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SHORT COMMUNICATIONS

Naturally occurring fatty acid methyl esters and ethyl esters in the green microalga *Chlamydomonas reinhardtii*

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Abstract Biodiesel can be defined as fatty acid methyl esters (FAMEs) or fatty acid ethyl esters (FAEE) obtained from the transesterification of triacylglycerides (TAGs) from vegetable oils and animal fats; however, oleaginous microalgae are emerging as potential substitutes of these feedstocks for biodiesel production. The use of the green microalga *Chlamydomonas reinhardtii* P. A. Dang as a model system for lipid metabolism studies offers the advantage that extensive physiological and genomic data are available. The presence of naturally occurring FAMEs has been reported previously in plants and microalgae. In this study, oil extracts of *C. reinhardtii* batch cultures at the end of the growth phase were analyzed before and after the transesterification reaction to investigate the presence of naturally occurring FAMEs in this microalga. As a result, the presence of these compounds was observed in hexane oil extracts of *C. reinhardtii* before transesterification. Five FAMEs were identified by gas chromatography-mass spectrometry (GC-MS) before and after transesterification, while one additional FAME appeared only after transesterification. Additionally, three FAEEs were also identified before and after the transesterification reaction. Therefore, naturally occurring FAMEs and FAEEs are reported in *C. reinhardtii*. These results will pave the way for further studies on the biosynthesis of these compounds in green microalgae, and their potential use as biofuels.

Keywords Microalgae · Biodiesel · Lipid · Triacylglyceride

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Introduction

Biodiesel [fatty acid methyl esters (FAMEs) or fatty acid ethyl esters (FAEE)] is considered a very attractive, renewable and non-toxic fuel (Meng et al. 2011), which can be used with existing technology for diesel consumption. Currently, biodiesel is produced from oil crops like rapeseed, soybean, oil palm and *Jatropha curcas* (Ma and Hanna 1999; Muniyappa et al. 1996; Myint and El-Halwagi 2009), and also from waste vegetable oils (Canakci 2007); likewise, algal oil has proved useful for this purpose (Xu et al. 2006). Furthermore, microalgae have been suggested as the only option with the potential to displace petroleum fuels without affecting food supply or the environment (Chisti 2008). Microalgae have high photosynthetic efficiencies, rapid growth, can be grown in treated waste waters, and can be used for CO_2 sequestration (Schenk et al. 2008). Both freshwater and marine microalgae have been reported as suitable renewable oil sources, and studies on their fatty acid composition have been reported (Chisti 2008; Gouveia and Oliveira 2009; Rodolfi et al. 2009). To achieve high levels of biomass and oil production from microalgae, culture conditions must be optimized (Xu et al. 2006); many microalgae produce substantial amounts of storage lipids [triacylglycerides (TAGs)] for energy storage under adverse environmental conditions such as nutrient

starvation, particularly nitrogen limitation (Khozin-Goldberg et al. 2002; Li et al. 2008; Mandal and Mallick 2009; Widjaja et al. 2009), or under specific conditions of salinity, temperature, pH, light intensity, and CO_2 concentration (Herrera-Valencia et al. 2011; Hu et al. 2008; Takagi et al. 2006). In addition, it is necessary to understand lipid metabolism and how fatty acids and TAGs are synthesized and accumulated within the cell. In plants, lipid metabolism is better understood (Durrett et al. 2008), while in microalgae the metabolic routes involved in lipid synthesis are less studied and need further investigation. *Chlamydomonas reinhardtii* is a model system for biological processes, for which extensive physiological and genomic data are available (Merchant et al. 2007; Harris 2009). Early studies in this microalga focused on its fatty acid composition using various culture conditions (El-Sheekh 1993) as well as the structural role of lipids in the membranes, the genes encoding proteins involved in membrane biogenesis and glycerolipid synthesis, and the function of lipids in signal transduction pathways (Riekhof et al. 2005; Guschina and Harwood 2006; Vieler et al. 2007). Recently, the mechanisms of TAG accumulation in *C. reinhardtii* have attracted more attention (James et al. 2011; Miller et al. 2010; Siaut et al. 2011) since the knowledge generated in this microalga could be applied to improve the accumulation of TAGs for biodiesel (FAMEs or FAEEs) production in other green microalgae. Biodiesel can be produced by transesterification reactions or enzymatic methods (Meng et al. 2011). Interestingly, FAMEs can be produced naturally by plant cells, as it has been reported for *J. curcas* (Annarao et al. 2008). The lipid profiling in developing seeds of *J. curcas* revealed the presence of FAMEs in hexane extracts of very young seeds (Annarao et al. 2008). In the case of microalgae, naturally occurring FAMEs have been reported previously in the freshwater green microalgae *Eudorina unicocca* and *Volvox aureus* (Zhang et al. 2009). Therefore, the aim of this study was to determine the presence of naturally occurring FAMEs in the model microalga *C. reinhardtii*. The results revealed the natural occurrence of FAMEs along with FAEEs at the end of the growth phase of *C. reinhardtii* cultures.

