MULTIVARIABLE AND SENSOR FEEDBACK BASED REAL-TIME MONITORING AND CONTROL OF MICROALGAE PRODUCTION SYSTEM

by

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ABSTRACT

A multi-wavelength laser diode based optical sensor was designed, developed and evaluated for monitoring and control microalgae growth in real-time. The sensor measures optical density of microalgae suspension at three wavelengths: 650 nm, 685 nm and 780 nm, which are commonly used for estimating microalgae biomass concentration and chlorophyll content. The sensor showed capability of measuring cell concentration up to 1.05 g L^{-1} without sample dilution or preparation. The performance of the sensor was evaluated using both indoor photobioreactors and outdoor paddle wheel reactors. It was shown that the sensor was capable of monitoring the dynamics of the microalgae culture in real-time with high accuracy and durability. Specific growth rate (μ) and ratios of optical densities (OD ratios) at different wavelengths were calculated and were used as good indicators of the health of microalgae culture. A series of experiments was conducted to evaluate the sensor's capability of detecting environmental disturbances in microalgae systems, for instance, induced by dust or *Vampirovibrio chlorellavorus*, a bacteria found to cause crash of microalgae culture. Optical densities measured from the sensor were insensitive to the amount of dust that consisted of 59.7% of dry weight of microalgae in the system. However, the sensor was able to detect multiple events of introduction of dust timely by μ and OD ratios. The sensor was also capable of detecting subtle changes of culture in color that leads to a total crash of the culture before it can be differentiated by naked eye. The sensor was further integrated into an existing outdoor raceway to demonstrate the sensor's potential of being a core component to control microalgae production system. A real-time monitoring and control program along with a graphical user

interface (GUI) was developed for a central control station aiming at improving resource use efficiency for biomass production.

1. INTRODUCTION

Microalgae are photoautotrophic microorganisms that convert solar energy into chemical energy by consuming carbon dioxide and water while release oxygen into the atmosphere as a byproduct. They have been commercially cultivated to produce antioxidants, antibiotics and toxins for pharmaceutical applications; long-chain polyunsaturated fatty acids (PUFAs), polysaccharides, vitamins, β-carotene and pigments for nutritional supplements and animal feed for decades (Spolaore et al. 2006; Harun et al. 2010). Certain strains of microalgae showed great potential as one of the renewable energy sources to limit the use of fossil fuels due to their high levels of lipids which can be extracted and converted into biofuels et al. 2010). The residual biomass after lipid extraction (lipid extracted algae - LEA) can further be converted to other forms of biofuels including ethanol, hydrogen and methane (Hernández et al. 2014). Microalgae are also used in wastewater treatment with their advantages of lower energy demand compared to conventional wastewater treatment methods and the ability to convert nitrogen and phosphorous into biomass.

1.1 MICROALGAE PRODUCTION SYSTEMS

1.1.1 Open pond raceways

Large-scale production facilities provide the possibility of delivering a continuous supply of high quality microalgae. Microalgae cultivation in commercial scales are conducted in open pond raceways or in closed photobioreactors (PBRs). There are several types of ponds are used in research and commercial applications including paddle wheel

raceways, shallow ponds and circular ponds (Chaumont 1993; Y. Lee 2001). In general, the physical culture conditions in open pond systems (temperature, lighting intensity and duration) are climate dependent (Waller et al. 2012). Microalgae are not always growing under the optimum condition for this reason, which results in a low productivity compare to the ones grown in closed systems (Richardson et al. 2012). This further increases the cost of the product by the large volume of culture going through the harvest process (Danquah et al. 2009; Knuckey et al. 2006). Furthermore, microalgae are more vulnerable to contaminants and predators because they are exposed to the environment (Soo et al. 2015; Velicer and Mendes-Soares 2009; Carney and Lane 2014; Rego et al. 2015). Therefore, only a limited range of species that can survive extreme culture conditions are suitable for production in open pond systems (Rodolfi et al. 2009). However, the initial investment on construction of open pond systems are significantly lower than that of closed systems since less expensive materials are being used and simplicity of reactor design. The operational and maintenance costs are lowered as well since less environmental conditions need to be controlled (Richardson et al. 2012).

1.1.2. Closed Photobioreactors (PBRs)

Closed PBR systems, on the other hand, have the advantages of higher areal productivities (3 times higher than that obtained in open pond systems) and wider selection range of cultivation species compared to open pond systems (Chaumont 1993; Carvalho et al. 2006). This is attributed to the ability of having total control over the cultivation condition that is optimal for the production strain including pH, temperature, lighting intensity, quality and duration (Saeid and Chojnacka 2015; Pirouzi et al. 2014; Huang et

al. 2014). The closed systems prevent contaminants and undesirable microorganism from entering the culture. This helps to improve the control of the quality of final products when they are highly susceptible to microbial degradation (e.g. amino acids and polysaccharides), as well as to reduce the possibility of a total crash of the culture. Furthermore, the close system can reduce the water evaporation and recover the CO_2 that was not used by the microalgae to improve the resource use efficiency. However, the capital investment and operational cost in of the closed systems are currently high. The biomass productivity and the value of the final products need to be much higher to offset the high production cost (Richardson et al. 2012).

1.2 MICROALGAE BIOMASS CONCENTRATION MEASUREMENTS

1.2.1 Ash Free Dry Weight (AFDW)

There are several methods commonly used in laboratory setup to determine microalgae biomass concentration. Ash free dry weight (AFDW) measurement is a direct measurement of the amount of dry biomass in a unit culture volume. The measurement of dry weight involves cell separation, washing and drying steps. Cells are normally separated from culture medium by filtration, followed by washing with diluted medium or deionized water for fresh water microalgae or by isotonic solution for marine algae. The wet biomass is then dried in an oven at a low temperature (60°C- 100°C) for at least 12 hours. The weight differential of the filter before and after low temperature drying process is measured by a high precision balance to determine the dry weight of the sample. The filter with dried algae is combusted in a furnace at 540°C for 4 hours to evaporate all organic matter leaving only the inorganic matter (ash) on the filter. The filter is transferred to a desiccator to be

cooled before making a measurement to avoid absorption of moisture in the air. The weight of ash is subtracted from the dry weight to obtain the ash-free dry weight (Zhu and Lee 1997). The whole process is laborious and time-consuming. Large quantity of biomass is necessary for an accurate measurement.

1.2.2 Cell counting

Cell count is a direct measurement method to determine cell concentration in a suspension. Microalgae cell suspension need to be diluted in order to form a single layer of cells in a cell counting chamber under a microscope. Then, the number of cells in a defined volume then is counted by human or image analysis software (Richmond 2004; Córdoba-Matson et al. 2009).

1.2.3 Spectrophotometry

Spectrophotometry has been widely used to estimate biomass concentration and chlorophyll content by measuring the absorbance, turbidity or fluorescence of the culture suspension. When a ray of straight light shined on a medium, fractions of the light can be absorbed, reflected and scattered by the material and the rest will pass through it. The absorbance measures the attenuation of the incident light due to absorption, scattering and reflection from the medium. It is also proportional to the light path length and the concentration of the material according to Beer - Lambert's Law (Lee 1999).

$$A = \alpha l c$$

A = Absorbance

α = Absorptivity of the medium

l = Light path length

c = Concentration of the medium

The absorptivity is an intrinsic property of a medium which is a constant. The light path length is normally fixed during a measurement. Therefore, the concentration of a medium can be estimated by measuring the absorbance and calculated using the Beer's law. However, the linear correlation only holds when the attenuating medium is homogeneous. Microalgae cell suspension can be considered as homogeneous at low concentrations. Therefore, the microalgae suspension sample need to be diluted to a low concentration in order to accurately estimate biomass concentration from absorbance measurement. Wavelengths of 650 nm, 680 nm and 750 nm are commonly used to estimate cell concentration of green algae. Measurement of light absorbance at 650 nm and 680 nm can be correlated to the intensity of green color of the algae which is mainly attributed to the concentration of chlorophyll (Das et al. 2011; Solovchenko et al. 2011; Nedbal et al. 2008). Light absorbance at 750 nm (Near Infrared) correlates to the total biomass because color has no effect on light absorbance in that wavelength range (Thomasson et al. 2010; Yao et al. 2012; Sandnes et al. 2006).

Table 1. Commonly used wavelengths for optical density measurements.

Wavelength (nm)	Advantages	Disadvantages
550 600	Minimum absorption by pigments	Does not reflect the viability of microalgae cells as dead cells have absorption on these wavelengths
630	Decided by extensive laboratory experimentation	
680 680 682 682	Commonly used in lab analysis, related to concentration of pigments	May not reflect the real biomass concentration due to the change of pigments concentration in cells during different growth phase and culture condition
750 750 750 870 880	Minimum absorption by pigments	Does not reflect the viability of microalgae cells as dead cells have absorption on these wavelengths
940	Decided by extensive laboratory experimentation	

1.2.4 Turbidity measurement

Turbidity measures the opaqueness or cloudiness of a liquid suspension by measuring the amount of light that was scattered by the particles at a certain angle. The intensity of the scattered light is dependent on the concentration and size of the particles. There are different standards for turbidity measurement. EPA method 180.1 requires the light source to be tungsten lamp with a color temperature between 2000 K and 3000 K, and a photodetector with a spectral peak response at 400-600 nm placed at 90 degree angle to the incident light (O'Dell 1993). ISO 7027 standard requires a monochromatic light source within a wavelength range of 830-890 nm, and a photodetector place at 90 degree angle to the incident light (ISO 1990). Measuring turbidity with a NIR light source has the

advantage of reduced error caused by colored particles (ISO 1990). Therefore, ISO 7027 standard becomes a better candidate for biomass concentration measurement of microalgae.

1.2.5 Chlorophyll fluorescence measurement

Chlorophyll fluorescence measurement is an established tool to estimate physiological state and photosynthetic rates of microalgae. Chlorophyll fluorescence is measured by the pulse amplitude modulation (PAM) technique. The efficiency of photosystem II can be calculated from maximum fluorescence and measured fluorescence using the equation $\Phi_{PSII} = \frac{(F_m{}' - F)}{F_m{}'}$, where F_m' is the fluorescence level of illuminated sample as induced by saturating pulses which temporarily close all PSII reaction centers and F is the fluorescence level at the time of measurement (Nedbal et al. 2008; Marxen et al. 2005; White et al. 2011).

1.2.6 Flow cytometry

Flow cytometry is the measurement of properties of a single cell in a flow system by measuring scattered light and fluorescence of different wavelengths. The value of this technique is the ability to make measurements on large numbers of single cells within a short period of time. Fluorescent chemicals are normally used to label cell components, such as DNA, directly; others are attached to antibodies against a wide variety of cellular proteins. A typical flow cytometer is consisted of light source, flow chamber, optical system, light detectors and computer. The flow chamber has a diameter of about 10 μm to allow a single cell pass at the point of measurement. When a cell flow through a ray of measurement light (UV, red or blue), the light scattered from the cell subsequently passes

through a series of long pass dichroic filter to be selected out at different wavelengths. The intensity and duration of the scattered light then is measured by a photodetector. As a high throughput technology, flow cytometry has been used by researchers in microalgae related studies. Hyka et al. (2013) used flow cytometric methods to characterize the behavior of particular microalgal species under different culture conditions, which will provide valuable information on design and optimize production strategies. Franqueira et al. (2000) used flow cytometry to analyze stress produced by copper or paraquat in two microalgal species for toxicity studies. Flow cytometry was also used to detect several common microalgal toxins that are known to be poisonous to human and wildlife (Fraga et al. 2014). Although flow cytometry has the advantages described above, the high cost of the instrument restrained its use in microalgae production applications.

1.3 REAL-TIME MONITORING AND CONTROL

A real-time monitoring system and strategy is desired for the study of microalgae growth and physiological dynamics under various culture conditions as well as optimizing resource use efficiency. For microalgae production settings, it is necessary to have accurate and timely measurement of biomass density, physiological status of the microalgae and use them as feedback to precisely control the growth of the culture and the quality of the products. For instance, a real-time monitoring system can be integrated into a microalgae production setup in order to maintain the cell density of the culture within an optimal range to maximize the productivity of the system. Too low of a cell density will increase the cost of harvesting, while cell density being too high can decrease the productivity by reducing the amount of light available to the culture. Contamination of microalgae by parasites,

grazers and pathogenic bacteria has always been an issue for large scale microalgae production since it can lead to the total crash of a cultivation system that results in a loss of biomass and desired bioproducts (Carney and Lane 2014). It becomes a main constraint of species selection for open pond production systems. The detection of microalgal parasites are currently relying on three technics: microscopy and staining, flow cytometry and molecular-based detection. However, none of these technics can detect the contamination event in-situ in real-time whereas effective remedies to parasites contamination (e.g., salvage harvest, ozone treatment, UVC treatment, abscisic acid and sonication) need to be executed in a timely manner to minimize the damage. A real-time sensor that is capable of early detection of microalgal parasites is desirable for large scale production applications.

1.4 COMMERCIAL MICROALGAE SENSORS

There are sensors designed to measure microalgae concentration exist on the market. Hydrolab (www.hydrolab.com), OSIL (www.osil.co.uk), YSI (www.ysi.com), OTT Hydromet (www.ott.com) and EXO (www.exowater.com) all developed blue-green algae sensors that have the same working principle. The sensors are essentially fluorometers that measures fluorescence of the chlorophyll a in the living algal cells. Since they are designed to measure microalgae in environmental levels (0 -- 2 x 10^6 cell mL^{-1}), they can't be used to monitor microalgae concentration in production applications where high concentration of biomass (> 1 x 10^7 cell mL^{-1}) is normally maintained. Thus, development of an integrated system for monitoring growth parameters is important for

commercial viability, providing the growers with valuable information to optimize production processes and reduce costs.

1.5 PROBLEM STATEMENT

Measurements of biological variables, including cell mass concentration, cell size, cell morphology, population composition (i.e. concerns with contamination), pigments and lipid content, are especially desirable because they are the direct indicators of the dynamics of a microalgae culture system. Standard methods developed for measurements of these variables are either too laborious or destructive to be employed for real-time monitoring and control purposes (Richmond 2004; Lee et al. 2015). Spectrophotometry has been widely used to estimate these biological variables by measuring the absorbance, turbidity or fluorescence of the culture suspension (Chen and Vaidyanathan 2012; Collos et al. 1999; Held 2011). As a non-destructive and rapid analytical method, spectrophotometry became a preferable candidate for real-time monitoring and control of microalgae culture systems.

There are some commercialized sensors to monitor microalgae concentration. However, most of them are designed to monitor microalgae concentration at an environmental level which is much lower than the cell concentration in microalgae production applications. Therefore, they are not practical to integrate into outdoor raceway or photobioreactor (PBR) based algae production systems. Therefore, there is no current optical sensor design exist in the market for microalgae production that was capable of measuring multiple biological parameters in real time within a high cell concentration range and without needing sample preparation (i.e. dilution, washing, filtration) for measurements. A real-time sensor that is capable of early detection of microalgal parasites will be desirable for large scale production applications to minimize the damage from culture crash. Furthermore, for microalgae production settings, it is necessary to have

accurate and timely measurement of biomass density, physiological status of the microalgae and use them as feedback to precisely control the growth of the culture and the quality of the products.

1.6 Research Objectives

There has been interest from research and commercial microalgae production settings for inexpensive, non-destructive and accurate monitoring system to provide real-time growth and health information from microalgae directly, and being able to manage the production system autonomously based on the feedback from the sensors. Therefore, the overall objective of this study was to develop an in-line multi-wavelength optical sensor that was capable of measuring dynamics of microalgae growth and health condition, and integrate it to a given cultivation system for control purposes. The specific objectives of the study were:

1. To design and develop a multi-wavelength, in-line optical sensor to monitor microalgae growth and physiological condition dynamics in real-time. Evaluate and improve the performance of the sensor in indoor PBR and outdoor raceway settings.
2. To evaluate multi-wavelength inline sensor's capability for autonomous detection of an abiotic and biotic disturbance in a microalgae culture system.
3. To develop sensor feedback based control strategy for culture condition adjustment and optimization of resource inputs.

2. LITERATURE REVIEW

Optical properties of microalgal cells can be measured by absorbance, turbidity and fluorescence that correlates to biomass concentration and health status of the culture. A few studies focused on real-time monitoring and control of microalgae cultivation by converting these measurement technics to in-line sensors, and utilize the sensors feedback for control purposes.

Sandnes et al. (2006) developed a near infrared (NIR) light sensor for real-time monitoring of algal biomass density in growing *Nannochloropsis oceanica* cultures. An array of 880 nm wavelength light emitting diode (LED) and photodiode were used as light source and photodetector respectively. Light transmittance was measured while microalgae suspension flew through a transparent "biofence" production tube with 10 mm light path length. The voltage generated from the photodiode, which was proportional to the light intensity passed through the sample, showed good correlation with biomass with maximum error of 8% of the total biomass. The sensor was used to monitor growth response from microalgae to the change of irradiance during 4 days of period. It was also used to monitor the diurnal patterns of microalgae growth under different culture light scheme in semi-continuous production mode. Lastly, the sensor was integrated into a microalgae production system as feedback to maintain the optimal population density of the culture by automatic injection of fresh growth medium. The study indicated that each sensor, system and species combination must be individually calibrated.

Briassoulis et al. (2010) developed an automated flow-through sensor for continuous cell concentration monitoring of *Nannochloropsis sp*. The LEDs paired with

photosensors were used to measure the light transmittance of cell culture at 470 nm (blue), 518 nm (green), 630 nm (red) and 940 nm (infrared). LEDs and photodiodes pairs were mounted on opposite sides of a tube with 32mm inner diameter where microalgae samples flew through. A neural network (NN) was employed to estimate biomass by associating a 4 x 1 vector consisted of voltage outputs from each photosensor with the cell concentration measured by cell count (up to $400x10^6$ cells mL^{-1}). Training of the NN was needed for different species. The sensor reported has an absolute estimation error below $8x10^6$ cells mL^{-1}, and a maximum error at 9% within interval of 5 to $145x10^6$ cells mL^{-1}.

