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Review

Cell Walls of Lipid-Rich Microalgae: A Comprehensive Review on Characterisation, Ultrastructure, and Enzymatic Disruption

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Abstract: Certain microalgae species have gained traction in the biofuel and food/feed sectors due to their ability to accumulate large amounts of intracellular lipids. However, the extraction of lipids from microalgae is hindered by the presence of complex and recalcitrant cell walls that act as a barrier to mass transfer. This paper examines the intricate details of microalgae cell walls of species belonging to three genera—*Nannochloropsis*, *Scenedesmus*, and *Schizochytrium*—known for their high total lipid contents and omega-3 polyunsaturated fatty acid contents, thus having dual potential for both biofuel and food/feed application. An overview of the techniques used to analyse the cell walls, followed by a detailed description of the cell wall architecture of the three genera and the growth conditions that affect the ultrastructure and composition of their cell walls, is presented. Since cell wall disruption is a crucial step in recovering intracellular products from microalgae biomass, different cell-disruption technologies are also reviewed, focusing specifically on approaches that can be applied directly to wet biomass without the need for biomass drying, thus exerting a low-energy footprint. Enzymatic treatment is operated under mild conditions and offers a promising wet route for targeted recovery of intracellular products from microalgae with minimal side reactions and risk of product degradation. The high cost of enzymes can be mitigated by reducing enzyme requirements through the adoption of a minimal design approach that uses the cell wall composition as the basis to direct enzyme choice and dosage. Different enzyme-recycling and immobilisation strategies to reduce enzyme requirements and improve commercial scalability are also reviewed. Finally, the paper provides a summary of the current state-of-the-art in direct biological approaches using algicidal bacteria and fungi to achieve cell disruption. Overall, the paper provides a roadmap for a more efficient cell disruption of microalgae.

Keywords: microalgae; *Nannochloropsis*; lipids; cell wall; biorefinery; enzymes

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1. Introduction

Fossil fuels contribute to global warming and are becoming increasingly less abundant [1]. Therefore, alternate forms of biofuels are needed to cater to the rising global demand for bioenergy. Lipid-rich microalgae have been recognised as a promising feedstock for biobased products (including biofuels) due to their high areal productivity and ability to capture CO_2 for greenhouse gas sequestration. Microalgae can also be cultivated without diverting valuable agricultural resources (e.g., arable land) from food production [2].

Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) are critical for human, animal, and fish health as they serve as essential precursors to immune-promoting bioactive molecules, known for their effect in aiding cognitive development and preventing cardiovascular and inflammatory diseases [3]. Fish oil mainly consists of C20:5 eicosapentaenoic fatty acid (EPA) and C22:6 docosahexaenoic fatty acid (DHA). It is currently the primary source of ω-3

PUFAs in human, animal, and aquaculture diets. Over the past 2 decades, the exponential increase in the global demand for ω-3 PUFAs within the food and feed sectors has expanded the fish oil industry. This, however, has contributed to the depletion of oceanic fish stock and placed an enormous strain on the marine ecosystem. Certain microalgae species are rich in ω-3 PUFAs and can replace fish oil as a more sustainable source of valuable fatty acids.

"Microalgae" is a common term used to name a group of mostly photosynthetic single-celled organisms in the micrometer range found in marine, brackish, freshwater, or soil habitats with diverse temperatures, illumination, pH, carbon concentration, and nutrient levels [4]. The kingdom Protista is characterized by metabolic diversity, ranging from autotrophic organisms (commonly referred to as microalgae) to fully heterotrophic organisms such as amoebas. Many protists, such as *Euglena* sp., exhibit dual metabolic strategies, such that they can perform photosynthesis and feed on organic matters.

To survive in such varied environments and protect themselves against predators and adverse environmental conditions, microalgae have developed a diverse array of cell walls [5]. The variation in layer composition and architecture can have major implications on the disruption propensity of microalgae biomass and extractability of intracellular products, which can in turn affect the selection and complexity of downstream processing methods [6]. Most microalgal products are intracellular and can only be recovered after being freed from cell wall encapsulation. Microalgae have developed a multilayered and intricate cell wall structure to (a) act as a barrier between the interior of the cell and the outside environment, regulating the transport of materials in and out of the cells, and (b) provide structural support and protection against external stresses, environmental changes, predators, and infections. Moreover, the cell wall in some algal species is known to regulate buoyancy. For example, the enhanced silicification found in the bipartite cell walls of diatoms raises the density and, consequently, the sinking rate of the cell, aiding in cell relocation to habitats with ample nutrients [7]. Microalgae cell walls are generally composed of carbohydrates, proteins, lipids, carotenoids, tannins, and even lignin [8], though significant variations are observed between genera and species. Polysaccharides are primary structural components of most algal cell walls, taking the form of cellulose, chitin-/chitosan-like molecules, hemicelluloses, pectins, fucans, alginates, ulvans, carrageenans, and lichenins [9]. The cell wall surfaces are also often decorated with functional groups that can serve as adsorption sites. Spain et al. demonstrated that heavy metals can adsorb to microalgal cell walls, helping to treat wastewater [10].

The total lipid content of microalgae like *Nannochloropsis*, *Scenedesmus*, and *Schizochytrium* ranges between 37–60, 30–50, and 50–77 wt.% of the dry weight of biomass, respectively [11,12]. This makes them desirable candidates for biofuel production. Microalgae exhibit considerable biotechnological promise for a range of industries beyond biofuel production, with applications ranging in food, feed, chemical, and personal care, nutraceuticals, and pharmaceutical sectors. A comprehensive understanding of the structure and composition of microalgal cell walls can contribute to a more informed selection of downstream processes for targeted extraction of biochemical components or a more efficient design of a cascading biorefinery approach for the fractionation of the biomass into multiple product streams.

Cell disruption is a critical step in the downstream process, enabling the release of intracellular products for further recovery or purification steps. The selection of cell-disruption methods is based on several factors, including the scale of production, the type of compounds to be extracted, and the structure and toughness of the cell walls. An ideal cell-disruption process should be rapid, efficient, and non-degradative to the target products while having a low energy footprint and cost requirements. To achieve a low energy footprint, it is critical that the selected disruption system can directly process wet biomass, thereby overriding the need for an energy-intensive drying step.

In this paper, the cell walls of microalgae belonging to the lipid-rich genera *Nannochloropsis*, *Scenedesmus*, and *Schizochytrium* are reviewed. The three genera have been

selected because of their dual potential in being able to accumulate lipid fractions for both biofuel production (e.g., triacylglycerols) and food/feed/nutraceutical applications (e.g., ω-3 PUFAs). An overview of the techniques used in the isolation and analysis of microalgal cell walls, followed by a detailed review of the cell wall architecture of these species and the growth conditions that affect the ultrastructure and composition of the walls, are presented. The study then reviews different cell-disruption technologies that can be applied for the targeted recovery of intracellular lipids from the biomass, focusing on enzymatic treatment and biological approaches as a novel processing route for wet biomass. The review aims to improve the current understanding of the state-of-the-art cell-wall characterisation, cell-wall architecture, and enzymatic cell disruption for lipid-rich species, paving the way for more efficient cell disruption and commercially viable biorefinery processes.

2. Microalgae Cell Wall

2.1. Cell-Wall Isolation

To study the cell wall of any microalgae, it is important to first isolate the cell wall from the rest of cellular components. This separation involves many steps, as the microalgae cells contain complex organic components. A summary of microalgal cell-wall isolation methods is provided in Table 1.

Table 1. Summary of microalgae cell-wall isolation methods.

Microalgae	Biomass State	Disruption Methods	Lipid-Extraction Solvents	De-Starching Method	Reference
Neochloris oleoabundans	Dry	Mechanical disruption—Milling	chloroform: methanol (2:1, *v/v*)	Enzymatic method—alpha-amylase in maleate buffer	[13]
Chlorella sorokiniana	Wet paste	Mechanical disruption—Mortar and pestle	chloroform: methanol (1:1, *v/v*)	Enzymatic method—amylase in phosphate buffer	[14]
Nannochloropsis gaditana	Lyophilized and in water	Mechanical disruption—French press and sucrose gradient	80% ethanol, *n*-hexane–acetone (1:1, *v/v*), and *n*-hexane	-	[9]
Chlorella vulgaris, *Scenedesmus* sp., *Haematococcus pluvialis*, and *Coelastrella* sp.	Dry	Chemical disruption—80% ethanol and 70% ethanol + Heating	chloroform: methanol (1:1, *v/v*)	Enzymatic method—α-amylase, and amyloglucosidase + 0.01% sodium azide	[15]

Cell-wall isolation generally begins with the disintegration of the cells. The commonly used methods are mechanical in nature, such as bead milling, ultrasonication, or simple homogenization using a mortar and pestle. In Rashidi and Trindade, dry *N. oleoabundans* biomass was milled for 1 min at a frequency of 25 s^{-1} [13]. Homogenisation of wet biomass paste was carried out using pestle and mortar in the presence of liquid nitrogen by authors Vojvodić et al. in their work [14]. Scholz et al. conducted experiments using a French press and sucrose gradient centrifugation to isolate cell walls. They used seven passes through the French press at 18,000 lb/in^2 pressure for nearly complete cell lysis. Multiple centrifugation cycles at 5000× *g* were used to separate cell walls from entire cells. The pressed cell walls were then layered onto sucrose gradients of 20, 30, 40, and 60% and centrifuged for 30 to 60 min at 10,000× *g*. One of the most effective methods for separating molecules is sucrose density gradient ultracentrifugation. Different-sized molecules sediment through the gradient at varying speeds during centrifugation. Larger macromolecules sediment at the bottom of the gradient while lighter ones stay near the top, separating them based on size [16]. Hence, the pressed walls migrated to the bottom, while shed cell walls and debris remained in the higher strata [9]. Alternatively, solvents can be used to initially break the cell walls. For example, in Spain and Funk, 80% ethanol was first added to dry microalgal biomass. The samples then underwent heating, cooling, and a 10-min centrifugation at 21,000× *g* centrifugation, were resuspended in 70% ethanol, and heated twice to 95 °C [15].

Ethanol and temperature fluctuations cause the cell wall and cell membrane to lose its integrity. It has been demonstrated that ethanol alters the proton motive force and increases metabolite leakage from cells [17].

The next steps involve the removal of intracellular components from the walls. The lipids are usually removed using a total lipid extraction method (e.g., Folch method or Bligh and Dyer method), which uses both polar and nonpolar solvents. The polar solvent disrupts the protein-lipid complexes, while the nonpolar solvent dissolves the neutral lipids [18]. Both the Folch method and Bligh and Dyer method use chloroform and methanol in different ratios for lipid extraction. Rashidi and Trindade removed all intracellular lipids in three cycles of chloroform: methanol (2:1 *v/v*) incubation, each lasting 30 min at 60 °C with constant shaking at 600 rpm [13]. In the total lipid extraction performed by Vojvodić et al., they used a 1:1 *v/v* ratio of chloroform and methanol and incubated the mixture overnight at 4 °C [14]. Similarly, Spain and Funk also used a 1:1 *v/v* ratio of chloroform and methanol solvent mixture and incubated the mixture at room temperature for 15 min [15]. Unlike the previously mentioned studies, which used chloroform/methanol extraction with varying ratios, Scholz et al. treated the pressed cell wall with n-hexane-acetone instead of chloroform and methanol and incubated for 15 min in a sonicating water bath to remove loosely bound proteins, lipids, carotenoids, and other soluble material [9].

Removal of starch is the next step in cell-wall isolation. Amylase enzyme or a cocktail of enzymes containing Amylase is generally used to remove starch from cell walls. For example, in Rashidi and Trindade, alpha-amylase was added to the *Chlorella sorokiniana* cell wall in maleate buffer with a pH of 6.5, and the mixture was then incubated for 24 h at 30 °C [13]. Vojvodić et al. treated the disintegrated lipid-free cell wall extract with an amylase enzyme in a phosphate buffer (pH 7.2) for 24 h at 30 °C in order to remove starch [14]. In Spain and Funk, the cell wall material was digested with 0.01% sodium azide, α-amylase, and Amyloglucosidase in a shaker for a duration of 24 h at 37 °C. The pellet was resuspended with 0.1 M potassium phosphate buffer after the samples were centrifuged, and 0.01% sodium azide, α-amylase, and Amyloglucosidase were added per gram. The digestion procedure was repeated three times for complete removal of starch. Once isolated, the cell walls can then be studied for their structure and composition [15]. In all examples provided, the reactions were carried out at a pH and temperature range of 6.5–7.2 and 30–37 °C, respectively. This is because it is the optimal pH and temperature range for the Amylase enzyme.

