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Growth of Microalgae in Spectrum-Neutral, Volume-Distributed Light Restriction as the Baseline of Wastewater Exploitation

Sed Giorgia*[a], Cicci Agnese[b], Bravi Marco[a]

[a] Dept. Chemical Engineering M. E., University of Rome La Sapienza, via Eudossiana 18, 00184 Rome (IT)
[b] Bio-P s.r.l. via di Vannina 88, 00156 Rome (IT)
giorgia.sed@uniroma1.it

Producing culture media for microalgae from wastewaters may help reduce the culture management and wastewater treatment costs, but concomitant light limitation, nutrient restriction, toxic and growth-promoting effects complicate the preliminary data analysis and their subsequent exploitation. Post-treating the experimental data with a light-centred growth model would warrant the process developer with a powerful tool, but also a difficult one to validate in the absence of an accurate description of the microalgal growth behaviour in a purely radiative (i.e., non toxic) light restriction condition. Furthermore, purely radiative light restriction cannot be obtained by simply adjusting the external irradiance since in this latter case local irradiance and biomass growth cannot be decoupled.
Aim of this work is, thus, carrying out an experimental investigation on the influence of purely radiative, chromatically neutral (grey), and volume-distributed light restriction on the observed growth rate of a microalgal culture. Semi-continuous cultures of the microalga Scenedesmus dimorphus (UTEX strain 1237) were realized in a synthetic culture media, with the addition of a non metabolised and non toxic black dye at different dilutions obtaining an array of equally spaced absorbances, ultimately yielding to a tailored light limitation. The cultures were grown in cylindrical and flat panel photobioreactors under artificial light.
The experimental results were analysed with a simple (Lambert-Beer + Monod) growth model to investigate the exploitation criteria for wastewaters as culture media for microalgae.

1. Introduction

Commercial algal farms require light, source of water, nutrients availability and fitness of culturing conditions as temperature, pH and carbon dioxide concentration. These are the main factors that contribute to increase the biomass production costs. One alternative to the use of synthetic fertilizers is to employ byproducts of processing in the agro-food and in ancillary domains. They are rich in inorganic pollutants such as nitrogen and phosphorus and have a big potentiality for the bioremediation of the sludge coupled with the production of potentially valuable biomass. For these reasons, they were frequently assayed for their suitability as growth media for various microbial biomasses, including microalgae, although they are almost always opaque. However, the suitability of such media, judged by the specific growth rate obtained therein may be misleading due to said low transparency of the byproduct itself and to the length of the light path even in laboratory scale photobioreactors (Cicci et al., 2014a). In synthetic media, substrates, nutrients and microelements supply is an additional cost of culturing, but normally they are transparent, with minimal absorbance, are devoid of toxic effects and do not add up heterotrophic growth in mixotrophic species. Liquid byproducts/wastewaters are almost for free but they do not resemble any of the synthetic media generally required by microalgae: often are non-transparent and heavily absorbing due to the presence of polyphenols, bile salts, riboflavin. Furthermore the nitrogen:phosphorus target balance is not respected, there are also several toxic action origins due to biotoxic agents and are present organic substrates like oligo- and polysaccharides. Some significant test cases were carried out for analysing nutritional scarcity and toxic effects on biomass growth in culture media based on anaerobic cattle digestate and olive mill wastewater (Cicci et al., 2014b, Cicci et al.,

2013). Algae growth is affected by several variables such as nutrient availability, inhibitors, temperature, and mixing, etc. However, being algae photosynthetic organism, light is the key variable determining growth, efficiency and kinetics. The relation between light intensity and growth is complex: while low light is growth-limiting, excess irradiation promotes the formation of reactive oxygen species and has an inhibitory effect (Li et al., 2009). In their natural environment microalgae are subjected to the heterogeneity of a light gradient and are constantly competing for this resource. In photobioreactors (PBRs), the exploitation of solar irradiation, presents the problem that the inner volume of the culture is darker than the external ones. Only a small portion of the suspension volume of a few millimetres thickness is actually able to carry out photosynthesis at an acceptable pace. An important fraction of the PBR volume becomes dark enough to cause photosynthesis to cease, due to mutual shading, while the cells placed near the external part of the PBR result exposed to over-saturating light that could cause photoinhibition and, eventually, to a photooxidation, which is usually destructive for the microalgal culture. An effective mixing, ensure spatial homogeneity and avoid limitations and toxicities, it can serve the purpose of avoiding the overload of photosynthetic by moving the cells between photic and dark zones of the PBR. Aim of the work was to unbundle energy effects from metabolic effects, analysing the growth rates of cultures cultivated in synthetic dark media, identifying the single contribution of light on the biomass growth, without inhibitor or promoter components. To reach this goal we design a scale of dark culture media with a neutral black dye; after we tested the light effects on the growth cultivating microalgae in two different reactor geometries, tubular and flat panel.

