American Journal of Analytical Chemistry, 2016, 7, 141-150
Published Online February 2016 in SciRes. http://www.scirp.org/journal/ajac
http://dx.doi.org/10.4236/ajac.2016.72012

Evaluation of Various Extraction Techniques for Efficient Lipid Recovery from Thermo-Resistant Microalgae, *Hindakia*, *Scenedesmus* and *Micractinium* Species

—Comparison of Lipid Extraction Methods from Microalgae

Melih Onay[1], Cagla Sonmez[2], Huseyin A. Oktem[2,3], Meral Yucel[2,3*]

[1]Department of Chemistry, Yuzuncu Yil University, Van, Turkey
[2]Department of Biology, Middle East Technical University, Ankara, Turkey
[3]Department of Biotechnology, Middle East Technical University, Ankara, Turkey
Email: melihonay@yyu.edu.tr, *meraly@metu.edu.tr

Received 23 December 2015; accepted 26 January 2016; published 29 January 2016

Copyright © 2016 by authors and Scientific Research Publishing Inc.
This work is licensed under the Creative Commons Attribution International License (CC BY).
http://creativecommons.org/licenses/by/4.0/
Open Access

Abstract

In recent years, photosynthetic microalgae regained attention for biodiesel production. For efficient utilization of microalgae, a number of criteria including a strain with high biomass and lipid productivities and employment of effective and reliable methods for oil extraction from the obtained biomass should be met. Recently, we have isolated and identified three thermo-resistant green microalgae strains, namely; *Scenedesmus sp.* ME02, *Hindakia tetrachotoma* ME03 and *Micractinium sp.* ME05. In this study, we compared percent lipid content of thermos-tolerant microalgal strains using the following solvent extraction methods: Soxhlet, Bligh and Dyer and Folch methods with or without assisted cell disruption techniques including lyophilization, homogenization, ultrasonication, bead and microwave-assisted. The highest increase in lipid yield was obtained with a combination of lyophilization and ultrasonication techniques together with Soxhlet method: 27% of total dry weight for *Micractinium sp.* ME05. We conclude that lyophilization and ultrasonication are effective assistance methods for lipid extraction from thermo-resistant microalgae.

*Corresponding author.

How to cite this paper: Onay, M., Sonmez, C., Oktem, H.A. and Yucel, M. (2016) Evaluation of Various Extraction Techniques for Efficient Lipid Recovery from Thermo-Resistant Microalgae, *Hindakia*, *Scenedesmus* and *Micractinium* Species. *American Journal of Analytical Chemistry*, **7**, 141-150. http://dx.doi.org/10.4236/ajac.2016.72012

Keywords

Lipid Extraction, Thermo-Resistant Green Microalgae, Cell Disruption Techniques, Ultrasonication, Biodiesel

1. Introduction

The global demand for fuels is at all time high due to the ever-increasing world population and energy consumption associated with it [1]. Currently, majority of world fuel demand is supplied by fossil-based fuels [2]. This type of fuel is problematic in many respects, particularly because of its detrimental effects on the environment and needs to be replaced by renewable energy sources in the near future [3] [4]. One form of renewable energy is derived from sustainable biological materials. Plants have been recognized as alternative sources of biofuels such as bioethanol production from corn or sugarcane and biodiesel production from oil crops like soybeans or oil palm [1]. However, the most important drawback of using crop plants for energy production is the competition with food consumption and limited availability of agricultural land. Photosynthetic microalgae are advantageous as an alternative source of biodiesel with little or no competition for arable land, adaptability to different growth environments, high biomass accumulation and lipid yield [5]. Oil content of some microalgae can be as high as 80% of dry weight [6]. Despite these advantages, microalgae are yet not commercially competitive with petroleum based fuel [1] [7]. Lipid yield can substantially vary depending on the type of microalgae used as well as cultivation conditions of the particular strain such as light intensity, temperature and growth media [8]. Thus, prior to large scale cultivation, each parameter needs to be optimized per microalgal strain.

One of the prerequisites for efficient utilization of microalgae for biodiesel production is to employ effective and reliable methods for oil extraction from the microalgal biomass [9]. Lipids generally dissolve in organic solvent such as hexane, ether and chloroform [10]. Particularly, the choice of solvent is of major importance for efficient lipid extraction. Traditional lipid extraction methods involve use of methanol and chloroform as solvents. Folch *et al.* were among the first to successfully isolate lipids from animal tissues in a methanol/chloroform/water phase [11]. A second method developed by Bligh and Dyer utilizes the same solvents for extraction at different initial solvent to sample ratios [12]. This method was originally used for lipid extraction from fish tissue but became a popular technique for lipid extraction from microalgae as well. Despite their widespread application, both Folch and Bligh and Dyer methods require use of high amounts of toxic chemicals, methanol and chloroform, which have adverse effects on human health and the environment. An alternative solvent used for lipid extraction is the non-polar solvent hexane. It is advantageous as it is less toxic, easily evaporated, relatively cheaper and highly selective for neutral lipids and it eliminates other non-lipid contaminants [9]. Due to these qualities, hexane can easily be scaled up for large amounts of lipid extraction. Besides choosing the right solvent, choice of extraction methodology and equipment is also important [13]. A classical method for lipid extraction involves the use of a specialized device known as the Soxhlet apparatus [14] [15].

