Research Paper

Metabolite profiling of *Schizochytrium* sp. by GC-MS, an oleaginous microbial source of biodiesel

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Abstract

The chemical screening carried out on *Schizochytrium* sp. biomass led the identification of 24 types of organic compounds belonging to *n*-alkanes, 1-alkenes, 1-alkanols, free fatty acids, methyl and ethyl esters of saturated and unsaturated fatty acids, saturated tri- and diglycerides, unsaturated monoglycerides, wax esters, sterols, triterpenes, and mono- and sesquiterpenes. Moreover, a sample containing fully saturated ethyl biodiesel was obtained experimentally with a yield of 28.72% w/w of the crude extract, and an average chain length of 15.52 carbons. This strain produced no toxins, but showed important nutrients, making it potentially applicable to the field of functional food, and biodiesel production.

Key words: *Schizochytrium* sp., zoosporic microorganism, organic compounds, renewable fuel.

Introduction

Schizochytrium sp. is a zoosporic organism that belongs to the Labyrinthulomycota Phylum, a known group of protists abundant in marine and estuarine environment (Porter, 1990). In the last decades, a particular attention has been given to this group of organisms, since it has been proven to be a very productive source of important primary metabolites of industrial interest (Yongmanitchai and Ward, 1989). These organisms are capable to produce, by *de novo* synthesis, both saturated and unsaturated fatty acids, particularly long chain polyunsaturated fatty acids from non-lipid conventional sources (Bowles *et al.*, 1999; Yokochi *et al.*, 1998).

Its importance has increased due to the growing demand for these marine natural products, potentially capable of generating commercial applications in nutraceutical, pharmaceutical and aquaculture (Lewis *et al.*, 1999, 2000; Nichols *et al.*, 1999). Beyond the role outlined to these lipids, *Schizochytrium* sp. can be similarly an interesting producer of secondary metabolites. So that, although their fatty acid profiles have been described (Ashford *et al.*,

2000; Barclay and Zeller, 1996), the bibliographic background indicate the presence of glycolipids, phospholipids, sphingolipids and sterols as cholesterol, stigmasterol and brassicasterol (Kendrick and Ratledge, 1992).

Furthermore, these organisms also become of industrial interest as a biodiesel source. Biodiesel, as an alternative fuel, has attracted increasing worldwide attention driven by factors such as oil price spikes, the need for increased energy security, and concern over greenhouse gas emissions from fossil fuels (Bondioli *et al.*, 2008). Oleaginous fermentations from microbial strains can generate high added-value biodiesel by using a large variety of material as glycerol and ethanol as a carbon source to produce single-cell biomass (Johnson and Takoni, 2007; Ochoa-Estopiera *et al.*, 2011).

The present work reports the study of the metabolites biosynthesized by the heterotrophic *Schizochytrium* sp. which was produced by fermentation, in accordance with Barclay procedures (Barclay, 1994). It was carried out a detailed screening of its lipo- and hydrosoluble fractions, and its compounds were identified by GC-MS and NMR spectroscopy, looking for to confirm those substances described

previously in the literature and isolate new structures that could show any interesting bioactivity, as well as, provide some type of industrial application as a biodiesel production, for instance.

Materials and Methods

Microorganism and heterotrophic production

The heterotrophic *Schizochytrium* sp. was purchased from Aquafauna Bio-Marine Inc., Hawthorne, CA, USA. The biomass fermentation was produced by Omega Tech Inc., Bounder, CO, USA, in accordance with Barclay procedures (Barclay, 1994). The biomass obtained was concentrate by centrifugation, spray-dried and vacuum packaging (Barclay and Zeller, 1996).

