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ARTICLE

Marine fungoid producers of DHA, EPA and carotenoids from central and southern Chilean marine ecosystems

Fungoides marinos productores de DHA, EPA y carotenoides provenientes del ecosistema marino de Chile central y sur-austral

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Resumen.- La creciente demanda de ácidos grasos poliinsaturados (AGPI) provocada por la escasez de pesca extractiva, ha impulsado la obtención de fuentes alternativas sustentables para abastecer su mercado. Los Thraustochytridos y levaduras marinas (fungoides marinos), son potenciales productores de lípidos y carotenoides de importancia comercial. Desde una colección de 41 cepas de fungoides marinos aisladas en el Sistema de Corriente de Humboldt, se determinó la capacidad de producir DHA (ácido docosahexanoico), EPA (ácido eicosapentanoico) y CT (carotenoides totales) en cultivos líquidos comercial (MS) y alternativos (PDB, GAM, BAM y MCM). Los resultados indican que el medio MS y PDB, presentaron las mayores velocidades de crecimiento (0,02 h^{-1}). No obstante, el medio MS presenta estos parámetros cinéticos a 16ºC y el PDB a 37°C. Trece de las cepas analizadas presentaron una alta capacidad para producir DHA (hasta 23% peso seco) y CT (hasta 18% peso seco), comparables a los niveles observados en *Schizochytrium* sp. KH105 y *Rhodosporidium toruloides*. Adicionalmente, todas las cepas ensayadas también produjeron pequeñas cantidades de EPA (hasta 0,3% peso seco). La microscopia electrónica revela que la cepa C36 es morfológicamente consistente con levaduras y su secuencia parcial del gen ribosomal 18s presenta un 97% de similitud con el género *Rhodotorula*, el que hasta ahora no ha sido reportado como productor de DHA y EPA. Finalmente, las cepas C36, C22 y C4 son interesantes objetos de estudio para escalar su producción y proyectar su uso comercial, como por ejemplo para suplementar en omega-3 y carotenoides alimentos para consumo humano, animales de corral o para estadios larvales de peces cultivados y pigmentar la carne del salmón, entre otros.

Palabras clave: Fungoides marinos, DHA, EPA, carotenoides totales, *Rhodotorula*, parámetros cinéticos

Abstract.- Declining fishing yields have pushed the search for sustainable alternative sources for polyunsaturated fatty acids (PUFAs). Thraustochytrids and marine yeasts (marine fungoid protists) are potential commercial sources of lipids and carotenoids. It was determined the capacity of a collection of 41 strains of marine fungoid isolated in the Humboldt Current System, to produce DHA (docosahexanoic acid), EPA (eicosapentanoic acid) and CT (total carotenoids) in commercial growing media (MS) and alternative growing mediums (PDB, GAM, BAM and MCM). The media MS and PDB exhibited the highest growth rate (0.02 h^{-1}), at 16 and 37°C, respectively. Thirteen of the studied strains showed high capacity to produce DHA (up to 23% dry weight) and CT (up to 18% dry weight), comparable to levels observed in *Schizochytrium* sp. KH105 and *Rhodosporidium toruloides*. Additionally, all studied strains produce small amounts of EPA (up to 0.3% of dry weight). Scanning electron microscopy reveals that strain C36 is morphologically consistent with yeasts, while partial sequencing of the 18s ribosomal gene shows 97% similarity to the genus *Rhodotorula*, which has not been reported until now as a producer of DHA and EPA. Finally, the strains C36, C22 and C4 offer promising potential for upscaling their production for commercial use for enriching human food and animal and larval fish feed with omega-3 and carotenoids, as well as being a source for food dyes for salmon and other products.

Key words: Marine fungoid protist, DHA, EPA, total carotenoids, *Rhodotorula*, kinetic parameters

INTRODUCTION

The main commercial source of omega 3 long-chain polyunsaturated fatty acids (PUFA) is fish oil obtained through industrial-scale fisheries (Jackson & Shepherd 2012). PUFAs are a major input in the production of feed for the fish farming industry. In the last two decades their cost has increase from US$300 to US$2300/ton owing to

diminishing fishing catches because of overfishing and due to increased demand for PUFAs as nutritional supplements (Valenzuela *et al.* 2012).

As well, the temporal variability of fishery products, for example as a result of the events 'El Niño' and contamination by heavy metals, dioxins and polychloride biphenyls (PCBs) (Vicente *et al.* 2008) undermine the sustainability of fishery production. This has led to the search for new raw materials, such as oils from plants and microorganisms, to minimize the impact of fluctuations in the availability of marine resources.

In search for sustainable PUFA sources, marine microorganisms of the Thraustochytriaceae family have been isolated (Miller *et al.* 2007). The classic technique to isolate these microorganisms is the use of pine pollen as bait (Raghukumar & Gaertner 1980). Through these techniques these types of microorganisms have been quantified in the world's oceans (Scheckenbach *et al.* 2010), indicating their importance in microbial carbon flows in marine environments (Raghukumar 2004).

The pollen bait technique has been used to isolate strains in the Humboldt Current System, with a collection of 129 strains, stored at the Marine Biotechnology Unit of the Universidad de Concepción that produce omega-3 polyunsaturated fatty acids (PUFAs), omega-3, docosahexanoic acid (DHA) and eicosapentanoic acid (EPA), and carotenoid pigments.

The objective of the present work was to identify and characterize marine fungoid strains in the collection that produce DHA, EPA and total carotenoids (CT). The optimal culturing conditions, substrate and temperature for the strains were determined in terms of production of the aforementioned substances. As well, the gene 18s rRNA was partially sequenced.

MATERIALS AND METHODS

BIOLOGICAL MATERIAL

Forty-one strains of marine fungoid were used from the collection of the Marine Biotechnology Unit of the Universidad de Concepción, Chile. The strains were isolated from coastal waters of the Biobío and Carlos Ibáñez del Campo Regions (Fig. 1). All the strains were isolated from seawater according to the method of Gaertner (1966) for selective isolation of Thraustochytrids using pine pollen as bait. Isolation consists of observing by optical microscopic the adherence of marine fungoid to pine pollen in a liquid culture medium and transferring bait with positive adherence until obtaining a pure culture. The isolated clones were transferred to solid Sabouraud medium for storing and subsequent analysis.

EXTRACTION OF POLYUNSATURATED FATTY ACIDS (PUFA)

The strains were multiplied in solid Sabouraud medium to obtain sufficient material for PUFA analysis. Fatty acids were extracted from 100 mg of biomass in the medium by saponification reaction with 1mL de NaOH at 0.5M in 96% of ethanol and homogenized with Ultra Turrax for 1 min. After cell rupture, the strains were centrifuged at 7000 x g for 5 min. and the pellet discarded. One mL of HCl at 0.6 N and 3 mL of ethyl acetate were added to the supernatant, vortex agitated and incubated for 30 min at ambient temperature. The treated samples were dried by nitrogen current ($N_{2\ (g)}$) to eliminate the organic solvent, then lyophilized to eliminate remaining water and stored at -20°C for subsequent chromatographic analysis.

FATTY ACID ANALYSIS

Fatty acids were analyzed in a HPLC Hitachi (model L-2000), using a UV detector (Hitachi, model L-2400), a gradient pump (Hitachi, model L-2100/2130) and a 15cm-x-4.6-mm LC-18 Supelco® column. A gradient A with 25% of acetonitrile (ACN) and 75% of Milli-Q purified water and a gradient B with pure ACN with 0.12% of acetic acid were used. The mobile phase used was 100% of A with a flow of 1 mL min^{-1} at 50% of B with a flow of 2 mL min^{-1} for 15 min, followed by a flow at 1 mL min^{-1} for 30 min. The samples were read at 195 nm and standards (Sigma-Aldrich®) with an injection volume of 10 µL to identify docosahexanoic and eicosapentanoic acids (Li *et al.* 2001). Based on the results, the strains with the highest quantities of DHA and/or EPA were selected.

