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Ahumada-Rudolph, Ramón; Cajas-Madriaga, Daniel; Rudolph, Anny; Reinoso, Rodrigo; Torres, Cristian; Silva, Mario; Becerra, José

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ARTICLE

## Variation of sterols and fatty acids as an adaptive response to changes in temperature, salinity and pH of a marine fungus *Epicoccum nigrum* isolated from the Patagonian Fjords

Variación de esteroides y ácidos grasos como respuesta adaptativa a cambios de temperatura, salinidad y pH de un hongo marino *Epicoccum nigrum* aislado desde los fiordos patagónicos

Ramón Ahumada-Rudolph<sup>1,4,5</sup>, Daniel Cajas-Madriaga<sup>1</sup>, Anny Rudolph<sup>2</sup>,  
Rodrigo Reinoso<sup>1</sup>, Cristian Torres<sup>3</sup>, Mario Silva<sup>1</sup> and José Becerra<sup>1</sup>

<sup>1</sup>Laboratorio de Química de Productos Naturales, Departamento Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción, Casilla 160-C, Chile. ramonahumada@udec.cl

<sup>2</sup>Facultad de Ciencias, Universidad Católica de la Santísima Concepción, Alonso de Ribera 2850, Concepción, Chile

<sup>3</sup>Laboratorio de Genómica & Biodiversidad (LGB), Departamento de Ciencias Naturales, Universidad del Bío-Bío, Dieciocho de Septiembre 580, Chillán, Chile

<sup>4</sup>EULA-Chile Environmental Sciences Centre, Universidad de Concepción, Chile

<sup>5</sup>Programa de Postgrados en Oceanografía, Departamento de Oceanografía, Universidad de Concepción, Casilla 160-C, Chile

**Resumen.** Se aisló e identificó una cepa hongo marino a partir de muestras de sedimentos obtenidas a partir de un sector de los fiordos patagónicos. La cepa LQRA39-P fue identificado como *Epicoccum nigrum* utilizando técnicas de microscopía y corroborada mediante técnicas moleculares. Se espera demostrar que las respuestas de variación metabólicas permiten hongos ubicuos desarrollen en diferentes ambientes como marinos, de agua dulce y ambientes terrestres. Se analizaron las variaciones en el contenido de ácidos grasos y esteroides, en una batería de medios de cultivo a diferentes temperaturas, salinidad y pH. Se analizó el contenido de ácidos grasos y esteroides, usando la cromatografía de gases acoplada a cromatografía de masas (GC-MS). Los cambios de temperatura no indujeron un efecto cambios significativos en el contenido de ácidos grasos; sin embargo, respecto del contenido de esteroides fue un factor determinante a pH 4 cuando fue crecido en agua dulce a 25°C, obteniéndose una mayor diversidad de esteroides no observados en otras condiciones. La salinidad generó cambios en la composición lipídica (*i.e.*, C16:0; C16:1; C18:2 y C18:3). En cuanto al pH, este factor tiene un efecto significativo sobre la composición de ácidos grasos y esteroides. Se demuestra que *E. nigrum* es capaz de cambiar su composición de esteroides y ácidos grasos como respuesta adaptativa para co-habitar sustratos marinos y dulceacuícolas.

**Palabras clave:** Ecología de hongos, hongos extremófilos, hongo marino, metabolitos secundarios

**Abstract.** We isolated and identified a marine fungus strain from samples of sediments obtained from a sector of the Patagonian fjords. The LQRA39-P strain was identified as *Epicoccum nigrum* using microscopy techniques and corroborated using molecular techniques. We expect to prove that metabolic variation responses allow ubiquitous fungi to develop in marine, freshwater, and terrestrial environments. We analyzed variations in the content of fatty acids and sterols, in a battery of culture media at different temperatures, salinity and pH. The content of fatty acids and sterols was analysed using gas chromatography coupled to mass chromatography (GC-MS). Changes in temperature showed no significant changes in the fatty acid content; nevertheless, sterol content was significantly affected by this factor at just pH 4, increasing diversity of sterols in freshwater at 25°C. Salinity generated changes in the lipid composition (*i.e.*, C16:0; C16:1; C18:2 and C18:3). As for pH, this factor has a significant effect on the composition of fatty acids and sterols. We demonstrate that *E. nigrum* is capable of changing its composition of sterols and fatty acids as an adaptive response that may enable co-inhabiting marine and terrestrial substrates.

**Key words:** Fungal ecology, extremophile fungus, marine fungus, secondary metabolites

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## INTRODUCTION

*Epicoccum nigrum* Link (Pleosporales, Ascomycetes) is a ubiquitous fungus, characterized by a colored mycelium which varies from yellow to dark red, produces conidia multi-septate with brown to black pigmentation with short conidiophores located in sporodochia (Mims & Richardson 2005). The biogeographical records show a wide distribution in all 5 continents. In Latin America, according to GenBank® records, it has been studied in Perú, Puerto Rico, Ecuador, Colombia and Chile (Fávaro *et al.* 2011). The substrates that it colonizes are several. Its presence has been reported in terrestrial environments with substrates such as soil and plant tissue, saprobe or parasitizing crops, *e.g.*, soybean (Larran *et al.* 2002, Pitt & Hocking 2009), as well as degrading wood of conifers in the south of Chile (Butin & Peredo 1986). In addition, its adaptive ability has allowed it to colonize marine environments, where it has been isolated from sponges (Hai-Hong *et al.* 2011) and algae of the genus *Fucus* and *Spartina* (Kohlmeyer 1974, Abdel-Lateff *et al.* 2003).

The *E. nigrum* strain studied in this work was isolated from sediments collected in the area of the Moraleda Channel, Aysén region of Chile, where the transport of organic matter is mainly of marine origin (*i.e.*, 74% autochthonous planktonic organisms) (Silva *et al.* 2011). In these Patagonian fjords, freshwater entry from the river to the head of the fjord forms a surface plume of low-density that connects to the open sea and results in layers of different densities (Silva & Guzmán 2006). The head of the fjord is associated to glaciers with detachments of ice and low temperatures, constituting a unique system that is directly disrupted by terrestrial, climatic and tidal influences (Armstrong *et al.* 1985).