Obtaining of the extract and fractionation procedure

A sample of 110 g of spray-dried *Schizochytrium* sp. was soaked in dichloromethane (x3, 24 h) and methanol (x3, 24 h). The extracts were filtered by Whatman paper (grade 1) and evaporated at reduced pressure in a rotary evaporator. Thus, they were combined, dried under high vacuum, and stored in the fridge under a nitrogen atmosphere. The resulting crude extract was, then, subjected to partition by polarity in accordance to a modified Kupchan solvent partitioning scheme (Kupchan *et al.*, 1973). See Figure S1, in the supplementary material.

Experimental

Normal-phase column chromatography was carried out on silica gel (Scharlau) with a 0.06-0.2 mm particle size as the adsorbent in the head of the chromatographic column and 0.04-0.06 mm for the stationary phase. The chromatography was performed either a medium pressure (Büchi Chromatography System) or a low pressure with a Fluid Metering Inc. motors connected in series with an Ace Glass Inc. column. Reverse-phase chromatography was achieved on LiChroprep RP-18 (Merck, 40-63 µm particle size) column connected with a low pressure chromatography system based in a Fluid Metering Inc. apparatus too. Size exclusion chromatography was carried out on lipophilic Sephadex® LH-20 (Sigma). The column was conditioned first with anhydrous methanol (2 h) and then with a mixture of CH_2Cl_2/CH_3OH (50:50, 2 h). The extracts were applied on the top of the column and eluted with CH_2Cl_2/CH_3OH (50:50) at a rate of 1.0 mL min^{-1}. Normal-phase TLC was performed on silica gel plates (0.25 mm diameter, Tracer Analitica) using a combination of hexane, ethyl acetate, chloroform and methanol as eluent, at the proportion detailed in each case. Reverse-phase TLC was carried out on RP-18F$_{254}$ plates (0.25 mm diameter, Merck) with the use of $CH_3CN/CH_3OH/H_2O$ (80:18:2) as a mobile phase. In all cases, the TLC spots were revealed by spraying with *oleum* (sulphuric acid, 4% + acetic acid, 80% + water, 16%) and heating at 120 °C for 20 min. Normal-phase semi-prepa-

rative HPLC were performed on an Alltech Econosphere C18 column (10 µm particle size, 250 x 4.6 mm, 100 Å pore size) and reverse-phase semi-preparative HPLC were performed on a Waters ODS column (10 µm particle size, 250 x 4.6 mm, 100 Å pore size). Both of them, were carried out on a semi-preparative HPLC apparatus accomplished to Spectra-physics P100 isocratic pump and used in line with a Hewlett Packard 1050 UV-VIS variable wavelength detector, working at room temperature (26 °C) and at λ = 254 nm. Analytical Chromatography was performed using a Shimadzu HPLC system with a LC-9A pump connected in line with a UV-VIS SPD-6AV detector (λ = 254 nm). The conditions used for the normal-phase analytical chromatography were combinations of hexane and ethyl acetate as eluent and for the size-exclusion chromatography column (Shodex OH Pak SB 806 HQ) were used a mixture of water and 0.05% of sodium azide as eluent. An eluent flow rate of 1.0 mL min^{-1} was used in all analysis. ^{1}H- and ^{13}C-NMR spectroscopy experiments were recorded at 250 or 300 MHz on AC or AMX Bruker apparatus, respectively. Tetramethylsilane was used as an internal standard for ^{1}H and deuterated chloroform (δ 77.00) or deuterated methanol (δ 49.00) for the calibration of the ^{13}C-NMR spectra. Gas chromatography-mass spectrometry (GC-MS) analysis was carried out on a chromatograph model Varian CP3800 with an ion-trap mass spectrometer model Saturn 2000 and under the following conditions: CP-Sil 8 low bleed capillary column. The injector temperature was kept isothermal at 270 °C; initial split conditions *on*; 0.01 min *off* and 5 min *on* with a split ratio 1:50; the oven was set at 50 °C for 5 min, and then ramped at 15 °C min^{-1} till 250 °C and held for 10 min (total run time of 28.33 min for each sample); flux of 1 mL min^{-1}; mass detector in the EI mode (the m/*z* range was 20 to 400). Relative GC retention times were obtained by comparison of authentic standard alkanes (Dr. Ehrenstorfer GmbH Alkanes-Mix 10), fatty acid methyl esters (Supelco® 37-Component FAME Mix), 1-alkenes and 1-alkanols (Chemika Fluka). The rest were assigned by similarity of the MS footprint observed with the registered ones in the NIST library.

