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Neochloris oleoabundans cell walls have an altered composition when cultivated under different growing conditions

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Abstract

The impact that environmental factors have on the intracellular components of microalgae has been the focus of research for a number of decades. Despite that, their effects on the cell wall have received very little attention. In this study, we investigated how different growing conditions affect the cell walls of *N. oleoabundans*. The results revealed that the cell wall composition varied in that the modifications were different in the four cultivation media: freshwater nitrogen-replete (optimum culture) and -depleted conditions, and seawater nitrogen-replete and -depleted conditions. Nitrogen deficiency in freshwater cultivation was the only condition that significantly ($p<0.05$) increased the total content of carbohydrates in the cell wall. The three most abundant components of freshwater-cultivated cell wall polysaccharides were rhamnose, galactose and glucuronic acid whereas in seawater media the main components of cell wall polysaccharides were rhamnose, glucose and galactose. The combined results of the biochemical analyses and monoclonal antibodies epitope-binding revealed that *N. oleoabundans* cell walls are likely composed of sulphated polysaccharides enriched in mannose, β-(1→4)-D-mannans, and glucose as they grow in seawater. Salinity and nitrogen deficiency also had an impact on the nitrogenous components of the cell wall. Under these conditions we observed a decrease in glucosamine in the cell wall. The analysis of specific binding of monoclonal antibodies, revealed that the cell wall of *N. oleoabundans* is possibly enriched in arabinogalactan proteins (AGPs). Under salinity and nitrogen deficiency *N. oleoabundans* increased the proportion of the non-polar to polar amino acids in the cell walls. An increase of leucine in the cell walls may suggest that *N. oleoabundans* contains leucine-rich repeat proteins which are known to play a vital role in stress responses. This report provides new insights into microalgae cell wall biology and how cell walls are remodelled when growing under different conditions.

1. Introduction

The impact that environmental factors have had on the intracellular components of microalgae has already been the subject of research for a number of decades. In contrast, variations in cell wall composition and structure due to the impact of different environments has received limited attention. Adverse environmental factors can influence physiological processes and consequently have an impact on the regulation of cell wall biosynthesis, at both transcriptional and biochemical levels [1, 2].

Nitrogen deficiency and salinity are two stress factors that have been used to modulate the intracellular composition in various microalgae species [3-7]. It is clear that these factors play a significant role in the cell carbon partitioning, and one would expect these alterations to be visible in the cell wall as well. A few reports are now available on the effects of stress conditions on the algae cell wall. These include alterations in the cell wall composition or structure such as increased cell wall thickness which is associated with nitrogen deficiency [8, 9].

A great example on effects of stress condition on green microalgae cell wall has been recently published [2]. It was revealed that the increased thickness of the cell wall caused by nitrogen deficiency in *Nannochloropsis salina* is associated with up-regulation of genes encoding for cellulose biosynthetic enzymes, which resulted in an increase the cellulose content of the cell walls. Another study demonstrated that transition of *N. salina* from high to low saline culture increased the cell wall thickness, although detailed changes in biochemical composition are not yet available [10].

Neochloris oleoabundans, is oleaginous unicellular green microalga belonging to the Chlorophyta phylum. Over the years, this microalga has been considered one of the most promising candidate industrial microalgae, due its high growth rate and biomass composition [11]. *N. oleoabundans* is an edaphic freshwater green microalga which was originally isolated

from the sand dunes in Saudi Arabia, an environment where the lack of water is a tenacious threat and cells can be exposed to saline or drought stress [12]. The cell wall is the outermost structure of the cell and the first part of the cell to be exposed to severe conditions. It therefore requires specific properties to guarantee cell viability. Owing to the plasticity of *N. oleoabundans* and its saline resistance mechanism, this alga can be cultivated in both freshwater or saline cultivation media, with seawater salt concentration [13-15].

Recently, the cell wall composition of *N. oleoabundans* growing in freshwater condition was biochemically characterized and the morphology of the cell wall dissected by means of electron microscopy [16]. This report revealed that 56% of the cell wall is composed of carbohydrates and nitrogenous components such as amino sugars and proteins.

In this manuscript we aim to evaluate the effects of different cultivation media, freshwater nitrogen-replete (optimum culture) and -depleted conditions, and seawater nitrogen-replete and -depleted, on cell wall composition, with the main focus on carbohydrate and protein compositions. We expect that a lack of nitrogen could not only affect the carbohydrates but also have a direct impact on the nitrogenous components of the cell wall, such as amino sugars and proteins. The results revealed considerable variations in the biochemical composition of cell walls grown under different culture conditions. These results provide important insights into the biology of *N. oleoabundans* and will greatly contribute to our understanding of cell wall remodelling in green microalgae when they are exposed to adverse environments.

