



Research article

Characterization and comparison of lipid and PUFA production by native thraustochytrid strains using complex carbon sources



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ABSTRACT

The thraustochytrid are marine heterotrophic protists that are widely distributed in the marine world. They are characterized by producing and accumulating great amount of lipids in their cells, especially long chain polyunsaturated fatty acids (LC-PUFA), highlighting the docosahexaenoic acid (DHA, 22:6, n-3), eicosapentaenoic acid (EPA, 20:5, n-3) and arachidonic acid (ARA, 20:4, n-6), as well as pigments of interest for human health and animal nutrition, such as carotenoids. Therefore, the objective of this study was to isolate and characterize three natives isolated of thraustochytrids and assess the potential of the by-products of the manufacture of beer (RB) and protein extraction of Lupine flour (RL) as complex carbon sources to produce biomass, lipid and polyunsaturated fatty acids. Three native strains of thraustochytrid (AS5-B2, IQ81 y VAL-B1), isolated from Chilean coastal waters were morphologically and genetically identified as thraustochytrid. For the determination of biomass production cultures were quantified by gravimetry and the fatty acids quantification and identification were carried out by gas chromatography (GC-FID). Our results show that the culture with any sources of complex carbon used, increased significantly the production of both biomass and total lipids in the strains IQ81 and VAL-B1, compared to glucose as pure carbon source. On the other hand, strain AS5-B2 showed a decrease in the total production of lipids in RB compared to the pure carbon source. For the production of fatty acids, the strains IQ81 and VAL-B1 showed a significant increase in DHA when growing in RB. In conclusion strains IQ81 and VAL-B1 can be used to biotransform industrial waste, such as RB and RL, into a more valuable product such as DHA, EPA, ARA and lipids.

1. Introduction

A sustainable food industry depends on improving the efficiency in the use of biomass produced. The Food and Agriculture Organization (FAO) of the United Nations estimated that 18% of all food produced in the world was lost or waste in 2019 (FAO, 2019). In Latin America it has been estimated that annually an average of 201 kg/per capita of food waste are produced (FAO, 2019). One of the strategies to reduce the loss of biomass is using the by-products of the food industry as raw materials to produce other products of interest such as methane, ethanol or nutraceuticals to name a few. Some microorganism can be used to transform agroindustry by-products, cheap and full of nutrients, into more valuable products.

The development of agroindustry brings an increase in the generation and variety of by-products containing significant amounts of carbon and nitrogen that are not used. Its inadequate disposal could be an environmental threat, increasing the contamination of water and

the uncontrolled release of methane. Thus, in recent years, the interest for converting residues and by-products of the food industry into products of added value has been promoted (Thyagarajan et al., 2014; Silva et al., 2015; Caamaño et al., 2017; Shene et al., 2018; Villarroel and Silva, 2018; Sato et al., 2019; Kothri et al., 2020; Singh et al., 2020).

One of these products with higher added value are bioactive compounds such as vitamins (provitamin A, vitamins B12, B6, biotin), phyosterols, terpenes, eicosanoids, carotenoids and polyunsaturated fatty acids (PUFA) among others (Volkman, 2003; Quilodrán et al., 2010; Miranda et al., 2020; Singh et al., 2020). From the commercial standpoint PUFA, specifically DHA, docosapentaenoic acid (DPA, 22:5 n-3), EPA, ARA and γ -linolenic acid (GLA, 18:3 n-6), have a potential application as nutraceuticals, pharmaceuticals and food ingredients (Lewis et al., 1999, 2000a) due to their ability to prevent various diseases in human as well as in animals. These fatty acids are essential for many animals and it must be obtained through the diet.

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DHA is a long-chain polyunsaturated fatty acid of marine origin fundamental for the formation and function of the nervous system, particularly the brain and the retina of humans. DHA is considered a critical nutrient during pregnancy and breastfeeding due their active participation in the development of the nervous system in early life (Echeverría et al., 2017). Another study demonstrated that the supplementation of 420 full term newborn children with n-3 LCPUFA (daily, 250 mg of DHA and 60 mg of EPA) from their birth until six months, showed a significant accretion in the DHA content of erythrocyte phospholipids and an early development of language and communication skills (Meldrum et al., 2012). This shows that DHA and EPA are necessary as supplements in the diet of both infants and their mothers, for good development and as protectors against degenerative diseases.

Marine microorganisms such as bacteria, algae, fungi and thraustochytrids has been proposed as a source of PUFA alternative to fish oil, which is nowadays widely used as a source of long chain PUFAs (LC-PUFAs) (Damare & Raghukumar, 2008; Ratledge, 2013; Adarme-Vega et al., 2014; Soressa et al., 2014; Manikan et al., 2015; Tocher, 2015; Byreddy et al., 2016; Silva et al., 2017).

Thraustochytrids (TH) are oleaginous protists microorganisms, that belong to Phylum Heterokonts (Labyrinthulomycota), Class Thraustochytridae (Labyrinthulomycetes), Order Thraustochytriales, Family Thraustochytriaceae including Genus *Thraustochytrium*, *Ulkenia*, *Aurantochytrium* (formerly known as *Schizochytrium*), *Japonochytrium*, *Althornia*, *Aplanochytrium* (Raghukumar, 2002; Yokohama and Honda, 2007). TH have a globose sporangium wall with a multi-layer laminate, which are immobile except in the biflagellate spore stage (Leander et al., 2004). TH are known because they high content of LC-PUFA, DHA and DPA.

Their lipid accumulation is a multifactorial process, which depends on the organism, culture conditions and the growth phase. Some TH can be grown to produce high quantity of biomass with considerable amounts of lipids rich in LC-PUFAs, the production of LC-PUFAs can be modified by manipulating the physical and chemical parameters of the culture medium (mainly: pH, temperature, nutrients, salinity and aeration) (Lewis et al., 2000a).

The TH play an important role in decomposition of organic macromolecules, such as those produced by macrophytes, or refractory substrates such as cell walls through the production of highly degradative enzymes (Santangelo et al., 2000). In addition, some TH are associated with bio-erosion of carbonate materials that are important parts of reef structure (Porter and Lingle, 1992).

These microorganisms have been found in different marine and estuarine habitats including live algae, marine detritus, phytoplankton aggregates in invertebrates into the water column of the Mediterranean, Atlantic Ocean, the North Sea, Arabian Sea, coastal waters of Japan and India and also in coastal waters of Argentina and Chile (Raghukumar, 2002; Raghukumar et al., 2000, 2001; Nagayama et al., 1998; Bremer, 2000; Mo et al., 2002; Rosa et al., 2006, 2011; Hinzpeter et al., 2009; Silva et al., 2015; Pino, 2013; Shene et al., 2013, 2018; Pino et al., 2015; Gupta et al., 2016; Caamaño et al., 2017). The TH can be isolated from various substrates and appear to be abundant in the sediments of coastal and estuarine environment (Raghukumar & Raghukumar, 1999; Bongiorno, 1998, 2012; Bongiorno et al., 2005; Singh et al., 2014; Caamaño et al., 2017). They have been found in diverse habitats such as the deep sea and anoxic waters. Many species seem to be substrate-specific in their occurrence. Some of the most common habitats are decaying mangrove leaves, decomposing algae and fecal pellets of marine invertebrates (Raghukumar and Damare, 2011; Silva et al., 2015; Kothri et al., 2020), coinciding though with higher sampling effort in these habitats.

