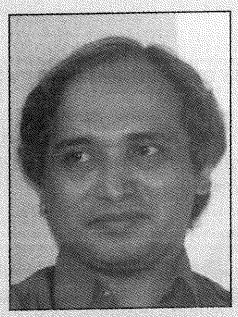
Biofuel Prospects & Phytoremediation Potential of Fresh Water Duckweed - Lemna minor



T.V. Ramachandra
Energy & Wetlands Research Group, Center for Ecological Sciences [CES], Centre for Sustainable Technologies (astra), Centre for infrastructure, Sustainable Transportation and Urban Planning [CiSTUP], Indian Institute of Science, Bangalore

Shri T. V. Ramachandra is a faculty at Centre for Ecological Sciences (CES) and Associate Faculty at Centre for Sustainable Technologies (astra) and Centre for infrastructure, Sustainable Transportation and Urban Planning (CiSTUP), Indian Institute of Science. His areas of research include energy systems, energy planning, renewable energy, aquatic ecosystems, ecological modelling, environmental management, regional planning, spatial decision support systems, GIS and remote sensing. He teaches principles of remote sensing, digital image processing, environmental and natural resources management. He has published over 238 research papers, 48 book chapters as well as fourteen books. He is a fellow of the Institution of Engineers (India) and Institution of Electrical Engineers (UK), Senior member, IEEE (USA) and AEE (USA), and many similar institutions. Details of his research and copies of publications are available at http://ces.iisc.ernet.in/energy

Saranya G
Energy & Wetlands Research Group, CES, (astra), Indian Institute of Science, Bangalore

Saranya G is currently pursuing her PhD at the Centre for Sustainable Technologies and associated with Energy and Wetland Research Group, Centre for Ecological Sciences, Indian Institute of Science - Bangalore. Her research interests include renewable bioenergy generation and development of sustainable and alternative energy technologies. She is currently working on biofuel prospects of estuarine diatoms.

Abstract

Energy security concerns with over exploitation of fossil fuels and increased Green House Gases (GHG's) emissions have necessitated exploration of technically feasible, economically viable and socially acceptable alternative energy sources. Biomass based fuels have emerged as viable renewable energy sources and are the potential alternatives to fossil based fuels in recent times. Biofuel feedstocks are quite diverse. Based on the type of feedstocks used, the biofuels are categorized as first, second and third generation. Food crops derived biofuels are first generation, wherein biofuels from macrophytes, lignocellulosic crops fall under second generation and algae (both micro/macroalgae) derived biofuels comprise of third generation. Duckweed, one of the smallest flowering macrophytes, usually called as an aquatic weed and this feedstock was assessed for its bioethanol production potential. These duckweeds usually proliferates in stratified water bodies especially during summer. In this study, duckweed Lemna minor that proliferated excessively in the centenary pond located inside Indian Institute of Science campus Bangalore was collected and investigated for its biochemical composition and bioethanol production. The duckweed samples were collected twice during the sampling period and the variations in biochemical composition and bioethanol yield was estimated and quantified. Bioethanol obtained from two samples collected at two different time periods ranges from 0.821 g/L - 1.02 g/L.

1. Introduction

Greenhouse gas (GHG) level in the atmosphere is increasing with urbanisation and industrialization and estimates reveal that about three-quarters anthropogenic carbon-dioxide are due to burning of fossil fuels. Fast dwindling stocks of fossil fuels and changes in the climate with enhanced GHG emissions have necessitated the exploration of cost effective sustainable energy sources. Estimates reveal that fossil fuels such as oil, coal and gas would deplete in 35, 107 and 37 years respectively, highlighting the impending energy crisis and the escalation of fuel prices with the dwindling of natural resources stock. Apart from this, nutrient accumulations in ecosystems due to indiscriminate disposal of liquid wastes have further enhanced GHG levels. Energy demand has increased with the

spurt in economic activities, bringing along a change in the consumption pattern, which in turn varies with the source and availability of its energy, conversion loss and end use efficiency. The growing demand of burgeoning population coupled with developmental activities based on ad-hoc decisions have led to resource scarcity in many parts of India. In this context, studies have shown that plants during their growth process synthesize carbohydrates during photosynthesis and then stock starch and lipids variably. They act as a solar energy driven cell factory converting CO_2 to O_2 thereby reducing the atmospheric CO_2 while trapping nutrients from the environment. Cleaner, sustainable and cost-effective energy alternatives can be accomplished through bioenergy.

