Utilization of quaternary solvent mixtures for extraction of lipids from Scenedesmus obliquus microalgae

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Abstract: Solvent toxicity is of major concern in the extraction of lipid from algae biomass via the solvent extraction technique. This study was carried out to determine the optimal solvent mixture (chloroform, methanol, ethanol, and dichloromethane) composition with less toxicity for the extraction of lipids from Scenedesmus obliquus microalgae. Optimization of the solvent mixture composition was performed using augmented simplex centroid design and the influence of cell disruption on lipid yield was assessed. The eco-toxicity of the solvent mixtures was assessed using thermodynamic prediction model. The optimal lipid yield of 19.4% lipid g-1 DCW (dry cell weight) was obtained using solvent mixture composition (1:5:1:1 v/v) chloroform/methanol/ethanol/dichloromethane. The cost estimation and environmental risk parameter values obtained from the use of proposed quaternary solvent mixture composition indicated that lower cost and less toxicity were achieved when compared with the commonly used chloroform-methanol mixture composition. Microwave-assisted lipid extraction gave 55.67% higher lipid recovery from microalgae and the quality of the extracted lipid was unaffected when compared with the conventional solvent extraction. The fatty acid profile revealed the extracted lipids as an appropriate feedstock for biodiesel production. Applicability of lipid extracted biomass obtained using the proposed technique is confirmed by SEM and FTIR analyses.

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The research interest in our laboratory range from catalysis for energy application, conversion of biomass to biofuels and other high-value products, thermal conversion processes such as pyrolysis, hydrothermal liquefaction, and gasification. This paper is part of our project on thermal conversion of algal biomass and its derivatives to biofuels and petrochemicals.

PUBLIC INTEREST STATEMENT

Microalgal biomass is considered to be very promising alternative renewable energy resources that can meet the global demand for fuels in the nearest future. As a result, microalgal biodiesel has gained interest as a potential alternative to petroleum diesel. The major process constraint in microalgal biodiesel technology is the cost-effective and efficient extraction of lipids. The use of single solvents or solvent mixtures for the extraction of microalgal lipids has been a method of choice for many years. However, cost-effectiveness, safety, and overall associated toxicities of the solvent mixtures are still of major concern. Here we propose a new alternative solvent mixture composition of chloroform, methanol, ethanol, and dichloromethane that is effective but less toxic for efficient lipid extraction from Scenedesmus obliquus microalgae.
1. Introduction

The utilization of single organic solvent or combined solvent mixtures for efficient lipid extraction from algal biomass has been an important area of interest to many researchers due to its ease of operation and scaling-up for industrial applications (Al-Ameri & Al-Zuhair, 2019; Ranjith-Kumar et al., 2015). The diverse polarities of microalgal lipids make it difficult to choose a single solvent that can extract the total lipids. For instance, non-polar solvents such as chloroform and dichloromethane are used mostly to extract neutral or storage lipids while polar solvents such as methanol and ethanol are used to disrupt the hydrogen bonds or electrostatic forces of membrane-associated lipid (also known as polar lipids) (Balasubramanian et al., 2013; Ishida & Chapman, 2009). The selection of the preferred extraction solvents is based on several criteria such as low cost, non-toxic, high efficiency, and relatively low boiling point; however, finding a single suitable solvent that meets all the specified conditions appears difficult (Arumugam et al., 2011).

In addition, the most widely used Bligh and Dyer (Bligh & Dyer, 1959) and Folch (Folch et al., 1957) extraction techniques for lipid from microalgae employ the use of chloroform-methanol in the different ratios. While Folch method used chloroform-methanol in the ration (2:1 v/v), the Bligh and Dyer method of extracting lipids from homogenized cell suspension used ratio of 1:2 (v/v) chloroform/methanol mixture. In spite of the effectiveness of the solvent mixtures for lipid extraction from microalgae, environmental and health risks remain a serious concern for industrial-scale application. Additionally, studies have shown that less toxic however less effective solvents such as dichloromethane, ethanol, petroleum ether, hexane, cyclohexane in various combinations have been employed for microalgae lipid extraction by many researchers (Jeon et al., 2013; Lae et al., 2019; Ramluckan et al., 2014; Ranjith-Kumar et al., 2015). Also, the efficiency of lipid extraction from microalgae was found to differ depending on microalgae species and solvent mixture composition (Lee et al., 2010).

For instance, Jeon et al. (Jeon et al., 2013) examined the efficiency of 15 different organic solvents in the extraction of lipids from Chlorella vulgaris microalgae and the gas chromatography (GC) analysis revealed methanol as the best solvent with greater efficiency for the extraction of fatty acids followed by dichloromethane. On the contrary, chloroform showed very low fatty acid content and a high level of unknown impurities although the yield of lipid obtained with the chloroform was equal or greater than using other solvents. But when solvent mixtures of chloroform, methanol, and dichloromethane were applied to determine the optimal composition for a high lipid extraction efficiency, the best solvent mixture composition was methanol: dichloromethane (1:1) and resulted in a 25% increase of lipid extraction yield when compared with the result obtained using Bligh and Dyers method. In another study reported by Ramluckan et al. (Ramluckan et al., 2014), 13 solvents of different polarities and solubility were used for the extraction of lipids from Chlorella sp. microalgae. The efficacy of the solvent individually and in the combined mixture was investigated. The authors concluded that ethanol, chloroform, and hexane were more efficient in the extraction of lipids than other solvents and the combination of chloroform and ethanol in the ratio (1:1) exhibited the greatest extraction efficiency. These findings contradict the outcome of Jeon et al. (Jeon et al., 2013) who reported methanol:dichloromethane (1:1) as the best binary solvent mixture for the extraction of lipids from Chlorella vulgaris microalgae. Chatsungnoen and Chisti (Chatsungnoen & Chisti, 2016) revealed that the optimal solvent mixture composition for the extraction of total lipids from Nannochloropsis salina microalgae was chloroform/methanol/water, in the volume ratio of 5:7:3:1. This shows that the optimum
solvent mixture composition for lipid extraction from microalgae depends on a number of factors such as microalgae species, growth conditions, source, or geographical location.

