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Research article

Cultivation of *Chlorella vulgaris* in aquaculture wastewater as alternative nutrient source and better treatment process

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A B S T R A C T

Together with the fastest growing of aquaculture production, aquaculture industry also produces a large amount of wastewater that results in not only eutrophication of waterbody but also increases the aquaculture cost and threatening global sustainability. To overcome those barriers in the aquaculture industry, application of microalgae for wastewater remediation, biomass production to produce value added products, and water quality control have been devoted as microalgae assimilate nutrients in a eutrophic water body and a good way for wastewater remediation. The objective of this study was to determine the growth and nutritional profile of *Chlorella vulgaris* culture in different percentage of aquaculture wastewater (ww) in combination with Conway media. For the experiment, *C. vulgaris* was cultivated in different wastewater concentration such as 25% ww + 75% Conway media (T2); 50% ww + 50% Conway media (T3); 75% ww + 25% Conway media (T4); 100% ww (T5) and 100% Conway media, considered as control (T1). The results of this study showed that the growth of *C. vulgaris,* in terms of cell density and optical density were maximum at day 5 in T3 (50% wastewater) and was significantly ($p > 0.05$) higher ($4.33x10^6$cellsml^{-1}) (0.400 Abs) compared to the other treatments. Highest amount of protein content was found in Conway media (T1) (42.69 % dry weight) whereas lipid content was highest in 100 % ww (17 % dry weight) and then in 75 % ww treatment (16 % dry weight) and carbohydrate content was maximum in T3 treatment (24 % dry weight) which were significantly higher ($p > 0.05$) then the other treatments. The study indicates that media enriched with aquaculture wastewater can enhance the cell growth and nutrient values of *C. vulgaris* which is a cost effective and environment friendly approach to minimize aquaculture waste loading in water body.

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1. INTRODUCTION

Waste water from aquaculture is usually high in waste nutrients such as nitrogen and phosphorus, total suspended solids, volatile suspended solids, biochemical demand for oxygen and chemical demand for oxygen (Mook et al., 2012). A build-up of solid waste inside the culture system should be avoided because when it decomposes, it can cause oxygen depletion and ammonia toxicity that contributes to eutrophication and nitrification of ecosystems receiving effluent (Xin et al., 2010). Moreover, the conventional treatment processes suffer from some important disadvantages: (a) variable efficiency depending upon the nutrient to be removed; (b) costly to operate; (c) the chemical processes often lead to secondary pollution; and (d) loss of valuable potential nutrients (N, P). To overcome the aforementioned problems, numerous laboratory and pilot studies have been constructed to control wastewater pollution and improve survival efficiency of aquatic animals (Shi et al., 2007; Zhu et al., 2008). In general, Biological treatments are better process compared to the chemical and physical processes, which is too costly and may lead to secondary pollution. With this aspect, over 50 years ago, Oswald and Gotaas (1957) has proposed the elegant concept of wastewater bio-treatment with algae to remove nutrients as well heavy metals in an impressive level. Wastewater provides all the essential nutrients required for the growth of algae (Khatoon et al., 2016). Waste water from municipal, agricultural and industrial activities is a source of nutrients for the cultivation of microalgae, which could significantly reduce the operating costs of algal production systems (Khatoon et al., 2018). Eutrophication of nearby water bodies could be caused by the mass volume of aquaculture wastewater containing high concentrations of nitrogen and phosphorus produced during the year and when released untreated. On the other hand, many methods and technology are used to extract these nutrients, and this method of treatment is extremely expensive (Yuan et al., 2011) because the wastewater requires a lot of energy and maintenance purposes. The use of waste water will reduce the need for nitrogen and phosphorus sources by around 55% (Yang et al., 2011) in microalgae culture media. In some early studies, *Chlorella vulgaris* has been appeared to perform well in the removal of nutrients. They reported that the results indicated in a nutrient removal efficiency up to 87.9% and 98.4% were recorded for total nitrogen and total phosphorous (Singh et al., 2017). It is recently reported that *C. vulgaris* cultivated by several streams from municipal wastewater yields microalgal biomass from 39 to 195 mg L-1 d-1 308 (Cabanelas et al., 2013). Cho et al. (2013) also revealed that *Chlorella* sp. yield the highest biomass production approximately 3.0 g L-1 310 using 10% anaerobic digestion tanks, and conflux line of the 90% wastewaters combined wastewater as nutrients for microalgae cultivation. It is very common in coastal area and an important species used as supplementary feed in aquaculture (Muys et al., 2019; Khatoon et al., 2013) because of its high protein, lipids and long chain polyunsaturated fatty acids (PUFA) contents (Radhakrishnan et al., 2015). In search for cost effective growth media, waste water has been found to be economically cheap method for mass culture of microalgae species. In Bangladesh, a huge quantity of aquaculture is being produced continuously as being the most suitable region for fisheries with the world's largest flooded wetland and aquatic biodiversity ranking 5th position in world aquaculture production (FAO, 2018). One of the main export products in Bangladesh is shrimp. Total production of shrimp and prawn from both capture and culture has increased from 1.60 lakh MT in 2002-03 to 2.54 lakh MT in 2017-18 (DoF, 2020). Discharge of farm wastewater with high nutrient concentration leads to high incidence of diseases occurrence and medicinal use which have residual effect on the environment (Lafferty et al., 2015).

