

3.1 MATERIALS

Table 3.1: List of All the Chemicals and Reagents used in the Complete Study

Chemicals and reagents	Make
Algae genomic DNA purification kit	Himedia
Antibodies	Sigma-Aldrich
Basic salt chemicals	HiMedia/ Fischer Scientific/ Merck/ Sigma-Aldrich
COD estimation reagents	Merck
DIG High Prime DNA labeling and detection starter kit I	Roche
High range standard DNA ladder	Thermo Fisher Scientific
Restriction enzymes	New England Biolabs
HPLC-grade chemical	Sigma-Aldrich
Nylon membrane	Roche
Organic solvent	HiMedia/ Sigma-Aldrich/ SRL
PCR reagents	HiMedia/ Thermo Fisher Scientific/ G-Biosciences
Plasmid extraction and purification kits	Thermo Fisher Scientific
Primers for PCR	Sigma-Aldrich
Protein marker	G-Biosciences
RNA extraction reagent	Thermo Fisher Scientific

3.2 MICROALGAE CULTIVATION AND MEASUREMENTS

3.2.1 Cultivation Medium and Operational Conditions

The green microalgae *Chlamydomonas reinhardtii* CC-503 (wall-less strain) was purchased from the Chlamydomonas Resource Center, University of Minnesota, USA, and *C. reinhardtii* WT CC-125 (cell-walled strain) was obtained from the Metabolic Engineering Group, ICGEB New Delhi [Ahmad *et al.*, 2015]. These microalgae strains were cultivated in a 250 ml Erlenmeyer flask at 25 ± 2 °C containing TAP medium at pH 7. The TAP media consisted of 1 M Tris base 20 ml/l, Phosphate Buffer II 1 ml/l (10.8 g K_2HPO_4 and 5.6 g KH_2PO_4 in 100 ml stock), Solution A 10 ml/l (20 g NH_4Cl , 5 g $MgSO_4 \cdot 7H_2O$ and 2.5 g $CaCl_2 \cdot 2H_2O$ in 500 ml stock), Glacial Acetic Acid 1 ml/l, and Hutner's trace elements 1ml/l as specified by Chlamydomonas Resource Center (<https://www.chlamycollection.org>).

For GM microalgae cultivation and maintenance, hygromycin B was used as a selection agent and supplemented as per dose (10 µg/ml) in TAP media on solid plates or in a liquid medium with shaking at 150 rpm. Steam autoclaving was applied to all microorganisms, including GM microalgae, for decontamination. The culture was subjected to intermittent agitation and an atmosphere of ambient air containing CO_2 . All the chemicals used in this study were of analytical grade.

3.2.2 Microalgae Biomass Harvesting and Drying

The microalgae biomass was harvested after 15 days of operation and was freeze-dried using a Lyophilizer (Lyodel freeze dryer, Delvac) and processed for further analysis, including lipid extraction.

3.2.3 Lipid Extraction and Gravimetric analysis

Lipid extraction involves dispersing the freeze-dried microalgae biomass into the chloroform-methanol (1:2) as per the modified Bligh and Dyer method [Vyas and Chhabra, 2019]. The dry microalgae cell pellet was briefly treated with 10 ml of 2 M HCl (for approximately 300 mg cell pellet) at 90 °C for 1 h in a water bath. The acid-hydrolyzed cells were then disrupted by sonication (20 kHz, 10 min) followed by stirring in 20 ml of 2:1 chloroform and methanol for 3 h. The mixture was centrifuged at 8000 rpm for 15 min to separate the aqueous and organic phases. The lower organic phase was aspirated, filtered, and dried to obtain the extracted lipids in a pre-weighed tube. The total lipid content (%DCW) was determined gravimetrically.

3.2.4 Microalgae Growth and Biomass Productivity

The microalgae cell counting was performed using a Neubauer hemocytometer under a light microscope (Leica). A linear regression equation between cell number vs Optical Density (OD) was derived and used to measure the microalgae growth. The OD₇₅₀ is an absorbance of 750 nm, which is a widely used spectrophotometric method to monitor microalgae cell growth [Chioccioli *et al.*, 2014]. The specific growth rate (μ) was calculated during the exponential phase using the stated equation:

$$\mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1} \quad (3.1)$$

Where, N_1 and N_2 are the number of cells at time t_1 and time t_2 (d), respectively.

The microalgae cells' doubling time and divisions per day were calculated using the method reported previously [Zohir *et al.*, 2022]. For measuring biomass productivity, a 2 ml sample was collected from the microalgae cultivation flask/PMFC cathode chamber at specific intervals in a pre-weighed microcentrifuge tube and harvested using a centrifuge at 12,000 rpm for 5 min. The microalgae pellet was dried at 80 °C in a hot air oven (Rescholar Equipment, India). After reaching the constant weight, the dried biomass was measured, and calculated as specified previously [Khandelwal *et al.*, 2020].

