

SCREENING OF LIPID CONTENTS OF MICROALGAE BY OPTIMIZATION OF OIL EXTRACTION PROCESSES

N. Munir, A. Imtiaz, N. Sharif, R. Haq and S. Naz

Department of Biotechnology, Lahore College for Women University, Lahore

Corresponding author e- mail: neelma.munir@yahoo.com

ABSTRACT: Currently microalgae are considered as an efficient biological producer of oil. Present study was conducted to explore the lipid yield percentage of three microalgal strains *Chlorella* sp., *Oedogonium* sp. and *Spirogyra* sp. Bligh and Dyer, Soxhlet, Folch and Microwave assisted extraction methods were employed for oil extraction. It was observed that Bligh and Dyer method with Chloroform-methanol (1:2, v/v) gave maximum extraction yield (15.46 ± 0.240 %) for *Chlorella* sp. whereas Folch method with chloroform-methanol (2:1, v/v) gave more lipid yield as compared to Bligh and Dyer method i.e 5.53 ± 0.240 % for *Spirogyra* sp., 13.67 ± 0.240 % and 19.69 ± 0.667 % for *Oedogonium* sp. and *Chlorella* sp. respectively. Soxhlet extraction resulted in less (14.5 ± 0.45) percentage yield as compared to Bligh and Dyer and Folch method (16.7 ± 0.24). Microwave assisted extraction with chloroform- methanol resulted in highest lipid yield (30%) with *Chlorella* sp. indicating that it contained the maximum lipid content among all the three species.

Key words: Algae, Biofuel, *Chlorella* sp., Lipids, *Oedogonium* sp., *Spirogyra* sp.

(Received 20-01-2014 Accepted 01-03-2016)

INTRODUCTION

Algae are capable to produce 10-100 times as abundant mass as terrestrial vegetation yearly (Schneider and Erez, 2006). Microalgae possess the capacity to yield energy-rich oils and a number of microalgae were found to naturally gather high oil content in their total dry weight (Rodolfi *et al.*, 2009). Some *Botryococcus* sp. was identified with 50 percent of their dry mass deposited as long-chain hydro-carbons (Knothe *et al.*, 2005). This is the reason that the microalgae are being considered as potential biofuel plants (Demirbas, 2010). The groups include are green algae, diatoms, golden brown, eustigmatophytes, prymnesiophytes and several cyanobacteria sp. Species from all of these groups have been studied as possible fuel production algal strains (Reijnders, 2011).

Lipids present in algal cells are generally hydrophobic molecules that interact with moderately non-polar solvents such as ethyl ether, chloroform, acetone and benzene. While membrane associated polar lipids need polar solvents such as ethanol and methanol to interrupt the hydrogen bondings and the electrostatic forces between the lipids and proteins (Freedman *et al.*, 1984) Most commonly used laboratory techniques for lipid extraction are the Soxhlet extraction method with hexane as extracting solvent. Folch method (Folch *et al.*, 1957) and Bligh and Dyer method (Bligh and Dyer 1959). Organic solvents such as benzene, cyclohexane, hexane, acetone and chloroform have shown to be effective when used for microalgae. These solvents degrade microalgal

cell wall and extract the oil which has a high solubility in organic solvents (Deng *et al.*, 2009).

An interesting new feature is the development of microwave-assisted extraction (MAE) of different compounds. Microwave methods have been used for extracting oils from various plants (Jocelyn *et al.*, 1994). Microwave assisted extraction procedures can also be important alternatives to formal extraction procedures in algae (Terigar *et al.*, 2010). Regardless of the wide range of oil extraction methods accessible, very little is known about the scalability of the procedures and the economics of their usage and application on large industrial scale (Cooney *et al.*, 2009).

There has been a recent resurgence interest in microalgae as an oil producer for biofuel applications. An adequate supply of nutrients and carbon dioxide enables algae to transform successfully the light energy of the sun into energy rich chemical compounds through photosynthesis. A strain with high lipids, successfully grown and harvested could logically provides the most oil for our process by volume, which in turn would be helpful to get the most profitable output. Significant advances have also been made in upstream processing to generate cellular biomass and oil. However, extracting and purifying oil from algae continues to prove a significant challenge in producing both microalgae bio products and biofuel, as the oil extraction from algae is relatively energy-intensive and expensive (Singh and Singh, 2014). The aim of this study is to assess the lipid contents of three different algal species and analyze the variation in oil yield using different extraction methods.

