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Potential of Fresh Water Green Microalgae Scenedesmus dimorphus in Producing High Quality Biodiesel

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ABSTRACT

Aim: *The present investigation was carried out to study the potential of green microalga Scenedesmus dimorphus as a suitable feedstock for biodiesel production.*
Methodology: The growth analysis pattern of this robust alga in the Bold Basal media showed that the specie is fast growing and reached a stationary phase on 14th day of incubation only and is suitable for high-density culture. Based upon its rapid growth this promising specie of microalgae was selected for large scale biomass production in indigenous made 25 liter lab scale photobioreactor. The microalga thrived very efficiently and harvested biomass was lypholized and subjected to lipid extraction by Soxhlet method (1875). The microalgal oil was subjected to fatty acid and physico-chemical analysis also.
Results: *The fatty acid methyl ester profile showed that the specie possess appreciable amounts of primary fatty acids with carbon chain length of C16 to C18 viz oleic acid 21.1 %, palmitic acid 18.9 % and linoleic 13.1 % making suitable feedstock for the production of good quality biodiesel. The quality parameters of the microalgal oil like degree of unsaturation, cetane number, iodine value, saponification value were within the limits of National petroleum agency(ANP 255), American society for testing and materials (ASTM D6751) and European biodiesel (EN 14214) international standards respectively.*
Interpretation: *The highly dense (0.980 g cc-1) and viscous (0.539 Pa s) oil of microalga reveal that transesterification is a crucial step in minimizing these characteristics of the oil and converting the algal oil into biodiesel. Overall, our results suggest that Scenedesmus dimorphus is the promising isolate for producing high-quality biodiesel.*

1. Introduction

The global energy demand has been increasing at an unprecedented rate with increasing pressure on fossil-based fuel utilities. However, the non renewable nature of fossil fuels has raised numerous problems like increasing crude oil prices and global warming (Malcata, 2011). Thus there is an urgent need to search for an alternative fuel source that is renewable, economical, sustainable and environmental friendly. Algae, one of the forms of biomass (Durakovic and Memon, 2016; Kumar *et al.,* 2016) can also be substantially used to produce domestic bio-fuels (biodiesel, bioethanol, biohydrogen etc). Algae and plants serve as a natural source of oil, which conventional petroleum refineries can convert into diesel fuel–a product known as biodiesel (NREL, 2006). As a biodegradable, renewable and non-toxic fuel, biodiesel emit fewer gaseous pollutants and reduces probable carcinogens in the environment (Chisti, 2007; Kumar *et al.,* 2018).

Algae are considered superior to oil yielding terrestrial plants because of their unicellular nature and faster multiplication rate (doubling time very short within 24 hours and can double their weight 3-5 times a day). Due to this reason, they are capable of synthesizing 30 times more oil per hectare than oleaginous terrestrial plants (Tickell, 2000; Chisti 2007). Moreover annual oil production of algae is around 90,000 L ha^{-1}compared to terrestrial plants

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(Schneider, 2006; Haag, 2007). As algae possess lipids (neutral and polar), storage products, metabolites, and other energy sources, they can transfer most of their energy into lipids as storage products for survival. Some species can accumulate more than 80 per cent of their weight as lipids under unfavourable environmental conditions (Metting, 1996; Spolaore *et al.,* 2006). Accumulation of neutral lipids mainly occurs in triacylglycerides that can be extracted and processed by transesterification with primary alcohols into diesel oil. Thus, high yield and high density algal biomass can be an excellent source for algal oil, which can be utilized to produce biodiesel.

Therefore a comprehensive research programme was carried out to study the biodiesel potential of fresh water green microalga *Scenedesmus dimorphus* by subjecting this alga to large scale biomass production, oil extraction, fatty acid profiling and assessment of algal oil for biodiesel production.