Results and Discussion

Chemical analysis of the microbial biomass

After extracting the microbial biomass and partitioned it in accordance to Figure S1 (see the supplementary material), all fractions were screened carefully by GC-MS for their volatile components as well by refractionation: TLC, column chromatography, size-exclusion chromatography and spectroscopic study (NMR), identifying the following substances:

Organic compounds

Organic compounds were identified by GC-MS (Table 1) and classified by structural criteria (Figures 1 and 2), as following: *n*-alkanes (1), 1-alkenes (5), 1-alkanols (2), saturated (3) and unsaturated (7) free fatty acids, saturated (4, 6) and unsaturated (8, 9, 10) methyl and ethyl esters of fatty acids, saturated triglycerides (12) and diglycerides (13, 14), unsaturated monoglycerides (15), wax esters (16),

Table 1. Organic compounds identified in the biomass of *Schizochytrium* sp. by GC-MS.

| No	Retention time min (mean ± SD)	Compound (structure)
1	12.377 ± 0.009	Tridecane (**1**; n = 10)
2	12.484	2H-Pyran-2-one, tetrahydro-4-hydroxy-4-methyl- (**17**)
3	12.789	Cyclohexanol, 2-methyl-5-(1-methylethyl)-, (1α, 2β, 5β)- (**19**)
4	12.803	4-Hydroxy-3,4,6-trimethylhept-5-enoic acid lactone (**20**)
5	12.837 ± 0.009	Cyclohexanol, 4-methyl-1-(1-methylethyl)- (**18**)
6	13.230 ± 0.006	1-Pentadecene (**5**; n = 12)
7	13.287 ± 0.012	Tetradecane (**1**; n = 11)
8	14.145 ± 0.012	Pentadecane (**1**; n = 12)
9	14.260	Trinonanoin (**12**; n = 7)
10	14.396 ± 0.053	Dodecanoic acid, methyl ester (**4**; n = 10)
11	14.412	Undecanoic acid, 10-methyl-, methyl ester (**11**; n = 8)
12	14.590	Stearic acid, 1,2,3-propanetriyl ester (**12**; n = 16)
13	14.812 ± 0.066	Tetradecanoic acid (**3**; n = 12)
14	14.875 ± 0.024	1-Hexadecene (**5**; n = 13)
15	14.885	Dodecanoic acid, ethyl ester (**6**; n = 10)
16	15.128	Tridecanoic acid, methyl ester (**4**; n = 11)
17	15.609	9-Hexadecenoic acid, tetradecyl ester, (Z)- (**16**; n = 13)
