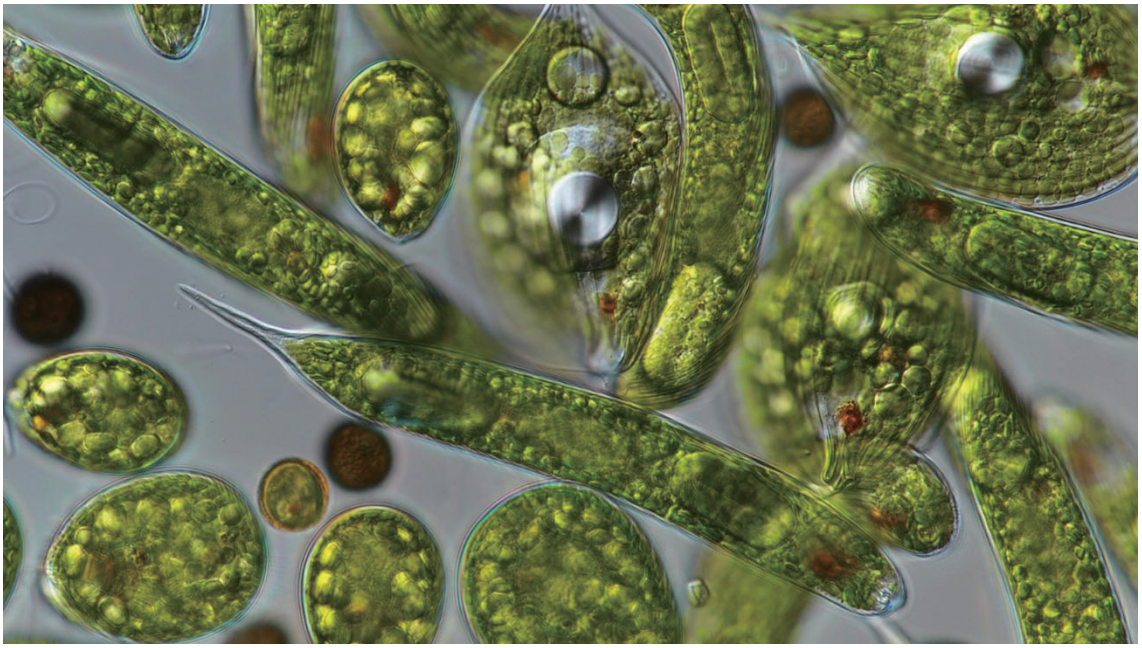
4th International Congress on Euglenoids (EIN2025) / 40th Annual Meeting of Japan *Euglena* Research Association

Hybrid format (online and in-person)



Book of Abstracts

30 June – 1 July, 2025

Osaka, Japan

(I-site Namba, Osaka Metropolitan University)

Zoom meeting information

https://omu-ac-jp.zoom.us/j/92209222246?pwd=bo1obsGhpBsk3oRb0HwI08rI2rfyvX.1

Meeting ID: 922 0922 2246
Passcode: 016621

Organizers

Masami Nakazawa
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Day 1

10:00–10:05 Welcome talk Masami Nakazawa

Session 1 **Chair: Yuichiro Kashiyama**

10:05–10:50 Keynote 1: Pierre Cardol
Evolutionary Remodeling of Respiration and Photosynthesis in *Euglena gracilis*

10:50–11:10 Oral 1: Rikuto Oishi
Understanding anaerobic metabolism and developing a co-production system of wax ester and succinate in *Euglena gracilis*

11:10–11:30 Oral 2: Oskar Fields
Enhancement of Omega-3 Production in the Microalga *Euglena gracilis*

11:30–11:50 Oral 3: Midori Nakamura
Photosynthetic Adaptation through Light- and CO_2-Dependent Remodeling of Antenna Proteins in Marine Diatoms

11:50–12:10 Oral 4: Takahiro Ishikawa
Identification of enzymes involved in hypoxic paramylon degradation in *Euglena gracilis*

12:10–13:30 Lunch Break

Session 2 **Chair: Takahiro Ishikawa**

13:30–14:15 Keynote 2: Jiangxin Wang
Euglena Research and Industrialization in China: From Genomic Insights to Biotechnological Challenges

14:15–14:35 Oral 5: Chengfu Sun
Deep sequencing analysis of chloroplast transcription and splicing in *Euglena gracilis*

14:35–14:55 Oral 6: Michael Henry Cagney
Developing *Euglena gracilis* as a Biotechnological Chassis for Metabolic Engineering and Recombinant Protein Expression

14:55–15:15 Oral 7: Honoka Suzuki
The mechanism of 2-oxoglutarate production by isocitrate dehydrogenase and aconitase in *Synechocystis* sp. PCC 6803

15:15–15:35 Oral 8: Sahutchai Inwongwan
Metabolic optimisation of *Euglena gracilis*

15:35–15:50 Break

15:50–16:50 Poster Session (Flexible start, ends at 16:50)

16:50-17:00 Closing

Moving to Networking dinner (within 10 min by walk)

18:00-20:00 Networking dinner

@ ALLY's PASTA SHOP NAMBA (on 1st floor of Hotel Keihan Namba Grande)

Day 2

Session 3 **Chair: Masami Nakazawa**

10:00–10:45 Keynote 3: Vladimír Hampl
Evolution of the protein import into euglenid chloroplasts

10:45–11:05 Oral 9: Anežka Konupková,
Breaking the barrier: *Euglena gracilis* expansion microscopy enables visualisation of organelles and detailed cytoskeletal structures

11:05–11:25 Oral 10: Vojtěch Žárský
A novel role of nucleotidyltransferases in the maturation of *Euglena gracilis* plastidial introns

11:25–11:45 Oral 11: Yuichiro Kashiyama
Considering the evolution of euglenid phototrophy based on N-terminal low complexity domain of kleptoplast-targeted proteins in *Rapaza viridis*

11:45–13:15 Lunch Break

Young Researchers' Lunch Social (@ S5 room)

Session 4 **Chair: Ellis O'Neill**

13:15–14:00 Keynote 4: Taeho Lee
Valorization of food-industrial byproducts for *Euglena gracilis*-based biorefinery: paramylon and wax ester production

14:00–14:20 Oral 12: Sutthiphat Sriwari
Mitochondrial Responses of *Euglena gracilis* to Varying Trophic Conditions

14:20–14:40 Oral 13: Haruka Higuchi
Improved fumarate titer in cyanobacteria via metabolic pathway engineering

14:40–15:00 Oral 14: Yuanguang Li
Industrialization of Heterotrophic Cultivation of *Euglena* and Production of Paramylon as well as Functional Applications

15:00–15:20 Oral 15: Keunho Kim
pH Regulation via Nitrogen Source Consumption in Fed-Batch Cultivation of *Euglena gracilis*: Toward Automated Bioprocessing

15:20–15:35 Break

15:35–16:35 Poster Session (Flexible start, ends at 16:35)

16:35–16:40 Talk Contest, Poster Contest

16:40–16:45 Introduction for 5th International Congress on Euglenoids & Closing

16:45 Wrap-up

Departure

Keynote talk and Oral presentations

Evolutionary Remodeling of Respiration and Photosynthesis in *Euglena gracilis*

Pierre Cardol

Genetics and physiology of microalgae, UR InBioS, University of Liège, 4000 Liège, Belgium

Euglena gracilis is a unicellular photosynthetic eukaryote from the Euglenozoa clade, related to trypanosomes but distinguished by its secondary green plastid acquired through endosymbiosis. This complex evolutionary origin has driven distinctive adaptations in both its respiratory and photosynthetic systems, leading to unique bioenergetic strategies.

Mitochondrial oxidative phosphorylation in *E. gracilis* relies on canonical complexes I–IV and ATP synthase (complex V), but with significant modifications. Proteomic and structural analyses have identified over 40 Euglenozoa-specific subunits in addition to ~50 conserved eukaryotic components. While the overall architecture of these complexes resembles classical oxidases, electron microscopy revealed lineage-specific features: an extended peripheral arm in complex I, a helmet-like domain in complex IV, and unique extended membrane-spanning region and peripheral stalk structures in Complex V.

Photosynthesis in *E. gracilis* is similarly divergent. Both PSI and PSII lack several canonical core subunits and instead incorporate a novel LHCE antenna family that binds red-shifted chlorophyll a. Cryo-EM analysis revealed a PSI–LHC supercomplex with a reduced core surrounded by 14 LHCE and 2 LHCBM proteins. One PSI subunit, PSAD, was acquired via lateral gene transfer from cyanobacteria and plays a structural role in antenna integration. Functional knockouts of genes such as CAO (involved in chlorophyll b biosynthesis) and CP29 (antenna stabilization) impair light harvesting and photoprotection under high-light conditions.

E. gracilis also exhibits tight coupling between respiration and photosynthesis. This interaction is modulated by trophic conditions: in mixotrophy, it is governed by chloroplast redox balance, while in photoautotrophy it is influenced by CO_2 limitation and photorespiration. Mitochondria–chloroplast contacts suggest strong metabolic integration. Under dark anoxic conditions, *E. gracilis* sustains photosynthesis via cyclic electron flow, likely relying on fermentation pathways such as wax ester fermentation in the absence of hydrogenase activity.

Understanding anaerobic metabolism and developing a co-production system of wax ester and succinate in *Euglena gracilis*

Rikuto Oishi, Ryunosuke Katayama, Mitsuhiro Ueda, Tatsuji Sakamoto, Masami Nakazawa

Graduate school of Agriculture, Osaka Metropolitan University, Japan

Biological carbon recycling systems offer a sustainable route to fuel and chemical production. We focused on *Euglena gracilis*, a microalga that accumulates wax esters (WEs) under anaerobic conditions. In our previous study, we showed that genome editing of WE metabolism genes enables modification of WE composition, supporting the potential for tailored production of biodiesel, biojet fuel, and chemical feedstocks. This study aimed to increase wax ester yield by analyzing carbon flow and improving production efficiency. In particular, we developed a system that enables the co-production of succinic acid, a valuable byproduct with industrial applications. By enhancing both the main product and a usable byproduct, we present a promising strategy for practical *Euglena*-based biomanufacturing.