2.2. Cell-Wall Characterization

Relevant methods used for studying microalgal cell walls, namely staining and labelling, component analysis, ultrastructure analysis, and spectroscopy, are reviewed in this section. A summary of the analytical methods can be found in Table 2.

Table 2. Microalgal cell-wall characterisation methods.

Method and Uses	Drawbacks	Microalgae Application Examples
1. Staining and overall labelling		
Staining Simple visual instructions and chemical staining. Identification of various components	Sample preparation can be cumbersome Low specificity Stain penetration can be influenced by cell-wall ultrastructure	Crystalline violet to detect the presence of algaenan [19] Calcofluor white to stain cellulose [20]
Immunolabeling Mono-/polyclonal antibodies attached to fluorochromes and fluorescent probes High-level resolution imaging of cell-wall microstructures	Need for destructive pretreatment High cost of commercial antibodies Limits on the spatial resolution and depth of penetration into samples	Monoclonal antibodies to reveal the presence of xylan/arabinoxylan in *Neochloris oleoabundans* cell walls [13] Polyclonal anti-xyloglucan antibody to stain primary wall of *Micrasterias* spp. [21]
Genetic encoding Target and fluorescent protein produced as a translational fusion Nonspecific labelling can be avoided	Genetic transformation required Non-quantitative Photobleaching of samples	Interspecies incorporation of frustulins in diatoms [22] Identification of proteins involved in diatom's silica wall formation [23]

Table 2. *Cont.*

Method and Uses	Drawbacks	Microalgae Application Examples
2. Chemical analysis		
Inductively coupled-plasma atomic emission spectroscopy (ICP-AES) Accurate and precise identification of elements (or ions) present in the cell wall	Need for cell wall isolation Requires digestion of the sample High operating cost	Identification of elements present *N. gaditana* cell wall [9]
High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) Separation of negatively charged molecules, such as mono-/oligosaccharide and glycoconjugate analysis	Need for cell wall isolation Requires solubilisation of target molecules via hydrolysation/digestion/derivatisation, which can lead to sample loss and reduced accuracy.	Identification of monosugars in *N. gaditana* cell wall [9] Identification of monosugars and uronic acid in 10 microalgae species' cell walls [24]
Gas Chromatography—Mass Spectrometry (GC–MS) Gas mobile phase separation Better at identifying more volatile compounds	Need for cell wall isolation Requires solubilisation of target molecules via hydrolysation/digestion/derivatisation, which can lead to sample loss and reduced accuracy. Non-volatile components have to be derivatised to a more volatile intermediate prior to analysis.	*C. vulgaris*, *Coelastrella* sp., *Scendesmus* sp., and *H. pluvialis* cell-wall sugar analysis [15] Identification of ester-bound ω-hydroxy fatty acids of *T. minimum*, *S. communis* and *P. boryanum* [25]
Liquid Chromatography—Tandem Mass Spectrometry (LC-MS/MS) Liquid mobile phase and ionization for separation. Better for thermally labile compounds	Need for cell wall isolation Requires solubilisation of target molecules via hydrolysation/digestion/derivatisation, which can lead to sample loss and reduced accuracy.	Identification of frustule-associated glycoprotein peptides [26] Analysis of polysaccharide-associated-protein peptides of *B. braunii* [27]
3. Ultrastructure analysis		
Transmission Electron Microscopy (TEM) Based on the transmission of electrons through the cell. Study of the fine internal structure/cross sections of cells and cell-walls	Expensive Long sample processing Ultrathin sections Possibility of artifact generation	Study of enzymatic treatment effect on *C. vulgaris* cell wall [28] Effect of N starvation on the cell wall of *N. oceanica* [29]
Scanning Electron Microscopy (SEM) 3-dimensional surface analysis based on scattered electrons, high throughput	Expensive Possibility of artifact generation Lower resolution than TEM	Study of frustule morphogenesis in diatoms [30] Effect of lysozyme on the outer cell wall of *Chlorella* sp. and *Nannochloropsis* sp. [28]
Atomic force microscopy (AFM) Non-destructive study of nanomechanical properties and/or interactions Fine spatial resolution	Limited vertical and magnification range Data dependent on the tip, which can be easily damaged Limited scanning speed	Study of *C. vulgaris* cell-wall roughness and rigidity [31] To monitor fibril layer changes during growth and enzyme treatment [32]
Block face imaging SEM (FIB-SEM) Study 3D architecture of large volumes by thin ablation of thin serial sections	Long preparation time to achieve smooth and consistent milling Limited availability of (semi)automated workflows	Effect of high pressure (Guo et al., 2022) and improper incorporation of cryoprotectant [33] on *C. pyrenoidosa* cell walls
Cryo-soft X-ray tomography (Cryo-SXT) Complementary to electron and visible light microscopy, to obtain the organization, distribution, and dimension of organelles.	The missing wedge when using flat support Glass absorption when using capillary supports	Study of the adsorption of gold nanoparticles to the cell wall of *C. utilis* [34]
4. Spectroscopy:		
Fourier-Transform InfraRed (FTIR) Detection of several biomolecules in one analysis and species-specific changes, through radiation absorption. Complements Raman spectroscopy.	Validity of Beer-Lambert's law within a narrow linear range Limited availability of chemometric predictive models	Identification of sporopollenin in the outer layer of *Chlorella protothecoides* cell wall [35] Characterisation of heterotrophically grown *Chlorella* cell wall [36]
Raman spectroscopy Detection of several biomolecules in one analysis and species-specific changes, through radiation scattering. Complements FTIR spectroscopy.	Some compounds are not Raman active Others may fluoresce and mask the Raman signal	Study of cell wall changes during *Mougeotia disjuncta* zygospore formation [37] Study of daughter cell wall formation in *Micrasterias* [38]
(Cryogenic) X-ray photoelectron spectroscopy ((Cryo-)XPS) Semi-quantitative determination of the composition of cell surfaces	X-rays cannot be focused in the same manner as electron beams Life span of biological materials under vacuum X-ray photon damage	Comparison of *Chlorella vulgaris*, *Coelastrella* sp. and *Scenedesmus obliquus* cell wall composition during stationary phase [39] Comparison of pH effect on *Chlorella* sp. and *N. oculata* cell wall [40]
Nuclear Magnetic Resonance (NMR) Study of the structure, conformation, and dynamic of biological molecules	Heterogeneity of samples and molecule complexity renders resonance assignment difficult Limited availability of (semi)automated workflows	Identification of glycans in *Parachlorella beijerinckii* cell wall [41] Comparison of *C. reinhardtii*, *P. lutheri*, and *N. ocultata* cell-wall characteristics [42]

2.2.1. Staining and Overall Labelling

Imaging techniques based on labelling are the classic approach to studying cell walls, as they allow for the identification and location of components within the cell wall. Common techniques include staining, immunolabeling, and genetically encoded techniques.

A commonly used dye is calcofluor white (CFW), a nonspecific fluorochrome that binds with cellulose and chitin. The dye has been used to stain *Botryococcus braunii* cell walls and assess cellulase treatment efficiency [13]. CFW was also used to study the cell

wall of *Haematococcus pluvialis*, in addition to primuline (Direct Yellow 7) to confirm the presence of algaenan and aniline blue in lactophenol to examine the trilaminar sheath [43].

Analogously, CFW labelling of cell walls has allowed for the confirmation of the presence of (1→3, 1→4)-β-glucan in the secondary cell wall of *Micrasterias* spp., with anti-xyloglucan staining the primary wall [21]. Polyphosphate in *Chlamydomonas reinhardtii* cell walls, whose abundance correlated with the cell cycle, was stained using a histidine-tagged exopolyphosphatase (EcPPXc) [44].

2.2.2. Chemical Analysis

The majority of methods used to characterise cell-wall components require prior isolation and purification of the cell wall. This is a critical step that needs to be carried out rigorously, as the isolation and purification steps can lead to unintended loss of molecules (e.g., polar lipids, weakly bound proteins). Once the cell walls have been isolated, they are subjected to a solvent-extraction process to remove impurities, followed by a digestion step to recover the target group of molecules.

For example, Scholz et al. were able to identify cellulose, amino acids, and minerals from isolated *Nannochloropsis gaditana* cell walls. Isolated cell walls were successively extracted with solvents of decreasing polarity to remove lipids, proteins, and organic matter, leaving behind pure cell wall materials that were ready to be digested and compositionally analysed. Cell wall monosaccharides were characterised by digesting the isolated cell wall with enzymes and analysing the resultant supernatant for released sugars using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Polysaccharide composition (e.g., cellulose) was then inferred based on monosaccharide profiles coupled with staining and microscopy results. Similarly, an amino acid analyser was used to characterise the HCl hydrolysate of the isolated cell walls, and inductively coupled plasma atomic emission spectroscopy (ICP-AES) was applied for elemental quantification of cell walls after HNO_3 digestion [9].

Similarly, Spain and Funk isolated the cell walls of *C. vulgaris*, *Coelastrella* sp., *Scendesmus* sp., and *Haematococcus pluvialis* from freeze-dried biomass. After extracting phenolics and degrading starches, the cell-wall isolates were analysed for sugar composition via trimethylsilyl derivatisation and gas chromatography–mass spectrometry (GC–MS) analysis. Bound proteins were analysed using acid hydrolysation, AccQ-Tag derivatisation, and measurement by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC/ESI-MS/MS) [15].

2.2.3. Ultrastructure Analysis

Microalgae sizes can range from 1–1000 µm (e.g., *Ostreococcus* spp. and *Ceratium* spp., respectively), which can pose a challenge particularly when studying the physiological characteristics of the cell wall of smaller species. The ultrastructure of microalgae cell walls is generally studied using different types of electron and atomic force microscopy, using either intact cells or isolated cell walls.

Transmission electron microscopy (TEM) is a technique used to observe cross-sections of fixed samples, providing valuable insights into the layer and structural configurations of cell walls. Early studies on microalgal ultrastructure established the existence of trilaminar structure in *Chlorella* sp. [45] and *Arthrospira ptatensis* [46] cell walls. More recently, this technique was employed to study cell wall modifications in industry-relevant species, such as *Nannochloropsis oceanica* upon nitrogen starvation [29] or *Chlorella vulgaris* after enzymatic treatment [28].

Scanning electron microscopy (SEM) is the method of choice to study the surface of cell walls. SEM was used to examine the intricate nanostructure of diatoms' silica exoskeleton (frustule), teratologies associated with contamination, and cell wall morphogenesis [30,47]. Surface analysis using SEM can also provide valuable information regarding disruption method efficiency and the effect of cell disruption on the morphology and intactness of cell

walls, such as the use of a lysozyme treatment to remove the outer layer of *Chlorella* sp. and *Nannochloropsis* sp. cell walls [28].

Atomic force microscopy (AFM) is a non-destructive technique used to measure rheological properties of living structures by measuring interactions between atoms, although its use in the microalgae field is still limited [48,49]. Nonetheless, AFM has been used in several studies to investigate the properties of microalgae's surface, such as the roughness and rigidity of the *C. vulgaris* cell wall when exposed to salinity stress [31], cell aggregation in the presence of microplastics [50], as well as changes in the fibril layer of cell walls during growth and lysozyme treatment [32].

3D electron microscopy (3D-EM) is a powerful technique to study not only the structure of the cell walls but also the interactions among the different organelles, providing a holistic understanding of biological processes. 3D-EM techniques rely on cryo-preservation, electron tomography [51], and volume EM [52] to overcome key limitations of TEM/SEM, such as nanostructure loss and artifact generation during sample preparation and the limited depth of 2D analysis. Included in this group are techniques such as serial block face imaging SEM (FIB-SEM), Cryo-soft X-ray tomography (Cryo-SXT), cryo-scanning transmission electron tomography (CSTET), phase contrast cryo-transmission electron tomography (PC-CET), and sub-tomogram averaging PC-CET (Subtomo Avg PC-CET). Since recent advances for all these techniques have been reviewed in Varsano & Wolf [53], only a brief description of FIB-SEM and Cryo-SXT will be provided in this review. FIB-SEM block face imaging is a technique that employs a beam of ions (e.g., gallium) to remove the top surface of a sample, exposing a new layer, which can then be imaged by SEM [54]. This process is repeated in an iterative manner, which allows for 3D reconstruction of a posteriori [55]. This technique has previously been used to generate 3D reconstruction of cells incorporating changes in cell wall architecture due to high-pressure freezing [56]. Cryo-SXT leverages the different linear absorption coefficients of carbon (proteins) and oxygen (water) to obtain high-resolution (dozen nanometres) and high-contrast images without the need to stain the samples [57]. Cell walls were expected to influence *x*-ray absorbance, but a study comparing wild-type and mutant cell wall-less *Chlamydomonas reinhardtii* strains showed no major impact of cell wall presence on tomogram resolution [58]. The same authors also reported the effect of the presence of ice in the samples, which can lower tomogram quality and deform the cell wall.