In the area, knowledge pertaining to the metabolic changes of fungi as an adaptive response in transition environments is scarce (Gunde-Cimerman *et al.* 2009). It is proposed that the basis of these changes relates mainly with the ability of the fungi to modify structures of the cell membrane, which acts as a selective barrier (Horvath *et al.* 1998, Bowman & Free 2006, Kavanagh 2011). Membrane proteins are essential elements involved in functions such as the maintenance of cell shape, adhesion processes, cellular protection of toxic substances, molecule absorption, signal transmissions to the cytoplasm, and synthesizing or remodelling the components of the wall (Bowman & Free 2006). Sterols are essential components of eukaryotic cell membranes, and therefore cells tightly regulate sterol levels and sterol metabolism. Alteration of sterol levels can have an effect

on the susceptibility of fungal cells, including osmotic and oxidative pressures (Zavrel *et al.* 2013). It is proposed that the ability to adapt and increase the chances of surviving and reproducing in marine conditions, is based mainly on the ability to regulate osmosis through modification of the sterol composition of the structural components of their cell membrane, and to variations in the synthesis of fatty acids, *i.e.*, increasing the content of unsaturated molecules at low temperatures (Nordström & Laakso 1992, Mysyakina & Funtikova 2007), that manages to keep the fluidity of the membrane and proper cellular function (Swan & Watson 1997).

Salinity and pH are factors that limit the growth of fungus species that generally grow and proliferate under optimum conditions. The fungal cell wall is the first line of defense against environmental stress, providing the capacity to protect against external physiochemical changes. As such, cell wall adaptation is expected to play one of the most important roles in the proliferation of the fungus. The capacity of the cell wall to resist both the water loss and salt toxicity in a minimum of water activity ( $a_w$ ) and fluctuations in alkalinity (Kapteyn *et al.* 1999, Mager & Siderius 2002), makes it essential for maintaining the osmotic homeostasis of cells due its ability to protect against mechanical damage and keeping the level of turgor pressure required for intracellular cytoplasm (Bowman & Free 2006).

Our objective was to analyze changes in the content of sterols and fatty acids of the ubiquitous fungus *E. nigrum* isolated from Patagonian fjords area, as adaptive responses to changes in the conditions of temperature, pH and salinity.

## MATERIALS AND METHODS

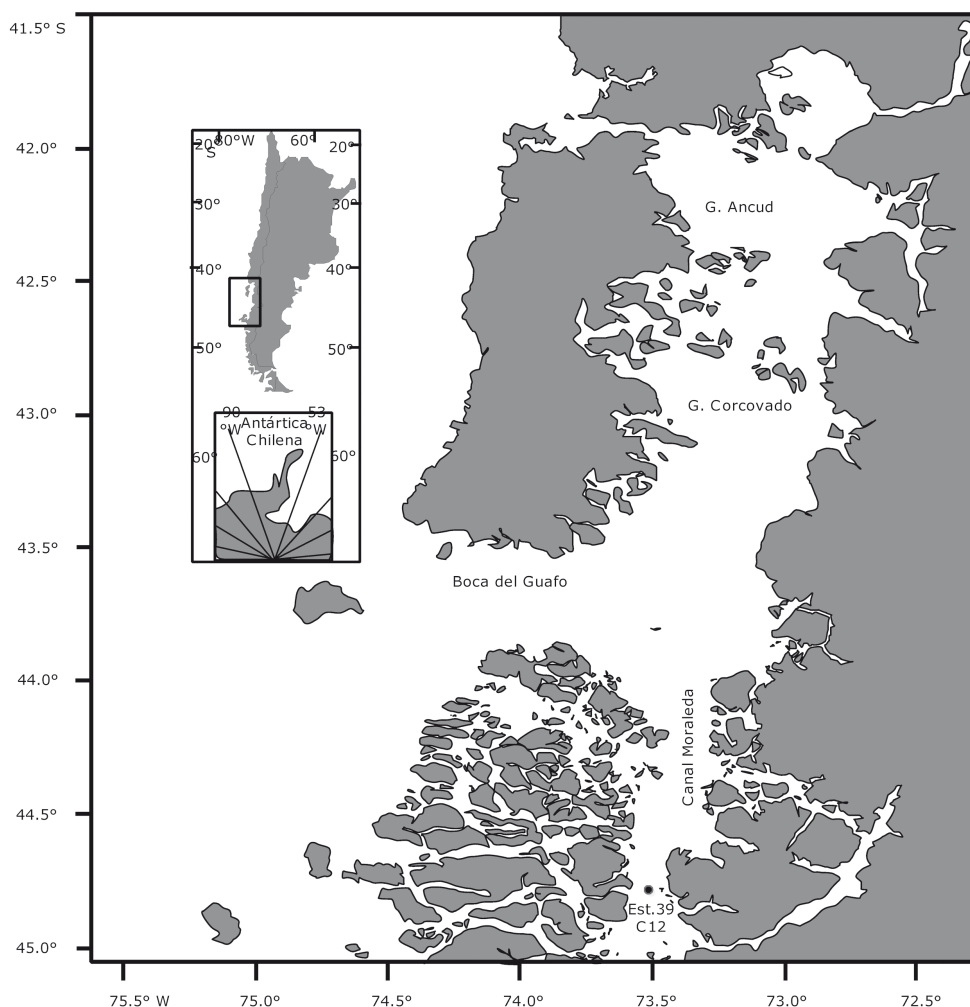
The marine strain *Epicoccum nigrum* LQRA39P (Fig. 2), was isolated from sediment (93% clay, 3-5% total organic matter) collected from Patagonian fjords region of the Moraleda Channel. The sampling point corresponds to station 39 C12-F (44°39'42"S - 73°30'06"W) at 300 m depth (Fig. 1). The sediment samples were collected from 3 cm using a box corer and stored at -18°C (Silva *et al.* 2011).

The strain was isolated from 10 g of sediment, which was diluted in 20 mL sterile seawater and left to decant for 15 min to eliminate the excess particulate material. The supernatant was subsequently diluted 1:10 with sterilized seawater, an aliquot of 100 µL was transferred to agar YMG seawater media with streptomycin (200 mg L<sup>-1</sup>) and spread over the surface using a sterile glass rod. The

growth of fungal colonies was evaluated after 5 days of growth at room temperature and pure strains were obtained from these agar plates (Webster & Weber 2007).

In order to validate the morphological identification of *E. nigrum* strain, we amplified the ITS (which include 5.8S gene) and the nuclear ribosomal large subunit rRNA (LSU) regions using ITS-5 (White *et al.* 1990) and LR6 (Vilgalys & Hester 1990) primers. DNA was extracted from fruit bodies using the E.Z.N.A. fungal DNA MiniKit (Omega-Biotek). PCR reactions were performed using ITS5 (5'-ggaagtaaaagtcgtaacaagg-3') as forward primer and

LR06 (5'-cgccagttctgcttacc-3') as reverse primer. Each reaction was conducted in a 15 µl volume containing 30-50 ng of DNA, 1x of PCR buffer, 2 mM MgCl<sub>2</sub>, 0.1 µM of each dNTP, 0.5 µM of forward and reverse primers, and 1 U of Taq DNA polymerase. PCR amplification was carried out with an initial denaturation step of 4 min at 94°C, and then 35 cycles of 30 s at 94°C, 60 s at 50°C and 60 s at 72°C s, followed by a final step of 5 min at 72°C. PCR product was sequenced in both senses at Macrogen® Inc. (Seoul, Korea).



