Production and characterization of Biodiesel from Oleaginous Freshwater microalgae *Scenedesmus obliquus* grown in Oil and Gas Produced water

[1]Rengonee Das, [2] Mohan Chandra Kalita,[3]Hamendra Chandra Das

[1]Research Scholar,[2] Proffesor,[3]Director
[1]Department of Biotechnology,
[1]Gauhati University, Guwahati, India

Abstract : Biodiesel is an attractive alternative fuel for diesel engines due to its technical, environmental and strategic advantages.The viability of production of Biodiesel from microalgae grown in waste water co-produced with crude oil and natural gas (termed as produced water), was studied in the present analysis. In the present work the growth patterns of the Freshwater oleaginous microalgae *Scenedesmus obliquusin* two different growth conditions i.e. one experimental set grown in only BG11 culture media and other in BG11 culture media coupled with Produced water (PW) were studied for a period of 30 days. Studies were also conducted on different extraction solvents used for lipid and transesterification process. The maximum yield biodiesel of *S. obliquus* grown in BG11 Media was found out to be 35.45 % and in 40% PW+ BG11 media biodiesel obtained using hexane in a single stage transesterification process was 48.32%.Gas chromatographic analysis data also showed that biodiesel derived from *S. obliquus* grown in 40% FW is richly composed of Oleic acid and linoleic acid methyl ester. Fuel properties showed that biodiesel were comparable in quality with that of other conventional biodiesel**.**

Index Terms **- Microalgae, *Scenedesmus obliquus*, Produced water, Transesterification, Biodiesel, Fuel characterization.**

I. INTRODUCTION

The rummage for alternative fuels from renewable resources to meet the challenges of global energy demand due to depletion of fossil fuel and environmental concerns has attracted considerable attention in recent years [1]. Methyl ester mixture is one of the most promising alternative fuels because it is renewable, biodegradable, and non-toxic and has almost very close properties to that of fossil diesel fuel [2]. Properties of microalgae biodiesel with respect to viscosity, flash point, calorific value, density and heating value are similar to petroleum diesel. Microalgae generally grow at a faster rate and most species have lipid content ranging from 25-45% of biomass [3]. Under favorable growth conditions like ideal physical parameter of light, temperature, CO2 and nutrient supply, biomass doubles during the exponential growth phase, in periods as short as 1.9 h.

PW co-extracted with oil and gas has a great potential economic value for algal biofuel production, and could reduce fresh water limitations to create a viable algal biofuel industry [4]. These waste nutrients are currently seen as a liability and are either lost to the atmosphere through denitrification or leached into the local environment where nitrogen enrichment and eutrophication problems can occur with global scale impacts. PW is a challenging water resource for this use because of variable salinity, geochemical complexity, and the presence of biologically toxic components. However, these excess nutrients need not be relegated to waste but rather could be recycled and utilised as an input resource for the large-scale cultivation of phototrophic organisms to be recycled again as bio products [5]. Inorganic nitrogen in particular is the primary limiting nutrient for the production of algae and as such this nitrogen waste could be an ideal resource for the large-scale production of algal biomass. The cultivation and subsequent on-site use of microalgae biomass at sites with high-nutrient waste streams, such as intensive livestock production, can close the loop between waste production, waste capture and re-use. The on-site use of cultured biomass has the additional benefit of reducing the energy and transportation costs associated with either bringing animal feeds to the farm or transporting algal biomass away. Microalgae can be grown intensively in tank or ponds if water supply and agitation is constantly provided to mimic that of natural environmental conditions. [6]. High concentration of inorganic nitrogen, phosphorous and carbon in PW supplies adequate amount of nutrient for high growth rate as well as it maintains the pH close to natural freshwater reservoirs.

The most comprehensive study performed to date utilizing PW medium was done as a thesis by Godfrey [7]. Strains tested included: *Amphora coffeiformis* UTEX 2039, *Chaetocerosgracilis* UTEXLB2658, *Phaeodactylumtricornutum* UTEX640, and several strains isolated from Great Salt Lake, Utah (western USA).Eight strains of microalgae were shown to successfully grow and produce some amount of neutral lipids. Growth and lipid production through nitrate and phosphate addition were initially optimized in the diatom organism *Amphora coffeiformis* UTEX 2039 which showed greatest lipid productivity with no phosphate addition and 150 mg L^{-1} sodium nitrate addition. Because of difficulties with benthic properties during batch growth, other strains were reconsidered. Most consistently, the diatom strain *C. gracilis* and the green alga *Chlorella sp*. USU080 have been shown to have high lipid and growth productivities with only addition of 300 mg L^{-1} sodium nitrate. Growth of diatom strain *C. gracilis* and the green alga *Chlorella sp*. USU080 did cause a reduction in several compositional elements of produced water, when compared the final concentration of elements against water quality standards, microalgae *Chlorella* sp. USU080 succeeded in reducing the levels of calcium, phosphate, manganese and *C. gracilis* growth reduced magnesium to below acceptable concentration ranges

The current study demonstrates on the ability of microalgae *S.obliquus* to grow for the purpose of lipid production for biofuel conversion on produced water with optimization of nutrient supplementation. Produced water for algae biomass production is expected to be a cheaper feedstock for biodiesel production as compared to pure cultures for cultivation. The growth patterns of the *S. obliquus* were studied with an aim to determine the maximum productivity. The biodiesel production was attempted via oil extraction and transesterification both in single stage and two stage reactor units in order to get the maximum biodiesel yield. The

present work investigated the usefulness of techniques like FTIR, NMR, GC and elemental analyses to understand the chemical properties of *S. obliquus* biomass, lipid and biodiesel. The fuel properties were also investigated. The results were then compared with conventional diesel in order to establish the potential of *S. obliquus* biomass for biodiesel production.

