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**Modelling *Tetraselmis* sp. growth-kinetics and optimizing bioactive-compound production
through environmental conditions**

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Abstract :

The aim of this study is to predict *Tetraselmis* cells growth-kinetic and to induce the synthesis of bioactive compounds (chlorophylls, carotenoids and starch) with high potential for biotechnological applications. Using the statistical criteria, the Baranyi-Roberts model has been selected to estimate the microalgae growth-kinetic values. The simultaneous effects of salinity, light intensity and pH of culture medium were investigated to maximize the production of total chlorophylls, carotenoids and starch. The optimal culture conditions for the production of these compounds were found using Box-Behnken Design. Results have showed that total chlorophyll and carotenoids were attained 21.6 mg·g⁻¹ DW and 0.042 mg·g⁻¹ DW, respectively. In addition, the highest starch content of 0.624 g·g⁻¹ DW has been obtained at neutral pH with high irradiance (182 μmol photons·m⁻²·s⁻¹) and low salinity (20). A highly correlation ($R^2 = 0.884$) has been found between the gravimetric and flow cytometric measurements of chlorophyll content.

Keywords: Microalgae; Optimal culture conditions; Carotenoids; Starch; Chlorophylls.

1. Introduction

Many microalgae species have been singled out as sources of value-added compounds such as vitamins, polyunsaturated fatty acids, proteins, pigments, and carbohydrates. Some of them have shown a wide range of biological activities (Khatoon et al., 2014; Delattre et al., 2016). *Tetraselmis* sp. is a marine microalga that has promising potential particularly in the domains of aquaculture and biofuel production (Hemaiswarya et al., 2011; Yao et al., 2013). *Tetraselmis* species are able to produce high amounts of starch, up to 42 - 62.1% of total dry weight (DW), which makes them a potential source of fermentable sugar for bioethanol production (Yao et al., 2012; Dammak et al., 2017). In order to improve the valorisation of *Tetraselmis* biomass economically in this field, it is necessary to enhance the contents of high value compounds and starch as well as the *Tetraselmis* productivity and the biomass treatment by a biorefinery strategy (Yao et al., 2012). Various environmental stress-conditions such as irradiance, salinity, pH and temperature have been described in literature as being parameters that might influence the production of some biochemical compounds in microalgae (García et al., 2012; Markou et al., 2012; Yao et al., 2013; Khatoon et al., 2014; Sun et al., 2014; Takeshita et al., 2014; Dammak et al., 2016). Several studies reported that under different stress conditions, the microalgae produce bioactive compounds as a cell-protecting mechanism (El-Kassas and El-Sheekh, 2016). Li et al. (2015) demonstrated that starch is the dominant sink for energy and short-term carbon storage in response to environmental stresses. Regarding carotenoids synthesis, the isomerisation of its precursor (isopentenyl pyrophosphate (IPP)), to dimethylallyl diphosphate, is catalysed by isopentenyl pyrophosphate isomerase (IPI), and transcripts of *IPI* genes increased in response to oxidative stress (Han et al., 2012). Indeed, the microalgae are harvested commercially to extract chlorophyll that is used in drugs and foods as a green colouring agent (E140). Chlorophylls, the essential photoreceptors in photosynthesis, are the principal pigments giving the characteristic green color of *Chlorophyceae* (Koca et al., 2007). Additionally, some researchers suggested that

chlorophyll metabolism influenced all of the whole photosynthetic machinery (Tanaka and Tanaka, 2006). Decreasing salinity was shown to enhance starch production by marine microalgae such as *Tetraselmis subcordiformis* (Yao et al., 2013) and *Dunaliella* (Chen and Jiang, 2009). Light and pH were reported to highly influence microalgae production (Juneja et al., 2013). Maximum starch accumulation has been attained when microalgae were exposed to stress conditions. They adapted themselves to these conditions shifting their metabolism towards storing energy compounds (Kirrolia et al., 2011). Recent studies demonstrated that chlorophyll and starch contents of *Tetraselmis subcordiformis* strains have been inversely correlated with NaCl concentration in the culture medium (Yao et al., 2013).

The present study aims at (i) analyzing kinetic models describing the *Tetraselmis* cell growth, (ii) optimizing the salinity, irradiance and pH to reach the highest content in value-added bioactive compounds (total chlorophyll, carotenoids and starch) by Response Surface Methodology (RSM) and (iii) studying their influence on the metabolism of *Tetraselmis* sp. grown under single-stage cultivation process. The correlation between spectrometry and flow cytometry methods for total chlorophyll estimation was also investigated.

2. Material and methods

2.1. Microalgal strain maintenance and growth conditions

The green microalga *Tetraselmis* sp. (V₂) was isolated from the Gulf of Gabes (Mediterranean Sea) along the coast of Sidi Mansour (Tunisia). The culture was maintained in F/2 medium (Guillard, 1975) at $25 \pm 1^\circ\text{C}$. Irradiance of $84 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with 12h-light/ 12h-dark photoperiod was provided by a white fluorescent lamp (Compact Fluorescent Lamp, Superlight, Tunisia).

2.2. Assays

The cell pellets from 1 mL culture were sonicated in 1 mL ethanol at 65°C for 30 min and the extracts were centrifuged at $5000 \times g$ for 5 min. The pellets were used for starch content

determination according to Xiao et al. (2006) and Hirst et al. (1972) using iodine reagent (2 % (w/v) potassium iodide and 0.2 % (w/v) iodine). The supernatants were used for absorbance measurements ($A_{666\text{nm}}$, $A_{653\text{nm}}$ and $A_{470\text{nm}}$) The total chlorophyll and carotenoid contents were calculated according to Lichtenthaler and Wellburn (1985) and Kumar et al. (2010).

The content determinations of lipids, proteins, ashes, potassium, magnesium, zinc, manganese, calcium, iron and copper were carried out according to protocols described by Dahmen et al. (2014) and Saddoud et al. (2009).

2.3. Experimental kinetics

The cell abundance was expressed as a function of the experiment duration. It was monitored by absorbance ($A_{680\text{nm}}$) measurement as described by Schmidt et al. (2016) and Nguyen and Rittmann (2016).

This experiment was achieved in duplicate and data were expressed as mean values with standard deviation (\pm SD).

2.4. Kinetic modelling

Four models were chosen to account for *Tetraselmis* cell growth kinetics: logistic, logistic with lag, modified Gompertz and Baranyi–Roberts (Mohamed et al., 2014). The sigmoid-shaped growth-model can precisely depict the lag, exponential and stationary phases of cell growth (Arbib et al., 2014).

