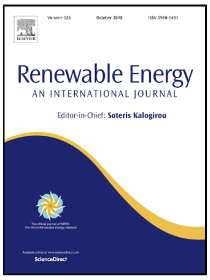

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Screening of different species of *Scenedesmus* isolated from Egyptian freshwater habitats for biodiesel production

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ABSTRACT

Nowadays, microalgae are widely discussed as a promising feedstock for biodiesel production due to the legitimate concerns about the consequences of using edible oils. Selection of the most suitable microalgal species relies on several key parameters such as growth rate, lipid productivity and fatty acid profile. In the present study, different species of *Scenedesmus* were isolated and compared for their efficiency as biodiesel feedstocks. *S. obliquus* showed the highest biomass productivity of 0.102 g CDW L^{-1} day^{-1} at stationary phase. However, *S. intermedius* showed the highest significant lipid content of 400.9 mg g^{-1} CDW. Regarding lipid productivity, *S. obliquus* was the most lipid productive strain at stationary phase with up to 24.94 mg L^{-1} day^{-1}, representing 23.9% significant increase over that of *S. intermedius*. In addition, cetane number and iodine value of *S. obliquus* FAMEs were 54.12 and 110.37 g I_2/100 g, respectively. Moreover, FAMEs of *S. obliquus* showed kinematic viscosity and specific gravity of 1.9–6.0 mm^2 s^{-1} and 0.88 g cm^{-3}, respectively, which are in accordance with the international standards. Among the different studied species of *Scenedesmus*, the present study nominated *S. obliquus* as a promising renewable feedstock for biodiesel production.

Keywords: Microalgae, *Scenedesmus* sp., Biomass productivity, Lipid productivity, Biodiesel.

1. Introduction

Due to the increased population and industrialization in recent years, the energy need is increasing continuously. The continued use of fossil fuel is now widely recognized unsustainable because of its depletion and significant contribution in carbon dioxide emission and, consequently, the global warming. During the last decade, many studies have been conducted on biofuel for substituting fossil fuel and reducing the greenhouse gas emissions [1,2]. However, biodiesel is gaining more attention as one of the most vital substitutions for the depleting fossil diesel. There are many advantages of using biodiesel; 1) it can be used directly in the existing diesel engines blended with petroleum diesel without any modifications in the infrastructure with better engine performance [3,4]; 2) biodiesel is highly biodegradable and is nontoxic as well as renewable; 3) the combustion properties of biodiesel are also very close to those of petroleum diesel [5]; and 4) the exhaust of biodiesel during combustion has lesser carbon monoxide, sulfur dioxide, hydrocarbons and particulate matter as compared to those of fossil diesel [3]. Using of crop seeds for biodiesel production dramatically affects huge land areas that are presently harnessed to human food production and, currently, has many ethical and environmental issues. One possibility to overcome this problem is to use microalgae for biofuel production.

Microalgae utilization as a feedstock for biodiesel has several advantages compared to higher plants, mainly they have higher photosynthetic activity and growth rates, higher ability to capture CO_2, and the ability to grow on arid lands using sea water or wastewater [6]. Recent research efforts have been focused on increasing lipid content of microalgae by optimization of growth conditions [7-11] or screening of freshwater and marine microalgae to select the most suitable organism [12,13]. Oil of microalgae has various advantages concerning degree of fatty acids saturation, chain length, proportion of triglycerides, and the close constitution with vegetable oils which makes it suitable, or sometimes superior, for biodiesel production [2]. Thus, microalgae presently receive an increasing interest as the most appropriate biomass for renewable energy production representing the third-generation biofuel feedstocks.

