Comparison of Four Lipid Extraction Methods from Microalgae *Dunaliella Sp.* for Biodiesel Production

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ABSTRACT: The present study attempts to investigate Solvent Extraction (SE), Ultrasonic Assisted Extraction (UAE), Microwave Assisted Extraction (MAE), and acid treatment lipid extraction (ATLE) methods for the extraction of oil from Dunaliella Sp. In doing so, the results showed that UAE, MAE, and ATLE led to an increase in lipid extraction compared to SE. The extracted lipid using MAE (with 17.83 % extraction efficiency) was significantly higher than UAE (14.5 %) and SE (9.16 %) methods. However, considering the energy aspects, ATLE method (17.06%) by omitting the algal biomass drying process and directly extracting lipid from wet biomass could be introduced as an effective method for lipid extraction and biodiesel production process from Dunaliella Sp cells. The fatty acid profile of extracted oil from different methods showed that the extraction method has not a significant effect on the fatty acid composition of the oil. Also, the produced biodiesel properties were according to ASTM standards.

KEYWORDS: Microalgae; Oil extraction; Ultrasonic; Microwave; Acid treatment; Efficiency.

INTRODUCTION
Microalgae are unicellular photosynthetic microorganisms, living in saline or fresh water and they make algal biomass using sunlight, water, and carbon dioxide [1]. In addition, they are useful in bioremediation applications and also used as nitrogen fixing biofertilizers. Microalgae can provide several different types of renewable biofuels. These include methane produced by anaerobic digestion of the algal biomass; biodiesel derived from microalgal oil, and photobiologically produced biohydrogen [2]. In the industry, microalgae have been used as source for a wide variety of food supplements, pharmacological substances, lipids, polymers, toxins, pigments, enzymes, biomass, wastewater treatment, and “green energy”. They are also important in aquaculture as a source of nutrients [3], production of oxygen, consumption of carbon dioxide, and nitrogen-based compounds [4-5].

During recent years, biodiesel production has gained extensive attention due to the depletion of fossil fuel [6].
Microalgae are one of the potential feedstock for production of fuel, because it has high growth rate and it does not compete with food crops [7]. However, the commercial production of biodiesel from microalgae is still challenging due to several technical and economic issues [8]. Oil extraction is one of the most challenging steps in biodiesel production [9]. The studies on life cycle assessment show that 90% of energy is consumed in oil extraction process [10]. Therefore, the economic viability of the process needs to be improved. The methods proposed by Folch et al. [11] and Bligh and Dyer [12] have been used for extraction of oil from microalgae [13, 14]. In these methods, specific composition of solvents is required in multiple steps. Moreover, the composition of solvents, temperature and duration in both methods must be optimized for each species of microalgae. In this regard, microwave and Ultrasonic pretreatments have been used to reduce energy consumption and increasing the extraction efficiency [15 -17]. In ultrasonic Assisted Extraction (UAE) method, the high frequency mechanical agitation releases oil from cells [18] by disrupting the cell walls and facilitating solvent access to the cell content. Microwave Assisted Extraction (MAE) process include the actions of heating whole sample simultaneously and disrupting weak hydrogen bonds by rotation of molecular dipoles which leads to the penetration of solvents into cells and increase in extraction efficiency [19]. The efficient sonication amplitude in (UAE) and microwave power in (MAE) processes as well as treatment duration depends upon the microalgae structure and species [16, 17].

In some extraction methods, microalgae biomass should be dried before extraction. In this respect, drying is considered as an energy consuming step which raises the cost of produced biodiesel. However, Acid Treatment Lipid Extraction (ATLE) is an alternative method for omitting the drying cost and saving energy. The difference between ATLE process and solvent extraction is in the presence of water. Furthermore, in the process of solvent extraction, the pigments are extracted which by itself leads to reduction in biodiesel quality [20].

The supercritical fluid technology and supercritical CO₂ extraction are other methods for wet algal oil extraction under high pressure and high temperature [21-23]. Although the results of research show that extraction efficiency increases slightly through the application of these methods, high cost of implementation has limited this process to lab scale which is costly to scale up and operate [22].