The aim of this study was to isolate, characterize and evaluate the ability of new strains of TH to use the by-products of the protein extraction of lupine flour and the brewery as complex carbon sources to produce biomass, lipids and polyunsaturated fatty acids.

2. Materials and methods

2.1. Chemicals

Monosodium glutamate (MSG), glucose, ethanol and methanol, were obtained from Merck (Darmstadt, Germany). Peptone was obtained from Oxoid (Basingstoke, Hampshire, England). Yeast extract and agar were obtained from Becton, Dickinson and Co. (Sparks, MD, USA). Streptomycin sulphate, penicillin G, thiamine, biotin and cobalamin, were obtained from Sigma–Aldrich Co. (Steinheim, Germany). Deoxynucleotide mix and DNA polymerase were obtained from Biotools B&M Labs (SA, Spain). FAME standards (Mix L209, FAME C20:0, C20:1, C20:4, C22:1, C22:6) was obtained from Alltech (USA). Composition of artificial sea water (ASW) for 1 L was: NaCl 27.50 g, MgCl₂×6H₂O 5.38 g, MgSO₄×7H₂O 6.78 g, KCl 0.72 g, NaHCO₃ 0.20 g and CaCl₂×2H₂O 1.40 g.

2.2. By-products of the brewery and protein extraction lupine

Lupine flour protein was extracted by ultrafiltration and isoelectric precipitation. Using the isoelectric precipitation method was obtained as by-product the supernatant liquid (RL). This by-product was used in the cultures. The brewery by-product (RB) was the filtrate obtained from wet brewer grains obtained after extracting the mashed malted grains. The liquids were filtered with cheesecloth.

2.3. Microorganisms isolation

The isolation was performed using the technique of pine pollen to fix microorganisms which are morphologically characteristic of TH. The isolation was performed on a series of 10 test tubes with sterile pine pollen and incubated at 25 °C (Stove QUIMIS incubation) for a period of 5–10 days before observe by microscopy (OLYMPUS Optical – Epifluorescence Arquimed INNOVATION). To continue the insulations pollen grains, containing microorganisms adhered, were removed from the test tubes with inoculating loop and streaked on solid medium (Agar TH: Yeast extract 2 g/L MO Bio yeast extract bacteriological grade, peptone 2 g/L BD Bactotm Peptone agar 10 g/L BD Difco TM granulated agar) all diluted in artificial seawater with a concentration of 29–31 ppm salinity and setting pH 6.5. To eliminate the bacterial flora resident in the sample 1 mL of sodium penicillin and streptomycin (Calbiochem) 1.5 mg/mL were incorporated into 50 mL culture under the same conditions of temperature (25 °C) for 5 days and performing successive platings grooves to obtain isolated colonies.

2.4. DNA extraction and genetics analysis

The TH organisms (isolated AS5-B2, IQ81 and VAL-B1) were isolated from samples collected from Chilean coast. The isolated were cultivated in 100 mL of growth medium at 25 °C and 180 RPM for 48–72 h. Cells were precipitated by centrifugation at 6000 g for 10 min, lyophilized and keep freeze until it uses.

2.5. DNA extraction

DNA extraction was performed starting from 60–100 mg of freeze sample, using the E.Z.N.A. Tissue DNA kit (Omega Bio-Tek), according to the manufacturer. The final elution was performed with 200 µL of molecular biology degree water preheated to 70 °C.

2.6. DNA amplification and sequencing

In order to sequence the 18S RNA we performed a PCR amplification (0.2 mM each dNTPs, 1.6 mM MgCl₂, Taq pol 0.012 U/µL and 0.2 µM each primer) using 100–200 ng DNA as templated and the oligonucleotides GC 37 and GC 38 (5'-GCCATGCATGTSTAAGTATAAGC-3' and 5'-

TTCAATCGGTAGGTGCGAC-3', respectively) for isolated AS5-B2 and IQ81; oligonucleotides GC46 and GC47 (5'-TAAGCCATGCATGTG-TAAGTATAAG-3' and 5'-TTCCTCTAAACAATAAGATTCACCCG-3', respectively) for isolated VAL-B1 were used. The PCR amplification program was 94 °C for 60 s, 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 120 s. The final extension was at 72 °C for 5 min. The PCR reactions were confirmed by agar TAE electrophoresis. The sequencing reaction was performed in both strands by Macrogen.

2.7. Culture conditions

The inoculum was prepared transferring cells from agar plates to 50 mL of sterile B2 medium (for 1 L: glucose (20 g), YE (2 g), MSG (2 g), ASW at 29%). Incubation was carried out for 48 h at 25 °C in shaking flasks (180 rpm). Sterile growth medium (100 mL) inoculated with 5% v/v was incubated at 25 °C in shaking flasks, for 3 or 5 days. In the experiments carried out to test the effect of the carbon source, growth medium contained either YE (2 g/L) or YE-MSG (both at 2 g/L) and ASW at 29%. Concentration of glucose, 20 g/L. Fermentation of glucose-YE-MSG was carried out as control medium. Solutions of carbon and nitrogen sources were sterilized (121 °C, 15 min) separately and mixed to give the required concentration. Before sterilization, pH of the media was fixed (NaOH or HCl) at 7.0, unless specified. Results are presented as mean \pm standard deviation of triplicate assays. Data for the growth curves was obtained from two flasks that were taken out every 24 h for analysis. Results are presented as mean \pm standard deviation of duplicate assays.

2.8. Analytical methods

2.8.1. Biomass and residual carbohydrate concentration

Biomass was recovered by centrifuging (4,000 g, 10 min, 4 °C), washed three times with sterile distilled water, frozen, lyophilized and its weight gravimetrically determined. Concentration of residual carbohydrate was determined using the anthrone method.

2.8.2. Total lipids

Extraction method Bligh & Dyer modified. A portion of lyophilized biomass of 50 mg was weighed and incorporated in a test tube with screw cap, initially adding chloroform-methanol (2:1), shaken vigorously on a vortex and sonicated, adding chloroform, water and stir again with vortex. Tubes were centrifuged (refrigerated centrifuge, HERMLE make, model Z 326 K) at 4000 rpm, and keep the organic phase. The extract was filtered and then lead to drying at 104 °C for 1 h to constant weight by gravimetric method.

