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Optimizing growth and pigment content of promising green microalgae and application of living microalgal cells as a sole practical diet for white shrimp larvae

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ABSTRACT

Microalgae are promising feed sources for aquaculture species as they contain high levels of pigments, which can help develop proper coloration. This study aimed to economically produce living microalgal cells and apply them as practical diets for white shrimp larvae. Among the strains screened, a halotolerant *Chlorella* sp. SHP isolated from shrimp culture pond was selected, as it contains the highest chlorophyll and carotenoid contents of 11.14 ± 1.15 and 4.89 ± 0.21 mg/g-cell, respectively. To reduce production costs, cheap fertilizer containing nitrogen, phosphorus, and potassium (NPK) was utilized as a nutrient source for microalgae cultivation. The optimal ratio of NPK fertilizer for microalgae cultivation was 1:0.25:0.25. Optimization using response surface methodology revealed that the optimal nitrogen and salt concentrations were 1.75 g/l and 1%, respectively. The optimal light intensity was 3,000 lux. The maximum microalgal biomass obtained was 1.45 ± 0.01 g/l with improved chlorophyll and carotenoid contents up to 24.51 ± 0.03 and 11.29 ± 0.28 mg/g-cell, respectively. Scaling up the process in an 8-l tank photobioreactor with the optimal aeration rate of 0.1 air volume per liquid volume per min (vvm) increased the biomass production up to 1.72 ± 0.06 g/l. The use of living microalgal cells as a practical diet for shrimp larvae not only improved the color of the shrimp but also increased dissolved oxygen and reduced sediments in the shrimp culture pond. These strategies may contribute greatly to the aquaculture sector as they are cost-effective and can help promote ecosystem balance and minimize waste.

1. INTRODUCTION

Microalgae are receiving increasing interest in various sectors including food and feed, biochemicals, biofuels, wastewater treatment, and carbon dioxide capture as they possess high photosynthetic efficiency and biomass productivity in comparison to other crops [1,2]. They are also reliable sources of lipids, proteins, carbohydrates, pigments, and vitamins [1–3]. Notably, the levels of these compounds are comparable or even greater than those found in plants [4]. Additionally, the microalgal cells are used as feed additives or practical diets for many aquaculture species [5]. They have been used as nutrients for fairy shrimp and diet supplements in most hatcheries due to their appropriate size (2–50 µm), high nutritional value, antioxidant properties, and property to increase disease tolerance of aquatic species, and so on [6,7]. It has been reported that the pigment contents in microalgae like carotenoids and chlorophylls influence nutrient assimilation and the immune system of the shrimp. Specifically, the functions of carotenoids include the role of provitamin A inducer and antioxidants, and those of chlorophylls include regulation of the gut microbiota and exhibiting antibacterial activity [8,9]. Additionally, in order to apply microalgae in aquaculture systems as living cells the halotolerant property of microalgae is also a crucial factor. To reduce the production costs of microalgae, low-cost media, and efficient culture conditions should be explored for their mass production and suitable biomass composition.

Cultivating microalgae on a large scale for mass production requires a significant amount of nutrients, specifically nitrogen, phosphorus, and potassium (NPK) [10]. The ratio of these nutrients influences biomass productivity, composition, and yield of specific products [11]. However, synthetic medium for microalgae can cost from US$ 2.6 to 18.3 each 1,000 l [12]. To minimize the production costs of microalgae, the cheaper inorganic fertilizer (US$0.5–1.0/1,000 l) can be a convenient alternative medium for microalgae cultivation. However, it is crucial to determine the suitable NPK ratio and culture

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conditions, including nutrient and salt concentrations, and light intensity for maximizing microalgal growth and pigment production. Moreover, in large-scale cultivation, adequate mixing and aeration should be provided to ensure adequate exposure to light, and the availability of nutrients [13]. Using living microalgal cells as a diet for shrimp larvae culture has advantages over the use of commercial feed at high concentrations. This is because the accumulation of feed residues in shrimp culture ponds may cause shrimp to grow slowly, consume less food, be stressed, have weak immunity, and be easily infected with disease [14]. In addition, it has been reported that the advantages of using living microalgae mainly include their metabolic activity in removing phosphorus and nitrogen compounds and preventing the accumulation of *Vibrios* in the environment [15].

This study aimed to screen promising microalgae with high pigment content, optimize medium and culture conditions, and apply living microalgal cells as a diet for shrimp larvae culture. To replace expensive synthetic medium for the microalgae, modified inorganic fertilizer containing NPK was used as a sole nutrient source to cultivate microalgae. The nutrient concentrations and light intensity were optimized using response surface methodology (RSM). The microalgae culture was scaled up in an 8-l tank photobioreactor, and the living microalgal cells were harvested and used as diets for white shrimp larvae. The effects on survivability, size, and color of the shrimp larvae, as well as on water quality, were studied.

2. MATERIALS AND METHODS

2.1. Microalgae and Medium

Oleaginous *Chlamydomonas* sp. WP1 and *Scenedesmus* sp. WP2 were isolated from wastewater treatment ponds in a seafood processing plant in Songkhla, Thailand. *Chlorella* sp. SHP was isolated from a local shrimp culture pond in Songkhla, Thailand. The modified Chu13 medium used for the preparation of microalgal inoculum was composed of 0.2 g KNO_3, 0.1 g citric acid, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.01 g Fe citrate, 0.04 g K_2HPO_4, 0.036 g $NaHCO_3$, and 1 ml of trace metal stock solution per 1 l. One liter of trace metal stock solution contained 2.85 g H_3BO_3, 1.8 g $MnCl_2 \cdot 4H_2O$, 0.05 g $Na_2MoO_4 \cdot 2H_2O$, 0.08 g $CuSO_4 \cdot 5H_2O$, 0.08 g $CoCl_2 \cdot 6H_2O$, and 0.02 g $ZnSO_4 \cdot 7H_2O$. The sodium chloride was added 0.5%. The pH of the medium was adjusted to 6.8 [16].

2.2. Selection of Microalgae With High Pigment Contents

Three microalgal strains, including *Chlorella* sp. SHP, *Chlamydomonas* sp. WP1, and *Scenedesmus* sp. WP2 was 10% inoculated in modified Chu 13 medium. The microalgae were cultured under a light intensity of 3,000 lux with a photoperiod of 16 hours per day at 30°C ± 2°C for 5 days. The cultures were aerated at 0.02 air volume per liquid volume per min (vvm). The samples were taken daily to measure pH and optical density (OD) at 660 nm. The biomass, lipid, protein content, carbohydrate, pigment, and antioxidant activity on day 5 were analyzed. The microalgal species with the highest pigment contents was selected for further experiments.

2.3. Use of Modified Fertilizer as a Low-Cost Medium for Microalgae Cultivation

The liquid fertilizer was prepared using commercial fertilizer that contains NPK in the forms of H_2NCONH_2, P_2O_5, and K_2O, respectively. The nitrogen concentration was set at 1 g/l, and the ratios of NPK were set at 1:0:0, 1:0.25:0.25, 1:0.5:0.5, and 1:1:1. The selected microalga was 10% inoculated and incubated under a light intensity of 3,000 lux with a photoperiod of 16 hours per day at 30°C ± 2°C for 7 days. The aeration rate was 0.02 vvm. The sampling and analysis were the same as mentioned above. The ratio of NPK that gave the highest microalgal growth and pigment contents was selected for further experiments.

2.4. Optimization of Culture Conditions

RSM and Box-Benhken Design (BBD) were employed to optimize the key operating factors, including nitrogen and salt concentrations and light intensity. Their individual and interaction effects on microalgal growth, chlorophyll ab, carotenoids, lipid, and protein contents were studied. Through this experimental design, seventeen runs involving triplicates of the central point were conducted. The relationship between operating factors (x_i and x_j) and responses (Y) were determined through a second-order polynomial quadratic equation as shown below:

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x^2_i + \Sigma\beta_{ij} x_i x_j \quad (1)$$

where β_0, β_i, and β_{ii}, and β_{ij} are coefficients representing offset, linear term, squared term, and interaction term, respectively.

2.5. Scale Up in an 8-l Photobioreactor and Effect of Aeration Rate

The selected microalgal strain was cultured in 7 l medium in an 8-l photobioreactor using different aeration rates of 0.02, 0.1, 0.3, and 0.5 vvm and incubated under light intensity at optimal levels with photoperiod (light:dark) of 16:8 hours for 7 days. The sampling and analysis were the same as mentioned above. The optimal aeration rate giving the highest growth and pigment contents was selected for further experiments.

