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Sulfate amendment improves the growth and bioremediation capacity of a cyanobacteria cultured on municipal wastewater centrate

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ABSTRACT

A series of experiments were performed to evaluate the growth of the model cyanobacterium *Synechocystis* sp. PCC6803 cultured on wastewater centrate sourced from a municipal water reclamation facility. Centrate is the liquid removed from sludge during sewage processing. The addition of 304 μM Na_2SO_4 to centrate diluted by 90% in water yielded final cell concentrations that were over 6 times higher than cultures grown on just centrate. This suggested that S is the primary limiting nutrient for photoautorophic growth on centrate. The expression of the sulfate transporter system encoded by the *spbA-cysTWA* operon was upregulated when cells were grown in non-amended centrate, confirming that *Synechocystis* sp. PCC6803 experienced S-limitation during growth on centrate alone. Elemental analysis of centrate further confirmed that the proportion of S relative to other macronutrients is lower in centrate compared to the measured elemental ratios found in cyanobacterial biomass. The cyanobacteria removed 69% of the total soluble nitrogen (TN) in S-amended centrate compared to 25% from cultures grown in centrate alone. It is proposed that S could be recycled from the production of H_2S during anaerobic digestion of wastewater, or it could be added from exogenous material such as gypsum. Overall, this work suggests that S-amendment of centrate could improve the sustainability of wastewater remediation and biomass production using photosynthetic microbes.

1. Introduction

Sustainable and cost-effective solutions for producing biofuels are necessary to reduce society's reliance on fossil fuels. Wastewater is currently being investigated as a nutrient source for biofuel feedstocks of microalgae and cyanobacteria. The cultivation of these organisms requires exogenous water, and macronutrients such as carbon, nitrogen, and phosphorus. Carbon may be supplied as CO_2 or HCO_3^- for photoautotrophic growth [1], or as organic carbon for species capable of performing heterotrophic or mixotrophic growth [2,3]. Nitrogen and phosphorus account for 1–14% and 0.5–3.3% biomass content of microalgae, respectively [4]. Phosphorus is typically sourced from non-renewable phosphate based minerals such as potassium-, sodium-, and ammonium-phosphates [1]. Nitrogen based fertilizers are largely produced using the Haber-Bosch process [5]. This process produces ammonia from atmospheric nitrogen using natural gas and it is estimated to generate 785–999 kg CO_2 per ton of ammonia produced and is therefore a considerable contributor to greenhouse gas emissions associated with fertilizer production [6]. Wastewaters have been proposed as renewable replacements of nitrogen and phosphorus for microalgal growth, with the added benefit of inorganic nitrogen and phosphorus removal [7].

Wastewaters are produced as a bi-product of industrial processes, as well as from human and animal activities [8]. Municipal wastewaters are produced from residential and business waste. They undergo extensive physical, chemical, and biological processing at treatment facilities in order to separate and remove the organic solids and nutrients from water [9,10]. Biological nutrient removal systems utilize bacterial communities to assist in this process [11,12]. Centrate is the supernatant following centrifugation of sludge formed during anaerobic digestion and it contains the highest concentrations of nitrogen and phosphorus within the wastewater treatment process [9,13–15]. Therefore, centrate may not be released from wastewater treatment facilities due to concerns associated with eutrophication of waterways [16–18]. For example, Colorado Regulation 85 limits the discharge of N and P to receiving water bodies to $< 15\ mg\ N\ L^{-1}$ and $< 1\ mg\ P\ L^{-1}$ [18]. This high N and P in centrate is therefore recycled back into anaerobic digesters, resulting in a reduced efficiency for overall

Abbreviations: DWRF, Drake Water Reclamation Facility; RT-qPCR, quantitative reverse transcription polymerase chain reaction; ICP-OES, inductively coupled plasma optical emission spectrometry; TN, total soluble nitrogen; TM, Trace Metals

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nutrient removal [19]. So, the application of centrate to supply nutrients to microbial autotrophs may not only increase the sustainability of the algal/cyanobacterial biomass production process, but it also could provide an additional bioremediation step for excess nutrients produced during wastewater treatment.

Several studies have evaluated the productivity of eukaryotic microalgae grown on wastewaters. *Chlorella* sp. [20,21], *Scenedesmus* sp. [22,23], *Nannochloropsis* sp. [24,25], and *Chlamydamonas* sp. [26], are among the species most commonly studied for biomass production. Investigators have also evaluated the ability for eukaryotic microalgae to remove dissolved nutrients from wastewater [27,28]. Furthermore, pilot-scale experiments of eukaryotic microalgae grown in raceway ponds using wastewater as the water and nutrient source have shown that wastewater based systems can be scaled up to industrially relevant conditions [22,25]. A novel single step wastewater treatment process has recently been demonstrated utilizing the mixotrophic growth of a thermo- and acidophilic red microalgae, *Galdieria sulphuraria*, directly on wastewater [29]. Large-scale cultures using this approach have been shown to reduce the nutrient load of urban wastewaters to concentrations permitting its discharge [30]. These studies show that the cultivation of photosynthetic eukaryotes on wastewaters is indeed a feasible approach for reducing the reliance of algal growth on non-sustainable fertilizers.

Cyanobacteria are promising organisms for advanced biofuel production. Cyanobacteria have simpler and more easily transformable genomes relative to eukaryotic microalgae – which make engineering of exogenous metabolic pathways for advanced biofuel production within cyanobacteria an attractive alternative to eukaryotes [31]. Several investigators have evaluated growth of cyanobacteria on centrate. For instance, Lynch et al. [32] evaluated eight different cyanobacterial isolates and *Synechocystis* sp. PCC6803 against one native Scenedesmaceae alga and *Chlorella vulgaris* for their ability to grow on synthetic growth media containing N and P concentrations commonly found in centrate. The Scenedesmaceae outperformed the cyanobacteria. *Synechocystis* sp. PCC6803 has also been shown to grow on artificial sea water amended with anaerobic digestion effluent (centrate) [33]. This study evaluated *Synechocystis* in a high salinity environment for the purpose of using seawater to dilute wastewater for cultivation, which would only be a feasible solution in select coastal regions.