DETERMINATION OF TOTAL CAROTENOIDS

Samples of 50 mg in biomass were drawn from the strains under study; to which 1000 µL of homogenized acetone was added with Ultra Turrax for 1 min. Samples were then incubated for 10 min at 37°C and then centrifuged at 11,000 x g for 10 min. The supernatant was extracted for spectrophotometric analysis.

The extracts obtained were analyzed by the method described by Rodher (1966). A potassium dichromate calibration curve was made considering 0.036% of potassium dichromate equivalent to 0.0026% mg mL^{-1} of total carotenoids (TC). The analysis was carried out in 450-nm glass cuvettes.

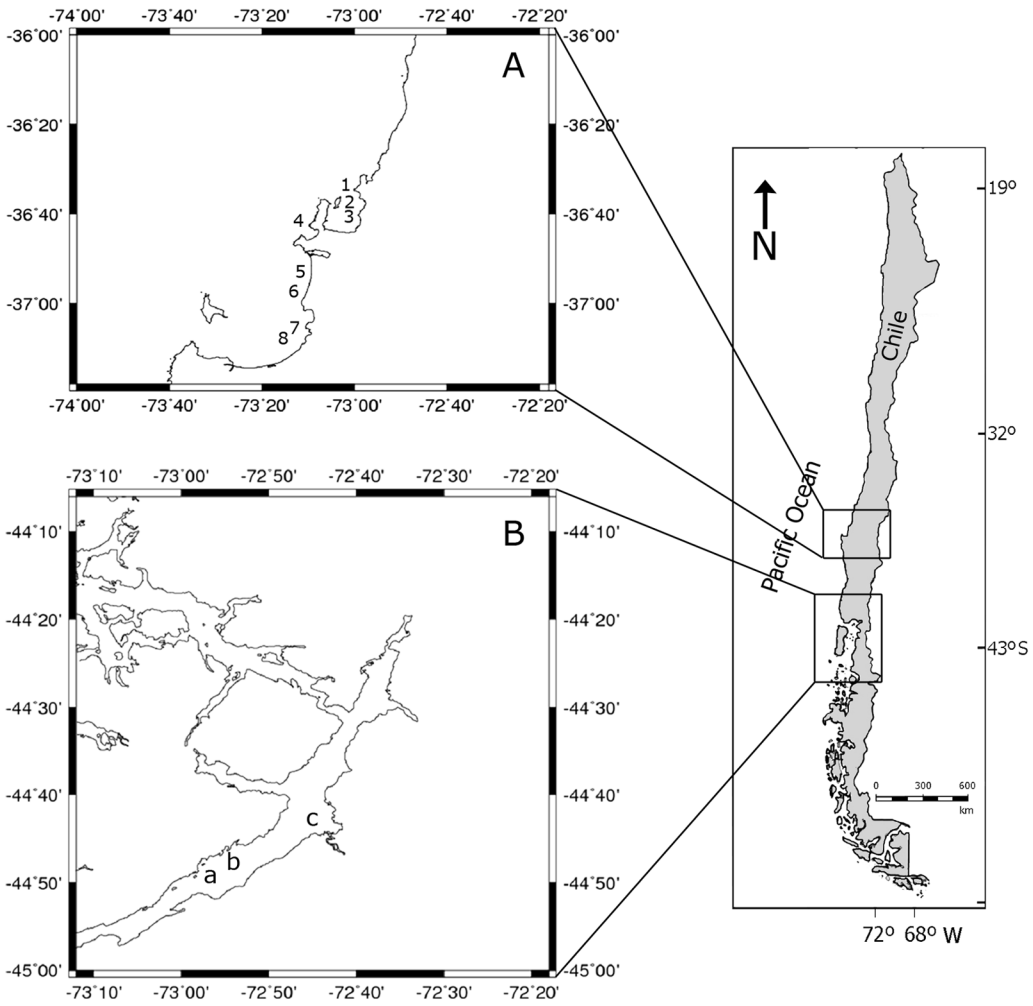

Figure 1. Geographic origin of the 41 strains of marine fungoid used in this study. A) Central Chile, Biobío Region: 1) Playa Dichato, 2) Playa Bellavista, 3) Playa Penco, 4) Playa Lenga 5) Caleta Maule, 6) Playa Blanca, 7) Playa Colcura and 8) Playa Laraquete. B) Austral-Southern Chile: sampling stations denominated a) E1, b) E18 and c) E22 in Canal Puyuhuapi; Carlos Ibáñez del Campo Region / Origen geográfico de las 41 cepas de fungoides marinos utilizadas en este estudio. A) Chile Central, Región del Biobío: 1) Playa Dichato, 2) Playa Bellavista, 3) Playa Penco, 4) Playa Lenga, 5) Caleta Maule, 6) Playa Blanca, 7) Playa Colcura y 8) Playa Laraquete. B) Chile Sur-Austral: estaciones de muestreo denominadas a) E1, b) E18 y c) E22, en el Canal Puyuhuapi, Región Carlos Ibáñez del Campo

PUFAs and total carotenoids were initially determined from the strains cultivated in solid media as a pre-quantification and pre-selection of potential producers of these substances. The selected strains were used in assays for quantification in liquid media.

Culture media

The biomass production of the selected strains was assessed in commercial and alternative liquid culture media at different temperatures. The media used were: green algae-based (GAM), brown algae-based (BAM), *Mazzaella laminaroides*-based (MCM), Sabouraud (MS) and potato broth supplemented with dextrose (PDB). Before evaluating the cultures, 10-mL of inoculum of each strain was developed and incubated in liquid glucose, yeast and peptone medium for 3 days at ambient temperature at 120 x *g*. Each medium was evaluated at three temperatures: 16, 25 and 37°C taking one 10 µL of inoculum. These growths were cultured for 10 days at 120 x *g* in 100-mL flasks.

Preparation of culture media

Algae based media

Fronds of *Ulva* sp., *Porphyra* and *Mazzaella laminaroides* were collected for the green alga media (GAM), brown alga media (BAM) and *Mazzaella laminaroides* media (MCM), respectively. All the algae were obtained from rocky intertidal pools on the coast in the Biobío Region, Chile during low tide, taking care to avoid adhesion of epiphytes to the fronds. The fronds were stored and transported to the laboratory, where they were submerged in seawater to remove stones, sand and adhering epiphytes, with 2 or 3 repetitions of this step. The fronds were cooked in a pressure pot for 15 to 20 min in seawater sieved at 100 µm. The alga was then triturated and transferred to 100 mL Schott flasks sieved at a 100 µm, maintaining pH at 7, and then autoclaved at 120°C for 15 min.

Sabouraud medium (MS)

This medium 700 mL was prepared with commercial reactive formulated with dextrose in the following quantities: 21 g of dextrose, 7 g of peptone and 7 g of Instant Ocean sea salt at pH 7. The mixture was dissolved by mild agitation and then autoclaved at 120°C for 15 min.

Potato and dextrose broth (PDB)

To prepare 700 mL of the medium, 400 g of potato were weighed, cleaned thoroughly to avoid leaving all remaining earth and then cooked until completely soft, but without breaking. The broth was transferred to 1000 mL Schott flasks, 14 g of dextrose at pH 7 was added and the mixture was autoclaved at 120°C for 15 min.

Once all the media had been autoclaved and allowed to cool, 200 µL of antibiotics were added (1000 U/mL of penicillin and 1000 U/mL of streptomycin).

Biomass production

To determine biomass production cells were quantified with a hemocytometer from 10 µL aliquots taken every 12 h from the culture media. The growth kinetics of the biomass or specific growth velocity (maximum µ) was then determined according to the following formula:

$$\mu\, max\ (h^{-1}) = \frac{\Delta\ biomass}{\Delta\ time\ (h)} = \frac{(Ln\ x - Ln\ x_0)}{\Delta t} = \frac{Ln\ 2}{td}$$

where, x is biomass production reached in a determined time, Δt is the cultivation time, and x_0 is initial biomass production.

