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# Optimization of *Moringa oleifera* seed extract and chitosan as natural coagulant in treatment of fish farm wastewater

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**ABSTRACT**

Holistic development of natural coagulants is always being promoted in many countries due to its high sustainability in wastewater treatment systems. Aquaculture field in Malaysia has generated huge wastewater volume and proactive methods must be taken to iron out the kinks of turbidity contamination which places the aqua-ecosystem at risk. In this study, *Moringa oleifera* seed extract and chitosan were utilized as the bio-coagulants via optimization of the coagulation–flocculation process with response surface methodology based on central composite design matrix. Interactions between the governing operating parameters such as pH, coagulant dosage, mixing time and settling time were all being meticulously examined and further validated using jar tests. As a result, 84% of turbidity removal was observed for water samples treated with chitosan at optimized condition of 100 mg/L coagulant dosage, pH 6, 15 min mixing time and 10 min settling time and 47% of turbidity removal for *M. oleifera* seed extract at optimized condition of 400 mg/L coagulant dosage, pH 10, 15 min mixing time and 10 min settling time. Comparing to *M. oleifera* seed extract, satisfactory removal performance (>64%) was achieved by chitosan when tested against chemical oxygen demand, total suspended solids, total volatile solids and phosphate content.

*Keywords:* *Moringa oleifera*; Chitosan; Central composite design; Turbidity removal; Fish farm wastewater

## 1. Introduction

Fish farming is the principal form of aquaculture, which involves raising fish commercially in tanks or enclosures, usually to increase the human food security. It is a common source of income in Malaysia as freshwater aquaculture had contributed 39.5% or 0.096 million tons to the entire fish production in 2008 and the total fishery production from aquaculture has been increased to approximately 0.2 million tons in 2017, turning the country to be the world's 15th largest producer of farmed seafood in 2019 supplying 0.427 million tons of seafood [1]. In view of the huge economic benefits contributed from this industry, fishermen in Malaysia were being urged by government to actively engage themselves in aquacultures to protect the depleting fish stocks in future due to long term overfishing. However, with the respect of the pollution generated from the aquaculture, huge water demand and effluent discharged into the receiving water bodies will reduce the dissolved oxygen level, inducing the build-up of bottom sediments and eventually impairing the water quality by stimulating excessive phytoplankton production due to high nutrient loading from aquaculture intensification [2]. Nitrogenous waste from the aquaculture systems is highly toxic to aquatic life, inadvertently elevating their blood ammonia and suspended solids present stimulate the bacterial growth which causes deoxygenation at the end.

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Various progressive approaches such as adsorption, ion exchange, membrane filtration and anaeraobic/aerobic biological systems have been implemented for wastewater clarification especially coagulation/flocculation (CF) process which utilizes natural plant/non-plant-based substituents to mitigate the toxicity rendered by conventional chemical coagulants such as alum and iron salts [3]. Wastewater treated with those low-cost natural coagulants poses no risk to biological organisms and the sludge produced is biodegradable, eliminating the environmental concerns. These environmentally benign alternatives are of great interest over the years due to their coagulation effectiveness as reported by Sćiban et al. [4] describing the high coagulation activities contributed by the seed extracts from European chestnut (80% turbidity removal) and common oak acorn (70% turbidity removal) at only 0.5 mL/L coagulant dosage. Similar investigation was performed onto the ability of *Pistacia atlantica* seed extract aid along with ferric chloride. Findings pointed out that 97.43% of turbidity removal efficiency could be obtained by combining 0.4 mL/L of natural coagulant and 10 mg/L of chemical coagulants at pH 9 [5]. Moreover, coagulation with pine cone extract was maximized to approximate 82% and could take place in the first hour at only 0.5 mL/L of dosage [6]. Other biopolymers include starches, sodium alginate, tannin, natural gum and xanthan were also some good choices for the replacement of chemical coagulants [7].

A plant-based coagulant that has been extensively studied, *Moringa oleifera*, is a multipurpose tree with remarkable potential. Its cultivation has been actively promoted in many developing countries and recommended for wastewater remediation purposes [7–11]. For example, Ugwu et al. [12] studied the effectiveness of the seeds in the treatment of domestic wastewater, comparing it with the usage of alum and the results obtained has been fairly impressive with almost all parameters (pH, BOD, nutrients, hardness and coli form) showing values within WHO tolerable level and less sludge production compared to alum. Assiduous studies were continuously carried out by researchers to claim its capability as natural coagulants in wastewater purification with the aid of membrane filtration [10]. Concomitantly, exploration of non-plant based materials such as chitosan (a deacetylated polymer obtained from the alkaline deacetylation of chitin extracted from shellfish sources) as coagulants has led to adequate works revolving around its coagulation performance. As a demonstration, surface water was successfully clarified by chitosan with results on par with alum [13]. Potential application of chitosan in coagulation–ultrafiltration hybrid process was discovered too to treat drinking water by releasing high-quality permeate [14].

Generally, the efficiency of CF treatment is highly depending on the operating factors such as optimal coagulant dosage to keep away from charge reversal and colloids re-stabilization [5]. Once the coagulants are overdosed and reach its critical coagulation point, insignificant CF effects would be discovered due to the disruption of the bridge formation between adjacent particles. Addition of acids and alkalis also promotes electrostatic attraction as the inter-particle force between the particles is eliminated due to reduced surface charges [15]. Majority of plant-based coagulants are usually effective at lower pH because the particles are more likely to coalesce into flocs at this pH range owing to the presence of positively loosed charged particles to bind with negatively-charged colloids [16]. An ideal CF process could be further enhanced by optimizing the mixing time via fast stirring to ensure even distribution of coagulant or slow stirring to encourage the size growing of the flocs, leading to easier settlement [17]. Particle transport and collision rate are both uplifted not only by mixing speed but also by temperature of the wastewater. Low temperature would decrease hydrolysis and hinder the flocs aggregation rate, eventually producing irregular and less compact flocs. A simple CF process with bio-based coagulants is capable to render significant breakthrough in sustainable environmental technology with growing importance of process parameters optimization.

However, most of the previous studies revolved around the comparison between commercial inorganic coagulants and natural organic coagulants though it was far known that bio-coagulants always offer higher versatility and efficacy [7,12,18]. To date, there is still a dearth of information reporting a direct comparison between bio-coagulants, especially in treating aquaculture wastewater found in Malaysia. Moreover, qualities of raw water can vary by region, which has an indirect impact on the optimum combination of targeted bio-coagulants treatment parameters. Hence, this current work shed light on utilization of two natural coagulants (chitosan and *M. oleifera*) in fish culture effluent treatment and solid waste removal, specifically tailored for catfish reared in Malaysia aquaculture ponds. In this study the treatment performance was optimized employing response surface methodology (RSM), a well-recognized flexible statistical tool to speed up the tine-consuming factor-by-factor assessment (pH, coagulant dosage, settling time and mixing time). A robust design matrix was map out to correlate the relationship between the input and output variables, thereby resulting in a good estimation of the optimal operating conditions for a system based on its surface topography.

## 2. Materials and methods

### 2.1. Sample collection

The fish farm wastewater was collected from Chop Aik Lee Fishery in Parit Buntar, Perak (GPS coordinate 5.1189, 100.4164) located approximately 10 km far from Engineering Campus, Universiti Sains Malaysia. Upon collection, it was stored in a cold storage room prior to analysis. The wastewater samples should be restored back to room temperature to mimic the real condition of wastewater treatment in the industry.

