J. Phycol. **56,** 1208–1215 (2020)

DOI: 10.1111/jpy.13007

OPTIMIZATION OF MEDIUM USING RESPONSE SURFACE METHODOLOGY TO ENHANCE THE GROWTH OF *EFFRENIUM VORATUM* (SYMBIODINIACEAE, DINOPHYCEAE)

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Survival of coral reef-associated Symbiodiniaceae is vital to maintain the healthy coral community in coral reefs. However, knowledge about cultivation of free-living or symbiotic Symbiodiniaceae has been limited. In this study, the response surface methodology was applied to optimize the medium for *Effrenium voratum*. The results showed that the impacts of nutrient components on algal growth were: $FeCl_3$> NaH_2PO_4>$MnSO_4$> $MgSO_4/CoSO_4$ > KCl>$ZnSO_4$> $CaCl_2/NaNO_3$, among which NaH_2PO_4 and $FeCl_3$ significantly affected algal growth. The optimal medium was: natural seawater supplemented with $NaH_2PO_4{\cdot}2H_2O$ 0.25 mM,$FeCl_3{\cdot}6H_2O$ 14.24 µM, $NaNO_3$ 0.94 mM, $MgSO_4{\cdot}7H_2O$ 40.63 mM, KCl 5.37 mM, $CaCl_2{\cdot}2H_2O$ 4.08 mM, $ZnSO_4{\cdot}7H_2O$ 0.35 µM, $MnSO_4$ 9.93 µM, and $CoSO_4$ 0.36 µM. The use of the optimized medium resulted in an increase of biomass yield (0.76 g dry weight $\cdot$ L^{-1}) by 46% over that using the initial medium, which agreed with the predicted value (0.71 g $\cdot$ L^{-1}). Additionally, fatty acids, mainly consisting of palmitic acid (C16:0) and ethyl carbonate (C20:0), accounted for approximately 50% of the total fatty acids in *E. voratum*. Interestingly, docosahexaenoic acid (DHA) accounted for 6% of total fatty acids, a high proportion that makes *E. voratum* a potential candidate feedstock in aquaculture for DHA production.

Key index words: ***Effrenium voratum*; fatty acid composition; growth; medium; response surface methodology**

***Abbreviations*: CV, coefficient of variation; DHA, docosahexaenoic acid; GC-MS, Gas Chromatography–Mass Spectrometer; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acids; RSM, response surface methodology; SFA, saturated fatty acid**

Zooxanthellae are autotrophic endosymbiotic dinoflagellate and belong to the family Symbiodiniaceae, the order Suessiales, and the class Dinophyceae (Trench 1993, LaJeunesse et al. 2018). Zooxanthellae are crucial components of reef ecosystems since they can form mutualistic relationships with scleractinian corals and other marine invertebrates. Through photosynthesis, Symbiodiniaceae species provide oxygen and organic compounds to their hosts and in return receive the living space that is a light-rich and well protected habitat, as well as a continuous supply of nutrients, like phosphate, nitrate, calcium, carbon dioxide, and other metabolic byproducts (Muscatine et al. 1981, Little et al. 2004, Boulotte et al. 2016). Reports have revealed that symbionts can provide 60–85% of the total nutrition of the hosts, the loss of which results in coral bleaching and eventual demise (Muscatine et al. 1981, Fujise et al. 2014, Ainsworth et al. 2016). Currently, there are a lot of unknowns on the symbiotic relationship between Symbiodiniaceae and their hosts. Additionally, Symbiodiniaceae species are rich sources of biologically active and structurally unique secondary metabolites (Tsunematsu et al. 2009, Kita et al. 2010). Therefore, it is important to investigate the algal culture, physiological property, and ecological functions considering their enormous ecological and economic importance.

There are nine large lineages (clades A–I) in the family of Symbiodiniaceae, among which the species from clade C are mostly investigated (Rowan and Powers 1991, Pochon and Gates 2010). Recently, divergent "clades" have been redefined and partitioned into multiple genera by LaJeunesse et al. (2018). The clades A, B, C, D, E, F, and G were classified into genera *Symbiodinium*, *Breviolum*, *Cladocopium*, *Durusdinium*, *Effrenium*, *Fugacium*, and *Gerakladium*, respectively. However, cultivation of Symbiodiniaceae species has been challenging. Until now, only a limited number of species can be cultivated in vitro under artificial conditions. To our knowledge, the literature about optimization of environmental conditions, particularly nutritional factors (e.g., nitrogen, phosphorus and iron), to promote the growth of Symbiodiniaceae species is rare (Rodriguez et al. 2016, Xiang et al. 2018, Langenbach and Melkonian 2019, Lin et al. 2019). Moreover, these studies mainly focused on single-

[1]Received 25 September 2019. Accepted 27 March 2020. Published Online 26 May 2020, Wiley Online Library (wileyonlinelibrary.com).
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Editorial Responsibility: S. Lin (Associate Editor)

factor experiments, which missed the important interactions among those factors (Towanda and Thuesen 2012, Benstein et al. 2014). The individual and interactive effects of various nutrients on the growth of Symbiodiniaceae species under artificial condition require more detailed research.