Another important parameter for successful extraction is the mode of cell disruption prior to addition of the extraction solvent. Microalgae contain rigid cell walls that need to be removed for efficient recovery of lipids. A commonly used method of cell disruption is mechanical disruption via homogenization, pressing or bead milling (bead beating). Homogenization and pressing rupture the cell walls by applying pressure, whereas bead milling damage the cell wall via agitation through grinding with small beads at high speed [1]. Other assisted techniques of lipid extraction include but are not limited to microwave, ultrasonication and lyophilization. The main principle of microwave technology is to generate thermal energy via friction of the polar water molecules inside the cells. Upon heating in the microwave oven, the cells will eventually rupture due to the pressure caused by evaporation of the water molecules [16]. During ultrasonication, high intensity sound waves cause agitation via creating micro-scale bubbles. These bubbles collapse and burst near cell walls in a high/low pressure cycle causing rupturing of the cells. By mechanical means, ultrasonication disrupts cell walls and allows release of the cellular content for efficient extraction [17]. Lyophilization or freeze-drying, on the other hand, refers to dehydration of frozen cells under a vacuum chamber via evaporation of ice crystals.

In a recent study, we isolated and characterized three thermo-resistant green microalgae, namely; *Scenedesmus sp.* ME02, *Hindakia tetrachotoma* ME03, and *Micractinium sp.* ME05 from thermal water flora of Central

Anatolia [18]. We identified these strains as potential candidates for large scale biodiesel production. In this study, we evaluated efficiencies of three solvent extraction methods, namely Soxhlet extraction, Bligh and Dyer and Folch methods with or without assisted cell disruption techniques including lyophilization, homogenization, ultrasonication, bead-assisted and microwave-assisted for lipid extraction from these microalgae. Lyophilization and ultrasonication or a combination of both techniques significantly improved the lipid yield in most cases.

2. Materials and Methods

2.1. Microalgal Strains and Cultivation Conditions

In this study, comparison of different lipid extraction methods from three green microalgal strains, *Scenedesmus sp.* ME02, *H. tetrachotoma* ME03 and *Micractinium sp.* ME05 were evaluated. All three strains were obtained from thermal water pools of Haymana, Ankara, Turkey and isolated in our laboratory [18]. For lipid extraction, 200 mL of microalgal cultures were grown in liquid Tris-Acetate-Phosphate (TAP, pH 6.8) or BG-11 [19] [20] media in one liter flasks with constant shaking at 24˚C ± 1˚C, 16 h light-8 h dark photoperiod with a light intensity of 54 $\mu E \cdot m^{-2} \cdot s^{-1}$ until cultures reached stationary phase. All chemicals used in this study were technical grade and obtained from Sigma-Aldrich.

2.2. Lipid Extraction Methods

One part of extract was dissolved in forty parts of solvent in three different modified extraction methods: Bligh and Dyer, Folch and Soxhlet extraction method [11]-[15]. For all three methods, initially two flasks of 100 mL of the same microalgal suspension were separated and centrifuged at 3000 *g* for 10 min at 4˚C. Then, for dry weight calculation, one tube containing the pellet was taken and oven-dried. The second tube was used for lipid extraction according to the method of choice. All experiments were performed with three biological replicates (n = 3).

2.3. Folch Method

This method was originally described by Folch [11]. Pellet of 100 mL microalgal suspension was mixed with chloroform and methanol in a 2:1 v/v ratio and mixed thoroughly in a vortexer. Then, methanol and water were added to the mixture to a final ratio of 1:1:0.9 v/v for methanol:chloroform:water, respectively. The final ratio of pellet to methanol, chloroform and water mixture should be 1:40 w/v. The mixture was shaken for 10 min in a separating funnel and incubated until two distinct phases were visible. The lower phase containing the microalgal lipids was separated and evaporated by a rotary evaporator (BUCHI Rotavapor R-200). Lipid content was quantified gravimetrically.

2.4. Bligh and Dyer Method

Bligh and Dyer method was carried out as described previously with some modifications [12]. Pellet of 100 mL microalgal suspension was mixed with methanol and chloroform in a 2:1 v/v ratio and mixed thoroughly in a vortexer. Then, chloroform and water were added to the mixture to a final ratio of 1:1:0.9 v/v for methanol: chloroform:water, respectively. The rest of the protocol is identical to that of Folch method as described above.

2.5. Soxhlet Method with Hexane

Soxhlet extraction method with n-hexane was performed with some modifications [14] [15]. One part of pellet was mixed with forty parts of n-hexane in the Soxhlet apparatus. The solvent was heated to reflux. Total oil of microalgae was dissolved in n-hexane. Pellet was left for 24 hours in Soxhlet apparatus and oil was taken from the distillation flask. Remaining n-hexane was evaporated by rotary evaporator (BUCHI Rotavapor R-200). Microalgae oil was weighed and lipid yield was calculated gravimetrically.

2.6. Cell Disruption Techniques of Microalgal Strains for Assisted Lipid Extraction

We carried out five different cell disruption methods, namely; homogenization assisted (H-A), microwave assisted (MW-A), ultrasonication assisted (US-A), bead assisted (B-A) and lyophilization assisted (L-A) and combinations of these together with the three solvent extraction procedures.

2.7. Homogenization Assisted (H-A) Method

100 mL of microalgal suspension was homogenized in tubes on ice by a homogenizer (Heideoph Diax-900) with 20 s homogenization time and 5 s interval. We tried three different time points; 5, 10 or 20 min for the total time of homogenization.

2.8. Microwave Assisted (MW-A) Method

100 mL of microalgal suspension was placed in a conventional microwave oven (Arcelik intellewave MD-599) and samples were disrupted at 900 W, 20 s microwave power and 5 s interval time for a total duration of 1, 5 or 10 min.

2.9. Ultrasonication (US-A) Method

100 mL of microalgal suspension was extracted on ice via an ultrasonicator (Cole Palmer Ultrasonic Processor CPT) at 400 W, 20 s ultrasonication, 5 s interval time for a total duration of 5, 10 or 20 min.

2.10. Glass Bead (B-A) Method

100 mL of microalgal suspension was mixed with 2 g glass beads (0.4mm diameter in size) and shaken for 5, 10 and 20 min at 1000 rpm rotation speed.

