Modeling and Optimization of Lipid Extraction Process from Municipal Secondary Sludge for Biodiesel Production

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ABSTRACT

In the current study, the potentiality and optimization of lipid extraction from secondary sludge for biodiesel production were investigated. Four lipid extraction parameters were examined and used for process optimization and model development using Design of Experiment (DoE) method (namely methanol to hexane ratio -%, solvent to sludge ratio -ml/g, temperature -oC and extraction time -h). During the optimization process, free fatty acid (FFA) and saponifiable lipids (SLs) content of the extracted lipid were analyzed. The results revealed that, the maximum lipid extraction yield (Y_{lipid}) predicted through numerically optimized conditions by the model for highest desirability (0.99) was 16.5% at methanol to hexane ratio (%) of 84%, solvent to sludge ratio (v/wt) of 45 ml/g, temperature at 90 oC for 6 hours extraction time. The extracted lipid contained a maximum amount of 31% (wt/wt) FFA, where palmitic acid was predominant. The FAMEs yield produced from ex-situ acid-catalyzed esterification/ transesterification of the methanol-hexane co-solvent extracted lipid ranged between 4.5-5% (wt/wt) based on sludge weight. Fatty acid profile of FAMEs was found to be dominated by methyl palmitate (C16:0) representing 36% of FAMEs composition, followed by palmitoleic acid methyl ester (C16:1), oleic acid methyl ester (C18:1) and stearic acid methyl ester (C18:1) representing 24%, 18% and 10% of the FFA composition respectively. PCA analysis showed that solvent to sludge ratio (ml/g) has the highest significant positive effect on FAMEs yield (p-value < 0.05) where methanol to hexane ratio (X1), temperature (X3) and extraction time (X4) were inversely correlated with FAMEs yield. The results indicated the feasibility of using secondary sludge as an alternative feedstock for biodiesel production. However, the optimized conditions for maximizing extracted lipid content should not be considered suitable for FAMEs yield as well.

Keywords: Lipid extraction; secondary sludge; saponifiable lipids; Biodiesel; FAMEs; Optimization

1. INTRODUCTION

Currently, most of the energy we use comes from fossil fuels (non-renewable energy sources) which will be depleted in the near future.1,2 Despite our reliance on fossil fuels as energy sources resulted in a number of serious environmental problems, the energy demand for fossil fuels has been increasing during the last few years, and expected to further increase in the future (85 million barrels of liquid fuel per day in 2006 and projected to grow to 107 million barrels of liquid fuel per day in 2030).3 All these factors have led to global interests in developing biodiesel as an alternative fuel. Biodiesel is a promising green fuel to displace an appreciable amount of petroleum-based diesel fuel. It is renewable, biodegradable, less toxic, and safe for storage and handling, and provides similar energy density to diesel and can be used directly without any engine modification and does not require new refueling stations.

Chemically, biodiesel fuels are fatty acid methyl or ethyl esters (FAME) that is produced via esterification and/or transesterification of various lipids sources in the presence of a base, acid, enzyme or solid catalyst. Biodiesel can be produced from vegetable oils or animal fats, have been used as fuel in diesel engines and heating systems for over 25 years.4-10 However, the competitive potential of biodiesel is currently limited by the high price of conventional lipid feedstocks, which constitutes between 70% and 85% of the overall biodiesel production cost.11 Furthermore, the cultivation of edible vegetable oils for biofuels raises the concerns of food shortage, which competes with fuel production, therefore, at present, biodiesels cannot compete economically in the market.4 Therefore, a low-cost and non-edible feedstock is required to reduce the production costs and to facilitate competitiveness with petroleum diesel.