3.2.5 Pigment Extraction and Quantification

Determination of pigment content was carried out using acetone/methanol extraction followed by spectrophotometric analysis. Briefly, the freeze-dried microalgae biomass was mixed with 80% acetone to extract the pigments. The pigment identification involved recording absorption spectra at 400 - 750 nm. Pigment concentrations (chlorophyll a, chlorophyll b, and carotenoids) were calculated using the given equations [Lichtenthaler and Buschmann, 2001]:

For methanol extraction

$$Chl\ a = 12.25 \times OD\ at\ 663.2nm - 2.79 \times OD\ at\ 646.8nm \quad (3.2)$$

$$Chl\ b = 21.5 \times OD\ at\ 646.8\ nm - 5.1 \times OD\ at\ 663.2\ nm \quad (3.3)$$

$$C\ (x + c) = \frac{1000 \times OD\ at\ 470\ nm - 1.82 \times Chl\ a - 85.02 \times Chl\ b}{198} \quad (3.4)$$

For acetone extraction

$$Chl\ a = 16.72 \times A_{665} - 9.16 \times A_{652} \quad (3.5)$$

$$Chl\ b = 34.09 \times A_{652} - 15.28 \times A_{665} \quad (3.6)$$

$$C\ (x + c) = \frac{1000 \times A_{470} - Chl\ a \times 1.63 - Chl\ b \times 104.96}{221} \quad (3.7)$$

Here chl a, chl b, and C (x+c) represent chlorophyll a, chlorophyll b, and total carotenoids respectively in µg/ml.

3.2.6 High-Performance Liquid Chromatography

HPLC was performed to separate and quantify the β-carotene and astaxanthin concentration by modifying the existing method [Tran and Kaldenhoff, 2020; Zohir *et al.*, 2022]. Briefly, microalgae biomass was lyophilized (Lyodel, Delvac), and sequentially extracted with methanol, acetone, and chloroform, mixed with deionized water. A lower layer containing pigments was pooled together and dried under dark conditions using a nitrogen gas stream. The dried pigments were reconstituted with HPLC-grade methanol and filtered with a 0.45 µm disposable PTFE syringe filter (Axivia). For each measurement, a 10 µl sample was injected into the column and run for 10 min. The quantification was carried out at 450 nm using Waters 2489 with a UV-visible detector HPLC system using a reverse-phase C18 column, and the mobile phase was pumped through a Waters 515 pump at a flow rate of 0.1 ml/min. The solvent system was prepared as described [Zohir *et al.*, 2022], and a flow gradient was set as described elsewhere [Tan *et al.*, 2007]. The data processing was done through Waters Empower Software. The β-carotene (C4582-5MG) and astaxanthin (SML0982-50MG) standards were procured from Sigma Aldrich, and standard solutions were prepared with a concentration range of 2-10 µg/ml and 0-15 µg/ml, respectively. A standard linear regression equation was obtained by following the above-mentioned protocol of HPLC.

3.2.7 Preparation of Algae Biomass Methanol Extract

Freeze-dried algae biomass (100 mg) was homogenized and extracted in 100 ml methanol. The extract was clarified by filtration using Whatman no.1 filter paper. The filtered extract, also called Algae Biomass Methanol Extract (ABME), was used for subsequent assays [Jayshree *et al.*, 2016].

3.2.8 Total Phenol Content and Total Antioxidant Activity of ABME

The total phenol content was measured using a standard method based on Folin-Ciocalteu's reagent (1:10 dilution). Gallic acid served as a standard phenolic compound for calibration curve preparation. The absorbance was read at 765 nm using a UV-vis Spectrophotometer (UV-1800, Shimadzu, Japan). The total phenols in microalgae biomass extract were expressed as mg/g gallic acid equivalent (GAE) of dry extract [Alhakmani *et al.*, 2013]. The antioxidant assay was done as described previously by using ascorbic acid as standard [Rao *et al.*, 2010].

3.2.9 Antimicrobial Activity of ABME using Agar Well Diffusion Assay

The assay was done using Gram-positive bacteria *Bacillus licheniformis*, and Gram-negative bacteria *Pseudomonas aeruginosa*, and *Escherichia coli*, respectively. Three broad-spectrum antibiotics, namely ampicillin, tetracycline, and chloramphenicol were acquired from HiMedia, and used as positive controls at 10 µg concentration. ABME was processed to remove methanol by drying at 45 °C in a rotary evaporator (Rotavapor® R-300, BUCHI) and suspending the dried extract powder in 100% Dimethyl Sulfoxide (DMSO) as described previously [El Shafay *et al.*, 2016]. The LB agar plates were prepared with wells in the center. The wells were supplemented with 100 µl of microalgae extract (1 mg/ml) and 100 µl of 100% DMSO as a negative control. The antibiotics were used as positive controls. The bacterial cultures were plated at 0.5 McFarland, followed by incubation of plates at 37 °C for 24 h (Rivotek, India). The diameter of the inhibition zone was measured against the positive controls.