**Figure 1. Oceanographic location of station 39 C12-F that sample was recollected during CIMAR 12 Fjords cruise, where we obtained samples of sediment from which the strain *E. nigrum* was isolated / Ubicación oceanográfica de la estación 39 C12-F donde fue recolectada la muestra de sedimento en la que se aisló la cepa *E. nigrum* durante el Crucero CIMAR 12 Fiordos**

Forward and reverse sequences were edited using Genious v5.4 software (Drummond *et al.* 2011). The sequence of our *E. nigrum* strain was analyzed with BLAST (Basic Local Alignment Search Tool, database nucleotide collection nr/nt)<sup>1</sup> using Megablast to determine the percentage of maximal identity with the sequences of that global database. Specifically, we performed a neighbor-joining distance tree based on ITS and LSU regions to determine the phylogenetic affinity of our isolated. Finally, the sequence obtained in the present study was deposited in GenBank® (CODE: KC164754).

Liquid cultures of the strain were carried out in 125 ml of YMG medium, with sterile distilled water (pH 4, 7 and 10) and sterile filtered seawater (pH 4, 7 and 10). Both distilled water and seawater mediums were modified with HCl acid and NaOH alkaline. Incubation was carried out at 6 and 25°C under constant agitation at 120 rpm for 21 days until the glucose was consumed. The cultures were subsequently filtered and the mycelium was washed with sterile distilled water 3 times to remove all traces of the culture medium.

The extraction of sterols was performed from 5 g of mycelium to which 25 mL solution methanol-water (1:1) with NaOH granules, resulting in a concentration of 2N. The mixture was heated for 30 min at 100°C, filtered and the supernatant extracted 3 times with a solution of diethyl ether-hexane (1:1), and finally concentrated on a rotary agitator (120 rpm) at 40°C.

The first steps in fatty acid extraction were similar to those of sterol extraction. However, after the mixture was heated for 30 min at 100°C, it was cooled to room temperature. Subsequently, a solution of HCl 2N was added until pH 2 was obtained. The mixture was then filtered and the supernatant was extracted 3 times with a solution of diethyl ether-hexane (1:1), and finally concentrated on a rotary agitator (120 rpm) at 40°C. All extracts were stored at 4°C for subsequent analysis (Stahl & Klug 1996).

The analysis of fatty acid and sterols were carried out by GC-Mass spectrometry (GC-MS) and recorded on a Shimadzu GC-17A/MS QP5050AGC-MS system (electron ionization mode 70 eV, source temperature 270°C, scanned mass ranged m/z 43-350). The operating conditions were as follows: 30 m HP-5MS (5%-diphenil-95%-dimethylsiloxane) capillary column; 0.25 mm id, thickness:

0.25 µm, helium as the carrier gas, flow rate of 1 mL min<sup>-1</sup> and with split ratio of 1:30, temperature was programmed as follows: from 100°C (5 min) to 260°C at 10°C min<sup>-1</sup>. The identification of the components of the sterol and fatty acid composition was accomplished by matching their mass spectra with those recorded in the NIST 05 (NIST / EPA/NIH MASS 2005 spectral Library). Spectra were considered coincident if the similarity index was higher than 95%.

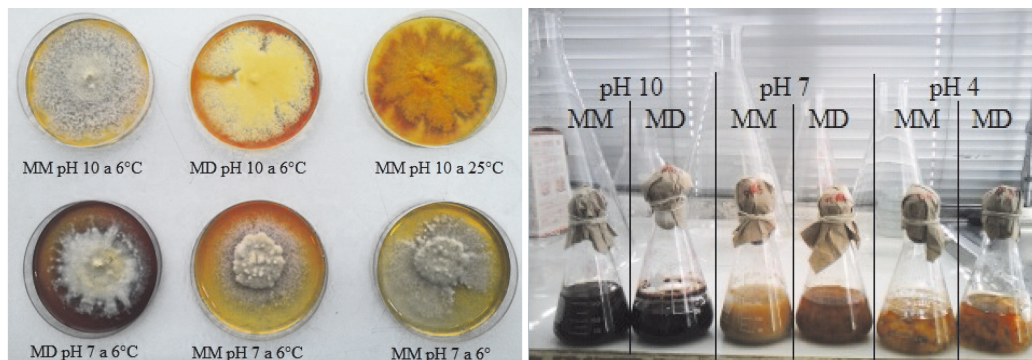
The Shapiro-Wilk test was used for testing normality of the data and Cochran's test for homogeneity of variances. The effect of cultivation conditions; salinity (*i.e.*, freshwater and seawater); pH (*i.e.*, pH 4, pH 6-7 and pH 10) and temperature (*i.e.*, 6-25°C), were compared using the parametric test one-way ANOVA in accordance with its respective response variable in fatty acids and sterols. All statistical calculations were performed using the software package STATISTICA version 6.0 (StatSoft Inc. 2005).

## RESULTS

Of the fungus extracted from the Patagonia region, over 100 strains were isolated and identified in the laboratory. Of these strains, the *E. nigrum* demonstrated changes in growth and pigmentation under different conditions of cultivation (pH, salinity) in both solid and liquid mediums (Fig. 2). In the figure, the 250 ml flasks show the intense red pigmentation in a culture with a basic pH level, in both seawater and freshwater. The pigmentation is a clear yellow color in an acidic culture. In the figure, it is observed that 2 of the solid marine mediums at pH 7 and 6°C, had similar types of growth (Fig. 2).

A 1517 bp PCR fragment was amplified that fit with the expected PCR product size (~1600 bp). This fragment, covered ITS1, 5.8S, ITS2 and 28S region (partial sequence). MegaBLAST analysis confirmed the identity of our isolated (GenBank® accession number: KC164754) was grouped with others *E. nigrum* accessions such as (GenBank accession number: GU183122, ITS region, Lee *et al.* 2007) and (GenBank® accession number: JN938882, LSU region, Schoch *et al.* 2012), showing high scores (1519 and 1104 bits, respectively) and high similitude percentages (100 and 99%, respectively).

