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ENVIRONMENTAL ENGINEERING

PhD Thesis

"Optimization of microalgae growth and separation processes for the production of high added value products"

«Βελτιστοποίηση διεργασιών ανάπτυξης και διαχωρισμού μικροφυκών για την παραγωγή προϊόντων υψηλής προστιθέμενης αξίας»

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Abstract

Microalgae are unicellular photosynthetic microorganisms that can grow in diverse environments, by developing adaptation mechanisms. They are found in freshwater, saline water, ice, soil, and even in wastewater. According to their pigmentation, arrangements of photosynthetic membranes or other morphological features, microalgae are classified as diatoms, green algae, golden algae, and cyanobacteria. Microalgae share similar growth mechanisms as terrestrial plants, with the difference that they are cellular microorganisms. They use sunlight to sequester CO_2 dissolved in the growth medium for biomass and O_2 production. Cultivation of microalgae takes place in open ponds or photobioreactors, comprising of various types. Sunlight or artificial light (e.g., fluorescent, LED) can be used for boosting photosynthesis. However, light can be a limiting factor for microalgae growth as high chlorophyll causes a "shadow effect" to microalgae growing near the center of photobioreactors. Microalgae have various applications such as biofuels, food supplements, cosmetics, and animal feed, being rich in proteins, lipids, carbohydrates, fatty acids, pigments, and vitamins. Production of microalgae biomass could be boosted by channeling CO_2 supply during cultivation. In this way, food productivity can be increased and CO_2 from flue gas be decreased. Furthermore, industrial flue gas typically contains 4-14% or more (v/v) CO_2 concentration and toxic compounds such as SO_x, NO_x, and trace elements, which are emitted at high flow rate, having high temperatures ranging from 80 to 120 ℃ or above. For this reason, microalgae should be able to withstand this extreme environment, to mitigate CO_2. Flue gas from natural gas could be used to lessen the negative effects on microalgae growth.

The present PhD thesis consists of four main research parts. The initial scope was the mutation of wild-type *Stichococcus* sp. microalgae strain which led to a new strain with reduced chlorophyll content, and increased biomass and lipids productivity (namely EMS1). Mutation was achieved by the chemical reagent ethyl methanesulfonate (EMS) which causes random mutations in the DNA. The purpose of the mutation is that high chlorophyll can inhibit light penetration in the inner part of the culture, cultivated in photobioreactors. Thus, leading to reduced biomass productivity. The mutant strain EMS1 showed 51% less chlorophyll, 12% higher biomass, and 45% higher lipids productivity, compared to the wild-type.

The second scope was to further investigate the strains and select one which will be optimized on small scale with the ultimate goal to be scaled up in pilot photobioreactors and fixate CO_2 from industrial flue gas. Experiments on wild-type and EMS1 *Stichococcus* sp. strains were conducted at laboratory scale (beaker vessels, 150 mL culture volume) with Bold's Basal growth medium diluted in artificial seawater. Microalgae were grown attached on horizontal sandblasted glass tiles placed at the bottom of the vessels, as a measure to minimize harvesting costs. Agitation, CO_2 capture efficiency, cultivation duration, nitrogen starvation duration, biomass, and bio-products production were examined. It was found that the absence of agitation had a minor effect on the reduction of biomass production. The addition of CO_2 to reach 5% concentration in the gaseous phase resulted in up to 300% increase in biomass, while microalgae required 25 days to grow sufficiently. Also, the application of nitrogen starvation three days before harvesting enhanced intracellular lipids production. Screening of the two strains resulted in the selection of mutant EMS1 strain for optimization of its biomass and bio-products production (i.e., lipids, pigments, proteins, and carbohydrates). Taguchi's Design of Experiments (DOE) was implemented to find the least required experimental runs. Also, microalgae were fed with synthetic flue gas (for CO_2 fixation), to simulate real conditions from combustion of natural gas from industrial power plants. Five optimal combinations of the growth parameters were found from the statistical analysis of the data, maximizing each of the measured characteristics. Changes in aeration rate did not have a significant effect in biomass and total bioproducts production. Higher illuminance intensity (6,600 lux), continuous lighting and higher $NaNO_3$ concentration (0.75 g L^{-1}) resulted in higher yield in biomass and bio-products. Maximum value for biomass was equal to 45.7 ± 1.3 g m^{-2}, while bio-products and their corresponding values were: lipids 11.6 ± 0.4 g m^{-2}, pigments 0.22 ± 0.02 g m^{-2}, proteins 9.5 ± 0.5 g m^{-2}, carbohydrates 19.0 ± 1.5 g m^{-2}, and total bio-products 34.8 ± 2.2 g m^{-2}. *Stichococcus* sp. was found to be an excellent carbohydrates producer, having content up to 52%. Lipids production constituted up to 40% and the total bio-products yield was up to 91% of the biomass. Flashing light (1,000 Hz) effect triggered chlorophyll production by an average 8%, while three-day nitrogen starvation increased lipids production by an average 22%. Experimental maximum values were compared with regression analysis models for each of the five optimal conditions. Theoretical and experimental results were similar, with R^2 of the models ranging from 84 to 99%. Following the lab-scale experiments, EMS1 *Stichococcus* sp. strain was scaled up in flat-panel photobioreactor (15 L culture volume) with the microalgae

immobilized on sandblasted glass tiles. The experimental conditions were selected for biomass maximization and results showed biomass production equal to 49.2 g m^{-2}, while total bio-products accounted for 41.7 g m^{-2}. Microalgae were also fed with synthetic flue gas.

The third scope of the PhD thesis was to test *Stichococcus* sp. in real conditions with an industrial flue gas supply for CO_2 mitigation. Experiments were conducted in Lavrio power station, located in the region of Attica in Greece. EMS1 *Stichococcus* sp. strain adapted to this extreme environment and produced biomass values up to 50.5 g m^{-2} and the total bio-products measured were equal to 39.8 g m^{-2}.

As a final scope of the present thesis, dewatering and drying processes of microalgae grown i) suspended and ii) immobilized were evaluated. Cultures of suspended microalgae were harvested by vacuum filtration, centrifugation, and chitosan flocculation coupled with the aforementioned processes. Immobilized microalgae were harvested by scraping of the sandblasted glass tiles, as a means of cultivating microalgae in specific places, thus lowering harvesting costs. Based on the experimental results, even though biomass scraping showed lower recovery rates by 18%, compared to harvesting of suspended microalgae, it showed the lowest energy consumption (0.7 ± 0.1 kWh kg^{-1} of dry biomass), which verified the scope of cultivating immobilized biomass. Growth medium removed from the bulk liquid of immobilized microalgae cultures could possibly be retained for future use, as it contains a portion of the previous culture and nutrients. Harvested biomass was further processed by drying of the excess liquid. Convective, solar, and freeze drying processes were tested for their efficacy and their influence on microalgae biomass and bio-products. Convective drying required the greater electrical energy consumption (58.5 kWh kg^{-1} of wet biomass), but it dried the biomass in the shorted period (5.5 hours). Extracted bio-products (i.e., lipids, pigments, proteins, and carbohydrates) were found to be linked with the drying processes. Lipids and total chlorophyll recovery were greater after the application of freeze-drying with their values equal to 0.26 ± 0.01 g g^{-1} and $0.70 \pm 0.04 \cdot 10^{-2}$ g g^{-1} of biomass. Carbohydrates recovery was maximized by applying solar and convective drying, with a maximum value of 0.45 ± 0.01 g g^{-1} biomass. In the case of proteins, each drying process showed similar recovery rates with an average value equal to 0.19 ± 0.02 g g^{-1} biomass. The above indicate that drying processes should be considered for maximization of the end-products. The main conclusions that derived from the present PhD thesis is that microalgae are a sustainable source of valuable products and

biofuels, through CO_2 conversion for greenhouse effect mitigation. Cultivation of immobilized microalgae in seawater with the appropriate conditions can significantly reduce cultivation costs, by maximizing biomass and bio-products yield.

Περίληψη

Τα μικροφύκη είναι μονοκύτταροι φωτοσυνθετικοί μικροοργανισμοί που μπορούν να αναπτυχθούν σε διαφορετικά περιβάλλοντα, αναπτύσσοντας μηχανισμούς προσαρμογής. Βρίσκονται σε γλυκό νερό, αλατούχο νερό, πάγο, έδαφος, ακόμη και σε υγρά απόβλητα. Σύμφωνα με τη χρώση τους, τις διατάξεις των φωτοσυνθετικών μεμβρανών ή άλλα μορφολογικά χαρακτηριστικά, τα μικροφύκη ταξινομούνται ως διάτομα, πράσινα φύκη, χρυσά φύκη και κυανοβακτήρια. Τα μικροφύκη μοιράζονται παρόμοιους μηχανισμούς ανάπτυξης με τα χερσαία φυτά, με τη διαφορά ότι είναι κυτταρικοί μικροοργανισμοί. Χρησιμοποιούν το ηλιακό φως για να δεσμεύσουν το CO_2 που είναι διαλυμένο στο μέσο ανάπτυξης για παραγωγή βιομάζας και O_2. Η καλλιέργεια των μικροφυκών λαμβάνει χώρα σε ανοιχτές λίμνες ή σε φωτοβιοαντιδραστήρες, που αποτελούνται από διάφορους τύπους. Το ηλιακό φως ή το τεχνητό φως (π.χ. φθορίζον, LED) μπορεί να χρησιμοποιηθεί για την ενίσχυση της φωτοσύνθεσης. Ωστόσο, το φως μπορεί να είναι ένας περιοριστικός παράγοντας για την ανάπτυξη μικροφυκών, καθώς η υψηλή χλωροφύλλη προκαλεί ένα «φαινόμενο σκίασης» στα μικροφύκη που αναπτύσσονται κοντά στο κέντρο των φωτοβιοαντιδραστήρων. Τα μικροφύκη έχουν διάφορες εφαρμογές όπως σε βιοκαύσιμα, συμπληρώματα διατροφής, καλλυντικά και ζωοτροφές, καθώς είναι πλούσια σε πρωτεΐνες, λιπίδια, υδατάνθρακες, λιπαρά οξέα, χρωστικές ουσίες και βιταμίνες. Η παραγωγή βιομάζας μικροφυκών θα μπορούσε να ενισχυθεί με τη διοχέτευση της παροχής CO_2 κατά την καλλιέργεια. Με αυτόν τον τρόπο, μπορεί να αυξηθεί η παραγωγικότητα των τροφίμων και να μειωθεί το CO_2 από τα καυσαέρια. Επιπλέον, τα βιομηχανικά καυσαέρια περιέχουν συνήθως 4-14% ή περισσότερο (v/v) συγκέντρωση CO_2 και τοξικές ενώσεις όπως SO_x, NO_x και ιχνοστοιχεία, τα οποία εκπέμπονται με υψηλή ταχύτητα ροής και υψηλή θερμοκρασία που κυμαίνεται από 80 έως 120 °C ή υψηλότερη. Για το λόγο αυτό, τα μικροφύκη θα πρέπει να είναι σε θέση να αντέξουν αυτό το ακραίο περιβάλλον, ώστε να μετριάσουν το εκπεμπόμενο CO_2. Τα καυσαέρια από φυσικό αέριο θα μπορούσαν να χρησιμοποιηθούν για τη μείωση των αρνητικών επιπτώσεων στην ανάπτυξη των μικροφυκών.

Η παρούσα διδακτορική διατριβή αποτελείται από τέσσερα κύρια ερευνητικά μέρη. Ο αρχικός στόχος ήταν η μετάλλαξη του άγριου στελέχους μικροφυκών *Stichococcus* sp. που οδήγησε σε ένα νέο στέλεχος με μειωμένη περιεκτικότητα σε χλωροφύλλη και αυξημένη

παραγωγικότητα βιομάζας και λιπιδίων (ονομασία EMS1). Η μετάλλαξη επιτεύχθηκε με το χημικό αντιδραστήριο Ethyl Methanesulfonate (EMS) που προκαλεί τυχαίες μεταλλάξεις στο DNA. Ο σκοπός της μετάλλαξης είναι ότι η υψηλή χλωροφύλλη μπορεί να αναστείλει τη διείσδυση φωτός στο εσωτερικό μέρος της καλλιέργειας, που αναπτύσσεται σε φωτοβιοαντιδραστήρες. Έτσι, οδηγεί σε μειωμένη παραγωγικότητα βιομάζας. Το μεταλλαγμένο στέλεχος EMS1 έδειξε 51% λιγότερη χλωροφύλλη, 12% υψηλότερη βιομάζα και 45% υψηλότερη παραγωγή λιπιδίων, σε σχέση με τον άγριο τύπο.

Ο δεύτερος στόχος ήταν η περαιτέρω διερεύνηση των στελεχών και η επιλογή ενός που θα βελτιστοποιηθεί σε μικρή κλίμακα με απώτερο στόχο την κλιμάκωση σε πιλοτικούς φωτοβιοαντιδραστήρες και τη δέσμευση CO_2 από βιομηχανικά απαέρια. Πειράματα με το άγριο και EMS1 στέλεχος *Stichococcus* sp. πραγματοποιήθηκαν σε εργαστηριακή κλίμακα (ποτήρια ζέσεως, όγκος καλλιέργειας 150 mL) με μέσο ανάπτυξης Bold's Basal αραιωμένο σε τεχνητό θαλασσινό νερό. Τα μικροφύκη αναπτύχθηκαν προσαρτημένα σε οριζόντια γυάλινα πλακίδια αμμοβολής στο κάτω μέρος των δοχείων, ως μέτρο για την ελαχιστοποίηση του κόστους συγκομιδής. Εξετάστηκαν η ανάδευση, η αποτελεσματικότητα δέσμευσης CO_2, η διάρκεια καλλιέργειας, η διάρκεια της πενίας αζώτου, η παραγωγή βιομάζας και βιο-προϊόντων. Διαπιστώθηκε ότι η απουσία ανάδευσης είχε μικρή επίδραση στη μείωση της παραγωγής βιομάζας. Η προσθήκη 5% αερίου CO_2 είχε ως αποτέλεσμα έως και 300% αύξηση της βιομάζας και τα μικροφύκη χρειάστηκαν 25 ημέρες για να αναπτυχθούν επαρκώς. Επίσης, η εφαρμογή της πενίας αζώτου τρεις ημέρες πριν από τη συγκομιδή ενίσχυσε την ενδοκυτταρική παραγωγή λιπιδίων. Ο έλεγχος των δύο στελεχών οδήγησε στην επιλογή του μεταλλαγμένου στελέχους EMS1 για τη βελτιστοποίηση της παραγωγής βιομάζας και βιο-προϊόντων (δηλαδή λιπίδια, χρωστικές ουσίες, πρωτεΐνες και υδατάνθρακες). Η μέθοδος Taguchi – Design of Experiments (DOE) εφαρμόστηκε για να βρεθούν οι λιγότερο απαιτούμενες πειραματικές εκτελέσεις. Επίσης, τα μικροφύκη τροφοδοτήθηκαν με συνθετικά καυσαέρια (για δέσμευση CO_2), με σκοπό την προσομοίωση πραγματικών συνθηκών από την καύση φυσικού αερίου από βιομηχανικούς σταθμούς ηλεκτροπαραγωγής. Από τη στατιστική ανάλυση των δεδομένων βρέθηκαν πέντε βέλτιστοι συνδυασμοί των παραμέτρων ανάπτυξης, μεγιστοποιώντας κάθε ένα από τα μετρούμενα χαρακτηριστικά. Οι αλλαγές στον ρυθμό αερισμού δεν είχαν σημαντική επίδραση στη βιομάζα και τη συνολική παραγωγή βιο-προϊόντων. Η υψηλότερη ένταση φωτισμού (6.600 lux), ο συνεχής φωτισμός και η υψηλότερη συγκέντρωση $NaNO_3$ (0,75 g L^{-1}) είχαν ως αποτέλεσμα την υψηλότερη

απόδοση σε βιομάζα και βιο-προϊόντα. Η μέγιστη τιμή για τη βιομάζα ήταν ίση με 45,7 ± 1,3 g m^{-2}, ενώ τα βιο-προϊόντα και οι αντίστοιχες τιμές τους ήταν: λιπίδια 11,6 ± 0,4 g m^{-2}, ολική χλωροφύλλη 0,22 ± 0,02 g m^{-2}, πρωτεΐνες 9,5 ± 0,5 g m^{-2}, υδατάνθρακες 19,0 ± 1,5 g m^{-2} και συνολικά βιο-προϊόντα 34,8 ± 2,2 g m^{-2}. Το στέλεχος *Stichococcus* sp. βρέθηκε ότι είναι εξαιρετικός παραγωγός υδατανθράκων, με περιεκτικότητα έως και 52%. Η παραγωγή λιπιδίων αποτελούσε έως και το 40% και η συνολική απόδοση βιο-προϊόντων ήταν έως και 91% της βιομάζας. Η επίδραση του φωτός με αναλαμπές (1.000 Hz) που εφαρμόστηκε αύξησε την παραγωγή χλωροφύλλης κατά μέσο όρο 8%, ενώ η πενία αζώτου τριών ημερών αύξησε την παραγωγή λιπιδίων κατά μέσο όρο 22%. Οι πειραματικές μέγιστες τιμές συγκρίθηκαν με μοντέλα γραμμικής παλινδρόμησης για καθεμία από τις πέντε βέλτιστες συνθήκες. Τα θεωρητικά και τα πειραματικά αποτελέσματα ήταν παρόμοια, με το R^2 των μοντέλων να κυμαίνεται από 84 έως 99%. Έπειτα από τα πειράματα εργαστηριακής κλίμακας, το στέλεχος EMS1 *Stichococcus* sp. κλιμακώθηκε σε επίπεδο φωτοβιοαντιδραστήρα (όγκος καλλιέργειας 15 L) με τα μικροφύκη ακινητοποιημένα σε γυάλινα πλακίδια αμμοβολής. Οι πειραματικές συνθήκες επιλέχθηκαν για τη μεγιστοποίηση της βιομάζας και τα αποτελέσματα έδειξαν παραγωγή βιομάζας ίση με 49,2 g m^{-2}, ενώ τα συνολικά βιο-προϊόντα αντιστοιχούσαν σε 41,7 g m^{-2} επιφάνειας. Τα μικροφύκη τροφοδοτήθηκαν επίσης με συνθετικά καυσαέρια.

Ο τρίτος σκοπός της διδακτορικής διατριβής ήταν η δοκιμή του *Stichococcus* sp. σε πραγματικές συνθήκες με παροχή βιομηχανικών καυσαερίων για τη δέσμευση CO_2. Πειράματα πραγματοποιήθηκαν στον ηλεκτροπαραγωγικό σταθμό Λαυρίου, που βρίσκεται στην περιοχή της Αττικής. Το στέλεχος EMS1 *Stichococcus* sp. προσαρμόστηκε στο ακραίο αυτό περιβάλλον και παρήγαγε τιμές βιομάζας έως 50,5 g m^{-2} και τα συνολικά βιο-προϊόντα που μετρήθηκαν, ήταν ίσα με 39,8 g m^{-2}.

Ως τελικό αντικείμενο της παρούσας διατριβής, αξιολογήθηκαν οι διαδικασίες αφυδάτωσης και ξήρανσης μικροφυκών σε i) εναιώρημα και ii) ακινητοποιημένα. Καλλιέργειες αιωρούμενων μικροφυκών συλλέχθηκαν με διήθηση κενού, φυγοκέντριση και κροκίδωση χιτοζάνης σε συνδυασμό με τις προαναφερθείσες διαδικασίες. Η συγκομιδή των ακινητοποιημένων μικροφυκών έγινε με απόξεση των πλακιδίων με αμμοβολή, μειώνοντας έτσι το κόστος συγκομιδής. Με βάση τα πειραματικά αποτελέσματα, παρόλο που η απόξεση βιομάζας έδειξε χαμηλότερα ποσοστά ανάκτησης κατά 18% σε σύγκριση με τη συγκομιδή αιωρούμενων

μικροφυκών, έδειξε τη χαμηλότερη κατανάλωση ενέργειας (0,7 ± 0,1 kWh kg^{-1} νερού που αφαιρέθηκε), η οποία επαλήθευσε το εύρος της καλλιέργειας ακινητοποιημένης βιομάζας. Το μέσο ανάπτυξης που αφαιρέθηκε από τις ακινητοποιημένες καλλιέργειες μικροφυκών θα μπορούσε ενδεχομένως να διατηρηθεί για μελλοντική χρήση, καθώς περιέχει ένα μέρος της προηγούμενης καλλιέργειας και θρεπτικών συστατικών. Η συλλεγόμενη βιομάζα υποβλήθηκε σε περαιτέρω επεξεργασία με ξήρανση της περίσσειας υγρού. Η ξήρανση σε κλίβανο, ηλιακή ξήρανση και λυοφιλίωση δοκιμάστηκαν για την αποτελεσματικότητά τους και την επιρροή τους στη βιομάζα και στα βιο-προϊόντα των μικροφυκών. Η ξήρανση στον κλίβανο απαίτησε τη μεγαλύτερη κατανάλωση ηλεκτρικής ενέργειας (58,5 kWh kg^{-1} υγρής βιομάζας), αλλά ξήρανε τη βιομάζα σε σύντομο χρονικό διάστημα (5,5 ώρες). Τα ανακτημένα βιο-προϊόντα (δηλαδή, λιπίδια, χρωστικές ουσίες, πρωτεΐνες και υδατάνθρακες) βρέθηκαν να συνδέονται με τις διαδικασίες ξήρανσης. Τα λιπίδια και η ολική ανάκτηση χλωροφύλλης ήταν μεγαλύτερη μετά την εφαρμογή λυοφιλίωσης με τις τιμές τους να είναι ίσες με 0,26 ± 0,01 g g^{-1} και 0,70 ± 0,04·10^{-2} g g^{-1} βιομάζας. Η ανάκτηση υδατανθράκων μεγιστοποιήθηκε με την εφαρμογή ηλιακής ξήρανσης και ξήρανσης σε κλίβανο, με μέγιστη τιμή 0,45 ± 0,01 g g^{-1} βιομάζας. Στην περίπτωση των πρωτεϊνών, κάθε διαδικασία ξήρανσης έδειξε παρόμοια ποσοστά ανάκτησης με μέση τιμή ίση με 0,19 ± 0,02 g g^{-1} βιομάζας. Τα παραπάνω δείχνουν ότι οι διαδικασίες ξήρανσης πρέπει να λαμβάνονται υπόψη για τη μεγιστοποίηση των τελικών προϊόντων. Τα κύρια συμπεράσματα που προέκυψαν από την παρούσα διδακτορική διατριβή είναι ότι τα μικροφύκη αποτελούν βιώσιμη πηγή πολύτιμων προϊόντων και βιοκαυσίμων, μέσω της μετατροπής του CO_2 για τον μετριασμό του φαινομένου του θερμοκηπίου. Η καλλιέργεια ακινητοποιημένων μικροφυκών σε θαλασσινό νερό με τις κατάλληλες συνθήκες μπορεί να μειώσει σημαντικά το κόστος καλλιέργειας, μεγιστοποιώντας την απόδοση βιομάζας και βιο-προϊόντων.

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Chapter 1: Introduction – Literature Review

1.1 Historical perspective

Algae are found in various habitats, ranging from freshwater to marine environments, and range in size, from unicellular microorganisms to larger seaweeds. Microalgae, with their remarkable cell structure and growth parameters, have been recognized as great "green" candidates for cultivation and production of high-value products.

Throughout human history, algae, both macroalgae and microalgae, have been used for food, medicine, and even as a biofertilizer. Coastal communities have long incorporated seaweeds, a type of marine macroalgae, into their diets due to their rich nutritional content. In Asian cultures, such as Japan, China, and Korea, algae like nori and kombu have been staples in traditional dishes, as it has been recognized that they provide essential vitamins, minerals, and dietary fiber that contribute to human health (Sánchez et al., 2019). There is a lack of extensive knowledge regarding the utilization of microalgae throughout the human history, however, some reports suggest their early usage from the Aztecs. They relied on *Arthrospira* (Spirulina) *maxima*, a cyanophycae, as a valuable nutritional resource. Similarly, the indigenous people of Lake Chad in Africa harvested *Aphanizomenon flosaquae*, a type of cyanobacteria, as a food source (Abdulqader et al., 2000). *Nostoc flagelliforme* microalgae have used from ancient Chinese civilizations as medicinal remedies as they were helpful in treating conditions such as diarrhea, hepatitis, and hypertension and as food for more than 2000 (Gao, 1998).

Research on microalgae began in the late 19th century, with the successful cultivation of pure cultures of *Chlorella vulgaris* (Beijerinck, 1890). Since then, various microalgal species have been employed in scientific research, with one of the most important being the understanding of the mechanisms underlying photosynthesis. Melvin Calvin and his team identified the Calvin cycle of photosynthetic CO_2 assimilation, for which Calvin received the Nobel Prize in Chemistry (Calvin, 1949). Later, during World War II, research on lipid production from microalgae for

biofuels focused on the potential use of microalgae for CO_2 capture to mitigate the greenhouse effect, and to produce high-value products.

Today, microalgae are extensively studied for their applications in environmentally friendly production of high-value products such as biofuels, nutraceuticals, pharmaceuticals, and applications such as wastewater treatment, and bioremediation. Thus, they offer a promising alternative to conventional resources, providing high-value products while minimizing environmental impact (Hsieh and Felby, 2017).

1.2 Algae

The term “Algae” refers to a heterogenous, polyphyletic group of photosynthetic organisms. They are primitive plants (thallophytes) lacking roots and they do not develop pure tissue structures like those that can be found in plants (Brennan and Owende, 2010). These organisms inhabit and can thrive in a very diverse range of environmental niches, from marine and freshwater environments to tree barks in the land. This means that are very adaptable to various environmental conditions, such as temperatures, salinity and pH levels, and very low light intensities. Algae can even tolerate very harsh environments as they have been found in dessert sand, in oceans in polar regions and in hot springs (Barsanti et al., 2008). Their ability to thrive in these environments can be attributed to a very wide range of forms and structures that can be found in algae species, which are adaptations to their specific environmental niches (Sánchez et al., 2019).

Algae have been an integral part of the Earth's ecosystem for billions of years, and have been a major contributor in shaping the environment (Falkowski and Raven, 2007). They are photosynthetic organisms as the use light, carbon dioxide, and nutrients to produce organic matter and release oxygen into the atmosphere. This process not only supports their own growth but also plays a crucial role in maintaining global oxygen levels and regulating carbon dioxide in the atmosphere. In fact, algae serve as the foundation for approximately 70% of biomass production on Earth and contribute to around 50% of the oxygen produced through photosynthesis (Andersen, 2013; Wiessner et al., 1995). In fact, the photosynthetic efficiency of microalgae is 10-15 times

higher than that of terrestrial plants (Cheng et al., 2019), while they have various advantages, compared to terrestrial plants. Microalgae require smaller cultivation areas, compared to terrestrial plants, while they may do not necessarily need freshwater consumption (Chisti, 2007).

Algae can be categorized into two main groups depending on their size and cellular structure: macroalgae and microalgae. Macroalgae, also known as seaweed, are multicellular organisms. While some species within the Phaeophyceae class can grow quite large, they do not possess the tissues or organs that land plants have (Figure 1). On the other hand, microalgae are microscopic single-celled organisms that can be further classified as either prokaryotic, resembling cyanobacteria (Chloroxybacteria), or eukaryotic, resembling green algae (Chlorophyta) (Khan et al., 2018).

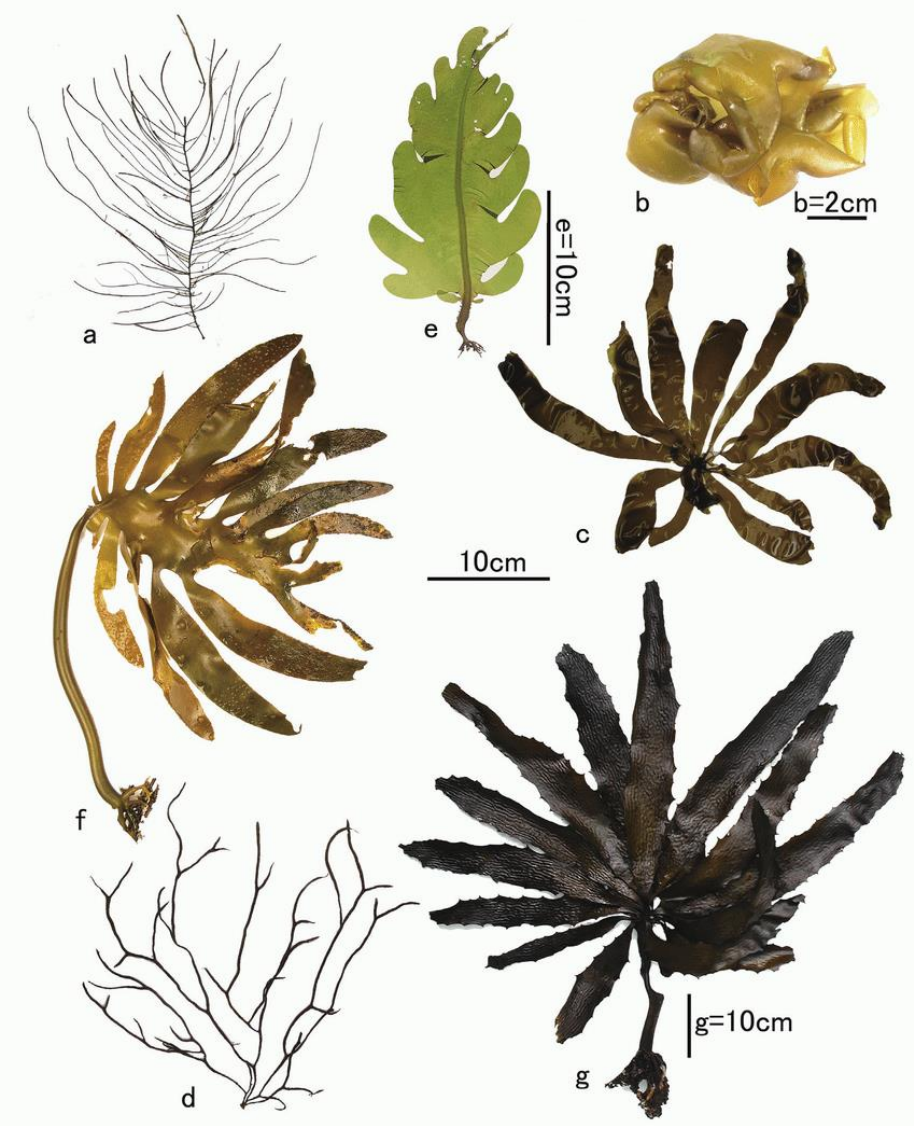

Figure 1. Specimens of Phaeophyceae **(a)** *Papenfussiella kuromo*, **(b)** *Colpomenia sinuosa*, **(c)** *Petalonia binghamiae*, **(d)** *Cutleria cylindrical*, **(e)** *Undaria pinnatifida*, **(f)** *Ecklonia cava*, and **(g)** *Eisenia arborea* (Kimbara et al., 2015).

As mentioned, “algae” encompass a diverse group of organisms originating from different evolutionary lineages. These taxa are classified into eukaryotic and prokaryotic categories:

- **Eucaryotic algae** These algae are further classified into various phyla based on their morphology, pigmentation, life cycle, and cellular structure. They include the red algae (Rhodophyta), brown algae (Phaeophyta), and green algae (Chlorophyta). Within the Plantae Kingdom, there are four phyla: Glaucophyta, Rhodophyta, Chlorophyta, and

Charophyta. In the Chromista kingdom, there are four phyla: Haptophyta, Cryptophyta, Ochrophyta, and Cercozoa. Additionally, two phyla, Mizozoa and Euglenozoa, belong to the Protozoa Kingdom (Khan et al., 2018; Masojídek et al., 2013).

- **Procaryotic algae** belong to the phylum Cyanophyta. They are photosynthetic Gram-negative eubacteria without membrane-bound organelles (plastids, mitochondria, nuclei, Golgi bodies, and flagella). They are further categorized into Cyanophyceae (blue-green algae) and Prochlorophyceae. Some commonly studied genera of Cyanophyceae used in microalgae cultures for various products include *Anabaena*, *Arthrospira (Spirulina)*, *Microcystis, Nostoc, Oscillatoria*, and *Phormidium* (Khan et al., 2018).

Algae can also be categorized based on their nutritional mode, meaning the carbon and energy sources they utilize, into autotrophic, heterotrophic, mixotrophic and photoheterotrophic as shown in Figure 2 (Hu et al., 2018). Autotrophic algae derive their energy for growth through photosynthesis, while heterotrophic algae depend on external sources from their surroundings of organic matter and nutrients for their metabolic requirements (Hu et al., 2018). Mixotrophic algae are able to perform photosynthesis, but simultaneously, they intake organic nutrients from exogenous sources to supplement their energy requirements. This adaptive strategy enables them to thrive in diverse environmental conditions and successfully exploit various sources of nutrients (Brennan and Owende, 2010). It is important to note that mixotrophic microalgae also possess the capability of photoautotrophic metabolism (Hu et al., 2018). Finally, photoheterotrophic utilize light to oxidize organic carbon sources to obtain carbon for their metabolic processes and energy needs (Zhou et al., 2017).

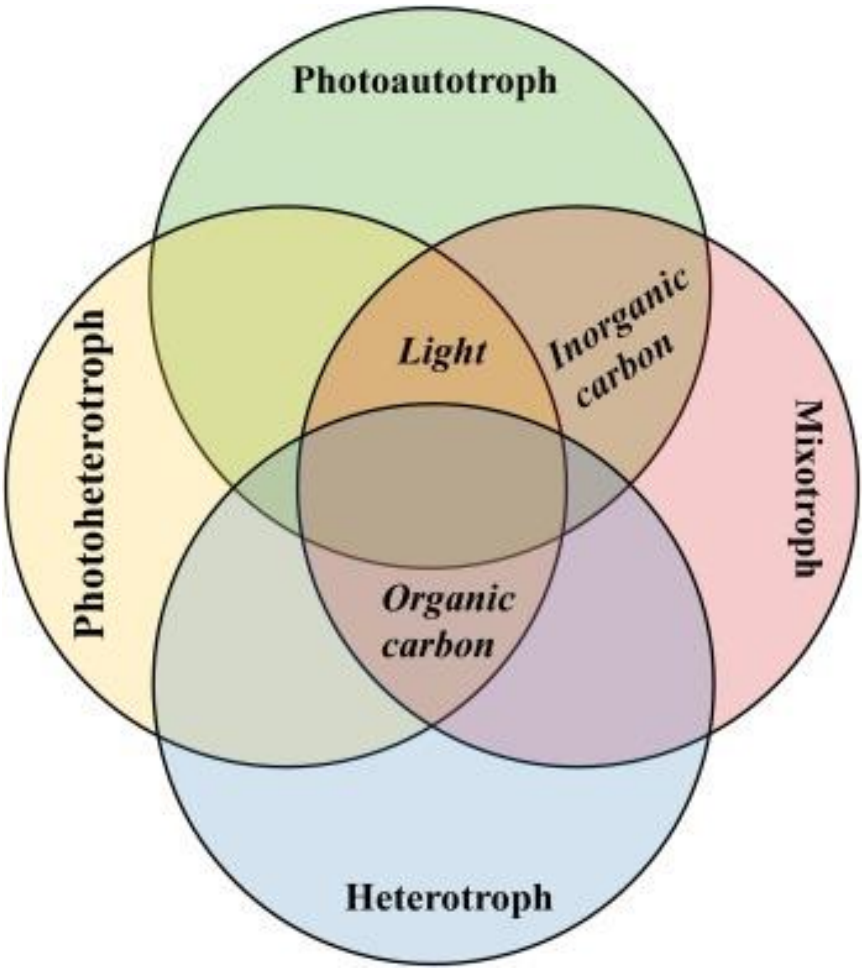

Figure 2. Light, inorganic and organic carbon needs of photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic nutritional modes of microalgae (Daneshvar et al., 2021).

Algae exist autonomously, but they are often involved in a wide array of interactions and connections with other organisms, including symbiotic associations, epiphytism and parasitism (Barsanti et al., 2008).

1.3 Microalgae

Microalgae are eukaryotic unicellular microorganisms, able to perform photosynthesis and to produce low molecular weight organic compounds. Some species are being exploited as a source of lipids, pigments and dyes in the industrial sector, while others are under investigation for the

production of next generation biofuels (O'Neill et al., 2016). They are an incredibly diverse group of organisms that are found everywhere on the earth in both aquatic and terrestrial habitats. Macroalgae in coastal regions, phytoplankton and benthic they serve as the primary producers in both marine and freshwater ecosystems, playing a vital role in sustaining life (Reynolds, 2006). Microalgae are able to adapt in extreme environments such as in hot springs (Brock, 1985) and glaciers (Bunt and Wood, 1963). It is estimated that the number of microalgae species exceeds 50,000, with approximately 30,000 of those have been studied (Richmond, 2013).

In terms of size, microalgae range from a few micrometers, typically around 0.250 μm while some diatom species can reach lengths of 1-2,000 μm (Khan et al., 2018). The diversity of microalgae extends beyond species variation, with distinct morphological characteristics within different life stages of the same species (Figure 3). The typical shapes of microalgae are amoeboid, palmelloid (also referred to as capsoid), coccoid, filamentous, flagellate, and sarcinoid microalgae (Andersen, 2013).

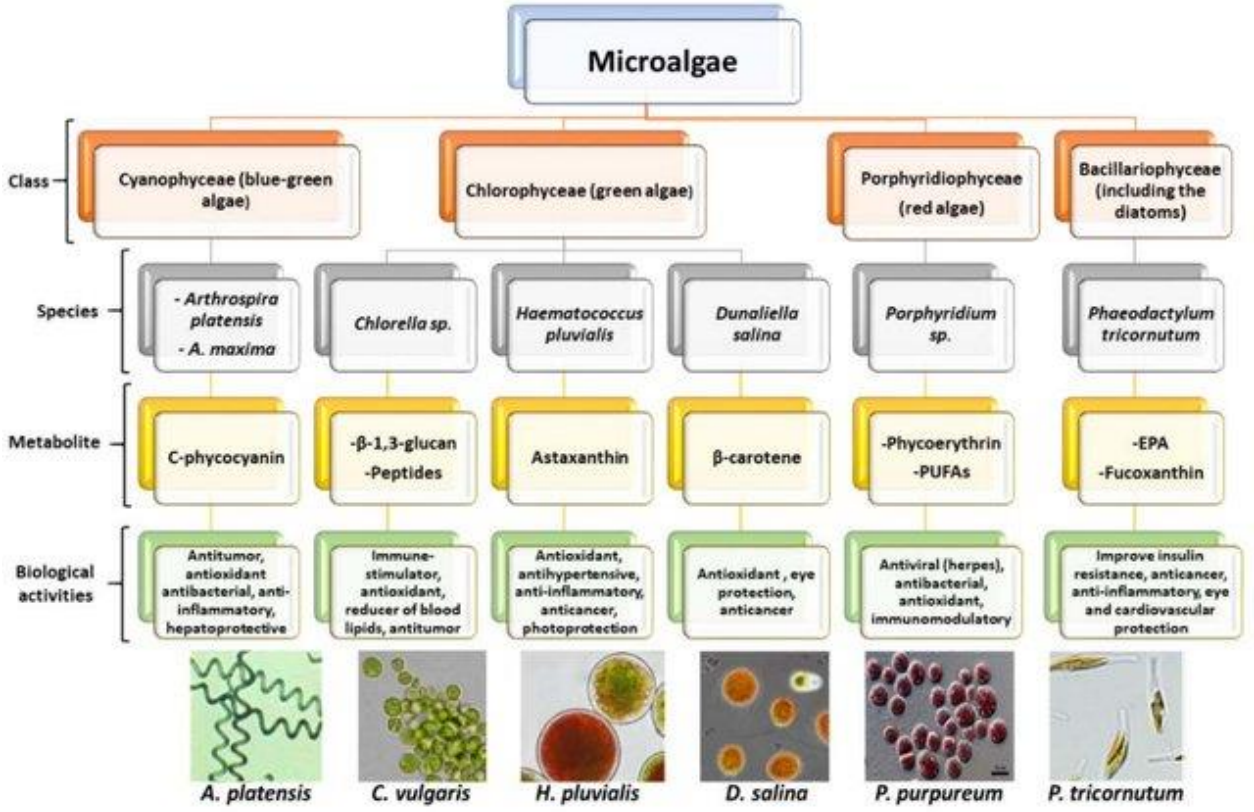

Figure 3. Main microalgae classes, their most important species, and associated biological activities (From: Enhance Microalgae Project available at https://www.enhancemicroalgae.eu/wp-content/uploads/2020/05/EMA-Strain-catalogue-2nd-Edition.pdf).

1.3.1 *Stichococcus* (Trebouxiophyceae, Chlorophyta) genus

The filamentous green algae taxa of *Stichococcus* belongs in the Prasiola clade of the Trebouxiophyceae (Nägeli, 1849; Pröschold and Leliaert, 2007). *Stichococcus*-like algae are widely distributed in various habitats, both terrestrial and aquatic ecosystems, such freshwater and brackish water, marine environments, including extreme ones such as hot acidic springs, and even snow (Butcher, 1952; Hodač et al., 2016; Pröschold and Darienko, 2020). Their small cell sizes and simple cell structure, as well as their resistance to abiotic stress, allows them to disperse over long distances (Sharma et al., 2007). They inhabit biofilms on natural and artificial substrates and in soils, with almost every soil containing *Stichococcus* sp. (Pröschold and Darienko, 2020). Morphologically, *Stichococcus* sp. has cylindrical or short-cylindrical cells with plate-like chloroplasts, sometimes with pyrenoids. They can form short to moderately long filaments and

reproduce through vegetative division (Figure 4) (Pröschold and Darienko, 2020). Certain strains of *Stichococcus bacillaris* isolated from polar terrestrial environments, have attracted attention for their metabolic capabilities and biotechnological potential, as they exhibit high resistance to stresses and metabolic versatility due to their adaptation to harsh conditions such as low temperatures, nutrient scarcity, and limited liquid water availability (Chen et al., 2012; Hodač et al., 2016). Some *Stichococcus* strains have even demonstrated extreme resistance by surviving exposure to space environments (Scalzi et al., 2012).

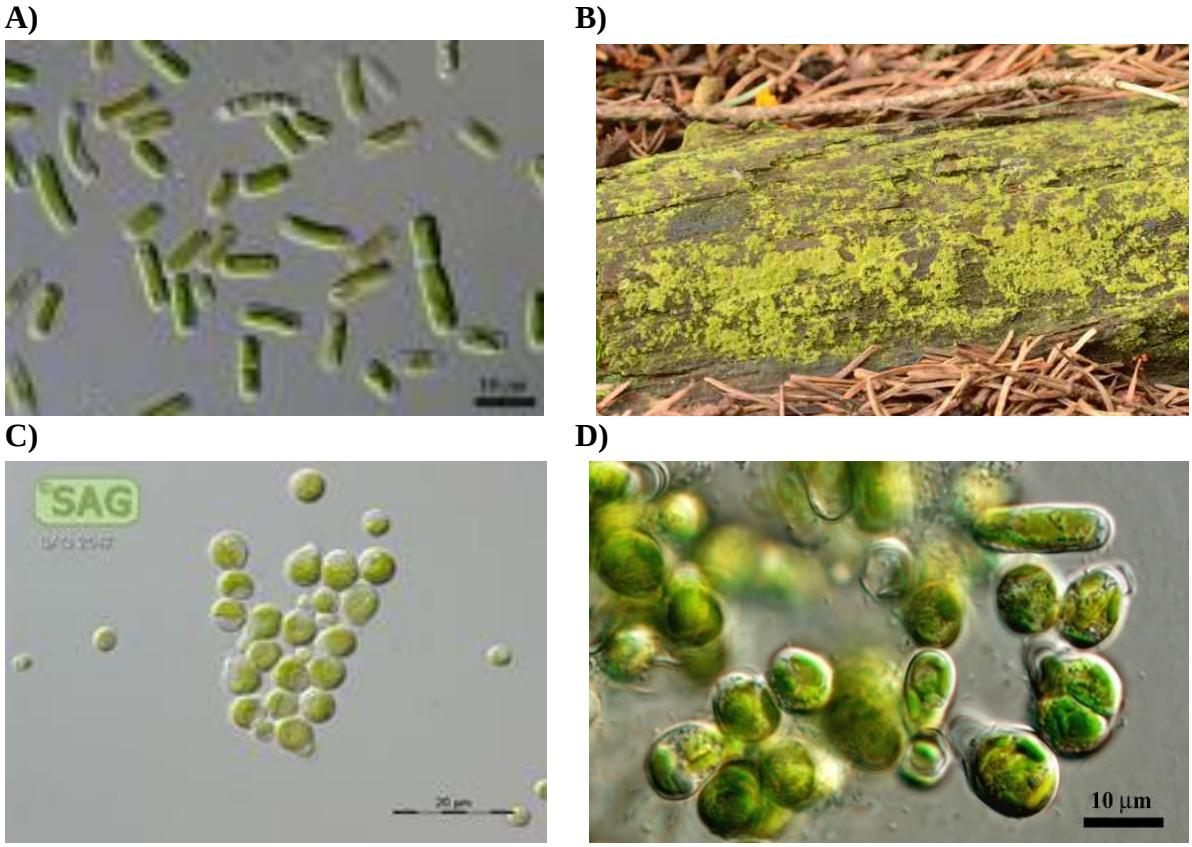

Figure 4. Morphology of various *Stichococcus* species **A)** *Stichococcus bacillaris* **B)** *Stichococcus bacillaris* growing on a tree bark **C)** *Stichococcus ampulliformis* **D)** *Stichococcus chlorelloides* (https://ccala.butbn.cas.cz/).