2.2.4. Spectroscopy

The use of spectroscopic analysis, particularly the non-destructive type, for molecular group identification (and thus, chemical composition) in the biological sciences has long been established [59]. In the particular case of microalgae cell-wall analysis, it allows for the study of cell-wall properties in isolated cell walls or directly in intact cells. The analysis provides qualitative and semiquantitative data, albeit with less detail when compared with chemical analysis.

Fourier–Transform InfraRed (FTIR) spectroscopy uses mid-infrared light absorption to interrogate the vibrational activity of molecular groups (e.g., OH, NH, CH, CH2, and >PO) [60,61] and generate a unique fingerprint of a compound. When used to analyse a biological sample (e.g., isolated cell walls), the method generates multiple fingerprints, which can be juxtaposed to produce a unique signature of the sample to reflect the specific lipid, protein, carbohydrate, and biopolymer contents of the sample. The signature can be used as the basis to monitor any changes in the chemical composition of the cell walls (e.g., before and after enzymatic treatment). FTIR spectroscopy was fundamental in the identification of algaenan in *N. gaditana* cell walls [9]. It was also used to study polysaccharide changes in *C. vulgaris*, *Coelastrella* sp., *Scenedesmus* sp., and *H. pluvialis* cell walls during growth [15].

Raman spectroscopy relies on the scattered light emitted by the biological molecules, each having a spectrum composed of overlapping bands related to specific molecular bonds (e.g., O–H, C=O, N=O, C≡C), to generate a signature spectrum that reflects the

composition of a complex biological sample, e.g., isolated cell walls [62]. The spectrum of experimental samples can then be compared to that of the control sample to detect any changes in chemical composition. Confocal Raman spectroscopy has been used to study changes in the cell- walls of *Mougeotia disjuncta* during zygospore formation, highlighting the presence of lipids and aromatic compounds [37]. Similarly, the development of the cell wall was studied in *Micrasterias* sp., which displayed two strong carbohydrate signals at the cell-wall level in mother cells but not in the developing cells [38].

Cryogenic X-ray photoelectron spectroscopy (cryo-XPS), an emerging technique in microalgae cell wall characterisation, as well as fungi and bacteria, can be used to study the surface chemical composition of hydrate samples without the need for prior cell-wall isolation [63]. This technique has the advantage of being fast and non-destructive, with an analysis depth of a dozen nanometres. Cryo-XPS has been used to study the surface of the whole *C. vulgaris* and *Coelastrella* sp. cells [39,64], as well as the effect of pH changes on cell wall polysaccharides and proteins of freshwater *Chlorella* sp. and marine *N. oculata* [40].

Nuclear Magnetic Resonance (NMR) is a powerful spectroscopic technique with subatomic resolution, which has been used to analyse biomolecules in many organisms and tissues since the middle of the last century [65,66]. Solid-state NMR (ssNMR) is an emerging technique in the study of cell walls and extracellular matrices of whole cells and organisms, as it overcomes many of the limitations of traditional solution NMR, such as chemical modification and solubilization of the matrices [67]. In the microalgae field, ssNMR has been used to characterise the composition of the cell wall, namely glycans in *Parachlorella beijerinckii* [41], the fibril and glycoprotein network in *C. reinhardtii*, the cellulose scales of *Pavlova lutheri*, and the rigid cellulose in *N. ocultata* [42].

2.3. Cell-Wall Ultrastructure and Composition

The cell-wall structure and composition of each microalgae genus is unique. The cell-wall features are detailed in this section and summarized in Table 3.

Table 3. Cell-wall ultrastructure and composition of *Nannochloropis*, *Scenedesmus*, and *Schizochytrium* genus.

Genus	Ultrastructure	Composition		References
		Amino Acids	Saccharides	
Nannochloropsis	Bilayered structure—cellulosic inner layer and ahydrophobic algaenan outer layer	Asparagine, glutamine, and other amino acids except cysteine, methionine, and tryptophan	Monosaccharides: glucose Polysaccharides: cellulose	[9,68,69]
Scenedesmus	Trilaminar cell wall—inner cellulose layer covered by algaenan layer. The cell walls form a pectic layer that separates individual cells from one another in a coenobium.	Glycine, glutamic acid, aspartic acid, threonine, and alanine.	Monosaccharides: glucose, galactose, and mannose. Polysaccharides: cellulose, hemicellulose and pectin	[15,70]
Schizochytrium	Thin non-cellulosic layer covered by overlapping scales. Contains sagenogenetosomes that develop from invaginations of the cell membrane.	Glutamic acid (main), aspartic acid and lysine, taurine (minor amounts), and hydroxyproline (minor amounts)	Monosaccharides: L-galactose (main), glucose, mannose, and xylose. Polysaccharides: pectin	[24,71,72]

2.3.1. Nannochloropsis

Many species of *Nannochloropsis* sp., part of the Eustigmatophyceae family, are being studied for their ability to produce biofuel-convertible lipids and long-chain PUFAs. For

many years, *Nannochloropsis* species have been employed to make feed additives and nutraceuticals because of their robust growth patterns and high lipid yield.

The cell walls of *Nannochloropsis* are relatively robust and thick (0.06–0.11 nm), conferring the cells with significant resistance to the externally applied cell-disruption step. The structure of the *Nannochloropsis* cell wall is illustrated in Figures 1 and 2. The walls are composed of two distinct layers, which shield the cells from outside forces and create mass transfer barriers that resist biomolecule extraction. The bi-layered cell wall consists of an internal cellulose layer with algaenan as an exterior layer. Algaenan, an insoluble, non-hydrolyzable biopolymer, is generally attributed for cell-wall hardness and resistance to disruption [68]. Fourier Transform Infrared Spectroscopy (FTIR) and electron microscopy (EM) reveal that algaenan is made up of straight-chain (C30), highly saturated aliphatic hydrocarbons joined by ether bonds at the terminal and one or two mid-chain positions. Cellulose is a homopolysaccharide of β-1,4-linked glucose units (Figure 3a) forming a long chain of varying lengths that resemble microfibrils when viewed under an electron microscope [73]. The plasma membrane and the inner cellulose layer of the cell wall are joined by struts [69]. "Interlayer gap" denotes the electron-sparse space between the cellulose layer and the algaenan layer [74].

Figure 1. Schematic representation of the *Nannochloropsis* sp. cell wall. Created with BioRender.com.

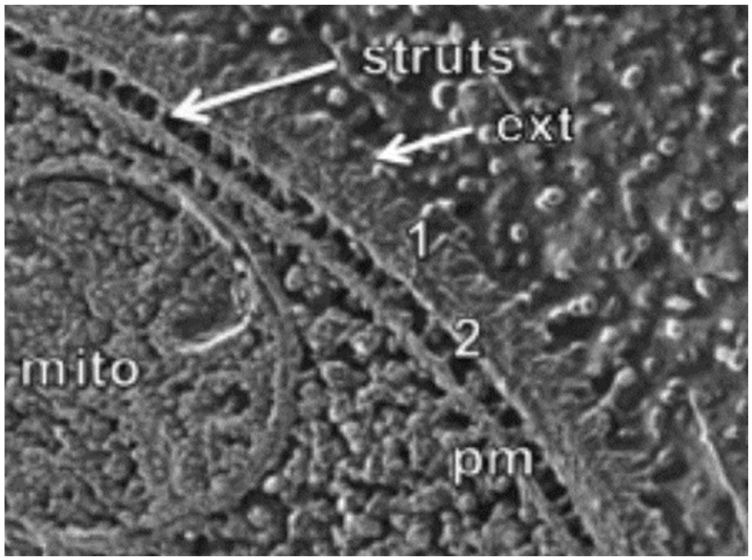


Figure 2. Quick-Freeze Deep-Etch Electron Microscopy (QFDE-EM) image of the cell wall of *Nannochloropsis*. 1,2—Layers of cell wall, ext—extensions, pm—Plasma Membrane, mito—Mitochondria. Extracted from [9].

Experiments performed by Scholz et al. showed that the cell walls of *Nannochloropsis gaditana* primarily contain glucose as the dominant monosaccharide, with a significant presence of 1,4-linked glucose confirmed through linkage analysis. These experiments provided concrete evidence of cellulose as the primary constituent of the *Nannochloropsis* cell wall. Two proteins identified as cellulose synthases, similar to those found in cyanobacteria, and nine proteins that the authors characterize as having strong similarities to plant endoglucanases, were obtained via bioinformatic analysis of the CCMP 1779 (a strain

of the *N. oceanica*) genome. As a proportion of the whole biomass, the cell wall protein content was 6.2 ± 1.7%. According to an amino acid analysis of the cell wall material, the most dominant amino acid was Asparagine/Aspartic Acid with 10.8 ± 0.7% of the total protein, followed by Glutamine/Glutamic Acid at 9.7 ± 0.8% of the total protein. Tryptophan, cysteine, and methionine were not quantitatively recovered [9].

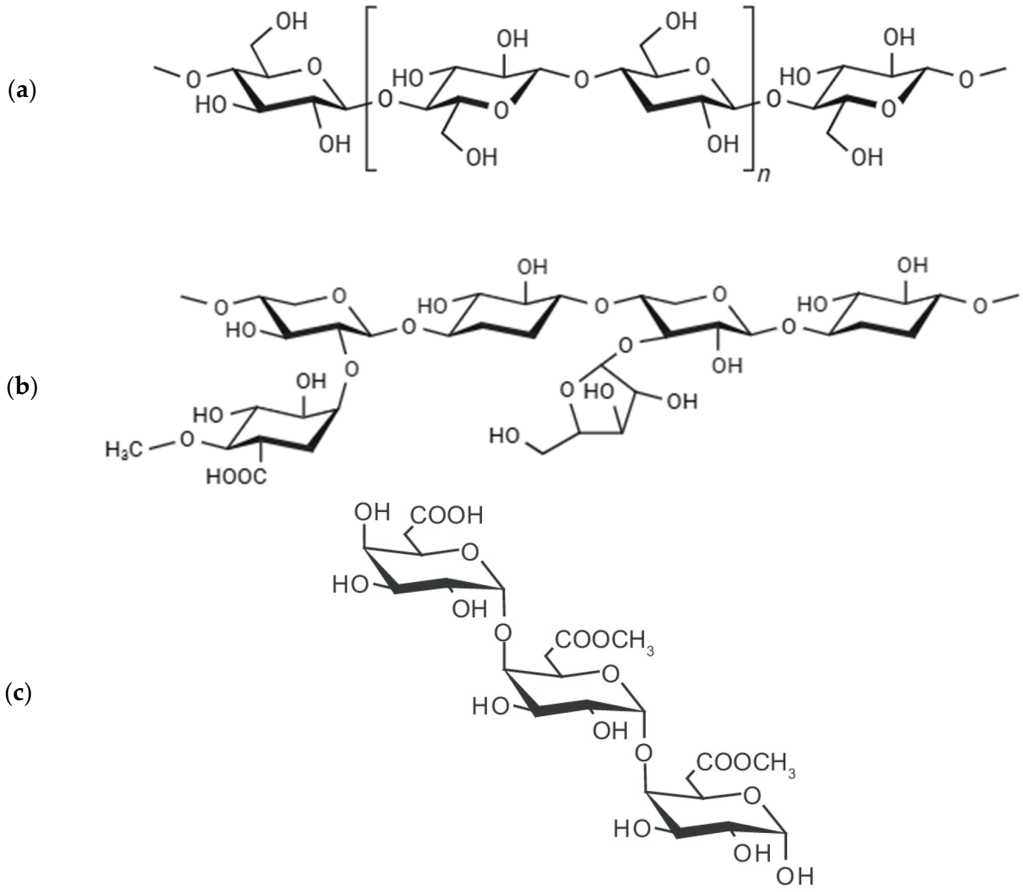


Figure 3. (**a**). Structure of Cellulose present in *Nannochloropsis* and *Scenedesmus*. Created with BioRender.com. (**b**). Structure of Hemicellulose present in *Scenedesmus*. Created with BioRender.com. (**c**). Structure of Pectin present in *Scenedesmus* and *Schizochytrium* [75].

