BIOFUEL PRODUCING COMPETENCE OF MICROALGAE DUNALIELLA SALINA

Abstract- Microalgae is considered as the advanced generation of biofuels, which is investigated as an alternative means of ecofriendly and renewable fuels source. Green energy is safe to meet the global demand of fossil fuel and mitigate the greenhouse gases emission due to their use. This study discusses the prospects of halophilic microalgae *Dunaliella salina* as a prospective feedstock for biodiesel manufacturing. In this respect *D. salina* microalgal strain was obtained Sambhar Lake, Rajasthan. It is cultured under controlled laboratory conditions. The biomass recovered at the end of exponential phase was 1.25g/l dry wt. and the oil was about 22.4 % of the biomass. The oil recovered was converted into biodiesel by acid catalysed transesterification and the yield was 60.18%. Some specific types of the Fatty acid methyl esters (FAMEs) formed were identified by GC/MS analysis. The considerable amount is accounted by four fatty acid palmitic acid (19.6%), oleic acid (25.6%), linolenic acid (27.0%) and linoleic acid (18.4%). The presence of these major fatty acid in the microalgal oil makes *D. salina* a favourable feedstock for biodiesel production.

Key words: Biodiesel, Blight and Dyer, *Dunaliella salina*, FAME, GC-MS.

Introduction

Utilization of fossil fuel has increased at a tremendous pace in recent years, which has triggered a necessity of an energy substitute to fulfill the present crises. An alarming situation that the world struggles to deal is the limitations of fuel stock, and the increase in the temperature of the environment due to emission of greenhouse gases resulting in global warming (Rasoul-Amini *et al*. 2011). The problems of sustainability of the reserve resources (Fossil fuel) and maintaining environmental equilibrium, demands an alternative source of energy to tackle these issues. A renewable clean energy source is pre-requisite to meet out the global energy demand and reduce the carbon foot print from the environment. In this context biodiesel has fascinated wide attention due to its renewability and eco-friendly nature. Biodiesel generally contains a low amount of nitrogen and sulfur and therefore, less nitrogen oxide (NO_x), sulphur oxide (SO_x), and carbon monoxide (CO) when compare with fossil fuels (Chen *et. al*. 2012). A variety of sources can be used to produce biodiesel, such as vegetable oil, waste oil, and microalgal oil. According to the raw feed used, the biodiesel was categorized into different generations. The first-generation oil was produced from biomass

often used for food such as corn, soy, sugarcane etc. The second-generation feedstock used the source not suitable for human consumption viz non edible crop and waste biomass. The third generation emphasized on use of algal biomass (Mehariya *et al.* 2021)

To produce biodiesel, cyanobacteria and microalgae can be suitable organisms to meet our need for clean and safe source of energy (Rasoul-Amini, *et al*. 2011). Algae are heterogeneous, predominantly eukaryotic aquatic organisms that vary from a single cell to highly differentiated plants. Microalgae can be found in salt or freshwater which could be a promising source of biodiesel. Microalgae manifest high efficiency of CO_2 fixation, depicts a fast growth rate and elevated lipid production (Rasoul-Amini *et al.* 2011), play a major role to inhibit the greenhouse effect. All these traits make microalgae a suitable prospect for the purpose of biodiesel production. (Sánchez *et al.* 2011). However, the price and supply of feedstock are still limiting factors in the production of biodiesel. This problem can be overcome by a large-scale cultivation of microalgae. Microalgal biodiesel production cost can be reduced by microalgal bio-refinery approach. (Devi *et al.* 2012)

D. salina is a green, unicellular, and halophilic microalgae which can be found in marine waters, salty ponds and in sea salt fields. According to Yu. *et al.* 2014 *D. salina* can accumulate about 35% of algae oil. *Dunaliella* is extremely salt-tolerant and can grow in salinities from 0.05 to 5.0M NaCl, with low intracellular NaCl concentration (Chen *et al*. 2015). The species of *Dunaliella* also have the potential to fix the atmospheric CO_2 and wastewater remediation (Andreotti *et al.* 2020). Some strains of *D. salina* accumulate a high concentration of total lipids and produce a high amount of β-carotene under stress conditions, and these strains used commercially in the production of different products (Chen *et al.* 2015; 9 Bonnefond *et al.* 2017). *D. salina* was used in the study because it utilizes inorganic nutrient from the medium. It is a motile species and can sustain high salt concentration, it can be cultivated easily, shows high content of oil and growth. The objective of the study is to

find the potential of *D. salina* for biodiesel production as well as evaluate the biomass and lipid content. The profile of the resultant fatty acid methyl ester (FAME) will be determined by the process of transesterification. The present study focuses on the cultivation, harvesting, lipid extraction and final production of biodiesel from *D. salina.*

