Bioresource Technology 398 (2024) 130513

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Outdoor cultivation and metabolomics exploration of *Chlamydomonas* engineered for bisabolene production

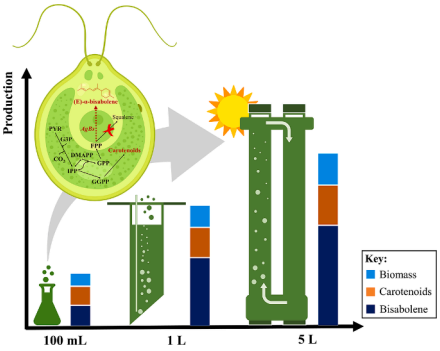
Kaustubh R. Sawant [a,1], Aditya P. Sarnaik [b,1], Rabinder Singh [c,d], Prashant Savvashe [a], Thomas Baier [e], Olaf Kruse [e], Pannaga Pavan Jutur [c], Arvind Lali [f], Reena A. Pandit [a,*]

[a] DBT-ICT Centre for Energy Biosciences, Institute of Chemical Technology, Matunga, Mumbai 400019, India
[b] School for Sustainable Engineering and the Built Environment, Arizona State University, The Polytechnic Campus, Mesa, AZ 85212, USA
[c] Omics of Algae Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India
[d] Laboratory of Cell Cycles of Algae, Centre Algatech, Institute of Microbiology of the Czech Academy of Sciences, 237, Trebon 379 01, Czech Republic
[e] Bielefeld University, Faculty of Biology, Center for Biotechnology (CeBiTec), Universitätsstrasse 27, 33615 Bielefeld, Germany
[f] Department of Chemical Engineering, Institute of Chemical Technology, Matunga, Mumbai 400019, India

HIGHLIGHTS

- Chlamydomonas as a one-cell two-wells (engineered and natural products) biorefinery.
- Autotrophic cultivation with added bicarbonate improved outdoor strain performance.
- Successful outdoor cultivation of engineered Chlamydomonas at incremental scales.
- Engineered strain produced 906 mg/L bisabolene and 54 mg/L carotenoids.
- Metabolomics and PAM fluorometry confirmed improved cellular metabolism.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Natural dynamic conditions
Algal bio-refinery
Growth engineering
Bicarbonate supplementation
Carotenoids

ABSTRACT

Demonstrating outdoor cultivation of engineered microalgae at considerable scales is essential for their prospective large-scale deployment. Hence, this study focuses on the outdoor cultivation of an engineered *Chlamydomonas reinhardtii* strain, *3XAgBs-SQs*, for bisabolene production under natural dynamic conditions of light and temperature. Our preliminary outdoor experiments showed improved growth, but frequent culture collapses in conventional Tris-acetate-phosphate medium. In contrast, modified high-salt medium (HSM) supported prolonged cell survival, outdoor. However, their subsequent outdoor scale-up from 250 mL to 5 L in HSM was effective with 10 g/L bicarbonate supplementation. Pulse amplitude modulation fluorometry and metabolomic analysis further validated their improved photosynthesis and uncompromised metabolic fluxes towards the biomass and the products (natural carotenoids and engineered bisabolene). These strains could produce 906 mg/

* Corresponding author.
E-mail addresses: kaustubhsawant17494@gmail.com (K.R. Sawant), asarnaik@asu.edu (A.P. Sarnaik), rabinderbiotech@gmail.com (R. Singh), prashantsavvashe@gmail.com (P. Savvashe), Thomas.Baier@uni-bielefeld.de (T. Baier), olaf.kruse@uni-bielefeld.de (O. Kruse), Pavan.Jutur@icgeb.org (P.P. Jutur), arvindmlali@gmail.com (A. Lali), drreenapandit@gmail.com (R.A. Pandit).
[1] Contributed equally as co-authors.

https://doi.org/10.1016/j.biortech.2024.130513
Received 19 December 2023; Received in revised form 27 February 2024; Accepted 29 February 2024
Available online 1 March 2024

L bisabolene and 54 mg/L carotenoids, demonstrating the first successful outdoor photoautotrophic cultivation of engineered *C. reinhardtii*, establishing it as a one-cell two-wells biorefinery.

1. Introduction

Algal systems are considered as sustainable platforms for bio-production because of their ability to convert CO_2 to chemicals (Arora et al., 2021). Over the past three decades, there have been several studies on algal engineering for the production of a variety of hydrocarbons and value-added compounds (Scranton et al., 2015). However, a very few investigations have reported their outdoor cultivation at incremental scales. Although the algal strains are usually isolated from natural water bodies, their mass production under natural dynamic conditions is challenging (Banerjee et al., 2016). The main reason is that these outdoor conditions are a combination of multiple factors, including dynamic light and temperature, which ultimately affect their growth, sustenance, and production (Davies et al., 2021; Schoepp et al., 2014). When light absorption exceeds the cellular photosynthetic capacity, which is a typical encounter under natural high light, terminal oxygen receives excess electrons, causing photo-oxidation and eventually leading to cell death (Virtanen et al., 2021). In addition, prolonged exposure to dynamic and high temperatures causes degradation of pigment proteins, thereby reducing photosynthetic efficiency (Sirikhachornkit and Napaumpaiporn, 2016). Other abiotic factors such as pH, photoperiod, and hydrodynamics can also affect the cell growth during outdoor cultivation. The cultivation system is another essential factor influencing algal growth. For instance, open pond cultivation has a higher possibility of contamination and evaporative water loss (Vishal et al., 2021). In contrast, a closed tubular photobioreactor (PBR) system can improve aerial productivity with fewer chances of contamination. Hence, to leverage these potential cell factories as industrial workhorses, it is paramount to perform multifactorial optimization and demonstrate their outdoor cultivation at higher scales.

Algae possess natural isoprenoid biosynthesis pathway for production of a number of isoprenoids or terpenoids. Terpenoids are a structurally diverse class of hydrocarbons that can serve as fuel precursors and are important constituents in the food, pharmaceutical, and cosmetic industries (Moser and Pichler, 2019; Scaife et al., 2015). With advancements in algal metabolic engineering, heterologous terpenoid pathways can be efficiently engineered in different algal systems (Pattanaik and Lindberg, 2015; Scranton et al., 2015; Vavitsas et al., 2018). Among these, the model photosynthetic microalga *Chlamydomonas reinhardtii* has been widely explored through metabolic engineering to produce different terpenoids such as bisabolene. Bisabolene has got several applications especially in flavour, fragrance, jet fuels and paint industries; with a projected market compound annual growth rate (CAGR) of 6 % and a market value of $12,000 per metric tonne, by 2027 (Zhao et al., 2021). Currently, bisabolene is commercially produced directly from plant tissues. However, the method has many disadvantages like limited raw material, low yields, etc (Zhao et al., 2021). With its growing market and from its sustainable production perspective, researchers have engineered various microbes to synthesize bisabolene. However, most of these studies are performed under controlled laboratory conditions (Lauersen et al., 2016; Wichmann et al., 2018, 2022). Current work has used one such engineered strain of *C. reinhardtii* by Wichmann *et al.*, that has been demonstrated to efficiently produce bisabolene (Wichmann et al., 2018). The study presents its outdoor cultivation at incremental scales and explores its bio-production potential, under outdoor dynamic conditions of light and temperature.

