Optimization of *Synechococcus* sp. VDW Cultivation with Artificially Prepared Shrimp Wastewater for Ammonium Removal and Its Potential for Use As a Biofuel Feedstock

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Abstract: To investigate the potential of application of marine cyanobacterium for concurrent biomass production and ammonium removal, *Synechococcus* sp. VDW was cultured under different conditions in medium containing varying concentrations of NH₄Cl. Response surface methodology (RSM) was then used to build a predictive model of the combined effects of independent variables (pH, inoculum size, ammonium concentration). At the optimum conditions of initial pH 7.4, inoculum size 0.17 (OD730) and ammonium concentration 10.5 mg L⁻¹, the maximum ammonium removal and biomass productivity were about 95% and 34 mg L⁻¹d⁻¹, respectively, after seven days of cultivation. The result of fatty acid methyl ester (FAME) analysis showed that the major fatty acids were palmitic acid (C16:0), linoleic acid (C18:2 n6 cis), palmitoleic acid (C16:1) and oleic acid (C18:1 n9 cis), which accounted for more than 80% weight of total fatty acids. Further, analysis of neutral lipid accumulation using flow cytometry revealed that the mean of the fluorescence intensity increased under optimal conditions. These results indicate that *Synechococcus* sp. VDW has the potential for use for concurrent water treatment and production of biomass that can be applied as biofuel feedstock.

Key words: *Synechococcus* sp. VDW, optimization; response surface methodology, ammonium removal, fatty acid profiles, biodiesel production

1 INTRODUCTION

The demand for energy is increasing continuously because of increases in population and industrialization. Most energy comes from fossil fuels such as coal, petroleum oil and natural gas, which are non-renewable resources. Therefore, finding alternative sources of energy has attracted attention. The potential for use of algae as a source of renewable energy has received considerable interest because they have many significant advantages⁴⁻⁵, including a rapid growth rate, high lipid content and the ability to grow without using arable land. Moreover, algae can effectively capture carbon dioxide through photosynthesis and produce polysaccharides and triacylglycerol (TAG). These compounds are the raw materials for production of bioethanol and biodiesel, which can be used in current engines without major modifications⁶⁻⁷. Although, many algae are accumulate significant quantities of TAG (20–50% of total dry weight) and has potential as a raw material for biodiesel production than cyanobacteria, which typically store carbon as glycogen and or polyhydroxybutyrate (PHB).

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However, cyanobacteria do contain lipids in thylakoid membranes and available for direct conversion to biodiesel\(^1\). Moreover, it can also provide valuable byproducts such as lubricants, bio-plastics, animal feed, nutraceuticals and pharmaceuticals\(^2\). However, the cultivation of microalgae requires large amounts of water and high initial investments. Microalgae culture coupled with wastewater treatment is considered one of the most promising routes to overcome these problems because it enables environmentally friendly and cost-effective production of biofuel.

Worldwide, many studies have shown that aquaculture production impacts the environment\(^3\). Specifically, aquaculture leads to enrichment of water by nutrients, especially ammonium compounds that may accumulate because of uneaten feed, feces and plankton die-offs in aquaculture ponds, which are eventually discharged into surrounding environments, where they have diverse impacts such as eutrophication and ecosystem damage\(^3,\)\(^4\). Ammonium levels in aquaculture effluent can vary widely, even in aquaculture source waters; however, levels are generally in the range of 1-10 mg L\(^{-1}\)\(^5\). It is well known that most algae could utilize ammonium into amino acids, but the excess ammonium concentration can cause the negative effect on microalgae growth. Several previous studies have successfully used microalgae cultures to remove ammonium from various types of wastewater\(^6\). For example, Lim et al.\(^1\) found that Chlorella vulgaris UMACC 001 could reduce NH\(_4\)-N concentrations in textile wastewater by 44.4\% -45.1\% (initial concentration = 6.5 mg L\(^{-1}\)), while Feng et al.\(^2\) reported that 97\% of NH\(_4\)-N was removed by C. vulgaris cultivated in artificial wastewater (initial concentration = 20 mg L\(^{-1}\)). Most of those species are suitable for culture in freshwater; thus, there is limited information available regarding their fatty acid profiles when grown in the presence of excess ammonium. Our previous studies have shown that Synechococcus sp. VDW is able to grow in excess ammonium condition which covers the range of shrimp farming wastewater sample\(^3\). Moreover, this strain is one of the fastest growing species of microalgae; therefore, they have been used as a model for synthetic biology studies and shown to have numerous biotechnological applications, including production of biochemicals and biofuels\(^4,\)\(^5\).