Once cellular growth was completed, total biomass production in dry weight was determined. The samples were washed with distilled water, centrifuged at 3600 x *g* for 20 min and lyophilized. The differences in production among the media were statistically analyzed with the Kruskal-Wallis test using XLSTAT2013 software.

Fatty acid and total carotenoid production

The levels of biomass productions of cultures in 100 mL flasks were determined in relation to the assessed culture media. Fatty acid and total carotenoid production were then determined in relation to the optimal growth conditions. The cultures were prepared in 500 mL matrices with agitation at 120 x *g* for 10 days, taking 5 mL aliquots every 24 h. The methodologies described above were followed to determine fatty acid and total carotenoid levels.

Marine fungoid identification - DNA extraction

The DNA of the marine basidiomycetes was extracted with the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories Inc.) following the supplier's instructions. The extracted DNA was quantified with a NanoDrop 2000® spectrophotometer (ThermoScientific), as well as its purity was determined using 260/280 nm ratio, where 50 µg mL^{-1} was the conversion factor for DNA. Once purified and quantified, the DNA was stored at -20°C until used.

PCR amplification of the 18S ribosomal gene

A polymerase chain reaction (PCR) was used to amplify the 18s rRNA gene. The primers used to identify the marine fungoid were 18S1 and 18S12 (Honda *et al.* 1999). The reaction mixture for the primers contained the following reactives: 5X buffer, 0,2 mM of a mixture of dNTPs, 2 mM of $MgCl_2$, 1 U of Go-taq polymerase, 0.5 µM of each primer and approximately 1.5 µL de DNA in a final volume of 25 µL. The amplification conditions were: initial denaturation at 95°C for 5 min, followed by 35 amplification cycles. Each cycle consisted of denaturation at 95°C for 30 s, alignment for 30 s at 55ºC and extension at 72°C for 1 min, with a final extension step of 72°C for 10 min. The PCR products were visualized by electrophoresis in agarose gel at 2%. The bands were visualized by gel staining with ethidium bromide (0.5 µg mL^{-1}) and exposure with a UV transilluminator.

Purification, cloning and extraction of plasmids

Having confirmed the expected size (>1500 pb) of the bands by visualization, the bands were cut away from the gel and purified using the E.Z.N.A® Gel Purification Kit, following the supplier's instructions. The purified PCR product was ligated to the pGEM®T-Easy vector (Promega) using a vector ratio of 3:1 in a final reaction volume of 10 µL and then incubated overnight at 4ºC.

Bacterial transformation was done with a vial of competent *Escherichia coli* cells (JM109® Promega) and the ligation product (recombinant plasmid), following the thermal shock methodology. The transformed bacteria were seeded in Petri dishes with LB agar and ampicillin (50 μgmL^{-1}), IPTG (0.5 mM) and α-galactosidase (50 mg mL^{-1}), which allowed for identifying the bacteria in the recombinant plasmid. The seeded plates were left to grow overnight at 37ºC. The positive clones were confirmed by conventional PCR in colonies and the bands were viewed by ethidium bromide staining with and exposure in a UV transilluminator. The positive colonies were grown in liquid LB medium with ampicillin, and the DNA plasmid was purified with the commercial kit commercial Plasmid Miniprep kit II E.Z.N.A.®, following the supplier's instructions.

Plasmid DNA sequencing and phylogenetic molecular analysis

The purified plasmids containing the cloned segment were sequenced by MACROGEN (South Korea). The sequences were used to identify the microorganisms by comparing them to sequences available in the GenBank Database[1]. The BLAST program was used to search for the closest homologue sequences (Altschul *et al.* 1990) available at the NCBI website. The data was then processed with the MEGA 6 (Tamura *et al.* 2013) and Geneious version 6.0.3 programs (Drummond *et al.* 2011).

The sequences obtained from the studied strains were entered to the Genbank database with the following access numbers: KJ530974 (C6), KJ530975 (16CC1), KJ530976 (C36), KJ530977 (C3), KJ530978 (C30), KJ530979 (C46), KJ530980 (C51), KJ530981 (C24), KJ530982 (P39) and KJ530983 (C4).

Scanning electron microscope

The micrographs from the scanning electron microscope were taken at the Spectroscopy and Microscopy Center of the Universidad de Concepción with the following procedure: strain C36 was placed on a Peltier heating and cooling pad for 2 h at 35°C and then fixed with glutaraldehyde and osmium tetroxide, washed, dehydrated in a series of concentrations of acetone, including polymerized in resin. Finally, the preparations were cutted with an ultramicrotome. Subsequently the samples were covered with a 100 Å thick layers of platinum from an injecting apparatus and observed with a scanning electron microscope (Morris *et al.*1997).

Results

Identification of fatty acid and carotenoid producing strains

Of the 41 strains of marine fungoid cultivated in solid media, the analysis for polyunsaturated fatty acids showed that 8 had high concentrations of DHA in the range of 0.5 to 5.6% of total dry weight biomass (Table 1).

Table 1. Concentration of docosahexaenoic acid (DHA) and total carotenoids (CT) in strains of marine fungoid isolated from different geographic areas grown in sabouraud solid media and selected as potential producers of these substances / Concentración de ácido docosahexanoico (DHA) y carotenoides totales (CT), en cepas de fungoides marinos aislados desde distintas áreas geográficas utilizando medio sólido sabouraud y seleccionados como potenciales productores de estas substancias

Samples	Area	% DHA in dry weight	% CT in dry weight
16CC1	Penco	Nd	0.56
C4	Caleta Maule	0.32	0.57
P39	PYPI-22-50m	Nd	1.11
C24	Caleta Maule	0.71	0.78
C46	Dichato	0.55	0.87
C51	Dichato	Nd	1.16
C6	Caleta Maule	0.68	Nd
C14	Caleta Maule	1.39	Nd
C22	Caleta Maule	0.66	Nd
C3	Caleta Maule	5.63	Nd
C30	Caleta Maule	0.84	Nd
O51	Laraquete	0.83	0.36
C36	Caleta Maule	Nd	0.77

Nd: not detectable

[1]National Center for Biotechnology Information, USA. NCBI. <http://www.ncbi.com>

The total carotenoid analysis indicated that 7 strains had concentrations in the range of 0.5 to 1.1% of CT in dry weight (Table 1). Based on these results, the 13 strains considered in Table 1 were subsequently cultivated in liquid media. The other strains that presented production levels of less than 0.01% of DHA and 0.05% of total carotenoids were not considered further.

Optimal culture conditions and biomass production in liquid media

Biomass production was low (Fig. 2) with the alternative alga-based culture media used in this study (GAM, BAM and MCM). Moreover, these media were not assayed with some strains until the end of the experiment (Fig. 3) because of the absence of any growth. The PDB medium was the only alternative media in which there was significant cellular growth ($P < 0.05$), reaching high cellular densities at 37ºC (Figs. 2 and 3).