II. MATERIALS AND METHODS

II. 1 MICROALGAE, GROWTH MEDIUM AND CONDITIONS

S. obliquus microalgae were obtained from Freshwater Microalgae Culture Laboratory of the Department of Biotechnology, Gauhati University, Assam (India). Initially the cultures were grown in sterile petri-dishes containing solid nutrient rich medium and 15% (w/v) agar and were maintained in a 24-hour 120µmol m^{-2} s^{-1}light setup. The colonies were then transferred to the baffled Erlenmeyer flasks containing 200 mL of nutrient rich medium (Blue green algal 11 media) and kept on an illuminated shaker table. The cultures were then transferred to a 1 L sterile Photobioreactors (PBR) illuminated at 156µmol m^{-2} s^{-1} on a 16/8 hour on/off duty cycle and maintained at 24 °C ± 1 °C [9]. The culture was mixed with air enriched with CO2 maintained at 1.5 L min^{-1} and 20 mL min^{-1}, respectively. Regular sub culturing was performed after every 15–18 days. The pH was maintained at 7.0 ± 0.1 through injection of CO_2 at the rates outlined above based on pH feedback control. Laboratory grade CO_2 (Assam air products) was utilized in all experimentation so that negligible levels of contaminants from CO_2 addition could be assumed. These conditions provided for a steady but relaxed growth rate for the microalgae culture with low light used to minimize light stress and support culture growth at low starting densities [8]. Experimental medium was made following the protocol by Blue Green algal (BG11) medium consisting macronutrients (g/L) $NaNO_3$ (1.5), $CaCl_2$•$2H_2O$ (0.036), $MgSO_4$•$7H_2O$ (0.075), KH_2PO_4 (0.04), $NaCO_2$ (0.020), EDTA-FeNa (0.001), citric acid (0.006), ammonium ferric citrate (0.006) and micronutrients(mg/L) H_3BO_3 (0.061), Na_2MoO_4•$2H_2O$ (0.013), $MnSO_4$•$5H_2O$ (0.169), $ZnSO_4$•$7H_2O$ (0.287), $CuSO_4$•$5H_2O$ (0.003), $Co(NO_3)_2.6H_2O$ (0.049). Analytical grade reagents (MERCK) were used, and the medium was autoclaved at 120 °C for30 min.

II. 2 COLLECTION AND PRE-TREATMENT OF PRODUCED WATER

The produced water used in this study was obtained from the Oil fields of Dulijan located in Oil India Limited, Upper Assam (India) during the period of December 2014-January 2016. Two-barrel sample was collected and then was subjected to filtration by the used of activated charcoal by Aquarium Pharmaceuticals® on a Whatman Filter papers (125mm), to filter out the dense layer of crude oil from the surface of the produced water and then autoclaved at 120 °C for 30 min. After the water samples cool down to room temperature, water is analyzed by ion chromatography for determination of compounds present in it.

II. 3 *S. OBLIQUUS* GROWTH STUDY IN DIFFERENT CONCENTRATION OF PRODUCED WATER

The *S. obliquus* was in grown in different concentration of produced water (10%, 20%, 30%, 40%, 50%, 60%) with2mM of Urea in each 500ml Erlenmeyer Conical flask and separate flask with no produced water with only 2mM Urea in BG11 media. The experiment was conducted in indoor culture condition, where the rise in pH is regularly monitored and adjusted with pure CO2 gas cylinder (Assam air products) to neutralize the algal culture. The experiment was conducted for 30 days. The media has been replenished with 2mM of Urea and BG11 media with PW every 3rd and 6th day in a week, after taking out 10ml algal culture from each flask for Biomass estimation, Biochemical analysis and further elemental analysis of the media water derived after the separation of biomass.

II. 4 MEASUREMENT OF CELL GROWTH

Determination of growth was done by direct cell count or by measuring the optical density of the sample at absorbance 680 nm using a UV-visible spectrophotometer (Sepecord®50 PLUS, analytikjena, Germany). Biomass yield was determined by measuring the dry mass of the cells gravimetrically. Equal volume of fresh media was added after each withdrawal to maintain the total volume of the culture. For each parameter of the studies average values were considered from the data generated from at least three replicates of each study. The cell concentration was determined by measuring the optical density at 680 nm and comparing with calibration curves.

i) Cell count and preparation of growth curve

Cell count by using Haemocytometer is widely used to determine the density of cells in a unit volume of fluid. Haemocytometer has nine squares (each an area of 1 mm^2) etched over it of which only one can be seen per field under low magnification of microscope (100×). The Haemocytometer is provided with 50 squares of three variable sizes: 9 large squares of 1.0 mm2 each, 25 squares of 0.2 mm^2 each within a large square and 16 small squares 0.05 mm^2 each within each medium sized square. The depth of the Haemocytometer is 0.1 mm as the cover glass is 0.1mm in diameter thick above the counting chamber. Thus each large square has a volume of, 1 mm × 1 mm × 0.1 mm or 1/10 cm × 1/10 cm × 1/100 cm or 1/ 10,000 cm^3.

The cell numbers were counted randomly in some of the medium sized squares of the haemocytometer at 400× magnification of a compound microscope. The average number of cells per medium is calculated and multiplied with 25 to obtain the number of cells per large square. The calculated number is again multiplied with 10^4 to obtain the total number of cells per cm^3 (mL).

ii) Determination of specific growth rate (µ/d), divisions per day (k) and doubling time (T2)

Specific growth rate represents the average growth rate of all cells present in a culture, which defines the fraction of increase in biomass over a unit time. In case of direct cell count method, the specific growth rate was calculated according to Equation (2), otherwise Equation (3) was followed for determination of growth by measuring the optical density.

$$\mu/d = [Ln (N_2 / N_1)] / (t_2 - t_1) \quad (1)$$

where, N_1 and N_2 = biomass at time (t_1) and time (t_2) respectively.

$$\mu/d = (\log OD_t - \log OD_o) / T \quad (2)$$

where, ODt, ODo were the absorbance at terminal and initial day respectively; and T was the duration (days) between the two measurements.

If "t" is expressed in days, the growth rate (μ) can be converted to divisions or doublings per day (k) by dividing (μ) by the natural log of 2 (= 0.6931), according to the Equation (4):

$$k = \mu / 0.6931 \quad (3)$$

The time required to achieve a doubling of the number of viable cells is termed as doubling time (T2), which was calculated following Equation (4):

$$T_2 = 0.6931 / \mu \quad (4)$$

II. 5 BIOMASS DETERMINATION

Biomass production was determined by measuring the dry cell weight gravimetrically. The dry weight of algal cells was measured by filtering an aliquot of culture suspension on pre-weighed Whatman filters (GF/C). The filters were rinsed with redistilled water, dried in a hot-air oven at 80°C for 6 hrs, cooled down to room temperature in desiccators over anhydrous silica gel until constant weight. Biomass productivity was expressed in gram per unit volume per day (g/L/d).

II. 6 HARVESTING OF CULTURE AND LYOPHILIZATION OF WET BIOMASS

Flocculation is used to facilitate the harvesting of biomass by aggregating the algal cells and increase the particle size. It is commonly combined with filtration, centrifugation or sedimentation. Flocculation was tested by the adjustment of culture pH and the effect of addition of ferric chloride ($FeCl_3$) on the recovery rate. Selection of this method was determined, based on the perspective that raising the culture pH to a high alkalinity (~pH 11 - 12) will act as a measure to check pre-cultivation contamination by treating large volume of culture in order to facilitate recycling of waste medium for cultivation. Moreover, addition of $FeCl_3$ (one of the mineral nutrient of the selected medium used to culture the microalgae strain) during harvesting will omit the necessity of further addition of the compound [9].

