Ecological Engineering 99 (2017) 47–53

Contents lists available at ScienceDirect

Ecological Engineering

journal homepage: www.elsevier.com/locate/ecoleng

Heterotrophic cultivation of microalgae using aquaculture wastewater: A biorefinery concept for biomass production and nutrient remediation

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ARTICLE INFO

Article history:
Received 13 July 2016
Received in revised form
21 September 2016
Accepted 13 November 2016
Available online 18 November 2016

Keywords:
Microalgae
Aquaculture wastewater
Heterotrophic
Biomass
Biorefinery

ABSTRACT

Cultivation of microalgae utilizing wastewater substrate could form a sustainable biorefinery with double benefit of biomass generation and nutrient remediation. In this study potential of aquaculture wastewater is evaluated for cultivation of *Chlorella sorokiniana* in heterotrophic mode for generation of high value biomass. Nutrient removal potential is also assessed. Aquaculture wastewater with 400 mgL^{-1} sodium nitrate supplementation resulted in biomass productivity of 498.14 $mgL^{-1}d^{-1}$. The biomass generated showed lipid productivity of 150.19 $mgL^{-1}d^{-1}$, carbohydrate productivity of 172.91 $mgL^{-1}d^{-1}$ and protein productivity of 141.57 $mgL^{-1}d^{-1}$. The nutrient removal efficiencies were 75.56% for ammonium, 84.51% for nitrates, 73.35% for phosphates and 71.88% for COD (chemical oxygen demand). The findings of this study underline the potential of aquaculture wastewater for production of valuable microalgal biomass which can be utilized for biofuels or feed application. This biorefinery concept also polished aquaculture wastewater which can be effectively reused.

1. Introduction

Microalgal biomass has been proven as a sustainable feedstock for biofuels, feed and numerous value added products pertinent to nutraceuticals and therapeutic industry. Microalgae can be effectively grown using wastewater for biomass generation and nutrient remediation (Ma et al., 2016; Rawat et al., 2011). Heterotrophic cultivation of microalgae is advantageous in terms of eliminating light dependency and higher biomass yields over phototrophic cultivation (Kim et al., 2013; Venkata Mohan et al., 2015). Microalgal biomass generated is rich in lipids, carbohydrates and proteins. The microalgal biomass can be utilized primarily for biofuels generation (biodiesel, biomethane, biohydrogen etc.) and for feed applications (aquaculture and animal) (Singh et al., 2015a). Alternatively, microalgal biomass can also be utilized for value added products such as pigments, nutraceuticals, bioplastic etc. (Suganya et al., 2016). Use of synthetic medium makes the commercial scale microalgal biomass generation unfeasible. Heterotrophic cultivation using different waste streams have been gaining interest from researchers as it reduces the production cost by dropping the usage of expensive inorganic chemicals (Medeiros et al., 2015; Rawat et al., 2011).

Biorefinery concept where different industries are integrated together for various products and mutual benefits could prove as a sustainable and economical approach for microalgal biomass generation. Aquaculture is one of the fastest growing food industries. This growing industry generates wastewater rich in nutrients such as ammonia, nitrates, phosphates and organic load (Gao et al., 2016; Lananan et al., 2014). Wastewater generated in aquaculture industry needs treatment prior to its reuse or release in environment to avoid the eutrophication. This wastewater treatment step adds to the production cost of aquaculture produce. Existing wastewater treatment processes used in aquaculture are denitrification process to release nitrogenous compounds in atmosphere and chemical precipitation using ferrous chloride to remove phosphorous compounds. These processes are not only adding to the cost but also lead to toxic by-products (Mook et al., 2012; Nasir et al., 2015). Microalgal cultivation using aquaculture could prove itself as a promising biorefinery for economical biomass generation and sustainable wastewater remediation. Microalgae can utilize the nutrients and organic load present in aquaculture wastewater to produce valuable biomass which can be subsequently utilized for biofuels or feed applications. Microalgae have been studied as a feed supplement and also as a whole feed for aquaculture industry. Microalgae contain proteins, long chain fatty acids, pigments which

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http://dx.doi.org/10.1016/j.ecoleng.2016.11.013
0925-8574/

are essential for fish nutrition. Whole microalgae or lipid extracted microalgae can thus be utilized in fish feed production (Ju et al., 2012).

There are several studies on utilization of wastewater for microalgal cultivation (Caporgno et al., 2015; Ramanna et al., 2014). However, using aquaculture wastewater for this purpose is not well explored area. There are very few studies which report the use of aquaculture wastewater for biomass production, but most of these studies mainly focus on phototrophic cultivation and nutrient removal. Very few studies report the biomass composition, which is important for its applications. Thus it is important to investigate the potential of aquaculture wastewater for heterotrophic cultivation of microalgae and evaluate the biomass composition. In this study, aquaculture wastewater has been used to cultivate *Chlorella sorokiniana* heterotrophically. Microalgal growth, nutrient removal and biomass composition were thoroughly investigated and compared with synthetic media cultivation. Sodium nitrate supplementation strategy has also been developed to achieve possible sealing biomass and primary metabolite productivities.