<sup>1</sup><<http://blast.ncbi.nlm.nih.gov/Blast.cgi>>



**Figure 2.** *E. nigrum* in solid and liquid cultured media YMG, with sterile distilled water (MD) and sterile filtered seawater (MM) in both cases pH was tested at 10, 7 and 4. Solid medium at pH < 5 does not coagulate agar / Medios de cultivos sólidos y líquidos de *E. nigrum* en medio YMG, con agua destilada estéril (MD) y agua de mar filtrada estéril (MM), en ambos casos se evaluó el efecto del pH (10, 7 y 4). El medio sólido, a pH < 5, no coagulan el agar

*E. nigrum* biomass cultivated in freshwater as well as seawater medium showed different proportions of fatty acids, with a carbon chain length of 14-18 carbon atoms. In relation to salinity, *i.e.*, cultures in freshwater and seawater medium, significant differences were observed in fatty acid content with: C16:0 ( $F_{(18,00)} = 0.001$ ) (Tukey's *a posteriori*  $P < 0.05$ ); C16:1 ( $F_{(6,6)} = 0.027$ ) (Tukey's *a posteriori*  $P < 0.05$ ); C18:2 ( $F_{(9,01)} = 0.013$ ) (Tukey's *a posteriori*  $P < 0.05$ ); C18:3 ( $F_{(8,25)} = 0.01$ ) (Tukey's *a posteriori*  $P < 0.05$ ). In regard to pH, this variable exerted a significant effect on the composition of fatty acids, observing differences in the fatty acids with: C16:0 ( $F_{(34,2)} = 0.0006$ ) (Tukey's *a posteriori*  $P < 0.05$ ); C16:1 ( $F_{(5,7)} = 0.024$ ) (Tukey's *a posteriori*  $P < 0.05$ ); C18:2 ( $F_{(16,45)} = 0.009$ ,  $P < 0.05$ ); C18:3 ( $F_{(11,44)} = 0.0003$ ) (Tukey's *a posteriori*  $P < 0.05$ ).

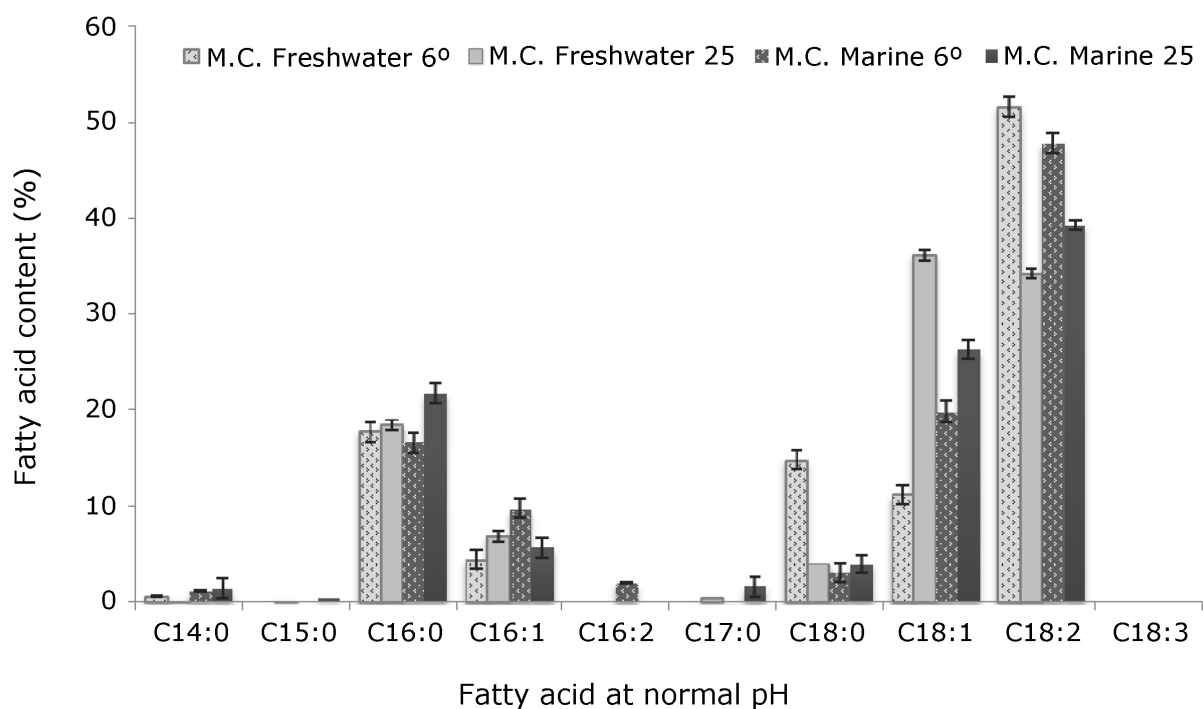
At the pH 6 of freshwater, we observed that the fatty acid composition varied with temperature. At 25°C unsaturated molecules C18:1 and linoleic acid C18:2 predominated in equal proportions. However, at 6°C fatty acid 18:2 predominated (Fig. 3).

At the normal pH of seawater medium (pH 7), at 6 and 25°C we observed no changes in fatty acid composition in contrast to what was observed in freshwater ( $F_{(24,08)} = 0.127$ ,  $P < 0.05$ ). In general, in normal pH, 60% of the observed fatty acid was with C18 unsaturated (*i.e.*, 18:2 and 18:1). In contrast, C16 fatty acid is predominantly saturated *i.e.*, 16:0 > C16:1 > C16:2. Fatty acid 16:2 was detected only at 6°C (low temperature) and in seawater medium (Fig. 3).

