Enzyme-assisted disruption of oleaginous microalgae to increase the extraction of lipids: Nannochloropsis as a case study

Peer-reviewed author version

MIENIS, Esther; VANDAMME, Dries & Foubert, Imogen (2023) Enzyme-assisted disruption of oleaginous microalgae to increase the extraction of lipids: Nannochloropsis as a case study. In: Current Opinion in Food Science, 51 (Art N° 101034).

DOI: 10.1016/j.cofs.2023.101034
Handle: http://hdl.handle.net/1942/40162

Enzyme-assisted disruption of oleaginous microalgae to increase the extraction of lipids: *Nannochloropsis* as a case study

Esther Mienis[a,b], Dries Vandamme[c], Imogen Foubert[a,b] *

[a] KU Leuven Kulak, Research Unit Food & Lipids, E. Sabbelaan, 8500 Kortrijk, Belgium

[b] Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven, Kasteelpark Arenberg 20, 3001 Leuven, Belgium

[c] Analytical and Circular Chemistry (ACC), Institute for Materials Research (IMO), Hasselt University, Martelarenlaan 42, 3500 Hasselt, Belgium

E-mail Addresses:

esther.mienis@kuleuven.be

dries.vandamme@uhasselt.be

imogen.foubert@kuleuven.be

* Corresponding author at: KU Leuven Kulak, Research Unit Food & Lipids, E. Sabbelaan, 8500 Kortrijk, Belgium. E-mail address: imogen.foubert@kuleuven.be (I. Foubert)

Abstract

Photoautotrophic microalgae such as the marine microalga *Nannochloropsis,* are unicellular organisms able to synthesise valuable lipids for application in food, aquaculture, agriculture, and bioenergy. Their rigid cell walls limit the extraction of lipids. Therefore enzyme-assisted disruption methods are often used to increase the lipid extraction yield. While enzyme-assisted disruption of *Nannochloropsis* can increase lipid extraction yield, the enzyme and solvent selection, as well as treatment conditions vary considerably in the literature. This review gives an overview of recent literature on enzyme-assisted disruption of *Nannochloropsis* to increase lipid extraction yield. Our aim is to provide guidelines and good practises for enzyme selection, pre-treatment, and post-treatments which can be extrapolated to other oleaginous microalgae.

Introduction

Oleaginous photoautotrophic microalgae are unicellular organisms able to synthesise valuable components for application in human nutrition, agriculture, aquaculture, and bioenergy. These microalgae accumulate lipids and only need light, carbon dioxide and an inorganic source of nitrogen and phosphorous to grow. They can be cultivated at locations unfit for conventional agriculture [1]. These microalgae are therefore an interesting, sustainable and novel biomass [2]. Several microalgae species have rigid cell walls (CW) which act as barrier to lipid extraction. CW can be disrupted to increase subsequent lipid extraction or to improve the bio-accessibility and digestibility of lipids in food applications [3]. Multiple disruption techniques have been studied with these aims. These include physical (e.g.: high pressure homogenisation, bead milling, ultrasonication, pulsed electric field) and (bio)chemical methods (e.g.: hydrothermal, acid or alkaline treatment, enzyme-assisted disruption) [4,5]. Enzyme-assisted disruption (EAD) has advantages compared to other cell disruption methods as it can be performed under milder operating conditions, requires less capital investment for specialised equipment [6], is associated with decreased energy costs [7], and can be tailored towards the disruption of specific microalgal species or components within the microalgal CW.

In this review, the oleaginous marine microalga *Nannochloropsis* (Eustigmatophycea) is used as case study of EAD to increase lipid extraction. Note that recently some *Nannochloropsis* species have been redefined into a new genus, *Microchloropsis* [8]. In this review we include both *Nannochloropsis* and taxa originally assigned to *Nannochloropsis* that have now been re-assigned to *Microchloropsis*. Hereafter, we refer to this ensemble as *Nannochloropsis*. *Nannochloropsis* has received interest for (potential) application in food due to its ability to synthesise nutritionally valuable omega-3 poly-unsaturated fatty acids, such as eicosapentaenoic acid (C20:5n-3) [9], carotenoids, α-tocopherol [10] and vitamin D_3 [11]. This genus also functions as a model organism for algal biofuel production as it can accumulate up to ~70% of its dry weight as lipids [12]. However, the lipids in *Nannochloropsis* are surrounded by an especially rigid CW acting as a barrier for their extraction. This necessitates the use of a cell disruption technique that liberates these lipids from the *Nannochloropsis* cell. This review aims to provide an overview of the recent advances in EAD of the microalga *Nannochloropsis* to increase lipid extraction yield (also called lipid yield, oil yield or lipid extraction efficiency by other authors) and to provide guidelines and good practises for enzyme selection, pre-treatment, and post-treatments (Figure 1). These guidelines and good practises can be extrapolated to other oleaginous microalgae.

