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Modeling and Experimental Investigation of the Effect of Nitrogen Starvation and pH Variation on the Cultivation of the Extremophile Microalga *Coccomyxa Melkonianii* SCCA048

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The effects of nitrogen concentration and pH on the growth kinetics of the extremophile microalgal strain *Coccomyxa melkonianii SCCA 048* in multiwell and batch photobioreactors are investigated in this work. Moreover, the experimental results were successfully interpreted by a simple, mathematical model which represents a starting point towards the development of a suitable tool for the design, control, and optimization of large scale photobioreactors where *C. melkonianii* would be cultivated. On the basis of fatty acids methyl esters (FAMEs) profile, potential properties of biodiesel have been predicted through suitable software.

1. Introduction

Extremophile microalgae have the ability to grow under specific extreme conditions that might help reducing contamination by other microorganisms. Accordingly, these algae could be cultivated in open ponds with lower contamination risks and exploit the economic advantages of using open raceways (Soru *et al.,* 2018a). Under abiotic stress, microalgae undergo strong metabolic changes in their physiology, and biochemistry for survival (Bermejo *et al.*, 2018). Microalgae exposed to nutrient starvation, excess of salt, UVA (ultraviolet A) light, or metal toxicity, show an increasing intracellular concentration of lipids, carotene, and antioxidant enzymes (Bermejo *et al.*, 2018). *Coccomyxa melkonianii* SCCA 048 is a novel heavy-metal-resistant microalga (Malavasi *et al.*, 2016) belonging to the Sardinian Culture Collection of Algae (SCCA), and is currently under investigation in the framework of a regional COMISAR project whose goal is to identify promising strains for the development of several technologies. This extremophile strain is currently under investigation to evaluate its ability to grow in culture media with different concentrations of heavy metals as Iron Sulfate ($FeSO_4$). In previous laboratory studies, this strain has shown to grow well in the pH range 4.0–8.0, with an optimal value for its growth at pH 6.8 (Soru *et al.*, 2018b). Under nitrogen manipulation, an increase in the total lipid content, including changes in the fatty acid methyl esters (FAME) profile, was observed (Soru *et al.*, 2018a). Moreover, Soru *et al.*, (2018a) proposed a novel mathematical model of the growth of *C. melkonianii* in batch photobioreactors. In this study, the pH-dependent growth kinetics of *C. melkonianii* obtained in multiwell devices has been compared with suitable batch experiments, with the aim of obtaining crucial information about the profitability of the cultivation of this strain in large scale devices under extreme conditions. Because lipid synthesis is affected by nitrogen availability, the nitrate-dependent growth kinetics in batch experiments was also investigated. The lipids synthesized during growth in nitrogen replete and starvation condition, were quantitatively characterized and profiled in terms of fatty acids composition to verify whether valuable chemicals could be produced through this alga. Finally, on the basis FAMEs profile, a wide range of biodiesel

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fuel properties have been predicted through suitable software and for the potential exploitation in the industrial system of Sardinia (Italy)

2. Materials and methods

The freshwater microalgal strain used in this work is *Coccomyxa melkonianii* SCCA 048, isolated from highly contaminated mine waters of river Irvi (SW Sardinia, Italy) (Malavasi *et al.*, 2016), and maintained at the Sardinian Culture Collection of Algae (SCCA) under axenic conditions (Malavasi and Cao, 2015). Preliminary 72 h growth test screenings were performed in 24-welled multiwell plates by imposing different nitrate concentrations and different pH values (Soru *et al*., 2018a). Batch experiments were then carried out in 2 L Pyrex bottles under different nitrogen concentrations (Soru *et al*., 2018a) as well as under specific pH values, i.e. 4.0, 6.8, and 8.0, that were kept constant during the cultivation (Soru *et al*., 2018b). Lipid extraction and analysis of fatty acids methyl esters were performed according to the method reported elsewhere (Soru *et al.*,2018a, b).

