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Cold stress stimulates algae to produce value-added compounds

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ABSTRACT

Two cold-tolerant microalgae, *Chlorella vulgaris* and *Scenedesmus* sp., were grown at 22 and 5 °C. At the lower temperature, the microalgae showed substantial biochemical and morphological changes. The soluble sugar profile in response to low-temperature cultivation was very different in the two strains. *C. vulgaris* increased both the sucrose and raffinose family oligosaccharides (RFOs) content at 5 °C while *Scenedesmus* sp. drastically reduced the sucrose content. Both strains increased the total fatty acid methyl ester (FAME) content when grown at 5 °C. However, the FAME profiles were very different: *C. vulgaris* mainly increased C18:1 and less so C18:3, while *Scenedesmus* sp. decreased C18:1 but greatly increased C18:3. The morphology of *C. vulgaris* changed slightly at the lower temperature, while *Scenedesmus* sp. showed substantial changes in the size and shape. Low temperature triggered the synthesis of unsaturated fatty acids that are essential for human nutrition.

1. Introduction

Microalgae are microorganisms that are very abundant in many ecosystems (Abdelaziz et al., 2013). In recent years, microalgae have received attention due to their potential applications in a wide range of industries, such as food and feed, biopharmaceuticals, nutraceuticals, and renewable energy sources. Biofuels, animal feeds, bioactive medicinal products, food ingredients, cosmetics, and health supplements are some of the applications where microalgae are considered a sustainable, economical, and renewable source (Khan et al., 2018; Silva et al., 2020. These fast-growing, unicellular organisms have the capacity to fix atmospheric carbon dioxide (CO_2) via photosynthesis, producing high-value biomass such as lipids, pigments, polysaccharides, bioactive compounds, biopolyesters, and bio-hydrogen (Koller et al., 2014; Silva et al., 2020).

Microalgae are not only good for products that can be produced after harvesting, they also have great ecological benefits during cultivation. Their ability to fix atmospheric CO_2 sustainably is of great significance to sustain Earth's ecosystem, to reduce greenhouse gases in the atmosphere, and to limit global warming (Kurano et al., 1995; Velea et al., 2009). Microalgae could also be used to fix CO_2 directly from industrial exhaust gases such as flue gas, which is a CO_2-rich source compared with atmospheric CO_2 and, therefore, better for microalgal cultivation and the environment (Wang et al., 2008). Their ability to remove nutrients from wastewater is another useful ecological benefit and has attracted the interest of the scientific community. Minimal care during cultivation and the ability to grow in harsh conditions make microalgae suitable for growth in wastewater and, as a result, they can remove mainly nitrogen and phosphorous from wastewater. Wastewater treatment with microalgae makes the process more environmentally sustainable and economical (Mata et al., 2010).

In a study dealing with microalgal cultivation for the production of bioenergy, *Chlorella vulgaris* was identified as the most promising of the 10 species tested (Cancela et al., 2019). In a recent work, a *C. vulgaris* strain obtained by ultraviolet (UV) mutagenesis could accumulate up to 50 % of its biomass as carbohydrates under photoautotrophic culture conditions (Cheng et al., 2022).

Sucrose is a known sugar in microalgae and can accumulate in response to salt, desiccation, osmotic, heat, or cold stress (Salerno and Pontis, 1989; Sanz Smachetti et al., 2020). Another sugar that accumulates in response to cold shock in *C. vulgaris*, and that is usually not considered to be present in algae, is raffinose (Salerno and Pontis, 1989). Raffinose is a trisaccharide with a α-1, 6-galactosyl extension to sucrose, meaning it is composed of galactose, glucose, and fructose, and it is part of the raffinose family of oligosaccharides (RFOs) that are all α-galactosyl derivatives of sucrose. These sugars are usually found in higher plants, in which they are suggested to have numerous important functions. Signal transduction, carbon transport and storage, messenger RNA

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(mRNA) export, and membrane trafficking are some of the important cellular functions of which RFOs are considered to be a part (Sengupta et al., 2015). In seeds, RFOs are stored and synthesised to protect the seed from desiccation, extending longevity and providing the seed with energy during germination (Downie et al., 2003). Moreover, raffinose accumulation in higher plants helps protect plants from oxidative damage triggered by salinity, methyl viologen treatment, or chilling (Nishizawa et al., 2008). A possible use for raffinose is in the functional food industry because it is a prebiotic that is beneficial to iron bioavailability, the function of the brush border membrane, and the gut microflora (Pacifici et al., 2017).