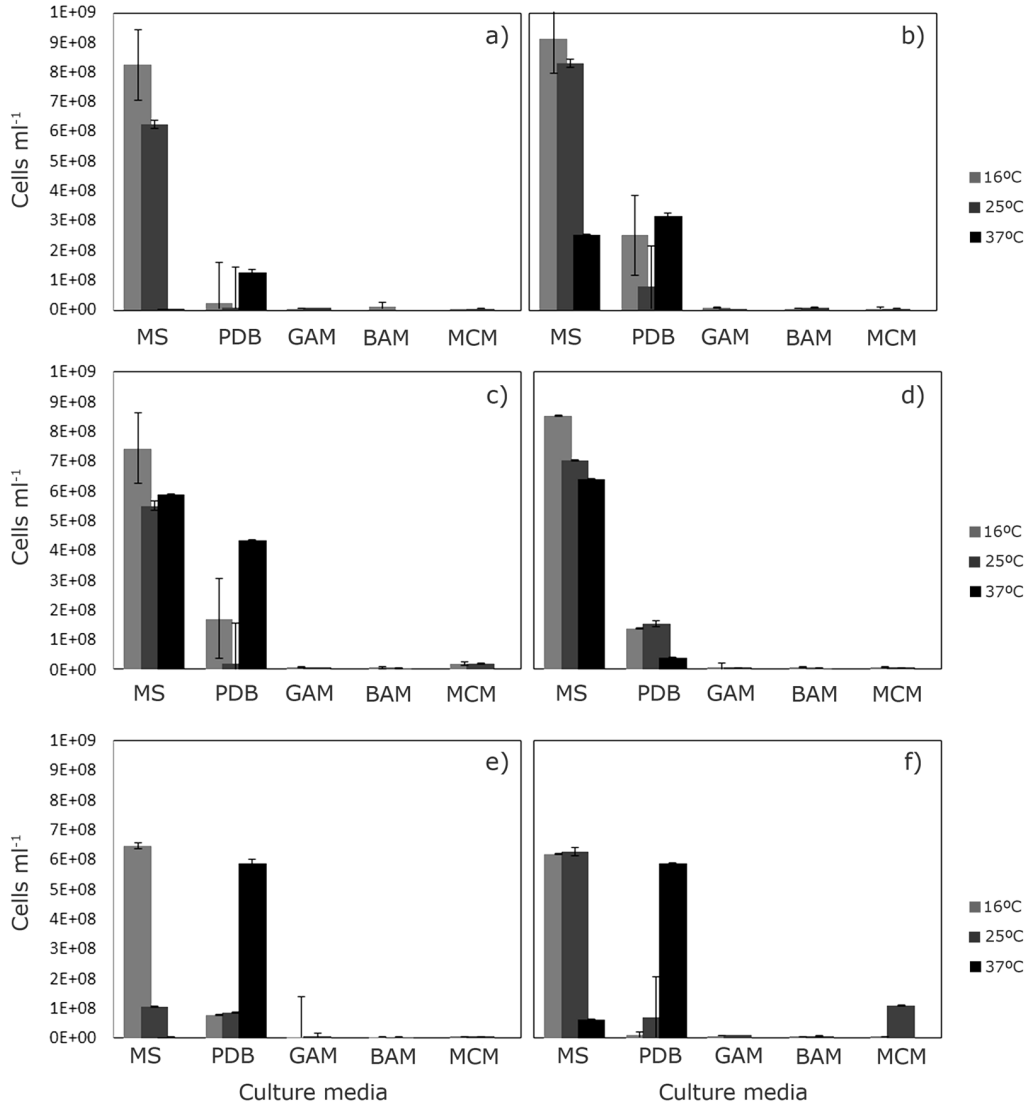

Figure 2. Maximum concentration of cells mL^{-1} in different culture media: Sabouraud medium (MS), potato broth supplemented with dextrose (PDB), green alga-based medium (GAM), brown alga-based medium (BAM) and *Mazzaella laminaroides*-based medium (MCM), at 3 different temperatures. Strains evaluated: a) 16CC1, b) C4, c) P39, d) C24, e) C46 and f) C51 / Concentración máxima de células mL^{-1}, en diferentes medios de cultivo: Medio Sabouraud (MS), Caldo de papa suplementado con dextrosa (PDB), Medio a base de algas verdes (GAM), Medio a base de algas pardas (BAM) y Medio a base de *Mazzaella laminaroides* (MCM), a 3 diferentes temperaturas. Cepas evaluadas: a) 16CC1, b) C4, c) P39, d) C24, e) C46 y f) C51

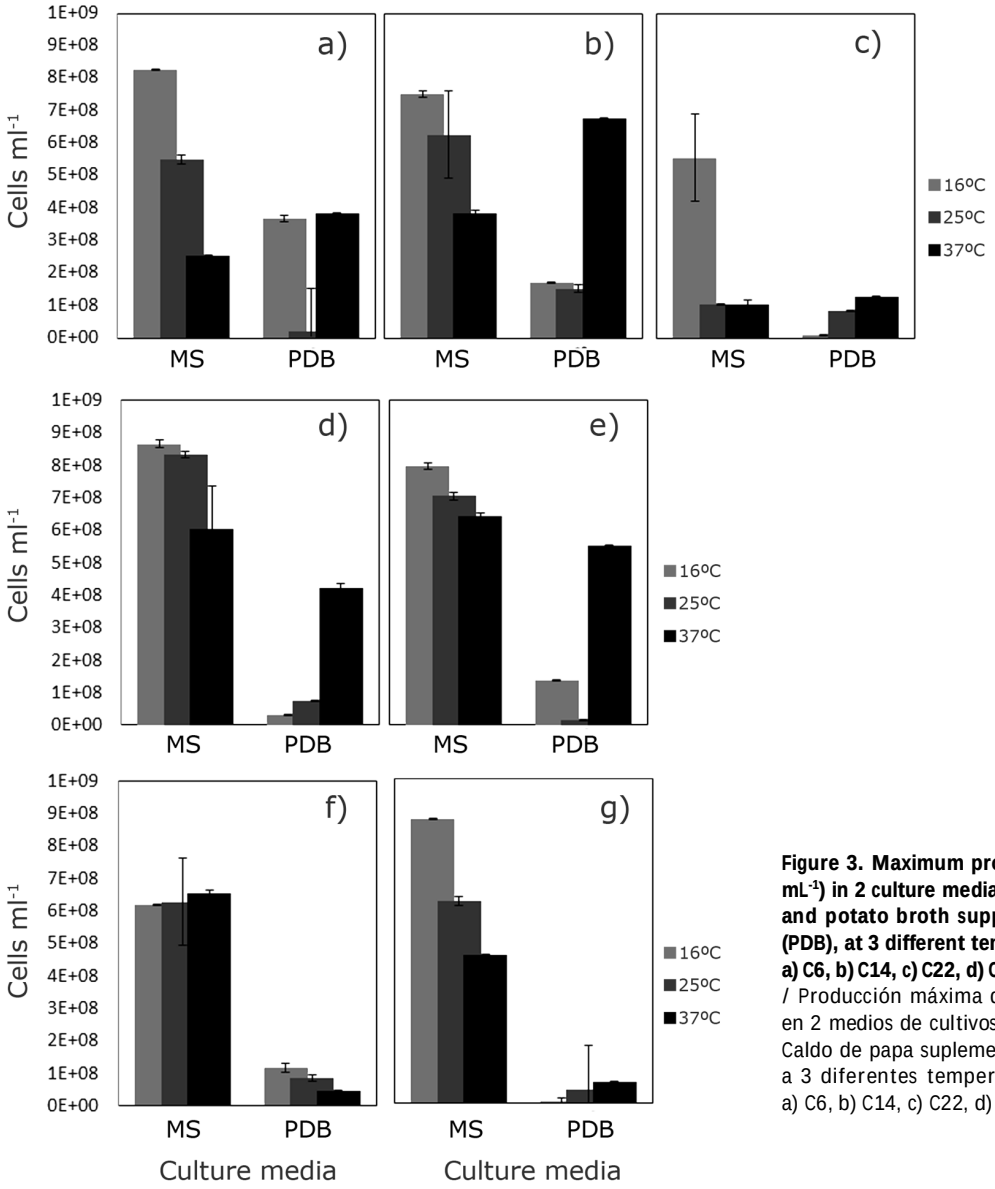

Figure 3. Maximum production of biomass (cells mL^{-1}) in 2 culture media: Sabouraud medium (MS) and potato broth supplemented with dextrose (PDB), at 3 different temperatures. Strains tested: a) C6, b) C14, c) C22, d) C3, e) C30, f) O51 and g) C36 / Producción máxima de biomasa (células mL^{-1}), en 2 medios de cultivos: Medio Sabouraud (MS) y Caldo de papa suplementado con dextrosa (PDB), a 3 diferentes temperaturas. Cepas evaluadas: a) C6, b) C14, c) C22, d) C3, e) C30, f) O51 y g) C36

The highest level of biomass production ($P < 0.05$) in the liquid media assays was obtained at 16ºC in Sabouraud liquid medium (MS), reaching cellular densities of 10^8 to 10^9 cells mL^{-1} (Figs. 2 and 3). The maximum growth velocities of the studied strains under the assay conditions were in the range of 0.02 h^{-1} to 0.03 h^{-1} with doubling times of 26 to 30 h (Table 2). Strain C4 (Fig. 2b) presented the highest total biomass production ($P < 0.05$), with a high maximum growth rate (Table 2).

