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Biodiesel production from microalgae 1 2 Abstract 3 Algal biodiesel represents a promising renewable alternative for reducing greenhouse gas emissions 4 and dependence on fossil fuels due to its exceptionally high oil productivity. In this study, seven 5 algal strains were isolated from diverse natural habitats, of which three microalgae (MG-1, MG-2, 6 and MG-3) were selected for oil extraction using the hexane–ether method. Oil yield was higher at 7 boiling temperature for all strains, with yields of 31 ml, 23 ml, and 16 ml from 2.5 g biomass of 8 MG-1, MG-2, and MG-3, respectively. 9 Microscopic identification revealed MG-1, MG-2, and MG-3 as Cyanobacteria spp.MG1 10 Phormidium spp., and Scenedesmus spp. Followed by molecular identification of most efficient one, 11 the sequence was submitted to GenBank under accession number Cyanobacteria spp.MG1 12 PP702711. Biodiesel was produced via alkali-catalyzed transesterification, yielding 4 ml biodiesel 13 from 5 ml oil of Cyanobacteria spp.MG1 which showed the highest oil and biodiesel productivity 14 among the tested strains. FTIR analysis confirmed the chemical composition of the biodiesel, and 15 partial purification was achieved using a charcoal column. Biodiesel characterization indicated a 16 cetane number of 54, demonstrating that algal biodiesel, particularly from Cyanobacteria spp.MG1 17 is an efficient and eco-friendly alternative to petroleum diesel. 18 Keywords: Microalgae, Algal biodiesel, Transesterification, Renewable biofuels, FTIR analysis
19 20 21 22 23 24 25 26 27 28 29 30 31 32

33 34 35 36 INTRODUCTION 37 The global demand for energy is increasing continuously due to rapid industrialization and 38 population growth. One of the major drawbacks of petroleum-based fuels is the severe atmospheric 39 pollution caused by their combustion. Petroleum diesel combustion is a significant source of 40 greenhouse gas (GHG) emissions and also releases other harmful air pollutants, including nitrogen 41 oxides (NOx), sulfur dioxide (SO_2), carbon monoxide (CO), particulate matter, and volatile organic 42 compounds. 43 Algae are considered one of the most promising feedstocks for biodiesel production due to their 44 exceptionally high oil yield. Algal biomass can produce

up to 250 times more oil per acre than 45 conventional crops such as soybeans and 7–31 times more oil than palm oil. In fact, biodiesel 46 derived from algae may be one of the few viable options capable of meeting current automotive fuel 47 demands. Among algae, microalgae are particularly advantageous because they contain higher lipid 48 content than macroalgae and can be grown rapidly and efficiently. Although the concept of using 49 microalgae as a fuel source is not new, it has gained renewed attention due to the escalating prices 50 of petroleum fuels and growing concerns about global warming associated with fossil fuel 51 consumption. However, limited literature is available on biodiesel production from macroalgal 52 species such as Oedogonium and Spirogyra. Therefore, the present study was undertaken to 53 investigate optimal transesterification conditions, biodiesel yield, and key physical properties— 54 including ester yield, glycerin formation, and sediment content—of biodiesel produced from algae 55 (A.B.M. Sharif et al., 2008). 56 Biodiesel is defined as mono-alkyl esters of long-chain fatty acids derived from vegetable oils, 57 animal fats, or waste cooking oil. It is renewable, non-toxic, biodegradable, non-flammable, and 58 environmentally friendly. Compared to conventional petroleum diesel, biodiesel offers several 59 advantages, including higher flash point, improved cetane number, and reduced exhaust emissions. 60 However, biodiesel production from edible crops has raised concerns regarding food-versus-fuel 61 competition. In contrast, algae can be cultivated on non-arable land with minimal freshwater 62 requirements and do not compete with food crops. Moreover, algal biomass productivity is several 63 times higher than that of terrestrial plants (Veeramuthu et al., 2014). 64 The increasing global demand for biofuels highlights the urgent need to identify highly productive, 65 non-food biomass sources for sustainable fuel production. The U.S. Department of Energy– 66 supported Aquatic Species Program (1978–1996) demonstrated the potential of microalgae as a 67 renewable and sustainable feedstock for biodiesel production (Keesoo et al., 2014). 68 Algae also play a crucial role in the global carbon cycle by capturing atmospheric CO_2 and 69 converting solar energy into chemical energy through photosynthesis. Chlorella vulgaris has been 70

