BIOMASS AND PHYCOCYANIN FROM OIL AND NATURAL GAS EXTRACTION PRODUCED WATER UTILIZING A CYANOBACTERIA DOMINATED ROTATING ALGAL BIOFILM REACTOR (RABR)

by

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A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Biological Engineering

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ABSTRACT

Biomass and phycocyanin from oil and natural gas extraction produced water utilizing a cyanobacteria dominated rotating algal biofilm reactor (RABR)

by

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The production of cyanobacterial biofilms and phycocyanin from Rotating Algal Biofilm Reactors utilizing undiluted produced water from oil and natural gas extraction as a culture medium was investigated in this study. Produced water is the largest waste stream generated by the oil and natural gas industries and represents a large volume of non-potable water that could be exploited for algae culture instead of freshwater resources. Phycocyanin production from cyanobacteria dominated biofilms cultured in produced water was examined, and phycocyanin yield enhancements were investigated with light limiting conditions. A novel Oscillatoriales strain was isolated from the Logan City Wastewater Treatment Facility in Logan, Utah and used in conjunction with the Rotating Algal Biofilm Reactor platform for the duration of this study.

Ash Free Dry Weight (AFDW) areal biomass productivities of up to 4.8±0.7 g/m²-day were observed using laboratory scale 1 L bioreactor units and 220 µmol
photons/m²-s PAR. Areal phycocyanin productivity was shown to be 84.6±9.3 mg/m²-day with an associated crude phycocyanin extract purity of 0.23±0.03. A lower light intensity of 40 µmol photons/m²-s PAR resulted in an average 87.6% increase in phycocyanin yield and a 230% increase in crude phycocyanin extract purity. A lower AFDW biomass productivity of 2.7±0.4 g/m²-day resulted in areal phycocyanin productivities that were statistically similar between the two light treatments.

An evaluation of growth substrata was conducted with cotton rope and conveyer cloth materials found to be the most durable and having the highest yields of harvestable biomass. The cotton rope and cotton conveyer cloth materials were evaluated on a Rotating Algal Biofilm Reactor operating in an outdoor 2000 L produced water pond. The cotton rope yielded a near 140% increase in AFDW biomass vs. the cotton cloth although the compositions varied greatly. The cotton cloth biomass showed a more robust phototrophic biofilm with higher phycocyanin yields and lower Autotrophic Indices (47.0 vs. 3.4 mg/m² and 127 vs. 507, respectively for cotton cloth vs. cotton rope). These results show promise for the utilization of produced water to culture cyanobacteria dominated biofilms with modifiable biomass characteristics as a source of high value phycocyanin pigments.
PUBLIC ABSTRACT

Biomass and phycocyanin from oil and natural gas extraction produced water utilizing a cyanobacteria dominated rotating algal biofilm reactor (RABR)

Jonathan L. Wood

The production of cyanobacterial biofilm biomass and phycocyanin from Rotating Algal Biofilm Reactors utilizing undiluted produced water from oil and natural gas extraction as a culture medium was investigated in this study. Produced water is the largest waste stream generated by the oil and natural gas industries and represents a large volume of non-potable water that may be available for algae culture with minimal impact on freshwater resources. Combining the use of produced wastewater as culture medium with the production of high value algal pigments, such as phycocyanin, may increase the economic viability of algae culture and wastewater purification. High value phycocyanin pigment production and methods to increase phycocyanin yields with light limitation were examined in this study. A unique cyanobacteria species was isolated from the Logan City Wastewater Treatment Facility in Logan, Utah and used in conjunction with the Rotating Algal Biofilm Reactor platform for the duration of this study.

Between the “high” and “low” light treatments used in this study, the high light treatment showed nearly twice the biomass production as the low light culture (4.8±0.7 vs. 2.7±0.4 g/m2-day). The low light biomass contained 87.6% more of the phycocyanin pigment, with a 230% increase in purity, then the biomass from the high light treatment.
The areal footprint productivity of phycocyanin per day was the same for both the light
treatments.

An evaluation of growth attachment materials was conducted with cotton rope
and cotton conveyor cloth materials found to be the most durable and having the highest
yields of harvestable biomass. The cotton rope and cotton conveyor cloth materials were
evaluated on a floating Rotating Algal Biofilm Reactor operating in a 2000 L outdoor
produced water pond. The cotton rope yielded a 140% increase in biomass vs. the cotton
cloth although the compositions varied greatly. The cotton cloth biomass was composed
of mainly healthy algae with higher phycocyanin yields while the cotton rope showed a
higher proportion of non-algae organisms and little phycocyanin. These results show
promise for the utilization of produced water to grow cyanobacteria biofilms with
modifiable biomass characteristics as a source of high value phycocyanin pigments.
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<th>Description</th>
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<tr>
<td>TDS</td>
<td>Total Dissolved Solids</td>
</tr>
<tr>
<td>PC</td>
<td>Phycocyanin</td>
</tr>
<tr>
<td>PCB</td>
<td>Phycocyanobilin</td>
</tr>
<tr>
<td>AFDW</td>
<td>Ash Free Dry Weight</td>
</tr>
<tr>
<td>PAR</td>
<td>Photosynthetically Active Radiation</td>
</tr>
<tr>
<td>RABR</td>
<td>Rotating Algal Biofilm Reactor</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis</td>
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CHAPTER I

INTRODUCTION

1.1 Produced Water

Produced water is often saline wastewater that is generated during the hydrocarbon extraction process and is the general term for any water that is produced from a hydrocarbon recovery well. Produced water is a combination of naturally occurring formation water and water/drilling additives injected during the initial drilling and later recovery processes. This wastewater is largely generated when a water/hydrocarbon mixture is brought from the subsurface and subjected to downstream separation processes to recover the valuable hydrocarbons, while discarding the co-extracted wastewater [1].

Produced water is the largest volumetric waste stream generated by the oil and gas industries and constitutes approximately 98% of the total volume of waste generated by the oil and gas industry in the United States [2]. On average, over 10 barrels of produced wastewater are generated for every barrel of oil in the United States, totaling over 21 billion barrels of produced water in 2012. As the age of an oil well increases, generally, there is a steady increase in the water: oil ratio produced from that well. Aging wells in Texas and California accounted for the largest portion of produced water generated in the United States in 2012. A substantial portion of this produced water brought to the surface was reinjected, about 91%, for either enhanced oil recovery in productive formations, or into disposal wells. The remainder was treated by evaporation, discharged to the surface, or saw beneficial reuse [1]. Much of the time that produced water spends on the surface is spent in lined ponds for either evaporation or temporary
Such an abundant wastewater may be useful as a prevalent non-freshwater medium for algal culture. Particularly in the often arid western United States, utilization of these wastewaters could reduce the freshwater requirements of a large-scale algae culture operation while providing an economic value in algal derived products [3]. Additionally, the use of produced water as an algal growth medium has the potential for biological treatment of the wastewater and the cogeneration of algal biomass that, in turn, may be used to produce high value and commodity products [4–8].

1.2 Algal Culture in Produced Water

The current physical downstream separation processes that are commonly used in produced water treatment do not excel in removing organics from the produced water at the low ppm level of contamination. Current literature suggests that an algal biofilm
Based bioreactor may provide the necessary ability and compartmentalization to handle low ppm level organics contamination in produced water waste streams, although more studies are needed on this topic and other potential target contaminants for remediation in produced water by algae based biofilms [6,9,10]. Although produced waters vary widely in their total dissolved solids content (1,000-400,000 mg/L TDS), many have the advantage of containing many of the inorganic nutrients needed in an algal growth medium and average under 100,000 mg/L TDS [1,6,11]. This potential for preexisting nutrients may reduce the need for micronutrient and buffer additions to make this wastewater a viable algal culture medium and highlights the need for characterization among produced water. Produced water utilized in these studies was aerated before being amended with nitrogen and phosphorous. Selected wastewater characteristics of the produced water used in this study are shown in Table 1, a more exhaustive analysis of composition is listed in Appendix B.

Table 1
Characteristics of produced water used in this study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Total Dissolved Solids (mg/L)</td>
<td>11,640</td>
</tr>
<tr>
<td>Conductivity (µmhos/cm)</td>
<td>19,400</td>
</tr>
<tr>
<td>pH</td>
<td>8.6</td>
</tr>
<tr>
<td>Oil and Grease (mg/L)</td>
<td>16</td>
</tr>
<tr>
<td>Hardness (mg eq. CaCO₃)</td>
<td>461</td>
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The extremely large genetic diversity of cyanobacteria has allowed them to become adapted to the varied and extreme environments that produced waters may display, often forming biofilm mats in hydrocarbon contaminated waters [12,13]. This trait may make cyanobacteria ideal candidates for produced water algae culture as well as future bioremediation studies. There are few published reports of utilizing produced water as an algal growth medium [6,8,11,14,15]. Of those studies, the majority focus on algae as a biofuel feedstock with limited studies on high value bioproducts from produced water grown algae [8]. High value product streams can be integrated into an algal biorefinery to improve the economics of algal derived biofuels, as well as provide a potentially higher margin revenue stream to offset costs [7]. Common high value products from algae include pigments, vitamins, and polyunsaturated fatty acids.

