Lipids and lipolytic enzymes of the microalga *Isochrysis galbana*

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**Abstract** – Marine microalgae are now well-known for their ability to produce omega-3 long chain polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Among these microalgae, *Isochrysis galbana* has received increasing interest especially because of its high DHA content and its common use in hatchery to feed fish larvae and clams. Moreover, lipolysis occurring from the biomass harvest stage suggests that *I. galbana* may contain lipolytic enzymes with potential interesting selectivities. For these reasons, the potential of this microalga for the production of valuable lipids and lipolytic enzymes was investigated. Lipid analysis revealed that DHA is mainly located at the sn-2 position of the phospholipids. Thus, *I. galbana* was considered as an interesting starting material for the lipase catalyzed production of 1-lyso-2-DHA-phospholipids which are considered as convenient vehicles for the conveyance of DHA to the brain. Lipids from *I. galbana* can also be used for the enzyme-catalyzed production of structured phospholipids containing one DHA and one medium chain fatty acid in order to combine interesting therapeutic and biological benefits. Starting from total RNA extract from *I. galbana*, coding sequences of putative lipolytic enzymes were obtained by RACE and Nested PCR. The heterologous expression of a sequence designated IgTeCe was implemented. An expression plasmid was constructed by ligation of the coding sequence to a plasmid vector and then cloned and expressed in *E. coli*. Results showed the effective functionality of plasmid construction for the production of a recombinant protein with the expected molecular mass. Moreover, local alignment using BLASTP and biochemical evidences support the hypothesis that the expressed protein is a thioesterase.

**Keywords:** microalgae / ω-3 polyunsaturated fatty acids / phospholipids / lipolytic enzymes

**Résumé** – Les microalgues marines sont maintenant bien connues pour leur aptitude à produire des acides gras à longue chaîne de la série ω-3 comme l’acide docosahexaénoïque (DHA) ou eicosapentaénoïque (EPA). Parmi ces microalgues, *Isochrysis galbana* connaît, ces dernières années, un intérêt croissant du fait d’un contenu lipidique riche en DHA et de son importante utilisation en écloserie pour nourrir les larves de poissons et les bivalves. D’autre part, la lipolyse souvent observée dès l’étape de collecte de la biomasse ainsi que la forte teneur en DHA suggère qu’*I. galbana* pourrait contenir des enzymes lipolytiques intéressantes en termes de sélectivité. Pour ces différentes raisons, le potentiel de cette microalgue pour la production de lipides d’intérêt et d’enzymes lipolytiques a été étudié. L’analyse des lipides d’*I. galbana* a tout d’abord révélé que le DHA était majoritairement greffé sur la position sn-2 des phospholipides. Dans ce contexte, les phospholipides d’*I. galbana* constituent une matière première intéressante pour la production, par voie enzymatique, de 1-lyso-2-DHA phospholipides, des composés intéressants pour optimiser le transport du DHA au niveau du cerveau. À partir des lipides d’*I. galbana*, on peut également envisager la production, toujours par voie enzymatique, de phospholipides structurés contenant du DHA et un acide gras à chaîne moyenne ce qui permet de combiner des intérêts thérapeutiques et biologiques intéressants. À partir des ARN totaux d’*I. galbana*, des séquences codant des enzymes lipolytiques putatives ont été obtenues par RACE et Nested PCR. L’expression hétérologue d’une séquence nommée IgTeCe a été initiée. Une construction plasmidique contenant la séquence codante a été clonée et exprimée avec *E. coli*. Les résultats ont montré que la
1 Introduction

Microalgae are unicellular photosynthetic and eukaryotic organisms with a wide range of morphologies, shapes, sizes and aspects. Actually, the total number of algae species has been recently estimated to 72,500 despite some uncertainties regarding what organisms should be included as algae or microalgae (Guiry, 2012). Primary producers of the aquatic ecosystems, microalgae belong, along with the cyanobacteria, to the so-called “phytoplankton” consumed by zooplankton, fish, larvae and clams. According to favorable conditions, microalgae have periods of fast growth called “bloom” that can cause red tides. Besides, these tides can sometimes be associated with the production of phycotoxins and lead to harmful algal blooms (HAB) and shellfish poisoning (Masó and Garcés, 2006).

