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Improving lipid and biodiesel production from ethyl methanesulfonate induced mutated strain of *Dictyosphaerium ehrenbergianum* Nageli

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Abstract
Biodiesel is a renewable, completely burning, non-toxic and eco-friendly fuel commonly derived from microalgal lipid. The present study aimed to isolate mutated strains of *Dictyosphaerium ehrenbergianum* (M-DE) and evaluated its enhanced lipid production and biodiesel efficiency. The mutagen ethyl methane-sulfonate (EMS) was used to induce mutation. The M-DE was mass cultivated in a bioreactor, and its fatty acid profile was analyzed using gas chromatography-mass spectroscopy (GC-MS). The total cytosolic lipid content was increased compared to the unmutated strain of *D. ehrenbergianum* (U-DE). In GC-MS analysis, a total of fifteen different fatty acids were identified from the lipid of M-DE, among which palmitic acid was recorded as a major fatty acid. The kinematic viscosity, flashpoint, density at 15°C, ash contents, and an acid number of M-DE were comparable with the recommended values of Indian and US petroleum, petroleum products, and lubricants sectional committee. The obtained results confirm that M-DE could be used as an efficient alternative source for the production of biodiesel on large scale.

Keywords: *Dictyosphaerium ehrenbergianum*, mutation, lipid production, biodiesel analysis

Introduction
In the last few decades, the overconsumption of fossil fuels leads to increased global warming, air pollution, acid precipitation, ozone depletion, and emission of radioactive substances [1, 2]. The emissions of toxic elements viz., carbon dioxide (CO_2), nitrogen dioxide (NO_2), sulfur dioxide (SO_2), and carbon monoxide (CO) during the combustion of fossil fuel cause serious health complications including chronic asthma, chronic bronchitis with low lung functioning, and cardiovascular diseases [3]. The rapid increase of these gaseous pollutants followed by depleted supplies of fossil fuels leads to an increased commercial interest in the production of alternative eco-friendly fuel [4]. Biodiesel is one of the renewable, completely burning, non-toxic and eco-friendly fuels commonly produced from microalgae [5].
Biodiesel production from microalgae has gained extensive interest in recent years due to its rapid and vigorous growth rate, high efficiency for lipid production, and a broad range of habitats with high adaptability to heat, cold, drought, salinity, osmotic pressure, and UV radiation [6]. Microalgae are autotrophic, fast reproducing, unicellular, prokaryotic, or eukaryotic microorganisms, and they have the potential to produce 5,000–15,000 gallons of biodiesel per acre per year without affecting the environment [7]. Microalgae are rich in unsaturated and saturated fatty acids such as palmitic, stearic, and linoleic acid which are essential for the production of high-quality biodiesel [7, 14]. The biodiesel property of U-DH has been previously reported in our study [14]. In the present investigation, an attempt has been made to evaluate the enhanced lipid production and biodiesel properties of M-DH and the obtained results are presented in this paper.

Materials and methods
Isolation of M-DH
The U-DH isolated from the freshwater sample was collected for mutation study. The complete methodology for isolation and identification of U-DH was published in our previous paper [14]. The identified U-DH was cultured in 500 mL BBM broth, providing 2% CO_2 and 2000 lx light intensity. The mid-exponential phase culture was subjected to mutation studies [15]. Briefly, five ml culture (6×10^6 cells/ml) was harvested by centrifugation at 10,000 rpm for 10 min (Avanti J-E centrifuge, Beckman Coulter Life Sciences, Indianapolis, USA), and the collected cell pellets were treated with 500 µl of 0.1 M sodium phosphate buffer (pH 7.0) and different concentrations of ethyl methane-sulfonate (EMS) (0.75, 1.25, 1.5 and 2.0 % w/v) (Sigma-Aldrich, USA) for 1 h with gentle agitation under dark condition. Then, EMS was inactivated by adding 500 µl of 10% (w/v) sodium thiosulfate and subjected to centrifugation. The collected cell pellet was washed twice with 0.1 M sodium phosphate buffer, then re-suspended in 1 ml of fresh BBM broth and incubated for 48 h. at 30ºC. After 48 h. incubation, again repeated the same process up to three times. The known amount of EMS treated cell suspension was spread uniformly on a BBM agar plate and kept for incubation up to 14 days at 30±2 °C. The colonies that appeared on BBM agar plates were isolated, sub-cultured in BBM broth up to 48h, centrifuged, and the cell pellet was collected.
The collected EMS treated cell pellet was washed separately by adding 1000 µl of sheath fluid and centrifuged at 2000 rpm for 10 min, then again re-suspended with 500 µl of sheath fluid and 50 µl of Nile red stain, and identified mutated cells by analyzing intrinsic structural parameters using a flow cytometer (BD Biosciences, San Jose, CA) [16]. The cells of M-DH were harvested and subjected for large-scale cultivation using a laboratory-scale bioreactor (5 L capacity). The cultural condition employed for cultivation is as follows: 4 L medium (BBM broth: sterile pond water 1:10 v/v), 2% CO_2, and 2000 lx light intensity was provided by four 1500 LMPF CFL fluorescent bulbs at 16:8 light/dark