Municipal sludge lipids are gaining more attention nowadays as promising lipid source which can make biodiesel production more profitable. As municipal sewage sludge is a waste, formed during the treatment of wastewater, it is a possible alternative source of lipids for the production of biodiesel, consequently lowering the wastewater treatment plant (WWTP) operation costs.11-16 However, there are still different technical and economical constraints required to be solved before scaling-up into industrial scale can be reached. Compared to microalgae, wastewater sludge has more potentiality for scaling-up due to the technical aspects (such as biomass cultivation, collection, and separation), which are already settled; also the availability of biomass in a large amount as the process by-product. However, there are key aspects
should be considered first to ensure successful scaling-up, these includes 1) an efficient lipid extraction process with high saponifiable lipids (SLs) content; 2) lowering the cost of sludge drying process and even more preferable to efficiently produce biodiesel from wet sludge. The second aspect is one of the crucial points when talking about cost reduction. However, in the current study, we will focus on the first aspect.

SLs are the lipids that can be transformed into biodiesel (FAMEs) through esterification process. Lipids in general can be classified into two major categories based on the polarity of the head molecules; those are: 1) polar lipids (PLs); and 2) Neutral lipids (NLs). Polar lipids (PLs) can further be classified into phospholipids and glycolipid which are also considered as saponifiable lipids (SLs) because they contain fatty acids. While Neutral lipids (NLs) comprise Neutral Saponifiable Lipids (NSLs) “e.g. acylglycerols and free fatty acids (FFA)”; and Unsaponifiable Lipids (USLs) “e.g. hydrocarbons, sterols, and waxes”. Therefore, assuming higher FAMEs production based on the amount of lipid extracted from the sludge is often a mistaken judgment which was observed in a number of research studies, where high FAMEs production was predicted based solely on the amount of lipid extracted. Furthermore, NSLs extraction is more interesting than PLs, because the former contains a fewer fraction of unsaturated fatty acids compared with the later. It has been reported that the higher the fraction of saturated fatty acids, the higher the oxidation stability of biodiesel and cetane number.

Lipids extracted from municipal sludge differ quantitatively as well as qualitatively according to the type of microorganisms in the sludge; type of wastewater being treated; operational condition of the wastewater treatment plant; and type of sludge “primary, secondary or scum sludge.” It has been demonstrated that municipal sludge is largely comprised of fatty acids “i.e. NLs”, predominantly in the range of C10 to C18 which are excellent for biodiesel production.12,18-20 However, bacterial species present in the sludge have a wider range of lipid fractions, due to the fact that bacteria are either assimilate lipids from the wastewater or synthesize them in situ and store them intracellularly as NSLs “e.g. triacylglycerides, TAGs”; or as USLs “e.g. waxes esters, WEs; or polyhydroxyalkanoates, PHAs”. Therefore, optimal extraction process to ensure maximum extraction of SLs for biodiesel production and to minimize co-extraction USLs from municipal sludge sources is required.

Lipid extraction is the first step for biodiesel production from wastewater treatment plant sludge. At present, several methods are available for lipid extraction from biological materials. Most of these methods use organic solvents, usually in mixtures.5,18,19,21,22 Extraction of lipids from sludge may be influenced by many variables such as the type of sludge, type and amount of solvent, extraction time, temperature, stirring rate, type of microorganisms present in the sludge, etc. Although several researchers have demonstrated the lipid extraction from wastewater sludge, the effects of different parameters and their interactions in relation to SLs have not been investigated.

In the current study, the process of lipid extraction from secondary sludge was optimized using Design of Experiment (DoE) approach. Four variables which are believed to play a major role in the extraction of lipid using organic solvents were optimized. The Four factors investigated in the current study are: 1) methanol to hexane ratio (X1); 2) solvent to sludge ratio (X2); 3) temperature (X3); 4) and extraction time (X4). Furthermore, FFA and SLs content of the extracted lipid were analyzed and assessed quantitatively and qualitatively.

2. EXPERIMENTAL

2.1. Sludge sample collection and preparation

The secondary sludge used in this study was collected from secondary sedimentation tank of municipal wastewater treatment located in Madinah, KSA, in Alkhaleel area north of the city. The treatment plant is a conventional activated sludge system (without tertiary treatment) and has a design capacity of 129,000 m³/d. In 2001, the WWTP was upgraded to a total capacity of 240,000 m³/d and include extended aeration activated sludge tank followed by sand filtration system (Fig. 1). The raw sludge samples were collected from the secondary clarifier in plastic bottles and kept on ice during transportation to the laboratory. Sludge samples were concentrated by gravitational settling at 5 °C for 12 hours. After settling the supernatant was discarded and the settled sludge was subjected to two rounds of centrifugation at 3000 rpm for 10 min for further dewatering. The thickened sludge samples were then frozen at -20°C and freeze-dried for 5 days. The freeze-dried samples were crushed using mortar and pestle, homogenized and then stored in the freezer until further use.