In recent decades, renewable and alternative means of energy generation, especially in the form of liquid transportation fuels has started to incline more towards biofuel, derived from the biomass that could be easily replenished within a short period of time. These biomass derived biofuels are now emerging as a vital substitute for non-renewable fossil fuels. Every possible sources of biofuels are being explored and exploited for its potential to be used as a fuel. Biofuels are termed based on the nature of the biomasses /feedstock as first, second and third generations. First generation feedstock are those derived from food crops and now considered unsustainable as it competed with food resources. Second generation biofuel substrates are the biomasses which does not have direct link with the human-food chain like lignocellulosic biomasses, macrophytes, aquatic weeds (such as duckweeds, etc.), and municipal solid wastes. Third generation feedstock are derived from algae. Duckweed are small free-floating aquatic plants belong to four genera (Lemna, Spirodela, Wolffia, and Wolfiella) and approximately 40 species are reported throughout the world. These plants usually grow on nutrient rich water of the wetlands, ponds and lagoons and do not have any direct human-food use values. The most preferred habitat of duckweed is tropical and sub-tropical regions and the species distribution in these areas are high, as well as diverse. Summer seasons of temperate/colder countries also favors the sudden emergence of this plant in the waterbodies[1]. Researches on duckweed revealed that it has a potential starch source for bioethanol production[2,3]. Depending on the duckweed species and the condition in which it inhabits, starch contents[4,5] varies from 3 to 75%. In this study, duckweed species Lemna minor proliferated in the centenary pond inside IISc campus were evaluated for biofuel prospects. Watrer and plant samples were collected to understand the variations in macrophytes spread biochemical composition of plant samples with physico-chemical parameters in the pond. These natural stresses generally triggers the accumulation of one biochemical component in excess when compared to others. Stompe et.al (2012)[6], studied the variation in accumulation of starch levels when duckweeds were subjected to nutrient starvation by transferring them from waste water to well water. He found that the accumulation of starch increased considerably, giving a starch yield of upto 94.6%. Thus the Objectives of this study were to:

i) understand the environmental parameters that favored the growth of duckweeds
ii) study the biochemical composition of duckweeds with respect to changes in the physico-chemical parameters of the lake and quantify the increase in amounts if any
iii) estimate the amount of bioethanol derived from collected duckweed and compare the variations in its ethanol yield with respect to time of collection.

2. Materials and Methods

2.1 Study Area: The study area is an artificially constructed lake, located between 13° 1'16.41"N and 77°34'14.96"E to 13°1'17.22"N and 77°34'15.28"E inside the Indian Institute of Science campus, Bangalore. The inlet source of water to the lake is the treated water from sewage treatment plant situated inside the campus which acts as a principal feeder to the lake during non-monsoon seasons. During monsoon seasons, the run-offs due to down-pour will also be an additional inlet source of water to this lake catchment.

2.2 Water sampling and Analysis: Water sampling was done during the summer (March – April) months on a weekly basis for a period of 5 weeks. The physico-chemical parameters were analyzed using standard protocols of APHA[7] and the parameters analyzed were: DO, TDS, pH, Nitrates, Phosphates, Total alkalinity, Chlorides, Total hardness, Calcium and Magnesium hardness, Sodium and Potassium.

