

Research Article

Molecular structure analysis of water-soluble polysaccharides from *Nannochloropsis oculata*

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ABSTRACT. *Nannochloropsis* species are highly productive and rich in carbohydrates, proteins, and polyunsaturated fatty acids. Nevertheless, in *Nannochloropsis* species, carbohydrates and proteins have been less explored; their lipids have been extensively tested for biofuel applications, and in the past decade, the carbohydrate content has been explored for biomedical applications. Despite this, knowledge about their composition and chemical structure remains insufficient. In this study, we characterized the water-soluble polysaccharides of *N. oculata* CIB76 using mass spectrometry and nuclear magnetic resonance spectroscopy. The analysis revealed that water-soluble polysaccharides from *N. oculata* CIB76 have a β -1,3/1,6 glucan structure, with a molecular weight of 4.2 to 6.2 kDa and a high degree of polymerization of 26 to 38. The type of β -glucans obtained from *N. oculata* CIB76 has been described as a high-value product with huge potential in biomedical applications. These types of β -glucans have applications in immune modulation, anti-inflammatory treatments, and anticancer therapies, positioning them as high-value in the pharmaceutical industry. Our findings provide the first detailed structural insight into polysaccharides from *N. oculata* CIB76, paving the way for further exploration in the biomedicine field. In conclusion, this work highlights the dual role of *Nannochloropsis*: as a pivotal renewable energy source in the bioeconomy and as an innovator in healthcare solutions that emphasize sustainability and the use of high-value resources.

Keywords: microalgae; carbohydrates; immunostimulants; β -glucan; potential biomedical

INTRODUCTION

Carbohydrates present in microalgae have generated interest in developing new biotechnologies, such as the

production of immunostimulants (Lee et al. 2024). Most of these compounds are polysaccharides (PS) that possess pathogen-associated molecular patterns (PAMPs) (Murphy et al. 2023), which bind to pattern recognition

receptors (PRR) and act as biological response modifiers (BRMs) (Shen et al. 2024). The molecular characterization of PS from microalgae, along with their beneficial properties, generates commercial interest as alternative products (Espinoza-Gallardo et al. 2017). Currently, the known microalgal PS are β -D-1,3 glucan structures, also known as β -glucans. Moreover, the β -D-1,3/1,6 glucans, such as laminaran (or laminarin) from the brown algae *Laminaria hyperborea*, chrysolaminaran (or chrysolaminarin) from marine diatoms (Størseth et al. 2004, 2006), and β -1,3 glucans of the unicellular freshwater algae *Euglena gracilis* named paramylon (Gottlieb 1850, Guo et al. 2025), are well known as immunostimulants. Despite this, knowledge of the composition and structure of microalgae PS remains scarce (Table 1).

For example, *Nannochloropsis* sp. is a high-value algae for research and aquaculture, and there has been considerable interest in its biotechnological applications (Espinoza-Gallardo et al. 2017). Still, the molecular structure of storage PS has not yet been confirmed. The genus *Nannochloropsis* belongs to the class Eustigmatophyceae, division Ochrophyta, order Eustigmatales, with five taxonomically accepted species: *N. australis*, *N. granulata*, *N. limnetica*, *N. oceanica*, and *N. oculata* (Guiry & Guiry 2018). This anterior taxonomic diversity, along with its biotechnological value, makes *Nannochloropsis* a key genus, and clarifying the molecular structure of its storage polysaccharides could open new doors for aquaculture, biotechnology, and biomedical applications.

Vogler et al. (2018) mention that it has been assumed that *Nannochloropsis* species utilize a storage PS structured similarly to its evolutionary neighbors, diatoms (chrysolaminarin; Myklestad 1989) and brown algae (laminarin; Read & Currie. 1996). As a result of a secondary endosymbiosis of a red alga, stramenopiles retained β -glucans as the primary soluble storage PS, in contrast to the starch and glycogen of green plants and animals. β -glucan has been identified and described in representative diatoms, brown algae, and oomycetes (Vogler et al. 2018). However, its biochemical characterization in all *Nannochloropsis* species remains unreported.