Dewatering is the process in which the supernatant i.e. biomass free medium is decanted and finally the biomass is collected separately. To achieve this, the biomass in agglomerate form was further centrifuged at 4000 RPM for 2 - 5 min. and the pellets were collected. The biomass in agglomerate form thus achieved first frozen at -20˚C followed by freeze drying at -110˚C using a freeze dryer (SCANVAC Cool safe, ALPHA 1-4, Germany) for 36 - 48 h[10].Lyophilized algal biomass samples were then finely grinded and stored in -20˚C until analysis.

II. 7 OIL EXTRACTION AND BIODIESEL PRODUCTION

i) Simultaneous oil extraction and transesterification (In single stage process):

Lepage et al. developed a single step method to prepare FAME from the total lipids of a sample [11]. The method, termed direct transesterification or in situ transesterification, allowed for the quantification and characterization of the total fatty acids of a sample without requiring a separate extraction method. Direct transesterification has been successfully used to produce biodiesel from materials for which traditional solvent extractions are not efficient the dried algae biomass was added to the reactor after fine grinding. The solvent and methanol along with the catalyst (conc. H_2SO_4) were also added to the reactor. The temperature was maintained at 60 °C for 1 h with mixing. After the reaction was completed, the products were allowed to cool down to room temperature and distilled water was added to the mixture. The products were transferred to the separating funnel which immediately resulted in the formation of two layers. The upper solvent layer with biodiesel was separated and subjected to distillation for biodiesel recovery. Percentage yield of biodiesel was then determined using the above Eq. (5). The effect of different solvents (chloroform, hexane, and no solvent) on biodiesel yield was also studied using the similar procedure

ii) Oil extraction followed by transesterification (In two stage process):

Lipid content from lyophilized biomass (5 g) was initially extracted with n-hexane (250 ml) by soxhlet extraction at 50 ºC. The soxhlet apparatus was run for 16 h in total with the interval of 15 - 20 min duration between two cycles. Dry weight of the extracted lipids was measured gravimetrically after the recovery of solvent using a rotary evaporator at 50 ºC under reduced pressure. The biomass residue was collected from the extraction thimble after cooled down to room temperature and dried in a hot air oven at 60 ºC for 1 h. A known amount (1 g) of the residual biomass was grounded using an ice cold mortar and pestle with the addition of solvent mixture (5 ml) of chloroform / methanol (2:1, v/v) containing Butylated Hydoxytoluene (BHT 0.2%, w/v)[12].Water was then added to the reactor and the mixture was transferred to a separating funnel. Phase separation was observed. Lower phase of biodiesel was collected and washed with distilled water. The percentage yield of biodiesel was calculated using the Eq. (5):

$$\textit{Yeild of biodiesel}\ (\%) = \frac{\textit{Grams of Biodiesel produced}}{\textit{Grams of Oil used}} \times 100 \quad (5)$$

II. 8 CHARACTERIZATION OF BIODIESEL FROM *S. OBLIQUUS*

The biodiesel from S. obliquus grown in control media (only BG11) and biodiesel extracted from *S.obliquus* grown in 40% PW media were characterized using techniques like FTIR, NMR, GC and elemental analyses to understand the chemical properties of biodiesel. The fuel properties of algae biodiesel were also investigated

i) CHNS analysis

The elemental analysis of carbon, hydrogen, nitrogen and sulphur of dried algae biomass, algae oil and biodiesel was carried out using CHNS analyser. It is performed to check carbonate and organic carbon content to get some idea of the composition of the organic matter (ElementarVario El Cube)

ii) Proton nuclear magnetic resonance (^{1}H NMR)

NMR has been shown to be an efficient technique for the evaluation of biodiesel quality. A typical ^{1}H NMR spectrum of petroleum diesel shows signals of aromatic (6.00–9.00 ppm) and aliphatic (0.00–3.30 ppm) hydrogen. The ^{1}H NMR analysis of *S.obliquus*

biodiesel was conducted using BrukerSpectrospin 300 operating at 300 MHz. [13]. The samples were prepared in $CDCl_3$ with the ratio of 1:1 by volume in 5 mm NMR tube and TMS (tetramethylsilane) was used as an internal standard.

iii) Fourier transform-infrared (FTIR) spectroscopy

Fourier transform infrared spectroscopy was employed as a fast and reliable analytical technique for the quantification of fatty acid methyl ester content in the produced biodiesel. Using FTIR spectrometer (THERMO NICOLET iS10 FT-IR SPECTROMETER) at room temperature [14].The dried biomass powder of *S. obliquus* was mixed with potassium bromide (KBr) powder and pressed into pellets before analysis. The liquid samples of algae oil and biodiesel were sandwiched between the two KBr pellets and then introduced in the spectrometer.

iv) Gas chromatography (GC) analysis

Lipid extract by n-hexane soxhlet extraction was subjected for GC analysis. Preliminary identification and quantitative estimation of the major hydrocarbons was achieved using an Agilent 6890N GC system equipped with a flame ionization detector (FID). A DB-1 fused-silica capillary column (15 m × 0.32 mm × ID 0.25 µm) was used. Oven temperature was programmed at 75°C to 230°C at a rate of 25°C / min and then to 340°C at a rate of 10°C / min with a holding time of 10 min at 340°C. The injector and detector temperatures were 300°C and 350°C respectively. Identification and quantification of the major hydrocarbon groups were done based on the comparison with authentic standards (nC8 to nC36 aliphatic hydrocarbons, Sigma Aldrich) and following the standard method according to TNRCC METHOD 1005,respectively[15]. The relative amount of individual components was expressed as percent peak areas relative to total peak area. Compounds were identified from the mass spectra based on comparison with the NIST and Willy GC-MS Libraries.

v) Fuel properties

The characteristic of fuel properties of *S. obliquus* oil in normal media and produced water were evaluated using standard methods and results were then compared to ASTM D 6571 and EN 1424 the European standard for ideal fuel properties [16]. The following fuel properties were evaluated: density (specific gravity bottle), viscosity (Fungi lab rotational viscometer), acid number (titrimetric analysis), calorific value (RSB Rajdhani digital bomb calorimeter), pour point (assembled pour point tester) and flash point (Pensky marten's flash point Tester-Biomate).

