



Neochloris oleoabundans from nature to industry: a comprehensive review

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Abstract Microalgae technology has been extensively studied during the last two decades. Thousands of species were isolated, and few are currently used in the market for multiple purposes. The current comprehensive review focuses on a promising species named *Neochloris oleoabundans*. It encompasses a historical overview of the species followed by a detailed description of its taxonomy, ecophysiology and morphology. Furthermore, a thorough screening is conducted to outline the production conditions employed to grow the microalga. In addition, multiple cell disruption and fractionation methods previously applied on *N. oleoabundans* are described and discussed together with the value chain creation after applying all the unit operations.

Keywords *Neochloris Oleoabundans* · Cell wall · Morphology · Cell disruption · Fractionation · Biorefinery

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1 Introduction

Microalgae have played a capital role during the process of evolution of life on earth. About 3.7 billion years ago, the Earth's atmosphere was composed of water vapor, ammonia, hydrogen, CO₂ and methane gas. Hence, an atmosphere that is unlikely to support any kind of life. Fossil records indicated that a microscopic organism emerged in the primordial soup (theory of the origin of life on earth) and initiated the extraordinary process of photosynthesis, and released the oxygen molecule to the atmosphere. From that moment, algal cells had begun their three billion years journey toward a drastic atmospheric transformation from the harsh environment—that does not sustain life—to an oxygen-rich atmosphere that gave birth to plants during the Paleozoic Era 450 million years ago (Chapman and Waters 2002; Safi et al. 2014; Avagyan 2021). The journey was full of obstacles due to the brutal environment in which these species were evolving. Therefore, the first algae cells evolved and diversified millions of times in order to adapt, thrive and survive. Furthermore, according to paleobotanist Russell Chapman, “*the first algae that managed to gain residence on terra firma must have come from freshwater, not the sea*”. Chapman also said, “*even though four distinct types of algae managed to come ashore, only one of them evolved enough complexity to eventually cover the land with vegetation, what we*

now call trees, shrubs, flowers, and grass” (Daily Mail 2001).

Despite the lack of major breakthroughs during the last few years, microalgal technology is still gaining interest every year, and due to their large diversity, it is plausible to provide a detailed overview on the promising species. Microalgae represent a large diversity that accounts for more than one million species (Barsanti and Gualtieri 2010), among them an interesting species called *Ettlia oleoabundans*, also known as *N. oleoabundans* (Komárek et al. 1989). It is a unicellular freshwater green species (Gatenby et al. 2003) that belongs to the class of Chlorophyceae.

So far, available reviews focused on microalgae in general, which provide limited information on the potential of each specific species. Only one review considered a detailed overview on a well-known species called *Chlorella vulgaris* (Safi et al. 2014). Therefore, the following review provides an in-depth overview of *N. oleoabundans* by describing its morphology, taxonomy, biochemical composition, eco-physiology and also explains the reproductive process of the cells. Furthermore, it highlights the applicative potential of *N. oleoabundans* focusing on production methods and other unit operations applied on this species to fractionate its valuable components.

2 Taxonomy

Between 1958 and 1960, seven new *Neochloris* species were described based on morphological attributes, cell dimensions, cell wall thickening, number of nuclei, position of pyrenoid and the kind of zoospores produced (Arce and Bold 1958; Bold 1958; Herndon 1958). Subsequently, Archibald (1973), described five new *Neochloris* species and provided a morphological key to all species (Archibald 1973). *N. oleoabundans* was first isolated sometime between 1958–1962 from the top of a sand dune (2° N; 55° E) in Rub al Khali in Saudi Arabia and named by Bold and Chantanachat (1962). It displayed the following characters: spherical cells, with a variable size, with a thin and smooth cell wall, single nucleus, parietal and cup-like chloroplast, two elongate pyrenoids, ovoid zoospores with two isokont flagella. Moreover, the authors reported that 3-weeks old cultures produced a considerable amount of oil droplets that coalesced in a single large drop during the

following weeks, thereby confining the chloroplast at one pole of the cell.

The use of ultrastructural and molecular characterisation, together with the continuous description of new taxa, has led to a deep taxonomical revision of many genera, including *Neochloris*. Starr (1955) erected the new genus *Neochloris*, which is morphologically close to *Chlorococcum*, due to the following diagnostic features: the occurrence of naked zoospores with isokont flagella, a hollow spherical chloroplast, and the presence of at least one pyrenoid in the chloroplast of vegetative cells. The type species, *Neochloris aquatica*, isolated from an aquarium of Indiana University, was multinucleate and showed an eccentric position of the pyrenoid (Starr 1957).

Its name was changed by Ríří Komárek in 1989 after conducting advanced taxonomic studies which led to its classification into a new special genus named *Ettlia* (zoospore Chlorellales). The genus *Neochloris* was subsequently split in two by Komárek (1989) who maintained the multinucleate species in *Neochloris*, transferring the uninucleate species in the new genus *Ettlia*. In the same year, Watanabe and Floyd (1989) proposed a taxonomic treatment of the genus, that was based on comparative ultrastructure, ending in three groups (Watanabe and Floyd 1989). The authors did not include in the study *N. oleoabundans*, but erected the new genus *Chlorococcopsis* with two species *Neochloris wimmeri* and *Neochloris minuta* whose morphological and ultrastructural characters are closely related to *N. oleoabundans* (Watanabe and Floyd 1989). Subsequently, Deason et al. (1991) tried to unify the taxonomic treatments proposed by Komárek (1989) and Watanabe and Floyd (1989), suggesting a different circumscription of the genera *Neochloris* and *Ettlia*, and acknowledging the priority of the name *Ettlia* over *Chlorococcopsis*, that was abandoned (Deason et al. 1991).