3.3 MFC REACTOR FABRICATION AND OPERATION

3.3.1 MFC Configuration

MFCs were made up of acrylic material with an anodic and cathodic chamber of 100 ml working volume **while the total volume of each chamber was 120 ml** [Khandelwal *et al.*, 2018]. Acrylic MFC chambers were fabricated at Mayur Plastic Industries Jodhpur. A gas exchange tube connected the two chambers from the top. The transparent acrylic material was selected for MFC fabrication to allow maximum light penetration for microalgae cultivation. The anode and cathode were graphite felt (Dimensions: 4 cm × 4 cm × 0.56 cm). The electrodes were connected through a copper wire (gauge 2 mm). Electrodes and Nafion membranes were procured from Nickunj Eximp Pvt. Ltd, Mumbai, India. All the fabrication accessories were procured from local vendors at Jodhpur. Nafion-117 (DuPont, USA) clamped between the two chambers and served as a proton exchange membrane. Electrodes were pretreated by acid-alkali treatment as described previously to remove contaminants followed by washing with DI water [Huang *et al.*, 2015]. The Nafion membrane was activated by subjecting it to boiling in H₂O₂ and mild H₂SO₄, followed by multiple rinses with boiled DI water, as described previously [Ghasemi *et al.*, 2013]. The spacing between the anode and cathode was kept at 2.5 cm to minimize the internal resistance. A fixed resistor of 1000 Ω was used to complete the circuit using a copper wire, which enabled the current flow. This MFC setup (Figures 3.1 and 3.2) was used in the experiments described in Chapters 4 and 6.

