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Culture and Biofuel Producing Competence of Microalgae *Dunaliella salina*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

In the present study halophilic microalgae *Dunaliella salina* obtained from Sambhar Lake, Rajasthan was cultured under controlled laboratory conditions. The biomass recovered at the end of the 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-catalyzed transesterification and the yield was 60.18%. Some specific types of Fatty acid methyl esters (FAMEs) formed were identified by GC/MS analysis. A considerable amount is accounted for four fatty acids palmitic acid (19.6%), oleic acid (25.6%), linolenic acid (27.0%) and linoleic acid (18.4%) The presence of these major fatty acids in the microalgal lipid and their transesterification gives the lipid the characteristic properties to be used for biodiesel.

Keywords: Biodiesel; blight and dyer; Dunaliella salina; FAME; GC-MS.

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ABBREVIATIONS

SFA : Saturated Fatty Acid
MUFA : Monounsaturated Fatty Acid
PUFA : Polyunsaturated Fatty Acid
ASWM : Artificial Sea Water Medium
GC-MS : Gas Chromatography-Mass Spectroscopy

1. INTRODUCTION

Global development has led to the utilization of fossil fuels at a tremendous pace in recent years, and the growing demands have caused the depletion of fossil reserves at a faster rate [1]. An alarming situation that the world struggles with is the rise in temperature of the environment due to an increase in CO_2 concentration from 280 ppm in 1900 to 419 ppm in 2019. In the year 2021 itself, 416.45 ppm of CO_2 concentration was measured, however, it is expected that in the next 25 years, the atmospheric temperature will increase by 1.5–5.9^{0}C [2]. The problems of sustainability of the reserve resources (Fossil fuel) and maintaining environmental equilibrium demand an alternative energy source to tackle these issues. A renewable clean energy source is a prerequisite to meet the global energy demand and reduce the carbon footprint of the environment. In this context, biodiesel has fascinated wide attention due to its renewability and eco-friendly nature [3].

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 a source not suitable for human consumption *viz.* nonedible crop and waste biomass. The third generation emphasized the use of algal biomass [4].

The biofuel produced from lipids derived from microalgae is considered an alternative feedstock to maintain a sustainable environment [5]. Besides being sustainable, microalgae-derived biofuels are non-toxic and biodegradable. Moreover, greenhouse gas (GHG) emission is minimized in microalgal-based biofuel than conventional fuels, establishing them as clean and safe alternatives to fossil fuels [6]. Algae are heterogeneous, predominantly eukaryotic aquatic organisms that vary from single-cell to highly differentiated plants. Microalgae can be found in salt or freshwater which could be a promising source of biodiesel. Microalgae species can accumulate up to 70 % lipid which has the potential to be converted into biofuel [7]. The other benefits of algal-based biofuel in comparison to fossil fuel are high oxygen levels in fuel combustion and very less sulfur emission [8]. However, the price and supply of feedstock are still limiting factors in the production of biodiesel. This problem can be overcome by large-scale cultivation of microalgae and integrating the biorefinery with some byproduct that would make the process cost-effective and provide a sustainable environment for increased microalgal biomass [9].

D. salina is a green, unicellular, and halophilic microalga that can be found in marine waters, salty ponds, and sea salt fields. According to Yu et al*.* [10] *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 [11]. The species of *Dunaliella* also have the potential to fix atmospheric CO_2 and wastewater remediation [12]. Some strains of *Dunaliella* accumulate a high concentration of total lipids and under stress conditions produce a high amount of β-carotene and these strains are used commercially in the production of different products [13]. *D. salina* was used in the study because it utilizes inorganic nutrients from the medium. It is a motile species and can sustain high salt concentrations, it can be cultivated easily and 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.*

2. MATERIALS AND METHODS

2.1 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 according to Stanier and Cohen- Bazime [14] of microalgae *D. salina* was performed on Artificial Sea Water Medium (ASWM) [15] by serial dilution. Purification cultures were identified by the

following Prescott [16] manual. 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 a culture room illuminated by a fluorescent lamp (4000 lux) for 12 hrs. The temperature was maintained in the range between 23-25°C for the entire period of culture. The cultures were constantly shaken and the salinity was maintained at 12% throughout the entire period of growth. All chemicals used were of analytical grade (Merck, India).

2.2 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 levels of growth in the culture medium. The Optical density was taken at 660 nm and the dry weight of the algal sample was measured. The cultures were incubated in the culture room and before the start of the 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.