Lipid productivity is defined as the mass of lipids produced per unit volume of the microalgal culture during a specific time, which depends on both oil content and algal growth rate [7]. Therefore, it is necessary to identify adequate species of oil-rich microalgae for economically-feasible biodiesel production [14]. A key challenge for microalgal biodiesel production is the isolation of microalgal species that can maintain a high growth rate with high lipid content. This major challenge can be addressed via extensive screening of microalgae to select the most appropriate species. Some species belong to the genus *Scenedesmus* were previously discussed as promising candidates for biodiesel production. For example; Abomohra et al. [12] screened thirteen freshwater microalgae for biodiesel production and suggested *S. obliquus* as a promising microalga for large-scale biodiesel because of its high growth rate which resulted in high lipid and fatty acid productivities. Gressler et al. [15] reported the efficiency of *S. subspicatus* as biodiesel feedstock due to its high oil content. Anand and Arumugam [16] and Sharma and Chauhan [17] confirmed the efficiency of *S. quadricauda* for biodiesel production under nitrogen starvation and genetic transformation, respectively, which led to significant accumulation of triglycerides. Moreover, Wibul et al. [18] concluded the high energy efficiency and positive environmental impact of biodiesel produced from *S. intermedius* through life cycle assessment. Furthermore, Mandotra et al. [19] and Mandotra et al. [20] confirmed the potential of *S. abundans* as a feedstock for biodiesel in terms of lipid yield and biodiesel properties. However, growth and lipid content significantly differ for the same species under different conditions. Therefore, it is important to comparatively screen different species of the same genus under the same growth and analytical conditions in order to nominate the most promising species for biodiesel production. For the best of authors' knowledge, isolation, screening and comparison of different species of the genus *Scenedesmus* under the same conditions was not previously studied. Thus, the present study aimed to screen some species of *Scenedesmus* isolated from local freshwater habitats in order to evaluate and compare their potential for biodiesel production.

2. Materials and Methods

2.1. Microalgae isolation and growth conditions

Microalgae used in the present study were isolated from water samples collected from River Nile at Benha city, Egypt. Petri-dishes containing sterilized Bold's Basal agar medium [21] were inoculated with the water sample, spread on the medium surface and incubated for 7 days at 28 ±2 ºC. Cultures were illuminated by tubular fluorescent lamps (PHILIPS Master 85W/840) with light intensity of 2500 Lux at the surface of the plates. After incubation, individual colonies were picked up and re-cultivated on fresh medium and incubated at the aforementioned conditions. Sub-culturing was performed for several times until complete purification of the desired microalgal species. Purification using antibiotic was used whenever required using a mixture of 30 ppm streptomycin and 30 ppm tetracycline for 20 minutes. Isolated *Scenedesmus* species were identified morphologically using light microscope and scanning electron microscope.

2.2. Microalgal identification

Microalgal species were identified using light microscope [22,23]; and the identification was confirmed using scanning electron microscope (SEM) [24]. Briefly, 300 µL of culture was filtered and washed 3 times in sterilized saline. Samples were dehydrated in a series of increasing alcohol concentrations (25%, 50%, 75%, 95%, and 100%; 10 min/step). Dehydrated cells were coated with a golden layer in a sputter system, then examined using SEM (JSM-6510LV, JEOL, USA).

2.3. Screening of microalgae for biomass and lipid accumulation

For each isolate, three 1 L conical flasks containing 700 mL of liquid BBM medium were inoculated at initial optical density (OD_{680}) of 0.05. All flasks were incubated at the pre-mentioned conditions and aerated through a narrow glass tube with a bacterial filter of 0.2 µm. Optical density, dry weight and lipid content were measured at four days intervals. All experiments were performed under sterilized conditions.

2.4. Growth measurement

Algal growth was monitored using optical density (OD_{680}) at the highest absorbance peak of chlorophyll [25], and by determination of algal cellular dry weight (CDW) [26]. Briefly, 10 mL of algal culture were collected, filtered using 0.45 µm filter, and then dried at 105 ºC until constant weight. Biomass productivity was calculated according to the following equation;

$$\text{Biomass productivity (g CDW L}^{-1}\text{ d}^{-1}) = (CDW_E - CDW_I) / T$$

where CDW_I and CDW_E represent the initial CDW (g L^{-1}) and CDW at the desired growth phase after time (T), respectively.

2.5. Determination of total lipid content

Extraction of lipids was done by the modified method of Folch et al. [27]. Briefly, 20 mL of algal culture were collected and centrifuged at 2000x *g* for 10 min. The supernatant was discarded, then 10 mL of chloroform/methanol (2/1, *v/v*) were added into the pellet and kept at room temperature for 2 h with shaking. The homogenate was centrifuged at 2000x *g* for 10 min to recover the liquid phase. The liquid phase was transferred to a new glass vial and washed with 2 mL of 0.9% NaCl (*w/v*) and centrifuged at 200x *g* for 2 min to separate the two phases. The organic lower phase was transferred into a pre-weighed vial, and the lipid extract was dried at 80 °C, cooled in a desiccator, and weighed again. The weight of lipid was determined, and the total lipid productivity was calculated as follows;

$$\text{Lipid productivity (mg L}^{-1}\text{ d}^{-1}) = (L_E - L_I) / T$$

where L_I and L_E represent the initial lipid content (mg L^{-1}) and lipid content at the desired growth phase after time (T), respectively.