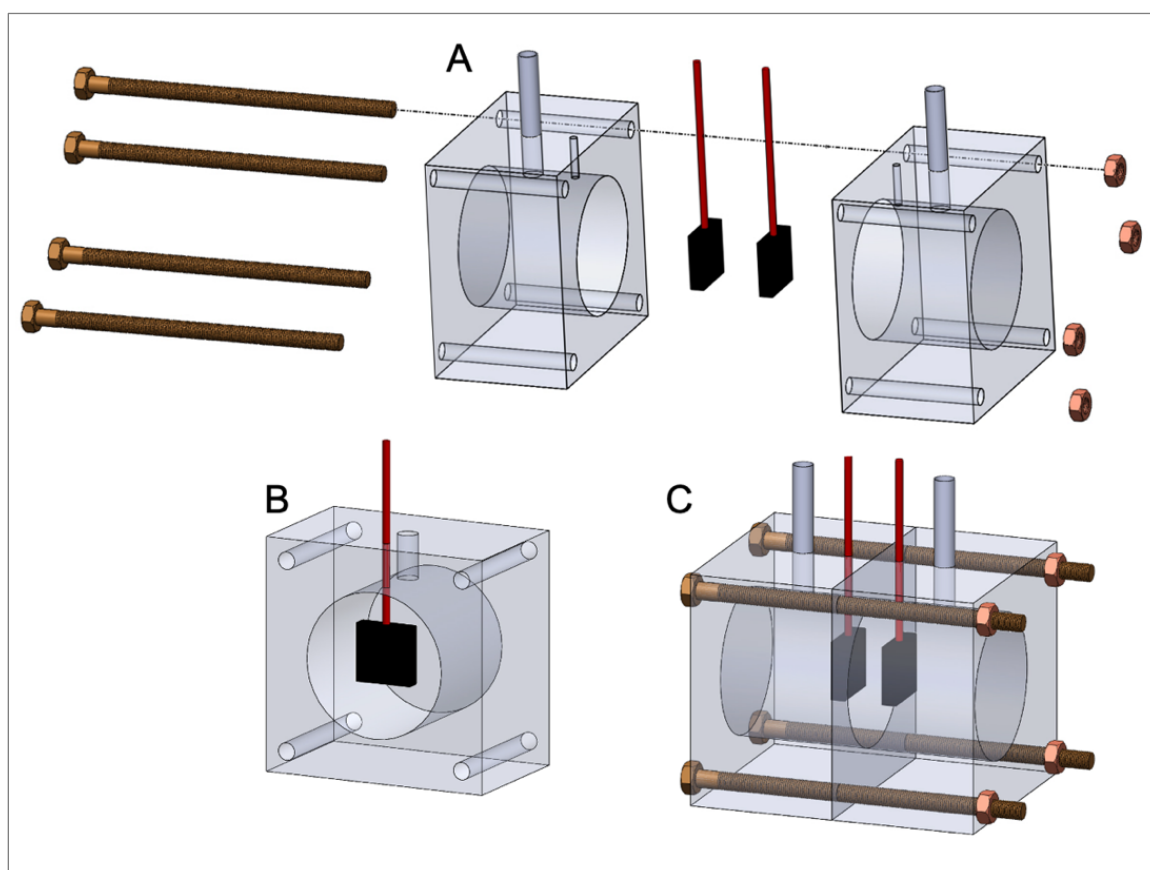


Figure 3.1 : The detailed configuration of the MFC reactor used in this study. A) disassembled view, B) half-cell view, and C) assembled view

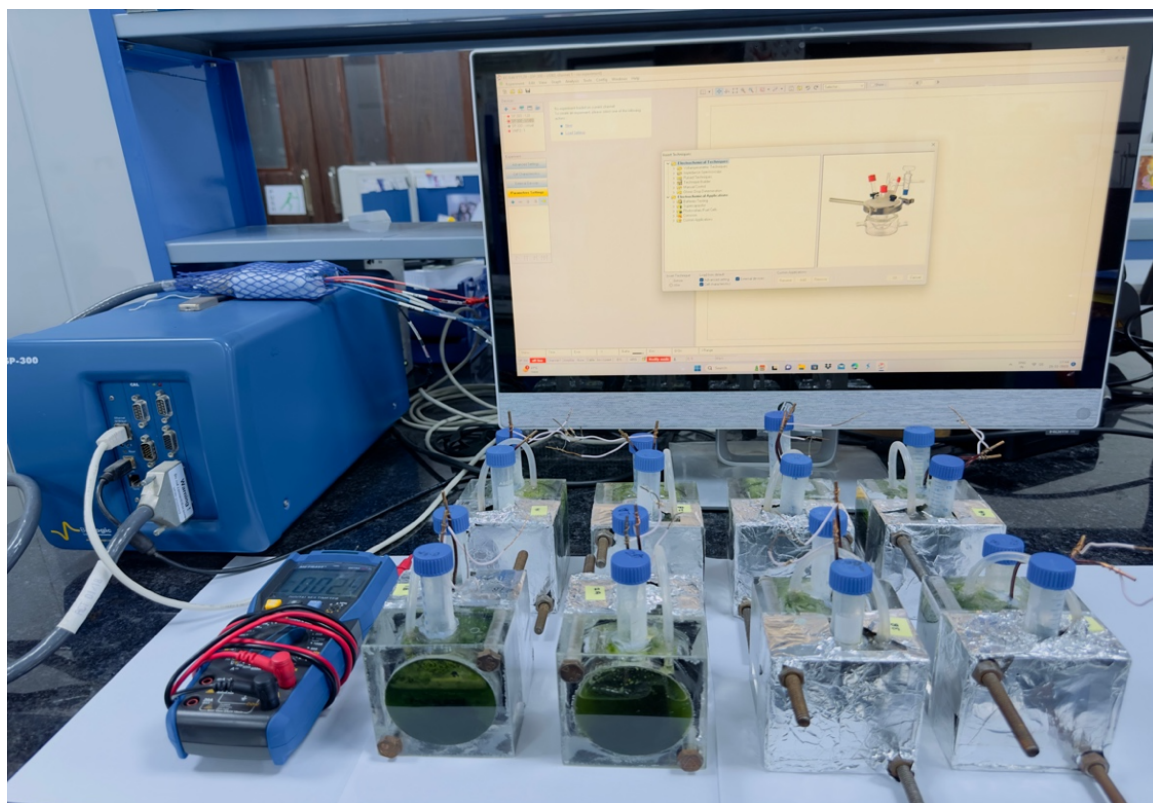


Figure 3.2 : The MFC reactors with potentiostat, and multimeter used in this study