The response surface methodology (RSM) is an effective and convenient method that can screen key factors from multiple factors rapidly and optimizes culture conditions, thus avoiding the defects brought by single-factor optimization (Qin et al. 2013, Yang et al. 2014). RSM has been successfully utilized in many fields (Hill and Hunter 1966, Martínez-Patiño et al. 2019), but remains underexplored for Symbiodiniaceae species. In this study, a strain of *Effrenium* (*Symbiodinium*) *voratum* isolated from *Galaxea fascicularis* coral was selected. *Effrenium voratum* is the only known species in culture in the genus of *Effrenium* (Jeong et al. 2014). It is difficult to culture the strain because of the low growth rate under the original condition used in our study. This study investigated a feasible method to promote the algal growth in vitro. Specifically, a Plackett–Burman design was applied to estimate comprehensively the significance of various media nutrient constituents toward growth. Then a Box–Behnken design was employed to identify the optimal strategy. Furthermore, the algal physiological properties were evaluated under the optimum conditions, which can not only help us to understand their ecological functions, but also provide a new approach to culturing Symbiodiniaceae species.

MATERIALS AND METHODS

Strain and culture conditions. *Effrenium voratum* used in this study was isolated from coral *Galaxea fascicularis* collected at 18°13′ N and 109°2′ E in the coast of Sanya City, South China Sea. The coral was washed with sterilized seawater gently to remove epiphytic organisms, and then the tissue was stripped from the coral with a dental washer filled with the filtered seawater. The tissue slurry contained zooxanthellae was homogenized, centrifuged, resuspended, and transferred to culture plates. *Effrenium voratum* was isolated with single-cell isolations, and cultivated in 48-well plates, 6-well plates, and 50 mL culture flask. Finally, the algal cells were cultured in 250 mL culture flask containing 150 mL of natural seawater supplemented with modified f/2 media at 26°C. Light intensity was maintained at 80 μmol photons · m^{-2} · s^{-1} with a 12:12 h light:dark (12L/12D) cycle.

Experimental design. Response surface methodology (RSM) with Plackett–Burman and Box–Behnken design was employed to optimize medium to accelerate the growth for *Effrenium voratum*. In this experiment, the algal cells were cultured in 250 mL culture flask containing 150 mL of natural seawater supplemented with modified f/2 media at 26°C. The cultivation lasted for 12 d. The light intensity was maintained at a photon flux of 80 μmol photons· m^{-2}· s^{-1} with a 12:12 h light:dark cycle. Three replicate flasks were assigned to each run. The response value was determined three times from each flask, and represented as the mean value.

The Plackett–Burman design was carried out to identify these parameters (variables) having significant effects on the growth of *Effrenium voratum*. The chosen variables included $NaNO_3$, $NaH_2PO_4{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, KCl, $CaCl_2{\cdot}2H_2O$, $FeCl_3{\cdot}6H_2O$, $MnSO_4$, $ZnSO_4{\cdot}7H_2O$, and $CoSO_4$. Two dummy variables were added to estimate the experimental error. Each independent variable was set at two levels: −1 for a low level and + 1 for a high level, according to the Plackett–Burman design and preliminary trials. The higher levels of the variables were chosen to equal six times their lower levels. A matrix of 12 runs was generated using software package, Design Expert 8.05. The actual factor levels corresponding to the coded factor levels, together with the experimental data, are shown in Table S1 in the Supporting Information. The growth, expressed as dry weight, was considered as the response value. The model was validated using various statistical parameters such as *p* value, F value, determination coefficients (R^2, adjusted R^2 and predicted R^2), and coefficient of variation (CV).

The coefficient estimate is an important parameter to assess the effects of variables on the response value. The response value enhances with increasing concentrations of the variable if the coefficient estimate is positive; conversely, it indicates that the value is negatively correlated with the variable levels (Jiang et al. 2013). *P*-values are applied to assess the significance of these variables in greater depth, which are calculated with software package, Design Expert 8.05 based on experiments. When the *p*-value of the variable is less than 0.05, it represents that the variable has a significant effect on the response value. Moreover, a higher contribution ratio is an indicator that the variable is more important.