widely recognized as a promising biodiesel feedstock due to its rapid growth and ease of 71 cultivation. However, under normal growth conditions, its lipid content is approximately 20% of 72 dry biomass, which may not meet industrial-scale requirements (Lenka et al., 2015). 73 Microalgae can assimilate CO_2 from various sources, including atmospheric CO_2, dissolved 74 carbonates, and industrial flue gases. Biomass energy accounts for approximately 10.4% of total 75 global energy consumption, and nearly 77.4% of renewable energy originates from biomass 76 sources. Algal biomass is considered one of the most efficient bioenergy options due to its 77 adaptability to diverse climatic conditions. Raceway pond systems are regarded as a techno78 economically feasible approach for large-scale algal cultivation compared to photobioreactors 79 (Neda et al., 2019). 80 Microalgae are regarded as an ideal biodiesel feedstock because they are aquatic, non-edible, 81 genetically modifiable, and fast-growing, with productivity 3–35 times higher than terrestrial plants 82 in terms of energy content. Additionally, their cultivation requires less water than conventional 83 crops (Vandana et al., 2015). Lipid content in microalgae generally ranges between 20–50%, 84 making them suitable for biodiesel production. Research has primarily focused on eukaryotic 85 microalgae such as Botryococcus braunii, Chlorella spp., Chlamydomonas reinhardtii, and 86 Nannochloropsis spp., 1 due to their high lipid accumulation. Cyanobacteria, although prokaryotic, 87 are also gaining attention for biodiesel production owing to their rapid growth rates and lipid 88 productivity (Keshini et al., 2014). 89 The efficiency of biodiesel production depends on both feedstock quality and cultivation conditions. 90 While replacing petroleum diesel with biodiesel offers environmental benefits, certain challenges 91 remain, including lower energy output and potential increases in NO_2 emissions. Nevertheless, 92 microalgae-derived biodiesel has gained significant interest due to higher biomass yield, superior 93 photosynthetic efficiency, and faster growth compared to traditional energy crops (Marium et al., 94 2020). On average, biodiesel yields from microalgae are 10–20 3 times higher than those obtained 95 from oilseed crops (Lusia et al., 2008). 96 Hence, the objective of the present

study is to produce renewable, non-toxic, cost-effective, and 97 eco-friendly biodiesel from microalgal biomass using an alkali-catalyzed transesterification process. 98 MATERIAL AND METHODS 99 1.1 Sample Collection 100 Samples were collected from different natural resources such as Lonar Lake, Farm pond, Salim Ali 101 Lake and local marshy (Drywall) places brought to laboratory processing. 102 1.2 Isolation of Microalgae from Natural Resources 103 Microalgae were isolated using the colony-picking method. BG-11 medium (pH 7.2) was inoculated 104 with small algal colonies obtained from collected natural samples (designated MG-1, MG-2, and 105 MG-3) using a sterile inoculation loop. The cultures were incubated under natural sunlight 106 conditions until visible algal growth was observed. Incubation was carried out under light intensity 107 of approximately 1500 lux with a 16:8 h light–dark cycle, and alternatively at 3000 lux under the 108