Microalgae are generally able to produce lipids at levels ranging from 1.5\% to 75\% (based on dry weight) depending on the culture medium and procedures\(^6\). High amounts of lipids are commonly found in microalgae grown under stress conditions, such as in conditions with limited nitrogen levels or high saline concentrations. However, high lipid content does not necessarily correspond to high biodiesel production\(^7,\)\(^8\) because fatty acid components such as steroids and pigments cannot be converted to biodiesel\(^9\). Characterization of fatty acid profiles may therefore help to predict biodiesel quality, particularly its oxidative stability, as well as the potential for cold filter plugging and mono-, di- and triglycerides content\(^2\). Thereby, this study aimed to investigate the optimal condition of media for enhancement of algal biomass production together with ammonium removal by Synechococcus sp. cultivated in the presence of different levels of ammonium using Response Surface Methodology (RSM) based on Central Composite design (CCD). Finally, characterization of the fatty acid profile in terms of the fatty acid methyl ester (FAME) content is analyzed and discussed.

2 EXPERIMENTAL

2.1 Microalgae strain and culture conditions

Marine cyanobacterium, Synechococcus sp. VDW was obtained from the Bioenergy Research Unit, King Mongkut’s Institute of Technology Ladkrabang, Thailand\(^10\). This strain was grown in a 250 mL Erlenmeyer flask containing 100 mL of BG11 medium supplemented with Turks Island salt solution (pH 7.5)\(^2\) while aerated by regular shaking at 150 rpm. Cells were cultivated under a fluorescent light irradiance of 30 \(\mu\)mol photon m\(^{-2}\)s\(^{-1}\) with a 12 h light/12 h dark cycle at 30 ± 2°C. Absence of microbial contamination was checked by the spread plate method. Normal medium (BG11 supplemented with Turks Island salt solution) consisted of 17.6 mM NaNO\(_3\), 30 mM MgSO\(_4\), 7H\(_2\)O, 0.189 mmol C L\(^{-1}\) Na\(_2\)CO\(_3\) and 0.5 M NaCl was used as the control. For optimization, NaNO\(_3\) was removed from the BG\(_11\) medium and replaced with various concentrations of NH\(_4\)Cl (Table 1).

The fragment of 16S rDNA of cyanobacterial isolates was amplified by polymerase chain reaction using the protocol previously described\(^1\). The nucleotide sequences of the 16S rDNA from Synechococcus sp. VDW have been deposited in the GenBank database under accession number MH393765.

2.2 Experimental design

The optimal culture conditions to achieve the maximum microalgae biomass and ammonium removal from synthetic shrimp farm effluent were estimated using the Design-Expert statistical package, version 10.0.6 (Stat-Ease, Inc. Minneapolis, MN, USA). To investigate the interactions between parameters, a central composite design (CCD) was applied. Three parameters, initial solution pH\((x_1)\), inoculum size\((x_2)\), and initial ammonium concentration\((x_3)\), were studied. Therefore, the full design matrix consisted of eight factorial points, six axial points and three replicates at the center points, resulting in a total of 17 experiments as shown in Eq. (1):

\[
N = 2^3 + 2^2 + n_c = 2^3 + 2(3) + 3 = 17
\]

where \(N\) is the number of experiments, \(n_c\) is the number of
independent variables and \( n \) is the number of center point.