The Box–Behnken design was utilized to optimize the levels of these variables having significant effects on dry weight. The chosen variables were $NaH_2PO_4{\cdot}2H_2O$ and $FeCl_3{\cdot}6H_2O$. Each independent variable was set at five levels. For this procedure, 13 runs, including five replicates of the center points and eight star points, were required. The concentrations of $NaH_2PO_4{\cdot}2H_2O$ and $FeCl_3{\cdot}6H_2O$ were given in Table S2 in the Supporting Information, while the levels of other nutrients were constant as described above. The growth, which was expressed as dry weight, was determined as the response value and analyzed by the Design-Expert program (8.05 version). The optimal response value and culture conditions were predicted. Meanwhile, a second-degree polynomial equation was obtained by software package, Design Expert 8.05as following:

$$Y = a_0 + a_1A + a_2B + a_3AB + a_4A^2 + a_5B^2$$

where, A and B stood for these variables having significant effects on the algal growth; Y represented the predicted value of dry weight; a_0 was intercept; a_1–a_5 were estimated coefficients. The variable had a significant effect on the response value if the estimated coefficient was less than 5%. The Design-Expert program (8.05 version) was employed to analyze the data.

The adequacy of the model was evaluated based on R^2, *p* -value and *F*-test. Additionally, the experimental response value obtained under the optimal conditions, was compared with the predicted value to further verify the reliability of RSM model.

Dry weight and pigment content. Algal growth was determined using the dry weight technique. Aliquots of 20 mL microalgal suspension were filtered by preweighed GF/C filter paper on the 12th d (Whatman, Poole, UK). The filter paper with algal biomass was then dried at 105°C to a constant weight. After cooling down to room temperature in a vacuum desiccator, the filter paper was reweighed to obtain the dry weight.

Chlorophyll *a* (Chl *a*) and Chlorophyll *c* (Chl *c*) were spectrophotometrically determined according to Jeffrey and Humphrey (1975). Specially, aliquots of 10 mL microalgal suspension were collected by mild centrifugation (3000*g*, 5 min), and then thoroughly grinded in of 90% (v/v) acetone and stirred for 24 h in the dark until the alga turn white. The extracts were centrifuged (5000*g*, 5 min) and supernatant were collected. The absorbance of filtered supernatant at 630 nm and 663 nm was determined using a Multiskan Ascent spectrophotometer (Thermo Labsystems, Waltham, MA, USA). The Chl *a* and Chl *c* concentrations were quantified with the equation of Jeffrey and Humphrey (1975), and normalized to the cell density to provide units of picogram Chl per cell. The cell density was assessed using a hemocytometer. All the treatments were repeated three times and data were reported as the mean ± SD values.

Fatty acid composition. The algal cells were collected by mild centrifugation (3000*g*, 5 min), and freeze-dried on the 12th d. About 2 mL of NaOH-CH_3OH and 4 mL of BF_3-CH_3OH were added to the dry algae (approximately 10 mg) for transesterification reaction at 75°C for 30 min. Then an additional 6 mL of hexane and 2 mL of deionized water were added, mixed, and centrifuged. The upper layer containing fatty acids was collected and analyzed by Gas Chromatography–Mass Spectrometer (GC-MS) with an Omegawax 250 polyethylene glycol capillary column (length, 30 m; diameter, 0.25 mm; 0.25 μm film thickness) using the method reported by Khozin-Goldberg et al. (2005). One milliliter of each sample was injected into the capillary column with a split ratio of 5:1. Helium was employed as the carrier gas with a flow rate of 1.5 mL · min^{-1}. The temperatures of the injector and detector were both maintained at 250°C. The column temperature was programmed from 130°C at 5°C · min^{-1} ramp rate to 250°C maintained for 5 min. The content of each component was determined according to the area normalization method. Data were reported as mean ± SD values.

RESULTS AND DISCUSSION

The impacts of medium compositions on the dry weight. As shown in Table S1, the Plackett–Burman design with two coded levels for all 12 runs was firstly utilized to evaluate the influence of nutrient components on dry weight, which was a reliable indicator for evaluating algal growth. The concentrations of nine variables including $NaNO_3$,$NaH_2PO_4{\cdot}2H_2O$, KCl, $FeCl_3{\cdot}6H_2O$, $MgSO_4{\cdot}7H_2O$, $MnSO_4$, $CoSO_4$, $CaCl_2{\cdot}2H_2O$, and $ZnSO_4{\cdot}7H_2O$ were all set at two levels: −1 for a low coded level and + 1 for a high coded level, according to the Plackett–Burman design and preliminary trials. The actual factor concentrations corresponding to the coded levels of these variables represented as X_1–X_9 were listed in Table S1. Generally, the experimental groups (runs) can be set as 12, 20, 24, 28, and 36 according to the number of variables. Therefore, a matrix of 12 runs was chosen and generated with software package, Design Expert 8.05 in this study. The experimental data in Table S1 were analyzed by the Design-Expert software. Various statistical parameters such as *F* ratio, *P*-value, and determination coefficients (R^2, R_{adj}^2 and R_{pred}^2) were calculated to check the fit of model. *P*-value less than 0.05 indicated model terms are significant. The *F*-ratio of 16.72 implied that the model was significant, as revealed by a *P*-value lower than 0.01 (df = 8,11 $P = 0.0008$). There was only a 0.08% chance that the model *F*-ratio could occur due to noise. R^2 was 0.8625, indicating that 86.25% of the data in Plackett–Burman design could be explained by the model; that is, the proposed model was reasonable. Furthermore, the R_{pred}^2 of 0.8109 was in good agreement with the R_{adj}^2, which further demonstrated that the model was fit to explain the data.