III. RESULTS AND DISCUSSIONS

III. 1 GROWTH STUDY OF *S. OBLIQUUS* IN BG11 MEDIA AND DIFFERENT CONCENTRATION OF PRODUCED WATER

The growth pattern of *S. obliquus* was studied for 30 days in two different growth media setup, one in normal BG11 media and other set with 10%-40% PW+ BG11 media. From Fig. 1 and 2, that the gradual increase in cells/ml with time indicates the growth and biomass productivity of *S. obliquus* was best in BG11 media and 40% PW. On comparison, *S. obliquus* in BG11 was observed to have the highest Cell count of 28.12 x 10^4 cells ml-1followed by Biomass of (1.2gL^{-1}) whereas growth in BG11 showed highest cell count of algae 22.12 x 10^4 cells ml^{-1}followed by Biomass of (1.4 gL^{-1}).The figures show that there was gradual increase in the growth of *S. obliquus* in BG 11 medium and in 40% PW+ BG11 media from 16-30 days after which growth was almost stable. In different concentration of PW, the specific growth rate, doubling time and rate of division differs. The highest specific growth rate (1.89 d^{-1}), least doubling time (0.23 days) and rate of division (4.17 d^{-1}) for *S. obliquus* growth in BG11 media was recorded on 3rd, 14th and 15th day respectively and highest specific growth rate (2.76 d^{-1}), least doubling time (0.224 days) and highest rate of division (3.75 d^{-1}) for *S. obliquus* in40% PW media was recorded on 12th, 17th and 28th day of study. Thus the growth study significantly indicates the potential of replacing costly BG11 media with 40% produced water for the better biomass productivity and cell growth by *S. obliquus* in the same period of growth study. The biomass and cell growth from 10%, 20%, 30%, 50% and 60% PW test studies were not satisfactory for the study, so their biomass were not considered for further biomass, lipid and biodiesel analysis.

Since *S. obliquus* growth rate and biomass production in BG11 media was almost similar to the growth in 40% PW, therefore both were considered for further comparisons in lipid content and biodiesel characterization.