We first investigated the roles of cytosolic malate synthesis and mitochondrial pyruvate transport in WE production. Knockout (KO) of the cytosolic malate dehydrogenase (cMDH) gene resulted in only a 10% decrease in WE content compared to the wild type (WT). However, when the mitochondrial pyruvate carrier (MPC) was inhibited by UK5099 in the cMDH-KO background, WE production dropped to 20% of the WT level under UK5099 treatment. These results indicate that cytosolic malate synthesis and mitochondrial pyruvate transport play complementary roles in providing carbon for WE production, depending on the availability of malate. In addition, succinate levels were decreased in the cMDH-KO strain, suggesting that the reductive TCA cycle contributes to succinate production under anaerobic conditions.

Interestingly, the WE content in MPC-KO strains was higher than that in WT. This was likely due to increased accumulation of storage polysaccharides, which may have resulted from suppressed cell growth when ethanol was used as the carbon source. These results suggest that blocking pyruvate transport alone does not significantly impair WE production.

Next, we aimed to enhance succinic acid accumulation without decreasing WE levels. Since odd-chain fatty acids in WEs are made from propionyl-CoA, which is derived from succinate, we knocked out the β-subunit isozyme 1 of succinyl-CoA synthetase (SCSβ1). This resulted in a decrease in odd-chain WEs, but even-chain WEs increased about twofold, so the total amount of WEs remained unchanged. The accumulation of succinic acid increased to 380 mg/L, about three times higher than in WT. To further enhance succinate accumulation, we used RNA interference to suppress malic enzyme (ME) expression in the SCSβ1-KO background. This raised succinate levels up to 1000 mg/L without affecting WE production.

In conclusion, this study provides new insights into the metabolic regulation of wax ester biosynthesis in *Euglena gracilis*. Furthermore, we demonstrate that succinic acid accumulation can be enhanced without reducing wax ester levels. This strategy enables efficient co-production of lipids and organic acids.

Enhancement of Omega-3 Production in the Microalga *Euglena gracilis*

Oskar Fields[1], Dr Donal McGee[2] & Dr Ellis O'Neill[1]
1. School of Chemistry, Biodiscovery Institute, University of Nottingham, NG7 2RD, 2. Algaecytes, Discovery Park, Kent, CT13 9ND

Omega-3 fatty acids are essential micronutrients with proven health benefits such as reduced cancer risk, neuroprotective properties and improved cardiovascular health.[1] Their production from renewable biological sources, such as microalgae, has gained attention as a sustainable alternative to traditional methods, such as fish or krill derived oils, that are also suitable for vegans and vegetarians. *Euglena gracilis* is a promising candidate for industrial scale omega-3 production due to its rapid growth and ability to produce eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA).[2] However, optimising the omega-3 yield from this organism remains a challenge, with the complex genome being difficult to manipulate and with market forces preferring non-GMO methodologies.[3]

This research focuses on three key strategies to increase omega-3 production in *E. gracilis*: 1) **optimising growth conditions**, 2) **random chemical mutagenesis**, and 3) **targeted gene editing**. First, growth parameters such as light intensity, media, pH and nutrient availability were optimized to enhance biomass and lipid production, resulting in increased omega-3 yields and providing the foundation for further experiments. Second, random mutagenesis was performed using two chemical mutagens, EMS (Ethyl Methanesulfonate) and colchicine, in order to generate diverse pools of mutants. Mutants with high potentials for omega-3 production were selected using FACS (Fluorescence Activated Cell Sorting). Finally, targeted gene editing using CRISPR/Cas9 was used to knockout genes involved in fatty acid and PUFA (Polyunsaturated Fatty Acid) metabolic pathways, aiming to enhance the accumulation of omega-3 fatty acids. This multi-faceted approach holds great potential for identifying the best methods for improving the omega-3 production in microalga and provides the basis for the commercial development of enhanced algal strains.

Key Words: Euglena, Microalga, PUFAs, Omega-3s, Algal Biotechnology, Chemical Mutagenesis, Gene Editing

References:

1 F. Shahidi and P. Ambigaipalan, DOI:10.1146/annurev-food-111317.
2 Y. Wang, et al, *PLoS One*, 2018, 13, e0195329.
3 O. Fields, et al, *Trends in Genetics*, 2025, 41, 251–260.

Photosynthetic Adaptation through Light- and CO_2- Dependent Remodeling of Antenna Proteins in Marine Diatoms

Midori Nakamura[1], Minoru Kumazawa[12], Ryo Nagao[3], Shoko Tsuji[1], Takehiro Suzuki[4], Noriko Ishikawa[1], Naoshi Dohmae[4], Seji Akimoto[5], Kentaro Ifuku[1]

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Diatoms, a major group of photosynthetic microalgae, are estimated to contribute approximately 20% of global carbon fixation and serve as key primary producers in marine ecosystems. Their ecological success is largely attributed to their ability to adapt to the highly dynamic light environments found in aquatic habitats. Diatoms utilize light-harvesting complexes (LHCs) to capture light energy for photosynthesis and to dissipate excess light energy as heat.

In the marine diatom *Chaetoceros gracilis*, we previously identified 46 LHC genes (Kumazawa et al., *Physiol. Plant.* 174: e13598, 2022). Among them, we found a unique LHC that is expressed under both red-light illumination and high CO_2 conditions (Ueno et al., J. *Phys. Chem. Lett.* 10: 5148–52, 2019; Nagao et al., *Photosynth. Res.* 146: 189–95, 2020). In this study, we performed detailed expression analysis of this LHC gene and conducted physiological characterization using knockout and overexpression strains. The results suggest that this LHC contributes to optimizing photosynthesis under variable environmental conditions.

Our findings provide insight into the flexible remodeling of light-harvesting systems in diatoms and shed light on the molecular strategies underlying their environmental adaptability.

Identification of enzymes involved in hypoxic paramylon degradation in *Euglena gracilis*

Yuji Tanaka[1], Kyo Goto[1], Shigeru Shigeoka[2], Takahiro Ishikawa[1]

[1]Grad. Sch. Nat. Sci. Technol., Shimane Univ., Japan, [2]Exp. Farm, Kindai Univ., Japan

Paramylon, a linear β-1,3-glucan, is a distinctive storage polysaccharide in *Euglena gracilis*. Under hypoxic conditions, *E. gracilis* degrades paramylon into glucose, which is subsequently metabolized through wax ester fermentation to generate energy and synthesize wax esters, mainly myristyl myristate (C28), as the final product. We previously identified Glucan Synthase-Like 2 (GSL2) as a key enzyme involved in paramylon biosynthesis [1]. In this study, we aimed to identify the enzymes responsible for paramylon degradation during the hypoxic response.

Initially, three major β-1,3-glucanases (Cel17A, Cel81A, and Cel81B) were purified from *E. gracilis* using column chromatography with laminarin as the substrate [2]. However, gene silencing analyses revealed that these enzymes are not directly involved in paramylon degradation under hypoxic conditions. To identify the enzymes responsible for this process, we conducted a proteomic analysis of paramylon-associated proteins before and after hypoxia treatment. Gene ontology analysis identified several sugar metabolism-related proteins, including β-1,3-glucanases and laminaridextrin phosphorylases [3]. Among them, we focused on three phylogenetically related glucanases (Eng81A, Eng81B, and Eng81C) and three phosphorylases (LDP1, LDP2, and LDP3). Simultaneous knockdown of *Eng81A* and *Eng81B* gene significantly suppressed paramylon degradation under hypoxia. Notably, concurrent knockdown of *LDP1* and *LDP3* resulted in more pronounced defects in both paramylon synthesis and degradation, suggesting that LDP1 and LDP3 cooperatively regulate paramylon metabolism.

[1] Tanaka, Y. et al., (2017). Glucan Synthase-Like 2 is indispensable for paramylon synthesis in *Euglena gracilis*. *FEBS Lett*., 591: 1360-1370.

[2] Takeda, T. et al. (2015) Identification and enzymatic characterization of an endo-1,3-beta-glucanase from *Euglena gracilis*. *Phytochemistry*, 116: 21-2.

[3] Tanaka, Y. et al., (2022) Identification of glucanases and phosphorylases involved in hypoxic paramylon degradation in *Euglena gracilis*. *Algal Res*., 67: 102829.

Euglena Research and Industrialization in China: From Genomic Insights to Biotechnological Challenges

Chao Li, Zixi Chen, Ming Du, Sheng Huang, Jiangxin Wang

Lab of Euglena Synthetic Biology, School of Life Sciences and Oceanogrpahy, Shenzhen University, Shenzhen, P. R. China

Euglena, a unique unicellular organism exhibiting both photosynthetic and heterotrophic traits, has gained significant attention in China as a promising chassis for synthetic biology and biotechnological innovation. In this keynote presentation, I will provide an in-depth overview of recent *Euglena* research and commercialization efforts in China. The first part of the talk will summarize recent advances in *Euglena gracilis* research by Chinese scientists. A chromosome-level genome assembly of a paramylon-producing strain was completed in 2024, providing crucial insights into metabolic pathways and gene regulation mechanisms. Gene expression and transcriptomic analyses have revealed how light, carbon sources, and environmental stresses regulate photosynthesis, lipid accumulation, and paramylon biosynthesis. Efforts to improve CRISPR-Cas9 delivery in *Euglena* using microinjection and RNPs have also been initiated. In parallel, metabolomic and proteomic studies are clarifying stress response networks and functional compound production under autotrophic and mixotrophic conditions. Collectively, these efforts are building a foundational toolbox for bioengineering and industrial application of *Euglena* in China. The second part will focus on the industrial landscape. Although *Euglena*-based products, such as dietary supplements, skin care ingredients, and live-cell aquaculture additives, have emerged in China, large-scale commercialization remains limited. I will present practical case studies from Shenzhen and Yunnan, analyze production bottlenecks such as strain instability, fermentation optimization, and cost-performance imbalance, and compare China's progress with Japan's well-established *Euglena* industry. By sharing both scientific breakthroughs and real-world challenges, this presentation aims to foster deeper collaboration within the *Euglena* International Network and identify shared opportunities for advancing *Euglena* biotechnology globally.

