**Electron partitioning in soluble organic products by wild-type and modified
Synechocystis sp. PCC 6803**

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13 **Abstract**

14 The partitioning of electrons photosynthetically fixed in carbon by the cyanobacterium
15 *Synechocystis* sp. PCC 6803 was evaluated for up to 24-day batch cultures continuously
16 exposed to incident light intensity (LI) from 111 to 598 $\mu\text{Em}^{-2}\text{s}^{-1}$. The fate of fixed
17 electrons was assayed by Chemical Oxygen Demand (COD) in biomass, generic soluble
18 microbial products (SMP), and excreted laurate (for a modified strain). Because SMP is
19 not a valuable product, light energy embedded in SMP is wasted. Normalized to the total
20 COD (TCOD), wild-type *Synechocystis* partitioned 75 to 84% of its TCOD in particulate
21 biomass and 16 to 25% in generic SMP. A strain modified to excrete lauric acid partitioned
22 6.6% to 10% of its TCOD in laurate, 21% to 30% in generic SMP, and 64% to 69% in
23 particulate biomass. The greatest electron partitioning in SMP occurred with the highest LI
24 tested, which suggests that moderating the LI in the photobioreactor is a promising mean to
25 accentuate the production of valuable forms of photosynthetically fixed electrons.

26 **Keywords:**

27 electron partition; laurate; light intensity; photoautotroph; SMP; *Synechocystis*;

1 Introduction

Eukaryotic algae and prokaryotic cyanobacteria, collectively called microalgae, have promise for generating renewable energy and reducing our society's reliance on fossil fuels [1,2]. Compared to terrestrial plants, microalgae have advantages of faster growth rates, simpler nutrient requirements, growth in suspension instead of on arable land, and amenability to genetic modification [3,4]. For example, microalgae can produce feedstock for liquid transportation fuel at about 20 times the areal rate of palm trees, the most productive biodiesel crop [5,6].

Photosynthetic organisms capture suitable electromagnetic radiation and store that energy in the electrons embodied in organic carbon. The efficiency of energy capture is key to cost-effective production of energy feedstock by photosynthetic reduction of carbon. For example, *Chlorella* converted 8 to 12% of the energy of a selected spectrum in biomass [7], and *Scenedesmus* achieved 5.1 to 6.7% [8] and 3.6% energy recovery from Photosynthetically Active Radiation (PAR) [9] in tubular pilot reactors.

Equally important to the overall efficiency of energy capture in reduced carbon is the fraction of electrons embodied in harvestable and desirable products. Separation techniques mainly harvest particulate biomass, and its valuable products are intracellular, such as lipids, glycogen, and poly-beta-hydroxybutyrate (PHB) [10], which the microorganisms typically produce when they experience nutrient stress [11]. For example, nutrient stress leads to the accumulation of triacylglycerides by eukaryotic algae when nitrogen is depleted [12]. Genetic engineering can enhance the accumulation of internal storage products. Wu *et al.* [10] engineered *Synechocystis* sp. PCC 6803 to enhance

production of PHB, and the engineered strain produced 20 to 25% more PHB than the wild-type strain, making PHB about 15% of the cells' dry weight.

Other valuable products can be excreted, such as aldehydes, fatty acids, or alcohols [13,14]. Engineered cyanobacteria excreted isobutyraldehyde at a rate equal to 5-6% of biomass production, which is equivalent to ~10 % electron partitioning [15]. Liu *et al.* [13] modified *Synechocystis* to excrete free fatty acids (mostly laurate). Excreted laurate accounted for ~13% of the C fixed in biomass or ~26% electron equivalents. Algenol Biofuels claims that its Direct to Ethanol[®] technology can produce annually 8,000 gallons per acre of ethanol [16], which is roughly 20 times higher productivity than corn-to-ethanol.