Method and materials

Microalgal strain and growth conditions

The strain *D. salina* was sampled from saline water of Sambhar Lake, Jaipur 26.9261° N, 75.0962° E, of Rajasthan state, India.The culture isolation and purification (Stanier and Cohen-Bazire, 1977) of microalgae *D. salina* was performed on Artificial Sea Water Medium (ASWM) (Harrison *et al*. 1980) by serial dilution. Purification cultures were identified by following (Prescot ,1982). The experimental cultures were grown on ASWM which contains the following ingredients (g L-1): NaCl (116.9), $NaHCO_3$ (4.2), $MgSO_4$ (1.23), KNO_3 (0.5), KH_2PO_4 (0.03), $CaCl_2$ (0.03), $FeCl_3.6H_20$ (0.08), $MnCl_2.4H_2O$ (0.04), $(NH_4)_2MoO_4$ (0.09), $ZnCl_2.4H_2O$ (0.014) $CoCl_2.6H_2O$ (0.02), Na_2EDTA (0.02) $CuCl_2.2H_2O$ (0.02), pH was adjusted to 8.1. The cultures were incubated in culture room illuminated by the fluorescent lamp (4000 lux) for 12 hrs. The temperature was maintained in the range between 23-25°C for entire period of culture. The cultures were constantly shake and the salinity was maintained at 12% throughout the entire period of growth.

Conditions for algal growth, lipid production and fatty acid composition

The stock culture at the exponential phase was inoculated in 250 ml ASWM in 500ml Erlenmeyer flask and the cell growth was determined by taking the optical density using UV/ Visible spectrophotometer (SL-177 Scanning mini spec) at various level of growth in the culture medium. The Optical density was taken at 660 nm and the dry weight of algal sample was measured. The cultures were incubated in the culture room and before the start of

stationary phase a known volume of culture was withdrawn, centrifugation at 4000 rpm for 10 min. The cell pellet obtained is washed two times with distilled water to remove the excess salt and finally it is stored at -20°C.

Lipid Extraction

The lipid extraction was done using Bligh and Dyer (1959) methods. The algal cells pellet stored at -20˚ C was used for the extraction of lipid. 20 g of wet algae sample was macerated in mortar and pestle for 20 min to disrupt the cells. Further the sample was sonicated. Chloroform and methanol used in the extraction of lipid is mixed in the ratio of 2:1. The lipids present are separated in chloroform layer (bottom layer), and aqueous methanol layer is formed as the top layer. The final volume of chloroform: methanol: water was made 1:1:1. (v/v/v) by adding methanol and water. The upper layer (methanol/water layer) was separated with the bottom chloroform layer contains the lipid. Finally chloroform layer is washed many times with 10% NaCl solution The solvent is removed by evaporation under reduced pressure and finally algal lipid is obtained. The lipid obtained was estimated and store at -20°C under nitrogen for subsequent analysis.

Fatty Acid Esterification and GC/MS Analysis

Acid catalysed transesterification method was used to convert algal oil into biodiesel. Lipids and methanol are mixed in the ratio 1:50 molar ratio. The reaction was performed at 60˚C for 3-4 hrs in the presence of sulphuric acid as the catalyst. The ratio of catalyst and lipid is kept equal. Afterwards the prepared FAMEs were extracted by adding 1 ml hexane to the reaction mixture. The FAMEs were analysed by a GCMS QP-2020 Plus (Shimadzu) system. A flow rate of 1 ml/min were maintained by a Rxi5 Si MS (Cross bond 0, 5% diphenyl/ 95% dimethylpolysiloxane) column (30.0m x.025 mm x 0.25 µm with a 66.8 kPa pre column pressure. The column temperature was maintained at 50 ˚C for 2 min, followed by an

increase at a rate of 6 ˚C /min upto 90˚C /min and then by an increase at a rate of 8 ˚C /min upto 280˚C for 2 min. The injection temperature and volume were 250˚C and 1 µl respectively with a split ratio of 15.0. The mass spectrometer operated with electron energy of 70 eV. The interface and ion source temperature were fixed at 250 ˚C. The mass spectra of the fatty acids present were compared with NIST libraries and identification was done.

Table 1. Fatty acid composition of *D.salina* under culture conditions

No of fatty acid	SFA	MUFA	PUFA	% of C16-C18
8	21.0±1.4	31.9±1.6	47.1±1.8	92.0±1.3

SFS-Saturated fatty acid
MUFA- Monounsaturated fatty acid
PUFA- Polyunsaturated fatty acid

Table 2. The composition and content of FAME in *D. salina*

Fatty acid methyl ester (FAMEs)	Content (%)
C16:0	19.6±2.1
C16:2	1.8±2.0
C18:3	27.0±1.6
C18:1	25.6±1.4
C18:2	18.0±2.1
C21:1	2.7±2.2
C23:0	1.4±1.5
C24:1	3.6±1.6

Statistical Analysis

The experiments were performed in triplicate and the results are expressed as mean value ± SD

Result and discussion

D. salina was cultured in *invitro* system. Maximum growth of the cells and the biomass is obtained on 13th day when the colour of the flask is dark green. The microalgal cells were harvested and the biomass dry wt. 1.25g/l was obtained.