Boosted by the attracting market of biofuels, microalgae have received increasing interest during the last decade and today they are considered as sustainable natural factories for the production of a wide range of molecules with potential applications in the field of feed, food, cosmetics, pharmaceuticals and energy (Mimouni et al., 2012; Person, 2010).

As photosynthetic organisms, microalgae are a source of pigments such as β-carotene, that can be found for instance in the halophilic strain Dunaliella salina cultivated on a large scale along the California coast. Other species like Porphyridium cruentum are also known to be sources of interesting polysaccharides. Nevertheless, the most promising biochemical compounds that can be found in microalgae are certainly the lipids for their ω-3 fatty acids (Mimouni et al., 2015; Poisson et al., 2002) and mainly for the production of biodiesel which has been an important topic for several years now (Cadoret and Bernard, 2008). However, according to many literature data, to be interesting from an economical point of view, biodiesel needs to be coproduced with other high added value molecules leading to the concepts of “bio-refinery” and “cellular milking” (Vinayak et al., 2015).

In this frame, the present paper is aimed at presenting the interest of the microalga Isochrysis galbana for the production of lipids and lipolytic enzymes with potential applications.

2 Isochrysis galbana: an overview

Isochrysis galbana is one of the species of the Isochrysidales order, belonging to the haptophyta division (phylum) (Fig. 1). Unlike other microalgae classified within the Isochrysidales such as Emiliania huxleyi, I. galbana has no dimorphic lifecycle and does not form calcareous cocoliths. Moreover, contrary to Emiliania huxleyi, I. galbana exhibits organic scales (Bendif et al., 2013).

First identified by Bruce, Knight and Parke in 1939, Isochrysis galbana is a rather small round to ovoid cell (2–5 μm; 40–50 μm²) bearing two smooth apical flagella (Fig. 1). In reference to the division name, the cells were also bearing a reduced haptonema said “vestigial” inserted between the two flagella (Robert et al., 2004).

Up to date, two strains of Isochrysis were mainly described and studied in literature: T-Iso and Parke. Isochrysis affinis galbana, more simply designated as T-iso, was formerly said to be a Tahitian clone conspecific with I. galbana Parke which originate from temperate zones. In link with its origin, T-Iso is capable of adapting to a much broader range of temperature and light conditions when comparing with I. galbana Parke (Ewart and Pruder, 1981; Tzovenis et al., 2003).

Apart from its plastid coloration which is more yellow, T-Iso is similar to Parke from a morphological point of view and no difference can be noticed nor by photonic microscopy nor by scanning electronic microscopy (SEM) (Robert et al., 2004).

In 2013, using, as molecular phylogenetic markers, the genes coding RNA small ribosomal subunit (SSU rRNA), Bendif et al. (2013) concluded that the genetic distance between T-Iso and I. galbana is sufficient to raise T-Iso as a new genus. This new genus was called Tsiochrysis lutea in reference to the former name of the strain and to the yellow color of the cells.

In the same area, Pseudoisochrysis paradoxus was also renamed Isochrysis galbana.

Before this recent revision, the strains of Isochrysis were not always fully characterized and some confusion may have occurred in literature as already pointed, more generally for whole microalgae, by Muller-Feuga (1997).

I. galbana and T. lutea can be obtained worldwide in several culture collections under different specific strain codes (Tab. 1). This can also be a source of error in literature.

To be suitable in mollusc hatchery, algal cells have to present four main features:
• a size below 30 μm or even 10 μm for the early larval stages;
• devoided of cell wall or organs making their ingestion difficult;
• an adequate nutritional value;
• easy to cultivate on a large scale (Robert and Trintignac, 1997).

Combining these four features, I. galbana and more particularly Tsiochrysis lutea were intensively used for years in hatchery to feed fish larvae and clams.