However, the phylogenetic relationships of the *Ettlia* species have been analysed on the basis of 18S rDNA (Pegg et al. 2015). Results have shown that if *E. carotina* is the typical species of the genus, the other three taxa included in *Ettlia* should be assigned to other genera (Pegg et al. 2015). *E. oleoabundans* is clearly separated from the other *Ettlia* species and is in a closer phylogenetic relation with taxa belonging to Micractinium and Actinastrum, members of family *Chlorellaceae* (*Chlorellales*, *Trebouxiophyceae*). Given these results, a taxonomic reassignment of *E.*

oleoabundans was required. Indeed, the most common name used nowadays in the literature is *N. oleoabundans* (UTEX 1185) instead of *Ettlia oleoabundans* (UTEX 1185) (Gatenby et al. 2003; Baldissierotto et al. 2014; Castro-Puyana et al. 2013; Li et al. 2008; Morales-Sánchez et al. 2013; Murray et al. 2011; Villa et al. 2014; Wang and Lan 2011).

3 Cell morphology

Cell morphology changes with respect to growth conditions applied for cell growth. In general, *N. oleoabundans* has a spherical shape with a diameter of 3–6 µm (Chantanachat and Bold 1962; Baldissierotto et al. 2014). The large part of its cytoplasm is occupied by a cup-shaped parietal chloroplast that embeds the thylakoids and a large pyrenoid. The latter is surrounded by a shell of starch granules, and it is crossed by one or two thylakoids (Baldissierotto et al. 2014; Giovanardi et al. 2013). The pyrenoid contains high levels of Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) and is the centre of carbon dioxide fixation (Safi et al. 2014). *N. oleoabundans* has a single nucleus that contains one nucleolus (Castro-Puyana et al. 2013), and has one or two mitochondria (Giovanardi et al. 2013). During cell maturation, cytoplasmic vacuolisation occurs and keeps increasing until four weeks of growth by compressing the cytoplasm at the periphery of the cells (Baldissierotto et al. 2012; Giovanardi et al. 2013).

4 Ecophysiology

N. oleoabundans reproduces asexually through formation of *zoospores* or *aplanospores*. To our knowledge, sexual reproduction has not been observed in *N. oleoabundans* (Chantanachat and Bold 1962). Zoospores are biflagellate and measure between 2 and 3.5 µm in width and 3.6 and 4.5 µm in length (Deason et al. 1991). The flagella allow them to swim to a favourable environment in which they can develop and mature. Zoospores form vegetative spherical cells after a short period of time, and lack a well-defined cell wall (Arce and Bold 1958; Deason et al. 1991). Nevertheless, zoospores fail sometimes to become motile and begin to mature within the confine of their sporangium until rupturing it after maturation. These

new born cells are aplanospores (Arce and Bold 1958), which accumulate in considerable numbers before they are liberated by rupturing the cell wall of the sporangium.

Their morphological simplicity has led to an undervaluation of their biological diversity, but different species not phylogenetically related are present in desert crusts, evidencing that the resistance to water stress is largely diffused among green algae (Gray et al. 2007). *Chlorophyta* from arid environments can modulate their osmotic balance either by expelling salts from the cell or accumulating compatible solutes, and can also counteract water loss by aggregating cells to colonies, producing extracellular polymeric substances (EPS) or reinforcing the cell wall with a sporopollenin layer (Bertuzzi et al. 2017). *N. oleoabundans* was isolated from the sand dunes of Rub al Khali desert, a hyper-arid environment where rainfalls are very scarce and water is available as dew, fog, humidity (Henschel and Seely 2008). Present knowledge on *N. oleoabundans* world distribution is very scanty, and it is not known if this species can be considered endemic to desert environments. Apparently, all the recent studies carried out on the biotechnological potential of this alga rely on the strain UTEX 1185. The continuous environmental changes that *N. oleoabundans* daily endures in its habitat accounts for its flexibility: freshwater or sea water can be used for its cultivation, and the alga can grow either in mixotrophic or heterotrophic conditions (Baldissierotto et al. 2016).

N. oleoabundans has a high growth rate (Gouveia et al. 2009), and showed an adaptive capacity to grow in marine water (Popovich et al. 2012), which is convenient for sustainable production. This ability can be explained by taking into consideration the peculiar habitat of this alga: the desert sand. Water shortage, in turn, induces matric and osmotic stresses that require adaptation strategies. A matric stress is defined as the direct contact with dry air, whereas a hyperosmotic stress occurs when cells are still submerged in a water solution (Billi and Potts 2000). In the first case, the dehydration is more rapid and severely affects the physiological machinery of cells (Gustavs et al. 2010).

The cellular activities triggered by the contemporary occurrence of different stresses are not fully understood: de Jaeger et al. (2018) report that under salt stress, proline is accumulated by *N. oleoabundans*, and that anti-oxidate pathways are activated to defend

the cells from oxidative stresses (de Jaeger et al. 2018). Moreover, also pH plays an important role, and values equal to 9.5 can mitigate stress effects (Peng et al. 2017). The lipid content is related to stress conditions and the occurrence of both nutrient starvation and high temperature stimulated by 66% the lipid production in laboratory tests carried out on *N. oleoabundans* (Kwak et al. 2015).