2.8.3. Fatty acid analysis

Lyophilized biomass (20–50 mg) was used for direct transesterification. This method referred by Lewis et al. (2000b) and modified by Burja et al. (2007). The FAMES were extracted into hexane and performed on an Agilent 7890 series gas chromatograph equipped with auto sampler, split injection, 4 mm ID inlet sleeve with glass wool plug, and flame ionization detector (FID) (Agilent, Alpharetta, GA, USA) set at 280 °C with an inlet temperature of 250 °C. The initial temperature of the oven started at 50 °C was held for 1 min, followed by temperature programming at 25 °C min⁻¹ to 175 °C then at 4 °C min⁻¹ to 230 °C where it was held for 5 min. The capillary column used was a DB-23, 30 m \times 0.25 mm ID \times 0.15 μ m film thickness, with a temperature range of 20–250 °C (Chromatographic Specialties, Brockville, Canada). Injections of 1 μ L of sample were used with a 50:1 split. The hot needle technique was used. For this the syringe was left inside the injector for 5 s, then the injection was quickly performed; the needle was kept inside the injector for 5 s. The column was held at 50 °C for one minute after injection, then ramped at 25 °C min⁻¹ to a final temperature of 230 °C and held for 5 min for a total run-time of 25 min. Helium was used as the carrier

gas at a flow rate of 30 mL min⁻¹ and H₂ gas and air detectors a flow rate of 40 mL min⁻¹ and 450 mL min⁻¹ respectively. The quantification of fatty acids was done by applying the area normalization and standard calibration methods (external standard method; Sigma-Aldrich Australia standard). For this purpose, the ChemStation chromatographic software (Agilent Technologies, US) was used and the areas were corrected by a response factor of each component, which was calculated and applied by it. Results are presented as mean \pm SD duplicates repeated twice.

2.9. Statistical analysis

The treatment of cultured TH strains with different carbon sources were performed in duplicate or triplicate as well as their controls. The results are expressed as mean \pm standard deviation. Statistical evaluation was performed with the software package GraphPadPrism 5 (GraphPad, La Jolla, CA, USA). The data normality was checked by D'Agostino test. The data of the parameters analyzed were transformed to a logarithmic scale prior to analysis because they did not pass the normality test. Biomass production, levels of DHA, EPA and lipids were assessed by analysis of variance one-way (ANOVA). A value less than 0.05 ($p < 0.05$) was considered statistically significant. The yield coefficients for biomass and products on total carbohydrates were calculated from the mass ratio between synthesized product and consumed carbohydrates.

3. Results

3.1. Characterization of chemical and proximal liquid waste

Characterization of by-products in their proximal composition presents significant differences ($p < 0.05$) in the protein percentage, no nitrogenous extracts (N.N.E) and total carbohydrate (CH), parameters that could have an effect on the C/N ratio to be submitted by culture media of thraustochytrids micro-organisms for their growth. In the two culture media consisting of RL and RB ion phosphate with significant difference is present ($p < 0.05$) in its concentration, RB has the largest concentration of this ion. The determined trace elements are found in the two-complex media RL and RB, with copper being present in lower concentration and magnesium in higher concentration, which could influence cell growth.

3.2. Morphological analysis and genetic identification

To identify the taxa corresponding to the isolated AS5-B2, IQ81 and VAL-B1, we performed a phylogenetic analysis using the Neighbor-Joining method of the *18S RNA* gene. In this analysis the isolated IQ81 and Val-B1 were identify corresponding to *Thraustochytrium Kinney*, while AS5-B2 correspond to *Aurantiocytrium sp.* (Figure 1).

3.3. Determination of the composition of fatty acids in different culture media

Table 2 shows that all strains contain DHA, and that ARA was not detected in AS5-B2 strain in the three-culture media used. In the sum of the LC-PUFAs the highest percentage corresponds to the VAL-B1 strain in the different culture media used, the greater percentage of unsaturated fatty acid that includes C18:1, C18:2 and C18:3 it presents the AS5-B2 strain in the three-culture media followed by the IQ81 strain. The lowest percentage value for the total of the unsaturated fatty acid corresponds to the VAL-B1 strain culture on glucose. On the other hand, the composition of saturated fatty acid produced by the strains IQ81 and VAL-B1 are similar when cultured in any of the three culture media previously described.

3.4. Effect of the composition of the culture media in the biomass production and total lipids

Knowing the fatty acids profile of the three native strains cultured with different carbon sources; glucose, by-product of beer brewing and by-product of the manufacture of lupine flour and according to previous studies, cultures were performed for 5 days to determine the concentrations of the three LC-PUFAs of interest and the concentration of the total lipids in the biomass. The Figures 2, 3, 4 and 5 show how change the concentration of biomass, DHA, EPA and total lipids. The LC-PUFA ARA for AS5-B2 strains were not detectable, however, IQ81 and VAL-B1 strains were detectable but not quantifiable because of their low concentrations at the 5 days of culture.

3.5. Effect of complex carbon sources on the biomass production

The results show a statistically significant increase in the production of biomass to the 5th day of culture at 25 °C with both complex carbon sources (RB & RL) in IQ81 and VAL-B1 strains, compared with the pure carbon source (Figure 4b, c). However, in the AS5-B2 strain a statistically significant increase is not observed using complex carbon sources (Figure 4a).

3.6. Effect of complex carbon sources on the production of lipids

Figure 5 shows statistically significant differences in the production of lipids in each of the evaluated strains at the 5th day of culture at 25 °C. In the case of the AS5-B2 strain a decrease significant of the production of