![A schematic layout for Madinah WWTP](Image)

Fig.1 A schematic layout for Madinah WWTP.
2.2. Lipid extraction from secondary sludge

Lipid extraction was conducted using different extraction variables as listed in Table 1, namely, methanol to hexane ratio (%), solvent to sludge ratio (ml solvent/g dried sludge), temperature (°C) and extraction time (h). Lipid extraction from secondary sludge was carried out according to the method described by Wang et al. The mixture of 5 g of sludge powder and solvents (based on the experimental design) was placed in a condenser attached 500 ml Erlenmeyer flask for sequential extraction. After extraction, the resulting mixture was filtered using Buchner funnel, Whatman filter paper No. 1 and water aspirator to remove the remaining solvents. The filtrates were further concentrated using a rotatory evaporator at 40°C and dried to a constant mass in a vacuum desiccator. The resulting lipid was weighed, and the yield of extracted lipid was then determined and expressed as a percentage of grams extractable lipid per gram dry sludge. The yield of extracted lipid was calculated according to the following formula:

\[
Y = \frac{\text{Residual weight (mg)}}{\text{Sludge solid weight (mg)}} \times 100
\]

(1)

All solvents (Methanol and n-Hexane) were HPLC-grade and purchased from Fisher Scientific (Atlanta, USA).

**Table 1.** The experimental variables, their low, high and center point levels for lipid extraction from secondary sludge.

| Variables                          | Low (-1) | High (+1) | Center point (0) |
|------------------------------------|----------|-----------|------------------|
| X1: Methanol to Hexane (%)         | 40       | 80        | 60               |
| X2: Solvent to Sludge ratio (ml/g) | 10       | 40        | 25               |
| X3: Temperature (°C)               | 30       | 80        | 55               |
| X4: Extraction Time (h)            | 1        | 6         | 3.5              |

2.3. DoE for lipid extraction from dried sludge

The Design of Experiment (DoE) approach allows investigating the influence of different factors on a given process, through conducting minimal number of trials. DoE as a statistical approach has been widely applied and successfully implemented for various bioprocess optimization purposes. Process optimization using DoE usually involves four main steps as follow: 1) independent variables selection and their variation ranges and defining dependent (response) variable(s); 2) data screening for determining the main effect of independent variables on response variable; 4) carrying out the statistically designed experiments in a randomized order and estimating the coefficients and the mathematical model; 5) checking, verifying and optimizing the resulting model. In the current study different statistical methods were used to investigate and optimize the four extraction variables to maximize the extracted lipid yield, \(Y_{\text{lipid}}\) (Fig. 2). The statistical methods used included 2\(^k\) factorial screening design to determine the main effects and Response Surface Method (RSM) for modeling the process and optimization. All statistical analysis was performed using the JMP@ software (Version 13.1.0, SAS Institute, Cary, NC, USA).

![Experimental Lipid Extraction Process](image)

**Fig. 2** Schematic representation of the research approach for experimental lipid extraction process and optimization.

2.3.1 Screening of extraction variables using 2\(^k\) factorial design

Screening the main effects of variables is the first step in the optimization process, in which the effect magnitude of every independent variable was estimated. 2\(^k\) factorial design as a screening method is a well-established statistical technique for screening the main effects of the independent variables based on first order model:

\[
Y = \beta_0 + \sum \beta_i X_i + \epsilon
\]

(2)

Where \(Y\) is the response variable (\(Y_{\text{lipids wt/wt %}}\), \(\beta_0\) is the model intercept, \(\beta_i\) is the linear coefficient, \(X_i\) is the magnitude of the independent variable, and \(\epsilon\) is the error factor.