conditions. Fourteen days old culture was used for the estimation of biomass and lipids.

Estimation of biomass and lipid of M-DH

The biomass was estimated using the procedure of Bagchi *et al*. [17]. Briefly, One hundred mL of culture broth was harvested by centrifugation at 10,000 rpm, dried at 105 °C overnight, and weighed for the determination of dry weights. The biomass yield was calculated and expressed as g/L. For lipid estimation, five hundred ml of M-DH cultures broth was harvested by centrifugation at 10,000 rpm, and the collected pellets were dried using a vacuum evaporator (Lyoquest-85, Telstar Technologies, S.L. Terrassa, Spain) and subjected to lipid estimation using the gravimetric method [14, 18]. The lipid yield (w/w) was expressed in percentage (g/100 g biomass)

Chemical profile and biodiesel properties analysis of fatty acid methyl ester (FAME) of M-DH

The FAME extracted from M-DH was subjected to GC-MS analysis for chemical profile analysis [14]. The fatty acids were identified by comparing the chromatogram peaks with the NIST Mass Spectral Library. Further, the FAME of M-DH was subjected to biodiesel property analysis following the procedure described by the American Society for Testing and Materials (ASTM) [19]. The kinematic viscosity was determined as per the procedure of IS:1448 (Part-25) and ASTM D 445. The flash Point was analyzed as per the test procedure of IS:1448(Part-66) and ASTM D 93. The total acid number was determined as per the test procedure of IS:1448(Part-2) and ASTM D 664. The ash content was analyzed using the procedure of ASTMD482. The density of the FAMEs was determined following the procedure of IS:1448(Part-16) and ASTM Method D1475. The obtained data were compared with Indian standard testing methods for petroleum, petroleum products, and lubricants sectional committee values (IS: 1448 (Part-25), IS: 1448 (Part-66), IS: 1448 (Part-2), IS: 1448 (Part-16) and US standard methods of testing for petroleum, petroleum products, and lubricants sectional committee values (ASTMD482).

Results and discussion

The U-DH isolated from the freshwater sample was identified using 18S rDNA nucleotide sequence analysis, and its sequence was submitted to NCBI gene bank (India) (accessory number SAMN07187741) [14]. In flowcytometric analysis, the EMS treated U-DH shows a twofold shifting of pick from left to right confirms the mutation of the cells (Figure 1). The number of viable cells in the control set was 1557 CFU, whereas in EMS treated sets were 867, 369, 125, and 0.0 CFU at 0.75, 1.25, 1.5, and 2.0 % treatments, respectively. No viable cells were observed in the samples treated with 2.0 % EMS. The 1.5% EMS treatment M-DH cells showed the presence of a significant amount of lipid in their cytosol were selected for mass cultivation. In microscopic evaluation, approximately 90-100 % of M-DH cells showed the presence of orange-yellow colored lipid granules, whereas around 40-65% of U-DH cells showed the presence of lipid granules. Similarly, increased lipid content was observed in M-DH (48.6±3.6%), compared to the U-DH (36.8±2.8%). The biomass productivity was slightly increased in U-DH (2.4 ± 0.3 g L^{-1}) compared to M-DH (2.3 ± 0.4 g L^{-1}). The fatty acid profiles of FAME of M-DH were evaluated using GC-MS. A total of 15 fatty acids were identified, among which palmitic acid (22.63%) and Linolenic acid (13.43) were detected as major fatty acids (Table 1). The FAME of M-DH was subjected to biodiesel property analysis, and the obtained values are presented in Table 2. The obtained results revealed that the FAME of M-DH showed kinematic viscosity 3.58 cSt at 40°C., the flashpoint was 103°C, density at 15°C was 0.86 kg/m³, and ash content was 0.016% with no acid number.

