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Biotransformation of lactose-free acid whey by *Chlorella vulgaris* and *Tetrademus obliquus* into value-added products

Duygu Ozcelik^a, Rocio Espinosa^a, Radhakrishna Shetty^b, Shyam Suwal^c, Henriette Lyng Røder^a, Charlotte Jacobsen^d, Colin Ray^a, Brijesh K. Tiwari^e, Poul Erik Jensen^a, Mahesha M. Poojary^{a,*}

^a Department of Food Science, University of Copenhagen, Rolighedsvej 26, 1958 Frederiksberg C, Denmark

^b Research Group for Microbial Biotechnology and Biorefining, National Food Institute, Technical University of Denmark, Henrik Dams Allé, Building 202, 2800 Kongens Lyngby, Denmark

^c Arla Innovation Centre, Arla Foods amba, Agro Food Park 19, 8200 Aarhus, Denmark

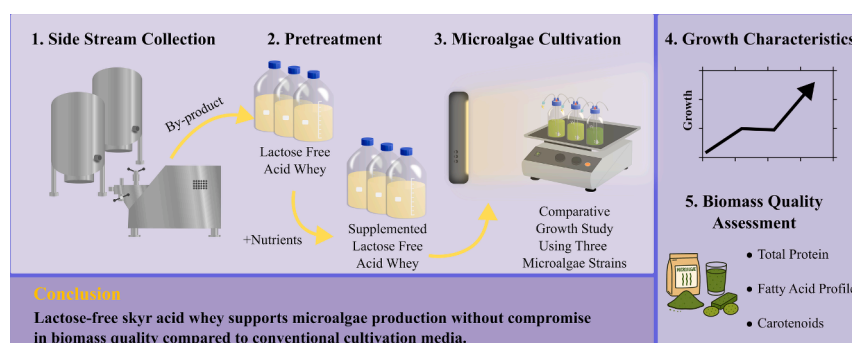
^d Research group for Bioactives – Analysis and Application, National Food Institute, Technical University of Denmark, Henrik Dams Allé, Building 202, 2800 Kongens Lyngby, Denmark

^e Department of Food Chemistry & Technology, Teagasc Food Research Centre, Ashtown D15 DY05 Dublin, Ireland

HIGHLIGHTS

- *C. vulgaris* and *T. obliquus* 3A are suitable for acid whey valorization.
- Growth in acid whey is shown to be highly strain-specific.
- Nutrient fortification of whey is key to unlocking microalgal productivity.
- Targeted nutrient supplementation reduces microbial contamination.

GRAPHICAL ABSTRACT



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ABSTRACT

Lactose-free acid whey (LFAW), an abundant byproduct from the production of skyr, was evaluated as a novel substrate for the mixotrophic cultivation of *Chlorella vulgaris* and two strains of *Tetrademus obliquus* (CCAP 2761A and CCPA276/3A). Nutrient profiling revealed that LFAW contained macronutrients such as calcium, magnesium, and potassium but was deficient in nitrate, phosphate, and trace elements, prompting the formulation of a supplemented medium (S-LFAW). Cultivation trials under mixotrophic conditions in 1 L photo-bioreactors demonstrated that S-LFAW significantly enhanced biomass productivity for *C. vulgaris* (140 mg/L/day) and *T. obliquus* 3A (79 mg/L/day), outperforming both standard Bold's Basal Medium (BBM) and LFAW alone. *C. vulgaris* grown in S-LFAW exhibited improved protein (13 mg/L/day) and total fatty acid content (31

* Corresponding author.

E-mail addresses: duygu@food.ku.dk (D. Ozcelik), rocie.espinosa@food.ku.dk (R. Espinosa), rads@food.dtu.dk (R. Shetty), shykr@arlafoods.com (S. Suwal), h.lyng.roeder@food.ku.dk (H. Lyng Røder), chja@food.dtu.dk (C. Jacobsen), colin.ray@food.ku.dk (C. Ray), brijesh.tiwari@teagasc.ie (B.K. Tiwari), peje@food.ku.dk (P.E. Jensen), mahesha@food.ku.dk (M.M. Poojary).

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mg/L/day), along with high carotenoid yields, indicating phototrophic metabolic dominance. *T. obliquus* 3A showed moderate performance with strain-specific sensitivity to nutrient composition, while *T. obliquus* 1A had limited growth in all LFAW-based media. The amplicon sequencing (i.e., 16S and 18S rRNA genes) of prokaryotic and eukaryotic contaminants revealed that the addition of limiting nutrients to the culture medium reduces the level of contamination. This study demonstrates the effective valorization of LFAW for sustainable microalgal biomass production, contributing to circular bioeconomy strategies and nutrient recovery from dairy side-streams.

1. Introduction

Microalgae are gaining attention as sustainable biomass producers due to their rapid growth, efficient CO₂ fixation, and ability to thrive on non-arable land using non-potable water, thereby minimizing competition with traditional agriculture. (Tahir et al., 2024). The protein content of microalgae can constitute 40–60 % of the dry biomass, with a well-balanced profile that includes all essential amino acids (Acquah et al., 2020). Furthermore, microalgae are primary producers of essential fatty acids like linoleic acid (LA) and alpha-linolenic acid (ALA), which are prominent in the Chlorophyceae class that includes this study's target organisms, *Chlorella vulgaris* and *Tetrademus obliquus* (Maltsev and Maltseva, 2021). These species also synthesize valuable carotenoids such as lutein, zeaxanthin, and β-carotene, known for their potent antioxidant properties, with reported lutein yields reaching up to 10 mg/g in *C. vulgaris* and 5 mg/g in *T. obliquus* under optimized conditions (Gong and Bassi, 2017; Ho et al., 2014). Despite this potential, the economic viability of commercial production is often hindered by high cultivation costs (Loke Show, 2022).

Mixotrophic cultivation has emerged as a promising strategy for improving growth and biomass yields, but it is constrained by its reliance on expensive organic carbon sources like glucose, which can add approximately 0.4 €/kg to biomass production costs and an increased risk of microbial contamination (Özcelik et al., 2024; Ruiz et al., 2022). To overcome the economic barriers, the valorization of agro-industrial by-products offers a sustainable and cost-effective solution. These nutrient- and organic carbon-rich side-streams have been successfully used to cultivate *C. vulgaris* and *T. obliquus*, in some cases achieving biomass concentrations as high as 12 g/L (Abreu et al., 2012; Cai et al., 2022; Piasecka et al., 2020).

Dairy side-streams have gained significant attention for microalgae cultivation due to rich composition of organic carbon, minerals, and phosphorus (Özcelik et al., 2024). For example, studies utilizing sweet whey (pH > 6) have demonstrated robust microalgal growth; for instance, cultivating *T. obliquus* in a medium containing 40 % sweet whey permeate yielded a biomass concentration of 5 g/L (Girard et al., 2014). Similarly, the use of hydrolyzed sweet whey powder resulted in a 3-fold increase in *C. vulgaris* biomass compared to photoautotrophic conditions (Abreu et al., 2012). Skyr is a popular high-protein, low-fat fermented dairy product in EU and USA. Industrial production of lactose-free Skyr generates unique and underutilized byproducts known as lactose-free acid whey (LFAW) (Ramos et al., 2015). LFAW is acidic (pH ≤ 4.5) and significant lactic acid content (9.4–9.7 g/L) compared to previously explored substrate, sweet whey (Nielsen et al., 2021). Furthermore, LFAW contains high concentrations of readily available monosaccharides (18–24 g/L of glucose and galactose), as lactose is hydrolyzed during manufacturing. This property could be advantageous for microalgae cultivation, as microalgae readily assimilate monosaccharides compared to lactose (a disaccharide), eliminating the need for substrate hydrolysis before cultivation. While several valorization strategies for acid whey are in development, including its use in baked goods and nutritional drinks, most of it is still used unprocessed for animal feed or biogas production (Ramos et al., 2015). Acid whey, including LFAW, is a challenging substrate due to its high acidity and lactic acid content, and its use for microalgae cultivation is therefore not well-documented. To the best of our knowledge, (Bentahar et al., 2019)

is the only study to explicitly report using acid whey permeate for the cultivation of *T. obliquus* reached to ~5 g/L in batch cultivation conditions in 16 days.

The present study aims to valorize LFAW as an underexplored substrate for cultivation of *C. vulgaris* and two strains of *T. obliquus* for potential food applications. *C. vulgaris* was selected as a robust and well-characterized microalgae, providing a strong benchmark for performance comparison against two strains of *T. obliquus* in this novel substrate. The composition of LFAW as a growth medium was investigated and a targeted micronutrient supplementation strategy to enhance the growth of the target microalgae was developed. The biomass obtained was systematically evaluated for protein, lipid and carotenoid composition. In addition, the relative abundance of other microorganisms in the culture media as contaminants was evaluated by sequencing the 16S and 18S rRNA genes. By integrating dairy side-stream valorization with microalgae-based food production, this research significantly contributes to the advancement of circular bioeconomy models and sustainable food systems.

2. Materials and methods

2.1. Microalgal strains and maintenance

The microalgal strains *Chlorella vulgaris* CCAP 211/11B, *Tetrademus obliquus* CCAP 276/1A (hereafter *T. obliquus* 1A), and *T. obliquus* CCAP 276/3A (hereafter *T. obliquus* 3A) were sourced from the Culture Collection of Algae and Protozoa (CCAP), Scotland, UK. These cultures were maintained in *Euglena gracilis* medium supplemented with Jaworski's medium (EG:JM), also procured from CCAP. Duplicate cultures were grown in either low-light conditions at room temperature or in a Forma Steri-Cycle i160 CO₂ incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA) set to 25 °C, without additional CO₂ supplementation. All three microalgae strains were regularly monitored for morphology, identification, and axenicity. *T. obliquus* 1A and 3A differ in morphology where 1A is unicellular and 3A forms a coenobium. Bold's Basal Medium (BBM) was selected as a control growth medium for all cultivation experiments in this study (see [supplementary data-sheet](#)). For heterotrophic and mixotrophic conditions, BBM was supplemented with organic carbon sources such as glucose, galactose, and lactose (see [supplementary datasheet](#)).