With this perspective and to address the challenge of converting sunlight into solar fuels and chemicals, under the mission innovation program, supported by the Govt. of India, collaborative efforts have been undertaken by DBT-ICT Centre for Energy Biosciences at Institute of Chemical Technology (ICT), Mumbai, IN; the International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, IN; with the German partner the Centre for Biotechnology (CeBiTec), Bielefeld University, DE. The consortium attempted to address this challenge by gathering insights into real-time outdoor cultivation of an engineered strain of *C. reinhardtii*, *3XAgBs-SQs* (Wichmann et al., 2021, 2018), demonstrated for its efficient bisabolene production. A quadruple mutant strain of *C. reinhardtii* UVM4U, constructed by CeBiTec, Bielefeld University, was engineered by sequentially incorporating codon-optimized triple copies of *Abies grandis* bisabolene synthase (*AgBs*), forming *3XAgBs*, followed by squalene synthase (SQs) knockdown using the amiRNA gene, ultimately forming a *3XAgBs-SQs* strain (Wichmann et al., 2018). Earlier laboratory studies conducted by CeBiTec showed bisabolene production by *3XAgBs-SQs* under mixotrophic conditions in Tris-acetate-phosphate (TAP) medium (Wichmann et al., 2018). The current investigation, has taken this investigation further, by transitioning these engineered strains from controlled laboratory (artificial light) to outdoor (sunlight) cultivation conditions at incremental volumetric scales, at ICT, IN. This study encompasses acclimatization of the strain to dynamic outdoor conditions, media optimization, and subsequent cultivation in closed photobioreactors constructed in-house by the DBT-ICT Centre for Energy Biosciences. Our results showed a 14-fold improvement in outdoor bisabolene titers compared to those from the incubator conditions. The metabolomics studies, conducted at ICGEB, further supported our findings. For complete value addition to this system, along with bisabolene (an engineered product), we also analysed the production of the natural carotenoids from the system. To our knowledge, this study is the first successful demonstration of outdoor cultivation of engineered *C. reinhardtii* as a one-cell two-wells (for natural and engineered products) biorefinery that sustains the conversion of CO_2 and sunlight into solar chemicals without compromising the biomass.

2. Materials & methods

2.1. Chemicals & reagents

Dodecane, glacial acetic acid, methanol, and antibiotics, such as paromomycin (*Paro*), bleomycin (*Ble*), hygromycin B (*Hyg*), and spectinomycin (*Spec*) were purchased from *HiMedia*. Salts for Tris-acetate-phosphate (TAP), Sueoka's high salt (HSM) medium, and sodium bicarbonate obtained from Sd fine chemicals were limited. Commercial bisabolene standard was purchased from *Alfa Aesar*.

2.2. Strain cultivation

C. reinhardtii strain, *3XAgBs-SQs* was earlier engineered and studied for bisabolene production by Wichmann et al. (Wichmann et al., 2018). The strain had a series of codon-optimized *AgBs* (*Abies grandis* bisabolene synthase gene, Uniport; 081086) engineered into the UVM4 strain for cytosolic bisabolene synthase overexpression, forming *3XAgBs*. The strain was then targeted for knockdown of SQs (*Squalene synthase*) using amiRNA, ultimately resulting in *3XAgBs-SQs*. These engineered strains were repeatedly passaged on TAP medium supplemented with 20 mg/L hygromycin in a 250 mL flask. The engineered strain was cultivated in incubator conditions (I_{55}) equipped with continuous illumination of 55 μmol photon m^{-2} s^{-1}, temperature maintained at 25 ± 2 °C and shaking at 100 rpm. Stock cultures were maintained on TAP agar supplemented with 20 mg/L hygromycin and then in a liquid medium. For seed culture, the acclimatized culture was passaged to 100 mL TAP medium in the 500 mL flask with an initial Abs_{750} of 0.4.

2.3. Experimental design: Growth condition and photobioreactor design

The *3XAgBS-SQs* were maintained, cultivated, acclimatized, and analysed under three different light, temperature, and mixing conditions, as follows:

Laboratory-artificial light conditions (A_{250}): A stacked tube light stand illuminated to produce a light intensity of 250 ± 5 μmol photon m^{-2} s^{-1}, the air-conditioned cabinet was maintained at 30 ± 2 °C. The *3XAgBs-SQs* were grown using a 2 L photo-bioreactor (PBR) polyethylene sleeve bag reactor with slant geometry at the bottom with spargers installed for culture suspension, Fig. 2A.

Environmental laboratory-natural light conditions (E_{1500}): A cutting-edge glasshouse facility supporting sunlight penetration and allowing experimentation under diurnal and seasonal variations of natural light (sunlight) with maximum light intensity reaching 1500 ± 300 μmol photon m^{-2} s^{-1}. The temperature was maintained at 25 ± 3 °C by using air conditioners and ventilators. The conditions were utilized for scale-up progression from 100 mL flask on the shaker at 120 rpm and 2 L PBR polyethylene sleeve bag reactor (1 L working volume) to 6 L PBR tubular glass reactor (5 L working volume). The 6 L tubular reactor constitutes a riser and downcomer, and the parallel vertical tubes are interconnected with the High-Density Polyethylene (HDPE) top and bottom connectors. The sparger aeration was set at the bottom, where the bottom connector was machined to the central part of the riser. The airflow was provided through an aquarium air pump and controlled with mechanical valves.

Outdoor-natural light cultivation condition (O_{1500}): An open-air rooftop facility, wherein cultures were cultivated under direct sunlight with diurnal and seasonal variations comprised of maximum light intensity 1500 ± 300 μmol photon m^{-2} s^{-1}; temperature ranges from 28 ± 2 °C to 38 ± 5 °C. The conditions were utilized for volumetric scale-up studies in a 6 L tubular glass PBR (5 L working volume) positioned with the help of a stand. The reactor was aerated using an aquarium air pump and was controlled with mechanical valves.

The diurnal light and temperature profiles of E_{1500} and O_{1500} over the culture period are provided (*see supplementary material*). An aeration rate of 0.05 vvm was maintained for all working conditions.

2.4. Growth and pigment estimation

The growth was evaluated daily by monitoring absorbance Abs_{750} using a UV–Vis spectrophotometer (Shimadzu, UV-2550), and pigment extraction was performed using protocol instructed by Wichmann *et al.* and measurements for chlorophyll *a* and total carotenoids were derived accordingly (Lichtenthaler et al., 2005).