Microalgae Dunaliella sp. is a very strong and tolerant species in harsh condition (contamination, low and high temperature, pH and salinity variations) compared with fresh water species [24]. In this regard, it can be easily cultured outdoor under low maintenance and conservation costs. Therefore, it could be considered as one of the best feedstock for production of fuels and bio crude oil. However, its oil content is less than 20% (w/w dry basis) and the saline nature of its medium inhibits the effective oil extraction.

Despite the various advantages of Dunaliella sp. for fuel production, no efficient oil extraction method was found in the literature review. Therefore, this study intended to introduce an efficient extraction process for these species of microalgae to produce biodiesel. To this end, this study attempted to investigate the efficiency of following oil extraction methods to produce biodiesel which is economically viable: Solvent Extraction (SE), Ultrasonic Assisted Extraction (UAE), Microwave Assisted Extraction (MAE) and Acid Treatment Lipid Extraction (ATLE).

**EXPERIMENTAL SECTION**

**Microalgae cultivation**

Dunaliella sp. M1 isolate was obtained from microalgae collection of branch for Northwest and West regions of Agriculture Biotechnology Research Institute of Iran. The strain was incubated in sterile jars (Fig.1a) in modified Johansson culture. The optimum temperature for Dunaliella is 26°C [13, 21] and the light intensity for microalgae growth ranges from 2500 ~ 5000 lux. In doing so, the light was supplied by fluorescent lamps and light intensity was set to 4000 lux by adjusting the distance between the lamps and medium. The aerating and mixing of culture was carried out by means of air pump (Hailea model 420 China). The appropriate pH for microalgae culture ranges from 7~7.5[14]. In this respect, the suitable pH was controlled by injecting CO₂ to the medium and it was kept around 7.5. Then, after 8 days and during logarithmic phase, the culture scaled up to 20 liters in the transparent PET tanks (Fig.1b). Finally, the culture scaled up to 300 liters in an open pond photo bioreactor (Fig.2).

**Microalgae harvesting**

Microalgae were harvested by electroflucculation method [25]. In this regard, microalgae culture was
transferred from pond to the electroflucculation tank at optical density of higher than 1.000. Then, an overhead mixer was used for mixing the medium (200 rpm). Two aluminum electrodes were placed in the medium and Dazheng ps-305D DC power supply made in China was used for coagulation of cells (Fig.3a). The voltage was set to 5.00 V and the current reached up to 1.00 A. During 30 min the microalgae cells were coagulated and moved to top of the medium. The coagulated microalgae were separated from the top surface of water (Fig.3b), dried in an oven (60°C) and then desalinated by de-ionized water. Afterward, the obtained biomass were dried again in 60°C and kept in 4°C refrigerator for experiments (Fig.3c).

**Solvent Extraction (SE)**

The modified Bligh and Dyer method was used for extraction of lipid from algal cells [12]. Based on this, 10.00 g of dry algal biomass were blended with 100.0 mL of methanol, 50.0 mL of chloroform and 40.0 mL of de-ionized water. The slurry was stirred for 2 h at 800 rpm with magnetic stirrer at room temperature. After that, 50.0 mL chloroform was added to the mixture and blended for 1 min. Then, the suspension was centrifuged (3500×g, 10 min). In this regard, the top layer (methanol/water) was discarded, the bottom layer (chloroform) was separated and collected, the middle layer (residual algal biomass) was retreated as described above and finally the bottom layer was collected. The residual algal biomass was extracted for third time and the collected chloroform layer derived from third step was combined with previous recovered chloroform. In the end, the whole extract was evaporated in the preweighed glass dish for 24h at room temperature in the fume hood. The lipid content was calculated as a weight percent of dry biomass as follows:

\[
L \left( \frac{w}{w} \right) \left( \% \right) = \frac{A}{M} \times 100
\]  

Where \( L \) is the percentage (% w/w) of lipid in the dry biomass, \( A \) (g) is the weight of extracted lipid and \( M \) (g) is the weight of dry biomass used in the extraction.

**Ultrasound assisted extraction (UAE)**

The Hielscher UP200S series ultrasonic device (200 Watts 24 kHz Germany) was used for pre-treatment of samples. The sonication amplitude was adjusted at two levels (50 and 100 percent) for tree time duration 3, 5 and 7 minutes exposure time [18]. For each test, the prepared
slurry using modified Bligh and Dyer method (10.00 g of algal biomass) was treated in 300 mL beakers. The temperature was kept around 90˚C by placing the beakers in the water bath with thermostat cooling system. After ultrasonic pre-treatment, the lipid extraction process was carried out according to procedure described in solvent extraction section.

