CHAPTER 3

Materials and methods

3. Materials and methods

This chapter describes the rationale behind the raw material selection and the adapted experimental methods to reach the objective. This chapter is subdivided into three sections based on the objectives of this research work. A brief description of each section is provided below.

3A. To design and develop a photobioreactor (PBR) for mass-scale microalgae cultivation.

Different microalgae culture systems were studied to find out the pros and cons of the systems currently being used, and based on that, microalgae culture system design and development was carried out. Based on the studies, Light Emitting Diode (LED) based illumination for the microalgae culture was chosen, and further developments were carried out. Three microalgae culture systems were designed, developed, and tested. Finally, a photobioreactor capable of mass-scale microalgae culture was designed and developed, prioritizing biofouling prevention, a significant issue in the success of wide-scale implementation of closed photobioreactor systems. The developed microalgae culture systems were tested by culturing microalgae and then harvesting and characterizing the final products.

3B. To optimize the culture conditions and improve the biomass and lipid productivity.

A statistical tool, Response Surface Methodology (RSM), was used to model and optimize the relationship between the independent parameters and the resultant response response in the LED-based microalgae culture experimentation. Face centered central composite design (FCCCD) approach of RSM was used with the independent variables, nitrogen concentration (sodium nitrate), light intensity, light duration, and air flow rate, to model and optimize for the highest possible resultant responses, i.e., biomass and lipid productivity of *Chlorella homosphaera*. FCCCD was chosen for this experiment as it requires only three levels, the highest, lowest, and midpoint of each experimental variable, making it simple. Additionally, it also has the most negligible probability of error.

3C. To analyze the algal biomass and biofuel properties produced from the microalgae cultured in the developed PBR.

The microalgae biomass produced in the developed photobioreactor was harvested using chemical flocculation, sedimentation, and centrifugation, followed by drying in a hot air oven. The dry microalgae biomass was analyzed for its physiochemical properties like calorific value,

ash, and lipid content. The lipid obtained was then further processed into biodiesel using transesterification, and the properties of the obtained biodiesel were investigated.

CHAPTER 3A

To design and develop a photobioreactor (PBR) for mass-scale microalgae cultivation.

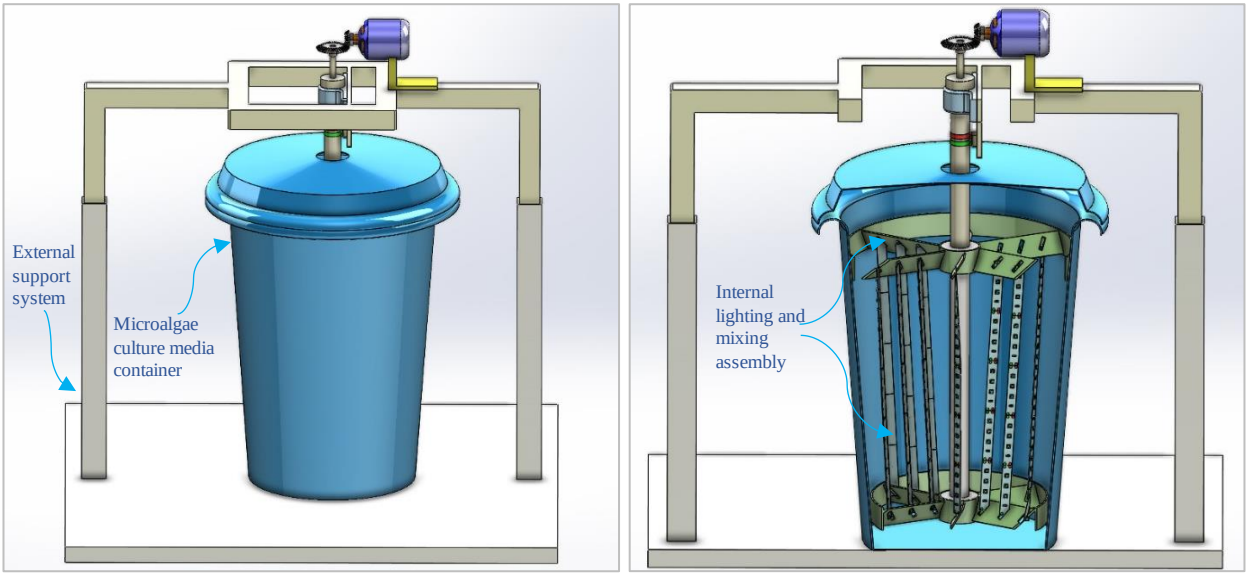
3A. To design and develop a photobioreactor (PBR) for mass-scale microalgae cultivation.

After a thorough review of the literature to figure out the limitations faced by photobioreactors in being able to be commercially successful, three photobioreactor designs were conceptualized and developed. The design's primary goal was to minimize the photobioreactors' capital and operational costs while having a high level of flexibility in modulating the culture conditions to achieve optimum microalgae productivity. The details of the three photobioreactor designs are described in the following sections.

3A.1. Design and development of Internally Illuminated Stirred Light Column Photobioreactor (IISLCP)

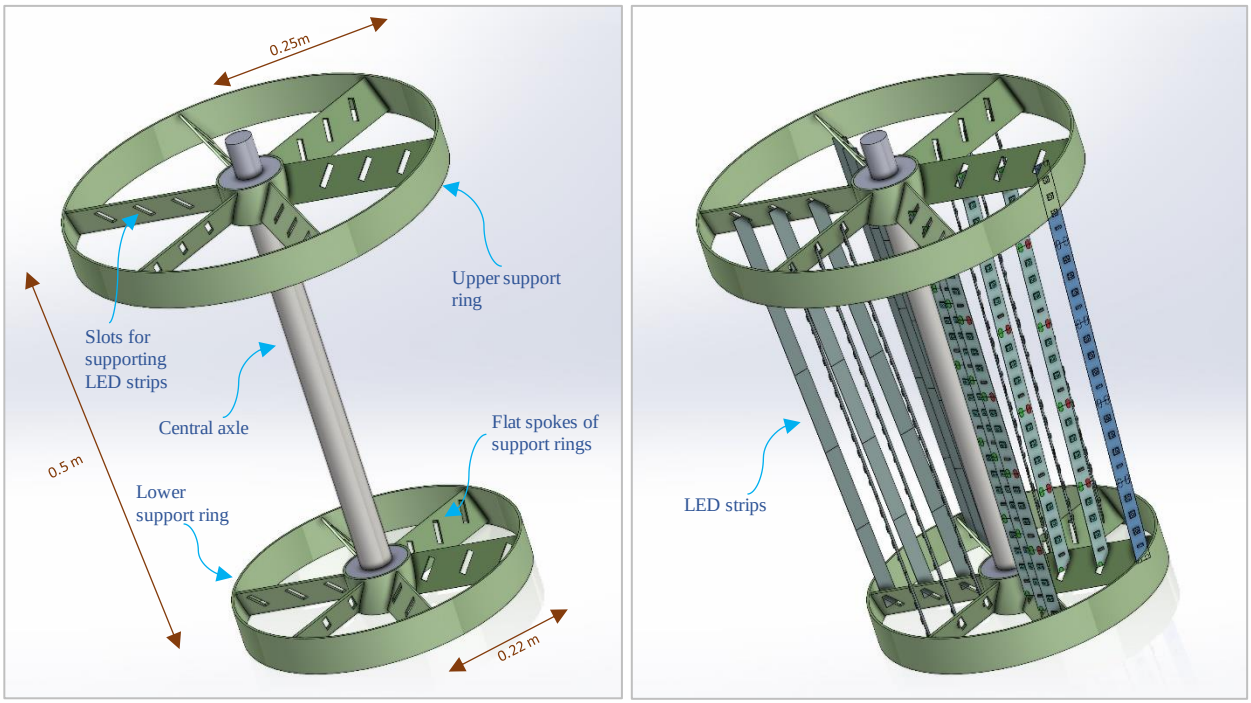
Internally Illuminated Stirred Light Column Photobioreactor (IISLCP) design emphasizes uniform light distribution and media circulation to improve microalgae production. LED illumination is considered while designing the system due to the small form factor of LED lights, making it easy to distribute the light evenly and with great flexibility. LEDs are also highly controllable in terms of wavelength, light intensity, and light duration, which play a vital role in microalgal growth and its characteristics. For instance, red light stimulates cell division [1], and blue light enhances lipid accumulation [2]. Another important consideration in the design of the IISLCP was to emphasize the multiple roles of the components to reduce the system's capital cost. For example, the LED support system was designed to work as propeller blades, helping circulate the microalgae culture.

IISLCP consists of an internal lighting and mixing system, an external support system, and a microalgae culture media container, as shown in Figure 3.1(a & b). The internal lighting and mixing system consists of a central revolving axel. The axel houses two support rings at the top and the bottom, as shown in Figure 3.2 (a), supporting the LED strips that illuminate the photobioreactor as shown in Figure 3.2 (b). The LED strips are enclosed in silicon tubing, making them IP68-rated waterproof, allowing them to be immersed in water media. The support rings have flat spokes serving as support structures for the LED strips as well as acting as fins or paddles to circulate the culture media. The LED strips are fixed in the slots made in the spokes of the top and bottom rings, as shown in Figure 3.2. The entire system, consisting of the central axel, the support rings, and the LED strips, is supported from the top using a bearing and a housing assembly fixed to an external support system, as shown in Figure 3.3.



(a) **(b)**

Figure 3A.1 (a): Schematic model of Internally Illuminated Stirred Light Column Photobioreactor (IISLCP). **(b):** Cross sectional view of the IISLCP model.



(a) **(b)**

Figure 3A.2 (a): Schematic of support for internal lighting and mixing system consist of a central revolving axel and LED support rings **(b):** Schematic of the support system with LED strips installed.

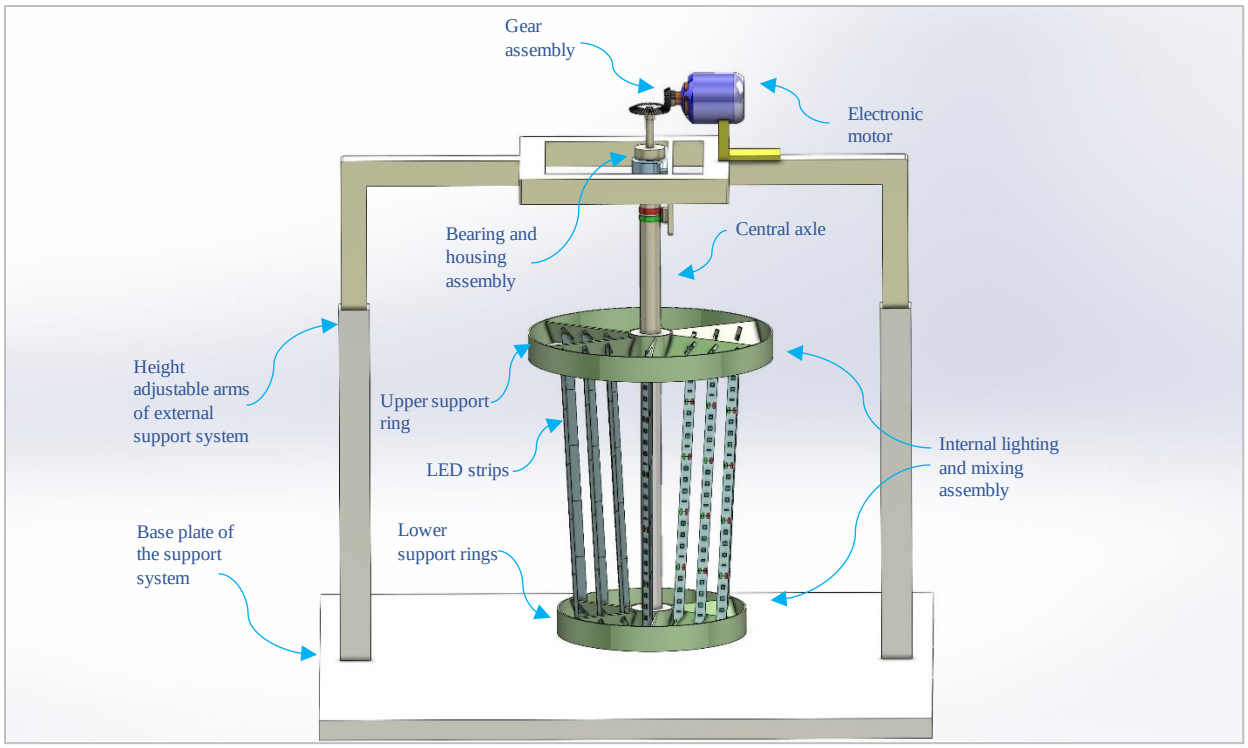


Figure 3A.3: Schematic of lighting and mixing assembly fixed to the external support system using bearing and housing assembly.

At the top of the central axle, a gear system connects the axle of the lighting and mixing system to the electronic motor assembly that rotates the entire lighting and mixing assembly, shown in Figure 3.4. (a). The gears can be easily replaced to control the speed of the lighting and mixing assembly. Below the bearing, two conducting rings are made on the axle and electronically connected to the external support system with the help of a graphite brush assembly, as shown in Figure 3.4 (b). The conducting rings transmit power to the LED strips on the lighting assembly. The microalgae culture media container is placed at the center of the external support system, enclosing the lighting and mixing assembly to complete the IISCLP system, as illustrated in Fig. 3.1 (a).

During the systems' operation, the central axel rotates, and the LED strips attached to it rotate along with it, resulting in uniform illumination inside the entire microalgae culture media. The fins of the support rings circulate the culture media. The fins at the bottom ring push the culture media upward and, thus, prevent the microalgae from settling to the bottom of the IISLCP. Multiple experiments were conducted to test the performance of IISCLP.

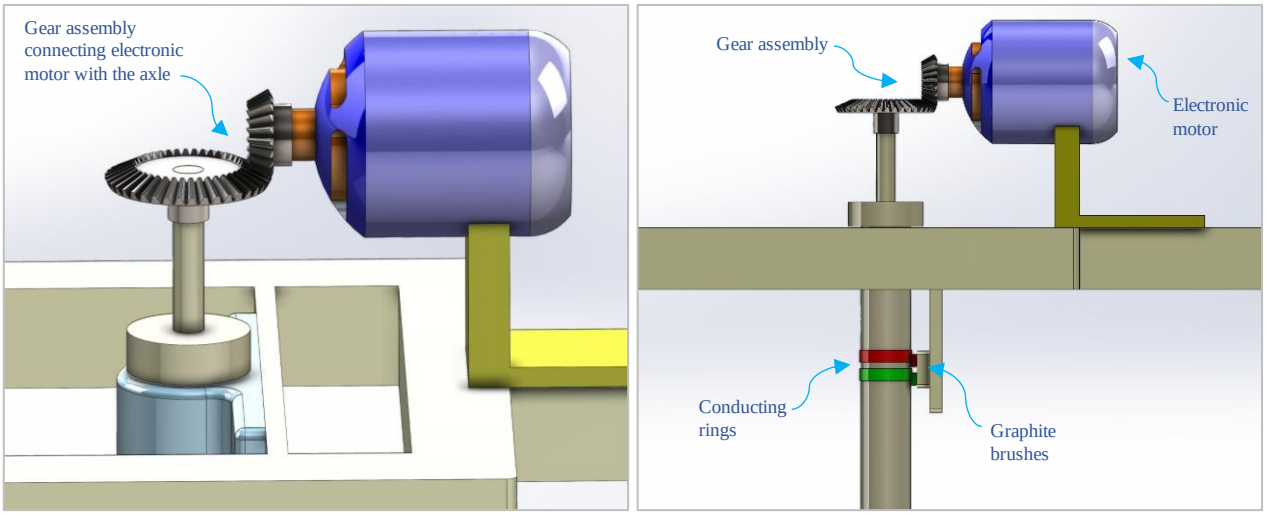


Figure 3A.4 (a): Schematic of IISCLP gear assembly **(b):** Schematic of the IISCLP LED connecting rings and graphite brush assembly.

A 100 L capacity IISCLP system was developed and tested in the Biomass Conversion Laboratory, Department of Energy, with assistance from the Central Workshop, Department of Mechanical Engineering, Tezpur University. The support structure was fabricated using MS sheets and square pipes. The 100 L capacity culture vessel was fabricated using a commercially available water tank with slight modifications. The LED support rings were made using 3mm thick aluminum sheets and were coated with a waterproof coating. Commercially available LED strips (Crimson LED Strips, Goldmedal Electricals, India) enclosed within transparent silicon tubes were used for the illumination. The central axel was made using a 30 mm diameter CPVC water pipe. The connectors and the gears were fabricated using Teflon rods. The motor was a generic 12V 60 rpm geared DC motor with a rated torque of 3.6 kg-cm, procured online (www.robu.in).

