Norwegian University of Life Sciences
Faculty of Chemistry, Biotechnology and Food Science

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Tailoring microalgal biomass for food applications: fermented beverage as a case study

Optimalisering av mikroalgebiomasse for anvendelser i mat: fermentert drikke som en eksempelstudie

Giorgia Carnovale

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Abbreviations and definitions

AGPase	ADP-Glucose-Phosphorylase
AMA	Alpha-amylase
AMB	Beta-amylase
BSPSR	Biomass Specific Photon Supply Rate
DBE	De-branching Enzyme
DHA	Doecohexanoid acid
DPE	Disproportionating Enzyme
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic Acid
FTIR	Fourier-Transform Infrared Spectroscopy
GBSS	Granule Bound Starch Synthase
HT-FTIR	High-Throughput Fourier-Transform Infrared Spectroscopy
IR	Infra-red
ISA	Isoamylase
IVD	In Vitro Digestibility
MUFA	Mono-Unsaturated Fatty Acids
PBR	Photobioreactor
PUFA	Poly-Unsaturated Fatty Acids
PWD	Phosphoglucan Water Dikinase
SBE	Starch Branching Enzyme
SFA	Saturated Fatty Acids
SPh	Starch Phosphorylase
SSS	Soluble Starch Synthase
TAG	Triacyl glycerides

List of papers

Paper I

Starch Rich *Chlorella vulgaris*: High-Throughput Screening and Up-Scale for Tailored Biomass Production.

G. Carnovale, F. Rosa, V. Shapaval, S. Dzurendova, A. Kohler, T. Wicklund, S.J. Horn, M.J. Barbosa, K. Skjånes. (2021) *Applied Sciences*, 11: 9025. https://doi.org/10.3390/app11199025.

Paper II

Metabolic pathways for biosynthesis and degradation of starch in *Tetraselmis chui* during nitrogen deprivation and recovery.

Giorgia Carnovale, Sonia Torres, Carmen Lama, Filipa Rosa, Lalia Mantecon, Svein Jarle Horn, Kari Skjånes and Carlos Infante. (2022). *Bioresource Technology*, 354, 127222. https://doi.org/10.1016/j.biortech.2022.127222

Paper III

Starch-rich microalgae as an active ingredient in beer brewing.

Giorgia Carnovale, Shaun Leivers, Filipa Rosa, Hans-Ragnar Norli, Edvard Hortemo, Trude Wicklund, Svein Jarle Horn and Kari Skjånes. (2022). *Manuscript accepted for publication in Foods*

Abstract

Microalgae are renowned as super-foods due to their unique nutritional profiles; however, their applications to human nutrition are still limited to nutraceuticals and food supplements. This is likely due to factors such as high production costs, the complexity of product development, and their impact on the technical and sensory properties of different foods. Producing algal biomass tailored to fit into established production processes is a possible way to tackle all of these issues, expanding the microalgal industry and leading to novel discoveries and improvements in sustainability.

This thesis discusses a holistic approach to tailoring microalgal biomass for application in novel products, providing an overview of the current status of microalgal food research and the related challenges, followed by a discussion of possible approaches to enhance biomass composition.

The three papers/ manuscripts included in this thesis explore the whole process leading to the development of a novel beverage for human consumption, producing microalgal starch-rich biomass and applying it as an active ingredient in beer brewing. In the first work, starch production in the freshwater species *Chlorella vulgaris* was studied using High Throughput FTIR spectroscopy to screen for optimal environmental and nutrient conditions on an extensive experimental design. Selected conditions were further tested in a flat panel reactor to assess the role of light through biomass specific photon supply rate, finally achieving a maximum starch concentration of up to 50% of the dry weight in up-scaled 25 L tubular photobioreactors. The second manuscript studied the starch metabolic pathways connected to nitrogen stress and recovery on salt-water species *Tetraselmis chui*, giving an overview of gene expression analysis during accumulation and subsequent degradation of 60% starch. Finally, the application of starch as an active ingredient was tested in brewing. Starch-rich biomass was developed in a 250 L pilot-scale photobioreactor, following indications from the first two papers, and brewing was successfully performed with support from an industrial partner, the Norwegian-based craft brewery Nøgne Ø.

Based on the experiments presented in the current thesis, the production of biomass tailored to a target product proved to be an efficient method to successfully implement microalgae in established food products.

Norsk sammendrag

Mikroalger er kjent som 'supermat' på grunn av høyt næringsinnhold, men innen human ernæring er bruken av mikroalger fortsatt begrenset til nutraceuticals og kosttilskudd. Dette skyldes sannsynligvis en kombinasjon av faktorer, som høye produksjonskostnader, kompleksitet i produktutvikling og innvirkning på de tekniske og sensoriske egenskapene til ulike matvarer. Å produsere algebiomasse som er skreddersydd for å passe inn i etablerte produksjonsprosesser har potensiale til å takle alle disse problemene, og dermed føre til en ekspansjon av mikroalgeindustrien, nye oppdagelser og forbedringer i bærekraft.

Denne oppgaven diskuterer en helhetlig tilnærming for å skreddersy mikroalgebiomasse for bruk i nye produkter, og gir en oversikt over dagens status innen forskning på mikroalgemat og relaterte utfordringer, etterfulgt av en diskusjon av mulige tilnærminger for forbedring av biomassekomposisjonen.

De tre artiklene/manuskriptene som er inkludert i denne oppgaven, utforsker hele prosessen som fører til utviklingen av en ny drikk for humant konsum. I prosessen blir stivelsesrik algebiomasse produsert og brukt som en aktiv ingrediens i ølbrygging. I det første arbeidet ble stivelsesproduksjonen i ferskvannsarten *Chlorella vulgaris* studert ved bruk av HT-FTIR spektroskopi for å screene for optimale miljø- og næringsbetingelser i et stort eksperimentelt design. Utvalgte dyrkingsbetingelser ble videre testet i flatpanelreaktorer for å vurdere lysets rolle gjennom biomassespesifikk fotontilførsel for til slutt å oppnå en maksimal stivelseskonsentrasjon på opptil 50 % av tørrvekten i oppskalerte 25 L rørbaserte fotobioreaktorer. Metabolismeveiene for stivelse som er knyttet til nitrogenstress og stressrestitusjon, ble studert i saltvannsarten *Tetraselmis chui*, og manuskript nummer to presenterer resultater fra genekspresjonsanalyse ved akkumulering og nedbrytning av 60 % stivelse. Til slutt ble bruken av stivelse som aktiv ingrediens testet i brygging. Utviklingen av stivelsesrik biomasse ble utført i en 250 L pilotskala fotobioreaktor, etter indikasjoner fra de to første arbeidene, og bryggingen ble vellykket utført med støtte fra en industriell partner, det norske håndtverksbryggeriet Nøgne Ø. Resultatene presentert i denne avhandlingen, viste at produksjon av mikroalgebiomasse med komposisjon tilpasset et spesifikt målprodukt, kan være en effektiv metode for å lykkes med implementering av mikroalger i etablerte matprodukter.

1 INTRODUCTION

1.1 Microalgae biotechnology

Unicellular photosynthetic organisms were considered historically to belong to the kingdom of Protists, which was defined for the first time in the late 19th century by the German scientist, philosopher, and artist Ernst Haeckel (Figure 1). The Protista kingdom conveniently involved anything that was not fungi, plants and animals; however, the limitations of this grouping were soon highlighted by advances in genetics and evolutionary studies, which led to the modern understanding of photosynthetic microorganisms as a complex, polyphyletic group, which encompasses a variety of species adapted to life in all environments on earth (Norton et al., 1996; Tirichine and Bowler, 2011). A conservative estimate considers the existence today of more than 72000 species of microalgae belonging to more than 15 phyla (Guiry, 2012). This diversity, together with the capacity of these organisms to store metabolites in large amounts, generated a great interest in the scientific community leading to the development of microalgal biotechnology and industry.

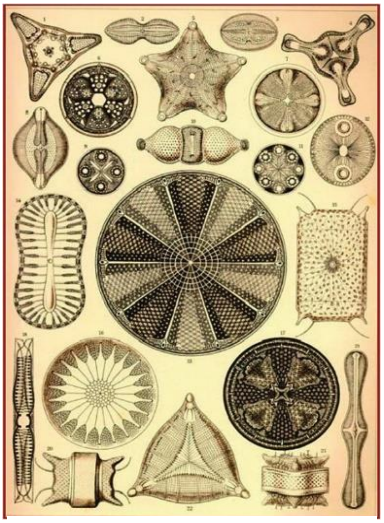

Figure 1: Illustration of diatoms. The 84th plate from Ernst Haeckel's ''Kunstformen der Natur'' (1904)

In biotechnology, the term “microalgae” is often used to refer both to procaryotic cyanobacteria such as *Arthrospira platensis* and to eukaryotic microalgae. This is because, despite the differences which are now clear between organisms belonging to separate domains, both microalgae and cyanobacteria are cultured using the same technologies, have similar properties and are studied for the same possible applications (Fernández et al., 2021).

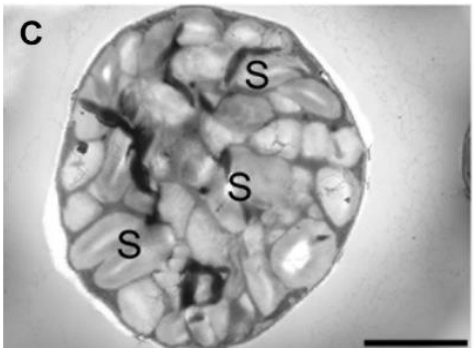

Figure 2: Electron microscopy image of starch granules in a nutrient deprived C. vulgaris cell. Starch granules are marked with an S. (Brányiková et al., 2011)

Interest in microalgal farming as a sustainable innovation is continuously growing, as cultivation can be performed without competing for resources required by other important production lines, such as fresh-water and arable land. Amongst a wide variety of metabolites and bioactive compounds, microalgal cells are known to store large amounts of fatty acids and carbohydrates in the form of lipid bodies or starch granules (Figure 2), attracting the interest of stakeholders and researchers in the fields of biofuels, pharmaceuticals, nutraceuticals, animal feed and human nutrition (Mata et al., 2010; Spolaore et al., 2006; Torres-Tiji et al., 2020).

The main obstacle to the global scale diffusion of the microalgae industry is cost-effectiveness, and though the latest techno-economical assessments are optimistic (Norsker et al., 2011; Ruiz et al., 2016), the current technologies mostly allow the for production of high-value products, mainly targeting aquaculture and human consumption (Acién et al., 2017).
Thus, the industrial application of microalgae at large scales is still in its infancy. Research needs to advance in parallel to the development of novel attractive products to lead the growth of microalgae as a sustainable technology in modern society. Integrating scientific research into industrial product development is the primary goal of the "Algae to Future - A2F" project (algae2future.no), which is the framework where this thesis was developed as a case study for microalgal application in the food industry.

1.2 Microalgae Application in Food

Microalgae and Cyanobacteria have been used as a food source by indigenous populations for thousands of years. Farming of *Spirulina* was an established production in Aztec Mesoamerica, where the algae were sold and consumed as loaves,

called Tecuitlatl, much resembling cheese (Farrar, 1966). *Spirulina* has also been consumed by African communities in the areas of Chad and Niger for centuries, and the production of semi-dried "dihe'" loaves continues in modern times (Abdulqader et al., 2000). Also, according to archaeological reports, *Nostoc* was consumed up to 2000 years ago in China to survive a famine (Spolaore et al., 2006). Despite the long history of blue-green algae farming, it was only in the 1950s that the scientific community started looking at microalgal and cyanobacterial production for human nutrition. Algae like *Chlorella vulgaris* (Farrar, 1966) were attractive due to their low land and water requirements and rich nutritional profiles.
Since then, research has explored multiple routes, and microalgae have been widely studied for their use as food supplements and nutraceuticals (Nicoletti, 2016) or to enhance existing foods by improving their nutritional qualities (Matos et al., 2017). Possible applications in malnourished populations, for example in developing countries (Hug and von der Weid, 2012) or as a food supplementation for the elderly (Santos et al., 2016) have also been evaluated.

Microalgal market size has been steadily growing and there are numerous reviews showing that in the last decades microalgae have been successfully introduced in a variety of foods and beverages, many of which are currently commercially available in some countries (Germany, France, Japan, USA, China, Thailand) (Caporgno and Mathys, 2018; Chacón-Lee and González-Mariño, 2010; Draaisma et al., 2013; Nicoletti, 2016; Torres-Tiji et al., 2020; Vaz et al., 2016) .
Commercialisation of microalgae follows different regulations worldwide, and while species with a rich usage history, such as *Spirulina sp.* and *Chlorella sp.* are typically not subjected to regulations, some stricter rules may apply for other species. For example, in the EU all novel foods (food that was not consumed to a significant extent in the EU before 15 May 1997) must undergo rigorous testing in order to be approved for human consumption by the European Food Safety Authority. Food safety regulations represent the principal constraint for the exploitation of microalgae in this industry. Industrial producers have to document safety themselves and file requests for approval. For example, Innovalg (France) has obtained food approval of the marine diatom *Odontella aurita* in the EC Regulation 258/97, whereas the use of *Tetraselmis chui* by Fitoplancton Marino S.L. (Spain) was approved more recently for human consumption through the EU 2017/2470 Regulation (EU Commission, 2017).

Besides the existing commercialised products, which often do not offer insights into production processes and properties, a whole body of research is available, reporting on challenges and benefits of microalgae addition in foods. Table 1 summarises different applications and species used in research, with focus on creating functional foods with improved health benefits. The species used, combined with the type of product and the amount of biomass included have major impacts on the technical properties, on the sensory profiles, and on the functional benefits of the food items.

Table 1 – summary of recent research studies about food application of microalgal species and amount of biomass used.

Product type	Species	Content	Reference
Dry baked goods	*Isochrysis galbana*	1-3%	(Gouveia et al., 2008b)
	Chlorella vulgaris	0.5-1-2-3%	(Gouveia et al., 2007)
	Arthrospira platensis	1.63 to 8.36%	(Singh et al., 2015)
	Arthrospira platensis *Chlorella vulgaris* *Tetraselmis suecica* *Phaeodactylum tricornutum*	2-6%	(Batista et al., 2017)
	Arthrospira platensis *Tetraselmis suecica*	2-6%	(Batista et al., 2019)
	Nannochloropsis sp. *Tetraselmis* sp.	1.25-2.5-3.75%	(Lafarga et al., 2019b)
	Nannochloropsis sp. *Tetraselmis sp.*	0.5-3%	(Hernández-López et al., 2021)
	Tetraselmis chui	0.5-1-1.5%	(García-Segovia et al., 2020)
Bread	*Chlorella vulgaris*	1%	(Nunes et al., 2020b)
	Chlorella vulgaris	1 to 5%	(Graça et al., 2018)
	Nannochloropsis sp. *Tetraselmis sp.*	1-2-3%	(Lafarga et al., 2019b)
	Tetraselmis chui	4-8-12-16%	(Qazi et al., 2021)
Gluten free	*Tetraselmis chui*	1-2-4%	(Nunes et al., 2020a)

	Arthrospira platensis	0.5 to 4%	(Selmo and Salas-Mellado, 2014)
Pasta	*Chlorella vulgaris* *Spirulina maxima*	0.5-1-2%	(Fradique et al., 2010)
	Arthrospira platensis	n.a.	(Grahl et al., 2018)
	Isochrysis galbana *Diacronema vlkianum*	0.5-1-2%	(Fradique et al., 2013)
Gluten free	*Arthrospira platensis*	1 to 15%	(Fradinho et al., 2020)
Cheese	*Chlorella vulgaris*	2-3%	(Shalaby and Yasin, 2013)
Yogurt	*Arthrospira platensis*	0.05% to 1%	(Barkallah et al., 2017)
	Arthrospira platensis *Chlorella vulgaris*	0.5 to 1%	(Beheshtipour et al., 2012)
Ice Cream	*Nannochloropsis oceanica* *Diacronema vlkianum* *Porphyridium cruentum*	0.1 to 0.3%	(Durmaz et al., 2020)
Emulsions, Gels and Creams	*Arthrospira platensis* *Haematococcus pluvialis*	0.75 to 3.25%	(Batista et al., 2012)
	Arthrospira platensis *Diacronema vlkianum*	0.1 to 0.75%	(Gouveia et al., 2008a)
	Chlorella vulgaris *Haematococcus pluvialis*	0.5 to 2%	(Gouveia et al., 2006)
	Chlorella vulgaris	1 to3%	(Raymundo et al., 2005)
	Chlorella sp. *Tetraselmis sp.*	0.5-1-2%	(Lafarga et al., 2019a)
	Arthrospira platensis *Chlorella vulgaris* *Tetraselmis chui* *Nannochloropsis oceanica*	1.5 to 3%	(Boukid et al., 2021)

1.2.1 Functional properties and digestibility

Microalgal biomass is renowned and studied as a food supplement due to its nutritional properties. Thus, it comes as no surprise that its addition as an ingredient imparts functional properties to foods. The addition of species such as *Arthrospira platensis*, *T. chui*, *C. vulgaris*, and *Phaeodactylum tricornutum* has been widely tested to improve protein content of the final product (Batista et al., 2017; Lafarga et al., 2019b; Nunes et al., 2020a; Qazi et al., 2021), with reported increases of the protein content, compared to control, of up to 20% (Selmo and Salas-Mellado, 2014). Some studies have also explored the antioxidant activity and presence of bioactive compounds, phenolics and phycocyanin consolidating the result that addition of microalgal biomass boosts the functional properties of foods (Batista et al., 2019, 2017; Fradinho et al., 2020; Lafarga et al., 2019a). For example, Gouveia and colleagues targeted specifically the enrichment of omega-3 and omega-6 fatty acids, successfully enhancing their content in biscuits through the addition of *Isochrysis galbana* biomass (Gouveia et al., 2008b, 2007).

The assessment of in vitro digestibility (IVD) of foods is performed to simulate gastrointestinal conditions and determine chemical or structural changes happening to the food matrix. Biscuits containing different amounts of microalgae have shown no significant differences in digestibility compared to controls (Batista et al., 2017). Further studies on crackers have reported a positive effect on IVD with higher values at 6% inclusion than at 2% with four different species tested (Batista et al., 2019). This is in contradiction with a study on *A. platensis* enriched pasta, which reports a decrease in IVD with increasing microalgal contents (Fradinho et al., 2020). Additionally, *A. platensis* biomass sourced from different producers had different digestibilities, indicating that the IVD is extremely dependent not only on the species but also on the overall composition of the biomass used.

1.2.2 Impact on technical properties

The inclusion of microalgae into foods has different effects on technical and structural properties based on the nature of the targeted product, thus multiple applications have been attempted in previous research to identify the best applications and evaluate challenges or criticalities (Figure 3). In the following paragraphs the different macro-categories of food products will be explored to highlight challenges and structural properties of microalgae-enhancement of foods.

Multiple studies have focused on the production of emulsions and gels. *C. vulgaris* biomass increases the viscosity of emulsions allowing to add less fat to the mixture and can act as an emulsifier due to its high protein content, though it can't completely substitute traditional emulsifiers such as pea protein (Raymundo et al., 2005). The use of *C. vulgaris* and *Haematococcus pluvialis* in food emulsions (Figure 3.e) also adds resistance to lipid oxidation of the final product, possibly increasing its shelf life (Gouveia et al., 2006). Microalgae have a significant effect also on the microstructure of biopolymer gels, improving their structure and firmness (Batista et al., 2011); interestingly, microalgae have been shown to interfere with the gelatinisation

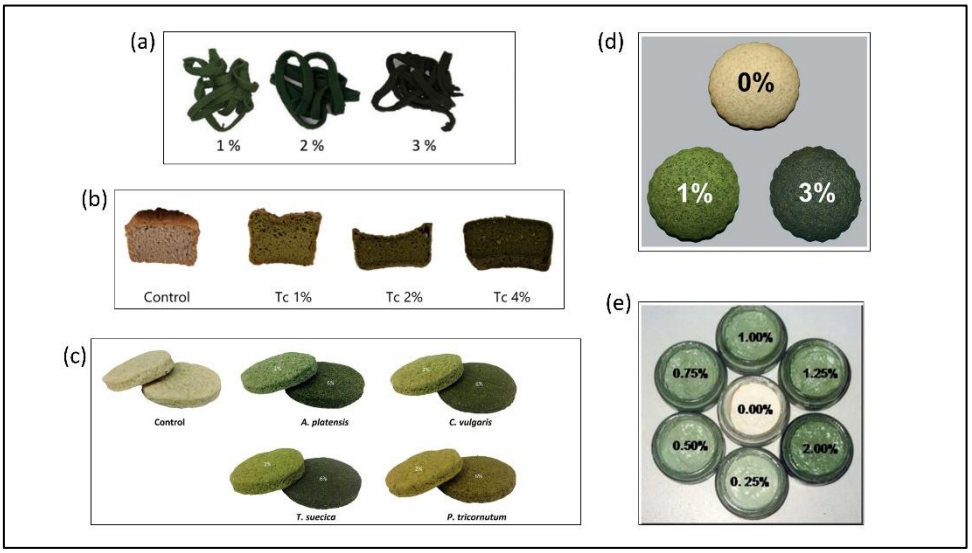

Figure 3 Examples of microalgae-enriched food products. (a) A. platensis pasta (Fradinho et al., 2020); (b) T. chui bread (Nunes et al., 2020a); (c) Biscuits with 2% and 6% incorporation of different species (Batista et al., 2017); (d) Biscuits with different amounts of I. gaditana biomass (Gouveia et al., 2008b); (e) Emulsions with different amounts of C. vulgaris biomass (Gouveia et al., 2006).

process during thermal treatment, with higher temperature yielding firmer gels in microalgae compared to controls (Batista et al., 2012), suggesting that processing is an additional parameter influencing microalgae addition in foods. Firmness of microalgae-gels varies also depending on the species used, thus on the quality and composition of the biomass added, highlighting the importance of pondered species and composition choice (Gouveia et al., 2008a).

In dry bakery goods, such as biscuits (Figure 3.c-d), the addition of microalgal biomass proportionally improved firmness and texture, possibly because of competition with wheat in the water absorption process (Batista et al., 2017; Singh et al., 2015). This could represent a benefit also on shelf life and preservation of biscuits, which remain fragrant over a longer timespan (Gouveia et al., 2008b). No impact on texture, moisture, stability or pH was instead recorded in wheat tortillas prepared with up to 3% *Nannochloropsis sp.* and *Tetraselmis sp.* biomass (Hernández-López et al., 2021). Microalgal addition to bread has proved to be more complex, as also in this case it tends to increase firmness, which is not a desirable trait for most bread types. Replacement of wheat flour in contents up to 16% (Qazi et al., 2021) was attempted; at lower doses (< 3-4%) the presence of microalgae does not affect the rheological properties of bread (Lafarga et al., 2019b), nor impact the fermentation process (Graça et al., 2018), resulting in bread loaves with appreciable texture. Higher contents often yielded inferior quality; this is probably due to the dilution effect on the gluten matrix by high protein microalgal biomass, which could additionally disrupt the gluten network by aggregating in the dough (Graça et al., 2018). An interesting attempt to overcome technical issues in bread was to focus on production of gluten-free bread, where microalgal biomass was tested as an ally in stabilising and structuring the dough in absence of gluten. This approach allowed to include up to 4% *A. platensis,* with the addition of transglutaminase and hydrocolloid methylcellulose to aid, respectively, the formation of protein network and the firmness of the crumb (Selmo and Salas-Mellado, 2014). Further work on gluten-free bread supplied with up to 4% *T. chui* biomass (Figure 3.b) confirmed that higher amounts of biomass can be added in gluten-free breads with a positive effect on the dough (Nunes et al., 2020a). Additionally, Qazi and colleagues attempted the pre-treatment of the biomass by ethanol extraction, which improved the overall quality of bread upon supply of higher microalgal contents, resulting in softer and more elastic breads than the non -extracted counterparts. The authors suggest this may be caused by the removal of fatty acids, which may act as plasticisers or disrupt the complex balances of reactions in the dough, but also by the high content of antioxidants, which may interfere with the development of gluten networks (Qazi et al., 2021).

