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Simultaneous nutrient removal and lipid production with *Chlorella vulgaris* on sterilized and non-sterilized anaerobically pretreated piggery wastewater

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ABSTRACT

Piggery wastewater is a potent nutrient source for microalgal lipid production. Wastewater has been usually sterilized when used for microalgal cultivation. This is uneconomical in large-scale applications. Therefore, lipid productivity of *Chlorella vulgaris* CY5 using sterilized and non-sterilized diluted anaerobically pretreated piggery wastewater was studied in batch reactors. The maximum average lipid productivity was obtained after 12 days of incubation and it was higher with the sterilized wastewater than with the non-sterilized one (117 g/L/d vs. 91.3 g/L/d), due to the higher biomass concentration. Because of the unexpected increase of dissolved organic carbon (DOC) in the cultures, second experiment was conducted to characterize the composition of produced DOC in non-sterilized wastewater. Carbohydrate content increased in the liquid phase but decreased in the biomass after nitrogen had been exhausted. After 12 days of incubation, soluble chemical oxygen demand (COD_s) was 414 ± 56 mg/L, biomass production was 2.8 ± 0.15 g/L, and lipid content was 30.3 ± 1.2 wt%. Average lipid productivity from day zero to day 12 was 70.5 ± 1.1 g/L/d. *C. vulgaris* removed nutrients from the non-sterilized wastewater and produced oleaginous biomass, although the lipid productivity was higher with sterilized wastewater.

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1. Introduction

The demand for more eco-efficient society is enormous. Industrial, agricultural and municipal systems require modifications, such as reusing wastewaters and flue gases in energy generation to become more sustainable. Production of lipid-rich biomass to generate biofuels is one example [1–4]. Lipid-rich biomass can be produced with various microorganisms, such as bacteria, yeasts, fungi, and microalgae, but only photosynthetic organisms can be used to simultaneously fix CO_2 from the flue gas and remove nutrients from the wastewater [4].

Several factors, such as various microalgal species, cultivation strategies, reactor designs and harvesting technologies, have been used to produce third-generation biofuels with microalgae [5]. *Chlorella vulgaris* is one the most studied microalga due to its ability to grow in relatively harsh conditions and in the presence of culture invaders [6]. Biomass composition of *C. vulgaris* is highly dependent on cultivation conditions and medium composition [7]. In stressful growth conditions, such as nitrogen starvation, *C. vulgaris* accumulates lipids mainly in the cytoplasm and chloroplasts [8], but it may also produce starch granules inside the chloroplasts [6]. *C. vulgaris* accumulated high concentrations of lipids, especially in mixotrophic conditions, where both light, CO_2, and organic carbon were provided simultaneously [6]. In mixotrophic conditions algae can grow both using light as energy source and CO_2 as carbon source (autotrophic growth), and using organic carbon as energy and carbon source (heterotrophic growth) [4].

Integration of nutrient removal and biodiesel production with *Chlorella* spp. and piggery wastewater has been reported [3,9–11]. Microalgal lipid studies on piggery wastewater have been mainly conducted with sterilized wastewater. In addition to autoclaving,

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Table 1
Composition of the undiluted anaerobically treated piggery wastewater before (non-sterilized) and after sterilization (sterilized) with autoclave (121 °C, 20 min).

Component	Anaerobically treated piggery wastewater	
	Non-sterilized	Sterilized
pH	7.7	9.8
COD_{tot} (mg/L)	332	377
COD_s (mg/L)	298	308
TKN (mg/L)	348	287
NH_4-N (mg/L)	233	210
NO_{3-} (mg/L)	5.5	7.1
PO_4- (mg/L)	101.4	28.4

sterile-filtration, UV irradiation and addition of sodium hypochlorite have also been used to eliminate bacterial and protozoal invaders in dairy wastewater [12].

The objective of this work was to compare cultivation of *C. vulgaris* CY5 in sterilized and non-sterilized anaerobically pretreated piggery wastewaters, as sterilization of large volumes of wastewater is very costly. These experiments aim to delineate microalgal ability to grow in non-sterilized wastewater environment, and simultaneously accumulate lipids and remove nitrogen from the anaerobically pretreated piggery wastewater.

2. Materials and methods

2.1. Microalgae

Chlorella vulgaris CY5 isolated from a freshwater area in Southern Taiwan was used in this work. Prior to the growth experiments in wastewater the strain was pre-cultured in BG-11 medium, the composition of which has been described in our previous study [13]. Between the experiments, the strain was maintained in BG-11 agar plates (BG-11 with 15 g/L agar).

2.2. Piggery wastewater

Wastewater of this study originated from a Taiwanese wastewater treatment plant treating piggery effluent. Treatment process consisted of a pH control basin, followed by four anaerobic basins, three aerobic basins and a settler. A grab sample of anaerobically pretreated piggery wastewater was taken after the fourth anaerobic basin prior to the aerobic treatment. The growth of *C. vulgaris* was examined in both sterilized (autoclaved 121 °C, 20 min) and non-sterilized anaerobically pretreated piggery wastewater. Composition of the wastewater before and after autoclaving was as given in Table 1.