Not all microbial products originating from photosynthesis are useful to humans. For example, generic soluble microbial products (SMP) are ubiquitously produced mixtures of polysaccharides, proteins, nucleic acids, and humic acids that are formed either by the hydrolysis of extracellular polymeric substances (EPS) or are released directly by microorganisms as part of their metabolism [17–19]. SMP contain carbon-embodied electrons and energy, but they are distinct from valuable products that can be excreted by modified phototrophs.

A main source of SMP is the hydrolysis of EPS [17], which are essential to aggregation, such as with flocs and biofilms, and EPS also protects cells from desiccation, predation, and biocides [20]. EPS is a solid-phase microbial product, and it can be a large fraction of total biomass in well-aggregated systems [21,22]. EPS is composed mainly of carbohydrates, proteins, and uronic acids and can represent 10% to 70% of biomass dry weight for phototrophs, depending on strain and growth condition [23–25].

For phototrophs, light intensity was reported to affect EPS production and more general excretion of organic carbon [26]. Wolfstein and Stal [27] studied a diatom, *Cylindrotheca*, and reported an upward increment of “colloidal EPS” from an LI of 84 to 571 $\mu\text{Em}^{-2}\text{s}^{-1}$. Colloidal EPS is a term sometimes used to mean SMP. A study on EPS production of a cyanobacterium, *Anabaena* sp. ATCC 33037, found a distinct increment of EPS between those culture illuminated by 115 to 345 $\mu\text{Em}^{-2}\text{s}^{-1}$ and those illuminated by 460 to 1840 $\mu\text{Em}^{-2}\text{s}^{-1}$ [28]. Again, EPS is a major source of SMP.

Cyanobacteria also are known to protect themselves against photochemical damage by producing chemicals that absorb UV to violet light. Some of these “sunscreens” are lipophilic, such as scytonemin and mycosporine-like amino acids (MAA) found in the sheaths of cyanobacteria [29,30]. Others are hydrophilic and soluble, such as glutathione, ascorbate, and urate [31]. The lipophilic materials may be considered part of the EPS, while the hydrophilics may be part of the SMP.

The partitioning of electrons in fixed carbon soluble and particulate biomass can be assayed directly by measurements of the Chemical Oxygen Demand (COD) [32]. We investigated the partitioning of COD by the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), including how the partitioning was affected by the incident light intensity (LI) and strain modification to release laurate (12-C carboxylate) to the medium. Wild-type and modified strains were grown in inorganic BG-11 growth medium and continuously exposed to irradiance by florescent bulbs with incident light intensity (LI) from 111 to 598 $\mu\text{Em}^{-2}\text{s}^{-1}$ over 3 weeks. The results show that *Synechocystis* partitioned up to 30% of the electrons it fixed in generic SMP (not laurate), and the greatest electron

95 partitioning in SMP occurred with the modified strain and with the highest irradiance we
96 tested.

2 Materials and Methods

We grew *Synechocystis* wild-type or modified strain with different incident LI in standard BG-11 growth medium and under continuous illumination by florescent light. Samples taken at regular intervals were assayed for total, soluble, and particulate COD, as well as laurate for the modified strain. We focused on the proportioning of COD among SMP (non-laurate soluble COD), biomass (particulate COD), and laurate (for the modified strain).

2.1 Inoculum preparation

We obtained wild-type and modified *Synechocystis* as pure colonies on agar plates provided by the laboratory of Dr. Willem Vermaas, School of Life Sciences, Arizona State University. The modified strain was designed to excrete mainly lauric acid to the medium [33]. The modified *Synechocystis* was created by inserting an acyl-ACP thioesterase that diverts part of the metabolic flux for lipid production to fatty acid synthesis [13,34]. The modified strain also has a deletion of acyl-ACP synthetase (*srl 1609*) to prevent re-uptake up of fatty acids. Lauric acid is passively excreted to the medium due to diffusion through the cell's membrane. Genes encoding for peptidoglycan layer were deleted to promote excretion of laurate by the modified strain [13].

