Extraction of Fatty Acids from Microalgae

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2013

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Introduction

Algae

Algae are the most often processed as a fast growing biomass with a high proportion of lipids. They have a big potential for commercial applications as nutraceuticals and pharmaceuticals or feed ingredients owing to the high content of proteins, vitamins, carotenoids, enzymes, polysaccharides or unsaturated fatty acids. Algae produce a great variety of fatty acids (FA) and lipids in dependence on the individual conditions of cultivation and biomass processing. The main fatty acids are saturated and unsaturated cis-isomers with 12 to 22 carbons with no more than six double bonds. Chlorella, the most intensively studied alga, is a single-cell freshwater green alga (Chlorophyta) with a spherical shape about 2 to 8 μm in diameter and the high content of lipids (14-22%) (Montes D'Oca, 2011). In comparison with the other plants, it contains the largest amount of chlorophylls (a and b), high percentage of proteins, vitamin A, H, D, C and B as well minerals. To allow the better penetration of solvents into the cells microalgae are often treated by grinding (bead mill), freezing, high-pressure homogenization, ultrasonication, microwave treatment etc. or their combination to break the cell structure (Šoštarič et al., 2012; Lee et al., 2010).

Extraction techniques

The solid–liquid extraction techniques are based on diffusion and osmosis. Maceration by organic solvents, such as cyclohexane, hexane, petroleum ether, ethyl acetate, diethyl ether, acetone, chloroform etc belong to the simplest and the cheapest technique. Bligh and Dyer (1959) procedure, based on extraction by chloroform/methanol/water mixture, is routinely used for lipid extraction from microalgae as the comparative method. This method primarily developed for determination of lipids in the cod muscle possesses some limitation for samples of plant/algae origin which contain pigments.

Hexane has been reported (Peralta-Ruiz et al., 2013; Ramírez-Fajardo et al., 2007) as the most efficient solvent owing to its high extraction capability, low cost and low volatility and toxicity. The comparable lipid yields obtained Halim et al. (2011) by hexane extraction from the dried microalgal powder as well the wet microalgal paste.

Combinations of co-solvents, especially alcohols, have been proposed for extraction of the microalgae lipids with the alcohol content as the main factor; the higher polarity the higher yield (Molina Grima et al., 1994).

Ethanol (96% yields) is considered as the more efficient solvent for fatty acid extraction in comparison with hexane/ethanol (1/2.5 v/v; 86% yield) due to its higher polarity (Molina Grima et al., 1994; Cartens et al., 1996).

In general, applications using the pure lower alcohols have performed similarly; if not slightly better, than alcohol/hexane mixtures, but nevermore than 90% of the Bligh and Dyer co-solvent method. Alcohol also aids dissolution of lipids in the organic phase. However, the alcoholic solvents also extract some cellular contaminants such as sugars, amino acids, salts, hydrophobic proteins and pigments (Kates, 1986a).

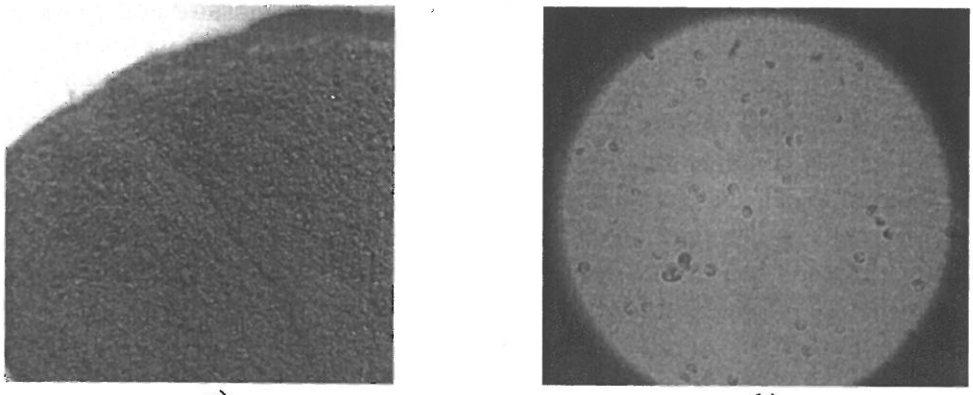
Besides conventional organic solvent extraction ionic liquids are recommended (Kates, 1986b). Ultrasound-assisted extraction (Cravotto et al., 2008) and microwave-assisted extraction (Virot et al., 2007) have been recognized as the efficient extraction techniques that shorter the working times and the increase yield of the extract. In recent years, efficient selective "green" methods that operate at elevated pressures and lower temperatures and thus do not cause degradation of the labile substances have been preferred.