It was hypothesized that neither nitrogen nor phosphorus limits the growth of biomass on centrate diluted with freshwaters. A bioassay approach was used to ascertain the limiting nutrient for biomass accumulation in centrate whereby differences in final biomass were compared after supplementation of centrate with components of the standard cyanobacteria culture media. Biomass accumulation of *Synechocystis* sp. PCC6803, as well as soluble nitrogen drawdown, was found to be enhanced when centrate was supplemented with sulfate. PCC6803 was confirmed to be sulfate limited in centrate by comparing the expression of sulfate transporter genes during stationary phase during growth on centrate compared to sulfate supplemented cultures. This study provides a simple approach to improve cyanobacterial biomass production on wastewater with minimal nutrient supplementation that could improve the sustainability of wastewater treatment and the production of biomass for biofuels or bioproducts.

2. Materials and methods

2.1. Culture conditions, standard growth media, and biomass measurements

2.1.1. Organism and normal growth media

The cyanobacterium *Synechocystis* sp. PCC6803 (hereafter referred to as PCC6803) was generously gifted from Dr. Jianping Yu of the National Renewable Energy Laboratory. Cells were maintained axenically in BG-11 (pH 8) growth media buffered with 10 mM TES-NaOH buffer (pH 8) [34]. All chemicals used for the BG-11 media were laboratory grade and purchased from either Thermo FisherScientific® or Millipore Sigma®. Unless otherwise noted, PCC6803 cultures were incubated under 165 μmol photons $m^{-2} s^{-1}$ of continuous light (Phillips F17T8/TL841/ALTO fluorescent lights) at 30 °C. Cells were cultivated in 125 ml Erlenmeyer flasks on an orbital shaker (VWR, Model 3500).

2.1.2. Wastewater centrate collection and standardized use

The supernatant from the wastewater dewatering process of anaerobically digested sludge was collected directly from a decanter centrifuge (Alfa Laval/Sharples® Model DS 706) at the City of Fort Collins Drake Water Reclamation Facility (DWRF; Fort Collins, CO) in 250 ml plastic bottles provided by the dewatering facility. This is hereafter identified as centrate. Immediately following collection from the centrifuge sampling port, the centrate was vacuum filtered through a 0.2 μm filter (Thermo Scientific™ Nalgene™ Rapid-Flow™ 75 mm Bottle Top Filter-500 ml) to remove any remaining solids and native microorganisms. Filtered centrate was kept at 4 °C until dilution and inoculation within 8 h of collection. Precipitates were observed in the bottom of centrate samples after 24 h in preliminary experiments indicating changing chemical composition.

2.1.3. Cell counts

Cell counts were done by either flow cytometry or via direct microscopic observation. Flow cytometry based cell counts were performed by first filtering diluted culture through a 30 μm pre-separation filter (Miltenyi Biotec Inc.; Auburn, CA) and then running samples through a BD Accuri™ C6 Plus Personal Flow Cytometer (BD Life Sciences; San Jose, CA) at a flow rate of 14 μl min^{-1}. The autofluorescence of chlorophyll *a*/phycobilisomes (640 nm excitation, 675 ± 25 nm emission detection) containing cells were gated from non-viable particles. Manual counts were performed using a Reichart Bright-Line Hemacytometer with Neubauer ruling (Hausser Scientific; Horsham, PA) under 400× total magnification with a bright field microscope.

2.2. Determining nutrient in centrate limiting biomass accumulation

In the following experiments, centrate was used at a concentration of 10% v/v in sterile 18.2 MΩ (MQ) H_2O.

2.2.1. Nutrient supplemented centrate media

The four stock components of BG-11 media [Part A (1.76 M $NaNO_3$, 30.4 mM $MgSO_4$, 24.5 mM $CaCl_2$, 3.12 mM Citric Acid, 2.29 mM Ammonium Ferric Citrate, 342 μM NaEDTA), Part B (105 mM K_2HPO_4), Part C (18.9 mM Na_2CO_3), and Trace Metals (46.3 mM H_3BO_4, 14.4 mM $MnCl_2$, 765 μM $ZnSO_4{\cdot}7H_2O$, 1.9 mM Na_2MoO_4, 320 μM $CuSO_4{\cdot}5H_2O$, 171 μM $Co(NO_3)_2{\cdot}6H_2O$)] were added individually to 10% centrate (% v/v centrate in sterile MQ water) to achieve the respective final stock concentrations found in BG-11 listed above (1% v/v for Parts A–C, 0.1% v/v for Trace Metals).

In subsequent experiments, Part A compounds were prepared as individual solutions and added to centrate at the concentrations they were in complete BG-11 media. Additionally, all three components associated with iron (citric acid, ammonium ferric citrate, and EDTA) were added as a single solution for this experiment.

Finally, the individual components of $MgSO_4$ (Mg^{2+} and $SO_4{}^{2-}$) were then evaluated to determine which is limiting in centrate. The effects of magnesium and sulfate were tested using 304 μM $MgCl_2{\cdot}7H_2O$ and 304 μM Na_2SO_4.

PCC6803 cultures were added to each centrate solution with a starting concentration of 5.5×10^5 cells ml^{-1}. The relative exponential growth rates were calculated for PCC6803 in different centrate medias by calculating the slopes from the natural log transformation of exponentially growing cell concentrations.