Seventeen experiments were conducted in 100 mL Erlenmeyer flasks containing culture medium of different pH, inoculum size and initial ammonium chloride (NH\(_4\)Cl) concentration as shown in Table 1. The percentage biomass production and ammonium removal were considered as dependent variables (response). The design matrix contained a \( 2^4 \) factorial design augmented by six axial points coded \( \pm a \) and three central points (all factors = zero). The value of \( a \) was calculated by Eq. (2):

\[
\alpha = 2^{n/4}
\]

where \( n \) is the number of factors in the design. Therefore, \( \alpha \) is equal to \( 2^{0.5} = 1.68 \) according to Eq. (2). The range and levels of all factors in coded and actual values were calculated by Eq. (3):

\[
X_i = \frac{X_i - X_{0i}}{\Delta X_i}
\]

where \( X_i \) is the coded value of the \( i \)th independent variable; \( X_i \) and \( X_{0i} \) is the actual value of the \( i \)th independent variable and the \( i \)th independent variable at the center point, respectively. \( \Delta X_i \) is the step change value.

The quadratic polynomial equation was fitted to correlate the relationship between independent variables and responses as shown in Eq. (4):

\[
Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i,j=1}^{n} \beta_{ij} x_i x_j
\]

where \( Y \) is the predicted response, \( x_i \) and \( x_j \) are input variables, \( \beta_0 \) is the intercept, \( \beta_i \) is the linear coefficient, \( \beta_{ij} \) is a quadratic coefficient and \( \beta_{ij} \) is an interaction coefficient.

### 2.3 Analytical method

#### 2.3.1 Biomass productivity

After seven days of cultivation, 10 mL aliquots of samples from each experimental run were collected and centrifuged at 12,000 x g for 15 min. The biomass concentration of cyanobacterium *Synechococcus* sp. VDW was measured by cell dry weigh (DW) and monitoring the optical density (OD) at 730 nm using a microplate spectrophotometer (Multiskan GO, Thermo Scientific, Japan) \(^{23} \). A linear correlation between DW and OD\(_{730}(\lambda)\) was calculated using Eq. (5). Biomass productivity (BP) was calculated from the variation in biomass concentration within a cultivation time according to Eq. (6):

\[
DW(\text{mg L}^{-1}) = 333.95 A + 152.46 \quad (R^2 = 0.996) \quad (5)
\]

\[
BP(\text{mg L}^{-1} \text{day}^{-1}) = \frac{333.95 \times (A_T - A_0)}{T_t - T_0} + 152.46
\]

where \( A_0 \) and \( A_T \) were initial and final of optical density (OD) at 730 nm on days \( T_0 \) and \( T_t \), respectively.

#### 2.3.2 Efficiency of ammonium removal

Ammonium concentrations were determined by the indophenol blue colorimetric method according to Koroleff \(^{26} \). The supernatants were filtered (GF/F grade), and the resulting filtrates were immediately analyzed. After 30 min of incubation at room temperature, the absorbance at 640 nm was used to calculate the ammonium concentrations using a standard curve of NH\(_4\)Cl. The ammonium removal was defined as the percentage of NH\(_4\)\(^+\)-N removed from the medium at day 7, which was calculated using Eq. (7):

\[
\text{Ammonium removal(\%)} = \frac{C_0 - C_T}{C_0} \times 100
\]

where \( C_0 \) is the initial ammonium concentration (mg L\(^{-1}\)) and \( C_T \) is the final ammonium concentration (mg L\(^{-1}\)).

#### 2.3.3 Neutral lipids estimation

The content of neutral lipids was estimated by using flow cytometry (BD FACSCalibur, BD Biosciences, Singapore) in combination with Nile red staining \(^{25} \). Microalgae cells (15-20 \( \mu \)L) were collected at 7 days old by centrifugation then re-suspended in 1 mL of 20 % DMSO containing 5 \( \mu \)L of Nile Red stock solution (0.10 mg mL\(^{-1}\) of Nile Red dissolved in methanol) and incubated at 40°C for 15 min. The stained pellets were then subjected to a flow cytometer. The emission signal was measured FL2 channel centered at 580/30 nm (indicative of neutral lipids of Nile Red stained cells). Each condition was tested at least three times, and relative fluorescence results are presented as means with 95% confidence intervals. The data were analyzed using FlowJo software.