In a previous study, researchers showed that the content of polyunsaturated fatty acids in *Scenedesmus* sp. increased with a decrease in temperature (Li et al., 2011). By changing the temperature from 20 to 10 °C, the polyunsaturated linolenic acid (C18:3) content increased dramatically. On the contrary, the long chain polyunsaturated fatty acid C22:3 was no longer present when the temperature increased from 10 to 20 °C (Li et al., 2011). The green alga *Acutodesmus obliquus* grown at 20, 30, and 35 °C had the highest polyunsaturated fatty acid content at the lowest temperature tested (Helamieh et al., 2021). Algal species respond to temperature changes in different ways – for example, two marine dinoflagellates belonging to the genus *Symbiodinium* showed an increase in fatty ethyl methyl esters (FAMEs) and docosahexaenoic acid (DHA) content when the temperature decreased from 27 to 22 and 17 °C (Tsirigoti et al., 2020). Another marine microalga, *Dunaliella tertiolecta*, showed an increase in C18:3 in a strain grown at a lower temperature compared with another strain grown at a higher temperature (Kim et al., 2014). In several different algal species, lowering the cultivation temperature induces an increase in polyunsaturated fatty acids that allows the algal cell to maintain membrane fluidity at lower temperatures (Sharma et al., 2012). In a previous study, other northern Sweden microalgae were screened for FAME production at 25 °C, showing that C16:0 and C18:3 were the dominant FAMEs (Nzayisenga et al., 2020).

The aim of this study was to highlight the potential biotechnological use of two cold-tolerant algae grown at a low temperature. The study hypothesis is that low temperature affects a) soluble sugar accumulation and b) the quality and quantity of fatty acids.

2. Materials and methods

2.1. Microalgae cultivation

Two cold-tolerant microalgal strains, *C. vulgaris* strain 13-1 and *Scenedesmus* sp. strain B2-2, isolated in Umeå, Sweden – with ability to efficiently remove nutrients in wastewater during cold-stress – were selected for this study (Ferro et al., 2018a, 2018b). The two microalgae were pre-cultivated in sterile 500-mL borosilicate glass bottles containing 400 mL of autoclaved Bold's Basal Medium (BBM; Bischoff and Bold, 1963) at pH 6.7. The bottles were inoculated with 6–8 loops (loop volume 5 μL) of algae grown on agar plates. Once inoculated, the bottles were bubbled with approximately 0.4 L air min^{-1} L^{-1} culture medium and placed on an orbital shaker at 105 rpm in a climate cabinet (A1000, Conviron, Winnipeg, Canada) at 22 °C with a 16-h:8-h light:dark cycle. The cultures were supplied with artificial light at a photosynthetically active radiation (PAR) of approximately 40–45 μmol m^{-2} s^{-1} measured at the middle height of the bottle. Pre-cultivation lasted for 8 and 5 days for the batches grown at 22 and 5 °C, respectively.

After pre-cultivation, sterile 1-L borosilicate glass bottles received 0.9 L of autoclaved BBM medium (pH 6.7) and were inoculated with 0.1 L of the pre-cultivated algae. The cultivation conditions were as described above except that the cultures were bubbled with approximately 0.7 L air min^{-1} L^{-1} culture medium. Each strain was cultivated in triplicate.

To monitor the growth phase, the optical density (OD) was measured at 750 nm, in an Epoch 2 microplate reader (BioTek, Winooski, VT, USA) every day during growth at 22 °C and every other or third day during growth at 5 °C. For algae grown at 22 °C, the start OD_{750} was 0.054 for *C. vulgaris* 13-1 and 0.045 for *Scenedesmus* sp. B2-2. For algae grown at 5 °C, the start OD_{750} was 0.070 for *C. vulgaris* 13-1 and 0.027 for *Scenedesmus* sp. B2-2. After 14 days of growth at 22 °C, the algae were harvested because both strains had reached the stationary phase. After 36 days of growth at 5 °C, *C. vulgaris* 13-1 had reached the stationary phase and was harvested. *Scenedesmus* sp. B2-2 was harvested after 46 days of growth at 5 °C, in the late exponential phase. Harvesting was done by centrifugation at 4000*g*. The supernatant was discarded, and then the pellets were quickly placed in liquid nitrogen and freeze-dried for approximately 48 h or until they reached a constant weight. The pellets were then stored in the dark under a dry atmosphere.

2.2. Morphological study

The morphology of the two strains cultivated at different temperature was studied by light microscopy using a Leica DMi1inverted microscope (Leica Microsystems, Wetzlar, Germany). Photographs of the two strains were taken at 400× total magnification. For *C. vulgaris*, 100 cells were measured for each cultivation temperature.

2.3. Biomass preparation for analysis

Freeze-dried algal biomass – three biological replicates for each strain – was transferred to 2-mL screw-cap plastic tubes containing ten 1-mm metal beads, and bead-milled for 3 × 30 s at 20 Hz with 1 min cooling between cycles (Bead Mill MAX, VWR, USA). Then, the beads were removed, and the milled biomass was stored in a dry, dark place until analysis.

2.4. Biochemical analyses

2.4.1. Soluble sugar analysis

Five-to-six milligrams of freeze-dried milled algal biomass was put in 1.5-mL screw-cap tubes. The sugars were extracted twice with 250 μL of 80 % ethanol and once with 250 μL of 50 % ethanol, at 90 °C for 30 min. The samples were vortexed before heating and once again after 15 min of heating. Then, the samples were put on ice to cool down and centrifuged at 13,000 rpm for 5 min (Universal 320, Hettich, Tuttlingen, Germany). The supernatants from all three extractions, containing the extracted sugars, were put in new 1.5-mL screw-cap tubes and stored at −20 °C until further analysis.

