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A Method of Central Composite Design (CCD) For Optimization of Biodiesel Production from *Chlorella vulgaris*

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Abstract

The direct transesterification of the oleaginous biomass using chloroform resulted in a high biodiesel yield and FAME content than the chloroform-based extraction transesterification method and the yield was almost 100%. Hexane (89%) and petroleum ether (80%) also produced biodiesel but the yield was low when compared with chloroform. Our study showed that the biodiesel yield and FAME content of the wet biomass in the direct transesterification was significantly lower than those obtained from freeze-dried biomass suggesting that drying the algae was necessary for direct transesterification. The optimization procedures were carried out with algae: ethanol ratio, catalyst amount, reaction temperature and reaction time. The central composite design (CCD) was used to optimize the various processes. The analysis of variance (ANOVA) was also carried out and the results were found to be significant. The ρ value less than 0.0001 indicated that the model was statistically significant. The optimization procedures revealed that a yield of 95% was obtained. The 3D plot was done by keeping two components constant and other two components variable. In GC-MS, the results showed that the main components found were tetradecanoic acid ethyl ester (C14:0) 2-5%, Hexadecanoic acid ethyl ester (C16:0) 26-45%, Hexadecanoic acid ethyl ester (C16:1) 25-38%, octadecanoic acid ethyl ester (C18:0) 1-2%, oleic acid ethyl ester (C18:1) 9-13%, Eicosapentaenoic acid ethyl ester (C20:5) 1.2-5.1%. As the temperature was increased the percentage of C20:5 were decreased to 1.2% which was a good indication for fuel properties.

Keywords: Biodiesel; *Chlorella vulgaris*; FAME; Microalgae

Introduction

Fossil fuels account for 88% of the primary energy consumption, with oil (35%), coal (29%) and natural gas (24%) as the major fuels, while nuclear energy and hydroelectricity account for 5% and 6% of the total primary energy consumption. The potential threat of global climate change has increased, and for a major part, this has been attributed to greenhouse gas emissions from fossil fuel usage [1]. One important goal is to take measures for transportation emissions reduction, such as the gradual replacement of fossil fuels by renewable energy sources, where biofuels are seen as real contributors to reach those goals. Biofuels production is expected to offer new opportunities to diversify income and fuel supply sources. They can be produced using existing technologies and be distributed through the available distribution system. For this reason biofuels are currently pursued as a fuel alternative that can be easily applied.

Although biofuels are still more expensive than fossil fuels their production is increasing in countries around the world. Biodiesel is produced from vegetable oils (edible or non-edible) or animal fats. Since vegetable oils may also be used for human consumption, it can lead to an increase in price of food-grade oils, causing the cost of biodiesel to increase and preventing its usage, even if it has advantages comparing with diesel fuel. According to the US standard specification for biodiesel [2], it is defined as a fuel comprised of mono alkyl esters of long chain fatty acids from vegetable oils or animal fats. Current policies at regional and national levels and the expected cost and difficulties in obtaining fossil fuels will necessarily lead to an increase in biodiesel production and of other types of renewable energy. The available quantities of waste oils and animal fats are not enough to match the today demands for biodiesel.

Thus transition to second generation biofuels, such as microalgae

can also contribute to a reduction in land requirements. A large investment in research and development (R&D) and strategies are still needed, for all stages of the biofuels value chain, from raw materials production to delivery and final consumption. Among the various possibilities currently being investigated and implemented at pilot scale or even at industrial scale concerning potential feedstocks, the more interesting ones are microalgae. Besides their cultivation is not directly linked to human consumption, they have low space requirements for its production. The reaction to make biodiesel, once the lipids from the algae are extracted, is the same as when using virgin vegetable oils, which has been in use for a number of years [3]. This reaction is known as transesterification.