The developed IISCLP system is shown in Figure 3.5 (a-c). The IISCLP system was tested by culturing *Chlorella homosphaera* microalgae species using BG11 media. Details about the culture methodology are described in the later section of this chapter.

(a) **(b)** **(c)**

Figure 3A.5 (a): Internal lighting assembly, the gear assembly and the support structure of the developed IISCLP system **(b):** The lighting assembly with LEDs powered ON of the IISCLP system **(c):** Top view of the interior of the culture tank and lighting assembly of the IISCLP system.

3A.2.1. Design and development of Internally Illuminated Airlift Photobioreactor (IIAP)

The Internally Illuminated Airlift Photobioreactor (IIAP) design is also an internally illuminated system like the IISCLP design, as shown in Figure 3.6, and its cross-sectional representation is shown in Figure 3.7 (a). However, IIAP is designed with a slightly different approach. IIAP design emphasized minimizing the number of moving parts in the photobioreactor. Reducing the number of moving parts reduces the wear and tear of the parts, thereby reducing the operational and maintenance costs. Thus, considering the long duration of continuous operation at commercial scale implementation, IIAP design was considered.

In the IIAP, vertically aligned waterproof LED strips are inserted into the microalgae culture vessel, as shown in Figure 3.7 (a). The LED strips are arranged to evenly illuminate the entire volume of the microalgae culture, as shown in Fig. 3.7 (b). The distance between the LED strips and their orientations is optimized so that the maximum light path to any point in the microalgae culture from its nearest LED surface is less than 10 cm. The distance is considered as per literature stating the optimum light path for microalgae culture is less than 10-15 cm [3, 4].

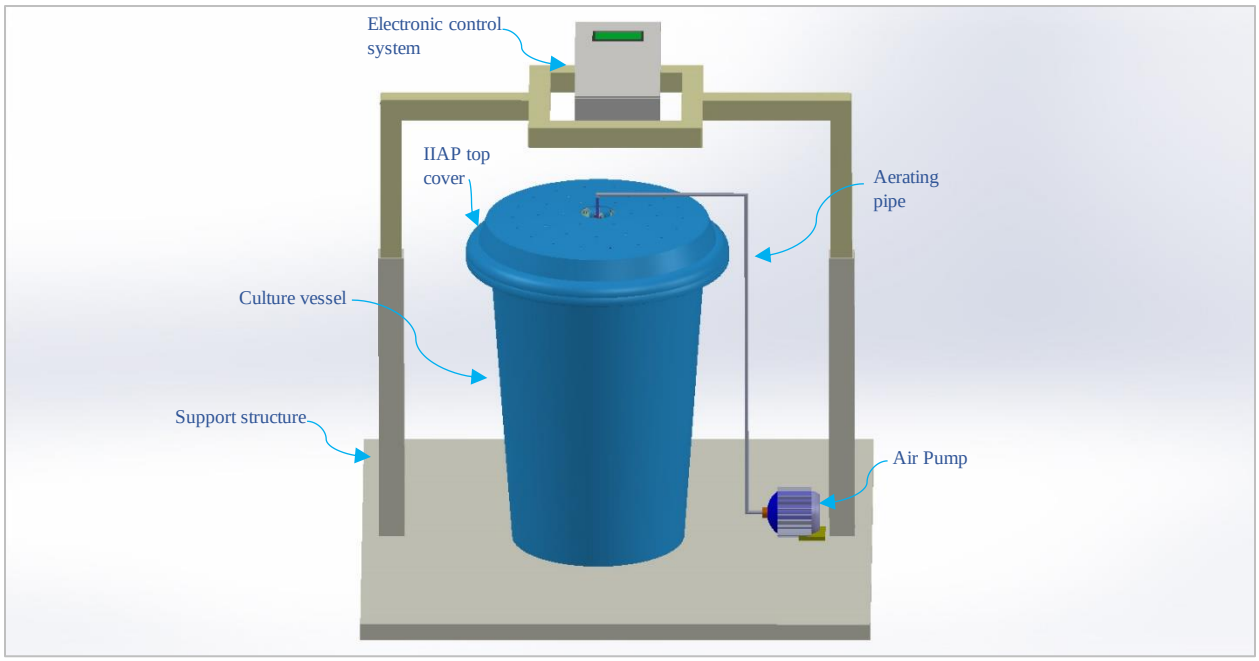
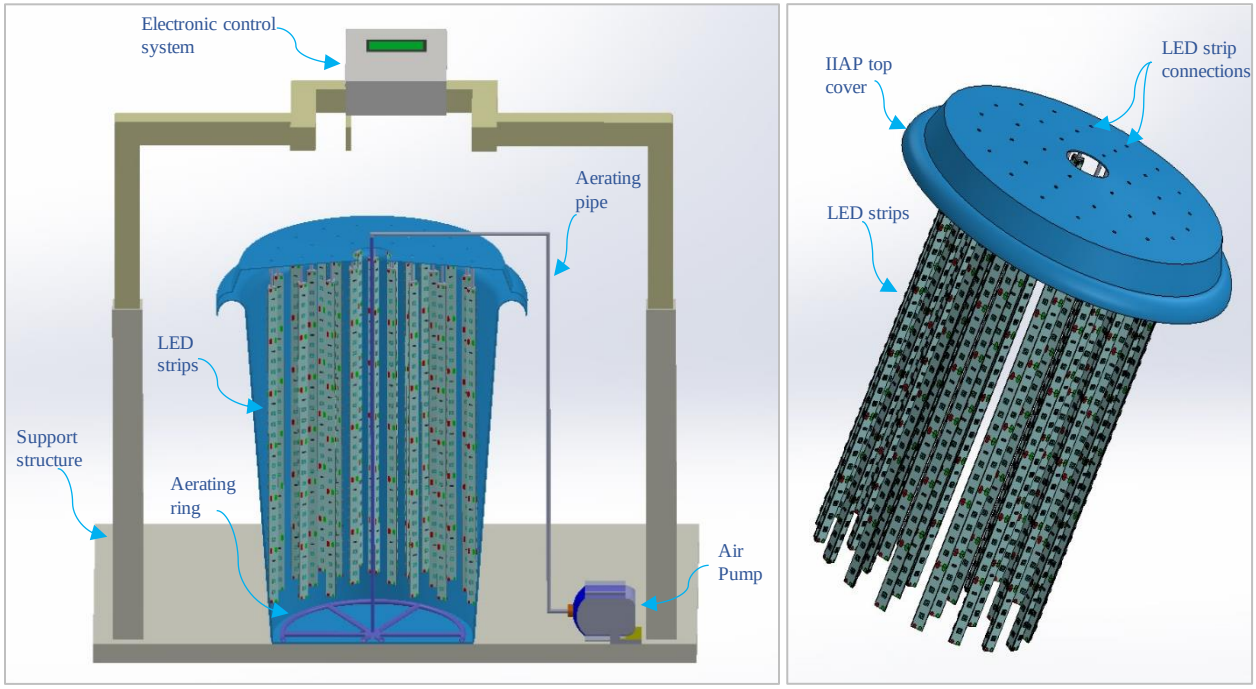


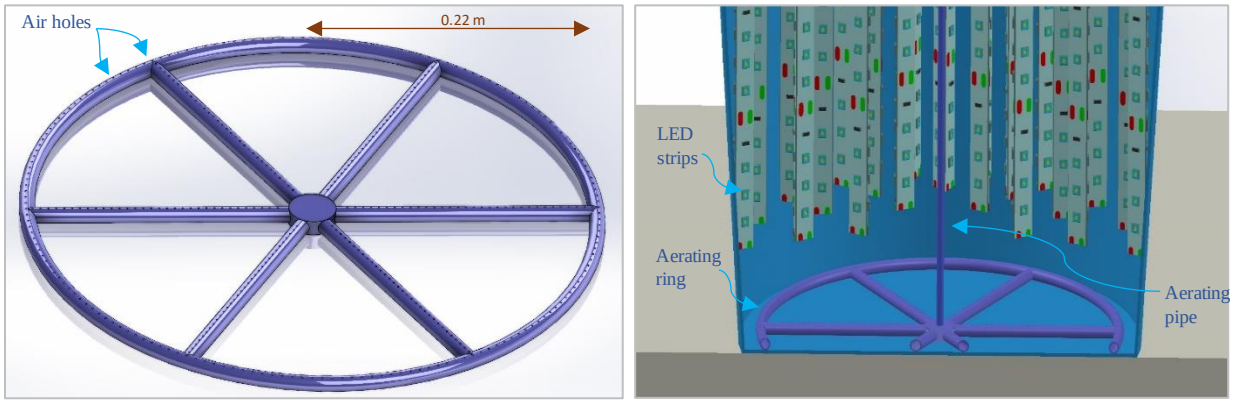
Figure 3A.6: Schematic of IIAP



(a) **(b)**

Figure 3A.7 (a): Schematic of the cross-sectional view of the IIAP. **(b):** Schematic of LED strip arrangement of the IIAP.

As shown in Figure 3.8 (a &b), an aerating ring structure made of perforated tubes is designed to be placed at the bottom of the microalgae culture vessel for aerating the microalgae culture. Aerating the culture helps dissolve carbon dioxide in the culture media, which is needed for microalgae growth. As shown in Figure 3.6, a reciprocating air pump is used to pump ambient air into the culture media. The air pump system has special valve to facilitate supplementing additional CO_2 with the ambient air into the culture medium. Supplementing CO_2 helps in regulating the pH of the microalgae culture [5], as well as helps in improving the productivity of the microalgae culture, as more quantity of CO_2 gets dissolved in the culture media [6] which is the primary food source of the microalgae being cultured. The CO_2 concentration in the airstream is planned to be optimized in further system studies. Additionally, the stream of tiny air bubbles rising from the bottom of the culture vessel to the top creates an upward draft carrying the microalgae upwards and preventing them from settling down. Thus, in the IIAP system, the air bubble circulates the culture media instead of moving parts like the rotating fins used in the IISCLP system.

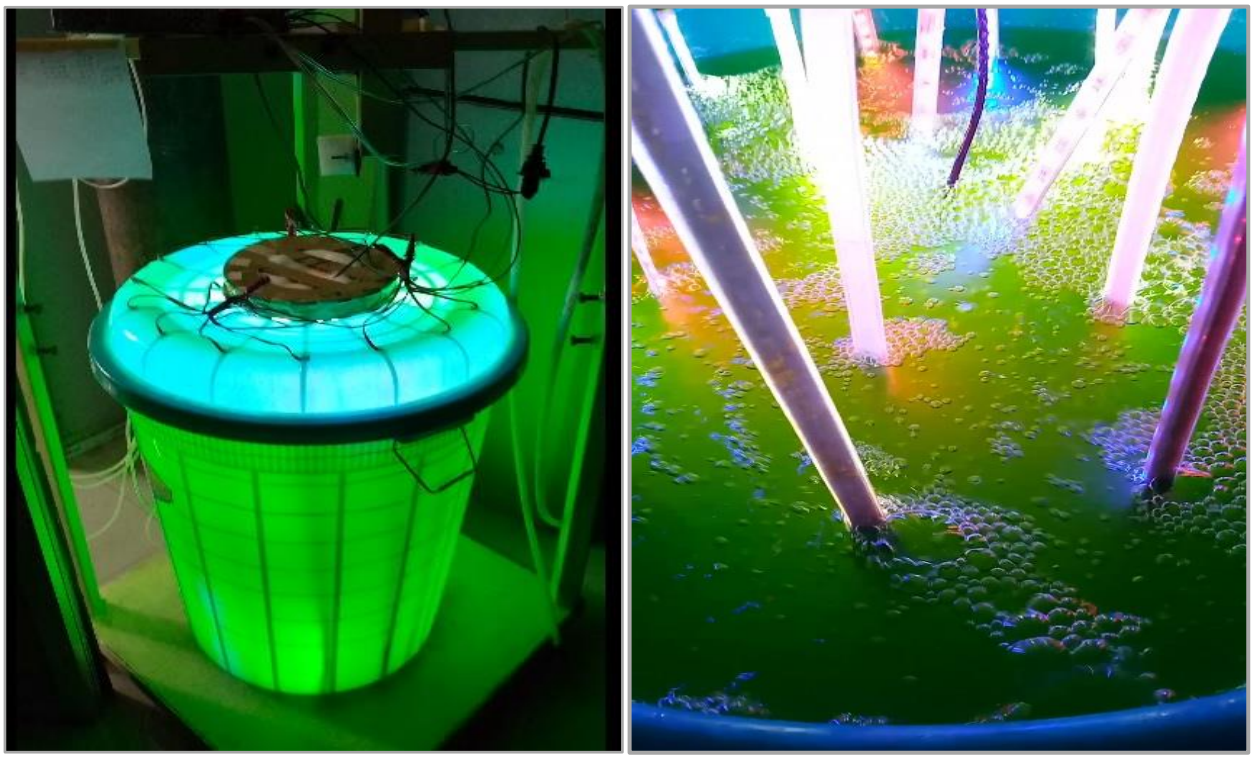


(a) **(b)**

Figure 3A.8 (a): Schematic of aerating ring of the IIAP in top isometric view. **(b):** Schematic of LED strip arrangement of the IIAP in bottom isometric view.

A 100 L capacity IIAP was fabricated and tested in the Biomass Conversion Laboratory, Department of Energy, Tezpur University. The support structure was made using 3 mm thick MS plate and square pipes. The culture vessel was made using a commercially available 100 L capacity water tank with required modifications. The LED strips used were commercially available LED strips (Crimson LED Strips, Goldmedal Electricals, India) enclosed within silicon tubes to make them waterproof. The aerating ring was made using a silicon pipe and perforated using needles.

The air pump was a reciprocating pump (Resun, Electro Magnetic Air Pump Aco 001) procured online. The developed system is shown in Figure 3.9 (a & b).



(a) **(b)**

Figure 3A.9 (a): The external view of the developed IIAP system. **(b):** Internal view of the developed IIAP system with LED strips and microalgae culture.

3A.2.2. Electronic control system to control the IIAP

A microcontroller-based electronic control system was designed to control the entire operation of the IIAP. Arduino microcontroller was chosen for the current prototype as Arduino is an open-source microcontroller platform, making it easily accessible and highly user-friendly. Additionally, Arduino has a diverse range of compatible hardware components like sensors, actuators, etc., readily available off the shelf, and it also has a vast user community support and free software libraries.

The control system designed and developed for IIAP was based on an Arduino Uno [7, 8] microcontroller system. The pin configurations of Arduino Uno are given in Annexure I. Additionally, the control system had temperature sensors (DB18B20) [9], a turbidity sensor (TS-300B) [10], a microSD card module [11], a real-time clock (RTC) module (DS3231) [12],

MoSFET switch [13], a relay switch [14] and an LCD display [15], connected to the Arduino Uno [8]. The developed control system for the IIAP system is shown in Figure 3.10.

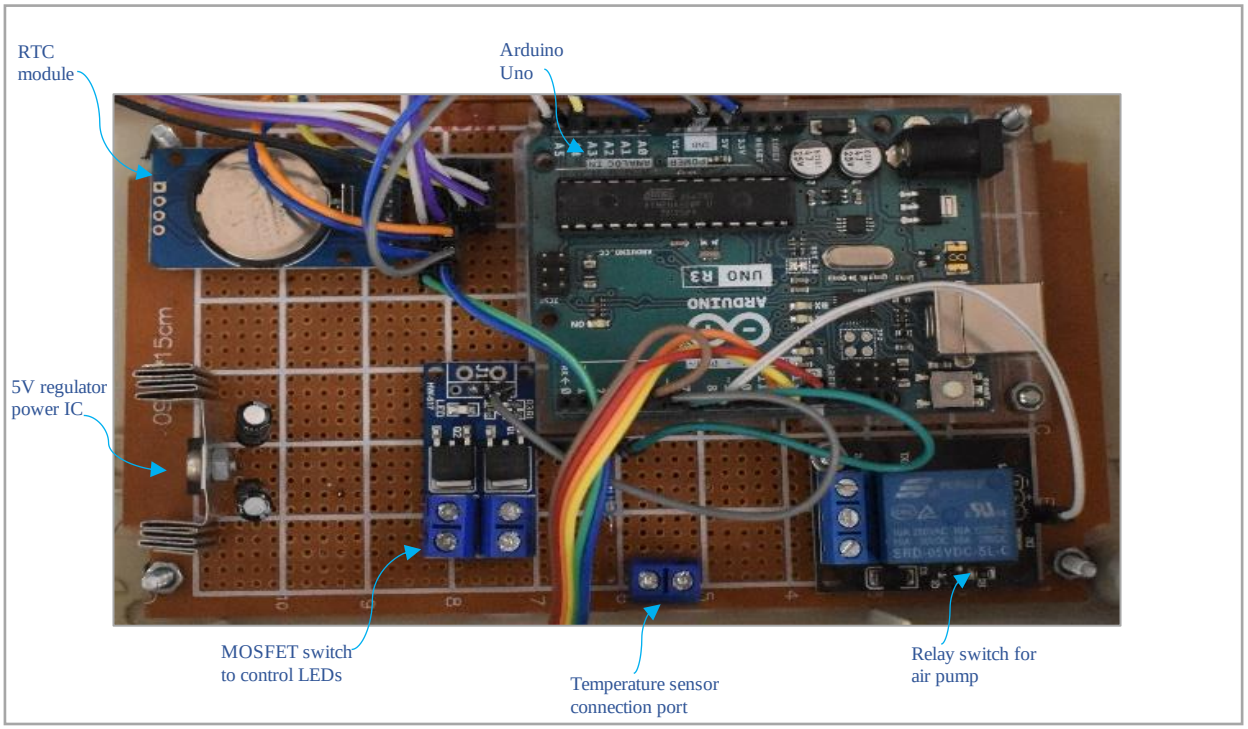


Figure 3A.10: IIAP control system developed using Arduino Uno microcontroller system.