An increased interest in dairy products has also been detected in research about microalgae addition to food, with articles mainly exploring applications in yogurts, ice cream and cheeses (Hernández et al., 2022). Microalgae addition to yogurt provides a favourable environment for probiotic and fermenting microorganisms

(Beheshtipour et al., 2012) and impacts buffering capacity (Barkallah et al., 2017). Further benefits of algae additions are that it is suggested to reduce the undesirable effect of serum separation in yogurt. Addition of *A. platensis* to ice cream could partially substitute stabilisers due to its water binding capacity, however it was also reported to reduce melting time, possibly because of its fat content (Durmaz et al., 2020). Also, in dairy products the effect of different biomass compositions was proved to have a significant impact. *A. platensis*, *C. vulgaris, T. chui* and *Nannochloropsis oceanica* were tested for the addition of 1.5-3% biomass in vegetable creams where interestingly it was shown how rheological properties are mainly impacted by the species used, and not by the level of addition (Boukid et al., 2021).

Pasta has also been extensively studied as a suitable product for microalga addition. The maximum content of 20% *A. platensis* was successfully added in wheat pasta with minor technical issues by De Marco and colleagues, however this study does not report on sensory evaluation of the product (Marco et al., 2014) and most studies focus on lower biomass contents. Addition of up to 2% microalgal biomass (*I. galbana* and *Diacronema vlkianum*) to pasta resulted in an increased firmness, with higher swelling, water absorption and lower or similar cooking losses to the controls (Fradique et al., 2013). Interestingly, another study highlighted how the firmness was mitigated upon cooking (*C. vulgaris* and *S. maxima)*, resulting in values which were comparable to the control wheat pasta (Fradique et al., 2010), proving once again how processing of the foods is a key parameter to consider for successful application of microalgae. Gluten-free fresh pasta was also prepared with *A. platensis*, attempting the addition of higher proportions (Figure 3.a), however already at 4-5% microalgal addition the doughs became difficult to manufacture, had strong adhesiveness and strong unpleasant odours (Fradinho et al., 2020) thus, the ideal content was identified to be 3%.

The state of art of microalgal impact on technical properties of food products leads to the conclusion that careful and pondered choices about biomass composition, species, amount included, and target product may dramatically change the outcomes of production.

1.2.3 Sensory profiles and consumer acceptance

The acceptance of novel foods by consumers is a key factor in their development and distribution (Siegrist, 2008). Studies conducted on costumer responses to

microalgae-based products have shown a positive response to breadsticks prepared with up to 1.5% *T. chuii* (García-Segovia et al., 2020) and to sushi, pasta and jerkies prepared with *A. platensis* (Grahl et al., 2018). In these studies consumers have shown that familiarity with the product and health benefit evaluation play an important role in acceptance, together with overall sensory profiles.

The enrichment of food products with microalgal biomass has a minor impact on sensory profiles when the biomass added is below a threshold of circa 3%, whereas the addition of higher amounts leads to strong odours and flavours that negatively impact consumer's acceptance (Gouveia et al., 2008b; Singh et al., 2015). Additionally, different microalgae have their characteristic odours and aromas. Thus, the choice of species may influence the acceptance. For example, cookies prepared with *A. platensis* had overall higher scores compared to those prepared with *C. vulgaris,* where the addition of 2% biomass was appreciated for both species. Increasing the content to 6% biomass resulted in poor scores for *C. vulgaris*, while *A. platensis* maintained similar scores despite the increase (Batista et al., 2017). This implies that the choice of microalgal species with the right aromas may allow the addition of higher biomass contents, allowing to improve significantly the bioactive profile of the final product.

Microalgae are known to have a strong aroma and flavour, due to the wide variety of volatile compounds. In a recent study, Qazi and colleagues achieved very promising results by pre-treating the biomass of *T. chui* with ethanol for further application in bread baking (Qazi et al., 2021). The resulting final products were more palatable and appealing, and not only the undesirable odours were reduced but the pre-treatment also benefitted the technical properties of the dough, allowing for higher contents to be successfully implemented in baking bread.

The most obvious result of addition of microalgae to food is the green colour, which varies in brightness and intensity depending on the amounts of biomass and the species used (Batista et al., 2019, 2017). Recent studies highlighted how colourful eating is associated with healthy eating, suggesting that greenness in food might induce healthier food choices in consumers (König and Renner, 2018). Microalgae also provide for a large variety of pigments which may be exploited with the purpose of food colouring, while enhancing the products with nutritional benefits. Interesting changes in colour may be achieved with very low amounts of biomass as it has been tested for example in food emulsions using up to 2% of both *C. vulgaris* and *H. pluvialis* (Gouveia et al., 2006) or in ice cream, where contents up to 0.3% of *N. oceanica*, *D. vlkianum* and *Porphyridium cruentum* provided the dessert with interesting shades of green and pink (Durmaz et al., 2020).

1.2.4 Challenges and criticalities

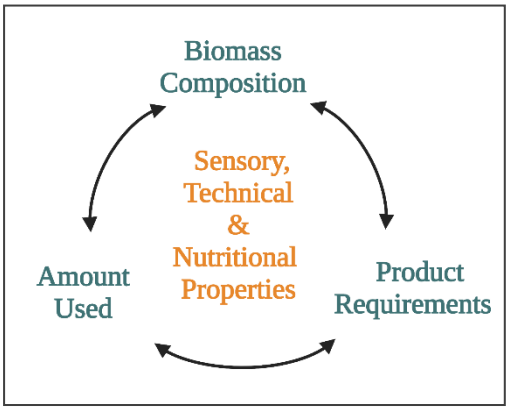

Figure 4: Key elements in microalgae application to foods

The body of research produced so far about enrichment of foods with microalgal biomass clearly depicts the complexity of the product development process. The biochemical profile of the biomass used, the amount to implement and the type of product chosen all influence each other, and a good product development must carefully consider all the factors to create a final product that is technically feasible, contains amounts of biomass that have a significant impact on the bioactive profile and is appealing for the final consumers (Figure 4). Thus, for example, all biomasses performed technically well -whole or disrupted- in the making of biscuits, which seem to be an excellent target product for microalgal addition. Some species, like *A. platensis* also performed well on sensory scores, allowing to create nutrient-rich baked goods that customers are likely to buy with a maximum addition of 6% microalgal biomass. Contrarily, in bread baking, or in pasta only low amounts of biomass could be added without impacting majorly the technical and sensory properties due to the high protein content of the biomass used, resulting in a final product that is less appreciable by possible customers.

1.3 Tailoring biomass composition for food industry: novel approaches and technologies

Tailoring biomass composition to meet the needs of specific products could ease the application of microalgae in foods. Microalgae have the capacity of accumulating reserve and antioxidant metabolites in response to changing environmental conditions; this property is what makes them “single-cell factories”, widely studied for their potential industrial applications (Eltanahy and Torky, 2021). The content of proteins, lipids, carbohydrates, pigments, and other compounds can be changed by manipulating the cultivation conditions. This may target accumulation of single or

multiple metabolites, as for example a combination of lipids and carotenoids (Minhas et al., 2016).

In this section several novel and established methods to manipulate biomass composition will be assessed, with a special focus on the species that are currently approved by EFSA for human consumption such as organisms of the *Chlorella* and *Tetraselmis* genera.

1.3.1 Screening of biomass composition

The accumulation of secondary metabolites or storage compounds and the response to different culture conditions can vary a lot between species and strains. Consequently, to efficiently tailor biomass composition, strains and growth conditions must be selected carefully, combining multiple parameters to identify the optimal production method. However, large screening experiments can be very cumbersome to perform and the analysis of composition through traditional analytical chemistry methods not only requires large amounts of biomass but is also time consuming (He et al., 2017).

High Throughput Fourier-Transform Infrared Spectroscopy (HT-FTIR) is a high-resolution, fast and non-disruptive method, which allows to simultaneously and accurately quantify multiple cell components using very small amounts of biomass, which are directly aliquoted on plates without cumbersome pre-treatment steps and let air dry before analysis (Figure 5) (Wagner et al., 2010).

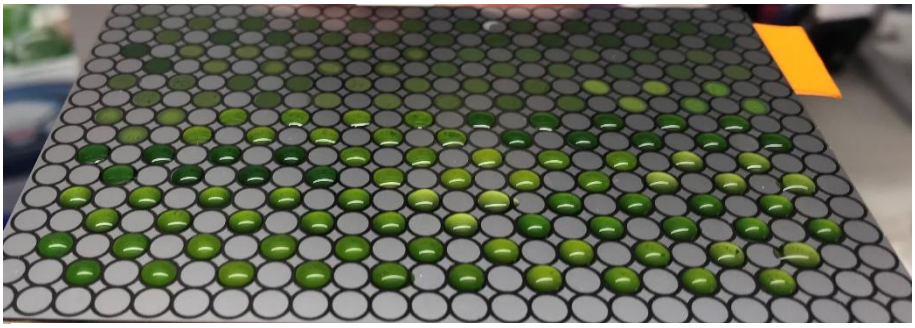

Figure 5: FTIR plate with aliquoted microalgal samples.

Different macromolecule groups constituting the biomass, such as lipids or carbohydrates, have characteristic chemical bonds and functional groups which absorb specific wavelengths in the IR radiation and the consequent absorption

spectra can be *de facto* considered a fingerprint of biomass composition. Since the first studies on FTIR application on microorganisms (Naumann et al., 1991) this tool has been adapted to also identify biomass components in microalgae and to determine composition and shifts in carbon metabolism under sub-optimal conditions such as nitrogen stress (Dean et al., 2010; Wagner et al., 2010). FTIR has been successfully used for species selection when screening for lipid-rich species (Meng et al., 2014) and in the last years particular attention has been given to the characterisation of lipids, allowing to quantitively assess the content of different fatty acids and triacylglyceride species (C. Esther Elizabeth Grace et al., 2020). Screening is the first essential step to identify how different growth conditions affect the biomass and to consequently choose the right protocol to maximise production of desired compounds. FTIR spectroscopy is thus an ideal starting point to develop protocols for tailored biomass production.

1.3.2 Manipulation of Environmental Conditions

For algae to grow and reproduce, certain environmental conditions are required. Every species has specific requirements for optimal growth. Irradiance, temperature pH and salinity are all factors that must be regulated to obtain good and stable growth. Additionally, macronutrients such as sulphur, nitrogen and phosphorous are needed for photosynthesis, biomass production and primary metabolism. All of these factors or combinations of them can be used to subject the cultures to stressful conditions, inducing the production of reserve compounds, such as lipids and carbohydrates (Markou et al., 2012; Sun et al., 2018) or antioxidants (Gauthier et al., 2020).

Cultivating microalgae under continuous stress condition, though, will have the side effect of reducing productivity and inhibiting growth. The most common solution to this issue is the use of a two-stage cultivation method (Cheng et al., 2017) where algae are grown in optimal conditions during a first phase, to achieve higher cell density, and in a second phase they are subjected to the stressing factors, which will stimulate the accumulation of the metabolites of interest.

1.3.2.1 Nutrient limitation

Nitrogen is a key element in cell metabolism, required for the synthesis of nucleic acids, amino acids, proteins and pigments, and it is the most studied nutrient stress factor for production of starch and lipids.

The complete absence of nitrogen will induce formation of storage compounds in most microalgal species. However, it has been shown that performing batch cultivation with some supply of nitrogen may be a preferable strategy, as it for example yields higher starch in *Tetraselmis subcordiformis* than the nitrate-free medium (Yao et al., 2012). Species from the *Chlorella* genus can accumulate starch concentrations of up to 40% of the biomass dry weight (Brányiková et al., 2011; Dragone et al., 2011). In this genus it has been reported multiple times that starch accumulation is a fast, transient response and that, under prolonged exposure to sub-optimal conditions, a rapid shift to lipid metabolism can happen (Brányiková et al., 2011; Fan et al., 2015; Zhu et al., 2018). Accumulation of starch in *Tetraselmis* species under nutrient deprivation is relatively slower and more stable, whilst still resulting in contents of up to 60% of the biomass (Dammak et al., 2017; Yao et al., 2012). The slower shift to lipid metabolism in these species allows for easier process control, compared to *Chlorella*, in cases where starch is the targeted metabolite.

Long-term stress by nitrogen deprivation will strongly affect growth and productivity rates. However, it can be an optimal method for fatty acid accumulation and for example *Chlorella* species, which are considered an oleaginous species, accumulate lipids under nitrogen deprivation reaching up to 65% of the biomass dry weight (Converti et al., 2009; Griffiths et al., n.d.).

Phosphorous is an essential element in the energetic metabolism of cells. Similarly to nitrogen deprivation, phosphorous deprivation has been shown to enhance accumulation of starch and lipids in microalgae and to strongly impact culture health (Ran et al., 2019). Yao and colleagues however have assessed that the presence of excessive phosphate in the medium is beneficial to starch accumulation under nitrogen deprivation, since it is a limiting factor for the functionality of several key enzymes in starch metabolism (Yao et al., 2018).

It should be noted that species from both the *Chlorella* and *Tetraselmis* genera have a mechanism to store inorganic phosphate through luxury uptake under optimal conditions, meaning that the response of these species to stress by phosphate deprivation will be slower, depending on how much phosphorous they have stored in inorganic granules within the cells (Powell et al., 2009; Zhu et al., 2015)(Powell et al., 2009; Zhu et al., 2015). Starch and lipid productivities, possibly because of this mechanism, are lower under phosphorous deprivation than through other stress factors, suggesting this approach is not ideal to induce the storage of these compounds (Brányiková et al., 2011).

Sulphur has been reported to be the most efficient stress factor for starch accumulation in *C. vulgaris*, leading to up to 60% starch accumulation and less severe impacts on culture health (Brányiková et al., 2011) and was further proved to be the most efficient approach to produce up to 50% lipids in the same species (Sakarika and Kornaros, 2017). Thus, the use of sulphur as a stress factor is considered a promising method for storage compound production in microalgae.

1.3.2.2 Light Stress

Microalgae use photosynthesis to transform CO_2 and light to glucose. Optimal irradiance, that meets the photosynthetic capacity of the cultures, is thus essential not only for basic cell metabolism but also for the synthesis of carbon-based storage molecules such as lipids and starch. High irradiation of microalgal cultures may result in photosaturation, where excessive energy captured by the antennas is dissipated as heat, or in photoinhibition, where the overabundant photons induce the formation of ROS species, causing cell damage. Microalgae have evolved to survive shifts in daily and seasonal light cycles. Thus, they have also developed the capacity to adapt to light stress by changing their pigment composition to protect the cells from damage through photoacclimation. Thus, the use of light as a source of stress has often been studied in connection to pigment production (Paliwal et al., 2017), with the most iconic example being the remarkable accumulation of astaxanthin in *H. pluvialis* (Harker et al., 1996).

Light stress has also been linked to the increase in accumulation of storage compounds. For example, He et al. reported in 2015 that *Chlorella sp*. shows lipid accumulation and FAME increase under the highest light intensity tested, when compared to lower irradiations (He et al., 2015). This is possibly connected to the antioxidant and photoprotective role that some lipid species have.

Amongst the stress factors, light may be the most difficult to study and to standardise, since the exposure of the antennas to photons depends not only on the irradiance but also on the density of the culture and on its depth. Though it is not often reported in the literature, the Biomass Specific Photon Supply Rate is a useful tool to understand, monitor and manipulate the response of cells to stress. Identification of the optimal BSPSR value can dramatically improve experimental procedures, because it permits the identification of the maximum flux of photons that a culture can use before becoming photoinhibited. For example, Janssen and colleagues have studied BSPSR

in connection to TAG synthesis in *N. oceanica*, subjecting cultures of different densities to identical irradiation values. At the lowest cell density, thus at the highest photon flux per cell, TAG synthesis decreased suggesting an inhibiting effect of excessive light. The optimal lipid yield was achieved at an intermediate photon flux per biomass unit (Janssen et al., 2018). Starch accumulation in *C. vulgaris* was similarly studied in connection to light penetration in cultures at different cell densities; in this study, a positive correlation between starch accumulation and average light intensity was seen. Thus, amongst the tested conditions the cultures which received the highest photon supply, produced the highest starch content (Brányiková et al., 2011).

1.3.2.3 Temperature Stress

Microalgae are very sensitive to temperature changes, and though they can survive in a wide range of conditions, each strain has an optimum temperature range that results in optimal growth. The response to temperature as a stress factor is highly species- and strain specific, and an increase in lipids has been reported both for low and high temperatures in microalgae (Minhas et al., 2016). In *T. subcordiformis* an increase in temperature yielded higher MUFAs and SFAa, accompanied by a decrease in PUFA content (Likun et al., 2015), changes in lipid composition with variations of temperature have been suggested to result from the need to adjust fluidity of cell membranes.

A study by Converti and colleagues has also highlighted the species-specific nature of responses to temperature. A temperature rise from 25 to 30 °C in fact resulted in an increased lipid content in *N. oculata.* However, the opposite response was reported for *C. vulgaris* (Converti et al., 2009).

1.3.2.4 Salinity Stress

Many seawater species can tolerate wide ranges of salinity and some of them are strongly halophilic like species from the *Dunaliella* genus, that have been isolated in evaporation ponds and are able to survive very harsh conditions. Excess salinity stress in those and in other species induces the accumulation of high amount of lipids, up to 70% in *Dunaliella tertiolecta* for example (Takagi et al., 2006). Salinity stress may influence greatly the fatty acid profile of microalgae and, for this reason, Minhas in his review suggests that combining salinity, light and nutrient stress may be an interesting way to enhance simultaneous productivity of lipids and carotenoids

Minhas 2016). Deviation from normal salinity limits starch accumulation in *T. subcordiformis,* however its use in combination with nitrogen stress improved the production of storage starch in the biomass (Yao et al., 2013). Instead, in *Tetraselmis marina,* high salinity values negatively influence growth and cell metabolism, limiting starch accumulation. However, in a reduced salinity environment, at 10 ppm NaCl, Dammak and colleagues detected an increase in starch accumulation (Dammak et al., 2018).

1.3.3 Study of Metabolic Pathways

Understanding the pathways behind metabolic changes in microalgae under sub-optimal growth conditions is important for choosing the optimal stress factors and to understand the timing of cell responses. For example, *C. vulgaris* is known to produce starch as a first response to nitrogen deprivation, whereas over prolonged stress it prefers to accumulate fatty acids. Transcriptome analysis with RNA-sequencing during these two stages of stress has been recently performed, confirming the existence of a clear shift in carbon partitioning from the expression of synthesis-related genes towards starch degradation metabolism, glycolysis, and lipid synthesis pathways between day 1 and day 4 of starvation. This approach also allowed to see changes in the regulation of photosynthesis related genes, showing a substantial downregulation induced by nitrogen stress and giving an overview of key processes in the cells (Nordin et al., 2020). Furthermore, gene expression analysis may help identify the expression profile of enzymes, which may also be a useful tool in the development of different foods, as for example in the case of amylases (Raveendran et al., 2018). Microalgae are in fact being studied for their potential as enzyme bio-factories However, the application of this technology is yet to be tested on food production (Brasil et al., 2017).

The study of gene expression has also proved to be a useful tool in choosing the right stress strategy. For example, phosphorous stress has been widely considered an efficient approach to regulate starch metabolism because its absence limits the activity of a key enzyme in starch synthesis, the AGPase. Jiang and colleagues have studied phosphorous deprivation and its effect on starch synthetic pathways in *T. subcordiformis* uncovering the existence of a possible alternative route to starch production through another enzyme, SPh, which is not affected by phosphate availability (Jiang et al., 2017). Thus, a later study from the same research group

uncovered how phosphorous presence may actually benefit the production of starch under nitrogen deprivation, allowing to fine tune the production process in this species (Yao et al., 2018).
A wide array of "-omics" tools is nowadays available to study the response of cells to changing environmental conditions, representing a valuable tool to understand metabolism under stress conditions. Further research in this direction may help uncover the optimal combination of growth conditions to enhance the production of compounds of interest in industrially relevant species.

1.3.4 Pre-treatment and Food Processing

Though pre-treatment of the biomass for food applications has not been widely studied there are hints that several protocols may be useful to improve the addition of microalgae in food products, allowing to overcome sensory and technical issues and possibly to improve sensory properties.
For example, milling biomass before addition in foods is an efficient way to improve the bioavailability of bioactive compounds, enhancing functional properties of the final product. A recent study by Nunes and colleagues studied the role of cell disruption on rheology and bioactivity on wheat bread, resulting in an overall improved antioxidant activity of bread, though yielding an increased firmness of the dough, possibly because of water sequestration (Nunes et al., 2020a). Heat treatments during food production have also been shown to impact the effect of microalgal addition in foods, for example microalgal-pasta texture properties improved upon boiling the pasta, partly remediating the increase in firmness that was detected in the dough (Fradique et al., 2010).
Additionally, the treatment of algal biomass with ethanol before addition in bread was shown to dramatically reduce the content of volatiles, with a positive impact on flavour, colour, and odour of the final product (Qazi et al., 2021).
Biomass pre-treatment may represent a valuable ally in adding higher microalgal contents in the production of foods and more ore research is needed in this regard to thoroughly assess the possibilities in this field.

1.4 Microalgae and Brewing

1.4.1 Brewing

Beer is an alcoholic beverage typically produced through alcoholic fermentation of barley malt by *Saccharomyces cerevisiae* or *Saccharomyces pastorianus*, flavoured and bittered through the addition of hops. It is one of the most widespread and ancient alcoholic beverages, with the first written records dated to 2800 BC in Mesopotamia and an estimated global consumption of billions of litres per year (Anderson et al., 2019; Pires and Brányik, 2015; Piron and Poelmans, 2016). Beer has one of the oldest food regulations concerning its composition, the "German (or Bavarian) Beer Purity Law", *Reinheitsgebot*, which dates back to 1487 and limits beer ingredients to water, malted barley, hops, and yeast. However, this rule is not set in stone and breweries often use starchy and sugars adjuncts or different starch sources to produce beers (Pires and Brányik, 2015). Additionally, with the current expansion of the craft brewing market and the increase of independent microbreweries worldwide the variety of novel ingredients and flavour combinations is also expanding (Baiano, 2021).

Figure 6 shows an overview of the beer production process. After germination, malting and milling, the malt goes through the process of mashing, where it is mixed with hot water to activate starch-degrading enzymes. The enzymes (e.g., amylases) are activated either using one fixed temperature (one-step mashing) or by increasing temperature through a series of enzymatic rests, to activate enzymes in a specific order, optimising the release of sugars. Once the starch has been degraded to fermentable sugars, the insoluble fraction (Brewers' spent grain) is filtered out from the sugar-rich wort through a process called lautering. The wort then goes through a boiling phase, where the hops are added to provide bittering and flavour notes, and, after cooling down, it is inoculated with yeast. Through alcoholic fermentation the yeast consumes fermentable sugars in the wort to produce ethanol, carbon dioxide, and other metabolites. After the fermentation is completed, the beers go through bottling and maturation before being ready to be consumed.

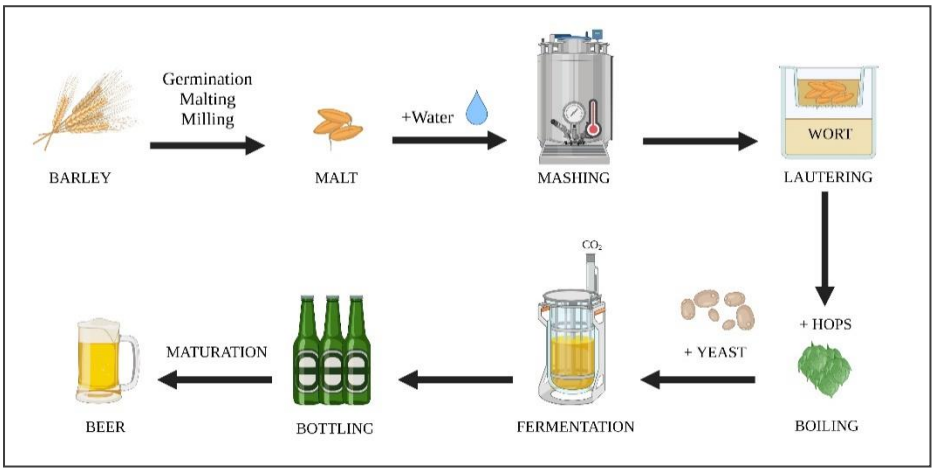

Figure 6: Schematic representation of the traditional brewing process

1.4.2 Alcohol, Brewing and Microalgae

Most literature about microalgae and alcohol refers to the production of bioethanol from species that accumulate large amounts of carbohydrates (de Farias Silva and Bertucco, 2016). Use of microalgal and cyanobacterial biomass for ethanol production has been explored through three main routes. The first, traditional, process involves pre-treatment of the carbohydrate-rich biomass, which is hydrolysed and saccharified before being subjected to alcoholic fermentation with yeasts (Ho et al., 2013). Another process which has been evaluated exploits the capacity of several species such as *C. reinhardtii, C. vulgaris* or *Synechococcus sp.* to execute dark fermentation. Dark fermentation is a process in which carbohydrates are directed to the production of hydrogen, acids and alcohols through fermentative metabolism when switching to a dark growth stage. The third route is "photofermentation", an innovative technology that uses genetic engineering to redirect microalgal photosynthetic pathways towards the production of ethanol, or "Photanol". This technology has acquired interest especially after the establishment of an industrial production plant where cyanobacteria were going to be used directly for ethanol production through a patented method (Piven et al., 2015).