Phycobiliproteins such as phycocyanin, phycoerythrin, and allophycocyanin, are a class of pigments that are readily water soluble and easily isolated, unlike the majority of high value algal products. A laboratory scale experiment using cyanobacteria isolated from the Logan City Wastewater Treatment facility in Logan, Utah was performed to determine the impact of a water based phycocyanin extraction on the total lipids fraction in the leftover biomass. Over 90% of the whole cell lipids were retained in the wet biomass phase following the phycocyanin extraction, demonstrating that phycocyanin extraction can be integrated into an algal biorefinery with minimal lipid loss (Figure 2) [16,17]. These properties associated with cyanobacteria make phycobiliprotein pigments such as phycocyanin an ideal candidate for integration into an algal biorefinery concept.
Phycocyanin is a blue accessory pigment to chlorophyll and is a phycobiliprotein found in many, if not all, cyanobacteria. Composed of a protein/chromophore complex, phycocyanin has increasing commercial value and is currently used as a natural food colorant, health supplement, fluorescent label in laboratory procedures, and as a feedstock to produce potential therapeutic agents [18–20]. The culturing of cyanobacteria on wastewater is a cost effective and potentially sustainable method to obtain large quantities of this bioproduct as evidenced by its commercial availability although purification processes can be time consuming and relatively tedious with low yields [7]. Cyanobacteria can naturally accumulate phycobiliproteins, such as phycocyanin, in quantities of up to a quarter of their dry weight and 40% of the total water-soluble protein under specific culture conditions [21].
Phycocyanobilin chromophores of phycocyanin have antitumor, antiviral, antioxidant, and anti-inflammatory effects [7,18,22,23]. The unbound chromophore phycocyanobilin may be used directly as a potential antioxidant compound and has been shown to inhibit NADPH dependent superoxide production in mammalian cells [24,25]. Additionally, Phycocyanobilin had been shown to stabilize human serum albumin in terms of thermal stability and resistance to proteolysis [26]. Binding of phycocyanobilin to bovine serum albumin also resulted in increased thermal stability as well as a protective effect from free radical induced oxidation. Minic et al. 2018 suggests that this effect could be used to stabilize phycocyanobilin in food colorant and therapeutic compositions [27]. Potential for an increased demand of phycocyanobilin is high due to the expanded understanding of its potential applications as both a colorant and antioxidant therapeutic agent.

Mesobiliverdin is another potentially high value, naturally occurring product that can be produced from cyanobacterial phycocyanin. Derived from the phycocyanobilin chromophore, the desirable properties of mesobiliverdin may include high antioxidant activity, cytoprotective, and anti-inflammatory properties similar to phycocyanobilin and biliverdin [28,29]. A study by Ito et al. 2013 suggests that mesobiliverdin may have higher human biliverdin reductase activity than phycocyanobilin and a study by Basdeo et al. 2016 showed a 5-fold reduction in activity for phycocyanobilin vs. biliverdin, the natural substrate for human biliverdin reductase [20,30]. Alternatively, a study performed by Terry et al. 1993 has suggested that phycocyanobilin has similar kinetics as biliverdin for rat liver derived biliverdin reductase [31]. Ito et al. 2013 also found that treatment with mesobiliverdin provided a significantly higher cell viability, even at lower
concentrations, than a biliverdin treatment during pancreatic islet cell isolation in rats [20]. This finding of increased islet cell viability may make this type I diabetes treatment much a much more viable treatment option. Varied and high demand therapeutic properties of these types increase the commercial potential of mesobiliverdin for many areas of application.

The utilization of oil and gas produced wastewater to produce high value pigments, such as phycocyanin and phycocyanobilin, have potential to reduce disposal costs and generate revenue for oil and gas operators and algae cultivators. Transfer of produced water to algal cultivators has the potential to save oil and gas operators up to $10 per barrel (~159 L) in disposal costs in ideal locations [6]. In addition, cyanobacteria derived phycocyanin has market values of $3-25/mg for food and cosmetic grades and as high as $1,500/mg for highly purified grades [32]. These economic incentives highlight the option to integrate high value pigments into an algal biorefinery set up around produced water as a culture medium. The following chapters are an investigation into the growth potential and phycocyanin production and enhancement of cyanobacteria dominated biofilms cultured on a Rotating Algal Biofilm Reactor operating in a produced water medium.
1.4 Objectives

A. Evaluate growth of cyanobacteria dominated biofilms in produced water using a Rotating Algal Biofilm Reactor (RABR).

B. Evaluate phycocyanin production of cyanobacterial biofilms grown in produced water.

C. Demonstrate phycocyanobilin production from cyanobacterial biofilms grown in produced water.
References


[32] A.A. Kenekar, M.A. Deodhar, Effect of varying physicochemical parameters on the productivity and phycobiliprotein content of indigenous isolate geitlerinema
CHAPTER II

BIOMASS AND PHYCOCYANIN PRODUCTION FROM CYANOBACTERIA
DOMINATED BIOFILM REACTORS CULTURED USING OILFIELD AND
NATURAL GAS EXTRACTION PRODUCED WATER

This chapter, with slight modifications is published with the following citation:


Abstract

The production of cyanobacterial biofilm biomass and phycocyanin from Rotating Algal Biofilm Reactors utilizing undiluted produced water from oil and natural gas extraction as a medium was demonstrated in this study. Oil and natural gas extraction produced water is the largest waste stream generated by these industries and may provide an abundant source of non potable water for the culture of cyanobacteria and phycocyanin. In the present study, a unialgal cyanobacteria isolate from the Logan City, Utah Wastewater Treatment Facility was shown to exhibit an areal ash free dry weight biomass productivity of $4.8\pm0.7 \text{ g/m}^2\text{-day}$ when cultured in produced water medium. The cyanobacterial biofilms yielded an areal phycocyanin productivity of $84.6\pm9.3 \text{ mg/m}^2\text{-day}$ with a maximum crude extract purity of $0.23\pm0.03$. The utilization of produced water for the production of cyanobacterial biofilm biomass and associated high value products could provide significant economic and bioremedial advantages to current produced water disposal technologies.
1. Introduction

Produced water is the largest waste stream generated by the hydrocarbon recovery industry [1]. Largely unsuitable for discharge, this wastewater is generally recycled or reinjected into disposal wells. Often, however, produced water is disposed of in large ponds for holding/evaporation. The large volumes of produced water held in open ponds represents a waste that is expensive to transport and dispose. Utilization of this produced water as an algal growth medium has the potential to remove chemicals from the produced water, generate useful algae biomass and other valuable products, and minimize the large volumes of freshwater resources required for algae culture.

The Rotating Algal Biofilm Reactor (RABR) is a novel algal biofilm reactor platform that utilizes a semi-submerged rotating drum with attached growth substrata and an integrated harvesting apparatus [2,3]. Algal growth in occluded waters is possible as the RABR rotates in and out of the water exposing the biofilm culture to light, nutrient, and gas exchange. The resulting algal biofilms may be harvested and dewatered with reduced operation costs when compared with traditional suspended culture [4]. Utilization of the RABR for the growth of algal biofilms may address the need for the economic treatment of produced water using immobilized biological films [1]. Cyanobacteria dominated biofilms have been shown to tolerate heavy oil pollution and degrade petroleum components [5,6]. Many Oscillatoriales in particular have been implicated in facilitating petroleum degradation directly and indirectly through oil droplet emulsification and the creation of oxic/anoxic zones within a biofilm [7]. Additionally, the resulting algal biofilms may be used to generate a variety of useful bioproducts.
including biofuel feedstock, high value chemicals, pharmaceutically active compounds, animal feed, and bioplastics [4,8].

Phycocyanin is a major blue phycobiliprotein pigment found in cyanobacteria that has many potential applications in cosmetics, foods, medicine, and biotechnology [9–11]. Widely used as a label for immunoassays and fluorescence diagnostics, phycocyanin contains covalently bound phycocyanobilin chromophores that have highly specific and intense fluorescent properties [12]. Production and accumulation of phycocyanin by cyanobacteria varies greatly and is regulated by many environmental factors including light intensity, temperature, and nutrient availability [13–16]. The degree of phycocyanin purity is dependent on cellular yields, lysis methods, pH of extraction solvents, use of cold temperatures and low light to reduce degradation, and the co-extraction of contaminants [17–19]. Production of high value phycocyanin is currently dominated by the outdoor culture of *Arthospira platensis* in open ponds and raceways [9]. This style of cyanobacteria culture generally requires large volumes of prepared growth medium, and expensive harvesting and drying operations. The RABR growth platform coupled with utilizing produced water as a growth medium may reduce the costs of phycocyanin production. The aims of this study were to determine the growth of cyanobacteria dominated biofilms using produced water as a growth medium, and to determine the production of phycocyanin by the resulting biofilms.
2. Material and Methods

2.1 Organism Isolation and Characterization

Cyanobacteria used in this study were obtained from harvesting the upper layer of a mixed culture algal biofilm from pilot scale RABRs operated at the Logan City, Utah municipal wastewater treatment facility, a 460 acre (1.86 km²) open lagoons system [2]. A unialgal biofilm forming culture was obtained on cotton rope in produced water supplemented with ACS grade 3.0 g/L NaNO₃, 0.5 g/L K₂HPO₄, and 50 mg/L cycloheximide [20].

