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POSGRADO EN BIOCIENCIAS

OBTENCIÓN Y CARACTERIZACIÓN DE POLISACÁRIDOS SULFATADOS DE LA MICROALGA *Navicula* sp. CULTIVADA EN DOS IRRADIANCIAS Y TRES LONGITUDES DE ONDA.

TESIS

que para obtener el grado de:

DOCTORA EN BIOCIENCIAS

presenta:

DIANA FIMBRES OLIVARRÍA

Hermosillo, Sonora, México **Enero de 2017**

DERECHOS DE AUTOR

El presente trabajo de tesis se presenta como uno de los requisitos parciales para la obtención del grado de **Doctora en Biociencias** de la Universidad de Sonora.

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APROBACIÓN

Los miembros del Comité designado para revisar la tesis titulada **Obtención y caracterización de polisacáridos sulfatados de la microalga *Navicula* sp. cultivada en dos irradiancias y tres longitudes de onda** presentada por **Diana Fimbres Olivarría**, la han encontrado satisfactoria y recomiendan que sea aceptada como requisito parcial para obtener el grado de Doctor en Biociencias.

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A mi Familia

"Para plantear nuevas preguntas, nuevas posibilidades, considerar los viejos problemas desde un nuevo ángulo... se requiere imaginación creativa y es lo que marca un verdadero avance en la ciencia"

Albert Einstein

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RESUMEN

Las microalgas se han utilizado para la obtención de compuestos bioactivos debido a que poseen un amplio rango de aplicaciones. Las condiciones de cultivo son el principal factor que afecta la composición bioquímica de las microalgas, siendo la luz una de las más importantes. Las diatomeas bentónicas se caracterizan por producir un mucílago constituido por un alto contenido de sustancias poliméricas extracelulares, incluyendo polisacáridos. El género *Navicula* ha adquirido gran interés debido a las propiedades bioactivas que poseen sus polisacáridos sulfatados. En este estudio se evaluó el efecto combinado de dos irradiancias (50 y 100 µmoles fotones $m^{-2}seg^{-1}$) y tres longitudes de onda: blanca, azul (430-480 nm) y roja (595-660 nm), en el crecimiento, biomasa y composición bioquímica de *Navicula* sp.; además de las características fisicoquímicas y posibles aplicaciones de sus polisacáridos sulfatados. Los conteos celulares se realizaron diariamente, la concentración celular fue superior en los cultivos expuestos a luz blanca; la biomasa se determinó gravimétricamente siendo superior en los cultivos expuestos a luz azul e irradiancia alta; la composición bioquímica se analizó utilizando micrométodos; el contenido más alto de proteínas se observó en luz azul, carbohidratos en luz blanca y lípidos en luz roja; todos obtenidos en irradiancia baja. Se detectaron diferentes concentraciones de glucosa, galactosa, ramnosa, xilosa y manosa mediante cromatografía de gases, en las tres longitudes de onda. Los espectros FT-IR mostraron el patrón típico de los carbohidratos. Los polisacáridos analizados mediante rodizonato de sodio presentaron bajo contenido de sulfato. Se observó que los polisacáridos sulfatados de *Navicula* sp. (1% p/v) son capaces de formar geles en presencia de $FeCl_3$ (0.4% p/v). Estos polisacáridos poseen actividad antioxidante DPPH y $ABTS^+$, por lo cual podrían ser utilizados como una fuente alternativa de antioxidantes.

ABSTRACT

Microalgae have been utilized to obtain bioactive compounds due their wide range of applications. The culture conditions are the main factor involved in the biochemical composition of microalgae, light being one of the most important. Benthic diatoms are characterized by producing a mucilage with a high content of extracellular polymeric substances, including polysaccharides. The *Navicula* genus is of great interest because of the bioactive properties of their sulfated polysaccharides. The present study evaluated the combined effect of two irradiances (50 and 100 µmol photon $m^{-2}sec^{-1}$) and three wavelengths: white, blue (430-480 nm) and red (595-660 nm), on the growth, biomass, and biochemical composition of *Navicula* sp.; the physical-chemical characteristics and the possible applications of their sulfated polysaccharides were also evaluated. Cellular counts were performed daily; the cellular concentration was higher in the culture exposed to white light; the biomass was analyzed by gravimetric methods, being higher in the culture exposed to high irradiances and blue light; the biochemical composition was analyzed by micromethods. The highest content of proteins was observed at blue light, carbohydrates in white light and lipids in red light; they all were obtained at low irradiance. Regarding to the sulfated polysaccharides analysis, different concentrations of glucose, galactose, rhamnose, xylose, and mannose were detected by gas chromatography in the three wavelengths analyzed. The FT-IR spectra showed the typical infrared footprint of carbohydrates. The polysaccharides analyzed by sodium rhodizonate method presented a low sulfate content. It was observed that sulfated polysaccharides (1% w/v) formed gels in the presence of $FeCl_3$ (0.4% w/v). The sulfated polysaccharides from *Navicula* sp. presented antioxidant activity DPPH and $ABTS^+$; these polysaccharides can be used as an alternative source of antioxidants.

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I. INTRODUCCIÓN

Las microalgas marinas han sido utilizadas durante décadas principalmente como alimento vivo para organismos acuáticos, debido a su alto contenido de proteínas, lípidos y carbohidratos Brown *et al*., (1997); sin embargo, en la actualidad se han desarrollado estudios para la obtención de sus compuestos bioactivos. Los compuestos con actividad biológica poseen un amplio rango de aplicaciones en la industria biotecnológica, especialmente en el área biomédica, farmacéutica, nutracéutica y cosmética; esto debido a sus propiedades antivirales, antimicrobianas, antioxidantes, antitumorales y otras (Staats *et al*., 1999; Lee *et al*., 2006; Melo *et al*., 2007; Laurienzo, 2010; Amaro *et al*., 2011; Raposo *et al*., 2013). Dentro de los principales géneros de microalgas utilizadas con fines biotecnológicos se encuentran *Dunaliella*, *Spirulina* y *Chaetoceros* (Abd El Baky *et al*., 2013; Hemalatha *et al*., 2013; Karthikeyan, *et al*., 2013).

Las características químicas de las microalgas son altamente variables, sin embargo, se conoce que las condiciones de cultivo son el principal factor que afecta su composición, siendo la calidad y cantidad de luz uno de los parámetros de mayor importancia (Markou *et al*., 2012). Autores como Jungandreas *et al*., (2014) reportan que la exposición a la luz roja produce un incremento en la concentración de carbohidratos, y que la luz azul promueve la producción de proteínas; mientras que Korbee *et al*., (2005) afirman que la exposición a la luz blanca representa un efecto combinado de las luces azul y roja.

Entre la gama de microalgas que se cultivan con propósitos biotecnológicos se encuentran las diatomeas; dentro de este género se encuentran las diatomeas bentónicas, las cuales se caracterizan por producir un mucílago que las ayuda a mantenerse unidas al sustrato y además las protege de las condiciones adversas que las rodean. Éste mucílago es una matriz constituida por un alto contenido de sustancias poliméricas extracelulares, como lípidos, proteínas, ácidos nucleicos y carbohidratos (Raposo *et al*., 2012).

Las microalgas pertenecientes al género *Navicula* poseen una amplia distribución geográfica; se han registrado a lo largo del Golfo de California y en las Costas del Pacífico y Atlántico de Norteamérica (Licea-Duran, 1974).

El género *Navicula*, perteneciente al grupo de las diatomeas, comprende alrededor de 1200 especies; son microorganismos unicelulares o coloniales y su tamaño varía con respecto a la especie. Son eucariotas fotosintéticos y su pared celular se encuentra constituida de sílice formando valvas que se sitúan en forma de caja. Su morfología celular es característica, dando el aspecto de un pequeño barco. Sus valvas son lanceoladas, estriadas transversalmente en la zona media, en sentido opuesto a los polos y los extremos de la célula son redondeados (Figura 1) (Round *et al*., 1990; Bruder y Medlin, 2008; Van de Vijver *et al*., 2011).

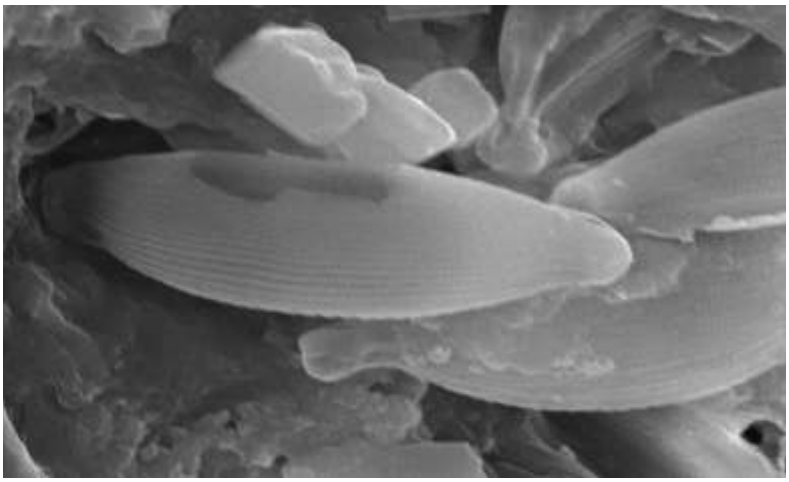

Figura 1. Morfología celular de la microalga *Navicula* sp. Micrografía SEM (Microscopía Electrónica de Barrido), x 5000 magnificaciones. Micrografía propiedad del Laboratorio de Análisis Químico y Microbiológico de la Universidad de Sonora (DICTUS).

Recientemente las especies del género *Navicula* han adquirido gran interés debido a las propiedades que poseen sus polisacáridos sulfatados, los cuales se caracterizan por su alto potencial biotecnológico (Lee *et al*., 2006; Jiao *et al*., 2011; Wang *et al*., 2012).

Los polisacáridos sulfatados son polímeros que contienen en su estructura una cantidad de grupos sulfatos unidos a los carbohidratos; se encuentran ampliamente distribuidos en la naturaleza, particularmente están presentes en organismos marinos como algas, microalgas y ciertos equinodermos (Abd El Baky *et al*., 2013). Dentro de las propiedades biológicas que poseen estos polisacáridos se encuentran las antioxidantes, antitumorales, anticoagulantes, antivirales, antimicrobianos, antilipémicas, capacidad de formar geles, entre otras (Running *et al*., 2012; Abd El Baky *et al*., 2013; Raposo *et al*., 2013).

A pesar de que se han explorado las distintas propiedades que poseen estos carbohidratos, aún no se conoce por completo la relación que existe entre la estructura de estos compuestos y su actividad biológica (Jiao *et al*., 2011); sin embargo, se ha reportado que su estructura y composición, el peso molecular, el contenido de sulfato, la distribución de los grupos sulfato, el tipo de monosacáridos, entre otras características, tienen una fuerte influencia en sus propiedades bioactivas (Qi et al., 2005; Jiao *et al*., 2011; Lo et al., 2011; Wang et al., 2015).

Actualmente no existen investigaciones en las que se haya evaluado el efecto combinado de la longitud de onda y la irradiancia en el crecimiento, producción de biomasa y composición bioquímica en microalgas del género *Navicula*, además de la producción de polisacáridos y la potencial utilización de estos compuestos en la industria biotecnológica.

Algunas investigaciones acerca de la capacidad antioxidante de extractos obtenidos a partir de microalgas se han enfocado principalmente en la actividad de sus pigmentos, tales como los carotenoides y las xantofilas; sin embargo, existen otros compuestos como los polisacáridos que poseen un alto poder antioxidante. En particular, se ha observado que los polisacáridos sulfatados de macroalgas y microalgas juegan un papel importante como antioxidantes al eliminar radicales libres, por lo cual su evaluación resulta de gran interés (Souza *et al*., 2012; Sun *et al*., 2014).

Aunque se han realizado distintos estudios acerca de las aplicaciones de los polisacáridos sulfatados de microalgas, no existen reportes acerca de la capacidad de gelificación de estos compuestos, particularmente de aquellos obtenidos a partir del género *Navicula*; sin embargo, existen evidencias de que estos polisacáridos pueden formar geles en presencia de iones trivalentes, como se ha observado en extractos de λ-carragenano obtenidos a partir de macroalgas (Running *et al*., 2012).

Por lo anterior, en la presente investigación se obtuvieron polisacáridos sulfatados de la microalga bentónica *Navicula* sp. en cantidades suficientes para extraerlos, caracterizarlos y evaluar su posible potencial como un producto de alto valor agregado.

II. JUSTIFICACIÓN

Las diatomeas bentónicas son un grupo poco investigado por lo cual su estudio resulta de gran interés debido a que representan una fuente alternativa para la obtención de polisacáridos. Las microalgas como *Navicula* producen un mucílago que las mantiene adheridas al sustrato; esta matriz está constituida por un alto porcentaje de sustancias poliméricas extracelulares, incluyendo a los polisacáridos sulfatados. Se ha demostrado que estos compuestos poseen una amplia gama de aplicaciones en la industria biotecnológica debido a sus propiedades bioactivas como antivirales, antibacterianas, antioxidantes, entre otras.

Actualmente, no existen investigaciones en donde se analicen en conjunto los efectos que produce la irradiancia y las longitudes de onda en estos organismos, particularmente sobre la producción de polisacáridos en especies del género *Navicula*, por lo cual no se han establecido las condiciones más adecuadas, tanto de irradiancia como de longitud de onda, que permitan obtener una producción adecuada de polisacáridos en cantidades suficientes para extraerlos, caracterizarlos y evaluar su potencial como un producto de alto valor agregado.

III. HIPÓTESIS

La exposición a distintas longitudes de onda y diferentes irradiancias en la microalga bentónica *Navicula* sp., causará un cambio en la cantidad de polisacáridos sulfatados extraíbles, así como en sus propiedades funcionales.

