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Scenedesmus incrassatulus CLHE-Si01: A potential source of renewable lipid for high quality biodiesel production

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HIGHLIGHTS

- *S. incrassatulus* oil has the potential for high quality biodiesel production.
- *S. incrassatulus* biodiesel properties meet international standards for biodiesel.
- Microalgal biodiesel synthesized using immobilized lipase without solvent.
- High amounts of oleic acid and palmitic acid were detected in *S. incrassatulus* oil.

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ABSTRACT

The potential of microalgal oil from *Scenedesmus incrassatulus* as a feedstock for biodiesel production was studied. Cell concentration of *S. incrassatulus* and lipid content obtained during mixotrophic growth were 1.8 g/L and 19.5 ± 1.5% dry cell weight, respectively. The major components of biodiesel obtained from *S. incrassatulus* oil were methyl palmitate (26%) and methyl linoleate (49%), which provided a strong indication of high quality biodiesel. Fuel properties were determined by empirical equations and found to be within the limits of biodiesel standard ASTM D6751 and EN 14214. The quality properties of the biodiesel were high cetane number (62), low density (803 kg/m^3), low viscosity (3.78 mm^2/s), oxidation stability (9 h) and cold filter plugging point (−4 °C). Hence, *S. incrassatulus* has potential as a feedstock for the production of excellent quality biodiesel.

1. Introduction

Global interest in biodiesel production has significantly increased in recent years. Traditional fossil fuel sources are being exhausted and fossil fuel combustion has caused the accumulation of greenhouse gases in the atmosphere and global warming. Biodiesel, in contrast, constitutes an alternative renewable source of energy that is eco-friendly. However, current technologies of biodiesel production have generated global debate because its production requires vast areas of farmland used at the expense of other food crops (Mata et al., 2010). Competition between biofuel raw material and food production has led to an increase in food prices that is not widely accepted. Therefore, it is important to find alternative feedstocks for biodiesel production that do not compete for farmland destined to food production.

Biodiesel is a mixture of fatty acid alkyl esters (FAAEs) obtained by transesterification of vegetable oils, animal fat or waste cooking oil (Dizge et al., 2009; Sarin et al., 2009; Chattopadhyay et al., 2011). Microalgae are currently promising sources of oil to obtain biodiesel. They offer several advantages, for instance, microalga can synthesize 20 times more oil per hectare than the terrestrial plants (Amaro et al., 2011). In addition, production of biomass from microalgae can be actually doubled in a few hours (i.e. as short as 3.5 h). Microalgae growth conditions can be optimized to obtain higher effective photosynthetic efficiency (4.5–7%) compared with other energy crops (Amaro et al., 2011). Some species can accumulate up to 50% (w/w DW) of triacylglycerols, and, above all, microalgae can grow on marginal land, fresh water or seawater without affecting farmland dedicated to food production. Moreover, it has been reported that microalgae can utilize industrial flue gas as a carbon source (Mata et al., 2010).

The synthesis of alkyl esters is accomplished by transesterification of oils and fats with alcohol of low molecular weight. Methanol

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is mostly used because of its lower cost compared with other alcohols, so FAAEs most commonly refers to fatty acid methyl esters (FAMEs). This reaction can be carried out in the presence of chemical (alkaline, acid) or enzymatic catalysts (Dizge et al., 2009; Chen et al., 2012). Chemical methods are widely used in many industrial processes and give high yields in short times. However, high energy consumption, difficulty in the recovery of the catalyst and glycerol and a high amount of alkali or acid waste water are major disadvantages in chemical catalysis (Tan et al., 2010). Recently, enzymatic transesterification using lipases has become more attractive for biodiesel production, as it allows the use of mild reaction conditions, the catalytic effectiveness is not affected by free fatty acids and water in the reactors, avoids the generation of wastewater and eases the recovery of high purity glycerol, which could solve downstream processing problems. However, the high cost of enzyme remains a barrier for its industrial applications. In order to decrease the cost of the process, the enzyme could be immobilized in a suitable carrier and reused (Tan et al., 2010).