The experimental inocula were prepared by transferring pure colonies of the wild-type or modified *Synechocystis* on agar plates to ~120 mL of sterilized BG-11 liquid medium and incubated for 3-5 days at 30°C and ~80 $\mu\text{Em}^{-2}\text{s}^{-1}$ to obtain a sufficient biomass to inoculate experiments.

2.2 Growth medium

We used standard BG-11 medium [35,36], which contains 17.6 mM NaNO₃, 0.23 mM K₂HPO₄, 0.30 mM MgSO₄, 0.24 mM CaCl₂, 0.01 mM citric acid, 0.021 mM ferric ammonium citrate, 0.0027 mM Na₂EDTA, 0.19 mM Na₂CO₃, and trace metals. Before use, the medium was steam-sterilized with a liquid cycle (121°C and 15 minutes) in a Tabletop Sterilizer (SterileMax, Thermo Scientific, U.S.).

2.3 Experiment setup

All experiments were carried in 1-L Erlenmeyer flasks with a 0.5-L working volume. Atmospheric CO₂ (~ 395 ppm CO₂) was supplied to the culture through a glass pipette tip after filtration through a 1.0-μm bacterial vent-filter and at a rate of ~1.5 L air/L liquid-minute. Experiment vessels were steam-autoclaved with unwrap cycle (135°C for 3 minutes, followed by 15-minute drying) before and were capped by cheesecloth during experiments.

Flasks were illuminated by T5 high-output daylight florescent bulbs, and the incident light intensity (LI) was adjusted by placing each flask a specific distance away from the shared bulb rack. The LI at the location of each flask was checked regularly using a multi-meter equipped with a 190 LI-COR PAR sensor intergraded with an U1252 Agilent micro-ammeter. The reported LI was measured on the front side of the flasks and represents the highest LI for each culture. The four incident LI values were 111, 225, 346 and 598 μEm⁻²s⁻¹. The experiments were run in a room that had a temperature of 25°C. Due to continuous illumination, the temperatures of the cultures were 26, 27, 29, and 33°C from the lowest to the highest incident LI.

We calculated the Average Internal Light Intensity (AILI) inside the reactor based on the Beer-Lambert Law, the biomass concentration, the coefficient of light absorption, and depth of the light path [37].

2.4 Analyses

Culture samples were taken with a sterile pipette (Fisher Scientific, U.S.) every two days and inside a laminar biosafety hood after the sterile deionized water was added to offset water loss due to evaporation. Samples were transferred into 50-ml screw-cap Falcon polypropylene tubes (VWR, U.S.) to measure optical density (OD) immediately and then stored in a 4°C for later assay.

OD was measured by a UV-Vis BioSpec-mini spectrometer at 730 nm (Shimadzu Corp., Japan) with an appropriate diluting to obtain an OD less than 0.7. The OD reading was converted to biomass dry weight using an empirical conversion factor of 254 mg dry weight biomass/L per unit of OD [38].

COD was measured by thermal digestion and colorimetry using a Kit 822, DRB200 digester, and DR2800 spectrophotometer according method 8000 (Hach, CO, U.S.). After being removed from storage, a sample was vortexed vigorously for 30 seconds, and a 2-mL aliquot was added to one vial to measure total COD (TCOD). Soluble COD (SCOD) was obtained by filtering a sample through a 0.45- μ m-pore PVDF GD/X syringes filter (Whatman GPX, VWR, U.S.). Then, 2 mL of sample was added to a glass kit vial, mixed, and oxidized at 150°C for 120 minutes in the DRB200 digester. Particulate COD (PCOD) was computed by the difference between TCOD and SCOD.