2.11. Lyophilization Assisted (L-A) Method

Microalgal suspension culture was centrifuged at 3000 *g* for 10 min at 4˚C and pellet was freeze-dried (lyophilized) in a lyophilizator (Christ D-37520 Alpha 1-4 LD plus) for 24 hours. The lyophilized samples were weighed and 4 mL of solvent was added per 0.1 g lyophilized tissue in each of the three solvent extraction methods. For a double combination of L-A technique with other cell disruption methods, lyophilized samples were used in each subsequent assisted technique (*i.e.* MW-A, US-A and B-A) prior to solvent extraction.

3. Results

3.1. Solvent Extraction Methods with No Assistance

In this study, we did a comparative analysis of three different solvent extraction methods with or without assisted techniques for lipid extraction from three thermo-resistant microalgae strains, namely; *Scenedesmus sp.* ME02, *Hindakia tetrachotoma* ME03, and *Micractinium sp.* ME05 isolated from thermal water flora of Central Anatolia [18]. Initially, three solvent extraction methods; Soxhlet method, Bligh and Dyer, and Folch methods were used with no additional assisted technique. Results are summarized in **Figure 1**. For *Scenedesmus sp.* ME02, percent lipid contents were as follows: 9.0%, 9.3% and 10.3% total lipid.gdryweight^{-1} with Bligh and Dyer, Soxhlet and Folch methods, respectively. For *H. tetrachotoma* ME03, final lipid yield was 4.7% total lipid·gdryweight^{-1} with the Folch method and 7.0% with Bligh and Dyer and Soxhlet methods. Finally, *Micractinium sp. ME*05 had the highest lipid content among three species with all three methods tested. Percent lipid content for *Micractinium sp.* ME05 was 13.7%, 13.3%, and 11.7% with Soxhlet, Bligh and Dyer and Folch methods, respectively.

3.2. Extraction Assisted Methods

Next, we employed five different cell disruption techniques for assisted extraction of lipids from microalgal cells. These techniques are as follows: Homogenization assisted (H-A), microwave assisted (MW-A), ultrasonication (US-A), glass bead (B-A) and lyophilization assisted (L-A) methods.

For four of these five methods, namely; H-A, MW-A, US-A and B-A, we tested three different time durations of cell disruption (**Table 1**). For the H-A method, increasing the time of homogenization from 5 min to 10 min slightly improved the lipid yield for all three species and solvent extraction methods. A further increase in homogenization time to 20 min, on the other hand, did not lead to further improvement in yield. Therefore, 10 min was determined as the total time of homogenization.

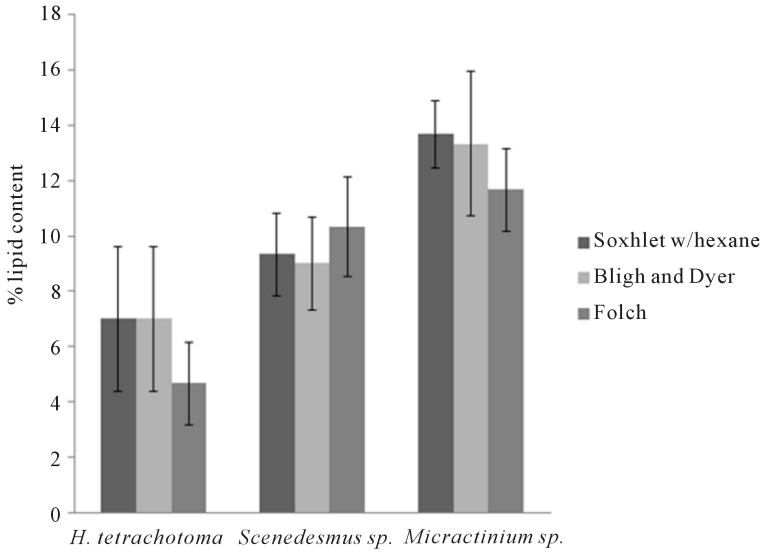

Figure 1. % lipid content of *H. tetrachotoma* ME03, *Scenedesmus sp.* ME02 and *Micractinium sp.* ME05 with Soxhlet, Bligh and Dyer and Folch methods. Error bars show SEM.

Table 1. Lipid contents (%) of *Hindakia tetrachotoma* ME03, *Scenedesmus sp.* ME02, *Micractinium sp.* ME05, at each time point of assisted technique together with the following solvent extraction methods: Soxhlet, Bligh and Dyer and Folch.

Species	Solvent extraction method	No assistance	Assisted technique											
			H-A			MW-A			US-A			B-A		
			5'	10'*	20'	1'	5'	10'*	5'	10'	20'*	5'	10'	20'*
H. tetrachotoma ME03	Soxhlet	7 ± 3	8 ± 2	8 ± 2	8 ± 1	7 ± 1	8 ± 2	8 ± 1	7 ± 1	8 ± 2	9 ± 2	7 ± 1	7 ± 1	8 ± 2
	Bligh and Dyer	7 ± 3	7 ± 1	8 ± 2	7 ± 1	6 ± 1	6 ± 2	6 ± 1	7 ± 2	6 ± 1	8 ± 2	5 ± 1	6 ± 1	7 ± 2
	Folch	5 ± 1	5 ± 1	6 ± 1	5 ± 1	5 ± 1	5 ± 1	5 ± 1	5 ± 1	6 ± 1	5 ± 1	6 ± 1	5 ± 1	5 ± 1
Scenedesmus sp. ME02	Soxhlet	9 ± 1	11 ± 1	11 ± 1	11 ± 1	11 ± 1	11 ± 2	9 ± 1	12 ± 2	11 ± 2	14 ± 2	10 ± 1	10 ± 2	11 ± 1
	Bligh and Dyer	9 ± 2	9 ± 1	12 ± 2	10 ± 1	9 ± 1	10 ± 1	11 ± 1	11 ± 1	12 ± 1	12 ± 1	9 ± 1	9 ± 3	9 ± 1
	Folch	10 ± 2	10 ± 2	11 ± 1	7 ± 1	9 ± 1	10 ± 1	8 ± 2	8 ± 1	11 ± 1	12 ± 1	8 ± 1	7 ± 1	11 ± 1
Micractinium sp. ME05	Soxhlet	14 ± 1	17 ± 2	20 ± 3	19 ± 2	14 ± 1	16 ± 1	16 ± 1	17 ± 1	17 ± 2	18 ± 1	13 ± 1	15 ± 1	16 ± 2
	Bligh and Dyer	13 ± 3	14 ± 2	17 ± 2	17 ± 1	12 ± 1	14 ± 2	13 ± 1	14 ± 1	12 ± 2	16 ± 1	13 ± 1	14 ± 2	17 ± 1
	Folch	12 ± 1	14 ± 2	16 ± 1	15 ± 1	11 ± 2	14 ± 1	14 ± 1	14 ± 1	14 ± 2	15 ± 1	10 ± 1	11 ± 1	13 ± 1