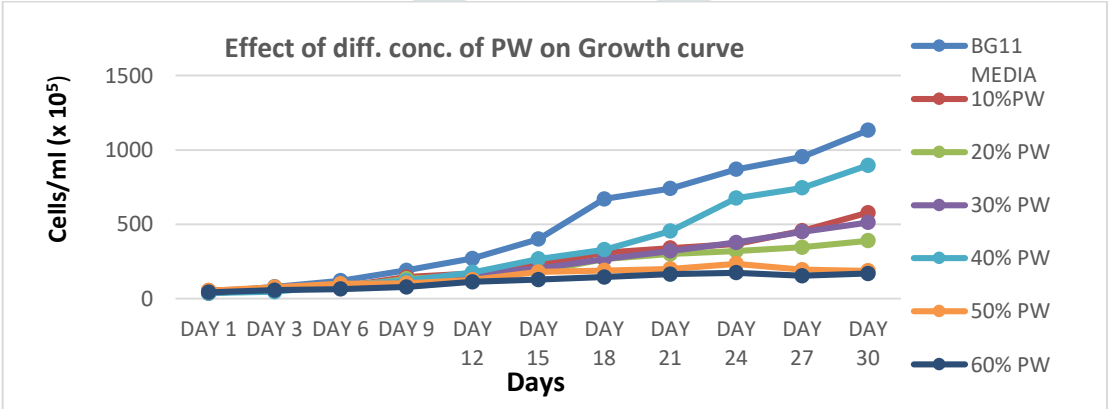

Figure 1: Effect of different concentration of PW on the growth of *S. obliquus*

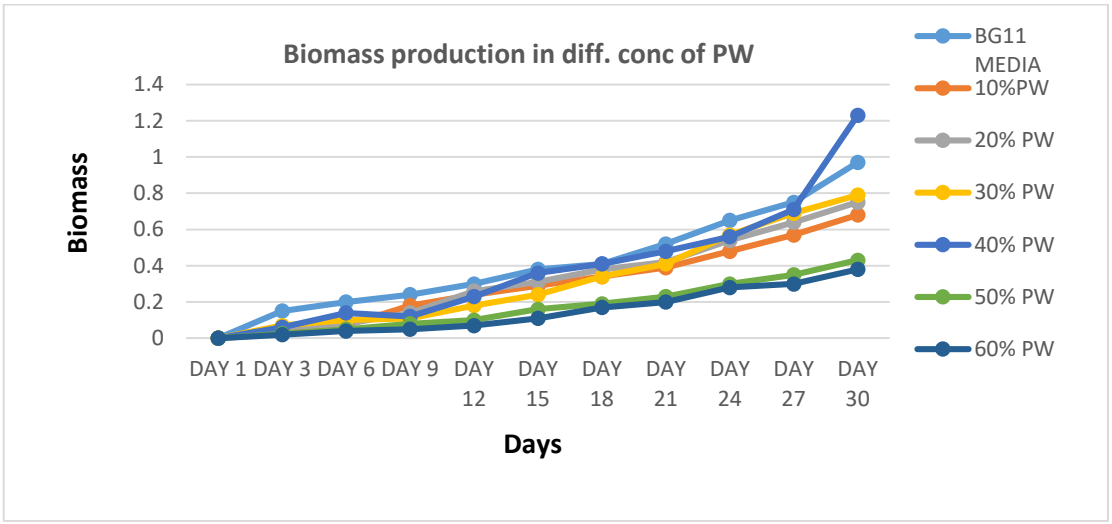

Figure 2: Biomass production of *S. obliquus* in different concentration of PW

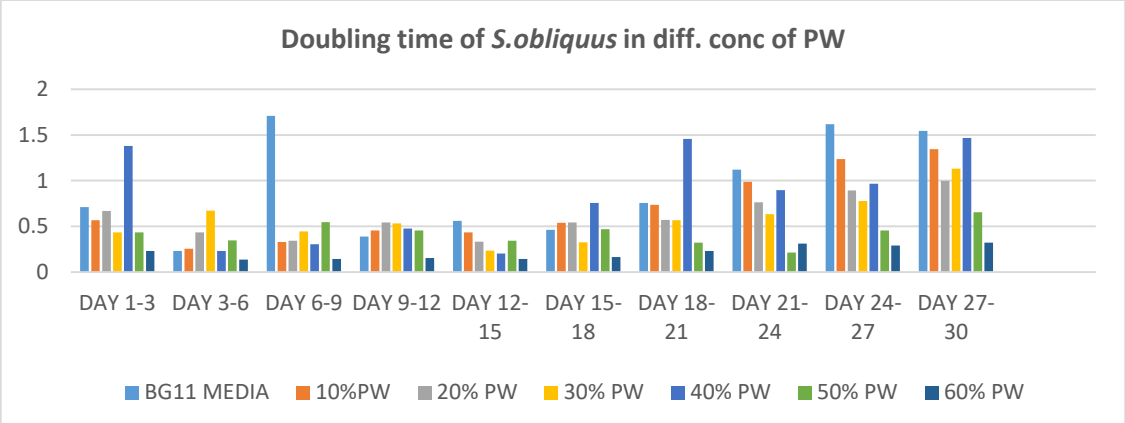

Figure 3: Doubling time of *S. obliquus* in different concentration of PW

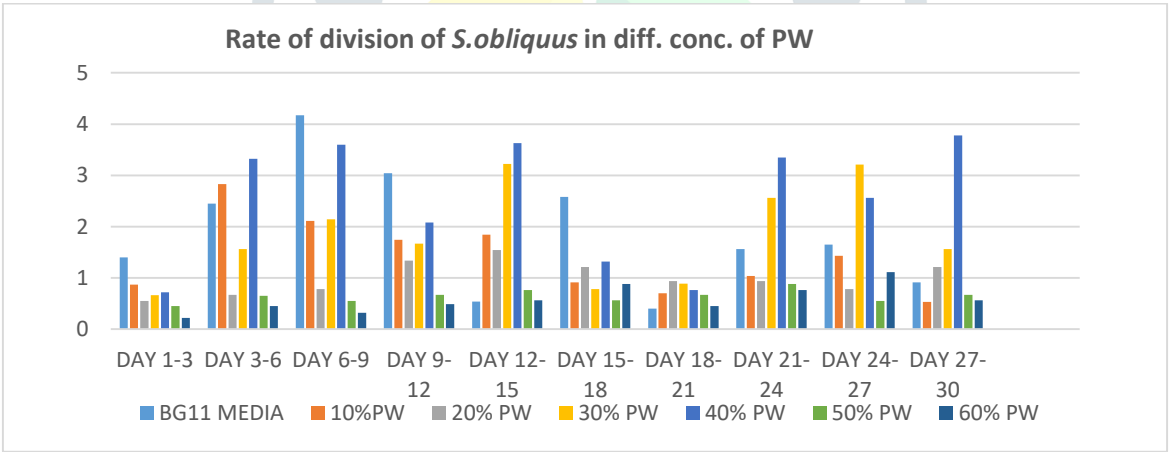

Figure 4: Rate of cell division of *S. obliquus* in different concentration of PW

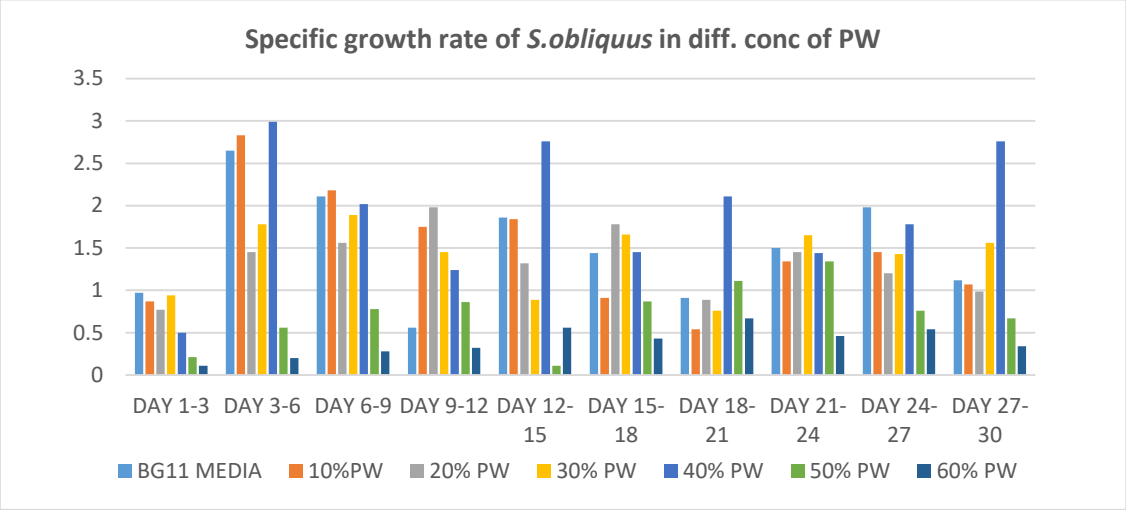

Figure 5: Specific growth rate of *S. obliquus* in different concentration of PW

III. 2 BIODIESEL PRODUCTION

The biodiesel yield obtained via a two stage process of oil extraction followed by transesterification was compared with that obtained by simultaneous oil extraction and transesterification, a single stage process as shown in Fig. 3 and 4. The biodiesel yield was expressed in the terms of relative weight of biodiesel obtained to that of oil present in algae biomass. It was observed that the single stage process resulted in higher biodiesel yield compared to two stage process. Direct transesterification was originally derived by Lepage and Roy (1984) [17] as a rapid method to analyze fatty acid contents of adipose tissues and milk. The method is highly desirable as it reduces the amount of downstream manipulation required for biodiesel production from any given biomass. The single-step procedure is less labour intensive and allows simultaneous recovery of total lipid extracts from multiple samples of green microalgae with quantitative yields and fatty acid profiles comparable to those methods devised by Folch et al. (1957), Bligh and Dyer (1959) [18].The method involves the simultaneous addition of acid catalyst and pure methanol to microalgal biomass (generally in the form of dried powder). The methanol extracts the lipids from the microalgal biomass and, catalyzed by the acid, concurrently transesterifies the extracted lipids to produce fatty acid methyl esters. The reaction mixture is first distilled to remove methanol. It is then left to settle under gravity to induce biphasic partitioning (top biodiesel/ un-transesterified lipids phase and bottom glycerol phase). The biodiesel/un-transesterified lipids phase is decanted off and washed repeatedly with water to eliminate any acid catalyst. It is noted that studies investigating direct transesterification of microalgal biomass have, so far, only used acid catalysts (acetyl chloride and H_2SO_4) [19].When methanol is used; the reaction produces fatty acid methyl ester (FAME) or biodiesel. Either an acid (such as H_2SO_4) or an alkali (such as NaOH or KOH) can be used as a catalyst for transesterification (Christie, 2007) [20]. Since alkali catalysts have faster reaction rates (estimated at 4000× faster) and higher conversions than acid catalysts for the transesterification of acylglycerols, they are commercially used in the chemical industry for conversion of plant and animal oils to biodiesel (Huang et al., 2010) [21]. During alkalinetransesterification of acylglycerols, the catalyst cleaves the ester bonds holding the fatty acids to the glycerol backbone (Chisti, 2007) [22]. The liberated fatty acids are then reacted with methanol to form FAME. In lab-scale experiments where only small amounts of crude microalgal lipids are available, a large amount of methanol (substantially in excess of stoichiometric requirement) is often added to ensure quantitative transesterification. Once transesterification is completed, the reaction mixture, containing biodiesel, glycerol, reformed alkali catalyst, excess methanol, and un-transesterified lipids, then undergoes posttransesterificationpurification to remove by-product contaminants (glycerol, alkali catalyst, and excess methanol). Analyses of the FAME composition of the purified biodiesel/untransesterified lipids phase are carried out using a gas chromatography (GC) system. Similar trends were obtained both for *Spirulina* and pond water algae when extraction was done by double and single step process. According to the studies conducted by Piyush Nautiyal *et. al* The maximum yield of *Chlorella* Sp. Biodiesel, *Spirulina* algae and *Ankistodesmus* sp. biodiesel obtained using hexane in a single stage process was 74.60% ,79.50% and 38.67% respectively [23].Comparing the effect of different solvents on the biodiesel yield, hexane was observed to give higher yield than chloroform. Hexane being more non-polar as compared to chloroform, lipids gets more effectively dissolved in hexane than in chloroform. Variables that affect FAME conversion during alkaline transesterification include the molar ratio of acylglycerol to methanol, the molar ratio of acylglycerol to catalyst, the reaction temperature, the reaction time, the FFA content of the crude lipids, and the water content of the Drastic reduction in biodiesel yield was observed when no solvent was used indicating that solvent is necessary for biodiesel production. The maximum yield of *S. obliquus* grown in only BG11 Media biodiesel and 40% FW+ BG11 media biodiesel obtained using hexane in a single stage process was 35.45 % and 48.32% respectively. Comparative study of Single step biodiesel extraction with two step biodiesel extraction yield of different strains of microalgae is compared with *S.obliquus* in table 1.