3. Mathematical model

The model is based on the consideration that the main limiting factors affecting microalgae growth during the experiments were nutrients concentration, light intensity, and medium pH, respectively. Accordingly, the mass balance of microalgal biomass under batch conditions could be written as follows

$$\frac{dC_x}{dt} = \mu_{\max} \cdot \frac{C_{NO_3^-}}{C_{NO_3^-} + K_{NO_3^-}} \cdot \frac{I_{av}^n}{I_{av}^n + I_K^n} \cdot h(pH) C_x - \mu_d \cdot C_x \tag{1}$$

along with the initial condition $C_x = C_x^0$. The symbol $\mu_{max}(h^{-1})$ is the maximum specific growth rate, $C_x\,(gL^{-1})$ represents the microalgal biomass concentration and $\mu_d\,(h^{-1})$ is the mass loss rate, i.e. a term which takes into account for all the phenomena that can lead to the reduction of microalgae cell mass such as catabolic and respiratory losses, apoptosis, lysis, etc. (Concas *et al*., 2013). The terms within parenthesis represent the functional dependence of the growth rate upon nitrogen concentration and light intensity while $h(pH)$ is a function describing the kinetic dependence upon pH. The symbol $K_{NO_3^-}(g\ L^{-1})$ represents the half saturation constant for nitrate, $I_K\ (\mu E\ m^{-2}s^{-1})$ represents the half saturation constant for light intensity, and $n\ (/)$ is a coefficient which allows to quantitatively simulate photo-inhibition phenomena potentially affecting microalgae when growth takes place under too high levels of incident light intensities. Finally, $I_{av}\ (\mu E\ m^{-2}s^{-1})$ represents the average photosynthetically active radiation within the growth media and was calculated through the following expression recently proposed in the literature (Soru *et al.*, 2018a)**:**

$$I_{av} = \frac{I_0}{\pi R^2} \int_0^{2\pi} \int_0^R \frac{2RI_0 \exp(-RK_c C_x)}{r} \cosh(rK_c C_x)\, r \cdot dr \cdot d\alpha \tag{2}$$

where $R\,(m)$ is the photobioreactor radius, $I_0\ (\mu E\ m^{-2}s^{-1})$ is the incident light intensity and $K_C\ (L\ g^{-1}\ m^{-1})$ is the optical extinction coefficient. The incident light intensity varied as a square wave function according to the mathematical relationship reported by Concas *et al.***,** (2016a). The dependence of growth rate on pH has been also taken in into account by this model by through the functional term $h(pH)$. The latter one was obtained by simply rearranging the formulation proposed in the literature (Tan *et al.*, 1998) for generic microorganisms so that the following dependence of growth rate on the molar concentration of protons was obtained:

$$h(pH) = h\left(\left[H^+\right]\right) = \left(\frac{\frac{k_0}{k_1} + \frac{1}{K_1}[H^+] + \frac{k_2}{k_1 K_1 K_2}[H^+]^2}{1 + \frac{1}{K_1}[H^+] + \frac{1}{K_1 K_2}[H^+]^2} \right) \tag{3}$$

where the symbol significance refer to model parameters. Such formulation permits to simulate different kinds of kinetic responses of microorganism to pH changes and thus is well suited for a quantitative interpretation of the acidophilic and/or alkaliphilic behaviour of algae. Since the obtained model of microalgae growth depends upon the nitrate concentration in solution, the corresponding mass balance which allows one to quantitatively describe its time evolution in the bulk liquid phase of a batch photobioreactor (PBR) can be written as follows:

$$\frac{dC_{NO_3^-}}{dt} = -Y_{NO_3^-}\frac{dC_x}{dt} \quad (4)$$

along with the initial condition $C_{NO_3^-} = C^0_{NO_3^-}$. In Eq. (4) the symbol $Y_{NO_3}(/)$ is the so called yield for nitrate, i.e. the weight of nitrate consumed for unit weight of microagal biomass produced. Finally, in order to simulate the evolution of lipid C_L $(g\ L^{-1})$, the corresponding mass balance recently proposed by Soru *et al.*, (2018a) was adopted:

$$\frac{dC_L}{dt} = \left[\psi_0 \cdot \mu_{\max}\left(\frac{C^0_{NO_3^-} - C_{NO_3^-}}{C_x}\right) - \alpha\frac{dC_x}{dt}\right]\left(1 - \frac{q_L}{q_L^{\max}}\right) \quad (5)$$