### 2.2. Preparation of Moringa oleifera seed extract and chitosan

The *M. oleifera* seeds were dried overnight in the oven at 55°C. The hulls and wings were manually removed from the kernels. Then, the kernels were crushed and grounded to the medium fine powder with a grinder and 2 g of the kernel powder was mixed with 40 mL distilled water. In order to extract the active ingredient from the kernel, the mixture of continuously stirred for 6 h and the

suspension was eventually made up to 100 mL to give a stock solution 2%. On the other hand, chitosan stock solution was prepared by dissolving 1 g of chitosan in 2.5 mL of 2 M HCl and 47.5 mL of distilled water. The mixture was stirred overnight at room temperature or mildly heated until it was completely dissolved. A 1% stock solution was eventually prepared by adding 50 mL distilled water. This solution was kept at a closed container inside the refrigerator to avoid any unnecessary deterioration.

### 2.3. Coagulation–flocculation experiments

CF performance of each natural coagulant was carried out using conventional jar-test. The glass beakers (500 mL) were filled with 300 mL of fish farm wastewater. All samples will undergo rapid and slow mixing. The rapid mixing speed was fixed at 250 rpm and then reduced to 40 rpm to allow coagulation process. Two layers could be observed in each beaker at the end and only the supernatant (3 cm from the water surface) was collected for pH, turbidity, ammoniacal nitrogen, chemical oxygen demand (COD), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) and phosphate content analysis.

The pH of each sample was measured by pH meter (H12020-02, Hanna Instruments, United States) using 0.1 M HCl and NaOH to regulate the solution pH. The turbidity was determined by a portable turbidity meter (HI 93703, Hanna Instruments, Italy) at 890 nm with a range of 0–1,000 NTU (Nephelometric Turbidity Units). The turbidity measurement was conducted following the ISO 7027 International Standard. Besides, 0.1 mL water sample was used for the determination of ammoniacal nitrogen (N–$NH_3$) concentration. Salicylate method (VARIO Ammonia Salicylate, Cyanurate, Powder Packs and VARIO am diluent reaction tube, detectable range of 0–50 mg/L N–$NH_3$) was employed for ammonia quantification with LOVIBOND MaxiDirect MD 600 Photometer at 660 nm. Aside from that, the COD was quantified by dichromate-sulfuric acid method (COD VARIO 0–1,500 mg/L Tube Test Reagent, detectable range of 0–1,500 mg/L COD/CSB) by LOVIBOND MaxiDirect MD 600 Photometer at 610 nm. These procedures applied complied with the standard methods for the examination of water and wastewater.

In order to test the MLSS, 30 mL of well-mixed water samples will be filtered through weighed standard glass microfiber filters (Whatman™, GF/C filter, 0.45 μm, 47 mm in diameter) with vacuum air pump. The residues remained on the filters would be dried in a 105°C oven for 1 h until achieving a constant weight. The total suspended particles were represented by the increment in recorded filter weight. Following that, MLVSS test was done by igniting the filters used for MLSS in a 550°C muffle furnace for 20 min. The fixed solids were indicated by the residual solids, whereas the volatile solids were indicated by the weight lost during ignition. APHA 2540D and APHA 2540E were used to complete this MLVSS analysis.

In compliance with USEPA method 365.2 and Standard Method 4500-P-E for wastewater, the determination of phosphate was conducted using ascorbic acid method (PhosVer®3 Phosphate Reagent Powder Pillows, detection range of 0.02–2.50 mg/L $PO_4^{3-}$) using HACH DR 2800 Spectrophotometer at 880 nm. All the results obtained were always compared with the control in this study (wastewater samples without addition of any coagulant). The percentage removal was calculated according to the equation:

$$\text{Percentage removal}(\%) = \frac{C_0 - C_i}{C_0} \times 100 \quad (1)$$

where $C_0$ is the initial concentration (mg/L) and $C_i$ is the final concentration (mg/L) for all the parameters except turbidity which the units are in NTU.

### 2.4. Design of experiments and statistical analysis

Design of experiment (DOE) is systematic planning and studies to delineate the relationship between the experimental variable and the effect of the response. There are several techniques of DOE, including best guess approach (trial and error), one factor at a time (OFAT) and response surface methodology (RSM). In Table 1, RSM design using central composite design (CCD) was employed to optimize the CF parameters over a range of independent input factors namely coagulant dosage, $X_1$ (mg/L), pH of wastewater, $X_2$, mixing time, $X_3$ (min) and settling time, $X_4$ (min). These systematic runs were configured through Design-Expert software version 7.1.5 (Stat-Ease Inc., Minneapolis, USA) to analyze the experimental condition with the highest desirability.

Based on the four variables studied, there were 8 axial or star points ($\alpha$ = +1) located at the center and both extreme levels of the experimental models and 6 central, replicates of the central point. As shown by the equation, total 30 experiments were calculated for *M. oleifera* seed extract and 30 experiments for chitosan.

$$N = 2^n + 2n + n_c \quad (2)$$

where $N$ is the total number of experiments required, $n$ is the number of factors and $n_c$ is the number of replicates at the center point.

Prior to the design of experimental runs, a preliminary study was used to initiate a narrower range of coagulant dosage. Coagulant dosages starting at 10 mg/L were explored, with incremental values following that until observing an appreciable turbidity reduction. As a result, the study ranges of coagulant dosage were chosen as 400–1,200 mg/L for *M. oleifera* seed extract and 100–800 mg/L for chitosan. The turbidity response was used to develop an empirical model using a second-degree polynomial equation as shown:

$$Y = b_0 + \sum_{i=1}^{n} b_i x_i + \left( \sum_{i=1}^{n} b_{ii} x_i \right) + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_i x_j \quad (3)$$

where $Y$ represents the predicted response, $b_0$ is the constant coefficient, $b_i$ is the linear coefficients, $b_{ij}$ is the quadratic coefficients and $x_i$, $x_j$ are the coded values of the process variables. The models developed were evaluated based on the correlation coefficients, $R^2$. Table 2 presents the CCD design matrix applied of the experiments.

Table 1
Range of parameters and the coded variables level used in the central composite design for natural coagulants, *M. oleifera* seed extract and chitosan

| Input factors | Unit | Symbol | Coded variables level | | |
|---|---|---|---|---|---|
| | | | Low (–1) | Center (0) | High (+1) |
| M. oleifera dosage | mg/L | $X_1$ | 400 | 800 | 1,200 |
| Chitosan dosage | mg/L | | 100 | 450 | 800 |
| pH | | $X_2$ | 4 | 7 | 10 |
| Mixing time | min | $X_3$ | 5 | 10 | 15 |
| Settling time | min | $X_4$ | 10 | 20 | 30 |

Table 2
Experimental design matrixes for (a) *M. oleifera* seed extract and (b) chitosan
(a)

| Run no. | Coagulant dosage, $X_1$ (mg/L) | pH, $X_2$ | Mixing time, $X_3$ (min) | Settling time, $X_4$ (min) | Turbidity, $Y_1$ (NTU) |
|---|---|---|---|---|---|
| 1 | 1,200 | 7 | 10 | 20 | 268 |
| 2 | 1,200 | 10 | 5 | 30 | 266 |
| 3 | 1,200 | 4 | 5 | 30 | 285 |
| 4 | 800 | 7 | 15 | 20 | 266 |
| 5 | 400 | 10 | 5 | 10 | 258 |
| 6 | 800 | 7 | 5 | 20 | 262 |
| 7 | 400 | 4 | 5 | 30 | 243 |
| 8 | 800 | 10 | 10 | 20 | 258 |
| 9 | 800 | 7 | 10 | 20 | 256 |
| 10 | 400 | 4 | 15 | 30 | 234 |
| 11 | 1,200 | 10 | 15 | 30 | 216 |
| 12 | 400 | 10 | 15 | 10 | 238 |
| 13 | 400 | 7 | 10 | 20 | 228 |
| 14 | 1,200 | 10 | 15 | 10 | 246 |
| 15 | 800 | 7 | 10 | 20 | 213 |
| 16 | 400 | 4 | 5 | 10 | 235 |
| 17 | 800 | 7 | 10 | 20 | 231 |
| 18 | 400 | 10 | 5 | 30 | 234 |
| 19 | 800 | 7 | 10 | 20 | 230 |
| 20 | 1,200 | 10 | 5 | 10 | 245 |
| 21 | 800 | 7 | 10 | 20 | 221 |
| 22 | 800 | 7 | 10 | 20 | 164 |
| 23 | 400 | 4 | 15 | 10 | 102 |
| 24 | 1,200 | 4 | 5 | 10 | 124 |
| 25 | 400 | 10 | 15 | 30 | 79.2 |
| 26 | 1,200 | 4 | 15 | 10 | 153 |
| 27 | 800 | 7 | 10 | 10 | 120 |
| 28 | 800 | 7 | 10 | 30 | 134 |
| 29 | 1,200 | 4 | 15 | 30 | 76.5 |
| 30 | 800 | 4 | 10 | 20 | 85.1 |