According to 2\(^k\) factorial screening design, four extraction variables were screened using full factorial analysis with 16 runs with two levels design: low (-1) and high (+1). The main effect of each variable was evaluated based on the average difference between the high and low levels measurements. All experimental measurements for the screening experiments were performed in triplicates and averaged. Analysis of variance (ANOVA) was conducted to evaluate variables with a significant effect on lipid yield from the extraction process (response variable, \(Y_{\text{lipid}}\)).

2.3.2 Optimization trajectory using steepest ascent method

The path of steepest ascent method was adopted to determine the trajectory of the increases in response
variable ($Y_{\text{lipid}}$). Determining the path of response increase helps to direct the experimental region towards optimum yield by changing the range of selected variables. The results obtained from the screening step, which identified the significant variables was used as a baseline for determining the trajectory of response increase. A step-wise steepest ascent was performed started from the variable levels that produced the maximum lipid yield in the screening design, and ended when a near optimal point was reached. The results from the steepest ascent step were used for further process optimization using response surface method (RSM).

### 2.3.3 Lipids extraction optimization using Box–Behnken design

For optimizing the lipid extraction yield from secondary sludge, Box–Behnken design and Response surface methodology (RSM) were applied. Box–Behnken design (BBD) was employed to optimize the most significant variables identified by factorial screening design step (section 2.3.1) using three center points. The Response Surface Method (RSM) was thus applied to visualize the experimental region. Predicting the optimal conditions can be estimated using the following second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_i X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j + \epsilon \quad (3)$$

Where $Y$ is the predicted response variable, $\beta_0$, $\beta_i$, $\beta_{ii}$ and $\beta_{ij}$ are the regression coefficients of intercepts, linear quadratic and interaction terms respectively. While $X_i$ and $X_j$ are the independent variables and $\epsilon$ is the error term. Measurements for optimization experiments were performed in triplicates and averaged except for the center points. The quality of the fit of the polynomial model equation was determined using regression coefficient ($R^2$) and adjusted $R^2$. The significance of the regression coefficients were checked using F-test.

### 2.4. Determination of FFA and SLs in extracted lipid

Lipids extracted from the steepest ascent step (section 2.3.2) were used for FFA analysis, and SLs estimates. FFA was analyzed using gas chromatography (GC) (HP 4890 D, Hewlett-Packard company, Wilmington, DE, USA) equipped with a capillary column and FID detector (Supelecowax: 30×0.53 mm; 0.25 µm, Agilent Technologies, USA). The carrier gas was nitrogen, with a flow rate of 1 mL min⁻¹ and sample injection volume was 1 µL, and injection split ratio 100:1. The temperatures of the injector, the detector, and the oven were held at 230, 250 and 210°C, respectively.

SLs of extracted lipid were converted into FAMEs (biodiesel) through esterification/transesterification in the presence of an acid catalyst ($\text{H}_2\text{SO}_4$) according to the method describes by Dufreche et al.². Twenty milligrams of lipids was dissolved in 1 mL of hexane containing and added to a vial with 2 mL of 1% sulfuric acid in methanol. The vial was then capped and heated overnight at 50 °C. Then, 5 mL aliquot of 5% NaCl in water was added, and the FAMEs were extracted with hexane, with vortexing the vial between extractions to provide efficient mixing. The hexane phase was washed with 2% sodium bicarbonate and dried over sodium sulfate.

The FAMEs produced by transesterification were analyzed, and the results of GC–FID were used to calculate the amount of saponifiable (transesterifiable/esterifiable lipids to FAMEs) material in the extracted lipid fraction and hence the maximum yield of biodiesel (FAMEs) that could be produced. The correlation between FAMEs and extraction conditions were assessed using principal component analysis (PCA) using XLSTAT® software (Addinsoft, Paris, France).