2.6. Fatty acid analysis

Esterified fatty acids were analyzed according to the method described by Abomohra et al. [28]. After lipid extraction from hot-water pretreated cells, lipid extracts were dried under a stream of argon. Dried lipid extract was mixed with 167 µL of 0.5 M sodium methoxide and 333 µL of methanol:toluene (1:1, *v/v*) at room temperature. After 20 min incubation, 50 µL of HCl (37%) and 500 µL of NaCl (1 M) were vortexed with the mixture, then fatty acid methyl esters (FAMEs) were extracted by 1.5 mL of hexane. After hexane evaporation under a steam of argon, FAMEs were resuspended in 40 µL of acetonitrile and subjected to analysis by GC-FID (Agilent 7890A) equipped with J&W HP-5 column (30 m x 0.32 mm with 0.25 µm inner film) using helium as a carrier gas. The oven temperature program was started at 140 °C for 3 min, increased at 20 °C min^{-1} until 140 °C, then increased at 4 °C min^{-1} until 260 °C, and then maintained at this temperature for 5 min. Injector temperature was adjusted to 280 °C, while detector temperature was kept at 300 °C.

2.7. Bioprospecting of biodiesel properties based on FAME profiles

Predictive models, based on lipid composition, were used in this study to calculate the important biodiesel properties. Recently, many studied suggested useful equations to calculate the properties of biodiesel based on fatty acid profile [29,30]. In the present study, the equations of Hoekman et al. [29] were selected to estimate the properties of biodiesel, since the calculated values using the equation were reported to be closer to the measured values from biodiesel [31,32]. The average degree of unsaturation (ADU), kinematic viscosity (KV), specific gravity (SG), cloud point (CP), cetane number (CN), iodine value (IV) and higher heating value (HHV) were calculated as previously described by Selvarajan et al. [33].

2.8. Statistical analysis

Experiments were performed in three replicates and results were presented as the mean ± standard deviation (SD). The software SPSS (IBM, v20) was used to perform the statistical analyses using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test at probability level $P \leq 0.05$.

3. Results

Eight species namely *S. opoliensis, S. subspicatus, S. quadricauda, S. microspina, S. acutiformis, S. obliquus, S. intermedius* and *S. abundance* were isolated from the collected water samples (Fig. 1). All species, except *S. quadricauda,* reached the stationary phase after 16 days of incubation (Fig. 2A). *S. obliquus* showed the maximum significant biomass productivity at stationary phase of 0.102 g CDW L^{-1} d^{-1}, while *S. intermedius* showed the minimum recorded biomass productivity (52.9% lower than that of *S. obliquus* at stationary phase, Fig. 2B).

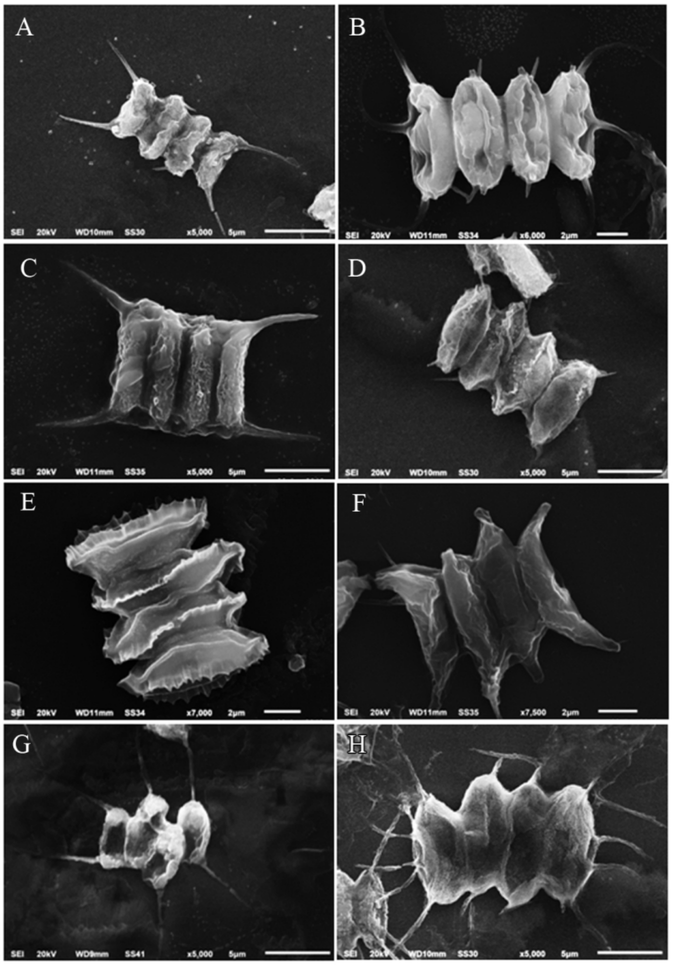

Fig. 1. Scanning electron microscopic examination of different isolated Scenedesmus species showing *S. opoliensis* (A), *S. subspicatus* (B), *S. quadricauda* (C), *S. microspina* (D), *S. acutiformis* (E), *S. obliquus* (F), *S. intermedius* (G) and *S. abundance* (H).