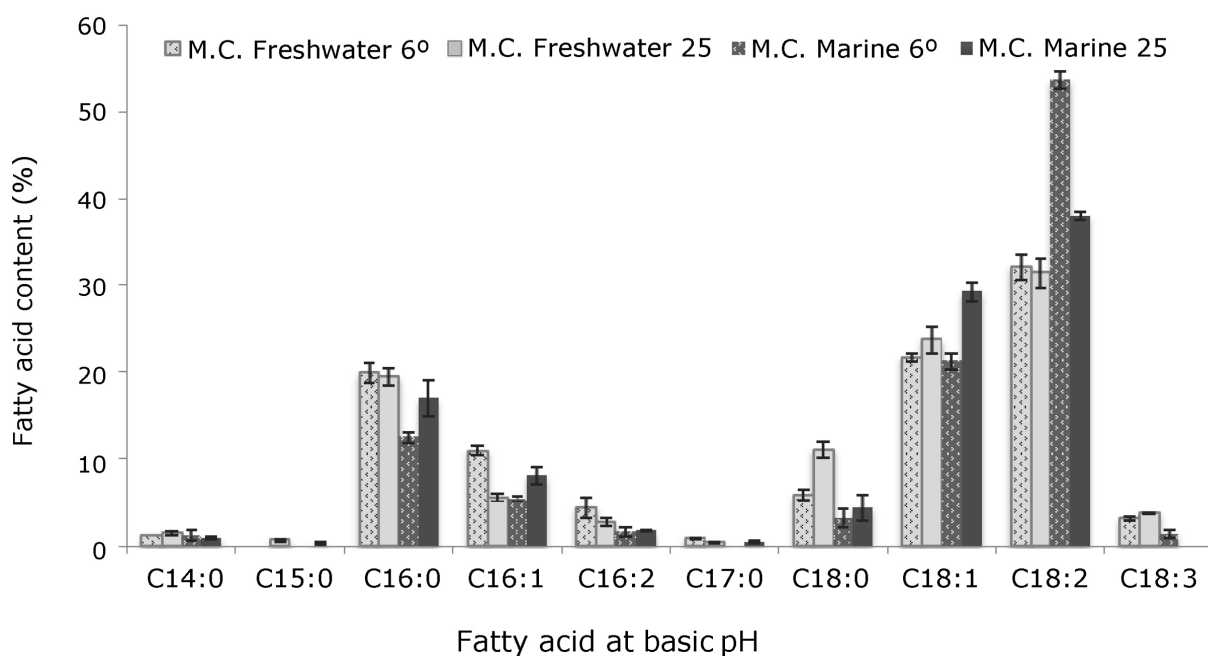
At the basic pH (pH 10) the biomass of *E. nigrum*, both in freshwater and seawater medium, displayed a great diversity of fatty acids of chain lengths C14, C15, C16, C17 and C18 with their respective unsaturation (*i.e.*, 16:1, 16:2, 18:1, 18:2 and 18:3). The contents of 18:1 and 18:2 were prominent at 53 and 74%, respectively (Fig. 4). In seawater medium, at a low temperature of 6°C, the percentage of unsaturated molecules C18 reached 68% and at a higher temperature of 25°C a higher percentage of 74% was reached, in contrast to observed in freshwater, where at a low temperature only 54 and 55% were respectively reached (Fig. 4)

The C16 fatty acids in both salinities showed a higher proportion of saturation. In contrast, the proportion of unsaturated molecules C16 was greater at low temperatures in freshwater and the reverse in seawater cultures ( $F_{(112,36)} = 0.059$ ) (Tukey's *a posteriori*  $P < 0.05$ ) (Fig. 4). However, differences in the percentages between the fatty acid molecules were not significant; a similar tendency was observed at basic pH between the different culture media and temperature.

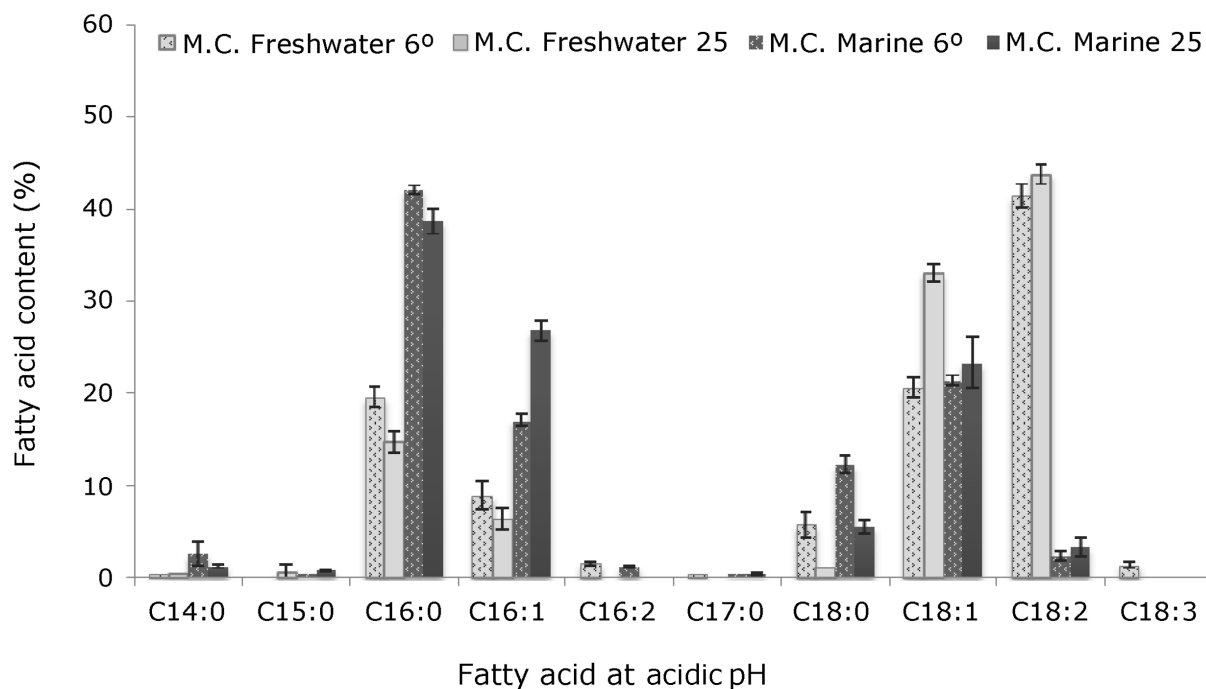
Fatty acids extracted from mycelium cultured in freshwater at pH 4, contained higher levels of unsaturated C18:1 and C18:2, with proportions of 64 and 77% at high and low temperatures, respectively. This is in contrast to what was observed in seawater medium where proportions of 24% were reached at low temperatures and 27% at high temperatures (Fig. 5). Unlike the previous cultures, the proportion of saturated fatty acid C16 with 66% at high temperature and 60% at low temperature was greater in



**Figure 3.** Fatty acid content in the *E. nigrum* biomass cultured at pH 7, in freshwater and seawater media at temperatures of 6 and 25°C ( $\pm$  s.d.) / Contenido de ácidos grasos en la biomasa de *E. nigrum* cultivado a pH 7, en medio con agua dulce y agua de mar, a temperaturas de 6 y 25°C ( $\pm$  d.e.)



**Figure 4.** Fatty acid content of *E. nigrum* biomass cultured with pH 10 in freshwater and seawater media at temperatures of 6 and 25°C ( $\pm$  s.d.) / Contenido de ácidos grasos en la biomasa de *E. nigrum* cultivado a pH 10, en medio con agua dulce y agua de mar, a temperaturas de 6 y 25°C ( $\pm$  d.e.)