Nedbal et al. (2008) described the monitoring of chlorophyll concentration and cell density of cyanobacteria *Cyanothece* sp. by a flat-cuvette photobioreactor (PBR) with built-in fluorometer and densitometer. The optical density of the suspension was measured at 680 nm and 735 nm with LED and photodiodes installed on opposite side of the cuvette PBR. Blue LEDs (455 nm) and orange LEDs (627 nm) were used for excitation of chlorophyll and phycobilins, respectively. The fluorescence emitted from chlorophyll and phycobilins were measured by the same photodiode with an optical filter that blocks the exciting lights. Cell counts and chlorophyll concentration were linearly proportional to optical density (OD) 680 in the range of 0.1–1.2 and to OD 735 in the range of 0.02–0.4 which can be exceeded in microalgae production. A non-linear calibration is necessary outside this range. They demonstrated the sensors capability of monitoring the dynamics cyanobacteria in a 6 day batch culture in terms of optical density, OD680/OD735, specific growth rate and effective quantum yield of photosystem II. They further use the sensor to compare cyanobacteria diurnal growth pattern in different media.

Marxen et al. (2005) developed a bioreactor system for the cultivation of the microalgae *Synechocystis* sp. PCC6803 under controlled physiological conditions. An optical density sensor that measures light absorbance at 870 nm and a fluorescent sensor was used to measure chlorophyll fluorescence by pulse amplitude modulation (PAM) technic were placed in a column PBR for inline measurements. A turbidostatic process was achieved by diluting the algal suspension in the reactor with the feedback from the optical density sensor to maintain the biomass concentration at a constant level. Furthermore, a new process strategy, physiostat, was developed aiming at maintaining a physiological parameter constant by modulating UVB-radiation level using chlorophyll fluorescence as a control parameter.

Shin et al. (2015) reported the development of a portable and low cost fluorescent sensing system with a disposable microfluidic chip for on-site detection of a microalgal sample and its concentration. The sensor system has multiple light emitting diodes (LEDs) for excitation at 448 nm and a photodetector for measuring a fluorescent signal at 680 nm from a microalgal sample. The concentration of a microalgal sample is determined by measuring the fluorescent signal emitted by chlorophyll a. A small volume of microalgal sample (<10 μL) was carried by a microfluidic chip consists of a glass slide and a PDMS channel with a vacuum pump. The photocurrent from the photodiode was calibrated to cell count of *Chlorella vulgaris* determine by a flow cytometer. A linear correlation between the two was shown with R^2 of 0.96 within cell concentration range of 0 to 1.9 x 10^7 cell mL^{-1}. The sensor was also tested with microalgal samples mixed with different turbidity water to validate its selectivity. Soil samples that consisted of sand, silt and clay with a

median grain size of 0.2 mm were used to achieve a turbidity level up to 157 NTU. The results show that the fluorescent detection of microalgal concentration is not influenced by the turbidity level of the sample solution. Improvements including signal noise reduction and integration of a detection circuit system were needed to enable the on-site measurement capability of the sensor.

Thomasson et al. (2010) developed an opto-electronic sensor for the purpose of in-situ measuring optical density of microalgae culture in real-time. The sensor system pumps aqueous algae through the sensor body and measures absorbance in two narrow wavebands in the red and near-infrared (NIR) regions. No further detail of the design of the sensor was revealed due to patent application reasons. The sensor was calibrated to a UV/VIS/NIR spectrophotometer with samples of *Nannochloropsis oculata* ranging from OD 0.05 to OD 0.5. A good linear correlation was shown with R^2 of 0.98. However, the linear correlation did not hold for measurements taken place in field test. Part of the cause was attributed to the increase of noise level in the detector signals. It was later reported that it was the temperature dependency of the sensor unit caused inaccurate measurement of algal biomass concentration (Yao et al. 2012).

Based on the literature reviewed and summarized above, it is determined that there is no current optical sensor design for real-time microalgae growth monitoring was capable of monitoring multiple biological parameters with high accuracy in a high cell concentration range, without sample preparation (i.e. dilution, washing, filtration) prior to measurements, and has the flexibility to be integrated to various forms of microalgae production systems.

Several researchers applied colorimetric methods to estimate biomass concentration, chlorophyll and lipid content of microalgae. The color variation in microalgae is mainly due to the change in the biochemical composition of the cells. Based on trichromatic theory, color can be decomposed into the primary colors and the intensity of an individual color can be represented by the number of pixels of brightness, in a digital image. Thus, the brightness values of the primary colors can be correlated to biomass concentration and biochemical contents of the microalgae.

Su et al. (2008) established a method of rapid determination of chlorophyll a and lipid contents of marine algae *Nannochloropsis oculata* by evaluating the brightness of the three primary colors (red, green, blue). A digital camera was used to capture image of microalgal samples contained in a quartz cuvette that has been diluted to a fixed biomass concentration (0.5 g L^{-1}). The image was decomposed and the brightness of each primary color was transformed to a 256 level scale. The brightness values of the three primary colors are modeled as two linear correlation functions (RGB model) for microalgal chlorophyll a and lipid contents, respectively with a squared correlation coefficient (R^2) of 0.99. The method was further applied to monitor chlorophyll a and lipid content of microalgae in a real culture system. The time-course chlorophyll a and lipid content change was observed in a batch culture that lasted 11 days. The manual sampling and sample preparation procedures were required for this detection method. Further development of the sensor is needed for use in on-line microalgae cultivation monitoring application.

López et al. (2006) developed methods to characterize *Haematococcus pluvialis* culture on both macroscopic and microscopic scales. The CIE-LAB system, the most

popular numerical color-space system, was used to extract color features from images taken by a CM-3500d Minolta spectrophotometer–colorimeter, then correlate color features to biomass concentration, and the chlorophyll, carotenoid and astaxanthin content of the biomass. The camera was able to estimate biomass concentration up to 2.0 g L^{-1} with a squared correlation coefficient (R^2) of 0.93. Image-Pro Plus 4.5.1 image analysis software was used to identify cell population, average cell size and population homogeneity from images taken by a CMOS camera (Evolution LC Color from Media Cybernetics) mounted on the a microscope. The sensors were further applied to monitor biomass concentration, pigment content and cell density of *H. pluvialis* in an airlift tubular PBR and a bubble column PBR for 16 days. The results were used to quantify the influence of design of the reactors on biomass productivity. All the measurement were taken placed either on a microscope or custom made cuvette which was not desirable for on-line monitoring application.

Córdoba-Matson et al. (2009) designed and constructed an inexpensive digital imaging system for counting microalgal cells. The images of *Isochrysis galbana* culture in an Erlenmeyer flask illuminated by an incandescent light bulb was taken by a CCD camera. All components were fixed in an opaque black enclosure to avoid any interference for ambient light. A program written in MATLAB converted RGB color images to gray scale which was further used to correlate to cell numbers of microalgae. It was concluded that *I. galbana* cell numbers could be measured with accuracies of less than 10% over the range of culture densities of 1.52×10^6 to 8.1×10^6 cells mL^{-1}. It was also found that precision values varied depending on cell density concentration. At high cell density concentration,

the precision was low (typically <4%), but at low cell concentration precision reached 20%. The system need to be re-calibrated to specific species of microalgae since the color of microalgae may vary depending on species.

Benavides et al. (2015) demonstrated the feasibility of a sensor based on an RGB color generator and a light sensor to detect the biomass concentration of microalgae *Dunaliella tertiolecta*. The sensor consisted of a sensor chamber, a data acquisition board and a computer for data processing. Inside the sensor chamber, a RGB sensor and a mirror were placed on opposite sides of a flow chamber that contains microalgae sample. A beam of light emitted from the RGB sensor was reflected back to the sensor by the mirror. The luminance of the reflected light was calculated as a weighted sum of each color component following the international standard recommendation ITU-R BT.709. The light absorbance was subsequently calculated using Beer's law. The sensor was calibrated against the biomass concentration of microalgae measured by a bench-top UV spectrophotometer, a good linear correlation was obtained with R^2 of 0.99. The performance of the sensor was also compared to a commercial NIR absorption probe in a batch culture of *D. tertiolecta*. The sensor was only able to accurately estimate biomass concentration up to 0.7 g L^{-1}.

Meireles et al. (2002) demonstrated an on-line optical density measurement system with flow injection analysis (FIA) device integrated spectrophotometer to monitor biomass concentration of *Pavlova lutheri*. The FIA device enabled automated dilution of microalgae samples to maintain the biomass concentration within the linear zone. The FIA also featured a washing mechanism that cleans the flow cell each time before and after a measurement was made. Two FIA loops with different dilution factors (1.88 and 4.56) were

used to measure optical density of microalgae in different concentration range. The sensor system was tested with batch and semi-continuous batch culture of microalgae, and the results showed good agreement with that from off-line measurements.

The parasites associated with algae has become a great interest and potential economic impact due to their devastating effect on commercial scale monoculture. Therefore, detection of microalgal parasites and solutions to parasites contamination became important to minimize the economic impact on microalgae production from contamination.

Gerphagnon et al. (2013) proposed a double staining method to assess chytrid infection rates of cyanobacteria using Calcofluor white and SYTOX green, a nucleic acid stain. The authors used a combination of UV and blue light to show chytrid zoospores (green) inside sporangia (blue). However, for some algae Calcofluor white is problematic when cellulose is the primary cell wall component, such as for *Haematococcus pluvialis*, because cellulose can be stained as well as chitin and may obscure detection (Damiani et al. 2006). However, Calcofluor white cannot stain fungi lacking chitin. As an alternative, staining chytrid sporangia with nile red, even at very young stages, can be used as an early detection method for algae (Gutman, Zarka, and Boussiba 2009).

Day et al. (2012) employed a Bench-top VS IV FlowCAM cytometer to detect grazers (size range 20–80 μm in length) in the presence of microalgae *Nannochloropsis oculata*. Detection limits were <10 cells mL^{-1} for both model grazers, *Euplotes vannus* (80 x 45 μm), and an unidentified holotrichous ciliate (~18 x 8 μm) respectively. Furthermore, the system can distinguish the presence of ciliates in *N. oculata* cultures with

biotechnologically relevant cell densities; i.e. $>1.4 \times 10^8$ cells mL^{-1} (>0.5 g L^{-1} dry weight).

3. PRESENT STUDY

3.1 Overall Summary

Appendix A, B and C of this dissertation present the manuscripts of the methods, results, and conclusions of objective one, two and three respectively. The following is a summary of the primary results of the research.

In Appendix A, the design of a multi-wavelength based optical density sensor unit to monitor microalgae growth in real time was described. The system consisted of five main components including (1) laser diode modules as light sources (2) photodiodes as detectors, (3) driver circuit, (4) flow cell and (5) sensor housing temperature controller. The sensor unit was designed to be integrated into any microalgae culture system for real time optical density measurements and algae growth monitoring applications. An indoor photobioreactor (PBR) and an outdoor open pond raceway were used to evaluate the performance of the optical sensor. Results showed that the optical sensor was capable of estimating biomass concentration accurately and was able to monitor the physiological status of the microalgae culture including the changes in growth rate and the change of chlorophyll content can serve as indicators of the health of the culture. During the outdoor open pond raceway test, a temperature regulation unit was integrated to maintain a constant temperature of the sensor housing. This also ensured a constant laser power output. The sensor was able to record the growth of microalgae in real-time under the dynamic change of lighting condition and temperature in outdoor environment. The growth rate of microalgae calculated from the real-time data was highly correlated to the photosynthetic active radiation (PAR) level. The sensor was able to monitor cell concentration as high as

1.05 g L^{-1} ($1.51x10^8$ cells mL^{-1}) during the culture growth without any sample preparation for the measurements.

The calibration of the optical sensor to environmental disturbances was undertaken in Appendix B. The capability of the optical sensor was evaluated on the application of early detection of microalgae culture failure associated with the introduction of the predator *V. chlorellavorus* to an experimental PBR. Also, the sensitivity of the sensor to the presence of dust in the PBR was investigated. In the dust test, field test dust with mean diameter of 25.3 μm, standard deviation of 11.8 μm was used to resemble the size distribution of dust that falls into the outdoor raceways in Arizona. The optical density didn't increase proportionally to the increase of dry mass, considering the amount of test dust added to the PBR which resulted a 59.7% increase of the total dry mass. Further analysis showed that the introduction of dust can be clearly indicated by the first derivative of OD_{780}. *V. Chlorellavorus* co-cultured with DOE 1412 was used to inoculate a healthy DOE 1412 culture for the bacteria contamination test that was replicated three times. Cell viability began to decrease two days prior to the rapid decline or 'crash' of the culture, the same time point at which a steep decrease in the OD_{685}/OD_{780} was also observed. A similar growth pattern was observed for each of the replicated experiments. Therefore, OD_{685}/OD_{780} was found to serve as an indicatory parameter for early detection of the crash of *C. sorokiniana* from *V. chlorellavorus* infection.

Finally, the optical sensor was integrated into an open pond raceway for the application of autonomous monitoring and control of microalgae production systems. The pH, electrical conductivity (EC), temperature (T), dissolved oxygen (DO), water depth

(WD), optical density of the culture (OD) and photosynthetic active radiation (PAR) were monitored and stored in a data acquisition system. The pH and water level of the system was control by the microcontroller based on the feedback from pH and water depth sensor respectively. The automation of harvesting was based on the optical density of the culture measured by the inline optical sensor. The water and nutrients addition following the harvest was automated as well. The communication between the data logger and the control station was established through a local network. Lastly, a graphical user interface (GUI) was created on the control station for real-time monitoring of the microalgae growth, resource input and environmental conditions of the culture system. The designed and developed real-time monitoring and feed-back based control system was capable of controlling the desired set points and culture conditions established by the operator and provide information on resource use in the microalgae culture in real-time.

3.2 Overall Conclusions and Recommendations

A multi-wavelength based optical density sensor was successfully designed, developed, and evaluated to monitor microalgae growth in real time. Algae biomass concentration was accurately estimated with optical density measurements at 650, 685 and 780 nm wavelengths used by the sensor. The sensor unit was able to monitor cell concentration as high as 1.05 g L^{-1} ($1.51x10^{8}$ cells mL^{-1}) during the culture growth without any sample preparation for the measurements. Growth rates and ratios calculated from optical density at each wavelength were good indications for monitoring of microalgae growth transitions and for detection of disturbances to the culture system (i.e change of light intensity, water addition, rain, and harvesting). The sensor showed low sensitivity to

the amount of dust that's 60% of the Ash Free Dry Weight (AFDW) of microalgae biomass. However, the sensor was able to clearly indicate the event of dust introduction to the culture system. The optical sensor for monitoring growth dynamics of microalgae in real-time described in this dissertation was capable of early detection of culture crash due to *V. chlorellavorus* infection while being insensitive to the dust content in a culture system. The inline optical sensor was integrated into an open pond raceway for automation of the biomass production operation. The harvesting, water and nutrients addition were completely automated based on the feedback from the optical sensor along with other sensors measuring key variables from the culture growing environment.

The sensor unit was operated continuously for 18 days without any visible microalgae biofilm deposit observed on the flow chamber of the sensor unit. In this design, the only sensor hardware part that had contact with culture medium was the flow chamber which can be easily replaced. For industrial microalgae production, the application of ultra-hydrophobic material (Hydrophobic glass coating, UltraTech International, Inc., USA) on the surface of flow chamber can further extend the maintenance interval. A temperature control device for the sensor is necessary, especially for outdoor applications where the air temperature can vary significantly, since the output power of laser diodes were temperature dependent. The light path was 5 mm in the current sensor design. Therefore, the cell concentration measurement range can be further improved by shortening the light path length of the flow chamber. Other laser modules and wavelengths of interest can be added to expand the number of biological variables and culture growth and health conditions measured by the sensor. With proper calibration, installation and operation, the optical

sensor described in this study can be integrated into microalgae culture systems for monitoring and control purposes at a relative low cost to ultimately help optimize product quality and quantity, and resource use efficiency.

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APPENDIX A - MULTI-WAVELENGTH BASED OPTICAL DENSITY SENSOR FOR AUTONOMOUS MONITORING OF MICROALGAE

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In submission: *Sensors*

ABSTRACT

A multi-wavelength based optical density sensor unit was designed, developed, and evaluated to monitor microalgae growth in real time. The system consisted of five main components including (1) laser diode modules as light sources (2) photodiodes as detectors, (3) driver circuit, (4) flow cell and (5) sensor housing temperature controller. The sensor unit was designed to be integrated into any microalgae culture system for both real time and non-real time optical density measurements and algae growth monitoring applications. It was shown that the sensor unit was capable of monitoring the dynamics and physiological changes of the microalgae culture in real-time. Algae biomass concentration was accurately estimated with optical density measurements at 650, 685 and 780 nm wavelengths used by the sensor unit. The sensor unit was able to monitor cell concentration as high as 1.05 g L^{-1} ($1.51x10^{8}$ cells mL^{-1}) during the culture growth without any sample preparation for the measurements. Since high cell concentrations do not need to be diluted using the sensor unit, the system has the potential to be used in industrial microalgae cultivation systems for real time monitoring and control applications that can lead to improved resource use efficiency.

KEYWORDS

Optical density; multi-wavelength; microalgae; real-time monitoring and control

1. INTRODUCTION

Microalgae have been successfully used as feedstock for the production of pharmaceutical products, nutritional supplements and chemicals [1-4]. Certain species of microalgae are candidates for the production of biofuels due to their high productivity and high oil content [5-7]. Producing sufficient amounts of biomass with controlled quality is the premise of production of microalgae derived products. Optimizing resource inputs and maintaining high productivity are the key components to control the quantity and cost of the algae production.

Real-time monitoring and control provides the platform to acquire the environmental and physiological dynamics of a microalgae culture system. For large scale microalgae production systems, effective decision making and overall production system management in terms of optimal resource use, harvesting and culture condition optimization (media composition, lighting, temperature, pH, dissolved oxygen levels etc.) is crucial in order to achieve maximum profit and to prevent or reduce economic losses in case of contamination [8].

Measurements of biological variables, including cell mass concentration, cell size, cell morphology, population composition (i.e. concerns with contamination), pigments and lipid content, are especially desirable because they are the direct indicators of the dynamics of a microalgae culture system. Standard methods developed for measurements of these variables are either too laborious or destructive to be employed for real-time monitoring and control purposes [9, 10]. Spectrophotometry has been widely used to estimate these biological variables by measuring the absorbance, turbidity or fluorescence of the culture

suspension [11-13]. As a non-destructive and rapid analytical method, spectrophotometry became a preferable candidate for real-time monitoring and control of microalgae culture systems.