2.4.1 Logistic model

The time dependent rise in biomass concentration following the Verhulst logistic function (Vogels et al., 1975) was represented by equation 1 (Eq. 1):

$$\frac{dX}{dt} = \mu_{max} \cdot \left(1 - \frac{X}{X_{max}}\right) \cdot X \quad (1)$$

where dX/dt is the microalgae growth rate (day^{-1}), μ_{max} is the microalgae maximum specific growth rate (day^{-1}), X and X_{max} refer to cell absorbance at time t and at the maximum, respectively.

The temporal increase of cell absorbance ($X(t)$) was determined by integrating the above equation (Eq. 1), as expressed by the logistic equation (Eq. 2) (Mohamed et al., 2014; Chang et al., 2016):

$$X(t) = \frac{X_0 \cdot e^{\mu_{max} \cdot t}}{1 - \frac{X_0}{X_{max}} \cdot (1 - e^{\mu_{max} \cdot t})} \quad (2)$$

where X_0 is the initial cell-suspension absorbance at time 0 ($X_0 = 0.0425$).

2.4.2. Logistic with lag model

A new parameter setting of the logistic model (Eq. 2) was applied by Zwietering et al. (1990). They incorporated an additional term λ (day), related to the first phase (lag phase) of microalgae growth kinetics as shown in (Eq. 3):

$$X(t) = X_0 + \frac{X_{max} - X_0}{1 + e^{\left\{ \left(\frac{4 \cdot \mu_{max}}{X_{max} - X_0} \right) (\lambda - t) + 2 \right\}}} \quad (3)$$

2.4.3. Gompertz model

The modified Gompertz model including the previous term, λ (day) relevant to biomass concentration parameters was rewritten by Mohamed et al. (2014) through (Eq. 4):

$$X(t) = X_0 + (X_{max} - X_0) \cdot e^{-e^{\left\{ \left[\left(\frac{\mu_{max} \cdot e^{\lambda}}{X_{max} - X_0} \right) (\lambda - t) + 1 \right] \right\}}} \quad (4)$$

2.4.4. Baranyi and Roberts model

A new family of bacterial growth models proposed by Baranyi and Roberts (1994) applied the first order differential function for the time dependent specific growth rate, $\mu(t)$, as defined by (Eq. 5):

$$\mu(t) = \frac{1}{X} \cdot \frac{dX}{dt} = \mu_{max} \cdot \alpha(t) \cdot f(t) \quad (5)$$

where $\alpha(t)$ is the adjustment function that represents the inoculum adaptation in the culture.

The above expression was transposed to a microalgae growth model through a linear equation by Mohamed et al. (2014), as shown in (Eq. 6) and (Eq. 7):

$$X(t) = X_0 + \mu_{max} \cdot B(t) - \ln \left(1 + \frac{e^{(\mu_{max} \cdot B(t))} - 1}{e^{(X_{max} - X_0)}} \right) \quad (6)$$

where

$$B(t) = t + \frac{1}{\mu_{max}} \cdot \ln \left(e^{-\mu_{max} \cdot t} + e^{-\mu_{max} \cdot \lambda} - e^{-\mu_{max} \cdot (t + \lambda)} \right) \quad (7)$$

2.4.5. Statistical treatment

A Matlab algorithm was carried out and applied to identify the parameters of Eqs. 2, 3, 4, 6 and 7, via the fitting procedure and to compare the experimental data and the fitted ones. The fitting procedure of *Tetraselmis* sp. growth data was established using non-linear least-square regression method. Four statistical parameters were chosen to check the model validation: the determination coefficient (R^2), the adjusted determination coefficient ($\text{Adj}R^2$), the sum of square error (SSE) and the root mean square error (RMSE).

All model coefficients were determined with 95% confidence level (i.e. P -value < 0.05).

2.5. Experimental design

Optimization experiments were carried out in two replicates and in 250 mL Erlenmeyer flasks with a working volume of 150 mL inoculated with 15 mL of culture. The chosen salinity, irradiance and pH values are reported in Table 1.

After growth (late logarithmic phase (15 days)), the biomass was harvested by centrifugation at $5000 \times g$ for 10 min and dried at 105°C for 24 hours until reaching a constant weight to quantify DW.

2.6. Statistical analysis

The influence of salinity, irradiance and pH on metabolite content of *Tetraselmis* sp. was assessed through a Box-Behnken Design (BBD) (Box and Behnken, 1960). Table 1 reports the selected values of variables by defining three experimental levels (-1: low, 0: medium and +1: high). The RSM was used to optimise pigment and starch productions as well as the interaction effects of the three factors on three responses: total chlorophyll, carotenoid and starch contents.

The multiple regression analysis was utilized for fitting the experimental results with a second order polynomial equation (Eq. 8).

$$\hat{Y}_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (8)$$

where \hat{Y}_i : calculated response variable; β_0 : constant; x_i and x_j : coded variable ranging between the minimum and the maximum levels of different factors; β_i , β_{ii} and β_{ij} : coefficients for the linear, quadratic, and interaction effects, respectively.

In this study, the experiment design was based on 15 combinations of variable values for optimising culture conditions (Table 2). Three replicates at central points were carried out to assess the experimental variance. The experiment design responses were the total chlorophyll (Y_1), carotenoid (Y_2) and starch (Y_3) contents. The model quality was evaluated by the R^2 and the Adj R^2 . The statistical significance of the model and the regression coefficients were tested by both Fisher-test (F -value and analysis of variance (ANOVA)) and Student-test (data not shown). Experimental results were analysed using STATISTICA Software 8.0 (Stat Soft. Inc 2008). The significant terms (regression coefficients and their interactions) correspond to those having a P -value < 0.05 (significance at 95%).

2.7. Flow cytometry (FCM)

Cultures of *Tetraselmis* sp. were analyzed using a LSR (Becton Dickinson Biosciences) flow cytometer equipped with a 488 nm argon laser, to optimize culture conditions with respect to cell red fluorescence intensity (chlorophyll content). The cell red fluorescence was collected through a 613/682 nm band-pass filter.

2.8. Microscopy

Total chlorophyll content in microalgae was quantified with an epifluorescent microscope, Observer Z1 (Carl Zeiss, Oberkochen, Germany), with a 470 nm excitation wavelength and collecting fluorescence at 700 nm.

