Valorization of regional crude glycerol. Culture media optimization for batch docosahexaenoic acid (DHA) production with *Aurantiochytrium* sp.

Valorización de glicerol crudo regional. Optimización de medios de cultivo para la producción batch de ácido docosahexaenoico (DHA) con *Aurantiochytrium* sp.

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Abstract

Docosahexaenoic acid (DHA) has many benefits for human health. Commercial DHA sources derive from marine fish but present several production challenges. *Aurantiochytrium* sp., an abundant marine microalga, becomes an alternative for DHA production. Crude glycerol produced by small-scale biodiesel refineries is a regional, available, and inexpensive waste that can be converted into value-added compounds. This study investigated crude glycerol as a potential carbon source for DHA-rich oil production using an aerobically isolated *Aurantiochytrium* sp. in batch shake flasks. We also optimized the culture medium formulation by varying carbon and nitrogen sources, thereby reducing medium costs while maximizing DHA production. A larger initial *Aurantiochytrium* sp. inoculum improved cell concentration and medium carbon depletion, increasing DHA productivity (P_{DHA}). Increasing culture time showed no differences in *Aurantiochytrium* sp. growth parameters, but reduced DHA production. The absence of yeast extract in the culture media resulted in faster substrate metabolism by *Aurantiochytrium* sp. and increased DHA production. Crude glycerol yielded the highest P_{DHA} (15.35 mg L^{-1} h^{-1}) at 120 h. Crude glycerol can be used as a cheaper carbon source in media formulation with *Aurantiochytrium* sp. cultures for DHA production.

Keywords
crude glycerol • *Aurantiochytrium* sp. • DHA • inoculum • culture medium

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Resumen

El ácido docosahexaenoico (DHA) posee muchos beneficios para la salud humana. Las fuentes comerciales de DHA se obtienen de peces marinos, pero presenta desventajas. *Aurantiochytrium* sp., una microalga marina abundante, surge como alternativa para la producción de DHA, solucionando los problemas de obtener DHA a partir de peces marinos. El glicerol crudo producido por pequeñas refinerías de biodiesel es un descarte regional, disponible y barato, capaz de ser transformado en compuestos de valor agregado. El objetivo de este trabajo fue investigar la potencial utilización de glicerol crudo regional como fuente de carbono para producir aceite rico en DHA, utilizando la cepa aeróbica aislada *Aurantiochytrium* sp. en matraces batch agitados; y optimizar la formulación de medios de cultivo, variando las fuentes de carbono y nitrógeno, para reducir costos y maximizar la producción de DHA. Los tamaños de inóculo iniciales más grandes de *Aurantiochytrium* sp mejoran la concentración celular y agotan la fuente de carbono, mejorando la productividad a DHA (P_{DHA}). El aumento del tiempo de cultivo no mostró diferencias en los parámetros de crecimiento de *Aurantiochytrium* sp., pero disminuyó la producción de DHA. La ausencia de extracto de levadura en los medios de cultivo produjo una metabolización más rápida del sustrato por *Aurantiochytrium* sp., mejorando la producción de DHA. Con glicerol crudo se alcanzó la mejor P_{DHA} (15,35 mg L^{-1} h^{-1}) a 120 h. El glicerol crudo se puede utilizar como fuente de carbono barata en la formulación de medios en cultivos con *Aurantiochytrium* sp. para producir DHA.

Palabras clave
glicerol crudo • *Aurantiochytrium* sp. • DHA • inóculo • medio de cultivo

Introduction

Polyunsaturated fatty acids (PUFA), particularly omega-3 (ω-3), play an important role in physiological functions (13). Docosahexaenoic acid (DHA, C22:6 ω-3) and eicosapentaenoic acid (EPA, C20:5 ω-3) have vasodilatory and anti-inflammatory capacity, prevent atherosclerosis and hypertension, reduce risk factors for thrombosis, arthritis, and Alzheimer's disease, and increase development of the central nervous system and retinal tissue, improving visual acuity and cognitive capacity in children (5, 33). DHA is commonly presented in pharmaceuticals (nutraceutical and functional products), medicine (Alzheimer's and cerebrovascular drugs), and food (soft drinks, dairy, and infant products) (12, 33).

Commercial sources of ω-3 DHA are obtained from marine fish and shellfish using a well-known technology that produces 600,000 tonnes annually (21). However, the process faces overfishing, strong seasonal dependence, low DHA yield (< 50% w/w), high levels of marine pollutants (dioxins, methylmercury, polychlorinated biphenyls, metals), fish odor, and low DHA stability (17). Given these problems and the constant demand for high-quality ω-3 DHA, new strategies like microalgae cultures become promising alternatives (6).

Microalgae cultures for DHA production offer high purity and good organoleptic properties, use renewable waste, are toxins-free, and have low fermentation costs (23). Heterotrophic strains of the Thraustochytriaceae family (*Aurantiochytrium*, *Schizochytrium,* and *Thraustochytrium*) have significant growth rates compared to photoautotrophic microalgal cultivation, producing more than 50% of their dry cell weight as lipids, with ω-3 DHA reaching 50% of total fatty acids in some species (8, 20). *Aurantiochytrium* sp., an aerobic thraustochytrid abundant in marine environments, has been an alternative source of ω-3 DHA. This microalga can grow readily on various carbon sources and has high P_{DHA}, replacing traditional marine fish oil production (20, 37).

Efforts regarding *Aurantiochytrium* sp. have focused on optimizing culture conditions and media (C, N, and micronutrients) to increase DHA production, mainly because costs highly depend on C sources (26, 28). Carbon concentration affects the synthesis of organic molecules and energy availability, while nitrogen concentration affects amino acids and nucleic acid synthesis (10). A traditional C source for fermentative DHA synthesis is glucose,

but in microalgal heterotrophic fermentation, glucose represents almost 80% of media cost (24, 37). The use of regional, highly available, and low-cost medium components with high C and/or N content instead of glucose economizes fermentation processes and provide energy for cell maintenance and biosynthesis (32).

In central Argentina, many companies produce large quantities of agro-industrial waste. The biodiesel industry produces crude glycerol (10% w/w) as a by-product. World biodiesel production will reach 40 million tonnes in 2025, producing 6.3 million tonnes of crude glycerol, and the market is estimated to grow to 3,670 million USD by 2030 (1, 16, 34). In biodiesel refineries, small-scale purification is not economically viable and the by-product is sold at low cost, depending on its quality level. This calls for a sustainable and economic process for converting crude glycerol into value-added compounds. Thus, crude glycerol could constitute a cheaper carbon source in microalgae cultures for ω-3 DHA production.

This study investigated two main points: i) the use of crude glycerol, without any purification treatment, as substrate for ω-3 DHA-rich oil production using a locally isolated oleaginous *Aurantiochytrium* sp. strain in batch cultures; and ii) the optimization of culture medium formulation, varying carbon and nitrogen sources, reducing costs and increasing ω-3 DHA-rich oil yield (P_{DHA}).