Mots clés : microalgues / acide gras polyinsaturés ω-3 / phospholipides / enzymes lipolytiques

Fig. 1. (A) Elements of classification; (B) photograph of Isochrysis galbana (photonic microscopy ×400).
Table 1. List of principal strain codes of *Isochrysis galbana* and relatives.

<table>
<thead>
<tr>
<th>Name</th>
<th>Strain code</th>
<th>CCAP</th>
<th>PLY</th>
<th>NCMA</th>
<th>UTEX</th>
<th>RCC</th>
<th>Isolator</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Isochrysis sp.</em></td>
<td>CCAP 927/12</td>
<td>PLY240</td>
<td></td>
<td></td>
<td></td>
<td>RCC3709</td>
<td>Butcher</td>
<td>/</td>
</tr>
<tr>
<td><em>Isochrysis galbana</em></td>
<td>CCAP 927/1</td>
<td>PLY565</td>
<td>CCMP1323</td>
<td></td>
<td>LB 987</td>
<td>RCC1348</td>
<td>Parke</td>
<td>/</td>
</tr>
<tr>
<td>Parke</td>
<td>CCAP 949/1</td>
<td>PLY507</td>
<td>CCMP715</td>
<td>LB 1988</td>
<td>RCC1353</td>
<td>/</td>
<td>Ott</td>
<td>Formerly <em>Pseudoisochrysis paradoxa</em></td>
</tr>
<tr>
<td><em>Tisochrysis lutea</em></td>
<td>CCAP 927/14</td>
<td>PLY506</td>
<td>CCMP1324</td>
<td>LB 2307</td>
<td>RCC1349</td>
<td>Haines</td>
<td>/</td>
<td></td>
</tr>
</tbody>
</table>

CCAP: Culture Collection of Algae and Protozoa (UK); PLY: Plymouth (UK); NCMA: National Center for Marine Algae and Microbiota (formerly CCMP) (USA); UTEX: University of Texas (USA); RCC: Roscoff Culture Collection (France).

In the frame of the study concerning lipolytic enzymes, *I. galbana* Parke (CCAP 927/1) was cultivated on a lab scale on Provasoli 1/3 medium in 500 mL Erlenmeyer flasks as previously described (Poisson and Ergan, 2001).

For the studies concerning lipids, larger amounts of biomass from *I. galbana* Parke (CCAP 927/1) and *Tisochrysis lutea* (CCAP 927/14) were obtained from Greensea (Mèze, France). The microalgae were cultivated in 100 L photobioreactors on sea water adjusted at 28 °C under continuous illumination of 100 μE.m⁻².s⁻¹. Agitation was provided by air bubbling and CO₂ injection. Microalgae were harvested by centrifugation in the early stationary phase when the lipid content is at the highest. Then, the vessel containing the harvested biomass was bathed in boiling water. The purpose of this step is to inhibit endogenous enzymatic activity; this is crucial since it allows to prevent the extent of lipolysis occurring in biomass during wet storage very shortly after harvest (Balduyck et al., 2016).

### 3 Lipids

Total lipids were extracted from the biomass using an optimized method derived from Folch et al. (1957) complemented by ultrasonic treatment (unpublished results).

Total lipids were classically separated by G60 silica gel chromatography using successively dichloromethane, acetone and methanol as eluting solvents. Total lipids and fatty acid analyses were carried out respectively by thin layer chromatography (TLC) and gas chromatography (GC) as previously described (Devos et al., 2006).

TLC of total lipids carried out by a double development in one dimension (Fig. 2) revealed the presence of three types of lipids:

- neutral lipids with sterol, triacylglycerol and a ghost spot of free fatty acids;
- glycolipids with in particular sulfoquinovosyldiacylglycerides (SQDG) an interesting compound which is reported to have anti-tumoral and antiviral activities (Hossain et al., 2005; Plouguené et al., 2013);
- phospholipids mostly containing phosphatidylcholine.