2. Materials and Methods

2.1. Biomass supply

N. oleoabundans (UTEX 1185, University of Texas Culture Collection of Algae) was pre-cultures in 100 ml sterilized fresh or seawater medium. Subsequent to reaching an optimum density (~ 0.2 g L^{-1}), the culture was used to inoculate a vertical tubular photobioreactor (VT

LGem, 1300 L working volume, Rotterdam, The Netherlands) installed inside a greenhouse at AlgaePARC facilities (Wageningen, The Netherlands). Details of the reactor are available on the manufacturer's website (www.lgem.nl). The same inoculations were used for the nitrogen-depleted experiments. The reactor was operated in a batch phase with the use of artificial light (7.2 kW), at an average temperature of 25°C and pH of 7.5. Concentration of the nutrients in the medium can be found in Table 1. The culture was monitored daily and the biological parameters including dry matter, optical density (530, 680, 750 nm) and quantum yield of the harvested points were recorded (Supplementary file 1). Additionally, daily microscopic visualization in order to assess the cell morphology and possible contamination was conducted. Details of the measurements have already been described [16]. Biomass from different points in time of this batch pipeline production was harvested, centrifuged (80 Hz, ~3000g, 0.75 m^3 h^{-1}) using a spiral plate centrifuge (Evodos 10, Raamsdonksveer, The Netherlands), rinsed with water and dried in an oven at 60°C until a constant weight was achieved. The dried biomass at different points of harvesting was pooled and used as a starting material. Cell wall extraction was carried out in three replicates. The extracted cell wall materials were combined and used for further biochemical characterization. All the biochemical analyses mentioned in this publication were performed at least with two technical replications and the values presented are the mean ± standard deviation (SD).

2.2. Preparing samples to extract the cell wall

Cell wall preparation was carried out as described in [16]. In brief, 1 g of dried biomass was mechanically disrupted in a mill for one min at a frequency of 25 s^{-1} (Mixer Mill MM 200 - Retsch, Germany). Following this, three incubation cycles of chloroform: methanol (2:1) was used in order to remove the intracellular lipids. Each cycle was conducted at 60°C for 30 min continuously shaking at 600 rpm. After each incubation, samples were centrifuged and the supernatant was discarded. Subsequent to the last extraction, the residual pellets were dried in

an oven at 60°C until a constant weight was achieved. When the lipids had been removed from the sample, the biomass was de-starched by incubation in a buffer containing a cocktail of alpha-amylase. In summary, 25 mL of maleate buffer (0.01 M $C_4H_4O_4$, 0.01 M NaCl, 0.001 M $CaCl_2$, and 0.05% $_{w/v}$ NaN_3) at pH 6.5 was added to the sample and then incubated 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 to the suspension which was then incubated for 24h at 30°C. Subsequently, samples were centrifuged and the supernatant containing glucose derived from starch was discarded. All the centrifugal steps mentioned in this publication were conducted at 4200 g for 10 min unless stated otherwise.

2.3. Neutral Detergent Fibre (NDF) extraction of the cell wall

Cell walls from the oil-free de-starched sample were extracted in accordance with the established protocol developed by Ankom Technology (ANKOM Technology Corporation, Fairpoint, NY) as described in [16]. In short, the biomass was incubated at 100°C for 1 h in a 25 mL NDF buffer (104 mM sodium dodecyl sulphate, 50 mM ethylenediaminetetraacetic disodium salt (dihydrate), 17.8 mM sodium borate, 32 mM sodium phosphate dibasic (anhydrous), 79 mM sodium sulphite and 10 g/L triethylene glycol) and stirred constantly at 600 rpm. As soon as the incubation was accomplished, the suspension was then centrifuged and the supernatant discarded. The remaining pellets were washed twice using ultrapure water (Milli-Q®) and once with ethanol (96%). Subsequently, the pellets were then dried in an oven at 60°C until a constant weight was achieved. These pellets corresponded to the total cell wall and will be further referred as the NDF-cell wall.

2.4. Sulphuric acid hydrolysis and characterization of the cell wall carbohydrates

A total amount of carbohydrates in the NDF-cell wall of *N. oleoabundans* were measured subsequent to the sulphuric acid hydrolysis of polysaccharides as described earlier [16]. Briefly,

20 mg of dried NDF-cell wall was incubated in 1 mL of 72% (V/V) H_2SO_4 for 1 h at 30°C. The acid concentration was then diluted by adding the right amount of ultrapure water (Milli-Q®) to reach the final concentration of 6% (V/V). Incubation with the diluted acid concentration was further continued for 1 h at 121°C (autoclave). Following the hydrolysis, the hydrolysates were cooled down, neutralized and after filtration using a 0.45 µm filter, sugar content was analysed by means of High Performance Anion Exchange Chromatography (HPAEC, Dionex ICS5000+DC, CarboPac PA1, 2 x 250 mm, Thermo Fisher Scientific, Waltham, MA, USA) as described in [16].