2.5. Media optimization

3XAgBs-SQs strain collapsed at multiple occasions in TAP medium under natural light conditions in E_{1500}. As an alternative, the culture was cultivated in Sueoka's high salt medium (HSM) (Abd El Baky and El Baroty, 2020). Under the current investigation, the *3XAgBs-SQs* was grown in HSM wherein different concentrations (mM) of nitrate (N), phosphate (P), and sulphate (S) were evaluated for improved biomass production: 1, 2, 4, 8 mM of sodium nitrate ($NaNO_3$); 0.25, 0.5, 1, 2 mM of phosphate buffer (K_2HPO_4 and KH_2PO_4); and 0.1, 0.25, 0.5, 0.75 mM of sodium sulphate (Na_2SO_4) were used for media optimization. The optimizations were performed in HSM formulated with 20 mg $MgSO_4{\cdot}7H_2O$, 10 mg $CaCl_2{\cdot}2H_2O$, and 1 mL Hutner (Stock: 50 g/L $Na_2EDTA.2H_20$, 22 g/L $ZnSO_4{\cdot}7H_2O$, 11.4 g/L H_3BO_3, 5.1 g/L $MnCl_2{\cdot}4H_2O$, 5 g/L $FeSO_4{\cdot}7H_2O$, 1.6 g/L $CoCl_2{\cdot}6H_2O$, 1.16 g/L $CuSO_4{\cdot}5H_2O$, 1.1 g/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$) solution dissolved in 1 L ddH_2O supplemented with 20 mg/L hygromycin (*Hyg*) with pH 8.0. Later, the strain was maintained on optimized HSM plates in I_{55}.

The medium was supplemented with inorganic carbon to improve the culture tolerance under natural light conditions. At lower CO_2 concentrations, cells can assimilate low levels of dissolved inorganic carbon (DIC), which is attributed to the high apparent affinity for DIC (bicarbonate or CO_2) (Sültemeyer et al., 1991). Therefore, bicarbonate or CO_2 was added to the HSM medium.

Different concentrations of these two inorganic carbon substrates were screened for their effects on cell growth and production: $NaHCO_3$ (0, 60, 120, 240, and 480 mM) and 3 % CO_2 in HSM, overlaid with 5 % dodecane in E_{1500} in a 1 L PBR. The cultures were monitored and evaluated for sustenance, biomass growth, pigment concentration, and photosynthetic efficiency. In addition, bisabolene productivities were estimated for different $NaHCO_3$ concentrations and 3 % CO_2 in HSM. All experiments were performed in biological triplicate.

Preliminary studies were conducted with *3XAgBs-SQs* acclimatized in a 250 mL culture volume in a 500 mL flask, followed by sequential increments in scale under E_{1500}, from 250 mL to 1 L in a PE bag (Heena plastics, diameter: 147 mm, thickness: 2.5 mm), 5 L photobioreactor (glass tubes interconnected with HDPE bottom C and top H-connectors, ID: 70 mm, thickness: 3 mm) in the optimized HSM medium. Finally, acclimatized *3XAgBs-SQs* were transferred to outdoor conditions using a 5 L photobioreactor. *3XAgBs-SQs* were evaluated for cell growth, biomass, pigment production, and bisabolene production.

2.6. Analytical techniques

2.6.1. Gas chromatography-mass spectroscopy

The culture was cultivated in HSM and covered with 5 % dodecane. After six days, the cells were centrifuged at 10,000×g for 5 min, and the dodecane layer was separated. The dry weight of *the 3XAgBs-SQs* strain was estimated using the protocol described by Sarnaik et al. (2017). The dodecane fraction was subjected to gas chromatography-mass spectroscopy (GC-MS) analysis, as described by Lauersen et al. (2016). For bisabolene production, measurements were conducted using GC–MS (5975C with a triple quadrupole axis detector, inert XL El/Cl MSD, equipped with an HP5 column, Agilent Technologies). The temperatures were set for the injector (250 °C), interface (250 °C), and ion source (220 °C). The oven temperature was held at 80 °C for 1 min and then ramped to 120 °C at a rate of 10 °C/min for 2 min. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The analysis was performed in triplicates. The extracted ion chromatograms were measured and reviewed using mass ranges of 91, 93, and 119. Bisabolene production is expressed in terms of mg/L and/or yield (mg/g DCW).

2.6.2. Pulse amplitude modulation fluorometry (PAM) analysis

PSII and PSI activities were measured in *3XAgBs-SQs* using dual-wavelength pulse-amplitude-modulated (PAM) fluorometry (Dual-PAM-100, Heinz Walz GmbH, Effeltrich, Germany). A 2 mL culture sample normalized to a pigment concentration of 40 μg/mL was collected and incubated in the dark for 10 min. PAM analysis of *3XAgBs-SQs* cells was performed using an optimized protocol described in previous studies (Sawant et al., 2022, 2021). The minimal fluorescence (F_o) was calculated using a modulated measuring beam of 3 μmol photon m^{-2} s^{-1}. The actinic light pulse was calculated using photon flux densities (PPFDs) recorded during sampling. Maximal fluorescence in the dark-adapted (F_m) and light-adapted (F_m') states was recorded before and after the addition of the actinic pulse (1 s) of saturating white light. The quantum yield of the energy conversion in PSII, Y(II), was calculated as previously described by Wang et al. (Wang et al., 2015). where ETR(I) and ETR(II) are the descriptive parameters for the electron transport from PS(I) and PS(II), respectively. These parameters were derived using Dual-PAM100 software, reflecting the physiological effects of *3XAgBs-SQs*.

2.7. Qualitative (untargeted) metabolomics

A total of 1 × 10^8 cells (corresponding to an OD_{750} of 2.0) were centrifuged at 8000 × g for 10 min at 4 °C, quenched in liquid nitrogen,

and resuspended in 1 mL ice-cold methanol, ethanol, and chloroform (2:6:2) before being subjected to sonication for 15 min in a sonication bath. The samples were centrifuged at 10,000×g for 15 min at 4 °C. The supernatant was filtered through a 0.2 mm filter and evaporated in nitrogen. Ribitol (10 mg/mL) was added as an internal standard, and the dried extract was mixed with a newly made methoxyamine hydrochloride solution (4 % w/v in pyridine) and incubated at 30 °C for 90 min. N-Methyl-N-(trimethylsilyl) trifluoroacetamide was added to the solution for derivatization and incubated for 30 min at 37 °C. After centrifuging the samples for 3 min at 14,000 × g, the supernatants were tested for various metabolites by gas chromatography-mass spectrometry (GC–MS/MS). All sample run circumstances and instrument setups have been reported in depth in prior studies (Mariam et al., 2021; Singh et al., 2022). Peaks in the samples were detected and matched using the NIST library based on the retention duration and mass spectral similarity (only hits with R-values greater than 750 were included in the analysis). The final analysis was performed using MetaboAnalyst 4.0 (https://www.metaboanalyst.ca, retrieved on 15th December 2022).