18	15.632	Tridecanoic acid, ethyl ester (**6**; n = 11)
19	15.672	Stigmasterol (**23**)
20	15.924 ± 0.055	Methyl tetradecanoate (**4**; n = 12)
21	16.316 ± 0.089	Hexadecanoic acid (**3**; n = 14)
22	16.330	1-Eicosanol (**2**)
23	16.378 ± 0.046	Tetradecanoic acid, ethyl ester (**6**; n = 12)
24	16.388	1-Octadecene (**5**; n = 15)
25	16.403 ± 0.072	7-Tetradecenoic acid, (Z)- (**7**; n = 5, m = 5)
26	16.629 ± 0.036	Pentadecanoic acid, methyl ester (**4**; n = 13)
27	17.071 ± 0.026	Pentadecanoic acid, ethyl ester (**6**; n = 13)
28	17.193 ± 0.053	9-Hexadecenoic acid, methyl ester, (Z)- (**8**; n = 5, m = 7)
29	17.303 ± 0.044	Hexadecanoic acid, methyl ester (**4**; n = 14)
30	17.311 ± 0.044	Pentadecanoic acid, 14-methyl-, methyl ester (**11**; n = 12)
31	17.427	Germanicol (**24**)
32	17.607 ± 0.037	Ethyl 9-hexadecenoate (**9**; n = 5, m = 7)
33	17.702 ± 0.044	Hexadecanoic acid, ethyl ester (**6**; n = 14)
34	17.735	Octadecanoic acid, 2-hydroxy-1,3-propanediyl ester (**13**)
35	17.735 ± 0.179	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester (**14**)
36	17.904	Heptadecanoic acid, methyl ester (**4**; n = 15)
37	18.413	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (**10**)
38	18.416	9-Octadecenoic acid, methyl ester (**8**; n = 7, m = 7)
39	18.459 ± 0.046	9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester (**15**)
40	18.514	11-Octadecenoic acid, methyl ester, (Z)- (**8**; n = 5, m = 9)
41	18.533	Octadecanoic acid, methyl ester (**4**; n = 16)
42	18.764	9-Hexadecenoic acid, eicosyl ester, (Z)- (**16**; n = 19)
43	18.838	Ethyl 9-octadecenoate (Z)- (**9**; n = 7, m = 7)
44	18.870	Cholesterol (**22**)
45	18.954	Octadecanoic acid, ethyl ester (**6**; n = 16)
46	19.038	1-Dodecanol, 3,7,11-trimethyl- (**21**)
47	19.206	Docosanoic acid, 1,2,3-propanetriyl ester (**12**; n = 20)
48	19.775	Erucic acid (**7**; n = 7, m = 11)
49	20.024	Eicosanoic acid, methyl ester (**4**; n = 18)
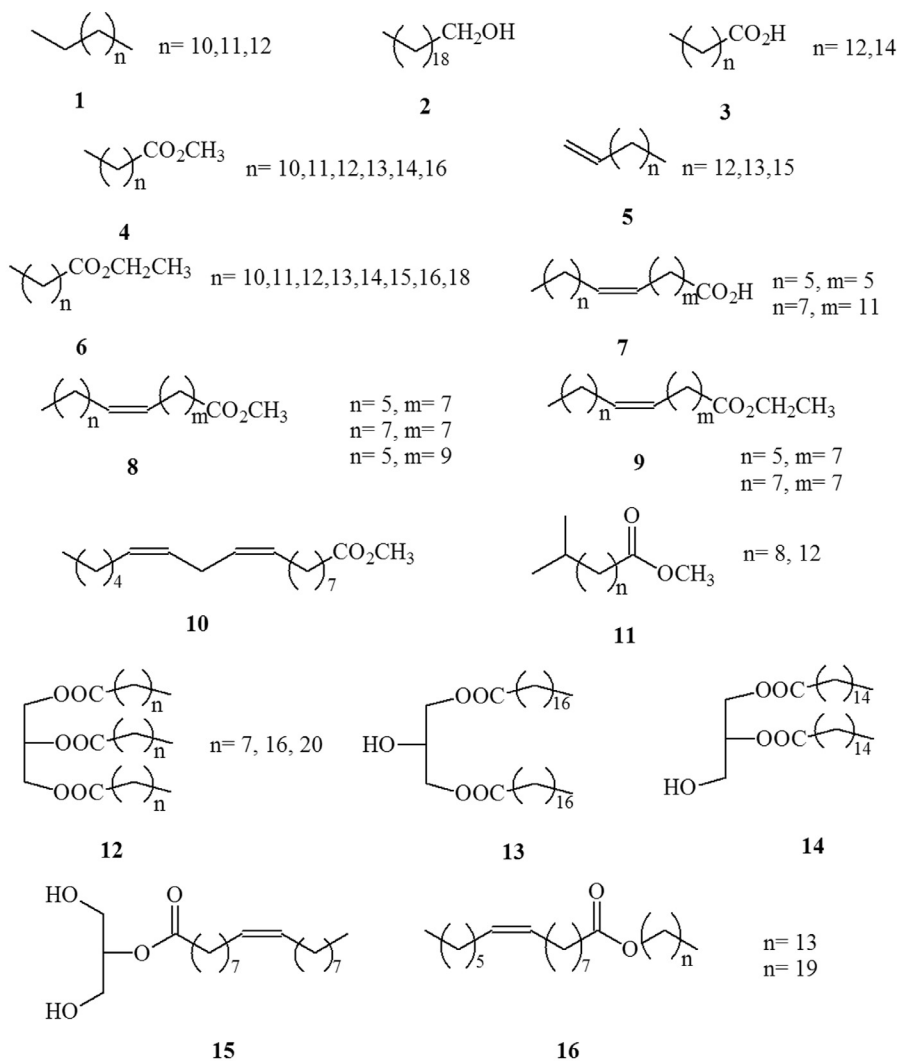
Figure 1 - Lipid compounds identified in *Schizochytrium* sp.