MATERIALS AND METHODS

Collection and growth of Algae: Algae samples were collected from Lahore College for Women University (LCWU); Jilani Park; Jinnah Garden; Gulshan-e-Iqbal Park; ponds in Punjab University and many other ponds and damp soil places of Lahore. Species were identified based on their morphological characteristics. BG medium and BB medium (Bold, 1949) were used for the algal growth. Orbital shaker (OS 5 KIKA - WERKE) was used for mixing and the flasks were kept over the shaker at 300 rpm for two weeks. Aeration was supplied through aerating pumps (CE – SB – 348A0. These cultures were placed indoor under constant light near a glass window.

Biomass processing: After the complete removal of algal cells by centrifugation from the culture medium, the cells were dried. Filamentous algae were dried by spreading them over blotting paper and placing the material under running fan until all water contents were removed. In case of unicellular algae the cell biomass was collected and dried in oven at 65 °C for 3 hours. All the types of algae were then ground to fine powder using motor and pestle.

Oil extraction

Bligh and Dyer extraction method: Extraction following Bligh and Dyer method was performed as originally outlined (Bligh and Dyer, 1959). Initially 5 g of the sample was used for filamentous as well as unicellular algae. Five grams of sample were mixed in 15 ml of chloroform-methanol (1:2, v/v), and mixture was homogenized and placed over shaker at 170 rpm for 20 min. This mixture was then centrifuged (MERMLE Z 300 K) for 10 minutes at 3000 rpm and was filtered. The residual biomass was re homogenized with 5 ml of chloroform and again centrifuged for 10 min at 3000 rpm. This mixture was filtered and collected together with the previous filtrate; and 5 ml of distilled water was added to this filtrate and shaken vigorously. A final biphasic form was achieved. This biphasic system was allowed to separate by centrifugation (10 minutes, 3000 rpm). The upper aqueous phase was eliminated as it contained only non-lipid material. The lower chloroformic phase was collected where the lipids were present. This material was then kept for drying to evaporate the chloroform and the remaining content was measured.

Folch extraction method: Folch extraction (Folch *et al.*, 1957) was performed by mixing 20 parts chloroform-methanol (2:1, v/v) to 1 part of algal sample. Initially, 5 g of sample was mixed with 100 ml of chloroform-methanol (2:1, v/v). This mixture was agitated for 20 minutes using a shaker. After homogenization the mixture was centrifuged for 10 minutes at 3000 rpm, and filtered. A 5 ml volume of distilled water was added to the filtrate and mixture was shaken. A biphasic form was

established and it was allowed to get separated completely by centrifugation for 10 minutes at 3000 rpm. The upper aqueous phase was removed and the lower chloroformic phase was filtered and dried for the lipid estimation.

Soxhlet extraction method: Soxhlet apparatus (Heidolph HB 4000) was used for the oil extraction from algae using various solvents, such as methanol, ethanol, hexane and chloroform-methanol. For extraction, 15 g of the dried algal biomass was weighed and placed in a thimble paper. Organic solvent (250 ml) was poured into the round bottom flask. Chloroform and methanol were used in equal volumes 1:1. The material was refluxed many times until all the solvent present over the thimble became clear. The extract was removed and poured into a pre-weighed china dish and was kept for drying under fan air. All the three algae samples were extracted with the above mentioned solvents and the extraction yields were compared. The experiments were carried out in triplicate.

Microwave assisted extraction method: Microwave extraction (Pasquet *et al.*, (2011) was performed using domestic microwave oven. The exposure time and power level were used as the variables. For extracting oil 0.1 g of the ground algal sample was used against 5 ml of organic solvent. The sample and solvent were placed inside the microwave oven. The power level and time was adjusted. After microwaving the solvent having the extract was collected with the help of syringe leaving behind the algal residue in the beaker. All of the algae samples were extracted in the same way and methanol, n-hexane, ethyl acetate, acetone:hexane, and chloroform:methanol solvents were used. Time of extraction also varied i.e. 60 sec, 120 sec and 180 sec. Similarly power level was also adjusted at 300, 600 and 900 watts.

Lipid content determination (percentage yield): The oil content was determined by using the following formula (Putri *et al.*, 2011):

Weight of lipid = (weight of container + extracted lipid) – (weight of container)

The contents of lipids in the sample were determined as follows:

Lipid contents (%) = amount of lipids extracted (g)/weight of original sample (g) × 100

Statistical analysis was performed by one-way ANOVA using SPSS software version 14.

