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OIL EXTRACTION AND FATTY ACID CHARACTERIZATION OF *NANNOCHLOROPSIS OCULATA* MICROALGAE FOR BIODIESEL APPLICATIONS

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ABSTRACT. Microalgae, the third generation biodiesel feedstock, have emerged as one of the most promising alternative sources of lipids that can be used in the production of biodiesel due to their advantages over conventional crops. The aim of this study was to obtain *Nannochloropsis oculata* microalgae oil using hexane extraction methods together with fatty acid characterization for biodiesel application. The chemical composition of microalgae showed a high total lipid content, *N. oculata* microalgae being a potential feedstock for biodiesel production. For algal oil extraction, dynamic extraction with Soxhlet apparatus proved more efficient, with a lipid yield of 0.190 g/g dried microalgae comparatively to only 0.136 g lipid/g dried microalgae for static hexane extraction. The main fatty acids for *N. oculata* microalgae are palmitic acid, palmitoleic acid, eicosatrienoic acid and eicosapentaenoic acid, the latest being also the major constituent, with a value of 48.86% (w/w).

Keywords: *microalgae, biodiesel, oil extraction, lipids, fatty acids*

INTRODUCTION

Microalgae, the third generation biodiesel feedstock, have emerged as one of the most promising alternative sources of lipids that can be used in the production of biodiesel due to their advantages: photosynthetic efficiency, high biomass production, higher growth rates and productivity when compared to conventional crops [1]. Microalgae use photosynthesis to convert solar energy into chemical energy in the form of oil, carbohydrates and proteins.

Biodiesel is a mixture of monoalkyl esters of long-chain fatty acids obtained in the transesterification reaction of vegetable oils or animal fats with short chain alcohols [2]. The cost of biodiesel production remains the

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major obstacle to its use at industrial scale, primarily because of the high cost of vegetable oils used as feedstock [3]. Another important reason is the inefficiency and unsustainability of first and second generation biodiesel [4].

The production of biodiesel from microalgal biomass consists in three steps: oil extraction, transesterification and separation/purification of biodiesel.

Lipid extraction from microalgal is the most important step because this process is one of the more costly processes which can determine the sustainability of algae-based biodiesel. Fatty acid methyl esters obtained from oil are known as biodiesel. Various methods for extracting oil from microalgae were developed, including mechanical pressing, homogenization, milling, solvent extraction, supercritical fluid extraction, enzymatic extractions, ultrasonic-assisted extraction and osmotic shock. Organic solvent extraction with hexane is the most common method for extracting oil from microalgae by repeated washing or percolation.

Total lipid extraction can be achieved by using appropriate methods of cell disruption to release cellular contents into the extraction medium. Numerous methods have been used for cell disruption, such as microwaves, ultrasonic, mechanical crushing [5]. For example, the use of microwaves proved to be an effective method for the extraction of vegetable oils [6], while ultrasonic disruption is widely used for microbial cells [7].

The purpose of this paper was to obtain *Nannochloropsis oculata* microalgal oil and to determine the fatty acid profile for further biodiesel production.

RESULTS AND DISCUSSIONS

N. oculata microalgae have been used for this study due to their commercial availability and for high lipid content. Rodolfi et al. reported that *Nannochloropsis* marine microalgae specie is one of the best candidates for algal oil production due to high biomass productivity and lipid content [8].

The microalgae were first analysed to determine its constituents. Microalgae contain lipids, proteins, carbohydrates and nucleic acids. Table 1 shows the average chemical composition of *N. oculata* microalgae.

Table 1. Average chemical composition of *Nannochloropsis oculata* microalgae on a dry matter basis. Values given are the mean of three replicates ± standard deviation.

Parameter	Content %(w/w)
Proteins	42.5 ± 1.8
Carbohydrates	18.0 ± 0.9
Lipids	32.8 ± 1.2

According to Brown et al. [9], microalgal composition varies by species in the proportion of protein (6 to 52%), lipid (7 to 23%) and carbohydrate (2 to 23%). In this study, the protein and carbohydrate content in *N. oculata* microalgae were within the ranges reported by Brown et al. (1997), while the lipid content was higher, but in accordance with other studies [8,10]. The high content of total lipids suggests that *N. oculata* microalgae used in this study is a potential feedstock for biodiesel production.

