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Production of Docosahexaenoic Acid and Odd-Chain Fatty Acids by Microalgae *Schizochytrium limacinum* Grown on Waste-Derived Volatile Fatty Acids

Laura Oliver [1,*], Laura Fernández-de-Castro [1], Thomas Dietrich [1], Maria Carmen Villaran [1] and Ramón J. Barrio [2]

[1] Health Division, TECNALIA, Basque Research and Technology Alliance (BRTA), Parque Tecnológico de Álava, c/Leonardo Da Vinci no. 11, 01510 Miñano, Spain; laura.fernandezdecastro@tecnalia.com (L.F.-d.-C.); thomas.dietrich@tecnalia.com (T.D.); mcarmen.villaran@tecnalia.com (M.C.V.)

[2] Department of Analytical Chemistry, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain; r.barrio@ehu.es

* Correspondence: laura.oliver@tecnalia.com

Featured Application: The study demonstrates the potential of industrial waste-derived volatile fatty acids to be used as a sustainable carbon source in the heterotrophic cultivation of *Schizochytrium limacinum*.

Abstract: Heterotrophic microalgae are recognized as a source of bioactive compounds. However, there are still some drawbacks for their use at an industrial scale associated with the high cost of glucose, the main carbon source in heterotrophic cultures. In recent years, significant efforts have been made to investigate more sustainable carbon sources to produce biomass. In this study, the capacity of *Schizochytrium limacinum* to grow on waste-derived volatile fatty acids and the effect that their use produces on biomass and fatty acids profiles were investigated. Acetic, propionic, butyric, valeric and caproic acid were evaluated independently, as well as in a synthetic mixture (VFA). The use of acetic and butyric resulted in a good biomass productivity, while the use of valeric and propionic acid resulted in higher content of odd-chain fatty acids (OCFA), increasingly investigated due to their potential benefits for human health. The use of industrial waste-derived VFA as a potential carbon source was validated through the utilization of biowaste derived effluents from a volatile fatty acid platform. The biomass produced was of 18.5 g/L, 54.0% lipids, 46.3% docosahexaenoic acid (DHA) and 25.0% OCFA, concluding that waste derived VFA can produce DHA and OCFA in a suitable ratio of DHA/OCFA with potential industrial applications.

Keywords: heterotrophic microalgae; volatile fatty acids; DHA; odd-chain fatty acids

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1. Introduction

Microalgae are a diverse group of organisms recognized for their capacity to produce biomolecules of interest for industrial applications (energy, food, nutrition, or pharma) [1–4]. Although most of them are cultivated under phototropic conditions and their products are already on the market, the industry is now focusing on heterotrophic cultivation due to the similar characteristics to traditional fermentation process.

Heterotrophic microalgae contain mainly proteins and lipids, Omega-3 polyunsaturated fatty acids (Omega-3 PUFA), in particular long chain PUFA docosahexaenoic acid, DHA (22:6 n3) and eicosapentadecaenoic acid, EPA (20:5 n3). They are essential compounds for nutrition and human health that the human body only produces in small quantities and therefore must be acquired through the diet. Traditional sources of these compounds are marine origin, fish and crustaceans in our diet since prehistory [5–7]. Nevertheless,

the progressive population increase and the knowledge about their effect in nutrition and health promote access limitations to traditional sources of Omega-3 PUFA [8].

To fill the gap between the demand and supply of Omega-3 compounds in the different industrial sectors, alternative sources for Omega-3 were investigated in recent years. In this context, heterotrophic microalgae represent an interesting alternative as they are also primary producers of Omega-3 [9–12]. The capacity of certain species of microalgae to produce high amounts of Omega-3 PUFA, in particular DHA under heterotrophic conditions, turn them into a very attractive source for the production of these compounds for industrial application. However, there are still challenges in the establishment of economic feasible production processes from microalgae [13–15]. The main drawback is the high cost associated with the use of glucose; the main carbon source used for the growth of microalgae in heterotrophic cultures. Techno-economic analysis carried out by experts indicated that the higher costs at industrial scale were mainly associated with the stage of cultivation, considering that glucose can constitute up to 80% of the cost of the medium. Therefore, it becomes a major disadvantage, from an economic point of view, to produce high added-value compounds by heterotrophic microalgae [16,17].