RESULTS AND DISCUSSION

Objective of this work was to extract the oil from algae using several extraction methods and to find out the best extraction method. The extraction methods used for oil production from algae included; Bligh and Dyer method, Folch method, Soxhlet extraction method

and Microwave assisted extraction method. Further, the effect of various extraction solvents on percentage yield was also compared. Results obtained by performing Bligh and Dyer extraction (figure 1) with chloroform and methanol gave less oil percentage i.e. $4.93 \pm 0.467\%$ for *Spirogyra* as compared to *Oedogonium* sp. and *Chlorella* sp. was $11.40 \pm 0.306\%$ and $15.46 \pm 0.240\%$ respectively. Similar results were reported by Hossain *et al.*, (2008) and Kholá and Ghazala (2012) who reported that biodiesel production was maximum in *Oedogonium* sp. and minimum in *Spirogyra* sp.. Bligh and Dyer method was used by Woertz *et al.*, (2009) and their results were in agreement with our findings. The total lipid contents were found to be 14% for *Oedogonium* sp. and 24% in *Chlorella* sp.

Folch method was used to compare the extraction yield among all types of above mentioned algae. The results given in figure 2 show that the percentage yield for lipid was much higher with Folch extraction method as compared to Bligh and Dyer method. *Spirogyra* sp. could give $5.53 \pm 0.240\%$ yield of lipids when extracted with 100 ml of chloroform-methanol (2:1, v/v). *Oedogonium* sp. was found to have higher lipid contents than *Spirogyra* sp. which was in accordance to the findings of Kholá and Ghazala (2012) which was calculated as $11.67 \pm 0.240\%$. The maximum yield ($17.69 \pm 0.667\%$) was obtained from *Chlorella* sp. The results from Bligh and Dyer extraction and Folch extraction method were compared and it was found that using the same solvents (Chloroform-methanol) with different volumes could give different lipids percentage (Figure 3). These results are highly in accordance with those depicted by (Palacios *et al.*, 2008). They have studied both the methods for determining the maximum %age yield of lipids. Almost all the products which they used in their work gave the maximum amount of lipids by Folch method. Iverson *et al.*, (2002) studied the total lipid content of algal species using Folch method, revealing that biological samples could be extracted for maximum lipids by this method.

Soxhlet extraction method was conducted to extract the lipids from algae using various solvents. Solvents used were methanol, ethanol, hexane and chloroform-methanol (1:1). All the three algal samples were extracted and the results obtained were compared for the percentage oil extraction. Methanol was used as extraction solvent in first experiment and the lipids percentage for *Spirogyra* sp. was calculated as 6.04 ± 0.083 while the percentage yield of *Oedogonium* sp. was $6.40 \pm 0.233\%$ which was slightly greater than *Spirogyra* sp. (Figure 4). During their work Ewald *et al.*, (1998) also reported the soxhlet extraction as an important method for lipid extraction. LeBlanc, (2011) has compared various solvents used for extracting algal biomass using Folch method and reported that chloroform and methanol are effective solvents. Likewise

the results obtained during present study also indicate that better lipid yield was obtained when chloroform and methanol were used in combination as compared to methanol alone.

MAE was performed using various solvents and then the yield was calculated. Microwave energy exposure time and power levels were the main factors which influenced the extraction efficiency. Algae were got extracted through microwave energy by applying various solvents. Alcohols like methanol and ethanol were used, similarly n-hexane, ethyl acetate, acetone-hexane and chloroform-methanol were also tested for better oil recovery from algae (Gude *et al.*, 2013). Initially 0.1 g of dried algal sample was used and extracted with 5 ml of organic solvent. All the extraction experiments were performed in randomized conditions. Three power levels (300 W, 600 W and 900 W) and different time intervals i.e. 60s, 120s and 180s were tested during the present study. The results have depicted that power level and exposure time had a significant effect on percentage yield of oil from algae. Using methanol as extracting solvent, power level of 300 W with time interval of 60, 120 and 180sec the percentage yield was obtained as 10.0 ± 1.571 . By increasing the power level and time of exposure the yield was also increased and it was calculated as $15.0 \pm 1.757\%$ with power level of 900 W and time of 120s. Maximum yield was obtained when time was further increased and at 900 W with 180s the yield was recorded as $20.0 \pm 1.751\%$ (figure 3).