*(Continued)*

Table 2 Continued
(b)

| Run no. | Coagulant dosage, $X_1$ (mg/L) | pH, $X_2$ | Mixing time, $X_3$ (min) | Settling time, $X_4$ (min) | Turbidity, $Y_2$ (NTU) |
|---|---|---|---|---|---|
| 1 | 100 | 10 | 5 | 10 | 246 |
| 2 | 450 | 7 | 10 | 30 | 57.5 |
| 3 | 450 | 7 | 10 | 10 | 53.5 |
| 4 | 100 | 4 | 5 | 30 | 58 |
| 5 | 800 | 10 | 5 | 30 | 159 |
| 6 | 800 | 7 | 10 | 20 | 33.2 |
| 7 | 450 | 4 | 10 | 20 | 51.3 |
| 8 | 450 | 7 | 10 | 20 | 47.3 |
| 9 | 450 | 7 | 10 | 20 | 65.1 |
| 10 | 450 | 10 | 10 | 20 | 145 |
| 11 | 450 | 7 | 5 | 20 | 95.5 |
| 12 | 450 | 7 | 10 | 20 | 44.3 |
| 13 | 800 | 10 | 5 | 10 | 131 |
| 14 | 800 | 4 | 15 | 30 | 46.5 |
| 15 | 100 | 10 | 5 | 30 | 238 |
| 16 | 100 | 10 | 15 | 10 | 201 |
| 17 | 800 | 10 | 15 | 30 | 131 |
| 18 | 100 | 4 | 5 | 10 | 127 |
| 19 | 800 | 4 | 5 | 10 | 72 |
| 20 | 800 | 4 | 5 | 30 | 57.6 |
| 21 | 450 | 7 | 10 | 20 | 53 |
| 22 | 100 | 4 | 15 | 10 | 82.1 |
| 23 | 100 | 4 | 15 | 30 | 48 |
| 24 | 100 | 7 | 10 | 20 | 143 |
| 25 | 450 | 7 | 15 | 20 | 68.8 |
| 26 | 450 | 7 | 10 | 20 | 56.5 |
| 27 | 100 | 10 | 15 | 30 | 176 |
| 28 | 800 | 10 | 15 | 10 | 134 |
| 29 | 800 | 4 | 15 | 10 | 51.9 |
| 30 | 450 | 7 | 10 | 20 | 47.6 |

### *2.5. Zeta potential measurement and Fourier-transform infrared spectroscopy*

The zeta potential values of both the natural coagulants were checked using Zetasizer Nano ZS (Malvern, UK) instrument equipped with a zeta cell at room temperature. Prior to measurement, suspension was ensured to be shaken well to avoid any undesirable re-stabilization of the particles that directly affect the data accuracy. Values were derived from the electrophoretic mobility using Smoluchowski equation. Moreover, the functional groups present in both *M. oleifera* seed extract and chitosan were determined using Fourier-transform infrared spectrometer (IRPrestige-21, Shimadzu, Japan) with spectral range varying between 4,000 and 600 $cm^{-1}$.

## 3. Results and discussion

### *3.1. Development of regression model equation and analysis of statistical significance*

Based on the sequential model sum of squares, quadratic models were selected as suggested by the Design-Expert software to correlate the data to the final turbidity ($Y_1$) and ($Y_2$) responses for jar tests using both *M. oleifera* seed extract and chitosan. The suggestion model was made by verifying lack-of-fit and model summary statistics as in Table 3. A second-order polynomial mathematical equation including linear and quadratic terms has been devised to manifest the relationship between the independent variables and response. The developed equations for *M. oleifera* seed extract and chitosan in terms of coded factors are shown by Eqs. (4) and (5) respectively.

$$Y_1 = 230.92 - 12.07A - 73.57B - 7.63C - 12.21AB - 7.71BC - 42.04B^2 \quad (4)$$

$$\mathrm{Sqrt}\left(Y_2\right) = 7.83 - 1.25A + 2.53B - 0.62C + 2.71B^2 \quad (5)$$

where $A$, $B$ and $C$ are the coded values of the process variables coagulant dosage, pH, and mixing time for *M. oleifera* seed extract and chitosan, respectively. Note that the process variable for settling time was missing here. This was due to the modification done by the analysis of

Table 3
Sequential fitting for jar test using (a) *M. oleifera* seed extract and (b) chitosan
(a)

| Sequential model sum of squares (Type I) | | | | | | |
|---|---|---|---|---|---|---|
| Source | Sum of squares | df | Mean square | *F*-value | Prob. > *F* | |
| Mean vs. Total | 1.27E+06 | 1 | 1.27E+06 | | | |
| Linear vs. Mean | 1.02E+05 | 4 | 25,366.88 | 33.38 | <0.0001 | |
| 2FI vs. Linear | 4,006.06 | 6 | 667.68 | 0.85 | 0.5504 | |
| ***Quadratic vs. 2FI*** | ***13,309.38*** | ***4*** | ***3,327.35*** | ***29.65*** | ***<0.0001*** | ***Suggested*** |
| Cubic vs. Quadratic | 802.72 | 8 | 100.34 | 0.8 | 0.624 | Aliased |
| Residual | 880.79 | 7 | 125.83 | | | |
| Total | 1.39E+06 | 30 | 46,325.3 | | | |
| Lack-of-fit tests | | | | | | |
| Source | Sum of squares | df | Mean square | *F*-value | Prob. > *F* | |
| Linear | 18,693.62 | 20 | 934.68 | 15.31 | 0.0034 | |
| 2FI | 14,687.56 | 14 | 1,049.11 | 17.18 | 0.0027 | |
| ***Quadratic*** | ***1,378.18*** | ***10*** | ***137.82*** | ***2.26*** | ***0.191*** | ***Suggested*** |
| Cubic | 575.46 | 2 | 287.73 | 4.71 | 0.0708 | Aliased |
| Pure error | 305.33 | 5 | 61.07 | | | |
| Model summary statistics | | | | | | |
| Source | Std. dev. | $R^2$ | Adj. $R^2$ | Pre. $R^2$ | PRESS | |
| Linear | 27.57 | 0.8423 | 0.8171 | 0.776 | 26,990.48 | |
| 2FI | 28.09 | 0.8755 | 0.81 | 0.658 | 41,205.45 | |
| ***Quadratic*** | ***10.59*** | ***0.986*** | ***0.973*** | ***0.9357*** | ***7,748.7*** | ***Suggested*** |
| Cubic | 11.22 | 0.9927 | 0.9697 | 0.1055 | 1.08E+05 | Aliased |