3. Results and discussion

3.1. Biochemical composition of *Tetraselmis* sp.

The biochemical analysis of *Tetraselmis* sp. grown under basic environmental conditions (salinity: 40; irradiance: $84 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; pH 7) highlight high protein ($250 \pm 2.4 \text{ mg}\cdot\text{g}^{-1}$ DW), lipid ($195 \pm 2.1 \text{ mg}\cdot\text{g}^{-1}$ DW), and starch contents ($340 \pm 1.2 \text{ mg}\cdot\text{g}^{-1}$ DW) as well as significant amounts of ashes ($166.81 \pm 2.49 \text{ mg}\cdot\text{g}^{-1}$ DW), total chlorophyll ($18.08 \pm 1.16 \text{ mg}\cdot\text{g}^{-1}$ DW) and carotenoids ($0.025 \pm 0.002 \text{ mg}\cdot\text{g}^{-1}$ DW). The investigated *Tetraselmis* sp. showed also higher magnesium ($14.263 \pm 0.041 \text{ mg}\cdot\text{g}^{-1}$ DW), calcium ($5.263 \pm 0.160 \text{ mg}\cdot\text{g}^{-1}$ DW) and manganese ($0.270 \pm 0.006 \text{ mg}\cdot\text{g}^{-1}$ DW) contents than those of *Picochlorum* sp. (0.254 ± 0.020 , 1.478 ± 0.250 , $0.008 \pm 0.001 \text{ mg}\cdot\text{g}^{-1}$ DW, respectively) reported by Dahmen et al. (2014).

3.2. Experimental growth data and kinetic model assessment

The maximum cells absorbance (X_{max}) and the maximum specific growth rates (μ_{max}) for *Tetraselmis* sp. growth-kinetics were determined using logistic, logistic with lag and modified Gompertz and Baranyi–Roberts models. The X_{max} and μ_{max} for predicted cell growth-kinetics, as well as the curve fitting quality of the four models are reported in Table 3. Fig. 1 illustrates the representation by the four models of *Tetraselmis* sp. experimental growth-kinetics with microalgae grown in F/2-medium under basic environmental conditions (salinity: 40; irradiance: $84 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; pH 7). The maximum specific growth-rate varied from 0.034 to 0.416 day^{-1} . The R^2 and the $\text{Adj}R^2$ were $> 95\%$ for the four models. The RMSE and SSE parameters supported a good fitting by all of the used models and they singled out the best two models (Baranyi–Roberts and Gompertz models) by their lowest values (Table 3).

From statistical considerations, Baranyi–Roberts and modified Gompertz models provided RMSE values of 0.018 and 0.019 respectively, and the same SSE (0.004). The growth-kinetic parameters distinguished the Baranyi–Roberts model as the one providing the best fit of the experimental *Tetraselmis* sp. growth-curve. The predicted culture yield derived from the Baranyi–Roberts model gave the highest μ_{max} of 0.206 day^{-1} (0.0085 h^{-1}) and the largest maximum absorbance

value, $X_{max} = 0.483$. Consequently, the Baranyi–Roberts model was selected to describe the *Tetraselmis* sp. growth-kinetics (Fig. 1).

The μ_{max} was higher than that determined by Mohamed et al. (2014) for *Tetraselmis* sp. FTC209 grown under the photoautotrophic conditions in W-20 medium (0.0045 h^{-1}).

3.3. Effect of independent variables on pigments production

3.3.1. Total chlorophyll production optimization

The experimental results of different BBD levels are reported in Table 2. A quadratic equation was used to represent the outputs of total chlorophyll (Y_I) production model (Eq. 9):

$$\hat{Y}_I = 12.67 - 2.19 x_1 + 2.43 x_1^2 - 4.17 x_2 - 3.50 x_3 - 1.45 x_3^2 - 1.03 x_1 x_2 - 1.05 x_2 x_3 \quad (9)$$

The R^2 of this quadratic equation from ANOVA output (Table 4) is high (98.87 %) and it draws attention to a good correlation between the experimental values of total chlorophyll production and the related values derived from Eq. (9). This relationship is supported by the result displayed in Fig. 2A. Indeed, the y -intercepts of the mathematical adjustment does not differ significantly from zero and the slope is close to unit. ANOVA test (Table 4) shows the non-significance of lack of fit for total chlorophyll production response (P -value > 0.05). This proves that the presented model is valid and it can be adopted in this work. When total chlorophyll content was used as response, all the linear effects were very significant, and irradiance (x_2 , P -value = $0.001 < 0.05$) was the most significant factor (Table 4). In addition, negative regression coefficients of all the linear effect factors were observed (Eq. 9), which indicates that the total chlorophyll production increased at low levels of salinity, irradiance and pH. The interactions between x_1 and x_2 and between x_2 and x_3 , also showed their significant and negative effects on total chlorophyll synthesis by *Tetraselmis* sp. (P -value < 0.05).

The optimal value of total chlorophyll content and interactions between the environmental culture conditions are shown in the two dimensions (2D) and three dimensions (3D) response surface plots (Fig. 3A). When the pH was fixed at a low level (pH 6.5), the highest total chlorophyll content of *Tetraselmis* sp. (from 19 to 21.6 mg·g⁻¹ DW) was obtained at low salinity (between 20 and 25) and irradiance (from 84 to 100 μmol photons·m⁻²·s⁻¹). The maximum total chlorophyll level was reached at pH 6.5; salinity: 20 and irradiance: 84 μmol photons·m⁻²·s⁻¹ (Fig. 3A).

This result is similar to that found by Yao et al. (2013) with the following culture-factors: salinity: 27; irradiance: 150 μmol photons·m⁻²·s⁻¹ being under nitrogen repletion. Our results showed an increase of total chlorophyll biosynthesis when cells were exposed to low salinity. In agreement with our results, El-Kassas and El-Sheekh (2016) reported that excess salinity (40 ppm) decreased chlorophyll a content in *Tetraselmis tetrathele*. This was consistent with the photosynthesis inhibition under high salinity observed with *Tetraselmis* species and other microalgae. Actually, this could result from protein-synthesis inhibition (Kirst, 1990; Sudhir and Murthy, 2004; Yao et al., 2013). Murata et al. (2007) also proposed that photosynthesis inhibition at high salinity might result from protein *de novo* bio-synthesis inhibition, especially D1 protein that could impair the photo-damaged PSII repair cycle.

3.3.2. Carotenoids production optimization

The experimental data for carotenoids production (Y_2 , Table 2) were fitted by the following quadratic equation (Eq. 10), established by the multiple regression analysis:

$$\hat{Y}_2 = 0.017 + 0.005 x_1 - 0.005 x_2 + 0.005 x_2^2 - 0.006 x_3^2 + 0.010 x_1 x_2 - 0.004 x_2 x_3 \quad (10)$$

The R² value of 92.10 % linked to carotenoids production according to Eq. (10) was relatively high (Table 5). In addition, the y-intercepts of the mathematical adjustment do not differ significantly from zero and the slope is close to the unit (Fig. 2B). So, these results express a good

agreement between both the experimental and the predicted data. The value-validity analysis revealed that the lack of fit for carotenoid production response was not significant (P -value > 0.05) which indicated also the good quality of the model (Table 5).