The control algorithm implemented by the control system, as shown in Figure 3.11, works as follows. Upon powering ON, the control system follows the following steps:

Step 1: the microcontroller checks the EEPROM for the experiment's status. If the experiment's status is turned ON, the microcontroller moves to Step 2. If the experiment's status in the EEPROM is turned OFF, the microcontroller checks the conditions for turning the experiment ON and keeps on looping.

Step 2: the microcontroller checks the time on the RTC module. If the current time satisfies the condition for the aeration system to be turned ON, the microcontroller turns ON the air pump using the relay module connected to it; else, it turns OFF the air pump and moves to Step 3.

Step 3: the microcontroller checks the current time with the condition for the illumination. If the current time satisfies the condition for the illumination to be turned ON, the microcontroller checks the temperature sensor to determine the temperature of the microalgae culture. Suppose the culture

temperature is higher than the set value. In that case, the microcontroller either turns OFF the LEDs or turns ON the LEDs in a preset dimmer value using the power MoSFET through which the microcontroller controls the LEDs. If the current time satisfies the condition for the illumination to be turned ON and the culture temperature is below the set value, the LEDs are turned ON with maximum power. And moves to Step 4.

Note: in the current iteration, the LED strip and the air pump control the microalgae culture's temperature. LEDs emit a certain amount of heat upon powering them with their rated power. Although the heat emitted is much less compared to other forms of illumination, the LEDs being submerged in the culture medium, the medium absorbs all the emitted heat, and thus, the temperature of the culture medium increases. Hence, to bring down the temperature of the culture medium, the LEDs are either turned OFF or dimmed to a certain level, and the heat is carried out by the air stream used to aerate the culture. Once the temperature comes down, the LEDs are turned back to their rated illumination.

Step 4: the microcontroller checks the time on the RTC module, and if the current time satisfies the time to save the culture parameters, the microcontroller collects the temperature and turbidity readings from the respective sensors and saves the data in the SD card module. Else if, the current time does not satisfy the condition to save the culture parameters, the microcontroller moves to step 5.

Step 5: the microcontroller checks the current time with the condition for terminating the experiment. Suppose the condition satisfies the termination of the experiment. In that case, the microcontroller turns off the air pump, the LEDs, and changes the status on the EEPROM to no experiment and end the routine. Else, if the condition for terminating the experiment is not satisfied, the microcontroller moves to Step 2 and continues the routine.

The algorithm was implemented by developing the required hardware interface and programming the Arduino Uno microcontroller using C++ programming language. The programming and uploading of the code to the microcontroller was done using the Arduino IDE [7]. The circuit diagram of the developed control system and the implemented C++ code are given in Annexure II. The *Chlorella homosphaera* microalgae strain was cultured using BG11 culture media in the

developed IIAP. Multiple culture experiments were carried out to determine the developed IIAP's performance and control system.

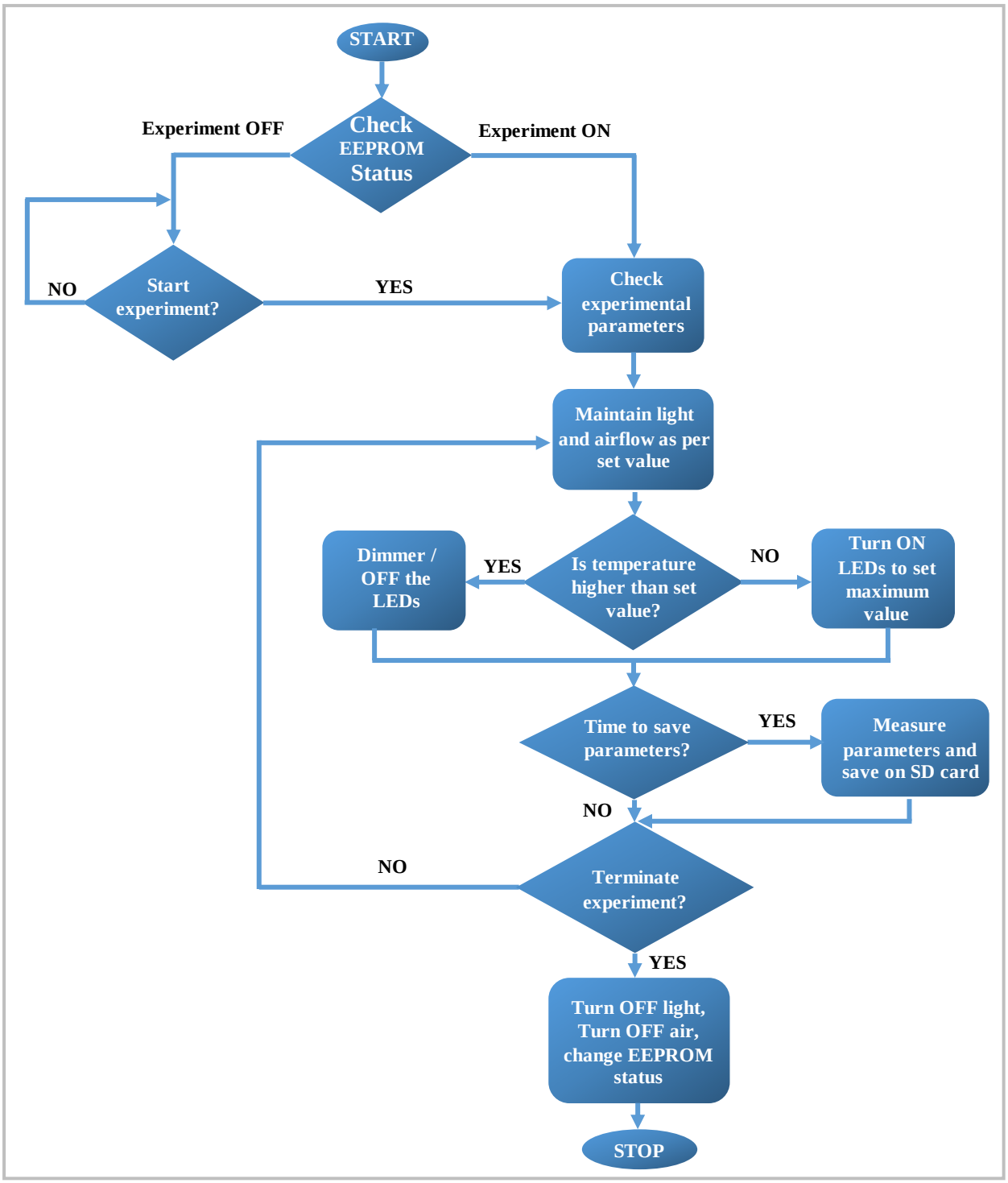


Figure 3A.11: The control algorithm for the IIAP system, developed using Arduino Nano microcontroller board.

3A.3.1. Design and development of Stacked Tray Automated Modular Photobioreactor (STAMP)

The Stacked Tray Automated Modular Photobioreactor (STAMP), as shown in Figure 3.12, is designed and developed with an entirely different design philosophy than IISCLP and IIAP. The primary design consideration of STAMP was to tackle the issue of biofouling.

The STAMP consists of microalgae culture trays (MCTs), as shown in Figure 3.13 (a), stacked one above another, as shown in Figure 3.12. The MCTs facilitate the photoautotrophic growth of the microalgae in the STAMP system. The MCTs are designed to have channels, as shown in Figure 3.13 (a). The channels improve the microalgae culture's flow characteristics while flowing within the MCT. The MCT's channels help maintain the microalgae flow velocity and prevent the microalgae from settling down. The channels are shallow, with a depth of less than 10 cm. The shallow depth is kept for having an optimal light path [16, 17], from top to bottom, within the microalgae culture. LED panels, as shown in Figure 3.13 (b), are used to illuminate the microalgae culture from the top, as shown in Figure 3.12. The LED panel is placed at an optimal height above the MCT, keeping an air gap between the two. The air gap has two significant roles: (i) prevents the microalgae culture from touching the LED panel, thereby preventing biofouling, and (ii) helps control temperature.

A raiser mixer tank is placed at the bottom of the STAMP system, as shown in Figure 3.12. A submersible water pump placed inside the raiser mixer tank, as shown in Figure 3.14, is used to pump the microalgae culture from the raiser mixer tank to the topmost MCT. The microalgae culture flows through the channels within the top MCT and exits the MCT to fall into the MCT placed below it. Thus, the microalgae culture gradually moves down each MCTs and finally reaches the raiser mixer tank and repeats the cycle. The microalgae flow path in the STAMP system and within the MCT is indicated with green and yellow arrows in Figure 3.12 and Figure 3.13 (a), respectively.

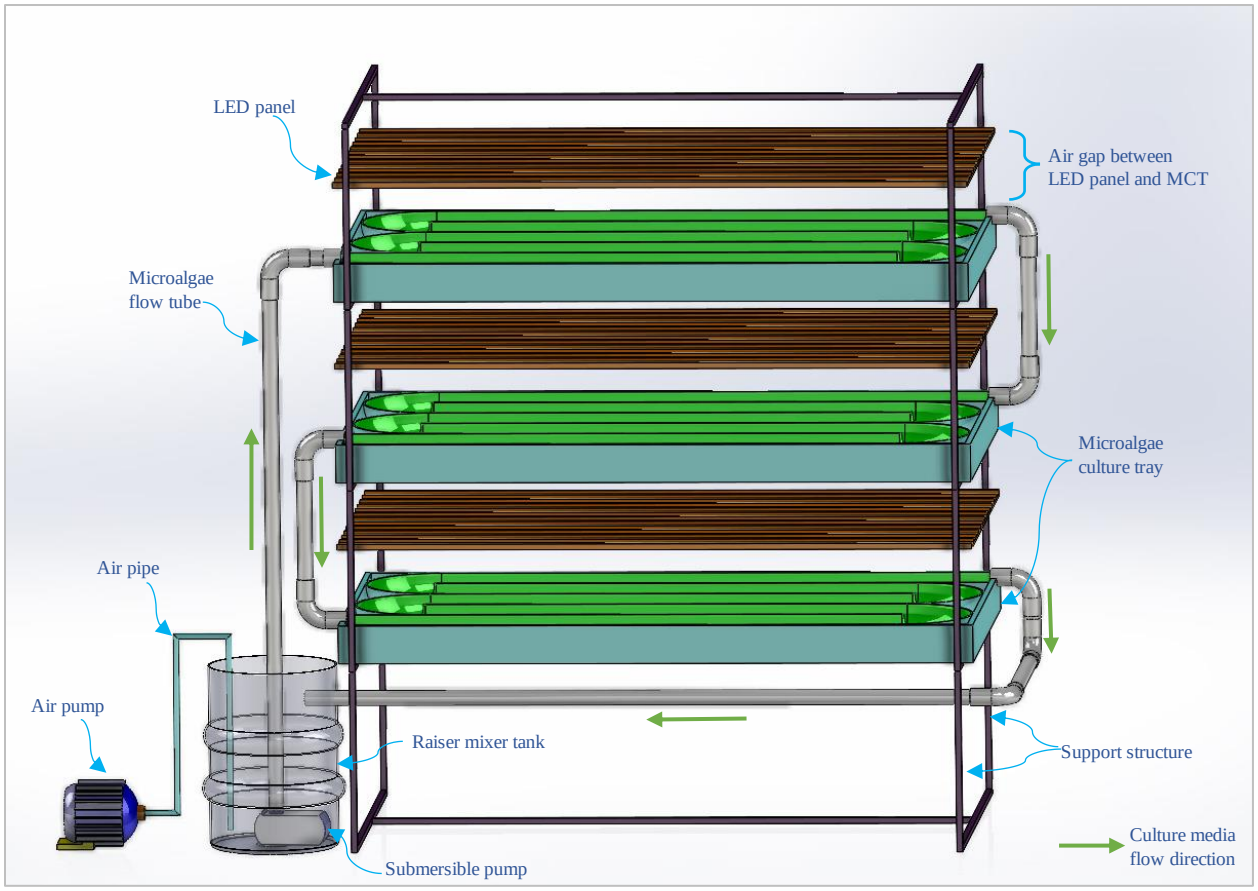
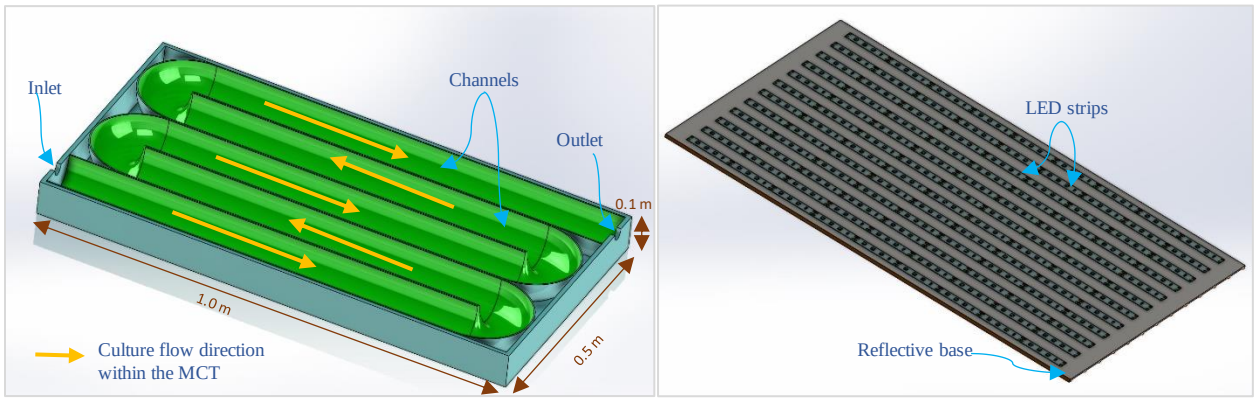


Figure 3A.12: Schematic model of Stacked Tray Automated Modular Photobioreactor (STAMP)



(a) **(b)**

Figure 3A.13 (a): Schematic view of the microalgae culture tray (MCT). **(b):** Schematic view of the LED panel used to illuminate the MCTs.