3.3.2 Anolyte and Catholyte Preparation

The anolyte (synthetic wastewater) consisted of sodium acetate, KH_2PO_4 , K_2HPO_4 , NaCl , MgSO_4 , CaCl_2 , and KNO_3 , and trace metal solution at a concentration 2 g/l, 4.4 g/l, 3.4 g/l, 0.5 g/l, 0.2 g/l, 0.014 g/l, 1 g/l and 1 ml/l, respectively [Vijay *et al.*, 2019]. The synthetic wastewater was used in experiments described in the Chapter 4. Oxygen-free nitrogen gas was purged into an anode chamber to make it anaerobic. The pre-treatment of cow manure was performed using a slurry of 20% wet weight of cow manure in 50 mM KH_2PO_4 (pH 4.5) and incubating it for 6 h under aerobic conditions to suppress methanogens. Subsequently, the slurry was diluted with 50 mM K_2HPO_4 to achieve a final pH of 7, followed by 30 min flushing with oxygen-free nitrogen to ensure anaerobic conditions. Although aerobic sparging inhibits rather than completely killing methanogens, it has been observed that restoring anaerobic conditions results in a reduced methane emission rate due to a long lag phase in the recovery of methanogens. Other important bacteria involved in hydrolysis, acidogenesis, and acetogenesis can withstand short-term air exposure [Kiener and Leisinger, 1983; Fetzer *et al.*, 1993]. Further, the anode chamber was inoculated with the pre-treated cow manure [Vijay *et al.*, 2016]. The MFC experiments were conducted in a fed-batch mode. One batch cycle sustained power production for 7-8 days. The medium was replenished following a voltage drop.

TAP media served as the catholyte in all the reactors used in this work. The cathode chamber was inoculated with *C. reinhardtii* from an exponential stage culture having an initial concentration of $18.00 \pm 2.49 \times 10^4$ cells/ml. All the experiments were done in triplicates, with results reported as an average of three values [Khandelwal *et al.*, 2018]. To limit the microalgae growth in the wastewater-fed anode chamber, the anode chamber was covered with aluminum foil to avoid any light entry points as MFCs were kept in an algal incubator with the above-specified operational conditions.

3.3.3 Wastewater Collection and Characterization

To provide the organic substrates at the anode chamber, grey water was collected from the inlet of a Decentralized Wastewater Treatment System (DEWATS) situated at the IIT Jodhpur campus [Sharma *et al.*, 2021]. Wastewater collection was done on the day of the experiment in the morning hours. The grey water was filtered using a muslin cloth mesh with a pore size of 2 mm, to remove large floating particles. It was used in the experiments described in Chapter 6.

3.4 ANALYTICAL TECHNIQUES

3.4.1 Chemical Oxygen Demand Estimation

The soluble Chemical Oxygen Demand (COD) at anode chambers was estimated using a photometric method (1.18572, COD cell test, Merck) [APHA, 1926] It is based on oxidizing the organic matter in the sample with acidified dichromate ($K_2Cr_2O_7$) solution in the presence of a silver catalyst (Ag_2SO_4). For this, 1.5 ml of solution A (contains $K_2Cr_2O_7$, $HgSO_4$, and H_2SO_4) was mixed with 3.5 ml of solution B (Ag_2SO_4 and H_2SO_4) in screw cap glass ampule followed by the addition of 2.5 ml of sample. This mixture of solution was vortexed vigorously and heated at 148 °C for 120 min in a digester. The ampules were cooled down, and the absorbance of the solution was read at 600 nm using a UV-vis spectrophotometer (Carry-4000 Varian).

3.4.2 pH Measurement

The pH was monitored using a multiparameter (PC-700, Eutech instruments), and adjustments were made accordingly.

3.5 ELECTROCHEMICAL ANALYSIS

3.5.1 Polarization Curve and Power Measurement

To assess the maximum current density and power density achieved in the MFC systems, a polarization curve was prepared by varying the external resistance from 40000 Ω to 40 Ω using a decade resistance box (Lutron, RBOX-408) and measuring the voltage with a digital multimeter (METRA safe-10). The smooth switching functions of the resistance box helped in changing the external resistance with ease [Logan, 2012]. All MFC systems were kept in an open circuit mode, for 2 h before starting the experiment when the system was at infinite resistance. The OCV is the maximum voltage MFC can achieve.

In between switching to the next resistance, systems were held for 5 - 10 min to regain the OCV. The current and power densities were calculated and normalized with the effective volume of the anode chamber as given below [Logan *et al.*, 2006]:

$$\text{Current density} = \frac{V}{R \times \text{Volume of anode chamber}} \quad (3.8)$$

$$\text{Power density} = \frac{V \times I}{\text{Volume of anode chamber}} \quad (3.9)$$

Where V is voltage in volt (V), R is external resistance applied in Ohms (Ω), and I is current in ampere (A).

The area under the power v/s time curve of the 5th batch cycle was used to calculate the electrical energy yield of the system [Khandelwal *et al.*, 2020].

3.5.2 Coulombic Efficiency

Coulombic Efficiency (CE), was calculated using the given equation [Logan, 2012]:

$$CE = \frac{M \int_0^t I dt}{FnV_{An}\Delta COD} \quad (3.10)$$

Where, where $M = 32$ (molecular weight of oxygen), F is Faraday's constant, $n = 4$ (number of electrons exchanged per mole of oxygen), V_{An} is the volume of the anode chamber, and ΔCOD is the difference in COD over a period.