*Indicates durations that were used as data points in **Figures 2-4**.

For the MW-A method, three different time durations of 1, 5 and 10 minutes were tested. 5 and 10 minutes of cell disruption in the microwave oven led to slightly higher percent lipid yield in most cases compared to one minute of disruption (**Table 1**). Between 5 and 10 minutes, we chose the latter as the total duration of microwave time due to the consistency of final percent yields between three biological replicates reflected by low standard error.

Finally, 20 minutes of incubation time was determined as the ideal duration of disruption for both US-A and B-A techniques.

Once the duration of disruption for each technique was determined, we compared all five extraction assisted techniques as well as double combinations of lyophilization assisted (L-A) method with MW-A, US-A and B-A in terms of percent lipid yield (**Figures 2-4**). For *Scenedesmus sp.* ME02, US-A together with the Soxhlet method resulted in an oil recovery of 14%. L-A and US-A combined further increased the lipid yield to 15.7%

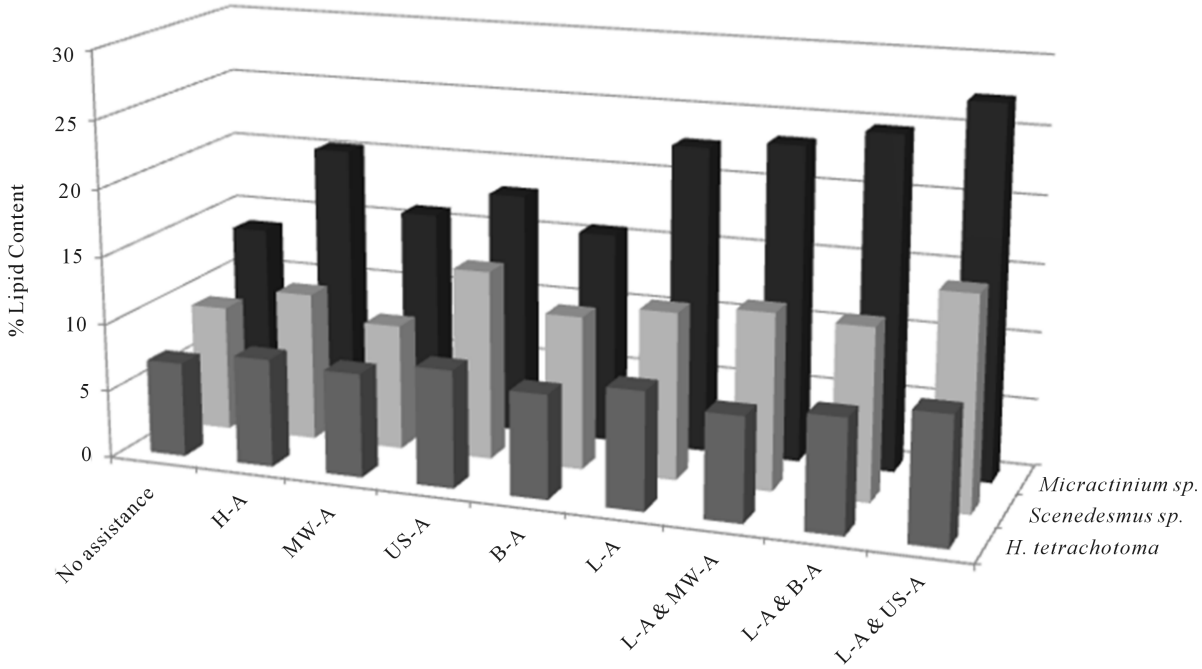

Figure 2. % lipid content (y axis) of *H. tetrachotoma* ME03, *Scenedesmus sp.* ME02 and *Micractinium sp.* ME05 with Soxhlet method together with no assistance, homogenization assisted (H-A), microwave assisted (M-A), ultrasonication assisted (US-A), bead-assisted (B-A), lyophilization assisted (L-A) and a combination of L-A and MW-A, L-A and B-A, and L-A and US-A techniques.

