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# Nutrient Removal Efficiencies of *Chlorella vulgaris* from Urban Wastewater for Reduced Eutrophication

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## Abstract

Urban wastewater contains both organic and inorganic nutrients and discharge of untreated water increases nitrogen and phosphorous content in water bodies leading to eutrophication problem. Physical and chemical treatment of urban waste water produces large quantities of waste sludge associated with secondary pollution. Microalgae can assimilate nutrients especially nitrogen and phosphorous from wastewater for their growth and produce valuable biomass and lipid. This study was performed to determine the growth of *Chlorella vulgaris* in urban wastewater (UWW) and Bold's basal medium (BBM) thereby identifying cost effective growth medium for microalga cultivation. In addition, nutrient removal abilities of *C. vulgaris* from various dilutions of urban waste water were explored at 10 days cultivation period. Specific growth rate, biomass and lipid content were higher in microalgae grown in urban waste water than BBM. The highest lipid productivity of 14.31 mg·$L^{-1}$·$day^{-1}$ was achieved in the culture grown in UWW medium which exceeded the BBM at 1.15 fold. The amount of nutrient removal tended to increase with higher dilutions of UWW. Removal rates of upto 87.9% and 98.4% were recorded for total nitrogen and total phosphorous by *C. vulgaris.* The results emphasized that urban waste water as a cost effective growth medium for higher biomass and lipid production accompanied with the nutrient removal efficiency of microalgae to reduce eutrophication.

## Keywords

*Chlorella vulgaris*, Wastewater, Nutrient Removal, Biomass, Lipid, Eutrophication

## 1. Introduction

Increasing industrialization and population explosion result in contamination of wastewater with available water resources. Discharge of urban waste water into water bodies introduces high levels of nitrogen and phosphorous which leads to eutrophication. Nutrient removal is an important aspect of wastewater treatment as eutrophication causes oxygen depletion in aquatic environments, increase in undesired vegetation, loss of aquatic flora and fauna. Phosphorous removal from waste water includes chemical treatment followed by physical treatment; however this method produces large quantities of waste sludge. Algae induced chemical processes are used to eliminate nitrogen and phosphorous [1] [2]. Microalgae require nitrogen and phosphorous as major nutrients besides other micronutrients and produce valuable biomass. Various synthetic media are used for growing microalgae but are associated with costs which exceed the value of final products under large scale cultivation [3]. For economic viability of algal biomass and lipid production, inputs to algal cultivation must be inexpensive. Waste water from urban areas is a cheap source of nutrients and could be used as growth medium for sustainable microalgal cultivation thereby reducing the need of resources like freshwater and fertilizers [4] [5]. Microalgae have adaptability and ability to utilize inorganic nutrients from wastewater. Cultivation of microalgae in wastewater reduces greater levels of fresh water usage and reduces the nitrogen inputs [6]. In addition to biomass and lipid productivities, microalgae are reported to have potential environmental benefits through removing nutrients from waste water [7] [8] [9] [10]. Integrating nutrient removal from wastewater with microalgae cultivation is one effective method to reduce the cost of large scale biofuel production [11] [12] and it would not cause secondary pollution [13].

Microalagae are used to treat various wastewaters in recent times [14] [15] [16] [17] [18]. Algae can assimilate nitrogen and phosphorous from wastewater for their growth at less operational costs [19] and also play an important role in elimination of contaminants from waste water. Utilization of wastewater for microalgal cultivation minimizes the nutrients supply and fresh water usage along with removal of organic and inorganic pollutants. In this study, comparative analysis of growth medium on the growth of microalgae was done using urban waste water and Bold's basal medium. Parallel analysis of nutrient removal capacity by the microalgae isolated from wastewater was also investigated.

## 2. Experimental Methods

### 2.1. Inoculum Collection, Identification and Scale-Up

Urban waste water (UWW) was collected from Bangalore and was characterized in terms of pH, electrical conductivity, temperature, biological oxygen demand, chemical oxygen demand, total organic carbon, total nitrogen and total phosphorous using APHA method [20]. The waste water was poured into a closed 250 ml bottles and exposed in sunlight for 3 weeks. The upper layer of the water was inoculated in agar plates enriched with BG11 medium containing ampicillin

(200 μg·ml$^{-1}$) to control the bacterial growth. Agar plating technique was used to isolate the microalgae and the plates were incubated at 25˚C ± 2˚C under cool white fluorescent light (40 μmol photons m$^{-2}$·s$^{-1}$; 15 h light/9 h dark) until algal growth was detected. The isolates were purified by streak plating and individual colonies were diluted in distilled water. Species of single cells were obtained using capillary pipette under microscope followed by inoculation into fresh media. After appropriate growth, cells were observed to confirm the single culture and the capillary method was repeated until pure culture was obtained. Identification of the algal isolates was carried out by following standard protocols [21] [22] and the database http://web.biosci.utexas.edu/utex/default.

