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Determination of Maximum Green Microalgae Biomass using Response Surface Methodology

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Abstract: Microalgae have been recently used as potential feedstock for many industries such as cosmetics, pharmaceuticals, nutritional, and biofuels production. Since nutrient stress has a major effect on biomass production and its characterizations, the dynamics of nutrient stress on microalgal growth must be taken into consideration. The study identifies two green algae species *Chlamydomonus variabills* and *Haematococcus pluvialis* isolated from River Nile. Response surface regression analysis was used to find the conditions that optimize biomass growth under nitrate starvation and salt stress. Four models describe the biomass growth for both species are presented. ANOVA analysis was used to validate these models. The analysis shows that the maximum biomass under nitrate starvation are 273 and 2116 mg/l for *Chlamydomonus variabills* and *Haematococcus pluvialis* occur for nitrate concentrations of 0.7 and 0.8 gm./l at 13 and 14 days respectively. The maximum biomasses are 670 and 211 mg/l at 14 and 15 days for *Chlamydomonus variabills* and *Haematococcus pluvialis* respectively.

Key words: Microalgae, green algae, biomass, nutrient stress, Response Surface Methodology.

1. Introduction

Microalgae are photosynthetic microorganisms that can grow rapidly and live in harsh conditions due to its unicellular or simple structure[1].It is renewable, environmentally friendly and it can contribute in reducing the CO_2 level at the atmosphere because microalgae consume CO_2 and converts it to oil[2].Microalgae biodiesel production per unit of area is many times higher than crops biodiesel. The productivity of diatom algae is about 46000 Kg of oil/hectare/year[3]. Some microalgae have oil content about 80% of dry weight[4].Microalgae biofuel is non-toxic, contains no sulphur and highly bio-degradable. After extracting oil the left material can be used as soil fertilizer or to produce ethanol[5].Several factors need to be considered in the cultivation of algae biomass. These include the provision of light, carbon and nutrients such as nitrate, phosphate and trace metals, the mixing regime, maintenance of optimal temperature, removal of O_2 and control of pH and salinity[6-7].The optimal and tolerated ranges tend to be species specific, and may vary according to the desired product[1].It is notedthat the increase in salinity can increase the lipid content of microalgae, but lowers the growth rate of a species. Therefore, the effect of salinity on the growth of microalgae species have to be investigated. The ability of cell to survive and flourish in saline environment under the influence of osmotic stress has received considerable attention[8-9].

In order to obtain the maximum biomass production using minimum amounts of substrates and time, response surface method (RSM) is an effective statistical technique for achieve this requirement. Building models and investigating them are complex processes for optimization[10].

The present study focuses on the development of a mathematical model for each studied species to describe the effect each investigated substrate concentration and time to obtain maximum biomass production. Through RSM technique, the degree and the significance of each term of the model were tested using both the error analysis, Fisher's test (*F*-test)[11] and the *P*-value. Hence, the optimum conditions are estimated for the highest biomass production using the contour plot of the RSM.

2. Material and Method

2.1 Algal isolation and cultures

The algal species used in this study were isolated from River Nile water using BG11 media as reported[12]. The isolated algal species are *Chlamydomonus variabills* and *Haematococcus pluvialis* (green algae). Normal condition for cultivation was carried out in sterilized one liter (l) conical shoulder flasks containing 600 ml of the corresponding culture medium under continuous illumination (24hrs.) with white fluorescent light intensity ≈2500Lux. The $NaNO_3$ and NaCl concentrations are 1.5 and 0.0 gm. /l respectively. Each strain was subjected to different conditions in order to study there effect on biomass production.

2.2 Algal Biomass harvesting

In order to harvest algal biomass, a suitable harvesting method may involve one or more steps and be achieved in several physical or chemical ways. Filter press operating under pressure was used with *Chlamydomonus variabills*, through membrane filter 0.8µm, while *Haematococcus pluvialis* harvested through settling then centrifugation at 2000 rpm for 10 minutes.

2.3Chlorophyll-a measurements

The fresh sample (25ml) of each strain was taken every 48 hr. and filtered through 0.45µm membrane filter and extracted with hot methanol[13]after the addition of 0.5ml magnesium carbonate solution (1%) in order to prevent chlorophyll degradation. Having the algal sample filtrated, the membrane filter was immediately soaked in little amounts (2-3ml) of hot methanol 90% for two minutes. The addition of methanol was repeated till complete extraction was assured. The extract was completed by methanol to a known volume, and then centrifuged for 10min. at 2000 rpm. The clear extract was transferred to a $1cm^3$ cuvette and absorbance at 664, 647 and 630 nm was determined using spectrophotometer. The following equation was used for calculating the concentration of chlorophyll C(µg/l)[14].