Salinity (x_1), irradiance (x_2) and their interactive effect (x_1x_2) have the most significant effects on carotenoid biosynthesis (P -value ≤ 0.05) (Table 5). The quadratic effect of irradiance (x_2^2) and pH (x_3^2) and their interaction (x_2x_3) were also significant, but the linear effect of pH (x_3) (Eq. 10), was not significant.

When testing the high range of salinity (x_1) and irradiance (x_2) at pH 7, the carotenoids content increased rapidly with both factors (Fig. 3B). The optimized carotenoids content could thus be obtained at neutral pH with a high salinity: 40 and irradiance: $182 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

3.3.3. Effect of factors on carotenoids/chlorophylls ratio (Y_2/Y_1)

In Table 2, run 3, salinity (20) was low as well as the produced carotenoid/chlorophyll ratio (Y_2/Y_1) (0.000428). In run 4, irradiance and pH were equal to those of run 3, salinity was fixed at 40 and Y_2/Y_1 increased (0.005479) about 13-fold in response to salinity stress. Our results are in agreement with those reported by Ben Moussa-Dahmen et al. (2016) who demonstrated that high salinity can induce oxidative stress and decrease total-chlorophyll production by reducing PSII activity. Likewise, Pancha et al. (2015) reported that high salinity in the culture medium of the *Scenedesmus* sp. and *Nannochloropsis* sp. increased the carotenoid/chlorophyll *a* ratio suggesting an antenna-size reduction and a better photosynthetic-activity protection against the photo-oxidative damages. Previous studies reported that for cultures upon increased salinity, the decrease in photosynthesis quantum-efficiency resulted in low total chlorophyll synthesis due to the high-salinity negative effect on photo-phosphorylation and /or electron transport processes (Kirrolia et al., 2011).

The carotenoid/chlorophyll ratio was high in run 4 with salinity and light intensity at high level (level +1), but the Y_2/Y_1 ratio decreased about 4-fold by setting the same parameters at level 0 and pH remaining neutral (run 13). The cells photo-protection against photo-damages was thus

enhanced under excess of irradiance (Yao et al., 2013). In contrast, high photosynthetic activity was observed under favourable conditions (low Y_2/Y_1 ratio) leading to a better use of irradiance for photosynthesis (runs 13, 14 and 15). In this respect, many previous studies reported that under light stress, the transcripts of *PDS* and *PTOX* genes, phytoene desaturase (PDS) and plastid terminal oxidase (PTOX), increased simultaneously in *H. pluvialis*. Such studies suggested that these two enzymes may dehydrogenate phytoene, remove excess electrons and then prevent the excess ROS formation and the photosynthetic electron transport chain over-reduction (Li et al., 2010).

3.3.4. Models validation

To confirm the relevance of pigment models for predicting maximal pigment synthesis, additional experiments using optimized culture conditions were made. The total chlorophyll content reached $21.6 \text{ mg}\cdot\text{g}^{-1}$ DW, expressing about 1.2- fold increase with respect to the total chlorophyll content obtained under basic environmental condition.

Under the optimized culture conditions, the carotenoids production reached $0.042 \text{ mg}\cdot\text{g}^{-1}$ DW, a value 1.68 times higher than under basic environmental conditions. The RSM statistical method using BBD was successfully applied to study the effect of independent variables and to optimise pigments production.

3.4. Effect of independent variables on starch production

3.4.1. Starch production optimisation

The starch content response of *Tetraselmis* sp. as derived from BBD experiments (Table 2), was affected by the most environmental factors. Runs 3, 6 and 14 gave the best starch content (Y_3), highlighting the strong influence of the three factors on starch biosynthesis (Table 2).

The experimental starch production (Y_3) in *Tetraselmis* sp. was represented by (Eq. 11).

$$\hat{Y}_3 = 0.50 + 0.13 x_2 - 0.12 x_2^2 - 0.05 x_3 - 0.12 x_3^2 - 0.12 x_1 x_2 - 0.07 x_1 x_3 - 0.05 x_2 x_3 \quad (11)$$

The variance analysis of starch production model (Table 6) showed that Eq. 11 yielded a high Adj R^2 (97.57 %) and a high R^2 (99.13 %), leading to a distribution of observed and predicted starch content values (Y_3) aligned along a straight line whose slope = 1 (Fig. 2C). This slope, highlighted the good quality of the regression model consistently with the non-significant lack of fit (P -value = 0.4 > 0.05; Table 6).

According to (Eq. 11) and the related variance analysis (Table 6), starch production was significantly affected by the linear and quadratic effects of irradiance and pH, as well as the interactive effects of all factors (P -value < 0.05).

The 2D and 3D graphs (Fig. 3C), display the interactive effects of salinity and irradiance at pH 7 on starch production. The hyperbolic shape of both term contour-plot (x_1 x_2), revealed the perfect interaction between both variables. As shown in Fig. 3C, starch synthesis increased continuously with an increasing irradiance and a decreasing salinity. The maximum starch content (0.624 g·g⁻¹ DW) was attained at neutral pH, low salinity: 20 and high irradiance: 182 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

In this research, different irradiances were chosen to ameliorate starch synthesis as illustrated in Fig. 3C, which illustrates starch accumulation after rising irradiance from 84 to 182 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. This finding is consistent with previous studies demonstrating that certain levels of irradiance should be required for high level of carbohydrate production. Ho et al. (2012) and Sun et al. (2014) also documented an increase of the carbohydrate content when rising irradiance from 60 to 420 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and from 50 to 100 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, respectively. In run 10 (Table 2), the increased irradiance (182 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) disturbed the cell photo-protection mechanism, which decreased the photosynthetic activity and led to *Tetraselmis* sp. photo-inhibition. Brányiková et al. (2011) reported that *Chlorella vulgaris* (Beijerinck CCALA 924) produced high starch content (60% DW) under high irradiance (780 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). It was also mentioned by Yao et al. (2012) that high irradiance (200 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and sulfur deprivation could induce starch accumulation (62.1% DW) in *T. subcordiformis*, while, upon

nitrogen deprivation and irradiance at $50 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, this strain reached starch content of 49.3% DW. A high value of starch content (38.6% DW) was also reported by Yao et al. (2013) with *Tetraselmis subcordiformis* grown under standard salinity (27), irradiance of $150 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and under nitrogen repletion. This level increased up to 58.2% DW in response to low culture salinity (5.4), irradiance of $150 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and nitrogen deprivation.