Laurate (for the modified strain) was measured by Gas Chromatography (GC2010, Shimadzu, Japan) using a polar Supelco Nukol column specifically coated for long-chained FFAs [39]. First, 20 μ L of 1-M EDTA was added to a 1.0-mL of culture sample, and the mixture was centrifuged for five minutes at 11,000 rpm with a Microfuge® 22R Centrifuge (Beckman Coulte., U.S.). Then, 1.0-mL supernatant aliquot was mixed with 80 μ L sulfuric acid (50%) to lower the pH to ≤ 1.0 , followed by adding 2.0 mL hexane (>99%), and the mixture was vortexed for at least two hours to achieve an optimal extraction. Lastly, 150 μ L of liquid in hexane was analyzed by GC, and the laurate concentration was computed using a calibration curve prepared with pure lauric acid. The laurate concentration was converted to laurate COD using the stoichiometric factor 2.72 g COD/g laurate based on its molecular formula for lauric acid ($C_{12}H_{24}O_2$) and the methods in Rittmann & McCarty [40]. When laurate was present, we computed the COD of SMP as the difference between the measured SCOD and COD of the laurate. Small concentrations of other fatty acids (Capric acid, Myristic acid and Palmitic acid) were detected in a few samples, but their CODs were insignificant compared to laurate.

We inspected the cultures using Light Microscopy. Cells were immediately fixed using freshly prepared 4% (v/v) formaldehyde in phosphate buffer (0.1M, pH 7.0) for two hours, followed by washing in phosphate buffer and stored at 4°C until imaged. Fixed samples were examined with an Axioskop light microscope (Carl Zeiss, Inc., Thornwood, NY, U.S.) with appropriate optics and record with a Roper Cool SNAP ES digital camera (Roper Scientific, Inc., Tucson, AZ, U.S.) and processed using MetaMorph 6.0/6.1 software (Universal Imaging Corporation, Downingtown, PA, U.S.). Imaging was carried out in the laboratory of Dr. Robert W. Roberson Laboratory, School of Life Sciences, ASU.

184 2.5 Electron partitioning

185 To evaluate electron partitioning, we normalized the non-laurate SCOD
186 (representing generic SMP), the COD equivalents from laurate, and the PCOD
187 (representing particulate biomass) to TCOD (representing all COD generated by
188 photosynthetic activity). From the 18th day onward, the modified culture illuminated by
189 incident LI of 111 $\mu\text{Em}^{-2}\text{s}^{-1}$ and 225 $\mu\text{Em}^{-2}\text{s}^{-1}$ showed rod-shaped bacteria indicative of
190 heterotrophic bacteria. Because heterotrophic bacteria could have consumed SMP and
191 laurate, we excluded all data beginning with day 18 for these experiments. For each LI, we
192 calculated COD ratios for each set of samples ($N = 7$ to 10), averaged the ratios, and report
193 the average value and its 95% confidence level.

3 Results

The electron-partitioning experiments were carried out up to 18 days with the wild-type strain and 24 days with the laurate-excreting strain, and the biomass-growth curves are presented in **Figure 1**. Wild-type *Synechocystis* (a panel) reached as much as 1350 mg/L dry weight after 12 days. The biomass grown with lower incident light intensities (111 and 225 $\mu\text{Em}^{-2}\text{s}^{-1}$) produced less biomass density than the higher ones (346 and 598 $\mu\text{Em}^{-2}\text{s}^{-1}$). The modified strain (b panel) grew considerably slower than the wild type, and the highest dry weight was 650 mg/L after 12 days with the highest LI. The wild-type biomass reached stationary phase by days 6-10, and the modified strain reached stationary phase later, around day 16.

Average Internal LI (AILI) also is shown in **Figure 1**. As biomass grew, the AILI declined quickly and then stabilized in the range of 6.5-20 $\mu\text{Em}^{-2}\text{s}^{-1}$, with the lowest AILI values associated with the lowest incident LI. Loss of biomass density at the end of the experiments with 598 $\mu\text{Em}^{-2}\text{s}^{-1}$ caused the AILI to increase to $> 30 \mu\text{Em}^{-2}\text{s}^{-1}$.