2.6. Application of Living Microalgal Cells as a Practical Diet for White Shrimp Larvae

After cultivation in photobioreactors, the microalgal cells were harvested by centrifugation prior to the application. The living and dried microalgal cells were applied as diets for growing white shrimp, *Litopenaeus vannamei* larvae. The experiment was divided into three experimental sets, each with three replicates. The first set of experiments was the control, which was the culture fed with commercial shrimp feed (Manee Samut Farm Co., Ltd., Songkhla, Thailand) (Table S2: composition of commercial feed). The second experimental set was the culture fed with dried microalgal power. The third experimental set was the culture fed with living microalgal cells at 10^8 cells/ml. To prepare the water for shrimp larvae culture, distilled water is sterilized by filtering through a 20-micron double nylon mesh and then adding BLUE OCEAN brand artificial seawater free of nitrates, nitrites, and phosphates. The composition of simulated seawater in 1 l was: salinity 1.5%, magnesium 670 ppm, potassium 205 ppm, and calcium 220 ppm [17].

The white shrimp larvae were reared in plastic tanks containing water that air-aerated with a rate of 0.05 vvm for 5 days to allow the shrimp larvae to acclimatize. The initial dissolved oxygen (DO) was 7 mg/l. The similar-sized shrimp larvae were selected and randomly weighed before starting the experiment. Twenty-five shrimp were cultured in 5-l plastic tanks for each experiment. Every 2 days, the sediment in the first and second experimental sets was removed and the water was 20% replaced with new water, while in the third experiment, there was much less sediment and there was no need to replace water throughout the 20 days of cultivation. In the first and second experiment sets, the feed was fed twice: at 8:00 a.m. and 6:00 p.m. The amount of feeding was set at 20% of the initial shrimp weight. The initial and final shrimp weight and shrimp number were measured and used to calculate

survival and growth rates. The color of the shrimp was measured by boiling and then measured with a color meter [17]. During shrimp larvae cultivation, the pH, DO, nitrates, nitrites, phosphorus, and chemical oxygen demand (COD) were analyzed.

2.7. Analytical Methods

The microalgal growth was monitored by measuring spectrophotometrically at 660 nm. The concentration of microalgal cells was measured using a hemocytometer. The pH change during microalgae cultivation was analyzed using a pH meter (F20 FiveEasy, Mettler Toledo, Thailand). DO was monitored by DO meter (EC900 Model, AMTAST, USA). The microalgal biomass was recovered by centrifugation at 8,000 rpm for 15 minutes, washed twice, and dried at 60°C until constant weight [16]. The microalgal lipids were extracted using a combination of chloroform and methanol solvents at a volumetric ratio of 2:1. After sonication for 30 minutes, the mixture was centrifuged at 8,000 rpm for 15 minutes to recover liquid fraction. The microalgal biomass was extracted twice. The extracted lipids after evaporation of the solvents were weighted. The chlorophylls and carotenoid contents were extracted from fresh microalgae cells by using acetone and the extracted pigments were spectrophotometrically and calculated following the previous report [18]. The antioxidant activity in terms of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was measured following the methods of Choochote *et al*. [19]. The carbohydrate and protein contents in the microalgal biomass were measured using proximate analysis and the total Kjeldahl Nitrogen method, respectively [20]. The water samples were characterized by COD, nitrates (NO_2^-), nitrites (NO_3^-), and phosphorus (PO_4^{3-}) [21].

To analyze the fatty acid compositions, the extracted lipids were hydrolyzed and esterified into fatty acid methyl esters (FAME) [22]. The FAME compositions were then determined using gas chromatography. A cross-linked capillary FFAP column (0.25 µm film thickness, 0.32 mm I.D., and 30 m length) and a flame ionization detector were equipped. The inlet temperature and detector temperature were set at 290°C and 300°C, respectively. The oven temperature was set at 210°C and held for 12 minutes before increased at 20°C/minute rate, and then held at 250°C for 8 minutes. Each type of FAMEs was quantified by comparing peak area with those of the standards. The fatty acid compositions and empirical equations were utilized to ascertain the fuel properties of the microalgae-derived biodiesel [18].

The experiments were conducted in triplicates. The means and standard deviations of the experimental data were calculated. One-way Analysis of Variance (ANOVA) was used to evaluate the statistical significance of the experimental data.

3. RESULTS AND DISCUSSION

3.1. Selection of Microalgae With High Pigment Contents

Figure 1 shows the results of the growth and pigment contents of three microalgal strains, including *Chlorella* sp. SHP, *Chlamydomonas* sp. WP1, and *Scenedesmus* sp. WP2 in modified Chu 13 medium. As shown in Figure 1a, the increase in OD represents the growth of microalgae. Among three microalgal strains screened, the OD values of *Chlamydomonas* sp. WP1 and *Scenedesmus* sp. WP2 increased faster than that of *Chlorella* sp. SHP. The initial culture pH was 8–9. The pH increased to 10–11 after the first day of cultivation (Fig. 1b). This might be due to the rapid use of carbonate (HCO_3^-) in the photosynthesis process. It should be noted that the microalgae could grow in the wide pH range of 6–11 [23]. The slight decrease in pH after day 4 might be due to the cell lysis and the components within the cells released into the medium [24]. The final microalgal biomass of three microalgal strains was in the range of 0.62–0.71 g/l (Fig. 1c), which were not significantly different ($p > 0.05$). When comparing the lipid content, *Scenedesmus* sp. WP2 yielded the highest lipid content of 37.94% ± 1.08% followed by *Chlamydomonas* sp. WP1 (34.34% ± 1.29%) and *Chlorella* sp. SHP (30.35%± 0.75%). The protein content of *Scenedesmus* sp. WP2 (32.65% ± 0.10%) was also higher than those of *Chlorella* sp. SHP (30.59% ± 0.40%) and *Chlamydomonas* sp. WP1 (28.09% ± 0.20%). The lower protein and lipid content often indicated the higher carbohydrate content of the microalgal biomass.

Figure 1d shows the pigment contents in three microalgal strains. The chlorophylls ab and carotenoid contents of *Chlorella* sp. SHP were 11.14 ± 1.15 and 4.89 ± 0.21 mg/g, respectively. These values were higher than those of the other two strains. The radical scavenging activity (%) of three microalgal strains was analyzed using the DPPH radical scavenging assay. *Chlorella* sp. SHP showed DPPH radical scavenging activity as high as 32.34% ± 0.17%, which was much higher than those of the other two strains. Safafar *et al.* [25] also reported that *Chlorella* sp. had high radical scavenging activity in the range of 28%–34%. Similarly, Choochote *et al.* [19] who studied the antioxidant activity of *Chlorella* sp. E53 and *Chlorella* sp. ED53, also found that the radical scavenging activity of these two microalgae was as high as 54%–58%. It was possible that the antioxidant activity of the microalgae might relate to their high pigment contents [26]. The pigment contents of *Chlorella* sp. SHP in this study were comparable to previously reported values [27]. As *Chlorella* sp. SHP showed comparable high final biomass and the highest pigment contents and antioxidant activity, it was then selected for further experiments.

3.2. Use of Inorganic Fertilizer as Low-Cost Medium for Cultivation of Microalgae

In this study, the low-cost inorganic fertilizer was used as the sole nutrient source for the cultivation of the selected *Chlorella* sp. SHP. The effect of the NPK ratio was studied based on an equal amount of nitrogen concentration of 1 g/l with different ratios of phosphorus and potassium by mixing commercial fertilizers. The effects of NPK ratio on cell growth and composition of microalgal biomass are shown in Figure 2 and Figure S1. It was found that the ratio of NPK at 1:0:0, which contains only nitrogen, gave the lowest growth of microalgae (Fig. 2a). The microalgae grew faster when phosphorus and potassium were added to obtain an NPK ratio of 1:0.25:0.25. However, the increased amount of phosphorus and potassium up to 1:0.5:0.5 and 1:1:1 did not further significantly promote the microalgal growth. The pH change during the first day was in the range of 7–8 and then increased to 9–10 after 3 days of cultivation. Figure 2c shows the biomass, lipid, and protein contents of *Chlorella* sp. SHP under different ratios of NPK. The NPK ratios of 1:0.25:0.25, 1:0.5:0.5, and 1:1:1 gave relatively high biomass of 0.7–0.8 g/l. It should be noted that the NPK ratio of 1:0:0 gave the lowest biomass but with the highest lipid content of 37% ± 6.20%. It has been reported that the limited or lack of phosphorus and potassium restricted the microalgal growth but increased lipid accumulation [28].

