Biofuel production, study & characterisation from macro-algae (*Azolla pinnata*)

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Abstract

The demands for energy and the scarcity in fossil fuel are constantly increasing. This has resulted in the search for sustainable, renewable, and low cost biofuel that has triggered the search for potential bioenergy crops. Aquatic plants that can grow rapidly with minimum resources and can produce biomass in bulk amounts are driving the attention of scientists and researchers throughout the world. The production of biofuels from such organic materials and waste components can result in developing of sustainable alternative that will not only be beneficial to the environment but also to public health. In this study, one such aquatic macro algae Azolla pinnata proved to be potential source for biofuel production. The evaluation of its growth was done and trans-esterification of *Azolla pinnata* lipid was carried out to produce biofuel. The species have a unique combination of physical, chemical and nutrients composition that makes it a boon to mankind. This macro algae was subjected to series of laboratory testing and evaluation for its characterization such as acid value test, trans-esterification, fatty acid methyl esters (FAMEs) test, gas chromatography which showed the feasibility of algal based biofuel. The comparison of properties of extracted biofuel (physicochemical) from *Azollla pinnta* was done with standardized ASTM D6751 values. The outcome of produced biofuel was very close to conventional fuel.

Keywords: *Azolla pinnata*, genus *Azolla*, biofuel, chlorophyll-*a*, trans-esterification.

Produção, estudo e caracterização de biocombustíveis a partir de macroalgas (*Azolla pinnata*)

Resumo

As demandas por energia e a escassez de combustível fóssil estão aumentando constantemente. Isso resultou na busca por biocombustíveis sustentáveis, renováveis e de baixo custo, o que desencadeou a busca por potenciais cultivos bioenergéticos. Plantas aquáticas que podem crescer rapidamente com recursos mínimos e podem produzir biomassa em grandes quantidades estão atraindo a atenção de cientistas e pesquisadores em todo o mundo. A produção de biocombustíveis a partir de tais materiais orgânicos e componentes de resíduos pode resultar no desenvolvimento de alternativas sustentáveis que serão benéficas não apenas para o meio ambiente, mas também para a saúde pública. Neste estudo, uma dessas macroalgas aquáticas *Azolla pinnata* provou ser uma fonte potencial para a produção de biocombustíveis. A avaliação de seu crescimento foi feita e a transesterificação do lipídio *Azolla pinnata* foi realizada para a produção de biocombustível. As espécies têm uma combinação única de composição física, química e de nutrientes que a torna um benefício para a humanidade. Esta macroalga foi submetida a uma série de testes laboratoriais e avaliações para sua caracterização, como teste de valor ácido, transesterificação, teste de ésteres metílicos de ácidos graxos (FAMEs), cromatografia gasosa que mostrou a viabilidade de biocombustível à base de algas. A comparação das propriedades do biocombustível extraído (físico-químico) de *Azollla pinnta* foi feita com valores padronizados ASTM D6751. O resultado do biocombustível produzido foi muito próximo ao do combustível convencional.

Palavras-chave: *Azolla pinnata*, gênero *Azolla*, biocombustível, clorofila-*a*, transesterificação.

1. Introduction

Azolla pinnata is a species of fern belonging to family Salviniaceae and is commonly known by various names like mosquito fern, water velvet, feathered mosquito fern and water fern. This macro algae is native to Africa, Asia, and parts of Australia; which is generally found floating upon the surface of the water such as ditches, ponds, and wetlands in temperate or tropical regions. This aquatic plant possesses capability of doubling its biomass in a week which makes it one of the fastest growing plants (Kollah et al., 2016).

The demand has risen for unconventional energy resources during the past few years. Scientists and researchers are constantly working to achieve alternative sustainable fuel. Biofuels are the fuels which are produced from renewable organic material and are gaining significance as a sustainable option as it has the potential to reduce some undesirable aspects of production and use of fossil fuel, including conventional and greenhouse gas pollutant emissions, depletion of exhaustible resource, and reliance on unstable foreign suppliers. These are considered more advantageous (Demirbas, 2009; Van, 2005) due to its compatible physical and chemical properties with traditional fuels. Biofuels can be put to direct use in engines thus eliminating engine modifications (Subramanian et al., 2005) The Properties which make biofuels eco-friendly are their biodegrading nature, renewability, and non-toxic behaviour.