(b)

| Sequential model sum of squares (Type I) | | | | | | |
|---|---|---|---|---|---|---|
| Source | Sum of squares | df | Mean square | *F*-value | Prob. > *F* | |
| Mean vs. Total | 2,684.57 | 1 | 2,684.57 | | | |
| Linear vs. Mean | 152.76 | 4 | 38.19 | 11.45 | <0.0001 | |
| 2FI vs. Linear | 9.38 | 6 | 1.56 | 0.4 | 0.8687 | |
| ***Quadratic vs. 2FI*** | ***60.62*** | ***4*** | ***15.16*** | ***17.01*** | ***<0.0001*** | ***Suggested*** |
| Cubic vs. Quadratic | 10.06 | 8 | 1.26 | 2.66 | 0.1071 | Aliased |
| Residual | 3.3 | 7 | 0.47 | | | |
| Total | 2,920.7 | 30 | 97.36 | | | |
| Lack-of-fit tests | | | | | | |
| Source | Sum of squares | df | Mean square | *F*-value | Prob. > *F* | |
| Linear | 82.02 | 20 | 4.1 | 15.19 | 0.0034 | |
| 2FI | 72.63 | 14 | 5.19 | 19.22 | 0.0021 | |
| ***Quadratic*** | ***12.01*** | ***10*** | ***19.22*** | ***4.45*** | ***0.0566*** | ***Suggested*** |
| Cubic | 1.95 | 2 | 4.45 | 3.62 | 0.1067 | Aliased |
| Pure error | 1.35 | 5 | 3.62 | | | |
| Model summary statistics | | | | | | |
| Source | Std. dev. | $R^2$ | Adj. $R^2$ | Pre. $R^2$ | PRESS | |
| Linear | 1.83 | 0.6469 | 0.5905 | 0.5033 | 117.28 | |
| 2FI | 1.97 | 0.6867 | 0.5218 | –0.0281 | 242.76 | |
| ***Quadratic*** | ***0.94*** | ***0.9434*** | ***0.8906*** | ***0.7556*** | ***57.72*** | ***Suggested*** |
| Cubic | 0.69 | 0.986 | 0.942 | 0.0049 | 234.98 | Aliased |

variance (ANOVA) by eliminating the terms found statistically insignificant. As the *p*-value for settling time was 0.1059 and 0.1593 for *M. oleifera* seed extract and chitosan respectively, it is deemed as insignificant ($p > 0.05$). Square root transformation was chosen for model implementing chitosan, as being recommended by the Box–Cox graph. All the coefficients with only one factor represent the individual effect of that particular factor, whereas the coefficients with two factors represent the mutual interactions between two independent variables as well as their respective quadratic effects. Terms with a positive sign in front of them are having synergistic effect, while those with a negative sign are having antagonistic effect.

The model fitting level was confirmed by the determination coefficient ($R^2$). The $R^2$ coefficient indicates the ratio of sum of squares due to regression (SSR) to total sum of squares (SST), reflecting the proportion of the total variation in the response predicted by the model. In this case, the value of predicted $R^2$ for *M. oleifera* seed extract was 0.9357 and for chitosan was 0.7556 while the adjusted $R^2$ for *M. oleifera* seed extract and chitosan were 0.9730 and 0.8960 respectively. High $R^2$ value for Eqs. (4) and (5) were 0.9860 and 0.9436, respectively, indicating that 98.60% and 94.36% from the total variation in the turbidity removal were in correlation with the experimental and predicted values respectively, ensuring a satisfactory adjustment of the quadratic models to the experimental data.

The model significancy and adequacy of both experiments were further justified using analysis of variance (ANOVA) using the calculated Fisher variation ratio (*F*-value) to compare the model variance with residual variance and the calculated *p*-value to test the significance level which was set to $p < 0.05$ (confidence level is more than 95%) in this study. As depicted in Table 4a, variables *A* (coagulant dosage), *B* (pH), *C* (mixing time) and *AB* (interaction term of coagulant dosage and pH), *BC* (interaction term of pH and mixing time) and $B^2$ (quadratic terms of pH) were significant model terms in this case. *D* (settling time), interaction terms (*AC*, *AD*, *BD*, and *CD*) and quadratic effects of other variables ($A^2$, $C^2$, and $D^2$) were insignificant terms. The model *F*-value of 135.24 and Prob. > *F* less than 0.05 implied that this model was significant. Meanwhile, *F*-value of 2.74 implies the lack-of-fit was not significant relative to the pure error, suggesting a great model fitting.

In Table 4b, variables *A* (coagulant dosage), *B* (pH), *C* (mixing time) and $B^2$ (quadratic terms of pH) were significant model terms in this case. *D* (settling time), interaction terms (*AB*, *AC*, *AD*, *BC*, *BD*, *CD*) and quadratic effects of other variables ($A^2$, $C^2$, $D^2$) were insignificant terms. For those values of "Prob. > *F*" less than 0.05, they can be

Table 4
ANOVA analysis for jar test using (a) *M. oleifera* seed extract and (b) chitosan
(a)

| Source | Sum of squares | df | Mean square | *F*-value | Prob. > *F* | Remarks |
|---|---|---|---|---|---|---|
| Model | 1.17E+05 | 6 | 19,524.33 | 135.24 | <0.0001 | significant |
| *A* – Coagulant dosage | 2,623.29 | 1 | 2,623.29 | 18.17 | 0.0003 | |
| *B* – pH | 97,416.98 | 1 | 97,416.98 | 674.77 | <0.0001 | |
| *C* – Mixing time | 1,047.29 | 1 | 1,047.29 | 7.25 | 0.013 | |
| *AB* | 2,383.88 | 1 | 2,383.88 | 16.51 | 0.0005 | |
| *BC* | 950.18 | 1 | 950.18 | 6.58 | 0.0173 | |
| $B^2$ | 12,724.33 | 1 | 12,724.33 | 88.14 | <0.0001 | |
| Residual | 3,320.52 | 23 | 144.37 | | | |
| Lack-of-fit | 3,015.19 | 18 | 167.51 | 2.74 | 0.1339 | not significant |
| Pure error | 305.33 | 5 | 61.07 | | | |
| Cor. total | 1.21E+05 | 29 | | | | |

(b)

| Source | Sum of squares | df | Mean square | *F*-value | Prob. > *F* | Remarks |
|---|---|---|---|---|---|---|
| Model | 203.09 | 4 | 50.77 | 38.42 | <0.0001 | significant |
| *A* – Coagulant dosage | 28 | 1 | 28 | 21.19 | 0.0001 | |
| *B* – pH | 115.21 | 1 | 115.21 | 87.17 | <0.0001 | |
| *C* – Mixing time | 6.88 | 1 | 6.88 | 5.2 | 0.0313 | |
| $B^2$ | 53 | 1 | 53 | 40.1 | <0.0001 | |
| Residual | 33.04 | 25 | 1.32 | | | |
| Lack-of-fit | 31.69 | 20 | 1.58 | 5.87 | 0.0294 | significant |
| Pure error | 1.35 | 5 | 0.27 | | | |
| Cor. total | 236.13 | 29 | | | | |

considered as significant, while for values greater than 0.10, it indicated that the model terms were not significant. However, values in between 0.05 and 0.1 was still under consideration [19]. The model *F*-value of 38.42 and Prob. > *F* less than 0.05 implied that this model was significant. *F*-value of 5.87 implies the lack-of-fit was significant relative to the pure error. The lack-of-fit should preferably be insignificant, as it implies that the model fits well and there is significant effect on parameters on output response. Higher-order terms might have to be included in the regression model if the lack-of-fit was significant [20].

In order to further investigate the model validity, the normality plot of residuals was used to confirm the normality assumption. Fig. 1 shows normal probability plots of the studentized residuals for jar test using *M. oleifera* seed extract and chitosan. A normal probability indicates that the residuals follow a normal distribution which data points should be linear while a non-linear pattern such as S-shaped curve shows non-normality in the error term. Obviously, all the residuals with the repeated points were close to the straight line with only few scattering points. However, some scattering was likely to happen even with the normal data. Thus, it can be assumed that both data were normally distributed and the quadratic models chosen in the ANOVA analysis was suitable. Fig. 2 depicts that the data points were distributed randomly on the 45° line, providing an agreement between the real experimental data and modelling data.