2.2. Characterization of lactose-free acid whey

For elemental analysis, samples (1 mL) were digested with concentrated nitric acid (70 % HNO₃) and hydrogen peroxide (15 % H₂O₂) in a pressurized microwave digester (ultraWAVE, Milestone Inc., Bergamo, Italy) at 240 °C and 200 bar for 20 min (Szameitat et al., 2021). Following digestion, samples were diluted to 3.5 % HNO₃. Elemental concentrations were determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (7900, Agilent Technologies, Santa Clara, CA, USA) operating in helium collision mode. Quantification was based on an external multi-element calibration curve. Quality control was ensured by processing blanks and a Certified Reference Material (CRM; NIST 1515 Apple Leaf) with all sample batches. The accuracy and precision of the method were confirmed to be within 5 % for most analytes, and instrumental drift was corrected by analyzing the CRM at regular

intervals during the analytical sequence.

Nitrate and orthophosphate levels in LFAW were quantified using Spectroquant photometric test kits (1.09713.0002 and 1.14848.0001, respectively). Triplicate samples were filtered through 0.2 µm PES syringe filters and appropriately diluted to fall within the standard calibration ranges. Total solid content was measured following the standard method (Rice et al., 2012).

2.3. Pre-treatment of raw lactose-free acid whey

Raw LFAW from skyr production was provided by Arla Foods a.m.b.a., Aarhus, Denmark. Preliminary empirical trials indicated that the target microalgae exhibited limited growth in undiluted LFAW. Therefore, the raw LFAW was diluted to 50 % with ultrapure water, adjusted to pH 6.4 with 6 N NaOH, and filter-sterilized through a 0.2 µm PES JetBiofil Filter Upper Cup sterile disposable bottle-top filter (Guangzhou Jet Bio-Filtration Co., Ltd., Guangzhou, China) (see [supplementary datasheet](#)).

2.4. Screening cultivation setup

Two screening experiments were conducted to assess the growth of the microalgae in different cultivation conditions. Cultivations were performed in 12-well, non-treated culture plates (VWR, Radnor, PA, USA) with 3 mL working volumes and an initial culture density of 10^7 cells/mL for 12 days, each performed in biological triplicates. Sample aliquots were collected every three days to analyze growth parameters.

2.4.1. Light intensity Screening

The initial experiment evaluated the effect of darkness (i.e. Heterotrophic conditions) and of different light intensities (50 or 100 µmol/m²/s) under mixotrophic conditions, using a growth light source (LUMERI PNLX600). The experimental design is detailed in the [supplementary datasheet](#). Based on the result of this screening (see [supplementary datasheet](#)), continuous light supplementation at 100 µmol/m²/s was selected for all phototrophic and mixotrophic cultivations in this study.

2.4.2. Nutrient supplementation Screening

The second experiment assessed the effect of nutrient supplementation in LFAW under heterotrophic and mixotrophic conditions (light source: 100 µmol/m²/s). Specifically, various combinations of nitrate, phosphate, trace metal solution, and vitamin mixtures were tested to identify the limiting nutrients affecting the microalgal growth (see [supplementary datasheet](#)).

2.5. Bottle photobioreactor setup

Scaling up from the screening experiments, cultivations were conducted in 1 L Pyrex bottle photobioreactors (PBRs) containing 600 mL cultures with an initial cell density of 10^7 cells/mL for 16 days. These were incubated on an orbital shaker at 50 rpm at room temperature (22 ± 2 °C). Mixotrophic and photoautotrophic cultures were illuminated continuously 100 µmol/m²/s using a LUMERI PNLX600 light source. Photoautotrophic conditions utilized BBM, while mixotrophic cultures were grown in LFAW, nutrient-supplemented LFAW (S-LFAW), or BBM enriched with glucose, galactose, and lactose at concentrations equivalent to those in LFAW (BBMGGL). At the end of the cultivation period, microalgae were harvested and freeze-dried for 48 h for biomass analyses.

2.6. Determination of growth rate

All conditions were tested in biological triplicates. Growth curves and rates were determined by sampling cultures every other day and measuring biomass concentration via absorbance at 750 nm using a UV1280 UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan) with 10

mm pathlength semi-microcuvettes (Sarstedt Inc., Nümbrecht, Germany). Specific growth rates (μ) were calculated using Equation (1) (Madigan et al., 1997):

$$\mu = \ln(N_2/N_1)/(t_2-t_1) \quad (1)$$

where N_1 and N_2 represent the optical densities at 750 nm at times t_1 and t_2 , respectively.

Final biomass yield (X_{final}) was determined by dry weight measurements. Samples were washed three times with ultrapure water to remove residual media sugars, followed by centrifugation at $9000 \times g$ for 5 min. The filtered samples were dried at 105 °C to a constant weight, using pre-weighed and pre-heated glass fiber filters (GF/F, 47 mm, 0.7 µm, Whatman Ltd., Maidstone, UK). Dry weights were calculated using Equation (2) (Zhu and Lee, n.d.).

$$X_{final} = \frac{W_f - W_i}{V_s} \quad (2)$$

where W_f is the final filter weight (g), W_i is the initial filter weight (g), and V_s is the sample volume (L).

Cell density (n) was determined using the MUSE cell analyzer (Fremont, CA, USA), with maximum cell concentration (n_{max}) reported in cells/mL. Doubling time (t_d) was calculated using Equation (3):

$$t_d = \frac{\ln(2)}{\mu_{max}} \quad (3)$$

where t_d is doubling time (days), and μ_{max} is the maximum specific growth rate. Biomass productivity was calculated by dividing the final biomass concentration (g/L) at the end of the batch cultivation by the total cultivation time of 16 days, expressed as mg/L/day. As daily growth measurements were not performed, the maximum biomass productivity, which occurs during the exponential growth phase, was not determined.

2.7. Quantification of glucose, galactose, and lactose

The consumption rates of glucose, galactose, and lactose were determined using ultra-high-performance liquid chromatography (UHPLC, Ultimate 3000, Thermo Scientific, Massachusetts, USA) with a refractive index detector (Shodex RI-101, Showa Denko K.K., Tokyo, Japan). The analysis employed an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm, Bio-Rad, California, USA) with an isocratic program featuring a column oven temperature of 60 °C and 5 mM H₂SO₄ as the sole mobile phase at 0.5 mL/min flow rate. Supernatants from the cultures were filtered through a 0.2 µm syringe filter and then directly injected into the system via an autosampler. The identification and quantification of carbohydrates were achieved by using pure standards of glucose, galactose, and lactose procured from Sigma-Aldrich and using Chromeleon 7 software. Rate of substrate consumption, specific substrate utilization rate, biomass to substrate yield, and percent removal of organic carbon were calculated using Equation (4), 5, 6 and 7, respectively.

$$\frac{dS}{dt} = \frac{S_f - S_i}{dt} \quad (4)$$

$$q_s = \frac{1}{X} \cdot \frac{dS}{dt} \quad (5)$$

$$Y_{X/S} = \frac{(X(t) - X_i)}{(S_i - S(t))} \quad (6)$$

$$\% \text{ Removal of Organic Carbon} = \frac{C_f - C_i}{C_f} \times 100\% \quad (7)$$

The initial and final substrate concentrations (g/L) were denoted as S_i and S_f , respectively and dt corresponds to the time interval of

calculation. The initial and final total substrate concentrations (sum of glucose, galactose, and lactose) were denoted as C_f and C_i .

2.8. Microbiome analysis

2.8.1. Library preparation and sequencing

Samples were collected from bottle PBRs (section 1.5) under sterile conditions and stored at $-20\text{ }^{\circ}\text{C}$ until processing. Total genomic DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Düsseldorf, Germany), following the manufacturer's recommendations. DNA concentration and purity were assessed using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis.

Library preparation was performed as previously described (Hui et al., 2021), with modifications. To target prokaryotic microorganisms, the 16S rRNA V1-V8 region was amplified using the following primers: 27Fa/b (5'-AGAGTTTGATYMTGGCTYAG, 5'-AGGGTTCGATTCTGGCTCAG), 338Fa/b (5'-ACWCCTACGGGWWGCAGCAG, 5'-GACTCCTACGGGAGGCWGCAG), 1391R (5'-GACGGGCGGTGTGTRCA), and 1540R (5'-TACGGYTACCTTGTACGACT). For eukaryotes, the V4 region of the 18S rRNA gene was amplified using primers (515F 5'-GTGCCAGCMGCCGCGGTAA and 119R 5'-GGTCCCTTCCGTC) to target eukaryotic communities (Popovic and Parkinson, 2018). Barcoding was performed by dual-stage PCR. Briefly, first PCR conditions for 16S primers were $95\text{ }^{\circ}\text{C}$ for 5 min, 2 cycles of $95\text{ }^{\circ}\text{C}$ for 20 s, $48\text{ }^{\circ}\text{C}$ for 30 s, $65\text{ }^{\circ}\text{C}$ for 10 s, $72\text{ }^{\circ}\text{C}$ for 45 s, and a final extension at $72\text{ }^{\circ}\text{C}$ for 4 min. First PCR conditions for 18S primers were $95\text{ }^{\circ}\text{C}$ for 5 min, 13 cycles of $95\text{ }^{\circ}\text{C}$ for 45 s, $57\text{ }^{\circ}\text{C}$ for 45 s, $72\text{ }^{\circ}\text{C}$ for 50 s, and a final extension at $72\text{ }^{\circ}\text{C}$ for 4 min. Second PCR step was done to barcode the first PCR products with the following conditions: $95\text{ }^{\circ}\text{C}$ for 2 min followed by 33 cycles of $95\text{ }^{\circ}\text{C}$ for 20 s, $55\text{ }^{\circ}\text{C}$ for 20 s, $72\text{ }^{\circ}\text{C}$ for 40 s. Amplicons were purified with AMPure XP Bead-Based reagents (Beckman Coulter, Brea, CA, USA) and quantified using a Qubit dsDNA HS Assay Kit. Libraries were pooled together for Oxford Nanopore Technologies (ONT) sequencing. The ONT sequencing libraries were prepared using the ligation sequencing kit SQK-LSK109 protocol, loaded in a R9.4.1 flow cell, and sequenced on a GridIONx5 platform (Oxford Nanopore Technologies, Oxford, UK). The sequencing was performed with MinKNOW (v21.05), and base-calling was carried out using Guppy (v5.0.11). The raw sequencing data from 16S and 18S rRNA amplicon sequencing have been deposited in the NCBI Sequence Read Archive (SRA) under Bio Project accession number PRJNA1330230.