Seed crops having edible oil can be used for generating biofuels. These are known as feed stocks for both first and second generation biofuels. In addition to this non-edible seed crop are coming into being for production of biofuels (Indhumathi et al., 2014), whereas the third-generation feed stock, focuses on macro algae. The production from macro algae has advantages like higher photosynthetic efficiency and biomass production. Macro algae has potential of flourishing in brackish saline waters which can be grown rapidly using CO_2 fixation and converts solar energy into chemical energy. The method is now being considered as viable method for generating biofuel and is supported by fatty acid profile of oil extracted from macro algae. Macro algae have enormous oil production capacity which attracts the usage of algal oil.

The concern about the negative environmental impact of fossil energy has increased globally in the last two decades that has drawn significant attention to renewable biofuels to replace the traditional petroleum based fuels. Biofuels are long chain alkyl esters that are renewable, biodegradable, and non-toxic in nature; these are derived from the trans-esterification of mono-, di- and triacylglycerides and the esterification of free fatty acids that occur naturally in animals, plants, and algae lipids. Thus, biofuel is a carbon neutral type of fuel (Lopez et al., 2005) which emits less environmental pollutants during combustion when compared to traditional petroleum biofuel (Swanson et al., 2007). As macro algae like *Azolla pinnata* can grow rapidly and has the potential of converting solar (light) energy to chemical energy via CO_2 fixation is taken into consideration as a potential source for biofuel production.

The main purpose of this paper is to utilise natural resources (*Azolla pinnata*) for production of sustainable alternative for mankind.

2. Materials and Methods

2.1 Algal sample

The sample for cultivation of *A. pinnata* used in the present study was collected from a water body in Ajmer, India. This macro algae was grown outdoors in mud pots having silpaulin sheets.

2.2 Cultivation of A. pinnata

For each mud pot a mix of 5 kg cow dung (2-7 days old) and 15 kg fine sieved garden soil was prepared and evenly spread and water was added in the pot upto 10 cm. After 3 days the pot was ready for inoculation of *Azolla* sample along with this 15 g of Single Super Phosphate (SSP) was also added and 5 g of SSP was also added on every 7^{th} day. The pH for growth was maintained between 6.5-7.5.

2.3 Cellulose extraction from A. pinnata

For extracting cellulose; fresh biomass of *A. pinnata* was harvested, washed, and dried at room temperature for 24-48 h. The dried biomass was then grinded and sieved for reducing the particle size and treated with alkali i.e., 2 L^{-1} 0.1 N NaOH in 40 g dried biomass at 40 °C for 4 h. Finally, the cellulose was extracted by filtering the prepared mixture after cooling at room temperature.

2.4 Dry cell method

The dry cell method is used for evaluating the growth of *A. pinnata*; in this method a known amount of biomass is taken from growing medium to remove salt traces where the biomass is thoroughly cleaned with purified water. For the filtration of biomass pre-weighed glass fiber and the same was dried at 60 °C overnight in oven and dry cells in it were then weighed (Zhu; Lee, 1997).

2.5 Chlorophyll-a content

For the entire duration, the chlorophyll-*a* (chl-*a*) content in *A. pinnata* helped in analysis of growth pattern. A known amount of biomass was grounded using methanol to determine the chl-*a* content and this mixture was subjected to water bath for 30 min at 60 °C (Chinnasamy S. et al., 2010). Now, the suspension was filtered using whattman glass fiber and the content of chl-*a* was checked using spectrophotometer. The chl-*a* content was determined by formula (Sumanta N. et al., 2014); Chl-*a* ($\mu g/mL^{-1}$) = 16.74 A665.3 - 9.17 A652.6. Where A665.3 and A652.6 are the absorbance value of algal biomass and methanol suspension at 665.3 nm and 652.6 nm respectively.