Recently, *N. oceanica* has been shown to accumulate β -glucans during the day of a diel light cycle and to consume this carbohydrate throughout the night (Poliner et al. 2015). Rojo-Cebreros et al. (2017) reported 1.1 pg cell⁻¹ of β -glucan individual content on *N. oculata* CIB76. Moreover, *N. gaditana* produces a soluble β -1,3-linked storage glucan with extremely

sparse β -1,6 branching (Vogler et al. 2018). However, an unusual report shows that the water-soluble PS fractions from *N. oculata* (Necton S.A. Olhão, Portugal; [https://necton.pt/]) are rich in mixed-linked (β 1-3, β 1-4)-glucans and (α 1-3)-, (α 1-4)-mannans (Pandeirada et al. 2019). Therefore, the high complexity of PS structures and the diversity among microalgae species limit their characterization and precise knowledge of their relationship with BRM activities, leaving most PS structures under-revealed (Pandeirada et al. 2019). Thus, the limited information available about PS in *Nannochloropsis* species constrains its reliable biotechnological application.

Furthermore, PS (like β -glucans) is considered a high-value byproduct that could also offset the excessive costs associated with the massive biomass production of distinct species of microalgae, such as *Nannochloropsis* (Espinoza-Gallardo et al. 2017). Despite the findings, there is little evidence supporting the natural molecular structure characterizing the water-soluble PS from *N. oculata*. Therefore, this study aims to analyze the molecular structure of water-soluble PS derived from viable microalgae biomass of *N. oculata* CIB76, as understanding the PS molecular structure promotes their successful biotechnological application.

MATERIALS AND METHODS

Microalgae culture conditions

The *N. oculata* strain CIB76 from the Collection of Microalgae Laboratory of the Centro de Investigaciones Biológicas del Noroeste (CIBNOR, S.C., México) was used (Fig. 1). Batch microalgae culture was performed in the Laboratorio de Ecofisiología de Organismos Acuáticos y Cultivos de Apoyo de la Facultad de Ciencias (FACIMAR, UAS, México). Successive transfers from 15, 200 mL, and 16 L carboys were performed (15 days) using F/2 medium (Guillard & Ryther 1962). A light intensity of 125 μ mol m⁻² s⁻¹ was maintained using six daylight white fluorescence lamps, which together consume 450 W and emit 6,000 lx, located on one side and close to the microalgae containers. Also, vigorous and constant aeration was supplied, but no CO₂ was added. The temperature (27.5 \pm 0.8°C) and pH (9.6 \pm 0.6) of water cultures were recorded daily.

Microalgal biomass production

The cell density of the microalgae culture was daily calculated using an improved Neubauer hemocytometer (0.1 mm depth, Brighline Optik Labor) under a light

Table 1. The molecular structure of water-soluble polysaccharides from *Nannochloropsis* species and other putative characteristics with biotechnology potential.

Species	Bond linkages	Branch linkages	G. Branch	Grade polymerization	Molecular weight (kDa)	Branch displacement (ppm)	Other characteristics	Potential	Cite
<i>N. gaditana</i>	β -1,3	β -1,6	0.028 - 0.105	10-20	< 5	4.2 - 4.6	A glycogenin-like glycosyltransferase family 8 (GT8) protein was also identified to be conserved among all species of <i>Nannochloropsis</i> , despite the lack of glycogen in the genus		Vogler et al. (2018)
<i>N. oculata</i>	Mixed-linked				< 10		Anionic sulphated heterorhamnans	The immunostimulatory assay showed that these fractions could also stimulate murine B lymphocytes	Pandeirada et al. (2019)
	β 1-3	β 1-4							
	α 1-3, mannans	α 1-4							
<i>N. oculata</i>	β -1,3	β -1,6		26 - 38	5.2	4.27			Present study