In brewing, malted barley provides both starch and the enzymes needed to degrade starch to fermentable sugars during the mashing process. This involves a range of different enzymes such as alpha- and beta- amylases, limit-dextrinases and alpha-

glucosidase. Microalgae have a similar repertoire of starch degrading enzymes and could potentially also provide enzymatic activity throughout the mashing process. However, this has not been explored in the scientific literature.

The majority of existing literature about microalgae and brewing concerns the application of this technology to wastewater treatment and purification of brewing effluents (Raposo et al., 2010). However, there are a few reports of the cyanobacterium *S. platensis* or microalgae being used as ingredients in beers. For example, Captain Lawrence Brewery in New York has been producing a beer called "Gimmicky Green" and the Freetail Brewing Company in San Antonio has been producing a semi-sweet Belgian-style wheat beer, the "*Spirulina* Wit" (Park et al., 2018). The choice of *Spirulina* as a colouring in beer was often justified by the desire of producing a natural product, thus avoiding synthetic or industrial food dye. In most cases it was added in the final steps of the process in quite low amounts.
A different case comes from the Netherlands, where the Delft microbrewery De Koperen Kat has been producing an *Algenbier*, replacing 5% of the malt with *C. vulgaris* biomass specifically produced for the purpose. No scientific data are available about this product. However, the beer is reportedly a success story, winning a Silver Award in the novel beer category at the Alltech Craft Beer Competition in Dublin in 2014, and still being produced today (DeKoperenKat.nl).

Microalgae are mostly famous for their health benefits, and with healthy lifestyles and habits becoming more popular in modern times it is interesting to note that a brewing company has created a beer containing 0.5% *Spirulina* extract, which was sold as "anti-aging beer" (Park et al., 2018), this is particularly interesting because of the association between microalgae and health, though the claims about aging are yet to be verified. The Delft microbrewery as well highlights in the label the nutritional properties of *Chlorella* biomass, and the success of their *Algenbier* leads to the conclusion that there is a potential market niche for beers with enhanced health benefits.
Despite these attempts the only evidence currently available about possible health benefits related to microalgae and alcohol in beverages comes from a study about the Brazilian spirit cachaça. Dantas and colleagues prepared a functional alcoholic beverage extracting *C. vulgaris* biomass with cachaça and compared its effect against regular cachaça. The experiments were conducted in vitro, testing antioxidant properties through radical scavenging tests, and in vivo on mice, modelling cortical

spreading depression in the brain. The results suggest that the extraction of *Chlorella* biomass confers to the beverage protective properties against the negative effects of alcohol, with strong antioxidant activity in vitro and in vivo, and with evidence of neuroprotective action on neural networks (Dantas et al., 2021).

2 Outline and aims of the thesis

The Algae to Future project, which this PhD thesis was a part of, had the ambitious objective of creating a bridge between microalgal research and industry through the development of algal production protocols of biomasses which could be easily inserted in an industrial setup. To achieve this goal the idea of a tailored biomass was developed: a microalgal biomass that is produced with a final product in mind, and thus has a chemical composition that is designed to fit both the production process and the properties of the final product.

The concept of tailoring microalgal biomass is not entirely new and can be found in the literature, where it generally defines the idea of improving biomass composition according to a special need, for example when targeting production of biofuels (Pavan Jutur et al., 2018). However, studies focusing on the development of a whole food production process including the application of tailored microalgal biomass are very limited.

The main aim of this thesis was to develop a production protocol for starch-rich algal biomass and apply it as an active ingredient in beer brewing. Each of the research papers presented in this thesis represents a different aspect of this process development.

In paper I optimisation of storage starch formation was studied in *C. vulgaris*. This was done applying a high throughput screening method of different cultivation conditions at small scale followed by up-scaling of production and fine-tuning of light conditions.

In paper II starch production was studied in *T. chui*, testing a nitrogen deprivation and replenishment protocol in 25 L tubular PBRs. The assessment of starch production was accompanied by an in-depth study of the expression patterns of genes related to starch synthesis and degradation during deprivation and replenishment of nitrogen.

In paper III starch production in *T. chui* was upscaled to 250 L tubular PBRs, and the harvested starch-rich biomass was used as an active ingredient in beer brewing. The impact of the microalgae on the brewing process and the product properties was investigated.

3 Main results and discussion

3.1 Optimisation of starch production in *Chlorella vulgaris* (Paper I)

Starch is one of the most interesting reserve metabolites produced by microalgae, and it has been mostly studied for its application in biofuel technologies (Chowdhury and Loganathan, 2019; Markou and Nerantzis, 2013; Mata et al., 2010; Milano et al., 2016; Onen Cinar et al., 2020). Nonetheless, recent studies are also beginning to hypothesise the use of starch-rich microalgae in human nutrition, as it proved to have good digestibility values (Ferreira et al., 2020), to have a role in gelatinisation of foods (Gifuni et al., 2018) and to have fat-mimicking properties, making it a potential functional ingredient in foods. Amongst microalgal species, *C. vulgaris* has been widely studied for its capacity of accumulating high amounts of starch, up to 60% of the dry weight, under different stress conditions (Brányiková et al., 2011). Growing microalgae under stress conditions however, is a renowned inhibiting factor for cell proliferation and health (Brányiková et al., 2011; García-Cubero et al., 2018), thus a careful approach is needed to maximise starch production while avoiding mortality and inhibition. Additionally, light irradiation needs to be especially optimised, as photosynthetic processes play a key role in carbohydrate metabolism, and irradiation strongly affects the cells (Janssen et al., 2019; Mulders et al., 2014).

Thus, in order to optimise starch accumulation in this species a three-step approach was applied. The first phase was performed through a screening of optimal starch-inducing conditions performed in erlenmeyer flasks with a full factorial design, crossing three light intensities with four nutrient conditions. Cultures were sampled closely during 96 h of starvation and analysed through HT-FTIR spectroscopy allowing to observe changes in the main biomass components. The results from this experiment allowed to see how both nitrogen and sulphur starvation yielded an increase in starch content within 12 hours from the onset of starvation (Figure 3, Paper I) whereas phosphorous deprivation yielded results similar to the control. This may be explained by the capacity of some algae, such as *Chlorella* , to accumulate phosphate reserves, thus phosphate deprivation was considered less interesting for short-term starch accumulation. In contrast to former studies reporting on the key role of light in starch storage (Brányiková et al., 2011; Cheng et al., 2017), light

intensity in this experiment did not have a significant impact on metabolism, leading to the hypothesis that in thick cultures the penetration of light in the vessels might have been limiting, requiring further optimisation studies.

The second experimental phase was consequently performed in 1.5 L flat panel photobioreactors (Skjånes et al., 2016) with the aim of optimising the irradiance in the cultures by studying the biomass specific photon supply rate. This parameter, which is based on the Lambert-Beer law, measures the penetration of light into the growth vessel keeping into account the light intensity, the light path and the attenuation caused by culture density (Eq.1, Paper I) (Zijffers et al., 2010). The biomass specific photon supply rate was studied in relation to starch accumulation and culture growth, with special focus on the first two days of starvation where starch peak has been identified to happen (Figure 4, Paper I). The results show how the BSPSR is a valuable tool to decide on the composition of microalgal biomass. Cultures subjected to high irradiation were the ones to mostly suffer in terms of cell viability, but yielded the strongest starch concentration over volume, instead at lower light penetration in the cultures higher biomass productivity was achieved, though with lower starch concentration. Irradiance has thus proved to be an interesting tool to define biomass composition which can be applied for tailoring biomass composition. The final step was to scale up the protocol deduced from the former experiments to 25 L tubular photobioreactors to evaluate the scalability of the final protocol in a lab-setup. The results from this preliminary scale-up trial were very promising, with a final productivity reaching up to 40% starch relative content over dry weight.

This research paper has proved that a step-wise method to optimise growth conditions is an efficient way to produce and scale-up a tailored biomass, with desired qualities. FTIR Spectroscopy proved to be an excellent qualitative method to observe the changes in ratio of different biomass components over time in large experiments. And finally, it was proved that to achieve high starch concentration in *C. vulgaris* nitrogen stress must be paired to high light intensity and low cell density, with an ideal BSPSR value above 200.

3.2 Metabolic Pathways of Starch Production in *Tetraselmis chui* (Paper II)

Knowledge about metabolic pathways leading to starch production may be a useful tool to understand how to fine tune stress and to learn which responses may be triggered by different conditions. Additionally, possibly relevant products which are not easily detectable through traditional biomass analysis, may be obtained by stressing microalgae, such for example enzymes.

T. chui, together with other species from the *Tetraselmis* genus, is one of the most promising microalgal species for the purpose of sustainable starch production. These salt-water species are known to accumulate up to 60% starch over dry weight (Yao et al., 2012), are characterised by high productivity rates (Reitan et al., 2021), and are renowned for its high antioxidant and bioactive content (Banskota et al., 2019; Lee et al., 2009; Sedjati et al., 2020).

Starch metabolism has been studied in depth in the model organism *C. reinhardtii* where a putative scheme of starch production pathways has been designed, becoming the reference model for starch metabolism in microalgae (Ball and Deschamps, 2009). Figure 7 reports a summary of this putative model, showing the main enzymes involved during all stages of synthesis and degradation of the granules. The gene expression of these enzymes was the main object of study of Paper II.

The microalga *T. chui* was cultivated in two parallel 25 L tubular photobioreactors in two phases. In the first phase the algae were grown under nitrogen limiting conditions to study the synthetic pathways of starch, then, after starch accumulation reached values above 40% the nitrogen source was resupplied to evaluate degradation and recovery of culture health. Throughout the experiment, growth parameters were closely monitored and samples for gene expression analysis were collected at each sampling point.

The nitrogen supplied was quickly exhausted and culture growth consequently halted as a result of stress conditions, while photosynthetic efficiency measured as *Fv/Fm* dropped (Paper II Figure 1). Consequently, accumulation of starch began, reaching a peak of 58.5% ± 2 of the biomass dry weight at the moment of nutrient resupply. A fast onset of recovery was visible after 12 h from nitrogen replenishment, when the degradation of starch began together with cell division and cultures recovered fully after 72 h.

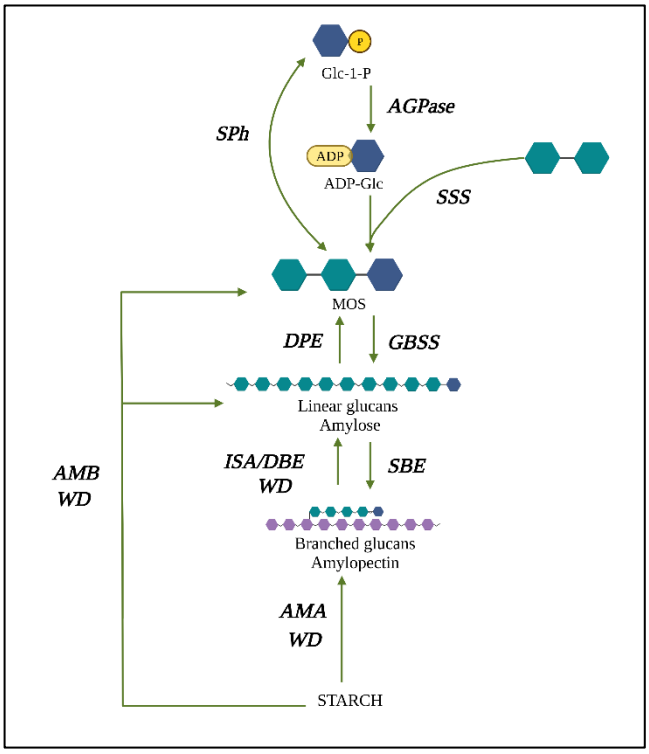

Figure 7: Traditional model of starch synthesis and degradation pathways in microalgae.

AGPase: ADP glucose-phosphorylase; AMA: alpha-amylase; AMB: beta-amylase; DBE: debranching enzyme; DPE: disproportionating enzyme; GBSS: granule bound starch synthase; ISA: isoamylase; MOS: maltose oligosaccharides; SBE: starch branching enzyme; SPh: Starch phosphorylase; SSS: soluble starch synthase; WD: water dikinases.

The gene expression analysis was performed sampling cultures for RNA extraction for further quantitative real-time reverse transcription PCR (RT-qPCR) analysis of constructed cDNA. Reference genes were selected amongst those evaluated in a former study on *T. chui* and were tested for their stability in the samples collected, using geNorm and Normfinder online tools (Paper II Figure 2). Quantitative analysis of gene expression was then carried out for 28 starch-related genes assessing changes in their activity in comparison to the activity at the start of the experiment.

From these results (Paper II Figure 3) a series of interesting remarks may be drawn out. First of all, a timely response to starvation and subsequently to replenishment was detected in most enzymes tested, showing a quick metabolic answer to changes in environmental condition is this species. Secondly, the expression of some key genes was quite distant from the expected results, as for example the SPh gene, which catalyses a key step in degradation of starch, was found to be strongly upregulated during both synthesis and degradation, whereas all the alpha amylase isoforms tested where upregulated during the synthetic phase. Despite this seemingly contradictory

result there is a large body of evidence pointing towards the expression of synthesis genes during degradation of starch, and vice-versa (Goodenough et al., 2014; Ikaran et al., 2015; Ran et al., 2019), with results similar to those reported in our study. This finding supports the idea that starch catabolism may be relevant during synthesis phases to maintain an active flux of metabolites within the synthetic pathway (Moseley et al., 2006).

A third interesting remark is that some other transcripts seemed to be substantially downregulated, despite their important roles in the traditional starch model. This is the case of the two AGPase subunits, which are considered to be the primary control step in starch metabolism (Ball and Deschamps, 2009). This suggests that *T. chui* under these conditions may either adopt different pathways, as it has been suggested SPh could represent an alternative to AGPase in a previous study on *T. subcordiformis* (Jiang et al., 2017), or that a number of underlying post transcriptional and additional regulatory steps may be at the basis of this discrepancy between gene expression and starch accumulation patterns.

3.3 Starch Rich *Tetraselmis chui* Application in Brewing (Paper III)

The application of *T. chui* for human nutrition has been recently approved by the European Union following an application from the Spanish microalgae producer Fitoplancton Marino S. L. (EU Commission, 2017). Thus, species from the *Tetraselmis* genus have been object of multiple studies concerning the application of microalgal biomass in foods, yielding interesting results in the production, for example, of bread, biscuits or soup (Batista et al., 2017; Lafarga et al., 2019a; Qazi et al., 2021).

Paper III has dealt with the case study of starch-rich *T. chui* application to beer brewing, to test the proof of concept that tailored microalgal biomass can be used as an active ingredient in industrial production.

Culturing was performed, following the findings of Paper I and II, in a 250 L tubular photobioreactor, achieving a final yield of 370 g of 50% starch-rich biomass. The biomass was freeze-dried and milled to break the cell walls and was firstly used to assess its behaviour during the mashing phase. In a second step, small scale brewing was performed in 1L flasks to test the effect of microalgal biomass addition to the technical properties of beer.

Firstly, a series of one-step mashing experiments in 15ml volumes (Figure 1, Paper III) at 67 °C was performed to establish challenges, criticalities and efficiencies of microalgal starch degradation to fermentable sugars. Efficient digestion of microalgal starch by barley starch-degrading enzymes was proved to happen within an hour, however it has also been observed how in absence of barley enzymes microalgal starch did not get degraded to fermentable sugars. This implies that endogenous microalgal enzymes were either not active at the given conditions or were not present in a sufficient amount in the biomass tested.

Microalgae-barley mixtures with 5%, 12.5% and 20% barley substitution were subsequently tested, achieving a release of fermentable sugars that was comparable to barley malt control (Figure 4, Paper III), provided that pH is adjusted with lactic acid. The 20% substitution samples yielded the highest result, further highlighting the active contribution of microalgal starch to the pool of fermentable sugars.

The information gathered from mashing trials were used to perform a small-scale brewing in 0.8 L volumes (Figure 8) using the same substitution rates.

Figure 8: Control and 20% microalgal biomass substitution brewing trials, performed in triplicate in 0.8 L volumes

Mixing during the mashing phase proved to be complex in this setup, since microalgae biomass visibly thickened and lumped the mash, thus lower sugar content was detected in microalgae enriched worts. Nonetheless, the results are in agreement with the 15 mL mashing experiments, since the 20% worts yielded the highest sugar content amongst the microalgae enriched samples. Lautering of the wort proved to be a critical step in the process due to the difficulties encountered in separating the thin algal biomass from the wort, resulting in an increased amount of suspension compared to the control. After boiling and adding the hops according to the recipe provided by partner brewery Nøgne Ø, the worts were set to ferment with brewer's yeast, monitoring sugar degradation over time. After 5-6 days the beers were bottled and stored for maturation to be finally run in an Anton Paar alcolyzer to assess their technical properties. The technical analysis showed that the beers were clearly affected by the addition of microalgae, resulting in higher colour values, haze and caloric content. Fermentation efficiency however decreased inversely to microalgal contents, together with CO_2 volume and alcohol content. Thus, further up-scaled testing needs to be performed to fine tune the protocol and improve these parameters.

Finally, tasting of the beers was performed by a small panel of expert brewers, which highlighted how the microalgae beers have a characteristic flavour with interesting marine, umami, and floral notes. The 20% microalgal beer was considered to have too strong seaweed flavours, leading to the conclusion that lower contents in the 5-15% range are the most palatable for the conditions tested.

Former studies on bread or pasta have shown how microalgal biomass may interfere with the doughs, yielding poor technical and sensory scores, unless a small amount of microalgae (<5%) is added to the product. Thickening of the mash and suspension formation were more significant at higher concentrations in this trial, and though they do not majorly hinder the production process at small scale their impact has to be assessed in larger productions. This case-study has however shown how a holistic approach may result in the successful application of larger amounts of biomass, without hindering technical properties and with no major impact on sensory profiles. Further testing is needed to improve the recipe and evaluate possible means to enhance and assess sensory and functional properties of microalgal biomass addition in beer.

4 Conclusion and future perspectives

The general trend emerging from the existing literature about microalgal application in food products is that the addition of microalgal biomass may have a strong impact on organoleptic and technical properties. This suggests that manipulating biomass composition may be beneficial to potentially mitigate these negative effects and improve production processes.
This thesis has explored the concept of tailoring biomass for food applications, focusing on the accumulation of starch in algal biomass with the final goal of applying it as an active ingredient in beer brewing. The main purpose of this study was to develop a proof-of-concept process, from production of tailored algal biomass to its application in brewing.

In Paper I, starch production in the fresh-water species *C. vulgaris* was addressed. Algal growth and productivity of starch was optimized by manipulating stress conditions, developing a solid production protocol which was successfully scaled up to 25 L photobioreactors, and further adjusted for future experiments. By applying FTIR as a screening protocol, the efficiency of nitrogen deprivation as a stress factor and the key role of light distribution in the cultures for successful starch accumulation were demonstrated, reaching up to 50% starch in the biomass. In **Paper II** the question of expanding our understanding of starch metabolism in *T. chui*, a species which is also approved for human consumption, was addressed. This work has underlined the complexity of metabolic pathways connected to stress responses and has clearly shown the species-specific nature of stress responses, which implies that a specific protocol needs to be designed for each species to achieve a desired composition. Though further studies are needed to extend or mapping of metabolism in *T. chui*, a wider understanding of metabolic pathways can be beneficial to further optimise production. In p**aper III**, a process for brewing beer with different microalgal concentrations was investigated, based on directions received from an industrial craft brewery. The experimental work performed allowed to overcome technical issues and challenges in product development, adjusting the method to the novel ingredient and proving the feasibility of microalgal starch implementation as an active element in fermentation.

This thesis has brought together several aspects of linking microalgal biotechnology advances to food industry. However, further research in up scaled conditions is needed to strengthen and confirm the results achieved, both for the brewing case study and for the biomass cultivation protocols. Economic assessments and life cycle analysis about production processes would also allow to evaluate the marketability of microalgal-starch, creating a framework to identify other possible food products where microalgal starch would be creating value, beyond the case study of beer.
An increased knowledge of metabolic pathways regulating storage of interesting compounds may be useful for the development of both high and low value products, thus research in this direction should be pursued implementing novel "-omics" technologies.
Finally, for the production of an optimised microalgae beer, further testing is required to mitigate the negative effects of biomass addition in larger amounts. This could be brought on by studying pre-treatment methods, evaluating the properties of different food-grade microalgal species and testing novel recipes that may complement or balance the sensory characteristics of algae beer.

In conclusion, the market size of microalgal food products is bound to increase in the years to come (Fernández et al., 2021), allowing for novel products to be developed. To successfully spread this sustainable technology throughout this high-value industrial sector, possibly paving the way for other industries to thrive as well, the knowledge base needs to be transferred outside of academia. Developing defined protocols that are potentially standardisable, in order to produce biomass which is tailored to fit into existing industrial lines, means that, ideally, the whole production process may happen outside of the safe haven of research institutes and highly specialised microalgal industry. This could bring microalgal biotechnology to a wider audience, increasing demand, and ultimately leading to novel discoveries and advances in the field.

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Starch Rich *Chlorella vulgaris*: High-Throughput Screening and Up-Scale for Tailored Biomass Production

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Abstract: The use of microalgal starch has been studied in biorefinery frameworks to produce bioethanol or bioplastics, however, these products are currently not economically viable. Using starch-rich biomass as an ingredient in food applications is a novel way to create more value while expanding the product portfolio of the microalgal industry. Optimization of starch production in the food-approved species *Chlorella vulgaris* was the main objective of this study. High-throughput screening of biomass composition in response to multiple stressors was performed with FTIR spectroscopy. Nitrogen starvation was identified as an important factor for starch accumulation. Moreover, further studies were performed to assess the role of light distribution, investigating the role of photon supply rates in flat panel photobioreactors. Starch-rich biomass with up to 30% starch was achieved in cultures with low inoculation density (0.1 g L^{-1}) and high irradiation (1800 µmol m^{-2} s^{-1}). A final large-scale experiment was performed in 25 L tubular reactors, achieving a maximum of 44% starch in the biomass after 12 h in nitrogen starved conditions.

Keywords: *Chlorella vulgaris*; starch; FTIR; photon supply rate; microalgae

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1. Introduction

Several microalgal species accumulate starch as a storage compound under sub-optimal environmental conditions and this property has made them extensively studied within the fields of biofuels, bioplastics and biorefining in general [1–5]. Microalgal biomass has the potential of being a sustainable substitute to fossil resources and research is consistently improving the feasibility and scalability of algal biorefining processes [6,7], from cultivation technologies to downstream processing. However, for a microalgal industry to thrive it is necessary to also target high-value compounds [8], and microalgal biotechnologists are starting just recently to valorise starch-rich biomass as a potential ingredient in the agri-food-tech sector, an important economical field that could make algal production economically viable [9] and thus implement a sustainable technology into novel industrial sectors.