Table I. Extraction of oil content

Microalgae	Soxhlet method			
	Amount of culture (ml)	Dried algal biomass (mg)	Algal oil (mg)	Oil (%)
S. dimorphus	150000	7889.3	2152.9	27.29

Materials and Methods

Sampling, growth conditions and identification

Scenedesmus dimorphus was isolated from Himalayan Dal Lake, Jammu and Kashmir (34º 07 N 74º 52 E), India. The isolation and purification of selected microalga were performed as per different Isolation techniques using BBM culture medium (Bold, 1949; Bischoff and Bold, 1963). Microalgae identification was carried out using advanced microscope (LEICA DM 500, U.K) connected with computer having digital image analyser and software (LAS EZ 1.8.0) and microphotographs were taken with attached camera LEICA EC3. The microalgae identification was also authenticated based upon standard keys for morphological characteristics (Tiffany, 1952; Prescott, 1970; Phillipose, 1967; Bellinger and Sigee, 2010). The cultures were grown autotrophically in the batch culture in 500 ml Erlenmeyer flasks containing 250ml media and 12% inoculum. These unialgal cultures were incubated in culture room under controlled temperature range of 27±0.5º C and continuous fluorescent illumination of 3000 lux with 14: 10 h light/ dark photoperiod.

Scanning and Growth measurement

The maximum absorbance was inspected by scanning a culture sample between 400 and 1100 nm using double beam UV Vis spectrophotometer (Chemito Spectrascan UV 2700, Thermo Scientific) loaded with Spectrum PC software. The highest absorbance peak value was then used to measure the optical density. The growth rate of cultures was determined by measuring the optical density (OD_{680nm}) every 24 hours at 11:00 am. For the measurement of OD, 3 ml culture was drawn from the flask and BBM media was used as a blank. OD was measured at 680nm as per the initial scanning process. The sample cultures were diluted to an OD of less than one, to fall within the linear measurement range. The actual OD was determined by multiplying the OD value with the dilution factor (Griffiths *et al.,* 2011).

Lab scale (self-made apparatus) photobioreactor for biomass production

The large scale biomass production was carried out using Haffkines flasks of 4 L and transparent tanks 25 L (lab scale photobioreactor) using BBM media plugged with non-absorbant cotton. 2 L unialgal inoculum was prepared as a starter inoculum in 4 L capacity Haffkines with the culture conditions same as above in culture room. After the late log phase, i.e. approximately two weeks, 10 % inoculum was used for the lab scale photobioreactor. This experimental setup was done for large scale biomass production and oil extraction from *S. dimorphus.* Two transparent plastic tanks (lab scale photobioreactor with 25 L capacity each) containing 15 L of BBM nutrient medium adjusted to pH 6.6 and 2 L inoculum of microalgae were taken. To avoid the sedimentation of microalgae and to speed up the biomass production aeration was provided with the help of aerators. These photobioreactors were kept under the indirect sunlight (8000 to10000 lux measured by lux meter) at the temperature range of 28 to 32^0 C. After five weeks the photobioreactors were maintained for 4 days under dark period and closed environment and upon microscopic examination it was observed that cells were much larger in size and shape and showed different morphology.

Harvesting of microalgal biomass

The algal biomass was harvested using different methods like flocculation, centrifugation and lypholisation.

Flocculation: In this process algal cells were forced to form lumps by the use of chemicals. Flocculation of the *S. dimorphus* was done using alum as a flocculating agent. With the help of thread alum was dipped into the tanks for about 10 minutes with simultaneous shaking of supernatant for complete sedimentation of microalgae. After 1 hour of time the upper phase (supernatant) in plastic tanks (transparent without algae) was removed carefully with the help of transparent plastic tubing and the bottom phase containing microalgae were centrifuged.

Centrifugation: Dewatering of flocculated microalgal biomass was carried out with the help of centrifuge (Thermo Scientific, Germany; Sorvall ST 16R) loaded with six transparent centrifuge tubes each having capacity of 100 ml. Centrifugation was carried out at 8,000 rpm for 8 minutes. To remove the salts, microalgal biomass was finally washed with distilled water three times.

Lypholisation: Biomass was first lypholized (Labconco Freezone 2.5, USA) to remove the moisture content at -50 ºC for 4 hours. The dried biomass was grinded using mortar pestle, converted to powder form, and subjected to oil extraction.

Oil extraction

The total oil content was determined from dried algal biomass using automatic Soxhlet technique (Soxhlet, 1879).

Table II. Quality parameters of oil from tested microalgae for biodiesel production

Oil	DU (%)	CN[1]	IV[2]	SV	SFAs	MUFAs	PUFAs	References
S. dimorphus	86.2	50.457	116.745	179.392	27	32.2	27	Present study
Chlorella species	74.1	56.7	65	217.8	-	-	-	(Francisco *et al.,* 2010)
Peanut	113.1	53	97	-	-	-	-	(Ramos *et al.,* 2009)

DU, degree of unsaturation; **CN,** cetane number; **IV,** iodine value; **SV,** saponification value (mg KOH g^{-1}); **SFAs,** saturated fatty acids; **MUFAs,** mono unsaturated fatty acids; **PUFAs,** poly unsaturated fatty acids.
[1]Minimum limit CN of European standards (EN 14214) – 47[2]Maximum limit of IV of European standards (EN 14214) – 120 g I_2 100 g^{-1}

The solvent chloroform: methanol (2:1) was used in the extraction method and the percentage of oil was calculated as per the equation of Abubakar *et al.,* 2012.