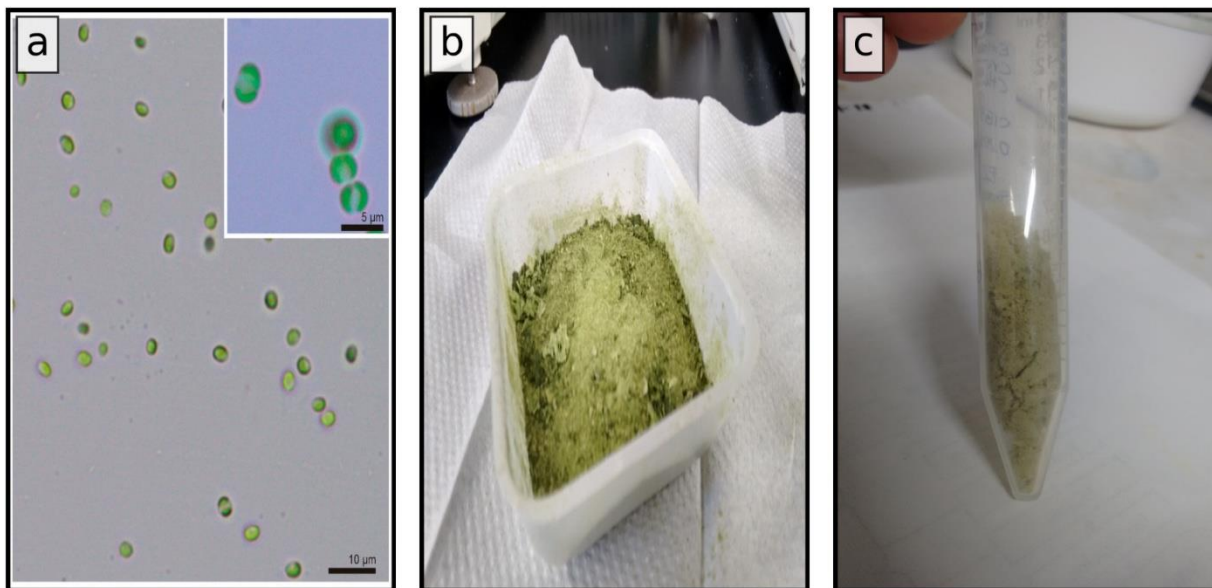


Figure 1. a) Photomicrographs of *N. oculata* CIB76 in the exponential growth phase (40x and 100x), b) biomass lyophilized sample from the microalgae, and c) crude carbohydrate as a white/yellow powder extracted from *N. oculata* CIB76.

microscope (Leica model CME) (Guillard & Sieracki 2005). The objective was to monitor every 24 h until the stationary growth phase, which we determined based on the maximum value of the cumulative cell division rate per day ($\Sigma\mu$) (Nieves et al. 1998). The microalgal biomass of *N. oculata* was centrifuged (3,600 rpm, 10 min, 10°C), and the supernatant was decanted (Eppendorf 5810R centrifuge). The biomass was then placed in an ultra-freezer at -70°C (Arctiko®) and after lyophilized (Labconco/208220®, 0.050 mmHg, -50°C).

Extraction of polysaccharides

Because storage and use of water-soluble PS by *Nannochloropsis* species are known, soluble carbohydrates were produced using the hot water-ethanol method described by Bobadilla et al. (2013) (Fig. 1), from the harvest of *N. oculata* CIB76 stationary growth phase in batch culture (10 L, 12 days, 33.9×10^6 cell mL⁻¹) (Fig. 1). First, a freeze-dried *N. oculata* microalgal paste (10 g) was subjected to a process of soaking and washing with 95% ethanol 1:10 (w/v) for 1 h for the depigmentation of the microalgal cells, subsequently, a fresh ethanol replacement was performed with vigorous shaking for 10 min, then it was left to rest overnight, the next day they were placed in a water bath for 1 h at 70°C, then centrifuged (4,550 rpm, 20 min, 10°C) to recover the supernatant, the cell pellet was once again resuspended in distilled water and

incubated in a water bath for 3 h, with shaking every 20 min for 5 min at a time, again a centrifuged, collecting the supernatant obtained which was cooled to 4°C and stocked at -70°C. The remaining solvent was removed using a lyophilizer (0.050 mmHg, -50°C) to give crude carbohydrate as a white/yellow powder (Fig. 2). The procedure is summarized as shown in Figure 2. The final product of the crude carbohydrate extract obtained was 1.2 g from 10 g of lyophilized biomass. The dried sample of water-soluble carbohydrates from viable *N. oculata* was subsequently analyzed by mass spectrometry and nuclear magnetic resonance (NMR) spectrometry.

