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Cultivation of *Chlorella vulgaris* on wastewater containing high levels of ammonia for biodiesel production

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HIGHLIGHTS

- Composition of microalgal cells depends on the composition of the wastewater feeds.
- FAME composition of the cultivated *C. vulgaris* is suitable for biodiesel production.
- Increasing NH_4^+-N yielded additional short-chain and saturated fatty acids.

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ABSTRACT

The feasibility of cultivating *Chlorella vulgaris* with wastewater containing high ammonia nitrogen concentrations was examined. The average specific growth rate of *C. vulgaris* was 0.92 d^{-1} at 17 mg L^{-1} NH_4^+-N, but declined to 0.33 d^{-1} at NH_4^+-N concentrations of 39–143 mg L^{-1}. At 39 mg L^{-1} NH_4^+-N, lipid productivity reached a maximum value (23.3 mg L^{-1} d^{-1}) and dropped sharply at higher NH_4^+-N levels, which demonstrated NH_4^+-N should be controlled for biodiesel production. C16 and C18 fatty acids accounted for 80% of total fatty acids. Increasing NH_4^+-N from 17 to 207 mg L^{-1} yielded additional short-chain and saturated fatty acids. Protein content was in positive correlation with NH_4^+-N content from 17 mg L^{-1} (12%) to 207 mg L^{-1} (42%). Carbohydrate in the dried algae cell was in the range of 14–45%, with a peak value occurring at 143 mg L^{-1} NH_4^+-N. The results demonstrate that product quality can be manipulated by NH_4^+-N concentrations of the initial feeds.

1. Introduction

Microalgae are a potential substrate for the production of biofuel, since it grows much faster than other energy crops (Minowa et al., 1995). Their oil contents are in the range of 20–50% of dry weight and the biodiesel can be consumed in a carbon-neutral manner (Chisti, 2007). Microalgae also represent a protein source in quality equal or superior to that of other high-quality plant proteins (Becker, 2004). The present cost of producing biodiesel from microalgae is ten times higher than the cost of crude oil (at 100$ per barrel) (Chisti, 2008), due in part to the cost of nutrients for microalgal cultivation (Chen et al., 2011).

The use of wastewater to cultivate microalgae provides an effective means of recycling nitrogen and phosphorus and to produce lipids and proteins (Spolaore et al., 2006). For example, microalgae were cultured on livestock fecal effluent following anaerobic digestion and lipid production at a rate of 17 mg L^{-1} d^{-1} was observed (Woertz et al., 2009). Feng et al. (2010) set up a semi-continuous process to cultivate *Chlorella vulgaris* on artificial wastewater, yielding a lipid production rate of up to 147 mg L^{-1} d^{-1}. Wang et al. (2010) cultivated *Chlorella* sp. on anaerobically-digested dairy manure and determined the lipid profiles of the cells. They found that the two most abundant fatty acids were octadecadienoic acid (27.2–33.4%) and hexadecanoic acid (20.6–26.0%) respectively. The potential of protein-rich microalgae residues as a nutrient additive in livestock feeds has also been investigated (Dubinsky et al., 1980) and Ehimen et al. (2011) studied the potential of recovering methane from post-transesterified microalgae residues.

Microalgae, cultivated under various feed conditions, can differ in their intracellular composition (Yeh et al., 2010). Since effluents

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of septic tanks are rich in ammonia, phosphorus, and nitrogen and organic compounds, they are a potential substrate for microalgal cultivation. In the present study, the biochemical composition of *C. vulgaris* cells grown under autotrophic conditions and fed with effluent mixes from a septic and a secondary sedimentation tank in a municipal wastewater treatment plant was examined.

2. Methods

2.1. Microalgal strain and culture

The *C. vulgaris* strain ESP-6 used in this study was obtained from the Department of Chemical Engineering, National Cheng Kung University, Tainan, Taiwan. Liquid Bold's Basal Medium (BBM) (Nichols, 1973) was used to cultivate *C. vulgaris*. *C. vulgaris* strain was inoculated into 1.5 L of BBM medium in a tubular photobioreactors (PBRs) consisting of vertical-columns with a diameter of 14 cm, a height of 24 cm and a normal working volume of 2 L and incubated at 27 ± 1 °C. The PBRs were continuously illuminated by 8000–10000 lux LED lights (WD-TM-D35W, Widen Photodiode Technology Co., China) and aerated with 10% vvm CO_2 (volumes of 5% CO_2 per total volume of the PBR per min). Magnetic stirrers at 50 rpm were used to agitate the media.

Samples of the *C. vulgaris* cultures were transferred to PBRs to achieve a microalgae concentration of 5 mg dry weight L^{-1} of medium composed of effluents prepared with different ratios of feeds from a septic and a secondary sedimentation tank. The effluents were filtered through 0.22-μm membrane filters prior to mixing. Initial concentrations of total nitrogen (TN) in the feeds were 29, 56, 72, 86, 99, 174 and 246 mg L^{-1}, and the corresponding feeds were designated as N30, N55, N70, N85, N100, N175 and N245. The characteristics of the feeds are listed in Table 1. All experiments were conducted in duplicate and mean values are reported.