Nutrients are vital factors which can affect the growth rate of coral and Symbiodiniaceae in coral reefs, but the effects are species-specific. Several studies observed that hosts were markedly affected by increased nutrient availability (Steven and Broadbent 1997, Ferrier-Pagès et al. 2000, Rädecker et al. 2017). However, the literatures about the impacts of nutrients on cultured Symbiodiniaceae species have been very limited (Sakami 2000, Karako-Lampert et al. 2005, Rodriguez et al. 2016, Rodriguez and Ho 2018, Lin et al. 2019, Newson 2019). In this study, the influences of nine variables including $NaNO_3$,$NaH_2PO_4{\cdot}2H_2O$, KCl, $FeCl_3{\cdot}6H_2O$, $MgSO_4{\cdot}7H_2O$, $MnSO_4$, $CoSO_4$, $CaCl_2{\cdot}2H_2O$, and $ZnSO_4{\cdot}7H_2O$ on the response value (dry weight) were investigated in *Effrenium voratum*. Coefficient estimate, *p*-value, and contribution ratio were used to assess the effects of these variables on the response value. As mentioned earlier, the negative coefficient estimate indicated that response value was negatively correlated with the variable levels, while the *p*-value less than 5% represented that the variable had a significant effect on the response value. Moreover, the higher contribution ratio implied the variable was more important (Jiang et al. 2013). As shown in Table 1, $NaNO_3$ expressed a negative effect on dry weight according to the coefficient estimate; however, it was insignificant with *p*-value more than 0.05. The result was consistent with the previous study, which reported that growth was not affected by the ammonium enrichment (Sakami 2000). Rosset et al. (2017) also suggested that symbiotic dinoflagellates were more flexible to different nitrogen concentrations. Additionally, KCl and $ZnSO_4$ shown negative effects, whereas other factors including NaH_2PO_4, $FeCl_3$, $CaCl_2$, $MgSO_4$, $MnSO_4$, and $CoSO_4$ exhibited positive effects on the response value.

Phosphorus is considered a vital limiting nutrient in coral systems, which can be used for energy transfer and nucleic acid synthesis (Tyrrell 1999, Ferrier-Pagès et al. 2016). Generally, Symbiodiniaceae species taken preferentially inorganic phosphates in the forms of $H_2PO_4^-$ and HPO_2^{2-}, which might have been experiencing phosphorus deficiency under an imbalanced nutrient regime. The photosynthetic capacity and growth can be repressed under P deficiency, as demonstrated in other algae, and P-replete condition supports higher growth rate of algae by enhancing photosystem quantum efficiency or RuBisCO abundance, and progressing the cell cycle (Li et al. 2015, 2016, Rosset et al. 2017,

TABLE 1. Statistical analysis of the Plackett–Burman experiment design.

Factor	Level		Contribution%	Coefficient Estimate	*P*-value
	−1	+1			
$NaNO_3$	80	480	0.02	-0.0017	—
$NaH_2PO_4{\cdot}2H_2O$	5	30	36.96	0.0717	0.0013[a]
$MgSO_4{\cdot}7H_2O$	4	24	4.50	0.0250	—
KCl	0.2	1.2	1.62	-0.0150	0.0582
$CaCl_2{\cdot}2H_2O$	0.2	1.2	0.02	0.0017	—
$FeCl_3{\cdot}6H_2O$	0.5	3	40.48	0.0750	0.0017[a]
$ZnSO_4{\cdot}7H_2O$	0.05	0.3	0.50	-0.0083	—
$MnSO_4$	0. 5	3	8.81	0.0350	—
$CoSO_4$	0.05	0.3	4.50	0.0250	—

[a]Significance level at a *P*-value less than 5%; —, significance level at a *P*-value of more than 5%; $R^2 = 0.8625$, $R_{adj}{}^2 = 0.8109$; Coefficient of variation (CV) = 5.09.