Table 1: Comparing different microalgae biodiesel concentration in two stage and single stage biodiesel extraction process using different extraction solvents

Microalgae (Freshwater)	***Two stage Biodiesel Prod.***	***Single stage Biodiesel prod.***
*S.obliquus*in BG11 media	*28.18%*	*35.45%*
S. obliquus in 40% PW media	*37.88%*	*48.32%*
Spirulinalipid	*68.65%*	*79.50%*
Chlorella sp. Lipid	*63.66%*	*74.60%*
Ankistodesmus sp.lipid	*30.57%*	*38.67%*

III. 3 CHARACTERIZATION

i). CHNS analysis of biomass and total lipid of S. obliquus grown in BG11 and 40% PW

Table 2 CHNS analysis shows the comparison of the elemental percentage of microalgae biomass, lipid and its biodiesel. An increase in the percentage of carbon and hydrogen was observed in biodiesel in both the cases, which depicts the higher heating value of microalgae biodiesel. The decrease in the percentage of sulphur, nitrogen and oxygen was also observed in biodiesel. The lower the nitrogen and sulphur percentages in the biodiesel, the lesser will be the exhaust emissions (NOx and SO_X) while using it in a diesel engine. The lower the oxygen percentage, the lesser will be the oxidative degradation of biodiesel, indicating higher stability of biodiesel.

Table 2: CHNS analysis (wt.%).

Experimental condition	**C**	**H**	**N**	**S**	**O**
***S.obliquus* Biomass in BG11**	43.04	7.45	9.87	0.56	32.45
***S.obliquus* Biomass in PW**	48.12	9.32	11.14	0.67	35.16
***S.obliquus* lipid in BG11**	49.09	12.45	0.16	0.35	25.23
***S.obliquus* lipid in PW**	59.12	14.26	0.43	0.14	19.12
***S.obliquus* Biodiesel in BG11**	57.54	10.76	0.35	0.10	15.23
***S.obliquus*Biodiesel in PW**	67.08	12.87	0.23	0.06	8.65

ii) 1H NMR of biodiesel from S. obliquus grown in BG11 and 40% PW

NMR is one of the most useful techniques to explain the structure of chemical compounds. On comparison of the NMR spectra (Fig.6and 7) of their respective biodiesel, a characteristic peak at 3.6 ppm (parts per million) could be observed in the biodiesel spectra of both NMR graphs due to the presence methyl group of ester. The NMR spectra of algae biodiesel was compared with that of karanja biodiesel which is a potential non-edible source of Biodiesel [24], which also showed the peak at 3.6 ppm. Other peaks observed in the ^{1}H NMR spectra of *S. obliquus* biodiesel and Karanja biodiesel were at 0.8 ppm (triplet, due to terminal methyl hydrogen), a strong signal at 1.2 ppm (strong singlet, due to the backbone methylenes of carbon chain), a multiplet at 1.6 ppm (multiplet, due to beta methylenes proton), 2.3 ppm (triplet, due to alpha methylene proton to ester), and some peaks because of unsaturation at 2.0 ppm (alpha methylene group to one double bond), 2.8 ppm (alpha methylene group to two double bonds) and 5.3 ppm (olefinic protons). The presence ofpeaks at 3.6 ppm and 2.3 ppm confirmed the presence of methyl esters in the sample. ^{1}H NMR spectra of *S. obliquus* oil grown in BG11 and *S. obliquus* oil grown in 40% FW biodiesel were similar to that of Karanja biodiesel supporting the fact that algae biodiesel has the potential as a potential substitute for diesel.