Materials and methods

The microalgal strain 137c (mt+) of *C. reinhardtii* was kindly donated by E.H. Harris (Chlamydomonas Center; http://www.chlamy.org/). All reagents and solvents were analytical grade. The culture system consisted of two 2 L Erlenmeyer flasks containing 1,800 mL complete Tris-acetate-phosphate (TAP) medium (Harris 1989). The flasks were inoculated with 10,000 cells mL^{-1}; culture conditions were 25±1°C, under a photoperiod of 16:8 light–dark cycle at light intensity of 20 μmol m^{-2} s^{-1}. For agitation, ambient air was pumped through a 0.7 μm sterile fiber glass disc (Millipore), bubbled through a humidifier and sparged at a rate of 1.1 vvm. This system proved to maintain the sterility of the culture. For the growth curve, samples were collected daily for 10 days. Cell concentration was determined using a Neubauer hemacytometer. Cells were harvested by centrifugation and dried using a freeze dryer for 24 h. The pellet was weighted and considered as dry biomass weight.

Prior to extraction of microalgal oil, biomass was collected on day 7; cultures were centrifuged at 4,000 rpm for 6 min at 25°C, the pellet obtained was kept at −80°C overnight and then freeze-dried for 24 h. The dry pellet was used for extraction with hexane in a Soxhlet apparatus for 8 h. The solvent was vacuum evaporated to obtain the algal oil extract.

Analytical thin-layer chromatography (TLC) was carried out on precoated silica gel aluminum plates (60 F_{254}, 0.2 mm thickness, Merck, Darmstadt, Germany) using hexane: ethyl acetate: acetic acid (90:10:1) as solvent system. Lipids were visualized by spraying a solution prepared with 20 g phosphomolybdic acid and 2.5 g ceric sulfate in 500 mL 5% aqueous H_2SO_4. Column chromatography was conducted with silica gel (60–200 mesh, J.T. Baker, Phillipsburg, NJ), and the fractions were eluted with hexane and different mixtures of hexane:ethyl acetate (90:10, 80:20, 70:30, 50:50 v/v) and ethyl acetate alone. The fractions were analyzed by TLC and the solvent vacuum evaporated. Gas chromatography-mass spectroscopy (GC-MS) analysis was carried out in an Agilent 6890 N chromatograph equipped with an Agilent 5975B mass selective detector. Compounds were profiled on a 30 m×0.32 mm ID×0.5 μm film Agilent DB-5 capillary column, carrier gas was He at 1.5 mL min^{-1}. Temperature conditions were: 120°C for 1 min, 15°C min^{-1} ramp to 180°C, 7°C min^{-1} ramp to 230°C and 10°C min^{-1} ramp to 300°C and hold for 60 min. Ionization voltage was 70 eV and sample injection volume was 2 μL.

For algal oil transesterification, the hexane fraction was dissolved in 2 mL heptane and 300 μL of a sodium metoxide solution (0.35% w/w) were added. In order to homogenize the reaction medium, 300 μL ethyl acetate was added. The reaction was carried out under agitation at 45°C for 90 min, then left to settle and biodiesel was recovered using a Pasteur pipette from the upper phase and analyzed.

Results and discussion

The *C. reinhardtii* growth curve is shown in Fig. 1. The end of the exponential phase was reached at day 7 (4.17×10^6 cells mL^{-1}), thus the microalgal biomass was collected for lipid extraction on this day. Five independent microalgae cultures were carried out and the following data were obtained: cell concentration at the end of exponential phase