same photoperiod at 28 °C for 14 days. After growth on Petri plates, individual colonies were 109 repeatedly sub-cultured onto freshly prepared BG-11 medium until pure single strains were 110 obtained (Naila et al., 2020; Krishna moorthy et al., 2020; Bhaskar, 2021; Keesoo et al., 2014). 111 1.3 Cultivation of Microalgae 112 Open ponds are the oldest and simplest systems [1] for mass cultivation of microalgae. In present 113 work, MG-1, MG-2 Algae were cultivated by the open pond method by using plastic tray of 114 near about 1foot depth under natural condition, and daily observed the algal growth. In this 115 system, the shallow pond is commonly with about 1foot deep; algae are cultured dependent 116 circumstances same to the natural environment. (Baig et al. 2022; Lenka et al., 2015). 117 1.4 Evaluation of Micro algal Growth Potential by Cell Dry Weight Measurement 118 Algal biomass was harvested and washed twice with distilled water, followed by drying in an oven 119 at 60 °C for 48 h. Based on dry biomass productivity, promising microalgal strains were selected for 120 further studies. (Zehra et al., 2022). 121 1.5 Identification of Efficient Microalgae 122 Cultivated algal cultures were initially identified based on morphological characteristics such as 123 color and growth appearance in open-pond cultivation (Fig. 5). This preliminary classification was 124 used only to differentiate

isolates at a basic level. Detailed morphological identification was carried 125 out based on cultural and microscopic characteristics. A drop of the water sample was placed on a 126 clean glass slide and examined under an inverted microscope at 10× and 40× magnifications. Algal 127 cell characteristics—including color, basal body, cell arrangement and pattern, length and width of 128 vegetative cells, sheath width, type and position of spores, presence or absence of hormogonia and 129 akinetes, nature of cell type, coil and helical shape, presence or absence of gas vacuoles, and 130 pigment color—were observed. Images were captured using a microscope-mounted camera 131 following the method described by Krishna Moorthy et al., (2020). The most efficient biodiesel132 producing algal isolate was further identified by 16S rRNA gene sequencing using the Sanger 133 sequencing method at Progenome Life Science, Chh. Sambhajinagar, and the obtained sequence 134 was submitted to GenBank under the accession number PP702711. 135 1.6 Algal Oil Extraction 136 Algal oil extraction was performed with minor modifications following Indumathi et al. (2014), 137 Harvind et al., 2014. And A.B.M. Sharif et al. (2008). Dried algal biomass (MG-1, MG-2, and MG138 3) was incubated at 80 °C for 20 min to remove residual moisture and ground thoroughly using a 139 mortar and pestle. The powdered biomass was mixed with hexane and ether (20 mL each) in clean, 140 dry bottles and heated in a water bath for 10 min. After cooling, the mixture was transferred to a 141 separating funnel and allowed to settle for 24 h. The oil layer was then separated and collected. 142 1.7 Biodiesel Production 143 Biodiesel was produced through an alkali-catalyzed transesterification process. Extracted algal oil 144 was heated to 45 °C in a water bath. Sodium methoxide was prepared by dissolving 0.25 g NaOH 145 pellets in 24 mL anhydrous methanol. The methoxide solution was added to the warm oil in a 3:1 146 ratio (methoxide: oil) and stirred vigorously for 90 min at 37 °C. The reaction mixture was 147 transferred to a separating funnel and allowed to settle for 24 h. The upper biodiesel layer was 148

separated, while the lower glycerol and soap layer was removed. Biodiesel yield was