Another solvent used for extraction was n-hexane and the results were same as methanol except for 600 W and the percentage yield was recorded as $20.0 \pm 1.571\%$ for all the time intervals. Chloroform and methanol were used in equal amount and at low power (300 W) the percentage yield was obtained as $10.0 \pm 1.667\%$ with all the time intervals. As the power level was doubled to 600 W the lipid yield was also doubled and obtained as $20.0 \pm 1.667\%$, this was continued with power level of 900 W. Thus maximum yield was obtained at both 600 W & 900 W with all the time intervals. Acetone and hexane (1:1) were tested together and the lipid percentage was recorded as 10.0 ± 0.000 with all power levels i.e 300 W, 600 W and 900 W with 69s, 120s and 180s as exposure time respectively. Statistical analysis revealed a non-significant effect of acetone: hexane. In contrast, ethyl acetate gave different a percentage yield with different power and time intervals. The lipid % age yield of *Spirogyra* sp. with 300 W was $20.0 \pm 1.571\%$, it was increased till $20.0 \pm 1.571\%$ with power level 600 W at a exposure time of 120s and 180s. But a decline in lipid yield was recorded when power level was further increased to 900 W and it gradually decreased to $16.67 \pm 1.571\%$, $13.33 \pm 1.571\%$ and $10.0 \pm 1.571\%$ with increased time 60, 120 and 180seconds.

Oedogonium sp. gave more lipid percentage yield when extracted with microwave technique using various solvents. Methanol gave yield up to 20% with power level of 600 W as well as 900 W. Yield was less when *Oedogonium* sp. was extracted with n-hexane and the lipid percentage yield was recorded as $10.0 \pm 0.000\%$ with all the three power levels and time intervals. Chloroform was used in combination with methanol in equal amounts and the solvent had significant effect on percentage yield at high power levels. At power of 300 and 600 W lipid percentage yield was calculated as $10.0 \pm 0.000\%$ with all the time intervals while the yield was doubled when power level was increased ($20.0 \pm 0.000\%$) to 900 (Figure 4). Acetone and hexane were used in equal volumes (1:1) and the yield was almost equal to obtained by chloroform:methanol. Similar effect of ethyl acetate was observed on extraction of lipids from *Oedogonium* sp. thus it was evident from results that power level and time play a significant role along-with the organic solvent. A power of 900 W was found to be good for microwave assisted oil extraction.

Chlorella sp. was observed to have high oil percentage when compared with other two algal species. Solvents used showed a significant effect on percentage yield e.g methanol was able to extract $20.0 \pm 0.000\%$ of oil with 300 W as well as with 600 W and by increasing the power to 900 W the yield was increased upto $30.0 \pm 0.000\%$. This was the maximum percentage yield obtained so far from all the algal samples, under present study by applying all the extraction methods. The yield recorded with n-hexane was not as high as it was with methanol but each power level showed a different effect on extraction and %age yield obtained at 300 W was $10.0 \pm 0.000\%$, at 600 W it was recorded as $20.0 \pm 0.000\%$ and hence with 900W and less exposure time the yield was $20.0 \pm 0.000\%$. it was evident that an increase in time period decreased the percentage yield i.e. $10.0 \pm 0.000\%$ with 120s and 180s using Chloroform-methanol in MAE,

the percentage yield of *Chlorella* sp. was as high as $30.0 \pm 0.000\%$ at power 600 W and 900 W. Similarly acetone: hexane have a significant effect and with low power the yield was also less as $10.0 \pm 0.000\%$, an increase in power have resulted increase in percentage yield. Thus at 600 W it was recorded as $20.0 \pm 0.000\%$ while at 900 W a further increase was recorded and yield became $30.0 \pm 0.000\%$ (Figure 5). Ryckebosch *et al.*, (2011) studied the effect of solvent mixtures and their ratios i.e dichloromethane–ethanol (1:1), chloroform–methanol (1:1), hexane–isopropanol (3:2), chloroform–methanol (2:1), acetone–hexane (1:1) on total lipid extraction from *Chlorella* sp. All these solvent were used to extract lipids from microalgae via MAE. Maximum lipid was extracted by chloroform–methanol (1:1) which was in agreement with our revealed findings. Similarly, Pasquet *et al.*, (2011) studied algal biomass for MAE. Two different *Chlorella* sp. were compared for lipid extraction by Singh and Singh, (2014). Acetone and chloroform-methanol (1:1) were used and maximum percent of lipids was found with chloroform-methanol using 800 W.

Chlorella sp. being rich in lipid content gave the maximum oil yield $9.43 \pm 0.145\%$. All the other solvents were compared in the same way and it was found that the lipid content of *Spirogyra* sp. was lesser than *Oedogonium* sp. which was lesser than *Chlorella* sp. Among the four solvents used, hexane was found to give lowest extraction yield i.e. 2.80 ± 0.069 , $3.47 \pm 0.202\%$ and $7.63 \pm 0.088\%$ for *Spirogyra* sp. *Oedogonium* sp. & *Chlorella* sp. respectively. Chloroform-methanol being the most suitable solvents for soxhlet extraction gave the maximum oil yield and it was $5.21 \pm 0.088\%$, $7.47 \pm 0.176\%$ and $14.0 \pm 0.642\%$ for *Spirogyra* sp., *Oedogonium* sp. and *Chlorella* sp. respectively. Hence our results indicated a significant role of solvents when used for extraction of algae.