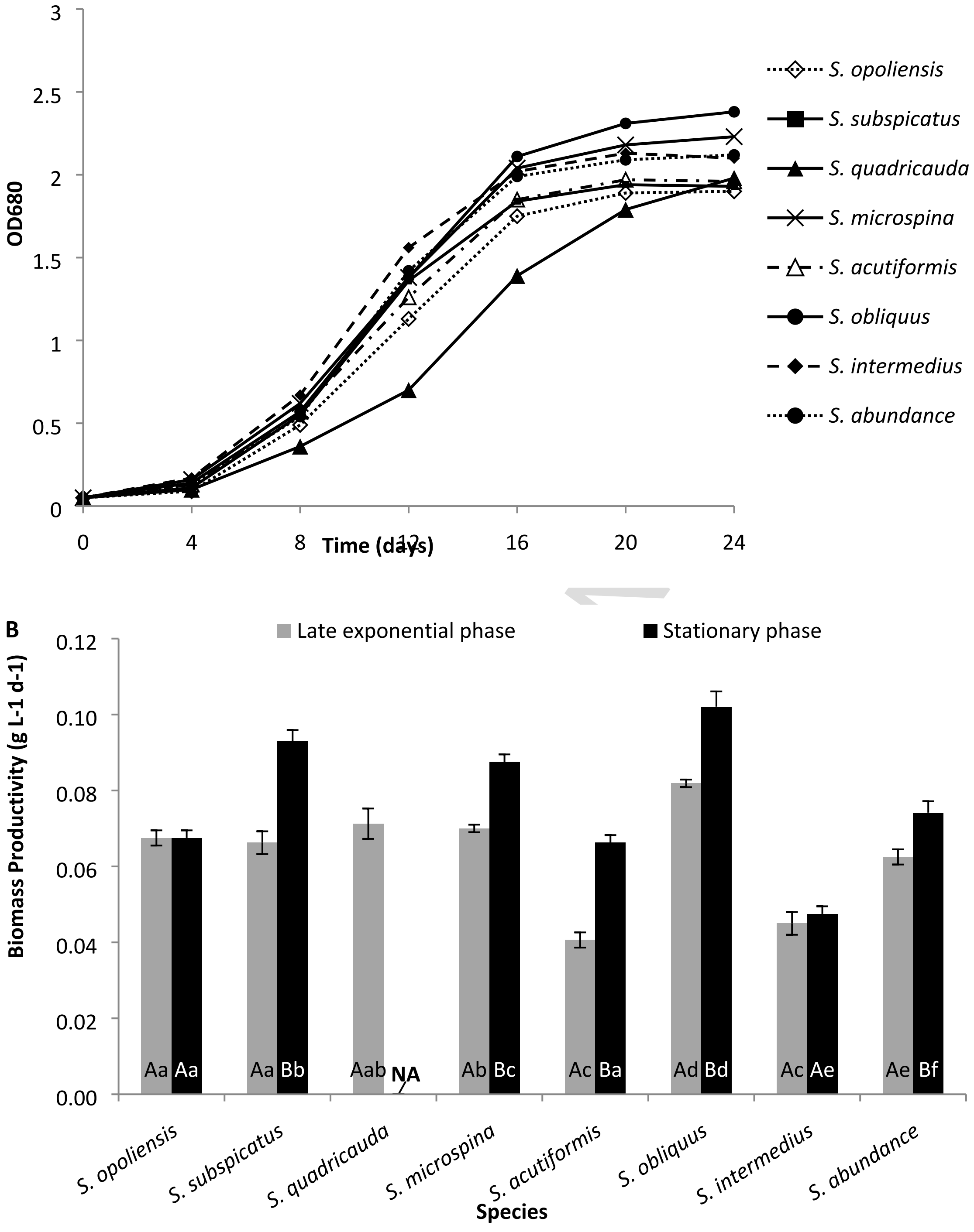

Fig. 2. Growth curves (A) and biomass productivities (B) of the studied isolates at late exponential phase and stationary phase. Error bars show the SD for three replicates. Columns of the same series with the same small letter showed insignificant difference at $P \leq 0.05$. Columns of the same organism at different growth phases with the same capital letter showed insignificant difference at $P \leq 0.05$. NA means not applied.