Furthermore, augmented simplex centroid design (ASCD) is one of the statistical tools used to design an experiment involving a mixture of components as well as the formulation of the empirical model relating the input variables to the response (Araromi et al., 2017; Chatsungnoen & Chisti, 2016; Jeon et al., 2013; Zuurro et al., 2016). Recently, Araromi et al. (Araromi et al., 2017) used the simplex centroid design (SCD) to optimize the mixing of petroleum ether, n-hexane, methanol, and ethanol for the extraction of oil (PLO) from Pitanga (Eugenia uniflora L.) leaves, via Soxhlet extraction. The experimental design matrix based on the simplex centroid design (SCD) produced different percentage compositions of the solvent mixtures. Although studies have been performed to identify solvent mixture composition with optimum lipid yield from microalgae; however, little attention has been given to finding effective but less toxic solvent mixtures. Besides, to the best of the authors’ knowledge, the optimization of solvent mixture composition for extraction of lipids from Scenedesmus obliquus microalgae has not been reported.

This study focused on the optimization of solvent mixtures (chloroform, methanol, ethanol, and dichloromethane) composition for efficient lipid extraction from Scenedesmus obliquus microalgae. The optimization studies were carried out using augmented simplex centroid design under the mixture methodology of the design of experiment (DOE). In order to assess the toxicity of the solvent mixtures, the environmental risk parameters such as octanol–water partition coefficient (Kow), soil adsorption potential (Koc), and bioconcentration factor (BCF) of the solvent mixture composition were determined using thermodynamic prediction model. Also, the economic feasibility of the proposed solvent mixture was evaluated.

2. Materials and methods

2.1. Chemicals and reagents
All chemicals used in this work were of analytical grade and were used without any further purification. All solvents used were of HPLC grade with purity ≥99.5% (Sigma Aldrich, USA). A mixed fatty acid methyl ester (FAME) standard made up of 37 components was acquired from Sigma–Aldrich, USA.

2.2. Microalgae cultivation and biomass collection
Microalgae S. obliquus was cultivated in outdoor raceway pond of about 2000 L working volume to generate biomass. The nutrient medium was modified BG11 with reduced nitrogen (750 mg/L) and iron supplementation (9 mg/L) (Cheah et al., 2016; Guldhe et al., 2017, 2014). The biomass was harvested on day 27 by gravitational settling followed by centrifugation to obtain thick biomass slurry. The harvested wet microalgal biomass was sun-dried at ambient temperature for 3 days using a drying bed covered with white plastic of 1500 µm thickness. The dried microalgal biomass flakes obtained were crushed using a stainless steel laboratory grinder and sieved to a particle size of <125 µm. The dry microalgal powder obtained was stored in a desiccator for further use. All the biomass used in this study was collected from one batch of the open pond cultivation.

2.3. Design of experiment (DoE) and modelling for lipid extraction
The augmented simplex centroid design (ASCD) under the mixture methodology of the Design-Expert software (version 11) was used to create the design of experiment (DoE) of the composition of quaternary solvent mixtures for lipid extraction from microalgae. The volume of the mixture constituents (chloroform, methanol, ethanol, dichloromethane) was each varied from volumes of 0 mL (0%) to 20 mL (100%) based on a preliminary experiment carried out to determine the effect of total solvent volume to biomass ratio on the lipid yield recovered from microalgae during which the maximum lipid yield was obtained at total solvent volume to biomass ratio of 20 mL: 1 g. This ratio was maintained all through the experiments. For a four mix-components scenario as the case in this study, the points chosen for ASCD are four pure blends (vertex points), six binary blends
(CentEdge points), four tertiary blends (TripBlend points), four quaternary blends (Axial points), and one centre point. Also, 5 replicates are added to the design of experiment by default consisting of 4 vertex points and one CentEdge point giving a total of 24 experimental runs as shown in Table 1.

The data of the response (lipid yield) obtained from the ASCD experiments were fitted to a regression model using Design-Expert software version 11. The regression model expression for the studied four-component mixture design is shown in equation 1.

\[
Y = b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4
\]  

(1)

Where, \(b_1, b_2, b_3\) and \(b_4\) are the linear coefficients; \(b_{12}, b_{13}, b_{23}, b_{24}, \) and \(b_{34}\) are the interaction coefficients; \(Y\) is the response (lipid yield) and \(X_1, X_2, X_3, \) and \(X_4\) are the terms of the solvent composition of chloroform (mL), methanol (mL), ethanol (mL), and dichloromethane (mL) respectively. The quality of fit of the developed model for the response was evaluated using significance and analysis of variance (ANOVA) test (Chartsungnoen & Chisti, 2016; Soji-Adekunle et al., 2018).

2.4. Lipid extraction

The lipid contents from the microalgae were extracted using the conventional solvent extraction method reported by Bligh and Dyer (Bligh & Dyer, 1959) with slight modifications. Specifically, 1 g of dry microalgal powder was mixed with a known volume of the individual components of the solvent mixture based on the design of experiments as shown in Table 1. The mixture was stirred for 3 h at 400 rpm with a magnetic stirrer (MS-H280-Pro digital magnetic hotplate stirrer) at room temperature and thereafter left for 24 h. The resulting mixture was centrifuged at 3490 x g for 5 min and filtered to separate solvent mixture and residual algae. The solvent was then removed to obtain the actual microalgal lipid using the oven at 70°C left overnight. The lipid content was measured gravimetrically and the yield of lipid extract was determined as the ratio of the mass of the extracted lipid to the mass of the microalgae powdered biomass used in the extraction (Equation 2):

\[
\% \text{Lipid Yield} = \frac{M_{\text{le}}}{M_{\text{b}}} \times 100
\]  

(2)

where \(M_{\text{le}}\) is the mass of the lipid extract, and \(M_{\text{b}}\) is the mass of dry microalgal biomass.

The determination of lipid yield was carried out in duplicate and average value reported.

2.5. Model validation experiment

The model validation was carried out using two (2) experiments of solvent mixture outside the studied solvent mixture composition. Lipid extraction experiment was carried out using the method earlier described and the percentage lipid yield was determined. The regression model obtained was also used to predict the percentage of lipid yield for each of the tested solvent mixture composition. The experimental lipid yield obtained was then compared with the predicted lipid yield.