The soluble sugar analysis protocol originates from Stitt et al. (1989). Before the enzymatic assay, the samples were diluted 50:50 with 80 % ethanol. A determination mix was prepared; it contained 15.5 mL of 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES)/KOH pH 7.0 (including 3 mM $MgCl_2$), 480 μL of 45 mM nicotinamide adenine dinucleotide phosphate (NADP), 480 μL of 100 mM adenosine triphosphate (ATP), and 80 μL of glucose-6-phosphate dehydrogenase (Merck KGaA, Darmstadt, Germany). Next, 160 μL of the determination mix was put in each well of a 96-well plate and 50 μL of the samples or glucose standard (0, 0.25, 0.5, and 1 mM in triplicate) was added. The glucose standards were used to construct a standard curve to calculate hexose units in the sugar extracts. The absorbance of dihydronicotinamide adenine dinucleotide phosphate (NADPH) was read at 340 nm with an Epoch 2 spectrophotometer and the Gen 5™ ver. 1.10 software (BioTek). Once the absorbance had stabilised (about 5 min), 3 μL of hexokinase (Merck KGaA) was added to all wells to detect glucose. The absorbance was measured until stabilisation (14 min) and then 2 μL of phosphoglucose isomerase (Merck KGaA) was added to detect fructose. After 7 min, the reaction had stabilised and 3 μL of invertase (Merck KGaA) was added to detect sucrose (the reaction had stabilised after ca. 1 h). As a final step, 2 μL of α-galactosidase (NEOGEN/Megazyme, Bray, Ireland) was added to detect raffinose and other RFOs; the reaction had stabilised after 50 min.

2.4.2. Lipid analysis

The lipids were extracted with a single-step method, developed by Axelsson and Gentili (2014) based on the method described by Folch et al. (1957). Twenty milligrams of freeze-dried biomass was mixed with 9 mL of 2:1 chloroform:methanol (v/v) and 2.4 mL of 0.73 % sodium chloride to achieve a ratio of 2:1:0.8 (v/v/v) chloroform:methanol: water. The mixture was vortexed and centrifuged for 2 min at 400*g* (Sorvall ST16R, Thermo Scientific, Waltham, MA, USA) to separate the phases; the lower hydrophobic lipid phase was transferred to a new glass tube. Two more extractions were performed by adding 2 mL of chloroform to the remaining sample; the lower phases were transferred to the same new glass tube. The solutions were vacuum dried in a multi-evaporator (Syncore® Polyvap, Büchi Labortechnik AB, Flawil, Switzerland) overnight at 45 °C, 275 mbar, and 180 rpm.

For esterification of the total lipids, 1 mL of toluene and 2 mL of 1 % sulfuric acid in dry methanol were added to the tubes, vortexed, and fluxed with N_2 before incubation for 2 h at 80 °C. N_2 was added to create an anaerobic environment during the esterification reaction to prevent the lipids from oxidising or carbonating. After incubation, 5 mL of 5 % NaCl solution was added and the FAMEs were extracted twice with 5 mL of hexane by vortexing, centrifuging (2 min, 400*g*), and transferring the upper hexane layer to a new tube (Lage and Gentili, 2018). Then, 3 mL of 2 % potassium bicarbonate solution was added to wash the hexane samples and a small amount of anhydrous sodium sulfate was added to remove any remaining water in the hexane layer. The hexane layer was transferred to a new glass tube and 50 µL of a methanol solution containing 10 µg μL^{-1} methyl pentadecanoic acid (C15:0) was added to each tube as an internal standard. The samples were vacuum dried in a multi-evaporator overnight at 45 °C, 275 mbar, and 180 rpm (as described above), and then at 55 °C, 100 mbar, and 180 rpm for 100 min to remove any remaining toluene residue. The dried FAME extracts were re-dissolved in 400 µL of heptane, transferred to gas chromatography (GC) vials, and used for GC analysis.

To determine the FAME profile, the samples were injected with a TriPlus RSH autosampler (Thermo Fisher Scientific, Hägersten, Sweden) into a gas chromatograph (TRACE 1310, Thermo Fisher Scientific) equipped with a flame ionisation detector and a 30 m FAMEWAX column (Restek Corporation, Bellefonte, PA, USA) with a film thickness of 0.25 µm and an internal diameter of 0.32 mm (Lage & Gentili, 2018). The added internal standard, methyl pentadecanoic acid (C15:0), was used to quantify the FAMEs, and an external FAMEs standard mixture was used to identify the FAMEs by retention time. The external FAME standard was a mixture of methyl tetradecanoate (C14:0), methyl hexadecanoate (C16:0), methyl palmitoleate (C16:1), methyl heptadecanoate (C17:0), methyl octadecanoate (C18:0), methyl oleate (C18:1), methyl linoleate (C18.2), methyl linolenate (C18:3), methyl eicosanoate (C20:0), and methyl docosanoate (C22:0) purchased from Larodan (Solna, Sweden). The program Chromeleon 7.2.7 (Thermo Fisher Scientific) was used to analyse the GC result and calculations were made in Microsoft Excel. The total amount of FAMEs was calculated as described by Breuer et al. (2013).

2.5. Statistical analysis

Data were analysed using a two sample *t*-test at a 95 % confidence level (Microsoft Excel).