Therefore, to reduce the post effects of aquaculture, current research work has investigated whether wastewater from shrimp hatchery is appropriate for microalgae cultivation with the goal of achieving substantial output of biomass and at the same time reducing the effect of wastewater on the ecosystem. Hence, the objective of this study was to evaluate the growth performance and nutritional composition of *C. vulgaris* grown in wastewater and compared with Conway media, to find out the commercial feasibility with lessening production cost and maintaining environmental sustainability.

2. MATERIALS AND METHODS

Wastewater collection and characterization

The aquaculture wastewater used in this study was obtained from Niribili Shrimp Hatchery (Cox's Bazar) and transported in 25 L containers. The collected wastewater was immediately filtered in the lab and sterilized with UV light for 48 hours. Then samples were taken for total ammonium nitrogen (TAN), nitrite nitrogen (NO_2-N) and soluble reactive phosphorous (SRP) determination following Parsons et al. (1984) analytical methods. Physical parameters were recorded in room temperature and stored in a cold room maintained at 20-21ºC.

Microalgae cultivation

Pure isolates of *C. vulgaris* was collected from Live Feed Research Corner of Department of Aquaculture, Chattogram Veterinary and Animal Sciences University (CVASU) and cultured in wastewater along with standard culture media where sub-culturing was performed in every two weeks to sustain a pure and stable stock culture.

Experimental design

The experiment was carried out in Live Feed Research Corner Laboratory of Faculty of Fisheries, CVASU. For this experiment, 1.5 L of media in 2 L Erlenmeyer flask was used based on culture media, the experimental design carried five treatments consisting of 100% wastewater as control (T1); Conway media with 25% wastewater replacement (T2); Conway media with 50% wastewater replacement (T3); Conway media with 75% wastewater replacement (T4) and 100% wastewater (T5) were evaluated in triplicate using a complete randomized design (Figure 1). The two culture variables- light and temperature (optimum for microalgal growth) were maintained in an insulated chamber and remained constant throughout the experimental period.

