Meso-biliverdin IXα production by conversion of cyanobacteria-derived phycocyanobilin

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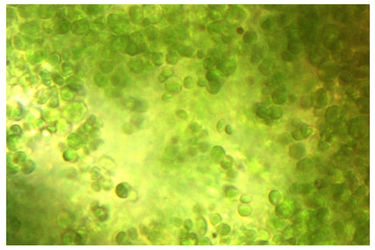
Introduction



Heme-derived bilins, such as meso-biliverdin IXα (MBV), are valuable bioproducts that are the subject of interest for both the medical and agricultural fields. MBV has been shown to have antioxidant and cytoprotective properties as well as benefits in livestock gut health and pancreatic transplantation procedures [1,2]. Development of a bioprocess that can produce MBV efficiently and effectively would be of interest for both commercial and medical applications. Additionally, produced water from the oilfield and natural gas extraction industries poses an expensive and hazardous waste problem [3]. Produced water is normally disposed of in large ponds to be evaporated, but this strategy results in a hazardous sludge that is environmentally detrimental. Utilizing produced water as a potential substrate for the growth of cyanobacteria, which produce bilin-derived pigments in energy harvesting protein complexes, would be a cost-effective way to capitalize on a waste-to-value bioprocess that has the potential to produce a marketable product (MBV) as well as minimize the detrimental effects of handling the disposal of produced water. Researchers at Utah State University isolated a novel strain of cyanobacteria from the Logan City, Utah municipal wastewater treatment plant referred to as Logan Lagoons Cyanobacteria 2 (LLC2). LLC2 has exhibited the ability to grow on produced water on novel rotating algal biofilm reactors (RABRs). LLC2 is an ample source of phycocyanin (PC) [4]. PC is the protein that binds to the blue pigment cofactor phycocyanobilin (PCB). PCB can be readily cleaved from PC and subsequently isomerized into MBV using a patented chemical synthesis process by Utah State University professors Drs. Jon Takemoto and Cheng-Wei Tom Chang [5]. MBV production from cyanobacteria has already been developed for the cyanobacteria *Limnospira fusiformis. L. fusiformis* produces MBV in appreciable amounts but does not have the ability to grow on produced water substrate. We aim to develop the waste-to-value bioprocess for MBV production by LLC2 as a potential alternative to the process already developed for L. fusiformis. We aim to develop the most effective engineering design for the production of MBV from LLC2 that will fit within the given constraints and that use scalable and efficient manufacturing processes. We also aim to seek commercial uses for MBV in the agricultural and medical industries. Developing this waste-to-value bioprocess would be of interest for both funders looking for a lucrative opportunity and for society in general due to this project's involvement in the reduction of hazardous waste, especially in the oil extraction industry.

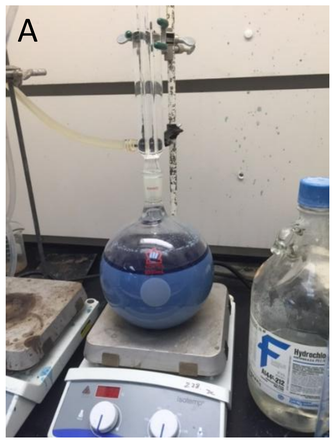
Culturing LLC2 on RABRs

To obtain LLC2 biomass, algae was first cultured on novel rotating algal biofilm reactors **(A)**. Methodology used is as follows: A liquid culture of LLC2 was first prepared with BG-11 media and 1% NaCl from a glycerol stock to obtain biomass for scale-up to benchtop-scale RABRs. 25 mL of inoculated BG-11 media in a 250 mL baffled flask was grown on a shaker plate at 100 RPM under artificial sunlight. After about seven days, a small LLC2 biofilm was visible on the wall of the flasks. To ensure no heterotrophic growth, the liquid media phases of the cultures were decanted, and 25 mL of fresh media was added periodically. Within ~5 weeks after inoculation, a biofilm large enough for RABR inoculation was generated. For RABR inoculation, liquid media was poured into RABR troughs and biofilm was scraped onto the cotton belts. RABRs were spun by an electric motor at 5 rpm to ensure gentle exposure to both nutrient media and light, with a PAR reading of approximately 300 lux at 22°C. Distilled water was added to the RABR every 3-4 days to ensure sufficient media volume after evaporative loss. Complete liquid media changes were performed biweekly to prevent heterotrophic growth and to ensure sufficient nutrient availability. The first benchtop RABR was allowed ~8 weeks to produce biomass, at which time the biomass was scraped off the roller and used to inoculate three additional benchtop RABRs. The three new RABRs were monitored and cared for in the same manner as the first.

Scale-Up of LLC2 Growths

In order to produce sufficient biomass for the MBV extraction process, a large greenhouse-scale RABR was constructed using a design created by USU SWBEC (A). Simulated produced water was used as the nutrient media (78 mg/L NH4Cl, 16 mg/L K2HPO4, 65 g/L NaCl, pH 8.4). The large RABR was constructed within a trough containing 100 L of media and used a cotton belt as a growth medium with ~ 0.6 m2 surface area. After sufficient biomass was present on the four benchtop RABRS, the biomass was scraped from the rollers and found to have a combined wet mass of 45 g. The wet biomass was then transferred to the belt on the large RABR which was grown in the USU APP greenhouse spun at ~1 rpm. This initial RABR design proved to be problematic, as the wood used was prone to expansion and contraction due to environmental conditions, causing excess stress on the frame and roller axels. Thus, a fully metal frame was fabricated to ensure equal expansion of all parts, which alleviated frame stress and allowed for proper function of the RABR.