2.5. Immunolabelling of the *N. oleoabundans* cell wall components

Comprehensive microarray polymer profiling (CoMPP) of *N. oleoabundans* grown in either fresh or seawater nitrogen-replete was performed as previously described in [17-19]. Cell wall was isolated using a alcohol insoluble residue (AIR) procedure and used as a starting materials to extract the carbohydrates microarrays [20, 21]. In brief, the cell wall polymers were extracted sequentially from 10 mg of the AIR-cell wall using 50 mM 1,2-diaminocyclohexanetetraacetic acid (CDTA), pH 7.5, followed by extraction with 4 M NaOH with 0.1% m/V NaBH4, and extractions printed in four dilutions and two replicates giving a total of 8 spots per sample. The same amount of cell wall material was used for each sample. Nitrocellulose microarrays were printed as described previously by Pedersen et al.(2012) and Moller et al.(2007). Briefly, the printed arrays were probed with a panel of anti-rat and anti-mouse monoclonal antibodies (PlantProbes and Biosupplies). Antibodies were diluted in PBS containing 5% w/v milk powder to 1/10 and 1/1,000, respectively. For secondary antibodies, anti-rat and anti-mouse secondary antibodies conjugated to alkaline phosphatase (Sigma) were diluted in MPBS to 1/5,000. Developed microarrays were scanned at 2,400 dpi (CanoScan 8800F), converted into TIFFs and signals were measured using Array-Pro Analyzer 6.3 (Media Cybernetics). Data are shown

in a heatmap in which the colour intensity is correlated to a mean spot signal value. A cut off, of 5 arbitrary units was applied.

2.6. Proteins and inorganic components of the cell wall.

Potential changes in the nitrogenous components of the cell wall due to the deficiency of nitrogen in the culture were further explored. To this end, the protein content was measured as described in [16]. In summary, a sample of 150 mg NDF-cell wall was dried in a convection oven for 1 h at 100°C and after cooling it down in a desiccator, the total nitrogen content was determined by combustion at 950°C using a LECO analyser (LECO CN 628 Dumas analyser, LECO Corporation, USA). Total protein content of the cell wall was assessed by multiplying the Nitrogen to Protein (NTP) conversion factor with the nitrogen content. This conversion factor was calculated specifically for the NDF-cell wall of *N. oleoabundans* as reported previously [16]. Prior to characterizing the amino acids, a 20 mg sample of the NDF-cell wall was hydrolysed in 6 N HCl for 24 h at 100°C. Subsequent to hydrolysis, the amino acid characterization was carried out using the Gas Chromatography (GC) technique based on the EZ:faast[TM] method (Phenomenex Inc.). All the steps were carried out in line with the protocol annexed to the kit [22].

Possible changes in the inorganic material of the cell wall under different environments were investigated. The inorganic content was assessed in line with the protocol described in [16]. Briefly, 100 mg ground dried NDF-cell wall in a pre-weighed glass tube was heated gradually in a muffle furnace for 5 h at 575°C and the contents were turned into ash. The inorganic residue was then cooled down to room temperature in a desiccator and the weight was recorded. To measure the cations and anions, approximately 3 mg of ash was dissolved in 1 ml of 3 M formic acid and incubated for 15 min at 99°C. Subsequent to dissolving, the solution was diluted 10 times with MQ water, filtered using a 0.45 µm filter and then the ions were characterized using

the Ion Chromatography (IC) system 850 Professional (Metrohm Switzerland). The anions were determined by means of a Metrosep A 150, 150/4.0 mm column equipped with a Metrosep C5/5 Supp 4/6 Guard column and the cations with a Metrosep C4 Supp 4, 250/4.0 mm column equipped with a Metrosep A Supp 4/6 Guard column.

2.7. Statistical analysis

Significant difference between the each trait under the different growing conditions were assessed by two-way analysis of variance (ANOVA). The Fisher's unprotected least significant difference values were calculated at 5% probability. All statistical analyses were performed using Genstat (19th edition software, VSN International, Hemel Hempstead, UK).

3. Results

3.1. Composition of the cell wall carbohydrates alters according to different growing conditions

Analysis of the *N. oleoabundans* cell cultivated in the 4 different media resulted in significant differences ($p<0.05$) in the total content of cell walls between the growing conditions evaluated (Figure 1). Nevertheless, the total amount of carbohydrates in the cell wall did not show any significant differences ($p<0.05$) between the different treatments with the exception of cell cultivated in fresh water with nitrogen deficiency (Figure 2). Under this condition, the total carbohydrates in the cell wall was about 14% (percentage of NDF-cell wall). Rhamnose, arabinose, glucosamine, galactose, glucose, mannose, xylose and glucuronic acid were the monosaccharides detected in the cell wall of *N oleoabundans* independently of the media used for cultivation (Figure 3 and Supplementary file 2). However, the proportion of the different monosaccharides was different. Rhamnose, galactose and glucuronic acid were the three most abundant monosaccharides in freshwater cultivations (~68% of total monosaccharides), whereas in seawater cultivations rhamnose, glucose and galactose constituted the main cell wall

monosaccharides (63% of total monosaccharides). The proportion of monosaccharides remained relatively constant when cells were cultivated in seawater conditions (Nitrogen-deplete *versus* nitrogen-replete). Nitrogen depletion in freshwater cultivations resulted in a significant increase in the amount of arabinose and glucuronic acid and a reduced galactose content ($p<0.05$). Except for xylose, of which the content was similar in all growing conditions, we observed that there were alterations in the relative amount of almost all monosaccharides in seawater cultivations, with or without nitrogen, as compared to the corresponding samples cultivated in freshwater conditions. Our results disclosed that glucose and mannose are the monosaccharides which increased the most in the cell wall carbohydrates of cells cultivated in saline media ($p<0.05$).