#### 2.3.4 Total lipid extraction and quantification

Total lipids were extracted from the dry biomass (dried at 60°C for 24 h) using a method applied from Axelsson and Gentili \(^{28} \). Briefly, Dry biomass (30 mg) was crushed and placed into test tubes (in three replicates)/with 2:1 parts of chloroform-methanol mixture (v/v). Cells were manually shaken for a few seconds. Finally 0.73 % NaCl water solution was added to produce a 2:1:0.8 system of chloroform : methanol : water (v/v/v). Next, the mixture was centrifuged for 2 min at 1851 x g. The chloroform layer was collected.

### Table 1 Level and range of variables in the experiments.

| Variables                   | Code levels       |
|-----------------------------|-------------------|
| \( X_1 \): pH               | -1.68  -1  0  +1  +1.68 |
| \( X_2 \): inoculums size (OD\(_{730}\)) | 6.66  7.00  7.50  8.00  8.34 |
| \( X_3 \): ammonium concentration (mg N L\(^{-1}\)) | 0.06  0.10  0.15  0.20  0.23 |
| \( X_4 \): ammonium concentration (mg N L\(^{-1}\)) | 4.96  7.00  10.00  13.00  15.04 |
carefully. The lipid extraction process was repeated five times, as described above. The liquid phase was transferred to previously weighed flasks. Afterwards, the solvent was evaporated using a rotary evaporator and the flasks were reweighed after being dried in an oven at 60°C and kept in a vacuum desiccator. The total lipid fraction was determined based on the flask weight differences, while the lipid content was calculated based on the percentage of initial dry weight.

2.3.5 Fatty acid characterization

The fatty acid components of Synechococcus sp. VDW under normal and optimal conditions were analyzed in term of fatty acid methyl ester (FAME) content by gas chromatography-mass spectrometry (GC-MS) based on the method described by Lepage and Roy.⁵⁷ First, seven-day-old microalgal cultures were collected, after which 3 g of sample were transferred into screw cap tubes. Next, 12.0 mL of dichloromethane : methanol (2 : 1 v/v) were added and samples were allowed to stand for 1 h, during which time they were vortexed every 15 min. The samples were subsequently filtered through Whatman No. 1 filter paper, after which 0.1 M KCl solution was added. The samples were subsequently vortexed briefly, then centrifuged at 2,000×g for 10 min and the upper phase was discarded. Samples were then methylated by adding 1 mL of 0.5 mol L⁻¹ NaOH-methanol to 200 μL of the product from the previous step and heating the tubes for 15 min at 100°C. Next, samples were allowed to cool to room temperature, after which 500 μL of hexane and 5 mL of saturated NaCl solution were added. The samples were then vortexed briefly and centrifuged at 2,000×g for 5 min, after which the organic (upper) phase was transferred to a fresh tube. GC-MS analyses were subsequently performed using Trace-GC Ultra (Thermo Scientific, Italy) equipped with a 60 m×0.25 mm TR-FAME capillary column (Thermo Scientific, USA) connected to a PolarisQ mass spectrometer (Thermo Scientific, USA). The fatty acid profiles were identified by comparison with commercial standards and the area normalization method was employed to quantify the relative percentage of individual fatty acids. All samples were analyzed in duplicate.

2.3.6 Degree of saturation (DU)

Degree of saturation parameter was determined by Eq. (8). Cetane number was estimated according to Ramos et al.⁶⁰

\[
DU = \text{(monounsaturated, wt. %)} + 2 \times \text{(polyunsaturated, wt. %)}
\]  
(8)

2.4 Statistical analysis

The Design Expert statistical package was used for regression analysis of variance (ANOVA). All experiments were conducted in triplicate, and the average values were recorded as the response. The quality of fit of the polynomial model equation was evaluated by the coefficient of determination (R²) and the adjusted R²(adj), and its statistical significance was assessed with a P-value. The optimum values of the selected variables were obtained by analyzing the contour plots and also by analyzing the quadratic polynomial equation.⁶⁰

3 RESULTS AND DISCUSSIONS

3.1 Optimization by response surface methodology

The maximum efficiency of ammonium removal and biomass productivity by Synechococcus sp. VDW were investigated under different operating conditions; namely, initial pH 6.66-8.34, inoculum size 0.06-0.23 and initial ammonium concentration 4.96-15.04 mg N L⁻¹. The design matrixes of the variables determined by CCD along with the predicted and experimental values of responses are given in Table 2. The results were analyzed by analysis of variance (ANOVA) as shown in Table 3.