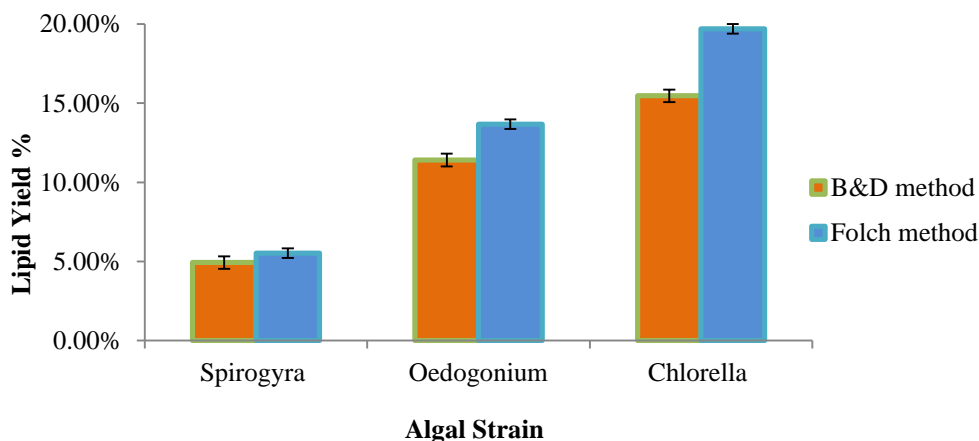


Figure 1. The comparison between B&D and Folch method for lipid extraction from algae

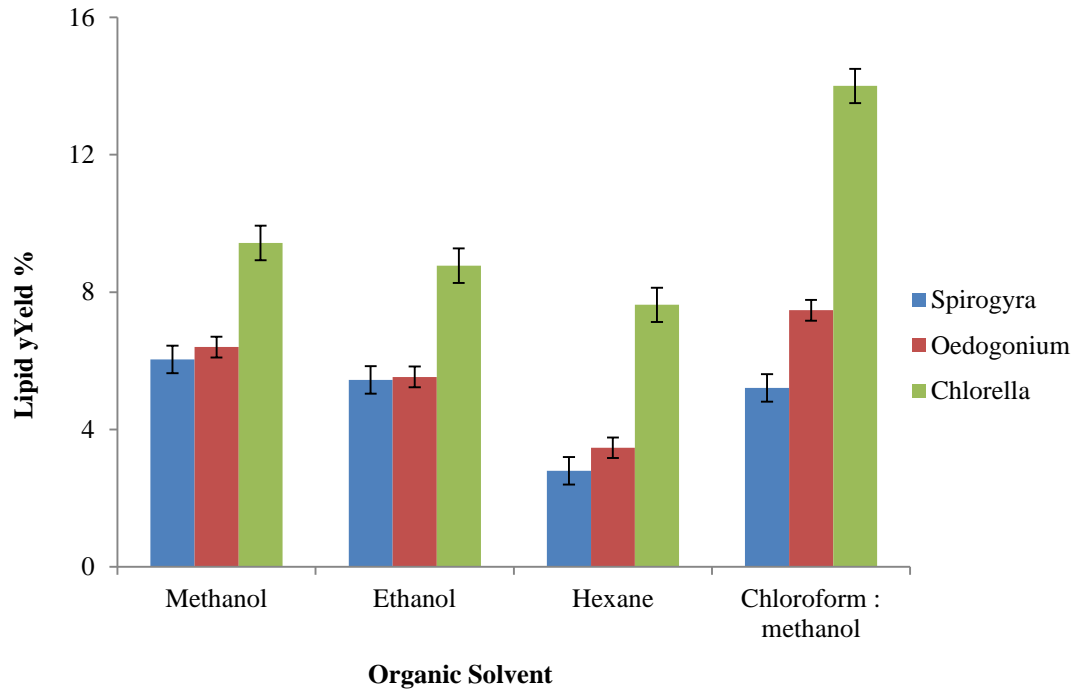


Figure 2. The comparison of various solvents used with soxhlet extraction of algae.