Lipid content was studied at two time points representing late exponential phases and stationary phase. Over all phases, *S. intermedius* showed the highest significant lipid content of 400.9 mg g^{-1} CDW representing 80.7 and 60.9% over that of *S. obliquus* at late exponential phase and stationary phase, respectively (Fig. 3). From late exponential phase to stationary phase, there was a statistically significant increase (one-way ANOVA, $P \leq 0.05$) in the lipid contents of *S. opoliensis, S. acutiformis* and *S. obliquus* by 14, 16 and 12 %, respectively (Fig. 3). Results of lipid productivity shown in Figure 4 revealed that *S. obliquus* was the most productive strain with up to 24.94 mg L^{-1} day^{-1} at stationary phase. However, significant lower productivities (one-way ANOVA, $P \leq 0.05$) were determined for *S. intermedius* by 19.2% with respect to *S. obliquus* (Fig. 4). Therefore, *S. obliquus* was selected as a promising candidate and used for further examination.

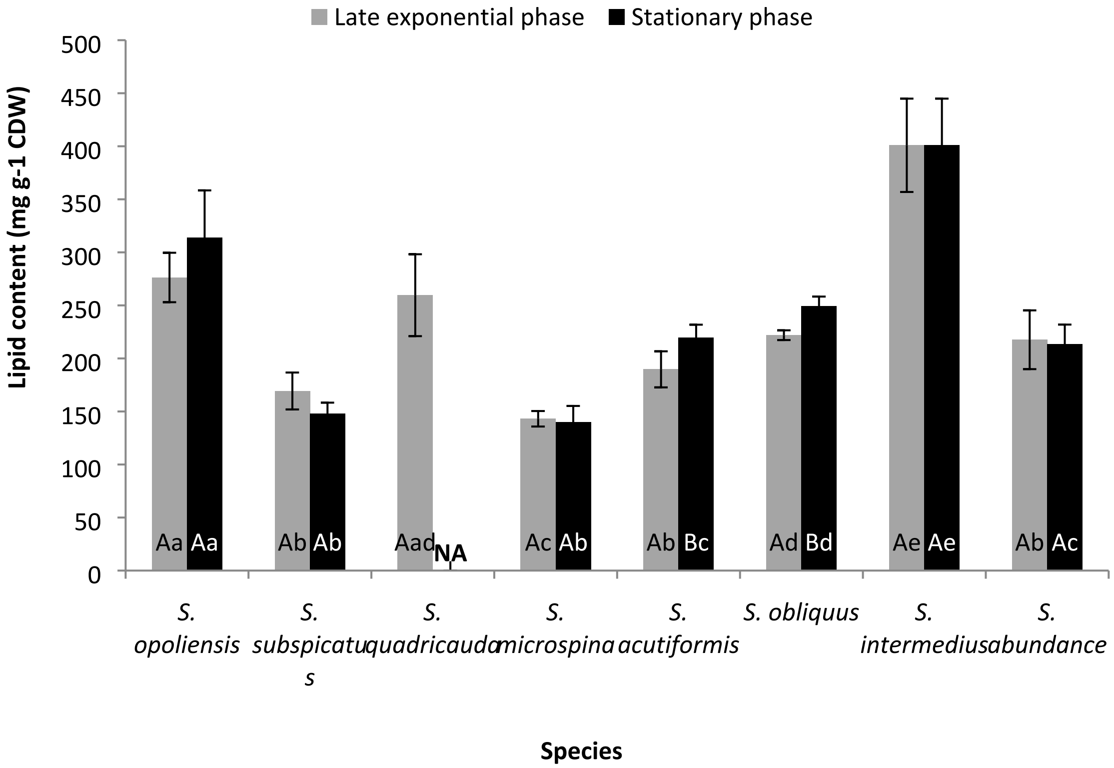

Fig. 3. Lipid content in different isolates during late exponential phase and stationary phase. Error bars show the SD for three replicates. Columns of the same series with the same small letter showed insignificant difference at $P \leq 0.05$. Columns of the same organism at different growth phases with the same capital letter showed insignificant difference at $P \leq 0.05$. NA means not applied.

