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ORIGINAL RESEARCH ARTICLE

Isolation and Identification of Microalgal Strains and Evaluation of Their Fatty Acid Profiles for Biodiesel Production

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ABSTRACT

Nowadays, increasing oil prices and climate change concerns, biodiesel has gained attention as an alternative energy source. Biodiesel derived from microalgae is a potentially renewable and carbon–neutral alternative to petroleum fuels. One of the most important decisions in obtaining oil from microalgae is the choice of algal species to use. Two microalgae from a total of 33 isolated cultures were selected based on their morphology and ease of cultivation. Two cultures were isolated from ponds and identified as strains of *Spirulina* sp. and *Chlorella* sp. *Spirulina* sp., reached a growth rate of 1.68 day^{-1} at 680nm and a biomass concentration of 1.57 g dwt L^{-1}, with a low lipid content of 20 %. Under similar environmental conditions, *Chlorella* sp. reached a growth rate of 2.3 day-^{1} and a biomass concentration of 2.28 g dwt L^{-1}, with a relatively high lipid content of 24 % w/w. The fatty acid compositions of the studied species were mainly myristic, palmitic, palmitoleic, oleic, linoleic, g-linolenic, and linolenic acids. Our results suggest that *Chlorella* sp. can be a possible candidate species for producing oils for biodiesel, due to its high lipid and oleic acid contents.

Key words: Biodiesel, Microalgae, *Spirulina* sp. and *Chlorella* sp.

1. INTRODUCTION

Current energy demand is mostly fulfilled by conventional energy resources, such as coal, petroleum, and natural gas. Petroleum- based fuels have limited reserves and are concentrated in certain regions of the world. According to many analysts, at the present staggering rates of consumption, the world fossil oil reserves will be exhausted in less than 50 years. The scarcity of known petroleum reserves makes renewable energy resources more attractive [1]. The most feasible way to meet the growing demand for energy is by utilizing alternative fuels. An alternative fuel to petrodiesel must be technically feasible, economically competitive, environmentally acceptable, and easily available [2]. One such fuel that exhibits great potential is biofuel, in particular, biodiesel [3]. Biofuels are generally considered to have many benefits, including sustainability, reduction of greenhouse gas emissions, regional development, social structure, agriculture and security of supply [4]. Usage of biodiesel will allow for a balance to be achieved between agriculture, economic development and the environment [5].

Extensive studies have been conducted on using vegetable oils as diesel fuel. The focus has mainly been on oils, like soybean, rapeseed, sunflower and safflower oils [2], which are essentially edible in nature. Most recently, research efforts have been aimed at identifying suitable biomass species which can provide high-energy outputs to replace conventional fossil fuels [6]. However, few attempts have been made to produce biodiesel from non-edible sources, like used frying oil, greases, tallow, lard, jatropha, and mahua oils [7–10]. Nevertheless, the cost of biodiesel production is still a major obstacle for large-scale commercial exploitation, mainly due to the high feed cost of vegetable oils [2].

Microalgae have been suggested as a potential feedstock for fuel production because of the number of advantages, including higher photosynthetic efficiency, higher biomass production, and higher growth rates, as compared to other energy crops [11]. Microalgae represent an exceptionally diverse but highly specialized group of microorganisms adapted to various ecological habitats. Many microalgae have the ability to

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produce substantial amounts (e.g., 20–50% dry cell weight) of triacylglycerols (TAG) as a storage lipid under photo-oxidative stress or other adverse environmental conditions. Microalgae with high oil productivities are desired for producing biodiesel. Depending on the species, microalgae produce many different kinds of lipids, hydrocarbons, and other complex oils [12,13]. Not all algal oils are adequate for making biodiesel, but suitable oils commonly occur. In this study, the algal growth rate, biomass production, lipid content, and productivity of microalgal cultures isolated from pond was determined. Furthermore, lipid extract from these isolates and its further converted to biodiesel was investigated.