2.3 Lipid Extraction

The lipid extraction was done using Bligh and Dyer's [17] methods. The algal cells pellet stored at -20˚ C were used for the extraction of lipids. 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 lipids are mixed in a ratio of 2:1. The lipids present are separated in the chloroform layer (bottom layer), and the 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 containing the lipid. Finally chloroform layer is washed many times with a 10% NaCl solution. The solvent is removed by evaporation under reduced pressure and finally, algal lipid is obtained. The lipid obtained was estimated and stored at -20°C under nitrogen for subsequent analysis.

2.4 Fatty Acid Esterification and GC/MS Analysis

Acid-catalyzed 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 analyzed by a GCMS QP-2020 Plus (Shimadzu) system. A flow rate of 1 ml/min was 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 an 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.

2.5 Statistical Analysis

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

3. RESULTS AND DISCUSSION

D. salina was cultured in an *invitro* system. In the present study, the exponential phase is obtained on the 13th day where maximum growth of the cells and the biomass is observed and the culture appears dark green in the flask. The microalgal cells were harvested at that time and the biomass dry wt. 1.25 g/l was obtained. The medium taken was Artificial Sea Water Medium (ASWM) and the culture condition with regular shaking was useful in the appropriate growth of the culture. The growth rate of the microalgae affects the quality and amount of the biomass and it is observed higher growth rate enhances the biomass in a short span of time [18]. Simultaneously the proper selection of microalgal strain and the cultivation medium and various

chemical and environmental factors play a major role in the accumulation of lipids in microalgal strains [19].

Table 1. 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

Table 2. 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

The lipid content for the dry biomass extracted by Blight and Dyer method was found to be 22.4 %. Similar results were reported by other scientists Rizwan et al. [20] reported 35% lipid content in *D. tertiolecta* and Fawzy and Alharthi, [21] examined optimum media for the accumulation of lipids in *D. parva*. Further, the lipid content was studied by other researchers in *D. salina* [22,23]. The lipid extracted was converted to FAME by trans-esterification with an average biodiesel yield of 60.18%. 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 1. 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. The transesterification of the triacylglycerol (TAG) and other lipids produced by microalgae into fatty acid methyl esters (FAME) are precursors of biodiesel [24]. The characteristic and rating of biodiesel depend on the quality and quantity of FAME, and the most common lipids which are accountable for giving the characteristic property are palmitic acid (C16:0), linoleic acid (C18:2) and γ-linoleic acid (C18:3) [25]. Similarly, Bredda et al. [26] reported the presence of two fatty acid majorly oleic acid (36.52%) and palmitic acid (36.52%) in the FAME analysis from *D. salina*. 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. [27] Pavon-Suriano et al. [28] disclosed that 30.35% and 21.61% of palmitic acid and palmitoleic acid is present respectively in *D. salina*. Arunachalam Sivagurulingam et al. [29] found that in *D. salina*, the major proportion is dominated by the presence of two important fatty acids viz palmitic and linolenic acids.

The lipid composition is evaluated for estimating the quality of biodiesel. Various factors such as length of the carbon chain, its branching pattern, number of double bonds all contribute to the characteristic of biodiesel [30]. The fatty acid composition of the microalgal lipid defines the properties of biodiesel. In the present study, the fatty acid composition is studied and the amount of saturated fatty acid (SFA) was found to be 21.0%, monounsaturated fatty acid (MUFA) (31.9 %), and polyunsaturated fatty acid (PUFA) (47.1 %) is estimated (Table 2). The fatty acid profile in the present study is dominated by saturated and monounsaturated fatty acids which give it the property of good biodiesel as stated by Khadim et al. [31]. The presence of a higher degree of unsaturated fatty acid in the present study is also supported by Islam et al.[32] suggesting the high performance of biodiesel prepared from a fatty acid having a higher percentage of unsaturation shows good performance at lower temperatures due to lower melting point and viscosity. The unsaturated fatty acid present in the lipid which is used in the production of biodiesel will emit a lesser amount of CO, hydrocarbons, and smoke compared to fuel produced from the higher percentage of saturated fatty acid lipids [33]. The presence of a lower level of saturated fatty acid 21% is supported by Sajjadi et al. [34] who found that at a lower temperature, the fuel having low saturated fatty acid content has better fuel properties as the pour point and cloud point of the biodiesel increases.

4. CONCLUSION

In the present study, *D. salina* isolated from Sambhar Lake is studied. It is a fast-growing strain that reaches an exponential phase on day 13$^{th.}$ Blight and Dyer's method was used for the extraction of lipids. 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 a hypersaline environment limits contamination and makes it fit to be commercially cultivated in a saline environment, which is inappropriate for other purposes. However, the low lipid content and productivity and the higher energy costs of harvesting are the main economic barrier in their biorefinery process. Efforts are needed to integrate the process of biodiesel production with the co-production of bioactive compounds.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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