CDTA and NaOH-extracted glycans of *N. oleoabundans* cell wall, fresh and seawater nitrogen-replete cultivations, were probed with 38 monoclonal antibodies (mAbs). Figure 4 shows a heatmap of the relative abundance of the mAbs binding to specific cell wall components. Overall, the majority of the mAbs did not bind to the *N. oleoabundans* cell wall polymers. Walls of cell cultivated in both nitrogen-replete freshwater and seawater conditions revealed that they were composed of (1→4)-β-D-xylan/arabinoxylan. Additionally, our results indicated the possible existence of arabinogalactan proteins (AGPs) in the cell wall of *N. oleoabundans*. Monoclonal antibodies of JIM16 and LM14 showed a high binding affinity with the NaOH-extract of the freshwater cell wall (nitrogen-replete), whereas JIM13 indicated a high affinity with the CDTA-extract of seawater cell wall (nitrogen-replete). Remarkably, our results demonstrated the accumulation of (1→4)-β-D-mannan/galactomannan in *N. oleoabundans* cell walls grown in seawater condition, whereas these epitopes were either absent or inaccessible in freshwater cultivation.

3.2. Cell walls have a varied protein composition when grown under different conditions

Analysis of the protein content in the cell walls of microalgae cultivated under different conditions revealed that cell wall accumulated a high amount of proteins in both sea and freshwater cultivation provided there was a sufficient amount of nitrogen in the medium (Figure 2 and Supplementary file 3). However, under nitrogen-depleted growing conditions, the protein content of the cell wall decreased significantly ($p<0.05$), in which nitrogen-depleted seawater showed the lowest content (19.3% of the NDF-cell wall). Further amino acid characterization revealed a decrease in the algae cell wall polar amino acids that were cultivated in fresh water under nitrogen depleted condition (Figure 5). This reduction was specifically on the positive-side-chain-amino acids, where lysine decreased dramatically and histidine reduced to undetectable amounts, as well as the polar-uncharged amino acids where the larger reductions were observed in serine and threonine. Cells cultivated in nitrogen-depleted seawater revealed a higher content of polar amino acids in the cell wall relatively to the nitrogen-replete culture ($p<0.05$). Positive-side-chain-amino acids were not detected in the cell wall of a nitrogen-depleted seawater cultivation. Aspartic acid and isoleucine remained constant in the different growing conditions.

Results of the inorganic portion of the cell wall revealed a higher accumulation of inorganic components in the nitrogen deficient cultures (Figure 2). Under nitrogen-replete growing conditions, the inorganic content of the cell wall decreased, with the lowest percentage being found in freshwater cultivation (~5%). Ion chromatography analysis revealed that the cell wall is primarily composed of sulphate and sodium, adding up to almost 85% of the total ash content in a nitrogen-replete freshwater culture and 98% in other growing conditions (Figure 6). Phosphate and magnesium were only detected in nitrogen-replete freshwater culture.

4. Discussion

Adverse growing conditions such as salinity and nitrogen depletion can influence cell physiology and consequently cell wall composition [1]. We observed significant variation in the total content of cell walls between the growing conditions assessed (4% - 19% g/g, $p<0.05$). Under different growing conditions *N. oleoabundans* cells were able to change the composition of their cell walls. This variation highlights an important property of *N. oleoabundans* cell walls, which are plastic and can adapt to different growing conditions. In this study we have characterized the content and composition of carbohydrates, proteins and inorganic components, though microalgae cell wall might have other components, such as lipid, that may as well vary under the adverse growing conditions, but these were not object of our study.

Our results revealed that walls of *N. oleoabundans* cells cultivated under nitrogen-depleted conditions accumulated a higher content of carbohydrates, although this increase was not significant ($p<0.05$) in the seawater cultivation (Figure 2). Previous studies of other microalgae indicate that when cells were cultivated under nitrogen-depleted conditions an increase in polysaccharide content of the cell wall was observed [2, 23, 24]. These results illustrate the ability of the *N. oleoabundans* cell wall to function as an additional type of sink for photo-assimilates accumulated under nitrogen stress. Seawater cultivation resulted in alterations in the monosaccharide profile of the cell wall as compared to the freshwater culture (Figure 3). Glucose and mannose content in the cell wall were abundantly increased when *N. oleoabundans* was cultivated in seawater ($p<0.05$). We have observed that the majority of the mAbs did not bind to the *N. oleoabundans* cell wall polymers. The most probable explanation is the absent of the epitopes in *N. oleoabundans* cell wall, which would not be surprising as these antibodies have been developed for cell walls of plants and other algae species. Another explanation could be that the epitopes might have been inaccessible and/or possibly degraded during the extraction procedure. Nevertheless, results from monoclonal antibodies disclosed that (1→4)-β-D-mannan/galactomannan are the possible epitopes of the accumulated mannan in the seawater

