Micronutrient Requirements for Growth and Hydrocarbon Production in the Oil Producing Green Alga Botryococcus braunii (Chlorophyta)

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Abstract

The requirements of micronutrients for biomass and hydrocarbon production in Botryococcus braunii UTEX 572 were studied using response surface methodology. The concentrations of four micronutrients (iron, manganese, molybdenum, and nickel) were manipulated to achieve the best performance of B. braunii in laboratory conditions. The responses of algal biomass and hydrocarbon to the concentration variations of the four micronutrients were estimated by a second order quadratic regression model. Genetic algorithm calculations showed that the optimal level of micronutrients for algal biomass were 0.266 µM iron, 0.707 µM manganese, 0.624 µM molybdenum and 3.38 µM nickel. The maximum hydrocarbon content could be achieved when the culture media contained 10.43 µM iron, 6.53 µM manganese, 0.012 µM molybdenum and 1.73 µM nickel. The validation through an independent test in a photobioreactor suggests that the modified media with optimised concentrations of trace elements can increase algal biomass by 34.5% and hydrocarbon by 27.4%. This study indicates that micronutrients play significant roles in regulating algal growth and hydrocarbon production, and the response surface methodology can be used to optimise the composition of culture medium in algal culture.

Introduction

Microalgae have recently been receiving much attention in an attempt to explore their use as a potential feedstock for biofuel production [1,2]. Botryococcus braunii is a green colonial microalgae found in freshwater lakes, reservoirs, and ponds [3,4] and is classified into A, B and L races depending on the type of hydrocarbons synthesized [5]. Race A produces C₃₇₋₃₃ odd numbered α-alkalenes, mono-, tri-, tetra-, and pentaenes and race B produces C₃₀₋₃₇ triperpenes while race L produces C₄₀ tetrapenenes [5]. This species is characterised by a conspicuous ability to synthesise and accumulate a variety of hydrocarbons [6,7,8]. These hexane-soluble hydrocarbons have the potential to be converted into biofuels by catalytic cracking [9]. However, the great variation of hydrocarbon content in B. braunii (0.1~86% of dry weight) provides an opportunity to explore the optimal growing conditions to maximise hydrocarbon production for a given B. braunii strain [10,11,12]. Therefore, it is necessary to identify the most efficient growing conditions for sustainable mass and hydrocarbon production in B. braunii.

The requirements for macronutrients by B. braunii have been intensively studied in the past a few decades. Largeau et al. [13] pointed out that the phosphorus (0.46 mM) in the Chlorella strain was not limiting through the stationary growth phase in B. braunii, while the nitrogen concentration of 0.5 mM NO₃⁻ is only adequate to sustain the growth of B. braunii for 10 days and the initial concentration of 8 mM NO₃⁻ is required to maintain the growth of B. braunii for 35 days. Ammonia can inhibit botryococcene biosynthesis in the B. braunii race B [14], but the replacement of nitrite nitrogen for nitrate nitrogen benefits the growth of race A B. braunii [15]. Air enriched with 1% CO₂ can enhance algal growth by doubling algal biomass and achieving 5-fold hydrocarbon production compared to aeration without CO₂ enrichment [16]. Dayanada et al. [17] reported that the N: P ratio played a significant role in both biomass and hydrocarbon production in B. braunii and the N: P ratio of 1:4 by weight favoured hydrocarbon production while the N:P ratio of 1:0.5 by weight increased the yield of algal biomass.