Species of *Chlorella*, *Scenedesmus*, *Pandorina* and *Oscillatoria* were the major isolates identified and cultivated using urban waste water as scale up medium for a period of 6 days at 24˚C ± 2˚C under illumination (12 h light/12 h dark). *Chlorella vulgaris* was selected and used for further studies as this strain could achieve relatively high biomass concentration among other isolates when cultivated in urban wastewater.

## 2.2. Influence of Growth Medium on Microalgae

Both Urban Waste Water (UWW) and Bold's Basal Medium (BBM) were used to determine the influence of growth medium on microalgal growth. Various parameters such as specific growth rate, biomass concentration, pigment concentration, total proteins and carbohydrates content and lipid productivity were determined. All experiments were performed in triplicates and the average values were statistically analyzed.

## 2.3. Specific Growth Rate and Biomass Productivity

Specific growth rate (μ·d$^{-1}$) of the microalgae was calculated according to the following formula [23].

$$\mu = \frac{\ln\left(N_t / N_0\right)}{T_t - T_0}$$

where, $N_t$ and $N_0$ are the dry cell weight concentration (g·L$^{-1}$) at the end ($T_t$) and start ($T_0$) of log phase respectively.

Biomass (g·L$^{-1}$) of *C. vulgaris* grown in waste waster medium and BBM was determined by measuring the optical density of samples at 600 nm ($OD_{600}$) using UV-Vis spectrophotometer. Biomass concentration was then calculated by multiplying $OD_{600}$ values with 0.6, a predetermined conversion factor obtained by plotting $OD_{600}$ versus dry cell weight (DCW). DCW was determined gravimetrically by centrifuging the algal cells (3000 × g, 10 min) and drying.

$$\text{Biomass concentration} = \text{OD}_{680} \times 0.6 \quad (1)$$

The biomass productivity (g·L$^{-1}$·d$^{-1}$) was calculated according to Equation (2),

$$\text{Biomass productivity} = \left(B_t - B_0\right)/d \quad (2)$$

where $B_t$ was the final biomass concentration, $B_0$ is the initial biomass concentration and $d$ is the cultivation time.

## 2.4. Pigment Assay

Cellular pigments were determined using a spectrophotometric method after extraction with 80% acetone [24] [25]. Briefly, microalgal pellet was resuspended in phosphate buffer (0.1 M, pH 7) and sonicated for 10 mins. This was followed by addition of 80% acetone, vortexing and incubation in the dark for 15 mins. The suspension was centrifuged and the supernatant was measured in a spectrophotometer.

Subsequently, the amount of pigments was calculated using the following formulae

$$C_a = 12.21A_{663} - 2.81A_{646} \quad (1)$$

$$C_b = 20.13A_{646} - 5.03A_{663} \quad (2)$$

$$C_t = 1000A_{470} - 3.27C_a - 104C_b/198 \quad (3)$$

where $C_a$ is the chlorophyll a, $C_b$ is the chlorophyll b, and $C_t$ is the total carotenoids ($\mu g \cdot mL^{-1}$).

## 2.5. Protein Assay

The extraction of proteins from microalgae was performed using alkali method. Aliquots of algal sample were centrifuged and 0.5 N NaOH was added to the pellet followed by extraction at 80˚C for 10 mins. The mixture was centrifuged and protein content of the supernatant was estimated using Bovine Serum Albumin (BSA) as standard [26].

## 2.6. Carbohydrate Assay

Cellular carbohydrates were estimated using the anthrone method [27] after hot alkaline extraction [28]. Briefly, microalgal pellets were resuspended in distilled water and then heated in 40% (w/v) KOH at 90˚C for 1 h. After cooling down, ice cold ethanol was added and stored at −20˚C overnight followed by centrifugation. The pellet was resuspended in distilled water and then reacted with anthrone reagent. D-glucose was used as standard and the colour development was read at 578 nm in a spectrophotometer.

## 2.7. Total Lipid Estimation

Lipid extraction from dried algal cells were carried out by chloroform:methanol extraction method [29]. Dried algal cells added with distilled water were ultrasonicated and mixed with chloroform: methanol (2:1). The mixture was left for 30 mins in a water bath (30˚C) and filtered through a Whatman No.1 filter paper. The filtrate was transferred to another screw cap tube containing NaCl solution (0.9%) and the purified chloroform layer was evaporated to a constant weight in a fuming hood under vacuum at 60˚C. The total lipid content of dry weight was calculated using the following Equation (3).

$$\text{Lipid content}(\%) = (m_2 - m_0)/m_1 \times 100\% \quad (3)$$

where $m_1$ is the weight of the dried algal cells, $m_0$ is the weight of the empty new screw cap tube and $m_2$ is the weight of the new screw cap tube with the dried li-

pids.