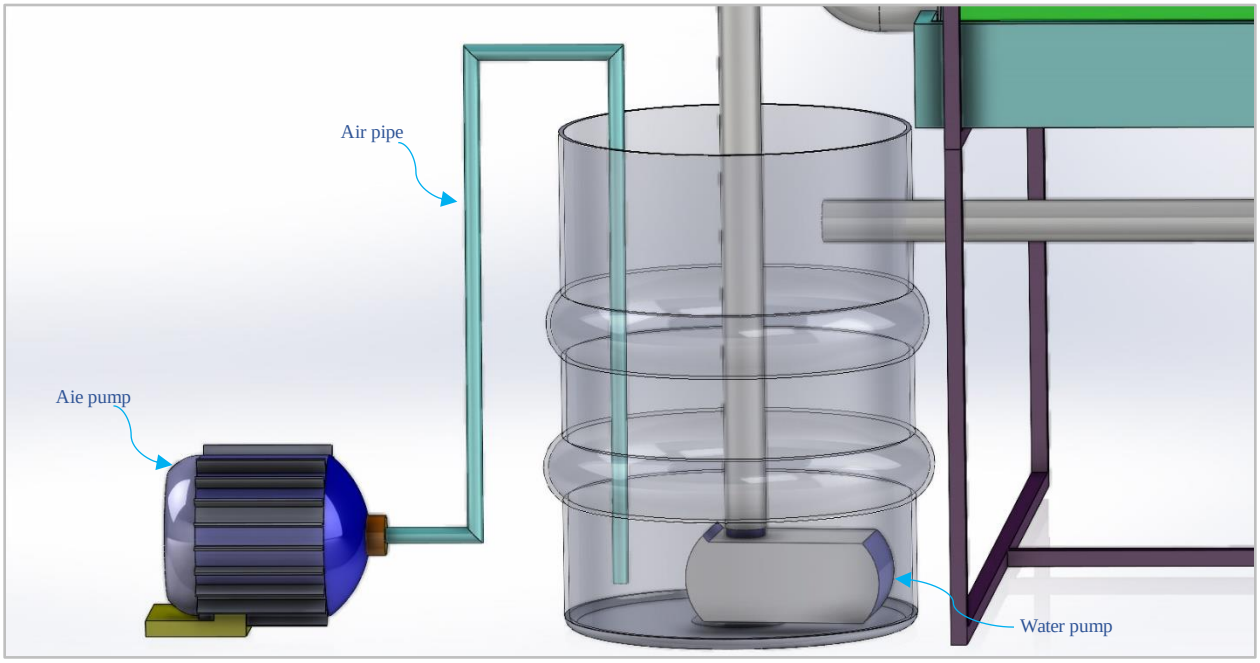


Figure 3A.14: Schematic representation of the raiser mixer tank of the STAMP system along with the submersible water pump and the reciprocating air pump.

The air pump, shown in Figure 3.14, aerates the microalgae culture in the raiser mixer tank. The water pump circulates the aerated microalgae culture in the STAMP system. The aeration dissolves carbon dioxide in the culture media required for microalgae growth. A pneumatic valve arrangement connected to a CO_2 source supplements the ambient air with additional CO_2 to improve the growth characteristics of microalgae. The CO_2 concentration is regulated by monitoring the pH of the culture media.

A 65 L capacity STAMP system was developed in the Biomass Conversion Laboratory, Department of Energy, Tezpur University. Two MCTs, 30 cm wide and 50 cm long, were constructed using 4mm flat glass sheets and placed one above the other with the help of a support structure. The support structure was built using 6 mm × 6 mm square cross-sectional MS rods. Two LED panels were made using 6 mm thick cardboard sheets, 30 cm wide and 50 cm long. The cardboard sheets were covered using aluminum foil for better reflectance. LED strips (Crimson LED Strips, Goldmedal Electricals, India) were attached on one side of the cardboard sheet lined parallelly along the length and at a gap of 3 cm between the LED strips. The LED panel thus formed was placed above the MCT with the LEDs facing towards the MCT, with the help of the support structure. A generic submersible pump (AC 240V 3W 220L/H 0.5MM Brushless

Submersible Pump) procured online (www.robu.in) was placed in the raiser mixer tank to circulate the microalgae culture. The raiser mixer tank was made using a commercially available 25 L water tank with required modifications. The aerating pump was a reciprocating air pump (Resun, Electro Magnetic Air Pump Aco 001) procured online. Figure 3.15 (a-e) details the developed STAMP system and its components. The entire operation of the STAMP is controlled using an electronic control system, described in detail in the following section.

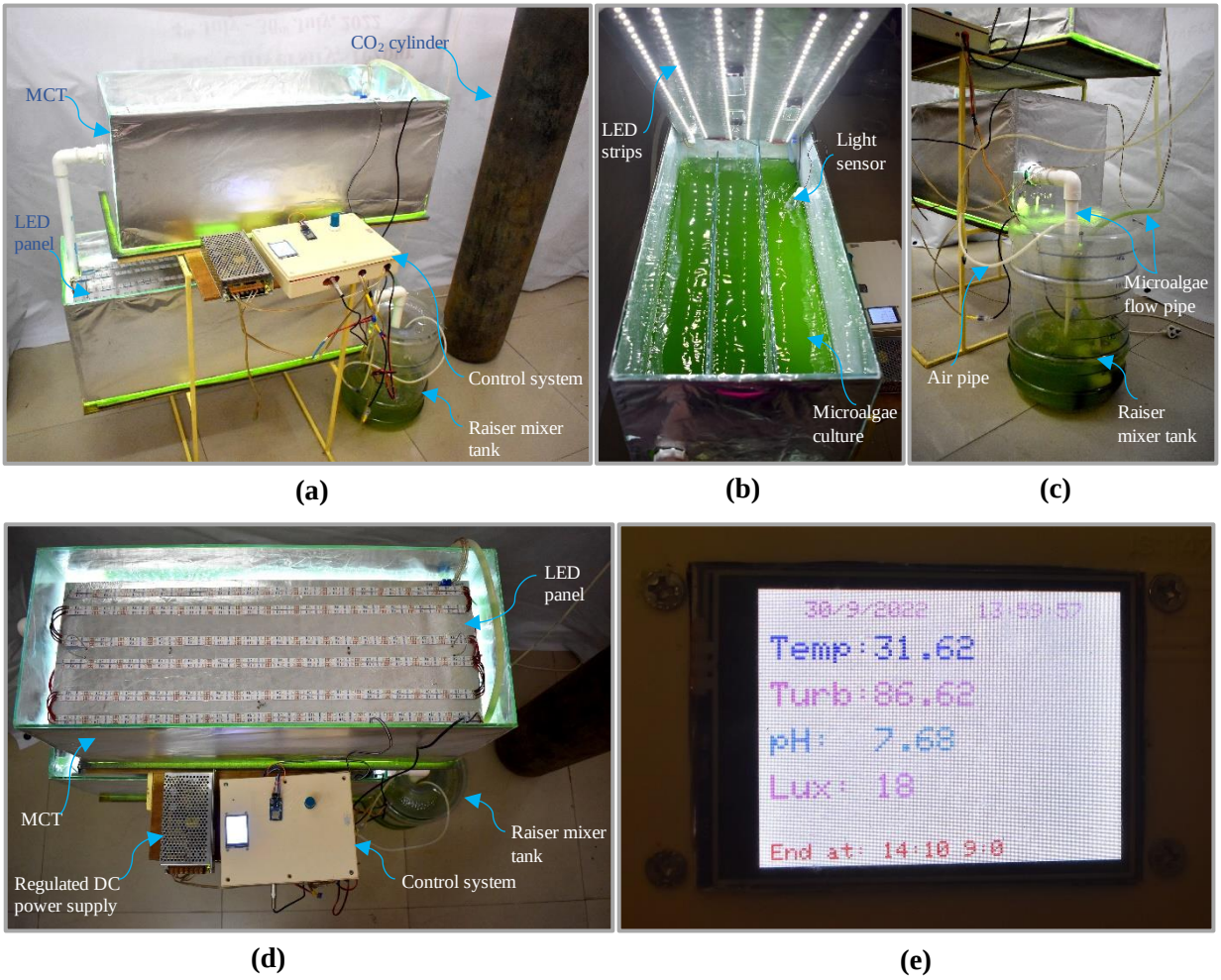


Figure 3A.15 (a): 65 L STAMP system developed at Biomass Conversion Laboratory, Department of Energy, Tezpur University. **(b):** Internal view of a MCT and the LED panel of the developed STAMP system. **(c):** Raiser mixer assembly of the developed STAMP system. **(d):** Top view of STAMP system. **(e):** Display panel of the developed STAMP control system.

3A.3.2. Electronic control system to control the STAMP

An electronic control system controls the STAMP system developed using an Arduino Mega microcontroller board [18] connected with a pH sensor [19], turbidity sensor (TS-300B) [10], and light intensity sensor (BH1750) [20] and touch screen display [21] to display different system parameters. A pneumatic valve (Saier, SEN-SZ06NA Solenoid Valve) to control the supply of CO_2, a relay switch to control the water pump (ELOVE, 18 Watt Submersible Water Pump) and air pump (Resun, Electro Magnetic Air Pump Aco 001) and a MoSFET switch [13] to control the LEDs were integrated into the circuit. The microcontroller keeps track of the current time using a real-time clock module (DS3231) [12]. An SD card module [11] is fitted to record the system parameters per the program. The control system controls different parameters of the STAMP system, such as light intensity, light duration, air duration, and culture circulation duration, and measures and records the parameters of the STAMP as per the algorithm illustrated in Figure 3.16 and described below.

Upon turning on the STAMP system, the control system follows the following steps.

Step 1: the microcontroller checks the experiment's status in the EEPROM of the microcontroller. If the experiment's status in the EEPROM is “Run Experiment,” the control moves to step 2. Else, if the status is “No Experiment,” the controller checks the condition to run the experiment and loops back to step 1.

Step 2: the microcontroller checks if the SD card is present. If an SD card is missing, an alarm is turned ON and loops to recheck the SD card. Else, it moves to step 3.

Step 3: the experimental parameters pH, temperature, turbidity, and light intensity are measured and stored in the SD card, and the program moves to Step 4.

Step 4: the microcontroller checks the current time and compares it to the condition for terminating the experiment. If the current time satisfies the condition to terminate the experiment, the microcontroller turns OFF the LEDs and pumps, changes the EEPROM status to NO Experiment, and terminates the process. Else, if the condition does not satisfy the condition to terminate the experiment, the control moves to step 5.

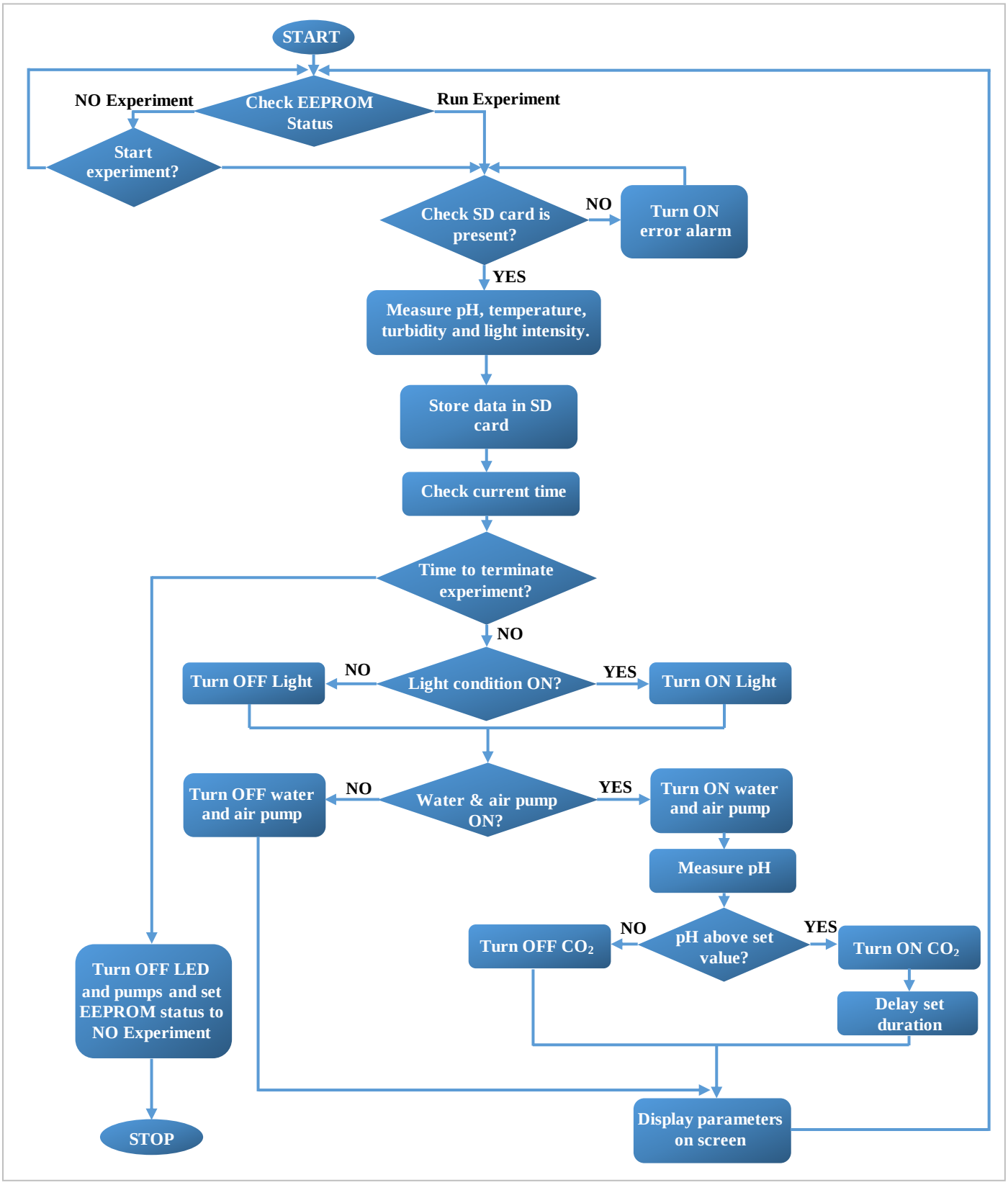


Figure 3A.16: Control algorithm of STAMP system, implemented using Arduino microcontroller system.

Step 5: the condition for the illumination is checked. If the illumination condition satisfies to be turned ON, the microcontroller turns ON the LEDs as per the preset illumination values. Else, it turns OFF the LEDs. The control then moves to step 6.

Step 6: the microcontroller checks the condition of the water pump and air pump status. If the condition satisfies the status of the pumps to be OFF, the microcontroller turns OFF the pumps and moves to step 8. Else, it turns ON the air and water pump and moves to Step 7.

Step 7: the microcontroller checks the pH of the microalgae culture. If the pH value exceeds the set pH value, the microcontroller turns ON the CO_2 supply for a set duration and moves to step 8. Else, the microcontroller turns OFF the CO_2 supply and moves to Step 8.

Step 8: the microcontroller displays the experimental parameter on the display system, and the control moves to Step 2.

The control algorithm of the STAMP system is implemented using an Arduino Mega microcontroller board, as shown in Figure 3.17. Annexure III contains the circuit diagram and the C++ program implementing the program. The STAMP and control systems were tested by culturing *Chlorella homosphaera* microalgae strain and cultured using BG11 culture media. Detailed culture methodology is described in later sections of this chapter.

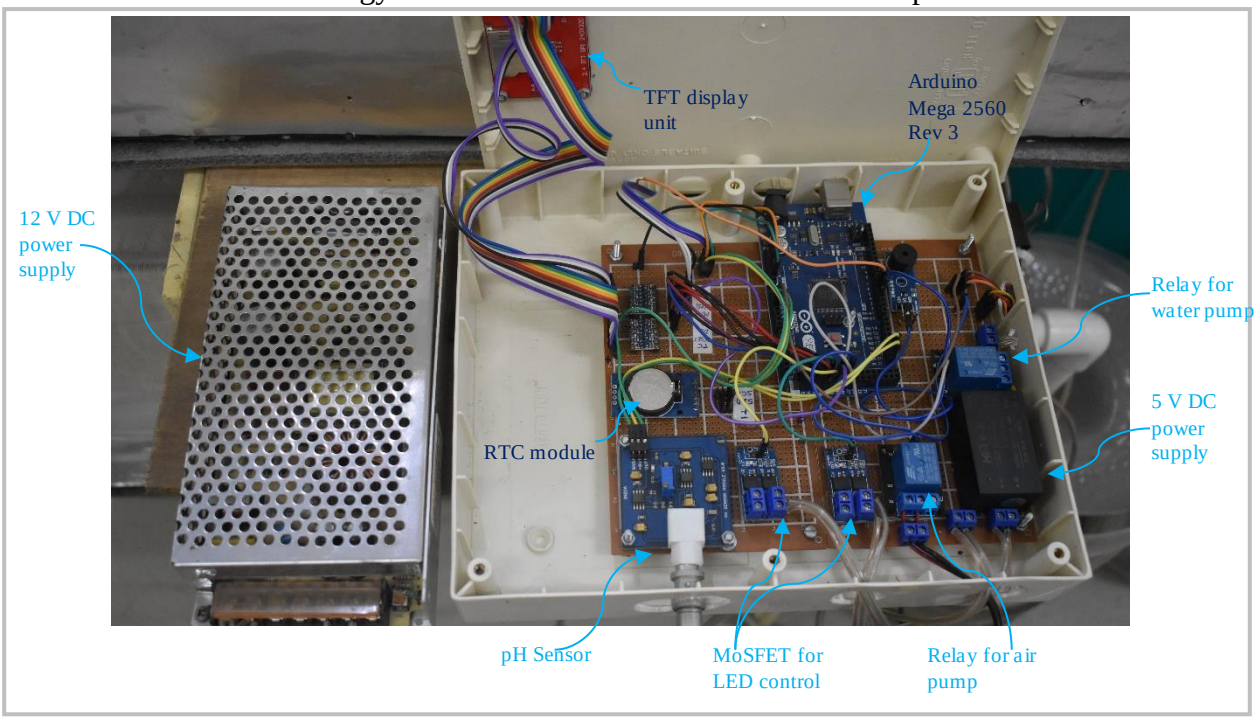


Figure 3A.17: Control system of the developed STAMP system

3A.3.3. Assessment of biofouling in the STAMP system

Biofouling is the growth or accumulation of microalgae on a solid surface, leading to mechanical or functional deficiencies. Biofouling is one of the significant limitations of an artificially illuminated microalgae culture system [22, 23]. Three glass slides were attached to the surface of the LED panel with the help of binder clips for easy detachment, as shown in Figure 3.18. The glass slides were used to assess the growth of microalgae on the surface of the LED panel, which has the potential to block light from reaching the microalgae culture. To assess biofouling, the transmittance of the glass slides was measured in the entire visible light spectrum (400 nm -700 nm) at regular intervals using a spectrophotometer (Shimadzu, Model UV-1700 Pharma Spec, Japan). The transmittance readings gave the rate of light being blocked, which can be assessed as the level of biofouling.