2.8.2. Amplicon-based microbial community analysis

Amplicon sequencing data targeting both the 16S and 18S rRNA gene regions were processed using the RubyRed pipeline (<https://github.com/reillyeo/RubyRed>). For 16S data, the standard RubyRed pipeline was used. For 18S data, the pipeline was adapted to improve the detection of microalgae by creating a merged database for 18S taxonomic assignment. Specifically, QIIME 2's RESCRIPt plugin was used to retrieve all available 18S sequences for microalgae from NCBI, which were then merged with the MIMt 18S database (<https://mimt.bu.biopolis.pt/18s>) to generate a more comprehensive training set. Downstream analysis was performed in RStudio (2025.05.0) using tidyverse, vegan, phyloseq, and ape packages. OTU tables were filtered to exclude samples with fewer than 1000 reads. Relative abundances of taxa were calculated per sample, and taxa with $< 1\%$ abundance were excluded and renormalized. Taxa were reported using the best available taxonomic resolution, allowing for mixed levels to preserve classification detail.

2.9. Characterization of biomass

2.9.1. Profiling of total amino acids

Lyophilized microalgal biomass ($10 \pm 0.5\text{ mg}$) was added with 3 mL of 4 M methanesulfonic acid containing 0.2 % tryptamine under a

nitrogen atmosphere for protein hydrolysis. Hydrolysis was conducted in a microwave reactor (Biotage Initiator+, Uppsala, Sweden), with heating to $150\text{ }^{\circ}\text{C}$ for 1 min, followed by $165\text{ }^{\circ}\text{C}$ for 10 min under continuous stirring (Poojary et al., 2020). Hydrolysates were neutralized with an equal volume of freshly prepared 4 M NaOH, mixed with 50 μM 6-aminocaproic acid (internal standard), filtered through 0.22 μm RC membrane filters, and analyzed via UHPLC-FLD Ultimate 3000 RS (Thermo Fisher Scientific Inc., Waltham, MA, USA). Separation was achieved using an AdvanceBio AAA column ($100\text{ mm} \times 3.0\text{ mm} \times 2.7\text{ }\mu\text{m}$, Agilent Technologies, CA, USA), with conditions as described in (Hildebrand et al., 2020). True protein content of the biomass was calculated by multiplying total amino acids count with a factor of $100/116$ (Feng et al., 2016).

2.9.2. Carotenoid analysis

Carotenoids were extracted from $50 \pm 0.5\text{ mg}$ of lyophilized biomass through saponification in methanolic KOH 40 % ($\text{g}_{\text{KOH}}/\text{g}_{\text{biomass}}$) at $80\text{ }^{\circ}\text{C}$ for 1 h in Pyrex tubes. The saponified material was extracted at $56\text{ }^{\circ}\text{C}$ for 30 min using 2 mL extraction solvent composed of ethanol: hexane: water (77:17:6, v:v:v) and 1 mL 1.3 % Na_2SO_4 solution, followed by five additional cycles of solvent extraction (Ceron-Garcia et al., 2018). Pooled extracts were dried under N_2 atmosphere and resuspended with mobile phase B containing *trans*- β -apo-8'-carotenal as an internal standard. Samples were filtered into HPLC vials by using a 0.22- μm RC membrane filters and analyzed by using a UHPLC system Ultimate 3000 RS (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a diode array detector (DAD). Separation of the analytes were achieved with a C18 reversed-phase column, $100 \times 2.1\text{ mm}$ Acquity UHPLC HSS T3, 1.8 μm , (Waters, Saint-Quentin-en-Yvelines; France). Chromeleon 7 software was used for instrument control, data acquisition, and data processing. Mobile phase B consisted of 75:10:15 mixture of acetonitrile, dichloromethane, and methanol, while mobile phase A was 0.05 M ammonium acetate. The gradient elution started at 75:25 (B:A) from 0 to 13.3 min, changed to 100:0 (B:A) from 13.3 to 14 min, then to 98:2 (B:A) from 14 to 30.7 min, and finally returned to the initial 75:25 (B:A) conditions for column equilibration from 30.7 to 37.4 min (Chauveau-Duriot et al., 2010). The flow rate was 0.4 mL/min. Column temperature was kept constant at $35\text{ }^{\circ}\text{C}$. Carotenoids were detected at 450 nm using a DAD that scanned between 210 nm and 600 nm and were further identified by comparing the retention times and visible spectra of analytical standards. Pure standards were dissolved in tetrahydrofuran containing 250 ppm butylated hydroxy toluene as a preservative, then, diluted to calibration curve concentrations (0.1–20 $\mu\text{g/mL}$) with mobile phase B.

2.9.3. Direct fatty acid methyl esters analysis

Fatty acid methyl esters (FAME) of the microalgae were prepared by transesterification of $25 \pm 0.5\text{ mg}$ of lyophilized biomass. Samples were treated with 1 mL of 1 M methanolic sodium hydroxide, 1 mL toluene, and 100 μL of 2 % C23 internal standard (GLC-68D, Nu-Check Prep), followed by ultrasonic incubation for 10 min and incubating in a boiling bath at $100\text{ }^{\circ}\text{C}$ for 2 min. After cooling, 2 mL of methanolic boron trifluoride was added, and the mixture was boiled again for 2 min. Upon cooling, saturated NaCl and heptane containing 0.01 % BHT were added, and the upper organic phase was collected for gas chromatography (GC) analysis. GC analysis conditions were maintained as described in the previous study (Safafar et al., 2019). Fatty acids were quantified as area percent of total fatty acids and reported as mg/100 g dry biomass.

2.10. Statistical analysis

All cultivation conditions were tested in biological triplicates. Fatty acids were extracted in duplicates for all biological triplicates. Amino acids and carotenoids were extracted as singlets and injected into the instrument in duplicates for all biological triplicates. Statistical analyses

were conducted using GraphPad Prism 10.3.1. Differences among experimental groups were evaluated using one-way ANOVA with Tukey's post hoc test ($p < 0.05$).

3. Results and discussion

3.1. Nutritional composition of culture media and side-stream

LFAW was evaluated as an alternative medium for cultivating the three selected microalgae, *C. vulgaris*, *T. obliquus* 3A, and *T. obliquus* 1A, due to its rich mineral and organic carbon content (see [supplementary datasheet](#) for the composition). Elemental analysis revealed that raw LFAW contains significantly higher concentrations of key macronutrients compared to the commonly used model microalgae growth medium BBM, making it a promising substrate for microalgal growth. For example, potassium was present at 1,416 mg/L (85 mg/L in BBM), calcium at 1,064 mg/L (7 mg/L in BBM), and magnesium at 93 mg/L (7 mg/L in BBM). Phosphorus was also abundant at 653 mg P/L, far exceeding 53 mg P/L of BBM. However, a substantial fraction of phosphorus, primarily orthophosphate (PO_4^{3-}), precipitated out of solution as insoluble calcium phosphate salts when the medium is adjusted to pH 6.4, thereby reducing its bioavailability. This may also create a significant challenge for industrial scale-up, where such precipitates can foul equipment and clog systems. Elevated levels of these salts also pose a risk of osmotic and ionic stress, potentially inhibiting algal growth. To mitigate this, the raw LFAW was diluted at a 1:1 ratio with ultrapure water prior to use (see Section 1.3), and the pH was adjusted from 4.3 to 6.4 using 6 N NaOH, during which visible salt precipitation was observed. Precipitate was removed during filter sterilization before the cultivation. Furthermore, salt precipitation was observed throughout the cultivations in all LFAW and S-LFAW reactors, which may have resulted from the gradual precipitation of excess calcium as calcium phosphate over time.

While LFAW contains most of the essential nutrients in excess, it is notably deficient in certain key components when compared to BBM. Specifically, nitrate was present at 33 mg/L, leading to an unbalanced C:N ratio of 2285:1, which is insufficient to support sustained algal growth. Furthermore, copper, a critical micronutrient involved in electron transfer and oxygen cycling as a cofactor of redox proteins (Kong, 2022), was not found in LFAW, unlike BBM which contains 0.01 mg/L of Cu as a micronutrient. The lack of copper and potentially other trace elements (e.g., cobalt, molybdenum, zinc) may limit microalgae metabolism and growth. To address these nutrient imbalances and to evaluate if addition of lacking nutrients improves the growth of microalgae in LFAW, the medium was supplemented with sodium nitrate (182.4 mg/L) to adjust its C:N ratio to 205:1, matching that of BBMGGL, and spiked with phosphate, trace metals, and vitamins at the equivalent concentration found in BBM and BBMGGL. The resulting medium is referred to as S-LFAW. The growth of target microalgae in LFAW and S-LFAW was then compared with that in BBM and BBMGGL (described in the following sections). BBMGGL was BBM supplemented with glucose (9 g/L), galactose (12 g/L), and lactose (0.1 g/L) at concentrations equivalent to those in LFAW and S-LFAW. Its high organic carbon content (C:N ratio = 205:1) makes it well suited for both heterotrophic and mixotrophic algal cultivation (see [supplementary datasheet](#)).