The most developed separation techniques for the crude extract purification include formation of urea inclusion compounds, low temperature fractional crystallization, solid-phase extraction, salt solubility methods, gas chromatography, thin-layer chromatography, liquid-liquid fractionation and liquid chromatography. These techniques usually fractionate the fatty acids based on the number of double bonds or chain length; few of those methods are capable of providing the highly pure individual fatty acids from a natural source (Robles Medina, 1998).

Materials and methods

Microalgal biomass

Lyophilized biomass of the single-cell algae *Chlorella vulgaris* (Figure 1) was used as a source of the lipid fraction. Any further disruptive technique was applied.



a) b)

Figure 1: *Chlorella vulgaris*. a) lyophilized dry microalgae, b) at × 2000 magnification (Microscope Arsenal).

Chemicals

Nine solvent systems were selected to extract the lipid fraction from studied microalgae in order to compare their extraction efficiency. Pure hexane, ethanol and acetone were tested. As hexane co-solvents methanol, ethanol, propan-2-ol, butan-1-ol, 2-methylpropan-1-ol and acetone at 2/3 or 3/4 (v/v) ratio were used. Biomass-to-solvent ration was 1:25 (w/v). The ratio of individual components of the extracted system was selected with regard to the type of used liquids and the maximisation of the lipid yield.

Lipid extraction

In each experiment, lyophilized microalgae were treated with the corresponding extraction system at 1:25 (w/v) ration. The mixture was heated under reflux condenser to boiling and maintained at that temperature for 60 min. The mixture was well-agitated during the extraction. Comparative experiments were performed. The sample of biomass was weighed into a closed Erlenmeyer flask with grinding, defined amount of hexane/ethanol mixture (2/3 v/v) was added and the mixture was stirred for 1 or 4 hours at the room temperature.

Individual phases (liquid organic extract and solid biomass) were separated from the obtained solutions by intensive centrifugation (4000 min^{-1} for 5 min). The upper organic phase (extract) was sucked off. Solvent was then evaporated from the organic phase by the

rotary evaporator under vacuum and thus extractable part (dry extract) was prepared. The weight of the crude lipid obtained from each sample was measured using an electronic scale. An aliquot of the dry extract was taken for the following analysis.

Analysis of fatty acid profile

Analysis of lipid content and 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. To determine FAs composition in lipids fraction extracted from algae, 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 flame ionization detector (FID).

Results and discussion

For an effective separation of the lipid part from microalgae the several studies with several types of extraction solvents were made using the single-stage extraction. This process ensured achievement of the equilibrium state in the system. Method of the biomass treatment at elevated temperature was used (Nagle and Lemke, 1990). Intensive mass transport was expected. Experiments compared the influence of the various extraction systems to yield of the extractable part and its composition. There is a lack of the comparable information in literature sources. Since many additives are prohibited in the food industry, no other reagents (acids, alkali) were added.

As a comparative experiment, extractable part of the microbial biomass was determined by chloroform/methanol/water mixture 1/2/0.8 v/v/v (modified Bligh-Dyer method). The 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. 17.8 % dry extract calculated to dry biomass was obtained.

Single solvents

The first extraction solvents tested were pure hexane, acetone and ethanol. Hexane is often recommended in literature for extraction of the lipid fraction from microalgae. Its advantage is the chemical stability and relatively low boiling point, which is favourable for separation/regeneration. For potential application of lipids/FAs in the food/pharmaceutical industry, this solvent is acceptable. Acetone and ethanol were chosen as representatives of the more polar organic substances. Relative yield of the extractable part (dry extract) in comparison with the chloroform/methanol/water method is shown in Table 1. Also percentage of the major FAs in the extractable part is given.