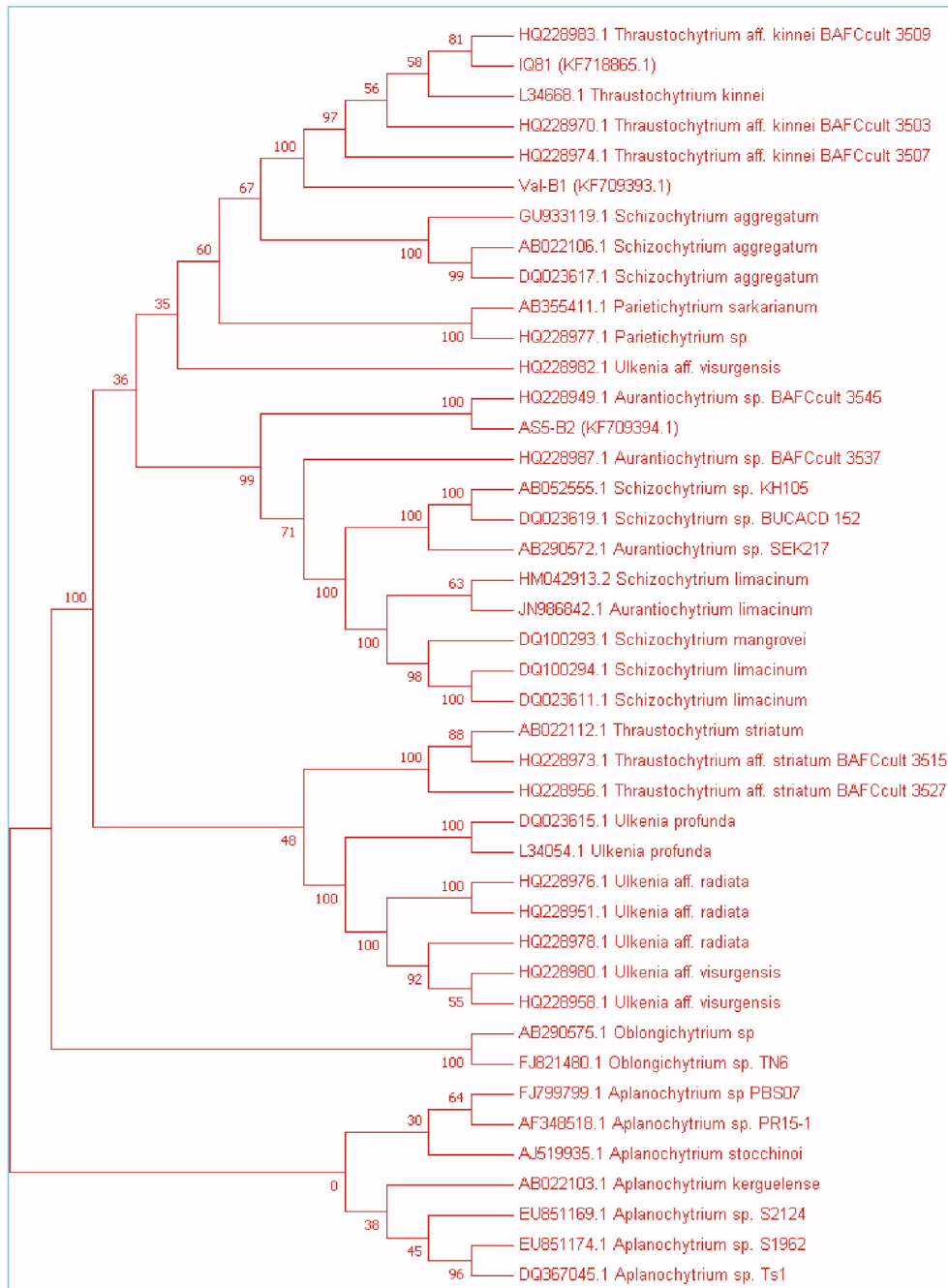


Figure 1. Evolutionary relationships of taxa.

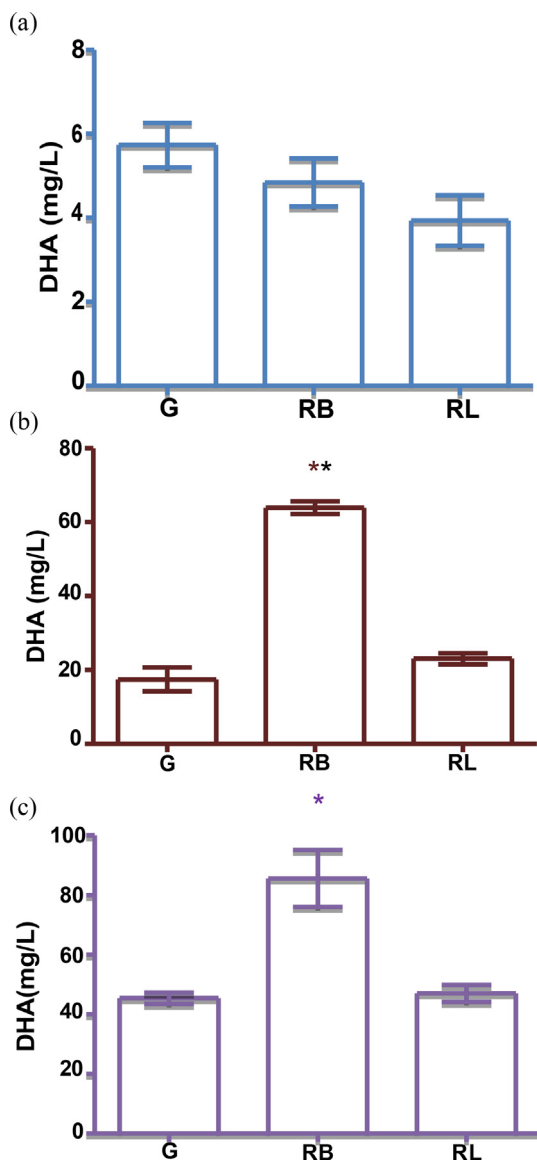


Figure 2. Evaluation of the production of DHA to the 5th culture day of: (a) AS5-B2, (b) IQ81 and (c) VAL-B1 strains. The strains were cultivated using G, RB and RL. (*) $p < 0.05$; (**) $p < 0.01$ both compared with respect to glucose control treatment.

lipids with the source of carbon complex RB is observed, with regard to the source of pure carbon (Figure 5a). However, the IQ81 and VAL-B1 strains, a significant increase in the production of lipids with both complex carbon sources (RL and RB) compared with the source of pure carbon was observed, under the same conditions of cultivation (Figure 5b, c). These results suggest that the application of alternative carbon sources has a positive impact on the production of lipids in the different strains studied, especially RL.

Figures 6, 7, and 8 show that to the 5th day of culture, the three strains increased the levels of LC-PUFAs with maximum RB consumption expressed as total carbohydrate. However, a variation of the parameters in the performance between the three strains studied was observed. Compared to the other two strains in study, AS5-B2 strain displayed the lowest values of productivity ($P_{DHA} = 1.116$ mg/L/d), biomass performance ($Y_{biom/CH} = 0.318$ g/g) and DHA ($Y_{DHA/CH} = 0.3791$ mg/g) performance based on the consumption of carbohydrates. The IQ81 strain have a yield of biomass of 0.420 g/g and VAL-B1 a yield of biomass corresponding to CH of 0.549 g/g. On the other hand, the performance of DHA for these two strains was $Y_{DHA/CH} =$

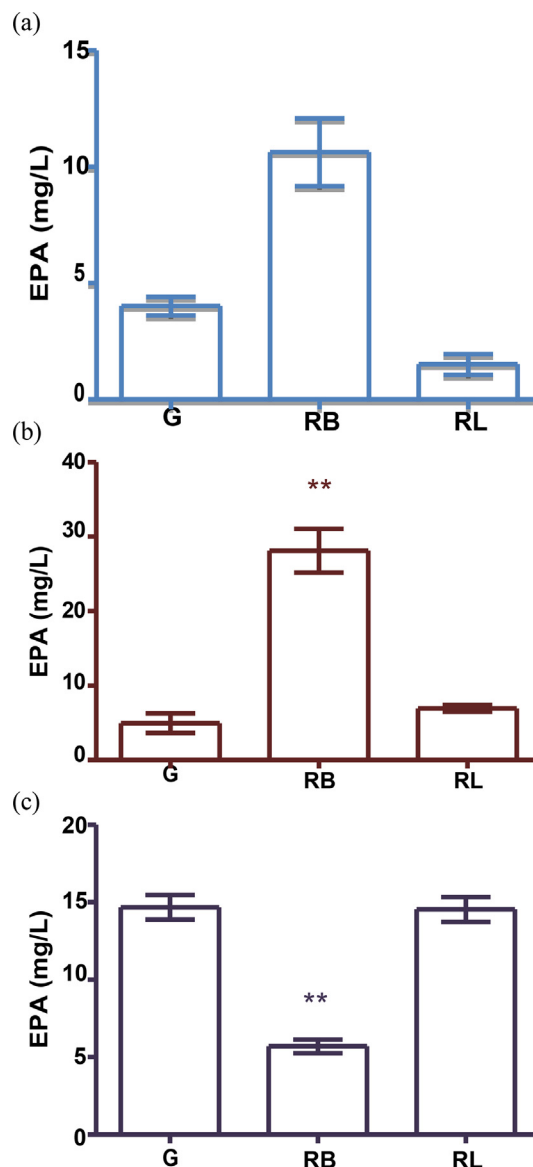


Figure 3. The evaluation of the production of EPA to the 5th day of culture the AS5-B2 (a), IQ81 (b) and VAL-B1 (c). Strains were cultured using G, RB and RL. (*) $p < 0.05$; (**) $p < 0.01$ both compared with respect to glucose control treatment.