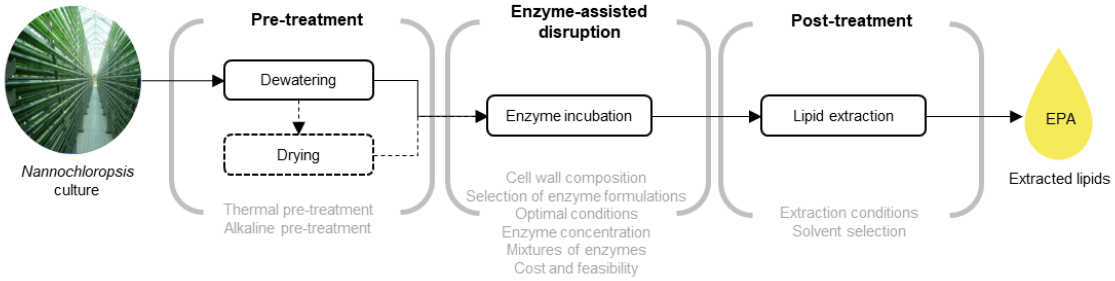

Figure 1 Processing of Nannochloropsis biomass from dilute culture to extracted lipids using enzyme-assisted disruption

Enzyme selection: cell wall composition is key

EAD is used to hydrolyse specific bonds in the CW constituents. Hence, a detailed understanding of the cell wall composition (CWC) and structure, as well as of the substrate and hydrolysis reaction specificity of the added enzyme(s) formulation (EF) is required. The *Nannochloropsis* CW (Figure 2) is composed

of a thinner, outer algaenan-based layer and a thicker cellulosic inner layer containing cellulose and hemicellulose [13]. The outer algaenan layer consists of highly saturated aliphatic material [13]. The main monosaccharide in the cellulosic layer is glucose (75-98%) [14–16]. Other monosaccharides originate from hemicellulose. Amino acids make up approximately 6% of the CW, indicating the presence of proteins [13]. *Nannochloropsis* CWC and thickness depends on the strain and the salinity of the cultivation media [16]. In addition, nitrogen limited cultivation conditions increase CW thickness due to an increase in cellulose content [14,17,18]. We suggest that one must determine the (monosaccharide) composition and the chemical bonds present in the CW, to select the appropriate EF which will specifically target and degrade CW components. Recent literature (Table 1) describing EAD of *Nannochloropsis* relies on a few articles (e.g. [13,19]) for their enzyme selection. All authors concluded that the applied EF increased the lipid extraction yield from *Nannochloropsis* (Table 2). However, there is no clear trend in the type and combinations of EF that are most successful in increasing the lipid extraction yield. A possible explanation for these variable results is the authors reliance on predetermined *Nannochloropsis* CWC which, as stated above, varies depending on strain and cultivation conditions. For example, Maffei et al. [20] combined cellulase with mannanase as hemicellulytic enzyme based on the results of Vieler et al. [19] who had shown that mannose is the second most abundant monosaccharide in the CW of *Nannochloropsis oceanica*. However, in the work of Bernaerts et al. [21], galactose is the second most abundant monosaccharide, followed by mannose and ribose in equal percentages. Therefore, we discourage reliance on previously reported CWC of *Nannochloropsis* for the selection of CW degrading EF. Rather we recommend determining the CWC using the monosaccharide composition and total protein or amino acid content e.g. as described by [21] and [13] respectively. Thereafter, this information can be used for the selection and subsequent interpretation of the mechanism causing the EAD. This approach should prevent unnecessary experimental set-ups containing EF that target components absent or present in minor quantities.

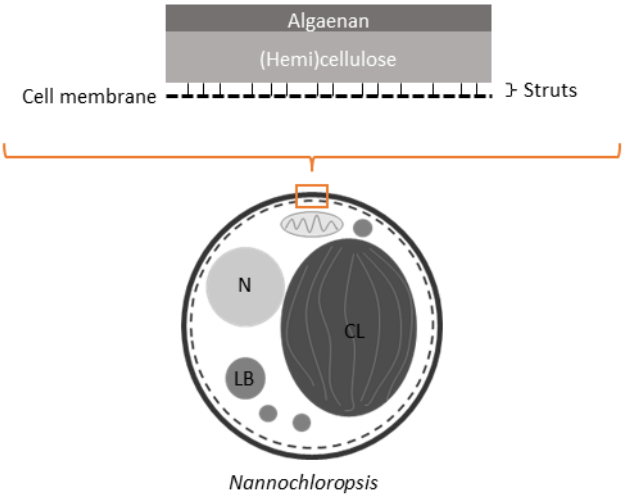

Figure 2 Schematic representation of the Nannochloropsis cell and cell wall, with N: nucleus, CL: chloroplast, LB: lipid body based on [13,15,17]