Deep sequencing analysis of chloroplast transcription and splicing in *Euglena gracilis*

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The transcription and splicing of the *Euglena gracilis* (*E. gracilis*) chloroplast have only been studied on individual genes and a genome-scale comprehensive analysis is lacking. Utilizing the transcriptomic RNA sequencing (RNA-seq) technology, we addressed this question in this study. Upon mapping tens of millions of RNA-seq reads onto the *E. gracilis* chloroplast genome, the transcription of protein-coding genes, including monocistronic genes and genes in operons, was visualized and expression levels of these genes were quantitated. The splicing of intron-containing genes on the *E. gracilis* chloroplast genome was also visualized and quantitated, with intron, including some twintrons, and exon being clearly discerned. The correct coding sequences of psbD, rpl23 and rpl16 were deduced based on reads coverage on respective genes and confirmed experimentally. Finally, Dimethyloxallyl glycine (DMOG), an α-ketoglutarate analog and competitive inhibitor of 2-oxoglutarate-dependent dioxygenases, was identified as a selective suppressor of chloroplast intron splicing in this protist. Further mechanistic studies are currently underway to elucidate the precise molecular basis of its inhibitory effects on splicing. This study updates the knowledge on the transcription and splicing of the *E. gracilis* chloroplast, and will be instrumental to mechanistic exploration of gene transcription and intron splicing in the *E. gracilis* chloroplast.

Developing *Euglena gracilis* as a Biotechnological Chassis for Metabolic Engineering and Recombinant Protein Expression

Michael Henry Cagney[1], Ellis O'Neill[1]

1. Biodiscovery Institute 1, School of Chemistry, University of Nottingham, Nottingham, United Kingdom

Euglena gracilis has great potential as a biotechnological chassis for producing industrially valuable compounds and recombinant proteins requiring post-translational modifications, such as *N*-linked glycosylations. Despite its advantages, the development of *E. gracilis* as a heterologous expression system has been hindered by the limited availability of characterised genetic regulatory elements for controlling transgene expression.

To address this, promoter and terminator sequences were identified from native *E. gracilis* genes encoding highly expressed proteins and were cloned into expression cassettes employing the fluorescent protein mTagBFP2 as a reporter system. The relative effectiveness of these promoter and terminators was assessed via flow cytometry. The results demonstrate strong and reliable promoter-terminator pairs capable of driving high levels of transgene expression, consistent with previous reports in *E. gracilis* and other microalgal species.

This study expands the molecular toolkit available for *E. gracilis*, providing essential regulatory elements for precise genetic control. It lays the groundwork for future metabolic engineering strategies aimed at the biosynthesis of high-value compounds and the production of complex recombinant proteins. By leveraging its unique cellular machinery, *E. gracilis* has the potential to emerge as a robust alternative to existing eukaryotic expression platforms such as *Leishmania tarentolae* (LEXSY), offering advantages such as reduced growth inputs. Further optimisation of genetic tools will enhance its applications in synthetic biology and biopharmaceutical production.

The mechanism of 2-oxoglutarate production by isocitrate dehydrogenase and aconitase in *Synechocystis* sp. PCC 6803

Honoka Suzuki[1], Masahiro Karikomi[1], Takashi Osanai[1,2]
1. Graduate School of Meiji University, Japan, 2. School of Meiji University, Japan

[Purpose]

Cyanobacteria are photoautotrophic prokaryotes that perform carbon fixation and nitrogen assimilation using ATP and reducing power generated through photosynthesis. Nitrogen assimilation requires a carbon skeleton such as 2-oxoglutarate (2OG), derived from carbon fixation. Thus, 2OG is a key metabolite connecting carbon and nitrogen metabolism.

2OG is synthesized from citrate by two enzymatic reactions, catalyzed by aconitase (AcnB) and isocitrate dehydrogenase (IDH). In the model cyanobacterium *Synechocystis* sp. PCC 6803, AcnB catalyzes the reversible isomerization of citrate to isocitrate, and exhibits higher activity in the reverse direction, from isocitrate to citrate. Hence, to produce 2OG as a substrate for nitrogen assimilation, IDH has to convert isocitrate to 2OG more rapidly than that AcnB converts isocitrate to citrate.

In this study, we aimed to elucidate the mechanism of 2OG production by comparing the enzymatic activity of IDH and AcnB with purified proteins and crude cell extracts.

[Results and Discussion]

IDH was expressed as a GST-fusion protein and purified by affinity chromatography. The kinetic parameters, "turnover number (k_{cat}), substrate affinity ($1/K_m$), and catalytic efficiency (k_{cat}/K_m)" of IDH for isocitrate, were superior to those of AcnB. IDH activity was inhibited by ATP, citrate, 2OG, and NADPH. The inhibitory effects of ATP and citrate on IDH activity were alleviated by increasing Mg^{2+} concentration, suggesting that IDH activity is regulated by modulating Mg^{2+} concentration. Furthermore, IDH activity in crude cell extracts was higher than that of AcnB.

These results show that in *Synechocystis* sp. PCC 6803, IDH can stably supply 2OG, which is subsequently utilized by nitrogen assimilation for biosynthesis of various biomolecules.

Metabolic optimisation of *Euglena gracilis*

Sahutchai Inwongwan[1]*, Sutthiphat Sriwari[1], Jeeraporn Pekkoh[1], and Chayakorn Pumas[1]

1. Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

This study aims to elucidate the metabolic networks of *Euglena gracilis*, emphasizing its notable metabolic flexibility and multifunctional capacity in comparison to other microorganisms. A systems biology framework was employed, integrating genome-scale metabolic modelling, ^{13}C metabolic flux analysis, and multi-omics approaches, including transcriptomics, metabolomics, and phenomics, to characterize the organism's metabolic responses under varying trophic conditions. The current focus involves a comparative analysis of *E. gracilis* cultivated under photoautotrophic, mixotrophic, and heterotrophic regimes, with particular attention to ethanol as a metabolically advantageous carbon source. The investigation assesses how distinct trophic strategies impact growth performance and CO_2 sequestration efficiency. Under ethanol-driven mixotrophy, *E. gracilis* demonstrated up to a 600% enhancement in CO_2 capture relative to photoautotrophic conditions, under identical environmental parameters. Integrated metabolic and transcriptomic analyses indicated that mixotrophic conditions induce a metabolic reprogramming, effectively combining elements of both autotrophic and heterotrophic metabolism, each associated with unique energy-harnessing strategies and metabolite production profiles. Furthermore, each trophic mode was found to generate distinct sets of bioactive metabolites, underscoring their potential for diverse biotechnological applications. These observations are consistent with prior findings that highlight the highly versatile metabolic flux capacity of *Euglena* and the efficacy of ethanol as a superior energy source for enhancing bioproduct synthesis.

Evolution of the protein import into euglenid chloroplasts

Anežka Konupková[1], Pricila Peña Diaz, Lucia Tomečková, Zoltán Füssy[2], Vladimír Hampl[1]

1. Department of Parasitology, Faculty of Science, BIOCEV, Charles University, Vestec, Czech Republic

2. Scripps Institution of Oceanography, University of California San Diego, La Jolla, California, USA.

Euglenophyceae have chloroplasts that originated through secondary endosymbiosis with a green alga from the Pyramimonadales lineage. Their envelope is composed of three membranes; the galactolipid composition indicates that two membranes evolved from green algal chloroplasts, while the third has an eukaryotic origin. The chloroplast proteome contains over 1,300 proteins, most of which are imported through an incompletely understood mechanism. They exhibit bipartite N-terminal pre-sequences, consisting of a signal peptide (SP) followed by a plant-like transport peptide, often ending with a hydrophobic stop-transfer sequence. Experimental data show that these proteins cross the outermost membrane in vesicles detaching from the endoplasmic reticulum (ER) and passing through the Golgi. Transcriptomic and proteomic studies suggest that the innermost membrane contains a highly modified "translocon at the inner chloroplast membrane" (TIC) complex derived from the endosymbiont, although only three paralogues of Tic21 subunits have been confidently identified. I will present evidence showing that chloroplast import relies significantly on membrane rhomboid pseudoproteases from the Derlin family identified in the chloroplast proteome. Their specific localization within the chloroplast remains unknown, and we hypothesize that they are components of an unidentified translocon across the middle membrane. A similar role for this family of proteins has been observed in complex plastids derived from red algae, suggesting remarkable molecular convergence. *Rapaza viridis* is the closest sister lineage to Euglenophyceae and possesses triple membrane-bound kleptoplasts harvested from its prey, *Tetraselmis* sp. Some proteins in *R. viridis* display N-terminal features similar to those of plastid proteins in *E. gracilis*, indicating their specific import. Furthermore, the transcriptome of *R. viridis* encodes putative components of translocons, some of which are also found in *E. gracilis* (Tic21), while others are uniquely present (such as Tic20 and Tic110) or absent (Derlins) in *R. viridis*. *R. viridis* thus offers an intriguing perspective on the evolution of chloroplast protein import in euglenids, which I will discuss together with future perspectives of this research.