2.3 Duckweed Sampling and analysis: Duckweed emerged in the lake due to changes in environmental conditions driven by seasonal variation in temperature that induced thermal stratification of the lake caused by higher temperature of water at the top layer and relatively colder temperatures at the bottom layers. Those profusely grown duckweeds were collected and brought to the lab for initial morphological characteristics study and subsequent identification to the species level. Based on its morphology and growth characteristics observed under light microscope using 40x resolution the duckweed species was identified to be Lemna minor by referring to the identification key "Aquatic and Wetland Plants of India" by Christopher (1996)[8] The Duckweed samples were collected twice during the

sampling period. The first sample collection was carried out during the 3rd week of March 2016, were there was a thick mat of duckweed growth in the pond with nutrient (Nitrate) and DO level considerably low. The second sample collection was done during the 5th week (April 2016) where there was a significant difference in the DO and nutrient levels due to higher rate and velocity of inflow to the Lake from the Sewage Treatment Plant which is located inside the IISc campus. This higher inflow of water considerably reduced the profuse growth of the duckweeds and pushed the duckweeds to a post blooming stage.

2.4 Duckweed Sample Preparation: The duckweeds thus collected were washed in running water and shade dried till the weight of the sample becomes constant and no more excess moisture was left to be removed. The dried duckweeds were then powdered using a mortar and pestle and a constant weight of 0.1 g was taken for the biochemical analysis of total carbohydrates, proteins, lipids, cellulose and starch. The methods followed for each of the bio-chemical analysis is listed in the figure 1.

i. **Biochemical Composition Analysis:** Total carbohydrates of duckweed was estimated by the Anthrone method of Hedge and Hofreiter[9], (1962). Protein estimation was carried out using biuret method as per Gornall10 (1949). Lipid estimation was done following the protocols of Folch et.al (1956)[11]. Estimation of cellulose was carried out following Updegraff[12] (1969). Estimation of starch is carried out by same Anthrone as that of total carbohydrates with little modification as suggested by Hodge and Hofreiter[13], 1962.

ii. **Optimization studies on acid pre-treatments for obtaining higher reducing sugars:** The powdered duckweed samples were subjected to acid pretreatment using two different acids (HCl and H_2SO_4) of varying concentrations, in-order to examine the increase in amount of reducing sugar which is effected by the dissolution of complex polysaccharides in the form of cellulose to simple monosaccharides. Pre-treatment is usually carried out in order to convert the complex sugars into simpler ones, thus making it amenable for the yeast to ferment upon. In this case, 0.1 g of duckweed sample was hydrolyzed using 1.5 N, 2 N and 2.5 N HCl and H_2SO_4 in a water bath for 3 hours.

iii. **Estimation of Reducing Sugar:** After acid hydrolysis, the samples were neutralized using sodium carbonate and then subjected to centrifugation at 300 rpm for 15 minutes. The supernatant formed was collected and analyzed for its amount of reducing sugar content using DNS method (Miller[14], 1972). Glucose standards were prepared using 0.2 to 1.2 ml aliquots and made upto 2 ml using dH2O. 2 ml of DNS reagent (1g of DNS powder was weighed and dissolved in 20ml of 2N NaOH. 30g of Sodium potassium tartarate was dissolved in 50ml of distilled water and both the solutions mixed up). 0.2 ml of sample aliquots were taken and absorbance readings were measured at 540 nm. Compared with the amount of reducing sugar obtained in sample 1 (3rd week sample) and sample 2 (5th week sample) with the reducing sugar quantity of non-pre-treated samples, the acid concentration which gave the highest reducing sugar percentage is taken for further downstream processing of fermentation and ethanol production.

iv. **Fermentation:** Fermentation of the acid pre-treated samples which yielded the highest percentage of reducing sugar (in this case, 2N HCl) for sample 1 and sample 2 was used for fermentation process using Baker's yeast Saccharomyces cerevisiae. Yeast inoculum was prepared in a conical flask by powdering baker's yeast and adding 2 g of yeast powder to 20 g sugar in 200 ml water. The conical flask was covered air-tight and left undisturbed for 24 hrs. Once the yeast started budding, the active and budding yeast inoculum of volume 1 ml was used for fermentation. Fermentation was carried out in a plastic vial of 125 ml capacity. 100ml of the acid pre-treated samples were kept in the 125ml tightly capped bottles with one outlet for carbon di-oxide produced during the process, by inserting a piston pulled-up syringe to hold the gas produced.