where the symbol q_L $(wt\%)$ is the intracellular content of lipid and can be evaluated time by time through the relationship: $q_L = C_L/C_x$. Besides, $q_L^{max}(wt\%)$ represents the maximum lipid accumulation within the cells while α and ψ_0 are model parameters. The strategy adopted to evaluate model parameters is described in what follows. First, experiments were performed in multiwell device in order to assess the effect of nitrates and pH on the growth rate and the corresponding kinetic parameters of Monod's and Tan's (Eq. 3) kinetics respectively. Subsequently, experiments performed in batch photobioreactors were interpreted through the model consisting of the ordinary differential Eqs (1), (4) and (5) while keeping fixed the above parameters and properly tuning the remaining ones.

4. Results and discussion

The effects of nitrate concentration and different pH on the growth kinetics of *C. melkonianii* cultivated in different devices were assessed. The resulting data of biomass concentration *Vs* time were then used to evaluate growth rate as a function of [NO_3] (cf. Figure 1a) and pH (cf. Figure **1**a) according to the equation $\mu = Ln(C_x/C_x^0)$.

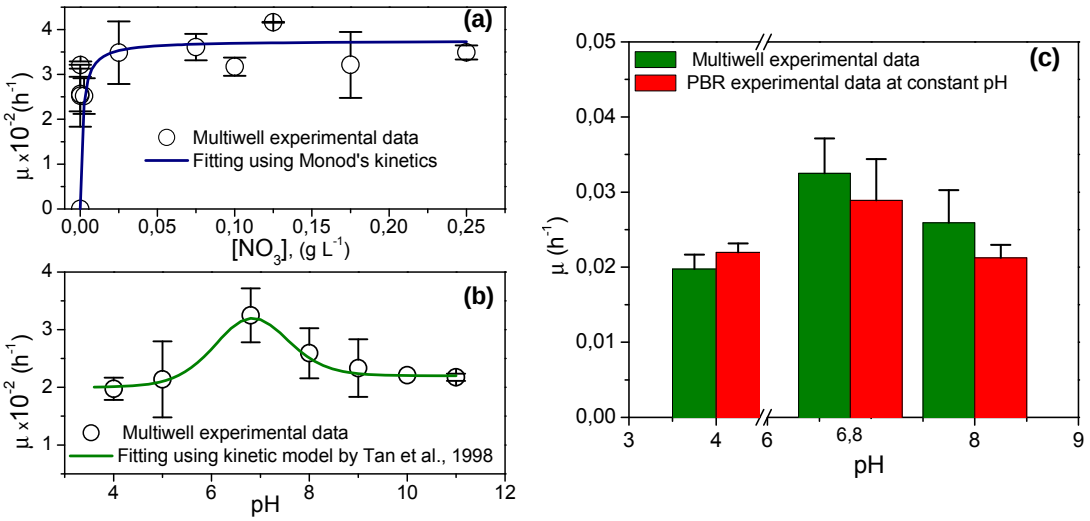

Figure 1. Evaluation of growth kinetics in multiwell devices as function of nitrate (a) and pH (b), respectively, and comparison with experimental results from batch photobioreactors in terms of μ vs pH (c).

The data reported in Figure 1a were then fitted through the Monod's kinetics by suitably tuning the parameters μ_{max} and $K_{NO_3^-}$ at the values of 3.75 x 10^{-2} (h^{-1}) and 1.0 x 10^{-5} (g L^{-1}). The data of Figure 1b were instead interpreted through the kinetics by Tan *et al.* (1998) while keeping fixed the obtained value of μ_{max} and $K_{NO_3^-}$ when tuning the model parameters in Eq. (3). The corresponding numerical values are reported elsewhere (Soru *et al*., 2018a). In Figure 1c, the pH-dependent growth rates of *C. melkonianii* obtained in multiwell screening tests are compared with those obtained in larger batch PBRs when operating at constant values of pH. As it can be observed, the multiwell results are quite well reproduced by the ones obtained in larger PBRs thus demonstrating the validity of the procedure to evaluate growth kinetics as a function of pH. The validated parameter values allowed to fit the subsequent experiments performed in larger PBRs through the developed model. In fact, in order to investigate the effects of nitrogen manipulation strategies on the prolonged growth and the lipid production of this strain, specific experiments were carried out by cultivating *C. melkonianii* in larger batch reactors where the initial concentration of dissolved nitrate was suitably changed (Soru *et al.*, 2018a). In the base-case, the growth and lipid accumulation kinetics were investigated using a nitrate