The coefficients and P-values of all the variables of linear (x₁, x₂, x₃), quadratic (x₁², x₂², x₃²) and interactions (x₁x₂, x₁x₃, x₂x₃) terms were determined according to ANOVA. Larger F-values and smaller P-values represent a high significance of the corresponding coefficient. P-values < 0.05 were considered to indicate significance.⁶⁰ The results of ammonium removal showed that x₁, x₂, x₁x₃, x₂x₃, x₁², x₂² and x₃² were significant terms; therefore, they were selected as the effective terms while other model terms were deleted. In addition, the model F-value of 133.44 for Y₁ (ammonium removal) and the P-value (< 0.0001) of the overall model is significant. The P-value of lack of fit was 0.0589 (non-significant), indicating that the model fit the experimental data well and the independent variables have considerable effects on the response. Analysis of variance of the biomass productivity showed that x₂, x₃, x₁², x₂² and x₃² were significant (p < 0.05). Moreover, the coefficient of determination (R²) was calculated as 0.9942 for ammonium removal and 0.9609 for biomass productivity, indicating that the regression model represented 96-99% of the experimental results, while about 1-4% of the variability in the response was not explained by this model. Additionally, the ANOVA table of the fitted model (Table 3) indicated that the regression was significant (p = 0.0001), while the lack of fit was not significant (p > 0.05). These results suggest that the design model adequately fits the experimental data. As a result, the following regression equations were obtained:

\[
Y₁ = 98.44 - 5.62x₁ - 22.5x₂ + 6.5x₁x₃ - 4.34x₂x₃ - 20.81x₁² - 5.86x₂² - 16.83x₃²
\]  
(9)

\[
Y₂ = 31.12 - 8.82x₂ - 4.30x₁² - 3.26x₃² - 3.40x₁²
\]  
(10)

where Y₁ and Y₂ are the predicted response of ammonium
Table 2  Responses for biomass production and ammonium removal.

| Run | pH  | inoculum size (OD<sub>730</sub>) | Ammonium concentration (mg N L<sup>-1</sup>) | Ammonium removal (%) | Biomass production (%) |
|-----|-----|---------------------------------|--------------------------------------------|----------------------|------------------------|
|     |     |                                 |                                            | Observed             | Predicted              |
| 1   | 7.50| 0.15                            | 10.00                                      | 99.07                | 98.10                  |
| 2   | 8.00| 0.20                            | 13.00                                      | 33.99                | 28.82                  |
| 3   | 8.00| 0.10                            | 13.00                                      | 30.70                | 37.32                  |
| 4   | 8.00| 0.20                            | 7.00                                       | 70.15                | 69.04                  |
| 5   | 7.00| 0.10                            | 7.00                                       | 83.33                | 85.12                  |
| 6   | 7.50| 0.15                            | 10.00                                      | 98.80                | 98.10                  |
| 7   | 7.50| 0.23                            | 10.00                                      | 87.34                | 81.61                  |
| 8   | 7.50| 0.06                            | 10.00                                      | 78.21                | 81.61                  |
| 9   | 7.00| 0.20                            | 7.00                                       | 95.12                | 93.62                  |
| 10  | 6.66| 0.15                            | 10.00                                      | 50.33                | 48.67                  |
| 11  | 8.34| 0.15                            | 10.00                                      | 97.13                | 98.10                  |
| 12  | 7.00| 0.10                            | 13.00                                      | 38.67                | 35.50                  |
| 13  | 7.50| 0.15                            | 4.96                                       | 88.56                | 88.38                  |
| 14  | 7.50| 0.15                            | 15.04                                      | 60.44                | 60.54                  |
| 15  | 7.50| 0.15                            | 10.00                                      | 97.13                | 98.10                  |
| 16  | 8.00| 0.10                            | 7.00                                       | 60.44                | 60.54                  |
| 17  | 7.00| 0.10                            | 13.00                                      | 22.13                | 27.00                  |