sterols (22, 23), triterpenes (24), mono- and sesquiterpenes (17-21).

Occurrence of branched fatty acid methyl esters

The methyl-branched fatty acids are widely distributed in the nature (Carballeira *et al.*, 2001; Nechev *et al.*, 2002). Now is known, that they are formed by the selective incorporation of the methylmalonyl-CoA, catalysed by the fatty acid synthetase enzyme (Seyama *et al.*, 1981), and that such bioenergetic pathway is characteristic from bacteria that produce relative high concentrations of these *iso*-methylbranched fatty acids. Which are, therefore, accepted as molecular markers of the organic matter produced by bacteria (Kaneda, 1991; Zabeti *et al.*, 2010). Therefore, the identification of the 10-methyundecanoic acid (11, n = 8) and the 14-methylpentadecanoic acid (11, n = 12) methyl esters, two derivatives from iso-methylbranched fatty acids, is an indirect evidence of the presence of *Mycobacterium* genus associated to *Schizochytrium* sp. biomass.

Apart of its GC-MS fingerprint, the *iso*-methyl-substitution proposed in 11 was confirmed by relatively intense fragment ion peak at M^+-43 observed by GC-MS together to the intensity diminution of the M^+-29 fragment (Andersson, 1978).

Absence of toxins

Although there are few studies on the chemistry of this organism is widely accepted that different metabolite profiles can result from each strain and, also, through of different fermentation conditions (Wang *et al.*, 1998). However, and under the present methodology applied, it was unsuccessful to find in the biomass possible toxics bioactive compounds harmful to humans or animals. Or, even, intermediary metabolites involved in a biogenetic pathway that could give rise to these substances. This suggests the potential use of this strain for food purposes, both animal and human.
