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

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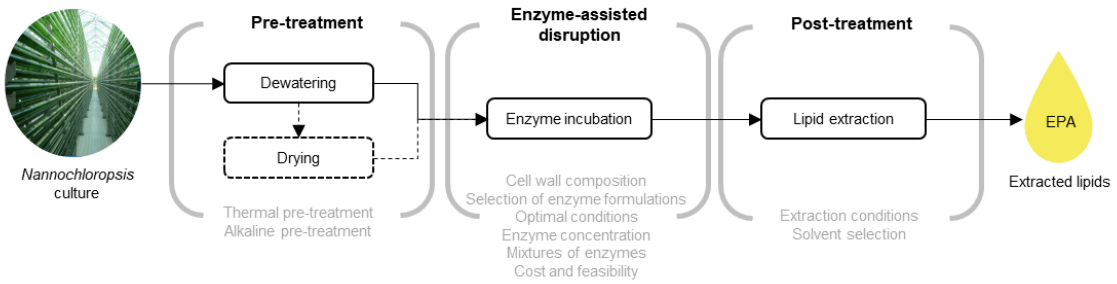
Abstract

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.

44 In this review, the oleaginous marine microalga *Nannochloropsis* (Eustigmatophycea) is used as case
45 study of EAD to increase lipid extraction. Note that recently some *Nannochloropsis* species have been
46 redefined into a new genus, *Microchloropsis* [8]. In this review we include both *Nannochloropsis* and
47 taxa originally assigned to *Nannochloropsis* that have now been re-assigned to *Microchloropsis*.
48 Hereafter, we refer to this ensemble as *Nannochloropsis*. *Nannochloropsis* has received interest for
49 (potential) application in food due to its ability to synthesise nutritionally valuable omega-3 poly-
50 unsaturated fatty acids, such as eicosapentaenoic acid (C20:5n-3) [9], carotenoids, α-tocopherol [10]
51 and vitamin D_3 [11]. This genus also functions as a model organism for algal biofuel production as it can
52 accumulate up to ~70% of its dry weight as lipids [12]. However, the lipids in *Nannochloropsis* are
53 surrounded by an especially rigid CW acting as a barrier for their extraction. This necessitates the use
54 of a cell disruption technique that liberates these lipids from the *Nannochloropsis* cell. This review aims
55 to provide an overview of the recent advances in EAD of the microalga *Nannochloropsis* to increase
56 lipid extraction yield (also called lipid yield, oil yield or lipid extraction efficiency by other authors) and
57 to provide guidelines and good practises for enzyme selection, pre-treatment, and post-treatments
58 (Figure 1). These guidelines and good practises can be extrapolated to other oleaginous microalgae.
59



60

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

62 Enzyme selection: cell wall composition is key

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

66 of a thinner, outer algaenan-based layer and a thicker cellulosic inner layer containing cellulose and
67 hemicellulose [13]. The outer algaenan layer consists of highly saturated aliphatic material [13]. The
68 main monosaccharide in the cellulosic layer is glucose (75-98%) [14–16]. Other monosaccharides
69 originate from hemicellulose. Amino acids make up approximately 6% of the CW, indicating the
70 presence of proteins [13]. *Nannochloropsis* CWC and thickness depends on the strain and the salinity
71 of the cultivation media [16]. In addition, nitrogen limited cultivation conditions increase CW thickness
72 due to an increase in cellulose content [14,17,18]. We suggest that one must determine the
73 (monosaccharide) composition and the chemical bonds present in the CW, to select the appropriate
74 EF which will specifically target and degrade CW components. Recent literature (Table 1) describing
75 EAD of *Nannochloropsis* relies on a few articles (e.g. [13,19]) for their enzyme selection. All authors
76 concluded that the applied EF increased the lipid extraction yield from *Nannochloropsis* (Table 2).
77 However, there is no clear trend in the type and combinations of EF that are most successful in
78 increasing the lipid extraction yield. A possible explanation for these variable results is the authors
79 reliance on predetermined *Nannochloropsis* CWC which, as stated above, varies depending on strain
80 and cultivation conditions. For example, Maffei et al. [20] combined cellulase with mannanase as
81 hemicellulytic enzyme based on the results of Vieler et al. [19] who had shown that mannose is the
82 second most abundant monosaccharide in the CW of *Nannochloropsis oceanica*. However, in the work
83 of Bernaerts et al. [21], galactose is the second most abundant monosaccharide, followed by mannose
84 and ribose in equal percentages. Therefore, we discourage reliance on previously reported CWC of
85 *Nannochloropsis* for the selection of CW degrading EF. Rather we recommend determining the CWC
86 using the monosaccharide composition and total protein or amino acid content e.g. as described by
87 [21] and [13] respectively. Thereafter, this information can be used for the selection and subsequent
88 interpretation of the mechanism causing the EAD. This approach should prevent unnecessary
89 experimental set-ups containing EF that target components absent or present in minor quantities.