Within the high-starch producers, *Chlorella vulgaris* is among the species enlisted in the novel foods catalogue by the European Food Safety Authority [10], it is approved for human consumption and is the most used species for food-related industrial production. The starch from *C. vulgaris* has an amylose-amylopectin ratio of 30–40%, which is similar to the ratio of potatoes and cereals and has similar crystallinity and thermal properties. However, the size of the starch granules in the algae is significantly smaller [11]. Algal

starch performs well in digestibility tests if compared to commercial potato starch [12], and may represent an easily accessible source of glucose. Extracted starch could also be evaluated as a functional food for processes needing gelatinisation [9] or fermentation steps since algal starch extraction only requires boiling. Despite the fact that the exact costs for starch production from microalgae are not known we can assume that these are higher than potato starch based on the existing techno-economic studies performed for biomass [13] and lipids (TAG) [14]. However, the use of microalgal starch in foods has huge potential in terms of sustainability since algae production has higher ground areal productivities, high CO_2 fixation rates and does not compete with crops for arable land and fresh water. There are also additional benefits related to the health-promoting properties of algal biomass as algae are rich in antioxidants and unsaturated lipids, even during starch accumulation [15].

Chlorella vulgaris is a well-studied microalgal species, reported to be an efficient starch producer. A variety of methods have been tested to induce starch accumulation and very diverse results, ranging from 20 to 60% starch in the dry weight, have been achieved. Nitrogen starvation has been extensively studied and reported to induce up to 55% starch in the biomass content [16,17] but is also renowned as a strong inhibitor factor for cell proliferation and biomass productivity [18,19]. Under sulphur starvation conditions *Chlorella vulgaris* was shown to accumulate up to 60% starch [18] with low short-term mortality, and thus better productivities [18,20,21]. The nutrient limitation has also been tested by diluting the whole medium, showing yet again high starch accumulation and low mortality rates [22].

While the modulation of nutrients is the most acknowledged trigger for metabolic switches, light availability also plays an essential role in the shift to starch accumulation within the cell. The biomass specific photon supply rate (BSPSR) is a parameter that allows the quantifying and standardisation of the light received from cultures in different setups, based on the surface irradiance, the depth and the density of the culture. Though it is not frequently reported in the literature, BSPSR has shown a strong effect on the accumulation of storage compounds for several species [23,24]. Thus, it can be inferred from the literature that high starch accumulation was frequently achieved in *Chlorella* species at low irradiance and low inoculum concentration [18,25,26], whereas the lowest reported starch contents have been reported either at a lower light irradiance or at high inoculum density or in the presence of wider light paths.

Considering the variety of parameters that may influence the accumulation of starch in *Chlorella vulgaris*, our investigations began with a high-throughput screening performed in a traditional shake flask system combined with a high-throughput Fourier Transform Infrared (FTIR) spectroscopy, to test different nutrient and light conditions. FTIR spectroscopy is a biophysical technique that allows the profiling of the main cellular components—lipids, proteins, polysaccharides, polyphosphates, pigments—in a rapid and high-throughput manner. The analysis of cell chemistry by FTIR is done on the intact cells and does not require tedious extraction of cell components, therefore, it has been positioned as a next-generation phenotyping technique suitable for extensive screenings in biotechnology [27–29] Recently, the technique was utilised for screening oleaginous yeast [30–38], filamentous fungi [39–41] and microalgae [42–45].

In this study, FTIR spectroscopy was used to map the chemical changes in the biomass of *Chlorella vulgaris* under multiple nutrient and light stress conditions using a factorial design on 12 different treatment combinations. Based on this, optimal parameters for starch production were selected and scaled to 1.5 L flat panel photobioreactors, to assess the effect of light distribution and BSPSR. Finally, the best conditions were translated to a 25 L tubular reactor, to evaluate the scalability and productivity of starch.

2. Materials and Methods

2.1. Algal Cultures

Chlorella vulgaris strain SAG 211-11b obtained from SAG Culture Collection of Algae (Göttingen, Germany) was maintained on agar plates on TAP medium [46] at a

20 μmol m^{-2} s^{-1} irradiance and 22 °C temperature. Prior to inoculation, the algae were scaled up in Erlenmeyer flasks or screw cap bottles, both supplied with 1% CO_2: air mixture (*v*/*v*) on a shaking table, at 50–100 μmol m^{-2} s^{-1}, 22 °C, at 110 rpm. The growth medium was M8a mineral medium, a version of the M8 medium developed by Mandalam and colleagues [47] based on *Chlorella vulgaris* elemental balancing, further modified by Kliphuis et al. [48].

For starvation experiments, the M8a medium was modified by removing either the nitrate, phosphorous or sulphur sources. To compensate the molarities of the other compounds in the salts, KNO_3 was substituted by KCl, and $MgSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$ were respectively substituted with $MgCl_2 \cdot 6H_2O$, $ZnCl_2$ and $CuCl \cdot 2H_2O$. In the experiments involving the removal of multiple macronutrients, the absence of a buffer in the P-deprived cultures was compensated by adding 3 g L^{-1} $NaHCO_3$ to all the cultures of the experiment. In all mediums, the initial pH was set to 7.5 with NaOH and HCl.

2.2. Experimental Designs

Three experiments were performed in our study to assess optimal conditions for starch production. The three experimental set-ups are illustrated in Figure 1.

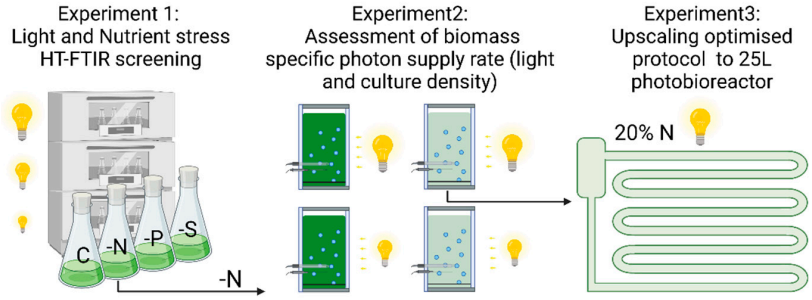

Figure 1. Experimental setup of the three experiments performed in the study.

In experiment 1, FTIR spectroscopy screening, a Multitron Incubator with three chambers (InforsHT, Mainz, Germany) was used to test different cultivation conditions in 36 cultures. The experiment was performed in 1 L Erlenmeyer flasks containing 500 mL culture, mixed by filter-sterilised air with 1% CO_2 and orbital shaking at 110 rpm, and the light was provided from two sides. Twelve cultures with a starting $Absorbance_{750\ nm}$ = 1.2 were cultured for acclimation in full M8a medium, increasing light intensity from 100 to 400 μmol m^{-2} s^{-1}. The starting pH was 7.5. After incubating for six days cultures were centrifuged and resuspended in 36 Erlenmeyer flasks at an $Abs_{750\ nm}$ = 0.5 in four medium treatments (control (C), nitrogen starved (-N), phosphorous starved (-P) and sulphur starved(-S) in triplicates. The temperature was set to 25 °C and the three incubator chambers were set respectively at 800, 1300 and 1800 μmol m^{-2} s^{-1}.

In experiment 2 the cultures were grown in autoclavable flat panel photobioreactors [49] to study the effect of light intensity and culture density on the production of starch. The reactors' volume capacity was 1.5 L and the light path 3 cm. The light was supplied by modulable LED panels, 5% CO_2: air mixture (v/v) was supplied on demand to maintain pH stable at 7.5 ± 0.1 and the temperature was set at 25 °C. Cultures were acclimated for four days, increasing light intensity daily from 100 to 500 μmol m^{-2} s^{-1}. The cultures were harvested and centrifuged, and the algae were resuspended in a nitrogen starvation medium at the desired density and aliquoted into six reactors. Two inoculation densities, 0.25 and 0.65 g L^{-1} were tested at two light intensities, 1300 and 1800 μmol m^{-2} s^{-1}. Each treatment combination was run three times.

For the scale-up trials, experiment 3, low inoculum (0.1 g L^{-1}) cultures were grown in LGemTube RD1-25, 25 L tubular photobioreactors (LGem BV, Rotterdam, The Netherlands)

in M8a medium containing 20% of the nitrogen source. The temperature was maintained at 25 ± 2 °C, pH maintained at 7.5 ± 0.2 controlled by CO_2 addition, and the light was increased daily (50 to 100 to 200 μmol m^{-2} s^{-1}) until the moment of starvation when it was finally set to 1300 μmol m^{-2} s^{-1}.

2.3. Growth Analysis

Algal growth was followed by spectrophotometry, measuring Optical Density as Absorbance at 750 nm using a Spark™ microplate reader, (Tecan®, Männerdorf, Switzerland).

Dry weight was measured filtrating a known volume, diluted in 20 mL distilled water, in pre-washed and weighed Whatman GF/F glass fibre filters 25 mm diameter with 0.7 μm pore size (Cytiva, Marlborough, MA, USA). Filters were subsequently dried in a drying oven at 105 °C for 24–48 h before weighing.

2.4. FTIR Spectroscopy of Algal Biomass

FTIR spectroscopy analysis of algal biomass for all screening samples was performed in the following way: (1) approximately 800 μL of cell culture, depending on the optical density, was centrifuged for 5 min at 4700 rpm (4816× *g*) and the supernatant was removed; (2) the obtained cell pellet was washed three times with distilled water; (3) the washed pellet was resuspended in 50 μL distilled water, to achieve a final density of Abs_{750} = 7–8, (4) 10 μL of cell suspension was transferred in triplicate on the 384 wells silicon FTIR plate and dried at room temperature.

FTIR Spectra were recorded in a transmission mode using a High Throughput Screening eXTension (HTS-XT) unit coupled to the Vertex 70 FTIR spectrometer (both Bruker Optik, Ettlingen, Germany). Spectra were recorded as the ratio of the sample spectrum to the spectrum of the empty IR transparent microplate in the region between 4000 cm^{-1} and 500 cm^{-1} with a spectral resolution of 6 cm^{-1}, a digital spacing of 1.928 cm^{-1}, and an aperture of 5 mm. For each spectrum, 64 scans were averaged. In total, 360 biomass spectra were obtained. The OPUS software (Bruker Optik GmbH, Ettlingen, Germany) was used for data acquisition and instrument control.

2.5. Light and Biomass-Specific Photon Supply Rate

The light was measured with a light meter (SpectroSense2, Skye instruments, Llandrindod Wells, UK), photon flux density was calculated averaging four different measurements taken in the same positions for all reactors. The biomass specific photon supply rate values at the beginning of starvation were calculated as described by Zijiffers et al. [50] using Equation (1).

$$r_{E,x} = PFD_{in}/C_x \times l \quad (1)$$

With PFD_{in} being the incoming photon flux density, expressed as μmol m^{-2} s^{-1}, C_x being the dry weight biomass concentration in g m^{-3} at the onset of starvation and *l* being the light path in meters.

2.6. Nutrient Consumption

The nitrogen consumption in experiments 2 and 3 was assessed on the spot with the colorimetric Nitrate Test strips, 10–500 mg L^{-1} (NO_3^-), MQuant® (Supelco, Bellefonte, PA, USA). Values were later confirmed with ion chromatography performed on 940 Professional IC Vario, (Metrohm AG, Herisau, Switzerland).

After centrifuging 15 mL, the supernatant was filtered through 0.22 μm cellulose acetate membrane filters (VWR International, Radnor, PA, USA). IC was calibrated for detecting three anions, NO_3^-, PO_4^- and SO_4^-.

2.7. Starch Analysis

Centrifuged pellets were freeze-dried, and 7 ± 1 mg biomass were weighed in triplicates. The samples were bead-beaten in ethanol to break the cells and remove interfering compounds and the starch was subsequently quantified with the Total Starch (AA/AMG)

Assay Kit (Megazyme, Wicklow, Ireland). Briefly, the pre-treated biomass was incubated with α-amylase and amyloglucosidase enzymes to degrade starch to monosaccharides. The total sugar content was then assessed with a colorimetric reaction against a glucose standard curve and the glucose content was transformed to starch multiplying by glucose/starch molecular weight conversion factor (0.9).

2.8. Data Analysis

The Following software packages were used for the analysis of FTIR spectra: Unscrambler X version 10.5.1 (CAMO Analytics, Oslo, Norway), and Orange (The Mathworks Inc., Natick, MA, USA) [51]. The pre-processing of FTIR spectra was performed in two ways: FTIR spectra of the algal biomass were first transformed to second-derivative spectra by the Savitzky-Golay algorithm using a polynomial of degree 2 and a window size of 11 in total. The second-derivative spectra were pre-processed by extended multiplicative scatter correction (EMSC), an MSC model extended by linear and quadratic components [52–54]. Technical replicates were averaged to remove the technical variability of the measurements. Then the spectra were cut and used for the principal component analysis (PCA) of lipid (3050–2800 cm^{-1}, 1800–1700 cm^{-1}), protein (1700–1500 cm^{-1}) and polysaccharide (1200–700 cm^{-1}) spectral regions.

Experiment 2 was analysed using R Studio version 3.6.1 (R. RStudio, Inc., Boston, MA, USA), one-way ANOVA was performed on the data after a Cochran test of homogeneity of variances. Where outliers were detected an sqrt transformation was applied, and where the transformation did not remove the effect of the outlier, the significance level to reject the null hypothesis was set at $\alpha < 0.001$.

3. Results and Discussion

3.1. Experiment 1: Effect of Light Stress and Nutrient Starvation

High-throughput cultivations are based on utilising microtiter plates or microbioreactor based systems, where it is possible to grow simultaneously many microbial species and strains under different conditions [36,37,55–58]. Such systems, as, for example, Bioscreen C, MTPS-Duetz or microbioreactors have not been evaluated for screening phototrophic microalgae because they often have limited light transparency which does not allow optimal lighting during cultivation. For this reason, we used a traditional shake flask system combined with the high-throughput FTIR spectroscopy to assess qualitatively how nutrient and light treatments affect the metabolism and composition of *C. vulgaris* biomass over time. FTIR detects the IR radiation, which is absorbed by specific molecular bonds, generating a spectrum that can be considered a chemical fingerprint of the biomass. The characteristic absorbance peaks of the different bonds, and the corresponding molecular components and spectral regions are detailed in Table 1 and Figure 2.

Table 1. Peak assignments of the FTIR-HTS spectra in the algal cells in experiment 1 (main chemical components are marked on Figure 2).

Chemical Components	Wavenumber (cm^{-1})	Peak Assignment	Reference
Polysaccharides (Red area on Figure 2)	3500–3200	O-H stretching	[59]
	1200–1000	C-O and C-O-C stretching	
	1080	C-O-H bending in starch	[12,60]
	1155	C-O stretching in starch	
Lipids (Blue area on Figure 2)	3010	=C-H stretching	[59]
	2955	-C-H (CH3) stretching	
	2925	>CH2 of acyl chain	
	2855	-C-H (CH2) stretching	
	1745	-C = O stretching in esters	
	1465	-C-H (CH2, CH3) bending	

Table 1. *Cont.*

Chemical Components	Wavenumber (cm^{-1})	Peak Assignment	Reference
Proteins (Green area on Figure 2)	1680–1630 1530–1560	-C = O stretching, Amide I C-N-H deformation, Amide II	[59]
Polyphosphate (Grey area on Figure 2)	1265 885	P = O stretching P-O-P stretching	[59]

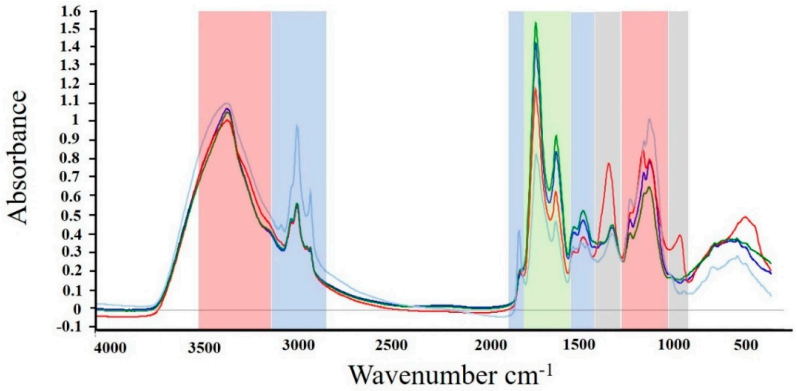

Figure 2. Preprocessed FTIR-HTS spectra of *Chlorella vulgaris* grown for 94 h on control medium (dark blue), nitrogen starvation (light blue), phosphorus starvation (green) and sulphur starvation (red) under light intensity 1800 μmol m^{-2} s^{-1} in experiment 1.

Figure 2 shows the spectral emission of cultures grown under different nutrient conditions using data from the last time point at a high light intensity and is an example of how the treatments generate different chemical fingerprints. The spectrum shows a marked difference in the starch peaks at the wavelengths of 1080 and 1155 cm^{-1}, with nitrogen starvation giving the highest signal, whereas the other polysaccharide region is homogeneous between treatments, thus we consider starch accumulation as a storage compound to be the main driver for differences in polysaccharide region, being starch the most abundant carbohydrate in *C. vulgaris* [12]. In the lipid region, there is again a strong signal for the nitrogen deprived culture which has a specific pattern and a higher peak at 1745 cm^{-1} related to ester bond stretching in acyl glycerides. Proteins have a higher signal at control and P- conditions though the peaks are similar for all treatments. Finally, the signals at 885 and 1265 cm^{-1} have been attributed to polyphosphates which are known to be accumulated in microalgae, including *C. vulgaris*, as inorganic IP3 granules [61,62].

As confirmed by further principal component analysis (PCA), the areas with higher variability are the lipid and polysaccharide regions. Their changes over time are detailed in Figure 3. The PCA results have shown significant chemical changes in the algal cells grown under different nutrient conditions, although no changes were detected for the different light intensities. Generally, among all tested conditions, nitrogen and sulphur starvation showed the most pronounced effect on the algal cellular chemical profile, while control and phosphorus did not show any significant effect. Moreover, lipids and carbohydrates were the most affected by the nitrogen and sulphur treatments, while changes in protein content and profile were not detected if not at the last time points, where cultures were also visibly stressed (data not shown).

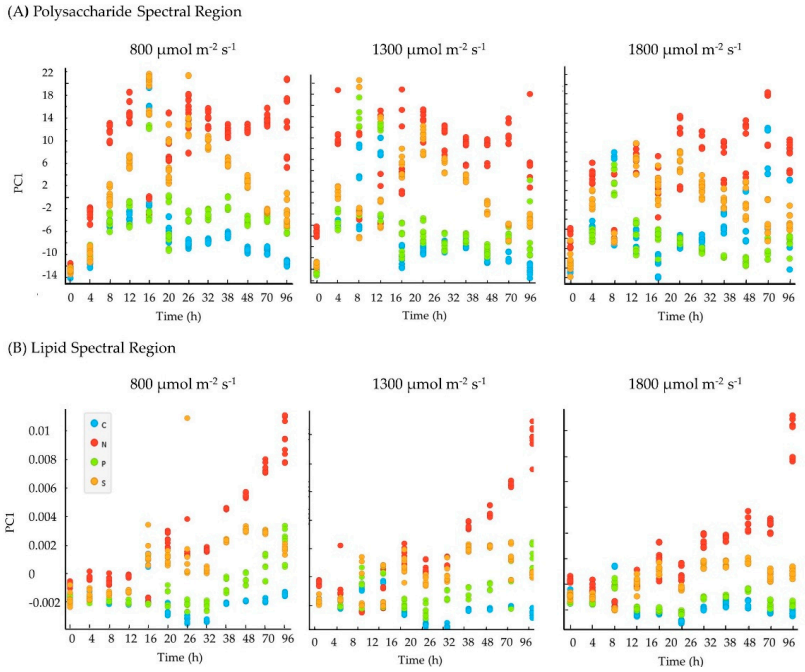

Figure 3. FTIR-HTS spectra PCA score plots of (**A**) polysaccharide (1200–900 cm^{-1}) and (**B**) lipid (3000–2800 cm^{-1} and 1800–1700 cm^{-1}) regions of *C. vulgaris* biomass grown on different nutrient conditions (blue for control, red for nitrogen starvation, green for phosphorus starvation and orange for sulphur starvation) and under three light intensities in experiment 1.

Thus, in Figure 3A, we can observe how nitrogen and sulphur starvation influenced polysaccharide accumulation in algal cells. In these conditions the polysaccharides increased rapidly from 4 h to 16 h, then it stabilized until 32 h and, finally, it dropped slightly for cells grown under nitrogen starvation, while in sulphur starvation, we can observe a considerable decrease. Additionally, a significant increase over time in the lipids (Figure 3B) can be seen under nitrogen starvation, with the highest value indicated after 94 h. Sulphur starvation also slightly affected total lipid with the highest content at 16 h–94 h while control and phosphorous conditions were shown not to be affecting the total lipid content.

Starch and lipid storage patterns in our study are in agreement with several examples existing in the literature that show how starch accumulation is a fast response to environmental changes, with an anabolic phase lasting 12–48 h followed by starch degradation and concomitant lipid synthesis [63–65]. This sequential accumulation is a mechanism that is activated when sub-optimal conditions are long-term, and the cells turn from starch to lipid synthesis, which is preferred as storage having higher energy density and stability [21,66].

Among the treatments, phosphorous starvation did not have an effect on the biomass composition, although Branyikova et al. in 2011 [18] reported it has a role in inducing starch accumulation. We theorise that this may be explained by the presence of polyphosphates, detected with FTIR spectroscopy, which may have been induced by high phosphorous content in the medium prior to starvation, through luxury uptake [61], whereas in the aforementioned study phosphorous in the growth medium was lower, possibly hindering IP3 uptake and resulting in stress.

Nitrogen and sulphur starvation proved to both be promising triggers for polysaccharide accumulation and, despite sulphur being reported to induce up to 60% starch in the literature [18], nitrogen led to a faster and steadier accumulation pattern. It is also worthy

to note that, according to the literature, sulphur limitation is associated with lower cell mortality and better growth performance over a long period of time [18,20]. However, since our work has focused on producing starch, which increased and should be harvested in a short time span, it was considered that the expected long term cell mortality and productivity loss in nitrogen starvation would not be deemed an issue for this specific purpose, and a faster and steadier accumulation would be preferable.

The tested light intensities did not result in any significant differences in the biomass composition within each treatment, which is in contrast to previously reported data [18,25] that show light as a major influence on starch concentration. Light is in fact necessary to fixate the carbon used for the starch synthesis [67]. These results led to the hypothesis that light penetration in this experimental setup may have been a limiting factor for polysaccharide production. Considering the difficulties encountered assessing light distribution in Erlenmeyer shake-flasks, further studies about the relationship between irradiation, culture density and starch concentration were performed in Flat Panel Photobioreactors under nitrogen starvation.

3.2. Experiment 2: Effect of Biomass-Specific Photon Supply Rate under Nitrogen Starvation

The light distribution per biomass unit was studied in flat panel reactors subjecting cultures inoculated at two different densities, 0.25 and 0.65 g L^{-1}, to two light intensities, 1300 and 1800 µmol m^{-2} s^{-1}. These conditions resulted, as shown in Table 2, in four biomass-specific photon supply rates (BSPSR) which have a clear effect on growth and starch accumulation under nitrogen deprivation.

Table 2. Experimental parameters and results from experiment 2. The experimental parameters were biomass-specific photon supply rate (BSPSR), light intensity and inoculum density, vs the resulting starch concentration in the biomass 12 h after N starvation, starch concentration in the culture 24 h after N starvation, and biomass productivities after 48 h. Average values ± SD.

BSPSR (µmol g^{-1} s^{-1})	Irradiance (µmol m^{-2} s^{-1})	Inoculum (g L^{-1})	$Starch\%_{12\,h}$ (g g^{-1}_{dw})	$Starch_{24\,h}$ (g L^{-1})	$Productivity_{48\,h}$ (g L^{-1} day^{-1})
102	1300	0.65 ± 0.01	15.0 ± 0.6	0.17 ± 0.03	0.42 ± 0.06
141	1800	0.65 ± 0.01	15.5 ± 3.0	0.15 ± 0.03	0.27 ± 0.10
268	1300	0.25 ± 0.04	28.9 ± 1.6	0.17 ± 0.01	0.25 ± 0.09
371	1800	0.25 ± 0.04	27.5 ± 0.2	0.13 ± 0.02	0.21 ± 0.05

Figure 4a,b, show the absorbance and dry weight over 96 h from starvation. The overall increase in dry weight and absorbance may be explained by the accumulation of metabolites such as starch and consequent increase in weight and granulometry [68] despite nitrogen starvation inhibiting growth.