2.3.2. Scenedesmus

The order Chlorococcales is home to the genus *Scenedesmus*. Species of this order can live as solitary cells, but later in their life cycles, they typically form coenobia, which are clusters of four to 16 cells. The structures of the *Scenedesmus* cell wall are illustrated in Figures 4 and 5. Members of the order Chlorococcales have rigid cell walls because of the presence of algaenans in the trilaminar outer layers of their cell walls. The cell walls also contain cellulose in the inner wall layers. Additional components of these tough cell walls include glycoproteins and biopolymers containing glucosamine [76]. The cell wall of *Scenedesmus* microalgae is also known to contain hemicellulose, a complex polymer that is linked with β-(1,4)-glycosidic bonds and contains pentoses and hexoses as the primary sugars. Its branched structure, which is characterised by the attachment of organic acids and saccharide residues to the main sugar chain, prevents the formation of microfibrils (unlike cellulose) and lowers the crystallinity of the wall [77,78]. This can be seen in Figure 3b. The cell walls form a pectic layer that separates each individual cell within the coenobium. The thickness and structure of this layer are dependent on the culture conditions. The pectic layer's outer surface is covered in ornaments like bristles, fangs, and spines. The coenobium's marginal cells are covered in lengthy spines, speculated to be a defence mechanism against grazing zooplankton. These spines and teeth-like structures

on the outside of *Scenedesmus*'s cell wall are embedded in the pectic layer and have been shown to be made of glycoproteins [15]. *Scenedesmus obliquus* has a multilayered cell wall that is ultra-structurally similar to *Chlamydomonas reinhardtii*'s cell wall [76].

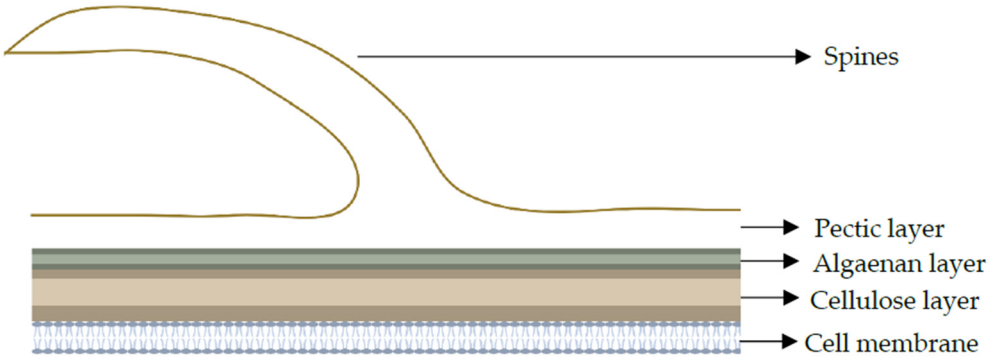


Figure 4. Schematic representation of the *Scenedesmus* sp. cell wall. Created with BioRender.com.

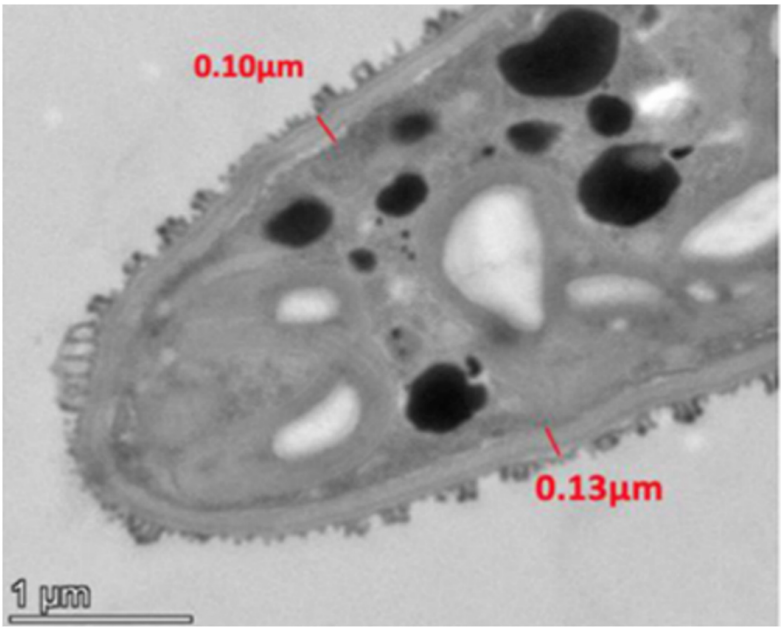


Figure 5. TEM image of the cell wall of *Scenedesmus* sp. Extracted from [15].

According to the study by Spain and Funk, fewer polysaccharides and more proteins are found at the surface of *Scenedesmus* sp. compared to other species they investigated. The spines in the outer cell wall layer are made up of glycoproteins. This would explain the significant level of proteins found in the *Scenedesmus* cell wall's outer layer. The three main monosaccharides found in *Scenedesmus* cell walls are glucose, galactose, and mannose, while the five most prevalent amino acids are glycine, glutamic acid, aspartic acid, threonine, and alanine. It has been noted that the cell walls of *Scenedesmus* sp. and *C. reinhardtii* share some structural characteristics, both having high glycine contents in their glycoproteins. The monosaccharide composition of the *Scenedesmus* cell wall varies among species. According to Takeda H, mannose is the main sugar in the wall matrices of *S. acutiformis*, *S. falcatus*, *S. obliquus*, and *S. wisconsiensis*, while glucose is the predominant sugar in the matrices of *S. acuminatus*, *S. armatus*, and *S. producto-capitatus* [70]. *Scenedesmus obliquus* has a significantly lower hydroxyproline content compared to *Chlamydomonas reinhardtii* [76].

2.3.3. Schizochytrium

Schizochytrium sp. is a heterotrophic microalga belonging to the order Thraustochytriales in the phylum Heterokonta. The species is rich in PUFAs, with DHA being the most

prevalent. The DHA-rich lipid extracted from *Schizochytrium* sp. is currently available as a commercial food/feed/nutraceutical ingredient to replace fish oil [79].

Literature regarding the makeup of the *Schizochytrium* cell wall is sparse. The wall is relatively thin and covered mostly in round scales. There are no fibrillar or firm structural elements in the scales. In addition to the wall, *Schizochytrium* also creates filamentous "rhizoids" known as sagenogenetosomes or bothrosome that develop from invaginations of the cell membrane [71]. This organelle is known to be involved in cell motility and the search for and attachment to food sources [72]. The primary components of *Schizochytrium* sp.'s cell wall are pectin and protein [80]. Pectins are heterogeneous polysaccharides. Three major structural domains make up pectins: homogalacturonan (Figure 3c), which alternates with two kinds of highly branched rhamnogalacturonan regions known as RG-I and RG-II [81]. The structure of the *Schizochytrium* cell wall is illustrated in Figures 6 and 7.

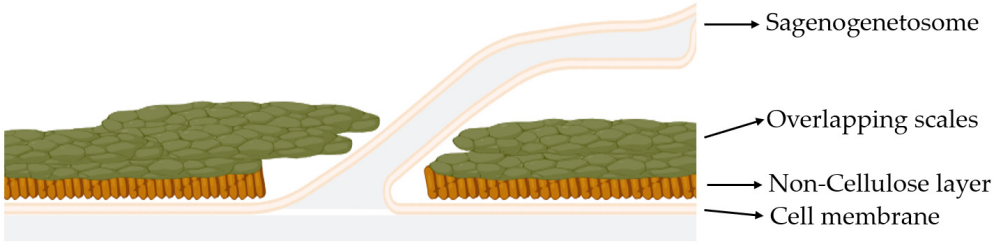


Figure 6. Schematic representation of the *Schizochytrium* sp. cell wall. Created with BioRender.com.

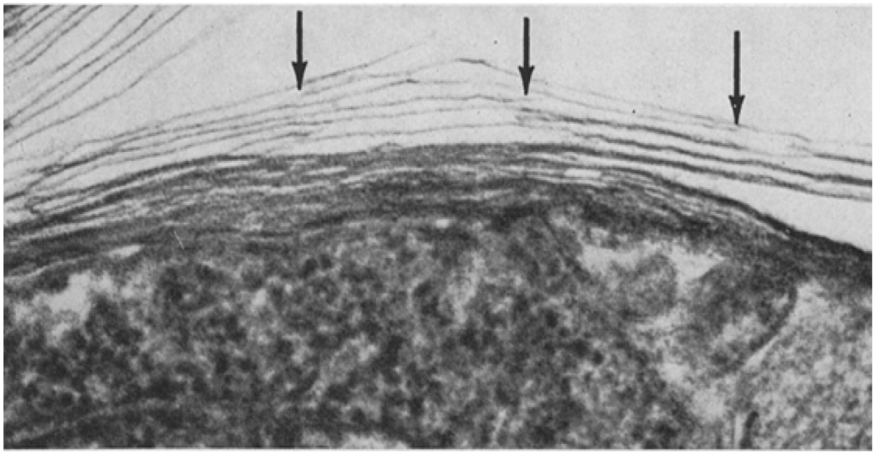


Figure 7. TEM image of the cell wall of *Schizochytrium* sp. The arrows in the figure indicate overlapping scales. Extracted from [71].

In terms of composition, the *Schizochytrium* cell wall comprises 30–43% protein and 21–36% carbohydrate by dry weight. Studies also suggest that pectin is a component of the *Schizochytrium* cell wall [80], with L-galactose being the primary monosaccharide constituent of the cell wall. Other monosaccharides like glucose, mannose, and xylose are also present. *Schizochytrium* sp. cell walls have no uronic acids and show a low degree of sulfation. The most prevalent amino acid present in the cell wall of *Schizochytrium* sp. is glutamic acid, which is followed by aspartic acid and lysine, with minor amounts of taurine and hydroxyproline [24,71,82].

2.4. Changes in Cell Wall Ultrastructure to Growth Environment

Table 4 provides a summary of the effect of growth parameters on cell-wall ultrastructure and composition.

Table 4. Effect of growth parameters on the ultrastructure and composition of cell walls.

Growth Parameters	Observed Changes	Species	References
Nitrogen	Nitrogen depletion increases the cell wall thickness	*N. salina*	[83]
Salinity	Less salinity can lead to increased cell wall thickness	*Schizochytrium*	[84]
Growth phase	From the exponential growth phase to the stationary growth phase, the cell wall thickness increased. The cell wall of cells exposed to an extended duration of intense light (orange cells) thickened by almost five times compared to that obtained from cells exposed to a shorter duration of intense light (green cells).	*S. abundans* *Scenedesmus komarekii*	[15,85]
CO_2 level	Increased CO_2 level led to an increased polysaccharide level in the cell wall.	*C. vulgaris*, *C. sorokiniana*, *C. minutissima*, and *C. variabilis*.	[86]

2.4.1. Nitrogen

Nitrogen availability plays an important role in the growth of microalgae, being a critical element in protein and nucleic acid needed for cell division. Research has shown that nitrogen content in the media has a significant impact on cell-wall thickness and composition in a microalgae cell, with nitrogen deprivation found to induce cell-wall thickening. In Halim et al., the thickness of the cellulose layer in the *Nannochloropsis* cell wall was shown to almost double under nitrogen starvation (from 33.3 ± 5.9 nm to 57.8 ± 9.6 nm). The biomass sugar content concurrently increased from 159.3 to 228.9 mg/g, validating the accumulation of structural polysaccharides [87]. TEM images of *N. salina* cells under nitrogen starvation showed that the N-limited cells exhibited a 1.54-fold thicker cell wall than that of N-replete cells due to the apparent swelling of the inner cell layer [83].

2.4.2. Salinity

Salinity of growth medium can have a significant impact on cell-wall thickness, with a decrease in salinity generally shown to induce cell-wall thickening. The low-salt treatment conducted by Dong and collaborators showed that the composition of the cell wall changes with salinity. Calcofluor-white was employed to stain *Schizochytrium* cell walls grown at 20 and 6 g/L of sea salt concentration. The fluorescence intensity of the 6 g/L culture was found to be substantially higher than that of the 20 g/L culture, indicating an enhancement in the concentrations of chitin and cellulose. These findings imply that *Schizochytrium* adapted to an environment with a low salt concentration by increasing the polysaccharides in its cell walls [84]. A study conducted by Beacham and collaborators showed that the increased osmotic potential under reduced saline growth circumstances led to a 20% thickening of the cell wall. There was a slight negative impact on lipid productivity due to the shift from 90% salinity to 10% salinity, suggesting that the cells adapted to low salinity by rerouting carbon from lipid synthesis to cell-wall accumulation. Salinity amendment can potentially be used as a growth strategy to reroute carbon from cell-wall production to lipid production for lipid-based biofuel. Growth under reduced salinity can lead to increased cell-wall thickness [88].