2.6. Environmental fate of the solvent mixture

Thermodynamic prediction model was employed to computationally assess the eco-toxicity of the solvent mixture. The model determines the thermodynamic properties of a solvent based on results obtained from quantum computation (Lee & Lin, 2014; Mustapha et al., 2013). In this study, environmental risk parameters such as octanol-water partition coefficient (Kow), soil adsorption potential (Koc), and bioconcentration factor (BCF) determined were used to evaluate the toxicity of the solvent mixture. The procedure reported by (Zakari et al., 2013) was adopted in this work. The Marvin sketch software was first used in sketching the structure of each molecule of
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Table 1. Experimental and predicted lipid yield values by ASCD for the extraction process

| Run | Composition of components % (mL) | Lipid yield (% lipid g\(^{-1}\) DCW) |
|-----|---------------------------------|-------------------------------------|
|     | Chloroform \((X_1)\) | Methanol \((X_2)\) | Ethanol \((X_3)\) | DCM \((X_4)\) | Actual | ASCD predicted |
| 1   | 100 (20) | 0 (0) | 0 (0) | 0 (0) | 8.20 | 7.49 |
| 2   | 0 (0) | 100 (20) | 0 (0) | 0 (0) | 17.60 | 17.25 |
| 3   | 0 (0) | 0 (0) | 100 (20) | 0 (0) | 6.40 | 6.59 |
| 4   | 0 (0) | 0 (0) | 0 (0) | 100 (20) | 6.00 | 5.73 |
| 5   | 50 (10) | 50 (10) | 0 (0) | 0 (0) | 20.20 | 20.16 |
| 6   | 50 (10) | 0 (0) | 50 (10) | 0 (0) | 13.80 | 13.30 |
| 7   | 50 (10) | 0 (0) | 0 (0) | 50 (10) | 7.80 | 7.23 |
| 8   | 0 (0) | 50 (10) | 50 (10) | 0 (0) | 15.40 | 15.51 |
| 9   | 0 (0) | 50 (10) | 0 (0) | 50 (10) | 18.00 | 18.07 |
| 10  | 0 (0) | 0 (0) | 50 (10) | 50 (10) | 12.00 | 11.60 |
| 11  | 33.3 (6.67) | 33.3 (6.67) | 33.3 (6.67) | 0 (0) | 18.20 | 18.28 |
| 12  | 33.3 (6.67) | 33.3 (6.67) | 0 (0) | 33.3 (6.67) | 17.60 | 16.82 |
| 13  | 33.3 (6.67) | 0 (0) | 33.3 (6.67) | 33.3 (6.67) | 10.80 | 12.08 |
| 14  | 0 (0) | 33.3 (6.67) | 33.3 (6.67) | 33.3 (6.67) | 16.40 | 16.80 |
| 15  | 25 (5) | 25 (5) | 25 (5) | 25 (5) | 16.80 | 16.83 |
| 16  | 62.5 (12.5) | 12.5 (2.5) | 12.5 (2.5) | 12.5 (2.5) | 12.80 | 13.94 |
| 17  | 12.5 (2.5) | 62.5 (12.5) | 12.5 (2.5) | 12.5 (2.5) | 19.40 | 19.64 |
| 18  | 12.5 (2.5) | 12.5 (2.5) | 62.5 (12.5) | 12.5 (2.5) | 15.20 | 13.64 |
| 19  | 12.5 (2.5) | 12.5 (2.5) | 12.5 (2.5) | 62.5 (12.5) | 12.40 | 12.55 |
| 20  | 0 (0) | 0 (0) | 0 (0) | 100 (20) | 5.40 | 5.73 |
| 21  | 100 (20) | 0 (0) | 0 (0) | 0 (0) | 7.00 | 7.49 |
| 22  | 0 (0) | 0 (0) | 100 (20) | 0 (0) | 6.20 | 6.59 |
| 23  | 0 (0) | 100 (20) | 0 (0) | 0 (0) | 17.00 | 17.25 |
| 24  | 50 (10) | 0 (0) | 0 (0) | 50 (10) | 7.20 | 7.23 |

the solvent. The TURBOMOLE Software which uses the xyz file generated from Marvin sketch was then used to perform quantum chemical/COSMO calculation to generate the screening charge densities of the molecules. The quantum chemical/COSMO computation on TURBOMOLE 5.6 was parameterized using DFT-level of computation on TZVP basis set. The file was then saved and used to determine the Octanol-Water Partition Coefficients (Kow) and soil adsorption potential (Koc). The bioconcentration factor (BCF) was determined using the correlation relating Kow and BCF (Gobas, 2001) as presented in equation 3.

\[
\log(BCF) = 0.79 \log(K_{ow}) - 0.4
\] (3)

2.7. Effect of cell disruption on lipid yield
Microwave and sonication assisted solvent extraction was carried out to investigate the effect of cell disruption on the yield of lipid using the proposed quaternary solvent composition as solvent mixture. The microwave-assisted solvent extraction was performed using the method reported by (Lee et al., 2010) with slight modification. Thus, 1 g of dry powdered biomass was added to specified volumes from the selected quaternary solvent mixture composition (Table 1) and exposed to cell disruption using a microwave oven (Milestone S.R.L., Italy, output power 1200 W)
operated at a temperature of 100°C for 10 min with 1 kW of microwave energy. The mixture was centrifuged and filtered to separate solvent mixture and residual algae. The solvent was evaporated from the solvent mixture using the oven at 70°C left overnight to obtain the actual lipid in the biomass. The crude lipid content was measured gravimetrically and the lipid yield (%) was determined. For sonication assisted extraction, microalgal biomass (1 g) was mixed with a known volume of the selected quaternary solvent mixture and the lipid was extracted in 50 ml tubes using a sonicator (SONIC—150 W ultrasonic homogenizer, frequency 20–25 kHz) at 15 kHz for 5 min. The resulting mixture was also centrifuged and filtered to separate solvent mixture with lipids and residual algae. The residual algae were then mixed with a known amount of the quaternary solvent mixture composition and again subjected to sonication followed by centrifugation and then filtered. The solvent mixture from both runs was combined and the solvent was then removed using the oven at 70°C left overnight to obtain the actual lipid (Guldhe et al., 2014). The crude lipid content was measured gravimetrically and the lipid yield (%) determined. The lipid yields obtained from the two cell disruption assisted solvent extraction techniques were then compared.

2.8. Analysis of the effect of disruption on cellular morphology
The effectiveness of the algal cell wall disruption was observed by conducting scanning electron microscopy (SEM) analysis on the lipid extracted algae obtained from both the conventional solvent extraction (CSE) without cell disruption and microwave-assisted solvent extraction (MASE). The samples were prepared before image analysis by sprinkling small quantity of the lipid extracted algae on carbon adhesive tape placed on the sample holder and sputter coated with Au–Pd using Quorum T150T for 5 min. The sputter-coated samples were then analysed using high resolution scanning electron microscope (HRSEM) operated with electron high tension (EHT) of 5 kV for imaging. Fourier-transform infrared (FTIR) measurements were also carried out on the samples from the CSE and MASE using a Bruker Vertex 70 spectrometer (Bruker Optics, Billerica, MA, USA) equipped with a Platinum ATR sampling module. FTIR spectra were acquired in the mid-IR region (4000–400 cm\(^{-1}\)).