Table 1: Lipid/FA fraction yield obtained by individual solvents in %.

Solvent	Hexane	Acetone	Ethanol
Extractable part[a]	35.2	56.6	94.3
Total fatty acids in extractable part	25.3	33.2	38.4
Total fatty acids/dry biomass	1.6	3.4	5.8
Oleic acid (C18:1n9c)[b]	4.1	2.8	3.3
Linoleic acid (C18:2n6c)[b]	25.1	27.9	33.9
α-Linolenic acid (C18:3n3)[b]	30.4	37.8	30.2

[a] relatively to chloroform/methanol/water method (content in dry microalgae)
[b] relatively to total fatty acids in extractable part

From a complex point of view, hexane is the most suitable solvent for the extraction due to its physical properties. Contrary, the amount of obtained extractables was the lowest

compared to all the tested solvents. Hexane, such as the non-polar substance has a low efficiency for the extraction of the neutral and polar lipid mixtures in an algal biomass.

According to the conclusions of Molina Grima et al. (1994), higher percentage of total lipids was extracted with the increasing extraction solvent polarity as follows: hexane < acetone < ethanol. It is obvious, that the total extractable part and content of the total FAs were included. However, no similar trend was observed in the yield of the selected individual FAs. The linoleic and α-linolenic acids were chosen as the dominant representatives of the essential acids necessary for the correct development of the human body. In our experiments unlike Kates (1986a) more than 90% of the Bligh and Dyer co-solvent method was reached.

Solvent mixtures

Generally, hydrocarbons require the use of the other solvents as de-emulsifiers to prevent formation of foams and stable emulsions, i.e. reduction of interfacial tension and improvement of fluidity of samples used in the extraction. In the patent literature two types of organic solvents – low-molecular ketone or alcohol are recommended. In our experiments, alcohol/ketone has been added to hexane to regulate the polarity of the system and thus increase the amount of extractables/FAs.

In the systems hexane/methanol > hexane/ethanol > hexane/propan-2-ol appreciable decrease (rel. 25%) in the total FA content in dry extract was detected. Slight decrease of total FA fraction in the hexane/butan-1-ol > hexane/2-methylpropan-1-ol systems was also observed. Both measurement series confirm the influence of diminishing polarity on efficiency of the lipid part extraction due to the length of the alcohol chain and its branching. As compared with results cited in Table 1, extractables/FA yield achieved with hexane/ethanol mixture fitted between hexane and ethanol values measured. Composition of FA fraction depending on co-solvents is depicted in Figure 2.

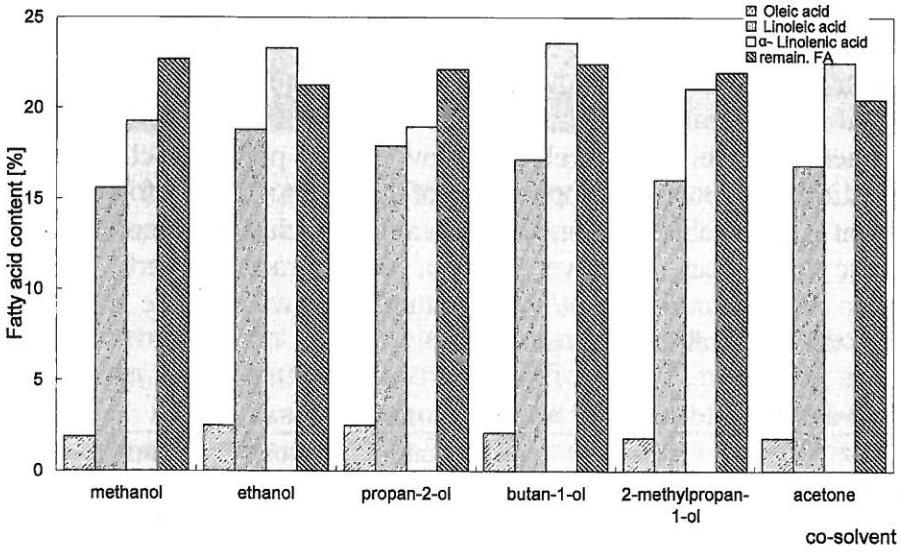


Figure 2: Fatty acids extraction efficiency according to hexane/co-solvent system.

