CHEMICAL ENGINEERING TRANSACTIONS
VOL. 38, 2014
A publication of AIDIC
The Italian Association of Chemical Engineering
www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Taj Keshavarz

ISBN 978-88-95608-29-7; **ISSN** 2283-9216
DOI: 10.3303/CET1438031

Mathematical Modeling of the Effect of Iron on the Growth and the Bio-Oil Productivity of *Chlorella Vulgaris*

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It has been recently shown in the literature that specific strains of microalgae are capable to simultaneously increase their growth rate and lipid content when cultured under suitable concentrations of iron. A mathematical model describing the effect of iron on all the complex phenomena affecting growth rate and lipid accumulation of *C. Vulgaris* is proposed in this work. Model results are successfully compared with experimental data which confirm the positive effect of growing iron concentrations on lipid productivity of *C. Vulgaris*.

1. Introduction

The potential exploitation of microalgae as renewable resource for the production of biofuels is receiving a rising interest mostly driven by the global concerns related to the depletion of fossil fuels supplies and the increase of CO_2 levels in the atmosphere. However, in view of industrial scaling-up, the current microalgae based technology should be optimized in terms of lipid productivities as well as design/operating parameters. In this regard, the identification of the optimal design and operating parameters that allow microalgae to increase their lipid content while maintaining an higher growth rate, may be accomplished by exploiting suitable process engineering techniques (Concas et al., 2010; 2012; 2014). Among them, the most widespread one consists of the induction of nitrogen starvation phenomena in the culture. Beside nitrogen starvation, several methods are currently being investigated for the induction of lipid biosynthesis in microalgae. These techniques are based on cultivating algae under extreme pH and temperature conditions, high radiation, osmotic stress, and high heavy metals concentration. However, the side effect of all the techniques above is the lowering of microalgae growth rate. For this reason the identification of suitable operating conditions that allow to increase at the same time both lipid content and biomass growth rate is one of the main challenges in the field of biofuels production through microalgae. Among the micronutrients which can improve microalgae growth rate, iron is well known to be one of the most important. In fact, iron limitation can result in the reduction of the rate of CO_2 fixation and nitrogen assimilation of microalgae by limiting the light reactions of photosynthesis (Buitenhuis and Geider, 2010). Moreover, recent results reported in the literature (Mata et al., 2013) seem to confirm that when the initial iron concentration is increased within a specific range, a simultaneous augmentation of growth rate and lipid content can be observed for specific strains. While these results are promising in the light of the microalgae technology, to the best of our knowledge, no comprehensive simulation models, which account simultaneously for all the phenomena taking place during lipid accumulation in microalgae when varying iron concentration, have been so far proposed in the literature. Consequently, the goal of the present work is to develop a mathematical model to quantitatively describe the growth of microalgae and their lipid accumulation as a function of iron concentration in solution. In order to validate model results, specific experiments were performed with *C. Vulgaris*, where iron concentration in solution was suitably changed.

Please cite this article as: Concas A., Steriti A., Pisu M., Cao G., 2014, Mathematical modeling of the effect of iron on the growth and the bio-oil productivity of chlorella vulgaris, Chemical Engineering Transactions, 38, 181-186 DOI: 10.3303/CET1438031

2. Materials and Methods

2.1 Microorganism and Culture conditions

The fresh water unialgal strain *Chlorella vulgaris* was investigated in this work. Growth experiments were performed in Erlenmeyer flasks and Pyrex bottles under axenic conditions. Algae were cultured at room temperature and under a photon flux density of 100 μE m^{-2} s^{-1} provided by six 11 W white fluorescent tubes and a light/dark photoperiod of 12 h. The culture media volumes were 250 mL and 1 L for flasks and bottles, respectively, which were agitated by a magnetic stirrer at 300 rpm..