3. Results and discussion

3.1. Soluble sugar analysis

3.1.1. Chlorella vulgaris

After soluble sugar extraction, an enzymatic assay was performed to study and compare the amount of glucose, fructose, sucrose, and RFOs present in *C. vulgaris* cultured at different temperatures. The glucose and fructose content in the algae was very low or negligible; however, glucose showed a limited but statistically significant increase when *C. vulgaris* was grown at 5 °C compared to 22 °C (Fig. 1). The sucrose content increased by 14.6 %, from 328.5 nmol/mg dry weight (DW) for algae grown at 22 °C to 376.6 nmol/mg DW for algae grown at 5 °C. This result is in agreement with what was found previously in *C. vulgaris* strain 11468, namely sucrose accumulation due to cold shock, indicating that algae and higher plants react to cold in similar ways (Salerno and Pontis, 1989). Sucrose is one of the primary cryoprotectants in plants and if sugar accumulation is blocked, the plants lose their ability to become freeze tolerant (Guy, 1990). The RFO content was greatly increased by 300 %, from 8.7 nmol/mg DW for algae grown at 22 °C to 35.4 nmol/mg DW for algae grown at 5 °C (Fig. 1). Salerno and Pontis (1989) were the first to report raffinose accumulation in response to cold shock when *C. vulgaris* strain 11468 was transferred from 24 to 4 °C. However, in the present study *C. vulgaris* was grown continuously at a low temperature, while Salerno and Pontis (1989) transferred *C. vulgaris* strain 11468 from 24 to 4 °C for 2 days, essentially stopping its growth. Raffinose is known to protect the cell and cellular metabolism from oxidative damage triggered by salinity, drought, methyl viologen treatment, or chilling by acting as an osmoprotectant, scavenging reactive oxygen species (ROS), and stabilising membrane (Nishizawa et al., 2008). Growing *C. vulgaris* strain 13-1 at low temperature that naturally occur at high latitudes could be of interest for the functional food industry because it increases the content of raffinose, a prebiotic with beneficial effects on mineral metabolism and intestinal health (Pacifici et al., 2017).

3.1.2. Scenedesmus sp.

The same enzymatic assay described above was used to study and compare the amount of soluble sugars present in *Scenedesmus* sp. cultured at different temperatures. Glucose and fructose content of *Scenedesmus* sp. followed a similar trend to what observed in *C. vulgaris* (Figs. 1 and 2). The fructose content in the algae grown at 22 °C was also undetectable, and no difference was observed when grown at 5 °C. Sucrose was the most abundant soluble sugar in *Scenedesmus* sp.; however, differently from *C. vulgaris*, it decreased by 79 %, from 107.8 nmol/mg DW when grown at 22 °C to 23.0 nmol/mg DW when grown at 5 °C (Fig. 2). This change is surprising because sucrose accumulation is a known response to cold stress in some microalgae and higher plants (Salerno and Pontis, 1989), although the change in sucrose in response to temperature changes seems to differ greatly among algal species. *Dunaliella* sp. accumulates sucrose at higher temperatures (Müller and Wegmann, 1978), while *C. vulgaris* accumulates sucrose at lower temperatures (Salerno and Pontis, 1989). The RFO content of *Scenedesmus* sp. increased in the algae grown at 5 °C, but only to 0.5 nmol/mg DW, which is too small of a difference to draw a conclusion. Hence, the change in the RFO content in response to low temperature was very different between *Scenedesmus* sp. and *C. vulgaris* (Figs. 1 and 2).

3.2. Total FAMEs and FAMEs profile

3.2.1. Chlorella vulgaris

The FAME profile and the FAME fractions (%DW) in *C. vulgaris* were altered when the temperature changed (Fig. 3A, B). The fatty acids present in *C. vulgaris* at both temperatures were C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, and C18:3 (Fig. 3A). The FAME fractions of C18:1 and C18:3 were 26.4 % and 19.8 %, respectively, when grown at 22 °C, and increased to 40.4 % and 23.0 %, respectively, when grown at 5 °C. The FAME fractions of C16:0, C18:0, and C18:2 were 17.1 %, 1.9 %, and 31.1 %, respectively, when grown at 22 °C, and decreased significantly to 14.2 %, 0.9 %, and 17.7 %, respectively, when grown at 5 °C. Of the tested FAMES, only C16:1 showed no significant difference when comparing the temperatures: 3.2 % when grown at 22 °C and 3.6 % when grown at 5 °C (Fig. 3A). The total FAMEs represented about 19.9 % of the dry biomass of algae grown at 22 °C and about 27.2 % of the dry biomass of algae grown at 5 °C (Fig. 3B). Hence, *C. vulgaris* grown at 5 °C