4.1 4.1 Cell wall

N. oleoabundans grown under normal growth conditions has a rigid 2-layered cell wall (200 nm thick) mainly composed of 24.3 % carbohydrates, 22.2 % lipids, 31.5 % proteins, and 7.8 % inorganic materials (Wang et al. 2015; Rashidi and Trindade 2018). Nevertheless, the composition of the cell wall is directly correlated to the growth conditions employed to grow the cells. It has been established that the cell wall thickness of *Chlorella* sp. - which has similar morphological characteristics as *N. oleoabundans* - increased by 70 % while grown under nitrogen deplete (ND) conditions (Yap et al. 2016). Moreover, the cell wall *N. oleoabundans* showed a significant increase in the concentration of carbohydrates when the microalga was grown under ND conditions (Rashidi et al. 2019). However, due to the scarcity of information in the literature about the physiological transformation of the cell wall of *N. oleoabundans* when grown in harsh conditions, it is plausible to consider the conclusion obtained on *Nannochloropsis salina* (Jeong et al. 2017). The latter concluded that the increase in the thickness of the microalga subjected to nitrogen deprivation is associated with the up-regulation of genes encoding for cellulose biosynthetic enzymes (Jeong et al. 2017), which leads to the accumulation of cellulose in the cell wall, thereby increase in rigidity.

4.2 4.2 Biochemical composition

The etymology of *N. oleoabundans* (Table 1) reflects the main reason scientists were initially interested in this species as it has the capacity to accumulate up to 55 % lipids on a dry weight basis (Tornabene et al. 1983; Li et al. 2008; Pruvost et al. 2009; Popovish et al. 2012) when grown under stress conditions. This large amount of lipids and more precisely triglycerides has attracted the attention of scientists to convert these

lipids into biodiesel (Avagyan 2018; Avagyan and Singh 2019). Nevertheless, *N. oleoabundans* composition is dependent on environmental factors in spite of its capacity to grow in harsh conditions, and/or to grow in marine or fresh water (Safi et al. 2020). Table 2 displays the primary composition of *N. oleoabundans* when grown under Nitrogen Replete (NR) or Nitrogen Deplete (ND) conditions. It can be observed that under nitrogen replete conditions, protein content increases up to $\pm 55 \text{ \%} \cdot \text{dw}^{-1}$, whereas under nitrogen deplete conditions lipid content increases up to $\pm 60 \text{ \%} \cdot \text{dw}^{-1}$ and protein content decreases significantly. This indicates that during nitrogen starvation the cells undergo biosynthesis changes and major ultrastructural changes such as chloroplast shrinkage and cell wall transformations (Sinetova et al. 2006). The shortage of nitrogen in the medium forces the cells to search for alternative sources of nitrogen by releasing nitrogen from the photosynthetic pigments such as chlorophyll, and utilize the same for the metabolic processes (Praveenkumar et al. 2012). Consequently, this leads to a cellular metabolic flux due to the decline in the rate of photosynthesis (Courchesne et al. 2009), and therefore, NADH would accumulate. Subsequently, the enzyme citrate synthase is inhibited and acetyl CoA ceases from entering the Krebs cycle. The increase in the level of acetyl CoA would lead to the activation of acetyl CoA carboxylase, which in turn would convert acetyl CoA into malonyl CoA (Praveenkumar et al. 2012). As a result, lipid synthesis increases resulting in significant storage lipid accumulation (Mandal and Mallick 2009), mainly composed of TAG (Table 2).

The fatty acid composition contains a range of saturated, monounsaturated and polyunsaturated fatty acids (Table 3). Similar to the aforementioned components, the fatty acid profile varies based on the growth conditions. However, regardless of the growth conditions employed, the predominant fatty acids are C16:0, C18:0, C18:1, C18:2 and C18:n3.

5 5. Production

The interest in the cultivation of *Neochloris* strains has significantly increased over the last decade due to its potential to treat wastewater treatment (Altunoz et al. 2020; Singh et al. 2020), to accumulate significant amounts of lipids suitable for biodiesel production

Table 1 Etymology and explanation of the former name of *E. oleoabundans*

<i>N. Oleoabundans</i> Etymology				
	Greek	Latin	Meaning	Remarks
<i>Neo</i>	<i>Neos</i> (νέος)	N/A	New	Due to its high concentration in chlorophyll
<i>Chloris</i>	<i>Chloros</i> (Χλωρίς)	N/A	Green	
<i>Oleo</i>	N/A	<i>Oleum</i>	Oil	Due to its capacity to accumulate large amounts of lipids
<i>Abundans</i>	N/A	<i>Abundō</i>	Overflowing	

(Banerjee et al. 2019), and its capacity to capture a substantial amount of CO₂ (Zhu et al. 2018; Sepulveda et al. 2019). In addition, the capacity of *Neochloris* to grow in fresh and marine medium has increased the interest in exploring its full potential (Safi et al. 2020). Indeed, multiple production methods can be employed to grow microalgae with mitigated rate of efficacy. Among the methods are open ponds and photobioreactors in which the species can be grown under heterotrophic, autotrophic and mixotrophic conditions (Avagyan 2010; Avagyan AB 2013; Avagyan 2018;). However, the effect of important operating conditions, such as light supply, nitrogen supply, medium composition and temperature play a crucial role in the healthy growth of *N. oleoabundans* and therefore these parameters have been extensively investigated over the last decade.

