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Hugo Mear

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Spécialité : Génie des Procédés et Bioprocédés

Par

Hugo MEAR

Extraction et purification de protéines fonctionnelles de microalgues pour l'alimentation humaine.

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Résumé en français

Le système agroalimentaire actuel est confronté à plusieurs défis, dont une population mondiale croissante, et donc une demande alimentaire croissante. De plus, il est un des principaux responsables d'impacts environnementaux majeurs tel que la déforestation, les émissions de gaz à effet de serre et la diminution des ressources en eau douce.

De nouvelles sources de protéines sont recherchées pour substituer en partie les sources actuelles et notamment les protéines d'origine animale qui contribuent significativement aux problèmes environnementaux. Les protéines alternatives identifiées comme étant les plus prometteuses sont les insectes, les levures, les champignons filamenteux et les microalgues et cyanobactéries.

Les microalgues et cyanobactéries sont intéressantes pour leurs hautes teneurs en protéines, leurs cultures sur des terres non arables et leurs hautes productivités surfaciques. Cependant, des défis à relever limitent leurs consommations comme le manque de maturité des procédés de culture et de transformation.

Pour répondre à ces défis et promouvoir l'utilisation des microalgues et cyanobactéries en alimentation humaine et animale, le projet européen H2020 ProFuture a été lancé. Ce projet vise à optimiser la culture de 5 espèces, *Arthrospira platensis* (aussi appelée spiruline), *Chlorella vulgaris*, *Tetraselmis chui*, *Nannochloropsis oceanica* et *Galdieria sulphuraria*.

Les trois premières espèces préalablement mentionnées sont également étudiées pour la production d'ingrédients protéiques après des étapes de transformation. Ces ingrédients sont caractérisés tant d'un point de vue nutritionnel que techno-fonctionnel. La chaîne de valeur complète, de la culture à la formulation de recettes contenant des microalgues entières séchées ou ingrédients, en passant par la transformation est analysée d'un point de vue économique et environnemental. Les procédés de culture, transformation et formulation sont également étudiés à plus grande échelle jusqu'à échelle industrielle. AlgoSource Technologies est un partenaire de ce projet et cette thèse s'inscrit dans le projet ProFuture et est en collaboration avec AlgoSource Technologies et le laboratoire GEPEA.

L'objectif de cette thèse est de développer un procédé afin de produire des extraits enrichis en protéines de microalgues faiblement colorés et solubles dans l'eau. Trois espèces de microalgues sont abordées, *A.platensis*, *C.vulgaris* et *T.chui* et le procédé est développé sur cette dernière et comparé aux autres espèces. Les extraits sont produits après des étapes de destruction cellulaire, clarification, concentration & purification et enfin une opération unitaire non étudiée dans cette thèse, le séchage. Les opérations unitaires sélectionnées dans le cadre de ce projet sont le broyage à billes et les champs électriques pulsés pour la destruction cellulaire, et la précipitation isoélectrique et la filtration membranaire pour la purification.

Tetraselmis chui est une microalgue marine autorisée pour une consommation alimentaire dans l'Union Européenne depuis 2014 comme condiments et sels spéciaux. Cette espèce, traditionnellement cultivée comme fourrage pour l'aquaculture possède des caractéristiques nutritionnelles intéressantes, comme une teneur en protéique pouvant varier de 35% à 56% de la matière sèche dans des conditions de culture standards. Elle possède également plusieurs avantages compétitifs en culture comme une haute productivité surfacique ou une résistance aux variations de salinité. L'utilisation de cette algue en alimentation a déjà été étudiée mais le goût, l'odeur et la couleur

limitent sa consommation. La transformation peut répondre à certains obstacles comme pour améliorer la digestibilité, éliminer la forte couleur ou augmenter les propriétés techno-fonctionnelles. Cependant, des progrès sont encore à réaliser pour développer un procédé couplant des opérations unitaires de destruction cellulaire et de purification.

Les microalgues ne possèdent pas de protéines de réserve connues comme les graines. Au contraire, les protéines de microalgues possèdent une grande diversité. Les protéines peuvent être classifiées en trois grandes catégories, les protéines globulaires, facilement soluble dans l'eau, membranaires soluble dans l'eau avec des détergents et fibreuses, très faiblement soluble dans l'eau. Les protéines globulaires de microalgues, solubles dans l'eau, sont majoritairement situées dans le cytosol, le stroma du chloroplaste et le pyrénôïde. Les protéines membranaires semblent localisées principalement dans les membranes des mitochondries et des thylakoïdes. Dans ces derniers, les protéines faisant partie du complexe de récolte de la lumière sont considérées comme les protéines membranaires les plus abondantes au monde et certaines d'entre elles sont liées aux chlorophylles. Enfin les protéines structurelles se trouvent notamment dans le cytosquelette.

Pour extraire les protéines intracellulaires, une opération de destruction cellulaire est nécessaire. Les microalgues ont des parois cellulaires très variées entre espèces, avec des résistances mécaniques diverses. Parmi les technologies de destruction cellulaire, le broyage à billes (BB) est une technologie mature et très utilisée pour détruire des microalgues. Le broyage à billes est une chambre remplie de billes mises en mouvement par un agitateur. Les contacts entre les billes et les particules à détruire permettent une réduction de taille des particules. Cette technologie permet de détruire complètement l'algue et d'accéder à toutes les protéines intracellulaires. En revanche, la réduction de taille des particules entraîne une dispersion d'une partie des microalgues, dont les protéines membranaires liées aux chlorophylles, entraînant un broyat vert. Cela nécessite une autre opération unitaire pour obtenir un extrait peu coloré.

Une autre technologie de destruction cellulaire, les champs électriques pulsés (CEP) peuvent être intéressants pour extraire des protéines solubles sans détruire complètement les cellules. Cette technologie repose sur l'électroporation de la membrane cellulaire, permettant la diffusion des protéines intracellulaires, particulièrement celles du cytosol et du stroma. L'électroporation peut être irréversible ou réversible suivant les conditions appliquées. Bien que les rendements obtenus soient faibles, l'extrait obtenu est peu coloré et peut potentiellement être purifié plus facilement. De plus, les coproduits peuvent être plus intéressants que les coproduits obtenus après BB où de complexes réorganisations moléculaires peuvent se produire.

Après destruction cellulaire, le lysat obtenu est clarifié par centrifugation et deux techniques sont envisagées pour purifier le surnageant obtenu, la précipitation isoélectrique et la filtration membranaire. La précipitation isoélectrique (PIE) repose sur la modification de la solubilité des protéines en fonction du pH. Quand le pH est proche du point isoélectrique des protéines majoritaires, la charge des protéines est neutre ce qui diminue les répulsions électrostatiques entre protéines, favorisant leurs agglomération et sédimentation. Cette opération unitaire est très couramment utilisée dans l'industrie agroalimentaire pour l'obtention d'extraits enrichis en protéines. Cette technologie permet de produire un extrait concentré à faible coût mais demande une forte quantité de produits chimiques, une augmentation de la salinité du produit et une dénaturation protéique possible. La PIE a été étudiée pour la purification de protéines microalgales mais son efficacité est débattue. Certaines études ont obtenu un précipitat vert enrichi en protéines, alors que d'autres études ont démontré une forte solubilité protéique, même à un pH proche du pI. La filtration membranaire est une alternative à la PIE permettant de produire un extrait protéique purifié concentré à faible couleur. En particulier, la microfiltration peut être utilisée pour séparer les débris cellulaires dispersés riches en chlorophylles

de l'extrait protéique, permettant la production d'un perméat faiblement coloré. L'ultrafiltration peut également être utilisée afin de retenir les protéines d'intérêt pour les concentrer et les purifier de plus petits composés.

Suite à la bibliographie réalisée, deux stratégies principales peuvent être appliquées. La première consiste à détruire complètement les cellules afin d'accéder à la majorité des protéines intracellulaires et obtenir un haut rendement sur l'étape de destruction cellulaire. En revanche, une partie des protéines dispersées peuvent être associées avec d'autres molécules, dont les chlorophylles. Afin d'obtenir un extrait purifié faiblement coloré, une opération unitaire est nécessaire après la centrifugation avec une étape de microfiltration. Cela permet de produire un perméat peu coloré, pouvant être purifié par plusieurs techniques. Une seconde stratégie peut viser à libérer les protéines intracellulaires solubles, sans détruire complètement les cellules, évitant de disperser les protéines membranaires des thylakoïdes, contenant des chlorophylles. Le surnageant obtenu après centrifugation peut contenir moins de protéines, mais peut être faiblement coloré, facilitant la purification et évitant une opération unitaire supplémentaire.

La destruction cellulaire de deux états physiologiques de *Tetraselmis* a été réalisée par broyage à billes. Différents lots de *Tetraselmis chui* ont été reçus du même fournisseur, avec des propriétés morphologiques différentes, probablement associées à une différence d'état physiologique : un lot contenait des cellules circulaires et en division et l'autre des cellules flagellées. Les lots ont été caractérisés biochimiquement (protéines, glucides, cendres) et l'aptitude à la rupture a été testée pour caractériser leur comportement à la rupture cellulaire. Le lot flagellé s'est avéré beaucoup plus fragile que l'autre. Le broyage à billes des deux lots a également montré de fortes différences en termes de cinétique de destruction cellulaire, de répartition de la matière sèche après centrifugation et de rendement de récupération des protéines dans le surnageant. Pratiquement aucune différence de rendement protéique n'a été observée après broyage à billes par rapport à la congélation-décongélation pour le lot résistant, avec un rendement final de 11 % des protéines initiales. Cependant, une forte libération des protéines dans le surnageant a été observée pour le lot fragile tout au long du broyage, avec un rendement final en protéines de 32%. Les protéines libérées ont été caractérisées par SDS PAGE et zymographie et des différences ont été observées, confirmant une différence de métabolisme. Plusieurs modifications physicochimiques ont été appliquées au lysat cellulaire pour augmenter les rendements en utilisant le pH, la température et la dilution. Cette dernière modification s'est avérée la plus prometteuse. Les résultats ont souligné qu'une différence d'état physiologique de *Tetraselmis chui* avait un impact significatif sur le bioraffinage, affectant directement la destruction cellulaire et les rendements de récupération des protéines. La valorisation de l'ensemble des microalgues nécessite donc un couplage très fin entre la culture et le bioraffinage. Ce travail a été valorisé par un article scientifique publié dans *Algal Research*: "Extraction of soluble proteins by bead milling from *Tetraselmis chui* in two different physiological states", Hugo Mear et al., *Algal Research*, volume 74, 103180, 2023 (Mear et al. 2023).

Le broyage de *Tetraselmis* a été comparé au broyage de *C.vulgaris* et *A.platensis*. Une destruction cellulaire plus rapide a été observée pour la destruction de *T.chui* en comparaison à la destruction de *C.vulgaris*. Une libération protéique plus rapide a également été observée pour le lot fragile de *T.chui* par rapport à *Chlorella*. La destruction cellulaire des différentes microalgues était très différente en fonction des espèces traitées. La destruction cellulaire de *A.platensis* était également fortement différente, avec une forte dispersion de la matière sèche dans le surnageant. Cela était lié à un rendement protéique et une pureté élevés car la biomasse initiale avait une forte teneur en protéines. En revanche, la forte dispersion peut compliquer une future purification.

Les champs électriques pulsés ont également été testés sur le lot résistant de *Tetraselmis* et *Chlorella vulgaris*. En revanche, aucune amélioration significative du rendement protéique a été observée après traitement en comparaison avec la décongélation.

Pour la libération des protéines de *Tetraselmis*, deux stratégies furent appliquées en fonction de l'état physiologique. La destruction cellulaire du lot résistant fut réalisée par décongélation car le broyage à billes et les champs électriques pulsés n'amélioraient pas significativement les rendements protéiques. Pour le lot fragile, une destruction cellulaire par broyage à billes peut être intéressante si couplée à une opération unitaire supplémentaire pour séparer les particules contenant des pigments présents dans le surnageant.

La solubilité des protéines du surnageant de *T.chui* après décongélation était très élevée à tous les pH testées, compliquant l'utilisation de la précipitation isoélectrique sur cet extrait. L'ultrafiltration à 10 kDa était efficace pour concentrer les protéines, avec un taux de rétention protéique élevé de 90% environ. De plus, une diafiltration permettait d'enlever efficacement les cendres, contenant principalement des sels, afin d'augmenter la pureté des protéines. Cette technologie fut sélectionnée pour le changement d'échelle.

En vue du changement d'échelle, la stabilité du surnageant dans le temps en fonction de la température fut testée afin de définir le temps de traitement acceptable et la température de filtration. Un plus faible développement bactérien a été observé à 12°C en comparaison avec 22°C et 47°C. De plus, le profil protéique restait constant à 12°C alors qu'il était modifié aux autres températures testées, ce qui pouvait témoigner d'une activité de dégradation des protéines par des protéases, ou par des microorganismes. La température de filtration fut fixée à 12°C afin de maintenir les propriétés initiales du surnageant.

Le procédé a été réalisé à échelle pilote, avec des flux obtenus similaires à ceux observés à échelle laboratoire. En revanche, la rétention des protéines était plus faible avec un taux de rétention entre 40% et 60% durant la concentration, et entre 60% et 90% pendant la diafiltration. Des améliorations auraient pu être réalisées à cette échelle mais un changement d'échelle fut réalisé à échelle semi-industrielle due aux contraintes du projet afin de produire suffisamment de produits pour les partenaires. Des densités de flux de perméat supérieures aux échelles précédentes ont été obtenues lors de la filtration à échelle semi-industrielle. En revanche, des taux de rétention des protéines faibles entre 15% et 25% ont été observés. Le taux de rétention des cendres était faible, permettant l'élimination des sels. La faible rétention des protéines compromettait l'efficacité du procédé. Une des sources de cette faible rétention peut provenir d'un lot de biomasse différent, dans un état physiologique différent de celui testé à échelle laboratoire. Des protéases ont été obtenues dans le surnageant après décongélation en quantité plus importantes sur le lot fragile par rapport au lot résistant. Cela pourrait expliquer la différence de rétention. Un essai de filtration du perméat de concentration acidifié a été réalisé et une amélioration significative du taux de rétention a été obtenu. Cela pourrait éventuellement être une piste pour améliorer la rétention des protéines.

Une autre stratégie consistant à broyer le lot fragile de *Tetraselmis*, centrifuger et filtrer le surnageant avec une microfiltration a été testée. Bien que le rendement de libération des protéines dans le surnageant soit élevé, le couplage avec la microfiltration est le point bloquant car une forte rétention des protéines est observée.

L'intégration de toutes les étapes, depuis l'amont (culture de la biomasse, récolte et stockage) jusqu'à l'aval (rupture cellulaire et purification), est une nécessité pour produire des extraits de microalgues. Les microalgues sont des micro-organismes très flexibles qui peuvent modifier leur composition en quelques heures ; cette flexibilité est très intéressante pour orienter leur métabolisme, mais cette qualité induit également un inconvénient majeur, la variabilité de la biomasse, en particulier lorsqu'elle est cultivée à l'échelle industrielle, comme cela a été mis en évidence dans ce travail. La sélection fine des technologies de destruction cellulaire est nécessaire afin de permettre la libération ainsi que la concentration et purification de protéines aux propriétés spécifiques. Le changement d'échelle réalisé a soulevé de nouveaux défis tels que la stabilité du produit du stockage à la purification, et la nécessité de prétraitements ou de contrôle de la température au cours du procédé.

List of communications

International communications:

Mear, Hugo, Pascale Gillon, Imma Gifuni, Laurence Lavenant, Alexia Poidevin, et Couallier Estelle. 2023. « Extraction of Soluble Proteins by Bead Milling from *Tetraselmis Chui* in Two Different Physiological States ». *Algal Research*, juin, 103180. <https://doi.org/10.1016/j.algal.2023.103180>.

Liu, Shuli, Imma Gifuni, Hugo Mear, Matthieu Frappart, et Estelle Couallier. 2021. « Recovery of Soluble Proteins from *Chlorella Vulgaris* by Bead-Milling and Microfiltration: Impact of the Concentration and the Physicochemical Conditions during the Cell Disruption on the Whole Process ». *Process Biochemistry* 108 (septembre): 34-47. <https://doi.org/10.1016/j.procbio.2021.05.021>.

Oral communication in an international congress:

Hugo Mear, Pascale Gillon, Imma Gifuni, Estelle Couallier “Integrated process conception to produce a soluble protein extract from *Tetraselmis chui* with low color for food application” 14th International Congress on Engineering and Food from the 20th to the 23 of June 2023 in Nantes.

Hugo Mear, Pascale Gillon, Imma Gifuni, Estelle Couallier “Extraction of soluble proteins by bead milling from *Tetraselmis chui* in two different physiological states” 8th European Phycological Congress from the 20th to the 26th of August 2023 in Brest.

Poster in an international congress:

Hugo Mear, Rémi Camus, Pascale Gillon, Imma Gifuni, Estelle Couallier “Pulsed Electric Field extraction of proteins from *Tetraselmis chui*” AlgaEurope 2021 from the 7th to the 10th of December 2021 Online.

Poster in a national congress:

Hugo Mear, Pascale Gillon, Imma Gifuni, Laurence Lavenant, Estelle Couallier « Destruction cellulaire de 3 souches de microalgues pour une intégration à un procédé global » 18^{ième} Congrès de la Société Française de Génie des Procédés 2022 from the 7th to the 11th November of 2022 in Toulouse.

List of abbreviations

BCA: BiCinchoninic Acid

BM: Bead Milling

BSA: Bovin Serum Albumin

CCM: Carbon Capture Mechanism

CFU: Colony Forming Unit

C_p: Heat capacity (J.K⁻¹)

C-PC: C-phycoerythrin

d_B: Bead diameter (m)

DI: Deionized

DM: Dry Matter

DV: DiaVolume (-)

E: Electric Field Strength (V.m⁻¹)

E_m: Specific energy for the milling (J.kg⁻¹)

f : bead filling ratio (-)

HPH: High Pressure Homogenizer

[I]: Ionic Strength (mol.L⁻¹)

IEP: IsoElectric Precipitation

k: Kinetic constant (min⁻¹)

LC MS/MS: Liquid Chromatography coupled with Mass spectrometry in tandem.

LHC: Light Harvesting Complex

MW: Molecular Weight (Da)

MWCO: Molecular Weight Cut Off (Da)

n: number of stirrer revolution per unit time (s^{-1})

P_{50} : bar

PEF: Pulsed Electric Field

PES: PolyEtherSulfone

pH(I): Isoelectric point

PS: PolySulfone

PSD: Particle Size Distribution

Q: volume flow rate ($m^3.s^{-1}$)

RCF: Rotational Centrifugation Force ???

RR: Retention Rate (-)

RuBisCO:

S: Surface (m^2)

SDS-PAGE: Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis

SE: Stress Energy (J)

SI: Stress Intensity ($J.m^{-1}$)

t: time (s)

T: temperature ($^{\circ}C$)

TMP: TransMembranePressure (bar)

U: Tension (V)

UF: UltraFiltration

v: Agitator speed ($m.s^{-1}$)

V: volume (m^3)

VRR: Volume Reduction Rate (-)

W: input specific energy ($kWh.kg_{DM}^{-1}$)

Y: Yield

YTZ: Yttria Stabilized Zirconium

Greek and Latin symbols

ϵ : porosity (-)

Φ : volumic ratio between the supernatant and the suspension (-)

ρ volumic density (kg.m^{-3})

σ : electric conductivity (S.m^{-1})

μ : dynamic viscosity (Pa.s)

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Chapter 1 Introduction

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1.1 Context of the PhD

1.1.1. General context

The current food system faces numerous challenges such as a growing population worldwide and a current inadequate protein intake for one in ten people while one out of four people is overweight. Based on numerous projections, the total global food demand is expected to increase by 35% to 56% between 2010 and 2050, and from 30% to 62% if climate change effects are taken into account (van Dijk et al. 2021). Moreover, the food system has major environmental impacts. It is already responsible for up to 30% of global anthropogenic greenhouse gas (GHG) emissions (Crippa et al. 2021). The food system is a major responsible for deforestation, with two third of the forest loss attributed to expanding cropland, pastures and tree plantations (Erb et al. 2016). It is also a major responsible for the depletion of freshwater resources (90% of freshwater consumption) and the pollution of aquatic and terrestrial ecosystems through excessive nitrogen and phosphorus inputs, as well as on biodiversity loss (Dalin et Outhwaite 2019 ; von Braun et al. 2021). Blue food production, defined as fish, shellfish, plants and algae from fishing and farming in marine or freshwater ecosystems, has growing environmental impacts (Scheffer, Carpenter, et Young 2005; Naylor et al. 2021; Ye et Link 2023) and is vulnerable to environmental disturbances (Cao et al. 2023). Drastic changes in the food system are needed in the future to tackle the several challenges.

Among the possibilities, a diet change can help to overcome part of the challenges since livestock production has many negative environmental impacts (Schiermeier 2019 ; Errickson, Kuruc, et McFadden 2021). A transition from animal-based proteins, and especially from ruminants, to plant-based proteins can for example significantly decrease greenhouse gas emissions as well as land and water use (Xu et al. 2021). New alternative protein sources are sought and need to be produced sustainably, grown on non-arable lands and have interesting nutritional properties. Among these, insects, yeasts, filamentous fungi and microalgae/cyanobacteria are seen as the most promising (Onwezen et al. 2021; Molfetta et al. 2022).

1.1.2. Microalgae and cyanobacteria, promising resources

Microalgae and cyanobacteria are unicellular photosynthetic microorganisms living in water. Microalgae are Eukaryotes, while cyanobacteria are Prokaryotes. They are very diverse genetically and can be promising for a wide range of applications.

Microalgae and cyanobacteria are interesting for food consumption as an alternative source of proteins. They do not necessitate arable land to grow, and they have a high protein content. A high aerial productivity of $30 \text{ t}_{\text{protein}} \cdot \text{ha}^{-1} \cdot \text{y}^{-1}$ may potentially be attained for microalgae compared to $1.1 \text{ t}_{\text{protein}} \cdot \text{ha}^{-1} \cdot \text{y}^{-1}$ for soy (Janssen, Wijffels, et Barbosa 2022; Barbosa et al. 2023). They can also use

marine water or food effluents, decreasing water consumption (Martins et al. 2018; Goswami et al. 2022).

However, several drawbacks limit their food consumption due to the immature cultivation and downstream processes, causing a high biomass cost.

1.1.3. The H2020 ProFuture program

To tackle these drawbacks and increase the environmental and economic sustainability of the microalgal value chain, the European Project H2020 ProFuture aims at improving the cultivation and use of microalgae as a source of proteins for the food and feed industries. The objectives of the project are:

- The improvement of five specie cultivation, ***Arthrospira platensis*** (also called spirulina), ***Chlorella vulgaris***, ***Tetraselmis chui***, ***Nannochloropsis oceanica*** and *Galdieria sulphuraria* (WP2),
- The development of innovative technologies to produce protein-rich ingredients as single-cell proteins (4 algae in bold) or protein isolates (3 algae underlined) (WP3),
- The ingredient characterization and evaluation for their nutritional, safety, techno-functional and organoleptic properties (WP4),
- The formulation of different types of foods and feeds, the evaluation of the consumer perception and acceptance and the regulatory point of view (WP4),
- The scale up of the culture, transformation, and reformulation processes to an industrial scale,
- And finally, the value chains are analyzed to identify the strengths and weaknesses with life cycle analysis and life cost analysis assessed to prepare a market implementation.

The side products are also identified and their possible uses are assessed. The consumer perception and acceptance of the products are also assessed to prepare a market implementation, especially from the regulatory point of view.

The different tasks of the project were distributed as Work Packages (WP) shared among the different partners and the value chain, and are presented in figure 1.1.

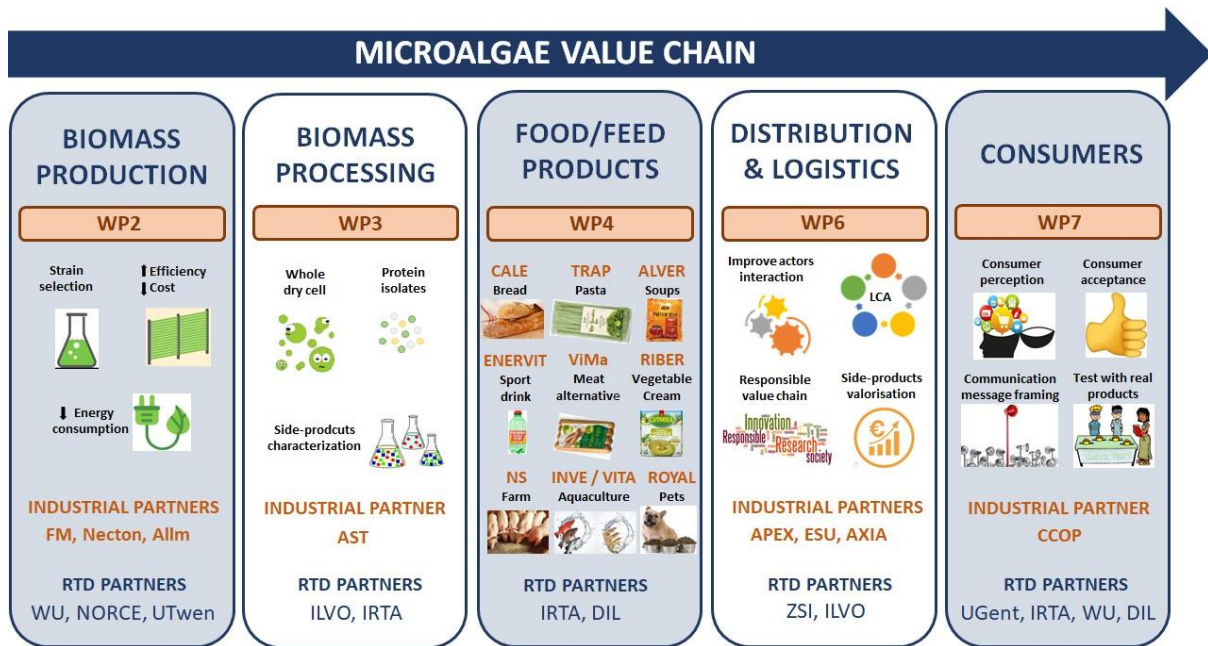


Figure 1.1 Innovations through the microalgae value chain in the Profuture project.

The partners are distributed in Europe and the organization necessitates complex logistics with sending and receiving of samples and products across partners. For example, the biomass is sent frozen from Portugal to the other partners.

The work presented in the following paragraphs takes part in the work package 3 and was performed in the context of a collaboration between AlgoSource Technologie and the GEPEA laboratory with a CIFRE (*Convention Industrielle de Formation par la REcherche*).

AlgoSource Technologies is part of the project ProFuture and is responsible for work package 3 on biomass processing. AlgoSource Technologies belongs to the group AlgoSource, a company in France specialized in the application of microalgae for health. AlgoSource is the fusion of two companies, Alpha Biotech and AlgoSource Technologies, and is located in Guérande and Assérac. Alpha Biotech is specialized in the cultivation and the biorefinery of microalgae and cyanobacteria for 30 years and produces active ingredients for cosmetic, nutraceutical and health nutrition. The main products are the Spirulysat, a liquid extract from *Arthrospira* obtained by a cold-water extraction process and a liquid extract from *Chlorella vulgaris*. AlgoSource Technologies is a research and consultancy company specializing in consulting for culture engineering, including the recovery of factory effluents for an industrial ecology, and for the biorefinery for the application of microalgae in several markets.

The GEPEA is a laboratory part of Nantes Université composed of 5 research teams, MAPS (Matrices et Aliments, Procédés, propriétés, Structure, Sensoriel/Food matrix, process, properties, structure and sensorial), TEAM (Traitement Eau Air Metrologie/Air/water treatments and metrology), OSE (Optimisation, Système, Energie/Optimization, System, Energy), VERTE (Valorisation Energie/matière

des Résidus et Traitement des Emissions/Energy and mass valorization of residus and emission treatment) and BAM (Bioprocédés Appliqués aux Microalgues/Bioprocess Applied to Microalgae). This thesis involves the team BAM in Saint-Nazaire. This team is focused on the optimization of photosynthetic reaction, the development, optimization and control of culture and biorefinery processes and the operation unit integration for optimized industrial exploitation in the context of industrial ecology. Several theses have been realized on microalgae biorefinery, especially on bead milling and membrane filtration and this thesis aims at improving the knowledge in microalgae biorefinery.

1.2 The objectives of the PhD and the global strategy

The objective of the PhD is to develop a process to produce protein-enriched extracts with low color and the general strategy is presented in figure 1.2. Three microorganisms were considered: *Tetraselmis chui*, *Chlorella vulgaris* and *Arthrospira platensis*. It has been chosen to deepen the process development on *Tetraselmis chui* and then to compare the obtained results with the two other species. The biomass processing to produce protein isolates consisted of mild cell disruption, fractionation, concentration, purification and drying. The drying of the protein isolates was performed by AlgoSource before sending to partners but not studied in this work. The processes selected in the ProFuture program for cell disruption are bead milling and pulsed electric fields to be compared with a simple thawing of the biomass. The isoelectric precipitation and membrane processes have been considered to concentrate and purify of the extracted proteins. Several criteria were fixed by the ProFuture project: the proteins that aimed to be extracted in our case were water-soluble proteins, the dry extract should be mostly white with an improved purity and digestibility compared to the initial biomass.

This manuscript is divided into 6 chapters. Chapter 1 is the present introduction. Chapter 2 is a bibliographic synthesis providing relevant backgrounds information on the studied strains and more specifically *Tetraselmis chui*, on the nature and location of proteins in microalgae, on microalgae cell disruption and separation processes useful for concentration and purification. In Chapter 3 the general materials and methods used for the PhD are described. Chapters 4 and 5 present specific materials and methods and the results devoted to the deepening of cell disruption processes and then the coupling of cell disruption and concentration/purification processes. In Chapter 6, the main conclusions of this work are summed up and the perspectives are drawn.

In Chapter 4, performances of the cell disruption techniques on *Tetraselmis chui* (bead milling and pulsed electric fields) are evaluated and compared to a simple thawing. The results are compared with the same processes to two other species (*Chlorella vulgaris* and *Arthrospira platensis*) for comparison. The performances of bead-milling are firstly deepened and the impact of the physiological state of

Tetraselmis chui on the cell disruption performances is highlighted. This original approach has been published in a dedicated scientific article in *Algal Research*: “Extraction of soluble proteins by bead milling from *Tetraselmis chui* in two different physiological states”, Hugo Mear et al., *Algal Research*, volume 74, 103180, 2023 (Mear et al. 2023). In a second part, the pulsed electric field process is studied. Finally, the selection of the cell disruption method is discussed, to produce protein extracts before the following concentration and purification steps.

Chapter 5 concerns the evaluation of isoelectric precipitation and membrane filtration to concentrate and purify proteins. After the selection of the cell disruption method depending on the physiological state, a comparison of isoelectric precipitation and microfiltration/ultrafiltration was performed at laboratory scale. The most promising coupling between cell disruption and concentration/purification was scaled up at pilot scale and semi-industrial scale to produce fractions for the ProFuture program partners. Finally, the choice of the coupling of cell disruption and separation is discussed versus the biomass physiological state and the objective of product quality.

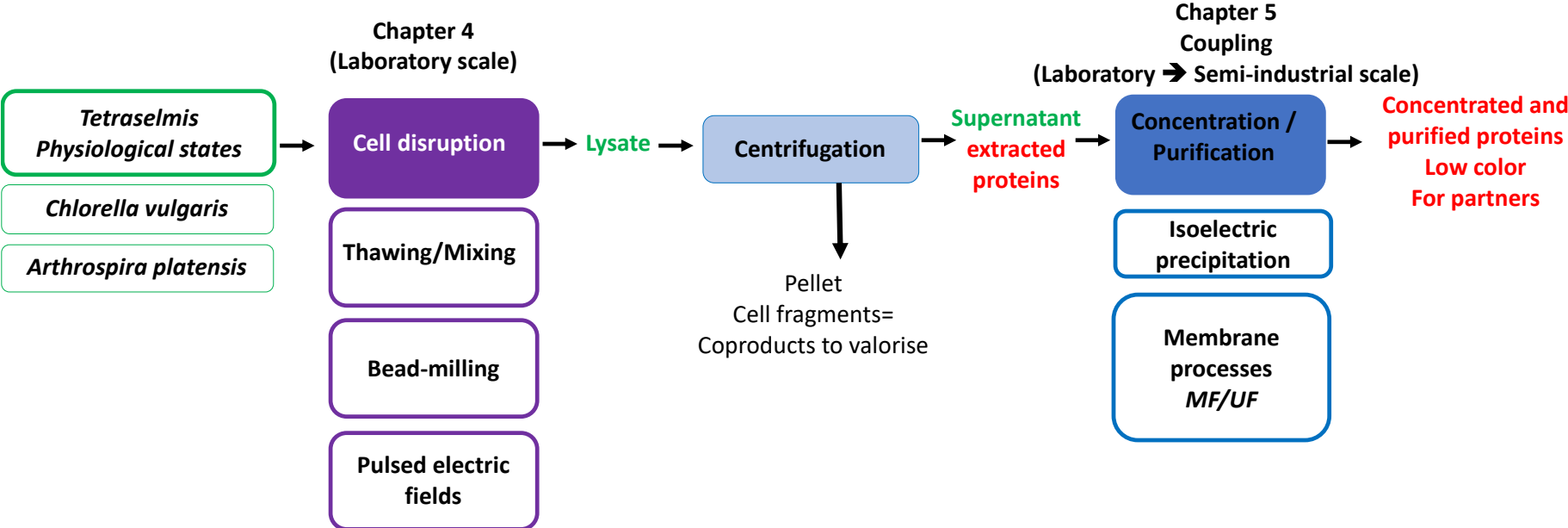


Figure 1.2 General strategy for the production of protein isolates and PhD related results chapters

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Chapter 2 Literature review

The objective of the PhD is to produce a protein-enriched extract from two microalgae, *Tetraselmis chui*, *Chlorella vulgaris*, and one cyanobacteria, *Arthrospira platensis*. In a first part, the microorganisms are presented, with a focus on *Tetraselmis chui*, the main strain of this work. In a second part, the nature, and properties of the proteins to be extracted are presented, and the main Profuture specifications and the constraints they generate on the production of isolate. The localization of the proteins in the microorganisms is detailed. Then the main processes selected to break the cells and access the intracellular compounds, bead milling and pulse electric fields, are presented. Following the process flow chart, the clarification by centrifugation is shortly explained before isoelectric precipitation and membrane filtration principles. For each process, the existing application on microalgae for protein purification is discussed. Finally, based on the literature review, the experimental strategy is drawn.

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2.1 Microalgae and cyanobacteria

2.1.1 General definition

Microalgae are unicellular photosynthetic micro-organisms belonging to the Eukaryotes and living in freshwater or saline environment while cyanobacteria are unicellular microorganisms belonging to the Prokaryotes. They are able to perform photosynthesis and are the base of the food web in aquatic environment. Microalgae have a rich biodiversity, with 40,000 to 50,000 described species over an estimation of 800,000 existing species (Barra et al. 2014). Of these species, only few are cultured in Europe and several methods of culture exists.

2.1.2 Trophic modes

While cyanobacteria are nearly always produced in photoautotrophy, often in solar condition, the cultivation mode of microalgae depends principally on the trophic mode chosen.

Several microalgae, such as *Chlorella* can use different types of carbon to grow because of their different trophic modes:

- Photoautotrophy, with the consumption of CO₂ and light to produce biomass and oxygen by photosynthesis. Two parts are present in photosynthesis, light-dependent reactions, and light-independent reactions. The light-dependent reactions consist of light harvesting by pigments in the thylakoid membranes to produce energy as ATP and reduced cofactor. The light-independent reactions consist of the fixation of CO₂ by the rubisco and the use of ATP and the reduced factor to produce carbohydrates. The algae are either cultivated in solar condition or with artificial light. In Profuture, *T.chui* and *A.platensis* are produced in photoautotrophy in solar condition.
- In heterotrophy, with the consumption of organic carbon (*e.g.* glucose, glycerol, acetate) by respiration, fermentation, or both. The algae are produced in fermentors. *Chlorella vulgaris* is cultivated in heterotrophy in this work.
- In mixotrophy, the two modes presented above are possible. The culture of some microalgae is possible in mixotrophy and is more and more studied but this mode is not used to produce the biomass treated in this work.

2.2 *Tetraselmis*

2.2.1 Presentation of *Tetraselmis*

Tetraselmis sp. is a unicellular microalga, part of a wide genus comprising very different species (>30). Most species are marine, either planktonic (floating in the ocean) or benthic (living on the seafloor), but several species are found in brackish water and freshwater such as *T.cordiformis* (John et al. 2002). Previously called *Platymonas*, *Tetraselmis* has a wide diversity including non-stalked species such as *T.suecica*, *T.chui* or *T.indica* and stalked species such as *T.marina* (Boraso 1975; Norris, Hori, et Chihara 1980). Specie *T.convolutae* is also a facultative endosymbiont of *Symsagittifera roscoffensis*, a flatworm in the tidal zone (Dupont, Moya, et Bailly 2012). A division of the genus in subgenus has been suggested based on the pyrenoid ultrastructure and *T.chui* belongs to the subgenus *Parviselmis* along with *T.levis*, *T.suecica*, *T.alacris*, and *T.striata* (Hori, Norris, et Chihara 1986).

Tetraselmis sp. is valorized industrially and constitutes the 4th most produced microalgae in Europe, with 5 tons of dry matter, behind *Chlorella* (82 tons), *Haematococcus pluvialis* (66 tons) and *Nannochloropsis/Microchloropsis* (21 tons) (Araújo et al. 2021). The species *T.suecica* and *T.chui* are widely cultivated in aquaculture to feed zooplankton, crustaceans and fish larvae, as well as to feed bivalve and mollusks and crustacean larvae (Michels et al. 2016; Vizcaíno et al. 2016; Sørensen et al. 2023) More recently, *T.chui* has been approved for food consumption by EFSA, enhancing the interest in this specie for other applications (Nunes et al. 2020).

2.2.2 Morphology and Life Cycle

2.2.2.1 General Characteristics

Tetraselmis chui is described as a compressed cell, elliptical to obovate being from 10-12 µm to 12-16 µm long and 7 to 10 µm broad (Hori, Norris, et Chihara 1986). The pyrenoid is irregular and small, being not larger than 1.1 x 2 µm and the specie possesses a large eyespot apparatus, a photoreceptive organelle, of 1.3 to 2.5 µm and 4 flagella (Hori, Norris, et Chihara 1986). This specie has been described in tide pools and estuaries in Europe and North America. No sexual reproduction has been observed in species from this genus (Norris, Hori, et Chihara 1980).

2.2.2.2 Life Cycle

The life cycle of *T. chui* has not been described in detail in literature to our knowledge, so the life cycle of different species of non-stalked *Tetraselmis* is described instead. *Tetraselmis* has at least three phases in its life (M. Arora 2016): a flagellate stage, a non-motile vegetative stage and a third stage, uncommon, as an aflagellate “cyst” with a thick cell wall. The most common phases are the first two phases and the term “cyst” is debated and concerns a thick-walled phase that is uncommon compared

with the other two phases (Norris, Hori, et Chihara 1980). The vegetative, non-motile phase was considered as the most dominant phase in some species under specific environmental conditions. In this phase, new walls are developed and the old walls cast-off and accumulated as concentric rings around the cell for non-stalked algae (Norris, Hori, et Chihara 1980).

2.2.2.3 Cell Wall

Tetraselmis has a cell wall, called theca that is considered a transition between microalgae with scaly and rigid cell walls. The theca is synthesized by the extracellular fusion of non-mineralized star-shaped scales synthesized in the Golgi apparatus (B. Becker, Marin, et Melkonian 1994). The rigid cell wall, called theca, covers the entire cell, including the pit. At the base of the pit a slit is present, from which four flagella emerge (Baudalet et al. 2017). *Tetraselmis* theca can contain several layers depending on the specie, with for example a double-layered theca (e.g. *T. striata*) and complex multilayered theca (e.g. *T. convolutae*).

The theca is mostly composed of carbohydrates and the theca of *T. striata* and *T. suecica*, close species from *T. chui* has mainly acidic polysaccharides, composing 82% of the dry weight of the cell wall (Burkhard Becker, Melkonian, et Kamerling 1998; Kermanshahi-pour et al. 2014).

The most important monosaccharides are:

- 2-keto-sugar acids 3-deoxy-manno-2-octulosonic acid, also called Kdo representing 54-60% molar of total carbohydrates in the cell wall of *T. striata* and 5% of the dry matter of *T. suecica* (Burkhard Becker, Melkonian, et Kamerling 1998; Kermanshahi-pour et al. 2014). Kdo is present in bacteria in LipoPolySaccharide (LPS), also called endotoxin, and in capsular polysaccharides (Orbe et Claesson 1989). This carbohydrate is also present in plants and algae, although its role is unclear. It is commercially available but is more than 2 million times more expensive per mole than glucose (Camci-Unal et al. 2012). This carbohydrate does not induce an endotoxic response and can be consumed since the response comes from lipid A and not from Kdo (Stewart, Schluter, and Shaw 2006). The enzymes involved in its synthesis are candidates as antibacterial compounds against Gram-negative bacteria (Baasov, Tkacz, et Belakhov 2001) and this compound can be interesting for research on carbohydrate-based therapeutic agents, vaccines, and diagnostic tools (Mi et al. 2017).
- 3-deoxy-5-O-methyl-manno-2-octulosonic acid, also called 5OMeKdo, representing 4% molar of the total carbohydrate in the cell wall of *T. striata* but not present in *T. suecica*.
- 3-deoxy-lyxo-2-heptulosaric acid or Dha, representing 6-8% molar of total cell wall carbohydrates of *T. striata* and 17% molar of total cell wall carbohydrates of *T. suecica*.

There are also galacturonic acid (20% molar of *T.suecica*), calcium (4%_{DM cell wall}), sulfate (6%_{DM cell wall}) and a little proportion of proteins (4%_{DM cell wall}) (Burkhard Becker, Melkonian, et Kamerling 1998; Kermanshahi-pour et al. 2014). The proteins in the cell wall are mainly constituted of a hydroxyproline glycoprotein having a molecular weight of 15.5 kDa.

Cell wall shed accumulates in the culture medium after cell division into daughter cells (Burkhard Becker et al. 1991). It accumulates in the form of "parchment/rolls" for *T. chui* (B. Becker, Marin, and Melkonian 1994). These objects can be several micrometers long and 1 micrometer wide.

2.2.3 Global Composition

2.2.3.1 Proteins

Tetraselmis chui has a protein content between 35% to 56.4% in standard culture conditions (Brown 1991; Khatoun et al. 2018). A Nitrogen deprivation can decrease the protein content down to 10% of the dry matter, which is linked with an increase in carbohydrate from 10% to more than 50% (Bondioli et al. 2012; Imma Gifuni et al. 2018). The protein content can also vary, when cultivated in solar conditions, with the harvesting time. The protein content increases during the night and is maximum at 6h-9h and decreases during the day with differences between day and night that can represent more than 10% of the total dry matter (Michels et al. 2014). This can be explained since the photosynthesis produces carbohydrates that are converted into proteins during the night. Moreover, the protein content was lower when the light was high (10 mol.d⁻¹) and the reverse when the light was low (3 mol.d⁻¹).

Overall, *Tetraselmis* is a good source of amino acids and especially aspartate and glutamate, as seen in Figure 2.1.

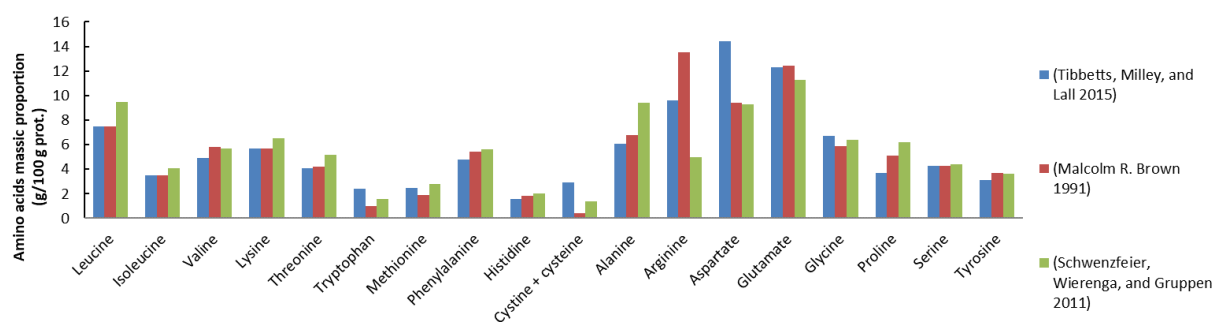


Figure 2.1 Amino acid composition of *Tetraselmis* (Brown 1991; Schwenzfeier, Wierenga, et Gruppen 2011; Tibbetts, Milley, et Lall 2015). The first 9 amino acids in the Figure, from leucine to histidine, are essential/indispensable and must come from the diet.

A high amount of glutamate is interesting because this amino acid is responsible for the umami taste, one of the five basic tastes (Kurihara 2009). The umami taste is also present in widely consumed products such as meat, fish, shellfish, and mushrooms.

The amino acids can be classified according to their side chain properties into polar, non-polar, basic, and acid, their percentage in *Tetraselmis* is shown in table 2.1.

Table 2.1 Composition of proteins from *Tetraselmis* in polar, non-polar, basic, and acid amino acids

Amino acid group	Polar	Non polar	Basic	Acid
(Tibbetts, Milley, et Lall 2015)	14.4	42.1	16.9	26.7
(Brown 1991)	12.6	42.9	21	21.8
(Schwenzfeier, Wierenga, et Gruppen 2011)	14.6	51.3	13.5	20.6

The proportion of acidic amino acids, aspartate and glutamate, is 23% on average, which is higher than in most proteins, with an average of 11.5% (Creighton 1993). The proportion of basic amino acids is also higher in *Tetraselmis* than the average of 13.6%. This knowledge could be useful to optimize extraction processes, even if it is only a general approach.

The amino acid nutritional quality can be evaluated by different ways. The Essential Amino Acid Index (EAAI) represents the geometric mean of the ratios of each essential amino acid in a protein in comparison with a reference protein, such as eggs. A high-quality protein has a EAAI>0.95 and a good protein quality has a score over 0.86. The EAAI in *T.chui* was good with 0.89 (Qazi, Ballance, Kousoulaki, et al. 2021; Tibbetts, Milley, et Lall 2015).

2.2.3.2 Carbohydrates

The carbohydrate content in *Tetraselmis* can vary from 7 to 50%, depending on the culture conditions (B. Becker, Marin, et Melkonian 1994; Schwenzfeier, Wierenga, et Gruppen 2011; Bondioli et al. 2012). More information on *Tetraselmis* polysaccharide characteristics can be found in literature (Brown 1991; Kermanshahi-pour et al. 2014).

The main carbon storage in *T. chui* is starch, accumulated as granules. It can be particularly abundant in certain conditions, with an accumulation of total carbohydrates of up to 50% of the dry matter (Imma Gifuni et al. 2018). The ratio starch over carbohydrates is included between 0.5 and 0.9 in *T.suecica* (Postma et al. 2017).

The starch content can increase when *Tetraselmis* is cultivated under low salinity (Yao et al. 2013; Trovão et al. 2019). A possible explanation of these authors is that the cells must increase their osmotic potential to reach an equilibrium with the surrounding medium. The cells limit the synthesis of small

osmolytes and focus on starch synthesis since it is osmotically inert because of its crystalline nature and poor water solubility.

2.2.3.3 Lipids

The lipid content in *Tetraselmis* is usually comprised between 7 to 22% (Rodolfi et al. 2009; Garcia et al. 2018; Trovão et al. 2019) but can be up to 50% for specific strains. In *T. chui* and *T. suecica*, the main fatty acids seem to be C16 with 35–37% of total fatty acids and C18s (C18, C18:1, C18:2, C18:3) with 37–43% of total fatty acids (8–12%) (Lim et al. 2012).

The lipid content of *Tetraselmis* after a nitrogen stress can increase depending on the strain and species from this genus have been considered for biofuel production (Bondioli et al. 2012; Lim et al. 2012; Pereira et al. 2016; Fon-Sing et Borowitzka 2016). Moreover, low salinity was associated with a lower total lipid content in *Tetraselmis* genus (Khatoon et al. 2014; Dammak et al. 2016; Trovão et al. 2019).

2.2.3.4 Minerals

The mineral compositions of *T. chui* have been analyzed and is presented in Table 2.2.

Table 2.2 Mineral composition of *T. chui* (Tibbetts, Milley, et Lall 2015)

Ca	P	Mg	K	Na	S	Cu	Fe	Mn
2.99%	1.46%	0.43%	1.86%	0.89%	1.38%	0.10 %	1.77%	0.19%

This specie has a very high calcium content in comparison with other strains. This can be linked to a biomineralization capacity in *T. chui* with the formation of intracellular mineral inclusions of hydrated amorphous calcium carbonates enriched in strontium, called micropearls (Martignier et al. 2018). These micropearls had a size of 0.7 μm and were elongated in the two strains of *T. chui* studied in a standard culture medium. They were mostly aggregated at one side of the cell at the apical side of the cell, near the flagella, and images after light microscopy using Nowarski Differential Interference contrast and scanning electron microscope are shown in Figure 2.2 (Martignier et al. 2020).

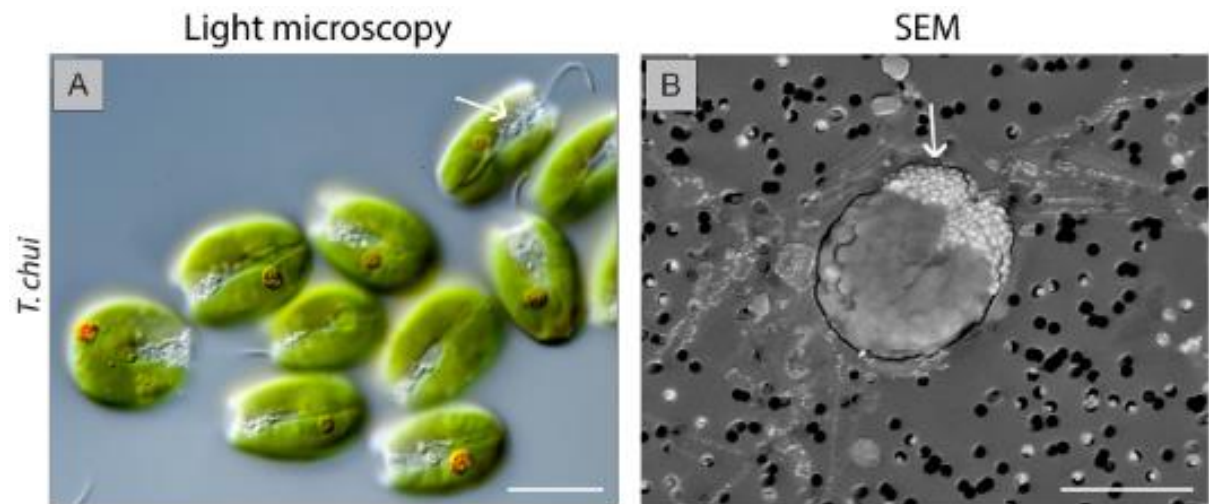


Figure 2.2 Micropearls of *T. chui* observed at light microscopy with DIC and at Scanning electron microscopy (SEM) with scale bars of 10 μm (Martignier et al. 2020).

The micropearls were present in 40% to 93% of the species of *T. chui* and the strontium enrichment was significant (up to 20 $\text{mg}_{\text{St.}}\cdot\text{g}_{\text{DM}}^{-1}$) and can take place in a few hours (Martignier et al. 2018; Segovia-Campos et al. 2022). These pearls may have different physiological functions, such as a help to sedimentate the cells or a calcium reserve and they may be used as a valid criterion to differentiate different phylogenetic groups (Martignier et al. 2018; 2020). More information on the micropearls can be found in the work of Martignier et al. (Martignier et al. 2018; 2020).

2.2.3.5 Polyphenols

Phenols are a class of chemical compounds containing one or more hydroxyl groups bonded to an aromatic hydrocarbon group. Polyphenols are secondary metabolites containing at least two phenol groups and are divided into 4 families, the flavonoids, phenolic acids, stilbenes and lignans. They are an important class of natural antioxidants, but they can react with proteins and complicate their extraction. The presence of polyphenols is one of the main obstacles to the use of rapeseed and sunflower cake proteins, with a polyphenol composition between 1-2% and 1-4% of the delipidated dry matter respectively (Laguna 2019).

The amount of phenolic compounds seems to vary in *Tetraselmis* with values of 3.74 mg gallic acid equivalent(GAE)/g DM in *Tetraselmis.spp* (Goiris et al. 2012), 10 mg GAE/g DM in *T.suecica* (Batista et al. 2017) and >20 mg GAE/g DM in *T.chui* (Tibbetts, Milley, et Lall 2015).

Polyphenols of *T. chui* were identified as capsaicin, cinnamic acid, 4-vinylphenol, dihydro-p-coumaric acid, sinapic acid and benzoic acid (Sørensen et al. 2023). Capsaicin was the main polyphenol after cell disruption, representing 16.4 mg/g DM. This quantity of polyphenols is significantly below the polyphenol content of rapeseed and sunflower and may less hinder protein extraction.

2.2.4 *Tetraselmis*, interesting for large-scale cultivation

The cultivation of *Tetraselmis* is very promising economically and environmentally at large scale in comparison with other microalgae.

2.2.4.1 High Biomass Productivity

It is a fast-growing algae since, for example, in photoautotrophy, 3 strains of *Tetraselmis* were among the most interesting for biomass production among the 30 tested, with biomass productivity of 0.28-0.32 $\text{g}_{\text{DM}}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ in flasks at 100 μmol Photosynthetically Active Radiation $\text{photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Rodolfi et al. 2009). The biomass productivity was also the highest for *Tetraselmis sp.* among four tested in another study (Huerlimann, de Nys, et Heimann 2010).

2.2.4.2 Multiple possible trophic modes

Species of *Tetraselmis* can grow in heterotrophy and in mixotrophy. The cultivation of *T.suecica* in heterotrophy was achieved successfully and a fermentor of 40 m^3 was operated (J. D. Day, Edwards, et Rodgers 1991). The cultivation in heterotrophy leads to a decrease in protein content from 66% in photoautotrophy to 12% in heterotrophy compensated by an increase in carbohydrates from 20% to 63%. The cultivation of *T. suecica* and *T. chui* in mixotrophy was also realized successfully (J. G. Day et Tsavalos 1996; Lu et al. 2016; Penhaul Smith et al. 2020).

2.2.4.3 High scale culture already conducted

The production of *Tetraselmis* at high scale has already been demonstrated in several conditions. *Tetraselmis* is produced at industrial scale on biofilm (Delran et al. 2023). In photoautotrophy, a culture of six 40,000 L semi-open raceway (240,000 L) was maintained all year long with similar productivity (W.-K. Lee et al. 2021). An industrial production of *Tetraselmis sp. CTP4* was also accomplished in 35,000 L and 100,000 L tubular photobioreactors (Pereira et al. 2018). An areal productivity of 20.3 $\text{g}_{\text{DM}}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ and a volumetric productivity of 0.08 $\text{g}_{\text{DM}}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ was obtained at the highest scale. The efficient production at high scale is promising because it can be a limiting point for several species. The shear stress in PBR for example can be problematic in certain species due to pump use (Jaouen, Vandanjon, et Quéméneur 1999; Wang et Lan 2018). However, *Tetraselmis* was able to withstand high shear stress in comparison with the other species studied, even in a centrifugal pump, with no difference in net volumetric productivity, but with a probable change in life cycle stage (Michels et al. 2016). The culture of *Tetraselmis sp.* in 25,000 L ponds was also accomplished and the average areal productivity was similar when the culture volume was maintained or not, leading to an increase in salinity in the latest case (Das et al. 2016).

2.2.4.4 Salinity tolerance, interesting for open pond culture and media recycling

The use of marine species is interesting to save fresh water and helps to reduce the water footprint of microalgae cultivation significantly (Yang et al. 2011). *Tetraselmis* species are also euryhaline, being able to resist very high variation of salinity such as from 20 to 60 g.kg⁻¹ for *T. suecica* (Pugkaew et al. 2019), until 1 to 100 g.kg⁻¹ for *T. striata* CTP4 (Pereira et al. 2016), between 30 to 110 g.L⁻¹ for *Tetraselmis* spp. (Fon-Sing et Borowitzka 2016), between 0.6 to 70 g.kg⁻¹ for *T. viridis* (Strizh, Popova, et Balnokin 2004) and from 3 to 31 g.kg⁻¹ for *T. jejuensis* (Hyung et al. 2021). This is a significant advantage since salinity can vary significantly with evaporation or precipitation and even more if part of the culture medium is recycled. It can affect the culture by changing the species to more halotolerant species or decrease the growth of the algae of interest (Fon Sing et al. 2014). Moreover medium recycling can also lead to a precipitation of salts such as calcium, causing a loss of other minerals such as iron and phosphorus (Shimamatsu 2004). The possibility to cultivate *Tetraselmis* in recycled media was successful at pilot scale (25 m²) in open raceway ponds for 5 months and even led to a higher biomass productivity (Fon Sing et al. 2014). It was also realized in open ponds at larger scale (120,000 L) in hypersaline water (Erkelens, Ball, et Lewis 2014).

2.2.4.5 Salinity tolerance, interesting to resist contamination

Tolerance to salinity variations can also be used to fight against culture contamination since the contaminants may not be resistant to high salinity variation. This was used with success for long-term large-scale cultivation to get rid of most of the contaminants (W.-K. Lee et al. 2021; Pereira et al. 2018). This is key for cultivation to enhance the biomass productivity but also to produce food respecting the microbiological norms. It is one of the major aspects limiting the large-scale culture of a wide variety of species. The algae cultivated at large scale withstand extreme conditions with a decrease in competition, such as high alkalinity (*Arthrospira*), high acidity (*Galdieria sulfuraria*), high salinity (*Dunaliella*) or with very high growth rate (*Chlorella*). *Tetraselmis* with its ability to withstand high salinity shifts and its good growth rate can also be cultivated successfully. *Tetraselmis* is even able to grow on saline industrial streams with liquid digestate as a nutrient source (Parsy et al. 2023). Another interesting aspect of withstanding high salinity variation is the possibility to lower the salinity before harvesting to decrease the salt content in the final biomass for downstream processes without cell lysis and to accelerate freezing. However, salinity modification can modify the cell physiology, modifying its composition (Strizh, Popova, et Balnokin 2004; Yao et al. 2013; Pugkaew et al. 2019).

2.2.4.6 Harvesting possible at low cost

Harvesting is a major cost for microalgae cultivation, representing up to 30% of the total cost depending on the process (A. I. Barros et al. 2015). *Tetraselmis* are large cells in comparison with other species and some species can settle naturally such as *T. striata* CTP4 (Pereira et al. 2016). A pilot scale

low-cost sedimentation harvesting by settling has been achieved on this specie with a 93% culture medium removal and either a highly concentrated culture at 20 g.L⁻¹ and a wet microalgal paste at 270 g.L⁻¹ (Trovão et al. 2019). Settling can also be induced after a stress such as a nutrient starvation (Lim et al. 2012) or a UV-C treatment (Sharma, Li, et Schenk 2014), maybe due to a life cycle stage modification from a flagellated cell to a settled cell. *Tetraselmis* can also be cultivated in biofilm, allowing easier harvesting (Delran et al. 2023).

2.2.5 Applications

The use of *Tetraselmis* for a wide range of applications has been studied, food applications will be the focus on this literature review since the final application of our work will be for food.

2.2.5.1 *Tetraselmis* as single-cell protein

2.2.5.1.1 Safety

Dried *Tetraselmis chui* by lyophilization is accepted for food consumption in the European Union as Novel Food for sauces (≤ 250 mg of dried *T. chui* per day), special salts ($\leq 1\%$) and condiments (≤ 250 mg per day). It is also considered as safe for food applications in Canada since 2021 and is sold for food consumption in the US since 2009. No pesticides, toxins, or heavy metals, that were looked for, were found in dangerous concentrations in the powder. This can open the way to an extension of these authorizations to other *Tetraselmis* species. Moreover, scientific studies can help, such as with the industrially produced *Tetraselmis striata* CTP4, for which the nutritional potential and toxicological evaluations were realized. No pathogenic bacteria, mycotoxins, cyanotoxins, heavy metals, polycyclic aromatic hydrocarbons and pesticides were detected in harmful concentrations. With the macromolecule composition and safety, *Tetraselmis* can become an interesting food product. However, its digestibility needs to be assessed since microalgae can have a resistant cell wall, limiting their absorption as single cell proteins.

2.2.5.1.2 Digestibility

The digestibility of *Tetraselmis* was assessed by different studies. An in vitro digestibility was carried out by an enzymatic method using pepsin and pancreatin on dried *T. suecica* and organic digestibility of 52% and 62% were found for the normal and starved algae (Niccolai et al. 2019). In comparison, the digestibility of *Arthrospira* was close to 90%. The in vitro protein digestibility (IVPD) of *T. suecica* was 62% and 65% for the normal and starved algae (Niccolai et al. 2019). Another study showed a IVPD of 82.5% after digestion of *T. chui* by trypsin, chymotrypsin and peptidase and an increase was observed when the lipids were extracted (Tibbetts, Milley, et Lall 2015).