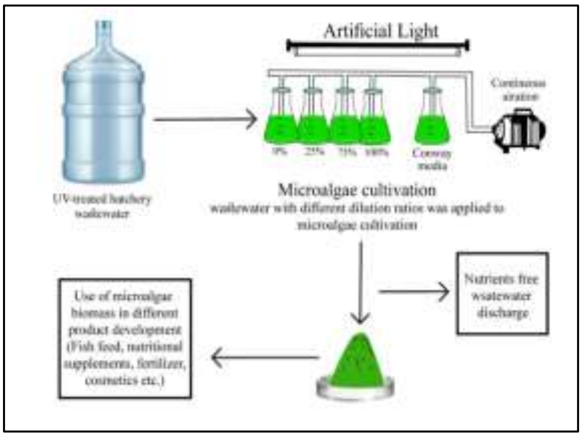

Figure 1. Schematic diagram of microalgae cultivation system in wastewater

Growth curve experiment

The experiment was conducted by preparing three replicates of sterilized Conway and waste water media with a volume of 200 mL in a 500 mL flask. The Conway medium was prepared by adding Solution A, Solution B and Solution C respectively 1 ml, 0.5 ml and 0.1 ml in 1 L of filtered and sterilized sea water (28 ppt) according to Tompkins et al. (1995). $1x10^3$ cells/ml *Chlorella vulgaris* were inoculated and slowly shaken from the stock culture into the flasks containing each culture medium. In the monitored culture room at 23-25°C with an artificial light range of 2000-3000 Lux (light: dark = 24 h: 0 h), the culture treatment was maintained on the rack. Throughout the experiment, aeration was continuously given. Cell density and optical density analysis were performed regularly, while biomass was performed in every alternate day until death phases were reached. For growth analysis, a 10 mL volume was taken daily. At the end of this experiment, the growth curve was plotted with cell density, optical density and biomass, and various growth phases of *Chlorella vulgaris*.

Harvest and collection of microalgae biomass

After the culture had reached stationary growth phase, aeration was stopped. Then the biomass was harvested with centrifuge machine (Hernle-Z206A), dried at 40°C for 24 h and kept in 4o C temperature in air tight glass vials.

Growth parameter analysis

Determination of cell density

Daily cell count was done by placing an aliquot of well-mixed culture suspension on a rhodium-coated haemacytometer (Hawksley AC1000, UK). The cell density of the microalgae culture was calculated according to the following formula (Clesceri et al., 1989)-

Cell count (cells/mL) for 25 squares:

$$\frac{\text{Total number of cell counted}}{10 \times 4 \times 10^{-6}}$$

Where, 10 = the squares of 2 chamber; $4x10^{-6}$ = the volume of samples over the small squares area which is equivalent to 0.004 mm^3 (0.2 x 0.2 x 0.1) expressed in cm^3 (mL).

Determination of optical density

The optical density of all cultures was determined daily using a UV-spectrophotometer (UV-VIS 1601, Shimadzu, Japan). The wavelength used here was 540 nm for *Chlorella vulgaris* (Lavens and Sorgeloos, 1996).

Proximate composition analysis

The algal protein content of different treatment was determined according to Lowry et al. (1951). The reading of the prepared sample was taken with spectrophotometer (UV-1601, Shidmadzu) at the wavelength of 750 nm. Based on Marsh and Weinstein (1966) the lipid analysis was performed with the sulphuric acid-charring method, following the carbonization method using tripalmitin as the standard after extracting lipids according to the method of Bligh and Dyer (1959). For carbohydrate analysis, samples were prepared based on the method of Dubois et al. (1956). The optical density was measured at 375 nm and 488 nm in a spectrophotometer respectively for lipid and carbohydrate analysis (Shimadzu UV-1601, Japan).

Statistical analysis

The parameters related to the experiment were analyzed using the analysis of variance. The significance of differences was evaluated using Tukey's honestly significant difference test at a level of $\alpha = 0.001$. All the statistical analyses were carried out using SPSS 26. Software by applying One Way ANOVA method.

3. RESULTS

Physicochemical parameters of aquaculture wastewater

The physicochemical characteristics of collected aquaculture wastewater were shown in Table 1 where small differences in physical and chemical parameters were observed before and after UV treatment due to UV-light exposure.

Growth parameter analysis

Figure 2(a) and Figure 2(b) showed the cell density (cells ml^{-1}) and optical density of C. vulgaris cultivated in controlled condition in different wastewater concentration against standard Conway media. The present study revealed that C. vulgaris cultivated under different wastewater concentrations had affected stationary phase where maximum cell density ($4.325 \pm 0.184 \times 10^6$ cells ml^{-1}) and optical density (0.40033 Abs) were observed at day 5 in T3 50% wastewater. This result revealed that C. vulgaris cultured in 50% wastewater concentration in cultured media had significantly affected the growth performance

Table 1. Physicochemical characteristics of the shrimp pond wastewater before and after UV