However, the high potential of heterotrophic cultivation leads the industry to search for more sustainable routes to produce Omega-3 PUFA though the identification of alternative carbon sources. Great efforts are being taken by the scientific community to investigate alternatives to the use of glucose, as is reflected in the increase in publications in this field in the last ten years. In our previous reports, a compilation of the state of the art on the use of alternative carbon sources was published [18]. Forest biomass, glycerol or pre-treated whey permeates were discussed as substitutes of glucose. Cane molasses and sugarcane waste was also investigated for this purpose, and it was concluded that the presence of toxic substances may cause inhibition and cane molasses might be used in combination with other carbon sources [19–24].

In recent years, the use of volatile fatty acids (VFA) as an alternative carbon source has been studied [15,25,26]. VFAs are short-chain fatty acids (two to six carbon atoms) that can provide microalgae a carbon chain ready to be used for further elongation, towards long-chain unsaturated Omega-3. However, current production routes using petrochemical feedstocks to obtain VFAs are considered as non-sustainable and therefore, more environmentally friendly pathways are being investigated. Based on the principle of circular bioeconomy, where the subproducts and waste from industry or human consumption must be considered as raw material for other industrial processes, VFAs can be obtained from organic side streams via anaerobic digestion and can be used as a carbon source to produce Omega-3 PUFA and other bioactive compounds through the heterotrophic cultivation of microalgae [27–30]. Several reports have been published in the last few years, indicating the capacity of certain species of microalgae to grow on VFAs [31–33]. In these studies, the authors focused on the culture conditions and DHA productivity, but the use of VFAs as a carbon source presents more advantages. The presence of odd chain VFAs (such as propionic or valeric acid) in the dark fermentation effluents and respective cultivation medium can modify the respective lipid profile in microalgae. The use of odd-chain short VFAs as a precursor for fatty acid synthesis leads to the formation of higher quantities of odd-chain fatty acids in the biomass.

Aside from PUFAs, odd-chain fatty acids have been receiving more attention in recent years. They consist of an odd chain of carbon, mainly pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) usually namely as OCFA. OCFA are presented in ruminants, bacteria and plants in small quantities. The interest in these compounds is based on pharmacology properties and their utility in industrial applications [34–36]. C15:0 was further studied by Venn-Watson et al. [37], and recent studies suggest that they can have a positive effect on membrane fluidity as they have lower melting points than closely even chain fatty acids, with particular interest for diseases like multiple sclerosis [38–40]. Diverse studies have revealed that the level of OCFA in Alzheimer patients was lower than in other groups and, therefore, their application is suggested in the treatment of this

type of disease [41]. Anticarcinogenic influence was also evaluated on carcinogenic cells by Mika et al. [42]. Different large-scale epidemiological studies have now shown that the plasma OCFAs levels found are associated with reduced disease risks for CHD and T2D [36,43].

The aim of the present study was to evaluate the capacity of *Schizochytrium limacinum* to grow on waste-derived VFA from dark fermentation effluents as an alternative carbon source, and the effect produced by its use on fatty acid profiles, in particular OCFA and DHA for the health benefits associated with them.

2. Materials and Methods

2.1. Chemicals and Microorganism

Schizochytrium limacinum (ATCC®® MYA1381TM) was procured from the American Type Culture Collection (ATCC) and it was cultivated and maintained by sub-cultivation on a medium recommended by the culture collection, containing 1 g/L yeast extract, 1 g/L peptone, and 5 g/L glucose in artificial sea water. Artificial seawater was made according to ASTM D1141-98 standard [44]. In addition, cryo-tubes were prepared for preservation at −80 °C. All chemicals used for culture media preparation were purchased from Scharlab (Barcelona, Spain). n-Hexane, dichloromethane, methanol, sulfuric acid and potassium hydroxide, used in the extraction and analysis of lipids, were analytical grade from Scharlab (Barcelona, Spain). Acetic, propionic, (iso)butyric, (iso)valeric and caproic acid used for synthetic VFA preparation were purchased from Merck Life Science (Madrid, Spain).

VFA rich dark fermentation (DF) permeate was derived from a vegetable, garden and food (VGF) waste stream treated via a VFA platform as described in [45]. The resulting effluent was purified using an ultrafiltration membrane system (Koch Membrane Systems, Inc., USA) with a ceramic membrane (1200 mm-length and 70 nm pore size). The permeate was further concentrated with a Rotavapor R-220 PRO (BÜCHI Labortechnik AG, Switzerland), reaching a final VFA concentration of 157.2 g/L. It consisted of acetic acid (41.0%), propionic acid (27.7%), butyric acid (18.2%), valeric acid (8.4%), caproic acid (1.6%), isobutyric acid (1.2%) and isovaleric acid (1.8%). A corresponding synthetic VFA feed solution was prepared (155 g VFA/L) to compare waste derived VFA with pure (clean) VFA.

