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Comparison of fatty acid analysis methods for assessing biorefinery applicability of wastewater cultivated microalgae

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ABSTRACT

In this study, we compared the performances of four different gas chromatography (GC) based microalgal fatty acid analysis methods that are typically applied to biorefinery research using wastewater-adapted microalgae. Compared with the HP-5-type non-polar column, WAX-type polar columns exhibited excellent abilities to quantitatively separate C_{16}-C_{18} polyunsaturated fatty acids (PUFAs) from selected wastewater-adapted microalgae (*Chlorella vulgaris*, *Ankistrodesmus gracilis* and *Scenedesmus quadricauda*) isolates. GC-mass spectroscopy (MS) using the WAX-type polar column provided the strongest detection sensitivity among the tested methods by lowest detection limit, and GC-flame ionized detector (FID) with the same polar column exhibited nearly consistent results to GC-MS analysis. Our statistical comparison of microalgal fatty acid composition profiles generated using various GC methods, microalgal resources and culture media (wastewater, BG11 and nitrogen limitation) suggested that an appropriate GC method and algal resource choice are more important than the optimization of culture conditions to evaluate the applicability of microalgal biorefinery using wastewater resources.

1. Introduction

To address the problems of global warming and fossil fuel exhaustion, bioenergy has been regarded as one of the most attractive alternatives among renewable energy strategies [1]. The first generation of bioenergy strategies achieved biofuel production based on sugar, starch and vegetable or animal oils using conventional technology [2], but these methods have been criticized because they competitively consume food resources [3]. To circumvent this problem, the second generation of bioenergy uses non-edible or waste vegetable oils and agricultural wastes such as lumber, straw and leaves [4]. Recently, algae have been proposed as another charming resource for renewable bioenergy, not only because algae remove carbon dioxide from the atmosphere but also because most microalgae contain a much higher lipid content per biomass than other plants [5–7]. Moreover, since Osward and Golueke [8] proposed the use of microalgae for nutrient removal from wastewater, the nutrient uptake and growth/adaptation of microalgae in wastewater environments have been well established in the literature [9–11]. These features promise a sustainable biofuel production strategy that uses green microalgae in wastewater resources [12,13].

Algal lipid production has been regarded as a key physiological factor in the choice of microalgal resources for biodiesel applications [14]. However, the evaluation of user acceptability of microalgal-based biodiesel has revealed algal fatty acid composition as a critical characteristic because the fatty acid methyl ester (FAME) composition of biodiesel candidates must comply with existing standards such as the American Society for Testing and Materials (ASTM) Biodiesel Standard D6751 and European Union EN standards [5,15]. In addition, the current biodiesel standards have been established mainly for plant-derived fatty acids, but microalgae contain more diverse fatty acids than plants [12]. Some microalgae contain a higher proportion of unsaturated fatty acids with a large number of double bonds than plant oils suitable for biodiesel [5,7,15,16]. Microalgal polyunsaturated fatty acids (PUFAs) such as linolenic acid ($C_{18:3}$), eicosapentaenoic acid (EPA, $C_{20:5}$) and docosahexaenoic acid (DHA, $C_{22:6}$) may be highly valuable materials not only for nutritional or medical purposes [17] but also for various oleochemical applications [18,19] even with tiny amount of compound. Fatty acid profiling is useful for evaluating the applicability of microalgal fatty acids to biorefinery and a useful tool to taxonomically characterize microalgal or

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microbial resources [16,20–22]. Thus, a quantitative and accurate assessment of microalgal fatty acid composition is important to make sound and profitable decisions concerning microalgal fatty acid biorefinery options.

Despite the general acceptance of the importance to accurately assess microalgal fatty acid composition, discrepancies in analysis methodologies exist in the literature. Quantification and composition analyses of algal fatty acids have been performed primarily using GC-flame ionized detection (FID) systems [16,23–25], GC-mass spectroscopy (MS) detection systems [26–28] or both [22,29]. In addition, different GC columns have been used in the literature and are classified as non-polar columns (e.g., phenyl dimethyl-polysiloxane columns such as HP-5, HP ultra-2 and DB-5), polar columns (e.g., polyethylene glycol or cyanoalky polysiloxane columns such as SUPELCOWAX-10, DB-WAX, CP Sil 88, SP2380, SP2560 and BPX-70) or both. Even though there have been some reviews on analytical methods for biodiesel characterization [30,31], to our knowledge, no attempt has been made to explore how different GC methods affect microalgal fatty acid profiling results. Variations in profiling introduce uncertainty for further engineering decisions regarding the feasibility of microalgal biorefinery options, particularly when using wastewater and physiological stress stimuli to produce valuable materials or fuels from microalgae. For instance, some reports claim that microalgal fatty acid composition shifts in response to wastewater [32,33] or to nitrogen limitation as a stress [34], whereas others describe only insignificant effects [15,35]. Because of the current lack of information regarding the potential impacts of fatty acid analysis methods, general conclusions about the feasibility of using wastewater resources with a stress factor cannot be drawn. To address this issue, a methodological exploration is necessary to compare fatty acid profiling performance among the microalgal fatty acid analysis methods typically used in the literature.