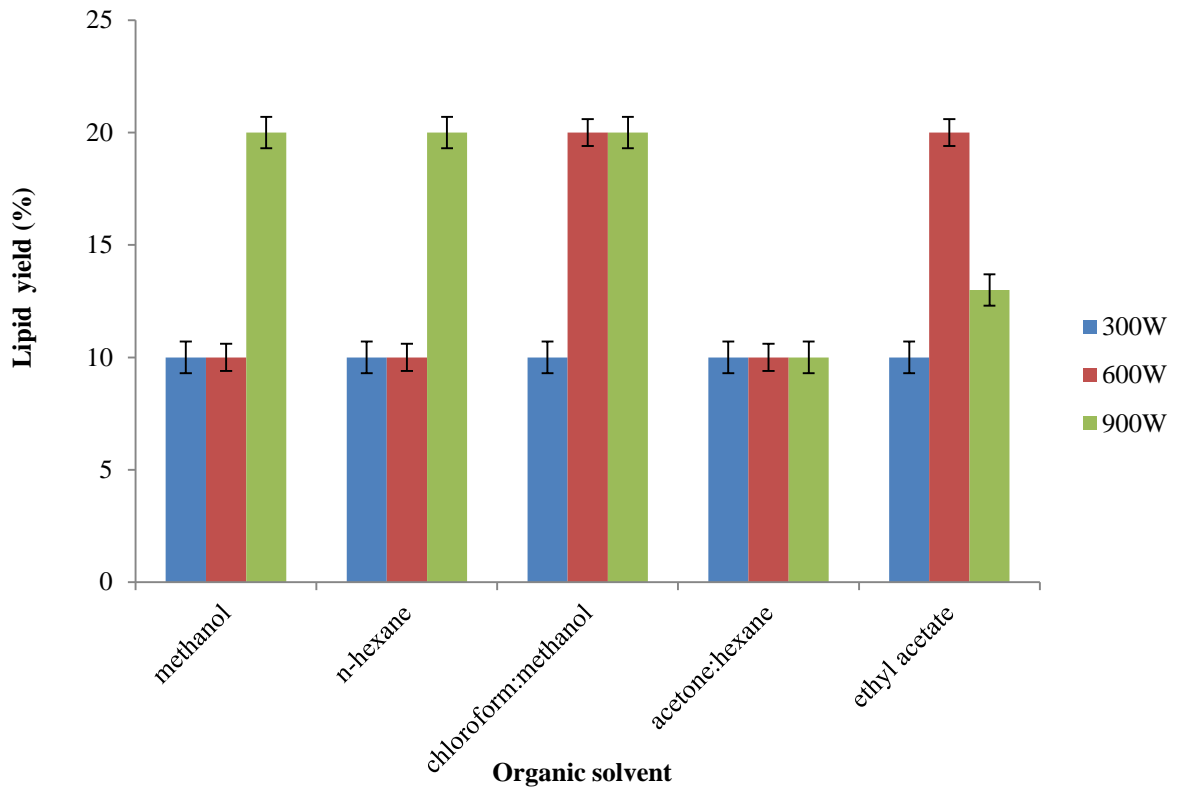


Figure 3. The oil %age yield from Spirogyra sp. with MAE using various solvents.