IV. OBJETIVOS

4.1. Objetivo General

Obtener y caracterizar los polisacáridos sulfatados producidos por la microalga *Navicula* sp. cultivada en dos irradiancias (50 y 100 µmoles fotones $m^{-2}seg^{-1}$) y tres longitudes de onda (blanca, azul: 430-480 nm y roja: 595-660 nm).

4.2. Objetivos Particulares

- Evaluar el crecimiento y producción de biomasa de *Navicula* sp. en tres longitudes de onda (blanca, azul y roja) a irradiancias de 50 y 100 µmoles fotones $m^{-2}seg^{-1}$.
- Determinar la composición bioquímica de *Navicula* sp. en tres longitudes de onda (blanca, azul y roja) a irradiancias de 50 y 100 µmoles fotones $m^{-2}seg^{-1}$.
- Obtener y caracterizar el contenido de polisacáridos sulfatados presentes en *Navicula* sp. expuestas a 50 µmoles fotones $m^{-2}seg^{-1}$ en las tres longitudes de onda.
- Evaluar las propiedades funcionales de los polisacáridos sulfatados extraídos para su posible aplicación en la industria biotecnológica.

V. ARTÍCULOS PUBLICADOS Y ENVIADOS

Tabla 1. Lista de artículos publicados y enviados durante el periodo 2015-16.

	ARTÍCULO	JOURNAL	ESTADO
5.1.	Growth and biochemical composition of *Navicula* sp. Cultivated at two light intensities and three wavelengths.	The Israeli Journal of Aquaculture (BAMIDGEH)	Publicado
5.2.	*Navicula* sp. Sulfated Polysaccharide Gels Induced by Fe(III): Rheology and Microstructure	International Journal of Molecular Sciences	Publicado
5.3.	Chemical characterization and antioxidant activity of sulfated polysaccharides from *Navicula* sp.	Food Hydrocolloids	Enviado

5.1. Artículo Publicado

Growth and biochemical composition of *Navicula* sp. Cultivated at two light intensities and three wavelengths

Fimbres-Olivarría, D.; López-Elías, J.A.; Martínez-Córdova, L.R.; Carvajal-Millán, E.; Enríquez-Ocaña, F.; Valdez-Holguín, E.; Miranda-Baeza, A.

The Israeli Journal of Aquaculture
(BAMIDGEH)

The Israeli Journal of Aquaculture - Bamidgeh, IJA_67.2015.1155, 7 pages

Growth and Biochemical Composition of *Navicula sp.* Cultivated at Two Light Intensities and Three Wavelengths

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Key words: *Navicula* sp., light intensities, wavelengths, LED, biochemical composition, growth

Abstract

Many studies have reported that the exposure of microalgae cultures to red light increases the production of carbohydrates, while blue light promotes the production of protein. There are several studies about *Navicula*, however there are few, if any, studies of the combined effects of wavelength and light intensity on the biochemical composition of this genus In this study we evaluated the combined effect of three wavelengths: white (400-750nm), blue (430-480nm), and red (650-750nm), at two light intensities (50 and100 µmol/m^2/sec) on the growth and biochemical composition of *Navicula* sp. cultured on a laboratory scale. The experiment was carried out under controlled conditions utilizing a factorial design 2x3 (light intensity and wavelength) with white light as the control. The cell concentration was measured daily. Dry biomass of filtered cells was incinerated at 450ºC in a muffle oven. The biochemical content was measured using micro methods. The cell concentration was higher with white light at both intensities (291,875 and 90,938 cells/mL at 50 and100 µmol photon/m^2/sec, respectively). Microalgae grown under blue wavelengths at 100 µmol photon/m^2/sec had the highest dry biomass (1607 pg/cell). The highest percentage of protein was obtained under the blue light (22.83%), carbohydrates under the white light (4.13) and lipids under the red light (35.25%) all these results were observed under the low light intensity (50 µmol photon/m^2/sec). The highest cell concentration and growth rate was observed under the low light intensity the largest proportions of which were proteins produced under the blue light. Lipid composition was not affected by light intensity or wavelength.

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Introduction

Microalgae are organisms of great interest due to the great variety of polysaccharides that can be extracted under culture conditions (Laurienzo 2010). Several studies have reported the effect of exposure to different wavelengths and light intensity on microalgae cultures. It has been observed that red light increases the production of carbohydrates and blue light promotes the production of protein in *Chaetoceros* cultures (Ramos-Lemuz 2000; Ramírez-Trejo 2002; Korbee et al. 2005). White light is the combined effects of red and blue wavelengths (Korbee et al. 2005).

Benthic microalgae have high potential for production of polysaccharides (Leal et al. 2010) because they generate mucilage which is high in extracellular polymeric substances (EPS), including lipids, proteins, nucleic acids, and carbohydrates (Staats et al. 1999; Leal et al. 2012). These compounds have a wide range of applications in biotechnological industries, such as gels production, cosmetics, antioxidants, antibacterials, antivirals, and others (Staats et al. 1999; Lee et al. 2006; Melo-Ruiz y Cuamatz, 2007; Laurienzo 2010; Amaro et al. 2011; Raposo et al. 2013).

Although there have been studies on the genus *Navicula* these have not investigated the combined effects of wavelength and light intensity on the biochemical composition of species of the genus. In this study we evaluated the effect of three wavelengths: white (400-750nm), red (650-750nm), and blue (430-480nm), at two light intensities (50 and100 µmol photon/m^2/sec) on the growth, biomass, and lipid, protein and carbohydrate content of *Navicula* sp.

Materials and Methods

Selection of microalgae strain. In this study we evaluated the benthic microalgae *Navicula* sp., using a strain from the collection of Department of Scientific and Technological Research of the University of Sonora (DICTUS).

Experimental design. The study was carried out under indoor controlled conditions. A factorial 3X2 (wavelengths X intensities) experimental design with 4 replicates per treatment was performed. Treatments consisted of three wavelengths: (control) white (400-750nm), red (650-750nm), and blue (430-480nm), and two light intensities (50 and 100 µmol photon/m2/sec). The experimental units consisted of transparent plastic containers with 10 L of F media (Guillard and Ryther 1962). The stocking density of microalgae was 35,000 cells/mL. Light was supplied by Light Emitting Diode lamps (LED) electronically controlled to the desired intensity. The irradiance was measured using a quantic spherical sensor Li-Cor 193SA to obtain the desired wavelength.

Cell count, biomass and proximate composition. The cell concentration was measured using a Neubauer chamber and an optical microscope (Carl Zeiss Axiostar plus) with the following equation:

$$\#\frac{\text{cells}}{\text{ml}} = (\text{Average number of cells in eight large squares})(10^4)$$

Samples for biomass evaluation were taken by filtering 250 mL culture water in Whatman GFC paper filters (diameter 47 mm) previously calibrated. The filters were dried for 8 h at 75ºC in a conventional oven, then incinerated for 12 h at 450ºC in a muffle oven, and finally weighed with a digital balance. Biomass was determined by difference in weight. The microalgae protein content was measured following the methodology described by Lowry and modified by López-Elías et al. (1995); the carbohydrate concentration was estimated by the "phenol-sulfuric acid" method reported by Dubois et al. (1956), and the lipid content was calculated by a colorimetric method described by Pande et al. (1963).

Statistical analysis. The cell concentration data was analyzed with descriptive statistics (average and standard deviation), in addition to an analysis of two-way ANOVA and a posteriori Tukey test to determine differences between treatments in terms of cell concentration, biomass, and biochemical composition of microalgae (Zar, 1999). For statistical analysis STATISTICA for Windows (StatSoft, 1995) was used.

Results

Cell concentration. Growth (measured as cell concentration) of control cultures (white light) and cultures treated with red light increased up to second day at light intensity of 50 µmol photon/m^2 /sec, while in cultures under blue light, they increased up to the third day at the same intensity. No significant differences in final cell concentration between blue light and the control (Figure 1a) were found (F=34.77, p>0.05). Growth of cultures in the control (white light) treatment, exposed to 100 µmol photon/m^2/sec was significantly higher on the first day, compared to the other wavelengths (F=34.77, p <0.05) (Figure 1b).

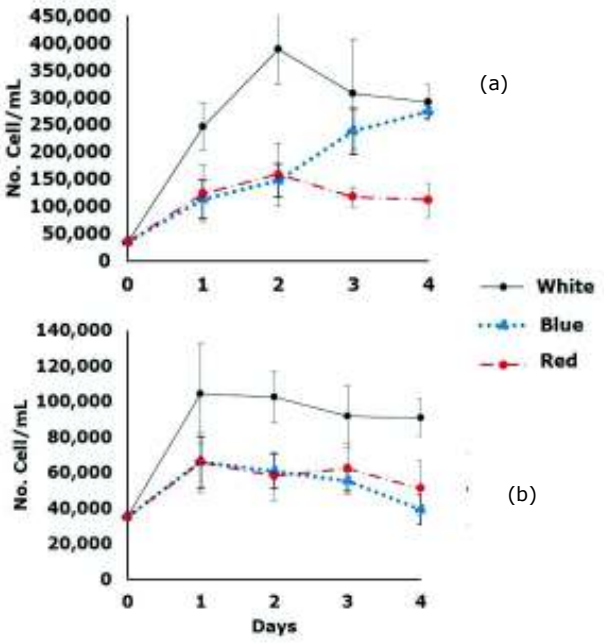

Fig. 1. Growth curves of the microalgae *Navicula* sp. cultivated at two light intensities:
(a) 50 µmol/m^2/sec and
(b) 100 µmol/ m^2/sec
at three wavelengths (white: 400-750nm, blue: 430-480nm and red: 650-750nm).

Cell concentration levels of microalgae at the end of the experiment were higher in white light at both intensities (291,875 cells/mL at 50 µmol photon/m^2/sec and 90,938 cells/mL at 100 µmol photon/m^2/sec), with significant differences ($F_{illumination}$ $F_{wavelength}$ between them (Table 1).

Table 1. Cell concentration and growth rates at the end of the culture of *Navicula* sp. cultivated at two light intensities (50 µmol photon/m^2/sec and 100 µmol photon/m^2 /sec) and three wavelengths.

Light intensities	*Wavelength (nm)*	*Final cell concentration (Cell/mL)*	*Growth rate (µmax)*	*Average growth rate*	*Accumulated growth rate*
50	400-750	291,875± 33,064^{d}	2.80±0.26^{b}	1.26±0.98^{b}	9.02±5.32^{a}
50	430-480	274,583±12,140^{d}	1.64±0.42^{a}	0.87±0.58^{ab}	5.07±3.23^{b}
50	650-750	112,500±31,225^{c}	1.72±0.71^{a}	0.75±0.68^{ab}	4.16±2.51^{ab}
100	400-750	90,938±10,625^{bc}	1.54±0.37^{a}	0.62±0.56^{a}	4.25±2.53^{ab}
100	430-480	39,063±8,189^{a}	0.88±0.31^{a}	0.31±0.36^{a}	1.91±1.41^{a}
100	650-750	51,250±15,579^{ab}	0.88±0.36^{a}	0.33±0.35^{a}	2.23±1.29^{ab}

Different letters in the same column indicate significant differences at P<0.01.

Final cell concentrations were higher in cultures grown under blue and red wavelengths at low intensities of 50 µmol photon/m^2/sec compared with the concentrations reached in cell cultures exposed to the higher light intensity of 100 µmol photon/m^2/sec at the same wavelengths. All cultures had the lowest concentration of cells at the highest light intensity during the experiment (Table 1).

Maximum growth rate was obtained at 50 µmol photon/m^2/sec light intensity under white light (2.80 divisions/day), significantly higher than the other treatments ($F_{illumination}$= 20.75 $F_{Wavelenght}$ significant differences (F=0.81, p>0.05) between maximum growth rates in cultures exposed to 100 µmol photon/m2/sec of light intensity at the three wavelengths.

Biomass production and biochemical composition. The highest production of dry biomass per cell was found in cultures exposed to light intensity of 100 µmol photon/m^2/sec at the three wavelengths, those grown at blue wavelengths being the greatest (Table 2), while the cultures exposed to 50 µmol photon/m^2/sec had the lowest dry biomass production, being greater in the cultures exposed to the red wavelength ($F_{illumination}$ $F_{wavelenght}$

Table 2. Biomass in pg/cell (picograms per cell) and final biochemical composition of the microalgae *Navicula* sp. cultivated at two light intensities (50 µmol photon/m^2/sec and 100 µmol photon/m^2/sec) and three wavelengths: white (400-750nm), blue (430-480nm), and red (650-750nm).

Light intensities	*Wavelength (nm)*	*Dry biomass (pg/cell)*	*Organic matter (pg/cell)*	*Protein %*	*Carbohydrate %*	*Lipid %*
50	400-750	199.14±11.35^{a}	99.7±3.40^{a}	16.54±2.13^{a}	4.13±0.70^{a}	25.40±2.60^{a}
50	430-480	178.22±68.16^{a}	132.6±14.09^{a}	22.83±2.42^{b}	3.06±0.86^{a}	25.32±5.32^{a}
50	650-750	423.56±25.17^{ab}	213.3±12.02^{ab}	15.12±4.79^{a}	3.33±0.78^{a}	35.25±4.54^{e}
100	400-750	592.16±105.98^{b}	320±59.96^{b}	15.02±2.12^{a}	3.49±0.85^{a}	30.34±6.20^{d}
100	430-480	1607.66±243.49^{c}	947.2±143.40^{d}	14.36±1.67^{a}	3.46±1.13^{a}	19.15±1.82^{ac}
100	650-750	1468.29±536.30^{c}	743.4±131.91^{c}	12.83±2.84^{a}	3.70±0.52^{a}	14.87±3.93^{b}

Different letters in the same column means significant differences at P<0.01.

As shown in table 2, a significant increase of dry biomass and organic matter production (pg/cell) was observed as the light intensity increased, with the greatest values for the microalgae cultivated at 100 µmol photon/m^2/sec.

The highest protein level was observed in cultures exposed at light intensities of 50 µmol photon/m^2/sec in blue light (23%) (Table 2). This was significantly different than in the other treatments ($F_{illumination}$= 8.76, p $F_{wavelenqth}$

The total carbohydrate content observed in cultures of *Navicula* sp., was higher at intensities of 50 µmol photon/m^2/sec in white light; however, no significant difference (Table 2).