Algal oil analysis

The different parameters of the algal oil were analysed by standard analysis methods (AOAC, 1995) and algal oil characters were compared with biofuel standards contained in IS 15607 and EN 14214.

Colour of algal oil was determined as per the colour codes, standards and terminology of Ridgway (1912).
Viscosity of the algal oils was estimated with the help of advanced digital Viscometer (Bohlin Visco 88, Malvern, U.K) attached with computer having latest digital Bohlin software (Visco 88 Julabo, V06.51).
Density, ρ, measurements as a function of temperature on the present microalgal oils has been performed by gravimetric method (Sankarappa *et al.,* 2005).

Determination of fatty acid composition using gas chromatographic method

The lipid extracts (algal oil) were converted into fatty acid methyl esters (FAME) by transesterification using 2% sulphuric acid in methanol reagent and the reaction products were examined. The fatty acid (FA) composition of algal FAME were analyzed quantitatively by gas chromatography (HP 6850 GC with flame ionization detector and fused silica capillary column DB -225) and qualitatively by mass spectrometry (Agilent 6890N GC-MS with Agilent 5973 mass selective detector and HP-5 capillary column) methods.

Assessment of microalgal oil quality for biodiesel production

The quality of microalgal oil was determined by assessing the saponification value (SV), iodine value (IV), cetane number (CN) and degree of unsaturation (DU) using standard protocols of IUPAC 2.201(1979), AOAC method 920.159 and AOAC method 920.160 respectively. These values were calculated using empirical equations of Francisco *et al.,* 2010; Osunkedo *et al.,* 2013.

Statistical analysis

In all the experimental setups, the measurements of the values were done in triplicates and the mean SD, SE were calculated using GraphPad Prism 5 statistical software.

Results and Discussion

Growth measurement of microalgae

The maximum absorbance was inspected by scanning a culture sample between 400 and 1100 nm and the highest absorbance peak value obtained at 680nm was then used to measure the optical density as shown in the Fig. 1. Therefore, growth of the specie was read in this wavelength. The specific data pertaining to the growth measurements of the tested microalgae *S. dimorphus* is shown in Fig. 2. During the experiment, the OD values at 680nm were taken in triplicates, the mean ±SE was calculated, and OD values in tabular form were converted into growth curve using GraphPad Prism 5 statistical software. The plot clearly shows distinct phases of a typical growth curve of microalga where the growth reached a stationary phase on 14th day of incubation and during the investigations it was found that the specie thrive well in the BBM media. During all the phases of growth curve it has been observed that *S. dimorphus* is fast growing. Initially the culture showed gradual growth rate and from the 4th day onwards, the alga had significant increase in total number of cells. As evident by the growth curve, the specie show lag phase of 5 days and on 6th day the culture showed signs of exponential phase. During the stationary phase, maximum growth with OD of 2.97 compared with initial reading of 0.045 was found in *S. dimorphus*.

In the present research work, green microalga *S. dimorphus* showed luxurious growth in the BBM media, which reveals its flexible nature to adapt the wide range of the environmental conditions as huge differences occurs in the climatic conditions of the places where sampling has been done (Himalayan Dal lake, Kashmir, India) and the place where all the experimental work was carried out (NBRI-Lucknow, UP, India).

As shown in the Fig. 1 maximum absorbance was inspected by scanning the culture samples and the highest absorbance value was then used to calibrate the curve of algal density. Standard procedures to estimate algal concentration include direct cell counts, chlorophyll content measurement and absorbance (EPA, 1994). When spectrophotometrical absorbance is the chosen method, a reading wavelength value is correlated to the light absorbance of chlorophyll (EPA, 1994; Eaton, *et al.,* 1995). Fig. 1 presents the pattern of light absorbance for *S. dimorphus* screened between 400 and 1100 nm. In the microalgal specie, peak could be observed with the highest absorbance obtained at 680 nm,

representing the wavelength of maximum sensitivity to quantify these *Scenedesmus* samples. Therefore, growth of the species was read in this wavelength.