Lipases from different sources like *Candida antartica*, *Candida* sp. 99–125, *Pseudomonas fluorescens*, *Pseudomonas cepacia*, *Rhizomucor miehei*, *Chromobacterium viscosum*, *Rhizopus oryzae*, *Thermomyces lanuginosus* and porcine pancreatic have been found suitable for biodiesel production (Hernandez-Martin and Otero, 2008; Tan et al., 2010; Chattopadhyay et al., 2011). The immobilized lipases that are commercially available are: Novozym 435 from *C. antarctica*, Lipozyme RM IM from *R. miehei* and Lipozyme TL IM from *T. lanuginosus*. In particular, the immobilized *C. antarctica* B lipase, commercially known as Novozym 435, has been investigated widely and is reported to exhibit the best performance (Shimada et al., 1999; Hernandez-Martin and Otero, 2008; Talukder et al., 2009). Optimum conditions for enzymatic conversion with Novozym 435 were found to be within the following ranges: alcohol:oil molar ratio, 1:1–25:1; enzyme loading, 4–50%; reaction time, 7–24 h; and temperature, 25–50 °C (Shimada et al., 1999; Hernandez-Martin and Otero, 2008; Talukder et al., 2009).

The most relevant properties of biodiesel are its density, kinematic viscosity, cetane number (CN), oxidation stability, combustion heat, and cold-flow properties such as the cloud, pour and cold filter plugging points. Previous studies have shown that these properties are strongly influenced by the chain length and degree of unsaturation of the fatty acid methyl esters (FAMEs) that constitute biodiesel (Knothe, 2009; Lapuerta et al., 2009; Ramos et al., 2009; Refaat, 2009). Generally, saturated fatty acids produce a biodiesel with high oxidative stability and cetane number but poor low-temperature properties, while unsaturated fatty acids provide better low-temperature performance (Knothe, 2009; Refaat, 2009). Cetane number, heat of combustion and viscosity increase directly with chain length and decrease with increasing degree of unsaturation, which means that long chain length (C16–C18) and low degree of unsaturation are preferable for biodiesel fuel (Knothe, 2009; Lapuerta et al., 2009; Ramos et al., 2009).

It should also be mentioned that density, kinematic viscosity and heating value are important properties in the design of fuel injection systems that affect engine performance and emission characteristics. The fuel density is generally related to the energy content; the heating values (HHV) increase with the decrease in the biodiesel density (Demirbas, 2008). Biodiesel with higher density and viscosity is more difficult to atomize. Low temperatures can affect the operation of fuel injection due to the delivery of a slightly greater mass of fuel, resulting in rich mixtures in the motor, which increase emissions and engine deposits (Refaat, 2009). Lower density biodiesel is more favorable because it can flow more easily. One of the difficulties encountered for biodiesel applications are its poor properties and performance when exposed to low temperatures. The key cold flow properties in biodiesel fuel specifications are the cloud (CP) and pour (PP) points as well as the cold filter plugging point (CFPP). At low temperatures, highest melting point (MP) components tend to nucleate, grow and form solid crystals. The temperature at which the crystals become visible and form a cloudy suspension is defined as the cloud point (CP). Prolonged exposure to temperatures at or below the CP causes crystal growth and formation of interlocking networks. The pour point (PP) is defined as the lowest temperature at which the biodiesel flows or can be pumped. Both CP and PP should be closely monitored by the user to ensure trouble-free operation in cold climates.

Despite current emphasis and larger investments in research and development of algal lipids as biodiesel feedstock, previous studies have focused on improving operating conditions for high lipid productivity and/or evaluate the triglyceride fraction profiles of algal lipids (Li et al., 2007, 2011; Mandal and Mallick, 2009; Ho et al., 2010, 2012; Yoo et al., 2010; Tan and Lin, 2011; Tang et al., 2011). Relatively few reports regarding the relevant fuel properties of biodiesel derived from microalgal oil have been published (Francisco et al., 2009; Vijayaraghavan and Hemanathan, 2009; Chen et al., 2012). The present study evaluated the potential of oil obtained from *Scenedesmus incrassatulus* as biodiesel production feedstock.