2.3. Experimental conditions

Experiments were conducted in 1 L glass vessels (15.5 cm in height and 9.5 cm in diameter) equipped with an external light source (14W fluorescent light/TL5). Light intensity was 150 $\mu mol/m^2/s$ and aeration rate 0.1 vvm with 2.5% CO_2. Initial culture volume was 800 mL and cultures were incubated for 16 days at room temperature (20-25 °C). Microalgal biomass from the pre-cultures was centrifuged (5000 rpm, 10 min) and the settled biomass was used as inoculum to minimize transfer of nutrients from pre-culture medium into the wastewater used in the microalgal growth experiments.

First *C. vulgaris* CY5 was cultivated in sterilized and non-sterilized 5× diluted wastewater using various inoculum concentrations (50 mg/L, 150 mg/L & 300 mg/L). The pH of the cultures with sterilized wastewater was adjusted to below 8, with 4 M H_2SO_4 (6 mL of 4 M H_2SO_4 into 800 mL of wastewater) at the beginning of the cultivation experiments, to minimize the effect of initial culture pH on microalgal growth in the experiment studying the effect of sterilization. NaOH was used to adjust the culture pH during the experiments if pH decreased below 4, because low pH has been shown to inhibit growth of *C. vulgaris* [14]. Aim of this experiment was to study the effects of sterilization and inoculum size on biomass and lipid production. As the COD_s concentration increased during the cultivation, the cultivation of *C. vulgaris* CY5 with non-sterilized 5x diluted wastewater was repeated in triplicate batch reactors to further delineate lipid production process in non-sterilized wastewater and to characterize the composition of released organic matter. Therefore, carbohydrates from the liquid phase and composition of the biomass were determined in the second experiment. Results for the triplicate batch reactors were calculated as average values ± standard deviation of the results from these three reactors.

2.4. Analyses

Light intensity was measured with LI-250 light meter and LI-190SA pyranometer sensor (LI-COR, Inc., USA). Culture pH was measured with PB-10 pH meter equipped with pH/ATC electrode (Sartorius, Germany). Cell size and morphology were studied using phase contrast microscopy (ECLIPSE 80i, Nikon, Japan). Lipid inclusions were identified with the same microscope using fluorescence light and Nile Red staining. The staining was conducted by adding 1 µL of Nile Red per 1 mL of sample and then incubated at room temperature (20-25 °C) for 5 min before microscopy examination with fluorescence light.

Biomass concentration was measured as optical density at a wavelength of 680 nm (OD_{680}) and as dried biomass (g/L). For biomass composition analyses, biomass was harvested with centrifugation (5000 rpm, 5 min) and washed thrice with reverse osmosis treated water. From the harvested biomass, the content of lipids, carbohydrates and proteins was analyzed. Lipids were determined as fatty acid methyl esters (FAMEs) after extraction from the biomass with direct transesterification method [13]. Extracted FAMEs were analyzed by gas chromatography using a flame ionization detector (GC-2014, Shimadzu, Japan). Carbohydrate content of the biomass was determined with the modified quantitative saccharification method [15] as described by Ho et al. [16]. Total protein content of the biomass was measured from acid hydrolyzed sample diluted with NaN_3 solution [17] as described by Ho et al. [18].

Total and soluble chemical oxygen demand (COD_{tot} and COD_s) were analyzed with the standard dichromate method 5520C: Closed Reflux, Titrimetric method [19]. COD_s sample was filtrated with 0.45 µm syringe filter prior to the analysis. Total Kjeldahl nitrogen and ammonium (TKN and NH_4-N) from the wastewater were analyzed with the standard method 4500-N(org)C: organic nitrogen/Semi-Micro-Kjeldahl [19]. NO_3^- and PO_4^- ions from the filtrated (0.20 µm) wastewater were analyzed with an Ion Chromatograph (ICS-5000, Dionex, Italy). During the experiments, ammonium and carbon concentrations were determined with TOC/TN_b analyzer (LiquiTOC II, Elementar, Germany) from filtrated samples (0.45 µm). $(NH_4)_2SO_4$ was used as a nitrogen standard. Total dissolved carbon (TDC) was determined as a sum of dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC). $KHC_8H_4O_4$ was used as the standard for DOC and Na_2CO_3 as the standard for DIC. Carbohydrate content of non-hydrolyzed and hydrolyzed liquid samples (centrifugation at 5000 rpm for 10 min) was determined by phenol-sulfuric acid method [20] and sugar column as described by Marjakangas et al. [13]. The non-hydrolyzed samples were analyzed to detect the monosaccharides present in the liquid phase, while the hydrolyzed samples were analyzed to detect the polysaccharides in the liquid phase by hydrolyzing the polysaccharides into detectable monosaccharides.

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