Jazzar et al., (2016) investigated the effect of nitrate and phosphate concentration on cultivation under indoor autotrophic batch cultivation in 1.8 L bubble column photobioreactor. The largest biomass productivity (0.115 g L⁻¹ day⁻¹) and concentration (1.69 g L⁻¹) were achieved in nitrate (0.99 g L⁻¹) and phosphate (0.085 g L⁻¹) rich medium at 23 °C and 200 μE m⁻² s⁻¹ light flux. However, lipid productivity (0.036 g L⁻¹ day⁻¹) and percentage within biomass (44%) were instead triggered in nitrate (0.074 g L⁻¹) and phosphate (0.016 g L⁻¹) deficient medium. Other (Morales-Sánchez et al. 2013, 2014) carried out similar investigations, but under heterotrophic conditions, and by separating the biomass growth from the lipid accumulation phase. Thus, the biomass was heterotrophically cultivated (10 g L⁻¹ glucose) batch-wise in a 20 L reactor under sufficient nitrogen supply, followed by the addition of 10 L medium under limited nitrogen condition and then a final resting phase with no further addition of medium. Consequently, due to the organic carbon supply, a larger biomass concentration was

achieved (5.6 g L⁻¹ day⁻¹) at the end of the fed-batch. Moreover, at the end of the resting phase, lipid productivity and percentage of the biomass were 1.9 g L⁻¹ day⁻¹ and 54%, respectively. Accordingly, the accumulation of lipid is often associated to the switch of the metabolism first from biomass to starch synthesis and then to lipid synthesis (Klok et al. 2013a; Morales-Sánchez et al. 2013, 2014; Baldissarotto et al. 2016).

Nonetheless, despite the fact that lipid synthesis and biomass growth are often considered decoupled, Klok et al. (2013a) proved that it is possible to simultaneously produce both in a continuous process. The production was therefore conducted in a 1.7 L flat panel turbidostat for autotrophic cultivation at a two inlet light fluxes (200 and 500 μE m⁻² s⁻¹). The system was run as a turbidostat, by adjusting the dilution on the basis of the light absorption. Biomass and lipid productivity ranged 0.26-1.29 g L⁻¹ day⁻¹ and 0.011-0.049 g L⁻¹ day⁻¹, respectively, while lipid content ranged 1.5 to 12.4%. The results were followed by a general metabolic model to demonstrate the possibility to customise the biomass composition on the basis of the dilution rate and inlet light flux Klok et al. (2013b). This was an important step to distinguish between nitrogen limited supply and completely depleted cultivation conditions, which is in agreement with the results obtained by Bona et al., (2014) in a semi-continuous cultivation condition (Bona et al. 2014).

Very few studies coupled the effect of nitrogen supply with the pH level in the culture. Therefore, it is worthwhile mentioning the study of Santos et al., (2014) because it showed that a step-wise increase of pH from the value taken during the growth (pH=8) to the value controlled during nitrogen starvation (pH=10) can optimise the lipid content up to 42%, while an initial level of 10-12% was measured during

Table 2 Biochemical composition of *N. oleoabundans* grown in nitrogen deplete and replete conditions

Components	Biochemical composition		
	N-Replete	N-Deplete	Reference
Proteins (%.dw ⁻¹) (Suarez Ruiz et al., 2020a) (Günerken et al., 2016); (Tibbetts et al., 2015); (Morales-Sánchez et al., 2014); (Garibay-Hernández et al., 2013); (Popovich et al., 2012)	27—55	17—26	(Safi et al., 2020);
Carbohydrates (%.dw ⁻¹) (Günerken et al., 2016); (Tibbetts et al., 2015); (Morales-Sánchez et al., 2014) (Sun et al., 2014); (Garibay-Hernández et al., 2013); (Popovich et al., 2012)	15—41	17—44	(Suarez Ruiz et al., 2020a);
Lipids (%.dw ⁻¹) (Suarez Ruiz et al., 2020a); (Chungjatupornchai et al., 2019); (Günerken et al., 2016); (Tibbetts et al., 2015); (Sun et al., 2014); (Garibay-Hernández et al., 2013); (Popovich et al., 2012); (Tornabene et al., 1983)	8—36	36—60	(Safi et al., 2020);
TAG (%/total lipids) (Sun et al., 2014); (Morales-Sánchez et al., 2014); (Popovich et al., 2012); (Tornabene et al., 1983)	9—19	21—80	(Günerken et al., 2016);
Glycolipids (%/total lipids) (Sun et al., 2014); (Popovich et al., 2012)	4—6	4—7	(Günerken et al., 2016);
Phospholipids (%/total lipids) (Sun et al., 2014); (Popovich et al., 2012)	1—11	2—12	(Günerken et al., 2016);

the growth at nitrogen sufficient conditions (Santo et al. 2014).