Mass spectrometry

The molecular weight was determined by matrix-assisted laser desorption/ionization coupled to time-of-flight analyzer (MALDI-TOF) in DMSO-d₆ using 2,5-dihydroxybenzoic acid (2,5-DHB) as the matrix at a 1:5 ratio, on a Bruker Daltonics Flex Analysis instrument.

NMR spectrometry

The crude freeze-dried carbohydrate extract (10 mg) was previously extracted with dichloromethane to remove fats and other nonpolar components. It was further dissolved in 0.5 mL of deuterated dimethyl sulfoxide (DMSO-d₆) and deuterium oxide (D₂O) in a 6:1 ratio, for hydrogen and carbon-13 NMR experiments. One-dimensional (1D) (¹H and ¹³C NMR and

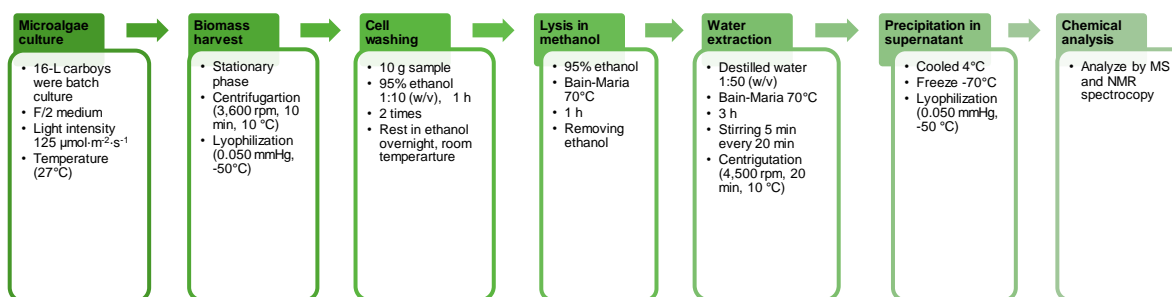


Figure 2. Extraction process and chemical analysis of soluble polysaccharide from *Nannochloropsis oculata* CIB76 (MS: mass spectrometry; NMR: nuclear magnetic resonance).

Attached Proton Test, APT) and two-dimensional (2D) spectra (COSY, HSQC, and HMBC) were determined in a 700 MHz spectrometer (Bruker Advance III HD). The residual DMSO- d_6 solvent was used as an internal reference ($\delta_{\text{H}} = 2.50$, $\delta_{\text{C}} = 39.52$). The spectra were processed using the Mestrenova software (Fig. 3). The degree of polymerization (DP) and degree of branching (DB) were calculated non-destructively from the ^1H NMR data according to Kim et al. (2000) using Lorentzian/Gaussian line-fitting.

RESULTS

Molecular structure of a water-soluble storage PS

The ^1H NMR spectra determined at 700 MHz showed proton signals of the basic skeleton of a β -1,3 glucan, with resonance signals in the range of 3.18 to 4.51 ppm (Fig. 3a). The anomeric proton signal ^1H corresponding to the main chain (β -1,3) was located as a doublet at δ 4.51 ($J = 7.2 \text{ Hz}$), the assignment of the remaining signals was made according to the couplings observed in the COSY spectrum (Fig. 3c), which were observed at δ 3.26 (m, H2, superimposed with H5), 3.46 (t, $J = 8.4$, H3), 3.18 (t, $J = 8.7$, H4), 3.23 (m, H5, superimposed with H2), the protons H6' and H6'' were found at δ 3.68 (d, $J = 10.7$) and 3.41 (m), respectively (Fig. 3c).