PCB Extraction, Purification, and MBV Production

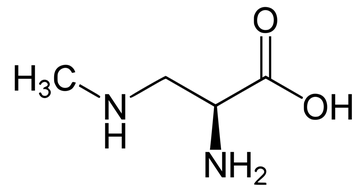


To extract PC from cyanobacteria biomass, either *Spirulina platensis* or LLC2 cells are first lyophilized. Cells are subsequently lysed using a series of freeze-thaw procedures and then centrifuged to isolate the PC-containing supernatant. PC is then extracted from the supernatant using a 65% ammonium sulfate precipitation method. PC-containing precipitant is is washed with methanol and stored at 5°C. The PC-containing pellet is then blended in methanol and boiled under reflux using a water-column condenser apparatus **(A)** for 16 h to cleave the PC-PCB linkages. The methanol-pellet solution is centrifuged to yield PCB-containing supernatant. Volume of the mixture is reduced by methanol evaporation using a rotary evaporator **(B)**. Once methanol is evaporated, PCB is recovered in the small remaining volume and added to acidified water (pH 4.5) to precipitate the PCB. The suspension is then centrifuged and the supernatant is decanted to produce a pure PCB pellet. The PCB pellet is suspended in a small amount of water and subsequently lyophilized and stored as a powder at -20°C. Purified PCB is then added to a mixture of ethanol, calcium carbonate, potassium carbonate, and sodium bicarbonate and boiled with constant stirring for 8 h at 80°C under reflux. Excess ethanol is evaporated by rotary evaporation and remaining residue is dissolved in new, small volume of ethanol and subsequently purified using Celite and silica gel.

Standards and Constraints

Growth of cyanobacteria and production of a cyanobacteria-derived pharmaceutical poses risks to the consumers of these products that must be considered for the design of a robust and safe bioprocess. Two of the main risks associated with the growth of the cyanobacteria LLC2 and the extraction of its blue-green pigment, phycocyanin, are the detectable levels of β-N-methylamino-L-alanine (BMAA) and heavy metals such as lead, cadmium, mercury, and arsenic. BMAA, whose chemical structure is shown in **(A)**, is a known neurotoxin that has been linked to the neurodegenerative diseases Amyotrophic Lateral Sclerosis (ALS) and Parkinsonism Dementia Complex (PDC)[1]. We plan to utilize high performance liquid chromatography (HPLC) **(B)** and mass spectrometry **(C)** to detect the presence of BMAA in our preparations. Additionally, consumption or exposure to high levels of lead can lead to lead poisoning, a type of metal poisoning that can result in a barrage of adverse health conditions such as anemia, hypertension, and birth defects (lead toxicity).

A



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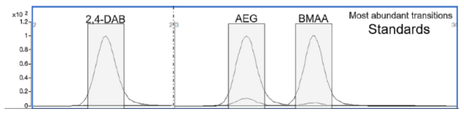

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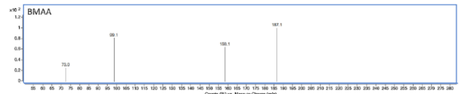
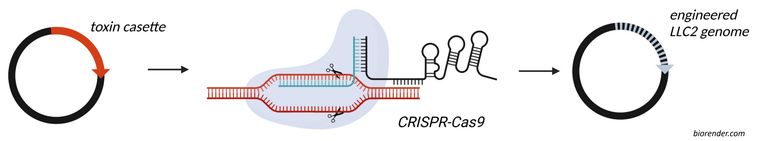


Conclusions and Future Directions



This project is focused on growing LLC2 Cyanobacteria and bioprocessing to produce MBV. This will enable greater production of MBV, which has valuable antioxidant and cytoprotective properties. Furthermore, since LLC2 is capable of growing on produced wastewater, this process offers a sustainable and profitable method for treating produced wastewater. The bioprocess will adhere to an array of standards and constraints. Lead levels within LLC2 growths cannot exceed a maximum value of 15 µg per amount of MBV consumed daily, and there must be no detectable BMAA levels. Additional economic and manufacturing constraints include wastewater processing to eliminate any potential toxins as well as scale-up to commercial-level production on rotating algal biofilm reactors (RABRs). Future directions of the project focus on solving problems related to further scale-up, optimization of biomass yield using environmental conditions, and maximization of the product yield.

- Establishing LLC2's ability to grow sustainably on produced water substrate
- Quantify PCB yield and compare to existing bioprocesses for PCB production
- Characterize toxin cassettes in LLC2 genome and develop plan for knocking out toxin genes using genetic editing techniques such as CRISPR – Cas9
- Further characterize optimal conditions for LLC2 growth on both laboratory scale and greenhouse scale RABRs, including ideal PAR, temperature, and nutrient conditions

References

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