2.9. Fatty acid profile of the extracted lipids
The fatty acid profile of the extracted lipids obtained from conventional solvent extraction method and microwave cell disruption assisted solvent extraction were compared. The acid catalysed transesterification process was used for the conversion of microalgal lipids to fatty acid methyl esters (FAME) using the method reported by Guldhe et al. (Guldhe et al., 2014). A gas chromatograph (Shimadzu GC-2014, Japan) equipped with a flame ionization detector and a capillary column (SP2380, Supelco Analytical, USA) operated at optimized conditions as reported by Guldhe et al. (Guldhe et al., 2014) was then used to analyse the fatty acid methyl esters (FAME) present in the extracted lipids.

3. Results and discussion
3.1. ASCD modelling for quaternary solvent composition
The augmented simplex centroid design (ASCD) as described earlier in section 2.3 was employed for the design of experiment with varying solvent mixture composition for lipid extraction from S. obliquus microalgae. Based on average permissible exposure limit (PEL) reported by California division of occupational safety and health for chloroform, methanol, ethanol, and dichloromethane (2 ppm, 200 ppm, 1000 ppm, and 25 ppm, respectively), the toxicity of the studied solvents can be classified as chloroform > dichloromethane > methanol > ethanol. Among all the above-mentioned solvents, chloroform remains the most toxic even at a very low concentration such that it could play the most significant role in reducing the overall toxicity of the solvent mixtures. The selection of a solvent mixture with the least possible amount of chloroform will be justified in terms of toxicity reduction. The results of the lipid extraction process are shown in Table 1. The minimum yield of 5.40% lipid g\(^{-1}\) DCW was obtained at 100% composition of dichloromethane solvent (Run 20). The maximum yield of 20.20% lipid g\(^{-1}\) DCW was obtained at the binary solvent composition
of 50% chloroform: 50% methanol (Run 5). These findings contradict the outcome of the study previously reported by (Jeon et al., 2013) and Ramluckan et al. (Ramluckan et al., 2014). For instance (Jeon et al., 2013) reported the best binary solvent mixture for the extraction of lipids from Chlorella vulgaris microalgae as methanol:dichloromethane (1:1) while (Ramluckan et al., 2014) found the combination of chloroform and ethanol in the ratio (1:1) to exhibit the greatest extraction efficiency for the extraction of lipids from Chlorella sp. microalgae. The work of Chatsungnoen and Chisti (Chatsungnoen & Chisti, 2016) investigated the optimal solvent mixture for the extraction of lipids from Nannochloropsis salina microalgae. Although the outcome of their findings showed similar optimal solvent mixture (chloroform/methanol) with the present study; however, the binary solvent composition differs. These differences in the optimal solvent composition could be as a result of the type microalgae species, growth conditions, source, or geographical location.

From Table 1, Run 5 with the maximum yield of 20.20% lipid g\(^{-1}\) DCW and composition 50% chloroform: 50% methanol (1:1) could serve as a benchmark for comparison in selecting solvent mixture composition with comparable lipid yield but less toxic. Considering the ternary solvent mixture (Run 11) with a composition of 33.33% chloroform, 33.33% methanol, 33.33% ethanol, gave a lipid yield of 18.20% lipid g\(^{-1}\) DCW. Similarly, the ternary mixture of chloroform, methanol, and dichloromethane (Run 12) gave a lower yield of 17.6% lipid g\(^{-1}\) DCW when compared with Run 5. In both cases, 33.4% reduction in the use of chloroform was achieved when compared to Run 5. From the quaternary solvent mixtures in Table 1, Run 18 is an interesting option with a composition of 12.5% chloroform, 12.5% methanol, 62.5% ethanol, and 12.5% dichloromethane. In this case, a 75% reduction in the use of chloroform was achieved compared to Run 5 and seems to be the best quaternary solvent mixture composition when considering toxic solvent replacement. However, the lower lipid yield of 15.20% lipid g\(^{-1}\) DCW obtained will be a limiting factor to the selection of this solvent mixture composition when compared to the lipid yield value of 20.20% lipid g\(^{-1}\) DCW obtained from Run 5. Another interesting option is the use of quaternary solvent mixture with composition of 12.50% chloroform, 62.50% methanol, 12.50% ethanol, and 12.50% dichloromethane (Run 17) which gave a comparable good yield of 19.40% lipid g\(^{-1}\) DCW when compared to the commonly used solvent mixture (Run 5). In this case, a 75% reduction in the usage of the extremely toxic chloroform solvent was achieved. This is an indication that this quaternary solvent mixture could be a better alternative solvent mixture composition to achieve a comparable lipid yield with reduced toxicity of the solvent mixture. Hence, the quaternary solvent mixture with composition of 12.50% chloroform, 62.50% methanol, 12.50% ethanol, and 12.50% dichloromethane was selected as the best solvent mixture composition for the extraction of lipid from microalgae to achieve a comparable lipid yield to that of the commonly used chloroform-methanol mixture.

The linear model obtained from ASCD modelling of the experimental data is shown in equation 4. This mathematical expression describes the relationship between the independent variables (solvent constituents) and the response (lipid yield)

\[
\%Yield = +7.49X_1 + 17.25X_2 + 6.59X_3 + 5.73X_4 + 31.16X_1X_2 + 25.02X_1X_3 + 2.46X_1X_4 \\
+ 14.35X_2X_3 + 26.31X_2X_4 + 21.76X_3X_4
\]

where \(X_1, X_2, X_3, \text{ and } X_4\) are the composition of chloroform, methanol, ethanol, and dichloromethane, respectively.