Microalgae can grow profusely under suitable conditions and sufficient nutrients (Chisti, 2007). Fig. 2 shows the enhanced growth rate of microalga with incubation time grown autotrophically during 17 days of batch cultures. Growth rate is an essential way of expressing species relative ecological success in adapting to its natural environment or the experimental environment imposed upon it. Microalgal growth is directly affected by the availability of nutrients, temperature, light and pH (Xun *et al.,* 2012). The present study revealed that cell growth started from the 1st day itself and reached its maximum at 14th day of the culture. The growth rate of *S. dimorphus* was fastest and higher. Similar results were also observed in *Nannochloropsis salina* and *Chlorella marina* culture by Muthukumar *et al.,* (2012). Our results indicate that the microalga is suitable for high-density culture.

Table III. Characteristics of oil obtained from the tested microalgae *S. dimorphus*

Parameters	Values
Density (g cc^{-1})	0.980
Viscosity (Pa s)	0.539
Physical appearance	Dark dull yellow-green
Solubility in water	Insoluble
Odour	Oily & fishy

Oil Extraction

The Soxhlet extraction method was employed to extract lipids from the tested microalgae (Soxhlet, 1879) and the comparison mean values of oil content are presented in Table 1. The microalgae *S. dimorphus* yielded 27.29% of oil content respectively.

Characteristics of microalgal oil

The oil obtained from microalga by Soxhlet extraction method was subjected to physico-chemical analysis and results are presented in Table 3. The colour (physical appearance) of oil obtained from the microalgal specie was dark dull yellow green from plate XXXII compared with the colour standards and nomenclature of Ridgway's (1912). The dynamic viscosity and density of oil was found to be 0.539 Pa s and 0.980 g cc^{-1}. The visual inspection test is a visual comparison method used to determine the odour of microalgal oil and its solubility in water. The microalgal oil was insoluable in water and shows oily and fishy odour.

Colour: The dark dull yellow green colour of algal oil might be due to abundant dark fats as algal oil obtained from Soxhlet extraction was not subjected to transesterification process. Transesterification process removes the glycerol content from fatty acids of the algal oil which can result in the shifting of colour. Sanford *et al.,* (2009) also carried out colour determination obtained from various feedstocks and he also found dark fats in the feedstocks obtained from algae. Our results of colour standards match with that of Sanford *et al.,* (2009) as he also found that during the esterification reactions, all fats and oils appeared to shift in colour.

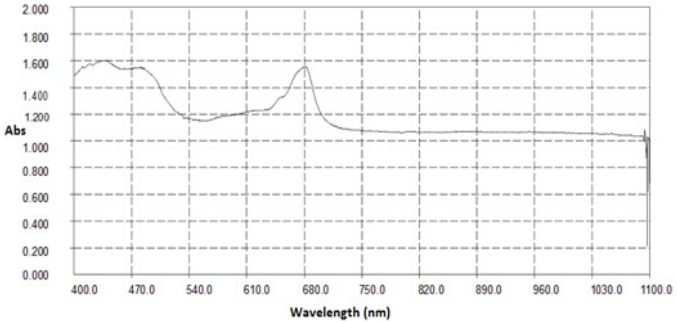

Fig. I. Spectrascan of *S. dimorphus* with scan speed of 1800 nm/min screened between 400 and 1100 nm