Lipid productivity ($g \cdot L^{-1} \cdot d^{-1}$) was determined using the following Equation (4).

$$\text{Lipid productivity} = \text{Biomass productivity} \times \text{Lipid content} \quad (4)$$

## 2.8. Nutrient Removal Efficiency

Urban waste water was diluted with three initial dilutions (10%, 20% and 30%) using distilled water to determine the nutrient removal efficiency of *Chlorella* in batch experiments for a period of 10 days. The contents of total phosphorous (P) and total nitrogen (N) before and after the cultivation period were determined [20] by centrifuging a 10 ml liquid culture sample at 8000 rpm for 10 min.

## 3. Results and Discussion

The urban wastewater was characterized for its physicochemical properties and the pH, electrical conductivity and temperature were 6.6 ± 0.13, 2.314 ($mS \cdot cm^{-1}$) and 30˚C. The COD, BOD and TOC of the wastewater were 697 ± 14.15 $mg \cdot L^{-1}$, 974 ± 22.04 $mg \cdot L^{-1}$ and 257 ± 27.98 $mg \cdot L^{-1}$. The total nitrogen and phosphorous were 192.27 ± 0.16 $mg \cdot L^{-1}$ and 39.97 ± 6.7 $mg \cdot L^{-1}$.

Microalgae isolated from natural environments could adapt better and produce good results [30]. It is reasonable to hypothesize that species that naturally develop in wastewater should perform better than most others in commercial scale cultivation on wastewaters. Hence urban waste water was chosen as growth medium in this study. The results showed that among the algal strains isolated from wastewater environment, *Chlorella* ranked among the top ones in terms of maximal growth rate and biomass productivity. The results from Table 1 indicate that the growth and biochemical composition of the *C. vulgaris* varied with the type of the medium used. After 10 days of cultivation, Urban Waste Water (UWW) medium obtained the maximum biomass concentration of 1.13 $g \cdot L^{-1}$ and biomass productivity of 0.19 $g \cdot L^{-1}$, which was higher than that of Bold's

**Table 1.** Effect of growth medium on biomass production, chlorophyll biosynthesis and lipid accumulation of *Chlorella vulgaris*.

| Parameters | Bold's basal medium | Urban waste water |
|---|---|---|
| Specific growth rate ($\mu\ d^{-1}$) | 1.12 ± 0.03 | 1.06 ± 0.02 |
| Total chlorophyll ($mg \cdot L^{-1}$) | 11.36 ± 0.92 | 9.37 ± 0.54 |
| Total carotenoids ($mg \cdot L^{-1}$) | 4.7 ± 0.10 | 4.8 ± 0.97 |
| Total protein ($mg \cdot L^{-1}$) | 58.7 ± 0.56 | 63.7 ± 1.76 |
| Total carbohydrates ($mg \cdot L^{-1}$) | 196 ± 1.64 | 187 ± 3.61 |
| Biomass ($g \cdot L^{-1}$) | 1.09 ± 0.04 | 1.13 ± 0.02 |
| Biomass productivity ($g \cdot L^{-1} \cdot d^{-1}$) | 0.17 ± 0.03 | 0.19 ± 0.01 |
| Lipid content (% DW) | 7.52 ± 0.31 | 8.31 ± 0.35 |
| Lipid productivity ($mg \cdot L^{-1} \cdot d^{-1}$) | 12.38 ± 1.01 | 14.31 ± 0.38 |

Basal Medium (1.09 $g \cdot L^{-1}$ and 0.17 $g \cdot L^{-1}$). This scenario was different with specific growth rate *i.e.* 1.12 $\mu \cdot day^{-1}$ was observed in BBM where as it was 1.06 $\mu \cdot day^{-1}$ in UWW. *C. vulgaris* has produced a biomass of 2.7 $g \cdot L^{-1}$ in urban waste water [14] and in this study 1.13 $g \cdot L^{-1}$ was obtained. Higher biomass was obtained in wastewater medium than BG11 medium [31] and similar results were produced in the present work. The chlorophyll biosynthesis increased in the Bold's Basal Medium (BBM). The maximum chlorophyll content (11.36 $mg \cdot L^{-1}$) was obtained in BBM whereas it was 9.37 $mg \cdot L^{-1}$ in UWW medium. Total carotenoids and protein were higher in cells grown in UWW medium and recorded 4.7 and 58.7 $mg \cdot L^{-1}$ respectively. However, total carbohydrates were increased in BBM (196 $mg \cdot L^{-1}$). The biomass productivity by *C. vulgaris* in the urban wastewater is 0.19 $g \cdot L^{-1} \cdot d^{-1}$and similar values were reported earlier [32] using municipal wastewater. The other species of *Chlorella* grown under municipal wastewater resulted in biomass productivity of 0.055 - 0.60 $g \cdot L^{-1} \cdot d^{-1}$ [30] [33] [34] [35].