The rapid increase of CO_2 concentration in the atmosphere combined with depleted supplies of fossil fuels has led to an increased commercial interest to produce alternative eco-friendly renewable fuel from natural resources. Biodiesel production from natural sources such as plants and microalgae has gained extensive interest in recent years [20]. Microalgae are more desirable for biofuel production than terrestrial plants because they have higher photosynthetic and oil productive potency than green plants [21, 26]. In the present investigation, we are the first time to report the enhanced lipid production of ethyl methanesulfonate induced mutated strain of *D. ehrenbergianum* (M-DH). The biodiesel property values obtained from the M-DH were comparable with the specified standard values prescribed by the Indian and US petroleum, petroleum products, and lubricants sectional committee. Hence, M-DH could be utilized as an alternative agent for biofuel production.

Table 1: Fatty acid profiles of the FAME of M-DH

Compound	Retention time	% Abundance
Hexanal	4.274	0.54
Dodecyl acrylate (2-Propenoic acid)	15.341	3.42
2,5-Cyclohexadiene-1,4-dione,2,6-bis(1,1-dimethylethyl)	17.87	0.18
Phytol	17.38	1.32
7,10-Hexadecadienoic acid, methyl ester	17.42	1.41
Methyl palmitoleate	17.68	2.67
Palmitic acid	17.86	22.63
Propionic acid	18.56	8.21
Stearidonic acid	19.13	12.8
Linolenic acid	19.28	13.43
9-Octadecenoic acid (Z) methyl ester	26.2096	2.106
Methyl stearate	26.516	3.931
Hexanedioic acid, bis(2-ethylhexyl) ester	29.382	3.575
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	30.402	6.788
Bis(2-ethylhexyl) phthalate	30.847	3.349

Table 2: Biodiesel properties of FAME of M-DH

Property	Biodiesel property	IS:1448 Test method	IS:1448 Limits	EN 14214(EU)	D-6751 (US)
Kinematic Viscosity @40 ◦C(cSt)	3.58	IS:1448(Part-25)	3.5-4.0	3.5-5.0	1.9-6.0
Flash Point(◦C)	103	IS:1448(Part-66)	Min.100	>101	>130
Total acid number (TAN) (mg KOH/g)	Nil	IS:1448(Part-2)	Max. 0.5	<0.5	<0.8
Density @ Room temperature (Kg/m^3)	0.86	IS:1448(Part-16)	0.84-0.90	0.86-0.90	–
Ash content (%)	0.016	ASTMD482	0.01-0.02	<0.02	<0.02