From the obtained linear model as shown in equation 4, the coefficients 7.49, 17.25, 6.59, and 5.73 obtained for chloroform, methanol, ethanol, and dichloromethane, respectively, indicate that the solvents have positive effects on the yield of lipid. The order of effect is given as methanol > chloroform > ethanol > dichloromethane and this is in agreement with the lipid yield of 17.60%, 8.20%, 6.40%, and 6.00% obtained experimentally for 100% composition of methanol, chloroform, ethanol, and
dichloromethane, respectively (Table 1). Furthermore, the model shows that all the interactions (X1X2, X1X3, X1X4, X2X3, X2X4, and X3X4) does not have negative effects on the lipid yield. This implies that the use of any of the solvent mixture is statistically well favoured toward the lipid yield with the interaction between chloroform and methanol having the highest effect (coefficients 311.16) and the interaction between chloroform and dichloromethane with the lowest effect (coefficient 2.46) and most times this is upheld experimentally (Runs 5–10) as shown in Table 1.

3.2. Statistical analysis of variance
The significance and accuracy of the model were subjected to analysis of variance test and the results are presented in Table 2. From the table, the f-value (104.28) obtained for the model and the low p-value (p < 0.0001) is an indication that the model is significant. At the confidence level of 95%, the model terms with p-values less than 0.05 are usually described as significant. In this case, all the model terms including the linear mixture of the solvent constituents and their interaction terms (X1X2, X1X3, X2X4, and X1X4) were found to be significant except for the interaction between chloroform and dichloromethane (X1X4) having p-value (0.3329) which is greater than 0.05. The lack of fit is not significant relative to the pure error with F-value of 3.03% and 11.74% chance that it could occur due to noise. The insignificant lack of fit further suggests that the model is significant.

The accuracy of the model was evaluated by determining the coefficient of determination (R²) and adjusted R². The value of 0.9853 obtained for R² is close to unity and indicates that 98.53% of the whole data distribution in the experiment is in agreement with values obtained from the predicted model. The alignment of the R² for the number of terms in the model and experimental data was assessed by determining the adjusted R² and the value of 0.9759 obtained is an indication of good fitness of the model. Also, the predicted R² value of 0.9637 was found to be in reasonable agreement with the adjusted R² (0.9759). The adequate precision was used to determine the measure of signal-to-noise ratio and the measure of its desirability is given as any value greater than 4 (Noordin et al., 2004). Therefore, the ratio of 29.099 obtained in this study indicates an adequate signal and that the model can be used to navigate the design space. The coefficient of variance obtained was 5.99% which indicates a low disparity between the experimental and predicted values (Betiku & Ajala, 2014).

The graphical representation of the interaction of the solvent mixture on lipid yield is shown in Figure 1. The two-component plot for the interaction of the solvents chloroform (X1) and methanol (X2) and their reciprocal effects on lipid yield while keeping ethanol and dichloromethane at 2.5% each is shown in Figure 1(a). On the other hand, Figure 1(b) illustrates the reciprocal interaction of chloroform (X1) and ethanol (X4) on lipid yield while keeping methanol at 12.5% and dichloromethane at 2.5%. Figure 1(c) indicates the two-component plot for the interactions of chloroform (X1) and dichloromethane (X4) on lipid yield while keeping methanol at 12.5% and ethanol at 2.5%. Similarly, Figure 1(d) illustrates the influence of interactions of methanol (X4) and ethanol (X1) on lipid yield while keeping both chloroform and dichloromethane at 2.5% each. Figure 1(e) shows the interactions between methanol (X4) and dichloromethane (X4) solvents on lipid yield while keeping chloroform and ethanol at 2.5%. The two-component plot shown in Figure 1(f) indicates the interactions between ethanol (X1) and dichloromethane (X4) on lipid yield while keeping chloroform at 2.5% and methanol at 12.5%. Summarily, from Figure 1(a–f), it can be seen that all the studied input parameters contributed to the yield of lipid either individually or by way of their combined interaction effects. However, the extent of contribution to lipid yield differs. For instance, the curvatures obtained for Figure 1 (a, d, and e) show that solvent methanol contributed the most to the yield of lipid and also, the contributions of the combined interactions between X1X2, X2X3, and X3X4 are far greater than that of X1X3, X1X4, and X3X4.
the experimental values. The model validation experiment was carried out and results presented in Table 3. The experimental lipid yields obtained were found to agree satisfactorily with the model predicted lipid yields, with error in the range of 1.06%—1.34% (Table 3). This also confirms the reliability of the obtained model in predicting lipid yield from S. obliquus microalgae at different solvent mixture compositions.

### 3.4. Environmental fate of the solvent mixture

The octanol-water partition coefficient (Kow), soil adsorption potential (Koc) and bioconcentration factor (BCF) of the solvent mixtures were determined from the thermodynamic model and the result is presented in Figure 3. The octanol-water partition coefficient (Kow) values are important for predicting a chemical’s potential impact on the aqueous environment. Kow values are also useful for estimating ecosystem risk factors such as bioconcentration factor (BCF) because partition coefficients in octanol-water systems display similarities to partition effects with natural organic substances and biological components in water (Gobas, 2001).

Understanding the way that solvents would impact the ecosystem will be helpful for regulatory agencies setting standards, such as maximum contaminant levels in aqueous waste streams. Kow, Koc, and BCF values of various solvent would also be of interest when selecting solvent or solvent mixture with minimal environmental impact prior to bulk or industrial implementation. As shown in Figure 3, the Kow, Koc, and BCF values of the proposed quaternary solvent mixture composition were compared with that of the values of the commonly used binary solvent mixture of chloroform and methanol in varying solvent composition of (1:2 v/v) (Bligh & Dyer, 1959), (1:1 v/v) (Lee et al., 2010), and (2:1 v/v) (Iverson et al., 2001). The result (as shown in Figure 3) shows that the proposed quaternary solvent mixture composition has the least values of 38.75, 51.02, and 5.11 for Kow, Koc, and BCF, respectively. When compared with the binary solvent mixture composition (1:2 v/v), (1:1 v/v), and (2:1 v/v) chloroform-methanol, significant reduction of Kow, Koc, and BCF values were observed confirming the proposed quaternary solvent mixture composition resulted in lower toxicity.