cultivated cell wall. Although results from monosaccharides composition of the cell wall revealed the presence of mannose in a freshwater cultivation (~7% of total monosaccharides, g/g), evaluated monoclonal antibodies were unable to recognize the mannose-epitope(s). This may be attributable to the existence of different epitope of mannose or inaccessibility of the same mannose-epitope in the cell wall of freshwater cultivation. Previous studies on marine algae established that some microalgae are enriched in sulphated polysaccharides including β-(1→4)-D-mannans, (1→3)- β -L-arabinopyranans and other sulphated polysaccharides containing galactose, glucose and arabinose [25-28]. Marine algae are able to resist the saline environment on account of particular mechanisms such as sodium exclusion or accumulation of sulphated polysaccharides [27-30]. The latter mechanism, which is unique to marine algae, is considered to be a strategy of adaptation in marine territories and is also existent in (some) halophyte terrestrial plants [27, 28]. It has been reported that sulphated polysaccharides, which provide a gel-like matrix for a fibrous and crystalline component of the cell wall, are part of the cell walls of some marine species belonging to the phylum Chlorophyta [28, 31]. Considering the abundance of mannose, β-(1→4)-D-mannans, and glucose together with a high content of inorganic sulphate in the seawater cultivated *N. oleoabundans* cell wall, it is tempting to hypothesize the presence of sulphated polysaccharides which may contribute to the adaptation of this species in a saline medium.

Nitrogen-containing biopolymers are one of the main components in the microalgae cell wall belonging to the Chlorococcaleae, therefore it is not surprising that a restricted amount of nitrogen in the medium caused a reduction of the cell wall fraction in the total cell mass of *N. oleoabundans* [32-34]. As depicted in Figure 3 there is a considerable reduction of glucosamine in the cell walls when they are cultivated under nitrogen depletion or saline medium. A recent transcriptomic study of *N. oleoabundans* cultivated under nitrogen depletion indicated a downregulation of glucosamine fructose-6-phosphate aminotransferase (GFAT) [35]. This

enzyme is involved in the hexosamine (amino sugar) biosynthesis pathway, synthesizing glucosamine-6-phosphate (GlcN-6P) from fructose-6-phosphate (Fru-6P) which is derived from glucose. It has been demonstrated that nitrogen depletion in *Chlorella vulgaris* resulted in morphological alteration of the cell wall [36]. *C. vulgaris* growing in nitrogen-replete conditions contains hair-like fibres mounted on the outer layer of the cell wall, referred to as hyaluron, an unsulphated glycosaminoglycan structure composed of β-1,4-glucuronic acid and β-1,3-N-acetylglucosamine [36-38]. Under nitrogen-depleted conditions *C. vulgaris* lacks these hair-like fibres.

The change in cell wall mass under nitrogen-depleted conditions was predominantly attributed to a reduction in the protein content of the cell wall (Figure 1 and Figure 2). Localized cell wall proteins comprise enzymes, both *in situ* enzymes for development and remodelling, and enzymes involved in biotic and abiotic responses; as well as glycine-rich proteins (GRPs), proline-rich proteins, extensins and hydroxyproline-rich glycoproteins (HRGPs) [39, 40]. Arabinogalactan proteins (AGPs) belong to the HRGPs and are found at the cell surface of a wide variety of plants and algae [34, 40, 41]. The positive signal of the antibodies JIM16, JIM13 and JIM14 toward the arabinogalactan/arabinogalactan-protein antigens revealed the possible existence of AGPs in *N. oleoabundans* cell walls. In line with previous studies, these monoclonal antibodies have an affinity to AGP1 and AGP2 immunogens [42, 43]. The likely existence of arabinogalactan proteins in the *N. oleoabundans* cell wall supports its taxonomical classification into the phylum Chlorophyta, in which cell walls are known to contain AGPs [44]. Despite these enlightening findings, questions still remain concerning the particular biological function of AGP1 and AGP2 in the *N. oleoabundans* cell wall. The exact function of AGPs in plant cells is still a matter of debate, yet cell division, cell extension, abiotic stress tolerance and cell viability are the main functions discussed in literature [41, 45, 46].

Polar amino acids were more abundant in the walls of cells cultivated in nitrogen-replete freshwater in comparison to all the other growing conditions (Figure 5). The final structure of a protein comprises polar/hydrophilic side-chain amino acids, which are normally located on the surface of the proteins and are in contact with the aquatic milieu, and non-polar/hydrophobic side-chain amino acids existing on the interface [47]. Changes in the abundance of amino acids, each with the different chemistry of side chain, alters conformation and structure of the protein. Amongst non-polar amino acids, the increase of leucine was considerable in the cell wall of *N. oleoabundans* grown in either seawater conditions or freshwater nitrogen-depleted culture. The significant increase of this amino acid ($p < 0.05$) could indicate that *N. oleoabundans* cell walls contain leucine-rich repeat proteins. These proteins are part of a large variety of organisms and are reported to be involved in many developmental processes and responses to biotic and abiotic stresses [48-50]. Due to the intrinsic limitation of the EZ:faast^{TM} method, we were unable to detect asparagine, glutamine and arginine in the *N. oleoabundans* cell wall. Furthermore, our results revealed a lack of cysteine and tryptophan in the *N. oleoabundans* cell wall that may well have been degraded during the acid hydrolysis process.