Breaking the barrier: *Euglena gracilis* expansion microscopy enables visualisation of organelles and detailed cytoskeletal structures

Anežka Konupková, Priscila Peña-Diaz, Vladimír Hampl

Faculty of Science, Department of Parasitology, BIOCEV, Charles University, Vestec, Czech Republic

This study explores the use of expansion microscopy, a technique that enhances resolution in fluorescence microscopy, on the autotrophic protist *Euglena gracilis*. A modified protocol was developed to preserve the cell structures during fixation. Using antibodies against key cytoskeletal and organelle markers, α-tubulin, β-ATPase, C-subunit of Photosystem I, and Rubisco activase, the microtubular structures, mitochondria, and chloroplasts were visualised. The organisation of the cytoskeleton corresponded to the findings from electron microscopy while allowing for the visualisation of the flagellar pocket in its entirety and revealing previously unnoticed details. This study offered insights into the shape and development of mitochondria and chloroplasts under varying conditions, such as culture ages and light cycles. This work demonstrated that expansion microscopy is a robust tool for visualising cellular structures in *E. gracilis*, an organism whose internal structures cannot be stained using standard immunofluorescence because of its complex pellicle. This technique also serves as a complement to electron microscopy, facilitating tomographic reconstructions in a routine fashion.

A novel role of nucleotidyltransferases in the maturation of *Euglena gracilis* plastidial introns

Vojtěch Žárský[1], Jayaram Moorthy[2], Jiří Pergner[1], Anna Vlčková[2], Karolína Kmeťová[2], Magdaléna Franková[1], Ragul Ravikumar[1], Marek Eliáš[1], Štěpánka Vaňáčová[2]

1. Department of Biology and Ecology, Faculty of Science, University of Ostrava, Czech Republic, 2. Central European Institute of Technology, Masaryk University, Czech Republic

We used reverse genetics of the photosynthetic alga Euglena gracilis to generate strains defective in two genes predicted to be plastid-targeted homologues of the terminal nucleotidyltransferase (pTNT). The apparent phenotype for both knock-out strains was the loss of photosynthetic pigments. To gain further insights into the molecular mechanisms involved, we determined and compared the transcriptomes and proteomes of the wild-type and knock-out strains. In the proteomics data of both mutant strains, we observed a strong down-regulation of plastidial proteins, especially of the plastid-encoded proteins of the photosynthetic apparatus. Interestingly, we discovered that two very specific and non-overlapping sets of plastidial group II introns were disrupted in the two knock-out strains. I will further discuss our working model of the E. gracilis pTNTs and the implications on the evolution and loss of photosynthesis in euglenophytes.

Considering the evolution of euglenid phototrophy based on N-terminal low complexity domain of kleptoplast-targeted proteins in *Rapaza viridis*

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The euglenozoan flagellate *Rapaza viridis* exhibits kleptoplasty, acquiring chloroplasts from the green alga *Tetraselmis* sp. through phagocytosis while discarding the algal nucleus. The retained chloroplasts remain photosynthetically active for approximately two weeks. Transcriptome analyses have revealed numerous highly expressed chloroplast-related genes in *R. viridis*, likely acquired via horizontal gene transfer, which may contribute to the maintenance of kleptoplast function [1]. In this study, we identified extended amino-terminal low complexity domains (NtLCDs) in the translated products of putative kleptoplast-targeted genes, selected based on high expression levels in quantitative transcriptome data. These NtLCDs (194 ± 37 amino acids; n = 35) are structurally similar to chloroplast-targeting presequences in *Euglena gracilis*, typically containing one or two transmembrane helices (TMHs) [2]. The most prevalent class features two TMHs flanking hydrophilic regions enriched first in basic residues and then in acidic residues. Functional analysis using a NanoLuc luciferase reporter demonstrated that the NtLCD of an *Rv*RbcS-like protein is sufficient to mediate translocation of a peptide into kleptoplasts. The structural similarity between *R. viridis* and *E. gracilis* targeting signals suggests a shared or conserved translocation mechanism. However, NtLCDs in *R. viridis* also contain features absent from green algal targeting sequences, including an acidic residue-rich segment before the second TMH, the second TMH itself, and a downstream low-complexity extension. These elements are inconsistent with a model relying partially on *Tetraselmis*-derived TOC/TIC complexes, as they would likely need to be removed for proper import into the chloroplast stroma, making that scenario less parsimonious. These findings support the hypothesis that the last common ancestor of *Rapaza* and Euglenophyceae had already evolved a protein translocation system, which was later adapted for the maintenance of both kleptoplasts and permanent plastids. This insight provides a new perspective on early molecular adaptations underlying the evolution of euglenid phototrophy.

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Valorization of food-industrial byproducts for *Euglena gracilis*-based biorefinery: paramylon and wax ester production

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The development of sustainable microalgal biorefineries is gaining attention for the production of biofuels and value-added biochemicals. *Euglena gracilis*, a metabolically flexible microalga capable of autotrophic, heterotrophic, and mixotrophic growth, stands out due to its ability to synthesize paramylon (β-1,3-glucan used in functional foods, cosmetics, and bioplastics) and wax esters (a key feedstock for sustainable aviation fuel, SAF). Despite its potential, large-scale cultivation is hindered by the high cost of culture media. To address these challenges, we have investigated heterotrophic fed-batch cultivation of *E. gracilis* using food-industry byproducts such as apple pomace and beet pulp. These residues provide fermentable sugars and nitrogen, replacing conventional substrates and reducing costs. In nutrient-rich, byproduct-based media, enhanced biomass production and higher yields of paramylon were achieved with UV-induced mutant strains. Anaerobic fed-batch strategies, with optimized CO_2 supply, apple pomace feeding, and pH control, resulted in an increase of over 20% in wax ester content. Notably, replacing glucose with apple pomace reduced cultivation costs by 30%, demonstrating the dual economic and environmental benefits of byproduct valorization. This approach aligns with circular bioeconomy principles by converting agro-industrial waste into valuable bioproducts. Future work should focus on integrated process development, including efficient substrate pretreatment, in situ product recovery, robust strain selection, and techno-economic evaluation. Leveraging food-industry residues for *Euglena* cultivation not only improves the sustainability of biofuel production but also supports carbon-neutral biomanufacturing through waste valorization.

Mitochondrial Responses of *Euglena gracilis* to Varying Trophic Conditions

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Abstract

Euglena gracilis is a metabolically versatile protist capable of surviving under diverse trophic modes, reflecting the adaptability of its mitochondrial system to different energy sources. This study investigates the mitochondrial physiology of *E. gracilis* cultured in Hutner's medium under three trophic conditions: photoautotrophic (light without external carbon), mixotrophic (light with a carbon source), and heterotrophic (dark with a carbon source). Key physiological parameters including growth, mitochondrial signals, fluorescence imaging, reactive oxygen species (ROS), and energy-related cofactors were analyzed. Cells grown under mixotrophic conditions reached stationary phase earliest and exhibited the strongest mitochondrial signals, followed by heterotrophic cultures; no mitochondrial signals were detected in photoautotrophic conditions. Fluorescence imaging confirmed this trend. Notably, photoautotrophic cultures produced the highest total ROS, hydrogen peroxide, and malondialdehyde levels, while hydroxyl radical levels peaked under mixotrophic conditions. Energy-cofactor analysis revealed that $NADP^+$ was undetectable across all conditions. Mixotrophic cultures showed the highest NADPH and NAD^+ levels, while NADH peaked in heterotrophic conditions. ATP, ADP, and AMP concentrations were also highest in heterotrophic cultures, moderate in mixotrophic, and lowest in photoautotrophic. These findings demonstrate that mitochondrial activity and oxidative stress responses in *E. gracilis* are highly influenced by trophic mode, providing valuable insights for optimizing biomass and bioactive compound production for food, energy, and pharmaceutical applications.

Improved fumarate titer in cyanobacteria via metabolic pathway engineering

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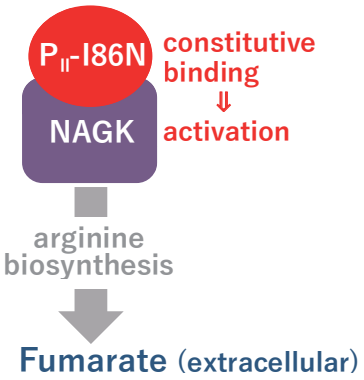

[Introduction] A cyanobacterium, *Synechocystis* sp. PCC 6803, is widely studied as a biorefinery host to produce useful chemicals such as fumarate directly from CO_2. Fumarate is an organic acid widely used as a raw material for bioplastics and as a sour flavoring agent.

Fumarate is a tricarboxylic acid (TCA) cycle metabolite. Previous studies have shown that, Δ*fumC* strain, lacking the fumarase (encoded by *fumC*, slr0018) in the TCA cycle, excretes fumarate from the cells under photoautotrophic conditions (Du et al., 2019). This process has not reached industrial levels, and we need to improve fumarate production in cyanobacteria.

In *Synechocystis* sp. PCC 6803, fumarate is generated as a byproduct of arginine biosynthesis (Du et al., 2019). N-acetylglutamate kinase (NAGK, encoded by *argB*, slr1898) catalyzes the bottleneck reaction of arginine biosynthesis. NAGK activity is regulated by a signal processor: P_{II} protein (encoded by *glnB*, ssl0707) (Heinrich et al., 2004). The P_{II}-I86N variant (encoded by *glnB-I86N*, a single amino acid substitution in *glnB*, Ile86 to Asp86) constitutively binds NAGK and enhances its activity (Fokina et al., 2010). We aim to improve fumarate production from arginine biosynthesis, by activating NAGK via the P_{II}-I86N variant.