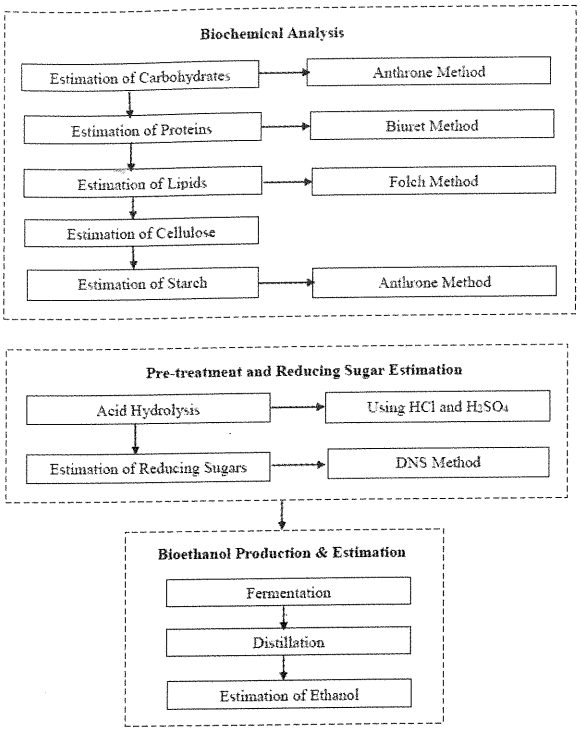
2.5 Ethanol Estimation: Potassium-di-chromate based spectrophotometric method (Caputi et al., 1968)[15] was used to estimate the amount of ethanol present in the duckweed sample after distilling it at a temperature of 70±2°C. Potassium-di-chromate reagent was prepared as per the protocol given by Caputi et al., (1968) and was mixed with absolute ethanol of concentrations ranging from 0 – 20% (v/v). 20 ml of distillate from duckweed samples was run as unknowns, after adding 25 ml of potassium dichromate solution, followed by heating it in a water bath at 60°C for 20 minutes and finally the volume was made up to 50 ml with distilled water. The absorbance of the standards and samples were measured at 600 nm which gave the estimate of ethanol present in the sample.

3. Results

3.1 Physio-chemical parameters of water

Air temperature: The monthly average variations in

Figure 1 : Biochemical Analysis, Reducing Sugar Estimation and Bioethanol Production



temperature recorded at sampling points ranged from minimum 25°C to maximum 33°C.

Water temperature: The average water temperature across the sampling points varied from 22.7°C to 27.9°C.

pH: The value of pH in the pond ranged from 7.8 to 8.5. The alkaline condition of the sample could be attributed to the presence of bicarbonates in higher amounts than the amount of CO_2 and carbonic acid equilibrium counterparts.

TDS: TDS of the pond was high across sampling points which varied from 328 ppm to 358 ppm.

DO: During the sampling period, very low DO levels were recorded during first three weeks of sampling which was 1.6 to 2 mg/L at S2, whereas it slowly started increasing from 4th week (7.22 mg/L at S2) due to higher inflow.

Turbidity: The average turbidity of the water samples at S2 were (4.15 – 5.45 NTU) and S3 were (3.85 – 5.62 NTU). The range of turbidity of S1 was quite low (0.48 – 0.9 NTU).

Alkalinity: The alkalinity values ranged from (230 – 288 mg/L) for S1, (272 – 312 mg/L) for S2 and (268 – 304 mg/L) for S3.

Total Hardness: Hardness in water is induced by cations such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Sr^{2+}, Zn^{2+} and Mn^{2+}. Though all these cations are responsible for hardness, the major contributors are Ca^{2+} and Mg^{2+}. The total hardness of the water samples at S1, S2 and S3 ranged from 150 – 180 mg/L. All the sampling points showed a similar range of total hardness with only slight variations. The calcium harness of the sample ranged from 80 mg/L to 120 mg/L and magnesium hardness of the water samples ranged from 9 mg/L to 20 mg/L.

Chlorides: Chlorides are the most common anions present in water and it ranged from 90 mg/L to 157 mg/L.

Sodium: Sodium salts have higher solubility in water and their sources are mainly from terrestrial leach outs to the water body. As sodium is highly reactive in its elemental state, it always exists in association with other elements or particulate matter. The sodium content of the water samples from sampling points (S1, S2 and S3) ranged from 90 mg/L – 157 mg/L.