measured and 149 recorded (Indumathi et al., 2014). 150 151 1.8 Characterization of Biodiesel 152 The produced biodiesel was characterized using various physicochemical analyses, including flame 153 test, pH, density, specific gravity, cetane number, aniline point, API gravity, diesel index, and FTIR 154 analysis (Indumathi et al., 2014). 155 1.8.1: Flame Test: 250 µL of biodiesel was ignited in a Petri plate, and the burning duration was 156 recorded. 157 1.8.2: Oil Extraction Efficiency (wt. %) = Mass of oil extracted (grams) / the total mass of dried 158 algae ×100 159 1.8.3: Density: Calculated as mass per unit volume of biodiesel. 160 1.8.4: Specific Gravity: Ratio of biodiesel density to water density. 161 1.8.5: Aniline Point: 162 Determined following standard procedures to assess aromatic content. Take 1 mL each of dry 163 aniline and algal biodiesel in a clean test tube and position the thermometer so that its immersion 164 mark is at the liquid level without touching the tube walls. Stir the mixture rapidly while avoiding 165 air bubbles. If the mixture is immiscible at room temperature, heat at a rate of 1–3 °C/min until 166 complete miscibility is achieved. Then allow the mixture to cool and record the temperature at 167 which uniform turbidity first appears; this temperature is noted as the aniline point 168 1.8.6: API Gravity: Calculated using the formula: = (141.5/Density of biodiesel) –131.5 169 1.8.7: Diesel Index: ={Aniline Point (0F)*0API}/100 170 1.8.8: Cetane Number: Cetane number = Density*Diesel Index+ 10. 171 1.8.9: FTIR Analysis 172 FTIR spectroscopy was performed to identify functional groups and assess the chemical 173 composition of algal biodiesel in comparison with petroleum diesel. FTIR analysis was conducted 174 at the Department of Chemistry, Dr. Babasaheb Ambedkar Marathwada University, Chh. 175 Sambhajinagar. 176 1.9 Partial Purification of Biodiesel 177 The crude biodiesel obtained through alkali-catalyzed transesterification was further purified by 178 column chromatography using activated charcoal as the stationary phase. The purified eluent was 179 collected and used as final biodiesel (Kulkarni et al., 2019). 180 181 182

Farm pond Salim Ali Lake 183 184 185 186 187 2. RESULTS and DISCUSSION 188 189 2.1 Collection of Microalgal samples: 190 In the present study, seven microalgal

samples (Fig. 1) were collected from diverse natural habitats, 191 including Lonar Lake, farm ponds, Salim Ali Lake, and local marshy (drywall) areas. The samples 192 were transported to the laboratory for further isolation and identification of microalgae. Similar 193 approaches for algal sampling have been reported by Baig et al., (2022) from Chasma Achusin, 194 Quetta (Pakistan), and by Hossain et al., (2008), who collected algal samples from the Phycology 195 Laboratory, University of Malaya, Malaysia. 2

196 197 198 199 200 201 202 Dry

wall Lonar Lake

203 204 205 Fig. 1: Microalgal samples collected from different marshy locations 206 2.2 Isolation of Microalgae 207 208 Out of the seven collected samples, three microalgal isolates—designated MG-1, MG-2, and 209 MG-3—were successfully isolated using the colony-picking method (Fig. 2). Isolation was 210 performed after incubation under a light intensity of 1500 lux with a 16:8 h light–dark 211

photoperiod for 14 days. The isolated strains were further cultivated to evaluate their 212 suitability for oil extraction and biodiesel production. Comparable isolation strategies were 213 reported by Ghani et al. (2020) while Dr. Uday Bhaskar (2020) in his work isolated four algae 214 (SP1, SP2, SP3&SP4) in Growth Chamber. 215 216

Fig. 2: Isolated microalgae in a growth chamber 217 2.3 Cultivation of Microalgae 218 The isolated microalgae (MG-1, MG-2, and MG-3) were cultivated using 1 an open pond system. 219 Profuse algal growth was observed after 15 days of incubation under natural light conditions (Fig. 220 3). Similar cultivation methods have been employed by Baig et al. (2022) and Lenka et al. (2015), 221 while Mahya et al. (2013) reported alternative systems such as photobioreactors, heterotrophic 222 cultures, and algae turf scrubbers.

223 224 225 226 227 228 Day1 (MG1) Day8

(MG1) Day15 (MG1) 229 Day1 (MG1) Day8 (MG1) 230

Day15(MG2) 231 Fig. 3: Day-wise growth of cultivated microalgae

232

233 2.4 Evaluation of Micro algal Growth Potential by Cell Dry Weight Measurement 234 After 15 days of cultivation, algal biomass was harvested, washed twice with distilled water, and 235 dried at 60 °C for 48 h (Fig. 4). Dry biomass estimation revealed that MG-2 exhibited the highest 236 biomass yield (20.8 g), followed by MG-3 (9.36 g) and MG-1 (7.45 g) (Table 1). All isolates were 237 selected for further analysis. Similar biomass productivity assessments were reported by Zehra et al 238 ., (2022).