[Results and Discussions] To investigate the effect of the P_{II} protein on fumarate production, we constructed two strains from the Δ*fumC* strain and measured fumarate levels of each strain in the media during photoautotrophic conditions. Compared fumarate titer to the Δ*fumC* strain, a strain overexpressing *glnB* showed no significant change. In contrast, a strain overexpressing *glnB-I86N* exhibited approximately a two-fold increase and reached the highest titer during photoautotrophic conditions reported to date. Our results indicate that the constitutive activation of NAGK by the P_{II}-I86N variant improved fumarate production from arginine biosynthesis. NAGK may act as a rate-limiting enzyme in the biosynthesis of fumarate under photoautotrophic conditions.

[Conclusion] Genetic engineering of the P_{II} protein to constitutively activate NAGK improved fumarate production and reached the highest titer to date. This study contributes to improving chemical productivity in cyanobacteria by genetic engineering of the P_{II} protein.

Industrialization of Heterotrophic Cultivation of *Euglena* and Production of Paramylon as well as Functional Applications

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Euglena gracilis is capable of growing under autotrophic, heterotrophic, and mixotrophic conditions. Compared with autotrophic cultivation, heterotrophic culture of *Euglena* offers a faster growth rate and higher cell density, making it particularly suitable for the large-scale production of high-value active compounds such as paramylon.

In this study, we successfully established a high-yield heterotrophic cultivation process for paramylon, scaling up from a 50 L fermenter to a 50 m^3 bioreactor. The maximum cell dry weight exceeded 100 $g \cdot L^{-1}$, and the paramylon content surpassed 75%. Furthermore, we developed a novel extraction process for paramylon that does not require organic solvents and successfully scaled it up to production levels. The produced paramylon powder had a paramylon content greater than 97%, with no detectable residues of pigments. We also established and scaled up the spray-drying processes for both *Euglena* biomass and paramylon. In production scale operations, the yield of *Euglena* powder exceeded 98%, while the paramylon yield exceeded 97% at the production scale. Ultimately, a complete production line was systematically integrated and optimized, achieving production capacities exceeding 300 tons per year for *Euglena* powder and 150 tons per year for paramylon.

This study also investigated the effects of different dosages of *Euglena* powder and paramylon on model mice. The results indicated that higher doses of *Euglena* powder and paramylon were more effective in protecting the kidneys from metabolic disease-induced damage.

Keywords: Euglena; Paramylon; Industrialization; Functional applications

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pH Regulation via Nitrogen Source Consumption in Fed-Batch Cultivation of *Euglena gracilis*: Toward Automated Bioprocessing

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Euglena gracilis, a microalga capable of utilizing organic wastes and wastewaters, synthesizes β-1,3-glucan under aerobic conditions and converts it into wax esters under anaerobic conditions, offering promise for functional materials and biofuel production. However, stable pH maintenance is critical for optimal growth and metabolite production, with typical cultivation pH ranging between 3.0 and 5.0. Conventional pH control using strong acids and bases presents challenges including safety concerns, cost, and equipment corrosion. This study explored a novel strategy for intrinsic pH regulation during fed-batch cultivation of E. gracilis by leveraging the opposing pH effects of two nitrogen sources: ammonium (NH_4^+) and glutamate.

Under heterotrophic conditions, NH_4^+ consumption led to acidification of the medium (pH drop to ~2), while glutamate utilization caused alkalization (pH rise to ~6.5), both negatively affecting growth beyond these limits. However, when the two nitrogen sources were appropriately utilized, pH control was achieved based on their distinct effects on the culture medium. Specifically, when the pH dropped below 3, the consumption of glutamate led to a pH increase, whereas when the pH rose above 5, the uptake of NH_4^+ caused acidification. This complementary dynamic enabled the culture pH to be maintained within the optimal range of 3 to 5 for heterotrophic cultivation of *Euglena gracilis*, without the need for external acid or base addition, even during repeated feeding cycles.

Building on these findings, a pH-responsive medium supply model was conceptualized, in which the timing and composition of feed solutions (carbon and nitrogen sources) can be automatically adjusted based on real-time pH monitoring. This model establishes the foundation for a closed-loop control system that enables cost-effective, scalable, and contamination-resistant *Euglena* bioprocesses, while minimizing labor requirements by automating pH-responsive medium supply.

Poster presentations

Core time Day 1

1P-01	Shun Tamaki
1P-02	Kanon Fukui
1P-03	Hiroaki Morita
1P-04	Jian Xing
1P-05	Takumi Shimotashiro
1P-06	Ryota Sakurada
1P-07	Kanako Iseki
1P-08	Xiao Xu
1P-09	Fengyang Sui & Yawen Fan
1P-10 (Oral 1)	Rikuto Oishi
1P-11 (Oral 7)	Honoka Suzuki

Core time Day 2

2P-12	Kengo Suzuki
2P-13	Masanobu Akimoto
2P-14	Takumi Ogawa
2P-15	Ryunosuke Katayama
2P-16	Jiwon Kang
2P-17	Kaisei Kurosaki
2P-18	Yuri Yanagihara
2P-19	Ryo Oda
2P-20	Bonhyeon Koo
2P-21	Hyeonbeen Seo
2P-22 (Oral 12)	Haruka Higuchi

Physiological and carotenoid responses of *Euglena gracilis* to low temperature stress

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Euglena gracilis is a unicellular microalga, making it a promising organism for various industrial applications. In particular, it holds significant potential for sustainable food and bioenergy production due to its ability to accumulate valuable nutrients and lipids under specific culture conditions. However, for large-scale outdoor cultivation of *E. gracilis* to be viable year-round, it is essential to understand how this alga responds to environmental stresses, especially low temperatures that often occur in temperate regions. To address this challenge, our study focuses on elucidating the biological and physiological responses of *E. gracilis* to low temperature stress, with the goal of identifying mechanisms that could be targeted to improve stress tolerance. In this study, we investigated the growth property and pigment composition, particularly carotenoids, of *E. gracilis* under autotrophic culture conditions at a low temperature (15°C), compared to a control temperature (26°C).

Our results showed that culturing *E. gracilis* at 15°C significantly impaired its growth, as evidenced by a substantial reduction in both cell concentration and dry biomass accumulation. Furthermore, cells grown at 15°C exhibited a visibly paler green color, which correlated with a 61% decrease in chlorophyll content, suggesting impaired photosynthetic capacity. Biochemical analyses suggested that oxidative stress may be a key limiting factor at low temperatures, as reflected in altered antioxidant enzyme activities. Notably, carotenoid profiling revealed a marked increase in diatoxanthin content, alongside decreases in other major carotenoids such as neoxanthin, violaxanthin, and β-carotene. These pigment shifts suggest an active role of diatoxanthin in mitigating low temperature-induced stress, potentially by contributing to reactive oxygen species (ROS) scavenging. Therefore, diatoxanthin may represent a useful molecular target for developing *E. gracilis* strains with enhanced tolerance to low temperature environments, which could improve biomass yields in outdoor cultivation systems.

Establishment of a transformation system that enables biomanufacturing in *Euglena gracilis*

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Tailored metabolic engineering tools will play a central role in enabling the broader use of *Euglena gracilis* for CO_2-based biorefinery applications. In our previous study (Nakazawa et al. 2023), we developed stable nuclear transformation methods for *E. gracilis* by electroporation of double-stranded DNA (dsDNA). The system is robust and traceable owing to the use of a small, bright NanoLuc luciferase (Nluc) fused with a neomycin resistance (Neo^r) protein. Nevertheless, challenges remain, including insufficient transgene expression to support metabolic engineering, truncation of inserted dsDNA ends, and inability to specify the genomic integration site. In this study, we aimed to improve nuclear genome transformation methods to overcome these limitations.

First, we investigated whether phosphorothioate (PS) modification at the ends of dsDNA could prevent terminal truncation during random integration. PS-modified dsDNA was introduced into *E. gracilis* by electroporation after amplification by PCR. Nano-Glo luciferase assays using mixed liquid cultures grown under G418 selection showed that cells transformed with PS-modified dsDNA exhibited approximately 10,000-fold higher luciferase activity compared to those transformed with unmodified dsDNA. Genomic DNA was extracted from individual clones isolated from the mixed culture, and insertion sites were identified by inverse PCR. The results indicated that the inserted DNA was largely intact, with minimal truncation at the ends.

Next, we performed site-specific insertion of long dsDNA near the stop codon of a highly expressed gene in *E. gracilis*, selected based on our expression analysis. A crRNA targeting the site was introduced with tracrRNA and Cas9 as an RNP complex, together with a PS-modified dsDNA containing an Nluc/Neo^r cassette flanked by 20 bp homology arms. Following G418 selection and clonal isolation, we obtained transformants with full-length insertion at the target site. These clones exhibited ~100-fold higher luciferase activity than those with random integration. To our knowledge, this is the first successful targeted insertion of long (>1 kb) dsDNA into the *E. gracilis* genome.

[Ref]

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Nakazawa M and Fukui K, JP Patent Application 2025-009199

Identification of fatty acid acyl-CoA reductase involved in wax ester production in *Euglena gracilis Z*

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Euglena gracilis adapts to hypoxic conditions *via* a distinctive metabolic pathway known as wax ester fermentation. In this process, the storage polysaccharide paramylon is degraded into glucose, generating pyruvate and yielding ATP through glycolysis. Subsequently, pyruvate is converted into acetyl-CoA within the mitochondria, which then undergoes a reverse β-oxidation pathway to form fatty acyl-CoA. A fraction of this fatty acyl-CoA is reduced to fatty alcohols, which are ultimately esterified and accumulated in the microsomes as wax esters, predominantly composed of myristyl myristate. Previously, we identified a wax ester synthase/diacylglycerol acyltransferase (WSD) that catalyzes the terminal step of wax ester biosynthesis (Tomiyama, et al., Sci. Rep., 7: 13504, 2017). Among the six WSD isoforms, WSD2 and WSD5 were found to play crucial roles in this process. In the present study, we directed our attention to fatty acyl-CoA reductase (FAR), the enzyme responsible for the penultimate reaction in the pathway, and sought to identify the FAR isoform implicated in wax ester biosynthesis.