6.298 mg/g, and $Y_{DHA/CH} = 10.217$ mg/g, respectively. Finally, IQ81 and VAL-B1 strains presented similar productivity values, corresponding to $P_{DHA} = 18.66$ DHA mg/L/d for IQ81 and $P_{DHA} = 21.20$ mg/L/d for VAL-B1. Figures 6, 7, and 8 show that to the 5th day of culture, the three strains increased the levels of LC-PUFAs with maximum RB consumption expressed as total carbohydrate. However, a variation of the parameters in the performance between the three strains studied was observed. Compared to the other two strains in study, AS5-B2 strain displayed the lowest values of productivity ($P_{DHA} = 1.116$ mg/L/d), biomass performance ($Y_{biom/CH} = 0.318$ g/g) and DHA ($Y_{DHA/CH} = 0.3791$ mg/g) performance based on the consumption of carbohydrates. The IQ81 strain have a yield of biomass of 0.420 g/g and VAL-B1 a yield of biomass corresponding to CH of 0.549 g/g. On the other hand, the performance of DHA for these two strains was $Y_{DHA/CH} = 6.298$ mg/g, and $Y_{DHA/CH} = 10.217$ mg/g, respectively. Finally, IQ81 and VAL-B1 strains presented similar productivity values, corresponding to $P_{DHA} = 18.66$ DHA mg/L/d for IQ81 and $P_{DHA} = 21.20$ mg/L/d for VAL-B1.

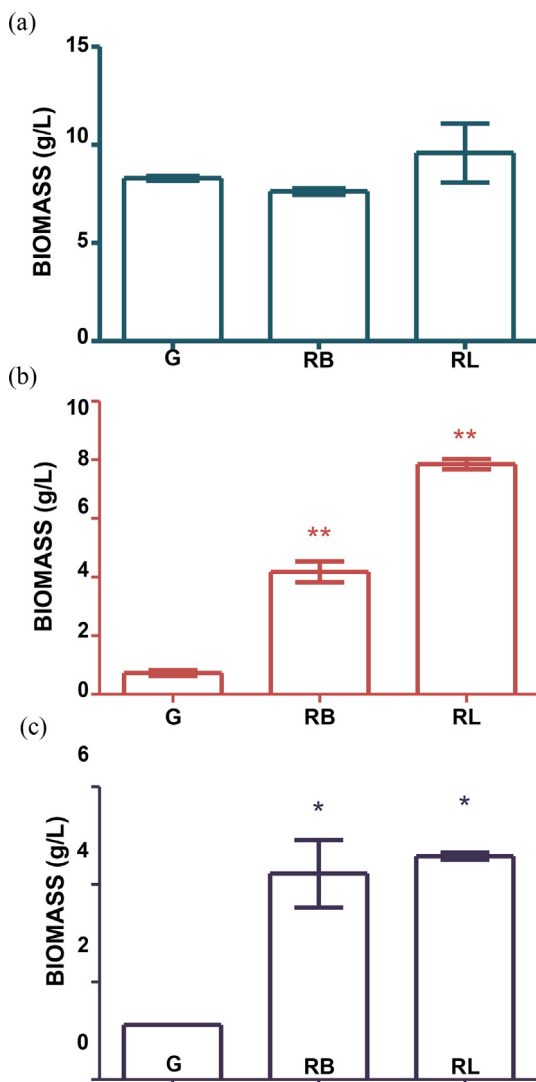


Figure 4. The evaluation of biomass production to the 5th culture day of: (a) AS5-B2, (b) IQ81 and (c) VAL-B1 strains. The strains were cultivated using glucose (G) and beer residue (RB) or lupine residue (RL). (*) $p < 0.05$; (**) $p < 0.01$ both compared with respect to glucose control treatment.

4. Discussion

The by-products from the food industry, food waste and sludge can be used as substrates for microbial growth to upgrade their nutritive value such as DHA, lipids, biomass-production. Our screening experiments showed that the strain *Aurantiochytrium sp.*, AS5-B2 and *Thraustochytrium sp.*, IQ81 are potential producer of lipids using any of the two by-products, further isolated *Thraustochytrium Kinney*, VAL-B1, is a potential DHA producer. The three isolated identified as TH strains were grown in different media, the composition of fatty acids does not vary with the environment, the percentage share of each fatty acid in each strain, presents differences associated with the carbon source used.

The AS5-B2 strain produces the highest concentration of total lipids in all the culture media, being the RL the main one. These results suggest that this strain grown in RL can be used to biofuel production rather than a LC-PUFA producer in the media tested.

4.1. Determination of fatty acid composition in the various culture media

In the determination the composition of the fatty acids in the different culture media for AS5-B2, IQ81 and VAL-B1 strains it can be seen that

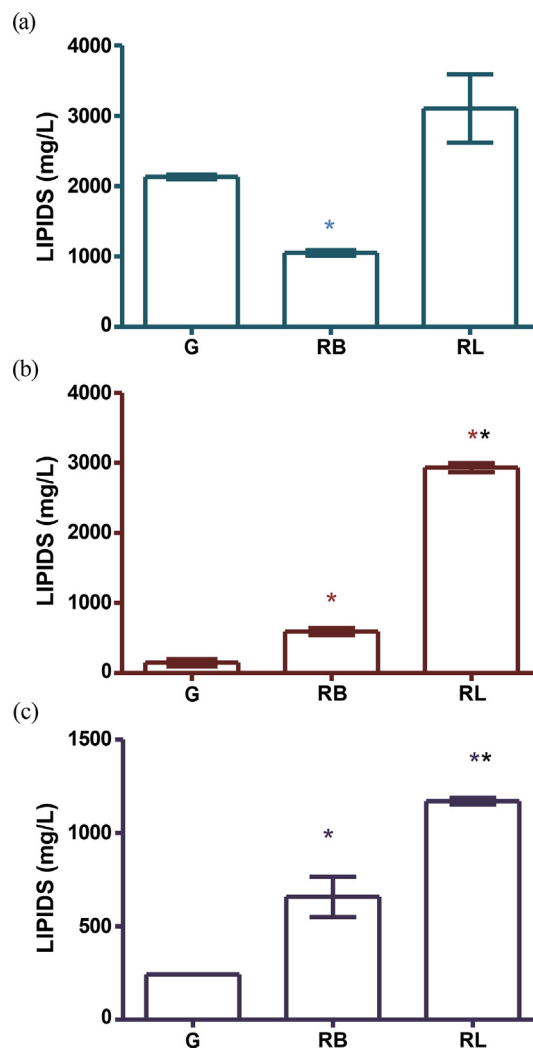


Figure 5. Evaluation of the production of lipids to the 5th culture day of: (a) AS5-B2, (b) IQ81 and (c) VAL-B1 strains. The strains were cultivated using G, RB and RL. (*) $p < 0.05$; (**) $p < 0.01$ both compared with respect to glucose control treatment.