3.2. Growth of microalgae in screening tests

The light intensity screening (see Section 1.4.1) showed that mixotrophic growth consistently benefited from light, particularly in galactose- and lactose-based media, leading to the selection of $100 \mu\text{mol}/\text{m}^2/\text{s}$ for subsequent experiments (see [supplementary datasheet](#)). The subsequent nutrient supplementation screening (see Section 1.4.2 and [supplementary datasheet](#)) in LFAW and S-LFAW revealed that *T. obliquus* strains adapted rapidly to LFAW with shorter lag phases than in standard media BBM and BBM containing glucose (BBMG), galactose (BBMA),

lactose (BBML), mixture of the three carbohydrates (BBMGGL). For both *C. vulgaris* and *T. obliquus* 3A, supplementing LFAW with trace metals and vitamins (i.e. S-LFAW) proved particularly effective for promoting robust growth. Therefore, the subsequent large-scale 1 L photobioreactor cultivations were conducted using S-LFAW.

3.3. Growth of microalgae in 1L photobioreactors

Among the three microalgal strains tested, *C. vulgaris* and *T. obliquus* 3A demonstrated successful mixotrophic growth in both LFAW and S-LFAW media at larger scale cultivation (Fig. 1). In contrast to the results observed in the nutrient supplementation screening (section 2.4.2), mixotrophic cultivations conducted in 1 L PBRs revealed that *T. obliquus* 1A exhibited limited growth in LFAW, regardless of whether the medium was supplemented with the previously identified limiting nutrients. See Table 1 for growth parameters.

Growth of *T. obliquus* 3A was minimal in BBM, but the addition of organic carbon (BBMGGL) led to an approximately 20-fold increase in final biomass (X_{final} from 0.04 to 0.7 g/L). In contrast, cultivating in LFAW suppressed growth, causing the final biomass to decrease approximately 3-fold compared to BBMGGL. Despite this poor growth, the biomass yield on substrate ($Y_{\text{X/S}}$) was 11-fold higher than that observed in the BBMGGL medium. This suggests a preference for phototrophic metabolism over utilizing the available organic carbon, a potential sign of catabolite repression, which is slightly different than the typical carbon catabolite repression where the presence of a preferred carbon source like glucose suppresses the utilization of other carbon sources (Gao, 2021). Supplementing the acid whey with nutrients (S-LFAW) promoted a significant recovery, increasing the final biomass by approximately 5.5-fold compared to LFAW and resulting in the highest overall biomass for this strain ($X_{\text{final}} = 1.3 \pm 0.2 \text{ g/L}$) with biomass productivity of 79 mg/L/day. Bentahar et al. (2019) reported a higher biomass of 5 g/L with *T. obliquus* in 16 days when acid whey permeate was supplemented with 20 % BBM. These comparative results suggest that the growth of *T. obliquus* could be further enhanced by optimizing the media composition, although this was not investigated in the present study.

T. obliquus 1A showed incompatibility with the acid whey media. While the growth of this strain was minimal in BBM, it increased substantially in BBMGGL, yielding an approximately 27-fold greater final biomass ($X_{\text{final}} = 0.8 \text{ g/L}$). However, it had limited growth in both acid whey media with the final biomass in both LFAW and S-LFAW was less than 1 % of that achieved in BBMGGL, indicating strong physiological inhibition due to cultivation conditions. This incompatibility may stem from a higher sensitivity of this specific strain to compounds present in LFAW, such as high concentrations of lactic acid. Furthermore, it is possible that *T. obliquus* 1A has specific macro- or micronutrient requirements that were not fully met even in the S-LFAW, leading to the observed lower growth. Consequently, nutrient utilization data could not be determined for these treatments due to the lack of significant growth.

C. vulgaris showed minimal growth in BBM, but the addition of sugars in BBMGGL led to an over 20-fold increase in final biomass. In BBMGGL, moderate carbon removal efficiency ($11.4 \pm 0.3 \%$) and near-complete nitrate removal ($99.4 \pm 0.0 \%$) were observed, indicating effective nitrogen utilization. Growth was further enhanced in LFAW, where the final biomass was approximately 1.5-fold greater than in BBMGGL. However, nitrate removal efficiency was slightly lower ($93.7 \pm 1.3 \%$) and organic carbon ($9.8 \pm 2.0 \%$) compared to BBMGGL. The highest growth efficiency was achieved in S-LFAW, which produced the highest final biomass ($X_{\text{final}} = 2.2 \pm 0.5 \text{ g/L}$) with 140 mg/L/day biomass productivity and a specific growth rate 2-fold higher than that in LFAW (1 day^{-1} , μ_{max}), confirming that supplementing the whey with limiting nutrients unlocked the full growth potential for this strain (see [supplementary datasheet](#)). This productivity (0.1 g/L/day) is promising, although lower than the 0.7 g/L/day with *C. vulgaris* under optimized,

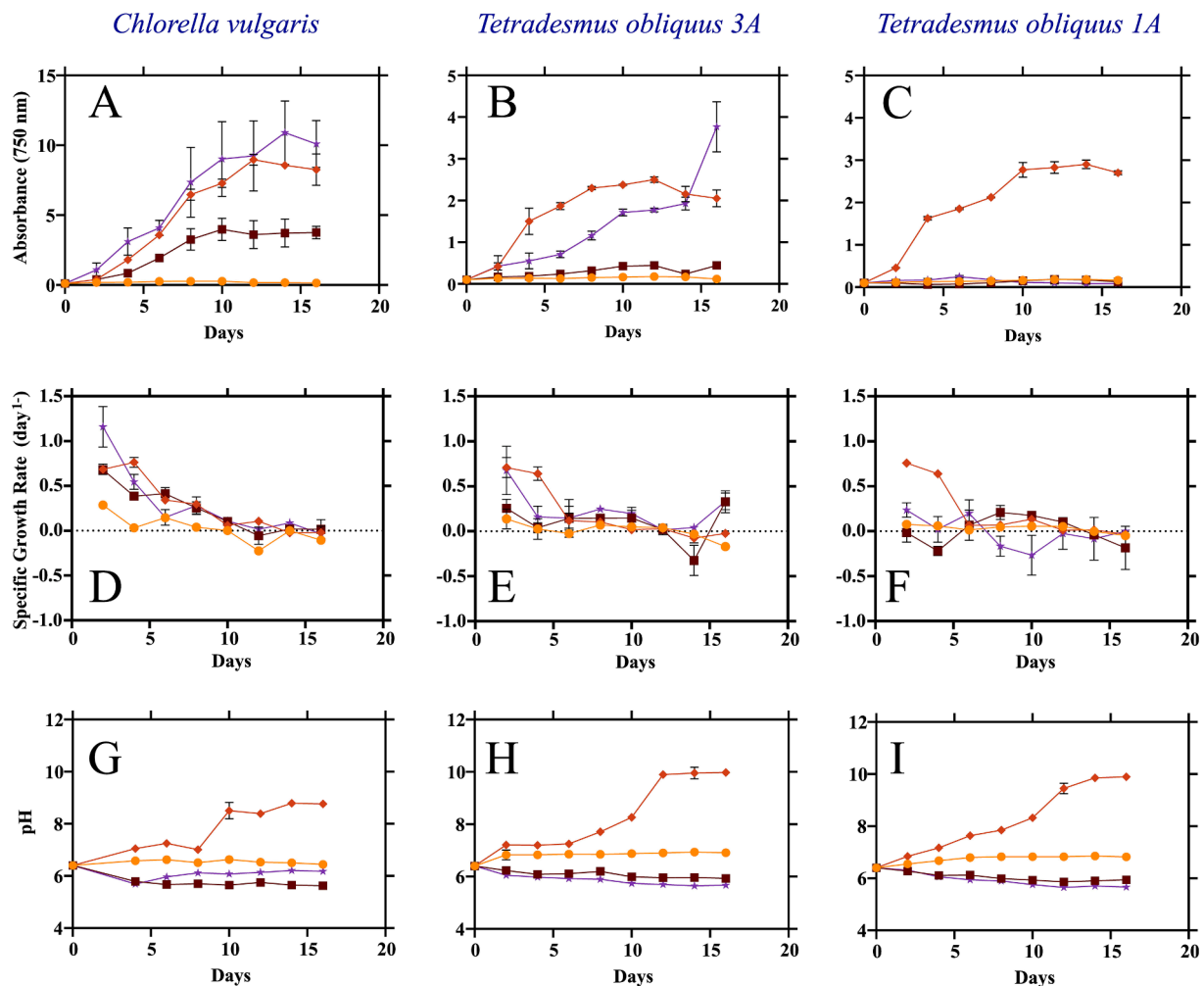


Fig. 1. Growth curves of *C. vulgaris* (A), *T. obliquus* 3A (B), *T. obliquus* 1A (C). Plots of specific growth versus time of *C. vulgaris* (D), *T. obliquus* 3A (E), *T. obliquus* 1A (F). pH versus time plots of *C. vulgaris* (G), *T. obliquus* 3A (H), *T. obliquus* 1A (I). Legend: Bold's Basal Medium (BBM) (—●—), BBM + glucose, galactose, lactose (equivalent concentrations in acid whey) (BBMGGL) (—◆—), Lactose-Free Acid Whey (LFAW) (—■—), Supplemented-LFAW (—▲—).