In *E. gracilis*, three putative orthologs of fatty acyl-CoA reductase (designated FAR1 through FAR3) were identified through database analysis. Three FAR isoforms exhibited low amino acid sequence identities, ranging from 9.9% to 15.7%, with overall similarities between 45.0% and 54.1%. Subcellular localization prediction using TargetP suggested that FAR1 and FAR3 are most likely cytosolic, whereas FAR2 is predicted to be mitochondrial. To investigate the specific roles of these FAR isoforms in wax ester biosynthesis, synthetic double-stranded RNA was introduced into the chloroplast-deficient *Euglena* strain SM-ZK through electroporation, resulting in the generation of targeted gene knockdown strains (*far1_KD*, *far2_KD*, and *far3_KD*). These knockdown strains were cultured under normoxic conditions until reaching the stationary phase, after which they were subjected to a 6-hour hypoxic treatment. Subsequently, the levels of myristyl myristate were quantitatively assessed. Remarkably, the *far2_KD* strain demonstrated an approximate 70% reduction in myristyl myristate accumulation relative to the control SM-ZK strain, thereby identifying FAR2 as the predominant isoform contributing to wax ester biosynthesis among the three candidates.

Mechanism of Non-Photochemical Quenching in the Diatom *Chaetoceros gracilis*

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Photosynthetic organisms convert light energy into ATP and NADPH, which drive CO_2 fixation into organic molecules and sustain life on Earth. However, when the absorption of photons by light-harvesting complexes (LHCs) exceeds the demand, it leads to the formation of reactive oxygen species, resulting in damage to the photosynthetic machinery. To avoid such damage, many photosynthetic organisms employ non-photochemical quenching (NPQ), a protective mechanism that dissipates excess light energy as heat. In diatoms, the major form of NPQ—energy-dependent quenching (qE)—requires the combined action of a trans-thylakoid proton gradient (ΔpH), de-epoxidized xanthophyll pigments, and Lhcx proteins. In this study, we investigated the function of light-harvesting complex (LHC) proteins in the diatom *Chaetoceros gracilis* using genome editing, molecular phylogenetics, transcriptome analysis, and structural analysis. Through this approach, we identified a novel protein that is essential for NPQ. Knockout of its gene completely abolished qE, indicating its indispensable role in photoprotection. Based on our findings, a model of NPQ in *C. gracilis* will be proposed and extended to other diatom species using phylogenetic analysis.

Rhodoquinone functions in anaerobic and newly identified aerobic pathways in *Euglena gracilis*

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Rhodoquinone (RQ) is a low-redox-potential quinone found in a limited group of anaerobically adapted organisms, including *Euglena*, nematodes, and the parasitic roundworm *Ascaris suum*. It is generally considered to function as an electron carrier in the reduction of fumarate to succinate via mitochondrial complex II under anaerobic conditions. In previous studies (Nakazawa et al., 2018), we demonstrated that silencing the RQ biosynthetic gene *rquA* decreased RQ content and impaired wax ester production under anaerobic conditions, indicating that RQ supplies electrons to the fatty acid biosynthesis pathway coupled with the anaerobic respiratory chain. In the present study, we generated *rquA* knockout strains (*ΔrquA*) in which RQ levels were below the detection limit, and performed biochemical analyses to further investigate the physiological roles of RQ in *Euglena gracilis*.

In *ΔrquA* strains, wax ester synthesis under anaerobic conditions decreased to nearly 10% of wild-type levels, confirming that RQ functions as a major electron carrier in anaerobic lipid synthesis. In contrast, anaerobic succinate secretion remained unchanged. When rotenone, a mitochondrial complex I inhibitor, was added during anaerobic incubation, approximately 60% of succinate secretion was still retained, suggesting that *E. gracilis* utilizes the RQ-dependent electron transport chain to reduce fumarate under anaerobic conditions, though its contribution is limited.

Notably, *ΔrquA* strains exhibited slower growth than the wild type even under aerobic conditions. This raised the question of whether RQ also contributes to aerobic metabolism. When cultured mixotrophically in Koren-Hutner medium under light at 50 μmol· m^{-2} sec^{-1} and 27 °C for seven days, *ΔrquA* strains secreted over 1000 mg/L of glycolate, while no glycolate was detected in the culture supernatant of the wild type. This secretion was abolished under dark, heterotrophic conditions, suggesting that glycolate production is linked to photosynthesis.

To determine the source of the glycolate, we examined the photorespiratory pathway, in which 2-phosphoglycolate generated by RuBisCO oxygenase activity is converted to glycolate by 2-phosphoglycolate phosphatase (2-PGP). We identified a candidate 2-PGP gene from RNA-seq data and silenced its expression by RNAi. Glycolate secretion decreased to nearly 10% of the mock control level, indicating that glycolate excretion in *ΔrquA* strains originates from 2-PGP. Taken together with previous studies, these findings suggest that in *E. gracilis* photorespiration, glycolate synthesized in the chloroplast via 2-PGP is transported to the mitochondria and oxidized to glyoxylate in a reaction to which RQ contributes, either directly or indirectly. This study provides the first evidence for an aerobic role of RQ and suggests a novel involvement in photosynthesis-related metabolism in *E. gracilis*.

Alternative electron sink reflected in extra O_2 evolution against CO_2 fixation in a variety of algae

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Following the principle of oxygenic photosynthesis, electron transport in the thylakoid membranes generates ATP and NADPH from light energy, which is subsequently utilized for CO_2 fixation in the Calvin-Benson-Bassham cycle. However, the coupling of these two reactions could discord when an alternative electron flow occurs with a rate comparable to the linear electron flow. Here, we quantitatively monitored O_2 and total dissolved inorganic carbon (DIC) during photosynthesis in the pennate diatom *Phaeodactylum tricornutum*, and found that evolved O_2 was larger than the consumption of DIC. In our measurements, the stoichiometry of O_2 evolution to DIC consumption (often termed as photosynthetic quotient) was always around 1.5 regardless of the DIC concentrations and supplied nitrogen forms in the growth conditions, except for the nitrogen-starved cells showing O_2 evolution 2.5 times larger than DIC consumption. Furthermore, we investigated the photosynthetic quotient in a variety of algae, including Cyanophyta, Glaucophyta, Chlorophyta, Rhodophyta, Euglenophyta, Eustigmatophyta, Bacillariophyta, and Dinophyta. Based on the present dataset, we propose that some eukaryotic algae largely develop the metabolic pathway(s) functioning as the O_2-independent electron sink under steady state photosynthesis that reaches nearly half of electron flux of the Calvin-Benson-Bassham cycle.

Enhancing Photosynthesis via Overexpression of Isocitrate Dehydrogenase in *Synechocystis* sp. PCC 6803

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Introduction:

Enhancing photosynthetic efficiency is a key strategy to reduce atmospheric CO_2, a major cause of global warming. Among photosynthetic organisms, cyanobacteria such as *Synechocystis* sp. PCC 6803 (*Synechocystis*) are widely used as model organisms for studying photosynthesis. While previous studies have focused on enhancing the activity of photosynthetic enzymes, the improvements in photosynthetic activity were limited. Recent research highlights the importance of consuming reducing power in photosynthesis regulation. Reducing power generated during the light reaction of photosynthesis and consuming it through alternative pathways improve these reactions. In this study, we focused on nitrogen assimilation to consume excess reducing power, particularly the glutamine synthetase-glutamate synthase cycle (GS-GOGAT cycle), which requires much reducing power. Among the three substrates of the GS-GOGAT cycle, 2-oxoglutarate (2-OG) levels is lower than glutamine and glutamate, suggesting that the 2-OG levels may limit this cycle. Since 2-OG is synthesized by isocitrate dehydrogenase (IDH, encoded by *icd*), we speculated that *icd*-overexpressing (*icd*ox) increases 2-OG levels, leading to enhance both nitrogen assimilation and photosynthesis.

Results and Discussion:

The generated *icd*ox strain showed higher IDH activity, but 2-OG levels in the cells were similar to the parental wild-type (WT) strain. This suggests that 2-OG is rapidly consumed and may be immediately converted into glutamine, glutamate, or other amino acids. Oxygen evolution was significantly higher in *icd*ox strain than WT, indicating enhanced photo photosynthetic activity by *icd*-overexpression. These results suggest that increasing 2-OG supply promotes nitrogen assimilation and improves photosynthesis by consuming reducing power. This study highlights metabolic control of reducing power as a strategy to enhance photosynthetic efficiency.

Exploring *Euglena gracilis*: Investigating Unique Proteins and Polysaccharides

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Euglena gracilis is a unicellular green microalgae, more related to kinetoplastid human parasites than with other algae and with many unique properties. Due to its ease of cultivation, extensive metabolic capabilities and complex biology, *E. gracilis* is considered a valuable biological model.[1] This study explores three interesting aspects of *Euglena gracilis*: the extracellular polysaccharide (EPS), extracellular proteins and a member of a novel protein family.

The EPS was extracted from a range of growth conditions and analysed using SDS-PAGE, which demonstrated them to be glycoproteins.[2] Further investigation will focus on determining the carbohydrate composition of the EPS. A highly abundant extracellular protein in *Euglena*,[3] known as protein 2713, was partially purified to determine the molecular weight, and analyse the structure and properties. Ten unique *Euglena* proteins were codon-optimised for *E. coli*, including an unexplored protein, 9594, which showed some expression in *E .coli*. The exploration of an unknown protein provides valuable insights into the cellular processes of *Euglena* and contributes to understanding its complex molecular functions. This research advances our understanding of *Euglena*'s unique biology and provides valuable insights for its future biotechnological applications.