Materials and methods

Materials

Crude glycerol (CG) was obtained as biodiesel by-product from Bolzán (Argentina). Pure anhydrous glycerol (AG), monohydrated glucose (MG) and methanol, ethanol, and isopropanol for extractive techniques were purchased from Cicarelli (Argentina). Peptone and yeast extract were purchased from Britania (Argentina). Salts for media preparation, KOH and *n*-hexane for fatty acid extraction, and Na_2SO_4 for total fatty acid determination were purchased from Research AG (Argentina). Fatty acid methyl ester (FAME) analytical standards were obtained from Merck (Argentina).

Isolation and identification of microorganisms

Aurantiochytrium sp. was obtained from seawater samples from the Argentinean coast and isolated in our laboratory. *Aurantiochytrium* sp. was isolated using the streak plate technique in Petri dishes (2). The culture medium was a synthetic AS100 (35) modified with 0.2 M NaCl (31), and supplemented with peptone (10 g L^{-1}), yeast extract (10 g L^{-1}), and monohydrated pure glucose (35 g L^{-1}), designed as PMG* medium. After adjusting pH to 7.00 and adding 1% agar, the medium was autoclaved at 115°C for 20 min. Cool but still liquid PMG* agar medium was distributed in sterile Petri dishes. Approximately 0.1 mL of the sample to be isolated was placed in these Petri dishes and spread with a sterilized bacteriological loop. Petri dishes were incubated at 28 ± 2°C for 3 to 4 days. Isolated colonies were then selected and transferred to another Petri dish until pure microalgae were obtained. Tubes containing 5 mL of liquid PMG* medium were prepared and autoclaved at 115°C for 20 min. Isolated microalgae were incubated at 28 ± 2°C for 2 to 3 days obtaining a higher-density culture for working stocks preparation. At all isolation stages, possible contamination with other microorganisms was detected by observing the microalgae under a 40X light microscope. The strain was stored in 25% (v/v) glycerol at -80 °C and cell viability was checked monthly.

Genomic DNA extraction was performed using the Easy Pure Plant Genomic DNA Kit (Transgene Biotech, China) identifying the microalgae at a molecular level. Polymerase chain reaction (PCR) was performed using a universal primer set (ITS Fo: 5' TCCGTAGGTGAACCTGCGG 3', ITS Rev: 5' TCCTCCGCTTATTGATATGC 3') to amplify the internal transcriber spacer (ITS) region at the ribosomal locus, including the 5.8S rRNA gene. The resulting PCR product was sequenced and the nucleotide sequence was analyzed with NCBI Basic Local Alignment Search Tool (BLAST). The sequence analysis showed 100% identity with the 5.8S rRNA gene of *Aurantiochytrium* sp. strain CCAP_4062/3 (MF766428.1). It also had a high percentage of identity (~98%) with *Aurantiochytrium limacinum* IMB188 (KP899823.1). Considering these results, the isolated organism would belong to the genus *Aurantiochytrium,* closely related to *A. limacinum*.

Batch shake flask cultures

Aliquots of 1.5 mL of cells preserved in 25% (v/v) glycerol were used to inoculate 125 mL shake flasks containing 15 mL PMG* medium and placed on a heated orbital shaker at 150 rpm and 28 ± 2°C for 48 h. Four fermentation media were used for screening assays in batch shake flasks. Table 1 shows substrate carbon composition for media preparation, comparing useful carbon used per L of culture medium. We prepared PMG* medium and three other media identically formulated to PMG* but without yeast extract. The first was supplemented with 35 g L^{-1} of pure monohydrate glucose (PMG medium), the second replaced glucose with 35 g L^{-1} pure anhydrous glycerol (PAG medium), and the third replaced glucose with 42.5 g L^{-1} biorefinery crude glycerol (BCG medium) achieving identical amounts of available carbon in each medium. Media pH was adjusted to 7.00 before autoclaving at 115°C for 20 min. Ten mL of 10% (v/v) inoculum medium were transferred to 250 mL shake flasks containing 90 mL medium and placed in a heated orbital shaker at 150 rpm and 28 ± 2°C for 120 and 240 h assays.

Table 1. Substrate carbon composition used in modified artificial seawater medium for cultures of *Aurantiochytrium* sp.

Tabla 1. Composición de carbón de los sustratos utilizados en el medio agua de mar artificial modificado para cultivos de *Aurantiochytrium* sp.

Substrate	% G	% W	% A	% MONG	% M	gC L^{-1}
MG	99.5	---	0.5	---	---	13.99
AG	99.5	---	0.4	0.1	---	13.69
CG	81.7	11.5	4.2	2.5	0.1	13.74

% G: % glucose or glycerol; % W: % water; % A: % ash; % MONG: % matter organic non-glycerol; % M: % methanol; gC L^{-1}: g of useful carbon per L of culture medium.

% G: % glucosa o glicerol; % W: % agua; % A: % cenizas; % MONG: % materia orgánica no glicerol; % M: % metanol; gC L^{-1}: g de carbono útil por L de medio de cultivo.

Cell disruption and extraction of algal lipids

Once cultures finished, a 40 mL aliquot was transferred to a glass beaker with 10 mL of distilled water. Cells were homogenized and lysed using an ultrasonic cell disruptor for 1.2 kW and 20 kHz (Bald Design, Argentina) at 25°C for 30 s. Then, 50 mL of pure *n*-hexane was added to the lysed cells, maintaining a 5:1 (v/v) ratio with respect to distilled water. The mixture was vigorously shaken with a magnetic stirrer for 10 min. Finally, the organic phase was transferred to a 250 mL balloon and total lipids were extracted by vacuum distillation at 55°C.

Analytical procedures

Biomass, glucose and glycerol determinations

Culture samples (1 mL) were periodically taken and centrifuged at 9000 g and 25°C for 10 min. The supernatant was stored at -20°C. Biomass concentration was determined by cell counting using a Neubauer chamber (Boeco, Germany). Glucose and glycerol concentrations were identified with Glycemia and TG colorimetric kits (Wiener Lab, Argentina), in a UV spectrophotometer at 505 nm.

Dry cell weight and total fatty acids

Culture samples (50 mL) were centrifuged at 3000 g and 25°C for 15 min. The supernatant was discarded and the biomass was washed twice with distilled water. Biomass was transferred to Petri dishes with filter paper (previously tared), oven-dried at 70°C for 12 h, and weighed until constant weight, obtaining dry cell mass. The filter paper (with dry biomass) was then removed from the Petri dishes and placed in a 50 mL Falcon tube for saponifiable lipid determination. The Falcon tube was filled with 5 mL of 30% w/v KOH and 5 mL of 96% v/v ethanol, and incubated in a thermostatic bath at 70°C for 16 h. The biomass-KOH-ethanol mixture was cooled to room temperature. Then 10 mL of *n*-hexane was added, the tube was vortexed for 1 min and the contents were centrifuged at 6000 rpm for 5 min, discarding the organic phase. This step was done twice.