### 3. RESULTS AND DISCUSSION

#### 3.1 Screening of variables for lipid extraction from primary sludge using factorial design

The $2^k$ screening designs experiments and resulting $Y_{\text{lipid}}$ of 16 runs are shown in Table 2. The effects of methanol to hexane ratio, solvent to sludge ratio, temperature and extraction time on lipid yield $Y_{\text{lipid}}$ (wt lipid /wt sludge %) are represented in Table 3. The analysis of variance (ANOVA) indicated that the resulting model fit for $Y_{\text{lipid}}$ was highly significant ($p$-value < 0.0001), besides the model lack of fit value > 0.05 ($p$-value = 0.6211) and $R^2 = 0.96$ confirming that the model fits the experimental data and explains 96% of the data variability.

| Run order | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $Y_{\text{lipid}}$ (wt/wt %) |
|-----------|------|------|------|------|-----------------------------|
| 1         | +    | +    | -    | -    | 13.6                        |
| 2         | -    | +    | -    | -    | 9.7                         |
| 3         | -    | -    | +    | +    | 13.5                        |
| 4         | +    | +    | -    | +    | 14.1                        |
| 5         | +    | -    | -    | -    | 11.9                        |
| 6         | +    | +    | +    | +    | 16.2                        |
| 7         | +    | +    | -    | -    | 14.8                        |
| 8         | +    | -    | +    | +    | 15.7                        |
| 9         | -    | -    | -    | -    | 9.4                         |
| 10        | +    | -    | +    | -    | 12.6                        |
| 11        | -    | +    | -    | +    | 11.4                        |
| 12        | -    | -    | +    | +    | 11.4                        |
| 13        | +    | -    | -    | +    | 14.9                        |
| 14        | -    | -    | +    | -    | 10.9                        |
| 15        | -    | -    | -    | +    | 9.8                         |
| 16        | -    | +    | +    | -    | 12.4                        |

According to Table 3, methanol to hexane ratio ($X_1$), solvent to sludge ratio ($X_2$), temperature ($X_3$) and...
extraction time (X4) showed a significant effect on lipid extraction from secondary sludge (p-value < 0.05). Methanol to hexane ratio and temperature showed the highest significant effect (p-value = 0.0002 and 0.0005 respectively). Where, extraction time estimated as the lowest significant effect on lipid extraction process within the examined range (p-value = 0.0144). The highly significant effect of methanol concentration, solvent to sludge ratio and temperature on lipid extraction from secondary sludge can be referred to the fact that secondary sludge consists of a high proportion of polar lipids that are fundamental constituents of the bacterial cell membrane (i.e. phospholipids). Therefore, using polar solvents like methanol has higher efficiency in extracting lipids from secondary sludge with increasing temperature and solvent to sludge proportion. Also, according to Olkiewicz et al., increasing the extraction time results in extracting more lipids from secondary sludge. All examined variable showed a positive effect on $Y_{\text{lipid}}$ as indicated by the sign of $\beta$ coefficient in Table 3. Since all tested variables showed a significant effect on lipid extraction from secondary sludge, all four variables were included in the steepest ascent test.

### 3.2 Steepest ascent path and response surface method (RSM) for process optimization

The path of the steepest ascent was applied to determine the appropriate direction of variables to maximize the lipid production based on the regression analysis of the screening design (Table 4). The steepest ascent experiments were designed based on the maximum $Y_{\text{lipid}}$ recorded during the screening step (run 6). Accordingly, levels of significant variables were increased towards maximum lipid extraction region. A stepwise increase in the levels of methanol to hexane ratio was performed to estimate the optimal ratio for the highest $Y_{\text{lipid}}$ (Table 4; runs from 1-3). Similarly, a stepwise increase in the levels of solvents to sludge ratio and temperature was carried out (Table 4; runs 1-4). From Table 4, the highest lipid extraction of 16.3% (wt/wt) was obtained by using methanol to hexane ratio of 80%, solvent to sludge ratio of 40 ml/g, the temperature at 82°C and 8 hrs extraction time. It can be clearly seen that the levels of the four variables initially screened were close to optimum.

Furthermore, response surface method (RSM) was conducted for optimization and modeling of lipid extraction ($Y_{\text{lipid}}$) using four significant independent variables. Box-Behnken design was applied for lipid extraction optimization using different levels of variables where a multiple regression analysis was applied to the experimental data to model $Y_{\text{lipid}}$. Box-Behnken design matrix as well as varying levels of variables used and statistics as shown in Table 5 and 6 respectively.