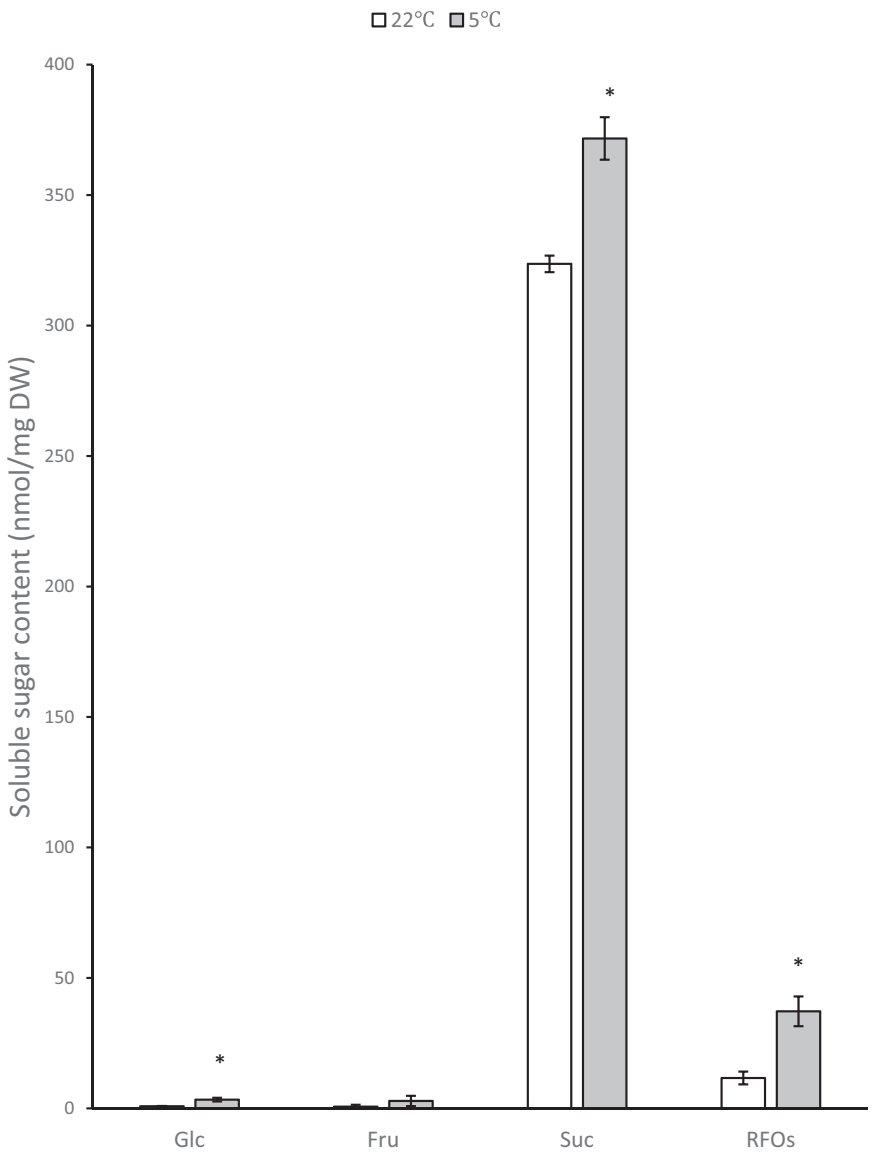

Fig. 1. Soluble sugar content of glucose (Glc), fructose (Fru), sucrose (Suc) and raffinose family oligosaccharides (RFOs) in *C. vulgaris* grown at 22 °C and 5 °C. Columns represent mean ± SD of three biological replicates. Asterisks indicate a significant difference between the two temperatures ($P < 0.05$, Student's *t*-test).

increased the total FAME content by 36.7 % compared with FAME production at 22 °C. However, as mentioned above the culture grown at the lower temperature needed longer time to reach the stationary phase compared with the culture grown at the higher temperature.

In a previous study on *C. vulgaris* strain CCTCC M 209256 grown at 20, 25, 30, and 35 °C, saturated fatty acids increased as the temperature increased and unsaturated fatty acids increased as the temperature decreased (Ma et al., 2014). One reason for this response to low temperature is that higher degree of unsaturated fatty acids increase membrane fluidity (Somerville, 1995; Sharma et al., 2012). The cold flow property of biodiesel is a critical factor; it is decreased with a high percentage of saturated fatty acids like C16:0 because these tend to solidify at cold temperatures (Dwivedi and Sharma, 2014). Hence, the increase in the percentage of the monounsaturated fatty acid C18:1 and the decrease in the percentage of the saturated fatty acid C16:0 would improve the cold flow property of biodiesel (Knothe, 2009). *C. vulgaris* strain 13-1 grown at 5 °C had a low percentage of C16:0, a high percentage of C18:1, and increased lipid production, all of which are important for biodiesel production. Of note, the C18:3 fraction also increased from 19.7 % to 23.0 % in *C. vulgaris* grown at 5 °C, which can cause biodiesel degradation and poor oxidation stability (Knothe, 2009). On the other hand, linolenic acid (C18:3) is an omega-3 fatty acid that, in addition to omega-9 fatty acid (C18:1), is essential for humans because they cannot be produced by the body and need to be retrieved from external sources such as food (Khan et al., 2018).

