Effect of Microalgae Cell-Disruption Method on Extraction Yield of Fatty Acids

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EFFECT OF MICROALGAE CELL-DISRUPTION METHOD ON EXTRACTION YIELD OF FATTY ACIDS

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Introduction

Microalgae possess advantages compared to the other crops, mainly a high growth rate, a short growth time, a high biomass production and a low land demand[1]. Their suitable composition of the lipid fraction can serve not only for the conversion to biofuels but also as the protein rich nutritional supplements and pharmaceuticals to a healthy diet or feed ingredients. Algae produce a great variety of the other valuable substances, such as fatty acids (FAs), proteins, vitamins, carotenoids, enzymes etc., depending on the individual algal species, conditions of the cultivation (e.g. composition of the medium, aeration rate, light intensity, duration of the photoperiod, temperature) and the processing of biomass[2]. The single-cell freshwater green alga *Chlorella sp.* belongs to the most intensively studied microalgae because of its rapid growth under suitable undemanding conditions and its relatively high lipid content.

The efficient lipid extraction from microalgae depends upon algal species and extraction method. Owing to that microalgae consist of individual cells surrounded by the thick cell walls of various compositions, appropriate cell-disruption may be important to increase the lipid extraction efficiency. Process of the cell wall disruption must meet several requirements: to be well defined, to disintegrate the maximum number of cells and to be gentle to the cell content (maintain its biological activity). Otherwise, undesirable oxidation of unsaturated fatty acids or their degradation can take place.

Various methods, such as lyophilisation[3,4], application of liquid nitrogen[5,6], autoclaving[7,8], microwave treatment[5,7-9], sonication[4-8], grinding[5], bead beating[4-8], osmotic shock[7,8], enzymatic lysis[5] etc. have already been tested. Usually, the solvent system of Bligh and Dyer[10] using chloroform/methanol mixture was applied. Although the total lipid content is determined gravimetrically, this method cannot be used without limitation. Samples of plant/algal origin besides lipids contain pigments and other soluble substances, significantly increasing the proportion of extractables.

There are diametrically different opinions about the most effective disruption method. Nielsen[3] stated that lyophilisation increased the surface area of the sample, leading to a better lipid extraction. Nevertheless, as results of Ryckebosh[6] showed, total lipids extracted from fresh and lyophilized algae were not significantly different. The fatty acid composition, also, was not significantly different. Therefore, lyophilisation can be considered as a pre-treatment that can be used without altering the lipid composition. Grinding in liquid nitrogen was identified as the most effective method in terms of disruption efficiency and time by Zheng[5]. The authors obtained 29 % lipid concentration in *Chlorella vulgaris*. Among the tested methods, the microwave disruptive method was found as the most simple, easy, and effective for lipid extraction from microalgae by Lee[7] with 10 % determined lipid content in *C. vulgaris*. The highest lipid content (19 %) from tested microalgae was obtained by Prabakaran[8] using the sonication method, which showed the highest efficiency for its recovery. The amount of total lipids extracted from *Phaeodactylum tricornutum* was used as an indication of the efficiency of the cell-disruption method used by Ryckebosh[6]. No significant differences were detected between the different cell-disruption methods. This indicated that the cell wall was penetrated or dissolved by the solvents used, so it did not need to be destroyed for optimum extraction.

To examine whether significant oxidation occurs during extraction, the effect of addition of the synthetic antioxidant TBHQ was tested by Ryckebosh[6]. Owing to that no oxidation of exogenous EPA was observed, it was supposed that no oxidation of endogenous fatty acids took place either. Addition of an antioxidant during lipid analysis was thus not necessary. This can be explained by the large antioxidant capacity of microalgal extracts, confirming that the large amount of natural antioxidants[11] (e.g. tocopherols, carotenoids, polyphenols), present in microalgae, protect the lipid extracts.

The main objective of the presented study was to examine the effects of the various cell-disruption methods (drying, lyophilisation, microwave treatment, sonication and high-pressure shock) using fresh *Chlorella vulgaris* suspension on the total lipid content (extractable part) and the fatty acid composition.

Materials and methods

Microalgal biomass

Inoculum of single-cell algae *Chlorella vulgaris* was obtained from the Culture Collection of Autotrophic Organisms (CCALA) of the Institute of Botany of the ASCR, Třeboň department. The microalgae were grown in 250 L continuous photo-bioreactor, equipped with automatic regulated data collection unit (designed at ICPF ASCR, v. v. i) with CO_2 (2%) aeration (200 $L.h^{-1}$) at 29 °C. The microalgal biomass was harvested by filtration in the early stationary phase (after 14 days). The fresh obtained algae were used directly for further processing.