The cultures which received fewer photons per biomass unit and are also those that had a higher density at starvation, had higher final biomass productivity, 0.2 and 0.3 g L^{-1} d^{-1}, compared to the lower irradiance treatments that were ranging between 0.12 and 0.15 g L^{-1} d^{-1}.

As reported in the FTIR screening, also in flat panels the starch content in the cells had increased significantly in all cultures within the first 12 h (Figure 4c) followed by degradation. Maximum starch concentration reached up to 30% in cultures with high BSPSR, whereas the cultures that received a lower light supply rate did not accumulate more than 15%. The relationship between BSPSR and starch content at 12 h was confirmed by a strong positive correlation of correlation coefficient 0.88 (Figure 5a).

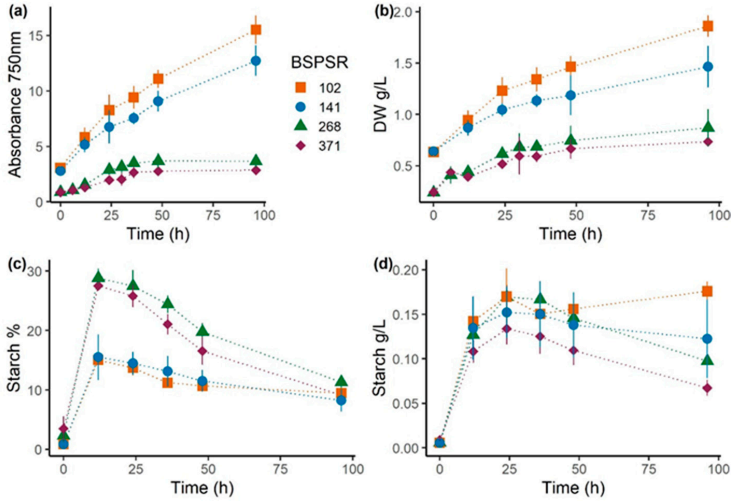

Figure 4. Time course over 96 h after N starvation expressed as average of triplicate cultures in experiment 2: (**a**) culture density expressed as absorbance at 750 nm; (**b**) culture density expressed as biomass dry weight; (**c**) starch concentration expressed as % of biomass dry weight; (**d**) starch concentration expressed as grams per litre.

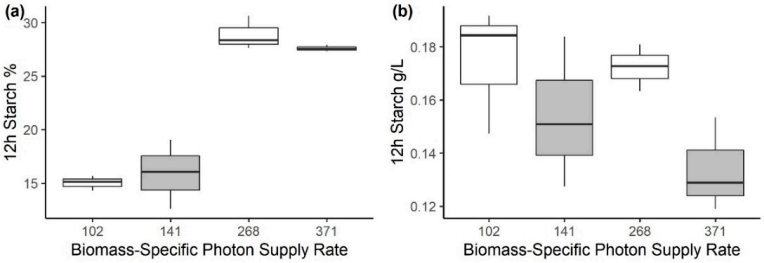

Figure 5. Starch maximum accumulation in *C. vulgaris* at different biomass specific photon supply rates in experiment 2. (**a**) peak of maximum concentration in the biomass, observed 12 h within starvation; (**b**) peak of maximum starch production in the culture, expressed as g L^{-1}, and observed 24 h within starvation.

The peak of starch concentration in the biomass did not coincide with the peak of maximum starch accumulation in the culture, expressed as g L^{-1}, which happened at 24 h rather than 12 h (Figure 4d), and was very similar for all four treatments. This discrepancy happened because in starch-rich cultures, at 24 h from starvation, the starch was still contained at a high percentage in the cells (>25%), while we see a small increase in biomass dry weight. At the same time, the cultures with lower starch content, which had a higher initial biomass, higher productivity and less cell mortality reached similar starch production values as the cultures with a high starch content, which on the other hand had lower productivity and suffered the impact of light stress on cell growth. The photon supply rate, in this case, showed the opposite effect with a weak negative correlation of −0.65 (Figure 5b). These two tendencies may both be interesting for industrial production depending on the desired final product. In a biorefinery context, where starch is to be extracted, a lower BSPSR may be chosen as a strategy to produce starch alongside other interesting metabolites. However, if the goal is to produce a starch-rich biomass with high percentage starch content, a higher BSPSR is to be preferred.

It is interesting to know that starch contents higher than what was achieved in our study have been previously reported in *Chlorella*, and have been often achieved combining high light and low inoculum density, reaching up to 55% in *C. vulgaris* [18] and even 77% in a CO_2 adapted strain grown in a 2-stage process with high dilution [25]. In continuous chemostat growth, it has also been reported a very high effect of light and inoculum density, with the best result of 25% starch-rich biomass [19]. However, Dragone et al. reported in 2011 a high starch content of 44% in *C. vulgaris* at a relatively low light intensity of 70 μmol m^{-2} s^{-1} [22]. Differences in mediums used and growth vessels may be the cause of those discrepancies, nonetheless, our study contributes to a solid body of literature that identifies *C. vulgaris* as a high starch producer, especially at higher photon supply rates.

3.3. Experiment 3: Upscaling Starch-Rich Biomass Production

Very few studies have tested starch production from *C. vulgaris* at a larger scale, and most experiments done so far, including our studies in flat panel reactors, have adopted a protocol where the cultures were centrifuged and resuspended in a starvation medium. Such protocols cannot be applied on large scales.

Thus, two further separate experiments were performed with *C. vulgaris* in 25 L tubular photobioreactors inoculated at a low density (<0.1 g L^{-1}) in a modified M8a medium containing 20% of the original nitrogen content. Giving a limited amount of nitrogen has allowed the algae to proliferate during the first cultivation days before exhausting the nitrogen and beginning the transition to starvation metabolism.

The difficulty when limiting the amount of nutrients supplied, rather than completely removing it at a known time point by centrifugation lays in identifying the exact moment of nutrient exhaustion. With a species like *C. vulgaris*, which in the present experiments has again been shown to accumulate maximum starch between 12–24 h from starvation, monitoring nitrogen consumption is essential to both (i) increase the light supply at the correct moment and (ii) harvest the algae before starch degradation begins.

To monitor consumption nitrogen was consequently checked daily with rapid test strips, and the results were subsequently confirmed with ion chromatography. In Figure 6a we see that the 20% of N source in these conditions was exhausted within three to four days from inoculation, allowing for the culture to acclimate and grow while the light was daily increased until it was finally set to 1300 μmol m^{-2} s^{-1} on t0 of starvation to support starch production. Starch follow up showed an increase over the 12 h after starvation up to 44% in both experiments performed.

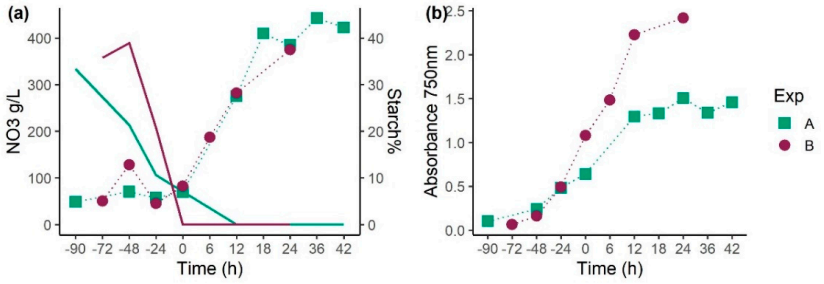

Figure 6. Time sequence of two experiments growing *C. vulgaris* in limiting medium containing 20% nitrogen in experiment 3. Experiment A was harvested after 24 h from starvation (x = 24), experiment B was harvested 36–48 h after starvation. (**a**) Nitrate content in the medium measured with ion chromatography (solid line) and starch accumulation in the cells (dotted line) (**b**) Absorbance at 750 nm.

Experiment A was followed up further to 48 h after starvation, and notably the starch content remained above 40% even after the 12 h peak. In experiment B, where starch-rich biomass was harvested between 12 and 24 h after starvation, the growth was altogether faster than in the former trial (Figure 6b) achieving higher biomass productivity and a final starch production upon the harvesting of 1 g L^{-1}. Harvesting 12–24 h after the onset of stress implies that nitrogen starvation mainly impacted starch accumulation, not other components of the biomass since other known effects of starvation usually require longer incubation times. Additionally, through a short-term stress phase, the supply of high light intensity, which is a factor that may greatly influence the cost of production, is limited to a brief time.

4. Conclusions

Starch accumulation above 40% was achieved at a large scale in *C. vulgaris*, through a protocol that could be suitable for industrial setups. FTIR spectroscopy proved to be an efficient tool for screening multiple stress conditions and characterising changes in biomass composition, identifying the stress conditions to be used for inducing starch production. Both nitrogen and sulphur starvation proved to be good methods for inducing starch and lipid accumulation, yet nitrogen as a stress factor led to faster starch accumulation (12 h), making it preferable for industrial production. To achieve efficient production, nutrient stress needs to be implemented in a protocol that uses a high light intensity to cell density ratio. Thus, a biomass specific photon supply rate above 200 is recommended to produce biomass containing high starch concentrations.

Future investigations involving novel approaches such as "omics" technologies may extend our understanding of metabolic responses to stress, allowing us to further improve the final composition of the biomass. To speed the transfer of knowledge to the industry, research should go forward in assessing the scalability of the process on one side, testing in pilot-scale reactors and in outdoors conditions to improve starch productivity and evaluating the properties of starch-rich algal biomass and testing the possible applications in the food sector.

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Paper II

Metabolic pathways for biosynthesis and degradation of starch in *Tetraselmis chui* during nitrogen deprivation and recovery

Manuscript under revision in Bioresource Technology

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Abstract

Tetraselmis chui is known to accumulate starch when subjected to stress. This phenomenon is widely studied for the purpose of industrial production and process development. Yet, knowledge about the metabolic pathways involved is still immature.

Hence, in this study, transcription of 27 starch-related genes was monitored under nitrogen deprivation and resupply in 25 L tubular photobioreactors. *T. chui* proved to be an efficient starch producer under nitrogen deprivation, accumulating up to 56% of relative biomass content. The prolonged absence of nitrogen led to an overall down-regulation of the tested genes, in most instances maintained even after nitrogen replenishment when starch was actively degraded. These gene expression patterns suggest post-transcriptional regulatory mechanisms playing a key role in *T. chui* under nutrient stress. Finally, the high productivity combined with an efficient recovery after nitrogen restitution makes this species a suitable candidate for industrial production of high-starch biomass.

Keywords

Microalgae; *Tetraselmis*; starch; gene expression; nitrogen

1. Introduction

Starch is the primary storage compound in plants and an essential carbohydrate source for human and animal nutrition. Production of starch as a reserve in microalgal cells has been widely studied for its potential use as a feedstock for bioethanol and other biorefinery processes (Chowdhury and Loganathan, 2019; Zhang et al., 2021), but also for applications in human nutrition, due to its putative functional and structural properties (Gifuni et al., 2017; Shahid et al., 2020). Regulation of starch synthesis and degradation in microalgae is linked to the shift between optimal and suboptimal growth conditions and has been the object of several studies (Ran et al., 2019; Shahid et al., 2020).

Species from the genus *Tetraselmis* are considered attractive for industrial production, having already been established as live feed in aquaculture of molluscs and shrimps and as an enrichment of other meals. Particularly, they are reported to have high specific growth rates (Reitan et al., 2021) and have shown to be high-starch producers under nutrient stress (Yao et al., 2018) and are reported to have high specific growth rates. A critical parameter for industrial production in semi-continuous modes is the recovery from nutrient-induced stressful conditions. In this regard, *T. subcordiformis* has shown a speedy recovery from nutrient stress, with starch being degraded within 24 h after nutrient restitution (Yao et al., 2012). It is important to note that, within this genus, *Tetraselmis chui* is the only species that has been authorised for human consumption (since 2014) as a novel food in the European Union (EU) and more recently (2017), also as a food supplement (Mantecón et al., 2019), widening the range of its possible industrial applications.

The traditional model of starch metabolic pathways in microalgae has been developed in the model organism *Chlamydomonas reinhardtii* (Ball and Deschamps, 2009; Busi et al., 2014), while an additional pathway map looking in depth at the fluxes of starch metabolism during day-night cycles was developed in *Ostreococcus tauri* (Sorokina et al., 2011). Both models identify the first regulatory step to be the phosphorylation of glucose-1-phosphate (Glc-1-P) to ADP-glucose by the ADP-glucose-pyrophosphorylase (AGPase). Different isoforms of starch synthases (SS) form crystalline starch using this basic building block. The granule-bound SS (GBSS) catalyses the formation of α(1-4) bonds, elongating existing glucan chains, and is strictly connected to the starch granules during metabolism. Short glucan chains are produced in the chloroplast lumen by soluble starch synthase isoforms (SSS). Starch branching enzymes (SBE) form

amylopectin or branched glucans by transferring linear glucan chains to the carbon 6 of an existing chain within the granule. This process is further assisted by the isoamylase 1 (ISA1) and 2 (ISA2), which are suggested to cleave branches that are improperly positioned for the sake of crystallisation, releasing soluble oligosaccharides, and by the disproportionating enzyme (DPE), which alters the chain length of such oligosaccharides recycling them to substrates useful for SS and SBE.

Starch degradation back to Glc-1-P requires the glucan chains to be phosphorylated by water dikinases (WD). After that, beta- and alpha-amylases (AMB and AMA) hydrolyse α(1-4) glycosidic bonds, while debranching enzymes, such as isoamylase 3 (ISA3), hydrolyse the α(1-6) bonds. Both processes release glucan chains that contribute to the pool of available maltose oligosaccharides (MOS) and water-soluble polysaccharides (WSP), whose chain length may be modified again by the DPE. Starch phosphorylase (SPh) finally catalyses the formation of Glc-1-P, which is then readily available again as substrate for the Calvin cycle and other metabolic processes.

The intricate mechanisms involved in starch synthesis and degradation in microalgae are, however, not yet understood completely. In this regard, a recent body of literature has consistently challenged the traditional *C. reinhardtii* model on the role of some enzymes (Ran et al., 2019), with studies performed on several genes, enzymes and species. Hence there is a clear need for further in-depth studies to shed light on responses to stress and recovery, especially in species of high commercial interest.

The aim of this work was to perform a comprehensive analysis of starch biosynthesis and degradation mechanisms in *T. chui* subjected to nitrogen starvation and subsequent recovery at a pilot-scale. Thus, growth and biomass composition of *T. chui* cultivated in

25 L tubular photobioreactors were monitored throughout the trial. In an attempt to understand the molecular basis underlying starch metabolism, transcriptional regulation of 27 genes involved both in starch synthesis and degradation was furthermore analysed by RT-qPCR. Results obtained in this study will widen the existing knowledge about starch metabolism in this economically relevant species.

2. Materials and Methods

2.1. Algae cultivation

T. chui SAG 8-6 obtained from SAG Culture Collection of Algae (Göttingen, Germany) was grown on agar plates on L1 medium at 22 °C and irradiated with 20 µmol m^{-2} s^{-1} with LED lights. The algae were scaled up in 2xF 50-100 µmol m^{-2} s^{-1} on a shaking plate with 1% CO_2:air mixture first to 500mL and then to 1L Erlenmeyer flasks. The cultures were used first to inoculate a 25L GemTube RD1-25, tubular photobioreactor (LGem BV, Netherlands) in 2xF medium and then for further scale-up in 250 L GemTube RD1−250 tubular photobioreactor (LGem BV, Netherlands). In the tubular reactors, temperature was set to 25 ± 3°C maintained via a room-temperature control; surface irradiance was set to 100 µmol m^{-2} s^{-1} and pH was set to 7.8 ± 0.2, controlled by CO_2 addition. After three days, the inoculum was sourced from the running 250 L reactor taking a 5 L culture sample which was gently centrifuged for 5 min at 2500 rpm. The pellet was rinsed with the new growth medium and used to begin the experiment, inoculating two 25 L photobioreactors (PBR 1 and PBR 2) at the same initial density of 0.5 ± 0.06 x 10^6 cells ml^{-1}. The medium used upon inoculation was a modified 2xF medium which contained only half of the nitrogen concentration (1.78 mM) to induce stress and starch accumulation gradually. Light was set to 150 µmol m^{-2} s^{-1}, and after

three days under nitrogen deprivation, all nutrient stocks were resupplied in non-limiting amounts (4xF medium) for the recovery phase of the experiment.

2.2. Cultivation monitoring

Algal growth was monitored daily by spectrophotometry, measuring absorbance at 750 nm with a SparkTM microplate reader (Tecan®, Switzerland). Data from the plate reader were converted to 1 cm cuvette light-path, with a calibration curve based on *T. chui* cultures.

Dry weight was assessed filtrating a known volume of culture, diluted in 20 mL ammonium formate, in pre-washed and -weighed Whatman GF/F glass fibre filters 25 mm diameter with 0.7 µm pore size (Cytiva, UK). Subsequently, filters were dried in a drying oven at 105 °C for 24-48 h before weighing.

Samples were stored in paraformaldehyde 2% for further analyses, and cell counts were performed using a Guava® easyCyte Flow Cytometer (Luminex Corporation, USA) with a minimum of 50,000 events screened per sample.

Quantum yield (*Fv/Fm*) was measured on the spot with Aquapen PAM fluorometer (Photon Systems Instruments, Czech Republic).

Nitrogen consumption was assessed on the spot using the colorimetric Nitrate Test strips, 10−500 mg L^{-1} (NO_3^-), MQuant® (Supelco). Values were later confirmed with ion chromatography performed with the 940 Professional IC Vario (Metrohm AG, Switzerland).

2.3. Biomass analysis

Biomass harvested at each timepoint was freeze-dried and then stored for analysis. For starch determination, 7 ± 1 mg were weighed in triplicates and exposed to bead−beating in ethanol to break the cell walls and remove interfering compounds. Starch was quantified using the Total Starch (AA/AMG) Assay Kit (Megazyme, Ireland). Protein content was analysed using 10 ± 1 mg freeze-dried biomass with the Bio-Rad protein assay dye reagent (Bio-Rad, USA) against a bovine serum albumin standard curve.

2.4. Total RNA isolation and cDNA synthesis

Cell lysis was performed using 0.2 mm stainless steel beads (Next Advance) for 3 min at speed 10 in the Bullet Blender® 24 (Next Advance, USA). Total RNA was isolated using the NucleoSpin® Plant II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. All RNA samples were treated twice with DNase I to ensure no further amplification of residual genomic DNA. RNA quantification was accomplished using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and quality was checked in agarose gels. The total RNA (1 µg) from each sample was reverse-transcribed using the iScript™ cDNA Synthesis kit (Bio-Rad, USA) in a reaction volume of 20 µL according to the manufacturer's protocol. All cDNA reactions were finally diluted 5-fold by adding 90 µL of nuclease-free water. Two randomly selected samples were amplified by PCR, in absence of cDNA synthesis, to confirm the lack of genomic DNA contamination.

2.5. Primer design and RT-qPCR

For gene expression normalisation, the stability of up to six potential reference genes was evaluated (Table 1). They were selected based on previously reported data (Torres

158 et al., 2021). For the target genes employed in this study, predicted coding sequences of
159 close to 22,600 transcripts identified in the *T. chui* strain PLY429 were retrieved from
160 the iMicrobe data set (www.imicrobe.us) and then annotated using the AutoFACT tool
161 (Koski et al., 2005). A total of 27 transcripts of interest were selected, and the predicted
162 encoding polypeptides were obtained with EditSeq v8.1.3 (DNASTAR). After that,
163 appropriate annotation was confirmed with BLASTp. The presence of putative
164 chloroplast signal peptides in the N-terminal portion of predicted polypeptide sequences
165 (when non-truncated) was analysed using the bioinformatic tool TargetP 2.0
166 (Armenteros et al., 2019).

167 Primer pairs for the candidate reference genes and the target genes *AGPLs* and *AGPSs*
168 were the same as previously reported (Torres et al., 2021). Primers for the remaining 25
169 target genes (Table 1) were designed using Oligo v7.60 software (Molecular Biology
170 Insights).

171 Appropriate performance of each primer pair was double-checked, first by PCR
172 amplification of the target amplicons (using the same conditions described below for
173 RT-qPCR) and then by loading the products in standard agarose gel electrophoresis. A
174 single DNA band of the expected sizes was obtained in each instance (data not shown).

175 RT-qPCR was performed using a CFX96™ Real-Time PCR Detection System (Bio-
176 Rad, USA). Each 10-µL reaction contained 5 µL of 2X iQ™ SYBR® Green Supermix
177 (Bio-Rad, USA), 300 nM of forward and reverse primers (0.3 µL of a 10 µM stock
178 each), 2 µL of cDNA (retrotranscribed from 20 ng of RNA), and 2.4 µL of nuclease-
179 free water. Reactions were run in duplicate and for further calculations, the mean
180 threshold cycle (C_T) was used.

Two different approaches were employed to select the most appropriate reference genes. The first one was geNorm (version 3.5), or pairwise comparison approach (Vandesompele et al., 2002), which ranks candidate genes according to their expression stability. The second software used in this study was NormFinder (version 0.953), a model-based approach that ranks the candidate reference genes according to their minimal combined inter- and intra-group expression variation (Andersen et al., 2004). To generate valid input data files for both geNorm and NormFinder, raw C_T values were first exported to a Microsoft Excel sheet and then transformed into relative quantities using the comparative C_T method. To achieve this and for each candidate reference gene, the lowest C_T value was subtracted from all other C_T values, thus transforming C_T values into ΔC_T values. After that, the formula $2^{-\Delta Ct}$ was applied to each data point to obtain relative quantities. As the formats for presenting input data were different in geNorm and NormFinder, relative quantities calculated as previously described were exported into new Excel datasheets and converted according to the specific software requirements.

Relative transcript levels of target genes were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The thermal cycling profile included a first incubation at 95 ºC for 3 min, followed by 40 x 15 s cycles at 95 ºC and 68 ºC for 30 s. For each primer pair, specificity was verified through a melting curve analysis from 70 ºC to 95 ºC, using a ramp speed of 0.5 ºC every 10 s. In each instance, a single and sharp peak was obtained.

2.6. Statistical analysis of RT-qPCR data

Statistical analyses were conducted using Prism 6 (GraphPad Software) after normalisation with *RPS10* and *UBCE* as previously indicated. In all instances, data were analysed using the Kruskal-Wallis test (non-parametric one-way ANOVA), and when significant, Dunn's multiple comparison test was performed. Significance was accepted for $P < 0.05$.

3. Results and discussion

3.1. Algal Cultivation

In this study, *T. chui* growth was monitored during two phases. First, cultures were inoculated in a modified 2xF medium containing a reduced nitrate content, equivalent to the proportion present in standard F medium (1.78 mM), to induce starch accumulation. Second, after the starch content reached values above 30% of the dry weight, nutrient stock solutions were resupplied in non-limiting concentrations (equivalent to 4xF medium) to examine how the cultures would recover.

The use of a reduced nitrogen content at inoculation was preferred over immediate and complete starvation. It has been previously shown that this approach induces a higher starch accumulation and better productivity, possibly due to the milder and more gradual advent of stressful conditions (Yao et al., 2017). The nitrogen supplied was consumed within the first 24 h (Fig. 1a), resulting in halted culture growth with no further increase in cell numbers (Fig. 1b). However, a slight increase in dry weight (Fig. 1c) and absorbance at 750 nm (Fig. 1d) were observed respectively, due to storage

compound accumulation and changes in cell granularity. After nitrogen restitution at high concentration, all growth parameters showed signs of recovery within 48 h.