C= 11.85(OD664) – 1.54(OD647) – 0.08(OD630)

C_c(Chlorophyll a µg/l) = C× extract volume(l)/ sample volume (l)

Where: OD664, 647 and 630 are the absorbance at 664,647 and 630.

Chlorophyll-a is used as an algae biomass indicator. Assuming that chlorophyll-a constitutes, on the average, 1.5% of the dry weight of organic matter of algae. The algae biomass is estimated using equation.

Biomass (µg/l) $=10^{Ca}$ (67/1000)

where $C_a = \log_{10} (C_c)$

2.4 Effect of sodium nitrate and sodium chloride on chlorophyll-a concentration.

The algal isolates were exposed to adaptation period on stress conditions. The adaptation period involved the re- culture of the algal isolates on the selected factors e.g. $NaNO_3$ starvation and NaCl stress until the algal isolates reach the stationary phase. The algae cells; then transferred to a new culture with the starvation and stress condition in order to collect algal biomass and evaluate the chlorophyll a content. The algal isolates were cultured in different $NaNO_3$ concentrations (0.3, 0.15, 0.075 and 0.0 gm/l) until stationary phase and the culture was collected to determine the effect of decreasing $NaNO_3$ concentrations on total biomass production. The effect of salt stress on algal biomass production were examined by adding NaCl through different concentrations (0.5, 1, 2.5, and 5 gm/l) the algae harvested at stationary phase to study the effect of salt stress on the total biomass production.

2.5 Regression model and Statistical analysis

The current experimental results are statistically analyzed and modeled applying the surface response method MATLAP R2012a. The general model equation is defined as:

$$Y=b_0+\sum_{i=1}^{n} b_i X_i+\sum_{ii}^{n} b_{ii}X^2+\sum_{i=1}^{n}\sum_{j>1}^{n} b_{ij}X_iX_j+\sum_{i=1}^{n}\sum_{j>1}^{n} b_{ij}X_i^2X_j+\varepsilon \quad (1)$$

where Y is response, the Chlorophyll-a content of the algae, b_0, b_i, b_{ii} and b_{ij} are the intercept, linear, quadratic and interaction coefficients respectively. The number of the independent is n namely the time, and the initial substrate concentration. The response of the model is the logarithmic based 10 of Chlorophyll-a content (C_a). The experimental parameters time and nitrate and salinity concentrations are encoded as X_i and X_j values between -1 and 1 based on the minimum and maximum values of the variable as shown in Table 1 and Table 2. The models' validity for data fitting is determined using correlation coefficient (R^2); adjusted statistic coefficient (R^2_{adj}); ration of variance, computed value (F-test); statistical estimator (*p*-value) using analysis of variance (ANOVA). The response surface and contour plots are developed using the proposed models' equations obtained by least square regression method. The significance of each term of the model is examined by evaluating the P-value of each term of the model. The non-significant terms which has P value higher than 0.05 are omitted from the model[15]. Based on the models, the optimum conditions for maximum Chlorophyll-a contents are deduced.

Table: 1 Independent variables and levels for both species under nitrate starvation

Symbol	coded	levels	
		-1	+1
Time (days)	X1	0.0	18.0
$NaNO_3$ (gm/l)	X2	0.0	1.5

Table: 2 Independent variables and levels for both species under salt stress

Symbol	coded	levels	
		-1	+1
Time (days)	X1	0.0	15.0
NaCl (gm/l)	X3	0.0	5.0

3. Results and discussion:

3.1 Algal growth rate

The growth rate of the selected isolates was determined as chlorophyll-a till reached the Stationary phase under control conditions (BG11 medium, white florescent light with intensity2500Lux for illumination period 24 hrs.)**.** The maximum standing biomasses of *Chlamydomonas variabills* and *Haematococcus pluvialis* were reached after 8days and continue to grow up to 16 days at these control conditions.

3.2 Development of regression models' equations.

Using Response Surface Methodology, four models are suggested to describe the relationship between biomass growth and the concentrations of both $NaNO_3$, and NaCl and time on that growth. Using the statistical analysis of variants (ANOVA), models that fitted the response (biomass growth) were carried out to determine the significant individual terms and their interactions, the models are not aliased. The models equations' based on the coded values (X_1, $X_2$and X_3 as time, $NaNO_3$ and NaCl substrates concentrations, respectively) for the

biomass growth (Y_1,Y_2,Y_3 and Y_4) are outlined. The insignificant terms (identified using *p*-value of terms less than 0.05) are omitted. Positive sign in front of the terms indicates synergistic effect which means increase the biomass production, whereas the negative sign indicates antagonistic effect[16].