2.8. Statistical analysis

All experiments were performed in triplicate, and the results were expressed as arithmetic mean values. Error bars represent the standard deviation (SD). Statistical differences were determined using single-factor analysis of variance (ANOVA) ($P < 0.05$). The statistical analysis was performed using Microsoft Excel.

3. Results & discussion

Chlamydomonas reinhardtii, a phototrophic cell factory, has been conventionally explored for the heterologous production of various chemicals. However, to leverage this for commercial applications, demonstrating its feasibility for outdoor cultivation is a prerequisite. Our previous experience recapitulated that the outdoor conditions for algae cultivation constitute numerous uncontrolled variables, including light, temperature, sparging rate, bubble size, and PBR design, at incremental scales, leading to a formidable challenge. In addition, oxidative stress, contamination, and photosynthetic responses to dynamic diurnal cycles; independently or collectively influence the cell sustenance (Schoepp et al., 2014). Hence, prior to outdoor scale-up cultivation, culture acclimatization to dynamic environmental conditions is imperative. Hence, this study aimed to implement this strategy for cultivating *3XAgBs-SQs*, an engineered strain of *C. reinhardtii* for bisabolene production, under dynamic outdoor conditions for demonstrating its effect on the growth and bisabolene production supported by its metabolomics.

3.1. Natural light acclimatisation of 3XAgBS-SQs

The engineered *Chlamydomonas* strain *3XAgBs-SQs* was grown in TAP medium under I_{55} conditions in an incubator. The strain had a biomass of 0.54 ± 0.03 g/L and the bisabolene titer of 6 mg/L (1 ± 0.2 mg/L.d productivity). Following this preliminary confirmation, the culture was grown on a 1 L scale in a photobioreactor (PBR) under A_{250} (250 μmol photon m^{-2} s^{-1} of artificial light). The results showed a slight improvement in the biomass (0.6 ± 0.03 g/L) with the bisabolene titer of 7.2 mg/L (1.2 ± 0.2 mg/L.d of productivity) than those under I_{55}. To further analyse the effect of outdoor natural dynamic conditions on the cell growth, the culture was acclimatized to natural light in E_{1500}, with three subsequent passages in TAP medium (250 mL). The results showed that the strain produced 1.5-fold higher biomass of 0.79 ± 0.04 g/L than I_{55}. However, *3XAgBs-SQs,* when grown in 1 L of TAP medium under E_{1500}, collapsed after 96 h. The collapse could be attributed to the presence of acetate in the TAP medium observed to increase cyclic electron flow in *C. reinhardtii*, which under higher light conditions would result in higher generation of reactive oxygen species and cause significant downregulation of CO_2 fixation by RuBisCO (Zamzam et al., 2022).

3.1.1. Media optimization under natural light conditions

To address the issue of culture collapse, the *3XAgBs-SQs* were grown in TAP medium supplemented with bicarbonate or with intermittent CO_2 purging. The supplements were prepared based on our earlier scale-up experiments using engineered cyanobacteria (Sawant et al., 2022, 2021). However, the cultures still collapsed on the 4th day. Hence, the culture was grown in a high-salt medium (HSM) as an alternative to TAP medium (Abd El Baky and El Baroty, 2020). The biomass in the HSM and TAP media were comparable (*see supplementary material*). However, better culture sustenance in HSM under natural light prompted further optimization of the medium for macronutrients (N ($NaNO_3$), P (K_2HPO_4 and KH_2PO_4), and S (Na_2SO_4)) for better growth, sustenance, and bisabolene production. Among different concentrations of N, P and S analysed, improved biomass production was observed with 4 mM N (0.75 ± 0.1 g/L of biomass), 0.5 mM S (0.77 ± 0.1 g/L of biomass) and 0.25 mM P (0.8 ± 0.1 g/L of biomass), Fig. 1A-1C. Therefore, HSM with optimized N, P, and S concentrations was used for further experiments. *3XAgBs-SQs* grown in optimised HSM supplemented with 3 % CO_2 displayed 1.8-fold improved biomass production (1.06 ± 0.5 g/L) and 1.2-fold improved bisabolene production (42 ± 5 mg/L), compared to the TAP medium (0.79 ± 0.3 g/L of biomass and 36 ± 4 mg/L of bisabolene). However, again we encountered the culture collapse after 96 h of cultivation.

Based on the earlier studies on natural scale-up cultivation of recombinant cyanobacteria (Sawant et al., 2022, 2021), the *3XAgBs-SQs* culture was supplemented with different bicarbonate concentrations. The results showed a significant increase in biomass production at E_{1500}. *3XAgBs-SQs,* when grown with 120 mM (10 g/L) $NaHCO_3$ supplementation in HSM, exhibited 3.2-fold improved biomass production (2.56 ± 0.1 g/L) compared to the TAP medium (0.8 ± 0.1 g/L), while 2.4-fold improvement compared to HSM medium supplemented with 3 % CO_2 (1.06 ± 0.5 g/L) in E_{1500}. At higher $NaHCO_3$ concentrations, that is 240 mM (2.2 ± 0.2 g/L) and 480 mM (0.8 ± 0.2 g/L), growth was negatively affected, Fig. 1D. The pH of the medium was consistent at 8 ± 1 throughout the cultivation, which emphasizes the buffering activity of bicarbonate (Shevela et al., 2020; Yamano et al., 2015). Unlike earlier encounters, the culture showed sustained growth even after 96 h of cultivation, with improved biomass, Fig. 1D. Hence, further studies were conducted using 120 mM (10 g/L) bicarbonate-supplemented HSM, referred to as optimized HSM, at incremental scales.

3.1.2. Natural light scale-up cultivation

3XAgBs-SQs cultures acclimatized in an optimized HSM were cultivated in a 5 L PBR under E_{1500} and analysed for culture sustenance, Fig. 2A. The strain exhibited 1.4-fold increase in biomass (3.6 ± 0.3 g/L), validating the successful transition of the engineered strain from 1 L to a 5 L PBR, Fig. 2B. Interestingly, medium optimization and culture acclimatization had positive effects on biomass generation and culture maintenance. Thus, the culture was transferred to a complete outdoor condition, O_{1500} with dynamic light and temperature.

The results showed a 1.7-fold improvement in biomass to 6 ± 0.3 g/L with culture sustenance for a month in O_{1500} compared to that in E_{1500}, Fig. 2B. These differences in production between E_{1500} and O_{1500} could be due to diurnal temperature variations synergistic with natural light. With the successful cultivation of *3XAgBs-SQs* in a modified HSM system under outdoor conditions, the strain was simultaneously assessed for its bio-production potential.