Another important parameter affects the biomass growth and lipid productivity: the supply of light. An extensive characterization of the dynamic of biomass composition of *N. oleoabundans* on the basis of the dynamic of light supply under different circadian cycles has been conducted in multiple studies (de

Winter et al. 2013; de Winter et al. 2014; de Winter et al. 2017a, b). The studies found that the duplication of *Neochloris* cells is naturally synchronised on a circadian cycle, despite the fact that the biomass was grown in a continuous flat panel photobioreactor operated as a turbidostat, and under constant light conditions. Small variations of the lipid content were also observed during the 24 h cell cycle, both at

Table 3 Fatty acid composition of *N. oleoabundans* grown in nitrogen deplete and replete conditions

Fatty acid composition			
Fatty acids	N-Replete	N-Deplete	References
C14:0 (Breuer et al., 2012)	trace	18	(Garibay-Hernández et al., 2013);
C16:0 (Sun et al., 2014); (Garibay-Hernández et al., 2013); (Santos et al., 2013); (Breuer et al., 2012)	24—31	2—24	(Morales-Sánchez et al., 2014);
C16:1 (Sun et al., 2014); (Garibay-Hernández et al., 2013); (Santos et al., 2013); (Breuer et al., 2012)	1—3	4	(Morales-Sánchez et al., 2014);
C16:2 (Sun et al., 2014); (Breuer et al., 2012)	2—6	6	(Morales-Sánchez et al., 2014);
C16:3 (Breuer et al., 2012)	12	5	(Sun et al., 2014);
C17:1 (Garibay-Hernández et al., 2013)	2—3	1—2	Morales-Sánchez et al., 2014;
C18:0 (Garibay-Hernández et al., 2013); (Santos et al., 2013); (Breuer et al., 2012)	1—11	3—11	(Sun et al., 2014);
C18:1 (Sun et al., 2014); (Garibay-Hernández et al., 2013); (Santos et al., 2013); (Breuer et al., 2012)	8—54	15—47	(Morales-Sánchez et al., 2014);
C18:2 (Sun et al., 2014); (Garibay-Hernández et al., 2013); (Santos et al., 2013); (Breuer et al., 2012)	17—30	16—26	(Morales-Sánchez et al., 2014);
C18:3n3 (Garibay-Hernández et al., 2013); (Santos et al., 2013); (Breuer et al., 2012)	3—4	3—16	(Sun et al., 2014);

nitrogen sufficient and limited cultivation conditions (de Winter et al. 2013, 2014; Krzemińska et al. 2014;). The supply of electrons for the biomass growth and lipid synthesis can be controlled by the level of oxygen concentration in the gas phase in contact with the medium. Sousa et al., (2012) showed that an increase

of the oxygen partial pressure from 0.24 up to 0.84 bar induced a 25% reduction of the growth rate (Sousa et al. 2013a). However, the study also showed that a parallel increase of the CO₂ partial pressure can mitigate this effect, provided to operate the photo-bioreactor at sub-saturating light intensity (Sousa et al.

2013a), while at saturating light intensity the inhibitory effect of oxygen by photorespiration cannot be overcome (Sousa et al. 2013a, b).

While the interest for lipid production is expected, in view of the lipid-accumulator feature of *Neochloris*, some other interesting applications of *Neochloris* strain have also been reported in literature. The superior efficacy of mixotrophic or even fully heterotrophic cultivation conditions (Giovanardi et al. 2013; Baldisserotto et al. 2016; Silva et al. 2016) has triggered the application of *N. oleoabundans* for waste treatment and valorisation. *Neochloris* biomass has been successfully cultivated on chicken manure (Altunoz et al. 2017), sewage sludge (Jeong et al. 2017), secondary wastewater from urban plants (Aravantinou et al. 2013), apple vinegar (Giovanardi et al. 2013), pig manure (Olguín et al. 2015a), vinasse digestate (Olguín et al. 2015b), pharmaceutical effluent (Singh et al. 2020), swine wastewater (Wang et al. 2017), biogas slurry (Zhao et al. 2015) and general municipal waste (Valev et al. 2020).

6 6. Downstream processing

6.1 Cell disruption

The cell wall rigidity of *N. oleoabundans* renders hard the extraction of the intracellular components. Hence, a unit operation of cell disruption is required to either weaken the integrity of the cell wall or to completely disrupt it to release the target components. Several cell disruption methods have been already tested on *N. oleoabundans* grown under different growth conditions (Safi et al. 2020), and showed a rather mitigated rate of success in terms of cell disruption effectiveness and release of components. The cell disruption methods consist of mechanical, enzymatic and chemical treatments.

6.1.1 Pulsed electric field

The method consists of creating pores on the cell membrane after applying intermittent electric field strength within a short period of time to release the intracellular components (Luengo et al. 2014). This method has been used to deactivate microbes in food products (juice, milk), to break the cell wall of yeast, as medical treatment such as electro-chemotherapy,

and for genetic transformation. Hence, given that Pulsed Electric Field (PEF) showed promising results in terms of cell disruption of microorganisms with minimal energy input. Therefore, it has been considered as a promising technique to break the cell wall of microalgae. In the case of *N. oleoabundans*, cell wall disruption efficacy by PEF was not as successful as expected. According to a study (Lam et al. 2017), PEF was efficient to perforate the cell wall of *N. oleoabundans* to release ions. However, the perforation was not efficient enough to release larger molecules such as soluble proteins even when significantly increasing the energy input. This poor efficacy corresponds to the outcomes obtained when testing PEF on other rigid cell walled species such as *Chlorella vulgaris* (Lam et al. 2017) and *Nannochloropsis gaditana* (Safi et al. 2017) where 3 to 10 % proteins (w/w) were released, respectively. Nonetheless, the poor efficacy of PEF to break the cell wall and release intracellular components of rigid cell walled microalgae does not necessarily imply that PEF will not be efficient on weak cell walled species (Safi et al. 2017). For instance, by means of PEF, highly pure β -Phycocyanin and C-Phycocyanin were successfully extracted from *Porphyridium cruentum* and *Arthrospira platensis*, respectively (Martínez et al. 2019; Akaberi et al. 2020).

