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Cultivation of a native alga for biomass and biofuel accumulation in coal bed methane production water

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ABSTRACT

Coal bed methane (CBM) production has resulted in thousands of ponds in the Powder River Basin of low-quality water in a water-challenged region. A green alga isolate, PW95, was isolated from a CBM production pond, and analysis of a partial ribosomal gene sequence indicated the isolate belongs to the *Chlorococcaceae* family. Different combinations of macro- and micronutrients were evaluated for PW95 growth in CBM water compared to a defined medium. A small level of growth was observed in unamended CBM water (0.15 g/l), and biomass increased (2-fold) in amended CBM water or defined growth medium. The highest growth rate was observed in CBM water amended with both N and P, and the unamended CBM water displayed the lowest growth rate. The highest lipid content (27%) was observed in CBM water with nitrate, and a significant level of lipid accumulation was not observed in the defined growth medium. Growth analysis indicated that nitrate deprivation coincided with lipid accumulation in CBM production water, and lipid accumulation did not increase with additional phosphorus limitation. The presented results show that CBM production wastewater can be minimally amended and used for the cultivation of a native, lipid-accumulating alga.

1. Introduction

Different diatoms and algae can produce and accumulate a variety of value-added precursors (*e.g.*, carbohydrates, fatty acids, and pigments) that can further contribute to reduced CO_2 emissions through utilization of atmospheric CO_2 [1]. Much of the reported algal biofuel research has focused upon exposing cultures to a range of environmental stresses prior to harvest ([1] and references therein), including nitrogen and phosphorus. In addition to carbon, nitrogen, and phosphorus, water is an inherent resource that has to be considered in the alternative energy sector. Water and nutrient availability are becoming an increasing concern in light of a rapidly increasing global population combined with decreasing reserves of fertilizer and the diminishing availability of clean water. The Committee on the Sustainable Development of Algal Biofuels (National Research Council) published a report in 2012 that indicated the large scale production of algal biofuels and bio-products would exert a significant strain on the already limited availability of nutrients and high-quality water resources. The report suggested that strategies were needed to reuse and recycle water and nutrients in future photosynthetically-driven production facilities [2].

The volume of water used in the energy sector represented 15% of global freshwater withdrawals in 2010 [3], and coal-bed methane (CBM) production produces large amounts of water that can negatively impact the environment and the economy. According to the Wyoming Oil and Gas Conservation Commission [4], in 2014 the Powder River Basin produced 246,601,576 Mcf (Mcf = 1000 cubic feet) of gas and an associated 256,305,005 Bbls (8,073,617,107 gal) of water. Water produced by CBM extraction is typically stored in lined or unlined impoundments but can be used for agricultural purposes or discharged into streams with proper monitoring and treatment [5,6]. However, the production water can be problematic due to the high concentrations of Na^+ and total dissolved solids (TDS), and low concentrations of Mg^{2+} and Ca^{2+} observed in the majority of the produced water ([7]). Therefore, integrated approaches are crucial when developing future water, energy, and climate policy [8], particularly in regions with changing water demands.

Recently, the growth of microalgae in low-quality water sources for the purposes of remediation and/or biofuel production have been reported in the literature, and include dairy wastewater [9] and municipal wastewater [10]. A primary concern when cultivating microalgae is selecting a species that is a good fit for the selected conditions in

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different parts of the country (e.g., water, light, nutrients). A recent study successfully grew a microalgal species in CBM production water in Australia in which a marine algae species was selected to accommodate the high salt content of the water [11]. However, the use of CBM production water from the Powder River Basin for this purpose has not been evaluated.

Identifying beneficial uses for low-quality production water from underground coal seams will become increasingly important as domestic energy production from subsurface reservoirs increases to meet growing energy demands. An alternative use for CBM production water could be the growth of microalgae for biodiesel and biomass production. Due to photoautotrophic growth, algae utilize solar energy for growth and CO_2 (HCO_3^-) as the carbon source, but require a low-quality water source that does not deplete already stressed water supplies. In addition, CBM production water is inherently high in bicarbonate (HCO_3^-) and pH, conditions that foster the utilization of dissolved inorganic carbon by photoautotrophs. Therefore, the low-quality water needs for algal biofuels and the wastewater streams from CBM production present an opportunity to couple water recycling, light-driven CO_2 utilization, and production of value added products. We have isolated a native green alga from a CBM production pond in northeastern Wyoming and evaluated the potential for growth in CBM production water with the ability to accumulate lipids that can be converted into biodiesel. A native species provides the benefit of an organism that is already adapted to the water and climate conditions of the region, and we have identified the key nutrients required for the species to grow photoautotrophically in CBM production water and accumulate lipids during N-deprivation.

2. Methods

2.1. Materials

The alga, PW95, was isolated from a CBM production water pond in the Powder River Basin of northeastern Wyoming (44° 52.613′N 106° 54.700′W). All chemicals were of highest purity available.

2.2. Culture methods

2.2.1. Culture isolation

Cultures were streaked on Bold's Basal Medium (BBM) agar plates in a 20 °C incubator with a 14:10 h light:dark cycle (7872 lx, cool white fluorescent). Subsequent colonies were streaked 3 times for the isolation of a single photoautotroph. DNA sequence analyses (18S and 16S) suggested a unialgal culture with no detected bacterial sequences, and heterotrophic plates (R2A) incubated at 20 °C in the dark did not display bacterial growth. Cultures were routinely checked for heterotrophic growth.