2. Methods

Experiments were done in a 22 L photobioreactor. After reaching the stationary phase of growth (30 days), the total lipid contents were extracted. These were first transesterified with methanol using an immobilized lipase from *C. antarctica*; then, the FAMEs were analyzed by gas chromatography. The FAMEs composition was used to predict any properties of the biodiesel. Finally, biodiesel quality was assessed by comparing the quality parameters to the two main standards (ASTM D6751 from the US, and EN 14214 from Europe). Details are provided in the following sections.

2.1. Biomass production and microalgae oil characterization

The microalga *S. incrassatulus* CLHE-Si01 used in the present work was provided by the culture collection of the Experimental Hydrobiology Laboratory ENCB-IPN, Mexico.

The strain was grown mixotrophically for 30 days in a 22 L flat-panel airlift photobioreactor (PBR) using PCG medium (Perales-Vela et al., 2007) modified with 1 g/L glucose. Growth conditions were: 22 °C, light intensity of 70 μmol photons m^{-2} s^{-1} and 12 h:12 h light/dark cycle. Cultures were continuously aerated at 0.5 vvm. Cells were harvested by centrifugation at 3500 rpm for 15 min, washed twice with distilled water and then lyophilized.

2.2. Analytical procedures

Lipids were extracted with chloroform–methanol–water (1:2:0.8, v/v/v) using a modified version of Bligh and Dyer's method (Molina-Grima et al., 1994). The mixture was centrifuged at 3500 rpm for 5 min to separate the biomass from the extracts. Residual lipids within the algal biomass were extracted once more. Next, the first and second extracts were mixed. The resulting extract mixture was separated into two layers, chloroform and aqueous-methanol, by addition of methanol and water, to give a final ratio of chloroform:methanol:water of 1:1:0.9. The upper layer (methanol layer including water) was removed and the lipid-containing chloroform layer was collected. Lipid content was measured gravimetrically and expressed as a dry weight percentage.

Fatty acid composition of the oil was determined using gas chromatography, after conversion of fatty acids to their corresponding methyl esters with a boron trifluoride/methanol solution (12%). The fatty acid methyl esters (FAMEs) were analyzed using gas chromatography (Perkin Elmer AutoSystem) equipped with a flame ionization detector (FID) and an HP-INNOwax capillary column: (30 m × 0.32 mm × 0.25 μm). The oven temperature was initially maintained at 150 °C for 4 min and then increased to 250 °C at a rate of 4 °C/min; this temperature was then maintained for 25 min. FAMEs were assessed by comparing their retention time with the standard (FAME Mix C_4-C_{24}, Cat: 18919 – 1AMP, Supelco Analytical®).

The molecular mass of lipids produced by *S. incrassatulus* was estimated according to the measured fatty acid composition of the microalgal oil, and this oil was then used in the transesterification experiments.

2.3. Enzymatic transesterification of microalgal oil

Novozym 435 (*C. antarctica* lipase B immobilized on acrylic resin) with activity of 10,000 PLU/g and methanol used as acyl acceptor were from Sigma®. All other chemicals were analytical grade.

The enzymatic transesterification reactions were carried out in test tubes containing 170 mg of *S. incrassatulus* oil, 39 mg of Novozym 435 (23%) and a 1:6 molar ratio of oil:methanol. *S. incrassatulus* oil was heated to 50 °C, lipase was added to the hot solution and the reaction was started by the addition of methanol to the system. Methanol was added in three successive steps (1/3 of the total amount each time) to avoid enzyme inhibition (Shimada et al., 1999). The reaction was left to take place for 6, 12 and 24 h at 50 °C, with constant stirring at 150 rpm. After running the reaction for a desired time, the whole sample was mixed with 3 ml n-hexane and centrifuged to separate the Novozym 435. FAMEs were analyzed by injecting diluted aliquots of the reaction mixture into a gas chromatograph. Conversion efficiency of biodiesel obtained from microalgal oil was expressed as weight percent of converted FAMEs against weight percent of oil content (wt.% oil). All experiments were performed in duplicate and results are reported as the mean ± standard deviation.