The standards for GC analysis FAME MIX, (C4-C24), DHA, C15:0, and C17:0 were purchased from Merck Life Science (Madrid, Spain) and prepared according to required concentration.

2.2. Shake Flask Culture Conditions

Schizochytrium limacinum cells were cultivated in 250 mL baffled flasks containing 50 mL of medium with 5 g/L yeast extract, peptone 5 g/L and the corresponding carbon source in each experiment—acetic, propionic, butyric, valeric acid or caproic acid prepared separately at a final concentration of 10 g/L in artificial sea water and pH adjusted to 6.8 prior to autoclaving (Presoclave-75 Selecta, Barcelona, Spain). The pH was analysed with a pH-meter (Basi 20; Crison Instruments S.A, ALELLA, Barcelona, Spain). The inoculum was 10% of the total culture volume and was 3-day-old static-grown cultures in basal medium (glucose 5 g/L, yeast extract 1 g/L, peptone 1 g/L in artificial sea water). The baffled flasks were incubated for 72–96 h, at 22 °C and 120 rpm. Dairy samples were collected to obtain cell dry weight, VFA consumption, and fatty acid profile.

2.3. Fed Batch Culture Conditions

Fed batch cultivations of *Schizochytrium limacinum* were carried out in a 2 L stirred tank reactor (Biostat B plus, Sartorius. BBI Systems GmbH, Melsungen, Germany) in DO-stat mode. The dissolved oxygen set-point was 20% saturation and the agitation speed varied from 200 rpm to 700 rpm. The aeration rate used was 2.0 L_{air}/min and the temperature 25 °C. The pH was controlled at 6.8 with 4 M NaOH and 2 M HCl. Innoculum (10% *v/v*) was prepared in 250 mL baffled flasks containing 50 mL of basal medium with 1 g/L yeast extract, 1 g/L peptone, 5 g/L glucose in artificial sea water. It was incubated for 72 h at 22 °C. The initial volume of the fed-batch culture was 1200 mL using as starting

medium 8 g/L yeast extract, 8 g/L peptone and 10 g/L VFAs (DF permeate or synthetic VFA solution) in artificial sea water.

The fed-batch operation mode was carried out using a VFA feeding regime based on the decrease in the stirring speed which occurred due to the increase in the dissolved oxygen concentration as a result of VFA exhaustion. The feed solution consisted either of a solution of synthetic VFA (155 g/L) or concentrated DF permeate (157.2 g $_{VFA}$/L) and was sterilized and adjusted to pH 6.8 before use. Every feeding pulse added 75 mL of VFA solution to the bioreactor. Culture samples were periodically harvested to analyse cell dry weight concentration, VFA consumption, as well as fatty acid profiles of the biomass lipid fraction.

2.4. Analytical Methods

2.4.1. Harvesting and Cell Dry Weight Determination

Culture samples were centrifuged at 3100× *g* for 15 min (Centrifuge 5810, Eppendorf, Merck Life Science, Madrid, Spain). The supernatant was collected, and the cell pellets were washed with deionized (DI) water, frozen and freeze-dried (Lyobeta 25; Telstar, Terrasa, Barcelona, Spain). The dry cell weight (DCW) was measured gravimetrically.

2.4.2. VFA Determination

Volatile fatty acids in samples (DF permeate, synthetic VFA solution, fermentation broth) were quantified by direct injection of supernatant after the centrifugation step. One millilitre of the sample was taken and then mixed with 50 µL of 1,3-butanediol (500 ppm) and 50 µL of ortho-phosphoric acid to ensure a pH below 2. Agilent 8890/5977B GC/MSD (Agilent Technologies, Inc., Palo Alto, CA, USA) (H_2 flow rate 30 mL/min, air flow rate 300 mL/min) and Agilent DB-FFAP column (30 m × 0.32 mm i.d., 0.5 mm film thickness, USA). Detector temperature and injector port temperature were 320 and 320 °C, respectively. Helium was used as the carrier gas at flow rate 2.5 mL/min. The oven temperature was programmed at 70 °C, raised to 130 °C at 15 °C/min, increased to 180 °C at 6 °C/min, then increased again to 240 °C at 30 °C/min for 5 min. Standards from each compound were used to perform calibration curves. The analysis of each sample was carried out in triplicate. Results are reported as mean ± standard deviation (SD) values.