Given the depth of understanding in the growth requirement for macronutrients in B. braunii, it is surprising that the requirements for trace elements are little known. Trace elements such as iron, molybdenum and manganese can play critical roles in a variety of metabolic pathways involving utilization of light, nitrogen, phosphorus, and CO₂ [18,19]. Among trace elements, iron is essential for photosynthetic electron transport, respiratory electron transport, nitrate and nitrite reduction, and detoxification of reactive oxygen species [20,21,22]. Mojaat et al. [23] demonstrated that the addition of iron to the Dunaliella salina culture medium stimulated β-carotene production. The iron enrichment in the Chlorella vulgaris culture could increase algal growth and lipid
accumulation [24], where the total lipid content of algae grown in the medium supplemented with 1.2×10^{-5} M FeCl₃ reached 56.6% of the dry biomass, which was a 3.7 fold increase compared to the medium without iron enrichment. Manganese is another important component in algal photosynthesis and also presents in enzymes to remove toxic superoxide radicals to sustain algal growth [25]. Chernikova et al. [26] reported that manganese (MnCl₂) enhanced the capacity to accumulate inorganic minerals coupled with iron in the enzymes for nitrate reduction, and its deficiency diminishes the nitrate uptake mechanism and interferes with lipid synthesis [27]. Nickel can facilitate nitrogen uptake to enhance the growth of Thalassiosira weissflogii when urea is the nitrogen source, suggesting the positive role of Ni in enhancing algal growth [28]. Berges et al. [29] also reported that the addition of nickel and molybdenum to the algal culture medium increased the overall primary productivity. Coincidently, in a field survey, Wake and Hillen [3] found that wherever the B. braunii bloom occurred in the Darwin River reservoir, the nickel concentration in the environment was always higher than that in adjacent water bodies where no B. braunii bloomed, suggesting this trace element may trigger the occurrence of B. braunii. However, no laboratory testing has been conducted so far to test the need of nickel to enhance the growth of B. braunii in the laboratory since the early field survey work of Wake and Hillen’s in the 1980’s.

Optimization of micronutrient requirements is an important undertaking prior to the establishment of sustainable production of B. braunii on a large scale. The conventional method to optimise the level of multiple nutrients in algal culture has been focussed on one-factor-at-a-time approach, studying the effect of one nutrient on the response of algae by keeping the other nutrients constant. However, this approach is time consuming and does not take into account interactions between nutrients, which usually results in poor optimization results [30,31].

Techniques in experimental design are critical to identify key nutrients required for algal growth. In this study we used the response surface methodology (RSM) [32] to explore the requirement of micronutrients in the culture of B. braunii because the RSM approach can optimise the nutrient requirement with low input of time and resources [33,34,35]. This approach has been widely used in optimization of plant nutrients [36,37], bacterial medium composition [38], enzymatic hydrolysis [39,40], synthesis of polymers [41], food processing [42,43] and operation conditions for photobioreactors [44]. The RSM approach has also been used for medium optimisation in algal culture. Azma et al. [45] optimised the culture medium for Tetraselmis suecica by RSM

![Figure 1. Illustration of the central composite design (only 3 out of the 4 dimensions are shown).](doi:10.1371/journal.pone.0041459.g001)
increased algal production by two times. Similarly, by using RSM, Isleten-Hosoglu et al. [46] optimised the carbon and nitrogen concentrations for *Chlorella saccharophila* and improved biomass production by 7.7 fold. The objectives of this study were to (1) estimate the roles of the four micronutrients iron, manganese, molybdenum, and nickel in regulating the responses of algal biomass and hydrocarbon, and (2) identify the optimum requirements of micronutrients for the cultivation of *B. braunii* to maximise hydrocarbon production.

**Methods**

**Materials and Procedures**

*B. braunii* UTEX 572 was obtained from the University of Texas Culture Collection, USA. The basic macronutrients for algal growth were adapted from the Bold 3N medium, which also contains micronutrients including 5.35 μM Fe, 6.36 μM Mn, and 0.31 μM Mo [47]. All chemicals were of analytical regent grade. To avoid the effect of other unknown trace elements, soil residuals were not added into the medium in this study. The experiment for model construction was conducted at 24 ± 1°C with illumination provided by fluorescent lights at 150 μmol/m²/s at 12 h light and 12 h dark. The algal growth experiments lasted 3 weeks.

The dry weight of algal cells was measured by vacuum filtration onto pre-weighed Whatman® GF/C filters [48]. The filters with algal cells were freeze-dried, weighed, and expressed as algal biomass (g/L). Hydrocarbons in dry biomass were extracted on glass filters using *n*-hexane [48]. Solvents were removed from the extracts by a rotary evaporator and the residues were rinsed with *n*-hexane. Hydrocarbon fractions were purified by passing the samples through an alumina gel plug and eluting with *n*-hexane. Solvents were evaporated under a stream of nitrogen to dry, and the pure hydrocarbon fractions were measured gravimetrically and expressed as hydrocarbon content (% w/w).