2.4.3. Growth Phase

Microalgal cells generally develop thicker cell walls as they enter the stationary phase or experience increasing levels of stress. Spain and Funk used TEM imaging to observe changes in cell wall shape and thickness under normal growth conditions in *S. abundans*.

The cell-wall thickness increased in all strains as they transitioned from the exponential growth phase to the stationary growth phase [15].

In the study conducted by Hanagata and Dubinsky, *Scenedesmus komarekii* cells were subjected to both high light intensity and nitrogen limitation. The cell colour changed from green to brown to orange/red as the cultivation progressed. The overall thickness of the cell wall of the orange cells was almost five times that attained in the green cells. The thickness of the inner polysaccharide layer of the cell wall, however, remained identical to that of the green cell. The cell-wall thickening can be attributed to the proliferation of electron-dense granules between the inner and outer cell wall layers [85].

2.4.4. Concentration of Carbon Dioxide

Carbon dioxide concentration can also affect the composition of microalgal cell walls. A study conducted by Cheng et al. showed that the cell walls of four different *Chlorella* species had a higher level of uronic acid when grown in 2% enriched CO_2 relative to ambient air. For *C. vulgaris*, *C. variabilis*, and *C. sorokiniana*, the amount of uronic acid significantly increased under 2% CO_2-enriched conditions, resulting in an increase in the cell-wall carbohydrate content as a proportion of the cell-wall dry weight [86].

3. Cell Wall Disruption

3.1. Cell-Disruption Technology

The selection of cell-disruption methods is based on several factors, such as the scale at which the biomass is processed and the type of compounds to be extracted. Cell-disruption methods can generally be classified under three types—Physical/mechanical, chemical, and biological (mainly enzymatic). The methods can be applied in combination with one another to disrupt the cell walls and extract valuable intracellular compounds. Each method has its own set of advantages and disadvantages (as displayed in Table 5).

Physical/mechanical methods use shear force (high pressure, high temperature, or a combination), electrochemical pulses, or electromagnetic radiation to break the cell walls. Chemical methods generally involve the use of chemicals to indiscriminately degrade the cell wall components, such as acid or alkali. Biological methods are mainly based on the use of enzymatic treatment to selectively target specific components in the cell wall to achieve disruption. Enzymatic treatment can be carried out using a single enzyme or a mixture of enzymes (known as enzyme cocktails) that bind to specific molecules on the cell wall.

3.1.1. Mechanical Methods

Physical or mechanical methods involve the application of force and/or energy on the cells directly to break the cell walls, such as bead-beating, milling, ultrasonication, high-pressure homogenization, spray-drying microwaving, autoclaving, and freezing. These methods are non-specific and may cause the cell wall and other lipid- and protein-storing cell compartments to degrade. As a result, it is difficult to achieve targeted extraction as all soluble intracellular components will be released into the suspension medium immediately upon disruption. A sequential biorefinery procedure that permits selective protein release prior to solvent extraction without adversely influencing biomass composition should be developed in order to extract both proteins and lipids from microalgae [89].

3.1.2. Chemical Methods

Chemical methods use chemicals, such as hydroxides, hypochlorites, chaotropes, chelating agents, and detergents, to degrade the components on the cell walls. The active chemical component contaminates the cell solution, requiring an additional recovery step that complicates downstream processes and adds to the overall cost of unit operations. In addition, the chemicals can also be highly corrosive and expensive [90].

3.2. Enzymatic Cell Disruption

Enzymatic treatment is one of the most promising approaches to disrupt microalgal cell walls. Enzymes target specific structures in the cell walls to achieve either cell wall disruption or cell permeabilization. Enzymes are biological catalytic proteins (or biocatalysts) with the capacity to lower the activation energy required for a reaction to happen, therefore speeding up the specific reactions needed to produce desired substances [91]. Enzymes are generally produced by fermentation using fungi or bacteria. These molecules have grown in importance in different types of industries for different reasons, as outlined below [91–93].

Chemical industry: enzymes make the process greener by replacing heavy metal catalysts and harmful solvents with greener alternatives [94] alongside the high selectivity for a specific enantiomer. Pharmaceutical industry: Enzymes offer a lower environmental impact on the production chain. Food industry: Enzymes provide the possibility to produce food enriched with various substances, such as prebiotics, and to have more efficient processes [95]. Feed industry: Enzyme mixtures help animals' digestion and protect them from harmful compounds. Cosmetic industry: Enzymes facilitate the production of natural products with greener processes.

A fundamental aspect that makes enzymes an attractive alternative to conventional mechanical or chemical disruption methods in microalgae is their mild operational conditions. Since enzymes are proteins, the settings required for them to be fully operational (pH, temperature, substrate availability) [96] are relatively mild, reducing the risk of product degradation while lowering the total energy cost of the process. Enzymes are also substrate-specific, able to attain disruption by attacking target components/linkages in the cell walls while preserving the rest of the cell walls and intracellular components.

Despite their considerable benefits, enzymes also draw various disadvantages [97]. The high cost of purified enzymes, relatively low efficiency, strict operational conditions for optimum operation, and long reaction time are some of the major roadblocks in the application of enzymes in microalgal biotechnology [98].

Maintaining optimal reaction conditions requires careful control and fine-tuning of process parameters [99]. A study by Lin et al. discovered that oil yield and DHA yield from microalgae *Schizochytrium* sp. fell when temperature either rose over 55 °C or dropped below 45 °C. The maximum enzyme activity had a sharp peak at 55 °C. The amount of DHA produced rose with the pH of the enzymatic treatment, peaking at pH 10 and then declining as the pH rose further. With an increase in enzymatic time, DHA yield peaked at 9 h and thereafter declined [80,100].

Enzymatic approaches to disrupt the microalgal cell wall need to be fine-tuned to achieve high disruption efficiency while keeping the enzyme cost to a minimum [101,102]. It is already known that disrupting microalgae cell walls using a mixture of enzymes (or enzyme cocktails) rather than a single enzyme can bring about higher disruption efficiency and, consequently, a higher yield of relevant biomolecules [98,101,103,104]. The selection of enzymes in the mixture, however, must be carefully considered to ensure that the enzymes do not produce molecules that hinder one another's catalytic activity, thus decreasing the overall enzymatic efficiency of the mixture [98].

The high cost of enzymatic treatment can potentially be mitigated by the adoption of a minimal design approach, where the enzyme types and dosage are carefully optimised so that disruption can be achieved at the lowest possible enzyme requirements. A minimal design approach avoids the addition of excessive/wasteful enzymes by configuring the enzyme cocktails to the specific ultrastructure and composition of the targeted cell walls. For example, enzyme cocktails targeting *Chlorella* cell walls should contain enzymes that attack N-acetylglucosamine polymers (e.g., Chitinases, Lysozymes), while those aimed at hydrolysing *Nannochloropsis* walls can be comprised of β(1->4) glucan-degrading enzymes (e.g., Cellulase). A detailed understanding of the cell-wall architecture and the composition of the target species is needed to design a tailored enzyme cocktail, underscoring the importance of cell-wall studies for the development of cost-effective microalgal biorefinery.

Table 5. Advantages and disadvantages of different cell-disruption methods.

Method	Advantages	Disadvantages
Physical/Mechanical—Bead milling, Autoclave, Microwave, Sonication, Ultrasonication, and High-pressure homogenization	1. The process is quick 2. High disruption efficiency 3. The process has been demonstrated at a large scale [105]	1. High infrastructure cost 2. High energy inputs 3. The heat produced can degrade the final products [106]
Chemical—Acids, Surfactants, Detergents	1. Low energy input 2. Low infrastructure cost 3. Easier to upscale compared to mechanical methods [106]	1. High cost of the chemicals 2. Use of corrosive chemicals 3. High risk of product degradation
Enzymatic (or Indirect Biological approach)	1. High specificity, which allows for exclusive targeting of cell-wall components and minimises the risk of product degradation 2. Low energy input 3. Gentle and mild operating conditions, preventing oxidation and denaturation of the product [107]	1. Enzymes are expensive 2. Enzyme recovery requires complicated downstream purification, adding to operational cost 3. Enzymes have a narrow optimal range and can slow the reaction if not provided

3.3. Enzymatic Cell Disruption for Nannochloropsis, Scenedesmus, and Schizochytrium

Studies investigating enzymatic cell disruption of *Nannochloropsis*, *Scenedesmus*, and *Schizochytrium* are reviewed in this section. Table 6 provides a summary of the treatment conditions (i.e., water content, enzyme type, pH, temperature, enzyme dosage, incubation time) and the key results obtained from the studies.

Table 6. Summary of studies investigating enzymatic cell disruption or enzyme-assisted lipid extraction of *Nannochloropsis*, *Scenedesmus*, and *Schizochytrium* biomass.

Species	Biomass State	Enzymes	Mode of Action	pH, Temperature, Enzyme Ratio	Incubation Time	Key Results/ Optimum Conditions	Reference
Nannochloropsis sp.	Lyophilized powder	Cellulase (Cellulyve 50LC), Mannanase (Feedlyve 50GMA)	Cellulase hydrolyzes β-1,4 glycosidic linkages in cellulose. Mannanase hydrolyses (1->4)-beta-D-mannosidic linkages in mannans.	55 °C pH 4.4, Cellulase: Mannanase = 1:9	24 h	Lipid yield: 70–75% of available lipids	[103]
Nannochloropsis sp.	Lyophilized powder	Cellulase (Cellulyve 50LC), Mannanase (Feedlyve 50GMA)	Cellulase hydrolyzes β-1,4 glycosidic linkages in cellulose. Mannanase hydrolyses (1->4)-beta-D-mannosidic linkages in mannans.	15, 30, 45, 60, 75 °C pH 2, 3.5, 6.5, and 8 Dosage of Cellulose: 0–5–10–15–20 mg/g biomass Dosage of Mannanase 0–0.5–1-1.5–2 mg/g biomass	30, 90, 150, 210, 270 min	Lipid yield: 36.6 g/100 g biomass 53 °C, pH 4.4, 210 min incubation time, 13.8 mg/g of Cellulase, and 1.5 mg/g of Mannanase	[96]
Nannochloropsis sp. NANNP2	Wet biomass on agar	Chitinase, Chitosanase, β-Glucosidase, β-Glucoronidase, Hyaluronidase, Lysozyme, Lyticase, Pectinase, Sulphatase, Trypsin, Zymolyase	Chitinase hydrolyses β-1,4 linkages of the N-acetylglucosamine units of chitin. Chitosanase endohydrolyses of-1,4-linkages between GlcN residues in a partially N-acetylated chitosan. β-glucosidase hydrolyses glucose dimers Lysozyme cleaves the β-(1,4)-glycosidic bonds in peptidoglycan. Lyticase is an enzyme complex of endoglucanase and protease. Pectinase acts in two different mechanisms, hydrolysis and trans-elimination lysis, in which they break the glycosidic bond by trans-elimination reaction Zymolase is a mixture of β-1,3-glucan laminaripentao-hydrolase and β-1,3-glucanase.	23 °C under light	5 days	-	[28]