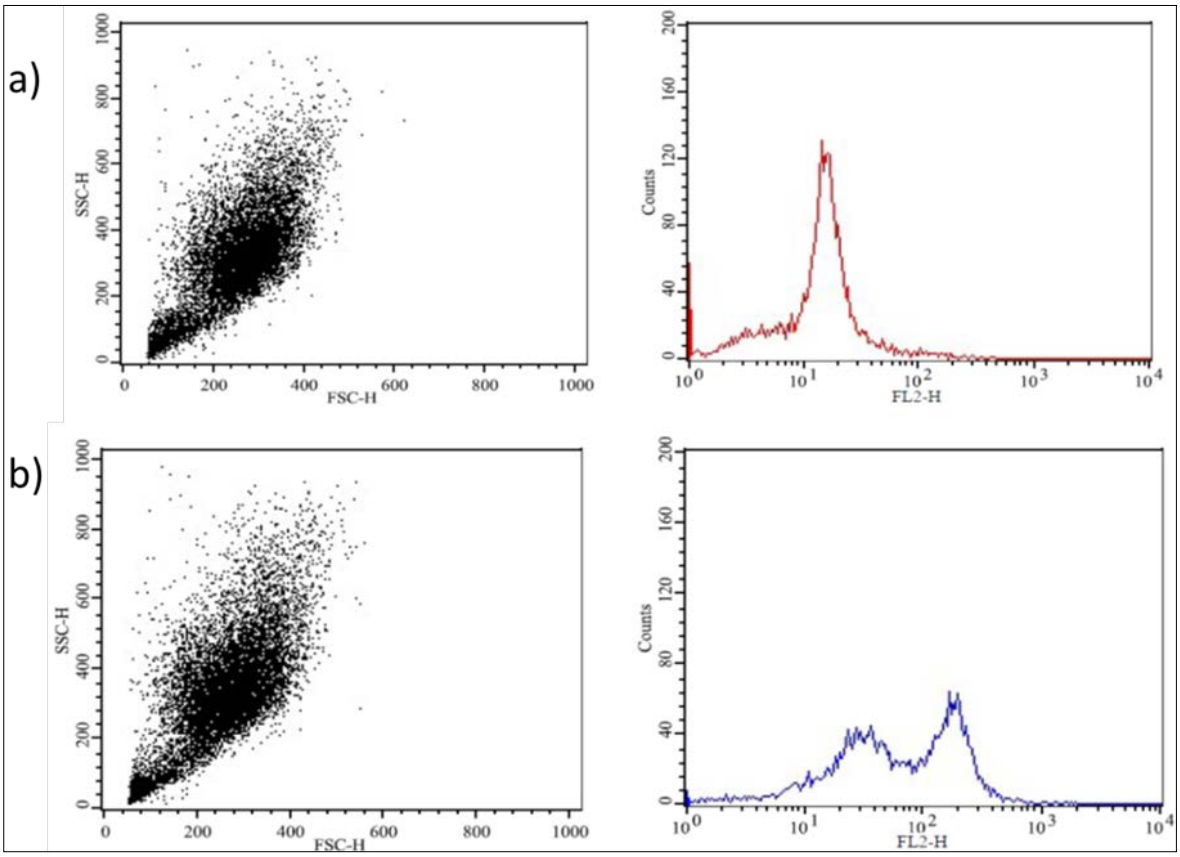

Fig: 1 Two-dimensional dot plots and flow cytograms of a) U-DH and b) M-DH

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Disclosure statement
No potential conflict of interest was reported by the authors.

References
1. Dincer I. Renewable energy and sustainable development: a crucial review. Renewable and sustainable energy reviews,2000:4(2):157-175.
2. Rastogi RP, Pandey A, Larroche C, Madamwar D. Algal Green Energy–R&D and technological perspectives for biodiesel production. Renewable and Sustainable Energy Reviews,2018:82:2946-2969.
3. McMillan JD, Beckham GT. Thinking big: Towards Ideal Strains and Processes for Large-Scale Aerobic Biofuels Production. Microbial Biotechnology,2017:10(1):40-42.
4. Raia RZ, da Silva LS, Marcucci SMP, Arroyo PA. Biodiesel production from *Jatropha curcas* L. oil by Simultaneous Esterification and Transesterification using Sulphated Zirconia. Catalysis Today,2017:289:105-114.
5. Liu J, Huang J, Chen F. Microalgae as Feedstocks for Biodiesel Production. Biodiesel-Feedstocks and Processing Technologies.2011:58:78.
6. Rawat I, Bhola V, Kumar RR, Bux F. Improving the feasibility of producing biofuels from microalgae using wastewater. Environmental Technology,2013:34:1765-1775.
7. Tyner WE. Biofuels and agriculture: a past perspective and uncertain future. International Journal of Sustainable Development and World Ecology,2012:19(5): 389-394.
8. Duong VT, Ahmed F, Thomas-Hall SR, Quigley S, Nowak E, Schenk PM. High protein-and high lipid-producing microalgae from northern Australia as potential feedstock for animal feed and biodiesel. Frontiers in Bioengineering and Biotechnology,2015:3:53.
9. Naghdi GF, Gonzalez ML, Chan W. Progress on Lipid Extraction From w*et al*gal Biomass for Biodiesel Production. Frontiers Microbial Biotechnology,2016:9(6): 718-72.