certain fatty acids are favored by the use of residues as a culture medium. For example, AS5-B2 strain increases by more than 67% linoleic acid (18:2) when using RL compared with RB or G (Table 2). In addition, we get the greatest value of linolenic acid (18:3) present for the three strains ($13.14 \pm 0, 24\%$), when RL is also used. This fatty acid is an omega-3 of commercial importance, due to it has important application as antioxidant in food and drug industry. The presence of this fatty acid is also interesting, since today has been proven that supplementing the culture medium with it, increased the biomass production, as well as the biosynthesis of others fatty acids and PUFAs (especially DHA) while increase the antioxidant capacity. In culture of *Schizochytrium sp.* supplemented with 0.05% of 18:3 rich flaxseed oil, achieving a significant DHA increased of up to 100.3 ± 4 mg/g biomass (Gaffney et al., 2014).

With the IQ81 strain, the high presence of linoleic acid in the three different mediums employed is standee out (Table 2). It is relevant to mention that on average, 18:2 is present at 35.76%, remaining relatively stable for the three experiments. However comparing the two-waste used, the greater presence of fatty acid in RB ($36.28 \pm 6.90\%$) can be seen, which differs with respect to the same fatty acid in AS5-B2, because even though the value is very similar ($36.57 \pm 7.2\%$) the greatest abundance occurs in RL. In the case of the RL values of 18:2 can be explained by the chemical composition of the lupine, because that is a legume that has high values of certain fatty acids, including linoleic acid;

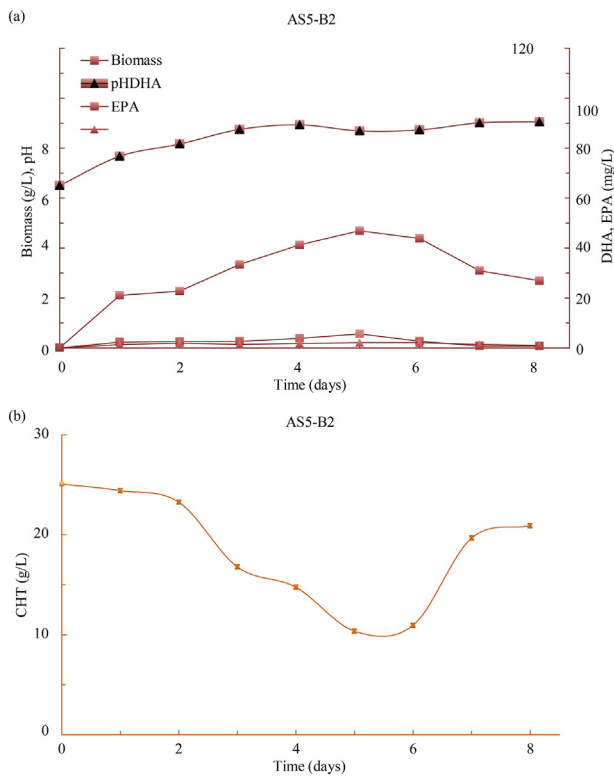


Figure 6. (a) Production of biomass, DHA, EPA and variation of pH of the culture medium based on RB by the strain AS5-2. (b) The complex carbon source such as carbohydrate consumption.

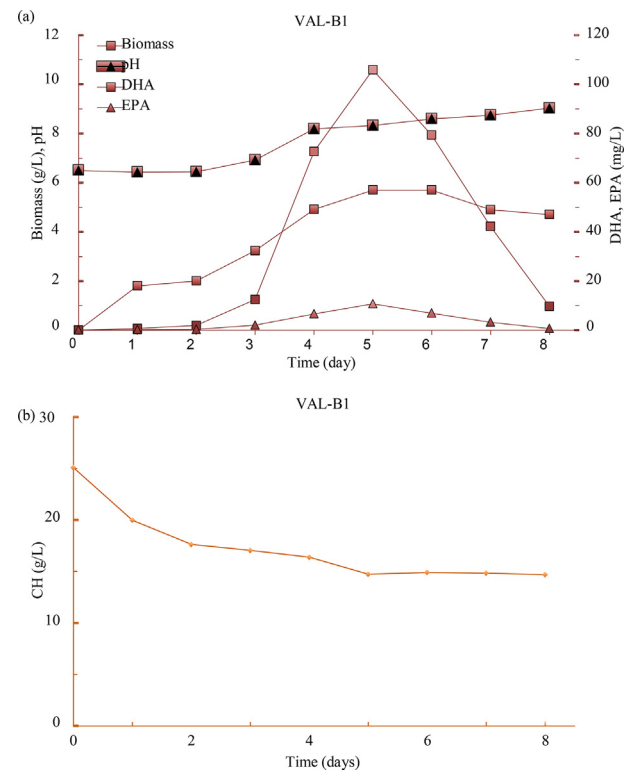


Figure 8. (a) Production of biomass, DHA, EPA, and variation of pH of the culture medium based on RB by the strain VAL-B1. (b) The complex carbon source such as carbohydrate consumption.

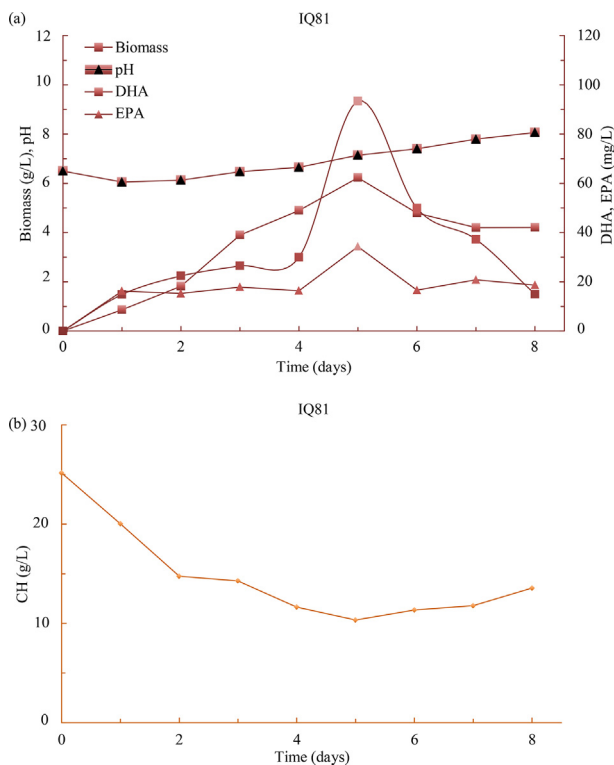


Figure 7. (a) Production of biomass, DHA, EPA and variation of pH of the culture medium based on RB by the strain IQ81. (b) The complex carbon source such as carbohydrate consumption.

a situation totally different for the RB case. The strain IQ81 is efficient in synthesize 18:2 fatty acid from RB as a carbon source; but still it does not reach the VAL-B1 level that obtained $49.31 \pm 1.09\%$ of 18:2 when using residual glycerol (Silva et al., 2015).