The cell disruption before digestibility seems to be efficient to increase digestibility in microalgae with resistant cell wall. For example, the in-vivo digestibility of *T. chui* by Atlantic salmon was improved from 11 to 42% in dry matter, from 53% to 69% in proteins and from 9% to 52% for lipids after bead milling (Sørensen et al. 2023). This was consistent with other studies on different microalgae such as *Chlorella*. The Protein Digestibility Corrected Amino Acid Score (PDCAAS) of *Chlorella vulgaris* and *Chlorella sorokiniana* improved from 0.63 and 0.64 respectively to 0.77 and 0.81 after mechanical cell disruption by High-Pressure Homogenization.

More information regarding digestibility can be obtained in the review of Van De Walle et al. (Van De Walle et al. 2023).

2.2.5.2 *Tetraselmis* as ingredient

The incorporation of *Tetraselmis* as an ingredient has been tried in several food products:

- Crackers were enriched with *Tetraselmis suecica* at 2 and 6% (w/w) by replacing wheat flour (Batista et al. 2019). The In Vitro Dry Matter Digestibility (IVDMD) of the crackers was not statistically different from the control. The 2% enriched crackers had low sensory analysis score in smell, color and taste compared to the control.
- Breads were enriched with 1%, 2% and 4% of *T. chui* and, as in the crackers, a strong darkening effect was seen when algae were added, even at low concentration due to the high chlorophyll content (Nunes et al. 2020). The sensory analysis of the bread showed similar acceptance at 1%, but lower at 4%, compared to the control due to the taste, the smell, and the color.
- Cookies were made with 2 and 6% *T. suecica* (w/w) and no difference in digestibility was found with the control but the cookies were less appreciated (Batista et al. 2017).
- Broccoli soup was also made from *Tetraselmis* from 0.5% to 2% but the acceptability index was below the control index and the purchase intention was low (Lafarga 2019).

The acceptability and the digestibility of *T. chui* in bread were improved by bead milling and drying (Tc), and when the dried powder was treated with ethanol to remove the pigments (TcEt), as seen in Figure 2.3 (Qazi, Ballance, Uhlen, et al. 2021).



Figure 2.3 Bread appearance with different addition of milled *T. chui* (Tc) and milled *T. chui* treated with ethanol (TcEt) (Qazi, Ballance, Uhlen, et al. 2021).

The extraction with ethanol improved significantly the sensory properties of the bread supplemented with *T. chui* on the smell, appearance and color, even at high addition (12%) (Qazi, Ballance, Kousoulaki, et al. 2021). However, lower protein digestibility was observed after ethanol treatment due to a reduced protein solubility. A need for appropriate microalgae species for food as well as the development of pre-processing methods were underlined to improve consumer acceptance.

2.2.6 Conclusion

Tetraselmis, in particular *T. chui*, is a promising specie being the 4th produced microalgae in Europe. It has a complex life cycle not fully identified in all species, including *T. chui* and not often mentioned. The cell wall is a theca composed of original acidic polysaccharides. *Tetraselmis* has a good protein content, with interesting amino acids. It can also be rich in carbohydrates and lipids, depending on the culture conditions. This alga is excellent for high scale culture in a wide variety of system production and low biomass price may be obtained in the future, enhancing its applicability in a wide range of sectors, including food. In food, its digestibility is better when disrupted and its acceptability needs to be improved, especially taste and color.

2.3 Comparison with other species

2.3.1 *Chlorella vulgaris*

2.3.1.1 General Description

Chlorella vulgaris is a unicellular freshwater microalga belonging to the phylum Chlorophyta. It is one of the most extensively studied species due to its wide distribution and diverse range of applications. *Chlorella sp.* has been present on earth since approximately 2.5 billion years ago (Safi, Zebib, et al. 2014). In 1890, Martinus Willem Beijerinck, a Dutch researcher, reported his discovery of *Chlorella*

vulgaris recovered from a small pond near Delft, with notably first microalgae with well-defined nucleus (Ru et al. 2020). This microalga is shaped resembling a sphere with a diameter of 2 to 10 μm and possesses organelles like those in plant cells (Ponnuswamy, Madhavan, et Shabudeen 2013; Borowitzka 1997). Since its discovery, *Chlorella vulgaris* gained research attention for its high nutritional value, being rich in proteins, vitamins, and essential fatty acids (Batista et al. 2017). However, multiple species have been called *C.vulgaris* because species were identified by morphological observations before and care must be taken since the taxonomy has evolved along the years with the apparition of new molecular biology techniques (Champenois, Marfaing, et Pierre 2015).

Chlorella vulgaris was among the first microalgae considered for industrial exploitation thanks to its high photosynthetic ability and rapid growth rate under various conditions (Safi, Zebib, et al. 2014). The cell and shape of *C. vulgaris* may vary according to the stage of development, nutrient availability, and other environmental factors.

2.3.1.2 Ultrastructure

The cell wall of *Chlorella vulgaris* is rigid and composed of cellulose, providing a good protection against contamination and environment (Safi, Zebib, et al. 2014). The cell wall composition and thickness are directly affected by the trophic mode, growth phase, light intensity, and nitrogen concentration (Soto-Ramírez et al. 2021).

For instance, during auto sporangia stage in photoautotrophy, the cell walls are more fragile, with a 2 nm thin electron-dense unilaminar layer (Yamamoto et al. 2004). After, the cell wall of the daughter cells will continue to increase in thickness and rigidity, reaching approximately 17-21 nm upon maturation, where it forms a microfibrillar layer in photoautotrophy (Yamamoto et al. 2004; Yamamoto, Kurihara, et Kawano 2005). The cell wall of *Chlorella vulgaris* lacks sporopollenin (Baudeflet et al. 2017), a resistant polymerized carotenoid, considered as one of the most resistant biological materials known (Mikhael et al. 2020).

The composition and the size of the cell wall change between a growth in photoautotrophy and a growth in heterotrophy. More information on the cell wall of *C.vulgaris* can be found in (Duval et al. 2022; Demir-Yilmaz et al. 2023) in photoautotrophy, in (Soto-Ramírez et al. 2021) during nitrogen limitation and (Canelli et al. 2021) in heterotrophy.

2.3.1.3 General Composition

2.3.1.3.1 Proteins

The composition of *C.vulgaris* depends heavily on medium composition and trophic condition. For instance, under autotrophic condition with no nutrient limitation, protein is the dominating biomolecule present in microalgae (Morris et al. 2008). In *Chlorella vulgaris*, protein represents

between 42% to 58% of the biomass dry matter. On the contrary, the protein content decreases during nitrogen limitation, as nitrogen is necessary for protein synthesis. *Chlorella vulgaris* possesses high concentration of protein, and its amino acid profile is adapted for human nutrition of World Health Organisation (WHO) and Food Agricultural Organisation (FAO), since it is able to synthesize both essential and non-essential amino acids (Safi et al. 2013; Naik et al. 2010).

The protein content in heterotrophy is often lower ($\approx 20\%_{DM}$) than in autotrophy (A. Barros et al. 2019) but mutants were developed with higher protein and low chlorophyll contents (Schüler et al. 2020).

2.3.1.3.2 Carbohydrates

The main polysaccharides in *C.vulgaris* are starch and cellulose. Cellulose acts as a structural and protective fibrous barrier in the cell wall while starch is present in the chloroplast as energy storage. During growth, starch will be broken down to glucose, hence during optimum autotrophic cultivating conditions, total carbohydrate content in *Chlorella vulgaris* is low. However, during nitrogen limitation, the concentration can reach up to 55% of its dry weight (Choix, de-Bashan, et Bashan 2012). The carbohydrate content is increased during a culture in heterotrophy compared to photoautotrophy (N. Arora et Philippidis 2021).

2.3.1.3.3 Lipids

Lipids in *Chlorella vulgaris* are synthesized in the chloroplast and are mainly present in the cell wall and the membranes. Lipids are composed of glycolipids, waxes, hydrocarbons, phospholipids, and small amount of free fatty acids (Hu et al. 2008). Under optimal growth conditions, the lipid content of *C. vulgaris* was reported to range from 5-40%_{DM} and under stress (light or nutrient limitation) it can reach up to 58%_{DM} (Walsby 1995; Mata, Martins, et Caetano 2010). The lipid content is also higher in heterotrophy compared to photoautotrophy (N. Arora et Philippidis 2021).

2.3.2 *Arthrospira platensis*

Arthrospira platensis, also called with the commercial name “spirulina”, is a gram-negative cyanobacterium traditionally used as human food for centuries such as in the lake Tchad (Masojídek et Torzillo 2008). *A.platensis* grows in very alkaline lakes at pH 9 to 10 which facilitates its cultivation in open ponds due to the low contamination at these extreme pH. the optimal growth temperature of this specie is between 35°C to 38°C and is generally cultivated in greenhouses in Europe (Gershwin et Belay 2007).

This is a filamentous cyanobacterium with cells having a diameter of 8 μm (Masojídek et Torzillo 2008) and are stacked in a colony called trichome usually organized as helically-coiled trichomes but sometimes organized linearly (Zhao et al. 2022). The cell wall is made of peptidoglycan (Zhao et al.

2022). This cyanobacterium is the most produced cyanobacteria and microalgae in the world for human nutrition, especially as a food supplement (>5000 t/year) (Habib 2008).

A. platensis is a promising protein source with a protein composition of up to 50-70%_{DM} (Chaiklahan et al. 2022). The main protein is a pigment, the C-phycoerythrin (C-PE), a phycobiliprotein present in the phycobilisome, as seen in Figure 2.4.

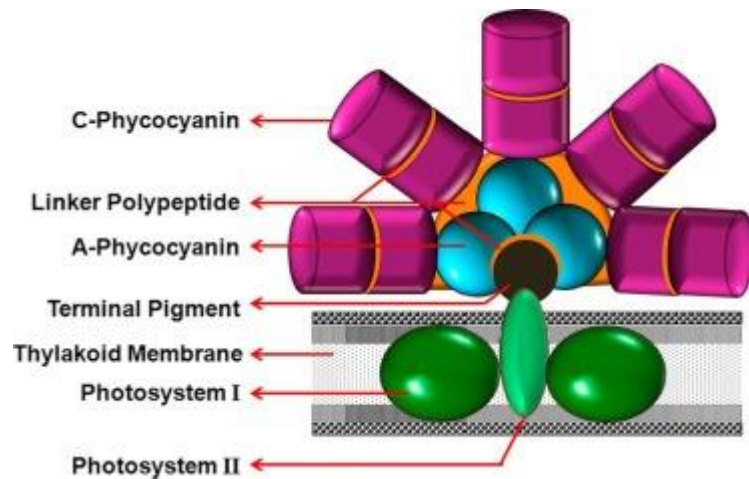


Figure 2.4 Structure of a phycobilisome (Tavanandi, Chandralekha Devi, et Raghavarao 2018)

2.4 Proteins

2.4.1 Amino acids, the building blocks of proteins

Proteins are biological macromolecules made of one or several chains of peptides, each made of a succession of amino acids linked by peptide bonds.

Amino acids are the building blocks of proteins and more than 300 different natural amino acids have been discovered, with 20 amino acids constituting the majority of the amino acids found in proteins and coded by the universal genetic code. Nineteen of these amino acids have the structure $H_2N-C_\alpha H(R)-COOH$, with different side chain R. The 20th, proline is an amino acid in which the side chain is bonded to the nitrogen atom. Apart from this specific amino acid, the carbon C_α has chirality and a majority of the amino acids in proteins have an L-form, the D-form being very rare.

Amino acids are amphoteric in aqueous solutions, and they possess an isoelectric point $pH(I)$ at which the amino acid carries no net electrical charge.

They can be classified in different groups, depending on their side chains:

- **Apolar:** glycine, alanine, proline, methionine, valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan

- **Polar:** cysteine, serin, threonine, glutamine, asparagine
- **Acid:** aspartic acid/aspartate and glutamic acid/glutamate
- **Basic:** lysine, arginine, histidine

The properties and the structure of the protein are highly dependent on the amino acid composition.

2.4.2 Proteins organized in different structure levels

Amino acids are precursors of proteins and are linked by peptide bonds to form short polymer called peptides, longer chain called polypeptides or proteins and the chain is linear and unbranched. Proteins fold into 3D structure and four different levels of protein structures are defined:

- A **primary structure**, representing the amino acid sequence, the disulfide bonds, and the post-translational transformations (methylation, glycosylation, phosphorylation). The disulfide bond joins two methionines. A post-translational modification can be an addition of a carbohydrate for example (glycosylation), the protein becoming a glycoprotein. A loss of the primary structure is called a protein hydrolysis.
- A **secondary structure**, representing the regular local conformation such as the main secondary structure, the alpha helix, and the beta sheets. The secondary structure is defined by patterns of hydrogen bonds. A loss of the secondary structure is called a protein denaturation which can happen due to different reasons such as extreme temperature or pH. It can be reversible or irreversible.
- A **tertiary structure** represents the protein folding in 3D.
- A **fourth structure** is optional and is present if several proteins, also called protein subunits, are associated. It is the case of the hemoglobin, phycocyanin, Rubisco.

2.4.3 Protein, a very diverse group

The different structure levels define the property of the protein and, while composed of the same building blocks, protein diversity is important. They can be divided by their shape, with globular, membrane, fibrous proteins as well as disordered proteins. They also have different biological functions such as enzymes, hormones, transport proteins, structural proteins, contractile proteins, storage proteins, regulatory proteins, defense proteins, etc.

For example, hemoglobin is an essential protein transporting oxygen in the body, having a quaternary structure made of 4 subunits composed of a globular protein, embedded with a heme group in humans. This protein is a globular protein soluble in the blood and has a transport function. Globular

proteins have in general buried hydrophobic amino acids, while their amino acid exposed to water are polar or charged (Yada 2017).

On the other hand, membrane proteins are part of or interact with biological membranes (Yeagle 2016) and represent between 20 to 30% of synthesized proteins in all proteomes (Krogh et al. 2001; Almén et al. 2009). They have fundamental roles in biological processes such as ion transport, signal transduction and photosynthesis. They can be classified as integral or peripheral membrane proteins. Integral membrane proteins can be subdivided into either polytopic membrane proteins (spanning the membrane multiple times, sometimes through the entire membrane becoming a transmembrane protein) or monotopic membrane proteins (exposed on one side of the membrane) (Allen et al. 2019). These proteins expose hydrophobic amino acids and are difficult to solubilize in an aqueous solution, necessitating detergents to solubilize them (Hering et al. 2020). Peripheral membrane proteins, on the other hand, interact indirectly with electrostatic or hydrophobic interactions and can be dissociated with the addition of salt in high quantity. Membrane proteins are thus very different from globular proteins and harder to solubilize in aqueous solution.

Fibrous proteins have very low water solubility and have protective and structural roles. For example, wool is made of 97% of keratin, it is also the main protein in nails, horns and hair (Sabatini et al. 2018). Silk is another example of a biomaterial made of two proteins, the glue-like sericin and the water-insoluble fibroin (Happey et Hyde 1952). In multicellular organisms, they can form a gel-like extracellular matrix that helps cells to bind together, forming a tissue, essentially made of collagen, the most abundant protein in mammals (Bielajew, Hu, et Athanasiou 2020). These proteins are very hard to solubilize in water and sometimes need very harsh extraction conditions such as alkali, acid, or enzymatic extractions, as used to produce gelatin, a protein extract derived from collagen.

The protein diversity outlines the need to know the type of proteins in the biomass as well as their localization to be able to optimize the process to extract and purify them.

2.4.4 Protein solubility

The process to extract proteins from seeds are often similar and aimed at increasing protein solubility and separate the proteins from the solid phase containing lipids and carbohydrates. Two main parameters strongly influence protein solubility, pH, and ionic strength.

2.4.4.1 pH

The most important parameter is pH since protein solubility usually follows a U curve, with higher solubilization at extreme pH due to an increase in the protein global charge. Proteins are the least soluble at a pH close to their pI, since hydrophobic interactions between the different proteins can

lead to protein aggregation, that may induce a protein precipitation. Traditionally, protein extraction relies on a pH shift, with extreme pH (usually basic) being used to solubilize a maximum of proteins. This is followed by the adjustment of the pH of the liquid to the pI of the majority of the proteins, and a solid/liquid separation, either by natural sedimentation or by centrifugation/filtration.

2.4.4.2 Ionic strength

Ionic strength is also a critical parameter for protein solubility. It is expressed in mol.L⁻¹ and is calculated by:

$$I = \frac{1}{2} \sum_i C_i z_i^2 \quad \text{Equation 2.1}$$

With C_i the molar concentration on the ion i and z_i its charge. An increase in ionic strength usually increases the solubility of proteins at low ionic strength, a phenomenon known as "salting in", whereas the solubility of proteins usually decreases at high ionic strength, a phenomenon known as "salting out". These reactions depend on the nature of the protein, the nature of the salt and the temperature (Okur et al. 2017).

2.5 Microalgal Protein Extraction

2.5.1 ProFuture Project Specifications of the Protein Extract

The choice of the cell disruption method depends highly on the objectives and several questions must be thought before choosing the appropriate operation unit since it will affect the whole process. Several criteria were fixed by the Profuture project: the proteins that aimed to be extracted in our case were water-soluble proteins, the extract should be mostly white with an improved purity and digestibility compared to the initial biomass.

2.5.1.1 Protein solubility

Protein solubility was an important request for the final extract as it is an important property affecting the technofunctional properties, the choice of the food engineering process, and finally the type of food produced (solid/liquid), etc. (Grossmann et McClements 2023). Solubility can have different definitions depending on the scientific field. It can be characterized by different methods, the relative solubility, the maximum solubility and the predicted solubility (Grossmann et McClements 2023). In bioprocess engineering and food processes, the relative solubility is the most frequent, and is defined as the protein fraction dispersed under specified conditions and not separated after a solid/liquid separation, nearly always centrifugation (Amorim et al. 2020). The other methods to characterize solubility are not presented here, but are well documented in the review of Lutz Grossmann et McClements 2023 (Grossmann et McClements 2023).

Centrifugation separation principle is based on sedimentation which can be described by Stokes law in centrifugation by assuming a creeping flow and its principle is detailed in Section 2.7.1.

In order to separate different compounds by centrifugation, volumetric mass difference and the particle diameter are the most important parameters. The particle diameter being at the power 2, it is the most significant and more information will be given Section 2.7.1 on the separation of proteins from other biomolecules. Nevertheless, particle diameter reduction is used to disperse insoluble molecules in solution as colloids to prevent their sedimentation in other applications such as heat exchanger fluids or pharmaceutical industries. For example, nanoparticle suspension of insoluble molecules such as sand or titanium dioxide ($\rho = 3,800\text{-}4,300 \text{ kg.m}^{-3}$) has been produced by “nanonization” with bead milling and HPH (Sivasubramanian et al. 2012; Silambarasan, Manikandan, et Rajan 2012; Nakach et al. 2018; Singhal et al. 2020).

To summarize, if the only criterion is solubility, its optimization relies on the fractionation of the cells in nanoparticles to avoid sedimentation and if all the proteins are dispersed in the aqueous phase, the solubility is maximal. If there is no solid phase and the whole biomass is fully solubilized, the protein yield obtained after the cell disruption and centrifugation operations is equal to 100%.

2.5.1.2 Purity

Purity was another criterion along the project to produce a product enriched in proteins. While no legal definition exists to the author knowledge, a protein concentrate is often defined as having more than 60% proteins and a protein isolate is defined as having more than 80% proteins. A high protein purity in an ingredient allows to increase significantly the protein content of the final food product with a small proportion of this ingredient in the final recipe (van der Goot et al. 2016).

The protein content of microalgae and cyanobacteria changes depending on the strain and the culture condition but is usually high in comparison with other food products (Grossmann, Hinrichs, et Weiss 2020). To summarize, it is possible to have a high protein purity and a high protein yield if the cells are completely dispersed in the supernatant and if the biomass has a high protein content. However, if the protein content in the microalgae is low (i.e. 20%), there is a need to purify proteins from the other molecules and the process becomes more complex.

2.5.1.3 Color

Another important criterion is color, especially the strong green color due to the chlorophylls. Color in microalgae comes from pigments such as carotenoids and chlorophylls. Microalgae have high chlorophyll content, with for example *C.vulgaris* having chlorophyll content from 1%_{DM} to 7%_{DM} in autotrophy (Nakanishi et Deuchi 2014; Paniagua-Michel 2015) while it is only from 0.8%_{DM} to 1.7%_{DM} in spinach leaves (Lefsrud et al. 2008). Chlorophylls can be good for nutrition and can be good

antioxidants (Lanfer-Marquez, Barros, et Sinnecker 2005; Ferruzzi et Blakeslee 2007). However, it causes a dark green color, which is one of the main drawback for microalgae consumption and integration at significant proportion in food according to sensory analysis (Chacón-Lee et González-Mariño 2010; Batista et al. 2017; Qazi, Ballance, Kousoulaki, et al. 2021).

Several ways were developed to decrease chlorophyll content in microalgae for food consumption. The growth of biomass in heterotrophy rather than with light helps to decrease the pigment content within the biomass (A. Barros et al. 2019) as in *Chlorella* produced in Profuture project. Moreover, it is possible to select mutant with low chlorophyll content, grown in heterotrophy. While GMO is forbidden in the EU, selection by chemical-induced mutagenesis is accepted and new mutants were selected with low chlorophyll content and approved for food consumption (Schüler et al. 2020). Protein extraction was planned on these strains along the Profuture project but it was not possible due to different timelines.

Finally, biorefinery can separate chlorophylls from the aqueous extract by purification operation units. Classical centrifugation is not sufficient to separate chlorophylls as they are adhered to fine particles, probably as a colloidal dispersion (Gerde et al. 2012). Solvent extraction was conducted in several studies but it uses a significant amount of solvents, can denature proteins, decreasing their solubility and can necessitate a drying step, generating a high energy consumption (Grossmann et al. 2018a; 2018b; Qazi, Ballance, Uhlen, et al. 2021; Qazi, Ballance, Kousoulaki, et al. 2021). It is also possible to process the biomass selectively to avoid the presence of chlorophylls in the aqueous phase while enriching the extract in proteins.

2.5.1.4 Circular Bioeconomy and Nutrition

The project Profuture also aimed at seeking co-products for the whole biomass and one work package was exclusively focused on this aspect in order to aim for a circular bioeconomy (Stegmann, Londo, et Junginger 2020). For co-products to be extracted, the extraction conditions are key to avoid their degradation so the use of solvents and harsh conditions with extreme pH and temperature were purposefully avoided. The nutritional quality aimed at being improved on several criteria such as digestibility or salt content. Digestibility for example is key since, as seen in Section 2.2.5.1.2, fibrous proteins such as keratin may not be digestible without extensive hydrolysis.

2.5.2 Where are the proteins from microalgae and cyanobacteria?

With the different criteria, it is important to identify and locate the main proteins and understand the cell ultrastructure to optimize protein extraction and choose appropriate operation units. Microalgae are single-cell non-specialized organisms, unlike seeds for example, specialized in embryo nourishment by storing nutrients including proteins. While in seeds, proteins are stored in protein bodies and a small variety of proteins are found, microalgae and cyanobacteria are more organized and proteins are diverse.

The nitrogen in cyanobacteria has been reported as mostly contained in phycocyanins, an accessory pigment, capturing light for photosynthesis and part of the phycobiliprotein family (Boussiba et Richmond 1980), or as cyanophycin, a pseudopeptidic polymer (Du, Li, et Zhou 2019) but this latest is not produced by *Arthrospira*. In *Arthrospira platensis*, C-phycocyanin is the main protein, constituting up to 20% of its dry matter and up to 30% of its total proteins (Chaiklahan et al. 2022). It is a water-soluble protein and is highly targeted for protein extraction from this cyanobacteria since it has interesting health properties (Akaberi et al. 2020; Fabre et al. 2022).

For eukaryotic microalgae, however, there is no known nitrogen storage as proteins and a wide protein diversity is present (Grossmann, Hinrichs, et Weiss 2020).

2.5.2.1 Classification of microalgal proteins

The presence of different protein pools was hypothesized by Suarez Garcia on *Tetraselmis* (Suarez Garcia et al. 2018), with 3 different fractions:

- An aqueous pool made of proteins from the cytosol and the pyrenoid. These proteins are extracted rapidly.
- A structural pool, made of proteins having a structural role, such as proteins in the cell wall, membranes and flagella. The extraction of these proteins necessitates detergent.
- A functional pool, corresponding to proteins present in the chloroplast and involved in the light-harvesting system of the cells. These proteins are non-covalently bound to an intricate assembly of pigments and have been shown to be soluble in detergent.

This protein difference is interesting and will be more detailed with the different organelles of *Tetraselmis*, with a figure of *Tetraselmis* cell scheme in Figure 2.5.

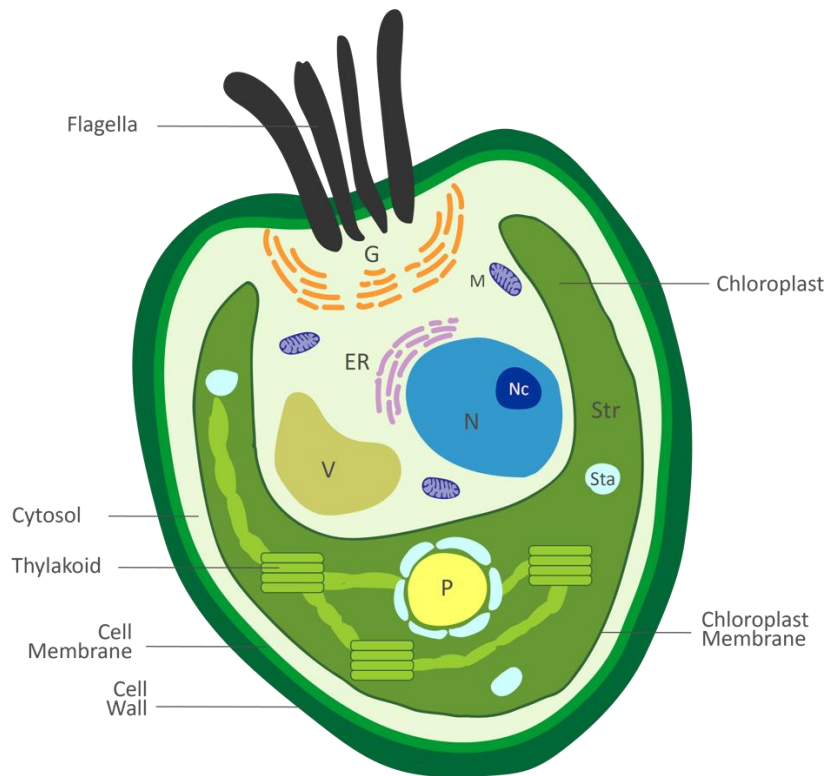


Figure 2.5 Ultrastructure of *Tetraselmis* with G the Golgi apparatus, ER the endoplasmic reticulum, M the mitochondria, N the nucleus, Nc the nucleolus, V the vacuole, P the pyrenoid, Sta in light blue the starch and Str the stroma, the liquid phase of the chloroplast.

Proteins are present in aqueous part of the cells in the cytosol, the stroma of the chloroplast and the pyrenoid and are mostly globular proteins. These proteins are usually soluble enzymes for metabolism, protein synthesis and signaling. For example, the ribulose 1,5 biphosphate carboxylase/oxygenase (EC 4.1.1.39), also called “rubisco” is considered as an important aqueous protein (Di Stefano et al. 2018). This enzyme is responsible for carbon fixation and is thought to be the most abundant protein on Earth, supporting nearly all life on Earth (Ellis 1979; Cooper 2000) and present in plant, eukaryotic algae, cyanobacteria and photosynthetic bacteria (Andersson et Backlund 2008; Ashida, Danchin, et Yokota 2005; Mueller-Cajar et Badger 2007; Tabita et al. 2008; Di Stefano et al. 2018).

The “functional” pool seems to represent membrane proteins involved in photosynthesis and while they have a function, it is also the case for the other proteins. Several membranes are present in an algae cell and can contain membrane proteins, such as the cell membrane, separating the cell wall from the protoplast, containing the other constituents. There are also the membrane-bound organelles, the chloroplast bi membrane, the mitochondria membrane, the Golgi membrane, the reticulum endoplasmic membrane and the nuclear membrane. While all organelles are important for algae growth, chloroplast is an important organelle in a microalgae cell, in terms of function but also in terms of volume in the cell, particularly when algae are autotrophically grown. It contains thylakoids, having membranes entangled with proteins to do the photosynthesis. Among these proteins, Light

Harvesting Complex (LHC) are the most dominant, being thought as the most abundant membrane proteins in the world (Drop et al. 2014). It is composed of several proteins such as D1, D2, but also chlorophyll-binding proteins (Palm et al. 2018).

The “structural” pool seems like a mix of membrane proteins and fibrous proteins in this classification. However, membrane proteins are not only structural since this function is also assumed by the membrane lipids but they can have other biological functions such as receptors or transporters (Cournia et al. 2015). Fibrous proteins are effectively structural but are not abundant in the cell wall of *Tetraselmis* and are present in other parts, such as in the cytoskeleton (Y. Liu et al. 2017).

Proteins can constitute up to 55% of total dry matter but their quantification in terms of globular/aqueous, membrane and fibrous proteins is difficult to perform and still lacking. Membrane proteins are hard to extract in water without solvents or detergents and can be associated with lipids. They can still be dispersed in water after strong size reduction (Buchmann, Brändle, et al. 2019). However, some membrane proteins are associated with chlorophylls such as CP43, complicating the purification (Murray, Duncan, et Barber 2006). Fibrous proteins are the most complicated to solubilize/disperse and were not aimed in this work.

Globular/aqueous proteins were the main target in a first approach and are deepened in the following paragraphs.

2.5.2.2 RuBisCO, a major soluble protein

The most important soluble protein in higher plants is the rubisco, composed of two different subunits, 8 long chains and 8 short chains. The rubisco accounts for 20 to 30% of total leaf nitrogen in C₃ plants and is present in the stroma (Amin et al. 2002). This protein is water soluble and is very interesting for food with a good composition of essential amino acids and promising technofunctional properties (Udenigwe et al. 2017; Ducrocq et al. 2020).

2.5.2.2.1 RuBisCO and Microalgae

This enzyme has two competitive functions, the carboxylation of the ribulose-1,5-biphosphate or its oxygenation, with CO₂ fixation or O₂ fixation respectively, depending on the gas composition (Bathellier et al. 2018). This enzyme led to the change of the atmosphere composition from CO₂ rich atmosphere to a O₂ rich atmosphere. In today's atmosphere, O₂ competes with CO₂ at rubisco's catalytic site, producing the toxic compound phosphoglycolate (Somerville et Ogren 1979). So several strategies were developed by photosynthetic organisms to enhance the carboxylation. In C₃ plants, representing 90% of the higher plants, rubisco is accumulated in abundance in the stroma of the chloroplast to cope with the low efficiency, explaining its high proportion in total proteins (Raghavendra 2003). However, other organisms have developed a Carbon Concentration Mechanism (CCM) in order to favor carbon

fixation, especially when the CO₂ concentration is low (Sage, Sage, et Kocacinar 2012; Giordano, Beardall, et Raven 2005). This is the case for most of the Eukaryotic microalgae due to the low gas diffusion in water in the pyrenoid, where the rubisco is mostly stored (Holdsworth 1971). The algae studied in the project, *T. chui* and *C. vulgaris*, possess a pyrenoid and algae possessing a pyrenoid are thought to mediate between 28-44% of global carbon fixation (Mackinder et al. 2016; R. E. Lee 2018).

2.5.2.2.2 Pyrenoid and RuBisCO

The pyrenoid is a proteinaceous body surrounded by starch sheath and the matrix of the pyrenoid is liquid-like (Rosenzweig et al. 2017). While the rubisco is predominantly in the pyrenoid, a portion is also in the stroma and the proportion depends on the environmental conditions such as CO₂ concentration or light intensity. For example, when the CO₂ concentration is enriched in the media surrounding the cell, 40% of the rubisco is in the pyrenoid while it is 90% when the media is not enriched (Borkhsenius, Mason, et Moroney 1998). This switch takes place within hours and also affects the starch surrounding the pyrenoid which disappears when the media is enriched in CO₂. Moreover, the proportion can also change with the trophic mode, with a higher rubisco fraction in the stroma when the algae is cultivated in mixotrophic growth (Borkhsenius, Mason, et Moroney 1998). These modifications, that can happen within hours, outline the difficulty of working with fast-changing biomass, with a difficult standardization and metabolic modifications that need very specific methods to be detected. Moreover, these studies have been conducted on *Chlamydomonas reinhardtii*, a biological model organism, and while it is genetically close to *C. vulgaris* and *T. chui*, differences may be present (Harris 2001).

2.5.2.2.3 RuBisCO Quantification in Total Proteins

The quantification of the RuBisCO from microalgae possessing a pyrenoid has been explored by authors such as Losh et al. 2013 on 8 species of algae, including one green algae (Losh, Young, et Morel 2013). The RuBisCO proportion in comparison with the total proteins was mostly between 1 and 4% and between 1 and 2% of the green algae. A decrease in the RuBisCO proportion is also seen in C₄ plants, having a CCM, in comparison with C₃ plants, without a CCM. In C₃ plants, RuBisCO contributes to 20-30% of leaf total nitrogen while it constitutes only 5-9% in C₄ plants (Feller, Anders, et Mae 2008). However, the total protein determination may have bias in the study of Losh et al. and no disruption techniques are specified. In diatoms, a low proportion of RuBisCO was also described but the cell disruption method was mild and the pyrenoid may not have been disrupted (Günerken et al. 2015; Valegård et al. 2018).

After bead milling and centrifugation of *C. vulgaris*, *N. oleoabundans* and *T. suecica*, the RuBisCO was identified as one of the main soluble protein observed by electrophoresis (darker band) in the supernatant containing 22 to 30% of total proteins (Postma et al. 2017). Similarly, the large unit of the

RuBisCO was identified by western blot and considered as one of the main protein in the supernatant of bead milled *T. impellucida*, this supernatant containing 21% of total proteins (Schwenzfeier, Wierenga, et Gruppen 2011). The rubisco seems to be an important fraction of the soluble proteins in microalgae, but its content seems to differ depending on the culture conditions and the cell disruption method.

To access to the intracellular proteins described in the former paragraphs, cell disruption processes are needed.

2.6 Cell Disruption

2.6.1 Microalgal cell wall, highly resistant compared to other biomass

Microalgae are very diverse with respect to the cell wall, with species lacking a cell wall such as *Dunaliella* or *Euglena*, or species having a mineralized cell wall such as Coccolithophores or diatoms for example (Alhattab, Kermanshahi-Pour, et Brooks 2019). The cell wall mechanical and chemical resistances are also very diverse, depending on the cell wall composition and thickness (Baudeflet et al. 2017).

Several techniques can be used to determine mechanical cell wall resistance such as compressing experiments. Different results have been obtained on several microalgae, and a comparison can be made with other cells in Table 2.3.

Table 2.3 Mechanical properties of different cells, including microalgae in bold.

Specie	Bursting energy per cell	Force required to break cells	Publication
Unit	pJ	μN	
<i>Chlorella vulgaris</i> (0 mosmol/kg)	22.3	49	(Günther et al. 2016)
<i>Chlorella vulgaris</i> (240 mosmol/kg)	81,6	116	(Günther et al. 2016)
<i>Chlorella vulgaris</i>	37	115	(Overbeck et al. 2017)
<i>Tetraselmis suecica</i>	17.43	7.1	(A. K. Lee, Lewis, et Ashman 2013)
<i>Thalassiosira punctigera</i>	-	180-260	(Hamm et al. 2003)
<i>Coscinodiscus granii</i>	-	90	(Hamm et al. 2003)
<i>Fragilariopsis kerguelensis</i>	-	730	(Hamm et al. 2003)

<i>Botryococcus braunii</i>		3563	(Tsutsumi et al. 2018)
<i>Sacharomyces cerevisiae</i>	82-132	129 - 220	(Overbeck et al. 2017)
<i>Mammalian cells: hybridoma</i>	-	1.5 – 4.5	(Zhang et al. 1991)
<i>Bacteria</i>	-	3 - 34	(Shiu, Zhang, et Thomas 2002)
<i>Yeasts</i>	-	55 - 175	(Mashmouhy, Zhang, et Thomas 1998)

Microalgae can have a very resistant cell wall and while *S.cerevisiae* had a higher absolute higher energy to bust one cell than *Chlorella*, more energy was needed to disrupt the cells in relation to the dry matter since *Chlorella* is smaller than the yeast (Overbeck et al. 2017). These results can be interesting to model and predict the cell disruption results of diverse species based on the mechanical properties of the cells and some models aimed at doing so (Suarez Garcia et al. 2019). *Tetraselmis suecica* was among the least resistant microalgae cells tested but no indication was given on the life cycle state. *Arthrospira platensis* on the other hand, should have results similar to bacteria but no data was found. Mechanical cell properties are very interesting and are an ongoing topic in biology (Viljoen et al. 2021).

However, the study of the cell mechanical properties is long and necessitates a specific material, so empirical approaches have been developed. One of them is to disrupt the algae by a High-Pressure Homogenizer with one pass at several pressures, changing the algae in between, and calculating the pressure to destroy 50% of the cells, the P_{50} (Spiden et al. 2013). A comparison of several algae is presented in Table 2.4 to illustrate results from this method.

Table 2.4 Mechanical sensitivity of microalgae in the literature

Microalgae	P_{50} (bar)	Publication
<i>Chlorella sp.</i>	1060	(Spiden et al. 2013)
<i>Tetraselmis suecica</i>	170	(Spiden et al. 2013)
<i>Nannochloropsis sp.</i>	2000 (extrapolated)	(Spiden et al. 2013)
<i>Porphyridium cruentum</i>	400	(Valéria Montalescot et al. 2013)
<i>Nannochloropsis oculata</i>	1400	(Valéria Montalescot et al. 2013)
<i>Parachlorella kessleri</i>	573	(Zinkone 2018)
<i>Chlorella sorokiniana</i>	830	(Zinkone 2018)

The high cell wall mechanical strength justifies the different processes being applied for microalgae cell disruption. The cell disruption processes generally applied to microalgae are well described in a review of Günerken et al. (Günerken et al. 2015).

2.6.2 Bead Milling

2.6.2.1 Global Mechanism

Bead milling is a type of milling used to grind, deagglomerate and disperse in a wide range of industries from paint, chemical, ceramic, pharmaceutical, food, nanotechnology (Kwade et Schwedes 2007). Beads are filled in a chamber and an agitator allows contacts between the beads and the particle to be treated to decrease the particle size, with a spacer to retain the beads. It has been used to disrupt microorganisms such as yeasts or bacteria (Currie, Dunnill, et Lilly 1972; Schütte et al. 1983; Bunge et al. 1992) and has been developed on microalgae the last 15 years (Doucha et Lívanský 2008; V. Montalescot et al. 2015; Postma et al. 2015; 2017; Zinkoné et al. 2018).

The bead mill is usually horizontal, and several agitation geometries exist, with discs, pin-counter pin, and annular gap. The mill we used had a disc agitator geometry and a scheme of it can be found in Figure 2.6.

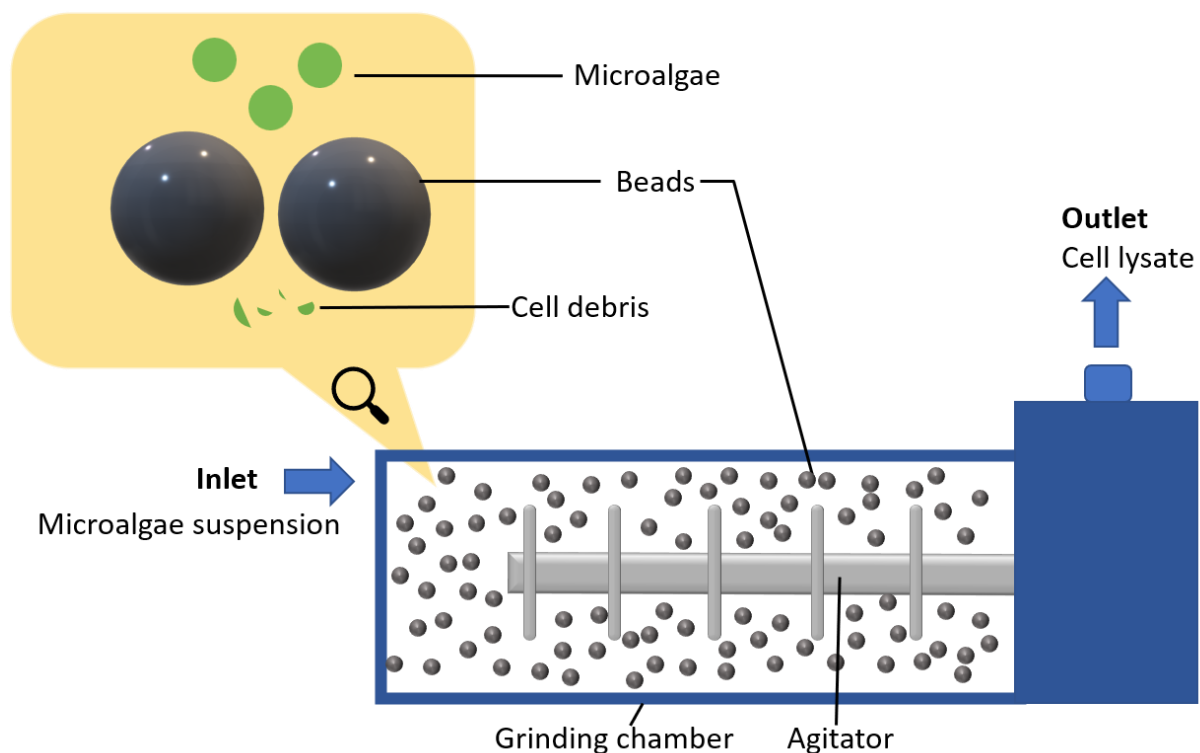


Figure 2.6 Bead milling scheme.

The bead milling has multiple parameters that can be used to tune the mild of disruption. The main parameter is the bead diameter d_b which usually ranges between 0.2 and 2 mm for microalgae cell

disruption (V. Montalescot et al. 2015). Another important parameter is the bead material, with two main choices, glass, or Yttria-stabilized Zirconium (YTZ). For food security results, glass was avoided and YTZ was selected. The agitator speed v and the bead filling ratio f are also key parameters, as well as the residence time within the bead mill.

2.6.2.2 Bead milling modeling

Bead milling mechanisms have been described by several models, some using fluid mechanics and others using a semi-empirical approach such as the stress model developed by Kwade and Schwedes (Kwade et Schwedes 2002). This model has been used to describe the comminution of ceramics, but also cells. It has been applied to microalgae cell disruption by Montalescot et al. (V. Montalescot et al. 2015) and has been used since in other studies (Zinkoné et al. 2018; Postma et al. 2017). It relies on two different parameters, the stress intensity, and the stress number.

The energy available for the milling was hypothesized to be proportional to bead kinetic energy. To estimate this latest, several hypotheses were taken (Kwade et Schwedes 2007). The bead tangential speed was proportional to the agitator tip speed. The diameter of the agitator was supposed to be constant. The suspension displacement between two beads was negligible on the bead speed. The material elasticity to be broken was negligible in comparison with bead elasticity.

The stress energy (SE , J) is proportional to the bead kinetic energy (SE_r J):

$$SE \propto SE_r = \rho_B d_B^3 v^2 \quad \text{Equation 2.2}$$

And the stress intensity (SI J.m⁻¹) is proportional to the stress energy with:

$$SI = \frac{SE}{d_p} \quad \text{Equation 2.3}$$

With d_p the particle diameter (m) (i.e., microalgae), ρ_B the bead specific density (kg.m⁻³), d_B The bead diameter (m) and v the agitator tip speed (m.s⁻¹).

The other parameter, the stress number, aimed at representing the number of stressing events happening in the bead milling and is calculated by:

$$SN \propto n t \zeta \frac{d_p}{d_B} \quad \text{Equation 2.4}$$

With $\zeta = f(1 - \varepsilon)$, n the agitation revolution (min⁻¹), t the milling time (s), f the bead filling ratio and ε the bead bed porosity.

The theoretical specific energy input E_m (kwh. $\text{kg}_{\text{DM}}^{-1}$) is proportional to the two parameters:

$$E_m \propto \frac{SN.SI}{M} \quad \text{Equation 2.5}$$

Where M is the dry matter of biomass in the system (kg_{DM}).

The stress model is a model useful to optimize the process by selecting the right parameters to optimize energy consumption and cell disruption/compound release. However, the disruption and liberation kinetics are important to be followed to compare different cell disruption conditions.

The cell disruption and compound release follow a first order kinetic (Currie, Dunnill, et Lilly 1972; Garrido et al. 1994; Doucha et Lívanský 2008; V. Montalescot et al. 2015; Postma et al. 2015):

$$C(t) = C_0 \exp(-k.t) \quad \text{Equation 2.6}$$

With k (min^{-1}) the kinetic constant, C_0 the initial concentration and $C(t)$ the concentration of cells or molecules as a function of time.

The residence time of the cells within the bead mill is linked to the space time t_s calculated by:

$$t_s = \frac{V}{Q} \quad \text{Equation 2.7}$$

With V (m^3) the volume accessible by the liquid and Q ($\text{m}^3 \cdot \text{s}^{-1}$) the flow rate.

The residence time was linked to the space time by a residence time distribution, and the bead mill was found to follow a series of two Continuous Stirred Tank Reactor (CSTR) by Montalescot et al. and Zinkoné et al. (V. Montalescot et al. 2015; Zinkoné et al. 2018).

The constant kinetic for a first order cell disruption in the bead mill used for this work can be calculated by:

$$k = \left(\frac{1}{\sqrt{\frac{C_f}{C_0}}} - 1 \right) \cdot \frac{2}{t_p} \quad \text{Equation 2.8}$$

With C_0 and C_f the initial and final concentration respectively.

The cell disruption rate X_{dis} was calculated as:

$$X_{\text{dis}} = 1 - \frac{C_s}{C_0} \quad \text{Equation 2.9}$$

With C_0 and C_s the intact cell concentration before and remaining after cell disruption.

The first-order kinetics can be compared to optimize the cell disruption and to compare the disruption of different microalgae or microalgae having different physiological states (V. Montalescot et al. 2015; Heredia et al. 2021).

2.6.2.3 Bead milling and microalgae

The cell disruption of microalgae, including *Chlorella* and *Tetraselmis*, has already been described, as seen in Table 2.5.

Table 2.5 Articles on bead milling of *Tetraselmis* for protein extraction.

Bead milling and centrifugation parameters	Physicochemical conditions	Protein recovery yield	Protein content in the initial biomass (% _{DM})	Article
0.3 L chamber 65% V/V 0.4-0.6 mm beads YTZ ¹ beads DM suspension: 120 g _{DW} .kg ⁻¹ <u>Centrifugation:</u> 40 000g, 30 min 4°C	Dilution with Tris HCl pH 8 EDTA DTT MgCl ₂	21% (calculation method not detailed)	36% Determined by amino acid analysis Frozen paste <i>T. impellucida</i>	(Schwenzfeier, Wiereng a, et Gruppe n 2011)
0.075 L chamber 65% V/V 0.3 mm 0.4 mm 0.65 mm 1 mm YTZ beads DM suspension: 90 g _{DM} .kg ⁻¹ <u>Centrifugation:</u> 20 000g 20 min	Dilution of the paste with phosphate-buffered saline (PBS) at pH 7	17.4% 21.5% 15.7% 21.1% Concentration ratio between the biomass and the supernatant.	43% 29% 37% 41% Colorimetric Fresh paste <i>T. suecica</i>	(Postma et al. 2017)
0.3 L chamber 65% V/V 0.4-0.6 mm beads YTZ ¹ beads DM suspension: 120 g _{DM} .kg ⁻¹	Dilution of the paste with PBS buffer at a final pH of 8	41%	36% ± 2% Amino acid analysis Frozen paste <i>T. impellucida</i>	(Teuling et al. 2017)

<u>Centrifugation:</u> 70 000g, 30 min 4°C				
0.075 L chamber 65% V/V 0.4 mm YTZ beads DM suspension: 100 g _{DM} .kg ⁻¹ <u>Centrifugation:</u> 20 000 g, 30 min, 20°C	Dilution of the paste with PBS at a final pH of 7	12%	37% Colorimetric Fresh paste <i>T. suecica</i>	Garcia 2018
0.075 L chamber 65% V/V 0.4 mm YTZ beads DM suspension: 100 g _{DM} .kg ⁻¹ <u>Centrifugation:</u> 14 000 rpm, 30 min, 20°C	Dilution with distilled water to pH 6.5	22.5%	54% Colorimetric Fresh paste <i>T. suecica</i>	Suarez Garcia 2018

¹ YTZ (Yttrium Stabilized-Zirconia beads); yield: $y = \frac{m_{\text{proteins}}^{\text{supernatant}}}{m_{\text{proteins}}^{\text{biomass}}}$

Tetrasemis cell disruption has been studied by several authors on different species (mainly *T. impellucida*, *T. suecica*) as seen in Table 2.5. The protein recovery yields, in the supernatant after bead milling and centrifugation, were distributed in 2 ranges, one with mass protein yields ranging from 12% to 21% of total proteins, and a second with a mass protein yield of 41%. Despite having similar biomass composition and process conditions, the yield showed significant differences, for example 21% with Schwenzfeier et al. (Schwenzfeier, Wierenga, et Gruppen 2011) and 41% with Teuling et al. (Teuling et al. 2017).

The bead diameter, by affecting the stress number and strongly the stress intensity, is one of the main parameters for cell disruption. Small beads were found to be more efficient to extract intracellular compounds in organelles than larger beads in several organisms (Schütte et al. 1983; Bunge et al. 1992; Postma et al. 2017). Moreover, the energy consumption can be decreased when the stress intensity SI is above an optimum stress intensity SI_{opt} (V. Montalescot et al. 2015). A better milling efficiency for cell disruption and protein release was found for $d_p = 0.3$ mm for *Chlorella vulgaris* and also correlated to a faster release of rubisco (Postma et al. 2017). This was also confirmed by Zinkoné et al., but the increase in protein release was associated to a higher dry matter release in general, including chlorophyll on *C.sorokiniana* (Zinkoné et al. 2018). At SI lower than $5 \cdot 10^{-4}$ J.m⁻¹ a high protein yield

recovery of 85% was obtained in the supernatant, as well as a high dry matter dispersion (up to 70%) and a high chlorophyll yield (up to 70%). On the contrary, at high SI, a lower protein yield recovery was obtained (60%) as well lower dry matter dispersion (40%) and chlorophylls (20%) in the supernatant. This is logical since bead milling is well known to prepare submicron dispersion to prevent sedimentation of several naturally insoluble materials including sand (Sivasubramanian et al. 2012) and TiO₂ (Silambarasan, Manikandan, et Rajan 2012). This is also the case on microalgae, with starch, a naturally insoluble polymer, recovered in the supernatant after *Tetraselmis* bead milling (Zinkoné et al. 2018; Suarez Garcia et al. 2018). Unlike other microalgae, bead diameter had a limited effect on the protein and especially the rubisco release kinetic from *T. suecica*, and on the final yield (Postma et al. 2017). It was hypothesized that the rubisco within the pyrenoid was released for *Chlorella* and *Tetraselmis* with small and large beads.

Another important parameter is the bead filling ratio, with 80% being optimal in several microalgae cell disruption to lower energy consumption and optimize cell disruption (V. Montalescot et al. 2015; Suarez Garcia et al. 2019).

The bead milling is very efficient to disperse a large quantity of proteins recovered in the supernatant, but the extract is mixed with several compounds such as chlorophyll or carbohydrates. However, the kinetic is different depending on the molecules and a faster release of proteins was compared to cell disruption kinetic and carbohydrate kinetic release (Postma et al. 2017). This was exploited to produce a supernatant enriched in proteins (50%_{DM}) and with a low carbohydrate content (26%_{DM}) (Suarez Garcia et al. 2018).

2.6.3 Pulsed Electric Fields

The bead milling is a mature technique within the microalgae industry and allows to access different organelles to extract a maximum of soluble proteins. However, proteins contained in the membrane of the thylakoid are also dispersed into the supernatant and an operation unit such as a microfiltration is needed to remove them (Grossmann, Hinrichs, et Weiss 2020). Moreover, the algae ultrastructure is disorganized and physico-chemical interactions can take place such as molecule complexation, molecule modification by enzymes (ex: lipid oxidation, protein hydrolysis, etc.). These modifications can reduce the value of the coproducts and/or complexify their recovery.

Other operation units exist, such as the Pulsed Electric Field (PEF), to extract soluble proteins without disrupting completely the cell.

2.6.3.1 Principle

PEF consists in the application of repeated short pulses of an electric field E created between two electrodes. Cells treated with PEF will present poration of the phospholipid bilayer if the modification of their transmembrane voltage reaches a critical value (Teissie, Golzio, et Rols 2005).

An uneven distribution of anions and cations on both sides of the cell membrane exists, generating an electric potential called Resting Transmembrane Voltage (RTV). When the cell is treated with a high electric field strength, the cell charges reorganize and accumulate on both sides of the membrane. The transmembrane voltage increases, being called Induced Transmembrane Voltage (ITV) (Tsong 1991). Above a limit, cell electroporation happens and, depending on the ITV applied and treatment time, the pores can be either reversible or irreversible (Teissie, Golzio, et Rols 2005). Moreover, the pore size increases with the electric field strength, allowing larger molecules to go through the membrane (Ivorra, Villemejeane, et Mir 2010). When the membrane is permeated, small soluble molecules such as small proteins and carbohydrates can diffuse out of the cell and be extracted without generation of fine debris and a strong reorganization of the cell, as described in Figure 2.7.

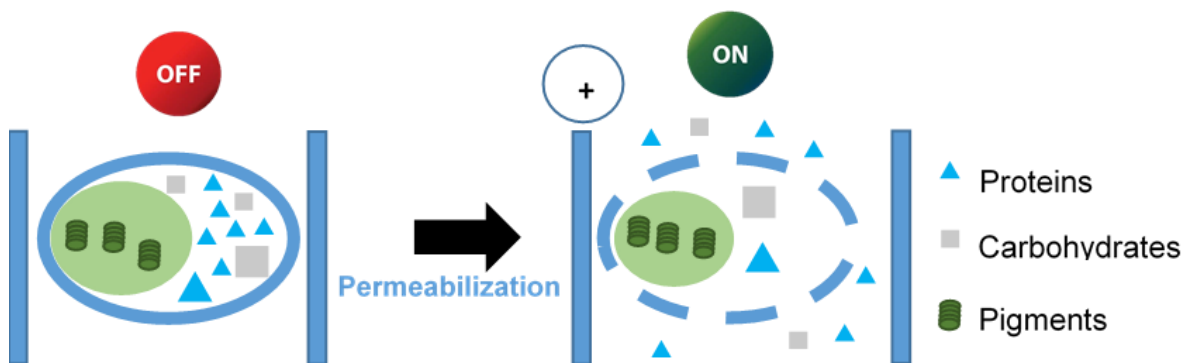


Figure 2.7 Pulsed Electric Field principle

Pulsed Electric Field is a scalable technique being used in food industry to inactivate microorganisms and spores or to increase the mass transfer from a biomass at reduced temperature. An example of the latest is the application of PEF to extract polyphenols and anthocyanins from grape skin and seeds (Maza et al. 2020). It can also be used before drying to increase the water diffusion out of the cell and reduce drying time and energy (Neri et al. 2021).

Several parameters affect PEF treatment and one of the most important is the specific energy delivered to the biomass. It depends on the electric field strength E applied. The electrodes are charged at certain tension U (V) and an electric field E ($V \cdot m^{-1}$) is developed between the electrodes. The electrical field strength that needs to be applied is increased when the size of the treated object (cell, organelle) is small (Coustets et al. 2013). The electric field is applied as repetitive pulses with duration between nanoseconds to few milliseconds (Raso et al. 2016) and due to the short pulse duration, the energy

consumption can be low despite a high voltage. The number of pulses is also an important parameter to take into consideration to optimize the treatment.

Secondary effects take place such as Joule heating where the temperature increase due to the propagation of the electric field in water (Lam et al. 2017). With the hypothesis of an adiabatic system, the temperature increase follows:

$$\Delta T_{pulse} = \frac{|E|^2 \cdot \Delta t_{pulse} \cdot \sigma_{medium}}{C_{p_{medium}} \cdot \rho_{medium}} \quad \text{Equation 2.10}$$

With ΔT_{pulse} (K) the increase in temperature after one pulse, E the electric field strength ($V \cdot m^{-1}$), Δt_{pulse} the pulse duration (s), σ_{medium} the medium conductivity ($S \cdot m^{-1}$), $C_{p_{medium}}$ the medium heat capacity ($J \cdot K^{-1}$), and ρ_{medium} the medium volumetric mass density ($kg \cdot m^{-3}$).

Different solutions were performed to avoid excessive heating for microalgae treatment, especially when the specie was marine. A first solution can be a buffer change with low conductivity (Coustets et al. 2013), another solution can be medium cooling (Sheng, Vannela, et Rittmann 2011).

2.6.3.2 PEF applied to protein extraction from microalgae

PEF has been applied to microalgae to facilitate the extraction of several compounds, water soluble (ie protein, carbohydrate) (Coustets et al. 2013) or water insoluble such as lipids before solvent recovery (Sheng, Vannela, et Rittmann 2011). Since the cell is not broken by PEF treatment, there is a need to assess that the treatment was sufficient to electroporate the cells. Several ways can attest cell perforation such as an increase in conductivity in the suspension, showing a leakage of intracellular ions (Goettel et al. 2013; Grimi et al. 2014), or by a staining of the electroporated cells by Evans Blue (Jacyn Baker et Mock 1994; Scherer et al. 2019).

Protein extraction has been realized by PEF and the protein yields were low on different fresh microalgae with 3.6% of total proteins on *N.salina* (Grimi et al. 2014), 4.9% of total proteins in *C.vulgaris* (Postma et al. 2016), 1% on *C.sorokiniana* (Leonhardt et al. 2020) and up to 13% on *C.vulgaris* and *N.oleoabundans* after very high energy consumption (Lam et al. 2017). An increase was observed after PEF and extraction at pH 11 on frozen-thawed *Nannochloropsis sp.* but chlorophyll was also extracted (Parniakov et al. 2015). The energy input was considered the main parameter affecting the protein yield after PEF treatment in several studies with a maximum yield at $4 \text{ kWh} \cdot \text{kg}_{DM}^{-1}$ on *C.sorokiana* and $4 \text{ kWh} \cdot \text{kg}_{DM}^{-1}$ on *Nannochloropsis sp.* for example (Goettel et al. 2013; Grimi et al. 2014; Postma et al. 2016; Carullo et al. 2018; Leonhardt et al. 2020). Different results were found on *C.vulgaris* with an increase of the protein concentration associated with an increase in electric field strength (from no protein extraction at $10 \text{ kV} \cdot \text{cm}^{-1}$ to $0.2 \text{ g} \cdot \text{L}^{-1}$ at $20 \text{ kV} \cdot \text{cm}^{-1}$) (Buchmann, Brändle, et al.

2019). An optimal electric field strength of 20 kV.cm^{-1} was also identified on *C.vulgaris* (Carullo et al. 2018).

The protein yields were lower than obtained by mechanical disruption such as bead milling or HPH (Lam et al. 2017; Carullo et al. 2018). However, specific type of proteins can be extracted. For example, a shotgun proteomic comparison of proteins extracted by HPH or PEF indicated that the majority of the additional proteins extracted by HPH were found in the thylakoid membrane, including chlorophyll-binding proteins (CP47 and CP43) (Buchmann, Brändle, et al. 2019). The other significant proteins extracted were found in the inner mitochondrial membrane and inside the chloroplast. However, a debate still exists on the localization of protein extracted by PEF, since nearly 50% of the proteins extracted by PEF was from the chloroplast after LC MS/MS identification on *Chlamydomonas* (Gateau et al. 2021). The comparison of PEF to other cell disruption techniques have been realized but without a comparison of a complete process, including the purification operation units.

Nonetheless, several ways exist to increase the protein yield such as maceration for 24 h after PEF extraction (Carullo et al. 2020). This increase has been observed in several studies and seems mediated by the release of proteases (Silve et al. 2018; Scherer et al. 2019; Canelli et al. 2022). The action of enzymes may help the extraction of proteins non-present in the cytoplasm and linked to organelles, realizing autolysis (Scherer et al. 2019).

The extraction of proteins by PEF from *Tetraselmis chui* was never reported to the author knowledge and has been only applied to extract antioxidant compounds (Kokkali et al. 2020). The PEF treatment of marine strains can be more difficult because of the salts in the water, a higher Joule effect and lower electric field strength on the cells, with a preferential path of the electric current in the liquid.