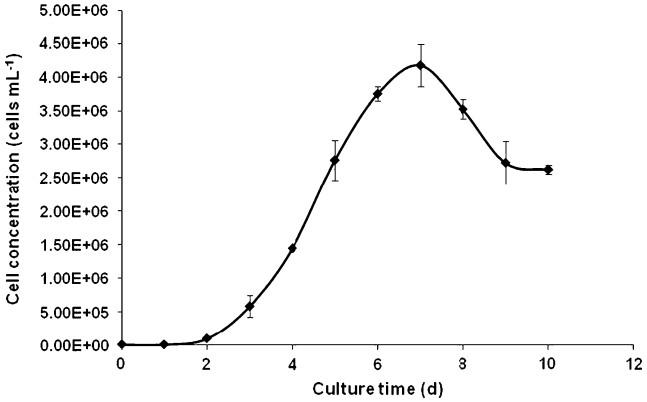

Fig. 1 Growth curve of *Chlamydomonas reinhardtii*

of the culture was 4.23×10^6 cells $mL^{-1}\pm7.88\times10^5$; dry biomass weight was 0.3 g $L^{-1}\pm0.054$; oil extract weight was 18 mg $L^{-1}\pm8.05$. A low yield in lipid extract was observed (6% of dry biomass weight), which is less than the 21 % of lipid content reported before for this microalga (Griffiths and Harrison 2009) or the 15% obtained when *C. reinhardtii* was cultured for hydrogen production (Torri et al. 2011). This low yield was probably due to different culture conditions and/or the strain used, but most likely due to the solvent used for lipid extraction. A mixture of chloroform-methanol (2:1 v/v) has been used in microalgae for total lipid extraction (Griffiths and Harrison 2009; Torri et al. 2011). This solvent mixture usually extracts all lipids present in the microalgal biomass including non polar lipids (TAGs) as well as membrane-related polar lipids (Cooney et al. 2009). Although lower lipid yields have been obtained when using hexane as solvent (Widjaja et al. 2009), we used it for lipid extraction since this solvent was used previously when the natural occurrence of FAMEs was reported in *J. curcas* seed oil (Annarao et al. 2008). Hexane has also been reported for microalgal oil extraction in *Chlorella protothecoides* (Miao and Wu 2006). Additionally, since we were investigating the natural occurrence of FAMEs in *C. reinhardtii*, we wanted to avoid any artifacts that could be produced during the extraction using methanol, since this solvent is usually used for transesterification of TAGs to obtain FAMEs (Casanave et al. 2007).

The crude oil extracts were analyzed by TLC. Commercial canola oil and biodiesel obtained from recycled oil in our laboratory were used as references. The algal crude oil extract contained compounds with the same retention factor (R_f) of biodiesel, as well as compounds with a R_f similar to that of TAGs (Fig. 2). The plate was similar to that presented by Shah et al. (2004), where the spot corresponding to biodiesel had a higher R_f than that of TAGs. The presence of putative FAMEs in *C. reinhardtii* crude oil extract was further corroborated using linoleic acid methyl ester as standard (Fig. 2).

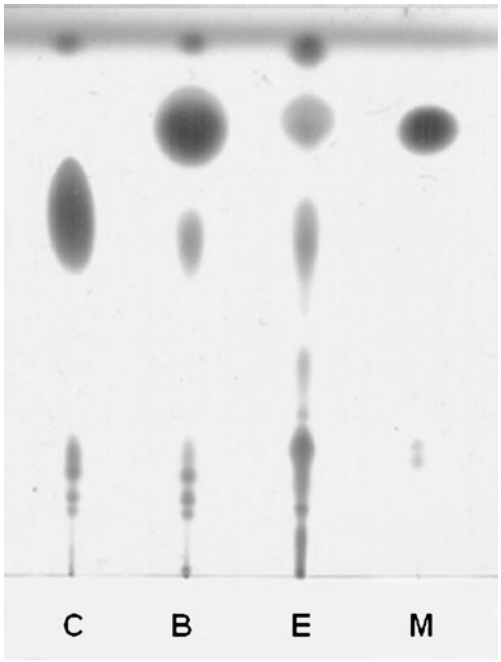

Fig. 2 Thin layer chromatography (TLC) analysis of *C. reinhardtii* crude oil extract showing the presence of both TAGs and putative FAMEs. Lanes: *C* Canola oil, *B* biodiesel from recycled vegetable oil, *E C. reinhardtii* crude oil extract, *M* linoleic acid methyl ester standard

The hexane fraction from the column chromatography contained mainly TAGs and putative FAMEs (Fig. 3). We used this fraction to obtain biodiesel by transesterification. The reaction medium was sampled after 5 min and then every 15 min. We found that the reaction was completed

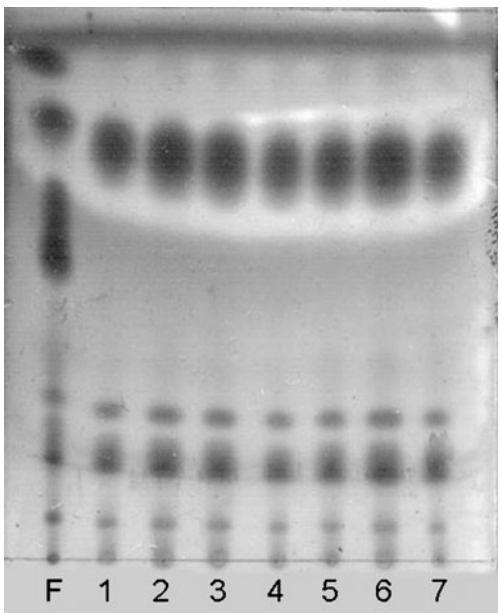

Fig. 3 TLC analysis of the transesterification reaction for the *C. reinhardtii* oil fraction. Lanes: *F* Hexane fraction before transesterification; *1–7* reaction mixtures after 5, 20, 35, 50, 65, 80 and 95 min, respectively