10. Barry A, Wolfe A, English C, Ruddick C, Lambert D. National Algal Biofuels Technology Review. Bioenergy Technologies,2016:1:69-198.
11. Marcon NS, Colet R, Bibilio D, Graboski AM, Steffens C, Dalla Rosa C. Production of Ethyl Esters by Direct Transesterification of Microalga Biomass Using Propane as Pressurized Fluid. Applied biochemistry and biotechnology,2019:187(4),1285-1299.
12. Rashid N, Rehman USM, and Sadiq M. Current status, Issues and Developments in Microalgae Derived Biodiesel Production. Renewable and Sustainable Energy Review,2014:40:760-778.
13. Lim S, Kuby M. Heuristic Algorithms for Siting Alternative-fuel Stations using the Flow-Refueling Location Model. European Journal of Operational Research,2010:204(1):51-61.
14. Nayana B, Venkatesh HN, Sudharshana TN, Manjunath K, Mohana DC. Isolation and Utilization of *Dictyosphaerium ehrenbergianum* Nageli for Biodiesel Production: Lipid Extraction and Biodiesel Property Analysis. Biofuels,2020:11(8):885-892.
15. Ngampauk V, Chookaew Y, Wipawee D. Ethyl Methane Sulfonate-Induced *Dunaliella salina* KU11 Mutants Affected for Growth rate, Cell Accumulation and Biomass. International Journal of Bioengineering and Life Science,2016:10(7):430-434.
16. Montero M F, Aristizábal M, Reina GG. Isolation of high-lipid content strains of the marine microalga Tetraselmis suecica for biodiesel production by flow cytometry and single-cell sorting. Journal of applied phycology,2011:23(6):1053-1057.
17. Bagchi SK, Rao PS, Mallick, N. Development of an oven drying protocol to improve biodiesel production for an indigenous chlorophycean microalga *Scenedesmus* sp. Bioresource technology,2015:180:207-213.
18. Sathya S, Srisudha S. Isolation and identification of freshwater microalgal strains- Potential for biofuel production. International Journal of Recent Scientific Research,2013:4:1432-1437.
19. ASTM D. Standard specification for biodiesel fuel blend stock (B100) for distillate fuels. In American Society for Testing and Materials,2015:6751-15.
20. Guzman-Lucero, Diego P, Flores T, Rojo R, Martínez-Palou. Ionic Liquids as Demulsifiers of Water-in-Crude oil Emulsions: Study of the Microwave Effect. Energy and Fuels,2010:24(6): 3610-3615.
21. Islam AM, Magnusson M, Brown JR, Ayoko AG, Nabi NM, Heimann. Microalgal Species Selection for Biodiesel Production Based on Fuel Properties Derived from Fatty Acid Profiles. Energies,2013:6:5676-5702.
22. Li T, Zheng Y, Yu L, Chen S. Mixotrophic Cultivation of a *Chlorella sorokiniana* Strain for Enhanced Biomass and Lipid Production. Biomass and Bioenergy,2014:66:204-213.
23. Mostafa SS, Gendy EI. Evaluation of Fuel Properties for Microalgae *Spirulina platensis* Bio-diesel and its Blends with Egyptian Petro-diesel. Arabian Journal of Chemistry,2017:10(2):2040-2050.
24. Babu AG, Wu X, Kabra NA, Kim PD. Cultivation of an Indigenous *Chlorella sorokiniana* with Phytohormones for Biomass and Lipid Production under N-Limitation. Algal research,2017:23:178-185.
25. Nagaraja YP, Biradar C, Manasa KS, Venkatesh HS. Production of biofuel by using micro algae (*Botryococcus braunii*). International Journal of Current Microbiology and Applied Sciences,2014:3(4):851-860.
26. Chi Y, Chen F, Takiguchi Y. Effect of nitrogen source on biomass and lipid production of a marine microalga, *Nannochloropsis oceanica* IMET1. Green Sustainable Chemistry,2015:05:101-106.