Enzyme selection: Enzyme formulation

To date, commercially available EF are used to weaken the *Nannochloropsis* CW. Usually, only the main enzymatic activity of these EFs is reported. The catalytic activity of pure enzymes is classified using EC numbers. Commercial EF are produced by micro-organisms and purified [22,23]. They are therefore often mixtures of several different EC enzymes. While some authors report the side activities of the EF used [24], this is only possible if the enzyme suppliers report the known side activities. These unknown or unreported enzymatic side activities may e.g., lead to undesired formation of free fatty acids (FFA) by lipase side activity. For instance, Blanco-Llamero et al. [25] observed an increase in FFA after ultrasound-assisted enzymatic pre-treatment compared to the control. Maffei et al. [20] observed polypeptide degradation or restructuring after treatment with (combinations) of cellulase and mannanase, indicating possible protease side activity. However, in both cases it was not established that these changes were caused by enzymatic side activity. It cannot be excluded that these changes were the result of e.g., endogenous lipases or proteases. Therefore, in an ideal case we suggest that anyone working with EF inform themselves about possible side activities by inquiring for information

with the EF supplier or by determining the side activity themselves. In addition, the experimental set-up itself should contain controls (blanks) in which the biomass is exposed to the same EAD conditions in the absence of enzymes to distinguish possible endogenous enzyme activities from the activities of added EF. Finally, the costs, feasibility, and availability of the EF must be considered. For instance, utilization of immobilized enzymes for CW disruption allows recovery and reuse of the enzymes, potentially enhancing economic viability and sustainability of the entire process [26].

Table 1 Pre-treatment, enzyme-assisted disruption conditions, enzyme(s), controls, post-treatment, and extraction solvent reported for the enzyme-assisted disruption of Nannochloropsis

Study (year)	*Nannochloropsis* biomass	Pre-treatment	Enzyme-assisted disruption					Post-treatment	
			Enzymes	pH modification	Temperature (°C)	Duration (h)	Control	Additional post-treatment	lipid extraction solvent
Zhao et al. (2022) [30]	N. *oceanica* powder	Not applied	Cellulase Laccase	Citric acid-sodium citrate buffer (pH 5.0)	50	24	Yes, not specified	Not applied	Ethanol-water (90% v/v)
Zhao et al. (2022) [31]	N. *oceanica* powder	Not applied	Cellulase Laccase Pectinase Mannanase Xylanase	Citric acid-sodium citrate buffer (pH 4.2 – 5.8)	40-60	1.5-24	Yes, not specified	Not applied	Ethanol-water (80% v/v)
Castéjon et al. (2022) [24]	Lyophilised N. *gaditana*	Not applied	Cellulase Carbohydrases Glucoamylase	Acetate buffer (pH 4.5)	50	1	Yes, a blank without enzymes	Ultrasound bath	Ethanol
Blanco-Llamero et al. (2021) [25]	Dry N. *gaditana*	Not applied	Carbohydrases Cellulase Protease	Sodium citrate buffer (pH 4.0- 5.0) pH 8.0, method not specified	35-55	0.25-24	Yes, a blank without enzymes	Ultrasound bath Ultrasound probe	Chloroform: methanol (2:1)
He et al. (2020) [33]	N. *oculata*	Not applied	Cellulase Papain Pectinase Hemicellulase	HCl (0.1M) (pH 5.5)	45	12	Yes, not specified	Not applied	Ethanol and dipotassium hydrogen phosphate
Qui et al. (2019) [32]	Wet *Nannochloropsis* sp.	Not applied	Cellulase Hemicellulase Papain pectinase	Phosphate buffer (pH 5.0)	50	4-12	Yes, not specified	Not applied	Tert-butanol and ammonium sulphate
Maffei et al (2018) [20]	Lyophilised *Nannochloropsis* sp. powder	Not applied	Cellulase Mannanase	pH 4.4, method not specified	53	24	Yes, control in pure water without enzymes	Not applied	Hexane:isopropanol (3:2)
Wu et al. (2017) [39]	*Nannochloropsis* sp.	Alkaline and thermal pre-treatment (pH 7.0-13.0)	Cellulase Protease Lysozyme Pectinase	Acetic acid (pH 3.5-6.0)	35-60	0.5-6	Not specified	Not applied	chloroform
Chen et al. (2017) [37]	*Nannochloropsis* sp.	Alkaline and thermal pre-treatment (pH 9.0)	Cellulase Pectinase Lysozyme Protease	Acetic acid (pH 3-7)	30-70	1-8	Yes, control not subjected to alkaline pre-treatment	Not applied	Ethanol and hexane
Chen et al. (2016) [34]	Wet *Nannochloropsis oceanica*	Thermal lysis	Cellulase Lipase Protease	pH 4-10, method not specified	37	5	Yes, not specified	Not applied	Aqueous surfactants, hexane, and chloroform:methanol (1:2)
Zuorro et al. (2016) [41]	Lyophilised *Nannochloropsis* sp.	Not applied	Cellulase Mannanase	pH 2-8, method not specified	15-75	0.5-4.5	Yes, control in pure water without enzymes	Not applied	Hexane:isopropanol (3:2)
Zuorro et al. (2016) [42]	Lyophilised *Nannochloropsis* sp.	Not applied	Lysozyme Cellulase Mannanase Galactanase Glucanase Xylanase	pH 5, method not specified	50	1	Yes, control in pure water without enzymes	Not applied	Hexane:isopropanol (3:2)
Zuorro et al. (2015) [44]	Lyophilised *Nannochloropsis* sp.	Not applied	Cellulase Mannanase	pH 2-10, method not specified	15-75	0.5-2.5	Not specified	Not applied	Hexane:isopropanol (3:2)