2.4.3. Lipids and Fatty Acid Profile

A modified Bligh and Dyer method was used to estimate the lipid content in dried microalgae biomass [46]. About 100–200 mg of dried biomass was put in contact with water at 50 °C under stirring, overnight, to induce autolysis of cells. After that, a dichloromethane/methanol (4:2) mixture was added to the sample and vortexed for 5 min. Dichloromethane/methanol (2:1) mixture was added again, and the sample was kept in an ultrasound bath (FB15054 Fisherbrand, Fisher Scientific, Madrid, Spain) for 10 min. After centrifugation at 3100× *g* for 15 min, the upper phase is discarded. The final extract with lipids is transferred to a pre-weighted vial and the process is repeated two more times. After evaporation of the solvent, the total lipid content was measured gravimetrically.

The dried lipids were mixed with 6 mL MeOH:KOH (88:12) and stirred at 55 °C for one hour and thirty minutes. A second step was performed with H_2SO_4 12 M, at 35 °C for one hour and thirty minutes. The methyl esters were extracted from the mixture with 1 mL of hexane. The extraction step was repeated three times.

FAME identification and quantification was carried out using a GC/MS/FID system with an Agilent 8890/5977B GC/MSD (Agilent Technologies, Inc., Palo Alto, CA, USA), and a ZB-FAME column designed for the specific purpose. The injection temperature was set at 250 °C, the carrier gas flow was 1.2 mL/min (Helium) and the column temperature rose from 100 °C (5 min) to 240 °C (30 min) with a rate of 4 °C/min. Samples were injected with a split ratio of 80:1. The Supelco 37 Component FAME mix, DHA, C15:0 and C17:0 standards were used for quantification. Results are reported as mean ± standard deviation (SD) values.

3. Results and Discussion

3.1. Schizochytrium Limacinum Growth on Synthetic VFAs and Resulting Fatty Acid Profile

3.1.1. Biomass Production and VFA Consumption in Batch Fermentations

The potential of *Schizochytrium limacinum* to produce DHA and OCFA utilizing volatile fatty acids was evaluated in a first step. Therefore, acetic, propionic, butyric, valeric and caproic acid, the main short-chain fatty acid present in the dark fermentation effluents, were used in separated cultures, and their effect on biomass production and related parameters was evaluated for each culture. At the same time, the rate of VFA consumption was also evaluated.

When the microalgae *Schizochytrium limacinum* was grown in batch culture on 10 g/L of each VFA for 72 h, acetic acid showed the highest dry cell weight reaching 3.2 g/L, while propionic acid showed the lowest production with 0.7 g/L. Butyric and valeric acid achieved 3.0 g/L and 2 g/L of dry cell weight, respectively. For caproic acid, no biomass growth could be observed after the time of growth. The evaluation of biomass productivity (g/L/h) and biomass yield ($g \cdot g^{-1}$substrate) resulted in a similar behaviour for acetic and butyric acid, 0.04 g/L/h and 0.3 $g \cdot g^{-1}$substrate in both experiments, as it can be observed in Table 1. On the other hand, propionic acid showed low productivity (0.01 g/L/h) and the yields obtained were below 0.1 $g \cdot g^{-1}$ substrate.

Table 1. Parameters relating to cultivation in flask of *Schizochytrium limacinum* on each VFA.

Parameters	Acetic Acid	Propionic Acid	Butyric Acid	Valeric Acid	Caproic Acid
Cell dry weight (g/L)	3.2 ± 0.05	0.70 ± 0.03	3.0 ± 0.05	1.2 ± 0.03	--
Biomass productivity (g/L/h)	0.04 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	--
Biomass yield ($g \cdot g^{-1}$substrate)	0.32 ± 0.01	0.07 ± 0.01	0.30 ± 0.01	0.12 ± 0.01	--

Every 24 h, an aliquot of each culture was analysed for the evaluation of VFA consumption. After 72 h of growth, 96.7% of the initial acetic acid concentration was consumed, leaving a residual concentration of 0.4 g/L. On the contrary, no consumption was observed after 72 h of growth on caproic acid. Propionic and valeric acid showed a similar rate of consumption, after 72 h of growth, 79.0% and 74.0%, respectively, of initial carbon source was still detected in the media (Figure 1). The evolution of valeric acid showed an interesting behaviour. As it can be observed in Figure 2, after 24 h, the VFA analysis revealed that at the same time that the valeric acid was disappearing in the culture media, propionic acid was beginning to be detected. The experiment was then allowed to continue for another 96 h and, after seven days of the experiment, the valeric acid was almost consumed (0.70 ± 0.03 g/L) and propionic acid reached a concentration of 5.69 ± 0.09 g/L in the media.