In this study, the lipid content was higher in cultures exposed to light intensities of 50 µmol photon/m^2/sec; the greatest lipid percentage was observed in growth under red light (Table 2).

Discussion

Light is one of the main factors involved in the development of microalgae; quality and quantity of light affects both, growth rate and biomass composition (Markou et al., 2012). In this study relatively low cell concentrations were obtained at 50 µmol photon/m^2/sec and 100 µmol photon/m^2/sec, respectively, cultivated under white light. Leal et al. (2013) reported higher cell concentrations (around 300,000 cells/mL) cultivating the microalgae *Navicula* at light intensities (between 120 and 130 µmol photon/m^2/sec) in white light. This difference is probably due to the different culture medium they used.

During our experiment all cultures of *Navicula* exposed to high light intensities (100 µmol photon/m^2/sec) produced the lowest cell concentrations. This intensity may have produced photo inhibition, causing a decrease in microalgae growth (Markou et al., 2012). Under natural conditions the benthic microalgae reach their maximum photosynthetic rate for a short period during the peak hours of light saturation, and after that go down to the sediment or produce cell aggregates to avoid photoinhibition (Blanchard et al., 2004; Cartaxana et al., 2013).

Biomass content in cultures exposed to 100 µmol photon/m^2/sec at white light, was similar to that found by Leal et al. (2013) who reported values of 410 pg/cell of dry biomass and 270 pg/cell of organic matter from the microalgae *Navicula germanolpolonica* cultivated at salinity of 35 at light intensities between 120 and 130 µmol/m^2/sec. Although the cell concentration was lower in the cultures exposed to higher intensities, the dry biomass was higher compared with those cultures exposed to lower intensity showing an increase as light intensity increased. There are no reports that explain the biomass enhancement in respect to light intensity stress. However this has been documented for other stressors. There was a reported increase of dry biomass in *Dunaliella* sp. when grown in a culture limited in nitrogen (Fimbres Olivarría et al., 2010). Morphological changes and increase in cell size in *Chaetoceros wighamii* and *Dunaliella parva*, respectively were observed when grown in a culture limited in phosphorus (de Castro Araújo and Garcia, 2005); Said, 2009).

Cell size of algae tends to increase as the salinity of the culture medium increases (García et al., 2012). This could indicate that cells such as microalgae, exposed to stressors like nutrient limitations, inadequate salinity, and even high light intensities, tend to increase in size in order to survive in stressful environments.

The values we obtained for biochemical composition of *Navicula* sp., are similar to those reported by other authors for marine microalgae. Brown et al. (1997) reported values between 6-34% protein, 7-23% lipids, and 5-23% carbohydrates, all of which are within the range of those found in the present study. Several studies have reported that the exposure of microalgae cultures to red light increases the production of carbohydrates, while blue light promotes the production of protein.

In this study, the protein content was higher in cultures exposed to blue light; the effect of this light on the photosynthetic cells has been studied mostly in unicellular green algae, and higher plants, showing that exposure to this wavelength promotes the synthesis of proteins, enzyme activation, and accumulation of nitrogenous compounds such as photoresist pigments (Ramos-Lemuz 2000; Ramírez-Trejo 2002; Korbee et al. 2005; Marchetti et al. 2013).

The total carbohydrate content of cultures of *Navicula* sp. was equal between all treatments, which suggests that exposure to these conditions does not influence the production of carbohydrates in this particular species. An increase in the content of total carbohydrates in cultures of microalgae exposed to red wavelengths, has been attributed to to the accumulation of carbon in the media (Ramos-Lemuz (2000); Ramírez-Trejo (2002); Korbee et al., (2005).

Benthic diatoms have been poorly investigated (Leal et al. 2010) even though there is increasing interest in analyzing their potential as a new source of polysaccharides with high added value (Melo et al. 2007).

The major component of the biochemical composition of *Navicula* sp. in this study was lipids. One of the main nutritional characteristics of benthic diatoms is the high content of these biomolecules (Leal et al., 2010). In our study the highest concentration of lipids (32.25%) was seen in cultures exposed to 50 µmol photon/m^2/sec under red light; these values are higher than those reported for *N. germanopolonica* cultivated between 120 and 130 µmol photon/m^2/sec under white light (Leal et al., (2013) and are also higher than those reported for other species of benthic diatoms (Lee et al. 2009); however, the values found in this study correspond with those obtained by Chen (2012) who evaluated biomass and total lipid content in 12 species of marine diatoms reporting values between 30 and 45%.

Conclusion

These results will facilitate studies related to the production of biomass from *Navicula,* rich in bioactive compounds like carbohydrates which despite being the minor components in this species, have great potential in obtaining high value added polysaccharides with antiviral, antioxidant, antibacterial, and others properties.

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5.2. Artículo Publicado

Navicula sp. Sulfated Polysaccharide Gels Induced by Fe(III): Rheology and Microstructure

Diana Fimbres-Olivarría, José Antonio López-Elías, Elizabeth Carvajal-Millán, Jorge Alberto Márquez-Escalante, Luis Rafael Martínez-Córdova, Anselmo Miranda-Baeza, Fernando Enríquez-Ocaña, José Eduardo Valdez-Holguín and Francisco Brown-Bojórquez

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Navicula sp. Sulfated Polysaccharide Gels Induced by Fe(III): Rheology and Microstructure

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Abstract: A sulfated polysaccharide extracted from *Navicula* sp. presented a yield of 4.4 (% *w*/*w* dry biomass basis). Analysis of the polysaccharide using gas chromatography showed that this polysaccharide contained glucose (29%), galactose (21%), rhamnose (10%), xylose (5%) and mannose (4%). This polysaccharide presented an average molecular weight of 107 kDa. Scanning electron microscopy (SEM) micrographs showed that the lyophilized *Navicula* sp. polysaccharide is an amorphous solid with particles of irregular shapes and sharp angles. The polysaccharide at 1% (*w*/*v*) solution in water formed gels in the presence of 0.4% (*w*/*v*) $FeCl_3$, showing elastic and viscous moduli of 1 and 0.7 Pa, respectively. SEM analysis performed on the lyophilized gel showed a compact pore structure, with a pore size of approximately 150 nm. Very few studies on the gelation of sulfated polysaccharides using trivalent ions exist in the literature, and, to the best of our knowledge, this study is the first to describe the gelation of sulfated polysaccharides extracted from *Navicula* sp.

Keywords: *Navicula* sp.; sulfated polysaccharide; gelation; trivalent ions

1. Introduction

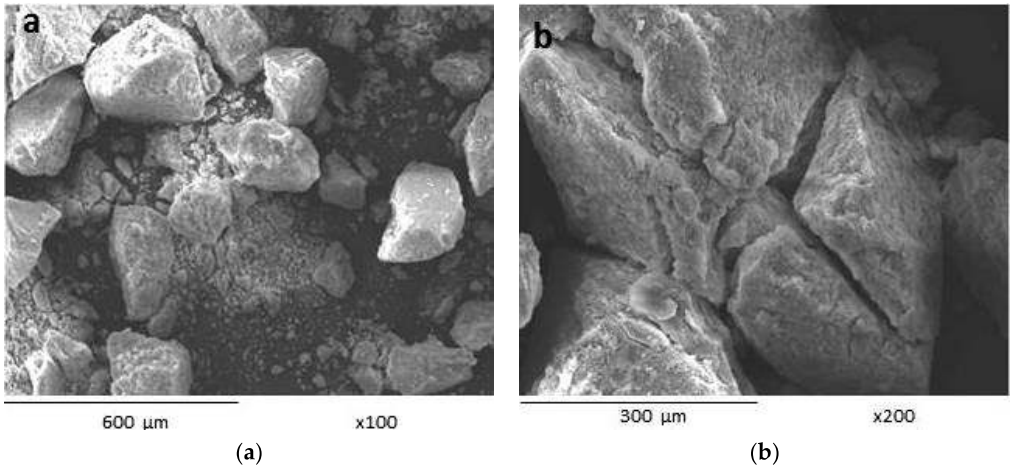
For several years, marine microalgae have been of great interest because they contain a great variety of bioactive compounds with biotechnological potential, especially in the biomedical, pharmaceutical, nutraceutical, and cosmetic areas. Among the wide variety of microalgae used for biotechnological purposes are the diatoms, whose principal purpose is the production of biodiesel due to their high lipid content [1]. Some diatoms are benthic microalgae; they produce mucilage that binds them to their substrate. This mucilage is a matrix with a high content of extracellular polymeric substances, including polysaccharides [2]. The marine microalgae of the *Navicula* genus are benthic diatoms, and several bioactive compounds of commercial interest can be obtained from them, including polysaccharides [3–5]. Several studies have proven that microalgae polysaccharides have great potential as antiviral, antibacterial, and antioxidant compounds, among other uses. Despite some research on their applications appearing already, information on sulfated polysaccharides from species of the genus *Navicula* is still scarce. Currently, no reports exist on the gelation behavior of sulfated polysaccharides from this genus. However, there is some evidence that sulfate polysaccharides can

form gels in the presence of trivalent ions, as shown for λ-carrageenan from seaweeds [6]. The aim of this study was to investigate the gelation of a sulfated polysaccharide from *Navicula* sp. in the presence of trivalent iron ions and to study the rheological and microstructural characteristics of the gel formed.

2. Results and Discussions

2.1. Polysaccharide Characteristics

The polysaccharide yield was 4.4 (% *w*/*w* dry biomass basis), nearest to the values reported in the diatom *Gomphonema olivaceum* (3% *w*/*w*) [7] and in the benthic seaweed *Sargassum qingdaoense* (7.2% *w*/*w*) [8], but lower than the values reported for the planktonic specie *Spirulina platensis* (13.6% *w*/*w*) [9]. These differences could be due to the extraction methods used and/or the type of species investigated. The extracted polysaccharide consisted of a white-colored powder with fine and granulated parts. Scanning electron microscopy (SEM) can be a useful tool to analyze the surface morphology of polysaccharide powder. The SEM micrographs showed that the lyophilized *Navicula* sp. polysaccharide is an amorphous solid (Figure 1). The observed particles were mostly aggregates of irregular shapes with sharp angles similar to those reported for other sulfated polysaccharides [10].



(**a**) (**b**)

Figure 1. Scanning electron microscopy (SEM) micrographs of lyophilized polysaccharide extracted from *Navicula* sp. at ×100 (**a**) and ×200 (**b**).

The main sugars present in the polysaccharide were glucose, galactose, rhamnose, xylose and mannose (Table 1), glucose being the most abundant, with ca. 30% of the polysaccharide dry weight. Staats et al. [11] found that extracellular polysaccharides from *Navicula salinarum* were mainly composed of glucose, galactose, mannose, rhamnose and xylose, with galactose concentrations similar to those found in the present study. On the other hand, Lee et al. [3] reported the presence of fucose, xylose, galactose, mannose and rhamnose in *Navicula directa* extracts, but at higher concentrations (% *w*/*w* dry weight basis) than those found in this study. A small amount of protein (0.48% *w*/*w*) was also detected in the polysaccharide from *Navicula* sp. (Table 1). However, a higher content of protein has been reported for polysaccharides from *Chlorella pyrenoidosa* at different ethanol concentrations (0.75%–11.21% *w*/*w*) [12]. The sulfate content found in the polysaccharide from *Navicula* sp. in this study (0.33%) (Table 1) was in the range reported for a sulfated galactan from the red algae *Ahnfeltia tobuchiensis* (0.2%–0.3% *w*/*w*) [13] but lower than that in other reports for *Navicula* species (8% and 11% *w*/*w*) [3,11]. However, it is well known that the sulfate content in microalgae is highly variable and can range from 0 to approximately 90% [14].

Table 1. Composition of sulfated polysaccharides from *Navicula* sp.

Compounds	% *w*/*w* Dry Weight Basis
Glucose	29.23 ± 2.04
Galactose	21.37 ± 2.27
Rhamnose	10.67 ± 2.66
Xylose	5.18 ± 1.09
Mannose	4.43 ± 0.79
Protein	0.480 ± 0.001
Sulfate	0.330 ± 0.004

All results were obtained from duplicates.

In the present study, the molecular weight (Mw) for the *Navicula* sp. sulfated polysaccharide was 107 kDa, lower than the reported value in another study with *Navicula directa* (222 kDa) [3]. However, it should be mentioned that the information and characterization of sulfated polysaccharides of the genus *Navicula* are still emerging. It should also be noted that the characteristics of microalgae and their biological compounds depend heavily on the culture conditions used and, to an even greater extent, on the species [15,16].

The Fourier transform infrared (FT-IR) spectrum of the sulfated polysaccharide extract showed five distinct bands at wave numbers ranging from 3405–821 cm^{-1} (Figure 2). The bands were assigned to particular functional groups according to previously published literature [17,18]. The spectrum of this polysaccharide showed the typical infrared footprint of carbohydrates. The band in the region of 3405 cm^{-1} corresponds to the stretching vibration characteristic of OH groups; a similar band around this wavenumber was observed for sulfated polysaccharides from green and brown seaweeds [19,20]. The band related to amides associated with the protein was detected at 1656 cm^{-1}; this band has also been detected in other microalgae [18]. The most important band was found at 1137 cm^{-1}, assigned to C–O–C bending; similar bands were reported for sulfated polysaccharides from brown and red seaweeds [21,22]. The band corresponding to the S=O vibration (1244 cm^{-1}) possesses a low intensity; this result could be due to the low sulfate content detected on the sample (0.33% *w*/*w*) as reported in Table 1. Some studies have reported the presence of this band in sulfated polysaccharides extracted from the diatom *Navicula directa* [3] and from the three major groups of seaweeds (green, brown and red algae) [19–21,23]. Finally, the band at 821 cm^{-1} was attributed to C–O–S stretching vibrations; sulfated polysaccharides extracted from some green and brown seaweed species also showed a band specific to the C–O–S group [19–21,23,24] around a similar wavelength as in our study.