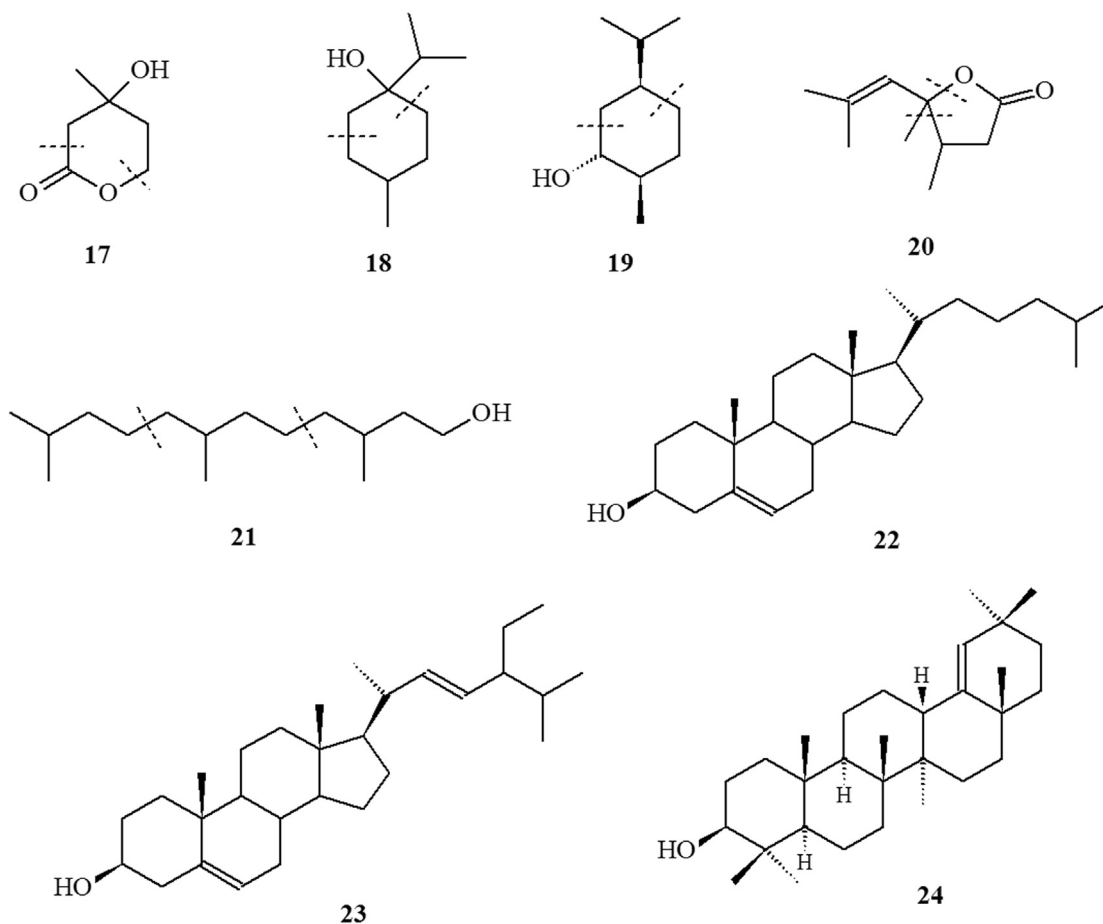
Figure 2 - Terpenoids identified in *Schizochytrium* sp.

Biodiesel production by transesterification of saturated esters

Lipid accumulation from oleaginous microorganisms is under investigation as an alternative to the use of food crop and oils plant as feedstock for the obtaining of renewable fuels and chemicals (Nigam and Singh, 2011). Fungi are especially of interest since they convert efficiently non lipid sources, as the use crop residues or industrial by-product, in cellular lipids. Thus, in order to provide data that could supply a reference to the applicability of *Schizochytrium* sp. in the manufacture of biodiesel, dried-cells were extracted with solvents to obtain a crude extract (15.77% w/w) which was submitted to column chromatography. Monitoring by TLC allowed a total of six fractions. The fourth was taken by the majority (68.69% w/w with respect to crude extract) and ^{1}H-NMR spectroscopy was made up mostly of triglycerides of saturated fatty acids. Transesterification with ethanol followed by column chromatography purification led to a sample of abundant saturated ethyl biodiesel (41.60% w/w compared to crude extract). Integral curve of ^{1}H-NMR spectrum (Figure 3) followed a chain length average of 15.52 carbons. In this experiment it was showed that *Schizochytrium* sp., which

was produced by fermentation and in accordance to Barclay procedures (Barclay, 1994), can be used to produce good yields of biodiesel by acid-catalyzed transformation with previous extraction of the lipids. Alternatively to this process, direct transformation should mean a cost savings for biodiesel production and lipid extraction can be increased, as reported previously for *Mucor circinelloides* (Vicente *et al.*, 2009).