2. MATERIALS AND METHODS

2.1. Isolation and identification of microalgae

Water samples for microalgae isolation were collected aseptically from sites that appeared to contain algal growth in a fresh water pond near Chidambaram. Ten mL of water samples were transferred to a 500 mL conical flask containing 200 mL of sterilized Zarrouk's medium and BBM medium, then incubated on a rotary shaker at 27 °C and 150 rpm under continuous illumination using white fluorescent light at intensities of 40 lmol m^{-2} s^{-1} for three weeks.

Every two days, the flasks were examined for algal growth using an optical microscope, with serial dilutions being made in Zarrouk's and BBM medium from flasks showing growth. Subcultures were made by inoculating 50 µL culture solution onto Petri plates containing Zarrouck's and BBM solidified with 1.5% (w/v) of bacteriological agar. Further, 50 µL aliquots of the same dilution were placed in the wells of 96 microtiter plates containing 200 µL Zarrouck's and BBM. These procedures were repeated for each of the original flasks. Both the Petri and microtiter plates were incubated at 27 °C under continuous illumination for two weeks. The purity of the culture was confirmed by repeated plating and by regular observation under a microscope. The microscopic identification was done using botanical approaches [15].

Cultivation and microalgal growth analysis

Microalgal suspension in Zarrouk's medium and BBM medium was adjusted to an absorbance of 0.05 at an OD of 680nm. Ten mL of each microalgal species was used as initial algal inoculums. The microalgae were cultivated at 27 °C in 250 mL conical flask containing a working volume (100 mL) of Zarrouk's medium and BBM medium at 150 rpm under continuous illumination using white fluorescent light at intensities of 40 µmol m^{-2} s^{-1} for three weeks. Algal growth was measured by daily changes in optical density at 680nm with a spectrophotometer, which was converted into dry cell weight. Each sample was diluted to give an absorbance in the range of 0.1–1.0 if the optical density was greater than 1.0. Microalgae dry weight per liter (g L^{-1}) was measured according to the method previously reported [21]. Microalgal cells were harvested by centrifugation and washed twice with deionized water. Microalgal pellets were dried overnight at 105 °C for dry weight measurements [22].

Lipid extraction and fatty acid analyses

The total lipids were extracted from the fresh microalgal biomass using a slightly modified method of Bligh and Dyer [23]. In brief, 50 mL of microalgae culture was harvested by centrifugation at 4000 rpm, re-suspended in 1 mL distilled water, the sample was then mixed with 1.25 mL chloroform and 2.5 mL methanol (1:2, v/v) and subjected to sonication for 30 min at maximum power. After sonication, the tubes were incubated overnight at 27 °C at 100 rpm. The next day, an additional portion of chloroform (1.25 mL) was added, and the extraction mixture was sonicated again for 30 min. separate the chloroform and aqueous methanol layers, 1.25 mL water was added and then centrifuged at 4000 rpm for 10 min. The chloroform layer was gently removed from the bottom, and a second extraction was performed by adding 2.5 mL chloroform and vortexing. The chloroform portions were collected and washed with 5 mL 5% NaCl solution and evaporated in an oven at 50 °C to dryness. Thereafter, the weight of the crude lipid obtained from each sample was measured gravimetrically.

Fatty acids were analyzed by modifying the method of Lepage and Roy [24]. The crude lipid (10 mg) was dissolved using 2 ml freshly prepared mixture of chloroform–methanol (2:1, v/v) and transferred to a 10 mL screw cap tube. One mL of chloroform containing nonadecanoic acid (500 g L^{-1}) as an internal standard, and 1 mL methanol and 300 µL sulfuric acid as transmethylation reagents were added to the tube and mixed for 5 min. The tube was incubated at 100 °C for 10 min and cooled to room temperature. One mL water was added for phase separation. The tube was mixed vigorously for 10 min and centrifuged at 4000 rpm for 10 min. After phase separation, the lower phase (chloroform) was transferred to another tube using a hypodermic disposable polypropylene syringe. The organic phase was

filtered. Methyl esters of fatty acids were analyzed with a gas chromatograph. Heptadecanoic acid, and g-Linolenic acid were used as standards. Other reagents used were of analytical grade.

