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Detailed biochemical and morphologic characteristics of the green microalga *Neochloris oleoabundans* cell wall

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ABSTRACT

Chlorophyta, the group of green algae of which there are > 6000 species, manifests a great diversity of intercellular and extracellular components. Building blocks in the cell walls of Chlorophyta are very distinct and they may contain various components. Here, we characterize the cell walls of *Neochloris oleoabundans*, a Chlorophyte microalga, both in terms of biochemical composition and morphology. *N. oleoabundans* cell walls are composed of about 24.3% carbohydrates, 31.5% proteins, 22.2% lipids and 7.8% inorganic material, which contrasts to the cell walls of (higher) terrestrial plants in which carbohydrates are by far the main component. We also observed that cell wall carbohydrates are mainly non-cellulosic polysaccharides, essentially composed of rhamnose, galactose, glucuronic acid and glucosamine, of which glucose is only a minor component. The lipids comprising the *N. oleoabundans* cell walls are generally wax/cutin-like. Electron microscopic studies revealed that *N. oleabundans* cell walls are approximately 200 nm thick and consist of two main layers: a thinner inner layer and a more electron-dense outer layer. On the outer layer are hair-like structures that are possibly rich in carbohydrates. These findings are an important contribution that enable us to understand the complexity of cell walls in green microalgae.

1. Introduction

Neochloris oleoabundans is a terrestrial microalga belonging to the Chlorophyta phylum. This species was first isolated from the sand dunes in Saudi Arabia, a very harsh environment where access to water is always a challenge [1]. Therefore, it must possess key properties to assure the viability of the cell. Depending on these specialized adaptations, *N. oleoabundans* can be cultivated in a freshwater medium as well as in saline water with seawater salt concentration [2,3].

Green microalgae, such as *N. oleoabundans,* is outlined by its cell wall; a dynamic and rigid structure, that determines cell viability in a wide-range of environments, defends a cell from biotic and abiotic stresses, and provides plasticity, enabling cells to expand and assume different shapes.

Despite the significance of a cell wall in microalgae, only limited information is available on its composition and structure for most of the species [4]. Chlorophyta, the largest group of green algae, displays a wide array of cell wall types regarding both chemical composition and morphology [4–6]. It is generally accepted that taxonomy can be a tool to speculate about the cell wall composition of algae and their related species [4,6,7]. A recent review indicated that Chlorophyta can be taxonomically divided into three main groups depending on their cell wall composition and structure, which are distinctly different from those of terrestrial plants. Group 1, namely algae belonging to the Prasinophytina and Chlorodendrophyceae, in which cell walls are mainly composed of 2-keto-sugar acids 3-deoxy-manno-2-octolusonic acid (Kdo), 3-deoxy-5-O-Methyl-manno-2-octolusonic acid (5OMeKdo) and 3-deoxylyxo-2-heptulosaric acid (Dha); group 2, unicellular algae related to Trebouxiophyceae and Chlorophyceae, in which cell walls are mainly composed of mannans, glucans, arabinogalactans, algaenans and less frequently chitin-like polysaccharides; and group 3 comprises green macroalgae, chiefly marine lineages, in which cell walls on the whole contain sulphated polysaccharides, xylan, mannan and glucan [4]. According to this taxonomical classification, *N. oleoabundans* belongs to “group 2” and is, therefore, expected to have a similar cell wall composition and structure as the other members.