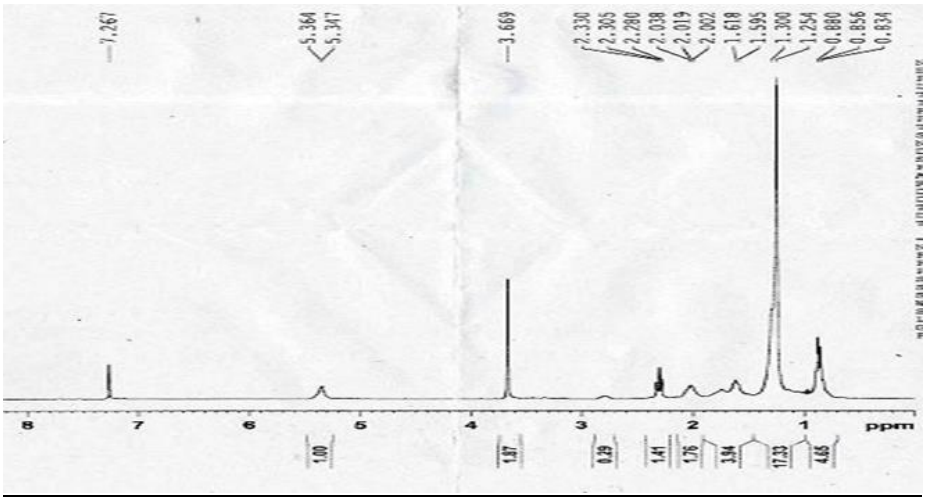

Figure 6. ^{1}H NMR spectra of *S. obliquus* oil grown in BG11

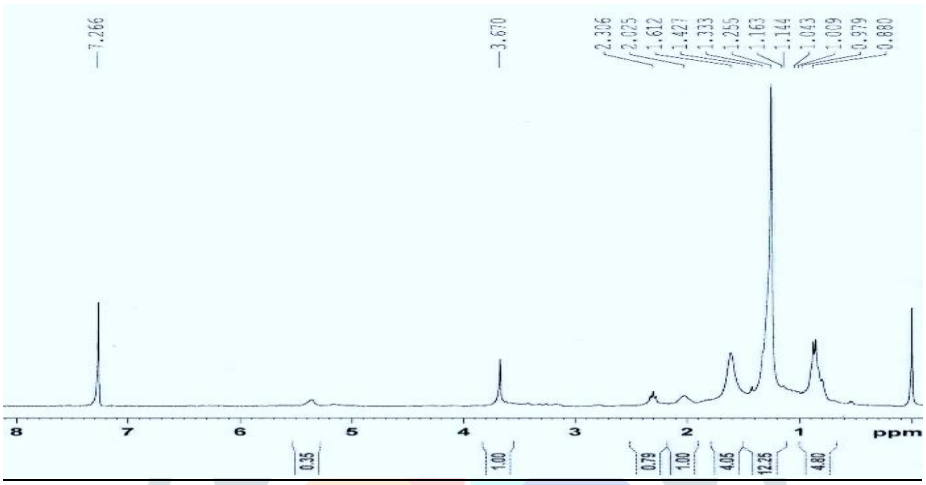

Figure 7. ^{1}H NMR spectra of *S. obliquus* oil grown in 40% FW biodiesel

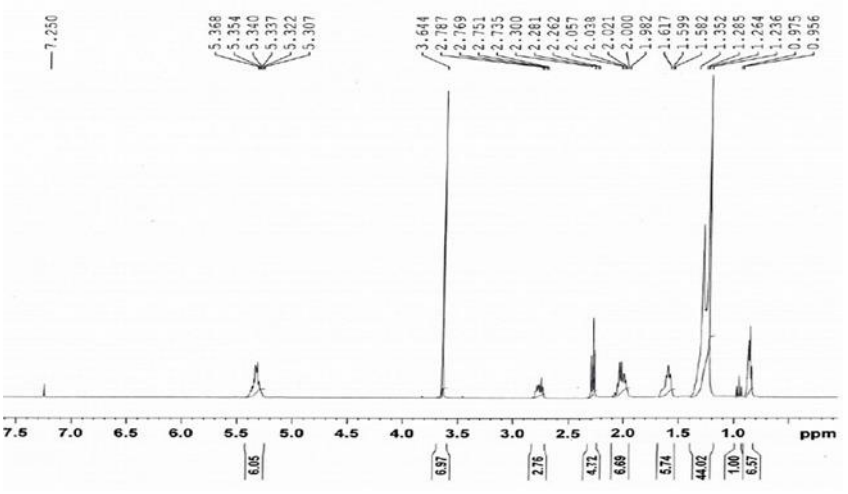

Figure 8. ^{1}H NMR spectra of Karanja biodiesel

iii) FTIR of biodiesel from *S. obliquus* grown in BG11 and 40% PW

The peaks or bands in the FTIR spectrum are due to the functional groups present in a particular sample. In the present study, mid FTIR region was selected to identify the functional groups in the particular sample because the functional groups run to 4000 cm^{-1} to 1450 cm^{-1}. The FTIR spectra (Fig.7 and 8) of biodiesel from *S. obliquus* grown in BG11 and in 40% PW were compared with their respective biodiesel spectra which showed the well absorbed regions of 3500–3000 cm^{-1}, 1800–1000 cm^{-1} and 800–700 cm^{-1}. The typical peaks at 2927 and 2860 cm^{-1} are due to symmetrical and asymmetrical stretching vibrations of $–CH_2–$ groups. The peaks at 3340 cm^{-1} due to double bond stretching and 1300–1100 cm^{-1} due to C\O bond (axial stretching) were also observed in the spectra of algae oil and biodiesel. Absorption peaks were also noticed at 699 cm^{-1} due to $–CH_2–$ bending out of the plane and at 1365–1377 cm^{-1} due to $–CH_3$ bond. Similar peaks were observed in the FTIR spectra of algae oil except a peak in the range of at 1442–1458 cm^{-1} in biodiesel spectra only, which is due to the methyl ester moiety and at 1714 cm^{-1} corresponding to the NC=O stretching. The presence of these peaks indicates the conversion of oil to biodiesel. Similar peaks were observed in the spectra of *S. obliquus* oil grown in 40% PW biodiesel. Comparing the FTIR spectra of conventional petroleum diesel with that of biodiesel, peak at 1743 cm^{-1} was not observed in the case of diesel, which is due to carbonyl group present only in biodiesel. Moreover, diesel is mainly composed of aliphatic hydrocarbon which shows absorption at 2924 cm^{-1} and 2853 cm^{-1}. [25]