Optimal enzymatic incubation conditions

Enzymes catalyse reactions within limited ranges of conditions such as appropriate pH, temperature, and ionic strength. Changes in protein structure occur outside this range resulting in loss of the enzyme activity [27]. The EF optimal temperature and pH are stated on the product data sheet. These optima are however determined under optimal conditions for the hydrolysis of standard products such as carboxymethyl cellulose [28] or by using filter paper activity [29]. Due to the standardised substrate and nature of these methods, these optimal conditions may deviate from the optimal conditions for the *Nannochloropsis* CW. To ensure constant and optimal pH, the pH of the *Nannochloropsis* algal biomass solution has been modified using several different buffers (citric acid-sodium citrate [30,31], acetate buffer [24], phosphate buffer [32]) (Table 1). pH adjustment using only acids (as applied by e.g. [33]) does not guarantee constant pH during the enzymatic incubation. In the latter case, we advise researchers to document pH progression throughout the enzymatic incubation as a slight change in pH may increase or decrease the enzyme activity. In addition, combining several commercial EF is only reasonable if their pH and temperature optima are similar. The type of agitation, distribution of the microalgal material in the buffer medium (e.g., fully dispersed as single cells, or as agglomerates of multiple cells), and the viscosity of the medium also plays a role in the ability of the EF to reach the *Nannochloropsis* CW substrate.

Enzyme concentration

The SI unit of enzyme activity is the katal (mol s^{-1}), but in practise, it is reported in U (µmol min^{-1}) [27]. Several authors (Table 1 and Table 2) applied cellulase to disrupt the *Nannochloropsis* CW, but they did not always clearly report the enzyme activity and dosage. When a dosage is reported, the component that is added, in this case enzymes, is reported in the numerator, while the biomass amount is reported in the denominator. In the case of EAD of *Nannochloropsis*, the values in the nominator are however reported as mg protein [24], mg enzyme [25] or in U per unspecified volume of wet N. *oculata* biomass (18% DM) [33]. The denominator is reported on dry weight [34], on volume

144 of biomass slurry [30], or per gram (presumably on dry biomass) [20,31]. These varying ways of
145 reporting the enzyme dosage and the lack of reporting on total volume of used biomass slurry or mass
146 of dry biomass make comparisons between the different studies almost impossible. We therefore
147 strongly recommend the requirement to report the enzyme activity, as well as enzyme dose and mass
148 or volume of the biomass. This requirement should ensure the feasibility of replication of experiments.
149

Table 2 Overview of enzyme (combinations) and enzyme-assisted disruption conditions leading to optimal lipid extraction yield

Study (year)	Enzymes	Enzyme dose and ratio	pH	Temperature (°C)	Duration (h)	Lipid extraction yield
Zhao et al. (2022) [30]	Cellulase Laccase	Cellulase 500 U/ml biomass slurry Laccase 0.24 U/ml biomass slurry	5.0	50	24	~26.3 wt% on microalgae powder
Zhao et al. (2022) [31]	Cellulase Laccase	21 mg enzymes /g biomass powder laccase:cellulase (1:2.5)	5.0	45	6	26.9 ± 0.2 wt% on microalgae powder
Castéjon et al. (2022) [24]	Glucoamylase	15 mg protein/g biomass	4.5	50	1	25.7 ± 0.5 wt% on dry biomass
Blanco-Llamero et al. (2021) [25]	Carbohydrases Cellulase Protease	46 mg enzyme/g biomass	5	55	6	28.9 wt% on dry biomass
He et al. (2020) [33]	Cellulase Papain Pectinase Hemicellulase	200 U of each enzyme/ unspecified volume of wet biomass	5.5	45	12	22.4 wt% on biomass
Qui et al. (2019) [32]	Cellulase	66.6 U/ g microalgal powder	5	50	12	24.84 ± 1.85 % of total fatty acids in the biomass
Maffei et al. (2018) [20]	Cellulase Mannanase	Cellulase 13.8 mg/g biomass Mannanase 1.5 mg/g biomass	4.4	53	24	73% of total lipids
Wu et al. (2017) [39]	Cellulase Lysozyme Pectinase Protease	Per enzyme: 200 IU/g dry biomass	4	50	0.5	90% of total lipids
Chen et al. (2017) [37]	Cellulase Lysozyme	Algae/enzyme mass ratio of 8:1 Mass ratio cellulase: lysozyme (2:1)	4.0	50	5	22.18 ± 0.26 wt% on dry weight of microalgae
Chen et al. (2016) [34]	Cellulase	1.3 %DW	5	37	5	88.3% of total lipids
Zuorro et al. (2016) [41]	Cellulase Mannanase	Cellulase: 13.8 mg/g dry biomass Mannanase 1.5 mg/g dry biomass	4.4	53	3.5	88% of total lipids
Zuorro et al. (2016) [42]	Cellulase Mannanase Glucanase	5 mg/g of total enzymes. With weight fraction of 0.187 cellulase, 0.156 glucanase and 0.657 mannanase	5	50	1	71.6% of total lipids
Zuorro et al. (2015) [44]	Cellulase Mannanase	1.3 mg enzymes /g dry biomass Mannanase:cellulase (9:1)	5	36	1.5	35.7 ± 2.5 wt% on dry biomass