6.1.2 Bead milling

The mechanism of this method consists of an agitator and a milling chamber. The slurry is first filled in a vessel connected to an agitator in order to keep a proper dispersion of the microalgal cells. Subsequently, the slurry is pumped into the milling chamber that contains the beads, which mechanically disrupt the microalgal cells by means of friction between the beads and the cells. The method is considered as highly efficient in terms of cell disintegration and release of intracellular molecules. Indeed, similar to bead milling, a cooling system is integrated to the machine in order to avoid overheating the solution and the denaturation of target components.

Bead milling is one of the most efficient cell disruption methods that inflicts severe damage to the integrity of rigid cell walled microalgae. It has been reported that > 99 % disintegration was reached after applying bead milling on *N. oleoabundans* with a specific energy input of < 0.5 kWh. kg⁻¹ (Postma et al.

2017). Nonetheless, the yield of soluble proteins released in the supernatant after bead milling (using 0.3 to 0.4 mm Yttrium stabilised ZrO₂ beads) was 50 % (Lam et al. 2017) to 59 % (Postma et al. 2017), and 68 % carbohydrates (Postma et al. 2017). The study also concluded that a selective protein extraction occurred during the first 10 minutes of bead milling (Postma et al. 2017).

Another study assessed the effectiveness of bead milling on *N. oleoabundans* grown under nitrogen replete (NR) and deplete (ND) conditions (Gürenken et al. 2016). The study reached 100 % cell wall disintegration, but the release of components differed between the cultivation conditions applied. Thereby, the release of proteins, carbohydrates and lipids for NR was 35 %, 33 %, 33 %, respectively. Whereas for ND the release of proteins, carbohydrates and lipids was 58 %, 65 %, 57 %, respectively.

6.1.3 6.1.3 High pressure homogenization

This method is commonly used to create emulsions, but also for cell disruption. It consists of forcing a solution through a system (shear forces, impact, cavitation) in order to homogenize it or reduce the particles size within the solution, thereby breaking the cell wall of microalgae. Indeed, similar to bead milling, a cooling system is integrated to the machine in order to avoid overheating the solution and the denaturation of target components.

High-pressure homogenization (HPH) was applied on several microalgae (Jubeau et al. 2012; Spiden et al. 2013; Safi et al. 2014; Yap et al. 2015; Safi et al. 2017) and showed high efficacy in terms of cell wall disintegration and release of intracellular components. With regards to *N. oleoabundans* grown under nitrogen replete condition, the disintegration rate was up to > 95 % (Karthikeyan and Prathima 2017), and yielded 69 % lipids (Wang et al. 2015) and 40 % proteins (Safi et al. 2020). Nevertheless, the disintegration rate and the effectiveness of releasing intracellular components are directly correlated to the growth conditions that affect the composition and the rigidity of the cell wall of microalgae (Yap et al. 2016; Jeong et al. 2017; Rashidi et al. 2019; Safi et al. 2020).

6.1.4 6.1.4 Ultrasonication

This method works by applying sound energy to produce cavitation on the cell wall, which facilitates the access to the intracellular components. Ultrasonication has shown mitigated results in terms of efficacy to break the cell wall of microalgae (Safi et al. 2014). For instance, this method was not efficient enough to break the cell wall and release the soluble proteins of green species like *Chlorella vulgaris*, *Nannochloropsis oculata* and *Haematococcus pluvialis*. Nevertheless, ultrasonication was more efficient on weak cell walled microalgae like *Arthrospira platensis* and *Porphyridium cruentum* (Safi et al. 2014). Although *N. oleoabundans* has a resistant cell wall, the microscopic observations showed that the cells were disintegrated (Karthikeyan and Prathima 2017) after applying ultrasonication, and the yield of lipids released was 53 % (w/w) (Wang et al. 2015).

6.1.5 6.1.5 Enzymatic treatment

The cell wall of *N. oleoabundans* contains proteins, cellulose and other components. This suggests that the choice of enzymes is an important factor given that the enzymatic approach focuses on hydrolysing one or several cell wall components to weaken the integrity of the cell wall, and have access to the intracellular molecules. SEM imaging showed intact cells after applying enzymatic treatment (Karthikeyan and Prathima 2017) on *N. oleoabundans*. This suggests that the enzymatic treatment softly disintegrates the cell wall but does not disintegrate the cell membrane, which results in the formation of fragile cell-like shaped protoplasts (Wang et al. 2015). Thus, by simply stirring the solution, the protoplasts will be easily disintegrated (Karthikeyan and Prathima 2017), and the intracellular soluble components will be released.

The enzymatic approach was also applied by combining multiple enzymes (cellulase and papain) in order to extract lipids from *N. oleoabundans* (Wang et al. 2015). The disintegration efficacy was not as high as bead milling, and the release of lipids was 46 % (w/w). Furthermore, the study also considered combining the enzymatic treatment with high-pressure homogenization or ultrasonication (Wang et al. 2015). Both combinations led to a high disintegration rate, and a higher release of lipids up to 93 % and 76 % (w/w).

w), respectively. This implies that combining two cell disruption methods is a plausible approach as long as the overall cost of the operation remains acceptable.