In recent years, research has been directed to explore alternate to diesel fuel, which is produced from oils via transesterification. Direct transesterification is a process that blends the microalgae with an alcohol and a catalyst without prior extraction. Number of acid catalysts have been investigated for heterotrophic microalgae biomass including hydrochloric (HCl) or sulphuric acid (H_2SO_4) but acetyl chloride (CH_3COCl) remains the catalyst producing the higher fatty acid methyl esters (FAME) yield of 56% (g FAME/g dry weight)

[4]. A less polar solvent, like hexane or chloroform, can be added to increase the yield of biodiesel production [5]. Direct transesterification using a heterogeneous catalyst could be more effective coupled with microwaves heating. As an example, using microwave with direct transesterification of Nannochloropsis in presence of a heterogeneous catalyst (SRO), Koberg et al., [6] reported an increase in the fatty acid methyl esters (FAME) yield from 7 to 37% (g FAME/g dry weight). Biodiesel is a potential substitute for conventional diesel fuel. This outlines the state-of-the-art in biofuel production from microalgae which gives high yield when compared to the soybean oil, waste oil, etc. This is because of the land utilized by the crops whereas the land for microalgae is only less.

The present work deals with the production of biodiesel from the microalga Chlorella vulgaris. Microalgae are preferred for macroalgae because they contain more amounts of lipids which are essential for extraction of biodiesel. Chlorella vulgaris is screened for study as they contain more oil per yield when compared to other microalgae. Biodiesel yield was also optimized with algae to ethanol ratio, reaction temperature, reaction time and catalyst amount.

Materials and Methods

Microalgal culture

The microalgae *Chlorella vulgaris* was collected from pond located near P. S. G. Arts and Science College campus and isolated using agar plating technique. Indoor algal stock culture was maintained according to Perumal et al., [7].

Preparation of biodiesel from algal biomass

Biodiesel (FAME) was prepared from algal biomass through two methods: (1) oil extraction from algal biomass followed by transesterification; and (2) direct transesterification from algal biomass. The algal oil extraction procedure was adapted from the protocol described by Bligh and Dyer [8]. Freeze-dried algal biomass (one gram) or wet algal biomass (with one gram dry weight equivalent) was placed in a solvent-proof chamber of a bead beater. Few ml of distilled water was added to the dry biomass. 2 ml of chloroform and 9 ml of methanol were then added to the chamber. 4 ml of distilled H_2O were then used to wash the chamber, and added to the tube followed by mixing. The contents were then centrifuged at 6000 g for 10 minutes. The organic layer containing the algal oil was transferred to a pre-weighed glass vial. The mass of lipids (oil) were determined after the solvent was evaporated.

Transesterification

A mixture of methanol, sulphuric acid and chloroform was added to the algal oil, and heated at 70°C for 25 minutes. After the reaction was completed, the samples were cooled and mixed with 1 ml distilled water. The lower phase containing FAME was collected and transferred to a pre-weighed glass test tube. The solvent was evaporated and the mass of biodiesel (FAME) were determined.

Direct transesterification

Freeze-dried algal biomass (one gram) or wet algal biomass (with one gram dry weight equivalent) was placed in a glass test tube and mixed with methanol and sulphuric acid. About 2.0 ml solvent was added to the tube, or there was no solvent addition; in this case, an additional methanol was added to keep the reaction volume consistent. The tubes were reacted at 70ºC for 25 minutes. After the reaction was completed, the tubes were removed from the water bath. The tubes

were allowed to separate, forming a biphasic solution. The organic layer containing FAME was collected and transferred to a pre-weighed glass vial. The solvent was then evaporated using oven, and the mass of biodiesel was determined via weighing.

Biodiesel yield evaluation

The yield of biodiesel produced from different transesterification methods was estimated as its weight relative to (1) the weight of total lipid/oil presented in the biomass, and (2) the weight of algal biomass.

Central Composite Design for biodiesel production

The experiments were initially considered to study the individual effects of the experimental factors on yield. A quadratic model was used to fit the data and tested for significance [9]. The analysis of variance over the quadratic model was conducted with 95% confidence level. Optimization of biodiesel production was carried out using Central Composite Design (CCD).

The four factors investigated were A: oil: alcohol ratio, B: Catalyst amount, C: Temperature and D: Time. CCD experimental design lead to a set of 30 experimental runs as shown in Table 5 and was used to optimize parameters for the production of biodiesel.