Table 3  Analysis of variance (ANOVA) of ammonium removal and biomass production by response surface quadratic model.

| Source   | d.f. | Ammonium removal (%) (<i>Y</i><sub>1</sub>) | Biomass production (%) (<i>Y</i><sub>2</sub>) |
|----------|------|---------------------------------------------|----------------------------------------------|
|          |      | Sum of square | Mean square | <i>F</i>-value | <i>P</i>-value | Sum of square | Mean square | <i>F</i>-value | <i>P</i>-value |
| Model    | 9    | 14299.52     | 1588.84     | 133.44         | <0.0001<sup>a</sup> | 8338.03     | 1023.03     | 19.14         | 0.0004<sup>a</sup> |
| x<sub>1</sub> | 1    | 441.42      | 441.42      | 37.07          | 0.0005<sup>a</sup> | 69.8        | 176.2       | 3.3           | 0.1123 |
| x<sub>2</sub> | 1    | 46.24       | 46.24       | 3.88           | 0.0894         | 11.71       | 3.81        | 0.071         | 0.7971 |
| x<sub>3</sub> | 1    | 6891.23     | 6891.23     | 578.77         | <0.0001<sup>a</sup> | 5909.43     | 6689.98     | 125.15        | <0.0001<sup>a</sup> |
| x<sub>1</sub>x<sub>2</sub> | 1    | 40.47       | 40.47       | 3.4            | 0.1078         | 221.03      | 71.22       | 1.33          | 0.2863 |
| x<sub>1</sub>x<sub>3</sub> | 1    | 337.91      | 337.91      | 28.38          | 0.0010<sup>a</sup> | 85.35       | 7.9         | 0.15          | 0.7121 |
| x<sub>2</sub>x<sub>3</sub> | 1    | 144.56      | 144.56      | 12.14          | 0.0102<sup>a</sup> | 11.02       | 9.66        | 0.18          | 0.6836 |
| x<sub>1</sub><sup>2</sup> | 1    | 4903.45     | 4903.45     | 411.83         | <0.0001<sup>a</sup> | 1409.03     | 1555.42     | 29.1          | 0.001<sup>a</sup> |
| x<sub>2</sub><sup>2</sup> | 1    | 383.12      | 383.12      | 32.18          | 0.0008<sup>a</sup> | 283.99      | 329.8       | 6.17          | 0.042<sup>a</sup> |
| x<sub>3</sub><sup>2</sup> | 1    | 3177.93     | 3177.93     | 266.91         | <0.0001<sup>a</sup> | 1217.16     | 1353.48     | 25.32         | 0.0015<sup>a</sup> |
| Residual | 7    | 83.35       | 11.91       |                |                | 225.72      | 53.45       |               |                |
| Lack of fit | 5   | 81.35       | 16.27       | 16.27          | 0.0589         | 217.72      | 73.07       | 16.56         | 0.0579 |
| Pure error | 2   | 2           | 1           |                |                | 8           | 4.41        |               |                |
| Total    | 16   | 14382.86    |             | 8563.75        |                |
| R<sup>2</sup> |    | 0.9942      |             | 0.9609         |                |
| R<sup>2</sup><sub>adj</sub> |    | 0.9868      |             | 0.9107         |                |

<sup>a</sup> Model terms are significant

d.f. = degree of freedom
Fig. 1  Response 3D-surface plots showing the combined influences of (a) inoculum size and pH (b) ammonium concentration and pH and (c) ammonium concentration and inoculum size on % ammonium removal. The combined influences of (d) inoculum size and pH (e) ammonium concentration and pH and (f) ammonium concentration and inoculum size on biomass productivity.
removal and biomass productivity; x₁ is the pH; x₂ is the inoculum size; x₃ is the ammonium concentration.