To characterize the unialgal isolate, referred to hereafter as Logan Lagoons Cyanobacteria 2 (LLC2), a crude cell lysate was used as template DNA for 23S plastid ribosomal DNA primers previously described by Sherwood & Presting (2007). The PCR mixture contained 5 μL of 10x PCR buffer, 8 μL of 25 mM MgCl₂, 2 μL containing 2 mM of each deoxynucleotide triphosphate, 1 μL dimethylsulfoxide, 1 μL each of 50mM forward and reverse primers (Eurofins Genomics, Huntsville, AL), 0.5 μL Taq DNA polymerase (Fermentas, Pittsburgh, PA), and 2 μL DNA template for a 50 μL reaction volume. PCR amplification was performed using the following conditions: 2 min denaturation step at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at 54.4°C, and 1 min at 72°C followed by a final elongation at 72°C for 10 min. PCR products were purified after agarose gel electrophoresis using Qiagen QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and sequenced by the Utah State University Center for Integrated Biosystems (Logan, UT). The resulting sequence chromatograms were examined for readout noise using 4Peaks (Netherlands Cancer Institute, Amsterdam, The Netherlands).
Forward and reverse 23S sequences were then aligned using BLASTn and then compared against NCBI’s nucleotide collection database, http://blast.ncbi.nlm.nih.gov (Table 1).

2.2 Growth Conditions

Rotating Algal Biofilm Reactors (RABRs) (8.9 cm dia. x 17.8 cm L) and associated 1 L working volume acrylic tanks were constructed and physically operated as described by Christenson and Sims (2012). The bioreactors were constructed with 3/16 in. dia. (0.476 cm dia.) solid braid cotton rope and operated in previously aerated produced water amended with 3.0 g/L NaNO₃ and 0.5 g/L K₂HPO₄ [22]. Produced water used in this study was obtained from the Southern Cross disposal facility near Baggs, Wyoming.

A 1000 W sodium vapor lamp coupled with a 24% transmittance neutral density filter (Rosco, Sun Valley, CA), provided 220 µmol photons m⁻²s⁻¹ of photosynthetically active radiation to the upper most surface of the RABR units over a 14 h on: 10 h off light cycle. The water temperature within the tanks averaged 20±2°C. A 1 g centrifuged wet weight inoculum, previously grown in produced water, was added to each RABR cotton rope substrata 15 min before beginning rotation of the reactors.

2.3 Biomass Determination and Phycocyanin Extraction

Biofilms harvested from cotton rope substrata were lyophilized for biomass determinations, ash free dry weight (AFDW) analysis, and phycocyanin extraction. AFDW was performed using lyophilized material at 550°C. AFDW and phycocyanin productivity were normalized to the areal view surface footprint of the operating reactor (0.0175 m²) while yields were normalized to available growth surface area on substrata.
Phycocyanin extractions were performed by first re-suspending lyophilized powdered biomass in E-Pure deionized water and rehydrating the material for 15 min. The samples were then subjected to two freeze/thaw cycles with a subsequent 2 h extraction by agitation on a Thermolyne Speci-Mix rocker table (Thermo Fisher Scientific, Waltham, MA). Following centrifugation for 15 minutes at 12,000 g, the crude extract supernatant phase was collected and analyzed for phycocyanin concentration and extract purity.

2.4 Phycocyanin Identification and Quantification

Phycocyanin concentration in the crude extract was determined by the methods of Bennett and Bogorad (1973). Phycocyanin purity in extracts was measured as the ratio of the optical absorbances at 620 nm and 280 nm [24]. Phycocyanin (PC) yields were calculated as PC Yield (mg PC/g AFDW) = PC conc. (mg PC/ml)* extraction volume (ml)/ AFDW of biomass (g).

SDS-PAGE was conducted on the crude phycocyanin extract of LLC2, Spirulina powder (Bio-Alternatives, Klamath Falls, OR) (positive control), *Chlorella vulgaris* UTEX 2714 (negative control), phycocyanin standard (AnaSpec, Fremont, CA), and a Bio-Rad Kaleidoscope Precision Plus protein standard ladder using a precast 10%-20% linear gradient Tris/HCl Ready Gel (Bio-Rad, Hercules, CA). Samples were boiled in water for 10 min in Laemmli sample buffer and β-mercaptoethanol. The resolved gel was rinsed and soaked for 5 min in a 20 mM zinc sulfate solution and phycocyanin subunit fluorescence was visualized over a 302 nm Benchtop 3UV Transilluminator lamp (UVP,
Upland, CA)[25]. Subsequently, the gel was rinsed in deionized water and stained with Coomassie Blue G-250 (Bio-Rad, Hercules, CA).

2.5 Statistical Analysis

All biomass and phycocyanin extraction experiments were conducted in triplicate with independent measurements. Error bars represent one standard deviation from the mean of the samples taken.

3. Results and Discussion

3.1 Organism Characterization

Inspection of the PCR products after agarose gel electrophoresis and the sequence chromatograms suggests that the Logan Lagoons Cyanobacteria 2 (LLC2) is a unialgal isolate. The majority of the top BLAST hits for the LLC2 sequence were organisms of the Order Oscillatoriales including the Genera Oscillatoria, Plectonema, Leptolyngbya, and Nodosiliena (Table 1). The LLC2 isolate 323 nucleotide 23S rDNA sequence does not have 100% identity to any organism in the NCBI database and is considered a novel cyanobacterial isolate capable of growth in produced water.
Table 1
Most significant BLAST hits with 100% query coverage for 23S rDNA primer amplicon of unialgal isolate Logan Lagoons Cyanobacteria 2 (LLC2).

<table>
<thead>
<tr>
<th>Organism</th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncultured algae L012</td>
<td>95</td>
</tr>
<tr>
<td>Nodosilinea sp. FI22HA2</td>
<td>94</td>
</tr>
<tr>
<td>Nodosilinea epilithica str. Kovacik</td>
<td>94</td>
</tr>
<tr>
<td>Nodosilinea sp. Rehakova 1960/20</td>
<td>94</td>
</tr>
<tr>
<td>Plectonema terebrans CCAP 1463/4</td>
<td>94</td>
</tr>
<tr>
<td>Leptolyngbya sp. PCC 7104</td>
<td>93</td>
</tr>
<tr>
<td>Uncultured organism clone C6.12</td>
<td>90</td>
</tr>
<tr>
<td>Oscillatoria acuminate PCC 6304</td>
<td>89</td>
</tr>
</tbody>
</table>

3.2 Biomass Production

The growth of LLC2 in amended produced water was evaluated over a culture period of 29 days. The produced water used in this study was shown to support the growth of LLC2 biofilms using the RABR platform without dilution. Figure 1 shows the average yield (AFDW), by growth surface area, of the biofilms harvested from the RABR platforms operating in produced water. After an initial 8-day lag period, the inoculated biofilm averaged a daily areal productivity of 4.8±0.7 g Ash Free Dry Weight/m²-day during the exponential growth phase. The biofilm growth rate then slowed after day 20 to reach an average maximum yield of 19.6±1.1 g AFDW/m². The areal productivity achieved in this study with the RABR platform compares favorably with other laboratory and bench scale studies on algal biofilm growth [2,3,26–28]. The utilization of undiluted produced water for cyanobacterial growth provides for the production of algal biomass
from a large waste resource stream that may then be employed for the production of valuable bioproducts [8].

![Graph showing growth surface area AFDW and phycocyanin (PC) yields](image)

**Fig. 1.** Growth surface area AFDW and phycocyanin (PC) yields of harvested cyanobacterial biofilms grown in produced water (standard deviation shown, n=3 for all measurements).

### 3.3 Phycocyanin Production and Analysis

Phycocyanin accumulated in the cyanobacterial biofilms harvested from RABR platforms operating in produced water. Figure 1 shows the phycocyanin yields of the harvested biofilms over the 29 day growth period. The phycocyanin content of the biofilms increased in a growth associated manner, with a maximum yield of 16.9±3.4 mg/g AFDW on day 26.

Similarly, the areal yield of phycocyanin reached a maximum of 1350±173 mg/m² during the stationary period of the growth (Figure 2). Under the growth conditions of this
study, the harvested biofilms averaged 84.6±9.3 mg/m²-day (areal view) of phycocyanin productivity. The phycocyanin productivity observed in this study was lower than achieved with *A. platensis* grown in outdoor raceways utilizing prepared growth medium (820 -850 mg/m²-day) [29,30]. As the cellular concentrations of phycocyanin are highly dependent on environmental growth factors, such as light and nutrient availability, the outdoor growth conditions and Zarrouk’s growth medium used in the previously cited studies with *A. platensis* may have provided growth parameters more favorable for phycocyanin accumulation [13,14]. Further studies are required to examine nutrient amendments for increased phycocyanin production from produced waters, as well as the influences of outdoor environmental conditions.