Two-dimensional (2D) homo- and heteronuclear spectroscopic correlation experiments revealed distinct couplings (Fig. 3b), facilitating the correct assignment of the signals. The APT experiment showed the number of carbons corresponding to the glucose monomeric structure that makes up the polymer, observing a shift of 103.32 ppm for the anomeric carbon one, identifying the remaining signals of 73.61, 86.08, 68.91, 76.79 and 61.39 ppm for carbons 2, 3, 4, 5 and 6 respectively, are shown (Fig. 3b). The carbon signal shifts were assigned according to the HSQC experiment. The long-range

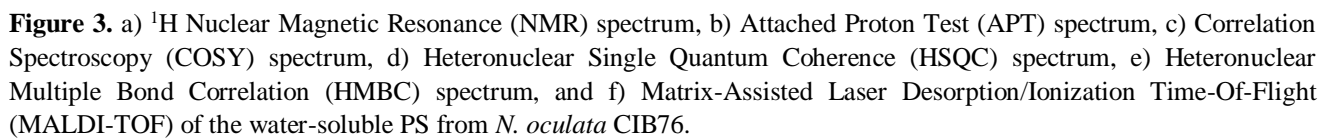
couplings (2J and 3J) observed in the HMBC experiment are consistent with the above (Fig. 3d).

Molecular weight and degree of polymerization (DP) of a water-soluble storage PS

The MALDI-TOF mass spectrum was analyzed, and a molecular mass for the PS was observed in the range of 4.2 to 6.2 kDa (Fig. 3f), with an estimated DP of 26 to 38 monomer units.

DISCUSSION

Although *Nannochloropsis* microalgae species have a high content of proteins and polyunsaturated fatty acids, especially eicosapentaenoic acid (Navalho et al. 2025), the nutritional and biotechnological utilization of carbohydrates from biomass *Nannochloropsis* species has not been significant. At the same time, they are the preferred substrate for significant biofuel production because, in some biomass conversion technologies, carbohydrates are the key component. Thus, biomass carbohydrate maximization might advance these biomass conversion technologies. Therefore, microalgae rich in carbohydrates may be a suitable feedstock for biofuel production (Markou et al. 2012). However, the most significant potential of microalgae carbohydrates lies in their biomedical applications as immunostimulants (Yaakob et al. 2014). Therefore, PS derived from *Nannochloropsis* biomass is crucial for the development of biotechnological processes (Lee et al. 2024). In this context, Rojo-Cebreros et al. (2017) state that *N. oculata* has excellent potential for the industrialized production of β -glucans because strains had 23-31% dry weight as carbohydrates, of which 14-21% are β -glucans, and with a maximum volumetric productivity of $81.55 \text{ mg L}^{-1} \beta$ -glucans.



for two species, *N. gaditana* (Vogler et al. 2018) and *N. oculata* (Pandeirada et al. 2019). *N. gaditana* presents

a structure consisting of a glucose chain of β -1,3 linkages with β -1,6 branches with a displacement of 4.2 to 4.6 ppm corresponding to the main chain, as well as the reducing terminals and the terminal of the side chain (Vogler et al. 2018). Regarding *N. oculata*, a different structure was reported; the water-soluble PS fractions were rich in (β 1-3, β 1-4)-glucans, (α 1-3)-, (α 1-4)-mannans, and anionic sulfated heterorhamnans. Compared with the characterization obtained in our analysis of PS from *N. oculata*, CIB76 was found to have a structure similar to that of *N. gaditana* (Vogler et al. 2018) and differs from that reported for *N. oculata* (Necton S.A., Olhão, Portugal, [https://necton.pt/]; Pandeirada et al. 2019). These differences can be explained by the procedure used to fractionate the soluble PS from *N. oculata*, as described by Pandeirada et al. (2019). Besides, it is believed that the last eukaryotic common ancestor had the tools for synthesizing both α -1,4- and β -1,3-glucans (Michel et al. 2010). However, β -1,3 glucans are the principal structural component reported in the molecular characterization of *Nannochloropsis* PS.

The presence of β -1,3-glucans as a storage carbohydrate in this evolutionary lineage is peculiar, as evidenced by the putative genes in *N. gaditana* that encode enzymes required for their synthesis. In *N. gaditana*, putative genes encoding enzymes needed for the synthesis of the carbohydrate oligosaccharide from glucose 6-phosphate were identified. A glycogenin-like glycosyltransferase family 8 (GT8) protein was also identified to be conserved among all species of *Nannochloropsis*, despite the lack of glycogen in the genus. Also, a set of three likely laminarinases is highlighted from the glycosyl hydrolases by phylogenetic analyses (Vogler et al. 2018).