3.2.2. *Scenedesmus sp.*

Scenedesmus sp. also showed an altered FAME profile and increased FAME fractions (%DW) as the temperature changed (Fig. 4A, B). The fatty acids present in *Scenedesmus* sp. at both temperatures were C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, and C18:3 (Fig. 4A). The FAME fractions of C14:0, C16:0, and C18:0 did not show significant differences between the two temperatures. The FAME fractions of C16:1, C18:1, and C18:2 were 1.7 %, 50.2 %, and 12.1 %, respectively, at 22 °C, and they decreased significantly when algae were grown at 5 °C, to 0.8 %, 40.3 %, and 7.8 %, respectively. Interestingly the content of unsaturated C18:2 decreased at 5 °C in both *C. vulgaris* and *Scenedesmus* sp. (Figs. 3A, 4A). Because fatty acid desaturases use C18:2 to generate C18:3 (He et al., 2020), the decrease in C18:2 at a low temperature could be due to enzymatic desaturation activity to produce C18:3. The FAME fraction of

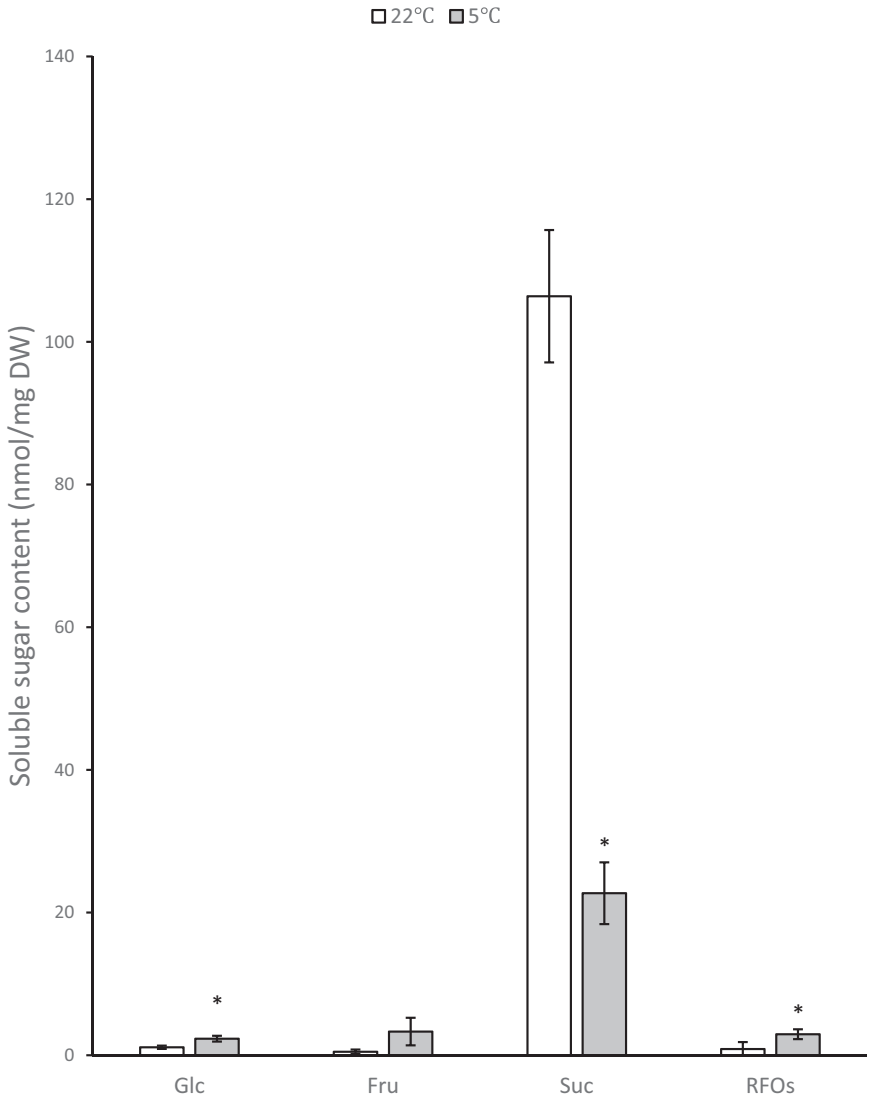

Fig. 2. Soluble sugar content of glucose (Glc), fructose (Fru), sucrose (Suc) and raffinose family oligosaccharides (RFOs) in *Scenedesmus* sp. grown at different temperatures. Columns represent mean ± SD of three biological replicates. Asterisks indicate a significant difference between the two temperatures ($P < 0.05$, Student's t-test).

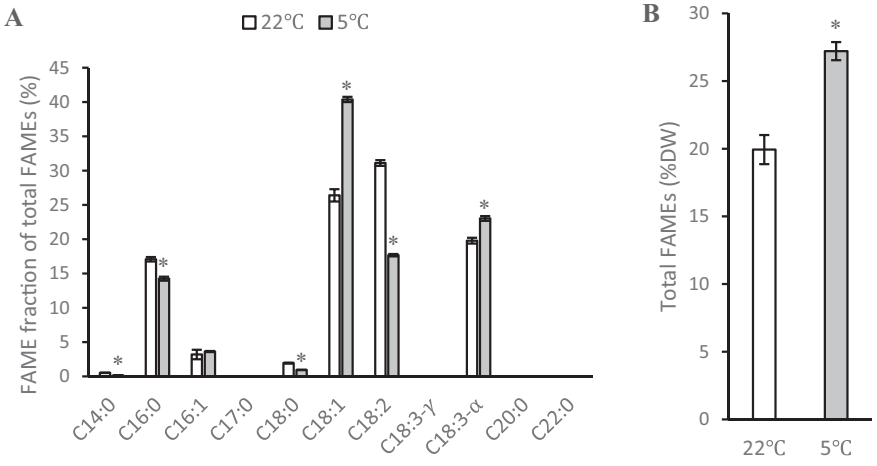

Fig. 3. Fatty acid methyl esters (FAMEs) profile (A), where each FAME is presented as FAME fraction of total FAMEs (%), and total FAMEs content (% DW) (B) of *C. vulgaris* grown under different temperatures, 22 °C and 5 °C. Columns represent mean ± SD of three biological replicates. Asterisks indicate a significant difference between the two temperatures ($P < 0.05$, Student's t-test).