Potassium: The potassium levels in the pond samples remained almost in a same range across all sampling points which ranged from 29.6 mg/L to 39.2 mg/L).

Nitrates: Nitrates (NO_3^-) are oxidized forms of nitrogen and sources of it to the water body are diverse. Duckweeds have the capability to take up nitrates and accumulate it at a higher rate which is known to have a check on the amount of nitrates in the water body. The nitrate levels of S1 (sampling location 1) ranged from 0.008 mg/L to 0.366 mg/L, for S2 it is 0.061mg/L to 0.25 mg/L and for S3 it ranged from 0.088 to 0.222 mg/L.

Phosphates: Phosphate values of the water samples ranged as low as 0.04 mg/L at S3 second week to as high as 0.141 mg/L at S1 during fourth week.

3.2 Biochemical Composition of Duckweed

Total Carbohydrate, Starch and Cellulose: Biochemical composition of duckweeds was analyzed considering the differences in the time of its collection. The total carbohydrates of duckweed was 16.06- 21.28% out of which 10.66- 13.27% contributed to its starch content. The cellulose content was 2.64-3.41%. Higher values were during lower levels of nitrate that acted as a natural starvation for the duckweeds. In other words, as the duckweeds were in their blooming stage during week 3, the amount of nitrates available for them to grow were not sufficient for its protein synthesis, but all other favorable conditions enhanced their proliferation but at the cost of higher accumulation of starch instead of proteins.

Protein: Protein content of samples were 19.26% and 21.24% respectively. Earlier studies on wild duckweed colony by Leng et al., (1999)[1] grown in nutrient poor water showed 15%-25% of protein. The protein content variation matches well with the higher levels of nitrates recorded in the lake during 5th week. The nitrate values during the 3rd week showed an average value of 0.064 mg/L across all the sampling points, wherein during the 5th week, the average nitrate values were found to be much higher, about 0.279 mg/L. The increase in the protein content of the duckweed population that are left behind in spite of duckweed's recession could be because of the higher nitrate levels in the system. Phillips et al., 2012[16] reported the protein content in Lemna minor grown in agricultural run-offs which are usually rich in Nitrogen due to its abundance of Nitrogenous fertilizers to be 32.3% Thus, the protein value of Lemna minor can be altered by controlling the amount of nutrient availability. Harvey and Fox 1976, reported that Lemna minor can be used for nutrient removal, due to which protein content of the plant increases and hence can be used as feed for poultry.

Lipid: The quantity of lipid estimated gravimetrically by modified folch method[17,18], showed the lipid content 0.0189 - 0.019 g.

3.3 Estimation of Reducing Sugar: The acid pre-treatment of duckweed samples carried out in-order to break the complex polysaccharides (Starch and Cellulose) into simple sugars with varying concentrations of HCl and H_2SO_4. Higher percentage of reducing sugar was observed for 1.5 N HCl treatment with reducing sugar ranging from 7.5 - 8.0%. Also, it was noticed that higher concentrations of acids were in-effective in actively converting the complex carbohydrates in the form of starch and cellulose into simple sugars. This reduction in percentage of reducing sugar could be attributed to the formation of certain inhibitory compounds like Hydroxy methyl furfurals (HMF) at higher concentrations of acid during digestion and hydrolysate formation.

3.4 Estimation of Ethanol: The estimation of ethanol carried out by potassium-di-chromate based spectrophotometric method gave 0.82 g/L - 1.02 g/L of ethanol depends on the quantity of total carbohydrate content, starch and cellulose.

4. Conclusions

The potential of an aquatic weed Lemna minor in the production of a useful biofuel (bioethanol), showed an average ethanol yield of 0.82 g/L - 1.02 g/L without subjecting the feedstock to any nutrient manipulation. The study reveals that nutrient starvation enhances starch accumulation. Duckweed are effective phytoremediation feedstock with advantage of biofuel production. The yield could be enhanced through an advanced pre-treatment processes like steam explosion, enzymatic saccharification and simultaneous saccharification and fermentation processes.