CO₂ supplemented conditions using hydrolyzed whey (Abreu et al., 2012).

The results of the 1L PBR cultivation of three microalgae strains highlight that the viability of using dairy acid whey for mixotrophic microalgae cultivation is profoundly species- and strain-dependent. *C. vulgaris* emerged as a highly robust candidate, achieving its highest biomass concentration and fastest growth rate in S-LFAW, aligning with literature that establishes *Chlorella* as a resilient genus for bioremediation (Coronado-Reyes et al., 2020). Its performance in LFAW surpassed that in a defined sugar medium BBMGGL, indicating its tolerance to the complex matrix of acid whey. In contrast, the two *T. obliquus* strains exhibited divergent outcomes. While strain 3A showed moderate success, particularly after nutrient supplementation in S-LFAW, strain 1A experienced growth limitation in both LFAW and S-LFAW. Successful large-scale implementation of these cultivation conditions will likely require additional optimization of both the growth medium and system design, and this aspect was not investigated in the present study.

3.4. Microbiological quality of the cultures

Microalgae cultivation is often associated with the growth of other prokaryotic and eukaryotic microorganisms as contaminants. These microorganisms may originate from the initial seed microalgae, from media components, or through contamination during cultivation. Such microbial communities can affect the growth of the target microalgae

and alter nutrient utilization. To assess their presence under different cultivation conditions, amplicon sequencing of the 16S (prokaryotic) and 18S (eukaryotic) rRNA genes was performed on the 1L PBR reactors on the last day (Day 16) of cultivation (Fig. 2). The 18S rRNA data confirmed the dominance of the target microalgae, which constituted 89–100 % of the eukaryotic reads in all non-contaminated samples. All cultures of *T. obliquus* 3A were found to be axenic, with no significant bacterial or fungal reads detected, indicating that any potential contamination was below the detection limit of the method. In contrast, reactors of *C. vulgaris* grown in LFAW were contaminated by the fungus *Talaromyces*, a genus known to contain food spoilage species (Pitt and Hocking, 2022). Fungal contamination was not present in the *C. vulgaris* cultures grown in S-LFAW and BBMGGL. This could indicate competitive exclusion (Grover, 1991), as the targeted supplementation created conditions that favored fast and robust growth of *C. vulgaris*, allowing the microalgae to effectively outcompete the fungus for limiting resources like nitrogen and phosphorus and maintain a stable, high-purity culture. In cultures where *T. obliquus* 1A growth was suppressed, a significant bacterial takeover was observed. Interestingly, *Methylobacterium* and *Lacrimispora* genera were found in LFAW reactors, *Bacillus* genus was abundant in S-LFAW reactors, suggesting the composition of bacterial community was media dependent. Further investigation is required to confirm whether these bacterial communities were responsible for the limited growth of *T. obliquus* 1A in LFAW and S-LFAW.

Table 1

Mixotrophic growth parameters for 1L photobioreactors cultivated in Bold's Basal Medium (BBM), Bold's Basal Medium with glucose, galactose, lactose (equivalent concentrations in acid whey) (BBMGGL), Lactose-Free Acid Whey (LFAW), and Supplemented-LFAW (SLFAW).

	μ_{max} (day ⁻¹)	X_{final} (g/L)	t_d (days)	dS/dt	q_s	$Y_{X/S}$	% Removal of Organic Carbon	% Removal of Nitrate	% Removal of Orthophosphate
<i>C. vulgaris</i>									
BBM	0.3 ± 0.0	0.1 ± 0.0	2.4 ± 0.1 ⁺	n.a.	n.a.	n.a.	n.a.	99.5 ± 0.1 ^ψ	70.8 ± 2.3
BBMGGL	0.8 ± 0.1 ⁺	1.3 ± 0.5 ⁺	0.9 ± 0.1 ⁺	0.4 ± 0.1	0.3	0.3 ± 0.1	11.4 ± 0.3 ⁺	99.4 ± 0.0 ^φ	73.9 ± 0.2
LFAW	0.7 ± 0.0 ⁺	2.0 ± 0.7 ⁺⁺	1.0 ± 0.0 ⁺	0.4 ± 0.1	0.2	0.1 ± 0.1	9.8 ± 2.0 ⁺	93.7 ± 1.3 ^{ψ φ †}	10.7 ± 3.1
S-LFAW	1.2 ± 0.2 ⁺	2.2 ± 0.5 [*]	0.6 ± 0.1 ⁺	0.4 ± 0.1	0.2	0.5 ± 0.2	14.6 ± 5.4 ⁺	99.8 ± 0.0 [†]	62.5 ± 3.6
<i>T. obliquus</i>									
3A									
BBM	0.1 ± 0.1 [*]	0.0 ± 0.0 [†]	7.3 ± 4.0	n.a.	n.a.	n.a.	n.a.	98.7 ± 0.0	49.5 ± 2.8
BBMGGL	0.7 ± 0.1 ⁺	0.7 ± 0.1	1.0 ± 0.2 ⁺	0.2 ± 0.1	0.3	0.2 ± 0.1	33.9 ± 6.2 ⁺	98.9 ± 0.0 ^φ	60.4 ± 1.1
LFAW	0.3 ± 0.1 [*]	0.2 ± 0.1 ^ψ	3.1 ± 1.5 ⁺	0.1 ± 0.0	0.6	2.0 ± 0.2	30.6 ± 7.6 ⁺	97.9 ± 0.2 ^{φ ψ}	7.1 ± 2.5
S-LFAW	0.7 ± 0.3 ⁺	1.3 ± 0.2 ^{† ψ}	1.1 ± 0.4 ⁺	0.2 ± 0.1	0.2	0.4 ± 0.1	31.3 ± 3.8 ⁺	99.2 ± 0.4 ^ψ	40.3 ± 13.1
<i>T. obliquus</i>									
1A									
BBM	0.0 ± 0.0	0.0 ± 0.0	10.5 ± 3.9	n.a.	n.a.	n.a.	n.a.	n.d.	n.d.
BBMGGL	0.8 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
LFAW	0.2 ± 0.1 ⁺	0.0 ± 0.0 ⁺	3.6 ± 1.1 ⁺	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S-LFAW	0.2 ± 0.1 ⁺	0.0 ± 0.0 ⁺	3.3 ± 1.4 ⁺	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.a. not applicable, n.d. not determined, [†], ^ψ, ^φ denote significant difference between pairs within groups of microalgae species. ^{*}, ⁺ no significant difference between pairs.

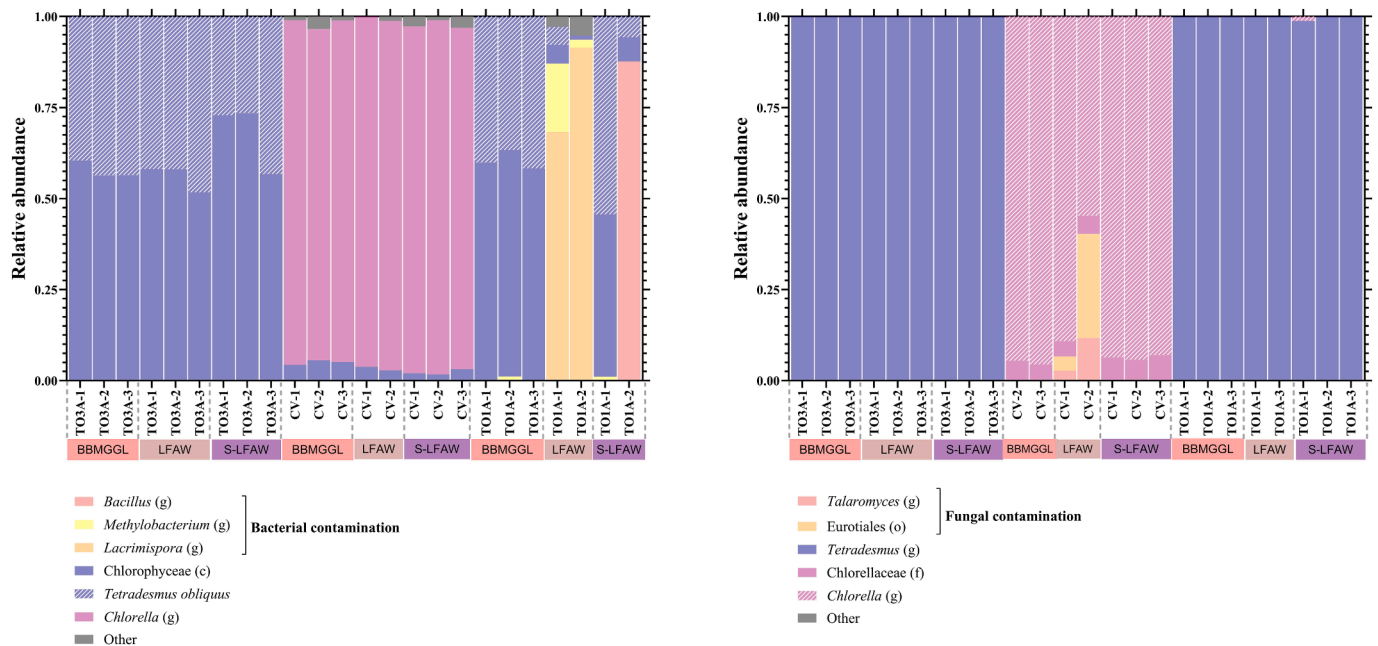


Fig. 2. Relative abundance of amplicon reads from 16S and 18S rRNA sequencing. The left panel shows the bacterial community composition and microalgae (identified by chloroplast genome) from 16S rRNA amplicon sequencing to visualize the proportion of bacteria. The right panel displays the fungal community profile and microalgae (from 18S rRNA) to assess fungal contamination. Each bar represents a sample, with taxa color-coded by classification. TO3A: *Tetradesmus obliquus* 3A, CV: *Chlorella vulgaris*, TO1A: *T. obliquus* 1A. Numbers adjacent to the strain abbreviations represent biological triplicate numbers. BBMGGL: Bold's Basal Medium with glucose, galactose, lactose, LFAW: Lactose-free acid whey, S-LFAW: Supplemented LFAW. Taxa are shown at the highest resolved rank available (class (c), order (o), family (f), genus (g), species).