**Microwave Assisted Extraction (MAE)**

The home appliance Feller microwave 3092 FS series (2.45 GHz, 900 W China) was used for pre-treatment of microalgae biomass samples. Treatment carried out at 5 levels exposure time (2, 4, 6, 8 and 10 minutes) [19]. For each test, 100 mL of sample was treated in 300 mL beakers. The temperature of samples was kept around 90˚C using ice bath while treatment. After microwave pre-treatment, the lipid extraction process was carried out according to procedure described in solvent extraction section.

**Acid Treatment Lipid Extraction (ATLE)**

To conduct the ATLE process, 10 g dry mass equivalent sample of wet algal biomass (80 % moisture content) was blended with 100 mL of 1 M sulfuric acid solution. The mixture was stirred and heated up to 90˚C for 30 min. This condition hydrolyzed complex algal lipids to free fatty acids. Afterward, 100 mL of 5 M sodium hydroxide solution was added to the mixture and heated again at 90˚C for 30 min. The sodium hydroxide made the mixture basic and transformed free fatty acids to salt forms and saponified remaining complex lipids. Then, the mixture was cooled and centrifuged to separate residual biomass. 3 mL of 0.5 M sulfuric acid solution was added to the collected supernatant in order to form solid precipitate. Then, decreasing the pH below seven allows the salts of free fatty acids to revert back to free fatty acid form and produce a solid precipitate. The solid-liquid suspension was centrifuged and the precipitated solid was collected and mixed with 5 mL of hexane. The mixture container was sealed, stirred and heated to 90˚C for 15 min. The presence of hexane led to the separation of lipids from solid phase. After that, the solution was cooled and centrifuged. In that case, the hexane phase was collected and heated gently under fume hood to vaporize the hexane. In this regard, the lipid content was calculated as a weight percent of dry biomass according to Equation (1).

**Electricity energy consumption**

For each method, the total electricity energy consumption for the extracted lipid was measured by Watt Meter K2000 series made in China for whole the process. Then this amount was scaled and calculated for 1 liter in each method.

**Statistical analysis**

All experiments were performed in triplicate. Analysis of variance (ANOVA) was performed by Minitab for Windows, version 16. Duncan test was applied to compare significant differences among the treatments (p<0.05).

**RESULTS AND DISCUSSION**

**Effect of UAE on lipid extraction yield**

The selection of solvent system for oil extraction from microalgal biomass is an important factor which would
allow cost-effective oil extraction without further expenses required for purification of the product. The extraction of lipids from microalgae is basically a mass transfer operation which depends on the nature of solute and solvent, selectivity of solvent and the level of convection in medium. Cracking of cell walls by mechanical methods would increase the accessibility of lipids for solvents. In UAE method, the disruption of cell walls causes the penetration of solvents into the cells and increase in the extraction efficiency. Fig. 4 shows the effect of sonication amplitude and time duration on lipid extraction yield from *Dnalliella* Sp. cells. As it is shown in the graph, the extracted lipid from cells is increased by raising the sonication amplitude from 50 to 100 % (p < 0.05). At both amplitudes the extraction yield increases by exposure time but significant variation was not observed from 7 to 9 min. It seems that the sonication ability at both amplitudes is limit and longer duration has no effect on increasing extraction efficiency because of microalgae structure. In the same vein, *Metherel et al.* (2009) showed that an increase in amplitude of exposure time can result in higher lipid recoveries [26].

**Effect of MAE on lipid extraction yield**

Fig. 5 shows the effect of microwave radiations on extraction yield. It is obvious that the extraction yield increases by exposure time from 2 to 8 min (p < 0.05). According to statistical analysis at durations longer than 6 min significant effect was not observed. *Balasubramanian et al.* (2011) demonstrated that the higher temperatures at longer times resulted in higher oil extraction efficiency when compared with standard methods like SE [27]. The extracted lipid using MAE was significantly higher than UAE method which means that the rupturing of cells in MAE was effective in comparison to UAE for 6 minutes treatment duration. But the power consumption in MAE was higher than UAE.