2.2 Growth medium

The culture medium consisted of a modified Kolkwitz medium (KTM-A) containing 2.5 g L^{-1} of KNO_3, 0.5 g L^{-1} of KH_2PO_4, 0.27 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 0.04 g L^{-1} of $CaCl_2 \cdot 2H_2O$, 1 g L^{-1} of $NaHCO_3$ and 1 mL of micronutrients solution. Iron was supplied in chelated form by adding to the culture medium suitable volumes from a solution containing 29.75 g L^{-1} of $Na_2EDTA \cdot 2H_2O$ and 24.90 g L^{-1} of $FeSO_4 \cdot 7H_2O$, respectively. Specifically, *C. Vulgaris* was cultivated in the above specified medium supplemented with $FeSO_4 \cdot 7H_2O$ at total iron concentration levels equal to 0.0, 10.0, 25.0 and 100 g_{Fe} m^{-3}.

2.3 Biomass concentration measurements

The growth of microalgae was monitored through spectrophotometric measurements of the culture media optical density (OD) at 560 nm wavelength (D560) with 1 cm light path. Biomass concentration C_b (g L^{-1}) was calculated from OD measurements using a suitable C_b vs. OD calibration curve.

2.4 Lipid extraction

In order to evaluate the lipid content of *C. Vulgaris*, the microalgae were first harvested and then centrifuged to obtain a wet biomass pellet characterized by a humidity of about 90 %wt/wt. Lipid extraction was performed directly on wet biomass. Subsequently the method proposed by Ibanez et al. (1998) was adopted for extracting lipids from microalgae through direct saponification.

3. Mathematical model

As shown in the experimental section, iron is added to the growth medium as $FeSO_4 \cdot 7H_2O$ mixed with suitable amount of $Na_2EDTA \cdot 2H_2O$. Several reactions taking place in solution are capable to allocate iron in different molecular species or complexes. Specifically, the relevant iron complexes which are formed as a result of the complexation-chelation phenomena taking place in solution can be defined as Fe^{2+} and Fe^{3+} free ions and inorganic complexes as well as Fe^{3+} organically chelated ones whose total concentrations will be hereafter indicated by [Fe(II)], [Fe(III)] and [Fe(III)-EDTA], respectively. In the conventional model used for simulating the effects of iron on microalgae growth, the corresponding rate is proposed to depend upon the concentration of unchelated Fe(III) species (Concas et al., 2014). According to this model, iron is bound as Fe(III) species to a cell surface ligand and is subsequently transferred across the plasma membrane. It is assumed that unchelated inorganic Fe(III) species are in chemical equilibrium with the chelate Fe(III)-EDTA through dissociation and chelation reactions whose rate constants are k_d and k_f, respectively. Upon illumination, photoreduction of Fe(III)-EDTA can also take place with a constant rate k_{hv} which leads to the production of unchelated reduced iron species, i.e. Fe(II), which are in turn quickly oxidized to inorganic Fe(III) with a rate constant k_{ox}. Under the assumptions above, the relevant material balances describing evolution of iron species in the bulk liquid can be written as follows:

$$\frac{d\left[Fe(II)\right]}{dt} = k_{hv}\left[Fe(III)-EDTA\right] - k_{ox}\left[Fe(II)\right] \quad (1)$$

$$\frac{d\left[Fe(III)\right]}{dt} = k_d\left[Fe(III)-EDTA\right] + k_{ox}\left[Fe(II)\right] - k_f\left[Fe(III)\right][EDTA] - \frac{v_{Fe}C_x}{MW_{Fe}} \quad (2)$$

$$\frac{d\left[Fe(III)-EDTA\right]}{dt} = -k_d\left[Fe(III)-EDTA\right] + k_f\left[Fe(III)\right][EDTA] - k_{hv}\left[Fe(III)-EDTA\right] \quad (3)$$

along with the corresponding initial conditions $[Fe(II)]^0=[FeSO_47H_2O]^0$, $[Fe(II)]^0=0$ and $[Fe(III)\text{-}EDTA]^0=0$ at t=0. Here, C_x represents the concentration of the non lipidic fraction of algal biomass. The term [EDTA] includes both the concentration of actually free EDTA ions as well as the EDTA ions that are bound to cationic species other than iron, namely Na^+, Ca^{2+}, etc., and thus can be evaluated as follows:

$$[EDTA] = \left[Na_2EDTA \cdot 2H_2O\right]^0 - \left[Fe(III)-EDTA\right] \quad (4)$$