2.4. Estimation of biodiesel properties from microalgal oil

FAME composition was used to predict quality properties of the biodiesel, such as density (ρ_B), kinematic viscosity (ν_B), cetane number (CN_B), higher heating value (HHV), oxidation stability and cold flow properties. These were then used to verify the suitability of the composition to be used as biodiesel by comparison with the specifications published by Biodiesel Standard of USA and European Standard Organization. To predict the value of most biodiesel properties, a number of empirical methods and models are available in the literature. For example, Clements (1996) proposed the blending rules equation (Eq. (1)) to estimate the density, heating value, viscosity, cetane number and cloud point of biodiesel as a function of its methyl esters profile. He found that the comparison between predicted and experimental data yielded typical average errors of less than 2%, with the exception of 10% for viscosity.

The blending rules equation is based on the main assumption that, since biodiesel mixtures of FAMEs can be regarded as near-ideal solutions, once the value of any specific property of each pure component is known, then the property for the mixture can be computed by employing a simple linear equation of the type:

$$M_B = \sum X_i M_i \quad (1)$$

where

X_i is the weight percentage of each methyl ester
M_i is the property of each methyl ester
M_B is the property of biodiesel.

The properties of each methyl ester were calculated using the models (Krisnangkura et al., 2006; Demirbas, 2008; Park et al., 2008; Lapuerta et al., 2009, 2010; Ramos et al., 2009; Sarin et al., 2009) shown in Table 1.

Table 1
Models employed for the determination of biodiesel properties.

Property	Correlations	Reference
Density 15 °C (kg/m^3)	$\rho_B = \sum X_i \rho_i$ $\rho_i = 851.471 + \frac{250.718\ db + 280.899 - 92.180(m-1)}{1.214 + n}$	Lapuerta et al. (2010)
Kinematic viscosity 40 °C (mm^2/s)	$\text{Ln}\ \nu_B = \sum X_i\ \text{ln}\ \nu_i$ $\text{Ln}\ \nu_{c12:0-c20:0} = -2.177 - 0.202n + \frac{403.66}{T} + \frac{109.77n}{T}$ $\text{Ln}\ \nu_{c18:1} = -5.03 + \frac{2051.5}{T}$ $\text{Ln}\ \nu_{c18:2} = -4.51 + \frac{1822.5}{T}$ $\text{Ln}\ \nu_{c18:3} = -4.018 + \frac{1685.5}{T}$	Krisnangkura et al. (2006)
Cetane number	$CN_B = \sum X_i CN_i$ $CN_i = -21.157 + (7.965 - 1.785\ db + 0.235\ db^2)n - 0.099n^2$	Lapuerta et al. (2009)
Oxidation stability (h)	Oxidation stability $= \frac{117.9295}{z} + 2.5905$	Park et al. (2008)
Cloud point (CP) (°C)	$CP = 0.526(PAME) - 4.992 \quad 0 < PAME < 45$	Sarin et al. (2009)
Pour point (PP) (°C)	$PP = 0.571(PAME) - 12.240 \quad 0 < PAME < 45$	Sarin et al. (2009)
Cold filter plugging point (CFPP) (°C)	$CFPP = 8.9243 * LCSF(A) - 19.325$ $LCSF(A) = MP_{c18} * C18(wt.\%) + MP_{c20} * C20(wt.\%)$	Ramos et al. (2009)
Higher heating value (HHV) MJ/kg	$HHV = 0.4625\nu + 39.45$	Demirbas (2008)

db number of double bonds in the acid molecule.
m number of carbon atoms in the original alcohol used for the transesterification process.
n number of carbon atoms in the original fatty acid.
T absolute temperature in Kelvin.
z content of the linoleic and linolenic acids (wt.%).
PAME content of palmitic acid methyl ester.
MW_i molecular mass.
LCSF(A) long chain saturated factor (A).
MP melting point.
wt.% composition of saturated fatty acids.

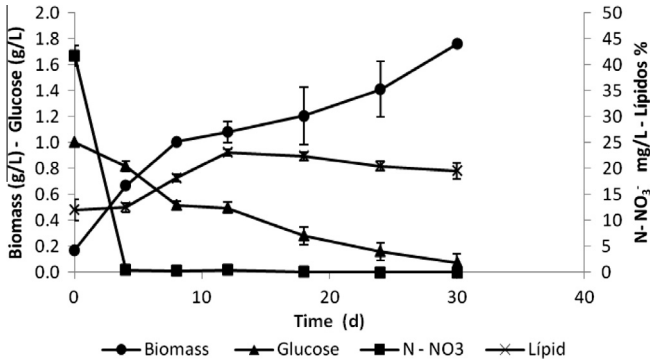

Fig. 1. Microalgal growth, lipid content and substrate consumption during the incubation period.