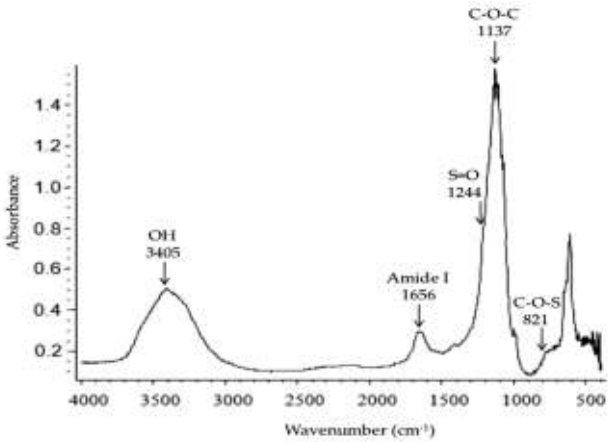


Figure 2. Fourier transform infrared (FT-IR) spectrum of sulfated polysaccharide from *Navicula* sp. The arrows indicate the principal absorption bands.

2.2. Sulfated Polysaccharide Gelation

Previous experiments were carried out in the present research in order to evaluate the gelation ability of the sulfated polysaccharide from *Navicula* sp. in the presence of mono and divalent cations (KCl and $CaCl_2$, respectively). However, for those cations no gelation was observed. When a 0.4% (*w*/*v*) $FeCl_3$ solution was dropped into a 1% (*w*/*v*) sulfated polysaccharide aqueous solution, a yellow-orange-colored gel-like substance precipitated as previously reported for λ-carrageenan [6]. The gel-like material was formed after 60 s of $FeCl_3$ addition. It has been suggested that trivalent iron metals promote appropriate ionic interactions between sulfated polysaccharide chains, causing their union and subsequent gelation. However, the gelling mechanism of sulfated polysaccharides in the presence of trivalent ions is currently unknown [6,25]. The precipitated coagulum formed in the present study was recovered for further rheological and microstructural characterization. The $FeCl_3$-induced gelation of the sulfated polysaccharide from *Navicula* sp. was rheologically investigated by small amplitude oscillatory shear. Figure 3 shows the changes in the elastic (G′) and viscous (G″) moduli of 1% (*w*/*v*) polysaccharide/$FeCl_3$ from 5 to 70 °C. The sample showed G′ and G″ values of 1.0 and 0.7 Pa, respectively, from 20 to 40 °C, indicating a gelation behavior. Higher G′ and G″ values were found in λ-carrageenan/$FeCl_3$ gels (G′ = 1200 Pa, G″ = 150 Pa) [6] which could be related to a higher sulfate content reported for that sample [26]. In the present study, the polysaccharide/$FeCl_3$ gel was thermally stable from 20 to 40 °C, as there was no crossover between G′ and G″ in this temperature region. The temperature at which this polysaccharide gel was thermally stable could allow its use in biomedical applications, where the implementation of organic material that supports the body temperature is needed. In a study by Vorvolakos et al. [27], it was observed that hyaluronic acid could form gels in the presence of trivalent cations, a behavior similar to the polysaccharide in our study. The hyaluronic acid gel can be utilized in laparoscopic surgeries to avoid adhesions [28]; because its gelling characteristics were similar to those in our study, *Navicula* sp. sulfated polysaccharide evaluation in that application could be of keen interest.

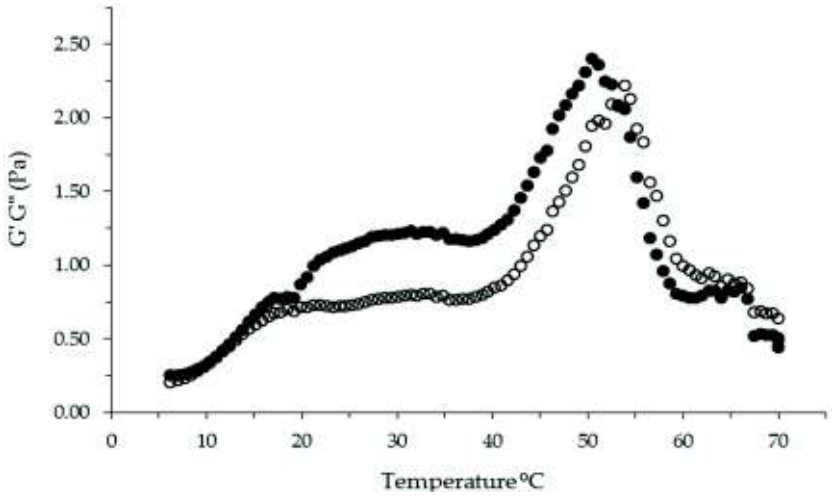


Figure 3. Temperature ramp for sulfated polysaccharide at 1% (*w*/*v*) in the presence of trivalent ions of $FeCl_3$ at 0.4% (*w*/*v*) at 1 Hz and 2% strain. G′ (●), G″ (○).

Mechanical spectra (Figure 4) of the gel were recorded at 25 °C, being typical of a solid-like material with a linear G′ independent of frequency and a G″ much smaller than G′ in the frequency interval from 0.1 up to 1.0 Hz [29]. At higher frequency values (from 1.0 up to 10 Hz), G′ and G″ enter into the non-linear range as a result of excessive oscillation frequency exposure, corresponding to a weak gel-like behavior [30]. The tangent delta values (tan δ = G″/G′) of the gel as a function of

frequency sweep are also presented in Figure 4. Under the experimental conditions used in the present study, the tan δ values registered varied from 0.46 to 0.12 when the frequency changed from 0.1 to 10.0 Hz. These tan δ values are typical of so-called weak gels [29]. When subjected to the strain sweep test, this polysaccharide gel showed a linear behavior from 1.5% to 10.0% strain (Figure 5). The elastic character of this gel could be attributed to the temporary association of sulfated polysaccharide chains during short oscillation periods. It has been suggested that trivalent ions could be more suitable than monovalent ions for balancing the three negative sulfate charges, per disaccharide repeat unit, of polysaccharides such as λ-carrageenan [6].

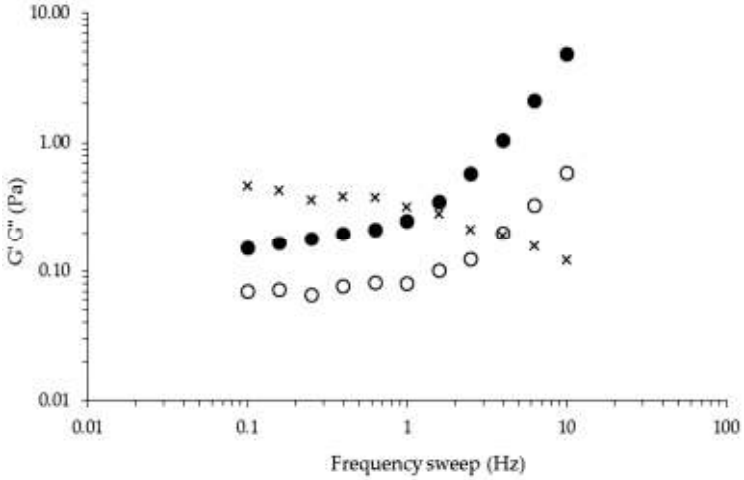


Figure 4. Mechanical spectra of sulfated polysaccharide gel at 1% (*w*/*v*) induced by $FeCl_3$ at 0.4% (*w*/*v*). Measurements at 2% strain and 25 °C. G′ (●), G″ (○), tan δ (×).

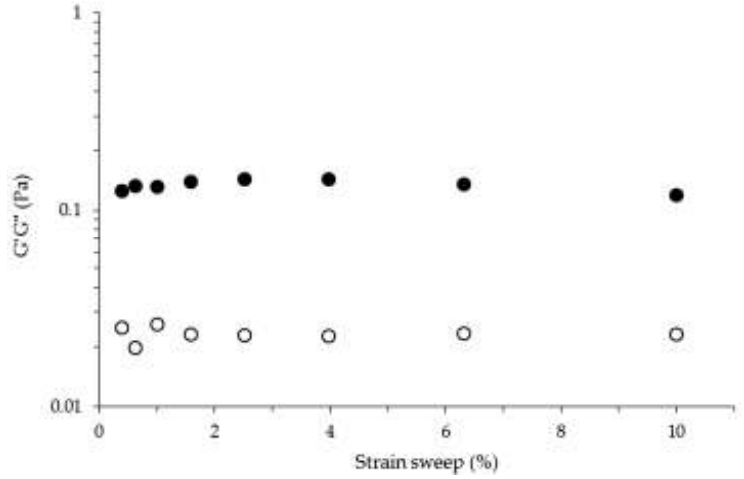


Figure 5. Strain sweep of sulfated polysaccharide gel at 1% (*w*/*v*) induced by $FeCl_3$ at 0.4% (*w*/*v*). Measurements at 1 Hz and 25 °C. G′ (●), G″ (○).

In Figure 6 the sulfated polysaccharide solution before (a) and after (b) $FeCl_3$ addition is observed. The yellow gel-like substance was lyophilized (Figure 6c) and analyzed by SEM (Figure 6d). SEM micrographs of the lyophilized polysaccharide gel present a compact pore structure, with an irregular pore size of approximately 150 nm. The gel formed with this trivalent metal consisted of

fine-stranded networks with strand thickness on the nm scale. In could be assumed that the SEM preparation method does not affect the sizes of the domains of the network structure. Nevertheless, it is important to note that lyophilized gel does not allow visualizing the original wet-polymeric network but it can be useful to investigate the dried microstructure of the polysaccharide gels.

Figure 6. Sulfated polysaccharide from *Navicula* sp. before (**a**) and after (**b**) the addition of $FeCl_3$; lyophilized gel (**c**); SEM micrograph of the lyophilized gel (magnification ×5000, scale bar 25 µm) (**d**).

3. Materials and Methods

3.1. Materials

The microalgae *Navicula* sp. was obtained and cultured as previously reported [31]. All chemical reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

3.2. Methods

3.2.1. Extraction of Polysaccharide

At the end of the microalgal culture, the full biomass was harvested by gravity sedimentation method [32] and lyophilized using a Freezone 6 freeze dry system (Labconco, Kansas, MO, USA). Once lyophilized, soluble sulfated polysaccharides were obtained by suspending the lyophilized total biomass in distilled water for 1 h at 30 °C, the suspended biomass was then centrifuged for 15 min at 20,000× *g*. Finally, the supernatant was separated and precipitated overnight under cold conditions with 96% (*v*/*v*) ethanol to allow for the precipitation of sulfated polysaccharides from *Navicula* sp. [11]. Precipitate was recovered and dried by solvent exchange (96% (*v*/*v*) ethanol and pure acetone) and the polysaccharide from *Navicula* sp. was obtained as reported for other marine sulfated polysaccharides [8,9].

3.2.2. Chemical Analysis

The sulfate content of the extracted polysaccharide was determined after hydrolysis with 1 N HCl at 100 °C for 1 h following the sodium-rhodizonate method proposed by Terho and Hartiala [33]. Na_2SO_4 was utilized as a standard. The protein content was analyzed using the Dumas method (Leco FP-528 nitrogen analyzer, St. Joseph, MI, USA) [34].

The monosaccharide content was analyzed by gas chromatography (Agilent HP 6890 GC Series, Santa Clara, CA, USA) [35]. Briefly, the polysaccharide sample was hydrolyzed with 3 N H_2SO_4 (98% *v*/*v*) at 100 °C, and inositol was added as the internal standard. The external standards were glucose, mannose, galactose, xylose and rhamnose (1 mg/mL, *w*/*v*), which were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Sugars were reduced to alditols with sodium borohydride, acetylated with acetic anhydride in the presence of methyl imidazole, and finally extracted with chloroform. After extraction, the alditol-acetates were injected (5 µL) in a DB 225 type column (50% cyanopropylphenyl-dimethylpolysiloxane, 30 m × 0.32 mm ID, 0.15 µm). The gas chromatography conditions were as follows: injection temperature 220 °C, detector temperature 260 °C, and oven temperature programmed to 205 °C at 10 °C/min. Nitrogen was used as the carrier gas and maintained at 1.0 mL/min.

3.2.3. Fourier Transform Infrared (FT-IR) Spectroscopy

The polysaccharide powder and the lyophilized trivalent gels were pressed into KBr pellets. A blank KBr disk was used as background. FT-IR spectrum was recorded on a Nicolet FT-IR spectrophotometer (Nicolet Instruments Corp., Madison, WI, USA). The FT-IR spectrum was measured in absorbance mode from 4000–400 cm^{-1}.

3.2.4. Molecular Weight Determination

The molecular characteristics based on the absolute weight-average molecular weight (M_W) of polysaccharide was analyzed by high-performance size-exclusion chromatography (HPSEC) attached to a multiangle laser-light scattering (MALLS) and refractive index (RI) detector (mini-Dawn®, Wyatt, Milford, MA, USA). The polysaccharide extract (1 mg/mL *w*/*v*) was dissolved in 100 mM $NaNO_3$, filtered through a 0.2 µm membrane, and injected at 25 °C. The RI increment (*dn*/*dc*) utilized for the polysaccharide extract was 0.147 mL/g.

3.2.5. Rheological Measurements

The gelation of the polysaccharide extract was carried out with the following reaction mixture: 1% *w*/*v* of polysaccharide solution with 0.4% *w*/*v* $FeCl_3$ in water. For rheological tests, the sulfated polysaccharide gel formation was followed using a strain controller rheometer (Discovery HR-2 rheometer; TA Instruments, New Castle, DE, USA) along with a parallel plate geometry with a plate diameter of 40 mm. A temperature ramp was carried out from 5 to 70 °C at a frequency of 1 Hz and 2% strain. Frequency sweep test was performed from 0.1 to 10 Hz at 2% strain and 25 °C. Strain sweep experiment was done from 0.4 to 10% strain at a 1 Hz frequency and 25 °C. All measurements were performed in duplicate.