The Yield of the biodiesel was calculated by the following equation,

$$Percentage\ Yield = \frac{Weight\ of\ biodiesel}{Weight\ of\ oil} \times 100$$

FAME analyses with GC-MS

The fatty acid ethyl esters were quantified with a gas chromatography–mass spectrometry (GC–MS) system composed of an Agilent 5975 C MSD and an Agilent 7890 A GC equipped with a capillary column (DB-23, 60 m X 250 μm X 0.15 μm nominal). Internal standard solution of ethyl heptadecanoate was prepared by dissolving 10.00 mg in 1 mL heptane. The weight percentages of fatty acids were approximated by the area of the detector response. The content of the fatty acid ethyl ester in the final product was calculated quantitatively by dividing the peak areas of fatty acid ethyl esters to the peak area of the internal standard obtained from GC-MS analysis.

Results and Discussion

Characteristics of algal biomass

The alga Chlorella vulgaris has established the ability of growing on biodiesel derived crude glycerol. The freeze-dried biomass appeared as green powder. The dried biomass contained 51% lipid, 14% proteins, and 24% carbohydrate, with 11% ash. The alga has a relatively simple fatty acid profile with myristic acid (C14:0), palmitic acid (C16:0), docosapentaenoic acid (C22:5). Previous work illustrated that total fatty acid accounted for 40-50% of dry biomass [10].

Comparison of extraction-transesterification and direct transesterification

The biodiesel yield of the algal biomass during extraction-transesterification and direct transesterification was compared. Similar to most reports on algal biodiesel production [11], the biodiesel yield was first expressed as its weight comparative to the algal oil present in the biomass.

Extraction-transesterification

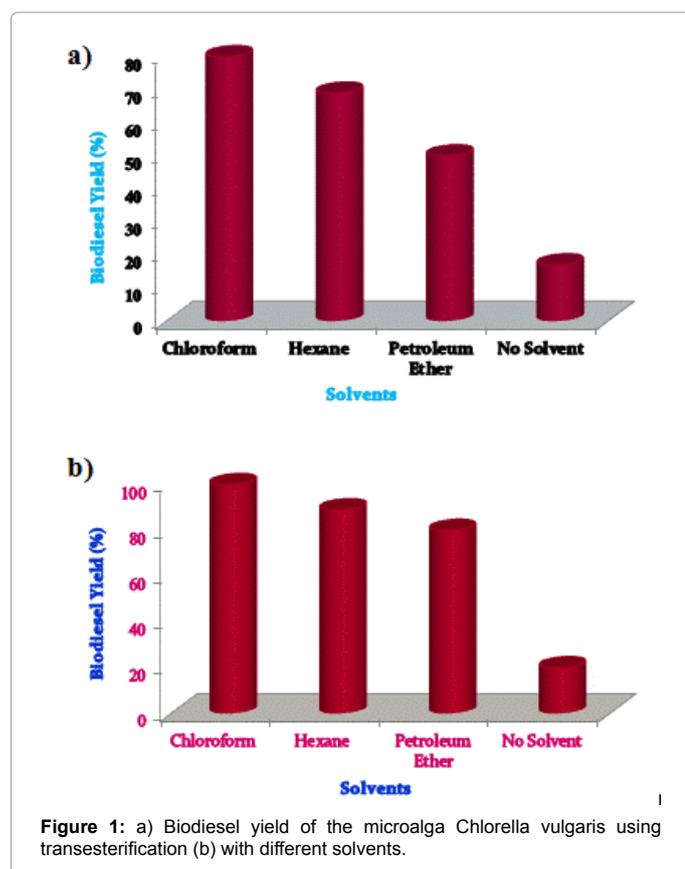As shown in Figure 1a, the extraction-transesterification resulted

Figure 1: a) Biodiesel yield of the microalga Chlorella vulgaris using transesterification (b) with different solvents.

in a biodiesel yield of 80%. When no solvent was used, however, biodiesel yield was very low, representing solvent was necessary for the transesterification reaction. The biodiesel yield was further estimated based on its weight comparative to the dry algal biomass. The transesterification reaction was carried out with different solvents such as hexane, chloroform and petroleum ether and there was also no solvent addition. Among these chloroform gave more yield of about 80%, hexane gave a yield of 69%, petroleum ether 50% and when no solvent was added the yield was reduced to about 17%. Demirbas [12] observed the similar results with chloroform in the extraction transesterification process.