In this study, we compared the performance of different GC methods to quantitatively assess the fatty acid composition of wastewater-adapted microalgal isolates. To evaluate the different GC methods typically used for microalgal fatty acid profiling in the literature, multiple FAME peak separation resolutions and quantitative detection sensitivities were examined. Differences in microalgal fatty acid profiling for different GC methods were statistically compared with profiles generated under different culture conditions (wastewater and nitrogen limitation) and using different microalgal organisms (*Scenedesmus quadricauda*, *Chlorella vulgaris* and *Ankistrodesmus gracilis*).

2. Materials and methods

2.1. Algal strains and culture conditions

Three microalgae, *Chlorella vulgaris* AG10032, *Ankistrodesmus gracilis* SAG278-2 and *Scenedesmus quadricauda* AG10308, were selected for study in this work. Algal strains were obtained from the Biological Resource Center of the Korea Research Institute of Bioscience and Biotechnology, South Korea. In aerated batch reactors, strains were cultured for 2 weeks at 25 °C with a continuous illumination and with 120 μmol m^{-2}/s in BG11 medium. The BG11 medium contained 1.5 g of $NaNO_3$, 0.04 g of K_2HPO_4, 0.075 g of $MgSO_4 \cdot 7H_2O$, 0.036 g of $CaCl_2 \cdot 2H_2O$, 0.058 g of $NaSiO_3 \cdot 9H_2O$, 0.006 g of citric acid, 0.006 g of ferric ammonium citrate, 0.001 g of EDTA (disodium salt), 0.02 g of $NaCO_3$ and 1 ml of trace metal mix A_5 in 1 L of distilled water. The trace metal mix A_5 contained 2.86 g of H_3BO_3, 1.81 g of $MnCl_2 \cdot 4H_2O$, 0.222 g of $ZnSO_4 \cdot 7H_2O$, 0.039 g of $NaMoO_4 \cdot 2H_2O$, 0.079 g of $CuSO_4 \cdot 5H_2O$, and 0.049 g of $Co(NO_3)_2 \cdot 6H_2O$ in 1 L of distilled water. After sterilization using a pressurized autoclave, the pH was adjusted to 8.4. After sufficient growth, algal samples were collected by centrifugation and freeze-dried.

To explore the effects of limiting nutrients, *C. vulgaris* AG10032 cultures were grown for 2 weeks in BG11 and then incubated in the nitrogen-limited condition for 8 day. For this nitrogen limitation, $NaNO_3$ was eliminated from the BG11 medium [36], and the other culture conditions were identical to those described above. To characterize the fatty acids in algae grown in real wastewater, municipal wastewater was collected from the influent point of the Seonam Municipal Wastewater Treatment Plant (Seoul, South Korea). The initial values for the total nitrogen, total phosphorus and pH of the wastewater were 37.5 ± 0.5 mg/L, 3.25 ± 0.05 mg/L and 7.35 ± 0.02 mg/L, respectively. The initial total nitrogen and total phosphorus in the wastewater were measured using a Spectroquant® NOVA 60 (Merck, Germany). The initial pH values were measured using an Orion 3-Star pH Meter (Thermo Scientific, Germany). The three microalgal strains were grown in wastewater with the same temperature, duration and illumination conditions as described above.

2.2. FAME extraction by in situ transesterification

By performing in situ transesterification, lipid extraction and transesterification steps were achieved simultaneously using the methods described by Moore et al. [20]. Fifty milli-grams of each freeze-dried algal sample was saponified with 1 ml of saturated KOH-CH_3OH solution at 100 °C for 30 min and then methylated with 2 ml of 5% HCl in CH_3OH at 80 °C for 10 min. After 1.25 ml of *n*-hexane and methyl-tert butyl ether (1:1) solution was added and mixed gently, samples were positioned until the upper and lower layers were separated. After the lower layer was discarded, each upper layer was washed with 3 ml of 1.2% KOH solution to eliminate any base residue. Finally, saturated NaCl solution was added until the KOH solution was completely separated from the *n*-hexane phase.

2.3. GC analysis

Four different GC methods were tested in this study (Table 1). For GC-FID methods, an Agilent 7890 GC was employed with three different columns: HP-5 (30 m, 0.32 mm i.d., 0.25 μm film thickness), SUPELCOWAX-10 (60 m, 0.32 mm i.d., 0.5 μm film thickness) or DB-WAX (30 m, 0.25 mm i.d., 0.5 μm film thickness). When the HP-5 column (Method 1) was used, the temperature began at 100 °C for 2 min, increased at a rate of 10 °C/min and was finally maintained at 280 °C for 20 min. The total analysis time of Method 1 was 40 min, and the flow rate was 2 ml/min

Table 1
GC methods employed in this study.

Methods	Detector	Identification	Column
Method 1	FID	Standard FAMEs	Non-polar, HP-5 (30 m, 0.32 mm, 0.25 μm)
Method 2	FID	Standard FAMEs	Polar, DB-WAX (30 m, 0.25 mm, 0.25 μm)
Method 3	FID	Standard FAMEs/MS libraries	Polar, SUPELCOWAX-10 (60 m, 0.32 mm, 0.5 μm)
Method 4	MSD	MS Libraries	Polar, SUPELCOWAX-10 (60 m, 0.32 mm, 0.5 μm)