Shi et al. 2017, Lin et al. 2019). In this study, *p*-value of 0.0013 revealed that the positive effect of NaH_2PO_4 on dry weight was significant. With increasing phosphate concentrations from 0.03 mM to 0.18 mM, the dry weight increased evidently. Moreover, NaH_2PO_4 was the second important factor after $FeCl_3$ since its contribution ratio was second with 36.96% among all variables. Similar results were reported by previous studies on other species of dinoflagellates (Li et al. 2016, Rosset et al. 2017, Lin et al. 2019).

Trace metals are not only essential components of electron transport chains in photosynthesis and respiration, but also vital factors in enzymes involved in variety biological processes, including photoprotection, photorepair, and chlorophyll synthesis (Twining and Baines 2013, Rodriguez et al. 2016, Rodriguez and Ho 2018). The trace metal requirements of some model microalgae have been well investigated (Cao et al. 2011, Sunda 2012, Pausch et al. 2019). However, there is a knowledge gap about the influences of trace metals on the growth of Symbiodiniaceae species (Rodriguez et al. 2016, Rodriguez and Ho 2018, Li et al. 2020). In this study, the quantitative importance of trace metals including Fe, Cu, Zn, Ca, Mg, and Co on the growth was comprehensively evaluated in *Effrenium voratum*. The results revealed that $ZnSO_4$ expressed adverse effects with the negative coefficient estimate, which was not consistent with several studies (Rodriguez et al. 2016, Li et al. 2020), mainly because of the difference in the concentration of $ZnSO_4$. $ZnSO_4$ was not a limiting factor for *E. voratum* growth in the test concentration range, the contribution ratio of which was only 0.5% with *P*-value more than 0.05. Conversely, $FeCl_3$, $CaCl_2$, $MgSO_4$, $MnSO_4$, and $CoSO_4$ displayed positive effects on dry weight. It implied that dry weight was enhanced with the improvement in concentrations of $FeCl_3$, $CaCl_2$, $MgSO_4$, $MnSO_4$, and $CoSO_4$. According to the contribution ratio and *p*-value, the effects of these trace metals on *E. voratum* growth were further investigated. $FeCl_3$, the contribution ratio of which was 40.48%, was the most important factor for the algal growth. The requirement for trace metals exhibited the following order: Fe ≫ Mn > Mg = Co > Zn > Ca. Similar results that Fe was more important than other trace metals (Cu/Zn/Mn/Co) for the growth were observed in *Fugacium kawagutii* (Rodriguez et al. 2016). Moreover, *P*-value revealed that only $FeCl_3$ had significant positive effects on dry weight among trace metals with *P*-value less than 0.01 in this study ($P = 0.0017$). It may be attributed that Fe enhancement could increase Chl *a* content, improve photosynthetic efficiency and photosynthetic carbon fixation capacity, resulting in an increase of algal dry weight (Rodriguez and Ho 2018). Further studies are needed to evaluate the importance of Fe as a limiting factor for Symbiodiniaceae species and analyze the Fe trafficking with the coral. Previous studies have shown that Mn availability was positively correlated with the growth rate of the dinoflagellate (Cao et al. 2011, Rodriguez and Ho 2018). In this study, Mn was the second needed trace metal after Fe for the growth of *E. voratum*, however, the positive effects were insignificant with *p*-value more than 0.05. The result was consistent with the report of Rodriguez and Ho (2018), which indicated that Mn was unlikely to become a limiting factor for *Symbiodinium kawagutii* growth in natural environments.

These results shown that the requirement for nutrients exhibited the following order: $FeCl_3$>-NaH_2PO_4≫$MnSO_4$> $MgSO_4/CoSO_4$ > KCl>$ZnSO_4$> $CaCl_2/NaNO_3$, among which NaH_2PO_4 and $FeCl_3$ had important positive impacts on the dry weight, whereas other variables were not limited in the growth process. Therefore, NaH_2PO_4, and $FeCl_3$ were chosen to further optimize using the Box–Behnken design.

Identifying the best culture conditions for growth. The Box–Behnken design was applied to confirm the optimal concentrations of $NaH_2PO_4{\cdot}2H_2O$ and $FeCl_3{\cdot}6H_2O$ to maximize the dry weight based on the results obtained by Plackett–Burman. In this experiment, five replicates of the center points and eight star points were required, resulting in a total number of 13 runs. Table S2 presents the experimental projects and the response values. Among 13

TABLE 2. Statistical analysis of the Box–Behnken experiment design.