The washed aqueous phase was incubated in an ice bath. Concentrated HCl was added until a pH of 1. Next, 10 mL of n-hexane was added, the tube was stirred vigorously for 1 min and the contents were centrifuged at 6000 rpm for 5 min to recover the organic phase. This step was also done twice. The two organic fractions (10 mL each) were transferred to a glass beaker (previously tared), oven-dried at 80 °C for 12 h, and weighed to constant weight. Saponifiable lipids obtained were compared with a calibration curve using crescent concentrations of standard fatty acid vs. the saponifiable fraction obtained by the described technique, obtaining total fatty acid (TFA) mass for each culture.

Fatty acids composition

Total lipids extraction employed a mixture of *n*-hexane:isopropanol (3:2) and 6% w/v Na_2SO_4 at room temperature (36). Extracted lipids were dried under a N_2 stream at 40°C. FAME was prepared following a cold method using *n*-hexane and KOH 2N in methanol (3). The obtained FAME fraction was quantified with a gas chromatograph GC-2014 (Shimadzu) equipped with a capillary column CP-Sil 88, 100 m x 0.25 mm ID (Varian) and a flame ionization detector (FID), using operating conditions as previously described (22).

Fatty acids were identified by comparing retention times of each peak and quantified from peak areas obtained from chromatograms with a FAME standard, using nonadecanoic acid methyl ester (19:0 ME) as internal standard (4, 11, 22).

DHA quality

The nutritional quality and sensory properties of DHA-rich oil are highly dependent on the efficiency of the extraction method used (29). Peroxide value (PV, AOCS Official Method Cd 8b-90), representing the amount of peroxide (meq of active oxygen per kg of lipids) in a sample was determined as follows. Hydroperoxides (dissolved in acetic acid and chloroform) reacted with iodide ions from KI to form iodine, and PV was determined by titration of the liberated iodine with a known concentration of Na2S2O3 solution, using starch as an indicator (27). Acid value (AV, AOCS Official Method Da 14-48), i.e. free fatty acids (% oleic acid) in a sample was determined by titration technique determining the mg of KOH (0.1 N, in ethanolic solution) required to neutralize free FA per g of sample, dissolved in a mixture of ethyl ether:ethanol, employing phenolphthalein as an indicator (14). Elemental analysis for metal identification (Pb, Cd, Hg, As, Ni, Cu, Fe, Cr, and Co) was performed using an Optima 2100 DV inductively coupled plasma optical emission spectrometer (ICP-OES) (Perkin Elmer) with a CCD detector. Limit values were extracted from the results published by the European Union report (9).

Kinetic and stoichiometric parameters

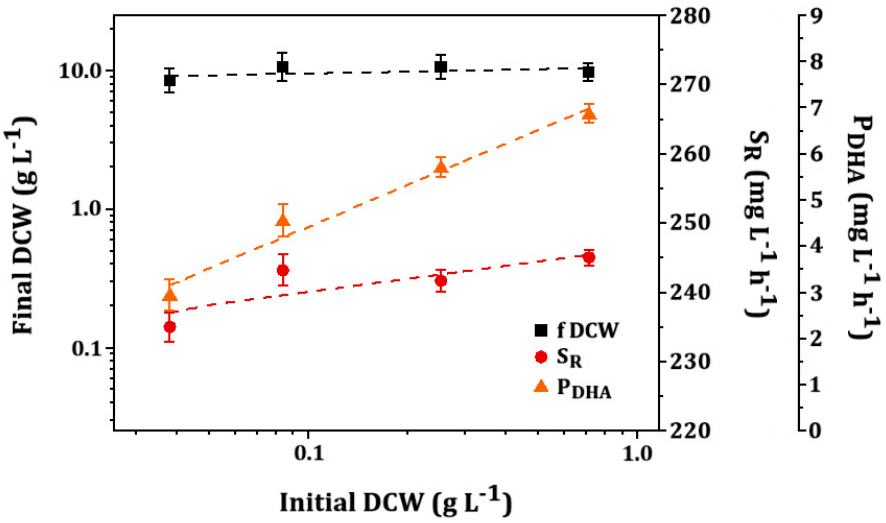
Specific growth rate (μ, h^{-1}), substrate consumption rate (S_R, mg L^{-1} h^{-1}), DHA productivity (P_{DHA}, mg L^{-1} h^{-1}) and yield ($Y_{X/S}$, g g^{-1}) were calculated from experimental culture data obtained from dry cell weight (DCW, g L^{-1}), total fatty acid concentration (C_{TFA}, g L^{-1}), ω-3 PUFA concentration (C_{PUFA}, g L^{-1}) and DHA concentration (C_{DHA}, g L^{-1}).

Results and discussion

Influence of inoculum size on culture parameters

To understand the influence of the initial inoculum size added to the media on main culture parameters, tests were developed in 250 mL batch shake flasks with 100 mL PMG* medium at 150 rpm and 28 ± 2°C for 120 h. Figure 1 (page XXX), shows the initial inoculum concentration of *Aurantiochytrium* sp. in relation to final cell concentration (f_{DCW}), $S_{R,}$ and P_{DHA}. Increasing the initial inoculum size lowered final *Aurantiochytrium* sp. concentration only for the first inoculum size (0.5% v/v), but did not significantly increase the last three inoculum sizes (2.5, 5.0 and 10.0% v/v), which showed similar DCW levels. As *Aurantiochytrium* sp. metabolism produces a maximum cell concentration after 5 culture days, this was not much affected when the inoculum size added was between 2.5 and 10.0% v/v of fresh culture medium. Larger inoculum sizes could be tested but increasing inoculum sizes would not be economically viable in a scale-up process. As expected, S_R slightly increased with increasing inoculum size given a greater number of *Aurantiochytrium* sp.

cells growing in the same volume of initial culture medium. Only the two largest initial inoculum sizes (5.0 and 10.0% v/v) practically exhausted the medium substrate. The P_{DHA} markedly increased with increasing inoculum size, due to higher S_R obtained by a greater amount of *Aurantiochytrium* sp. cells available in culture. P_{DHA} was 2.85 and 6.84 mg L^{-1} h^{-1} at inoculum size of 0.5 and 10.0% v/v fresh culture medium, respectively.



Dashed lines show linear adjustment for each parameter. Tests were conducted in triplicate.

Las líneas discontinuas muestran el ajuste lineal para cada parámetro. Los ensayos fueron realizados por triplicado.