Chemicals

Hexane, ethanol, chloroform and methanol (p. a., Lach-Ner, CR) were used as solvents. The ratio of individual components of the extracted system was selected with regard to the type of used liquids.

Methods of cell-disruption

The concentrated algal biomass suspension was disrupted using various methods as follows: (i) drying (60 °C to the constant weight), (ii) lyophilisation (-70 °C under a vacuum), (iii) microwave treatment (2.45 GHz, 550 W, 30 min), (iv) sonication (35 kHz, 800 W, 30 min), (v) high-pressure shock (550 MPa, 10 min).

Lipid extraction

Dry matter in algal suspension was determined before each extraction to assure the exact content of the dry algal biomass in solution (105 °C, 7 h to the constant weight).

In each experiment, microalgal suspension (containing 10 g dry biomass) was treated by the single-stage extraction with the hexane/ethanol mixture (2/3 v/v) at 1/15 (dw/v) ratio in continuously stirred Erlenmeyer flask for 4 h under inert atmosphere in the absence of light at the ambient temperature. Individual phases (liquid organic extract, water phase and solid biomass) were separated from the obtained solutions by intensive centrifugation (4000 rpm for 5 min). The upper organic phase (extract) was sucked off. The solvent was then removed from the organic phase by rotary evaporation at 40 °C after which the total lipid content (extractable part) was determined gravimetrically. An aliquot of the dry extract was taken for the following analysis.

Analysis of fatty acid profile

Analysis of FA profile in the extracts of the tested microalgae was performed at the Department of Food Analysis and Nutrition of the Institute of Chemical Technology Prague. Accredited (ISO 17025) gas chromatographic (GC) method was used. Briefly, following the release of FAs from ester bonds by saponification, their methylation was performed. Target analytes were separated on capillary column and detected by the flame ionization detector (FID). Quantitative determination was carried out by the inner standard technique performed by direct comparison of the addition of the inner standard nonadecane acid (C19:0).

Results and discussion

Solvent selection

Comparison of the individual solvent systems was executed by the single-stage extraction according literature, where the single-stage extraction was found as sufficient for determination the complete lipid profile of the microalgae with no effect on the proportion of the individual fatty acids[4]. Moreover, in our previous study[12] variation of the extraction system polarity was studied using lyophilised microalga *Chlorella vulgaris*. It was proved[6,12] that the extracted total lipid percentage and total fatty acid composition were highly dependent on the used solvent system and the highest yields of lipids/FAs were obtained by the extraction with the most polar solvent system.

Experiments in our present study were performed using concentrated water suspension of fresh microalgae. Each set of experiments was executed with homogenised solution, where dry matter was precisely determined. Content of dry matter varied in the range of 13.4-15.2 %.

As a comparative experiment, extractable part of algal biomass was determined using chloroform/methanol solvent mixture (modified Bligh-Dyer method). Mentioned method has been used exclusively for analytical purposes. The referential extraction was carried out at ambient temperature in a mixer–settler in a single-stage under moderate stirring with the extraction solvent for 1 h. 24.3 % extractable part (Figure 1) calculated to dry biomass was obtained. It can be considered to be maximal proportion of extractable components. Samples of plant/algal origin, which contain proteins, pigments and other soluble substances, significantly increase the proportion of extractables when Bligh-Dyer method is used. Among disadvantages of the procedure the toxicity, aggressivity and flammability of the solvents belong, too.

