The Kv ensures adequate fuel supply reaches injector at different operating temperatures. The predicted value of Kv in compliance to biodiesel standards is mainly due to the balanced contents of SFAs and MUFAs. Higher SFAs may cause oil polymerization at lower temperatures. Thus, increase in SFAs alone should not be construed an efficient indicator for quality biodiesel. This study showed a

balance between SFAs and MUFAs at all the tested conditions in such a way that predicts the Kv values in the specified range of different fuel standards. This parameter is mainly important for defining cold flow properties (CFPP) and thus determines the suitability of biodiesel operations in low temperatures. The CFPP values in the present study were in the range of -2.51 to -8.48 °C and are similar to the same defined for globally accepted biodiesel of *Botryococcus braunii* [57]. Another important parameter deciding the quality of biodiesel, OS, mainly depends upon DU of FAME. The recorded balanced decline in the DU for the FAME derived from *C. vulgaris* NIOCCV biomass cultivated in wastewater enriched medium over F/2 medium and at different salt concentrations and light intensities, except the light intensity of 200 $\mu\text{ m}^{-2}\text{ s}^{-1}$, attributes to the improvement in OS (Table 2). Furthermore, a decline in DU supports the decline in IV values in FAMES derived from *C. vulgaris* NIOCCV grown in wastewater enriched medium. The low IV values are indicative of the least tendency of oil polymerization thereby preventing their deposition in engine [54]. The increase in salt concentrations and light intensities, though, decrease the lipid accumulation but resulted in an increase in PUFA for *C. vulgaris* NIOCCV grown in wastewater. This increase in PUFAs was found to be significantly lower than the same in F/2 medium. Though the change in FAME composition was observed at different salt concentrations and light regimes, the biodiesel quality was not predicted to get affected significantly and remained in the prescribed range of different biofuel standards. The investigated microalgal strain *C. vulgaris* NIOCCV thus represents unique features of growth in different salt concentration and light intensities and producing FAME profile suitable for biodiesel production. Nevertheless, the change in biomass productivity needs to be considered while designing and developing the suitable cultivation of this alga. In summary, cultivation of *C. vulgaris* NIOCCV in wastewater enriched medium improves the FAMES quality for biodiesel production with quality parameters in accordance to different fuel standards.

3.8. Strategic advantages

The economic sustainability towards microalgal cultivation using fish processing industry wastewater is appeared to be the major advantage in this study because no additional nutrient supply is needed. Heterotrophic and mixotrophic cultivation of microalgae using different carbon sources, preferably glucose, has shown as a promising approach for attaining the economic sustainability [7,41,58]. However, the supplementation of glucose alone overheads to 80% of the total cultivation medium cost [7]. It has been suggested that the other carbon sources such as digested starch and cellulose may reduce 60-70% cost of growth medium [7,41,58]. The direct utilization of seafood processing industry wastewater as cultivation medium, without any supplementation of carbon or any other nutrient, in this study may 100% offset the cultivation medium cost. In an estimate,

production of 1 Kg of biodiesel, require about 3726 kg of water, 0.33 kg of nitrogen, 0.71 kg of phosphorous [6]. These facts indicate that the cultivation of bioresource is the major economic constrain towards the feasibility and life cycle assessment of biodiesel production, not the transesterification process [58]. This study showed the utilization of wastewater from the fish processing industry for improved biomass productivity and lipid accumulation in *C. vulgaris* NIOCCV as a cost effective approach. Yang et al. [6] while assessing the life cycle for biodiesel production from microalgae indicated that the use of sea/ wastewater might decrease water requirement up to 90% and eliminate the need of all the nutrients except phosphate. Thus, reduction in water and nutrient use in this study are indicative of economic sustainability with another strategic advantage towards waste management.