3.5.3 Cyclic Voltammetry

Electrochemical performances were studied using SP-300 potentiostat (Biologic). SP 300 has a 2-channel modular electrochemical workstation for fast, sensitive, and stable analysis with a compliance of $\pm 12\text{V}$ and an accuracy of $<1\text{ mV} \pm 0.03\%$ of reading (<https://www.biologic.net/products/sp-300/>). To assess the effect of the cathodic community on the redox activity of the MFC, CV was performed using a three-electrode system. For the CV measurement at the anode, the anode served as the working electrode, and the cathode was used as the counter electrode. For CV measurement at the cathode, the cathode was used as the working electrode, and the anode was connected as the counter electrode. In both configurations, an Ag/AgCl electrode (012167, RE-1B, ALS Co., Ltd., Japan) served as a reference electrode. All the systems were kept in an open circuit for 2 h before performing the voltammetric experiments [Zhao *et al.*, 2009].

3.6 SEM Analysis

The anode and cathode electrodes were removed from the MFC systems after the end of the study. For Scanning Electron Microscopy (SEM) analysis electrode was completely dried overnight in the oven. The electrode was kept at the stub using the carbon tape and it was fixed on the stub. The sample was charged with gold particle sputter coating. The anodic and cathodic biofilms were studied by imaging under the scanning electron microscope (SEM, SEM-EVO 18, Zeiss) [Vijay *et al.*, 2019].

3.7 MOLECULAR BIOLOGY TECHNIQUES

3.7.1 Vector Construction

The expression vector for microalgae (pUC57_ *bkt_crt_hyg*, 8325 bp) was designed *in silico* (Figure 3.3) and synthesized by Genescript, USA. The vector has an *aph7* gene driven by the β -tubulin promoter to provide resistance to hygromycin selection (pChlamy_1 vector). The *Hpbkt* (Accession no. AY603347.1) and *Hpcrt* (Accession no. KP866868.1) gene sequences from *H. pluvialis* were obtained from NCBI. These genes were codon-optimized and integrated into the nuclear transformation vector. The codon-optimized genes, namely *Hpbkt* and *Hpcrt* are driven by constitutive 35Sde (Accession no. JQ693016.1) and chimeric Hsp70A/RbcS2 (Accession no. LC150884.1) promoters, respectively. Other essential elements were incorporated into the vector, including 5' and 3' UTRs from *Hpbkt* gene (Accession no. AY603347.1), and Nos-Poly A (Accession no. KX683862.1). The genes were tagged with 10X HIS tag at the C-terminus of *Hpbkt* gene and a FLAG tag at the C-terminus of the *Hpcrt* gene. The synthesized vector was used in the experiments described in Chapter 5.

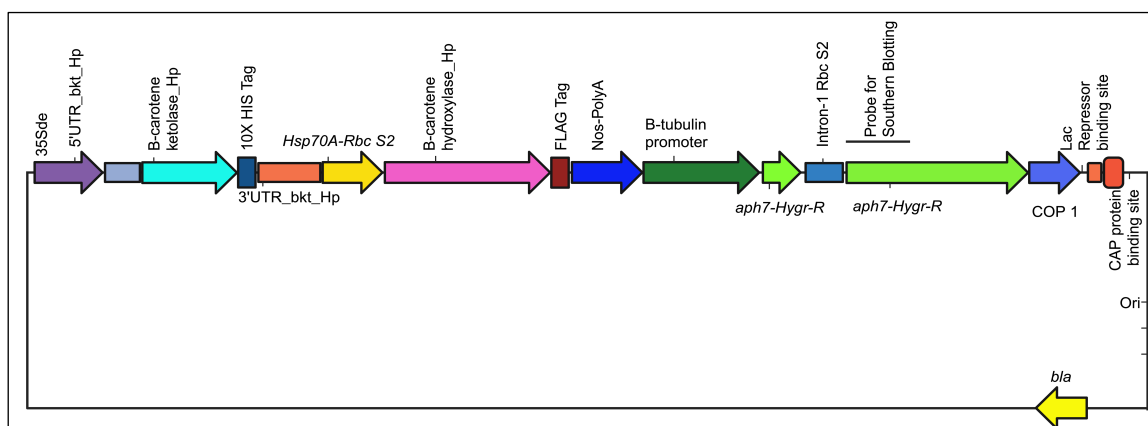


Figure 3.3 : The schematic showing the vector map for the plasmid (pUC57_ *bkt_crt_hyg*)