There are some commercialized sensors to monitor microalgae concentration [14-17]. However, most of them are designed to monitor microalgae concentration at an environmental level which is much lower than the cell concentration in microalgae production applications. Furthermore, these sensors are too expensive for low added value product applications. Therefore, they are not practical to integrate into outdoor raceway or photobioreactor based algae production systems.

There have been only few studies on development and evaluation of self-constructed optical sensors for microalgae monitoring and control applications [18-25]. For instance, Sandes et al. (2006) [23] focused on measuring the intensity of light transmitted through a transparent production tube with a 10 mm light path length containing a microalgae suspension using a LED (880 nm) and photodiode pair mounted on the opposite side of the tube. The sensor was able to estimate the cell concentration of *Nannochloropsis oceanica* and correlated both with dry weight (up to 2.0 g L^{-1}) and cell count. Briassoulis et al. (2010) [18] developed an automated flow-through density sensor and harvesting system for *Nannochloropsis* sp. The LEDs paired with photosensors were used to measure the light transmittance of cell culture at 470, 518, 630 and 940 nm. A neural network was employed to estimate biomass concentration by associating the voltage readings from each photosensor with the cell concentration measured by cell count. The sensor reported has an absolute estimation error below $8x10^6$ cells mL^{-1}, and a maximum error at 9% within

interval of 5 to $145x10^6$ cells mL^{-1}. Nedbal et al. (2008) [22] described the monitoring of chlorophyll concentration and cell density of a cyanobacterial suspension by a flat-cuvette photobioreactor with a built-in fluorometer and densitometer. Blue LEDs (455 nm) and orange LEDs (627 nm) were used for excitation of blue absorption and phycobilins, respectively. The optical density of the suspension was measured at 680 nm and 735 nm. Cell count and chlorophyll concentration were linearly proportional to optical density (OD) 680 in the range 0.1–1.2 and to OD 735 in the range 0.02–0.4; these values of OD or cell density are typically exceeded in microalgae production systems. Furthermore, the sensor unit was designed for a specific PBR, re-configuration and re-calibration of the sensor will be necessary if it were to be integrated into other culture systems. Marxen et al. (2005) [20] developed a bioreactor system for the cultivation of *Synechocystis* sp. PCC6803. Dry mass of microalgae was estimated by the measurement of optical density of the suspension at 870 nm. Chlorophyll concentration was determined by the pulse amplitude modulation (PAM) technique. Yao et al. (2012) [25] developed and tested an optical density based sensor using a LED and photodiode based unit at two wavelengths (Red and NIR) to monitor algae growth. The sensor was able to estimate biomass concentration ranging from 0.05 to 0.50 OD in indoor conditions. The study reported temperature dependency of the sensor unit that caused inaccurate measurement of algal biomass concentration when tested in outdoor conditions.

To our knowledge, there is no current optical sensor design that exists in the market for measuring multiple biological parameters in real time both in an indoor PBR and outdoor raceway system within a high cell concentration range and without needing sample

preparation (i.e. dilution, washing, filtration) prior to measurements. Therefore, we describe here the design, development and evaluation of a relatively low cost multi-wavelength laser diode-photodiode based sensor applicable for use both in an indoor photobioreactor system and an outdoor raceway system to monitor optical density and growth of microalgae in real time.

2. MATERIAL AND METHODS

2.1. Optical density measurement sensor

The growth dynamics of the microalgae culture was measured using the real-time optical density sensor (Fig. 1.) developed in this study. Light absorbance of microalgae suspensions at multiple wavelengths correlate to different characters of microalgae cells. The 650 (650nm-10mW, US-Lasers Inc., USA), 685 (HL6750MG, Oclaro Inc., USA) and 780 (780nm-10mW, US-Lasers Inc., USA) laser diodes were used in the developed sensor unit for this study. These three wavelengths have been commonly used to estimate the cell concentration of microalgae suspension [11-13]. Light absorbance at 780 nm estimates the turbidity of the suspension since the color of microalgae has no effect on the absorbance, whereas, light absorbance at 650 and 685 nm correlates to both intensity of the color (i.e. chlorophyll content) and cell concentration.

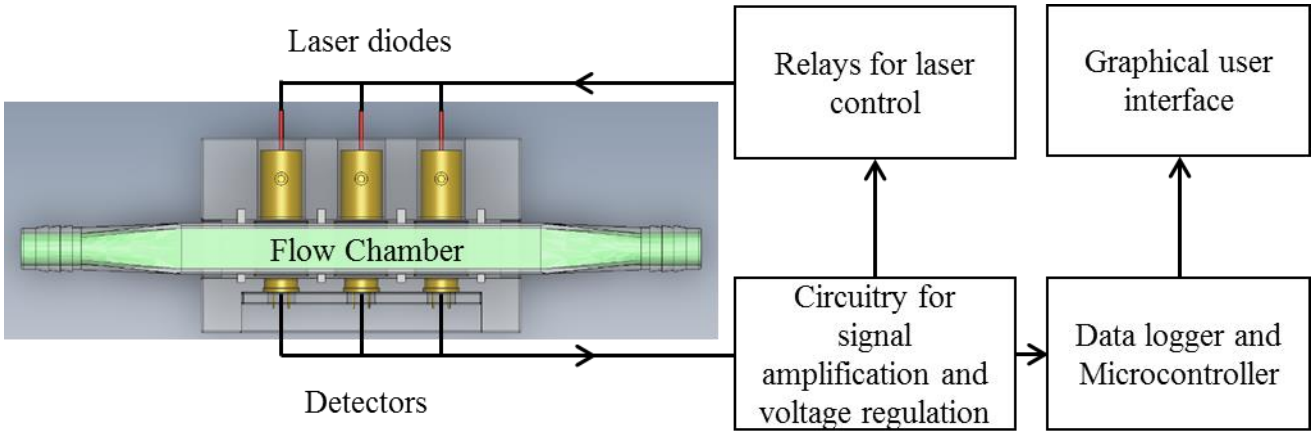

Figure 1. Component layout of the optical sensor unit. Three laser diodes at wavelengths of 650 nm, 685 nm and 780 nm were aligned with 3 photodiodes with a detection range of 350-1100 nm. The flow chamber window was perpendicular to the laser beam.

The optical sensor unit consisted of laser diode modules as light sources, a photodiode as a detector and custom-made fixtures to house them. Laser diode modules consisted of laser diodes, driver circuit (iC-WK BMST WK2D, iC Haus LLC., USA) and brass housing with adjustable optical lenses (10.4mm Module Housing Kit, US-Lasers Inc., USA). An optical filter (86734, Edmund Optics Inc., USA) was placed in front of the 685 nm laser diode to allow only the light with wavelength from 680 to 690 nm to pass through. The system design enabled adjustment of the output power of the modules by a potentiometer connected to a 5 VDC power source. The photodiodes (FDS100, Thorlabs Inc., USA) with a detection range of 350-1100 nm were connected to a zero-bias amplification circuit. Three pairs of laser diode modules and photodiodes were placed in a linear pattern in the fixture. Each pair was aligned and placed 15 mm apart. The diameter of the circular light beam from the laser diode modules was adjusted to be slightly smaller than the size of detection window on the photodiode. The optical sensor unit was designed

to enable measurements from either standard cuvettes or custom made flow chambers with a light path length of 5 mm. Cuvettes and flow chambers were placed perpendicular to the laser beam and 1 mm away from the window of photodiodes. When used for real-time monitoring, laser diodes were powered sequentially by the data logger's control module to avoid light noise from individual laser light sources. The voltage generated from the photodiodes was amplified and recorded by a data logger and controller (CR3000, Campbell Scientific Inc., UT, USA). The entire sensor unit was mounted in a weather proof enclosure enabling connection of tubes for algae flow through the sensor flow cell and signal cables for the laser diodes and photo diodes.

The voltage output of the photodiode is proportional to the intensity of incident light. According to Beer-Lambert law, the light absorbance of the sample was determined by,

$$Abs = -ln(V_s/V_b)$$

Abs = Light absorbance

V_b = Output of the photodiode from growth media (mV)

V_s = Output of the photodiode from a sample (mV)

2.2. Cultivation conditions and organisms

2.2.1 Indoor photobioreactor (PBR) cultivation

Chlorella sorokiniana Beijerinck, 1890 (DOE 1412) received from Pacific Northwest National Laboratory, WA, USA [26] was cultivated in local well water enriched with Peters professional 20-20-20 general purpose water soluble fertilizer 0.26 g L^{-1}

(E99290, Peters Professional, USA), Citraplex 20% iron 0.053 g L^{-1} (Citraplex 20% Iron, Loveland Products, Inc., USA) and trace elements solution (H_3BO_3 0.0029 g L^{-1}, $MnCl_2 \cdot 4H_2O$ 0.0018 g L^{-1}, $ZnSO_4 \cdot H_2O$ 0.00014 g L^{-1}, $Na_2MoO_4 \cdot 2H_2O$ 0.00039 g L^{-1}, $CoCl2 \cdot 6H_2O$ 0.000055 g L^{-1}) under illumination intensity of 200 µmol m^{-2} s^{-1} or 400 µmol m^{-2} s^{-1} in rectangular air lift photo bioreactors (PBRs). The algae culture temperature was light intensity dependent and was stabilized at 30±2 °C. The pH of the medium was controlled at 7±0.3 by injecting CO_2 from a pressurized liquid CO_2 tank into PBRs.

2.2.2 Outdoor open pond raceway cultivation

Scenedesmus obliquus was used in the outdoor open pond raceway cultivation experiments. *Scenedesmus obliquus* was received from Texas A&M AgriLife Research (Texas, USA) and was cultivated in local well water enriched with Pecos medium, trace metal solution and 5g L^{-1} NaCl. The Pecos medium contained 0.1 g L^{-1} urea ($(NH_2)_2CO$), 0.012 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.035 g L^{-1} $NH_4H_2PO_4$, 0.175 g L^{-1} Potash (KCl), 0.0054 g L^{-1} $FeCl_3$ and 0.02 g L^{-1} Na_2CO_3. The culture was maintained in an open pond paddle wheel raceway with a surface area of 3 m^2 located in Tucson, Arizona, USA. The culture depth was maintained at 10 cm and increased to 15 cm later in the experiment. The pH of the medium was controlled at 8±0.05 by injecting 95% CO_2 through an air sparger.

2.3 Offline biomass concentration measurement

Biomass concentration of microalgae was determined by both cell counting and ash-free dry weight (AFDW) measurements. Cell suspension was diluted to a concentration between 10^6 and 10^7 cells mL^{-1} for cell counting by a neubauer chamber hemocytometer (Hy-Lite Ultra-plane, Clayadams, USA) under a microscope (XSZ-138, AOK International

Group Ltd., China). The AFDW of the cells was measured following the method described by Zhu and Lee (1997) [27]. The light absorbance of the cells suspension was measured at 650, 685, 750 and 780 nm by a spectrophotometer (DR 3800, HACH, USA) using a 10 mm light path length cuvette. Samples were diluted with deionized water when necessary to keep the absorbance reading below 0.5.

2.4 Real-time monitoring of microalgae growth dynamics

2.4.1 Indoor PBR cultivation

The microalgae culture system consisted of an air lift flat panel PBR illuminated by a fluorescent lighting system. The pH (HI1001, Hanna Instruments, USA), electrical conductivity (HI3001, Hanna Instruments, USA), dissolved oxygen (DO1200/T, Sensorex, USA) and thermocouple temperature probes (Type T, Omega Engineering Inc., USA) were placed in the PBR for monitoring and control by a CR3000 datalogger. Each sensor was scanned every second and 10 minute averaged data was stored in the datalogger.

Flat panel PBRs with dimensions of 61 (H) x 61 (L) x 7.6 cm (W) were built using 6.35 mm thick clear acrylic panels (ACRYCLR0.250PM48X48, Plexiglas, USA). Air was constantly injected into the PBR via a 45.7 cm long air sparger mounted at the bottom of PBR for aeration and to achieve proper mixing of the microalgae culture. Carbon dioxide injection was controlled by the datalogger to maintain a desired pH level (7±0.3) in the PBR. The lighting system consisted of sixteen 61 cm 17-watt fluorescent light tubes (F17T8/741, Litetronics International, Inc., Illinois, USA) mounted on a supporting structure. Two levels of light intensity (200 and 400 µmols m^{-2} s^{-1}) were achieved by adjusting the number of lights used. The light remained on 24 hours per day, no dark period

was used. A small centrifugal pump (Seltz 20, Hydor, CA, USA) was used to recirculate cell suspension through the inline optical density measurement unit for the PBR. The optical density sensor was connected to the PBR system for continuous monitoring of OD and microalgae growth (Fig. 2).

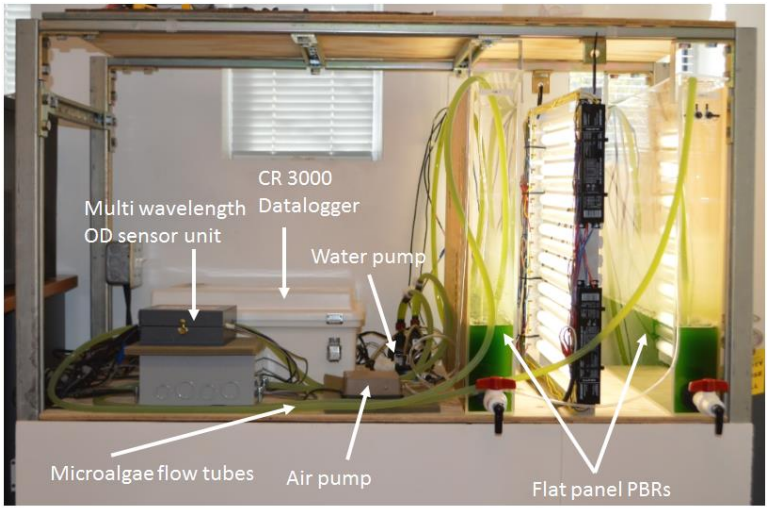

Figure 2. Multi wavelength optical sensor integrated into air-lift flat panel photobioreactors for real-time microalgae growth monitoring.

2.4.2 Outdoor open pond raceway cultivation

The optical density sensor was also integrated into an outdoor raceway system for continuous monitoring of microalgae growth (Fig. 3). Since sensor electronics maybe sensitive to environmental conditions, the optical sensor with its housing and the datalogger were placed in a location at the outdoor raceway site to minimize direct exposure to sunlight. The laser output is also temperature dependent (5-15 mV/ ^{o}C, vary with lasers). Therefore a temperature control unit was installed and consisted of a small heater plate (HT24S, Thorlabs, NJ, USA) and heat sink (55 mm Fan Heatsink, USA) to maintain a constant temperature (25±0.1 ^{o}C)inside the sensor box. This also ensured a constant laser

power output. The paddle wheel in the raceway system was operated 24 hours a day for continuous culture mixing. The CO_2 injection was turned off during night time. In addition to the measurement data collected for the indoor experiment, photosynthetically active radiation (PAR) was also measured using a quantum sensor (SQ-110, Apogee instruments, USA) at the level of the raceway system. All variables were recorded at the same frequency for sensor scanning and data averaging as described for the indoor cultivation experiment. The experiment occurred from 2/25 to 3/15 for a total of 18 days.

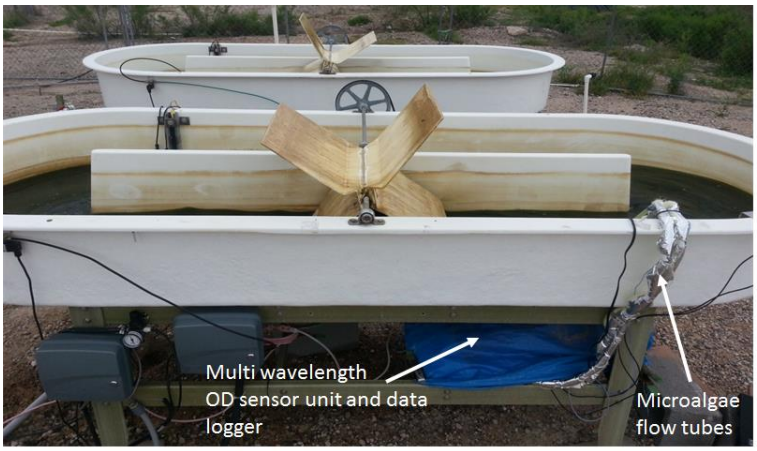

Figure 3. Optical sensor integrated into an open pond raceway for real-time microalgae growth monitoring.

3. Results and Discussion

3.1 In situ calibration of the optical density measurement unit

Light absorbance from a flowing cell suspension can be different from static samples due to cell movement and potentially the presence of fine air bubbles. Therefore, a calibration of the unit using flowing microalgae culture is necessary. In order to achieve in-line real-time monitoring, sample preparation needs to be eliminated or automated.

Cuvettes with 10 mm path length has been commonly used for optical density measurement. However, the OD does not increase proportionally to the increase of biomass concentration beyond a certain point. Therefore, dilution of the sample is necessary to keep the measurement within the linear correlation range for high concentration microalgae samples. According to Beer's law, the same results can be achieved by shortening the light path length of the measurement chamber. A preliminary experiment was conducted to prove this theory (Fig 4). Linear correlation between OD and AFDW held from the measurements made in shortened light path length flow chamber (5 mm). In contrast, OD started to saturate as biomass concentration increase when using 10 mm flow chamber. In this study, flow chambers with light path lengths of 5 mm were used to extend the measurement range of the unit without requiring sample dilution.

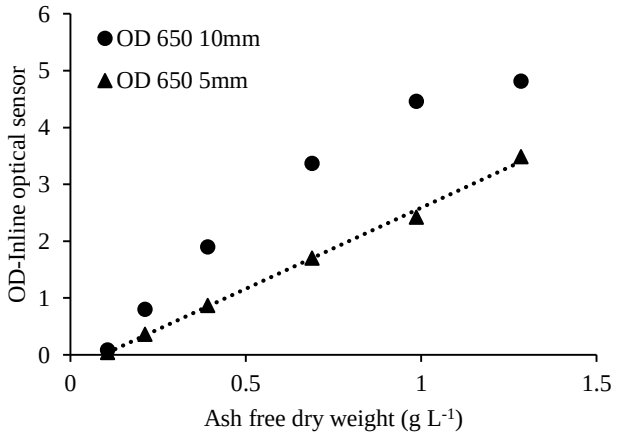

Figure 4. Correlation between OD measured by the inline optical density and AFDW using two flow chambers with 10 mm and 5 mm light path length respectively.