3. Results and discussion

3.1. Growth and lipid characteristics of S. incrassatulus

S. incrassatulus was cultured in a photobioreactor for 30 days. The results of microalgal growth, glucose and nitrate consumption, and lipid content are shown in Fig. 1. The microalgae showed an initial fast growth rate that was maintained for eight days and then was followed by a slow rate-growing phase, reaching a biomass concentration of 1.8 g/L at 30 days. Nitrogen was quickly depleted after 4 days of microalgae growth. The results also indicated that glucose was not the limiting substrate because it was not completely consumed until day 30.

As shown in Fig. 1, nitrogen stress and culture time caused a remarkable effect on the lipid content of *S. incrassatulus*. The *S. incrassatulus* cell lipid content increased from 12.0 ± 2.1% to 12.5 ± 0.8% after 4 days. As microalgae growth increased, nitrogen was depleted and lipids rapidly increased up to 23.1 ± 0.6% (day 12) and then decreased to 19.5 ± 1.5% on day 30. The results are consistent with the fact that *Scenedesmus* is capable of increasing its lipid content as a consequence of nutrient deficiency (Ho et al., 2012; Tan and Lin, 2011). The maximum lipid content observed for *S. incrassatulus* was 23.1%, which is higher than that found in *Scenedesmus obliquus* CNW-N (12.3%) and *S. obliquus* SJTU-3 (15.15%) (Ho et al., 2010; Tang et al., 2011) and falls within the range of the *Scenedesmus* species (20–25%) (Ho et al., 2012; Wu et al., 2012) The maximum lipid productivity obtained in this work was 18.96 mg L^{-1} d^{-1} on day 8. This result indicated higher lipid productivity than the values reported in previous works by Tang et al. (2011) for *S. obliquus* SJTU-3 (12.57 mg L^{-1} d^{-1}) and lower than that observed by Yoo et al. (2010) for *Scenedesmus* sp. (20.65 mg L^{-1} d^{-1}), and by Ho et al. (2010) for *S. obliquus* CNW-N (35.1 mg L^{-1} d^{-1}).

Table 2
Fatty acids composition of *Scenedesmus incrassatulus* oil.

Fatty acid	Distribution (%)	Molecular mass (g/mol)	Molecular mass contribution (g/mol)
Myristic acid (C14:0)	0.79	228.4	1.80
Myristoleic acid (C14:1)	0.93	226.4	2.10
Palmitic acid (C16:0)	24.93	256.4	63.93
Palmitoleic acid (C16:1)	3.73	254.4	9.48
Heptadecanoic acid (C17:0)	3.10	270.4	8.38
Heptadecenoic acid (C17:1)	1.08	268.4	2.90
Stearic acid (C18:0)	4.17	284.5	11.87
Oleic acid (C18:1)	45.36	282.5	128.15
Linoleic acid (C18:2)	6.60	280.5	18.52
Linolenic acid (C18:3)	5.97	278.5	16.63
MW_{FFA}			263.75

MW_{FFA} is the mean molecular mass of the constituent lipid fatty acids.

Table 2 shows the *S. incrassatulus* oil fatty acid profile. The major components are C16/C18 fatty acids, comprising up to about 95% of total fatty acids, and almost 84% of the acids were either saturated or monounsaturated. The results indicated that oleic acid was the most abundant fatty acid in the sample with over 45% of the total fatty acids. These results are consistent with those of Tan and Lin (2011) who indicated that the main fatty acids present in the lipids of *Scenedesmus rubescens* are C16 and C18 (95.8%) and the best-represented is oleic acid (43.3–53.5%). In contrast, Yoo et al. (2010) and Tang et al. (2011) reported that the main fatty acid components of *S. obliquus* SJTU-3 and *Scenedesmus* sp. are fatty acids with C16–C18 (>94%) but oleic acid were found in the order of 0.67% and 25.9%, respectively. The fatty acid composition was used to determine the average molecular weight of microalgal oil. Triglycerides, the major component of microalgal oil, are formed by the combination of three molecules of fatty acid (FA) and one molecule of glycerol with the condensation of three molecules of water, following the reaction indicated below (2).