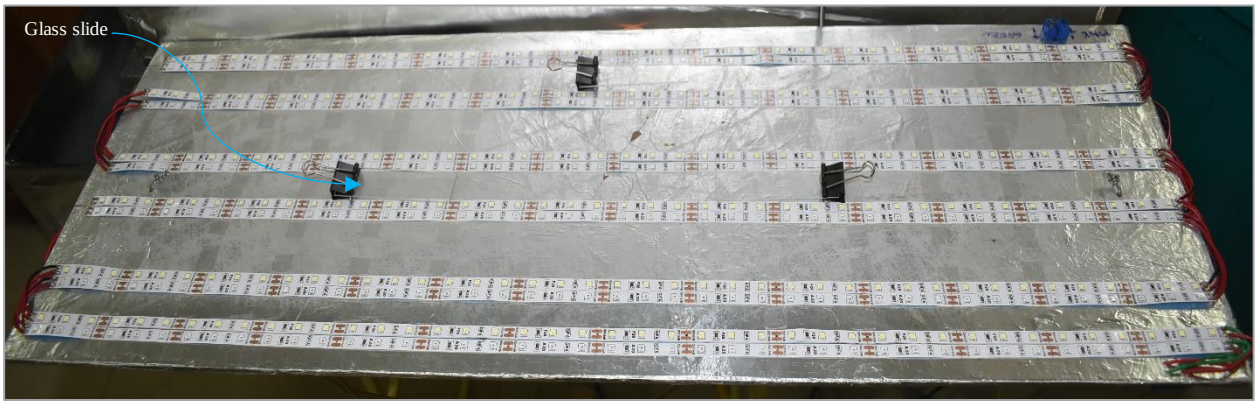


Figure 3A.18: The LED panel of the STAMP system with glass slides to monitor biofouling

3A.4. Microalgae strain and culture conditions

Chlorella homosphaera microalgae strain was provided by Prof. Mohan Chandra Kalita, Department of Bioengineering and Technology, Gauhati University, Assam, India. The morphological characteristics of the obtained microalgae strain were observed using an optical microscope (Radical, RXLr-4, India) connected with a fixed camera (Radical Procam, India), as shown in Figure 3.19. The obtained microalgae strain was grown axenically using BG11 media [24] and gradually increasing the culture volume from 500 mL to 2 L Erlenmeyer flasks and finally in 20 L volume transparent plastic water bottles. The BG11 media composition is given in Table 3.1 [24]. The BG11 media consists of two groups of nutrients: macronutrients and micronutrients.

The macronutrients are dissolved in distilled or UV-treated filtered water with the help of a magnetic stirrer till adequately dissolved. The quantity of macronutrients required to be dissolved per liter of BG11 media is given in Table 3.1. The micronutrients as given in Table 3.1 are dissolved in 1 L of distilled water to prepare the sock solution. The stock micronutrient solution is then added into the prepared BG 11 media at a volume of 1mL per liter of BG 11 prepared. The prepared BH 11 is autoclaved before inoculating with the microalgae strain.

The inoculated culture was maintained at pH 7.0 ± 0.5 (adjusted using 0.1 M NaOH or 0.1 M HCl), temperature of 28 ± 2 ℃, and illuminated with florescent lamps at an intensity of 3000 Lux (Metravi 1332 Fc/Lux meter). The microalgae culture was aerated with pre-filter air (Moxcare, 0.22µm pore size) at a constant airflow of 3.5 SLPM (Mass Flow Meters, Alicat Scientific, Tucson, USA) using reciprocating air pumps (Venus Aqua Air Pump Model AP-208A, China). Microalgae culture was taken from the 20 L flask as and when required for experimental purposes, and fresh BG11 medium was filled in the 20 L flask to maintain the culture volume.

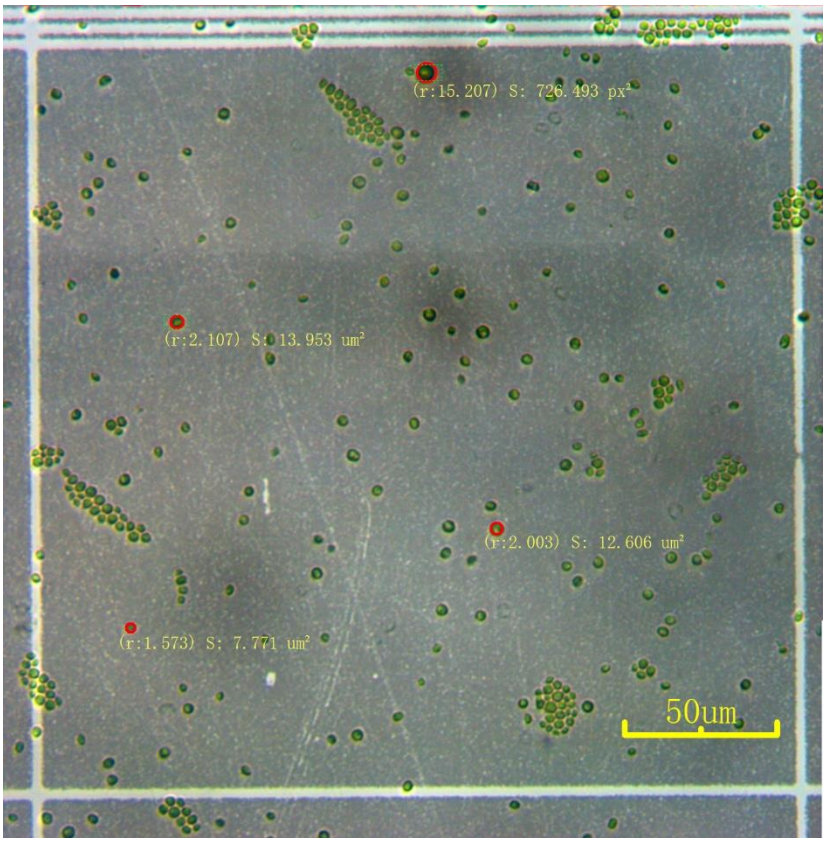


Figure 3A.19: *Chlorella homosphaera* microalgae strain viewed under compound microscope at ×40 magnification.

Table 3A.1: Chemical composition of Blue Green 11 (BG-11) culture media.

Macro nutrients (g/L of BG 11)		
Compounds		**Quantity**
Name	**Chemical formula**	**(gL^{-1})**
Calcium chloride dihydrate	$CaCl_2.2H_2O$	0.036
Magnesium sulfate heptahydrate	$MgSO_4.7H_2O$	0.075
Dipotassium hydrogen phosphate	K_2HPO_4	0.04
Sodium Nitrate	$NaNO_3$	1.5
Ferric Ammonium Citrate	$(NH_4)_5[Fe(C_6H_4O_7)_2]$	0.006
Citric acid	$HOC(CH_2CO_2H)_2$	0.006
Sodium Carbonate	Na_2CO_3	0.02
EDTA disodium salt dihydrate	$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$	0.001

Micro nutrients (1mL/L of BG11)		
Compound		**Quantity**
Name	**Chemical formula**	**(g)**
Molybdic acid sodium salt dihydrate	$NaMoO_4.2H_2O$	0.39
Zinc sulfate heptahydrate	$ZnSO_4.7H_2O$	0.222
Manganese (II) chloride tetrahydrate	$MnCl_2.4H_2O$	1.81
Boric acid	H_3BO_3	2.86
Cobaltous Nitrate Hexahydrate	$Co(NO_3)_2.6H_2O$	0.049
Copper sulfate pentahydrate	$CuSO_4.5H_2O$	0.079
Distilled water	H_2O	1000

3A.5 Chemicals used for the study

Chemicals used for the current study were used as received from the supplier without any modification or purification. Calcium chloride, magnesium sulfate, dipotassium hydrogen phosphate, sodium nitrate, citric acid, sodium carbonate, sodium molybdate, zinc sulfate, manganese (II) chloride tetrahydrate, boric acid, cobalt nitrate, copper sulfate, sodium hydroxide

and sulfuric acid (98%) were bought from Merck (Mumbai, India). EDTA-Na and ferric ammonium citrate were procured from Rankem (Maharashtra, India).

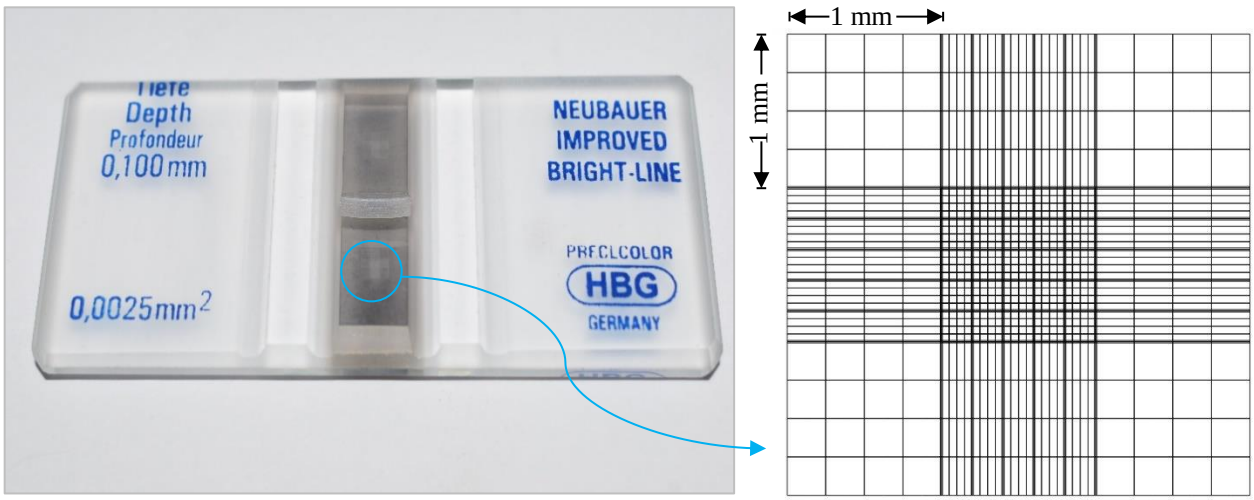
Solvents, including methanol (≥99.0%), ethanol (ACS grade), chloroform (≥99.0), petroleum benzene (ACS), and toluene (≥ 99.9%) were supplied by Merck (Mumbai, India). Acetone (ACS), hexane (99.0%), and heptane (99.8%) were bought from Rankem (Maharashtra, India). Deuterated methanol (99.0 atom% D), Isopropanol (99.5%), and deuterated chloroform (99.8 atom% D) were procured from Sigma-Aldrich (St. Louis, MO, USA).

3A.6 Microalgae growth monitoring

The microalgae growth in the photobioreactor system was measured at regular intervals to evaluate the performance of the developed photobioreactors. There are numerous ways to assess microalgae concentration in a culture media, including dry cell weight, cell count, spectrophotometry, dielectric permittivity, oxygen production rate, flow cytometry, etc. [25]. For the current study, three methods of microalgae growth estimation were initially considered according to available laboratory infrastructure. Those methods included (i) manual cell count, (ii) gravimetric analysis, and (iii) optical density. However, optical density measurement was extensively used throughout the study because it was a fast and reliable method of microalgae growth determination. The three methods are elaborated below.

3A.6.1 Microalgae cell count based growth estimation

Microalgae growth can be monitored by counting the number of cells present per unit volume of culture media. This is done with the help of a specially designed device called a hemocytometer [26], which is placed and observed under a microscope. A hemocytometer is a specially designed device, as shown in Figure 3.20 (a), that contains a cavity called a Nebular chamber capable of holding a specific volume of microalgae culture. Microalgae culture samples diluted as required for ease of calculation can be loaded into the hemocytometer using a pipette and then placed under a microscope to count the number of microalgae cells present within that volume of microalgae culture. The cell concentration in the measured culture sample is then evaluated based on the cell count of that particular volume of microalgae culture. In brief, the Nebular chamber has built-in grid lines marked on it, as shown in Figure 3.20 (b). Microalgae suspension loaded into the nebular chamber is counted with the help of the reference grid lines.



(a) **(b)**

Figure 3A.20 (a): Hemocytometer, **(b):** Nebular chamber.

As seen in Figure 3.20 (b), the larger squares are 1 mm wide and 1 mm long with a depth of 0.1 mm. Thus, the total volume of the microalgae culture present within a single large grid square is 0.1 mm^3 or 0.1 µL. The cells present within four or five such cells are counted, and the average is taken [27]. The total cell count in the culture is thereby determined using the equation Eq. 3.1:

$$\text{Total cells per mL} = N_{avg} \times D.F \times 10^4 \qquad \text{Eq. 3.1}$$

where, N_{avg} is the average number of cells counted in the 1 µL volume grid, and D.F is the dilution factor, which is 1 if the sample is not diluted.

For the current study, the Neubauer chamber was placed under the microscope (Radical RXLr-4, 40x). Photos were taken instead of direct cell count to minimize the error in cell count. The images were taken using ProCam software, which comes with the microscope. Ten images of 0.25mm × 0.25mm size grids were taken for each sample. The cell count was done using free software called DotDotGoose. The cell concentration was then determined using equation Eq. 3.1.

3A.6.2 Microalgae growth estimation by gravimetric analysis

In the gravimetric method, glass microfiber filters (Whatman Glass Microfiber Filters 110mm diameter) were dried at 60-80˚C for 12-24 hours to remove any moisture from the sheets, and the initial weight (W_i) was recorded. The filter sheets were then placed in glass/plastic funnels and

placed in an empty conical flask. A measured volume (V_s) of the sample (microalgal culture) were poured into the filter sheet and set aside undisturbed to filter the media for some time, as shown in Figure 3.21. After completion of filtration, the filter sheets were kept in the oven at 60-80˚C for 12-24 hours, and the final weight of the filter sheets (W_f) was recorded. The dry weight of algae per liter of culture is calculated by equation Eq. 3.2, as shown.

$$W_{dry/L} = ((Wf - Wi)/Vs) \quad \text{Eq.3.2}$$

The biomass productivity was calculated by equation Eq 3.3, where CDW_{final} and CDW_{intial} represent the cellular dry weight (gL^{-1}) at the start of the culture (T_{intial}) and end of the experiment (T_{final}), respectively.

$$\text{Productivity } (g\ L^{-1}d^{-1}) = (CDW_{final}\text{-}CDW_{intial})/(T_{final}\text{-}T_{intial}) \quad \text{Eq. 3.3}$$

Figure 3A.21: Microalgae culture filtered using glass microfilters for microalgae growth measurement using gravimetric method.

3A.6.3 Microalgae growth estimation by optical density

Microalgae growth estimation by optical density method was done with the help of a Spectrophotometer[28, 29]. The current study used a UV-Vis Spectrophotometer (Shimadzu, Model UV-1700 Pharma Spec, Japan) for the measurement of optical density (OD). The microalgae sample was taken in a quartz cuvette having a path length of 1 cm, and the absorbance measurement was done at a wavelength of 560 nm due to the least absorbance of light by chlorophyll and other pigments at this wavelength, giving accurate OD measurements [27, 30, 31]. Initially, the absorbance (OD_{560}) of BG11 medium without inoculum was taken and marked as blank (reference sample) tared to OD_{560} of 0, and after that, the absorbance (OD_{560}) of the samples was measured. The OD_{560} readings were taken at specific time intervals (12 hours) and used to calculate the specific growth rate using the equation Eq. 3.4 [32, 33].

$$\mu = \ln(OD_{t1}/OD_{t2})/(t_2-t_1) \quad \text{Eq. 3.4}$$

where, μ is the specific growth rate, OD_{t1} is OD_{560} at time t_1 and OD_{t2} is the OD_{560} at time t_2.