However, what most stands out with respect to the production of fatty acids are the values obtained for PUFAs (Table 2). Here, the production of DHA for VAL-B1 is remarkable with glucose as carbon source ($43.40 \pm 7.7\%$). Nevertheless, when residues are compared, it can see that RB generates higher DHA ($25.07 \pm 4.71\%$) and EPA ($3.67 \pm 0.68\%$), two omega-3 fatty acids of great importance today. These two PUFAs are remarkably observed in VAL-B1 with respect to the other two strains in this study, in which the values of DHA and EPA are very low, and could therefore be considered that AS5-B2 and IQ81 could be used better for biofuels production, due to their high content of saturated and unsaturated Fatty acid (16:0; 18:1 and 18:2, respectively).

By the other hand, the high values of PUFAs of VAL-B1 (30.19%) obtained with RB made this strain could be used for the production of DHA and EPA, two LC-PUFA important from the point view clinical (Valenzuela et al., 2019) because they have a positive effect on the decrease of hepatic steatosis. In addition, the production levels are equivalent to that observed in others strains: *Aurantochytrium* sp. genus 37.8% DHA (Lee Chang et al., 2015) using crude glycerol as carbon source; *A. limacinum* SR21 produced 34.3% DHA (Liang et al., 2010) using juice from sorghum, *Thraustochytrium* sp. AH-2 produced 23.74% of DHA (Thyagarajan et al., 2014) with bread crumbs in submerged substrate and *Thraustochytrium* Kinney VAL-B1 generated 35.06% of DHA (Silva et al., 2017) with residual glycerol. Recently Nazir et al. (2020) generated extracts with rejected pineapple and banana fruits, and used them as a substrate for *Aurantochytrium* SW1 producing 44.86% DHA, being one of the highest values found so far.

Table 1. Proximal chemical analysis of liquid waste used for the growth of native strains of TH. Liquids waste: by-product of protein extraction lupine flour, RL; by-product of brewing (filtering bagasse), RB.

RL	RB	
Solids (% [*])	3.58	3.22
Ash (% ^{**})	13.12	5.9
Protein (% ^{**})	38.56	7.8
Fats (% ^{**})	0.39	1.2
N.N.E (% ^{**})	52.3	85.1
CH (g/L)	17.3	29.4
CDO (mg/L)	14,000	37,800
Phosphate (mg/L)	113.2	295.2
Mg (mg/L)	1.2	0.87
Mn (mg/L)	0.13	0.052
Fe (mg/L)	0.19	0.23
Cu (mg/L)	0.029	0.03

^{*} In the liquid.

^{**} In the soluble solids.

For this reason, the culture of VAL-B1 using waste instead of pure carbon sources could be a good alternative for producing DHA at low cost, since the utilization of pure carbon source, increase the cost of the industrial production processes. It can also be highlighted that within the three analyzed strains in this research, only VAL-B1 registered the presence of the arachidonic (ARA, omega-6) acid fatty, which is other fatty acid of commercial interest. The importance of both DHA and ARA, which are fundamental for brain development and function, is recognized. However, a low PUFA n-6/n-3 ratio is essential for brain development and structural integrity (Echeverría et al., 2017) which makes this strain a good producer of these fatty acids to be used in food development and/or nutraceuticals of different presentations and content of DHA.

4.2. Effect of complex carbon sources on the production of lipids

The production of lipids in the case of the three strains studied shows the highest results when the RL is used as a substrate. It is even highlighted that AS5-B2 and IQ81, produce on average 3000 mg/L of lipids,

being an outstanding value (Figure 4). When the composition of RL is analyzed, a high value is observed in the percentage of proteins, mainly contributed by nitrogen (more than 38%, Table 1), which suggests that both strains use this source to a large extent to generate the values of lipids obtained.

In the same way, Li et al. (2015), using the *Aurantiochytrium limacinum* SR21 strain, reached a lipid production of 49.6% when was cultured in a carbon-nitrogen rich medium (residual glycerol). Due to that lupine is a legume, and like all of their species, it has a high protein contents, increased the presence of soluble protein in RL that might favor the production of biomass and lipids. Burja et al. (2006) performed tests with three different genera of TH (*Schizochytrium*, *Aurantiochytrium* and *Thraustochytrium*), using a medium in which nitrogen consumption was quite high, their results showed that *Aurantiochytrium* consumes nitrogen from the medium more rapidly than *Thraustochytrium*, which results in the strain does not have much time to reach nitrogen stress, so the accumulation of lipids in the cell is carried out faster. This is in agreement with what was obtained in this study, where AS5-B2 (*Aurantiochytrium* sp.) accumulates 5% more lipids than IQ81 and 50% more than VAL-B1 (*Thraustochytrium Kinney*) (Figure 1). Miranda et al. (2020) use the strain of *Aurantiochytrium* sp. (MAN69) and obtained a 33% lipid production using glycerol as substrate, and with *Thraustochytrium* sp. (MAN37) using malt extract produced 28% lipids. This is in agreement with the results obtained by the IQ81 and VAL-B1 strains of the genus *Thraustochytrium*, which show increase in lipid production.

The consumption of nitrogen in the medium is directly associated with the microorganism's growth, so if there is a high ratio of C:N that is favorable for TH it also promotes lipids production. Gupta et al. (2016) used two strains of TH (DBTIOC-1 and DBTIOC-18) and studied the effect of the C/N ratio on biomass and lipids. When the ratio increased from 12 to 24, the DBTIOC-18 strain had a slight increase in lipids from 64% to 64.5%. In the case of DBTIOC-1 the increase in lipid production was also minimal (less than 1%). However, the increase in biomass in DBTIOC-1 was double when going from a ratio of 12–24, where the values obtained were 8.43 g/L to 17.09 g/L. In this study, the C/N ratio for RL is 3 and for RC 2.8. Nazir et al. (2020) using *Ananas comosus* extract as a substrate for *Aurantiochytrium* sp. SW1 increased lipid production by 15% and biomass by 12%, compared to glucose as a pure carbon source.

Table 2. Profiles of fatty acids produced by AS5-B2, IQ81 and VAL-B1 grown with different carbon sources (glucose, G; by-product brewing, RB; by-product of protein extraction lupine flour, RL).