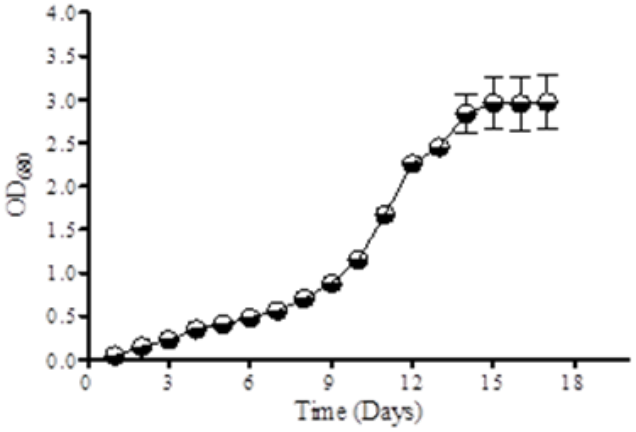

Fig. II. Growth curve of microalgae under batch mode

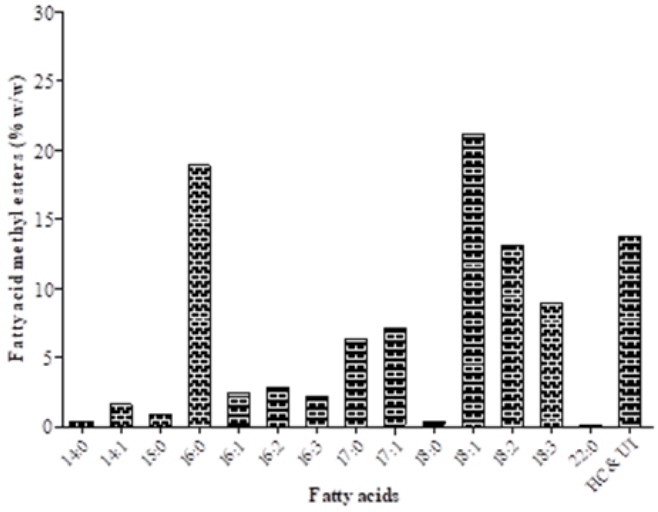

Fig. III. Fatty acid profile of *S. dimorphus*

Density: The density of the microalgal oil in our study was higher than 0.900 g cc^{-1} and may not pass EN 14214 Standard, which specifies the density at 15°C to be 0.860-0.900 g cc^{-1}. Xu *et al.,* (2006) reported 0.864 kg l^{-1} density of biodiesel obtained from *Chlorella protothecoides*. The higher density of algal oil indicates that transesterification step is required to minimize the density because of high amounts of hydroxyl esters. Sanford *et al.,* (2009) also found that high amounts of hydroxyl esters may be associated with higher density. Stanley *et al.,* (2010) extracted oil from *Chaetoceros species* and found it highly dense with 1.305 g ml^{-1}. In view of higher value of density parameter they found that microalgal oil obtained from the same algae can be

efficiently used for biodiesel production.

Viscosity: The microalgal oil exhibited the higher dynamic viscosities. Stanley *et al.,* (2010) extracted oil from *Chaetoceros* sp. and found it highly viscous. One possible reason for this observation is that the oil possesses high concentrations of hydroxy containing fatty acids capable of forming hydrogen bonding (Firestone, 2006). The higher viscosity of microalgal oil indicates that transesterification step is required to minimize the viscosity because of presence of high amounts of hydroxyl esters and make this algal oil suitable for biodiesel production. Kumar *et al.,* (2016) also reported the highly viscous oil (0.3 Pa s at shear rate of 500 s^{-1}) obtained from the consortia of algae collected from natural water bodies of Himachal Pradesh, India. Kerschbaum and Rinke, (2004) measured dynamic viscosity of rapeseed oil at shear rate of 420.3 s^{-1} from 323.15 K up to 258.15 K and found viscosity continuously increases from 23.2 mPa s up to 489 mPa s which indicates that, at the lower temperatures viscosity of algae oil becomes higher. Similar findings were reported in algal oil, 5-30 times viscous than that of Cashew nut oil, Palm Kernel and Peanut oil (Davis, 2009; Latinwo, 2010).

Fatty acid estimation

FA profile of *S. dimorphus* was determined and FA composition was calculated as percentage of the total fatty acids present in the microalgae from the peak areas. In the tested microalga the dominant FAs were oleic acid (18:1), palmitic acid (16:0), linoleic acid (18:2) and linolenic acid (18:3) and cis-10-heptadeceoic acid (17:1). The total FAME of the *S. dimorphus* was 86.2% with 13.8% hydrocarbons and unidentified respectively. Arachidic acid, gadoleic acid, eicosapentaenoic acid and heneicosanoic acid were absent in the species. The most dominant fatty acid in *S. dimorphus* was oleic acid (21.1%) followed by palmitic acid (18.9%), linoleic acid (13.1%), linolenic acid (8.9%), cis-10-heptadeceoic acid (7.1%), margaric acid (6.3%) and the FA present in least concentrations were behenic acid (0.1%) and myristic acid (0.4%) as shown in Fig. 3.