Figure 2 presents results for TCOD, PCOD, and soluble components of COD (generic SMP and laurate for the modified strain) produced by *Synechocystis* wild-type (panels a1 – a3) and the modified strain (panels b1 – b4). TCOD is the direct measure of total electron equivalents fixed in organic C by photosynthesis. For both strains, higher incident LI led to more electron fixation (panels a1 and b1), but the differences were more dramatic for wild-type *Synechocystis*. Also, photosynthetic electron capture leveled off by 12 days for the wild type, while it continued almost linearly for the modified strain out to 15 to 18 days. This difference in the generation of TCOD was driven in large part by the

much faster growth of biomass, shown as PCOD in panels a2 and b2. Higher biomass concentrations for the wild type increased the overall rate of photosynthesis to TCOD.

The COD in SMP (panels a3 and b3) rose continually during the experiments, roughly in proportion to the PCOD. Except for the highest incident LI, SMP-COD concentrations for wild-type *Synechocystis* were similar to or smaller than SMP-COD concentrations for the modified strain, despite the higher production rate of PCOD by the wild-type strain. Laurate COD produced by the modified strain (panel b4) increased in parallel with biomass growth and SMP-COD, but it was produced in considerably smaller concentration than SMP-COD and PCOD.

Panels a3, b3, and b4 identify SMP-COD and laurate-COD samples for which LM detected heterotrophic bacterial rods. **Table S1** in Supplementary Information (SI) reports the LM results. These samples in b3 and b4 showed loss of SMP-COD and laurate COD. All samples showing evidence of heterotrophs were excluded from the analyses of electron partitioning.

The average COD partitionings over the duration of experiments without signs of heterotrophic bacteria (N = 7 to 10) are presented in **Figure 3**. Electron equivalents embodied in SMP by the wild type ranged from 16% to 25% of TCOD, while PCOD was 75% to 84% (a panel). Thus, wild-type *Synechocystis* released a substantial portion of the electrons it fixed in C as SMP. For modified *Synechocystis* (b panel), laurate comprised 6.6% to 10% of the TCOD, while non-laurate SMP comprised 21% to 30% of the TCOD, leaving PCOD as 64% to 69% of TCOD. The lower partitioning of TCOD in PCOD explains part of the slower specific growth rate of modified *Synechocystis*.

4 Discussion

From the point of view that the valuable products are in harvestable biomass and laurate, partitioning 16% to 30% of TCOD in SMP means that *Synechocystis* was significantly “wasteful.” For the modified strain, SMP-COD was 2-fold to 4-fold more than the COD invested in the desired soluble product, laurate. Furthermore, the fraction in SMP-COD was 25% to 30% higher for the modified strain than for *Synechocystis* wild type. Perhaps SMP release was exacerbated as an unwanted side effect of modification to enhance the excretion of lauric acid by weakening the peptidoglycan layer [13]. Alternately, the modified strain produced more EPS that was hydrolyzed partly to SMP.

Another clear trend for both strains is that they partitioned significantly more electrons to SMP when exposed to the highest incident light. For instance, $598 \mu\text{Em}^{-2}\text{s}^{-1}$ generated about 1.4-fold more SMP-COD than did $111 \mu\text{Em}^{-2}\text{s}^{-1}$ for the wild-type strain. The modified strain showed a sharp increment in SMP-COD at $346 \mu\text{Em}^{-2}\text{s}^{-1}$. A few studies reported that light intensity affects carbohydrate production, a main component of EPS and, consequently, SMP [24,28,41]. For example, the cyanobacterium *Microcoleus vaginatus* exposed to 40 and $80 \mu\text{Em}^{-2}\text{s}^{-1}$ produced SMP at 8.0% and 14.0% of cell dry weight, respectively [23]. The nitrogen-fixing microalgae *Anabaena* sp. exhibited a 4-fold increment of EPS when its incident light intensity increased from 345 to $460 \mu\text{Em}^{-2}\text{s}^{-1}$ [28]. The flasks with the highest incident LI had higher temperatures (increasing from 26°C to 33°C). Although 44°C increased unsaturation of fatty acids in wild-type *Synechocystis* sp. PCC 6803, the range of 25 to 33°C showed almost no effect on the fraction of total lipids to biomass, indicating that this modest temperature range caused no profound impact on the physiology of *Synechocystis* [42]. In addition, Wolfstein and Stal [27] reported no change

in the production of an SMP-liked pool by a diatom grown at 25 and 35°C. Thus, the small temperature increase likely was not the cause of increases in SMP production for the highest incident LI.