Mixtures of enzyme formulations and synergy

Due to the diverse composition of the *Nannochloropsis* CW, combinations of EF have often been used to disrupt the CW (Table 2). For example, Blanco-Llamero et al. [25] found that the use of only cellulase

157 does not increase the lipid extraction yield compared to control extractions. However, Zhao et al.
158 [30,31] showed that cellulase alone increases the lipid extraction yield, but the highest lipid extraction
159 yield was obtained when cellulase was combined with laccase. He et al. [33] and Maffei et al. [20] also
160 concluded that cellulase alone can increase the lipid extraction yield, but combining cellulose with
161 papain, hemicellulase and pectinase, or with mannanase respectively, increases the lipid extraction
162 yield. This increase in lipid extraction yield is attributed to synergistic effects between the enzymes
163 [30,31,34]. A synergy of enzymes is 'the ratio of the rate or yield of product release by enzymes when
164 used at the same time to the sum of rate or yield of these products when the enzymes are used
165 separately in the same amounts as they were employed in the mixture' [35]. A synergy observation
166 during EAD of *Nannochloropsis* CW is however often based on an increase in lipid extraction yield
167 [31,33]. This is often an indirect method for measuring the reaction that is catalysed by the enzymes.
168 When claims regarding synergy are made, direct methods measuring the targeted product release are
169 advised. For instance, when (hemi)cellulases are used, a more correct method to claim synergy would
170 be to measure the release of specific saccharides. We conclude that statements about the synergy of
171 enzymes should thus be avoided if not substantiated with measurements of the targeted product
172 release. In contrast, some combinations of enzymes did not result in increased lipid extraction yield
173 and so-called 'synergy'. Castejón and Marko [24] observed that a mixture of three commercially
174 available EF composed of several carbohydrases, cellulase and glucoamylase respectively, does not
175 significantly increase the lipid extraction yield compared to control extractions, while the individual EF
176 were able to increase the lipid extraction yield. The latter results indicate a possible inhibitory
177 interaction between the three commercially available EF. It has been reported that cellobiose inhibits
178 cellulase activity and glucose can inhibit ß-glucosidase [36].
179 In addition, simultaneous or sequential application of enzymes to the *Nannochloropsis* CW should be
180 considered when applying multiple EF. If proteases, as e.g. in the work of Chen et al. (2017) [37], were
181 added to combinations of pectinase, lysozyme and cellulase, the lipid extraction yield declined. The

authors attributed this to unfavourable pH conditions, however it could also be possible that the protease is acting on the pectinase, lysozyme and cellulase itself as they are proteins.

Pre-treatment

Pre-treatment of the biomass includes any step in the microalgal processing chain between the stage where the dilute *Nannochloropsis* culture is ready for harvest and the start of the enzymatic treatment (Figure 1). Pre-treatment of lignocellulose-rich biomass before enzymatic incubation is a well-known method to remove lignin and increase the susceptibility and accessibility of the enzymes towards the cellulose biomass [38]. Analogous, pre-treatments of *Nannochloropsis* biomass before enzymatic incubation have been applied (Table 1). Chen et al. (2017) [37] performed simultaneous alkaline and thermal pre-treatment at pH 9.0 for 5h at 80 ^{o}C before enzyme treatment. They showed that the alkaline pre-treatment in combination with enzymatic incubation led to the extraction of more than double the amount of fatty acids. However, they did not report the effect of the alkaline pre-treatment. It can therefore not be excluded that the attributed effect can be allocated to the alkaline pre-treatment itself. A similar remark can be made about the work of Wu et al. [39], who also observed the highest lipid extraction yield when alkaline pre-treatment was applied before EAD without reporting the effect of the individual treatments. Chen et al (2016) [34], observed that thermal lysis before enzymatic cell disruption increased the lipid extraction yield compared to either method alone. We advise that when applying EAD, a pre-treatment of *Nannochloropsis* can be used, but experimental designs should allow for distinction between the influence of the pre-treatment and the influence of the EAD on lipid extraction yield.