3A.7 Microalgae lipid extraction and quantification

The lipid was estimated using the method of Bligh and Dyer [34]. Briefly, 50 mL of algal culture was centrifuged, washed with phosphate buffer, and dried at 60 °C. The dried biomass was treated with a mixture of methanol and chloroform in a 2:1 (v/v) ratio and then incubated under continuous shaking conditions for 24 hours. Subsequently, chloroform and saline water (0.73% of NaCl w/v) were added to the mixture to adjust the final ratio to 2:2:1.8 (v/v/v), chloroform, methanol, and saline water. The mixture was mixed well and kept vertically until three layers were distinct. Next, the lower layer (lipid dissolved in chloroform) was collected, the chloroform evaporated, and the lipid was measured gravimetrically. The equation, Eq. 3.5 evaluated the lipid content.

$$LC\ (\%) = M_{lip}/M_{bio} \times 100 \quad \text{Eq. 3.5}$$

where, LC(%) is the lipid content expressed in %, M_{lip} is the mass of extracted lipid, and M_{bio} is the mass of biomass from which the lipid was extracted.

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CHAPTER 3B

To optimize the culture conditions and improve the biomass and lipid productivity.

3B. To optimize the culture conditions and improve the biomass and lipid productivity.

After developing a photobioreactor capable of commercial-scale microalgae cultivation, its operational parameters were optimized for efficient operation and high productivity. The investigation studied and optimized the effect of five parameters: light intensity, light duration, light wavelength, air flow rate, and nitrogen concentration on microalgae biomass and lipid productivity. There are other parameters affecting microalgae biomass and lipid productivity; however, due to experimental constraints and technical limitations, the effect of only these five parameters on a single microalgae species, i.e., *Chlorella homosphaera*, was investigated in the current study. Optimization was carried out using a statistical tool called Response Surface Methodology (RSM), which was implemented, and a special culture setup was developed to perform the optimization experiments, as discussed in the following section. The culture parameters obtained using the optimized process were then experimentally verified in the developed photobioreactor system.

3B.1. Response Surface Methodology (RSM) based optimization of microalgae culture parameters

To model and optimize the relationship between the independent parameters and the resultant response in the LED-based microalgae culture experimentation, a statistical tool, Response Surface Methodology (RSM) [1, 2], was used. Face Centered Central Composite Design (FCCCD) approach of RSM was used with the independent variables, nitrogen concentration (sodium nitrate), light intensity, light duration, and air flow rate, to model and optimize for the highest possible resultant responses, i.e., biomass and lipid productivity of *C. homosphaera*. FCCCD was chosen for this experiment as it requires only three levels, the highest, lowest, and the midpoint of each experimental variable, making it simple, and it also has the most negligible probability of error [3]. The highest and lowest values of nitrogen concentration and light durations were chosen based on data from preliminary investigations carried out to study the effect of these parameters. The highest and lowest values of airflow and light intensity were based on the maximum values possible with the developed experimental setup, details of which are described in the following section.

To compare the performance of different LEDs based on their power consumption, the light intensities were considered in percentage based on the input power. 10W is the maximum power that can be applied in the developed setup; thus, it is considered 100% intensity, and 2W was the least power applied, being 20% intensity, and so forth. The values of the independent variables used to model the parameters are given in Table 3B.1.

Table 3B.1: Actual and coded values of the independent variables of *C. homosphaera*

Experimental variables	Code	Coded levels		
		-1	0	1
Light duration (h)	A	0	6	24
Light intensity (%)	B	0	20	100
Airflow (Ls^{-1})	C	0	0.4	1.6
Nitrogen content (g L^{-1})	D	0	0.1	10.0

The experiment was designed using the Design Expert software (Version 13, Stat-Ease Inc., USA). The experiments had 30 runs with six center points, eight axial points, and sixteen factorial points, shown in Table 3B.2. To overcome the limitations of running maximum four experiments in the developed experimental setups at a time and to conduct the experiments efficiently, the experiments were grouped into 11 groups, from A to K, as shown in Table 3B.2, in a similar manner as done by Hosseini et al. [4] to overcome the limitations of available bioreactors.

The experimental variables were coded as per Eq. 3B.1

$$y_j = \frac{Y_j - Y_{j0}}{\triangle Y_j} \qquad \text{Eq. 3B.1}$$

where, y_j, Y_j, and Y_{j0} represent the coded value, experimental value, and actual experimental value at the central point of the j^{th} experimental variable, respectively, and $\triangle Y_j$ indicates the step change of the value.

Table 3B.2: Four-factor FCCCD based experimental design

Four-factor FCCCD experimental design					
Exp. No.	**Intensity (%)**	**Duration (Hours)**	**Air (L min^{-1})**	**Nitrogen (g L^{-1})**	**Group**
1	20	6	0.4	0.1	A
2	20	6	0.4	10	
3	20	6	1.6	0.1	
4	20	6	1.6	10	
5	20	15	1	5.05	B
6	20	24	0.4	0.1	C
7	20	24	0.4	10	
8	20	24	1.6	0.1	
9	20	24	1.6	10	
10	60	6	1	5.05	D
11	60	15	0.4	5.05	E
12	60	15	1	0.1	
13	60	15	1	5.05	
14	60	15	1	5.05	
15	60	15	1	5.05	F
16	60	15	1	5.05	
17	60	15	1	5.05	G
18	60	15	1	5.05	
19	60	15	1	10	
20	60	15	1.6	5.05	
21	60	24	1	5.05	H
22	100	6	0.4	0.1	I
23	100	6	0.4	10	
24	100	6	1.6	0.1	
25	100	6	1.6	10	
26	100	15	1	5.05	J
27	100	24	0.4	0.1	K
28	100	24	0.4	10	
29	100	24	1.6	0.1	
30	100	24	1.6	10	

3B.1.1. Biomass productivity

The microalgae biomass productivity was first evaluated by determining the culture's dry cell weight (DCW). To determine the DCW, 25 ml of the algae culture was filtered using a pre-dried and pre-weight glass fiber filter (Whatman, GF/A, 1.6 µ, 110 mm diameter). The filtered cells were

dried at 80℃ till a constant weight was achieved. The dry cell weights were then determined gravimetrically. After the determination of the DCW, the biomass growth was calculated using the following (Eq. 3B.2) equation:

$$\text{Biomass productivity (mg L}^{-1}\text{ day}^{-1}) = \frac{(W_{end} - W_{start})}{\triangle T \times V} \times 1000 \quad \text{Eq. 3B.2}$$

where, W_{end} and W_{start} are the biomass dry weight at the end and start of the experiment, $\triangle T$ is the duration of the experiment, and V is the volume of culture used to calculate the dry weight, which is 25 mL in the current experiment.

3B.1.2. Lipid productivity

Lipid productivity was determined by extracting the lipid using the Blight and Dyer method [5]. The lipid productivity was evaluated using the following (Eq. 3B.3) equation:

$$\text{Lipid productivity (mg L}^{-1}\text{ day}^{-1}) = \frac{W_{tl} - W_{te}}{\triangle T \times V} \times 1000 \quad \text{Eq. 3B.3}$$

where, W_{tl} and W_{te} are the weight of the test tube with lipid and the weight of the empty test tube, $\triangle T$ is the duration of the experiments, and V is the volume of the microalgae culture used to extract the lipid, which is 50 mL in the current scenario.

3B.1.3. Statistical analysis

The experimental data were analyzed, and the responses were predicted by the response surface regression procedure using the following (Eq. 3B.4) second-order quadratic equation,

$$y = \beta_o + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum\sum_{i<j} \beta_{ij} x_i x_j \quad \text{Eq. 3B.4}$$

where, 'y' indicates the response, 'β_o' indicates the graph intercept, 'β_i', 'β_{ii}', and 'β_{ij}' are the mean values of linear, quadratic, and interaction constant coefficients, respectively, and 'x_i' and 'x_j' are the coded input variables. The output responses of biomass and lipid were all desired, with desirability set to maximum. The overall desirability (D) was evaluated by using the following (Eq. 3B.5) equation:

$$D = (d_1 y_1 \times d_2 y_2)^{1/2} \quad \text{Eq. 3B.5}$$

where, d_1 and d_2 are desirability of the responses y_1 and y_2 [6].

3B.2. Development of experimental setup for RSM-based microalgae culture experiments

An experimental setup was designed to perform the RSM-based experimentations, and four such setups were developed, as shown in Figure 3B.1. The setup consisted of a microalgae culture chamber (MCC), an electronics control circuity, and air pumps for the air supply.

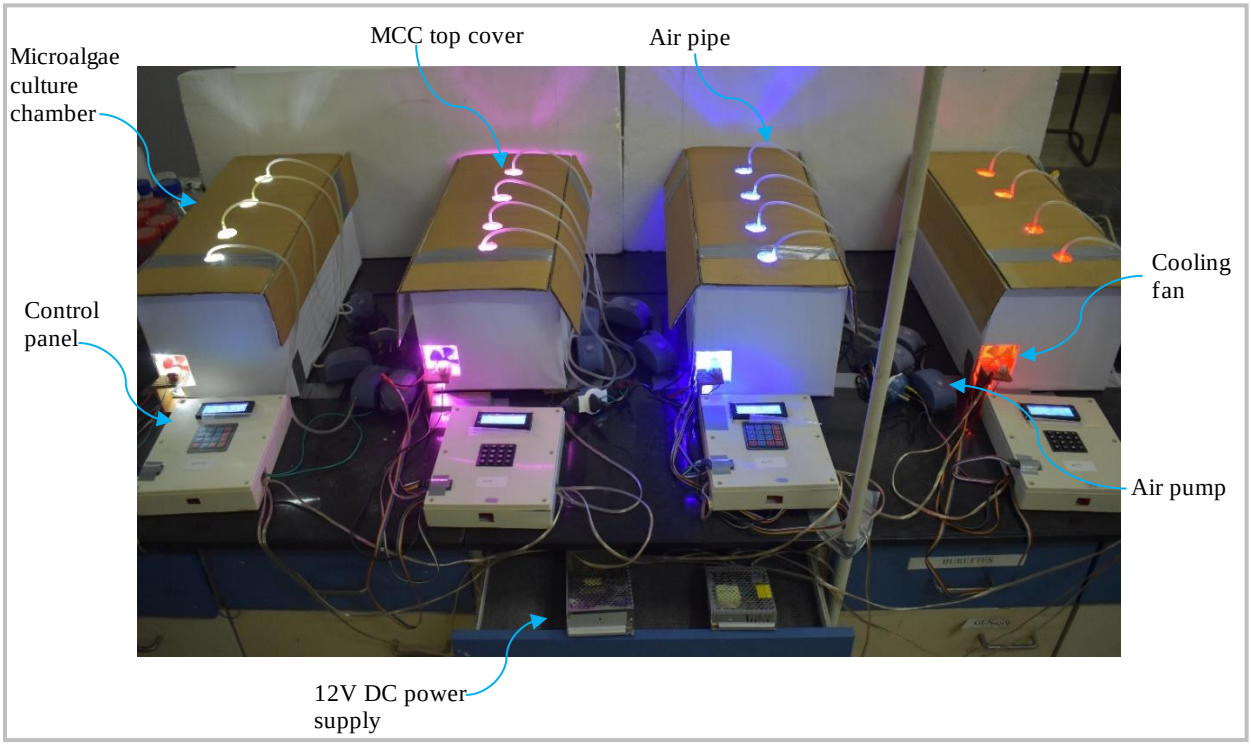


Figure 3B.1: Four experimental setups, fitted with cool-white, pink, blue and red LED illuminations.

3B.2.1. Design and development of the microalgae culture chamber (MCC)

The MCC, as shown in Figure 3B.2 (c), is made using cardboard, as shown in Figure 3B.2 (a), to keep costs low and construction easy. The dimension of the box is 0.5 m in length, 0.25 m in width, and 0.25 m in height. These dimensions are chosen to accommodate four 500 mL Erlenmeyer flasks (culture vessels) inside it. As shown in Figure 3B.2 (b), the cardboard box is lined with aluminum foil for uniform light distribution. Light uniformity inside the box is verified by measuring the light intensity at different locations, equidistant from the light source, using a Lux meter (METRAVI Lux/Fc model 1332, Taiwan). Six LED strips, each 0.5 m in length, are fixed inside the chamber, as seen in the image of the MCC in Figure 3B.2 (c).

(a) **(b)** **(c)**

Figure 3B.2 (a): Cardboard sheet cut into shape and dimension to build microalgae culture chamber (MCC) **(b):** Cardboard cut for MCC lined with aluminum foil **(c):** The MCC fitted with LED strips, sensors and accommodating four 500 mL capacity Erlenmeyer flasks.

Commercially available LED strips (Crimson LED Strips, Goldmedal Electricals, India) were used. Every meter of the strip consisted of 60 LEDs, totaling 180 LEDs inside the chamber. The number of LEDs was chosen based on future electrical power and microalgal growth experiments. This is the minimum quantity of LEDs required to provide a maximum of 10 W of electrical power.

The top cover of the MCC, as shown in Figure 3B.1, is made using cardboard sheets. Four holes are made on the top cover for the air pipes from the air pumps to be put inside the MCC. The air pipes are then let into the microalgae culture inside the Erlenmeyer flasks.

The MCC is also equipped with a light intensity sensor (BH1750) to measure the light intensity inside the MCC, a temperature sensor (DS18B20) to measure the temperature inside the MCC, and a fan (12V DC) to regulate the temperature inside the MCC by blowing in ambient air if the internal temperature of the MCC raises above the desired temperature due to the heat emitted by the LEDs. The electronic control system described in the following section controls the entire operation of the electronic components, such as the LED's light intensity and duration of illumination, turning ON and OFF the air pumps, measuring and storing the sensors readings, temperature control, etc.

3B.2.2. Design and development of the electronic control system

The entire operation of the developed microalgae culture experimental setup was controlled using an Arduino microcontroller [7] based control unit. The control unit had the primary function of controlling the light intensity, light duration, air flow duration, and temperature inside the MCC as per the preset experimental parameters and monitoring parameters like temperature and light

intensity using the sensors attached to it and saving the sensor readings to the memory device connected to the system. The control circuitry and the program were designed based on the algorithm shown in Figure 3B.3.

As per the algorithm, once the circuit is powered ON, the microcontroller checks the experimental status in its non-volatile memory, i.e., its EEPROM. Suppose the experiment's status in the EEPROM indicates that there is no experiment at the current time. In that case, the microcontroller waits and keeps rechecking the EEPROM status till the status indicates that the experiment is ON. Once the status of the EEPROM indicates that the experiment is ON, the microcontroller checks the experimental parameters like light intensity, light duration, and airflow duration and turns ON the LEDs and air pumps accordingly. Next, the microcontroller checks the temperature inside the MCC. If the temperature inside the MCC is higher than the set value, the microcontroller turns ON the cooling fan. The cooling fan is turned OFF if the temperature is within the set value range. Following the temperature control routine, the microcontroller checks if it is time to measure the sensor readings. Suppose the current time satisfies the condition for recording the sensor readings. In that case, the microcontroller collects the temperature and light intensity sensors' readings and saves those readings to the memory device incorporated in the system. Next, the microcontroller checks the conditions to terminate the experiment. If the conditions satisfy the termination of the experiment, the microcontroller terminates the experiment by turning off the LEDs and air pump and setting the EEPROM status to NO experiment.

The control algorithm described above was implemented by developing an electronic circuit using an Arduino Nano microcontroller [8]. The pin configurations of Arduino Nano are given in Annexure I. The control system is fitted with temperature sensors (DB18B20) [9] to monitor the temperature inside the MCC, light intensity sensor (BH1750) [10] to monitor the light intensity inside the MCC, microSD card module [11] to record the sensor readings, real-time clock (RTC) module (DS3231) [12] to keep track of the current time, MoSFET switch [13] to control the LED light intensity and cooling fan, a relay switch [14] to control the air pumps, two 5V voltage regulator (LM7805) to power the microcontroller and the auxiliary components, an LCD [15] to display the experimental parameters and a buzzer to sound alerts for system malfunction events. The developed electronic control system is shown in Figure 3B.4. The circuit diagram and the C++ code of the system are given in Annexure IV.