6.2 Fractionation

6.2.1 Ionic liquids (IL) and aqueous two-phase system (ATPS)

ATPS is a liquid-liquid extraction method that is based on employing two immiscible aqueous solutions to separate components (Yau et al. 2015). According to Asenjo and Andrews (2011) ATPS exploits the incompatibility between aqueous solutions of two polymers, or a polymer and a salt at high ionic strength. Hence, as the polymers are mixed, large aggregates form and the two polymers will tend to separate into two different phases due to steric exclusion (Asenjo and Andrews 2011). Moreover, ATPS is described as an efficient, scalable and mild technique that preserves the functional integrity of the extracted molecules (Rosa et al. 2011) such as proteins and pigments. Therefore, the extraction of proteins by means of ATPS (Iolilyte 221PG – citrate system or PEG 400 – potassium citrate system) showed that *N. oleoabundans* proteins migrate efficiently to the upper phase (80 to 100 % extraction efficacy) during phase separation (Figure 1). It has been implied that proteins favour the upper phase during the partitioning process due to their net charge and not their molecular weight (Suarez Garcia et al. 2018). On the other hand, the high affinity of sugars to water and their lack of charge triggered their migration to the bottom phase (> 90 % extraction efficacy), thereby inducing an efficient

separation from proteins (Suarez Garcia et al., 2018). Moreover, ATPS can take multiple forms based on the target components that require separation. For instance, Suarez-Ruiz et al. (2018) separated pigments (chlorophyll and lutein) from proteins by using a polymer (PEG 400) and an ionic liquid (Ch Dhp). However, in this case, pigments migrated to the upper polymer phase with an extraction efficacy of 97 % and 52 % for lutein and chlorophyll, respectively, whereas proteins were detected in the interface with an extraction efficacy of 92 % (Suarez Ruiz et al. 2018). Furthermore, Suarez-Ruiz et al. (2020b) fractionated pigments, carbohydrates, proteins and lipids by means of ATPS followed by a purification process using ultrafiltration (10 kDa regenerated cellulose membrane) (Suarez Ruiz et al. 2020). Desai et al. (2019) separated the hydrophobic fraction from the hydrophilic fraction by using the ionic liquid tributylmethylphosphonium methylsulfate (TBP SO₄). The study concluded that TBP SO₄ was able to permeabilise the cell wall of fresh intact cells of *N. oleoabundans* to release 68% of total lipids (Desai et al. 2019). Subsequently, the hydrophilic fraction (proteins and carbohydrates) was further extracted in an aqueous buffer after applying bead milling.

6.2.2 Membrane filtration

Further fractionation or purification of the crude extracts without employing toxic chemicals could be achieved by filtration. The unit operation consists of passing the solution through a filter on which some components will be retained based on their molecular weight. Multiple parameters should be taken into

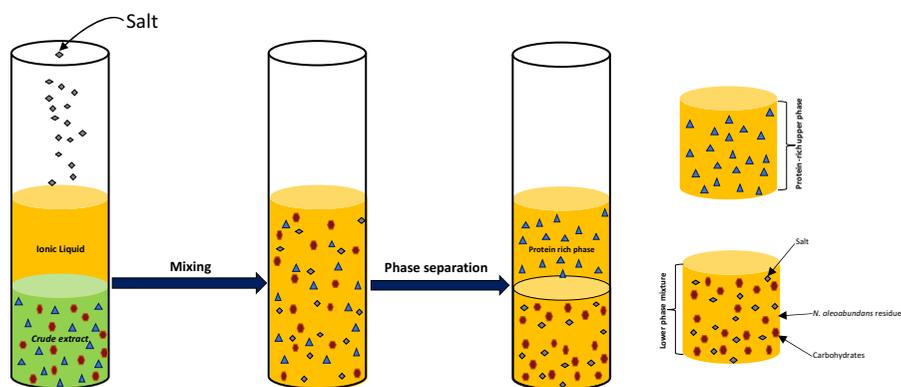


Fig. 1 Schematic example of an aqueous two-phase system to separate proteins from carbohydrates by means of ionic liquids and salts