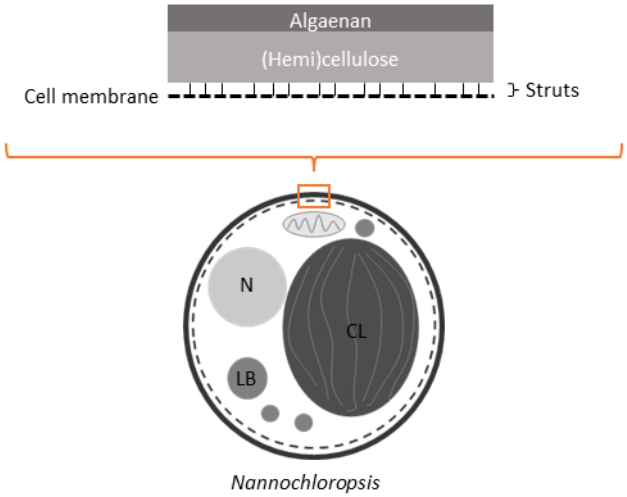


Figure 2 Schematic representation of the Nannochloropsis cell and cell wall, with N: nucleus, CL: chloroplast, LB: lipid body based on [13,15,17]

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)

118 ## Optimal enzymatic incubation conditions

119 Enzymes catalyse reactions within limited ranges of conditions such as appropriate pH, temperature,
120 and ionic strength. Changes in protein structure occur outside this range resulting in loss of the enzyme
121 activity [27]. The EF optimal temperature and pH are stated on the product data sheet. These optima
122 are however determined under optimal conditions for the hydrolysis of standard products such as
123 carboxymethyl cellulose [28] or by using filter paper activity [29]. Due to the standardised substrate
124 and nature of these methods, these optimal conditions may deviate from the optimal conditions for
125 the *Nannochloropsis* CW. To ensure constant and optimal pH, the pH of the *Nannochloropsis* algal
126 biomass solution has been modified using several different buffers (citric acid-sodium citrate [30,31],
127 acetate buffer [24], phosphate buffer [32]) (Table 1). pH adjustment using only acids (as applied by e.g.
128 [33]) does not guarantee constant pH during the enzymatic incubation. In the latter case, we advise
129 researchers to document pH progression throughout the enzymatic incubation as a slight change in pH
130 may increase or decrease the enzyme activity. In addition, combining several commercial EF is only
131 reasonable if their pH and temperature optima are similar. The type of agitation, distribution of the
132 microalgal material in the buffer medium (e.g., fully dispersed as single cells, or as agglomerates of
133 multiple cells), and the viscosity of the medium also plays a role in the ability of the EF to reach the
134 *Nannochloropsis* CW substrate.

135

136 ## Enzyme concentration

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

182 authors attributed this to unfavourable pH conditions, however it could also be possible that the
183 protease is acting on the pectinase, lysozyme and cellulase itself as they are proteins.
184

185 Pre-treatment

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

203 Post-treatment

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

208 instance (Table 1), Zhao et al. [30,31] used ethanol to extract lipids from *Nannochloropsis*, while this
209 solvent co-extracts many non-lipid components [40]. With hexane/isopropanol, used by Maffei et al.
210 and Zuorro et al. [20,41,42], co-extraction is less pronounced [40]. Optimised protocols using
211 chloroform and methanol that can extract all lipids from the microalgal biomass directly should be
212 avoided as they do not give insight in the increase in lipid extraction yield caused by EAD. However,
213 these protocols should be used to analytically determine the total lipid content in the biomass, so that
214 the increase in lipid extraction yield by EAD can be compared relative to the total lipid content, which
215 aids interpretation of the effectiveness of EAD [43]. Maffei et al. and Zuorro et al. [20,41,42] apply the
216 latter, but others (e.g. [24,25,30,31]) define the lipid extraction yield based on mass of the biomass.
217 The use of different extraction solvents (Table 1) which themselves influences the type and total mass
218 of lipids extracted (Table 2), and the varying definitions of 'lipid extraction yield', complicate comparing
219 the effectiveness of the EAD between various studies. Finally, it is recommended to extract the lipids
220 under temperature and time conditions that do not affect the lipids themselves.
221

222 Conclusion and perspective

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

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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.

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