The optical sensor unit (Fig. 1) developed in this study (referred as IOS hereafter) was calibrated by comparing the reading from the sensor unit to that from a bench-top

spectrophotometer (referred as BT hereafter) at 650, 685 and 780 nm. The bench-top spectrophotometer (DR3800, Hach, CO, USA) was calibrated to both ash-free dry weight (AFDW) and cell count (CC) for *C. sorokiniana* at all three wavelengths: AFDW= $0.188*OD_{650}+0.0453$ g L^{-1} ($R^2=0.96$), AFDW= $0.161*OD_{685}+0.0292$ g L^{-1} ($R^2=0.96$), AFDW= $0.205*OD_{780}+0.0546$ g L^{-1} ($R^2=0.95$), CC=$(28.6* OD_{650}+1.13)$ 10^6 cells mL^{-1} ($R^2=0.91$), CC=$(26.8* OD_{685}-3.92)$ 10^6 cells mL^{-1} ($R^2=0.95$), CC=$(29.8* OD_{780}+3.96)$ 10^6 cells mL^{-1} ($R^2=0.90$). The optical density readings measured from the spectrophotometer using standard 10 mm cuvettes were compared to the results obtained from optical sensor unit using 5 mm flow cell. Strong linear correlations between the two measurement units were obtained at all wavelengths examined (Fig. 5). A linear correlation was tightly followed ($R^2=0.99$) between the optical density measurements obtained from IOS and BT units at 780 nm with cell concentration up to 1.05 g L^{-1} ($1.51x10^8$ cells mL^{-1}). Linear correlations hold for OD_{650} ($R^2=0.98$) and OD_{685} ($R^2=0.99$) for cell concentrations below 0.592 g L^{-1}. However, beyond this range while below 1.05 g L^{-1}, different linear correlations were observed for these two wavelengths (Fig. 5). Compared to the results from Nedbal et al. (2008) [22], the optical sensor unit showed the capability of measuring cell concentration over a wide range without dilution of the samples. The same calibration procedure was performed for *S. obliquus* during outdoor testing.

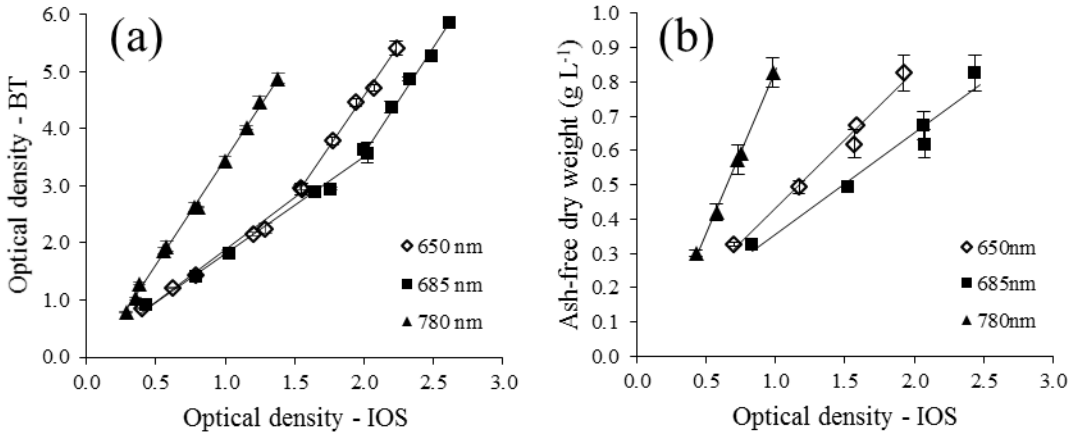

Figure 5. (a) Correlation between the optical densities of DOE 1412 in the PBR measured by a bench-top spectrophotometer (BT) and by the inline optical sensors (IOS). $OD_{650\ (BT)} = 1.82 \times OD_{650\ (IOS)} + 0.056$ (AFDW < 0.592 g L^{-1}), $OD_{685\ (BT)} = 1.70 \times OD_{685\ (IOS)} + 0.11$ (AFDW < 0.592 g L^{-1}), $OD_{650\ (BT)} = 3.54 \times OD_{650\ (IOS)} - 2.51$ (0.592 g L^{-1} < AFDW < 1.05 g L^{-1}), $OD_{685\ (BT)} = 3.72 \times OD_{685\ (IOS)} - 3.88$ (0.592 g L^{-1} < AFDW < 1.05 g L^{-1}), $OD_{780\ (BT)} = 3.71 \times OD_{780\ (IOS)} - 0.2445$ (AFDW < 1.05 g L^{-1}). **(b)** Correlation between optical density (IOS) and AFDW, $AFDW = 0.96 \times OD_{780\ (IOS)} - 0.12$ ($R^2 = 0.99$); $AFDW = 0.40 \times OD_{650\ (IOS)} + 0.032$ ($R^2 = 0.98$); $AFDW = 0.30 \times OD_{685\ (IOS)} + 0.061$ ($R^2 = 0.96$).

The OD readings from the optical sensor unit measured using 5 mm path length flow cell should be half of that from the spectrophotometer using a standard 10 mm cuvette in theory. However, the results did not show an exact correlation between the two units. This was because of the light quality from the laser diodes wasn't the same as that in a spectrophotometer where a monochromatic light was generated. Fig. 6 shows the spectra of the laser diodes used in the developed sensor unit, measured by a spectroradiometer (PS-300, Apogee Instruments, UT, USA) and the optical density spectra of DOE 1412. The peak wavelengths of each laser diode were slightly shifted from what was claimed by the manufacturers. An optical filter (86734, Edmund Optics, NJ, USA) was used to narrow the

band width of 685 nm laser diode from 80 nm to 10 nm and corrected the peak wavelength back to 685 nm from 688 nm. Despite the inferiority of the light beam generated from laser diodes, the strong linear correlations proved that the optical sensor unit was able to estimate the cell density as accurate as a spectrophotometer via calibration (Fig. 5).

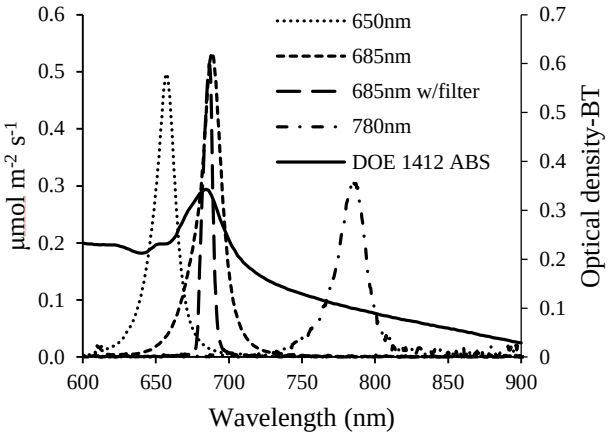

Figure 6. Light absorbance spectrum of DOE 1412 and light spectra of laser diodes used on the optical sensor.

3.2 Real-time microalgae growth monitoring

The optical sensor unit along with other sensors to monitor algae culture environment was integrated into a PBR to monitor the dynamics of a microalgae culture system. Fig. 7a shows the growth dynamics of semi-continuous culture of DOE 1412 as measured by the optical sensor unit over a period of 10 days. Sensor output shown in Fig.7a was calibrated to optical density reading from a bench-top spectrophotometer. The optical sensor unit showed the capability to capture the growth phases during semi-continuous operation, and the sudden change of cell concentration due to harvesting and addition of fresh media (indicated with arrows on the figure). A maximum cell concentration of 1.05

g L^{-1} ($1.51x10^8$ cells mL^{-1}) was observed during the cultivation experiment without any sample preparation and dilution for the measurements.

Growth dynamics of the microalgae was quantified by the growth rate. The growth rate was determined by the following equation with Δt of 2 hours (0.08 days).

$$\mu = \frac{ln(OD_2)_\lambda - ln(OD_1)_\lambda}{\Delta t}$$

μ = Growth rate (day^{-1})

OD = Optical density of microalgae at different time points (λ=780 nm)

Δt = Difference between the two time points (day)

The change of growth rate was clearly demonstrated by plotting the growth rate (μ) of DOE 1412 over time (Fig. 7b). The initial lag phase was followed by an increase in cell growth. Microalgae culture reached maximum growth rate soon after the lag phase when there is no light limitation. The growth rate then gradually decreases as the culture becomes light limited. When the illumination intensity was increased from 200 μmol m^{-2} s^{-1} to 400 μmol m^{-2} s^{-1} on 3/2/2014 an increase in growth rate was observed (Fig 7b). The growth rate dropped down to the level prior the alternation of light intensity as the culture again became light limited. These events were detected by the optical sensor unit (Fig. 7a and Fig. 7b). Although real time growth rate is not required for microalgal biomass production purposes, data with such high resolution provided a great tool for studying the fast response of microalgae to sudden change of the environmental conditions.

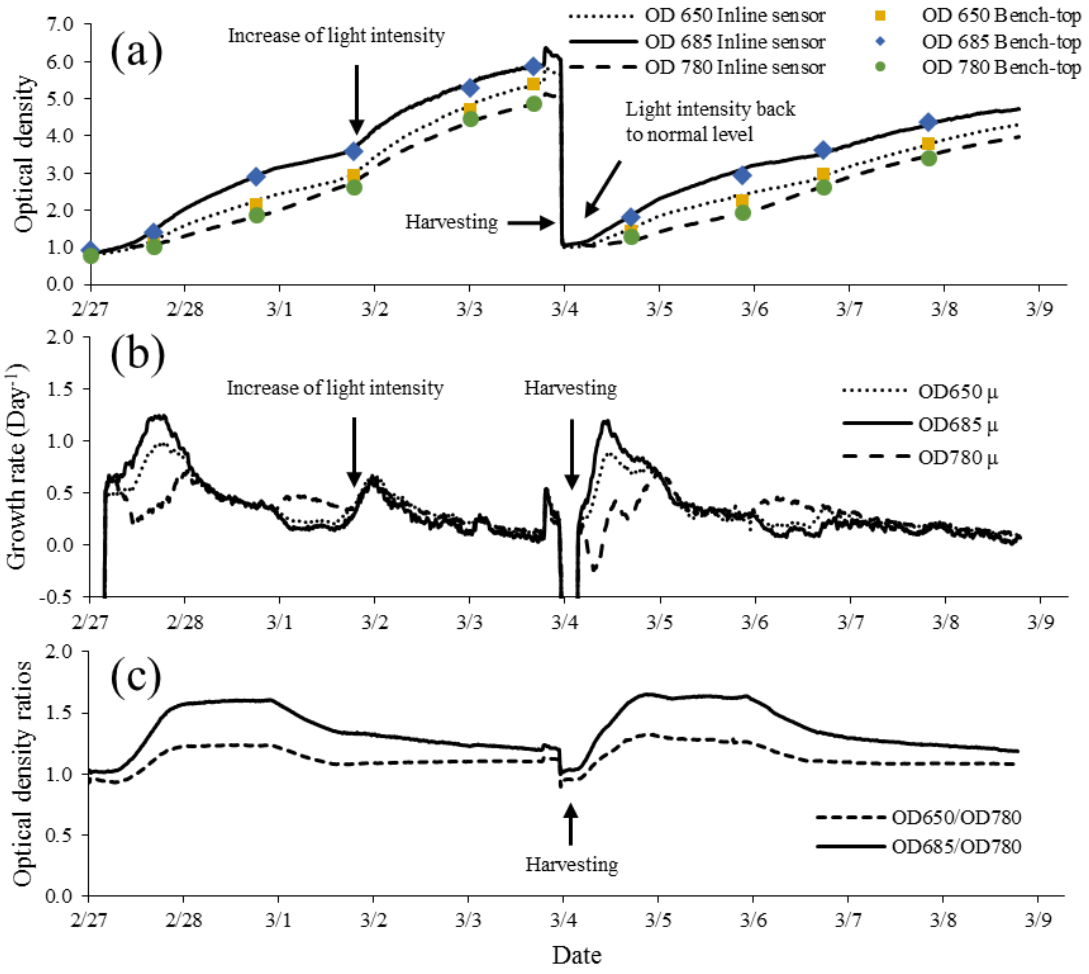

Figure 7. (**a**) Dynamics of optical density at 650 nm, 685 nm and 780 nm during semi-continuous culture of DOE 1412 run for 10 days. Illumination intensity was increased from 200 µmol m^{-2} s^{-1} to 400 µmol m^{-2} s^{-1} during the first batch on 3/2, it was then reduced to 200 µmol m^{-2} s^{-1} by the end of the batch. (**b**) Growth rate of DOE 1412 at 650, 685 and 780 nm and (**c**) ratios of optical densities at 650/780nm and 685/780nm.

Monitoring not only the cell concentration change, but also the dynamic physiological status of the microalgae culture including the changes in growth rate and the change of chlorophyll content can serve as indicators of the health of the culture. This is

important for cultivation of microalgae production when it is desirable to control conditions to produce a product of interest. For example, some microalgae produce more lipids when nutrients such as nitrogen are limiting. The ratios of optical densities at different wavelengths (685/780nm and 650/780nm) are shown in Fig. 7c. The ratios remained constant during lag phase, followed by a rapid increase during the exponential growth phase and stabilized at a higher level throughout the linear growth phase. The ratios then started to decrease as the growth of cells slowed down which indicated the transition from linear to stationary phase. The pattern of the ratio change occurred repeatedly over the time course of the experiment regardless of the growth pattern change induced by increased light intensity during the first batch. Signaling of this transition indicated that there is a decrease of chlorophyll content which absorbs most of the red light during the period indicated by the decreasing optical density ratios [29]. This might have been due to nitrogen limitation, since nitrogen is often rapidly consumed by algal cells during exponential growth according to López et al. (2006) [19]. Similar results for the change of OD 680/ OD 735 was reported by Nedbal et al. (2008) [22].

The optical sensor unit was also integrated into an outdoor open pond raceway for stability testing under highly dynamic outdoor weather conditions such as large temperature variations between daytime and nighttime periods. For instance, a 20 ^{o}C temperature difference were measured inside sensor box from daytime to nighttime when the temperature control system was not activated (Fig 8). The resulted inaccurate OD measurement by the inline optical sensor was shown in figure 8. The actual OD of the

culture was determined by a bench-top spectrophotometer. The steep decrease of OD during the nighttime was due to the increase in laser diode power that corresponded to the decrease of temperature. This did not reflect the actual OD dynamic of the microalgae culture in outdoor conditions. Thus, it is necessary to integrate a temperature control unit into the optical sensor for accurate OD measurements.

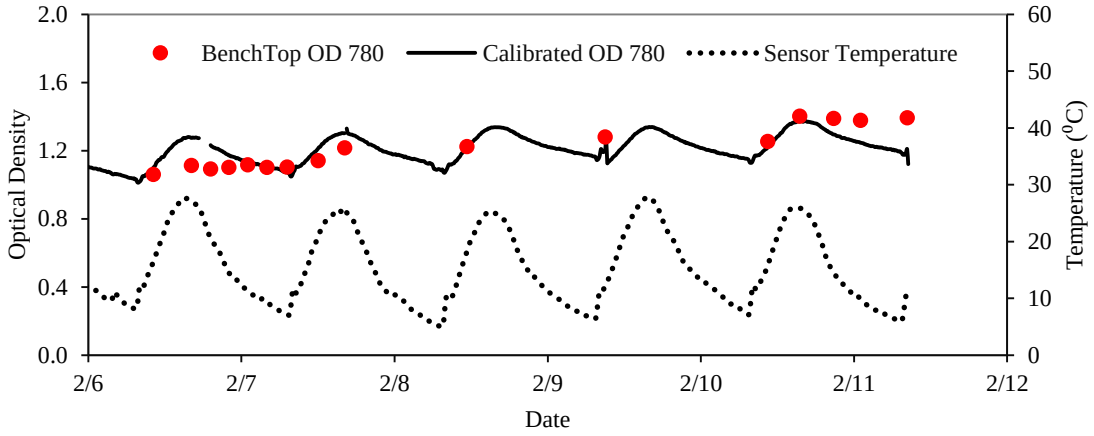

Figure 8. Optical density change of *S. obliquus* in open pond raceway over 5 days without temperature control unit on the inline sensor.

After the implementation of the temperature control unit, the temperature of the optical sensor was maintained at a constant level that ensured a consistent level of power output from the laser diodes. The optical density of the culture of *S. obliquus* during a period of 18 days recorded by the optical sensor is shown in Fig.9. The real-time optical density shows repeatedly an increase OD reading indicating the biomass increase during the day time due to photosynthesis. A small decrease in optical density was observed during the nighttime since photosynthetic microorganisms metabolize intracellular carbohydrate to sustain their metabolic activity as described by Ogbonna and Tanaka (1996) [28].

Sudden decreases of optical density of the culture due to water addition, precipitation (rain) and biomass harvesting were clearly shown in the figure labeled by arrows.

The growth rate of *S. obliquus* was compared to photosynthetic active radiation (PAR) measured at the raceway (Fig. 10). The growth rate of *S. obliquus* was dependent on the PAR level except during the water addition time period. This set of high resolution data enables one to evaluate in detail about how *S. obliquus* responds to solar radiation level in a sunny day. The correlation between PAR and growth rate can be used for the prediction of biomass production rate based on historical weather data for a given region.