Interestingly, in contrast to starch contents reported in the literature, our study achieved a higher content (62.4 % DW) when *Tetraselmis* sp. was exposed to the following conditions: salinity: 20, irradiance: $182 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and pH 7. In fact, the starch content produced by the isolated *Tetraselmis* sp. V2 strain was higher than those obtained with *Tetraselmis subcordiformis* (Yao et al., 2012; 2013), *Chlorella vulgaris* Beijerinck P 12 (Dragone et al., 2011), *Chlorella vulgaris* Beijerinck CCALA 924 (Brányiková et al., 2011) and *Dunaliella tertiolecta* ATCC 30929 (Ike et al., 1997) . These experiments suggest that *Tetraselmis* sp. has a relevant potential for starch production.

3.4.2. Effect of factors on starch and chlorophyll contents relationship

The low total chlorophyll ($7.3 \text{ mg}\cdot\text{g}^{-1}$ DW) production and starch content (35% DW) in run 4 under high salinity (40), high irradiance ($182 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and neutral pH could result from the extrusion of excess sodium ions (Na^+) from microalgae cytoplasm and therefore mitigate the negative effect of high salinity on cells. Indeed, Strizh et al. (2004) found two isomers of the Na^+ -ATPase in the green microalgae *Tetraselmis viridis* whose Na^+ pumps regulated the osmotic pressure. The Na^+ extrusion requires ATP which reduces ATP supply for the cells needs such as CO_2 fixation and this decreases biomass production and starch accumulation.

Similarly, Yao et al. (2013) showed that the high salinity of the culture medium decreased the growth of *Tetraselmis subcordiformis* and the protein and chlorophyll biosynthesis by this strain. These conditions induced starch catabolism by up-regulating respiration. The green microalgae *Tetraselmis subcordiformis* cultivated for 6 days under high salinity (54 and 67.5), $150 \mu\text{mol}$

photons·m⁻²·s⁻¹ and with nitrogen repletion, produced a lower starch level than under standard salinity (27). This phenomenon was explained by the allocation of fixed CO₂ from photosynthesis to the production of low-molecular-weight organic osmolytes to acclimate cells to hyper-osmotic stress. This hypothesis was supported by Richter and Kirst (1987) who reported that in hypersaline environments, the microalga *Platymonas subcordiformis* accumulated the mannitol compounds for osmo-regulation.

Chen and Jiang (2009) also found low starch content in *Dunaliella* sp. biomass obtained from culture media with high salinity. According to Khatoon et al. (2014), the *Tetraselmis* sp. showed higher carbohydrate contents when grown with a salinity of 30 than 40 and 20. In contrast, Siaut et al. (2011) reported that starch production in the freshwater microalgae *Chlamydomonas reinhardtii* raised by increasing salinity.

In run 6 (Table 2), total chlorophyll content reached a high value (15 mg·g⁻¹ DW) as well as starch content (50% DW) under high salinity (40), irradiance (133 μmol photons·m⁻²·s⁻¹, zero level) and pH 6. However, in run 8 where salinity and irradiance were maintained, increasing pH to 8 lowered the photosynthetic activity and starch synthesis.

In this study, at low salinity (20 and 30) and at high irradiance (182 μmol photons·m⁻²·s⁻¹), the starch production under alkaline medium (28% DW- run 12) was lower than those established under neutral pH (64% DW- run 3) and under acidic medium (49% DW- run 10) (Table 2). The present results are similar to those of Khatoon et al. (2014) who obtained higher carbohydrate content when *Tetraselmis* sp. was grown at pH 7.5. By contrast, *Chlorella ellipsoidea* yielded a high carbohydrate content at pH 9, while this level decreased at pH 10 and 4 (Khalil et al., 2010).

3.4.3. Model validation

In this study, cultivating *Tetraselmis* sp. under the optimized culture conditions for 15 days leads to a maximum (62%) starch content that was about twice that under basic environmental

condition. This result demonstrates the high efficiency of the Box-Behnken Design to achieve the highest starch production.

3.5. Flow cytometry and gravimetric for chlorophyll estimation

In this study, cell chlorophyll content was quantified through auto-fluorescence by flow cytometry. Fig. 4B displays the ratio of chlorophyll autofluorescence optimal value (= 79.36 arbitrary units (a.u.)) results related to the RSM runs. The analysis of total chlorophyll production by FCM for the RSM experimental conditions (Fig. 4B) showed an increase in total chlorophyll content correlated with a decrease of salinity (from run 2 to run 1, for example, Table 2), irradiance (from run 3 to run 1) and pH (from run 7 to run 5). These results are similar to those obtained after estimation of total chlorophyll content by the gravimetric method (Table 2 and Fig. 4A). The lowest total chlorophyll content was related to cultures of *Tetraselmis* sp. exposed to high irradiance, as seen in runs 4 and 12 (Fig. 4). These results are consistent with the morphological changes linked to the chlorophyll content in *Tetraselmis* sp. cells corresponding to conditions 1, 12 and 14 of RSM cultures. An important fluorescence intensity, cell size and number of chlorophyll biomolecules were reported for *Tetraselmis* cells cultivated under conditions 1 (salinity: 20; irradiance: $84 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; pH 7) but these granulation fluorescences decreased under conditions 14 (salinity: 30; irradiance: $133 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; pH 7) applied to cells cultivated under conditions 12 (salinity: 30; irradiance: $182 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; pH 8). Similar to findings reported in Table 2 and illustrated by Fig. 4, these results suggest that the total chlorophyll production increased after the decrease of pH, salinity and irradiance.

As shown in Fig. 5, a significant degree of correlation was achieved with an R^2 of 0.884 between the FCM red fluorescence signal (613/682 nm) and the total chlorophyll content estimated by the gravimetric method (653/666 nm). Hence, the present study illustrates the possibility of estimating total chlorophyll content in green microalgae in response to different environmental culture

conditions by FCM. However, the chlorophyll red-fluorescence signal, expressed in a.u., is highly dependent on the instrument and its setting. One step ahead in that direction could be to normalise the sample red fluorescence signal by that of standards beads added to the sample. Such normalisation would enable the comparison of observations made at different times or even with different instruments. Nevertheless, in order to benefit from the capacity of FCM, analyze a large quantity of samples cleanly and rapidly (Davey and Kell, 1996) and achieve a valid and rapid method for chlorophyll estimation in response to different environmental culture conditions, the calibration of the red fluorescence signal with conventional techniques (spectro-fluorometry, HPLC) will remain necessary.