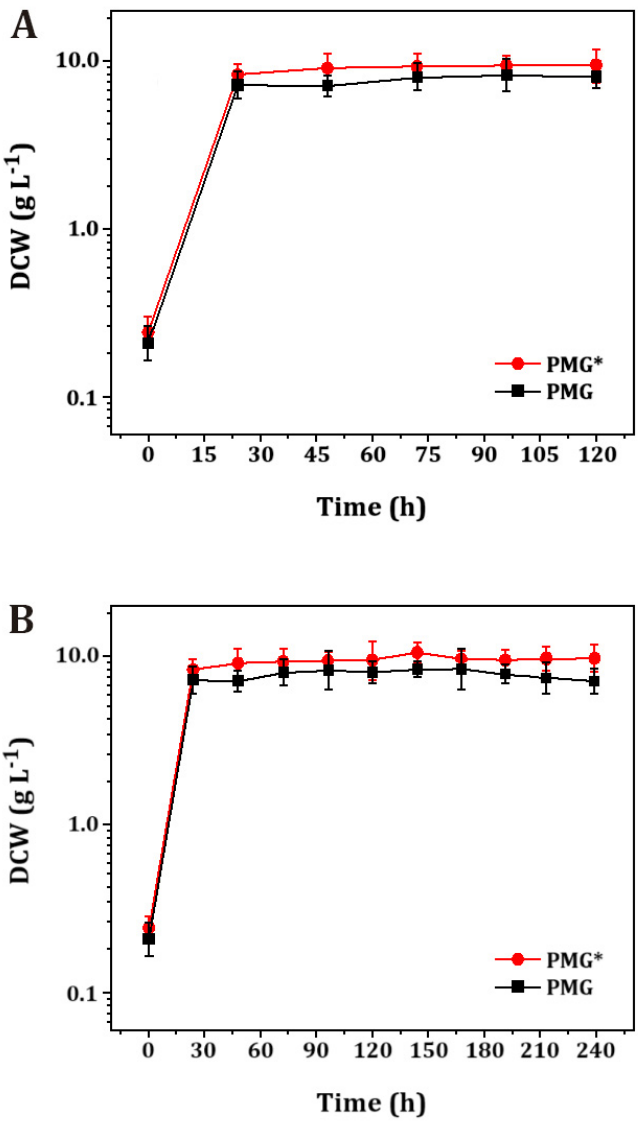
Figure 1. Final dry cell concentration (fDCW), S_R, and P_{DHA} vs. initial dry cell concentration (iDCW) for *Aurantiochytrium* sp. in 250 mL batch shake flasks with 100 mL PMG* medium, tested at 150 rpm and 28 ± 2 °C for 120 h.

Figura 1. Concentración celular final en base seca (fDCW), S_R y P_{DHA} vs. Concentración celular inicial en base seca (iDCW) para *Aurantiochytrium* sp. en matraces batch agitados de 250 mL con 100 mL de medio PMG*, testeados a 150 rpm y 28 ± 2 °C durante 120 h.

Previous results with *A. limacinum* SR21 demonstrate that inoculum preparation is fundamental for DHA production, with inoculum size having a significant effect on biomass production. High lipid accumulation was obtained with 10.0% v/v inoculum size, with higher P_{DHA} and DHA production (11 g L^{-1}) in a shorter time (30). In addition, the greatest increase occurred when inoculum size changed from 0.5 to 2.5% v/v of fresh culture medium, obtaining 60% more P_{DHA}. When inoculum size was doubled, from 2.5 to 5.0% v/v, and then from 5.0 to 10.0% v/v, 25% increase in P_{DHA} was achieved in both cases. Increasing initial inoculum size to 10.0% v/v had a direct effect on P_{DHA} and S_R, as a greater number of *Aurantiochytrium* sp. cells metabolize high amounts of substrate at the same medium volume and culture time.

Influence of yeast extract and test time on culture parameters

The development of *Aurantiochytrium* sp. cultures in 250 mL batch shake flasks at two final culture times was analyzed using PMG* and PMG media. Figure 2 (page XXX), shows cell concentration curves at 120 h (figure 2a, page XXX) were slightly higher for PMG* than for PMG medium, but showed similar behavior. An increase in culture time until 240 h (figure 2b, page XXX) displayed the same behavior as for 120 h, with no major difference in DCW, and remaining constant during the stationary phase.



Tests were conducted in duplicate.

Los ensayos fueron realizados por duplicado.

Figure 2. Cell concentration curves of *Aurantiochytrium* sp. cultures in 250 mL batch shake flasks with 100 mL PMG* and PMG, tested at 150 rpm and 28 ± 2 °C for: a) 120 h and b) 240 h culture times.

Figura 2. Curvas de concentración celular de cultivos *Aurantiochytrium* sp. en matraces batch agitados de 250 mL con 100 mL de medios PMG* y PMG, ensayados a 150 rpm y 28 ± 2 °C durante: a) 120 h y b) 240 h de cultivo.

Table 2 (page XXX), exhibits the main parameters obtained during fermentation of *Aurantiochytrium* sp. with 100 mL medium in batch shake flasks at 120 to 240 h culture times. The μ parameter was practically similar for all tests, but DCW decreased by 12.3 and 18.1% in PMG* and PMG media respectively, between 120 and 240 h culture time. Cultures with yeast extract did not deplete the supplied glucose, consuming 82.5 and 83.5% of added glucose at 120 and 240 h, respectively. PMG cultures also showed higher S_R than PMG* cultures. With PMG medium, consumed glucose reached 98.1% of the total added substrate at 120 h, and was practically consumed (99.7%) at 240 h. A preliminary screening revealed that adding 3.0% w/v glucose concentration from 2 to 10% w/v showed optimal culture parameters for *Aurantiochytrium* sp. cultures (18). In our tests, adding 35 g L^{-1} glucose (3.5% w/v) as initial substrate was appropriate.

Table 2. Culture parameters obtained during *Aurantiochytrium* sp. tests in 250 mL batch shake flasks with 100 mL PMG* and PMG, tested at 150 rpm and 28 ± 2 °C for two culture times.

Tabla 2. Parámetros de cultivo obtenidos durante los ensayos con *Aurantiochytrium* sp. en matraces batch agitados de 250 mL con 100 mL de medios PMG* y PMG, testeados a 150 rpm y 28 ± 2 °C a dos tiempos de cultivo.