3.2.6. Scanning Electron Microscopy Imaging

The polysaccharide powder and the lyophilized gel were all analyzed by field emission scanning electron microscopy (SEM) (JEOL 5410LV, JEOL, Peabody, MA, USA) using a voltage of 10 kV and ×100, ×200 or ×5000 magnifications. SEM images were obtained in secondary and backscattered electrons imaging modes.

4. Conclusions

The present study demonstrated that the sulfated polysaccharide from *Navicula* sp. can form gels in the presence of trivalent iron cations and showed the basic viscoelastic and microstructural characteristics of this material. This finding has the potential to expand the utility of sulfated polysaccharides from microalgae in different biotechnological applications and provides a basis for further structural analysis and evaluation of the bioactivities of this sulfated polysaccharide and its trivalent gel.

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Author Contributions: Diana Fimbres-Olivarría performed the experiments, analyzed the data and wrote the paper draft. José Antonio López-Elías and Elizabeth Carvajal-Millán conceived and designed the experiments and edited the paper. Jorge Alberto Márquez-Escalante performed the rheological experiments. Anselmo Miranda-Baeza, Luis Rafael Martínez-Córdova, Fernando Enríquez-Ocaña and José Eduardo Valdéz-Holguín analyzed the data and collaborated to edit the paper. Francisco Brown-Bojórquez performed the SEM analysis.

Conflicts of Interest: The authors declare no conflicts of interest.

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5.3. Artículo Enviado

Chemical characterization and antioxidant activity of sulfated polysaccharides from *Navicula* sp.

Diana Fimbres-Olivarría, Elizabeth Carvajal-Millan, José Antonio López-Elías, Karla Guadalupe Martínez-Robinson, Anselmo Miranda-Baeza, Luis Rafael Martinez-Córdova, Fernando Enríquez-Ocaña, José Eduardo Valdez-Holguín

Food Hydrocolloids

Chemical characterization and antioxidant activity of sulfated polysaccharides from *Navicula* sp.

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Abstract: Sulfated polysaccharides were extracted from *Navicula* sp. cultivated at three wavelengths: white (WSPN), red (RSPN) and blue (BSPN) with yield rates of 3.4, 3.9 and 4.4 (% *w*/*w* dry biomass basis) respectively. Analysis of these polysaccharides using gas chromatography showed that they contain glucose, galactose, rhamnose, xylose and mannose as main neutral sugars. The amount of rhamnose was higher in WSPN. The molecular weight (*Mw*) value was 17, 107 and 108 kDa for WSPN, BSPN and RSPN, respectively. The sulfate content in WSPN was higher (0.40% *w*/*w)* than in the BSPN and RSPN. The polysaccharides recovered from *Navicula* sp. presented antioxidant activity, which could be related to the molecular structural characteristics such as *Mw* and sulfate content. The scavenging activity was higher in WSPN (DPPH 49% and $ABTS^{+}$ 68 μmol Trolox/g), than in the BSPN and RSPN samples. The WSPN possess a high antioxidant capability, thus this sulfated polysaccharide might be a potential antioxidant for biotechnological applications.

Keywords: *Navicula* sp.; sulfated polysaccharides; chemical characterization; antioxidant activity.

1. Introduction

The bioactive compounds from microalgae have a wide range of applications such as antivirals, antimicrobials, antioxidants among others, which has been attributed to their biological activities and chemical structures (Sun, Wang, Guo, Pu, & Yan, 2014). The microalgae culture conditions are the main factor affecting their biochemical composition, being the light one of the most important variables (Markou, Angelidaki, & Georgakakis, 2012). Jungandreas et al., (2014) documented that exposition of microalgae to red light produces an increment in the carbohydrates concentration, while blue light promotes the proteins content. However, authors such as Korbee, Figueroa, & Aguilera (2005) report that the effect of white light represents the combined effect of red and blue light. The marine microalgae have been mainly used as a live food for cultivable aquatic organisms, due their rich biochemical composition; however, nowadays these microorganisms are being used to obtain bioactive compounds with high biotechnological potential in biomedical, pharmaceutical, nutraceutical and cosmetics industries, being the antioxidant activity one of the most studied aspects of these compounds. Some of the main genera of marine microalgae used for this purposes are *Dunaliella*, *Spirulina*, (Abd El Baky, Hanaa El Baz & El-Latife, 2013; Hemalatha, Girija, Parthiban, Saranya, & Anantharaman, 2013; Karthikeyan et al., 2013) and recently the species from the genus *Navicula* (Affan, Karawita, Jeon, & Lee, 2007; Hemalatha et al., 2013). Some investigations about the antioxidant activity from extracts of microalgae have focused on the activity of their pigments such as carotenoids and xanthophylls; however, there are other compounds such as the polysaccharides with high antioxidant activity that should be evaluated. The microalgal sulfated polysaccharides have shown to play an important antioxidant role with effective scavenging activities on different radicals (Souza et al., 2012; Sun et al., 2014). It is well known that the benthic diatom *Navicula* sp. is characterized by producing a mucilage with a high content of extracellular polymeric substances included lipids, proteins and polysaccharides (Raposo, De Morais, & De Morais, 2013). Although there are some investigations about the properties of the sulfated polysaccharides of this microalgae, the information is incipient and there is no research on the effects of different wavelength in these organisms, particularly on the production of polysaccharides from *Navicula* and their antioxidant activity. The aim of the present study was to characterize and evaluate the antioxidant activity of sulfated polysaccharides extracted from the benthic diatom *Navicula* sp. cultivated at three wavelengths.

2. Materials and Methods

2.1 Materials

The marine diatom *Navicula* sp. was obtained from the strain collection of the Laboratory of Chemical Analysis and Microbiology of the University of Sonora. All chemical reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

2.2. Methods

2.2.1. Culture conditions

The experiment was carried out under indoor controlled conditions by quadruplicate, using tubs containing 10 L of culture medium. For their culture, the "F" medium from Guillard & Ryther (1962) was utilized. Cell counts were performed daily and biomass was harvested at the stationary phase. Total biomass was quantified by gravimetric methods. Microalgae were cultured at 50 μmol photon $m^{-2}sec^{-1}$ of irradiance in white (400-750 nm), blue (430-480 nm) and red wavelength (595-660 nm); light was supplied by Light Emitting Diode lamps (LED) electronically controlled to the desired irradiance.

2.2.2. Extraction of sulfated polysaccharides from microalgal culture

Once finished the microalgal culture, the total biomass of each treatment was full harvested by gravity sedimentation method (Shelef, Sukenik, & Green, 1984) and lyophilized using a Freezone 6 Freeze dry System (Labconco, Kansas, MO, USA). Subsequently, the lyophilized biomass was suspended in distilled water for 1 h at room temperature to obtain the soluble fraction of sulfate polysaccharides, the suspended biomass was then centrifuged for 15 min at 20,000x *g*. The supernatant was separated and precipitate overnight on cold conditions with ethanol 96% (v/v) to allow the precipitation of sulfated polysaccharides (Fimbres-Olivarría et al., 2016) from *Navicula* sp. The resultant extracts were named as follow: WSPN (sulfated polysaccharide from *Navicula* sp. in white wavelength), BSPN (sulfated polysaccharide from *Navicula* sp. in blue wavelength) and RSPN (sulfated polysaccharide from *Navicula* sp. in red wavelength).

2.2.3. Chemical analysis

The sulfate content of the extracted polysaccharides was determined after hydrolysis with 1 N HCl at 100 ºC for 1 h following the sodium-rhodizonate method proposed by Terho & Hartiala (1971). Sodium-rhodizonate forms a colored compound with the barium ion; when sulfate is present, $BaSO_4$ is formed and the intensity of the color decrease; the sulfate amount can be calculated from this reduction. Na_2SO_4 was utilized as a standard. The protein content was analyzed using the Dumas method (Leco FP-528 nitrogen analyzer, St. Joseph, MI, USA) (AOAC, 1995).

The monosaccharides content of polysaccharides was analyzed by gas chromatography (GC) (Agilent HP 6890 GC Series, Santa Clara, CA, USA) (Rouau & Surget, 1994). The samples were hydrolyzed with 3 N H_2SO_4 (98% *v*/*v*) at 100 ºC, and inositol was added as the internal standard. The external standards were glucose, mannose, galactose, xylose and rhamnose (1 mg/mL, *w*/*v*), which were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Sugars were reduced to alditols with sodium borohydride, acetylated with acetic anhydride in the presence of methyl imidazole, and finally extracted with chloroform. After extraction, the alditol-acetates were injected (5 μL) in a DB 225 type column (50% cyanopropylphenyl-dimethylpolysiloxane, 30 m x 0.32 mm ID, 0.15 μm). The GC conditions were as follows: injection temperature 220 ºC, detector temperature 260 ºC, and oven temperature programmed to 205 ºC at 10 ºC/min. Nitrogen was used as the carrier gas and maintained at 1.0 mL/min. A flame ionization detector was used.

2.2.4. Fourier Transform Infrared (FT-IR) Spectroscopy

The polysaccharide powders were pressed into KBr pellets. A blank KBr disk was used as background. FT-IR spectrums were recorded on a Nicolet FT-IR spectrophotometer (Nicolet Instruments Corp., Madison, WI, USA) and measured in absorbance mode from 4000–400 cm^{-1}.

2.2.5. Molecular Weight Determination

The molecular characteristics based on the absolute weight-average molecular weight (*Mw*) of polysaccharide was analyzed by high-performance size-exclusion chromatography (HPSEC) attached to a multiangle laser-light scattering (MALLS) and refractive index (RI) detector (mini-Dawn®, Wyatt, Milford, MA, USA). The polysaccharides (1 mg/mL *w*/*v*) were dissolved in 100 mM $NaNO_3$, filtered through a 0.2 μm membrane, and injected at 25 ºC. The RI increment (*dn*/*dc*: 0.147 mL/g) utilized in the present study, was an average of different *dn*/*dc* values employed in

polysaccharides from several algae (Geresh, Adin, Yarmolinsky, & Karpasas, 2002; Ammar et al., 2015; Holtkamp, Kelly, Ulber, & Lang, 2009; Saboural et al., 2014).

2.2.6. Antioxidant activity of microalgal sulfated polysaccharides

2.2.6.1. DPPH (2,2-diphenyl-1-picrylhydrazyl radical scavenging activity assay)

This assay involved the scavenging of stable DPPH radicals by the radical scavenging components of sulfated polysaccharides from *Navicula* sp. cultivated at the three wavelengths. An ethanolic DPPH solution (0.1 mM) was prepared. An aliquot of each sample (25-200 μg/mL) was added to DPPH solution (1:1 v/v) (Hou, Hsu, & Lee, 2002). The absorbance was measured at 517 nm in a GENESYS™ 10UV spectrophotometer (Thermo Scientific), after incubation for 30 minutes in the dark at room temperature. Vitamin C was used as a positive control. Measurements were performed in triplicate. The scavenging activity of DPPH radicals by the sulfated polysaccharides was calculated according to the next equation:

DPPH-scavenging activity (%) = $[1-(A_{sample517nm}-A_{blank517nm})/ A_{control517}] \times 100$

2.2.6.2 $ABTS^{+}$ (2,2'-Azino-bis 3-Ethylbenzthiazoline-6-Sulphonic acid radical scavenging activity assay)

The antioxidant activity of sulfated polysaccharides from *Navicula* sp. cultivated at three wavelengths was measured using the $ABTS^{+}$ method as described by Martinez-Lopez, Carvajal-Millan, Lopez-Franco, Lizardi-Mendoza, & Rascon-Chu (2014). The absorbance was measured at 734 nm in a GENESYS™ 10UV spectrophotometer (Thermo Scientific). All measurements were performed at 7, 15 and 30 min after mixing the samples with ABTS reagent. The antioxidant activity was expressed as μmol of Trolox equivalent antioxidant activity (TEAC) per gram of sample by means of a dose-response curve for Trolox. Measurements were performed in triplicate.

2.3. Statistical analysis

All the results were presented with descriptive statistics as the media and standard deviation. Results from polysaccharides, proteins and sulfate were analyzed by one-way ANOVA and mean difference test of Duncan with $p \leq 0.05$.

3. Results and Discussion

3.1. Growth and biomass of microalgae

At the stationary phase the maximum cell concentration was higher on white wavelength with 291,875 cells/mL at irradiance of 50 μmol photon $m^{-2}sec^{-1}$, nevertheless no differences were found at blue wavelength (274,583 cell/mL); meanwhile, red wavelength had the lower concentration (112,500 cell/mL). Microalgae grown under red wavelength had the highest dry biomass (423 pg/cell); however, no significant differences were found in the dry biomass from white (199 pg/cell) and blue (178 pg/cell) wavelength. These results indicate that the red wavelength had a strong influence on the growth and in the biomass content of *Navicula* sp.

The *Navicula* sp. cultures showed typical characteristics of benthic microalgae growth, being mucilaginous and dispersed on surface and walls of the tub of culture (Fig. 1a). It is well known that mucilage generated by benthic microalgae, help to bind it to substrate and protect them of the adverse conditions of aquatic environment (Raposo et al., 2013). Fig. 1b show the micrograph of *Navicula* sp. recorded in a Carl Zeiss microscope; the morphological characteristics of the microalgae presented the typical boat-shape. The individual cells were in the range of 19 μm on average. Microalgae of the genus *Navicula* can range between 15 and 55 μm in length on depending specie (Van de Vijver et al., 2011).