### *3.2. RSM predictions and the influence of process variables towards the turbidity removal efficiency*

#### *3.2.1. M. oleifera seed extract*

Three-dimensional response surface plots were plotted based on the predicted model equation as in Eqs. (4) and (5). These graphical representations of the regression equations were used to allow numerical optimization and also surface visualization with a view to maximize the turbidity removal. From the analysis, pH was the most significant parameter, followed by coagulant dosage and mixing time on the coagulation efficiency of *M. oleifera* seed extract. The settling time was insignificant ($p > 0.05$) as predicted by the ANOVA. Fig. 3a, d and e explain that with increasing amount of pH until pH 10, the turbidity will decrease until

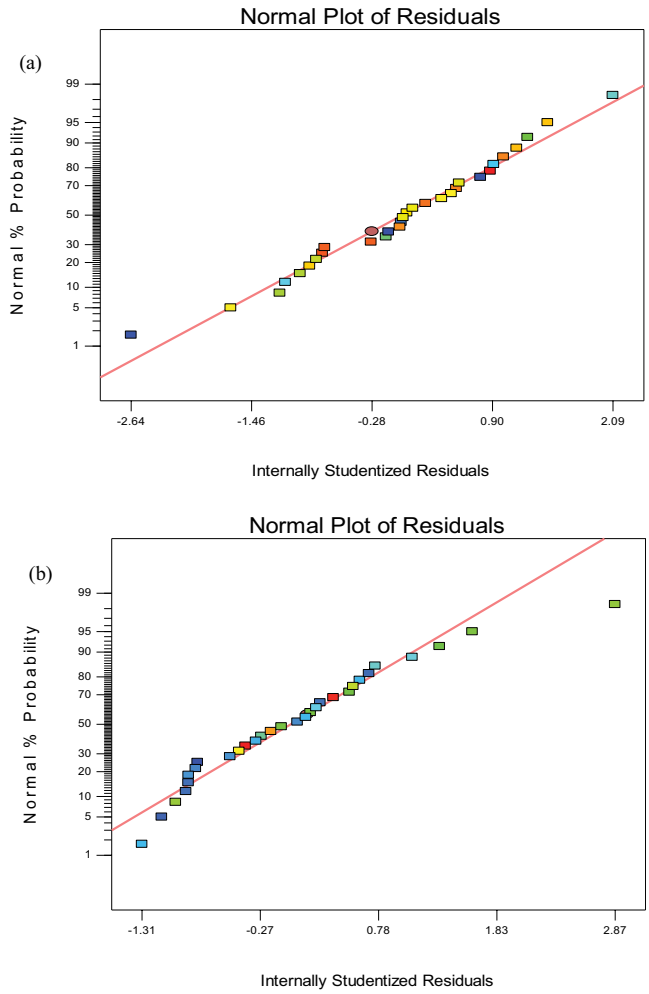


Fig. 1. Normal plot of residuals for (a) *M. oleifera* seed extract and (b) chitosan.

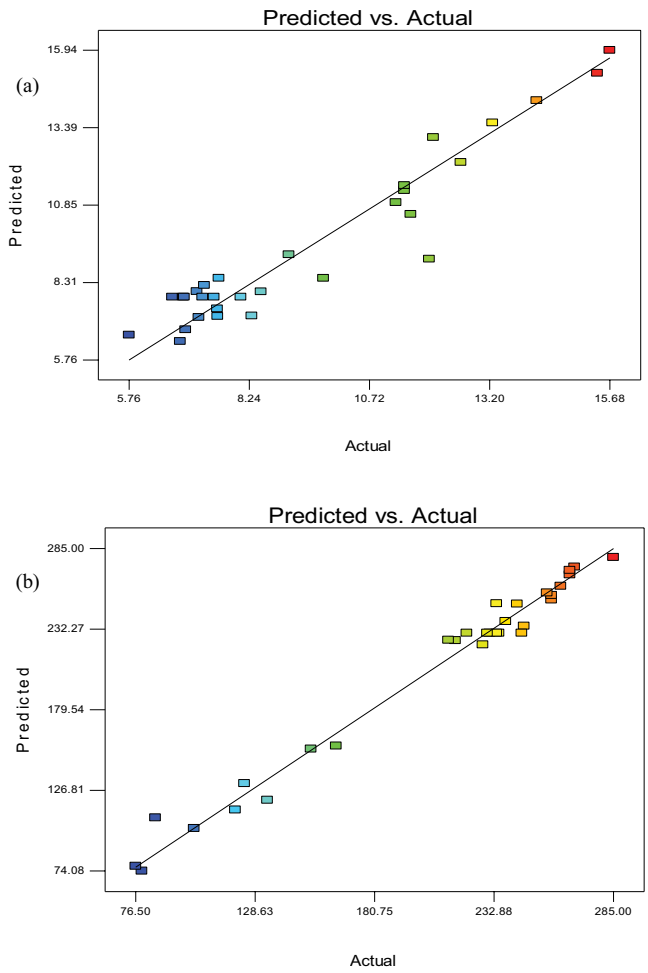


Fig. 2. Predicted vs. actual for (a) *M. oleifera* seed extract and (b) chitosan.