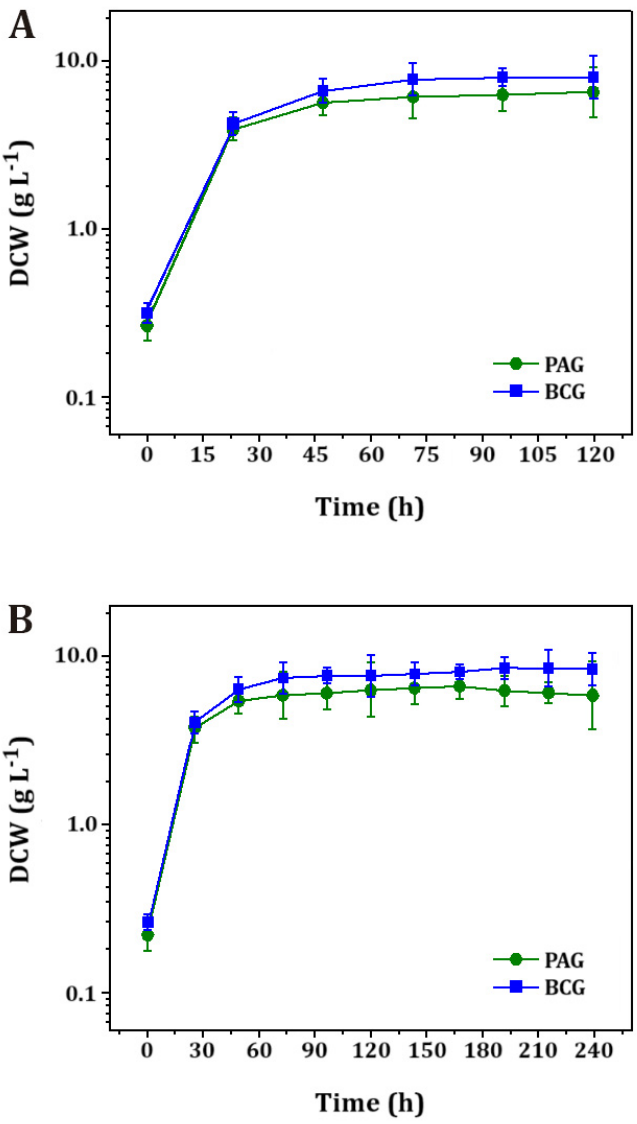
Parameter	120 h		240 h	
	PMG*	PMG	PMG*	PMG
μ (h^{-1})	0.72	0.72	0.69	0.70
DCW ($g\ L^{-1}$)	11.01	10.42	9.66	8.37
S_R ($mg\ L^{-1}\ h^{-1}$)	243.83	290.75	120.41	141.60
C_{TFA} ($g\ L^{-1}$)	1.55	5.47	0.32	2.55
C_{PUFA} ($g\ L^{-1}$)	1.17	4.21	0.24	1.96
C_{DHA} ($g\ L^{-1}$)	0.71	2.65	0.15	1.22
P_{DHA} ($mg\ L^{-1}\ h^{-1}$)	6.04	21.80	0.63	5.07
$Y_{X/S}$ ($g\ g^{-1}$)	0.38	0.30	0.33	0.20

Eliminating yeast extract (an expensive ingredient) in culture media causes *Aurantiochytrium* sp. to metabolize glucose 1.2 times faster. These results agree with a previous study where optimal culture conditions were achieved only with peptone as N source in culture media, and cell concentration and DHA yield were significantly increased (7). Another work replaced yeast extract with discarded orange peel extract and nitrates ($NaNO_3$, NH_4Cl, urea) for a more economical culture formulation in microalgae DHA production (25). Finally, when culture time was increased to 240 h, S_R was reduced by half for both evaluated cultures. Interestingly, absent yeast extract increased C_{DHA} by 3.6 times at 120 h and 8.1 times at 240 h. However, at longer culture time, C_{PUFA} and C_{DHA} were less than half than at 120 h for both cultures tested.

Decreased P_{DHA} was also observed when culture time increased from 120 to 240 h, resulting in 6.04 and 0.63 mg L^{-1} h^{-1} for PMG* medium, and 21.80 and 5.07 mg L^{-1} h^{-1} for PMG medium. This suggests that increasing culture time to 240 h was not appropriate, in agreement with previous results (7). Although DCW remains at the same level in all cultures, *Aurantiochytrium* sp. metabolism changes by decreasing lipid synthesis while some reserve substances are used for cell maintenance. Therefore, no yeast extract was added to media for the next tests.

Influence of glycerol quality and assay time on culture parameters

Pure and crude glycerol was used as a carbon source in *Aurantiochytrium* sp. cultures to evaluate the differences between glycerol qualities, analyzing crude glycerol as an economical and accessible substrate from regional small-scale biodiesel refineries. Figure 3 (page XXX), shows cell concentration curves of *Aurantiochytrium* sp. cultures at 120 and 240 h in 250 mL batch shake flasks with 100 mL PAG and BCG media, without yeast extract addition. The best performance was observed with BCG medium compared to PAG medium at 120 h (figure 3a, page XXX), becoming more evident at 240 h (fgure 3b, page XXX). Cell concentration curves obtained with glycerol cultures and PMG medium showed similar behaviors but were slightly higher with PMG. Table 3 (page XXX) shows the main parameters obtained in *Aurantiochytrium* sp. cultures at 120 and 240 h with PAG and BCG media in batch shake flasks. The μ parameters are practically similar for all tests, with values close to those reported with PMG medium, demonstrating that glycerol was an adequate substrate for *Aurantiochytrium* sp. Concerning DCW, BCG showed the best performance at both culture times, and lower values were observed at 240 h culture, with a decrease of 10 and 24% for PAG and BCG, respectively.



Tests were conducted in duplicate.

Los ensayos fueron realizados por duplicado.

Figure 3. Cell concentration curves of *Aurantiochytrium* sp. cultures in 250 mL batch shake flasks with 100 mL PAG and BCG media, tested at 150 rpm and 28 ± 2 °C for: a) 120 h and b) 240 h culture times.

Figura 3. Curvas de concentración celular de cultivos *Aurantiochytrium* sp. en matraces batch agitados de 250 mL con 100 mL de medios PAG y BCG, testeados a 150 rpm y 28 ± 2 °C durante: a) 120 h y b) 240 h de cultivo.

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Tabla 3. Parámetros de cultivo obtenidos durante los ensayos con *Aurantiochytrium* sp. en matraces batch agitados de 250 mL con 100 mL de medios PAG y BCG, testeados a 150 rpm y 28 ± 2 °C a dos tiempos de cultivo.