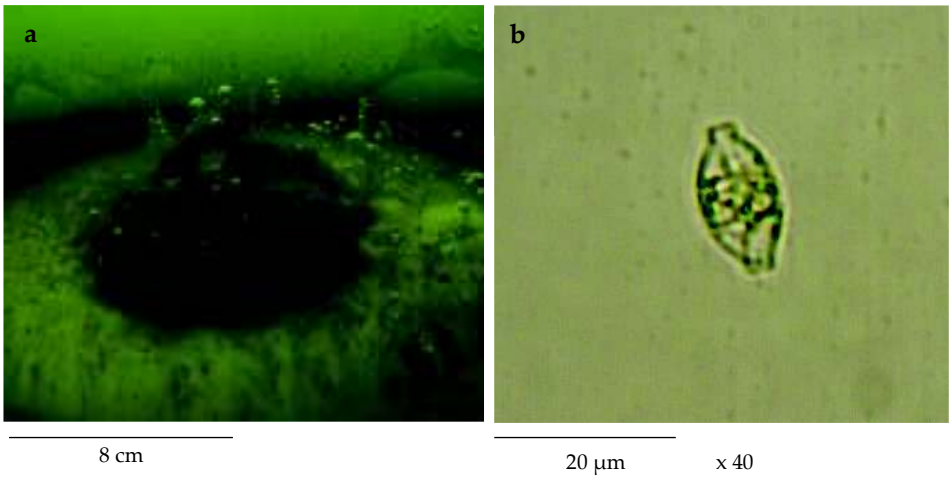

Fig. 1. Wet biomass from *Navicula* sp. (a); Micrograph of *Navicula* sp. recorded in a Carl Zeiss microscope (magnification x40, scale bar 20 μm) (b).

3.2. Polysaccharides yield

The polysaccharide yield in the cultures exposed to white wavelength was 3.4 (% *w*/*w* dry weight basis), meanwhile, for the cultures exposed to red and blue wavelength were 3.9 (% *w*/*w*) and 4.4 (% *w*/*w*), respectively. These values are similar to the reported in the diatom *Gomphonema olivaceum* (3% *w*/*w*) (Huntsman & Sloneker, 1971); however, the yield of polysaccharides from microalgae depend on environmental factors and the specie (Lahaye & Robic, 2007). The effects of the wavelengths on the characteristics and biochemical composition of microalgae do not follow a defined pattern, thus cannot be generalized (Carmona, Vergara, Lahaye, & Niell, 1998). In a study by Carmona et al., (1998) it was observed that the red algae *Gelidium sesquipedale* showed the same behavior in response to wavelength than in our study, being the yield from culture at blue wavelength higher than those under red and white wavelengths.

3.3. Polysaccharides charactetistics

The monosaccharide composition of WSPN, BSPN and RSPN is presented in Table 1. The main sugars present in the polysaccharides from each wavelength were glucose, rhamnose, galactose, mannose and xylose. Staats, De Winder, Stal & Mur (1999) reported the presence of the same monosaccharides in the extracellular polysaccharides extracted from the specie *Navicula salinarum.* No significant differences were found in mannose through the three wavelengths. Staats et al., (1999) and Lee et al., (2006) reported higher concentrations of mannose than those found in the present study; however, the mannose quantified in WSPN, BSPN and RSPN are in the range reported for microalgae (Brown, Jeffrey, Volkman, & Dunstan, 1997). WSPN presented a high rhamnose content (35.34 % *w*/*w*) in relation to BSPN and RSPN. Haug & Myklestad (1976) found a high concentration of rhamnose (34-39% *w*/*w*) in the extracellular polysaccharides from species of diatoms. Nevertheless, the rhamnose content in BSPN (10.67% w/w) was similar to those reported by Lee et al., (2006) in *Navicula directa* (8.7% *w*/*w*). The galactose content obtained in WSPN, BSPN and RSPN were in the range reported for *Navicula salinarum* (19.1% *w*/*w*) and *Navicula directa* (20.7% *w*/*w*) (Lee et al., 2006; Staats et al., 1999). On the other hand, the xylose content was lower than the reported by Staats et al., (1999) and Lee et al., (2006), however the results of the present study are in the range for microalgae (Brown et al., 1997). Finally, the glucose content in the three wavelengths were lower than those reported by Staats et al., (1999) in *Navicula salinarum*, but higher than the value reported in *Navicula directa* (Lee et al., 2006). Small amounts of proteins were also detected in all the polysaccharide samples, being higher in WSPN when compared to the other wavelengths (Table 1).

These protein contents were lower than the values reported for other species of *Navicula* (4.9% and 15.1% *w/w*) (Lee et al., 2006; Staats et al., 1999).

The sulfate content was higher in WSPN than in the rest of the polysaccharide samples. In general, the sulfate content in the three polysaccharides analyzed were similar to those reported for a sulfated galactan from the red algae *Ahnfeltia tobuchiensis* (0.2%-0.3% *w/w*) (Truus et al., 2006), but lower than the levels reported for *Navicula* species (6.3% and 8% *w/w*) (Lee et al., 2006; Staats et al., 1999). It is well known that the sulfate content in microalgae is highly variable and can range from 0 to 90% (Pérez Loyola, Popowski Casaña, Pérez Castillo, & Alonso Romero, 2003).

Table 1. Composition of polysaccharides from *Navicula* sp. cultivated at three wavelength

	WSPN	BSPN	RSPN
Glucose	15.46±5.89^{a}	29.23±2.04^{b}	17.41±1.20^{a}
Rhamnose	35.34±1.53^{c}	10.67±2.66^{a}	19.81±0.29^{b}
Galactose	24.48±5.62^{b}	21.37±2.27^{ab}	16.82±0.54^{a}
Mannose	4.89±0.58^{a}	4.43±0.79^{a}	5.07±1.99^{a}
Xylose	9.28±1.25^{b}	5.18±1.09^{a}	10.38±0.97^{b}
Protein	1.65±0.10^{b}	0.48±0.001^{a}	0.55±0.03^{a}
Sulfate	0.40±0.004^{b}	0.33±0.004^{a}	0.32±0.002^{a}

Results are expressed in g/100 sulfated polysaccharide dry matter. All results were obtained from duplicates. Different letters in the same line means significant differences at $p \leq 0.05$.

The molecular weight (*Mw*) of the WSPN (17 kDa) was lower than the BSPN (107 kDa) and the RSPN (108 kDa). These values were lower than the reported by Lee et al., (2006) for the sulfated polysaccharide from *Navicula directa* (222 kDa).

It is important to mention that the information and characterization of sulfated polysaccharides of the genus *Navicula* is yet emerging (Fimbres-Olivarría et al., 2016). It should be noted that the physical-chemical composition and characteristics of microalgae depends heavily of the culture conditions and the differences are always species-specific (Dautania & Singh, 2014; de Morais, da Silva Vaz, Etiele Greque, & Vieira Costa, 2015).

3.4. Fourier Transform Infrared (FT-IR) Spectroscopy

The Fourier transform infrared (FT-IR) spectrum was recorded for all sulfated polysaccharide extracted from *Navicula* sp. (Fig. 2). All they showed similar FT-IR spectrum with five distinct bands from the wave numbers ranging from 3413-818 cm^{-1} and showed the typical infrared footprint of carbohydrates. The bands were assigned to particular functional groups according to published literature (Socrates, 2004; Stehfest, Toepel, & Wilhelm, 2005). The bands at 3413-3405 cm^{-1} region were attributed to the stretching vibration of O-H groups; a similar band around these wavenumbers were observed for polysaccharides from the microalgae *Isochrysis galbana* (Sun et al., 2014) and *Spirulina platensis* (Abd El Baky et al., 2013). Less intense bands were detected at 1646-1656 cm^{-1}, these bands were attributed to amide I vibrations, which are typical for proteins (Goo et al., 2013); this band has also been detected in other microalgae (Stehfest et al., 2005). Comparing these vibrations with the rest of the regions, is evident that the polysaccharides are the major component, while the proteins are present as the impurities (Goo et al., 2013).

The strongest absorption bands were found at 1142-1135 cm^{-1}, and were assigned to glycosidic linkages (C-O-C) stretching vibrations; similar bands were reported in the polysaccharide from microalgae *I. galbana* (Sun et al., 2014) and for the sulfated polysaccharides from several seaweeds (Abdala-Díaz, Chabrillón, Cabello-Pasini, López-Soler, & Figueroa, 2010; Morimoto et al., 2014). In regard to the S=O vibration low intensities bands were detected at 1250-1244 cm^{-1}, this results are related to the low sulfate content detected on the samples (Table 1). This band was also reported in sulfated polysaccharide from *Navicula directa* (Lee et al., 2006) and from the three major groups of seaweeds (Morimoto et al., 2014; Saboural et al., 2014; Shao, Chen, Pei, & Sun, 2013; Souza et al., 2012). The bands detected at 832-818 cm^{-1} corresponds to C-O-S stretching vibrations, and a similar band was reported in sulfated polysaccharides from *Spirulina platensis* (Abd El Baky et al., 2013), and in the polysaccharides extracts from several seaweeds (Morimoto et al., 2014; Saboural et al., 2014; Shao, Chen, Pei, et al., 2013; Shao, Chen, & Sun, 2013; Souza et al., 2012).

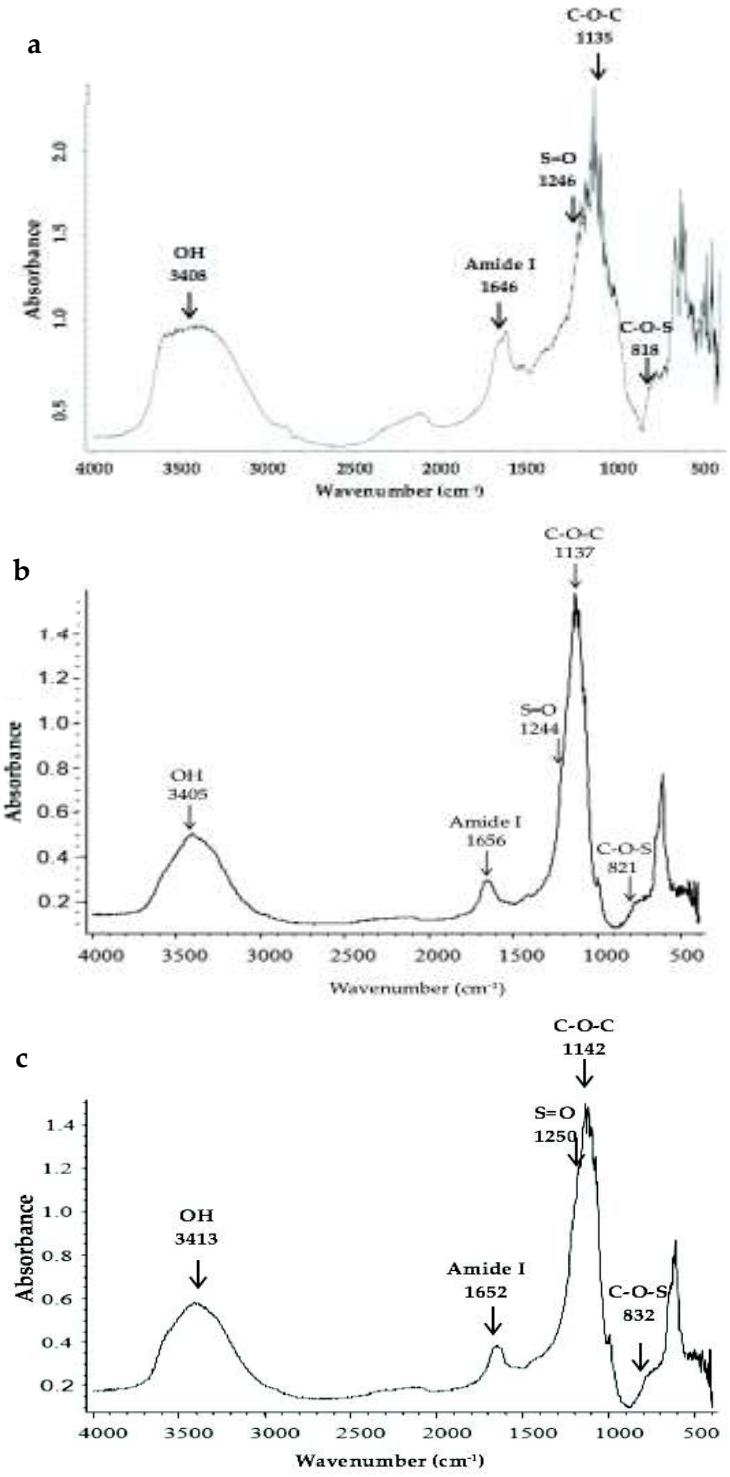

Fig. 2. FT-IR spectra of a) WSPN, b) BSPN and c) RSPN. The arrows indicate the principal absorption bands of the sulfated polysaccharides from *Navicula* sp.

3.5. Antioxidant activity of microalgal sulfated polysaccharides

The demand by natural products that satisfy the need of human being is increasing. Microalgae as a naturally source have a great potential for synthesis of products with high added value and high bioactive capacity as antioxidants; thus, it is essential to develop and utilize effective natural antioxidants such as the polysaccharides from microalgae to protect against free radicals.

Antioxidants are substances that can prevent the oxidation of cells. Their effects are on the scavenging of Reactive Oxygen Species (ROS) (Qi et al., 2005). The antioxidants can minimize the oxidative damage by increasing natural defenses and by scavenging the free radicals (Sun et al., 2014).

All the polysaccharide samples (WSPN, BSPN and RSPN) showed DPPH scavenging activity at various concentrations. The DPPH free radical scavenging rate varied from 14.0% to 48.7% when the concentration augmented from 25 up to 200 μg/mL (Fig. 3). The BSPN showed a clear increment of the antioxidant activity when the concentration increased from 25 up to 200 μg/mL, meanwhile WSPN and RSPN samples remained almost constant across the concentrations evaluated. The highest scavenging activity was observed at 200 μg/mL for the three samples being higher for WSPN (48.7 ± 3.1%) in relation to BSPN (35.5±3.2%) and RSPN (27.2± 2.1%). Lee et al., (2009) and Affan et al., (2007) reported DPPH scavenging activity values between 27.3 to 81.6% for polysaccharide from *Navicula* sp. and *Navicula incerta*. In the present study, DPPH scavenging activity values were in the range reported by these authors. The lowest percentages were observed at 25 μg/mL, being lower for BSPN (14.01%) in comparison to the rest of the samples (Fig. 3). Abd El Baky et al., (2013) reported 28.05% of DPPH scavenging activity from *Spirulina platensis* at 200 μg/mL polysaccharide, being similar to the antioxidant activity found in the present study for BSPN and RSPN (Fig. 3).