2.2.2. Culture conditions

Liquid BBM was used routinely for culture maintenance and biomass production at 20 °C. Flasks were placed on a shaker rotating at 125 rpm to encourage mass transfer of CO_2. Flask experiments were inoculated from a stock culture of PW95 grown in liquid BBM. Each inoculum was centrifuged (1750 ×*g* for 5 min) and washed twice with sterile CBM water or BBM depending on the intended use. CBM water was supplied from well FG-09 (45° 26′ 5.8914″N 106° 23′ 31.416″W) in the Powder River Basin, and this water was used for all experiments. CBM production water was filter sterilized using a bottle top 0.2 μm, polyethersulfone (PES), Nalgene Rapid-Flow vacuum filter (Thermo Scientific) in order to better understand the direct responses of the algal isolate in CBM production water. Growth experiments at 20 °C were performed with biological duplicates in 500 mL Erlenmeyer flasks containing 250 mL of sterile CBM water or Bold's Basal Medium (BBM; 0.5 mM nitrate, 0.05 mM phosphate). Five different nutrient conditions were evaluated using CBM water: CBM water without additions; CBM water with 0.5 mM nitrate; CBM water with 0.5 mM nitrate and 0.05 mM phosphate; CBM water with 0.5 mM nitrate, 0.05 mM phosphate, and 0.3 mM magnesium sulfate; and CBM water with 0.5 mM nitrate, 0.05 mM phosphate, 0.3 mM magnesium sulfate, and micronutrients (i.e., trace vitamins and minerals) used in BBM [12]. All flasks were inoculated with 15 mL of inoculum from the same stock culture.

2.3. Analysis methods

2.3.1. Determination of biomass dry weight

Nitrocellulose or acetate cellulose 0.2 μm filters (25 mm diameter) were used to periodically determine the dry weight biomass of the cultures. Culture samples were vacuum filtered and rinsed with water to remove excess salts before air drying in an oven set at approximately 90 °C for 48 h. Filters were weighed before and after use to determine dry weight biomass.

2.3.2. Determination of chlorophyll

Culture samples (1 mL) were centrifuged (16,162 ×*g* for 15 min) in microcentrifuge tubes and 950 μL of supernatant was frozen at −80 °C for water chemistry analysis. The cell pellet was submerged in 1 mL of 100% methanol, disrupted by sonication and vortexing, and stored at 4 °C for at least 24 h in a covered box. After 24 h, the samples were centrifuged (16,162 ×*g* for 5 min). The supernatant was extracted and analyzed in disposable plastic cuvettes using a Shimadzu UV-1700 UV–VIS spectrophotometer at wavelengths of 665.2 nm, 652 nm, and 632 nm. Chlorophyll concentrations in μg/mL were estimated as previously described by Ritchie [13].

2.3.3. Determination of lipid content

Lipid accumulation was tracked using a Nile Red staining method [14]. Culture samples (1 mL) were stained with Nile Red stock solution (4 μL of 0.25 mg Nile Red/mL of acetone). Samples were stored in the dark for 5 min. The set time of 5 min was determined by performing a time assay with PW95 to determine the optimum time to measure Nile Red fluorescence as described by Chen et al. [15]. After 5 min, 200 μL duplicates were pipeted into a 96 well plate and fluorescence was read using a Synergy H1 hybrid flourometer/spectrophotometer reader. Gen5 microplate reader software was used to evaluate the fluorescence at an excitation of 480 nm and emission of 580 nm. Samples were diluted 2, 4, or 10 times as necessary to attempt to keep Nile Red measurements within a linear range [15].

2.3.4. Determination of anion concentrations

Nitrate and phosphate concentrations were determined with a DIONEX ICS-1100 and Chromeleon Chromatography Management System with an ASRS 4 mm suppressor. A 4.5 mM sodium carbonate and 1.4 mM sodium bicarbonate eluent mix was used. Values for pH were measured using a standard bench top Oakton pH 11 Series meter.

2.3.5. Determination of FAME content via GC-MS

Cultures were harvested once a Nile Red peak was observed and processed as described previously [16]. Flasks were transferred to 50 mL sterile Falcon tubes and centrifuged (4816 ×g for 10 min). The supernatant was decanted and the biomass was flash frozen at −80 °C until the sample was lyophilized. After lyophilization, biomass was weighed in glass tubes, and submerged in 1 mL of toluene and 2 mL of sodium methoxide. The samples were vortexed and placed in a 90 °C oven for 30 min (samples were vortexed every 10 min). After 30 min, 2 mL of 14% boron trifluoride in methanol were added to each sample and vortexed. Samples were again incubated for 30 min at 90 °C (samples were vortexed every 10 min). When finished, 0.8 mL of hexane and 0.8 mL of 15% NaCl were added to each sample. Samples were incubated for 10 min and centrifuged (2500 ×*g* for 2 min). The top layer in the tube (containing the lipids) was extracted and analyzed on an Agilent