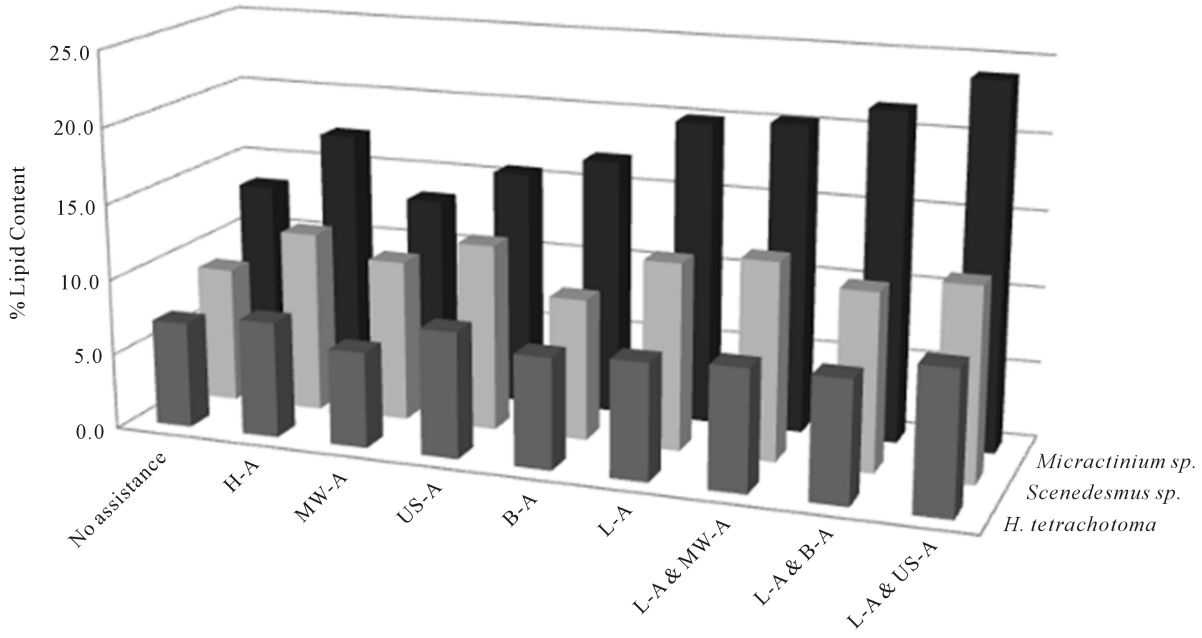

Figure 3. % lipid content (y axis) of *H. tetrachotoma* ME03, *Scenedesmus sp.* ME02 and *Micractinium sp.* ME05 with Bligh and Dyer method together with no assistance, homogenization assisted (H-A), microwave assisted (M-A), ultrasonication assisted (US-A), bead-assisted (B-A), lyophilization assisted (L-A) and a combination of L-A and MW-A, L-A and B-A, and L-A and US-A techniques.

(**Figure 2**). For *H. tetrachotoma* ME03, US-A and L-A in combination with the Soxhlet method (n-hexane) were equally effective leading to an increase in lipid yield from 7.0% to 8.7%. For the same species, using the

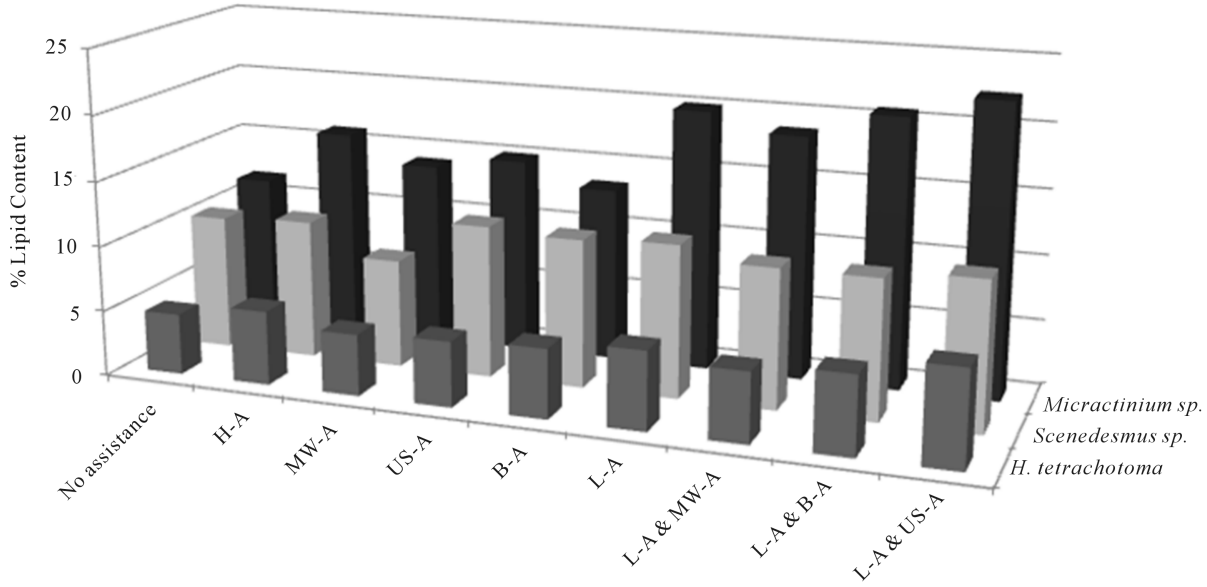

Figure 4. % lipid content (y axis) of *H. tetrachotoma* ME03, *Scenedesmus sp.* ME02 and *Micractinium sp.* ME05 with Folch method together with no assistance, homogenization assisted (H-A), microwave assisted (M-A), ultrasonication assisted (US-A), bead-assisted (B-A), lyophilization assisted (L-A) and a combination of L-A and MW-A, L-A and B-A, and L-A and US-A techniques.

Soxhlet method together with L-A and US-A techniques combined further increased the lipid yield to 9.3%. Finally for *Micractinium sp.*, Soxhlet extraction using the lyophilized samples (*i.e.* L-A technique) yielded in a nearly two fold increase in total lipid content (22.7%) compared to Soxhlet extraction alone (13.7%). However, the highest increase in percent lipid yield was achieved with L-A and US-A combined (27.3%) (**Figure 2**).

Next, we compared the effectiveness of each assisted or a combination of assisted technique(s) together with the Bligh and Dyer solvent extraction method for all three species used in our study. The results were similar to those of Soxhlet method with minor differences (**Figure 3**). A combination of L-A and US-A resulted in the highest increase in percent lipid yields in *Scenedesmus sp.* ME02 (12.7%), *H. tetrachotoma* ME03 (9.3%) and *Micractinium sp.* ME05 (24%) compared to Bligh and Dyer method alone.