**Figure 5.** Fatty acid content of *E. nigrum* biomass cultured with pH 4 in freshwater and seawater media at temperatures of 6 and 25°C ( $\pm$  s.d.) / Contenido de ácidos grasos en la biomasa de *E. nigrum* cultivado a pH 4, en medio con agua dulce y agua de mar, a temperaturas de 6 y 25°C ( $\pm$  d.e.)

seawater medium. Additionally, the proportion of molecules was less for C18:2, with percentages of only 2 and 3% at low and high temperatures respectively (Fig. 5). The differences observed in the production of fatty acid cultures with freshwater and seawater mediums to acidic pH, was statistically significant, differences that would be given by the ratio of fatty acids C18:2 ( $P = 0.012$ , d.f. = 1 and C16:0,  $P = 0.0016$ , d.f. = 1).

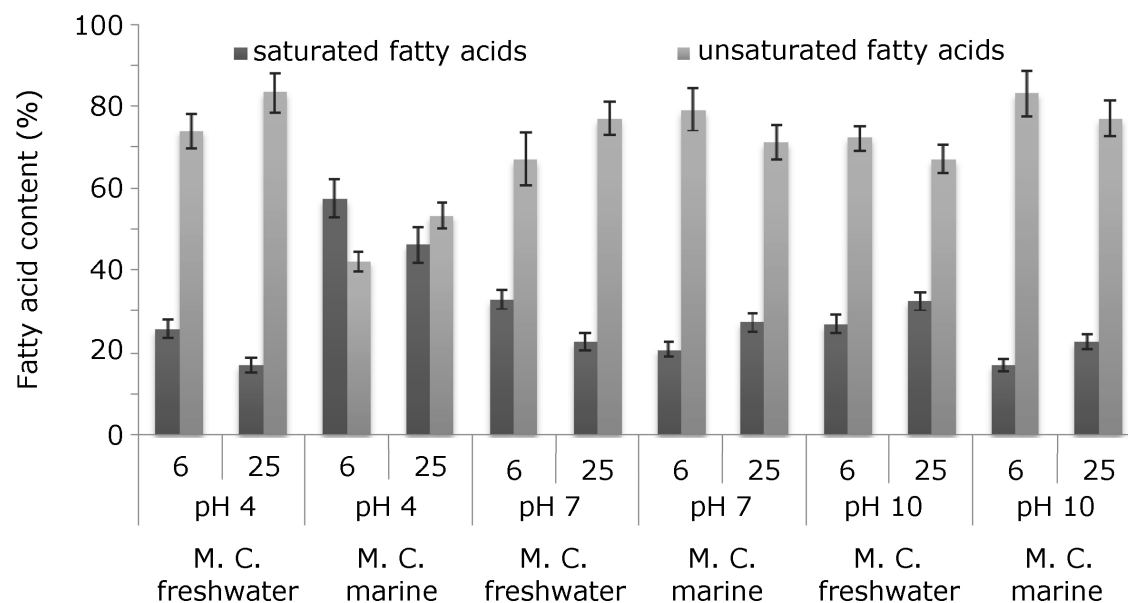
The fatty acids C14:0, C15:0, C17:0 and C18:3 were present in low percentages (0.16 to 3.69%). C15:0 was found in percentages between 0.16 and 0.73%, only at 25°C in both salinities and pH, and was also found at 6°C only in culture seawater medium and in low proportion. Unsaturated fatty acid C16:2 was detected in slightly higher proportions (which ranged between 1.25 and 4.4%) at pH 10 in both temperatures. However, at pH 7 and pH 4 it was only detected at 4°C. Thus, the proportion of this fatty acid is significantly different depending on the temperatures ( $P < 0.01$ , d.f. = 2). C14:0 was found under all conditions of cultivation in trace amounts. C16:2 was only observed in low percentages from 0.16 to 2.65%. C17:0 was also found in trace amounts (0.16 to 1.5%) under all culture conditions except freshwater pH 7 at 6°C, freshwater pH 4 at 25°C and

seawater medium pH 10 at 6°C. Finally, C18:3 was found in freshwater pH 10 at both 6 and 25°C (3.15 and 3.69%), in freshwater pH 4 at 6°C (1.41%) and seawater pH 10 at 6°C (1.39%) (Figs. 3, 4 and 5).

The graphical representation of the proportions of fatty acids in the 12 culture media (*i.e.*, salinity, temperature and pH) of *E. nigrum*, show that in general the proportion of unsaturated fatty acids was greater ( $F_{(8,97)} = 0.007$ ) (Tukey's *a posteriori*  $P > 0.05$ ) with percentages between 67 to 83%. The exception was observed in 2 seawater media at pH 4 (Fig. 6). *E. nigrum* cultivated at two temperatures 6 and 25°C, did not present significant differences in the total percentages of saturated and unsaturated fatty acids ( $F_{(3,1)} = 0.124$ ) (Tukey's *a posteriori*  $P > 0.05$ ).

The *E. nigrum* cultures, in both freshwater and seawater medium, show to contain as main sterols 9(11)-dehydroergosterol and ergosterol, both characteristic components of the fungi, as well as the content of 8 other minor sterols (Table 1, Fig. 7). The strain showed significant differences in the quantity of sterols, depending on the salinity of the culture medium, sterols





**Figure 6. Graphical representation of the proportions of saturated and unsaturated fatty acids of *E. nigrum* biomass cultured at different salinity, pH and temperature ( $\pm$  s.d.) / Representación gráfica de la proporción de ácidos grasos saturados e insaturados en la biomasa *E. nigrum* cultivado a diferentes salinidades, pH y temperatura ( $\pm$  d.e.)**

**Table 1. Percentages of extracted sterols from the biomass of *E. nigrum* cultivated in culture media at different salinity, pH and temperature / Porcentajes de esteroides extraídos desde la biomasa de *E. nigrum* cultivados en medios de cultivo con diferentes salinidad, pH y temperatura**

			Percentages of sterols (Fig. 7)									
Culture Mediums		T (°C)	a	b	c	d	e	f	g	h	i	j
pH 4	M. C. freshwater	6	100	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
		25	18.1	65.1	2.4	1	3.8	2.2	2.6	4	N.D.	N.D.
	M. C. seawater	6	38.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	61.2	N.D.
		25	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
pH 7	M. C. freshwater	6	56.9	43.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
		25	100	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	M. C. seawater	6	65.5	34.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
		25	100	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
pH 10	M. C. freshwater	6	43.7	37.3	7.9	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	11.1
		25	32.8	52.1	5.3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	9.8
	M. C. seawater	6	100	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
		25	100	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

in freshwater culture medium being more diverse *i.e.*, 8 different types of sterols were detected, as opposed to what was observed in the seawater medium, where only up to 3 different types of sterols were observed ( $F_{(10,66)} = 0.01$ ,  $P > 0.05$ ). Similarly, the pH generated significant differences in the content and diversity of the sterols ( $F_{(11,77)} = 0.016$ ,  $P > 0.05$ ).