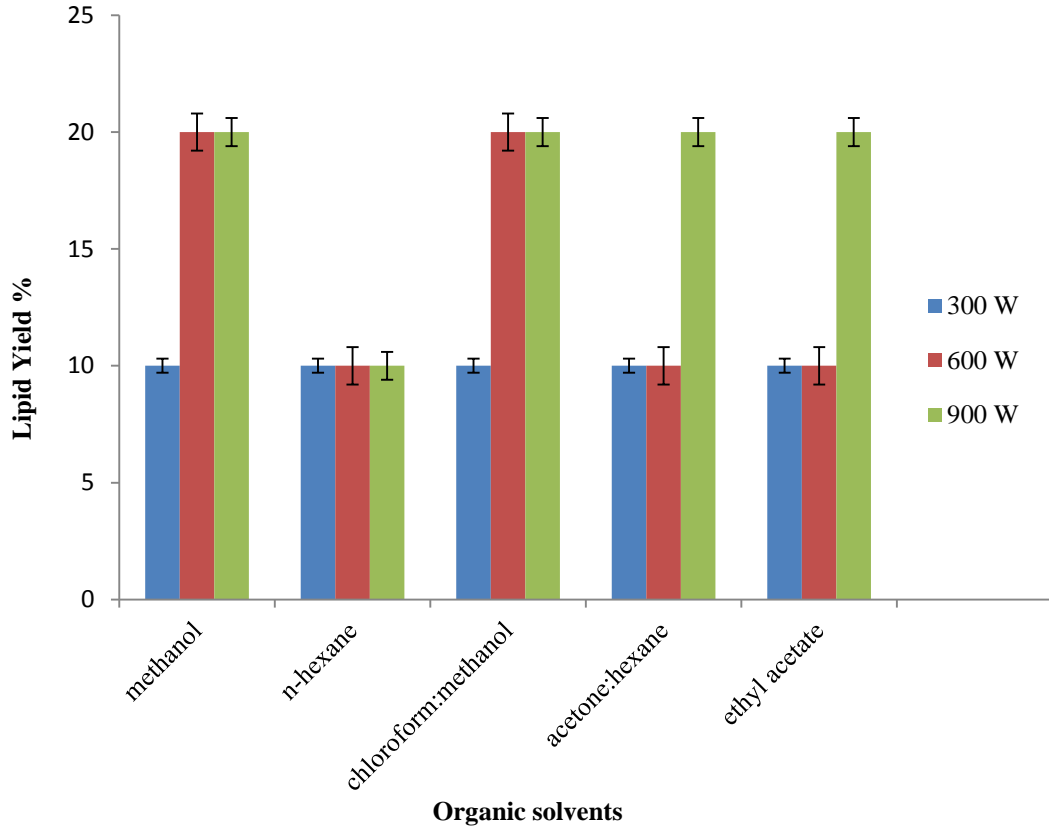


Figure 4. The oil %age yield from *Oedogonium sp.* with MAE using various solvents

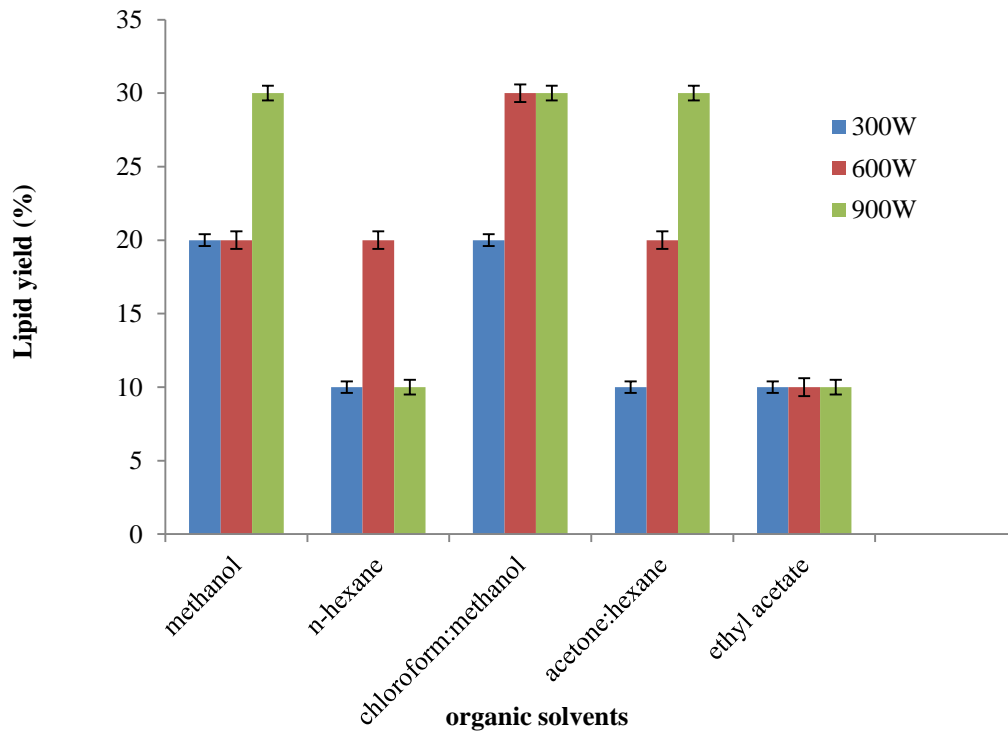


Figure 5. The oil %age yield from *Chlorella sp.* with MAE using various solvents.

Conclusion: Current study revealed that the highest oil content were found in *Chlorella* sp. i.e 30% achieved during microwave extraction method using chloroform and methanol as solvents. Hence it was concluded that among the three algal species studied *Chlorella* sp. had maximum oil content.