In order to simulate microalgal growth and lipid production, the following assumptions are taken into account. The microalgal cell consists of two distinct compartments, i.e. the non lipidic fraction whose

concentration is indicated by C_x (g_{dw} m^{-3}) and the lipidic fraction whose concentration is indicated by C_ℓ (g_{dw} m^{-3}). Thus, the total concentration of algal biomass, C_b is the sum of the concentrations of the two compartments. By considering that under the experimental conditions adopted microalgae growth can be limited by nitrogen, iron or light, according to the Liebig's law of the minimum, the material balance for the batch growth of the non lipidic fraction of microalgae can be written as follows:

$$\frac{dC_x}{dt} = \mu \cdot C_x = \min\left\{ \mu_{\max}\left(1 - \frac{Q_N^{\min}}{q_N}\right) ; \mu_{\max}\left(1 - \frac{Q_{Fe}^{\min}}{q_{Fe}}\right) ; \frac{P}{\chi} \right\} \cdot C_x \tag{5}$$

along with the initial condition $C_x=C_x^0$ at t=0. The iron and nitrogen quotas appearing in eq. (5), namely q_{Fe} (g_{Fe} g_{dw}^{-1}) and q_N (g_N g_{dw}^{-1}), vary with time according to the following equations (Concas et al., 2014):

$$\frac{dq_{Fe}}{dt} = \nu_{Fe} - \mu \cdot q_{Fe} = \nu_{Fe}^{\max} \cdot \left(\frac{Q_{Fe}^{\max} - q_{Fe}}{Q_{Fe}^{\max} - Q_{Fe}^{\min}}\right) \cdot \frac{C_{Fe(III)}}{K_{Fe} + C_{Fe(III)}} - \mu \cdot q_{Fe} \tag{6}$$

$$\frac{dq_N}{dt} = \nu_N - \mu \cdot q_N = \nu_N^{\max} \cdot \left(\frac{Q_N^{\max} - q_N}{Q_N^{\max} - Q_N^{\min}}\right) \cdot \frac{C_N}{K_N + C_N} \cdot \gamma_{Fe} - \mu \cdot q_N \tag{7}$$

along with the corresponding initial conditions: $q_{Fe}=q_{Fe}^0$ and $q_N=q_N^0$ at t=0, respectively. The terms v_{Fe}, v_{Fe}^{max} (g_{Fe} $g_{dw}^{-1}min^{-1}$) and v_N, v_N^{max} (g_N $g_{dw}^{-1}min^{-1}$) in equations (6) and (7) represent the effective and the maximum uptake rate of iron and nitrogen, respectively. Moreover $C_{Fe(III)}$ and C_N are the total mass concentrations, respectively, in liquid bulk while the symbols K_{Fe} (g_{Fe} m^{-3}) and K_N (g_N m^{-3}) refer to the corresponding half saturation constants. Finally the function expressing the cell satiation state, reported within parenthesis in eqs. (6) and (7), is zero when the cell quota q_{Fe} (or q_N) is equal to its maximum allowed value, i.e. Q_{Fe}^{max} (or Q_N^{max}), while it reaches the value 1 when q_{Fe} (or q_N) approaches the minimum allowed value, i.e. Q_{Fe}^{min} (or Q_N^{min}), below which no cell growth can take place. It is worth noting that the maximum uptake rate of nitrogen appearing in eq. (7) is multiplied by a term γ_{Fe}, which is a function of the iron cell quota, in order to take into account that nitrogen uptake is greatly enhanced by intracellular iron. According to Ward et al (2012), the term γ_{Fe} can be expressed as follows:

$$\gamma_{Fe} = \frac{q_{Fe} - Q_{Fe}^{\min}}{Q_{Fe}^{\max} - Q_{Fe}^{\min}} \cdot \frac{Q_{Fe}^{\max}}{q_{Fe}} \tag{8}$$