For lipid contents, no statistically significant differences between the UWW and BBM were observed. However, the volumetric lipid productivities of the cultures grown in UWW were enhanced notably due to their high cell density. The highest lipid productivity of 14.31 $mg \cdot L^{-1} \cdot day^{-1}$ was achieved in the culture grown in UWW medium which exceeded the BBM at 1.15 fold. In a study using artificial wastewater medium by Feng *et al*., [36], 42% lipid content and 147 $mg \cdot L^{-1} \cdot d^{-1}$ of lipid productivity were obtained from *C. vulgaris* whereas 8.31% and 14.31 $mg \cdot L^{-1} \cdot d^{-1}$ were produced in this study using urban waste water as growth medium.

Urban waste water contains both organic and inorganic phosphate which can be readily assimilated by algae. Phosphorus (P) is an important macronutrient for microlalgal growth and microalgae assimilate phosphorus as inorganic orthophosphate for production of phospholipids, ATP and nucleic acids [37]. Phosphorous uptake by microalgae is affected by algal physiology as well as P concentrations and its chemical forms. Waste water contains nitrogenous matter in the form of ammonium ions, ammonia and organic nitrogen along with other oxidized form of nitrogen. The presence of various forms of nitrogen and phosphorous in wastewater leads to eutrophication [38]. *C. vulgaris* has a high potential to reduce nutrients in secondary waste water treatment plants effluents [39] [40] [41] while simultaneously producing algal biomass with sufficient lipids content and an acceptable fatty acids profile for use as a biodiesel feedstock [42]. Nutrient uptake is realized via the active transport of ions across the cell membrane. The efficiency of nutrient removal by microalgae is related to its ability to reduce the nitrogen and phosphorous levels in the wastewater. Nutrient removal efficiency depends on the concentrations of nitrogen and phosphorous present in water [43]. Urban wastewater often contained high concentrations of nitrogen and phosphorus, and dilution with fresh water is needed before use to increase the transmission of light in while cultivation. Urban waste water was diluted as the initial concentrations of nitrogen and phosphorous of the waste water was higher than the recommended concentration of microalgae hence various

**Table 2.** Nutrient removal performance of *Chlorella vulgaris* in different UWW concentrations at 10 days cultivation period.

| | Initial concentration (mg/l) | | Final concentration (mg/l) | |
|---|---|---|---|---|
| | Total N | Total P | Total N | Total P |
| 10% | 21.37 ± 0.25 | 1.84 ± 0.22 | 3.61 ± 1.31 | 0.12 ± 0.04 |
| 20% | 37.46 ± 1.62 | 4.12 ± 0.14 | 7.25 ± 0.47 | 0.09 ± 0.06 |
| 30% | 59.31 ± 2.74 | 9.61 ± 1.23 | 7.14 ± 2.54 | 0.15 ± 0.03 |

dilutions of wastewater was prepared using distilled water. Both nitrogen and phosphorous removal amounts in different initial concentrations of UWW for 10 days cultivation are represented in Table 2.

The total nitrogen removal efficiencies recorded were 83.1% ± 0.14%, 78.3% ± 0.06%, 87.9% ± 0.11% in 10%, 20% and 30% dilutions. Up to 84.11% nitrogen uptake from urban waste water was reported by *Chlorella* [44] and an 87.9% nitrogen removal was found in this study. The amount of removed total phosphorous tended to increase with higher initial dilutions of UWW. Approximately, 93.4, 97.8 and 98.4% were removed from the growth medium containing 10%, 20% and 30% of UWW respectively. Hongyang *et al*., [45] reported the removal of nitrogen (77.8%) and phosphorous (88.8%) from soybean processing wastewater using *Chlorella pyrenoidosa*. *C. vulgaris* grown in tertiary municipal wastewater with initial concentration of 8.7 and 1.71 $mg \cdot L^{-1}$ of total nitrogen and phosphorous has completely removed the nutrients at the end of 4 days cultivation [46]. In other studies, removal rate of total nitrogen was observed between 61.1% - 91.8% by *Chlorella* sp. grown in municipal wastewater [47] [48] [49] whereas 87.9% removal was recorded in this study. It was observed that 98.4% of total phosphorous was removed which is higher than removal rate reported in previous studies [46] [50] [51].

## 4. Conclusion

The results clearly emphasized that urban waste water is a cost effective growth medium for higher biomass and lipid production in microalgae. The nutrient removal efficiency by *C. vulgaris* isolated from wastewater proved that microalgae are potential in removing nitrogen and phosphorous from highly concentrated nutrient rich urban wastewater. This study highlights the more effective alternative ecofriendly approach for wastewater treatment using microalgae to eliminate eutrophication.

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