2.2. Analytical methods

2.2.1. Characteristics of microalgal suspensions

Optical density (as an indicator of cell density) of microalgal samples was determined at 685 nm (OD_{685}) using a spectrophotometer (752 Grating Spectrophotometer, Shandong GaomiCaihong Analytical Instrument Factory, China). Preliminary tests established the following correlation between cell dried weight and OD_{685} at $r^2 = 0.9992$:

$$\text{Dry cell weight } (\text{mg L}^{-1}) = 443.0\ \text{OD}_{685} \quad (1)$$

For each test, a 60-ml liquid sample was centrifuged (15 min at 3600*g*). The sediment was washed twice with deionized water and the solids were dried at 105 °C.

Table 1
Characteristics of wastewater feeds used for the cultivation of *Chlorella vulgaris*.*

Components	N30	N55	N70	N85	N100	N175	N245
pH	6.47	6.96	7.12	7.10	6.82	7.42	7.19
TN	29	56	72	86	99	174	246
NO_3^--N	6.3	10.6	10.4	10.9	8.4	10.6	11.6
NO_2^--N	1.60	0.16	0.15	0.14	1.16	0.10	0.49
NH_4^+-N	17	39	52	65	77	143	207
COD	99	170	225	279	391	602	1032
BOD	23	84	118	151	200	350	596
TOC	32	61	82	104	125	231	340
TP	1.4	3.9	5.0	6.2	7.1	13.1	19.6

* Initial concentrations of total nitrogen (TN) in tested feeds were 29, 56, 72, 86, 99, 174 and 246 mg L^{-1}, designated as N30, N55, N70, N85, N100, N175 and N245 tests, respectively. (The values were the average of duplicate experiment and the error bars represented the upper and lower value.)

For measurements of pigment concentrations, 5 ml of the culture was centrifuged (15 min at 3600*g*). Pigment in the cell pellet was extracted with methanol in the dark for 30 min at 45 °C. The mixture was centrifuged, at 3600*g* for 15 min, and concentrations of *chlorophyll-a* (*CCHL-a*) and *chlorophyll-b* (*CCHL-b*) in the supernatant were determined according to Ritchie (2006), using Eqs. (2) and (3).

$$CCHL - a(\mu\text{g ml}^{-1}) = -8.096A_{652} + 16.52A_{665} \quad (2)$$

$$CCHL - b(\mu\text{g ml}^{-1}) = 27.44A_{652} - 12.17A_{665} \quad (3)$$

Photoprotective carotenoids (*CPPC*) were determined according to Strickland and Parsons (1968), using the following equation:

$$\text{Carotenoids } (\mu\text{g ml}^{-1}) = 4A_{480} \quad (4)$$

2.2.2. Determination of carbohydrates, proteins and lipids

An 8-ml sample was centrifuged (15 min at 3600*g*) with the supernatant discarded. The cell pellet was re-suspended with pure water and was centrifuged again at 3600*g* for 15 min. The sediment was suspended in 50-ml deionized water and treated with ultrasound (480 W) for 10 min before determination of carbohydrate, using the phenol–sulfuric acid method (Su et al., 2007). Solids, extracted by centrifugation (15 min at 3600*g*) from 100-ml of cultures, was used for determination of protein by the Kjeldahl method (Kjeltec 800 Analyzer Unit, FOSS Company, Denmark).

A modified Floch protocol, as proposed by Christie (1989), was used for the determination of total lipids. In general, a 100-ml microalgae culture was centrifuged (15 min at 3600*g*). The cell pellet was extracted with 8 ml methanol for 1 h with shaking, followed by the addition of 16 ml of chloroform. The solution was mixed for 2 h, followed by centrifugation at 3600*g* for 15 min. The supernatant was transferred to another tube and the solids extracted with 12 ml of a mixture of methanol/chloroform (v/v, 1/2) for 30 min. After centrifugation (15 min at 3600 g), the supernatant was transferred to the previously used tube and washed with 9 ml of 0.88% KCl solution. The washed solids were collected by centrifugation (15 min at 3600*g*) and washed with a mixture of 3 ml of methanol and 3 ml of 0.88% KCl solution. The mixture was centrifuged for 15 min at 3600*g*. The lower layer was transferred into another pre-weighed tube and centrifuged again to further remove water and solids. The solvent was evaporated by nitrogen at 40 °C for 4 h and weighed (total lipid).

2.2.3. Fatty acids methyl esters (FAMEs) determination

The lipid composition was determined as FAMEs by direct transesterification (Su et al., 2007). An 8-ml aliquot of 0.5 M methanolic NaOH solution was added to the extracted total lipid of 100 ml sample mentioned in Section 2.2.2 under nitrogen flow and shaken for 30 min before heating in a water bath at 100 °C for 30 min. After cooling to room temperature, 8 ml of 0.7 M HCl methanolic solution and 8 ml of a 14% (m/v) methanolic BF_3 solution (Sigma, USA) was added under nitrogen flow and mixed. After heating in a 100 °C water bath for 30 min and cooling to room temperature, 2 ml of a saturated NaCl solution and 10 ml of hexane were added prior to centrifugation (15 min at 3600*g*). The supernatant was collected for analysis of FAMEs.

FAMEs were detected by gas chromatography, using a FOCUS GC (Thermo Electron Corporation, Milan, Italy) equipped with a flame-ionization detector and a 60 m long capillary column (Type no. 260M154P, Thermo Fisher Scientific, Waltham, MA) with an internal diameter of 0.25 mm. For each measurement, 2 μl of the sample was injected into the GC. Helium was used as the carrier gas at a flow rate of 1.5 ml min^{-1}. The temperatures of injector and detector were maintained at 260 °C. The oven temperature