For microalgal biodiesel production the ideal lipid extraction method should not only be lipid-specific – in order to minimize co-extraction of non-lipid contaminants, but also selective – towards only a few lipid fractions, e.g. neutral lipids like triacylglycerols [11]. Even though the classic chloroform-based lipid extraction protocol is particularly suitable for most microalgal lipid analyses, alternative organic solvents that are less toxic are preferred prior to scale-up. Hexane is less efficient than chloroform for extraction of oils from microalgae, but is also less toxic – and has a marginal affinity for non-lipid contaminants, and an apparently higher selectivity for neutral lipid fractions [11]. This is why hexane was chosen for oil extraction from *N. oculata* microalgae.

In this paper two methods have been tested for oil extraction from *N. oculata* microalgae: static hexane extraction and dynamic hexane extraction (Soxhlet). The lipid yield obtained for each method was compared to total lipid content determined using the combination of microwaves with chloroform-methanol extraction. Soxhlet extraction with hexane was found to be significantly more efficient than static hexane extraction, with a lipid yield of 0.190 g/g dried microalgae comparatively to only 0.136 g lipid/g dried microalgae. This improvement was expected since Soxhlet operation, through solvent refluxing, constantly exposed a fresh batch of hexane to the microalgae biomass and enabled continuous re-establishment of mass transfer equilibrium [12].

Microalgae synthesize fatty acids as building blocks for the formation of various types of lipids. The most commonly synthesized fatty acids have chain lengths that range from C16 to C20, similar to those of higher plants [13]. Saturated fats come from animal products such as meat and dairy while most vegetable oils are unsaturated. Fatty acids are either saturated or unsaturated, and unsaturated fatty acids may vary in the number and position of double bonds on the carbon chain backbone. Polyunsaturated fatty acids (PUFAs) contain two or more double bonds.

Fatty acid composition is the most important parameter for a biodiesel feedstock because the properties of the corresponding fatty esters that comprise biodiesel determine the overall fuel properties of biodiesel. Fatty acid composition of *N. oculata* algal oil was determined using a gas chromatograph with flame ionization detector, the chromatogram being presented in Figure 1. The results are given in Table 2, data for each fatty acid being expressed as percentage of the total fatty acids in the microalgal oil.

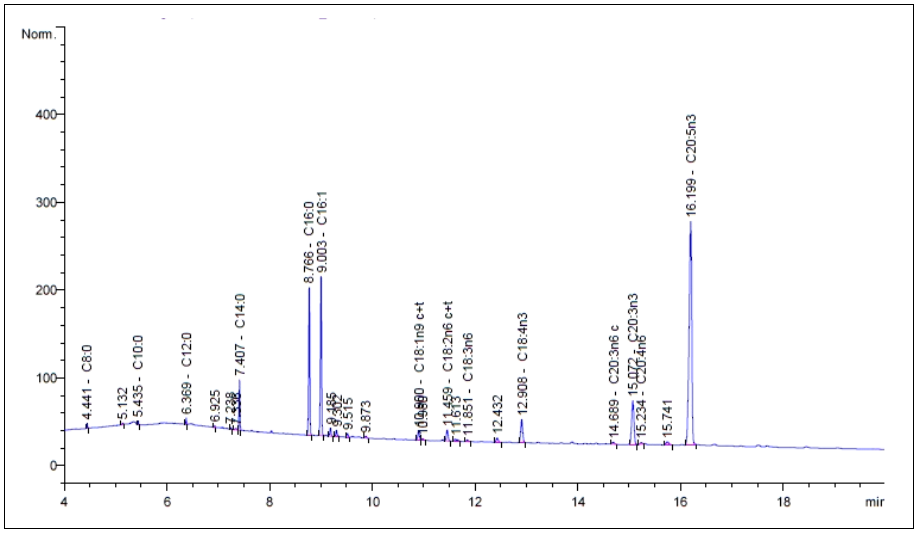

Figure 1. The GC chromatogram obtained for the fatty acid profile of *Nannochloropsis oculata* microalgae.