1.4 Microalgae cellular structure

The cellular structure of microalgae exhibits remarkable diversity, surpassing that observed in animal and plant cells, as it is directly associated with the phylogenetic origins of microalgae taxa which reflects evolutionary changes, they have undergone over a period of 3.5 billion years and also their adaptability to dynamic environments (Masojídek et al., 2013). Procaryotic algae, are characterized by relatively simple structure with DNA and numerous thylakoids lying in the cytoplasm without membrane organelles (Tomaselli, 2003). On the contrary, eukaryotic algae display a much more complex cellular organization as they have various membrane-bound organelles that are needed for their highly specialized cellular and metabolic functions (Andersen, 2013; Tomaselli, 2003). Both procaryotic and eukaryotic microalgae have a cell wall, that provides structural support and protection to the cell. It consists mainly of microfibriles of cellulose or a layer of peptidoglycan but the biochemical composition of cell walls can vary between different algal groups (Andersen, 2013; Tomaselli, 2003). The chloroplast, is the dominant organelle in eukaryotic algae as it encloses the thylakoid membranes where light-harvesting pigments such as chlorophylls and phycobiliproteins are located and thus where photosynthesis occurs (Tomaselli, 2003). In eukaryotic algae inside the cytoplasm as mentioned before there are various organelles, each with specific functions. These are Golgi apparatus, endoplasmic reticulum, ribosomes, mitochondria, vacuoles, various plastids and lipid globules (Tomaselli, 2003).

Some microalgae form specialized structures such as cysts or spores and flagella (Tomaselli, 2003). Spores are cells with thickened cell walls that represent dormant stages that enabling the survival in difficult environments (Andersen, 2013). Flagella, serve as locomotory organs in microorganisms, featuring a complex structure that includes an axoneme composed of nine peripheral double microtubules surrounding two central microtubules. The entire structure is enveloped by the plasma membrane (Tomaselli, 2003).

1.5 Growth parameters

Microalgae cultivation has gained a lot of scientific and commercial traction due to their potential use for producing high-value products. In order to achieve successful and productive

cultivations of microalgae several key parameters affecting the growth of microalgae need to be carefully controlled to achieve optimal productivity. These parameters include carbon dioxide (CO_2), inorganic nutrients, light, temperature, pH, and other factors.

1.5.1 CO_2

Carbon dioxide is a vital substrate for phototrophic and mixotrophic microalgae photosynthesis and hence algal growth and reproduction, as it used by the algal cell for respiration, energy production, or the synthesis of macromolecules (Berman-Frank and Dubinsky, 1999). The rate of carbon fixation directly affects the growth rate of algae. Carbon can be obtained and utilized in various forms, depending on the growth mode of the algae. For autotrophic growth, algae can utilize carbon in the form of CO_2, carbonate, or bicarbonate while in heterotrophic growth, algae rely on organic carbon sources, such as acetate or glucose (Juneja et al., 2013). The form of carbon that is utilized also depends on the specific environmental conditions, such as pH, temperature, and nutrient content. It is often supplied through gas bubbling or using flue gases from industrial processes (Juneja et al., 2013). Biological CO_2 fixation by microalgae is a promising and novel tool to reduce CO_2 emission (Goswami et al., 2022; Khoobkar et al., 2022; Ou et al., 2021; Wang et al., 2018). Microalgae generate organic materials by converting sunlight into carbohydrates and oxygen. The sequestrated CO_2 is converted into high added value products (i.e., lipids, proteins, carbohydrates, pigments) without emitting pollutants (Vuppaladadiyam et al., 2018). Microalgae biomass is consisted of 50% carbon and 1.83 kg CO_2 can be fixed by only 1 kg microalgae (Ji et al., 2013; Pavlik et al., 2017). Some microalgae species can have high CO_2 tolerance capacity. Microalgae such as *Scenedesmus obliquus*, *Chlorella* sp., *Chlorococcum littorale*, and *Desmodemus* sp. have been reported to tolerate 34% (Molitor et al., 2019), 40% (Park et al., 2021), 70% (Ota et al., 2009), and even 100% (Kativu et al., 2012).

1.5.2 Inorganic nutrients

Microalgae require plenty of inorganic nutrients, including nitrogen and phosphorus, and micronutrients, for their metabolic processes. Nitrogen is an essential constituent of all structural and functional proteins in algal cells and it constitutes approximately 7% to 20% of the cell's dry weight (Hu, 2004). It also plays an important role in lipid and carbohydrate synthesis (Yodsuwan

et al., 2017; Zarrinmehr et al., 2020). Algae can rapidly assimilate inorganic nitrogen from their surroundings, converting it into biologically active compounds. Microalgae then can easily recycle it in order to meet the physiological demands (Hein et al., 1995). Therefore, nitrogen starvation can reduce the photosynthetic capacity of the algal cells and alter the enzyme balance, resulting in higher lipid production and decreased chlorophyll and pigment accumulation (Juneja et al., 2013).

Phosphorus is another vital nutrient for algae, as it is a fundamental component of DNA and RNA. Additionally, phosphorus is fundamental in the formation of phospholipids, the building blocks of cellular membranes (Hu, 2004). Phosphorus content in algae is typically around 1% of their dry weight (Vymazal, 1988). Phosphorus limitation leads in reduced photosynthetic capacity, as the synthesis and regeneration of substrates in the Calvin-Benson cycle is reduced (Barsanti and Gualtieri, 2022). Also, it may result in the buildup of lipid and reduced protein content in the algal cell (Kilham et al., 1997).

Trace metals are essential micronutrients for algal growth that are present in very small quantities (<4 ppm) within the cells. The six most important trace metals for algae are iron (Fe), manganese (Mn), cobalt (Co), zinc (Zn), copper (Cu), and nickel (Ni), as they serve various metabolic functions (Bruland et al., 1991). Insufficient levels of trace metals can restrict algal growth, while excessive or high concentrations of metals beyond the toxicity threshold can inhibit growth, hinder photosynthesis, deplete antioxidants, and damage the cell membrane (Bruland et al., 1991).

1.5.3 Light

Light enables the photosynthetic processes in phototrophic and mixotrophic microalgae (Figure 5). Intensity, duration, and quality of light affect microalgae growth, as they directly impact their photosynthetic activity and, consequently, influence their biochemical composition and biomass productivity (Krzemińska et al., 2014). Optimal growth of microalgae is achieved within specific ranges of light intensity, as both extremely low and high intensities can hinder their efficient growth (Sforza et al., 2012). Photoperiod, also affects microalgal growth, with studies suggesting that a 16-hour light/8-hour darkness cycle is optimal for many species (Amini Khoeyi et al., 2012). Therefore appropriate light intensity and duration is a crucial parameter for successful

cultivation, as it helps prevent photo-oxidation and growth inhibition (Amini Khoeyi et al., 2012). For optimal growth, most microalgae species require light intensities ranging from approximately 200 to 400 μmol photons·m^{-2}·s^{-1} (Huesemann et al., 2013; Khan et al., 2018; Schuurmans et al., 2015).

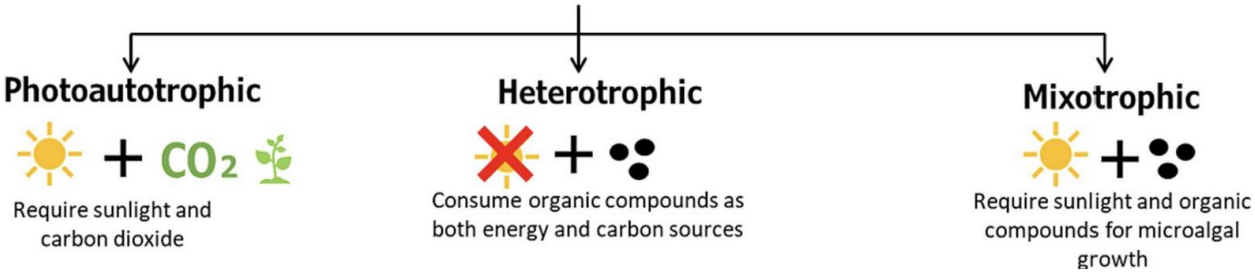

Figure 5. Microalgae cultivation technologies (Wan Mahari et al., 2022).

Light can be obtained from natural sources, like sunlight, or artificial sources, such as lamps (i.e., fluorescent, LEDs) (Abu-Ghosh et al., 2016; Blanken et al., 2013). While artificial light is more expensive than sunlight, it offers precise control over microalgal growth which is required when microalgal biomass is used for high-added value products production (Abu-Ghosh et al., 2016; Blanken et al., 2013). In recent years, a method has been developed that aims in decreasing energy consumption while increasing microalgae photosynthetic efficiency using flashing light. Flashing lights included illumination of microalgae cultivation systems light-emitting diodes (LEDs) (Park and Lee, 2001). These lights are applied in short and intense flashes, allowing light to penetrate deeper into the culture and ensuring that the photosynthetic apparatus of microalgae is operating near its maximum capacity, a phenomenon known as the "flashing light effect" or "light integration effect" (Abu-Ghosh et al., 2016). Under this type of light, cultures can even achieve higher photosynthetic rates compared to continuous light at the same light intensity (Terry, 1986). This novel approach, therefore, can provide the means for “greener” methodology for optimizing phototrophic cultivation, resulting in higher growth performance and reduced photodamage and energy costs compared to continuous illumination (Schulze et al., 2017).

1.5.4 Temperature

Temperature is another critical factor that affects microalgae growth and biochemical composition. Each microalgae species has an optimal temperature range for growth, with deviations for the optimal range impacting algal growth and activity, or even leading to cell death (Béchet et al., 2017). In cultivation systems, the appropriate temperature conditions should be ensured in order to maximize productivity, which for most algal species is 20-30 °C (Ras et al., 2013), but thermophilic species can endure up to 80 ºC (Covarrubias et al., 2016). Temperature is, also, one of the main factors that affects large scale open cultivation systems (Béchet et al., 2010). It can, also, serve as means for inducing the production of valuable metabolites as manipulating temperature conditions can influence the synthesis of specific compounds in microalgae (Converti et al., 2009).

1.5.5 pH

The pH of the culture media is another factor that affects the growth of microalgae. Microalgae species exhibit varying pH preferences for optimal growth, and alterations in environmental pH can significantly impact their enzymatic and protein activities. While most microalgae thrive in neutral pH conditions, certain species have been observed to grow under highly acidic (pH < 5) or alkaline (pH > 9) conditions (Li et al., 2020). It has also been observed that pH influences the solubility and availability of CO_2 and other essential nutrients and the salinity of the culture media (Chen and Durbin, 1994; Juneja et al., 2013). Moreover, the growth of microalgae can influence the pH of the surrounding medium, subsequently influencing their own growth dynamics (Yu et al., 2022). High dissolved CO_2 concentrations significantly affects water chemistry by decreasing pH and increasing dissolved inorganic carbon (Brown et al., 2020). However, the pH of microalgal cultures rises gradually during the day due to the uptake of inorganic carbon by microalgae. Cell growth can be inhibited by higher pH, which limits CO_2 availability. (Azov, 1982; Chen and Durbin, 1994; Qiu et al., 2017). Contrary to that, high pH can suppress undesired biological contaminants (Bartley et al., 2014).

1.5.6 Other factors

Additional factors, such as salinity and agitation, impact microalgae growth. Salinity, refers to the concentration of sodium chloride in the growth medium, which directly influences the growth rate. It has been observed that relatively high salinity might increase lipids production (Almutairi et al., 2021; Renaud and Parry, 1994; Zhila et al., 2011). Agitation ensures mixing and aeration in the culture which are important for uniform distribution of nutrients, air and CO_2 (Show et al., 2017). It also facilitates the penetration and even distribution of light within the culture, preventing biomass settling and aggregation. Thus, it has been observed that without proper mixing, even if all other requirements are met, the productivity of biomass will be significantly reduced (Khan et al., 2018).

1.5.7 Growth under nutrient stress

Microalgae can synthesize and produce a wide range of secondary metabolites when they are subjected to extreme environmental conditions or grow under stress (Figure 6). Secondary metabolites are organic compounds that that do not directly participate in the primary processes of growth and development, but they have significant functions in the adaptation and survival of microorganisms in harsh environments (Markou and Nerantzis, 2013). Microalgae in their natural environment often undergo nutrient limitation, particularly concerning nitrogen and phosphorus availability. Limitation of these inorganic nutrients can lead to a shift in the metabolic pathway of the organism. Depending on the degree and type of nutrient limitation, considerable variation in the biochemical composition can be observed in the microalgae culture (Minhas et al., 2016; Titman, 1976).

Nitrogen deficiency impacts algal cultures as it leads to an increase in the biosynthesis and accumulation of lipids and triglycerides, while simultaneously reduces protein content (Juneja et al., 2013). This results in a higher lipid/protein ratio negatively affecting the growth rate (Li et al., 2008). Low nitrogen concentration can also divert their photosynthetically fixed carbon to carbohydrate synthesis, although the implications of this remain unclear (Hu, 2004). In some algal genera, such as cyanobacteria and red algae, nitrogen limitation leads to degradation of phycolbillisomes, the light harvesting antennae for photosystem II, and so, photosynthetic rate

drops (Collier and Grossman, 1992). Therefore, nitrogen starvation may lead to a decrease in photosynthetic activity due to reduced carbon dioxide fixation and chlorophyll content (Richardson et al., 1969). Finally, in the case of *Spiriluna platensis* nitrogen starvation can result in the accumulation of excess (Baky et al., 2020), while *Dunaliella* sp. and *Haematococcus pluvialis* accumulate high amounts of pigments and acyl esters (Ben-Amotz and Avron, 1990; Borowitzka et al., 1991)

Phosphorus deprivation has also been found to cause lipid accumulation and astaxanthin production, while reducing chlorophyll α and protein content, which in turn increases the amount of carbohydrates in algal cells (Juneja et al., 2013). Overall it decreases cell growth, and so, careful evaluation is necessary when attempting to increase lipid concentration via nitrogen or phosphorous limitation to ensure high lipid productivity (Metting, 1996).

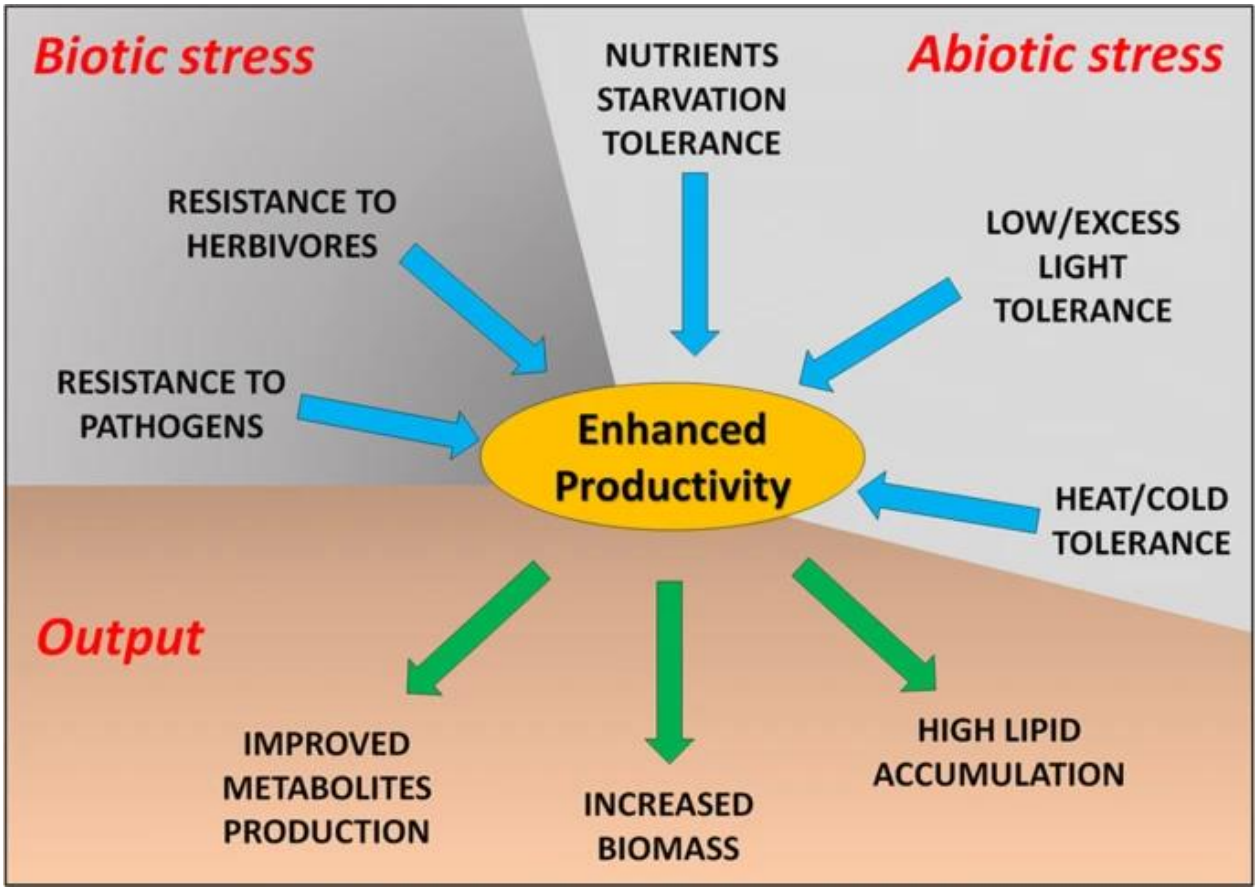

Figure 6. Illustration of stress inducing strategies implemented in microalgal cultivation and their beneficial outcomes (Benedetti et al., 2018).

1.6 Bio-products and applications

Microalgae, as very diverse group of organisms are of commercial significance due to their distinct features such as unique biochemical and physiological properties (Richmond, 2013). Due to their vast and unexplored nature, microalgae are considered an untapped resource for numerous valuable products (Rizwan et al., 2018). Microalgae are utilized in two primary applications: the generation of biomass, and the cultivation of microalgae for the isolation of biologically active substances (Figure 7) (Dolganyuk et al., 2020).

Microalgae are rich in biologically active compounds (Figure 7). These metabolites can be categorized as primary (i.e., essential compounds for the survival of microorganisms such as lipids, proteins, and carbohydrates) and secondary (i.e., pigments like carotenoids, astaxanthin, and polyhydroxyalkanoates-PHA) (Japar et al., 2021). A lot of these possess antioxidant, antibacterial, antiviral, antitumor, and various medicinal properties that are high in demand in medicine, in cosmetics industry, in agriculture and in food industry (Gürlek et al., 2020).

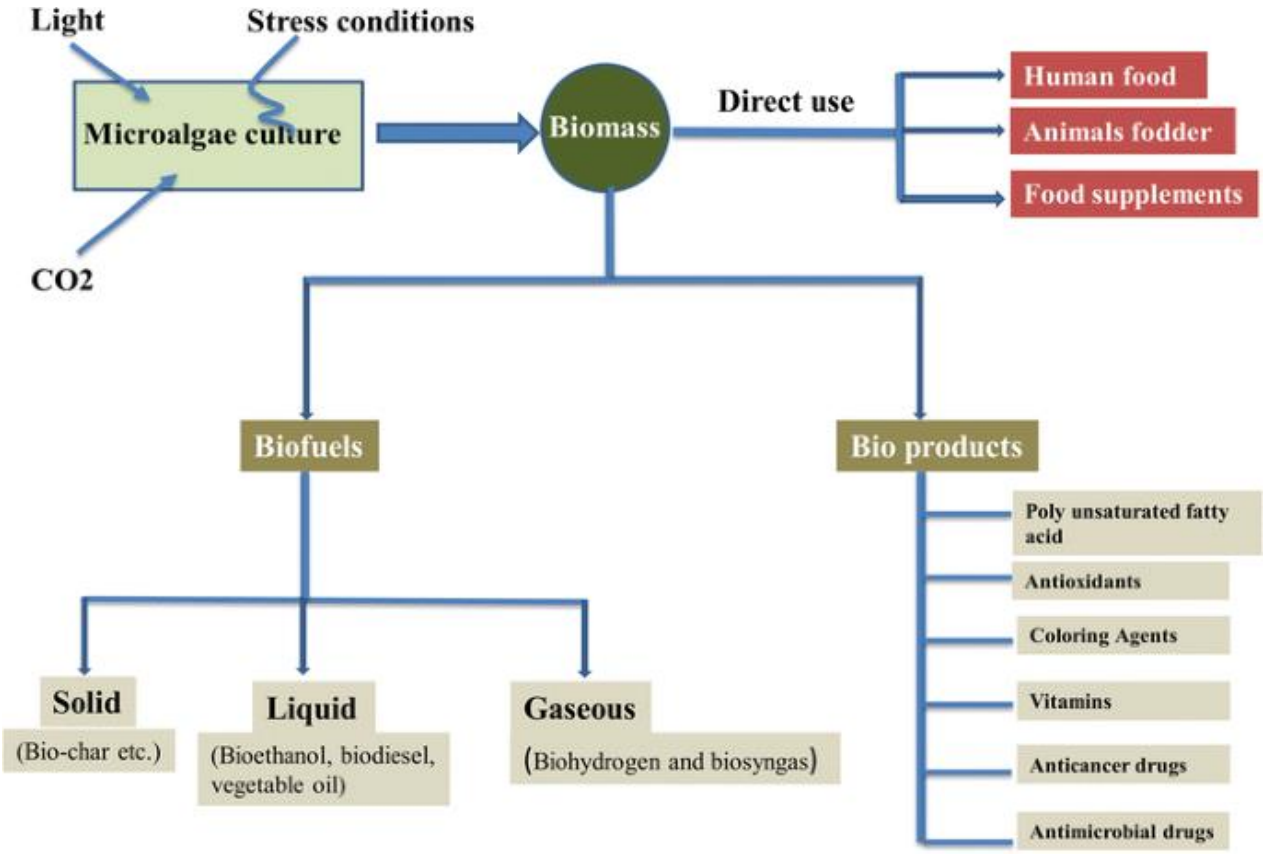

Figure 7. Microalgae photosynthesize and convert atmospheric CO_2 to biomass that can be directly used or utilized for bio products and biofuels production (Khan et al., 2018).

1.6.1 Proteins

Proteins derived from microalgae have gained attention as an alternative protein source in food and animal feed industries. Microalgae are a rich resource of proteins, with some species such as *Arthrospira, Chlorella, Aphanizomenon*, and *Nostoc* having up to 70% of their biomass dry weight comprised of this macronutrient (Caporgno and Mathys, 2018). Microalgae protein exhibits a balanced amino acid profile and contain all essential amino acids for human consumption, making it a potential substitute for traditional protein sources like soy and animal-derived proteins (Henchion et al., 2017). Additionally, a number of microalgae species produce enzymes with a lot of commercial applications, including enzymes with antioxidant properties such as superoxide dismutase, catalase, and peroxidase activities (Toyub et al., 2012). Finally, microalgae are a valuable source of peptides, which are short sequences of amino acids, that have been proven beneficial for human nutrition and medicine as they possess significant antimicrobial, antioxidant, anti-inflammatory, anti-hypertensive, and anti-atherosclerotic properties (Echave et al., 2021; Sathya et al., 2021).

1.6.2 Carbohydrates

Carbohydrates are another valuable microalgal bioproduct. Carbohydrates form a significant portion of microalgae biomass, making up roughly 20% of it, stored in the form of starch or other polysaccharides, including β-glucans, sulfated polysaccharides, and exopolysaccharides (Eltanahy and Torky, 2021). Starch and cellulose can be used for biofuels production while some polysaccharides and oligosaccharides have been previously examined for their prebiotic effects (Eltanahy and Torky, 2021). Other carbohydrates, such as agars, agarose, are valued for their gelling and thickening properties in the food and pharmaceutical sectors, and some such as alginates and carrageenans have been used as preservatives (Radmer, 1996).

1.6.3 Lipids

Microalgae-derived lipids have been extensively investigated for their potential commercial use (Maltsev and Maltseva, 2021). They can be utilized in dietary supplements, functional foods, and pharmaceuticals. The lipid content in algae usually ranges from 20 to 50%, but can reach up to 80% depending on the strain and cultivation method (Sun et al., 2018). Compared to terrestrial crops, microalgae can produce oil yields that are significantly higher and faster with similar land occupation (Shahid et al., 2020), which has led to significant interest in their potential use environmentally friendly producer of biodiesel (Sajjadi et al., 2018).

The lipids produced by microalgae are composed of fatty acids and glycerol, and they come in a variety of forms including triacylglycerols (TAGs), phospholipids, and carotenoids. TAGs are the main form of lipid storage in microalgae and are of particular interest as a source of biofuel because they can be converted into biodiesel. It is worth noting that microalgae are a great source of Polyunsaturated fatty acids (PUFAs). This fatty acids, namely Linoleic acid (C18:2 or Omega-6), linolenic acid (C18:3 or Omega-3) and others, offer various health benefits to humans including cardiovascular and cognitive health (Oliver et al., 2020). Furthermore, Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) are widely used in aquaculture as they are essential in feeding aquatic organisms (B. R. Kumar et al., 2021). Finally, microalgae are an excellent source of phytosterols, that are beneficial human health as they help with cholesterol reduction, and possess anti-inflammatory and even anti-cancer properties (Randhir et al., 2020).

1.6.4 Pigments

Microalgal pigments are involved in the photosynthetic apparatus. These pigments exist in the form of antennae complexes inside the thylakoid membranes (Pagels et al., 2020). Pigments are broadly classified into three categories, namely chlorophylls (chl), carotenoids and phycobiliproteins (Masojídek et al., 2013). Every algae group produces a different combination of pigments; for example, Chlorophyta produce chlorophyll-a,-b, and carotenoids (Moheimani and Parlevliet, 2013). Photosynthetic pigments absorb light in the visible spectrum 400 – 700 nm, which is designated as the Photosynthetically Active Radiation, often abbreviated as PAR. Light is captured by the cells, for the synthesis of $NADPH_2$, ATP and various biochemical, then through

enzymatic reactions $NADPH_2$ and ATP utilize CO_2 for the production of carbohydrates (Moheimani and Parlevliet, 2013).

Carotenoids, which are yellow, orange, or red terpenoid pigments. More than 600 compounds have been recognized that are broadly categorized into two types: carotenes, such as α and β-carotene, and xanthophylls, such as astaxanthin and lutein (Pagels et al., 2020). Carotenoids have great commercial value and so microalgae have garnered increasing attention as a sustainable source. Of the carotenoids derived from microalgae, β-carotene, lutein, and astaxanthin are the most commercially valuable, given their antioxidant, anti-inflammatory, vitamin A precursor, and neuroprotective properties (Cezare-Gomes et al., 2019; Rammuni et al., 2019). These pigments have already found extensive use in diverse industries such as food, nutraceuticals, pharmaceuticals, aquaculture, and cosmetics (Dufossé et al., 2005).

1.6.5 Biofuels

Great scientific research has recently been focusing on implementing microalgae biofuel production Because of their elevated lipid levels. Algal biomass is a sustainable and renewable resource for the production of various biofuels, such as biodiesel, bioethanol and biobutanol, biogas, biohydrogen, and biochar (Figure 8) (B. R. Kumar et al., 2021). Microalgae have been shown to be able to produce bioelectricity through Microbial Fuel Cells, as well as for environmental remediation purposes (Figure 8). However, it should be noted that these research efforts are still in their early stages (Reddy et al., 2018).

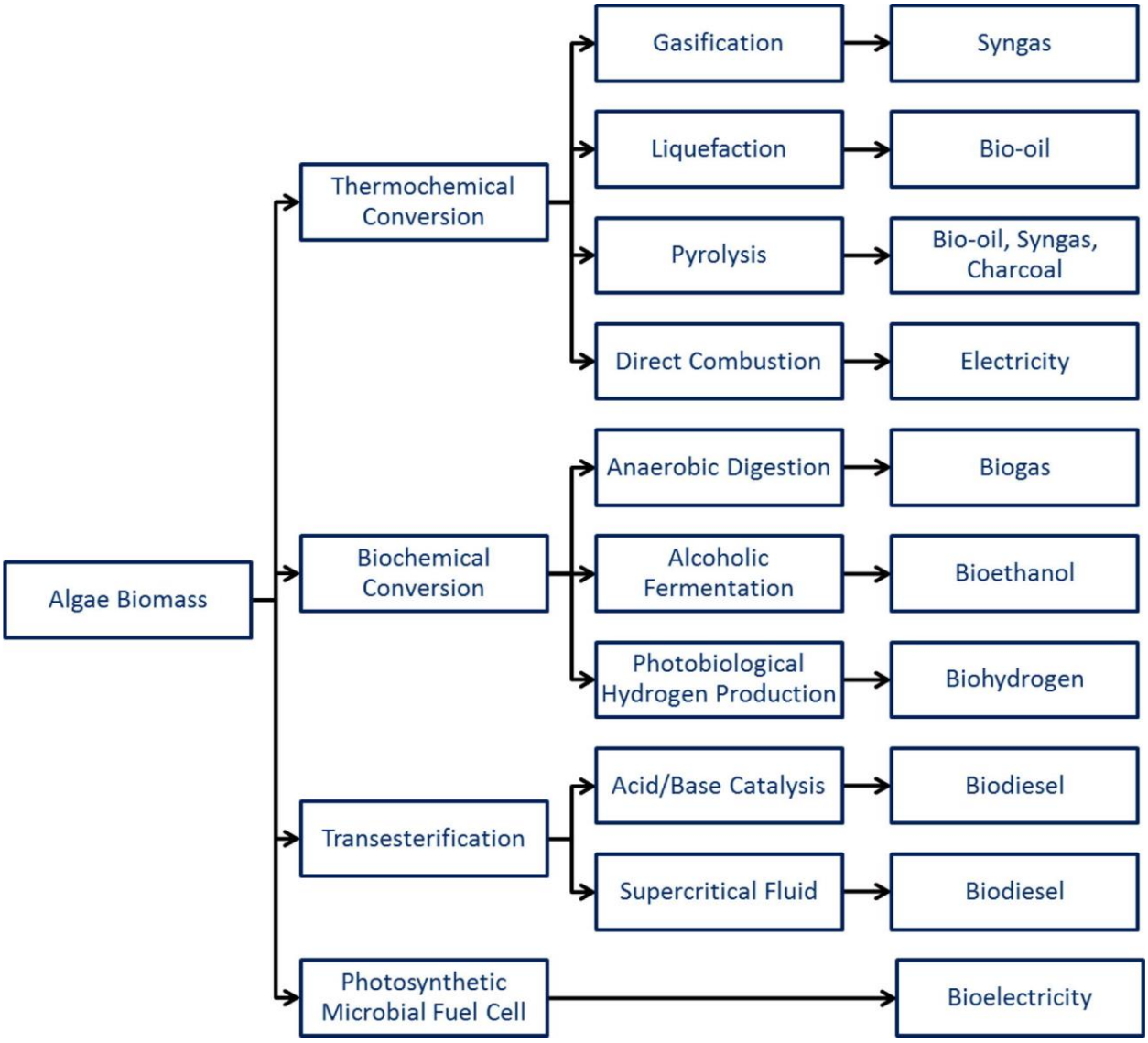

Figure 8. Microalgae biomass conversion processes for biofuels production (Chew et al., 2017).

1.6.6 Bioplastics

Microalgae are a good potential producer of bioplastics, that are biodegradable polymers obtained from various biological resources such animals, plants, algae, and microorganisms (Calijuri et al., 2022). Plastics, which are typically derived from petroleum contribute to greenhouse gas emissions and are very timely to degrade in nature. On the contrary, bioplastics are derived from sustainable sources and can be used as conventional plastics. Bioplastics today are commonly produced from agricultural crops such as corn, wheat, soy proteins, milk, collagen,

and gelatin. However, there are concerns about the sustainability of this bioplastic feedstocks due to competition for land and water resources (Calijuri et al., 2022; Onen Cinar et al., 2020). Microalgae serve as a greener alternative for bioplastics production. Microalgae can synthesize biopolymers like polyhydroxyalkanoates (PHA), starch, and protein. Stress inducing cultivation have been show to enhance biopolymer accumulation as they promote PHA accumulation as microalgae produce carbon-rich compounds for energy storage (Gifuni et al., 2017; Mathiot et al., 2019; Vieira de Mendonça et al., 2021). Thus, utilizing microalgae for bioplastics production on difficult substrates such as eutrophic waters and waste waters offers a promising way for sustainability in the plastics industry (Castro et al., 2020).

1.6.7 Others

Certain types of microalgae are rich in diverse water-soluble and lipid-soluble vitamins, such as vitamins A, B-complex, C, D2, D3, E and K (Eltanahy and Torky, 2021). For instance, *Chlorella* sp. are known to accumulate considerable amounts of vitamin C and B12 (Watanabe et al., 2002). Microalgae also produce different kinds of antioxidant, enzyme polymers and toxins which can find several applications in industrial products (Moreno-Garcia et al., 2017). It should be also noted here, that, there are numerous biochemical compounds that remain undiscovered within microalgae, as it is difficult to isolate, for example from microalgae that form symbiotic relationships with sponges (Wijffels, 2008).

1.7 Mutation processes

It has become apparent that microalgae are an excellent source of renewable energy, nutrition, and pharmaceuticals, and are now beginning to be utilized as a raw material in both economic and ecological terms due to the high volumetric productivity and minimal environmental impact of their cultivation. The goal, therefore, is to achieve the economically viable use of microalgae in the industry. This required optimized biotechnological processes by enhancing the productivity and robustness of the cell “factories”. Apart from ensuring biological and abiotic parameters in their cultivation process, another essential step is ensuring that a robust, high-performance strain is used (Brar et al., 2021). Mutation processes help identifying or creating these

robust strain (or species) as they can result in the emergence of new strains that can be used commercially (Bleisch et al., 2022). By inducing or selecting for specific mutations, robust microalgae strains with desirable traits, such as increased growth rates and biomass productivity, enhanced lipid or pigment production, improved tolerance to environmental stressors, or altered biochemical compositions, are selected through screening and selection processes (Olabi et al., 2023).

1.7.1. Types of mutation processes

There are two main approaches to enhance the performance of cellular factories metabolic engineering and random mutagenesis (Woolston et al., 2013). It is important to note that to successfully implement these strategies a thorough understanding of microbial physiology, genetics, metabolomics, and environmental interactions is needed.

Spontaneous or Random mutagenesis is a strategy that can overcome the regulatory concerns of the utilization of genetically modified organisms (GMOs) in industrial sectors (Bleisch et al., 2022). These strategies aim to achieve desired metabolic capabilities without relying on genetic modification. Such approaches might involve the utilization of natural genetic variation, directed evolution, or the engineering of non-GMO microbial strains. Through random mutagenesis the rich biodiversity and adaptability of microorganisms is implemented to identify or engineer cellular factories with improved properties (Bleisch et al., 2022). They can involve screening and selecting naturally occurring microorganisms or their derivatives with desirable traits (Acevedo-Rocha et al., 2014). The concept of random mutagenesis involves an iterative exposure to physical or chemical mutagens, such as UV radiation, ionizing radiation, such as gamma irradiation, X-rays, alkylating and intercalating agents, yielding a genetic and phenotypic diversity of mutants, which have to be screened for the desired cell properties and improved metabolic functions (Bleisch et al., 2022). Chemical methods include ethyl methanesulfonate (EMS) or nitrosomethyl guanidine) (Sivaramakrishnan and Incharoensakdi, 2017). Ethyl methanesulfonate ($CH_3SO_3C_2H_5$) is a volatile organic solvent which is mutagenic and carcinogenic. EMS is an ethylating agent which produces random mutations in the DNA and RNA by nucleotide substitution, specifically by guanine alkylation. Thus, at the presence of EMS, cytosine in DNA is frequently replaced by thymine (while in RNA cytosine is replaced by uridine),

which results to the production of mutant cells (Sarmiento et al., 2011). Ethyl methanesulfonate is more potent mutagen compared to UV radiation, for the generation of mutated microalgae strains (Tillich et al., 2012).

Laboratory research has also been conducted on genetically engineered microalgae. Metabolic engineering involves targeting specific parts of metabolic pathways within microalgae cells to alter the flux of the metabolites and target a specific bioproducts, such as the fatty acid or isoprenoid biosynthetic pathways. Another application of genetic engineering is protein recombination. An exogenous gene encoding for the desired protein is inserted into the algal genome in a strategic way, which enables the protein to be increased and targeted to a specific subcellular region of the algae cells (Naghshbandi et al., 2020).

1.7.2. Mutation as a strain selection mechanism

Metabolic engineering focuses on optimizing metabolic pathways through targeted manipulation of enzymatic activities. This involves techniques such as gene editing, gene insertion, knock-out or overexpression, or introducing new enzymatic conversions or enhancing desired metabolic pathway in the cell (Gimpel et al., 2015). These sophisticated metabolic engineering tools are still not extensively used in microalgae improvement. Conventional genetic tools such as episomal plasmid expression and homologous recombination are difficult to apply to microalgae (Specht et al., 2010). However, development of precise engineering tools, such as CRISPR/Cas9, combined with advancements in various omics fields facilitates research in microalgae metabolic engineering (Brar et al., 2021).

1.8 Cultivation systems

Over the years, various systems have been used for microalgae cultivation, each offering distinct advantages and challenges (Randrianarison and Ashraf, 2017; Tan et al., 2020). An ideal microalgae culture system should possess certain characteristics. These include an adequate light source for photosynthesis, effective transfer of materials across the liquid-gas barrier, a simple operation procedure, minimal contamination rate, low overall building and production costs, and

high land efficiency (Lam and Lee, 2012a). Two main categories of microalgae culturing systems are commonly used: open ponds and photobioreactors. The term "bioreactor" is commonly used to describe containers are used for the growth of microalgae. Photobioreactors specifically, is a type of bioreactor designed to provide light to photoautotrophic microalgae, which rely on light as their energy source (Daneshvar et al., 2021) (Figure 2).

1.8.1 Open-type photobioreactors

Open pond cultivation is a traditional and cheap method for large-scale microalgae cultivation (Figure 9). It involves the use of natural or artificial bodies of water, such as lakes, ponds, circular ponds, or raceway ponds. Open type systems have large surface areas where microalgae can move freely and utilize natural sunlight. Benefits of open pond systems are lower construction, maintenance, and operation costs, as well as simplicity of operation and scalability (Hannon et al., 2010).

Figure 9. Commercial microalgae production systems: ponds and photobioreactors. **(a–c)** Commercial production systems using paddle wheel mixed raceway pond systems. **(d)**, circular ponds, each ~500 m^2, with central pivot mixing; **(e)** unmixed ponds **(f-g)** tubular photobioreactors (Benemann, 2013).

One type of open pond is the circular pond, which features a rotating agitator at the center to ensure effective blending and avoid settling of algal biomass (Figure 9d) (Hamed, 2016). However, larger ponds may experience increased water resistance and additional stress on mechanical components. Moreover, this design required high energy usage driving up the construction and operating cost (Hamed, 2016). Another common open pond design is the raceway pond, which consists of closed-loop channels and paddlewheels for biomass recirculation and

equal nutrient distribution (Figure 9a-c) (Rogers et al., 2014). Raceway ponds are energy-efficient, with a single paddlewheel typically sufficient for a 5-hectare pond (White and Ryan, 2015).

Open pond systems have several drawbacks. Firstly, the quality of water sources may result in lower microalgae cell concentrations, which required efficient and usually expensive harvesting methods. Additionally, there are often issues with rapid light and temperature changes, salinity, pH changes, as well as erosion and water turbidity, can impact microalgae productivity (Stark and O'Gara, 2012; Tan et al., 2020). Contamination is also a concern. In these types of ponds, cultivating microalgae that can thrive under extreme alkaline or saline conditions can help mitigate these concerns (Tan et al., 2020).

1.8.2 Closed-type photobioreactors

Photobioreactors, enclosed systems used for microalgae cultivation, offer specific advantages over open pond cultures, such as smaller size, controlled and contamination-free growth conditions, and higher nutrient efficiency (Posten, 2009). However, their scalability is limited, and they come with high operating costs (Gupta et al., 2015). There are a lot of photobioreactor types, such as tubular photobioreactors that use long transparent tubes for culture circulation. These bioreactors usually suffer from poor mass transfer. Vertical column photobioreactors have good gas-liquid mass transfer efficiency but lack sufficient light and are expensive to construct (Figure 9f) (Tan et al., 2020). Finally, flat-plate photobioreactors provide high photosynthetic efficiency but microalgae cells can easily be damaged and require innovative designs for improved performance (Figure 9g) (Hannon et al., 2010; Huang et al., 2017).

1.8.3 Attached cultivation

Recently, a third cultivation method, the attached growth system, has gained attention as a promising cultivation method for microalgae. It involves immobilizing microalgae on a support material, such as ropes, fibers, or solid surfaces forming a biofilm on a substrate, which simplifies the separation of microalgae from the growth medium (Figure 10) (Jafari et al., 2018). Compared to suspended growth systems, the attached growth system requires less water, shorter retention periods, lower initial investments, and decreased monitoring requirements. Besides achieving high

microalgae productivity, the attached growth system enables the efficient concentration of microalgae through biofilm formation, facilitating easier separation processes (Zhuang et al., 2018).

There are three main types of microbial biofilm designs in the attached growth system: permanently immersed biofilms, biofilms between liquid and gaseous phases, and permeated biofilms (Mantzorou and Ververidis, 2019; Zhuang et al., 2018). The specific design and supply of the liquid medium determine the arrangement of the biofilm. Various environmental factors can influence biofilm growth in the attached cultivation, including the microalgae strains used, the supporting material for attachment, the flow rate of the liquid medium, nutrient concentration, and light intensity (Zhuang et al., 2018).

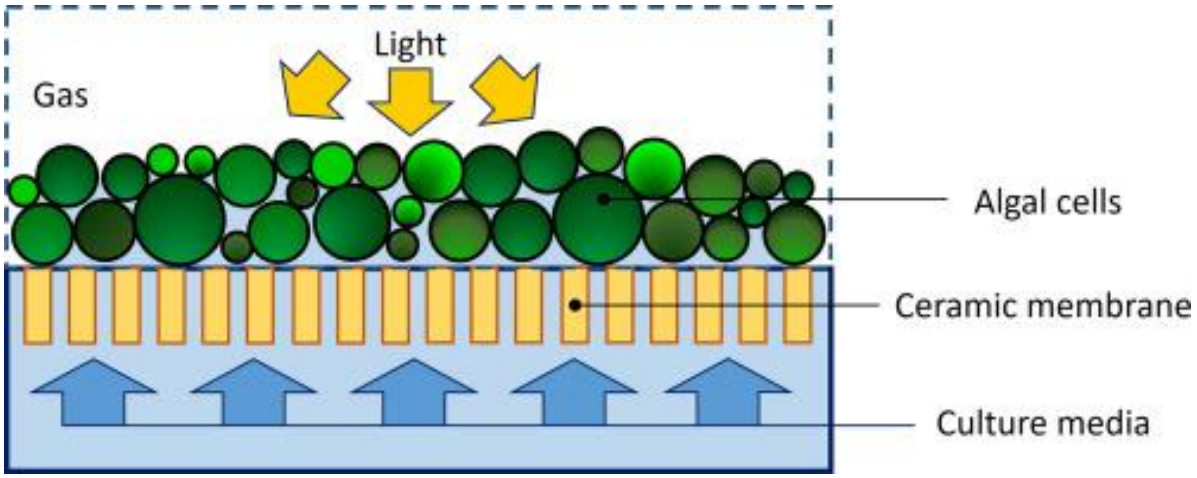

Figure 10. Attached growth cultivation method (Dalirian et al., 2021).

1.9 Microalgal production chain

There is a number of different strategies for microalgae harvesting from cultures, each with its advantages and drawbacks (Figure 11). The reduction of harvesting costs a crucial aspect for the environmentally friendly production of microalgal biomass on commercial scale (Christenson and Sims, 2011).

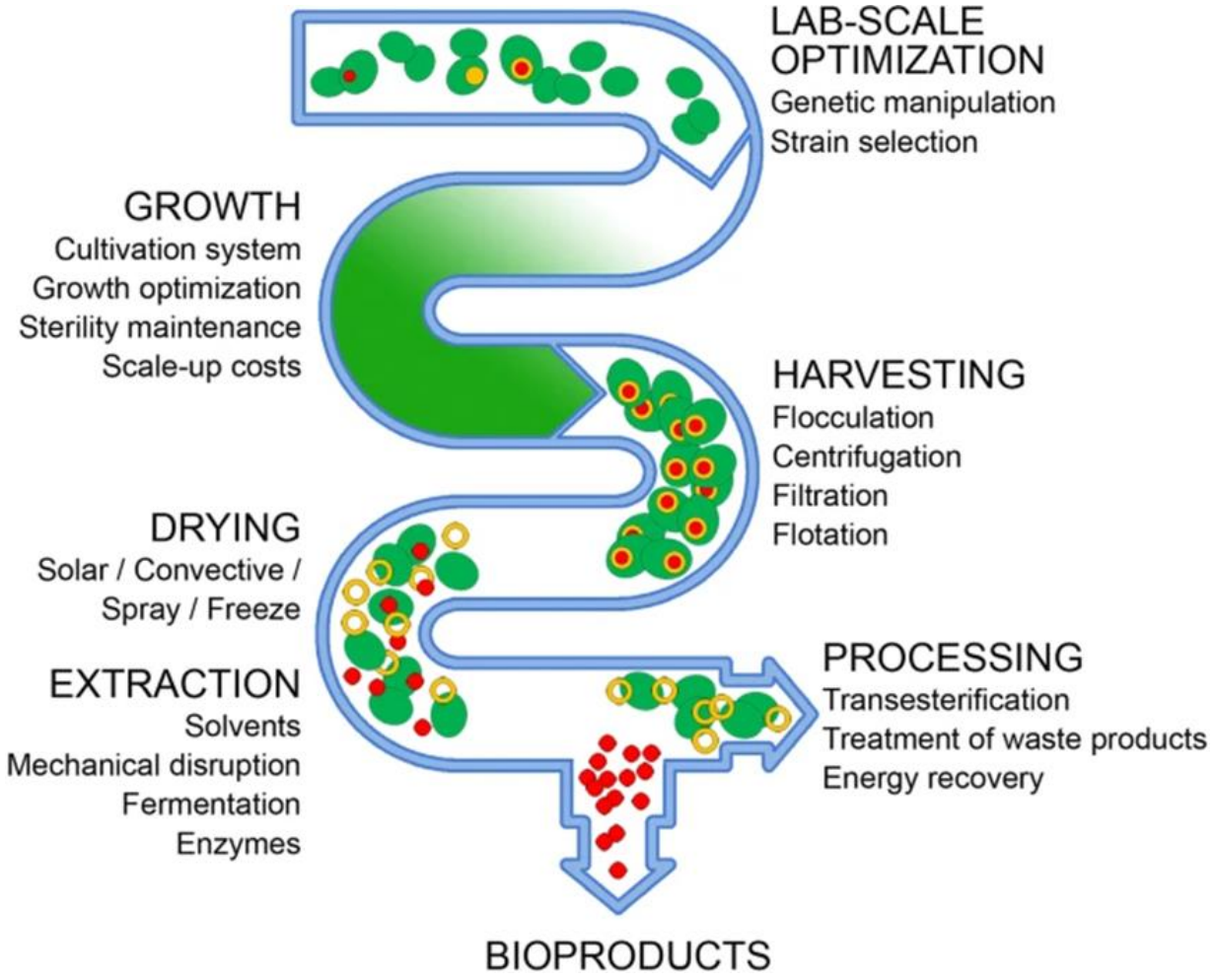

Figure 11. Schematic depiction of methods used in microalgal production chain (Benedetti et al., 2018).