$$3(\mathrm{FA}) + \mathrm{Glycerol} \rightarrow \mathrm{TG(triglyceride)} + 3\mathrm{Water} \quad (2)$$

Using mass balance, the molecular mass of microalgal oil (triglyceride) was estimated by relating the molecular mass of H_2O, FA, and glycerol (Eq. (3)) (Ehimen et al., 2010).

$$MW_{oil} = 3MW_{FA} + MW_{glycerol} - 3MW_{water} = 829\ \mathrm{g\ mol^{-1}} \quad (3)$$

MW_{oil} is the average molecular mass of the microalgae oil. MW_{FA} (Table 2) was calculated from the respective contributions of the different fatty acids of the microalgal oil, and $MW_{glycerol}$ and MW_{water} represent the molecular masses of glycerol and water, respectively. The average molecular mass of the *S. incrassatulus* oil of 829.33 g mol^{-1} is lower than that reported for *S. obliquus* of 917 g mol^{-1} (Mandal and Mallick, 2009) and *Chlorella* of 880 g mol^{-1} (Ehimen et al., 2010) but higher than that estimated for *Chlorella vulgaris* ESP-31 (789.3 g mol^{-1}) (Tran et al., 2012). These differences are due to the fact that the fatty acid composition can change significantly with growth conditions, which is clearly shown by their FAME profiles.

3.2. Biodiesel production from microalgal oil

Biodiesel produced via transesterification of vegetable oils (triacylglycerols) with methanol is a mixture of methyl esters of saturated and unsaturated long-chain fatty acids (FAMEs). Novozym 435, a *C. antarctica* lipase supported on a macroporous resin, which has shown to be a robust and stable catalyst compared to other lipases in the presence of short chain alcohols, was selected to be used in this study.

Conversion of the extracted microalgal oil to biodiesel (FAMEs) was determined at different reaction times (6, 12 and 24 h). It was found that the FAMEs yield increased quickly up to 67.9 ± 3% after 6 h of reaction time and then very slowly to a maximum value of 71.7 ± 0.3% at 24 h. A further increase in the reaction time from 6 to 24 h, caused a less effect on conversion of *S. incrassatulus* oil. This result clearly demonstrates that a higher FAMEs yield can be obtained in a shorter reaction time.

Table 3
Composition of biodiesel samples.

Fatty acid methyl ester	Relative content (%)		
	6 h	12 h	24 h
Methyl myristate (C14:0)	0.93	0.84	0.48
Methyl palmitate (C16:0)	25.98	25.77	26.94
Methyl palmitoleate (C16:1)	3.14	3.22	3.37
Methyl margarate (C17:0)	1.36	1.33	1.43
Methyl stearate (C18:0)	4.55	4.19	4.39
Methyl oleate (C18:1)	48.54	48.37	48.89
Methyl linoleate (C18:2)	3.45	3.32	3.37
Methyl linolenate (C18:3)	3.68	3	2.82
Others	8.37	9.96	8.31
Saturated	32.82	32.13	33.24
Monounsaturated	51.68	51.59	52.26
Polyunsaturated	7.13	6.32	6.19

After converting *S. incrassatulus* lipid to FAMEs by enzymatic transesterification, the composition of biodiesel was analyzed by gas chromatography (GC). The fatty acid methyl esters (FAMEs) of the biodiesel produced for the three reaction times (6, 12 and 24 h) are listed in Table 3. It was found that palmitate (C16:0), palmitoleate (C16:1), stearate (C18:0), oleate (C18:1), linoleate (C18:2) and linolenate (C18:3) were the major components, which accounted for more than 88% of the total methyl esters. This profile was similar to the extracted oil (Table 2). FAMEs profile obtained of biodiesel from *S. incrassatulus* oil indicates that this biomass could be a good feedstock for biodiesel production due to the high proportion of saturated and monounsaturated methyl esters (>83%), which have been considered ideal for biodiesel production because it provides better cold flow properties without losses of oxidative stability and retaining the cetane number at an acceptable level (Knothe, 2009).