### 3.5. Total solvent cost estimation

In this study, the solvent composition in the given solvent mixture was of interest. The methodology used in the lipid extraction was the same and the total solvent volume to biomass ratio of
20 mL: 1 g was constant all through the experiment. With all other factors remaining constant, a simple cost estimation approach was used to determine the total cost of solvents in the solvent mixture simply by estimating the sum of the product of the unit cost of each solvent present in the solvent mixture and their respective solvent composition. The unit price per litre for the solvents; chloroform, methanol, ethanol, and dichloromethane solvents was obtained as shown in Table 4. The summary of the cost estimation for the proposed quaternary solvent mixture and those of the commonly used binary solvent mixture of chloroform and methanol with solvent composition (1:1 v/v), (1:2 v/v), and (2:1 v/v) are presented in Table 5. As shown in Table 5, the highest cost of solvent mixture ($ 63.93 per litre) was obtained from the binary mixture of chloroform and
methanol (2:1 v/v) while the lowest cost of solvent mixture ($47.89 per litre) was obtained from the proposed quaternary solvent. The proposed quaternary solvent mixture was able to achieve a total solvent cost reduction of 11.62%, 24.74%, and 34.48% when compared with (1:2 v/v), (1:1 v/v), and (2:1 v/v) chloroform-methanol mixture, respectively. This is also a justification that the proposed quaternary solvent is not only found to be an efficient replacement for lipid extraction with lower toxicity but also an economically viable option.

| S/N | Solvent Mixture          | Solvent Composition (v/v) | Lipid yield (% lipid g⁻¹ DCW) | Predicted | Experimental | Error (%) |
|-----|--------------------------|---------------------------|-------------------------------|-----------|--------------|-----------|
| 1   | Chloroform-Methanol      | 1:2                       | 20.92                         | 20.70     | 1.06         |           |
| 2   | Chloroform-Methanol      | 2:1                       | 17.66                         | 17.90     | 1.34         |           |

Figure 2. Plot of predicted lipid yield from ASCD against actual lipid yield.

Figure 3. Plot of predicted lipid yield from ASCD against actual lipid yield.

Table 3. Model validation for the lipid extraction experiment

Figure 3. Plot of predicted lipid yield from ASCD against actual lipid yield.

Figure 3. Plot of predicted lipid yield from ASCD against actual lipid yield.

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### Table 4. Prices of solvents (Merck KGaA, Germany, 2020)

| Description       | CAS No. | Product Number | Unit Price, UP ($/L) |
|-------------------|---------|----------------|----------------------|
| Methanol          | 67-56-1 | 1,060,181,000  | 30.91                |
| Ethanol           | 64-17-5 | 1,117,271,000  | 45.70                |
| Chloroform        | 67-66-3 | 1,024,441,000  | 80.42                |
| Dichloromethane   | 75-09-2 | 1,060,441,000  | 54.43                |

### 3.6. Effect of cell disruption on lipid yield

The extraction of lipid from microalgae was carried out via cell disruption assisted solvent extraction using the selected quaternary solvent mixture composition from ASCD (Table 1). The effect of cell disruption (microwave and sonication) technique on lipid yield was investigated and the results showed a higher yield of lipid was achieved with microwave (Figure 4). A substantial yield of 30.20% lipid g⁻¹ DCW was achieved with the microwave-assisted solvent extraction compared to sonication assisted solvent extraction where the yield obtained was 20.97% lipid g⁻¹ DCW and the conventional solvent extraction with a yield of 19.40% lipid g⁻¹ DCW.

Microwave-assisted lipid extraction resulted in 55.67% higher yield as compared to the conventional solvent extraction and an increment of 44.02% lipid yield was observed when compared to the sonication assisted lipid extraction. The efficient heating achieved as a result of electromagnetic waves penetrating the cell matrix and their interaction at the molecular level can be responsible for higher lipid yield with the microwave technique (Manco et al., 2012). The microwave energy usually aids the rotation of the molecular dipole thereby disrupting weak hydrogen bonds. This effect will cause movement of dissolved ions which tends to increase the solvent penetration into the microalgae biomass and improves lipid extraction yield (Cravotto et al., 2008; Dai et al., 2014). The higher lipid yield from the microwave-assisted solvent extraction may be as a result of the non-fatty acid lipids such as chlorophyll pigments, proteins, and other contaminants co-extracted with neutral/nonpolar lipids and polar lipids. Results from this present study were in agreement with the previous work reported by Guldhe et al. (Guldhe et al., 2014) and their findings also showed that cell disruption combined with solvent extraction promotes significant structural changes in the microalgal biomass thereby increasing the lipid yield.

### 3.7. SEM and FTIR Analysis of the lipid extracted biomass

The scanning electron microscopy (SEM) was used to analyse the effect of disruption on the cell wall of the microalgae materials as shown in Figure 5. For the conventional solvent extraction (Figure 5a), the cellular morphology of algal material was observed to be fully intact with no signs of damage to the cell wall which explains the lower lipid yield. On the

### Table 5. Summary of cost estimation for some selected solvent mixtures

| Solvent Mixture            | Solvent Composition v/v (%) | Total Solvent Mixture Cost ($/L) $^\text{a}$ |
|----------------------------|----------------------------|-----------------------------------------------|
| Chloroform-Methanol        | 1:1 (50:50)                | 55.67                                         |
| Chloroform-Methanol        | 1:2 (33.33:66.67)          | 47.40                                         |
| Chloroform-Methanol        | 2:1 (66.67:33.33)          | 63.93                                         |
| Chloroform-Methanol-Ethanol-Dichloromethane | 1:5:1 (12.5:62.5:12.5:12.5) | 41.89$^b$                                    |

UP: Individual solvent unit price in the mixture, $x$: Solvent volume fraction for each solvent in the mixture; b: Proposed solvent mixture
other hand, with microwave-assisted solvent extraction (Figure 5b), the algal cells appeared fragments and a lot of cell wall debris occurred on the surface of the algae cells. This shows that cell disruption promotes significant microalgae biomass structural changes resulting to increase lipid yield. Disrupted cell walls in lipid extracted biomass could also aid in its application for extraction of carbohydrates and proteins. Microwave-assisted lipid extraction also acts as a pretreatment step for further application of lipid extracted biomass for biomethane production.