1.9.1 Strain selection

The initial step in microalgal production chain is species selection. It is a step of upmost importance in microalgae cultivation as it determines the sustainability and economic feasibility of the cultivation system. It involves identifying robust microalgae species that can thrive in various environmental conditions, including “extreme” ones, characterized by high light intensity, nutrient limitation and rapid temperature and salinity fluctuations and strain that are compatible

with high production abilities of the desired products (Bhushan et al., 2020; Geada et al., 2017; Rizwan et al., 2018).

1.9.2 Biomass flocculation and thickening

Moving on to the harvesting stage, it is also very important and can determine the success of downstream steps. There are two main methodologies: a two-step concentration process and a single-step concentration process. The choice of method relies on the final product and its properties. The two-step concentration method, which entails thickening and dewatering, is the most is widely strategy utilized for microalgae harvesting (Uduman et al., 2010).

Thickening techniques, such as biomass flocculation, are employed to facilitate the separation of microalgae cells from the cultivation medium in order to concentrate the solids in the medium and minimize the volume of biomass that needs to be processed (Show et al., 2019). Most used thickening approaches are coagulation/flocculation, gravity sedimentation, flotation, and electrical methods (Uduman et al., 2010). Chemical flocculation is particularly important for economically optimizing microalgal harvesting processes due to its applicability to a wide range of species and large volumes of microalgal cultures (Uduman et al., 2010). It involves the coalescence of small particles into larger aggregates, which then settle to the bottom of the vessel or the reactor (Uduman et al., 2010). pH adjustment, use flocculation agents, such as chitosan, salts, polyelectrolytes and cationic polymers are some of the techniques employed in coagulation/flocculation as reduce electrostatic repulsion between negatively charged cell surfaces, and therefore inducing the formation of the aggregates (Horiuchi et al., 2003; Papazi et al., 2010). It has also been suggested that bioflocculants, natural polymers produced by microorganisms, including bacteria and fungi, can be utilized for biomass flocculation, offering unique potential advantages in terms of sustainability and cost-effectiveness, as they can be produced using renewable resources (Barros et al., 2015; Christenson and Sims, 2011).

Other methods have been explored. Some are beneficial for certain applications, but they usually offer lower yields and unreliable microalgal concentration. Sedimentation, is an energy-efficient method for microalgal harvesting that can be effective for various types of microalgae, particularly when the end product is biofuel (Molina Grima et al., 2003; Rawat et al., 2011).

However, the slow settling rates of microalgae, make the sedimentation process inefficient and prone to biomass deterioration (Christenson and Sims, 2011). Electrical approaches are another alternative environmentally friendly method. They include the use of an electrical field to the culture broth, microalgal cells, which carry a negative charge, can be separated either by forming precipitates on electrodes (electrophoresis) or by accumulating at the bottom of the vessel (electro-flocculation) (Uduman et al., 2010). This method is not widely used as it is difficult to be used commercially.

1.9.3 Biomass harvesting processes

After concentrating the microalgae cells the next process is harvesting. Common techniques include mechanical methods like filtration, centrifugation, and sedimentation, as well as thermal processes such as evaporation. Filtration is the most commonly used after coagulation/flocculation, but it can be hindered by fouling/clogging issues, which increase operational costs (Show et al., 2019). Centrifugation, on the other hand, is the quickest yet costliest (Molina Grima et al., 2003; Rawat et al., 2011). It is typically used for high-value products and can be efficient as a one-step separation process. Some issues that have been reported with this method is that centrifugation may result in cell structure damage due to the exposure of microalgal cells to high gravitational and shear forces (Barros et al., 2015).

1.9.4 Biomass drying processes

After the separation and concentration process, microalgal biomass contains a significant amount of water, making it susceptible to microbial growth and enzymatic activity (Morist et al., 2001). Therefore, the next step is drying the microalgal biomass to extend its shelf life and to allow it to be further processed (Show et al., 2019). Various drying techniques have been developed, including rotary drying, spray drying, solar drying, crossflow drying, vacuum shelf drying, flashing drying, and incinerator drying. Rotary drying involves using a rotating cylinder to move the algae from one end to the other by gravity (Soeder and Pabst, 1975). Spray drying is a widely employed industrial drying method for microalgae biomass. It involves atomizing the biomass suspension into fine droplets, which are then dried rapidly in a hot air stream. The droplets undergo rapid evaporation, resulting in the formation of dry particles. Spray drying offers rapid and

efficient drying, although it can potentially damage the integrity of the cells and negatively impact product quality (Barros et al., 2015; Shelef et al., 1984). Drum drying is recommended as an alternative with lower energy requirements. In areas without access to a common energy supply, solar heat drying becomes a feasible option. Algae drying can be achieved through direct solar radiation or solar water heating. However, direct sun radiation can lead to the dehydration and degradation of chlorophyll, affecting the texture and color of the final product, while also the risk of fermentation and spillage during prolonged drying hinders the wide usage of this method (Becker and Venkataraman, 1982). Flash drying, originally developed for wastewater sludge drying, is another method used for algae drying. It involves spraying or injecting a mixture of dried and wet algae into a hot gas stream, which serves as a carrier for moisture removal. The quality and cost of drying are greatly influenced by the hot gas source (Barros et al., 2015; Shelef et al., 1984). Finally, incinerators can be utilized for algae drying by providing hot gases and, thus ensuring the uniform combustion and separation of the dried algae from the sand or other materials used in the process (Barros et al., 2015).

1.9.5 Biomass processing and extraction of high added value bio-products

The final step in the microalgal production chain is biomass processing and extraction of fuels, foods, feeds, and high-value products from microalgae. The extraction process is highly specific and varies depending on the desired products. To purify bioactive compounds from microalgae, the extraction process involves disrupting the algal cell wall (Tan et al., 2020). Various strategies have been proposed that can be broadly categorized into four groups. Firstly, mechanical extraction methods involve the application of shear forces, electrical pulses, waves, or heat to disrupt the cellular structure (Lee et al., 2017). On the other hand, chemical extraction techniques utilize various solvents such as polar or non-polar organic solvents, supercritical carbon dioxide, and ionic liquids (ILs) to extract the intracellular compounds (Mubarak et al., 2015). Thirdly, physical extraction involves the application of microwave and ultrasound operations (Mubarak et al., 2015). Lastly, enzymatic lysis utilizes enzymes like trypsin to digest the tough cell walls of microalgae (B. R. Kumar et al., 2021). These different approaches enable the release and isolation of bioactive compounds from microalgae for further purification and utilization.

1.10 Bioproducts and Applications – Commercial Use

Microalgae, with their unique functional properties are a valuable component for a wide range of industrial applications. They have the potential to contribute ecologically to various sectors by meeting the increasing demand for plastics, providing products that promote human and animal health, such as nutraceuticals and pharmaceuticals, and serving as a renewable energy source with high energy potential.

1.10.1 Food, Beverages, and Nutraceuticals

Microalgae have gained recognition as valuable food sources due to their unique nutritional composition and potential health benefits. Microalgae have been used as additives in various food products, either as extracts or dried biomass (Rizwan et al., 2018). They can be utilized in various forms such as tablets, capsules, liquids, and as additives in snack foods, candy bars, pastas, and beverages under strict usage regulations (Pulz and Gross, 2004; Rizwan et al., 2018). They can also be used as preservatives and for the production of supplements, food dyes, vitamins, polyphenolic compounds etc. (Apt and Behrens, 1999). *Spirulina, Chlorella, Dunaliella*, and *Nostoc* are some of the microalgae species commonly used in human food products due to their nutritional value and potential health benefits. Their biomass offers a protein quality that is superior to vegetables, rice, and wheat but lower than animal proteins such as milk and meat (Shahidi, 2007). Yet, they also produce sterols, such as clionasterol, which have been associated with the prevention of cardiovascular diseases (Sharma, 2007). Microalgae are rich sources of antioxidant compounds, including astaxanthin, β-carotene, and mycosporines, which protect against oxidative stress (Rizwan et al., 2018; Shahidi, 2007). These traits have been utilized by the newly emerged nutraceuticals industry.

1.10.2 Pharmaceuticals

Microalgae are natural sources of bioactive molecules due to their ability to produce compounds that are challenging to synthesize through chemical methods. Microalgae metabolism can generate a wide range of bioactive compounds, including algicides, antibiotics, toxins, plant

growth regulators, and pharmaceutically active substances (Borowitzka et al., 1991), with diverse chemical structures. They exhibit a wide range of activities, including antiviral, anticancer, anti-HIV, neurological, and antimicrobial properties. Research is currently focused on investigating the potential of microalgal biosynthetic pathways for the development of novel pharmaceuticals. Microalgae biomass, as well, can improve immune response, and aid with fertility and weight control (Pulz and Gross, 2004).

1.10.3 Cosmetics

Microalgae, such as *Spirulina* and *Chlorella*, have found applications in the production of skin care products and cosmetics, with companies establishing their own microalgal production systems (Stolz and Obermayer, 2005). Microalgae are incorporated into cosmetic formulations due to their various properties, such as antioxidant activity, thickening capabilities, and water-binding properties. They serve as natural ingredients that offer beneficial effects. Microalgal extracts are utilized in face, hair and skin care products particularly in sun protection products such as sunscreens and sun protective oils (Spolaore et al., 2006). Furthermore, manufacturers have developed numerous commercial products utilizing microalgae, with specific claims such as anti-aging, tissue regenerating and collagen production stimulating properties (Stolz and Obermayer, 2005).

1.10.4 Animal feed

Microalgae are the primary producers of our ecosystems and growing in aquatic environments they are an essential source of nutrients for aquatic animals (Becker, 2013). Various species of microalgae, such as *Chlorella, Tetraselmis, Isochrysis, Pavlova*, and *Nannochloropsis*, are widely implemented as feed and food additives in both freshwater and marine aquacultures, as they contribute to the growth, the quality and external appearance of fish, including the coloring of farmed salmonids. Additionally, they are shown to enhance the immune systems in fish (Certik and Shimizu, 1999; Rizwan et al., 2018).

Microalgae can be incorporated as a protein source in poultry feed, with careful consideration to avoid adverse effects, such as changes in the color of broiler skin and egg yolk

(Becker, 2013). *Arthrospira,* is one of the most common microalgae that is cultivated and utilized as feed supplements for various animals, including aquarium fish, livestock, pets, and ornamental birds. Despite the benefits of using microalgae as feed supplements, challenges such as expensive production and the potential for toxic contamination, have limited their widespread use in food production (Certik and Shimizu, 1999).

1.10.5 Environmental applications

Microalgae have found applications in environmental biotechnology, particularly in the areas of environmental toxicants monitoring, bioremediation, and bioassay (Japar et al., 2021; Phang et al., 2000). Microalgae can be used for domestic wastewater treatment and the recycling of livestock wastes. *Spirulina platensis* and *Chlorella vulgaris* has been efficiently utilized for wastewater treatment, eliminating contaminants and generating biomass that can be utilized as animal feed and for biofuel production (Lim et al., 2010; Phang et al., 2000). Additionally, microalgae are commonly used in bioassays to assess environmental pollutants and evaluate nutrient levels, particularly nitrogen and phosphorus, in freshwater ecosystems (Lim et al., 2010; Vannini et al., 2011). Utilizing the inorganic nutrients such as nitrogen and phosphorus that are commonly found in wastewater, makes them effective by mitigating the negative effects of sewage and industrial wastewater and eliminating eutrophication issues by removing nitrogen and phosphorus (Mata et al., 2010). Microalgae also show potential for the removal of heavy metals, organic pollutants and pathogens from wastewater when co-cultivated with bacterial species (Muñoz and Guieysse, 2006). Finally, microalgae can be utilized a biological method for atmospheric CO_2 mitigation, as they naturally fix CO_2 during photosynthesis. This approach could potentially help balance CO_2 emissions from power plants when microalgae are used for biofuel production, resulting in a net-zero release of CO_2 (Chisti, 2007; Hall and House, 1993).

1.10.6 Agricultural applications

Finally, microalgae find a lot of applications in agricultural practices, specifically as soil conditioners and biofertilizers. Cyanophycae, in particular, have been shown to enhance biomass yield, reduce the need for nitrogen-based fertilizers, and improve the physicochemical properties

of soil (Rizwan et al., 2018). Biochar, as well, is very useful as a biofertilizer and as a means of carbon sequestration (Marris, 2006).

Chapter 2: Objectives of PhD thesis and methodology

2.1 Objectives

Algae cultivation shows ever-increasing growth not only due to the demand for cultivated species for human consumption and the manufacture of pharmaceuticals and supplements but also due to new applications, such as biofuels. An important role is finding optimal methods for biomass production, recovery, and drying, as well as extraction of bioactive compounds (e.g., proteins, carbohydrates, photosynthetic pigments, lipids). Microalgae cultivation commonly takes place in photobioreactors, with the cells mainly in suspension, rather than immobilized on a specific surface.

The present PhD thesis aims to find innovative methodologies for optimal production of high-added value products from microalgae, with parallel CO_2 capture from industrial the flue gas emissions. The thesis can be divided into four main research parts:

(i) Selection and cultivation of a *Stichococcus* sp. mutant microalgae strain, with higher biomass productivity and lower chlorophyll content, as high chlorophyll concentration reduces light transmission into the bioculture, thus reducing biomass productivity.

(ii) Screening and optimization of biomass and bioactive components production of the most promising strain of *Stichococcus* sp. (wild-type or mutant), initially on a laboratory scale and later, on a pilot scale photobioreactor under optimal conditions. Microalgae will be cultivated immobilized on horizontal sandblasted glass tiles to facilitate biomass harvesting at the end of cultivation period. At the same time, the possibility of capturing CO_2, responsible for the greenhouse effect, which is contained in the exhaust gases of industrial units will be investigated.

(iii) Pilot scale experimental investigation of the potential for CO_2 capture from flue gas emitted from a power plant utilizing natural gas.

(iv) Finding optimal methods for separating biomass from the culture liquid, drying, and recovering its bioactive components, at minimal financial and energy requirements. Another goal is the comparison of alternative processes for the removal of immobilized cells, to conventional methods (i.e., filtration, centrifugation, flocculation). A comparison between convective drying, solar drying, and freeze-drying will also be examined.

2.2 Methodology

The methodology that was followed throughout the experiments was based on the entire microalgae cultivation process. From the selection of the appropriate strain to finding the optimal cultivation parameters in photobioreactors, and finally harvesting and drying of the biomass for conversion into valuable products. The extracted products have the potential to be used as human or animal food, self-care products, nutraceuticals, biofuels.

The wild-type strain of microalgae *Stichococcus* sp. was selected, as it was isolated from a nearby stream to the University, in Souda bay located near the city of Chania in the region of Crete, in Greece. Earlier research had been carried out in the laboratory, for the selection of a suitable material for *Stichococcus* sp. biofilm formation and its capability in treating winery wastewater.

The first step of the experimental process of the current PhD thesis was initially the cultivation of *Stichococcus* sp. wild-type to establish the basic cultivation parameters and acclimate the strain to laboratory conditions. To achieve the first goal of the thesis, the wild-type strain was subjected to random mutation using the chemical agent “Ethyl Methanesulfonate (EMS)”. After successful mutation, strains with lower chlorophyll content were further examined and compared with the wild-type for the verification of increased biomass and bioactive compound characteristics. One mutant strain was selected, which exhibited lower chlorophyll content and increased biomass and lipids productivity.

The wild-type and mutant strains were then examined in terms of: i) optimal days of cultivation for the maximization of biomass production and ii) biomass productivity growing in an atmosphere with 5% (v/v) CO_2 concentration. Experiments were conducted on a laboratory scale (beaker glassware) with a Modified version of Bold’s Basal Medium. From the experimental

results, one strain was selected (i.e., the mutant one) and was further examined for the optimization of biomass and bioactive compounds (i.e., lipids, carbohydrates, total chlorophyll, and proteins) on a laboratory scale and then it was scaled-up in photobioreactor scale. Microalgae were cultivated immobilized on horizontal sandblasted glass surfaces and were supplied with synthetic flue gas for CO_2 sequestration, simulating gaseous mixtures of natural gas exhaust gases from electricity production power plants. Aeration rate, illumination type and intensity, $NaNO_3$ concentration in the growth medium, and nitrogen starvation pattern were examined as growth parameters for the optimization of biomass and bio-products production. The experimental data were then processed with appropriate statistical tools, such as ANOVA and regression model analysis, to find optimal procedures for the maximization of biomass and each bioactive component production. The optimal cultivation parameters for biomass maximization were selected for scaling-up to photobioreactor size, to fixate carbon dioxide, pumped directly from the chimney of the Lavrio Power Station (located in the region of Attica, Greece), which operates using natural gas. The efficacy of *Stichococcus* sp. on flue gas was examined.

The 3rd research part of the PhD thesis investigated the efficiency of different harvesting/dewatering and drying processes for microalgae cultivations. To examine the above, filtration, centrifugation, and flocculation from microalgae in suspension were examined as harvesting/dewatering processes and were compared to biomass scraping from an immobilized microalgae system photobioreactor, as an alternative and possibly cost-efficient method. As for the drying processes, a comparison between convective drying, solar drying, and freeze-drying was performed. After each drying process, the bioactive compounds (i.e., lipids, carbohydrates, pigments, and proteins) were quantified, to find out the influence of each process on the production of each component.

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Chapter 3: Chemical mutation of *Stichococcus* sp.

3.1 Introduction

The aim of the present chapter is the production and isolation of a mutant strain of the microalgae *Stichococcus* sp., with reduced chlorophyll content and increased biomass/lipids augmentation, compared to the wild-type strain. High chlorophyll content results to an increased "shadow effect" in microalgae, cultivated in photobioreactors (Beacham et al., 2017), therefore, the higher the chlorophyll content of the cell, the more intense "shadow effect". The phenomenon is more pronounced in cell growing on biofilms, as the suspended cells in the bulk medium obstacle the light penetration onto the biofilm. *Stichococcus* sp. has been selected, as previous studies have revealed that wild-type strains of *Stichococcus* sp. have a high affinity to form biofilms on solid surfaces (Makaroglou et al., 2021).

3.2 Materials and Methods

3.2.1 *Stichococcus* sp. cultivation

Stichococcus sp. was isolated from the North coast (Souda bay) of the island of Crete in Greece. *Stichococcus* sp. was identified using polymerase chain reaction (PCR) and Sanger sequencing of the 18S-rRNA gene (Makaroglou et al., 2021). Sterile seawater was selected as a cultivation medium for *Stichococcus* sp., as it is abundant and easily accessible in coastal areas. In addition, the use of seawater aids in the conservation of valuable freshwater resources. Salinity level was approximately 35 g L^{-1}, which was measured in situ, using a Hach HQ40D Portable Multimeter. Cultures were enriched with modified Guillard's (1975) F/2 medium for the autotrophic cultivation (Guillard, 1975). The medium was ready-to-use and filter-sterilized (Sigma-Aldrich). $NaNO_3$ concentration was based on modified Bold's Basal Medium (MBBM) used in previous studies regarding *Stichococcus* sp. (Karapatsia et al., 2016; Psachoulia and Chatzidoukas, 2021). At the final dilution, growth medium contained per liter: $NaNO_3$ 750 mg,

$NaH_2PO_4H_2O$ 5 mg, Trace metals solution ($FeCl_3 \cdot 6H_2O$ 0.00315 mg, $Na_2EDTA \cdot 2H_2O$ 0.00436 mg, $CuSO_4 \cdot 5H_2O$ 0.0098, $NaMoO_4 \cdot 2H_2O$ 0.0063 mg, $ZnSO_4 \cdot 7H_2O$ 0.022 mg, $CoCl_2 \cdot 6H_2O$ 0.01 mg, and $MnCl_2 \cdot 4H_2O$ 0.18 mg), and Vitamins solution (Thiamine HCl [B1] 0.2 mg, Biotin [H] 0.001 mg, and Cyanocobalamin [B12] 0.001 mg). The microalgal strain was initially cultivated in 600 mL glass beakers with a working volume of 200 mL. All glassware and seawater were autoclaved beforehand at 121 ^oC for 20 min. The starting optical density (measured at 750 nm) of the cultures was 0.2. Beakers were placed on a moving shaker with mild shaking, to ensure the appropriate mixing of the medium and to avoid the formation of aggregates. Cultures were illuminated with warm (3,000 K) and cool (6,500 K) fluorescent light lamps (2x18 W) adjusted to 60 μmol m^{-2} s^{-1} (4,400 lux) and with a photoperiod of 16:8 hours (Day:Night). The temperature was set to 25±0.5 ^oC. The pH values of the cultures were between 7.5-8.0. When *Stichococcus* sp. growth reached the stationary phase, mutagenesis took place.

3.2.2 Mutagenesis

The mutation of *Stichococcus* sp. was performed with the chemical reagent, Ethyl methanesulfonate (EMS, Sigma-Aldrich) using a modified protocol of (Mehtani et al., 2017). EMS induces random mutations in the DNA and RNA of microalgae, causing alterations in their characteristics, thus, different concentrations of EMS were tested to identify the optimal concentration of EMS, to which mutation induces a 5% survival rate. Specifically, calculated quantities of EMS were injected into 5 mL of microalgae culture, so the final concentrations of EMS in the culture were: 1%, 1.25%, 1.50%, 1.75%, and 2.0% v/v. Samples were kept in a dark environment under shaking conditions for 2 hours. After the 2-hours interval, 1 mL of 7% w/v sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$, Sigma-Aldrich) was added to the treated cultures in order to inactivate the remaining EMS and to avoid any further mutations. Each of the five samples were plated on solid media agar with dilutions up to 10^{-5}. At the same time, untreated *Stichococcus* sp. was plated on petri dishes with solid agar enriched with F/2 medium, as a positive control. The incubation period lasted 14 days and the growth parameters (i.e., illumination, temperature) were the same as mentioned in Section 3.2.1. Control and mutated colonies were compared visually. Survival of approximately 5% was achieved with 2% v/v of EMS. Out of the surviving cells, light-green colonies were selected and cultivated for three consecutive generations, in order to verify

the mutation. The selected strains were then cultivated in triplicates, in 600 mL beaker glassware with aquatic media (seawater + F/2 medium) at a working volume of 200 mL. Cultivating conditions were identical as described in Section 3.2.1.

3.2.3 Biomass and Chlorophyll protocols

During the cultivation of wild-type and mutated strains of *Stichococcus* sp., biomass samples were collected to quantify biomass production, and chlorophyll content.

Biomass production was measured every 48 h by filtrating 5 mL of culture with dry, pre-weighed glass fiber filters (Whatman, 1.2 μm pore diameter). Filters were then oven-dried at 64 ^{o}C for 24 h.

Total chlorophyll was extracted from the filtrated biomass using a modified version of the protocol described by (Mehtani et al., 2017). Specifically, 5 mL of cell suspension were centrifuged at 4,000 rpm for 10 min and then, 5 mL of methanol (Honeywell, 99.8%) were added to the pellet. Samples were incubated at 45 ^{o}C with mild shaking. After 24 h, the optical density of the samples was measured with a UV/Vis spectrophotometer (Shimadzu UVmini-1240) at 665.2 nm and 652.4 nm. The concentrations of chlorophyll a (Chl a), chlorophyll b (Chl b), and total chlorophyll (Tot Chl) were calculated using equations 1-3 (Lichtenthaler, 1987):

$$Chl\ \alpha\ (\mu g \cdot mL^{-1}) = 16.72 \cdot A_{665.2} - 9.16 \cdot A_{652.4} \quad (1)$$

$$Chl\ \beta\ (\mu g \cdot mL^{-1}) = 34.09 \cdot A_{652.4} - 15.28 \cdot A_{665.2} \quad (2)$$

$$Tot\ Chl = Chl\ \alpha + Chl\ \beta \quad (3)$$

where $A_{652.4}$ and $A_{665.2}$ are the absorbance at 652.4 and 665.2 nm.

To be noted that the equations mentioned above, only apply when using methanol as a solvent for extracting photosynthetic pigments.

3.2.4 Lipids quantification

Lipids content was estimated using a modified version of the rapid colorimetric method Sulfo-Phospho-Vanillin (SPV) (Mishra et al., 2014). This protocol is divided into two sections, the creation of a calibration curve for calculating lipid content of the samples and the preparation of Sulfo-Phospho-Vanillin reagent.

A. Calibration curve

A standard solution was prepared out of 20 mg of commercial olive oil, mixed with 10 mL of chloroform (99.8%) to achieve a concentration of 2 mg mL^{-1}. Appropriate dilutions were made for creating the calibration curve, starting from 0 to 0.7 mg mL^{-1}.

B. Sulfo-Phospho-Vanillin method

SPV reagent was prepared by mixing 1.2 g of vanillin (99%), 20 mL of ethanol, 180 mL of diH_2O, and 800 mL of orthophosphoric acid (85+%, Extra Pure). The SPV reagent was kept in a dark environment under continuous mixing before use.

For lipids quantification, 2.5 mL of filtered cell suspension and 2 mL of chloroform were placed in vials. All vials were heated to 60 ℃, until the evaporation of chloroform. Subsequently, 100 μL of diH_2O and 2 mL of sulfuric acid (95-97% v/v) were added to the vials. Solutions were then heated to 100 ℃ for 10 min and cooled in an ice bath, until they reached room temperature. 5 mL of phospho-vanillin reagent were added in each vial and proceeded with heat treatment at 37 ℃ for 15 min. The concentration of lipids was determined spectrophotometrically at 530 nm.

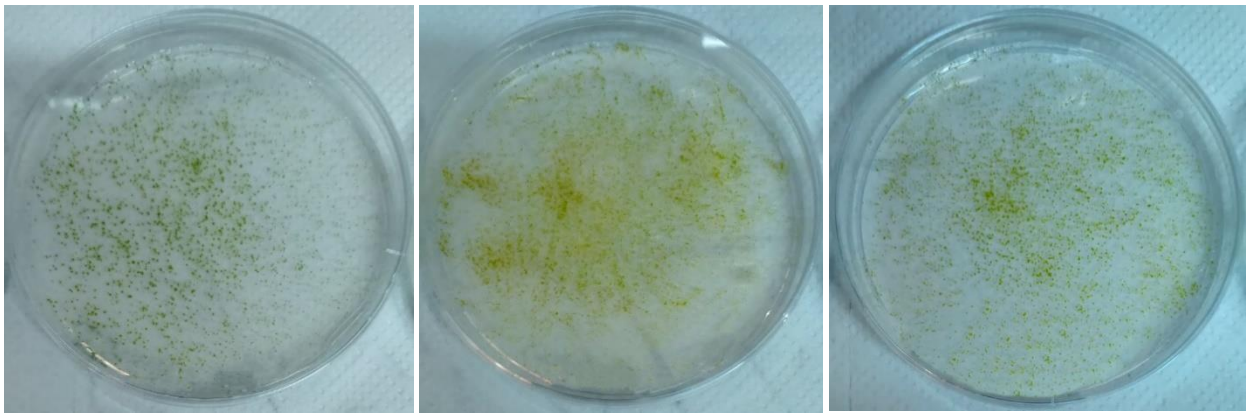
3.2.5 Statistical analysis

One-way analysis of variance (ANOVA) and Tuckey HSD method using Minitab 17.0 (Minitab LLC, State College, PA, USA) statistical software were selected for comparing the results and for investigating the statistical differences of the means. A probability value of 0.05 was used for analyzing the difference between sample means. Data are represented as the mean ± standard deviation (SD).

3.3 Results and Discussion

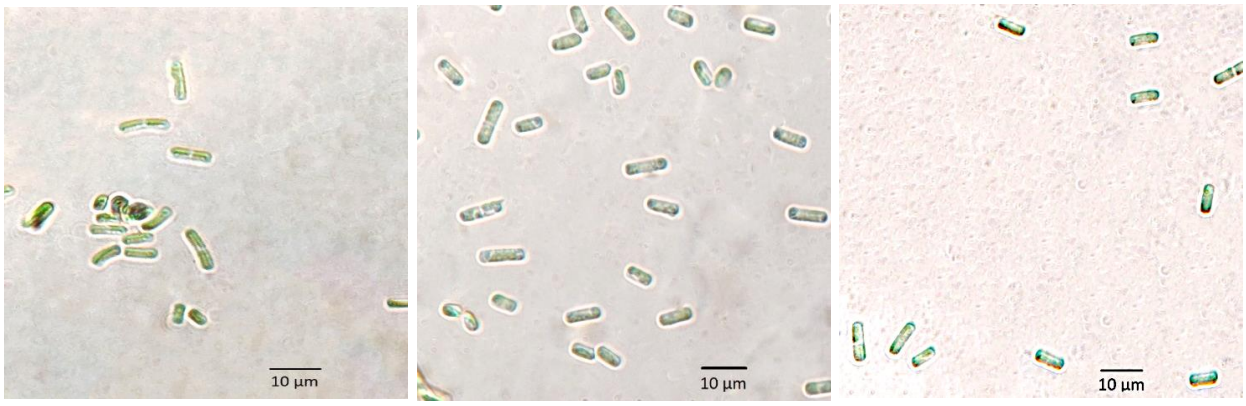
3.3.1 Characteristics

Mutagenesis is a simple method for altering microalgae characteristics, such as biomass production, lipids, pigments accumulation, and thermal/heavy metals tolerance (Mehtani et al., 2017). Targeting the increase or decrease of specific characteristics in desired microalgae strains, can lead to intensification of large-scale cultivations, for the extraction of respectable quantities of biomass and bio-products. Random mutagenesis is still considered a useful technique in the genome editing era for providing strains with desired characteristics (Park et al., 2019). The mutation of *Stichococcus* sp. strain was succeeded at 2.0% v/v concentration of EMS. At this level of the mutagen, many cells that survived formed colonies with lighter green color, compared to the wild-type strain. Two mutant strains (named EMS1 and EMS2) and were visually selected as they formed the less bright green colonies, compared to the other mutant colonies. The colonies formed on petri dishes are shown in Figure 12. As it can be seen, EMS1 and EMS2 colonies have a lighter green color, in comparison to the wild-type strain, which is a qualitative indication of the reduced chlorophyll content in the two mutant strains. Colonies from the three examined strains were collected and dispersed into liquid cultivation medium (F/2 medium). To further qualitatively verify the color of individual strains, the cells were checked under a microscope at a magnification of 100x (Figure 13). The cells are rod-shaped and their length varies from 3 to 18 μm (Figure 13). Wild-type cells clearly have a darker green color, compared to EMS1 and EMS2 cells.



Wild **EMS1** **EMS2**

Figure 12. Visual differences between the wild-type and the mutant strains EMS1 and EMS2 of *Stichococcus* sp. colonies.



Wild **EMS1** **EMS2**

Figure 13. Wild, EMS1 and EMS2 *Stichococcus* sp. under microscope.

3.3.2 Biomass production

The three examined strains were evaluated in terms of biomass production, chlorophyll, and lipids content. Figure 14A shows the growth curves of wild, EMS1 and EMS2 *Stichococcus* sp. strains. EMS1 displayed higher growth rate compared to the wild-type and EMS2, with a maximum value of 2.47 ± 0.10 g L^{-1} on the 14th day of the cultivation. The sharp increase in growth rate on day 6 of EMS1, may indicate an increase in metabolic rate. On the other hand, the wild-type strain, from the first day of cultivation exhibited the slowest growth, meeting its maximum growth on the 14th day, with a value of 2.20 ± 0.06 g L^{-1}. EMS2 growth rate was between the two other strains until the 12th day of cultivation. On the 14th day, it noted its highest growth at 2.18 ± 0.11 g L^{-1}, slightly lower than that of the wild-type strain. The latter shows that even if EMS2 had a higher growth rate compared to the wild, they reached similar final biomass concentrations. Significant differences between results were statistically proven by the ANOVA test (p-value = 0.013). EMS1 dry cell weight was significantly higher compared to the dry biomass of the other strains examined (Figure 14B).

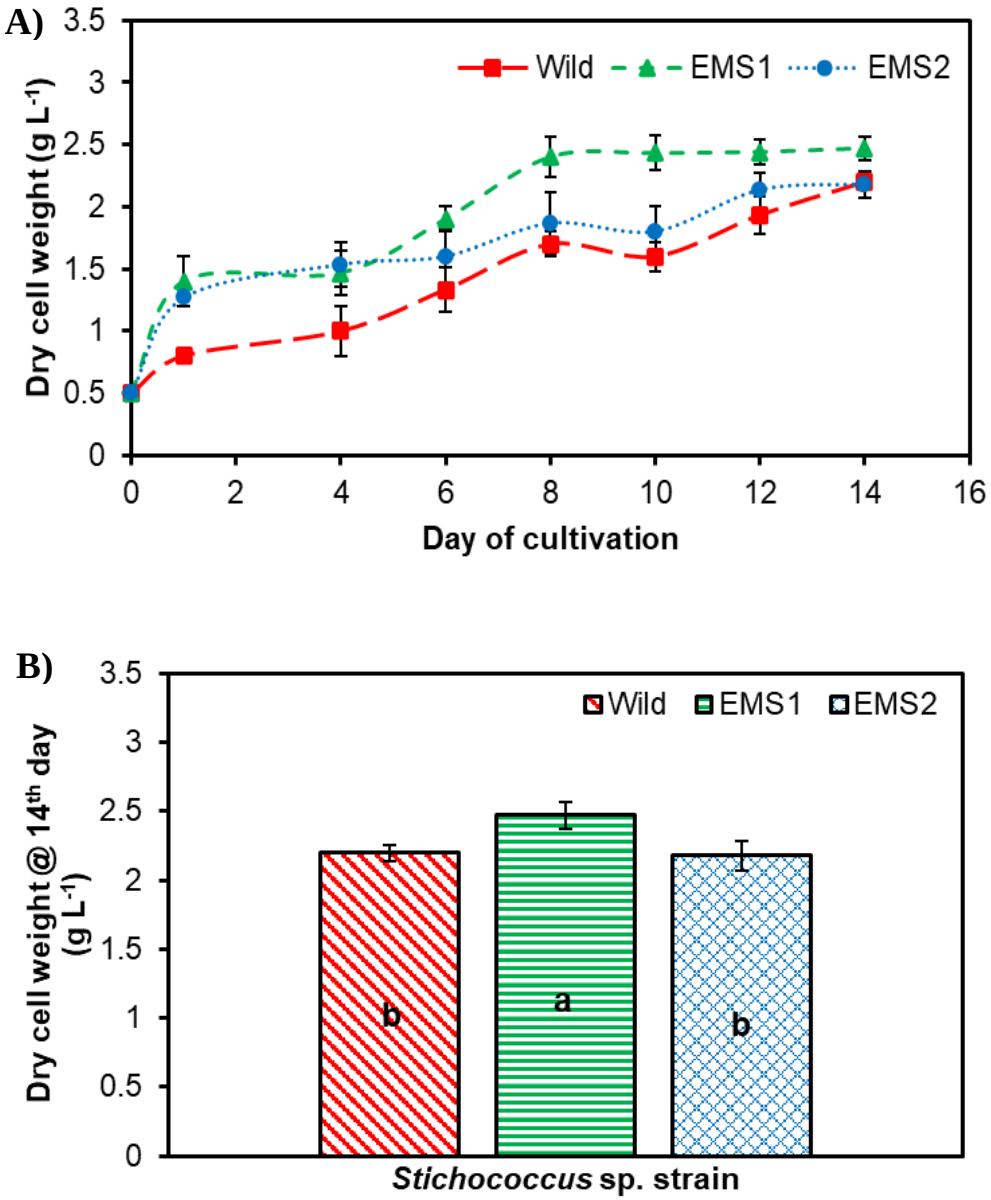


Figure 14. A) Dry cell weight (DCW) growth curve of *Stichococcus* sp. during 14 days of cultivation and **B)** DCW on the 14th day of cultivation (p-value = 0.013, n = 3). Vertical bars represent standard deviation. Results are represented as Mean ± Standard Deviation. One-way ANOVA was used for analyzing the data. Means that share the same letter do not have significant difference, according to Tuckey HSD method at p-value < 0.05.

3.3.3 Chlorophyll content

Photosynthetic pigments were extracted in order to explore the difference in yield, among the wild-type and mutant strains. The mutant strains of *Stichococcus* sp., especially EMS1, showed a considerable difference in chlorophyll content compared to the wild-type strain. Chlorophyll content was measured every 2 days during of the cultivation period (i.e., 14th day). Figure 15A shows the chlorophyll content of biomass (dry basis) during cultivation, for the three examined strains of *Stichococcus* sp. Strains EMS1 and EMS2 noted lower chlorophyll content, compared to the wild-type strain (Figure 15B, p-value = 0.009), (except for a short period at the beginning of the cultivation period). Maximum chlorophyll values were 6.99 ± 0.83 mg g^{-1} of biomass for EMS1, followed by EMS2 with a value of 10.98 ± 0.51 mg g^{-1} and 14.22 ± 1.26 mg g^{-1} of biomass for the wild-type strain. On the last day of biomass recovery (Figure 15A-B), chlorophyll plummeted to 6.29 ± 0.52, 8.39 ± 1.23, and 11.00 ± 1.63 mg g^{-1} correspondingly for EMS1, EMS2, and wild-type *Stichococcus* sp. strains. As EMS1 mutant strain displayed significantly lowest chlorophyll content compared to the other strains, thus it was selected for further exploitation in photo-bioreactors.

According to (Rinawati et al., 2020), the chlorophyll content in the stationary phase is greater than the content of chlorophyll pigments in the logarithmic phase. Following logarithmic growth phase, cell mass starts decaying, thus the pigments content is reduced. In various studies, researchers have used EMS induced mutations for reducing the pigments' content (Kim et al., 2017; Patil et al., 2020; Schüler et al., 2020; Shin et al., 2016; Vani et al., 2022). Patil et al. (2020) used EMS to mutate *Chlorella saccharophila*; the mutant strain indicated reduced chlorophyll content and increased biomass productivity up to 27%. Similarly, Vani et al. (2022) achieved a significant decrease of 28.83% in chlorophyl a and 54.03% in chlorophyl b for *Chlorella pyrenoidosa*. Dry biomass of the mutants was increased by approximately 1.32-fold, compared to the wild-type strain. Furthermore, it was observed that many studies have conducted experiments with the genus *Nannochloropsis* to induce chemical mutation for increasing specific characteristics of the microalgae (V. Kumar et al., 2021). Perin et al. (2015) chemically mutated *Nannochloropsis gaditana* which leaded to the production of strains with greater photosynthetic efficiency and reduced pigment content, while they also identified one mutant with greater biomass productivity. Shin et al. (2016) chemically mutated *Chlorella vulgaris* with EMS and reported the production

of mutants with truncated antenna-sized molecules, which is an effective mechanism to improve photosynthetic efficiency and overall biomass productivity (V. Kumar et al., 2021). *Chlorella vulgaris* mutants showed reduced chlorophyll a and b content, with increased biomass productivity approximately 44% compared to the wild-type. The aforementioned studies indicate that chemical mutation is an effective way, for the manipulation of chlorophyll content, with ultimate target the production of strains with high biomass and lipids production.

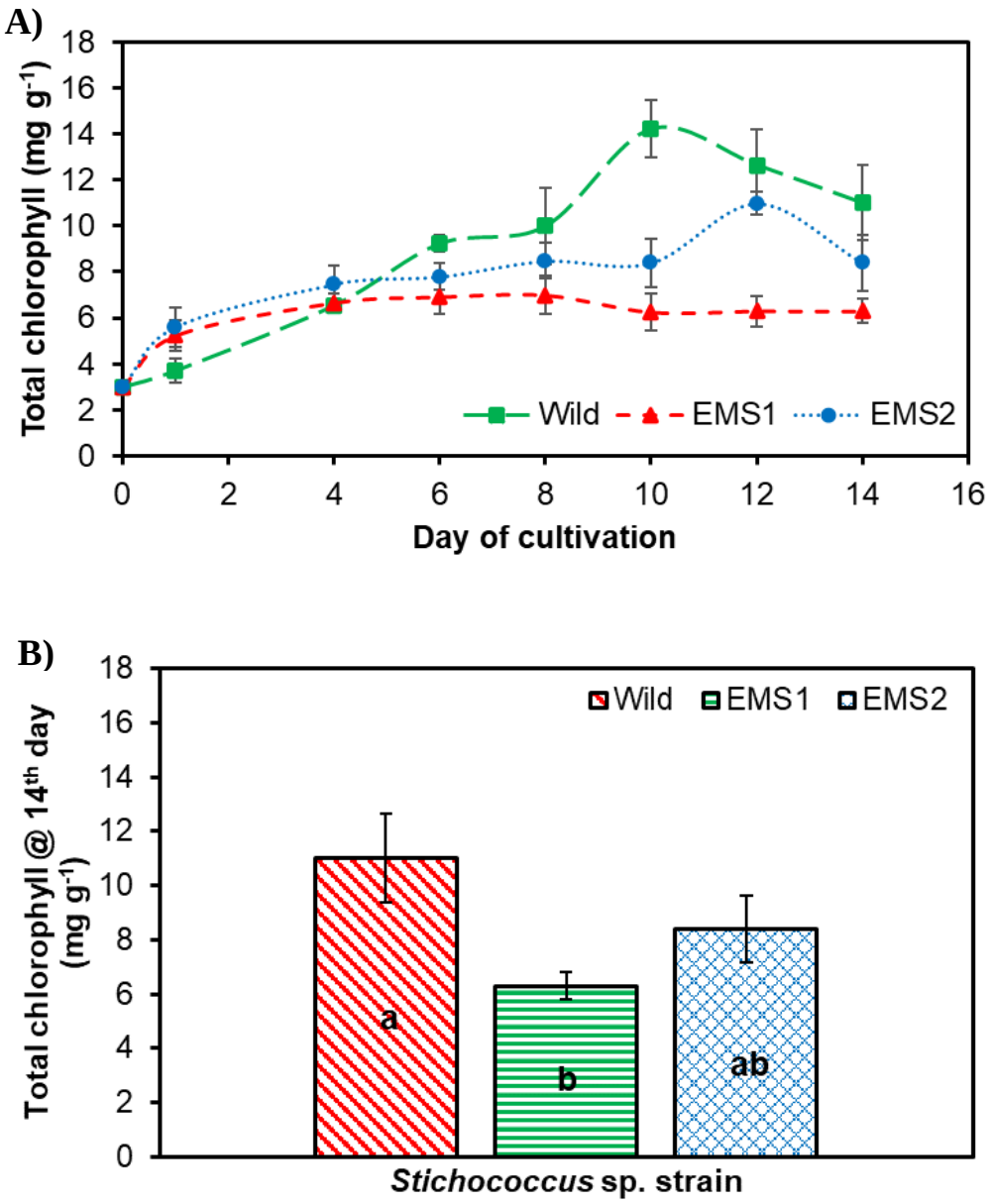


Figure 15. A) Total chlorophyll growth curve of *Stichococcus* sp. during 14 days of cultivation and **B)** Total chlorophyll on the 14th day of cultivation (p-value = 0.009, n = 3). Vertical bars

represent standard deviation. Results are represented as Mean ± Standard Deviation. One-way ANOVA was used for analyzing the data. Means that share the same letter do not have significant difference, according to Tuckey HSD method at p-value < 0.05.

3.3.4 Lipids content

Microalgae lipids are a type of organic compound that are produced by microalgae and play a crucial role in their biology. These lipids are of great interest to researchers and industry due to their potential to be used as a renewable source of energy and as a raw material for the production of biofuels, cosmetics, and other products.

Figure 16 shows the production of lipids on the 14th day of cultivation for the examined strains: wild, EMS1, and EMS2 of *Stichococcus* sp. EMS1 *Stichococcus* sp. showed the highest lipid content, with a value of 214 ± 28 mg g^{-1}, followed by EMS2 with 179 ± 16 mg g^{-1} and wild-type with 148 ± 17 mg g^{-1} of biomass. ANOVA statistical method and Tuckey HSD methods were implemented for evaluating the data. Results showed that the observed difference between EMS1 and wild-type strain is statistically significant (p-value = 0.025). EMS2 strain shares similar letters with EMS1 and wild-type strain (Figure 16), so it cannot be considered to prevail over the aforementioned strains. Lipids content could be linked to biomass production. According to Figure 14, EMS1 noted the greatest biomass production, followed by EMS2 and wild-type. Therefore, as the biomass yield is increased, so as the lipids content. The percentage of lipids, in relation to dry biomass was approximately 21%, 18%, and 15%, for EMS1, EMS2, and wild-type, respectively.