4. Conclusions

The Baranyi-Roberts model was found to be efficient for fitting *Tetraselmis* growth kinetic through different statistical validations. *Tetraselmis* sp. showed also a high (62.4% DW) starch content under low salinity, high irradiance and neutral pH. Thus, it can be suitable for bioethanol production. The *Tetraselmis* cells yielded a total chlorophyll content of 21.6 mg·g⁻¹ DW under salinity: 20; irradiance: 84 μmol photons·m⁻²·s⁻¹ and pH 6.5. The carotenoid content was 0.042 mg·g⁻¹ DW under high salinity and irradiance and neutral pH. Therefore, the green microalga *Tetraselmis* sp. represents an attractive feedstock for human nutrition, aquaculture or bioethanol production.

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Figure captions

Fig. 1. Cells absorbance measurements and the four models (logistic, logistic with lag, modified Gompertz and Baranyi–Roberts, respectively) predicted values for *Tetraselmis* sp. growth cultured under basic environmental conditions (salinity: 40; irradiance: $84 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; pH 7; temperature: 25°C). Bars are means of duplications \pm SD

Fig. 2. Comparison between experimental and predicted data. Total chlorophyll (A), Carotenoid (B) and Starch (C) contents.

Fig. 3. Contour plots and the corresponding response surface plot showing the effect of environmental culture conditions on total chlorophyll content ($\text{mg}\cdot\text{g}^{-1}$ DW) at pH 6.5 (A), carotenoids content ($\text{mg}\cdot\text{g}^{-1}$ DW) at pH : 7 (B) and starch content ($\text{g}\cdot\text{g}^{-1}$ DW) at pH 7 (C).

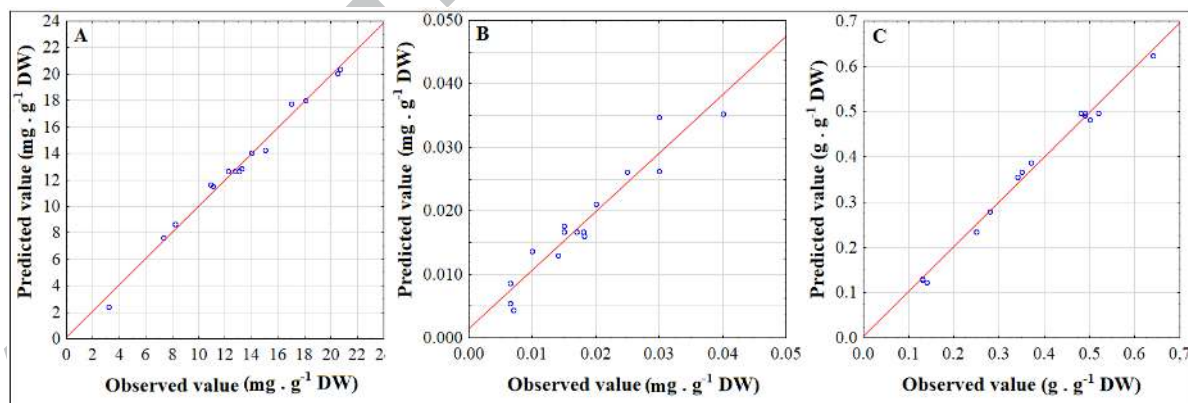
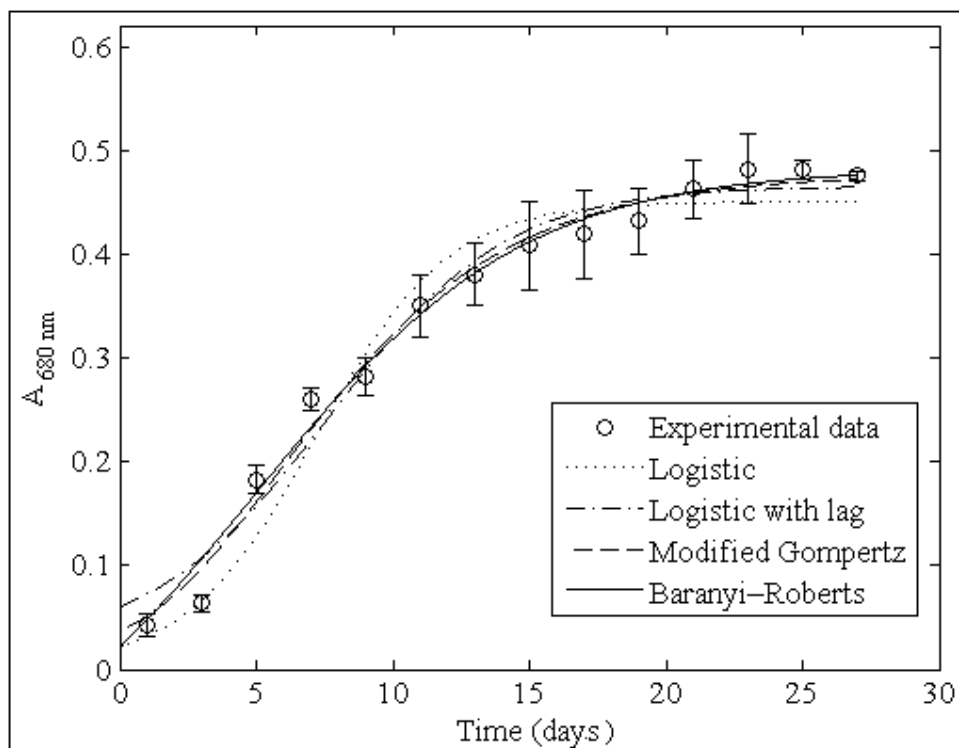
Fig. 4. The ratio of chlorophyll content / optimal value results (A) and the ratio of chlorophyll autofluorescence / optimal value results (B) related to the RSM runs.