Parameter	120 h		240 h	
	PAG	BCG	PAG	BCG
μ (h^{-1})	0.70	0.73	0.74	0.74
DCW ($g\ L^{-1}$)	9.72	10.60	6.51	8.08
S_R ($mg\ L^{-1}\ h^{-1}$)	351.67	350.42	150.84	169.93
C_{TFA} ($g\ L^{-1}$)	3.25	3.86	0.61	0.63
C_{PUFA} ($g\ L^{-1}$)	2.49	2.96	0.47	0.48
C_{DHA} ($g\ L^{-1}$)	1.55	1.84	0.29	0.30
P_{DHA} ($mg\ L^{-1}\ h^{-1}$)	12.89	15.35	1.21	1.25
$Y_{X/S}$ ($g\ g^{-1}$)	0.23	0.25	0.12	0.12

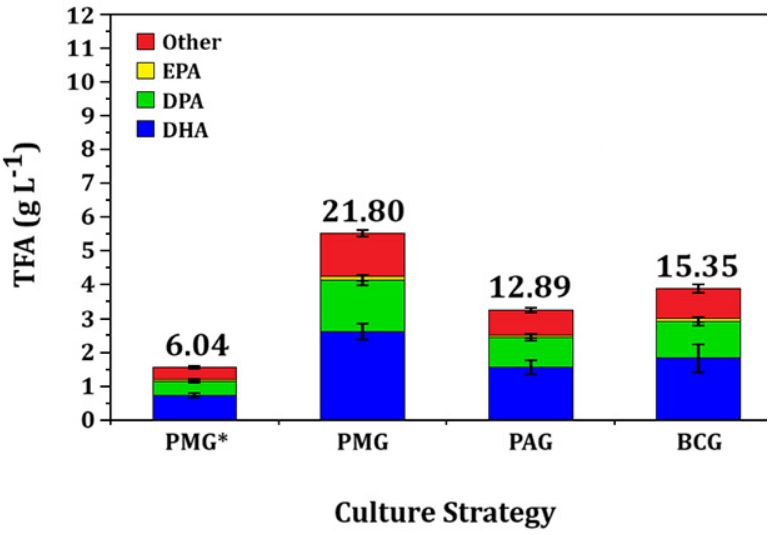
PAG and BCG cultures have a high S_R but decreased by 43% for PAG and 54% for BCG at 240 h. The S_R values in cultures with glycerol are higher than with glucose. Adequate C_{TFA} and C_{PUFA} were obtained at 120 h but strongly decreased when the culture was extended to 240 h. Higher C_{DHA} (1.84 g L^{-1}) was reached with BCG medium at 120 h, whereas with PAG C_{DHA} was 1.55 g L^{-1}, a value 16% lower than with BCG medium. The best P_{DHA} (15.35 mg L^{-1} h^{-1}) was produced with BCG medium at 120 h, slightly lower than the value obtained with PMG (21.80 mg L^{-1} h^{-1}) at 120 h. The use of glycerol or monosaccharides (fructose, glucose, mannose) in culture media resulted in higher concentrations of DCW and DHA than with disaccharides (maltose, lactose, sucrose) and polysaccharides (starch) (7). At 240 h, C_{DHA} decreased between 5 and 6 times with respect to 120 h. This result suggests that longer culture times cause *Aurantiochytrium* sp. to consume lipid reserves given lower carbon availability in batch culture. Lipids are accumulated as carbon and energy sources in the first culture stage, maybe after the faster cell growth (*i.e.*, culture time higher than 15 h). Some authors reported that glucose promotes rapid cell growth and lipid synthesis in early fermentation stages and glycerol produces late DHA accumulation with *A. limacinum* SR21 (15). Times over 240 h were not tested, as no clear improvement in culture parameters could be demonstrated with *Aurantiochytrium* sp.

These results are key since no prior (and more expensive) purification step, and no conditioning treatment was necessary before using crude glycerol as substrate in culture media. The good performance reached with BCG medium was explained by glucose being a C-6 compound, and glycerol a C-3 compound, easier to incorporate and assimilate by *Aurantiochytrium* sp. Increasing C_{TFA} and C_{DHA} induced by glycerol in *Aurantiochytrium* sp. cultures could be explained by the up-regulation of two important metabolites, oxalic acid (intermediate in citric acid cycle) and myo-inositol (growth promoter) (18). The cumulative effect of these metabolites produces an up-regulation of the citric acid cycle directly affecting cell metabolism, together with the pentose phosphate pathway, which generates reducing power, causing dynamic changes at a molecular level in enzymatic activities (18, 19).

Lipid composition and quality

Fatty acid production by *Aurantiochytrium* sp. cultures was analyzed with different substrates in a modified artificial seawater medium, prepared from a mixture of salts and nutrients in water. Figure 4 (page XXX) shows these strategies concerning total fatty acid production expressed as DHA, DPA (docosapentaenoic acid), EPA, and other fatty acid concentrations. The most efficient culture for C_{DHA} was PMG medium, with a maximum C_{DHA} of 2.65 g L^{-1} and C_{TFA} of 5.47 g L^{-1} (table 2, page XXX). C_{DHA} increased 3.7 times in PMG compared to PMG* medium, while P_{DHA} is about 3.5 times higher, indicating that absent yeast

extract greatly favors cellular machinery for lipid production, allowing C_{TFA} to increase. It should be noted that the DPA concentration (healthy ω-3 PUFA) also increases in the PMG culture and represents almost half of the total amount of DHA obtained. The highest C_{DHA} for glycerol cultures was obtained with BCG, reaching 1.84 g L^{-1}, and C_{TFA} of 3.86 g L^{-1}. In this medium, DPA concentration was more than half of the total amount of DHA obtained, and the P_{DHA} reached a maximum of 15.35 g L^{-1} h^{-1}, superior to PAG medium. In all tests, EPA concentration (another healthy ω-3 PUFA) is negligible, reaching a maximum value of 0.10 g L^{-1} with PMG medium.



Numbers on bars represent P_{DHA} (mg L^{-1} h^{-1}) for each culture strategy. Assays were run in duplicate.

Los números sobre las barras representan la P_{DHA} (mg L^{-1} h^{-1}) para cada estrategia de cultivo. Los ensayos fueron realizados por duplicado.

Figure 4. C_{TFA}, as concentration of DHA, DPA, EPA, and other fatty acids, obtained in *Aurantiochytrium* sp. cultures in 250 mL batch shake flasks with 100 mL media, tested at 150 rpm and 28 ± 2 °C for 120 h culture time.

Figura 4. Concentración de ácidos grasos totales (C_{TFA}), expresada como concentración de DHA, DPA, EPA y otros ácidos grasos, obtenidos en cultivos *Aurantiochytrium* sp. en matraces batch agitados de 250 mL con 100 mL de medio de cultivo, testeados a 150 rpm y 28 ± 2 °C durante 120 h de cultivo.

The obtained DHA-rich oil constitutes a safe product for human and animal food formulation. However, it requires certain quality parameters to be a stable product. After extraction from the obtained TFA, DHA quality parameters were obtained by titrimetric assays and metal identification. For PV and AV assays, DHA products comply with the limit (< 1.20 meq O_2 kg^{-1}, and < 0.10 meq KOH g^{-1}, respectively) set by the European Union standards for algal oils (9). Since the main difference between ω-3 DHA obtained from microalgae and marine fish was the presence of heavy metals, ICP-OES analysis of DHA products obtained from *Aurantiochytrium* sp. cultures showed that metal contents (Pb < 0.001, Cd < 0.020, Hg < 0.015, As < 0.030, Ni < 0.135, Cu < 0.090, Fe < 0.505, Cr < 0.060, Co < 0.135, expressed in ppm) were below the maximum levels established by the European Union legislation (9). This ω-3 DHA-rich oil obtained from microalgae was safer and had greater competitiveness and commercial advantage than marine fish DHA.