For the Folch method, although some improvement was achieved particularly with L-A and US-A combination in *H. tetrachotoma* (7.3%), increase in lipid yield was not nearly as high as either the Soxhlet or the Bligh and Dyer methods in *Scenedesmus sp.* (11.3%) (**Figure 4**).

Taken together, extraction assisted techniques significantly improve the percent lipid yield when combined with a solvent extraction method. Lyophilization and ultrasonication and a combination of both proved to be particularly effective for three green microalgae species used in this study.

3.3. Calculation of Lipid Productivities of Microalgae

We calculated lipid productivities of *Scenedesmus sp.* ME02, *H. tetrachotoma* ME03, and *Micractinium sp.* ME05 as a factor of gram per liter dry weight per day (**Table 2**). We previously determined and described growth characteristics of each three species [18]. Based on the results, *Scenedesmus sp.* ME02 had the lowest lipid productivity (0.006 $g \cdot L^{-1} \cdot day^{-1}$), whereas *Micractinium sp.* ME05 showed the highest lipid productivity rate (0.05 $g \cdot L^{-1} \cdot day^{-1}$). Lipid productivity of *H. tetrachotoma* ME03 was calculated to be 0.01 $g \cdot L^{-1} \cdot day^{-1}$.

4. Discussion

4.1. Solvent Extraction Methods with No Assistance

The choice of an effective extraction method depends on both the type of microalgae used and the solvent of choice. Previous studies emphasized importance of selecting an optimal extraction method suitable for different microalgae with different cell sizes, cell wall structure, lipid and fatty acid characteristics [21]-[23]. Lee *et al.*

Table 2. Lipid productivities of *Hindakia tetrachotoma* ME03, *Scenedesmus sp.* ME02 and *Micractinium sp.* ME05 based on percent lipid contents (%) of dry weight obtained with Soxhlet method combined with lyophilization and ultrasonication.

Name	Lipid content (%)	Lipid productivity ($g \cdot L^{-1} \cdot day^{-1}$)
H. tetrachotoma ME03	9 ± 2	0.01 ± 0.002
Scenedesmus sp. ME02	16 ± 2	0.006 ± 0.003
Micractinium sp. ME05	27 ± 3	0.05 ± 0.003

observed significant differences in lipid content from *Botryococcus sp.*, *Chlorella vulgaris* and *Scenedesmus sp.* using different extraction methods [21]. All microalgal strains used in this study belong to different taxonomic groups (*i.e. Scenedesmus*, *Hindakia* and *Micractinium* genera). However, the extraction method of choice did not show a significant difference among any of the three species used (**Figure 1**).

An appropriate solvent extraction method may be selected on a number of criteria. For instance, in a comparative study, Guckert *et al.* found Soxhlet method with methylene chloride:methanol (2:1) to be the most selective method for extraction of neutral lipids among others [24]. However, Soxhlet method is not suitable for extraction of unsaturated lipids due to instability of these lipids at elevated temperatures during reflux. High temperature can also accelerate transesterification of lipids in the presence of methanol, changing their natural form. In this respect, Bligh and Dyer method with chloroform and methanol causes less artifacts, therefore; results obtained with this method are considerably more reliable and reproducible [24]. However, Bligh and Dyer method is practically not convenient for large scale extractions due to high amounts of toxic waste generated, which requires expensive recycling methods and compromises user safety [25].

Hexane as an extraction solvent, on the other hand, is advantageous for large scale extractions due to being cheaper and less toxic compared to other solvents like chloroform and methanol [26] [27]. Additionally, it has no affinity for non-lipid contaminants during extraction. In this study, the solvent extraction method of choice alone did not differ significantly for any of the three species used.

4.2. Extraction Assisted Methods

In the present study, lipid extraction from dry material (*i.e.* lyophilized samples) proved to be more effective than using wet material. Among other benefits like easy handling and storage [28], lyophilization may also assist in direct and rapid contact of samples with the extraction solvent by increasing the surface area [29] without altering the initial solvent to water ratio in contrast to using wet material.

Several studies tested the effectiveness of microwave-assisted disruption for lipid extraction from microalgae. In a previous study, Lee *et al.* found among other methods, microwave to be the most effective in oil extraction from *Botryococcus sp.*, *Chlorella vulgaris* and *Scenedesmus sp.* [21]. In yet another study, Balasubramanian *et al.* showed a 30% increase in lipid yield using the microwave assisted extraction compared to control from *Scenedesmus obliquus* [30].

In a recent study, Koberg *et al.* successfully employed a one-step biodiesel production method via microwave and ultrasonication directly from *Nannochloropsis* biomass [31]. In yet another study, Cui *et al.* used microwave irradiation for direct transesterification of *Cryptococcus curvatus* and effectively determined optimum conditions of biodiesel production [32]. In addition, Cheng *et al.* studied direct transesterification of wet *Chlorella pyrenoidosa* via microwave and compared it to two-step process of biodiesel production. They found that one step process displayed six-fold higher biomass than that of two-step process for wet *Chlorella pyrenoidosa* [33]. In our study, microwave assisted technique did not result in significant improvement of lipid yield. Increasing the microwave processing time to 20 minutes or higher and using a high-power industrial scale microwave oven could help in improving the results.

Shen *et al.* showed that various cell disruption techniques including bead-beating, French press, sonication and wet milling followed by solvent extraction had different effects on the lipid contents of *Scenedesmus dimorphous* and *Chlorella protothecoides* [34]. For instance, in *S. dimorphus*, wet milling resulted in more than five fold increase in lipid yield compared to solvent extraction alone. In *C. protothecoides*, on the other hand, bead beating was the most effective technique in lipid recovery. In a previous study, Araujo *et al.* studied different lipid extraction methods and found the Bligh and Dyer method as suitable extraction method for *Chlorella vulgaris* [35].