3.2 Graphical description of the model equation

According to regression equation, Design-Expert 10.0.6 software was used to make the three-dimensional surface of the response surface. The effect of three independent variables (pH, inoculum size and ammonium concentration) and the interaction on the response surface were shown in Fig. 1. The optimal pH, inoculum size, and ammonium concentration were found to be 7.4, 0.17 (OD₃₅₀), and 10.5 mg L⁻¹, respectively, which yielded an optimal algal productivity of about 34 mg L⁻¹ d⁻¹ and an average ammonium removal of 95%. These results agreed well with the predicted value of algal productivity and ammonium removal of 31.65 mg L⁻¹ d⁻¹ and 93%, respectively. An abrupt slope on the RSM curve indicates this factor largely influence on the response, while a gentle slope suggests this factor does not largely affect the response. Clearly, the combined effects of pH and ammonium concentration were found to be the dominant factor influencing ammonium removal (Figs. 1a-c). Previous studies have reported that ammonium removal rate is highly varied in different microalgae. De Alva et al.⁵² found that above 92% of ammonium removal when treating municipal wastewater with Scenedesmus sp. which initial ammonia was in a range of 27-49 mg L⁻¹ while Chlorella vulgaris was dramatically removed ammonium from wastewater effluent (initial concentration of 7.7 ± 0.19 mg L⁻¹ in 48 h⁵⁰). This result suggested that the ammonium removal rate for microalgae depend on species. Figures 1d-f showed the interactive effect of three factors on the biomass productivity. From the figures, it was observed that the biomass productivity decreased with the rise of ammonium concentration (above 10 mg L⁻¹). These findings are consistent with those of Chen et al.⁴⁰, who found that the specific growth rate of Chlorella pyrenoidosa decreased by more than 50% and 80% when the ammonia concentration increased to 30-40 and 50-60 mg L⁻¹, respectively. Conversely, the specific growth rate decreased approximately 12.7%-23.1% at a pH of 8.5-9.0, while pH variations between 6.5 and 8.5 had no significant influence on growth. In general, there are two forms of ammonia, ionized (NH₄⁺) and un-ionized (NH₃). As pH increases, the toxicity of ammonia increases because the relative proportion of unionized ammonia, which is known to be toxic to several photosynthetic organisms, increases. However, Caicedo et al.⁵⁵ found that both ammonia forms caused growth inhibition via inhibition of photosynthesis. For the persistence of microalgae cells in excess ammonium condition, the boosting of their inoculum size has been noticed to be helpful. Low inoculum sizes were deemed more susceptible to ammonia inhibition compared to high inoculum sizes, as stated by Markou et al.⁴⁹. Typically, inoculum size is clearly linked to the amount of cells contributing to the population expansion, causing increased biomass manufacture. Conversely, cell growth rate will be influenced by excessive inoculum size based on the limitation of nutrient and light. Meanwhile, the findings in this work have proposed preliminary cell inoculation of OD₃₅₀ = 0.17, which comprises the most appropriate condition for algae growth in high ammonium conditions using small-scale laboratory cultures. On the other hand, the inoculum size could vary for large-scale cultures, meaning further study is needed.

3.3 Total lipid and neutral lipids estimation

The results of growth performance in terms of biomass productivity for Synechococcus sp. VDW under control and optimized conditions were 34.36 and 30.36 mg L⁻¹ d⁻¹, respectively. Meanwhile, the percentage of total lipid was higher when compared with control (Table 4). The flow cytograms have shown significant fluorescence intensity higher than the control indicating high neutral lipid accumulation in the cell cytoplasm (Fig. 2). These results suggest that lipid accumulation in Synechococcus sp. VDW was likely related to ammonium concentrations. However, a number of studies have reported that lipid production could be triggered under nitrogen starvation. Sassano et al.⁴⁷ observed that the highest lipid contents could be obtained by increasing the nitrogen supply to a maximum limit value, beyond the value at which the excess nitrogen source becomes toxic to the cell. However, the effect of variations in nutrient concentrations on microalgae biomass and lipid content is probably due to differences in strain, nitrogen source and culture conditions, which should remain to be ascertained.