According to RSM, the quadratic equation model for the optimized significant variables was found to be as follow:

$$Y_{\text{lipid}}=-44.6055+1.123001X_1+0.147778X_2+0.219653X_3+0.15832X_4-0.00684X_1^2-0.00078X_2^2-0.00114X_3^2-0.01569X_4^2-0.00005X_1X_2+0.00029X_1X_3-0.00012X_1X_4-0.00078X_2X_3-0.00039X_2X_4+0.00069X_3X_4$$

Where, $Y_{\text{lipid}}$ is the corresponding extracted lipid yield (wt/wt %), $X_1, X_2, X_3$ and $X_4$ represents the values of methanol to hexane ratio (%), solvent to sludge ratio (ml/g), temperature (°C) and extraction time (h) respectively.

### Table 3. Levels of variables examined and screening statistical analysis

| Code | Variable | Low Level (-1) | High level (+1) | F-ratio | $\beta$ coeff. | p-value |
|------|----------|----------------|----------------|---------|---------------|---------|
| X1   | Methanol to Hexane ratio (%) | 40 | 80 | 328.93 | 1.1581 | 0.0002* |
| X2   | Solvent to sludge ratio | 10 | 40 | 42.554 | 0.5687 | 0.0058* |
| X3   | Temperature (°C) | 30 | 80 | 153.807 | 1.0813 | 0.0005* |
| X4   | Extraction time (h) | 1 | 6 | 25.904 | 0.4438 | 0.0144* |

*indicates variables with significant effects on $Y_{\text{lipid}}$ (p-value < 0.05)

### Table 4. Steepest ascent experiments for maximizing lipid extraction from Secondary sludge and the FAMEs content (% of extracted lipid).

| Runs | X1 | X2 | X3 | X4 | $Y_{\text{lipid}}$ (wt/wt%) | FAMEs % (wt/wt) |
|------|----|----|----|----|-----------------------------|---------------|
| 1    | 82 | 40 | 82 | 6  | 16.16                       | 4.52          |
| 2    | 87 | 40 | 82 | 6  | 16.18                       | 4.69          |
| 3    | 90 | 40 | 82 | 6  | 16.19                       | 4.70          |
| 4    | 80 | 50 | 82 | 6  | 16.28                       | 4.84          |
| 5    | 80 | 55 | 82 | 6  | 16.29                       | 5.05          |
| 6    | 80 | 40 | 85 | 6  | 16.2                        | 4.54          |
| 7    | 80 | 40 | 90 | 6  | 16.4                        | 4.64          |
| 8    | 80 | 40 | 82 | 6  | 16.23                       | 4.74          |
| 9    | 80 | 40 | 82 | 8  | 16.29                       | 4.69          |
| 10   | 80 | 40 | 82 | 9  | 16.30                       | 4.77          |
was found to be 0.916, explaining 91.6% of the variability in lipid yield. While the linear effect of time (h) was not significant, the quadratic effect of methanol to hexane ratio (%) was as well highly significant (p-value < 0.001) indicating a significant effect of methanol to hexane ratio on lipid extraction from secondary sludge. The linear effect of temperature (°C) to sludge ratio (ml/g) were highly significant with p-values=0.0001, 0.00019 and 0.0049 respectively. Also, the quadratic effect of methanol to hexane ratio (%) was as well highly significant (p=0.00053) indicating a significant effect of methanol to hexane ratio on lipid extraction from secondary sludge. While the linear effect of time (h) was insignificant (p-value = 0.9591). The model coefficient (R²) was found to be 0.916, explaining 91.6% of the variability of the response variable (Y lipid) and with the Adj R² = 0.8173.