Table 1 Composition of fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEE) from the *Chlamydomonas reinhardtii* hexane fraction before and after the transesterification reaction. *BT* Before transesterification, *AT* after transesterification

Compound	Retention time (min)	C atoms: double bonds	Relative abundance[a] (%)	
			BT	AT
7,10-hexadecadienoic acid methyl ester	10.60	16:2	nd[b]	1.9
hexadecanoic acid methyl ester	10.99	16:0	8.9	22.8
hexadecanoic acid ethyl ester	11.83	16:0	6.5	2.3
9,12-octadecadienoic acid (Z,Z)-, methyl ester	13.07	18:2	7.5	14.8
9-octadecenoic acid (Z)-, methyl ester	13.14	18:1	51.7	39.1
10-octadecenoic acid methyl ester	13.20	18:1	4.7	5.7
octadecanoic acid methyl ester	13.42	18:0	6.4	7.0
9,12-octadecadienoic acid (Z,Z)-, ethyl ester	13.82	18:2	4.8	1.4
9-octadecenoic acid (Z)-, ethyl ester	13.89	18:1	9.5	4.9

[a] From total methyl and ethyl esters

[b] Not detected

after 5 min, as it was observed by TLC (Fig. 3). Furthermore, we were able to produce biodiesel using the crude algal extract under the same conditions (data not shown).

The hexane fraction was analyzed by GC-MS before and after the transesterification reaction. Overall, our results showed the natural occurrence of FAMEs and also FAEEs in *C. reinhardtii*. Five FAMEs and three FAEEs were identified by their MS spectra before and after the transesterification reaction (Table 1). An additional FAME, 7,10-hexadecadienoic acid methyl ester, appeared only after transesterification, which indicates that it came from TAGs and was not present as a free methyl ester molecule. Major FAMEs were C16:0 and C18:1 (Table 1), in agreement with a previous work from this microalga (Tatsuzawa et al. 1996). The natural occurrence of C16 and C18 FAMEs has also been reported in the green microalgae *E. unicocca* and *V. aureus* (Zhang et al. 2009). We also found FAEEs (C16:0, C18:1 and C18:2) which have not been reported before for *C. reinhardtii* (Table 1). Other compounds like methyl branched fatty acids have been found in *Botryococcus braunii* (N-836), which were identified as 16-methyl heptadecanoic (0.1% w/w total fat) and 5,9,13-trimethyl tetradecanoic acids (trace amounts) (Dayananda et al. 2006). Two types of C-methyltransferases that act on lipids have been identified in plants: sterol methyltransferases (Nes 2003) and cyclopropane fatty acid synthases (Bao et al. 2002; Bao et al. 2003). Nevertheless, to date no report on a FAME synthetase has been reported in plants or microalgae.

In the present study, we found the presence of FAMEs along with TAGs in oil extracts of *C. reinhardtii*, while in the case of *J. curcas* seeds, these compounds were found at different stages of maturation (Annarao et al. 2008). Naturally occurring FAMEs were reported in small amounts at early stages of seed maturation along with sterols in *J. curcas*, but they almost disappeared while TAG content increased as fruit ripened (Annarao et al. 2008). Since the natural occurrence of FAMEs has been reported in plants (Annarao et al. 2008) and microalgae (Zhang et al. 2009), several questions arise regarding these observations. For example, what would be the biological function of FAMEs in the plant or microalgal cells? And what enzyme would be responsible for the synthesis of these compounds?

Regarding FAEEs, these compounds are produced as secondary metabolites in plants and microorganisms like bacteria and fungi (Saerens et al. 2006), and it is known that their biosynthesis is carried out by two different enzymatic mechanisms, esterification or alcoholysis. However, knowledge of their biological role is still limited, and to date no enzymes involved in their biosynthesis have been characterized in plants (Neal et al. 2006; Saerens et al. 2006) or microalgae. Since both FAMEs and FAEEs can be used as biodiesel, further research will be necessary to study the biosynthesis of these compounds, and to investigate the potential to over produce, extract and use them as biodiesel.

In conclusion, naturally occurring FAMEs and FAEEs were detected by GC-MS in oil extracts of *C. reinhardtii*. These new insights will pave the way for further research in this model microalga regarding the biosynthesis of both FAMEs and FAEEs, and their potential use as biofuels.

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