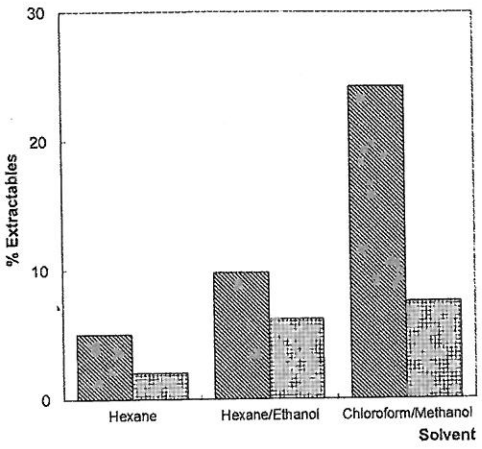

Figure 1

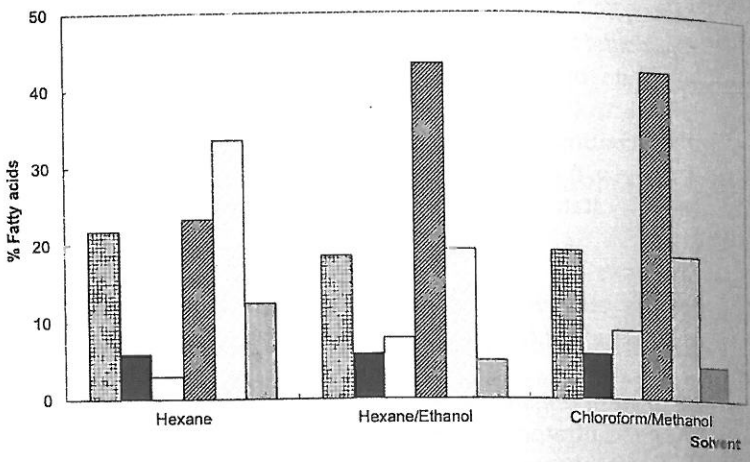

Figure 2

Figure 1. The effect of solvent mixture (hexane, hexane/ethanol, chloroform/methanol) composition (different polarity of solvents) on lipid (extractable part) and sum of FAs content related to dry matter. Extractable part, sum of FAs.

Figure 2. The effect of solvent mixture composition (hexane, hexane/ethanol, chloroform/methanol) on the profile of fatty acids. FAs: C16:0, C18:0, C18:1n9c, C18:2n6c, C18:3n3c, remaining FAs.

First, the effect of solvent polarity on the extractable part and the total fatty acids in fresh microalgae was tested (Figure 1). Maximal extractable part was obtained using chloroform/methanol system (24.3 %), while the highest proportional content of fatty acids in the lipid fraction was included using hexane/ethanol mixture (63.2 %). Pure hexane extracted 40.6 % FAs in the lipid fraction. Similar trends were measured previously[12].
The aim of experiments was not to extract total lipids/FAs from microalgae studied, but mainly to compare yields of the various cell-disruption techniques. Extracted biomass was intended for subsequent use as the poultry feed supplement. For this reason, as the solvent system chloroform/methanol should be avoided.
Green alga *Chlorella sp.* is generally declared as an important source of α-linolenic acid (C18:3n3). Our experiments have established the most represented acids, such as palmitic (C16:0), linoleic (C18:2n6c) and α-linolenic (C18:3n3). Stearic (C18:0) and oleic (C18:1n6c) acids were analysed in much lower percentage.
As can be seen in Figure 2, systems chloroform/methanol and non-toxic hexane/ethanol provided comparable FAs profile with high content of essential unsaturated linoleic acid (C18:2), >40% of total FAs. On the other hand, hexane provided the highest yield of essential α-linolenic acid (C18:3), but its total extraction capacity was too low.

Further testing was performed with a solvent system that provided FAs profile comparable with chloroform/methanol procedure. The solvent system had to be environmental friendly with the relatively high extraction capability, moreover low cost with lower volatility and toxicity for humans. The hexane/ethanol system was proved to be a suitable alternative for extraction of lipids/FAs from dry microalgal biomass[12] as well as water algal suspension.

Cell-disruption methods
The disruption methods efficiency was investigated by means of the total extractables and the profile of selected fatty acids. Solvent system hexane/ethanol 2/3 (v/v) was applied. Drying, lyophilisation, microwave treatment (MW), sonication (US) and high-pressure shock (550 MPa) as disruption methods were applied. Results were compared with extraction yields of untreated fresh biomass.
To exclude reaction with lipid fraction and/or decomposition of fatty acids, no chemical procedure of disintegration of algal cells (acids, alkali, salts, enzymes etc.) was executed. There is a risk of imperfect separation of used chemicals from biomass. Therefore, many additives are prohibited in food industry or as feed additives. Released cell content can be sensitive to enzymatic lysis or oxidation, especially under the influence of other chemicals. Moreover, for our purposes, extraction of total lipids from microalgae is not essential and the presence of chemicals is unacceptable for further application of remaining biomass as feeding mixtures.