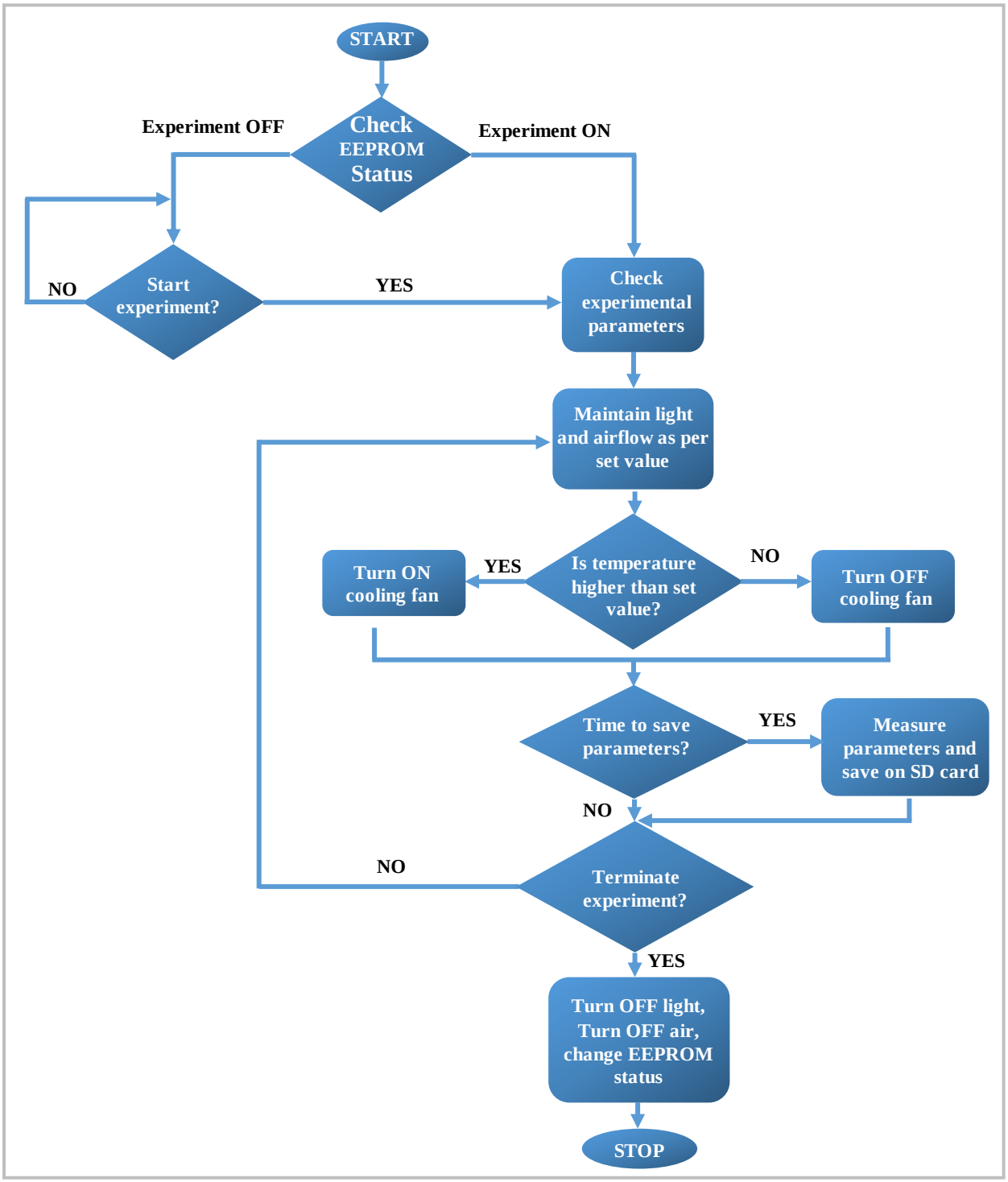


Figure 3B.3: Flow chart of the developed control algorithm.

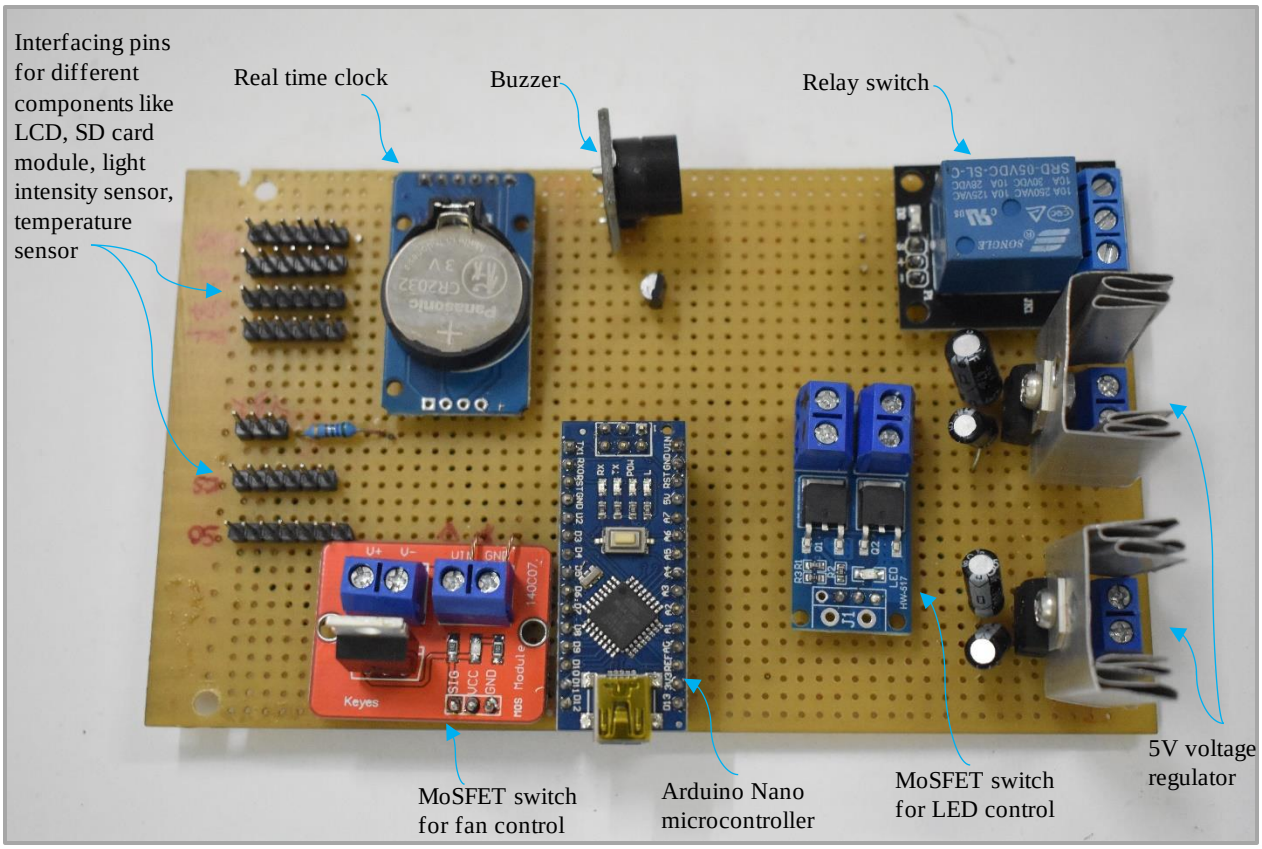


Figure 3B.4: Arduino Nano based control circuit developed for the microalgae experimentation setup

3B.2.3 Selection of LEDs for optimization

Six types of commercially available LED strips were procured for the LED-based optimization and analysis of the effect of different wavelengths on the microalgae culture. The six LED strips (Crimson LED strip light, Goldmedal, India) were cool-white, warm-white, blue, red, pink, and green colored LED strips. The spectral range of the LEDs was measured using a ThorLab Compact CCD Spectrometer (Model CCS200/M). Based on the spectrum, four LEDs were chosen to be fitted to the four culture setups developed. The light intensities of the LEDs were measured using Apogee MQ-510 full spectrum quantum sensor at various power levels for further correlation.

3B.3. Experimentation methodology of the RSM-based experiments

The experiments for the RSM-based experimentation were done as per the methodology illustrated in Figure 3B.5. Initially, a large volume, around 6-10 L, of BG 11 [16] media was prepared, as described in the previous chapter. However, in this case, the BG11 media was prepared without adding any nitrogen source. The prepared BG 11 medium was then divided into smaller portions,

and a nitrogen source was added per the experimental requirement. Microalgae inoculum from stock microalgae culture was centrifuged, and the pallet was washed with distilled water. The process was repeated 2-3 times to remove residual nutrients from the microalgae. The washed microalgae were mixed with distilled water to obtain a clean, dense microalgae inoculum.

The required volume of the cleaned microalgae inoculum was then added to the prepared BG 11 medium containing nitrogen source to obtain the starting culture having an initial OD_{560} of approximately 0.1.

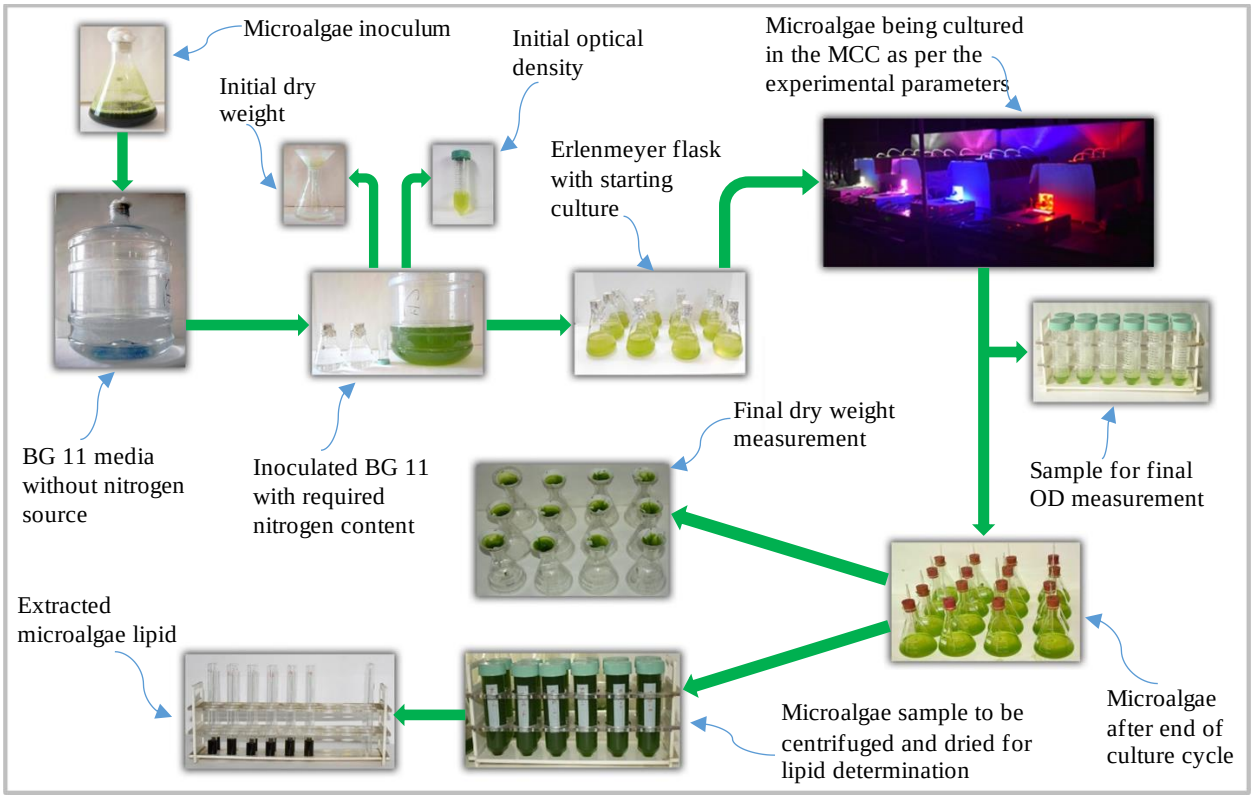
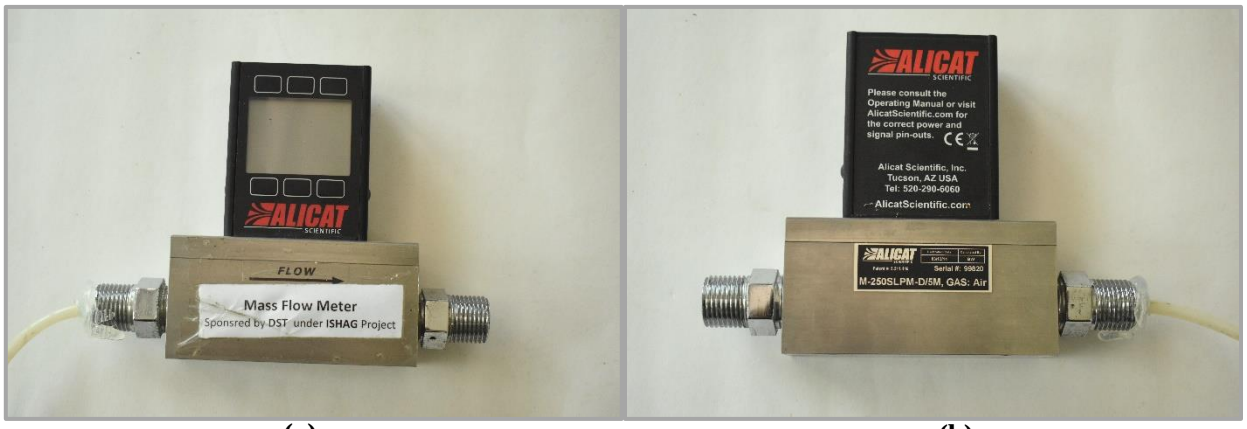


Figure 3B.5: Flow diagram of RSM based microalgae culture experimentation

The initial OD_{560} and the initial dry weight of the starting culture were measured and recorded. An equal volume, 300 mL, of inoculated microalgae cultures was then transferred to 500 mL volume Erlenmeyer flasks. Glass tubes specially designed to connect air pipes were put into the flasks, with the bottom end of the glass tubes being submerged in the microalgae culture. The mouth of the flasks was then sealed with cotton, fixating the glass tubes firmly inside the Erlenmeyer flasks.

The flasks were then transferred into the respective microalgae culture chambers and inoculated for the specified duration, i.e., 120 hours, per the required experimental parameters like light intensity, light duration, and air flow rate. The developed control system controlled the parameters like the light intensity and light duration. The airflow rates were controlled manually using generic air flow valves connected to the airline, and the airflow was measured using a mass flow meter (3.2 SLPM, Alicat Scientific, USA), as shown in Figure 3B.6.



(a) **(b)**

Figure 3B.6 (a): Front side of the Alicat Scientific mass flow meter used for air flow analysis **(b):** Rear side of the mass flow meter used.

After completion of the culture cycle, the final OD_{560}, final dry weight, and lipid content of the culture are evaluated and recorded for further analysis using RSM.

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CHAPTER 3C

To analyze the algal biomass and biofuel properties produced from the microalgae cultured in the developed PBR.

3C. To analyze the algal biomass and biofuel properties produced from the microalgae cultured in the developed PBR.

Microalgae biomass cultured in the developed photobioreactor system was harvested for further analysis. Two harvesting methodologies, centrifugation, and chemical flocculation, were primarily employed. Harvested microalgae biomass was dried in a hot air oven for preservation and further analysis. The calorific value and ash content of the dried microalgae biomass were evaluated. After drying, microalgae lipid was extracted by solvent extraction, i.e., the Bligh-Dyer extraction method [1]. The composition of the extracted lipid was evaluated using GC-MS. The extracted lipid was further processed into biodiesel using transesterification methodology. The biodiesel thus formed was verified by ^{1}H-NMR analysis, and then further physiochemical properties like acid value, density, calorific value, and carbon residue of the biodiesel were evaluated. Detailed methodologies are presented in the following sections.

3C.1. Harvesting microalgae biomass

Different harvesting procedures like sedimentation [2], flotation [3], flocculation [4], filtration [5], centrifugation [6], etc., can be applied for harvesting microalgae biomass from the culture media. The choice of harvesting technique depends on various parameters like the size of microalgae cells to be harvested, purity of biomass required, the sensitivity of microalgae biomass to the harvesting technique, the volume of culture to be harvested, the economics of the operation, etc. In the current experimentation, two harvesting methods were employed depending on the volume of microalgae culture needed to be harvested: (i) Centrifugation & (ii) Chemical flocculation.