Factor	Sum of squares	Degree of Freedom	Mean square	*F*-value	*P*-value
Model	0.0234	3	0.0047	35.86	<0.0001
$NaH_2PO_4{\cdot}2H_2O$	0.0152	1	0.0152	70.23	<0.0001
$FeCl_3{\cdot}6H_2O$	0.0028	1	0.0028	13.02	0.0057
$NaH_2PO_4{\cdot}2H_2O$*$FeCl_3{\cdot}6H_2O$	0.0000	1	0.0000	0.0000	1.0000
$NaH_2PO_4{\cdot}2H_2O^2$	0.0051	1	0.0051	24.32	0.0008
$FeCl_3{\cdot}6H_2O^2$	0.0000	1	0.0000	0.0065	0.9382
Residual	0.0020	9	0.0003		
Lack of Fit	0.0014	5	0.0005	1.81	0.2935
Pure Error	0.0006	4	0.0002		
Cor Total	0.0253	12			

Determination coefficients of the model: R^2 = 0.9228; R_{adj}^2 = 0.8971; Coefficient of variation (CV) = 2.22. $NaH_2PO_4{\cdot}2H_2O$*$FeCl_3{\cdot}6H_2O$ represented the interactions between NaH_2PO_4 and $FeCl_3$ while $NaH_2PO_4{\cdot}2H_2O^2$ and $FeCl_3{\cdot}6H_2O^2$ represented quadratic effects of NaH_2PO_4 and $FeCl_3$, respectively.

runs, experiment four ($NaH_2PO_4{\cdot}2H_2O$ and $FeCl_3{\cdot}6H_2O$ concentrations of 0.26 mM and 14.80 μM, respectively) offered the highest dry weight (0.71 g · L^{-1}), while experiment five ($NaH_2PO_4{\cdot}2H_2O$ and $FeCl_3{\cdot}6H_2O$ concentrations of 0.01 mM and 9.25 μM, respectively) provided the lowest dry weight (0.54 g· L^{-1}).

Multiple regression analyses were applied to investigate the data in Table S2 and to confirm the adequacy of the model. As shown in Table 2, the multiple correlation coefficient R^2 was 0.92, indicating that 92% of the data in Box–Behnken design could be explained by the model. R_{pred}^2 of 0.72 was in good agreement with the R_{adj}^2 of 0.90, implying that the quadratic polynomial model was suitable for revealing the mutual relationship of varieties, and predicting the response values. An F-ratio of 35.86 implied the model was significant, as revealed by *P*-value lower than 0.0001. Additionally, an *F*-ratio of "Lack of Fit" with 1.81 was not significantly relative to the pure error, which further demonstrated that the model was fit to explain the data.

As shown in Table 2, the individual effects and their interactions of all variables on dry weight were analyzed. NaH_2PO_4 exerted significant individual and quadratic effects, respectively (*P*-values less than 0.05). $FeCl_3$ was significant (*P*-values less than 0.05), yet with non-significant quadratic effects for the response value (*P*-value more than 0.05). The interactions between two parameters (NaH_2PO_4 and

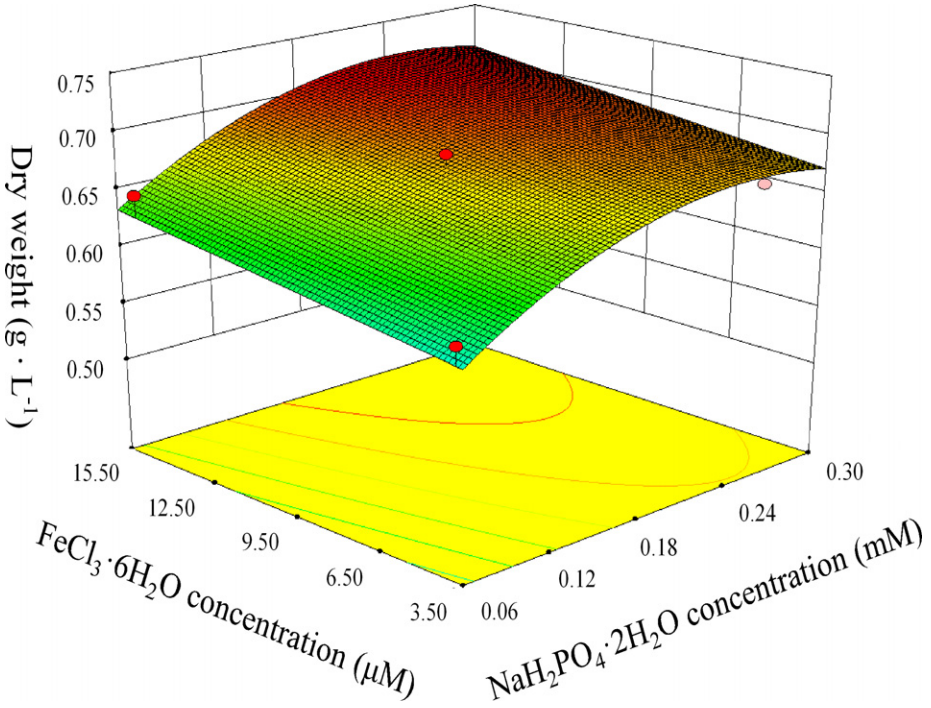

FIG. 1. Three-dimensional response surface plots for dry weight showing the interaction effects of NaH_2PO_4 and $FeCl_3$. The X and Y axes represent the concentrations of $FeCl_3{\cdot}6H_2O$ and $NaH_2PO_4{\cdot}2H_2O$ respectively with the vertical Z axis representing dry weight. [Color figure can be viewed at wileyonlinelibrary.com]