3. RESULTS AND DISCUSSION

Isolation and identification of the microalgae

Microalgae are present in all existing earth ecosystems, not just aquatic, but also terrestrial, representing a large variety of species living in a wide range of environmental conditions [25]. It is estimated that more than 50,000 species exist, but only a limited number, of around 30,000, have been studied and analyzed [26]. In this study, a total of 33 microalgal cultures were isolated, two isolates were selected based on their purity and growth rates. Microscopic observation of the algal isolates revealed its colonial existence and purity (**Table 1**). The two isolated microalgal cultures were identified as the genera *Spirulina* and *Chlorella*, by morphological examination under a microscope based on cell shapes. Chinnasamy *et al*. [27] found that about 27 species of green algae, 20 species of cyanobacteria, and 8 species of diatoms were observed in both treated and untreated wastewaters. Metzger and Largeau [13] reported that, within in each chemical race and for the same strain, the morphology of the alga could vary in relation to age and culture conditions. The morphological heterogeneity of the alga makes the microscopic examination difficult.

Growth rate of microalgal strains

Under suitable conditions and sufficient nutrients, microalgae can grow profusely. Commonly, they double their biomass within 3.5 h or 24 h during the exponential growth phase [29]. The net growth rate differed among the examined microalgal species (**Fig 1**). Algal growth is directly affected by the availability of nutrients, light, the stability of pH, temperature, and the initial inoculum's density [30]. Under similar environmental conditions, the average specific growth rates of 1.06 ± 0.03 and 1.19 ± 0.17 days^{-1} at 680nm were found for *Spirulina* sp and *Chlorella* sp. The growth rates of *Spirulina* sp and *Chlorella* sp compared with 0.152 ± 0.01 and 0.168 ± 0.004 at an OD of 680 nm after 20 days of incubation. This result indicates that the *Spirulina* sp and *Chlorella* sp strains were suitable for high-density cultures.

Biomass, lipid content and lipid productivity

The two microalgal species were tested for their lipid production by evaluating biomass productivity and lipid content in 250-mL flask laboratory cultures after 21 days of incubation under similar conditions (**Table 2**). Chlorella sp showed the highest biomass productivity at 1.65g dwt L^{-1}, while Spirulina sp showed the lowest biomass productivity at 1.37 g dwt L^{-1}, when compared to the other species. The lipid productivity of Chlorella sp was highest at 0.74 g L^{-1}, when compared with the other tested microalgal species (Table 2). Lipid content data for different algal species are readily available and have been consistently reported in the literature [31]. Many microalgae species can be induced to accumulate substantial quantities of lipids [1], contributing to a high oil yield. In previous studies [32,33], total lipid contents representing 20– 50% of the dry biomass weight were found to be quite common, and some microalgae even exceeded 90% as a response to different culture conditions [34]. Lipid content of freshwater microalgae was near 20%, and the best biomass producers, Chlorococcum sp. UMACC 112 and Scenedesmus sp. DM (0.28 and 0.26 g L^{-1} d^{-1}, respectively), were also the best lipid producers (54 mg L^{-1}d^{-1}) [35].

Fatty acid composition

Fatty acids in the two strains of microalgae were primarily esterified, and the major fatty acid compositions were determined using GC analysis (**Table 3**). In the two tested microalgae, linoleic acid (C18:2n6c), palmitic acid (C16:0), linolenic acid (C18:3n3), oleic acid (C18:1n9c) and g-linolenic acid (C18:3n6) were commonly dominant, which ranged from 11% to 40%, 18% to 33%, 10% to 33%, 2% to 26% and 5% to 17%, respectively, whereas the palmitoleic acid (C16:1) and eicosatetraenoic acid (C20:4) existed as minor fatty acids. The fatty acids Nonanoic (C9:0) and Undecanoic (C11:0) were not detected in any of the tested microalgal species. The most commonly synthesized fatty acids have chain lengths that range from C16 to C18, similar to those of higher plants [36]. Specifically, the major fatty acids were C16:0 and C16:1 in the Bacillariophyceae, and C16:0 and C18:1 in the Chlorophyceae [37].