Direct transesterification

The direct transesterification resulted in a higher biodiesel yield when compared with extraction transesterification. As shown in Figure 1b, the algae can make 10-20% higher biodiesel yields by means of direct transesterification with solvent rather than when applying the extraction-transesterification method. When no solvent was used in the direct transesterification, however, biodiesel yield was very low, representing solvent was necessary for the direct transesterification. Figure 2a also explains that when chloroform was employed, the biodiesel yield went beyond 100% due to the partial oil extraction. The yield of hexane was about 89% and that of petroleum ether was about 80%. When no solvent was added the yield was reduced to 20%. Thus addition of solvent was essential to get more yields. Johnson and Wen [5] used direct transesterification in Schizochytrium limanicum and obtained greater yield with chloroform as a solvent.

The categorization of the FAME composition and the total FAME content of the biodiesel were presented in Table 1. The major methyl

esters contained in the biodiesel fuel were derived from myristic acid (C14:0), palmitic acid (C16:0), docosapentaenoic acid (C22:5). These compositions are in agreement with the major fatty acids profile of the algal biomass. The degree of unsaturation of the FAME was in the range of 2.04–2.26 (Table 2). The content of total FAME varied significantly with the different transesterification methods and the solvents used. The direct transesterification with chloroform resulted in highest FAME yield. When hexane and petroleum ether were used as solvent, the FAME yields were significantly reduced (Table 3). The results indicate that the biodiesel quality (heating value, based on FAME) was much higher when using chloroform rather than hexane or petroleum ether.

Algal biodiesel production was typically performed by extraction of algal oil followed by transesterification [11,13]. Direct methylation producing FAME from biomass has also been reported in some algal species [14] and fungi [13]. The direct transesterification was much less time consuming than the traditional oil extraction and transesterification. Transesterification of neutral lipids (triacylglycerides) into FAME requires a catalyst for the reaction. Currently, alkali-catalyst is commonly used by commercial biodiesel producers with pure vegetable oil as feedstock. However, when microbial sources such as fungi or microalgae were used as a biodiesel feedstock, acid proved a more effective catalyst for the transesterification because of the high free fatty acid value and thus soap formation in the alkali transesterification [11].

Previous reports on biodiesel production commonly used gravimetric weight determination relative to the oil as biodiesel yield. While this definition was still used in this work, the biodiesel yield was also evaluated based on algal biomass, which provides another indication on the potential of algal biodiesel production. Biodiesel yield and the FAME composition are significantly influenced by the transesterification conditions such as the methanol to oil ratio, the catalyst loading, reaction time, and temperature. Complete mixing of the reactants is another important parameter influencing the fuel quality. Previous results on direct transesterification have been reported using dried biomass [13-15].

When direct transesterification was used, the biodiesel yield from wet algal biomass was similar to that obtained from dry feedstock in terms of yield relative to oil and biomass, but was much reduced when compared to FAME yield. The characterization of FAME of the wet biomass derived biodiesel was presented in Table 1. The relative FAME

Contents	Extraction – Transesterification		Direct transesterification (with different solvent)	
	Wet biomass	Dry biomass	Wet biomass	Dry biomass
Oil content (% dry biomass)	62.25	58.19	-	-
Biodiesel yield	94.2	94.06	97.3	96.9
% of algal oil	60.18	61.9	68.67	66.47
% of biomass				
FAME Composition	4.20	5.40	ND	5.40
C14:0 (% FAME)	48.8	57.4	42.7	56.9
C16:0 (% FAME)	5.94	5.33	5.20	5.15
C22:5 (% FAME)	37.3	30.2	26.27	29.0
C22:6 (% FAME)	8.90	3.56	47.7	3.20
Others (% FAME)				
Total FAME content	68.62	61.68	11.1	68.65
% of algal oil (w/w)	44.2	40	7.03	44.7
% of biomass (w/w)	2.43	2.10	1.84	2.045

Table 1: Biodiesel yield and its FAME composition prepared from wet and dry biomass using extraction- transesterification and direct transesterification.