It was reported that suitable NPK ratios depend on the microalgal strains. Kumari *et al*. [29] reported that the microalga *Spirulina platensis* grown on fertilizer with NPK ratio of 10:26:26 at 0.76 g/l added with 10 g/l sodium bicarbonate, 1 g/l sodium chloride, and micronutrients (1.0 ml/l), gave the biomass of 1.22 g/l, chlorophylls of 8.92 g/l, protein, and lipid contents of 52.35% and 14.84%, respectively. *Scenedesmus* sp. IMMTCC-6 could also grow well on medium added

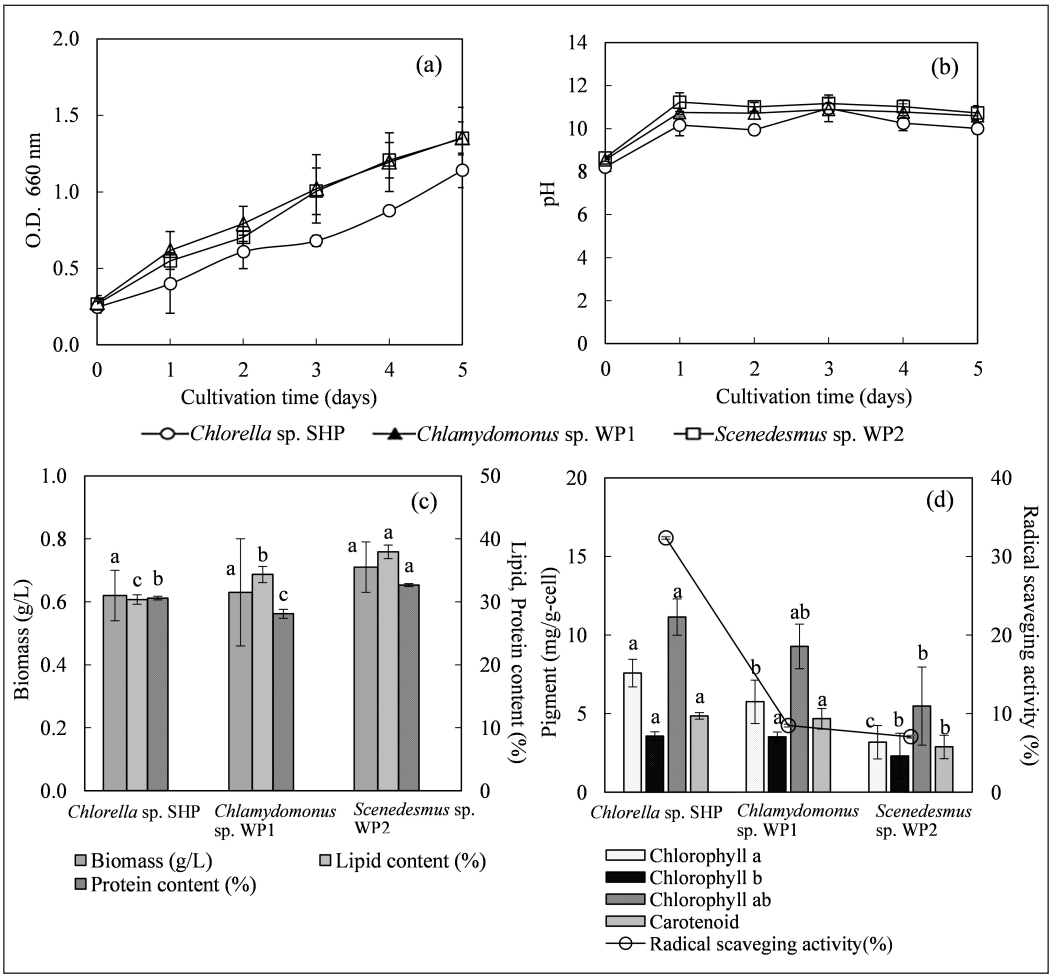

Figure 1. Screening microalgal strains cultivated in modified Chu 13 medium. Time courses of growth (a), pH (b), composition of biomass, lipid and protein (c) and pigment contents and radical scavenging activity (d) of microalgae strains. Different letters on the bars indicate significant differences between microalgae.

with 0.1 g/l urea and 1.0 g/l NPK fertilizer (0.146:0.26:0.26) giving the biomass productivity of 50 mg/l.day [8]. Mahmood and Khudhair [11] found that *Chlorella vulgaris* grew well on NPK fertilizer giving biomass of 0.33 g/l which was higher than that grown on synthetic Chu 10 medium (0.21 g/l). It was suggested that the composition of NPK fertilizer was more suitable for microalgae growth than the Chu 10 medium. In this study, the NPK ratio of 1:0.25:0.25 was found most suitable for biomass and pigment production by *Chlorella* sp. SHP. Therefore, the NPK ratio of 1:0.25:0.25 was selected for further optimization of culture conditions.

3.3. Optimization of Culture Conditions Through RSM

3.3.1. Statistical analysis

The independent variables include nitrogen concentrations of 0.25, 1.00, and 1.75 g/l (A), salt concentrations of 0%, 1%, and 2% (B), and light intensity of 3,000, 5,000, and 7,000 lux (C). Their values were coded at three levels of −1, 0, and +1 and their combinations were designed using BBD experimental design (Tables 1 and 2). The experimental responses were: biomass (g/l; Y_1), chlorophyll ab (mg/g-cell; Y_2), carotenoids (mg/g-cell; Y_3), lipid content (%; Y_4), and protein content (%; Y_5). All responses were fitted with the second-order polynomial quadratic Equations (2–6) as follows:

$$Y_1 = 0.78+0.25A-0.012B-0.074C+0.11A^2- 0.066B^2+0.061C^2-0.016AB-0.13AC-0.029BC \quad (2)$$

$$Y_2 = 32.44+4.56A-3.25B-1.04C+4.82A^2-1.05B^2-17.60C^2-6.74AB-2.07AC+1.78BC \quad (3)$$

$$Y_3 = 9.88+1.94A-1.05B-1.00C+1.28A^2-0.72B^2-3.90C^2-4.09AB-1.27AC+0.45BC \quad (4)$$

$$Y_4 = 37.93-8.33A+0.41B+2.55C-8.86A^2-5.09B^2-0.46C^2-0.88AB+7.03AC+1.28BC \quad (5)$$

$$Y_5 = 52.70-4.79A-2.67B+5.81C-9.65A^2-11.38B^2-5.80C^2-6.89AB+6.40AC+0.48BC \quad (6)$$

As depicted in Tables 3 and 4, the correlation coefficients (R^2) of the regression equations were 0.87, 0.88, 0.95, 0.87, and 0.99, respectively. This indicates that up to 87%, 88%, 95%, 87%, and 99% of the response variations can be described by the models. The relatively high values of the adjusted determination coefficients (adjust R^2) and the Fisher's *F*-test with $p \leq 0.0001$ also indicated the significance of the models. The coefficient value (C.V.) less than 20 indicated the reliability of the experimental results. The lack of fit with $p > 0.05$ suggested that the pure error and the residual error from the replicates were not significantly different. The relationships between the operating factors and the responses could be accurately described by these quadratic