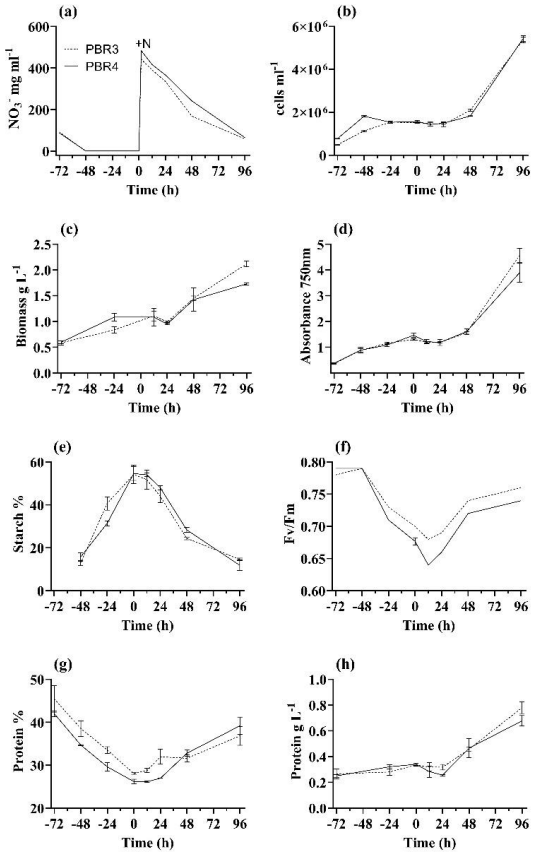


Figure 1: Time course of cell growth and biomass changes in *Tetraselmis chui* in two parallel 25 L tubular photobioreactors, PBR 1 (dotted line) and PBR 2 (solid line). Timepoint 0 on the x axis corresponds to the moment of nitrogen replenishment. (a) flow cytometry cell counts; (b) nitrate concentration in culture medium; (c) biomass dry weight; (d) absorbance at 750 nm; (e) starch concentration expressed as % of biomass dry weight; (f) quantum yield; (g) protein concentration expressed as % of biomass dry weight; (h) protein concentration expressed as grams per litre.

In the pilot-scale set-up assessed in this work, nitrogen starvation led to a 3.5-fold increase in the relative starch content (Fig. 1e), reaching 58.5% ± 2 of the biomass dry weight. Species from the genus *Tetraselmis* are known to produce such high storage contents at lab-scale, and up to 62% of starch has been previously found (Yao et al., 2012). During the replenishment phase, starch degradation started after 12 h. The decrease proceeded slowly to reach 25% of starch content within 48 h and finally returned to basal level after 96 h.

Nitrogen is a primary constituent of proteins, and its absence results in reduced cell functions and protein synthesis. In the first phase, as expected, relative protein content in the biomass decreased concomitantly to an increase in starch reserves, whereas the protein content in the cultures (expressed as g L^{-1}) was halted (Fig.1 g-h). In the second phase, recovery started after 24 h from replenishment, in parallel with cell division. Overall, a very similar performance of the two parallel 25L reactors could be observed (Fig. 1).

Photosynthetic activity has also proved essential for starch metabolism during stress conditions (Carnovale et al., 2021; Janssen et al., 2018; Li et al., 2015), therefore, it becomes crucial to ensure that cultures are not limited or inhibited by light and receive an adequate irradiance dose. Quantum yield (*Fv/Fm*) has been studied as a proxy for the activity of Photosystem II, in combination with stress-induced starch accumulation. Its threshold for efficient starch production has been identified to be above 0.60 for both *Chlorella vulgaris* (Bránykiová et al., 2011) and *T. subcordiformis* (Yao et al., 2012). In this study, *Fv/Fm* decreased after nitrogen consumption from 0.79 ± 0.01 to 0.68 ± 0.03

(Fig. 1f). Such values did nonetheless not hinder starch accumulation. Quantum yield further decreased after nitrogen resupply. However, it recovered faster than other parameters monitored, with values returning to levels above 0.70 within 48 h.

Former studies on phosphorus deprivation in *T. subcordiformis* have shown a faster recovery from stress (Yao et al., 2012). Thus, it could be hypothesised that nitrogen deprivation has a more substantial impact on cell metabolism. It is noteworthy that in the pilot-scale experiment nutrient replenishment did not directly result in significant changes in culture density over 48 h.

3.2. Selection of candidate reference genes for RT-qPCR

Gene expression analysis by quantitative real-time reverse transcription PCR (RT-qPCR) is considered a valid and commonly used tool to provide information concerning molecular regulatory mechanisms associated to cellular processes owing to its speed, high sensitivity, cost, accuracy, reliability and reproducibility (Gao et al., 2020; Mou et al., 2015). However, appropriate selection of reference genes is crucial in order to normalise RT-qPCR thus minimising the effect of potential interfering factors. Hence, stability of candidate reference genes has to be validated under specific experimental conditions as expression profiles are not always constant (Chapman and Waldenström, 2015; Radonić et al., 2004). With this aim, different mathematical algorithms have been developed to evaluate the suitability of reference genes, with geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) among the most widely employed tools. As an appropriate selection of stable genes is a major factor to ensure gene expression data reliability, a high number of studies have been conducted in different organisms and cell types, including microalgae (Cao et al., 2012; Guo et al., 2013; Liu

et al., 2020). Particularly, the expression stability of up to 18 different candidate reference genes has been recently evaluated in the green microalgae *T. chui* using samples collected from large scale industrial photobioreactors and indoor cultures (Torres et al., 2021). Taking advantage of the results obtained in that previous report, a set of six genes that were revealed to be highly stable (*RPS10*, *EFL*, *ACT*, *rbcL*, *UBCE* and *cdkA*; see Table 1) were selected to check suitability as reference genes using geNorm and NormFinder in samples collected in this study.

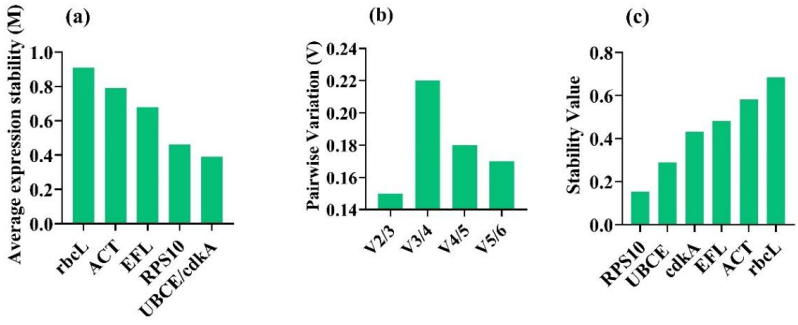
Table 1: List of reference and target genes in *Tetraselmis chui* and the respective primers used for RT-qPCR. (*) sequence retrieved from GenBank.

CANDIDATE REFERENCE GENES		
Gene symbol	Sequence Acc. No.	Sequence ID(AutoFact + BLAST)
CT	MMETSP0491_2-20121128|5070_1	Actin
EFL	MMETSP0491_2-20121128|385_1	Elongation factor-1 alpha like
cdkA	MMETSP0491_2-20121128|4820_1	Cell division control protein 2 homolog A isoform X1
rbcL	HF931099*	Chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
UBCE	MMETSP0491_2-20121128|9783_1	Ubiquitin-conjugating enzyme
RPS10	MMETSP0491_2-20121128|7667_1	40S ribosomal protein S10
TARGET GENES		
Gene symbol	Sequence ID (*T. chui* strain PLY429)	
AGPLs	MMETSP0491_2-20121128|23823_1	Glucose-1-phosphate adenylyltransferase large subunit
AGPSs	MMETSP0491_2-20121128|4141_1	Glucose-1-phosphate adenylyltransferase small subunit
GBSS1	MMETSP0491_2-20121128|5277_1	Granule-bound starch synthase
GBSS2	MMETSP0491_2-20121128|1231_1	Granule-bound starch synthase
SSS1	MMETSP0491_2-20121128|9227_1	Soluble starch synthase
SSS2	MMETSP0491_2-20121128|10712_1	Soluble starch synthase
SSS3	MMETSP0491_2-20121128|5181_1	Soluble starch synthase
SSS4	MMETSP0491_2-20121128|8254_1	Soluble starch synthase
SSS5	MMETSP0491_2-20121128|5721_1	Soluble starch synthase
SBE1	MMETSP0491_2-20121128|5189_1	Starch branching enzyme
SBE2	MMETSP0491_2-20121128|6860_1	Starch branching enzyme
AMA1	MMETSP0491_2-20121128|7949_1	Alpha amylase
AMA2	MMETSP0491_2-20121128|21071_1	Alpha amylase
AMA3	MMETSP0491_2-20121128|15281_1	Alpha amylase
AMA4	MMETSP0491_2-20121128|10987_1	Alpha amylase

AMA5	MMETSP0491_2-20121128|11752_1	Alpha amylase
AMB1	MMETSP0491_2-20121128|2795_1	Beta amylase
AMB2	MMETSP0491_2-20121128|4712_1	Beta amylase
AMB3	MMETSP0491_2-20121128|22759_1	Beta amylase
DPE	MMETSP0491_2-20121128|19976_1	Disproportionating enzyme
SPh	MMETSP0491_2-20121128|6575_1	Starch phosphorilase
ISA1	MMETSP0491_2-20121128|9150_1	Isoamylase 1
ISA3	MMETSP0491_2-20121128|11209_1	Isoamylase 3
PWD	MMETSP0491_2-20121128|4718_1	Phosphoglucan water dikinase
GWD	MMETSP0491_2-20121128|3077_1	Alpha-glucan water dikinase
DBE1	MMETSP0491_2-20121128|24193_1	Starch debranching enzyme (Limit dextrinase)
DBE2	MMETSP0491_2-20121128|26458_1	Starch debranching enzyme (Alpha-1,6 glucosidase, pullulanase-type)

First, the average expression stability (M) values of the six candidate reference genes were determined using the geNorm algorithm (Fig. 2a). All the genes exhibited an M value below the 1.5 geNorm threshold. The most stable genes across timepoints were *UBCE*/*cdkA*, followed by *RPS10*. Next, pairwise variation values (V) were calculated with a cut-off suitability set at 0.15 (Fig. 2b) to identify the ideal number of reference genes needed for accurate expression normalisation. The combination of two genes rendered this value of 0.15, but the addition of more genes revealed to increase V. Thus, only two genes were used for normalisation. Stability was also evaluated using the NormFinder software (Fig. 2c). In line with geNorm, the most stable genes were *RPS10*, *UBCE* and *cdkA*. Given the known sensitivity of geNorm to co-expressed genes, *RPS10* (the most stable gene in NormFinder) and *UBCE* (one of the two most stable genes with geNorm, and the second most stable gene with NormFinder) were finally selected for gene expression normalisation. Results obtained in this work are in agreement with those previously reported in a comprehensive ranking of 18 potential reference genes in *T. chui*, showing *RPS10* and *UBCE* among the four most stable genes (Torres et al.,

303 2021).



304

305 **Figure 2:** Selection and stability of candidate reference genes in *Tetraselmis chui*. (a)
306 average expression stability (M) and (b) pairwise variation (V) extrapolated from
307 geNorm, and (c) stability value extrapolated from NormFinder. Detailed information
308 about target genes is listed in Table 1.

309 ## 3.3. Expression of target genes

310 Quantitative analysis of gene expression is undoubtedly a powerful tool in order to
311 unravel the molecular and biochemical events that underlie physiological characteristics
312 and responses to environmental changes in any given organism. Fortunately, genetic
313 resources have been exponentially increasing during the last decade thanks to Next
314 Generation Sequencing technologies, and thus complete genomes as well as
315 transcriptomes obtained by RNA sequencing (RNA-Seq) of many different organisms
316 and cell types are nowadays available in databases. Regarding microalgae, this last high
317 throughput technology has been applied with success to analyse the whole adaptive
318 physiological responses to changes in varying culture conditions (Corteggiani Carpinelli
319 et al., 2014; Scarsini et al., 2022). However, to study particular metabolic pathways, the
320 construction of RT-qPCR platforms for the expression analysis of a variable number of

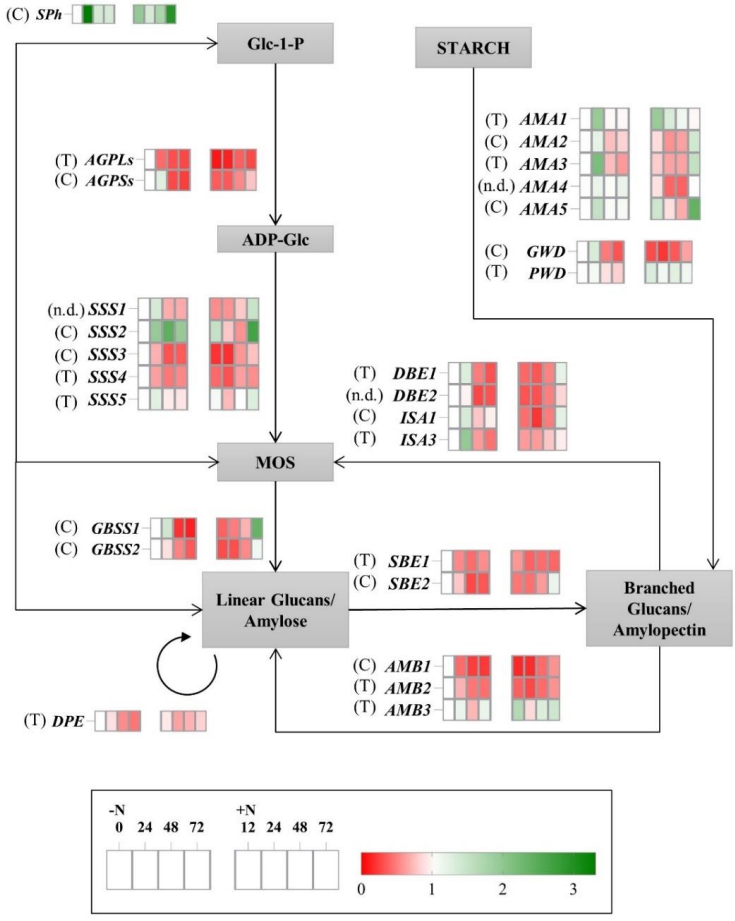
321 selected and representative genes becomes a valuable approach. In this regard, and
322 owing to the reliability and accuracy of RT-qPCR, this strategy has been followed, for
323 instance, to validate RNA-seq data in the green microalgae species *Chlorella*
324 *sorokiniana* (Li et al., 2016), *Tetraselmis* sp. M8 (Lim et al., 2017) and *Tetraselmis*
325 *suecica* (Lauritano et al., 2019). Moreover, the influence of light quality on metabolism
326 has been studied using a RT-qPCR platform containing more than 100 selected genes in
327 the Eustigmatophyceae *Nannochloropsis gaditana* (Patelou et al., 2020).

328 These studies highlighted how gene expression analysis of selected markers by RT-
329 qPCR can be used in microalgae to study the modulation of metabolic pathways in
330 response to environmental changes. In the present study, availability of a full *T. chui*
331 transcriptome (strain PLY429; see Materials and Methods) allowed identification of a
332 set of 27 genes potentially involved in starch biosynthesis and degradation (Busi et al.,
333 2014). Thus, primer pairs could be designed to create a specific RT-qPCR platform to
334 study starch metabolism during nitrogen deprivation and further recovery.

335 Gene expression results had a low variation between the two parallel culture units. As a
336 whole, expression patterns in *T. chui* during nitrogen deprivation showed an overall
337 down-regulation of most target genes, especially after prolonged stress exposure
338 (timepoint −N 48 to 72h). A closer look at the single expression profiles over time (See
339 supplementary material Table S2) provided insights about the regulatory starch
340 metabolic pathways under nitrogen deprivation, with different trends during starch
341 synthesis and degradation phases.

342 The reaction catalysed by AGPase is considered the primary control step for starch
343 synthesis (Ball and Deschamps, 2009). It is also one of the main regulatory steps, with

its expression peaking at the beginning of the starch synthesis phase in *O. tauri* (Sorokina et al., 2011). Previous studies on microalgae have reported that under stress conditions such as nutrient deprivation, a transient increase both in AGPase activity (Li et al., 2011; Zhu et al., 2015) and transcript amounts (Goodenough et al., 2014; Jaeger et al., 2017; Juergens et al., 2015; Li et al., 2015; Tan et al., 2016) was concurrent to the process of starch accumulation. In the present study, both *AGPLs* (the gene encoding the large subunit of the AGPase) and *AGPSs* (the gene encoding the small subunit of the enzyme) were significantly down-regulated during nitrogen deprivation, while a slight increase was detected after nitrogen replenishment (Fig. 3). Transcription of these genes did not completely recover to the levels observed before stress, particularly in the case of *AGPLs* suggesting that even if some starch was being synthesised through this pathway, anabolism was slower than catabolism. Down-regulation of AGPase activity (Yao et al., 2018) and transcription (Rismani-Yazdi et al., 2016) has been reported in microalgae under nitrogen starvation, and in *Chlorella sorokiniana* was demonstrated to be associated with an excessive orthophosphate presence (Zhu et al., 2015). In *T. subcordiformis*, the decline in AGPase activity during starch accumulation led to identifying a second regulatory mechanism based on SPh (Jiang et al., 2017; Yao et al., 2018). SPh in plants is involved in the last degradation steps, where it catalyses glucose phosphorylation to Glc-1-P. However, its upregulation has been shown in connection to starch accumulation in multiple studies and species (Ikaran et al., 2015; Jaeger et al., 2017; Jiang et al., 2017; Juergens et al., 2015).



365

366 **Figure 3:** Proposed regulatory model of starch synthesis and degradation pathways in
367 *Tetraselmis chui* based on gene expression data obtained in this study. The expression
368 profile of each gene is represented in two boxes, one for the nitrogen deprivation phase
369 (-N, 0-72 h) and one for the recovery phase (+N 12-72 h). Transcriptional regulation
370 relative to timepoint zero (-N 0; white) is represented as a colour coded heat-map (Red=
371 down-regulated, Green= up-regulated). In the genes marked with the (C) symbol, a

putative chloroplast signal peptide was found in the predicted polypeptides, suggesting a putative location of the enzymes in the chloroplast. The symbol (T) indicates truncated sequence in the amino portion, and hence the analysis could not be performed, and (n.d.) indicates no signal peptide detected. The image is based on a figure by Ran et al. (2019) and Jaeger et al. (2017). Detailed information about target genes is listed in Table 1.

The *SPh* expression profile found in this study supports a role for this gene in starch production under nitrogen deprived conditions since it exhibited an early significant increase in transcripts 24 h upon starvation and, despite being down-regulated during prolonged exposure to nutrient stress, transcript amounts remained above basal levels (Fig. 3). Interestingly, the transcriptional response to nitrogen re-supply also suggests a role for *SPh* in starch breakdown in *T. chui*, in agreement with proposed models for starch metabolism in microalgae (Busi et al., 2014; Ran et al., 2019) as a rapid up-regulation of *SPh* was observed just after 12 h.

As theoretically might be expected, most studies looking into starch pathways under nutrient stress in microalgae have detected an up-regulation of different *SSS* and *GBSS* genes during starch synthesis, especially in the early phases of stress (Blaby et al., 2013; Goodenough et al., 2014; Juergens et al., 2015; Moseley et al., 2006; Toepel et al., 2013). In the present study, several starch synthase genes (*GBSS1*, *SSS1*, *SSS2*, and *SSS5*) were up-regulated during the first 24 h of the trial (Fig. 3), coinciding with the depletion of nitrogen and with the beginning of starch storage. However, transcripts of *SSS3, SSS4* and *GBSS2* decreased during prolonged nitrogen deprivation, whereas *SSS2* was up-regulated throughout the starvation period. Such results suggest *SSS2* as a key *T. chui* isoform involved in starch synthesis under nitrogen deprivation. Toward the end of

396 the trial (72 h after nitrogen replenishment), a recovery in transcript abundance was
397 detected in almost all synthase genes. This final increase, observed also in other genes
398 such as *SPh*, might represent an adaptive but transient state in response to full (or close
399 to) recovery after the previous stressful conditions.

400 The cleavage of α(1-6) bonds by isoamylases is functional to structural rearrangements
401 during both synthesis and degradation of starch. In *C. reinhardtii, ISA1* mutants are
402 unable to produce starch and, instead, divert carbon metabolism to lipid synthesis (Kato
403 et al., 2021). The expression of *ISA1*, *ISA2* and *ISA3* was reported to be up-regulated
404 within 24 h from stress in *C. reinhardtii* (Juergens et al., 2015), whereas, in *C.*
405 *sorokiniana*, the regulation of *ISA1* was studied over a more extended starvation period
406 (up to 6 days), showing an increase in transcripts in parallel to degradation of starch (Li
407 et al., 2015). In the present study, similarly to what was observed for some starch
408 synthases, both *ISA1* and *ISA3* genes were up-regulated at the onset of starvation and
409 subsequently down-regulated during prolonged exposure to stress (Fig. 3). The
410 expression of both genes slowly increased during starch degradation. Thus, it was not
411 possible to deduce a clear and direct role of *ISA1* and *ISA3* in starch metabolism
412 according to their gene expression profiles.

413 Despite their key role in starch catabolism, amylases have been often reported to be
414 positively regulated also in the phases of starch accumulation. For instance, an increase
415 in transcripts encoding alpha-amylases has been reported under sulphur and phosphorus
416 deprivation in *C. reinhardtii*, in parallel to an increase in starch content (Juergens et al.,
417 2015; Moseley et al., 2006; Nguyen et al., 2008; Toepel et al., 2013). A transient up-
418 regulation of several genes encoding alpha and beta amylases was also detected during
419 the transition to lipid accumulation in *M. neglectum* under nitrogen deprivation though

starch content did not consequently decrease In this study, the five AMA genes analysed exhibited an unexpected transient up-regulation after 24 h of nitrogen deprivation. Then, two of them (*AMA2* and *AMA3*) showed transcript levels below starting basal levels whereas the others returned to values close to the basal level. During the starch degradation phase *AMA1* was the only alpha amylase consistently up-regulated. The other genes were all down-regulated, with the exception of *AMA5* that showed an initial transient up-regulation after 12 h of nitrogen replenishment. It has been previously stated that the existence of post-transcriptional regulatory mechanisms modulating protein abundance or enzymatic activity of amylases could explain unexpected expression profiles during starch accumulation (Jaeger et al., 2017; Nguyen et al., 2008). Moreover, it cannot be ruled out a potential role of these enzymes in cleaving glycosidic bonds during starch accumulation, which might be linked to the rearrangement of the crystal structure. As such, differential expression patterns in both alpha and beta amylases encoding genes have been reported concerning the starch flux during the dark/light cycle in *O. tauri* (Sorokina et al., 2011).

The role of SBE and DPE in starch metabolism has also been questioned as different expression patterns have been detected for these enzymes during starch production in microalgae (Ran et al., 2019). Up-regulation of SBE genes has consistently been found in *C reinhardtii* during nitrogen-induced starch accumulation (Blaby et al., 2013; Goodenough et al., 2014; Juergens et al., 2015) and similar results were also observed in *D. tertiolecta* (Tan et al., 2016). However, down-regulation of *SBE2* was also observed under sulphur deprivation conditions in *C. reinhardtii* (Toepel et al., 2013). Differential expression of DPE isoforms was instead observed under nitrogen stress in *C. reinhardtii*, with *DPE1* being down-regulated after 24 h of stress, whereas *DPE2*

444 transcripts increased (Juergens et al., 2015). In the present study, *SBE1, SBE2* and *DPE*
445 were all significantly down-regulated during the starvation phase. Once nitrogen was
446 resupplied in the medium, *DPE* transcripts almost returned to basal levels, whereas
447 *SBE1* and *SBE2* remained down-regulated. This somewhat unexpected result could be
448 explained either by the presence of additional isoforms being active in this process, or
449 by post-transcriptional regulatory mechanisms that could play a role in the regulation of
450 these genes, as suggested for isoamylases or amylases encoding genes (Ikaran et al.,
451 2015; Jaeger et al., 2017; Nguyen et al., 2008).