**Experimental Design**

Central composite design (CCD) is one type of RSM approach [49] which allows estimating the polynomial regression between independent variables and dependant variables [50]. In this study, a 2⁴ CCD with 24 runs and six replications of the centre points were used to determine the optimal concentrations of iron, manganese, molybdenum, and nickel on the yield of algal biomass and hydrocarbon production (Fig. 1). The coded and corresponding actual values are given in Table 1. The corresponding central composite experimental design and their values are shown in Table 2. All the design points except the centre point (0, 0, 0, 0) were run in three replications. Due to the restriction of modeling protocol, only one mean value of the three replicates for each

| **Table 3.** Analysis of variance (ANOVA) for the fitted quadratic polynomial regression model for optimization of the algal biomass production. |
| --- |
| **Source** | **Sum of squares** | **df** | **Mean square** | **F-value** | **Probability P-value** |
| Model | 0.162049 | 14 | 0.011575 | 31.64 | <0.001 |
| Residual | 0.005488 | 15 | 0.000366 | | |
| Lack of fit | 0.005354 | 10 | 0.000535 | 20.08 | 0.002 |
| Pure error | 0.000133 | 5 | 0.000027 | | |
| Cor. total | 0.167537 | 29 | | | |
| $R^2=0.967$ |

| **Adj. $R^2=0.937$ Pred. $R^2=0.824$** |
| --- |

| **Table 4.** Analysis of variance (ANOVA) for the fitted quadratic polynomial regression model for optimization of the hydrocarbon production. |
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| **Source** | **Sum of squares** | **df** | **Mean square** | **F-value** | **Probability P-value** |
| Model | 218.69 | 14 | 15.621 | 36.58 | <0.001 |
| Residual | 6.406 | 15 | 0.427 | | |
| Lack of fit | 4.127 | 10 | 0.413 | 0.91 | 0.584 |
| Pure error | 2.279 | 5 | 0.456 | | |
| Cor. total | 225.096 | 29 | | | |
| $R^2=0.972$ |

| **Adj. $R^2=0.945$ Pred. $R^2=0.875$** |

| **Table 5.** Concentration of micronutrients in different algal culture media. |
| --- |
| **Culture media** | **Fe** | **Mn** | **Mo** | **Ni** |
| Original Bold 3N | 2.150 | 1.240 | 0.099 | 0.00 |
| Modified Bold 3N-1 | 0.276 | 0.707 | 0.624 | 3.38 |
| Modified Bold 3N-2 | 10.430 | 6.530 | 0.012 | 1.73 |

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dependent variable was allowed to enter the model. Therefore, the
degree of freedom of the triplicate for each non-centrepoint could
not be used for pure error calculation. Experiments were repeated
six times at the central point to provide an estimate of pure error
[51,52,53,54] thus providing adequate degree of freedom (df = 5 )
for pure error calculation (Tables 3 and 4).

Data from the CCD experiment were analysed by RSM. A
mathematical model with a second-order polynomial regression
was developed to describe the relationships between the predicted
response variables (biomass or hydrocarbon) and the independent
variables (Fe, Mn, Mo and Ni). The regression equation was
described as follows (Eq. 1):
where $y$ is the predicted response variables (biomass or hydrocarbon production); $b_0$ is a constant, $b_i$ is the linear coefficient, $b_{ii}$ is the quadratic coefficients, $b_{ij}$ is the interaction coefficients of the model, respectively; $x_i$ and $x_j$ ($i=1, 4; j=1, 4; i\neq j$) represent the non-coded independent variables (micronutrient concentrations).

Model Validation

The predicted models on algal biomass and hydrocarbon production of $B. braunii$ were validated in an independent experiment using optimized micronutrient concentrations from the genetic algorithms calculations [55]. A flat plate photobioreactor (3.2 L) was used as the culture vessel under a light intensity of 300 $\mu$mol/m$^2$/s and a mixing rate of 1.10 L/L/min. The $B. braunii$ cells were separately inoculated into the original Bold 3N medium, the modified Bold 3N-1 medium for producing algal biomass, and the modified Bold 3N-2 for producing hydrocarbon with different micronutrient compositions (Table 5). The experimental protocols in the validation study were the same as those in the model construction. Algal biomass and hydrocarbon content were separately measured at 3-day intervals over 12 days to assess the response of algal performance to modified media. The productivities of algal biomass and hydrocarbon during the experimental period were also calculated and expressed as g/L/day. All data points in the figures were the mean of three replicates to provide a better estimate of the response of each dependent variable.