3.4 Fatty Acid Composition

The fatty acid composition of Synechococcus sp. VDW was evaluated in the early stationary growth phase by GC-MS analysis. Total fatty acid profiles indicated seven fatty acids in normal condition, while six were found in cells cultivated under optimal conditions. Under both conditions, dominant long-chain fatty acids consisted of 16 and 18 carbon atoms with four major constituents as palmitic acid (C₁₆:0), linoleic acid (C₁₈:2 n6 cis), palmitoleic acid (C₁₆:1) and oleic acid (C₁₈:1 n9 cis) accounting for more than 80% of the total (Table 4). Percentages of saturated fatty acids and monounsaturated fatty acids decreased (≤ 5 wt. %) with increasing polyunsaturated fatty acids (~14 wt. %) compared to normal condition. Previous studies have shown that various stresses such as nitrogen concentration, pH, salinity and light are commonly used to induce polyunsaturated fatty acid (PUFA). PUFA play a prominent role as the osmo-protecting molecules may help microalgae cells confront environment stresses, and these mechanisms, in turn, stimulate the biosynthesis of lipids as energy-rich storages in microalgae cells⁵⁵,⁵⁸. Although giving
slightly lower saturated fatty acids when compared to the control, palmitic acid content showed similar to fatty acid in palm oil (Table 5). Palmitic acid has a higher cetane number (CN) as the most important indicator of diesel in terms of ignition quality with a higher value indicating better grade. Degree of unsaturation (DU) indicates the number of double bonds present in the fatty acid chain with a high number of double bonds representing a high degree of unsaturation. Ramos et al.28 suggested that higher unsaturation in oil was associated with higher CN and iodine value. In comparison with other feedstocks, the DU value of Synechococcus sp. VDW was similar to olive oil but higher than the commercial biodiesel feedstock palm oil. Ramos et al.28 reported that the CN of olive oil presented near to palm oil at 57 and 61 respectively, while soybean and sunflower oil presented CN values lower than the European Standard (minimum CN of 51). As a result, oils with DU higher than 137 do not meet European Standards for cetane number and iodine value. Low CN were associated with more highly unsaturated components such as esters of linoleic (C18:2) and linolenic (C18:3) acids40. Li et al.41 discovered that higher DU values resulted in increased nitrogen oxide (NOx) emissions. High levels of unsaturation adversely affect engine performance and exhaust gases with the degree of biodiesel unsaturation resulting in small variations of oxygen content, heating value and viscosity42. Results indicated that biodiesel quality from Synechococcus sp. VDW in terms of CN and iodine values almost attained the European Standards criteria; hence, this strain shows promise for use in biodiesel production.

**4 CONCLUSIONS**

This study evaluated the feasibility of ammonium treatment with simultaneous biomass production by marine cyanobacterium Synechococcus sp. VDW. RSM was used to build a predictive model of the combined effects of independent variables (pH, inoculum size, ammonium concentration). The predicted value agreed well with the experimental value, as determined by validation experiments,
which confirmed the availability and accuracy of the model. Under optimum conditions, *Synechococcus* sp. VDW was able to grow effectively in a high concentration of ammonium and was almost completely removed. The result of total lipid and neutral lipid content were higher than control um and was almost completely removed. The result of total which confirmed the availability and accuracy of the model. Under optimum conditions, *Synechococcus* sp. VDW was able to grow effectively in a high concentration of ammonium and was almost completely removed. The result of total lipid and neutral lipid content were higher than control conditions. The fatty acid profiles revealed high levels of saturated and monounsaturated fatty acids, which together comprised more than 60% of the total fatty acids. Especially, large amount of palmitic acid may positive correlation with cetane number. Overall, the results indicated that using wastewater contaminated with ammonium as a nitrogen source under optimal conditions is beneficial to the growth of this microalgae and biofuel production.

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