Conclusions

It was identified in the biomass of *Schizochytrium* sp. 24 classes of volatile compounds including *n*-alkanes, 1-alkenes, 1-alkanols, saturated and unsaturated free fatty acids, saturated and unsaturated methyl and ethyl esters of fatty acids, saturated triglycerides and diglycerides, unsaturated monoglycerides, wax esters, sterols, triterpenes, and mono- and sesquiterpenes. Considering the biomass oil extraction (9.47-15.77%) it was concluded that this organism can be used for industrial production of biodiesel once that the "fully saturated ethyl biodiesel" obtained experimentally gave yields of 41.6% w/w regarding the crude extract, with an average chain length of 15.52 carbons.
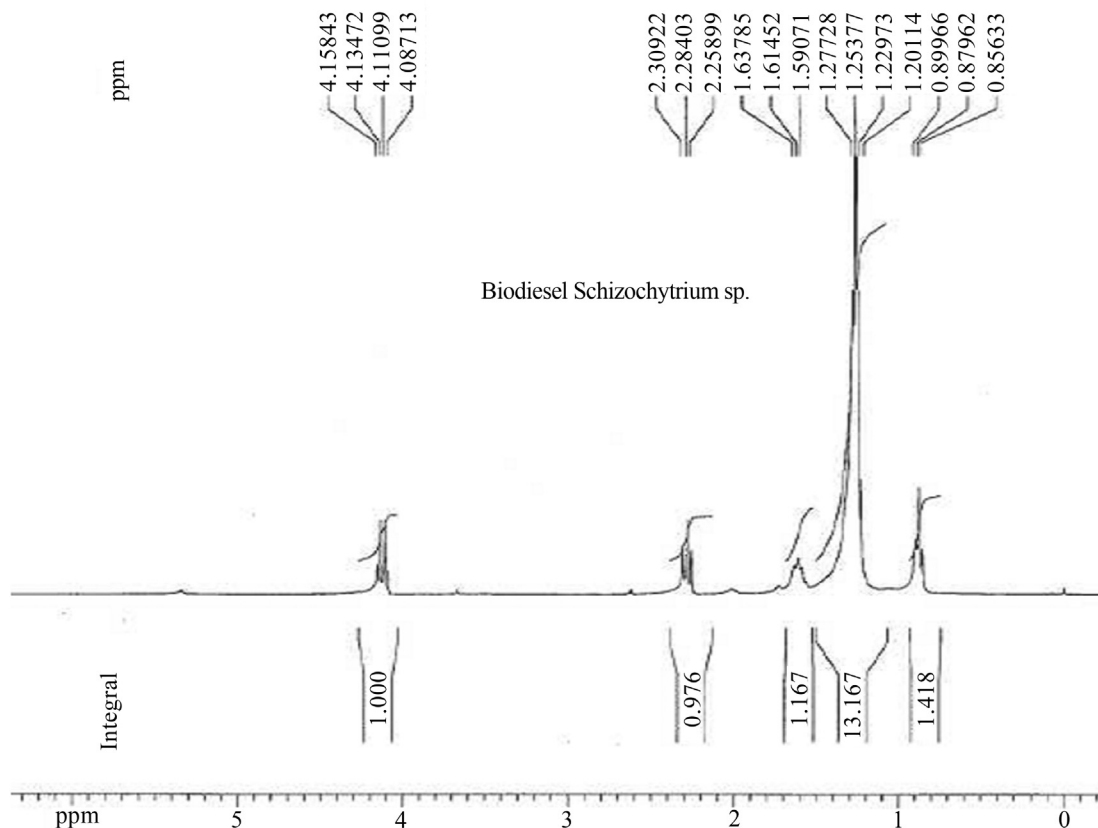
Figure 3 - ¹H-NMR spectrum of saturated ethylic biodiesel obtained from *Schizochytrium* sp.

Acknowledgments

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Supplementary Material

Figure S1 - Solvent-solvent processing scheme for partitioning of *Schizochytrium* sp. biomass, adapted from Kupchan (Kupchan *et al.*, 1973).