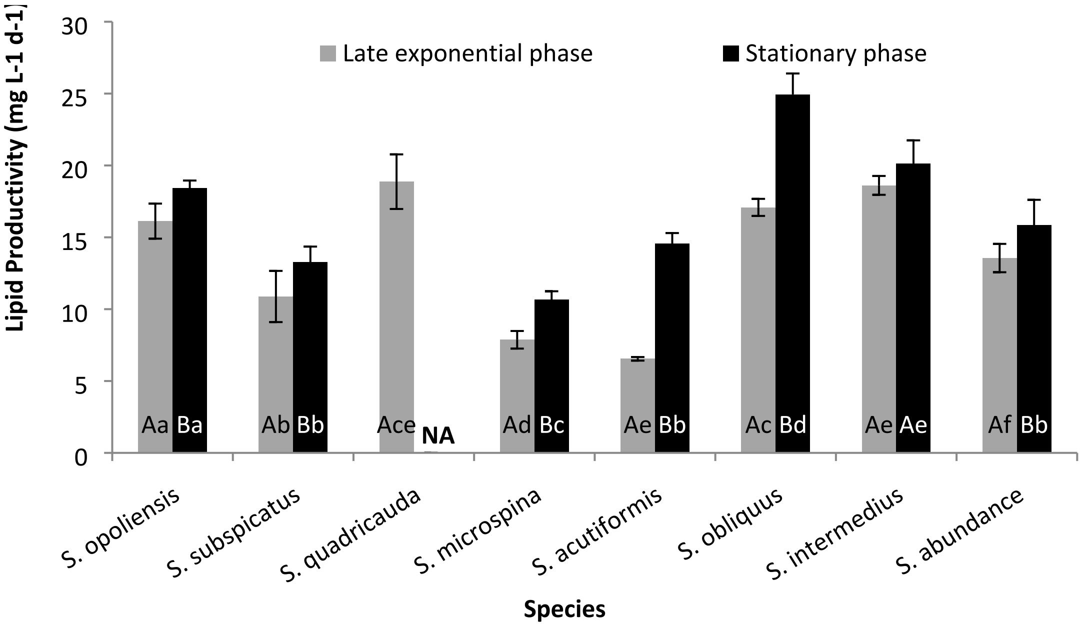

Fig. 4. Lipid productivities of different isolates at late exponential phase and stationary phase. Error bars show the SD for three replicates. Columns of the same series with the same small letter showed insignificant difference at $P \leq 0.05$. Columns of the same organism at different growth phases with the same capital letter showed insignificant difference at $P \leq 0.05$. NA means not applied.

The relative proportions of fatty acids in *S. obliquus* harvested after 24 days of cultivation are presented in Table 1. In general, 7 fatty acids ranging from palmitic acid (C16:0) to linolenic acid (C18:3) were identified. The recorded dominant fatty acids were palmitic acid (C16:0), stearic acid (C18:0), linoleic acid (C18:2) and linolenic acid (C18:3). Polyunsaturated fatty acids (PUFAs) content was 44.22% of total fatty acids, while the content of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) together was 55.78% of total fatty acids (Table 1).

Table 1

Fatty acid percent composition of *Scenedesmus obliquus* grown for 24 days in Bold's basal medium.

Fatty acids	Name	Percent
C16:0	Palmitic acid	16.10
C16:1	Palmitoleic acid	3.51
C17:0	Margaric acid	5.20
C18:0	Stearic acid	16.43
C18:1	Oleic acid	14.54
C18:2	Linoleic acid	19.40
C18:3	Linolenic acid	24.82
Saturated fatty acids (SFAs)		37.73
Monounsaturated fatty acids (MUFAs)		18.05
Polyunsaturated fatty acids (PUFAs)		44.22

Structural features of fatty acids; such as chain length and degree of unsaturation; significantly influence the physical and chemical properties of biodiesel. Physical and chemical properties of biodiesel of the promising species, *S. obliquus*, are shown in Table 2 with that of the ASTM D6751-08 [34] and EN14214 [35] standards for biodiesel characterization. The minimum CN value should be 47.0 and 51.0 according to ASTM D6751-08 and EN14214 standard, respectively; and the maximum IV should be lower than or equal to 120 g I_2/100 g according to EN 14214. Values of CN and IV in *S. obliquus* showed 54.12 and 110.37 g I_2/100 g, respectively. KV and SG limits are 1.9–6.0 mm^2 s^{-1} and 0.85–0.9 kg^{-1}, respectively, as for ASTM6751-08. FAMEs of *S. obliquus* showed KV and SG of 1.9–6.0 mm^2 s^{-1} and 0.88 kg^{-1}, respectively. Furthermore, the values of four or more double bonds complied with that of ASTM standard of ≤1 wt% (Table 2).