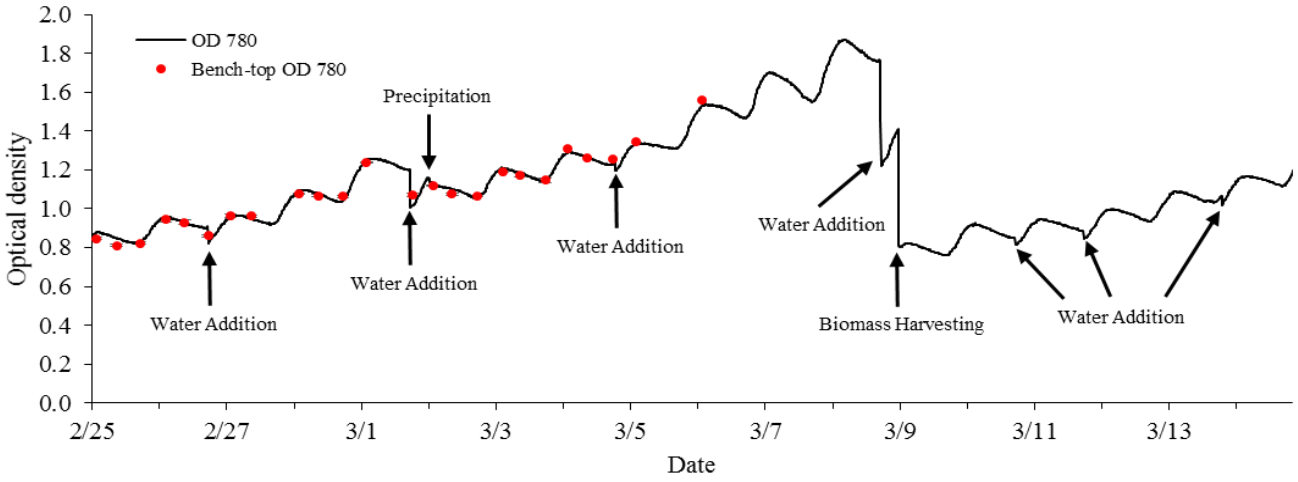

Figure 9. Optical density change of *S. obliquus* in open pond raceway over 18 days. Black arrows indicate events of water addition, precipitation and biomass harvesting.

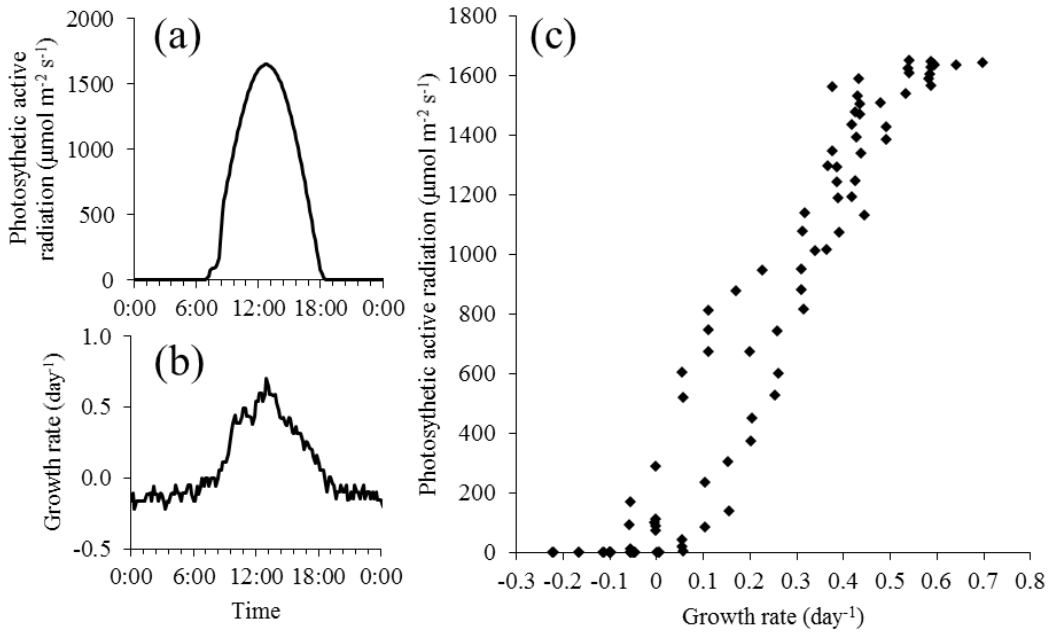

Figure 10. (**a**) Photosynthetic active radiation (PAR) of a sunny day in Tucson, AZ, USA. (**b**) Growth rate (µ) of *S. obliquus* in open pond raceway of the same day. (**c**) Scattered plot of PAR and µ from the data presented in (**a**) and (**b**).

4. CONCLUSIONS

The multi-wavelength laser diode based optical sensor unit was designed, developed and evaluated for the monitoring of microalgae culture dynamics in real-time both in a PBR and in an outdoor raceway system. The optical sensor unit prototype demonstrated the capability of estimating cell concentration and changes of the physiological status of the microalgae culture in real-time. The sensor unit was operated continuously for 18 days without any visible microalgae biofilm deposit observed on the flow chamber of the sensor unit. In this design, the only sensor hardware part that had contact with culture medium was the flow chamber which can be easily replaced. For industrial microalgae production, the application of ultra-hydrophobic material

(Hydrophobic glass coating, UltraTech International, Inc., USA) on the surface of flow chamber can further extend the maintenance interval. Biomass concentration was accurately estimated by optical density measurement at 650, 685 and 780 nm wavelengths. The sensor was capable of measuring maximum optical density of 5.41, 5.86 and 4.88 without sample preparation at 650 nm, 685 nm and 780 nm respectively. Growth rates and ratios calculated from optical density at each wavelength were good indications for monitoring of microalgae growth transitions and for detection of disturbances to the culture system (i.e change of light intensity, water addition, rain, and harvesting). A temperature control device for the sensor is necessary, especially for outdoor applications where air temperature can vary significantly, since the output power of laser diodes were temperature dependent. The cell concentration measurement range can be further improved by shortening the light path length of the flow chamber. Other laser modules and wavelengths of interest can be added to expand the number of biological variables measured by the sensor which is our focus for future studies. The real-time monitoring data from the optical sensor can be valuable for microalgae modeling studies both for PBR and outdoor raceway based production systems. With proper calibration, installation and operation, the optical sensor described in this study can be integrated into microalgae culture systems for monitoring and control purposes at a relative low cost to ultimately help optimize product quality and quantity.

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APPENDIX B - AUTONOMOUS DETECTION OF AN ABIOTIC AND BIOTIC DISTURBANCE IN A MICROALGAL CULTURE SYSTEM USING A MULTI-WAVELENGTH OPTICAL DENSITY SENSOR

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ABSTRACT

The development and calibration of an autonomous detection of environmental (abiotic and biotic) disturbances in an experimental microalgal culture system was undertaken using a multi-wavelength laser diode optical sensor. The goal was to develop a sensor capable of real time detection of fluctuations in algal cell number (density) indicative of the physiological (growth) status of a suspension culture of the microalga *Chlorella sorokiniana* (isolate DOE 1412). The rapid decline of a DOE 1412 culture infected by *V. chlorellavorus* was detected 2 days prior to the rapid death of the culture by parameters such as ratios of OD_{685} and OD_{780} indicating color features of microalgae culture. The sensitivity of the sensor to the presence of particulates in an indoor experimental continuous, temperature and light-controlled cultivation system was tested by introducing test 'field dust' like that from agricultural land in Arizona. The sensor showed relatively low sensitivity to a microalgal suspension containing particulates comprising 60% of the AFDW of microalgae biomass, however, it clearly indicated the field dust introduction 'event' to the culture system. Both types of 'invasions' were detectable using this early detection system.

KEYWORDS: *Chlorella sorokiniana*, early detection, multi-wavelength optical density sensor, real-time monitoring, *Vampirovibrio chlorellavorus*

1. INTRODUCTION

Microalgae have been commercially cultivated to produce protein, poly-unsaturated fatty acids (PUFAs), pigments and chemicals mainly for human nutrition and animal feed application since 1960s (Spolaore et al. 2006; Harun et al. 2010; A. Carlsson et al. 2007). Because certain microalgae species can achieve high productivity and moderate to high oil content compared to terrestrial oil crops, they are good candidates as raw material for biofuel production (Y. Li et al. 2008; Chisti 2007; Mata et al. 2010). Microalgae cultivated at commercial scales are typically grown in open pond raceways or closed photobioreactors (PBRs). Many commercial production settings have adopted open pond raceways because the financial feasibility has been shown to be substantially greater than that of PBRs (Richardson et al. 2012). In general, the physical culture conditions in open pond systems, including temperature, lighting intensity, and duration are climate dependent (Waller et al. 2012), and when conditions are not optimal, microalgal productivity can be negatively affected (Richardson et al. 2012). Sub-optimal productivity increases the cost of the product because large volumes of water must be processed to harvest to sufficient biomass (Danquah et al. 2009; Knuckey et al. 2006). However, the most prominent drawback of open pond raceway systems is cultivation failure due to the vulnerability of microalgae to biotic disturbances caused by the invasion of grazers, predators, and pathogens (Soo et al. 2015; Velicer and Mendes-Soares 2009; Carney and Lane 2014; Rego et al. 2015).

The fluorescence excitation of chlorophyll *a* molecules associated with microalgal cells grown in suspension cultures is commonly monitored to assess microalgal density in

near real-time because optical density (OD) is linear with cell number, and can be used to rapidly assess cell growth, and to estimate time of harvest and potential yield. Also, because measurements can be obtained for a very small sample size, the process is minimally destructive (Briassoulis et al. 2010; Sandnes et al. 2006; Thomasson et al. 2010; Marxen et al. 2005; Nedbal et al. 2008).

Dust and dirt suspended in microalgae cultures can cause inaccurate estimations of biomass concentration by increasing light absorption and alternating color profile of the culture suspension. This becomes a concern for microalgae production located in arid and semi-arid regions where a considerable amount of dust and dirt can fall into the cultivation raceways carried by dust storms or other causes of air disturbance.

Contamination of microalgae by parasites, grazers and pathogenic bacteria has always been an issue for large-scale microalgae production since it can lead to a rapid death of a culture that results in a loss of biomass and desired bioproducts. It also became a main constraint of species selection for open pond production systems. The detection of microalgal parasitic microorganisms such as bacteria rely on either microscopy and staining, flow cytometry, and molecular detection (Day et al. 2012; Gerphagnon et al. 2013). However, none of these approaches can detect the bacteria or the timing of the contaminating event *in-situ* in real-time. Several effective approaches for contending with parasite contamination have been tested, including salvage harvesting, ozone and UVC treatments, the addition of abscisic acid, and sonication. However, early detection is required for any abatement measure(s) to minimize damage (Webb et al. 2012; Benderliev et al. 1993; Shurin et al. 2013; Woo and Kamei 2003; Rego et al. 2015). Feasibly, real-time

sensors (Jia et al., 2015) that are capable of detecting 'contamination events', including predators, parasites, and pathogens, could be applied to their early detection and potentially result in rapid response time for abatement, particularly in large-scale production facilities.

The microalgal, *Chlorella sorokiniana* isolate 1412, is one of several robust candidate algae identified for use in lipid-based biofuel feedstock production (Lammers, P. et al. 2015). However, the bacterium, *Vampirovibrio chlorellavorus* (Gromov&Mamkaeva, 1972) (class Melainabacteria; Cyanobacteria), is a damaging, microbial predator of *C. sorokiniana*, and also of the related species, *C. vulgaris and C. kessleri* (Coder and Goff 1986). *V. chlorellavorus* is thought to destroy the host microalgal by adhering to and penetrating the cell, and utilizing its cellular contents by implementing a Type IV secretion system (T4SS), to deliver two conjugative plasmids that integrate into the genome (where they replicate and express essential pathogenicity proteins, such as an efflux pump) through the channel in the T4SS apparatus, along with proteins and hydrolytic enzymes made by the bacterium that digest the cell contents (Soo et al., 2015). Although *Chlorella* cells remain intact after *V. chlorellavorus* attack for about one week, the color of the cells fades due to the absence of pigments (Soo et al. 2015; Velicer and Mendes-Soares 2009).

Monitoring microalgal biomass concentration can be monitored using light scattering measurements based on the diffraction of incident light. Light scattering is measured using optical density, which increases as the number of cells increase. Optical density measurements at various wavelengths offers a rapid approach for assessing algal

growth and health in bioreactors and outdoor cultivation systems (Das et al. 2011; Solovchenko et al. 2011; Nedbal et al. 2008).

In this study, a multi-wavelength optical sensor was developed and tested for its ability to monitor microalgal performance in a near real-time capacity, and specifically, prior to the result of such disturbances being detectable by visual inspection. Two kinds of 'contamination' experiments were carried out to investigate the use of the sensor to perform real-time 'smart' monitoring of a *C. sorokiniana* suspension culture grown in a temperature and light-controlled bioreactor. The first involved the intentional application of 'field dust' to the bioreactor containing algal culture, and the second utilized the inoculation of the algal culture with the predator, *V. chlorellavorus*.

2. MATERIAL AND METHODS

2.1 Cultivation conditions and organisms

The DOE 1412 culture of *Chlorella sorokiniana* Beijerinck, 1890 was obtained from Pacific Northwest National Laboratory, WA, USA was used in all experiments (Jones et al. 2014). DOE1412 was cultivated in indoor experimental photobioreactors (PBRs) under illumination at 200 µmol m^{-2} s^{-1}, and the pH of the medium was controlled at 7±0.3 by injecting CO_2 from a pressurized liquid CO_2 tank into PBRs for both experiments.

For the 'field dust' experiment, DOE 1412 was cultivated in water pumped from a local well, enriched with Peters general purpose water soluble fertilizer (20-20-20) at a concentration of 0.26 g L^{-1} (E99290, Peters Professional, USA), Citraplex 20% iron 0.053 g L^{-1} (Citraplex 20% Iron, Loveland Products, Inc., USA), and a trace element solution

(H_3BO_3 0.0029 g L^{-1}, $MnCl_2•4H_2O$ 0.0018 g L^{-1}, $ZnSO_4•H_2O$ 0.00014 g L^{-1}, $Na_2MoO_4•2H_2O$ 0.00039 g L^{-1}, CoCl2•$6H_2O$ 0.000055 g L^{-1}). The temperature of the DOE 1412 suspension culture in the PBR was held constant at 30±1 ^{0}C. The purpose of the field dust experiment was to simulate the cultivation condition of microalgae in an open pond cultivation system, such as the DOE RAFT project ARID raceway at the University of Arizona (Waller et al. 2012). To this end, soluble fertilizer was used at the same rate in the experimental PBR system to simulate the open pond conditions for the 'field dust' experiment.

For the *Vampirovibrio chlorellavorus* inoculation test, the DOE 1412 was cultivated in local well water enriched with Pecos medium and the trace metal solution, per above, to simulate the laboratory conditions under which the *V. chlorellavorus* culture was maintained. The Pecos medium contained 0.1 g L^{-1} urea ($(NH_2)_2CO$), 0.012 g L^{-1} $MgSO_4•7H_2O$, 0.035 g L^{-1} $NH_4H_2PO_4$, 0.175 g L^{-1} Potash (KCl), 0.0054 g L^{-1} $FeCl_3$ and 0.02 g L^{-1} Na_2CO_3. The temperature of the media was maintained at 34 ± 0.1 ^{o}C. DOE 1412 was inoculated to the algal culture at this temperature because observations by our group demonstrated that it was most susceptible to attack and rapid decline by *V. chlorellavorus* (Park et al., in preparation).

2.2 Offline biomass concentration measurement

The biomass concentration of DOE 1412 was determined by cell counts, and by determining the ash-free dry weight. The algal cell suspension was diluted to different cell concentrations ranging from 10^6 and 10^8 cells mL^{-1}, and the number of total cells and live

cells was determined using an automated cell counter (Cellometer Vision, Nexcelom Bioscience, MA, USA). The ash-free dry weight of the cells was determined, following the method described by Zhu & Lee (1997). The OD of the cell suspension was measured at 650, 685, 750 and 780 nm by a bench-top spectrophotometer (DR 3800, HACH, CO, USA) using a 10 mm light path length cuvette. Samples were diluted with deionized water (as necessary) to achieve an absorbance reading of approximately 0.5 or less, to be within the range of measurable biomass density, which is expected to be linearly proportional to the OD concentration based on Beer's law (Lee 1999).

2.3 PCR detection of *V. chlorellavorus* and *C. sorokiniana*

The *V. chlorellavorus* infected DOE 1412 biomass pellet collected daily from the experimental PBRs was stored in -80 ^{0}C freezer prior to DNA extraction. Total DNA was isolated from the pellet using the CTAB method (Doyle and Doyle 1987) with slight modifications, as described below. . One milliliter of microalgae suspension was pelleted and resuspended in 1 mL of CTAB. A quantity of glass beads (G-8772, Sigma Chemical Co., St. Louis, MO, USA) sufficient to fill the conical portion of the centrifuge tube was added, and the tube was agitated on a bead beater (Mini-Beadbeater, BioSpec, OK, USA) for 5 min. The mixture was centrifuged, and supernatant was extracted with an equal volume of chloroform: isoamyl alcohol (24:1). The preparation was centrifuged in a benchtop microcentrifuge (5415C, Eppendorf, Germany) at 9000 RPM (6611 x g) for 10 min. The upper aqueous phase was removed and mixed with 2/3 volume cold isopropanol, and held a t -20^{o}C for a minimum of 20 min. The DNA was precipitated with 1 mL of 70%

ethanol and collected by microcentrifugation at 9000 RPM (6611 x g) for 10 min. The pellet was washed with 70% ethanol and resuspended in low TE (10 mM Tris-EDTA buffer, pH 8.0) (Maniatis et al. 1982), and held at -20 °C until used for polymerase chain reaction (PCR) amplification(Mullis et al. 1986).