Statistical Analysis

The data analyses for model construction were performed with MINITAB 16, based on the response surface methodology. The $F$-test for the analysis of variance (ANOVA) was performed on experimental data to evaluate the statistical significance of the model. The significance of regression coefficients was evaluated using $t$-test. The contour plots described by the regression model were drawn using MATLAB 7 to illustrate the effects of the independent variables and interactive effects of each independent variable on the response variables.

Optimisation of nutrient composition in the medium was determined by the procedure of genetic algorithms (MATLAB 7), which is a computer simulation program based on the best fit theory of natural selection to generate optimal solutions to problems [55]. In simulations, the program selected the best-fit concentration of each nutrient to maximise the algal response such as biomass and hydrocarbon production. In the validation experiment, data from the original 3N medium and modified medium were analysed by quadratic regression to compare the significant differences of curves. The probability level for significant difference was set at $P<0.05$.

Results and Discussion

Model Fitting

The application of RSM yielded the following regression equations for biomass (Eq. 2) and hydrocarbon production (Eq. 3). A central composite design (CCD) with five coded levels for all the four factors: iron, manganese, molybdenum, and nickel were used for model simulations. The range of variables, experimental designs and results for biomass and hydrocarbon production are presented in Table 2. The second order polynomial regression equations were used to fit the dependent variables ($Y_{biomass}$ and $Y_{hydrocarbon}$) to the independent variables $x_1$ (iron), $x_2$ (manganese), $x_3$ (molybdenum) and $x_4$ (nickel).

![Contour plot showing hydrocarbon prediction from Mn ($x_2$) and Ni ($x_4$) with other independent variables Fe ($x_1$) and Mo ($x_3$) being constant.](https://doi.org/10.1371/journal.pone.0041459.g003)
The significance and adequacy of the regression model were tested using ANOVA. These two regression models could significantly predict algal biomass (P<0.001) and hydrocarbon production (P<0.001) from the four micronutrients (Tables 3 and 4). The predicted $R^2$ (0.824 for Eq. 2 and 0.875 for Eq. 3) agreed well with the adjusted model $R^2$ (0.937 for Eq. 2 and 0.945 for Eq. 3), suggesting a close correlation between the observed values and the predicted values. Therefore, we can use the regression models to predict algal biomass and hydrocarbon production from the amount of micronutrients in the culture medium.

Effect of Micronutrients on Algal Biomass

The regression coefficients of the model for biomass prediction are presented in Table 6. The linear effect of $x_1$ and the quadric effect of $x_1^2$ and $x_4^2$ had significant effects (P<0.001) on $Y_{\text{biomass}}$, followed by the interaction effect of $x_1x_4$ (P=0.019). Other terms of the model had no significant effect on $Y_{\text{biomass}}$. Negative coefficients of $x_1$ and interaction term $x_1x_4$ decreased $Y_{\text{biomass}}$. However, the quadratic terms of $x_1^2$ and $x_4^2$ had positive effects on $Y_{\text{biomass}}$.

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![Figure 4. Regression plots of biomass (A) and hydrocarbon (B) productions in the modified and original Bold 3N media.](doi:10.1371/journal.pone.0041459.g004)

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Effect of Micronutrients on Hydrocarbon Production

The regression coefficients of the model for hydrocarbon production are presented in Table 7. The linear effect of \( x_2 \) and \( x_4 \) and the quadratic effect of \( x_2^2 \) and \( x_4^2 \) had significant effects \((P<0.001)\) on \( Y_{\text{hydrocarbon}} \). Other terms of the model had no significant effect on \( Y_{\text{hydrocarbon}} \). Positive coefficient of \( x_2 \) and \( x_4 \) indicated their role to enhance \( Y_{\text{hydrocarbon}} \). However, the quadratic terms of \( x_2^2 \) and \( x_4^2 \) had negative effects on \( Y_{\text{hydrocarbon}} \).