Our results demonstrated that crude glycerol is an adequate, cheap, and available substrate resource for producing ω-3 DHA-rich oil employing the microalgae *Aurantiochytrium* sp. in batch cultures. Applying simple culture strategies allowed increasing C_{TFA}, $C_{PUFA,}$ and C_{DHA} production in batch shake flasks, reducing culture costs by eliminating useless components and employing a cheaper and widely available regional waste, presenting identical substrate consumption and product yield rates as those using purified sugars. Our next challenge is to develop future assays for ω-3 DHA-rich oil production in bioreactor systems at 10X or 100X

scale factor. These experiences will allow the implementation an integrated process also able to evaluate other valuable wastes from regional agro-industries minimizing effluent production in a circular economy system.

Conclusions

Crude glycerol has great potential as substrate for ω-3 DHA-rich oil production using a locally isolated strain of *Aurantiochytrium* sp. in batch cultures. The largest initial inoculum (10.0% v/v) of *Aurantiochytrium* sp. improved DCW, practically exhausted glucose substrate, and enhanced P_{DHA}. Absent yeast extract in culture media enabled faster glucose metabolism in *Aurantiochytrium* sp. favoring lipid production and improving C_{TFA} and C_{DHA}. Longer culture times showed no differences in μ and DCW but decreased S_R and C_{DHA} due to *Aurantiochytrium* sp. consumed lipid reserves for cell maintenance. Better DCW performance was observed with BCG medium, with μ values similar to those reported with PMG medium. For glycerol cultures, the best C_{DHA} and P_{DHA} (1.84 g L^{-1} and 15.35 mg L^{-1} h^{-1}, respectively) were reached with BCG at 120 h, and S_R was higher than in glucose cultures. Quality assays showed ω-3 DHA-rich oil product is safer for human and animal food formulation. This study confirms that crude glycerol, without prior and expensive purification steps or conditioning treatment, constitutes a cheap and highly available carbon source for media formulation in *Aurantiochytrium* sp. cultures for ω-3 DHA-rich oil production. The next experiences for ω-3 DHA-rich oil production will focus on evaluating other wastes from regional agro-industries and developing scale-up assays in bioreactor systems.

References

1. Attarbachi, T.; Kingsley, M. D.; Spallina, V. 2023. New trends on crude glycerol purification: A review. Fuel 340: 127485. DOI: 10.1016/j.fuel.2023.127485
2. Band Schmidt, C. J. 2007. Aislamiento, purificación y mantenimiento de cepas de microalgas. In: Arredondo-Vega, B. O.; Voltolina, D. (Eds.), Métodos y herramientas analíticas en la evaluación de la biomasa microalgal. La Paz (Mexico), Centro de Investigaciones Biológicas del Noroeste. p. 1-11.
3. Bannon, C. D.; Breen, G. J.; Craske, J. D.; Hai, N. G.; Harper, N. L.; O'Rourke, K. L. 1982. Analysis of fatty acid methyl esters with high accuracy and reliability. III. Literature review of and investigations into the development of rapid procedures for the methoxide-catalysed methanolysis of fats and oils, J. Chromatogr. 247(1): 71-89. DOI: 10.1016/S0021-9673(00)84857-8
4. Bautista-Martínez, Y.; Granados-Rivera, L. D.; Jimenez-Ocampo, R.; Maldonado-Jáquez, J. A. 2024. Morphostructural composition and meat quality in local goat kids from the northeastern region of Mexico. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 56(1): 127-137. DOI: https://doi.org/10.48162/rev.39.129
5. Campoy, C.; Escolano-Margarit, M.; Anjos, T.; Szajewska, H.; Uauy, R. 2012. Omega-3 fatty acids on child growth, visual acuity neurodevelopment. Br. J. Nutr. 107: S85-106. DOI: 10.1017/S0007114512001493
6. Chauton, M. S.; Reitan, K. I.; Norsker, N. H.; Tveteras, R.; Kleivdal, H. T. 2015. A techno-economic analysis of industrial production of marine microalgae as a source of EPA and DHA-rich raw material for aquafeed: research challenges and possibilities. Aquaculture. 436: 95-103. DOI: 10.1016/j.aquaculture.2014.10.038
7. Chen, X.; Sen, B.; Zhang, S.; Bai, M.; He, Y.; Wang, G. 2021. Chemical and physical culture conditions significantly influence the cell mass and docosahexaenoic acid content of *Aurantiochytrium limacinum* strain PKU#SW8. Mar. Drugs. 19(12): 671. DOI: 10.3390/md19120671
8. Du, F.; Wang, Y. Z.; Xu, Y. S.; Shi, T. Q.; Liu, W. Z.; Sun, X. M.; Huang, H. 2021. Biotechnological production of lipid from thraustochytrids. Biotechnol. Adv. 48(8): 107725. DOI: 10.1016/j.biotechadv.2021.107725
9. EFSA Panel on Nutrition. Novel Foods and Food Allergens. 2020. Safety of *Schizochytrium* sp. oil as a novel food pursuant to regulation EU 2015/2283. EFSA J 18(10): e06242. DOI: 10.2903/j.efsa.2020.6242
10. Furlan, V.; Maus, V.; Batista, I.; Bandarra, N. 2017. Production of docosahexaenoic acid by *Aurantiochytrium* sp. ATCC PRA-276. Braz. J. Microbiol. 48(2): 359-365. DOI: 10.1016/j.bjm.2017.01.001