Production of docosahexanoic (DHA) and eicosapentanoic acids (EPA) and total carotenoids (CT) under optimal liquid culture conditions

The cultures of the 13 selected strains cultivated at 16ºC in 500 mL of liquid MS medium presented a total dry biomass of 82 to 126 g L^{-1} of culture medium (Table 2). The dry biomass had a content of 1.1 to 23% of DHA, 0.03 to 0.1% of EPA and 4 to 18% of CT (Table 2).

Table 2. Production of eicosapentanoic acid (EPA), docosahexanoic acid (DHA) and total carotenoids (CT), of the 13 selected strains grown at 16°C, Sabouraud liquid medium in bioreactors in batches of 500 mL. Results are expressed as concentrations of the substances in g of dry weight of biomass and the percentage in g of such biomass. Maximum growth rates (μmax), grown in liquid Sabouraud medium at 16°C / Producción de ácido eicosapentanoico (EPA), ácido docosahexanoico (DHA) y carotenoides totales (CT), de las 13 cepas seleccionadas y cultivadas a 16°C, medio líquido Sabouraud en biorreactores en batch de 500 mL. Los resultados se expresan como concentración de las substancias por g de biomasa seca y su porcentaje en gramos para dicha biomasa. Tasas de crecimiento máxima (μmax), cultivadas en Medio líquido Sabouraud a 16°C

	Strains												
	C24	C46	C6	C14	C22	C3	C30	O51	C4	P39	C51	16CC1	C36
Total production (500-mL bioreactor)													
EPA (mg EPA g^{-1} biomass)	0.3	1.5	0.4	0.7	2.5	0.3	0.4	1	1	0.3	0.4	0.4	2
DHA (mg DHA g^{-1}) biomass	180	136	27	53	234	136	62	170	98	40	27	11	234
CT (mg total C. g^{-1} biomass)	59	101	83	59	123	59	125	59	179	41	45	134	106
% EPA in biomass in dry weight	0.03	0.1	0.04	0.07	0.3	0.03	0.04	0.1	0.1	0.03	0.04	0.04	0.2
% DHA in biomass in dry weight	18	14	3	5	23	14	6	17	10	4	3	1.1	23
% CT in biomass in dry weight	6	10	8	6	12	6	12	6	18	4	5	13	11
Biomass in dry weight (g L^{-1})	9.6	9.2	12.2	10.6	12.6	11.8	9.8	9.0	9.4	12.2	9.2	10.6	8.2
Growth parameters													
Maximum μ (h^{-1})	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.03	0.02	0.03

Strains C36 and C22 produce DHA in up to 23% of dry weight, which is significantly higher ($P < 0.05$) than the levels produced by the other strains (Table 2). Strain C4 produced the highest levels of CT ($P < 0.05$), reaching 18% of dry weight (Table 2). The 13 cultivated strains can produce eicosapentanoic acid (EPA), although in lower concentrations than the DHA produced (Table 2).

Molecular characterization of the thirteen marine fungoid strains selected as highly productive of DHA and CT

The selected 13 strains were positively amplified by conventional PCR with the primers described by Honda *et al*. (1999). This fragment was sequenced with an amplicon of 1500 pb.

The BLAST analysis of all the sequenced strains from the coasts of the Biobío and Carlos Ibáñez del Campo Regions had 91 to 97% genetic similarity to *Rhodotorula* and *Rhodosporidium*. They also shared 81% similarity with species of the Thraustochytriceae, thus ruling out that the strains are Thraustochytrids due to the low percentage of similarity to this family. Two clusters were observed in the phylogenetic tree (Fig. 4) of the sequenced strains and sequenced microorganisms of the aforementioned family. The strains C36, C24, C22, C3 and C30 are 97% related to *Rhodotorula* sp. and *Rhodosporidium diobovatum*, forming a robust cluster. The strains C6, 16CC1, C14, P39, C51, C46, C4 and O51 form another cluster related with 91% to *Rhodotorula* and *Rhodosporidium* and 81% similarity to the Thraustochytriceae family. The strains C3-C30 and C22-C24 are similar to each other, as are the strains C6-16CC1-C4. Both clusters are different from *Schizochytrium* sp. and *Prorocentrum micans*, the latter used as a reference.

Characterization of strain C36 by scanning electron microscope

Given the similarity of the molecular information for genera *Rhodotorula* and *Rhodosporidium*, scanning electron micrographs were obtained of the strain C36 cultivated in Sabouraud and Vishniac media (Vishniac 1956). The micrographs of the cultures in Sabouraud medium show cells approximately 3 to 5 μm in size, with a morphology like that of yeast, including some are in gemmation (Fig. 5a). The micrographs of cultures in Vishniac medium also show cells with yeast-like morphology and slightly larger than those in Sabouraud medium. An ectoplasmic network similar to that described for the Thraustochytride group can also be observed (Fig. 5b).

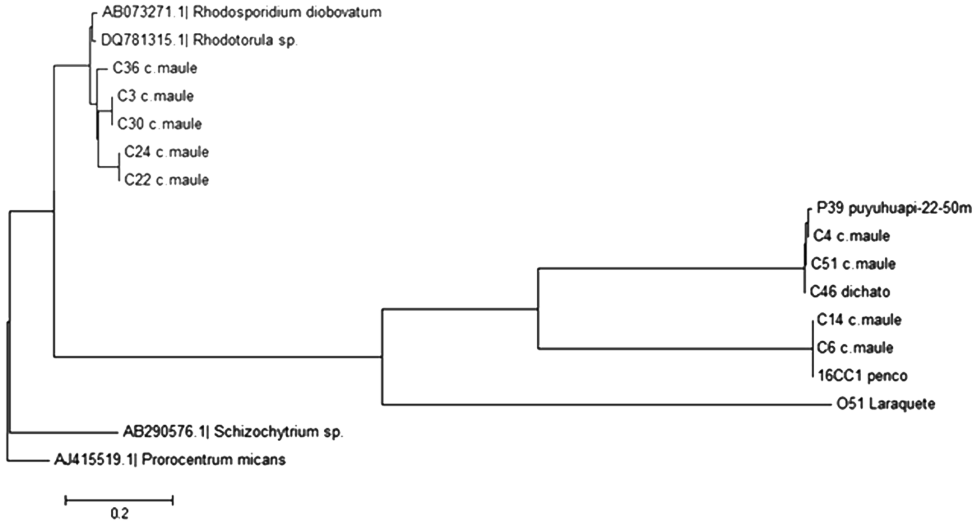

Figure 4. Phylogenetic tree of the partial sequence of 18S RNA gen of the 13 marine fungoid strains selected and isolated from the coast of the Biobío Region (C. Maule, Dichato, Laraquete and Penco) and Carlos Ibáñez del Campo (Canal Puyuhuapi, E22). The following species were used for comparisons: *Rhodotorula* sp., *Rhodosporodium diobovatum* and *Schizochytrium* sp., which represent the gender *Rhodotorula*, *Rhodosporidium* and family Thraustochytriaceae, respectively. *Prorocentrum micans*, was used as out group. The tree was constructed using the neighbor-joining method (NJ) and MEGA program 6 / Árbol filogenético basado en la secuencia parcial del gen 18S RNA de fungoides marinos obtenidos desde las costas de la Región del Biobío (C. Maule, Dichato, Laraquete y Penco) y Carlos Ibáñez del Campo (Canal Puyuhuapi, E22). Para comparar se utilizaron las especies *Rhodotorula* sp., *Rhodosporodium diobovatum* y *Schizochytrium* sp., representantes de los géneros *Rhodotorula*, *Rhodosporidium* y familia Thraustochytriaceae, respectivamente. *Prorocentrum micans*, fue utilizada como outgroup. El árbol fue construido mediante el método de Neighbor-joining (NJ) y programa MEGA 6