The IC_{50} was utilized to compare the radical scavenging activity from different extracts. This value expresses the concentration of the sample required to scavenge 50% of the free radicals. The WSPN presented the lowest IC_{50} value (238 μg/mL) compared with BSPN (326 μg/mL) and RSPN (3066 μg/mL). The RSPN IC_{50} value was 9 and 13 times higher than those found for WSPN and BSPN, respectively. In a previous investigation, Kokabi, Yousefzadi, Ali ahmadi, Feghhi, & Keshavarz (2013) reported an IC_{50} of 62.13 μg/mL in polysaccharides from the green alga *Ulva lactuca*. Gopinath &

Sampathkumar (2014), registered an IC_{50} value of 70.83 μg/mL in polysaccharides from the benthic diatom *Nitzschia longissima.* The values reported by Kokabi et al. 2013 and Gopinath & Sampathkumar (2014) were lower than those found in the present investigation . However, Zhang, Wu, Wang, & Lan (2012) reported an IC_{50} of 600 μg/mL for polysaccharides of *Sargassum graminifolium* which are higher to the values found for WSPN and BSPN .

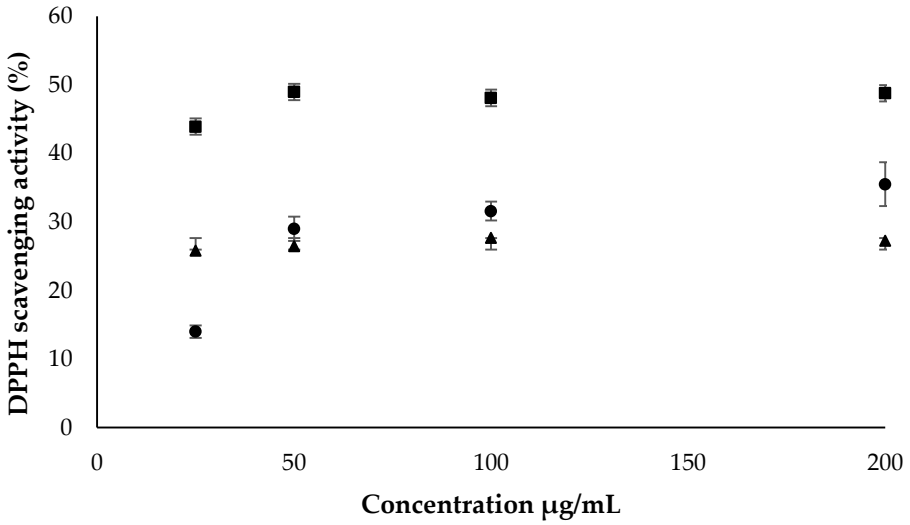

Fig. 3. Antioxidant activity from sulfated polysaccharides from *Navicula* sp. cultivated at three wavelengths assessed by DPPH. The values represent the mean ± SD determined from triplicate samples. WSPN (■), BSPN (●) and RSPN (▲).

Regarding to the antioxidant activity of sulfated polysaccharides from the microalgae *Navicula* sp. assessed by $ABTS^{+}$ method, the WSPN showed higher activity than BSPN and RSPN, as well as observed in the DPPH scavenging activity. The BSPN and RSPN showed similar values (Table 2). Díaz-Bayona et al., (2012) reported a lower antioxidant activity value in a sulfated polysaccharide from the red algae *Porphyridium cruentum* (40.46 μmol Trolox/g) in comparison to the levels found in the present study (Table 2). However, Leung, Zhao, Ho, & Wu, (2009) and Yan, Wang, Ma, & Wu (2013) reported similar values of antioxidant activity (35-40 and 66.5 μmol Trolox/g, respectively) in sulfated polysaccharides from the mushroom *Cordyceps sinensis*.

Table 2. Antioxidant activity of sulfated polysaccharides from *Navicula* sp. cultivated at three wavelengths assessed by $ABTS^{+}$.

Sample	*$TEAC_{/ABTS}$ (μmol Trolox/g)
WSPN	68.17±4.68
BSPN	53.85±0.45
RSPN	53.69±2.89

All values are means ± standard deviation from triplicates.
*Antioxidant activity expressed as μmol of Trolox equivalent antioxidant activity (TEAC) by gram of sample.

It is well known that the composition, molecular weight, structure, sulfate content, type of monosaccharides among other characteristics have great influence on the biological activities of polysaccharides (Lo, Chang, Chiu, Tsay, & Jen, 2011; Qi et al., 2005; Wang et al., 2016).

In both methods (DPPH and $ABTS^{+}$), the WSPN scavenging activity was higher than in the BSPN and RSPN; this could be related with the low *Mw* (17 kDa), sulfate content (0.40% *w*/*w*) and the amount of rhamnose (35% *w*/*w*) of the WSPN. Sun, Wang, Shi, & Ma (2009) investigated polysaccharides of different *Mw* (6.55-256 kDa), extracted from the red algae *Porphyridium cruentum* and found higher antioxidant activity values for low *Mw* polysaccharides. In other investigation by Sun et al., (2014), it was found that low *Mw* polysaccharides (15.93 kDa) from the marine microalgae *Isochrysis galbana* present higher antioxidant activity values than high *Mw* polysaccharides.

It is widely known that polysaccharides with low *Mw* have the strongest reducing power, and it is due to the polysaccharide chains. The low molecular weight polysaccharides present a higher content of reducing end (reductive hydroxyl group terminals) to eliminate and accept the free radicals (Qi et al., 2005; Wang et al., 2016).

Although the role of monosaccharides in the antioxidant activity of polysaccharides remained unclear, there are evidences that the composition and ratios of the type of monosaccharide influence in the antioxidant properties (Wang et al., 2016). An investigation by Lo et al., (2011) found that the rhamnose content in the polysaccharide from the edible mushroom *Lentinula edodes* (shiitake), was the most significant determinant factor associated with the antioxidant properties, which is consistent with the main findings in the present study, with respect to the amount of rhamnose in the WSPN sample (Table 1).

It has been widely documented that there is a positive correlation between sulfate content and antioxidant activity in sulfated polysaccharides (Souza et al., 2012; Sun et al., 2014); the higher the content of sulfate, greater the antioxidant capability (Jiao, Yu, Zhang, & Ewart, 2011; Qi et al., 2005), as observed in the present study.

4. Conclusions

The sulfated polysaccharides recovered from *Navicula* sp. cultivated at different wavelength contain glucose, rhamnose, galactose, mannose and xylose as main sugars. The polysaccharide obtained under white light (WSPN) present a low molecular weight (17 kDa) and high sulfate content (0.4% w/w) in relation to the rest of the wavelength evaluated in the present study. Polysaccharides extracted from *Navicula* sp. present antioxidant activity. White light (WSPN) and blue light (BSPN) polysaccharides register IC_{50} values lower than those reported in the literature for other sulfated polysaccharides like *Sargassum graminifolium*. These sulfated polysaccharides might be a potential antioxidant for biotechnological applications.

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VI. DISCUSIONES

La luz es la principal variable en el desarrollo de las microalgas; tanto la calidad (longitud de onda) como la cantidad de luz (irradiancia) afectan la tasa de crecimiento, composición bioquímica y biomasa de estos microorganismos (Markou *et al*., 2012).

En este estudio se observaron concentraciones celulares superiores en los cultivos expuestos a luz blanca en irradiancias de 50 µmol fotones $m^{-2}seg^{-1}$. Leal *et al*., (2013) reportaron concentraciones similares en la microalga *Navicula germanopolonica* cultivada en luz blanca, entre 120 y 130 µmol fotones $m^{-2}seg^{-1}$.

Los cultivos expuestos a altas irradiancias (100 µmol fotones $m^{-2}seg^{-1}$), en las tres longitudes de onda, presentaron las concentraciones celulares más bajas, lo cual pudo deberse al estrés lumínico (Markou *et al*., 2012). Bajo condiciones naturales, las microalgas bentónicas alcanzan su máxima tasa fotosintética por un corto periodo durante las horas de mayor irradiancia, posteriormente descienden al sedimento y segregan sustancias que las protegen del exceso de iluminación (Blanchard *et al*., 2004; Cartaxana *et al*., 2013).

Los valores de biomasa observados en los cultivos expuestos a 100 µmol fotones m^{-2} seg^{-1} en luz blanca, fueron similares a lo observado por Leal *et al*., (2013) en la microalga *Navicula germanopolonica*.

La concentración celular alcanzada en este estudio fue menor en los cultivos expuestos a irradiancias superiores, sin embargo, la biomasa fue mayor comparada con los cultivos sometidos a irradiancias más bajas. Actualmente no existen reportes que relacionen el incremento de biomasa con respecto a la iluminación; sin embargo, ha sido reportado para otros factores de estrés. Fimbres-Olivarría *et al*., (2010) reportaron un incremento en el contenido de biomasa seca en *Dunaliella* sp., cultivada en medios limitantes en nitrógeno.

Por otro lado, autores como de Castro Araújo y García, (2005) y Said (2009) reportaron cambios morfológicos e incremento en el tamaño celular de las microalgas *Chaetoceros wighamii* y *Dunaliella parva* respectivamente, al ser cultivadas en medios limitantes en fósforo. En su estudio, García *et al*., (2012) observaron que el tamaño celular de la diatomea *Thalassiosira weissflogii* tiende a incrementar conforme lo hace la salinidad del medio de

cultivo. Lo anterior indica que las células al estar sujetas a factores de estrés, tales como la limitación de nutrientes, salinidades extremas, e incluso irradiancias extremas, tienden a incrementar su tamaño como una forma de sobrevivir a las condiciones adversas que las rodean.

En cuanto a la composición bioquímica de *Navicula* sp., el contenido de proteínas fue superior en los cultivos expuestos a longitudes de onda azul en bajas irradiancias, similar al reportado por Brown *et al*., (1997) para microalgas marinas, encontrando valores entre 6-34%. El efecto de esta longitud de onda en células fotosintéticas ha sido estudiado principalmente en algas verdes unicelulares y plantas superiores, en donde se ha observado que la exposición a esta luz promueve la síntesis de proteínas, la activación enzimática y la acumulación de compuestos nitrogenados (Korbee *et al*., 2005; Marchetti *et al*., 2013).

En lo que respecta al contenido de carbohidratos, no se observaron diferencias significativas entre las interacciones (irradiancias x longitudes de onda), lo cual sugiere que la exposición a estas condiciones de cultivo no influye en la producción de carbohidratos totales para esta microalga en particular. Sin embargo, el contenido de carbohidratos obtenidos en el presente estudio, se encuentra dentro del rango reportado para microalgas marinas (5-23%) (Brown *et al*., 1997).

Los lípidos fueron los componentes mayoritarios en la composición bioquímica de *Navicula* sp.; una de las principales características de las diatomeas bentónicas son las altas concentraciones de estos compuestos (Leal *et al*., 2010). En este estudio las concentraciones más altas de lípidos se observaron en los cultivos expuestos a bajas irradiancias y longitud de onda roja; estos valores fueron superiores a los reportados para la especie *Navicula germanopolonica* cultivada en longitud de onda blanca (Leal *et al*., 2013) e incluso superiores a los reportados para otras especies de diatomeas bentónicas (Lee *et al*., 2009).

Con base en los análisis químicos y las concentraciones celulares obtenidas en el presente estudio, se tomó la decisión de cultivar la microalga *Navicula* sp. en las tres longitudes de onda e irradiancia de 50 μmol fotones $m^{-2}seg^{-1}$, con la finalidad de obtener biomasa en cantidades suficientes para extraer polisacáridos, posteriormente caracterizarlos y evaluar sus posibles aplicaciones en la industria biotecnológica.

El rendimiento de polisacáridos en los cultivos expuestos a longitud de onda azul (4.4% p/p) fue superior a las longitudes de onda roja (3.9% p/p) y blanca (3.4% p/p). Estos valores son similares a lo reportado en la diatomea *Gomphonema olivaceum* (3% p/p) (Huntsman y

Sloneker, 1971); sin embargo, el rendimiento de polisacáridos de microalgas depende del método de extracción y de la de especie (Lahaye y Robic, 2007). La investigación de Carmona *et al*., (1998), mostró que el extracto de la macroalga roja *Gelidium sesquipedale* presentó una tendencia similar a la observada en el presente estudio en respuesta a la longitud de onda, siendo superior el rendimiento de polisacáridos obtenido en luz azul con respecto a la luz roja y luz blanca.

En cuanto a las características de los polisacáridos de *Navicula* sp., se detectaron cinco azúcares neutros en distintas concentraciones para cada longitud de onda; los principales monosacáridos encontrados fueron glucosa, ramnosa, galactosa, manosa y xilosa. Staats *et al*., (1999) reportaron la presencia de éstos azúcares en extractos de polisacáridos extracelulares de la microalga *Navicula salinarum*.

La composición de monosacáridos en las microalgas varía entre especies, siendo la glucosa el monosacárido predominante (21-87% p/p), seguido por galactosa (1-20% p/p) y manosa (2-46% p/p), mientras que ramnosa, xilosa, fucosa y ribosa pueden encontrarse en concentraciones variadas (0-17% p/p) (Brown *et al*., 1997).

Las concentraciones de manosa observadas en los polisacáridos de *Navicula* sp., no presentaron diferencias significativas entre los tratamientos. En investigaciones similares, Staats *et al*., (1999) y Lee *et al*., (2006) reportaron concentraciones de manosa superiores a las del presente estudio; sin embargo, los resultados obtenidos en este trabajo se encuentran dentro de los valores reportados para microalgas (Brown *et al*., 1997).