The extracted lipid contained a maximum amount of 31% (wt/wt) FFA, where palmitic acid was predominant. Similar findings were reported in other studies.22,33,34 The FAMEs yield (which represents the SLs content of the extracted sludge) produced from ex-situ acid-catalyzed esterification/transesterification of the methanol-hexane solvent extracted lipid, was ranging between 28-31% (wt/wt) based on the lipid weight (data not shown), which represents 4.5-5% (wt/wt) based on sludge weight. These results were close to the findings reported by Revellame et al., who reported FAMEs yield 4.7% (wt/wt) from secondary sludge using in-situ transesterification. The fatty acid profile of FAMES produced from the extracted lipid using acid-catalyzed ex-situ esterification/transesterification was found to be dominated by methyl palmitate (C16:0) which represented 36% of FAMES composition, followed by palmitoleic acid methyl ester (C16:1), oleic acid methyl ester (C18:1) and stearic acid methyl ester (C18:0).

3.3 FFA and SLs composition of extracted lipid

The analysis of variance ANOVA showed that the model fit was highly significant (p-value=0.0002) and the lack of fit was not significant (p-value=0.5356), indicating that the model represents the experimental data. The linear effect of methanol to hexane ratio (%), temperature (°C) and solvent to sludge ratio (ml/g) were highly significant with p-values=0.0001, 0.00019 and 0.0049 respectively. Also, the quadratic effect of methanol to hexane ratio (%) was as well highly significant (p=0.00053) indicating a significant effect of methanol to hexane ratio on lipid extraction from secondary sludge. While the linear effect of time (h) was insignificant (p-value = 0.9591). The model coefficient (R²) was found to be 0.916, explaining 91.6% of the variability of the response variable (Y lipid) and with the Adj R² = 0.8173.

The results showed that maximum yield of lipid from secondary sludge achieved by increasing methanol to hexane ratio, solvents to sludge ratio, temperature and time was 16.4% of dried secondary sludge. This was in accordance with what was reported by Wang et al., and relatively higher than what was recorded by Siddiquee and Rohani, who reported a maximum lipid extraction of 10.04% from dry secondary sludge using only methanol as an extraction solvent. The maximum lipid extraction yield (Y lipid) predicted through numerical optimized conditions by the model for highest desirability (0.99) was 16.528% at methanol to hexane ratio (%) of 84%, solvent to sludge ratio (v/wt) of 45 ml/g, temperature at 90°C for 6 hours extraction time. The three-dimensional response surfaces which represents the effects of different variables on Y lipid based on equation (4) is shown in fig. 3. Response surface shows the interactions between methanol to hexane ratio and solvent to sludge ratio, methanol to hexane ratio and temperature and solvent to sludge ratio and temperature (Fig. 3 A-F). A clear peak was observed in the response surface for variables combinations indicating optimal conditions were achieved which also was confirmed by the model prediction results. Thus, there was a significant increase in lipid extraction under optimized conditions and clearly indicate the importance of process optimization via statistical experimental design.

### Table 5. Levels of extraction variables used in Box-Behnken design and statistics

| Code | Variable                  | Low Level (-1) | High level (+1) | Center point | F-ratio | p-value |
|------|--------------------------|----------------|-----------------|--------------|---------|---------|
| X1   | Methanol to Hexane ratio (%) | 73             | 87              | 80           | 54.780  | 0.0001* |
| X2   | Solvent to sludge ratio   | 25             | 55              | 40           | 22.378  | 0.0005* |
| X3   | Temperature (°C)          | 73             | 90              | 73           | 28.160  | 0.0002* |
| X4   | Extraction time (h)       | 3              | 9               | 6            | 0.0027  | 0.9591  |

*indicates variables with significant effects on Y lipid (p-value<0.05)