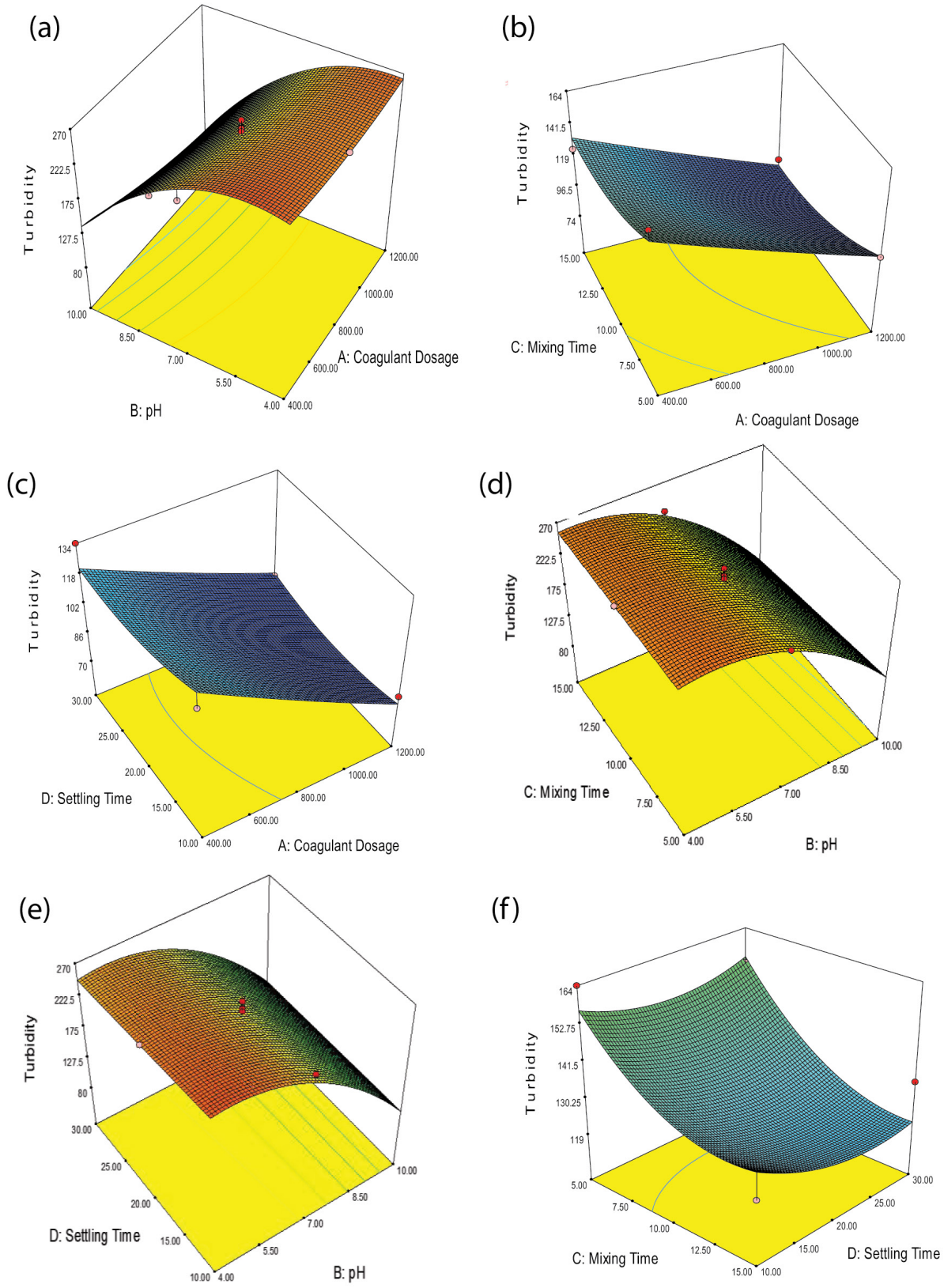


Fig. 3. Response surface plot showing the interaction between two parameters: (a) pH and coagulant dosage, (b) coaguant dosage and mixing time, (c) coagulant dosage and settling time, (d) pH and mixing time, (e) pH and settling time, and (f) mixing time and settling time using *M. oleifera* seed extract as the natural coagulant.

its optimum turbidity value (128 NTU) at optimum coagulant dosage of 400 mg/L, 15 min of mixing time and settling time. The effectiveness in removing turbidity from fish farm wastewater was highly depending on the coagulant dosage as a clear decrease in turbidity values was observed by enhancing the coagulant dosage up to 1,200 mg/L in Fig. 3b. Similar trend could be seen in Fig. 3c, with settling time showing insignificant effect here. However, mixing time was playing a more dominant role over settling time with the turbidity reduction as shown in Fig. 3f.

An optimum dosage of coagulants decided a successful CF process by avoiding undesirable extra dosage cost as over-dosing would only lead to the downtrend of coagulation efficiency. Each coagulants have their own critical inflection point and the aggregated particles would re-disperse and thereby disturbing the particle settling beyond that point [21]. When there is an exceed of coagulant dosage, it instead causes a turbidity rise in the water samples as all the excess coagulants do not interact with oppositely charged colloidal particles anymore when most of the colloids have undergone sufficient neutralization and precipitation [22]. Likewise, an optimum degree of acidity is long-known to significantly affect the CF process too so that the alkaline amino acids from *M. oleifera* protein could ionize to produce carboxylate ion and proton, allowing the colloids carrying electrons to be drawn closer and eventually forming neutral group, floc. However, it must be taken note that the addition of *M. oleifera* seed extract has always induced alkaline condition in the wastewater due to the presence of cationic water-soluble protein found in its skin and seeds [22]. Individually, the effect of increasing mixing time was relatively mild with only slight decrease in its turbidity value, showing that the rate of collision did not assist much in CF process under the condition of insufficient coagulant dosage. Flocs might not be dense enough for them to undergo rapid settling; hence giving a less remarkable effect towards the turbidity removal even though providing a long duration for particle settlement.

#### 3.2.2. Chitosan

Similar to *M. oleifera* seed extract, pH was the most crucial factor affecting the coagulation efficiency followed by coagulant dosage, mixing time and settling time the only insignificant contributor ($p > 0.05$) according to the contour diagrams in Fig. 4. As explained by the charge neutralization mechanism, the protonation of amine groups in chitosan at lower pH would led to favorable condition for neutralization of the anionic charges held by the colloids [23]. From Fig. 4a, the turbidity reduced to 73 NTU at optimum pH of 6 and optimum dosage of 100 mg/L. Comparing with pH, the coagulant dosage was in fact just having a minor effect towards the CF performance which directly proved that charge neutralization was the only dominant mechanism. In this scenario, turbidity reduction could only be seen at the low pH value where the amine groups were highly likely to protonate. However, from Fig. 4b, the significant effect of coagulant dosage could be seen in which the turbidity of the fish farm wastewater has experienced a downtrend and remained constant upon reaching an optimum coagulant dosage of 625 mg/L. This scenario mentioned that the coagulant dosage has reached a critical coagulant concentration and thereby the turbidity only jotted down small changes once the dosage was applied beyond this point. Both mixing time and settling time were not bringing about any key results as seen in Fig. 4c–f.

In the profiles of chitosan dosage vs. turbidity as studied by Ruelas-Leyva et al. [11], a qualified flocculation process must ensure that the polymeric flocculants are having adequate freely-available surface for the adsorption of the extended chains or the surface saturation would occur if excess polymer is adsorbed. In their work, they suggested that only adsorption charge neutralization and adsorption bridging were displaying a stoichiometric relationship of colloid concentration and coagulant dosage. Current CCD obtained stated that mixing time was inconsequential due to exposure of aggregates to fluid shear rates as a result of prolonged mixing. The floc growth process will be delayed as longer time is necessary to form larger structures prior to fragmentation induced by the high shear stress [24]. Furthermore, rate of floc breakup will also increase under intense agitation, resulting in re-dispersion of the adsorbents in the suspension [25]. Therefore, combination of rapid and slow mixing is encouraged before attaining an optimal dosage. In this study, it could be concluded that the settling speed of the floc particles was too slow hence a 10–20 min increment in the settling time might not end up in a significant collection of the sediments. In other words, the floc size was not big enough to induce a free settling velocity increment and the sedimentation was mainly driven by gravitational force. Throughout all the times, optimization of all the operational conditions involved in CF process was indispensable and typically carried out by the simplest jar tests as in this work.

### 3.3. Optimization of coagulation/flocculation process

The canonical analysis from the RSM surface plots promised the optimum combination of factors in this study. The desired goal for two factors, coagulant dosage ($A$) and settling time ($D$) were set to minimum to obtain a cost-effective condition with the highest performance while mixing time and pH value were defined to be within the studied level range to achieve the highest performance. Table 5 shows the solutions generated by the software based on the desirability. The wastewater samples were being treated using the optimized condition obtained from the software to be compared with the control sample having the same operating conditions. It was observed that the experimental value obtained for *M. oleifera* seed extract in Table 6 was in good agreement with the value predicted from the model with only 0.66% such small error happening between the predicted and the experimental value. In the contrary, the experimental value for jar test using chitosan displays a fairly high percentage of error (49.79%). It might be due to the low accuracy of the model prediction, as the lack-of-fit for the model was significant. Nevertheless, since the experimental result recorded (36.7 NTU) was better than the one predicted by the software (73.09 NTU), the optimized condition proposed was still being used for further analysis on its water quality. Obviously, chitosan exhibited higher