2.6 Oil extraction

The dried biomass of *A. pinnata* was pulverized for the ease of oil extraction; this biomass was now subjected to continuous extraction using soxhlet apparatus. A mixture of chloroform: methanol 2:1 (*v/v*) was used as a solvent at a temperature of 56 °C. The excess solvent present in the crude lipids were separated from oil through distillation process.

2.7 Acid value

The acid value of *A. pinnata* lipids was determined using volumetric analysis. The analysis was done by preparing a mixture of 0.1 g lipid sample with 50 mL ethanol and few drops of phenolphthalein indicator; the prepared mixture was titrated against standardized NaOH solution until permanent pink colour was obtained. Following this, the titrated solution was subjected to boiling, shaking, and mixing. Now, few more drops of phenolphthalein indicator were added and again titrated against NaOH solution until permanent reddish-pink colour was obtained. Similarly, the analysis with blank (without sample) was also carried out for comparison.

2.8 Melting point and specific gravity

The melting point of lipids derived from *A. pinnata* was determined by using capillary tube method and was estimated to be 97.5 °C. On the other hand, the specific gravity of lipids was determined using pkynometer method and estimated to be about 1.03 at 29 °C.

2.9 Trans-esterification

To produce biofuel, the lipids were subjected to trans-esterification process. Methanol and conc. H_2SO_4 were added to crude lipids and maintained at 60 °C for one hour and subjected to continuous stirring in reactor to convert mono-, di-, tri-glycerides and fatty acids to fatty acid methyl esters (FAMEs) in the laboratory of Institute for Innovative Learning and Research (IILR) Academy, Indore, India. After completion of trans-esterification process, the mixture was allowed to cool at room temperature after which the mixture was transferred to separating funnel of other 24 h.

2.10 Water wash and drying

Through trans-esterification process the biofuel obtained was impure as it contained impurities like methanol, catalysts, glycerin, and soaps which affect the quality of biofuels. These impurities were removed by mixing trans-esterified biofuel with warm water (around 45 °C) as it allows catalysts, soluble materials like soaps and other impurities to dissolve with water and to settle at the bottom of the vessel. It is possible that after removal of impurities the trans-esterified biofuel can retain cloudy appearance because of trace amounts of water in it which can be removed by gentle heating to 100 °C until the moisture present gets totally evaporated (Nautiyal et al., 2014).

2.11 Fatty acid value

Fatty acid and methyl ester composition was analysed and confirmed by gas chromatography which showed the presence of saturated (having single bond) and unsaturated (having double bonds) fatty acids. Among these the saturated fatty acid methyl esters are Butyric, Capric, Myristic, Palmitic, Margaric and Steric acid; all these saturated fatty acids shows decent oxidative stability and ignition properties, whereas the unsaturated fatty acids are Oleic, Linoleic, Eicosapantaenoic and Erucic acid which depicts poor oxidative stability due to double bond presence and have low energy content. The details of these saturated and unsaturated fatty acid methyl esters (FAMEs) are shown in Table 1.

3. Results and Discussion

The growth of biomass was regularly checked using dry cell method by weighing the dried cell mass of *Azolla*; the rise in weight of dried cell with respect to time was observed and was estimated as 0.249 g/L^{-1}, 0.279 g/L^{-1}, 0.310 g/L^{-1}, 0.400 g/L^{-1}, 0.613 g/L^{-1}, 0.820 g/L^{-1} on 5, 10, 15, 20, 25 and 30 days respectively as depicted in (Figure 1).

The amount of mass produced by algae in biofuel production is directly porportional to the energy matrix and production. Various algal species show satisfactory biomass yield, which is important factor in production of biofuel. This mass production is an attraction for the new industries of renewable fuels and, also we observe attractive characteristics of algae (Sialve et al., 2009; Ullah et al., 2014). The average biomass yield observed is 20 to 30 times higher and rapid when compared to vegetable crops, providing up to 30 times more fuel compared to other biofuel sources. Several studies show positive and fascinating results when evaluated on the biomass produced, up to 60% in the form of oil or carbohydrates from which biofuels can be obtained (Ullah et al., 2014; Bugs et al., 2018).