Table 2 shows fatty acid profiles obtained by GC. In total lipids, eight main fatty acids can be found from myristic acid (C14:0) to docosaheaxenoic acid (C22:6-DHA) which represents 13.5% of the total fatty acids in *I. galbana* Parke (CCAP 927/1). DHA is a ω-3 polyunsaturated fatty acid (PUFA) known for its health benefits on cardiovascular system and cognitive functions. However, its consumption is less than the recommended daily intake in most occidental countries (Barnathan, 2007; Bourre, 2005).

When comparing the fatty acid composition of the different lipid fractions, it can be assumed that the phospholipid composition is quite different from the two others with 25% of myristic acid and especially 50% of DHA which is pretty high. Similar results were obtained with *T. lutea* except for the content of linoleic acid which appeared to be higher when comparing with *I. galbana* Parke. Concerning *T. lutea* lipids, it can also be noticed that a mutant has been recently obtained by IFREMER Nantes leading to an increase of 80% for neutral lipids productivity (Bougaran et al., 2012).

To go further with *I. galbana* lipids, the location of DHA on the phospholipid backbone was determined. The phospholipids from *I. galbana* were submitted to hydrolysis catalyzed by a phospholipase A2 (PLA2). The time course of this reaction clearly showed that DHA was preferentially released so it can be concluded that DHA is mainly located at the sn-2 position (Devos et al., 2006).
Starting with this raw material, a selective deacylation can be implemented to produce lysophospholipids rich in DHA (Fig. 3A). In the last decade, several studies have contributed to suggest that lyso-PLs are convenient vehicles for an optimal absorption of DHA in the brain. Moreover, DHA is reported to be less suggested to oxidation when associated with phospholipids comparing with triglycerides (Pencréac'h et al., 2013).

Phospholipids from *I. galbana* appear also to be convenient for the production of structured phospholipids containing DHA and a medium chain fatty acid like caprylic acid (C8:0) with a view to combine rapid energy intake with health benefits (Fig. 3B). Moreover, it has been reported in literature that medium-chain fatty acids (MCFA) may improve bioavailability of PUFAs (Lemarié et al., 2016). The production of DHA lysophospholipids as well-structured phospholipids can be achieved by enzymatic catalysis using more particularly lipases (Devos et al., 2006; Vikbjerg, 2006).

### 4 Lipolytic enzymes

As mentioned above, when biomass was not treated with boiling water, important amounts of free fatty acids are detected in the total lipid extracts: this may suggest the presence of endogenous lipolytic enzymes. Moreover, the high proportion of PUFAs in *I. galbana* could also suggest attractive enzyme specificities. Very few data can be found in literature concerning this point. For these reasons, the exploration of lipolytic enzymes from *I. galbana* Parke (CCAP 927/1) was implemented.

### Table 2. Main fatty acids in lipids from *Isochrysis galbana* Parke (927/1) and *Tisochrysis lutea*.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Common name</th>
<th>wt% total fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>I. galbana</em> Parke</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TL</td>
</tr>
<tr>
<td>C14:0</td>
<td>Myristic acid</td>
<td>18.0</td>
</tr>
<tr>
<td>C16:0</td>
<td>Palmitic acid</td>
<td>8.8</td>
</tr>
<tr>
<td>C16:1</td>
<td>Palmitoleic acid</td>
<td>2</td>
</tr>
<tr>
<td>C18:1</td>
<td>Oleic acid</td>
<td>13.5</td>
</tr>
<tr>
<td>C18:2</td>
<td>Linoleic acid</td>
<td>4.3</td>
</tr>
<tr>
<td>C18:3</td>
<td>Linolenic acid</td>
<td>11.4</td>
</tr>
<tr>
<td>C18:4</td>
<td>Stearidonic acid</td>
<td>9.5</td>
</tr>
<tr>
<td>C22:6</td>
<td>Docosahexaenoic acid</td>
<td>13.5</td>
</tr>
</tbody>
</table>

TL: total lipids; NL: neutral lipids; GL: glycolipids; PL: phospholipids; /: not detected or not taken into account.