Lee *et al.* found bead-beating and microwave methods to be the most efficient methods for *Botryococcus sp.* compared to homogenization, French press and lyophilization while sonication was the least effective [26]. Cell wall structure, cell size, bead size and duration and speed of beating are important parameters affecting the efficiency of the bead-beating method. Smaller bead size can be tried for more effective extraction as 4 mm bead size used in our study may have been less effective for disruption of cells of approximately 2 - 3 µm in size [18]. Optimization of these parameters in future studies may improve the lipid yield.

5. Conclusion

In this study, we did a comparative analysis of three methods, namely Soxhlet extraction, Bligh and Dyer with chloroform and methanol (initial ratio of 1:2) and Folch with chloroform methanol (initial ratio of 2:1) methods for effective lipid recovery from thermo-resistant green microalgal species, *Scenedesmus sp.*, *Hindakia tetrachotoma* and *Micractinium sp.* The highest increase was obtained with a combination of lyophilization and ultrasonication techniques together with Soxhlet extraction with hexane. This study has practical applications for large scale biodiesel production from microalgal biomass as lyophilization and ultrasonication are suitable for scaling up for large scale lipid extraction for biodiesel production.

Acknowledgements

We would like to thank METU Central Laboratory Research and Development, Molecular Biology and Biotechnology Sections for technical support. This work was supported by METU-BAP, TUBITAK BIDEB 2211 fellowship to MO and TUBITAK 2232 fellowship to CS. Authors declare no conflict of interest.

References

[1] Jones, C.S. and Mayfield, S.P. (2012) Algae Biofuels: Versatility for the Future of Bioenergy. *Current Opinion in Biotechnology*, **23**, 346-351. http://dx.doi.org/10.1016/j.copbio.2011.10.013

[2] Demirbas, A. and Demirbas, F. (2011) Importance of Algae Oil as a Source of Biodiesel. *Energy Conversion and Management*, **53**, 163-170. http://dx.doi.org/10.1016/j.enconman.2010.06.055

[3] Veljkovic, V.B., Stamenkovic, O.S. and Tasic, M.B. (2014) The Wastewater Treatment in the Biodiesel Production with Alkali-Catalyzed Transesterification. *Renewable and Sustainable Energy Reviews*, **32**, 40-60. http://dx.doi.org/10.1016/j.rser.2014.01.007

[4] Yaakob, Z., Narayanan, B.N., Padikkaparambil, S., Unni, S.K. and Akbar, M.P. (2014) A Review on the Oxidation Stability of Biodiesel. *Renewable and Sustainable Energy Reviews*, **35**, 136-153. http://dx.doi.org/10.1016/j.rser.2014.03.055

[5] Chisti, Y. (2008) Biodiesel from Microalgae Beats Bioethanol. *Trends in Biotechnology*, **26**, 126-131. http://dx.doi.org/10.1016/j.tibtech.2007.12.002

[6] Chisti, Y. (2007) Biodiesel from Microalgae. *Biotechnology Advances*, **25**, 294-306. http://dx.doi.org/10.1016/j.biotechadv.2007.02.001

[7] Boadu, V.A., Pfromm, P.H. and Nelson, R. (2014) Economic Feasibility of Algal Biodiesel under Alternative Public Policies. *Renewable Energy*, **67**, 136-142. http://dx.doi.org/10.1016/j.renene.2013.11.029

[8] Chen, C.Y., Yeh, K.L., Aisyah, R., Lee, D.J. and Chang, J.S. (2011) Cultivation, Photobioreactor Design and Harvesting of Microalgae for Biodiesel Production: A Critical Review. *Bioresource Technology*, **102**, 71-81. http://dx.doi.org/10.1016/j.biortech.2010.06.159

[9] Mercer, P. and Armenta, R.E. (2011) Developments in Oil Extraction from Microalgae. *European Journal of Lipid Science and Technology*, **113**, 539-547. http://dx.doi.org/10.1002/ejlt.201000455

[10] Ríos, S.D., Castaneda, J., Torras, C., Farriol, X. and Salvado, J. (2013) Lipid Extraction Methods from Microalgal Biomass Harvested by Two Different Paths: Screening Studies toward Biodiesel Production. *Bioresource Technology*, **133**, 378-388. http://dx.doi.org/10.1016/j.biortech.2013.01.093

[11] Folch, J., Lees, M. and Sloane-Stanley, G.M. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. *The Journal of Biological Chemistry*, **226**, 497-509.

[12] Bligh, E.J. and Dyer, W.J. (1959) A Rapid Method of Total Lipid Extraction and Purification. *Canadian Journal of Biochemistry and Physiology*, **37**, 911-917.

[13] Sathish, A. and Sims, R.C. (2012) Biodiesel from Mixed Culture Algae via a Wet Lipid Extraction Procedure. *Bioresource Technology*, **118**, 643-647. http://dx.doi.org/10.1016/j.biortech.2012.05.118

[14] Soxhlet, F. (1879) Die gewichtsanalytische Bestimmung des Milchfettes. *Dinglers Polytechnisches Journal*, **232**, 461-465.

[15] Luque de Castro, M.D. and Garcia-Ayuso, L.E. (1998) Soxhlet Extraction of Solid Materials: An Outdated Technique with a Promising Innovative Future. *Analytica Chimica Acta*, **369**, 1-10. http://dx.doi.org/10.1016/S0003-2670(98)00233-5

[16] Mandel, V., Mohan, Y. and Hemalatha, S. (2007) Microwave Assisted Extraction—An Innovative and Promising Extraction Tool for Medicinal Plant Research. *Pharmacognosy Reviews*, **1**, 7-18.