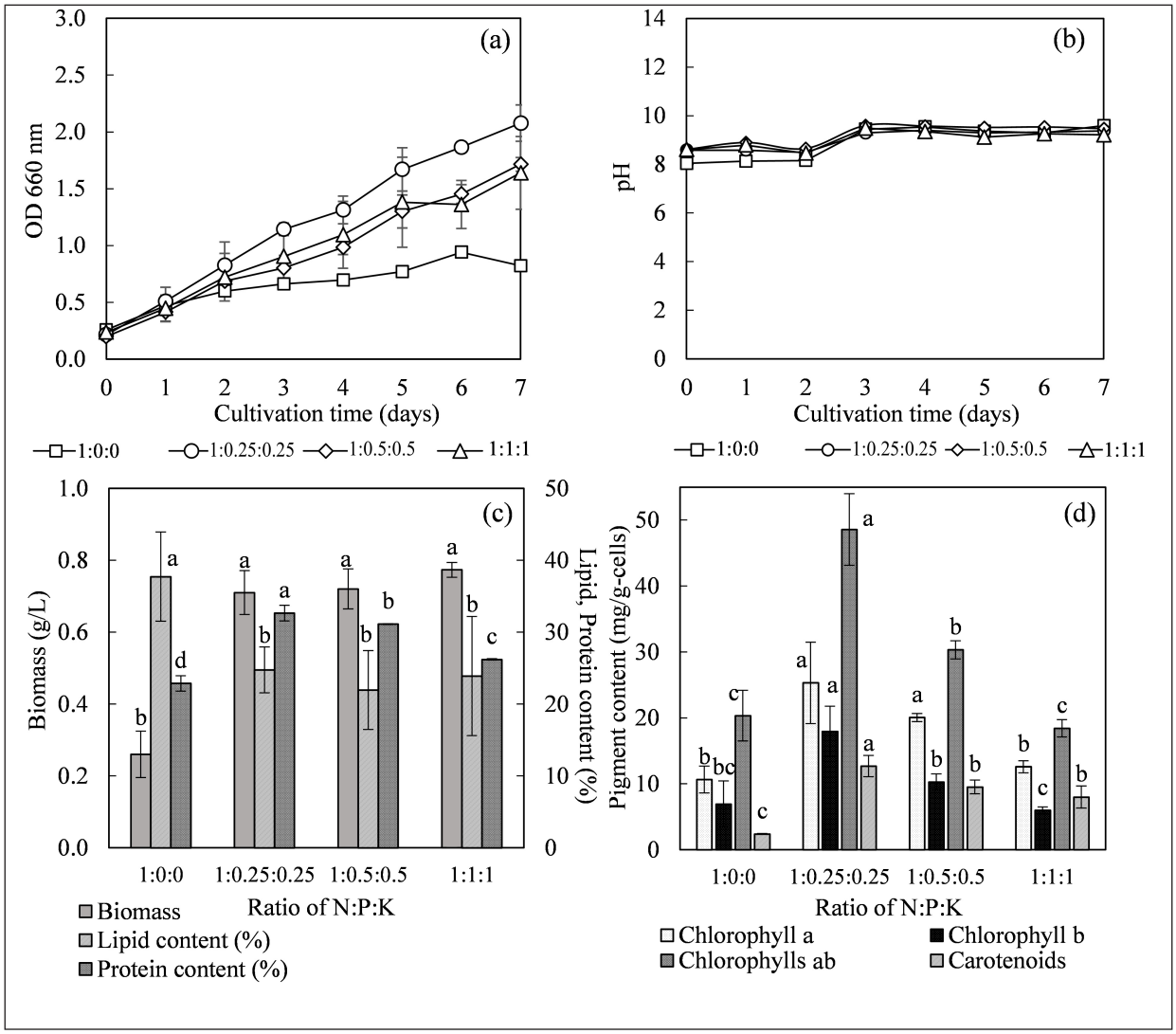

Figure 2. Optimization of NPK fertilizer for cultivation of *Chlorella* sp. SHP. Time courses of growth (a), pH (b), biomass, lipid and protein content (c) and pigment contents as chlorophylls and carotenoids (d). Different letters on the bars indicate significant differences between microalgae.

equations. Additionally, the individual and interaction effects of the operating factors on the responses could be explored.

3.3.2. Effect on microalgal biomass

Figure 3a–c shows the contour plots and three-dimensional (3D) plots based on Equation (2), in which one factor was set constant at the center point and the other two factors varied within the ranges tested. Through response surface analysis, the optimum levels of operating factors can be calculated to maximize the target responses. The significances of the model terms were evaluated by the probability values (p values) listed in Table 3. From Table 3 and Figure 3b, it can be observed that the significant operating factors for microalgal biomass were nitrogen concentration (A) and light intensity (C). The biomass increased when the nitrogen concentration was increased to the maximum level. As nitrogen concentration is a fundamental nutrient for protein synthesis, higher nitrogen concentration leads to faster cell growth and higher biomass [29]. Light intensity affects the photosynthetic and metabolic activities and the production of various components within the cells [30–32]. At an insufficient level of light intensity, the microalgal growth is limited, but at a light intensity beyond the saturation level, photoinhibition would occur [33]. In this study, the optimum light intensity was found at 3,000 lux. The light intensity above this level decreased biomass production (Fig. 3b). The biomass production slightly improved with increasing salt concentration up to 1%. Although the salt concentration was increased to 2%, no obvious growth inhibition occurred, indicating the halotolerant property of this strain (Fig. 3c). By solving the derivatives of Equation (2), the optimum conditions could be calculated to obtain the maximum biomass production. Those were a nitrogen concentration of 1.75 g/l, salt concentration of 1%, and light intensity of 3,000 lux.

3.3.3. Effect on pigment contents

The experiment results show that the contents of chlorophylls and carotenoids of *Chlorella* sp. SHP ranged from 10–50 and 4–18 mg/g-cell, respectively (Table 1). Table 3 shows that the linear terms of operating factor and the quadratic term of light intensity (C^2) significantly affected the chlorophyll and carotenoid contents ($p < 0.05$). Figure 3d–i shows the contour plots and 3D plots based on Equations (3, 4). The chlorophyll and carotenoid contents increased when nitrogen concentration and light intensity were

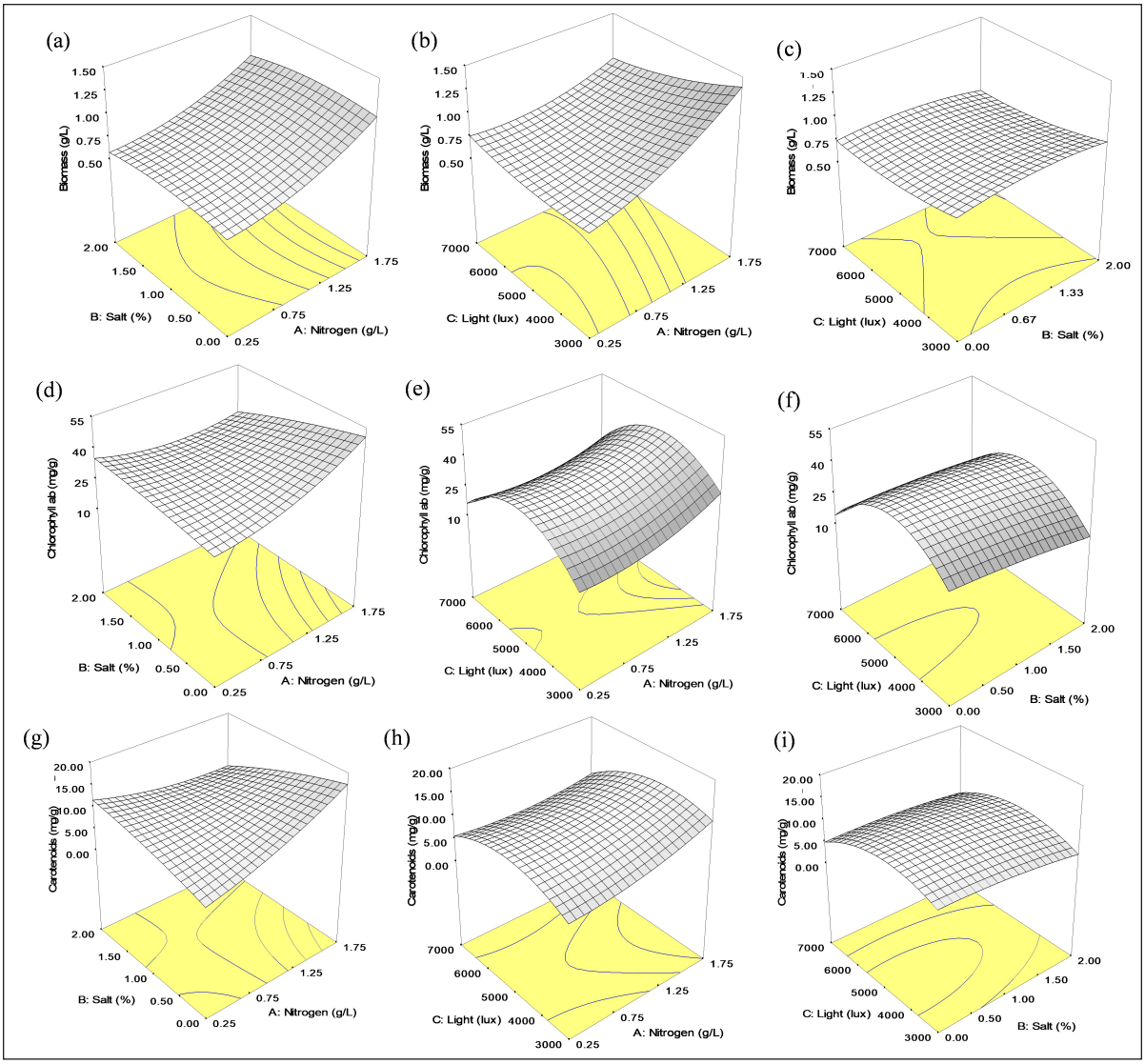

Figure 3. 3D surface plots showing effects of nitrogen and salt concentrations and light intensity on biomass production (a–c), chlorophyll ab (d–f) and carotenoid contents (g–i) of *Chlorella* sp. SHP.

increased up to 1.75 g/l and 5,000 lux, respectively. While salt concentration had negative effect on the pigment content. Nitrogen is an important component for chlorophyll formation that is bound to the magnesium molecule and affects the synthesis of protein chloroplasts (chloroplastic proteins), which are reactive proteins in photosystem I and photosystem II [34,35]. The chlorophyll and carotenoid contents were as low as 5–12 mg/g-cell when using low concentrations of nitrogen (Runs 3, 5, and 7), while the high chlorophyll and carotenoid content (>18 mg/g-cell) was obtained when using high nitrogen concentrations (Runs 2, 4, 6, and 8).