3.2. Quantification of production titers for bisabolene and carotenoid

Compounding to the successful acclimatization of *3XAgBs-SQs* in the optimised HSM medium under outdoor conditions, the strains were also evaluated for their ability to produce bisabolene and total carotenoids;

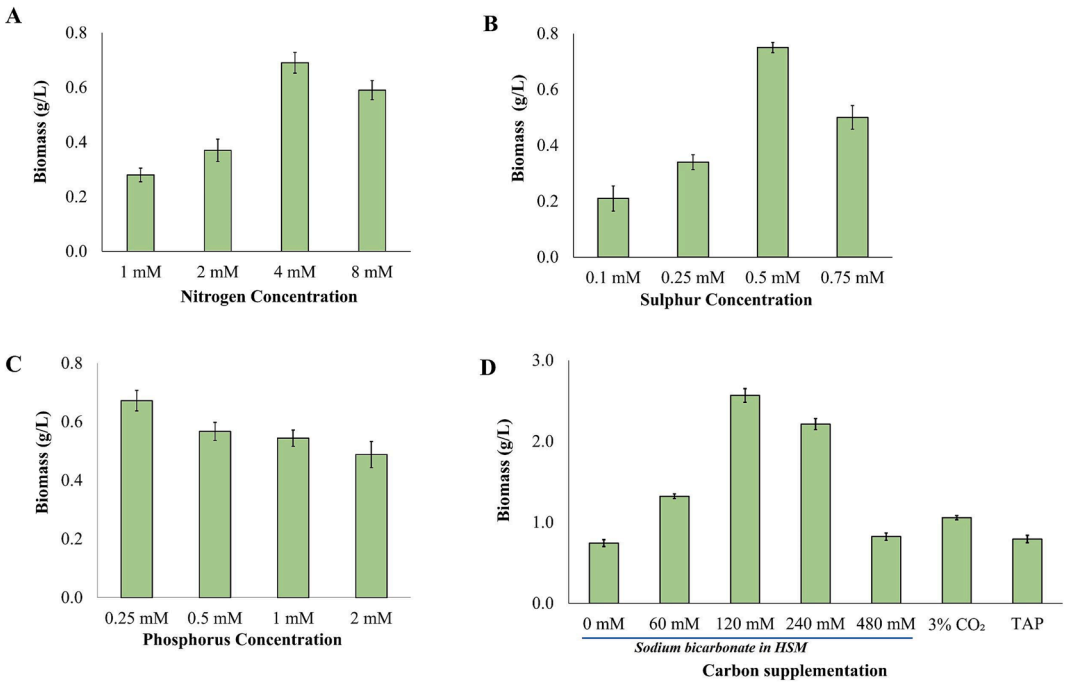


Fig. 1. Optimization of the media components under outdoor conditions. High Salt medium, used for cultivating *3XAgBs-SQs* was optimized for A) Nitrogen (1–8 mM $NaNO_3$), B) Sulphur (0.1–0.75 mM Na_2SO_4) and C) Phosphorus (0.25–2 mM K_2HPO_4 and KH_2PO_4) concentration (mM), along with D) inorganic carbon supplementation (NaHCO3 (60 to 480 mM), and 3 % CO_2). Data are shown as the mean ± SD, n = 3, $P < 0.01$.

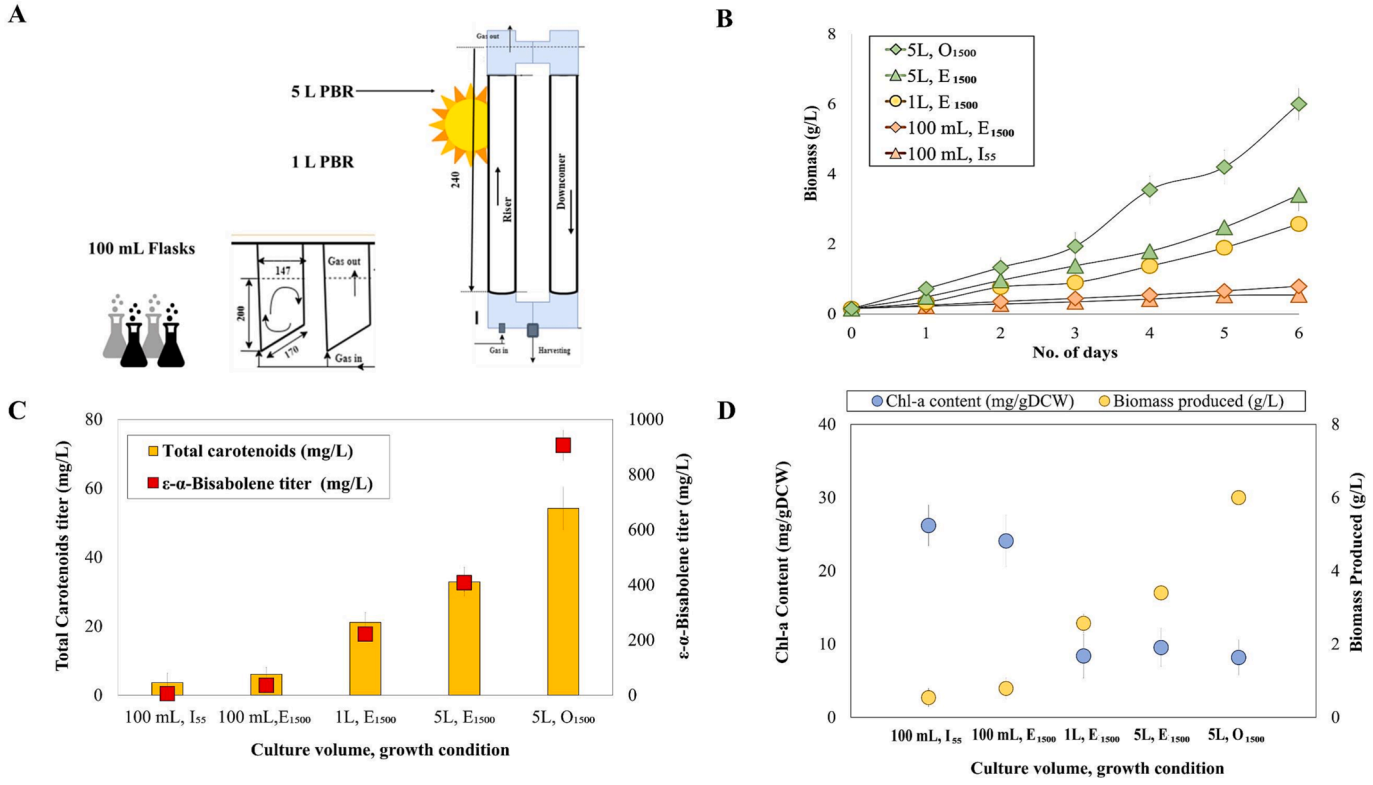


Fig. 2. Outdoor scale-up cultivation of 3XAgBs-SQs. A. Schematic representation of the reactors (100 mL in flasks, 1 L polythene and 5 L glass photo-bioreactors) used for laboratory and outdoor cultivation of *3XAgBs-SQs*. B. Comparative growth analysis under variable growth conditions. C. Production titers for bisabolene and total carotenoids after 144 h of cultivation from I_{55}, E_{1500} to O_{1200} with incremental scale 100 mL, 1 L to 5 L PBR. D. Chl-a content (mg/gDCW) profile for *3XAgBs-SQs* in different cultivation and growth condition. Data are shown as the mean ± SD, n = 3, $P < 0.01$.