452 Compared to most starch-related genes included in the present study, regulation under
453 stress conditions of water dikinases, PWD and GWD, has been tackled much less in
454 scientific literature. In *C. reinhardtii*, two different *GWD* genes and *PWD* were down-
455 regulated under nitrogen starvation 24 h after the onset of stress, when starch content
456 was increasing (Juergens et al., 2015). In contrast, in the green microalga *M. neglectum*
457 three *GWD* genes exhibited a significant up-regulation in nitrogen-deprived cells after
458 24 h of stress when starch content peaked, whereas *PWD* remained stable. Moreover,
459 during the recovery phase after nitrogen resupply, transcript levels of *GWD* were
460 unaffected, whereas those of *PWD* genes were down-regulated and returned to basal
461 levels (Jaeger et al., 2017). In the present study, the expression of *GWD* exhibited an
462 up-regulation at the beginning of the starvation phase. Still, it was then strongly
463 repressed after 48 h and onto the starch degradation phase. However, *PWD* remained
464 relatively stable throughout the trial, with transcript amounts slightly decreasing under
465 nitrogen starvation, but increasing after nitrogen replenishment. Altogether, these
466 findings reveal significant differences in the transcriptional regulation of dikinases in
467 microalgae and potentially suggest phosphorylation of glucan chains as a process

occurring in all stages of starch metabolism. In support of this idea, in the *O. tauri* day-night cycle model, *PWD* was shown to have a 12 h circadian rhythm. Expression peaks were thus observed both in the middle of daylight and night-time, potentially underpinning the importance of this enzyme in starch anabolism and catabolism (Sorokina et al., 2011).

As a whole, findings obtained in this study suggest firstly that the green microalgae *T. chui* displays species-specific transcriptional regulatory pathways in starch metabolism. This is in agreement with a recent review showing that the expression of starch-related genes during stress differs greatly between species and conditions, and that the activity of typical anabolic enzymes can be detected during catabolism and vice versa (Ran et al., 2019). Secondly, up-regulation of degrading enzymes during starch production as similarly reported in multiple species (Nguyen et al., 2008; Zhang et al., 2004, Ikaran et al., 2015), supports the hypothesis that catabolic enzymes may play a role in maintaining an active flux of substrates in the starch biosynthetic pathway (Moseley et al., 2005). In this regard, the expression profile of *SPh* detected in *T. chui* suggests a potential role of this enzyme in starch synthesis, in agreement with the results of a former study performed on *T. subcordiformis* (Jiang et al., 2017). Finally, the results of this study allow to formulate the hypothesis that post-transcriptional regulatory mechanisms may play a critical role in starch metabolism in *T. chuii*, as was formerly suggested for other species (Nguyen et al., 2008; Ikaran et al., 2015; Jaeger et al. 2017). Further studies could explore this route, possibly using "-omics" technologies to better understand and characterise the accumulation of reserve starch in this industrially relevant species.

4. Conclusions

T. chui proved to be an efficient and fast starch producer under nitrogen deprivation in the pilot-scale trial here conducted, with a maximum relative content of 59% storage starch. A full recovery was achieved between 48-96 h after nitrogen replenishment, with a speedy degradation of starch and an upturn of photosynthetic activity. However, no clear relationships between evolution of starch content and expression profiles of target genes involved in starch metabolism could be established during starch synthesis and degradation phases, which strongly suggest that specific post-transcriptional regulatory mechanisms may play a crucial role in starch metabolism in *T. chui.*

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6. Data availability

Data available from authors.

7. References

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Metabolic pathways for biosynthesis and degradation of starch in *Tetraselmis chui* during nitrogen deprivation and recovery

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Supplementary Material

716 **Supplementary Table S1** List of primers used for qRT-PCR.

CANDIDATE REFERENCE GENES

Gene symbol	Primer sequences	Amplicon length (bp)	Tm (ºC)
ACT	F: 5'-AGAAGACCTATGAGCTGCCCGACG-3'	168	64.0
	R: 5'-GGTCCTTACGGATATCGACATCGCACT-3'		
EFL	F: 5'-CCGGCGAGATCAAGGTCGGCTAC-3'	171	63.9
	R: 5'-AGGGCTTGAAGACAATGGTGGATACCTC-3'		
cdkA	F: 5'-ACCGCAGAACTTACTGATTGACCGT-3'	123	61.2
	R: 5'-CGGTACCACAGAGTCACAACCTCGT-3'		
rbcL	F: 5'-ACGTAAATTCACAAGCTTTCATGCGTTGG-3'	140	57.0
	R: 5'-CATCATTTCTTCACAAGTCCCAGCCGTT-3'		
UBCE	F: 5'-CCAAACATCAACAGCAACGGCAGCA-3'	149	64.1
	R: 5'-TGCGCAATCTCGGGCACCAG-3'		
RPS10	F: 5'-CAAGAAGAACCGCCGCGAGGTGT-3'	172	63.0
	R: 5'-CCACGCAAAACGCTCAGTGACCAG-3'		

TARGET GENES

Gene symbol	Primer sequences	Amplicon length (bp)	Tm (ºC)
AGPLs	F: 5'-CCGCCACCATCACTCCCGAAT-3'	164	63.2
	R: 5'-CCGCACGAGGTCCTGATAGTCCAT-3'		
AGPSs	F: 5'-GACTTTCTCATCCTCTCCGGCGACCA-3'	163	66.5
	R: 5'-TCCGCCCGCTGTCGTCGATC-3'		
GBSS1	F: 5'-GCGCTGACTTTATGCTCGTTCCGT-3'	125	64.4
	R: 5'-CTCCTTCACCGTGTCGACCAGTCC-3'		
GBSS2	F: 5'-GGCTGCACTCCTCGTCCACC-3'	155	63.4
	R: 5'-AAGCAACCTCCGTCCATGTTTCGAT-3'		
SSS1	F: 5'-CGCCATCAACTACAGCAACTGGGTC-3'	158	65.2
	R: 5'-ACTCTTCCGTGTCGATGCCGTTC-3'		
SSS2	F: 5'-ATCAAGGAAGCCGAACTGAGAATAAGGT-3'	149	63.1
	R: 5'-CTCAGACGCCACGAACACCAAGC-3'		
SSS3	F: 5'-CATGAACATCCTGAAGGCGGGCAT-3'	132	65.9
	R: 5'-TTCCAGCTCATGTCGCGCAGGT-3'		
SSS4	F: 5'-GACGCCAAGATGATGGATACCGTGTT-3'	180	62.7
	R: 5'-CATGCCGCCGACCTTTGCAAT-3'		
SSS5	F: 5'-AGGAATTTGACGCGACCACCACT-3'	183	65.1
	R: 5'-TCAGCTCCCACCTGTTGAATGCGTA-3'		
SBE1	F: 5'-CGCAATTACAATCGCAGAGGATGTCAGT-3'	137	62.1
	R: 5'-CCACGGTGTTCTTTAGCAAGTCGG-3'		
SBE2	F: 5'-CCTACAAGCTCGTCCTCTCCTCCG-3'	140	63.4
	R: 5'-GCTTGTCGTGTAAACCTGGAAGGAGT-3'		
AMA1	F: 5'-GTCTGCATTGGACCGGACGCCAAG-3'	126	65.4
	R: 5'-GCAGTGCAGAGACTCGTCCACCTTC-3'		
AMA2	F: 5'-GCCAGATGTTAGCCTTACCGAGTCAGC-3'	126	65.3
	R: 5'-GACTCGACTGACCAGGGCTCGGA-3'		
AMA3	F: 5'-GCCTTTCCCCTCCTACCATGTTGC-3'	152	63.9
	R: 5'-TGATGCCGTTGCGCTTCCGGATG-3'		
AMA4	F: 5'-AGCTCATCCGCAAGGCACAC-3'	165	64.4
	R: 5'-CGCCGTAGTCTTCGCTGTCGTT-3'		
AMA5	F: 5'-GACGCCCGTGCCATTGTGTCT-3'	146	64.9
	R: 5'-CGAAGCCACTGCAACCACTCGC-3'		
AMB1	F: 5'-GCAGTAGCAACACGCCGTATGGAT-3'	128	65.8
	R: 5'-TCGGGTGGTGAATCCCTGTACTCC-3'		

TARGET GENES

Gene symbol	Primer sequences	Amplicon length (bp)	Tm (ºC)
AMB2	F: 5'-ACAGCCAGTGGTTCGACTATGCC-3'	148	62.9
	R: 5'-GCCTTCACATCCTCGACCAAAGTCT-3'		
AMB3	F: 5'-GCAGAGGCGAACTCCGACCTT-3'	163	61.2
	R: 5'-TGTGATCCTCAAACTCGTCCCTGAATGC-3'		
DPE	F: 5'-CCCCTCCAACCCCATCTTCGC-3'	173	65.0
	R: 5'-GAAACTCCAGCTGCCCGTCGT-3'		
SPh	F: 5'-CCGCACCATGAACTTCACCAACCAC-3'	180	63.7
	R: 5'-TTGGCAGCGTCCTCCTCATCCTT-3'		
ISA1	F: 5'-GTACCTACGCCGCACTCACCCA-3'	120	64.2
	R: 5'-TGCCCGGCTTGATCTCGTAGTACTCC-3'		
ISA3	F: 5'-AACTATTACGGCCACGACAGCGAA-3'	188	63.3
	R: 5'-TCCTCGTCGTCCCAGTTGCTCT-3'		
PWD	F: 5'-CTTCTCCGCCGAGTTTACCGCTT-3'	158	64.2
	R: 5'-CCTCCGTGGCAATCAGGTCCTC-3'		
GWD	F: 5'-ACGCCATTCAGGACGAAGTGTACGA-3'	143	66.0
	R: 5'-AGCGCGTACGGATACGTGAGACA-3'		
DBE1	F: 5'-TCCGCAGAACATCAGAAACACGCACA-3'	177	63.8
	R: 5'-GCATCCGCGCTTGTCATCTCCAT-3'		
DBE2	F: 5'-GCAACCTGGCCTCGTACTCCT-3'	150	63.7
	R: 5'-ACAGATCGAAGAGCGTCTCGTTATCGT-3'		

717

Supplementary Table S2. Average relative expression values of synthesis and degradation related genes during nitrogen deprivation (−N 0 to 72 h) and restitution (+N 12 to 72 h) of *Tetraselmis chui*. Means denoted by different letters indicate a significant difference between timepoints ($p<0.05$). The putative location of the enzymes, analysed on the TargetP-2.0 bionformatic online tool, to identify chloroplast signal peptides in the N-terminal portion of predicted polypeptide sequences (when non-truncated) (CP=chloroplast; TR=truncated sequence; ND= non detected). Detailed information about target genes is listed in Table1 of the article.

Gene	*Location*	*-N 0h*	*-N 24h*	*-N 48h*	*-N 72h*	*+N 12h*	*+N 24h*	*+N 48h*	*+N 72h*
AGPLs	TR	1^{a}	0.42^{a}	0.31^{ac}	0.28^{ac}	0.09^{bc}	0.14^{cd}	0.38^{ad}	0.29^{ac}
AGPSs	CP	1^{ab}	1.27^{a}	0.29^{bc}	0.24^{c}	0.36^{abc}	0.35^{abc}	0.54^{abc}	0.77^{abc}
aGWD	CP	1^{ab}	1.34^{b}	0.48^{bc}	0.33^{ac}	0.30^{ac}	0.21^{c}	0.35^{ac}	0.64^{ab}
AMA1	TR	1^{ab}	1.91^{a}	0.98^{bc}	0.97^{bc}	1.92^{a}	1.33^{ab}	1.12^{ab}	0.97^{b}
AMA2	CP	1^{ab}	1.2^{a}	0.75^{ab}	0.83^{ab}	0.87^{ab}	0.57^{b}	0.63^{b}	1.41^{a}
AMA3	TR	1^{abc}	2.18^{a}	0.74^{abc}	0.60^{b}	0.80^{abc}	0.64^{bc}	0.64^{bc}	1.52^{ac}
AMA4	ND	1^{ab}	1.18^{a}	1.03^{ab}	1.19^{a}	0.89^{ab}	0.40^{b}	0.39^{b}	0.99^{ab}
AMA5	CP	1^{abc}	1.51^{ab}	1.04^{abc}	1.09^{abc}	1.43^{ab}	0.88^{ac}	0.68^{c}	2.38^{b}
AMB1	ND	1^{a}	0.42^{ac}	0.24^{abc}	0.22^{bcd}	0.16^{b}	0.18^{bc}	0.42^{ac}	0.57^{ad}
AMB2	TR	1^{a}	0.70^{a}	0.47^{ab}	0.44^{ab}	0.38^{ab}	0.29^{b}	0.43^{ab}	0.57^{a}
AMB3	TR	1^{abc}	1.18^{abc}	0.72^{a}	1.17^{abc}	1.67^{b}	0.86^{ac}	1.3^{abc}	1.43^{bc}
DPE	TR	1^{a}	0.88^{a}	0.55^{ab}	0.47^{b}	0.91^{a}	0.64^{ab}	0.70^{ab}	0.83^{ab}
DBE1	TR	1^{ab}	1.29^{a}	0.49^{ab}	0.32^{b}	0.41^{ab}	0.34^{b}	0.52^{ab}	1.21^{a}
DBE2	ND	1^{ab}	0.95^{a}	0.28^{c}	0.32^{bc}	0.33^{abc}	0.32^{abc}	0.50^{abc}	0.84^{abc}
GBSS1	CP	1^{ac}	1.43^{a}	0.2^{bc}	0.13^{b}	0.39^{abc}	0.49^{abc}	0.70^{abc}	2.36^{a}
GBSS2	CP	1^{abd}	0.88^{ad}	0.51^{abcd}	0.35^{abc}	0.30^{c}	0.31^{bc}	0.54^{abcd}	1.11^{d}
ISA1	CP	1^{ab}	1.35^{a}	0.78^{ab}	0.94^{ac}	0.44^{bc}	0.21^{b}	0.49^{bc}	1.24^{ac}
ISA3	TR	1^{ab}	1.95^{b}	0.61^{acd}	0.45^{c}	0.61^{acd}	0.61^{acd}	0.78^{abc}	0.93^{bd}
PWD	TR	1^{ab}	1.09^{ab}	0.88^{ab}	0.82^{a}	1.32^{b}	1.11^{ab}	1.25^{ab}	1.12^{ab}
SBE1	TR	1^{a}	0.54^{ab}	0.43^{ab}	0.56^{ab}	0.59^{ab}	0.39^{b}	0.43^{ab}	0.40^{b}
SBE2	CP	1^{a}	0.78^{a}	0.29^{b}	0.34^{b}	0.47^{ab}	0.44^{ab}	0.61^{ab}	1.18^{a}
SPh	CP	1^{a}	3.31^{b}	1.37^{ac}	1.35^{ac}	1.95^{abc}	1.37^{ac}	1.76^{abc}	2.94^{bc}
SSS1	ND	1^{ab}	1.37^{b}	0.68^{ab}	0.67^{ab}	0.55^{ac}	0.57^{ac}	0.79^{ab}	1.48^{b}
SSS2	CP	1^{ab}	1.93^{ab}	2.43^{a}	1.93^{ab}	1.51^{ab}	0.78^{b}	0.57^{b}	2.66^{a}
SSS3	CP	1^{a}	0.69^{a}	0.32^{ab}	0.36^{ab}	0.20^{b}	0.19^{b}	0.59^{a}	0.76^{a}
SSS4	TR	1^{a}	0.62^{ab}	0.47^{abc}	0.53^{abc}	0.41^{bc}	0.32^{c}	0.62^{ab}	0.56^{ab}
SSS5	TR	1^{ab}	1.25^{a}	0.92^{ab}	$0.90a^{b}$	1.08^{ab}	0.73^{b}	1^{ab}	1.29^{a}

Paper III

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Article

Starch-rich microalgae as an active ingredient in beer brewing

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Abstract: Microalgal biomass is widely studied for its possible use in food applications and has been added to wheat and other ingredients in staple foods, mostly for nutritional purposes. In our study we attempted to integrate microalgal biomass as an active ingredient in an established industrial process: brewing. The starch-producing species *Tetraselmis chui* was cultivated under nitrogen deprivation to induce starch accumulation. The properties of microalgal carbohydrates in traditional mashing was then assessed to identify critical steps and challenges, to test the efficiency of fermentable sugar release and to develop a protocol for small scale brewing trials. Finally, *T. chui* was successfully integrated at small scale in the brewing process as an active ingredient, with a distinct effect on colour and beer properties. Regulation of pH proved to be a key parameter in the process.

Keywords: *Tetraselmis*; microalgae; brewing; food; beverage; starch

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1. Introduction

Microalgae cultivation is one of the emerging technologies in food sciences because of the rich nutritional profiles, impressive health benefits and positive environmental impact of these photosynthetic organisms [1,2]. Amongst the wide diversity of microalgal species, *Tetraselmis chui* is particularly interesting for industrial applications in food as its upscaled cultivation is already well established for the production of feed ingredients [3]. Biomass from *Tetraselmis* species was proved to be notably rich in antioxidant content and activity [4–6] and species from this genus are reported to have high productivity rates [7]. Additionally, *T. chui* has been proved to be safe for human consumption [8] and has recently been authorised as a novel food and as a food supplement in the European Union (EU 2017/2470 Regulation). Species from the genus *Tetraselmis* have been successfully introduced as a novel ingredient in a wide variety of applications such as bread [9,10], soup [11], gluten free bread [12] or savoury biscuits [13]. Products enriched with up to 4% *T. chui* show enhanced bioactive properties and good technical properties, proving the feasibility of microalgal biomass use as an ingredient in staple foods [9,14].

With the rapid development of the brewing industry and of its environmental impact, microalgae are being widely studied for CO_2 capture and wastewater bioremediation [15], mainly with the focus on removing excessive nutrients and converting the biomass to biofuels [16]. However, microalgal biomass is rarely used as an ingredient in the brewing process and mainly *Chlorella vulgaris* and the cyanobacteria *Spirulina* were considered as an additive to beers and alcoholic beverages in contents going up to 5% [17,18]. Microalgal biomass has the potential to be introduced as an active ingredient in brewing

since microalgae are known to be highly efficient starch producers [19]. Under environmental stress conditions the carbon partitioning in microalgal cells is redirected towards starch metabolism. *Tetraselmis* species have been widely studied for their potential to produce high starch content under nitrogen deprivation, reaching up to 60% of the total dry weight [20].

The aim of this study was to test the feasibility of starch-rich microalgal biomass used as an active ingredient in fermented beverages. The effect of different algae to malt ratios on brewing protocols and on the final product was assessed by monitoring the release of fermentable sugars during mashing and fermentation phases and finally qualifying the beer characteristics and fermentation efficiencies. Providing insights and methods to implement microalgal starch in brewing may pave the way for an increased microalgal presence in industrial setups and in everyday products.

2. Materials and Methods

2.1 Biomass production

2.1.1. Microalgal cultivation

Tetraselmis chui SAG 8–6 from the SAG Culture Collection of Algae (Göttingen, Germany) was kept on L1 medium agar plates [21] at 22 °C with 20 μmol m^{-2} s^{-1} irradiation. The algae were scaled up, maintaining exponential growth, to 0.1–0.25–0.5–1 L Erlenmeyer flasks in 2x F medium [22], at 50–100 μmol m^{-2} s^{-1} irradiance, on a shaking plate and supplied with 1% CO_2 : air mixture diffused through a needle. Erlenmeyers were then used to inoculate first an GemTube RD1–25, 25 L tubular photobioreactor (LGem BV, Netherlands) at 25 ± 2 °C, pH 7.8 ± 0.2 (controlled by CO_2 addition), and 100 μmol m^{-2} s^{-1} irradiance with 2x F medium. The 25 L culture was then finally scaled up to a GemTube RD1–250, 250 L tubular photobioreactor (LGem BV, Netherlands) at 25 ± 2 °C, pH 7.8 ± 0.2 (controlled by CO_2 addition), and 100 μmol m^{-2} s^{-1} irradiance.

The cultures were monitored daily by measuring absorbance at 750 nm and nitrogen content with nitrate strips 10–500 mg L^{-1} (NO_3^-), MQuant® (Supelco).

Once the nitrate dropped below 50 mg L^{-1}, samples were extracted daily for starch analysis.

2.1.2. Microalgal biomass harvesting and milling

The culture was harvested after a total 8 days of growth, when starch content higher than 40% had been measured. The algae biomass was centrifuged at 3000 xg with an Evodos 10 centrifuge (Evodos BV, Raamsdonksveer, The Netherlands). The concentrated algal paste (~15–20% dry weight) was frozen at −20 °C and further freeze dried in a FreeZone freeze dryer (Labconco, USA). Subsequently, the algae powder was milled in a Planetary Ball Mill PM400 (Retsch GmbH, Germany) at 400 rpm, for three cycles of the duration of 6 minutes (3+3 in each direction). Between cycles the containers were cooled down on ice to prevent biomass overheating by friction. Milled biomass was mixed and aliquoted in 2 ml Eppendorf tubes according to protocols for compositional analyses and experiments.

2.1.3. Microalgal biomass analysis

Starch content was assessed using the Megazyme Total Starch (AA/AMG) Assay Kit (Megazyme, Ireland) on 10 ± 1 mg biomass, following the protocol supplied by the manufacturer adapted to remove chlorophyll interference, as previously reported [23]. Protein content was assessed on 10 ± 1 mg biomass with the Bio-Rad protein assay dye reagent, following the protocol provided by the manufacturer (Bio-Rad, USA). Bovine serum albumin was used to create a standard curve. Additionally, 30 ± 1 mg biomass were assessed for fatty acid profile by use of gas chromatography mass spectrometry (GC-MS). The fatty acids were identified and quantified as fatty acid methyl esters (FAME), against FAME-

mix 37, CRM47885 (Sigma-Aldrich, Wyoming USA). All biomass analysis was performed on quadruplicate samples.

2.2 Mashing experiments with algal biomass

2.2.1. Microalgal starch degradation by barley enzymes

The first trials aimed at understanding whether the tightly packed, small, microalgal starch granules would be efficiently degraded by barley starch degrading enzymes at mashing conditions.

Nine samples of milled starch-rich algal biomass and nine samples of barley malt standard (K-MALTA kit, Megazyme, Ireland), as a control, were prepared weighing 10 ± 0.2 mg in 2 ml screw-cap tubes. Enzyme-rich wort was prepared separately with a solid:liquid ratio of 0.24 Kg L^{-1}, thus adding 24 g of milled Pilsner malt (Weyermann, Bamberg, Germany) in 100 ml water. The flask was incubated for 1 hour in a water bath set at 67 °C, shaking the bottle every 5 minutes. After incubation the wort was centrifuged at 4700 rpm for 5 minutes and 1 ml of the enzyme-rich supernatant was pipetted into each of the pre-weighed microalgae and barley malt standard samples. The tubes were then incubated for 0, 1 and 2 hours at 67 °C. At each timepoint, triplicates of each treatment were inactivated at 95 °C for 5 minutes, then centrifuged at 15000 rpm for 5 minutes. The supernatant was removed, and the pellets were analysed with the protocol described in section 2.1.3.

2.2.2. Mashing with malt and microalgae

After studying microalgal starch degradation by malt enzymes, mashing trials were carried out with microalgae (starch rich *T. chui)*, barley malt, (Pilsner malt, Weyermann, Bamberg, Germany), and mixtures of microalgae plus barley malt, as shown in Figure 1. The aim of these experiments was to evaluate the behaviour and the effect on fermentable sugar yields of microalgal biomass alone (M1-3) and of 20%, 12.5% and 5% microalgae substitution of total solids during mashing, compared to barley malt alone (B1-4). A one-step mashing at 67 °C was thus carried out in triplicate, in falcon tubes. Figure 1 reports the exact grams of biomass which were used in 15ml volume for each trial, where the control has a final solid:liquid ratio of 0.24 Kg L^{-1}. Microalgae addition in the mash increased the pH to 8 ± 0.2, thus microalgae plus barley malt mixtures were tested with and without pH adjustment to pH 5.5 ± 0.1 with lactic acid, sampling at 0, 1, 2, 3 and 5 hours from incubation.