Table 2 Fatty acid composition of *Nannochloropsis oculata* microalgae on a dry matter basis (%).Values given are the mean of three replicates ± standard deviation.

Systematic name	Common name	Abbr.	Content % (w/w)
Octanoic acid	Caprylic acid	C8:0	0.30 ±0.02
Decanoic acid	Capric acid	C10:0	0.19 ± 0.02
Dodecanoic acid	Lauric acid	C12:0	0.35 ±0.01
Tetradecanoic acid	Myristic acid	C14:0	3.87 ±0.04
Hexadecanoic acid	Palmitic acid	C16:0	14.14 ±1.09
9-cis-Hexadecenoic acid	Palmitoleic acid	C16:1	17.02 ± 2.50
cis-9-Octadecenoic acid	Oleic acid	C18:1n9	1.20 ± 0.04
cis,cis-9,12-octadecadienoic acid	Linoleic acid	C18:2n6	1.47 ± 0.03
6,9,12-octadecatrienoic acid	γ-Linolenic acid	C18:3n6	0.25 ± 0.02
(6Z,9Z,12Z,15Z)-6,9,12,15-octadecatetraenoic acid	Stearidonic acid	C18:4n3	3.88 ± 0.04
all-cis-8,11,14 -eicosatrienoic acid	Dihomo-gamma-linolenic acid (DGLA)	C20:3n6	0.35 ± 0.02
all-cis-11,14,17-eicosatrienoic acid	Eicosatrienoic acid (ETE)	C20:3n3	7.74 ± 0.03
all-cis-8,11,14,17-eicosatetraenoic acid	Eicosatetraenoic acid (ETA)	C20:4n6	0.27 ± 0.02
all-cis-5,8,11,14,17-eicosapentaenoic acid	Eicosapentaenoic acid (EPA)	C20:5n3	48.86 ± 1.77

As seen in Table 2, the main fatty acids for *Nannochloropsis oculata* are palmitic acid, palmitoleic acid, eicosatrienoic acid and eicosapentaenoic acid EPA. From these acids the major PUFAs for *N. oculata* microalgae are: eicosatrienoic acid (20:3*n*3), stearidonic acid (18:4*n*3) and eicosapentaenoic acid EPA (C20:5*n*3), the latest being also the major constituent of *N. oculata* microalgae, with a value of 48.86% (w/w). The high level of polyunsaturated acids in *N. oculata* microalgae may cause stability problems, the biodiesel resulted being more susceptible to oxidation process. However, polyunsaturated acids also have much lower melting points than monounsaturated or saturated acid, thus, algal biodiesel should have much better cold weather properties than many other biodiesels.

CONCLUSIONS

Nannochloropsis oculata microalgae have been used for this study to obtain microalgal oil and to determine the fatty acid profile. The chemical composition showed a high total lipid content that proved *N. oculata* microalgae is a potential feedstock for biodiesel production. The fatty acid composition of *N. oculata* microalgae showed a high level of polyunsaturated acids which may cause stability problems, but, in the same time, the cold weather properties should be improved comparatively to other biodiesels.

EXPERIMENTAL SECTION

Materials

All chemicals were analytical reagent grade. *Nannochloropsis oculata* microalgae were purchased from Astaxa GmbH (Germany Milz Gerbergrasse). Hexane, chloroform, methanol, acetic acid, sodium hydroxide, sulphuric acid, boric acid and copper sulphate were purchased from Merck (Darmstadt, Germany). Sodium chlorite (80%) was purchased from Alfa Aesar (Germany). Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Methyl ester standards were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were chromatographically pure.