concentration equal to 0.25 g L^{-1}. The time evolution of nitrate concentration and pH were also monitored during this experiment. In Figure 2 the results obtained are shown. The base case results, showed that, pH increases from the initial value of 6.8 to a constant value of about 9.5 after about 25 days of cultivation (cf. Figure 2a). From Figure 2b it can be observed that after a short lag phase, the culture started growing almost exponentially for about 33 d (~800 h), when a decelerating growth took place. After about 45 d (~1080 h) the culture achieved a stationary state when the biomass concentration was about 1.4 g L^{-1}. This behaviour was the result of the corresponding lack of nitrogen in the solution.

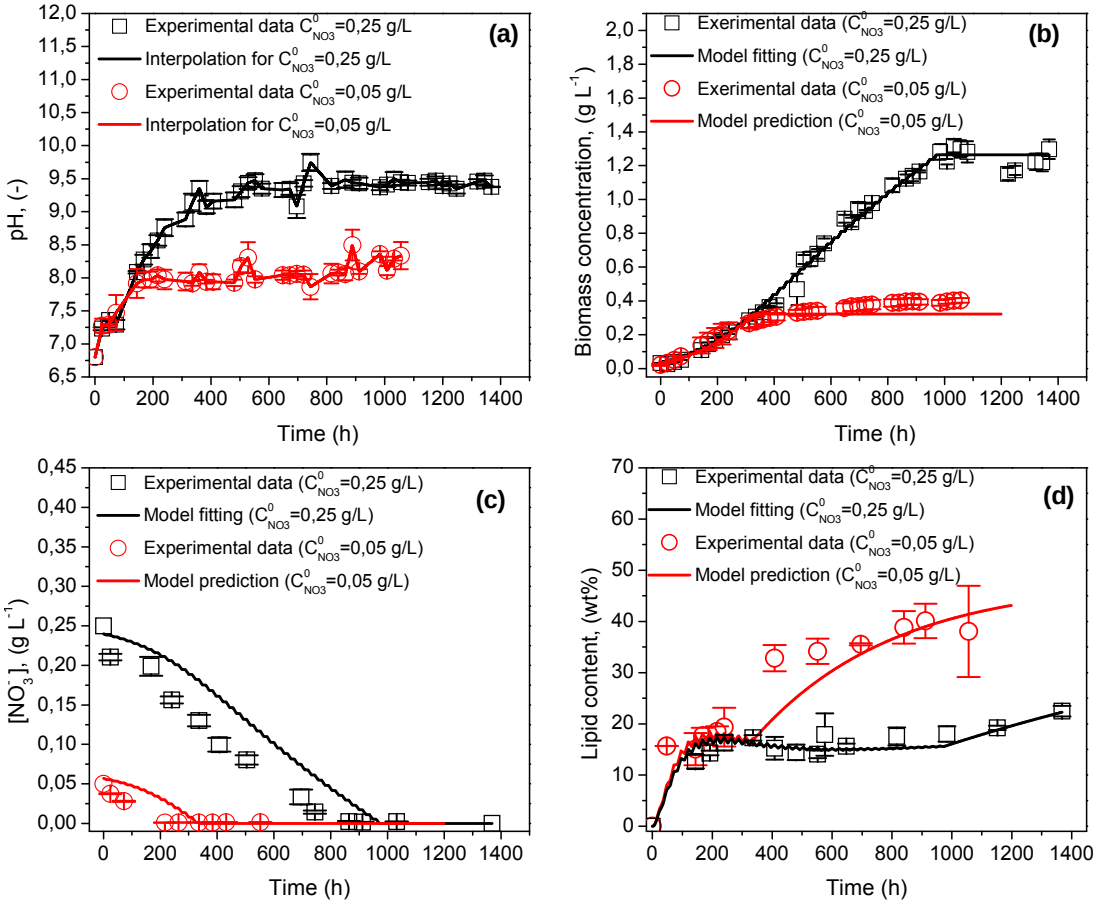

Figure 2. Comparison between experimental data and model results in terms of pH evolution (a), total dry biomass concentration (b), dissolved nitrate concentration (c), and lipid content (d) as a function of time during batch experiments with initial nitrate concentration of 0.25 g L^{-1} and 0.05 g L^{-1}.