239 Fig. 4: Dried algal biomass in powdered form

240 Table 1. Dry biomass yield of isolated microalgae 241 Sample Dry weight MG-1 7.45g MG-2 20.8g MG-3 9.36g 2.5 Identification of Efficient Microalgae: 242 Preliminary identification of MG-1, MG-2, and MG-3 was performed based on morphological 243 characteristics such as color, growth pattern, and microscopic features observed under an inverted 244 microscope. Based on cellular morphology—including cell size, sheath structure, pigmentation, 245 presence of akinetes, gas vacuoles, and filament structure—the isolates were identified as 246 Cynobacteria spp. (MG-1), Phormidium spp. (MG-2), and Scenedesmus spp. (MG-3) (Fig. 5). 247 Similar identification methods were reported by Ghani et al. (2020), Keesoo et al. (2014), and 248 Zehra et al. (2022). The most efficient isolate (MG-1) was further confirmed by 16S rRNA gene 249 sequencing. Genomic DNA was extracted using a Nucleospin Microbial DNA Kit and amplified 250 using specific primers. Sequencing was performed using the Sanger method, and the consensus 251 sequence was analyzed using BLAST against the NCBI GenBank database. MG-1 showed 99.17% 252 similarity with an uncultured cyanobacterial clone, and the sequence was submitted to GenBank 253 under accession number Cyanobacteria spp.MG1 PP702711. 254 255

MG-1 (Cynobacteria spp.) MG-2 (Phormidium spp.) MG-3(Scenedesmus spp.) 256 Fig. 5: Inverted microscopic

images of isolated microalgae 257 2.6 Algal Oil Extraction 258 Oil extraction was carried out from all three isolates using a hexane–ether solvent mixture under 259 both room temperature and boiling conditions (Fig. 7). Higher oil yields were observed under 260 boiling conditions for Cyanobacteria spp.MG1 PP702711 and Scenedesmus spp., whereas oil 261 extraction from Phormidium spp. was unaffected by temperature (Table 2). The hexane–ether 262 solvent system yielded higher oil recovery compared to acetone or hexane alone. 5 To the best of our 263 knowledge, this study is the first to report algal oil extraction using a hexane–ether mixture under 264 boiling conditions. 265 266 Microalgae +Hexane & Ether solution Oil separation 267 268 2 269 270 271 272

Extracted micro algal oil 273 Fig. 6: Algal oil extraction using hexane–ether solvent 274 Table 2. Oil yield from different microalgae 275 Sr. No. Algae Name Amount of Algae (in gram) Oil extracted(in ml) Room Temp. Boiled 1) Cyanobacteria spp.MG1 PP702711 2.5 20 23 2) Phormidium spp. 2.5 21 16 3) Scenedesmus spp. 2.5 14 31 276 2.7 Biodiesel Production 277 Biodiesel was produced from the extracted oils of all three microalgae using an alkali-catalyzed 278 transesterification process (Fig. 8). Among the isolates Cyanobacteria spp.MG1 PP702711 yielded 279 the highest biodiesel output and was selected for further characterization. From 5 mL of algal oil, up 280 to 4 mL of biodiesel was obtained, demonstrating the efficiency and cost-effectiveness of the 281 process (Table 3). Similar methodologies were reported by Sharif et al. (2008), Indumathi et al. 282 (2014), and Baig et al. (2022). 283 284 285 Fig.No.7: Trans-esterificationofMG-1, MG-2, MG-3. 286 287