**Fig. 2.** Areal yield of phycocyanin (PC) from cyanobacteria based RABR system utilizing produced water (standard deviation shown, n=3).
Higher degrees of phycocyanin purity in extracts (measured spectrophotometrically as the ratio of absorbance values at 620 nm and 280 nm) were achieved as the cellular phycocyanin content increased (Figure 3). A maximum average phycocyanin purity absorbance ratio of 0.23±0.03 was achieved on day 26 of the growth cycle corresponding to the peak phycocyanin yield of the harvested biomass. Harvesting the biofilm when cellular concentrations of phycocyanin are the highest will result in lower downstream phycocyanin purification processing costs. Much phycobiliprotein production research and economic analysis has focused on scalable downstream processes with goals of achieving phycocyanin food, reagent and analytical grade purities with absorbance ratios of 0.7, 3.9 and 4.0, respectively [9].

![Fig. 3. Crude phycocyanin extract purity of RABR harvested cyanobacterial biomass grown in produced water (standard deviation shown, n=3).](image-url)
Zinc staining of the SDS denaturing gel displayed the bright orange fluorescence of phycocyanobilin and other porphyrins when associated with zinc ions and UV light [17]. The alpha and beta subunits of phycocyanin in the LLC2 crude extract and standard can be seen as predominant in both the zinc and Coomassie Blue stained gel (Figure 4) suggesting that phycocyanin makes up a large percentage of the water-soluble protein fraction from the LLC2 dominated biofilm.

Fig. 4. SDS-PAGE of aqueous crude extract of cyanobacteria based biofilm cultivated in produced water visualized by Coomassie Blue (A) and zinc sulfate (B) stain. Lane 1: Kaleidoscope Precision Plus standard ladder (Bio-Rad), Lane 2: Crude extract of Logan Lagoons Cyanobacteria 2, Lane 3: Phycocyanin standard (AnaSpec), Lane 4: Crude extract of Spirulina powder (Bio-Alternatives) (positive control), Lane 5: Crude extract of Chlorella vulgaris UTEX 2714 (negative control).

4. Conclusions

The growth of Logan Lagoons Cyanobacteria 2 and its production of phycocyanin have been demonstrated in undiluted produced water utilizing a Rotating Algal Biofilm
Reactor. A biomass areal productivity of 4.8±0.7 g AFDW/m²-day and areal phycocyanin productivity of 84.6±9.3 mg/m²-day were observed. Produced water utilization for the production of cyanobacterial biomass and high value products, such as phycocyanin, may increase the value of this waste stream that is produced during hydrocarbon extraction. Future studies utilizing produced water are planned that will focus on increasing biomass and phycocyanin yields using the scalable and sustainable biofilm based RABR processes developed in this present research work.

Acknowledgments

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References


CHAPTER III

MICROALGAE-BASED BIOFILM PHOTOBIOREACTORS: INFLUENCE OF LIGHT INTENSITY AND GROWTH SUBSTRATA ON PHYCOCYANIN AND BIOMASS PRODUCTION FROM PRODUCED WASTEWATER

Authors: Jonathan L. Wood, Jon Y. Takemoto, and Ronald C. Sims

Abstract

The production and enhancement of high value phycocyanin pigment from microalgal biofilms cultured on oilfield and natural gas produced wastewater were investigated. Logan Lagoons Cyanobacteria selection 2 was cultured in amended produced water using rotating algal biofilm reactors. The bioreactors were operated under “low” and “high” light conditions and biomass and phycocyanin content were compared. Phycocyanin content was enhanced by growth under low light conditions to a maximum of 31.7 mg/g AFDW biomass for an 87.6% increase in phycocyanin yield. Phycocyanin productivity was equivalent for both the low and high light treatments (327±81 and 305±39 mg/m², respectively), due to the significantly lower AFDW biomass productivity of the low light treatment (2.7±0.4 g/m²-day). An evaluation of 14 growth substrata showed that cotton rope and cotton belt material were the most durable and provided the highest biomass yields. Further evaluation in an outdoor produced water pond showed that the biomass characteristics from the two substrata differed. The corrugated surface area of the cotton rope cultured a biofilm with a large community of non-photosynthetic organisms having an autotrophic index of 507 and a low phycocyanin yield (3.4 mg/g
AFDW). However, the cotton belt substratum cultured a healthy photosynthetic biofilm with an autotrophic index of 127 and a phycocyanin yield of 47.0 mg/g AFDW. These results show that phycocyanin and biomass yields from a rotating algal biofilm reactor operating in produced water can be modified utilizing bioreactor design and operation parameters.

1. Introduction

“Produced water” disposal from oil and natural gas extraction is a growing problem in the U.S.A. and around the world. Oil extraction in the U.S.A. generated an average ratio of over 10:1 liters of produced water to oil, for an average of just over 3.3 million megaliters of produced water in 2012. Aging wells in arid regions such as Texas accounted for the largest portions of produced water generated. Over 91% of this wastewater is reinjected in disposal wells or into formations for enhanced recovery while much of the remainder is stored in lined ponds before further treatment [1]. This portion of produced water represents an opportunity for algal cultivation and beneficial use of the biomass generated.

Sullivan et al. [2] have conducted a thorough review on produced water as a growth medium for microalgal cultivation. They found that although produced water can have high salinity ranges and organic chemical constituents detrimental to growth of microalgae, it has the advantage of containing inorganic nutrients needed for microalgal growth. This reduces the need for expensive micronutrients and buffer additions. The authors also indicate that microalgal cultivation in produced water represents an opportunity for wastewater treatment and biofuels generation [2]. Few reports of using
produced water as a microalgal cultivation medium have been published. Most report using endogenous environmental microbes, highlighting the need to explore microalgal strains optimized for produced water chemical compositions [2–5].

Currently, most of the investigations into produced water as a cultivation medium focus on microalgae as a biofuel feedstock with limited published work on generating high value bioproducts [6]. High value bioproduct side streams can be integrated into an microalgal biorefinery to potentially improve the economics of microalgal biomass derived biofuels as well as offset capital costs [7].

Phycobiliproteins and their derivatives have been identified as high value products and their recovery can be integrated into a microalgae biorefinery operation [7]. Phycobiliproteins are water soluble and their easy extraction in biorefinery operations will have minimal impacts on the recovery efficiencies of energy dense lipids. In cyanobacteria, phycobiliproteins are assembled in large complexes (phycobilisomes) that harvest and funnel light energy to chlorophyll. The phycobiliprotein phycocyanin is used as a natural blue food dye as well as a laboratory fluorescent agent; and it also has antioxidant, antitumor, antiviral, and anti-inflammatory effects [7,8]. Phycocyanin’s brilliant blue color is due to its phycocyanobilin chromophore. In addition to color, phycocyanin derives many of its therapeutic effects from phycocyanobilin [7–9]. Therapeutic compositions of phycocyanobilin have been reported to be effective at low micromolar concentrations, making them potent antioxidants [10].

In limited light conditions, Oscillatoriales cyanobacteria increase the size and number of phycobilisomes present on their cellular thylakoid membranes to maximize light energy capture for photosynthesis [8,11]. This biological strategy is complemented
by using the high efficiency energy absorption and transfer capabilities of phycobilisome
phycocyanin in the upper red-orange end of the visible light spectrum.

A Rotating Algal Biofilm Reactor (RABR) platform was used as a microalgal
photobioreactor for this study due to the generation of high solids content, its ability to
use high turbidity produced water, and the bias toward the selected cyanobacteria strain
used to form biofilms. As previously described, the RABR can yield a harvested biomass
slurry with a solids content of up to 12-16% with a cotton rope growth substratum [12].
For increased ease of scale up, harvesting, and bioreactor maintenance, flat-belt growth
substratum materials as opposed to previously used rope substrata [12–15] were
investigated.

The main purposes of this study were to evaluate the influences of light intensities
and growth substrata materials on biomass and phycocyanin production by RABR
microalgal biofilms operating in produced water.

2. Materials and Methods

2.1 Laboratory Growth Conditions

Logan Lagoons Cyanobacteria selection 2 (LLC2), described previously [6], was
cultured using 1 L Rotating Algal Biofilm Reactors (RABRs) with a bioreactor areal
footprint of 0.0175m² [6]. The bioreactors were fitted with 0.476cm dia. solid braid
cotton rope (Knot and Rope Supply, Perrysburg, OH) as a growth substratum. Produced
water (Southern Cross, Baggs, WY) with a conductivity of 19400 µmhos/cm was
amended with 3.0 g/L NaNO₃ and 0.5 g/L K₂HPO₄ for use as a growth medium. Physical
operation was performed as in Christenson & Sims (2012) [6,12] with 16-21º C daily
growth medium temperatures. Light was provided on a 14 hrs. on: 10 hrs. off cycle by 1000 W sodium vapor lamps and fluorescent bulbs fitted with neutral density filters (Rosco, Sun Valley, CA) to provide “low” (40 μmol photons m\(^{-2}\)s\(^{-1}\) PAR) and “high” light (220 μmol photons m\(^{-2}\)s\(^{-1}\) PAR) growth conditions. An inoculum (1 g centrifuged wet weight) of LLC2, previously grown in produced water medium, was added to the bioreactors before operation.