In fact, as shown in Figure 2c, nitrate concentration decreased from the initial value of 0.25 g L^{-1} to 0 after about 1080 h as a result of algae uptake. The lipid content significantly increased during the first cultivation days, achieving a value close to 17 %wt after only 12 d (~300 h) of cultivation. Subsequently, after a slight decrease, it reached an almost constant value of about 15 %wt until total consumption of nitrogen occurred, i.e. 1080 h. Next, photosynthesis and growth became decoupled and thus lipids started to increase at a higher rate by reaching the value of about 22.5 %wt at the end of cultivation. In Figs 2b, 2c, and 2d the comparison between experimental data and model results is also shown. The pH values used in Eq. 3 for each cultivation time were obtained by interpolating the corresponding experimental data, as shown in Figure 2a. It should be noted that model parameters were taken from the literature (Soru *et al.*, 2018a and references therein) except for $\psi_0, \alpha, Y_{NO_3^-}$ and I_K whose values were suitably tuned in order to fit the experimental data. It can be seen that even if with some difference, the proposed model quantitatively captures the experimental behaviour in terms of biomass and nitrogen evolution as well as lipid accumulation dynamics.

In order to evaluate the predictive capability of the model, further experiments were performed using an initial concentration of nitrate that was reduced by five times (0.05 g L^{-1}) with respect to the one of the base-case. From Figure 2a it can be observed that pH achieved an almost constant value of 8 after about 240 h (10 d) that is quite lower than the one attained in the base case experiment. This is because under this operating condition microalgae growth stopped after about 10 days of cultivation when the biomass concentration

achieved the value of about 0.4 g L^{-1}. Accordingly, CO_2 consumption was reduced and thus the pH increase was inhibited. As shown from Figure 2c, this early achievement of culture stability was caused by the total consumption of nitrogen after about 10 days. The evolution of lipid showed a growing trend for the entire investigated period of time. During the first 15 days lipid accumulation dynamics was almost similar to the one already observed under the base case conditions. When nitrogen was consumed, lipid concentration greatly augmented reaching the value of ~40 %wt at the end of cultivation. Such behaviour is due to the fact that since microalgae stopped growing due to the consumption of nitrogen, all the assimilated carbon was converted into lipids rather than into proteins (Concas *et al.*, 2017). As it can be seen from the Figs. 4b-4d, the model well simulates these experimental results without adjusting any parameter thus demonstrating a good predictive capability. Accordingly, it might represent a useful tool to design, control and optimize the growth and lipid production *of C. melkonianii* in different devices. togliere uno spazio In particular, by combining model outputs related to lipid productivity with the experimental results obtained from the analysis of FAMEs reported by Soru *et al.*, (2018a), it was possible to evaluate the productivity of the different fatty acids recovered from *C. melkonianii* cultivated under the abovementioned operating conditions. The corresponding results are summarized in Figure 3.