The FTIR spectra confirmed the similarity in the quality of the lipid extracted biomass from both the convention solvent extraction method and microwave-assisted solvent extraction is shown in Figure 6. For instance, the broad spectra band ascribed to O-H stretching and N-H stretching was at 3431.39 cm\(^{-1}\) for algae biomass from microwave-assisted extraction and 3417.19 cm\(^{-1}\) for the conventional extraction biomass. The sequence sharp bands which described the C-O groups were at 2924.84 cm\(^{-1}\) and 2852.17 cm\(^{-1}\) for MASE residual biomass. The C-O group was also found to be in the conventional residual biomass at sequence bands 2923.21 cm\(^{-1}\) and 2851.15 cm\(^{-1}\). Similarly, the C = O group was observed at spectra bands 1653.30 cm\(^{-1}\) for microwave and 1653.34 cm\(^{-1}\) for conventional residual biomass. Furthermore, the 1542.02 cm\(^{-1}\) and 1534.44 cm\(^{-1}\) spectra observed from the residual
biomass from the microwave and conventional solvent extraction process was band due to N-H bend. The spectrum attributed to C-O group in alcohols was observed at 1055.20 cm\(^{-1}\) and 1024.91 cm\(^{-1}\) for microwave and conventional residual biomass, respectively. Therefore, the surface-active groups such as O-H, N-H, C=O, C-O are confirmation that carbohydrates and proteins are majorly present in the residual biomass obtained from both microwave and conventional extraction. The FTIR spectra clearly showed that microwave cell disruption method does not have a deteriorating effect on the composition of lipid extracted biomass and thus can be utilized for carbohydrates and proteins based applications.

### 3.8. Fatty acid profile of the extracted lipids

The fatty acid profile of the extracted lipids from Scenedesmus sp. biomass by microwave-assisted solvent extraction (MASE) and conventional solvent extraction (CSE) is presented in Figure 7. From the figure, the most dominant fatty acid in the extracted lipids for both MASE and CSE technique was observed to be palmitic acid (C16:0) and oleic acid (C18:1). The other important fatty acids identified in the extracted lipids were C14:0, C16:1, C18:2, C18:3, and C20:0. In this study, a slight variation was observed in the percentage composition of the fatty acids present in the lipid extracts from both techniques. Although 55.67% higher lipid yield was obtained with MASE as compared to the CSE, the closeness of their fatty acid profile is an indication that similar magnitude of saponifiable lipids was present in the overall extracted lipids of both MASE and CSE. For instance, lipids extracted by microwave technique showed 45.63% palmitic acid (C16:0) while lipids extracted by conventional extraction showed 45.37%. The linolenic acid (C18:3) concentration of 3.47% was obtained in MASE and 6.38% in CSE. The values obtained from both MASE and CSE for the linolenic acid (C18:3)

![Figure 6. FT-IR spectra of lipid extracted algae from (a) conventional solvent extraction (b) microwave-assisted solvent extraction.](image-url)
are within the desirable limit of below 12% specified by EN 14,214 (Singh et al., 2014). The fatty acid composition has been reported to have a great effect on biodiesel fuel property (Misra et al., 2014; Sharma et al., 2008; Singh et al., 2014). In order to achieve better oxidation stability of biodiesel, high content of saturated fatty acid is desirable. On the other hand, high content of unsaturated fatty acid in the lipid is favourable for the biodiesel cold flow properties (cloud point, cold filter plugging point, and pour point). From this study, the total saturated fatty acid and unsaturated fatty acid for MASE were found to be 59.43 ± 1.58% and 40.57 ± 1.54%, respectively, while the total saturated fatty acid and unsaturated fatty acid for the CSE were obtained to be 55.58 ± 0.55% and 44.42 ± 0.52%, respectively. This shows that in both of the MASE and CSE technique, there is a good proportion of the mixture of saturated and unsaturated fatty acids in the lipid oil thereby creating a balance between the oxidation stability and cold flow property. Hence, it may be concluded that the fatty acid profile from both the MASE and CSE extracted lipids is suitable feedstock for biodiesel production.