Fig. 3. Enzyme-catalyzed modification of phosphatidylcholine from *Isochrysis galbana* to obtain DHA-lysophosphatidylcholine (A) and structured phosphatidylcholine containing DHA (B). Rx: nonspecific fatty acid; MCFA: specific medium-chain fatty acid.
Starting from the total mRNA extract, complete cDNA of putative lipolytic enzymes were obtained after RACE and Nested PCR (Fig. 4). Specific primers used for RACE and Nested PCR were designed by consulting *I. galbana* Express Sequence Tag Database. This protocol allowed to obtain three coding sequences. *In silico* analysis using BLASTP alignment led to assign the first sequence as being a putative esterase designated “Igest1” and registered under the accession number FR870450. The second sequence showed close relationships with fungal lipases and has been designated “Iglip” and recorded under the accession number FR870450. The third sequence was designated as “IgTeCe” standing for *I. galbana* thioesterase-carboxylesterase (Godet et al., 2010; 2012; Kerviel et al., 2014).

To go further, the heterologous expression of IgTeCe was then implemented. IgTeCe was ligated in pJET plasmid and cloned into *E. coli* strain TOP 10 (Fig. 4). Cloning products were verified by sequencing and the expression of IgTeCe was performed into *E. coli* strain Rosetta2 using the plasmid pET 52. It has to be noticed that pET 52 contains an induction system working with IPTG and allows for the production of a protein containing a deca-histidine C-terminal tag (Kerviel et al., 2014).

After expression, soluble proteins were submitted to SDS-PAGE analysis (Fig. 5). No significant additional band can be clearly noticed on lane corresponding to the induced cells when comparing with the non-induced cells. Nevertheless, when the proteins were blotted and detected with Anti-His antibodies, the membrane revealed two bands only with the induced cells (Fig. 5). The major one agrees with the predicted size of the recombinant protein, that is to say 46.18 kDa. To explain the second band, the hypothesis of a phosphorylated form of the protein can be made. Reverse Transcriptase PCR implemented with primers specific of the IgTeCe gene, confirm that a transcript was only present when IPTG was added but not in the absence of IPTG (Fig. 6).
By local alignment using BLASTP, the obtained protein displays similarities with thioesterases. To confirm this finding, the fatty acid profile of E. coli IPTG induced cells has been compared with E. coli non-induced cells. A fatty acid analysis of both cell types was carried out by GC (Fig. 7). A high proportion of palmitic acid and the presence of six other fatty acids can be noticed. Two other fatty acids remain unknown when comparing with all fatty acid standards available in the lab. Presumably, they could be branched-chain fatty acids commonly found in the lipids of bacteria. It appeared that IgTeCe expression led to an increase in palmitoleic acid (C16:1) from around 2% to 10% and an increase in oleic acid (C18:1) from 15% to 21%. On the other hand, the proportion of the two unknown fatty acids decreased. In literature, similar modification was observed with heterologous expression of acyl ACP thioesterase. Indeed, Chen et al. (2012) published very similar results with a thioesterase from peanut. Thus, the hypothesis that the expressed protein is a thioesterase appears to be consistent. Actually, thioesterase are quite interesting enzymes since they can be used to improve the release of free fatty acid and to alter the fatty acid profile with a view to obtain a particular lipid composition needed for a specific application such as biofuel production (Chen et al., 2012; Kerviel et al., 2014).

5 Conclusion

Apart from their potential in aquaculture I. galbana Parke and T. lutea also constitute an original source of phospholipids and enzymes with interesting applications in human nutrition and biotechnology. Nevertheless, to take the most of these molecules, they need to be integrated in a more complete valorization of high value compounds leading to a kind of “microalga cracking”. To make it feasible, an integrated approach needs to be implemented to explore I. galbana Parke and T. lutea from biomass production to product extraction and valorization. This may go through collaborative works specifically dedicated to these microalgae and through the building of comprehensive genomic and transcriptomic data catalogues.

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References


Fig. 7. Fatty acid composition of Escherichia coli strain expressing pET IgTeCe. Data are means of triplicates (from Kerviel et al., 2014).


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