3.5. Protein and total amino acid profile of the biomass

Phototrophic cultivation of *T. obliquus* 3A in BBM resulted in the highest yield of protein content with 19 g_{protein}·100g_{biomass}⁻¹ (Fig. 3) with 0.4 mg/L/day protein productivity. *T. obliquus* 3A produced a protein content of 15 g_{protein}·100g_{biomass}⁻¹ when cultivated in BBMGGL with 7 mg/L/day productivity. Compared to LFAW, *T. obliquus* 3A accumulated

additional 3 g_{protein}·100g_{biomass}⁻¹ when cultivated in S-LFAW which is also higher than control group (BBMGGL). Due to very limited sample amount, *T. obliquus* 1A biomass could not be analyzed. *C. vulgaris* accumulated the highest protein when cultivated in LFAW (11 g_{protein}·100g_{biomass}⁻¹), however, it was substantially lower compared to previously reported data in the literature (Cai et al., 2022). *C. vulgaris* accumulated a substantially higher protein content in S-LFAW (9

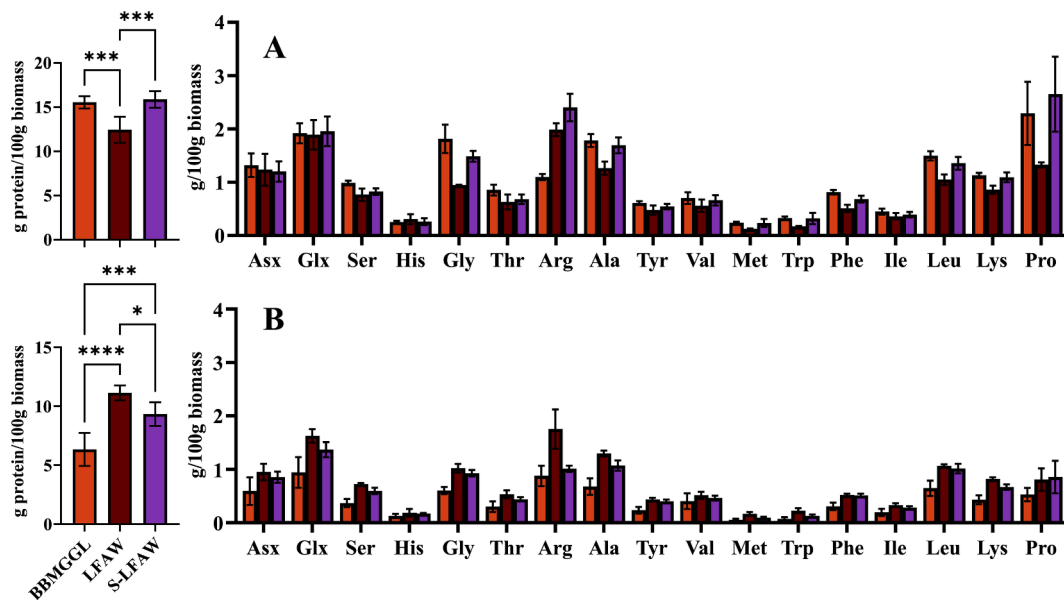


Fig. 3. Total protein and amino acid content of (A) *T. obliquus* 3A cultivated in Bold's Basal Medium with glucose, galactose, lactose (equivalent concentrations in acid whey) (BBMGGL) (red), Lactose-Free Acid Whey (LFAW) (burgundy), and Supplemented-LFAW (SLFAW) (purple) and (B) *C. vulgaris* cultivated in BBMGGGL (red), LFAW (burgundy), and S-LFAW (purple). Cystein (Cys) was not quantified. Glx denotes the sum of Glutamate (Glu) and Glutamine (Gln), Asx denotes the sum of Asparagine (Asn) and Aspartate (Asp).

$\text{g}_{\text{protein}} \cdot 100 \text{g}_{\text{biomass}}^{-1}$) compared to the BBMGGGL control. Protein productivities of *C. vulgaris* were 14 and 13 mg/L/day in LFAW and S-LFAW, respectively. Using hydrolyzed cheese whey in batch cultivation mode, Abreu et al. (2012) reported a significantly higher protein content (64 %) and a protein productivity of 474 mg/L/day for *C. vulgaris*. This superior performance is likely due to the use of CO₂ supplementation to boost photosynthesis.

The protein and amino acid profiles of *C. vulgaris* and *T. obliquus* 3A reveal the relationship between nutrient availability, metabolic strategy, and strain-specific stress responses, providing deeper insight into the growth results observed previously. Nitrogen is the most critical macronutrient for protein synthesis, and its availability dictates the metabolic compromises between producing nitrogen-rich compounds like proteins versus nitrogen-poor storage molecules like lipids and carbohydrates. Nitrate concentrations in LFAW, S-LFAW, and BBMGGGL were 0.02 g/L, 0.18 g/L, and 0.18 g/L, respectively (see [supplementary datasheet](#)). Since these media contained lower nitrogen concentrations than previously reported near optimal media, reduced protein accumulation was expected for both *C. vulgaris* and *T. obliquus* (Griffiths et al., 2014; Rani and Maróti, 2021; Xie et al., 2017). Several studies have reported that nitrate concentrations varying between 1.2–2.4 g/L depending on the cultivation mode, resulted in maximal protein yields for *C. vulgaris* (Griffiths et al., 2014; Rani and Maróti, 2021; Xie et al., 2017). Two-step heterotrophic cultivation of *C. vulgaris* with nitrogen depletion followed by nitrate feeding resulted in maximal protein yield of 44.3 % with nitrate concentration of 2.4 g/L in the nitrogen feeding phase and initial nitrate level of 0.18 g/L (Xie et al., 2017). Another study reported that protein content increased when nitrate concentration increased from 0.3 g/L to 0.6 g/L but decreased at 0.9 g/L suggesting that optimal protein production in mixotrophic conditions for *Chlorella* sp. occur around 0.6 g/L nitrate supplementation in TAP medium (Rani and Maróti, 2021). Under nitrogen replete conditions (specifically 1.2 and 2.0 g/L), *C. vulgaris* maintained a stable biochemical composition with high protein content. In contrast, under nitrogen-limited conditions (0 to 0.4 g/L nitrate), a rapid decline in protein content was observed (Griffiths et al., 2014).

Studies on *Scenedesmus* (*T. obliquus* was formerly classified in the *Scenedesmus* genus) genus have shown that decreasing nitrogen concentration leads to a significant drop in growth and protein content

while increasing lipid accumulation by as much as 2.5-fold (Akgül and Akgül, 2022). The observed increased recovery in protein content when moving from the nitrogen-starved LFAW to the supplemented S-LFAW further validates this principle, showing a classic cellular response to the partial mitigation of nitrogen limitation. However, the performance of *T. obliquus* 3A in the whey-based media appears to be constrained by more than just nitrogen availability. Even in the supplemented S-LFAW, protein levels did not reach those seen in the BBM medium. This is particularly noteworthy because mixotrophic cultivation, where algae utilizes both light and an external organic carbon source, is generally expected to produce protein levels that are similar to or even significantly higher than those from phototrophic cultivation. For example, a study by Piasecka et al. found that the protein content of *T. obliquus* increased from 24.1 % in phototrophic culture to 45.2 % in mixotrophic culture, nearly doubling the protein content and quadrupling the daily protein productivity (Piasecka et al., 2020). Similarly, another study reported a protein content of 44 % in mixotrophic and only 32 % in autotrophic cultivation of *S. obliquus* (Silkina et al., 2025). The failure of *T. obliquus* 3A to approach its phototrophic benchmark in S-LFAW, despite the anticipated advantage of mixotrophy, strongly suggests further nitrogen deficiencies for optimal protein biosynthesis or the influence of additional environmental stressors inherent to this side-stream.

The protein quality of *T. obliquus* 3A and *C. vulgaris* was evaluated by comparing their essential amino acid (EAA) composition in mg/g protein to the established FAO/WHO dietary requirement pattern for adults and soybean (*Glycine max*) (Kuiken and Lyman, 1949; WHO, 2007). Soybean data is used as the benchmark, as soybean is widely regarded as a high-quality protein source and classified by the WHO as a complete plant-based protein. (WHO, 2007). Both microalgal species demonstrated promising protein quality, with variability depending on the cultivation medium. The EAA profiles were largely comparable to or exceeded those of optimally grown *C. vulgaris* reported in the literature (Becker, 2007). In *T. obliquus* 3A, EAA profiles were similar across all media, with Leu (84–96 mg/g protein), Lys (69–72 mg/g protein), aromatic AAs (77–91 mg/g protein), Thr (43–55 mg/g protein), and Trp (13–21 mg/g protein) all exceeding both soybean and FAO/WHO requirements. Similarly, *C. vulgaris* exhibited superior levels of Leu (95–109 mg/g protein), Lys (6873 mg/g protein), and aromatic AAs

(85–97 mg/g protein) across all media. However, consistent with many literature reports for *C. vulgaris*, methionine was the primary limiting amino acid in both species (Becker, 2007; Safi et al., 2014), with concentrations ranging from 8 to 15 mg/g protein, well below the FAO/WHO threshold of 16 mg/g. In *T. obliquus* 3A, Met ranged from 10 mg/g protein (LFAW) to 15 mg/g (BBMGGL), with an increase observed in S-LFAW (15 mg/g protein).