Table 6. *Cont.*

Species	Biomass State	Enzymes	Mode of Action	pH, Temperature, Enzyme Ratio	Incubation Time	Key Results/ Optimum Conditions	Reference
N. gaditana CCMP 526	not stated	Cellulase (0615 Sigma), Cellulase (16419.02 Serva), Chitinase, Chitosanase, Lyticase, Protease, Zymolyase	Protease. hydrolyses peptide bonds in protein	Room Temperature pH 7	24 h	Cellulase 1 and Cellulase 2, hydrolyzed up to 76% of the mass of the cell wall	[9]
Nannochloropsis sp.	not stated	Cellulase, Esterase, Mannanase or Cellulase, Esterase, Galactanase	Esterase catalyses the de-esterification of pectin by the removal of methoxy esters. Galactanase catalyses the hydrolysis of β-1,4 galactosidic bonds in arabinogalactan and galactan side chains.	50 °C pH 5	1 h	Lipid yield: 37.8 g/100 g biomass or 34g/100 g biomass	[101]
Nannochloropsis sp.	fresh paste	Cellulase, Snailase, Neutral protease, Alkaline protease, Trypsin	Snailase is a complex mixture of more than 20 enzymes, including Cellulase, Invertase, Hemicellulase, Pectinase, Polygalacturonase, Protease.	55 °C, pH 4.8 37 °C, pH 5.8 50 °C, pH 7 55 °C, pH 8.5 37 °C, pH 8 Ultrasonication pretreatment	not stated	Lipid yield: 12% of available lipids	[108]
Nannochloropsis gaditana	dry biomass	Viscozyme, Celluclast (cellulase), Alcalase	Viscozyme is a mixture of Arabanase, Cellulase, β-Glucanase, Hemicellulase, and Xylanase. Alcalase is an endo-protease.	pH 5, 55 °C	6 h	Lipid yield: 29% of available lipids	[109]
Nannochloropsis oculata Nannochloropsis sp. *Nannochloropsis oceanica*	wet biomass	Cellulase, Papain, Hemicellulase, Pectinase	Papain is a protease.	Equal ratio of all enzymes. pH 5.5, 45 °C, 150 rpm	12 h	Three Phase Partitioning extraction method used. Lipid yield = 221.4 mg/g biomass. Combination of enzymes were more efficient than single enzymes.	[110]
Nannochloropsis oceanica	powder	Cellulase, Laccase		Laccase: Cellulase = 1:2.5. pH 5, 45 °C	6 h	Lipid yield: 26.9% (unclear if this is % of biomass or % of available lipids) EPA contents—20.7 ± 0.13 g/100 g of lipids	[111]
Nannochloropsis oceanica	powder	Cellulase, Laccase	Laccase catalyses oxidation reactions of lignin.	500 U/mL Cellulase 0.24 U/mL Laccase, pH5, 50 °C	24 h	Lipid yield—66.29% EPA content—42.63% (relative to the control)	[88]
Nannochloropsis oceanica	spray-dried biomass	Cellulase		pH 5, 37 °C pH 5, 50 °C	5 h	Lipid yield: 57.6% of available lipids Carotenoid yield: 38.8 mg g^{-1} Lipid yield: 62.1% of available lipids Carotenoid yield: 28.3 mg g^{-1}	[112]
Scenedesmus sp.	Freeze-dried biomass	Cellulase, Xylanase, Pectinase	Xylanase hydrolyses β-1,4 glycosidic bond of xylan backbone	pH 4.4, 45 °C	180 min	Lipid yield -13.8g/100g of biomass, Lipid recovery 86.4%	[113]
Scenedesmus sp.	Wet biomass	Lysozyme, Cellulase		pH 7.48, 37 °C	30 min	Lipid yield: 16.6 and 15.4% using Lysozyme and Cellulase, respectively of the available lipids	[114]

Table 6. *Cont.*

Species	Biomass State	Enzymes	Mode of Action	pH, Temperature, Enzyme Ratio	Incubation Time	Key Results/ Optimum Conditions	Reference
Scenedesmus dimorphus	Fresh paste (18% solid content)	Snailase, Cellulose, Neutral protease, Alkaline protease, Trypsin	A broad class of enzymes known as proteases is responsible for catalyzing the breakdown of peptide bonds found in proteins and polypeptides. Acid proteases are defined as those with a pH optimal range of 2.0–5.0. Neutral proteases have a pH optimal range of 7.0. Trypsin breaks proteins into smaller peptides by catalyzing the hydrolysis of peptide bonds.	4, 37 °C	12 h	Lipid yield (Sonication + enzyme treatment)—46.81% total lipids	[108]
Scenedesmus quadricauda CASA CC202	Dry biomass	Pectinase and Cellulase		5, 50 °C	14 h 24 h	Pectinase treatment, total reducing sugar 129.82 mg/g. Sonication + pectinase +Cellulase—Total reducing sugar yield was 379.45 mg/g.	[115]
*Scenedesmus obliquus*CCAP 276/3A	Fresh paste (15% solid content)	Cellulose, Neutral protease, Lysozyme Pectinase, and Trypsin		7.0, 37 °C (only for Neutral protease)	72 h	Neutral protease-assisted cell disruption effectively extracted 75% lipids from 15% S. obliquus CCAP 276/3A	[116]
Schizochytrium sp. ATCC20888	-	Alkaline protease	Alkaline proteases are proteases that have an ideal pH range of 8.0–11.0.	8, 55 °C	9 h	The oil yield and DHA yield were 14.52 g/L and 7.12 g/L.	[80]
Schizochytrium sp.	Spray-dried biomass	Hemicellulase	Enzymes called Hemicellulases degrade substances that are normally associated with cellulose. These enzymes include Pectinase, Arabinase, Xylanase, Beta-glucanase, beta-mannanase, Pectin methylesterase, Pectin lyase, and Polygalacturonases	-, 55 °C	2 days	Lipid yield: 21.72 ± 0.74% of total lipids	[117]

The genus *Nannochloropsis* is composed of six different species (*N. oculata*, *N. oceanica*, *N. salina*, *N. gaditana*, *N. limnetica*, and *N. granulata*) with the common feature of being able to produce high quantities of lipids, above all omega-3 polyunsaturated fatty acids (ω-3 PUFAs) in the form of eicosapentaenoic acid (EPA) [118]. Furthermore, these species can produce other high-value products, such as essential amino acids, carbohydrates, pigments, and vitamins [118,119]. These classes of compounds should also be taken under consideration when thinking about the industrial use of *Nannochloropsis*, as they would increase the value of the biomass through the adoption of a multi-stream biorefinery fractionation process [118]. Despite their potential, *Nannochloropsis* sp. has a highly resistant cell wall with the ability to withstand both mechanical and chemical disruption [103]. Generally, the enzymes used to treat and degrade *Nannochloropsis* cell walls act on the principal sugar and protein components of the cell walls (i.e., Cellulase, Mannanase, and Lysozyme) [101,103,104].

In Scholz et al., isolated *Nannochloropsis* cell walls were treated with enzymes (such as Chitinase, Chitosanase, Lysozyme, Lyticase, Protease, Sulfatase, Cellulase, and Cellulase Onozuka R10) at room temperature and pH 7. The two most efficient Cellulase formulations, Cellulase and Cellulase R10 hydrolyzed up to 76% of the mass of the cell wall [9], confirming Cellulose as the primary component of the *Nannochloropsis* sp. cell wall. Gerken et al. was able to identify the components of *Nannochloropsis* cell walls by assessing its sensitivity to various enzymes. Lysozyme, Chitinase, and Sulfatase were able to permeabilise most cells,

indicating the presence of an N-acetylglucosamine-containing polymer in the cell walls of *Nannochloropsis* sp. [28].

A study conducted by Maffei et al. investigated the effect of Cellulase and Mannanase on the cell wall of lyophilized powder of marine *Nannochloropsis* sp. These enzymes had been selected based on preliminary experiments that showed mannan-type hemicelluloses and cellulose as the primary component of the inner wall of *Nannochloropsis* sp. cells. In the study, 0.2 g of microalgae biomass was treated with 10 mL of enzyme solution prior to lipid extraction. Lipid yield was found to increase from 40.8% for the control sample to over 73% in the treated sample [103]. Similarly, in a study by Zuorro et al., the temperature, pH, enzyme dosage, and incubation time for Cellulase and Mannanase enzyme treatment of *Nannochloropsis* biomass was optimised using response surface methodology and central composite design. Optimal enzyme concentration was found to be 13.8 mg/g biomass for Cellulase and 1.5 mg/g biomass for Mannanase [96]. Lipid extraction using hexane/isopropanol mixture on the enzyme-treated biomass successfully recovered 90% of available lipids (or 36.6 g lipids/100 g of biomass).

In another study by Zuorro et al., they investigated enzyme-assisted lipid extraction from *Nannochloropsis* sp. For every 0.2 g of microalgae biomass,10 mL of enzyme solution was used. Treated biomass was then subjected to lipid extraction using a hexane/isopropanol mix. An enzyme cocktail consisting of Cellulase, Endo-1,3(4)-β-glucanase, and Endo-β-1,4-mannanase was shown to have a greater lipid-extraction yield (37.8 g lipids/100 g of biomass) compared to a Cellulase, Endo-1,3(4)-β-glucanase, and Endo-1,4-β-galactanase enzyme mix (34 g lipids/100 g of biomass). The authors demonstrated that successful disintegration of the *Nannochloropsis* cell wall required the simultaneous presence of different forms of Cellulase and Hemicellulases [103]. In the study conducted by Blanco-Llamero et al., *Nannochloropsis gaditana* biomass was treated with three commercially available enzymes—Viscozyme, Celluclast, and Alcalase. For each gram of dried microalgal biomass, 46 mg of a single enzyme or a combination of enzymes was added [109]. He et al. investigated enzymatic hydrolysis of three species of *Nannochloropsis* (*N. oculata*, *Nannochloropsis* sp., and *N. oceanica*) for biodiesel production. A mixture of four enzymes—Cellulase, Papain, Hemicellulase, and Pectinase—was applied on *N. oculata* biomass. The highest lipid yield of 221.4 mg lipids/g of biomass (equivalent to a fatty acid methyl esters recovery of 50.21%) was achieved at an equal ratio of all enzymes and incubation conditions of pH 5.5, 45 °C, 150 rpm [110].

Zhao et al. optimised enzymatic pretreatment to weaken the cell wall of *Nannochloropsis oceanica* using Cellulase, Laccase, Pectinase, Mannanase, and Xylanase enzymes prior to lipid extraction with ethanol. The response surface method was used to develop a predictive model. As described in Table 6, the dose of the enzyme was 21 mg/g of biomass (in powder form). They showed that the enzyme mixture containing Laccase and Cellulase in the ratio of 1:2.5 produced the highest lipid yield (26.9 ± 0.20% of available lipids) [111]. These findings were further supported in a follow-up study by Zhao et al., which confirmed that a Cellulase (500 U/mL) and Laccase (0.24 U/mL) cocktail at optimal parameters (Table 6) was able to hydrolyse *Nannochloropsis* biomass and increased lipid yields by 69.31% and EPA content of the extracted lipids by 42.63% [102]. Gallego et al. studied enzymatic treatment as a means to weaken the cell walls of *Nannochloropsis oceanica* before pressurized liquid extraction (Table 6). Enzymatic treatment was carried out at 37 °C with an enzyme dosage of 500 µL of Cellulase per g of spray-dried biomass. The process resulted in the recovery of 57.6% of available lipids. The lipid extracts also contained Carotenoid, up to 25.3 ± 0.2 mg Carotenoid/g extract [112].

Liang et al. investigated enzyme-assisted extraction of lipids from wet microalgal biomass using Hexane as an extraction solvent. The following enzymes were used to treat the fresh paste (biomass concentration = 18 wt.%) of *Scenedesmus dimorphus* and *Nannochloropsis* sp.: Cellulose, Snailase, neutral Protease, alkaline Protease, and Trypsin. Prior to that, the samples were sonicated for 15 min at 600 W. The enzyme concentration, combination, pH, and duration of incubation during enzymatic treatment were optimised

to obtain maximum lipid yield. The incubation temperature was set at each enzyme's optimal temperature. Trypsin and Snailase achieved the highest lipid yield (11.73% of available lipids for *Nannochloropsis* sp. and 46.81% of available lipids for *Scenedesmus dimorphus*) at an enzyme dose of 4% (Table 6) [108].

In Trivedi et al., five different enzymes (Cellulase, neutral Protease, Lysozyme, Pectinase, and Trypsin) were investigated as a pre-treatment for lipid extraction from wet *S. obliquus* biomass using a combination of water-immiscible ethyl acetate and chloroform. The enzymatic treatments were compared with surfactant-based cell disruption. *S. obliquus* slurry at 15% dry biomass concentration was treated enzymatically at the optimum pH of each enzyme (Table 6). A maximum lipid recovery of 75.7% of total lipid was obtained by adding a neutral protease enzyme to *S. obliquus* slurry at a dosage of 5% (*w*/*w* of biomass). The authors ascribed the high lipid yield obtained from *S. obliquus* when treated with neutral protease to the high protein content in the biochemical makeup of the targeted microalgae cell wall [116]. Reshma and Arumugam examined the effect of Pectinase and Cellulase on the cell wall of *Scenedesmus quadricauda.* They selected these enzymes based on the structure of the cell wall of *Scenedesmus quadricauda*, which is made up triple layers, with the innermost layer being Cellulose and the outermost layer being Pectin. Initially, they optimized the dosage of the enzyme, pH, incubation temperature, and incubation time to increase the lipid yield (Table 6). Incubation time of 14h with 1:5 [Enzyme]:[biomass] ratio attained the highest total reducing sugar yield of 129.82 mg/g biomass using only Pectinase. This yield can be increased to 352.44 mg/g biomass when Cellulase and Pectinase enzyme mixture was used, and the enzymatic treatment was coupled with sonication for 2 min [115].