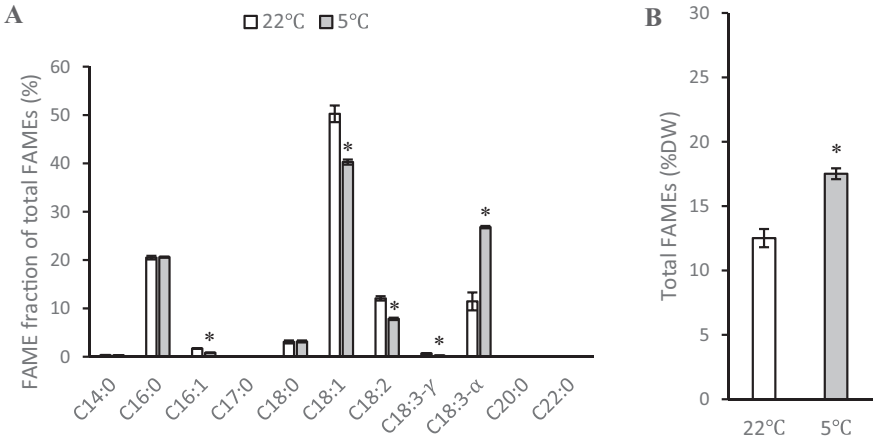

Fig. 4. Fatty acid methyl esters (FAMEs) profile (A), where each FAME is presented as FAME fraction of total FAMEs (%), and total FAMEs content (% DW) (B) of Scenedesmus sp. grown under different temperatures, 22 °C and 5 °C. Columns represent mean ± SD of three biological replicates. Asterisks indicate a significant difference between the two temperatures ($P < 0.05$, Student's t-test).

C18:3 was 11.5 % at 22 °C and increased to 26.8 % in the *Scenedesmus* sp. grown at 5 °C. Hence, C18:3 was the only FAME that increased significantly in *Scenedesmus* sp. grown at 5 °C. As mentioned, a high fraction of C18:3 is not optimal for biodiesel production because it decreases biodiesel quality, but the aforementioned health effects of this omega-3 fatty acid could make this alga beneficial for human health.