Protein extraction from fresh *Arthrospira* by PEF has been applied and unlike in microalgae, high electric field strengths ($20\text{-}25$ to 40 kV.cm^{-1}) were needed to extract C-phycoyanin, the main protein (Martínez et al. 2017; Akaberi et al. 2020; Käferböck et al. 2020). Moreover, phycoyanin was not released immediately and needed at least 150 min of extraction but a plateau was reached after 330 min and higher temperatures (40°C) during maceration improved significantly the C-phycoyanin yield (Martínez et al. 2017).

PEF for milking is also a promising way to extract proteins and the milkings of *C.vulgaris* and *H.pluvialis* were successfully conducted without killing the cells (Buchmann, Brändle, et al. 2019; Gateau et al. 2021). However, few studies were conducted to date and the biomass needs to be alive which was not the case after the necessary freeze-thawing due to the Profuture project organization.

Once the cells are disrupted and the biomolecules recovered in the aqueous phase, several processes are needed to separate them from the cell fragments or other extracted compounds, to be concentrated and to eliminate the salts.

2.7 Separation Processes

2.7.1 Centrifugation

The first operation unit after cell disruption to extract proteins is most often clarification by centrifugation to separate the protein-rich aqueous phase from the solid phase containing insoluble particles (Amorim et al. 2020). The centrifugation separation principle is based on sedimentation which can be described by Stokes law by assuming a creeping flow. The terminal velocity can be calculated by:

$$v_s = \frac{1}{18} \frac{(\rho_s - \rho_L) G d_s^2}{\mu_L} \quad \text{Equation 2.11}$$

With v_s the terminal velocity ($\text{m}\cdot\text{s}^{-1}$), ρ_s and ρ_L the solid and liquid density respectively ($\text{kg}\cdot\text{m}^{-3}$), G the centrifugal acceleration ($\text{m}\cdot\text{s}^{-2}$), d_s the particle diameter (m) and μ_L the liquid viscosity (Pa.s). G is equal to $G = RCF \cdot g$ with RCF the relative centrifugal force and g the Earth's gravitational acceleration ($\text{m}\cdot\text{s}^{-2}$) (Leung 2007).

The main objective of the centrifugation is to separate insoluble particles, including proteins present in aggregates, from water-soluble proteins. The volumetric mass difference should be above 2% and is better above 10% to ensure an efficient separation at large scale (Leung 2007). The average protein volumetric mass is $1,350 \text{ kg}\cdot\text{m}^{-3}$ so it is above water volumetric mass from more than 10%.

Depending on the RCF and on centrifugation time, different sizes of suspended particles can be separated, and this has been used to study biological macromolecules, including proteins by ultracentrifugation. For example, the proteins of seeds have been classified using their physico-chemical properties using Osborne classification (Osborne 1909), but also by their sedimentation coefficient in Svedberg S (Shewry et al. 1986). In industrial centrifugation, however, a RCF higher than 10,000 g is rarely seen to fractionate proteins from solid residue and a particle diameter limit of 0.5 μm under which the centrifugation hardly separate industrially can be hypothesized (Leung 2007; Amorim et al. 2020).

In microalgae biorefinery, a wide range of parameters are used for centrifugation with sometimes high RCF (e.g. 70,000 g) (Teuling et al. 2017) or long time (e.g. 30 min) (Garcia et al. 2018). While analytical centrifugation is interesting to discriminate "soluble" and "dispersed" particles, high RCF or time are not compatible with a pilot and industrial scale centrifuge (Zinkoné et al. 2018; Amorim et al. 2020).

When the cell disruption is realized with HPH and bead milling, the supernatant most often contains chlorophylls. However, this depends on the centrifugation conditions, as supernatant containing nearly no chlorophylls were obtained after analytical centrifugation (Schwenzfeier, Wierenga, et Gruppen 2011; Teuling et al. 2017).

Several operation units have been applied to purify proteins from the supernatant but the final objective can be different. In this work we aimed at testing and characterizing two operations units widely used for protein purification, IsoElectric Precipitation (IEP) and membrane filtration. The objectives were to produce a protein-rich aqueous extract with low color and characterize the operation unit to be able to scale-up the process to supply the project partners with sufficient material for their analysis.

2.7.2 Isoelectric Precipitation

2.7.2.1 Principle

The operation unit of isoelectric precipitation relies on the low protein solubility at a pH close to the $pH(I)$, as seen in Part 2.4.4.1 of this chapter. The net charge of the proteins is neutral, which decreases the electrostatic repulsion of the different proteins and the attractive hydrophobic forces become more important. The proteins can start to agglomerate, enhancing the size of the particle. This can be used to recover the proteins by solid/liquid operation units such as gravitational sedimentation, centrifugation, or filtration.

This technique is widely and traditionally used to produce a wide range of protein-enriched ingredients such as protein concentrates or protein isolates by adjusting the pH to the $pH(I)$ of the dominant proteins and separating the precipitate from the liquid. It was used traditionally for the production of cheese, called acid-set cheese or sour milk cheese (ex: queso blanco, quark, cream cheese...) obtained by coagulation of milk protein either by acid addition or lactic acid bacteria metabolism (Fox et al. 2016). The non-precipitated proteins are found in a product called whey and this name is sometimes also used to characterize the liquid product after IEP of plant proteins.

IEP is easily used for seed proteins since only few different proteins are present as storage proteins. For example, glycine and β conglycinin are storage proteins representing up to 80% of the total protein of soybean (Krishnan et al. 2007; Luthria et al. 2018). Hence, a high yield is obtained by choosing a pH near the $pH(I)$ of the major proteins and the choice of the pH can change the proportion of the precipitated protein types (Verfaillie et al. 2023).

This technique has different benefits such as a high yield, a low cost, and an easy protocol. However, it has some disadvantages such as a high use of solvents, a high amount of wastewater, an increase in

salt content and a possible denaturation of proteins (Cruz-Solis et al. 2023). The denaturation can result in a loss of protein solubility, an important techno-functional property (Nash, Kwolek, et Wolf 1971; Salt et al. 1982).

2.7.2.2 Isoelectric precipitation applied to microalgal extract

This technique has also been recently used for the purification of protein from algae. It is an efficient technique for cyanobacteria because of their storage proteins as phycocyanin or cyanophycin and a pH(I) of 3 was identified and used for protein purification of *Arthrospira platensis* extract (Devi et al. 1981; Benelhadj et al. 2016; Buchmann, Bertsch, et al. 2019). For microalgae, however, this technique can be more complex to use due to the diversity of proteins with different pH(I) and no known storage protein (Ursu et al. 2014). This operation unit has different outcomes for microalgae and a table referencing the main outcomes can be seen in Table 2.6.

Table 2.6 IEP of protein extracts from different microalgae and the main results

Species	Disruption process	Solubility/yield	Comment	Article
<i>Tetraselmis impellucida</i>	Bead milling	pH(I) = 3.5-4 at [I]=0.03M and 2.5-4 at [I]=0.5M	Presence of glycoproteins in high quantity. No well-defined minimum solubility.	(Schwenzfeier, Wierenga, et Gruppen 2011)
<i>Nannochloropsis sp.</i>	Ultrasound and pH 11	pH(I) = 3.2 Protein yield of the IEP as an operation unit was only 50%		(Gerde et al. 2013)
<i>Chlorella vulgaris</i>	HPH and pH 7/12	pH(I) between pH 3 and 5.	Diverse protein pH(I)	(Ursu et al. 2014)
<i>Nannochloropsis oculata</i>	Bead milling and pH 7/10	pH(I) between pH 1 and 4. Protein yield of this operation unit near 100%	The ratio volume of precipitate over the volume of extract went from 88% at pH 5 to 47% at pH 3 and 40% at pH 1. Precipitate dark green with a protein purity 23% and a dry matter of 130 g _{DM} .kg ⁻¹	(Cavonius, Albers, et Undeland 2015)

<i>Auxenoclorella prothotecoides</i>	HPH	pH(I) at pH 2	High protein solubility at acidic pH, probably due to polysaccharides	(Grossmann, Hinrichs, et Weiss 2019a)
<i>Chlorella sorokiniana</i> (Cs), <i>Phaeodactylum tricornutum</i> (Pt), <i>Nannochloropsis oceanica</i> (No)	HPH	pH(I) at pH 4 for Cs and pH 2 for Pt and No	Presence of glycoproteins in Cs extract, little in Pt and No. Presence of hydrophilic amino acids. Possible formation of electrostatically stabilized soluble complexes.	(Grossmann, Hinrichs, et Weiss 2019b)
<i>Arthrospira maxima</i> (Am), <i>Macrochloropsis gaditana</i> (Mg), <i>Tetraselmis impellucida</i> (Ti), <i>Scenedesmus dimorphus</i> (Sd)	Bead milling	pH(I) at pH 4-4.5	Glycoproteins found in all purified extracts. The uronic acids may decrease the pH(I) by 0.24–0.52 pH units. Protein solubility of the purified extracts decreases when the [I] increase	(Teuling et al. 2017)

The presence of charged polysaccharides modified the protein solubility when it was compared with the pH(I) of proteins obtained by a 2D electrophoresis in several studies. The efficiency of the purification depends on the process and the strain used, with an efficient precipitation obtained in a study by Ursu et al. 2014 and Cavonius et al. 2015, but difficulties on studies of Grossmann 2018, 2019a,b.

Moreover, IEP was able to purify proteins in some cases but was not able to obtain an extract with low color (Cavonius, Albers, et Undeland 2015). The disruption by energy-intensive methods such as HPH or bead milling has been shown to disperse membrane proteins, including chlorophyll binding proteins (Buchmann, Brändle, et al. 2019). It is likely that these proteins precipitate also with the other proteins, producing a very green extract. While a low color precipitate was obtained on *T. impellucida*, it was after an ultracentrifugation leading a low color supernatant. Hence, isoelectric precipitation of a low-colored fraction should be used to have a low color precipitate, but it can still be tested on a green supernatant since it may depend on the algae and the type of extracted proteins.

2.7.3 Filtration

2.7.3.1 Principle

Membrane processes are also promising operation units to obtain protein-rich extract with low color. A membrane process is an operation unit allowing a physical separation of molecules based on either different sizes or physical characteristics (charge) by a selective membrane. The mass transfer is carried out by a driving force, either by a pressure gradient (membrane filtration), a concentration gradient (dialysis) or an electric potential gradient (electrodialysis). Membrane process selectivity depends mainly on the particle size and geometry but also on their physicochemical interactions (polarity, charge).

This work will focus on membrane filtration. Membrane filtration can be classified by the molecular weight cut-off (MWCO), especially for ultrafiltration and microfiltration, defined as the size of the molecules that will be retained at more than 90% and has consequences on the applied pressure and the flux, as seen in Table 2.7.

Table 2.7 Membrane filtration classification as a function of the MWCO, according to Synder.

	MWCO	Average L_{p_w} (25°C) $L \cdot h^{-1} \cdot m^{-2} \cdot bar^{-1}$	Operating pressure (bar)	Type of retained molecules
Microfiltration (MF)	800 kDa – 0.2 μm	300 – 1500	0.5 – 1	Bacteria, colloids
Ultrafiltration (UF)	1-300 kDa	50 – 100	1 – 5	Proteins, sugars
Nanofiltration (NF)	100 – 800 Da	2.5 – 5	10 – 40	Small proteins, multivalent ions
Reverse osmosis (RO)	<100 Da	0.5 – 1.5	30 – 80	Monovalent ions

Membrane filtration can also be classified into two categories depending on the direction of the filtrated suspension compared to the membrane: dead-end filtration and cross-flow filtration. In dead-end filtration, the suspension is injected perpendicular to the membrane while in cross-flow filtration, the suspension flows tangentially to the membrane. The tangential flow allows reducing material accumulation on the membrane surface, limiting fouling, and this configuration will be applied in this work. There is one inlet, called feed, and two outlets called retentate, corresponding to the product retained by the membrane, and permeate, corresponding to the product that goes through the membrane.

The membrane used can also be classified depending on its composition into two categories, organic and mineral membranes. Mineral membranes are interesting due to their high thermal and chemical

resistance but have a higher cost. Organic membranes, on the other hand, are the most used membranes due to their low cost and availability in a wide range of pore size and geometry (Berk 2009) and will be used in this work.

Membrane filtration is carried out in membrane modules that can be provided with different geometries (Berk 2009). Four main geometries are used in the food industry: plate membranes, spiral wounds, tubular membranes and hollow fibers. Plate modules represent plane membranes stacked in a vertically or horizontally. A plate membrane can also be used at laboratory scale to study the membrane performance. Tubular membranes with a cylindrical geometry (diameter few cm) are suitable for applications where the feed has a high dry matter content. The spiral wound configuration consists of a perforated tube to collect the permeate with rolled-up membrane sheets separated by a mesh spacer to provide a flow channel to the retentate. They have a high compactness, characterized by a high surface-to-volume ratio. A hollow fiber module contains an array of narrow fibers with a thin tubular geometry (few mm). This type of module is extremely compact, but they have a higher fouling and clogging susceptibility compared to the other geometries.

2.7.3.2 Filtration characterization

Membrane filtration can be characterized by different parameters important to understand the mechanisms, optimize the process and acquire data to scale up the process.

Membrane productivity is determined by the permeate flux density J ($L \cdot h^{-1} \cdot m^{-2}$), calculated by the ratio of the permeate flow rate by the membrane surface. In the case of a pure solvent, Darcy law allows to rely on the flux density J to the transmembrane pressure (TMP):

$$J_w = TMP \cdot L_w = \frac{TMP}{\mu_{perm} \cdot R_m} \quad \text{Equation 2.12}$$

With TMP the transmembrane pressure (Pa), L_w the membrane hydraulic permeability ($m^3 \cdot h^{-1} \cdot m^{-2} \cdot Pa^{-1}$), R_m the hydraulic membrane resistance (m^{-1}) and μ_{perm} the permeate viscosity (Pa.s).

The transmembrane pressure is the driving force of the filtration and is calculated as follow:

$$TMP = \frac{P_f + P_r}{2} - P_p \quad \text{Equation 2.13}$$

With P_f the feed pressure, P_r the retentate pressure and P_p the permeate pressure.

The filtration of a suspension yields to the accumulation of material due to the retained molecules which leads a to flux decrease. Two phenomena can occur, concentration polarization and membrane fouling.

Concentration polarization is the progressive accumulation of material near the membrane surface creating a concentration gradient from the bulk to the surface. Under pressure, a higher solute concentration is present at the surface than in the suspension and when the pressure decreases, the concentration polarization will disappear. Membrane fouling is a solute deposit on the membrane surface or inside the pores, adding a resistance to the water permeation that can be either reversible by cleaning or irreversible, leading to a decrease in membrane performances.

One of the goals of membrane filtration optimization is to avoid fouling to keep membrane performance across time. Fouling may be prevented by choosing a TMP below a critical pressure of which the flux is no longer proportional to the TMP (Bacchin et al. 2006). The filtration of pure water has a flux density proportional to the TMP, as shown in the equation below. During the filtration of a suspension, the flux is proportional to the TMP at low pressure until the deposit becomes important, and the flux is no longer proportional to the TMP, until that J no longer increases with an increase in TMP.

The selectivity is also a key parameter to be optimized for membrane filtration and refers to the membrane property of retaining or not a compound and is defined by the retention rate. Several methods are possible to define the retention rate, in this work it is the observed retention rate RR, characterized experimentally by:

$$RR = 1 - \frac{C_p}{C_R} \quad \text{Equation 2.14}$$

With RR (%), C_p the permeate concentration of a compound (g.L^{-1}), C_R the retentate concentration of a compound (g.L^{-1}), in the bulk solution.

Depending on the objectives, the compound RR can be aimed at being high when the compound needs to be concentrated or low to get rid of a small size compound, or to purify the small size compound of interest from bigger contaminants.

Cleaning is an important criterion in membrane filtration and should not be overlooked on the long-term system operation. The membrane water permeability must be measured before filtration and after cleaning to measure a potential modification of the membrane properties. However, cleaning optimization was not realized during this study.

2.7.3.3 Filtration modes

Membrane filtration can be performed in several modes to filtrate a suspension such as in batch, as seen in Figure 2.8, in fed-batch or in continuous.

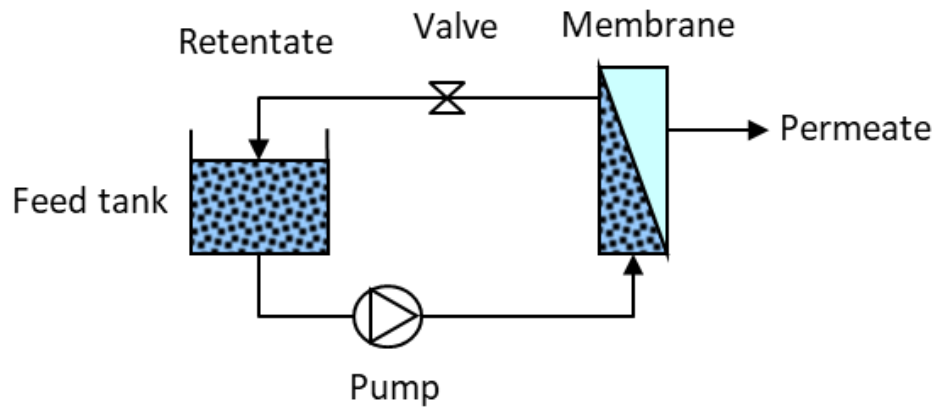


Figure 2.8 Scheme of filtration in batch mode

The volume reduction during a concentration in batch can be described by the Volume Retention Rate (VRR) calculated as:

$$VRR = \frac{V_0}{V_r} \quad \text{Equation 2.15}$$

With V_0 the initial suspension volume and V_r the retentate volume.

The filtration can also be performed in diafiltration which consists of the addition of a solution, such as water, as a function of the permeate flow, as seen in Figure 2.9. It is often realized to remove small molecules or salts from the compounds of interest.

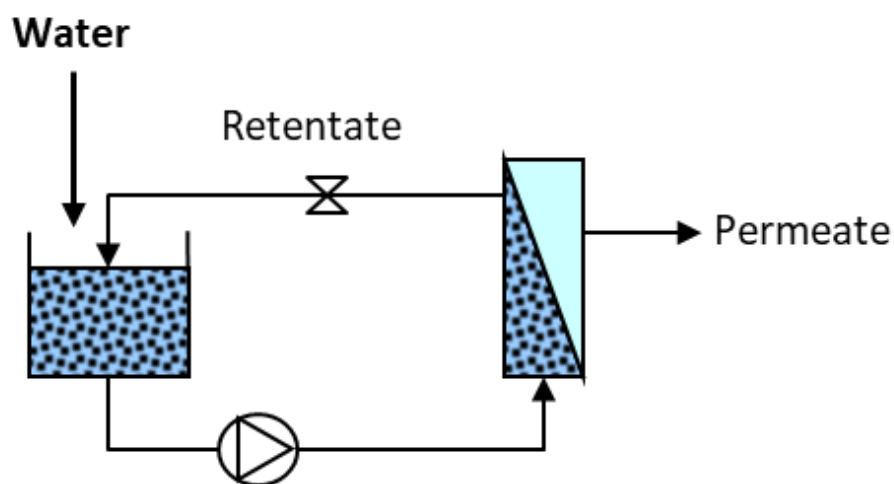


Figure 2.9 Scheme of diafiltration.

The diafiltration is often performed at a constant retentate volume and the diafiltration operation can be described with the diavolume DV calculated as:

$$V_w = V_p \quad \text{Equation 2.16}$$

$$DV = \frac{V_w}{V_r} \quad \text{Equation 2.17}$$

With DV the diavolume number, V_r and V_p the retentate and permeate volumes respectively and V_w the added solution volume.

2.7.3.4 Membrane filtration and microalgae biorefinery

Membrane filtration has been used for different goals in the microalgae field and was well studied for harvesting and/or media recycling (Rossi et al. 2004; 2008; Babel et Takizawa 2010; Wicaksana et al. 2012). In microalgal biorefinery, however, the studies are less frequent and the necessity to improve these processes has been outlined by several studies (Gerardo, Oatley-Radcliffe, et Lovitt 2014; Soto-Sierra, Stoykova, et Nikolov 2018; Grossmann, Hinrichs, et Weiss 2020; Bussa, Zollfrank, et Röder 2021). In microalgal biorefinery for protein extraction, membrane filtration was used for several objectives:

- The removal of cell debris containing chlorophyll and the increase of extract purity.
- The concentration of the proteins

The elimination of the cell debris has been studied by some studies for protein purification after cell disruption by bead milling or HPH and mild centrifugation. The cut-off used ranged from 100 kDa to 0.45 μm (Safi, Liu, et al. 2014; Garcia et al. 2018) and different systems were used, from manual dead end filtration (Garcia et al. 2018), agitated dead-end filtration (S. Liu et al. 2021; 2022), tangential filtration (Ursu et al. 2014; Safi, Liu, et al. 2014; I. Gifuni et al. 2020; S. Liu et al. 2022) to dynamic filtration (S. Liu et al. 2022). In most cases, a high protein retention rate was observed after cell disruption and mild centrifugation (RRprot 78% (I. Gifuni et al. 2020), RRprot 85-90% (S. Liu et al. 2021), 85-87% (S. Liu et al. 2022)). A low colored permeate was obtained with a cut-off equal or lower than 0.22 μm in the different studies (Safi, Liu, et al. 2014; I. Gifuni et al. 2020; S. Liu et al. 2021; 2022) and chlorophyll was present in the permeate after a MF at 0.45 μm (Garcia et al. 2018).

Several protein fractions are present in the supernatant after cell disruption, with “free” proteins and aggregated proteins with other compounds, including chlorophylls. This is particularly visible on results from Ursu et al. (Ursu et al. 2014) who obtained a protein yield of 98% in the supernatant after HPH at pH 11 and mild centrifugation, as shown by the Size Exclusion Chromatography (SEC) result in Figure 2.10.

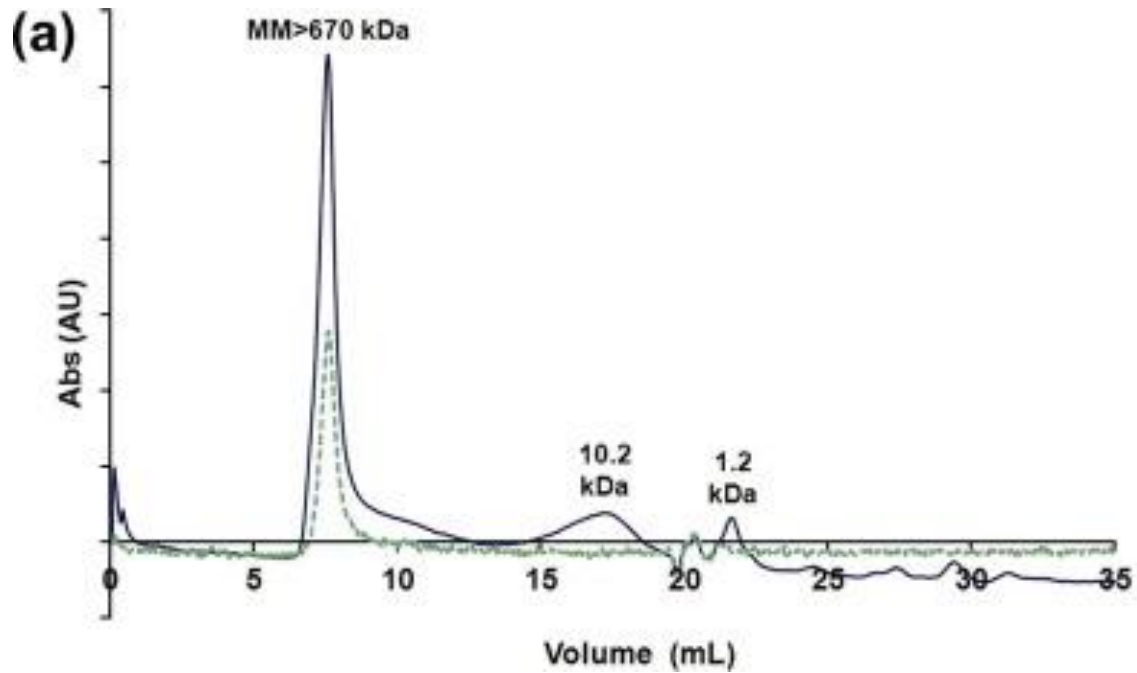


Figure 2.10 SEC chromatogram from (Ursu et al. 2014) of 300 kDa retentate after cell disruption and centrifugation. The proteins are detected using A280 (-) in black and the chlorophylls are detected at A649 (--) in green.

While the fractions at 1.2 kDa and 10.2 kDa get through the membrane and were not linked with chlorophyll, the major fraction with high molecular weight (>670 kDa) seems linked with chlorophylls. The used cell disruption process dispersed nearly all the proteins, including the membrane proteins associated with chlorophylls. The coupling between bead milling and microfiltration for soluble protein extraction and purification is interesting but still necessitates improvement.

Protein concentration by membrane filtration has also been studied by several authors and can be a promising operation unit to concentrate before a possible drying step and purify proteins from other compounds such as salt. Several cut-offs have been studied, 3 kDa (Kulkarni et Nikolov 2018; I. Gifuni et al. 2020) and 10 kDa (Safi, Liu, et al. 2014; Garcia et al. 2018) and 300 kDa (Garcia et al. 2018). Ultrafiltration with a cut-off of 3 kDa had excellent protein retention (Kulkarni et Nikolov 2018; I. Gifuni et al. 2020), with a protein RR of 91% on proteins from *C. vulgaris* (I. Gifuni et al. 2020). A cut-off of 10 kDa was able to retain all proteins while discarding part of the carbohydrates, enhancing the protein purity of the extract (Safi, Liu, et al. 2014). However, this was not the case for proteins extracted from *T. suecica* with a protein yield on the operation unit of 30% in the retentate and 52% in the permeate (Garcia et al. 2018). A high peptide content was also noticed, with a low retention rate during dialysis at 14 kDa of other microalgal extracts (Schwenzfeier, Wierenga, et Gruppen 2011; Teuling et al. 2017). A protein yield on the operation units of 62% and 66% were retained on *T. impellucida* extracts, and independently of the protein yield after bead milling of 21% and 41% respectively (Schwenzfeier, Wierenga, et Gruppen 2011; Teuling et al. 2017)

Several reasons may explain differences in protein retention such as a difference in protein extract, the presence of free amino acids (Derrien et al. 1998), although negligible in comparison with protein amino acids (Araya et al. 2021), or the presence of proteases degrading the extracted proteins. Nevertheless, the use of ultrafiltration can be very interesting to concentrate and purify proteins.

2.8 Experimental Strategy

Based on the literature review, we can conclude that to extract intracellular proteins from microalgae, two main strategies can be applied, as seen in Figure 2.11.

The first one is to perform a very soft cell disruption or permeabilization to extract proteins from aqueous part of the cells, mostly the cytoplasm, and avoid the dispersion of thylakoid membrane to limit green chlorophyll contamination of the samples. In this case, after clarification to remove cell fragments, a concentration can be directly performed by isoelectric precipitation (IEP) or by ultrafiltration (UF) and the main compounds to be removed should be polysaccharides or salts. In this first case, the protein yield will be limited because the proteins present inside organelles may not be recovered, but the number of operation unit is limited and the side-products should be easier to use for the partners.

The second strategy is to break the whole cell to access most of the proteins to recover a high protein yield but also to control the conditions to allow an appropriate clarification. In this case, a part of the green color due to chlorophylls should be present in the supernatant and an additional separation step is necessary. A microfiltration could potentially separate the green aggregates from the uncolored proteins before concentration but with a cut-off smaller than 0.45 μm . The IEP can also be interesting but led to a green protein precipitate in previous studies on other algae (Cavonius, Albers, et Undeland 2015), so it needs to be tested on the specie of interest.

To select the best strategy, the first step is to evaluate the performances of the cell disruption processes: bead milling (BM) and pulse electric fields (PEF). An in-depth investigation on the BM and a complementary study of PEF is performed in Chapter 4, where the consequences of physiological state of the microorganisms are highlighted. Based on the results of Chapter 4, the first strategy is deepened in Chapter 5 on *T. chui*, and studied from laboratory scale to semi-industrial scale with the aim to produce fractions for Profuture partners. The second strategy is also evaluated at laboratory scale.

After a presentation of the general materials and methods in the Chapter 3, the results are presented in Chapters 4 and 5 before a general conclusion in Chapter 6 on the strategy to adopt to produce enriched-protein extracts from microalgae.

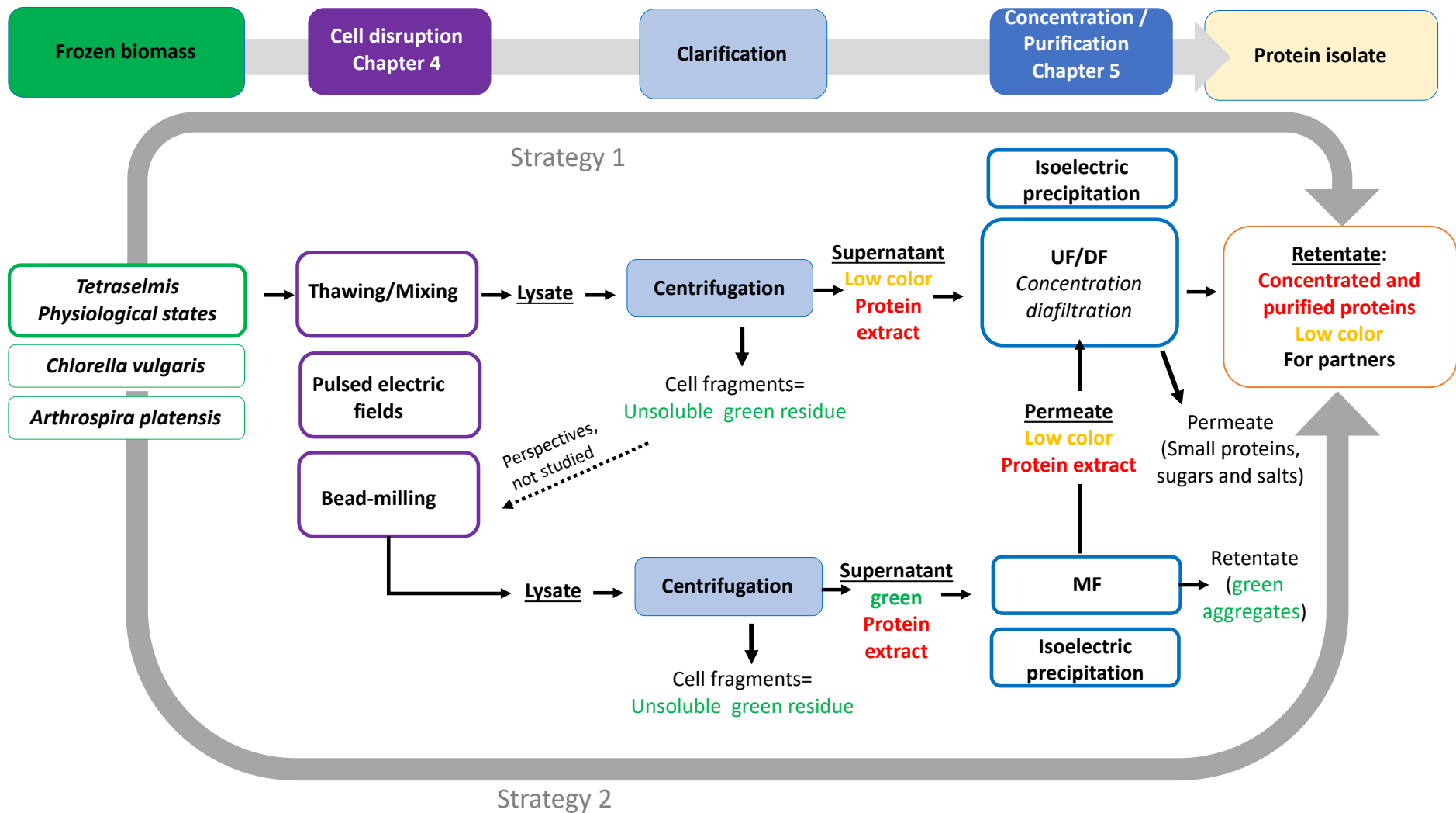


Figure 2.11 Experimental strategy

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Chapter 3 Materials and methods

This chapter describes the materials and methods used during the thesis. The aim of this study was presented in figure 2.11 in chapter 2.

In the following paragraphs will be presented the biomass treated during this work, the cell disruption and purification processes, and the analysis techniques used to evaluate the processes performances.

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3.1 Biomass

The biomass was produced by partners of the Profure project or by Algosource.

Tetraselmis chui was grown photoautotrophically in tubular photobioreactors by Necton (Portugal) under solar conditions. The biomass was harvested by centrifugation to obtain a paste at 20%_{DM}, conditioned in 1 kg plastic bags and frozen immediately. Several batches were received. Two biomass batches were treated by bead milling as frozen paste at least 20%_{DM} from the producer and called batch A, produced in December 2019 and batch B, produced in May 2020. They were characterized by microscopy and biochemically in terms of proteins, carbohydrates and ashes, following protocols in the analysis sub-section.

Chlorella vulgaris was grown heterotrophically in Allmicroalgae facilities (Portugal). The heterotrophic cultivation leads to a very concentrated culture (150-200 g/L) which is directly conditioned as a frozen paste at 15%_{DM}.

Arthrospira platensis was grown autotrophically at Algosource facilities (France) in open raceways, harvested by vibrating sieving, concentrated by a hydraulic press and conditioned in plastic bags as a frozen paste at 20%_{DM}.

3.2 Cell disruption

Three methods were compared to perform the cell disruption: a simple thawing of the frozen biomass or the coupling of thawing and bead-milling or pulsed electric fields.

3.2.1 Freeze-thawing and suspension preparation

The initial paste at 20%_{DM} was diluted at 10% and thawed overnight for the different scales with different biomass quantities presented in **Table 3.1**.

Variations of dry matter were present and the percentage provided by the suppliers was assumed for the preparation of the suspension at 10%. An experimental measure of the dry matter content was performed on the suspension after thawing to assess it and correct it if needed.

A possibility could have been to measure it beforehand on the paste but it was unreliable because of the cryoconcentration of the dry matter due to freezing. The principle of cryoconcentration is that when ice crystals appear, they expel impurities to build up pure crystals during the freezing process. The solution around the ice crystals is hence concentrated (Nakagawa, Maebashi, et Maeda 2010). In our case, a small sample of the biomass at the periphery of the paste was not representative of the whole paste.

The laboratory scale thawing was done in a 5 L beaker, the pilot scale was done in 20 L buckets and the semi-industrial was in a tank of 100 L.

Table 3.1 Different masses of paste of *T. chui* and suspension as well as the dry matter concentration in the suspension for the different scales.

	Chapter 4		Chapter 5		
Operation unit/Scales	PEF	BM	IEP / UF lab	pilot	semi-industrial
Batch of biomass used	A	A&B	A	C	D & E
Mass of paste (kg)	0.04 tot.	4 tot.	0.7-0.75	6	31 - 25 (tot. 56)
Mass of suspension (kg)	0.38 tot.	13.3 tot.	2.5	20	163 – 116 (tot. 279)
Dry matter concentration (g _{DM} .kg ⁻¹)	10 – 30	60	50	90 (error)	50 - 60
Thawing temperature (°C)	4°C	4°C	4°C	4°C	20°C
Suspension agitation	Magnetic stirrer + baffles			Manually	Pale stirrer

3.2.2 Bead milling

The bead mill used was a Dyno-mill multi-lab of Willy A Bachofen AG (Muttens, Switzerland) with a milling chamber of 0.6 L. It was supplied in biomass with a peristaltic pump with a flow rate calibrated with the suspension in order to take into account the density. The bead mill was cooled by a double wall supplied with glycerol with an external cooler in order to limit the product temperature to 25°C and the system can be seen in **Figure 3.1**. The bead millings were operated in pendulum mode.

In all experiments, the bead diameter was chosen at 0.5-0.7 mm on the basis of previous experiences and based on literature (Postma et al. 2017; Garcia et al. 2018; Zinkoné et al. 2018; S. Liu et al. 2021; 2022). A smaller diameter could have been chosen but the spacer separating the beads from the product leaving the chamber was too wide for smaller beads if a scale-up at pilot scale was realized. The bead material in yttrium-stabilized zirconium with a density of 6 g.cm⁻³ was also kept and chosen for its food compatibility and high life expectancy. A bead filling ratio of 80% was selected as this was considered as optimal in several studies on microalgae cell disruption (Montalescot et al. 2015; Suarez Garcia et al. 2019). A same dry matter concentration at 60 g_{DM}.kg⁻¹ was chosen for the different biomasses. A higher dry matter could have decreased energy consumption (Bussa, Zollfrank, et Röder 2021). However, lower protein yield in the supernatant could be observed (Postma et al. 2015) and the supernatant distribution is lowered, necessitating a dilution before the centrifugation (S. Liu et al. 2021). Moreover, the bead mill has a large volume milling chamber so a significant amount of biomass

would have been needed. Further optimization of this dry matter concentration could be explored in the future.

The rotational speed of the impellers was fixed at 14 m.s^{-1} for the Eukaryotic microalgae *T.chui* and *C.vulgaris* because of their tougher cell wall while it was fixed at 8 m.s^{-1} for *A.platensis*. The flow rate was fixed at 200 mL.min^{-1} for *T.chui* while a lower flow rate of 150 mL.min^{-1} was chosen to compare to previous studies and to preserve the pump.

Cell disruption, dry matter, protein, and Particle Size Distribution (PSD) were measured every pass by the methods presented in the analysis section. The proteins extracted of *Chlorella* and *Tetraselmis* were analyzed by SDS-PAGE to determine the electrophoretic differences and the presence of proteases evaluated by a zymogram.

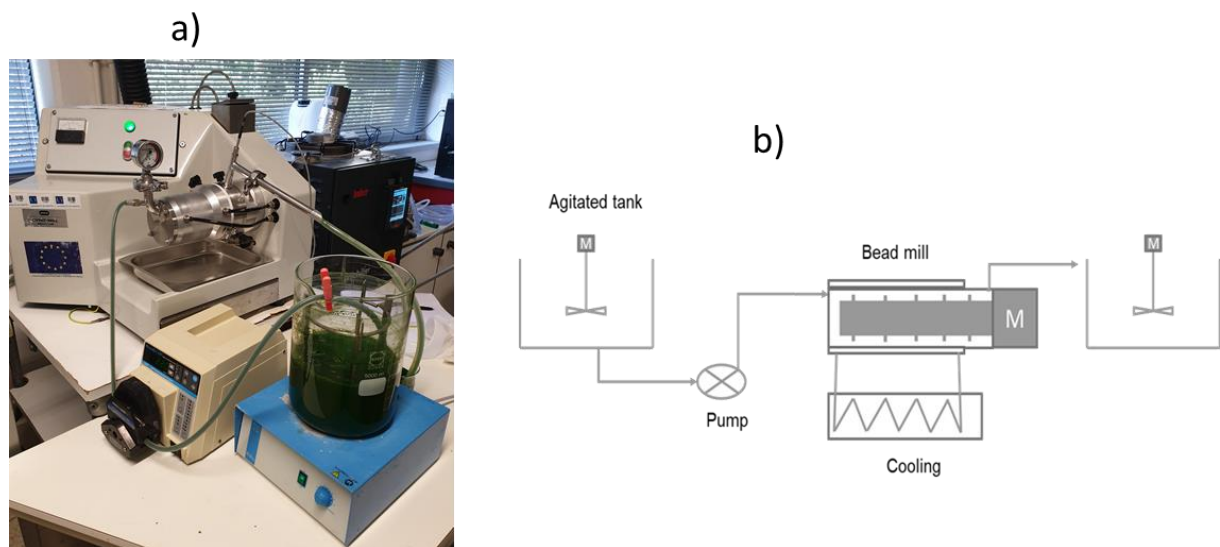


Figure 3.1 a) Image of the bead mill associated with the cooling system and the peristaltic pump, and b) the process scheme.

3.2.3 Pulsed Electric Fields

The PEF treatment was realized in a PEF-Cellcrack III equipment (Elea-Technology, Quakenbrück, Germany) seen in Figure 3.2, with a fixed maximum voltage of 30 kV and a fixed pulse duration of $40 \mu\text{s}$. The treatment was realized in batch at small pilot scale in a 0.2 L chamber with an electrode gap of 2 cm and a maximum electric field strength possible of 15 kV.cm^{-1} . This electric field strength was chosen to ensure a permeation for most of the algae (Pataro et al. 2017; Carullo et al. 2018) but also to be able to be scaled-up at industrial scale. Moreover, it was the highest possible by the equipment.

The electric field strength (E , kV.cm^{-1}) and total input specific energy (W , in $\text{kWh.kg}_{\text{DM}}^{-1}$) were deduced from the applied voltage, the chamber used, the number of pulses and the dry matter of the

suspension, as described by Parniakov et al. 2015 (Parniakov et al. 2015). The treatments of suspensions with different dry matter were performed at $10 \text{ g}_{\text{DM}}\cdot\text{kg}^{-1}$ and $30 \text{ g}_{\text{DM}}\cdot\text{kg}^{-1}$ for *T.chui* and $10 \text{ g}_{\text{DM}}\cdot\text{kg}^{-1}$, $30 \text{ g}_{\text{DM}}\cdot\text{kg}^{-1}$ and $60 \text{ g}_{\text{DM}}\cdot\text{kg}^{-1}$ for *C.vulgaris*.

The pulse number was fixed at 50 while total specific energy W was set by varying the applied voltage E to the suspension. After each series of 50 pulses, a sample was collected and the temperature was measured. The warming of the suspension is inherent to the treatment due to the Joule effect and it was controlled manually. The suspension was cooled in a cool water, itself cooled by an external thermal exchanger bath, if the suspension temperature reached 40°C to limit the potential degradation of the proteins. Moreover, the temperature was quickly decreased due to the low volume in comparison with the bead milled lysate ($> 2.5 \text{ L}$). The cell electroporation was followed by measuring the suspension conductivity before and after treatment by a conductimeter (Mettler Toledo, Columbus, Ohio, USA). The electroporation was also followed by Evan blue staining as shown in the analysis section.



Figure 3.2 Pulsed Electric Field equipment used.

3.3 Clarification by centrifugation

3.3.1 Context

Centrifugation is an important operation unit in the process since it ensures a primary clarification after cell disruption and the centrifugation efficiency and reliability ensure the success of the following purification units. Three scales were considered for the centrifugation: laboratory, pilot scales, and semi-industrial scale. The clarified supernatant obtained by centrifugation was then characterized and used for the following purification processes, while the pellet was frozen for storage. This coproduct was not considered afterwards in this work.

3.3.2 Laboratory and pilot scale

The centrifugations at laboratory and pilot scale were realized at the GEPEA on a Sorvall Lynx 6000 superspeed centrifuge (Thermo Scientific) at room temperature.

The operating conditions were 5,000 g for 4 min in 50 mL Falcon tubes for the characterization of the cell disruption performances and to produce supernatant for isoelectric precipitation. To produce supernatant for filtration experiments, at laboratory and pilot scale, it was realized at 5,000 g for 6 min in 900 mL pots. The increase in centrifuge residence time being necessary since the width of the pot was larger and a common residence time on 50 mL tubes and 900 mL pots could have led to differences in supernatant clarification. The operating conditions were chosen for their compatibility for a possible scale-up of a semi-industrial scale centrifuge.

3.3.3 Semi-industrial scale

The centrifugation at semi-industrial scale took place in Algosource (Asserac) using a Westfalia KA6-86-076 concentric chamber centrifuge. It had a working volume of 9 L and a RCF of 7,400 g. The centrifuge was operated in feed batch mode, with the agitated suspension added to the centrifuge by a peristaltic pump, and the pellet emptied when the chamber destined to the solid residue was filled (appearance of green color in the supernatant). A flow rate of 86 L.h⁻¹ was selected and the centrifugation was stopped, to remove the pellet.

At this scale, the heat generated was important, with an increase in temperature from 9 °C to 35 °C at the end of the centrifugation. The supernatant was cooled down to 4 °C using an external heat exchanger and then stored at 4 °C. This issue was not observed at the lower centrifugation scale.

3.3.4 Supernatant processing

At laboratory scale the centrifugation was performed in 30 min while it was performed in 2 h at pilot scale. At semi-industrial scale, the centrifugation was processed in one day.

In addition, at laboratory and pilot scale, the supernatant was directly processed to the next operation unit after centrifugation. However, the semi-industrial scale centrifugation being performed at Asserac, a transportation of the supernatant of 40 min was needed before storage overnight at 4 °C before filtration.

3.3.5 Calculations

Mass compound recovery yield (Y) was defined as the ratio between the mass of the considered molecules in the supernatant, for example proteins ($m_{p.Sup}$), and the mass of the same molecule in the lysate, ($m_{p.Lysate}$):

$$Y_p = \frac{m_{p.Sup.}}{m_{p.Lysate}} = \frac{C_{p.Sup.} \times V_{Sup.}}{C_{p.Lysate} \times V_{Lysate}}$$

with the supernatant protein concentration $C_{p.Sup}$, the volume of supernatant V_{sup} , the lysate protein concentration $C_{p.lysate}$, the lysate volume V_{Lysate} .

The efficiency of the separation of the supernatant from the lysate during the centrifugation was defined by the volume ratio ϕ between the supernatant volume and the suspension volume (S. Liu et al. 2021).

3.4 Concentration and purification of the proteins

Two processes were compared on *T.chui* for the concentration and the purification of the proteins from the supernatant after cell disruption by thawing: the isoelectric precipitation and the ultrafiltration. A complementary test of microfiltration after cell disruption by bead-milling was conducted to drive perspectives. These three steps are detailed in the following paragraphs.

3.4.1 Isoelectric precipitation

The isoelectric precipitation was tested at laboratory scale.

A suspension of *T.chui* at 50 g_{DM}.kg⁻¹ was prepared on batch A and centrifuged at 5000 g for 4 min to recover the supernatant S0-A and pH, conductivity and dry matter were characterized. The supernatant was then redistributed in 50 mL Falcon tubes and the pH acidified with hydrochloric acid at 1M under agitation, and the pH was measured across the experiment and adjusted if needed (tampon effect of the proteins). The selected pH were 2, 4, 5 and 6 for the first experiment and the pH range narrowed to pH 2, 2.5, 3 and 3.5 and a control at native pH was also present. The added acid volume was considered negligible in protein concentration modification. The acid was added while maintaining the tube agitated to avoid a local zone with a very low pH which could denature proteins, affecting their solubility, but mixing was stopped after acid addition to allow protein aggregation.

The supernatant S1-A was sampled after settling for one hour at room temperature.

The proteins were quantified in triplicate by BCA in the supernatant S1-A without centrifugation and pH modification and after centrifugation at 11,000g for 10 min leading to the supernatant S2-A. The turbidity of the supernatant S1-A before centrifugation was measured at 600 nm with a spectrophotometer (Jasco V630).

The proportion of proteins still soluble after pH modification at pH X was expressed as:

$$Protein\ solubility\ (pHX) = \frac{[Proteins]_{S2-A\ pHX}}{[Proteins]_{S0-A\ initial\ pH}} \quad \text{Equation 3.1}$$

3.4.2 Ultrafiltration

The membrane filtration was performed after cell disruption and clarification with two objectives: (1) to concentrate the proteins and (2) to eliminate the salts using the diafiltration mode.

The process was performed at the laboratory, pilot and semi-industrial scales at 10 kDa. The choice of the cut-off was based on previous results of native electrophoresis. The material and methods used for the three scales are detailed below.

The materials and methods of the different scales are summed up in table 3.3.

Table 3.2 Summary of the parameters used for the different scales with in green the suspension preparation, in orange the centrifugation and in blue the ultrafiltration.

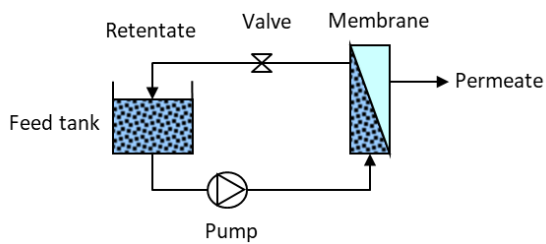
Scale	Laboratory	Pilot	Semi-industrial
Batch of biomass	A	C	D and E
Dry matter (kg)	0.139	1.7	10.2
Paste (20% DM)	0.695	8.5	51
Suspension mass (kg)	2.9	19.8	163
DM susp (g _{DM} .kg ⁻¹)	49	91	55
Agitation type	Magnetic stir bar		Pale stirrer rotated by compressed air
Centrifuge type	Sorvall Lynx 6000 superspeed centrifuge		Westfalia KA6-86-076
Centrifuge volume	6 x 0.9L		9L
Treated supernatant	S0-A-1 S0-A-2	SO-C-1	S0-D-SI 1, S0-E-SI 2
Experiments	L1, L2	P1	SI1, SI1', SI2
Membrane supplier	Synder		Koch
Geometry	Plane	Spiraled	Hollow fibers
Material	PolyEtherSulfone (PES)		PolySulfone (PS)

Surface (m ²)	0.013	0.269	2.3
Temperature (°C)	30	12	12
Cut-off (kDa)	10 kDa		
Crossflow speed (m.s ⁻¹)	1		
TMP (bar)	1		

For the different experiments the following steps were considered:

- membrane conditioning
- water flux with clean membrane
- measurement of the critical pressure and selection of the used transmembrane pressure
- cleaning of the membrane
- concentration step to reach the appropriate volume reduction rate ($1 < VRR < 3$) (figure 3)
- diafiltration for purification with the appropriate number of diavolumes ($0 < DV < 2$) (figure 3)
- membrane cleaning
- verification of the water flux recovery

Concentration in batch:



Diafiltration:

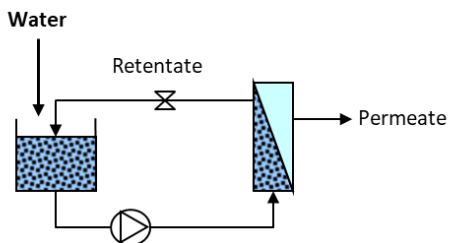


Figure 3.3 Filtration modes: concentration (a) and diafiltration (b)

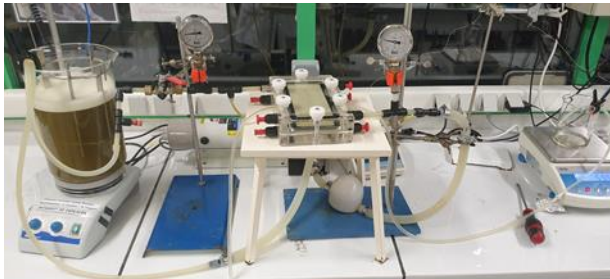
The flux density was standardized with the temperature at all scales by calculating the viscosity as a function of the temperature, as shown as follows (Kestin, Sokolov, et Wakeham 1978):

$$\log\left(\frac{\mu(T)}{\mu(T_{nom})}\right) = \frac{20-T}{T+96} * (1.2348 - 0.001467 * (20 - T)) - \frac{20-T_{nom}}{T_{nom}+96} * (1.2348 - 0.001467 * (20 - T_{nom}))$$

equation 3.2

With T the temperature measured, T_{nom} the normalized temperature, μ the viscosity at the temperature T and $\mu(T_{nom})$ the viscosity at the temperature T_{nom} . The viscosity of the permeate was assumed to be equal to the viscosity of water since it contains less than 2% of dry matter.

Differences of equipment and operation conditions, as seen in **Figure 3.4** during the experiments were due to the differences in the volumes treated and the operation time and are detailed in the following paragraphs.



Laboratory



Pilot



Semi-Industrial

Figure 3.4 Images of the filtration systems at laboratory, pilot and semi-industrial scales.

3.4.2.1 Laboratory scale

3.4.2.1.1 Equipment

At laboratory scale, the cross-flow ultrafiltration was carried out in a plane system, the Rayflow (Orelis), fed by a peristaltic pump, with a flat sheet membrane having a surface of 0.013 m². The membrane was in PolyEtherSulfone (PES) with a cut-off of 10 kDa from Synder and a spacer with a diamond geometry and a thickness of 1 mm. The system can be seen in **Figure 3.4**.

The same membrane was used for all the experiments and cleaned between the different trials. The mass, the pressure and the temperatures were recorded online by homemade software on LabVIEW.

3.4.2.1.2 Conditioning

Before usage, the lab scale membrane was gently rinsed with 96% ethanol and water to remove the glycerol, conditioned in the system and cleaned by a basic bleach (10 ppm NaClO at pH10.5) at 30 °C for 30 min at 1 bar, followed by water rinsing until no pH variation was seen in the permeate. This step was followed by a cleaning with 0.2% (v/v) Ultrasil110 (Ecolab, USA) at 45 °C for 30 min at 1 bar before final water rinsing at 30°C for at least 30 min.

Compaction was realized at a PTM of 1 bar for 12 h with DI water at room temperature until no decline in flux was observed.

3.4.2.1.3 Cleaning

The membrane was first rinsed with Deionized (DI) water in an open system for 30 min. It was followed by a cleaning with 0.2% (v/v) Ultrasil110 (Ecolab, USA) at 45 °C for 30 min at 1 bar before final water rinsing at 30°C for at least 30 min. Then the system was washed by a basic bleach cleaning (10 ppm NaClO at pH 10.5) at 30 °C for 30 min at 1 bar, followed by water rinsing until no pH variation in the permeate.

3.4.2.2 Pilot scale

3.4.2.2.1 Equipment

At pilot scale, the cross-flow ultrafiltration was carried out in a membralox Xlab5 pilot from Pall which was revamped to adapt a spiral membrane of 0.269 m². The membrane material was PES, conserving a MWCO of 10 kDa and the membrane was supplied by Synder. A spacer with a diamond geometry with a thickness of 1.17 mm was used to promote turbulence. The system can be seen in Figure 3.4. The system was disinfected before use with a disinfectant approved for food contact and rinsed with deionized water.

3.4.2.2 Conditioning

The procedure to install and clean the membrane to remove the preservative solution was realized following the advices of the provider (Synder). In summary, the membrane was rinsed with Deionized water at 50°C in non-recirculating mode for 30 min. A caustic wash was also realized at pH 10 at 46°C for 30 min and a water rinse to neutral pH.

3.4.2.3 Cleaning

The cleaning procedure applied was given by the supplier and was processed as follows. The membrane was rinsed in the system with DI clean water at 50 °C in non-recirculation mode to remove the remaining fouling. Then water at 50 °C was circulated in the system at a PTM of 1 bar and a retentate circulation flow rate of 0.8 m³.h⁻¹ was selected as advised by the supplier to ensure a cross-flow speed of 1 m.s⁻¹. Sodium hydroxide was added slowly to achieve a pH of 10.5 and the solution was circulated in the pilot for 30 minutes. The solution was discarded and flushed with clean water. The presence of an orange compound found in the paste seemed to be a mineral since it was also found in the ashes. Hence, an acid-cleaning step was added to avoid mineral fouling. A solution of hydrochloric acid at pH 2.2 and 50°C was circulated for 30 min in the system at a pressure of 1 bar. The solution was discarded and the system was rinsed with hot DI water.

Warm water was added and circulated in the system, then sodium hydroxide was added until pH 10.5, followed by the addition of chlorine until a concentration of 150 ppm. The solution was circulated for 30 min, discarded and flushed with clean water until the pH in the system returned neutral.

3.4.2.3 Semi-industrial scale

3.4.2.3.1 Equipment

The crossflow ultrafiltration was carried out in a Polypilote supplied by Orelis at semi-industrial scale at the Algosolis platform. The membrane was supplied by Koch, it was made of PolySulfone (PS) with a cut-off of 10 kDa, a surface of 2.3 m² and a hollow fibers geometry. The membrane is able to withstand a pH range of 1.5 to 13 at 54°C maximum in operating conditions. A flow rate of 3000 L.h⁻¹ was chosen to ensure a cross-flow speed of 1 m.s⁻¹. The system can be seen in Figure 3.4. The system was disinfected before use with a disinfectant approved for food contact and rinsed with deionized water. Such filtration experiments coincided with the first use of the filtration unit so efforts have been needed for training and know-how acquisition.

3.4.2.3.2 Conditioning

The membrane was cleaned before the experiments following the advice of the supplier. The membrane was first rinsed with DI water and then cleaned with a sodium hydroxide solution at pH 10 and 50°C in circulation for 15 min at a TMP of 1 bar. Following this step, the solution was discarded and the system was flushed with DI water at 50°C for 10 min, with more than three times the hold-up volume. The system was washed at 1 bar for 15 min with a sodium hydroxide solution at pH 10, 50°C and 180 ppm of total chlorine. The solution was washed with DI water at 50°C until the pH in the system became neutral.

3.4.2.3.3 Cleaning

The membrane was washed following the instructions of the supplier. A DI water rinsing at 50°C was applied before cleaning with a solution at pH 10 at 50°C and with U110 at 0.2% for 15 min at 1 bar. The cleaning solution was discarded and the system was rinsed with DI water at 50°C for 10 min at 1 bar. The membrane was washed for 15 min at 1 bar with a sodium hydroxide solution at pH 10 and 50°C completed with chlorine until a concentration of 180 ppm of total chlorine. After cleaning, the solution was discarded and the system rinsed with DI water until the pH became neutral.

3.5 Coupling of bead milling, centrifugation and microfiltration

Taking into account the results on the fragile cells from batch B that were acquired lately during this work and presented in chapter 4, it was also decided to evaluate the coupling of bead-milling and microfiltration through laboratory scale experiments to recover proteins.

The bead milling experiment was conducted in the same conditions as described in chapter 4 on fragile cells. Briefly, the chamber was filled with 80% of beads having a diameter of 0.5-0.7 mm, in Yttrium stabilized zirconia. The dry matter content of the suspension was 60 g_{DM}.kg⁻¹ and the bead mill temperature was controlled by an external exchanger so that the product temperature stayed lower than 25 °C.

After cell disruption, the lysate was diluted by half to facilitates the separation between the supernatant S_{BM} and the pellet during centrifugation according to results from chapter 4 and Liu et al since the dilution allowed a higher protein yield and a filtration more efficient (S. Liu et al. 2021)ref). The centrifugation of the samples for analysis was carried out at 5,000g for 4 min in the 50 mL tubes while the entire suspension was centrifuged at 5,000g for 6 min in the 900 mL pots for the microfiltration.

The cross-flow microfiltration was carried out using the Rayflow system described in the previous paragraph. The membrane selected for this study was a plane membrane in PES (KOCH) with a cut-off of 0.1 μm and a surface of 0.013 m^2 . This cut-off was chosen with the results of particle size distribution within the supernatant and to compare the performances of protein purification with former studies from Liu et al (S. Liu et al. 2021; 2022). The membrane was prepared with the same protocol described for the laboratory scale ultrafiltration membrane.

The filtration of the supernatant S_{BM} was realized at a TMP of 0.3 bar and with a crossflow speed of 1 $\text{m}\cdot\text{s}^{-1}$ and a temperature of 25°C. The TMP and cross-flow speed were chosen according to results of Liu et al. (S. Liu et al. 2021; 2022).

3.6 Analysis

The objectives of the analysis were to quantify the components from the whole biomass and the fractions obtained after processing, as well as to characterize the cell disruption. The results were used to characterize the process performances. More detailed analysis was performed on the recovered protein fractions in order to understand the interaction between molecules and their effect on process performances.

3.6.1 Global characterization

3.6.1.1 Total microbial count

The total microbial count was performed after sampling in sterile equipment and inoculation on Plate Count Agar in a Petri dish incubated at 30°C for 72 hours following the norm EN ISO 4833-1:201. The medium is not selective and widely used in the food industry. The microorganisms counted are often referred as total plate count or total aerobic mesophilic colony count. The number of microorganisms is expressed as colony-forming units (cfu) per mL of liquid sample and provides an estimation of the numbers of microorganisms that can grow aerobically at mesophilic temperatures (Mendonca, Thomas-Popo, et Gordon 2020).

3.6.1.2 Particle size distribution

The particle size distribution was realized by laser refraction using a Malvern MasterSizer 3000. Particle sizes are calculated by measuring the angular variation in intensity of light scattered of a laser beam in the sample. The small particles scatter light at large angles to the laser beam while large particles scatter light at small angles. The Mie theory is used to calculate the particle size as a volume equivalent sphere diameter using the refractive and the absorption indexes.

3.6.1.3 Cell disruption rate

Cell disruptions of *Tetraselmis* and *Chlorella* were characterized as described by Zinkoné et al. (Zinkoné et al. 2018). Briefly, cell counting was performed using image analysis with ImageJ software. The images were taken with a Carl Zeiss optical microscope equipped with an AxioScope A1 camera, after fixation with Lugol iodine to enhance the contrast. A sufficient number of pictures (>20) were taken to ensure statistical representability.

The cell disruption rate Dis was calculated as follows:

$$Dis = 1 - \frac{N_t}{N_0} \quad \text{Equation 3.3}$$

With N_t and N_0 the intact cell number at milling residence time t and initially respectively.

The cell disruption of *Arthospira* is extremely difficult to follow by cell counting since it is a multicellular colony. Its disruption was followed by the increase in optic density (OD) at 560 nm in the cell lysate as the ratio between OD at milling residence time t and the maximum (Chaiklahan et al. 2022).