On the basis of alkali extraction, algae cell walls could further be classified in accordance with the sugar composition of the alkali soluble part, hemicellulose, and the remaining residue, known as the “rigid cell wall”. To exemplify this classification, cell walls of the unicellular *Chlorella* strains, belonging to “group 2”, could be categorised into two distinct groups based on the presence or absence of glucosamine in the “rigid cell wall” [8,9]. For instance, *C. sorokiniana*, C. *vulgaris* and *C. kessleri* (currently known as *Parachlorella kessleri*, Trebouxiophyceae)

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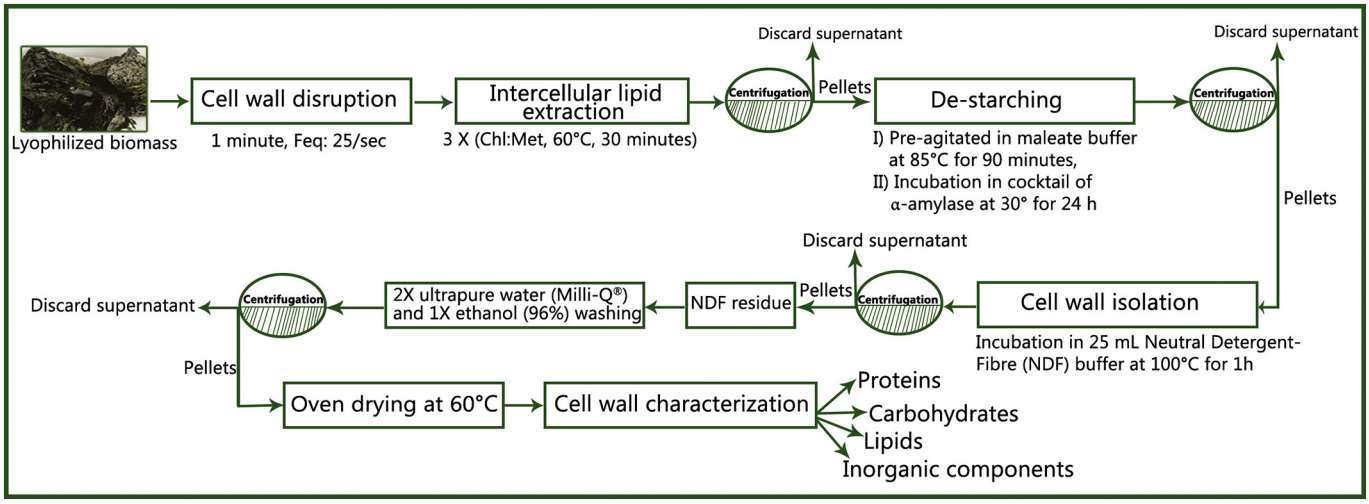

Fig. 1. Schematic diagram of the cell wall extraction procedure.

belong to the glucosamine-rich rigid cell wall, whereas *C. fusca* (currently known as *Scenedesmus fuscus*, Chlorophyceae) lacks glucosamine and its "rigid cell wall" contains mannose and glucose [7,10]. Rhamnose and galactose are the main sugars in the hemicellulose fraction of the glucosamine-rich rigid cell wall [7].

Cell walls in the Chlorophyta phylum are very diverse and in addition to carbohydrates they comprise several other components. As far as protein is concerned, its abundance in the cell wall can differ substantially. This can be illustrated in *C. fusca* (currently known as *Scenedesmus fuscus*) which has approximately 7% protein content compared to *C. sorokiniana* with 17%. The amino acid profile of these two species appeared to be correspondingly different [10–12]. Alongside the aforementioned components, the presence or absence of algaenan, an acetolysis-resistant-biopolymer, in the cell wall, is yet another feature that creates diversity within the Chlorophyta species [13,14]. With few exceptions, such as species in the family Chlorellaceae, most of the microalgae belonging to "group 2" have an algaenanic layer, whereas this layer is absent in the cell wall of the other two groups of Chlorophyta [4].

Apart from their composition, Chlorophyta cell walls display different structures; some possess a single microfibrillar layer, referred to as the Inner layer (I-layer), while others have an additional layer, known as the Outer layer (O-layer). The O-layer, depending on the species, can be a mono-electron-dense layer or composed of three sublayers, so called the trilaminar O-layer [14–16]. *C. vulgaris* C-30, *C. sorokiniana* and *C. fusca* (currently known as *Scenedesmus fuscus*) are examples of microalgae that have a single microfibrillar I-layer, a mono O-layer and a trilaminar O-layer, respectively [12,17].