The net carbon fixation rate of microalgae through photosynthesis P (g_C g_{dw}^{-1} min^{-1}), appearing in equation (5), has been evaluated as Poisson function of average light intensity (I_{av}) modified in order to take into account the dependence upon the iron quota of the initial slope of the photosynthesis-irradiance (P-I) curve, namely ($\beta \cdot \gamma_{Fe}$), and the chlorophyll-a cell quota q_{Chla} (Ward et al., 2012):

$$P = P_C^{\max} \cdot \min\{\gamma_{Fe}, \gamma_N\} \cdot \left[1 - \exp\left(-\frac{\beta \cdot \gamma_{Fe} \cdot q_{Chla} \cdot I_{av}}{P_C^{\max} \cdot \min\{\gamma_{Fe}, \gamma_N\}}\right)\right] \tag{9}$$

where P_C^{max} represents the maximum carbon-specific light-saturated photosynthetic rate while the term γ_N can be written according to Ward et al. (2012) as follows:

$$\gamma_N = \frac{q_N - Q_N^{\min}}{Q_N^{\max} - Q_N^{\min}} \tag{10}$$

Since the nitrogen uptake by algae leads to its consumption in the growth medium, the following mass balance can be written to describe the time evolution of its concentration in the bulk liquid phase:

$$\frac{dC_N}{dt} = -\nu_N \cdot C_x \tag{11}$$

along with the initial condition $C_N=[NO_3^-]^0$ at t=0, where $[NO_3^-]^0$ is the initial molar concentration of nitrates in the growth medium. In this regard, it is worth noting that the material balances describing the evolution of iron species in the liquid bulk have been already reported in equations (1-3) in terms of molar concentrations. In eq. (9), the term q_{Chla} (g_{Chla} g_{dw}^{-1}) represents the amount of chlorophyll-a (Chla) per unit of microalgal biomass, namely the chlorophyll cell quota, which can vary with time due to photo-acclimation phenomena taking place as a result of light intensity changes within the culture. Moreover, chlorophyll content evolution is affected by nitrogen uptake and iron availability and thus can be evaluated according to Concas et al. (2013) as follows:

$$\frac{dq_{Chla}}{dt} = \theta_{Chla}^{N,\max} \cdot \left(\frac{P}{\beta \cdot \gamma_{Fe} \cdot q_{Chla} \cdot I_{av}}\right) \cdot \nu_N - \mu \cdot q_{Chla} \tag{12}$$

where $\theta_{Chla}^{N,max}$ (g_{Chla} g_N^{-1}) is the maximum amount of chloropyll synthesized for unit weight of nitrogen assimilated while I_{av} is the average light intensity. The variation of I_{av} in the conical volume of microalgal culture within the flasks has been evaluated according to Concas et al. (2013) as:

$$I_{av}(t) = \frac{1}{h} \cdot \int_0^h \frac{2 \cdot I_0(t)}{R(z) \cdot \tau_a \cdot C_b(t) \cdot \pi} \left[1 - \int_0^{\frac{\pi}{2}} \cos(\omega) \cdot \exp\left(-2 \cdot R(z) \cdot \tau_a \cdot C_b(t) \cdot \cos(\omega)\right) \cdot d\omega \right] \cdot dz \quad (13)$$

where τ_a is the optical extinction coefficient, h is the height of the conical volume of the flask and R(z) is the radius of the flasks at an height equal to z. In addition, I_0 is the incident light intensity which varies with time as a square wave having amplitude equal to 100 $\mu E\ m^{-2}\ s^{-1}$ and a photoperiod T_p equal to 12 h.
In order to quantitatively describe the lipid production by microalgae, it is assumed that it can be either primary, i.e. directly related to metabolism and growth, or secondary, i.e. not coupled to cellular growth and typically occurring in response to a stress or external stimuli. Specifically, the material balance for lipid production can be expressed as follows (Concas et al., 2014):

$$\frac{dC_l}{dt} = \alpha \cdot \frac{dC_x}{dt} + \eta \cdot C_x = \frac{q_l^0}{1 - q_l^0} \cdot \frac{dC_x}{dt} + \eta_m \cdot C_{Fe(III)} \cdot C_x \quad (14)$$