3.6.1.4 Evan blue staining

An Evan Blue staining was realized on the suspension after PEF treatment to quantify the permeabilization efficiency. The staining solution was prepared with 0.25% Evan blue solution in a 0.1 mM $CaCl_2$ solution. The colorant was added to the suspension before and after PEF with a 1:10 (v/v) ratio, mixed with the pipette and incubated for 5 min. The permeabilized cells were quantified on a Malassez cell at a X400 magnification. If the dye enters the cells, the cells were considered electroporated.

3.6.1.5 Colorimeter

The color of the extracts was characterized using the CIELAB classification in by a colorimeter PCE – XXM20 on freeze-dried samples. The color is expressed by three values $L^*a^*b^*$, as seen in **Figure 3.5**, and aimed at quantifying color perceived by humans (Eissa et al. 2013).

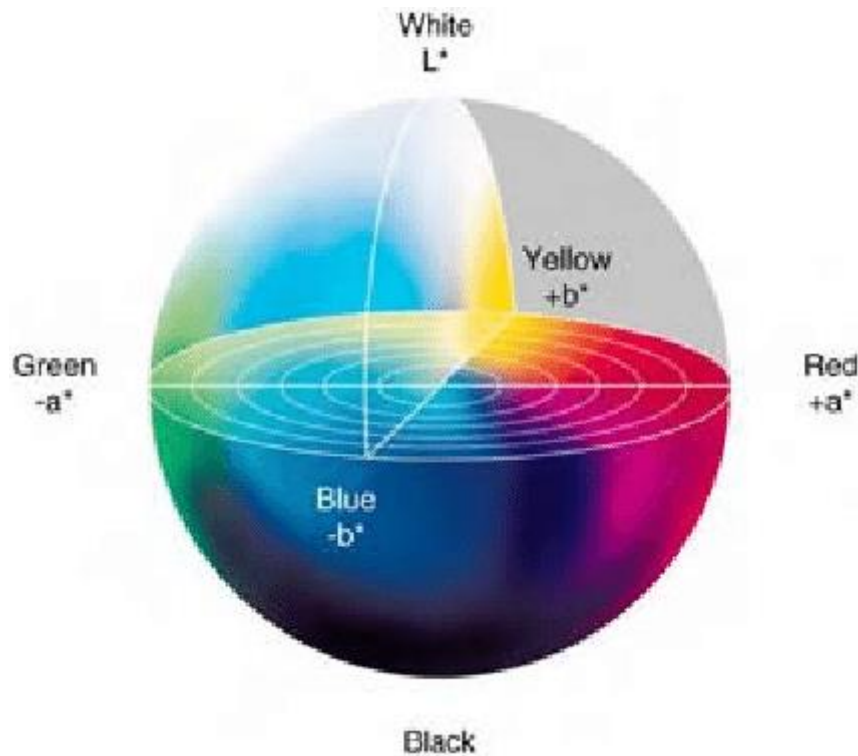


Figure 3.5 CIELAB system (Eissa et al. 2013)

3.6.2 Quantification

The dry matter, the ashes, the proteins and the carbohydrates were quantified all along the processes. The concentration of the different components quantified in the supernatant recovered after 5000g centrifugation on the laboratory centrifuge previously described was defined as 'total' concentration of the corresponding compound. This was used to predict the supernatant obtained after scale-up on an industrial centrifuge with limited residence time and RCF.

An analytical centrifuge at 11,000 g for 10 min in 50 mL Falcon tubes on the laboratory centrifuge previously described was also performed on the supernatant obtained at 5,000 g. The goal was to evaluate the concentration of finer particles, representing the 'soluble' fractions as performed by Liu et al. (S. Liu et al. 2021; 2022).

3.6.2.1 Dry matter

Two methods were used to measure the dry matter content in the samples: the classical method and the thermobalance method. The second one was used to rapidly quantify the dry matter in the thawed biomass prepared at a concentration near 10%w before dilution, to take into account the variation of dry matter content in the frozen paste ($\geq 20\%w$).

3.6.2.1.1 Classical method by oven measurement

A sufficient volume, depending on the dry matter concentration of the sample, was added to crucibles and dried at 105°C for 48h in an oven with forced air ventilation.

3.6.2.1.2 Thermobalance method

After thawing, a sample was taken under agitation to quantify the suspension dry matter by thermobalance. A quantity of 1 g to 2 g of suspension was heated at 105°C until no mass variation was quantified by the thermobalance Mettler Toledo. An overestimation of the dry matter compared to the traditional technique exists but was corrected by a factor acquired experimentally.

3.6.2.2 Ashes

The ash content was evaluated after dry matter determination and consisted of heating the sample at 600°C for 16 h in a muffle furnace following the method of Liu et al. (K. Liu 2019).

3.6.2.3 Proteins

The initial biomass was characterized by diluting the suspension to 30 g_{DM}.L⁻¹ and disrupting it by a high-pressure cell disrupter (HPH, TS series 2.2 kW from Constant Systems Limited, Daventry Northants, UK) 3 passages at 2.7 kbar. The lysate was solubilized in 2 N NaOH at 95 °C for 5 min, mixed with 1.6 N HCl (1v:1v:1v) and subsequently quantified by BiCinchoninic acid Assay (BCA) kit (Thermo Scientific) in 96 well microplates with Bovine Serum Albumin (BSA) as standard. The spectrophotometer used was a Synergy LX from Biotek.

This method is able to characterize biomass without the need for an expensive apparatus. However, this technique can be controversial to realize a mass balance since it can be difficult to quantify the insoluble proteins efficiently (Gerde et al. 2013; Angell et al. 2016). The alkaline treatment can release more proteins and in theory, solubilize them entirely, but the use of a strong basis at hot temperature can also degrade proteins in some algae, affecting their quantification by colorimetric methods (Rausch 1981). The protein quantification by colorimetric methods on the initial biomass was verified with an analysis of the total Nitrogen content with a Dumas method, corrected by a conversion factor of *Tetraselmis* of 4.4 (Lourenço et al. 2004; Schwenzfeier, Wierenga, et Gruppen 2011; Teuling et al. 2017).

The proteins in the liquid fractions were directly quantified by BCA in 96 well microplates with Bovine Serum Albumin (BSA) as standard.

3.6.2.4 Carbohydrates

The carbohydrates in the initial biomass were quantified by the Dubois method (Dubois et al. 1956) on the lysate after cell disruption with HPH three times at 2.7 kbar. The fractions were directly analyzed by the Dubois method. The standard used was glucose and the spectrophotometer was a Jasco V630.

3.6.3 Electrophoresis and zymograms

Electrophoresis and zymograms were used to characterize the proteins from the biomass and the extracted fractions.

3.6.3.1 Sodium Dodecyl Sulfate PolyAcrylamid Gel Electrophoresis (SDS PAGE)

3.6.3.1.1 Principle

This electrophoresis separates proteins as a function of their molecular weight. This is realized by linearizing and charging proteins with SDS and making them migrate through a polyacrylamide gel under an electrical current. After migration, the gel is stained by a protein-selective colorant and rinsed with water to remove the excess. The bands corresponding to the proteins can be compared to standards to evaluate their molecular weight.

The electrophoresis can be realized under non-reducing conditions, in which the disulfide bonds stay intact, or under reducing conditions to break the disulfide bonds. The rupture of disulfide bonds can linearize 3D structure and separate subunits held together by disulfide bonds (Pickles et al. 2014). The electrophoresis was carried out under reducing conditions.

3.6.3.1.2 Materials and methods

The protein composition was characterized by SDS-PAGE with Mini-Protean TGX Precast gels gradient 4-20% using Mini-Protean Tetra Cell system (Bio-Rad Life Science, France). All reagents were from Bio-Rad Life Science, France. All samples were diluted with 2x Laemmli sample buffer in reducing conditions with 2-mercaptoethanol 5%. The migration buffer contained 25 mM Tris, 192 mM glycine, 0.1% SDS according to Laemmli (1970) protocol (Laemmli 1970). The samples were heated at 100°C for 5 min. Each protein sample was then loaded on a sample well of the gradient gel 4-20% polyacrylamide gel electrophoresis. Molecular weight (MW) protein markers from MW 10 to 250 kDa (Precision Plus Protein Standards, All Blue, Bio-Rad Life Science, France) and from MW 14.4 to 116 kDa (unstained Molecular weight marker, Euromedex, Souffelweyersheim, France) were used for MW calibration. The migration was carried out under standard conditions at 200 V for 45 min. The gel was stained with Coomassie Brilliant Blue G-250 staining solution for 2h with gentle agitation on a platform rocker

according to Lawrence and Besir (2009). The gel was rinsed with distilled water before scanning on a flatbed scanner (Image Scanner iii, GE Healthcare Europe, Velizy-Villacoublay, France).

3.6.3.2 Zymogram

3.6.3.2.1 Principle

Zymography is an electrophoretic technique to detect hydrolytic enzymes from the proteins separated by molecular mass. An SDS PAGE is performed on the sample in non-reducing conditions and the gel is embedded with a substrate of which the lytic activity aimed at being shown. In our case we chose gelatin to identify proteases. After electrophoresis, the SDS is removed to allow the separated proteins to recover their active conformation. The gel is incubated and then stained by a gelatine-selective colorant. The gel in presence of gelatin is blue everywhere except in cleared spots, revealing the presence of gelatin-digestion by an active protease.

3.6.3.2.2 Methods

In detail, the protease identification was performed by 10% zymogram which 1% gelatin was incorporated into the gel providing a substrate for proteases. Samples were diluted 50% v/v with a sample buffer in non-reducing conditions and incubated 10 min at 25 °C. Then 10 µg of the sample was loaded in each well and the electrophoresis was run using Tris Glycine SDS buffer migration at 35 mA for 1h30. Molecular weight (MW) protein markers from MW 10 to 250 kDa (Precision Plus Protein Standards, All Blue, Bio-Rad Life Science, France) was used for calibration. After migration, sample proteases must be renatured by placing the gel in renaturing solution containing 2.5% Triton X100 three times for 15 min and rinsed twice for 10 min with MilliQ water. The gel was incubated for 16 h at 37 °C with the development solution phosphate buffer 50mM, pH 7.8. Then the gel was stained with Coomassie Brilliant Blue R-250 0.5% in ethanol/acetic acid/water, v/v, 30/5/65 for 2 h at room temperature. The gel was placed in destaining solution ethanol/acetic acid / water until clear bands appeared against the blue background and finally the gel was scanned.

3.7 References

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Chapter 4 Cell disruption

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4.1 Introduction

Cell disruption is a key step in microalgae downstream processing and is often a prerequisite to extract intracellular compounds such as lipids, carbohydrates or proteins. Different techniques exist to access such molecules and some of them have been presented in chapter 2. Cell disruption operation units have a determining impact on the whole process since it defines the initial yield of the process, but it also determines the quality of the feed that will enter the following separation steps.

The soluble proteins of microalgae are mostly in the cytoplasm, in the stroma of the chloroplast and in the pyrenoid. However, the main pigments in microalgae, the chlorophylls, are present in the thylakoids, themselves present in the chloroplast. Moreover, in the case of *Tetraselmis* and *Chlorella* the pyrenoid is surrounded by starch plates and is present in the chloroplast, along with the thylakoids, as seen in Figure 4.1.

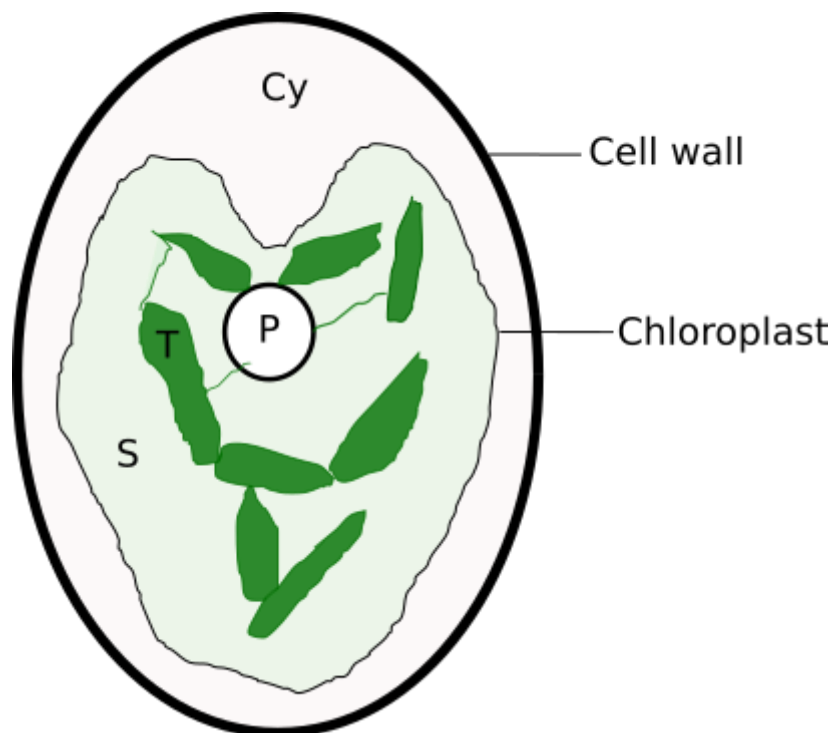


Figure 4.1 Eukaryotic microalgae like *Tetraselmis* or *Chlorella*. The cell is surrounded by a cell wall. The chloroplast is present in the cytoplasm (Cy) and composed of a liquid phase, the stroma (S), thylakoids (T) who contains pigments and the pyrenoid P which contains proteins.

In order to access the pyrenoid, the cell wall, the chloroplast and the starch sheath need to be broken. This complete disruption allows the release of most of the globular proteins in the supernatant and

can be performed by bead milling or HPH for example. However, the thylakoids are also destroyed and the chlorophylls can be dispersed in the aqueous phase and form colloids, complex to separate from other compounds (Gerde et al. 2012). Hence further operation units are necessary to obtain low-colored soluble proteins. After a first clarification by centrifugation other techniques have already been tested such as microfiltration (Garcia et al. 2018; Liu et al. 2021; 2022), dialysis (Schwenzfeier, Wierenga, et Gruppen 2011; Teuling et al. 2017) and protein precipitation by solvent (Grossmann et al. 2018).

Three different cell disruption techniques were tested in order to release proteins. The first one, the bead milling, is considered as very efficient for cell disruption and protein release and has been widely used for microalgal biorefinery (Günerken et al. 2015). This technique allows a complete cell disruption, access the stroma and, in theory, the pyrenoid, releasing all uncolored soluble proteins. However, a high protein release has been found to be proportional to a high pigment release (Zinkoné et al. 2018), hence an additional operation unit is needed to obtain low-colored protein extract. Another technique was tested, the pulsed electric fields (PEF), that aimed to permeabilize the cell membrane in order to extract soluble proteins in the cytoplasm and the stroma. These proteins are thought to be globular proteins and thus should not necessitate a further separation from the chlorophylls. While the amount of proteins extracted is lower, it can contribute to a profitable process by reducing the number of operation units, which in turn lowers the investment and the operational cost. Those techniques were compared to a simple thawing of the biomass.

Moreover, in a global biorefinery, a multiproduct valorization is aimed for a wide variety of products and industries (fuel, cosmetic, food), enhancing the profitability of the costly microalgal biomass (Gifuni et al. 2018; Bussa, Zollfrank, et Röder 2021). This allows diluting the overall costs within a wide range of products and helps the transition towards a bioeconomy. In this approach, the extraction of proteins, being a fragile and costly ingredient is prioritized over the other compounds such as lipids, which necessitate solvents. Indeed, a first extraction of proteins facilitates a lipid extraction, whereas protein denaturation can happen if the lipids were extracted first, decreasing their values (Préat et al. 2020).

In this chapter, the performances of the cell disruption techniques of *Tetraselmis chui* (bead milling and pulsed electric fields) were evaluated and compared to a simple thawing. The same processes were also applied to two other strains (*Chlorella vulgaris* and *Arthrospira platensis*) for comparison.

The performances of bead-milling are firstly deepened and part of this work has been published in a dedicated scientific article in *Algal Research*: “Extraction of soluble proteins by bead milling from *Tetraselmis chui* in two different physiological states”, Hugo Mear et al. (Mear et al. 2023). Then, the PEF process is studied. The experiments were performed in collaboration with Remi Camus, during his master internship (2021). The thawing process and the effect of pH and dilution during this step have been investigated. Finally, the selection of the cell disruption method is discussed.

4.2 Materials and methods

Most of the material and methods were described in the corresponding chapter. Here the process is summed up and specific experiments dedicated to the study of the cell disruption are added (rupturability, maceration).

4.2.1 Biomass

The biomass was produced by partners of the Profure project. *Tetraselmis chui* was grown autotrophically in photobioreactor by Necton (Portugal), under solar conditions. The biomass was harvested by centrifugation to obtain a paste at 20%_{DM} and frozen immediately. Two biomass batches were received frozen from the producer and called batch A, produced in December 2019 and batch B, produced in May 2020. They were characterized by microscopy and biochemically in terms of proteins, carbohydrates and ashes, following protocols in the analysis sub section.

Chlorella vulgaris was grown heterotrophically in Allmicroalgae facility (Portugal), harvested and conditioned as a frozen paste at 15%_{DM}.

Arthrospira platensis was grown autotrophically at Algosource facility (France), harvested and concentrated as a frozen paste at 20%_{DM}.

4.2.2 Bead milling

4.2.2.1 Rupturability measurement

The rupturability of *Tetraselmis* was assessed following the protocol described by Spiden et al (Spiden et al. 2013) by High-Pressure Homogenization (HPH). The biomass was at first diluted at $10 \text{ g}_{\text{DW}}.\text{kg}^{-1}$ with deionized water containing 30 g.L^{-1} of NaCl to avoid an osmotic stress. A high pressure homogenizer (TS Series 2.2 kW fabricated by Constant Systems Limited (Northants, UK)) was used. Each sample was sent to the HPH with one pass per tested pressure. The pressure allowing 50% of cell disruption was called the P_{50} and used for the empirical comparison of specie rupturability (Spiden et al. 2013; Montalescot et al. 2015) or physiological states.

4.2.2.2 Bead milling

The bead mill used was a Dyno-mill multi-lab of Willy A Bachofen AG (Muttenz, Switzerland) with a milling chamber of 0.6 L operated in pendulum mode, as described in chapter 3.

In all experiments, the bead diameter was chosen at 0.5-0.7 mm on the basis of previous experiences and based on literature (Postma et al. 2017; Garcia et al. 2018; Zinkoné et al. 2018; S. Liu et al. 2021; 2022). A smaller diameter could have been chosen but the spacer separating the beads from the product leaving the chamber was too wide for smaller beads if a scale-up at pilot scale was realized. The bead material in yttrium-stabilized zirconium with a density of 6 g.cm^{-3} was also kept and chosen for its food compatibility and high life expectancy. A bead filling ratio of 80% was selected as this was considered as optimal in several studies on microalgae cell disruption (Montalescot et al. 2015; Suarez Garcia et al. 2019). A same dry matter concentration at $60 \text{ g}_{\text{DM}}.\text{kg}^{-1}$ was chosen for the different biomasses. A higher dry matter could have decreased energy consumption (Bussa, Zollfrank, et Röder 2021). However, lower protein yields in the supernatant could be observed (Postma et al. 2015) and the supernatant distribution is lowered, necessitating a dilution before the centrifugation (S. Liu et al. 2021). Moreover, the bead mill has a high volume milling chamber so a significant amount of biomass would have been needed. Further optimization of this dry matter concentration could be explored in the future.

The rotational speed of the impellers was fixed at 14 m.s^{-1} for the Eukaryotic microalgae *T.chui* and *C.vulgaris* because of their tougher cell wall while it was fixed at 8 m.s^{-1} for *A.platensis*. The flow rate was fixed at 200 mL.min^{-1} for *T.chui* while a lower flow rate of 150 mL.min^{-1} was chosen to compare to previous studies and to preserve the pump.

4.2.2.3 Macerations

Modifications were performed on the cell lysate after 4.2 min of residence time in the bead mill, in order to improve protein extraction. Four conditions were tested after cell disruption of batch A biomass: 1) a control with the lysate at 60 g_{DM}.kg⁻¹ at 20°C and native pH (8.3); 2) a diluted condition, with the lysate diluted at 30 g_{DM}.kg⁻¹, using deionized water at 20°C and native pH; 3) a diluted condition at 30 g_{DM}.kg⁻¹ at 40°C and native pH; 4) a diluted condition at 30 g_{DM}.kg⁻¹ at 40°C and pH 10.

Three conditions were tested after cell disruption of the biomass from the batch B: 1) a control with the lysate at 60 g_{DM}.kg⁻¹ at 20°C and native pH (8.7); 2) a diluted condition, with the lysate diluted at 30 g_{DM}.kg⁻¹, using deionized water at 20°C and native pH and finally 3), a diluted condition, with the lysate diluted at 30 g_{DM}.kg⁻¹, using deionized water at 20°C and pH 10.

The maceration has been performed in beakers with an operating volume of 300 mL under agitation. The pH was corrected with a solution of 1M NaOH and the samples were collected at initial time, 30 minutes, 1 hour and 1.5 hour.

4.2.2.4 Centrifugation

Centrifugation was conducted in a Sorvall Lynx 6,000 centrifuge (Thermo Scientific) at a relative centrifugal force of 5,000g for 4 min at 20°C in order to simulate an industrial centrifuge and to acquire data for a potential scale-up (Amorim et al. 2020; Liu et al. 2021).

4.2.2.5 Calculation methods

Mass compound recovery yield (Y) was defined as the ratio between the mass of the considered molecules in the supernatant, for example proteins ($m_{p.Sup}$), and the mass of the same molecule in the lysate, ($m_{p.Lysate}$):

$$Y_p = \frac{m_{p.Sup.}}{m_{p.Lysate}} = \frac{C_{p.Sup.} \times V_{Sup.}}{C_{p.Lysate} \times V_{Lysate}}$$

with the supernatant protein concentration $C_{p,Sup}$, the volume of supernatant V_{Sup} , the lysate protein concentration $C_{p,Lysate}$, the lysate volume V_{Lysate} .

The efficiency of the separation of the supernatant from the lysate during the centrifugation is defined by the volume ratio ϕ between the supernatant and the lysate suspension $\phi = \frac{V_{Sup}}{V_{Lysate}}$.

Protein purity is defined as:

$$Protein\ purity\ (\%) = \frac{C_{p.sup}}{C_{DM.sup}}$$

With $C_{DM,Sup}$ the supernatant dry matter concentration.

4.2.3 Pulsed electric fields

The PEF treatment was realized in a PEF-Cellcrack III equipment (Elea-Technology, Quakenbrück, Germany), with a fixed maximum voltage of 30 kV and a fixed pulse duration of 40 μ s. The treatment was realized in batch at small pilot scale in a 0.2 L chamber with an electrode gap of 2 cm and a maximum electric field strength possible of 15 kV.cm⁻¹. This electric field strength was chosen to ensure a permeation for most of the algae (Pataro et al. 2017; Carullo et al. 2018) but also its ability to be scaled-up at industrial scale. Moreover, it was the highest possible value for the equipment.

The electric field strength (E, kV/cm) and total input specific energy (W, in kWh/kg DM) were deduced from the applied voltage, the chamber used, the number of pulses and the dry matter of the suspension, as described by Parniakov et al. 2015 (Parniakov et al. 2015).

The pulse number was fixed at 50 while total specific energy W was set by varying the applied voltage E to the suspension. After each series of 50 pulses, a sample was collected and the temperature was measured. The warming of the suspension is inherent to the treatment due to the Joule effect and it was controlled manually. The suspension was cooled in a cool water, itself cooled by an external thermal exchanger bath, if the suspension temperature reached 40°C to limit the potential degradation of the proteins. Moreover, the temperature was quickly decreased due to the low volume in comparison with the bead milled lysate (> 2.5 L). The cell electroporation was followed by measuring

the suspension conductivity before and after treatment by a conductimeter (Mettler Toledo, Columbus, Ohio, USA). The electroporation was also followed by Evan blue staining as described in chapter 3.

Experiments were performed on batch A of *Tetraselmis chui* and on *Chlorella vulgaris*.

4.2.4 Statistical analysis

The experiments in this study were carried out in triplicate for rupturability, and in duplicate for bead milling for *T.chui* with each identified batch of harvested biomass, due to the limitation of biomass quantity for each batch with exactly the same properties, and the biomass quantity needed to perform the following separation and analysis. The physico-chemical modifications were then limited to one. The bead milling experiments were carried out once with *C.vulgaris* and *A.platensis* due to similar reasons. The Pulsed Electric Field experiments were carried out once and the conditions changed between the different experiments. All the values resulting from the samples analysis (dry weight, ashes, proteins and carbohydrates) were based on triplicate measurements. The standard errors (SE) were calculated as the standard deviations of the triplicate. The combined standard error of any value y was calculated by Equation 1 for additions or 2 for multiplications or divisions of two variables X_1 and X_2 :

$$\text{If } y = X_1 + X_2, SE_y = \sqrt{SE_{X_1}^2 + SE_{X_2}^2} \quad \text{Equation 4.1}$$

$$\text{If } y = X_1^{\pm 1} \times X_2^{\pm 1}, SE_y = y \cdot \sqrt{\left[\frac{SE_{X_1}}{X_1}\right]^2 + \left[\frac{SE_{X_2}}{X_2}\right]^2} \quad \text{Equation 4.2}$$

4.3 Results

Here are presented the results on the BM, PEF and thawing processes.

4.3.1 Bead milling

In the BM part, the impact of the different physiological states of *Tetraselmis chui* on the BM performances were firstly deepened. Then the performances of this process for different microalgae species were compared.

4.3.1.1 *Tetraselmis chui* disruption with different physiological states

The objective here was to evaluate the performances of BM for the recovery of soluble proteins from two different batches of frozen *Tetraselmis chui* produced at industrial scale, characterized by significant physiological differences. The first step was to evaluate the rupturability of the two batches based on a method described by Spiden et al. (Spiden et al. 2013). The second was the characterization of the kinetic of cell disruption and protein release by bead milling. In the last one, the physicochemical conditions (pH, temperature, ionic strength) were modified to increase protein release.

4.3.1.1.1 Biochemical characterization

The paste belonging to the two different batches (A and B) has been analyzed in terms of proteins, carbohydrates and ashes as a function of the paste dry matter, as shown in Figure 4.2. The ash content was higher in batch A than in batch B. The percentage of ashes in batch B (23%) was similar to what was found by Teuling et al. on an industrial paste of *T.impellucida*, with an ash content of 17%, among which 11.8% of ashes being considered as intracellular ashes (Teuling et al. 2017). However, ash content was significantly superior to the 8% found on laboratory paste of *T.suecica* (Garcia et al. 2018; Suarez Garcia et al. 2018). The ash content can come from intracellular minerals or can be contained extracellularly, such as salt from the culture media. The percentage of proteins was between 28 and 34%, corresponding to the description in literature.

The carbohydrate content (13-16%) was in agreement with the 5 – 25% found in the literature (Michels et al. 2014). Such values were generally associated with biomasses which are not under Nitrogen starvation, where carbohydrate content can reach up to 50%, mainly as starch (Bondioli et al. 2012; Gifuni et al. 2018).

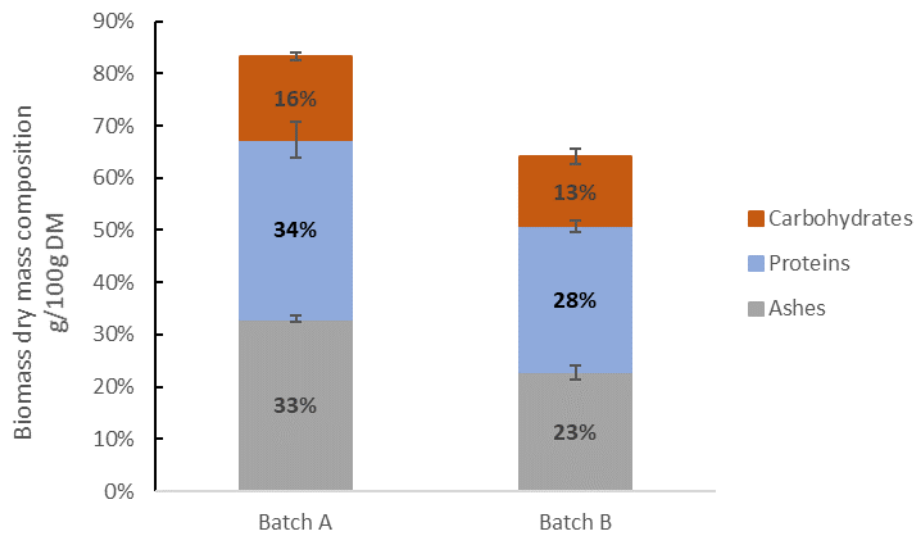


Figure 4.2 Biomass composition of batch A and batch B pastes in percentage of the dry matter (quantification was performed in triplicates).

4.3.1.1.2 Physiological characterization

The cells were observed by microscopy, as shown in Figure 4.3. A large proportion of the cells in batch A were in cell division ($\approx 80\%$), shown by 2 protoplasts in one cell (figure 4.3 A1 and A2) while the others were circular and more voluminous than batch B cells. The cells in batch B presented a compressed shape with the presence of a depression near the apex sometimes visible and a single large chloroplast containing one pyrenoid (figure 4.3 B1 and B2). Some cells were dividing ($\approx 1\%$) but unlike batch A, it was not the majority.

No flagella can be found in batch A. Flexible translucent filamentous objects were present in the liquid phase of batch B, having a length close to the algae, and being absent in batch A. It is highly possible that these objects were flagella that have been detached due to the upstream process (harvesting, freezing etc.) since flagella have been found to have a length similar to *T. chui* cells, according to Norris et al. (Norris, Hori, et Chihara 1980).

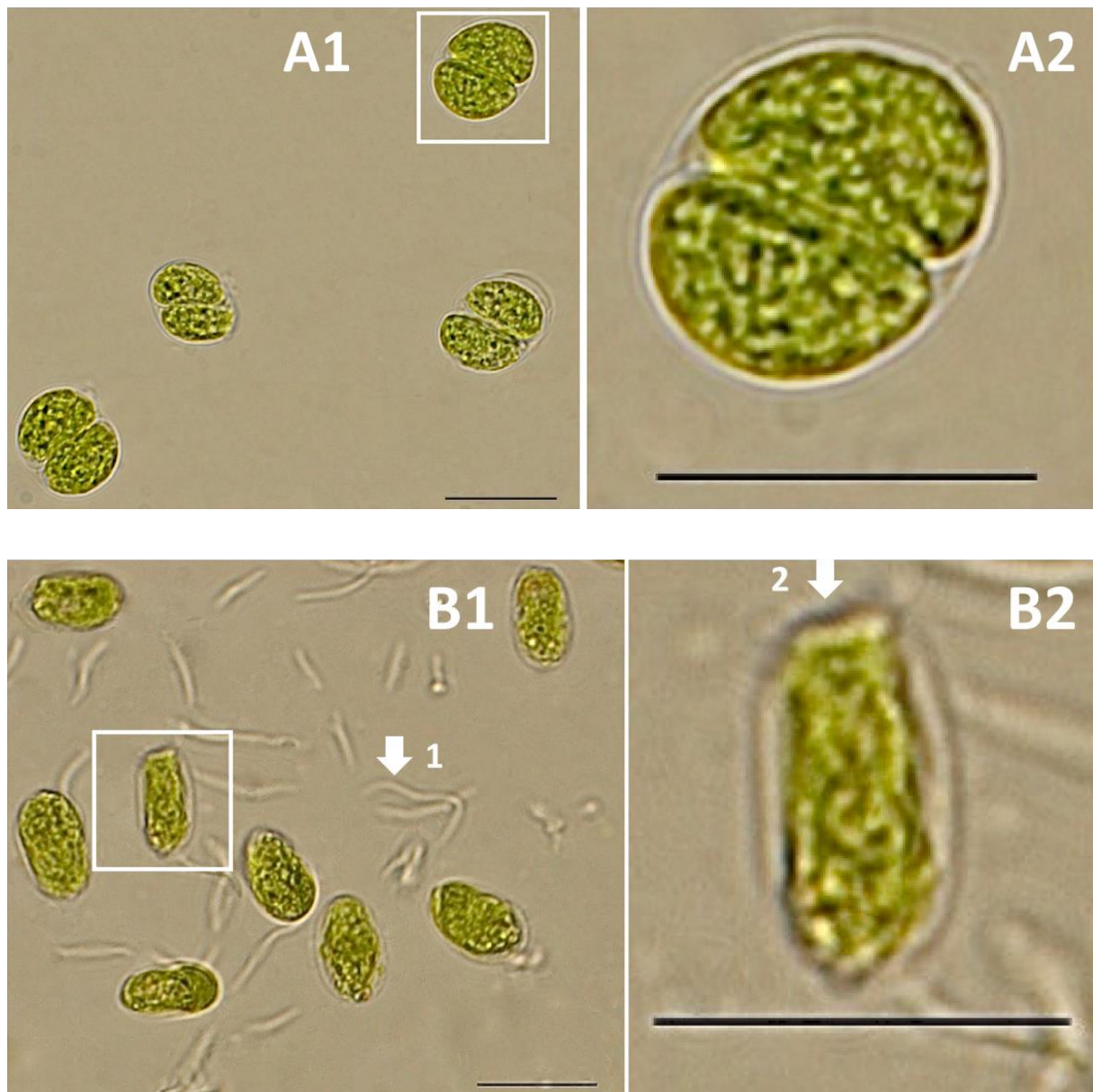


Figure 4.3 Light microscopic images of *Tetraselmis chui* of batch A cells (A1 and A2) and batch B cells (B1 and B2). The scale bars are 10 μm for all pictures. The first arrow shows objects corresponding to flagella, and the second arrow shows a pit, where flagella are usually inserted.

Tetraselmis showed different biological characteristics that may influence biorefinery. In our case, batch A differed from batch B in size, morphology and composition. Cells from the genus *Tetraselmis* are commonly represented as quadriflagellate individual cells, with a compressed shape and a single large chloroplast, containing one pyrenoid (Paterson et al. 2023). However, the presence of a circular shape has recently been identified in *T.chui* by Lu et al. 2016 who showed different cellular aspects after a culture in autotrophy and a culture in mixotrophy (Lu et al. 2016). Their cells grown under mixotrophic condition looked like the cells of batch A, with a circular shape, while their cells grown in

autotrophic condition, looked like the cells from batch B. However, in the case of batch A and batch B, *T.chui* cells were both cultivated in autotrophic mode.

Phycologists, such as Norris, Hori, and Chihara described different life cycle stages of *Tetraselmis* in the 80's (Norris, Hori, et Chihara 1980) with the presence of at least 3 phases, a flagellate phase i.e. with motile cells, a non-motile vegetative phase in which cells settle and replicate and a phase where the cells become aflagellate cysts (Figure 4.4). According to the authors, cells are in flagellate form and then undergo a physiological change to become non-motile vegetative cells, also called resting cells, just before replicating, or in unfavorable conditions. The authors also identified the presence of cysts in very unfavorable conditions. The presence of different life cycle phases in *Tetraselmis* has also been observed by different authors on *T.indica* (Arora et al. 2013; Naik et Anil 2018) and *T.jejuensis* (Hyung et al. 2021) with circular shape associated with the resting cells.

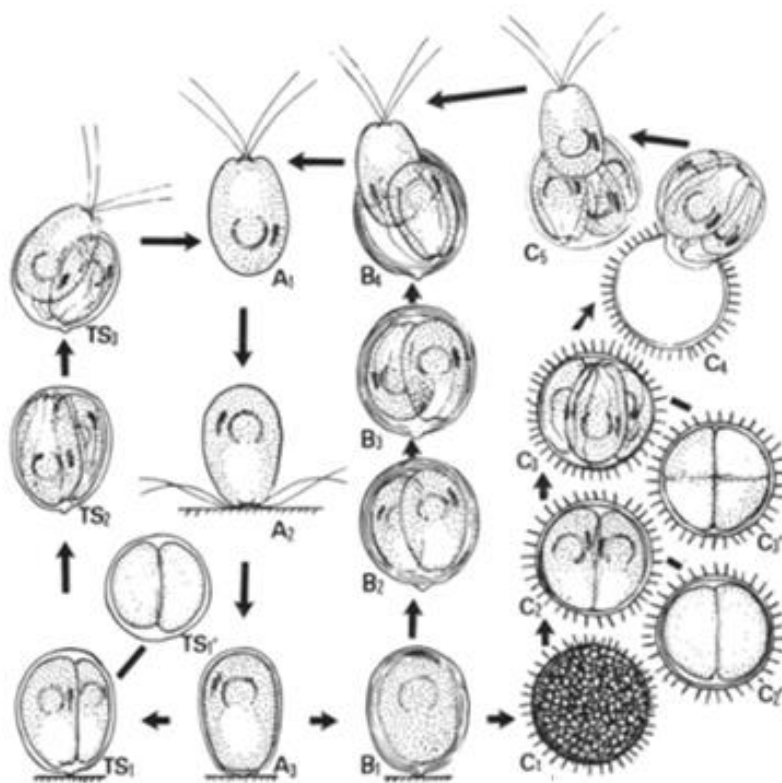


Figure 4.4 Life cycle stage of *Tetraselmis* (from Norris, Hori, et Chihara 1980)

Cells present in batch A looked like resting cells, before and during reproduction as shown by Norris, Hori, and Chihara (Norris, Hori, et Chihara 1980), as seen in figure 4.4 (TS₁ and TS₂ or B₁ to B₃). On the contrary, cells present in batch B looked like flagellate cells, with the flagella being released in the

medium. No images of cyst on *T.chui*, to the author knowledge, have been yet published. Nevertheless, the batch A cell pictures did not look like cysts present in other algal life cycle, such as *Haematococcus pluvialis* (Orosa et al. 2005). Moreover, the biochemical composition did not show an accumulation of carbohydrates that could be induced by stress, for example due to Nitrogen and/or Phosphorus starvation.

Consequences of what seemed to be a life cycle stage shift have been noticed and used: a loss of flagella and settling was identified following a nutrient starvation stress (Lim et al. 2012) or UV C treatment (Sharma, Li, et Schenk 2014), allowing an easier harvesting. Natural settling has been found in the production of *Tetraselmis sp.* CTP4 (Pereira et al. 2016). It was exploited for low-cost biomass harvesting up to pilot scale, reaching a concentration in dry matter 20 times higher than the culture and removing 97% the total biomass without energy input but the life cycle stage was not detailed (Trovão et al. 2019). Moreover, what seemed to be a modification in life cycle stage was observed when the cells were submitted to a high shear stress, the cells going into a rest phase and settling at the bottom of the PBR (Michels et al. 2016).

Another possible explanation for the high number of cells in division can be a possible natural synchronization caused by the light and dark cycle, which has been found to be possible on *Tetraselmis suecica* (Fábregas et al. 1995). The cells can spontaneously synchronize their cell cycle states. An example of the evolution of a synchronous culture as a function of time on the microalgae *Medakamo hako* can be found in figure 4.5 (Kato et al. 2023). The cell underwent different cell stages, from I to IV and some of these stages take place in the dark for a synchronized culture. The cells observed in batch A look like the cells in stage II. At 16h, 90% of the cells are in stage II, followed by the stage III briefly and the stage IV and I. While most of the time, the cells are in stage I and IV, a fraction of the time, most of the cells are in stage II and III. The synchronized *Tetraselmis* also showed a nuclear division and a cytokinesis during darkness, but cell separation only occurs when the light period begins, the cells being a single theca (Ricketts 1977a; 1977b).

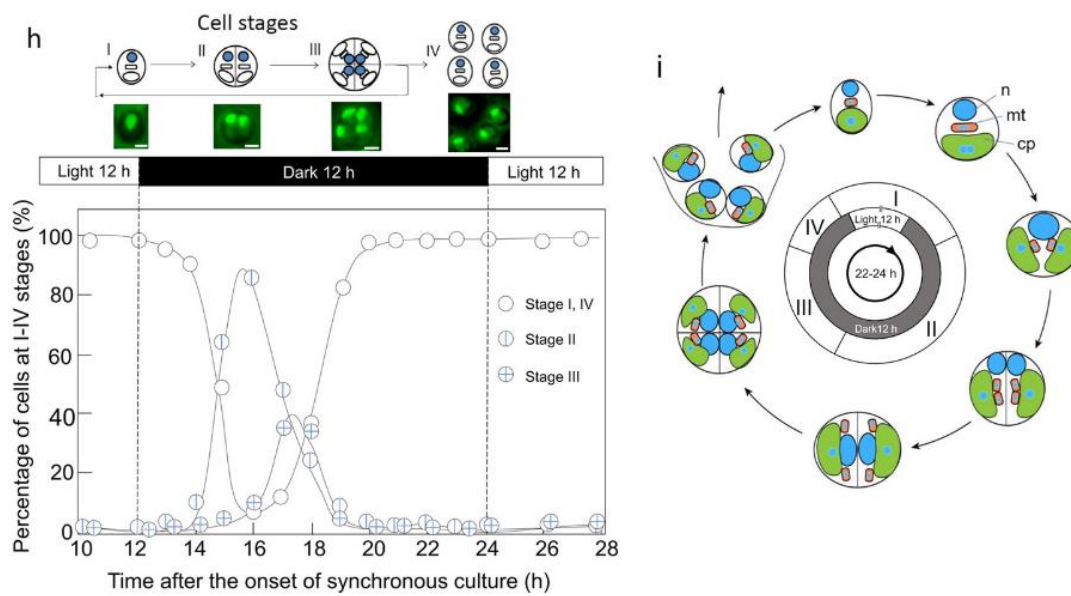


Figure 4.5 Synchronization culture of *M. hakoo*. h) The schematic diagram describes the following cells stages: single-cell stage (I), two-cells-combined stage (II), tetrad stage (III), and dissection stage (IV). i) Schematic diagram of the *M. hakoo* lifecycle; n, mt, and cp indicate the nucleus, mitochondrion, and chloroplast, respectively. Figure from Kato et al. 2023.

The life cycle in *Tetraselmis* has been poorly described in bioprocess engineering related to culture (Bondioli et al. 2012; San Pedro et al. 2013; Fon Sing et al. 2014; Pereira et al. 2016; 2020; Penhaul Smith et al. 2020) or biorefinery. However, in more intensively studied algae like *Haematococcus pluvialis*, some life cycle stages are known to negatively affect cell disruption and the extraction of target molecules (Abu-Ghosh et al. 2021; Kobayashi et al. 1997).

The physiological state may have different effects on cell disruption and compound release which will be deepened in the following paragraphs.

4.3.1.1.3 Rupturability characterization

Mechanical cell resistance is a key parameter to take into account for microalgal and cyanobacterial biorefinery, especially because of their wide variety of cell wall composition (Baudelet et al. 2017; Alhattab, Kermanshahi-Pour, et Brooks 2019). For example, *Arthrospira platensis* does not need an intensive cell disruption technique unlike other strains such as *Microchloropsis* or *Chlorella*.

Cell rupturability was assessed by HPH and a P_{50} between 400 and 1000 bars was obtained for batch A, while P_{50} of batch B was 190 bars \pm 46 bars, much lower than the batch A. Intact cells were still present at 1200 bars within batch A. Despite the uncertainty in cell rupturability of batch A, probably due to difficulties in full-cell identification by image analyses, a different behavior was obtained for the

different physiological states. The difficulties in full-cell identification was also observed when *Tetraselmis* was in doublets form by other authors (Ricketts 1977a).

The P_{50} of batch B was close to what Spiden et al. (Spiden et al. 2013) found on *T.suecica*, with a P_{50} of 170 bar. Magpusao et al. (Magpusao et al. 2021) observed similar results on *Tetraselmis sp.* Their intact cells looked like batch B cells and were completely disrupted at 300 bars, as for batch B.

Different algal species have been evaluated by this method, with a P_{50} of 1060 bar for *Chlorella sp.* (Spiden et al. 2013), 400 bar for *Porphyridium cruentum* and 1400 bar for *Microchloropsis oculata* (Montalescot et al. 2015).

Compared to other species, *T.chui* seems to be fragile in batch B and moderately resistant in batch A. This difference might be caused by the presence of accumulated concentric cast-off walls in non-motile vegetative phase (Norris, Hori, et Chihara 1980).

4.3.1.1.4 *Bead milling*

The first bead milling experiment was conducted to assess the optimal residence time. The different liberation kinetics of proteins and dry matter were also evaluated to find a compromise between protein recovery yield and protein purity since the latter can facilitate the protein purification step for the achievement of protein isolates.

4.3.1.1.4.1 Cell disruption

The disruption of *Tetraselmis* cells was very fast with a disruption rate of more than 70% after 1.4 min residence time for the milling of the batch A and more than 90% for the batch B as seen in figure 4.6. A limited difference of cell disruption between the two batches was evaluated by cell counting. However, more differences can be seen using complementary analysis.

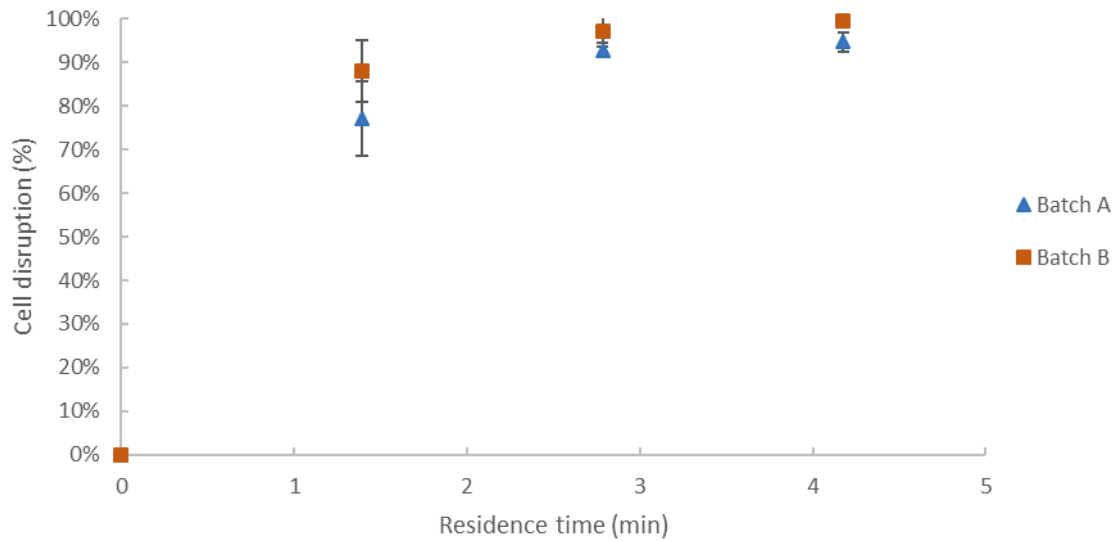


Figure 4.6 Cell disruption as a function of the milling residence time for the two different batches (each experiment was performed twice, mean value and standard deviations presented).

This result can be compared to particle size distribution (PSD) of cell lysates along bead milling residence time, as shown in figure 4.7.A1 and B1. During batch A cell disruption, a clear diminution of the maximum peak is seen, from 13.6 μm before milling (original cell diameter), to 10.5 μm after 1.4 min and 7.2 μm after 4.2 min residence time. After 4.2 min residence time, 2 peaks remain, one with a maximum at 7.2 μm , representing big cell debris and one at 0.8 μm , which represent small cell fragments. However, no further diminution in cellular fragment size can be seen after 4.2 min residence time for the batch A cells (results not shown).

The PSD is different for the milling of the batch B as seen in figure 4.7 B1, with a widening of the bigger cell debris peak along the milling toward smaller sizes, especially visible at 4.2 min. The generation of fine debris is also more important in batch B, compared to batch A. These results show that the cells from batch B are easier to break than the cells from batch A, linking the rupturability results and the bead milling results. The presence of fine debris will have an influence on the purification step since centrifugation at industrial scale has limited acceleration (< 10,000 g) and so a limited settling power. Hence, the smaller debris (<1 μm) are more difficult to separate with this operation unit (Leung 2007) and a finer technique has to be used to remove them, such as microfiltration.

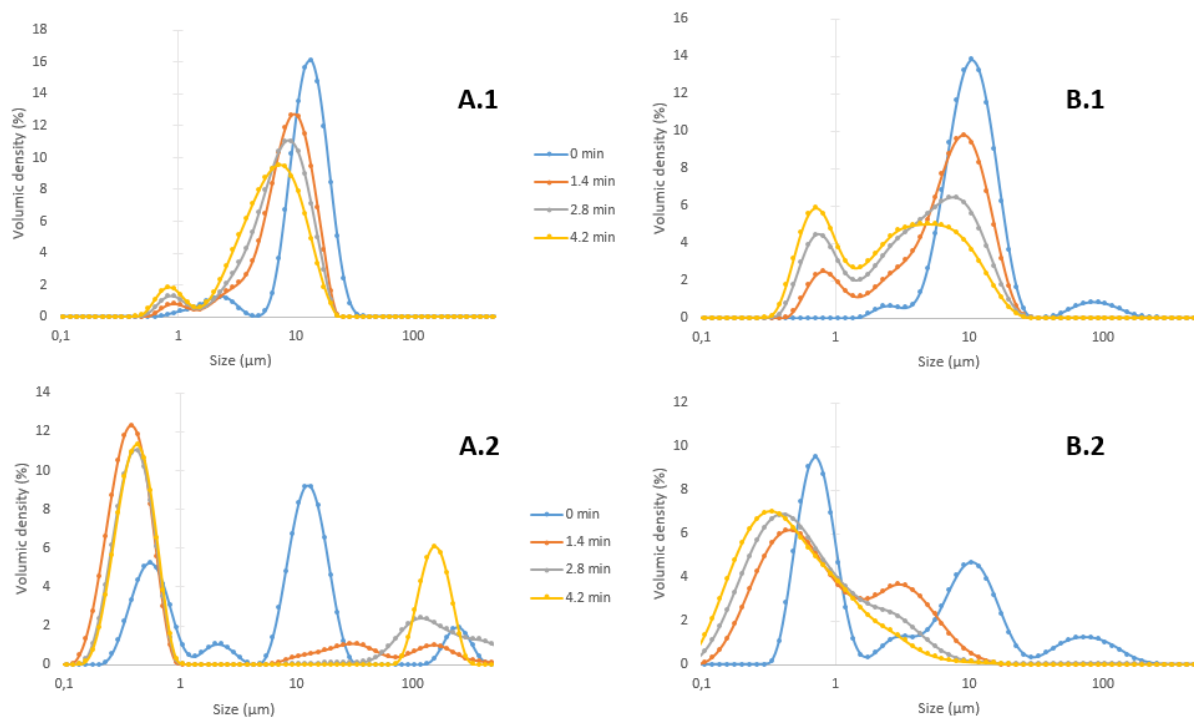


Figure 4.7 Particle size distribution of cell lysates (A.1 and B.1) and clarified supernatants (A.2 and B.2) as a function of the residence time in the bead mill for batch A and batch B, measurements were performed in triplicates.

The PSD of clarified supernatant after bead milling has been characterized for the two batches and reported in figure 4.7 A.2 and B.2, respectively. For batch A (figure 4.7 A.2), the proportion of fine particles increases after 1.4 min of milling residence time and stays constant after. The peak is well defined and is comprised between 0.1 to 1 μm, with a mode at 0.49 μm. A peak around 10 μm, corresponding to few intact cells in the supernatant, is present before milling but disappears after and no particles with a size between 1 to 10 μm are detected after milling. Peaks, present around 100 μm, are suspected of being rare agglomerates. For batch B (figure 4.7.B2), a peak of fine particles appears after milling, being wider and less defined than the peak in figure 4.7.A2. Some particles are present between 1 to 10 μm and their proportion decreases during milling, while the proportion of fine particles increases, showing a further comminution.

4.3.1.1.4.2 Dry matter and protein release

The dry matter released in 5,000 g and 11,000 g supernatants was followed as a function of the milling residence time as seen in Figure 4.8.

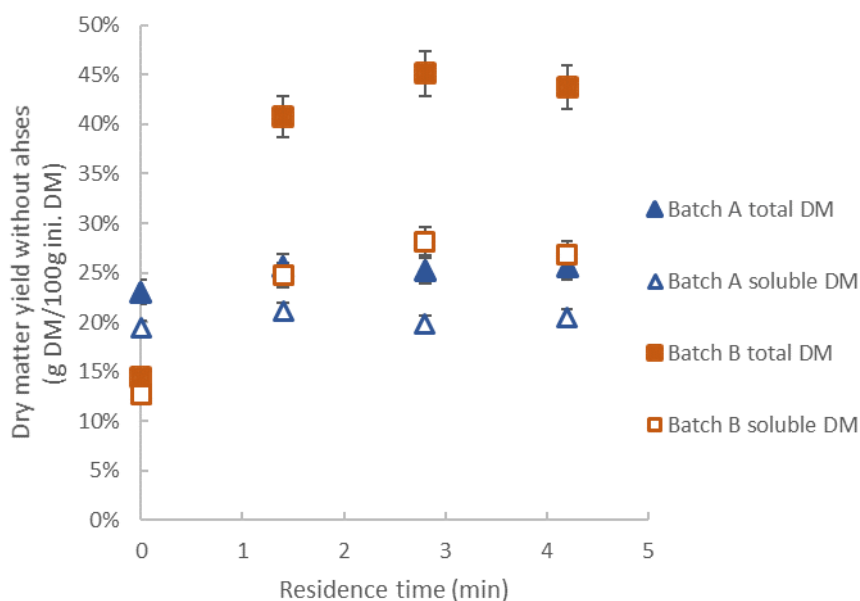


Figure 4.8 Total and soluble dry matter yields without ashes (ashes being removed to consider only organic compounds) in supernatants as a function of the residence time within the bead mill for the two conditions, each experiments were performed twice, analysis in triplicates, mean value and standard deviations presented.

The objective of cell disruption by bead milling is the comminution of cells to release water-soluble compounds. At the same time, cell fragments are dispersed within the aqueous phase. The separation of particles from the aqueous phase by centrifugation depends on different criteria, such as the diameter and density of the small debris and the centrifugation parameters (relative centrifugal force, time and temperature). Since different PSD were obtained for different lysate batches, it is interesting to evaluate the quality of this separation with the analysis of dry matter repartition within the supernatant for different conditions, as seen in figure 4.8.

Total dry matter yield without ashes recovered in batch A supernatant increases slightly from 23% after thawing to 26% after 4.2 min of milling residence time. This little difference is also observed on soluble dry matter measured after centrifugation at 11000 g of batch A supernatant with a slight increase from 19 to 20%. The negligible effect of bead milling on the dispersion of dry matter in the liquid phase for batch A cells is very contrasted with the results for batch B. In the supernatant from batch B, total dry matter recovery yield increases from 14% to 44% in supernatants along the milling

time. The soluble dry matter yield of batch B is also very different from batch A, with an increase from 13% to 27%.

Total protein recovery yield in supernatant from batch A increases from 9% after thawing to 11% of total proteins after 4.2 min of milling as seen in Figure 4.9, and an increase in protein concentration from 1.6 g.L^{-1} to 2.1 g.L^{-1} . Moreover, no statistical difference is observed in total and soluble protein yields, and no increase of protein yield was observed when milling residence time was increased to 8.2 min (results not shown). On the contrary, the total protein recovery yield in the supernatant, after cell disruption of batch B, increases from 7% of initial proteins after thawing to 48% after only 1.4 min of milling residence time and stabilizes after. In terms of protein concentration, this represents an increase from 1.5 g.L^{-1} to 12.3 g.L^{-1} . The soluble protein recovery yield increases continuously, from 6% to 32% after 4.2 min of milling residence time, so an increase in concentration from 1.4 g.L^{-1} to 10.8 g.L^{-1} .

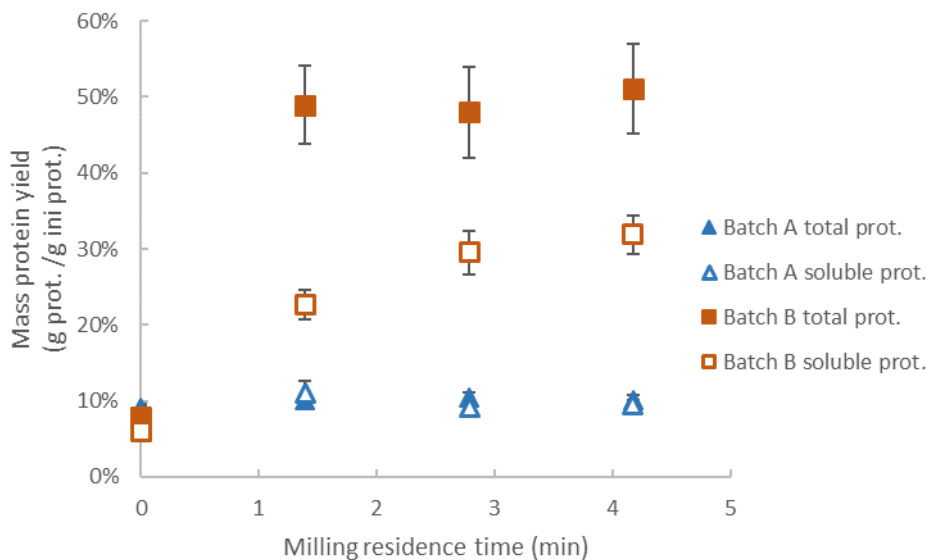


Figure 4.9 “Total” and “soluble” protein recovery yields in supernatant as a function of the milling residence time for batch A and batch B, each experiments were performed twice, analysis in triplicates, mean value and standard deviations presented.

The differences in the release of proteins and dry matters between batches are probably related to the generation of more fine particles in batch B, more difficult to remove from water than for batch A. As seen in figure 4.7 A2, milling residence time has little influence on particle size in batch A supernatants, leading to a similarity between “total” and “soluble” proteins versus residence time. However, for batch B, milling residence time induces a comminution of larger cell debris as seen in

figure 4.7 B2, leading to an increase in submicrometric particles in the supernatant. This increase of fine particles, which should contain proteins, increases the “total” protein yield in the supernatant. Since more fine particles are generated, some of them can also be found in the supernatant at 11,000 g, increasing the “soluble” protein yield. Proteins can be associated with fine cell debris containing chlorophylls and being hard to centrifuge, even at high relative centrifugal force (Gerde et al. 2012; Ursu et al. 2014).

The soluble protein yields obtained, 11% for batch A and 32% for batch B are aligned with results presented within literature. Most of the yields presented are comprised between 12 to 22% of total proteins (Schwenzfeier, Wierenga, et Gruppen 2011; Garcia et al. 2018; Suarez Garcia et al. 2018), while one study mentions a protein yield of 41% (Teuling et al. 2017). No data on the life cycle stage of *Tetraselmis* has been given by the different authors.

4.3.1.1.4.3 Differences between proteins released from batches A and B

The protein profile of the supernatants, before and after bead milling of the different batches of biomass, were analyzed by SDS-PAGE, as seen in figure 4.10 (1). The presence of protease was characterized by a zymogram (figure 4.10 (2)).

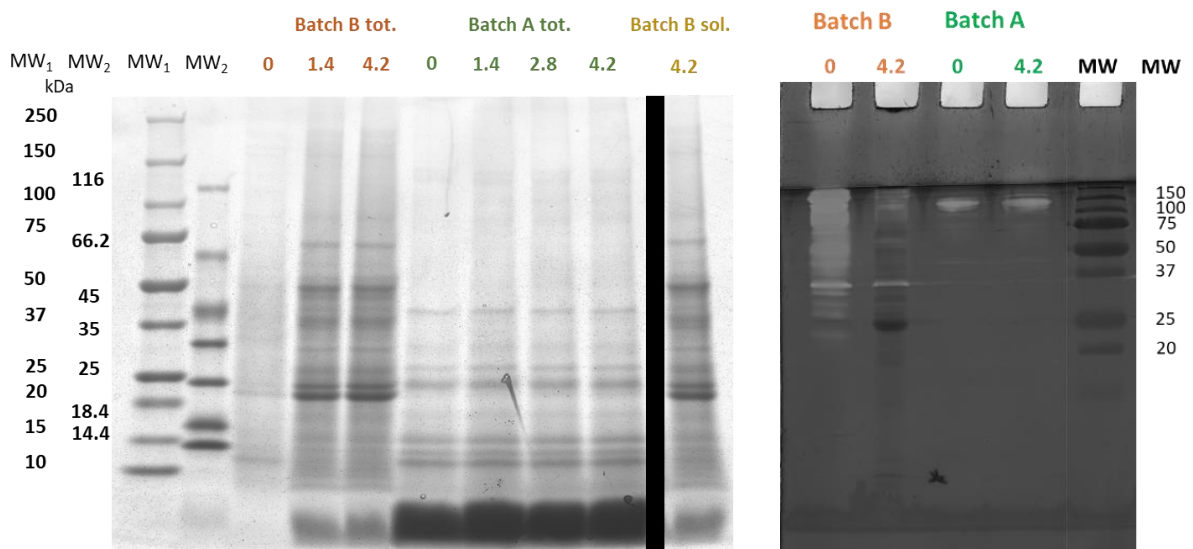


Figure 4.10 (1) Electrophoresis SDS PAGE in reducing conditions of total (tot.) and soluble (sol.) and (2) zymogram of supernatants after bead milling and centrifugation of *Tetraselmis*, batch A and batch B. The residence time (0 to 4.2 min) in the bead mill is shown above each line.