The interaction effects of two independent variables (Mn and Ni) on the response variable (hydrocarbon) are shown by the contour plots generated by keeping the independent variables (Fe and Mo) as constants (Fig. 3). Hydrocarbon production was more sensitive to the change of Mn and Ni concentrations. An increase in hydrocarbon production was observed with the increase of Mn concentrations. But this trend was reversed when the Mn concentration was above 9 \( \mu \text{M} \). The effect of Ni on \( Y_{\text{hydrocarbon}} \) followed the similar trend. With the increase of Ni concentration, \( Y_{\text{hydrocarbon}} \) firstly increased and then decreased as a result of excessive Ni concentration. The circular profile of the contour plots indicated that the interaction between the Mn and Ni concentrations on hydrocarbon was negligible (Fig. 3).

The composition of the culture medium affects not only algal productivity, but also secondary metabolites [50]. This finding was consistent with result of Wang et al. [59] who found that the increase of Fe and Mn concentrations stimulated the growth of blue green algae, while a further increase in their concentrations inhibited algal growth. Cloez et al. [60] found that lipid synthesis increased by three times after adding manganese, copper and nickel at 2 mM. On the other hand, Mohammad and Fathy [61] reported that the total lipid content in Dunaliella salina cultivated in nickel supplemented media (0.5 mg/L NiCl\(_2\)) has reduced in comparison to the control. In another study, Roush and Sommerfeld [62] found that manganese had stronger impact on the growth of a green alga (Ulothrix sp.) than nickel. However, in this study, both nickel and manganese regulated the production of hydrocarbon, though the algal biomass was only affected by nickel.

Optimisation of Micronutrients

The concentrations of these four micronutrients for producing algal biomass were optimized using the genetic algorithm calculation. The optimal medium for biomass consisted of 0.266 \( \mu \text{M} \) Fe, 0.707 \( \mu \text{M} \) Mn, 0.624 \( \mu \text{M} \) Mo and 3.38 \( \mu \text{M} \) Ni. By running the optimization simulation within the experimental range, the optimal medium for hydrocarbon production is recommended to contain 10.43 \( \mu \text{M} \) Fe, 6.53 \( \mu \text{M} \) Mn, 0.012 \( \mu \text{M} \) Mo and 1.73 \( \mu \text{M} \) Ni. It is worth noting that the optimal composition of these four micronutrients for algal biomass was different from that for hydrocarbon production. This difference highlights the importance of selecting culture medium to achieve different objectives in algal culture since the nutrient requirement differs for algal cell division and accumulation of secondary metabolites [63].

Validation of Algal Growth and Hydrocarbon Production

The reliability of nutrient requirement generated from the predicted models and the genetic algorithm calculations for biomass and hydrocarbon production in B. braunii was validated in an independent photobioreactor study. From day 3 to day 12, the algal biomass produced in the Bold 3N medium supplemented with 0.266 \( \mu \text{M} \) Fe, 0.707 \( \mu \text{M} \) Mn, 0.624 \( \mu \text{M} \) Mo, 3.38 \( \mu \text{M} \) Ni was significantly higher than that produced in the original Bold 3N medium \((P<0.05, \text{Fig. 4A})\). The maximal algal biomass productivity \((1.300 \pm 0.176 \text{ g/L/day})\) in dry weight with modified media...
was significantly higher than that (0.967 ± 0.033 g/L/day) in the original media (P<0.05, Fig. 5A).

The hydrocarbon production of algae in the Bold 3N medium supplemented with 10.45 μM Fe, 6.53 μM Mn, 0.012 μM Mo and 1.73 μM Ni was significantly higher than that in the original medium from day 3 to day 12 (P<0.05, Fig. 4B). The maximal hydrocarbon productivity (0.110 ± 0.003 g/L/day) in the modified media was significantly higher than that (0.087 ± 0.002 g/L/day) in the original media (P<0.05, Fig. 5B).

The biomass and hydrocarbon productivity are key parameters affecting the economic feasibility of producing bioproducts from algae. The micronutrient concentrations optimised by modelling were validated in a photobioreactor, and the accuracy and reliability of the model in predicting nutrient requirements for producing algal biomass and hydrocarbon have been confirmed.