3C.1.1 Microalgae harvesting using centrifugation

Centrifugation was employed for microalgae harvesting when a small volume of microalgae culture was needed to be harvested for analytical purposes. A benchtop centrifuge (MF8R, Thermo Scientific) available in the Biomass Conversion Laboratory, Department of Energy, was used to harvest microalgae biomass by centrifugation. The centrifugation was done by taking 50 mL of microalgae culture in 50 mL volume centrifuge tubes. Four such tubes can be placed in the centrifuge at a time. Thus, 200 mL of culture can be harvested at a time. The centrifugation was done at a speed of 8000-10000 rpm and for 5-10 minutes. After the completion of the centrifuge

cycle, the supernatant is discarded, and the microalgae pallet formed at the bottom of the centrifuge tube is taken out on a petri dish and dried in the hot air oven.

3C.1.2 Microalgae harvesting using chemical flocculation

Chemical flocculation was employed when a large volume of microalgae culture was needed to be harvested. Different flocculating chemicals like sodium hydroxide (NaOH), alum ($KAl(SO_4)_2$), ferric chloride ($FeCl_3$), chitosan, Mimusops elengi extract, etc., were explored initially. Finally, ferric chloride was chosen as the preferred flocculating chemical for its better flocculating characteristics in comparison to the rest, which were found during initial experimentations.

The chemical flocculation harvesting was done in a makeshift 100 L capacity flocculating arrangement, as shown in Figure 3C.1 (a-c). The arrangement consisted of a 100 L water tank, a stirring mechanism with a speed-adjustable motor and stirrer setup, a support system with adjustable height, and a CO_2 cylinder with manual control valves.

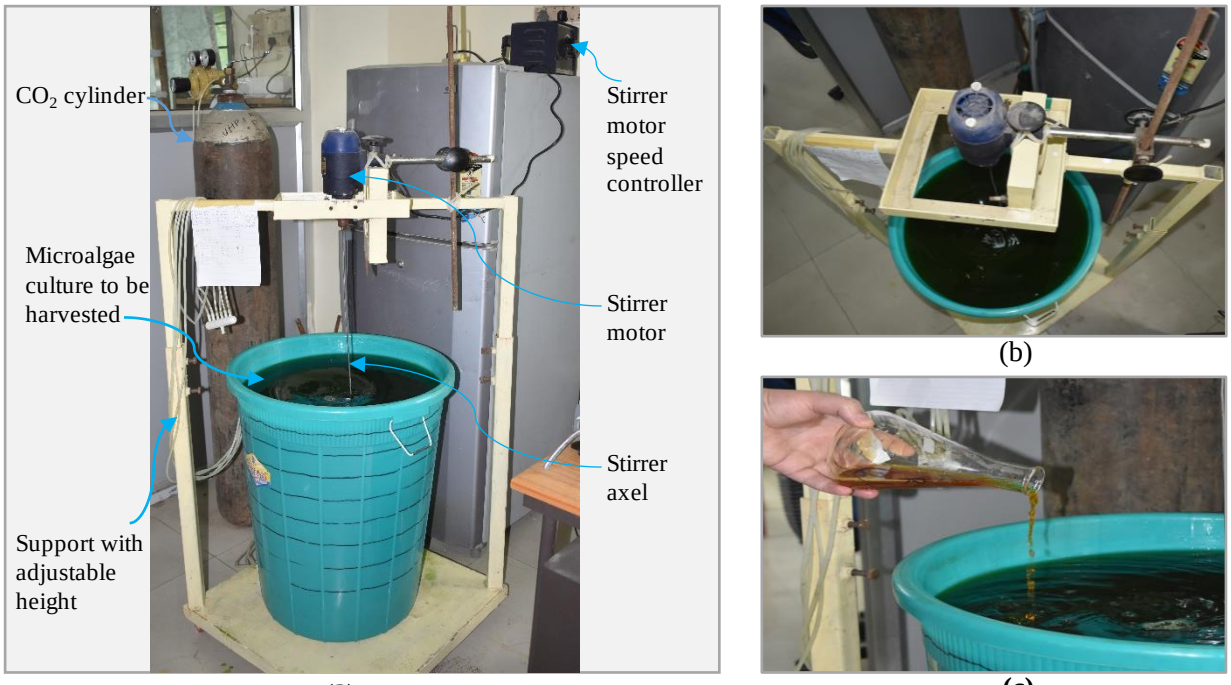

Figure 3C.1 (a): Microalgae harvesting setup of 100 L harvesting capacity. **(b):** Top view of the harvesting100 L microalgae culture setup. **(c):** Adding ferric chloride solution for microalgae harvesting.

During the initial experimentations, as well as from published literature [7, 8], it was found that ferric chloride flocculation worked best at a slightly acidic condition (pH less than 6). Thus, for

harvesting the 100 L culture, the pH was brought to a range of 5.5-6 by sparging CO_2 from the CO_2 cylinder and monitoring the pH with a pH meter at regular intervals. Once the pH was in the range of 5.5-6, the stirrer was turned ON, and the speed was adjusted to achieve approximately 200-300 rpm. Once the culture achieved a steady rotational speed, the required volume of ferric chloride solution was added. The ferric chloride solution was prepared by mixing 10g of ferric chloride with 1 L of distilled water to achieve a 10 mg/mL concentration. A concentration of 50 mg/L of ferric chloride per liter of microalgae culture was used to harvest the microalgae. Thus, for 100L microalgae culture, 500 mL of the ferric chloride solution was poured into the container.

After adding the ferric chloride solution, the stirring was continued for 5-10 minutes for the solution to be mixed homogeneously. Then, the stirring was stopped, and the stirrer was removed from the culture. The culture was then left to rest undisturbed. After 2-3 hours, the microalgae settle to the bottom of the tank. The supernatant was discarded, and the bottom layer of approximately 2-3 L containing dense algal suspension was processed by centrifugation harvesting, as described in the previous section.

3C.2. Drying harvested microalgae biomass

After harvesting the microalgae biomass per the procedures described in section 3C.1, it was dried to remove the remaining moisture from the microalgae biomass. The drying was done by keeping the centrifuged microalgae biomass on Petri-dishes or other glass containers having large open surfaces suitable for accommodating the microalgae biomass for drying and placing them in the hot air oven at 60°C. The microalgae biomass was initially kept in the drier for 24 hours. After 24 hours, the weight of the biomass was monitored periodically, at 30-minute intervals, till a constant weight was achieved in a consecutive weighting regime. Once the constant weight was achieved, the microalgae biomass was removed from the hot air oven and kept in air-tight containers for further analysis. The final weight of the dried microalgae biomass and the volume of culture from which it was harvested were recorded for further study.

3C.3. Calorific value of microalgae biomass

The calorific value of the microalgae biomass was evaluated to determine the potential of the microalgae biomass as a direct combustible fuel source. A dried microalgae biomass sample was crushed in ceramic mortar pastel to obtain a fine powder. The microalgae powder obtained was

palletized to make it suitable for analysis in the bomb calorimeter (SE-1AC/ML, CKIC, China) used to evaluate the calorific value. The weighted microalgae pallet was oxidized in an adiabatic bomb pressurized to 3 Mpa with oxygen from an oxygen cylinder. Once the bomb and the water jacket achieved temperature equilibrium, the equipment automatically fired the algal biomass to evaluate the calorific value of the biomass.

3C.4. Ash content of microalgae biomass

Ash content of microalgae biomass was evaluated by ASTM D 3174 protocol. As per the protocol, approximately 1 g of dry microalgae biomass, pulverized and sieved to pass through the No. 60 (250 mm) sieve, was taken in a pre-weight crucible. The biomass sample was heated in an electronic muffle furnace at 900°C for two hours. After two hours, the sample was left to cool down, and the weight was recorded to determine the ash content of the sample using the formula Eq. 3C.1.

$$M_{ash} = (W_{CBI} - W_{CBF}) - W_C \qquad \text{Eq. 3C.1.}$$

where, M_{ash} is the mass of ash, W_{CBI} is the initial mass of the crucible with microalgae biomass, W_{CBF} is the final mass of the crucible after the muffle furnace, and W_C is the mass of the empty crucible.

3C.5. Lipid Extraction

Microalgae lipid was extracted by the Blight and Drier [1] method. In short, dry microalgae biomass was pulverized for lipid extraction using an electronic mixer grinder setup (Philips 1659, 350 mL). The pulverized microalgae biomass was then transferred to a cellulose thimble, and the mouth of the thimble was sealed with cotton. The thimble was then transferred to the extraction tube of the Soxhlet apparatus. The extraction was carried out using a mixture of chloroform & methanol solvents in the ratio of 1:2 taken in the round bottom flask of the Soxhlet apparatus. The volume of biomass to solvent ratio was kept at 1:3. The solvent in the round bottom flask was heated to approximately 65°C to evaporate the solvent, allowing it to rise to the extraction tube through the bypass side arm of the extraction tube and come in contact with the condenser present at the top of the extraction tube. The solvent vapor cools down in the condenser, changes its phase back to liquid, and falls back at the cellulose thimble containing the microalgae biomass. The

solvent extracts the lipid from the biomass and fills the extraction tube gradually. Once the solvent containing the dissolved lipid reaches the level of the reflux side arm of the extraction tube, the solvent and the lipid mixture siphons to the round bottom flask present at the bottom. The solvent evaporates back to the extraction tube, keeping the lipid, which has a higher boiling point, behind at the round bottom flask, and the process keeps on repeating. The extraction was carried out for about 24 hours or until the solvent appeared colorless in the extraction tube of the Soxhlet apparatus. After the completion of the extraction, the round bottom flask containing the chloroform, methanol, and lipid mixture was taken, and 0.73% (w/w) of saline water and chloroform was added to it to achieve 0.9: 1: 1 ratio of saline-water: methanol: chloroform in the final mixture. The mixture was shaken vigorously, transferred into a separating funnel, and left undisturbed until three distinct forms were formed. The bottom layer containing the chloroform lipid mixture was separated, and the chloroform was evaporated to obtain the microalgae lipid.

3C.6. Acid value and free fatty acid of microalgae lipid

The acid value of the microalgae lipid was estimated using the ASTM D974 methodology [9]. In short, a 100 ml homogenate mixure of water, isopropanol, and toluene mixture (1:99:100) were mixed with 2 grams of microlage lipid and 0.5 mL of the p-naphtholbenzein indicator, and tritrated against 0.01M KOH solution. The titration was carried out till the a solution containing the indicator change the color from orange to green. After the titration the acid value (mg KOG/g) and the free fatty acid content (%) were estimated using the formulas Eq. 3C.2 and 3C.3 respectively [10, 11].

$$\text{Acid value (mg KOH/g)} = \frac{V \times M \times 56.1}{W} \qquad \text{Eq. 3C.2}$$

Where, V is the volume of KOH solution required for the titration, M is the molarity of the KOH solution used and W is the mass od the same in grams.

$$\text{Free fatty acid content (\%)} = \frac{\text{Acid value (mg KOH/g)}}{1.99} \qquad \text{Eq. 3C.3}$$

3C.7. GC-MS analysis of microalgae lipid

The lipid profile analysis of the obtained lipid was done using a GC-MS [12, 13] (PerkinElmer, Model: Clarus680 GC/600C MS) present at Guwahati Biotech Park, Guwahati, Assam. A 60 m × 250 µm column was used to analyze helium as the carrier gas with a 1.0 mL/min flow rate. The oven temperature profile was kept as an initial temperature of 60°C for 1 min, ramp 7°C/min to 200°C, hold for 3 min, ramp 10°C/min to 300°C, hold for 5 min. The sample was auto-injected with a split of 10:1.

3C.8. Transesterification for biodiesel production

The extracted microalgae lipid was subjected to a transesterification reaction for biodiesel production. The microalgae lipid was transesterified by taking 10 g of microalgae lipid in a 100 ml volume round bottom flask. The microalgae lipid was then heated to 100°C to remove any moisture. The microalgae lipid was then let to cool down to 60°C, and methanol was added to the microalgae lipid at 8:1 (methanol: lipid) molar ratio. The mixture was maintained at 60±0.5°C and stirred continuously. Sulfuric acid was added to the mix as an acid catalyst for the transesterification reaction. Sulfuric acid was added in 2.5 wt.% of the mixture. The mixture was maintained at 60±0.5°C with continuous string for another 2.5 hours. After 2.5 hours, the stirring was stopped, and the mixture was transferred to a separating funnel and let to rest until two distinct layers formed. The upper layer is collected and washed with hot (70°C) distilled water and then analyzed for biodiesel formation.

3C.9. ^{1}H-NMR analysis of microalgae lipid and microalgae biodiesel

^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy is a powerful tool to characterize the chemical structure of lipids and biodiesel. In the current investigation, the biodiesel formed by the transesterification reaction described in the previous section was characterized using ^{1}H NMR (Jeol, ECS-400, JAPAN). For ^{1}H NMR analysis, deuterated methanol was used as a solvent. Biodiesel formation is confirmed by the ^{1}H NMR from the chemical shift observed at ~ 3.6 ppm and ~2.3 ppm due to methoxy and α-methylene protons present in biodiesel, respectively [14, 15]. Based on the ^{1}H NMR spectroscopy the FAME conversion was calculated using the formula Eq. 3C.4 [16].

$$\text{FAME conversion percentage (\%)} = \frac{2\times X}{3\times Y} \times 100\% \qquad \text{Eq. 3C.4}$$

Where X and Y are the integral value of methoxy proton ($-OCH_3$) peak at ~3.6 ppm and α-methylene proton ($-CH_2-$) peaks at ~2.3 ppm, respectively.

3C.10. Acid value of microalgae biodiesel

The acid value of the obtained biodiesel was determined by the titration method described by ASTM D974 [9]. In short, a 100 mL mixture of toluene, isopropanol, and water (100:99:1) was used as a solvent to dissolve 2 g of biodiesel in an Erlenmeyer flask, and 0.5 mL of p-naphthol-benzene was mixed into it as the color indicator. The homogeneous mixture thus obtained was titrated against 0.01 M KOH solution. Titration was carried out till the color of the solution changed from orange to green. The volume of the KOH solution was recorded. The acid value was determined by the formula Eq. 3C.5.

$$\text{Acid value (mg KOH/g)} = \frac{V\times M\times 56.1}{W} \qquad \text{Eq.3C.5}$$

where, V is the volume of KOH required for the titration, M is the molarity of the KOH, and W is the sample volume in g.

3C.11. Density of the microalgae biodiesel

The biodiesel density was determined using the API (American Petroleum Institute) hydrometer following the ASTM D287-92 procedure [17]. The biodiesel sample is taken in a clear cylindrical vessel and let settle until it is clear of any air bubbles. The temperature of the biodiesel was adjusted to 15°C using a water bath. The API hydrometer, which consists of a floating body, is put into the biodiesel sample and kept in a vertical position so that it doesn't touch the walls of the cylinder. Once the hydrometer stabilized, the reading on the hydrometer scale was noted. Following that, the temperature of the sample being measured was recorded. The density was noted from the hydrometer scale, and the temperature was noted for any variation from the standard 15°C that will require correction in the density value as per the standard chart available with the hydrometer.

3C. 12. Calorific value (CV) of the microalgae biodiesel

The biodiesel's calorific value was estimated using a bomb calorimeter (SE-1AC/ML, CKIC, China). A weight sample of microalgae biodiesel was taken in an adiabatic bomb pressurized to 3 Mpa using oxygen gas. The machine oxidized the biodiesel sample once the temperature equilibrium was achieved between the bomb and the surrounding water jacket. From the rise in water temperature, the calorimeter determined the CV of the sample, i.e., the biodiesel in this case.

3C.13. Carbon residue of the microalgae biodiesel

The carbon residue test determines the carbonaceous compounds left after the combustion of the biodiesel under specific conditions. This test indicates the tendency of the fuel to deposit in the engine under high-temperature conditions. The ASTM D 189-01 method was utilized for the carbon residue analysis. A clean silica crucible was taken and its weight was recorded for the test. Then, a weight volume of the biodiesel was taken in the crucible and placed in the muffle furnace. Thermal decomposition of the sample was achieved by gradually heating the sample to 500°C and maintaining that temperature for another 30 minutes. Then, the sample was taken out and let cool down in a desiccator. Once the sample cooled down, the weight of the crucible containing the carbon residue was noted. The carbon residue was estimated using the formula Eq. 3C.6.

$$\text{Carbon residue (\%)} = \frac{\text{Wc-Wi}}{\text{Ws}} \times 100\ \% \qquad \text{Eq.3C.6}$$

where, W_c is the weight of the crucible with carbon residue, W_i is the weight of the empty crucible recorded initially, and W_s is the weight of the biodiesel sample.

3C.14. Statistical Analysis

The experiments were carried out in triplicate, and the results are expressed in mean ± standard error. One-way ANOVA using SPSS software version 16.0 (SPSS Inc, Chicago, IL, USA) with Duncan's post hoc to evaluate differences between groups of discrete variables. $p < 0.05$ was considered a statistically significant difference.

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