2.2 Greenhouse RABR Growth Substratum Experiments

Growth substratum testing was performed using laboratory scale RABRs as in Christensen and Sims (2012) in a greenhouse at Utah State University’s Innovation Campus during the month of May 2013. Substrata materials tested for microalgal biofilm biomass yields included four polyester, one acrylic, one ethylene vinyl acetate foam, seven cotton, and one burlap of various construction, surface characteristics, and durability. All materials tested were in a sheet configuration except for the 0.476 cm dia. cotton rope. After an initial inoculation of LLC2, the biofilms were allowed to seed and develop over a 29-day period before harvesting. Biomass yields are reported as the mean of duplicate measurements with error bars showing one standard deviation from the mean. Durability is reported as a qualitative assessment after the 29-day growth period after biomass harvest with a flat scraping blade.

2.3 Outdoor RABR Growth Conditions

A 2000 L outdoor produced water pond was constructed at the Algae Processing and Products facility on Utah State University’s Innovation Campus in Logan, Utah and operated during the months of August and September 2013. The pond was filled with
produced water from the Southern Cross produced water facility and amended with 1.5 g/L NaNO₃ and 0.5 g/L K₂HPO₄. The floating RABR unit was constructed with two 0.51 m² areal footprint drums to compare the biomass characteristics of the two best performing growth substrata materials previously tested, cotton conveyor belt and cotton 0.476 cm dia. rope, in an outdoor pond environment. Drum rotation was geared to provide the same peripheral velocity as laboratory scale units. The bioreactor units and pond were shaded with Gardener Sun Screen Fabric. For inoculation, 13 g wet centrifuged weight of LLC2 cyanobacteria was distributed along the bioreactor surface area at the start of the 45-day growth period. Biomass was harvested using a spool harvester (Christenson and Sims, 2012) for the rope and a flat scraping blade for the conveyor belt material [12].

2.4 Biomass, Phycocyanin, Phycocyanobilin, and Chlorophyll a Determinations

Biomass harvested from the growth substratum was lyophilized and powdered for Ash Free Dry Weight (AFDW) and phycocyanin analysis. Percent total solids, ash content, chlorophyll a, and Autotrophic Index of the harvested biomass were performed as in Eaton et al. (2005) [16]. Biomass and phycocyanin yields are defined based on surface area available to light exposure.

Phycocyanin extractions were performed by first resuspending lyophilized powdered biomass in E-Pure deionized water and rehydrating the material for 15 min. The samples were then subjected to two freeze/thaw cycles with a subsequent 2 hr extraction by agitation on a Thermolyne Speci-Mix rocker table (Thermo Fisher Scientific, Waltham, MA). Following centrifugation for 15 min at 12,000 g, the crude
extract supernatant phase was collected and analyzed for phycocyanin concentration and extract purity.

Phycocyanin (PC) concentration in the crude extract was determined by the methods of Bennett and Bogorad (1973) using the equation \[ PC = \frac{A_{620} - 0.474(A_{652})}{5.34} \] [17]. Phycocyanin purity in extracts was measured as the ratio of the optical absorbances at 620 nm and 280 nm [18]. Phycocyanin (PC) yields were calculated as PC yield (mg PC/g AFDW) = PC conc. (mg PC/ml) * extraction volume (ml)/ AFDW of biomass (g).

Phycocyanobilin was extracted from the unwashed and lyophilized outdoor pond RABR biomass as described previously [19] using 0.05 M sodium phosphate buffer (pH 7) as phycocyanin extraction buffer. The resulting mixture was centrifuged at 4500 g and 4° C for 90 min and the crude phycocyanin extract was used to form a 50% saturated solution of ammonium sulfate. After the phycocyanin solution was allowed to precipitate for 1 hr at 4° C, the mixture was centrifuged at 4500 g and 4° C for 1 hr and the phycocyanin pellet was washed with methanol 7X, until the supernatant was clear. The resulting phycocyanin pellet was heated at 60° C for 16 hrs in methanol and centrifuged to obtain the cleaved phycocyanobilin (PCB) chromophore in the supernatant. The PCB content of the crude extract was estimated in a 2% HCl/methanol solution using a molar attenuation coefficient of \( \varepsilon_{680}=37.9 \) mM\(^{-1}\)cm\(^{-1} \) [20–22].

3. Results and Discussion

3.1 Laboratory RABR Phycocyanin and Biomass Yields

Low light LLC2 biofilms produced nearly twice the amount of phycocyanin per biomass amount (maximum of 31.7±1.9 mg/g AFDW) compared to high light LLC2
biofilms (Figure 1). This increase in phycocyanin yield was accompanied by an increase in crude extract purity to just above the benchmark standard for food grade quality phycocyanin ($A_{620}/A_{280} = 0.7$) (Figure 2) [8,23]. These results highlight the malleability of the phycobilisome apparatus to differing light intensities which may be configured or controlled at large scale by the RABR photobioreactor design and operation.

**Fig. 1.** Phycocyanin (PC) Ash Free Dry Weight (AFDW) yields from harvested algal biomass with low (♦) and high (■) light growth conditions (one standard deviation shown, n≥3).
LLC2 grown with low light achieved a maximum areal Ash Free Dry Weight (AFDW) biomass productivity of 2.7±0.4 g/m²-day and a maximum growth surface area yield of 10.6±1.4 g/m² over a 38-day growth period (Figure 3). In contrast, growth with high light yielded nearly twice the ADFW productivity (4.8±0.7 g/m²-day) as compared to cultures grown with low light.
However, similar phycocyanin maximum surface area yields of 327±81 and 305±39 mg/m² were obtained, respectively, for low and high light (Figure 4). Both light conditions yielded statistically similar phycocyanin areal productivities equivalent to 94.0±31.4 mg/m²-day during exponential growth. Therefore, phycocyanin AFDW yields and purity varied inversely as a function of light intensity, but total phycocyanin productivity did not. As a consequence, different production goals may be achieved by varying the biofilm culture light level. Operations with a high demand for biomass and less need for phycocyanin purity would operate with higher light levels. Conversely, operations with a demand for high purity phycocyanin and less need for biomass would operate under lower light levels. Operating at a low light level would reduce biomass processing costs for high-purity phycocyanin extraction by reducing the volumes of processed biomass [7].

**Fig. 4.** Growth surface area yields of phycocyanin (PC) from low (♦) and high (■) light conditions (one standard deviation shown, n≥3).
3.2 Greenhouse Growth Substratum Evaluation

Growth substrata were evaluated for biomass growth and durability (Table 1 and Figure 5). Natural materials displayed better biofilm attachment and harvestable growth, as seen in previous studies, although with varying degrees of durability over a 29 day growth period [12,14,24]. Of all substrata tested, cotton rope gave the highest harvestable biomass yield of 34.3±5.9 g/m² AFDW, or a nearly 225% increase in biomass compared with the next highest biomass yield from cotton conveyor belt material (15.3±2.8 g/m² AFDW). Other natural materials: burlap, black cotton broad cloth, and duck cotton materials gave lower levels of biomass. Black cotton broad cloth yielded five times the AFDW biomass compared with white cotton broad cloth (12.6±3.5 g/m² AFDW and 2.2±0.5 g/m² AFDW, respectively) (Figure 5). Synthetic materials such as polyester, acrylic, and ethylene vinyl acetate did not yield harvestable growth. The exception was Pellon Peltex 70 which provided low levels of harvestable growth at 1.6±1.0 g/m² AFDW. Substrata durability was observed after 29 days of greenhouse growth with the highest durability observed for thick belt and cotton rope materials (Table 1).
Fig. 5. Growth Substratum Evaluation of biomass Ash Free Dry Weight (AFDW) yields (■) (one standard deviation shown, n=2).

Table 1
Qualitative durability assessment of different growth substrata.