4. Conclusion

The increase in biomass productivity and lipid accumulation for *C. vulgaris* NIOCCV after cultivation in wastewater from fish processing plant than in F/2 medium was recorded in this study. These results, therefore, confirms the suitability of wastewater from fish processing industry as cultivation medium without the need of additional nutrients. The efficiency of wastewater as cultivation medium was further confirmed by scale-up cultivation with no change in biomass productivity than the small-scale experiments. No requirement of additional nutrients indicates fish processing industry wastewater as cost-effective feedstock for microalgal cultivation. Further, the determined euryhaline physiology of *C. vulgaris* NIOCCV together with its nutrient removal efficiency are indicative to its growth and treat different salinity wastewaters which otherwise can be difficult . Further, the biodiesel properties derived from the relative change in FAME composition were improved in wastewater grown alga than in F/2 medium and were in compliance to international fuel standards. Thus *C. vulgaris* NIOCCV can be an effective bioresource for realizing the concept of biorefinery in an economically feasible manner utilizing fish processing industry wastewater. Since, sterilization of wastewater was performed in this study to avoid any effect of contaminants which could be an additional cost to biodiesel production despite use of wastewater as a cost effective cultivation medium. Therefore, outdoor cultivation of *C. vulgaris* NIOCCV in untreated wastewater without any treatment should be established in order to realize the economic sustainability of this bioresource for biodiesel production.

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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

Figure 1: Effect of different concentrations of untreated wastewater on biomass productivity of *Chlorella vulgaris* NIOCCV after different cultivation periods.

Figure 2: Effect of different salt concentrations on biomass productivity of *Chlorella vulgaris* NIOCCV after different cultivation periods.

Figure 3: Effect of different light intensities on biomass productivity of *Chlorella vulgaris* after 8 days of cultivation period.