[17] Cravotto, G., Boffa, L., Mantegna, S., Perego, P., Avogadro, M. and Cintas, P. (2008) Improved Extraction of Vegetable Oils under High-Intensity Ultrasound and/or Microwaves. *Ultrasonics Sonochemistry*, **15**, 898-902. http://dx.doi.org/10.1016/j.ultsonch.2007.10.009

[18] Onay, M., Sonmez, C., Oktem, H.A. and Yucel, A.M. (2014) Thermo-Resistant Green Microalgae for Effective Biodiesel Production: Isolation and Characterization of Unialgal Species from Geothermal Flora of Central Anatolia. *Bioresource Technology*, **169**, 62-71. http://dx.doi.org/10.1016/j.biortech.2014.06.078

[19] Gorman, D.S. and Levine, R.P. (1965) Cytochrome F and Plastocyanin: Their Sequence in the Photosynthetic Electron Transport Chain of *Chlamydomonas reinhardi*. *Proceedings of the National Academy of Sciences of the United States of America*, **54**, 1665-1669. http://dx.doi.org/10.1073/pnas.54.6.1665

[20] Stanier, R.Y., Kunisawa, R., Mandel, M. and Cohen-Bazire, G. (1971) Purification and Properties of Unicellular Blue-Green Algae (Order Chroococcales). *Bacteriological Reviews*, **35**, 171-205.

[21] Lee, J.Y., Yoo, C., Jun, S.Y., Ahn, C.Y. and Oh, H.M. (2010) Comparison of Several Methods for Effective Lipid Extraction from Microalgae. *Bioresource Technology*, **10**, 75-77. http://dx.doi.org/10.1016/j.biortech.2009.03.058

[22] Ryckebosch, E., Muylaert, K. and Foubert, I. (2012) Optimization of an Analytical Procedure for Extraction of Lipids from Microalgae. *Journal of the American Oil Chemists' Society*, **89**, 189-198. http://dx.doi.org/10.1007/s11746-011-1903-z

[23] Guschina, I.A. and Harwood, J.L. (2006) Lipids and Lipid Metabolism in Eukaryotic Algae. *Progress in Lipid Research*, **45**, 160-186. http://dx.doi.org/10.1016/j.plipres.2006.01.001

[24] Guckert, J.B., Cooksey, K.E. and Jackson, L.L. (1988) Lipid Solvent Systems Are Not Equivalent for Analysis of Lipid Classes in the Micro-Eukaryotic Green Alga, Chlorella. *Journal of Microbiological Methods*, **8**, 139-149. http://dx.doi.org/10.1016/0167-7012(88)90015-2

[25] Sahena, F., Zaidul, I.S.M., Jinap, S., Karim, A.A., Abbas, K.A., Norulaini, N.A.N. and Omar, A.K.M. (2009) Application of Supercritical CO_2 in Lipid Extraction: A Review. *Journal of Food Engineering*, **95**, 240-253. http://dx.doi.org/10.1016/j.jfoodeng.2009.06.026

[26] Lee, S.J., Yoon, B.D. and Oh, H.M. (1998) Rapid Method for the Determination of Lipid from the Green Alga *Botryococcus braunii*. *Biotechnology Techniques*, **12**, 553-556. http://dx.doi.org/10.1023/A:1008811716448

[27] Medina, A., Grima, E., Gimenez, A. and Gonzalez, M. (1998) Downstream Processing of Algal Polyunsaturated Fatty Acids. *Biotechnology Advances*, **16**, 517-580. http://dx.doi.org/10.1016/S0734-9750(97)00083-9

[28] Ausborn, M., Nuhn, P. and Schreier, H. (1992) Stabilization of Liposomes by Freeze Thaw and Lyophilization Techniques: Problems and Opportunities. *European Journal of Pharmaceutics and Biopharmaceutics*, **38**, 133-139.

[29] Nielsen, S.S. (2003) Food Analysis. 3rd Edition, Kluwer Academic/Plenum, New York.

[30] Balasubramanian, S., Allen, J.D., Kanitkar, A. and Boldor, D. (2010) Oil Extraction from *Scenedesmus obliquus* Using a Continuous Microwave System-Design, Optimization, and Quality Characterization. *Bioresource Technology*, **102**, 3396-3403. http://dx.doi.org/10.1016/j.biortech.2010.09.119

[31] Koberg, M., Cohen, M., Ben-Amotz, A. and Gedanken, A. (2011) Bio-Diesel Production Directly from the Microalgae Biomass of *Nannochloropsis* by Microwave and Ultrasound Radiation. *Bioresource Technology*, **102**, 4265-4269. http://dx.doi.org/10.1016/j.biortech.2010.12.004

[32] Cui, Y. and Liang, Y. (2014) Direct Transesterification of Wet *Cryptococcus curvatus* Cells to Biodiesel through Use of Microwave Irradiation. *Applied Energy*, **119**, 438-444. http://dx.doi.org/10.1016/j.apenergy.2014.01.016

[33] Cheng, J., Yu, T., Li, T., Zhou, J. and Cen, K. (2013) Using Wet Microalgae for Direct Biodiesel Production via Microwave Irradiation. *Bioresource Technology*, **131**, 531-535. http://dx.doi.org/10.1016/j.biortech.2013.01.045

[34] Shen, Y., Pei, Z., Yuan, W. and Mao, E. (2009) Effect of Nitrogen and Extraction Method on Algae Lipid Yield. *International Journal of Agricultural and Biological Engineering*, **2**, 51.

[35] Araujo, G.S., Matos, L.J.B.L., Fernandes, J.O., Cartaxo, S.J.M., Gonçalves, L.R.B., Fernandes, F.A.N. and Farias, W.R.L. (2013) Extraction of Lipids from Microalgae by Ultrasound Application: Prospection of the Optimal Extraction Method. *Ultrasonics Sonochemistry*, **20**, 95-98. http://dx.doi.org/10.1016/j.ultsonch.2012.07.027