Properties	Before UV Treatment	After UV Treatment
Physical properties		
pH	7.9	7.6
Temperature(^{o}c)	24.2.	22.7
Dissolved oxygen(mg/L)	5.26	5.1
Salinity(ppt)	30.0	31.2
Chemical properties(mg/L)		
Total Ammonium Nitrogen(TAN)	4.72	4.42
Nitrite nitrogen (NO2-N)	2.82	2.21
Soluble Reactive Phosphorus(SRP)	4.32	5.46

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Proximate composition

Protein content, lipid content and carbohydrate content (% dry weight) of *C. vulgaris* cultivated in standard Conway media and different wastewater media were showed in Figure 3. According to the findings, highest protein content in *C. vulgaris* was observed in Conway media (42.70 ± 0.2 % of dry weight) where protein content was lowest in T2 25% wastewater (19.89 ± 0.26 % of dry weight) which stated that protein content in *C. vulgaris* was significantly higher ($P < 0.05$) when cultured in Conway medium.

On the other hand, highest lipid content was found in T5 (17 ± 0.2 % dry weight) and T4 (16 ± 0.3 % dry weight) where lipid content was lowest in Conway medium (13 ± 0.018 % of dry weight) and revealed that lipid content was significance higher ($p > 0.05$) in 100% wastewater.

Moreover, carbohydrate content was maximum in T3 50% ww (24 ± 1.37 % dry weight) and minimum in T4 (75% ww) and T5 (100% ww) which concluded that carbohydrate content was significantly different ($p > 0.05$) among Conway media and media containing different concentrations of wastewater.

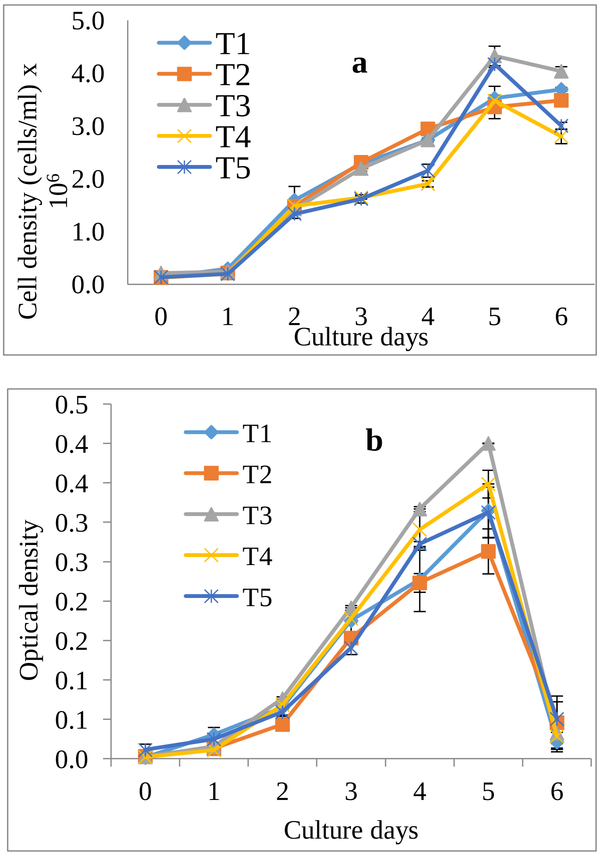

Figure 2. Cell density (a) and optical density (b) of *Chlorella vulgaris* cultured in wastewater and conway medium

4. DISCUSSION

Availability of both macro and micro nutrients plays a major role on the growth and biochemical composition where adequate supply of nutrients mainly nitrogen, phosphorus is crucial to achieve higher growth rates in microalgal cells (Xia et al., 2013). The growth rate declines when the metabolic requirements and supplied nutrients are not balanced properly (Zarrinmehr, 2019). There are also other important factors like temperature, light, salinity, pH etc. which might distress the growth and biochemical compositions of microalgae (Yeh and Chang, 2012). However in this study, different percentage of UV treated wastewater (25%ww, 50%ww and 75%ww) were applied as a source of nutrients along with commercial Conway media and compared with 100% commercial Conway media.