Run No	Oil: Alcohol Ratio	Catalyst Amount	Time	Temperature	% Y Expected	% Y Predicted	Residue
1	5	0.3	90	40	88.4	89.208	0.808
2	5	0.3	90	60	94	92.825	-1.175
3	5	0.3	90	60	94.5	94.532	0.032
4	7	0.1	150	40	76.5	76.152	-0.348
5	5	0.1	90	60	88	87.755	-0.245
6	7	0.1	30	70	70	68.597	-1.403
7	3	0.1	150	70	68	67.596	-0.404
8	5	0.3	90	60	93.7	91.8	-1.9
9	5	0.7	90	60	87.5	88.042	0.542
10	3	0.1	30	60	69.5	70.087	0.587
11	7	0.5	150	50	86.1	85.92	-0.18
12	5	0.3	150	60	86.7	86.887	0.187
13	7	0.5	30	70	80	81.35	1.35
14	5	0.3	90	60	94	92.825	-1.175
15	3	0.1	30	50	64	64.018	0.018
16	5	0.3	90	60	95	96.239	1.239
17	3	0.5	150	50	78	78.77	0.77
18	5	0.3	30	60	95	94.316	-0.684
19	5	0.3	30	60	95	96.239	1.239
20	3	0.5	30	70	74	73.313	-0.687
21	3	0.5	150	70	73.5	73.486	-0.014
22	3	0.5	150	50	62.7	61.383	-1.317
23	5	0.3	30	60	81.6	81.365	-0.235
24	3	0.1	30	50	74.5	73.31	-1.19
25	7	0.1	150	70	65.3	66.792	1.492
26	7	0.5	30	50	73.5	73.559	0.059
27	7	0.1	30	50	67.5	68.394	0.894
28	7	0.5	150	70	78	78.808	0.808

Table 2: Results of central composite design (CCD) showing observed and predicted response for optimization of parameters for biodiesel production from algal oil.

FAME Composition	Extraction – Transesterification	Direct transesterification (with different solvent)		
		Chloroform	Hexane	Petroleum ether
C14:0 (% FAME)	5.30	5.35	4.52	4.57
C16:0 (% FAME)	56.76	56.76	55.03	55.10
C22:5 (% FAME)	5.32	5.15	5.98	5.81
C22:6 (% FAME)	30.72	29.80	32.97	32.72
Others (% FAME)	3.56	3.20	2.89	4.85
Total FAME content				
% of algal oil (w/v)	60.68	68.65	9.75	10.30
% of biomass (w/v)	39.35	44.17	6.20	6.51
Degree of unsaturation	2.10	2.045	2.30	2.26

Table 3: FAME composition and total FAME content of the biodiesel produced from extraction-transesterification and direct transesterification methods.

Properties	ASTM method	Limits	Units	Actual value
Free glycerin	D-6584	0.02 Max	Mass %	0.007
Total glycerin	D-6584	0.24 Max	Mass %	0.099
Moisture	D-1796	Report	Mass %	0.11
Water & sediment	D-2709	0.05 Max	Volume %	0.09
Acid number	D-664	0.50 Max	mg KOH/g	0.21
Corrosiveness to copper	D-130	3 Max	ASTM standard	1
Flash point (closed cup)	D-93	130 Min	°C	204
Particulate matter check	C-100	Yes	visual appearance Yes	Yes
Kinematic viscosity (40°C)	D-445	1.9-6.0	mm²/s	4.87
Soap	Cc-17-79	Report	PPM	ND
Sulfur Content	D- 7039	15 Max	PPM	50

Table 4: Characters of biodiesel produced in a scale up of methylation of chloroform.

compositions were similar to those obtained from the dry biomass during the extraction-transesterification, but were different when using direct methylation. Also, the total FAME content, particularly obtained from the direct transesterification, was much lower than the FAME content from the corresponding dry biomass.

Biodiesel characteristics

The scaled-up biodiesel production resulted in approximately 1200 ml of liquid fuel from 400 g of algal biomass. The ASTM standard test of this liquid fuel indicated that the free glycerol, total glycerol, acid number, soap content, flash point, viscosity, and particulate free test meet the standard. However, the water and sediment and sulfur content did not meet the ASTM standard (Table 4).