3.2.1. Adequacy of Biomass growth models under nitrate starvation.

RSM suggested models' Eq. 2 and Eq. 3 for *Chlamydomonus variabills* and *Haematococcus pluviali* with Y_1 andY_2 as their responses (Chlorophyll-a content) respectively under nitrate starvation. The models' R squared, adjusted R, *F*-test and *p*-value are presented in Table 3, these values for both species show that the models fit the experimental data.

$$Y_1 = 3.40 + 0.79\ X_2 - 0.096\ X_1 - 0.76\ X_2 + X_2^2 - 0.50\ X_1^2 \quad (2)$$

$$Y_2 = 3.83 + 1.66\ X_2 + 0.2\ X_2X_1 - 0.74\ X_2^2 - 0.79\ X_1^2 - 0.90\ X_2^2X_1 \quad (3)$$

Table: 3 Models' ANOVA analysis output for *both* species under nitrate starvation

Species	R^2	R^2_{Adj}	*F*-tests	*p*-value
Chlamydomonas variabills	0.8715	0.8601	76.33	< 0.0001
Haematococcus pluvialis	0.9264	0.9180	110.71	< 0.0001

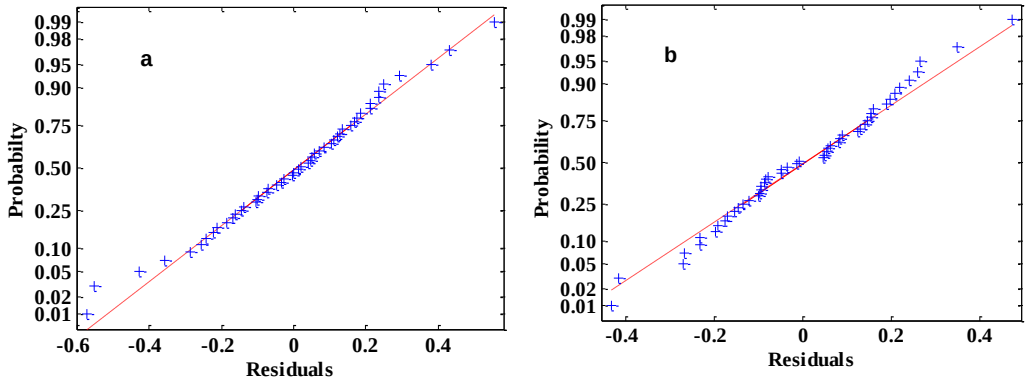

Fig.1 Normal probability plots of the residuals for both strains *Chlamydomonus variabills* (a) and *Haematococcus pluvial* (b) under nitrate starvation.

The analysis was examined using the normal probability plots of the residuals (Figure 1 a and b) for *Chlamydomonus variabills* and *Haematococcus pluviali* respectively. The normal probability plots of the residuals for suggested models indicate that the errors are distributed normally in straight line hence those models adequately represent the experimental data for both strains[17].

3.2.2. Adequacy of Biomass growth models under salinity stress.

Table: 4 Models' ANOVA analysis output for *both* species under salt stress

Species	R^2	R^2_{adj}	*F*-test	*p*-value
Chlamydomonas variabills	0.8374	0.8261	74.65	< 0.0001
Haematococcus pluvialis	0.8255	0.8091	50.87	< 0.0001

Suggested models for *Chlamydomonus variabills* and *Haematococcus pluviali* are presented in Eq. 4 and Eq. 5 with Y_3 andY_4 as their responses (Chlorophyll-a content)respectively. Values of R squared, adjusted R, *F*-tests and *p*-value are shown in Table 4.These values revealed that these models are accepted as a good fit of the data.

$$Y_3 = 2.72 + 1.17X_3 + 0.25\ X_1\ X_3 \quad (4)$$

$$Y_4 = 2.56 + 0.93\ X_1 - 0.55\ X_3 + 0.53\ X_1^2X_3 \quad (5)$$

The normal probability plots of the residuals for *Chlamydomonus variabills* and *Haematococcus pluviali* strains grth rate models are illustrated in Figure 2 a and b. The plots show a linear relation of the residuals and the normal probability which indicates adequate agreement between the experimental data and the data calculated according to the models.