Taher et al. researched enzymatic disruption of *Scenedesmus* sp. using Lysosyme and Cellulase. The researchers used wet biomass to avoid the expensive drying step. Using enzymatic hydrolysis followed by lipid extraction with hexane or supercritical carbon dioxide, lipid extraction yields of 16.6% and 15.4% of total lipid content using Lysozyme and Cellulase, respectively, were obtained [114].

Zhang et al. investigated enzymatic hydrolysis for cell disruption of *Scenedesmus* sp. biomass. They used Cellulase, Xylanase, and Pectinase for cell disruption. An experimental framework based on central composite design was conducted to investigate the effect of temperature, pH, length of pre-treatment, and amount of enzymes during enzymatic treatment of *Scenedesmus* sp. biomass on lipid yield with a Chloroform/Methanol mixture. Lipid extraction yields of 13.8 $\pm$ 0.4 g/100 g of biomass (equivalent to 86.4% of total available lipids) were achieved under optimum enzymatic treatment conditions outlined in Table 6 [113].

Lin et al. studied *Schizochytrium* sp. cell wall disruption using an alkaline protease enzyme. Studies were conducted to optimize pH value, enzyme dose, enzymatic lysis incubation temperature, and enzymatic lysis duration to increase the lipid and DHA yields by subsequent freeze drying and extraction with Soxhlet. The results demonstrated that lipid yield and DHA yield achieved their maximum levels at 14.52 g/L solvent and 7.12 g/L solvent, respectively, under the following enzymatic treatment conditions: lysis temperature of 55 °C, lysis duration of 9 h, enzyme dosage of 3% of biomass, and pH 8 [80].

The study by Hac İsa et al. compared acid treatment, osmotic shock, enzyme applications, and ultrasonication to increase the lipid yield from the spray-dried *Schizochytrium* biomass. They compared two lipid-extraction methods: Bligh and Dyer and Soxhlet. Biomass treatment with 10% Hemicellulase enzyme mixture coupled with Bligh and Dyer extraction achieved a lipid yield of 21.72 $\pm$ 0.74% of total available lipids with a DHA content of 19.25 $\pm$ 0.09% in the extract. This pre-treatment and lipid-extraction combination was able to produce the highest lipid yield when compared to other methods [117].

Across all of the studies shown in Table 6, enzymatic treatment was carried out on both wet and dry algal biomass. While sun drying can be used as a low-cost method to dry microalgae biomass prior to biorefinery processing (such as enzymatic treatment and lipid extraction), this method is time-consuming, can introduce significant biomass

degradation, and relies on uncertain and seasonal weather conditions. Other drying methods (e.g., thermal drying or spray drying) are energy-intensive and can account for up to 89% of the necessary energy input and 70–75% of the total processing cost. The high energy cost associated with biomass drying renders their commercial use in microalgal processing prohibitive, particularly when the target biorefinery end-products are fuels (where achievement of a favourable overall energy balance is paramount) and high-volume, low-value products (e.g., animal feeds and aquaculture feed). Additionally, thermal drying can also inadvertently degrade lipid structure and denature protein [114]. Future studies on microalgal biomass processing should therefore focus their investigation on wet extraction pathways, where cell disruption is directly applied on wet biomass rather than dried biomass form, in order to emulate industrially relevant conditions. A study by Lardon et al. showed that wet oil extraction (i.e., lipid extraction on wet microalgal biomass) greatly lowered heat and cost requirements [120].

Across all studies reviewed in Table 6, higher cell disruption is generally achieved when a combination of enzymes was used instead of a single enzyme, confirming the benefits of using enzyme cocktails with multiple modes of action. Even when a single enzyme was used, it was often accompanied by other mechanical (e.g., sonication) or chemical cell-disruption methods to enhance cellular disintegration and enzyme-substrate contact. Improved lipid yield and EPA content were obtained when Laccase and Cellulase were combined to pretreat *N. oceanica* compared to pretreatment with individual enzymes. This can likely be attributed to Laccase ability in hydrolysing the outermost algaenan layer, exposing the inner amorphous cellulose layer to Cellulase activity [111,112].

3.4. Use of Enzymes on Other Microalgal Species

Microalgal cell walls are varied in architecture, both in terms of ultrastructure and composition, with many genera displaying considerable interspecies variation. For example, in *Chlamydomonas* sp., the cell wall is composed of six distinct layers, some of them made of intricate Cellulose-Pectin complexes while others are made of Hydroxyproline-rich glycoproteins [121]. Another example is *Chlorella* sp., whose cell wall can be composed of either a single microfibrillar layer or two separate layers, with an inner microfibrillar layer and an outer trilaminar layer [28]. The enzyme mixture used to disrupt microalgal cell walls will have to be tailored to the specific ultrastructure and composition of the target microalgae species. Various studies have used alpha-amylase and proteases, as well as Autolysin, Carbohydrases, and Lipases/phospholipases for *Chlamydomonas*. For *Chlorella*, the most commonly used enzymes are Amylase, Xylanase and Cellulase, with Chitinases and Lysozyme added to enhance performance [28,121,122]. The major parameters that ensure the success of an enzymatic treatment of the cell wall are the enzyme dosage, incubation time, incubation temperature, and pH of the media. Most of the studies summarized in Section 3.3 have selected optimum pH and temperature of Cellulase (pH 5 and 50–55 °C) for their treatment given that the enzyme was used as the primary component of their mix.

3.5. Recycling and Immobilization

Enzyme recycling and/or immobilisation are often used to reduce the quantity (and thus, the cost) of enzymatic treatment. Enzyme recycling generally consists of recovering the biocatalysts from the different phases generated during biomass processing steps. In order to be recoverable, the enzymes need to be reversibly bound to the substrates and must be stable throughout the entire process [123]. The easiest enzymes to recover tend to be those which remain soluble in water and have partitioned in the liquid fraction. During enzyme reaction, an equilibrium will be reached where certain amounts of enzymes will bind to the substrate while the rest remains free in the medium. The first strategy of enzyme recycling is based on the sole recovery of the fraction suspended in the medium. In the case of cellulase used to hydrolyze lignocellulose at an industrial scale, the efficiency of the recovered enzymes varies from 48–59% after the first round to 35/40% after up to five rounds of hydrolysis reactions [123–126]. The second strategy of enzyme recycling

generally consists of recovering enzymes from the spent biomass. This involves using solvents or reagents to modify environmental conditions (pH and temperature) to facilitate the desorption of enzymes from the spent biomass back into the medium, which can then be recovered together with other soluble enzymes. In the third strategy, fresh substrate (e.g., a new batch of biomass) is added to the spent substrate (e.g., spent biomass) to promote the transfer of the catalysts from the spent substrate to the fresh substrate [124,125].

Enzyme immobilisation is another industrially relevant method that can be applied to reduce enzyme requirements. Compared to recycling, these techniques are better characterized and consequently, more frequently utilized. Briefly, the enzymes are first physically trapped in a non-reactive matrix (normally made of silica) prior to the reaction, which in turn allows for facile post-reaction separation from the liquid medium or the spent substrates. The enzymes can also be chemically immobilized in the matrix. In this case, immobilization is obtained by making the enzymes react with the stationary phase to form a covalent bond (reversible or irreversible) that traps the proteins. Either way, the goal for both of these alternatives is to trap the enzymes so that they can catalyse the desired reaction without being suspended in the solution and being washed away in further processing steps. Enzyme immobilisation offers several advantages over enzyme recycling. In addition to conferring simpler and, consequently, cheaper downstream processing for enzyme recovery, the process is also more readily transferable to a continuous operation mode, which in turn allows for higher throughput. The main disadvantages of enzyme immobilisation, however, reside in the hydrolytic efficiency of these methods. Since enzymes are immobilized on a solid surface, the total surface available to bind the substrate will be steric, which will hinder reaction and slow down enzyme activity. Moreover, the solid phase used for immobilisation also has to be frequently replenished, adding to the overall cost of the operation [127].

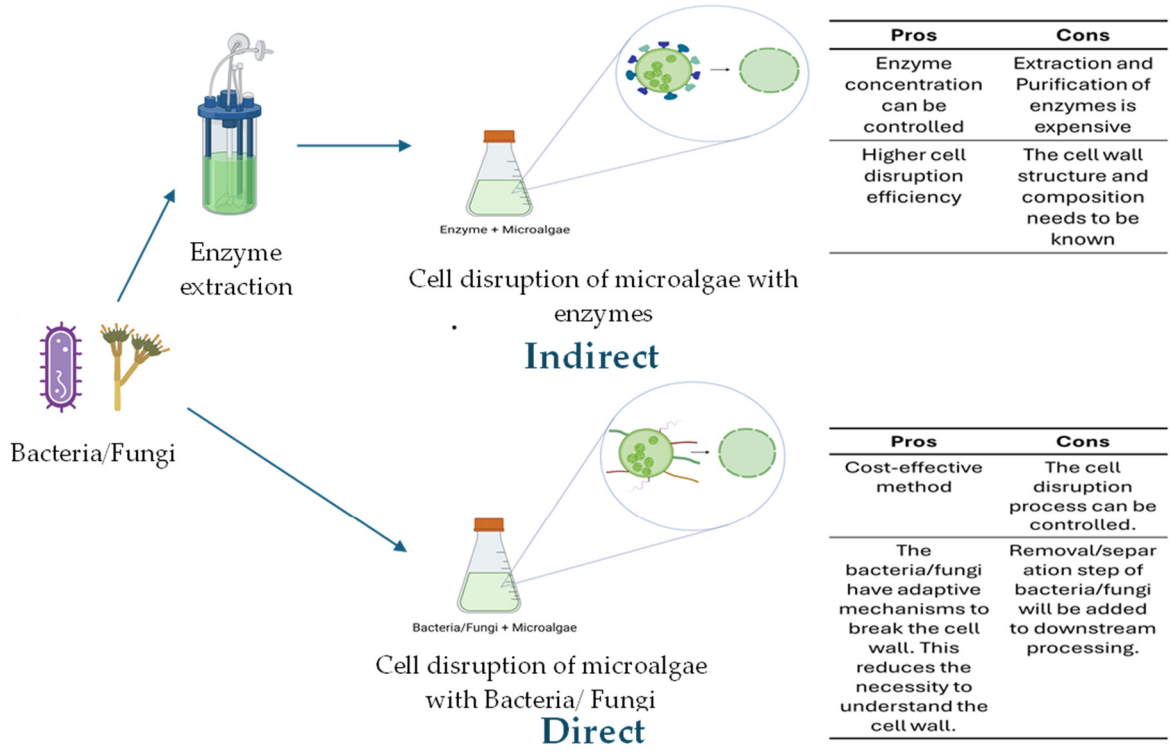
Blanco-Llamero et al. investigated carrier-free immobilization of commercial enzymes Viscozyme, Celluclast, and Alcalase for microalgal processing. They suggested a method to catalyze the enzymatic breakdown of the *N. gaditana* cell wall, which involved combining three enzymes in an enzyme mix. The immobilized enzymes were shown to be 10 times more stable than soluble enzymes. When applied on microalgae biomass as a pre-treatment, immobilized enzymes attained a lipid yield ranging from 27.15% to 33.23% [128].

Fu et al. immobilized cellulase onto an electrospun polyacrylonitrile (PAN) nanofibrous membrane and investigated their application for the hydrolysis of *C. pyrenoidosa* cell walls, which are made up of 45% cellulose. PAN nanofibers' nitrile groups were made active by amidination and covalent bonding to the amino groups of enzymes. The microalgal cell walls were then hydrolyzed using the immobilized cellulase under ideal circumstances, including temperature, pH, and substrate concentration. The immobilized cellulase exhibited a stable structure and strong activity in the pH range of 4.6 to 6.6 [129].