288 Cyanobacteria spp.MG1 Phormidium spp. Scenedesmus spp. 289 Fig.8: 1 Biodiesel production from microalgae 290 291

TableNo.3: Amount of biodiesel produced different sample 292 Sr. No . Microalgae name Amount of algal oil (in ml) Amount of Biodiesel(in ml) Room temp. solution Boiled solution Room temp. solution Boiled solution 1 Cyanobacteria spp.MG1 PP702711 5 14 4 5 2

Phormidium spp. 5 14 - 7 3 Scenedesmus spp. 5 14 1.4 0.58 2.8 Characterization of Biodiesel 293 The produced biodiesel was evaluated for physicochemical properties including flame test, 294 density, specific gravity, pH, and cetane number (Table 4). Oscillatoria spp. biodiesel exhibited 295 the highest cetane number (54), indicating superior ignition quality. Density values ranged from 296 0.67 to 0.77 kg/L, consistent with reported biodiesel standards. 297 Cyanobacteria spp.MG1 Phormidium spp. Scenedesmus spp. 298 Fig. 9: Flame test of algal biodiesel 299

TableNo.4: Characterization of Biodiesel 300 Sr. No . Biodiesel obtained from microalgae pH Specific gravity Density /L Cetanenu mber 1 Cyanobacteria spp.MG1 PP702711 RT 6 0.611 0.68 44 Boiled 6 0.772 0.77 54 2 Phormidium spp. Boiled 6 0.672 0.67 49 3 Scenedesmus spp. RT 5 0.722 0.72 29 Boiled 6 0.732 0.73 36 2.9 FTIR Analysis 301 FTIR spectroscopy confirmed that the functional groups present in algal biodiesel closely resembled 302 those of petroleum diesel (Fig 10). Characteristic absorption peaks corresponding to alkanes, 303 amines, nitro groups, and alkenes were observed, validating the chemical compatibility of algal 304 biodiesel as a diesel substitute. Similar observations were reported by Rahman et al. (2017). 305 306 307 Fig No. 10: FTIR Analysis of Produced Biodiesel 308 2.10 Partial Purification of Biodiesel 309 Crude biodiesel was partially purified using column chromatography with activated charcoal. Post310 purification, the biodiesel showed reduced impurities, improved clarity, and increased ignition time, 311 confirming enhanced fuel quality (Table 5). Similar purification strategies were reported by 312 Kulkarni et al. (2019). 313

Fig. No.11: Partial purification of Fig. No. 12: Purified & unpurified Biodiesel 320 Biodiesel column chromatography 321 322 Table No.5: Effect of Purification 2 323 324 325 326 327 328 329 CONCLUSIONS 330 Seven microalgal strains were successfully isolated from diverse natural sources, including Lonar 331 Lake, a farm pond,

marshy dry walls, Panchakki, Salim Ali Lake, Deogiri Fort, and a dry wall 332 sample from Salim Ali Lake. Among these, the most promising isolates—MG-1, MG-2, and MG333 3—were identified under an inverted microscope as Cyanobacteria spp.MG1 PP702711 334 Phormidium spp., and Scenedesmus spp., respectively. 335 Oil extraction using the hexane–ether method at boiling temperature resulted in higher lipid 336 recovery from all three microalgal species. The extracted oils were efficiently converted into 337 biodiesel via an alkali-catalyzed transesterification process. The produced microalgal biodiesel was 338 characterized using key fuel parameters, including cetane number, aniline point, density, diesel 339 index, and FTIR analysis, which collectively confirmed its suitability as a diesel fuel. 340 Among the evaluated strains, Cyanobacteria spp.MG1 PP702711 demonstrated the highest oil yield 341 and biodiesel production, followed by Phormidium spp. and Scenedesmus spp. Partial purification 342 of biodiesel was successfully achieved using activated charcoal. The high cetane number (54) 343 obtained indicates that algal-based biodiesel is an eco-friendly, cost-effective, and promising 344 alternative to conventional petroleum diesel. 345 Unpurified Biodiesel Purified Biodiesel Amt. of Biodiesel Ignition Time Amt. of Biodiesel Ignition Time 100µl 7 sec. 100 µl 15 sec.

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