In *N. oleoabundans,* research has generally focussed on studying the intercellular components. As far as we are aware this study is the first report on the detailed biochemical characterization and morphological appearance of the *N. oleoabundans* cell walls. The results provided herein will greatly contribute to advance our knowledge on the cell wall complexity in Chlorophyta.

2. Materials and methods

2.1. N. oleoabundans cell culturing

Biomass was produced in a 280 L indoor tubular photobioreactor at AlgaePARC facilities (Wageningen, The Netherlands). Details of the reactor have already been described [18]. *N. oleoabundans* (UTEX 1185, University of Texas Culture Collection of Algae) was cultivated in a freshwater medium with the following components: 0.023 M $NaNO_3$, 0.017 M NaCl, 2.49 mM $MgSO_4$, 0.9 mM $CaCl_2$, 5.95 mM $NaHCO_3$, 282 μM $Na_2EDTA.H_2O$, 108 μM $FeSO_4.7H_2O$, 11 μM $MnCl_2.4H_2O$, 2.3 μM $ZnSO_4.7H_20$, 0.24 μM $Co(NO_3)_2.6H_2O$, 0.1 μM $CuSO_4.5H_2O$, 1.1 μM $Na_2MoO_4.2H_2O$. The culture was operated in a batch state without the use of artificial light, at a temperature of 25 °C and pH of 7.0. The reactor was monitored daily by measuring the optical density and dry weight as described earlier [19]. Additionally, quantum yield of the culture was monitored daily using portable AquaPen-P AP-P 100 (Photon Systems Instruments, Czech Republic) based on the manufacturer's protocol. Biomass was harvested several times at different points in time, centrifuged (80 Hz, ~3000*g*, 0.75 $m^3 h^{-1}$) using a spiral plate centrifuge (Evodos 10, Raamsdonksveer, The Netherlands), rinsed 3 times with water and lyophilized. The dry weight and quantum yield of the harvested biomass were approximately 0.5 g/L and 0.69, respectively. All the experiments were conducted with two biological replicates and four technical replicates. The lyophilized biomass from different harvest points of each biological replicate was pooled and used as material to commence the cell wall biochemical characterization.

2.2. Cell wall extraction

Fig. 1 depicts the simplified schematic diagram of the cell wall extraction procedure used in this study. The extraction process began with 1 g lyophilized biomass that was mechanically disrupted for 1 min in a mill (Mixer Mill MM 200 -Retsch, Germany) at a frequency of 25 s^{-1}, followed by the removal of intercellular components including lipids, starch and soluble sugars. Cell wall disruption and the removal of intercellular components were carried out following a slightly modified version of the established protocol [13]. In brief, the biomass was incubated in 25 mL of chloroform: methanol (2:1) at 60 °C for 30 min continuously shaking at 600 rpm. The samples were then centrifuged and the supernatant was discarded. All the centrifugal steps mentioned in this manuscript were conducted at 4200*g* for 10 min unless stated otherwise. The intercellular lipids from the remaining pellets were extracted during two more cycles following the same process. Subsequent to a third extraction, the residual pellets were dried in an oven at 60 °C until a constant weight was achieved. After removing the lipids, the biomass was then incubated in 25 mL of a maleate buffer at pH 6.5 (0.01 M $C_4H_4O_4$, 0.01 M NaCl, 0.001 M $CaCl_2$, and 0.05% $_{W/V}$ NaN_3) and agitated for 90 min at 85 °C. Once the sample had cooled down to room temperature, a cocktail of alpha-amylase (50 μL/25 mL, ANKOM Technology Corporation, Fairpoint, NY) was added in which it was incubated for 24 h at 30 °C. Following the centrifugal stage, the supernatant containing glucose derived from starch was discarded. During the next stage, cell walls were extracted from the oil-free de-