4. Direct Biological Approach for Cell-Wall Disruption

Conventional cell-wall disruption involves the use of mechanical methods (such as ultrasonication and microwaves). They are effective but can be energy-intensive, have limitations regarding scalability, and can often lead to product degradation. Therefore, research on the disruption of microalgae cell walls by biological organisms is crucial for developing efficient and sustainable extraction methods. Biological disruption can be divided into two types: a direct biological approach, whereby microalgal cells are exposed to the lysing organisms, or an indirect approach, where microalgal cells are exposed to enzymes that have been isolated from the enzyme-secreting organisms. The indirect enzymatic approach is more commonly applied, as discussed in Section 3. The direct biological approach can potentially be more effective than the indirect approach as the enzyme-secreting or algicide-secreting organisms naturally adapt to co-existence with microalgae and thus produce enzymes or algicides that are tailored to disrupt the cell walls. The indirect approach, on the other hand, relies on the design and selection of enzyme cocktails based on known cell wall composition (or intelligent guessing, in the case

that cell wall composition has not been fully elucidated). The direct approach generally uses either symbiotic bacteria or fungi to disrupt microalgal cell walls (Figure 8). The scope of this section has been expanded to include other microalgae species beyond *N.*, *Scenedesmus*, and *Schizochytrium* due to limited literature found on algicidal bacteria and fungi for these genera. Table 7 summarises different studies that have investigated direct biological approaches for microalgal cell disruption.



Pros	Cons
Enzyme concentration can be controlled	Extraction and Purification of enzymes is expensive
Higher cell disruption efficiency	The cell wall structure and composition needs to be known

Pros	Cons
Cost-effective method	The cell disruption process can be controlled.
The bacteria/fungi have adaptive mechanisms to break the cell wall. This reduces the necessity to understand the cell wall.	Removal/separation step of bacteria/fungi will be added to downstream processing.

Figure 8. Comparison between direct and indirect methods of enzymatic cell-wall disruption.

Table 7. Summary of studies investigating a direct biological approach for cell disruption.

Microalgae	Organism	Overall Results	References
	Bacteria		
Nannochloropsis salina	Bdellovibrio-and-like-organisms	The contents of the cells are broken down, leaving behind empty cell walls.	[130]
Nannochloropsis oculata and *Dunaliella salina*	*Sagittula stellata*	After 6 days, the algicidal rate on *N. oculata* and *D. salina* was 64.7% and 52.4%, respectively. Crude lipid yield of *N. oculata* and *D. salina* increased from 19.6% to 36.4% and from 32.9% to 45.7%, respectively, following a 6-day incubation by *S. stellata*.	[131]
Chlorella sp.	*Aeromonas hydrophila*	The findings showed that bacteria can break *Chlorella* sp. cells and release lipids. The longer the co-culture period, the higher the proportion of cell disruption.	[132]
	Fungi		
Microcystis aeruginosa, *Microcystis flos-aquae*, *Oocystis borgei*, and *M. aeruginosa*	*Trichaptum abietinum*	All algal cells from cultures were destroyed within 48 h of co-incubation with the fungi.	[133]
Chroococcus sp.	*Aspergillus lentulus*	After being incubated for 48 h at 30 °C, the fungal enzyme caused 100% disruption of microalgal cells. Additionally, the fungal enzyme's activity was able to solubilize up to 44% and 46% of the biomass's total sugar and COD.	[134]
Chlorella sorokiniana and *Scenedesmus obliquus*	*Aspergillus niger*	Catalytic/hydrolase activity on glycosyl and O-glycosyl compounds was observed.	[135]

4.1. Algicidal Bacteria

Microalgal cells are surrounded by a microscale environment named "phycosphere", a layer enriched in exudates that forms a concentration gradient of dissolved organic material (DOM) [136]; the phycosphere is also the layer where most associated bacterial cells reside in the culture and interact with microalgal cells [137]. These symbiotic associations are both complex and dynamic and can range from mutualism to parasitism. For cell-wall destruction, the most interesting aspect is antagonistic interactions in bacteria–algae relationships. There are two terms for the description of these kinds of interactions. The broad term "algicidal" or "pathogenic" refers to interactions that kill algae, while the term "algistatic" more specifically denotes interactions that inhibit algal growth [138].

One possible mechanism for cell disruption using live bacteria (such as those in the phycosphere) involves the infection of the microalgal cells through direct physical contact [90]. Lee et al. described the application of a new predatory bacterium belonging to the Bdellovibrio-and-like-organisms (BALOs) group isolated from *N. salina* culture. Light and TEM microscopy revealed the presence of multiple bacterial morphologies, including free-swimming and those attached to the host cell surface. The bacteria were inoculated in *N. salina* culture to induce infection. As the infection progressed, the colour of the algal cells appeared to recede. Eventually, the cellular contents were completely degraded, leaving behind hollow cell walls [130]. Furusawa et al. identified a bacterium from the sea, *Saprospira* sp. SS98-5, which was able to destroy diatom cells *Chaetoceros ceratosporum* upon contact [139].

Another possible mechanism for cell disruption using live bacterial cells is through the secretion of enzymes or algicides by bacteria to attack microalgal cell walls [90]. The bacteria releasing chemicals to adversely affect the growth or structural integrity of microalgal cells are often referred to as algicidal bacteria. The algicidal action often relies on specific microalgal-bacterial interactions for delivery.

The main component of the algicidal activity of *Alteromonas* FDHY-03 is the enzyme Beta-glucosidase, whose level increased while being co-cultured with dinoflagellate *Prorocentrum donghaiense* [140]. *Alteromonas* was also able to produce algicidal compound Questiomycin A when exposed to *Chattonella antiqua* cells [141]. Algal cues appear to play a role in inducing algicidal properties for these bacteria. For instance, the haptophyte algal species *Emiliania huxleyi* produced dimethyl sulfoniopropionate (DMSP), which triggered algicidal actions by the alpha proteobacterium *Sulfitobacter* D7 [142]. The lysing effect of *Alteromonas* and *Pseudoalteromonas* species on algae occurs through the extracellular secretion of a complex mixture of agarases, toxins, bacteriolytic substances, and other enzymes [143]. Yang et al. showed that two different bacterial strains were able to cease the growth of *M. aeruginosa* and achieve an algicidal effect within a short time frame by damaging the photosynthetic system and lowering the rate of photosynthetic activity [144].

Algicidal bacteria can release a wide range of extracellular substances to damage algal cell walls, including lipid peroxidases, proteins, enzymes, bacillamide, amino-peptidase, lipase, glucosaminidase, alkaline phosphatase, and antibiotics [145]. The bacteria *Sagittula stellata* showed strong algicidal activity against two microalgal species, *Nannochloropsis oculata* and *Dunaliella salina*. The algicidal rate reached 64.7% for *N. oculata* and 52.4% for *D. salina* in 6 days. A decrease in chlorophyll-a fluorescence of both algae upon bacteria addition in a co-culture system was observed. The bacterial pre-treatment was found to have a positive impact on the lipid yield of the biomass when subjected to subsequent hexane extraction. The crude lipid yields of *N. oculata* and *D. salina* biomass that had been incubated with *S. stellata* for 6 days increased from 32.9% to 45.7% and from 19.6% to 36.4%, respectively, relative to those obtained from control biomass not previously treated with bacterial addition. These results demonstrated the capacity of algicidal bacteria *S. stellata* in promoting cell disruption and lipid recovery [131].

Deng et al. measured the percentage of cell disruption and lipid extraction of *Chlorella* when treated with five different algicidal bacteria and their cell-free supernatants. The results demonstrated that both bacteria cells and supernatant can induce cell disruption

and lipid release from *Chlorella* biomass. Supernatant isolated from the bacteria *Aeromonas hydrophila* achieved the highest percentage of cell disruption and lipid extraction. The extent of cell disruption increased with incubation time [132].

Even though algicidal bacteria have commonly been applied in the management of harmful algae bloom in aquatic ecosystems, a scalable application of this technology for sustainable cell disruption in microalgal biorefinery settings still requires significant process development and a deeper understanding of the mechanism that governs algae/bacteria interactions and the effect on algicidal activity on the integrity of target products (e.g., lipids).

4.2. Fungal Enzymes

Fungi is another promising group of organisms with the capacity to exert direct biological cell disruption in microalgae. Fungi are better known as hydrological enzyme producers than bacteria and are being actively studied for microalgae biomass processing. The majority of studies investigating indirect enzymatic approaches for disrupting microalgae cells have used enzymes previously isolated from fungi. Researchers have reported that fungal extracellular enzymes, particularly Cellulase, Amylase, Lipase, and Xylanase, can efficiently dissolve the cellulose and hemicellulose structure present in algal cell walls, allowing for the release of lipids from algal cells [146].

Jia et al. reported that all microalgal cells in the cultures of *Microcystis aeruginosa*, *M. flosaquae*, *Oocystis borgei*, and *M. aeruginosa* were destroyed within 48 h of co-incubation with the fungus strain *Trichaptum abietinum* [133]. *Aspergillus niger* demonstrated excellent catalytic/hydrolase activity on glycosyl and O-glycosyl compounds when used to treat *Chlorella sorokiniana* and *Scenedesmus obliquus* biomass [135].

A 20% (*v/v*) dosage of *Aspergillus lentulus* crude enzyme (AL2) was shown to have a considerable impact on *Chroococcus* sp. biomass. Within 48 h of incubation at 30 °C, AL2 resulted in nearly 100% cell death of *Chroococcus* sp. Furthermore, the activity of AL2 led to the solubilisation of up to 44% and 46% of total sugar and COD in the biomass, respectively [134]. Direct co-incubation of the fungi with microalgae cells, however, was not tested in the study.

The addition of fungus *Trametes versicolor* broth to a mixed algae biomass (mainly *Oocystis* sp.) harvested from cultivation in raceway ponds was shown to successfully increase the methane potential of the algal biomass. When subjected to a subsequent digestion step, the fungal-treated biomass produced 74% more biogas than untreated microalgal biomass. On the other hand, the addition of commercial laccase to microalgal biomass only managed to increase the methane yield by 20%, demonstrating that a direct isolate of an enzymatic cocktail from fungus can potentially be more effective in disrupting microalgal biomass compared to a single purified enzyme [147]. Direct co-incubation of the fungi with microalgal cells, however, was also not tested in the study. Despite these early promises, the application of fungal-based processes for direct biological disruption of microalgal cells is still at an early stage of development, with significant efforts required to elucidate the underlying mechanism of algae/fungi interaction and the associated enzyme production or algicidal activity.

5. Conclusions and Perspectives

This review provides an overview of the microalgae cell walls and approaches used for their characterisation and cell disruption, focusing on three lipid-accumulating genera, namely, *Nannochloropsis*, *Scenedesmus*, and *Schizochytrium*. Enzymatic cell disruption is a gentle and non-degradative process that can be directly applied on wet biomass and finetuned to target specific components in the cell wall, thus potentially achieving cell disruption or permeabilization at low energy and infrastructure costs. Numerous studies undertaken over the past 2 decades have attested to the effectiveness of enzymatic treatment in disrupting/lysing or permeabilizing microalgal cell walls, leading to increased product recovery (such as lipid) when applied as a pre-treatment to solvent extraction. Despite the promising nature of enzymatic treatment in disrupting microalgal cell walls over

mechanical and chemical methods, their use for commercial-scale microalgae processing has been hampered by the high cost associated with enzyme purchase. To overcome this limitation, it is imperative to reduce enzyme requirements. This can potentially be achieved through the adoption of a minimal design approach that uses the cell wall composition of the target species as the basis for direct enzyme choice and dosage, enabling maximum cell disruption at minimum enzyme amounts. The design of enzymatic treatment should, therefore, be based on the cell wall ultrastructure and composition of the target species, signifying the need to understand better the cell wall architecture of microalgae and their morphological changes under different growth conditions, particularly those that are conducive for product accumulation. Another method to reduce the process cost is developing an efficient method for recycling and immobilizing enzymes that can be optimized to minimize the enzyme needs; however, this approach necessitates an extra purification step and may unintentionally reduce enzymatic performance over multiple cycles. To avoid using pure enzyme cocktails or recycling enzymes, a direct biological approach can be adopted. Using this approach, microalgal cells are directly contacted with algicidal bacteria or fungi that secrete enzymes to break down cell walls. Implementation of this strategy in a biorefinery setting, however, is still in its infancy and will require significant process optimisation and mechanistic understanding of the interactions between microalgae and the algicidal organisms to progress the process through technology-readiness levels. This study provides guidance for scientists and manufacturers who are considering the use of lipid-rich microalgae for the production of biofuels and food/feed on (a) cell wall structures of lipid-rich microalgal species, (b) the importance of determining an optimal cell-disruption strategy based on target products and cell-wall architecture of the species, and (c) state-of-the-arts in enzymatic cell disruption and direct biological cell disruption for a more ecologically friendly biorefinery process.

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