The electrophoresis profiles of batch A were similar before (0 min) and after bead milling. An important quantity of peptides with a molecular weight under 10 kDa was observed. Intense bands were present between 10 and 15 kDa, one at 25 kDa and one at 45 kDa.

The electrophoresis profiles of the batch B supernatants were different before (0 min) and after bead milling. Before cell disruption, the most intense bands were present at a molecular weight around 12 kDa, and between 20 and 25 kDa. The peptides under 10 kDa were not visible. After bead milling, some peptides appear. The two bands between 20 and 25 kDa are far more intense. Among several new bands, three ones are more intense at 37, 50 and 70 kDa. The corresponding proteins were released massively during cell disruption. The electrophoretic profile of the soluble proteins from the batch B supernatant after 4.2 min of residence time is similar to the profile of the total proteins. No specific band was removed due to stronger centrifugation conditions.

The protein profiles are very different between the two batches. However, some common bands could be attributed to the small subunits (near 20 kDa), and large subunits (between 35 and 50 kDa) of the RuBisCO, a water-soluble enzyme found in the stroma of the chloroplast and in the pyrenoid in Eukaryotes (Lacoste-Royal et Gibbs 1987). The subunits of the Rubisco of *Tetraselmis chui* are indeed respectively 22.6 kDa (Corre et al. 2021) and 40.4 kDa (Corre et al. 2021). However, a Western blot using specific antibodies against the RuBisCO should be used to prove it. The RuBisCO was already observed during the bead milling of other strains such as *C.vulgaris*, *T.suecica*, *N.oleoabundans* (Postma et al. 2017).

Green spots corresponding to the presence of chlorophyll were also present in the electrophoresis gel for the batch B at 1.4 and 4.2 min in high quantity, as well as in the supernatant after 11,000g centrifugation. The presence of chlorophyll in the supernatant after cell disruption of microalgae was largely reported in literature as colloids (Ursu et al. 2014; Gerde et al. 2012). This can be due to the presence of chlorophyll a-b binding protein. These latest are part of Light Harvesting Complexes, considered as one of the most abundant membrane protein in the world (Scherer et al. 2019).

The presence of proteases among the proteins in the supernatant of different extracts was also followed by a zymogram, as seen in figure 4.10 (2). The zymogram of batch A supernatant showed a band at 150 kDa before and after bead milling. The supernatant of batch B after freeze-thawing showed a high quantity and diversity of proteases. It may testify a high metabolic activity, with different proteases

being produced. After bead milling, the overall quantity of proteins increases, hence the sample had to be diluted, thus the relative proportion of proteases decreases. Some proteases still remain visible at 37 kDa and 100-150 kDa.

The presence of proteases in the supernatant of batch A and batch B has been confirmed, before and after bead milling. A part of these enzymes is extracted after the freeze-thawing step. The presence of different proteases confirms the presence of two different physiological states, as already mentioned in literature. Indeed, proteases, such as autolysins, capable of degrading cell walls, were found to be life cycle stage-specific in *C.reinhardtii* (Matsuda, Saito, et Taketoshi 1994; Buchanan et al. 1989). Moreover, the localization of these proteases in the cell and their solubility were changing. Their release in the soluble phase was found difficult when the cells were vegetative whereas it was not the case for gametic cells (Matsuda et al. 1987). The presence of proteases can be detrimental from a purification process point of view because of a possible protein hydrolysis. To avoid it, a treatment at high temperature during a low time, blanching, can be used for different biomass (Xiao et al. 2017).

A significant quantity of released enzymes can deteriorate compounds and change the product. For example, two enzymes often targeted for inactivation are the peroxidase (POD), causing undesirable changes in products like color and flavor changes, and nutrient degradation in plants, and the polyphenol oxidase (PPO), present in plants, fungi, bacteria and insects, and responsible for a browning reaction. The presence of proteases can also be detrimental to our process. A lower protein size can be detrimental to ultrafiltration since the proteins will not be retained and to IEP since it can affect their aggregation behavior, enhancing the protein solubility and reducing the effectiveness of this operation unit. In literature, the majority of the studies are not applying a blanching step before protein extraction (C. Safi et al. 2017; Suarez Garcia et al. 2018; Liu et al. 2021) and only one study, to our knowledge, realized a blanching step before protein extraction of *Microchloropsis* (Gerde et al. 2013). The paste was blanched at 80°C during 5 min to minimize possible enzymatic proteolysis.

While proteases were found in microalgae, no degradation of endogenous proteins by these proteases were found in extracts from *Dunaliella tertiolecta*; a Chlorophyte like *Chlorella* and *Tetraselmis* (Berges et Falkowski 1996).

Considering the reduced protein release for batch A cells, macerations of the lysate after bead milling were carried out, changing different physicochemical conditions: dilution, pH and temperature. Indeed, 90% of the cells being disrupted, the reason for little protein release was most likely to be a

biochemical lock. The preselected conditions were also applied to batch B cells, taking into account the previous results, to further improve protein extraction for comparison.

4.3.1.1.5 *Bead milling: modified conditions with macerations*

Physicochemical conditions such as temperature or pH can strongly affect protein extraction (Gerde et al. 2012; Liu et al. 2021). Several conditions were tested on the cell lysate after 4.2 min of milling residence time and “total” and “soluble” protein recovery yields obtained in supernatants were measured as a function of the maceration time, between 30 min, 1h and 1.5 h (figure 4.11). No significant differences were measured between 30, 1 and 1.5 h of maceration. Thus, the following figure presents the mean protein yields for the different conditions.

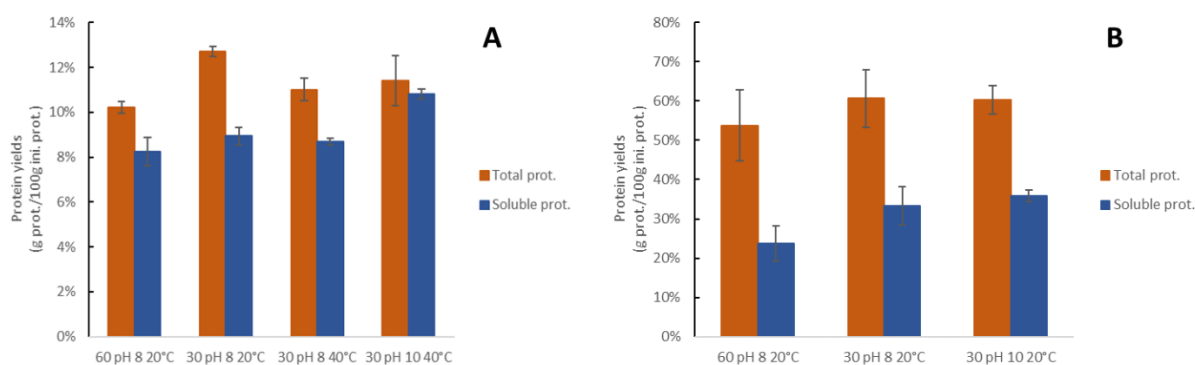


Figure 4.11 “Total” and “soluble” protein yields for different conditions applied on the final cell lysate of batch A (A) and batch B cells (B) (quantification was performed in triplicates)

A control at 60 $\text{g}_{\text{DM}}\cdot\text{kg}^{-1}$ and pH 8 at 20°C was tested on batch A final cell lysate and compared to the other conditions. The dilution at 30 $\text{g}_{\text{DM}}\cdot\text{kg}^{-1}$ (native pH, 20°C) improved the protein yield for total proteins, from 10.2% to 12.7%, while the increase was less significant for soluble proteins, from 8.2% to 8.9%. The rise of temperature between 20°C and 40°C had a negative impact on total protein yield and a limited impact of soluble proteins at native pH. However, pH 10 and 40°C slightly improved the protein yield on soluble proteins, but with a 2-3% of protein yield increase only.

On batch B, dilution to 30 $\text{g}_{\text{DM}}\cdot\text{kg}^{-1}$ had an impact on “soluble” protein release, compared to the control, with an increase from 24 to 36%. However, the increase in pH from 8.7 to 10 did not improve protein solubility, with no statistical differences observed.

The pH modification did not induce a strong rise in protein recovery yield as expected according to the literature. Its effect on protein solubility is theoretically strong but is a function of the protein

isoelectric point and solubility. A complementary analysis of the protein solubility extracted from batch A showed that they were 100% soluble at pH above 5. This is coherent with a limited effect of a rise of pH from 8 to 10. Moreover, protein solubility is also strongly affected by ionic strength, as shown by Teuling et al. on *Tetraselmis impellucida*, *Scenedesmus dimorphus*, *Microchloropsis gaditana* and *Arthrospira maxima* extracts (Teuling et al. 2017). Protein solubility was affected by ionic strength in all tested extracts, with a higher ionic strength leading to a decrease in protein solubility.

Since *Tetraselmis* is a marine species, the paste is salted, as shown in figure 4.2, with 33% and 23% of ashes in batch A and batch B paste, respectively. Seawater is mainly composed of NaCl, contributing over 85% of the charges and a part of it is present in the paste. Thus, considering sodium chloride as the main salt in the supernatant, an ionic strength close to 0.3 M can be estimated for batch A supernatant while it is 0.1 M for batch B supernatant. This difference might be significant, according to Teuling et al. results, however, it does not explain entirely the gap between batch A and B protein yields.

Thus, the desalination of the biomass could be a solution to facilitate protein release. However, desalination of the frozen paste at industrial scale necessitates another operation unit such as microfiltration or centrifugation, to recover the desalted biomass in the retentate or the pellet respectively. The medium salts would hence be washed out in the liquid phase, as well as the proteins released after freeze-thawing, inducing a significant protein loss. A washing step after harvesting and before freezing might be a more suitable option, or a culture at lower salinity if the strain allows it.

The increase in protein yield after dilution was already noticed also because the separation between pellet and supernatant was more efficient (Liu et al. 2021). Thus, a dilution can be interesting to produce protein extracts, but this conclusion could be mitigated depending on the purification step applied, because a larger volume to be treated would also induce larger costs. If microfiltration is selected to purify soluble proteins, the dilution can be useful, increasing membrane performances by enhancing permeation, as seen by Liu et al. (Liu et al. 2021).

4.3.1.1.6 *Different process conditions for different biomass characteristics*

The bead milling operation unit increases the yield of soluble protein from 9% after freeze-thawing to 11% after batch A bead milling and no further improvement can be achieved increasing the residence time. Since freeze-thawing is a mandatory operation unit in our case, the biomass being frozen, the CAPEX, the OPEX, and the environmental costs associated with the bead mill in comparison with simple freeze-thawing do not seem reasonable. Moreover, while the dry matter repartition was slightly increased after bead milling, the supernatant after simple freeze-thawing contained less chlorophyll. This was shown by the lightly green color compared to the strong green color obtained in the supernatants after bead milling. The light presence of chlorophyll in the supernatant after freeze-thawing can avoid an additional purification unit if a lightly green product is acceptable. Food products such as bread, with *T.chui* containing low chlorophyll after ethanol-extraction have been seen as more acceptable than using raw disrupted algae (Qazi et al. 2021). Hence, to produce low color protein extract from *T.chui*, using biomass in the same physiological state as batch A, a simple thawing would be recommended and a final protein yield of 9% can be expected. Bead milling on batch B cells releases a more significant protein quantity than freeze-thawing, so the addition of the operation unit could be useful to produce proteins extracts. However, the recovered protein extract shows a green color thus a purification step may be needed, depending on the application target. A dilution before centrifugation can be effective but it leads to a higher volume to be purified. However, pH and temperature did not improve significantly the protein yields in the tested conditions.

Defining the characteristics of the biomass that can change is important on one hand to adapt the biorefinery process to it and, on the other hand, to identify the culture conditions that can act on these characteristics or not.

4.3.1.1.7 *Conclusion on the bead-milling of *Tetraselmis chui**

Two batches of *Tetraselmis chui* paste were received and characterized. They presented difference in aspect, with batch A, having mostly dividing cells or circular cells, while batch B contained compressed cells with a depression at the apex. The differences were attributed to different physiological states of *Tetraselmis*. The rupturability of batch A was achieved at higher pressures compared to batch B. This was aligned with a faster cell disruption by bead milling for batch B compared to batch A.

The differences of dry matter and protein recovery yields in the supernatant after centrifugation between the two batches were strikingly different as well. An increase from only 19 to 20% of initial soluble dry matter without ashes within the supernatant was observed for batch A, while it was 13 to 27% for batch B. The soluble mass protein yields only improved from 9% after thawing, to 11% after milling for batch A, while it increased from 6% to 32% after milling for batch B. The difference of physiological state was confirmed through the supernatant analysis by electrophoresis and zymograms. Physicochemical modifications were applied on the lysate, with an improvement of protein solubility when the extract was diluted by half, but without a significant effect of temperature or pH.

Through these results, it was demonstrated that the microalgal physiological state deeply impacts the biorefinery performances. A better understanding of *Tetraselmis* physiology and its links to the culture process should facilitate the selection of the best harvesting conditions (period, duration etc.) to recover fragile cells and improve biorefining. The coupling of upstream (culture, harvesting) to downstream processes for microalgae valorization is still a challenge. The assessment of the correlation between the physiological state of the biomass and its extraction performances can definitively contribute to unlock the potential of microalgae protein isolates. The characteristics of the target product, mainly driven by consumer demand, will deeply affect the number of operations and the cost.

4.3.1.2 Comparison of *Tetraselmis chui* bead-milling with the other algal species

4.3.1.2.1 Cell disruption

The cell disruption by bead-milling of *T. chui* (batches A and B) was compared to other species widely used in the microalgae industry, *C. vulgaris* and *A. platensis*. The results are presented in figure 4.12.

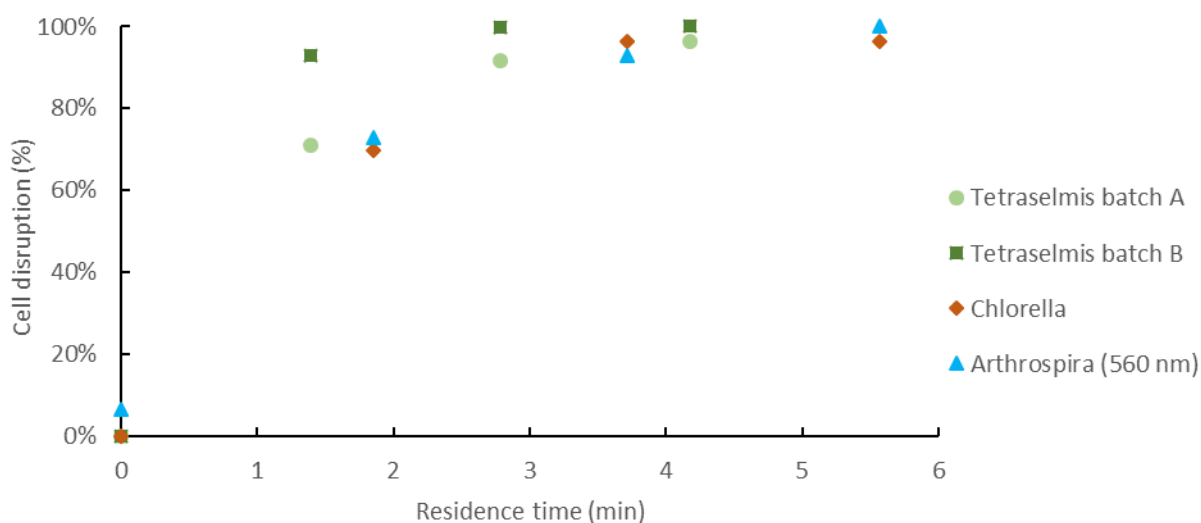


Figure 4.12 Kinetics of cell disruption as a function of the residence time in the bead milling for *C. vulgaris*, *T. chui* and *A. platensis*.

Arthrospira sp. is a filamentous cyanobacteria complicating heavily cell counting in comparison with the two other species. It was chosen instead to measure the dispersion of the cell debris in the suspension by measuring the turbidity using absorbance at 560 nm. It followed the destruction of the colony in single cells and the breakage of the cells in small cell debris, dispersed in suspension. The comparison between the strains has to be carefully interpreted.

Tetraselmis chui cell disruption is faster compared to other strains such as *Chlorella*, as already shown by (Postma et al. 2017; Suarez Garcia et al. 2019). A disruption rate of above 80% was found after 2.8 min while it happened only after 5.2 min for *Chlorella vulgaris* milled by (Liu et al. 2021).

Cell disruption by bead milling follows a first-order kinetic which can be described as follows:

$$Dis = 1 - e^{-k_{dis} t} \quad \text{Equation 4.3}$$

With Dis the disruption rate, k_{dis} the disruption kinetic rate constant and t the residence time.

Cell disruption kinetic constants of 1.9 min^{-1} for the milling of the batch A of *Tetraselmis* and 0.95 min^{-1} for the bead milling of *Chlorella* have been calculated. The cell disruption kinetic constant of the batch B of *Tetraselmis* was not possible to calculate in these conditions since the cell disruption was too fast. A higher flow rate can be applied in the future to optimize its cell disruption and to calculate a cell disruption kinetic constant. The kinetic constant of *Tetraselmis*, calculated on the same bead mill, was higher than with different species such as *C.vulgaris*, with kinetic constants ranging from 0.24 min^{-1} to 1.0 min^{-1} (Liu et al. 2021), *C.sorokiniana*, ranging from 0.18 min^{-1} to 1.0 min^{-1} (Zinkoné et al. 2018), *Microchloropsis oculata* with a kinetic constant of 0.2 min^{-1} and 0.46 min^{-1} on *Porphyridium cruentum*. However, the kinetic constant of *Chlorella* grown in heterotrophy was aligned with other constants obtained after cell disruption of *Chlorella* grown in autotrophy (Zinkoné et al. 2018).

The constants have been calculated for the disruption of *T.suecica* and a kinetic constant of 2.12 min^{-1} has been found for the same bead material and similar bead size (0.65 mm beads) but with a smaller bead mill (0.075 L compared to 0.6 L) (Postma et al. 2017). A higher constant, 3 min^{-1} , was obtained at a bead size of 1 mm. The constant on *Tetraselmis* was also superior to the cell disruption kinetic constant of *Chlorella*, with a constant of 2.46 min^{-1} at a bead diameter of 0.3 mm. Smaller beads can lead to a higher cell disruption in some cases, for *Chlorella* for example, due to a higher capture frequency. However, a limitation on the bead diameter would have been reached with lower bead sizes than 0.5-0.7 mm, since the spacer limit and the bead size was too close. Some beads could have gone through the spacer, inside the product.

Differences in cell disruption between species can be due to different parameters, mainly the cell size and the cell wall composition. *Tetraselmis* has a diameter between 12 to 14 μm (Brown et Blackburn 2013) while *Chlorella* has a diameter between 2 to 10 μm (Carl Safi, Zebib, et al. 2014) and *Microchloropsis* has a cell diameter of 2-5 μm (Gwo et al. 2005). A bigger cell diameter is often associated with a faster cell disruption since the capture probability between two beads is improved. This is also the case for bigger cells in the same suspension, as seen by Zinkone et al. on *Chlorella* (Zinkoné et al. 2018). *Arthrospira* has a different ultrastructure than the other algae, being a cyanobacteria. The trichomes have a variable length depending on the growth conditions (Bronnenmeier et Märkl 1982), between 50 μm to 1 mm if the shear stress during cultivation is high, and a mean value of 200 μm . The cells have a cell diameter of 8 μm (Zhao et al. 2022). A decrease in trichrome size was observed as a function of the milling, and individual cells appeared. However, at

the latest residence time, very few cells, entire and broken, were observed in the suspension, as seen in figure 4.13.

Differences in cell wall composition can also be significant between algae since the genetic biodiversity is important. The cell wall of *Tetraselmis* is composed of acidic polysaccharides organized as a theca, while the cell wall of *Chlorella vulgaris* is mainly composed of neutral sugars, especially glucosamine (Alhattab, Kermanshahi-Pour, et Brooks 2019). Thus, differences in cell wall resistance exist between different microalgae and a table presenting the cell wall mechanical resistance was presented in chapter 3.

The force to break *Tetraselmis* can be considered small among the different microalgae, but the physiological state is not specified. *Chlorella vulgaris* needs a higher force than *Tetraselmis* to break the cells at microscopic scale on one cell. *Arthrospira* being a cyanobacteria, the force is expected to be close to the one of bacteria. These results are expected to be important with a suspension containing a large quantity of cells and the tuning of the environment to decrease this force can lead to promising results. This was realized by Günther et al. for example, who modified the osmolality of the medium and obtained different forces depending on the osmolality (Günther et al. 2016). A lower force was associated with a low osmolality, because of a cell turgor. The easiness of cell disruption at the scale of one cell with a low osmolality was also observed during cell disruption by bead milling.

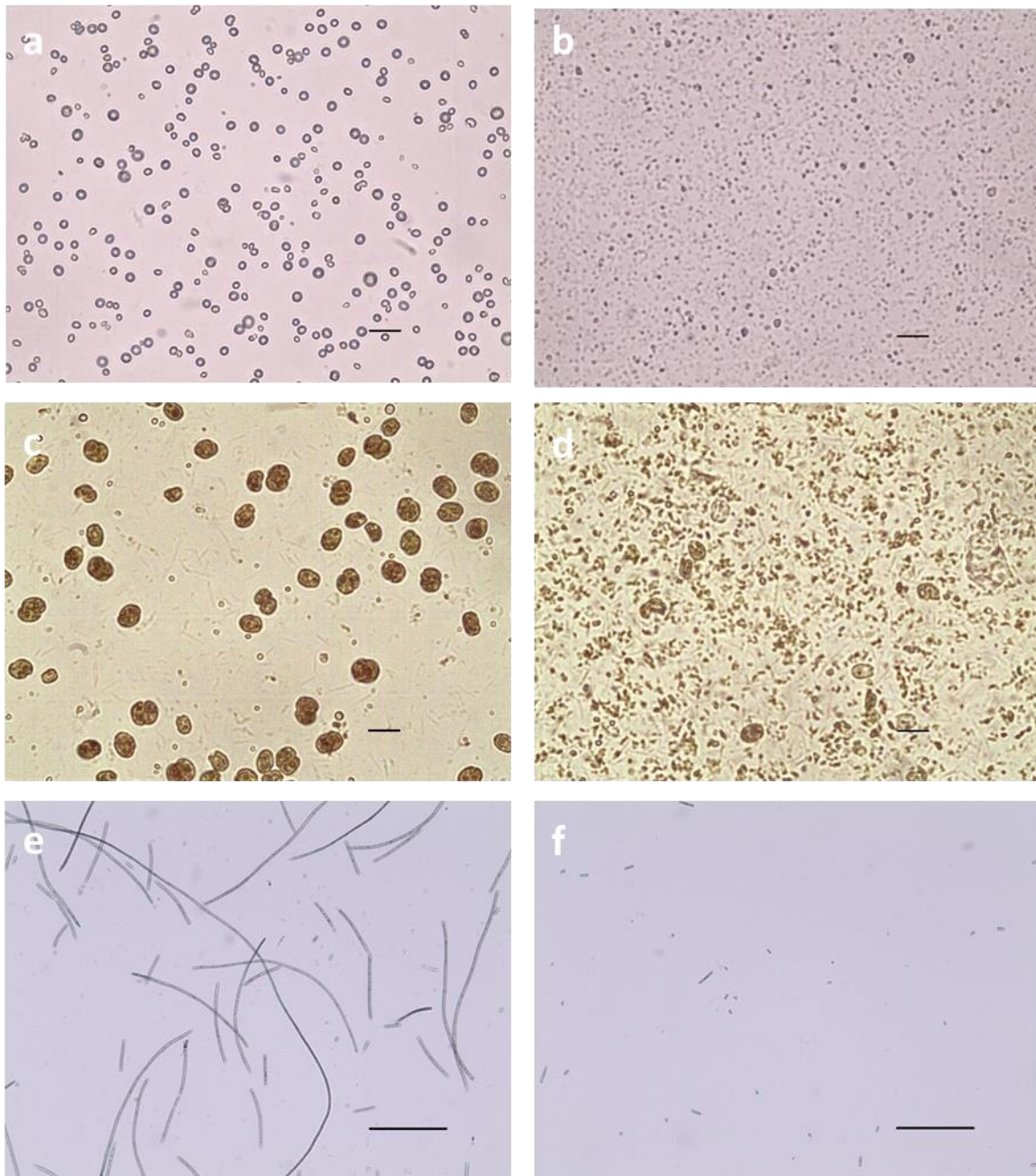


Figure 4.13 Light microscope images of *Chlorella vulgaris* before (a) and after bead milling (b) (scale: 10 μm , x40); of batch A of *Tetraselmis chui* before (c) and after bead milling (d) (scale: 10 μm , x40); *Arthrospira platensis* before (e) and after bead milling (f) (scale: 100 μm , x10).

The cell lysates of *Tetraselmis* and *Chlorella* were analyzed by laser diffraction in order to obtain the Particle Size Distribution, as seen in figure 4.14. The analysis of the cell lysate of *Arthrospira* needs to

be optimized because this technique is adapted to the analysis of spherical or close to spherical particles, hence hampered by the filament aspect.

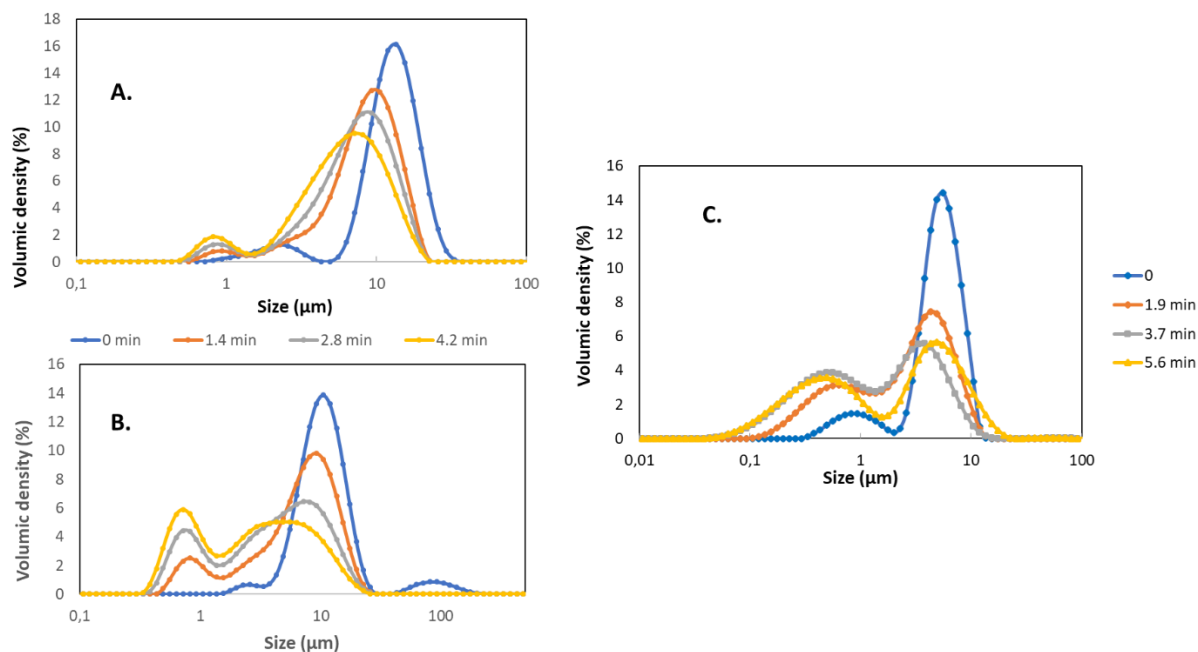


Figure 4.14 Particle size distribution of *Tetraselmis* (batch A (A) and batch B (B)) and *Chlorella* (C) lysates as a function of the residence time within the bead mill.

The cell lysate PSD differences between the different batches of *Tetraselmis* were already compared previously. The PSD of *Chlorella* lysates shows a cell diameter reduction as a function of the time from 5.6 μm to 4.9 μm after 5.6 min and no further decrease was seen after 7.4 min (data not shown). This size seems to be the limit of comminution for this bead diameter and this specie. The cell disruption was associated with a strong increase of small debris along time with a size distribution between 0.1 μm to 1 μm . The cell debris were thinner than what has been found in *Tetraselmis*, where the smaller cell debris had a size of 0.49 μm .

4.3.1.2.2 Dry matter liberation

Cell disruption leads to the production of fine debris, as seen previously. These fine debris can behave like a colloidal suspension, being difficult to centrifuge with parameters suitable for industrial scale. The dispersion of the initial dry matter within the 5,000 g supernatant is seen in figure 4.15. The dispersion of dry matter within the supernatant was high with *Arthrospira*, reaching 89% after 3.7 min of residence time. In comparison, the dispersion was lower for the Eukaryotic microalgae *Chlorella* and

Tetraselmis. The dispersion of *Chlorella* seems to be stabilized at a residence time of 5.6 min with a dry matter yield of 43%, close to the yield obtained on the batch B of *Tetraselmis* where a dispersion of 44% was obtained. The behavior of batch A *Tetraselmis* is unique compared to the other species with the release of only 25%DM.

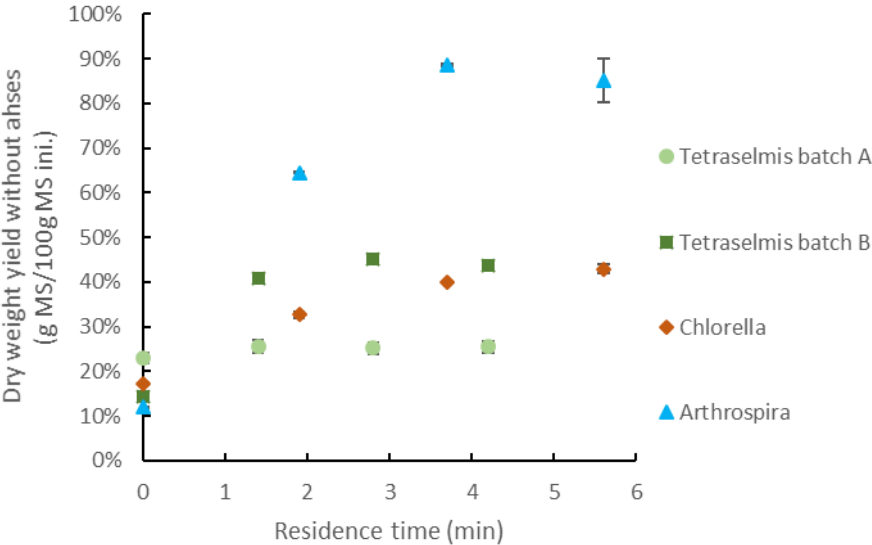


Figure 4.15 Dry matter dispersion as a function of the milling residence time for 3 different species.

4.3.1.2.3 Protein liberation

The protein liberation as a function of the milling residence time was followed and is shown in Figure 4.16.

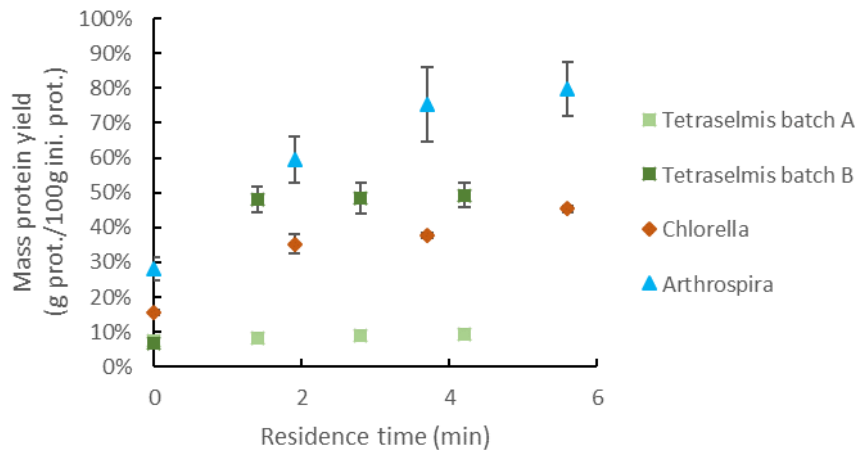


Figure 4.16 Protein yield in the supernatant as a function of the residence time

The release of proteins after freeze thawing was different among species, it was high in *Arthrospira*, with 28% of the total proteins liberated, while it was lower for *Chlorella* and *Tetraselmis* with 16 and 8% respectively. This fast liberation was also observed by Safi et al. 2014 who saw a strong effect of the freeze-thawing on the protein release of *Arthrospira* in comparison with *Chlorella* (Carl Safi, Ursu, et al. 2014).

The bead milling succeeded to release up to 80% of total proteins of *Arthrospira* in the supernatant, with a fast liberation after 1.9 min, with 60% of the initial protein being released. This release is promising in comparison with the other algae, where a maximum of 49% of total proteins was released in batch B *Tetraselmis*. The protein yield of *Chlorella* reached 45% of total proteins after 5.6 min of milling and 35% after the first passage. The fastest protein extraction of all the species was achieved with batch B *Tetraselmis*, which is promising for this specie.

Strong differences exist between protein release results from the tested microalgae, *Tetraselmis* and *Chlorella* and from the tested cyanobacteria, *Arthrospira*. This was expected since this latest specie is

less resistant than Eukaryotes (Fabre et al. 2022) with a different structure since it is a Prokaryote, with a more fragile cell wall and a less organized ultrastructure.

Several authors aimed at extracting proteins from microalgae and different results were obtained on *Chlorella* and *Arthrospira* depending on the studies. On *Chlorella*, Postma et al. 2015 calculated a ratio between the supernatant and the initial biomass of 40% while it was 76% in our case after 3 passages (Postma et al. 2015). This is probably due to a lower bead diameter used in our study since it was also observed by (Postma et al. 2017; Zinkoné et al. 2018).

The disruption of *Arthrospira* led to a high dispersion of the dry matter within the supernatant. Since the biomass is rich in proteins, a high protein yield and a high protein purity were attained after bead milling. A high protein extraction was also obtained on *Arthrospira* after bead milling in another study with a final extraction of $46 \text{ g}_{\text{proteins}} \cdot 100 \text{ g}^{-1}_{\text{dry matter}}$ (Jaeschke et al. 2019). A protein yield of 74% was also obtained after bead milling on *A. maxima* by Teuling et al., similar to the protein yield obtained in this study (Teuling et al. 2017). Moreover, the main storage proteins in *A. platensis*, phycocyanins, were released along the milling and represented 40% of all the extracted proteins after 1.9 min and 35% of all the extracted proteins after 5.6 min. Thus, this water-soluble pigment was mostly released in the first passage (74% of total phycocyanins) while other proteins were released after. It is possible that the released proteins were membrane proteins from the thylakoid.

The objective of the cell disruption is to facilitate the release of proteins in water, for a future separation and enrichment of the protein fraction. With an intensive cell disruption like High Pressure Homogenization, the whole biomass is dispersed in a suspension difficult to separate. Thus, the bead-milling should offer a progressive cell disruption to limit this phenomenon.

According to our results, for *Arthrospira*, while the high protein extraction is promising, nearly no pellet was present at the end of the milling, 92% of the suspension volume becoming the supernatant and 80% of the dry matter being in the supernatant. The clarification under the tested conditions was not efficient to selectively separate proteins from other cell constituents such as chlorophyll, lipids or carbohydrates. Moreover, the strong miniaturization of the cell debris can hinder a following separation and purification. This coupling may finally lead to a lower yield if the whole process is considered and a higher energy consumption. So the criterion of protein recovery yield only is not sufficient. Another criterion could be the purity of the proteins in the extracted supernatant. The protein purity as a function of the residence time is shown in Figure 4.17. The purity of the proteins

from *Chlorella* in the supernatant as a function of the residence time increased slightly after 1.9 min and stabilized until 22%. It was slightly low compared to the purity of the extract from batch B *Tetraselmis*, where a purity of 35% was achieved. This was due to a release in other compounds different from proteins in the supernatant. The evolution of the purity of proteins from *Arthrospira* was promising, reaching a purity of 62%. However, the biomass being at 67% of proteins, this result was linked to the dispersion of a large quantity of the dry matter within the supernatant. Thus the purity is not an ideal criterion either.

The ratio between the phycocyanines, soluble proteins, was the highest after 1.9 min, and decreased after. A shorter residence time may be a better way to release soluble proteins, with less dispersed dry matter. It is highly possible that the bead milling release at first the soluble proteins, and then disperse the biomass, including the insoluble proteins in the supernatant due to the high shear stress. So the yield and the purity are interesting parameters but are not sufficient. The possibility to separate the released proteins from the rest of the biomass afterwards is very important.

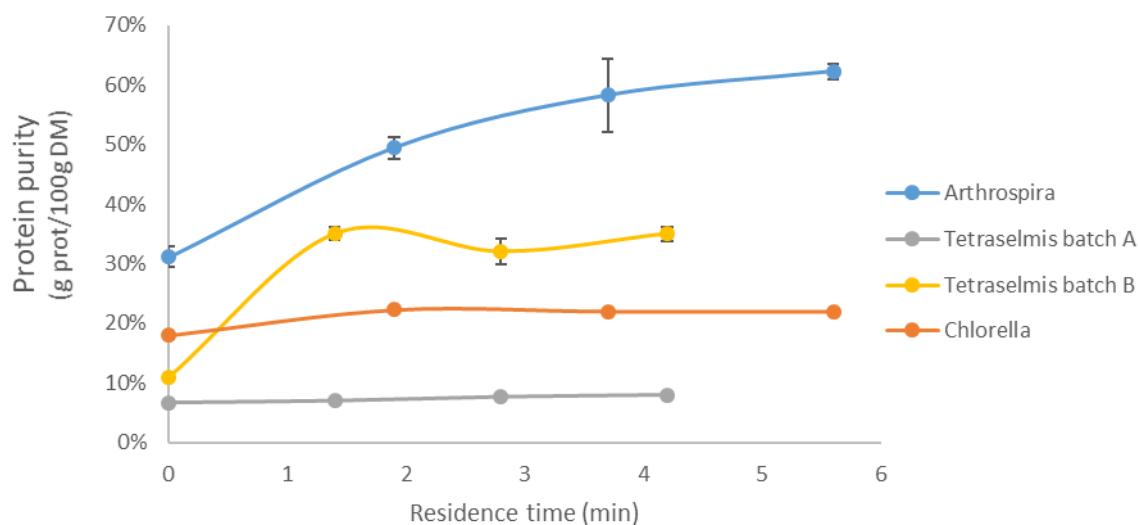


Figure 4.17 Protein purity for 4 different biomass

4.3.1.2.4 Different species, different protein profiles

The protein composition released in the supernatant after bead milling of the two eukaryotes microalgae, *Tetraselmis* and *C.vulgaris*, were compared using SDS PAGE electrophoresis and zymograms as seen in Figure 4.18.

We can see that the protein electrophoretic profile from *Chlorella* is different from *T.chui* batch B, and even more from batch A. The zymograms are also very different: no protease was seen in the zymogram of *Chlorella* on the contrary of *Tetraselmis*.

The electrophoretic profile of the proteins after bead milling of *Chlorella vulgaris*, grown in heterotrophy, was very diverse and bands corresponding to the RuBisCO (22.98 kDa for the small subunit and 52.57 kDa for the large subunit) may be seen but are not intense. The electrophoretic profile is different from what has been observed in *Chlorella vulgaris* grown in autotrophy, where bands that should correspond to the Rubisco were really intense compared with the rest (Rakleova et al. 2013). This microalgae was grown in heterotrophy, which changes the microalgae ultrastructure, such as the cell wall composition (Canelli et al. 2021). In *C.reinhardtii*, a culture on acetate in mixotrophy switched the RuBisCO to the stroma (Borkhsenius, Mason, et Moroney 1998). The ultrastructure changes in microalgae grown in heterotrophy are still not well known.

No proteases were seen in the supernatant of bead-milled *Chlorella* after 5.6 min of residence time. However, the proteins with a molecular weight above 150 kDa were not seen because of a too-short migration time. Proteases from autotrophically grown *C.vulgaris* isolated from Antarctica, after cell disruption, were found at a molecular weight near 200 kDa (Rakleova et al. 2013). However, their activities were weak. Extracellular proteases were also characterized, from 20 to 40 kDa and from 50 to 200 kDa for the mesophilic strain but they were not found in our zymogram.

These results show that the different species and physiological states may strongly impact the processes needed to extract and then purify the proteins. The stability of the extracts may also be directly impacted.

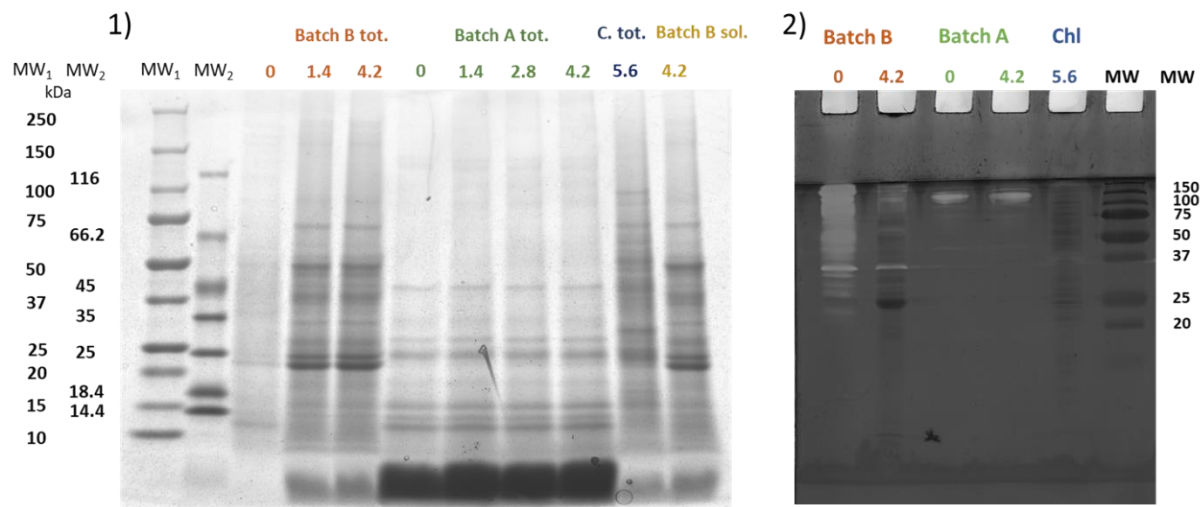


Figure 4.18 (1) Electrophoresis SDS PAGE in reducing conditions of total (tot.) and soluble (sol.) of *Tetraselmis* and *Chlorella* (C.tot) and (2) zymogram of supernatants after bead milling and centrifugation of *Tetraselmis*, batch A and batch B, and *Chlorella* (Chl). The residence time (0 to 4.2 min) in the bead mill is shown above each line.

The electrophoretic profile of the batch B supernatant of the soluble proteins after 4.2 min of residence time is similar to the profile of the clarified proteins. No specific band was removed due to stronger centrifugation conditions.

4.3.1.2.5 Conclusion on the comparison of bead milling for different strains

The cell disruption of *T.chui* was compared with two other species, *C.vulgaris*, a microalgae, and *A.platensis*, a cyanobacteria. A faster cell disruption was observed for *T.chui* compared to *C.vulgaris*. The faster cell disruption may be explained by a higher cell diameter and a weaker cell wall, important parameters for bead milling cell disruption. However, smaller cell debris were observed for *C.vulgaris*, above 0.1 μm compared to cell debris having a size higher than 0.49 μm *T.chui*. It is possible that the clarification may be easier with *T.chui* supernatant due to the lower quantity of fine cell debris. A faster protein release from *T.chui* was observed, compared to *C.vulgaris*. Moreover, the proteins present in the supernatant were different. While these strains belong to the Chlorophyta division, their cell disruption is very different and the extracted proteins are also different. The cell disruption of *Arthrospira* was also different from *T.chui*, with a strong dry matter dispersion within the supernatant. This was linked with a high protein yield and a high protein purity since the initial biomass had a protein content of $72\% \pm 7\%$. However, the supernatant was very green and nearly no pellet was obtained.

While this can be interesting for certain applications, the purification of *A.platensis* proteins released by bead milling can be difficult due to the high organic content in the supernatant.

4.3.1.3 Conclusion on the bead-milling process

Bead milling is an interesting cell disruption technology with a fast cell disruption and a possibility to disrupt cells having a resistant cell wall. However, it can also disperse the biomass nearly completely within the supernatant on fragile species such as *Arthrospira*, leading to a challenging separation if needed. The proteins released by this technology are comprised of soluble free proteins, such as proteins within the cytoplasm, but also of dispersed proteins present in cell debris, such as membrane proteins linked with chlorophylls. Depending on the final objectives, the dispersion of the biomass can be wanted, such as increase extraction by increasing the surface contact, but a high dispersion can also complicate purification. Moreover, depending on the species and on the physiological state, completely different results can be obtained on the dry matter yield and protein yield. This technology can be interesting to extract proteins from *Chlorella* and the fragile cells of *T.chui* but does not seem adapted for the resistant cells, due to the small yield increase. This technology seems also too destructive and energy intensive for *A.platensis* in comparison with traditional methods with freeze-thawing and maceration (Fabre et al. 2022).

4.3.2 Pulsed electric fields

A less disruptive operation unit can be interesting to extract only the soluble proteins present in the cytoplasm and the stroma, and avoid the formation of cell debris. Pulsed electric fields were tested to electroporate the cells without disrupting them. It is a technology widely used to increase the extraction of several compounds such as polyphenol from grapes (Bouras et al. 2015; 2016). This technology has been applied to extract proteins to several microalgae but not to *Tetraselmis chui*. Moreover it was mostly realized on fresh biomass, either to extract irreversibly proteins (Lam et al. 2017) or to do milking, extract proteins while keeping the cells alive (Gateau et al. 2021). In the following paragraphs, the results of the PEF disruption method on the resistant cells of *T.chui* and to *C.vulgaris* are compared.

The dry matter used for PEF can have an important effect on its specific energy consumption. Moreover, a higher dry matter concentration can also lead to a higher protein concentration, decreasing the need for a concentration by other operation units.

The effects of the variations of the dry matter for different electric field strengths E as a function of the specific energy input W on the protein yield were followed and summarized in Figure 4.19.

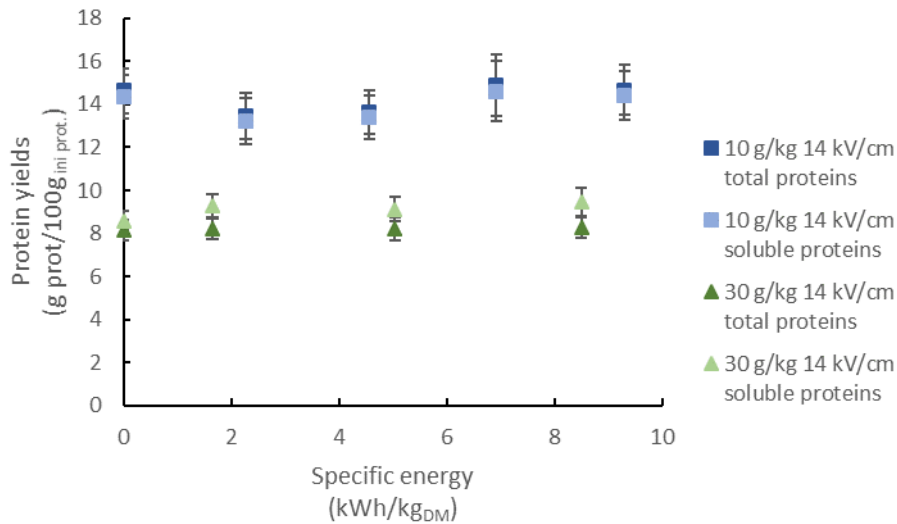


Figure 4.19 Protein yields of treated *T.chui* at two different dry matter as a function of the specific energy.

In comparison with the control after freeze-thawing, no statistical increase in protein yield was observed, for total and soluble proteins and for both dry matters according to ANOVA tests (5%). This could be explained since while PEF can permeabilize the cells due to the electric field strength but freeze-thawing can also permeabilize the cells due to the formation of ice crystals. This result shows that the extraction was not increased after PEF, probably because the proteins targeted by PEF were already extracted by freeze-thawing. This assumption was tested by staining the cells with Evans blue, a dye that does not penetrate intact cells but can penetrate permeabilized cells (Jacyn Baker et Mock 1994). The following of stained cells after PEF treatment has been found to be effective to ensure membrane permeabilization according to other authors on fresh *Chlorella* (Scherer et al. 2019). In our case, the cells of the control were stained in blue, proving that the cells were already permeabilized after freeze-thawing and no change was seen after PEF processing as seen in Figure 4.20. Another way of attesting the permeabilization of cells is the measurement of the electrical conductivity before and after PEF treatment (Goettel et al. 2013; Grimi et al. 2014; Pataro et al. 2017; Carullo et al. 2018). An

increase attests the leaking of ionic intracellular components released in the suspension. In our case, no significant differences were obtained before and after PEF treatment, being in agreement with the blue Evans test.

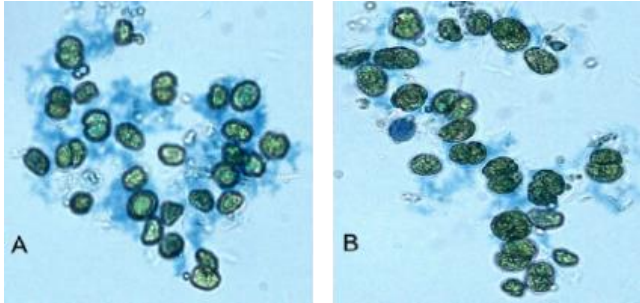


Figure 4.20 Evans blue staining of *T.chui* cells before (A) and after (B) PEF treatment (magnification $\times 400$). The PEF treatment had an electric strength field of 12.5 kV.cm^{-1} and a specific energy of $11.2 \text{ kWh.kg}_{DM}^{-1}$

Moreover, bead milling only showed a small increase in protein yields despite a complete cell disruption on this batch in comparison with freeze-thawing, hence a low PEF protein extraction was expected on the resistant batch.

A difference in protein yield was observed for the two different dry matter concentrations, with higher values obtained at 10 g.kg^{-1} compared to 30 g.kg^{-1} . This can be explained by two main factors. First, the protein yield is affected by the protein ratio between the supernatant and the suspension, but also by the volume distribution between the supernatant and the suspension. This latest is increased when the dry matter decreases, hence less pellet and more supernatant were obtained when the dry matter increases. The second factor can be the fact that the physicochemical environment is different, such as the ionic strength but also the other constituents. The solubility of one protein can be lowered by the presence of other proteins, but also other molecules. It is possible that a limit of solubility of important proteins was attained at 30 g.kg^{-1} was not attained at 10 g.kg^{-1} .

The pulsed electric field extraction of thawed *Chlorella vulgaris* was also tested and the results are presented in Figure 4.21.

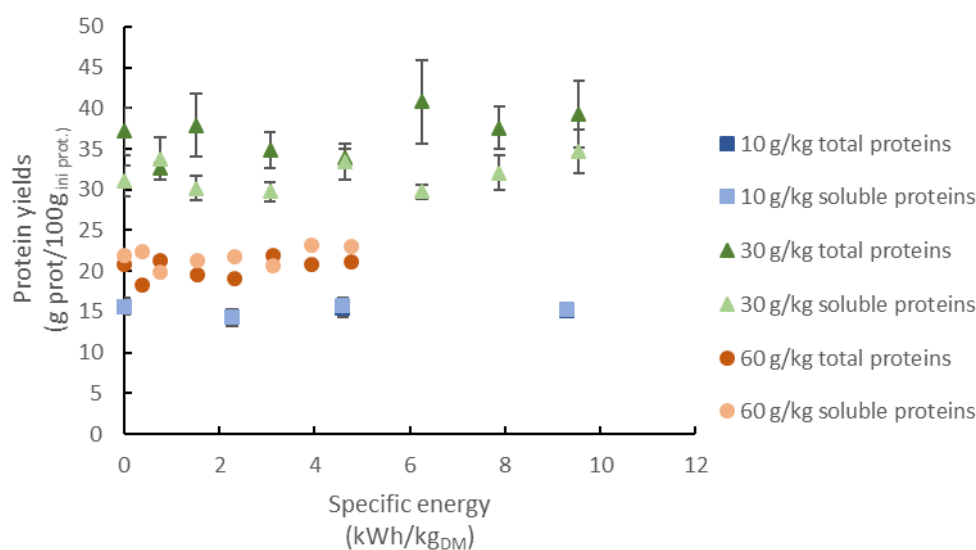


Figure 4.21 Protein yields of PEF processed *Chlorella* at two different dry matter concentrations.

In comparison with the control after freeze-thawing, no statistical increase in protein recovery yield from *Chlorella* was observed, for total and soluble proteins and for the three dry matters according to ANOVA tests (5%). An increase in protein recovery yield from *Chlorella* was observed when the dry matter was increased from 10 to 30 g_{DM}.kg⁻¹. This is in contradiction with what has been found on *Tetraselmis* and may be linked with the different properties of the extracted proteins.

The protein yields observed are higher than the yields reported in the literature. For example, Postma et al. 2016 did not exceed a protein yield of 13% of total protein on fresh *Chlorella* (Postma et al. 2016). The extraction of proteins from fresh *Chlorella vulgaris* has also been attempted by Carullo et al. 2018 and a low yield has been obtained, lower than 8% (Carullo et al. 2018). However, in all reported cases that we are aware of, the biomass was produced in autotrophy. One study applied PEF on a *Chlorella* grown in heterotrophy, but the goal was the extraction of lipids (Canelli et al. 2022).

Several possibilities can explain the higher yields obtained, in comparison with the other studies. The higher protein yield obtained in comparison with other studies could come from a lower protein content within the biomass (20-25%) in comparison with other studies, in which *Chlorella* is grown in autotrophy (40-50%). While the total yield is higher, the quantity of protein extracted may be similar.

Macerations of the cell lysate for 6 hours were tried after the extraction at 10 g.kg⁻¹ on *Tetraselmis* and 10 and 30 g.kg⁻¹ on *Chlorella* but no significant improvement was observed on the macerated control and PEF-treated sample. An improvement of the protein yield has been reported after PEF processing and maceration at room temperature by several authors (Buchmann et al. 2019; Scherer et al. 2019). For example, Scherer et al. 2019 observed an improvement of the protein yield after PEF, followed by an increase for 24 h (Scherer et al. 2019), with most of the increase seen during the first 6 h of extraction. The protein yield due to the maceration was strongly decreased with the addition of a protease inhibitor (cOmplete Plus by Roche). Moreover, some proteins were not released as a function of time when a protease inhibitor was present, such as a membrane-associated protein from mitochondria. The release of compounds after PEF mediated by endogenous enzyme was also observed on *Xanthophyllomyces dendrorhous*, a yeast (Aguilar-Machado et al. 2020) and on *Chlorella vulgaris* grown in heterotrophy (Canelli et al. 2022).

The presence of a wide variety and quantity of proteases after freeze-thawing of resistant *Tetraselmis* was already attested by zymogram, as seen previously. However, the improvement was not significant on either *Tetraselmis* or *Chlorella*, on the contrary to what other authors have found on fresh *Chlorella*.

The PEF treatments on the resistant cells of *Tetraselmis* and *Chlorella* did not lead to an increase in the protein release in comparison with freeze-thawing. The permeabilization of the cells of the two species was not modified before and after treatment according to the conductivity measurement and the Evan Blue staining. The conducted macerations did not lead to a significant increase in the protein yield for the two species.

4.4 Conclusion

The cell disruption of different species of algae was realized by two different cell disruption technologies, bead milling and pulsed electric fields, and compared to freeze-thawing.

Regarding bead milling, the cell disruption of two physiological states of *T.chui* had significant differences in protein yield and dry matter yield within the supernatant. With the fragile cells, a significant increase of the soluble protein yield from 6% to 32% was obtained after bead milling while it increased only from 9% to 11% on resistant cells between freeze-thawing and bead milling. The extracted proteins were different in both cases and characterized by SDS PAGE and zymograms. A higher protease content was obtained in the supernatant of fragile cells in comparison with resistant cells. The results obtained on *T.chui* were compared to *C.vulgaris* and *A.platensis*. The cell disruption and the protein release from *T.chui* were faster than for *C.vulgaris*. Moreover, the cell debris generation was finer in *C.vulgaris* compared to *T.chui*, which should necessitate a decrease of the microfiltration cut-off to retain them, and may lead to a decrease of the flux density. The cell disruption on *A.platensis* led to a strong dispersion of the dry matter within the supernatant. This led to a high protein yield of 80% and a high protein purity due to the high initial protein content within the biomass.

Cell disruption of *C.vulgaris* and *T.chui* by PEF were also compared but no significant improvement of the protein yield was observed between PEF and freeze-thawing. Moreover, no permeabilization differences were observed on the two species by following the intracellular ions leakage through an increase in conductivity, or by Evan Blue staining.

On *Tetraselmis chui*, the selected cell disruption methods were chosen depending on the physiological state. On the one hand, with the fragile cells, bead milling led to a high protein release within the supernatant. While this high protein yield is promising, the proteins extracted can be free globular proteins, or proteins dispersed in the supernatant and associated with cell debris, for example membrane proteins associated with chlorophylls. Thus an operation unit is needed to separate the membrane proteins associated with chlorophylls from the other proteins. On the resistant cells, on the other hand, bead milling led to a little increase in protein yield compared to freeze-thawing and led to a green supernatant. The investment and use of the bead milling were not economically justified in comparison to the little benefits of this technique on the resistant *T.chui* thus freeze-thawing was selected for to extract the soluble proteins. The resistant cells were described to be the most produced

so in prevision for large scale experiments the purification of proteins from this physiological state was more studied.

4.5 References

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5.1. Introduction

The process to obtain an enriched protein extract was focused on *Tetraselmis chui* and especially the resistant cells since it was assumed as the most frequent according to the producer. Following the studies in chapter 4, a simple thawing step was applied to *Tetraselmis* because Pulsed Electric Fields did not increase statistically the yield, and bead milling was not increasing sufficiently the protein yield compared to a simple thawing (8% compared to 11%). Moreover, the extract obtained after bead milling was strongly green while the extract after freeze-thawing was very slightly green. Several techniques have been used to remove most of the chlorophyll after intense cell disruption by bead milling or HPH, such as strong centrifugation conditions (70,000g 30 min, 4°C) (Teuling et al. 2017), microfiltration (Liu et al. 2022) or protein precipitation by organic solvent (Grossmann et al. 2018b). Given the low gain in protein yield with bead milling on the resistant *Tetraselmis*, our approach was to use a minimal processing and reduce the number of operation units.

Since the goal was to produce a low-color extract, a simple thawing was producing a low-colored protein extract with no need for further operation unit to specifically remove chlorophyll.

Traditional purification techniques such as isoelectric precipitation or ultrafiltration could be used to concentrate and purify proteins but not to remove chlorophylls (Ursu et al. 2014; Cavonius, Albers, et Undeland 2015).

The extract from frozen-thawed biomass aimed to achieve a protein purity of 60%_{DM} and a concentration as high as possible to decrease the cost and the environmental impacts of the following drying. New techniques exist for protein purification and are being developed such as chromatography (Schwenzfeier, Wierenga, et Gruppen 2011) or aqueous two-phase partitioning (Moldes et al. 2022). However, they are not yet mature in the food industry and are complicated to scale up. To supply the Profuture project partners with sufficient product quantity, isoelectric precipitation (IEP) and membrane filtration, already used industrially on other biomass, were chosen. The applied purification process is shown in Figure 5.1.

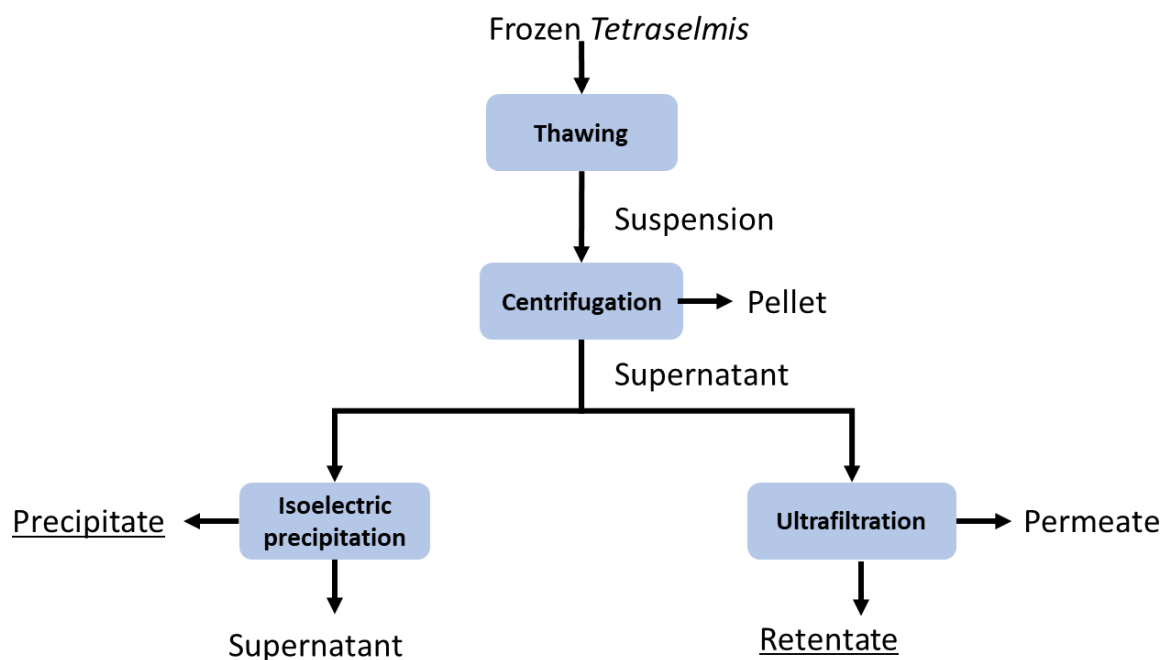


Figure 5.1 Process diagram of protein purification from *Tetraselmis* by IEP and UF. The proteins are recovered in the products underlined, the precipitate after IEP and the retentate after UF.