Olivieri et al. (2013) experimented with *Stichococcus bacillaris* in a vertical bubble column and in an inclined bubble column and found that the lipids content was approximately 29% and 28% of the biomass, respectively. In a similar study, Gargano et al. (2016) experimented with 6 species of *Stichococcus* and showed that lipid yield ranged from 8.6% (*S. fragilis*) up to 35.5% (*S. bacillaris*), while through the transesterification process, the strains exhibited bio-oil yield ranging from 1.4% (*S. jenerensis*) up to 9.5% (*S. deasonii*). The impact of light intensity is also reflected in the lipid content of the microalgae. Sufficient light was found to increase lipids biosynthesis and biomass production, reported in several species of microalgae (V. Kumar et al., 2021; Zhu et al., 2016). This may justify the fact that lipids of EMS1 and EMS2 mutants of *Stichococcus* sp. show increased concentrations.

Successful mutagenesis has been performed in the literature using EMS to generate mutant microalgae with increased lipid concentration. For example, *Nannochloropsis oculata* and *Chlorella* sp. showed a high potential for lipid production as well as high growth rates compared to the wild-type strains (Al-Saedi et al., 2019). Further studies on chemical mutation performed on *Nannochloropsis* sp. (Doan and Obbard, 2012; Kawaroe et al., 2015). Similarly, mutation of *Botryosphaeria* sp. and *Chlorococcum* sp. strains was found to result in increased biomass and lipids production (Nojima et al., 2017). The results of the present work regarding the mutation of *Stichococcus* sp. are in accordance with the findings of the literature on chemical mutagenesis aimed at improving the biomass and lipid productivity of microalgae such as *Nannochloropsis* sp. and *Chlorella* sp. (Doan and Obbard, 2012; Shin et al., 2016).

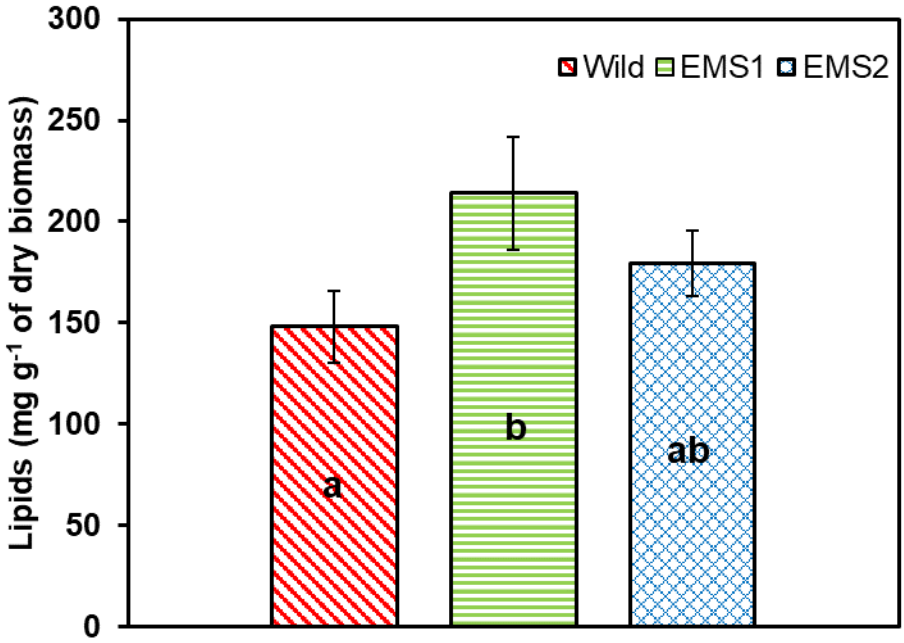


Figure 16. Lipids content of *Stichococcus* sp. on the 14th day of cultivation (p-value = 0.025, n = 3). Vertical bars represent standard deviation. Results are represented as Mean ± Standard Deviation. One-way ANOVA was used for analyzing the data. Means that share the same letter do not have significant difference, according to Tuckey HSD method at p-value < 0.05.

3.4 Concluding Remarks

The photosynthetic cultivation process of microalgae is highly dependent on growth parameters and access to sufficient light. However, high chlorophyll content in microalgae enhances the "shadow effect", thus reducing the yield at the photo-bioreactor in areas away from the light source. The latter is particularly pronounced in the growth of cells immobilized on solid surfaces. Selection of mutated microalgae strains, baring low chlorophyll content, may also increase biomass production and yield of various bio-chemicals, such as intracellular lipids. Optimization of microalgae growth strategies is critical for optimizing the performance of 3rd generation biorefineries.

The present study focused on the random mutation of *Stichococcus* sp. microalgae, using EMS at different concentrations, to generate mutants with lower chlorophyll content. It confirmed the hypothesis that mutants with lower chlorophyll content may also exhibit increased biomass and bio-chemicals production. Two best performing mutant strains (EMS1 and EMS2) with lighter green phenotypes were selected for further investigation, in terms of biomass production, chlorophyll and lipid content and were compared to the wild-type strain. The mutant strain EMS1 contained the least chlorophyll content, compared to the rest of the strains examined. It also exhibited the highest dry cell yield (2.47 ± 0.10 g L^{-1},) the highest lipid content (21%), and the highest carbohydrates content (44% of dry biomass). The proteins content of the mutant strains was similar to the one of the wild-type strain. EMS random mutagenesis has been proved a suitable and efficient technique for the improvement of strain characteristics.

Chapter 4: Biomass and bio-products optimization in lab- and pilot-scale photobioreactors

4.1 Introduction

The aim of the present chapter is initially the optimization of biomass and bio-products of a selected strain of *Stichococcus* sp. on a laboratory scale and then its scaling up to a pilot photobioreactor. Microalgae were grown attached to sandblasted glass, with respect to simplifying harvesting steps and minimizing capital costs (Zhuang et al., 2018). On both scales, a gas mixture was fed to the microalgae that simulated flue gas from a natural gas power plant, by aiming to the reduction of CO_2 content.

4.2 Materials and Methods

4.2.1 *Stichococcus* sp. cultivation

Two strains of *Stichococcus* sp. were used; the wild-type and the mutant "EMS1", described in Chapter 3. Both strains were cultivated in modified Bold's Basal Medium, which is different from the one used in the experiments of Chapter 3. The new composition in $g \cdot L^{-1}$ is: $NaNO_3$ 0.75, KH_2PO_4 0.175, K_2HPO_4 0.075, $MgSO_4$ 0.075, $CaCl_2$ 0,025, and NaCl 35. BBM medium was supplemented with a solution of trace metals and vitamins, in accordance with the F/2 medium (Guillard, 1975). Initial pH of the BBM growth medium was adjusted to 6.5 using a phosphate buffer K_2HPO_4/KH_2PO_4 (pH=8.0). BBM was freshly prepared and autoclaved for 20 min.

For the scope of the experiments, two identical closed-type and flat-panel photobioreactors were designed and manufactured from polymethyl methacrylate (Plexiglas®) with dimensions of 150x40x15 cm (Figure 17). The surface area is 0.6 m^2 and the working volume was 15 L. Temperature of the cultures was regulated through an A/C unit. A LED strip lighting system with

natural light (4,500 K) was placed on top of the photobioreactors to illuminate the cultures. The system was built in the laboratory and had the capability of adjustable illuminance, Light-Dark ratio, and frequency of blinking. A light time profile of 16 h, followed by 8 h dark was used for all cultures. Different algae species require different light intensities for their optimum growth, so experiments are required for determining light intensity and duration to avoid photo-inhibition. However, most studies on microalgae lighting conditions have found that 16 h light and 8 h dark are the most appropriate (Chew et al., 2017). Furthermore, the photobioreactors were connected to a gas supply, giving the capability of selecting air, N_2, CO_2, and O_2 ratios. The gas mixture was sterilized using a 0.2 µm PTFE filter before entering the photobioreactor.

Flashing light effect is characterized by two parameters: the frequency of blinking "f" in Hz and the Light-Dark ratio "Φ" in %, expressed by the following formulas (Yoshimoto et al., 2005).

$$f\ [Hz] = \frac{1}{T_L + T_D}$$

$$\Phi\ [\%] = \frac{T_L}{T_L + T_D} \times 100$$

Where:

- T_L, the duration of light
- T_D, the duration of dark

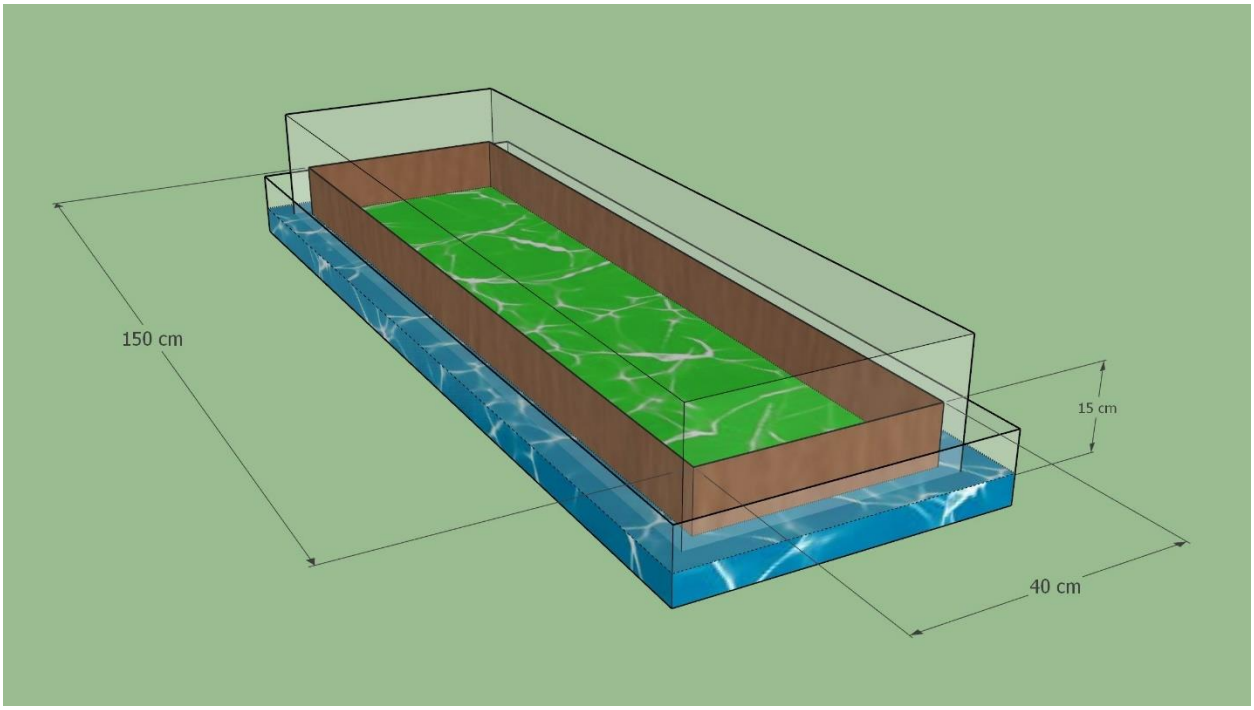


Figure 17. Flat-panel photobioreactor designed for microalgae cultivation. The photobioreactor has internal dimensions 150x40x15 cm and the working volume is 15 L. The internal photobioreactor is surrounded by an outer vessel, filled with tap water, which is used to prevent cross-contamination and isolate the gas mixture contained in the photobioreactor.

4.2.2 Lab-scale cultivation optimization

Optimization of *Stichococcus* sp. cultivation was achieved using stepwise experiments and was conducted in beaker vessels. To begin with, agitation experiments were conducted on wild-type *Stichococcus* sp. to investigate its influence on biomass production. After that, wild-type and EMS1 *Stichococcus* sp. were cultivated under different gas mixtures and cultivation days to find out the one corresponding to biomass maximization. Experiments were also conducted to select one strain, which was further examined for optimization of its biomass and bio-products. The above-mentioned process is described extensively in the following sections.

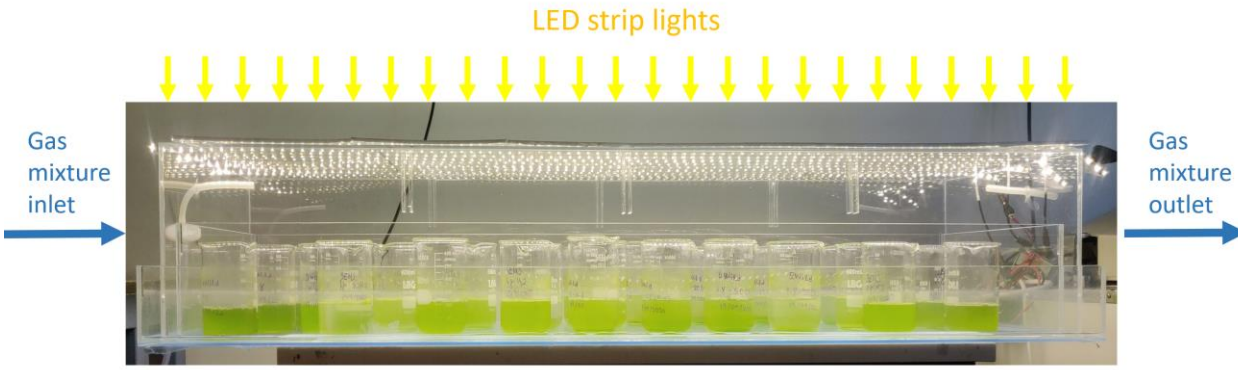
4.2.2.1 Agitation

Eight samples of wild-type *Stichococcus* sp. were agitated at 100 rpm, along with 8 samples without agitation, which were used as controls. Microalgae were cultivated for 25 days attached

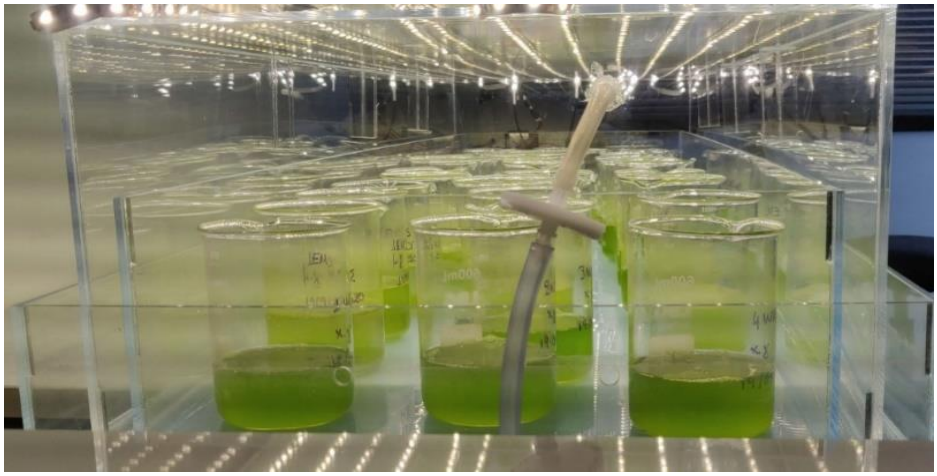
on sandblasted glass with 150 mL of modified Bold's Basal medium (see Section 4.2.1 for its composition). Culture medium depth was approximately 4 cm, surface area was 38.5 cm^2, and the ratio between liquid medium to surface used for attachment was measured as 39 L m^{-2}, similar to (Makaroglou et al., 2021).

All vessels were placed inside an empty photobioreactor, to achieve identical cultivation conditions (Figure 18A-B). Temperature was set to 25 ± 1 ºC. Microalgae were aerated with atmospheric air at a flowrate of 0.8 L min^{-1}. The illuminance intensity was set to 100 μmol/m^{-2}/s^{-1}, corresponding 6,600 lux for daylight 4,500 K LED strip lights. Lux is the measure of how bright a surface will be. Strip lights were mounted on top and in parallel, along the length of the photobioreactors. Luminance intensity was adjusted using a portable luxmeter. Microalgae were grown attached on removable sandblasted glass, placed in a horizontal position, covering the entire bottom of the culture vessels (Figure 18C). According to the study of Makaroglou et al. (2021), sandblasted glass was compared with ceramic tile and HD-polyethylene, and it was found to be an appropriate material for biofilm formation of *Stichococcus* sp. At the end of each cultivation period, immobilized biomass was scraped from the sandblasted glass (Figure 18D) and was filtered with pre-weighted glass fiber filters (1.2 μm) to quantify dry cell weight. The filters were oven-dried at 60 ºC for 24 hours. Results were compared using ANOVA method to find any statistical difference between treatments.

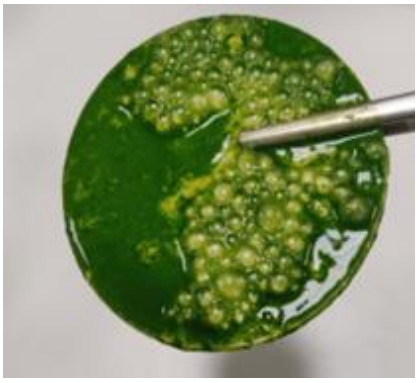
A)



B)



C)



D)

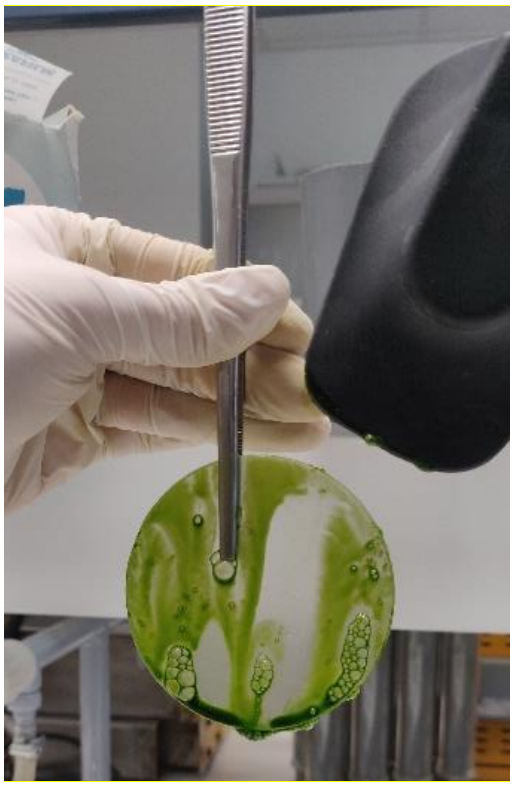


Figure 18. A, B) Lab-scale cultivation in beaker vessels, enclosed in the pilot photobioreactor to simulate scale-up conditions. Microalgae were illuminated by LED strip lights (4,500 K) on top of the photobioreactor. Gas mixture inlet and outlet are located on opposite sides to achieve uniform aeration of the cultures. **C)** Microalgae biomass attached on sandblasted glass during cultivation (left) and upon harvesting (right). **D)** Biomass scraping from sandblasted glass, as a harvesting technique.

4.2.2.2 Atmospheric air & Enriched atmospheric air cultivation

Since one major scope of the study is to examine the influence of CO_2 (contained in flue gas), wild-type and EMS1 *Stichococcus* sp. were cultivated in triplicates, with atmospheric air enriched with 5% (v/v) CO_2. Microalgae were also aerated with plain atmospheric air, to compare CO_2 influence on biomass productivity. The experimental conditions were the same, as mentioned in the previous section 4.2.2.1. At the end of the cultivation period, biomass was harvested and the dry cell weight was measured. ANOVA and Tuckey HSD statistical methods were used for comparing experimental results. It is worth mentioning that due to the accumulation of OH^- during

photosynthesis, the pH of the culture can increase up to 11, which is not favorable. By supplying CO_2, the pH can be stabilized (Khoo et al., 2020).

4.2.2.3 Selection of cultivation days

Triplicates of wild-type and EMS1 *Stichococcus* sp. strains were cultivated for 15, 20, and 25 days in 600 mL beakers with 150 mL of modified Bold's Basal medium. Microalgae were aerated with a gas mixture with its composition being: CO_2 at 5%, O_2 at 14%, and the remainder N_2 at 81%. This composition was based upon flue gas data from a natural gas-fired power plant, as the ultimate scope of the research is to fixate CO_2 from real-time industrial flue gas. The total aeration rate was adjusted to 0.8 L min^{-1}. Details about the cultivation conditions were mentioned in the Section 4.2.1. After 25 days, harvested biomass was dried to quantify dry cell weight and results were processed with ANOVA and Tuckey HSD methods.

4.2.2.4 Nitrogen starvation

Nitrogen starvation was implemented to microalgae cells as a method to increase intracellular lipids production (Al-Rashed et al., 2016; Breuer et al., 2012; Chu et al., 2019; Wang et al., 2015). To achieve that, 2 or 3 days prior to harvesting, the supernatant culture medium was pumped out and the vessels were replenished with fresh BBM without the addition of $NaNO_3$. Experiments with wild-type and EMS1 *Stichococcus* sp. strains were performed in triplicates, as mentioned in the previous paragraphs. Triplicates without nitrogen starvation were used as controls. At the end of the cultivation period, samples were collected to quantify intracellular lipids production using a modified version of Folch method (Folch et al., 1957). The analytical protocol is presented in Section 4.2.4.3.

4.2.2.5 Strain selection

After the selection of optimal cultivation period, experiments were conducted with wild-type and EMS1 strains cultivated in beaker glassware (in triplicates). Microalgae were cultivated with 150 mL of BBM, as mentioned in section 4.2.1. Both strains were grown immobilized for 25 days with the addition of synthetic flue gas. At the end of the cultivation period, biomass was

harvested from each vessel to quantify dry cell weight, lipids, pigments, proteins, and carbohydrates. Analytical protocols are mentioned in Section 4.2.4. The strain which showed higher biomass and bio-products production under the atmosphere enriched with CO_2 was selected as a promising strain for 3rd generation biorefineries and the following step was the optimization of its overall production.

4.2.2.6 Optimization of the selected strain

To optimize growth on the laboratory scale of the selected *Stichococcus* sp. strain, different values of growth parameters were examined (Table 1) and the experiments were performed in triplicates. All samples were placed inside the photobioreactor to achieve identical cultivation conditions and simulate cultivation in scale-up situation (Figure 18A-B). Two-level Taguchi's Design of Experiments (DOE) method was implemented to find the optimal combination of experimental runs. Microalgae were cultivated with synthetic flue gas (5% v/v CO_2), while the aeration rate was regulated to 0.4 and 0.8 L min^{-1}. For illumination, two types of LED lights were implemented, one being continuous and one being flashing at a frequency of 1,000 Hz and duty cycle Φ of 0.5. Frequencies greater than 1,000 Hz, which is less than 1 ms (i.e., the electron turnover time in the electron transport chain of photosynthesis) enhance photon absorption and increase the photosynthetic efficiency (Martín-Girela et al., 2017; Park and Lee, 2000; Takache et al., 2015). Also, flashing effect reduces electricity cost, as LED lights are not switched on continuously. Flashing light effect was achieved by using an Arduino module and appropriate electronic circuits to control the pulse width modulation (PWM) signal of LED strip lights. Illuminance intensity was 3,300 and 6,600 lux, corresponding to 50 and 100 μmol/m^{-2}/s^{-1}. Furthermore, two $NaNO_3$ concentrations were examined, one being 0.25 g L^{-1} and the other 0.75 g L^{-1}. To increase lipids production, microalgae were also subdued to nitrogen starvation three days prior to biomass recovery. Nitrogen starvation induces stress to the microalga cells, by reducing cell growth. Hence, microalgae are shifting their metabolic pathways into neutral lipids production and maximize the production of lipids (Suastes-Rivas et al., 2020). Temperature was adjusted to 25 ± 1 ºC and the initial pH 6.5 was adjusted during the preparation of the Bold's Basal medium. Experimental results were analyzed with Analysis of Variance (ANOVA) and Tuckey

HSD method, analysis of means, and linear regression models were implemented to find optimal cultivation conditions.

Table 1. Growth parameters (factors) and their corresponding levels that were examined for microalgae cultivation optimization.

Aeration rate (L min^{-1})	**Illuminance (lux)**	**Illumination type**	**$NaNO_3$ concentration (g L^{-1})**	**Nitrogen starvation (days)**
0.4	3,300	Continuous	0.25	0
0.8	6,600	Flashing	0.75	3

4.2.3 Biomass measurement

Microalgae were cultivated attached on removable sandblasted glass tiles, as a promising method to lower harvesting costs (Zhuang et al., 2018). In order to harvest the biomass and quantify dry cell weight, the supernatant was initially pumped out from the vessels, to provide access to the sandblasted glass (Figure 18C). Then, the biomass was easily scraped from the surfaces (Figure 18D) and further dewatered by vacuum filtration using pre-weighted 1.2 μm glass fiber filters with porous size of 1.2 μm (Figure 19). Dry cell weight was calculated according to the following formula:

$$DCW\ [g \cdot L^{-1}] = \frac{(m_f - m_i)}{V_s}$$

Where:

- m_f, the final weight of the filter after filtration and drying,
- m_i, the initial weight of the filter,
- V_s, the sample volume.

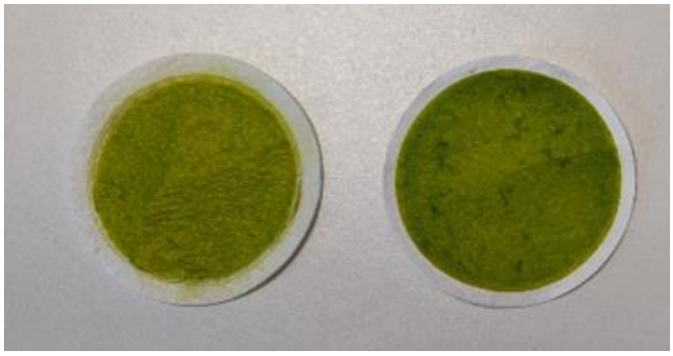


Figure 19. Filtered biomass after vacuum filtration.

4.2.4 Bio-products measurement

Dried biomass was further processed and converted into the selected bio-products (i.e., carbohydrates, proteins, lipids, and total chlorophyll). The biochemical composition of microalgae is consisted of these components (Chen and Vaidyanathan, 2013), that is why they were selected to be measured in the present study. Each protocol that was used is a modified version of (Karapatsia et al., 2016; Psachoulia and Chatzidoukas, 2021). Detailed information is given in the following sections.

4.2.4.1 Carbohydrates

For the quantitative determination of carbohydrates, solutions of 2.5 mol L^{-1} HCl and 2.5 mol L^{-1} NaOH were prepared. Then, 5 mL of HCl (2.5 mol L^{-1}) were added to 1.5 mg of freeze-dried biomass and the suspension was incubated at 100 ^{o}C for 3 h. To neutralize the acid, 5 mL NaOH (2.5 mol L^{-1}) were added. For the quantitative determination of carbohydrates, the phenol-sulfuric acid method was used (Nielsen, 2010). More specifically, for this method 100 mg of glucose were dissolved in 1 L of diH_2O. To this solution, 0.05 mL of 80% phenol and 5.0 mL of H_2SO_4 (95-98%) were added. The solution was vortexed and allowed to settle for 10 min. Then, it was dipped into a water bath at 25 ^{o}C for 10 min. Finally, the carbohydrates content in samples (Figure 20) was measured calorimetrically using known sample concentrations between 0 to 100 μg glucose/2 mL. The spectrophotometer was zeroed with a blank sample containing 0 μg glucose/2 mL. The absorbance of all samples was measured at 490 nm.

Figure 20. Microalgae samples during their conversion into traceable carbohydrates.

4.2.4.2 Proteins

To extract the proteins, first a solution of 0.5 NaOH with 5% MeOH (hereafter R1) was prepared. Then, a phosphate buffer solution (PB) 0.05 M (pH = 7.4) was added to R1 at a ratio of 1% (v/v). 3 mg of freeze-dried biomass were then dispersed in 10 mL of the R1 mixture and ultrasound was used to disrupt the cell walls. The ultrasound intensity was set to 50% and the operation was intermittent for a total duration of 10 min. Then, solution R1 was added to the mixture until the final volume reached 15 mL. The suspension was then heated to 100 ºC in an oil bath for 30 min under continuous stirring at 280 rpm. After that, centrifugation was performed for 10 min at 4,000 rpm. Proteins were measured in the recovered supernatant solution with the micro-BCA analytical method (MilliporeSigma™ Novagen™ BCA Protein Assay Kit), which is based on the conversion of Cu^{2+} to Cu^{+} under alkaline conditions. Cu^{+} is detected after its reaction with BCA and in the presence of proteins the solution becomes intensely colored. The resulting purple color absorbs at 562 nm using a visible-UV spectrophotometer. The method was calibrated using serum albumin (BSA) as a standard protein, included inside the kit. Then, 8 standard solutions of known concentration of BSA were prepared, at the range of 0.5-200 μg mL^{-1} (Figure 21). In addition, a zero BSA sample was prepared to serve as blank. Standard solutions contained 1% MeOH in 0.1 N NaOH with 0.534% (v/v) PB as solvent.

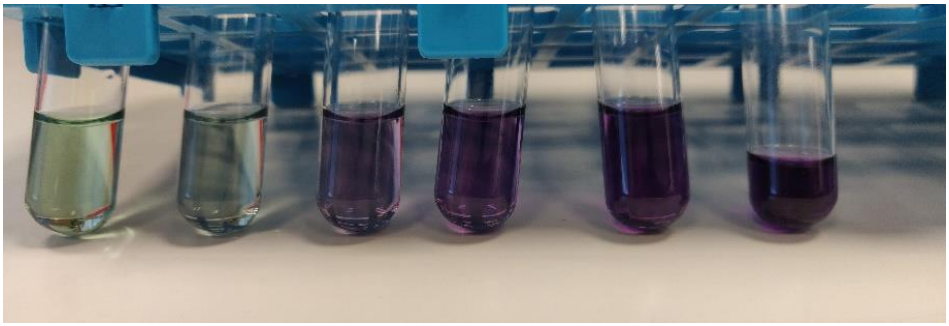


Figure 21. Standard solutions of BSA for proteins quantification.

4.2.4.3 Lipids

The method of Folch et al. (1957) was used for the quantitative determination of lipids in microalgal cells. Initially, 100 mg of freeze-dried biomass were weighed and transferred to a 50 mL falcon tube. Then, 20 mL of 2:1 chloroform:methanol were added. Immediately after, the tubes were placed in an ultrasonic device for 10 min. Centrifugation was then applied for 10 min at 4,000 rpm. The supernatant was removed and transferred to a 100 mL or 250 mL separation funnel (depending on the amount of extracted lipids) (Figure 22A). Then, 20 mL of 2:1 chloroform:methanol were again added to the falcon tube. The process from centrifugation onwards was repeated until the color of the solvent was light green and remained the same as of the previous extraction. The aim was to recover from the biomass, as much lipids as possible. After all the supernatant was collected in the separation funnel, aqueous solution of KCl (0.74% w/v) equal to 0.2 x amount (mL) of solvent was added. The separation funnel was shaken (2-3 shakes) and the tap was opened to release air. Once the two phases were separated, each phase was collected in a 100 mL Erlenmeyer's flask. The chloroform with the lipids was returned to the separatory flask and KCl is added again, thus achieving the second separation. The lower phase was then collected in a 250 mL Erlenmeyer's flask containing Na_2SO_4 to remove moisture. Then, the upper phase was also collected, which was placed in the flask where the 1st upper phase from the 1st extraction was collected. Both upper phases were returned to the separatory funnel and KCl was added. The lower phase was collected in the 250 mL flask, where the chloroform and lipids from the previous lower phases were already present. The lower phases collected in the flask, were placed in a suitable spherical flask (50 or 100 or 250 mL, depending on the amount of lipids). A glass funnel with filter paper containing Na_2SO_4 was placed on top of the spherical flask. The

conical flask was then washed with Na_2SO_4 using pure chloroform, which had also been dried with Na_2SO_4. This was done for as many times as it was required for the Na_2SO_4 to "whiten" again. The spherical flask was placed in a rotavaporator, at a water temperature of 58 ºC and a vacuum of minus (-) 450 mbar. A small quantity of moisture-free chloroform was placed in each dry spherical flask and the liquid was transferred (with a glass Pasteur pipette) to dry, pre-weighed 4 mL vials (Figure 22B). The vials were placed on a hot plate, at 58 ºC and with a stream of nitrogen to achieve chloroform evaporation. The process was repeated until the spherical flask was completely washed. Finally, a gravimetric calculation of the lipids in each 4 mL vial was performed.

A)

B)

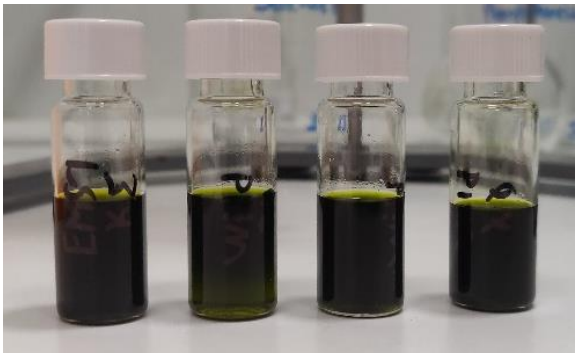


Figure 22. A) Lipids extraction from microalgae biomass and **B)** Extracted lipids.

4.2.4.4 Photosynthetic pigments

To determine the chlorophyll concentration, 2 mL of methanol (99.8%) were added to 5 mg of freeze-dried biomass. The suspension was left to rest for 20-30 min. After the time has elapsed, the suspension was centrifuged at 4,000 rpm for 10 min. The absorbance of the supernatant solution was then measured at 470, 652 and 665 nm with a UV-Vis spectrophotometer (Figure 23). Before measurement, a dilution of 1:4 or other suitable was made so that the measured values were within the measuring range of the device (0-1). Finally, for the determination of total chlorophyll (chlorophyll a, chlorophyll b) and β-carotene, the following equations are used (Lichtenthaler, 1987):

$$Chl_a \left[\frac{\mu g}{mL\ culture}\right] = (16.72 \cdot A_{665}) - (9.16 \cdot A_{652}) \quad (1)$$

$$Chl_\beta \left[\frac{\mu g}{mL\ culture}\right] = (34.09 \cdot A_{652}) - (15.28 \cdot A_{665}) \quad (2)$$

$$Chl_{total} = Chl_\alpha + Chl_\beta \quad (3)$$

$$Carot_\beta \left[\frac{\mu g}{mL\ culture}\right] = \frac{(1000 \cdot A_{470}) - (1.63 \cdot Chl_a) - (104.96 \cdot Chl_\beta)}{221} \quad (4)$$

Where:

- A_{665}, A_{652}, *and* A_{470}, the absorbance at 665, 652, and 470 nm.

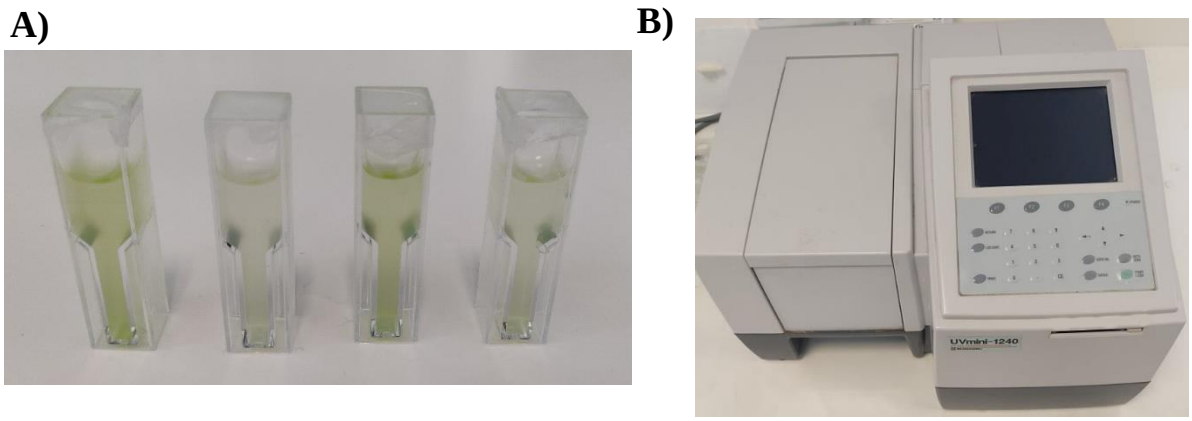


Figure 23. Photosynthetic pigments measurement from microalgae biomass.

4.2.5 Statistical analysis

Experimental data were processed using One-way Analysis of Variance ANOVA and Tuckey HSD method to find the significant difference among treatments. ANOVA is a statistical tool which interprets the significance of experimental data and indicates the optimal performance (Mbachu et al., 2023). Sample means were analyzed with a probability value (P-value) of 0.05.

Taguchi's Design of Experiments (DOE) was used to find the minimum number of experiments to be performed within the permissible limit of factors and levels. Factors correspond to growth parameters (i.e., aeration rate, illuminance, illumination type, $NaNO_3$, and nitrogen starvation) and levels to their values (Table 1). Taguchi's DOE defines the influence of levels of process parameters, whereas ANOVA indicates the contributions of the parameters (Mbachu et al., 2023).

A linear regression model was used to describe the relationship between dependent variables y (responses) and the independent variables x (factors). The dependent variable is also called the response variable. Independent variables are also called explanatory or predictor variables. Continuous predictor variables are also called covariates, and categorical predictor variables are also called factors. The matrix X of observations on predictor variables is usually called the design matrix (The Mathworks, Inc., 2023). Linear model equation is given below:

$$y_i = \beta_0 + \beta_1 x_{i1} + \beta_2 x_{i2} + \cdots + \beta_p x_{ip} + \varepsilon_1,\ i = 1,\ldots,n$$

Where:

- n, the number of observations,
- y_i, the i^{th} response,
- β_k, the k^{th} coefficient, where β_0 is the constant term in the model,
- X_{ij}, the i^{th} observation on the j^{th} predictor variable, j = 1, ..., p,
- ε_i, the i^{th} noise term, that is, random error.

All statistical analysis was carried out in Minitab 17.0.

4.3 Results and Discussion

4.3.1 Lab-scale cultivation

To select and optimize one of the two strains of *Stichococcus* sp., namely wild-type and EMS1 (see Chapter 3), experiments were initially performed to determine the effect of agitation on biomass production of immobilized microalgae in lab scale cultivation. Figure 24 shows the experimental results of wild-type *Stichococcus* sp. under no agitation and under 100 rpm. The maximum biomass production was achieved when implementing agitation of the culture at 17.2 ± 5.6 g m^{-2}. However, there is no significant difference among the samples, as the P-value is 0.171, above the threshold of 0.05. This means that without agitation, significant amounts of biomass can also be achieved, by reducing high energy usage in the agitation process (Fu et al., 2023; Hamed, 2016; Singh and Dhar, 2011).

Note that all results are expressed in grams per sq. meter (g m^{-2}), since microalgae were grown immobilized on sandblasted glass and not in suspension.

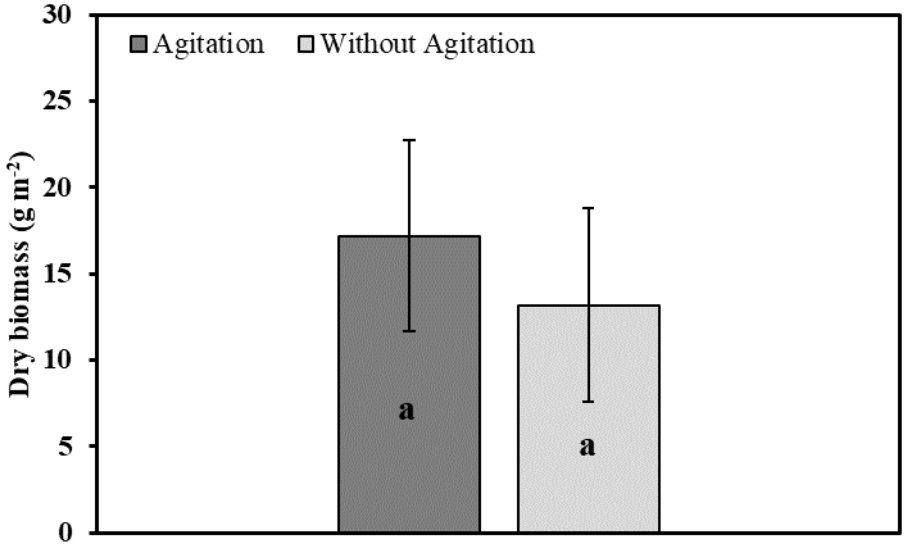


Figure 24. Dry biomass of wild-type *Stichococcus* sp. cultivated without and with agitation at 100 rpm. Values are presented as the mean ± standard deviation (n = 8, p-value = 0.171). Values with the different letters represent a significant difference (p-value < 0.05) between treatments.

As the scope of the present study was the cultivation of *Stichococcus* in elevated CO_2 concentrations, wild-type and EMS1 strains were cultivated with atmospheric air enriched with 5% (v/v) CO_2 and with plain atmospheric air as control, containing approximately 0.04% CO_2 (Global Monitoring Laboratory, 2023). CO_2 addition significantly increased biomass productivity up to approximately 300% (Figure 25), showing that both strains were grown effectively under elevated CO_2 concentrations. In the case of plain atmospheric air, maximum dry biomass production was achieved with EMS1 *Stichococcus* sp. (11.7 ± 0.8 g m^{-2}), whereas in the case of enriched atmospheric air, dry biomass production was equal to 47.4 ± 4.6 g m^{-2}. Higher biomass productivities under CO_2 addition were also found in various studies (Gonçalves et al., 2016; Hu et al., 2012; Hussain et al., 2017; Uggetti et al., 2018). Especially, Gonçalves et al. (2016) and Hu et al. (2012) found that microalgae showed greater biomass productions under 5% (v/v) CO_2. ANOVA statistical tool was applied independently for both CO_2 levels as there is a significant difference between them. Strains cultivated under both aeration conditions didn't show any statistical difference, with P-values being 0.156 and 0.297.

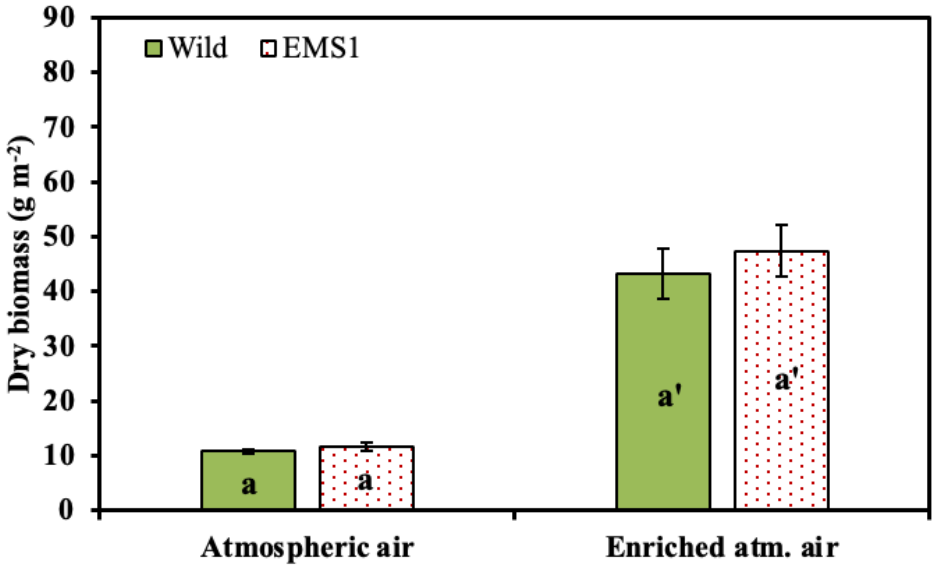


Figure 25. Dry biomass of wild-type *Stichococcus* sp. cultivated with plain atmospheric air and atmospheric air enriched with 5% (v/v) CO_2. Values are presented as the mean ± standard deviation. Values with the different letters represent a significant difference (p-value < 0.05) between treatments. ANOVA was implemented independently for each condition, indicated on the plot by a-a and a'-a'. N = 3 and p-value = 0.156 for plain atmospheric air, and n = 3 and p-value = 0.297 for enriched atmospheric air.

Cultivation period is an important parameter to be determined, for achieving maximum biomass productivity. Experiments with wild-type and EMS1 *Stichococcus* sp. were conducted for 15, 20, and 25 days (Figures 26-27). For both strains, the maximum value of the biomass concentration was obtained at 25 days and the corresponding values were equal to 42.6 ± 2.2 and 44.3 ± 1.6 g m^{-2}. P-values were equal to 0.005 and 0.002, indicating that both strains had a significant difference at 25 days of cultivation. According to the literature, cultivation period varies with microalgae species and cultivation conditions. Similar studies conducted on *Stichococcus* sp. have shown that cultivation period up to 32 days (Olivieri et al., 2011), with others ranging from 15 to 19 days (Karapatsia et al., 2016; Maršálek et al., 1992; Psachoulia and Chatzidoukas, 2021; Safonova et al., 2004). Given the experimental and ANOVA results, 25 days were selected for future experiments, as both strains noted greater biomass productivity.

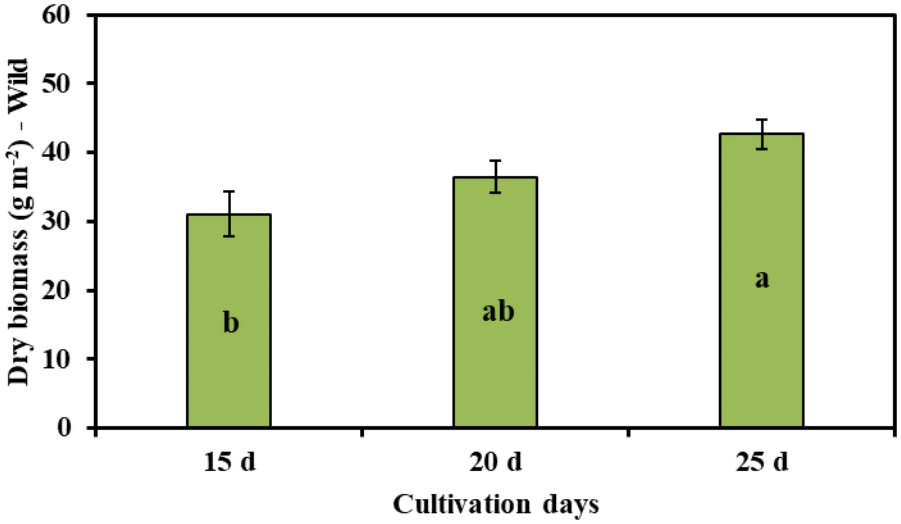


Figure 26. Dry biomass of wild-type *Stichococcus* sp. in relation to 15, 20, and 25 days of cultivation. Values are presented as the mean ± standard deviation (n = 3, p-value = 0.005). Values with the different letters represent a significant difference (p-value < 0.05) between treatments.