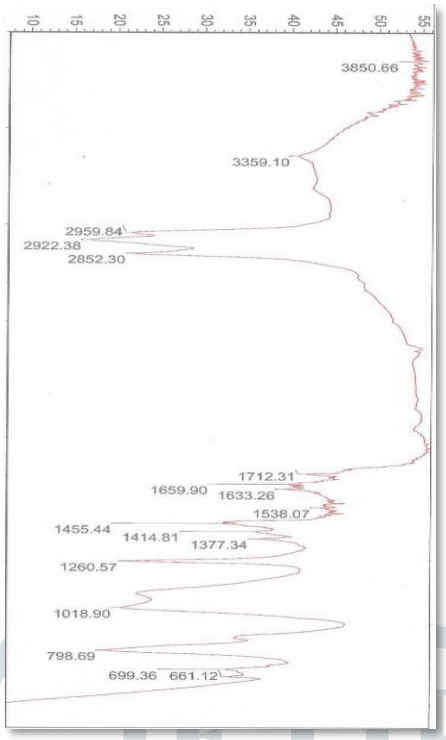

Figure 9 FTIR Spectra of Biodiesel from grown in BG11 media

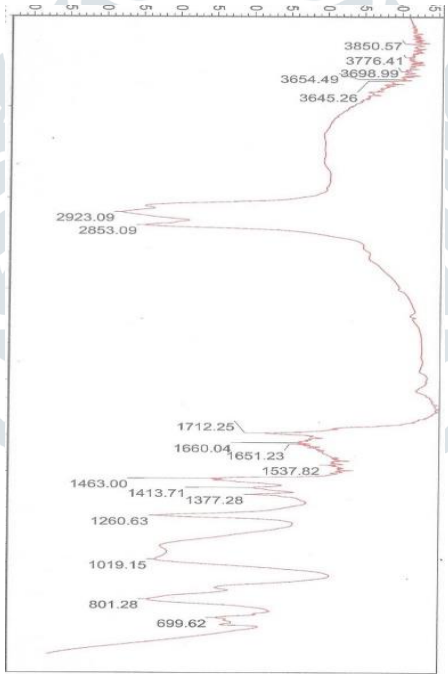

Figure 10 FTIR Spectra of Biodiesel from S. obliquusgrown in 40% PW media

iv) GC analysis

GC analysis was used to study the chemical composition of algae biodiesel produced from *S. obliquus* grown in BG11 and *S. obliquus* grown in 40% PW. The peaks in the chromatograms of biodiesel samples and the standard were compared and their respective retention time was used to identify and quantify the peaks. The analysis majorly showed the presence of five saturated (palmitic, caprylic, myristic, lauric, and stearic) and five unsaturated (linolenic, linoleic, oleic, palmitoleic, and pentadecanoic) methyl esters in both the algae biodiesel chromatograms. However, one extra peak of polyunsaturated fatty acid (Eicosapentaenoic) was observed in the case of *S. obliquus* oil grown in 40% PW biodiesel. Table 3 shows that the composition of fatty acid methyl esters present in both cases, biodiesel is different with respect to the sum percentage of saturated and unsaturated fatty acid methyl esters. *S. obliquus* grown in 40% PW biodiesel is rich in oleic and palmatic methyl esters. The fatty acid composition is also compared with that of karanja biodiesel (Table 3). Polyunsaturated fatty acid (Eicosapentaenoic) was observed in the case of *S. obliquus* oil grown in 40% PW biodiesel. Table 3 shows that the composition of fatty acid methyl esters presents in both cases, [26] biodiesel is different with respect to the sum percentage of saturated and unsaturated fatty acid methyl esters. *S. obliquus* grown in 40% PW biodiesel is rich in oleic and palmatic methyl esters. The fatty acid composition is also compared with that of tallow biodiesel (Table 3).

Table 3: FAMEs (fatty acid methyl esters) composition of biodiesel (a) *S. obliquus* oil grown in BG11 (b) *S. obliquus* oil grown in 40% FW (c) Karanja oil

Fatty acid profile	*S.obliquus* grown in normal BG11 media (wt. % of FAME in biodiesel)	*S.obliquus* grown in FW water media (wt. % of FAME in biodiesel)	Karanja FAME (wt. % of FAME in biodiesel)
Caprylic	3.56	3.95	-
Lauric	2.12	1.12	-
Myristic	3.12	2.54	6.54
Cis-10-Pentadecanoic	2.22	3.12	-
Palmitic acid (16:0)	35.43	41.24	32.35
Stearic acid (18:0)	3.23	2.67	4.65
Oleic acid (18:1)	10.45	34.65	35.23
Linoleic acid (18:2)	5.45	12.21	15.32
Linolenicacic (18:3)	3.12	16.7	1.65
Eicosapentaenoic	-	0.08	-

v) Fuel properties

The fuel properties of biodiesel of *S. obliquus* grown in BG11 and *S. obliquus* grown in 40% PW were determined and compared with commercial diesel in Table 4. Most of the properties satisfy the standards prescribed by the American and European specific to various test methods to be used in the determination of certain properties for biodiesel blends. The density of the biodiesel sample in the present study *S. obliquus* grown in BG11 biodiesel and *S. obliquus* grown in 40% PW biodiesel was 855 kg/m^3 and 872 kg/m^3 respectively, which satisfies the European standards. Specific gravity of *S. obliquus* grown in BG11 biodiesel and *S. obliquus* grown in 40% PW biodiesel was 0.860 and 0.877 respectively. Density and viscosity of particular a fuel affect the brake specific fuel consumption. The higher the density and viscosity, the higher will be the mass of fuel injected which will increase the brake specific fuel consumption. The density and viscosity of conventional diesel is 842 kg/m^3 and 3.34 mm^2/s respectively [27]. These values are comparable to the density and viscosity values of algae biodiesel from *S. obliquus* grown in BG11 biodiesel and *S. obliquus* grown in 40% PW which can be easily blended with diesel for running the engine. Acid value is related to long term stability of biodiesel against corrosiveness. The lower the acid value, the better is the quality of biodiesel. The acid value for *S. obliquus* grown in BG11 biodiesel and *S. obliquus* grown in 40% PW biodiesel was determined to be 0.48 mg KOH/g and 0.42mg KOH/g respectively. The acid value of diesel (0.2 mg KOH/g) is less as compared to biodiesel due to presence of acidic components (esters) in the chemical structure of biodiesel [28]. Calorific value also affects the brake specific fuel consumption because more fuel with lower calorific value is required to maintain the specified power. Calorific value of biodiesel from *S. obliquus* grown in BG11 biodiesel and *S. obliquus* grown in 40% FW was found to be 30.56 MJ/kg and 38.36 MJ/kg respectively which lower as compared to calorific value of 39.112 MJ/kg for karanja biodiesel. Flash point of conventional diesel is about 78 °C as compared to 114 °C of biodiesel from *S. obliquus* [29]. The higher the flash point, the lower will be the chances of premature ignition. Pour point of a liquid fuel is the lowest temperature at which it loses its flow properties. Pour point is the lowest temperature at which a fuel will ceases to flow or pour when cooled and tested under prescribed conditions. It is an index of lowest temperature limit for utility as lubricating oil. It also indicates dissolved wax concentration of lubricating oil. Pour point of *S. obliquus* grown in BG11 biodiesel and *S. obliquus* grown in 40% FW biodiesel was in the specified range laid down by the American and European standards

Table 4: Fuel properties of S. obliquus biodiesel compared with conventional diesel standard properties

Parameter	*S.obliquus* grown in normal BG11 media biodiesel	*S.obliquus* grown in FW water media biodiesel	Diesel	ASTM D6751	EN 14214
Density (kg/m3)	855	872	832	-	860-900
Viscosity (mm2/s) at 40 °C	5.55	5.77	3.22	1.9-6.0	3.5-5.0
Specific gravity	0.860	0.877	-	0.88	-
Acid number (mg KOH/g)	0.48	0.42	0.17	0.50 max	0.50 max
Calorific value (MJ/kg)	30.56	38.36	42.65	-	-
Pour point (°C)	-19	-15	-17	-	-
Flash point (°C)	114	109	78	Min 100-170	Min 120

IV. Conclusion

From the growth study in the present work, it can be concluded *S. obliquus* grown in 40% PW biodiesel showed the maximum growth rate based on the cell proliferating rate and dry cell weight. *S. obliquus* grown in 40% PW biodiesel can be considered as a viable feedstock for biodiesel production as well successful handling to waste of refinery from oil fields. The single stage process of simultaneous extraction and transesterification resulted in higher biodiesel yield as compared to extraction followed by transesterification in two stages. The spectra of NMR and FTIR and gas chromatographic analysis data showed very clearly that biodiesel obtained from *S. obliquus* grown in 40% PW biodiesel biomass were composed of rich fatty acid methyl esters. Gas chromatographic analysis data also showed that biodiesel is richly composed of oleic acid and linoleic acid methyl ester. Fuel properties of algae biodiesel show that biodiesel from *S. obliquus* grown in BG11 and in 40% PW biodiesel were comparable in quality with that of other conventional biodiesel. Based on the fatty acid composition and the fuel properties, it is concluded that biodiesel produced from *S. obliquus* grown in 40% PW biodiesel could be a good alternate to conventional diesel.

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