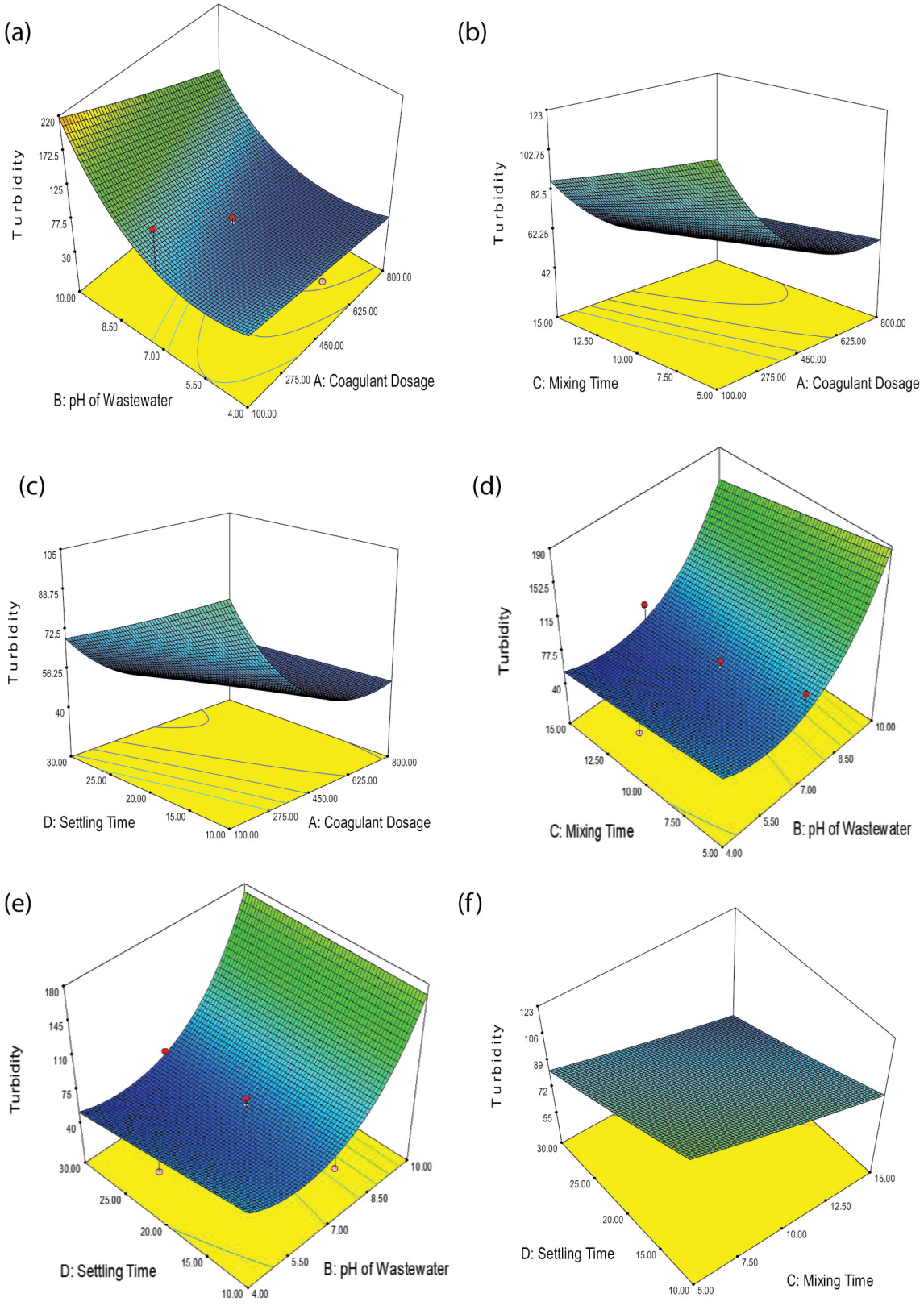


Fig. 4. Response surface plot showing the interaction between two parameters: (a) pH and coagulant dosage, (b) coaguant dosage and mixing time, (c) coagulant dosage and settling time, (d) pH and mixing time, (e) pH and settling time, and (f) mixing time and settling time using chitosan as the natural coagulant.

Table 5
Solutions generated from Design-Expert based on desirability for (a) *M. oleifera* seed extract and (b) chitosan
(a)

| No. | Coagulant dosage | pH | Mixing time | Settling time | Turbidity | Desirability | |
|---|---|---|---|---|---|---|---|
| 1 | 400 | 10 | 15.38 (15) | 10 | 128.854 | 0.903 | Selected |
| 2 | 400.02 | 10 | 15.42 | 10 | 128.853 | 0.903 | |
| 3 | 400.14 | 10 | 15.07 | 10 | 128.856 | 0.903 | |
| 4 | 400 | 10 | 13.9 | 10 | 128.858 | 0.903 | |
| 5 | 400 | 10 | 13.77 | 10 | 128.872 | 0.903 | |

(b)

| No. | Coagulant dosage | pH | Mixing time | Settling time | Turbidity | Desirability | |
|---|---|---|---|---|---|---|---|
| 1 | 100 | 5.6 (6) | 15 | 10.16 (10) | 73.0963 | 0.861 | Selected |
| 2 | 100 | 5.6 | 15 | 25.84 | 73.0963 | 0.861 | |
| 3 | 100 | 5.6 | 15 | 29.14 | 73.0964 | 0.861 | |
| 4 | 100 | 5.6 | 15 | 17.50 | 73.0964 | 0.861 | |
| 5 | 100 | 5.6 | 15 | 16.24 | 73.0962 | 0.861 | |

efficiency in the turbidity removal than *M. oleifera* seed extract (84% vs. 47%).

In order to coagulate, the zeta potential of the wastewater must be close to zero [9]. Majority of the particulates in natural wastewater are negatively charged in pH ranging from 6 to 8 [26]. Hence, when the positively charged coagulant such as chitosan with a zeta potential of 22.1 ± 3.29 mV (Fig. S1) was added into it, the repulsive charges would be neutralized resulting a strong van der Waals force, eventually inducing agglomeration. Chitosan has a significant effect of turbidity removal when its pH is around 5–7 and this pH range is attributed to the pKa value of chitosan (~6.0) [27,28]. At this value, chitosan would have a better ability to donate protons and stronger positive charges are carried by chitosan especially at lower pH. Reducing the pH up to 6 is not only enhances the solubility of chitosan but also increases the protonation of the chitosan surface. When the wastewater was in a slightly acidic condition, a reasonably high removal efficiency of turbidity could be observed. Furthermore, due to the action of polymer bridging, high-molecular-weight chitosan was able to achieve better turbidity removal performance under constant pH [29].

On the other hand, the coagulation performance of *M. oleifera* seed extract is known to be efficient at alkaline condition, as the isoelectric point (IEP) of the proteins in the seeds is identified to be between 10 and 11 [30]. As depicted in Supplementary data S1, the zeta potential of *M. oleifera* seed extract at its optimized condition has shown to own a negative value of –8.62 ± 5.60 mV in extreme pH conditions (pH 10 in this study). Findings were in an agreement with previous study citing that turbidity could be reduced at extreme pH such as pH 2 or 10. Colloids were possibly entrapped in the hydroxide precipitation due to excessive addition of alkali followed by settlement. During the settling process, any colloids came onto contact would be then captured along, hence lowering down the turbidity value [31].

Surprisingly, the main functional groups present in *M. oleifera* seed extract have depicted a high similarity index with that of chitosan from Supplementary data S2. The broadband centered at 3,302 $cm^{-1}$ attributed to OH stretching in the carbohydrates, proteins, fatty acids and lignin units. The peaks appeared in 1,639 $cm^{-1}$ can be attributed to the C=O stretching which correspond to the carbonyl group as part of fatty acids or proteins [32].

Generally, leftover feed and biological metabolites derived from the organisms are the principal sources of N-containing compounds in the aquaculture water. Among the compounds, $NH_3$ is more toxic to aquatic organisms than $NH_4^+$ or $NO_3^-$. Both *M. oleifera* seed extract and chitosan were not so effective in removing the ammonia content with their removal efficiency only about 30% and far less than that of the control (56%). The final ammonia concentration was still way above the recommended discharge limit. Presence of amine group in the chitosan was somehow contributing to the increased concentration of ammonia in the water sample but its removal efficiency heavily depended on its source, molecular weight and degree of deacetylation. To further increase the efficiency of ammonia removal, lower molecular weight of chitosan [29] and increasing degree of deacetylation [33] was preferred. As reported by previous study, more positive charges were carried by chitosan at higher degrees of deacetylation and the average turbidity removal efficiency reached 87.6% for chitosan with 98% deacetylation degree [33].