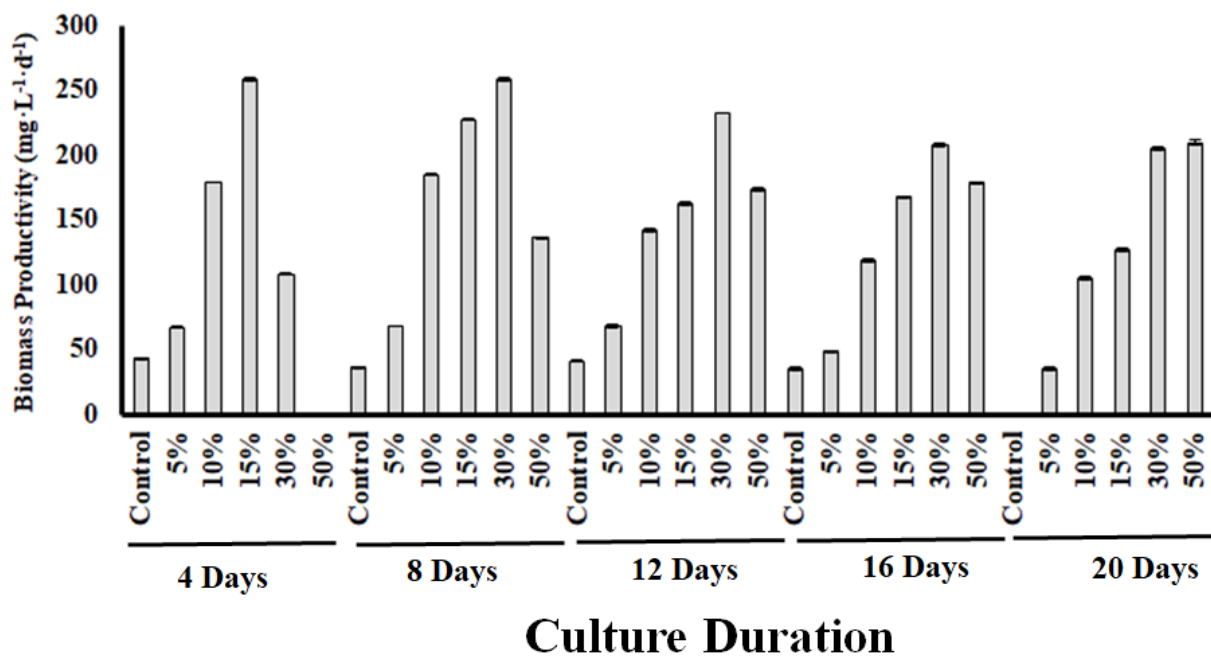


Figure 1

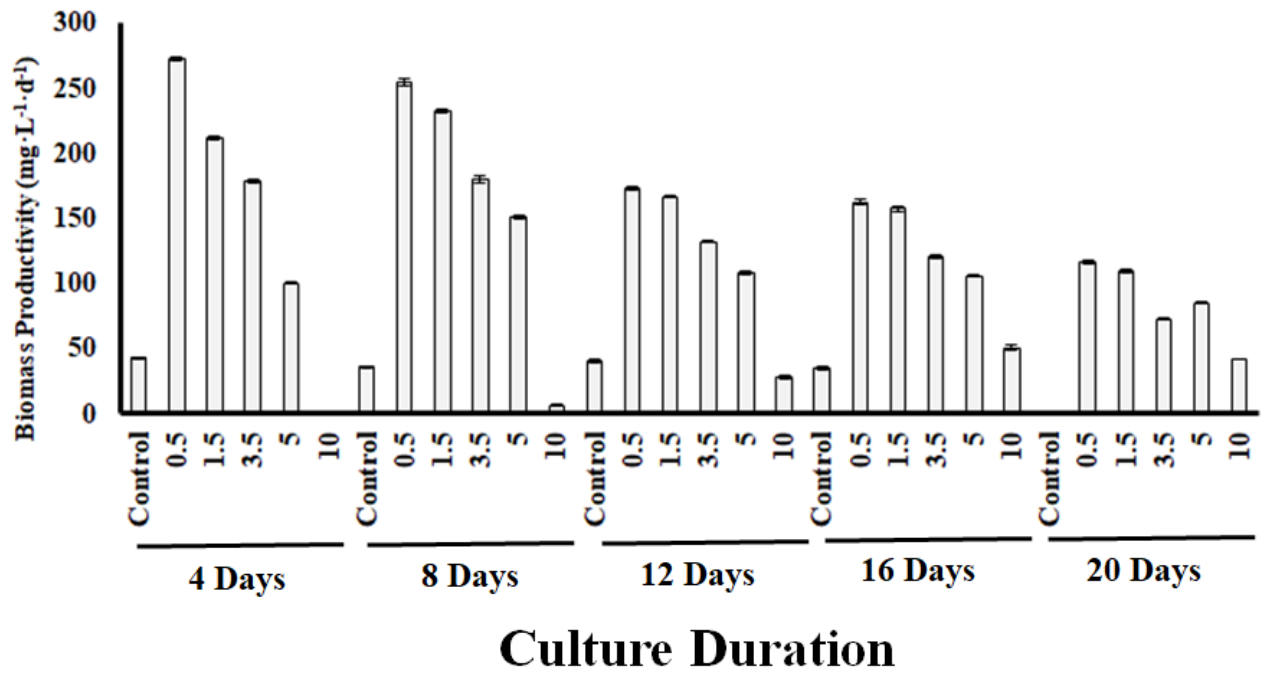


Figure 2

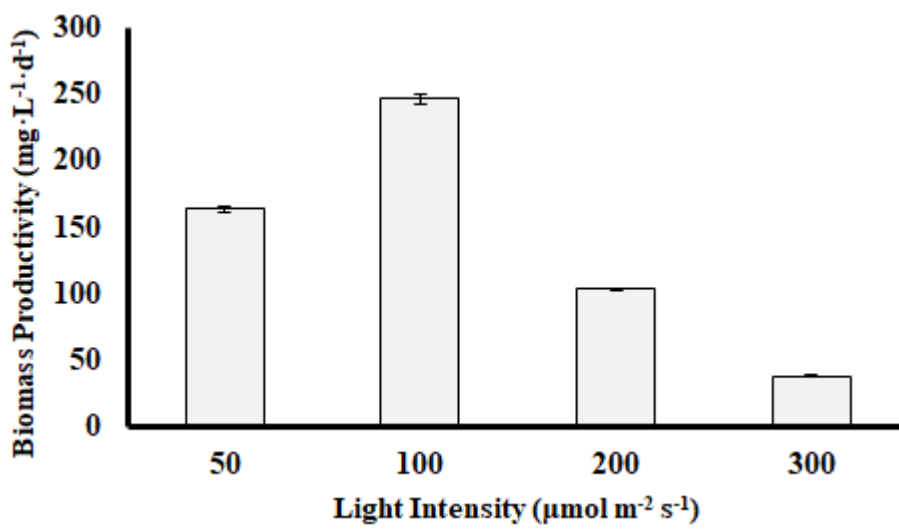


Figure 3

Table 1

The total lipid content (% on dry weight basis) and fatty acid composition (relative %) of *Chlorella vulgaris* NIOCCV cultivated in control F/2 and wastewater enriched medium at different salt concentrations and light intensities.