<table>
<thead>
<tr>
<th>Material</th>
<th>Durabilitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>solid polyester belt</td>
<td>++++</td>
</tr>
<tr>
<td>cotton-polyester blend belt</td>
<td>++++</td>
</tr>
<tr>
<td>cotton conveyor belt</td>
<td>++++</td>
</tr>
<tr>
<td>Pellon Peltex 70</td>
<td>+++</td>
</tr>
<tr>
<td>cotton rope</td>
<td>+++</td>
</tr>
<tr>
<td>duck cotton</td>
<td>+++</td>
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<tr>
<td>Pellon 931TD Mid wt. fusible</td>
<td>+++</td>
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<tr>
<td>ethylene vinyl acetate foam</td>
<td>++</td>
</tr>
<tr>
<td>cotton broad cloth (white)</td>
<td>++</td>
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<tr>
<td>cotton broad cloth (black)</td>
<td>++</td>
</tr>
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<td>polyester cloth</td>
<td>+</td>
</tr>
<tr>
<td>acrylic felt</td>
<td>+</td>
</tr>
<tr>
<td>burlap</td>
<td>+</td>
</tr>
<tr>
<td>muslin</td>
<td>+</td>
</tr>
<tr>
<td>cotton batting</td>
<td>+</td>
</tr>
</tbody>
</table>

a. High durability = ++++, low durability = +
3.3 Outdoor RABR Biomass Composition and Phycocyanobilin Extraction

Based on the above described results and of other studies [12,14], cotton rope and cotton conveyor belt materials were selected for experiments with a larger size floating RABR in an outdoor pond. Biomass harvested from the cotton belt substratum vs. the cotton rope substrata differed greatly in yield and composition at the end of a 45-day growth period (Table 2). Visually, the cotton belt material yielded deep blue green biomass with a thick consistency, while the cotton rope yielded biomass in shades of red, brown, and green with a thin watery consistency. After microscopic inspection, contamination by “weed” algal species not was observed on a large scale although a large non-algal microbial community was observed on the rope substrata. The AFDW growth surface area biomass yield of the cotton rope material (24.2 g AFDW/m²) displayed a near 140% increase in AFDW biomass yield when compared to the cotton belt material (10.1 g AFDW/m²), similar to findings with the smaller RABR system in the greenhouse trials. The lower biomass yields of the outdoor RABR compared to yields for the greenhouse RABR may be due to the 10-15 °C colder average daily temperatures observed during the outdoor testing period in August-September. These results highlight the importance of a regional and seasonal evaluation of microalgal growth performance when considering outdoor microalgal growth systems.
Table 2
Comparison of properties of biomass cultivated on cotton rope versus flat cotton belt substrata on an outdoor floating RABR.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Cotton Belt&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cotton Rope&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Dry Biomass Yield (g/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>14.7</td>
<td>42.1</td>
</tr>
<tr>
<td>AFDW Biomass Yield (g/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>10.1</td>
<td>24.2</td>
</tr>
<tr>
<td>% Solids Harvested</td>
<td>6.90%</td>
<td>4.20%</td>
</tr>
<tr>
<td>% Ash Content</td>
<td>31.5%</td>
<td>42.5%</td>
</tr>
<tr>
<td>Phycocyanin Yield (mg PC/g dry biomass)</td>
<td>32.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Phycocyanin Yield (mg PC/g wet biomass)</td>
<td>2.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Phycocyanin Yield (mg PC/g AFDW biomass)</td>
<td>47.0</td>
<td>3.4</td>
</tr>
<tr>
<td>Phycocyanin Extract Purity (A&lt;sub&gt;620/A&lt;sub&gt;280&lt;/sub&gt;)</td>
<td>0.432</td>
<td>0.077</td>
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<tr>
<td>Chlorophyll a + pheophytin a (mg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>84.9</td>
<td>71.3</td>
</tr>
<tr>
<td>Chlorophyll a (mg/g dry biomass)</td>
<td>5.4</td>
<td>1.1</td>
</tr>
<tr>
<td>Chlorophyll a (mg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>79.4</td>
<td>47.7</td>
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<tr>
<td>Autotrophic Index (AI)</td>
<td>127</td>
<td>507</td>
</tr>
<tr>
<td>664nm/665nm of Chl a extract</td>
<td>1.67</td>
<td>1.42</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average value shown, n=3-4.

While the cotton rope substratum produced significantly more AFDW biomass than the cotton conveyor belt material, the rope also displayed 11% higher ash content and 2.7% lower percent solids in the recovered biomass when compared to the belt material (Table 2). The higher saline water content in the harvested rope biomass may be due in part to the spool harvester method of harvesting biomass versus the simplified flat scraper blade used for the belt material. The increased water and ash content of the rope material significantly increases the cost of processing by increasing both the overall process input volumes and the potential low value waste volumes in ash.
The phycocyanin yield from the two materials differed greatly with the cotton belt material displaying 47.0 mg PC/g AFDW when compared to the cotton rope material at 3.4 mg PC/g AFDW (Table 2). Similarly, the crude phycocyanin extract purity was 5.6 times higher with the cotton belt material, but not to the food grade purity level of 0.7 without further purification steps [18]. Due to the low phycocyanin levels and purity in the biomass from the cotton rope material, phycocyanobilin (PCB) content was evaluated solely in the biomass from the cotton belt material. The crude PCB extracts from the cotton belt material yielded an average of 0.34±0.01 mg PCB/g AFDW biomass, or roughly 3.4 mg PCB/m² of growth surface area. This corresponds to a 13.7±0.5 percent of theoretical yield of PCB from the LLC2 biomass, similar to those found by D. J. Chapman et al., 1967 [20,25].

Phycocyanin and phycocyanobilin and its derivatives have possible applications as antioxidants, anti-inflammatories, fluorescent labels, and coloring agents [8,10,26]. Phycocyanobilin can be converted to mesobiliverdin IXα which has similar cytoprotective and therapeutic potential as its close analog, biliverdin IXα [19]. High value phycocyanin and phycocyanobilin, after additional purification, may help offset wastewater treatment costs, or provide a revenue stream for produced water disposal operations.

Chlorophyll a extractions of the harvested biomass showed that the cotton belt material yielded nearly five times the chlorophyll a content than that of cotton rope in mg/g of biomass (Table 2). From a growth surface area perspective, the chlorophyll a yield of the cotton belt was 79.4 mg/m² and the cotton rope 47.7 mg/m². When combined
with the 664nm/665nm ratios of the cotton belt and cotton rope, 1.67 versus 1.42 respectively, the overall photosynthetic physiological condition of the cotton belt biomass was superior to that of the cotton rope [16]. The autotrophic index was calculated as 127 and 507 for the cotton belt and cotton rope, respectively. Values between 50-200 are typical of autotrophic biofilms, with higher values indicating a large consortium of heterotrophs. These results suggest that the cotton rope material supported a substantial portion of heterotrophs in the attached biofilm when compared to the cotton belt despite both materials being 100% cotton in construction.

Differences in the surface characteristics may be related to the different biomass characteristics in the recovered biomass with respect to AFDW yield, phycocyanin, autotrophic index, and chlorophyll a content. While the cotton belt provides a uniform growth surface area with regards to exposure to light, the cotton rope substratum creates voids with limited and no light exposure. The limited light exposure in these areas are an ideal settling area for heterotrophic bacteria, detritus, and grazers. These areas of limited light exposure are then harvested when using the spool harvester design that is avoided with the flat scraper blade used in harvesting the cotton conveyor belt material. Heterotrophic biofilms are widely documented in rotating biological contactors without exposure to a light source and are essential to many wastewater treatment processes including hydrocarbon removal, volatile organic compound control, and heavy metals remediation [27–31]. With a relatively large biomass yield and both phototrophic and heterotrophic biomass zones, the rope substratum may be beneficial for operations where wastewater treatment and/or large biomass yields is the primary goal. Alternatively, the cotton belt substratum may be better suited to operations with goals of harvesting
phototrophic cyanobacterial biomass in good physiological condition for downstream product development.

4. Conclusions

It was shown that the phycocyanin content of LLC2 cyanobacteria can be modified by varying the light intensities provided during growth. The increase in phycocyanin content for light limited cultures, was accompanied by a lowered biomass productivity which resulted in an areal phycocyanin productivity equivalent to the productivity of high light cultures. Cotton rope growth substratum showed the highest biomass yields of all materials tested, although a large non-photosynthetic community was observed. Cotton cloth growth substratum was shown to be a better choice for phycocyanin and a lower autotrophic index for microalgal biofilm production in produced water. These results were likely due to the surface area available for light penetration and the biofilm harvesting mechanism used in this study. Future studies should be conducted to assess other high value products, such as other phycobiliproteins, pigments, and metabolites from microalgal based biofilms cultured in produced water. Analysis of downstream purification and potential contaminants should be evaluated for produced water cultured microalgae bioproducts. Additionally, the potential value of microalgal based biofilms in terms of biofuels, fertilizer, feed, and biogas potential should be assessed. To the best of the authors knowledge, this is the first reported application of cyanobacteria-dominated biofilms cultured in an outdoor produced water pond for the production of high value pigments.
Acknowledgments

The authors would like to thank the Environmental Department of the City of Logan, Utah (Contract #090203), the Utah Water Research Laboratory (Contract #WR-1089), and the Huntsman Environmental Research Center (Grant #A17779) for providing financial and material support for this study. We would also like to acknowledge the Utah Science Technology and Research (USTAR) initiative and the Sustainable Waste to Bioproducts Engineering Center (SWBEC) and Dr. Dong Chen for the generous access to their laboratory facilities and ongoing support. Alan Hodges, Tyler Gladwin, and Cody Maxfield were instrumental in their supporting roles.
References


CHAPTER IV

ENGINEERING SIGNIFICANCE AND RECOMMENDATIONS FOR FUTURE RESEARCH

The culture of cyanobacteria dominated biofilms in produced water to produce high value bioproducts has not been previously reported in the peer reviewed literature to the best of the author’s knowledge. This potential application of algal biotechnology may provide an additional economic opportunity in many oil and gas regions. Algal culture utilizing produced water as a growth medium is a very new field with many opportunities for wastewater characterization, water conservation, bioremediation, bioreactor design, and bioproducts development. The following is a discussion of the engineering significance this work, observations, and recommendations for future work in this field.