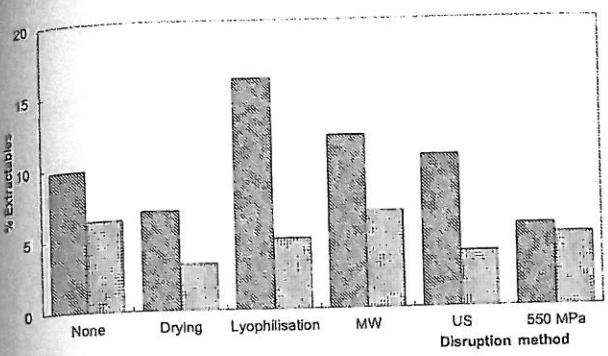

Figure 3

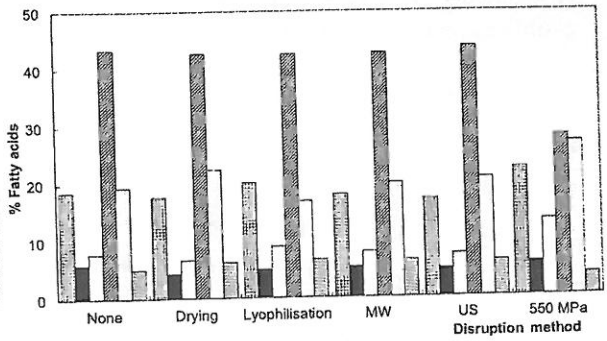

Figure 4

Figure 3. The effect of disruption method on the content of lipids (extractable part) and sum of fatty acids related to dry matter. Extraction system hexane/ethanol, ▨ extractable part, ▦ sum of FAs.

Figure 4. The effect of disruption method on the FA's profile. Extraction system hexane/ethanol. FAs: ▦ C16:0, ■ C18:0, □ C18:1n9c, ▨ C18:2n6c, ⬚ C18:3n3c, ▥ remaining FAs.

Application of the individual disruptive methods shows the different ratio of extractables and FAs (Figure 3). The highest proportion of lipids was extracted, when lyophilisation as a disruption method was used. It is in contradiction with results of Ryckebosh[6], who stated that lyophilisation did not lead to better lipid extraction. By this way, 63 % more lipid fraction was isolated in comparison with untreated algae. The content of FAs in extractables was relatively low, 30.5 %. The proportion of the extracted fatty acids ranged from 3.2 to 6.7 % related to dry biomass for all disruptive methods. Quantitatively the highest amount of fatty acids was obtained with the application of microwave processing, 6.7 %, representing 55.9 % of the total extractable part. The lowest yield of lipids as well as FAs was observed for drying at 60 °C. This heat treatment had probably no effect on the cell wall disintegration; furthermore, undesirable degradation of the cell content could take place. Thus, the partial oxidation destruction should be assumed.

Profiles of selected fatty acids (Figure 4) contained in the extractable part were comparable for studied disruption methods covering the various processing: none, drying, lyophilisation, microwave treatment and sonication. Content of individual FAs varied in the range of 17.1-20.1 %, 4.3-5.9 %, 7.4-9.0 %, 42.6-43.7 % and 16.8-22.4 % for C16:0, C18:0, C18:1n9c, C18:2n6c, C18:n3, respectively. In smaller quantity palmitoleic acid (C16:1; 0.3-0.5 %), heptadecanoic (C17:0; 0.2-0.3 %), γ-linolenic (C18:3n6; 0.1-0.4 %), EPA (C20:5n3; 0.2-1.5 %), lignoceric (C24:0; 0.1-0.8 %) and nervonic (C24:1n9; 0.1-0.5 %) acids were detected. Therefore, pre-treatment procedures are considered to have no significant effect on the individual fatty acid composition. However, the applied disruption method affected total FAs content obtained from the fresh microalgal suspension. It corresponds with results measured by Ryckebosh[6], who detected no significant differences between the different cell-disruption methods.

In comparison with already mentioned results, only high-pressure shock (550 MPa) showed dramatically different data. Total FAs proportion represented the highest yield of extractable part (88.4 %). Also all monitored FAs, except C18:0 and C18:2, occurred in the highest concentrations. Higher amount of palmitic and oleic acids were determined, but mainly the content of unsaturated α-linolenic acid.

Nevertheless, our results demonstrated that any of the tested disrupting methods contributed to the expected significant increase in extractables in comparison with the simple solvent extraction of untreated microalgae.

Conclusions

Extraction using three solvent systems showed that in comparison with toxic chloroform/methanol system environmental friendly hexane/ethanol mixture provided the similar FAs profile with the high content of essential unsaturated linoleic acid (C18:2). The hexane/ethanol system was proved to be a suitable alternative for extraction of lipids/FAs from dry microalgal biomass as well as water microalgal suspension.

To determine the effects of the cell-disruption pre-treatments on the total extractable part and the fatty acid composition, fresh microalgal suspension of *Chlorella vulgaris* was processed by drying, lyophilisation, microwave treatment, sonication and high-pressure shock. No significant difference was observed between the total lipid content with or without cell-disruption. Therefore, cell-disruption is not essential for obtaining

adequate fatty acid profile. Unsaturated linoleic (C18:2) and α-linolenic (C18:3) acids approx. 43 % and 20 %, respectively, were dominant in tested systems. Only the high-pressure shock (550 MPa) provided different FA profile as opposed to the other disruption methods.

Acknowledgments

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Keywords

solvent extraction, *Chlorella*, microalgae, fatty acids, cell-disruption

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