	Salinity (%)					Light Intensity ($\mu\text{mol m}^{-2} \text{s}^{-1}$)			
	F/2 medium	0.5	1.5	3	5	50	100	200	300
Lipid	36±1.86	48±2.75	46±3.25	38±2.95	36±3.45	48±2.75	36±1.95	32±2.45	30±2.75
Fatty acids (%)									
C14:0	0.34±0.085	1.21±0.25	ND	ND	ND	1.21±0.12	ND	0.72±0.18	0.55±0.2
C16:0	13.1±0.55	29.44±2.45	24.53±1.25	24.08±2.05	23.85±2.23	29.44±3.45	25.46±3.52	23.48±2.35	19.302±1.3
C18:0	2.525±0.12	3.0±0.4	1.93±0.5	1.52±0.25	1.45±0.21	3.0±0.47	1.48±0.84	0.3953±0.025	1.8±0.74
C20:0	4.85±0.15	2.4±0.25	2.15±0.4	1.95±0.12	2.01±0.42	2.4±0.13	1.87±0.72	0.72±0.11	2.3±0.16
SFA	20.815	36.05	28.61	27.55	27.31	36.05	28.81	25.33±2.36	24.04
C16:1 n-9	1.15±0.24	2.51±0.58	1.81±0.085	3.45±0.33	3.56±0.65	2.15±0.17	1.0±0.35	1.52±0.24	2.73±0.89
C18:1	2.47±0.25	10.14±0.85	9.21±0.36	8.15±0.34	6.5±0.48	10.14±1.26	4.29±0.39	2.56±0.25	3.16±0.11
MUFA	3.62	12.65	11.02	11.6	10.06	12.65	5.29	4.09±0.88	5.90±0.44
C16:2	9.36±1.12	8.5±1.25	11.13±0.92	13.24±0.87	15.25±1.11	8.5±0.78	9.74±1.24	9.6±1.23	7.09±1.01
C16:3	13.98±1.56	8.85±0.75	8.99±0.54	8.47±0.91	7.85±0.89	8.85±1.01	9.53±1.65	13.8±1.65	11.3±1.4
C18:2	16.78±1.85	18.86±1.85	25.25±2.35	24.44±3.41	26.75±2.35	18.86±1.26	24.89±2.85	19.7±1.53	21.2±3.62
C18:3	32.97±3.25	13.17±1.23	13.72±1.33	12.89±1.23	12.85±2.12	13.17±0.85	19.09±2.35	23.42±3.42	26.11±3.2
PUFA	73.09	49.38	59.09	59.04	62.7	49.38	63.25	67.56918	67.85592

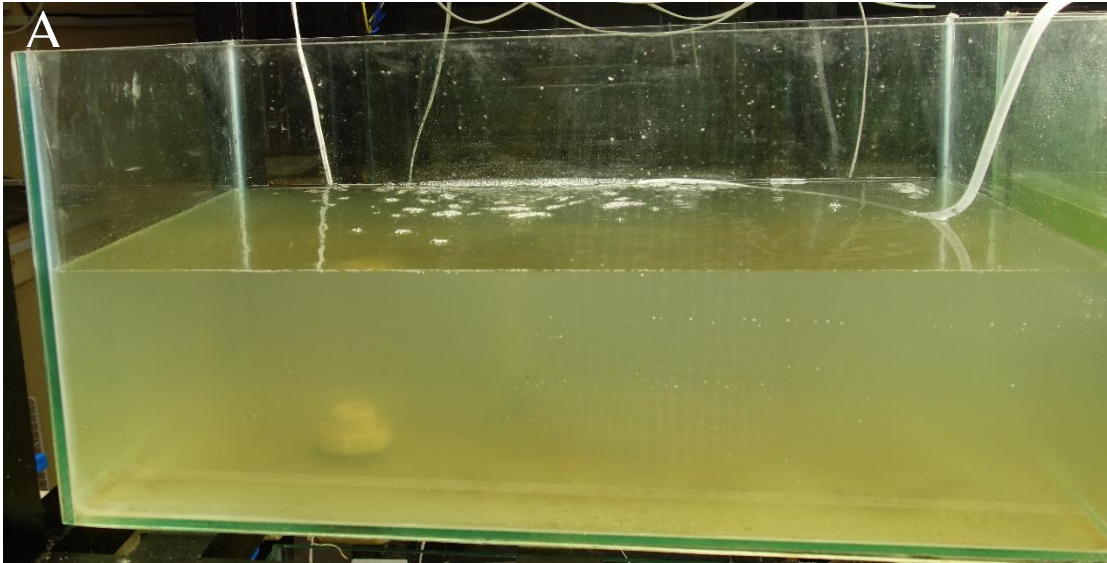
C14:0-myristic acid; C16:0-palmitic acid; C18:0-stearic acid; C20:0-eicosanoic acid; C16:1 n-9-palmitoleic acid; C18:1-oleic acid; C16:2- 7,10-hexadecadienoic acid; C16:3- 7,10,13-hexadecatrienoic acid; C18:2-Linoleic acid; C18:3-linolenic acid; SFA-saturated fatty acids; MUFA-monounsaturated fatty acids; PUFAs-polyunsaturated fatty acids