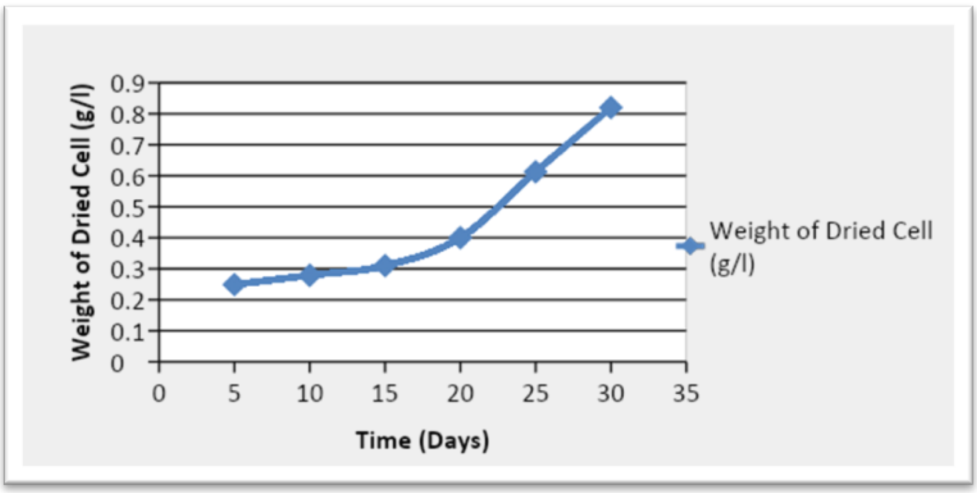

Figure 1. Representation of dry cell weight of *Azolla pinnata*, Source: Authors, 2022.

From the growth pattern, it is observed that the chlorophyll-*a* content gradually increased with time. This shows the growth is algal colony which was estimated as 0.140 $\mu g/mL^{-1}$, 0.202 $\mu g/mL^{-1}$, 0.271$\mu g/mL^{-1}$, 0.820 $\mu g/mL^{-1}$, 1.115$\mu g/mL^{-1}$ and 1.227 $\mu g/mL^{-1}$ for 5, 10, 15, 20, 25 and 30 days respectively (Figure 2).

Chlorophylls belong to an important group of special metabolites present in plants and are pigments responsible for absorbing sunlight and producing O_2 alongwith various types of sugars through photosynthesis (Maestrin et al., 2009). The sources of absorption in the blue and red regions of the electromagnetic spectrum imparts intense green colour to chlorophylls, causing them to transmit in the green region (Maestrin et al., 2009). It is known that chlorophyll *a* is the most common pigment that forms around 75% of the green pigments present in plants, being used in pharmacy, cosmetology, oral hygiene material, diets, cooking, detergents, agriculture and in the production of biofuels (Hardy; Castro, 2000; Menezes-Filho et al., 2021).

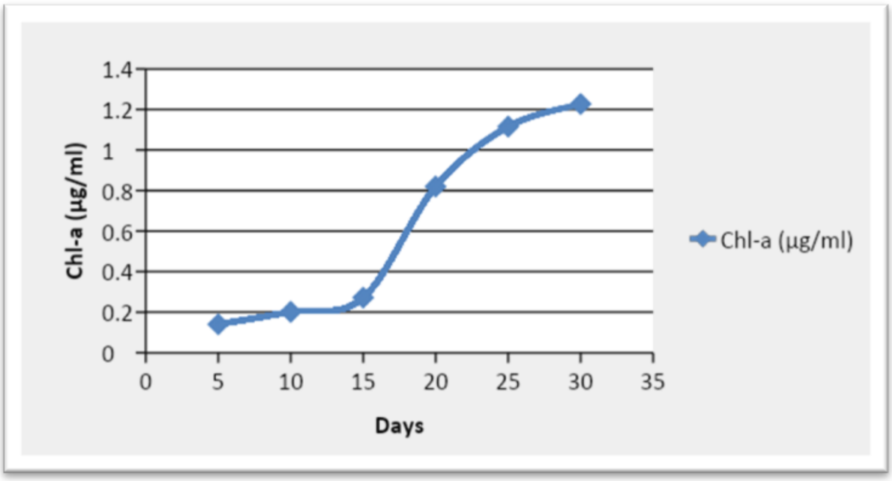

Figure 2. Representation of growth in Chl-*a* content in *Azolla pinnata*. Source: Authors, 2022.