the engineered and natural products from the system. Earlier, the *3XAgBs-SQs* acclimated in TAP medium, showed bisabolene production of 36 mg/L (6 ± 0.5 mg/L.d of productivity). This bisabolene titer was nearly 5-fold higher than that obtained under continuous light in A_{250}, Fig. 2C. In addition, the natural value-added total carotenoids from the cells were analysed, showing a total yield of 7.7 ± 3 mg/gDCW and a titer of 13.5 ± 0.3 mg/L. Subsequently media conditioning of the acclimatized *3XAgBs-SQs* in 120 mM (10 g/L) bicarbonate-supplemented HSM under E_{1500} conditions produced 222 ± 23 mg/L of bisabolene, which was almost 20-fold higher than the originally reported bisabolene titer from *C. reinhardtii* of 11.0 ± 0.5 mg/L, in TAP medium with CO_2 purging under continuous artificial light (200 μmol photon m^{-2} s^{-1}) (Wichmann et al., 2018). The corresponding total carotenoid yield was 8.23 ± 4 mg/gDCW, with the titer of 21.5 ± 0.5 mg/L, under E_{1500}, Fig. 2C. Consequently, the strain was transitioned to a higher scale PBR in 5 L optimized HSM medium. The results showed 1.8-fold improvement in the bisabolene titer (408 ± 31 mg/L, with a productivity of 68 ± 4 mg/L.d), and 1.5-fold higher total carotenoids (32.9 mg/L, with a yield of 9.68 ± 4 mg/gDCW), than those obtained from 1 L PBR in E_{1500}, Fig. 2C. This was consistent with previous reports that *C. reinhardtii* strains tend to increase carotenoid production as a protective mechanism under environmental stress (Wichmann et al., 2021, 2018).

Furthermore, when these cultures were ultimately transitioned to O_{1500}, bisabolene and carotenoid production by *3XAgBs-SQs* was significantly enhanced, with a 2.2-fold increased bisabolene titers (906 ± 55 mg/L, with a productivity of 151 ± 5 mg/L.d), and 1.7-fold increase in total carotenoids (54 ± 2 mg/L, with a yield of 12.9 ± 3 mg/gDCW) compared to the 5 L of E_{1500} culture, Fig. 2C. These results suggested the positive effect of natural light acclimatization, media optimization, and incremental scale-up on bio-production potential of the engineered *C. reinhardtii*.

3.3. Photosynthetic efficiency in natural light condition

With a goal to establish a practicable framework for outdoor culture sustenance and maintaining higher production titers, the cultures were also assessed for their photosynthetic efficiency using PAM fluorometry. The engineered strain *3XAgBs-SQs*, grown in TAP medium under I_{55} conditions in an incubator had 14 mg/L (26 ± 3 mg/gDCW) of total chlorophyll, Fig. 2D. The strain transferred to a 1 L PBR under A_{250} (250 μmol photon m^{-2} s^{-1} of artificial light) showed a significantly reduced total chlorophyll yield of 12 ± 3 mg/g DCW, Fig. 2D. This modulation could be attributed to increased light intensity and a photoprotective adaptation of cells (Erickson et al., 2015; Niyogi, 2009; Sarnaik et al., 2019). The *3XAgBs-SQs* cultivated in E_{1500} under diurnal natural light conditions in the optimized HSM, exhibited an inverted bell-shaped curve with respect to the diurnal period. In principle, chl-a content decreased at higher light intensities (with the lowest amount at noon) and increased with a decrease in the light intensity, exhibiting circadian modulation (Sarnaik et al., 2017), Fig. 3A. ETR I and ETR II, on the other hand, displayed a bell-shaped pattern with values increasing till noon and gradually decreasing thereafter, as shown in Fig. 3B-3C. This pattern indicated an uninterrupted cyclic and linear electron transport. This trend was opposite to the quantum photochemical yield Y (II) pattern, Fig. 3D, indicating adaptation of the culture for regulating cellular energy and photoprotection under natural light conditions.

These results indicate that dynamic light and temperature synergistically influenced the photosynthetic efficiency of *3XAgBs-SQs* in O_{1500}, contemplating the overall improved culture sustenance in $O_{1500} > E_{1500}$