3.3 Response of isolated strains to nitrate starvation.

Predicted values of chlorophyll-a as response surfaces of nitrate starvation and time as well as contour plots for both strains *Chlamydomonus variabills* and *Haematococcus pluviali* are shown in Figure 3. The figures show the effect of nitrate starvation with time on the chlorophyll-a content. The chlorophyll-a content represents the biomass growth directly, since a linear relation between them is defined by Eq. 1. The figures show biomass increase with time until 13 days with maximum biomass of 273 mg/l at 0.7 gm/l $NaNO_3$for *Chlamydomonus variabills* strain(Figure 3a), while it increased until 10 days with maximum biomass of 2116 mg/l at 0.7 gm/l $NaNO_3$ for *Haematococcus pluviali* strain (Figure 3b).

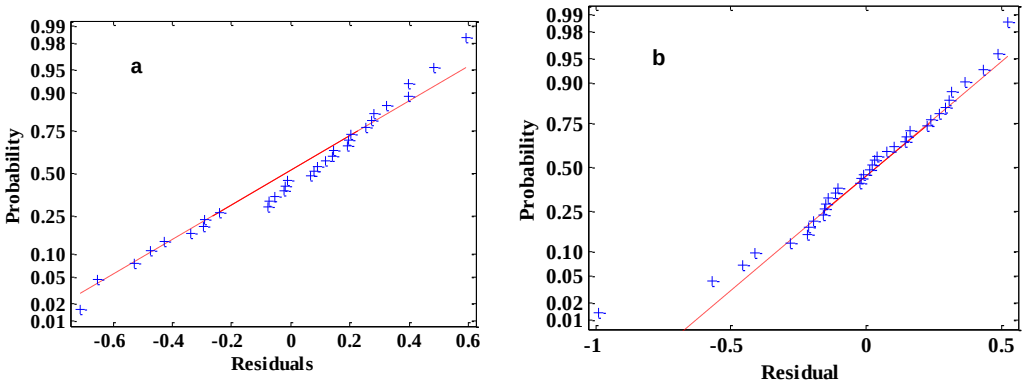

Fig. 2 Normal probability plots of the residuals for both strains *Chlamydomonus variabills* (a) and *Haematococcus pluvial* (b) under salinity stress.

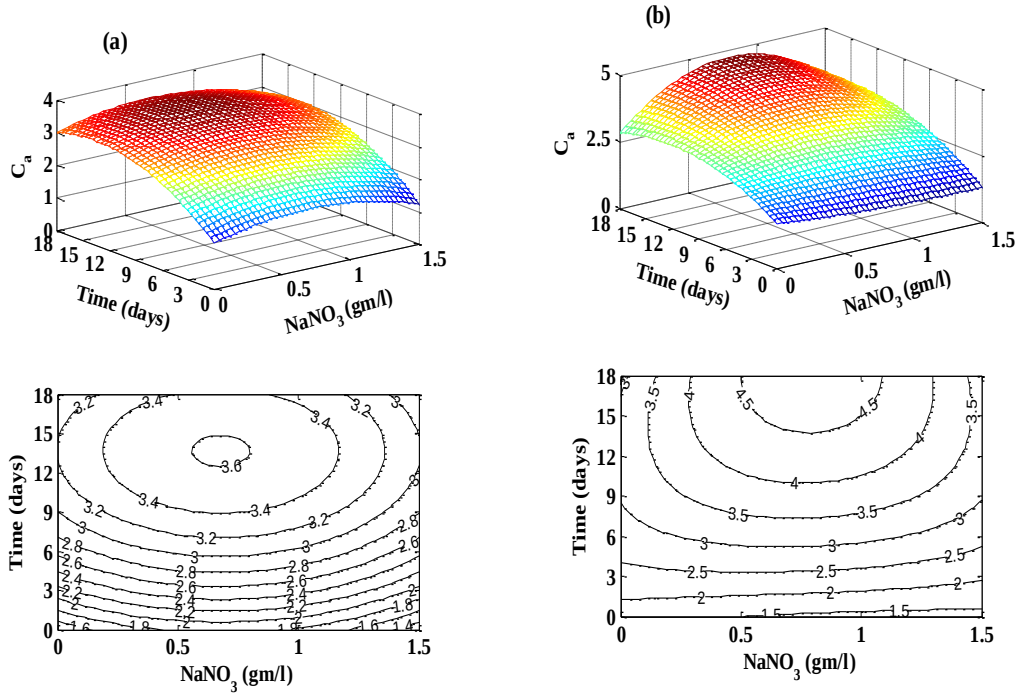

Fig. 3 Response surface and contour plots of biomass production for both strains *Chlamydomonus variabills* (a) *and Haematococcus pluvial* (b)under nitrate starvation.