Table 2

Properties of biodiesel obtained from *Scenedesmus obliquus* grown for 24 days in Bold's basal medium.

Properties	*S. obliquus*	ASTM D6751-08 [34]	EN14214 [35]
ADU	1.31	-	-
Kinematic Viscosity (mm^2 s^{-1})	4.38	1.9–6.0	3.5–5.0
Specific gravity (kg^{-1})	0.88	0.85–0.9	-
Cloud point (ºC)	2.46	-	-
Cetane number	54.12	Minimum 47	51-120
Iodine value (g I_2/100 g)	110.37	-	Maximum 120
HHV (MJ kg^{-1})	40.85	-	-
Db ≥ 4 (wt %)	0.00	≤1	-

ADU: average degree of unsaturation
HHV: higher heating value
Db: Double bonds

4. Discussion

Microalgae are widely discussed as an important source for third generation biofuels, especially as renewable and environmental-friendly alternative feedstock for biodiesel production [36]. In the present work, different species of the freshwater green microalga *Scenedesmus* were isolated and screened not only on the basis of high lipid content, but on the basis of lipid productivity which is controlled by the lipid content and growth rate. *S. intermedius* showed the maximum recorded lipid content at both late exponential phase and stationary phase. However, the maximum biomass productivity was recorded for *S. obliquus* which resulted in the highest lipid productivity. This is in agreement with a previous study of Abomohra et al. [12] who screened 13 freshwater microalgae for their efficiency as biodiesel feedstock. They named the green microalga *S. obliquus* as a promising candidate for large-scale lipid production due to its high biomass yield which resulted in high lipid productivity. However, lipid productivity of *S. obliquus* at stationary phase was significantly higher than that at late exponential phase which is in disagreement with our previous finding where *S. obliquus* SAG276-10 was grown in synthetic waste water and showed significant reduction in biomass and lipid productivities during stationary phase [37]. The recorded difference in both studies might be attributed to the difference in medium composition and growth conditions which results in different behavior in cell physiology and metabolic pathways.

Biodiesel properties can vary substantially from one feedstock to another. Therefore, not all oils extracted from algae are suitable or compatible to be used for biodiesel production [38]. The properties of biodiesel are determined mainly by its FAMEs profile [39,40]. The degree of unsaturation plays a significant role in fuel properties as the higher the degree of unsaturation of the FAMEs, the higher oxidation tendency of the biodiesel [41]. On the other hand, shorter and more unsaturated fatty acids increase the viscosity and flow characteristics at low temperatures, which are undesirable characteristics. Therefore, a proper ratio between saturated and unsaturated fatty acids should be maintained in order to obtain biodiesel with appropriate characteristics [42]. The present results revealed that SFAs and MUFAs content of *S. obliquus* was 37.73 and 18.05 % of total fatty acids, respectively, with no parinaric acid (C18:4) contents which complied with the European standard specifications EN14214 and results in oxidative stability of the biodiesel [35]. In addition, the IV of *S. obliquus* FAMEs was significantly lower than the limit established by the EN14214. The conversion of triglycerides into FAMEs through the transesterification process reduces the viscosity by a factor of about eight [43]. The recorded KV of *S. obliquus* biodiesel was found to be 4.38 $mm^2 s^{-1}$, which is comparable to that obtained by Suganya et al. [43]. One of the most important features of biodiesel is the CN which indicates the longer the fatty acid carbon chains and more saturated molecules present in the obtained biodiesel. Biodiesel with high CN gives better ignition properties [5]. The recorded CN was 54.12, while minimum acceptable value is 47 according to ASTM D6751-08. Another important parameter for low-temperature applications of a biodiesel is CP at which crystallization begins and is related only to the amount of saturated methyl esters. The recorded CP of *S. obliquus* FAMEs allows it to be used safely and comparable to that of the conventional diesel.

5. Conclusions

Eight strains of the genus *Scenedesmus* were isolated and their efficiency for biodiesel production was investigated in terms of lipid productivity and biodiesel quality. Among the studied species, *S. obliquus* showed the highest significant lipid productivity with predominance of SFAs and MUFAs corresponding to a favorably biodiesel properties. Therefore, the present study suggests *S. obliquus* as an attractive alternative renewable feedstock for biodiesel production. Further studies are in progress to optimize the growth conditions and enhance the lipid productivity of *S. obliquus* for large scale outdoor cultivation and bioenergy production.