In biodiesel, the FA profile is considered to be the most important as that of the total fatty acid content. The FA profile of the microalga reveals the appreciable amounts of FA with carbon chain length of C16 and C18. It was previously reported that oleic acid, palmitic acid, stearic acid and linolenic acid were recognized as the most common fatty acids in biodiesel (Knothe, 2008).In the present study these FA were also present in the tested microalga. Oils with high oleic acid content have been reported to have a reasonable balance of fuel, including their ignition quality, combustion heat, oxidative stability, viscosity and lubricity, which are determined by the structure of their component fatty esters (Knothe, 2008). In our study the alga also contain appreciable amounts of oleic acid content making it suitable feedstock for producing good quality biodiesel. Prabakaran and Ravindran (2012) concluded that *Scenedesmus* sp. showed the highest oleic acid content among their tested microalgal species. Also Xu *et al.,* (2006) studied the FA composition of algal oil from *Chlorella protothecoides* using GC analysis. According to EN 14214 standards, the percentage of linolenic acid and polyunsaturated FA (≥4 double bond) should not exceed 12 and 1% respectively for an ideal biodiesel (Gouveia and Oliveira, 2009; Pereira *et al.,* 2013). In our study the contribution of linolenic acid is 8.9% of the FAME, whereas, polyunsaturated FA with ≥4 double bond were completely absent. However, the previous studies have also found that all these characteristics primarily depends upon the microalgal strain selected (Romano *et al.,* 2000), culture conditions and biomass preservation techniques employed (Zepka *et al.,* 2008). Over all, the tested microalgae is the best suitable for producing good quality biodiesel.

Quality parameters of microalgal oil for biodiesel production

The quality parameters of microalgal oil for biodiesel production are presented in the Table 2. The DU, CN, IV and SV for *S. dimorphus* were 86.2, 50.46, 116.74 and 179.40. The total amount of SFAs, MUFAs and PUFAs were 27 %, 32.2% and 27% respectively (Table 2).

Quality parameters of oil for biodiesel production

DU is one of the important properties that influence the oxidative stability of biodiesel (Francisco *et al.,* 2010). When there is presence of large quantities of polyunsaturated (more than one double bond) FAME, it will negatively affect the biodiesel's oxidative stability because they contain reactive sites that are susceptible to free radical attack. In case of the tested microalga *S. dimorphus* the FAME profile obtained possess appreciable amount of saturated and monounsaturated FAMEs which shows its suitable nature to be a good biodiesel fuel as these FAMEs improve oxidative stability without greatly affecting cold flow properties of biodiesel (Table 2) (Wahlen *et al.,* 2013).

CN is also one of the important fuel properties highly influenced by the FA profile. The higher value of Cetane number is the indicator of better combustion, easier engine start-up, less knocking and low NO_x emission (Arias-Penarands *et al.,* 2013). The diesel fuel with large quantities of saturated and monounsaturated FAMEs have higher value of CN. The minimum cetane value of ASTM D6751, EN 14214 and ANP 255 standards are 47, 51 and 45 respectively (Francisco *et al.,* 2010). In the present study, the values of CN calculated for *S. dimorphus* were in accordance with the standards reported (Table 2).

IV is used to determine the unsaturation of biodiesel oil, more the double bond in the fatty acid chain, higher the IV for that oil (Knoth, 2012). The EN 14214 standard specifies the maximum limit of IV to be 120 g I_2 100 g^{-1}, whereas DIN 51606 and SANS 1935 standards for IV are 115 and 140 g I_2 100 g^{-1} respectively. The higher IV of biodiesel oil may result in the polymerization of glycerides and lubricant deposition in the engine (Francisco *et al.,* 2010). A study was performed by Predojevic *et al.,* (2012) on sunflower oil suggests that higher IV does not necessarily indicate the unsuitability of oil for biodiesel. The higher IV of several promising biodiesel feedstock sources such as soyabean, sunflower seed oil and linseed oil were calculated as 128, 132 and 184.5, which do not satisfy the European biodiesel standards (Giakoumis, 2013). In the present study, as shown in Table 2, the IV of *S. dimorphus* shows that oil from the microalgae is a much better biodiesel source than sunflower seed, soybean, and

linseed oil.

SV has not been included in the European and Serbian biodiesel standards as a restricted property of biodiesel oil (Predojevic *et al.,* 2012). In the present study the SV for *S. dimorphus* is less than the SV of *Chlorella* sp. (217.8 mg KOH g^{-1}) calculated by Francisco *et al.,* (2010). However, SV is comparable to Stanley *et al.,* (2010) who reported SV value of 173.56 in *Chaetoceros* species.

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