Although these techniques are traditional to purify protein for the food sector, they have not been widely used on microalgae. IEP and UF techniques can also be used in other application fields, such as the purification of a particular protein for pharmaceuticals or the removal of proteins to avoid emulsion during biofuel production. A better knowledge of these operation units in microalgae biorefinery can also help these applications.

These techniques presented in chapter 2 have different advantages and disadvantages that are interesting to compare. Briefly, isoelectric precipitation relies on protein solubility and can be used to produce a low-cost purified product with a high concentration. However, if a high amount of chemicals is used, the proteins may be denatured, losing functionality, and salt is generated after neutralization. Membrane filtration, on the other hand, can preserve protein integrity, with less solvent consumption and its study for microalgal protein purification was considered promising from a life cycle analysis point of view (Bussa, Zollfrank, et Röder 2021). Moreover, no salt is added and the salt already present can even be removed by a successive diafiltration step. However, the energy consumption can be higher than IEP due to the high tangential flow rate to limit polarization concentration. In addition, the final dry matter concentration can also be lower than IEP.

5.1.1. Product and salt content

Tetraselmis is a marine alga, consequently, the biomass contains salt and in particular sodium chloride in a non-negligible amount. A majority of the ash content can be assimilated as salt since the studied algae do not mineralize their cell wall (unlike diatom or coccolithophore) and most of it is sodium chloride according to analysis realized in Profuture. The ash content can be up to 30% of the dry matter. Since it is highly water soluble and outside the cell, sodium chloride is evenly distributed between the supernatant and the water trapped extracellularly in the pellet. Ash represents the main component in the supernatant S_1 obtained by the latest process diagram. It has to be removed to enhance protein purity and obtain a protein concentrate. Moreover, for food applications, the sodium content should be limited since excessive sodium consumption increases the risks of cardiovascular disease and hypertension (He et MacGregor 2018). The Europeans have already a too-high sodium consumption and it should be reduced to avoid related health issues hence a decrease in the ash content of the final ingredient had another benefit, other than purity (Turck et al. 2019). The food formulations targeted in Profuture were very diverse, ranging from sausages, energy drinks, soup, pasta, so the reduction of salt content is crucial to ensure the compatibility to all recipes, especially for energy drinks. According to the French food composition database CIQUAL, the mean ash content for energy drinks is 1.1 g.kg^{-1} while it ranges from 4 to 12 g.kg^{-1} for soups (ANSES 2023). Given that the ingredient would be diluted and would represent a small fraction of the final ingredient, the maximum ash content of 2 g.kg^{-1} was fixed as the objective. To achieve this goal, a diafiltration step could be added after the UF on the protein concentrate. The number of diavolumes (the volume of water) to add should be assessed to optimize the salt removal and minimize the protein loss.

5.1.2. Scale-up

Several studies were conducted on protein ingredient production from microalgae in literature. However only few of them were carried out at a large scale. The scale-up of the process to pilot and industrial scale is crucial to produce representative product prototypes for deeper characterization of digestibility, detailed composition and techno functional properties. These analyses necessitate between 500 g to 2 kg, depending on the conditions used. For example, techno functional properties can include protein solubility, often as a function of the pH, but it can also be realized at different ionic strengths. It also includes emulsifying, gelling and foaming properties as well as water-holding capacities. The determination of the techno-functional properties guides the incorporation of these ingredients into a food matrix. Plus, specific techno functional properties can be an added value more desired than purity for food formulation (van der Goot et al. 2016).

The upscaling of a process is complex subject which entails

the re-evaluation of the process performances using large-scale equipment which often differs from the lab scale ones. For example, the simple mixing of the suspension follows different mass transfer kinetic if applied to a Becher of limited volume agitated by magnetic stirrer or to an industrial tank agitated by impellers. Another operation unit that is worth considering is the centrifugation, since relevant differences exist between lab scale batch centrifuge and large-scale centrifuge. Differences geometries and rotational speeds can lead to difference performances and the scale-up of lab scale conditions is far to be obvious.

The management of the temperature and the time frame of the operations along the process of large volumes. In fact, the development of the process on several days and the increase of the temperature due to longer treatments (centrifugation, filtration) can affect the product quality (protein denaturation) and product stability (bacterial growth).

The microalgae industry is not fully matured like others, such as the dairy or the soy industry. As such, operation units already mature and used industrially such as IEP or UF has to be adapted to the specificity of the new biomass. A scale-up of the downstream processes is still a challenge and more work in downstream processes at higher scales, especially in membrane filtration, is needed (Bussa, Zollfrank, et Röder 2021).

5.1.3. Strategy

The IEP and the UF were compared at laboratory scale and characterized, to concentrate and purify proteins. Their performances in terms of protein yield and purity were compared.

The objective of the isoelectric precipitation study was to evaluate the production of a precipitate allowing an easy and low-cost upscaling of the protein purification. The isoelectric precipitation has already been used for protein purification but the operation unit was not always characterized and its efficiency on microalgae extracts is debated in the literature, as seen in chapter 2. The first objective was to evaluate the feasibility of this operation unit in the condition considered and the minimization of processing steps and added chemicals. The solid/liquid separation attended as a first approach was gravitational separation, which was tested with a separation expected 1h after pH modification according to methods from literature (Grossmann et al. 2018a). A centrifugation step was also tested as a second approach to assess its feasibility. The feasibility, and if positive, the protein yield, purity and concentration as well as the color and the composition of the final product needed to be characterized. This product can be salty because of the salt already present in the supernatant

microalgae extract before precipitation but also because of the correction of the pH to be neutral, however, a cheap product was expected.

Ultrafiltration has already been used for protein concentration and purification from microalgae extracts but a limited number of studies are available in literature, as seen in chapter 2. The results on this operation unit often lack detailed characterization of the process parameters such as the selection of the critical pressure, the productivity (flux density), or selectivity (retention rate). In this work, the productivity and the selectivity were characterized as a function of the Volume Reduction Ratio (VRR) during the concentration step and as a function of the number of diavolume (DV) during the diafiltration step. These parameters presented in Chapter 2 were calculated as follows in Table 5.1.

Table 5.1 Parameters and their associated equations to characterize the filtration with P_f , P_r and P_p , the pressure of the feed, retentate and permeate respectively, Q_p the permeate flow rate, S the membrane area, C_p and C_r the permeate and retentate concentrations, V_0 , V_r , V_w are respectively the initial volume to be treated, the retentate volume and the added water volume.

Parameters	Equations
TransMembrane Pressure: TMP (bar)	$TMP = \frac{P_f + P_r}{2} - P_p$
Permeate flux density: J (L.h ⁻¹ .m ⁻²)	$J = \frac{Q_p}{S}$
Observed Retention Rate: RR	$RR (\%) = 1 - \frac{C_p}{C_r}$
Volume Reduction Ratio: VRR	$VRR = \frac{V_0}{V_R}$
DiaVolume number: (DV)	$DV = \frac{V_w}{V_r}$

These parameters allow an appropriate characterization of the filtration and are needed to assess its performances and guide the scale up. Ultrafiltration can be more expensive in investment cost than IEP, but a premium product, with less salt and hopefully higher protein purity, was targeted. The most promising operation unit was scaled up at pilot scale and semi-industrial scale.

Taking into account the results on the fragile cells that were acquired lately during this work and presented in chapter 4, it was also decided to evaluate the coupling of bead-milling and microfiltration through laboratory scale experiments to recover proteins, as seen in figure 2.11.

In the following paragraphs, the experimental strategy is described, and the protocols are summed up. Then the results are presented on isoelectric precipitation and ultrafiltration to concentrate and purify proteins from a supernatant after biomass thawing and the performances are compared. The most promising technique is scaled up at a pilot scale and at a semi-industrial scale and the results are

described. The first results are also presented on the coupling of bead-milling and microfiltration to recover proteins from fragile *Tetraselmis*. Finally, the choice of the coupling of cell disruption and separation is discussed versus the biomass physiological state and the objective of product quality.

5.2. Materials and Methods

The details of the material and methods are given in Chapter 3. In the following paragraphs, the strategy of the experiments is presented, and the protocol summed up. The experimental design on the freeze-thawed biomass to compare IEP and UF is presented in Figure 5.2.

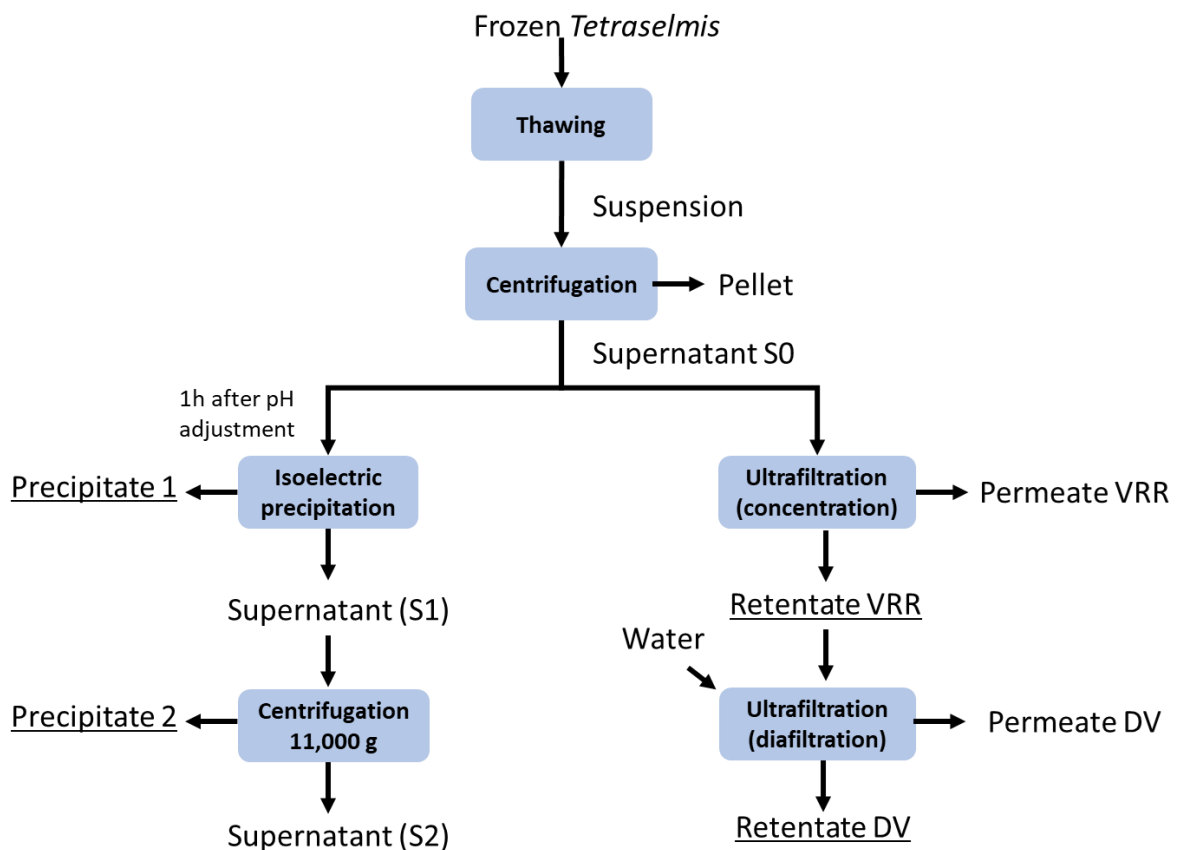


Figure 5.2 Process diagram of protein purification from *Tetraselmis* by IEP and UF with the fractions containing the proteins underlined. IEP is realized by adjusting the pH and waiting 1h for settling and recover proteins in the precipitate 1, while a centrifugation is tested to precipitate the proteins remaining in the precipitate 2. The UF is realized by a first concentration, followed by a VRR.

5.2.1. Suspension and centrifugation

The frozen paste of *Tetraselmis* at 20%_{DM} was thawed at 60 g_{DM}.kg⁻¹ and centrifuged at 5000g for 4 min to recover the supernatant.

5.2.2. Isoelectric precipitation

5.2.2.1. Objectives and strategy

The objective is to precipitate the proteins contained in a mixture by finding the pH at which most of the compounds are near their isoelectric point $pH(I)$. The pH is also key to determine the functionality of the precipitate obtained. In general, less interesting functionalities after a precipitation close to the $pH(I)$ can be observed, such as a lower solubility of the proteins from the recovered precipitate when the pH is sent back to neutral pH (Nash, Kwolek, et Wolf 1971).

To evaluate the optimal pH to precipitate the proteins, the minimum protein solubility has to be identified. The solubility of the proteins as a function of pH was evaluated after gravitational separation in the supernatant S1, and after an analytical centrifugation in the supernatant S2, as seen in Figure 5.2. Moreover, the turbidity in the supernatant S1 was also characterized, since an increase in turbidity was hypothesized to be mainly due to protein aggregation and was used as a marker of isoelectric precipitation in other studies (Grossmann et al. 2018a).

5.2.2.2. Methods

The frozen paste of *Tetraselmis* of batch A at 20%_{DM} was thawed at 50 g_{DM}.kg⁻¹ and centrifuged at 5000 g for 4 min to recover the supernatant S0. The pH, conductivity and dry matter of the supernatant were characterized. The supernatant was then redistributed in 50 mL Falcon tubes for the first experiment. The pH was adjusted at pH 2, 4, 5 and 6 with hydrochloric acid at 1M under agitation and the pH was measured across the experiment and adjusted if needed (tampon effect of the proteins). The added acid volume was considered negligible in protein concentration modification.

The acid was added while maintaining the tube agitated to avoid a local zone with a very low pH which could denature proteins, affecting their solubility. Samples were taken after an hour at room temperature without agitation.

The experiment was done a second time, narrowing the pH tested to 2, 2.5, 3, 3.5 and at native pH to determine precisely the $pH(I)$.

The proteins were quantified in triplicate by BCA in the supernatant S₁ without centrifugation and after centrifugation at 11,000g for 10 min leading to the supernatant S₂. The turbidity of the supernatant S1 before centrifugation was measured at 600 nm with a spectrophotometer (Jasco V630). The proportion of proteins still soluble after pH modification was expressed as:

$$Protein\ solubility = \frac{[Proteins]_{S_2}}{[Proteins]_{S_1}} \quad \text{Equation 5.1}$$

5.2.3. Ultrafiltration

5.2.3.1. Objectives and strategy

The membrane filtration was performed after cell disruption and clarification with two objectives: (1) to concentrate the proteins and (2) to eliminate the salts using the diafiltration mode.

The commercial membrane cut-off was chosen based on native electrophoresis. The ultrafiltration performance was characterized by different parameters after the membrane preparation from the storage, applied as previously presented in chapter 3. The concentration of the proteins in the final retentate, their recovery yield and purity as well as the color and the time needed to obtain the desired product were explored.

The clean membrane permeation flux at the initial state was characterized by the determination of water permeability. The best membrane operating conditions were determined by a product permeability to reach the critical conditions and to deduce the operating TMP. This was done only at laboratory scale to avoid irreversible fouling of larger-scale membranes, and the membrane was thoroughly washed after. The fluxes and the retention rates were quantified along the concentration step. The reduced volume allowed a lower water consumption during the diafiltration. The retention rates and the flux of this latest step were characterized as a function of the diavolume used to decrease salinity and increase protein purity.

The volume to be treated by filtration is highly function of the membrane surface, which is fixed for the equipment, and also of the flux density, which strongly depends on the product characteristics. Time is the parameter that is the most easily adaptable and the relation between the time t , the VRR, the membrane surface S , the initial volume V_0 and the flux J is shown in the following equation:

$$t = \int_{VRR=1}^{VRR} \frac{V_0}{JS(VRR)^2} d(VRR) \quad \text{Equation 5.2}$$

Since the goal was to produce a protein extract for food applications, several aspects have to be taken into account for the scaling up. A biological extract can be prone to changes such as the development of microorganisms or/and of enzymatic degradation that can modify the product along filtration time. Microbial contamination can degrade proteins and are in general not acceptable above a certain limit in food. The contamination of the protein product by important microbial load can also hinder their use for food reformulation by the ProFuture partner's. Moreover, an unpleasant smell can be developed, which can repel the consumer, not because of the ingredient, leading to wrong decisions about the applicability. The enzyme activity, such as proteases, can degrade the product. The proteases may hydrolyze the proteins in smaller fragments, leading to a lower recovery because of a lower retention rate. The microbial development and the enzyme activity are highly dependent on

temperature so the stability of the extract along the time was tested as a function of temperature. The tested parameters were the protein concentration since it was our compounds of interest, the total aerobic microorganisms, protein types by electrophoresis, pH, and sensory aspects such as smell.

5.2.3.2. Methods

5.2.3.2.1. *Laboratory scale (L)*

5.2.3.2.1.1. Suspension and centrifugation

The suspension was prepared as described in detail in Chapter 3 from the paste to a first dilution at 10%. After dilution and thawing, the dry matter was measured and the suspension was diluted to 50-60 g_{DM}.kg⁻¹. The suspension was centrifuged at 5000g for 6 min in 1 L buckets. The biomass used for these tests belonged to the resistant batch A since the resistant cells were supposed to be the most frequent at industrial scale according to the supplier.

5.2.3.2.1.2. Ultrafiltration

The membranes were cleaned from the storage solution and prepared as shown in detail in Chapter 3 at all scales. The same coupon was used for all the experiments.

5.2.3.2.1.3. Water permeability

The water permeability was measured at 30°C with deionized water and by recording the mass of the permeate as a function of the time at different TMP. Each TMP was held for 5 to 10 minutes to ensure correct measurement. The water permeability was measured before filtration and after cleaning to verify the membrane flux performance. The water permeability was carried out with the same protocol if an exception was not mentioned at the other scales.

5.2.3.2.1.4. Product permeability in full recycling mode

The product permeability in full recycling mode was measured at 30°C since the following filtrations at this scale were carried out at this temperature. This temperature was chosen to increase the flow rate in order to test higher VRR and DV at laboratory scale in prevision for a scale-up.

5.2.3.2.1.5. Concentration mode

The product was concentrated at 30°C at a TMP of 1 bar to reach a high VRR to decrease the volume, increase the concentration and if possible, increase the purity. The flux density and the retention rates were followed as a function of the VRR to assess the filtration performances when the product was concentrated. A concentration until a VRR of 2.7 was obtained for the first filtration L1 while the second filtration L2 aimed at adding a diafiltration step with experiments performed in the same day, so a lower VRR of 1.8 was obtained.

5.2.3.2.1.6. Diafiltration mode

The final retentate was diafiltrated at constant volume, 30°C and a TMP of 1 bar to eliminate salts and increase protein purity. The flux density and the retention rates were followed as a function of the DV to assess the filtration performances when the product was diafiltrated. The second filtration F2 aimed at diafiltrating the retentate to a DV of 0.75.

5.2.3.2.2. *Scale up*

5.2.3.2.2.1. Supernatant stability along time as a function of the temperature

A suspension of *Tetraselmis* from the resistant batch A cells was diluted with distilled water at 30 g_{DW}.kg⁻¹ and was centrifuged at 5,000 g for 4 min in 50 mL falcon tubes. The supernatant was transferred into beakers were covered by parafilm to avoid evaporation as well as external contamination and agitated with magnetic stirrers. The beakers in duplicate were stored at 3 different temperatures, 12°C, room temperature (20°C) and 47°C and sampled 3 times per day (morning, midday, and evening). A control was followed at 4°C and sampled the morning and the evening.

The proteins were quantified by BCA analysis and the pH followed. The total aerobic flora was quantified following the norm EN ISO 4833-1:2013 and the sampling was carried out with sterile material.

SDS PAGE in reducing condition was realized in order to follow a possible modification of the type of proteins (protease hydrolysis, microbiological development). The electrophoresis was conducted following the protocol of Lawrence et Besir, 2009 (Lawrence et Besir 2009) and revealed by Coomassie Brilliant Blue G 250.

5.2.3.2.2.2. Pilot scale (P) Suspension and centrifugation

The experiment P was performed using the biomass from batch C. The suspension was prepared as described in detail in Chapter 3 from the paste to a first dilution at 10%. After dilution and thawing, the dry matter was controlled, and the suspension was diluted to 50-60 g_{DM}.kg⁻¹. The suspension was centrifuged at 5000 g for 6 min in 1 L buckets. After centrifugation, an orange compound was present at the bottom of the tank containing the suspension. It was partly water-soluble and was also present in the supernatant, with a different color than at laboratory scale.

5.2.3.2.2.3. Ultrafiltration

The water permeability and the product permeability were conducted as shown in the laboratory scale part at 12°C. The product was concentrated at 12°C at a TMP of 1 bar to a VRR of 3.4. The final retentate was diafiltrated at constant volume at a TMP of 1 bar until 2 DV. The conductivity of the retentate was

measured across time to reach a conductivity equivalent to a sodium chloride solution at 2 g.L^{-1} . The temperature of the permeate never reached a temperature higher than 13°C .

5.2.3.2.3. *Semi-industrial scale (SI)*

5.2.3.2.3.1. Suspension and centrifugation

The suspension was prepared in Algosource as described in detail in Chapter 3 from the paste to a first dilution at 10%. After dilution and thawing, the dry matter was controlled and the suspension was diluted to $50\text{-}60 \text{ g}_{\text{DM}}.\text{kg}^{-1}$. The suspension was centrifuged. The process led to the increase in the product temperature from 9°C to 35°C . The supernatant was then cooled and stored at 4°C .

Two different batches of supernatant were produced from two biomass batches, D and E, for the supernatants S0-D and S0-E respectively. The orange compound present during the experiment at pilot scale was present in small quantity in the paste but discarded to avoid interference with the carbohydrate quantification.

5.2.3.2.3.2. Ultrafiltration

The supernatant S0-D-1 represented a too large volume to be treated all in one day with the membrane surfaces available. It was treated in two filtrations SI 1 and SI 1' during two successive days. Before the first filtration SI 1, the membrane was cleaned. However, the membrane was only rinsed with water between SI 1 and SI 1'.

The supernatant S0-E-1 was treated at once during filtration SI 2. Taking into account the retention rates measured during the former experiments, an additional experiment on the SI 2 permeate of the concentration was performed.

The water permeability was conducted at 12°C before each filtration. The product permeability was conducted just before the concentration at 12°C to reach a TMP slightly higher than 1 bar in order to assess the presence of an important concentration polarization. The supernatants were concentrated at different VRR as seen in Table 5.2, depending on the initial volume. This was carried out at 12°C and a TMP of 1 bar.

Table 5.2 Semi-industrial scale filtrations and their VRR.

Filtration	SI 1	SI 1'	SI 2	Acid Permeate
Supernatant	S0-D-SI 1	S0-D-SI1	S0-E-SI 2	S0-E-SIé-permeate
VRR	2.6	3	3.5	3

The final retentates of SI 1, SI 1' and SI 2 were diafiltrated at constant volume at a TMP of 1 bar until 2 DV. The conductivity of the retentate was measured across time to reach a conductivity similar to a sodium chloride solution at 2 g.L⁻¹. The temperature of the retentate was maintained below 15°C (slight warming due to the added water).

The concentration of the acidified permeate from the concentration step from S2 was tested after the diafiltration step. The semi-industrial pilot was only emptied and rinsed with water between the two experiments. The permeate from S2 was recovered from the concentration step and acidified at pH 3 under agitation with 1M HCl. The choice of acidification at pH 3 was carried out to aggregate the proteins since an increase of turbidity was shown at this pH during the study of isoelectric precipitation. Moreover, pH 3 limited the risk for the membrane degradation and the amount of acid added.

5.2.3.2.4. Experiment organization

The materials and methods of the different scales are presented in Table 5.3.

Table 5.3 Summary of the parameters used for the different scales with, in green, the suspension preparation, in orange the centrifugation and in blue the ultrafiltration.

Scale	Laboratory	Pilot	Semi-industrial
Biomass	A	C	D and E
Dry matter (kg)	0.139	1.7	10.2
Paste (20% DM)	0.695	8.5	51
Suspension mass (kg)	2.9	19.8	163
DM susp (g _{DM} .kg ⁻¹)	49	91	55
Agitation type	Magnetic stir bar		Pale stirrer rotated by compressed air
Centrifuge type	Sorvall Lynx 6000 superspeed centrifuge		Westfalia KA6-86-076
Centrifuge volume	6 x 0.9 L		9 L
Treated supernatant	S0-A-1 S0-A-2	S0-C-1	S0-D-SI1, S0-E-SI2
Ultrafiltrations	L1, L2	P1	SI1, SI1', SI2, perm.acid.
Membrane supplier	Synder		Koch
Geometry	Plane	Spiraled	Hollow fibers
Material	PolyEtherSulfone (PES)		PolySulfone (PS)
Surface (m ²)	0.013	0.269	2.3

Temperature (°C)	30	12	12
Cut-off (kDa)	10 kDa		
Crossflow speed (m.s ⁻¹)	1		
TMP (bar)	1		

5.2.4. Bead milling and microfiltration

The experiments presented in this part were performed at the end of the project, using the results from Chapter 4 on the cell disruption of fragile cells. These experiments were not repeated due to a time constraint but can be useful to compare to the process thawing/centrifugation/UF and suggest a new approach.

5.2.4.1. Bead milling and centrifugation

The bead milling experiment was conducted in the same conditions as described in Chapter 4 on fragile cells. Briefly, the chamber was filled with 80% of beads having a diameter of 0.5-0.7 mm, in Yttrium stabilized zirconia. The dry matter content of the suspension was 60 g_{DM}.kg⁻¹ and the bead mill temperature was controlled by an external exchanger so that the product temperature stayed lower than 25°C. After cell disruption, the lysate was diluted by half to facilitate the separation between the supernatant S_{BM} and the pellet during centrifugation according to results from Chapter 3 and Liu et al. (Liu et al. 2021).

The centrifugation of the samples for analysis was carried out at 5,000g for 4 min in the 50 mL tubes while the entire suspension was centrifuged at 5,000g for 6 min in 900 mL pots for the microfiltration.

5.2.4.2. Microfiltration

The cross-flow microfiltration was carried out at 0.1 µm with a plane membrane in PES (KOCH) and a surface of 0.013 m² and in the Rayflow system, in order to compare with former studies from Liu et al (Liu et al. 2021; 2022). The membrane was prepared with the protocol described for the laboratory scale ultrafiltration membrane preparation, as described in Chapter 3.

The filtration of the supernatant S_{BM} was realized at a TMP of 0.3 bar and with a crossflow speed of 1 m.s⁻¹, corresponding to experimental conditions proposed by Liu et al. and a temperature of 25°C (Liu et al. 2022).

5.3. Results

5.3.1. Isoelectric precipitation of resistant *Tetraselmis chui* freeze-thawed supernatant

5.3.1.1. Performances of the IEP

The protein solubility in the supernatant as a function of the pH was evaluated to determine the pH with a minimum protein solubility, the pI of a majority of the proteins, in the process conditions considered.

The resistant batch A of *Tetraselmis chui* was frozen-thawed and centrifuged to produce the supernatant S0-A. It contained 1.0 g.L⁻¹ of proteins for the supernatant of the first IEP experiment and 1.3 g.L⁻¹ for the second, in the same proportions as found in chapter 4 after thawing of batch A biomass. It was acidified at different pH to characterize the isoelectric point. The turbidity was followed in the supernatant S1-A after 1 hour of pH modification and is seen in **Figure 5.3**. Despite an evolution of the turbidity, no gravitational protein precipitation or settling was observed at all and S0-A and S1-A had similar protein concentrations on the studied pH range. An additional solid/liquid operation unit seemed to be necessary. To test it further, a centrifugation at 11,000 g was added to make the formed aggregates settle and quantify the remaining soluble proteins in supernatant S2-A as seen in **Figure 5.3**.

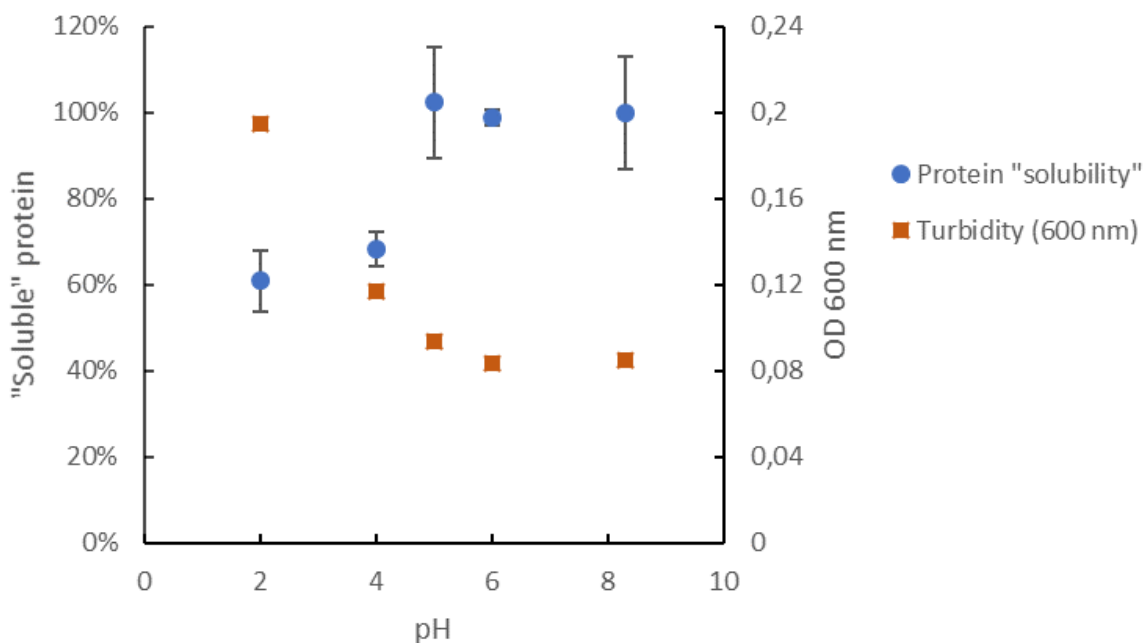


Figure 5.3 Protein solubility in the supernatant S2-A after centrifugation at 11,000g 10 min centrifugation and the turbidity in the supernatant S1-A as a function of the pH, one hour after pH adjusting,

The protein solubility decreased from 100% at pH 5 to 60% at pH 2. The turbidity of S1-A was the highest around pH 2. The decrease of protein solubility in S2-A was correlated with an increase in

turbidity in S1-A. This may be due to an increase in light dispersion due to an increase in particle size caused by protein aggregation. This behavior was also observed by Grossmann et al. 2019 (Grossmann, Hinrichs, et Weiss 2019b). A low turbidity of the aqueous extract at neutral pH may be attributed to a high protein solubility and a low size of the dispersed proteins. The protein solubility was also tested after 24h of pH modification, but no differences were seen in comparison with the results obtained after 1h. Another experiment aiming at determining more precisely the pH(I) between pH 2 and 3.5 is presented in **Figure 5.4**.

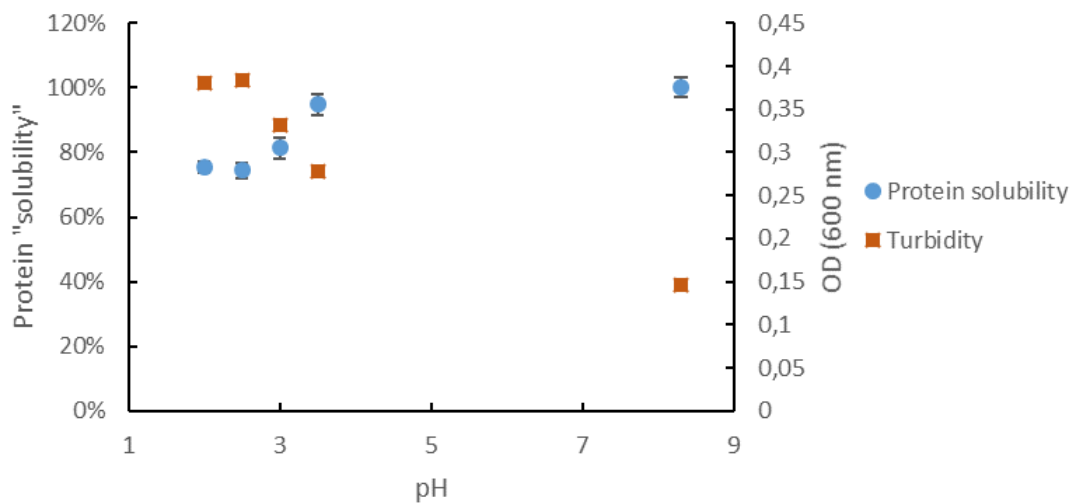


Figure 5.4 Protein solubility in the supernatant S2-A after centrifugation at 11,000g 10 min centrifugation and the turbidity in the supernatant S1-A, one hour after pH adjusting.

The protein solubility dropped from pH 3.5 to a minimum, the pH(I), at pH 2.5 and was stabilized at pH 2. The turbidity had an opposite behavior with an increase from pH 3.5 to a maximum at pH 2.5 and was stabilized at pH 2. The linked behavior between the remaining soluble proteins in the supernatant S2-A and the turbidity in the supernatant S1-A was also observed by different authors (Grossmann, Hinrichs, et Weiss 2019a). The high stability of the proteins from the supernatant whatever the pH complicates the recovery of the proteins by this operation unit in these conditions.

5.3.1.2. Discussion

Overall the proportion of *Tetraselmis* proteins from the supernatant after thawing that remains soluble whatever the pH was high compared to other protein sources such as soybeans or peas, even at the pH(I). For example, only a protein solubility of 5% at the pH(I) of pH 4.5 was obtained on soy isolate (Ali et al. 2010).

The protein solubility was also high in Figure 5.3 and Figure 5.4 in comparison with what has been obtained by Schwenzfeier et al. 2011 on bead-milled *Tetraselmis* extract as seen in Figure 5.5 (Schwenzfeier, Wierenga, et Gruppen 2011).

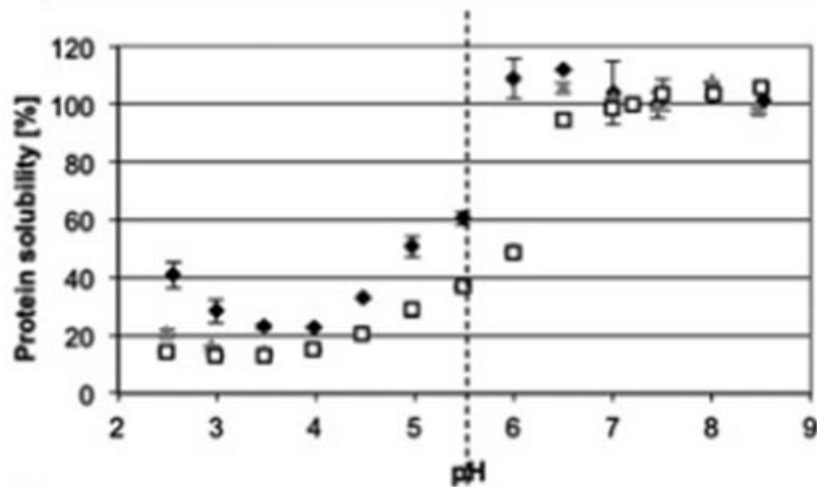


Figure 5.5 Protein solubility as a function of the pH at three different ionic strengths (0.03M, represented by the diamonds, 0.2M represented by the triangles and 0.5 M represented by the squares), from (Schwenzfeier, Wierenga, et Gruppen 2011).

This difference may come from different types of proteins that were extracted between freeze-thawing and bead milling since this latter technique can disperse proteins associated with chlorophyll that may be hydrophobic.

High microalgal protein solubility in acid conditions has already been observed by Grossmann et al. for proteins extracted from different microalgae (Grossmann, Hinrichs, et Weiss 2019a; 2019b). Different reasons were identified such as a high hydrophilicity of the amino acids, but also the presence of highly glycosylated proteins. The presence of glycoproteins was hypothesized to be a reason for a low pH(I) at pH 2 in their extracts. Most of the amino acids have higher pKa values than 3 so the low pH(I) was assumed to be complexes of proteins with charged polysaccharides, such as pectin-like polysaccharides (Schwenzfeier, Wierenga, et Gruppen 2011; Schwenzfeier et al. 2014; Teuling et al. 2017; Grossmann, Hinrichs, et Weiss 2019a; 2019b). This was also shown by negative ζ potentials at pH 3, at a lower pH than the pKa of proteins (Grossmann, Hinrichs, et Weiss 2019b).

The presence of charged polysaccharides enhancing the protein solubility of our extract could explain the low pH(I). Moreover, the zeta potentials of our extract were also tested and were negatives and similar from pH 8.3 to pH 3.5, with a shift from pH 3.5 (-13.1±0.9 mV) to pH 2.5 (-9.5±0.5 mV), strengthening an action of charged polysaccharides. It is in agreement with Schwenzfeier et al. (Schwenzfeier, Wierenga, et Gruppen 2011) who found glycoproteins in *Tetraselmis*. Moreover, *Tetraselmis* has a great proportion of galactose in the whole biomass (Brown 1991), a monomer of pectin, and its cell wall is composed of 82%_{DW} of acidic polysaccharides.

While the presence of acidic polysaccharides or hydrophilic amino acids may explain the absence of precipitation in the conditions tested, it is possible that the protein concentration was not high enough to settle in the centrifugation conditions tested. However low protein concentration (0.6 g/L) of glycinin, a major protein of soy seed was used for IEP and led to a precipitate with a very high yield. So a high solubility of the extract obtained from *Tetraselmis* may still be the most supported hypothesis (Lakemond et al. 2000). Moreover, the goal was to use this operation unit as a succession of the freeze-thawing step so the low protein concentration was a constraint of the integration.

The use of multivalent ions is well known to promote precipitation and could be tested in the future. For example, magnesium or calcium are used for the production of tofu, a soy protein precipitate from soy milk (Arii et Takenaka 2013). However, this may have resulted in an enriched ash fraction, which was sufficiently high in the initial product. More research is needed to better understand it and since the objective was to obtain a precipitate, this approach was not investigated for the resistant *Tetraselmis*. A pH(I) was identified at pH 2.5, with a high protein solubility in comparison with the supernatant obtained by other authors on bead-milled *Tetraselmis* and in comparison with other plant proteins.

5.3.2. Ultrafiltration at laboratory scale

The concentration and diafiltration of the proteins present in the supernatant by ultrafiltration were tested at laboratory scale. The material and methods are presented in part II and detailed in chapter 3. A cut-off of 10 kDa was selected based on native PAGE realized on the supernatant. The composition of the supernatants produced for the filtrations L1 and L2 is given in Table 5.4.

Table 5.4 Supernatant compositions before ultrafiltration obtained on the batch A of T.chui. NA: Non Analyzed.

Supernatants	Dry Matter	Organic Matter	Ashes	Proteins	Carbohydrates
S0-A-1 (L1)	23.52 ± 0.02	9.44 ± 0.03	14.08 ± 0.05	1.36 ± 0.15	2.78 ± 0.4
S0-A-2 (L2)	19.7 ± 0.33	7.9 ± 0.16	11.8 ± 0.14	1.00 ± 0.02	NA

The protein concentration was close to the supernatant concentration obtained at IEP so the products treated were comparable. The objectives of this study at laboratory scale were at first to characterize the initial membrane performances by calculating the water permeability and then to evaluate the product permeability in total recycling mode as well as to choose an optimal TMP. The fluxes could help us scale up the process and choose the volume that can be treated, even if caution must be taken since with the small surface being used, variation in membrane performances can be seen. Moreover,

plane geometry is not very used at industrial scale, hence a change of geometry can change the hydrodynamic and can change the performances. A first range of flow density is nonetheless key to plan future experiments at higher scale.

Finally, the other objective was to evaluate the retention rates of the main compounds (dry matter, ashes, proteins and carbohydrates) to characterize the selectivity of the membrane. A high protein retention rate was expected to limit the protein loss and a low ash retention rate was expected to be able to eliminate salts and increase protein purity.

5.3.2.1. Permeability and flux density

5.3.2.1.1. Product and water permeabilities

The membrane performance was first tested with a water permeability, followed by a product permeability. The product permeability allows the choice of an optimal TMP since at high TMP, the flux density is not increasing proportionally to the pressure due to a polarization of concentration. Even worst, at a pressure too high, defined as the critical pressure, the permanent fouling can appear, limiting the membrane performances. The initial water permeability and the product permeability are shown in Figure 5.6.

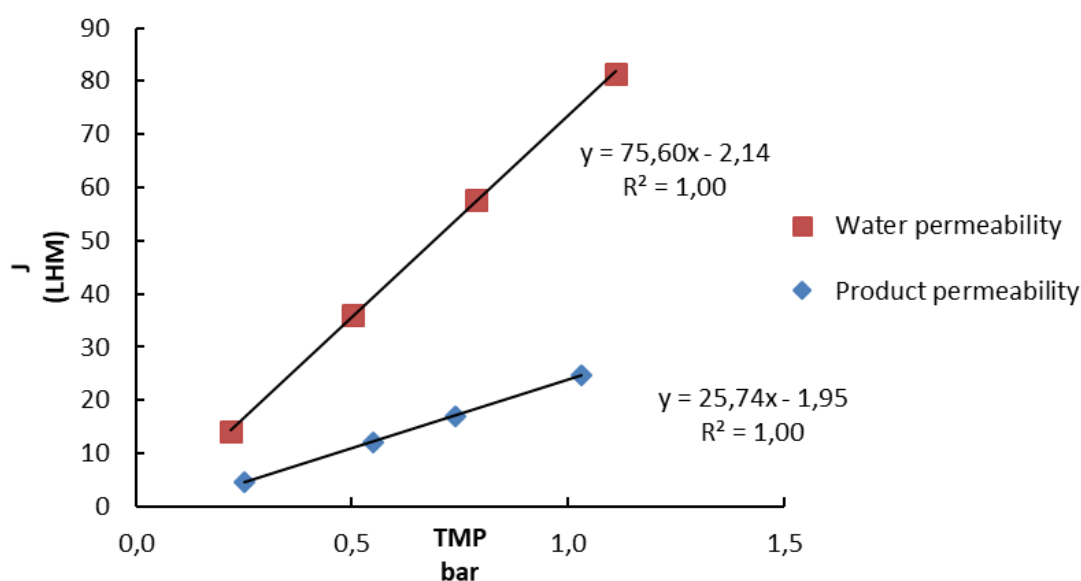


Figure 5.6 Water and product flux densities as a function of the TMP at 30°C

In this case, the volumetric water permeability at 30°C was 76 L.h⁻¹.m⁻².bar⁻¹ and the product permeability was linearly function of the TMP, with a permeability of 26 L.h⁻¹.m⁻².bar⁻¹ and a R² of 1. No decline in flux density as a function of the TMP was observed for the product in the range of tested pressures so the critical pressure was not attained and a higher pressure could have been applied.

However, due to the pressure limit of the system and especially the peristaltic tube, the product was filtered at a TMP of 1 bar.

A filtration in recycle mode was also realized to quantify the flux behavior when this product was filtered for a longer period when a deposit layer was present. A decline in flux from 0.3 to 0.28 J_{sup}/J_w in 70 min, hence a decline in flux from 26 to 20 $L \cdot h^{-1} \cdot m^{-2}$ was observed.

5.3.2.1.2. Concentration of the supernatant

The extract was concentrated in batch mode and the impacts of the volume reduction on the filtration performances were followed. The evolution of the flux density as a function of the VRR during the filtrations L1 and L2 is presented in Figure 5.7.

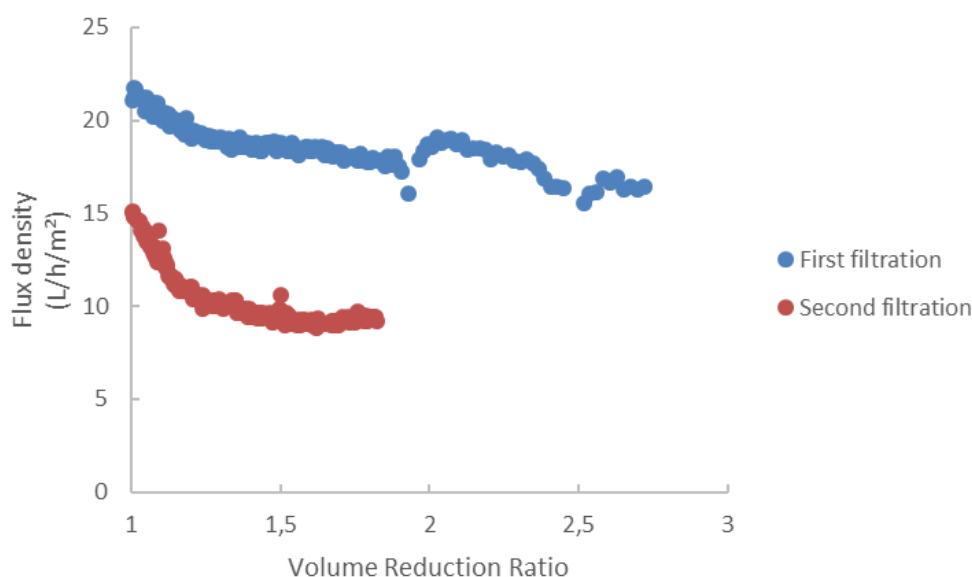


Figure 5.7 Flux densities of filtrations L1 and L2 as a function of the volume reduction ratio at 30°C.

The flux decreased as a function of the VRR from 21 to 16 $L \cdot h^{-1} \cdot m^{-2}$ for L1 from VRR 1 to VRR 2.7, and this was especially seen during L2 filtration, with a decrease from 15 to 9 $L \cdot h^{-1} \cdot m^{-2}$ from VRR 1 to VRR 1.8. The evolution of the ratio of the product flux density over water flux density as a function of the VRR was similar to figure 5.6 since the water permeability was recovered. The variation of $J_{sup} \cdot J_w^{-1}$ was from 0.27 to 0.20 for L1, from VRR 1 to 2.7, and from 0.20 to 0.12 for L2, from VRR 1 to VRR 1.8.

Several hypotheses may explain the differences in flux densities between the two filtrations. The supernatants were not identical between the different experiments and may contribute to differences. Moreover, the membrane was slightly used before the filtration of the first product, with only water and product permeabilities as well as a quick filtration in recycle mode and a cleaning step. On the other hand, the second filtration was realized on the same coupon, thus a membrane with an older history. While the initial water permeability was recovered after L1 filtration, it does not imply that the

membrane has recovered its initial characteristics. Hydrophilic compounds may have fouled the membrane, not affecting the water permeability but affecting the product fluxes. Nevertheless, the fluxes were in range with flux densities obtained on the UF concentration of permeate from microfiltration of the supernatant from bead-milled *Chlorella sorokiniana*, with fluxes from 10 to 8 L.h⁻¹.m⁻².bar⁻¹ (Gifuni et al. 2020). It was also the case with other food products filtrated with similar cut-offs and the same membrane material such as skim milk (Luo, Ramchandran, et Vasiljevic 2015).

5.3.2.1.3. Diafiltration of the retentate

A diafiltration was achieved after the concentration during L2 filtration and the evolution of the flux density as a function of the diavolume was decreasing from 9 to 8 L.h⁻¹.m⁻² at a 0.75 diavolume. The flux density stayed relatively constant during the diafiltration and did not change strongly from the fluxes obtained after concentration.

5.3.2.2. Retention rates and selectivity

The retention rates of the main categories of compounds were characterized and are presented in Figure 5.8.

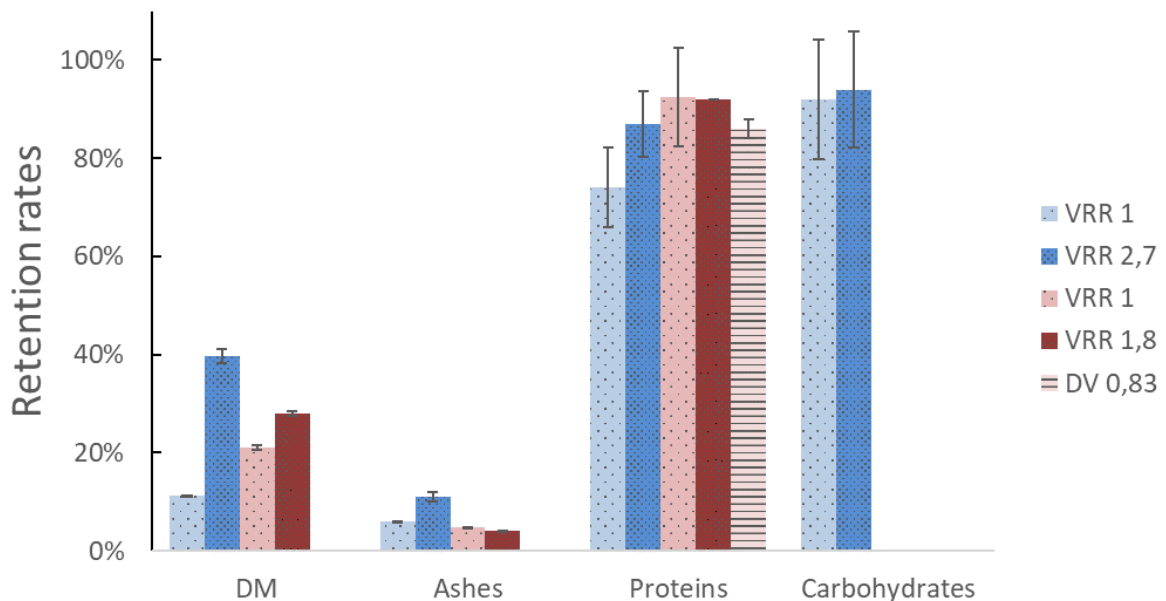


Figure 5.8 Retention rates of the dry matter, ashes, proteins and carbohydrates for two different filtrations (green and orange) at different filtration time. The retention rates of the first filtration L1 in green were taken during the concentration (VRR 1 → 2.7) while the retention rates of the second filtration L2 in orange were taken during the concentration (VRR 1 → 1.8) and the diafiltration (DV 0.83).

The retention rates of the proteins were high and close between the different filtrations with 87% for L1 at a VRR of 2.7 and 92% for L2 with a VRR of 1.8. It stays rather high, 86%, during diafiltration. Since

the retention rate of the proteins is higher than the retention rates of the dry matter (30-40%), a purification of the proteins can be achieved by concentration.

The retention rates of the dry matter during both concentrations increased with an increase in VRR, this can be due to a deposit layer that increases the retention. This was especially the case for L1 with an increase in the retention rate of the proteins, from 74% to 84%, and the ashes from 6 to 10%, over filtration time.

The retention rates of ashes for both filtrations were low (5-10%) and since the proteins have a higher retention rate, the two compounds can be separated. The ashes were the main component in the supernatant since *T. chui* is a marine species and it has to be removed for a higher protein purity. Moreover, sodium chloride is expected to contribute to most of the ashes in the supernatant. This assumption was verified by analysis part of the ProFuture project. The intake of sodium can contribute to higher cardiovascular disease risks and concerns are rising about an excess intake of sodium in the European population. The concentration followed by a diafiltration can contribute to a lower ash content and a higher protein purity and is technically feasible.

However, the retention rate of the carbohydrates was high and reached 94% at VRR 2.7 for L1 and since the retention rate of the proteins is close to the retention rates of the carbohydrates, they cannot be separated by this operation unit in these conditions.

Two different studies aimed at dialyzing at 12-14 kDa the supernatant obtained after bead milling of *T. impellucida*. The first study had a protein yield in the supernatant of $21 \text{ g}_{\text{prot.sup.}} \cdot 100 \text{ g}_{\text{prot.ini}}^{-1}$ and the dialysis step had a protein yield of $62 \text{ g}_{\text{prot.retentate}} \cdot 100 \text{ g}_{\text{prot.supernatant}}^{-1}$, so 38% of the proteins probably had a molecular weight under 12-14 kDa (Schwenzfeier, Wierenga, et Gruppen 2011). The second study had a protein yield after cell disruption of $41 \text{ g}_{\text{prot.sup.}} \cdot 100 \text{ g}_{\text{prot.ini}}^{-1}$ and the dialysis step led to a protein yield of $65 \text{ g}_{\text{prot.retentate}} \cdot 100 \text{ g}_{\text{prot.supernatant}}^{-1}$ (Teuling et al. 2017). This quantity of small proteins that get through the membrane contrasted with our results.

A low protein retention in the 10 kDa ultrafiltration retentate seems to also be obtained after bead milling, centrifugation and microfiltration of *Tetraselmis suecica* (Garcia et al. 2018). They obtained a protein yield on the ultrafiltration step of $29 \text{ g}_{\text{prot.retentate}} \cdot 100 \text{ g}_{\text{prot.permeate.MF}}^{-1}$ in the retentate and $52 \text{ g}_{\text{prot.retentate}} \cdot 100 \text{ g}_{\text{prot.permeate.MF}}^{-1}$ in the permeate. This is lower than observed in our case on a similar membrane surface (0.05 m^2 versus 0.03 m^2 in our case), however no VRR or RR were given to characterize the ultrafiltration.

5.3.3. Choice of the purification operation unit

The two operation units, i.e IEP and ultrafiltration, are used and mature to obtain a protein isolate and were compared at laboratory scale.

5.3.3.1. The IEP operation unit

The IEP did not lead to a precipitate despite high centrifugation conditions (11,000 g 10 min). Moreover, the use of a microfiltration at 0.2 μm by a syringe filtration was tested and the protein solubility was identical to the protein solubility after centrifugation (data not shown). This operation unit was not more studied and upscaled and more research is needed to better understand it as a debate is present in literature on microalgal protein solubility.

While on the process point of view, this result hinders the production of a protein-rich product, it is very interesting on the technofunctional point of view. The absence of precipitation at pH 3-5 is very interesting for food applications and is something sought, especially for plant proteins where there is often a very low solubility at acidic pH (Lan, Chen, et Rao 2018). Hence, this extract can potentially be used in acidified beverages with low to medium viscosity for which an addition of a low quantity of plant protein extract can be good candidates (Grossmann, Hinrichs, et Weiss 2019a). However, this extract is not unique since several natural extracts can still be candidates such as rapeseed albumin, napin, which have a pI of 11 and has been shown to be very soluble in acid conditions. They were successfully extracted and purified by membrane filtration recently (Beaubier et al. 2022) and may be complementary to the protein extract from *Tetraselmis* for other applications. Moreover, research is ongoing to increase acid solubility of traditional proteins with the use of acidic polysaccharides such as pectin, and has been tried on proteins from lentil (Aryee et Nickerson 2012), pea (Lan, Chen, et Rao 2018) or soy (Jaramillo, Roberts, et Coupland 2011) for example. Our extract could be a natural protein extract highly soluble, without the need of other additives and may have value for certain applications.

5.3.3.2. The UF operation unit

Ultrafiltration, on the other hand, seemed promising with a high protein retention rate, allowing high yields and a selective purification from ashes. However, several points can be discussed.

5.3.3.2.1. Mass balance on the whole process coupling, thawing, centrifugation and ultrafiltration, and final recovery yield

The succession of operation units affects different indicators such as the protein yield, the protein purity and the protein concentration, with some indicators often being antagonists. The effects of the concentration and the diafiltration on protein yield, concentration and purity are summarized in Figure 5.9.

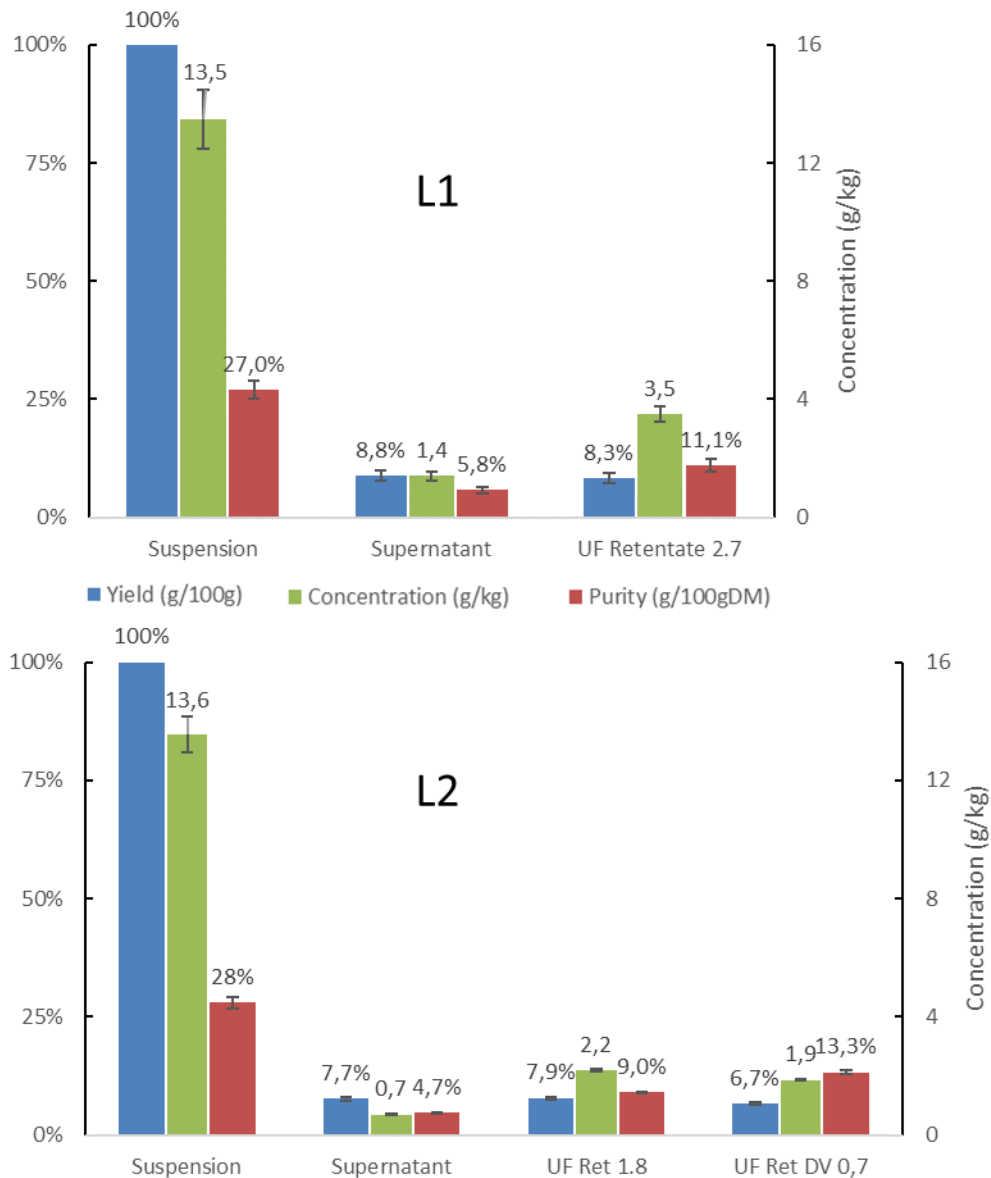


Figure 5.9 Protein yield, concentration and purity for different products along the process for the first filtration (L1) and the second filtration (L2): the initial suspension of biomass, the supernatant after thawing and centrifugation at 5000g, the retentate after the UF concentration step and the retentate after diafiltration.

The protein yield was low after the freeze-thawing step, as detailed in chapter 3. The concentration in both filtrations decreases the yield but increase the concentration and the protein purity. The

diafiltration, on the other hand, decreases the protein yield and protein concentration, while increasing the protein purity.

A high yield is desired for reducing the costs and use the biomass efficiently. The microalgal biomass is expensive in comparison to other biomass with a more mature cultivation such as soy or pea. For example, the price of *Nannochloropsis* in Europe is 30-100€/kg_{DM} in business-to-business (Araújo et al. 2021) compared to 0.4 €/kg_{DM} for soybean meal, a coproduct of the soy industry (Pope et al. 2023). Hence, this is a key indicator for the economic sustainability of the process and aimed to be maximized. In this case a protein retention rate of 90% is considered as satisfactory and the yield of the purification, from the supernatant to the diafiltration was sufficient. The protein yield of the first step from the suspension to the supernatant, one of the main goals of chapter 4, must still be highly improved to increase the sustainability of the whole value chain.