Post-treatment

After EAD, the lipids must be extracted from the *Nannochloropsis* biomass. The extraction solvent used greatly influences the type and quantity of lipids extracted [40]. In addition, the extraction solvent should be selective for the extraction of lipids and have minimal co-extraction of ‘impurities’ such as proteins and polysaccharides as the lipid extraction yield is often determined gravimetrically. For

instance (Table 1), Zhao et al. [30,31] used ethanol to extract lipids from *Nannochloropsis*, while this solvent co-extracts many non-lipid components [40]. With hexane/isopropanol, used by Maffei et al. and Zuorro et al. [20,41,42], co-extraction is less pronounced [40]. Optimised protocols using chloroform and methanol that can extract all lipids from the microalgal biomass directly should be avoided as they do not give insight in the increase in lipid extraction yield caused by EAD. However, these protocols should be used to analytically determine the total lipid content in the biomass, so that the increase in lipid extraction yield by EAD can be compared relative to the total lipid content, which aids interpretation of the effectiveness of EAD [43]. Maffei et al. and Zuorro et al. [20,41,42] apply the latter, but others (e.g. [24,25,30,31]) define the lipid extraction yield based on mass of the biomass. The use of different extraction solvents (Table 1) which themselves influences the type and total mass of lipids extracted (Table 2), and the varying definitions of 'lipid extraction yield', complicate comparing the effectiveness of the EAD between various studies. Finally, it is recommended to extract the lipids under temperature and time conditions that do not affect the lipids themselves.

Conclusion and perspective

EAD of *Nannochloropsis* can improve the lipid extraction yield. Many different (mixtures of) EF at varying pH, temperatures and treatment times have been applied. No standard solvent is used to extract the lipids to determine the EAD efficiency. In addition, appropriate control extractions without enzymes are often lacking or not well defined. For progress in the field of EAD, a thorough selection of appropriate EF based on CW composition should be implemented. Side activities of the EF, especially related to lipid degradation, should be investigated. Activity and dosage of the enzymes should be reported to ensure replication. Optimal conditions for EAD should be sought for. When a pre-treatment method is applied, the individual contribution of the pre-treatment should be distinguishable from the EAD effect on lipid extraction yield. We believe that following these recommendations will allow researchers to make strides in this field.

Acknowledgements

This work was supported by the EffSep project, VLAIO (c-SBO project HBC.2019.0012). The authors sincerely thank Dr. Elaine Kozma for revising the language of the manuscript.

Conflict of interest statement

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

Data availability

No data was used for the research described in this paper.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest.

1. • Torres-Tiji Y, Fields FJ, Mayfield SP: **Microalgae as a future food source**. *Biotechnol Adv* 2020, **41**, Article 107536.

recent review providing an overview of the nutritional composition of microalgae. It specifies the strategies that can be used to improve the organoleptic properties, nutritional content, productivity, and the production process of microalgae as new food crop.

2. Nethravathy MU, Mehar JG, Mudliar SN, Shekh AY: **Recent Advances in Microalgal Bioactives for Food, Feed, and Healthcare Products: Commercial Potential, Market Space, and Sustainability**. *Compr Rev Food Sci Food Saf* 2019, **18**:1882–1897.

3. • Bernaerts TMM, Verstreken H, Dejonghe C, Gheysen L, Foubert I, Grauwet T, Van Loey AM: **Cell disruption of *Nannochloropsis* sp. improves in vitro bioaccessibility of carotenoids and ω3-LC-PUFA**. *J Funct Foods* 2020, **65**, Article 103770.

Research article indicating the importance of cell disruption of *Nannochloropsis* for increased lipid digestibillity and bioaccessibility *in vitro.*

4. • Zhou J, Wang M, Saraiva JA, Martins AP, Pinto CA, Prieto MA, Simal-Gandara J, Cao H, Xiao J, Barba FJ: **Extraction of lipids from microalgae using classical and innovative approaches**. *Food Chem* 2022, **348**, Article 132236.

Review article providing an overview of traditional and emerging lipid extraction approaches of microalgae and possible applications of extracted microalgal lipids in food, feed, fuel, and cosmetics.

5. •• Nitsos C, Filali R, Taidi B, Lemaire J: **Current and novel approaches to downstream processing of microalgae : A review**. *Biotechnol Adv* 2020, **45**, Article 107650.

Review article discussing microalgae in the context of biorefinery, highlighting the importance of the microalgal cell wall composition. It provides an overview of microalgae harvesting and disruption methods and the associated energy use.

6. Sivakumar R, Sachin S, Priyadarshini R, Ghosh S: **Sustainable production of eicosapentaenoic acid-rich oil from microalgae: Towards an algal biorefinery**. *J Appl Microbiol* 2022, **132**:4170–4185.

7. • Russell C, Rodriguez C, Yaseen M: **Microalgae for lipid production: Cultivation, extraction & detection**. *Algal Res* 2022, **66**, Article 102765.

Review article discussing the cultivation, disruption and lipid extraction of oleagenous microalgae. It provides detailed information on (organic) solvent extraction and detection and quantification of lipids and highlights the influence of morphological differences between microalgal species.

8. Fawley MW, Jameson I, Fawley KP: **The phylogeny of the genus *Nannochloropsis* (Monodopsidaceae, Eustigmatophyceae), with descriptions of *N. australis sp. nov.* and**

Microchloropsis gen.nov. *Phycologia* 2015, **54**:545–552.