Conclusion

The application of response surface methodology (RSM) is a reliable approach to model and optimize the requirements for iron, manganese, molybdenum, and nickel in producing algal biomass and hydrocarbon in B. braunii. Nickel and iron played significant roles but manganese and molybdenum had a trivial role in algal biomass production. In contrast, nickel and manganese were more important than molybdenum and iron in regulating algal hydrocarbon production. The production of algal biomass and production of hydrocarbon require different micronutrients in the culture medium. The recommended levels of micronutrients in the Bold 3N medium are 0.266 μM iron, 0.707 μM manganese, 0.624 μM molybdenum and 3.38 μM nickel for B. braunii biomass and 10.45 μM iron, 6.53 μM manganese, 0.012 μM and 1.73 μM nickel for hydrocarbon production. The model validation showed that by using modified algal culture media, algal biomass productivity increased 1.345 fold and hydrocarbon productivity increased 1.274 fold compared with the original Bold 3N medium without addition of the trace elements.

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Author Contributions

Conceived and designed the experiments: LS JGQ SC. Performed the experiments: LS. Analyzed the data: SS JX YS. Wrote the paper: LS JGQ JX SC.

References

1. Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25: 294–306.
2. Qin JG (2016) Hydrocarbons from algae: In: Timmis KN editor. Microbiology of hydrocarbons, oils, lipids, and derived compounds, vol 4: Consequences of microbial interactions with hydrocarbons, oils and lipids. Berlin: Springer, 2017–2016.
3. Wake LV, Hillen LW (1949) Study of a “bloom” of the oil-rich alga Botryococcus braunii in the Darwin River reservoir. Biotechnol Bioeng 22: 1637–1656.
4. Wake LV, Hillen LW (1949) Nature and hydrocarbon content of blooms of the alga Botryococcus braunii occurring in Australian freshwater lakes. Aust J Mar Freshwater Res 32: 353–367.
5. Metzger P, Largeau C (2005) Botryococcus braunii: a rich source for hydrocarbons and related ether lipids. Appl Microbiol Biot 66: 486–496.
6. Brown AC, Knights BA, Conway E (1969) Hydrocarbon content and its relationship to physiological state in green algae Botryococcus braunii. Phytochemistry 8: 543–547.
7. Knights BA, Brown AC, Conway E, Middlebush BS (1970) Hydrocarbon in the green form of the freshwater alga Botryococcus braunii. Phytochemistry 9: 1317–1324.
8. Li Y, Qin JG (2005) Comparison of growth and lipid content in three Botryococcus braunii strains. J Appl Physiol 17: 551–556.
9. Hillen L, Pollard G, Wake LV, White N (1982) Hydrocracking of the oils of the oils of Botryococcus braunii to transport fuels. Biotechnol Bioeng 24: 193–205.
10. Qin JG (2008) Bio-hydrocarbons from algae: impacts of temperature, light, and salinity on algae growth. Development 3: 1–26.
11. Qin JG, Li Y (2006) Optimization of the growth environment of Botryococcus braunii strain CHN 357. J Freshwater Ecol 21: 169–176.
12. Metzger P, Largeau C (1999) Chemicals of Botryococcus braunii. In Cohen Z editor. Microorganisms from microalgae. London: Taylor & Francis. 205–260.
13. Oliveira R, Almeida MF, Santos L, Madeira LM (2006) Experimental design to optimize the oxidation of orange II dye solution using a clay-based Fenton-like catalyst. Ind Eng Chem Res 45: 204–294.
14. Oliveira R, Almeida MF, Santos L, Madeira LM (2006) Experimental design of 2, 4-dichlorophenol oxidation by Fenton’s reaction. Ind Eng Chem Res 45: 1286–1276.
15. Myers RH, Montgomery DC (2002) Response surface methodology: process and product optimization using designed experiments, 2nd ed. New York: John Wiley & Sons. 704 p.
16. Ren J, Lin WT, Shen YJ, Wang JF, Lou XC, et al. (2008) Optimization of fermentation media for nitrite oxidizing bacteria using sequential statistical design. Bioreour Technol 99: 602–5609.