Our findings herein clearly confirm that the cell wall composition of *N. oleoabundans* varies depending on the cultivation medium. Remodelling of this dynamic structure is key for the plasticity of this species to survive in a wide range of growing conditions.

5. Conclusion

In this study we have highlighted the importance of the *N. oleoabundans* cell wall in response to high saline and/or nitrogen-deficient mediums. Cell wall remodelling under saline conditions comprises the possible accumulation of sulphated polysaccharides enriched in mannose, β-(1→4)-D-mannans, and glucose. The likely abundance of sulphated polysaccharides together with non-polar amino acids, especially leucine, could well enable the cell to resist the saline

environment. Nitrogen depletion also has an important effect on the cell wall composition, being the primary effect of this stress a substantial reduction in the nitrogenous components of the cell wall. This is the first study of this kind on the cell wall biology of *N. oleoabundans* which has enabled us to understand the complexity of remodelling the cell wall in response to salt and/or nitrogen deficiency..

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Author contributions

B.R performed the experiments, analysed data and wrote the paper. L.M.T wrote and coordinated the project, supervised the research and help shaping the manuscript. A.D help developing protocols and assisted in the chemical analysis. M.G.R and B.J performed the carbohydrate microarray and the glycomic profile and revised the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interest for this paper.

Informed consent

The authors declare that there are no conflicts, informed consent, human or animal rights applicable for this paper.

Authorship consent

All authors read, approved the final manuscript and agreed for its submission for peer review.

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Table 1. Concentration of nutrients in the nitrogen-replete medium. For a nitrogen-depleted medium, amount of $NaNO_3$ is reduced and half of the concentration was used. Macronutrients and micronutrients are indicated based of mM and μM, respectively.

	Component	Freshwater	Artificial seawater
Macronutrients	$NaNO_3$	11.765	11.765
	$MgSO_4.7H_2O$	0.811	-
	$MgCl_2$	-	102.929
	$NaCl$	17.111	419.233
	$NaHCO_3$	9.523	9.523
	KH_2PO_4	2.939	1.469
	K_2HPO_4	-	1.148
	$CaCl_2.2H_2O$	0.680	-
	$CaCl_2$	-	4.775
	K_2SO_4	-	4.877
	$NaSO_4$		26.878
Micronutrients	$NaEDTA$	31.722	95.168
	H_3BO_4	141.329	128.481
	$FeSO_4.7H_2O$	20.142	60.493
	$MnSO_4.H_2O$	2.366	-
	$MnCl_2.4H_2O$	-	6.992
	$ZnSO_4.7H_2O$	0.765	2.292
	$CoCl_2.6H_2O$	0.210145	0.630
	$CuSO_4.5H_2O$	0.0800	0.236
	$(NH4)Mo_7O_{24}.4H_2O$	0.040	-
	$Na_2Mo_4.2H_2O$	-	0.619
	KI	0.301	-