9. Calder PC: **Chapter 2 - Health benefits of omega-3 fatty acids**. In *Omega-3 Delivery Systems*. Edited by García-Moreno PJ, Jacobsen C, Moltke Sørensen A-D, Yesiltas B. Academic Press; 2021:25–53.

10. •• Zanella L, Vianello F: **Microalgae of the genus *Nannochloropsis*: Chemical composition and functional implications for human nutrition**. *J Funct Foods* 2020, **68**, Article 103919.

Detailed review of the composition, toxicology, safety and nutritional value of *Nannochloropsis* sp.

11. Ljubic A, Jacobsen C, Holdt SL, Jakobsen J: **Microalgae *Nannochloropsis oceanica* as a future new natural source of vitamin D3**. *Food Chem* 2020, **320**, Article 126627.

12. Xu Y: **Biochemistry and Biotechnology of Lipid Accumulation in the Microalga *Nannochloropsis oceanica***. *J Agric Food Chem* 2022, **70**:11500–11509.

13. Scholz MJ, Weiss TL, Jinkerson RE, Jing J, Roth R, Goodenough U, Posewitz MC, Gerken HG: **Ultrastructure and composition of the *Nannochloropsis gaditana* cell wall**. *Eukaryot Cell* 2014, **13**:1450–1464.

14. Jeong SW, Nam SW, HwangBo K, Jeong WJ, Jeong B, Chang YK, Park Y-I: **Transcriptional Regulation of Cellulose Biosynthesis during the Early Phase of Nitrogen Deprivation in *Nannochloropsis salina***. *Sci Rep* 2017, **7**, Article 5264.

15. Simionato D, Block MA, La Rocca N, Jouhet J, Maréchal E, Finazzi G, Morosinotto T: **The response of *Nannochloropsis gaditana* to nitrogen starvation includes de novo biosynthesis of triacylglycerols, a decrease of chloroplast galactolipids, and reorganization of the photosynthetic apparatus**. *Eukaryot Cell* 2013, **12**:665–676.

16. Beacham TA, Bradley C, White DA, Bond P, Ali ST: **Lipid productivity and cell wall**

ultrastructure of six strains of *Nannochloropsis* : Implications for biofuel production and downstream processing. *Algal Res* 2014, **6**, **Part A**:64–69.

17. Halim R, Hill DRA, Hanssen E, Webley PA, Martin GJO: **Thermally coupled dark-anoxia incubation: A platform technology to induce auto-fermentation and thus cell-wall thinning in both nitrogen-replete and nitrogen-deplete *Nannochloropsis* slurries**. *Bioresour Technol* 2019, **290,** Article 121769.

18. Roncaglia B, Papini A, Chini Zittelli G, Rodolfi L, Tredici MR: **Cell wall and organelle modifications during nitrogen starvation in *Nannochloropsis oceanica* F&M-M24**. *J Appl Phycol* 2021, **33**:2069–2080.

19. Vieler A, Wu G, Tsai C-H, Bullard B, Cornish AJ, Harvey C, Reca I-B, Thornburg C, Achawanantakun R, Buehl CJ, et al.: **Genome, Functional Gene Annotation, and Nuclear Transformation of the Heterokont Oleaginous Alga *Nannochloropsis oceanica* CCMP1779**. *PLOS Genet* 2012, **8**, Article e1003064.

20. Maffei G, Bracciale MP, Broggi A, Zuorro A, Santarelli ML, Lavecchia R: **Effect of an enzymatic treatment with cellulase and mannanase on the structural properties of *Nannochloropsis* microalgae**. *Bioresour Technol* 2018, **249**:592–598.

21. Bernaerts TMM, Gheysen L, Kyomugasho C, Jamsazzadeh Kermani Z, Vandionant S, Foubert I, Hendrickx ME, Van Loey AM: **Comparison of microalgal biomasses as functional food ingredients: Focus on the composition of cell wall related polysaccharides**. *Algal Res* 2018, **32**:150–161.

22. Patel AK, Singhania RR, Pandey A: **Chapter 2 - Production, Purification, and Application of Microbial Enzymes**. In *Biotechnology of Microbial Enzymes*. Edited by Brahmachari G. Academic Press; 2017:13–41.

23. Srivastava N: **Chapter 43 - Production of food-processing enzymes from recombinant**

microorganisms. In *Enzymes in Food Biotechnology*. Edited by Kuddus M. Academic Press; 2019:739–767.

24. Castejón N, Marko D: **Fatty Acid Composition and Cytotoxic Activity of Lipid Extracts from *Nannochloropsis gaditana* Produced by Green Technologies**. *Molecules* 2022, **27**, Article 3710.

25. Blanco-Llamero C, García-García P, Señoráns FJ: **Combination of synergic enzymes and ultrasounds as an effective pretreatment process to break microalgal cell wall and enhance algal oil extraction**. *Foods* 2021, **10**, Article 1982.

26. Nadar SS, Rao P, Rathod VK: **Enzyme assisted extraction of biomolecules as an approach to novel extraction technology: A review**. *Food Res Int* 2018, **108**:309–330.