These results are in line with those obtained for the *Thraustochytriacea and Crypthecodiniacea* family, that are the two main families most studied for their capacity to grow under heterotrophic conditions. In particular, *schizochytrium* and *ulkenia* species are able to produce high Omega-3 PUFA content, especially DHA [47]. The evaluation of the effect of every short chain fatty acid that comprises VFA on the growth of *Schizochytrium limacinum,* resulted in acetic acid and butyric acid appearing to be the most suitable fatty acid to be used when a concentration of 10 g/L of VFA was studied. Meanwhile, propionic acid resulted in a very low capacity to produce biomass by itself (0.78 g/L). *Aurantiochytrium* sp. from *Thraustochytrid* exhibited similar behaviour when Patel et al. studied its growth on VFA [26]. Acetic and butyric acid resulted in a higher cell dry weight (2.10 g/L and 3.23 g/L, respectively) at 10 g/L carbon source [26].

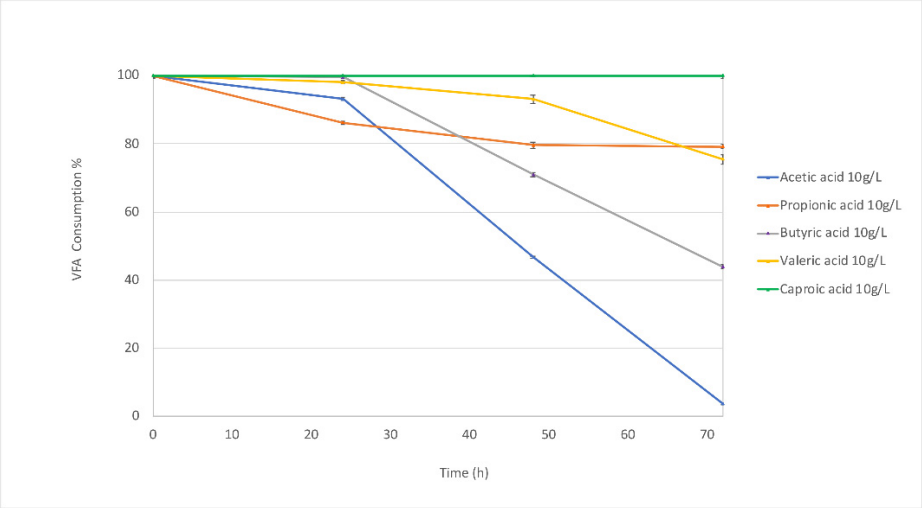

Figure 1. Evolution of VFA (%) during experiments on batch cultivation of S. Limacinum at 10 g/L VFA.

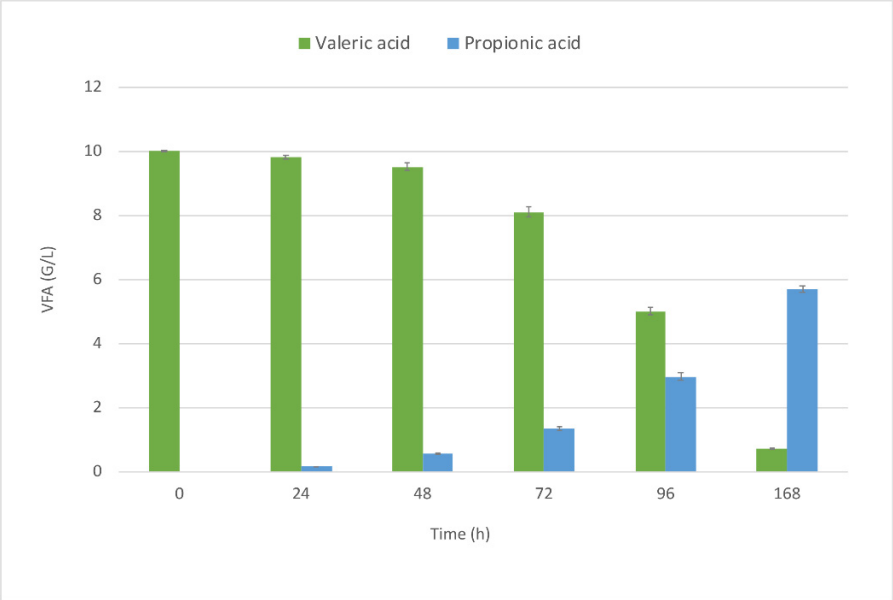

Figure 2. Evolution of carbon source consumption on valeric acid feed 10 g/L.

Cryptecodinium cohnii was also deeply investigated by Chalima et al., reaching similar results, with the acetic acid consumed relatively fast with good biomass productivity [15]. Despite the different published studies, all of them mainly focused on the use of acetic and butyric acid for the production of DHA or biodiesel [48–52]. The production of OCFA was studied by Wang et al. [50] and Lee Chang et al. [14], but the effect of using short chain fatty acids (propionic and valeric acid) on biomass growth and fatty acid profile have not yet been studied for this species.