In addition, the light intensity is also one of the key factors that stimulate the production of pigments. Chlorophylls and carotenoids are constituents of light-harvesting complexes involving in light reaction of photosynthesis and contribute in electron transport chains. During the photosynthesis reaction, with light energy and water, carbon dioxide is converted to geranylgeranyl pyrophosphate (geranylgeranyl-PP), which is then reduced to form phytyl pyrophosphate (phytyl-PP). Phytyl-PP is attached to the derivatives of protoporphyrin IX which contain magnesium ions, and these complexes lead to the formation of chlorophylls. In addition, geranylgeranyl-PP is also a carotenoids precursor [36]. As a result, when both nitrogen concentration and light intensity were increased, the chlorophyll and carotenoid contents also increased.

The calculated optimal conditions for maximizing chlorophyll and carotenoid contents were: nitrogen concentration of 1.75 g/l and moderately high light intensity of 5,000 lux without the addition of salt. The positive effect of high light intensity on pigment content in the microalgae has been reported likely due to the photoprotective function of carotenoids to stabilize light-harvesting. However, when light energy exceeds the photoadaptive capacity of the microalgae it will cause photooxidative damage and the pigment contents drastically decrease [18]. It should be noted that under these conditions, the microalgal biomass obtained was only 1.18 g/l likely due to the photoinhibition effect on the microalgal growth.

3.3.4. Effect on lipid content

Table 4 presents that the linear and quadratic terms of nitrogen concentration (A) and (A^2) significantly affected lipid content (p <0.05). The contour plots and 3D plots based on Equation (5) are

Table 1. Experimental design and results of biomass, chlorophylls and carotenoids of selected *Chlorella* sp. SHP.

Run	A: Nitrogen concentration (g/l)	B: Salt concentration (%)	C: Light intensity (lux)	Biomass (g/l)		Chlorophylls (mg/g)		Carotenoids (mg/g)	
				Actual value	Predicted value	Actual value	Predicted value	Actual value	Predicted value
1	−1 (0.25)	−1 (0)	0 (5,000)	0.65	0.56	28.34	28.14	5.78	5.47
2	1 (1.75)	−1 (0)	0 (5,000)	1.18	1.10	52.56	50.76	18.41	17.54
3	−1 (0.25)	1 (2)	0 (5,000)	0.49	0.57	33.34	35.14	10.67	11.54
4	1 (1.75)	1 (2)	0 (5,000)	0.96	1.04	30.58	30.78	6.94	7.25
5	−1 (0.25)	0 (1)	−1 (3,000)	0.69	0.64	12.85	14.06	5.44	5.06
6	1 (1.75)	0 (1)	−1 (3,000)	1.45	1.40	24.51	27.32	11.29	11.48
7	−1 (0.25)	0 (1)	1 (7,000)	0.70	0.75	18.94	16.13	5.78	5.59
8	1 (1.75)	0 (1)	1 (7,000)	0.94	0.99	22.32	21.11	6.56	6.94
9	0 (1)	−1 (0)	−1 (3,000)	0.69	0.83	20.85	19.84	7.08	7.77
10	0 (1)	1 (2)	−1 (3,000)	0.89	0.86	12.80	9.79	5.26	4.77
11	0 (1)	−1 (0)	1 (7,000)	0.71	0.74	11.20	14.21	4.37	4.87
12	0 (1)	1 (2)	1 (7,000)	0.79	0.66	10.27	11.28	4.35	3.66
13	0 (1)	0 (1)	0 (5,000)	0.78	0.78	27.78	32.44	9.05	9.88
14	0 (1)	0 (1)	0 (5,000)	0.87	0.78	43.31	32.44	11.81	9.88
15	0 (1)	0 (1)	0 (5,000)	0.78	0.78	27.78	32.44	9.05	9.88
16	0 (1)	0 (1)	0 (5,000)	0.80	0.78	35.54	32.44	10.43	9.88
17	0 (1)	0 (1)	0 (5,000)	0.65	0.78	27.78	32.44	9.05	9.88

shown in Figure 4. Figure 4a presents the effect of nitrogen (A) and salt (B) concentrations on lipid content. The low nitrogen concentration and the presence of salt did increase the lipid content, even though they limited microalgal growth. This was likely because, under unfavorable conditions for cell growth, protein synthesis was limited, and the photosynthetic products, i.e., glucose and starch, were then utilized to synthesize lipids through the Kennedy pathway [31]. Another possible pathway would be that with low nitrogen concentration, the cellular content of thylakoid membrane is likely to decline, and the acyl hydrolase enzyme that hydrolyzes phospholipid is activated. These lead to the high amount of the lipid precursor in β-oxidation, fatty acid acyl-CoA, and consequently increased lipid synthesis [37]. Figure 4b depicts the interaction effect of nitrogen concentration (A) and light intensity (C) on the lipid content. At 1% salt concentration, the impact of light intensity on lipid content was more obvious when compared to that of nitrogen concentration.

Figure 4c depicts the interaction effect between salt concentration (B) and light intensity (C) on the lipid content. When nitrogen was set at an optimal level for lipid accumulation (0.75 g/l), the highest lipid content could be obtained when using moderate salt concentration (1%) and a relatively high light intensity (5,000 lux). The optimal light intensity for lipid content was the same for pigment content. It has been revealed that salt stress can increase the lipid production of microalgae. The addition of high salt might induce oxidative stress and cause microalgae to produce more lipids in order to act as an osmoprotectant and help them survive salt stress [18,38]. Notably, the optimal conditions for microalgal growth and lipid accumulation are normally different. The culture stresses restricted cell growth and limited biomass productivity but increased lipid accumulation [18].

3.3.5. Effect on protein content

The protein content ranged from 18.83% to 53.76% (Table 2). Table 4 shows that the nitrogen concentration (A), salt concentration (B), and light intensity (C) significantly affected protein content ($p < 0.05$). The quadratic terms of nitrogen (A^2), salt (B^2) concentration, and light intensity (C^2) were also significant for the protein content ($p < 0.05$). The contour plots and 3D plots based on Equation (6) are shown in Figure 4d–f. Figure 4d shows the interaction effect between nitrogen (A) and salt (B) concentrations on protein content. The protein content increased with a moderate salt concentration of 1%. The dissociation of salt into sodium and chloride ions causes osmotic stress and stimulates photosynthetic organisms to synthesize low molecular mass-compatible solutes or osmolytes such as protein, proline, glycine, sorbitol, and so on, to reduce the effect of salt stress [39]. Figure 4e reveals the interaction effect of nitrogen concentration (A) and light intensity (C) on protein content. Figure 4f reveals the interaction effect of salt concentration (B) and light intensity (C). High nitrogen concentration coupled with high light intensity, improved the protein content. The optimal conditions for maximizing the protein content of *Chlorella* sp. SHP were: moderate nitrogen concentration of 1 g/l, salt concentration of 1%, and relatively high light intensity of 5,000 lux. Noteworthy, the light intensity affected protein content more obviously than the salt concentration did.

3.3.6. Optimal conditions for biomass and pigment production

Based on the preliminary experimental findings, Design-Expert statistical software was employed to optimize the culture conditions. The optimum conditions for the production of various substances are shown in Table S1. A high nitrogen concentration of 1.75 g/l was found to be most suitable for the production of microalgal cells and pigment contents, while a low nitrogen concentration was more suitable for lipid production. The salt concentration of 1% was suitable for the production of microalgal growth, lipids, and proteins. The use of a relatively high light intensity of 5,000 lux enhanced the production of pigments, lipids, and proteins but suppressed microalgal growth. Therefore, the optimal conditions for microalgal growth were selected, which were 1.75 g/l nitrogen concentration, 1% salt concentration, and 3,000 lux light intensity. Under these conditions, the maximum