Figure 1. Percentage of the isolated cell wall in four different growing conditions. Values are the average from two technical replicates of pooled biomass and error bars represent the standard deviation (SD). Letters are based on the Fisher's unprotected least significant difference of cell walls amongst the different growing conditions.
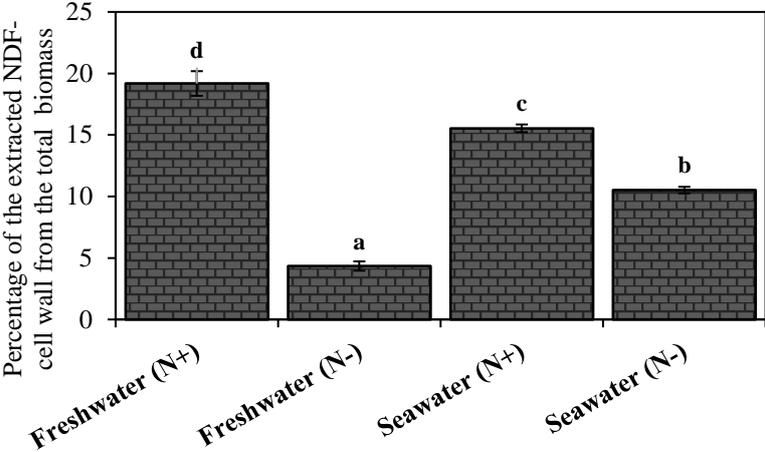
Figure 2. Percentage of the cell wall components in four different growing conditions. Values are the average from two technical replicates of pooled biomass and error bars represent the standard deviation (SD). "Others" fraction stand for the portion of the cell wall composition that remained uncharacterized. Letters are based on the Fisher's unprotected least significant difference of each cell wall component amongst cultures.
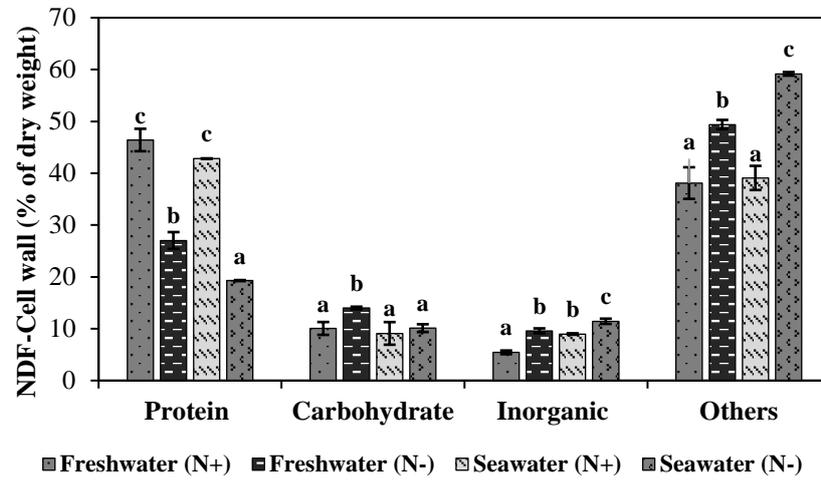
Figure 3. Monosaccharides composition of *N. oleoabundans* cell wall polysaccharides. Bar chart represents a relative amount of each sugar to the total monosaccharides. Values indicates the average from two technical replicates of pooled biomass. Letters are based on the Fisher's unprotected least significant difference of each sugar amongst cultures and error bars represent the standard deviation (SD).
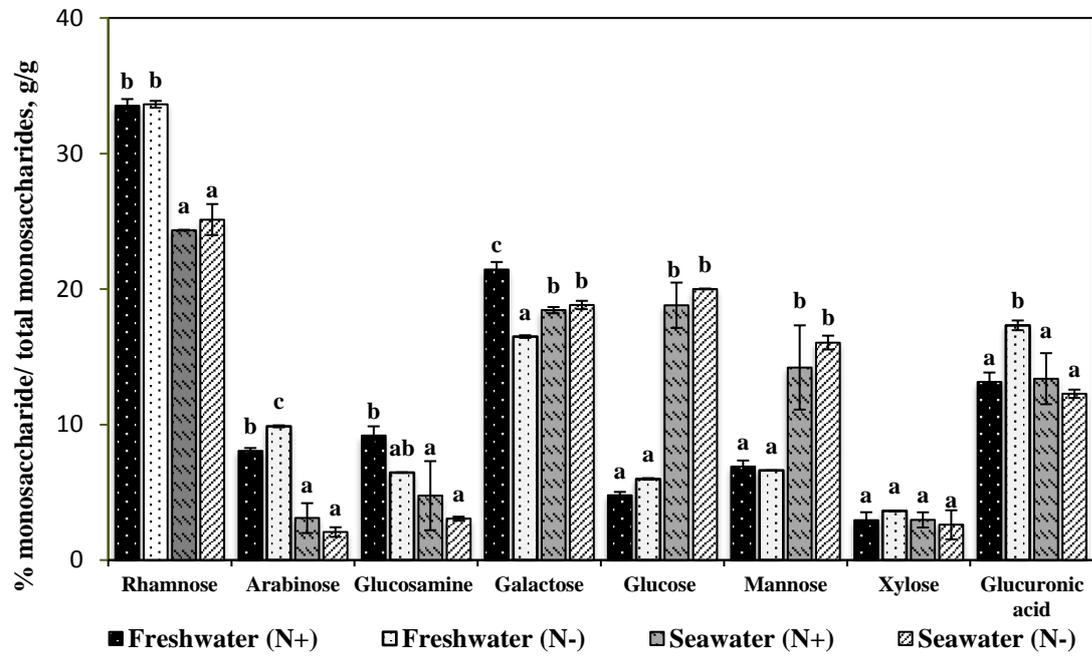
Figure 4. Heatmap showing the binding of monoclonal antibodies (mAbs) to the extracted cell wall materials. Values are the average of the replications and indicate the relative abundance of the antibodies to glycan epitope of *N. oleoabundans* cell wall. Colour intensity in the heatmap is correlated to mean spot signals. The highest signal in each dataset was set to 100, and all other values were normalized accordingly as indicated by the colour scale bar.