Acknowledgments

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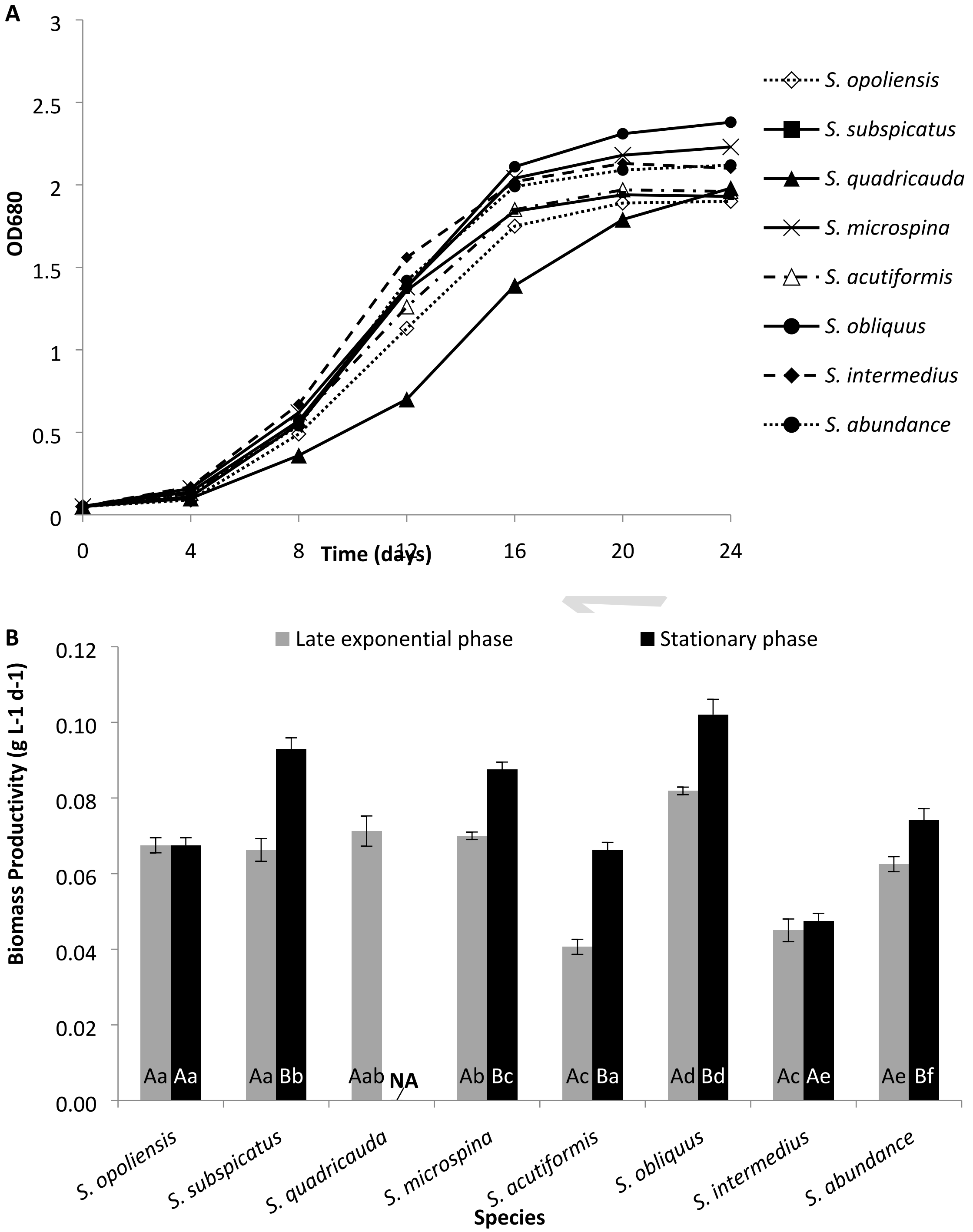

Fig. 2. Growth curves (A) and biomass productivities (B) of the studied isolates at late exponential phase and stationary phase. Error bars show the SD for three replicates. Columns of the same series with the same small letter showed insignificant difference at $P \leq 0.05$. Columns of the same organism at different growth phases with the same capital letter showed insignificant difference at $P \leq 0.05$. NA means not applied.

Highlights

1. *Scenedesmus* species were screened for biodiesel production.
2. *Scenedesmus obliquus* showed the highest biomass productivity.
3. *Scenedesmus intermedius* showed the highest lipid content.
4. *Scenedesmus obliquus* was the most lipid productive strain with up to 24.94 mg L^{-1} day^{-1}.
5. FAMEs specifications of *Scenedesmus obliquus* complied with US and EU standards.