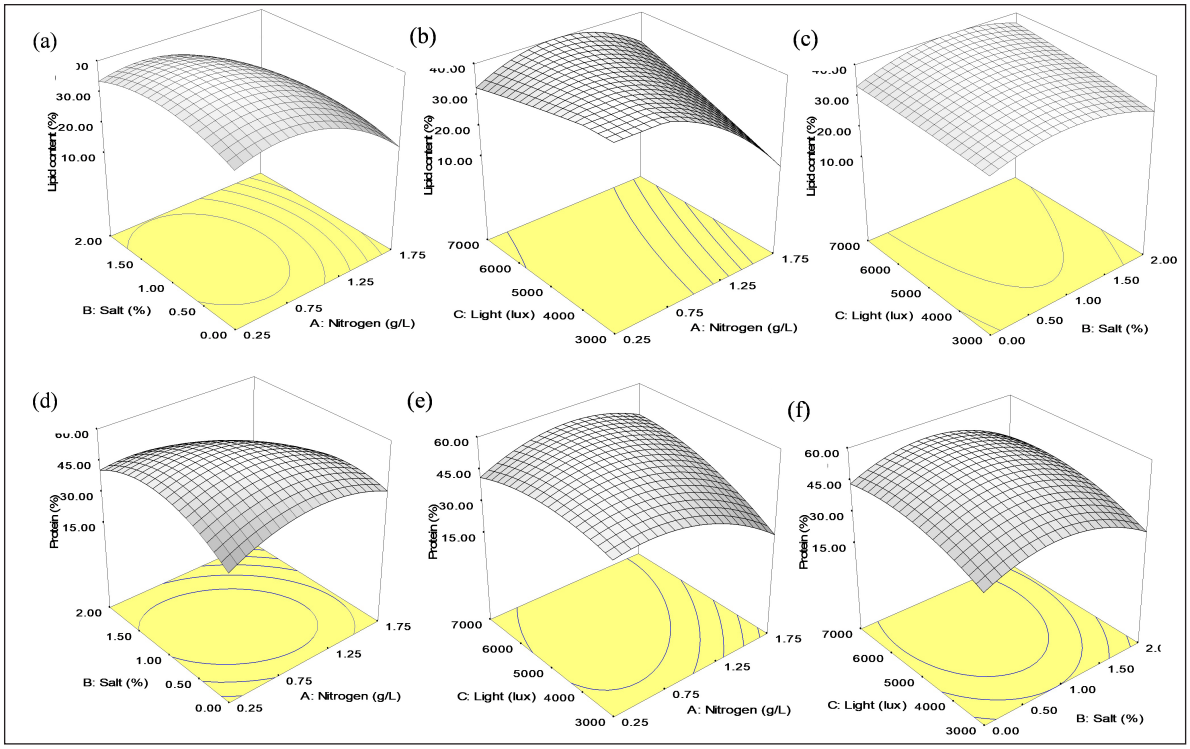

Figure 4. 3D surface plots showing effects of nitrogen and salt concentrations and light intensity on lipid (a–c) and protein contents (d–f) of *Chlorella* sp. SHP.

Table 2. Experimental design and results of lipid and protein contents of *Chlorella* sp. SHP.

Run	A: Nitrogen concentration (g/L)	B: Salt concentration (%)	C: Light intensity (lux)	Lipid (%)		Protein (%)	
				Actual value	Predicted value	Actual value	Predicted value
1	−1 (0.25)	−1 (0)	0 (5,000)	29.03	31.02	30.68	32.25
2	1 (1.75)	−1 (0)	0 (5,000)	16.09	16.12	37.30	36.43
3	−1 (0.25)	1 (2)	0 (5,000)	33.63	33.60	39.83	40.69
4	1 (1.75)	1 (2)	0 (5,000)	17.17	15.18	18.90	17.33
5	−1 (0.25)	0 (1)	−1 (3,000)	44.78	41.42	43.66	42.65
6	1 (1.75)	0 (1)	−1 (3,000)	12.10	10.71	18.83	20.25
7	−1 (0.25)	0 (1)	1 (7,000)	31.08	32.47	42.87	41.45
8	1 (1.75)	0 (1)	1 (7,000)	26.51	29.86	43.66	44.67
9	0 (1)	−1 (0)	−1 (3,000)	29.33	30.70	33.42	32.86
10	0 (1)	1 (2)	−1 (3,000)	25.58	28.96	26.41	26.56
11	0 (1)	−1 (0)	1 (7,000)	36.62	33.24	43.66	43.51
12	0 (1)	1 (2)	1 (7,000)	37.97	36.61	38.58	39.14
13	0 (1)	0 (1)	0 (5,000)	35.29	37.93	52.00	52.70
14	0 (1)	0 (1)	0 (5,000)	35.29	37.93	52.00	52.70
15	0 (1)	0 (1)	0 (5,000)	35.29	37.93	53.76	52.70
16	0 (1)	0 (1)	0 (5,000)	35.29	37.93	53.76	52.70
17	0 (1)	0 (1)	0 (5,000)	48.48	37.93	52.00	52.70

microalgal growth would be obtained along with acceptable contents of pigments, lipids, and proteins.

3.4. Scale Up in an 8 l-Photobioreactor and Effect of Aeration Rate

The culture of microalga *Chlorella* sp. SHP was cultured in an 8-l photobioreactor using the optimum conditions from the previous section. The suitable aeration rate defined as the gas fraction within the entire liquid volume is a key characteristic for aerated culture systems as it impacts mass transfer and liquid circulation velocity. The aeration rate was varied at 0.02, 0.1, 0.3, and 0.5 vvm (Fig. 5 and Fig. S2). The OD and pH slightly increased with increasing aeration rates. The aeration rate of 0.1–0.3 vvm gave indifferent biomass productions, which were in the range of 1.7–

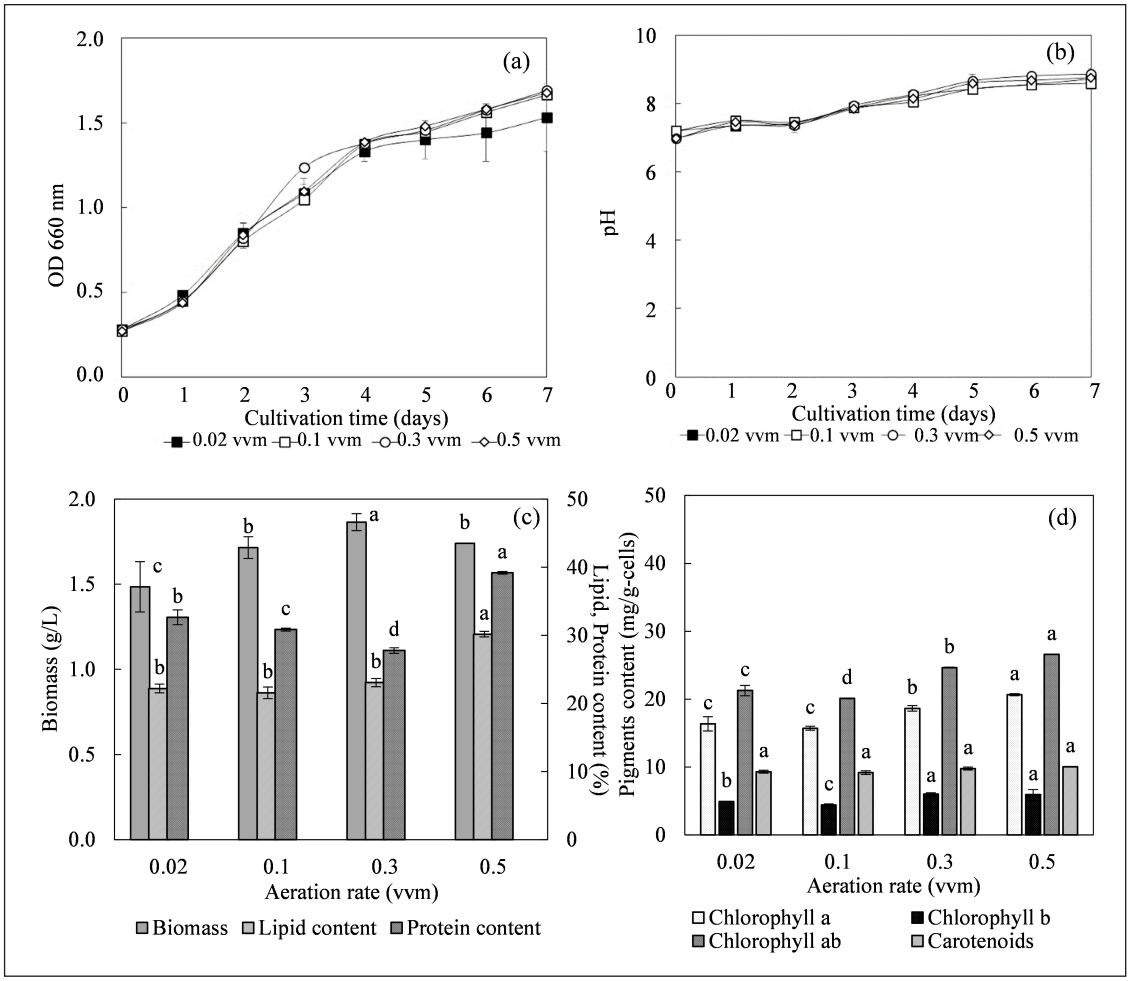

Figure 5. Effects of aeration rate on growth (a), pH (b), biomass, lipid and protein contents (c), and pigment content (d) of *Chlorella* sp. SHP cultivated in an 8-l photobioreactors.