3.1.2. Effect on Fatty Acid Production

After 72 h of growing experiment, the culture medium was centrifuged and freeze dried for biomass and fatty acid profile evaluation. According to the chromatographic results obtained from batch experiments, propionic acid reached a higher unsaturated fatty acids ratio (63.8%), close to the butyric acid experiment that showed 58.7% unsaturated fatty acids. Regarding acetic and valeric acid, an equal distribution between unsaturation and saturation was obtained, but significant differences can be observed in the type of fatty acid in each group. Palmitic acid (C16:0) was the principal contributor to total saturated

fatty acids concentration during the acetic experiment (40.6% of TFA), while for the valeric acid experiment, OCFA (C15:0 and C17:0) were the main contributors to saturation on fatty acid composition. The 20.34% TFA corresponded to C15:0 and 16.03% corresponded to C17:0 fatty acid. Moreover, the use of propionic acid reported the highest DHA ratio, 50% of TFA (Figure 3). The use of propionic acid and valeric acid (C3 and C5) as a carbon source can result in a higher concentration of DHA and OCFA. However, the biomass productivity obtained using these carbon sources was low and consequently it would be needed to introduce acetic or butyric acid to increase the productivity. In comparison with a standard feed of glucose, the results are very promising. A similar fatty acid profile was observed when glucose, acetic or butyric acid was used for culture growing, but the contribution of OCFA was significatively lower when glucose is used as feedstock. A combination of the different VFAs could enhance the production of DHA and OCFA.

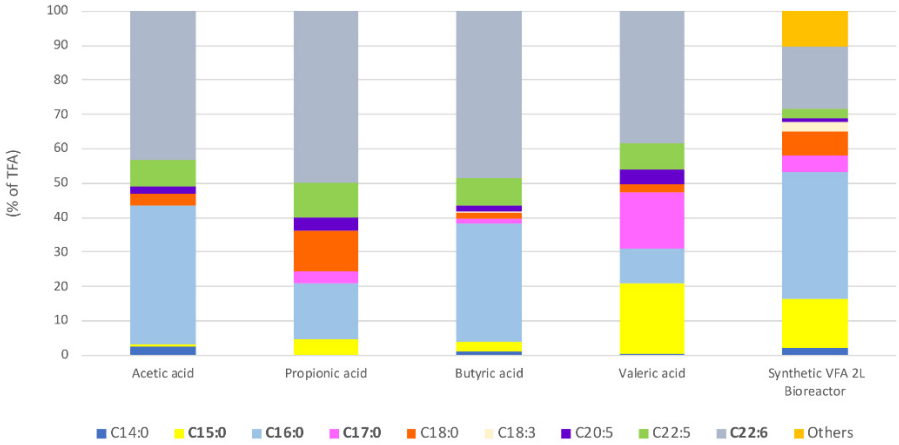

Figure 3. Distribution of fatty acids on acetic, propionic, butyric, valeric and synthetic VFA mixture experiments.

Once the effect of each VFA was evaluated in 250 mL flasks, fed-batch cultivation of *Schizochytrium limacinum* in 2 L bioreactor on a synthetic mixture of VFA in a similar ratio as that usually found in dark effluents was performed [45]. The purpose of this study was to evaluate the possibility of competition between VFAs that could result in any growth inhibition or any change in expected final fatty acid composition. Synthetic VFA media was prepared according to Section 2.3.

In the bioreactor, the use of a mixture of synthetic VFA resulted in a good biomass productivity (12.3 g DCW/L), high lipid content 45.9 %, and a high quality fatty acid distribution, with 18.3% of DHA and 19.2% of OCFA (Table 2). In comparison to the results obtained by using separated VFA in batch experiments, the fatty acid profile distribution seemed to follow the same pattern. Acetic and butyric acid contributed to the biomass growth, C16:0 and DHA production, meanwhile, valeric and propionic acid contributed to the production of OCFA, C15:0 and C17:0 (Figure 3).

Table 2. Synthetic VFA 2L bioreactor.