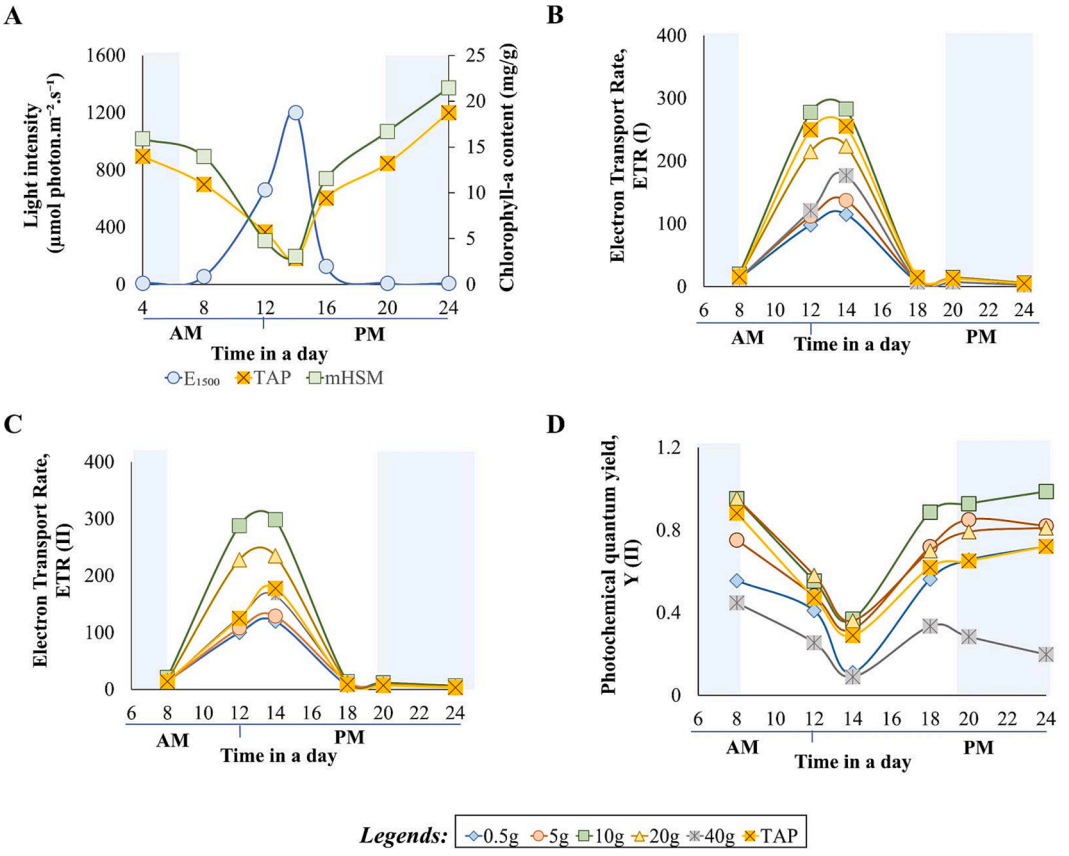


Fig. 3. PAM analysis of 3XAgBs-SQs under natural light conditioning in the bicarbonate-integrated system. (A) Dynamic light intensity (μmol photons m^{-2} s^{-1}) and corresponding Chl-a content (mg/gDCW) of *3XAgBs-SQs* over 24 h duration. Comparative electron transport rates from (B) PS I and (C) PS II of *3XAgBs-SQs*, and (D) Effective photochemical yield (Y (II)) of engineered strain grown in in E_{1500} for different bicarbonate concentrations in HSM in comparison with the TAP medium.

> A_{250}.

3.4. Comparative global metabolomics

Multiple metabolic pathways originating from central C metabolism were traced based on the concentrations of their intermediate metabolites, to estimate the effects of environmental parameters under A_{250}, E_{1500}, and O_{1500}. The metabolic profile of the engineered *C. reinhardtii* in the optimised HSM exhibited differential responses to the central carbon pathway under different cultivation conditions. Profiles of approximately 40 metabolites were analyzed, showing significant upregulation of amino acids, sugars, organic acids, fatty acids, alcohols, antioxidants, and sterols under natural light conditions, especially O_{1500} (*See Supplementary material*), compared to A_{250} and E_{1500}. Differential patterns exhibited by the metabolites, influenced by both, outdoor transition and engineering heterologous terpenoid biosynthesis pathway, have been reported as the concentrations, normalized to the cell densities.

Similar to higher plants, in *C. reinhardtii* central C pathway metabolites serve as the sinks for photosynthetically fixed carbon (Wichmann et al., 2021, 2018). Glucose is central to different cellular processes, including photosynthesis and respiration. The results showed 1.5-fold increase in glucose concentration from A_{250} to E_{1500}, and a 3.3-fold increase from E_{1500} to O_{1500}, Fig. 4. Glucose is further metabolized to glucose-6-phosphate (G6P) and partitioned into multiple pathways, including glycolysis, oxidative pentose phosphate pathway, and biosynthesis of stress-responsive metabolites such as sucrose, glycerol, and myo-inositol (MI) (Qian et al., 2021). Hence, the trend O_{1500} > E_{1500} > A_{250} exhibited by glucose was reflected in all subsequent metabolites, including fructose, sucrose, galactose, MI, and succinic acid, Fig. 4.

Glycerol is another important metabolite produced from glycerol-3-phosphate, a direct product of dihydroxy acetone phosphate (DHAP), and it is synthesized in response to counteracting environmental and osmotic stress (Demmig-Adams et al., 2017). Thus, its increased concentration under O_{1500} conditions would have elicited a protective mechanism, indicating the importance of seed culture acclimatization during outdoor cultivation. In addition, glycerol-3-phosphate, synthesized from glycerol, is an essential precursor for glycerolipid and free fatty acid (palmitic acid, palmitoleic acid, oleic acid, linoleic acid) synthesis (Driver et al., 2017). Hence, in O_{1500}, increased glycerol levels could be correlated with improved flux towards fatty acid synthesis, particularly in the development of cellular membrane systems, Fig. 4.

Further analysis revealed similar trends for MI and galactose, Fig. 4. MI has been observed to activate the enzymes involved in chlorophyll-a biosynthesis, balancing the loss of these pigments (Qian et al., 2021). MI could also trigger various algal receptors and pathways, promoting cell growth and division. Galactose usually participates in the biosynthesis of galactolipids, which are essential for the interaction with photosynthetic proteins (Hö lzl et al., 2006). Altogether, these metabolic modulations improve photo-protection, and hence, their concentrations were higher under O_{1500}, potentially as a response to natural light acclimatization, resulting in the improved cell sustenance and hence, the growth.

In addition, flux towards certain crucial metabolites was corroborated based on the concentrations of amino acids synthesized from them (Batista et al., 2019). For instance, comparative analyses of alanine and