Selection of an microalgal strain for biodiesel production is based on the criteria that it contains high oil content and shows rapid growth. (Chisti *et al.* 2007). During the period of growth of microalgae, the presence of various nutrients and the environmental conditions plays a major role in determining the amount of fatty acid content in microalgal oil (Gouveia and Oliveira, 2009). Different classes of lipids are present, viz. cholesterol, triglycerides, and phospholipids. Among the different polar and non-polar lipids present triglycerides are the crucial raw lipid precursor to produce biodiesel. In the present study the fatty acid composition is studied and the amount of saturated fatty acid (SFA), mono saturated fatty acid (MUFA) and polysaturated fatty acid (PUFA) is estimated (Table 1).

In the algal biomass assessment of the amount of FAME is required to suggest the amount of optimal lipid present which can be transformed into biodiesel. (Li *et al*. 2011)

The depiction of fatty acid methyl esters (FAMEs) was done by comparing the mass spectrum with the structures present in NIST 17 libraries. The FAME profile is shown in Table 2. Different type of FAME was detected in GC/MS analysis. The result showed that the four major fatty acid *viz.* palmitic acid (16:0), 19.6%, linolenic acid (18:3), 27.0 %, oleic acid (18:1), 25.6% and linoleic acid (18:2), 18.4% were present in the highest amount. Comparing our study with the work of other scientist it is observed that these four fatty acids are

predominantly found in other *Dunaliella* species which are found accountable for biodiesel production. Devi *et al.* 2012 in *D. salina* found that the fatty acid methyl esters (FAME) of three major fatty acid linolenic acid (18:3), oleic acid (18:1) and palmitic acid (16:0), are responsible for enhancing the yield of fatty acid. The fatty acid composition in the native strain of *D. salina* from Maharlu Salt Lake (Iran) has the maximum amount of hexadecanoic acid (palmitic acid ,23.7%) and octadecanoic acid (stearic acid, 20.3%) as reported by Rasoul- amini *et al.* 2014. Many reports define that palmitic acid also known as hexadecanoic acid, is an important constituent of biodiesel (Rasoul- Amini *et al.* 2011). Hanna H *et al.* 2014 found the major constituent of fatty acid was C16:0. Besides this, the two other important fatty acids are C18:3 and C18:2. Pavon-Suriano *et al.* (2018) disclosed that 30.35% and 21.61% of palmitic acid and palmitoleic acid is present respectively in *D. salina*. Arunachalam Sivagurulingam *et al.* 2022 found that in *D. salina*, the major proportion dominated by the presence of two important fatty acid viz palmitic and linolenic acids.

The important factors that are majorly responsible for the quality of biodiesel are length of carbon chain and the degree of saturation of the fatty acid. The quality of biodiesel is affected by the constituent of the lipid. The important characteristic of algal oil is shared by the triglycerides (Griffiths and Harrison, 2009). Presence of unsaturated fatty acid in algal biodiesel is responsible for lower viscosity and lower melting point. The improved low temperature performance is also a beneficial property marked by the presence unsaturated fatty acid. (Hoekman *et al.* 2012; Islam *et al.* 2013). The presence of higher percentage of unsaturated fatty acid in the biodiesel is responsible for the emission of lesser amount of CO, hydrocarbons, and smoke. (Gopinath *et al.* 2010). The algal oil contains some natural antioxidants which provide oxidative stability to the unsaturated fatty acid. (Frankel *et al.* 2002).

Conclusion

The depleting petroleum reserves and hazardous environmental outcome caused by exhaust gases from fossil fuel, has instigated an urge of renewable energy resource. In this context biodiesel has captivated attention as a potent source of renewable, biodegradable, and ecofriendly fuel. In the present study *D. salina* isolated from Sambhar Lake is studied. It is a fast-growing strain which reaches exponential phase on day 13$^{th.}$ Blight and Dyer (1959) method was used for the extraction of lipid. The FAME showed ideal fatty acid composition which is accountable for *D. salina* as a competent feedstock for biodiesel production. Its ability to grow in hypersaline environment limits contamination and makes it fit to be commercially cultivated in the saline environment which is not appropriate for other purposes. However, the low lipid content and productivity and the higher energy costs of harvesting are the main economic barrier in their bio refinery process. Efforts are needed to integrate the process of biodiesel production with the co-production of bioactive compounds. Moreover, alteration in physical factors and using advance molecular technology, can make the process more economically feasible.

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