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Enhancing Photosynthetic Performance and Carbon Metabolism Efficiency of *Euglena gracilis* via Microwave Mutagenesis Combined with Carbon Dioxide Gradient Acclimation

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Abstract: Microalgae, with their high photosynthetic efficiency, play a crucial role in global carbon fixation and are potential sources for biofuels, nutraceuticals, and biomaterials. *Euglena gracilis*, a mixotrophic microalga, has advantages in carbon sequestration but faces challenges in industrial - scale cultivation due to its limited adaptability to high carbon dioxide (CO_2) concentrations in flue gases. This study aimed to develop a novel strategy by integrating microwave mutagenesis and CO_2 gradient acclimation for engineering *E. gracilis* strains. After microwave mutagenesis with optimized parameters (output power 300 W, irradiation time 45 s) and six-cycle CO_2 gradient acclimation (from 5% to 10% CO_2), the mutant strain LE-VB was obtained. Compared with the wildtype, LE-VB showed significantly improved growth performance, photosynthetic efficiency, and carbon sequestration rate at 10% CO_2. The content of photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoid) increased, and the activities of key photosynthetic carboxylases (Rubisco and PEPC) were regulated. Transcriptome analysis revealed that genes related to photosynthesis, carbon metabolism, and central carbon metabolism were differentially expressed. This research demonstrated that the combined treatment enhanced the photosynthetic activity, carbon assimilation, and central carbon metabolism of *E. gracilis*. It provides a simple, cost-effective, and efficient approach for cultivating microalgal strains adaptable to high-CO_2 environments, which has great potential for industrial carbon capture and bioresource production.

Keywords: *Euglena gracilis*; Microwave mutagenesis; CO_2 gradient acclimation; Photosynthesis; Carbon assimilation

Enhancing Fermented Food Functionality through the Incorporation of Edible Euglena Biomass during Fermentation Processes

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We are currently exploring a novel biotechnological approach to fermented food production by introducing edible algal biomass, particularly Euglena gracilis, during the fermentation process. In this study, we investigated the impact of incorporating Euglena powder during the fermentation of tempeh, a traditional soy-based fermented food. Comparative metabolomic profiling was conducted using liquid chromatography-mass spectrometry (LC-MS) to analyze changes in the chemical composition of the fermentation products. Our results demonstrated a marked increase in the production of certain metabolites, including allantoin, in the Euglena-supplemented samples compared to the control.

Development of a Safe and Simple Method for Cultivation and Flocculation of Microalgae Using Food and Food Additives

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[Purpose]

Foaming and solidification inhibit microalgal growth and effective ways to prevent them are required, as well as effective recovery of microalgal cells. In this study, we have developed a safe and simple method to produce microalgae while preventing foaming and sticking using food or food additives.

[Materials and Methods]

Synechocystis sp. PCC 6803 (hereafter GT6803), and *Chlorella sorokiniana* were used to evaluate various chemicals in preventing foaming and sticking during cultivation. To prevent foaming and sticking of cell cultures, silicon solution and a food additive were used. For cell flocculation and recovery of the cells, Mixture 1, a mixture of equal amounts of chitosan and acetic acid, and Mixture 2, a mixture of equal amounts of sodium hydroxide and ethanol, were used. The effects of foaming and anti-sticking were examined on the inner wall piping in the culture vessel, and the effects of flocculation were visually examined. The effect of flocculation was evaluated by measuring the OD_{730} of the supernatant via centrifugation at 50 g × 10 min after flocculation with different amounts of Mixture 1 and Mixture 2 to be added.

[Results and Discussion]

The addition of silicon solution at 165 mg/L for GT6803 and 50 mg/L for *C. sorokiniana* prevented both foaming and sticking and did not inhibit microalgal growth. In flocculation, a final chitosan concentration of 15 mg/L resulted in precipitation of both cells after several minutes of standing. The OD_{730} of the supernatant after centrifugation decrease in the presence of >5 mg/L and almost all the cells were flocculated at 25 mg/L.

In this way, we developed a safe method for preventing foaming and sticking and for accelerating flocculation and recovery of microalgal cells using reagents used for food products.

Metabolic profiling of *Euglena gracilis* treated with 1,4-diaminoanthraquinone, a compound that enhances wax ester accumulation

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Euglena gracilis, a photosynthetic protist, produces wax esters under anaerobic conditions by utilizing stored polysaccharides as a primary carbon source. The wax esters produced by *E. gracilis* are attracting attention as a biodiesel feedstock. We previously found that treatment with 1,4-diaminoanthraquinone (referred to as OATQ008) promotes wax ester accumulation in *E. gracilis* [1]. In this study, we investigated the effects of OATQ008 on *E. gracilis* metabolism by metabolic profiling using capillary electrophoresis–mass spectrometry (CE–MS). When comparing the effects of OATQ008 between light and dark conditions, the wax ester accumulation was enhanced more strongly in the light (11-fold) than in the dark (2.6-fold), suggesting that OATQ008 may influence light-dependent metabolic processes. To further characterize this effect, we analyzed the metabolic profiles of *E. gracilis* cells treated with DMSO or 100 μM OATQ008 (dissolved in DMSO) under light conditions for 6 hours using CE–MS. The levels of 25 compounds, including sugars, amino acids, and compounds related to nucleotide metabolism, differed significantly between the two treatments. Myristic acid, a building block for the most abundant wax ester species (myristyl myristate), was significantly increased in OATQ008-treated cells compared to DMSO-treated controls. Furthermore, vitamin B6, a coenzyme primarily involved in amino acid metabolism, and 5-phosphoribosyl 1-diphosphate (PRPP), a key precursor in nucleic acid biosynthesis, were detected only in DMSO- and OATQ008-treated cells, respectively. The present study reveals that OATQ008 treatment affected amino acid and nucleic acid metabolism in *E. gracilis*. These metabolic pathways are promising regulatory points that may lead to enhanced wax ester accumulation in *E. gracilis*. The detailed effects of OATQ008 on light-dependent metabolic processes remain unexplored and require further studies to better understand the underlying mechanisms.

[1] Ogawa *et al*. (2022) *J Biosci Bioeng*, **133**, 243–249.

Anaerobic biosynthesis of odd-chain fatty acids and distinct enzyme roles in propionate assimilation in *Euglena gracilis*

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Euglena produces wax esters (WE) under anaerobic conditions, which are considered promising biofuels and chemical feedstocks. These WE include a high proportion of odd-chain fatty acids, but the biosynthetic pathway for their precursor, propionyl-CoA, remains poorly understood. Propionyl-CoA is hypothesized to be generated via the methylmalonyl-CoA pathway, which involves several enzymes, including succinyl-CoA synthetase (SCS), methylmalonyl-CoA mutase (MCM), methylmalonyl-CoA epimerase (MCE), and propionyl-CoA carboxylase (PrCC). In this study, we used CRISPR/Cas9-mediated genome editing to knock out the genes encoding these enzymes in *Euglena* and analyzed their roles in odd-chain WE biosynthesis and propionate metabolism.

Guide RNA-Cas9 ribonucleoprotein complexes targeting the SCSα, SCSβ1, SCSβ2, MCM, MCE, PrCCα, and PrCCβ genes were introduced into wild-type *E. gracilis* Z strain by electroporation (Nomura et al., 2019). Mutant strains were identified by PCR screening and maintained in KH medium. Cells were first cultured aerobically until the late logarithmic phase and then incubated anaerobically under argon gas for 24 hours. Gas chromatography analysis revealed that knockout of SCSα, SCSβ1, and MCM led to a drastic reduction in the proportion of odd-chain WE to approximately 1.5%, compared to ~44% in the wild type. In contrast, knockouts of MCE, PrCC, and SCSβ2 had little effect on WE composition, suggesting that SCSα, SCSβ1, and MCM are essential for anaerobic biosynthesis of odd-chain fatty acids.

To assess their roles in propionate metabolism, the mutant strains were cultured in the medium supplemented with sodium propionate. The growth of MCM-, MCE-, and PrCC-deficient strains was significantly impaired, while the SCSα and SCSβ2 mutants exhibited moderate growth defects. Interestingly, the SCSβ1 knockout showed growth comparable to that of the wild type. These findings suggest that SCSα and SCSβ2 contribute to propionate assimilation through an alternative, possibly irreversible route of succinyl-CoA synthesis, independent of odd-chain fatty acid biosynthesis.

These results demonstrate that the methylmalonyl-CoA pathway enzymes serve distinct and partially overlapping roles in anaerobic odd-chain fatty acid biosynthesis and propionate assimilation in *Euglena*.

Enhancing Anaerobic Wax Ester Production in *Euglena gracilis* by Optimizing Organic and Inorganic Carbon Sources

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Euglena gracilis is known to produce wax esters under anaerobic conditions by utilizing paramylon. Although the demand for wax esters as a raw material for sustainable aviation fuel is increasing, the optimal conditions for their production remain poorly understood. Therefore, this study investigated anaerobic culture conditions, with a focus on carbon source requirements, to enhance wax ester production.

Separate experiments were conducted to evaluate the requirements for organic and inorganic carbon sources under anaerobic conditions. For the organic carbon requirement, glucose (15 g/L) was added to the anaerobic medium to assess its effect on wax ester production. For inorganic carbon, either bicarbonate (100 mM) or CO_2 gas (approximately 10%, balanced with N_2) was tested at two different pH levels (pH 4 and 8). For anaerobic cultivation, aerobically grown cells were inoculated at 5×10^6 cells/mL and either sealed or supplied with CO_2 gas, depending on the experimental condition.