Carbon Source	DCW (g/L)	Biomass Productivity (g/L/h)	Biomass Yield ($g \cdot g^{-1}$ Substrate)	Lipids (%)	DHA (%TFA)	C15:0 (%TFA)	C17:0 (%TFA)
Synthetic VFA	12.3 ± 0.1	0.23 ± 0.1	1.23 ± 0.01	45.9 ± 1.0	18.3 ± 0.9	14.3 ± 0.7	4.9 ± 0.6

3.2. Waste-Derived VFA for DHA and OCFA Production in Fed-Batch Fermentations

Biomass Production and VFA Consumption Pattern

Since the capacity of *Schizochytrium limacinum* to grow on both single and VFA mixtures was verified in batch culture and bioreactor, the purpose of this study was to evaluate the cultivation of this microalgae on VFA from an industrial effluent with the composition

defined in point 2.3. The capacity of waste derived VFAs as a carbon source resulted in a lipid accumulation of 54% *w/w*, 18.5 DCW/L of biomass, and a fatty acid profile containing 51.6% saturated fatty acids and 47.9% polyunsaturated fatty acids. OCFA are presented in 25.0% of TFA, comprising C15:0 and C17:0 at 18.1 and 6.9%, respectively. DHA as the main fatty acid compound found in *Schizochytrium limacinum* resulted in almost 50% of TFA and 46.3% of TFA (Table 3).

Table 3. Waste derived VFA 2L bioreactor.

Carbon Source	DCW (g/L)	Biomass Productivity (g/L/h)	Biomass Yield ($g \cdot g^{-1}$ Substrate)	Lipids (%)	DHA (% TFA)	C15:0 (% TFA)	C17:0 (% TFA)
Waste-derived VFA	18.5 ± 0.1	0.35 ± 0.1	1.85 ± 0.01	54.0 ± 1.2	46.3 ± 0.8	18.1 ± 0.7	6.9 ± 0.7

The analysis of VFA throughout the experiment showed that during the first 24 h of cultivation, over 75% of the carbon source was consumed, and it was practically exhausted at 53 h where 93.2% VFA was already consumed by microalgae (Figure 4). However, as it was described in Section 3.1., VFAs were not consumed in the same way. Acetic and butyric acid were consumed faster than odd-chain VFA, propionic and valeric acid. Meanwhile, most of them were consumed at 49 h of the growth experiment, while propionic acid exhibited 23.9% of its initial content at this time in the experiment (Figure 5).

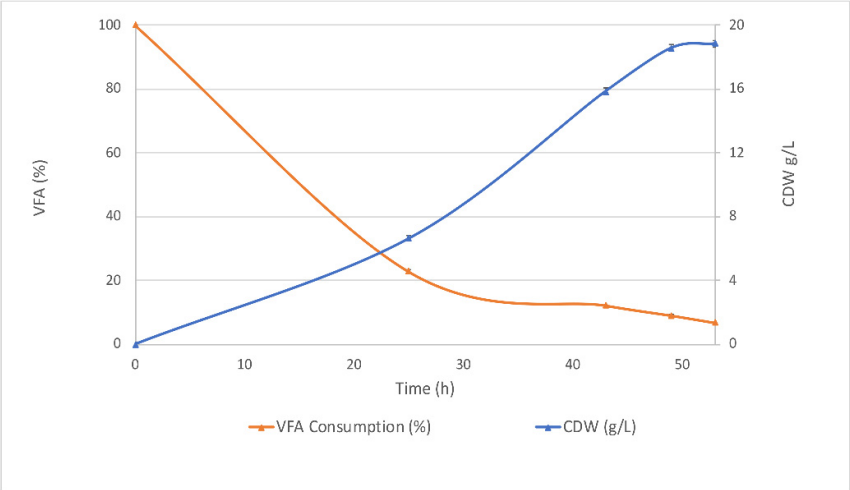

Figure 4. Biomass production vs. waste-derived VFA consumption.

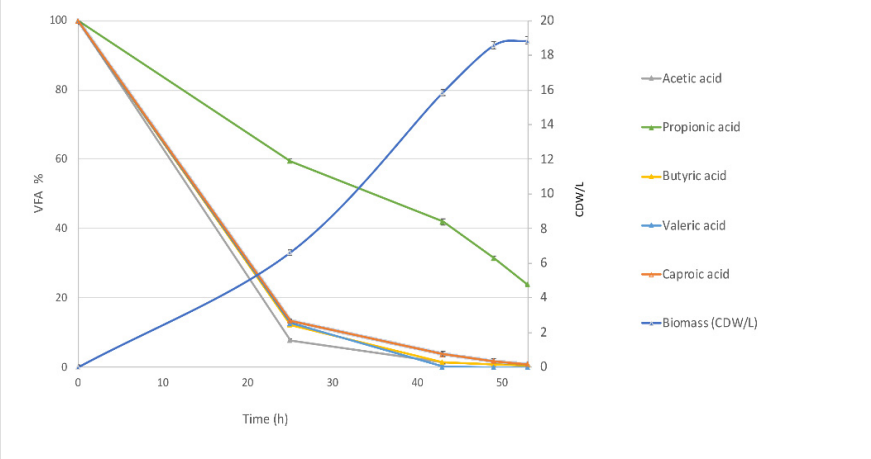

Figure 5. Evolution of each VFA during fermentation.

