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.*, 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. apparatus too. Size exclusion chromatography was carried out on silica gel plates (0.25 mm diameter, Tracer Chem) with the use of CH2Cl2/CH3OH (50:50) at a rate of 1.0 mL min-1. Normal-phase TLC was carried out on the top of the column and eluted with CH2Cl2/CH3OH (50:50, 2 h). The extracts were applied 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>
<thead>
<tr>
<th>No</th>
<th>Retention time min (mean ± SD)</th>
<th>Compound (structure)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.377 ± 0.009</td>
<td>Tridecane (1; n = 10)</td>
</tr>
<tr>
<td>2</td>
<td>12.484</td>
<td>2H-Pyran-2-one, tetrahydro-4-hydroxy-4-methyl- (17)</td>
</tr>
<tr>
<td>3</td>
<td>12.789</td>
<td>Cyclohexanol, 2-methyl-5-(1-methylethyl)-, (1α, 2β, 5β)- (19)</td>
</tr>
<tr>
<td>4</td>
<td>12.803</td>
<td>4-Hydroxy-3,4,6-trimethyloct-5-enolic acid lactone (20)</td>
</tr>
<tr>
<td>5</td>
<td>12.837 ± 0.009</td>
<td>Cyclohexanol, 4-methyl-1-(1-methylethyl)- (18)</td>
</tr>
<tr>
<td>6</td>
<td>13.230 ± 0.006</td>
<td>1-Pentadecene (5; n = 12)</td>
</tr>
<tr>
<td>7</td>
<td>13.287 ± 0.012</td>
<td>Tetradecane (1; n = 11)</td>
</tr>
<tr>
<td>8</td>
<td>14.145 ± 0.012</td>
<td>Pentadecane (1; n = 12)</td>
</tr>
<tr>
<td>9</td>
<td>14.260</td>
<td>Trinonanoin (2; n = 7)</td>
</tr>
<tr>
<td>10</td>
<td>14.396 ± 0.012</td>
<td>Dodecanoic acid, methyl ester (4; n = 10)</td>
</tr>
<tr>
<td>11</td>
<td>14.412</td>
<td>Undecanoic acid, 10-methyl-, methyl ester (11; n = 8)</td>
</tr>
<tr>
<td>12</td>
<td>14.590</td>
<td>Stearic acid, 1,2,3-propanetriyl ester (12; n = 16)</td>
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<td>13</td>
<td>14.812 ± 0.066</td>
<td>Tetradecanoic acid (3; n = 12)</td>
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<tr>
<td>14</td>
<td>14.875 ± 0.024</td>
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<td>14.885</td>
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<td>16</td>
<td>15.128</td>
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<tr>
<td>17</td>
<td>15.260</td>
<td>9-Hexadecenoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester (14)</td>
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<td>18</td>
<td>15.362</td>
<td>Tridecanoic acid, ethyl ester (6; n = 11)</td>
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<tr>
<td>19</td>
<td>15.672</td>
<td>Stigmasterol (23)</td>
</tr>
<tr>
<td>20</td>
<td>15.924 ± 0.055</td>
<td>Methyl tetradecanoate (4; n = 12)</td>
</tr>
<tr>
<td>21</td>
<td>16.316 ± 0.089</td>
<td>Hexadecanoic acid (3; n = 14)</td>
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<td>22</td>
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<td>1-Eicosanol (2)</td>
</tr>
<tr>
<td>23</td>
<td>16.378 ± 0.046</td>
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<td>24</td>
<td>16.388</td>
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<td>25</td>
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<td>7-Tetradecenoic acid, (Z)- (7; n = 5, m = 5)</td>
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<td>16.629 ± 0.036</td>
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<td>27</td>
<td>17.071 ± 0.026</td>
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<tr>
<td>28</td>
<td>17.193 ± 0.053</td>
<td>9-Hexadecenoic acid, methyl ester, (Z)- (8; n = 5, m = 7)</td>
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<tr>
<td>29</td>
<td>17.303 ± 0.044</td>
<td>Hexadecanoic acid, methyl ester (4; n = 14)</td>
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<tr>
<td>30</td>
<td>17.511 ± 0.044</td>
<td>Pentadecanoic acid, 14-methyl-, methyl ester (11; n = 12)</td>
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<tr>
<td>31</td>
<td>17.427</td>
<td>Germanicol (24)</td>
</tr>
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<td>32</td>
<td>17.607 ± 0.037</td>
<td>Ethyl 9-hexadecenoate (9; n = 5, m = 7)</td>
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<td>33</td>
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<td>34</td>
<td>17.735</td>
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<tr>
<td>35</td>
<td>17.735 ± 0.179</td>
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<td>17.904</td>
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</tr>
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<td>37</td>
<td>18.413</td>
<td>9,12-Octadecadienoic acid (Z,Z), methyl ester (10)</td>
</tr>
<tr>
<td>38</td>
<td>18.416</td>
<td>9-Octadecanoic acid, methyl ester (8; n = 7, m = 7)</td>
</tr>
<tr>
<td>39</td>
<td>18.459 ± 0.046</td>
<td>9-Octadecanoic acid (Z), 2-hydroxy-1-(hydroxymethyl)ethyl ester (15)</td>
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<td>40</td>
<td>18.514</td>
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<td>41</td>
<td>18.533</td>
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<td>42</td>
<td>18.764</td>
<td>9-Hexadecenoic acid, eicosyl ester, (Z)- (16; n = 19)</td>
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<tr>
<td>43</td>
<td>18.838</td>
<td>Ethyl 9-octadecenoate (Z)- (9; n = 7, m = 7)</td>
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<td>44</td>
<td>18.870</td>
<td>Cholesterol (22)</td>
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<td>45</td>
<td>18.954</td>
<td>Octadecanoic acid, ethyl ester (6; n = 16)</td>
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<td>46</td>
<td>19.038</td>
<td>1-Dodecanol, 3,7,11-trimethyl- (21)</td>
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<td>47</td>
<td>19.206</td>
<td>Docosanoic acid, 1,2,3-propanediyl ester (12; n = 20)</td>
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<tr>
<td>48</td>
<td>19.775</td>
<td>Erucic acid (7; n = 7, m = 11)</td>
</tr>
<tr>
<td>49</td>
<td>20.024</td>
<td>Eicosanoic acid, methyl ester (4; n = 18)</td>
</tr>
</tbody>
</table>
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.
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 1H-NMR spectrosopy 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 1H-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.
Acknowledgments

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References

Dried mycelium (118.00 g)

Soak in CH₂Cl₂ (x3, 24 h), CH₃OH (x3, 24 h), filtration and rotavap down

Crude extract (10.850 g)

H₂O (200 mL): CH₂Cl₂ (x3, 200 mL)

Aqueous phase
Sa-H-0
Extracted with BuOH (x3)

Organic phase
Sa-L-0 (1.673 g)

BuOH phase
Sa-H-1 (0.806 g)

H₂O phase
Discarded

Partition 90% CH₃OH: H₂O, extracted with Hexane (x3)

Hexane phase
Sa-L-1 (1.298 g)

Methanolic-aqueous

Partition 50% CH₃OH: H₂O, extracted with CH₂Cl₂ (x3)

CH₃OH/ H₂O phase
Sa-L-3 (0.023 g)

CH₂Cl₂ phase
Sa-L-2 (0.331 g)

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