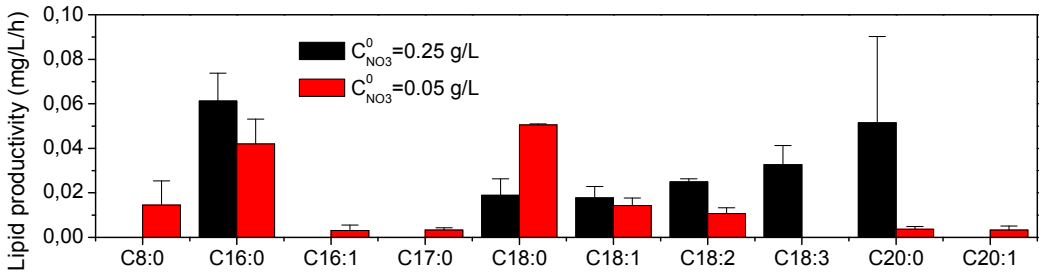

Figure 3. Evaluated productivity of fatty acids

As it can be observed, the productivities obtained by changing operating conditions were quite different for almost all the detected FAMEs. In particular, the BBM standard medium yielded the highest productivities of C16:0 (palmitic), C18:3 (linolenic), and C20:0 (arachidic) acids. On the other hand, when using a lower initial concentration of nitrogen (1/5N-BBM) the main FAMEs produced were C16:0 (palmitic), C18:0 (stearic), and C18:1 (oleic). An unusual high production of C8:0 (caprylic) acid was observed when using the lower initial concentrations of nitrogen. The possibility of using the extracted lipids for producing biodiesel was evaluated on the basis of the FAMEs profile by taking advantage of the software Biodiesel Analyzer© Ver. 2.2, according to a procedure reported elsewhere (Concas *et al.*, 2016). By means of suitable mathematical relationships, the latter one permits to evaluate the relevant characteristics of biodiesel which would be obtained from the concerned FAMEs mixture. The obtained results are summarized in Table 1.

Table 1. Estimated values of the main parameters of the biodiesels obtainable from Coccomyxa melkonianii SCCA 048 and comparison with the European standard for quality biodiesel

Symbol	Biodiesel Parameter	Units	BBM	1/5N	EN 14214 limits
SFA	Saturated Fatty Acid	%	63.900	67.000	-
PUFA>1 D.B.*	Poly Unsaturated Fatty Acid >1	%	27.960	6.290	-
LAC18	Linolenic acid content	%	15.770	0.000	12
SV	Saponification Value	mg g^{-1}	202.121	190.226	-
IV	Iodine Value	-	73.074	22.364	< 120
CN	Cetane number	-	56.862	69.960	> 51
LCSF	Long Chain Saturated Factor	-	32.558	19.458	-
CP	Cloud Point	°C	10.646	7.990	-
PP	Pour Point	°C	4.736	1.852	-
OS	Oxidation Stability	hours	6.808	21.339	> 8
HHV	Higher Heating Value	MJ kg^{-1}	39.768	33.328	-
ν	Kinematic Viscosity	mm^2s^{-1}	4.122	3.040	3.5 4 5.0
ρ	Density	g cm^{-3}	0.878	0.743	0.86 4 0.90

* Double Bonds ** Limits are those by EN 590 standards for temperate zones

It should be noted that, while some parameters of the resulting biodiesel would comply with the range of values prescribed by the Europeans standard for quality biodiesel (EN 14214), some others are quite far from their optimal range. In particular, BBM and 1/5N-BBM mixtures show a very high cold filter plugging point (CFPP), thus hindering the possibility of using the resulting biodiesel in compression ignition engines. The density and kinematic viscosity of biodiesels obtained from 1/5N-BBM cultures is out of the corresponding optimal range. The BBM standard growth medium would be suitable to produce a biodiesel compliant with the European standard. In general, the obtained lipids are not appropriate for producing biodiesel. However it might be converted into viable biodiesel through suitable pre-treatment such as hydrogenation or through blending with fossil diesel before the use in internal combustion engines. Table 1 shows that a heating value similar or higher than that one of antracite (33 MJ/kg) has been achieved in all cases, thus allowing the use of this product for producing green energy. However, it seems that the best use of the lipids obtained from *C. melkonianii* would be in the food and cosmetic sector considering the high oxidative stability of the obtained fatty acids which would facilitate their storage.

5. Conclusions

The nitrogen and pH dependent growth kinetics of *Coccomyxa melkonianii* SCCA 048 were evaluated through suitable experiments in multiwell. The obtained results, corroborated by further experiments performed in batch photobioreactors at constant pH, confirmed the reliability of multiwell screening test for the evaluation of growth kinetic parameters. The effect of nitrogen concentration on the growth and lipid production was investigated in larger batch photobioreactors. The obtained results showed that under nitrogen starvation conditions the lipid content of microalgae increased, while the achieved biomass concentration was very low. The best operating condition requires a sufficient concentration of nitrogen. These results were successfully simulated through a suitable mathematical model which might allow developing specific optimization strategies for the scale-up of photobioreactors. The FAMEs composition of lipids extracted from *C. melkonianii* would permit the production of a biodiesel only after a suitable pre-treatment or blending.

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