The ASTM standard tests of the algae-derived biodiesel show that water and sediment failed the test. Since the liquid fuel was free of particulate matter but had 0.111% (by mass) of moisture content, water could contribute significantly (or fully) to the water and sediment content. In the future, the water and sediment content can be improved by a water removal step. In previous studies, excess water was removed via heating at 70°C under vacuum [16] or by drying the biodiesel over anhydrous sodium sulfate [17].

A final option, often used in industry, was to perform a dry wash using chemicals such as magnesium silicate so that water won't be added to the biodiesel during the wash steps. The other ASTM test that the liquid fuel failed was the sulfur content. This was probably due to the use of MgSO4 in the algal culture media, and the uptake of this sulfur by the algal biomass. Indeed, high sulfur content has been reported in this algal species [10]. From an ASTM standard point of view, the medium for this alga needs to be modified to use low sulfur/sulfate composition. Another important parameter in the ASTM test was cloud point, which indicated the cold flow properties of the
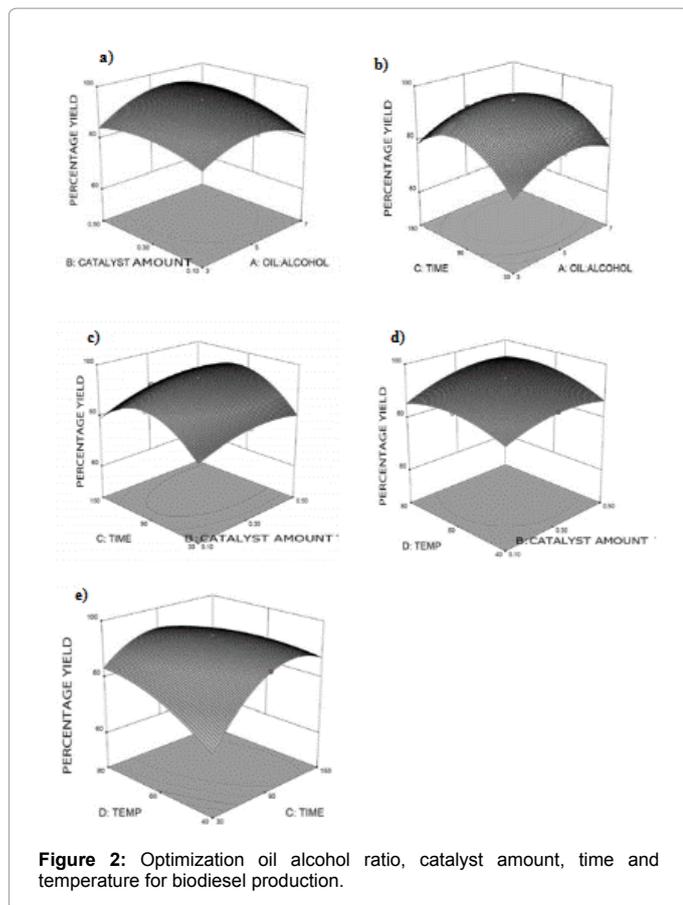
Figure 2: Optimization oil alcohol ratio, catalyst amount, time and temperature for biodiesel production.
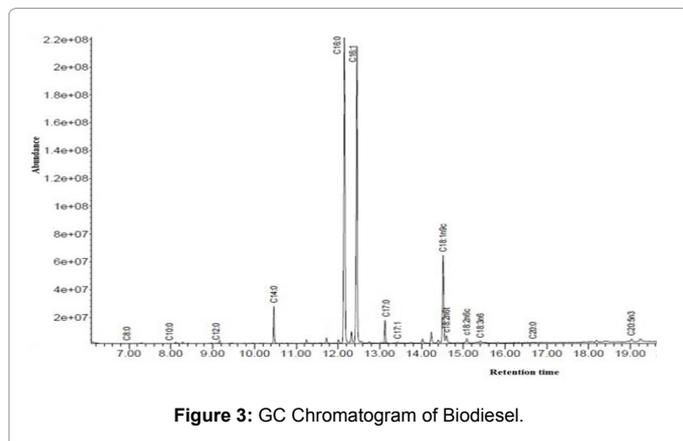

Figure 3: GC Chromatogram of Biodiesel.