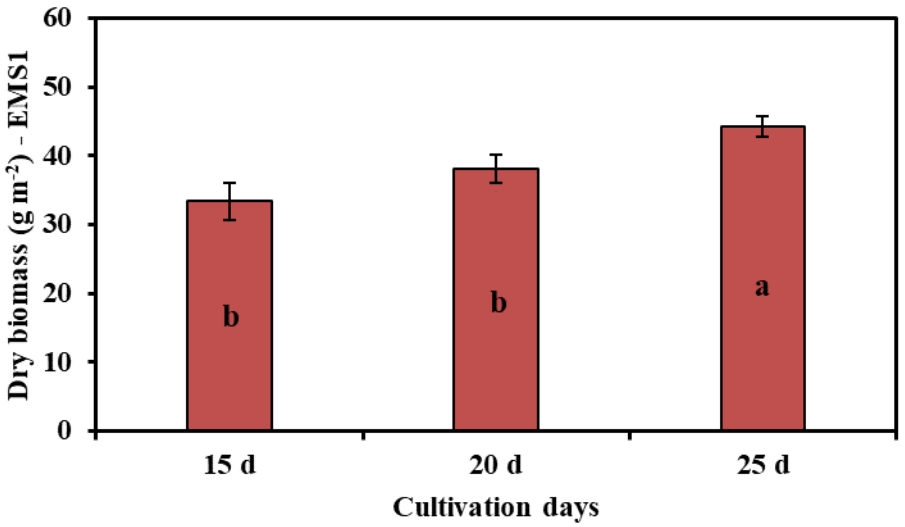


Figure 27. Dry biomass of mutant EMS1 *Stichococcus* sp. in relation to 15, 20, and 25 days of cultivation. Values are presented as the mean ± standard deviation (n = 3, p-value = 0.002). Values with the different letters represent a significant difference (p-value < 0.05) between treatments.

Next step of the experimental process was the implementation of nitrogen starvation prior to harvesting, inducing cell stress, which in turn increases intracellular lipids production (Suastes-Rivas et al., 2020). The following figures present the experimental results of wild-type (Figure 28) and EMS1 (Figure 29) *Stichococcus* sp. treated with 0, 2, and 3 days of nitrogen starvation. As can be seen in both figures, the application of 3-day nitrogen starvation, showed the highest lipids concentration, having significant difference compared to 0 and 2 days (p-value = 0.013 for wild-type and p-value = 0.005 for EMS1). The corresponding values were equal to 10.5 ± 0.4 g m^{-2} (Figure 28) and 13.9 ± 1.0 g m^{-2} (Figure 29), respectively. Also, it is evident that nitrogen starvation increases lipids production in both 2-day and 3-day applications, which has been reported extensively in the literature (Al-Rashed et al., 2016; Breuer et al., 2012; Chu et al., 2019; Suastes-Rivas et al., 2020; Wang et al., 2015). Based on the present statistical results on both strains, 3-day nitrogen starvation was selected for the following experiments on the optimization of *Stichococcus* sp. (see Section 4.3.2).

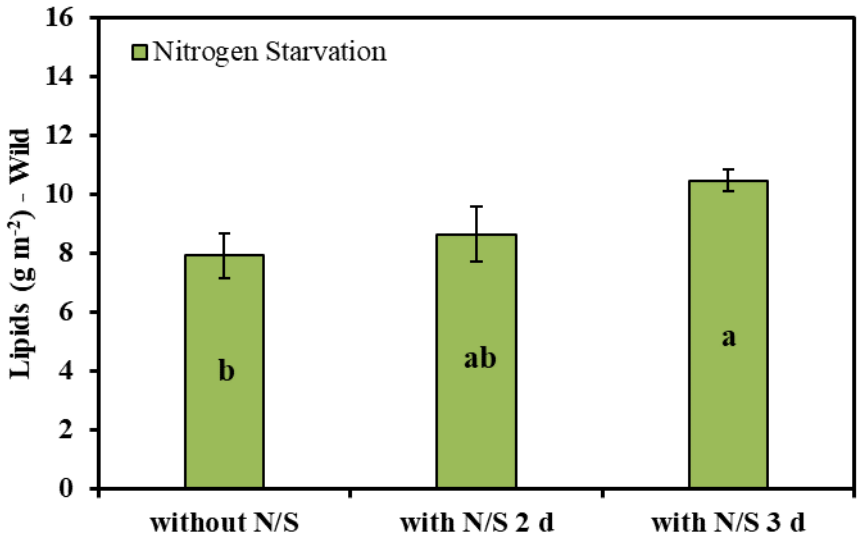


Figure 28. Lipids production of wild-type *Stichococcus* sp. in relation to 0, 2, and 3 days of nitrogen starvation (N/S). Values are presented as the mean ± standard deviation (n = 3, p-value = 0.013). Values with the different letters represent a significant difference (p-value < 0.05) between treatments.

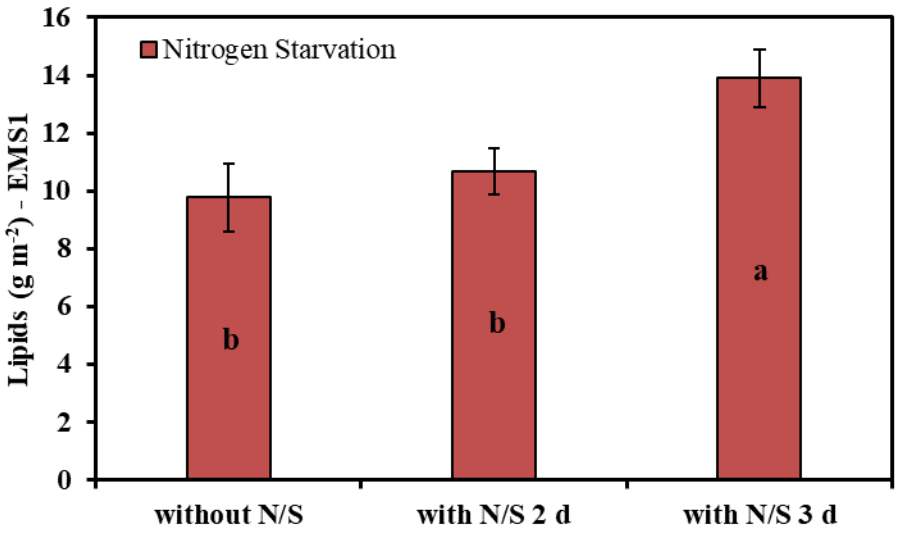


Figure 29. Lipids production of mutant EMS1 *Stichococcus* sp. in relation to 0, 2, and 3 days of nitrogen starvation (N/S). Values are presented as the mean ± standard deviation (n = 3, p-value = 0.005). Values with the different letters represent a significant difference (p-value < 0.05) between treatments.

After the completion of the abovementioned experiments, the final step was the selection of a promising strain, which could be used in 3rd generation biorefineries. To achieve that, both strains were cultivated in lab-scale and at the end of the cultivation period, dry biomass, lipids, pigments, proteins, and carbohydrates were measured to assess each strain's potential. Figures 30-35 show the results of each measurement mentioned above.

Dry biomass of EMS1 strain showed statistical significance (p-value = 0.006) over the wild-type, with the value being equal to 44.9 ± 1.1 g m^{-2}, compared to 40.7 ± 1.0 g m^{-2} of the wild-type. The above is also verified by the experimental results of Figures 14, 25-27.

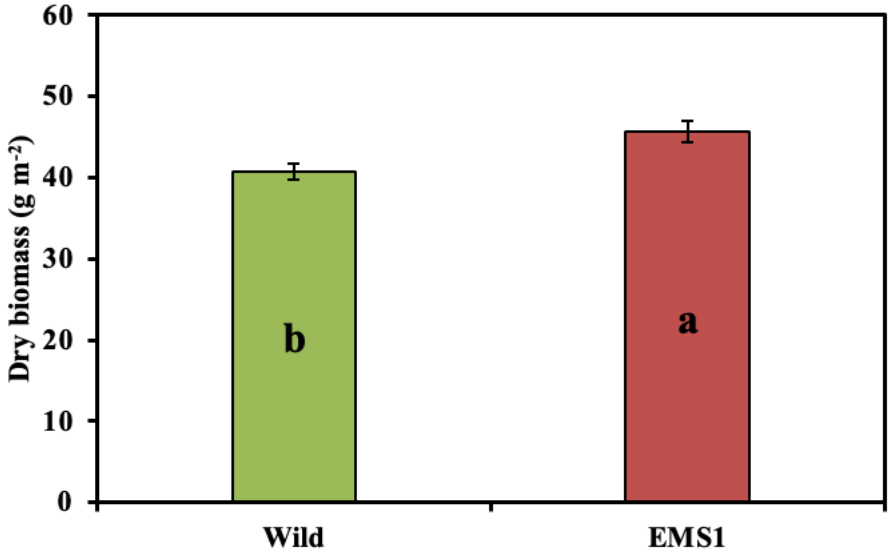


Figure 30. Dry biomass of wild-type and mutant EMS1 *Stichococcus* sp. Values are presented as the mean ± standard deviation (n = 3, p-value = 0.006). Values with the different letters represent a significant difference (p-value < 0.05) between treatments.

Lipids content (Figure 31) followed the same pattern as the dry biomass with EMS1 strain having a statistical difference (p-value = 0.042) and a value of 8.5 ± 0.7 g m^{-2}. The above was also mentioned in the initial study for the mutation of wild-type *Stichococcus* sp. (Chapter 3, Figure 16). The percentage of lipids to biomass was equal to 19% for EMS1 strain and 17% for wild-type *Stichococcus* sp. Similar studies on *Stichococcus* have shown corresponding results, ranging from as low as 6%, up to 46%, after optimization of cultivation conditions for maximizing lipids

production (Gargano et al., 2016; Karapatsia et al., 2016; Mutaf et al., 2019; Psachoulia and Chatzidoukas, 2021).

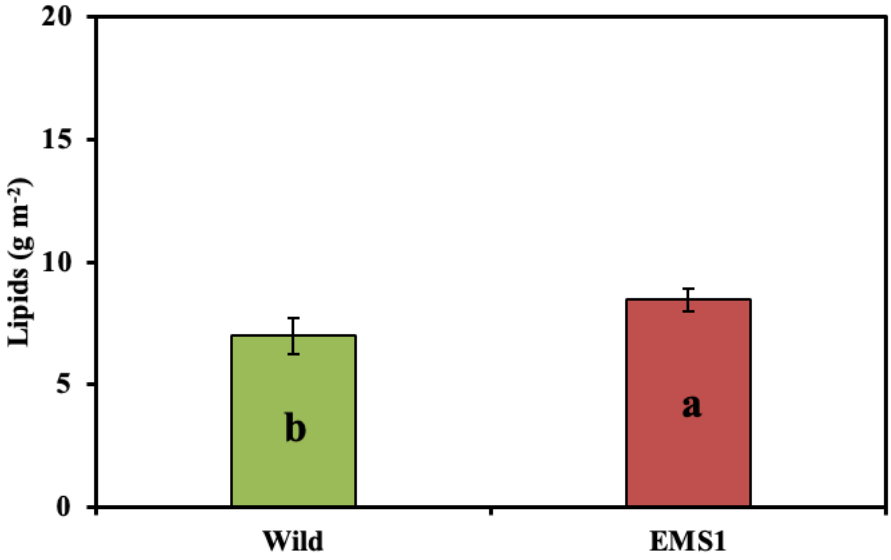


Figure 31. Lipids content of wild-type and mutant EMS1 *Stichococcus* sp. Values are presented as the mean ± standard deviation (n = 3, p-value = 0.042). Values with the different letters represent a significant difference (p-value < 0.05) between treatments.

Figure 32 shows the total chlorophyll (chl a and chl b) of the wild-type and EMS1 strains. Chlorophyll is the main distinguishing characteristic of the aforementioned strains, as the EMS1 was mutated from the wild-type (see Chapter 3) with the goal to have lower chlorophyll content. High chlorophyll constitutes a limiting factor in large-scale photo-bioreactors, causing a "shadow effect" as light penetration in the culture decreases, which negatively influences biomass productivity (Beacham et al., 2017). To this extent, EMS1 noted statistical significance (p-value = 0.008), with the value of total chlorophyll equal to 0.17 ± 0.01 g m^{-2}. Statistical significance was also noted in Figure 15.

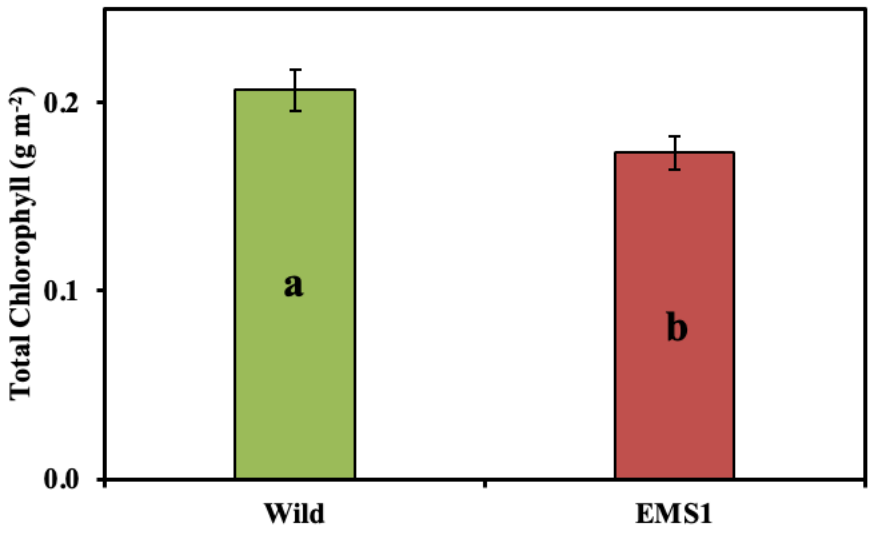


Figure 32. Total chlorophyll content of wild-type and mutant EMS1 *Stichococcus* sp. Values are presented as the mean ± standard deviation (n = 3, p-value = 0.008). Values with the different letters represent a significant difference (p-value < 0.05) between treatments.

Wild-type strain noted higher proteins production at 10.3 ± 0.5 g m^{-2}, having statistical significance (p-value = 0.023) compared to EMS1 (Figure 33).

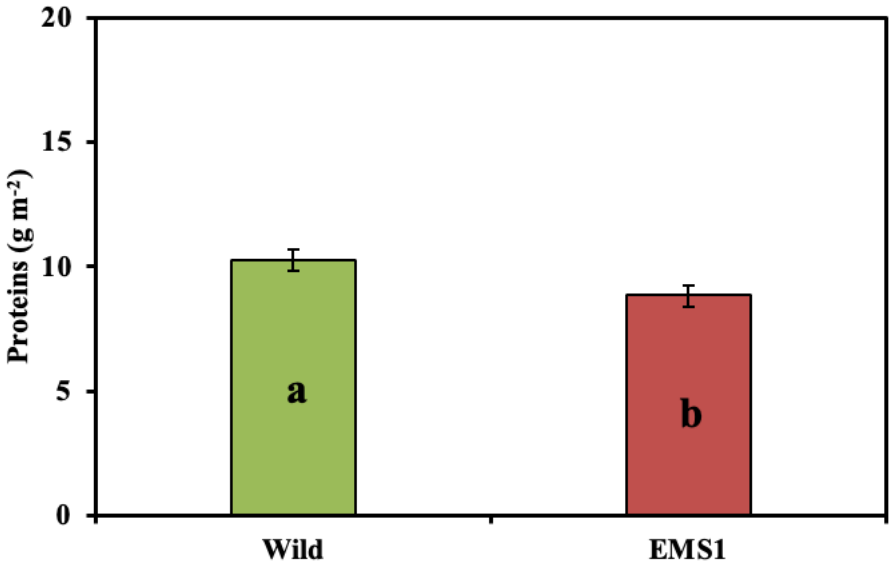


Figure 33. Proteins content of wild-type and mutant EMS1 *Stichococcus* sp. Values are presented as the mean ± standard deviation (n = 3, p-value = 0.023). Values with the different letters represent a significant difference (p-value < 0.05) between treatments.

The final bioactive compound that was measured was carbohydrates content in wild-type and EMS1 *Stichococcus* sp. (Figure 34). EMS1 strain carbohydrates were found equal to 16.5 ± 1.8 g m^{-2}. However, the respective value for wild-type was equal to 14.9 ± 1.5 g $m^{-2,}$ which based on ANOVA statistical tool and Tuckey HSD test, does not have a statistical significance on EMS1 (p-value = 0.233). Reviewing the values of the aforementioned bio-products (i.e., lipids, total chlorophyll, proteins) in Figures 31-33, it is evident that carbohydrates are the dominant bio-product of *Stichococcus* sp. The above, was also observed in the studies of Karapatsia et al. (2016), and Psachoulia and Chatzidoukas (2021). Therefore, both strains of *Stichococcus* sp. could potentially be exploited from industries for their carbohydrates content.

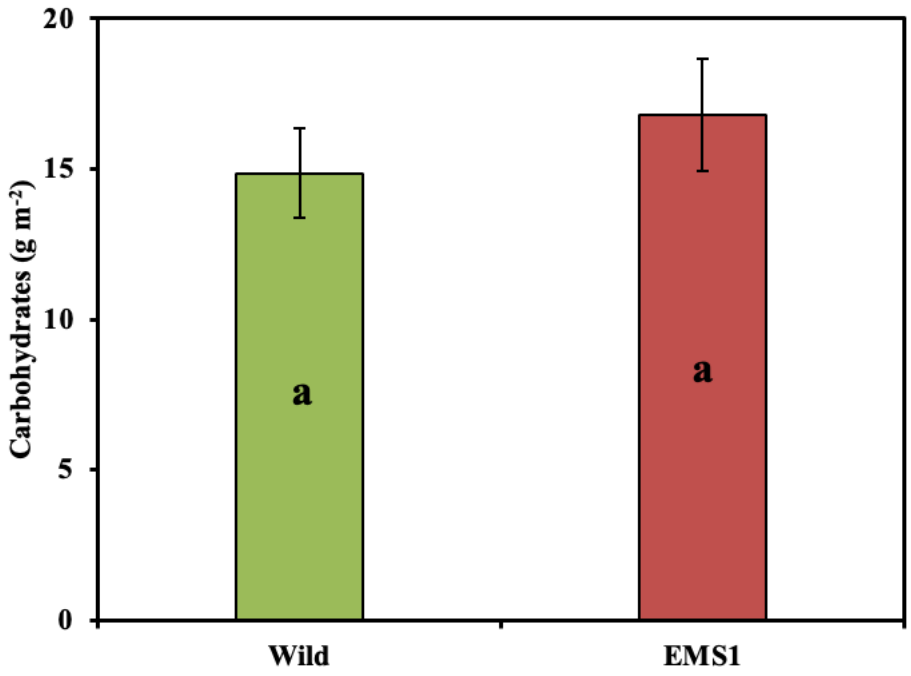


Figure 34. Carbohydrates content of wild-type and mutant EMS1 *Stichococcus* sp. Values are presented as the mean ± standard deviation (n = 3, p-value = 0.233). Values with the different letters represent a significant difference (p-value < 0.05) between treatments.

The total sum of bio-products for each strain examined is shown in Figure 35. As can be seen, strains do not show statistical difference, as the p-value was equal to 0.287. Maximum value of bio-products was met on EMS1 strain, equal to 34.0 ± 3.0 g m^{-2}, while on the wild-type it was determined as 32.3 ± 2.5 g m^{-2}. The corresponding percentages of bio-products to dry biomass

were 79% and 76% for wild-type and EMS1 strains, respectively. Even if the aforementioned percentage of wild-type is greater than of EMS1, the total amount of bio-products in grams per unit area is less than EMS1. Consequently, the overall performance of the strains is similar, with EMS1 being slightly more superior over the wild-type. Karapatsia et al. (2016) examined different combinations of growth parameters and found that wild-type *Stichococcus* sp. can deliver up to 98% in bio-products with an average value equal to 76%, similar to the present study.

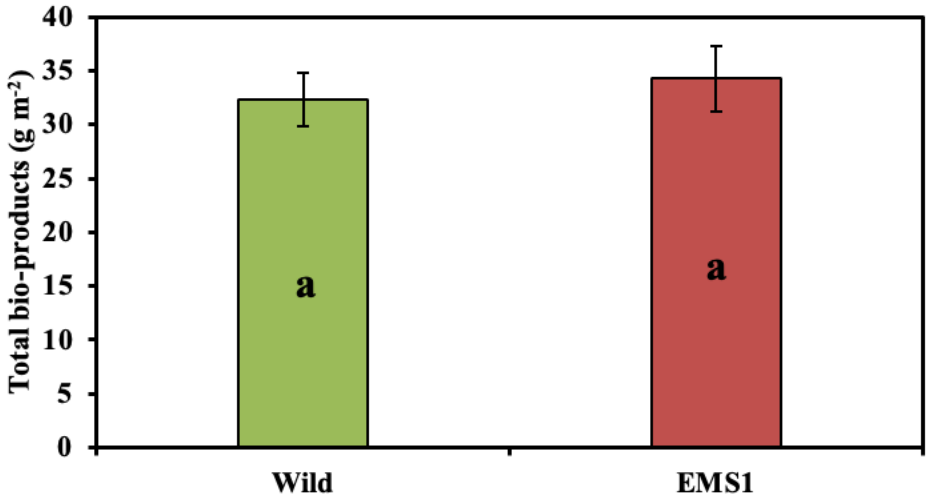


Figure 35. Total bio-products content of wild-type and mutant EMS1 *Stichococcus* sp. Values are presented as the mean ± standard deviation (n = 3, p-value = 0.287). Values with the different letters represent a significant difference (p-value < 0.05) between treatments.

Taking into account the experimental and statistical data mentioned in Figures 30-35 (i.e., dry biomass and bio-products of the selected strains), conclusions were made on the selection of the most promising strain which could be potentially used in 3rd generation biorefineries. EMS1 *Stichococcus* sp. strain was the one which showed increased biomass and bio-products productivity and at the same time contained less chlorophyll, thus minimizing the "shadow effect" in photobioreactors. To further increase the strain's potential, experiments were conducted for the optimization of EMS1 strain cultivation, by examining various growth parameters.

4.3.2 Optimization of a selected *Stichococcus* sp. strain

In section 4.3.1 experiments were conducted to compare the characteristics of wild-type and EMS1 *Stichococcus* sp. microalgae. Note that the latter is a mutant strain, derived from the wild-type (see Chapter 3). EMS1 was selected for continuing with the optimization experiments. In particular, the scope of the present study was the optimization of biomass and bio-products (i.e., lipids, pigments, proteins, and carbohydrates) in lab scale for scale-up in photo-bioreactors with optimal conditions. Another scope was the fixation of CO_2 contained in flue gas, emitted from thermal power plants.

To perform optimization on EMS1 strain, five growth parameters (factors) were examined. These were, i) aeration rate, ii) illuminance, iii) type of illumination, iv) $NaNO_3$ concentration in growth medium, and v) nitrogen starvation. Two levels were selected for each factor (Table 1). Specifically, levels i) for aeration rate were 0.4 and 0.8 Liters per minute (L min^{-1}), ii) for illuminance were 3,300 and 6,600 lux, which can be converted into 50 and 100 μmol of photons m^{-2} s^{-1}, iii) for illumination type, two discrete values of continuous and flashing light were implemented, iv) for $NaNO_3$ concentrations were 0.25 and 0.75 g L^{-1}, and v) for nitrogen starvation 0 and 3 days were selected. Detailed information on the growth parameters is mentioned in section 4.2.2.6.

Taguchi's Design of Experiments method was applied to find the optimal experimental runs, based on the given growth conditions. Taguchi results reported 12 runs (L_{12}) for the five factors and two levels, mentioned above. The order of experimental runs and combinations are mentioned in Table 2. Note that the 1st and 2nd runs have identical values, according to the method's results. Therefore, the actual runs were 11.

Table 2. Taguchi - Design of Experiments (DOE) results L_{12} based on the selected cultivation parameters of EMS1 *Stichococcus* sp. (Table 1).

Run#	Aeration rate (L min^{-1})	Illuminance (lux)	Illumination type	$NaNO_3$ Concentration (g L^{-1})	Nitrogen starvation (days)

1	0.4	3,300	Continuous	0.25	0
2	0.4	3,300	Continuous	0.25	0
3	0.4	3,300	Flashing	0.75	3
4	0.4	6,600	Continuous	0.75	3
5	0.4	6,600	Flashing	0.25	3
6	0.4	6,600	Flashing	0.75	0
7	0.8	3,300	Flashing	0.75	0
8	0.8	3,300	Flashing	0.25	3
9	0.8	3,300	Continuous	0.75	3
10	0.8	6,600	Flashing	0.25	0
11	0.8	6,600	Continuous	0.75	0
12	0.8	6,600	Continuous	0.25	3

Note: *Runs 1 and 2 are identical, based on the Taguchi's DOE results.*

Table 3 contains the experimental results of the five selected cultivation parameters reported in Table 2. For each run, six responses were measured, namely biomass, lipids, total chlorophyll, proteins, carbohydrates, and the sum of the aforementioned bio-products. The highest values for biomass and total bio-products were found in Run #11 and were equal to 45.7 ± 1.3 and 34.8 ± 2.2 g m^{-2}. Lipids maximization was achieved in Run #3 at 11.6 ± 0.4 g m^{-2}. Furthermore, the maximum concentration for total chlorophyll and carbohydrates was measured in Run #6 and the corresponding values were equal to 0.22 ± 0.02 and 19.0 ± 1.5 g m^{-2}. Finally, proteins content showed a maximum value of 9.5 ± 0.5 g m^{-2} in experiment #9.

Table 3. Experimental results of EMS1 *Stichococcus* sp. regarding Biomass, Lipids, Total Chlorophyll, Proteins, Carbohydrates, and Total bio-products, based on Taguchi – Design of Experiments (DOE) L_{12} reported in Table 2.

Run#	Biomass (g m^{-2})	Lipids (g m^{-2})	Total chlorophyll (g m^{-2})	Proteins (g m^{-2})	Carbohydrates (g m^{-2})	Total bio-products (g m^{-2})
1	16.5 ± 0.6	4.6 ± 0.3	0.05 ± 0.01	3.3 ± 0.4	6.2 ± 0.6	14.1 ± 0.9
2	16.5 ± 0.6	4.6 ± 0.3	0.05 ± 0.01	3.3 ± 0.4	6.2 ± 0.6	14.1 ± 0.9

3	28.2 ± 1.7	11.6 ± 0.4	0.10 ± 0.02	5.5 ± 0.5	8.5 ± 1.0	25.7 ± 0.5
4	42.1 ± 0.6	9.4 ± 0.1	0.15 ± 0.02	6.5 ± 0.4	15.2 ± 1.4	31.2 ± 1.6
5	19.9 ± 3.1	5.9 ± 0.2	0.06 ± 0.00	3.6 ± 0.2	6.5 ± 0.7	16.0 ± 0.8
6	36.5 ± 0.5	6.7 ± 0.5	0.22 ± 0.02	6.0 ± 0.4	19.0 ± 1.5	31.9 ± 0.7
7	29.7 ± 1.3	7.8 ± 0.4	0.14 ± 0.02	9.2 ± 0.7	6.5 ± 0.4	23.7 ± 1.3
8	14.8 ± 1.0	5.9 ± 0.1	0.09 ± 0.02	2.8 ± 0.1	4.3 ± 0.7	13.1 ± 0.8
9	32.8 ± 2.0	10.8 ± 0.5	0.12 ± 0.01	9.5 ± 0.5	7.5 ± 0.3	27.9 ± 0.9
10	24.9 ± 0.8	5.6 ± 0.2	0.20 ± 0.01	4.3 ± 0.5	7.4 ± 0.2	17.5 ± 0.4
11	45.7 ± 1.3	10.0 ± 0.1	0.17 ± 0.01	7.9 ± 0.4	16.8 ± 1.9	34.8 ± 2.2
12	25.7 ± 0.3	6.6 ± 0.2	0.11 ± 0.01	3.1 ± 0.5	7.5 ± 0.2	17.3 ± 0.4

Note: *Runs 1 and 2 are identical, based on the Taguchi's DOE results. Maximum value of each response is highlighted in grey color.*

Analysis of Variance (ANOVA) on each of the six process responses was performed using Minitab 17.0 statistical software. Results of the ANOVA tool are reported in Table 4. It is worth mentioning that when p-value is less than 0.05, the results are statistically significant. Degrees of freedom are calculated by subtracting one (1) from the number of levels (i.e., 2 levels in this case). R^2 ranged from 80.7% up to 98.7%, indicating that the experimental data fit very well into a linear regression.

Table 4. Analysis of Variance (ANOVA) results of EMS1 *Stichococcus* sp. for the mean values of dry biomass, lipids, total chlorophyll, proteins, carbohydrates, and total bio-products.

Biomass						
Source	**DF**	**Seq. SS**	**Adj. SS**	**Adj. MS**	**F-Value**	**P-Value**
Aeration rate (L min^{-1})	1	0.2	13.7	13.7	5.4	0.068
Illuminance (lux)	1	180.8	226.4	226.4	89.2	<0.0005
Illumination type	1	99.9	46.2	46.2	18.2	0.008
$NaNO_3$ concentration (g L^{-1})	1	691.8	665.3	665.3	262.1	<0.0005
Nitrogen starvation (days)	1	2.8	2.8	2.8	1.1	0.342
Residual Error	5	12.7	12.7	2.5		
Total	10	988.2				
R^2 (%)	**98.7**					
Lipids						

Source	DF	Seq. SS	Adj. SS	Adj. MS	F-Value	P-Value
Aeration rate (L min^{-1})	1	0.1	1.1	1.1	0.7	0.450
Illuminance (lux)	1	1.7	0.1	0.1	0.1	0.813
Illumination type	1	3.3	0.4	0.4	0.2	0.643
$NaNO_3$ concentration (g L^{-1})	1	34.2	38.5	38.5	23.7	0.005
Nitrogen starvation (days)	1	8.6	8.6	8.6	5.3	0.070
Residual Error	5	8.1	8.1	1.6		
Total	10	56.0				
R^2 (%)	**85.5**					

Total chlorophyll

Source	DF	Seq. SS	Adj. SS	Adj. MS	F-Value	P-Value
Aeration rate (L min^{-1})	1	0.002	0.003	0.003	2.6	0.166
Illuminance (lux)	1	0.008	0.009	0.009	8.0	0.037
Illumination type	1	0.001	0.002	0.002	1.5	0.283
$NaNO_3$ concentration (g L^{-1})	1	0.010	0.009	0.009	7.4	0.042
Nitrogen starvation (days)	1	0.003	0.003	0.003	2.6	0.166
Residual Error	5	0.006	0.006	0.001		
Total	10	0.030				
R^2 (%)	**80.7**					

Proteins

Source	DF	Seq. SS	Adj. SS	Adj. MS	F-Value	P-Value
Aeration rate (L min^{-1})	1	3.7	5.4	5.4	3.5	0.121
Illuminance (lux)	1	1.4	0.3	0.3	0.2	0.668
Illumination type	1	1.5	0.3	0.3	0.2	0.700
$NaNO_3$ concentration (g L^{-1})	1	44.1	41.7	41.7	26.7	0.004
Nitrogen starvation (days)	1	0.6	0.6	0.6	0.4	0.553
Residual Error	5	7.8	7.8	1.6		
Total	10	59.1				
R^2 (%)	**86.8**					

Carbohydrates

Source	DF	Seq. SS	Adj. SS	Adj. MS	F-Value	P-Value
Aeration rate (L min^{-1})	1	20.7	9.7	9.7	1.6	0.261
Illuminance (lux)	1	74.4	78.7	78.7	13.0	0.015

Illumination type	1	7.7	3.9	3.9	0.6	0.459
$NaNO_3$ concentration (g L^{-1})	1	101.1	89.6	89.6	14.8	0.012
Nitrogen starvation (days)	1	11.6	11.6	11.6	1.9	0.226
Residual Error	5	30.3	30.3	6.1		
Total	10	245.7				
R^2 (%)	**87.7**					

Total bioproducts

Source	DF	Seq. SS	Adj. SS	Adj. MS	F-Value	P-Value
Aeration rate (L min^{-1})	1	5.5	0.1	0.1	0.0	0.866
Illuminance (lux)	1	39.0	65.3	65.3	21.6	0.006
Illumination type	1	33.5	9.4	9.4	3.1	0.138
$NaNO_3$ concentration (g L^{-1})	1	512.7	493.9	493.9	163.4	<0.0005
Nitrogen starvation (days)	1	1.7	1.7	1.7	0.6	0.484
Residual Error	5	15.1	15.1	3.0		
Total	10	607.6				
R^2 (%)	**97.5**					

Note: ***DF (Degrees of freedom)*** *= maximum number of logically independent values;* ***Seq. SS (Sequential sums of squares)*** *= the reduction in the error sum of squares (SSE) when one or more predictor variables are added to the model or the increase in the regression sum of squares (SSR) when one or more predictor variables are added to the model;* ***Adj. SS (Adjusted sums of squares)*** *= measures the reduction in the error sum of squares (or increase in the regression sum of squares) when each predictor is added to a model that contains all of the remaining predictors;* ***Adj. MS (Adjusted mean squares)*** *= adjusted sum of squares / the degrees of freedom;* ***F-Value*** *= variation between sample means / variation within the samples;* ***P-Value*** *= Probability value;* ***Residual Error*** *= the differences between the observed values and the estimated values;* ***R^2*** *= percentage of variation in the response that is explained by the model (PennState Eberly College of Science, 2023).*

Figures 36-41 show the main effect plots of the six responses (i.e., dry biomass, lipids, total chlorophyll, proteins, carbohydrates, and total bio-products) in relation to the five factors (i.e., aeration rate, illuminance, illumination type, $NaNO_3$ concentration, and nitrogen starvation). The values for each level of the aforementioned figures are mentioned in Table 5. Main effect is the mean value of each level of the corresponding factor. Therefore, the following plots could give a better insight into the effects of different cultivation condition on EMS1 *Stichococcus* sp.

Table 5. 1st and 2nd level values of the five growth parameters (factors) that were examined for microalgae cultivation optimization.

	Aeration rate ($L \cdot min^{-1}$)	**Illuminance (lux)**	**Illumination type**	**$NaNO_3$ Concentration ($g \cdot L^{-1}$)**	**Nitrogen starvation (days)**
Level-1	0.4	3,300	Continuous	0.25	0
Level-2	0.8	6,600	Flashing	0.75	3

The higher level of $NaNO_3$ concentration (0.75 g L^{-1}) seemed to have the greatest influence on biomass production (Figure 36). The main effect for this level of $NaNO_3$ was equal to 35.8 ± 7.0 g m^{-2}. Biomass increase with the application of 3-fold $NaNO_3$ concentration has been also reported in the literature for the strains *Stichococcus* sp. (Karapatsia et al., 2016), *Haematococcus pluvialis* (Meryem et al., 2019), *Chromochloris zofingiensis* (Minyuk et al., 2019), and *Ankistrodesmus* sp. (Pan-utai et al., 2019) cultivated in Bold's Basal medium. When applying the higher level of illuminance (6,600 lux) and continuous lighting, it appeared that biomass was maximized, with mean values of 32.5 ± 10.4 and 32.6 ± 11.9 g m^{-2}, respectively. Higher frequencies or continuous lighting show increased biomass production, compared to 1,000 Hz (Hu and Sato, 2017). Aeration rate seemed to have low effect on biomass production. This may be justified as microalgae were receiving adequate amount of carbon, even with the lower aeration rate of 0.4 g L^{-1}. Finally, the application of nitrogen starvation resulted in lower biomass recovery, which was anticipated as during pumping of the initial growth medium (and the filling with fresh medium), a fraction of the floating microalgae cells is also removed.

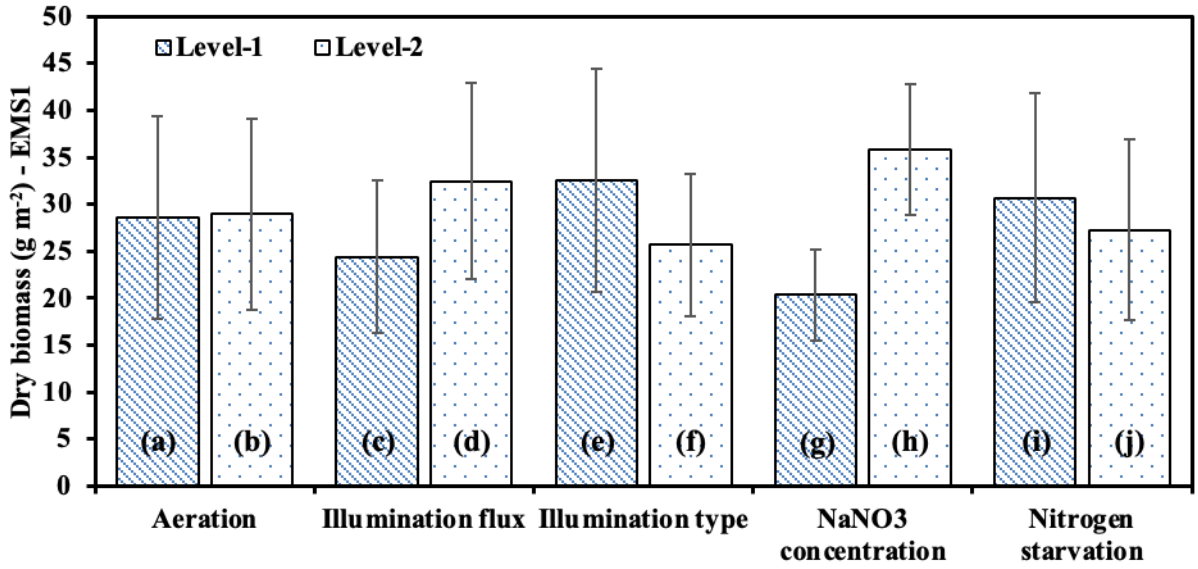


Figure 36. Main effect plot of dry biomass of EMS1 *Stichococcus* sp., in relation to the experimental parameters, based on the results shown in Table 3. **Level-1: (a)** aeration rate 0.4 L min^{-1}, **(c)** illuminance 3,300 lux, **(e)** continuous lighting, **(g)** $NaNO_3$ concentration 0.25 g L^{-1}, and **(i)** nitrogen starvation 0 days. **Level-2: (b)** aeration rate 0.8 L min^{-1}, **(d)** illuminance 6,600 lux, **(f)** flashing lighting, **(h)** $NaNO_3$ concentration 0.75 g L^{-1}, and **(j)** nitrogen starvation 3 days.

As in the case of biomass, lipids were mostly affected by the higher $NaNO_3$ concentration in the growth medium, having a main effect of 9.4 ± 1.8 g m^{-2} (Figure 37). Microalgae accumulate more lipids under continuous light than under flashing light, as a response to high-light stress (Abu-Ghosh et al., 2016). Three-day nitrogen starvation positively affected intracellular lipids production by 22% increase in the mean values. This effect is also reported in the literature (Al-Rashed et al., 2016; Breuer et al., 2012; Chu et al., 2019; Suastes-Rivas et al., 2020; Wang et al., 2015) and in Figure 29. Aeration rate, illuminance and illumination type showed minor effects on lipids production. Cheirsilp and Torpee, 2012; Han et al., 2015 mentioned that even if higher aeration rates result in higher growth rates, lipids production is reduced because the cells use more energy for proliferation, instead of lipids production.

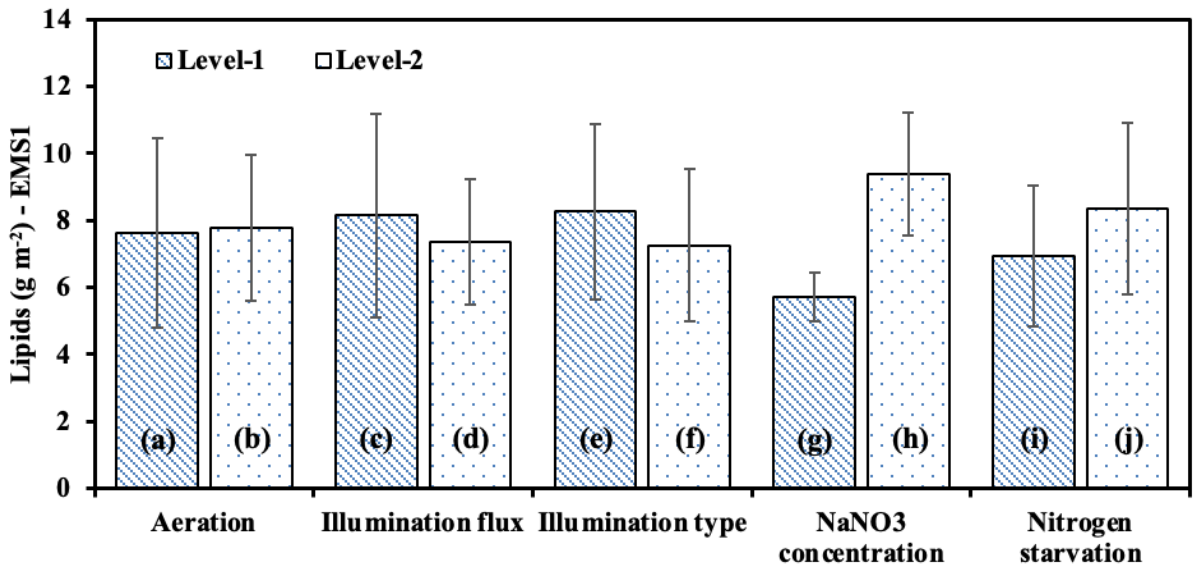


Figure 37. Main effect plot of lipids of EMS1 *Stichococcus* sp., in relation to the experimental parameters, based on the results shown in Table 3. **Level-1: (a)** aeration rate 0.4 L min^{-1}, **(c)** illuminance 3,300 lux, **(e)** continuous lighting, **(g)** $NaNO_3$ concentration 0.25 g L^{-1}, and **(i)** nitrogen starvation 0 days. **Level-2: (b)** aeration rate 0.8 L min^{-1}, **(d)** illuminance 6,600 lux, **(f)** flashing lighting, **(h)** $NaNO_3$ concentration 0.75 g L^{-1}, and **(j)** nitrogen starvation 3 days.

Figure 38 shows the main effects of the responses on total chlorophyll production. Illuminance, $NaNO_3$ concentration, and nitrogen starvation were the main contributors to chlorophyll production. Higher $NaNO_3$ concentration was also found to increase chlorophyll a in the marine microalga *Chroomonas* sp. (Bermúdez et al., 2004). Higher levels of illuminance, $NaNO_3$ concentration, and zero nitrogen starvation resulted in 50 to 60% increase in main affect values. Martín-Girela et al. (2017) conducted experiments with immobilized microalgae illuminated with continuous and flashing light at 3 frequencies of 0.1, 1, and 10 kHz and 4 light fractions of 5, 25, 50, and 75%. They found that flashing light causes higher photosynthetic activity, compared to continuous light. Chlorophyll molecules are highly efficient and possess the ability to absorb almost all irradiated light, however not all light is used for photosynthesis. For this reason, several studies have examined the use of flashing effect rather than continuous light (Kim et al., 2006; V. Kumar et al., 2021; Park and Lee, 2001; Tennessen et al., 1995; Zarmi et al., 2020). Also, higher photosynthetic activity is met with higher illuminance.

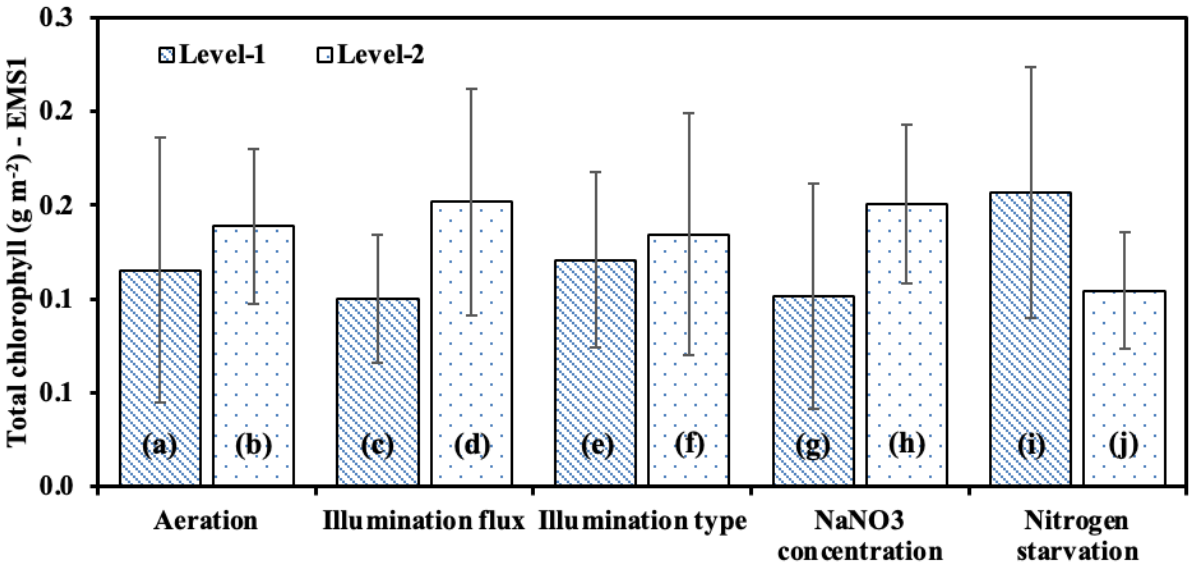


Figure 38. Main effect plot of total chlorophyll of EMS1 *Stichococcus* sp., in relation to the experimental parameters, based on the results shown in Table 3. **Level-1: (a)** aeration rate 0.4 L min^{-1}, **(c)** illuminance 3,300 lux, **(e)** continuous lighting, **(g)** $NaNO_3$ concentration 0.25 g L^{-1}, and **(i)** nitrogen starvation 0 days. **Level-2: (b)** aeration rate 0.8 L min^{-1}, **(d)** illuminance 6,600 lux, **(f)** flashing lighting, **(h)** $NaNO_3$ concentration 0.75 g L^{-1}, and **(j)** nitrogen starvation 3 days.