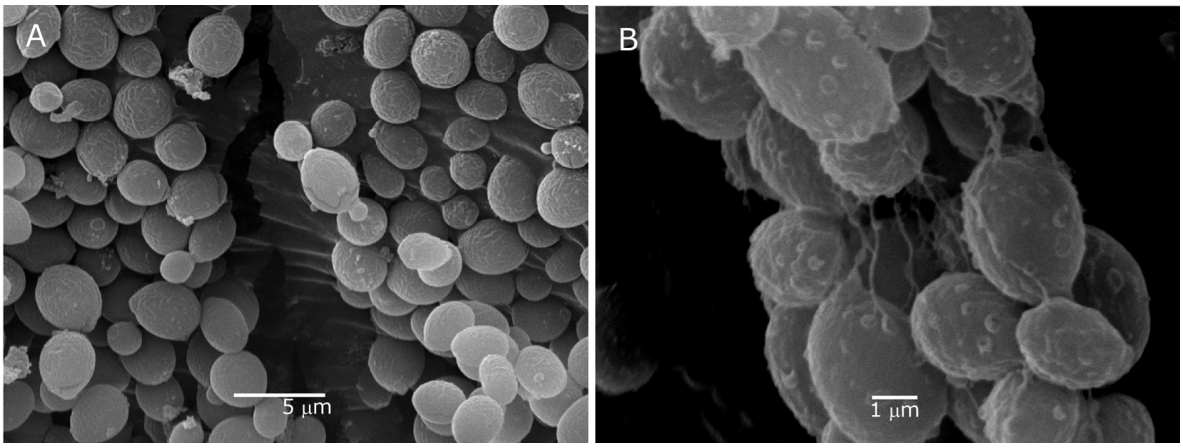

Figure 5. Scanning electron micrographs of strain C36. A) yeast-like cell structures approximately 3 to 5 µm long. Gems or sterigmata and B) ovoid cellular structures linked by an ectoplasmic-like net can be observed. Approximately 4-6 µm long / Micrografías electrónicas de barrido de la cepa C36. A) estructuras celulares levaduriformes de aproximadamente 3 a 5 µm de largo, con presencia de gemas o esterigmas y B) estructuras celulares ovoidales con presencia de una red superficial similar a una red ectoplasmática. Tamaño aproximado de 4 a 6 µm de largo

DISCUSSION

The pine pollen bait technique (Gaertner 1966) consists of directly isolating microorganisms from seawater that can be grown on pine pollen and that have morphology consistent with that of the Thraustochytrids group widely described through this technique (Bongiorni 2012). This technique was chosen because the microorganisms isolated with it are potential producers of substances of commercial interest such as DHA, EPA and carotenoids (Yamasaki *et al.* 2006) and allowed for constructing the strain collection used to investigate the presence of such substances. The results obtained from the solid media (SM) of the microorganisms of this collection show the existence of 13 DHA and CT producing strains from the Biobío and Carlos Ibáñez del Campo Regions. Eight of these strains produce DHA and 6 produce CT, varying in color from fuchsia (magenta), pink to orange, a characteristic of PUFA and carotenoids-producing marine fungoid (Pemán *et al.* 2007). Marine fungoid present high omega-3 PUFA concentrations and several strains can accumulate lipids up to 70% (Ratledge & Wynn 2002, Li *et al.* 2007). Species of the genera *Rhodotorula* and *Rhodosporidium* produce carotenoids at commercially interesting levels, with colonies that present reddish-orange and fuchsia coloring, which are similar to the colors of the studied strains. The intensity of the coloring varies depending on the strain owing to the presence of pigments such as astaxanthin (Andrews *et al.* 1976). However, these species have been isolated from terrestrial ecosystems and consequently no marine species has been described as highly productive of lipids or carotenoids. In the case of the Thraustochytriidae, high levels of PUFA production, such as DHA, have been observed, reaching 26% of dry weight biomass (Yamasaki *et al.* 2006). This establishes them as potentially sustainable and commercially important alternative for PUFA production, as are the strains studied in this work.

Given the biotechnological interest in these marine fungoid, it is necessary to optimize biomass production to obtain substances of commercial interest that they synthesize (DHA, EPA and CT). In this context, identifying liquid culture media alternative to commercial media could reduce production costs of these microorganisms for their upscaling to the industrial level (Quilodrán *et al.* 2010). The highest level of biomass production reached in this study was with Sabouraud liquid media (MS) at 16ºC. The biomass production of the medium of potato broth supplemented with dextrose (PDB), at 37ºC was similar to that of MS at 25ºC, which suggests that starchy substrates are an alternative for cultivating the studied strains using the waste from other productive activities involving starch. Growth levels in marine alga-based media were low compared to levels in PDB and MS. The strain C51 in *Mazzaella laminaroides*-based medium (MCM) at 25ºC yielded 10^8 cells mL^{-1}, similar to the levels obtained in MS and PDB at 16 and 37ºC, respectively. The limited growth in alga-based media (MCM, GAM and BAM) was probably the result of a substance in the media like polysaccharides of the alga or phenols that are enzymatic inhibitors (Ríos *et al.* 2009). The use of disaccharides or polysaccharides as carbon sources for some marine fungoid results in low growth levels, although other fungoid can grow with cellobiose, maltose and soluble starch (Goldstein 1963a, b). Several authors have also reported the use of alternative media for growing marine fungoid (Quilodrán *et al.* 2010). The use of substrates based on residues from industrial processes is a viable alternative for the mass production of microorganisms. Based on the resulted of the present work, it is necessary to improve the composition of alternative media (MCM, GAM, BAM and PDB), incorporating determined quantities of micronutrients such as iron, zinc, magnesium and vitamins, among others, as these are essential for the growth of eukaryotes and optimizing marine fungoid production (Cartagena *et al.* 2013).

Growth kinetics parameters are important variables in selecting the optimal biomass production conditions (Ertola *et al.* 1994). The use of the parameter maximum growth velocity in the present work allowed for discriminating among the formulated media (Table 2). Generally, the growth curves obtained for the assayed strains in Sabouraud media reached maximum growth velocities between 0.02 and 0.03 h^{-1} with concentrations of 10^8 to 10^9 cells mL^{-1}. The cellular concentrations reached by the strains in the present study are approximately on the same order of magnitude as those reported in studies of *Rhodotorula* and *Rhodosporidium* (Martearena *et al.* 2009, Martínez 2012). The 13 strains studied here present maximum production levels of up to 8.5 x 10^8 cells mL^{-1}, with the exception of strain C4, which reached 1.5 x 10^9 cells mL^{-1}. These cellular densities are similar to those reached by *Rhodotorula mucilaginosa*, which are on the order of 1.3 x 10^6 to 1.8 x 10^8 cells mL^{-1} (Martearena *et al.* 2009, Martínez 2012).

The 13 marine fungoid strains cultivated in MS at 16ºC in 500 mL bioreactors de 500 mL produced between 1.1 and 23% of DHA in dry weight. The strains C36 and C22

for their high DHA concentrations, which were higher than what is described in the literature (Bajpai *et al.* 1991, Burja *et al.* 2006, Yamasaki *et al.* 2006, Chi *et al.* 2007). *Rhodosporidium toruloides* and *Rhodotorula glutinis* produce around 70 and 33.7% of total lipids in dry weight, respectively. However, the fatty acids produced by these species include myristic, palmitic, palmitoleic, steric, oleic, linoleic and α-linoleic acids (Li *et al.* 2006, Freitas *et al.* 2014), in contrast to the strains studied here that produce high concentrations of DHA and EPA, neither of which have been described in *Rhodotorula* and *Rhodosporidium* (Li *et al.* 2007, Hu *et al.* 2009).