Microalgal lipid extraction

Static hexane lipid extraction

For static hexane lipid extraction, a quantity of 4 g microalgal powder was used. A volume of 300 mL n-hexane was added to the microalgal powder in a 500 mL Erlenmeyer flask. In order to reduce solvent evaporation, the flask was sealed with a ground joint. The extraction mixture was agitated at 800 rpm

at ambient conditions for 8 h. After extraction cell residues were removed by filtering through Whatman GF/C paper. The hexane phase was collected in a pre-weighed flask and then submitted to vacuum evaporation using a rotational evaporator to enable gravimetric quantification of the lipid extract.

Dynamic hexane lipid extraction

The performance of static hexane extraction with dynamic hexane extraction was compared using a Soxhlet apparatus. A quantity of 4 g microalgal powder was packed in a cellulose thimble inside the extraction chamber of the Soxhlet unit. A volume of 300 mL *n*-hexane was used to extract the lipid and the extraction was performed for 8 h at the rate of approximately 10 refluxes per hour. The extracted lipid collected in a pre-weighed flask and then submitted to vacuum evaporation using a rotational evaporator to enable gravimetric quantification of the lipid extract**.**

Analytical methods

Determination of carbohydrates content

Composition of carbohydrates from microalgae was determined according to Teramoto method, method used for determination of carbohydrates from wood [14]. Carbohydrates content was determined by treated microalgae with sodium chlorite in acetic acid solution (10%): 5 g sample was treated with 5 g $NaClO_2$ in 375 ml glacial acetic acid. The sample was mixed at 75 °C for 1h (repeated for three times). The product was filtrated, washed with water and acetone, dried at 105 °C for 24 h in vacuo, and weighed. The dried solid was treated with 17.5% NaOH at 20 °C for 40 min, and 25 ml water was added it. The residue was filtrated, washed with 40 ml 10% glacial acetic acid and 1L boiling water. The carbohydrates residue was filtrated, dried at 105 °C for 48 h in vacuo, and weighed.

Determination of protein content

The protein content of *Nannochloropsis oculata* microalgae was determined using Kjeldahl destruction method, all proteins being degraded to NH_3 that was quantified titrimetrically with sulphuric acid. An amount of 1 g microalgae biomass was destructed at 390°C with 10 mL 96% H_2SO_4 in the presence of $CuSO_4$ as catalyst for approximately 2 hours, until the residue became white and the supernatant clear. After cooling down, the sample was filled up to 100 mL with distilled water, distilled and the resulted NH_3 was captured with boric acid (H_3BO_3) and quantified titrimetrically with H_2SO_4. The amount of protein was calculated by multiplying the measured nitrogen concentration with 6.25.

Determination of total lipid content

Total lipid content was determined using the combination of microwave and liquid-liquid extraction. 0.5 g dry microalgae biomass was mixed with 20 ml distilled water. The mixture was further subjected to cell disintegration using a Speedwave MWS Berghof microwave digester (2450MHz) at 100 °C for 5 min. Total lipids were extracted from microalgae biomass using a modified version of Bligh and Dyer method [15]. Lipids were extracted with a mixture of chloroform-methanol 2:1 (v/v). The volume ratio of biomass subjected to the extraction and the organic solvent mixture is 1:1. The mixture was subjected to stirring for 5 minutes in a separating funnel. After extraction the mixture was introduced over a further 10 ml of methanol to separate the two phases: aqueous and organic. The chloroform organic phase was washed with 10 ml 5% sodium chloride and then submitted to vacuum evaporation using a rotational evaporator to enable gravimetric quantification of the lipid extract**.**

Determination of Fatty Acid Composition using GC-FID

The fatty acid profile was determined using an Agilent 7890A GC gas chromatograph equipped with a DB-WAX capillary column (30m × 0.25mm × 0.25µm) and a flame ionization detector. The column oven temperature was kept at 50°C for 1 min, heated to 200°C at 25°C/min, then to 230°C at 3°C/min and finally maintained for 18 min. The injector and detector temperatures were set to 250 and 280°C, respectively. Helium was utilized as a carrier gas. The gas chromatography calibration was conducted via the analysis of standard solutions of methyl esters and heptadecanoate methyl ester was used as internal standard.

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