along with the initial condition $C_\ell = q_\ell^0 C_b^0$, where q_ℓ^0 is the initial lipid content of biomass, C_b^0 is the initial concentration of the biomass including non-lipidic and lipidic fraction, while α (g_{dw} g_{dw}^{-1}) and η (g_{dw} g_{dw}-1 min^{-1}) represent the primary and secondary production rate, respectively. The value of the parameter α, has been evaluated accordingly to Concas et al. (2013) as shown in Eq. (14) as a function of the initial lipid content of algae, i.e. q_ℓ^0. Finally, in this first attempt to model the effect of iron on lipid accumulation, the non-growth associated production rate of lipids η has been evaluated as a linear function of the concentration of the bioavailable iron, i.e $C_{Fe(III)}$ in the medium (cf. eq. (14)). The system of ordinary differential equations (1-3, 5-7, 11-12, 14) was numerically integrated as an initial value problem through the Gear method by means of the subroutine DIVPAG of the standard numerical libraries (IMSL). Subsequently, the total biomass concentration C_b can be evaluated as the sum of the lipidic, C_ℓ and non-lipidic, C_x concentration of microalgae, while their lipid content at each cultivation time can be obtained as $q_\ell = C_\ell / C_b$.

4. Results and discussion

The effect of iron concentration on the growth kinetics of *C. Vulgaris* and its lipid content was quantitatively interpreted in this work. In order to demonstrate the reliability of the proposed model, the corresponding results were compared with suitable experimental data. To this aim, specific experiments were carried out by cultivating *C. Vulgaris* in batch stirred flasks where the initial concentration of dissolved iron was suitably changed while keeping fixed the initial molar ratio between [$FeSO_4.7H_2O$] and [$Na_2EDTA{\cdot}2H_2O$]). In Fig. 1a the time evolution of total biomass concentration obtained when cultivating *C. Vulgaris* in absence of dissolved iron is shown. It can be observed that the culture starts growing without showing a significant lag phase despite the absence of iron in solution. Thus, the initial intracellular content of iron was high enough to permit *Chlorella* cells to grow and duplicate for a specific time interval, as shown in Fig. 1a. In fact, it can be observed that culture grows until about 10 days, while after 20 days of cultivation it reaches a sort of “plateau” when the biomass concentration is about 320 g m^{-3}. Such stationary phase is reached due to the consumption of the available intracellular iron, since, being $C_{Fe(III)}$=0, the iron cell quota q_{Fe} decreases according to eq. (6), until the minimum allowed value Q_{Fe}^{min} is reached. Under such conditions, microalgae growth is stopped according to eq. (5). In Fig. 1a, the comparison between experimental data and model fitting is also shown. All model parameters values are taken from the literature (cf. Concas et al., 2014) except for the initial slope of the iron-dependent photosynthesis irradiance (P-I) curve β, the maximum iron uptake rate v_{Fe}^{max} as well as the maximum rate of secondary (iron induced) production of lipids η_m which have been suitably tuned through a nonlinear fitting procedure in order to quantitatively interpret the experimental data. Specifically, their values are obtained through direct comparison, based on the least squares methodology, of model results with experimental data in terms of biomass concentration evolution during microalgal growth as well as of lipid content at the end of cultivation. In particular, it is worth noting that the only parameter which was tuned to fit the experimental data of Fig. 1a and the corresponding final lipid content of Fig. 1b, obtained in absence of dissolved iron, was β since, under this operating conditin, the model does not require the knowledge of the values of v_{Fe}^{max} and η_m. The relative error obtained by the fitting procedure is equal to 6.2% while the value of β