Water quality parameters need to be maintained within the optimal range for the better growth of microalgae where the recommended ranges of temperature is 16-27^{o}C, salinity 12-40 ppt and pH 7-9 (FAO, 1996). Chisti (2008) suggested the ideal growth temperature is usually between 20 and 30^{o}C for most marine microalgae. However, many algal species with reduced growth rates, can withstand temperatures up to 15^{o}C lower than their optimum. But a temperature only a few degrees higher than optimal can cause cell death (Mata et al., 2010). In this study, the pH (7.6), temperature (22.7^{o}C) and salinity (31.2 ppt) (Table 1) of wastewater after UV treatment which were used to replace

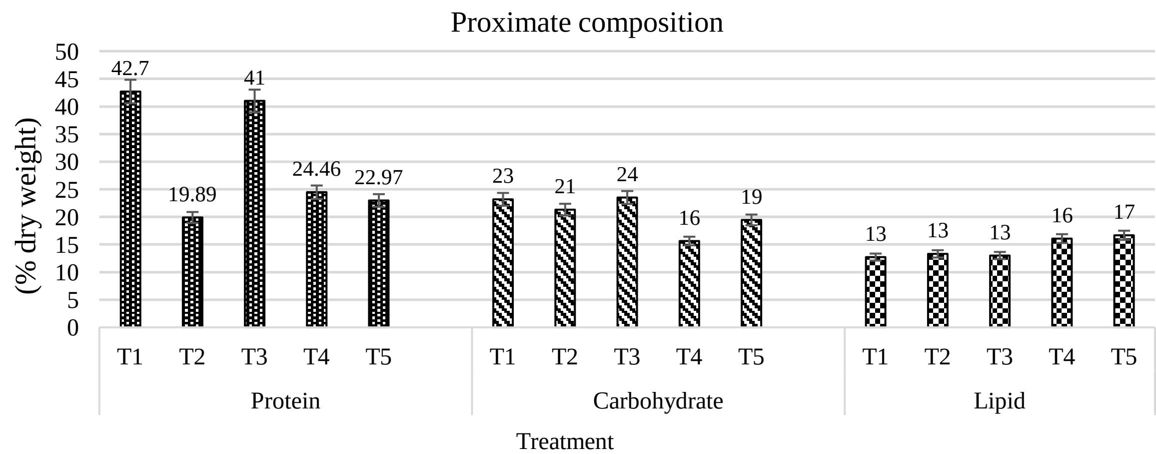

Figure 3. Protein, carbohydrate and lipid content of *C. vulgaris* in wastewater and Conway media

commercial media for microalgae culture found within the optimum range. Culture treatment with an artificial light range of 2000-3000 Lux (light: dark = 24 h: 0 h) was maintained in the experiment because phototrophs like the phytoplankton must obtain ample light for their net growth to reach their light compensation point.

After exposure to UV light, all nutrients (TAN, NO_2-N and SRP) decreased in concentration. In this study, after exposure to UV-light, the nutrient concentration also decreased significantly as substantial quantities of ammonia can be lost from medium through volatilization (McLachlan, 1973) and phosphorus level can also be decreased after wastewater sterilization (Sriram and Seenivasan, 2012). But wastewater contained enough essential nutrients for microalgae growth where Total Ammonium Nitrogen (TAN) is 4.42 mg/L, Nitrite Nitrogen (NO_2-N) is 2.212 mg/L and Soluble Reactive Phosphorous (SRP) is 4.32 mg/L. Marine microalgae can utilise the inorganic nitrogen for their growth, metabolic activities and increase their cell density throughout the nitrogen-enriched condition. The wastewater medium provided nitrogen as nitrates, nitrites and ammonium salts and they were readily available in the inorganic form. The number of nitrate-nitrogen (NO_3-N), nitrite-nitrogen (NO_2-N), ammonia-nitrogen (NH_3-N) and organically bonded nitrogen is Total Nitrogen (TN). Many of these nitrogen sources have been used for the processing of microalgae (Becker, 1994). Most ammonium in water is converted to toxic ammonia (NH_3) at high pH (>9), which can endanger marine species. In addition, for many types of microalgae, ammonium is preferable because it does not have to be reduced prior to amino acid synthesis and better biochemical enrichment in algal cells (Probert and Klaas, 1999).