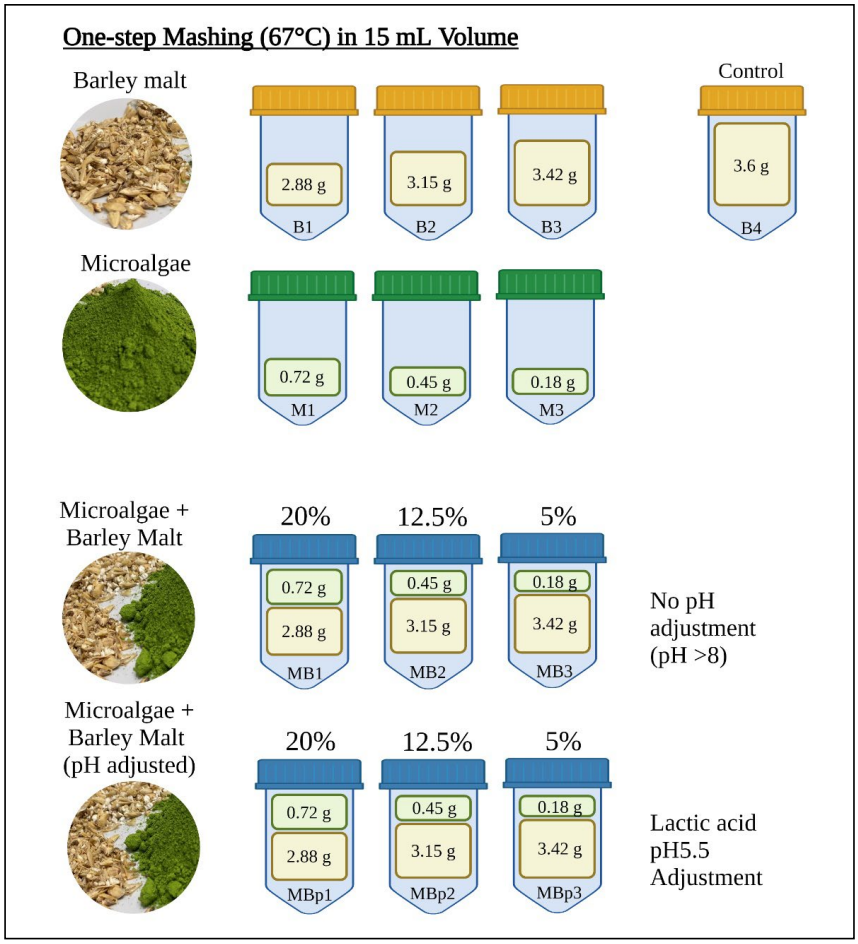


Figure 1: Mashing experiment design. Each tube represents a treatment (performed in triplicate). Yellow tubes (B1-4) represent barley malt mashing trials with content reported in grams; Green tubes (M1-3) represent microalgae mashing trials with *T. chui* content reported in grams; Blue tubes represent mashing trials with a mixture of barley malt and *T. chui*. Substitution of 20, 12.5 and 5% of the total solids with microalgal biomass, at a solid:liquid ratio of 0.24 Kg L^{-1}, was performed with (MBp1-3) and without (MB1-3) pH adjustment. Created with BioRender.com.

At each time point, all triplicates were sampled by aliquoting 1 ml to an Eppendorf tube and incubated at 95 °C in a heating block for 5 minutes to inactivate all enzymes. The samples were then centrifuged at 15000 rpm for 2 minutes and the supernatant was stored 1:1 in 10 mM sulphuric acid (H_2SO_4) for further HPLC quantification of maltose and glucose.

2.3 Brewing Experiment

Brewing with three malt:algae ratios was tested at a small scale against a 100% malt as control. Each treatment was done in triplicate and the twelve beers were prepared over two consecutive days. Table 1 reports the ingredients for 0.8 L volume, including the hops added at the beginning (60 min) and end of boiling (0 min). Both Pilsner malt and Cara malt were sourced from Weyermann (Bamberg, Germany).

Table 1. Brewing ingredients for 0.8 L volume. Four beers containing different malt:algae ratios were tested in triplicate.

	Beer 1	**Beer 2**	**Beer 3**	**Beer 4**
Malt %	100	95	87.5	80
Algae %	0	5	12.5	20
Pilsner Malt (g)	182.4	173.28	159.6	145.92
Cara malt (g)	9.6	9.12	8.4	7.68
Tetraselmis chui (g)	0	9.6	24	38.4
Northern Brewer 60 min (g)	0.4	0.4	0.4	0.4
Cascade 0 min (g)	1.6	1.6	1.6	1.6
Saaz 0 min (g)	1.6	1.6	1.6	1.6

For each batch, malt and algae biomass were weighed according to the values in Table 1 and added to 800 ml water, adjusting pH with lactic acid to the starting value of 5.7 ± 0.1 in 1 L borosilicate glass bottles. The bottles were placed in a water bath set at 67 °C and temperature increase inside the bottles was measured with an immersion temperature probe. The bottles were incubated for 1 hour after the internal temperature of the mash reached 66 ± 2 °C (45 minutes from start). Samples for High Performance Liquid Chromatography (HPLC) were extracted at the start, at temperature stabilization and then after half an hour and one hour incubation at 66 ± 2 °C. Each mash was then transferred into a 2 L Erlenmeyer flask, filtering out the solids with a 2 mm pore size colander. Proper lautering and sparging were not easily performable at such a small scale thus the final wort contained a significant amount of sediment. The worts in 2 L Erlenmeyer flasks were set to boil on heating plates for an hour, Northern Brewer hop (CraftCo, Norway) was added at the beginning and Saaz and Cascade (CraftCo, Norway) at the end of the boiling period. The worts were then adjusted, diluting them with water, to reach a gravity of 12 ± 1% w/v, measured with a Brix refractometer, and were finally transferred into clean 1 L glass bottles after sampling for further HPLC analysis. After the brew had cooled down to 21 ± 1 °C, *Saccharomyces cerevisiae* commercial strain US-05 (Fermentis, Lesaffre, France) was added, according to manufacturer's instructions, and the bottles were closed with yeast locks.

The brews were incubated at 20 ± 1 °C in the dark and sampled daily for brix refractometry to evaluate progression of the fermentation (results not shown). Fermentation in both batches stopped after 5 days, however the batches were bottled on the same date, thus on day 5 for Beers 3 and 4 and on day 6 for Beers 1 and 2. Upon bottling samples for HPLC analysis were extracted and the brews were gently decanted into 500, 330 and 250 ml dark glass bottles, removing most of the sediment. Carbonating sugar (sucrose 5 g L^{-1}) was added to the beers before capping and the bottles were incubated for 12 days at room temperature and for three weeks at 4 °C for maturation.

The beer was finally tasted by a small group of professional brewers from Nøgne Ø brewery, and brewing research scientists from NMBU and NIBIO that provided some general remarks about the taste profile.

2.4 Ethanol and Apparent Degree of Fermentation (ADF)

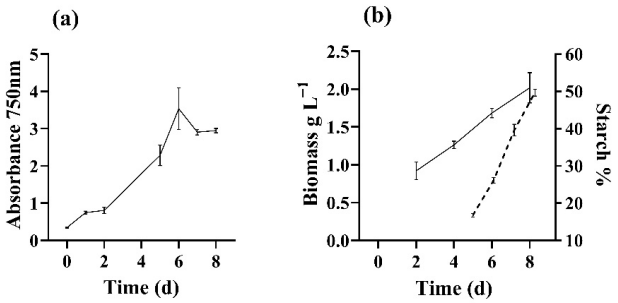
After maturation the beers were characterized using a Packaged Beverage Analyzer for Beer (PBA-B) instrument (Anton Paar, Graz, Austria), consisting of a DMA 4500 M density meter, an Alcolyzer Beer ME module, a CarboQC ME module and a PFD filling device. The instrument was operated with the aid of the Generation M software v2.42 (Anton Paar, Graz, Austria).

190 *2.5 HPLC analysis of Glucose and Maltose*

191 All samples were initially diluted 1:1 with 10 mM H_2SO_4 and stored at 4 °C before
192 further analysis. In some instances, where sediments occurred in storage over time, sam-
193 ples were centrifuged at 14800 rpm for 2 minutes prior to analysis. Sugar concentrations
194 were determined by HPLC using an Agilent Ultimate 3000 (Agilent Technologies, Santa
195 Clara, CA, USA) coupled to an ERC RefractoMax 520 (Shodex, Germany) refractive index
196 (RI) detector. An organic acid resin column (Rezex ROA-Organic Acid H^+ (8%), 300 ×
197 7.8 mm, Phenomenex Inc., United States) was used for separation at a temperature of 65
198 °C and a flow rate of 0.6 ml min^{-1}. As the mobile phase, 5 mM H_2SO_4 was used. Separation
199 was performed in isocratic mode.

200 **3. Results and Discussion**

201 *3.1 Microalgal biomass production and characteristics*



202 **Figure 2:** Time course of biomass growth and starch accumulation in *T. chuii* cultivated in a 250 L
203 tubular photobioreactor over 8 days. (a) Absorbance at 750_{nm} wavelength; (b) biomass dry weight
204 expressed as g L^{-1}(solid line) and starch accumulation expressed as % of biomass dry weigh (dotted
205 line). All data are average ±SD of triplicate measurements.

206 Figure 2a shows culture growth measured through Absorbance 750_{nm} and Figure 2b
207 shows dry weight and starch accumulation during cultivation of *T. chuii* in a 250 L photo-
208 bioreactor. After five days of cultivation most of the medium nitrogen was consumed
209 (NO_3 < 50 mg L^{-1}) and monitoring of starch content in the biomass was started. After three
210 days of starvation, starch content had quadrupled, reaching 50% of the total dry weight
211 (Figure 2b). This is in agreement with previously reported data which indicate *Tetraselmis*
212 species under nitrogen deprivation can accumulate up to 62% of starch [20]. The culture
213 was harvested and freeze-dried yielding a total 370 g of dry algal powder. The analysis of
214 biomass composition, reported in Table 2, shows that the biomass had a relatively low
215 protein content of 20%, *Tetraselmis* species grown under optimal conditions can reach up
216 to 30% protein [24,25]. The lower level of protein in our study can be explained by the fact
217 that the exhaustion of medium nitrogen reduces cell functions and protein synthesis but
218 also by the dilution effect of starch accumulation on relative content of other components
219 in the cells. The total fatty acid content in the microalgal biomass was circa 5.2%, this is in
220 agreement with previous studies on *Tetraselmis marina* which have shown a reduction of
221 fatty acid content under nutrient stress [26]. Polyunsaturated fatty acids, known for their
222 antioxidant properties, represent 52% of the total content. The oxidation of lipids during
223 brewing may result in undesirable flavours, thus it is often important to reduce their con-
224 tent [27], however novel research is shedding light on the role of fatty acids in malt bio-
225 mass, which is circa 1-3% of the total, proving they influence properties and qualities of
226 the grains in brewing [28].
227

Table 2. Composition of starch-rich *T. chui* biomass

	% dry weight	SD
Starch	49.42	0.82
Proteins	22.41	1.57
Fatty Acids	5.20	0.56
SFA	1.13	
MUFA	1.35	
PUFA	2.71	

3.2 Mashing trials

Firstly, it was tested whether the starch granules produced from the microalgae were efficiently digested by barley starch-degrading enzymes activated during the mashing phase (Figure 3). At the incubation temperature of 67 °C our results show an effective digestion of both the algae starch and the malt control after 1 hour of incubation, when the 88% and 95% of the total initial starch content in the *T. chui* and Barley Malt had been degraded. After two hours incubation, the starch in both treatments had nearly been depleted.

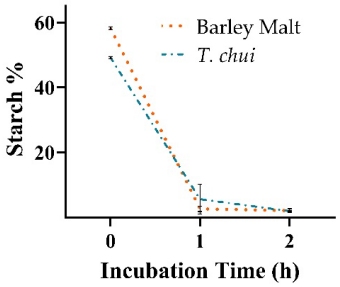


Figure 3: Starch degradation in Barley malt (dotted line) and *T. chui* (dashed line) biomass by enzyme-rich wort at 67 °C incubation over two hours. All data are average ±SD of triplicate measurements

Mashing experiments were then performed on barley malt, microalgae and microalgae-barley mixtures, with and without pH adjustments. In the malt-only controls the sugar release was directly proportional to the amount of malt used in the mashing, both in glucose and maltose results (Figure 4a-b).

To test the presence and activity of endogenous algal starch degrading enzymes, a second mashing trial was performed with only starch-rich algal biomass, incubated in suspension at 67 °C for 5 hours. Though under nitrogen deprivation endogenous starch degrading enzymes may be synthesized in several microalgal species [29], in this study either they were not present in significant amounts, or they were deactivated by temperature, since no significant increase in fermentable sugars was detected with HPLC (Figure 4c-d). The measured sugar concentrations were low and close to the detection limit of the HPLC, explaining the relatively high standard deviation. The three amounts of algae tested correspond to the final contents to be added in mashing and brewing trials with algae-malt mixtures (20%, 12.5% and 5% of the total solids).

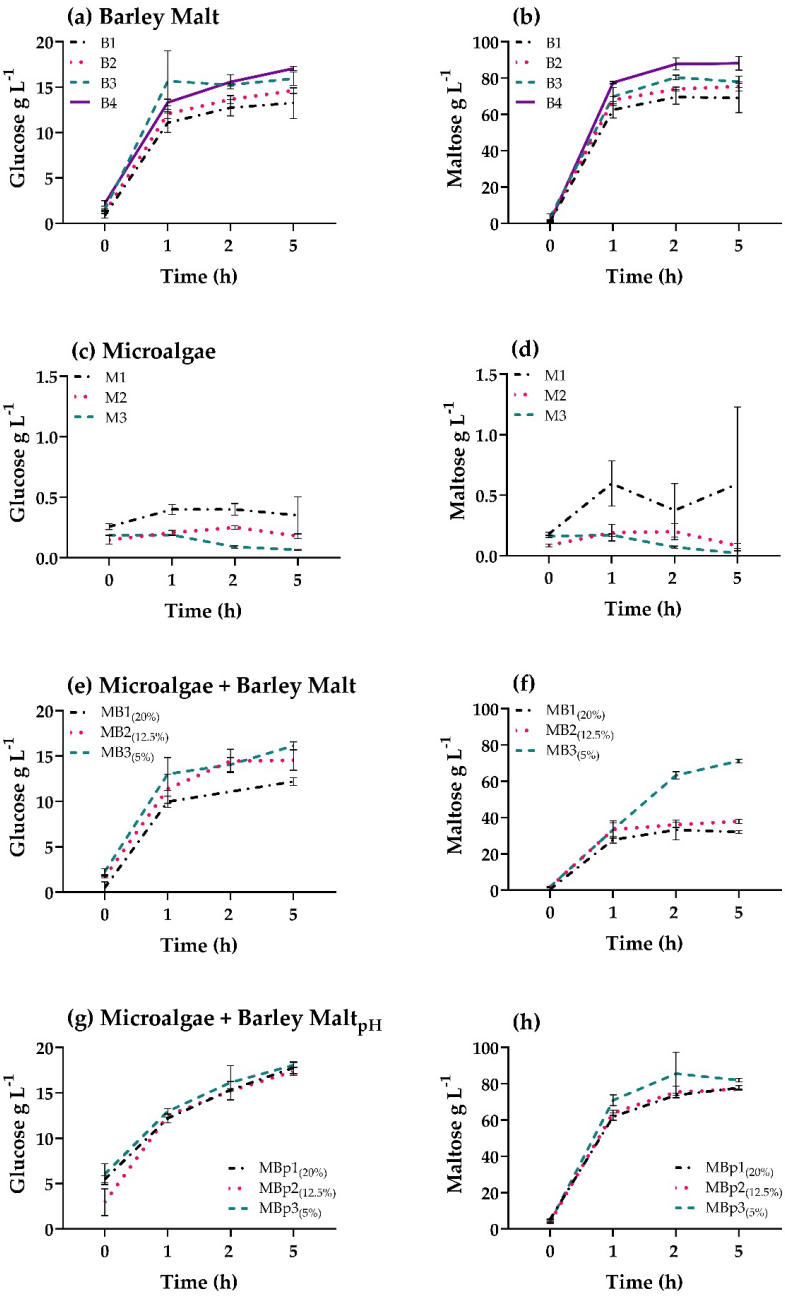


Figure 4: Glucose and Maltose accumulation in the wort during mashing experiments with barley malt and *T. chui* starch-rich biomass. Samples were incubated over 5 hours at 67 °C. Detailed information about samples is described in Figure 1. All data are average ±SD of triplicate measurements.

Measurement of pH upon mashing with barley malt and algae mixtures has highlighted a strong effect of algal biomass, resulting in pH values up to 8.0 ± 0.2. In traditional mashing the pH is usually between 5.2-5.6, and in our experiments, samples containing barley malt only (B1-4) fall within this range. Compared to the barley malt mashing (Figure 4a-b), the addition of algal biomass to barley malt without the adjustment of pH (Figure 4e-f) resulted in an overall lower yield of fermentable sugars. This is likely to be a consequence of the increase in pH. Highest yields of glucose and especially maltose was achieved in the samples containing the lowest algal biomass. Consequently, an additional mashing experiment was performed on barley malt and microalgae mixtures adjusting the pH to a value of 5.5 ± 0.1 with lactic acid at the start of incubation, yielding excellent results with regards to the production of fermentable sugars (Figure 4g-h). The addition of algal biomass in those samples resulted in an overall reduction of the differences between the three ratios, compared to the former trials, suggesting microalgal starch is contributing to the pool of total fermentable sugars. Additionally, the final sugar concentrations of both glucose and maltose are closer to the 100% malt control. Samples with the lowest algae content are however still the ones achieving the highest sugar concentration; this may be due to the fact that the barley malt used in this trial contains circa 60% of starch, whereas the algae biomass contains 50%.

3.3 Brewing trials

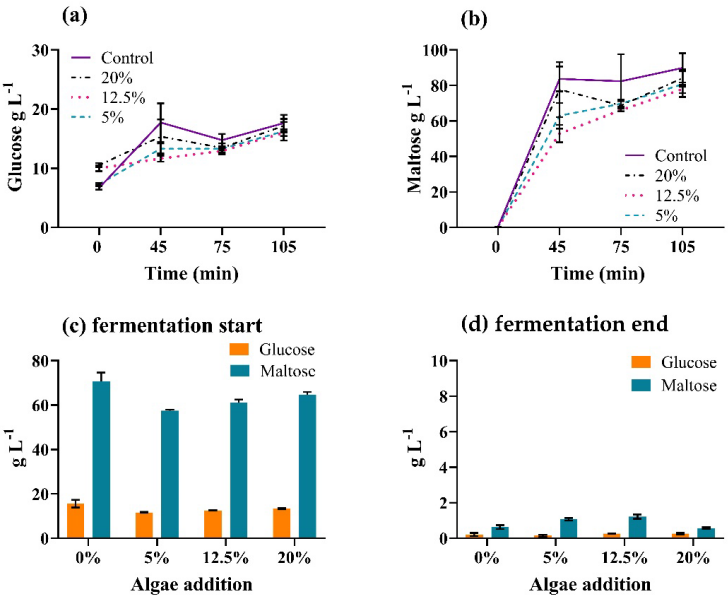


Figure 5: Mashing and fermentation sugar content during brewing trials with *T. chuii*. (a) glucose and (b) maltose detection during progression of one-step mashing at 67 °C; (c) fermentable sugars detection on the first and (d) last day of fermentation. All values are averages ± SD of triplicate samples.

The results from the former trials were used to scale the experiment to 1 L bottles, adjusting the pH at the beginning of mashing to 5.5 ± 0.1. Mashing was performed by placing the bottles in a water bath set at 67 °C. The mashes were incubated for one hour after the temperature inside the mashes reached 66 ± 1 °C (45 min from starting point)

The high deviations visible in the results (Figure 5a-b) are imputable to difficulties in mixing evenly the replicate bottles, however the results are in agreement with former mashing experiments, with the 20% algae enriched brew yielding the highest glucose and maltose content amongst the algae-containing worts. Fermentation was completed in 5-6 days, when on average 99% of both glucose and maltose present in the first day of fermentation were consumed by yeasts (Figure 5c-d). After maturation the beers were analysed in the Anton Paar Alcolyser (Table 3).

Table 3. Anton Paar measurement results from brewing using 3 different algae:malt mixtures vs control. Asterisk mark values with relative standard deviation higher than 5%.

Microalgae content	Alcohol	Haze	Density	Er	Ea	Colour	Calories	pH	CO2
	%v/v	EBC	g cm^{-3}	%w/w	%w/w	EBC	kJ $100ml^{-1}$	-	vol
Control, 0%	5.62	4.10*	1.01	4.13	2.10	7.55*	191.09	4.15	1.88
5%	5.61	3.23*	1.01	4.41	2.38	8.22	195.08	4.40	1.88
12.5%	5.46	3.47	1.01	5.04	3.09	9.30	201.41	4.60	1.80*
20%	5.11	4.38	1.01	5.45	3.62	9.69*	199.83	4.87	1.72*

Despite algal biomass not hindering fermentable sugar release during mashing, the results clearly show an effect on the final product. Higher content of algae resulted in stronger colour values, haze and in a higher caloric content. Additionally, pH values were higher in all algae beers compared to the control, whilst remaining below pH 5. Further testing is needed to better isolate and evaluate the effect of adjustment with lactic acid and improve the pH management protocols.

The alcohol content was lower in beers supplied with higher algal content. This is in agreement with the real and apparent degrees of fermentation, where the lowest algal content achieved similar values as the control, whereas high algal addition resulted in lower fermentation efficiency. The volume of CO_2 is also consequently lower in high-algae beers.

Microalgal biomass application in food has proven to be a complex process because of its impacts on technical and sensory properties of the final products. For example, in savoury biscuits, up to 8.3% *Arthrospira platensis* biomass was added to the doughs resulting in enhanced protein profile of the foods, however at this concentration the sensory profile was not appealing [30]. Similar results were reported for other species, with the addition of microalgal biomass in contents up to 6% in biscuits resulting in improved bioactive profile, although lower biomass additions yielded better sensory scores [13]. In baking goods addition of microalgal biomass above 3% had a disruptive effect on dough rheology, with optimal contents often ranging between 1-2% for the different species used [10,31]. In gluten free bread it was possible to successfully integrate 4% *T. chui* biomass with a stabilising effect on dough, however sensory scores were lower if compared to the 1-2% *T. chui* substitutions [12].

In our trials, the addition of higher microalgal content was aided firstly by the nature of beer brewing, which happens in suspension, reducing the impact of microalgae on texture. Secondly, some of the unpleasant aromas, which were clearly perceived at the beginning of brewing, were lost throughout the mashing, boiling and fermentation. Finally, a proportion of microalgal biomass sedimented, and was partially removed from the final product during lautering and later bottling, thus possibly reducing the impact on taste and texture. It is also noteworthy that the addition of higher amounts of biomass resulted in thicker mashes, with some difficulties in the mixing and major issues in the lautering

process, which led to consistent sediment formation. Thus, despite production at a small scale being successful, upscaling of the process is yet to be optimised.

The tasting by brewing experts highlighted how the algae beers had a characteristic flavour compared to the control, with increasing sourness upon higher microalgal biomass addition. The taste of algae was almost negligible at 5 % addition, giving the beer a slight floral flavour, with hints of umami, while the algae taste was more distinct with increasing amounts. The addition of 12.5% had a clear umami taste with seaweed and marine flavours. Additionally hints of a sweet aroma, resembling canned corn, could be perceived. The 20% beer had the most intense profile, with a stronger seaweed flavour and aroma, marked syrupy notes and strong umami taste. In the 5% and 12.5% beers appearance was similar to the control, whereas green hues were visible in the 20% beer.

Based on this trial an amount ranging between 5-10% microalgae could be the most suitable for commercial purposes. However, testing on a larger scale, with specific brewing equipment is necessary to define in more detail colour, texture, and flavour in a final product.

5. Conclusions

T. chui biomass was grown under nitrogen deprived conditions in 250L reactors to specifically produce biomass with the desired characteristics for brewing. Stepwise trials were performed to assess the effect of microalgae on the mashing process, optimizing the introduction of up to 20% algal biomass as an active ingredient in brewing. Adjustment of pH before mashing proved to be a key step for successful beer brewing with microalgae. Starch from microalgae was successfully transformed into fermentable sugars during mashing and further small-scale brewing trials proved the feasibility of microalgae-beer production, yielding a final product which is not only palatable, but pleasant to taste.

Further experiments will need to be performed to characterize the beers with a professional tasting panel, to evaluate possible bioactive properties of the final product and to establish production at a larger scale.

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Errata

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