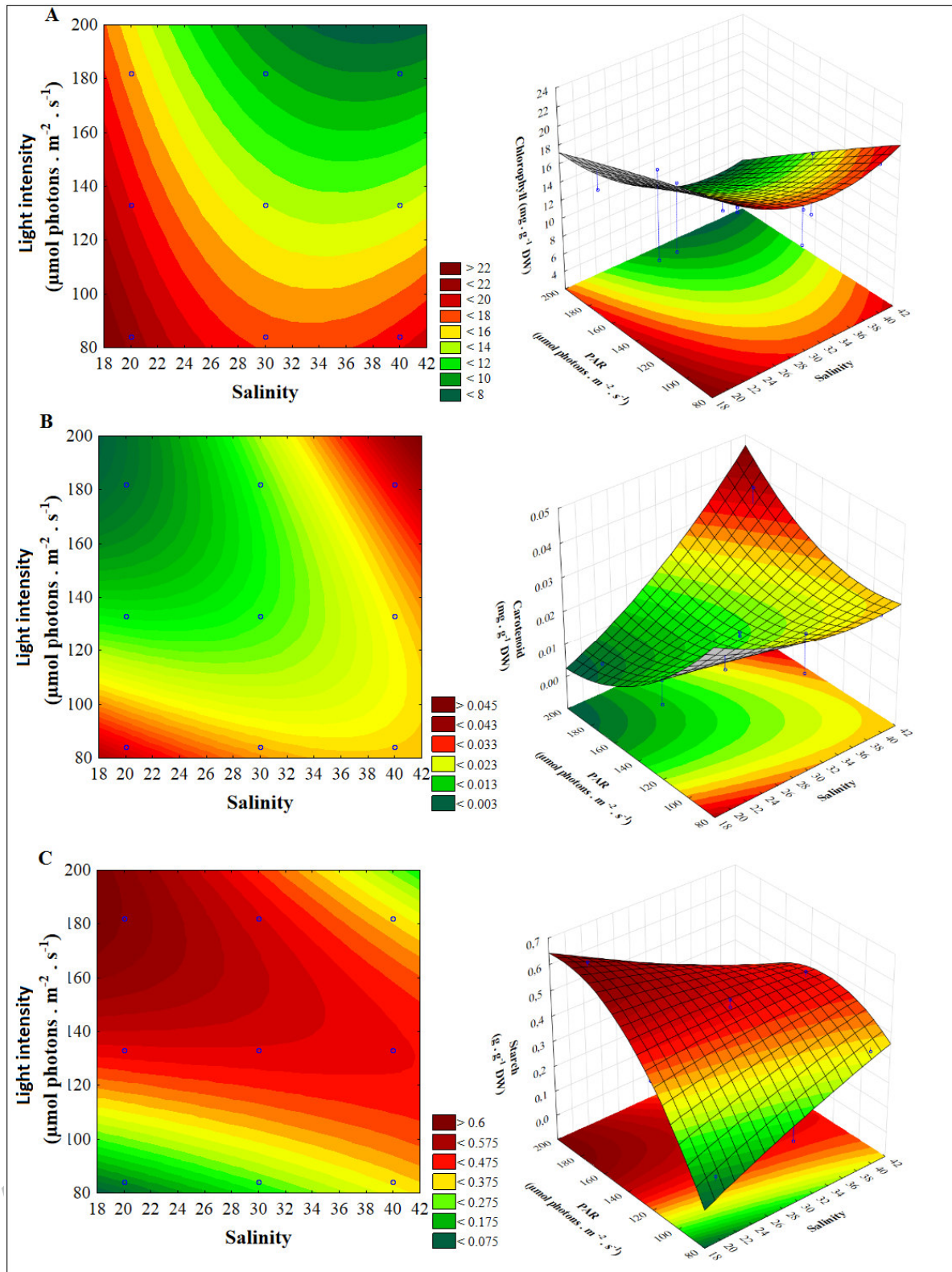
Fig. 5. Correlation between cellular contents of total chlorophylls estimated by gravimetric analysis and by flow cytometry ‘FL3 channel’ (excitation, 488; emission wavelengths, 613/682 nm).

Highlights

- Four non-linear models were evaluated for predicting cell-growth kinetic.
- Baranyi-Roberts model was selected to assess the microalga growth-kinetic values
- The greatest content-values achieved for chlorophyll and carotenoid compounds
- Combination of decreased salinity and light stress contributed to starch accumulation

ACCEPTED MANUSCRIPT





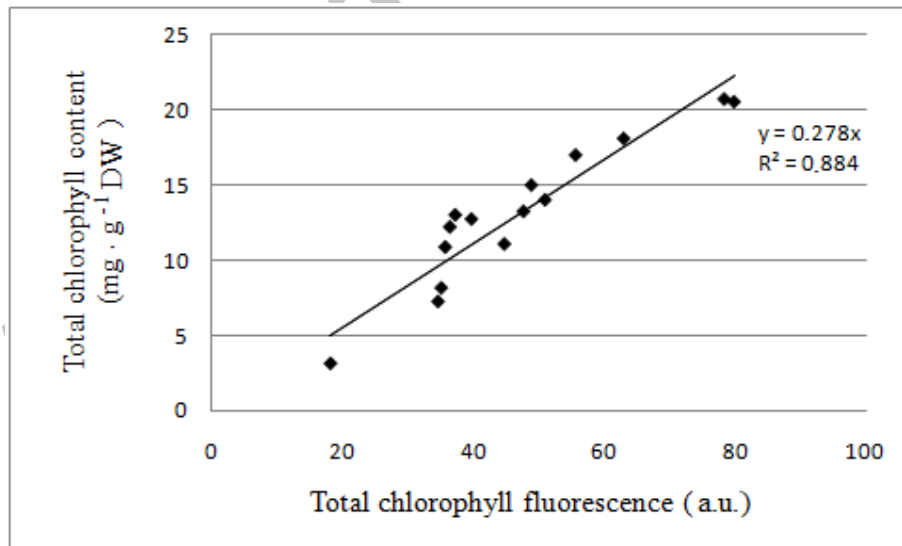
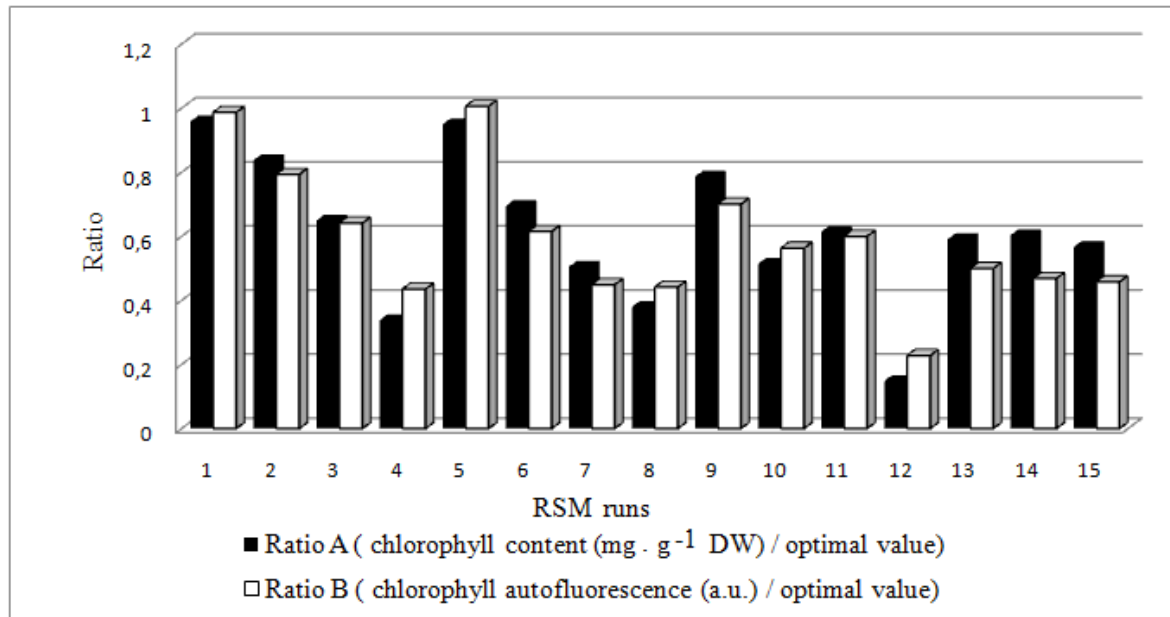


