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Effect of salt type and concentration on the growth and lipid content of *Chlorella vulgaris* in synthetic saline wastewater for biofuel production

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HIGHLIGHTS

- Salt concentration had more of an effect than salt type on algal metabolisms.
- Salinity stress reduced the growth of *Chlorella vulgaris*.
- Salinity stress increased total lipid content and saturated portions of fatty acids.
- Algae settling was improved by 33–83% with increased NaCl concentrations.

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ABSTRACT

Microalgae can offer several benefits for wastewater treatment with their ability to produce large amounts of lipids for biofuel production and the high economic value of harvested biomass for biogas and fertilizer. This study found that salt concentration (~45 g L^{-1}) had more of an effect than salt type on metabolisms of *Chlorella vulgaris* for wastewater treatment and biofuel production. Salinity stress decreased the algal growth rate in wastewater by 0.003 day^{-1} per mS cm^{-1} and slightly reduced nutrient removal rates. However, salinity stress was shown to increase total lipid content from 11.5% to 16.1% while also increasing the saturated portions of fatty acids in *C. vulgaris*. In addition, salinity increased the algal settling rate from 0.06 to 0.11 m day^{-1} which could potentially reduce the cost of harvesting for algal biofuel production. Overall, *C. vulgaris* makes a suitable candidate for high salinity wastewater cultivation and biofuel production.

1. Introduction

Saline wastewater is produced by several industrial processes including food processing, wine production, tanneries, textiles, aquaculture, and oil production (Lefebvre and Moletta, 2006). Treatment of these wastewaters is necessary to reduce pollution and prevent eutrophication; however, high salinity has been shown to strongly inhibit the metabolisms of non-halophilic bacteria. In particular, high salinity has been shown to strongly inhibit the metabolism of nitrifying bacteria which are responsible for removing nitrogen (N) from wastewater (Dincer and Kargi, 1999; Uygur and Kargı, 2004). In addition, landfill leachate and sea water infiltration into sewage systems can increase municipal wastewater salinity to levels where conventional wastewater treatment is affected (Linarić et al., 2013). As a result, most saline effluents are treated using physico-chemical techniques including evaporation, ion exchange, and membrane processes (Lefebvre and Moletta, 2006). However, physico-chemical techniques are energy intensive and operational costs are high. Recently, salt tolerant microbes including *Pseudomonas aeruginosa, Bacillus flexus, Exiguobacterium homiense* and *Staphylococcus aureus* have been studied as a biological treatment of saline wastewaters, but have not been extensively evaluated for nutrient removal (Abdel-Raouf et al., 2012; Sivaprakasam et al., 2008). Marine microalgae are reported to be efficient in removing N and phosphorus (P) from wastewater and represent a promising new organism for saline wastewater treatment (Craggs et al., 1997; Hoffmann, 1998).

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Microalgae offer several benefits for saline wastewater treatment; mainly, their ability to produce large amounts of lipids for biofuel production and the high economic value of harvested biomass for biogas and fertilizer (Safi et al., 2014). There are many studies which have been invested in the development of microalgae-based biofuels and the integration of algal biofuel production with wastewater treatment (Hwang et al., 2016). In particular, wastewater can be used as a low-cost carbon source for mixotrophic cultivation which can lead to higher biomass productivity compared to autotrophically cultivated microalgae (Praveenkumar et al., 2014).

Several studies suggest that salinity stress can improve algal lipid production (BenMoussa-Dahmen et al., 2016; Mohan and Devi, 2014). Heredia-Arroyo et al. (2011) reported that *Chlorella vulgaris* lipid content increased from 15.4% to 25.6% at 35 g L^{-1} NaCl rather than no NaCl; but the algal growth was totally inhibited at 35 g L^{-1} NaCl. Similar results of salinity effect on lipid contents were also observed at lower salinity concentrations where total lipid content increased from 15.2% to 23.4% as NaCl concentration increased from 0 g L^{-1} to 1 g L^{-1} NaCl (Mohan and Devi, 2014). While these studies show that salinity can impact lipid production, further research is needed to evaluate microalgae as a suitable method for treating high salinity waste streams. For example, only a few studies have looked at N removal by microalgae in high salinity wastewater and fewer have studied P removal. Furthermore, studies that investigate the effect of salinity on lipid production in microalgae consider NaCl only and the effects of other salts have not been fully investigated. A study by Mohleji and Verhoff (1980) investigated the effect of several salts (NaCl and KCl) on P uptake by the green alga *Selenastrum capricornutum* and observed that the sodium ion (Na^+) had a significant effect on phosphorus uptake compared to potassium ions (Mohleji and Verhoff, 1980). It is hypothesized that specific ions may have more of an effect on algal metabolisms than salinity as a whole. In this study, the effects of specific salts on the integration of wastewater treatment and biofuel production were evaluated under different salt concentrations. The specific objectives of this study were (1) to investigate the growth kinetics and nutrient removal of *C. vulgaris* in high salinity wastewater, and (2) to evaluate the lipid production and the harvesting of *C. vulgaris*.