27. Belitz H-D, Grosch W, Schieberle P: **Chapter 2 - Enzymes**. In *Food Chemistry*. Edited by Belitz H-D, Grosch W, Schieberle P. Springer Berlin, Heidelberg; 2009:93–157.

28. Siccama JW, Oudejans R, Zhang L, Kabel MA, Schutyser MAI: **Steering the formation of cellobiose and oligosaccharides during enzymatic hydrolysis of asparagus fibre**. *LWT - Food Sci Technol* 2022, **160**, Article 113273.

29. Abbas Al Talebi Z, Saad Al-Kawaz H, Kadhim Mahdi R, Al-Hassnawi AT, Hassan Alta'ee A, Hadwan AM, Abbas Khudhair D, Hussein Hadwan M: **An optimized protocol for estimating cellulase activity in biological samples**. *Anal Biochem* 2022, **655**, Article 114860.

30. Zhao K, Zhang M, Tian H, Lei F, He D, Zheng J, Zhang L: **Effects of Structural and Compositional Changes of *Nanochloropsis oceania* after Enzyme Treatment on EPA-Rich lipids extraction**. *Mar Drugs* 2022, **20**, Article 160.

31. Zhao K, Zhang L, Zhang M, Tian H, He D, Zheng J: **Response Surface Optimization of Enzyme Pretreatment Improves Yield of Ethanol-Extracted Lipids from *Nannochloropsis oceanica***. *Eur J Lipid Sci Technol* 2022, **124**, Article 2100200.

32. Qiu C, He Y, Huang Z, Li S, Huang J, Wang M, Chen B: **Lipid extraction from wet *Nannochloropsis* biomass via enzyme-assisted three phase partitioning**. *Bioresour Technol* 2019, **284**:381–390.

33. He Y, Zhang B, Guo S, Guo Z, Chen B, Wang M: **Sustainable biodiesel production from the green microalgae *Nannochloropsis*: Novel integrated processes from cultivation to enzyme-assisted extraction and ethanolysis of lipids**. *Energy Convers Manag* 2020, **209**, Article 112618.

34. Chen L, Li R, Ren X, Liu T: **Improved aqueous extraction of microalgal lipid by combined enzymatic and thermal lysis from wet biomass of *Nannochloropsis oceanica***. *Bioresour Technol* 2016, **214**:138–143.

35. Kumar R, Wyman CE: **Effects of cellulase and xylanase enzymes on the deconstruction of solids from pretreatment of poplar by leading technologies**. *Biotechnol Prog* 2009, **25**:302–314.

36. Sun C, Meng X, Sun F, Zhang J, Tu M, Chang J, Reungsang A, Xia A, Ragauskas AJ: **Advances and perspectives on mass transfer and enzymatic hydrolysis in the enzyme-mediated lignocellulosic biorefinery : A review**. *Biotechnol Adv* 2023, **62**, Article 108059.

37. Chen Q, Liu D, Wu C, Xu A, Xia W, Wang Z, Wen F, Yu D: **Influence of a facile pretreatment process on lipid extraction from: *Nannochloropsis* sp. through an enzymatic hydrolysis reaction**. *RSC Adv* 2017, **7**:53270–53277.

38. Van Dyk JS, Pletschke BI: **A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-Factors affecting enzymes, conversion and synergy**. *Biotechnol Adv* 2012, **30**:1458–1480.

39. Wu C, Xiao Y, Lin W, Li J, Zhang S, Zhu J, Rong J: **Aqueous enzymatic process for cell wall degradation and lipid extraction from *Nannochloropsis* sp.** *Bioresour Technol* 2017, **223**:312–

316.

40. Ryckebosch E, Bermúdez SPC, Termote-Verhalle R, Bruneel C, Muylaert K, Parra-Saldivar R, Foubert I: **Influence of extraction solvent system on the extractability of lipid components from the biomass of *Nannochloropsis gaditana***. *J Appl Phycol* 2014, **26**:1501–1510.

41. Zuorro A, Maffei G, Lavecchia R: **Optimization of enzyme-assisted lipid extraction from *Nannochloropsis* microalgae**. *J Taiwan Inst Chem Eng* 2016, **67**:106–114.

42. Zuorro A, Miglietta S, Familiari G, Lavecchia R: **Enhanced lipid recovery from *Nannochloropsis* microalgae by treatment with optimized cell wall degrading enzyme mixtures**. *Bioresour Technol* 2016, **212**:35–41.

43. Ryckebosch E, Muylaert K, Foubert I: **Optimization of an analytical procedure for extraction of lipids from microalgae**. *JAOCS, J Am Oil Chem Soc* 2012, **89**:189–198.

44. Zuorro A, Lavecchia R, Maffei G, Marra F, Miglietta S, Petrangeli A, Familiari G, Valente T: **Enhanced lipid extraction from unbroken microalgal cells using enzymes**. *Chem Eng Trans* 2015, **43**:211–216.