The total amount of fatty acid of the 2 algae ranged from 0.7 to 4.7 mg g^{-1} dwt. The highest amount of oleic acid (1.2 mg g_1 dwt) and palmitic acid (1.3 mg g_1 dwt) was detected in Chlorella sp. The percentage of the oleic acid, which is known to be a main component of biodiesel, was 26% and 24% of total fatty acids in *Chlorella* sp and *Spirulina* sp. This proportion was closely similar to the 24.8% of oleic acid reported by Ranga Rao *et al*. [38]. Oils with high oleic acid content have been reported to have a reasonable balance of fuel, including its ignition

quality, combustion heat, cold filter plugging point (CFPP), oxidative stability, viscosity, and lubricity, which are determined by the structure of its component fatty esters [39,40]. Therefore, among the tested microalgal species, Chlorella sp showed the highest oleic acid content (1.2 mg g^{-1} dwt), making it the most suitable for the production of good quality biodiesel.

Table: 1 Microscopic observations of Microalgae

S. No	Micro algae	Morphological character
1	*Chlorella* sp.	Oval shape
2	*Spirulina* sp.	Spiral shape

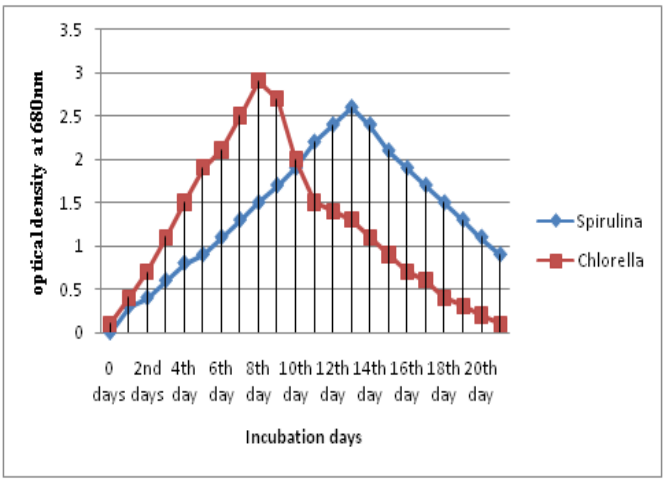
Table 2: Physiological characters of Microalgae

S. No	Microalgal strain	Biomass productivity (g dwt L^{-1})	Lipid Productivity (g L^{-1})	Lipid content (% biomass)
1	*Spirulina* sp.	1.37	0.66	20
2	*Chlorella* sp.	1.65	0.74	26

Table 3: Fatty acid composition of the different microalgal species

S. No	Fatty acid	Fatty acid composition (wt %)	
		***Spirulina* sp.**	***Chlorella* sp.**
1	C9:0	0	0
2	C11:0	0	0
3	C14:0	1 (0.05)[a]	2 (0.05)
4	C16:0	23 (1.2)	20 (0.6)
5	C16:1	0	2 (0.04)
6	C18:0	0	0
7	C18:1n9t	0	0
8	C18:1 n9c	24 (1.2)	17 (0.5)
9	C18:2 n6c	14 (0.6)	18 (0.5)
10	C18:3n6	5 (0.2)	8 (0.2)
11	C18:3n3	33 (1.5)	33 (1)
12	C20:4	0	0
	Total	100 (4.7)	100 (2.9)

Figure 1: Growth rate of microalgae in different incubation time



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