Other marine fungoid protist like Thraustochytrids are also potentially important alternatives to fish oil, with levels of DHA production similar to those of the strains studied here, with DHA concentrations of 26% in dry weight for *Schizochytrium limacinum* (Yaguchi *et al.* 1997). This microorganism, unlike the strains in this study, comes from a warm ecosystem. The strain *Thraustochytriidae* sp. AS4-A1 (Quilodrán *et al.* 2010), which was isolated in southern Chile, presents DHA concentrations of between 0.1 and 14% in dry weight, has been cultivated with alternative media composed of commercial residues from producing potato chips and beer. However, *Thraustochytriidae* sp. AS4-A1 produces less DHA than the levels determined for the strains C24, C46, C22, C3, O51 and C36 from the present study. The 13 marine fungoid also produce EPA (0.3 to 0.03% of biomass in dry weight), while *Thraustochytriidae* sp. AS4-A1 does not (Quilodrán *et al.* 2010). The production of this PUFA makes the studied strains even more interesting, presenting the advantage of offering 2 polyunsaturated fatty acids of commercial importance, making them comparable to already described species of Thraustochytrids; *Ulkenia* sp. KF13, *T. striatum* KF9, *S. mangrovei* KF5 and *Schizochytrium* sp. KF1, which yield concentrations of 1.4, 8.6, 7 and 12.5% of dry weight, respectively (Fan *et al.* 2001).

The total carotenoid production of the cultivated strains (41 to 179 mg CT g^{-1} of biomass in dry weight) is higher than that described for *Rhodotorula mucilaginosa* and *Rhodotorula glutinis* (21.4 and 35mg CT g^{-1} of biomass in dry weight, respectively), both considered highly productive of carotenoids (Aksu & Eren 2005, Saenge *et al.* 2011). The strains reported in the present work can potentially be used to produce natural pigments that are currently in high demand by the aquaculture industry (Aki *et al.* 2003, Wassef *et al.* 2010).

The molecular characterization of the 13 studied strains shows consistency between the sizes of the PCR products and those described by Honda *et al.* (1999), and Yokoyama *et al.* (2007). The primers used by Honda *et al.* (1999) are characterized as well for presenting specificity with marine fungoid given that they do not amplify for *Saccharomyces cerevisiae* (Socias 2012).

The molecular systematization of this group of marine fungoid isolated from marine ecosystems of central and southern Chile established their genetic similarity to strains described in the Genbank database, characterized by their lipid and carotenoid producing capacity. The closest species genetically to those studied in this work are *Rhodotorula* and *Rhodosporidium* (97%), both characterized as lipid and carotenoid producers (Saenge *et al.* 2011, Freitas *et al.* 2014). A more distant similarity (81%) was found with species of the Thraustochytriaceae family, characterized by high levels of DHA production (Chi *et al.* 2007). The strains C22, C24, C30, C3 and C36 share the same cluster with *Rhodotorula* sp. and *Rhodosporidium diobovatum*, in the phylogenetic tree, the strains in the present study being highly productive of DHA as a common biochemical characteristic among them. Although the other cluster observed are associated with production of substances of interest, more study is required to correlate the partial sequence of the gene 18s rRNA to the productive attributes discussed here.

The phylogenetic information obtained in the present work shows that the strains isolated from Canal Puyuhuapi are genetically similar to microorganisms isolated from the coast of the Biobío Region, indicating that these microorganisms are widely distributed latitudinal along the coast and fjord systems of central and southern Chile. The wide distribution of the marine fungoid studied here is consistent with what was described by Bongiorni *et al.* (2004, 2012).

A high degree of similarity to the family Thraustochytriceae was expected given that the strains were isolated using the pollen bait technique used for Thraustochytrides. However, only 81% genetic similarity to *Schizochytrium* sp. was obtained, a lower percentage than for the genera *Rhodotorula* and *Rhodosporidium*, which are good candidates for classifying the 13 strains. This information indicates that the studied strains belong to the genus *Rhodotorula* or *Rhodosporidium*. The genera *Rhodotorula* and *Rhodosporidium* are characterized by their ovoid shape, with vegetative states that are reproduced predominantly by gemmation or fission. They

are classified in the taxonomic division Eumycota, belonging to the subdivision Basidiomicotin, which are characterized by external spores located on basidia or sterigmata (Ochoa & Juárez 2004). Microscopic information obtained for strain C36, characterized in the present study as being highly productive of DHA and CT, are morphologically similar *Rhodotorula glutinis* and *Rhodosporidium diobovatum* (Ochoa & Juárez 2004). Ovoid-shaped cells were observed in the micrographs, in some of which gemmae are forming. A surface network was also observed covering cells similar to those in Thraustochytrids termed an ectoplasmic network (Perkins 1972). The information obtained here raises the question of whether microorganisms quantified and isolated through the pollen bait technique are all Thraustochytrides or whether they belong to other uncharacterized fungoid protist families (Bongiorni 2012).

In conclusion, the present study shows that strains from central and southern Chile are a potential source of DHA, EPA and carotenoids, highlighting the strains C36, C22 and C4 as producers of these substances under optimal growth conditions in liquid media.

Strain C36 is morphologically and genetically similar to *Rhodotorula*, which are terrestrial lipid and carotenoid producers, but there is no record indicating that the lipids produced are associated with DHA and EPA (Martínez 2012). Finally, this strain of marine origin may be the best option to upscale production at the level of a prototype and subsequent use commercially.

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Literature cited

Aki T, K Hachida, M Yoshinaga, Y Katai, T Yamasaki, S Kawamoto, T Kakizono, T Maoka, S Shigeta, O Suzuki & K Ono. 2003. Thraustochytrid as a potential source of carotenoids. Journal of the American Oil Chemists' Society 80: 789-794.

Aksu Z & TA Eren. 2005. Carotenoids production by the yeast *Rhodotorula mucilaginosa*: Use of agricultural wastes as a carbon source. Process Biochemistry 40(9): 2985-2991.

Andrews AG, HJ Phaff & MP Starr. 1976. Carotenoids of *Phaffia rhodozyma*, a red pigmented fermenting yeast. Phytochemistry 15: 10003-10007.

Bajpai P, P Bajpai & OP Ward. 1991. Optimization of production of docosahexaenoic acid (DHA) by *Thraustochytrium aureum* ATCC 34304. Journal of the American Oil Chemists' Society 68: 509-514.

Bongiorni L. 2012. Thraustochytrids, a neglected component of organic matter decomposition and food webs in marine sediments. Biology of Marine Fungi 53(1): 1-13.

Bongiorni L, L Pignataro & G Santangelo. 2004. Thraustochytrids (fungoid protist): an unexplored component of marine sediment microbiota. Scientia Marina 68(S1): 43-48.

Burja AM, H Radianingtyas, A Windust & CL Barrow. 2006. Isolation and characterization of polyunsaturated fatty acid producing *Thraustochytrium* species: screening of strains and optimization of omega-3 production. Applied Microbiology and Biotechnology 72: 1161-1169.

Cartagena EG, LY Britto, LJ Rodríguez & CL Zapata. 2013. Efecto de la Zeolita natural clinoptilita sobre el crecimiento de *Dunaliella salina* (teodoresco, 1905) cultivada en un fotobiorreactor de múltiples cámaras oscilantes. BIOCYT: Biología Ciencia y Tecnología 6: 20-24.

Chi Z, D Pyle, Z Wen, C Frear& S Chen. 2007. A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerolby microalgal fermentation. Process Biochemistry 42: 1537-1545.

Drummond A, B Ashton, S Buxton, M Cheung, A Cooper, C Duran, M Field, J Heled, M Kearse, S Markowitz, R Moir, S Stones-Havas, S Sturrock, T Thierer & A Wilson. 2011. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12): 1647-1649.

Ertola R, O Yantorno & C Mignone. 1994. Microbiología industrial, 104 pp. OEA, Washington.

Fan KW, F Chen, EB Jones & LL Vrijmoed. 2001. Eicosapentaenoic and docosahexaenoic acids production by and okara-utilizing potential of thraustochytrids. Industrial Microbiology and Biotechnology 27: 199-202.