3.4 Response of isolated strains to salinity stress.

The response surfaces and contour plots of the predicted chlorophyll-a values as functions of salinity stress and time for both strains *Chlamydomonus variabills* and *Haematococcus pluviali* are shown in Figure 4 a and b. The *Chlamydomonus variabills* response to salt stress shows that maximum biomass is 670 mg/l after 14 days (Figure 4a).The response of *Haematococcus pluviali* shows that the maximum biomass is 211 mg/l after 15 days (Figure 4b). There are two regimes; before day 7, the biomass growth rate decreases with increasing salt concentration while increasing after that day with increasing the salt concentration in the substrate. This verifies that this algae species adapt itself with the unusual presentation (increase) of salt in the media. The response of *Haematococcus pluviali* to salt stress is shown in the contour plot (Figure 4b).

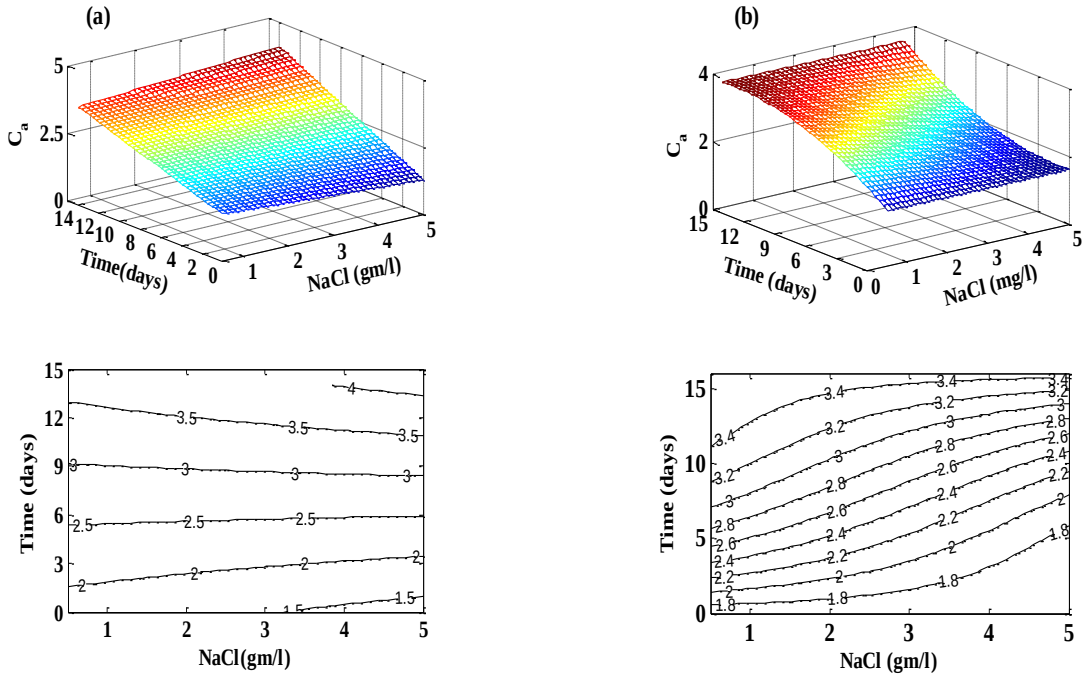

Fig. 4 Response surface and contour plots of biomass production for both strains *Chlamydomonus variabills* (a) *and Haematococcus pluvial* (b) under salinity stress.

4- Conclusion

Algal biomass is a potential source for many fine products and industries so, it is important to control its growth. The influence of nitrate starvation and salinity stress on biomass growth of algal strains *Chlamydomonus variabills and Haematococcus pluvial* are represented by mathematical models applying response surface methodology. According to ANOVA analysis outputs such as R squared, adjusted R, *F*-tests and *p*-value have assisted these models. Normal probability plots proofed that the models are adequate to represent the biomass growth. Response surfaces and contour plots displayed that maximum growth of both strains *Chlamydomonus variabills and Haematococcus pluvial* are 273 and 2116 mg/l for nitrate concentrations of 0.7 and 0.8 gm /l at 13 and 14 days respectively. The maximum biomasses were 670 and 211 mg/l at 14 and 15 days for *Chlamydomonus variabills* and *Haematococcus pluvialis* respectively under salinity stress.

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