Proteins production was mostly affected by $NaNO_3$ concentration in the growth medium, achieving up to 7.4 ± 1.7 g m^{-2} (Figure 39). Barman et al. (2022) applied 25, 50, 100, 200, and 500 mg L^{-1} of $NaNO_3$ in the growth medium for the cultivation of *Tetraselmis chuii*. They reported that $NaNO_3$ concentration of 500 mg L^{-1} had significantly increased proteins concentration. However, carbohydrates content was maximized with 25 mg L^{-1}, showing an inversely proportional effect of $NaNO_3$. Higher aeration rate and illuminance, along with continuous lighting, and zero nitrogen starvation seemed to favor proteins production. Gris et al. (2014) conducted experiments with different illumination intensities (10, 50, 150, 200, 350, and 1,000 μmol m^{-2} s^{-1}) and flashing light effect (5, 10, and 15 Hz) on *Scenedesmus obliquus* found that proteins are not affected by the different levels. However, Lima et al. (2021) mentioned that flashing effect with frequencies ranging from 0.05 to 300 μmol m^{-2} s^{-1} enhanced the protein content of *Nannochloropsis gaditana*, *Koliella antarctica*, and *Tetraselmis chui*. With respect to illuminance, Karapatsia et al. (2016) studied the optimization of wild-type *Stichococcus* sp. and found that the main effect of proteins at 8,800 lumens was lower than at 4,400 lumens under

fluorescent lighting, indicating that proteins content of *Stichococcus* sp. is inversely proportional to illuminance.

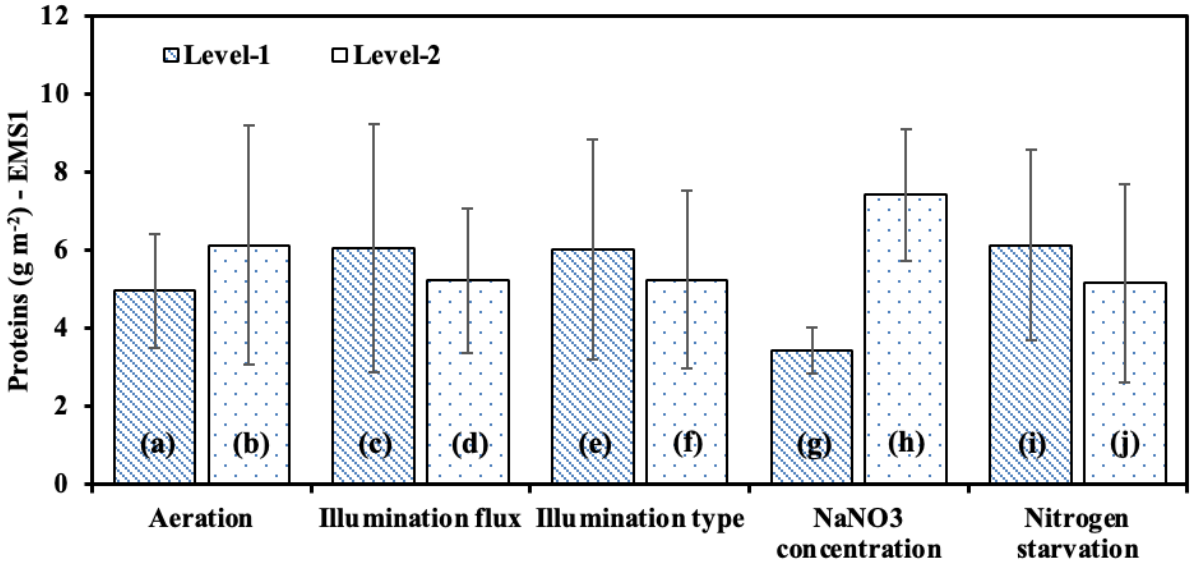


Figure 39. Main effect plot of proteins of EMS1 *Stichococcus* sp., in relation to the experimental parameters, based on the results shown in Table 3. **Level-1: (a)** aeration rate 0.4 L min^{-1}, **(c)** illuminance 3,300 lux, **(e)** continuous lighting, **(g)** $NaNO_3$ concentration 0.25 g L^{-1}, and **(i)** nitrogen starvation 0 days. **Level-2: (b)** aeration rate 0.8 L min^{-1}, **(d)** illuminance 6,600 lux, **(f)** flashing lighting, **(h)** $NaNO_3$ concentration 0.75 g L^{-1}, and **(j)** nitrogen starvation 3 days.

Figure 40 shows the main effects of EMS1 *Stichococcus* sp. carbohydrates production. As in the case of lipids, total chlorophyll, and proteins, $NaNO_3$ was the main contributor to carbohydrates maximization. The mean value of the main effect was determined as 12.3 ± 5.4 g m^{-2}, which is a 92% greater than the use of 0.25 g L^{-1} $NaNO_3$. Also, illuminance showed great increase in carbohydrates production equal to 83%. Especially, Psachoulia and Chatzidoukas (2021) mentioned that carbohydrate accumulation serves as a direct access energy store, so as biomass increases, so does carbohydrates content. Increased aeration rate did not seem to favor carbohydrates production, as well as flashing lighting and nitrogen starvation. The decreased carbohydrates content in *Stichococcus* sp. with increasing aeration rate (from 0.1 to 0.2 L min^{-1}) was also reported in the study of Karapatsia et al. (2016). However, *Chlorella sorokiniana* was found to be unaffected by aeration rate (Magdaong et al., 2019). Comparing Figures 36-40, it can

be seen that carbohydrates were the main bioactive compound of *Stichococcus* sp., which was mentioned in Karapatsia et al. (2016); Psachoulia and Chatzidoukas (2021).

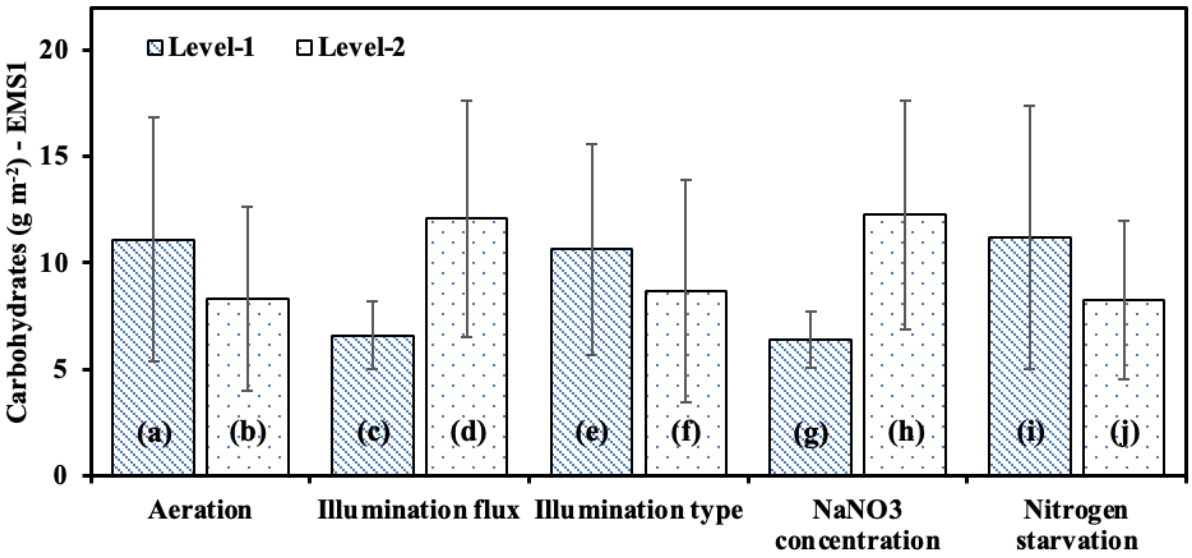


Figure 40. Main effect plot of carbohydrates of EMS1 *Stichococcus* sp., in relation to the experimental parameters, based on the results shown in Table 3. **Level-1: (a)** aeration rate 0.4 L min^{-1}, **(c)** illuminance 3,300 lux, **(e)** continuous lighting, **(g)** $NaNO_3$ concentration 0.25 g L^{-1}, and **(i)** nitrogen starvation 0 days. **Level-2: (b)** aeration rate 0.8 L min^{-1}, **(d)** illuminance 6,600 lux, **(f)** flashing lighting, **(h)** $NaNO_3$ concentration 0.75 g L^{-1}, and **(j)** nitrogen starvation 3 days.

Figure 41 contains the sum of all main effects of each bioactive compound examined (Figure 36-41). As mentioned in the previous paragraphs, $NaNO_3$ was the main ingredient for increasing biomass and bio-products production, with the main effect value reaching 29.2 ± 4.2 g m^{-2}. Barman et al. (2022) applied 25, 50, 100, 200, and 500 mg L^{-1} of $NaNO_3$ in the growth medium for the cultivation of *Tetraselmis chuii*. The higher concentration of 500 mg L^{-1} $NaNO_3$ resulted in higher cell density, biomass, optical density, specific growth rate, and division rates compared with the other concentrations. Higher aeration did not seem to have a major effect on *Stichococcus* bio-products. Tang et al. (2016) supplied microalgae cultures with 0.2 and 0.4 L min^{-1} and reported that aeration rate higher than 0.2 L min^{-1} inhibited algae growth and then limited nutrients uptake for biomass accumulation. Similar effect was reported by Karapatsia et al. (2016). The use of 0.2 L min^{-1}, instead of 0.1 L min^{-1} induced approximately 15% decrease in the main effects of total bio-products. Also, the supply of 50 L min^{-1}, instead of 25 L min^{-1} CO_2 (10% v/v) did not have

any significant difference in total bio-products production. This may be justified as microalgae receive sufficient CO_2 from the gas supply. Several studies have mentioned that 50% of microalgae biomass is carbon and for the production of 1 kg microalgae biomass, 1.83 kg of CO_2 are required (Cheah et al., 2015; Ighalo et al., 2022; Ji et al., 2013; Khoobkar et al., 2022; Pavlik et al., 2017). So, if one considers the lower aeration rate 0.4 L min^{-1} and CO_2 density 1.83 kg m^{-3}, then the supplied mass of CO_2 for 25 days of cultivation period is equal to 1.35 kg. The total microalgal biomass was less than this amount, therefore microalgae were receiving sufficient CO_2 in either case of aeration rate. When applying 6,600 lux of LED lighting, there was also a 19% increase in bio-products production. Continuous lighting offered greater results, compared to flashing lighting, which was described in the aforementioned results of biomass, proteins, lipids, and carbohydrates. Only pigments showed increase as flashing light promotes photosynthetic activity (Martín-Girela et al., 2017). Nitrogen starvation only enhanced lipids production (Figure 37). Zarrinmehr et al. (2020) investigated the effect of different $NaNO_3$ concentrations (0, 36, 72, 144, and 288 mg L^{-1}) on *Isochrysis galbana*. Diminishing nitrogen concentration decreased cell growth, pigments, and protein content. The same effect was reported in the present study showing that the absence of nitrogen negatively affects microalgae growth and biochemical composition. However, one would implement nitrogen starvation as a measure to further increase intracellular lipids production (Figure 37) two or three days prior to harvesting.

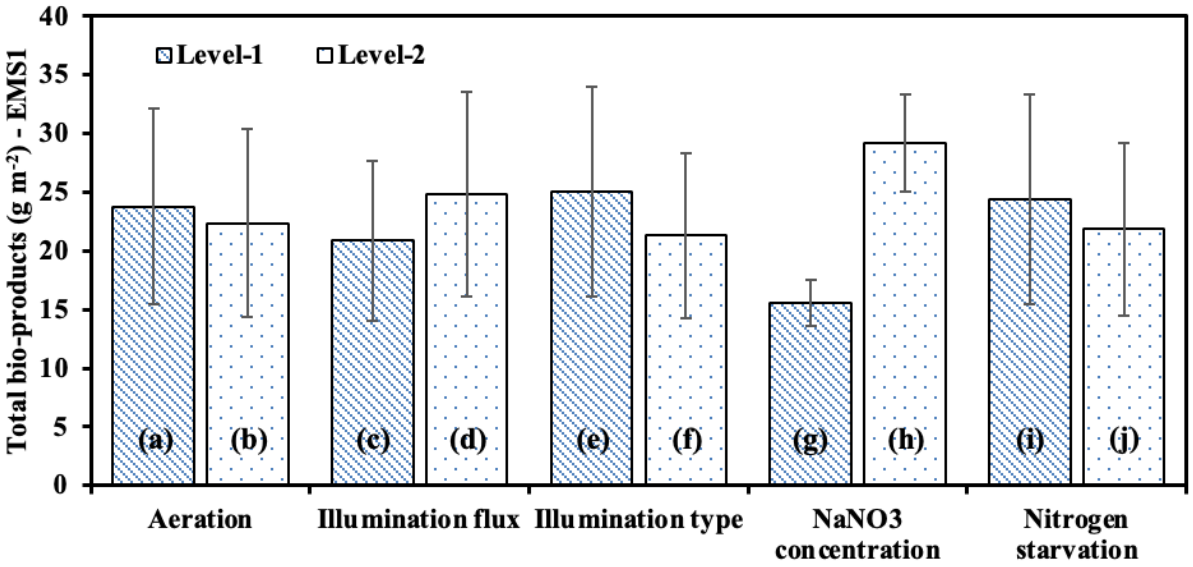


Figure 41. Main effect plot of total bio-products of EMS1 *Stichococcus* sp., in relation to the experimental parameters, based on the results shown in Table 3. **Level-1: (a)** aeration rate 0.4 L min^{-1}, **(c)** illuminance 3,300 lux, **(e)** continuous lighting, **(g)** $NaNO_3$ concentration 0.25 g L^{-1}, and **(i)** nitrogen starvation 0 days. **Level-2: (b)** aeration rate 0.8 L min^{-1}, **(d)** illuminance 6,600 lux, **(f)** flashing lighting, **(h)** $NaNO_3$ concentration 0.75 g L^{-1}, and **(j)** nitrogen starvation 3 days.

Based on EMS1 *Stichococcus* sp. experimental data, response tables for means were created using Minitab 17.0 statistical software (Table 6). The present table summarizes the main effects (Figure 36-41), delta values, and rankings of each response and factor. As can be seen, $NaNO_3$ was the dominant factor, being in 1st rank in most responses except total chlorophyll. This was also reported in Figure 38. The following factor was illuminance. It was ranked 2nd in dry biomass, total chlorophyll, carbohydrates, and total bio-products. To note that delta values indicate the differences between #1 and #2 values of each response and ranking highlights the significance of each factor.

Table 6. Response tables for means of dry biomass, lipids, total chlorophyll, proteins, carbohydrates, and total bio-products.

Level	Aeration rate (L min^{-1})	Illuminance (lux)	Illumination type	$NaNO_3$ concentration (g L^{-1})	Nitrogen starvation (days)
Dry biomass					
1	28.6	24.4	32.6	20.4	30.7
2	28.9	32.5	25.7	35.8	27.3
Delta	0.3	8.1	6.9	15.4	3.4
Rank	5	2	3	1	4
Lipids					
1	7.6	8.1	8.3	5.7	6.9
2	7.8	7.4	7.3	9.4	8.4
Delta	0.1	0.8	1.0	3.7	1.4
Rank	5	4	3	1	2
Total chlorophyll					
1	0.12	0.10	0.12	0.10	0.16
2	0.14	0.15	0.13	0.15	0.10

Delta	0.02	0.05	0.01	0.05	0.05
Rank	4	2	5	3	1
Proteins					
1	5.0	6.1	6.0	3.4	6.1
2	6.1	5.2	5.2	7.4	5.2
Delta	1.2	0.8	0.8	4.0	1.0
Rank	2	4	5	1	3
Carbohydrates					
1	11.1	6.6	10.7	6.4	11.2
2	8.3	12.1	8.7	12.3	8.2
Delta	2.8	5.5	2.0	5.9	2.9
Rank	4	2	5	1	3
Total bio-products					
1	23.8	20.9	25.1	15.6	24.4
2	22.4	24.8	21.3	29.2	21.9
Delta	1.4	3.9	3.7	13.6	2.5
Rank	5	2	3	1	4

***Note: Delta** = the overall change in a value; **Rank** = the significance of each factor.*

Table 7 shows the optimal combination of cultivation conditions on EMS1 *Stichococcus* sp. dry biomass, lipids, total chlorophyll, proteins, carbohydrates, and total bio-products. The presented results were based on the process factors of Table 5 and response tables of Table 6. Five optimal cultivation combinations were found, with carbohydrates and total bio-products sharing the same conditions. Specifically, the optimal level for dry biomass maximization (optimal condition I) was met when the aeration rate was set to 0.8 L min^{-1}, illuminance to 6,600 lux, illumination type to continuous, $NaNO_3$ to 0.75 g L^{-1}, and zero nitrogen starvation days. Similarly, the conditions that maximized lipids (optimal condition II) were: aeration rate 0.8 L min^{-1}, illuminance 3,300 lux, continuous lighting, $NaNO_3$ 0.75 g L^{-1}, and three days of nitrogen starvation. Optimal condition III for total chlorophyll maximization were equal to aeration rate 0.8 L min^{-1}, illuminance 6,600 lux, flashing lighting, $NaNO_3$ 0.75 g L^{-1}, and zero nitrogen starvation. Proteins (optimal condition IV) were maximized when applying aeration rate 0.8 L min^{-1}, illuminance 3,300 lux, continuous lighting, $NaNO_3$ 0.75 g L^{-1}, and zero nitrogen starvation. The

fifth combination of optimal conditions for carbohydrates and total bio-products maximization was achieved with aeration rate 0.4 L min^{-1}, illuminance 6,600 lux, continuous lighting, $NaNO_3$ 0.75 g L^{-1}, and zero nitrogen starvation. Carbohydrates were the main bio-product produced from EMS1 *Stichococcus* sp. biomass (Table 3), indicating that if one selects the fifth optimal combination it could offer both carbohydrates and total bio-products maximization.

Table 7. Optimal cultivation conditions of EMS1 *Stichococcus* sp. for each response (i.e., dry biomass, lipids, total chlorophyll, proteins, carbohydrates, and total bio-products), based on the experimental conditions (Table 5) and response tables (Table 6).

	Aeration rate (L min^{-1})	**Illuminance (lux)**	**Illumination type**	**$NaNO_3$ concentration (g L^{-1})**	**Nitrogen starvation (days)**
Biomass maximization – Optimal condition I					
Optimal level	0.8	6,600	Continuous	0.75	0
Lipids maximization – Optimal condition II					
Optimal level	0.8	3,300	Continuous	0.75	3
Total chlorophyll maximization – Optimal condition III					
Optimal level	0.8	6,600	Flashing	0.75	0
Proteins – Optimal condition IV					
Optimal level	0.8	3,300	Continuous	0.75	0
Carbohydrates – Optimal condition V					
Optimal level	0.4	6,600	Continuous	0.75	0
Total bio-products – Optimal condition V					
Optimal level	0.4	6,600	Continuous	0.75	0

Twelve linear regression models were generated using Minitab 17.0 for the six process responses of dry biomass, total chlorophyll, carbohydrates, and total bio-products (Table 8). The models were used to quantify the effects of the five factors (i.e., aeration rate, illuminance, illumination type, $NaNO_3$ concentration, and nitrogen starvation). Six out of twelve models correspond to continuous lighting and the other to flashing lighting. Illumination type (continuous/flashing) is consisted of discrete values, so separate models had to be generated. Table 8 presents the model terms (i.e., constant values and x_i) and coefficients (i.e., β_i). Note that, the

levels of factors had high order of magnitude, so in order for the model to work, the model terms were brought to similar order of magnitude. This is mentioned with multiplications of "10" and "10^{-3}" in model term column of the table below.

The model equation that was used is mentioned under Table 8. It is a general linear equation as each factor had two levels. However, there is a limitation of the model, because of the two-level design experiments. If one considers a x-y graph, then the linear regression line intercepting the experimental data passes from two points. Three or more points would have a better fit of the regression line. The reason that two levels were selected is that the data represent a linear form, so there will not be a major difference in the model outcome if a third level was to be introduced. Furthermore, two-level design values resulted from the experiments conducted on wild-type and EMS1 strains for the selection of the most promising (see Section 4.3.1). Specifically, for aeration rate, 0.8 L min^{-1} were used in the experiments of Section 4.3.1, so the same value was examined along with half of it. The same pattern was followed for the illuminance as 6,600 lux were used to illuminate the cultures of experiments of Section 4.3.1. The initial continuous lighting system was compared to the effect of flashing lights. 0.75 g L^{-1} of $NaNO_3$ in the growth medium were compared to the original composition of the Bold's Basal Medium (Bischoff and Bold, 1963). The last factor examined was nitrogen starvation of microalgae. From the experimental results of Figure 28, it was found that three days had a significant difference over two-day nitrogen starvation. Therefore, two days were eliminated from the experiments. All the above, led to the selection of two levels for the generation of linear regression models. Also, by considering the R^2 values of the models, it can be seen that most of the models have values above 85%, except in the case of total chlorophyll with a value equal to 83.7%. Therefore, the experimental data have a good fit in the model.

Table 8. Linear regression model parameters of EMS1 *Stichococcus* sp. for each of the six responses, according to the four experimental conditions. Two separate models were generated for each lighting condition.

Model parameter	Model term	Biomass	Lipids	Total chlorophyll	Proteins	Carbohydrates	Total bio-products

Continuous lighting							
α	Constant	-3.2	2.18	-0.0734	0.14	-0.31	1.94
β_1	Aeration (x_1) (·10)	0.577	0.163	0.00861	0.363	-0.486	0.048
β_2	Illuminance (x_2) ($\cdot 10^{-3}$)	2.843	-0.06	0.0182	-0.108	1.677	1.527
β_3	$NaNO_3$ concentration (x_3) (·10)	3.217	0.774	0.01156	0.806	1.18	2.772
β_4	Nitrogen starvation (x_4)	-0.348	0.611	-0.01148	-0.165	-0.707	-0.273
Flashing lighting							
α	Constant	-7.44	1.79	-0.0479	-0.18	-1.54	0.02
β_1	Aeration (x_1) (·10)	0.577	0.163	0.00861	0.363	-0.486	0.048
β_2	Illuminance (x_2) ($\cdot 10^{-3}$)	2.843	-0.06	0.0182	-0.108	1.677	1.527
β_3	$NaNO_3$ concentration (x_3) (·10)	3.217	0.774	0.01156	0.806	1.18	2.772
β_4	Nitrogen starvation (x_4)	-0.348	0.611	-0.01148	-0.165	-0.707	-0.273
R^2		**98.9**	**87.5**	**83.7**	**87.8**	**88.2**	**97.8**

General linear model equation for each response: $y_i = \alpha_i + \sum_{j=1}^{4} \beta_j x_{ij}$

The optimal conditions mentioned in Table 7, coupled with the linear regression models in Table 8 were used to find the model-based maximum values of dry biomass and bio-active compounds. Model-based results are mentioned in Table 9 and values highlighted in grey represent the maximums for continuous and flashing light models. To verify the model-based optimal conditions, experiments were conducted in triplicates for each of the five optimal conditions (Table 7) and the results are mentioned in

Table ***10***. Specifically, based on Optimal condition I for biomass maximization the maximum experimental value for dry biomass was determined as 44.1 ± 1.1 g m^{-2}, while the model-based value was 44.3 g m^{-2}. The respective experimental value for lipids production (Optimal condition II) was 12.0 ± 0.5 g m^{-2}, while the model-based 10.9 g m^{-2}. Total chlorophyll (Optimal condition III) was found to be 0.26 ± 0.02 g m^{-2} in real conditions, while the model-based was equal to 0.23 g m^{-2}. Proteins (Optimal condition IV) were 9.8 ± 0.7 g m^{-2}, compared to 8.7 g m^{-2}. Furthermore, carbohydrates (Optimal condition V) were found to be 21.2 ± 1.2 g m^{-2} and the model-based value equal to 17.7 g m^{-2}. The last response was total bio-products. The maximum experimental value (Optimal condition V) was equal to 35.9 ± 2.6 g m^{-2}, while the model-based was 33.0 g m^{-2}. As can be seen, the experimental values surpass the predicted values with errors ranging from 0.9 to 16.7%. For reliable statistical analyses, error values must be less than 20% (Cetin et al., 2011). Therefore, the results obtained from the confirmation tests reflect successful optimization of biomass and bio-products production of EMS1 *Stichococcus* sp. strain.

Table 9. Model-based results of Table 8, based on the optimal conditions of Table 7.

Response – Optimal condition	Illumination type	Model-based results (g m^{-2})
Biomass maximization – Optimal conditions I	Continuous	44.3
	Flashing	40.1
Lipids maximization – Optimal conditions II	Continuous	10.9
	Flashing	10.5
Total chlorophyll maximization – Optimal conditions III	Continuous	0.20
	Flashing	0.23
Proteins – Optimal conditions IV	Continuous	8.7
	Flashing	8.4
Carbohydrates – Optimal conditions V	Continuous	17.7
	Flashing	16.4
Total bio-products – Optimal conditions V	Continuous	33.0
	Flashing	31.1

Note: *Maximum value of each model is highlighted in grey.*

Table 10. Predicted and experimental values based on regression model equations. The predicted values are also mentioned in Table 9.

	Predicted	Experimental	Error (%)
Dry biomass	44.3	44.7 ± 1.1	0.9
Lipids	10.9	12.0 ± 0.5	8.9
Total chlorophyll	0.23	0.26 ± 0.02	12.4
Proteins	8.7	9.8 ± 0.7	10.9
Carbohydrates	17.7	21.2 ± 1.2	16.7
Total bio-products	33.0	35.9 ± 2.6	8.1

Contour plots were generated using the model-based results of dry biomass (Figure 42) and total bio-products (Figure 43), so that an overall picture of the products is presented. Contour plots represent the optimal areas for a pair of growth parameters, where dry biomass and total bio-products are maximized. These areas are marked with dark green color, while blue areas represent the lowest dry biomass and bio-products values. Highest areas of dry biomass (>39 and >40 g m^{-2}) are met i) in Figure 42B, where $NaNO_3$ concentration in the growth medium is above 0.75 g L^{-1} and aeration rate is approximately 0.8 L min^{-1}, and ii) in Figure 42C where $NaNO_3$ concentration in the growth medium is approximately 0.75 g L^{-1} and illuminance is approximately 6,600 lux.

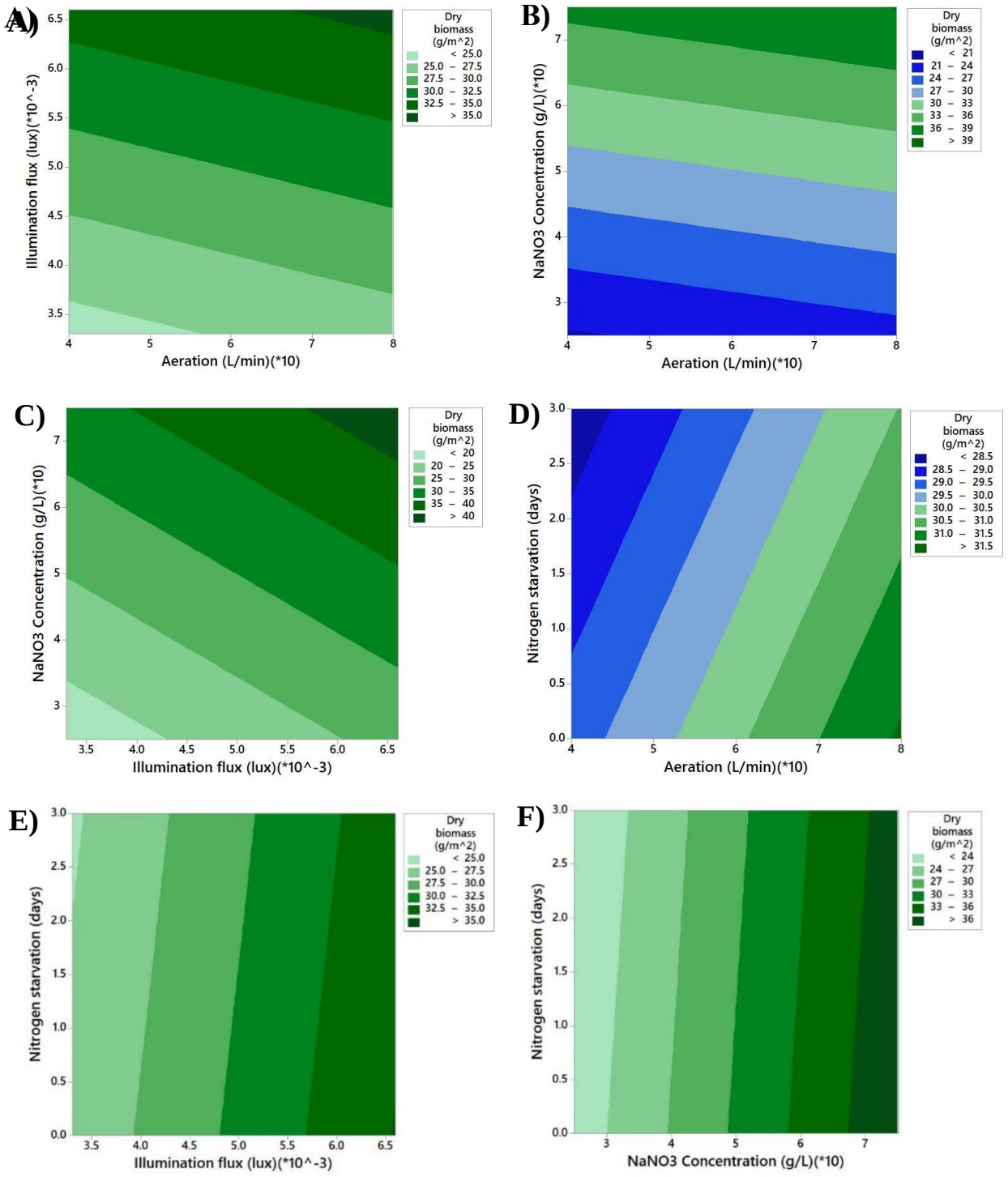


Figure 42. Contour plots of model-based results from the optimization of dry biomass, depending on: **A)** illuminance vs aeration rate, **B)** $NaNO_3$ concentration vs aeration rate, **C)** $NaNO_3$ concentration vs illuminance, **D)** nitrogen starvation vs aeration, **E)** nitrogen starvation vs illuminance, and **F)** nitrogen starvation vs $NaNO_3$ concentration.

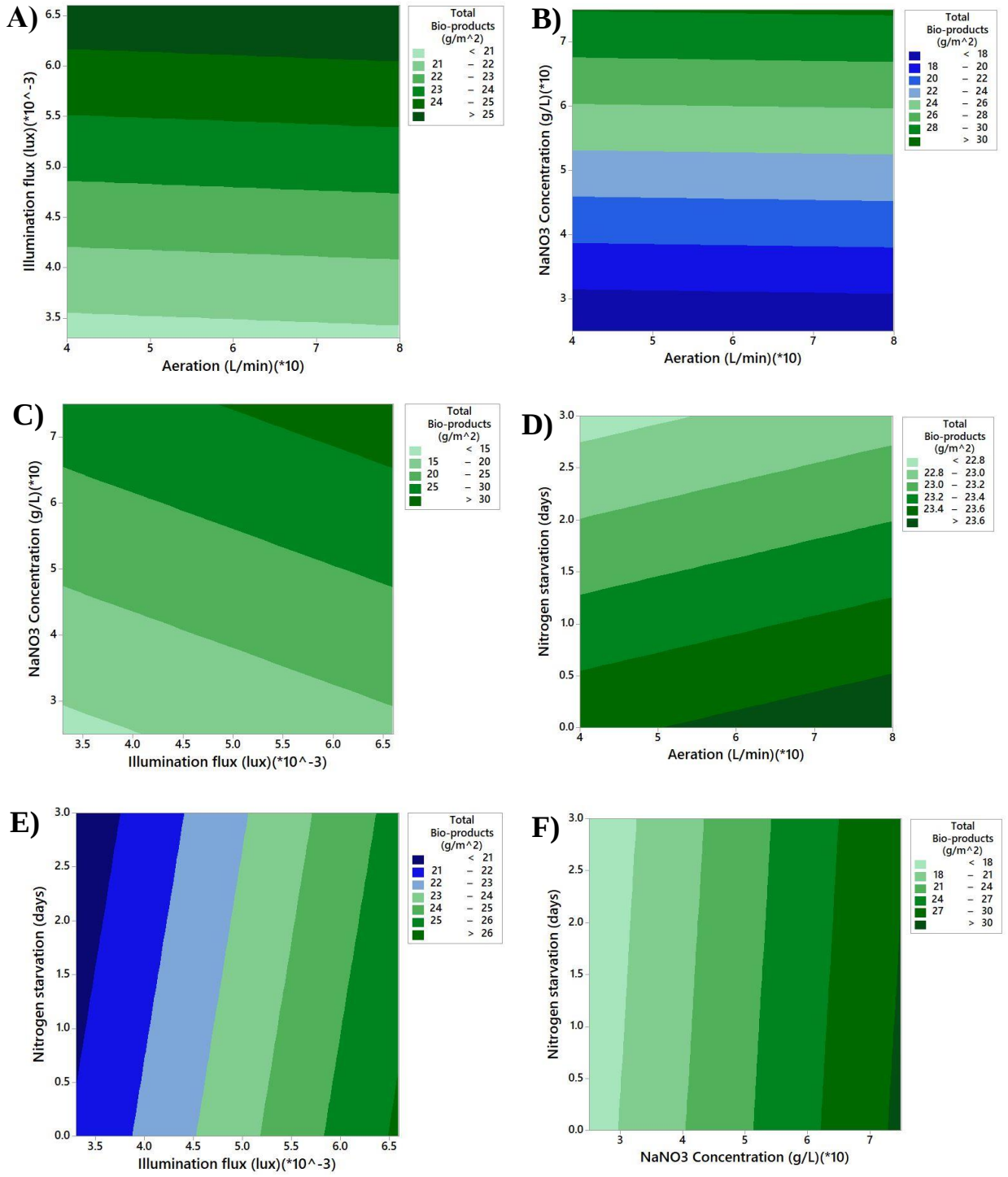


Figure 43. Contour plots of model-based results from the optimization of total bio-products, depending on: **A)** illuminance vs aeration rate, **B)** $NaNO_3$ concentration vs aeration rate, **C)** $NaNO_3$ concentration vs illuminance, **D)** nitrogen starvation vs aeration, **E)** nitrogen starvation vs illuminance, and **F)** nitrogen starvation vs $NaNO_3$ concentration.

4.4 Concluding Remarks

Wild-type and EMS1 *Stichococcus* sp. microalgae strains were screened to find out which strain is the most promising for scale up in industrial photobioreactors representing 3rd generation biorefineries. The scope was to cultivate immobilized microalgae as a means of lowering cultivation costs and at the same time feeding them with natural gas flue gas to lower CO_2 emissions. Both strains were subjected to a series of tests to exclude the least promising. For this reason, strains were tested in terms of: i) agitation and non-agitation conditions, ii) plain atmospheric air and atmospheric air enhanced with 5% (v/v) CO_2, iii) different cultivation days, and iv) nitrogen starvation days. Then, both strains were cultivated under the optimal conditions that were found from the aforementioned experiments and at the end of cultivation cycle dry biomass, lipids, pigments, proteins, and carbohydrates were measured. EMS1 *Stichococcus* sp. strain showed better results, compared to the wild-type, therefore it was selected for further optimization of its biomass and bio-products production in lab-scale. Taguchi's Design of Experiments was implemented to find the least required experimental runs, which could give adequate results. Aeration rate, illuminance, two types of illumination, $NaNO_3$ concentration in the growth medium, and nitrogen starvation were examined as growth parameters. At the end of cultivation cycle, dry biomass, lipids, pigments, proteins, and carbohydrates were again measured. Nitrate nitrogen in the growth medium was found to be the most important factor for the growth of Stichococcus cultures, as the triple amount of nitrogen significantly increases biomass and bio-products production. Five optimal conditions were found for each of the process responses, according to the statistical analysis of the experimental data. Finally, twelve linear regression models were generated to find the maximum optimized values for the optimal conditions. Verification experiments agreed with model-based optimal parameters, indicating successful optimization of EMS1 *Stichococcus* sp. strain.

Chapter 5: Scale-up cultivation and real-time CO_2 capturing from industrial flue gas of a thermal power station

5.1 Introduction

Most microalgal species isolated from natural streams, lakes or oceans have been pre-adapted for the living environment through artificial domestication and have been successfully used for fixation of atmospheric CO_2. However, unlike atmospheric air which has low CO_2 content (0.04% v/v), post-combustion flu gas typically contains 4-14% (v/v) or higher CO_2 and possibly toxic compounds (such as SO_x, NO_x, and trace elements). This means that the microalgal species must be able to tolerate the harsh flue gas composition and conditions, in order to capture CO_2. Microalgal growth under coal combustion flue gas is usually more complex than under atmospheric conditions (Zhang, 2015). Power plants are moving away from coal and toward natural gas as a source of fuel, so experimenting with natural gas flue gas could have more uses in future applications and give a better insight into its effect on microalgae growth.

The purposes of the current research are initially the scale-up cultivation of EMS1 *Stichococcus* sp. strain in a lab-scale flat-panel photobioreactor for CO_2 fixation. The following step is CO_2 sequestration from real-time flue gas emitted from a natural gas-fired power plant. Microalgae were also cultivated in flat-panel photobioreactors.

5.2 Materials and Methods

5.2.1 Pilot-scale cultivation in laboratory environment

Following the screening of wild-type and EMS1 *Stichococcus* sp. strains, and the optimization experiments of EMS1 mutant strain (see Chapter 4), EMS1 strain cultivation was scaled up in a laboratory photobioreactor (Figure 44). The strain was cultivated with a modified version of Bold's Basal Medium, as mentioned in Section 4.2.1. Culture volume was 15 L (2.5 cm

water depth, 25 L m^{-2}) out of which, approximately 14 L were the BBM with the concentration of $NaNO_3$ being 0.75 g L^{-1} and the remaining 1 L was the inoculum of EMS1 *Stichococcus* sp. Cultivation conditions were identical to the ones mentioned in Section 4.2. Specifically, cells were grown for 25 days (based on the results of Figure 27) immobilized on sandblasted glass, which covered the bottom of the photobioreactor. Temperature was kept at 25 ± 1 ^oC using an A/C unit. Light-Dark ratio was 16 h:8 h and microalgae were receiving synthetic flue gas as carbon source. The composition of the gases was: 5% CO_2, 14% O_2, and 81% N_2. Growth parameters were set according to the "Optimal condition I" for biomass maximization derived from the experimental results mentioned in Section 4.3.2. "Optimal condition I" was selected in order to test the scale-up ability of *Stichococcus* sp. The corresponding growth parameters are summarized in Table 11.

Table 11. Optimal growth conditions that were selected for EMS1 *Stichococcus* sp. scale-up cultivation, according to "*Optimal condition I*" described in Section 4.3.2.

Aeration rate (L min^{-1})	**Illuminance (lux)**	**Illumination type**	**$NaNO_3$ Concentration (g L^{-1})**	**Nitrogen starvation (days)**
0.8	6,600	Continuous	0.75	0

Harvesting was performed by first pumping out the supernatant liquid and then by mechanical scraping of the biomass, which was grown attached on sandblasted glass tiles (Figure 45). The collected biomass was further dewatered by centrifugation at 4,000 rpm for 10 min and was dried with freeze-drying for 24 h to quantify dry cell weight. Furthermore, lipids, total chlorophyll, proteins, and carbohydrates were measured as bio-products to find out the characteristics of EMS1 *Stichococcus* sp. in pilot-scale photobioreactors. The protocols that were followed are mentioned in Sections 4.2.3 & 4.2.4.

Figure 44. Photograph of the pilot-scale flat-panel photobioreactor that was used in the cultivation experiments of EMS1 *Stichococcus* sp.

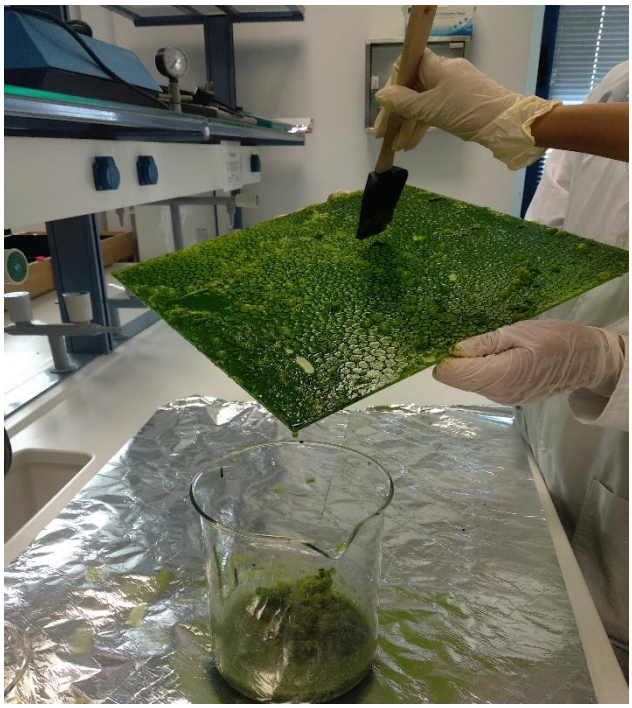


Figure 45. Mechanical scraping of EMS1 *Stichococcus* sp. biomass, attached on sandblasted glass tile and cultivated in the closed-type flat-panel photobioreactor (Figure 44).

5.2.2 Pilot-scale cultivation in real conditions

The final goal of the optimization series was to conduct experiments in real conditions for CO_2 sequestration of industrial flue gas. To achieve that, the two identical Plexiglass®

photobioreactors (Figure 47D) were moved to Lavrio power station, located in the region of Attica in Greece (Figure 46). The photobioreactors were placed inside a small air-conditioned prefabricated enclosure to provide protection from environmental conditions (Figure 47B-C). There, microalgae were receiving a fraction of real-time flue gas, emitted from a natural gas power plant (Figure 47A) of Lavrio power station. Flue gas stream was supplied from nearby instruments of the power plant used for analyzing flue gas composition. The final gas flow rate before entering photobioreactors was controlled with regulators. The measured CO_2 concentration was approximately 3.5%. For the repeatability of the experiment, cultivation conditions (i.e., growth medium, culture volume, pH, and temperature) were kept identical to the ones mentioned in the previous section 5.2.1. However, growth parameters (i.e., aeration rate, illumination, $NaNO_3$ concentration in the growth medium, and nitrogen starvation) were different. The scope was to increase the biomass and total bio-products production, mentioned in Section 4.3.2. Also, continuous light was compared to flashing light, in order to investigate the effect of different lights and flue gas on biomass and bio-products productivity. Flashing lighting was adjusted to 1,000 Hz and duty cycle 0.5. The 3rd run investigated lipids maximization, by implementing Optimal condition II. Growth parameters for the three experimental runs are summarized in Table 12. The supply of flue gas was set to 0.6 L min^{-1} in all experimental runs, as this was the maximum flow that the installed pumping system could convey from the plant exhaust gases. Also, $NaNO_3$ concentration was found to promote biomass and bio-products maximization when the concentration in the growth medium was 0.75 g L^{-1}. During the cultivation period, the pH was monitored every two days to investigate the effects of CO_2 to the pH of the growth medium.

Table 12. Growth parameters that were selected for EMS1 *Stichococcus* sp. cultivation with real-time flue gas sequestration.

Run#	Optimization policy	Aeration rate (L min^{-1})	Illuminance (lux)	Illumination type	$NaNO_3$ concentration (g L^{-1})	Nitrogen starvation (days)
1	Biomass/Total bio-products maximization	0.6	6,600	Continuous	0.75	0
2	-	0.6	6,600	Flashing	0.75	0
3	Lipids maximization	0.6	3,300	Continuous	0.75	3

Harvesting was again performed (section 4.2.3) by initially pumping out the supernatant liquid and then by mechanical scraping of the biomass, attached on sandblasted glass tiles (Figure 45). The collected biomass was transferred to the laboratory for further dewatering and drying (see Section 5.2.1). Lipids, total chlorophyll, proteins, and carbohydrates were measured to quantify the produced bio-products. Figure 48 shows a flowchart of the experimental procedure that was followed from carbon fixation of industrial flue gas through microalgae, to the conversion of the produced biomass into lipids, pigments, proteins, and carbohydrates. The protocols that were followed are mentioned in Sections 4.2.3 & 4.2.4.