Table 2

Biodiesel properties of fatty acid methyl esters derived from the biomass of *Chlorella vulgaris*NIOCCV cultivated in F/2 medium and wastewater enriched medium at different salinities and light intensities.

Parameters	F/2 grown	Salinity (%)				Light intensity ($\mu\text{mol m}^{-2} \text{s}^{-1}$)			
		0.5	1.5	3.5	5	50	100	200	300
DU	103.12	76.71	88.96	86.26	89.26	76.71	93.25	92.28	104.81
SV (mg g⁻¹)	141.611	162.943	157.628	154.06	154.923	162.943	157.318	150.644	158.624
IV (max)	123.996	81.83	93.375	90.32	93.021	81.83	102.186	106.244	121.31
CN	56.943	61.385	59.916	61.406	60.601	61.385	58.002	58.626	53.414
CFPP (°C max)	-8.434	-2.515	-5.739	-6.524	-6.706	-2.515	-6.153	-8.488	-7.476
CP (°C)	1.899	10.493	7.911	7.674	7.553	10.493	8.4	7.358	5.16
PP (°C)	-4.76	4.57	1.767	1.51	1.378	4.57	2.298	1.167	-1.22
APE	101.97	74.2	87.15	82.81	85.7	74.2	92.25	90.76	102.08
BAPE	82.72	45.2	52.69	50.22	52.45	45.2	63.07	68.46	77.72
OS (h min)	4.961	6.272	5.617	5.75	5.569	6.272	5.272	5.265	4.975
HHV	27.192	30.772	30.047	29.282	29.447	30.772	29.926	28.542	30.238
Viscosity mm² s⁻¹	2.235	2.687	2.598	2.529	2.535	2.687	2.538	2.382	2.492
Density (g cm⁻³)	0.615	0.688	0.673	0.656	0.66	0.688	0.643	0.643	0.682

Du: Degree of unsaturation; SV: Saponification value; IV: Iodine value; CN: Cetane number; CFPP: Cold Filter Plugging Point; CP: Cloud point; PP: Pour point; APE: Allylic Position Equivalent; BAPE: Bis-Allylic Position Equivalent; OS: Oxidation Stability; HHV: Higher Heating Value



Supplementary Figure 1: Scale up heterotrophic cultivation of *Chlorella vulgaris* in wastewater enriched medium (A) 0 day and (B) after 4 days of culture duration.

Supplementary Table 1: Water quality parameters before and after microalgal cultivation for 4 days.

Sample Identity	Total Organic Carbon (μM)	NO^2+NO^3 (μM)	PHOSPHATE (μM)	AMMONIA (μM)	SILICATE (μM)	Turbidity (NTU)
Untreated Effluent	272.7	331	1894	41278	ND	75.8
Diluted Effluent (15% v/v)	130.6	224	894	6167	ND	33.7
Microalgal cultivation	99.5	61.1	418	1241	ND	5.4
% Reduction	23.46	72.73	53.24	79.87	ND	84