In freshwater, normal pH at 25°C, the presence of the sterols 9(11)-dehydroergosterol (57%) and ergosterol (43%) were principally detected. This proportion was repeated in the seawater cultures with normal pH at 25°C, with a slight proportional increase of 9(11)-dehydroergosterol. After lowering temperature (6°C) in the cultures with both salinities, 9(11)-dehydroergosterol was only detected (Table 1).

At the basic pH (pH 10) no differences were observed between the cultures at the different temperatures (6 and 25°C), however, differences were observed between the cultures prepared in freshwater and seawater medium. In freshwater medium, at both temperatures the sterols observed were: 9(11)-dehydroergosterol; ergosterol; ergosta-5,7,22-trien-3-ol, acetate (3 $\beta$ ,22E) and pregn-1,4,6-triene-3,20-dione,6,16-dimethyl. The 9(11)-dehydroergosterol remains predominant at 25°C and the ergosterol is predominant at 6°C. In contrast, in seawater medium at the same pH, in the cultures at 6 and 25°C, only 9(11)-dehydroergosterol (Table 1) was detected.

In the biomass cultivated at pH acidic (pH 4), a different behavior in the proportion of sterols in relation to the change in temperature and salinity was observed. In seawater at 6°C no sterols were detected, and at 25°C the presence of 39% of 9(11)-dehydroergosterol and 61% of the stigmast-4-en-3-one were detected. The latter not detected in any other culture medium. In freshwater at 6°C only the 9(11)-dehydroergosterol was detected, in contrast to 25°C the proportions of sterols was different with ergosterol (65%) predominating, followed by proportions of 9(11)-dehydroergosterol and in smaller amounts ergosta-5,7,22-trien-3-ol,acetate (3 $\beta$ ,22E). In addition, other sterols undetected the previous cultures were found, such as: anthiaergostan-5,7,9,16,22-penten; 3 $\beta$ ,5-cyclo-5 $\beta$ -ergosta-6,8(14),22t-triene; neoergosterol; (3 $\beta$ ,22E) ergosta-7,22-dien-3-ol and ergosta-4,6,8(14),22-tetraen-3-one (Table 1).

In the *E. nigrum* cultures the temperature was the determining factor in the production of sterols. Significant differences were observed at higher temperature ( $F_{(12,23)} = 0.007$   $P > 0.05$ ), where the following were detected: anthiaergostan-5,7,9,16,22-penten; 3 $\beta$ ,5-Cyclo-5 $\beta$ -

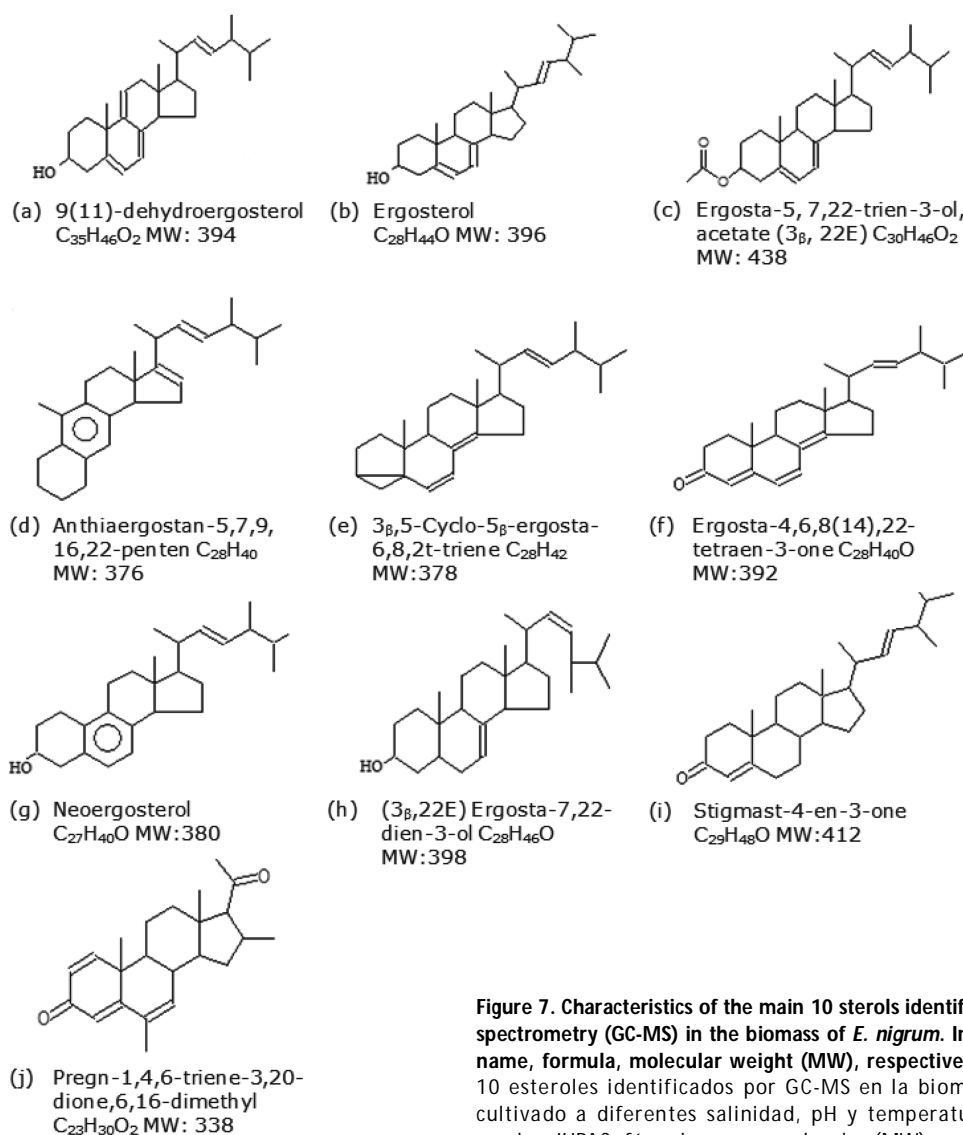
ergosta-6,8(14), 22t-triene; neoergosterol; (3 $\beta$ ,22E) ergosta-7,22-dien-3-ol, and ergosta-4,6,8(14), 22-tetraen-3-one and stigmast-4-en-3-one (Fig. 7).