Source	Sum of Squares	df	Mean Square	F Value	ρ Value Pro>F	
Model	3460.8	14	247.2			
Residual	2.92	15	0.19	1271.94	<0.0001	Significant
Pure Error	1.88	6	0.31			
Cor Total	3463.71	29				
				$R^2 - 0.9992$ Adj $R^2 - 0.9984$ Pre $R^2 - 0.9966$		

Table 5: Analysis of Variance (ANOVA) for the Fitted Quadratic Polynomial Model.

CCD plots: Figure 2a showed the 3D plot for the interaction effect between alcohol oil ratio (A) and Catalyst amount (B) toward biodiesel yield. The reaction time and reaction temperature were fixed at 90 minutes and 60°C respectively [18]. The 3D response surface showed the maximum biodiesel of 95.47% at oil: alcohol ratio of 1:5 and catalyst amount of 0.39. High yields of biodiesel were obtained at low catalyst concentrations because the reaction medium consisted of a three-phase system (oil-methanol-catalyst) in which the reaction would be slowed down when the catalyst amount is high.

In Figure 2b, the reaction temperature (D) and Catalyst amount (B) was kept constant at 60°C and 0.3% respectively and the plot was drawn between (C) reaction time and (A) alcohol-oil molar ratio. The maximum biodiesel of 94.01% was obtained at temperature of 60°C methanol: oil ratio of 1:5 concentrations. The 3D plot showed a sharp decline in the biodiesel yield when the molar ratio decreased [19].

In Figure 2c the temperature and oil: alcohol ratio was kept constant at 1:5 and the plot was drawn between reaction time and catalyst amount. As the reaction time increased to 30 minutes, the biodiesel yield started to increase and maximum yield of 95.17% was in 89 minutes at a catalyst amount of 0.38 [20].

In Figure 2d oil: alcohol ratio and time were kept constant at 1:5 and 90 minutes and the effect of reaction temperature and catalyst amount were studied on the biodiesel yield. It was found that the yield started to increase as the temperature was increased from 40°C and reached its maximum at 60°C and showed a decline at increasing temperature. The decline was due to evaporation of methanol as it is above its boiling point the optimum biodiesel yield of 95.099% was obtained at a catalyst amount of 0.36 and at a temperature of 60°C. From Figure 2e, methanol-oil molar ratio (A) and catalyst amount (B) were kept constant at 1:5 and 0.30% respectively and the plot was drawn between reaction temperatures (°C) and time (D). The graph showed that a maximum yield of 94.06% was obtained at temperature 60° C and time 60 minutes.

Analysis of fatty acid ethyl esters with GC-MS: The GC chromatogram data disclosed the occurrence of a major proportion of saturated and mono unsaturated fatty acid ethyl esters (Figure 3). Depending upon the reaction circumstances the main components found were tetradecanoic acid ethyl ester (C14:0) 2-5%, Hexadecanoic acid ethyl ester (C16:0) 26-45%, Hexadecanoic acid ethyl ester (C16:1) 25-38%, octadecanoic acid ethyl ester (C18:0) 1-2%, oleic acid ethyl ester (C18:1) 9-13% and Eicosapentaenoic acid ethyl ester (C20:5) 1.2-5.1%. As the temperature increased it was observed that the percentage of C20:5 present in the biodiesel was decreased almost to 1.2% which is a good indication for fuel properties. Other studies show that Chlorella species contains majority of short-chain fatty acids (C14–C18) which are main components of biodiesel.

Conclusions

The biodiesel was analyzed both by extraction- tranesterfication

biodiesel fuel. Although this parameter was not tested, it is believed the algal biodiesel will have a low cloud point, i.e. a superior cold flow property, due to the high unsaturated level of the FAME.