3.7.2 Plasmid DNA Extraction from Recombinant *E. coli* Cells

The synthesized plasmid was cloned into competent *E. coli* Top10 cells via the heat-shock method, facilitating the maintenance of the plasmid stock for subsequent applications. The transformed *E. coli* Top10 cells were propagated in Luria-Bertani (LB) media supplemented with 100 µg/ml ampicillin and 50 µg/ml streptomycin. Cultivation was carried out at 37 °C in an incubator shaker operating at 150 rpm to enable plasmid extraction. The GeneJET Plasmid Midiprep Kit (K0482, Thermo Fisher Scientific) was employed for the isolation and purification of plasmid (Vector) DNA from bacterial cells, following the manufacturer's instructions. The resultant purified plasmid DNA was subsequently utilized in the experiments detailed in subsequent sections.

3.7.3 Genetic Transformation using Electroporation

The plasmid (pUC57_ *bkt_crt_hyg*, 8325 bp) was linearized with the enzyme EcoRI (located in the vector backbone), as nuclear transformation is more efficient using linear plasmids [Kindle, 1990]. For electroporation, the logarithmic phase microalgae cells were harvested at 0.6 OD₇₅₀ (1×10⁶–2×10⁶ cells/ml) and washed gently with sterile MilliQ water to remove medium salts. The washed cells were resuspended in 10 ml of GeneArt® MAX Efficiency® Transformation Reagent (Invitrogen), and centrifuged at 2500 rpm for 5 min. The supernatant was discarded carefully, and the process was repeated once more. Finally, the pellet was resuspended into the transformation reagent to achieve a final concentration of 2×10⁸–3×10⁸ cells/ml. Linearized plasmid (2 µg per 250 µl cells) was added in cell suspension and incubated at 4 °C for 5 min. Electroporation was performed using Gene Pulser® II (BioRad) as per the manufacturer's instructions. In a pre-chilled electroporation cuvette (4 mm, BioRad), 250 µl of the cell-DNA mix was added and electroporated as per the instrument operation manual. The electroporation parameters were set as 800 V, 50 µF capacitance, and 800 Ω, which resulted in a time constant of around 50 ms. Cells were electroporated without DNA for the negative control and processed similarly, as discussed above.

3.7.4 Microalgal Cells Recovery and Selection after Transformation

After electroporation, cells were allowed to recover on ice for 15 min. Subsequently, the cells were transferred to 10 ml TAP + 40 mM sucrose media at room temperature. The cells were placed in the algal chamber and incubated overnight. The next day, the cells were collected using centrifugation, resuspended in 200 µl TAP medium, and then plated on hygromycin B (antibiotic) containing selective TAP agar plates. These plates were incubated in the algal chamber for 7-15 days. The hygromycin-resistant colonies were picked up and analyzed further [Nawarkar *et al.*, 2023].

3.7.5 DNA Extraction from Microalgal Cells

Colonies picked from selection plates were transferred into liquid TAP media (10 ml) and scaled up to 50 ml for DNA extraction. The Cetyl Trimethyl Ammonium Bromide (CTAB) method was used to lyse cells and isolate DNA [Doyle and Doyle, 1987]. The composition of the CTAB extraction buffer used was 2% w/v CTAB, 100 mM Tris- HCl (pH 8), 1.4 M NaCl, and 20 mM Ethylenediaminetetraacetic Acid (EDTA), with freshly added β-mercaptoethanol (BME). The DNA isolation was performed using phenol-chloroform-isoamyl alcohol extraction (25:24:1) as described previously [Cordero *et al.*, 2011]. The DNA pellet was resuspended in Tris EDTA (TE buffer, pH 8) (Thermo Fisher Scientific), and quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific).

3.7.6 Polymerase Chain Reaction for Gene Amplification

The Polymerase Chain Reaction (PCR) mixture includes PCR buffer, dNTP mix, MgCl₂, Taq DNA Polymerase, template DNA, forward and reverse primers, nuclease-free water, and Bovine Serum Albumin (BSA). Initial positive transformants were confirmed by PCR using the *aph7* primers (Hyg_F: TGTCTCAAGCAGCATCTAACC and Hyg_R: TCTCCTCGAACACCTCGAA). The PCR reaction cycles were as follows: 95 °C for 5 min; 35 cycles of 95 °C for 45 s; 58 °C for 45 s; 72 °C for 90 s, and a final extension at 72 °C for 10 min. PCR

products were analyzed on 1% agarose gel and identified using 1 Kb DNA marker. The plasmid (pUC57_ *bkt crt hyg*) containing *aph7* gene was used as a positive control, the WT genomic DNA was used as a negative control, and a No Template Control (NTC) without any template DNA was also run with the samples. The hygromycin-positive transformants were scaled up to assess the pigment production and extract total genomic DNA.