results to be $1.05 \cdot 10^{-3}$ (g_C m^2 g_{Chla}^{-1} μE^{-1}). From Fig. 1a it is worth noting that the proposed model quantitatively captures the growth trends from day to day in absence of iron.
In order to evaluate the effect of iron on microalgae growth rate and lipid accumulation further experiments were performed by setting the initial concentration of total iron in solution $C_{Fe}^{tot,0}$ equal to 25 g_{Fe} m^{-3}. From the analysis of experimental data shown in Fig. 1a it can be observed that under such operating condition the culture keeps growing during the whole investigated time interval. Such a behavior is due to the fact that microalgae can prevent the decrease of their iron cell quota by taking advantage of iron available in solution. Therefore, the iron cell quota remains always greater than the minimum value Q_{Fe}^{min} as a result of the uptake of iron from solution. In Fig. 1a, the comparison between experimental data and model fitting, for the case where $C_{Fe}^{tot,0}$ is equal to 25 g_{Fe} m^{-3}, is also shown. In this case the values of β obtained as above reported was kept fixed while the kinetic parameters v_{Fe}^{max} and η_m were suitably tuned in order to fit the experimental data obtained in the presence of iron, i.e. using both the experimental data shown in Figs. 1a and 1b corresponding to a total initial iron concentration of 25 g_{Fe} m^{-3}. The relative error obtained by the fitting procedure is equal to about 6% while the fitted values of v_{Fe}^{max} and η_m were $5.5 \cdot 10^{-9}$ (g_{Fe} g_{dw}^{-1} min^{-1}) and $1.11 \cdot 10^{-7}$ (g_{dw} m^3 g_{dw}^{-1} min^{-1} g_{Fe}^{-1}), respectively.

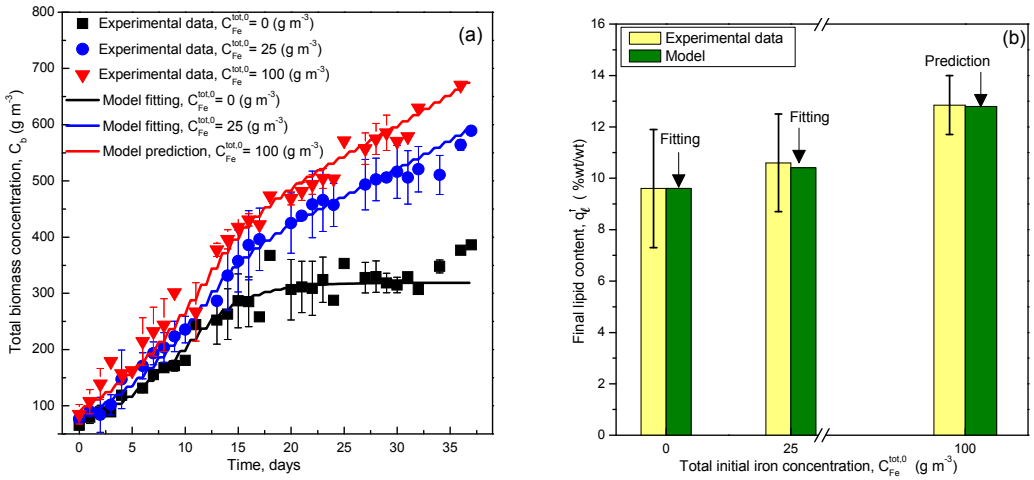

Figure 1 Comparison between model results and experimental data in terms of biomass concentration evolution (a) and final lipid content of algae (b) when varying the initial concentration of total dissolved iron.

As it can be observed from Fig. 1a, the experimental results obtained under $C_{Fe}^{tot,0}$=25 g_{Fe} m^{-3} are well captured by the proposed model also in terms of the change of the slope of the growth curve which is due to the fact that, under iron-replete conditions, at the start of the experiment, nitrogen becomes the main limiting nutrient and thus the value of the growth rate is dictated by nitrogen cell quota according to eq. (5). However, as the culture grows, its optical density increases and consequently, the light intensity that is available for microalgae, decreases. As a result the carbon specific photosynthetic rate, *P* decreases according to eq. (9) so that when very low values are reached, the culture becomes light-limited instead of nitrogen-limited and consequently the variation law of the growth rate changes according to eq. (5). This phenomenon provokes the change in the slope of the growth curve related to the experiment with $C_{Fe}^{tot,0}$=25 g m^{-3} which may be observed in Fig. 1a after 15 days of cultivation. From Fig. 1b, it can be seen that also the final lipid content is well fitted by the proposed model when total initial iron concentration in solution is equal to 25 g_{Fe} m^{-3}. Moreover, the experimental data confirm that total lipid content increases when the iron concentration in solution is augmented. Specifically, the lipid content increased from 9.6% to 10.6% by dry weight when the total initial iron concentration in solution was increased from 0 to 25 g_{Fe} m^{-3}, respectively. To test the predictive model capability, the experimental data, obtained when cultivation was carried out by starting with total iron concentration equal to 100 g_{Fe} m^{-3}, were simulated. It is important to remark that in this case no parameter has been adjusted. The comparison between model predictions and experimental data is shown in Fig. 1a in terms of biomass concentrations as a function of time as well as in Fig. 1b for the case of final lipid content. As it can be seen, the proposed model predicts the experimental behaviour both in terms of biomass concentration and final lipid content with sufficient accuracy. Moreover, when comparing the experimental results with those ones obtained with total iron concentration equal to 25 g m^{-3}, it can be observed that, while the growth rate increases slightly (cf. Fig. 1a) a significant augmentation of the final lipid content can be observed. With the aim of identifying the mechanisms and