We also calculated the production rates of biomass PCOD, SMP-COD, and laurate-COD per unit of PCOD, and the results are presented in **Figure S1**. The biomass-normalized production rates of PCOD, SMP-COD, and laurate-COD correlated to AILI. Consistent with **Figure 3**, the rate analyses show that the laurate-excreting strain produced less PCOD, but more SMP-COD than did the wild-type strain. Both strains, grown under a high incident LI ($598 \mu\text{Em}^{-2}\text{s}^{-1}$) had reduced productivity of biomass and laurate (for the modified strain), but increased SMP production. Comparing the slopes of the overall fits reveals that about 0.2 mg SMP-COD and 0.8 mg PCOD were produced per mg of TCOD by wild-type *Synechocystis*. For the modified strain, for every mg of TCOD generated, 31% was embodied in SMP, 67% in particulate biomass, and 10% in laurate, all proportions that are consistent with **Figure 3**.

A serious consequence of exposing *Synechocystis* to LI higher than about $400 \mu\text{Em}^{-2}\text{s}^{-1}$ is photoinhibition [43], in which phycobilisome is degraded, turning the culture color from dark green to yellowish green [44]. Exposure to inhibitory LI leads to deactivation of the electron carrier system and, in extreme case, damage to the integrity of the cell membrane [45]. We took regular color photographs of the cultures, and **Figure S2** in SI shows a yellowish color in the culture exposed to $598 \mu\text{Em}^{-2}\text{s}^{-1}$, indicating pigment degradation due to photoinhibition.

The higher proportion of electrons partitioned in SMP with exposure to the highest light intensity also might have led to a sunscreen response to photoinhibition. Compounds

such as scytonemin and mycosporine-like amino acids (MAA) are found in the outer membrane and S-layer of cyanobacteria. Previous research found only a small portion of those sunscreen compounds within the total EPS extracted from cells [29,30]. Nevertheless, increased production of those compounds in the EPS could ultimately lead to their hydrolysis and release to the medium as SMP [46].

5 Conclusion

A significant portion of photosynthetically harvested electron equivalents was embedded in organic carbon fixed in SMP, and the SMP proportion was larger for *Synechocystis* modified to release laurate to the medium. The wild-type and modified strains partitioned more electron equivalents in SMP when exposed to higher light intensity, which identifies the value of moderating the light intensity in the photobioreactor in order to minimize the diversion of electrons and fixed carbon to non-valuable products.

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435

436 **Figure Captions**

437 **Figure 1:** Biomass dry-weight density (closed symbols, left axis) and AILI (open symbols,
 438 right axis) of the *Synechocystis* sp. PCC 6803 wild type (a panel) and modified strain (b
 439 panel). The numbers on top of each panel give the incident light intensity in $\mu\text{Em}^{-2}\text{s}^{-1}$.

Figure 2: Panel a1-a3: Total COD, calculated COD of particulate biomass, and generic SMP COD of wild-type *Synechocystis*. Panel b1-b4: Total COD, calculated COD of particulate biomass, generic SMP CD, and COD equivalent of laurate for modified *Synechocystis*. The open symbols with an asterisk (*) show data that had heterotrophic rods identified by LM.

Figure 3: a panel: COD fractions of SMP and particulate biomass normalized to the total electron equivalents. b panel: COD fractions of SMP, particulate biomass, and laurate normalized to the total electron equivalents harvested by photosynthesis by modified *Synechocystis* sp. PCC 6803. The bars represent two standard deviations, or the 95% confidence level for each component. Numbers on the top of columns give the number of samples used for each incident LI; values less than 8 for wild type and less than 10 for the modified strain indicate the exclusion of samples due to the presence of heterotrophic bacteria (Table S1).