El monosacárido más abundante obtenido en luz blanca fue ramnosa (35% p/p); éste fue superior al contenido observado en longitud de onda azul y roja. Autores como Haug y Myklestad (1976) reportaron un alto contenido de ramnosa (34-39% p/p) en los polisacáridos extracelulares de distintas especies de diatomeas; sin embargo, la concentración de ramnosa del polisacárido obtenido en luz azul fue similar a la reportada por Lee *et al*., (2006) en *Navicula directa* (8.7% p/p).

El contenido de galactosa observado en las tres longitudes de onda fuer similar al reportado para *Navicula salinarum* (19.1% p/p) y *Navicula directa* (20.7% p/p) (Staats *et al*., 1999; Lee *et al*., 2006).

Por otro lado, el contenido de xilosa fue inferior al obtenido por Staats *et al*., (1999) y Lee *et al*., (2006); sin embargo, las concentraciones de xilosa se encontraron dentro del rango

reportado para microalgas marinas (Brown *et al*., 1997). Finalmente, las concentraciones de glucosa en las tres longitudes de onda, fueron inferiores a las reportadas por Staats *et al*., (1999) en *Navicula salinarum*, pero superiores a las reportadas para *Navicula directa* (Lee *et al*., 2006).

Se detectaron pequeñas cantidades de proteína en los extractos de los polisacáridos obtenidos en las tres longitudes de onda, encontrándose diferencias significativas entre la luz blanca y las otras longitudes de onda. El contenido de proteína fue inferior al reportado para otras especies de *Navicula* (4.9% p/p y 15.1% p/p) (Staats *et al*., 1999; Lee *et al*., 2006).

El contenido de sulfato fue más alto en los polisacáridos obtenidos en longitud de onda blanca (0.4% p/p); estas concentraciones fueron similares a las reportadas para el galactano sulfatado del alga roja *Ahnfeltia tobuchiensis* (0.2%-0.3% p/p), pero inferiores a lo observado para otras especies de *Navicula* (6.3% y 8% p/p) (Staats *et al*., 1999; Lee *et al*., 2006). El contenido de sulfato en microalgas es altamente variable y puede oscilar entre 0 a 90% (Pérez-Loyola *et al*., 2003).

El peso molecular obtenido en luz blanca (17 kDa) fue menor al obtenido en luz azul (107 kDa) y luz roja (108 kDa). Estos valores son inferiores a lo reportado por Lee *et al*., (2006) para el polisacárido sulfatado obtenido de *Navicula directa* (222 kDa).

Es importante mencionar que la información relacionada con la caracterización de polisacáridos sulfatados del género *Navicula* aún es incipiente. Cabe señalar que la composición físico-química y características de las microalgas dependen de las condiciones de cultivo y las variaciones en los resultados son con frecuencia especie-dependientes (Dautania and Singh, 2014; de Morais *et al*., 2015).

Se detectaron 5 bandas similares en los polisacáridos sulfatados de *Navicula* sp. en las tres longitudes de onda, mediante análisis de Espectrometría Infrarroja por Transformadas de Fourier (FT-IR). Las bandas detectadas se encontraron en un rango entre 3413-818 cm^{-1}, mostrando el patrón típico de los carbohidratos. Las bandas correspondientes a la región 3413-3405 cm^{-1}, fueron atribuidas a la vibración de estiramiento de los grupos O-H; se observó una banda similar alrededor de éste número de onda en los polisacáridos de la microalga *Isochrysis galbana* (Sun *et al*., 2014) y *Spirulina platensis* (Abd El Baky *et al*., 2013).

Se detectaron bandas de menor intensidad a 1646-1656 cm^{-1}, las cuales fueron atribuidas a la vibración del grupo amida I, correspondiente a las proteínas (Goo *et al*., 2013); esta banda ha sido observada en polisacáridos de distintas microalgas (Stehfest *et al*., 2005). Comparando

estas vibraciones con el resto de las regiones, es evidente que el polisacárido es el componente mayoritario mientras que las proteínas están presentes como impurezas (Goo *et al*., 2013).

Las bandas de mayor intensidad se observaron a 1142-1135 cm^{-1}, las cuales fueron asignadas a las vibraciones correspondientes a enlaces glicosídicos (C-O-C); se han reportado bandas similares en los polisacáridos de la microalga *Isochrysis galbana* (Sun *et al*., 2014) y en los polisacáridos sulfatados de varias algas (Morimoto *et al*., 2014 y Abdala-Díaz *et al*., 2010).

En cuanto a la vibración correspondiente a los grupos S=O, se detectaron bandas de baja intensidad a 1250-1244 cm^{-1}, estos resultados coinciden con el bajo contenido de sulfato detectado en los polisacáridos sulfatados de *Navicula* sp. en las tres longitudes de onda. Esta banda ha sido reportada en los polisacáridos sulfatados de *Navicula directa* (Lee *et al.*, 2006) y en los tres grupos principales de macroalgas (Souza *et al*., 2012; Shao *et al*., 2013; Morimoto *et al*., 2014; Saboural *et al*., 2014). Las bandas detectadas a 832-818 cm^{-1} corresponden a las vibraciones de C-O-S; se reportaron bandas similares en los polisacáridos sulfatados de *Spirulina platensis* (Abd El Baky *et al*., 2013) y en los polisacáridos de varias macroalgas (Souza *et al*., 2012; Shao *et al*., 2013; Shao *et al*., 2013b; Morimoto *et al*., 2014; Saboural *et al*., 2014).

Posterior a la caracterización parcial de los polisacáridos sulfatados de *Navicula* sp., se analizaron sus posibles aplicaciones. Se realizaron experimentos previos para evaluar la capacidad de gelificación de los polisacáridos en presencia de iones monovalentes (KCl) y divalentes ($CaCl_2$), sin embargo estos iones no mostraron resultados satisfactorios. Running *et al*., (2012) observaron que los polisacáridos sulfatados (λ-carragenanina) obtenidos de macroalgas son capaces de formar geles en presencia de iones trivalentes. Debido a estos antecedentes, en el presente estudio se evaluó la capacidad de formar geles en los polisacáridos sulfatados (1% p/v) de *Navicula* sp. en presencia $FeCl_3$ (0.4% p/v). Dado a que el rendimiento más alto de polisacáridos se obtuvo en longitud de onda azul, éstos fueron utilizados para evaluar su capacidad de gelificación en presencia de iones trivalentes.

La formación del gel trivalente se analizó mediante reología dinámica de baja deformación, la cual mostró los cambios en los módulos elástico (G') y viscoso (G'') a través de una rampa de temperatura de 5-70 ºC. El comportamiento de los módulos (G': 1 Pa > G'': 0.7 Pa) fue térmicamente estable entre 20-40 ºC, confirmando la formación del material visco-elástico, con características de un gel débil. En investigaciones anteriores se ha sugerido que el hierro

trivalente promueve las interacciones iónicas entre las cadenas de los polisacáridos sulfatados; sin embargo, el mecanismo de gelificación aún se desconoce (Le Nguyen *et al*., 2012; Running *et al*., 2012).

El carácter elástico del gel obtenido en el presente estudio, podría atribuirse a la asociación temporal de las cadenas de polisacáridos sulfatados durante cortos períodos de oscilación. Se ha sugerido que los iones trivalentes podrían ser más adecuados que los iones monovalentes para el equilibrio de las tres cargas negativas del sulfato de los polisacáridos, tales como la λ-carragenina (Running *et al*., 2012). En la literatura hay pocos estudios sobre la formación de geles a partir de polisacáridos sulfatados nativos en presencia de iones trivalentes (Le Nguyen *et al*., 2012; Running *et al*., 2012).

Debido a que el gel es estable entre 20-40 ºC, podría ser utilizado en aplicaciones biomédicas donde se requiera la implementación de material orgánico que soporte la temperatura corporal. En el estudio de Vorvolakos *et al.,* (2011), se observó que el ácido hialurónico puede formar geles en presencia de iones trivalentes, similar a los polisacáridos sulfatados de la presente investigación. El ácido hialurónico puede ser utilizado en cirugías laparoscópicas para evitar adherencias (Farell-Rivas *et al*., 2014), debido a que las características de gelificación son similares a las de esta investigación, por lo que la evaluación de los polisacáridos sulfatados de *Navicula* sp. resulta de gran interés.

Dada la creciente demanda por productos de origen natural, surge la necesidad de encontrar fuentes alternativas que satisfagan estos requerimientos. Las microalgas representan una opción viable para la obtención de compuestos antioxidantes. Estas sustancias pueden prevenir la oxidación celular, actuando sobre las especies reactivas de oxígeno (ROS) (Qi *et al*., 2005). Los antioxidantes minimizan el daño oxidativo incrementando las defensas naturales, eliminando radicales libres (Sun *et al*., 2014). Por lo anterior, en esta investigación se evaluó la capacidad antioxidante de los polisacáridos sulfatados de la microalga *Navicula* sp.

La mayor actividad antioxidante se observó en la concentración de 200 µg/mL en las tres longitudes de onda, siendo superior en los polisacáridos sulfatados obtenidos en longitud de onda blanca (48.7 ± 3.1%). Lee *et al*., (2009) reportaron valores de actividad antioxidante (DPPH) entre 31.6-63.2% en extractos de *Navicula* sp.; mientras que Affan *et al*., (2007) reportaron valores entre 27.3-81.6% para *Navicula incerta*. Los resultados obtenidos en el presente estudio se encuentran dentro del rango reportado por estos autores.

Al igual que el análisis DPPH, la mayor actividad antioxidante analizada mediante $ABTS^{+}$ se observó en los polisacáridos sulfatados obtenidos en longitud de onda blanca; esto podría estar relacionado con el bajo peso molecular (17 kDa), contenido de sulfato (0.40% p/p) y el contenido de ramnosa (35% p/p) del polisacárido obtenido en luz blanca. Reportes previos han indicado que la composición, peso molecular, estructura, contenido de sulfato, tipo de monosacáridos, entre otras características, influyen en la actividad biológica de los polisacáridos (Qi *et al*., 2005; Lo *et al*., 2011; Wang *et al*., 2015).

Sun *et al*., (2009) dividieron los polisacáridos extraídos del alga roja *Porphyridium cruentum* en fragmentos con distintos pesos moleculares y reportaron una alta actividad antioxidante en los polisacáridos con bajo peso molecular (6.55 kDa). Sun *et al*., (2014) reportaron que los polisacáridos de bajo peso molecular (15.93 kDa) de la microalga *Isochrysis galbana* presentaron mayor actividad antioxidante que los de peso molecular superior.

Los polisacáridos con bajo peso molecular poseen un fuerte poder reductor, y esto es debido a las cadenas de los polisacáridos. Se conoce que los polisacáridos de bajo peso molecular presentan un mayor contenido de extremos reductores para eliminar y aceptar radicales libres (Qi *et al*., 2005; Wang *et al*., 2015).

A pesar de que la función de los monosacáridos en la actividad antioxidante no ha sido esclarecida, existen evidencias de que la composición y el tipo de monosacárido influyen en las propiedades antioxidantes (Wang *et al*., 2015). En la investigación de Lo *et al*., (2011) reportaron que el contenido de ramnosa en los polisacáridos del hongo comestible *Lentinula edodes* (shiitake), fue el factor determinante en sus propiedades antioxidantes.

En lo que respecta al contenido de sulfato, se ha comprobado que existe una correlación positiva entre su concentración y la actividad antioxidante de los polisacáridos sulfatados (Souza *et al*., 2012; Sun *et al*., 2014); entre más alto es el contenido de sulfato, mayor es la capacidad antioxidante (Qi *et al*., 2005; Jiao *et al*., 2011), lo cual coincide con los resultados observados en el presente estudio.

VII. CONCLUSIONES

La exposición tanto a distintas irradiancias como a diferentes longitudes de onda influyen en la concentración celular de *Navicula* sp.

El contenido de biomasa incrementa en los cultivos expuestos a irradiancias altas en las tres longitudes de onda

La exposición a bajas irradiancias favorece el aumento de proteínas y lípidos de *Navicula* sp.; mientras que el efecto de las longitudes de onda e irradiancias no influye en la producción de carbohidratos totales para esta microalga en particular.

Los principales azúcares neutros presentes en los polisacáridos de *Navicula* sp., son glucosa, ramnosa, galactosa, manosa y xilosa.

El contenido de sulfato es superior en los cultivos expuestos a longitudes de onda blanca e irradiancia baja.

Los polisacáridos extraídos de *Navicula* sp. presentan el patrón típico de bandas FT-IR observadas en polisacáridos sulfatados.

Los polisacáridos sulfatados de la diatomea bentónica *Navicula* sp. son capaces de formar geles en presencia de iones trivalentes.

Los polisacáridos sulfatados extraídos de *Navicula* sp. presentan actividad antioxidante DPPH y ABTS+.

Los polisacáridos sulfatados extraídos de *Navicula* sp. podrían ser una fuente potencial para diversas aplicaciones biotecnológicas, tales como farmacéutica, biomédica y nutracéutica.

VIII. RECOMENDACIONES

Se recomienda establecer un método más eficiente de cosecha de microalgas para mejorar el aprovechamiento de la biomasa seca.

Es conveniente evaluar la producción de polisacáridos sulfatados de *Navicula* sp., bajo distintos factores de estrés como temperatura, salinidad y limitación de nutrientes durante su cultivo.

Es recomendable evaluar distintas longitudes de onda e irradiancias a las empleadas en esta investigación.

Es importante evaluar la capacidad de gelificación de los polisacáridos sulfatados obtenidos de *Navicula* sp. cultivada en longitudes de onda blanca y roja.

Dadas las propiedades bioactivas de los polisacáridos sulfatados de *Navicula* sp., se recomienda evaluar su posible actividad antiviral y antibacteriana.

Se recomienda analizar a profundidad la estructura química y molecular de los polisacáridos sulfatados de la diatomea bentónica *Navicula* sp.

Es importante analizar otras especies de microalgas bentónicas, tales como *Amphora* sp. y *Nitzschia* sp., para evaluarlas como fuentes potenciales de polisacáridos sulfatados.

IX. LITERATURA CITADA

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