5.3.3.2.2. *The high purity objective*

Another objective is a high protein purity, which is necessary to obtain a protein concentrate/isolate and is aimed to produce protein-enriched ingredients such as plant-based products. The protein purity improved with the concentration since the protein retention rate was higher than the dry matter retention rate. Moreover, the diafiltration, while decreasing the yield and the concentration, helped to increase the purity.

Protein purity can be interesting since new sources of proteins are sought for environmental reasons, such as wished lower CO₂ emissions, lower deforestation or overfishing. One way is to replace traditional animal food products by plant-based products. While plant-based meat is more and more researched, with some products already on the market, plant-based seafood is still in development and few are present on the market in comparison with terrestrial animal product replacements (Kim, Caputo, et Kilders 2023). The purified extract of *Tetraselmis*, with its strong sea food flavor, and a potential high protein purity can be a very promising ingredient for the production of plant-based seafood such as fish filet or fish soup. However, highly pure ingredient consumption is debated by nutritionists who criticize the use of nutrient balance only in food and the impact of food ultratransformation on health. The effect of the food matrix on nutrition is more and more recognized on satiety and glycemic potentials for example and may be partly responsible for the global obesity epidemic and chronic disease burden (Fardet et al. 2017; Fardet et Rock 2022; Kliemann et al. 2022). While important, purity was chosen to be less considered than the yield in our strategy after these results. However, one way to further purify the retentate from the carbohydrates that are not covalently linked with the proteins could have been to use IEP. Isoelectric precipitation alone was not able to recover a precipitate with this product and neither the solid/liquid separation techniques

tested, centrifugation and microfiltration at 0.2 μm . However, it can be used to increase particle size and apply other solid/liquid separation techniques such as ultrafiltration. If the free carbohydrates are smaller than the aggregated proteins, a cut-off higher than 10 kDa may be used and the selectivity of the proteins in comparison with the carbohydrates could have been increased.

5.3.3.2.3. *The necessity to reach a sufficient concentration*

The protein concentration is also important in an integrated process since the purified product is most often dried directly and this operation unit represents most of the energy consumption (Prabhuzantye, Khaire, et Gogate 2019). Moreover, the dry matter content is an important parameter influencing strongly the efficiency of the drying step such as with a spray drier. If the product is not enough concentrated, it can be concentrated in a supplementary operation unit in a falling-film evaporator for example, usually under vacuum, to reach a sufficient concentration before drying. Nonetheless, the evaporation of water is very energy consuming and a preconcentration step is most often desired for the product of protein concentrate or isolate (Jinapong, Suphantharika, et Jamnong 2008). A 7% dry matter was for example the lowest concentration tried before spray drying, with a highest at 20.7%, after UF concentration of soy milk by Jinapong et al. 2008.

The protein concentration can be increased thanks to a high protein retention rate, however, the initial concentrations were low ($\approx 1 \text{ g.L}^{-1}$). A theoretical VRR of 64 would be necessary to achieve the protein concentration of 42 g.L^{-1} corresponding to 7% dry matter, neglecting all the other components and a constant retention rate of 90%.

5.3.3.2.4. *Towards the upscaling*

A concentration and a purification by ultrafiltration were selected for the scale-up. However a decrease of the pH as a function of time was observed for the second filtration L2 since it lasted two days, as seen in Figure 5.10.

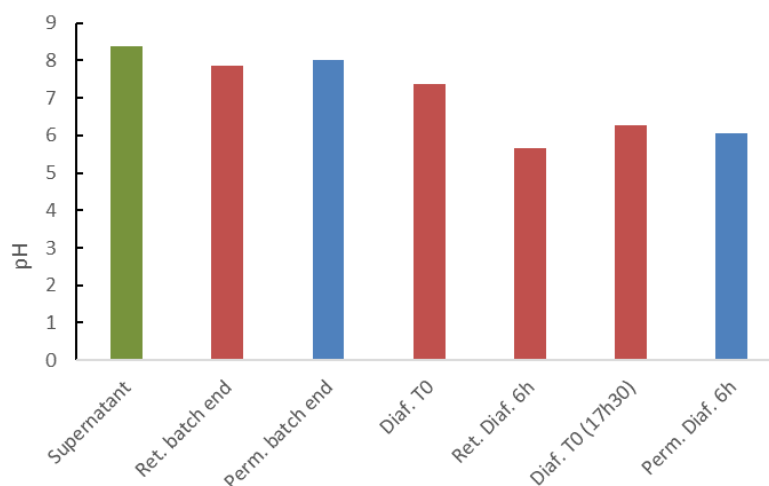


Figure 5.10 Evolution of the pH in different products as a function of the filtration L2.

This decline was seen during the diafiltration and also happened when the initial feed of the diafiltration was maintained for 8 h at room temperature. The higher decrease in pH in the retentate of the diafiltration might be due to the impact of a higher temperature on the reactions. A decrease in pH may be associated with a microbial development (Fotos, Diaz-Arnold, et Williams 1990) or/and a protein hydrolysis (Yada 2017). A microbial development can affect the product negatively since it should be consumed but also the operation unit itself, with a possible flux decline, a modification of the filtration selectivity and an altered product shelf life. Thus before the upscale, the stability of the supernatant over time at different temperatures had to be verified.

5.3.4. Supernatant stability

To prevent product degradation overtime, different parameters can be monitored such as the temperature and the treatment time. Since the treatment time was short for the centrifugation step and since the thawing step was achieved in cold conditions, the most critical step was the membrane filtration. With a fixed membrane surface, the choice of the batch volume to be treated was highly dependent on the filtration time allowed and the flux density. A filtration at 30 °C in laboratory conditions was possible and allowed a higher flux density, hence a higher VRR. However, this temperature is not optimal for the process because it favors both microbiological development and protease activity. This latest seemed less intense for the resistant *Tetraselmis* extract compared to the fragile *Tetraselmis*, hence no blanching was achieved. This was chosen to avoid another operation unit and another source of variation in the process. However, the risk of protease degradation was still present. In order to select a filtration temperature and an acceptable filtration time, a product sensitivity evaluation was achieved on the extract after freeze-thawing and centrifugation and is presented in the following section.

5.3.4.1. Bacterial development

The experiment aimed at testing the supernatant stability after thawing, without adding another cell disruption step, to define the filtration temperature and time. The microbiology was a key parameter to take into account for this experiment and the evolution of the total aerobic flora in the extract is represented as a function of time, for different temperatures, in Figure 5.11.

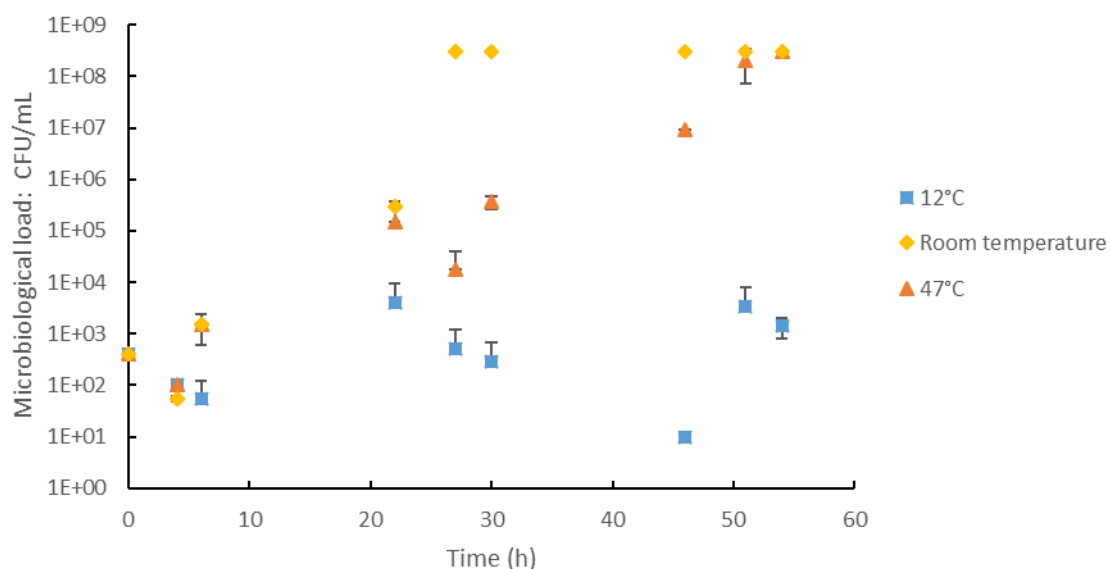


Figure 5.11 Microbiological development in the extract as a function of time at 12 °C, room temperature (≈22 °C) and 47 °C.

The condition at 12 °C showed the lowest microbial development in the extract, which stayed under $4 \cdot 10^3$ CFU.mL⁻¹ during the experiment. The bacterial growth was contained and might be acceptable during a long filtration on this criterion. In France a norm (CSHPF 01/1990 BID 1987) advises maximum aerobic mesophilic bacteria of 10^5 CFU.g_{DM}⁻¹ in the dry product. Since the product has a dry matter concentration of 20 g_{DM}.L⁻¹, a maximal theoretical microbial development of $2 \cdot 10^3$ CFU.mL⁻¹ should be reached to avoid the use of an operation unit for microbial stabilization (such as pasteurization, UHT, sterile filtration or pascalization). However, while the test was achieved on the supernatant, the concentration of the product by UF may modify the microbial development rate because of a concentration of the bacteria but also on the organic matter, substrate for microbial growth. Thus it is possible that a microbial stabilization may still be needed to conserve the product.

The condition at room temperature had a high increase in bacterial count, reaching the maximal counted amount, $3 \cdot 10^8$ CFU.mL⁻¹ after 27 h only, the fastest of all conditions. This condition, while maybe sufficient at laboratory scale if the filtration lasts only 6 hours, is not sufficient if the filtration needs to be prolonged during 30 or 54 h. As such, it might not be acceptable at higher scale, with a harder cleaning and disinfection, which could lead to higher contamination.

The condition at 47 °C had an intermediate bacterial growth compared to the other conditions. While the growth was not contained like the 12 °C condition, the growth was still slower compared to the room temperature condition, with 4.10^5 CFU.mL⁻¹ obtained after 30 h, respecting the hypothesis that a growth at 47°C was slower than a growth at room temperature. The bacterial growth was still high, between 22 to 30 h compared to the 12°C condition, but water viscosity is 2.1 higher at 12°C than at 47 °C, theoretically increasing the filtration time by 2.1 also. Moreover, long filtration time can also lead to a storage during the night if the filtration is done in 2 days, adding potentially more contamination risks.

The pH was also followed during the experiment because it can indicate a microbial development or a protein hydrolysis by protease activity (Yada 2017) but little differences of the pH was observed for the conditions at 12 °C and 47 °C, and was decreasing only for the condition at 20 °C, from pH 8.3 to pH 7.9.

5.3.4.2. Protein concentration

The protein concentration was also a key indicator for the product stability and was followed as a function of time, as seen in Figure 5.12.

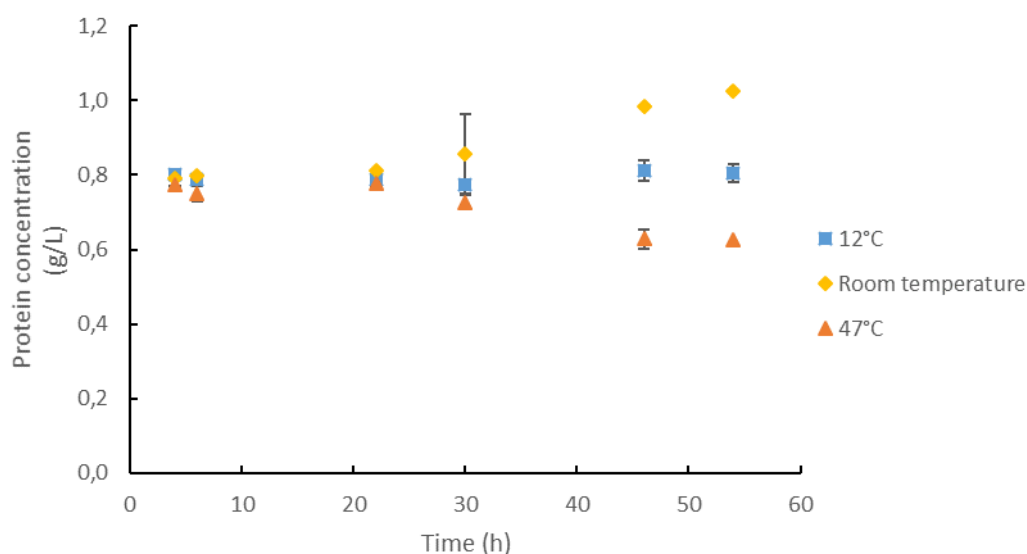


Figure 5.12 Evolution of the protein concentrations in the extract as a function of time at 12 °C, room temperature and 47 °C.

The protein concentration at 12 °C stayed stable during the time of the experiment. However, the protein concentration was changing at room temperature and at 47 °C, increasing at room temperature and decreasing at 47 °C. The differences may have different explanations. A microbial development, with a consumption of the carbohydrates present in the supernatant to make protein may explain the increase in protein concentration observed at room temperature. While a microbial development was also seen at 47 °C, a denaturation contributing to a little sedimentation, although

not seen, may have contributed to lower the protein concentration. A protein hydrolysis by protease may also have contributed to a decrease in protein concentration since the BCA quantify proteins by several amino acids and peptide bonds (Smith et al. 1985). Less peptide bonds may have contributed to a decrease in concentration by BSA equivalent.

5.3.4.3. Electrophoretic profile

To further investigate the evolution of the protein in the extract for the different temperatures, SDS-PAGE electrophoresis were realized on the extract along the time, as seen in Figure 5.13.

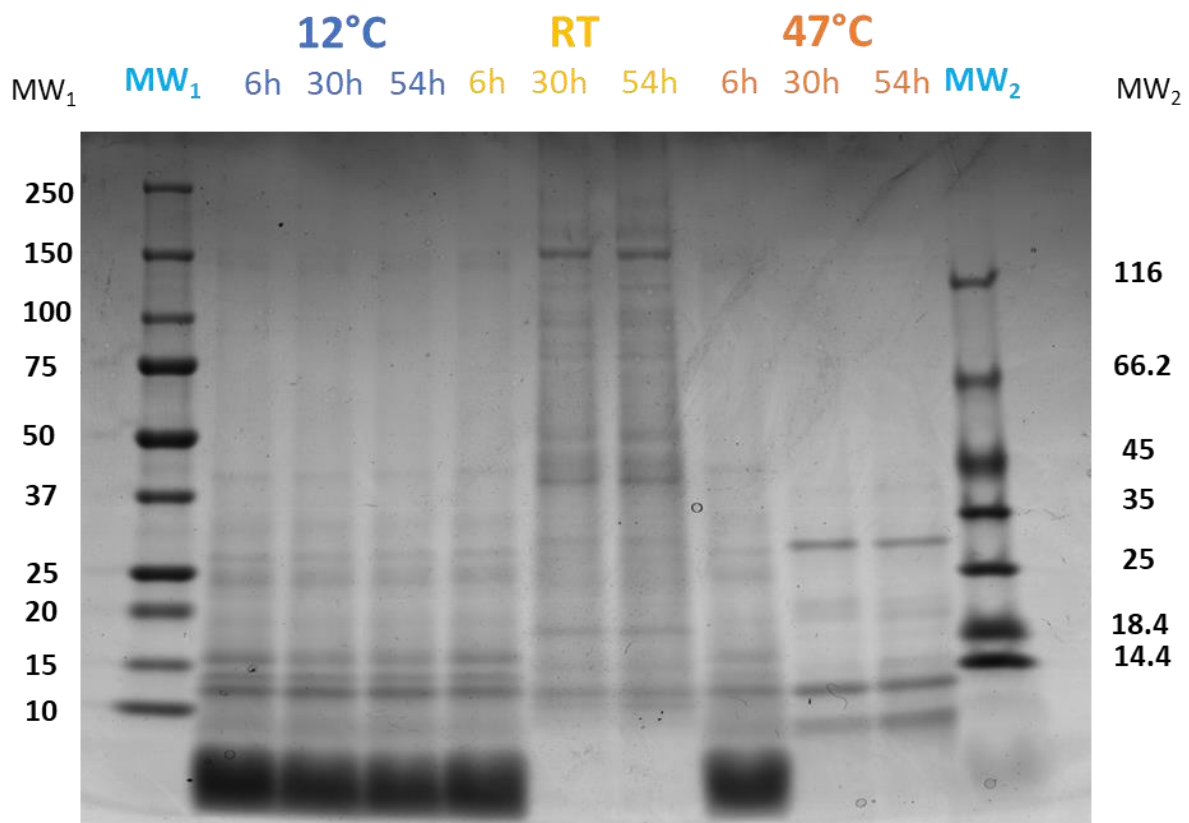


Figure 5.13 SDS PAGE electrophoresis in reducing conditions of the extract after 6 h, 30 h, and 54 h at 12 °C, room temperature (RT) or 47 °C.

The protein electrophoresis profile at 12 °C was stable through time and did not show a degradation overtime from 6 h to 54 h. The protein electrophoresis profile at room temperature, on the other hand, had a clear change from 6 h to 30 h and 54 h. The 6 h electrophoresis profile at room temperature was similar to the profiles at 12 °C. However, strong differences existed between the 6 h profile and at 30 h and 54 h, with a disappearance of the band below 10 kDa and the appearance of new bands, for example at 150 kDa. The bacterial growth was already at 3×10^8 CFU.mL⁻¹ at 30 h, hence it is possible that the bacteria consumed the small proteins, taken preferentially over bigger proteins, and released new proteins. The protein electrophoresis profile at 47 °C and 6 h was slightly different from the others

since, while 3 bands between 10 to 15 kDa were visible at 12°C and at the initial room temperature condition, the central band was less visible. A hypothesis can be a beginning of protease hydrolysis or the beginning of a protein denaturation. A band under 10 kDa appeared stronger as the time increases and among the 3 bands between 10 to 15 kDa, the upper band was not visible after 30 h. Moreover, the small macropeptides at the bottom of the gel disappeared after 30 h, as in the room temperature condition. It strengthens the hypothesis of a consumption by microorganisms since a growth was also observed at 47 °C, while they were still present at 12 °C, where the microorganism growth was limited along time.

5.3.4.4. Sensorial aspects

A characteristic smell of sea food, similar to surimi, was observed with the supernatants at 12 °C and room temperature, but the sea food smell vanished after 22 h at 47 °C and was replaced by an unpleasant smell. Fishy smells were seen as an obstacle to widen microalgae consumption by humans by Lafarga 2021 but it depends strongly on the desired type of food (Lafarga et al. 2021). The partial replacement of fish consumption by preparation completed by these types of ingredients can be a possible option. This replacement can be due to veganism but also for environmental reasons to fight against overfishing, induced either by wild capture for direct human consumption, either induced by capture to make fish meal and fish oil for aquaculture (Vaughan 2021). The demand for blue food, defined as aquatic food captured or cultivated in freshwater or marine systems, is expected to double for 2050 (Naylor et al. 2021).

5.3.4.5. Conclusion

Filtration at room temperature has to be avoided due to a high microbial contamination, leading to a strong modification of the protein profile. While the filtration at 47 °C might have been interesting due to an increase in permeate flux density, the apparition of an unpleasant smell and the development of microorganisms, as well as a modification of the protein profile led to the 12 °C condition being selected during membrane filtration. This preliminary study was essential to select the correct conditions to process the supernatant at larger scale.

5.3.5. Scale up

The process was studied at higher scale and more enriched protein extract was produced. The suspension preparation and the centrifugation were studied, followed by ultrafiltration.

5.3.5.1. Biomass and product variabilities

The supernatant to be purified had a variability due to the different biomass composition or centrifugation equipment used. In order to compare the different experiments, the biomass compositions as well as the centrifugation performance and the supernatant compositions were compared in the following paragraphs.

5.3.5.1.1. Suspension composition comparison

The suspension dry matter composition was slightly different between the different experiments, as seen in figure 5.14. The laboratory experiments were realized on the same biomass, the batch A. The other scale was realized on batch C at pilot scale and batch D and E at semi-industrial scale SI 1/1' and SI 2 respectively.

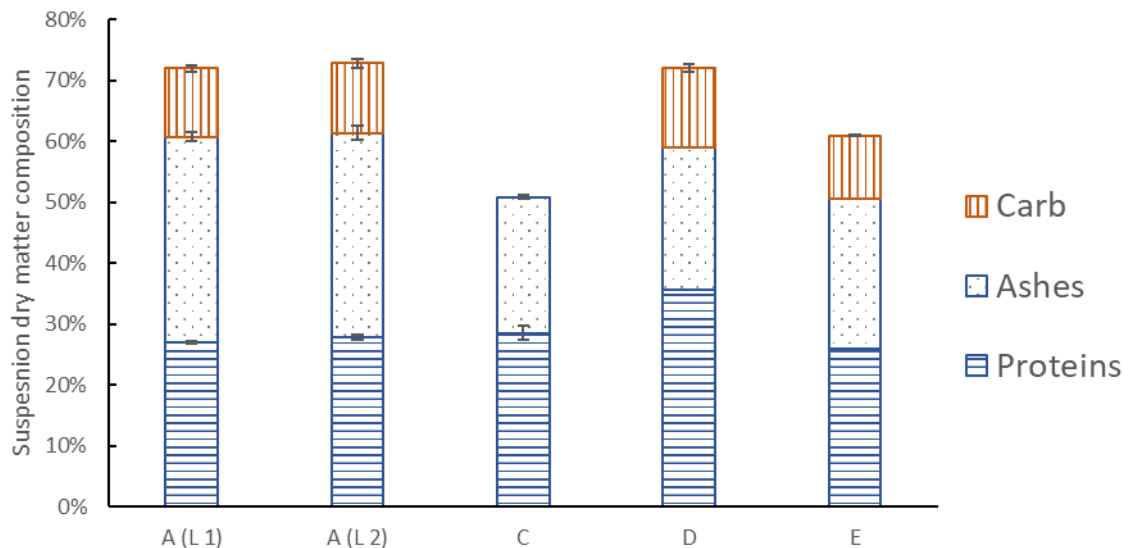


Figure 5.14 Suspension compositions in proteins, ashes and carbohydrates for the different biomass used for the filtration experiments at the different scales. The experiment L1 and L2 were realized on batch A, P1 on batch C, SI 1 on batch D and SI 2 on batch E.

A high ash content of 34% was observed at laboratory scale on batch A biomass while it was less the case at the other scales (22-25%). These differences can come from the harvesting process with differences if the biomass sent to be frozen was pumped at the top or the bottom of the tank. A part of the ashes contains the media that was used for cultivation hence the dry matter represents the microalgae cells in addition to some remaining salts from the medium between the cells.

This may explain the low carbohydrate content in the dry matter of 10 to 13% since it is a relative composition. The protein content was close for the different scales studied, with a higher protein composition for the batch D of the experiment SI 1 of 36% instead of 26-28% for the other batches. The biomass composition can change with environmental parameters such as temperature or light as

seen in chapter 2, which can explain differences in the suspension composition between the different experiments.

5.3.5.1.2. Supernatant repartition

A scale-up was realized to centrifuge the biomass from a laboratory centrifuge to a process centrifuge with a change of centrifuge technology. One way of ensuring the good separation by centrifugation is to calculate the ratio ϕ between the volume of supernatant and the volume of suspension (Leung 2007). The pellet build-up in the centrifuge can be a limiting parameter at higher scale since in batch there is a necessary removal of the pellet. Since the semi-industrial scale centrifuge used was a fed batch centrifuge, the residence time of the particles in the centrifuge could be regulated through the flow rate in order to achieve pellet compaction and no leaks of pellet in the supernatant. The different ratios ϕ have been calculated for the experiments at different scales and the results are presented in Table 5.5.

Table 5.5 Dry matter and ratio supernatant volume over suspension volume for the different scales used. The experiments L1, L2 and P1 were using the laboratory centrifuge while the experiments SI 1 and SI 2 were using the semi-industrial centrifuge.

Experiment	Centrifuge scale	Dry matter ($\text{g}_{\text{DM}} \cdot \text{kg}^{-1}$)	ϕ
L1	Laboratory	49.9	87%
L2	Laboratory	48.8	88%
P1	Laboratory	91	62%
SI 1	Semi-industrial	49.5	82%
SI 2	Semi-industrial	60.6	75%

The pellet was sufficiently packed with the parameters used and no strong differences were observed between the laboratory and semi-industrial centrifuge.

However, the supernatant was heated from 9 °C to 35 °C when using the pilot centrifuge and needed to be cooled after. The supernatant can be heated up by more than 20 °C from the rotating bowl because of the windage, which is the air resistance of the rotating bowl from the air mass trapped between the stationary centrifuge casing and the rotating bowl. The air mass adjacent to the rotating bowl is forced to rotate, while the air mass adjacent to the stationary casing is held stationary. The airflow pattern generates vortices, turbulence, and energy dissipation in the form of heat. A high rotation speed is able to generate high G-force for difficult separation, but it increases heat dissipation (Leung 2007). There are two ways to avoid it, one is to introduce a cold feed into the centrifuge. The second way is to have a specific centrifuge with a vacuum to avoid trapped air.

5.3.5.1.3. Compound yields in the supernatant

The repartition of the different macromolecules between the supernatant and the suspension was also studied and is shown in Figure 5.15.

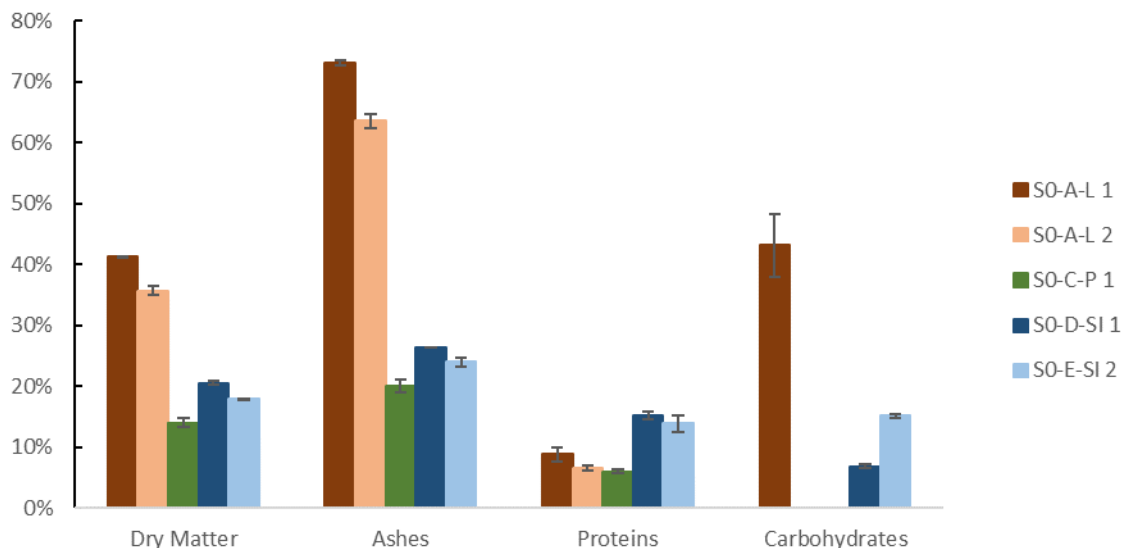


Figure 5.15 Mass yields between the supernatant and the suspension of dry matter, ashes, proteins and carbohydrates between the different experiments with laboratory experiments (L1/L2), pilot experiment (P1) and semi-industrial experiments (SI 1/SI 2).

The repartition of ashes was very different between lab scale and the other scales. *Tetraselmis chui* can accumulate strontium-rich amorphous calcium carbonate inclusions called micropearls inside the cell (Martignier et al. 2018) but this is not present in all cells. It might be possible that some lots have accumulated micropearls, leading to a difference in ashes repartition. This hypothesis was strengthened by a strong quantity of strontium and calcium, determined qualitatively by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP AES). Another explanation can come from a difference in harvesting between the bottom or the top of the tank, with nutrient precipitate that can deposit in the bottom.

The protein yields were higher at the highest scale on the supernatants S0-D-SI 1 and S0-E-SI 2, with a protein yield of 14% compared to 6-8% at the other scales. This outlines the impact of biomass variability on the process and the difficulty of scaling up when the biomass is changing and not well understood. The carbohydrate yields were also very different between laboratory scale to semi-industrial scale.

5.3.5.1.4. Comparison of the supernatant composition at the different scales

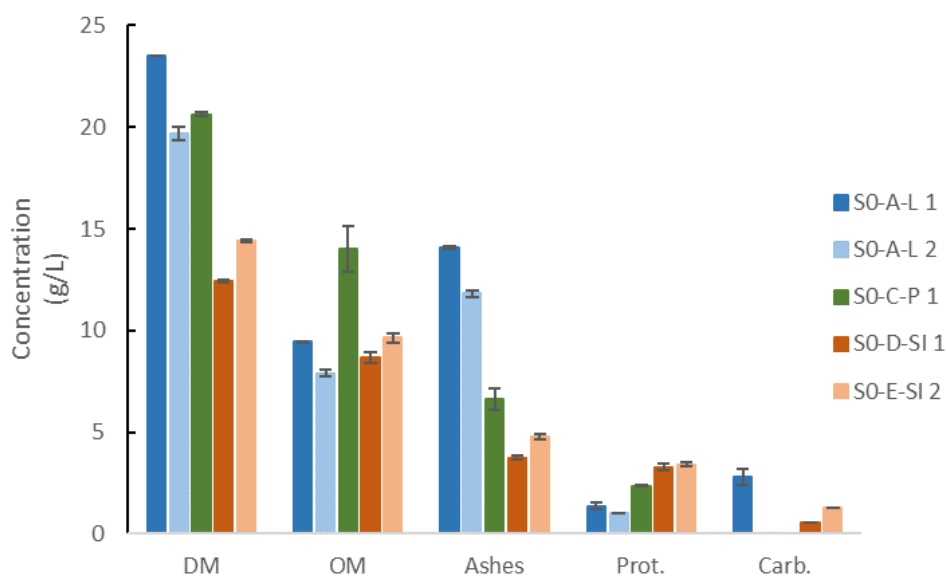


Figure 5.16 Supernatant composition before filtration for the different trials in dry matter (DM), organic matter (OM), representing the dry matter subtracted by the ashes, the ashes, the proteins (prot.) and the carbohydrates (carb.). The different supernatants are obtained at laboratory (L), pilot (P) and semi-industrial (SI) scales.

The supernatant compositions were compared for the different trials used in order to assess their variability, as seen in Figure 5.16. The concentration of the main components of the supernatant during the filtrations at laboratory scale was close, with a low variability among the trials due to the same batch A of biomass used. The variability was low at semi-industrial scale (SI 1 and 2) despite a higher variation of lots used. The organic matter concentration was similar between the trials at laboratory and semi-industrial scale, with a change at pilot scale probably due to a difference in the dry matter of the suspension ($90 \text{ g}_{\text{DM}} \cdot \text{kg}^{-1}$ instead of $50 \text{ g}_{\text{DM}} \cdot \text{kg}^{-1}$ for the other trials). This is important since differences in organic matter will probably have significant importance on the filtration results in ultrafiltration, in terms of flux density, retention rates and cleanability. However, significant differences in protein concentrations were obtained with a three-time increase at semi-industrial scale compared to laboratory scale. Moreover, the ash content was lower at pilot and semi-industrial scale in comparison with the laboratory scale, with two to three times less. The carbohydrate content was also lower at semi-industrial scale compared to laboratory scale and since the ratio proteins over carbohydrates was higher, a higher protein purity can theoretically be achieved.

5.3.5.2. Ultrafiltration upscale

In the context of the Profuture project, one of the objectives was to produce a sufficient quantity of protein extracts for characterization. The upscaling was thus a necessity. This upscaling was performed in two steps: the test of filtration at pilot scale, and then the production of protein extract at semi-industrial scale. All the filtrations were performed at 12 °C according to the preliminary stability test.

5.3.5.2.1. Pilot scale

The pilot scale filtration was key to verify the results obtained at laboratory scale and prepare the scale-up at semi-industrial scale. The flow density as well as the retention rates were quantified.

5.3.5.2.1.1. Permeabilities and flux densities

The water permeability at pilot scale was low compared to laboratory scale with 75 L.h⁻¹.m⁻² at 30 °C compared to 32 L.h⁻¹.m⁻² at pilot scale after conversion at 30 °C. The product permeability was not improving proportionally to the TMP, sign of a strong polarization concentration, as observed in Figure 5.17.

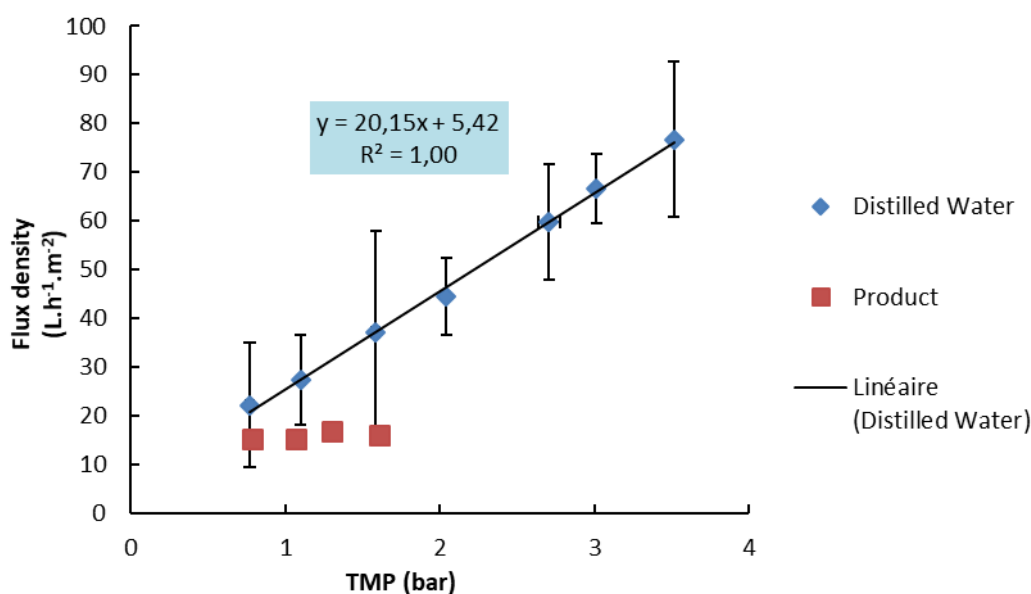


Figure 5.17 Water and product permeability as a function of the TMP at pilot scale at 12°C.

The critical TMP was reached and a lower TMP than 0.8 bar should have been used. However, the pressure induced by the flow only at the chosen membrane cross-flow velocity was already 0.8 bar. Hence, a pressure of 1 bar was chosen to compare to the other filtrations. Ideally, a lower TMP, obtained by restricting the permeate flow rate should have been used, limiting fouling. However, this was not present on the equipment used. A higher organic matter of the extract to be treated compared to the extracts at laboratory scale could be a reason why the product permeability was different from laboratory scale.

The filtration in concentration mode was carried out in fed-batch mode since the volume of the pilot tank was not sufficient to be directly used in batch mode. It is a usual method at higher scales since a big tank directly associated with the pilot may be associated with a high dead volume, limiting the achievement of high VRR. The permeate flux density for the concentration is shown in Figure 5.18.

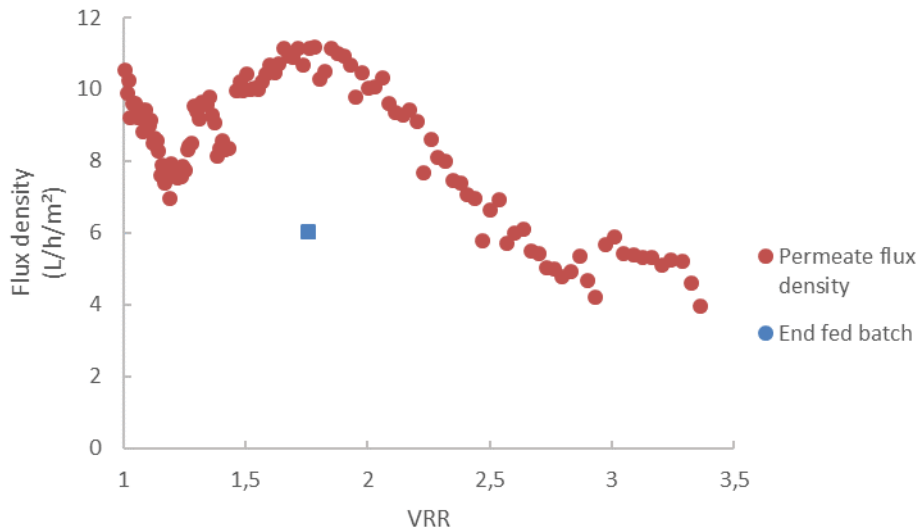


Figure 5.18 Permeate flux density as a function of the VRR at the small pilot scale

The flux density was decreasing from 10 to 5 L.h⁻¹.m⁻², and from 0.48 to 0.2 in flux ratio J.J_w⁻¹. The fluxes were similar to the fluxes obtained at laboratory scale, leading to a higher ratio J.J_w⁻¹ compared to laboratory scale. A diafiltration was also achieved to eliminate the salts to improve the extract purity.

The flux density as a function of the diavolume can be seen in Figure 5.19, with the conductivity measured in the retentate.

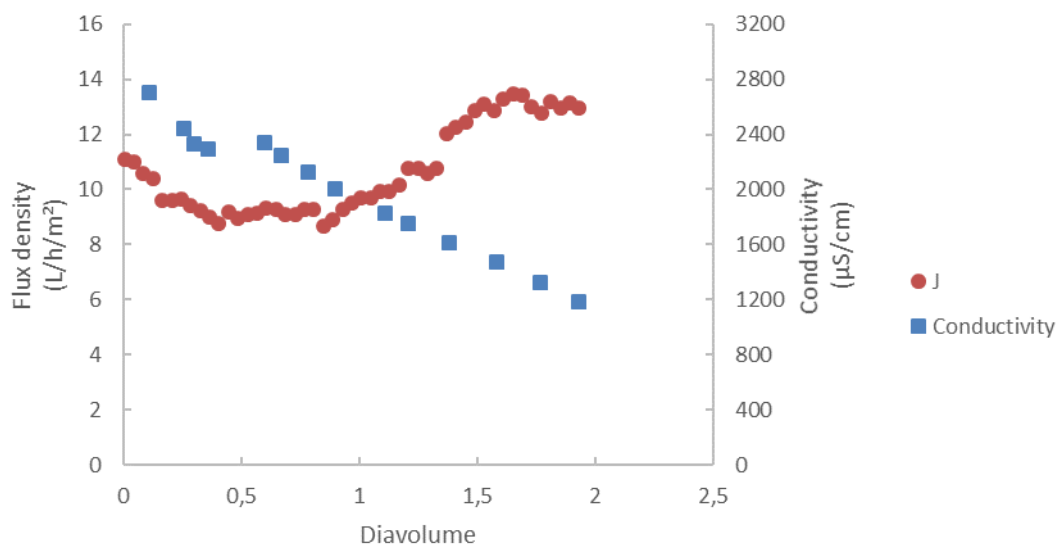


Figure 5.19 Flux density and retentate conductivity as a function of the diavolume during the diafiltration

The flux densities were high, taking into account the low water permeability, with the ratio J_p/J_w starting at 0.6 and increasing to 0.69.

5.3.5.2.1.2. Retention rates and selectivity

The retention rates at different steps of the filtration are presented in Figure 5.20.

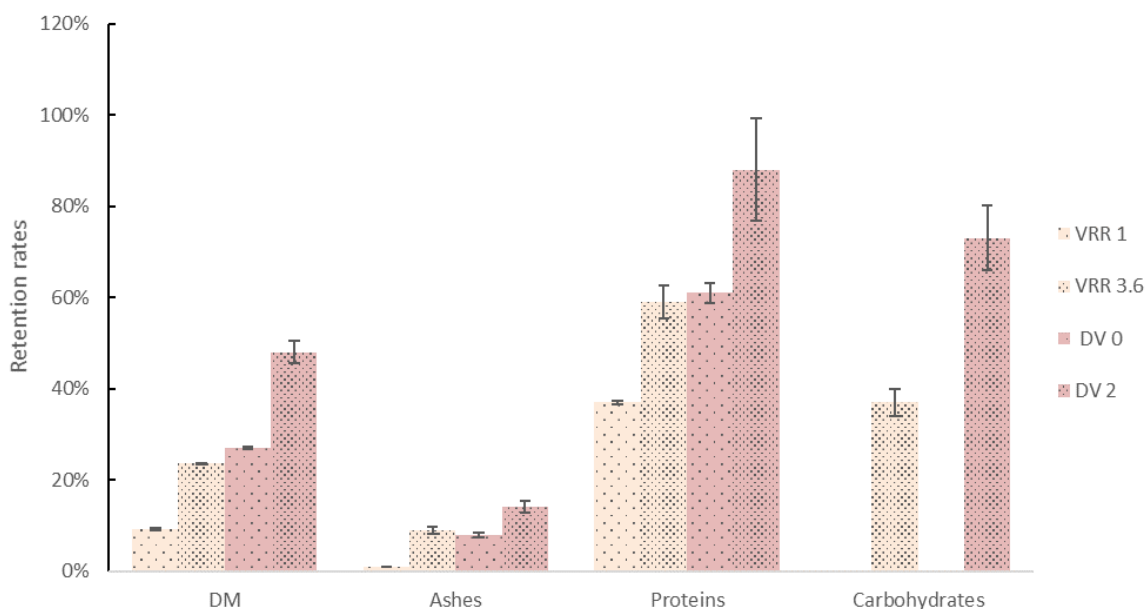


Figure 5.20 Retention rates as a function of the main macromolecules at different steps of the filtration

The retention rates of ashes were similar to the laboratory scales, with a retention rate between 5 to 15%. It increased during the concentration and during the diafiltration, probably because of the retention of compounds containing minerals, such as metalloproteins. A final concentration of 1.16 g.kg^{-1} of ashes has been obtained, so under the maximum limit of 2 g.kg^{-1} fixed within the project.

The protein retention rates increased along the filtration, with 40% to begin, and 60% at the end of the concentration. This difference could be attributed to the formation of a deposit layer, increasing the retention of macromolecules. A further increase of the retention rates can be observed during the diafiltration, from 61% to 88%. This increase can be explained by the heterogeneity of the protein sizes in the sample, and thus the heterogeneity of their retention rates, as summarized in Figure 5.21.

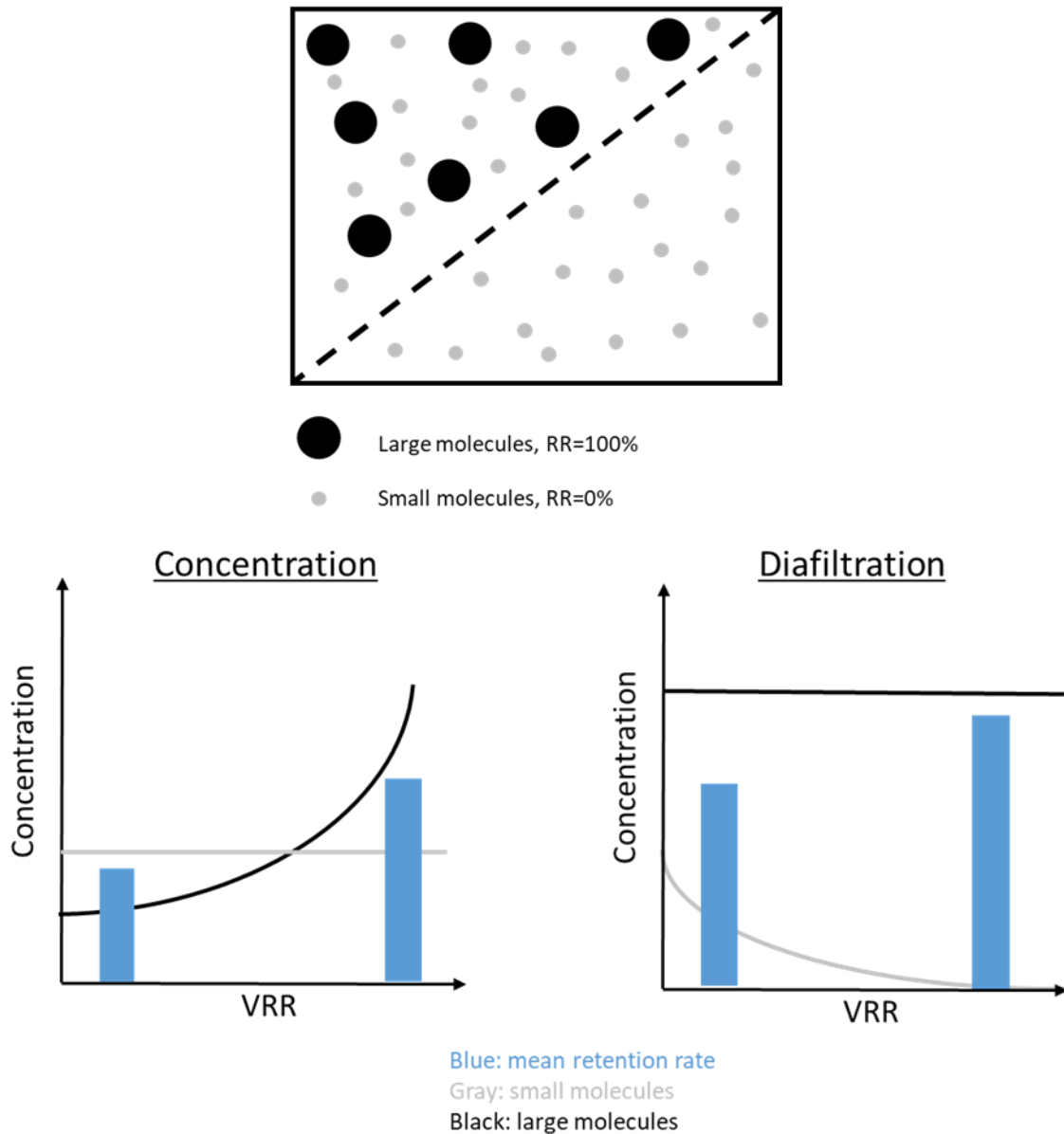


Figure 5.21 Scheme explaining the difference in retention rates when two different populations are present, themselves having two different retention rates.

The protein retention rate was lower than obtained at laboratory scale which were more than 90%. Since the retention rate of the proteins were higher than the retention rates of the dry matter, a purification of the proteins can be achieved by concentration. Analytical issues did not allow the determination of carbohydrates in this experiment.

The retentate after the concentration was stored overnight at 4°C but modifications in product composition happened during the night.

The concentration of dry matter decreased from 25.3 g.kg⁻¹ to 12.3 g.kg⁻¹, the concentration of ashes decreased from 7.4 g.kg⁻¹ to 3.3 g.kg⁻¹ and finally the concentration of proteins decreased from 4 g.kg⁻¹ to 1.8 g.kg⁻¹. This decrease could be due to several reasons such as microbial development, but

a decrease in ashes would not be explained. The most coherent reason would be a precipitation of the proteins, associated with other compounds overnight. This can happen with multivalent ions such as calcium, iron, or magnesium. An orange compound was present in the supernatant tank. It was also present in the retentate before diafiltration, and in the final retentate and in the permeates. This compound, present in the initial paste of batch C biomass, was not taken during the further experiments.

5.3.5.2.1.3. Conclusions

The experiment at pilot scale had differences in operating conditions, with a high dry matter content being used and the presence of an unidentified orange compound. The fluxes were similar than obtained at laboratory scale, if the latest were theoretically converted at 12 °C. However, the protein retention rates were lower, at 40 to 60% during the concentration and from 60 to 90% during the diafiltration.

More work could have been realized at this scale in order to improve the protein retention rates during the concentration and try with the correct suspension concentration. However, being in an interconnected research project, we had a pressure to produce more extract for the partners, even if our process was not fully mature. This was done in order to avoid blocking other partners and to optimize the process along the project.

5.3.5.2.2. *Semi-industrial scale*

The parameters selected at the previous scales were used for filtration at semi-industrial scale. The goals were to produce an extract for the European partners as well as validate the results obtained at the previous scales.

Several experiments were performed: SI 1, SI 1', SI 2. and SI 2 permeate. A trial called SI 2 permeate was also conducted on the permeate from the concentration step of SI 2, with acidification to aggregate the proteins, as seen in the IEP section, and increase the protein retention rate.

In the following paragraphs, the filtrations with the native supernatants are presented, before the filtration of acidified permeate.

5.3.5.2.2.1. Permeabilities and flux densities

The permeabilities of water and the product were quantified in order to characterize the membrane and to choose the optimal TMP.

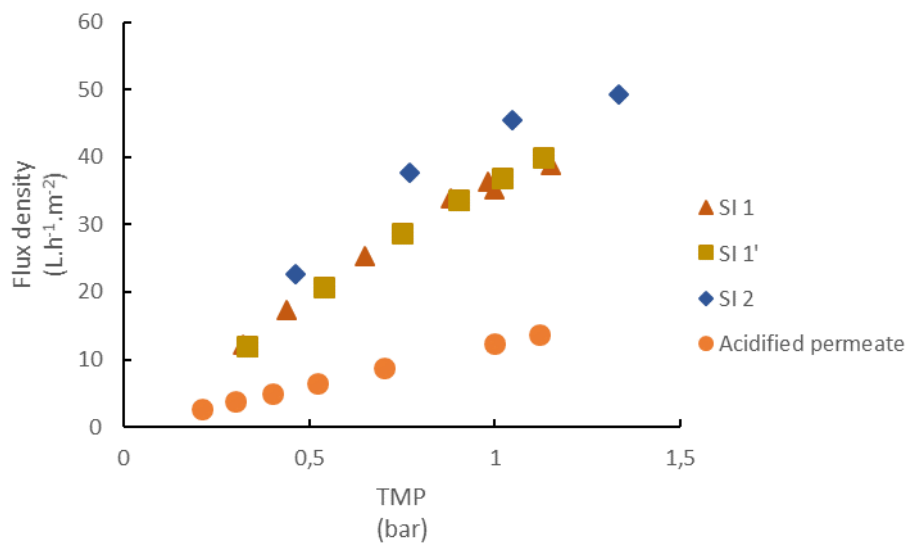
The water permeabilities of the membrane were characterized before filtration and are shown in Table 5.6.

Table 5.6 Water permeability at pilot scale of the different filtrations at 12°C.

Filtrations	SI 1	SI 1'	SI 2	SI2-Acidified permeate
Water permeability at 12°C (L.h ⁻¹ .m ⁻² .bar ⁻¹)	60	43	79	48

Since the membrane was only washed with water between SI 1 and SI 1' as well as between SI 2 and the acidified permeate filtration, the water permeability was lower before this latest and SI 1'. The initial water permeability was higher than before SI1 of 32%. This increase may be due to the adsorption of hydrophilic fouling materials such as proteins on the surface of the membrane, enhancing the water flow rate.

However, the differences in water permeability seemed to have little impacts on the product permeability, as seen in Figure 5.22. The product permeability of SI 1 and SI 1' were identical despite a water washing only before filtration SI 1', which decreased the water permeability from 60 to 43 L.h⁻¹.m⁻².bar⁻¹. The product properties seemed to have more effects on the flux density than the retrieval of the initial water permeability.

**Figure 5.22** Product flux density as a function of the TMP for the different filtration feed at semi-industrial scale at 12°C.

The filtration SI 2 showed a higher product permeability than SI 1 and 1' but a decline in linearity was observed above 1 bar. The decrease of the flux density as a function of the TMP due to a concentration polarization was explored and can be seen in Figure 5.23.

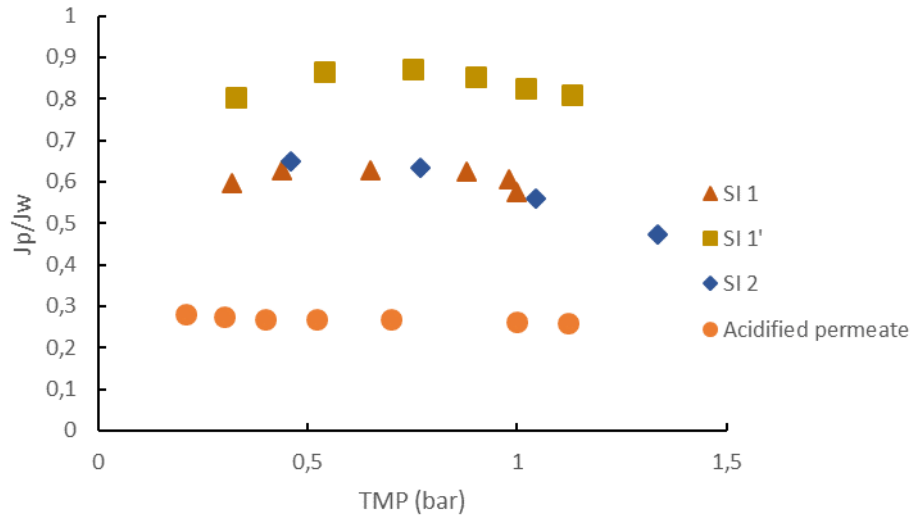


Figure 5.23 Ratios $J_p.J_w^{-1}$ for the filtrations at semi-industrial scale (SI) 1, 1' and 2, as well as the acidified permeate of SI 2. The membrane was rinsed with a simple water wash between SI 1 and SI 1' and between SI 2 and the filtration of the acidified permeate.

The ratios $J_p.J_w^{-1}$ were similar for SI 1 and SI 2 over the studied pressure range. The ratios started to decline at 0.88 bar for SI 1 and 0.77 for SI 2 and a TMP too high should be avoided. However a TMP of 1 bar seems to be acceptable. The ratios obtained with the clean membrane (SI1) were higher than the one obtained at laboratory scale ($J_p.J_w^{-1} = 0.3$). The variation of the products, the operating conditions (temperature) impacting the product, and the different geometries may explain the differences. The ratio $J_p.J_w^{-1}$ of SI 1' was higher, because of a lower water permeability.

The evolution of the flux density as a function of the VRR is shown in Figure 5.24.

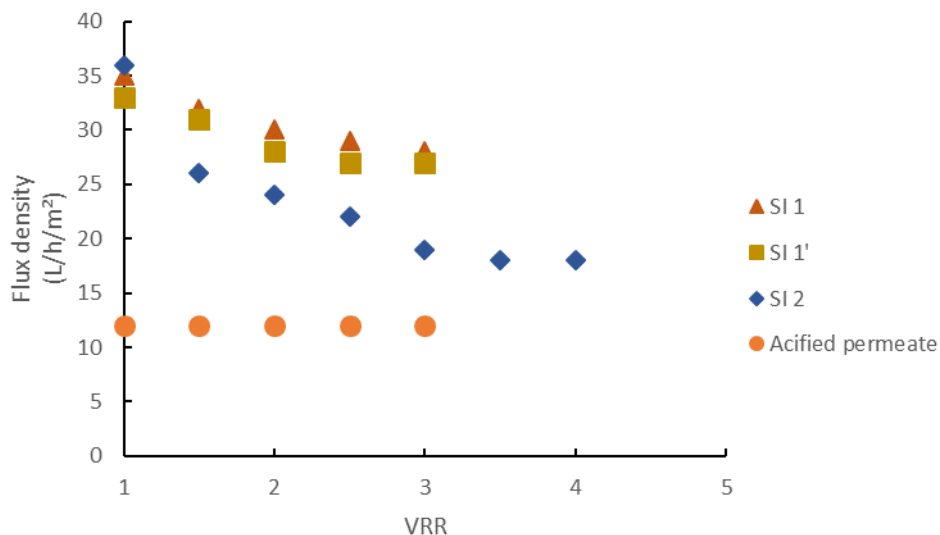


Figure 5.24 Flux densities as a function of the VRR for different filtrations at semi-industrial scale (SI 1, SI 1' and SI 2, as well as on the acidified permeate of SI 2). The membrane was only rinsed between SI 2 and the acidified permeate filtration.

The flux densities of the filtrations SI 1 and SI 1' were very close, despite a difference in initial water permeability, due to a water rinsing only before SI 1'. This behavior was also seen during the evaluation of the product permeability, as seen figure 5.21, and shows a filtration flux that seem driven by the product.

The lowest filtration flux of SI 2 may be linked to the membrane modification during the first filtration, but also to the higher carbohydrate content in SI 2 supernatant, 1.32 g.L^{-1} instead of 0.53 g.L^{-1} in SI 1, while the other compound concentrations were relatively similar. *Tetraselmis* exopolysaccharides have been found to be mostly acidic polysaccharides, being 'pectin-like' (Kermanshahi-pour et al. 2014). This type of polysaccharides are known to hinder filtration, of apple juice for example, and pectinase has been used to increase the fluxes (Rao et al. 1987).

5.3.5.2.2.2. Diafiltration of the retentate

The flux densities increased slightly in all cases along the diafiltration, as seen in Figure 5.25.

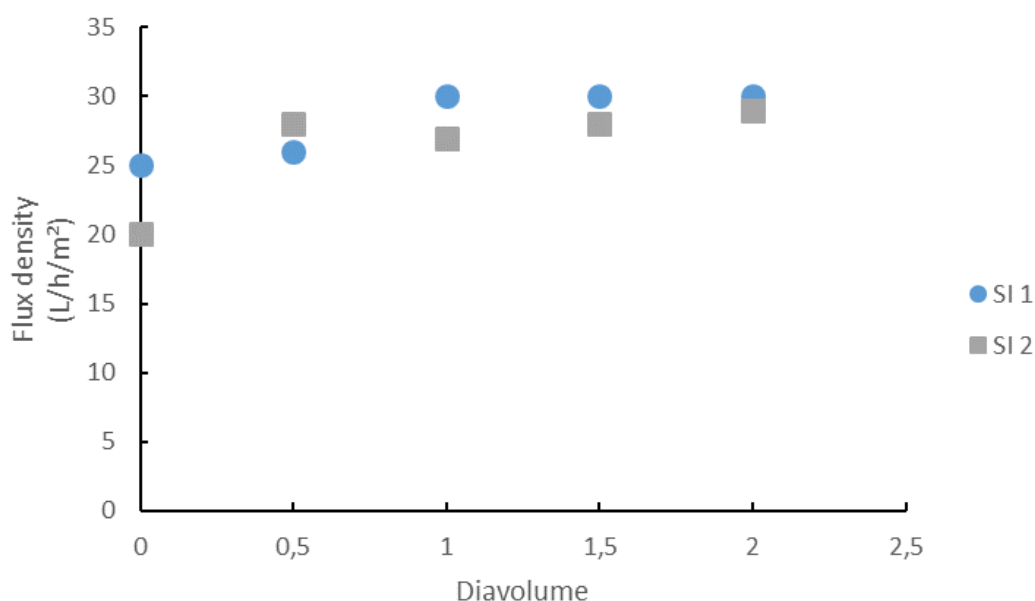


Figure 5.25 Flux density as a function of the diavolume during the diafiltration of the retentates following the concentration of SI 1 and 2 experiment.

One explanation can be the removal of multivalent ions, like calcium, that may generate membrane fouling. *Tetraselmis* possess relatively high quantity of calcium, which can represent up to 3% of the total dry matter (Tibbetts, Milley, et Lall 2015). Pectin is known to form a gel when calcium is present and the improvement may be linked. However, more experiments are needed to confirm it.

5.3.5.2.2.3. Retention rates and selectivity

The selectivity of the filtration can be determined by the retention rates, presented in Figure 5.26. The retention rates of the different macromolecules during SI 1 and 2, performed on the supernatant from thawed *T.chuij*, were close on most of the constituents. The retention rates obtained on the filtration SI 1 and 1' were very similar, so the retention rate of SI 1 only was represented to be clearer.

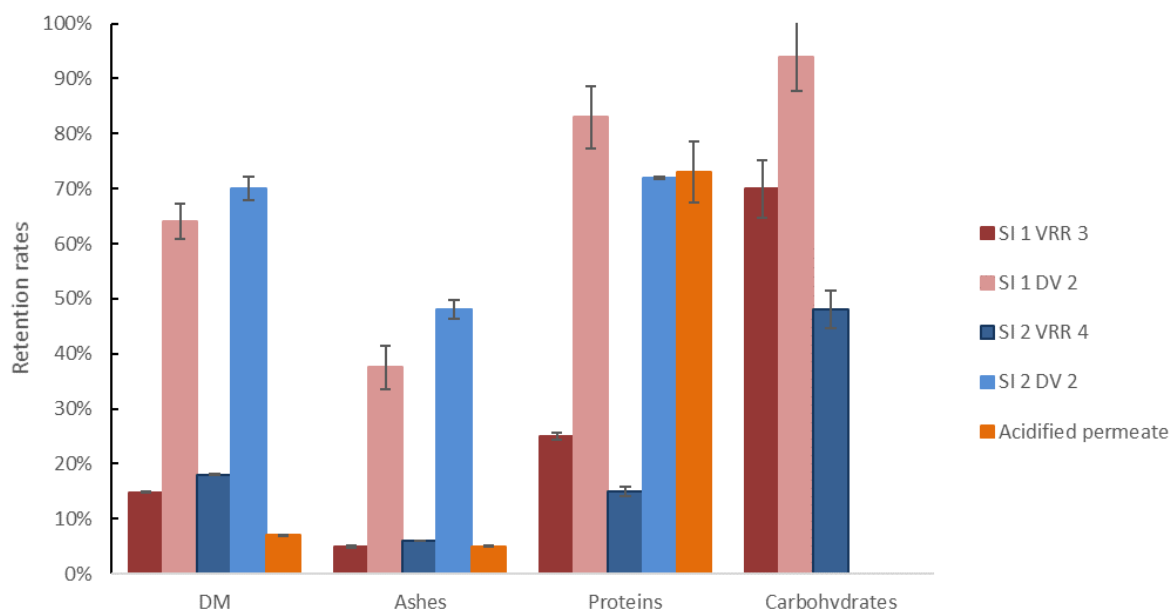


Figure 5.26 Retention rates of different compounds for the filtrations at semi-industrial scale (SI) 1 and 2 after concentration at the final VRR and at the final diavolume. The filtration of the acidified permeate of SI 2 was also compared.