Freitas C, TM Parreira, J Roseiro, A Reis & TL da Silva. 2014. Selecting low-cost carbon sources for carotenoid and lipid production by the pink yeast *Rhodosporidium toruloides* NCYC 921 using flow cytometry. Bioresource Technology 158: 355-359.

Gaertner A. 1966. Vorkommen, physiologie und verteilung, mariner niedererpilze (Aquatic Phycomycetes). Veröffentlichungen des Instituts für Meeresforschung in Bremerhaven (Suppl.) 2: 221-236.

Goldstein S. 1963a. Development and nutrition of a new species of *Thraustochytrium*. American Journal of Botany 50: 799-811.

Goldstein S. 1963b. Studies of a new species of *Thraustochytrium* that displays light stimulated growth. Mycologia 55: 799-811.

Jackson AJ & CJ Shepherd. 2012. The future of fishmeal and fish oil. In: Ryder J, L Ababouch & M Balaban (eds). Second international congress on szeafood technology on sustainable, innovative and healthy seafood, FAO/The University of Alaska, 10-13 May 2010, Anchorage. FAO Fisheries and Aquaculture Proceedings 22: 187-206. FAO, Rome.

Honda D, T Yokochi, T Nakahara, S Raghukumar, A Nakagiri, K Schaumann & T Higashihara. 1999. Molecular phylogeny of labyrinthulids and thraustochytrids based on the sequencing of 18S ribosomal RNA gene. Journal of Eukaryotic Microbiology 46: 637-647.

Hu C, X Zhao, J Zhao, S Wu & ZK Zhao. 2009. Effects of biomass hydrolysis by-products on oleaginous yeast *Rhodosporidium toruloides*. Bioresource Technology 100(20): 4843-4847.

Li Z, T Gu, B Kelder & J Kopchick. 2001. Analysis of fatty acids in mouse cells using reversed- phase high-performance liquid. Chromatographia 54: 463-467.

Li YH, BO Liu, ZB Zhao & FW Bai. 2006. Optimization of culture conditions for lipid Production by *Rhodosporidium toruloides*. Chinese Journal of Biotechnology 22(4): 650-656.

Li YH, ZB Zhao & FW Bai. 2007. High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. Enzyme and Microbial Technology 41: 312-317.

Martearena MR, E Scaroni & S Locatelli. 2009. Producción de lípidos a partir de levadura oleaginosa *Rhodotorula glutinis*. Segundo Congreso Iberoamericano, Hidrogeno y Fuentes Sustentables de Energía, HYFUSEN, Córdoba, Argentina, pp. 12-73.

Martínez R. 2012. Producción de lípidos por cepas de *Chlorella pyrenoidosa* y *Rhodotorula mucilaginosa* y generación de un consorcio microbiano. Doctoral dissertation, Universidad Autónoma de Nuevo León, Nuevo León, 111 pp.

Miller M, P Nichols & C Carter. 2007. Replacement of fish oil with thraustochytrid *Schizochytrium* sp. L oil in Atlantic salmon parr (*Salmo salar* L) diets. Comparative Biochemistry and Physiology 148: 382-392.

Morris CE, JM Monier & J Marie-Agne. 1997. Methods for observing microbial biofilms directly on leaf surfaces and recovering them for isolation of culturable microorganisms. Applied and Environmental Microbiology 63: 1570-1576.

Ochoa JL & RV Juárez. 2004. Las levaduras marinas como herramienta científica y biotecnológica, 12 pp. Red Universidad y Ciencia, La Paz.

Pemán J, E Martín-Mazuelos & MC Rubio-Calvo. 2007. Guía práctica de Identificación y diagnóstico en micología clínica. Identificación de levaduras. Revista Iberoamericana de Micología de Bilbao 11: 143-155.

Perkins FO. 1972. The ultrastructure of holdfasts, 'rhizoids', and slime tracks in thraustochytriaceous fungi and *Labyrinthula* spp. Archiv für Mikrobiologie 84: 95-118.

Quilodrán B, I Hinzpeter, E Hormazabal, A Quiroz & C Shene. 2010. Docosahexaenoic acid (C22:6n-3, DHA) and astaxanthin production by *Thraustochytriidae* sp. AS4-A1 a native strain with high similitude to *Ulkenia* sp.: Evaluation of liquid residues from food industry as nutrient sources. Enzyme and Microbial Technology 47: 24-30.

Raghukumar S. 2004. The role of fungi in marine detrital processes. In: Ramaiah N (ed). Marine microbiology: Facets & opportunities, pp 91-101. National Institute of Oceanography, Goa.

Raghukumar S & A Gaertner. 1980. Ecology of the thraustochytrids (lower marine fungi) in the Falden Ground and other parts of the North Sea II. Veröffentlichungen des Instituts für Meeresforschung in Bremerhaven 18: 289-308.

Ratledge C & JP Wynn. 2002. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Advances in Applied Microbiology 51: 1-51.

Ríos N, G Medina, J Jiménez, C Yánez, MY García, ML Di Bernardo & M Gualtieri. 2009. Actividad antibacteriana y antifúngica de extractos de algas marinas venezolanas. Revista Peruana de Biología 16(1): 97-100.

Rodher A. 1966. Determinación de carotenoides. Revista de Agroquímica y Tecnología de Alimentos 6(2): 24-27.

Saenge C, B Cheirsilp, TT Suksaroge & T Bourtoom. 2011. Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. Process Biochemistry 46(1): 210-218.

Scheckenbach F, K Hausmann, C Wylezich, M Weitere & H Arndt. 2010. Large-scale patterns in biodiversity of microbial eukaryotes from the abyssal sea floor. Proceedings of the National Academy of Sciences 107: 115-120.

Socias C. 2012. Thraustochytridos: un protista fungoide como alternativa biotecnológica marina para la producción sustentable de ácidos grasos poliinsaturados (PUFAs). Tesis de Ingeniero en Biotecnología Marina y Acuicultura, Universidad de Concepción, Concepción, 47 pp.

Tamura K, G Stecher, D Peterson, A Filipski & S Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis Version 6.0. Molecular Biology and Evolution 30: 2725-2729.

Valenzuela A, J Sanhueza & F Barra. 2012. El aceite de pescado: Ayer un desecho Industrial, hoy un producto de alto valor nutricional. Revista Chilena de Nutrición 39(2): 201-209.

Vicente J, M Galindo, M García & M Granado. 2008. Bioavailability of heavy metals monitoring water, sediments and fish species from a polluted estuary. Journal of Hazardous Materials 162: 823-836.

Vishniac HS. 1956. On the ecology of the lower marine fungi. The Biological Bulletin 111: 410-414.

Wassef EA, S Chatzifotis, EM Sakr & NE Saleh. 2010. Effect of two natural carotenoid sources in diets for gilthead seabream, *Sparus aurata*, on growth and skin coloration. Journal of Applied Aquaculture 22(3): 216-229.

Yaguchi T, S Tanaka, T Yokochi, T Nakahara & T Higashihara. 1997. Production of high yields of docosahexaenoic acid by *Schizochytrium* sp. strain SR21. Journal of the American Oil Chemists' Society 74(11): 1431-1434.

Yamasaki T, T Aki, M Shinozaki, M Taguchi, S Kawamoto & K Ono. 2006. Utilization of Shochu distillery wastewater for production of polyunsaturated fatty acids and xanthophyll's using thraustochytrid. Journal of Bioscience and Bioengineering 102: 323-327.

Yokoyama R, B Salleh & D Honda. 2007. Taxonomic rearrangement of the genus *Ulkenia sensu lato* based on morphology, chemotaxonomical characteristics, and 18S rRNA gene phylogeny (Thraustochytriaceae, Labyrinthulomycetes): emendation for *Ulkenia* and erection of *Botryochytrium*, *Parietichytrium*, and *Sicyoidochytrium* gen. nov. Mycoscience 48(6): 329-341.

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