Another main element necessary for normal growth of microalgae is phosphorus. The source of phosphorus was discovered naturally in waste water in the form of inorganic phosphate. Inorganic phosphate is a type of phosphate that is commonly added to the culture media and is favorable for the growth of microalgae. In the present experiment, total amount of nitrogen and phosphorus in the collected wastewater samples were sufficient and therefore supported good growth of *C. vulgaris*, because nitrogen and phosphorus are the main nutrients for algal growth through the process of photosynthesis in presence of light (Helena et al., 2018). Moreover, the conway media contained all the balance nutrients recommended which also ensured proper growth of experimental algal strain.

The growth of *C. vulgaris* was determined through the measurement of cell density and optical density to verify the consistency of the result as each technique has its limitations. Based on Figure-2, there were no significant differences ($p > 0.05$) in terms of cell density and optical density in *C. vulgaris* when cultured either in Conway or wastewater media. Chen (2011) performed experiments to encourage the development of *Chlorella* sp. by cultivating microalgae in waste water from a fish farm and obtained a high growth rate. Chopin et al. (2012) stated that the cultivation of some of the selected microalgae from the fish and shrimp farm in commercial medium and waste water showed a similar growth pattern. Here in this study, *C. vulgaris* passed lag phase period by 2 days prior to starting both in Conway and wastewater media and after that, they started quick division and the cell density and optical density rapidly picked onward days until the culture reached stationary condition which was reported by Barsanti and Gualtieri (2006), and stated that microalgae need a certain time period to physiologically adjust and adapt to the new environment even though the cells were viable as they were not in the state to undergo division yet.

Biochemical compositions are the means to evaluate the quality of cells in terms of nutrient accumulation and potential further utilization. Proteins, carbohydrates and lipids are the key components of algal cells. Some of the algae species (*N. oculata* and *T. chuii*) are interesting and important microorganisms in the field of biotechnology because of their high lipid content, higher proteins and essential fatty acids (Ghezelbash et al., 2008). However, based on Figure 3, protein content (% dry basis) in *C. vulgaris* had significant difference ($p > 0.05$) when cultured in wastewater medium (24.4555% dry weight) compared to Conway medium (42.6951% dry weight). But, 50%ww treatment had no significant difference in % protein content with the commercial Conway medium as protein synthesis is directly affected by nitrogen consumption. Along with this, there was significant difference ($p > 0.05$) in terms of lipid content in *C. vulgaris* either in wastewater or Conway where lipid content was highest in 100 % ww (17 % dry weight) and in 75 % ww treatment (16 % dry weight) where average 25% lipid content of C. vulgaris is found when grown in swine wastewater (Amini et al., 2016). Furthermore, there was significant difference ($p > 0.05$) in terms of carbohydrate content in *C. vulgaris* cultured either in wastewater or Conway medium (Figure 3). The highest carbohydrate content was found in 50%ww (24 % dry weight) followed by Conway medium and lowest in 75%ww. However, all the values of carbohydrate content in five different treatments were within the recommended range demonstrated in previous studies.

5. CONCLUSION

The idea of wastewater based microalgae cultivation is a model platform to simultaneously achieve waste reduction and potential microalgal biomass production. Based on current technologies for wastewater treatment alone is unlikely to be economically viable or provide a positive energy return, wastewater-grown microalgae suggests that this microalgal cultivation strategy offers real potential for wastewater related problems remediation. The present study showed that wastewater from aquaculture has great potentiality as an alternative medium for the cultivation of *C. vulgaris* under laboratory conditions. Our findings also reveal that aquaculture wastewater could promote *C. vulgaris* good algal growth to a similar extent as observed in the Conway medium. The ammonium concentration in wastewater would be the dominant factor for the results of microalgal *Chlorella* biomass and lipid production. Nutrients are used in waste water which would otherwise have been discarded, thus lowering operating costs and protecting the world. Properly planned use of wastewater from aquaculture alleviates issues with water contamination and not only conserves important water supplies, but also takes advantage of the nutrients in the effluent. However, more efforts on further investigation should be devoted before the economical and sustainable microalgal biomass production as growing microalgae with wastewaters is very challenging. If such work is carried out, then the resulting increase in bio-treatment of wastewater could help reduce the use of excess energy and cost, thus helping to meet global energy demand and aiding environmental sustainability.

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