The presence of *V. chlorellavorus* in the inoculated PBR suspension culture of DOE 1412 was detected by polymerase chain reaction (PCR) amplification (Mullis et al. 1986) using the isolate-specific forward (F) primer, F5'- GCCAGAGTGGGACTGAGA–3', with the reverse (R) primer, R-5'- GGGTTCGATTCCGGAGAG-3' to amplify a fragment of the *V. chlorellavorus* 16S subunit of the ribosomal DNA gene (rDNA) to yield an expected size product of 543 base pairs (bp). The following primers were used to detect the presence of DOE 1412 by PCR-amplification of a fragment of the 16S chloroplast rDNA gene: F16SW-5'- AGAGTTTGATCMTGGCTCAG-3' and R16SW-5'- ACGGTTACCTTGT TACGACTT -3', yielding an expected amplicon of 1500 bp. (Park et al., in preparation). The reactions were carried out in a final volume of 25 μL, containing 12.5 μL of JumpStart RED Taq ReadyMix Reaction Mix (P0982, Sigma-Aldrich, MO, USA), 0.2 μL of 20 μM each primer (forward and reverse), nuclease-free water, and 1 μL of DNA template.. The analogous DOE 1412 *and V. chlorellavorus* 16S rDNA fragments cloned separately into the pGEM-T Easy plasmid vector were used as the positive control, respectively, to test for DNA integrity. The addition of double distilled water to the reaction, instead of the DNA template, was used as a negative control for the PCR reaction. PCR parameters consisted of the initial denaturation at 95 °C for 10 min, followed by 25 cycles of amplification at 94 °C for 30 s (denaturation), hybridization at 58 °C for 45 s, and elongation at 72 °C for

90 s, and a final extension step at 72 °C for 10 min. PCR amplification reactions were carried out in a cycler (Mastercycler EP, Eppendorf, Germany). The PCR products were electrophoresed in a 0.8% (w/v) agarose gel in 1 X TAE buffer (40 mM Tris-OH, 20 mM acetic acid and 1 mM of EDTA; pH 8.0) at 80 V for 40 min.. A 1-kbp DNA ladder (Cat No.10787-026, Invitrogen, NY, USA) was used as an internal marker for determining the size(s) of amplicons. Amplicons and the bands of the ladder were stained using an inert red dye contained in the JumpStart RED Taq ReadyMix Reaction Mix, and visualized using a UV imager (Gel Doc XR+, BIO-RAD, CA, USA) with Image Lab software.

2.4 Real-time monitoring of microalgae growth dynamics

The growth dynamics of the DOE 1412 culture were measured using a real-time OD sensor described in a previous study (Jia et al., 2015, submitted). The absorbance of the DOE 1412 cells in the algal suspension was measured at the wavelengths, 650 nm, 685 nm, and 780 nm. The absorbance at 780 nm was used to estimate the turbidity of the suspension, because the green chlorophyll pigment of the microalgal cells does not absorbance light at this wavelength. Absorbance at 650 and 685 nm was used to measure the intensity of color associated with the algal chlorophyll (Solovchenko et al. 2011), and determine algal cell concentration (Das et al. 2011).

The pH (HI1001, Hanna Instruments, USA), electrical conductivity (HI3001, Hanna Instruments, USA), dissolved oxygen (DO1200/T, Sensorex, USA) and thermocouple temperature sensors (Type T, Omega Engineering Inc., USA) were placed in the indoor experimental photobioreactor (PBR), and connected to a data logger

(CR3000, Campbell Scientific Inc., UT, USA) for monitoring and control of the culture system. Each sensor was scanned every second and 10 min averaged data was stored in the data logger.

2.4.1 Experimental setup for dust test

DOE 1412 was cultivated in a PBR located in an indoor laboratory. A semi-continuous batch culture of DOE 1412 were conducted for comparison purpose. The 'test field dust' (Nominal 0-70 micron ATD, Powder Technology, Inc., MN, USA) having a mean diameter of 25.32 μm (σ = 11.8 μm) was used to simulate field dust that blows into the outdoor raceway in Tucson, Arizona. A total of 4 gms of the test field dust was added to the PBR during the second batch of semi-continuous culture. The test field dust was added in 4 occasions with 1 gm added each time. The negative experimental control consisted of the addition of no dust to the first batch of the semi-continuous culture. The pH of the algal culture in the PBR was maintained at 7±0.3 by injecting CO_2 from a pressurized liquid CO_2 tank into the PBR through a sparger. The OD of the culture was monitored and logged continuously by the optical sensor at multiple wavelengths. Microalgae samples were taken 30 min after the introduction of dust to ensure an even distribution of dust in the PBR. Fifty milliliters of sample from each PBR was used for ash-free dry weight measurement and OD validation by a bench-top spectrophotometer.

2.4.2 Experimental design for the V. chlorellavorus inoculation

The PBR (as described above) was inoculated with a *V. chlorellavorus*-free suspension culture of DOE1412 to achieve an inoculation OD of 0.1 at OD750nm in 5 liters DOE1412 culture volume. The culture was replenished with 500 mL of fresh Pecos

medium on the 4th day of the culture to ensure nutrients were not a limiting factor on microalgae growth. The culture was inoculated with 200 mL of *V. chlorellavorus* infected DOE1412 culture on the 5th day of the culture. Half of the biomass was harvested and replenished with fresh media to the original volume on the 6th day of the culture. The culture was maintained in the PBR until the biomass (OD reading) decreased by 25% in cell number was observed. The culture was sampled daily and tested for cell viability using chlorophyll fluorescent imaging method by the cellometer, which used an excitation wavelength of 470 nm and an emission wavelength at 535 nm, and the OD was measured using an off-line bench top spectrophotometer at 650 nm, 685 nm and 780 nm. Both instruments are described in section 2.2. An aliquot (1 mL) of algal suspension culture was collected daily from the PBRs and subjected to DNA isolation as described above, and tested for the presence of *V. chlorellovorus* by PCR analysis. Three experiment replicates were conducted.

3. Results and Discussion

3.1 Dust test

Optical density readings can be affected by the presence of abiotic factors, including particulates that enter the system, such as dust and other suspended solids, including algal cells themselves, based on the absorption and/or the scattering of light (Lee 1999). Outdoor raceway systems are more problematic than closed PBRs because dust can readily be deposited by blowing wind and other local disturbances into the algal suspension culture system, especially in arid or semi-arid regions of the world.

To investigate the sensitivity of the in-line optical sensor to the presence of dust in an outdoor raceway system, 4 gms of Arizona test dust were added to microalgae cultured in an indoor PBR in 4 occasions shown in Figure 1. **Error! Reference source not found.** listed the percentage of dry mass increase in the PBR due to the addition of test dust and the increase of OD_{780}. The OD did not increase proportionally to the increase of dry mass, considering the amount of test dust added to the PBR resulted a 59.7% increase of the total dry mass. An OD change associated with the introduction of dust was expected that would be comparable to changes caused by the inadvertent introduction of dust or other particulates that could cause cell density fluctuations in a natural system. Thus, the introduction of the test field dust had no apparent effect on the OD reading measured by the optical sensor.

Table 1. The increase of dry mass in the PBR and corresponding increase of OD 780 due to the accumulation of field test dust

Test dust in the PBR (g)	AFDW of DOE 1412 (g L^{-1})	Test dust concentration (g L^{-1})	Increase of dry mass	Increase of OD_{780}
1.0	0.386	0.088	22.8%	2.8%
2.0	0.460	0.176	38.3%	3.0%
3.0	0.542	0.264	48.7%	2.1%
4.0	0.590	0.352	59.7%	2.3%

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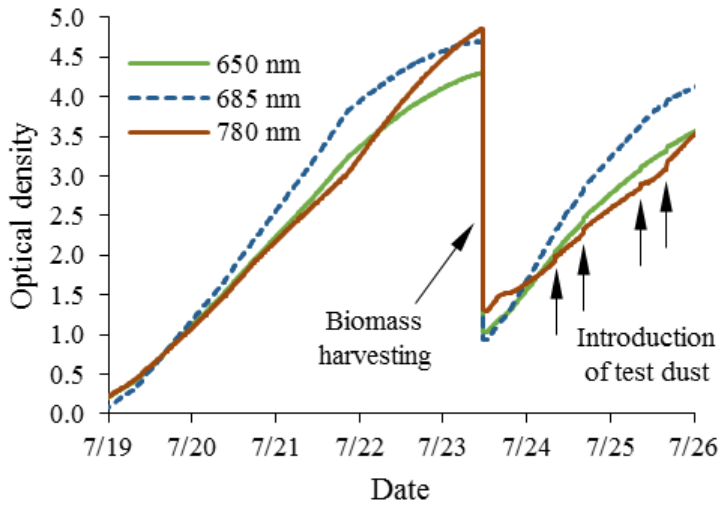

Figure 1. Dynamics of OD at 650 nm, 685 nm and 780 nm during semi-continuous culture of DOE 1412 in an indoor PBR. Arizona test dust was added to the culture at four time points indicated by arrows.

Further analysis showed that the introduction of dust was clearly detectable by analysis using the first derivative of OD_{780} (μOD_{780}). The four distinctive peaks showed the response of the optical sensor due to the introduction of dust as seen in Figure 2. These peak signals were resulted from the change in OD_{780} between two data points by definition. However, a fluctuation of μOD_{780} occurred constantly during the measurement process due to microalgal cell concentration change. Thus, an algorithm is needed in a monitoring and control strategy to differentiate the signal fluctuation from the signal peaks caused by the introduction of the test dust. The difference of two adjacent μOD_{780} were calculated ($\Delta \mu OD_{780}$), and a histogram of the absolute value of $\Delta \mu OD_{780}$ was plotted in Figure 3. A

total of 527 data points were taken during the 2-day period, as shown in Figure 2(b). The $\Delta \mu OD_{780}$ had an average of 0.51 and standard deviation of 0.53. There were 91.8% of $\Delta \mu OD_{780}$ had the value less or equal to 1.2, and 98.1% of $\Delta \mu OD_{780}$ had the value less or equal to 1.6.

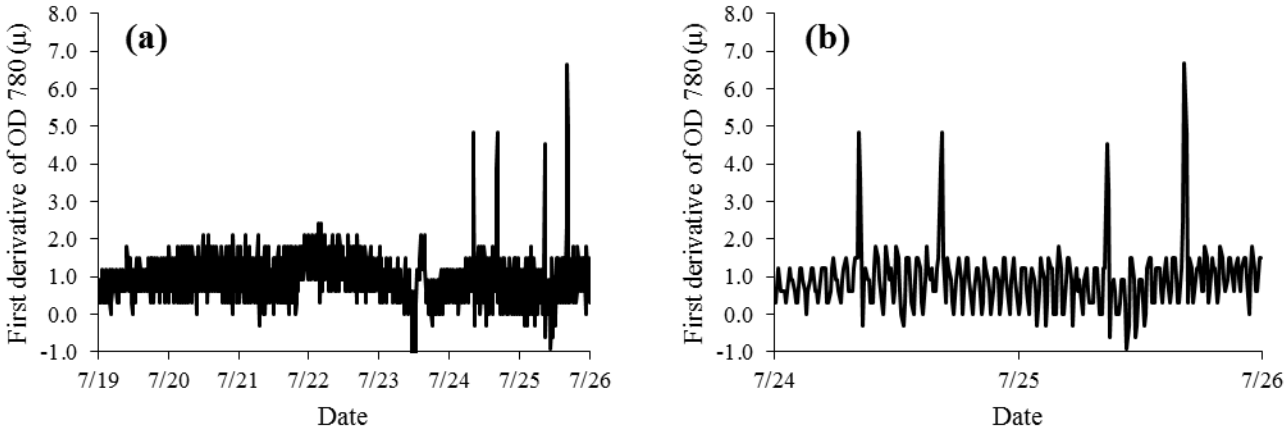

Figure 2. (a) First derivative of OD780 during 2 semi-continuous batch culture of DOE 1412. The first batch of culture served as a negative control with no field test dust introduced. The introduction of field test dust was detectable as 4 distinctive peaks, post-harvest. **(b)** The enlarged portion of the 4 peaks in **(a)** from 7/24 to 7/26.

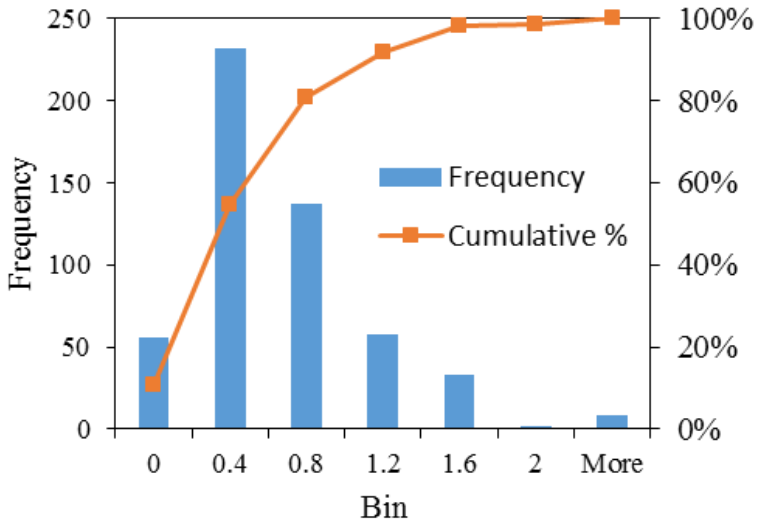

Figure 3. Histogram and cumulative frequency of $\Delta \mu OD_{780}$, which represents the change of two adjacent μOD_{780} from 7/24-7/26.

3.2 *V. Chlorellavorus* test

A field isolate of *V. chlorellavorus* was obtained from naturally-infected DOE 1412 cells from an outdoor culture system in Tucson, Arizona (Park et al., in preparation). It was maintained in a laboratory culture by mixing it with live cells of DOE 1412 maintained in BG-11 media at 24 ºC with a 12:12 light cycle (Park et al., in preparation). After confirmation of *V. chlorellavorus* presence in the culture by polymerase chain reaction (PCR) (Park et al., in preparation), was used to inoculate a healthy DOE 1412 culture at late linear growth phase. The culture was replenished with fresh media prior to the inoculation to eliminate the stress on microalgae from lack of nutrients (Figure 4). The growth of DOE 1412 immediately slowed down after the *V. chlorellavorus* inoculation.

The presence of *V. chlorellavorus* presence in the culture after inoculation of the suspension culture growing in the PBR was confirmed by PCR (

Table **2**). To reduce the effect of light attenuation on the growth of DOE 1412 due to high cell density, 50% of the culture was harvested on day 6, post-inoculation, and replaced with fresh media one day after the *V. chlorellavorus* inoculation. The suspension culture remained growing (based on increasing OD of live cells) for 5 days before the algal population rapidly declined. During that period, the CO_2 supply was interrupted for 14 hours from 5/13 to 5/14, which resulted in the decrease of algal cell density, as shown in Figure 4. To confirm that the attenuated CO_2 supply was a possible cause of the rapid decline of the algal culture, instead of to attack by *V. chlorellovorus,* the experiment was repeat 3 times. A similar growth pattern of DOE 1412 was observed for each of the replicated experiments, based on the assessment of algal cell viability, which was measured as the percentage of live cells and the OD_{685}/OD_{780} ratio were plotted together with OD780, as shown in Figure 5. The concentration of live DOE 1412 cells reached 99.4% on the second day of the culture and continued to drop throughout the first the batch. Concentration of live cells was reduced by 8% after the harvest, but recovered to 90% two days thereafter. This pattern is thought to reflect the re-resuspension of dead cells from the bottom of the PBR during harvesting of DOE 1412, a scenario that is supported by a sudden decrease in the OD_{685}/OD_{780} ratio at the time of harvest. Concentration of live cells began to decrease two days prior to the rapid death of the culture, the same time point at which a steep decrease in the OD_{685}/OD_{780} ratio also was observed, as is shown in (Figure 5). This observation suggested the occurrence of decreasing chlorophyll content of the algae cells, and is reminiscent of a similar pattern reported by Nedbal et al. (2008).

Accordingly, the predatory life cycle of *V. chlorellavorus*, as described by Soo et al. (2015), involves the utilization of cytoplasmic contents, including the chlorophyll, and its depletion is indicative of the cell contents having been released and/or consumed by the bacteria, prior to leaving the destroyed but intact cell as a large vacuolated area and membranous structures 5 to 7 days after *V. chlorellavorus* attachment. The dead cells however contribute to the light absorbance at 780 nm (NIR), but not at 685 nm (red). Therefore, the sudden decrease of the OD_{685}/OD_{780} ratio was found to serve as an indicator of the impending destruction of the suspension culture of DOE 1412 associated with *V. chlorellavorus* predation.

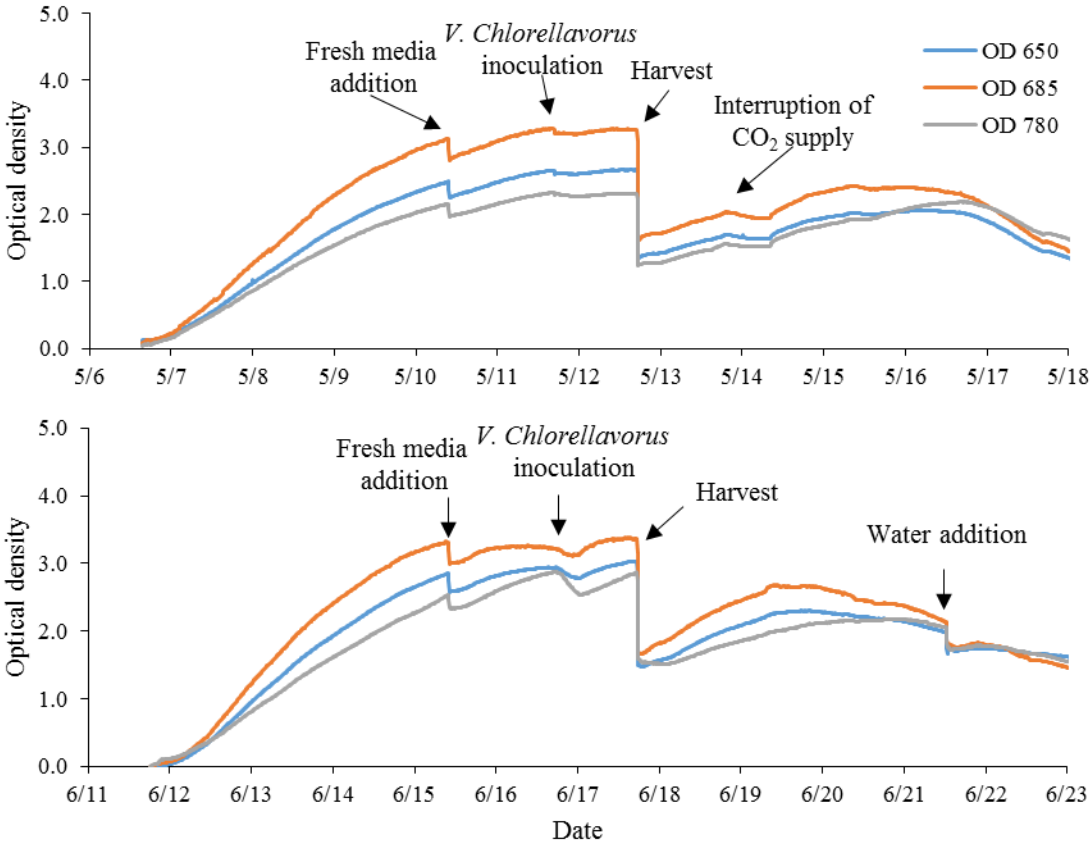

Figure 4. OD at 650 nm, 685 nm and 780 nm of semi-continuous growth of the DOE1412 suspension culture inoculated with *V. chlorellavorus* in the indoor experimental PBR system. Events of fresh media addition, *V. chlorellavorus* inoculation, harvesting, and the interruption of CO_2 supply are indicated by an arrow.