Table 1

Selected variable values and experimental levels for optimizing environmental conditions.

Factors	Symbol	Coded levels		
		1	0	+1
Salinity	x_1	20	30	40
Irradiance ($\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	x_2	84	133	182
pH	x_3	6	7	8

Table 2 Experimental results of Box-Behnken Design.

Run	Factors			Responses		
	x_1	x_2	x_3	Y_1	Y_2	Y_3
1	20 (-1)	84 (-1)	7 (0)	20.69	0.030	0.14
2	40 (+1)	84 (-1)	7 (0)	18.08	0.025	0.34
3	20 (-1)	182 (+1)	7 (0)	14.02	0.006	0.64
4	40 (+1)	182 (+1)	7 (0)	7.30	0.040	0.35
5	20 (-1)	133 (0)	6 (-1)	20.51	0.007	0.34
6	40 (+1)	133 (0)	6 (-1)	15.00	0.020	0.50
7	20 (-1)	133 (0)	8 (+1)	10.90	0.014	0.37
8	40 (+1)	133 (0)	8 (+1)	8.20	0.015	0.25
9	30 (0)	84 (-1)	6 (-1)	16.98	0.018	0.13
10	30 (0)	182 (+1)	6 (-1)	11.10	0.010	0.49
11	30 (0)	84 (-1)	8 (+1)	13.26	0.030	0.13
12	30 (0)	182 (+1)	8 (+1)	3.20	0.006	0.28
13	30 (0)	133 (0)	7 (0)	12.75	0.018	0.49
14	30 (0)	133 (0)	7 (0)	13.03	0.015	0.52
15	30 (0)	133 (0)	7 (0)	12.23	0.017	0.48

Y_1 : Total chlorophyll content ($\text{mg}\cdot\text{g}^{-1}$ DW); Y_2 : carotenoid content ($\text{mg}\cdot\text{g}^{-1}$ DW); Y_3 : starch content ($\text{g}\cdot\text{g}^{-1}$ DW).

Table 3Performance parameters and kinetic data of the four *Tetraselmis* sp. growth models.

Models	Logistic	Logistic with lag	Modified Gompertz	Baranyi–Roberts
Parameters				
Kinetic parameters				
X_{max} (A _{680nm})	0.451	0.466	0.477	0.483
μ_{max} (day ⁻¹)	0.416	0.034	0.035	0.206
λ (day)	-	1.145	1.028	5.367
Statistical parameters				
R ²	0.961	0.976	0.987	0.987
Adj R ²	0.957	0.971	0.985	0.985
SSE	0.012	0.007	0.004	0.004
RMSE	0.031	0.026	0.019	0.018

Table 4 Variance analysis of total chlorophyll production model.

Source	SS	Df	MS	F value	P value*
x_1	38.45	1	38.45	233.35	0.004*
x_2	139.36	1	139.36	845.64	0.001*
x_3	98.21	1	98.21	595.935	0.002*
x_1x_2	4.223	1	4.223	25.625	0.036*
x_1x_3	1.974	1	1.974	11.978	0.074
x_2x_3	4.368	1	4.368	26.505	0.035*
x_1^2	21.89	1	21.89	132.84	0.007*
x_2^2	0.025	1	0.025	0.152	0.733
x_3^2	7.789	1	7.789	47.268	0.020*
Lack of fit	3.309	3	1.103	6.694	0.132
Pure error	0.329	2	0.164		
Total	322.199	14			

R² = 98.87 %; AdjR² = 96.84 %; * Significant at P-value < 0.05.

Table 5 Variance analysis of carotenoid production model.

Source	SS	Df	MS	F value	P value*
x_1	0.0002	1	0.0002	96.30	0.01*
x_2	0.0002	1	0.0002	86.57	0.01*
x_3	0.00001	1	0.00001	5.79	0.13
x_1x_2	0.0004	1	0.0004	158.81	0.006*
x_1x_3	0.00004	1	0.00004	15.68	0.058
x_2x_3	0.00006	1	0.00006	25.08	0.037*
x_1^2	0.00004	1	0.00004	17.01	0.05
x_2^2	0.0001	1	0.0001	46.64	0.02*
x_3^2	0.0001	1	0.0001	55.47	0.017*
Lack of Fit	0.000099	3	0.000033	14.10	0.06
Pure error	0.000005	2	0.000002		
Total	0.0013	14			

$R^2=92.10\%$; $AdjR^2=77.87\%$; * Significant at P -value <0.05 .

Table 6 Variance analysis of starch production model.

Source	SS	Df	MS	F value	P value*
x_1	0.0003	1	0.0003	0.721	0.485
x_2	0.130	1	0.130	300.115	0.003*
x_3	0.023	1	0.023	53.336	0.018*
x_1x_2	0.060	1	0.060	138.519	0.007*
x_1x_3	0.020	1	0.020	45.230	0.021*
x_2x_3	0.011	1	0.011	25.442	0.037*
x_1^2	0.0004	1	0.0004	1.0000	0.422
x_2^2	0.052	1	0.052	119.313	0.008*
x_3^2	0.054	1	0.054	124.408	0.008*
Lack of fit	0.002	3	0.0007	1.634	0.401
Pure error	0.0008	2	0.0004		
Total	0.345	14			

$R^2=99.13\%$; $AdjR^2=97.57\%$; * Significant at P -value <0.05 .