4. Conclusion
The optimal lipid content of 19.4% lipid g-1 DCW was extracted from Scenedesmus obliquus microalgae using solvent mixture composition (1:5:1:1 v/v) chloroform/methanol/ethanol/dichloromethane. The efficiency of lipid extraction from microalgae was found to differ depending on microalgae species and solvent mixture composition. Based on environmental risk factors (Kow, Koc, and BCF) assessed, the proposed solvent mixture composition in this study showed lower toxicity when compared to the commonly used chloroform-methanol solvent mixture (1:2 v/v), (1:1 v/v), and (2:1 v/v). Microwave-assisted solvent extraction resulted in higher lipid yield when compared with the conventional solvent extraction. The fatty acid profile results revealed the extracted lipid is suitable for biodiesel production. This study has shown the potential of using less toxic and lower cost solvent mixture composition for efficient lipid extraction from Scenedesmus obliquus microalgae.
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References
Al-Amani, M., & Al-Zuhair, S. (2019). Using switchable solvents for enhanced, simultaneous microalgae oil extraction-reaction for biodiesel production. Biochemical Engineering Journal, 141, 217–224. https://doi.org/10.1016/j.bej.2018.10.017
Araromi, D. O., Ablade, A. O., Bello, M. O., Bokere, T., Akınwande, B. A., Jameel, A. T., & Adégbola, S. A. (2017). Optimization of oil extraction from Pitanga (Eugenia uniflora L.) leaves using simplex centroid design. Separation Science and Technology, 52(8), 1341–1349. https://doi.org/10.1080/01496395.2017.1287199
Arumugam, M., Agarwal, A., Arya, M. C., & Ahmed, Z. (2011). Influence of organic waste and inorganic nitrogen source on biomass productivity of Scenedesmus and Chlorococccus sp. Journal homepage: www.ijee.iie.foundation. org, 2(6), 1125–1132.
Bolasubramanian, R. K., Doon, T. T. Y., & Obbard, J. P. (2013). Factors affecting cellular lipid extraction from marine microalgae. Chemical Engineering Journal, 215, 929–936. https://doi.org/10.1016/j.cej.2012.11.063
Betiku, E., & Ajala, S. O. (2014). Modeling and optimization of Thvetio peruviana (yellow elder) oil biodiesel synthesis via Musa paradisiaca (plantain) peels as heterogeneous base catalyst: A case of artificial neural network vs. response surface methodology. Industrial Crops and Products, 53, 314–322. https://doi.org/10.1016/j.indcrop.2013.12.046
Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37(8), 911–917. https://doi.org/10.1139/o59-099
Chatsungnoen, T., & Chisti, Y. (2016). Optimization of oil extraction from Nannochloropsis salina biomass paste. Algal Research, 15, 100–109. https://doi.org/10.1016/j.algal.2016.02.015
Cheah, W. Y., Ling, T. C., Show, P. L., Juan, J. C., Chang, J.-S., & Lee, D.-J. (2016). Cultivation in wastewaters for energy: A microalgae platform. Applied Energy, 179, 609–625. https://doi.org/10.1016/j.apenergy.2016.07.015
Cravotto, G., Boffa, L., Mantegna, S., Perego, P., Avogadro, M., & Cintas, P. (2008). Improved extraction of vegetable oils under high-intensity ultrasound and/or microwaves. Ultrasonics Sonochemistry, 15(5), 898–902. https://doi.org/10.1016/j.ultraschon.2007.10.009
Doi, Y.-M., Chen, K.-T., & Chen, C.-C. (2014). Study of the microwave lipid extraction from microalgae for biodiesel production. Chemical Engineering Journal, 250, 267–273. doi:https://doi.org/10.1016/j.cej.2016.04.031
Folch, J., Lees, M., & Stanley, G. S. (1957). A simple method for the isolation and purification of total lipides from animal tissues. Journal of Biological Chemistry, 226(1), 497–509.
Gobas, F. A. (2001). Assessing bioaccumulation factors of persistent organic pollutants in aquatic food-chains. In Persistent organic pollutants (pp. 145–165). Springer.
Guldhe, A., Moura, C. V., Singh, P., Rawat, I., Moura, E. M., Sharma, Y., & Bux, F. (2017). Conversion of microalgal lipids to biodiesel using chromium-aluminum mixed oxide as a heterogeneous solid acid catalyst. Renewable Energy, 105, 175–182. https://doi.org/10.1016/j.renene.2016.12.053
Guldhe, A., Singh, B., Rawat, I., Ramluckan, K., & Bux, F. (2014). Efficacy of drying and cell disruption techniques on lipid recovery from microalgae for biodiesel production. Fuel, 128, 46–52. https://doi.org/10.1016/j.fuel.2014.07.015
Ishido, B. K., & Chapman, M. H. (2009). Carotenoid extraction from plants using a novel, environmentally friendly solvent. Journal of Agricultural and Food Chemistry, 57(3), 1051–1059. https://doi.org/10.1021/jf8026292
Iversen, S. J., Lang, S. L., & Cooper, M. H. (2001). Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. Lipids, 36(11), 1283–1287. https://doi.org/10.1007/s11745-001-0843-0
Jeon, J. M., Choi, H. H., Yoo, G. C., Choi, Y. K., Choi, K. Y., Park, H. Y., ... & Lee, E. Y. (2013). New mixture composition of organic solvents for efficient extraction of lipids from Chlorella vulgaris. Biomass and Bioenergy, 59, 279–284. doi:10.1016/j.biombioe.2013.09.009
Loe, K. Z. W., Su, S. S., Win, N. N., Than, N. N., & Ngwe, H. (2019). Isolation of lasiodiplodin and evaluation of some biological activities of the stem barks of Phylanthus Albizia (Kurz) Hook. f. SciMedicine Journal, 1(4), 199–216. https://doi.org/10.28991/Scimedj-2019-0104-5
Lee, B.-S., & Lin, S.-T. (2014). A priori prediction of the octanol–water partition coefficient (Kow) of ionic liquids. Fluid Phase Equilibria, 363, 233–238. https://doi.org/10.1016/j.fluid.2013.11.042
Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y., & Oh, H.-M. (2010). Comparison of several methods for effective lipid extraction from microalgae. Bioresource Technology, 101(1), 575–577. https://doi.org/10.1016/j.biortech.2009.03.058
Monaco, L., Giordani, L., Vaccari, V., & Oddone, M. (2012). Microwave technology for the biodiesel production: Analytical assessments. Fuel, 95, 108–112. https://doi.org/10.1016/j.fuel.2011.09.047
Misra, R., Guldhe, A., Singh, P., Rawat, I., & Bux, F. (2014). Electrochemical harvesting process for microalgae by using nonsacrificial carbon electrode: A sustainable approach for biodiesel production. Chemical Engineering Journal, 255, 327–333. https://doi.org/10.1016/j.cej.2014.06.010
Mustapha, S.I., Okonkwo, P.C., & Waziri, S.M., 2013. Improvement of carbon dioxide absorption technology using conductor-like screening model for real solvents (COSMO-RS) method. Journal of Environmental Chemical, 5(4), doi:pp.96-105.105. doi:10.5897/JECE12.057
Noordin, M. Y., Venkatesh, V. C., Sharif, S., Elting, S., & Abdullah, A. (2004). Application of response surface methodology in describing the performance of coated carbide tools when turning AISI 1045 steel. Journal of Materials Processing Technology, 145(1), 46–58. https://doi.org/10.1016/j.jmatpro.2003.07.107

Ramluckan, K., Moodley, K. G., & Bux, F. (2014). An evaluation of the efficacy of using selected solvents for the extraction of lipids from algal biomass by the soxhlet extraction method. Fuel, 116, 103–108. https://doi.org/10.1016/j.fuel.2013.07.118

Ranjith-Kumar, R., Hanumantha Rao, P., & Arumugam, M. (2015). Lipid extraction methods from microalgae: A comprehensive review. Frontiers in Energy Research, 2, 61. https://doi.org/10.3389/fenrg.2014.00061

Sharma, Y., Singh, B., & Upadhyay, S. (2008). Advancements in development and characterization of biodiesel: A review. Fuel, 87(12), 2355–2373. https://doi.org/10.1016/j.fuel.2008.01.014

Singh, B., Guldeh, A., Rawat, I., & Bux, F. (2014). Towards a sustainable approach for development of biodiesel from plant and microalgae. Renewable and Sustainable Energy Reviews, 29, 216–245. https://doi.org/10.1016/j.rser.2013.08.067

Soji-Adedunne, A. R., Asere, A. A., Ishola, N. B., Oloko-Oba, I. M., & Betiku, E. (2018). Modeling of synthesis of waste cooking oil methyl esters by artificial neural network and response surface methodology. International Journal of Ambient Energy, 1–34. https://doi.org/10.1080/01430750.2017.1423378

Zakari, A. Y., Waziri, S. M., Aderemi, B. O., & Mustapha, S. I. (2013). Computational study of environmental fate of ionic liquids using conductor-like screening model for real solvents (COSMO-RS) method. Journal of Environmental Chemistry and Ecotoxicology, 5(4), 90-95. doi:10.5897/JEC2113001

Zuarro, A., Miglietta, S., Familiarisi, G., & Lavecchia, R. (2016). Enhanced lipid recovery from Nannochloropsis microalgae by treatment with optimized cell wall degrading enzyme mixtures. Bioresource Technology, 212, 35–41. https://doi.org/10.1016/j.biortech.2016.04.025