The calculated total FA content varied in the range of 2.8-5.8% dry biomass. Although the content of total FAs showed the dependency on polarity of the used extraction system, composition of solvents had practically no significant effect on the yield of the studied individual FAs. In the hexane/co-solvent system palmitic and stearic acid content ranged from 12.2 to 16.8% and 3.0 to 5.8% of the total extracted acids, respectively. The content of other acids (lauric, palmitoleic, γ-linolenic, eicosapentaenoic etc.) varied in tenth of percent. The lipid fraction was composed of 59.4 to 67.8% total FAs, remaining components were glycerol (~5%) and fragments possibly resulting from the destructive autocatalytic reactions. Epoxy-,

hydroperoxy-, ketonic and/or aldehydic groups were identified.

Further, variation of the extraction system polarity was studied by modification of the hexane/alcohol ratio. As a co-solvent ethanol and propan-2-ol were selected due to their relatively low boiling points and favourable regeneration conditions.

Table 2: The effect of hexane/alcohol ratio (2/3 or 3/4) on the extractables/FA yield in %.

Solvent system	Dry extract/dry biomass	Total FA/dry extract
Hexane/ethanol 2/3	16.8	27.2
Hexane/ethanol 3/4	14.3	26.3
Hexane/propan-2-ol 2/3	12.3	24.1
Hexane/propan-2-ol 3/4	11.0	23.9

Slight increase of the hexane content in the hexane/alcohol systems (2/3 to 3/4), which caused a decrease in polarity of the mixture, resulted in decrease of the extractable part of the dry biomass about 15 or 11% (hexane/ethanol and hexane/propan-2-ol, respectively). The lower amount of the extractable part obtained by the hexane/propan-2-ol system can be caused by the lower polarity of propan-2-ol in comparison with ethanol. The lower co-solvent content reflected the lower amount of polar lipids. The effect on FA content in the dry extract was negligible. For more evident influence, the larger difference in the individual component ratio should be tested.

Since the decompositive products of FAs in dry extracts were identified, biomass at moderate conditions (stirring at ambient temperature) was treated (Table 3).

Table 3: Comparison of various temperature treatment effects on yield of the extractable part/FAs related to the dry biomass in %. Hexane/ethanol ration = 2/3.

Procedure	Dry extract/dry biomass	Total FA/dry biomass
Boiling for 1 h	16.8	4.5
Stirring for 1 h (ambient temperature)	12.0	5.1
Stirring for 4 h (ambient temperature)	15.6	6.2

In comparison with experiments at elevated temperature, biomass treatment at the ambient temperature had a negative effect on the extractable part yield. On the contrary, the total FA amount related to dry microalgae increased with extraction time extension. It can indicate that stirring for 1 h is insufficient for equilibrium state achievement and that diffusion can inhibit the extraction process. Also the percentage of the separated FAs was higher. It is evident, that the thermal treatment affected reactions in extracted system destructively and it caused the loss of the valuable components.

Conclusions

The presented work was focused on clarification of the discrepancies in the literature sources regarding the effect of extraction system polarity on the yield of microalgal lipids. In all experiments the positive effect of higher polarity on the yield of extractable substances, in particular higher fatty acids, was proved.

Three single solvents were tested, where the yield of the extractable part/total FAs increased in the series hexane < acetone < ethanol. The same trend was observed for hexane < hexane/ethanol < ethanol systems. When hexane/alcohol was tested, the slight dependence in series of the co-solvents methanol > ethanol > propan-2-ol > butan-1-ol > 2-methylpropan-1-ol was observed. Polarity of the solvent mixture played also the role in efficiency of the extraction process for various hexane/ethanol and hexane/propan-2-ol ratios. Despite the

higher content of FAs in extracts, the high extractable part could be caused by increased amount of the undesirable soluble components, such as pigments (chlorophylls), proteins etc. Other experiments were led to compare delicacy of the extraction method to the total FA yield when microalgae are heated or moderately stirred at ambient temperature. Biomass treatment at the ambient temperature appeared to be more efficient in obtaining the higher amount of the FAs.