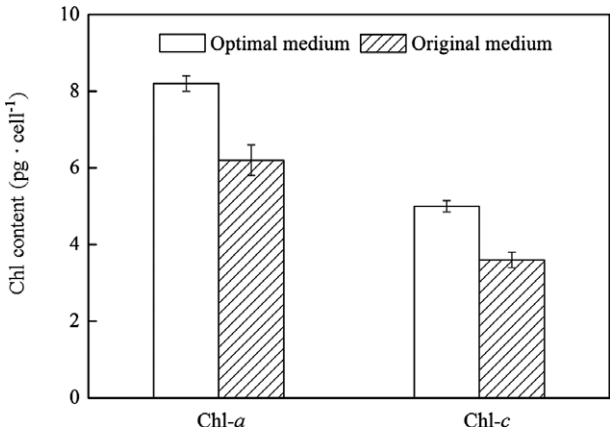

FIG. 2. The contents of chlorophyll *a* (Chl *a*) and chlorophyll *c* (Chl *c*) under the optimal and original conditions. The values were expressed as the means ± standard deviation ($n = 3$).

$FeCl_3$) and dry weight were further revealed by response surface plots and contour plots, as shown in Figure 1. The dry weight enhanced with the increase in the concentrations of NaH_2PO_4 and $FeCl_3$, respectively. However, the mutual interactions of these variables did not have a significant effect on dry weight. Experimental data in Table S2 were fitted to a second-degree polynomial model by software package, Design Expert 8.05 aiming at an optimal response value. The predicted second-degree polynomial model used in the response surface regression analysis could be described as follows:

$$\text{Dry weight} = 0.68 + 0.042NaH_2PO_4 \cdot 2H_2O$$
$$+0.018FeCl_3 \cdot 6H_2O - 0.025NaH_2PO_4 \cdot 2H_2O_2$$

The validation of the model. According to the attained results and equation, the model predicted the maximum dry weight of 0.71 $g \cdot L^{-1}$ in the concentration of 0.25 mM$NaH_2PO_4{\cdot}2H_2O$ and 14.24 μM$FeCl_3{\cdot}6H_2O$. The final optimal condition was as follows: natural seawater supplemented with $NaH_2PO_4{\cdot}2H_2O$, 0.25 mM; $FeCl_3{\cdot}6H_2O$, 14.24 μM; $NaNO_3$, 0.94 mM; $MgSO_4{\cdot}7H_2O$, 40.63 mM; KCl, 5.37 mM; $CaCl_2{\cdot}2H_2O$, 4.08 mM; $ZnSO_4{\cdot}7H_2O$, 0.35 μM; $MnSO_4$, 9.93 μM; $CoSO_4$, 0.36 μM. In order to confirm the model, culture experiments based on the optimal concentrations were performed. The dry weight was 0.76 $g \cdot L^{-1}$ under optimum conditions, which was agreed well with the predicted value, indicating that the model was valid.

Growth and pigment content. Culture experiments based on the original and optimal mediums were both performed. The growth and pigment content were compared to evaluate the optimum medium. The dry weight reached 0.76 $g \cdot L^{-1}$ under optimal conditions, which increased 46% more than that of the original conditions (0.52 $g \cdot L^{-1}$). As shown in Figure 2, the content of Chl *a* significantly enhanced under the optimal conditions, which increased to 8.24 $pg \cdot cell^{-1}$ from 6.32 $pg \cdot cell^{-1}$. A similar trend with a 2-fold increase was also observed in Chl *c* content, which could be related with the increase of Fe concentration in medium, which was a vital component in enzymes involved in chlorophyll synthesis.