In this study, the effect of carbon source consumption on the fatty acid profile was also evaluated. As shown in Figure 6, when the carbon source associated with butyric and valeric acid was consumed, and propionic and valeric acid were still in the media, *Schizochytrium limacinum* used these short fatty acids as a carbon source and, consequently, the fatty acid profile showed higher content in OCFA, C15:0 and C17:0.

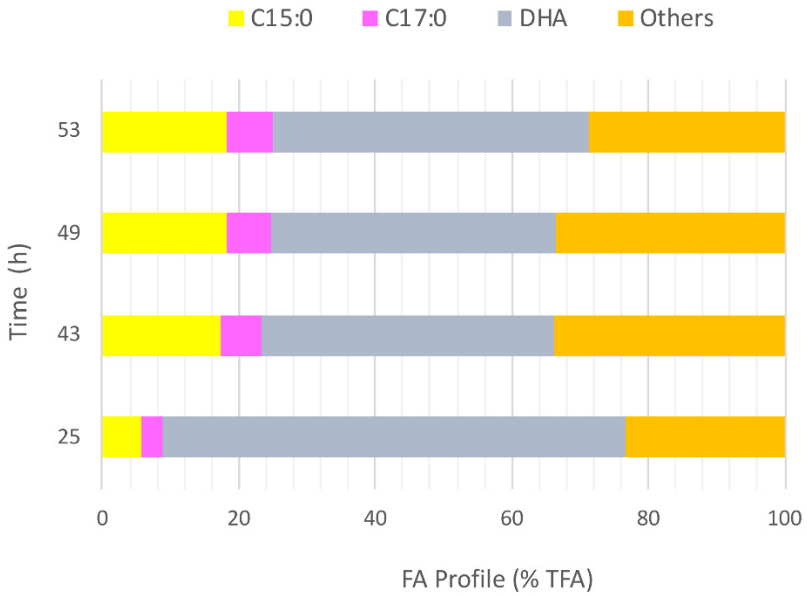

Figure 6. OCFA and DHA evolution on waste-derived VFA carbon source cultivation.

The parameters related to the two types of cultivation are summarized in Table 4.

Table 4. Parameters of Schizochytrium Limacinum grown on VFA carbon source.

Parameters	VFA Synthetic Media 2L	Waste-Derived VFA Media 2L
Cell dry weight (g/L)	12.3 ± 0.1	18.5 ± 0.1
Biomass yield ($g \cdot g^{-1}$ substrate)	1.23 ± 0.01	1.89 ± 0.01
Lipid content (%)	45.9 ± 1.0	54.0 ± 1.2
C14:0 (%)	2.2 ± 0.1	1,4 ± 0.1
C15:0 (%)	14.3 ± 0.7	18.1 ± 0.7
C16:0 (%)	36.9 ± 0.3	23.3 ± 0.3
C17:0 (%)	4.9 ± 0.6	6.9 ± 0.6
C18:0 (%)	6.8 ± 0.1	1.4 ± 0.1
C18:3 (%)	2.7 ± 0.1	0.3 ± 0.05
EPA (%)	1.2 ± 0.3	1.5 ± 0.3
DPA (%)	2.5 ± 0.3	2.2 ± 0.3
DHA (%)	18.3 ± 0.9	46.3 ± 0.8

4. Conclusions

The present study demonstrates the potential of waste derived VFA to be used as a sustainable carbon source in the heterotrophic cultivation of *Schizochytrium limacinum*. The presence of odd-chain fatty acids provides a very interesting final fatty acid composition, favouring the production of OCFA and DHA in proportions that are not achieved when glucose is used as a carbon source.

The results did not show an inhibitory effect on growth, suggesting that there were no toxic substances in the media. This is particularly interesting as other alternative carbon sources need to be used in a very diluted or highly purified state to avoid this inhibitory

effect. Moreover, the comparison between growth with synthetic VFA and growth with waste-derived VFA showed that the use of waste- derived VFA provided a balanced content of OCFA and DHA, which can be a good opportunity for the obtention of bioactive compounds for food, pharma or health applications.

Further research should be done regarding the effect of odd chain short fatty acids on biomass and the fatty acid profile produced by heterotrophic microalgae.

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