2. Materials and methods

2.1. Algal species and culture

Chlorella vulgaris (UTEX 2714, UTEX Algae Culture Collection, Austin, TX) was selected as a model microalga to represent algae which can be found in wastewater treatment plants. Initially, the *C. vulgaris* was inoculated in 1 L glass bottles (13951L, Corning Inc.) containing 500 mL of Bold's Basal Medium (BBM) (Bischoff and Bold, 1963). The bottles were incubated at room temperature (23 °C) under 2000 lx continuous cool-white fluorescent illumination and stirred using a magnetic stirrer at 50 rpm. The light intensity was measured by a dual-range digital light meter (06-662-63, Fisher). 2000 lx is about 27–33 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) (Tibbits, 1994). The culture was aerated using an aquarium air pump to supply CO_2 (0.04%) to the algae. After reaching the stationary phase (3 days), the culture was used to inoculate batch experiments.

2.2. Experimental design

The microalgae were grown in batch mode using artificial wastewater and varying amounts (0, 15, 30 and 45 g L^{-1}) of NaCl and KCl to supply salinity. Conductivity was measured using a four-pole conductivity probe (IntelliCAL™ CDC401, Hach) before and after each batch test. The experiments were conducted in 1 L glass bottles (13951L, Corning Inc.) with caps that were modified with a 0.22 μm filter to allow access to atmospheric air. The artificial wastewater used in the batch experiments was prepared by dissolving the following chemicals in deionized (DI) water; glucose (0.4125 g L^{-1}), $MgSO_4$ (0.013 g L^{-1}), $CaCl_2{\cdot}2H_2O$ (0.043 g L^{-1}), $FeSO_4{\cdot}7H_2O$ (0.005 g L^{-1}) and trace metal (Trace metal mix A5 with Co, 92949, Sigma-Aldrich) (1 mL L^{-1}) (Feng et al., 2011). Stock solutions of ammonia (10.0 g L^{-1} NH_4Cl) and phosphate (10.0 g L^{-1} $K_2HPO_4{\cdot}3H_2O$) were used to adjust the solution to 50, 40 or 20 mg N L^{-1} of ammonia and 6, 4 or 2 mg P/L of total phosphorus (TP). 500 mg L^{-1} $KHCO_3$ was added as an inorganic carbon source. Initial pH of the mixture of algae and synthetic wastewater ranged from 8.0 to 9.4. All batch experiments began with an OD_{600} of 3.00 (approximately 500 mg L^{-1} dry biomass) and tested for 8 days. The bottles were subjected to continuous 2000 lux fluorescent light and stirring (50 rpm). Samples were taken daily for analyses.

2.3. Analytical methods

2.3.1. Nutrient analysis

40-mL samples were taken for water quality analyses including lipid content measurements: (10 mL for pH and NH_4^+ + 5 mL for TP + 2 mL for OD + 1 mL for lipid) × 2 for duplicates. The samples were centrifuged at 10,000 rpm for 10 min to separate the algae from the bulk water. After centrifuging, the supernatant was obtained for analyzing pH, NH_4^+, and TP. Ammonia was measured using an ammonia probe (IntelliCAL™ ammonia probe, ISENH318101, Hach). TP was measured using ascorbic acid method test in tube kit from Hach (Method 8180). All samples were taken in duplicate for each sample time.

2.3.2. Biomass analysis

For biomass determination, a 50-mL sample was filtered through a pre-weighed 45 μm glass fiber filter paper (934-AH, Whatman) and rinsed 3 times with 20 mL of DI water. The sample was then dried at 105 °C until a constant weight was achieved. Optical density (OD) at 600 nm was used as an indicator of cell density using a spectrophotometer (DR1900, Hach). A relationship between OD_{600} and dry biomass weight in synthetic wastewater with various salt concentrations was determined as Eq. (1).

$$\text{Dry biomass weight } (\text{g L}^{-1}) = 0.1836 \times OD_{600} (R^2 = 0.996) \quad (1)$$

The average growth rate (μ:h^{-1}) was calculated using the following equation (Converti et al., 2009):

$$\mu = \frac{\ln X_{max} - \ln X_0}{t - t_0} \quad (2)$$

where X_{max} equals the maximum dry biomass weight at time t and X_0 represents the initial concentration of dry biomass weight at time t_0. GraphPad Prism 6.0 statistics software (GraphPad Software, Inc., La Jolla, CA) was used to run a 2-way ANOVA statistical analysis on the data.

2.3.3. FAME analysis

The lipid content of the microalgal biomass was estimated by the fatty acid methyl ester (FAME) concentration via the direct transesterification method followed by gas chromatography (GC; Agilent 7890, Agilent Technologies, Wilmington, DE, USA) (Praveenkumar et al., 2014). Samples were taken from each batch tests at the end of the 8-day batch experiment and centrifuged at 10,000 rpm for 10 min to separate the algae from the bulk water. After the supernatant was removed, the remaining pellet was rinsed with 50 mL of DI water. The sample was centrifuged again