Fatty acid composition. The major fatty acid compositions were determined by gas chromatography coupled to mass spectrometry (GC-MS). As shown in Figure 3a, saturated fatty acid (SFA) accounted for about 59.21% of total fatty acids in *Effrenium voratum*. The contents of monounsaturated fatty acid (MUFA) and polyunsaturated fatty acids (PUFA) were 23.73% and 10.95% of total fatty acids, respectively. Palmitic acid (C16:0) and eicosanic acid (C20:0) were the major fatty acids, accounting for approximately 50% of total fatty acids. Other fatty

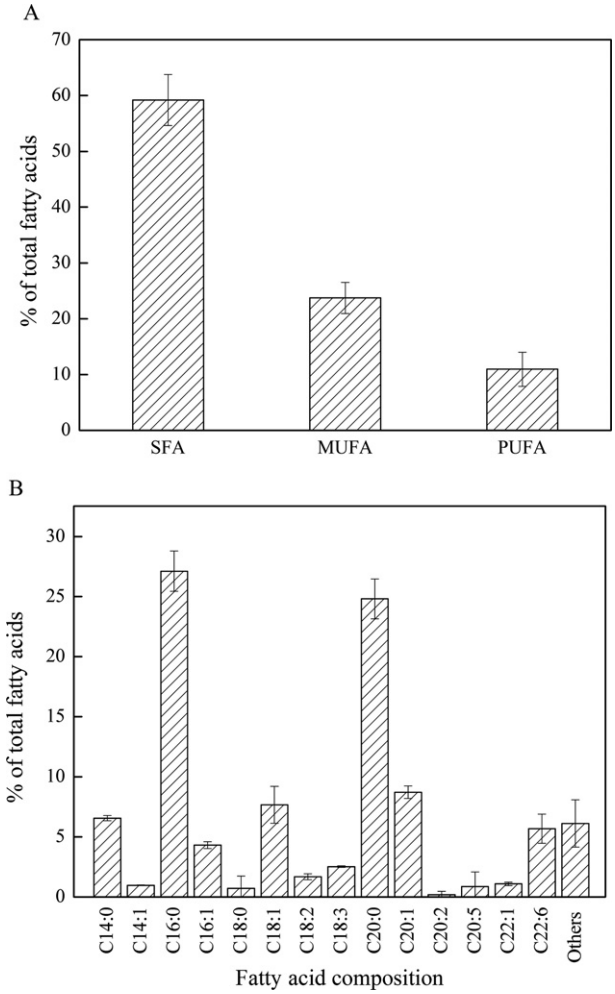

FIG. 3. The proportions of (A) SFA, MUFA, and PUFA; (B) major classes of fatty acids (carbon number: unsaturated bond number) of *Effrenium voratum* under optimal conditions. The values are the means ± standard deviation ($n = 3$). SFA, MUFA, PUFA represented saturated fatty acid, monounsaturated fatty acid, and polyunsaturated fatty acids, respectively.

acids, such as oleic acid (C18:1), Octadecanoic acid (C18:0), linoleic acid (C18:2), and linolenic acid (C18:3) were present in smaller quantities (Fig. 3b). Similar fatty acid compositions were observed in Symbiodiniaceae species (Meyers 1997, Weng et al. 2014). However, the contents of fatty acids varied with some researches who reported that C16:0, C18:0 and C18:1 were most identified fatty acid classes. In this study, the contents of C18:0 and C18:1 were only 0.72% and 7.67% of total fatty acids, respectively. Correspondingly, C20:0 was second with 24.82% after C16:0. It is worthwhile to note that about 6% of total fatty acids were observed in DHA (C22:6), implying that *E. voratum* may be a potential source of DHA, which in turn plays an important role in human health.

CONCLUSIONS

This study provides a useful approach to cultivating and enhancing the growth of Symbiodiniaceae species. The medium composition was optimized to accelerate the growth of *Effrenium voratum* by response surface methodology. These results showed that the requirement for nutrients decreased in the following order: $FeCl_3$>NaH_2PO_4> $MnSO_4$> $MgSO_4$/$CoSO_4$ > KCl>$ZnSO_4$> $CaCl_2$/$NaNO_3$, among which NaH_2PO_4 and $FeCl_3$ had important positive impacts on the dry weight. The medium containing $NaH_2PO_4{\cdot}2H_2O$, 0.25 mM; $FeCl_3{\cdot}6H_2O$, 14.24 μM was considered to be optimal for growth by *E.voratum*, which improved the dry weight by 46%. Additionally, palmitic acid (C16:0) and ethyl carbonate (C20:0) were dominant fatty acids. Meanwhile, DHA was also observed, implying that *E. voratum* may be a candidate feed in aquaculture as a source of DHA.

This research was supported by the National Natural Science Foundation of China (41806145), the Science and Technology Program of Guangzhou (201707010174), and the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA13020203).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Table S1. Plackett–Burman experimental design for nine variables (X_1−X_9) and the response value–dry weight. A matrix of twelve runs was generated. Each independent variable was set at two levels. The higher levels of each variable were chosen to equal six times their lower levels.

Table S2. Experimental design and dry weight in the Box–Behnken design. Each independent variable was set at five levels.