17. Peers GS, Price NM (2004) A role for manganese in superoxide dismutases and the growth of iron-deficient diatoms. Limnol Oceanogr 49: 1174–1173.
18. Derham DM, Sargent MW, Middleditch BS (1970) Hydrocarbons from the green form of the freshwater alga Botryococcus braunii. Phytochemistry 9: 1317–1324.
19. Niedz RP, Hyndman SE, Evens TJ (2007) Using a gestalt to measure the quality of macro- and micronutrients, and the eicosapentaenoic acid and docosahexaenoic acid contents of Passola lathami Enzym Microb Techn 38: 350–366.
20. Price NM, Morrel FMM (1991) Co-limitation of phytoplankton growth by nickel and nitrogen. Limnol Oceanogr 36: 1071–1077.
21. Bergs JA, Franklin DJ, Harrison PJ (2004) Evolution of an artificial seawater medium: improvements in enriched seawater, artificial water over the last two decades. J Phycol 37: 1130–1145.
22. Price NM, Morrel FMM (1991) Co-limitation of phytoplankton growth by nickel and nitrogen. Limnol Oceanogr 36: 1071–1077.
23. Peers GS, Price NM (2004) A role for manganese in superoxide dismutases and the growth of iron-deficient diatoms. Limnol Oceanogr 49: 1174–1173.
24. Carvalho AP, Pontes J, Gaspar H, Malcata FX (2006) Metabolic relationships between macro- and micronutrients, and the eicosapentaenoic acid and docosahexaenoic acid contents of Passola lathami Enzym Microb Techn 38: 350–366.
25. Myers RH, Montgomery DC (2002) Response surface methodology: process and product optimization using designed experiments, 2nd ed. New York: John Wiley & Sons. 704 p.
26. Ren J, Lin WT, Shen YJ, Wang JF, Lou XC, et al. (2008) Optimization of fermentation media for nitrite oxidizing bacteria using sequential statistical design. Bioreour Technol 99: 7923–7927.
27. Kammoun R, Nabi B, Bejar S (2008) Application of a statistical design to the optimization of parameters and culture media for a-amylase production by Aspergillus oryzae CBS 1957.2 grown on gruel (wheat grinding by-product). Bioreour Technol 99: 602–5609.
28. Pan CM, Fan YF, Xing Y, Hou HW, Zhang ML (2008) Statistical optimization of biodegradation of 2,4-dichlorophenol by Fenton’s reaction. Ind Eng Chem Res 45: 1286–1276.
29. Myers RH, Montgomery DC (2002) Response surface methodology: process and product optimization using designed experiments, 2nd ed. New York: John Wiley & Sons. 704 p.
30. Ren J, Lin WT, Shen YJ, Wang JF, Lou XC, et al. (2008) Optimization of fermentation media for nitrite oxidizing bacteria using sequential statistical design. Bioreour Technol 99: 7923–7927.
31. Pan CM, Fan YF, Xing Y, Hou HW, Zhang ML (2008) Statistical optimization of biodegradation of 2,4-dichlorophenol by Fenton’s reaction. Ind Eng Chem Res 45: 1286–1276.
39. Kunammeci A, Singh S (2005) Response surface optimization of enzymatic hydrolysis of maize starch for higher glucose production. Biochem Eng J 27: 179–190.
40. Nilsang S, Lertsiri S, Suphantharika M, Assaravichian A (2005) Optimization of enzymatic hydrolysis of fish soluble concentrate by commercial proteases. J Food Eng 70: 571–578.
41. Shieh CJ, Lai YF (2000) Application of response surface methodology to the study of methyl glucoside polymer synthesis parameters in a solvent-free system. J Agr Food Chem 48: 1124–1129.
42. Castro IA, Tirapegui J, Silva RSSF (2000) Protein mixtures and their nutritional properties optimized by response surface methodology. Nutr Res 20: 1341–1353.
43. Ozor EA, Herken EN, Guzel S, Ainsworth S, Ibanoglu S (2006) Effect of extrusion process on the antioxidant activity and total phenolics in a nutritious snack food. Int J Food Sci Tech 41: 209–293.
44. Jacob-lopes E, Lacerda LMCF, Franco TT (2008) Biomass production and carbon dioxide fixation by *Aphanizophyceae microscopica* nageli in a bubble column photobioreactor. Biochem Eng J 40: 27–34.