### Table 6. Box-Behnken design matrix for optimization step and the response variable (Y lipid) from secondary sludge

| Run order | X1  | X2  | X3  | X4  | Y lipid (wt/wt %) |
|-----------|-----|-----|-----|-----|------------------|
| 1         | 0   | -   | 0   | 0   | 15.6             |
| 2         | 0   | 0   | 0   | 0   | 16.2             |
| 3         | 0   | 0   | 0   | 0   | 16.2             |
| 4         | 0   | 0   | 0   | 0   | 16.1             |
| 5         | -   | -   | 0   | 0   | 15.1             |
| 6         | 0   | +   | 0   | 0   | 16.4             |
| 7         | -   | 0   | 0   | 0   | 15.2             |
| 8         | 0   | 0   | +   | 0   | 16.2             |
| 9         | 0   | -   | 0   | 0   | 15.5             |
| 10        | 0   | 0   | 0   | +   | 15.47            |
| 11        | 0   | 0   | 0   | 0   | 16.2             |
| 12        | 0   | 0   | 0   | 0   | 16.1             |
| 13        | 0   | 0   | 0   | 0   | 15.68            |
| 14        | 0   | 0   | 0   | 0   | 16.15            |
| 15        | 0   | 0   | 0   | 0   | 15.5             |
| 16        | 0   | 0   | 0   | 0   | 15.7             |
| 17        | 0   | +   | 0   | 0   | 15.8             |
| 18        | 0   | 0   | +   | 0   | 15.8             |
| 19        | 0   | 0   | 0   | +   | 15.5             |
| 20        | 0   | 0   | 0   | 0   | 16.37            |
| 21        | 0   | 0   | 0   | 0   | 16.3             |
| 22        | 0   | 0   | 0   | 0   | 16.26            |
| 23        | 0   | 0   | 0   | 0   | 15.7             |
| 24        | 0   | 0   | 0   | 0   | 15.57            |
| 25        | 0   | 0   | 0   | 0   | 16.18            |
| 26        | 0   | 0   | 0   | 0   | 15.5             |
| 27        | 0   | 0   | 0   | 0   | 16.4             |
Fig. 3 Three-dimension surface plot and contour plot (a-f), showing levels of variables for maximizing lipid extraction yield from secondary sludge. (X1, X2, X3 and X4 represents the values of methanol to hexane ratio (%), solvent to sludge ratio (ml/g), temperature (°C) and extraction time (h) respectively)
ester (C18:1) and stearic acid methyl ester (C18:1) representing 24%, 18% and 10% of the FFA composition respectively (Fig. 4). These results were found to be in agreement with the findings reported by other researchers.5,7,22,30 The percentage of unsaturated fatty acids in the extracted lipid was found to be 44.5%, similar to the findings of Wang et al.,24 reporting that the percentage of unsaturated fatty acid ester in FAMEs from secondary sludge was found to be 44.4%. Besides, extracted lipids from secondary sludge constituted fatty acids of higher hydrocarbons and represented 3% of the extracted lipid.

However, methanol to hexane ratio (X1), temperature (X3) and extraction time (X4) were inversely correlated with FAMEs yield. In Fig. 5, the arrows indicate the correlations, the closer the variable arrow to the FAMEs yield arrow indicates a positive relationship and vice versa. Hence, increasing solvent to sludge ratio would result in an increase of FAMEs (i.e. SLs), while increasing temperature, methanol to hexane ratio or extraction time would reduce FAMEs yield (i.e. SLs). Similar finding was also observed by Olkiewicz et al.,13 reporting that increasing methanol concentration, temperature and/or extraction time resulting in the extraction of more unsaponifiable lipid (USLs) which is not convertible to FAMEs and hence reducing the overall percentage of FAMEs of extracted lipid, but no correlation between solvent to sludge ratio and FAMEs yield was observed which contradicts with other.30

4. CONCLUSIONS

The results presented in the current study revealed the potentiality of secondary sludge as a feedstock for biodiesel production. Also, the study has demonstrated the usefulness of applying Design of Experiment (DoE) approach for optimizing lipid extraction process variables. The highest amount of extracted lipids (16.5 wt%, lipid/sludge) was achieved using 84:45:90:6 independent variables conditions (methanol to hexane ratio, solvent to sludge ratio, temperature and extraction time respectively). The results have revealed that the yield of extracted lipids positively related with increasing the investigated extraction conditions (methanol to hexane ratio, solvent to sludge ratio, extraction temperature and time) within the studied range. However, examining the relationship between FEMAs and the extraction variables revealed that increasing temperature, methanol to hexane ratio and extraction time reduced FAMEs yield (i.e. SLs). Also, this was indicated from the PCA analysis, showing the need for considering saponifiable lipid fractions of the extracted lipid in the optimization process rather than focusing only on the total amount of lipid extracted since not all extracted lipids can be converted into biodiesel.

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