Keywords: solvent extraction, chlorella, microalgae, fatty acids, hexane, alcohol

Literature

Bligh E.G., Dyer W.J.: A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* (1959), 37, 911-917.

Cartens M., Molina Grima E., Robles Medina A., Gimenéz Gimenéz A., Ibáñéz González J.: Eicosapentaenoic acid (20:5n-3) from the marine microalga Phaeodactylum tricornutum. *Journal of the American Oil Chemists' Society* (1996), 73(8), 1025-1031.

Cravotto G., Boffa L., Mantegna S., Perego P., Avogadro M., Cintas P.: Improved extraction of vegetable oils under high-intensity ultrasound and/or microwaves. *Ultrasonics Sonochemistry* (2008), 15, 898–902.

Halim R., Gladman B., Danquah M.K., Webley P.A.: Oil extraction from microalgae for biodiesel production. *Bioresource Technology* (2011), 102, 178–185.

Kates M. (1986a): Techniques of Lipidology: Isolation, Analysis, and Identification of Lipids. In Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 3, Part 2, 2nd ed.; Burdon, R.H. and van Knippenberg, P.H. (eds.) Elsevier: pp. 100–111.

Kates M. (1986b): Techniques of Lipidology: Isolation, Analysis and Identification of Lipids, Burdon, R.H. and Van Knippenberg, P. H. (eds.), 2nd edition, Elsevier, pp. 186-278. Separation of lipid mixtures.

Lee J.-Y., Yoo, C., Jun S.-Y., Ahn C.-Y., Oh H.-M.: Comparison of several methods for effective lipid extraction from microalgae. *Bioresource Technology* (2010), 101, S75–S77.

Molina Grima E., Rubies Medina A., Giménez Giménez A., Sánchez Pérez J.A., Garcia Camacho F., García Sánchez J.L.: Comparison between extraction of lipids and fatty acids from microalgal biomass. *Journal of the American Oil Chemists' Society* (1994), 71, 955-959.

Montes D'Oca M.G., Viêgas C.V., Lemões J.S., Miyasaki E.K., Morón-Villarreyes J.A., Primel E.G., Abreu P.C.: Production of FAMEs from several microalgal lipidic extractsand direct transesterification of the Chlorella pyrenoidosa. *Biomass and Bioenergy* (2011), 35, 1533-1538.

Nagle N. and Lemke P.: Production of methyl ester fuel from microalgae. *Applied Biochemistry and Biotechnology* (1990), 24/25, 355-361.

Peralta-Ruiz Y., González-Delgado A.-D., Kafarov V.: Evaluation of alternatives for microalgae oil extraction based on exergy analysis. *Applied Energy* (2013), 101, 226–236.

Ramírez-Fajardo A., Esteban-Cerdan L., Robles-Medina A., Acién-Fernández F.G., González-Moreno P.A., Molina-Grima E.: Lipid extraction from the microalga Phaeodactylum tricornutum. *European Journal of Lipid Science And Technology* (2007), 109, 120–126.

Robles Medina A., Molina Grima E., Gimenéz Gimenéz A, Ibáñéz Gonzáles M.J: Downstream processing of algal polyunsaturated fatty acids. *Biotechnology Advances* (1998), 16 (3), 517-580.

Šoštarič M., Klinar D., Bricelj M., Golob J., Berovič M., Likozar B.: Growth, lipid extraction and thermal degradation of the microalgae Chlorella vulgaris. *New Biotechnology* (2012), 29 (3), 325-331.

Virot M., Tomao V., Colnagui G., Visinoni F., Chemat F.: New microwaveintegrated Soxhlet extraction, an advantageous tool for the extraction of lipids from food product. *Journal of Chromatography*. A (2007) 1174, 138–144.

Acknowledgement

The financial support of the Technology Agency of the Czech Republic, project "Biorefinery research centre of competence Bioraf" (TE01020080) is acknowledged.