11. González-Martinez, A.; Angón, E.; González, M. A.; Rodríguez Tobar, J. M.; Barba Capote, C.; García Martínez, A. R. 2021. Effect of rearing system and sex on the composition and fatty acid profile of *Andinoacara rivulatus* meat from Ecuador. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 53(2): 232-242. DOI: https://doi.org/10.48162/rev.39.056
12. Hermida, L. G.; Gallardo, G. 2015. Food applications of microencapsulated omega-3 oils. In: Sagis L.M.C. (Ed.), Microencapsulation and microspheres for food applications. Academic Press. p. 271-299. DOI: 10.1016/B978-0-12-800350-3.00018-2
13. Janssen, C. I.; Kiliaan, A. J. 2014. Long-chain polyunsaturated fatty acids (LC-PUFA) from genesis to senescence: the influence of LC-PUFA on neural development, aging, and neurodegeneration, Prog. Lipid Res. 53: 1-17. DOI: 10.1016/j.plipres.2013.10.002
14. Kamal Eldin, A. 2010. Methods to determine the extent of lipid oxidation in foods. In: Decker, E.; Elias, R.; McClements, D. (Eds.). Oxidation in foods and beverages and antioxidant applications. Understanding mechanisms of oxidation and antioxidant activity. Sawston (UK), Woodhead Publishing. p. 181-195.
15. Li, J.; Liu, R.; Chang, G.; Li, X.; Chang, M.; Liu, Y.; Jin, Q.; Wang, X. 2015. A strategy for the highly efficient production of docosahexaenoic acid by *Aurantiochytrium limacinum* SR21 using glucose and glycerol as the mixed carbon sources. Biores. Technol. 177: 51-57. DOI: 10.1016/j.biortech.2014.11.046
16. Liu, Y.; Zhong, B.; Lawal, A. 2022. Recovery and utilization of crude glycerol, a biodiesel byproduct. RSC Adv. 12: 27997-28008. DOI: 10.1039/D2RA05090K
17. Lu, Q.; Li, H.; Xiao, Y.; Liu, H. 2021. A state-of-the-art review on the synthetic mechanisms, production technologies, and practical application of polyunsaturated fatty acids from microalgae. Algal Res. 55: 102281. DOI: 10.1016/j.algal.2021.102281
18. Mariam, I.; Kareya, M. S.; Nesamma, A. A.; Jutur, P. P. 2021. Delineating metabolomic changes in native isolate *Aurantiochytrium* for production of docosahexaenoic acid in presence of varying carbon substrates. Algal Res. 55: 102285. DOI: 10.1016/j.algal.2021.102285
19. Martínez-Reyes, I.; Chandel, N. S. 2020. Mitochondrial TCA cycle metabolites control physiology and disease. Nat. Commun. 11(1): 102. DOI: 10.1038/s41467-019-13668-3
20. Martins, D.; Custódio, L.; Barreira, L.; Pereira, H.; Ben-Hamadou, R.; Varela, J.; Abu-Salah, K. 2013. Alternative sources of n-3 long-chain polyunsaturated fatty acids in marine microalgae. Marine Drugs. 11(7): 2259-2281. DOI: 10.3390/md11072259
21. Nazir, Y.; Halima, H.; Al-Shorgani, N. K. N.; Manikan, V.; Hamid, A. A.; Song, Y. 2020. Efficient conversion of extracts from low-cost, rejected fruits for high-valued docosahexaenoic acid production by *Aurantiochytrium* sp. SW1. Algal Res. 50(5528): 101977. DOI: 10.1016/j.algal.2020.101977
22. Negro, E.; González, M. A.; Bernal, C. A.; Williner, M. R. 2016. Saturated and trans fatty acids content in unpackaged traditional bakery products in Santa Fe city, Argentina: nutrition labeling relevance. Int. J. Food Sciences Nutr. 68(5): 546-552. DOI: 10.1080/09637486.2016.1268100
23. Ochsenreitheri, K.; Gluck, C.; Stressler, T.; Fischer, L.; Syldatk, C. 2016. Production strategies and applications of microbial single cell oils. Front. Microbiol. 7: 1539. DOI: 10.3389/ fmicb.2016.01539
24. Oliver, L.; Dietrich, T.; Marañón, I.; Villarán, M.; Barrio, R. 2020. Producing Omega-3 polyunsaturated fatty acids: A review of sustainable sources and future trends for the EPA and DHA market. Resources 9(12): 148. DOI: 10.3390/resources9120148
25. Park, W. K.; Moon, M.; Shin, S. E.; Cho, J. M.; Suh, W. I.; Chang, Y. K.; Lee, B. 2018. Economical DHA (Docosahexaenoic acid) production from *Aurantiochytrium* sp. KRS101 using orange peel extract and low cost nitrogen sources. Algal Res. 29: 71-79. DOI: 10.1016/j.algal.2017.11.017
26. Patel, A.; Rova, U.; Christakopoulos, P.; Matsakas, L. 2020. Mining of squalene as a value-added byproduct from DHA producing marine thraustochytrid cultivated on food waste hydrolysate, Sci. Total Environ. 736: 139691. DOI: 10.1016/j.scitotenv.2020.139691
27. Patterson, H. B. W. 2011. Quality and control. In: List, G.R.; King, J.W. (Eds.). Hydrogenation of fats and oils. Theory and practice. AOCS Press. p 329-350.
28. Pleissner, D.; Lam, W. C.; Sun, Z.; Lin, C. S. 2018. Food waste as nutrient source in heterotrophic microalgae cultivation. Bioresour. Technol. 137: 139-146. DOI: 10.1016/j.biortech.2013.03.088
29. Plua Montiel, J.; Neira Mosquera, J. A.; Sanchez Llaguno, S. N.; Aldas Morejon, J. P.; Revilla Escobar, K. Y.; Caicedo-Álvarez, E. (en prensa). omparison of fatty acid profiles of sacha inchi oil (*Plukenetia huayllabambana*), sesame oil (*Sesamum indicum*), and peanut oil (*Arachis hypogaea*) using two extraction methods for food purposes. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina.
30. Rosa, S.; Soria, M.; Vélez, C.; Galvano, M. 2010. Improvement of a two-stage fermentation process for docosahexaenoic acid production by *Aurantiochytrium limacinum* SR21 apply statistical experimental designs and data analysis, Biores. Technol. 101(7): 2367-2374. DOI: 10.1016/j.biortech.2009.11.056
31. Santos Jesus, S.; Maciel Filho, R. 2010. Modeling growth of microalgae *Dunaliella salina* under different nutritional conditions. Am. J. Biochem. Biotechnol. 6(4): 279-283. DOI: 10.3844/ajbbsp.2010.279.283

32. Sartori, M.; García, D.; Grossi Vanacore, M. F.; Fessia, A.; Nesci, A. (en prensa). Biofungicide formulation based on *Bacillus velezensis* EM-A8 for control of maize foliar diseases. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina.
33. Siriwardhana, N.; Kalupahana, N. S.; Moustaid-Moussa, N. 2012. Health benefits of n-3 polyunsaturated fatty acids: eicosapentaenoic acid and docosahexaenoic acid. Adv. Food Nutr. Res. 65: 211-222. DOI: 10.1016/B978-0-12-416003-3.00013-5
34. Verified Market Research. 2023. Global glycerin market size. Report ID 17400. 202 p. www.verifiedmarketresearch.com/product/glycerin-market. Accessed December 2023.
35. Vonshak, A. 1986. Laboratory techniques for the cultivation of microalgae. In: Richmond, A. (Ed.), Handbook of microalgal mass culture. CRC Press. p. 117-145.
36. Wolff, R. L. 1995. Content and distribution of trans-18:1 acids in ruminant milk and meat fats. Their importance in European diets and their effect on human milk. J. Am. Oil Chem. Soc. 72(1): 259-272. DOI: 10.1007/BF02541081
37. Yu, X. J.; Liu, J. H.; Sun, J.; Zheng, J. Y.; Zhang, Y. J.; Wang, Z. 2016. Docosahexaenoic acid production from the acidic hydrolysate of Jerusalem artichoke by an efficient sugar-utilizing Aurantiochytrium sp. YLH70. Ind. Crops Prod. 83: 372-378. DOI: 10.1016/j.indcrop.2016.01.013

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