A prominent issue when considering the use of produced water as an algal growth medium is wastewater characterization and supply. A robust partnership with the oil and gas produced water industries is important for the free flow of information and possible co-location of facilities. Advantages to both the algal industry and produced water generators are the reduction of freshwater use for algal culture and beneficial reuse of produced waters. For thriving algal culture operations, produced waters must be characterized and mapped regionally for their resulting formational constituents such as salinity. The varied nature of regionally produced water compositions highlights the selection of algal strains that can thrive and produce bioproducts in an array of regional produced waters.

One option to standardizing produced water characteristics is the blending of
regional produced waters in equalization ponds. The benefit of this method is that most produced water disposal operations have storage ponds that currently fit this purpose. A second likely required option for many produced water regions is pretreatment of produced waters to make them suitable for algal culture medium. Throughout this study, produced water was aerated thoroughly before mixing in nitrogen and phosphorous amendments. Aeration removed or reduced many of the volatile dissolved organics that can be toxic to algal growth at high levels as well as oxidized reduced compounds and stabilized pH. Another consideration is the co-precipitation of added nutrient mixes with constituents in the produced water, particularly divalent ions and phosphates. Careful balancing of mineral additions with the make-up of the selected produced waters is needed to minimize nutrient precipitation.

Scale up of suitable bioreactors is another essential consideration for algal cultivation in produced waters. High turbidity levels in many produced waters will necessitate pretreatment for clarification when using suspended algal cultivation systems. Common pretreatments, depending on produced water constituents, are flocculation, filtration, centrifugation, pH adjustment, EDTA addition, and ultraviolet light or ozone treatment. These pretreatments can be an added economic burden and increase additional unit processes to the cultivation stream. In order to circumvent the need for clarification of produced water before use, the Rotating Algal Biofilm Reactor (RABR) was envisioned as a modular addition to existing deep produced water ponds with minimal additional footprint needed for algal cultivation units. Small RABR units were constructed for laboratory scale studies with larger floating units being constructed for outdoor and field evaluations (Figure 1).
Important engineering considerations with the RABR platform are the rotational speed, surface area: media volume ratio, and design construction. Laboratory scale and floating RABR units were constructed with a similar tangential velocity to keep biofilm behavior constant under the physical rotating operation of the RABR. One factor that may be considered with large diameter units is the increased “sheeting” velocity of culture media as the unit rotates in and out of the culture medium basin. This was not observed at the largest scale used in this study, the floating RABR units, and may be mitigated by the proportionally slower rotation speeds needed to maintain a constant tangential velocity as the RABR radius increases (dimensions in Appendix C). Rotational speeds and duty cycles may be optimized for algal growth but must be balanced with operational costs and motor start up energy requirements.

Optimization of the growth surface area: culture media volume ratio is another important consideration in both bioremediation potential and bioproducts generation. High growth surface to culture media ratios will increase biomass yields if sufficient light and nutrient loading rates are able to be maintained. A high growth surface to culture
media ratio will also increase bioremediation potential as contaminants are removed or metabolized by the growing biofilms for a given loading rate. For example, a two-stage scenario can be envisioned for the enhancement of lipid yields from RABR biofilms operating in produced water, or other culture media, where nutrient loading rates are increased for a growth phase to develop a high biomass yield, and then a second phase of limited nutrient loading (or combined with bicarbonate addition) is implemented to increase lipid yields.

For phycocyanin and phycocyanobilin generation, an important parameter is light availability and shading. As demonstrated in this study, lower light intensities will increase the phycocyanin yield and will also lower growth rates providing areal phycocyanin productivities similar to those at higher light intensities. This adjustment of phycocyanin content may be used to economic advantage to generate higher purity phycocyanin and to process less biomass volume with a light limited RABR. Alternatively, an algal biorefinery would highly value the residual biomass left over from phycocyanin extraction where it may be used for biofuels, or additional bioproducts. In the biorefinery scenario, a higher light supplying design and placement would generate more algal biomass although with lower phycocyanin crude extract purity and higher extraction costs due to increased biomass process volumes.

Phycocyanin bioproduct development and downstream processing is another area of opportunity for development at laboratory and large scale. There are many opportunities for research in phycocyanin extraction from the RABR system and the cost analysis of its different forms. Of particular need are studies on the potential contaminants carried throughout the selected purification processes. Contaminants of
concern are metals, toxic organic constituents, cyanotoxins, and beta-methylamino-L-alanine.

As laid out in this study, scale up of phycocyanin crude extraction from RABR biofilms will include at least four unit processes: a buffer mixing or biomass washing step, a cell lysis method, a phycocyanin extraction step, and a solids separation step. An advantage of the RABR system is the high solids content of the harvested biomass, reducing the need to concentrate the biomass slurry further unless the culture medium is incompatible with the downstream processes or final product. A buffer mixing step will be needed to increase yields and prevent degradation of the phycocyanin crude extract, if the biomass is not washed thoroughly of residual salts or the lysate pH is determined to be suboptimal. A lysis method for the resulting biomass slurry is generally needed on most cyanobacteria species, with high pressure homogenization being the easiest to scale up but with a generally lower crude extract purity. A thorough investigation into scalable, but gentle, lysis methods is needed for efficient and selective extraction of phycocyanin, with each strain of cyanobacteria generally having optimally unique requirements. Extraction times and methods will depend on the lysis techniques used, but generally can be short as phycocyanin is highly water soluble. A simple mixing tank or mixing pipe system will provide efficient extraction. Finally, a separation process will be needed with centrifugation or cross flow filtration being the two most promising options.

The cleavage of phycocyanobilin (PCB) from phycocyanin was achieved with a boiling methanol separation of chromophore and apoprotein. This method is by far the most widely used for chromophore separation in phycocyanin yet yielded only 13.7±0.5% of the theoretical yield of PCB, similar to values found in literature. Further
investigations into more efficient extraction methods of PCB from phycocyanin are needed as values range from 10.3% to 100% depending on the chromophore cleavage method used [1,2]. An economic and market analysis of phycocyanin derived products would be of value to researchers and industry to determine the profitability and feasibility of integrating these high value products into algal culture in produced waters.
References


The utilization of produced water as an algal growth medium is a new field with few studies reporting growth of algae in produced waters for bioproduct generation. Descriptions of growth rates and biomass productivities from biofilm or algal mat cultures in produced waters are sparse in the professional refereed literature and generally focus on bioremediation potential and tolerance to organic chemicals. The large supply of produced wastewater highlights its potential as a non-freshwater algal growth medium. Further studies on the integration of an algal biorefinery into produced water treatment and beneficial reuse will be valuable to produced water generators and the algae industry.

In satisfying Objective A, the growth of cyanobacteria dominated biofilms on a Rotating Algal Biofilm Reactor (RABR) platform was demonstrated using produced water as an algal growth medium. The novel Logan Lagoons Cyanobacteria selection 2 (LLC2) strain used in this study was isolated from pilot scale RABRs operating at the Logan City Wastewater Treatment Facility. Ash Free Dry Weight biomass areal productivities of up to 4.8±0.7 g/m²-day were reported from growth on laboratory scale RABRs operating in produced water medium. Although higher in biomass productivity than many other lab scale wastewater algal biofilm studies reported, optimization of biomass yields is needed to explore the possibility of increased yields.

An evaluation of 14 different growth substrata materials was performed with natural cotton materials, such as cotton conveyor cloth and cotton rope, showing the highest biomass yields and durability. Biomass characterization from a floating RABR
operating in an outdoor produced water pond showed that cotton rope had an AFDW biomass yield of near 140% more than cotton cloth. Due to growth surface characteristics and spool harvesting technique, the biomass from the cotton rope displayed a lower algal pigment content and high heterotroph: autotroph ratio when compared to the biomass harvested by a flat scraping method from the cotton cloth. Although little to no biomass growth was observed in this study with synthetic materials, a thorough investigation into synthetic growth substrata may yield promising results and increased long term durability.