As expected in the previous experiments, the retention rates of ashes during the concentration were low, allowing a possible purification of the extract. On the other hand, the proteins in SI 1 and 2 concentration steps had lower retention rates than shown at lower scale, with retention rates of 25% and 15% instead of 59% at pilot scale and more than 80% at laboratory scale. The carbohydrates, however, were retained SI 1, with a retention rate of 70% to 94%, similar to laboratory scale (RR > 90%), but not for SI 2 with a retention rate of 48%. The difference during the filtrations SI 1 and SI 2 can come from the difference in carbohydrate concentration, with a higher concentration in SI 2 supernatant. It is possible that the added carbohydrates were small and went through the membrane, leading to a difference in retention rates.

The diafiltration led to a higher retention rate of all the macromolecules. Indeed, the bigger macromolecules, (i.e. proteins) were retained by the membrane while the smaller were washed out. The retention rates of ashes also increased, but ashes can also be present as ions, which can be linked to other molecules, such as metalloproteins. The final concentration of 0.67 g.kg⁻¹ of ashes has been obtained, so under the maximum limit of 2 g.kg⁻¹ fixed within the project. A lower diavolume could have been used to respect the ash limit but the goal was also to improve protein purity.

The low retention of the proteins in the semi-industrial experiments was unexpected. This can be due to the modification of the products, or the change in the membrane properties (the available membrane at large scale was made of another material and the supplier was different). However, the cut-offs were the same for all the experiments. Thus a more detailed characterization of the supernatant was performed to verify the first hypothesis.

5.3.5.2.2.4. Characterization of the filtrated products

The electrophoretic profiles of different fractions from the filtrations at different scales were compared as seen in figure 5.27.

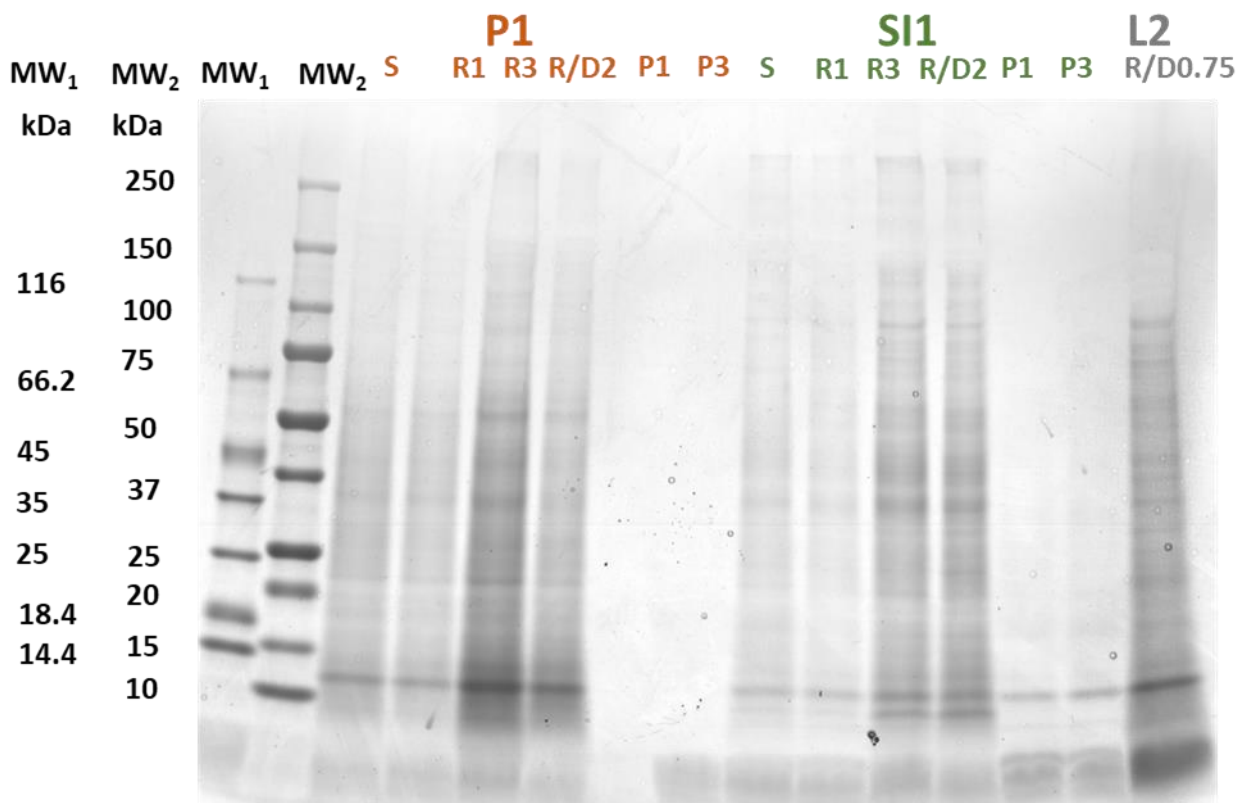


Figure 5.27 SDS PAGE electrophoresis in reducing conditions of fractions from different filtration scales. The filtrations of the laboratory scale, L2, the pilot scale P1 and the semi-industrial scale SI 1 were compared. The supernatant (S), the retentates at different VRR (R1, R3,) and after different diavolumes (R/D2, R/D0.75) and the permeates (P) were compared.

The bands were overall very similar between the different scales, with comparable products between the different scales. A band at 10 kDa was present in the product at semi-industrial scale but not at the lower scales. This may indicate a variation of the composition. Most of the lines were conserved throughout the filtration. No strong protein degradation was observed.

The small proteins with a size below 8 kDa were found in retentate and permeate, showing their low retention. This validates the membrane cut off.

A band was present at 12 kDa in every sample except the permeates at the pilot scale, while it was present in the permeate at semi-industrial scale. It can mean that the cut-off was higher at semi-

industrial scale than at pilot scale, leading to its presence in the permeate. This can be explained by a different membrane supplier. Since the MWCO determination is not standardized, differences can be observed. There was also a strong band at 10 kDa at semi-industrial scale, stronger in the retentates at VRR 3 and after DV 2. The band was more colorful at DV 2 than at VRR 1 in the retentate, despite a lower protein content in total in the DV2 retentate. It could be an effect of a protease during the filtration.

The presence of proteases in the retentates of the semi-industrial scale experiments were analyzed by a zymogram, as seen in Figure 5.28.

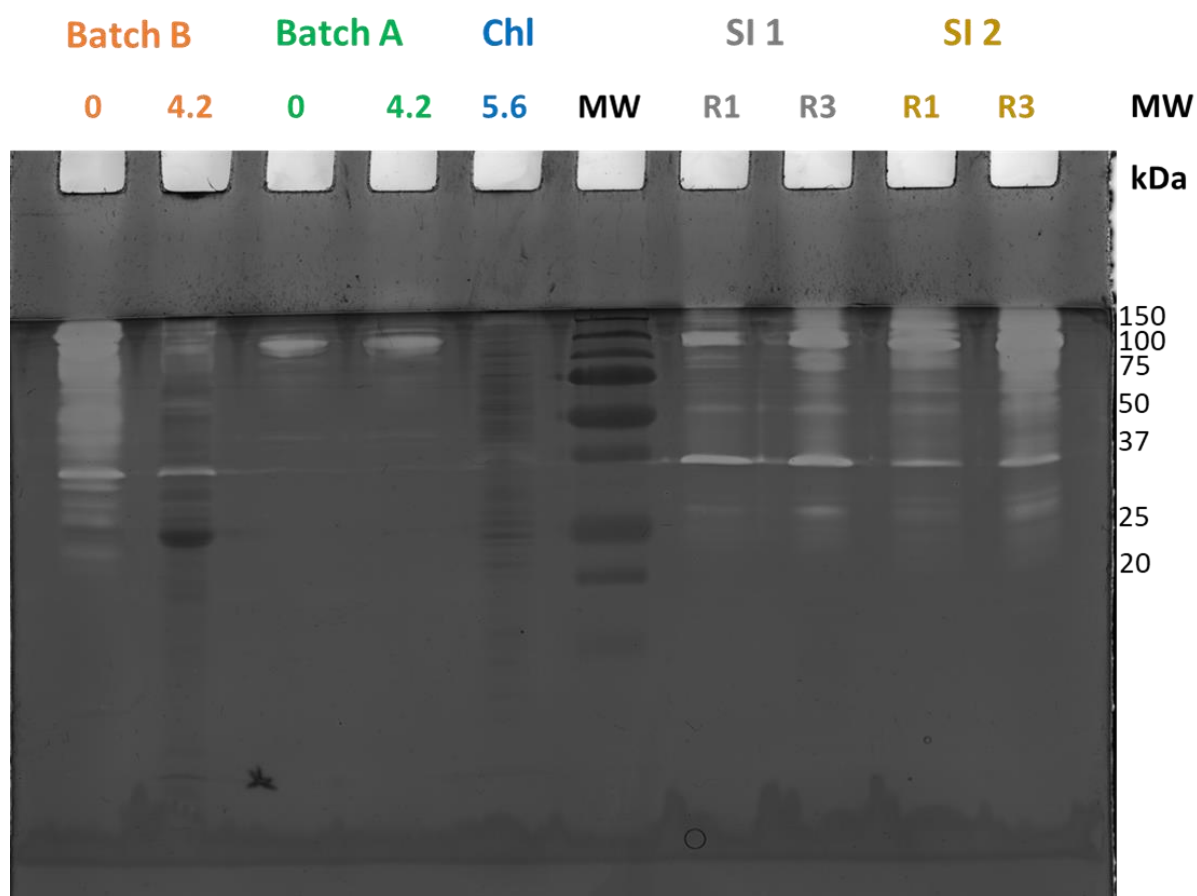


Figure 5.28 Zymograms of different products from microalgae. The supernatants from batch A and batch B *Tetraselmis* were characterized after bead milling at 0 and 4.2 min of residence time as *Chlorella* (Chl), as seen in chapter 4. The retentates after a VRR of 1 and 3 (R1, R3) of the filtrations at semi-industrial scale SI 1 and SI 2 were also characterized. The marker had visible bands having a molecular weight (MW) from 20 to 150 kDa.

Proteases were present in the supernatant (corresponding to retentate at VRR 1) and in the retentate at VRR 3 of the filtrations SI 1 and SI 2. The protease profile of the supernatant from batch D was closer to batch B supernatant after thawing, without bead milling than to batch A. This was also attested by a posteriori microscope images, with most of the cells looking like in batch B. The presence of more proteases in the supernatant treated at semi-industrial scale, according to the stronger digested bands,

may partly explain the difference in protein retention rate with the other scales. The proteases probably degrade proteins that are less retained.

Unfortunately, no strong differences in protein profile due to proteases were seen during the product stability experiment presented before, justifying not blanching the biomass to avoid an additional operation unit and a possible variability (protein denaturation). However, this was realized with batch A cells, since it was supposed to be the most frequent at higher scale and the zymogram analysis were not yet realized. Different results may have been obtained with the other batch, advising a blanching step for a future process. Moreover, the concentration by filtration in SI 1 from VRR 1 to 3 increased the protease concentration, as highlighted in the zymograms with more bands being digested. More proteases were seen in the retentates of SI 2 compared to SI 1. This might explain the lower protein retention rate observed for SI 2 compared to SI 1. The stronger digestion in SI 1 retentate seemed to be around 37 kDa and 100 kDa. The protease responsible for the degradation at 100 kDa seems to be conserved between batch A and B (resistant and fragile).

To evaluate the possibility to enhance the retention of the proteins that went through the membrane at semi-industrial scale, an acidification of the permeate was tested based on the results of IEP and the acidified permeate was filtered on the same filtration system.

5.3.5.2.2.5. Filtration of the acidified permeate at semi-industrial scale

The acid permeate filtration permeability was strongly lower than the other filtrations, but seemed to have a constant permeability as seen in Figure 5.21. A lower flux density was expected with the acidified permeate compared to the supernatants.

The acidified permeate filtration of SI 2 was also realized after a water washing after SI 2. However $J_p.J_w^{-1}$ was lower compared to the native supernatant, as seen in Figure 5.22. No inflection of the ratio was observed on the pressure range considered. This was also observed on protein extracted from soy, where while soluble proteins saw a classic flux limitation with pressure, following concentration polarization, the precipitated proteins did not show this behavior (Devereux et Hoare 1986). A higher pressure than 1 bar could have been used to increase the flux proportionally to the TMP on the acidified permeate. However a preliminary study could have been interesting to see its impact on membrane fouling since higher protein adsorption is also common at a pH close to the isoelectric point, so the membrane life expectancy may be lower (Bentham et al. 1988).

The concentration of the acidified permeate from the third filtration showed lower flux densities compared to the other filtrations but it was constant during the full concentration, as shown in Figure 5.23. A flux reduction at the isoelectric point of proteins is common in other biomass extract filtrations as well, and no variation of the flux along the concentration has also been observed with Bovine Serum

Albumin (Fane, Fell, et Waters 1983) or water treatment containing proteins (Ding et al. 2019). A higher protein adsorption is also common, at a pH close to the isoelectric point, so the membrane life expectancy may be lower (Bentham et al. 1988).

The filtration of the acidified permeate had a higher protein retention rate compared to the other filtrations, 73% instead of 15-25% as seen in figure 5.24. This allowed a higher selectivity and a higher yield. However, this method presents several disadvantages such as lower fluxes, probably a higher membrane fouling and the functionality of the proteins needs to be ensured. The acidification of a protein solution with a strong acid like the hydrochloric acid can lead to the denaturation of proteins where the acid is added, leading to a possible loss of solubility (Nash, Kwolek, et Wolf 1971). The hydrophobic amino acids that are normally buried within the proteins are exposed, thus the repulsion between proteins is minimized.

5.3.5.2.2.6. Summary of the UF performances

The summary of the protein yield, concentration and purity at the different process steps is presented in Figure 5.28.

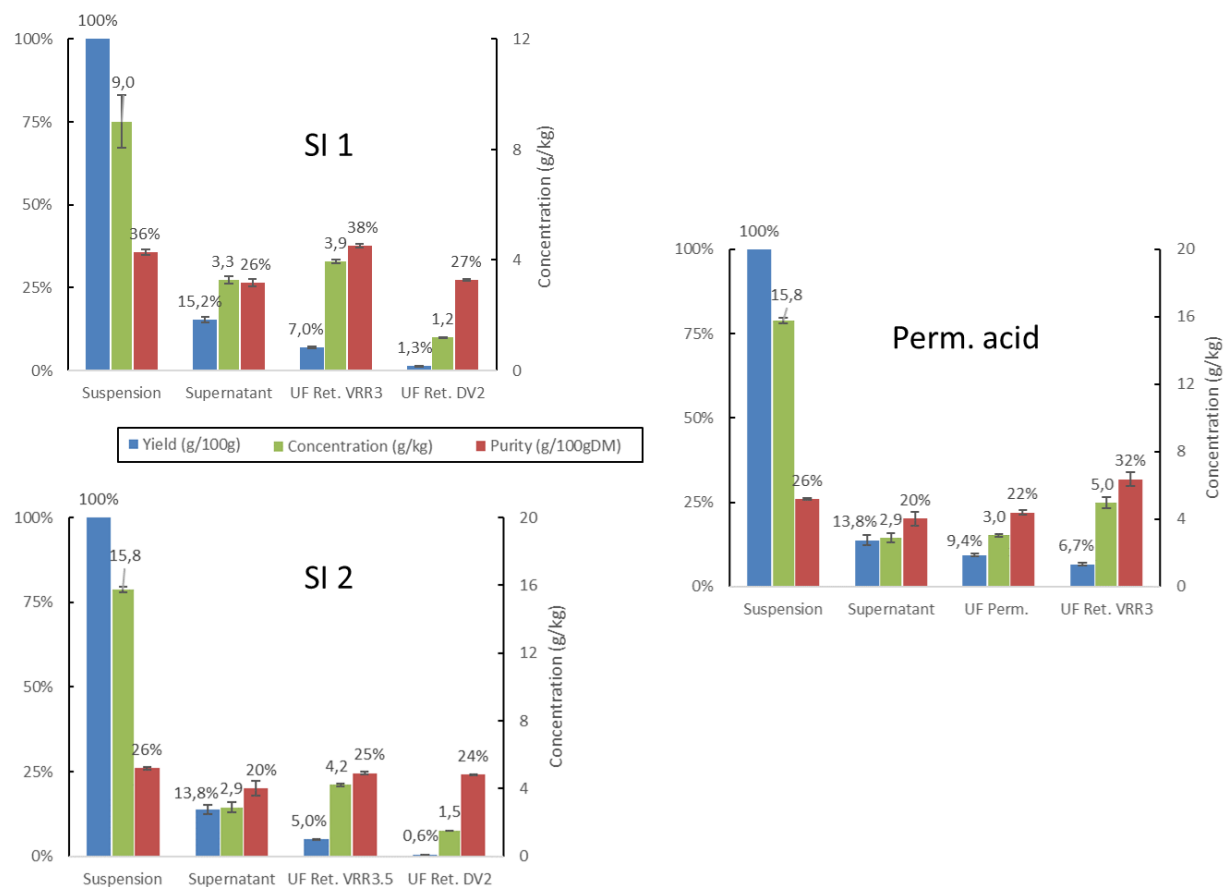


Figure 5.29 Summary of the filtrations at large scale with SI 1 and SI 2 as well as the filtration of the acidified permeate of SI 2 (Perm. acid). The protein yield, concentration and purity at different products along the process are presented for the filtration SI 1 and SI 2 at semi-industrial scale and on the acidified permeate of the concentration of SI 2. The products

presented are the initial biomass, the supernatant after thawing and centrifugation, the retentate after the UF concentration step and the retentate after diafiltration.

In comparison with the laboratory scale, ultrafiltrations at large-scale SI 1 and SI 2 had a lower protein retention yield, which led to lower protein yield and protein purity during concentration. The diafiltration led to a washing out of the proteins and the retention of the large proteins only.

The filtration of the acidified permeate was interesting, with a higher protein yield, a higher protein concentration and a higher protein purity. A possible improvement would be to acidify the supernatant in order to increase the protein retention rate and maybe to limit protease activity. However, the aroma of the extract before acidification smelled like seafood such as crab spiders, while the aroma of the acidified permeate smelled like heart of palm. Hence the application may be different, and the solution non optimal if a sea food aroma is aimed.

5.3.5.2.2.7. Color of the final product between the different scales

Color was key in the specifications that we defined with a targeted low-colored product. To measure color, the final products of different experiments at the different scales were lyophilized and analyzed by a colorimeter, the results are presented in Table 5.7.

Table 5.7 Colorimetric characterizations of different purified retentates after concentration and diafiltration at different scales (laboratory L, spiraled S and hollow fiber HF). The extracts were compared to a lysate after HPH and a precipitate after IEP of *N. oculata*. The $L^*a^*b^*$ values were converted in matlab to RGB values to give an overview and compare the different extracts, although the color depends on the computer.

Product	L*	a*	b*	RGB and color
L1 Ret. DV 0.75	62	-11	34	R:151 G:153 B:88
P1 Ret. DV 2	64	6	23	R:180 G:149 B:105
SI 1 Ret. DV 2	70	-9	28	R:172 G:173 B:119
Lysate (Cavonius et al.2015)	19	-7	9	R:40 G:48 B:33
Precipitate (Cavonius et al.2015)	23	-8	13	R:48 G:57 B:35

The extracts obtained after freeze-thawing and centrifugation followed by concentration and diafiltration by ultrafiltration were slightly colored, as seen in Table 5.8. The presence of green in the retentates of P1 and SI 1 showed that the formation of ice crystals probably broke the thylakoid

membrane. The extract obtained from S1 filtration was orange and probably due to the presence of a compound that was present in the paste.

The lysate obtained by Cavonius et al. 2015 was very dark green, with a low modification from the lysate obtained by HPH (Cavonius, Albers, et Undeland 2015). Hence while the protein yield obtained by this method was higher, with 86% of the initial proteins, very low color modification was not seen. On the other hand, soluble extracts after HPH of different algae were low colored after HPH and a filtration with a folded filter at 5-8 μm , depending on the specie (Grossmann et al. 2018b). It can be a suitable option of chlorophyll removal while conserving a higher protein yield. However, no protein yields were given between the suspension, the supernatant and the filtrate.

5.3.6. Purification of the supernatant after bead milling of the fragile *Tetraselmis chui*

5.3.6.1. Objectives

The presence of two batches of *Tetraselmis* and the bead milling experiment on the fragile batch was realized at the end of the PhD since the resistant batch was supposed to be the most frequent batch. However, with the results presented in Chapter 4 on the bead milling difference between the two different batches, the bead milling was demonstrated as interesting on the fragile batch. The purification of the proteins after bead milling of this strain was tested, as shown in Figure 5.30.

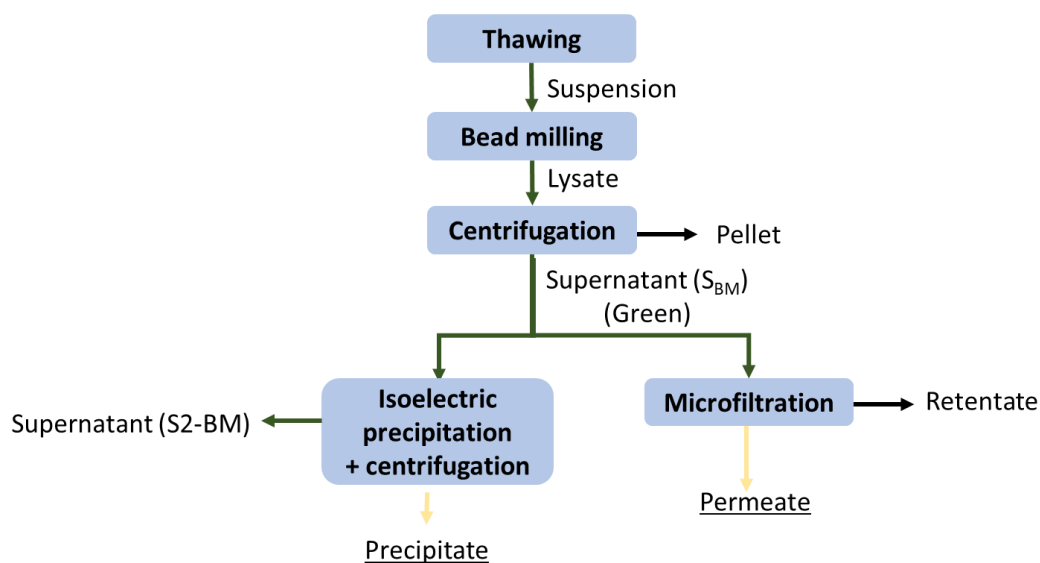


Figure 5.30 Process flow of the purification of proteins after the bead milling of fragile *Tetraselmis*. The products targeted after IEP and MF are the precipitate and the permeate respectively. In this case, a centrifugation of the supernatant S_{BM} was realized 1 h after pH adjustment to compare to the experiment realized on the resistant cells after freeze-thawing.

After bead milling, the suspension was diluted to $30 \text{ g}_{\text{DM}}.\text{kg}^{-1}$ and centrifuged and the supernatant purified by isoelectric precipitation and microfiltration at $0.1 \mu\text{m}$. The supernatant was also purified by isoelectric precipitation in order to observe the difference with the experiment on the resistant batch after freeze-thawing. The microfiltration, on the other hand, was aimed to retain the cell debris suspended within the supernatant and to let the protein go through as purified permeate.

5.3.6.2. Bead milling results

The bead milling was realized in the same conditions as the experiments in Chapter 3 and gave similar results, as seen in Figure 5.31.

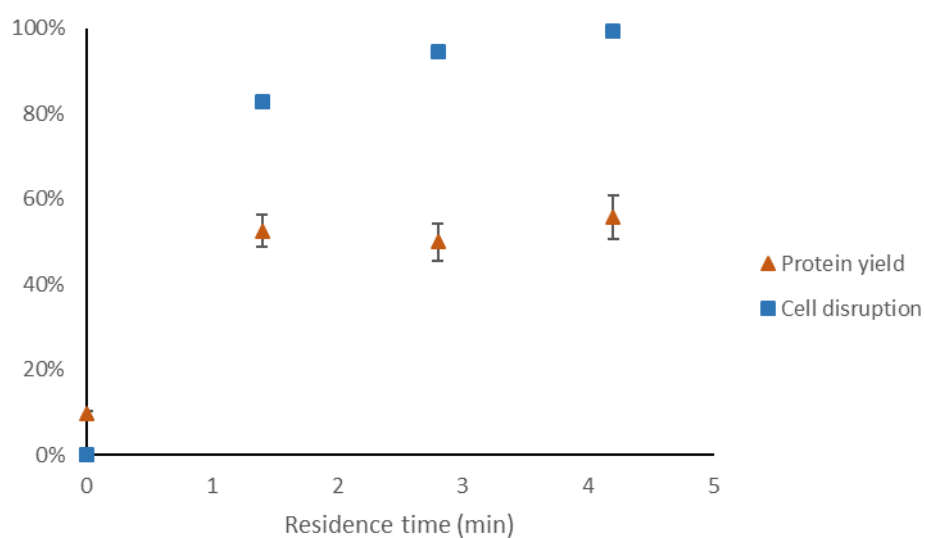


Figure 5.31 Cell disruption and protein yield in the supernatant as a function of the milling residence time.

A high protein release was obtained, with a protein yield of 50% of the initial proteins after the first passage in the bead mill.

The particle size distribution is similar to the PSD obtained on batch B in chapter 4, the cells are broken in the lysate after bead milling as seen in figure 5.32. The supernatant contains particles with sizes above $0.1 \mu\text{m}$ and we can suppose that the chlorophylls could be contained in these particles whereas the soluble proteins could be released in the aqueous phase. In these conditions, the microfiltration at $0.1 \mu\text{m}$ should allow an efficient protein purification.

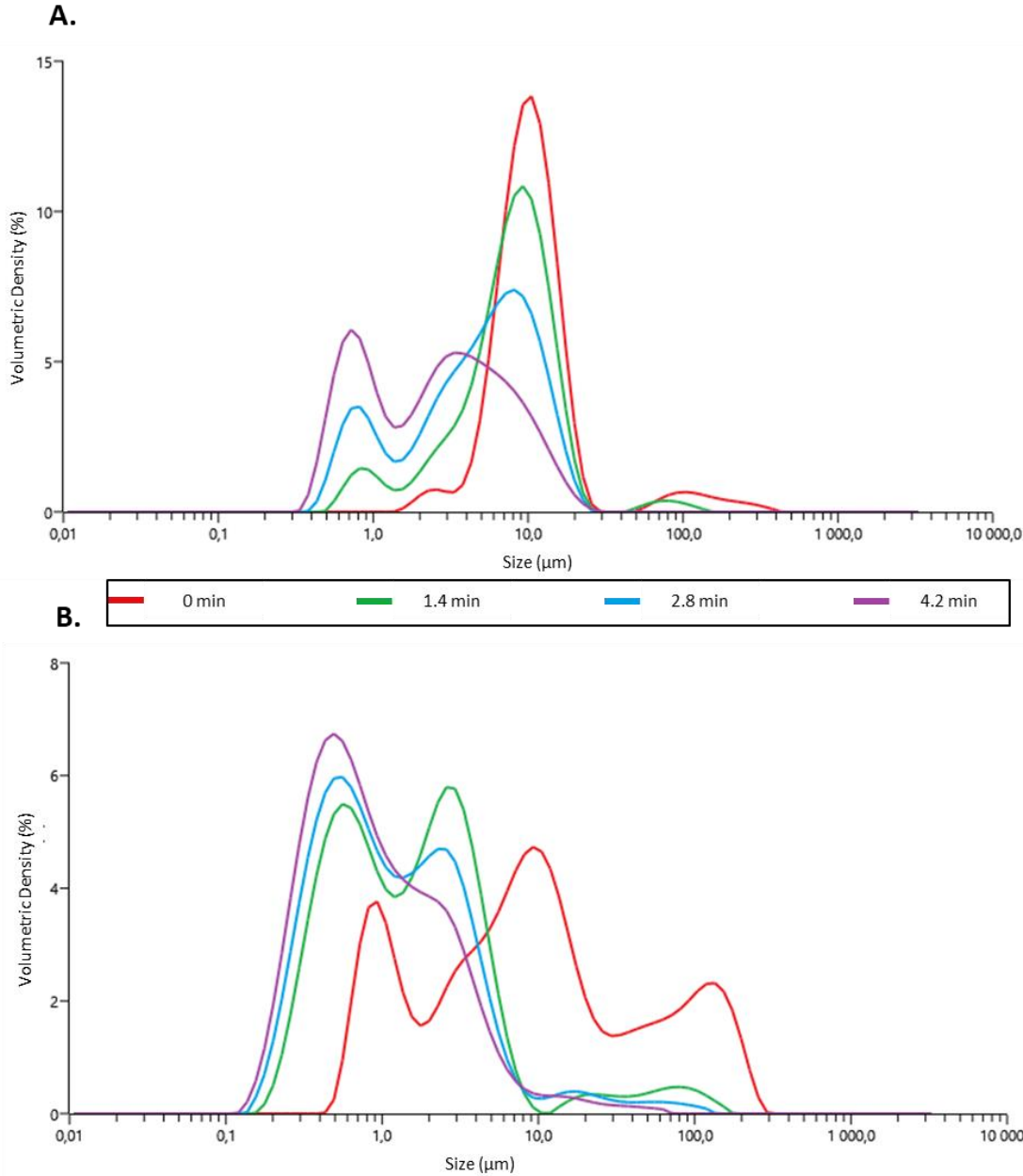


Figure 5.32 Particle size distribution of cell lysates (A) and clarified supernatants (B) as a function of the residence time in the bead mill for batch B before microfiltration, the measurements were performed in triplicates.

5.3.6.3. Isoelectric precipitation

An isoelectric precipitation was conducted on the supernatant after bead milling and the results are presented in Figure 5.33.

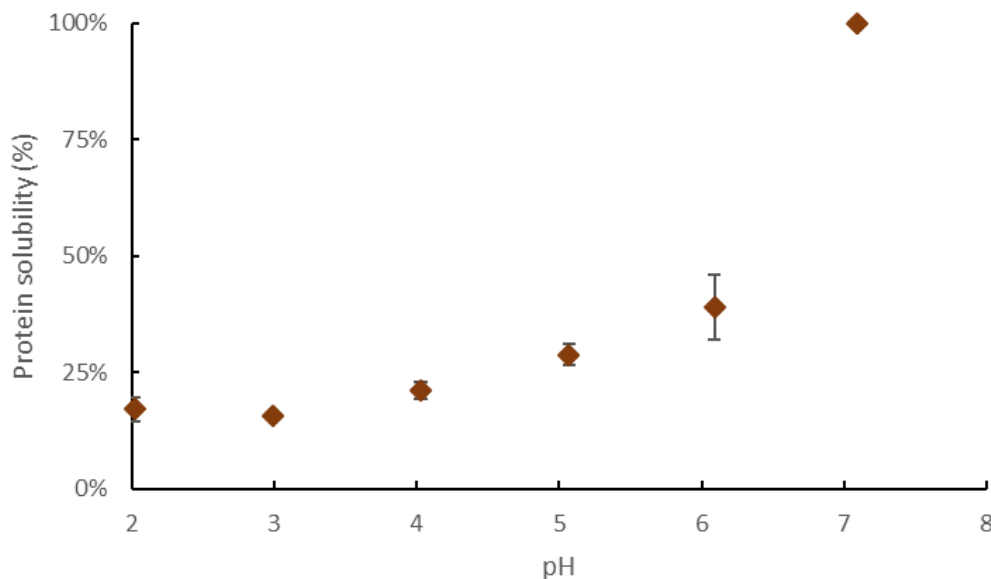


Figure 5.33 Protein solubility of the supernatant S2-BM after bead milling of fragile *Tetraselmis* as a function of the pH.

The protein solubility of the supernatant as a function of the pH was very different compared to the solubility presented after freeze-thawing on the resistant cells and the pI of the proteins was pH 3. This can be due to the difference in protein types released between the different batches, as seen in chapter 4.

While the precipitation was possible, with a clear precipitate after 1 h and a bigger pellet after centrifugation, the solid fraction was green/dark and did not satisfy the desired white soluble proteins planned in the context of the ProFuture Project.

5.3.6.4. Microfiltration

5.3.6.4.1. Cut-off choice

The supernatant was treated with microfiltration at 0.1 μm and 0.3 bar of PTM, in the same conditions as realized by Liu et al. 2021 (Liu et al. 2021). This cut-off was selected in order to retain the cell debris higher than 0.1 μm observed by PSD shown in Figure 4.7, presented in Chapter 4.

The little amount of cell debris at 0.1 μm could allow a higher flow rate and less probability of fouling present in the pores of the membrane.

The PSD of the supernatant obtained in chapter 4 for the fragile cells was similar to the PSD obtained in this experiment, confirming the cut-off choice.

5.3.6.4.2. Filtration performances

The water permeability was $70 \text{ L}\cdot\text{h}^{-1}\cdot\text{m}^{-2}$ at $25 \text{ }^\circ\text{C}$ and the product was filtrated at a TMP of 0.3 bar. The flux densities were ranging from 13 to $20 \text{ L}\cdot\text{h}^{-1}\cdot\text{m}^{-2}$ at $25 \text{ }^\circ\text{C}$ from VRR 1 to VRR 2, so ratio $J\cdot J_w^{-1}$ of 0.19 to 0.29. This was comparable to the microfiltration of a supernatant from bead milled *Chlorella*, which showed a flow rate of $13 \text{ L}\cdot\text{h}^{-1}\cdot\text{m}^{-2}$ at $30 \text{ }^\circ\text{C}$ when filtrating a supernatant of a $30 \text{ g}_{\text{DM}}\cdot\text{kg}^{-1}$ suspension (Liu et al. 2021).

The protein retention rates were also characterized and increased from 82% to 89% from VRR 1 to VRR 2 so only between 11 to 18% was passing through the membrane.

We can calculate a theoretical recovery yield of proteins in the permeate from the supernatant with the application of the following equation on the microfiltration.

$$Y_{MF} = 1 - VRR^{RR-1} \quad \text{Equation 5.3}$$

The protein yield on the microfiltration step was close to 7.3% by applying the retention rate obtained at VRR 2, so a total protein yield of $3.7 \pm 0.3 \text{ g}_{\text{protein,permeate}}\cdot 100\text{g}_{\text{initial,protein}}^{-1}$. In contrast with the high protein yield obtained after bead milling, the protein yield of the microfiltration was low. However the extract was not green and was lightly yellow.

A comparison of the processes between the freeze-thawing coupled with the centrifugation and the bead milling of the fragile strain coupled with the centrifugation and a $0.1 \text{ }\mu\text{m}$ microfiltration can be seen in Figure 5.34.

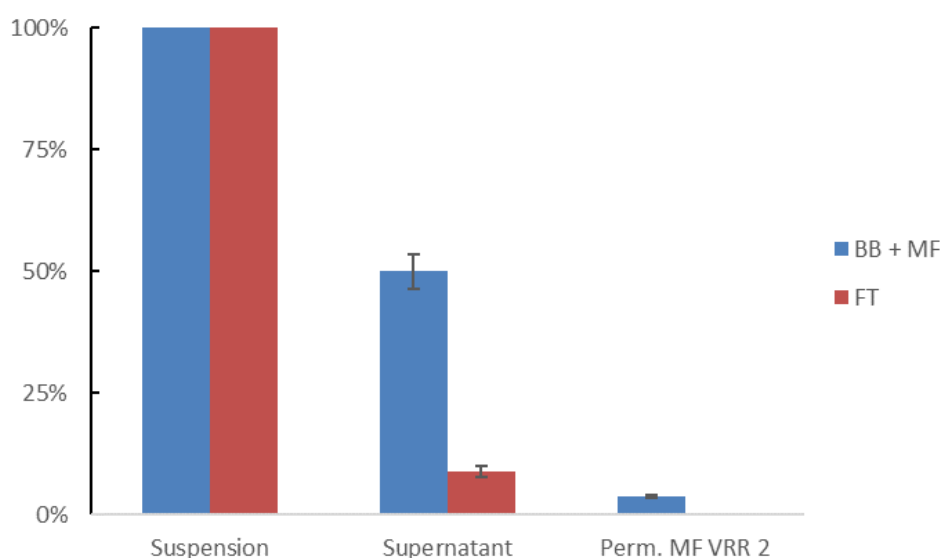


Figure 5.34 Protein yield comparison between the process with the fragile *Tetraselmis* destroyed by bead milling, centrifuge and filtrated by microfiltration at VRR 2 (BB + MF) and the process with a freeze-thawing only (FT).

The protein yield obtained in the supernatant was higher after bead milling compared to freeze-thawing only. However, part of these proteins were only dispersed in the supernatant, and were retained by the microfiltration at 0.1 μm . It has to be noted that a higher VRR and the use of a diafiltration to enhance the protein yield of the microfiltration may be performed (Gifuni et al. 2020). In addition, while a higher protein yield seemed promising after bead milling, the purification of the proteins from the supernatant still needs to be improved.

The protein retention rate obtained in our work was comparable to the results of a microfiltration at 0.1 μm of a supernatant after the bead milling of *Chlorella*, with protein retention rate between 86 to 89% (Liu et al. 2021). Similar results were obtained on *Parachlorella kessleri* after cell disruption by bead milling, centrifugation and microfiltration at 0.1 μm and 1 bar (Liu et al. 2022). No change in protein retention rate was observed when either agitated dead end filtration, plane cross-flow microfiltration and dynamic cross flow filtration were used.

On *Tetraselmis*, a study conducted by Suarez et al. 2018 aimed at purifying proteins after bead milling and centrifugation by microfiltration (Garcia et al. 2018). A total protein yield of $11.9 \text{ g}_{\text{protein, sup.}} \cdot 100 \text{ g}_{\text{initial, protein}}^{-1}$ was obtained in the supernatant after bead milling and a 20,000g 30 min centrifugation while a microfiltration in dead end cellulose filters at 0.45 μm had a yield of $11.9 \text{ g}_{\text{protein, sup.}} \cdot 100 \text{ g}_{\text{initial, protein}}^{-1}$. Hence, a very low protein retention was observed but their extract was green, sign that not all the cell debris containing chlorophyll were retained. The choice of a larger cut-off may be interesting but will have an impact on the purification quality.

5.4. Conclusion

The purification of the supernatant from frozen-thawed *Tetraselmis chui* was compared using two purification techniques, isoelectric precipitation and ultrafiltration. The resistant *Tetraselmis* was chosen as a basis for the scale-up since it was the most frequent at large scale according to the producer. The IEP was not effective on the protein purification of proteins from the resistant *Tetraselmis*, with no protein precipitation observed after centrifugation. However, a pI was identified at pH 2.5 based on the protein solubility and turbidity. On the other hand, UF at laboratory scale was giving satisfactory results with a protein retention rate close to 90% while the ash retention rate was low, 5-10%, allowing a good selectivity. However, the MWCO chosen, 10 kDa, was not able to separate proteins from carbohydrates. A diafiltration was also successfully implemented after the concentration, allowing an increase in protein purity and a decrease in salinity. The final lyophilized product was slightly green, which was still considered as acceptable in comparison with the SCP.

A scale-up of the process using the ultrafiltration was planned, and the filtration temperature and the batch volume needed to be addressed. A filtration temperature of 12°C was adopted because of a product modification on the supernatant from frozen-thawed resistant cells, probably due to a microbial development and/or a protein hydrolysis at room temperature and 47°C.

A pilot scale process was used to assess the results previously obtained at laboratory scale. The cells were partly resistant and fragile and the protein retention rate was between 40% to 60%, so lower than previously obtained. However, due to a need to supply a sufficient quantity of product in an appropriate timeline in regards with the project, these results seemed acceptable to scale up the process.

Finally, the semi-industrial scale was achieved to obtain a high product quantity for the project partners and to validate the results. However, unlike at laboratory scale, the protein retention rates were low during the different concentrations (RR ≈ 30%).

5.5. **References**

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6.1 Objectives

The main objective of this thesis was to compare different processes to produce a low-color microalgal extract enriched with soluble proteins for food applications. The three steps considered were: cell permeation/disruption, clarification by centrifugation, and concentration/purification (figure 2.11).

Two strategies were thought to extract proteins to attain this objective and were presented in chapter 3. The first strategy aimed at extracting aqueous proteins without dispersing the biomass to avoid the production of a green supernatant difficult to purify. The protein release was realized by Pulsed Electric Fields and the process compared to freeze-thawing. A low protein recovery yield in the aqueous phase was expected after clarification but with a low color, less operation units and the possibility to valorize more easily the side-product. The second strategy aimed at disrupting the biomass and macerate the cell lysate in order to release the intracellular proteins. The cell disruption was carried out by bead milling compared to freeze-thawing. The goal was to improve the protein recovery yield in the aqueous phase and to access most of the globular proteins and possibly extract some membrane proteins. However, the supernatant after clarification was green due to the presence of chlorophylls, so an additional operation unit such as microfiltration was needed to separate green aggregates containing chlorophylls from the protein extract. The study of these strategies led to highlight the key scientific question of the impact of the physiological state of the biomass on the performances of both the cell disruption and the concentration/purification processes.

6.2 Main results

6.2.1 Two different physiological states of *T. chui*

Upon the reception of *T. chui* biomass, two different physiological aspects of the cells were observed and the origin was unknown of the producer. After a literature review of the possible main reasons explaining the physiological differences, two main factors were outlined. The first reason can come from different environmental conditions during culture, harvesting or freezing, which can induce a change in the life cycle stage of *Tetraselmis*. The life cycle stage of *Tetraselmis chui* has not been studied precisely yet, to the author knowledge. However, the effects of salinity, bacterial contamination, pH, shear stress, light and temperature were environmental conditions triggering a life cycle stage change in several studies on different *Tetraselmis* species (Kirst 1977; L. et Montoya 2009; Michels et al. 2016). The other reason can come from a difference in the cell cycle stage if the culture was naturally synchronized, due to the influence of day and night cycles in solar culture. The synchronization of the cells can potentially have a positive aspect for biomass production since a better

light utilization of 10-15% was observed on synchronized algae in comparison with a non-synchronized culture (de Winter et al. 2017). However, the harvesting moment can determine the cell cycle state if the culture was in a synchronous state, and this may have an effect during biorefinery. Nevertheless, due to the industrial scale and since the culture happened a long time before biorefinery, the exact cause leading to the physiological difference of the biomass received cannot be identified. The different batches were characterized biochemically and a cell fragility difference was noticed, with one physiological state more fragile than the other.

6.2.2 Bead milling of *T.chui*

The bead milling of these different physiological states led to small differences in cell disruption kinetics with the same bead milling conditions. However, striking differences were obtained in dry matter release in the supernatant between the two physiological states, despite a complete cell disruption.

The soluble dry matter increased only from 19% after thawing to 20% after bead milling on the resistant state while it increased from 13% to 27% for the fragile state. The difference was also seen in the total dry matter with small increase from 23% after thawing to 26% on the resistant state and from 14% to 44% on the fragile state. These differences can come from a difference in comminution, and this was confirmed by the Particle Size Distribution of the lysates and supernatants. A higher fine particle generation was observed for the fragile state in comparison with the resistant state, probably leading to the difference in dry matter dispersion.

On protein release precisely, the difference in total protein yields were 9% after thawing to 11% after bead milling on the resistant state, while it was between 7% to 48% on the fragile state. Similarly, on soluble protein yields, the differences were from 9% to 11% for the resistant state and from 6% to 32% on the fragile state. Moreover, the types of extracted proteins were also different among the different states according to SDS PAGE analysis. The proteases released were also different between the different physiological states, which suggests a difference in metabolism between the two batches. The fragile cells seem to include more protease in the supernatant after thawing and bead milling than the resistant cells.

These results are significant for biorefinery in general since, while the resistant cells were completely disrupted, very low soluble compounds were released after complete cell disruption. The biomass physiological state of this specie is very important to take into account.

The release of proteins from *T.chui* by bead milling was significantly affected depending on the physiological states. The fragile state had a high protein release after bead milling compare to thawing so bead milling was promising. On the other hand, the resistant state had a very low protein yield increase after bead milling in comparison with thawing. The bead milling is energy intensive and the supernatant after bead milling was green so this operation unit does not seem appropriate to release proteins from the resistant cells.

6.2.3 Comparison with the bead milling of other species

A comparison was made with two well-known species for food application, *Chlorella vulgaris* and *Arthrospira platensis*. The cell disruption of *Chlorella* by bead milling showed a release of proteins similar to what has been shown in literature (Zinkoné et al. 2018). Bead milling led to a strong protein release in comparison with freeze-thawing but the coupling with another operation unit needs to be deepened (Gifuni et al. 2020; Liu et al. 2021; 2022). The protein release as a function of the milling residence time was slower than the protein release from the fragile cells of *T.chui*.

The dispersion of *Arthrospira* was very strong with bead milling and the protein yield as well as the protein purity were very high. However, this led to a green supernatant and nearly no pellet. The supernatant may be difficult to purify due to the high quantity of organic matter as colloids. Milder methods are used industrially to extract C-phycoyanine, the main protein and an important antioxidant in *Arthrospira*. The traditional method consists on freezing and thawing the biomass and macerating it, releasing the proteins overtime. A milder method seems more suited to extract protein from this specie.

6.2.4 Pulsed Electric Fields

Pulsed electric fields studied on the resistant *T. chui* and *C. vulgaris* led to a low protein extraction yield and the improvement was not significant compared to freeze-thawing in the tested conditions. The freeze-thawing performed before the PEF, due to the frozen biomass storage, permeated the cells before the process, limiting the interest of the added step.

6.2.5 Conclusion on the cell disruption method and consequences for the purification

Two different downstream processing of *T. chui* were proposed depending on the physiological state, considering different cell disruption methods and consecutive concentration and purification processes:

- For the resistant state, the freeze-thawing was chosen, followed by a clarification by centrifugation and IEP or UF to concentrate and purify proteins,
- For the fragile state, the bead milling was selected, coupled to centrifugation for clarification, and microfiltration to remove green aggregates, before concentration/purification by IEP or UF of the MF permeate.

The first strategy led to a low protein yield for the first operation unit but could provide a low colored extract and a decrease in operation unit number. Moreover, the side product may have more value since no complex molecular reorganization took place in comparison with a mechanically treated pellet.

The second strategy relies on a cell disruption method leading to the release of more proteins, however, some of them are membrane proteins from the thylakoids, linked with chlorophylls. To eliminate the chlorophylls, an additional operation unit as microfiltration is needed on the supernatant but more uncolored soluble proteins may be extracted compared to a simple freeze-thawing.

First strategy

The first strategy was tested at laboratory scale since the biomass to be sent by the European partners for large scale experiments was described by the producers as in the resistant physiological state. The concentration and purification were tested. A high protein solubility was obtained at all pH tested and IEP could not be used to purify the proteins extracted. However, ultrafiltration at 10 kDa was very efficient with a high protein retention rate and correct fluxes so this operation unit was chosen for the scale-up.

A product stability test was conducted in prevision of the scale-up on the resistant biomass supernatant after freeze-thawing. Three different temperatures were tested, 12°C, room temperature ($\approx 22^\circ\text{C}$) and 47°C. The filtration time could have been decreased with higher filtration temperatures but the microbiological content and the protein stability were not ensured at room temperature and 47°C. On the other hand, the stability of the supernatant at 12°C was ensured for 72 h for the microbiological load, the protein profile and the smell.

A scale up was realized at pilot scale and gave a lower protein retention rate compared to laboratory scale. However, the results were still considered as sufficient and the necessity to produce ingredients for the other European partners led to a scale up at semi-industrial scale.

The scale up at semi-industrial scale led to higher fluxes than obtained at other scales. However, the protein retention rate was unexpectedly low (30%) during the concentration, decreasing the protein recovery yield in the retentate. Several reasons could explain the lower protein retention rate. A main reason could be the variability of the biomass, since most of the cells were finally fragile at semi industrial scale and pilot scale, while they were resistant at laboratory scale and during the supernatant stability test. More proteases were present in the supernatant after thawing the fragile cells than in the resistant cells. The scale was also an important factor since due to the increase of temperature after centrifugation, the supernatant temperature rose up to 35°C and decreased slowly due to the inertia induced by the high volume. This higher temperature could have led to an increase in protease activity.

In order to increase the protein yield, the filtration was tested at acid pH. The permeate of the concentration at semi-industrial scale was acidified in order to aggregate the proteins and increase their retention rate. This helped to increase the global retention rate and improve the proteins yield. However, the smell of the product changed from a seafood flavor to a heart of palm flavor, which can change the acceptability by the consumer.

Second strategy:

Protein extraction from the fragile cell was also performed by bead milling at laboratory scale and a high protein yield was obtained, as presented in chapter 4. The supernatant was purified from the aggregates containing chlorophylls by microfiltration at 0.1 µm. A low colored permeate was produced, however a high protein retention rate (82 to 89%) was obtained on this operation unit leading to a low protein recovery yield in the microfiltration permeate (3.7% of initial proteins after a VRR of 2). The addition of a microfiltration diafiltration can be implemented to increase the protein recovery in permeate but, overall, it stays low in comparison with other biomass such as soy or pea.

The IEP was possible on the supernatant of the bead milled fragile *Tetraselmis*, which was not the case on the resistant cells, however a green precipitate was obtained. This could be due to the sedimentation of membrane proteins containing chlorophylls.

6.3 Original contributions of the study

6.3.1 The physiological state of the cells after harvesting directly influenced the following biorefining strategy

The effect of the physiology of *T.chui* on cell disruption and protein recovery was outlined by this study. Several methods were used to characterize the physiological state, such as the morphology analysis by microscopy, the characterization of its fragility by HPH, the identification of the protein extracted by SDS PAGE or the analysis of the proteases.

The bead milling of *T.chui* in different physiological states led to significant differences during cell disruption and implied different choices for the following fractionation strategy. However, the physiological state was not mentioned in other studies on *Tetraselmis* cell disruption (Schwenzfeier, Wierenga, et Gruppen 2011; Postma et al. 2017; Teuling et al. 2017; Garcia et al. 2018; Suarez Garcia et al. 2018) and scarcely mentioned in recent literature in culture. The high variability of protein yields obtained after *Tetraselmis* bead milling across the different studies may come from a difference of physiological state. The physiological state of *Tetraselmis* should be taken into account due to this work in the following studies.

6.3.2 Pulsed Electric Fields

Pulsed Electric Fields did not show significant improvements in comparison with freeze-thawing on *Tetraselmis chui*. However, this technology could be adapted on fresh microalgae to extract soluble proteins before an additional molecule extraction such as lipids, for other applications. PEF can also be promising for microalgae milking.

6.3.3 The nature of the extracted proteins, depending on the physiological state and the cell disruption process, modified the usual purification process performances

Isoelectric precipitation was conducted in two different conditions, on the supernatant of the resistant cells of *T.chui* after freeze-thawing or on the supernatant of the fragile cells after bead milling. In the first case, no precipitation was observed and the reasons could be the extraction of very soluble proteins from the cytoplasm or the stroma, or the association between proteins and charged polysaccharides, that may create coacervates. In the second case, proteins extracted after bead milling had a low solubility close to the pI and a green precipitate was obtained. Various proteins were probably extracted among which membrane proteins, and especially proteins linked to chlorophylls, explaining its color.

Ultrafiltration at 10 kDa can be successful at laboratory scale to retain proteins from *T.chui*. However, depending on the biomass physiological state, protease may be present and strongly modify the process performances, especially if the temperature is not well controlled at each step of the biorefining, as illustrated in the experiments at semi-industrial scale. The importance of taking into account the physiological state on the presence of protease and thus on protein purification of *T.chui* was outlined in this work.

To date very few protease study using zymograms have been conducted on microalgae, and not for protein purification but for cellular biology. This original method usually used for pure analytical characterization could become a useful tool to help biorefining processes development and understanding.

6.3.4 One of the first high scale biorefining study to produce protein extracts was conducted

Few studies coupling cell disruption and membrane filtration were conducted at such a high scale for the production of an extract rich in proteins. The first limitation is usually the biomass availability. The Profuture European program offered the opportunity to reach this high scale by giving us access to large biomass quantities produced at industrial scale ($>10 \text{ kg}_{\text{DM}}$), with resulting variability questions. The collaboration between Algosource and the GEPEA, with Algosolis platform, also allowed access to the appropriate semi-industrial units, equipment that is not common in all laboratories. For example, filtration was usually studied with a membrane surface between 0.005 m^2 to 0.019 m^2 (Garcia et al. 2018; Liu et al. 2022), instead of 2.3 m^2 used in this study.

6.3.5 Conclusion of this work

Finally, we can conclude from this work that the integration of all the steps, from the upstream (culture of the biomass, harvesting and storage) to the downstream processing (cell disruption and purification), is a necessity to produce well controlled microalgae extracts. The microalgae are very flexible microorganisms that can modify their composition in few hours; this flexibility is very interesting to orientate their metabolism, but this quality also induce a large drawback, the biomass variability, especially when cultured at industrial scale, as highlighted in this work. The fine selection of the disruption techniques is needed to allow the concentration and purification of proteins with specific properties, and progressive cell lysis could facilitate the following steps. The upscaling of the downstream processing with large biomass quantities to be treated, will raise new challenges like the product stability from the storage to the purification, and the related necessity of pretreatments or temperature control.

6.4 Opening and perspectives

Research on the physiology of *T.chui* could be deepened, especially the life cycle stage. The differences between the different physiological states could have significant impacts, other than on biorefinery. For example, cell digestibility by humans and animals can be heavily impacted by a cell wall modification. Since *Tetraselmis* is mainly used for feed for aquaculture, the physiological differences could have significant effects on the growth of the fish. This will be tested by other partners of the project. The origin of the physiological state difference between the different batches will also be researched by the producers to optimize culture and understand the differences. The variations of biomass complicate studies at high scale, especially if it is unknown, and studies on the physiology of this promising specie can improve its utilization on a various range of applications.

For protein extraction and purification, the low protein yields obtained outline the difficulties of realizing a low color enriched-protein extract using biorefinery processes on *Tetraselmis chui*. The species targeted are microscopic organisms, complicating the recovery in comparison with other biomass, due to the higher cost associated with the separation. Moreover, a multitude of proteins are present within the biomass, with a large range of functions, structures and cellular localization. While some of these proteins are globular water soluble proteins, others are membrane proteins linked with chlorophylls in the thylakoid membrane. The operation units usually used to break the algae, containing a resistant cell wall, can also be used to produce nanoparticles, suspended in aqueous solutions. Thus the proteins extracted can correspond to globular proteins, but also to naturally insoluble proteins, dispersed into the aqueous phase. For an efficient use of cell disruption operation units, the goals should be clearly stated, which physiological state is needed and which proteins are targeted. This question necessitates to go further in the knowledge of the protein repartition in the different parts of the microalgae during its life cycle.

The use of advanced analytical methods can help to understand more the action of these operation units, and to facilitate the coupling with purification operation units. For example, analytical techniques such as Size Exchange Chromatography (SEC) or Asymmetrical flow field-flow fractionation (AF4) can discriminate the different proteins extracted and their possible association with chlorophylls. This latest has been used for different trials not presented in this work. The use of mass spectrometry can also be a key to improve biorefinery since it identifies precisely the extracted proteins and their localization in the cell. This has for example been used by Gateau et al. to understand the milking of *H.pluvialis* by PEF (Gateau et al. 2021). A quantification of different proportions of proteins depending on their localization in the cells (e.g. 10% in the cytoplasm, 5% in the stroma, 30% in the thylakoid membrane, 10% in the mitochondria membrane etc.), or between their structure (e.g. globular, membrane, fibrous) can help significantly improving the processes for protein extraction from

microalgae. For example, the isolation of proteins from cellular fractions such as the cytosol, membrane-bound organelles and the nucleus was realized by gentle buffer with increasing detergent strength and could be applied to microalgae (Baghirova et al. 2015). The results can be interesting with biomass in different trophic modes, to study their impacts on the extracted proteins.

The removal of chlorophylls from microalgae can also be performed by appropriate culture selection and has been applied recently to grow microalgae with very low or nearly no pigments in heterotrophy on *C.vulgaris* (Schüler et al. 2020). However, this culture modification could modify the physiological state of the microalgae. This can modify the microalgae composition, the pigments may not be produced but also other compounds, which can modify the nutritional interest that should be verified. Bioprocess engineering could be used to produce protein extract from different cellular localization, with potentially different technofunctional properties. These latest could be the parameters used to select which fractions are interesting for food application, and defines the process used to obtain it.

The use of microalgae for food application can be interesting in the future as Single Cell Proteins, and biorefinery can be used to produce interesting fractions with special properties. The significant biodiversity in microalgae and cyanobacteria could lead to yet unknown protein properties, that could be promising for food application. However, challenges are still present due to the yet immature culture and biorefinery processes. Moreover, more studies in biology and biochemistry are needed to enhance significantly microalgae industry for protein valorization since fundamental questions such as where are the main proteins of microalgae are still unclear.

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Titre : Extraction et purification de protéines fonctionnelles de microalgues pour l'alimentation humaine.

Mots clés : Bioraffinage, Microalgue, *Tetraselmis*, Protéines, Filtration membranaire

Résumé : Les microalgues sont des sources prometteuses de protéines pour l'alimentation humaine mais des progrès sont à réaliser pour augmenter l'acceptabilité par les consommateurs. Pour ce faire, les objectifs de cette thèse sont d'identifier les verrous afin d'obtenir un ingrédient protéique soluble dans l'eau et faiblement coloré de *Tetraselmis chui*. L'intégration des opérations unitaires de destruction cellulaire avec des opérations unitaires de purification est étudiée et un changement d'échelle du procédé prometteur réalisé. La décongélation, le broyage à billes (BB) et les champs électriques pulsés (CEP) sont comparés pour la libération de protéines de *T.chui*, et comparés à *Chlorella vulgaris* et *Arthrospira platensis*. Lors de l'étude du BB, des résultats originaux sont obtenus sur l'impact de l'état physiologique de *Tetraselmis chui* sur la récupération des protéines.

Les techniques retenues pour la production d'extraits protéiques de cette algue sont la décongélation et le BB, selon l'état physiologique. La purification des protéines des surnageants est étudiée par précipitation isoélectrique (PIE) et filtration membranaire. L'ultrafiltration (UF) démontre une meilleure efficacité que la PIE sur le surnageant obtenu après décongélation et centrifugation. Le procédé est transféré jusqu'à une échelle semi-industrielle, échelle rarement mise en œuvre à ce jour sur des extraits de microalgues. Les contraintes liées au changement d'échelle affectent les performances du procédé mais des solutions sont proposées. Après broyage à billes *T.chui* fragile, la microfiltration est prometteuse pour l'obtention d'un produit protéique faiblement coloré mais des progrès restent à réaliser.

Title: Extraction and purification of functional proteins from microalgae for food application.

Keywords: Biorefinery, Microalgae, *Tetraselmis*, Proteins, Membrane filtration

Abstract: Microalgae are promising sources of protein for human consumption, but progress needs to be made to increase their acceptability to consumers. To this end, the objectives of this work are to identify the bottlenecks in obtaining a water-soluble, lightly colored protein ingredient from *Tetraselmis chui*. The integration of cell disruption with purification operations units is studied and the most promising process is scaled-up. Thawing, bead milling (BM) and pulsed electric fields (PEF) are compared for the release of proteins from *T.chui*, and compared with *Chlorella vulgaris* and *Arthrospira platensis*. During the BM study, original results were obtained on the impacts of the physiological state of *Tetraselmis chui* on protein recovery.

The techniques selected were thawing and BM, depending on the physiological state of *T.chui*. The purification of proteins from supernatants after clarification was studied using isoelectric precipitation (IEP) and membrane filtration. Ultrafiltration (UF) was more efficient than IEP on supernatant obtained after thawing and centrifugation. The process has been transferred to a semi-industrial scale for way R, a scale that has rarely been studied to date in microalgae biorefinery. The constraints associated with the scale-up affect the performance of the process, but solutions have been proposed. After bead milling of fragile *T.chui*, microfiltration is a promising technique to obtain a lightly colored protein product.