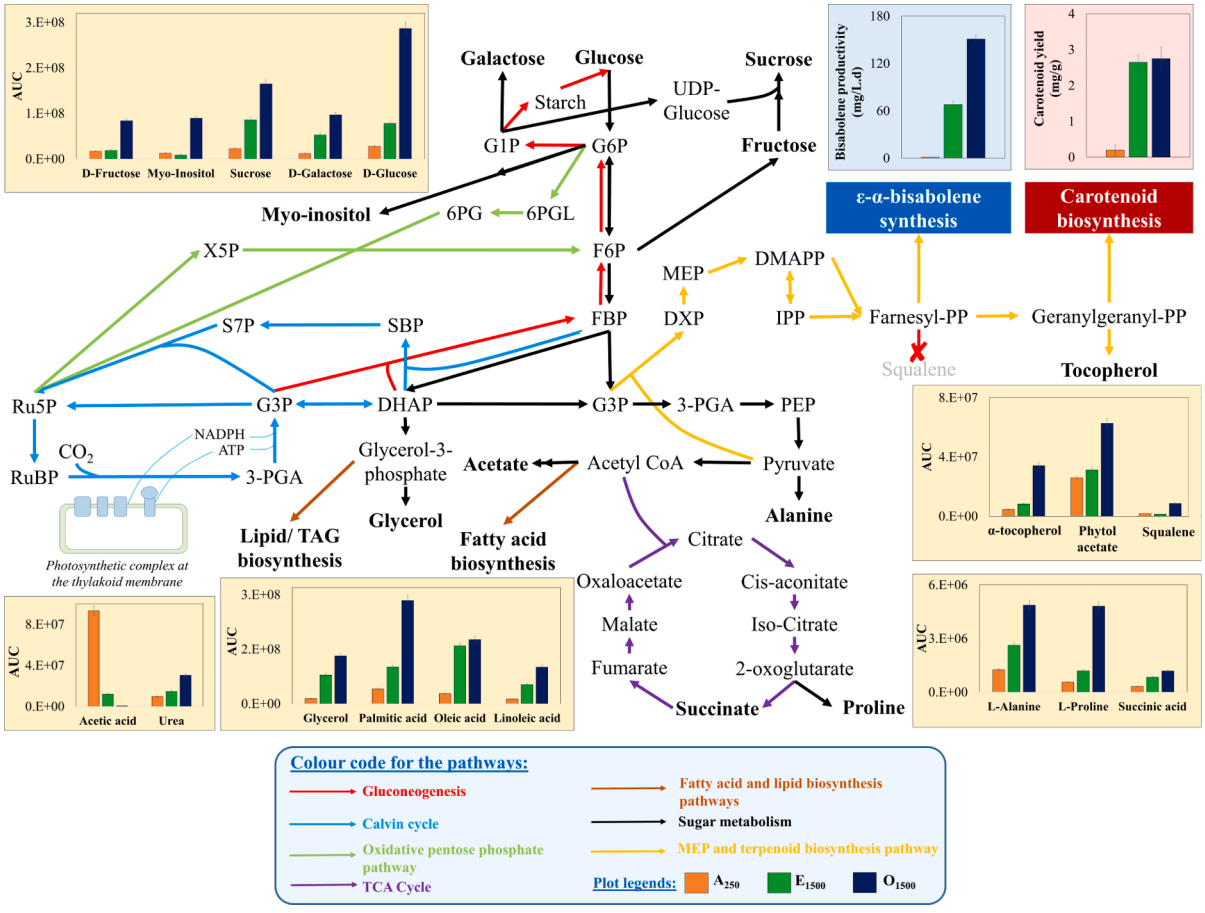


Fig. 4. Metabolomic expression profile of *3XAgBs-SQs*. Schematic representation for comparative metabolomics of *3XAgBs-SQs* grown under A_{250}, E_{1500} and O_{1200}. The intermittent metabolites normalised with the culture density and analysed using GCMS are represented in bold with their corresponding cellular fraction. Data are shown as the mean ± SD, n = 3, $P < 0.01$.

proline have been performed to estimate the effects of different environmental conditions on pyruvate and 2-oxoglutarate (2-OG). As the metabolites were the products of glycolysis and TCA cycle, they exhibited the same trend: $O_{1500} > E_{1500} > A_{250}$, as previously mentioned. In addition, proline exhibits metal chelation and antioxidant activity, and functions as a signalling molecule (Hayat et al., 2012). This protective role could be one of the reasons for its relatively higher fraction under O_{1500}, Fig. 4. In addition, the increasing proportion of urea is commensurate with the improvement in the protein: amino acid ratio, which ultimately leads to higher intracellular protein content (Batista et al., 2019). Consequently, higher protein biosynthesis in actively growing cells in O_{1500} could have led to the increased concentrations of these amino acids (Uslu and Güvenaltin, 2010).

In contrast, the comparative acetate concentrations exhibited the exact reverse trend: $O_{1500} < E_{1500} < A_{250}$. Acetate is a product of the cell's dark fermentative metabolism (Yang et al., 2014). Second, Heifetz *et al.* demonstrated that in the presence of acetate and saturating light intensities, photosynthesis was inhibited in *C. reinhardtii* (Heifetz et al., 2000). This indicated that external environmental conditions might directly affect acetate production along with other factors such as intracellular metabolic compartmentalization and acetate assimilation. This effect was also observed in this study. When the cultures were grown outdoors in HSM supplemented with 2 g/L acetate, culture collapse was observed on multiple occasions (data not shown). Hence, the scale-up was performed under autotrophic conditions, Fig. 4.

Apart from these central carbon metabolites, steroid production was also influenced in response to oxidative stress under natural dynamic conditions (Potijun et al., 2021). For example, carotenoids protect the photosystem II under stressful conditions (Sirikhachornkit and Napaumpaiporn, 2016). Hence, their yield was found to increase from A_{250} to E_{1500}, as compared to E_{1500} and O_{1500}, Fig. 4. Similarly, increased tocopherol production could be correlated with various abiotic stresses, including high light, salinity, drought, and temperature (Singh et al., 2020). Tocopherol production increased during temperature stress, suggesting a compensatory mechanism. Overall, the improved flux through central C metabolism, synergistic with photosynthesis, significantly contributed to the cell sustenance and increased carbon availability for producing fatty acids, carotenoids, and α-bisabolenes (Valenzuela et al., 2012). This upregulation indicated increased flux towards the precursor farnesyl pyrophosphate (FPP). FPP is also an essential precursor for bisabolene production; hence, bisabolene concentration was found to improve under outdoor conditions (Rodrigues and Lindberg, 2021; Wichmann et al., 2022, 2018).

Recognizing the metabolomic profile, it is evident that outdoor conditions have significantly enhanced bisabolene production. Although the productivities of 25.6 ± 0.8 g/m^2.d for 5 L obtained in the current study are equivalent to the conventional plant sources (Steele et al., 1998), further studies are necessary to establish the feasibility of this strategy. Additionally, commercial viability requires considering ethical and ecological concerns surrounding the cultivation of genetically modified organisms (GMOs) at incremental scales under outdoor conditions. Therefore, the adoption and design of well-isolated, large-scale closed photobioreactor system is crucial to minimize the risk of contamination and to maintain greater control over cultivation parameters (Abdullah et al., 2019).

4. Conclusions

The *3XAgBs-SQs* strain, engineered for bisabolene production, was successfully scaled up to 5 L under dynamic outdoor conditions. Natural light acclimatization and media optimization improved biomass (6 g/L) and bisabolene production (906 mg/L), outdoor. Metabolomic analysis and PAM fluorometry studies further supported these findings. The strain was simultaneously explored for production of natural carotenoids (54 mg/L) as value added-chemicals, making it a potential one-cell two-wells biorefinery. To our knowledge, this is the first successful demonstration of outdoor incremental scale-up of engineered *C. reinhardtii,* as bioproduction platform.

CRediT authorship contribution statement

Kaustubh R. Sawant: Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation. **Aditya P. Sarnaik:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation, Conceptualization. **Rabinder Singh:** Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Prashant Savvashe:** Writing – review & editing, Validation, Resources. **Thomas Baier:** Writing – review & editing. **Olaf Kruse:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition. **Pannaga Pavan Jutur:** Writing – review & editing, Validation, Supervision, Project administration, Investigation, Funding acquisition. **Arvind Lali:** Writing – review & editing, Supervision, Resources, Project administration. **Reena A. Pandit:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

The authors acknowledge the Department of Biotechnology, Ministry of Science and Technology, Government of India for the support of the work under the "Mission Innovation" scheme with project title- "Demonstration of algal chassis for the photo-autotrophic production of isoprenoids" and grant no. BT/PR31155/PBD/26/725/2019 and BT/EB/ICT-Extension/2012.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.130513.

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