**Comparison of UAE, MAE, ATLE and SE.**

The comparison of four extraction methods efficiency was shown in Fig. 6. It seems that 6 minutes treatment of samples with 2.45 GHz microwave radiations has the best yield among other methods (p < 0.05). ATLE and UAE with 17.06 and 14.5 % were at second and third order respectively. UAE can reduce extraction time; facilitate mass transfer and penetration of solvent into the cells. This method is efficient due to low scale up cost and fast operation time. The present study shows that MAE reduces the extraction time and is more effective than UAE. Furthermore, in MAE, the contact between polar material and oscillating electric field generates heat as a result of frictional forces between inter- and intra-molecules. Therefore, water vapor begins to be formed inside the cell which eventually ruptures it and opens up the cell membrane and releases the intra-cellular content [27,28]. Similarly, *Pohndorf et al.* (2016) reported that microwave improves lipid extraction compared to non-disruption or autoclaving method [29]. In this respect, *Yoo et al.* (2012) evaluated lipid recovery from wet biomass of *Chlamydomonas reinhardtii* by wet extraction method and polar and non-polar organic solvents [30]. The results of their study suggested that, wet extraction could increase...
Table 1: Fatty acid composition of extracted lipid from microalgae by UAE, MAE, ATLE and SE methods.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Extraction method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UAE</td>
</tr>
<tr>
<td>Myristic Acid</td>
<td>C14:0</td>
</tr>
<tr>
<td>Palmitic Acid</td>
<td>C16:0</td>
</tr>
<tr>
<td>Palmitoleic Acid</td>
<td>C16:1</td>
</tr>
<tr>
<td>Stearic Acid</td>
<td>C18:0</td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td>C18:2</td>
</tr>
<tr>
<td>Linolenic Acid</td>
<td>C18:3</td>
</tr>
<tr>
<td>Arachidic Acid</td>
<td>C20:0</td>
</tr>
<tr>
<td>Other</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6: Comparison of lipid extraction efficiency by UAE, MAE, ATLE and SE methods.

lipid recovery approximately twice. Despite being a simple method for cell disruption, ATLE is not widely employed because it depends highly on cell wall properties [31, 32]. For *Dunaliella* *Sp.*, the ATLE method was effective and the efficiency was close to MAE method.

**Electrical energy consumption**

The electrical energy consumption in each method was calculated per liter and shown in Fig. 7. Considering the energy aspects, ATLE with lowest energy consumption could be introduced as an efficient and cost-effective method for extraction of lipids from *Dunaliella* *Sp.* cells. Due to the omission of drying step in this method, the energy consumption is significantly lower than UAE, MAE and SE methods. *Roux et al.* also has shown that wet route skipping drying step is the only way to produce viable microalgae based biofuels considering scalability, improve lipid accessibility and energy consumption [32].

Fig. 7: Comparison of energy consumption for UAE, MAE, ATLE and SE methods.

**Fatty acid composition of extracted oil**

Table 1 shows the fatty acid composition of extracted lipid from microalgae by UAE, MAE, ATLE and SE methods. According to statistical analysis significant variation was not observed in fatty acid profile using these methods.

**Microalgae biodiesel properties**

The microalgae biodiesel properties have been shown in Table 2. All of the biodiesel properties were according to ASTM standards for biodiesel. Higher flash point improves the safety of transportation and storing. Also high viscosity causes increasing in lubricity of fuel and therefore increasing engine life time.

**CONCLUSIONS**

The microwave and ultrasonic assisted extraction, typical solvent extraction and acid treatment lipid extraction methods have been demonstrated for extraction
of lipids from Dunaliella Sp cells. Among these methods, microwave-assisted extraction had the best extraction yield (17.83%) while in second order, with respect to energy aspect, acid treatment lipid extraction method (17.06%) by omitting the algal biomass drying process and directly extracting lipid from wet biomass could be introduced as one of effective methods for lipid extraction and biodiesel production process from Dunaliella Sp. cells. Fatty acid composition of all extracted oil showed that the extraction method has not a significant effect on fatty acid profile and in all methods Palmitic acid forms the main portion of fatty acid composition.

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