Strains Fatty acids %	AS5-B2			IQ81			VAL-B1		
	G	RB	RL	G	RB	RL	G	RB	RL
C12:0	0.45 ± 0.08	1.91 ± 0.21	0.20 ± 0.33	0.41 ± 0.07	0.23 ± 0.03	0.13 ± 0.02	0.50 ± 0.11	1.38 ± 0.4	0.74 ± 0.13
C14:0	3.02 ± 0.43	5.98 ± 0.78	0.68 ± 0.12	0.82 ± 0.14	0.59 ± 0.11	0.65 ± 0.18	2.4 ± 0.38	3.82 ± 0.51	2.73 ± 0.36
C15:0	0.74 ± 0.08	1.51 ± 0.66	0.11 ± 0.01	6.22 ± 1.19	5.15 ± 0.98	3.12 ± 0.59	1.80 ± 0.24	0.71 ± 0.15	0.91 ± 0.17
C16:0	15.92 ± 2.21b,c	21.53 ± 3.1	16.14 ± 2.7a	26.67 ± 5.41a	25.88 ± 4.14	22.24 ± 4.1c	21.0 ± 4.08c,d	24.3 ± 5.0	27.60 ± 4.8a
C17:0	0.22 ± 0.03	0.70 ± 0.09	0.17 ± 0.03	0.30 ± 0.05	0.18 ± 0.02	0.20 ± 0.02	0.20 ± 0.04	0.27 ± 0.03	0.41 ± 0.07
C18:0	3.0 ± 0.54	3.17 ± 0.61	2.31 ± 0.37	4.91 ± 0.72	3.32 ± 0.41	3.90 ± 0.5	8.00 ± 1.51	3.61 ± 0.58	5.85 ± 0.93
C18:1	31.88 ± 5.79a	26.27 ± 4.9a	17.96 ± 3.44	8.57 ± 1.82c	7.92 ± 0.60	14.99 ± 4.7	5.10 ± 1.14d	11.68 ± 2.4b	16.83 ± 3.46
C18:2	10.9 ± 1.99b,c,d	12.48 ± 2.3b	36.57 ± 7.12	38.29 ± 7.71a	36.28 ± 6.90a	32.81 ± 6.71	5.00 ± 0.98d	8.24 ± 0.66d	8.77 ± 1.79c,d
C18:3	15.3 ± 2.79a	9.60 ± 1.15	13.14 ± 0.24b	4.97 ± 0.74c,d	3.58 ± 0.62d	3.88 ± 0.61d	0.20 ± 0.02d	8.6 ± 1.71	7.51 ± 1.36c,d
C20:0	0.11 ± 0.06	0.39 ± 0.51	4.43 ± 0.19	0.29 ± 0.03	0.24 ± 0.04	0.87 ± 0.44	1.10 ± 0.26	0.29 ± 0.04	0.37 ± 0.05
C20:3	0.10 ± 0.01	0.29 ± 0.03	0.12 ± 0.02	< 0.01	< 0.01	0.05 ± 0.00	0.50 ± 0.12	0.43 ± 0.08	0.81 ± 0.17
C20:4 (ARA)	n.d	n.d	n.d.	< 0.01	< 0.01	0.04 ± 0.00d	0.10 ± 0.00	1.02 ± 0.08	1.19 ± 0.1a
C20:5 (EPA)	0.27 ± 0.02b	1.07 ± 0.19	1.05 ± 0.24	0.64 ± 0.11b	0.54 ± 0.11d	0.80 ± 0.12	6.20 ± 1.4a	3.67 ± 0.68a	3.80 ± 0.61a,b
C22:6 (DHA)	0.31 ± 0.03d	2.34 ± 0.33b	2.38 ± 0.45c	1.01 ± 0.17b,c	0.44 ± 0.09d	1.46 ± 0.21d	43.40 ± 7.7a	25.07 ± 4.71a	19.44 ± 4.12a
Others	17.52	13.14	14.74	6.9	15.65	14.85	9.09	6.91	3.04
ΣSFA	23.46	35.19	24.04	39.62	35.59	31.11	35.0	34.38	38.6
ΣUFA	58.08	48.35	67.67	51.83	47.78	51.68	10.3	28.52	33.11
ΣLC-PUFAs	0.68	3.7	3.55	1.3	1.0	2.35	50.2	30.19	25.24

< 0.01 = trace; n.d. = not detected. Within a row, means without a common superscript letter differ significantly (p < 0.05).

The highest values of biomass production are given for strain AS5-B2 when grown in RL, which is also repeated for IQ81. The application of adequate means for heating and heating is C/N. In the case of the strains AS5-B2 and IQ81, the ratio is favorable for nitrogen, explained by the high definition of proteins in the RL since lipids represent the total composition of oils presents in the TH, the character of the accumulated fatty acids could indicate what would be the most appropriate use for each of the studied strains. The data presented in Figure 2 and Table 2 indicate, that AS5-B2 and IQ81 could be cultivated in RL in order to be used for biofuels (67.67% and 51.68% total unsaturated fatty acids respectively), due to its high amounts of total lipids. For VAL-B1, the best application is to guide it to the of polyunsaturated fatty acids production (PUFA), specially DHA and EPA, using as RB (25.07% DHA) and RL (19.44%) waste substrates. Despite the above, the best source to get DHA in the three strains, occurs when used glucose as pure carbon source, which is achieved with a 43.40% in VAL-B1 strain. This allows us to indicate, that the strains studied could have a dual function, on the basis that the content of total lipids are high.

According to the results the three strains grown in this medium shows the highest concentration of biomass, DHA and EPA at 5 days of cultivation, while the consumption of total carbohydrates reached their maximum at five days for the three strains under study too.

5. Conclusions

It is interesting that the three strain shows their own fatty acid profile, even when IQ81 and VAL-B1 were phylogenetically classify as *Thraustochytrium Kinney*. The strain VAL-B1 produce from 20 to 43% of DHA, depending on the carbon source, while IQ81 produce from 0.4 to 1.4% of DHA. These differences may be an ecotype variation, since the strain VAL-B1 were isolated in Valparaíso and the strain IQ81 in Iquique, two locations that are separated by 1,720 km.

However, we identify small quantity of C20:4 (ARA) in strains IQ81 and VAL-B1, both identify as *Thraustochytrium Kinney*, while it was undetectable in strain AS5-B2 (identify as *Aurantiochytrium* sp., this result is in agreement with Singh et al., 2020). The isolated AS5-B2 and IQ81 are good lipids producer (3 g/L), with a good proportion of unsaturated fatty acids of less 20 carbon atoms; these isolated are good candidate for biofuel. By the other hand VAL-B1 produce few quantities of unsaturated fatty acids of less 20 carbon atoms and a good proportion of PUFA, becoming VAL-B1 as a good candidate as food supplement and DHA producer.

Declarations

Author contribution statement

Benita Quilodrán: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Gabriel Cortinez: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Anita Bravo: Performed the experiments; Contributed reagents, materials, analysis tools or data.

David Silva: Analyzed and interpreted the data; Wrote the paper.

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Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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