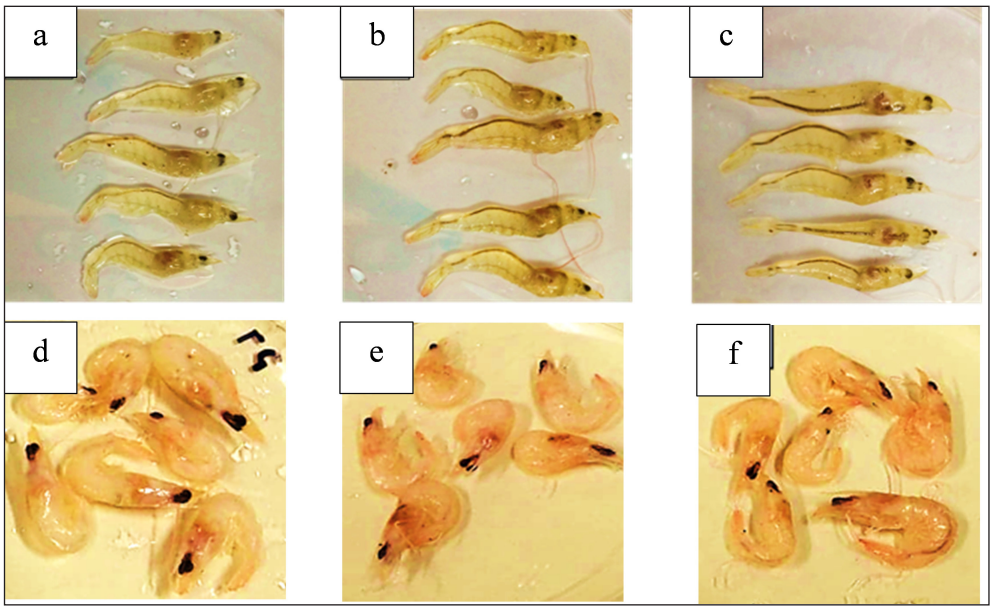

Figure 6. White shrimp *L. vannamei* after cultivation for 20 days in different experimental treatments; (a): feeding with commercial feed; (b): feeding with dried *Chlorella* sp. SHP biomass; (c): feeding with living *Chlorella* sp. SHP cells; (d): cooked shrimp those fed with commercial feed: (e): cooked shrimp those fed with dried *Chlorella* sp. SHP biomass; (f): cooked shrimp those fed with living *Chlorella* sp. SHP cells.

Table 3. Model coefficient and analysis of response variance of biomass production, chlorophylls and carotenoids content estimated by ANOVA.

Source	Biomass production		Chlorophyll content		Carotenoid content	
	Coefficient	*p*-value	Coefficient	*p*-value	Coefficient	*p*-value
Model intercept	0.63	<0.0203	81.76	<0.0135	−20.49	<0.0008
A	0.39	0.0023	4.83	0.0627	7.69	0.0022
B	0.21	0.9785	3.40	0.1598	4.70	0.0386
C	−0.00009	0.0204	0.0439	0.6313	0.0098	0.0462
A^2	0.19356	0.0124	8.57	0.1339	2.28	0.0591
B^2	−0.066125	0.3379	−1.054	0.7219	-0.71	0.2503
C^2	0.000000015	0.0301	−0.0000044	0.0004	−0.00000097	0.0002
AB	−0.021667	0.8403	−8.99	0.0541	−5.45	0.0002
AC	−0.000085	0.1414	−0.0013	0.5010	−0.00084	0.0673
BC	−0.000014	0.7222	0.00089	0.5611	0.00022	0.4665
R^2	0.87	-	0.88	-	0.95	-
Adjusted R^2	0.71	-	0.74	-	0.89	-
C.V.	14.50	-	20.51	-	14.09	-
Lack of fit	-	0.1091	-	0.8156	-	0.5684

Table 4. Model coefficient and analysis of response variance of lipid content and protein content estimated by ANOVA.

Source	Lipid content		Protein content	
	Coefficient	*p*-value	Coefficient	*p*-value
Model intercept	4.99	<0.0228	94.86	< 0.0001
A	19.44	0.0031	76.22	< 0.0001
B	0.047	0.8346	23.59	0.0018
C	1.82	0.2193	111.79	< 0.0001
A^2	11.56	0.0114	162.38	< 0.0001
B^2	3.83	0.0913	226.14	< 0.0001
C^2	0.031	0.8649	58.79	0.0001
AB	0.11	0.7520	78.60	< 0.0001
AC	6.91	0.0339	68.02	< 0.0001
BC	0.23	0.6473	0.39	0.5541
R^2	0.87	-	0.99	-
Adjusted R^2	0.69	-	0.98	-
C.V.	17.16	-	3.88	-
Lack of fit	-	0.6570	-	0.0839

Table 5. Fatty acid composition of lipids from *Chlorella* sp. SHP.

Fatty acid compositions	Composition (%)
Capric acid (C10:0)	9.12 ± 3.54
Tridecanoic acid (C13:0)	6.02 ± 0.88
Myristic acid (C14:0)	9.91 ± 2.04
Palmitic acid (C16:0)	43.08 ± 4.40
Stearic acid (C18:0)	9.96 ± 0.21
Oleic acid (C18:1)	5.94 ± 0.95
Linoleic acid (C18:2)	14.09 ± 1.62
Linolenic acid (C18:3)	3.77 ± 0.00

1.8 g/l. It should be noted that the aeration at 0.5 vvm gave the microalgal biomass with the highest contents of chlorophyll ab and carotenoid at 26.58 ± 0.62 and 10.03 ± 0.60 mg/g-cell, respectively.

Table 6. Growth performance, color of cooked, and water quality parameters of white shrimp *L. vannamei* after cultivation for 20 days.

Parameters	Experimental set		
	Control	Dried microalgal cells	Living microalgal cells
Weight (g)	0.38 ± 0.06^a	0.35 ± 0.08^a	0.38 ± 0.11^a
Size (cm)	4.06 ± 0.28^a	3.71 ± 0.57^a	3.83 ± 0.49^a
Survival rate (%)	96 ± 1.73^a	96 ± 1.00^a	100 ± 0.00^a
L*	56.3 ± 1.45^a	46.97 ± 1.69^b	46.85 ± 1.34^b
A*	3.88 ± 0.65^b	3.83 ± 0.96^b	5.05 ± 0.19^a
B*	10.29 ± 0.72^a	11.15 ± 0.50^a	11.83 ± 0.51^a
DO (mg/l)	6.33 ± 0.06^b	6.37 ± 0.06^b	7.07 ± 0.12^a
pH	7.71 ± 0.06^a	7.74 ± 0.02^a	7.83 ± 0.05^a
Temperature (°C)	28.6 ± 0.00^a	28.6 ± 0.00^a	28.6 ± 0.00^a
NO_2^- (mg/l)	0.39 ± 0.01^a	0.41 ± 0.01^a	0.23 ± 0.01^b
NO_3^- (mg/l)	2.3 ± 0.14^{ab}	3.7 ± 0.03^a	1 ± 0.00^b
PO_4^{3-} (mg/l)	3.134 ± 0.002^a	3.302 ± 0.004^a	1.536 ± 0.004^b
COD (mg/l)	100 ± 1.41^b	120 ± 0.00^a	108 ± 2.12^b

Control: the shrimp culture fed with commercial shrimp feed.
Data are average (n = 10). Different superscript letter in the same column indicate significant difference between treatments (p < 0.05).

This might be because an increasing aeration rate increases the available amount of carbon dioxide in the liquid phase and hence promotes the photosynthetic activity that relates to the production of pigments [40,41]. In addition, increasing the aeration rate also improved the mass transfer rate and allowed the microalgae to assimilate more nutrients and absorb more light, thereby enhancing the microalgal growth. Table 5 shows the profiles of fatty acids found in the microalgae cultivated using aeration rate of 0.1 vvm. The microalgal lipids contained mainly long-chain fatty acids including palmitic acid (43.08% ± 4.40%), linoleic acid (14.09% ± 1.62%), stearic acid (9.96%± 0.21%), oleic acid (5.94% ± 0.95%), and linolenic acid (3.77% ± 0.00%). These fatty acids have been recommended as supplements in the diet of shrimp [42].