3.7.7 Southern Hybridization for Copy Number Determination

The copy number of the integrated transgene was determined using Southern blot analysis. For this, the total genomic DNA was extracted from the transgenic and WT lines of *C. reinhardtii* using the CTAB method as aforementioned. For Southern blot analysis, the probe labeling and hybridization were carried out using DIG High Prime DNA labeling and detection starter kit I (Roche) following the manufacturer's instruction [Nawkarkar *et al.*, 2023]. The probe was prepared using a PCR amplicon of about 900 bp of *aph7* gene amplified from the plasmid pUC57_ *bkt crt hyg*. The PCR product was purified using the GeneJet PCR purification Kit (Thermo Fisher Scientific), and subsequently labeled. For random transgene integrations, the genomic DNA (20 µg for each sample) was digested using restriction enzymes EcoRI and BamHI (zero cutter enzymes, New England Biolabs) and separated by electrophoresis on 0.8% agarose gel for 5-6 h. The digested DNA was transferred to a positively charged nylon membrane (Roche) by capillary transfer and hybridized with a DIG-labeled *aph7* probe at 42 °C. The probe was detected using NBT/BCIP reagent after incubation with the anti-digoxigenin-antibody (11093274910, Roche) provided as per the manufacturer's instructions.

3.7.8 Western Blot Analysis to Study Protein Expression

The microalgae biomass was harvested on day 5 (at the late exponential phase) by centrifugation at 2000 rpm for 10 min to concentrate cultures to about 15 OD₇₅₀. The pellet was resuspended in protein extraction buffer, which consists of 800 mM Tris HCl (pH 8.0), 200 mM Sorbitol, 600 mM NaCl, 1% (V/V) Triton X 100, 1% (w/V) SDS, 4% (V/V) β-mercaptoethanol (freshly added), protease inhibitor, and 100 mg/ml Phenyl Methyl Sulfonyl Fluoride [Hurkman and Tanaka, 1986]. The mixture was subjected to sonication for 10 min (30 s, switched on/off cycle) and repeated thrice. Next, the supernatant was centrifuged at 12000 rpm for 1 h at 4 °C to separate soluble protein fraction. The protein concentration was determined with Bradford assay, using BSA as a standard protein.

To run the protein gel, the protein samples were denatured by adding an SDS sample buffer and boiled at 95 °C for 5 min. Subsequently, 30 µg proteins of each sample were separated on 15% SDS-PAGE gels, and for all the samples, duplicates were run. One protein gel was stained with Coomassie staining solution: Coomassie Brilliant Blue R-250 (0.12% w/v) in O-phosphoric acid (10% v/v), ammonium sulfate (10% w/v), and methanol (20% v/v) [Nawkarkar *et al.*, 2023]. Other gel was transferred to a nitrocellulose membrane (Hybond C-super, Amersham) using a semi-dry method (Trans-Blot SD, Bio-Rad, USA).

Purified monoclonal ANTI-FLAG® M2-peroxidase primary conjugate antibody (A8592-1MG, Sigma-Aldrich) raised in mice, was diluted to 1:1000 to detect the recombinant proteins carrying FLAG-tags at the C-terminus of *Hpcrt* gene. In addition, anti-polyHistidine peroxidase primary conjugate antibody (A7058-1VL, Sigma Aldrich) raised in mice, was diluted to 1:2000 to detect recombinant proteins carrying 10X-His-tags at the C-terminus of *Hpbkt* gene in the transformed microalgal cells. Both anti-His and anti-FLAG antibodies were conjugated with Horseradish Peroxidase to detect the signal. After blot transfer, the membrane was kept for blocking in 3% BSA in 1X PBS at room temperature for 1.5 h. The membrane was washed with 1X PBST (2 times) and 1X PBS (one time) for 5 min each to remove unbound proteins. The blot was then hybridized with diluted anti-His antibody for 2 h at room temperature. The protein spots were developed using chromogenic dye 3,3'-Diaminobenzidin (Sigma-Aldrich) and Hydrogen Peroxide (Qualigens). For FLAG-tagged protein, blocking was performed with 5% BSA in PBST, and washing cycles were increased to 5 to minimize the non-specific bindings. The

antibody hybridization was carried out for 2 h at room temperature. The blots and stained gel images were captured using an iPhone 14 pro camera (Apple, California, USA).

3.8 STATISTICAL ANALYSIS

All the experiments were carried out with three replicates, and the results are reported with a mean value $\pm$ standard deviation using MS Excel. The data was analyzed by ANOVA (GraphPad Prism 8.0.1 Software). Statistical significance was defined as p-value < 0.05 .