		(1→4)-β-D-mannan (mAb BS400-4)	(1→3)-β-D-glucan (mAb BS-400-2)	(1→3)(1→4)-β-D-glucan (mAb BS-400-3)	Backbone of rhamnogalacturonan I (mAb INRA-RU1)	Backbone of rhamnogalacturonan I (mAb INRA-RU2)	HG with a low DE (mAb JIM5)	HG with a high DE (mAb JIM7)	HG Partially methylesterified (mAb LM18)	HG Partially methylesterified (mAb LM19)	HG Partially methylesterified (mAb LM20)	Xylogalacturonan (mAb LM8)	Alginate? (mAb LM7)	(1→4)-β-D-galactan (mAb LM5)	Feruloylated (1→4)-β-D-galactan (mAb LM9)	(1→5)-α-L-arabinan (mAb LM6)	Linearised (1→5)-α-L-arabinan (mAb LM13)	(1→5)-α-L-arabinan) (mAb LM16)	Feruloylate on any polymer (mAb LM12)	(1→4)-β-D-mannan (mAb LM21)	(1→4)-β-D-mannan/galactomannan (mAb LM22)	No defined epitope (Anti callose like binding) (mAb JIM6)	Xyloglucan (XXXG motif) (mAb LM15)	Xyloglucan (mAb LM24)	Xyloglucan (mAb LM25)	(1→4)-β-D-xylan (mAb LM10)	(1→4)-β-D-xylan/arabinoxylan (mAb LM11)	(1→4)-β-D-xylan (mAb LM23)	Glucuronosyl-containing epitope of heteroxylan (mAb LM28)	Extensin (mAb LM1)	Extensin (mAb JIM12)	Extensin (mAb JIM19)	Extensin (mAb JIM20)	AGP (mAb JIM4)	AGP (mAb JIM13)	AGP (mAb JIM15)	AGP (mAb JIM16)	AGP (mAb JIM17)	AGP (mAb LM14)
CDTA	Freshwater	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0
NaOH	Freshwater	2	0	7	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	9	0	2	0	0	0	0	0	0	0	20	0	13
CDTA	Seawater	44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	2	0	0	0	0	0	0	0	10	0	0	0	0
NaOH	Seawater	71	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	24	9	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0

Scale Bar   0   25   50   75   100

Figure 5. Detected amino acids composition of *N. oleoabundans* cell walls using EZ:faast[TM] method. Bar chart signifies a percentage of molarity (%M) of each amino acid to the total identified amino acid content. Values indicates the average from two technical replicates of pooled biomass and letters are based on the Fisher's unprotected least significant difference of each amino acid amongst cultures. Error bars represent the standard deviation (SD). Due to the intrinsic limitation of the EZ:faast[TM] method we were unable to detect asparagine, glutamine and arginine. Asparagine and glutamine are quantitatively converted to aspartic acid and glutamic acid during acid hydrolysis. Therefore, the absolute value of these two amino acids might be overrepresented. Cysteine and tryptophan were not detected in the cell wall, which is most probably due to the degradation during HCl hydrolysis.
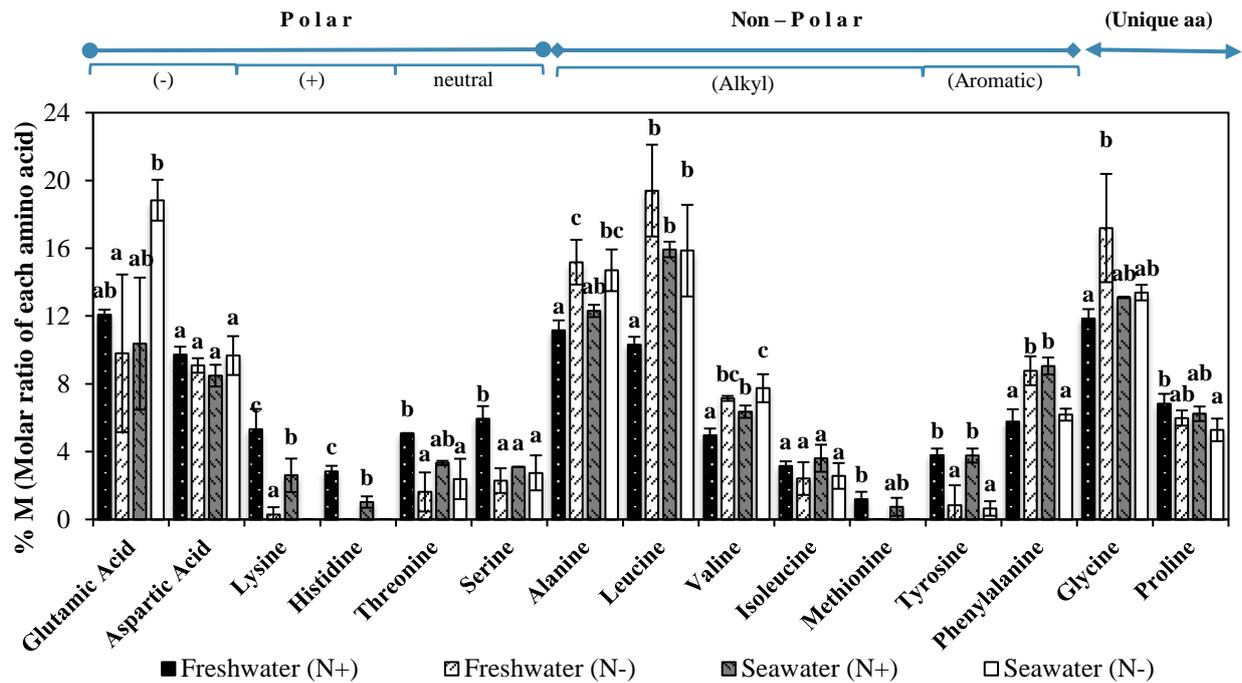
Figure 6. Percentage of each ion to the total inorganic content of NDF-cell wall (g/g). Bars represent the percentage of each ion in the total ash. Values are the average from two technical replicates of pooled biomass and error bars indicate the standard deviation (SD). Letters are based on the Fisher's unprotected least significant difference of each ion amongst cultures.