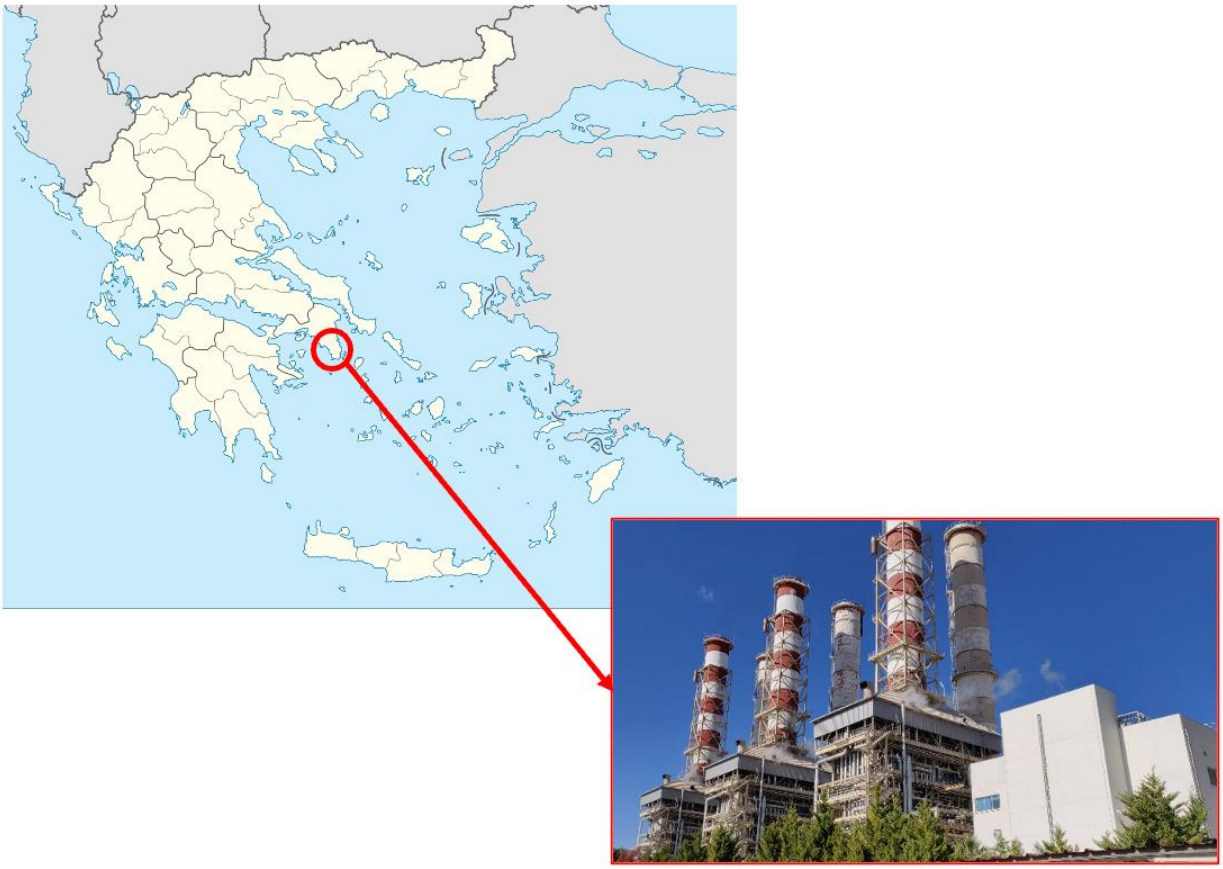


Figure 46. Map of Greece and pointing location of Lavrio power station, where experiments took place with real-time flue gas feed for microalgae cultivation.

A)

B)

C)

D)

Figure 47. A) Natural gas power plant at Lavrio power station used for flue gas feed, **B)** & **C)** Shed that was used for providing protection from environmental conditions, and **D)** EMS1 *Stichococcus* sp. culture in flat-panel photobioreactor, grown under industrial flue gas.

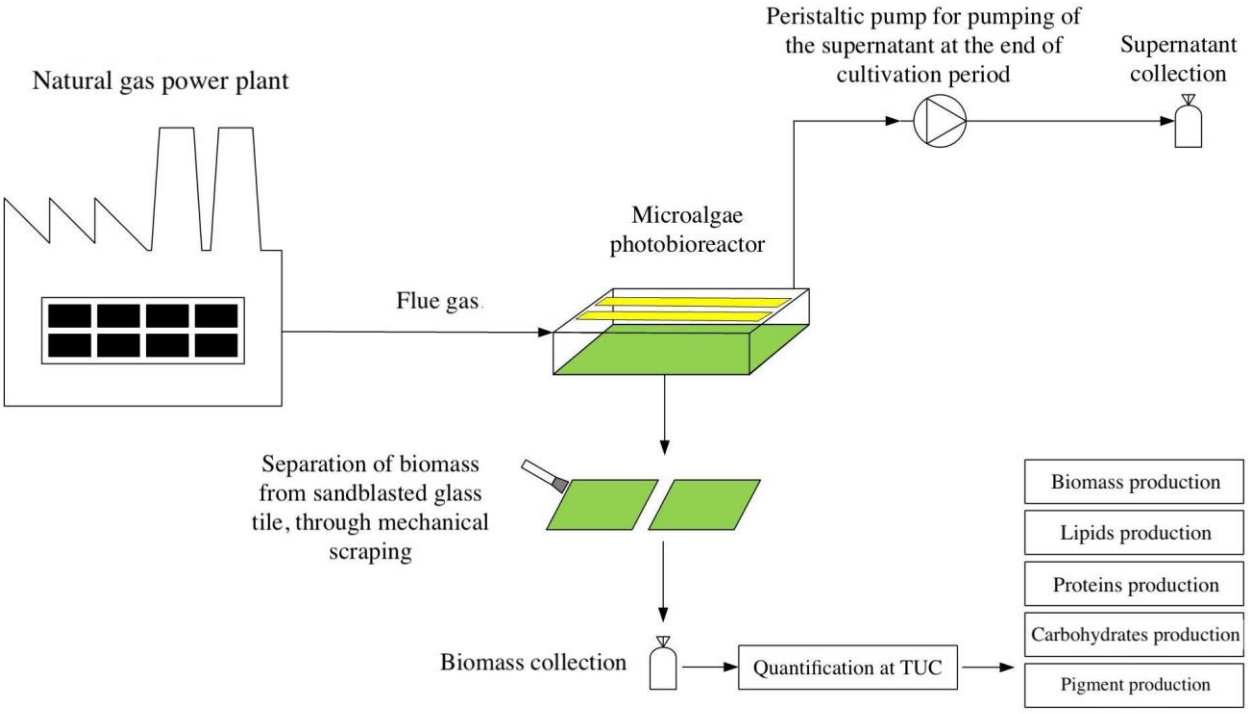


Figure 48. Flow chart of microalgae cultivation with carbon fixation contained in industrial flue gas and bioconversion of the produced biomass into valuable bio-products. To note that "TUC" stands for "Technical University of Crete".

5.3 Results and Discussion

5.2.1 Pilot-scale cultivation in laboratory environment

Scale-up cultivation of EMS1 *Stichococcus* sp. strain was performed in a closed-type, flat-panel lab-scale photobioreactor (Figure 44). Microalgae were grown immobilized on sandblasted glass tiles to test the possibility of reducing the cost of biomass harvesting steps. The effect of synthetic flue gas on biomass and bio-products production was also examined.

Dry biomass production of EMS1 *Stichococcus* sp. strain was determined as 49.2 g m^{-2}. The present results can be compared very well with the results of dry biomass in lab-scale cultivation experiments, presented in Section 4.3. In fact, by comparing the maximum produced biomass shown in Table 3 (45.7 ± 1.3 g m^{-2}), it can be seen that *Stichococcus* was able to grow successfully in larger scale and in synthetic flue gas environment, at relatively high biomass yields. Average CO_2 uptake was calculated as 90.0 g m^{-2}, based on the ratio: 1 kg of dry biomass per 1.83 kg of CO_2 consumption (Cheah et al., 2015; Ighalo et al., 2022; Ji et al., 2013; Khoobkar et al., 2022; Pavlik et al., 2017). Huang et al. (2016) conducted experiments with *Chlorella vulgaris* cultivated in attached and suspended systems with a continuous light intensity of 120 ± 3 μmol m^{-2} s^{-1} and 10% CO_2 (v/v). They reported that attached biomass was equal to 103 g m^{-2}, which was 30.4% higher than that in the suspended system. Huang et al. (2016) also reported that *C. vulgaris* grown under 5% or 10% CO_2 contained between 52-53% carbon and 7%. Commonly used microalgae and cyanobacteria for CO_2 sequestration include *Botryococcus braunii*, *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Chlorella kessleri*, *Chlorella* sp., *Chlorocuccum littorale*, *Monoraphidium minutum*, *Scenedesmus obliquus*, *Scenedesmus* sp., *Spirulina* sp., and *Tetraselmis* sp. (Ho et al., 2011; Kumar et al., 2014; Lam and Lee, 2012b; Zhang, 2015).

The production of lipids, carbohydrates, proteins, total chlorophyll, and total bio-products per m^2 of cultivated EMS1 *Stichococcus* sp. are shown in Figure 49. The total amount of bio-products was 41.7 g m^{-2}, which corresponds to 85% of the biomass on dry basis. As can be seen, carbohydrates account for the highest portion of the bio-products, with a value equal to 24.2 g m^{-2}. This was also noted in the results of optimization experiments, mentioned in Section 4.3.2, which shows that *Stichococcus* follows the same growth pattern in scale-up conditions. Carbohydrates were followed by proteins, being equal to 9.6 g m^{-2} and lipids were 7.6 g m^{-2}. The total chlorophyll production was $15 \cdot 10^{-2}$ g m^{-2}. By comparing photobioreactor results to lab-scale experiments for the optimization of EMS1 *Stichococcus* sp. (see Section 4.3.2, Table 3), it can be seen that in the case of photobioreactor cultivation, higher values of bio-products production had been achieved. Specifically, maximum total bio-products value mentioned in Table 3 was equal to 34.8 ± 2.2 g m^{-2}. Therefore, it has been observed about 20% increase of total bio-products production in photobioreactor scale, compared to optimization experiments of Chapter 4. Similarly, carbohydrates which report the highest content showed 27% increase. All the above, indicate that

cultivation of EMS1 *Stichococcus* sp. in a larger vessel seem enhance biomass and bio-products production.

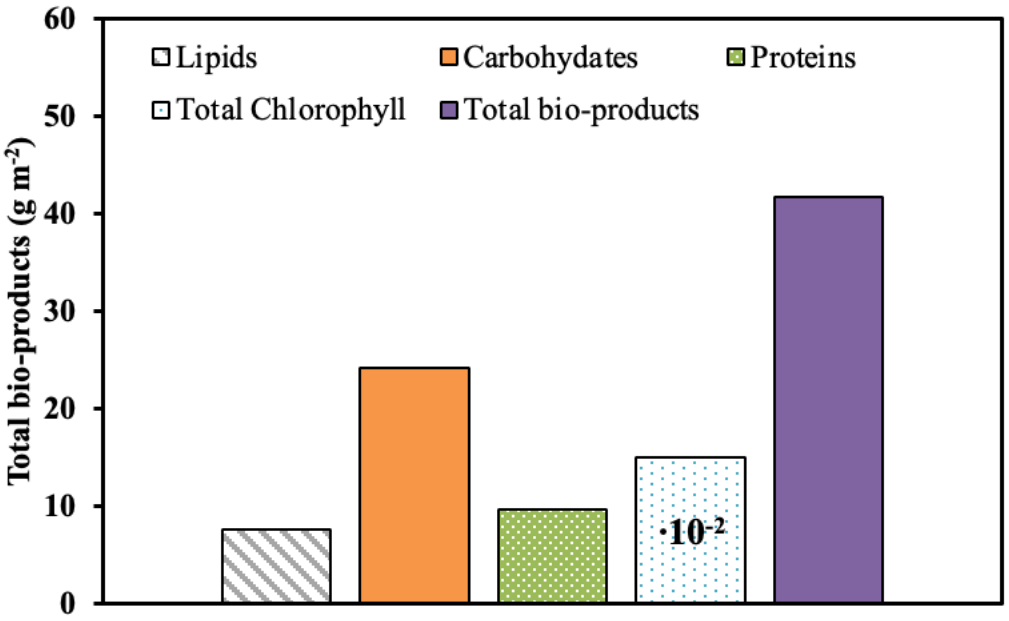


Figure 49. i) Lipids, ii) Carbohydrates, iii) Proteins, iv) Total chlorophyll, and v) Total bio-products from EMS1 *Stichococcus* sp. biomass, cultivated in laboratory flat-panel photobioreactor with synthetic flue gas feed. Culture volume was 15 L. Note that Total chlorophyll value was multiplied by 100, as it was very low and could not be illustrated properly compared to the other values.

5.2.2 Pilot-scale cultivation in real-time conditions

Experiments were conducted for the maximization of biomass/total bio-products and lipids production (Table 12). Also, flashing light was implemented in an experimental run. Flashing lighting was adjusted to 1,000 Hz and duty cycle 0.5. pH was monitored regularly. pH range of *Stichococcus* sp. grown under autotrophic conditions is from 3.0 to 8.5 (Olivieri et al., 2011). According to the recipe of Bold's Basal Medium, the initial pH was regulated to 6.5. However, as can be seen in Figure 50, pH values at day 0 were 6.3, and 6.4, and 6.3 for the three experimental runs, respectively. This may be justified by the fact that the growth medium was autoclaved before cultivation, which results to pH reduction (Skirvin et al., 1986). pH reached a maximum value of

7.7, which was determined during 1st run (Biomass/Total bio-products maximization). The reported pH values are within the range 3.0 to 8.5 mentioned by Olivieri et al. (2011) in which *Stichococcus* sp. can be cultivated. Therefore, even though the cultures were supplied with flue gas, the pH values in the growth medium were within acceptable limit and did not affect the growth.

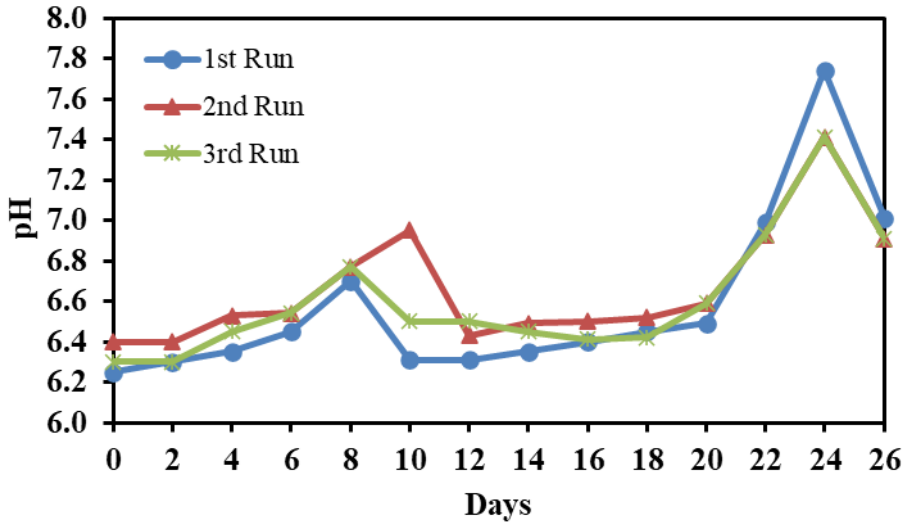


Figure 50. pH variation of EMS1 *Stichococcus* sp. strain, cultivated in flat-panel photobioreactor with real-time industrial flue gas feed. Culture volume was 15 L. Samples were collected every two days, during 26 days of cultivation period. Blue line with circular markers shows pH results of microalgae cultivated for biomass/total bio-products maximization, red line with triangle markers shows the results of microalgae illuminated by flashing lighting, and green line with asterisks shows pH results of microalgae cultivated for lipids maximization. The frequency of the flashes was 1,000 Hz and the duty cycle Φ was 0.5. Light:Dark ratio was 16:8 h.

Dry biomass production in the experimental runs was 50.5 and 47.9, and 38.3 g m^{-2} (Figure 51). In 1st and 2nd run, the illumination intensity was adjusted to 6,600 lux, justifying the increased biomass production. The implementation of flashing light induced a reduction in biomass production, which was also noticed in lab-scale experiments (see Section 4.3.2). However, both values were higher, compared to biomass results of Section 5.2.1 .lpand Table 3, showing that industrial flue gas increased biomass productivity of EMS1 *Stichococcus* sp. As in the case of lab-scale experiments, the implementation of lipids maximization (3rd Run) results in limited biomass

production, because of the lower illumination intensity (3,300 lux) and also the application of three-day nitrogen starvation removed a part of the suspended microalgae cells. Other microalgal species have been also found to grow with flue gas. Especially, microalgae isolated from the regions near the thermoelectric power plants may be more tolerant to flue gas than other native species (Brown, 1996; Chu et al., 1996; G. Huang et al., 2016). de Morais and Costa (2007) isolated the microalgae *Scenedesmus obliquus* and *Chlorella kessleri* from ponds near a thermal power plant and reported that the species were able to grow in 18 % (v/v) CO_2. Similarly, Choi et al. (2017) examined *Haematococcus pluvialis* under 5, 10, and 15% (v/v) CO_2 and resulted in higher CO_2 sequestration when the strain was exhibited to 15%. In another study by Kao et al. (2014), *Chlorella* sp. was fed with industrial flue gas from a steel plant containing up to 25% CO_2. It is worth mentioning that cement, iron and steel production also account for large proportions of world CO_2 emissions (US EPA, 2011). However, microalgae can also grow in flue gas emitted from other important industries, such as petrochemicals, sugar, tires, carbon black, aluminum, paper, inorganic chemicals, fertilizers, breweries, and mining (Chae et al., 2006; US EPA, 2011). As can be seen, microalgae are able to sufficiently grow in CO_2 rich environments, minimizing the adverse effects of this gas and converting it into valuable products which can be beneficial for human use.

Another parameter that was taken into consideration is electricity consumption, which was measured with a power consumption meter interconnected between the power supply and the wall outlet. After 26 days of cultivation, maximum energy consumption met with 6,600 lux light intensity was 33.1 and 25.7 kWh for the continuous and flashing lights, respectively. Based on the current cost of industrial electricity in Greece which is 0.167 € kWh^{-1} (Public Power Corporation S.A., 2023), the aforementioned consumptions cost 5.5 and 4.3 €. The corresponding cost per kg of dry biomass was 0.11 and 0.09 € kg^{-1}. So, even if the application of flashing lights resulted in lower biomass production, the cost of production was also lower. Lu et al. (2022) also investigated the electrical demand of continuous and flashing lighting. They reported that for the same light intensity conditions, flashing light consumed about 1.1–5.0 kWh g^{-1} of biomass produced, which was 6.2–32.7% lower than those of constant light.

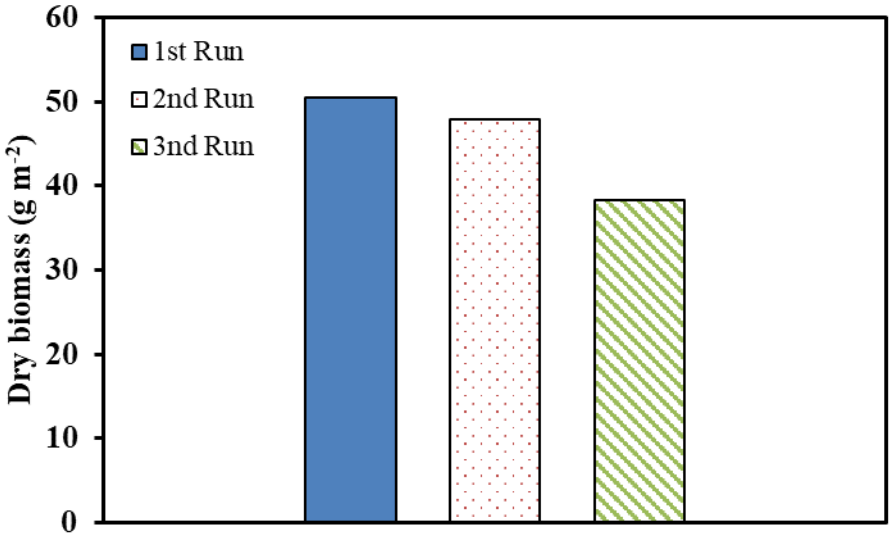


Figure 51. Dry biomass of EMS1 *Stichococcus* sp. cultivated in flat-panel photobioreactor with real-time industrial flue gas feed. Culture volume was 15 L. Solid blue bar shows dry biomass results of microalgae cultivated for biomass/ total bio-products maximization, red dot bar shows the results of microalgae illuminated by flashing lighting, and green line bar shows dry biomass results of microalgae cultivated for lipids maximization. The frequency of the flashes was 1,000 Hz and the duty cycle Φ was 0.5. Light:Dark ratio was 16:8 h.

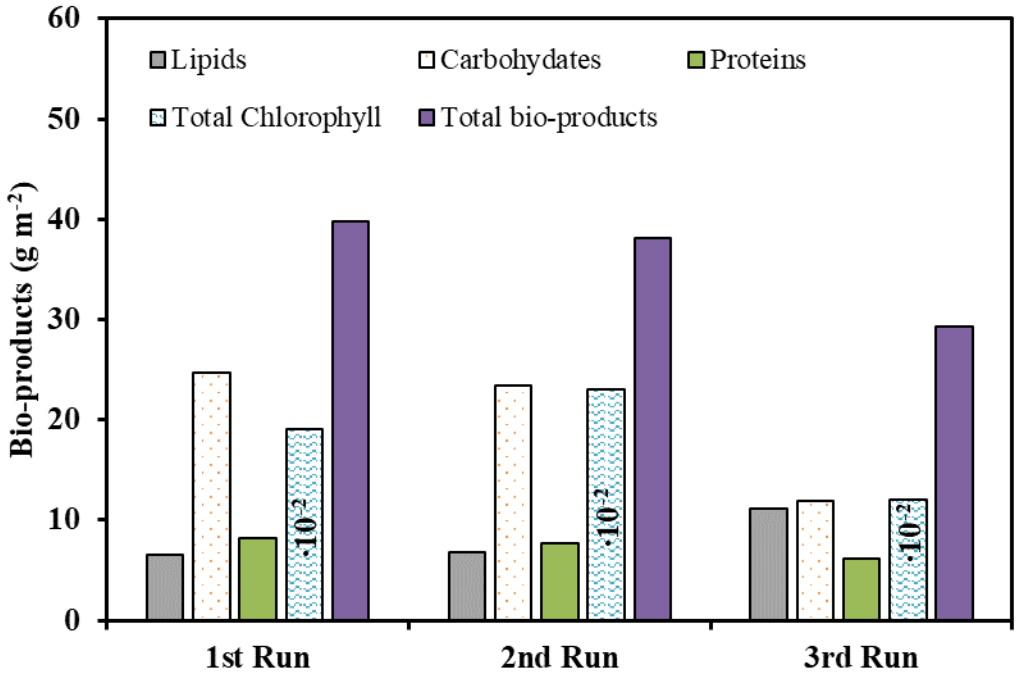


Figure 52 shows the results of lipids, carbohydrates, proteins, total chlorophyll, and total bio-products content of EMS1 *Stichococcus* sp. biomass, cultivated for lipids maximization (Run #3) and biomass/total bio-products maximization (Run #1), and the effect of flashing light at 1,000 Hz and duty cycle 0.5 (Run #2). Total bio-products were equal to 39.8, 38.1, and 29.3 g m^{-2}, for runs #1, #2, #3, respectively. These quantities correspond to approximately 79, 80, and 77% of dry biomass. Carbohydrates were the prevailing bio-products in all occasions as in the case of Figure 49, with values equal to 24.8, 23.4, and 11.9 g m^{-2}. Similarly, proteins content was 8.3 and 7.6, and 6.1 g m^{-2}. Lipids content was increased by the use of three-day nitrogen starvation (Run #3), with a value equal to 11.2 g m^{-2}. While, lipids content for runs #1 and #2 was 6.6. and 6.8 g m^{-2}. Total chlorophyl for each experimental run was equal to $19 \cdot 10^{-2}$, $23 \cdot 10^{-2}$, and $12 \cdot 10^{-2}$ g m^{-2}. Flashing light proved to increase pigments production, also verified by the lab-scale experiments. The overall results can be compared very well with the results of the lab-scale experiments (see Section 4.3.2). *Stichococcus* was able to grow both in small culture volumes and in larger photobioreactors with the supply of industrial flue gas, thus indicating a versatility of the strain to adapt in various environments.

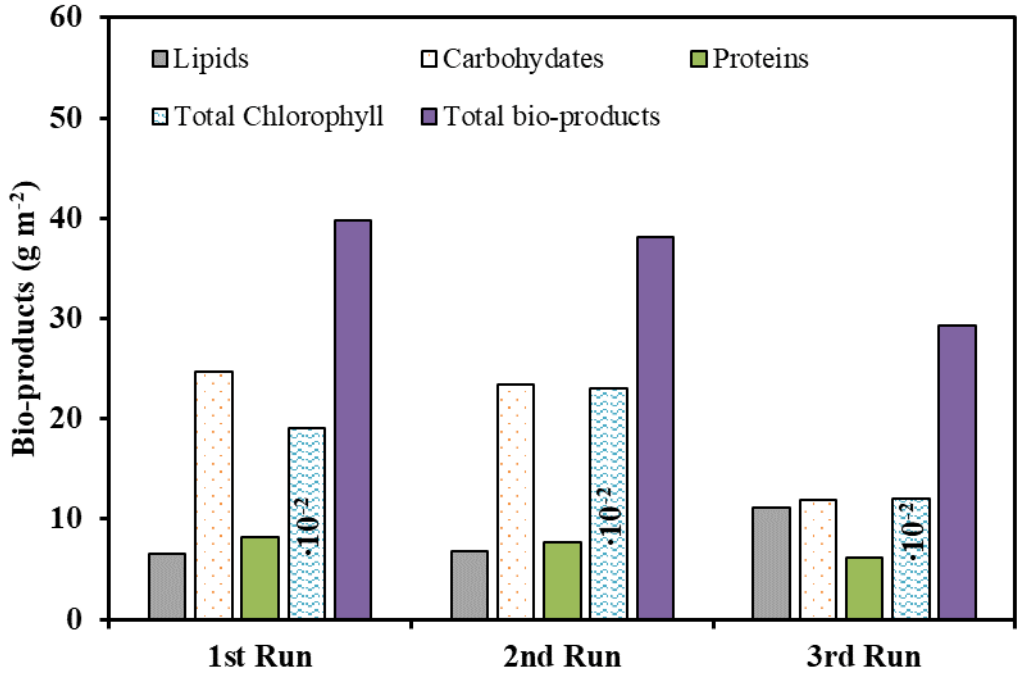


Figure 52. i) Lipids, ii) Carbohydrates, iii) Proteins, iv) Total chlorophyll, and v) Total bio-products produced by EMS1 *Stichococcus* sp. Culture volume was 15 L. 1st Run was conducted

for biomass/bio-products maximization, 2nd Run implemented the use of flashing lighting, and 3rd Run was conducted for lipids maximization. The frequency of the flashes was 1,000 Hz and the duty cycle Φ was 0.5. Light:Dark ratio was 16:8 h. To mention that Total chlorophyll value was multiplied by 100, because it was very low and could not be illustrated properly, compared to the other values.

5.4 Concluding Remarks

EMS1 *Stichococcus* sp. microalgae strain was initially scaled-up in the laboratory, in a flat-sheet photobioreactor, following its optimization for the maximization of biomass and bio-products. Cells were grown attached on sandblasted glass tiles, which covered the bottom of the photobioreactor. Carbon was supplied in the form of synthetic flue gas, which simulated natural gas flue gas emitted from thermoelectric power plant. *Stichococcus'* results were higher in the larger scale, compared to the small-scale experiments, producing sufficient amounts of biomass and bio-products. Carbohydrates were the prevailing bio-products, which could possibly be exploited for further processing and production of pharmaceuticals or biofuels. The aforementioned positive results, led to experiments in real conditions with industrial flue gas feed. Specifically, experiments took place in Lavrio power station, one of Greece's largest power station, owned by the Public Power Corporation S.A. EMS1 *Stichococcus* sp. was successfully grown under industrial flue gas, containing about 3% CO_2, producing high biomass. At the end of each cultivation cycle, biomass was collected from sandblasted glass tiles and lipids, pigments, proteins, and carbohydrates were quantified.

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Chapter 6: Biomass separation and drying processes

6.1 Introduction

Cultivation is the main cost factor for microalgae-based products (Barros et al., 2015; Norsker et al., 2011; Ruiz et al., 2016). However, the harvesting and dewatering processes are also considered a significant factor in the overall cost. Several studies report that harvesting costs account for 20–30% of the total production costs (Barros et al., 2015; Brennan and Owende, 2010; Fasaei et al., 2018; Molina Grima et al., 2003; Rawat et al., 2011; Uduman et al., 2010). The high capital cost and energy consumption result from the dilute algal solutions, the large volumes to be processed, and the small size of the microalgal cells (Draaisma et al., 2013; Molina Grima et al., 2003; Pahl et al., 2013). Finding efficient harvesting and drying processes could significantly reduce the production costs and minimize the environmental impacts while preserving the quality of the final added value products.

The objective of the present research was the development of optimal methods for biomass recovery by comparing the conventional processes of i) filtration, ii) centrifugation, and iii) flocculation, with biomass scraping from an immobilized-cell system, Experiments were conducted in small scale (glass beakers, 150 ml culture volume) and in pilot-scale flat-panel photo-bioreactor filled with 15 L of modified Bold's Basal Medium (see Section 4.2.1 for its composition). The processes of i) convective drying, ii) freeze drying, and iii) solar drying were also compared, to investigate their effect on biomass quality. For this reason, harvested biomass was converted into lipids, proteins, carbohydrates, and pigments to assess the influence of each drying process.

6.2 Materials and Methods

6.2.1 Biomass harvesting

Experiments were conducted using EMS1 *Stichococcus* sp. strain grown in modified Bold's Basal Medium (BBM) (see Section 4.2.1 for its composition) for 25 days. The culture temperature was kept constant at 25 ± 1 °C and microalgae were illuminated by LED strip lights with illuminance intensity 6,600 lux and photoperiod 16 h day – 8 h night.

For the scope of this research, two types of microalgae cultivation systems were examined: i) cell growth in suspension and ii) immobilized on sandblasted glass. Microalgae in suspension were cultivated in 600 mL glass beakers with of 150 mL BBM and in a 2 L batch photobioreactor with 1.5 L of BBM (Figure 53). The batch photobioreactor was utilized for conducting flocculation experiments. As for the immobilized cell system, microalgae were also grown in glass beakers and in the flat-panel photobioreactor with 15 L of culture medium (Figure 44). ANOVA and Tuckey HSD statistical methods were used for comparing experimental results. To achieve similar growth conditions in beaker cultures, microalgae were placed inside the flat-panel photobioreactor mentioned in section 4.2.1 (Figure 17, Figure 18A-B). Microalgae received carbon (CO_2) from synthetic flue gas with its composition being: 5% CO_2, 81% N_2 and 14% O_2. The gas mixture was sterilized using a 0.2 μm PTFE filter before entering the photobioreactor and the total flow rate was 0.8 L min^{-1}.

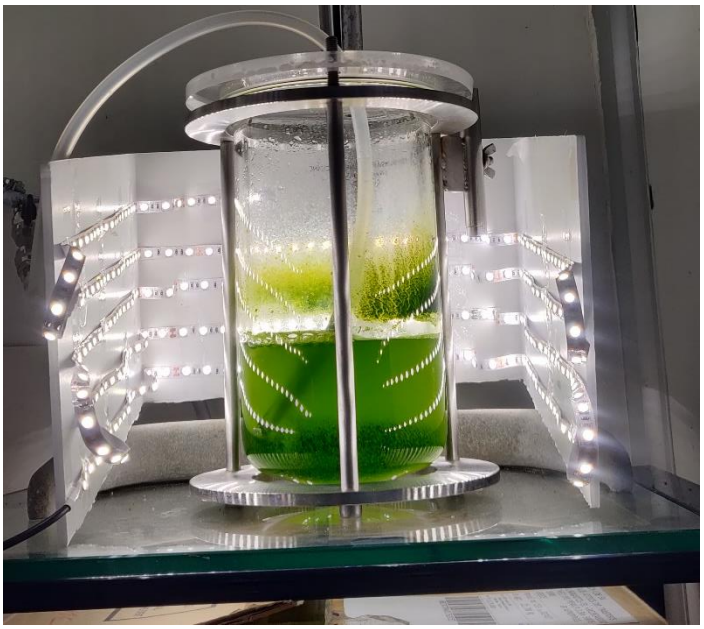

Figure 53. Batch photobioreactor used for biomass production in flocculation experiments.

To investigate the optimal separation of biomass from the culture medium, a simple and cost-effective method was used. Immobilized cells were harvested by mechanical scraping after the supernatant liquid was pumped out of the vessels. The efficacy of the process was compared with the conventional methods of centrifugation, vacuum filtration and flocculation coupled with centrifugation or vacuum filtration.

6.2.1.1 Immobilized biomass scraping

As mentioned in the last paragraph of Section 6.2.1, EMS1 *Stichococcus* sp. strain was grown attached on sandblasted glass tiles placed at the bottom of beakers and flat-panel photobioreactor. To recover microalgae biomass, the supernatant was first pumped out and then the sandblasted glass tiles were removed. The immobilized biomass was collected using a mechanical scraper (Figure 18D and Figure 45), dewatered by centrifugation or vacuum filtration and freeze-dried or oven-dried for 24 h to calculate dry biomass in grams per unit area (g m^{-2}).

6.2.1.2 Centrifugation

Centrifugation was conducted by collecting 45 mL of suspended biomass. Each was then centrifuged (Hettich ROTOFIX 32A) (Figure 54) at 4,000 rpm for 10 min and further dewatered with freeze-drying for 24 h. Finally, dry cell weight was calculated by weight measuring.

Figure 54. Centrifuge device used to separate the microalgal biomass from the culture medium.

6.2.1.3 Vacuum filtration

For vacuum filtration (Figure 55), 45 mL of biomass were collected and filtered using 1.2 µm glass fiber filters (Whatman). The recovered biomass was dried using convective drying for 24 h.

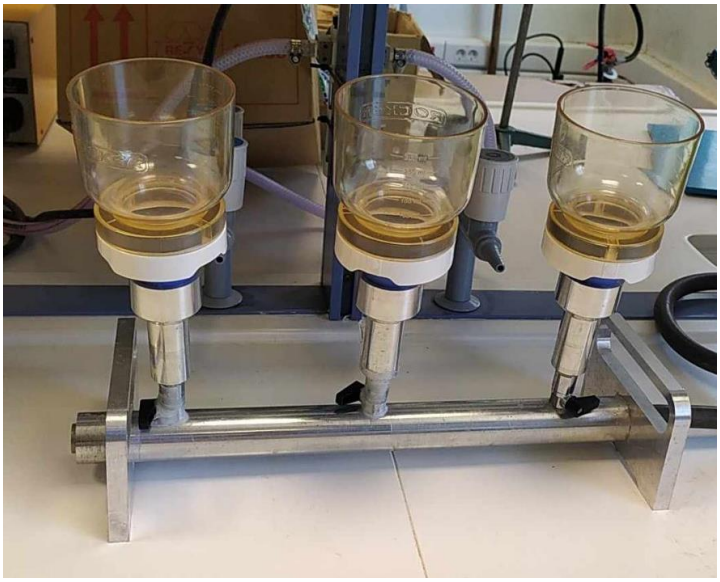

Figure 55. Triple-branch lab vacuum filtration system used for separation of microalgae biomass from culture medium.

6.2.1.4 Flocculation

Chitosan was selected for the flocculation of microalgae biomass. It is a cationic biopolymer that is a polyelectrolyte composed of a linear poly amino sugar chain. Chitosan, compared to other biopolymers (e.g., polyacrylamide, chitosan (poly-(D)glucosamine), cationic starches, and modified tannin) is relatively less toxic, more biodegradable, and less inexpensive, as it is prepared by treating the chitin shells of shrimp and other crustaceans with an alkaline substance, such as sodium hydroxide (Min et al., 2022; Xu et al., 2013). Microalgae aggregation is done by adsorption, crosslinking, sweeping, and neutralization (Toh et al., 2018). Biomass harvesting capacity depends on algal species, ionic strength, cell density, growth stage, temperature, and pH (Matter et al., 2019). Chitosan solution was prepared according to the protocol of Mohd Yunos et al. (2017). Specifically, stock solution of 1 mg mL^{-1} was prepared by dissolving 100 mg of chitosan in 10 mL of 0.1 M HCl. Chitosan is more soluble in 0.1 M hydrochloric acid solution compared to 0.1 M citric, nitric, and phosphoric acid solutions, achieving flocculation efficiency up to 84.5% (Rashid et al., 2013). The solution was stirred until the powder dissolved and diluted to 100 mL with diH_2O. The solution should be freshly prepared for each experiment.

To find the optimal dose of the flocculant, Jar-test experiments (JP Selecta Flocumatic) were conducted at 150 rpm for 5 min, then 10 rpm for 30 min, and settling for 30 min (Figure 56) (Mohd Yunos et al., 2017). Concentrations of 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 mg L^{-1} were tested. After flocculation, samples were either vacuum filtrated or centrifuged to further dewater the biomass. The recovered biomass was then dried to quantify dry cell weight. Turbidity and optical density at 680 nm were also measured for each flocculant dosage to find the optimal dosage. Percentage removal was calculated according to the following formula:

$$Removal\ (\%) = \frac{OD_i - OD_f}{OD_i}$$

Where:

- OD_i, the optical density of the culture at 680 nm before addition of chitosan.
- OD_f, the optical density of the culture at 680 nm after addition of each chitosan concentration.

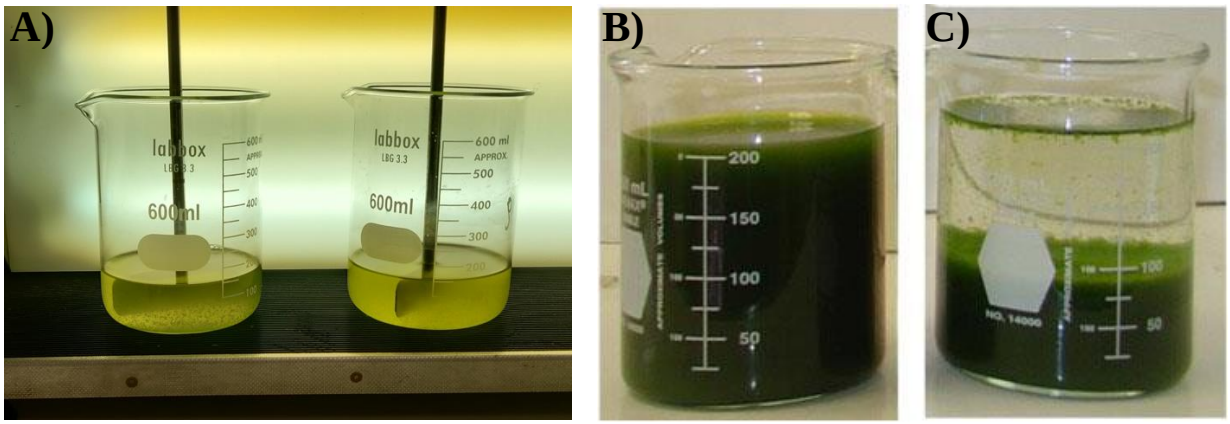

Figure 56. A) Jar-test for microalgae biomass flocculation, **B)** Microalgae biomass before, and **C)** after flocculation.

Considering the above, Figure 57 illustrates the processes and combinations of these that were applied for the separation of biomass from the culture medium. The effectiveness of biomass scraping was compared to the conventional processes of i) vacuum filtration, ii) centrifugation, iii)

chitosan flocculation couple with vacuum filtration, and iv) chitosan flocculation couple with centrifugation.

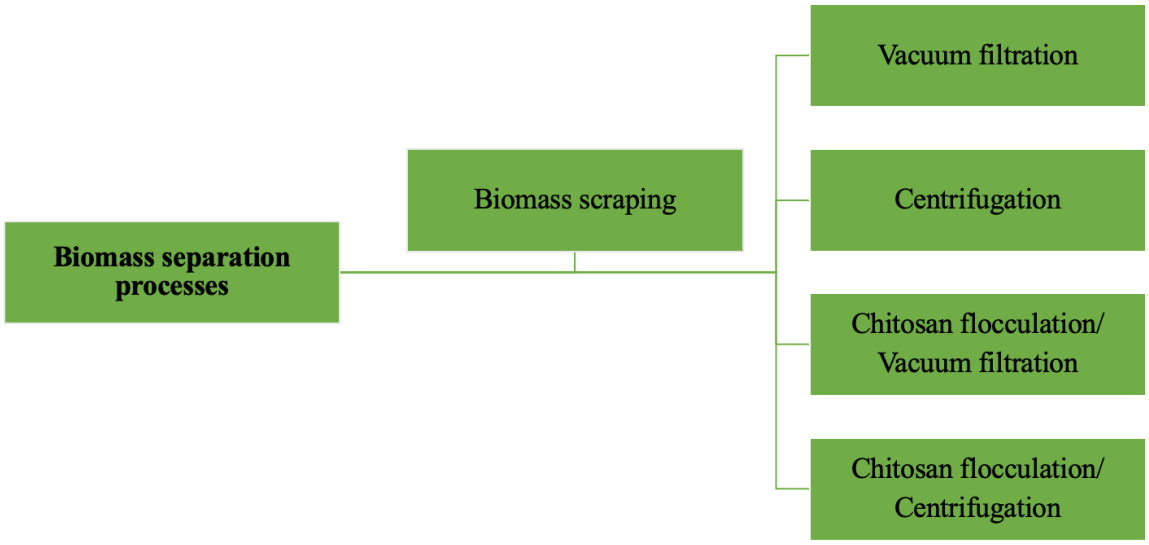

Figure 57. Methods for biomass separation from the culture medium, used in the present study.

6.2.2 Biomass drying

Microalgae biomass drying was conducted in three ways:

- Convective drying (oven-drying)
- Solar drying
- Freeze-drying (lyophilization)

To achieve similar drying conditions, an equal amount of 21 g of wet biomass with a moisture content of 39% was spread on a petri dish (90 x 15 mm), creating a thin layer. Experiments were conducted in triplicate. Samples were weighed at regular intervals until a constant weight was determined. The following sections 6.2.2.1 to 6.2.2.3 specify the conditions of each method. ANOVA and Tuckey HSD statistical methods were used for comparing experimental results.

6.2.2.1 Convective drying

A hot air oven (Memmert UL30) was used for convection drying (Figure 58). Temperature was set at 60 °C to match with the temperature reached inside the solar dryer (approximately 58 to 60 ºC, see Section 6.2.2.2). Also, 60 ºC are beneficial for microalgal lipids yield. Higher temperatures (i.e., 80, 100, 120 ºC) have been reported to decrease lipids extraction (Halim et al., 2011; Mubarak et al., 2015).

Figure 58. Laboratory hot air oven used for microalgae biomass convective drying.

6.2.2.2 Solar drying

A solar dryer (Figure 59) was made in a rectangular wooden shape and painted black to better absorb solar energy. The dryer is passive type, meaning that it does not require electricity as microalgae biomass is dried by natural ventilation and the increased temperatures inside the dryer. A Plexiglass® lid covered the wooden chamber, but not entirely, leaving a small gap for ventilation which can be seen in the schematic of Figure 59. Internal temperature was measured using a

HOBO® MX2202 portable data logger which reached peak temperatures of approximately 58 to 60 ºC.

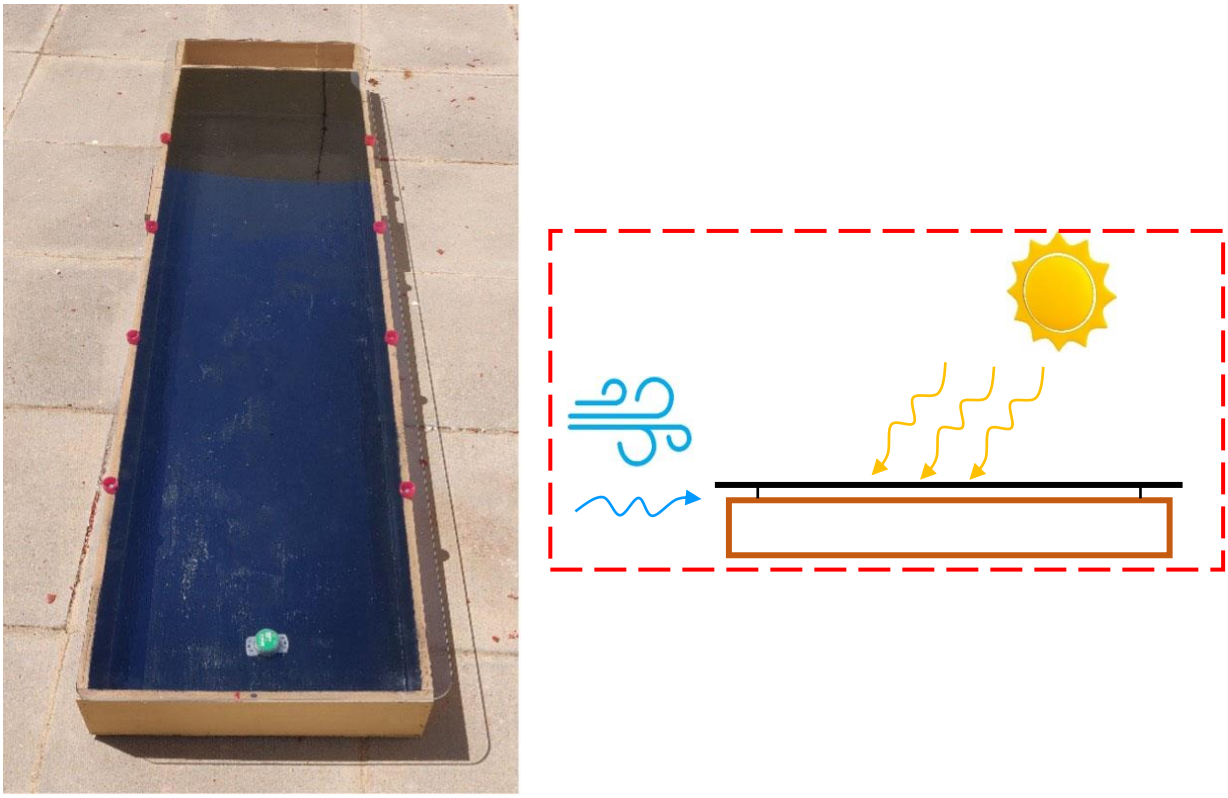

Figure 59. Rectangular wooden solar dryer used for microalgae biomass drying.