interactions that affect biomass and lipid productivity, a sensitivity analysis on model parameters is performed. In particular, in Fig. 2 it is shown the effect of tuneable model parameters β, v_{Fe}^{max} and η_m respectively, on the final biomass concentration and the final lipid content obtained for the case where $C_{Fe}^{tot,0}$=25 g m^{-3}.

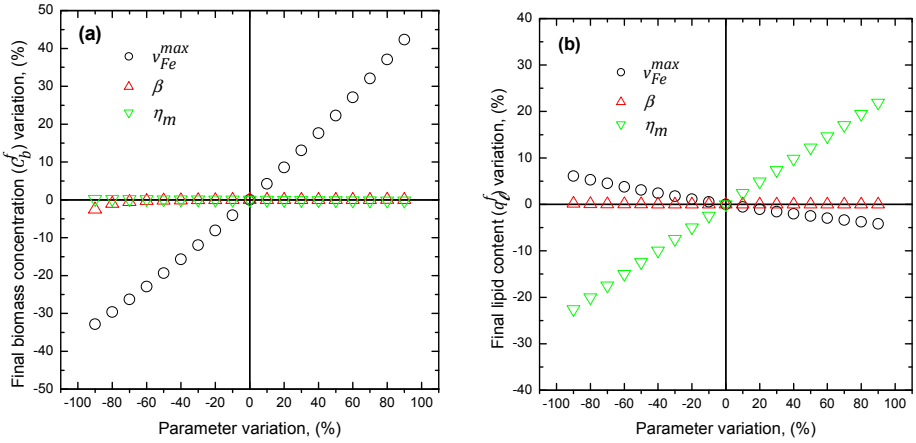

Figure 2. Model sensitivity analysis in terms of percentage variation of final biomass concentration (a) and final lipid content (b) with respect to the values obtained for the base case (i.e. $C_{Fe}^{tot,0}$=25 g m^{-3}), when the considered parameters values are modified up to ±100% with respect to the values obtained by tuning experimental data.

It may be observed from Fig. 2a that when the model output C_b^f is considered, the model is highly sensitive to the variations of the parameter v_{Fe}^{max}. On the contrary, from Fig. 2b it can be seen that, when the final lipid content is accounted for as model output, the most important parameter is represented by η_m. In both cases, the model parameter β does not seem to significantly affect the considered model outputs. These results highlight that while microalgae growth rate is strongly influenced by the amounts of iron that are uptaken by the cells, the lipid synthesis is influenced mostly by the iron concentration remaining in solution, which in fact has an important effect on the establishment of oxidative environment that, in turn, is capable to trigger the lipid synthesis.

5. Concluding remarks

A comprehensive mathematical model for the simulation of the effect of dissolved iron on the growth and lipid accumulation of *C. Vulgaris*, is proposed in this work. By comparing model results with experimental data a good matching is obtained. Moreover, the experimental results have shown that a simultaneous increase of the growth rate and lipid content of *C. Vulgaris* can be achieved by increasing the dissolved iron concentration within a specific range. Therefore, the mathematical model might permit to identify the iron concentrations that optimize the lipid productivity of *C. Vulgaris* in batch photobioreactors.

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