Acknowledgements: We are grateful to (i) Gautam and Vasantha Jagadisan Endowment– Lake Rejuvenation Indian Institute of Science, Bangalore 560012; (ii) The Ministry of Science and Technology, Government of India; (iii) The Ministry of Environment, Forests & Climate Change, Government of India and (iv) ISRO-IISc Space Technology Cell for financial and infrastructure support. Ms. Varsha Bolamma MM helped in carrying out the experiment.

References

[1] Leng, R.A. (1999) Duckweed: A tiny aquatic plant with enormous potential for agriculture and environment. Chapter 1-Chapter 4, University of Tropical Agriculture Foundation, Phnom Penh (Cambodia)
[2] J.J. Cheng, A.-M. Stomp, Growing duckweed to recover nutrients from wastewaters and for production of fuel ethanol and animal feed, CLEAN-Soil, Air, Water, 37 (2009), pp. 17–26
[3] Jiele Xu, Weihua Cui, Jay J. Cheng, Anne M. Stomp, Production of high-starch duckweed and its conversion to bioethanol. Biosystems engineering 110 (2011) 67 – 72
[4] Landolt, E., & Kandeler, R. (1987). The family of Lemnaceae monographic study. In. Phytochemistry, physiology; application; bibliography, vol. 2... Zurich, Switzerland: Vero¨ ffentlichungen des Geobotanischen Institutes ETH, Stiftung Rubel.
[5] Reid, M. S., & Bieleski, R. L. (1970). Response of Spirodelaoligorrhiza-M to phosphorus deficiency. Plant Physiology (Rockville), 46, 609 - 613.
[6] Stomp, Anne-M., Xu, Jiele. Zhao, Hai and Cheng, Jay, J (2012).The production of duckweed as a source of biofuels. Biofuels 3(5), 589–601.
[7] APHA, AWWA, WEF, (2005) Standard Methods for the Examination of Water and Wastewater, 20th ed. APHA, Washington, DC, USA.
[8] Cook, Christopher, D.K., (1996) Aquatic and wetland plants of India.p.228.
[9] Hedge, J.E. and Hofreiter, B.T., (1962). In: Carbohydrate Chemistry, 17 Academic Press, New York.
[10] Gornall, AG, CS Bardawill, and MM David. J. Biol. Chem. 177: 751. 1949.
[11] Folch, Jordi. LeesM. & Stanle, Sloane, G.H., (1956) A simple method for the isolation and purification of total lipids from animal tissues.
[12] David M. Updegraff, Semi micro Determination of Cellulose in Biological Materials, Analytical Biochemistry 3:2, 420 - 424 (1969)
[13] Hodge, J.E. and Hofreiter, B.T. (1962). In: Methods in Carbohydrate Chemistry, Academic Press, New York
[14] Miller, G.L. (1972). Anal. Chem., 31, p. 426.
[15] Caputi, A., Ueda.M.T. Brown, T., (1968).Spectrophotometric determination of ethanol in wine. American Journal of Enol.Vitic 19:L160-165.
[16] Phillips, Gregory C, Ge Xumeng., Zhang, Ningning., Xu, Jianfeng. (2012) Growing Lemna minor in agricultural wastewater and converting the duckweed biomass to ethanol Bioresource Technology 124:485–488.
[17] Harvey R. & Fox, J. (1973).Nutrient Removal Using Lemna Minor. Journal (Water Pollution Control Federation), 45(9), 1928-1938.
[18] Ramachandra T. V., Durga Madhab Mahapatra, Sudarshan P. Bhat and Joshi N. V., 2016. Biofuel Production along with Remediation of Sewage Water through Algae, Algae and Environmental Sustainability (eds: Bhaskar Singh, Kuldeep Bauddh, Faizal Bux), Volume 7 of the series Developments in Applied Phycology pp 33-51
[19] Ramachandra T V., Durga Madhab Mahapatra, Karthick B., Richard Gordon, 2009. Milking Diatoms for Sustainable Energy: Biochemical Engineering vs Gasoline Secreting Diatom Solar Panels, Ind. Eng. Chem. Res., Article ASAP, 10.1021/ie900044j