## DISCUSSION

In the present study, we observed variations in the composition of fatty acids and sterols of *E. nigrum* biomass cultivated under different conditions of salinity, temperature and pH. *E. nigrum* was shown to grow under the different conditions to which it was exposed, demonstrating adequate cell function (Fig. 2). In this study, the most abundant fatty acids were 16:0, 16:1, 18:0, 18:1 and 18:2 which represent approximately 88% of the total fatty acid content. These results are similar to the findings by Stahl & Klug (1996), who determined that the aforementioned fatty acids were the most common and abundant, constituting 95% of the total after analyzing 100 strains of filamentous fungi, including Oomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and Mycelium Esterilia. He also found a higher proportion of linoleic acid (18:2), followed by oleic acid (C18:1), with both of these unsaturated fatty acids accounting for approximately 54% of the total amount of fatty acids when normal conditions were used. However, we observed that after changing the culture conditions, the growth and development of *E. nigrum* was significantly altered, resulting in a modified fatty acid composition, which has not been previously reported.

The different culture treatments showed that the proportion of unsaturated fatty acids was significantly greater than saturated (Fig. 6), with percentages ranging between 67 to 83%, except in the seawater medium with pH 4, in which the proportion of saturated fatty acids corresponded to 57% at low temperature (6°C) and 47% at high temperature (25°C). This is explained by the condition of stress to maintain the osmotic conditions of the cell. Experimentally, it was observed that cultures in acid medium showed a reduced mycelial growth. This variation is attributable to increased synthesis of saturated fatty acids (*i.e.*, 16C:0 and 18C:0) that increase the rigidity of the cell membrane and make it less fluid, in order to maintain proper cell function during conditions of stress (Lösel 1990).

With respect to salinity, biomass cultured in fresh and sea water had significant differences in the content of fatty acids C16:0; C18:2; C18:3. Therefore, it is possible to assume that a single species of fungus can produce different fatty acids depending on the salinity of the



**Figure 7. Characteristics of the main 10 sterols identified by GC-Mass spectrometry (GC-MS) in the biomass of *E. nigrum*. Indicated: IUPAC name, formula, molecular weight (MW), respectively / Principales 10 esteroides identificados por GC-MS en la biomasa *E. nigrum* cultivado a diferentes salinidad, pH y temperatura. Se indica: nombre IUPAC, fórmula y peso molecular (MW), respectivamente**

medium, based on the adaptability to change its cell membrane. These results are consistent with the arguments raised by Swan & Watson (1997) in relation to the response of filamentous fungi to changes in the salinity of the medium, and to what was proposed by Turk *et al.* (2004) for halotolerance yeasts. In both cases, an increase in salinity to of medium involves an enrichment in C18:2. These alterations in the composition of fatty acids allow the plasma membrane to maintain its fluidity in a broad range of salinities, which is essential for normal cell function, since the physical properties of the lipid matrix of the membrane depends on the

composition of the fatty acids involved (Quinn 1981, Mysyakina *et al.* 2012). These studies were conducted under normal pH conditions in which a noticeable increase in C18:2 was observed in relation to the increase in salinity. However, increasing or decreasing the pH dramatically resulted in a decrease of C18:2 and an increase in C16:0 and C16:1, which does not coincide with work performed with yeast halotolerants in relation to an increase in the salinity (Hosono 1992) and leaves open questions pertaining to the metabolic response of these microorganisms under extreme changes in the pH of the medium.

In relation to pH, this variable has a significant effect on the composition of the fatty acids differences that were found in C16:0, C16:1, C18:2; C18:3. Therefore, in the same species of fungus, different fatty acids could be produced in response to environmental stress generated by a change in pH levels (Figs. 3, 4 and 5). A different response was obtained when the fatty acids increased for C16:0 and C16:1, and the decline of C18:2, in the cultures grown at pH 4. This may be related to alterations in the composition of the cell membrane that allows it to maintain a normal and stable cell function in culture medium conditions (Hazel & Williams 1990). Another explanation raised by Rolling (2003) where changes can occur in the enzymatic synthesis of filamentous fungi, is through the regulation of gene expression (gen *pac1*, among others) of these enzymes via variations in extracellular pH.

Authors have documented that temperature has a direct effect on the composition of fatty acids, with the majority of fungi showing increases in the synthesis of unsaturated molecules at low temperatures (Mysyakina *et al.* 2012). In this study no significant differences were observed between saturated and unsaturated fatty acids from the analysis of the total percentage of these molecules in the cultures of *E. nigrum* conducted at 6°C and 25°C. However, the temperature was a determining factor in the production of sterols since significant differences were observed at a higher temperature, noting the presence of sterols: anthiaergostan-5,7,9,16,22-penten; 3 $\beta$ ,5-cyclo-5 $\beta$ -ergosta-6,8(14),22t-triene; neoergosterol; (3 $\beta$ ,22E) ergosta-7,22-dien-3-ol, ergosta-4,6,8(14), 22-tetraen-3-one and stigmast-4-en-3-one (Table 1). This is important when it is required to produce a special type of sterol.

In relation to the steroidal composition, according to Parks (1978) the content of sterols in fungi depends on cultivation conditions. It was observed that the strain *E. nigrum* presented significant differences in the amount of sterols depending on the salinity of the culture medium. Sterols were the most diverse in freshwater, where 10 different types of sterols were detected in contrast to what was observed in the seawater medium where up to 3 different types of sterols were observed (Table 1).

Similarly, the pH generated significant differences in both the content and diversity of sterols (Table 2). This concurs with the arguments raised by Horvath *et al.* (1998), who proposed that the microorganisms would be capable of osmoregulation by modifying the structure of their cell membrane by altering the sterol composition.

The conditions of slower growth of mycelia of the *E. nigrum* strain was determined by the acidic pH, which in addition to the diversity of sterols observed in cultures of acid medium it could also be a protective function. These changes in the composition of sterols in the membrane allow for maintaining the high fluidity of the plasma membrane in a wide range of environmental stressors, which is essential for the normal functions of the cell (Hazel & Williams 1990).

It can be concluded that the strain of *E. nigrum* isolated from the Patagonian fjords is an appropriate model of research in the analysis of the fluctuations of the steroid and lipid synthesis resulting from the changes produced by the different culture conditions; considering this study as a starting point to understand the adaptive response of fungi that survive appropriately to continuous environmental changes in transition zones between terrestrial and marine environments.

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