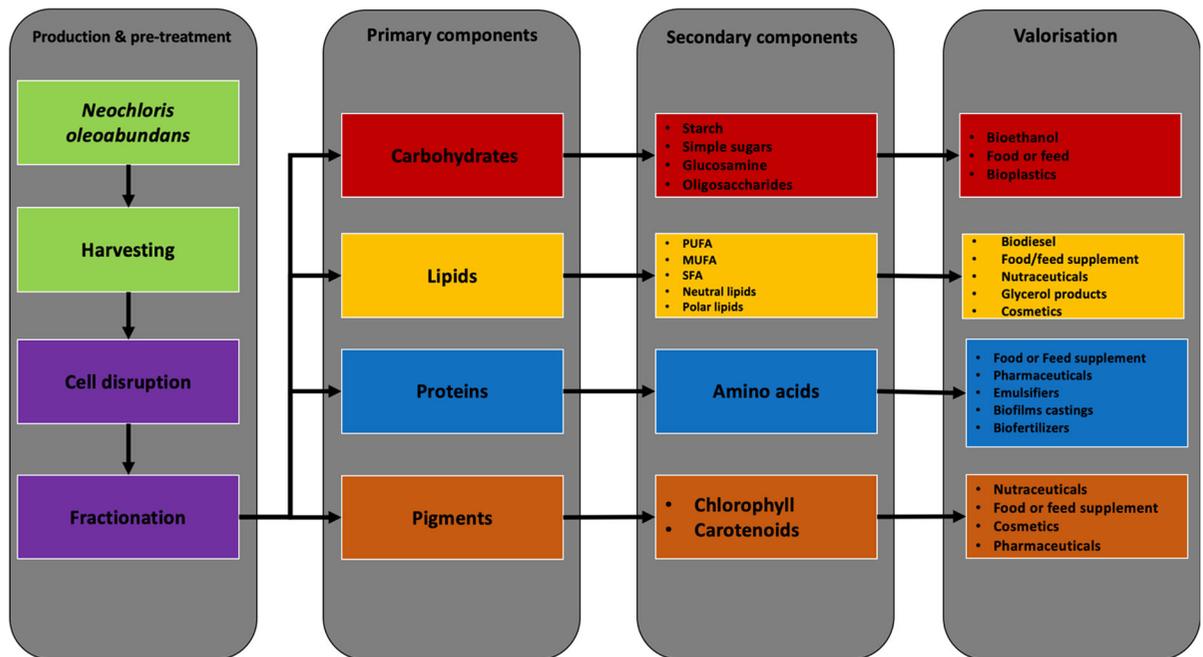


Fig. 2 Schematic description of the value chain of *N. oleoabundans*

consideration while operating this unit operation such as filter composition, transmembrane pressure, cut-off of the membrane and flow velocity, turbulent cross-flow. Therefore, a correct combination of parameters is always required to properly operate a filtration unit and limit the fouling phenomenon. In downstream processing of microalgae, filtration is used to either concentrate or to purify a target component obtained from the crude extract. Suarez Ruiz et al. (2020a) recovered 96% of the ionic liquid in the permeate, and retained 82% of the proteins obtained from the crude extract after ionic liquids extraction by using a 10 kDa polyethersulfone membrane (Suarez Ruiz et al. 2020a). Indeed, the literature on filtration of *N. oleoabundans* molecules is poor, but many studies applied this specific unit operation on other microalgal species, and therefore it can be extrapolated to *N. oleoabundans*. For instance, ultrafiltration was carried out to purify polysaccharides from *Porphyridium cruentum* (Patel et al. 2013; Macarti et al. 2014), *Spirulina platensis* and *Chlorella pyrenoidosa* (Pugh et al. 2001), or to concentrate proteins from *Chlorella vulgaris* and *Haematococcus pluvialis* in the retentate (Ursu et al. 2014; Ba et al. 2016). Other studies adopted a different approach when purifying the components of on *Tetraselmis suecica* and

Nannochloropsis gaditana (Safi et al. 2014; Safi et al. 2017) by obtaining the proteins in the filtrate instead of the retentate.

7 Valorisation track

N. oleoabundans gathers many features that make it an excellent candidate for a full valorisation. Basically, this species attracted attention for its capacity to accumulate lipids suitable for biodiesel production, to grow in marine and fresh water, and to clean wastewater. Nevertheless, looking at all the high-value components present in *N. oleoabundans* (Fig. 2), it would be more reasonable to valorise the remaining components (proteins, PUFAs, carbohydrates and pigments), which will increase profitability, promote the concept of circularity and most importantly, it is more sustainable. The complete valorisation approach has been previously studied (Wijffels et al. 2010) and showed that with a wider biomass valorisation, the process can significantly increase the profit compared to simply valorising it for biodiesel. Therefore, by employing a proper combination of unit operations, and by valorising the valuable components, the value of the microalgal biomass has the

potential to reach $\pm 1600\text{€}/\text{ton}$ after a complete valorisation (Wijffels et al. 2010).

Figure 2 represents the potential economic exploitation of *N. oleoabundans*. Starting from large scale production, the fractionation in the main component classes (lipids, carbohydrates and proteins) is followed by the purification in sub-fractions (soluble and insoluble proteins, starch, polar and neutral lipids), and the possible chemical conversion of some of them into other added values (protein hydrolysis into amino-acids, lipids esterification in fatty acids methyl-esters). All these fractions might be addressed to different markets, provided a product validation is performed at a demonstrative scale.

Currently, the status of any *Neochloris*-based value chain is at TRL not larger than 5 and few proofs of purification of secondary components are reported at that level. Indeed, despite the versatility in production conditions and in accumulation of either lipids or protein rich fractions, only few projects focused their efforts on *Neochloris* (Fuel4Me, AlgaePARC Biorefinery, Greenbiorefinery). Thus, it would be advisable that future projects take into consideration *Neochloris* in order to properly exploit it and valorise its valuable components.

8 Conclusion

N. oleoabundans has the capacity to grow efficiently in saline, fresh and wastewater. It accumulates valuable components that can be fractionated by employing multiple unit operations. The ratio of these components can vary based on the growth conditions. Although *N. oleoabundans* has a resistant cell wall, the growth conditions applied affect its cell wall physiology, making it less or more resistant to cell disruption. Moreover, the value chain scheme showed all the possible valorisation paths that lead to the development of end-products.

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Declarations

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

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