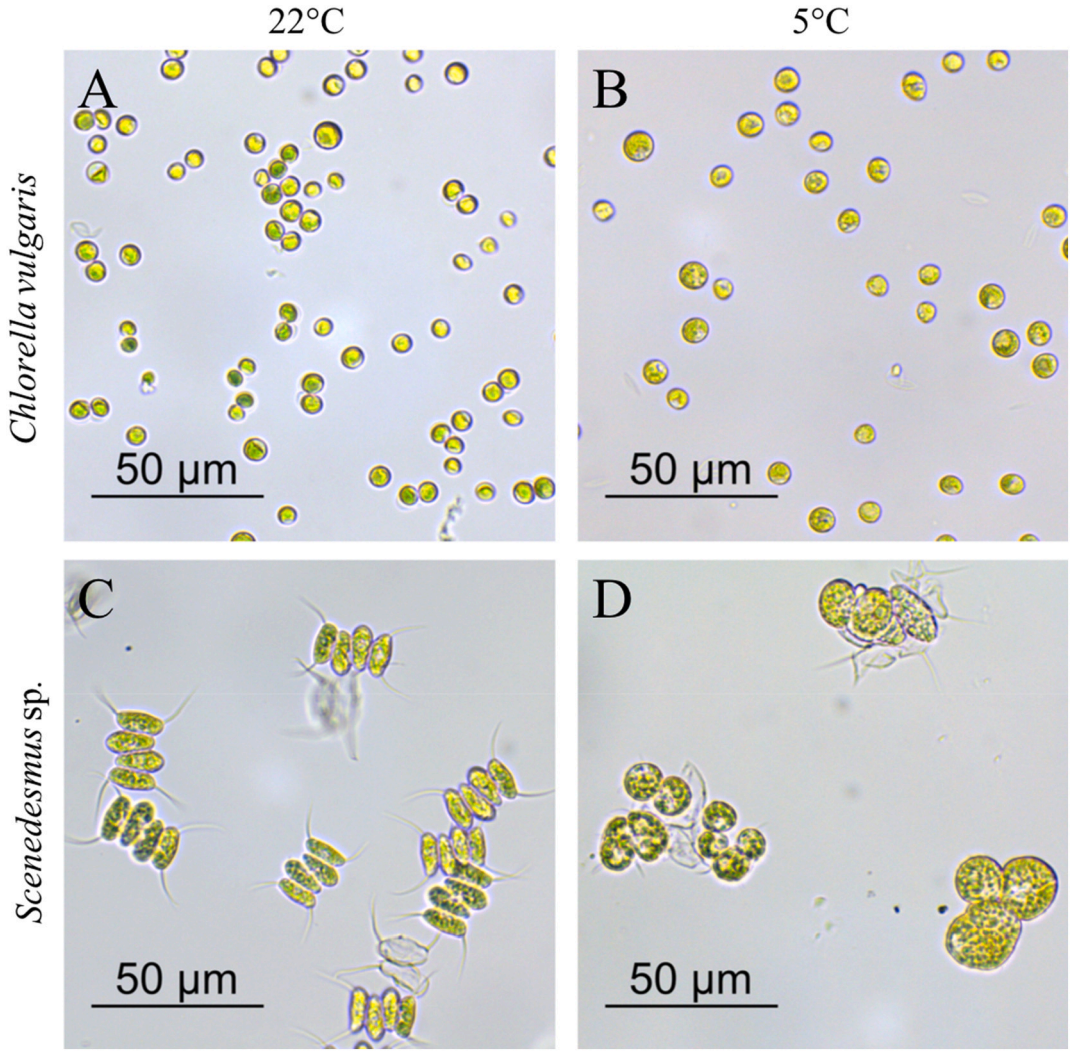

Fig. 5. Morphological changes observed by light microscopy in *C. vulgaris* (A, B) and in Scenedesmus sp. (C, D) at 22 °C (A, C) and 5 °C (B, D) with 16 h:8 h light:dark cycles. Magnification: 400×.

However, when grown at 22 °C, the C18:3 fraction of *Scenedesmus* sp. was below the 12 % threshold that is, according to European standard EN14214, the maximum percentage of C18:3 allowed in biodiesel, meaning that *Scenedesmus* sp. grown at 22 °C could potentially be a biodiesel source. The total FAMEs represented 12.5 % of the algal biomass when grown at 22 °C and increased significantly to 17.5 % of the dry biomass when grown at 5 °C (Fig. 4B). Hence, total FAMEs increased by 40 % when *Scenedesmus* sp. was grown at 5 °C compared with 22 °C, but the former culture required a longer cultivation time.

3.3. Morphological changes

The morphology of both strains grown at 22 and 5 °C was studied and compared microscopically. Both strains had distinct morphology and different cell sizes and shapes (Fig. 5). *C. vulgaris* grown at 22 °C appeared as single spherical cells with a diameter between 3 and 8.5 μm. *C. vulgaris* grown at 5 °C still appeared as single spherical cells with a diameter of 5–9 μm. *Scenedesmus* sp. grown at 22 °C had an ellipsoidal cell shape and mainly formed colonies of four cells, but there were also single cells and colonies of two cells. The cell length on the longer axis of *Scenedesmus* sp. grown at 22 °C was approximately 11–14 μm while the shorter axis was approximately 5–7 μm. There were spines 7–10 μm long on the colonies of four cells. *Scenedesmus* sp. grown at 5 °C had a very different morphology compared with that grown at 22 °C. The cell shape changed from ellipsoidal to almost round, and the cells were bigger. Most cells were 10–16 μm in diameter, but in some case they reached around 20 μm in diameter. The spines were mostly gone, or at least shorter, and granules were present in the cytoplasm. The typical formation of colonies of four cells was no longer seen in the *Scenedesmus* sp. grown at 5 °C; instead, they were clustered in many different ways, often two, three, or four together. This considerable change in morphology could be the reason why *Scenedesmus* sp. grew much slower than *C. vulgaris*. It seems that *Scenedesmus* sp. required considerable reprogramming of its cell morphology, and the large cell size formation might be the reason for the slower growth compared with *C. vulgaris*. The observed granules could be a way to accumulate storage products like starch or lipids at a low temperature. *Scenedesmus* sp. follows the temperature-size rule proposed by Atkinson (1994): many living organisms increase their cell size when exposed to cooler temperatures. Moreover, phenotypic plasticity has been observed in the *Scenedesmus* and *Desmodesmus* genera (Lürling, 2003).

4. Conclusions

In conclusion, the two cold-tolerant strains, *C. vulgaris* and *Scenedesmus* sp., responded to cold temperatures and produced different valuable compounds. *C. vulgaris* slightly changed its morphology, did modify its fatty acid profile, and accumulated sucrose as well as RFOs in response to cold. *Scenedesmus* sp., on the other hand, changed its morphology considerably, modified its fatty acid profile, particularly increasing the fraction of the omega-3 fatty acid linolenic acid (C18:3), and decreased its sucrose content in response to cold. Future studies on these cold-tolerant microalgal strains should focus on their potential industrial application as food and feed, biopharmaceuticals, nutraceuticals, and renewable energy sources.

CRediT authorship contribution statement

A. Lindberg: Investigation, Visualization, Writing – original draft. **C. Niemi:** Investigation, Supervision, Writing – review & editing. **J. Takahashi:** Method development, Writing – review & editing. **A. Sellstedt:** Conceptualization, Supervision, Writing – review & editing. **F.G. Gentili:** Investigation, Funding acquisition, Conceptualization, Supervision, Writing – review & editing.

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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