Surprisingly, using *M. oleifera* seed extract as coagulants demonstrated a low percent removal in COD concentration, from 394 to only 385 mg/L while chitosan achieved a great COD reduction from 375 to 135 mg/L but it was still above the discharge limit set by the Environmental Quality Acts 1974, which is 50 mg/L for Standard A and 100 mg/L for Standard B [34]. Thus, the removal efficiency can be further improved by increasing the chitosan dosage, which is related to the increase of available exchangeable reaction sites for the coagulation process, resulting in the cut down of organic matter in the wastewater [35]. There was always a disadvantage of using the crude water extract of *M. oleifera* as

Table 6
Comparison of water quality characteristics between control sample and samples using *M. oleifera* seed extract and chitosan and the deviation error for value estimated from Design-Expert (DE)

| Component | Control | | | *M. oleifera* | | | | Chitosan | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Before | After | Percent removal (%) | Before | After | Percent removal (%) | Predicted (deviation error) | Before | After | Percent removal (%) | Predicted (deviation error) |
| pH | 7.27 | 7.75 | | 10 | 8.02 | | | 6 | 7.15 | | |
| Turbidity (NTU) | 226 | 158 | 30.09 | 242 | 128 | 47.11 | 128.85 (0.66) | 227 | 36.7 | 83.83 | 73.09 (49.79) |
| Ammonia (mg/L) | 56 | 24.4 | 56.43 | 54.6 | 37.2 | 31.87 | | 49.4 | 36.8 | 25.51 | |
| COD (mg/L) | 352 | 271 | 23.01 | 394 | 385 | 2.28 | | 375 | 135 | 64.00 | |
| TSS (mg/L) | 140 | 110 | 21.43 | 200 | 140 | 30.00 | | 200 | 60 | 70.00 | |
| TVS (mg/L) | 140 | 100 | 28.57 | 200 | 120 | 40.00 | | 140 | 50 | 64.29 | |
| Phosphate (mg/L) | 1.12 | 0.99 | 11.61 | 1.72 | 0.76 | 55.81 | | 1.86 | 0.38 | 79.57 | |

the organic suspended solids did not participate well in the coagulation process and remained in the treated wastewater. To rectify this problem, it was suggested that *M. oleifera* could be used as a coagulant in wastewater treatment, only after an adequate purification of the active proteins [30].

Fine particles could easily block the gill lamella as well as attach to the fish skin, leading to dissolved oxygen shortage in the aquatic environment and threatening the aquatic life. However, fine particles are more difficult to be removed via CF process especially in the aquaculture wastewater which is subjected to suspended solids less than 100 μm in size [33]. In term of both total suspended solids (TSS) and total volatile solids (TVS), the water sample treated with chitosan exhibited the highest removal efficiency, followed by *M. oleifera* seed extract and control the last. In order to ensure that the final concentration of TSS comply with the discharge limit set by the Environmental Quality Acts 1974, the water samples would need to undergo secondary physico-chemical treatment or step up the coagulant dosage. The results were still correlating well with turbidity results as turbidity corresponded to the amount of total suspended solids found in wastewater. Similarly, chitosan has managed to get rid of the phosphate content by attaining a high removal efficiency of 79.57%. Chitosan possesses a high charge density per unit, which allows it to electrostatistically attract the counter-charged phosphate, resulting in its considerable removal from the aquaculture wastewater [29,36]. On the other hand, the lowest efficiency of phosphate adsorption was observed in the pH range of 8–11. In the alkaline conditions, chitosan surface attains the negative charge, as a result of which it repulses electrostatically the anionic P–$PO_4^{2-}$ and prevents their sorption [36]. Result from *M. oleifera* in this study was not in line with results obtained from other researches who pointed out an elevation in phosphate concentration (Sotheeswaran et al. 2011; Ndabigengesere et al. [30]). This was because of the crude water extract of *M. oleifera* containing a significant amount of orthophosphates, which might present as leachates in the wastewater samples, as well as different wastewater quality from the past researches.

## 4. Conclusion

*M. oleifera* seed extract and chitosan, these two abundantly available bio-coagulants in Malaysia have been investigated about its coagulation–flocculation efficiency in treating the fish farm wastewater collected. Estimation of turbidity removal response was well established via the formulation of reliable response surface model. The quadratic models developed in this study have the $R^2$ values of 0.9860 and 0.9436 for jar test using *M. oleifera* seed extract and chitosan respectively, suggesting a highly significant quadratic regression models. Optimum conditions for fish farm wastewater treatment by *M. oleifera* seed extract was at 400 mg/L, pH 10, 15 min mixing time and 10 min settling time while for chitosan was at 100 mg/L, pH 6, 15 min mixing time and 10 min settling time. Under optimization of relevant process parameters, the turbidity results were well matched for *M. oleifera* seed extract with only 0.66% error but did not tally with chitosan sample which registered a 49.79% error. Overall, chitosan exhibited higher reduction

of ammonia (25%), COD (64%), TSS (70%), TVS (64%) and phosphate (80%) than that of *M. oleifera* seed extract which only revealed a comparatively higher ammonia removal (32%). This analysis successfully implied that chitosan owns a promising ability to render reasonably efficient treatment of fish farm wastewater. Future pilot scale studies using chitosan as bio-coagulant could be practiced in clarifying fish farm wastewater. Moreover, modification of chitosan via chemical derivatization could be targeted for enhancement in treatment efficiency in order to meet the discharge limit stipulated by environmental legislations.

## Declaration of competing interests

The authors declare that they have no competing interest to influence the work reported in this paper.

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## Supplementary information

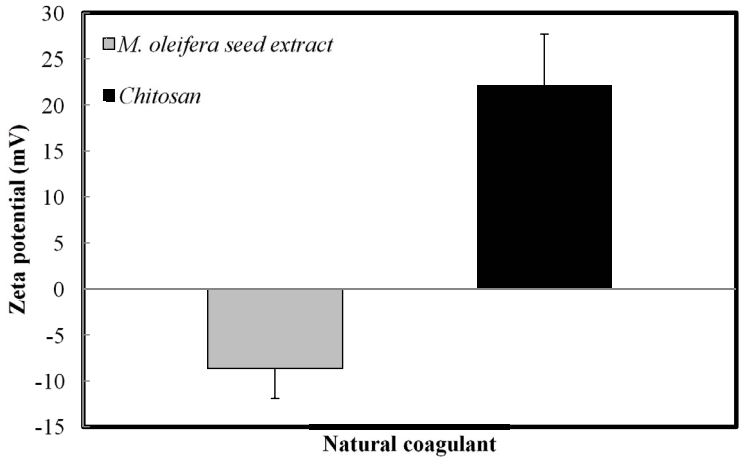


Fig. S1. Zeta potential of 400 mg/L *M. oleifera* seed extract at pH 10 and 100 mg/L chitosan at pH 6. Suspensions were prepared according to the optimization outputs obtained from Design-Expert.

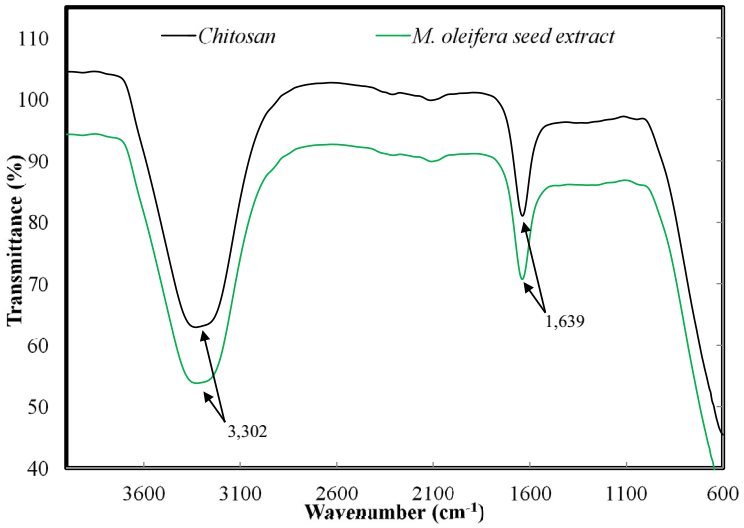


Fig. S2. Fourier-transform infrared spectra of both *M. oleifera* seed extract and chitosan.