6.2.2.3 Freeze-drying

Freeze-drying or otherwise lyophilization (Figure 60) of the samples was carried out at minus (-) 55 °C and under vacuum. The device that was used was Cooling Trap Pro by ScanVac.

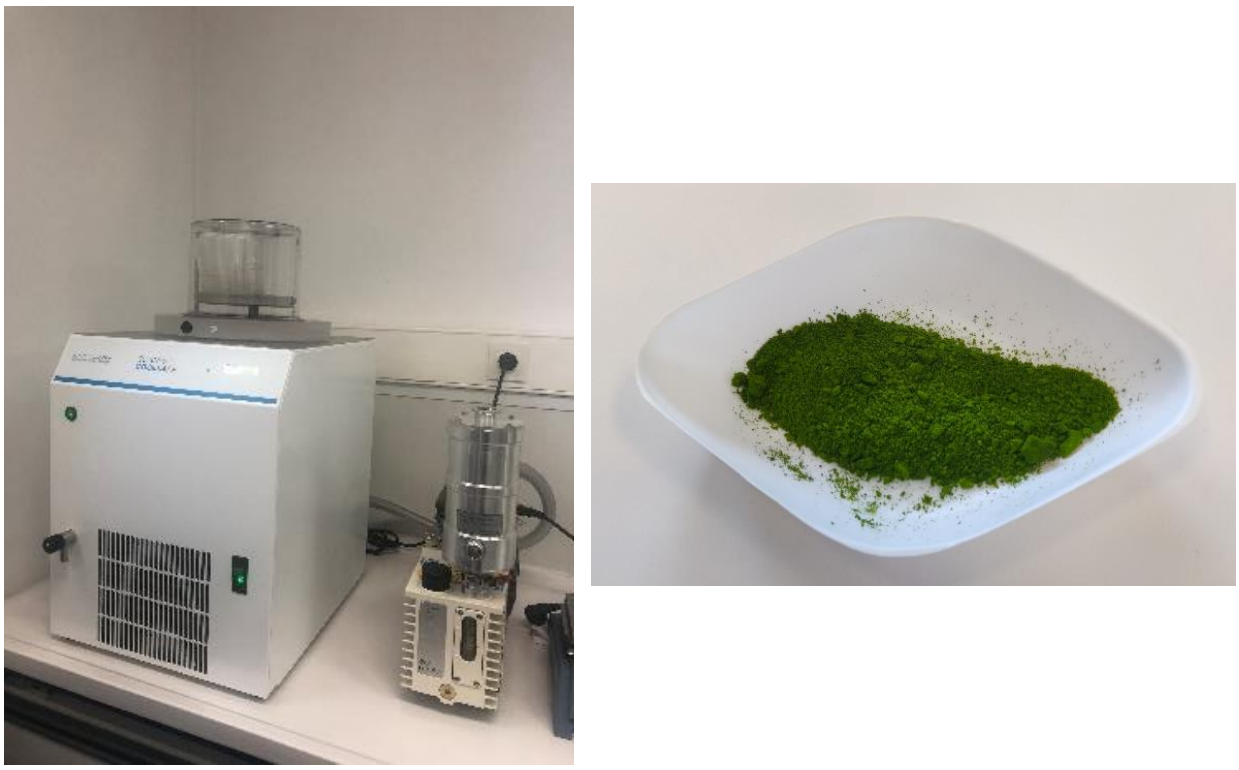

Figure 60. Laboratory freeze-drying (or lyophilization) device used for microalgae biomass drying (left) and lyophilized biomass in powder form (right).

After each drying process, samples of dry biomass were used for quantification of lipids, pigments, proteins, and carbohydrates. Detailed protocols are mentioned in Section 4.2.4. The scope of the measurements was to test the influence of each process on the quality of the produced bioproducts.

6.3 Results and Discussion

For the separation of biomass from the culture medium, five processes were examined as mentioned in Section 6.2.1. Specifically, the conventional dewatering processes of centrifugation, vacuum filtration, and flocculation applied on suspended microalgae cultures were compared with biomass scraping of immobilized microalgae cultures. Flocculation was combined with centrifugation or vacuum filtration. The following section presents the experimental results for determining the optimal flocculant dose.

6.3.1 Flocculant optimal dose

In the present study, chitosan was used as flocculating material. Chitosan flocculation requires lower dosage compared to cationic flocculants, resulting in the reduction of cultivation costs (Fasaei et al., 2018; Roselet et al., 2015; Vandamme et al., 2010). However, in marine conditions the effect of chitosan relative to freshwater is lower, so it requires higher concentrations and pH adjustment (Farid et al., 2013). Figure 61A-B show the experimental results of turbidity and optical density in relation to chitosan concentration (mg L^{-1}) in the growth medium. Figure 61C shows the percentage removal of suspended cells, in relation to chitosan done in mg per grams of dry biomass It was observed that the minimum concentration of flocculant was 80 mg L^{-1} (Figure 61A-B), achieving sedimentation of the cells by 80% (Figure 61C). In this case, turbidity declined from 64 to 11 NTU, while optical density measured at 680 nm declined from 0.43 to 0.09. Applying up to 120 mg L^{-1} of chitosan, induced 87% optical density removal of the suspended microalgae. Blockx et al. (2018) reported that the optimum dose for *Nannochloropsis* in seawater environment was 75 mg L^{-1}, close to the optimum of 80 mg L^{-1}, found in the present study. Flocculation efficiency was about 90%. Lubián (1989) investigated the flocculation of 11 marine microalgae species and found that chitosan concentration ranged from 40 to 80 mg L^{-1}. Also, when pH was adjusted to 8.0, flocculation efficiencies were equal or greater than 75%. Figure 61D shows the dry biomass and pH of the culture supernatant before and after flocculation with the optimal dose of 80 mg L^{-1}. Dry biomass decreased from 3.9 ± 0.3 g L^{-1} to 0.8 ± 0.2 g L^{-1}, with the flocculation efficiency being 80% The flocculated biomass was then recovered by centrifugation or vacuum filtration, as described in the following sections. pH of the samples before flocculation was 8.03 ± 0.04, which according to Blockx et al. (2018) should be greater than 7.5 for efficient seawater microalgae flocculation, as in this case. pH after flocculation decreased to 6.18 ± 0.14. Mohd Yunos et al. (2017) also reported a slight decrease in pH, resulted from chitosan flocculation. Maximum biomass recovery reported in this study was 81.35% for *Chlorella* sp. and when applying chitosan concentration equal to 80 mg L^{-1}. Alkaline conditions (pH 9.9) are proposed by Şirin et al. (2012) to improve the degree of algae recovery. However, they can cause mineral precipitation, thus contaminating microalgal biomass (Vandamme et al., 2013). Therefore, neutral pH is preferred to avoid contamination, while having the drawback of lower biomass recovery and the harvesting/dewatering costs increase by 25–30% (Fasaei et al., 2018).

Flocculation with chitosan and neutral pH is preferred (applied in this study) to prevent any contamination. However, the lower recovery of biomass at neutral condition increases the costs of harvesting and dewatering by 25–30%.

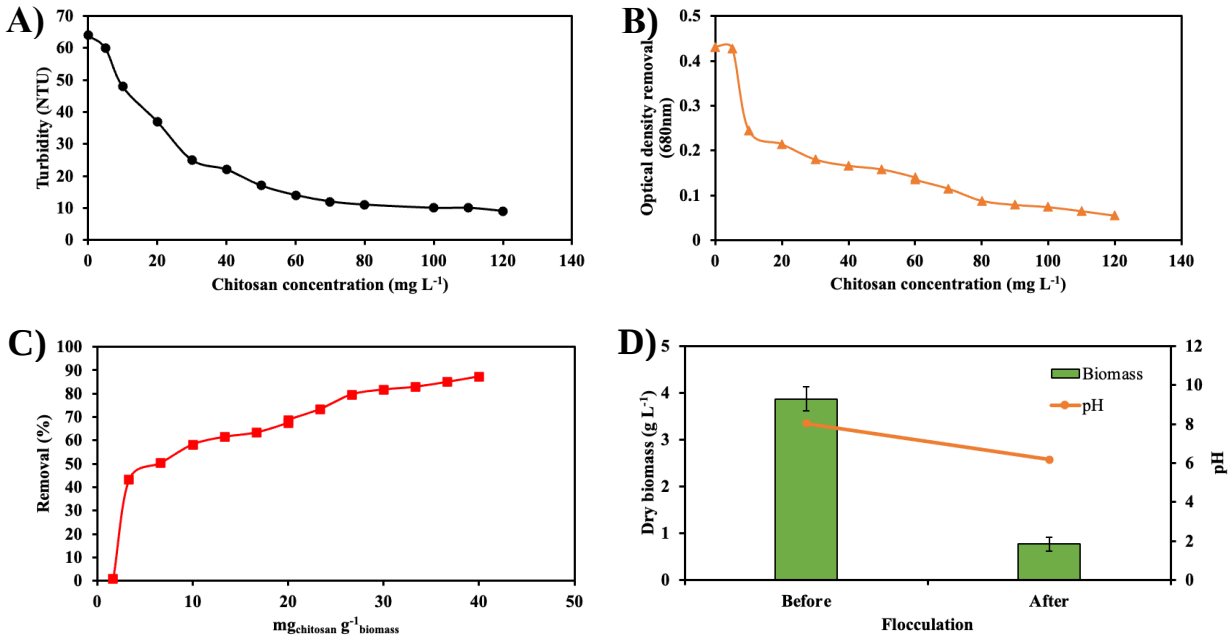

Figure 61. Turbidity **A)** and Optical density **B)** reduction in relation chitosan concentration in the culture. **C)** Percentage increase in removal of microalgae cells from culture supernatant, in relation to chitosan dose per gram of biomass. **D)** Dry biomass concentration and pH in culture supernatant, before and after flocculation by adding 80 mg L^{-1} of chitosan flocculant. Values in **D)** are presented as the mean ± standard deviation (n = 3).

6.3.2 Biomass harvesting

Five processes for biomass separation were examined (Figure 62). The principal methods were vacuum filtration, centrifugation, chitosan flocculation of suspended microalgae (Figure 62A), and scraping of immobilized microalgae (Figure 62B). To note that all dry biomass values are expressed in grams per 150 mL of culture volume, to provide a measure of comparison between suspended and immobilized microalgae. Filtration or centrifugation were also coupled with flocculation. Regarding mechanical scraping, dry biomass was quantified in both the recovered biomass and supernatant. The supernatant was initially pumped out to provide easier access to the

removable sandblasted glass tiles, placed at the bottom of the culture vessels. It was observed that the application of vacuum filtration and centrifugation methods resulted in maximum recovery rates, equal to 0.40 ± 0.03 and 0.39 ± 0.03 g/150 mL of culture, respectively. This is justified by the fact that the cultures were homogenized and processed for cell recovery. Also, filters that were used, retain almost all *Stichococcus* sp. biomass and by implementing centrifugation, almost all biomass is formed into a pellet. Therefore, both processes resulted in similar biomass recovery results. By coupling the abovementioned methods with chitosan flocculation, the recovery was reduced by 13-20%, which is justified by the results of Figure 61, as after the application of flocculation biomass recovery was 80%. Analysis of Variance (ANOVA) and Tuckey HSD method showed that flocculation coupled with centrifugation did not have significant difference over filtration alone (p-value = 0.023). Centrifugation and flocculation/filtration share the same letters as filtration and flocculation/centrifugation. Regarding the recovery of immobilized cells from the glass surface (Figure 62B), it was observed that the dry biomass noted the lowest value, equal to 0.22 ± 0.01 g/150 mL of culture, while in the supernatant was equal to 0.12 ± 0.01 g/150 mL of culture. This may be justified by the fact that during pumping of the supernatant liquid, a part of the suspended microalgae that were not adhered to the sandblasting glass were also removed. However, the recovered supernatant could be used as an inoculum for the next culture cycle as it contains a sufficient amount of biomass or it could be further processed for microalgae harvesting. Also, the supernatant does not contain residues from previous treatment, such as from the flocculation process, which will contaminate future cultures. In addition, the removal of retained flocculants will increase dewatering costs and energy use. If the retained culture cannot be reused for cultivation, a significant value loss of biomass occurs, resulting in higher operation costs. For example, 90% and 80% recovery rates can result in 0.56 €·kg^{-1} and even 1.25 €·kg^{-1} in lost value, respectively (Fasaei et al., 2018). Furthermore, the presence of flocculants may affect the performance of biomass conversion into bio-products. Especially, polymeric flocculants can alter the carbon profile composition, thus limiting biomass utilization (Borges et al., 2011).

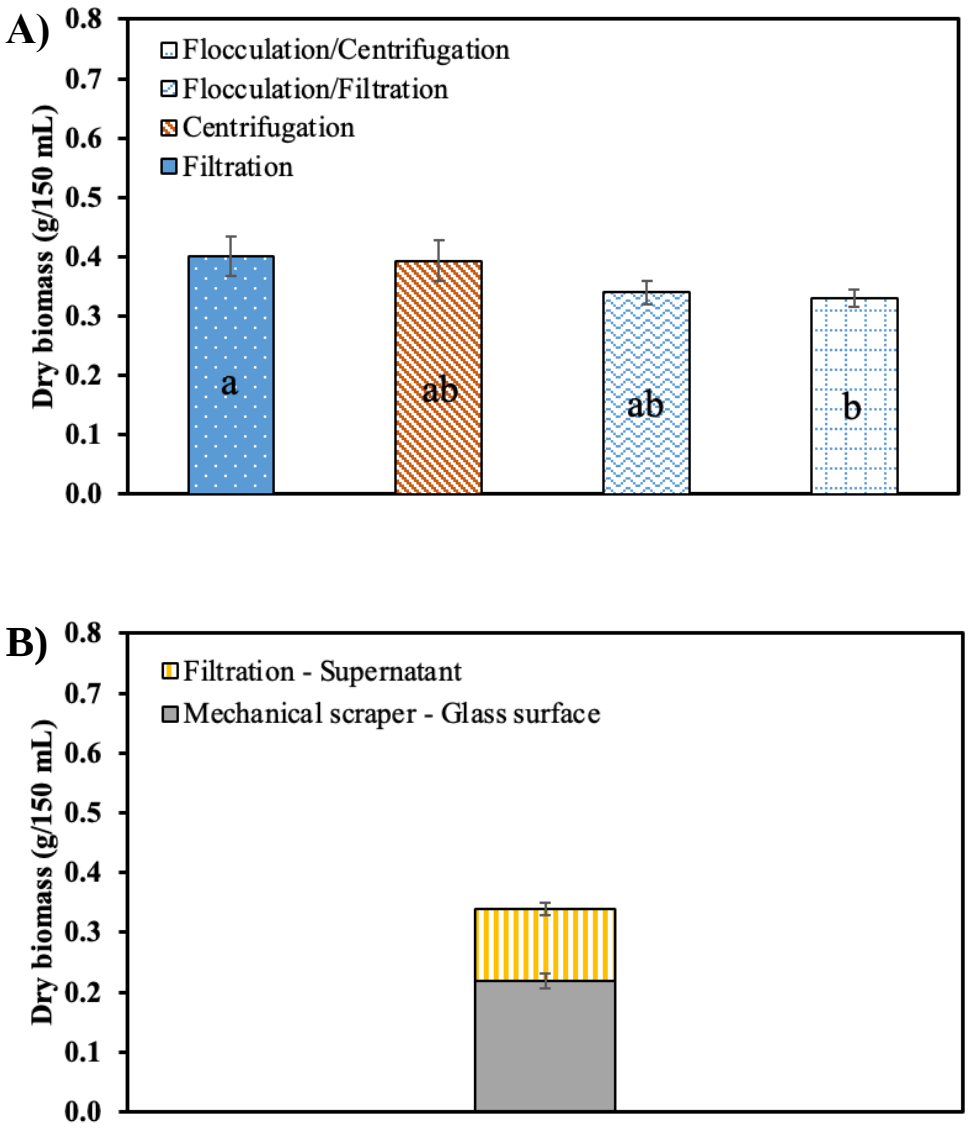

Figure 62. A) Dry biomass data of EMS1 *Stichococcus* sp. in g per 150 mL culture volume, applying different harvesting/dewatering protocols: (from left to right) i) Filtration, ii) Centrifugation, iii) Flocculation/Vacuum filtration, iv) Flocculation/Centrifugation, and **B)** Mechanical scraper. Values are presented in **A)** as the mean ± standard deviation (n = 3, p-value = 0.023). Values with the different letters represent a significant difference (p-value < 0.05) between treatments. Values are presented in **B)** as the mean ± standard deviation (n = 3). Note that all dry biomass values are expressed in grams per 150 mL of culture volume, to provide a measure of comparison between suspended and immobilized microalgae.

To perform a better comparison of the biomass separation processes from the culture medium, the energy requirement was estimated based on the laboratory equipment used (Figure

63). Specifically, the energy consumption in kWh per kg of wet biomass was examined for each process applied. As can be seen, vacuum filtration noted the highest electrical energy consumption, with a value equal to 12.0 ± 0.5 kWh kg^{-1} of dry biomass, followed by centrifugation with a value equal to 10.0 ± 0.6 kWh kg^{-1}. In contrast, the lowest consumption was met with biomass scraping of immobilized biomass from sandblasting glasses, with a value equal to 0.7 ± 0.1 kWh kg^{-1} of dry biomass. P-value was close zero (<0.0005), as mean values have great difference. Means that do not share the same letter, have statistical difference compared to the other processes.

Based on Figures 62-63, even if the recovery of immobilized biomass through mechanical scraping had low efficiency compared to the conventional processes applied on suspended microalgae (Figure 62), it noted the lowest energy requirement, as can be seen in Figure 63. Also, the recovered culture medium can be reused in future cultures by adding fresh nutrients, as mentioned earlier in the present section. Flocculation compared to vacuum filtration and centrifugation alone, required considerably less energy, specifically an order of magnitude lower since the culture medium has already been pretreated with an initial separation of the biomass from the culture medium. Therefore, not all culture material is processed, but only the flocculated biomass (via filtration or centrifugation), explaining the lower biomass recovery results. The aforementioned outcomes were also determined in the studies of Fasaei et al. (2018); Min et al. (2022), which contain a detailed review on harvesting and dewatering techniques used for microalgae cultivation. Similar effect to flocculation occurs in the case of immobilized microalgae scraping, meaning that harvesting takes place in one part of the photobioreactor. However no extra materials are used and lower energy consumption can be achieved. Thus, based on the experimental results, immobilized microalgae cultures could potentially reduce cultivation steps and harvesting costs.

Biomass dewatering is followed by further drying, as the moisture content was still high. The following section presents the experimental drying processes examined.

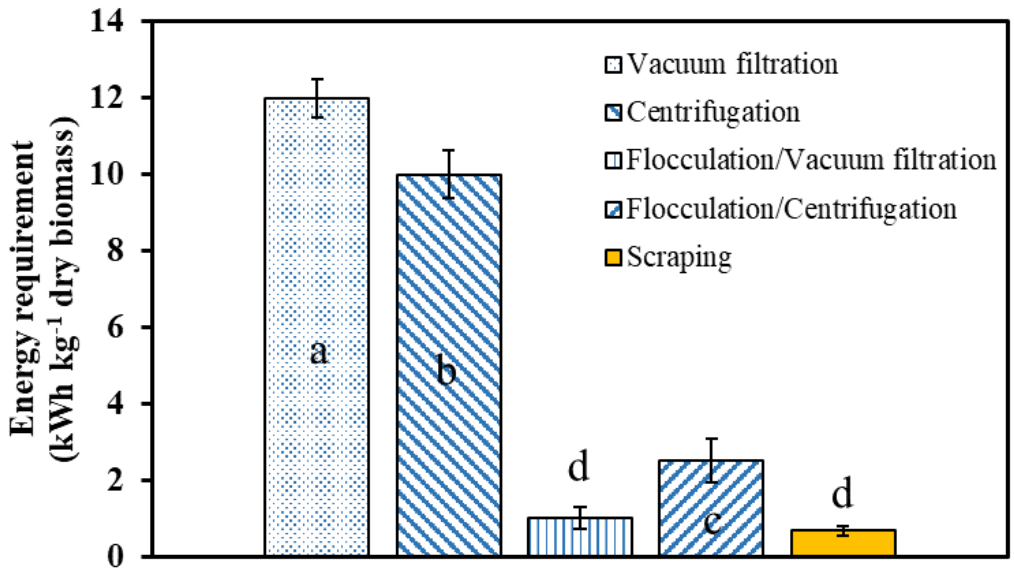

Figure 63. Electrical current energy requirement (kWh) per kg of dry biomass for each harvesting/dewatering processes. Values are presented as the mean ± standard deviation (n = 3, p-value = <0.0005). Values with the different letters represent a significant difference (p-value < 0.05) between treatments.

6.3.3 Biomass drying

Following microalgae recovery or harvesting through separation from the culture medium, the dewatered slurry was further dried to increase microalgae content. This provides more stability to the end product, which could be further processed for extraction of bioactive compounds used in nutraceuticals, cosmetics, biofuels or dehydrated microalgae could have other uses, such as animal feed. Furthermore, according to Show et al. (2013), feed of dried algae into a press can greatly facilitate yield of algal oil which could then be processed into biodiesel. Feasible drying techniques should be designed to eradicate possible deterioration of the delicate algae quality arising from the dehydration process. Microalgae drying exerts a major economic constraint as it constitutes up to 75% of the total cultivation costs (Show et al., 2015). Selection of drying processes depends on the production scale and the applications of the extracted biomass. One should consider the cultivation of microalgae with reasonable cost and with simple operation, to process large amount of microalgae cost-effectively (Chen et al., 2011; Show et al., 2013). A

notable mention is that most of the microalgae drying processes derived from wastewater sludge dewatering (Show et al., 2015).

The recovered EMS1 *Stichococcus* sp. biomass was dried by applying three different processes: i) convective drying, ii) solar drying, and iii) freeze-drying. An equal amount of 21 g biomass with a moisture content of 39% was spread on petri dishes (in triplicates) to investigate the duration of drying (Figure 64). The shortest drying method was found to be convective drying, requiring approximately 5.5 hours to dry the samples. Similarly, solar drying required 6.5 hours, while freeze-drying, 8.0 hours. Agbede et al. (2020); Behera and Balasubramanian (2021) both conducted experiments with convective drying at 60 ^{o}C and resulted in a duration of 4.2 and 7.5 hours, respectively. The results of the present study fall between the results of the aforementioned studies. The overall duration depends on the quantity of microalgae biomass, but mostly on water content, as biomass is spread evenly creating a thin layer. During the experiments, the maximum temperature inside the solar dryer ranged between 58-60 °C, which was close to the temperature selected for convective drying (60 °C). Light intensity of the sunlight during drying was measured at 80,000 lux. Convective drying and solar drying results are comparable to Agbede et al. (2020). It should be mentioned that solar drying is highly dependent on environmental conditions, so it cannot be applied uniformly throughout the year. Also, there is a risk of fermentation and spillage under prolonged drying. Solar drying is not recommended for microalgae used for human consumption, as the slow process of dehydration invariably emits unpleasant odor which affects the quality of microalgae. Solar drying could be applicable in microalgae intended for animal use. In order for the microalgae to maintain their nutritional value and safety, they should be subjected to a short duration of high heat at l20 °C (Show et al., 2015). Becker and Venkataraman (1982) applied a similar solar drying technique, implementing a wooden chamber covered with a glass plate, as in the case of the present study. Microalgae required 5–6 hours to dry to about 4–8% water content and temperatures ranged between 60 to 65 °C. Similarly, Prakash et al. (1997) solar dried *Spirulina* and *Scenedesmus* biomass in 3-5 hours, resulting in products with less than 10% moisture content and biologically of good quality.

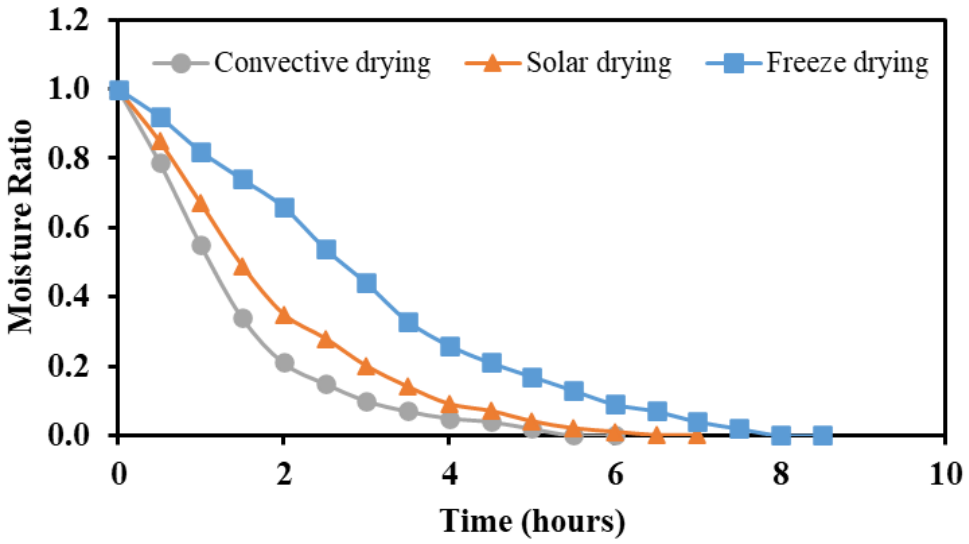

Figure 64. Moisture ratio reduction curves with respect to time, applying different microalgal biomass drying processes.

Comparing energy requirements of the aforementioned drying processes, it was found that convective drying, although it was the shortest method (Figure 64), it has the highest electrical energy demand, with a value equal to 58.5 ± 1.3 kWh kg^{-1} of wet biomass (Figure 65). Freeze-drying was found to be statistically significant with p-value equal to 0.002. Solar drying has zero consumption, as the dryer did not have any electrical ventilation system. Min et al. (2022) reported that about 45.7 kWh kg^{-1} are required for freeze-drying, 55.9–60.7 kWh kg^{-1} for convective drying, and 0.01-0.1 kWh kg^{-1} for solar drying. Similar results for convective drying were also been found by Agbede et al. (2020); Behera and Balasubramanian (2021). As can be seen, microalgae drying is the most energy demanding process. Therefore, appropriate drying processes should be selected by taking into account microalgae species, water content, quality of the end-product, and usage. To further investigate the effect of the drying process examined in the present study, the recovered biomass was processed and converted into bioactive compounds.

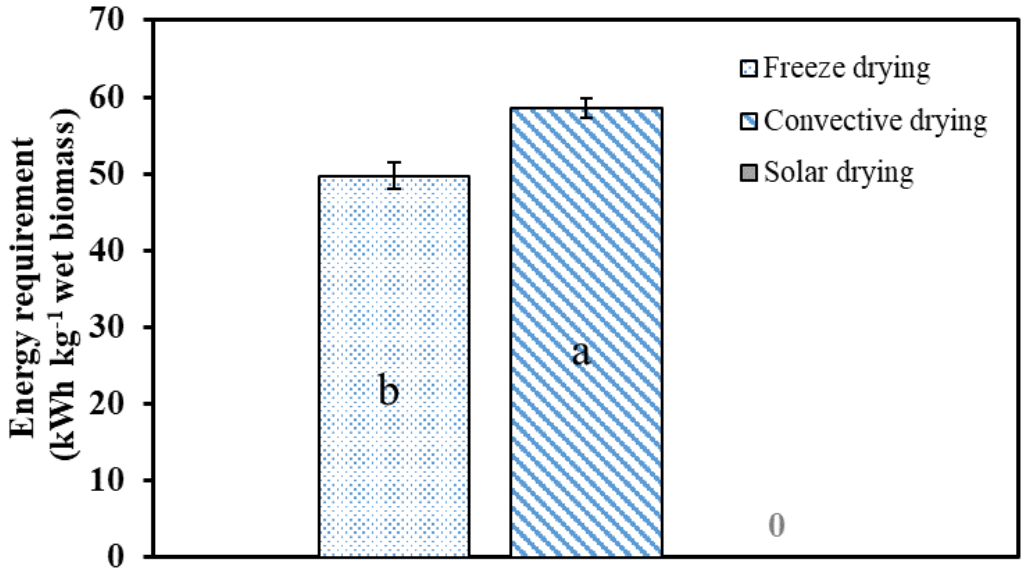

Figure 65. Energy requirement of electricity per kilogram of liquid biomass for each of the considered dehydration processes. Values are presented as the mean ± standard deviation (n = 3, p-value = 0.002). Values with the different letters represent a significant difference (p-value < 0.05) between treatments.

The final processing stage of the recovered biomass was its conversion into bio-products. Lipids, proteins, carbohydrates, and total chlorophyll were measured after each drying process. The results are presented in Figure 66. By applying freeze-drying, a statistically significant difference was noted in the results of lipids (p-value = 0.029) and total chlorophyll (p-value = <0.0005), with the maximum values being 0.26 ± 0.01 g g^{-1} and $0.70 \pm 0.04 \cdot 10^{-2}$ g g^{-1} of biomass. According to Show et al. (2015), direct sun radiation causes chlorophyll to dehydrate and disintegrate, which alters the texture and color of the microalgae biomass. The same effect was noticed in the samples of the present study (Figure 67). Specifically, only freeze-dried biomass had a vibrant green color, while solar and oven dried biomass was darker and more condensed. Also, when convective drying is applied, oxidative degradation of the cells is caused resulting in lower recovery of total chlorophyll, compared to freeze-drying (Schumann et al., 2005; Shekarabi et al., 2019). That is why chlorophyll from freeze-dried biomass was much higher, compared to the other processes. The same was verified in the case of lipids, according to Behera and Balasubramanian (2021); Hosseinizand et al. (2018). Regarding carbohydrates, maximization was achieved by applying solar and convective drying, with a maximum value of 0.45 ± 0.01 g g^{-1}

biomass (p-value = 0.005). Finally, for proteins there was no statistically significant difference (p-value = 0.125) observed between the drying processes, with the maximum value being equal to 0.19 ± 0.02 g g^{-1} biomass. Similar results of a null protein effect were found by Hosseinizand et al. (2018). Overall, the total bioproducts were not statically different, with the p-value being equal to 0.984. Values was equal to 0.80 - 0.81 ± 0.1 g g^{-1} of biomass.

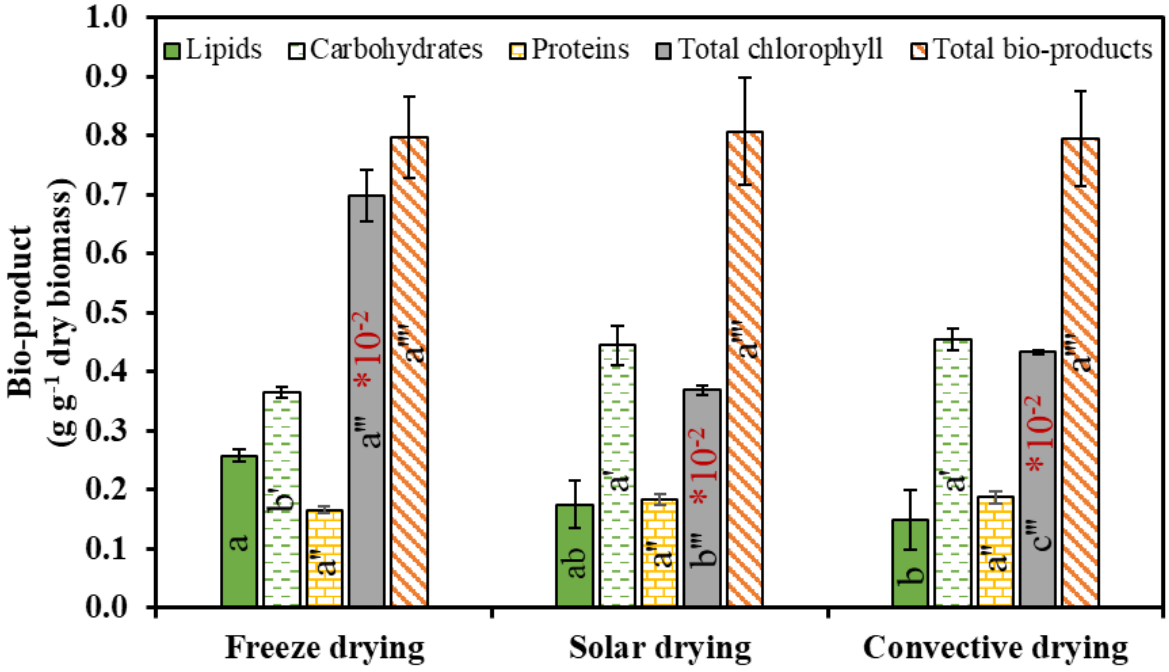

Figure 66. Production of bio-products from *Stichococcus* sp. biomass, after the application of different dehydration methods. Values are presented as the mean ± standard deviation (n = 3). ANOVA and Tuckey HSD method were applied independently for each bio-product and are marked with different apostrophes. P-values for each bio-products are as follows: Lipids p-value = 0.029, Total chlorophyll p-value = <0.0005, Proteins p-value = 0.125, Carbohydrates p-value = 0.005, and Total bio-products p-value = 0.984. Values with the different letters represent a significant difference (p-value < 0.05) between treatments.

Figure 67. Freeze-dried (left), convective dried (center), and solar dried (right) EMS1 *Stichococcus* sp. biomass.

6.4 Concluding Remarks

The results of the present study are useful for the development of efficient microalgae biomass recovery and drying processes, with low environmental impact. The efficiency of the following processes was examined: i) separation of biomass from the culture medium (i.e., filtration, centrifugation, flocculation of suspended microalgae and scraping of immobilized microalgae), ii) drying of biomass (i.e., freeze-, convective-, and solar-drying) and iii) quantification of high added value products (i.e., lipids, proteins, carbohydrates, and chlorophyll) after the application of each drying process. Biomass scraping from an immobilized cell system, although it showed low recovery, it appeared to be the most energy efficient, requiring the lowest electrical energy demand, as processing steps were reduced and the culture supernatant could be reused as a pre-culture in subsequent culture cycles. In terms of drying processes, convective drying was the fastest but most energy-intensive process, followed by freeze-drying. Although solar drying had zero energy requirements, it lacks consistency due to changing weather conditions and it affects the quality of the end product. Drying processes are linked to the recovery of bio-products as they affect their recovery performance. Choosing the right biomass recovery and drying processes could significantly reduce production costs, maximizing revenue from high value-added products in scale-up cultivation where operation costs are significant.

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Chapter 7: Conclusions and ideas for further research[1]

The present PhD thesis dealt with the cultivation of microalgae strain *Stichococcus* sp. and examined all cultivation steps, from selection of a suitable strain to optimization of its cultivation and finally extraction of valuable bio-products which could be used in various sectors, such as nutraceuticals, biofuels, animal feed, and others. This thesis is comprised of four research parts: i) selection and cultivation of a *Stichococcus* sp. strain with lower chlorophyl content and higher biomass production, ii) optimization of a selected *Stichococcus* sp. strain on a laboratory scale and pilot scale photobioreactors with the microalgae immobilized under optimal conditions. Another scope is CO_2 sequestration from synthetic natural gas industrial flue gas, iii) pilot scale experimental investigation of the potential for CO_2 capture from flue gas emitted from a power plant utilizing natural gas, and iv) finding optimal methods for separating biomass from the culture liquid, drying, and recovering its bioactive components, at minimal financial and energy requirements. Another goal was the comparison of alternative processes for the removal of immobilized cells, to conventional methods (i.e., filtration, centrifugation, flocculation).

The main conclusions that were derived from the experiments and data processing are the following:

- Chemical mutation of wild-type *Stichococcus* sp. strain using Ethyl methanesulfonate (EMS) was effective and resulted in a strain (namely EMS1) with reduced chlorophyll content, and increased biomass and lipids productivity. Increased chlorophyll causes a "shadow" effect in photobioreactors, thus inhibiting light penetration in the culture. In this case, productivity can be increased without altering any of the growth parameters.
- Experiments conducted in laboratory scale (beaker vessels, 150 mL culture volume) compared the wild-type and EMS1 strains in terms of agitation, CO_2 uptake, cultivation duration, nitrogen starvation duration, biomass, and bioproducts production. It was found that the absence of agitation had little effect on reducing biomass production. The addition of 5% CO_2 gas resulted in up to 300% increase in biomass and microalgae

[1] Ideas for further research of the present chapter were published in Makaroglou et al. (2021).

required 25 days to grow sufficiently. Also, the application of nitrogen starvation three days before harvesting enhanced intracellular lipids production.

- EMS1 *Stichococcus* sp. strain was selected and further optimized in terms of biomass and bio-products production (i.e., lipids, pigments, proteins, and carbohydrates). Microalgae were fed with synthetic natural gas flue gas for CO_2 fixation. Aeration rate, illumination, $NaNO_3$ concentration and nitrogen starvation were examined in small scale experiments (beaker vessels). Five optimal combinations of the aforementioned growth parameters were found from the statistical analysis of the data, maximizing each of the measured characteristics. Changes in aeration rate did not have a significant effect on biomass and total bioproducts production. Higher illuminance intensity, continuous lighting and higher $NaNO_3$ concentration resulted in a higher overall yield in biomass and bio-products. Flashing light effect triggered chlorophyll production. Three-day nitrogen starvation increased lipids production.
- EMS1 *Stichococcus* sp. strain was scaled up in flat-panel photobioreactor (15 L culture volume) with the microalgae immobilized on sandblasted glass tiles, producing high amounts of biomass and bio-products. Microalgae were also fed with synthetic flue gas. As a final step, the selected strain was tested with real-time flue gas in Lavrio power station, located in the region of Attica in Greece.
- The selected strain was grown effectively in real conditions and adapted with the extreme environment of industrial flue gas. Flue gas provided a thriving environment, as biomass and bio-products in conjunction with the appropriate cultivation conditions, resulted in high yield.
- In conclusion, the efficacy of dewatering and drying processes was evaluated. Biomass scraping of immobilized microalgae although it showed lower recovery rates compared to the recovery of suspended microalgae using conventional processes, it offered the lowest energy consumption. That is because microalgae are gathered on a specific area in the photobioreactors, which can be easily separated from the culture medium. Also, the supernatant could possibly be reused as preculture. Convective drying required the highest electrical energy demand, but it dried the biomass in shortest time. Bio-products are linked with the drying process. Appropriate methods should be selected according to the desired goals of a microalgae producer.

Microalgae cultivation is recent and still under development when compared to conventional agriculture. Future ideas for further research would be to examine other microalgae strains, which might be more effective than *Stichococcus* and/or examine additional parameters. Since *Stichococcus* sp. proved to grow in lab-scale photobioreactors with industrial flue gas feed, additional experiments could be conducted in real-scale photobioreactors to sequester larger amounts of CO_2 contained in flue gas and aid in the reduction of CO_2 emissions. Also, the produced bioactive compounds could be further processed to increase their value, for example raw lipids can be converted into biodiesel through transesterification, carbohydrates can be used in the food industry as animal feed and nutraceuticals, and also as biofuel (bioethanol, biobutanol, biomethane, and biohydrogen) (de Carvalho Silvello et al., 2022). Finally, microalgae have many applications in the wastewater treatment sector as they contain plenty of organic and inorganic nutrients which can used as a source of food (Srimongkol et al., 2022). Another interesting research would be to conduct a life cycle assessment for an industrial scale biorefinery which will include cultivation, dewatering, drying and bio-conversion of microalgae, based on the experimental results of *Stichococcus* sp.

A pilot plant cultivation concept of *Stichococcus* sp. was described in Makaroglou et al. (2021), treating winery wastewater and using seawater as the base culture medium to lower cultivation costs. The following paragraphs provide detailed information on the pilot plant concept.

For a pond with area of 1,000 m^2 (i.e.: 20 × 50 m) (Figure 68), the total volume of water inside the pond will be about 100 m^3, to achieve an average water height of 10 cm. For each batch, 165 L of winery wastewater along with about 100 m^3 of seawater (so to achieve 1:600 winery wastewater:seawater) and 1 m^3 of inoculum (which will contain 60 g (dry basis) of wild-type *Stichococcus* sp.). The inoculum may be prepared in a designated tank, by suspending 60 g (dry basis) of microalgae (produced during the previous batch cultivation) into 1 m^3 of seawater. Following cultivation (18 days), the treated effluent may be drained into the sea through a discharge valve, as BOD would have reached the discharge limit (e.g.: < 20 mg L^{-1}). Part of the treated mixture may be stored in a dedicated tank to be used as inoculum for the next batch.

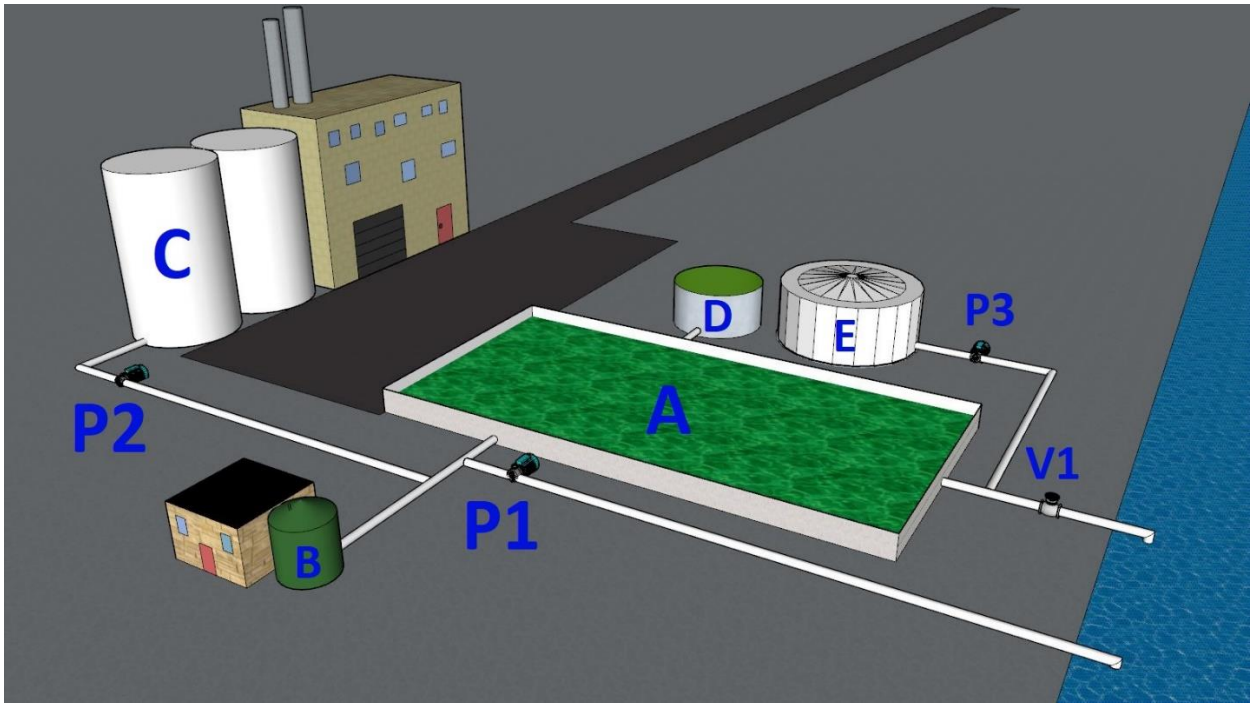

Figure 68. Layout of a pilot plant for cultivation of *Stichococcus* sp. in biofilms using seawater and industrial wastewater for a cost-effective biomass production. **A.** Pond for microalgae cultivation as an open-air batch photo-bioreactor with a bottom surface of sandblasted glass. **B.** Vertical bioreactor of *Stichococcus* sp. to provide the inoculum for the cultivation inside the pond. **C.** Industry from which the wastewater will be used. **D.** Tank for the collected biomass of *Stichococcus* sp. **E.** Tank to store the seawater and wastewater mix after the cultivation for reuse in next cultivation. **P1.** Pump driving seawater to the pond photo-bioreactor. **P2.** Pump driving wastewater from the industry to the pond. **P3.** Two-way pump for the collection of seawater and wastewater after each cultivation to tank E and for reuse in the next batch **V1.** Valve for the discharge of seawater and wastewater into the sea after the cultivation, under specific conditions and limits of deposition.

The produced immobilized biomass can be then collected easily, by surface scraping or vacuum pumping in a biomass collection tank for immediate transportation at a corresponding facility for downstream processing. The quantity of the produced biomass is estimated to range between 28 to 40 kg (dry basis) per batch for a pond with area of 1,000 m^{-2}. Nitrogen starvation may be achieved (if opted), by carefully pumping the treated growth medium into the sea, two days before the completion of the process, with parallel introduction of equal amount of seawater into the pond. The cultivation can be repeated every 18 days with on need for major maintenance. The remaining biofilm on the sandblasted glass, after biomass collection, may also serve as an

additional inoculum for the next batches. During periods of intense sunshine, freshwater may be added in the pond, to account for water evaporation and to diminish potential problems of increased salinity. During rainy periods, on the other hand, the salinity and the volume of the growth medium may be controlled by pumping calculated amount of growth medium from the pond into a tank, with parallel introduction of similar amount of sea water into the pond. The effect of temperature changes to the yield, may be counterbalanced by adjusting accordingly the duration of the cultivation periods per batch. Such environmental changes are inevitable in open-type cultivation systems, like the proposed one. However, they are expected to have minimal impacts on wastewater treatment capabilities and biomass production from *Stichococcus* sp. biofilms, because *Stichococcus* sp. is highly tolerant to various types of environmental fluctuations.

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