The production of high value products like phycocyanin and phycocyanobilin have the potential to make an algal biorefinery more economical. In satisfying Objectives B and C, phycocyanin content was evaluated and enhanced in algal biofilms cultured in produced water and phycocyanobilin production was demonstrated. An areal phycocyanin productivity of to 84.6.0±9.3 mg/m²-day was reported from laboratory scale RABRs operating in produced water. Enhancement of phycocyanin yields by light limitation was investigated with a resulting increase in phycocyanin yields from 16.9±3.4 mg/g AFDW to 31.7±1.9 mg/g AFDW and an increase in $A_{620}/A_{280}$ phycocyanin purity of 0.23 to 0.76. The increase in phycocyanin yields during light limitation was offset by a lowered areal biomass productivity of 2.7±0.4 g AFDW/m²-day. This resulted in statistically similar phycocyanin areal productivities for the two light intensity levels used in this study. Further investigation into phycocyanin enhancement of algal biofilms may be particularly beneficial to phycocyanin specific producers who wish to maximize phycocyanin yield and purity while keeping biomass processing volumes to a minimum. Integration of the RABR platform with phycocyanin enhancement designs is needed for
the flexible scale up of high value phycobiliprotein pigment production.
APPENDICES
APPENDIX A

OPTIMIZATION OF PHYCOCYANIN EXTRACTION METHODS AND BUFFER CONCENTRATION

Figures A1-A9 show method development for phycocyanin extraction from unwashed Logan Lagoons Cyanobacteria selection 2 (LLC2) grown as a biofilm in produced water medium. Figures A1-A6 show the optimum phycocyanin extraction procedure in terms of yield and purity to be using 0.05 M sodium phosphate buffer at pH 7 with either a 2 hour extraction time for higher purity and slightly less yield, or a 16 hour extraction time for a slightly higher yield and less purity. Mixing method had no impact on the yield or purity of the crude extracts (Figures A3 and A4). Figures A7 and A8 show that two freeze thaw cycles provided the highest yields and purities with the least variation. Figure A9 shows the impact on pH of residual salts from unwashed LLC2 biomass, showing the importance of extraction buffers if biomass is left unwashed. Figure A10 shows the phycocyanin standard curve used to verify the accuracy of the equation from Bennett and Bogorad 1973 that was used in this study. The comparison showed no statistical difference in phycocyanin yield between the method of Bennet and Bogorad 1973 and the method of generating a linear equation based on a phycocyanin standard curve.
Figure A1. Sodium phosphate buffer pH 7 molarity vs. phycocyanin yield (n=3-4).

Figure A2. Sodium phosphate buffer pH 7 molarity vs. phycocyanin extract purity (n=3-4).
Figure A3. Mixing method vs. phycocyanin yield (n=3-4).

Figure A4. Mixing method vs. phycocyanin extract purity (n=3-4).
Figure A5. Extraction time vs. phycocyanin yield (n=3-4).

Figure A6. Extraction time vs. phycocyanin extract purity (n=3-4).
Figure A7. Phycocyanin extraction optimization, lysis method yield (n=3-4).

Figure A8. Phycocyanin extraction optimization, lysis method purity (n=3-4).
Figure A9. Phycocyanin extraction buffer molarity, pH of biomass/extraction buffer.

Figure A10. Phycocyanin standard curve used to compare accuracy of phycocyanin estimation methods (phycocyanin standard from AnaSpec).
APPENDIX B

PRODUCED WATER COMPOSITION

Produced water used in this study was obtained from the Southern Cross Environmental Services produced water disposal facility in Baggs, WY. The following are inorganic and organic analysis of the bulk wastewater.
Figure B1. Produced water inorganic composition after aeration.
**Livestock Water Report and Interpretations**

**USU Analytical Labs**
Utah State University
Logan, Utah 84322-9400
(435) 797-2317
(435) 797-2117 (FAX)
www.usu.edu

**Date Received:** 8/24/2012
**Date Completed:** 11/29/2012
**Name:** JONATHAN WOOD
**Address:** 4105

**Lab number:** 1202-2658
**Identification:** NEW NON-AERATED
**Comments:** ICP DIGESTED THEN ANALYZED UNFILTERED
Arsenic: 0.01 mg/L
Selenium: <0.001 mg/L

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A value of zero indicates that the concentration was below the detection limit for that element.

If you have questions or if your water exceeds the safe levels, please contact:
Dr. Kerry Rodd, USU Extension Veterinarian, at 435-797-1982 or kerry.rodd@usu.edu
Dr. Jeff Hall, USU Veterinarian and Diagnostician, at 435-797-0238 or 435-797-1695

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**Figure B2.** Produced water inorganic composition prior to aeration.
Figure B3. Produced water oil and grease, diesel-range, gasoline-range, semi-volatile, and volatile organics composition (continued next page).
Figure B3. Produced water oil and grease, diesel-range, gasoline-range, semi-volatile, and volatile organics composition (continued next page).
Figure B3. Produced water oil and grease, diesel-range, gasoline-range, semi-volatile, and volatile organics composition (continued next page).
Figure B3. Produced water oil and grease, diesel-range, gasoline-range, semi-volatile, and volatile organics composition.
Figure C1. Phycocyanobilin extract from LLC2 cyanobacteria in methanol. Left: after HCl addition, Right: before HCl addition.
Figure C2. Laboratory scale 1 L Rotating Algal Biofilm Reactor dimensions.
Figure C3. 2000 L outdoor produced water pond floating Rotating Algal Biofilm Reactor dimensions.
Figure C4. 1 L RABR units operating with produced water, BG-11+1% NaCl, and DI water mediums (Left: day 0, Right: day 12).

Figure C5. Rotating Algal Biofilm Reactors operating in an outdoor produced water pond (Left: day 0, Right: day 45).
Figure C6. Elsevier (Algal Research) reprint permissions.
CURRICULUM VITAE

Jonathan L. Wood
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RELEVANT EXPERIENCE

Research Engineer, Synthetic Biomanufacturing Facility, Logan, UT 2015-17
- Management of an R&D team of up to four fermentation technicians, one research engineer, and multiple interns
- Primary customer point of contact for private contract bioprocess/downstream processing development and production
- Lab to pilot scale high density culture and downstream processing of E. coli to produce transgenic spider silk protein for large public project
- Development of professional network to create potential customer relationships
- Installation, operation, and maintenance of several laboratory and production facilities
  — BioCommand control software with 1L-400L bioprocess fermenters
  — Continuous flow stacked disk/tubular bowl centrifuges and high-pressure homogenizers
  — Tangential and normal flow filtration
  — CIP protocol development
  — SOP and batch record development
  — Initiation of cGxP-like protocols and practices
- Over 1 million dollars awarded through public and private funding sources FY17

Graduate Research Assistant, Utah State University, Logan, UT 2011-15
- Utilization of produced water (oilfield wastewater) to culture cyanobacterial biofilms
- Production of high-value bio-products from mixed and single strain culture phototrophic biofilms
- Bioremediation of wastewaters utilizing novel bioreactor platforms at scale
  — Aqueous protein extraction and purification techniques
  — DNA and protein gel interpretation
  — UV-VIS spectral analysis
  — Macronutrient analysis and method troubleshooting

Laboratory Technician, Bradley University, Peoria, IL 2009-10
- Investigation into the chemotaxis of nitrogen fixing soil bacteria towards secondary metabolites of invasive Brassicaceae (garlic mustard)
- Bacterial isolate culture maintenance and propagation

Laboratory Technician, Harold Washington College, Chicago, IL 2006-07
- Phytoremediation of heavy metal contaminated soils
- Growth and positive selection of cadmium tolerant Arabidopsis seedlings
- Assisted with grading, preparation, and execution of Biology I and II lab sessions

Tutor, Harold Washington College, Chicago, IL 2006-07
- Selected by Department of Chemistry for tutoring position in mathematics, biology, and chemistry
EDUCATION

M.S. Biological Engineering. Utah State University. Logan, Utah  Present
B.S. Cellular and Molecular Biology (Chemistry minor). Bradley University. Peoria, IL  2010

SKILLS

• Process design scale-up from flask to pilot plant
• Microbial culture, aseptic bioprocessing, and contamination mitigation
• Operation and maintenance of bioprocess fermenters from 2L to 400L scale
• Design, installation, operation, and maintenance of pilot-scale centrifuges, filtration systems, CIP systems, high-pressure homogenization systems, and supporting utility systems
• Conceptualization, implementation, and development of customer specific process designs
• Negotiation with vendors for equipment and supplies

SELECTED PUBLICATIONS AND CONFERENCE PRESENTATIONS


PATENT


ACTIVITIES AND LEADERSHIP

Mentoring:
- Managed and directed three undergraduate volunteer teams with specific team project goals for the development of novel algal biofilm harvesting devices.
- Mentored multiple undergraduate research teams conducting research projects on wastewater remediation and high-value bio-product production.
  - Alan Hodges- Utah State University, Biological Engineering. Nov 2013-2015
  - Tyler Gladwin- Utah State University, Biological Engineering. Jan 2014-2015
  - Cody Maxfield- Utah State University, Biological Engineering. Feb 2014-2015
  - Sean Bedingfield- Utah State University, Biological Engineering. Jan 2014-June 2014
  - Micah Rasmsussen- Utah State University, Biological Engineering. Jan 2014-June 2014
  - Adam Jones- Utah State University, Biological Engineering. Jan 2014-June 2014

Activities:
Engineering State: Planned and executed activities for a weeklong summer camp designed to inform statewide high school students about the engineering programs at Utah State University. 2011/2012/2017.

Discover BE: Presented non-technical overviews of research areas and findings that are pursued by the Department of Biological Engineering at Utah State University. 2011-2012.

Adventures in Biological Engineering: graduate speaker for an audience of high school students interested in the field of Biological Engineering. 2011/2013

AWARDS AND SCHOLARSHIPS

In-State Tuition Award for Graduate Students. Utah State University, 2013-2015
Out of State Tuition Award for Graduate Students. Utah State University, 2011
Graduate Research Assistantship. Utah State University, 2011-2014
Provost-Garrett Scholarship. Bradley University, 2007
Transfer Scholarship. Bradley University, 2007
Johnetta-Haley Scholarship. Southern Illinois University, 2004