The acid value of *Azolla* lipid was estimated to be 185.13. According to Schalargemann et al. (2012) the lipids extracted from algae starts from the biomass, separation, and dehydration of the cells. Observing an alternative means of producing biofuels, the process of obtaining the lipid fraction must be evaluated, which must be as economical as possible. Therefore, we have some forms of extraction, such as pressing the biomass, disposing of the biomass in a supercritical fluid, or a combination of pressing and the use of solvents. In the pressing process, up to 75% of the total oil present in the algae biomass can be obtained. In the process that combines solvent, for example: hexane and pressing, it is possible to obtain up to 95% of lipids. Supercritical fluid extraction has the highest and maximum extraction yield of up to 100%, using CO_2 as carrier gas (Defanti et al., 2010).

In our findings, 10 types of fatty acids were obtained from the mass of *A. pinnata* (Table 1). Lipids, among the most valuable algal products, comprise mostly C_{14}-C_{18} fatty acids destined for jet biofuel and biodiesel production, while omega-3 fatty acids, like the C_{18} alphalinolenic acid (ALA), the C_{20} eicosapentaenoic acid (EPA), and the C_{22} docosahexaenoic acid (DHA), are excellent sources of nutraceuticals and dietary supplements (Tsarpali et al., 2021).

Table 1. Representation of fatty acid methyl ester (FAMEs) composition *Azolla pinnata*.

S.No.	Fatty acid methyl ester	Structure
1.	Butyric acids	C4:0
2.	Capric acid	C10:0
3.	Myristic acid	C14:0
4.	Palmitic acid	C16:0
5.	Margaric acid	C17:0
6.	Oleic acid	C18:1
7.	Linoleic acid	C18:2
8.	Stearic acid	C18:3
9.	Eicosapantaenoic acid	C20:5
10.	Erucic acid	C22:1

Source: Authors, 2022

The chemical properties of biofuel produced from *Azolla pinnata* is compared with standardized ASTM D6751 Biofuel (bio-diesel fuel) values that are represented in Table 2.

Table 2. Representation of physical properties of produced biofuel with respect to ASTM standards.

S.No.	Properties	Test Method	ASTM D6751 Biofuel (diesel) standard	*Azolla pinnata* biofuel value
1.	Viscosity (cP)	ASTM D6751	1.9-6	4.4
2.	Calorific Value (MJ/Kg^{-1})	EN 14213	Min 35	38.1
3.	Flash Point (°C)	ASTM D93	100-170	110
4.	Fire point (°C)	ASTM D93	106-180	123
5.	Cloud point (°C)	ASTM D2500	-3 to 15	7
6.	Pour Point (°C)	ASTM D97	-5 to 10	2

Source: Authors, 2022.

4. Conclusions

In the present work, *Azolla pinnta* a macro algal species is cultivated for extraction of biofuel. Overall, the need for sustainable options for energy in day-to-day life and the concern for public health and environment have led us to think critically. The increase in population has also increased environmental pollution which causes many health hazards, mainly for respiratory health.

The usage of conventional fuels can have ill effects on health, can be cancerous, causes ozone layer depletion, global warming, brings about extreme changes in weather patterns, disturbs the ecosystem flow and much more. Hence, these traditional resources such as coal or petroleum are considered no longer sustainable. As there is always a room for better alternative, the biofuel generation from natural organic resources can be used to help safeguard the environment from further destruction for future generations. This biofuel produced from *Azolla pinnata* can be very promising due to its various qualities like being organic, natural, affordable, atmosphere-friendly, non-toxic, less or no side effects on human health.

5. Acknowledgments

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6. Auhors' Contributions

Porshia Sharma: Concieved and designed the analysis, Contributed data, wrote the paper. *Puja Biswas*: Performed the analysis. *Satya Tamrakar*: Collected the data. *Yogesh Choudhary*: Analysis Tools.

7. Conflicts of Interest

No conflicts of interest.

8. Ethics Approval

Not applicable.

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