REFERENCES

- Bligh, E. and W.A. Dyer (1959). A rapid method for the total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37: 911 - 917.
- Bold, H.C. (1949). The morphology of *Chlamydomonas chlamydogama* sp. *Bull. Torrey. Bot. Club.* 76: 101-108.
- Cooney, M., G. Young, and N. Nagle (2009). Extraction of bio- oils from microalgae. *Sep. Purif. Rev.* 38: 291-325.
- Demirbas, A. (2010). Use of algae as biofuel sources. *Energy Convers. Manage.* 51: 2738 - 2749.
- Deng, X., Y. Li, and X. Fei (2009). Microalgae: a promising feedstock for biodiesel. *Afric. J. Microbiol. Res.* 3: 1008-1014.
- Ewald, G., G. Bremle, and A. Karlsson (1998). Differences between Bligh and Dyer and Soxhlet extractions of PCBs and lipids from fat and lean fish muscle: Implications for data evaluation. *Marin. poll. bull.* 36: 222-230.
- Folch, J., M. Lees, and G. S.Stanley (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. chem.* 226: 497-509.
- Freedman, B., E. Pryde, and T. Mounts (1984). Variables affecting the yields of fatty esters from transesterified vegetable oils. *J. Amer. Oil. Chemist. Soc.* 61: 1638-1643.
- Gude, V.G., P. Patil, E. Martinez-Guerra, S.Deng, and N. Nirmalakhandan (2013). Microwave energy potential for biodiesel production. *Sustain. Chem. Proces.* 1: 5.
- Hossain, A., A. Salleh, A.N. Boyce, P. Chowdhury, and M. Naqiuddin (2008). Biodiesel fuel production from algae as renewable energy. *Am. J. Biochem. Biotech.* 4: 250-254.
- Iverson, S.J., K.J. Frost, and S. Lang (2002). Fat content and fatty acid composition of forage fish and invertebrates in Prince William Sound, Alaska: factors contributing to among and within species variability. *Mar. Ecol. Prog. Ser.* 241: 161-181.
- Jocelyn Paré, J., J.M. Bélanger, and S.S. Stafford (1994). Microwave-assisted process (MAP™): a new tool for the analytical laboratory. *TrAC.* 13: 176-184.
- Khola, G. and B. Ghazala (2012). Biodiesel production from algae. *Pak. J. Bot.* 44: 379-381.
- Knothe, G.J., V.J. Krahl, and Gerpen (2005). Biodiesel processing and production. *Fuel. Proc. Technol.* 86: 1097-1107.
- Kumari, P., M. Kumar, V. Gupta, C. Reddy, and B. Jha (2010). Tropical marine macroalgae as potential sources of nutritionally important PUFAs. *Food. Chem.* 120: 749-757.
- LeBlanc, O. (2011). Comparison of different solvents used in microalgae biomass extraction. *Appl. Biochem. Biotechnol.* 24: 355-361.
- Palacios, G., J. Druce, L. Du, T. Tran, C. Birch, T. Briese, S. Conlan, P.L. Quan, J. Hui, and J. Marshall (2008). A new arenavirus in a cluster of fatal transplant-associated diseases. *New. Eng. J. med.* 358: 991-998.
- Pasquet, V., Chérouvrier, J.-R., Farhat, F., Thiéry, V., Piot, J.-M., Bérard, J.-B., Kaas, R., B. Serive, T. Patrice, and J.P. Cadoret (2011). Study on the microalgal pigments extraction process: Performance of microwave assisted extraction. *Pro. Biochem.* 46: 59-67.
- Putri, E.V., Din, M.F.M., Ahmed, Z., Jamaluddin, H. and S. Chelliapan (2011). Investigation of microalgae for high lipid content using palm oil mill effluent (POME) as carbon source: *Int. Con. Environ. Ind. Innov.* 12: 85-89.
- Reijnders, L. (2011). Health Council of the Netherlands follows the ecological trail: a new venture. *Ned. Tijdschr. Geneesk.* 155: A3784.
- Rodolfi, L., G.C. Zittelli, N. Bassi, G. Padovani, N. Biondi, G. Bonini, and M. R. Tredici (2009). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnol. Bioeng.* 102: 100.
- Ryckebosch, E., M. Drouillon, and H. Vervaeren, (2011). Techniques for transformation of biogas to biomethane. *Biomass. Bioene.* 35: 1633-1645.
- Schneider, K. and J. Erez (2006). The effect of carbonate chemistry on calcification and photosynthesis in the hermatypic coral *Acropora eurytoma*. *Limnol. Oceanogr.* 51: 1284 - 1293.
- Singh, S. and P. Singh (2014). Effect of CO₂ concentration on algal growth: A review. *Renew. Sus. En. Rev.* 38: 172-179.
- Terigar, B., S. Balasubramanian, D. Boldor, Z. Xu, M. Lima, and C. Sabliov (2010). Continuous microwave-assisted isoflavone extraction system: design and performance evaluation. *Bioresour. Technol.* 101: 2466-2471.
- Woertz, I., A. Feffer, T. Lundquist, and Y. Nelson (2009). Algae grown on dairy and municipal wastewater for simultaneous nutrient removal and lipid production for biofuel feedstock. *J. Environ. Eng.* 135: 1115-1122.