The results confirmed that an organic carbon source is essential for wax ester production. The titer was significantly higher with glucose supplementation (1.00 ± 0.06 g/L) than without glucose (0.37 ± 0.01 g/L), and 6.0 g/L of glucose was consumed during cultivation. Additionally, elevated levels of dissolved CO_2 enhanced wax ester production. While bicarbonate addition had no effect, CO_2 gas supplementation markedly increased the titer to 3.91 ± 0.20 g/L under low pH conditions, suggesting that the dissolved form of CO_2 plays a crucial role.

These findings suggest that optimizing carbon supply can enhance wax ester production from *E. gracilis*, providing both a sustainable alternative to fossil-based fuels and a practical strategy for CO_2 utilization to address climate change.

Potential Advantages of Freshwater Cyanobacteria in Seawater Cultivation

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<Purpose>

Cyanobacteria are promising organisms for CO_2 fixation and the biosynthesis of valuable compounds, offering potential benefits for a low-carbon society. However, large-scale cultivation is limited by the high demand for freshwater. This study investigates seawater-based cultivation as a sustainable alternative to reduce freshwater usage.

<Materials & Methods>

The freshwater cyanobacterium *Synechocystis* sp. PCC 6803 was cultivated in either freshwater or seawater media. BG-11 medium was used as the freshwater medium, while the seawater medium was prepared by replacing deionized water in BG-11 with artificial seawater. Marine Art SF-1 (Osaka Yakken, Japan) was used as the artificial seawater. Growth, glycogen accumulation, and photosynthetic activity were measured to assess differences between the two conditions. Growth was monitored at an optical density of 730 nm using a UV-2700 spectrophotometer (Shimadzu, Japan). Glycogen content was quantified using the LabAssay Glucose (FUJIFILM Wako Pure Chemical Corporation, Japan). Photosynthetic activity was evaluated using a Clark-type oxygen electrode (Oxygraph & Oxytherm System, Hansatech Instruments, UK).

<Results>

Seawater cultivation of *Synechocystis* sp. PCC 6803 resulted in a prolonged logarithmic growth phase and a higher maximum optical density compared to freshwater conditions. Glycogen content in cells grown under seawater conditions was more than twice that observed under freshwater conditions. Additionally, while photosynthetic activity declined rapidly under freshwater conditions, it remained at a high level for a longer duration in seawater.

<Discussion>

Increased biomass, elevated glycogen accumulation, and sustained photosynthetic activity are critical factors contributing to the improved efficiency of biosynthesis. These favorable effects are presumably linked to the high concentrations of inorganic constituents in seawater, particularly magnesium and sulfate ions. Nevertheless, the underlying mechanisms remain unclear, and further investigations are currently underway to elucidate their roles.

Species-dependent preference and behavioral responses to *Tetraselmis* plastid donors in the euglenoid *Rapaza viridis*

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It is widely accepted that plastids in modern eukaryotes originated through secondary endosymbiosis, in which a photosynthetic eukaryote was engulfed and retained by another eukaryotic host. This acquisition of plastids was a major evolutionary event that occurred multiple times. Understanding the mechanisms of plastid acquisition could inform future technologies for manipulating plastid function. Among unicellular eukaryotes, which represent the majority of eukaryotic diversity, uptake of other cells has typically been studied in the context of nutrient acquisition. However, the mechanisms underlying plastid donor selectivity remain largely unknown, and no suitable model systems for biochemical analysis have been established.

In this study, we focused on *Rapaza viridis*, a euglenoid protist that performs kleptoplasty. *R. viridis* acquires plastids from certain *Tetraselmis* species for use as functional kleptoplasts. We aimed to investigate its behavioral and species-specific responses of *R. viridis* toward a broad range of potential plastid donors. We systematically tested 17 *Tetraselmis* species, obtained from the Algal Culture Collection of NIES (Japan) and from Dr. Pierre Cardol at the University of Liège (Belgium). Of these, *R. viridis* fully engulfed the cells of four species and selectively retained their plastids, enabling photoautotrophic growth. It exhibited no behavioral response to three species. For the remaining ten species, *R. viridis* showed clear behavioral responses, such as approaching and physical contact, but did not proceed to engulfment. Phylogenetic analysis based on 18S rDNA revealed that these behavioral differences broadly corresponded to the evolutionary relationships among the *Tetraselmis* species.

These findings provide the first systematic demonstration of plastid donor preference in a kleptoplastidic eukaryote. *R. viridis* represents a promising model for investigating the molecular mechanisms of plastid recognition and selective retention. Currently, we are extending this analysis by differentially labeling *Tetraselmis* strains with fluorescent dyes to further quantify plastid donor preference.

Generation and characterization of Environmental Stress-resistant *Euglena* Mutants for Biofuel Production

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Background and Purpose

The depletion of fossil fuels has accelerated the demand for alternative energy sources. Wax esters produced by *Euglena* possess fatty acid compositions similar to those of jet fuel and are thus regarded as promising candidates for biofuel production. However, commercial application requires the development of stable, large-scale outdoor cultivation systems. A major limitation is high sensitivity of *Euglena* to environmental stress, particularly heat. Temperatures exceeding 30 °C result in chloroplast malformation and cell etiolation, severely restricting cultivation in warm climates. This study aimed to generate heat-tolerant *Euglena* strains suitable for outdoor cultivation and to elucidate the underlying mechanisms of their stress tolerance.

Methods and Results

Wild-type (WT) *Euglena* cells were grown in KH medium under heterotrophic conditions until the stationary phase and then subjected to mutagenesis with 0.01–0.1% ethyl methanesulfonate (EMS) for one hour. Treated cells were washed, plated on KH agar, and incubated at 32°C. Colonies exhibiting growth without etiolation were subcultured weekly, and three strains showing normal chloroplast development were selected as heat-tolerant mutant (KH-HT1–3). WT and KH-HT1–3 strains were subsequently cultured for one week at 25°C and 32°C for one week. At 25°C, cell morphology, photosynthetic activity, chlorophyll fluorescence, and ascorbate peroxidase (APX) activity were comparable between WT and KH-HT1–3 strains. In contrast, at 32°C, WT cells exhibit etiolation, whereas KH-HT1–3 strains maintained green pigmentation and photosynthetic activity, as evidenced by $NaHCO_3$-dependent O_2 evolution. Chlorophyll fluorescence analysis showed that KH-HT1–3 maintained higher maximum quantum yields of PSII (Fv/Fm) than WT, indicating preserved photosynthetic function under heat stress. Notably, APX activity in KH-HT3 was 57% higher than in WT, suggesting enhanced antioxidant activity may contribute to observed heat tolerance.

Optimizing Fed-batch Conditions for enhancing Paramylon Productivity of *Euglena gracilis* in Lab-scale Fermentor

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Euglena gracilis is a microalga capable of accumulating high levels of paramylon, a β-1,3-glucan with immunomodulatory properties, making it a promising functional material for applications in the food, cosmetic, and feed industries. While *E.gracilis* can grow under both photoautotrophic and heterotrophic conditions, paramylon accumulation is significantly enhanced under heterotrophic cultivation. This study aimed to optimize high-yield heterotrophic cultivation conditions for the industrial production of paramylon.

Initially, flask-scale experiments were conducted to evaluate the effects of carbon sources, nitrogen sources, and initial pH on cell growth and paramylon accumulation. The optimal conditions identified were subsequently scaled up to a 5 L fermentor. To minimize cell damage caused by shear stress during fed-batch cultivation, agitation speed (RPM) and aeration rate were optimized. In addition, to prevent growth inhibition due to high salinity resulting from repeated medium feeding, the composition and concentration of the feeding medium were adjusted.

As a result, biomass and paramylon productivities increased by 3.29-fold (10.87 g/L/day) and 3.56-fold (5.59 g/L/day), respectively, compared to flask cultivation. The efficient fed-batch strategy for *E.gracilis* established in this study provides a valuable foundation for improving paramylon productivity in pilot-scale or large scale bioreactor systems for industrial applications.

Sustainable and Cost-Effective β-Glucan Production from *Euglena gracilis* Cultivated in Fed-Batch System Using Apple and Beet Pomace

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Euglena gracilis can grow under heterotrophic conditions and accumulate intracellular β-glucan, making it a valuable resource for the functional food and cosmetic industries. However, the high cost of synthetic media, particularly glucose and monosodium glutamate, remains a major barrier to commercial-scale production. To address this challenge, nutrient-rich food byproducts have been proposed as sustainable and economical alternatives. Among them, apple pomace is rich in sugars, while beet pomace is a good source of nitrogen. To evaluate the potential of combining apple and beet pomace as a low-cost medium for efficient β-glucan production, this study investigated their use in fed-batch cultivation of *E. gracilis*.

E. gracilis was initially cultivated in the media containing different ratios of apple and beet pomace to identify the optimal composition for β-glucan production. The selected medium was then applied in batch and fed-batch cultivation to evaluate productivity and cost-efficiency. A medium containing apple and beet pomace at a 7:3 ratio resulted in the highest β-glucan concentration (10.41 g/L) and productivity (3.16 g/L/day). For fed-batch cultivation, each byproduct was freeze-dried to prepare concentrated media, and various feeding conditions were tested. The most effective strategy was supplying beet pomace medium at 0.3× concentration, which resulted in the β-glucan concentration (14.49 g/L) and productivity of (3.45 g/L/day), without the addition of synthetic nutrients. Economic analysis showed that the apple and beet pomace medium reduced the medium cost from $0.94/L to $0.40/L. Compared to synthetic medium, this approach not only reduced the medium cost by 2.36-fold but also increased β-glucan concentration from 5.38 g/L to 10.41 g/L. These results demonstrate the feasibility of sustainable and cost-effective β-glucan production using *E. gracilis* and valorized apple and beet pomace, contributing to the advancement of a circular bioeconomy.

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