Statistical analysis

Analysis of Variance (ANOVA): The results of analysis of variance (ANOVA) were shown in Table 5 which indicated that the predictability of the model was at 99% confidence level. The predicted response fitted well with those of the experimentally obtained response. ρ value less than 0.0001 indicated that the model was statistically significant. The model was found to be adequate for prediction within the range of variable chosen.

and direct tranesterfication. Different solvents like petroleum ether, chloroform and hexane were used for extraction process. The yield of chloroform was 80%, hexane was 69% and that of petroleum ether was 50%. The direct transesterification of the oleaginous biomass using chloroform resulted in a high biodiesel yield and FAME content than the chloroform-based extraction transesterification method and the yield was almost 100%. Hexane (89%) and petroleum ether (80%) also produced biodiesel but the yield was low when compared with chloroform. The optimization procedures revealed that a yield of 95% was obtained. The 3D plot was done by keeping two components constant and other two components variable. The present study, central components design revealed that the detailed method providing mathematical modeling for optimization of biodiesel production from Chlorella vulgaris.

Acknowledgment

References

1. Ugarte DG, Walsh ME, Shapouri H, Slinsky P (2003) The economic impacts of bioenergy crop production in US agriculture, USDA Agricultural Economic Report, USA.

2. ASTM D6751-15 (2015) Standard Specification for Biodiesel Fuel Blend Stock (B100) for Middle Distillate Fuels, ASTM International, West Conshohocken, PA.

3. Apt KE, Behrens PW (1999) Commercial developments in microalgal biotechnology. Journal of Phycology 35: 215-226.

4. Cooney M, Young G, Nagle N (2009) Extraction of bio-oils from microalgae. Separation and Purification Reviews 38: 291-325.

5. Johnson M, Wen Z (2009) Microalgae as a feedstock for biofuel production. VCE Publication 442-886.

6. Koberg M, Much AR, Gedanken A (2011) Optimization of bio-diesel production from soybean and wastes of cooked oil: combining dielectric microwave irradiation and a SRO catalyst. Bioresour. Technol 102: 1073-1078.

7. Perumal P, Prasath B, Santhanam P, Ananth S, Devi SA, et al. (2012) Manual on Advances in Aquaculture Technology. In: Isolation and Culture of Microalgae 166-181.

8. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37: 911-917.

9. Joshi H, Moser BR, Toler J, Walker T (2010) Preparation and Fuel Properties of Mixtures of Soybean Oil Methyl and Ethyl Esters. Biomass and Bioenergy 34: 14-20.

10. Pyle DJ, Garcia RA, Wen Z (2008) Producing docosahexaenoic acid (DHA)-rich algae from biodiesel derived crude glycerol: Effects of impurities on DHA production and algal biomass composition. Journal of Agricultural and Food Chemistry 56: 3933-3939.

11. Xu H, Miao X, Wu Q (2006) High quality biodiesel production from a microalga Chlorella protothecoides by heterotrophic growth in fermenters. Journal of Biotechnology 126: 499-507.

12. Demirbas A (2005) Biodiesel production from vegetable oils via catalytic and non-catalytic supercritical methanol transesterification methods. Pror. Energy Combust Sci. 31: 466-487.

13. Liu B, Zhao Z (2007) Biodiesel production by direct methanolysis of oleaginous microbial biomass. Journal of Chemical Technology and Biotechnology 82: 775-780.

14. Lewis T, Nichols PD, McMeekin TA (2000) Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs. Journal of Microbiological Methods 43: 107-116.

15. Miao X, Wu Q (2006) Biodiesel production from heterotrophic microalgal oil. Bioresource Technology 97: 841-846.

16. Ma FR, Hanna MA (1999) Biodiesel production: a review. Bioresource Technology 70: 1-15.

17. Veljkovic VB, Lakicevic SH, Stamenkovic OS, Todorovic ZB, Lazic ML (2006) Biodiesel production from tobacco (Nicotiana tabacum L.) seed oil with a high content of free fatty acids. Fuel 85: 2671-2675.

18. Patil F, Adhikari B, Dhara S (2011) Development of chitosan-tripolyphosphate fibers through pH dependent ionotropic gelation. Carbohydr. Res. 346: 2582-2588.

19. Balat M (2008) Energy Sources Part A-Recovery Utilization and Environmental Effects 30: 429-440.

20. Gui MM, Lee KT, Bhatia S (2009) Supercritical ethanol technology for the production of biodiesel: Process optimization studies. J Supercrit Fluids 49: 286-292.