The results of this study are consistent with previous results reported for oils from *Scenedesmus* sp. (Chen et al., 2012), *S. obliquus* CNW-N (Ho et al., 2010, 2012), *Scenedesmus* sp. LX1 oil at high culture temperature (30 °C) (Li et al., 2011) and FAME composition of *Chlorella protothecoides* by transesterification of the microalgal oil with immobilized lipase from *Candida* sp. 99–125 (Li et al., 2007).

3.3. Fuel properties of algal biodiesel

Biodiesel as a substitute for fossil diesel must adhere to international biodiesel standard specifications. The quality of biodiesel obtained from *S. incrassatulus* oil was theoretically evaluated versus the American Standards for Testing Materials ASTM 6751 and the European Union Standards EN 14214 for biodiesel fuel and other feedstocks. Table 4 shows the main parameters of *S. incrassatulus* biodiesel and compares them with those of biodiesel standards. The main properties of *S. incrassatulus* biodiesel were calculated based on its FAME composition using models available in the literature (Table 1).

Table 4
Properties of *S. incrassatulus* oil methyl esters with comparison to biodiesel standards.

Property	EN 14214:2008	ASTM D 6751	*S. incrassatulus* FAME
Density at 15 °C (kg/m^3)	860–900	ND	803
Kinematic viscosity at 40 °C (mm^2/s)	3.50–5.00	1.9–6.0	3.78
Heating value (MJ/kg)	35 (calculated)	ND	41
Cetane number	min 51	min 47	62
Oxidation stability, 110 °C (h)	min 6	min 3	19
Cloud point (°C)	CS[a]	Report[b]	9
Pour point (°C)	ND	ND	3
Cold filter plugging point (°C)	Variable	ND	−4
Linolenic acid methyl ester % (m/m)	max 12	ND	4

min = minimum and max = maximum.
[a] Country specific.
[b] Low temperature properties are not strictly specified, but should be agreed upon by the fuel supplier or purchaser.

As shown, the properties of *S. incrassatulus* oil biodiesel are within the limits established by the standards related to biodiesel quality. It has low values of density and kinematic viscosity due to the presence of a lower proportion of saturated esters (<31%), in accordance with Demirbas (2008). Higher heating value (HHV) is a measure of the energy produced when the fuel is burned completely, which also determines the suitability of biodiesel as an alternative of diesel fuels. The HHV value of biodiesel produced from *S. incrassatulus* oil was 41 MJ/kg. Neither the U.S. nor European biodiesel standards include a specification for heating value but in EN 14213 (biodiesel for heating purpose) a minimum of 35 MJ/kg is required. The CN of *S. incrassatulus* oil biodiesel was calculated at 62 and slightly exceeds the minimum specifications of 47 and 51 by both ASTM D6751 and EN 14214 biodiesel standards. This high CN value can be specifically attributed to the abundance of saturated and monounsaturated methyl esters (>83%). Higher CN causes better combustion, improving engine motor efficiency and reducing nitrogen oxides (NO_x) in exhaust emissions (Knothe, 2009; Ramos et al., 2009).

Biodiesel oxidation stability is affected by its content of polyunsaturated compounds, which can increase its tendency to oxidize during storage and is critical for biodiesel use. Some studies have identified an inverse relationship between the oxidation stability of biodiesel and the degree of unsaturated compounds, especially of polyunsaturated FAMEs (C18:2, C18:3) (Ramos et al., 2009). This is attributed to the fact that these unsaturated fatty acid chains contain reactive sites that are particularly susceptible to free-radical attack. The oxidative stability of *S. incrassatulus* oil biodiesel determined in the present study was of about 19 h, which is higher than the minimum time specified in ASTMD 6751 (3 h) and EN 14214 (6 h), and can be attributed to the extremely low content of polyunsaturation (<7.5%). Cold flow properties (CP, PP and CFPP) of biodiesel depend mainly on its composition. Because of the large seasonal and geographic temperature variability, neither the U.S. nor European biodiesel standards have sharp specifications for these low temperature properties. However, these are among the most important properties that define the suitability of biodiesel fuels. Knothe (2009) suggested that each country should specify certain temperature limits for different times of the year, depending on climate conditions. For instance, for Spain, a country with a relatively mild climate, a CFPP of below 0 and −10 °C is specified for summer and winter, respectively (Ramos et al., 2009). *S. incrassatulus* biodiesel is composed of significant quantities (58%) of FAMEs with unsaturations (MPs < 21 °C) compared with saturated compounds (<33%), whose MPs are very high (18.5–39.1 °C). As a result, the CP, PP and CFPP of *S. incrassatulus* biodiesel were relatively low and the last one (−4 °C) meets the CFPP maximum limit requirements established in Spain for summer. According to EN 14214, the permissible level of C18:3 (linolenic acid) is <12%; in this study, the C18:3 content was less than 4%.