The protein from *T. obliquus* 3A and *C. vulgaris* exhibits a favorable amino acid profile, suggesting it could serve as a nutritionally valuable protein source. Comparing the protein qualities of LFAW and S-LFAW biomass demonstrates that the cultivation media can be modified to tune the final protein quality. In BBMGGL, *T. obliquus* 3A produces a protein with a Lys content (72 mg/g protein) that exceeds average soybean levels, demonstrating its capacity to serve as a standalone, high-quality protein source. S-LFAW-grown *T. obliquus* 3A and *C. vulgaris* produce protein that is rich in Leu (85 mg/g protein and 109 mg/g protein, respectively), a key EAA for stimulating muscle protein synthesis in older adults (Wilkinson et al., 2023). These concentrations surpass those found in average soybean meal and FAO/WHO requirement (59 mg/g protein), suggesting that microalgae grown on this substrate could be a premium plant-based ingredient for functional foods when proteins are

isolated. While Met was identified as the limiting amino acid, unlike the relatively unchanged profile of soybean, microalgae offer a dynamic system where cultivation on by-products can be optimized to produce specialized proteins, creating high-value nutritional ingredients.

3.6. Fatty acid profile of the biomass

The effect of different cultivation media with differing C:N ratios and elemental composition on fatty acid composition of microalgal biomass was investigated. Total fatty acid (TFA) composition of the freeze-dried biomass was quantitatively analyzed using the direct-FAME method. The complete fatty acid profile of *C. vulgaris* and *T. obliquus* 3A biomass harvested from 1L PBRs is shown in Fig. 4. Fatty acid productivities are given in brackets where relevant with the unit mg/L/day.

The TFA content, productivity, and composition of *C. vulgaris* varied significantly across the three tested culture media: BBMGGL, LFAW, and S-LFAW. Cultivation in BBMGGL yielded the highest TFA content at 22 g/100 g dry matter (18 mg/L/day productivity), which was similar to that achieved in S-LFAW (22 g/100 g dry matter and 31 mg/L/day). In contrast, cells grown in LFAW accumulated only 11 g/100 g dry matter, representing a two-fold decrease in fatty acid yield compared to the

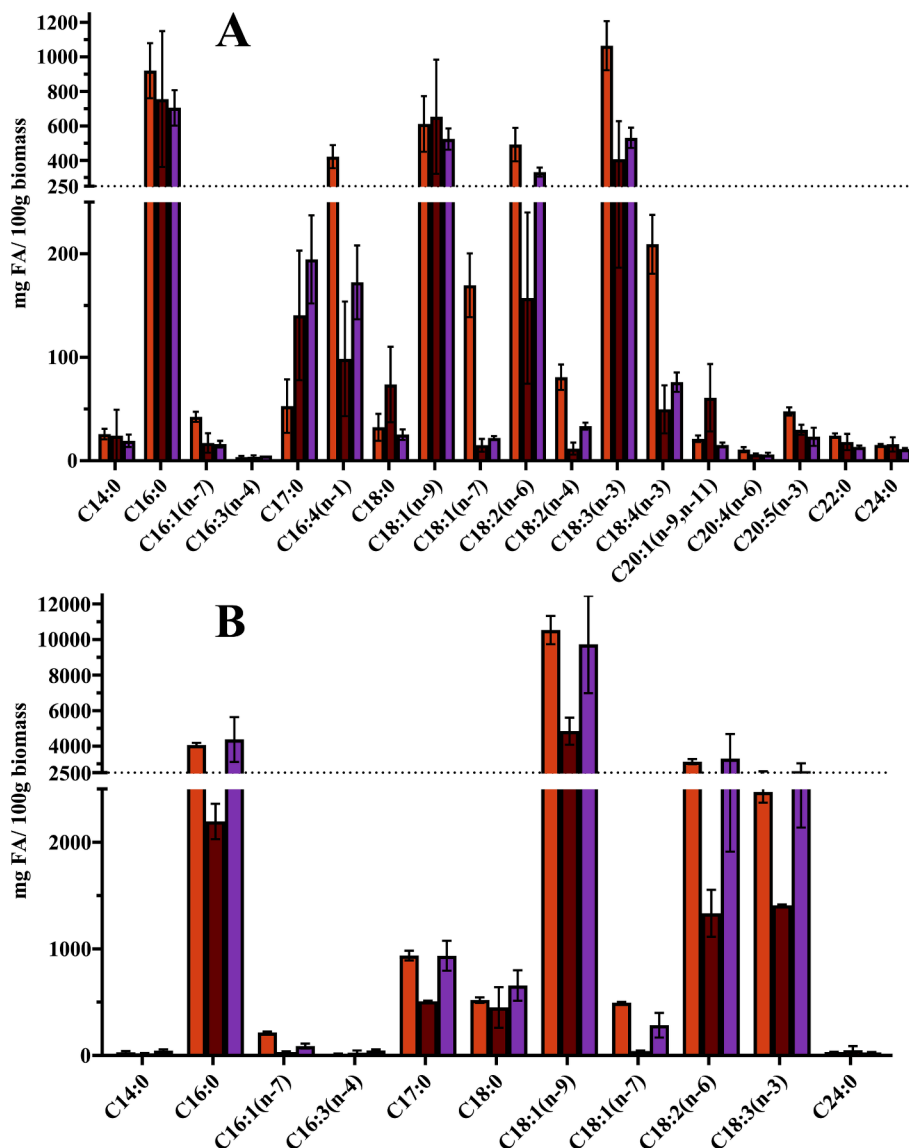


Fig. 4. Fatty acid profile of *T. obliquus* 3A (A), and *C. vulgaris* Bold's Basal Medium with glucose, galactose, lactose (equivalent concentrations in acid whey) (BBMGGL) (red), Lactose-Free Acid Whey (LFAW) (burgundy), and Supplemented-LFAW (S-LFAW) (purple).

other two media with 14 mg/L/day of productivity which is substantially lower than productivities in other media. This trend was consistent across all fatty acid classes, with significantly lower levels of both monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) in the LFAW-grown *C. vulgaris*. Specifically, MUFA content in *C. vulgaris* was 11 g/100 g dry matter in BBMGGGL, 5 g/100 g dry matter in LFAW, and 10 g/100 g dry matter in S-LFAW. PUFA levels followed a similar pattern, with 6 g/100 g dry matter in BBMGGGL, 3 g/100 g dry matter in LFAW, and 6 g/100 g dry matter in S-LFAW. Omega-3 fatty acids, primarily ALA, were also affected by the culture medium. In *C. vulgaris*, omega-3 content was 2 g/100 g dry matter in BBMGGGL, 1 g/100 g dry matter in LFAW, and 3 g/100 g dry matter in S-LFAW. The results corroborate earlier reports on *C. vulgaris* lipid profiles where oleic acid was the dominant MUFA, total MUFAs were 0.7 g/100 g dry matter and PUFA content of *C. vulgaris* was reported as 0.3 g/100 g dry matter (Cabrita et al., 2022).

T. obliquus 3A displayed relatively low and invariant TFA content across all conditions, with yields of 4 g/100 g dry matter in BBMGGGL (2 mg/L/day), 2 g/100 g dry matter in LFAW (1 mg/L/day), and 3 g/100 g dry matter in S-LFAW (2 mg/L/day). MUFA levels were low across all treatments, ranging from 0.6 to 0.8 g/100 g dry matter, while PUFA content varied from 0.8 g/100 g dry matter in LFAW to 2 g/100 g in BBMGGGL. Omega-3 fatty acid content in *T. obliquus* remained below 1 g/100 g dry matter in all cases, with BBMGGGL showing the highest value (1 g/100 g dry matter), followed by S-LFAW (0.6 g/100 g dry matter) and LFAW (0.5 g/100 g dry matter).

The modest lipid yields in *T. obliquus* 3A suggest a metabolic diversion of fixed carbon away from lipids and towards polysaccharides, such as starch, as the primary energy and carbon reserve under these specific cultivation conditions (Yu et al., 2011). This metabolic response is a well-documented characteristic of *Scenedesmeaceae* family, where during active growth or under moderate nutrient availability, carbon is preferentially channeled into rapidly metabolizable starch granules (León-Saiki et al., 2017). Furthermore, high carbon loading has been shown to increase polysaccharide accumulation up to 20 % of dry biomass in *T. obliquus* (Chen et al., 2023). A significant shift towards triacylglycerol (TAG) synthesis typically occurs only under more severe or prolonged stress, particularly nitrogen deprivation, often following the degradation of the initially synthesized starch reserves (Breuer et al., 2015). The consistently low lipid accumulation in this study indicates that *T. obliquus* 3A did not experience significant nutrient stress, such as nitrogen depletion, that is necessary to activate large-scale lipogenesis. The current study shows that the nutrient composition and C:N ratio of the culture medium have a great influence on lipid accumulation in *C. vulgaris*, while *T. obliquus* 3A appears less responsive to such nutritional variations.