Table 2. Results of PCR detection of *V. chlorellavorus* and DOE 1412, for which DOE 1412 presence was confirmed in all PBR samples that were inoculated with the bacterium. The presence of measurable *V. chlorellavorus* was detected only after *V. chlorellavorus* inoculation.

Date	*V. chlorellavorus*	DOE 1412	Date	*V. chlorellavorus*	DOE 1412
5/6	-	+	6/11	-	+
5/7	-	+	6/12	-	+
5/8	-	+	6/13	-	+
5/9	-	+	6/14	-	+
5/10	-	+	6/15	-	+
5/11	+	+	6/16	+	+
5/12	+	+	6/17	+	+
5/13	+	+	6/18	+	+
5/14	+	+	6/19	+	+
5/15	+	+	6/20	+	+
5/16	+	+	6/21	+	+
5/17	+	+	6/22	+	+
5/18	+	+	6/23	+	+

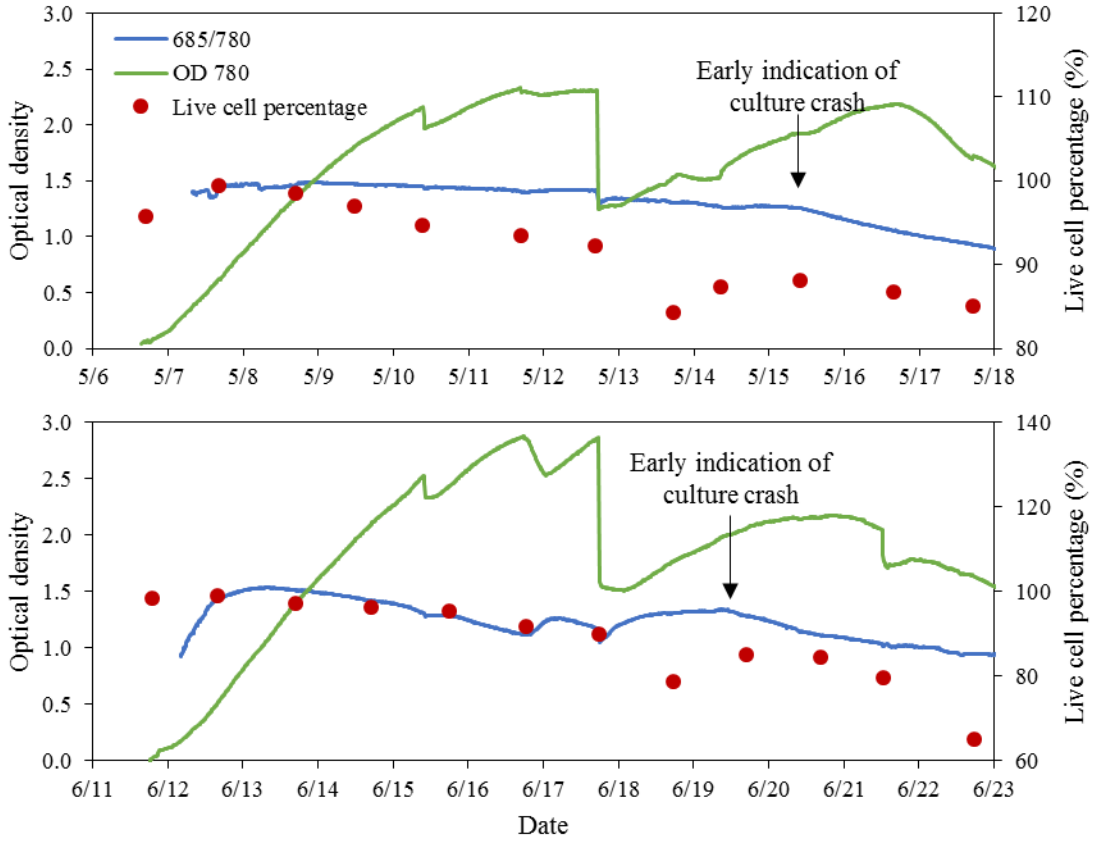

Figure 5. Cell viability and OD_{685}/OD_{780} trend change during the semi-continuous culture of DOE 1412 inoculated with *V. chlorellavorus* in the indoor, experimental PBR system.

4. CONCLUSIONS

A multi-wavelength laser diode based optical sensor was evaluated for its ability to detect an abiotic and a biotic environmental disturbance, before it was possible to detect such disturbances by visual inspection. A microalgal suspension culture of *C. sorokiniana* isolate DOE 1412 was cultivated in a controlled PBR system and experimentally perturbed by the addition of 'test field dust' (abiotic) and a highly virulent predator of DOE 1412 and several other *Chlorella* spp., *V. chlorellavorus*. The optical sensor was capable of

estimating cell concentration and changes in the physiological status of the microalgae culture in real-time. The sensitivity of the sensor to the presence of dust and dirt in a culture system was tested using test field dust that resembles the size distribution of dust from agriculture lands in Arizona. The sensor showed low sensitivity to the presence of the test dust when the test dust comprised approximately 60% of the AFDW of microalgae biomass. However, the sensor was able to clearly detect (indicate, based on the first derivative output) the event of the introduction of the test dust to the culture system. The decline and death of the DOE 1412 culture associated with the introduction of the predator *V. chlorellavorus* to the PBR, was detected (indicated) repeatedly by a decrease in the OD_{685}/OD_{780} ratio, and by concentration of live cells 2 days prior to the rapid decline, or 'crash' of the suspension culture. The parameters measured in this study were found to serve as effective indicators for the early detection of an impending loss of a microalgal culture due to the invasion and subsequent predation by *V. chlorellavorus*, a scenario that was confirmed by molecular detection of the predator using *V. chlorellavorus*-specific PCR primers. This optical sensor described here, and designed to monitor the growth dynamics of microalgae in real-time, was capable of the early-detection of the impending rapid decline of the culture due to biotic invasion e.g. by *V. chlorellavorus*, while at the same time, it was much less sensitive to the abiotic dust introduced into the experimental PBR culture system used here. This is possibly due to a different optical absorption property of the abiotic dust from that of microalgae cells.

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APPENDIX C - AUTONOMOUS MONITORING AND CONTROL OF MICROALGAE PRODUCTION SYSTEM

F. Jia, M. Kacira
Journal TBD

ABSTRACT

An automated monitoring and control system for microalgae production application was developed and tested on an open pond raceway. The key component of the system was an inline optical sensor that measures the biomass concentration in real-time. Environmental parameters such as pH, electrical conductivity (EC), dissolved oxygen (DO), temperature (T) and photosynthetic active radiation (PAR) were monitored and recorded. The harvesting procedure was fully automated through feedbacks from the optical sensor and water depth sensors. Resource inputs including water, nutrients, CO_2 and electrical power were accounted for resource management purposes. Internet connectivity was enabled on the microcontroller so that the microalgae production system, key culture growing and aerial environmental conditions, and resources used can be remotely monitored and controlled.

KEYWORDS:

Real-time monitoring and control, automation of microalgae production, inline optical sensor, resource management

1. INTRODUCTION

Large scale microalgae production is costly and laborious (Lee 2001). In order to maximize the productivity of a microalgae cultivation system, the resource and labor input need to be minimized while the cultivation conditions need to be maintained at an optimum level to achieve the maximum biomass production rate. Automation of the cultivation system can significantly reduce the operational cost of the production that includes harvesting, fertilizing and culture volume maintenance. Harvesting is an important procedure to maintain the biomass concentration in an optimum range for rapid microalgae growth and to prevent or reduce economic losses in case of contamination. Real-time monitoring and control provides the platform to acquire the environmental and physiological dynamics of a microalgae culture system that will be used for control and decision making purposes. Measurements of biological variables, including biomass concentration, cell size, population composition (i.e. concerns with contamination), pigments and lipid content, are especially desirable because they are the direct indicators of the dynamics of a microalgae culture system.

There have been few applications of microalgae harvesting control based on the feedback from real-time biomass concentration sensors. Sandnes et al. (2006) demonstrated automatic density control of microalgae culture using a custom made near infrared (NIR) optical density sensor. Three tubular photobioreactor (PBR) biofence systems were used to cultivate *Nannochloropsis oceanica* in a climate-regulated greenhouse. The pH of the culture was regulated between 7.3 and 7.8 by a controller unit that records the pH as well. Temperature and solar radiation were also measured every 15

seconds and averaged over 5 minutes. A turbidostatic culture control was demonstrated by injecting water/nutrients mix into the PBR on demand to maintain a constant optical density. The volume of the effluent of the culture was used to calculate the biomass productivity. Briassoulis et al. (2010) designed and constructed an automated harvesting system based on a flow-through cell concentration sensor integrated into a helical-tubular PBR. The pH of the culture was controlled between 8.3 and 8.6 by supplying CO_2 through the air phase of the system. The temperature was kept between 23.4 and 28.3 ºC through a cooling system. The harvesting system operates based on the cell concentration estimated from the flow-through sensor. An average 13.3% of total volume of the culture was harvested daily by the automated harvesting system during an 8 day experiment. The mean cell density was equal to $337.2 \pm 6.0 \times 10^6$ cells mL^{-1}. Nedbal et al. (2008) demonstrated turbidostatic control of microalgae growth in a commercially available flat panel PBR by a built-in densitometer. A peristaltic pump was automatically controlled by one of the programmable bioreactor outputs (OD680) to add fresh medium so that the optical density of the growing culture was maintained in a preset range (±2.5 %). The productivity was calculated for the curve of OD680 slope between the dilutions. The pH was regulated by injecting air enriched with 2% CO_2. Temperature, irradiance were also regulated by the PBR. Marxen et al. (2005) developed a bioreactor system for the cultivation of the microalgae *Synechocystis* sp. PCC6803 under controlled physiological conditions. A turbidostatic process was achieved by diluting the algal suspension in the reactor with the feedback from an optical density sensor that measures light absorbance at 870 nm to maintain the biomass concentration at a constant level. The pH was regulated by a pH

controller. Irradiance intensity and irradiance quality were adjustable through the process control system. However, none of the control applications listed above were carried out in an open pond raceway system. There were studies focused on the control of environmental conditions of microalgae cultivation in raceways. San Pedro et al. (2015) found that dilution rate has a high impact on maximum productivity of microalgae in raceway ponds. Pawlowski et al. (2014) utilized a Generalized Predictive Controller (GPC) aiming to improve the pH control accuracy and save control resources for a raceway reactor. However, there was no harvesting control strategies implemented that was based on the biomass concentration.

In this study, a novel multi-wavelength based inline optical sensor that measures biomass concentration in real-time along with sensors that measure key parameters for microalgae production were integrated into an open pond raceway for automation of operation as well as resource input management.

2. MATERIAL AND METHODS

2.1 Cultivation conditions and organisms

Chlorella sorokiniana Beijerinck, 1890 (DOE 1412) received from Pacific Northwest National Laboratory, WA, USA (Jones et al. 2014) was cultivated in local well water enriched with Pecos medium, trace metal solution and 5g L^{-1} NaCl. The Pecos medium contained 0.1 g L^{-1} urea ($(NH_2)_2CO$), 0.012 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.035 g L^{-1} $NH_4H_2PO_4$, 0.175 g L^{-1} Potash (KCl), 0.0054 g L^{-1} $FeCl_3$ and 0.02 g L^{-1} Na_2CO_3. The culture was maintained in an open pond paddle wheel raceway (Figure 6) with a surface

area of 3 m^2 located at Algae Research Facility in University of Arizona, Tucson, Arizona, USA. The pH of the culture was maintained at 8±0.05.

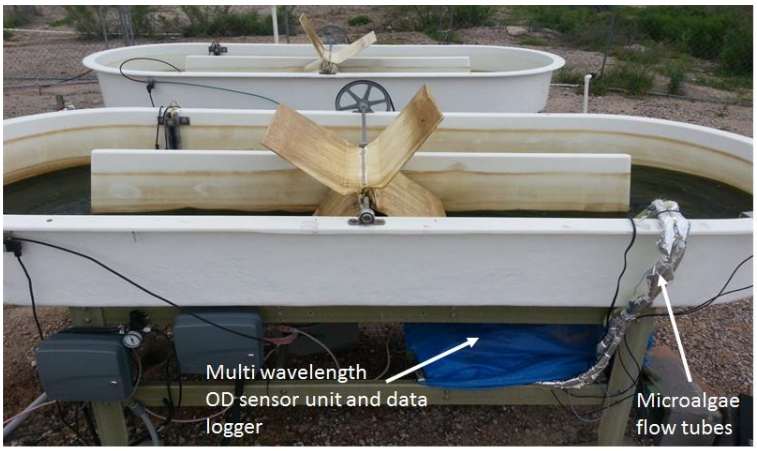


Figure 6. An open pond raceway with integration of inline optical sensors for real-time microalgae growth monitoring and control application.

2.2 Open pond raceway monitoring and control

The growth dynamics of the microalgae culture was measured using a real-time optical density sensor (Figure 7) developed in a previous study (Jia et al., 2015). The device measured light absorbance of microalgae cells at 650 nm, 685 nm and 780 nm. Since sensor electronics maybe sensitive to environmental conditions, the optical sensor with its housing and the datalogger were placed in a location at the outdoor raceway site to minimize direct exposure to sunlight. The laser output is also temperature dependent (5-15 mV/ ^{o}C, vary with lasers). Therefore a temperature control unit was installed and consisted of a small heater plate (HT24S, Thorlabs, NJ, USA) and heat sink (55 mm Fan Heatsink, USA) to maintain a constant temperature (40±0.1 ^{o}C)inside the sensor box. This also ensured a constant laser power output. Electrical conductivity (HI3001, Hanna Instruments, USA), pH (HI1001, Hanna Instruments, USA), dissolved oxygen (DO1200/T,

Sensorex, USA), photosynthetically active radiation (PAR) (SQ-110, Apogee instruments, USA), temperature (Type T, Omega Engineering Inc., USA) and water depth sensors (PN-12110215TC-12, MILONE Technologies, NJ, USA) were used to monitor the environmental conditions of the culture system (Figure 8). Each measurement was taken every second and 10 minute and averaged data was stored in a datalogger and microcontroller (CR3000, Campbell Scientific Inc., UT, USA). The real-time data was then transmitted to a central control station through Ethernet communication.

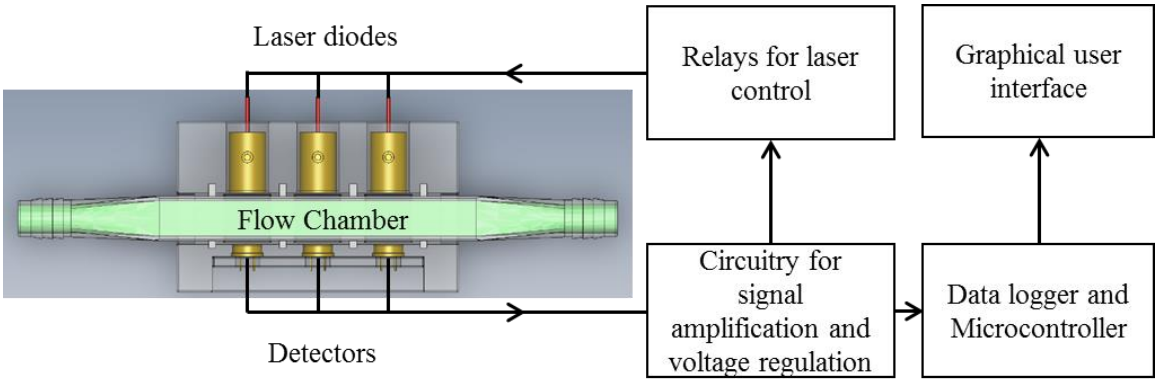


Figure 7. Component layout of the optical sensor unit. Three laser diodes at wavelengths of 650 nm, 685 nm and 780 nm were aligned with 3 photodiodes with a detection range of 350-1100 nm. The flow chamber window was perpendicular to the laser beam.

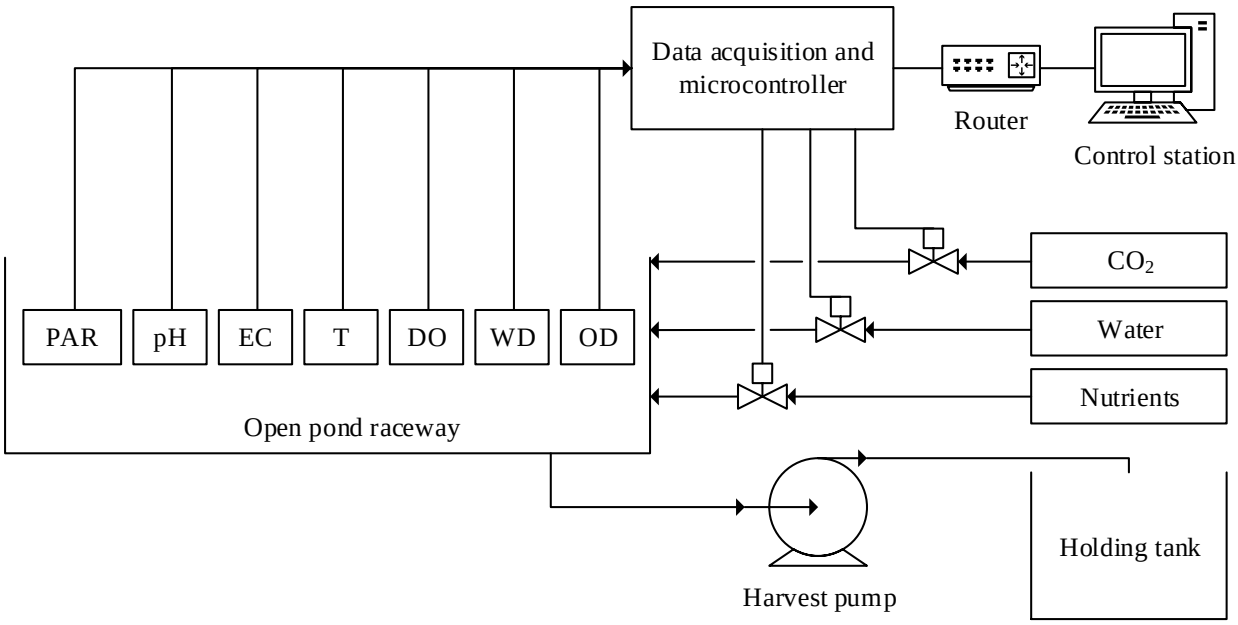


Figure 8. Schematic diagram of the open pond raceway monitoring and control system. The pH, electrical conductivity (EC), temperature (T), dissolved oxygen (DO), water depth (WD), optical density of the culture (OD) and photosynthetic active radiation (PAR) are monitored and stored in the data acquisition system. The microcontroller regulates CO2 injection base on pH value. Optical density values measured from the inline OD sensor controls the harvesting. The holding tank temporarily contains the harvested microalgae for further processing. Water and nutrients injection were controlled by water depth and optical density of the culture. The data acquisition and microcontroller communicates with the control station through a local network.