Likewise, molecular weight values and DP have been reported in different β -1,3 structures and β -1,6 branches obtained from other organisms. For example, Kao et al. (2012) reported the presence of a low molecular weight β -glucan extracted from the fungus *Ganoderma lucidum*, with an average molecular weight of 1.5 kDa and a DP ranging from 6 to 15 units, with an average of 9 units, as determined using the MALDI-TOF technique. For example, the diatom *Odontella aurita* (10-95 μ m, cell size) has a high molecular weight of 7.75 kDa (Xia et al. 2014). In contrast, Sadoshkaya et al. (2014) report a molecular weight range of 1.2-5.2 kDa in *Isochrysis galbana* (4-8 μ m, cell size). In species such as *Chaetoceros debilis* (cell size 10-40 μ m), a molecular weight of 4.9 kDa has been reported (Størseth et al. 2006). Similarly, in the present study, molecular weight values ranged from 4.2 to 6.2 kDa,

with an average of 5.2 kDa, and a DP of 26 to 38 units in β -glucans from *N. oculata* CIB76. In turn, reports of these compounds for *N. gaditana* indicate a molecular mass of <5 kDa with a DP between 8-9.2 (Vogler et al. 2018) (Table 1).

The highly branched β -1,3/1,6 glucans from microalgae have been proposed with important biotechnological potential (PAMPs and BRMs), for example: mixed-linked (β 1-3, β 1-4)-glucans and (α 1 \rightarrow 3)-, (α 1 \rightarrow 4)-mannans from *N. oculata* are a source of dietary fibers with prebiotic activity. They could also stimulate murine B lymphocytes (Pandeirada et al. 2019). Also, β -1,3/1,6 glucans isolated from *I. galbana* directly inhibit the proliferation of U937 human leukemic monocyte lymphoma cells and therefore have potential anti-tumor activity (Sadoshkaya et al. 2014). Another example is the evaluation of β -1,3/1,6 glucans isolated from *Chaetoceros mulleri* as immunostimulants in Atlantic cod larvae (*Gadus morhua*), which increased survival and growth, attributing the immunostimulant effect to their molecular characteristics (Skjermo et al. 2006). Also, β -glucans are considered nutraceutical compounds because their consumption has been associated with the prevention and/or treatment of diseases (Veverka et al. 2014) or with the health management of aquatic animals (Kumar et al. 2013). Thus, β -glucans present in *Nannochloropsis* species have potential biomedical applications, such as immunostimulants, prebiotics, and adjuvants for the treatment of cancer and infections, and possibly for inflammatory or neurodegenerative diseases (Novák & Vetricka 2008). These nutraceutical properties make it valuable for both human health and aquaculture (Murphy et al. 2023, Shen et al. 2024).

CONCLUSION

The molecular structure analysis of water-soluble polysaccharides from marine microalgae *N. oculata* CIB76 presented a structure with β -1,3-linked and β -1,6 branches with a molecular weight in the range of 4.2 to 6.2 kDa and a DP of 26 to 38. Also, *Nannochloropsis*-derived PS and glucans could revolutionize fields ranging from biotechnology to sustainable health management.

Credit the author's contribution

A. Rojo: conceptualization, validation, methodology, formal analysis, and writing-original draft; M. Morales: methodology, data curation, formal analysis, and review; L. Quijano: methodology, validation, and

supervision; L. Ibarra: funding acquisition, project administration, supervision, review, and editing; J.M. Martínez: formal analysis, review, and editing & Á. Escamilla: formal analysis, review, and editing. All authors have read and accepted the published version of the manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest.

ACKNOWLEDGMENTS

We want to express our gratitude to the staff at the Laboratorio de Ecofisiología de Organismos Acuáticos y Cultivos de Apoyo (FACIMAR-UAS), especially Pablo Piña, for providing access to the laboratory facilities, technical assistance, and valuable suggestions that contributed to the successful development of this study. The authors acknowledge Beatriz Quiroz, Martha Elena García, Erendira García, Adriana Romo, and Rocío Aguilar of UNAM's NMR lab, LURMN at IQ-UNAM, for collecting MS and NMR data.

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Received: April 22, 2025; Accepted: October 23, 2025