3.5. Application of Living Microalgal Cells as Practical Diets for White Shrimp Larvae

The microalgal cells of *Chlorella* sp. SHP with high pigment content from the previous experiment were used as diets for the aquaculture of 15-day-old white shrimp *L. vannamei* larvae in an open system. After raising white shrimp larvae for 20 days, it was found that three feeding strategies did not significantly influence the weight and body length of the shrimp, as shown in Figure 6a–c. The control gave an average shrimp weight of 0.38 ± 0.06 g/shrimp, the dried microalgae powder gave an average weight of 0.35 ± 0.08 g/shrimp, and the living microalgal cells gave an average shrimp weight of 0.38 ± 0.11 g/shrimp. The length of the shrimp in the control was 4.06 ± 0.28 cm/shrimp. The experiment using dried microalgae had an average length of 3.71 ± 0.57 cm/shrimp, and the experiment using living microalgal cells had an average length of 3.83 ± 0.49 cm/shrimp. The survival rates of control and dried microalgae cultures were 96% ± 1.73% and 96% ± 1.00%, respectively. The experiment using living microalgal cells had 100% survival rates, as shown in Table 6. Figure 6d–f shows the color of boiled shrimp. Table 6 also shows the measured color of cooked white shrimp under different experimental treatments. The control experiment had lightness (L*) greater than dried and living microalgal cells ($p < 0.05$) with values of 56.30 ± 1.45, 46.97 ± 1.69, and 46.85 ± 1.34, respectively. When comparing the red values (A*) of the three experiments of shrimp, it was found that the color of shrimp fed with living microalgal cells had an enhanced red value up to 5.05 ± 0.19 when compared to the control (3.88 ± 0.65) and the use of dried microalgae powder (3.83 ± 0.96). While the yellow values (B*) of the three experiments were insignificantly different. These results confirmed that the living microalgal cells could increase the red color of the shrimp.

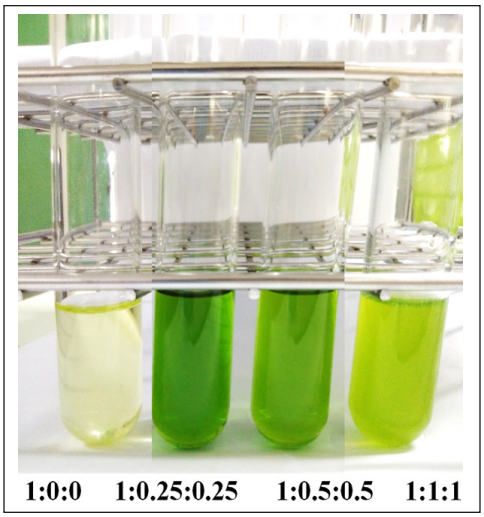

Figure S1. The pigments extracted from *Chlorella* sp. SHP cells cultivated using different ratio of N:P:K fertilizer.

In addition, after culture for 20 days, the water quality was examined for pH, DO, nitrate, nitrite, phosphate, and COD. The experiment using living microalgal cells had higher DO values than the control and the experiment using dried microalgae, which were 7.07 ± 0.12, 6.33 ± 0.06, and 6.37 ± 0.06 mg/l, respectively (Table 6). This was likely due to the ability of living microalgal cells to photosynthesize and produce oxygen. The pH values were insignificantly different ($p > 0.05$). The nitrate, nitrite, and phosphate values of the water obtained from the experiment using living microalgal cells were lower than those of the control and that using dried microalgae powder. It should be noted that the use of living microalgal cells did not show the obvious sediment, and therefore there was no need to remove sediment and replace the culture water. It has been reported that the use of living microalgae for white shrimp aquaculture could reduce the amount of ammonia, nitrate, and nitrite and increase the DO in the shrimp culture water without replacing the water throughout the 84 days of culture [43]. As the culture of white shrimp using the living microalgal cells did not require water replacement throughout the culture period, this reduced the cost of water used for the culture.

The types of feed used as commercial feed are easy to prepare for the aquaculture process, easy to control the amount of feed, and high-nutrient feed that is suitable for larvae shrimp. The disadvantages is that it requires frequent water replacement, is easy to contaminate, and the cost of food is higher than using microalgae. The cultivation of white shrimp larvae using microalgal cells in dried powder has advantages and disadvantages similar to those using commercial feed. But the advantage is that the cost of food is cheaper. On the other hand, the cultivation of white shrimp larvae by using living microalgal cells also has disadvantages, such as that it is quite difficult to control the amount of living microalgal cells. The advantage is that it does not require

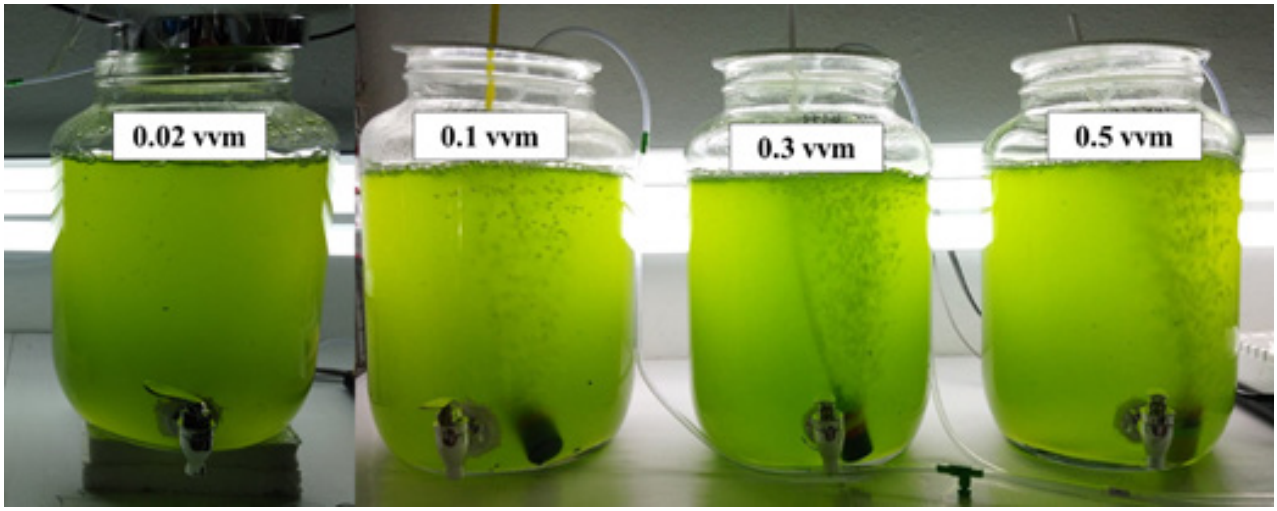

Figure S2. Cultivation of *Chlorella* sp. SHP in 8-l photobioreactors using different aeration rate.

Table S1. Optimal conditions from RSM.

Factors	Optimal condition for			
	Biomass	Pigments	Lipids	Protein
Nutrients concentration (g/l)	1.75	1.75	1	1
Salt concentration (%)	1	0	1	1
Light intensity (lux)	3,000	5,000	5,000	5,000

Table S2. Composition of shrimp commercial feed.

Composition	Amount (%)
Protein	35.69 ± 0.12
Fat	6.59 ± 0.02
Moisture	7.97 ± 0.03
Ash	12.38 ± 0.03
Total carbohydrate	37.38 ± 0.08

changing the water until the process is finished, which could reduce the cost of culture and the risk of contamination. Moreover, this method also increased the amount of oxygen in the water, which reduced the number of pumps required for aeration, thus reducing costs. Moreover, the water quality was also better than that of those using commercial feed and hence required a minimum cost for water treatment. In addition, living microalgal cells also increased the red color of the shrimp.

4. CONCLUSION

Among the microalgal strains screened, *Chlorella* sp. SHP was chosen considering its high biomass and high pigment production. With an optimal NPK ratio of 1:0.25:0.25 and optimized conditions through RSM, the maximum microalgae biomass of 1.45 ± 0.01 g/l was achieved with improved chlorophyll and carotenoid contents up to 24.51 ± 0.03 and 11.29 ± 0.28 mg/g-cell, respectively. The scale-up in an 8-l photobioreactor with the optimal aeration rate at 0.1 vvm did enhance biomass production up to 1.72 ± 0.06 g/l. The use of living microalgal cells as a diet for the cultivation of white shrimp *L. vannamei* larvae, gave shrimp size and weight comparable to those using commercial feed, but with a better red color quality after cooking. Moreover, the use of living microalgal cells also provided higher DO during cultivation and cleaner water quality. This study has shown that NPK fertilizer can be used as a sole nutrient source for low-cost production of living microalgal cells as practical diets for shrimp larvae. These strategies not only reduce the costs of feed and water replacement during cultivation but also maintain a balanced ecological system and minimize waste in the aquaculture sector.

5. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

6. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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8. ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

9. PUBLISHER'S NOTE

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10. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

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