Some properties of biodiesel from different strains of microalgae are compared in Table 5. Biodiesel from microalgae meets several specifications of the EN 14214 and ASTM D6751 standards. The properties of the biodiesel obtained from the oil of different microalgae meet international standards for biodiesel quality except for that produced from *Nannochloropsis* sp., lipids which have oxidation stability of 1.93 h, a lower value than those required by the EN14214 (6 h) standard and by ASTM D6751 (3 h) and that

Table 5
Properties of *S. incrassatulus* oil methyl esters with comparison to biodiesel from other microalgae.

Property	Freshwater algae[a]	*Chlorella protothecoides*[b]	*Nannochloropsis* sp.[c]	*Scenedesmus* sp.[c]	*Scenedesmus incrassatulus*[d]
Density at 15 °C (kg/m^3)	801	864	854	852	803
Kinematic viscosity at 40 °C (mm^2/s)	–	5.2	5.76	4.15	3,78
Heating value (MJ/kg)	40	41	39.81	39.76	41
Cetane number	52	–	–	–	62
Oxidation stability, 110 °C (h)	–	–	1.93	5.42	19
Cloud point (°C)	–	–	–	–	9
Pour point (°C)	−14	–	–	–	3
Cold filter plugging point (°C)	–	−11	–	–	−4
Linolenic acid methyl ester % (m/m)	–	–	–	–	4

[a] Vijayaraghavan and Hemanathan (2009).
[b] Miao and Wu (2006).
[c] Chen et al. (2012).
[d] This work.

produced from *Scenedesmus* sp. oil, showed oxidation stability higher than 5 h, which meets the European standard.

The biodiesel obtained from *S. incrassatulus* oil showed better values of density and viscosity, an equal or slightly higher HHV value, improved CN and oxidation stability and lower performance to cold temperatures compared with biodiesel obtained from other microalgae in previous studies (Vijayaraghavan and Hemanathan, 2009; Chen et al., 2012). This will be reflected in better combustion, lower emissions, excellent oxidation stability and reasonable flow properties at low temperatures. In comparison, Francisco et al. (2009) analyzed the quality of biodiesel obtained from six different species of algae and found a CFPP of 20.8 °C for *S. obliquus*. This value is probably due to its high content of saturated fatty acids (18.09%), mainly represented by heptadecanoic (C17:0) and stearic acid (C18:0). These differences in the fuel properties may be attributed to the variation in fatty acid content of the oil used as feedstock, which in turn depends on the culture conditions of microalgae and has great impact on fuel quality.

The above results clearly shown that the properties of the biodiesel obtained from *S. incrassatulus* oil meet the major biodiesel standard international specifications of the USA (ASTM 6751) and the European Standard Organization (EN 14214).

4. Conclusions

The utilization of *S. incrassatulus* oil, as a source of renewable lipid for biodiesel production was investigated in the present study. Biodiesel produced meets the specifications of current standards (ASTM Biodiesel Standard D6751 in the US and Standard EN 14214 in the EU). The most conspicuous properties of biodiesel derived from *S. incrassatulus* oil were its high cetane number, low viscosity and low oxidative potential with excellent CFPP retention. The lipid profile and high oleic acid content of *S. incrassatulus* oil provided a strong indication of its potential as a feedstock for the production of excellent quality biodiesel.

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