The operation of the raceway was automated in terms of pH control, water level control and biomass harvesting. The control of pH was achieved by controlling the injection of 95% CO_2 by the microcontroller based on the instantaneous pH value feedback from the pH sensor (**Figure *9***). The CO_2 injection was turned off during night time. The CO_2 usage was measured by a CO_2 mass flow meter in liters and accumulated amount was monitored and recorded by the monitoring algorithm and the datalogger. Water depth of the culture was controlled by a liquid level sensor through a feedback control loop shown

in **Figure *10***. The water level was compared to the set point (10 cm / 15 cm) at 8 am every morning. The water was added to the desired level through a solenoid valve when the level was lower than the set point. The harvesting of the culture was automated by the feedback from OD780 of the microalgae measured by the inline optical sensor (**Figure *11***). A harvest pump placed in the raceway was activated when OD780 of the culture exceeded 2.5 corresponding to AFDW of 0.57 g L^{-1}. The harvest was deactivated when water level in the raceway was less than 5 cm (50 % of the culture volume is harvested) followed by addition of nutrients solution concentrated and water simultaneously. Both were shut off once water level reached to the set point of 15 cm mark based on the sensor feedback. Water and nutrients usage were calculated by multiplying the time of addition and the flow rates of each in liters. The amount of biomass harvested in grams was calculated by multiplying the biomass concentration (g L^{-1}) before the harvest and the harvest volume (L). The paddle wheel in the raceway system was operated 24 hours a day for continuous culture mixing except for the duration of water addition and harvesting. This was for an accurate water level reading from the water level sensor. The energy usage for the paddle wheel was calculated based on the power consumption from the driving motor in kWh. The experiment was conducted on 7/9/2015 for a total of 10 days.

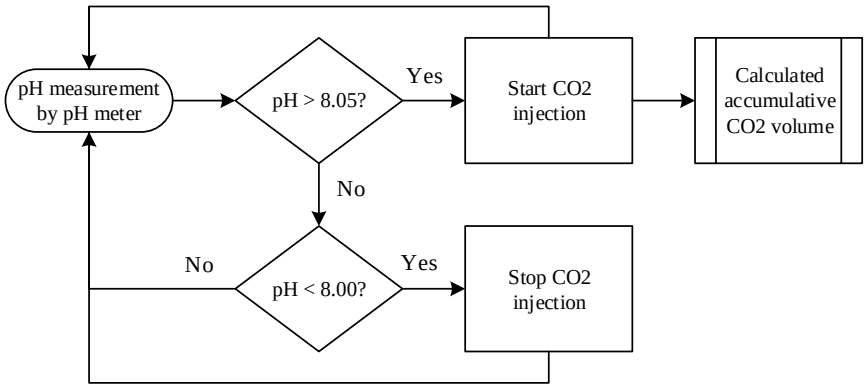


Figure 9. Logic flow chart of pH control in the culture system

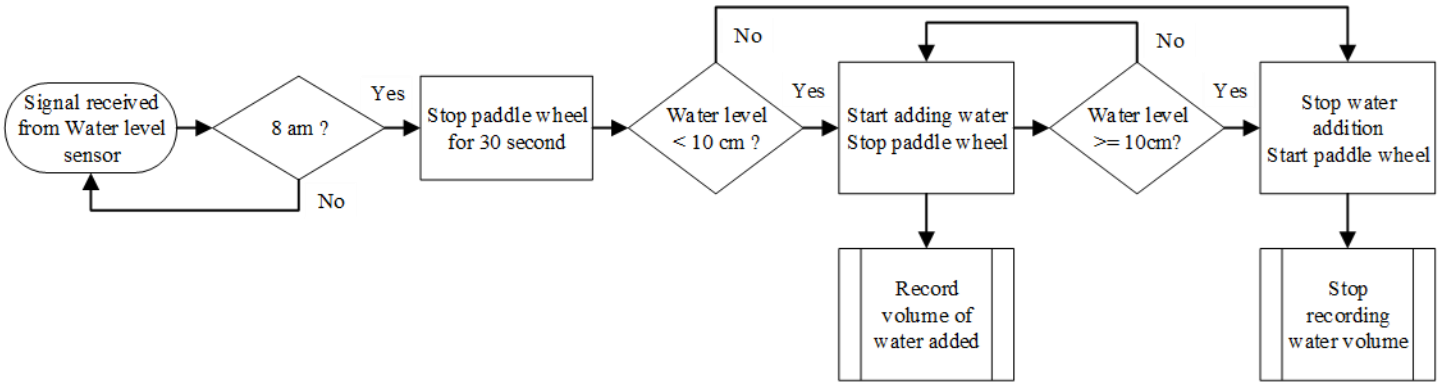


Figure 10. Logic flow chart of automated water addition in the culture system

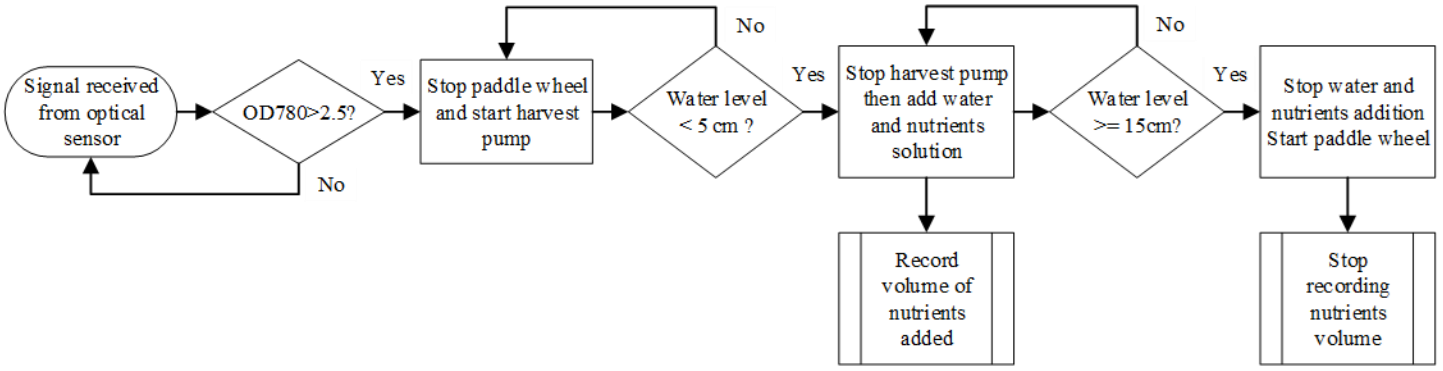


Figure 11. Logic flow chart of automated biomass harvesting and nutrients addition in the culture system.

2.3 Offline biomass concentration measurement

Biomass concentration of microalgae was determined by both cell counting and ash-free dry weight (AFDW) measurements. Cell suspension was diluted to a concentration between 10^6 and 10^7 cells mL^{-1} for cell counting by a neubauer chamber hemocytometer (Hy-Lite Ultra-plane, Clayadams, USA) under a microscope (XSZ-138, AOK International Group Ltd., China). The AFDW of the cells was measured following the method described by Zhu & Lee (1997). The light absorbance of the cells suspension was measured at 650, 685, 750 and 780 nm by a spectrophotometer (DR 3800, HACH, USA) using a 10 mm light path length cuvette. Samples were diluted with deionized water when necessary to keep the absorbance reading below 0.5.

3. Results and Discussion

The cultivation of DOE 1412 in an open pond raceway was monitored and automated by the control system. The optical density dynamics of the culture at 685 nm and 780 nm were shown in **Figure *12***. The real-time optical density shows repeatedly an increase of optical density indicating the biomass increase during the day time due to photosynthesis. A small decrease in optical density was observed during the nighttime since photosynthetic microorganisms metabolize intracellular carbohydrate to sustain their metabolic activity as described by Ogbonna and Tanaka (1996). Sudden decreases of optical density of the culture due to water addition at 8 am daily and one biomass harvesting performed on 7/15 were clearly shown in the figure. The temperature of the sensor was controlled at 40±0.1 ^{o}C to ensure a constant laser power output (**Figure *12***). The accumulated water and CO_2 input and the corresponding water level and pH change were

shown in **Figure *13***(a) and 8(b) respectively. Water consumption due to evaporation averaged 70 L m^{-2} day^{-1} excluding the amount of water added after the harvest which was 280 L. CO_2 consumption averaged 53.3 L m^{-2} day^{-1} during the culture except for the interruption of CO_2 supply on 7/11 and 7/12. This resulted in unregulated pH and a decrease of productivity due to no CO_2 supply during the 2 days. Twenty liters of 15X concentrated nutrient solution was added to replenish the culture medium. The electrical energy consumption due to operation of paddle wheel was 0.21 kWh per day. The total amount of dry biomass produced during the 10 day period was 306.7 g. This resulted in a productivity of 10.2 g m^{-2} day^{-1} dry biomass in an open pond raceway system. The CO_2 consumption was 6.86 L per gram of microalgal dry mass produced. The dynamic change of environmental parameters were measured and presented in **Figure *14***. The temperature of the raceway fluctuated from 20 to 35 °C daily. The concentration of dissolved oxygen increased in the daytime as a result of photosynthesis. The automated microalgae production monitoring and control system was able to operate the raceway with no labor input on water maintenance and harvesting procedures. All resource inputs were accounted for further calculation of overall productivity of the raceway.

Table 1. Summary of resource use for DOE 1412 cultivation in open pond raceway.

Average water consumption (L m^{-2} day^{-1})	Average CO2 consumption (L m^{-2} day^{-1})	Average electrical power consumption (kWh m^{-2} day^{-1})	Productivity (g m^{-2} day^{-1})	CO2 consumption (L g dry $biomass^{-1}$)
70	53.3	0.69	10.2	6.86

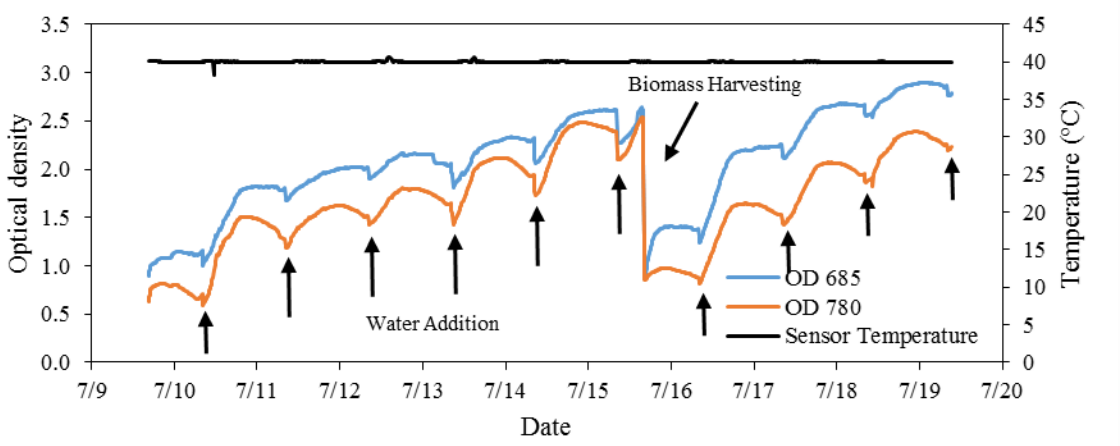
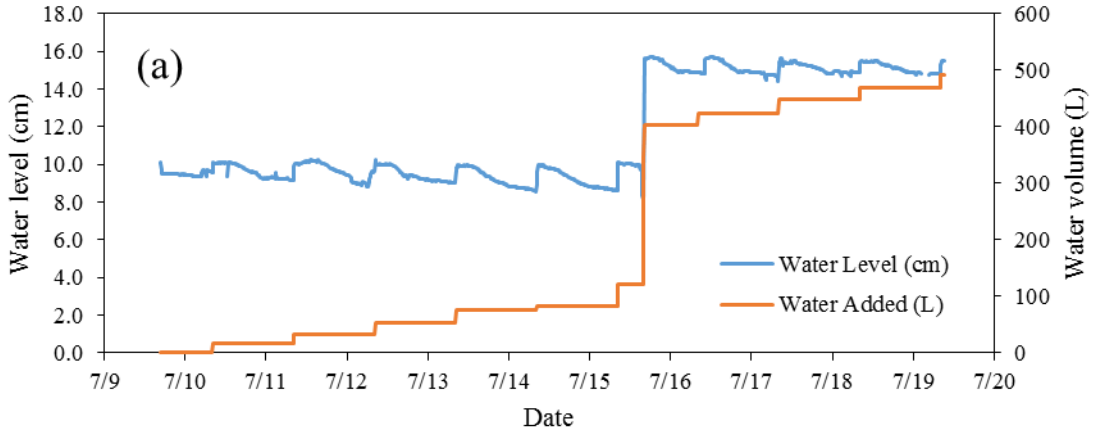


Figure 12. Optical density change of DOE 1412 in open pond raceway over 10 days. Black arrows indicate events of water addition and biomass harvesting. The sensor temperature was regulated and maintained at 40℃.



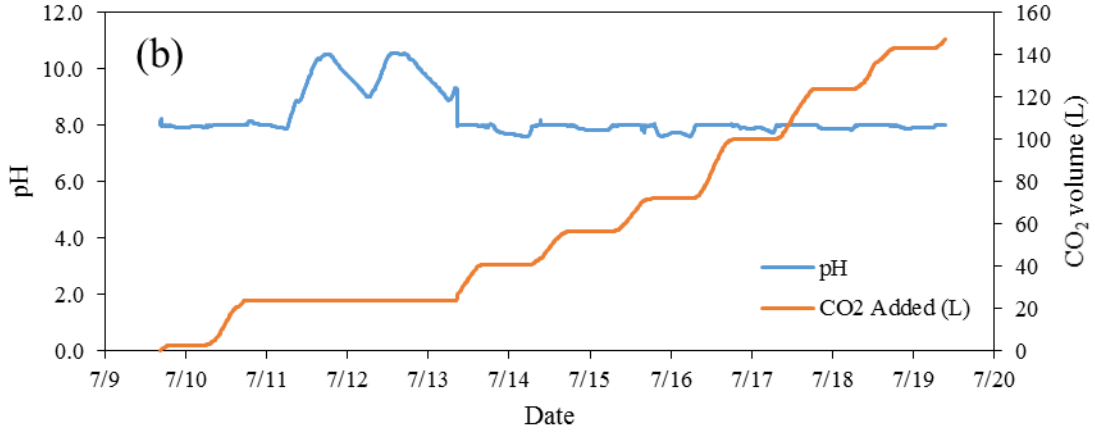


Figure 13. (a) Water level of the raceway and the cumulative water usage over 10 days. The initial water level was set at 10 cm and increased to 15 cm after the harvest. **(b)** pH of the culture and the cumulative CO_2 usage over 10 days. There CO2 supply was interrupted for 2 days from 7/11 to 7/13, resulted in an unregulated pH during that time period.

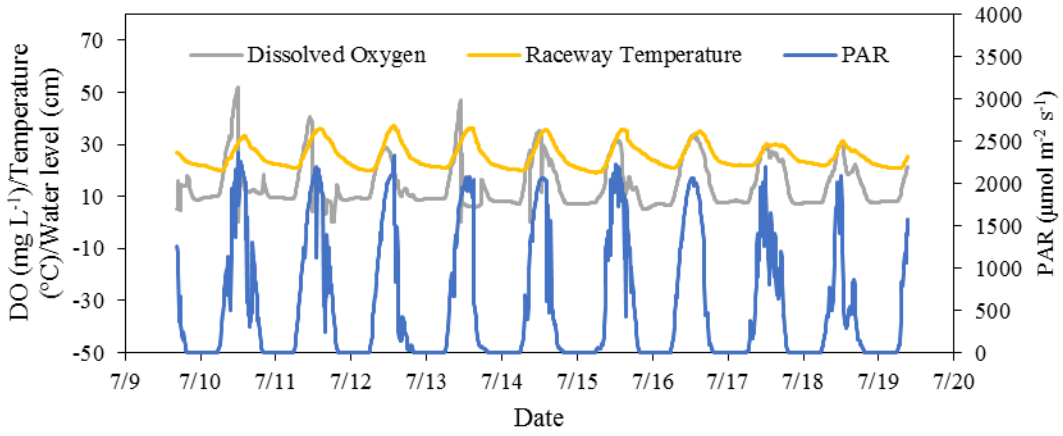


Figure 14. Photosynthetic active radiation (PAR), raceway temperature and dissolved oxygen dynamic of the system over 10 days.

4. CONCLUSIONS

The inline optical sensor integrated microalgae production monitoring and control system successfully monitored the dynamics of microalgae growth, key environmental parameter (pH, EC, DO, T, PAR) and automated the operation of an open pond raceway. The system regulated the volume of the raceway by using a water depth sensor. Being able to measure the biomass concentration of microalgae in real-time, the harvesting procedure was fully automated by utilizing the feedback from the optical sensor and the water depth sensor. All the resource input for the raceway operation were monitored, recorded, controlled, and the continuous data and key culture environment and aerial data were made available for users to account and determine the productivity of the system in real-time and to better manage the resource input for further improvement of the raceway.

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