2. Materials and methods

2.1. Wastewater collection and characterization

The aquaculture wastewater was collected from aquaculture research facility, Durban, South Africa. At this facility Nile tilapia are reared in 5000 L tanks in controlled temperature (27–32 °C) with continuous aeration. A biofiltration system was used to remove nutrients and organic load from recycled water at regular interval. For this study wastewater was sourced from the collection tank before the treatment. Aquaculture wastewater (AW) was collected in 25 L containers and brought to laboratory immediately after collection. The pH, electrical conductivity, temperature, salinity, dissolve oxygen (DO), dissolve oxygen percentage were measured at the time of sample collection by YSI MP – AES. The total solid and total dissolve solid were calculated by standard methods of APHA 2005 (APHA-AWA-WEF, 2005). The chemical oxygen demand (COD) was determined by closed refluxed method. Centrifuged sample were used to analyze ammonia (NH_4^+), nitrates (NO_3^-), nitrites (NO_2^-) and phosphates (PO_4^{3-}) concentration by Gallery™ Automated Photometric analyzer (Thermo Scientific, USA). For the heavy metals analysis, sample were digested in microwave (Milestone S.R.L., Italy, output power 1200 W) at 180 °C for 20 min at 1000 W using acid mixture (15 mL HNO_3 and 4 mL $HClO_4$). After cooling the solution was allowed to evaporate from digested samples until the volume reduced to 5 mL. The samples were filtered through filter paper and further diluted using deionized water to 50 mL for heavy metals analysis using microwave plasma atomic emission spectrometry (Agilent Technologies 4200 MP-AES). Bacterial count was determined by determined by heterotrophic plate count method.

2.2. Microalgae cultivation

Chlorella sorokiniana strain was used for the heterotrophic cultivation using aquaculture wastewater (AW). The AW was first filtered using glass fiber filter papers and then autoclaved prior to microalgal inoculation (Nasir et al., 2015). The microalgal cultures were maintain in 1 L conical flask with 500 mL working volume. The cultivation conditions were: temperature 25 °C, shaking at 110 rpm in complete dark phase. Microalgal culture flasks were wrapped in aluminum foil and were kept in dark conditions. These conditions were kept constant for all the experiments. Microalgae were also cultivated in BG11 nutrient medium supplemented with glucose for comparative analysis. Microalgae were also grown in BG11 medium and AW under phototrophic condition for comparative analysis. Each set of experiment was done in duplicate. Supplementation experiments were carried out by adding 200, 400, 600 and 1500 mgL^{-1} sodium nitrate in AW.

2.3. Analytical methods

2.3.1. Growth and biomass analysis

Microalgal growth was monitored daily by determining optical density at 680 nm using spectrophotometric method. Biomass was estimated gravimetrically at initial, middle and late log phases of growth. Biomass productivity ($mgL^{-1}d^{-1}$) was calculated at late log phase gravimetrically (Singh et al., 2015b). Biomass was harvested using centrifuge and freeze dried using lyophilizer (Mini lyotrap, LTE scientific Ltd., United Kingdom) for further analysis.

2.3.2. Nutrient removal

The nutrients removal efficiency was determined on every alternate day. For analysis 10 mL of sample were collected from culture flask and centrifuged. The samples were then filtered using 0.45 μm syringes filters. These samples were analyzed for nitrates (NO_3^-), nitrites (NO_2^-), TON, ammonia (NH_4^+) and phosphates (PO_4^{3-}) using Gallery™ Automated Photometric analyzer (Thermo Scientific, USA) (Gupta et al., 2016). The chemical oxygen demand (COD) was analyzed by closed refluxed method. The removal efficiency in percentage was determined by using following equation

$$\text{Percentage removal} = \frac{\text{Initial concentration} - \text{Final concentration}}{\text{Initial concentration}} \times 100 \quad (1)$$

2.3.3. Biochemical composition of biomass

Microalgal biomass collected from each experiment was analyzed for lipids, carbohydrates and proteins. Lipids were extracted from the harvested biomass using microwave assisted solvent extraction. Dried biomass was mixed with chloroform and methanol (2:1 v/v) and then subjected to microwave treatment (100 °C for 10 min at 1000 W) for cell disruption (Guldhe et al., 2014). Biomass residues were removed by filtration. The organic layer was collected and oven dried at 70 °C for lipid recovery. The lipids obtained were measured gravimetrically and the percentage lipid content was determined based on lipid recovered from known weight of dry biomass. Lipids productivity was calculated according to equation described by (Singh et al., 2015b)

$$\text{Lipid productivity} = \text{biomass productivity} \times \frac{\text{lipid content}}{100} \quad (2)$$

Where, biomass productivity is in $mgL^{-1}d^{-1}$, lipids content is in percentage per dry biomass weight.

Proteins extraction was done following the method given by Lopez et al. (Lopez et al., 2010). The quantitative analysis of proteins was done by Lowry's method. A spectrophotometer (Spectroquant-Pharo 300, Merck) was used to measure the absorbance of the extraction mixture at 750 nm. Standards for calibration were prepared by using bovine serum albumin (BSA) in lysis buffer. The standard calibration curve prepared using BSA was used for proteins quantification. Proteins productivity was determined using equation 3.

$$\text{Protein productivity} = \text{biomass productivity} \times \frac{\text{protein content}}{100} \quad (3)$$

Where, biomass productivity is in $mgL^{-1}d^{-1}$, proteins content is in percentage per dry biomass weight.

Total carbohydrates were quantified using the phenol-sulfuric acid method (Prajapati et al., 2013). Dried biomass was mixed with sulfuric acid (2% v/v) and autoclaved for 30 min at 121 °C for hydrolysis. The mixture was then neutralized with 1 M $NaOH/H_2SO_4$. Supernatant was collected by centrifugation at 1509 × *g* for 10 min.