Nitrogen limitation and starvation are effective strategies to enhance lipid accumulation in microalgae. A starvation period for three days resulted in 31 % increase in lipid content of *C. vulgaris* but lower growth rate (Liu et al., 2022). Likewise, under nitrogen starvation, mixotrophic cultivation of *C. vulgaris* with acetate supplementation resulted in higher biomass and fatty acid productivity compared to both heterotrophic and phototrophic cultures (Shen et al., 2019). Also, while a C:N of 10:1 favored carbohydrate synthesis, a C:N of 30:1 was found to favor lipid biosynthesis of *C. vulgaris* (Grubišić et al., 2024). Previous work identified BBM as a medium yielding the highest lipid content (14.6 g/100 g dry matter), outperforming alternative media like BG-11 and M-8 (Yadav et al., 2024). The high TFA yields observed in the current study using BBMGGGL and S-LFAW reaffirm that media with moderate C:N ratios and sufficient macro- and micronutrients are sufficient for lipid productivity for *C. vulgaris*. BBMGGGL and S-LFAW share the same C:N ratio of 205:1 and provided more balanced nutrient profiles, leading to higher TFA accumulation. Cultivation of *C. vulgaris* under mixotrophic conditions, glucose at a concentration of 20 g/L combined with a C:N 30:1, resulted in maximal lipid content (37 g/100 g dry matter) whereas biomass from C:N 10:1 cultures accumulated around 22 g/100 g dry

matter (Grubišić et al., 2024). Heterotrophic cultivation of *C. vulgaris* with 35 g/L glucose led to 7.26-fold and 1.05-fold increase in lipid productivity compared to phototrophic and mixotrophic conditions (Zhao et al., 2013). This supports earlier findings that mixotrophic cultures of *C. vulgaris* under nitrogen-replete or mildly deficient conditions can achieve superior lipid productivity, especially when acetate or glucose is supplemented (Grubišić et al., 2024; Liang et al., 2009). By contrast, LFAW had a substantially higher C:N ratio (2285:1) but was limited in phosphate and trace metals, explaining its reduced lipid productivity. Thus, the type and concentration of the carbon source, as well as the trophic mode of cultivation, impacts lipid productivity significantly.

3.7. Carotenoid content and composition of the biomass

C. vulgaris and *T. obliquus* are known to synthesize various carotenoids, including the primary photosynthetic pigment lutein, which is integral to the light-harvesting complexes, and the secondary photoprotective pigments β -carotene and zeaxanthin, which are central to antioxidant defense and dissipating excess light energy. This study investigated how pigment biosynthesis is modulated by nutrient availability and carbon sources under the continuous illumination in a mixotrophic setting. Absolute quantification of lutein, zeaxanthin isomers, and β -carotene in the microalgal biomass revealed that the pigment composition of *T. obliquus* 3A and *C. vulgaris* varied significantly across the four different cultivation media, highlighting the influence of nutrient availability and carbon sources on pigment biosynthesis under mixotrophic conditions with continuous light supply (Fig. 5).

In *T. obliquus* 3A, lutein content was highest in the defined media (BBM: 165 ± 8 mg/100 g; BBMGGGL: 153 ± 6 mg/100 g), indicating robust photosynthetic machinery under these conditions. However, lutein levels decreased in LFAW (19 ± 3 mg/100 g). This sharp decline is likely a direct consequence of severe limitation of essential nutrients (i.e. nitrogen, phosphorus, and trace metals), which halted the ability of microalgae to build and maintain its photosynthetic apparatus, leading to decreased photosynthetic function under continuous light, hence limited growth in this media. The partial recovery of lutein in S-LFAW, where these nutrients were replenished, supports this interpretation (Fig. 5).

C. vulgaris exhibited its highest lutein content in S-LFAW (109 ± 12 mg/100 g dry matter) and lowest in BBMGGGL (32 ± 5 mg/100 g dry matter). The enhanced pigment accumulation in nutrient-supplemented acid whey indicates a more efficient photoprotective and photosynthetic response to continuous light stress, especially under nutrient-rich conditions. Zeaxanthin isomers showed moderate levels across media in both species but increased in S-LFAW-grown *T. obliquus* (24 ± 7 mg/100 g dry matter), suggesting the ability to activate the xanthophyll cycle under stress when supplied by limiting nutrients.

These divergent pigment profiles are directly linked to the distinct metabolic strategies of the two species. The low organic carbon removal by *C. vulgaris* (10–15 %), even in carbon-rich media, suggests a predominantly phototrophic metabolism. It maintains a strong photosynthetic metabolism and uses external organic carbon opportunistically. For *T. obliquus* 3A, the significantly higher organic carbon removal (31–34 %) in BBMGGGL indicates a more flexible mixotrophic strategy. However, this strategy was unsuccessful in LFAW, where the lack of essential inorganic nutrients hindered the synthesis of photoprotective pigments, creating a bottleneck that inhibited cell proliferation despite the availability of organic carbon.

As a specific nutrient stress, nitrogen deprivation is known to alter the carotenoid profile in microalgae, though the response is highly species dependent. Studies on *Chlorella* genus shows nitrogen limitation generally leads to an increase in total carotenoids (Yong et al., 2019). This increase, however, often reflects a strategic reallocation of resources from primary to secondary carotenoids. For instance, a detailed study on *Chlorella zofingiensis* demonstrated high nitrate concentrations

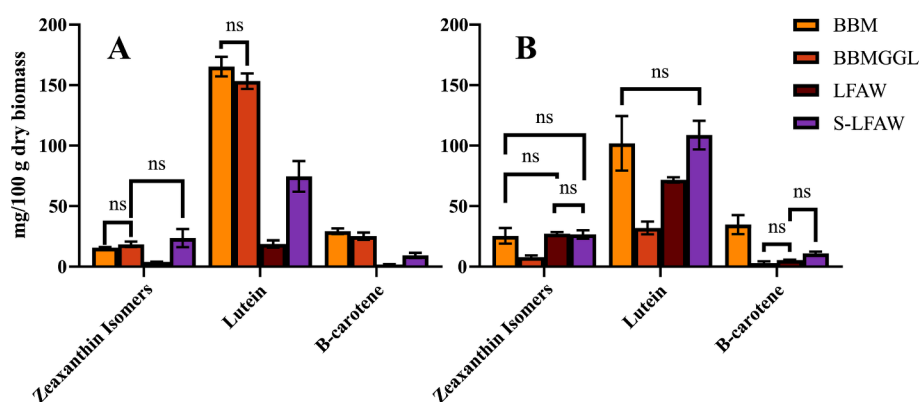


Fig. 5. Pigment content of *T. obliquus* 3A (A) and *C. vulgaris* (B) cultivated in Bold's Basal Medium with glucose, galactose, lactose (equivalent concentrations in acid whey) (BBMGGL) (red), Lactose-Free Acid Whey (LFAW) (burgundy), and Supplemented-LFAW (SLFAW) (purple). ns: not significant, the rest of the group comparisons are all significant ($p < 0.05$).

promoted the accumulation of lutein (a primary carotenoid essential for photosynthesis), whereas nitrogen deficiency triggered a significant shift towards the production of the secondary, protective carotenoid astaxanthin (Del Campo et al., 2004). This metabolic shift to synthesize protective pigments occurred even as low nitrate availability suppressed overall cell growth, highlighting an adaptive strategy that prioritizes survival over proliferation under stress. This aligns with other findings where doubling the nitrogen concentrations led to a doubling of lutein content, reinforcing the role of primary carotenoids in active growth phases (Del Campo et al., 2000). On the other hand, the response in *T. obliquus* is also complex; research shows that the relationship between nitrogen and lutein is not linear, with lutein concentration peaking at an optimal nitrogen supply (1 mg N/L) and then decreasing as nitrogen levels increase further (An et al., 2020). In another study, it is reported that 2 days of nitrogen starvation resulted in a decrease of total chlorophyll and carotenoid contents by 15 % and 17 %, respectively (Roy et al., 2021). This is also supported by the current study.

4. Conclusion

This study demonstrates a successful route for valorizing lactose free acid whey as a substrate for microalgae cultivation. However, the success was found to be strain specific. *C. vulgaris* and *T. obliquus* 3A achieved high biomass yields while *T. obliquus* 1A was intolerant. Supplementing the side-stream enhanced biomass productivity and reduced microbial contamination. Cultivation in supplemented medium yielded biomass with favorable carotenoid, amino acid and fatty acid profiles suitable for food applications. Although the protein content was modest, the inherent physiological ability of these microalgae for high protein synthesis presents an opportunity for future optimization to maximize this component. This study fills a research gap by being the first to characterize and optimize LFAW through targeted nutrient supplementation for microalgae cultivation. It provides a proof-of-concept for integrating the dairy and food industries within a circular bioeconomy that justifies future research into process optimization using this underexplored feedstock. Successful industrial application depends on selecting specific strains with high natural tolerance to acid whey and on targeted medium optimization to resolve any remaining nutrient imbalances.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used Microsoft Copilot version 1.25064.139.0 in order to check grammar and coherence of the text. Additionally, author(s) used the assistance of Cursor (v. 1.2.4) AI code editor to partially edit the script for the bioinformatics.

After using these tools, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

CRediT authorship contribution statement

Duygu Özcelik: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Rocio Espinosa:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Radhakrishna Shetty:** Writing – review & editing, Methodology, Formal analysis. **Shyam Suwal:** Writing – review & editing, Resources, Conceptualization. **Henriette Lyng Røder:** Writing – review & editing, Supervision, Resources. **Charlotte Jacobsen:** Writing – review & editing, Supervision, Resources. **Colin Ray:** Conceptualization, Writing – review & editing. **Brijesh K. Tiwari:** Writing – review & editing, Funding acquisition, Conceptualization. **Poul Erik Jensen:** Writing – review & editing, Supervision, Resources, Conceptualization. **Mahesha M. Poojary:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biortech.2025.133559>.

Data availability

Data will be made available on request.

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