45. Azma M, Mohamed MS, Mohamad R, Rahim RA, Ariff AB (2011) Improvement of medium composition for heterotrophic cultivation of green microalgae, *Tetraselmis suecica*, using response surface methodology. Biochem Eng J 53: 187–195.
46. Isleten-Hosoglu M, Gultepe L, Elibol M (2012) Optimization of carbon and nitrogen sources for biomass and lipid production by *Chlorella vulgaris*. Bioresour Technol 105: 252–248.
47. Provasoli L, McLaughlin JJA, Droop MR (1957) The development of artificial media for marine algae. Arch Microbiol 25: 392–428.
48. Okada S, Devarene TP, Murakami M, Abe H, Chappell J (2004) Characterization of botryococcene synthase enzyme activity, a squalene synthase-like activity from the green microalga *Botryococcus braunii*, race B. Arch Biochem Biophys 422: 110–118.
49. Wang JP, Chen YZ, Ge XW, Yu HQ (2007) Optimization of coagulation–flocculation process for a paper-recycling wastewater treatment using response surface methodology. Colloids Surf. A: Physicochem Eng Asp 302: 204–210.
50. Zheng ZM, He QJ, Hao J, Xu F, Gao NN, et al. (2000) Statistical optimization of culture conditions for 1,3-propanediol by *Klebsiella pneumonia* AC15 via central composite design. Bioresource Technol 99: 1052–1056.
51. Ghadge SV, Raheman H (2006) Process optimization for biodiesel production from mahua (*Madhuca indica*) oil using response surface methodology. Bioresource Technol 97: 379–384.
52. Cui FJ, Li Y, Xu ZH, Xu HY, Sun K, et al. (2006) Optimization of the medium composition for production of mycelial biomass and exo-polymer by *Geotrichum floridanum* GF9801 using response surface methodology. Bioresource Technol 97: 1209–1216.
53. Gu XH, Zheng ZM, Yu HQ, Wang J, Liang FL, et al. (2005) Optimization of medium constituents for a novel lipopeptide production by *Bacillus subtilis* MO-01 by a response surface method. Process Biochem 40: 3196–3201.
54. Kaushik R, Saran S, Isar J, Saxena RK (2006) Statistical optimization of medium components and growth conditions by response surface methodology to enhance lipase production by *Aspergillus carneus*. J Mol Catal B: Enzyme 40: 121–126.
55. Goldberg ED (1989) Genetic algorithms in search optimization and machine learning. Boston: Addison-Wesley Longman Publishing. 412 p.
56. Jin X, Nalewajko C, Kushner DJ (1996) Comparative study of nickel toxicity to growth and photosynthesis in nickel-resistant and -sensitive strains of *Scenedesmus alatus* f. alternans (Chlorophyceae). Microbial Ecol 31: 103–114.
57. Wong JPK, Wong YS, Tam NYF (2000) Nickel biosorption by two chlorella species, *C. vulgaris* (a commercial species) and *C. minuta* (a local isolate). Biorew Technol 73: 133–137.
58. Shah LK, Hunt HR, Wegner GH (1987) Highproductivity fermentation process for cultivation industrial microorganisms. J Ind Microbiol 2: 79–85.
59. Wang Z, Chen S, Cao X (2010) Micro-nutrients effects on algae colony: growth rate and biomass response to various micro-nutrients and competitive inhibitions among multi-microelements. Symposium of 4th Internat Con Bioinformat Biomed Eng 1–8.
60. Cloez I, Dumont O, Picioni M, Bourre JM (1987) Alterations of lipid synthesis in the normal and dysmyelinating trembler mouse sciatic nerve by heavy metals (Hg, Pb, Mn, Cu, Ni). Toxicology 46: 65–71.
61. Goldberg ED (1989) Genetic algorithms in search optimization and machine learning: Boston: Addison-Wesley Longman Publishing. 412 p.
62. Wang Z, Chen S, Cao X (2010) Micro-nutrients effects on algae colony: growth rate and biomass response to various micro-nutrients and competitive inhibitions among multi-microelements. Symposium of 4th Internat Con Bioinformat Biomed Eng 1–8.
63. Cloez I, Dumont O, Picioni M, Bourre JM (1987) Alterations of lipid synthesis in the normal and dysmyelinating trembler mouse sciatic nerve by heavy metals (Hg, Pb, Mn, Cu, Ni). Toxicology 46: 65–71.
64. Lee YK, Ding SY (1994) Cell cycle and accumulation of astaxanthin in *Haematococcus lacustris* (Chlorophyta). J Phycol 30: 445–449.