2. Materials and Methods

2.1 Microalgal cultivation and culture media

Scenedesmus dimorphus (UTEX 1237) was obtained from the Culture Collection of Algae at the University of Texas at Austin, USA. The strain on agar was inoculated into 3NB culture medium that in a liter contains: CaCl2 0.17 mmol, NaNO3 8.82 mmol, MgSO4•7H2O 0.3 mmol, K2HPO4 0.43 mmol, KH2PO4 1.29 mmol, NaCl 0.43 mmol, Na2EDTA•2H2O 2 mmol, FeCl3•6H2O 0.36 mmol, MnCl2•4H2O 0.21 mmol, ZnCl2 0.037 mmol, CoCl2•6H2O 0.0084 mmol and Na2MoO4•2H2O 0.017 mmol. The original culture media pH was adjusted at 6.25 and then it was sterilized in autoclave at 1.2 bar and 121 °C for 20 minutes. The cellular density of *S. dimorphus* was inferred from the absorbance measured at a wavelength of 690 nm (corresponding to the absorption peak of chlorophyll *a* for the aquatic microorganisms living in turbid waters, according to Dall'Olmo et al. 2003. Measurements were taken every 24 or 48 hours from the onset of the culture. Our set-up consisted in: an array of media created from a non toxic opaque dye at different dilution ratios in an optimal medium producing an array of culturing conditions with different light restrictions. The absorbance of the dark media was measured spectrophotometrically with the instrument UV1800PC by Shanghai Mapada Co., Ltd. It resulted to be constant in the visible spectrum from the wavelength of 780 nm to 350 nm. Our opaque media were classified according to their absorbance at the wavelength of 690 nm. We produced a medium with a starter absorbance of 1.6 at 690 nm, then we diluted it in order to create other culture media with an absorbance of 0.8, 0.6, 0.4, 0.2, 0.1. The dark culture media were performed staining the 3NB media with a concentrated sterile solution of distilled water and lamp black watercolour (Pigment PBk6, PBk7, vehicles Arabic Gum and Dextrin). S. dimorphus is an autotrophic microalga (Lavens and Sorgeloos, 1996) and showed no evidence of ability to metabolize dextrin or Arabic gum (assessing the quantity of sugars in culture media with Dubois assay (Dubois et al., 1956), it remains constant by the time). Cultures were grown in 400 mL cylindrical glass tubes, with a diameter of 6 cm, fed with filtered and humidified air; flow rate was $130*10^3$ Nm^3/h, one culture in transparent 3NB media (the control) and two replicas in 3NB with an addiction of black watercolour at the various optical densities showed before. A preliminary growth test in a flat panel PBR consisted in one culture grown in 3NB (control) and one in a media with an absorbance of 0.8 at 690 nm. PBR volume was 850 mL, PVC tubes (3/4 inches diameter) were maintained in the dark by coverage and the polycarbonate panel was exposed to the light. Enlightened region consists in seventeen cells 1(l) x 0,6 (b) x 37 (h) cm. The exposed volume was 377.4 cm^3. The cultures were fed with filtered and humidified air, flow rate was 90*103 Nm^3/h. The air supply was ensured by an air lift system and the bubbles diameter was reduced by a porous stone. Photoperiod of 16 h of light provided by cold white fluorescent lamps (400-700 nm, 865 K, 32 W, 80 µmol m^{-2} s^{-1}) followed by a period of darkness equal to 8 h. The temperature was maintained constant at 24 ±1 °C.

2.2 Cellular concentration and growth rate

Chapter 2 According to the Lambert-Beer's law, microalgae concentration is directly proportional to the absorbance. The cellular density was correlated with the absorbance measured spectrophotometrically at a wavelength of 690 nm, close to the absorption peak of chlorophyll. In the experiments was chosen a semi-

continuous mode of growth: after a period of time (48 or 72 hours) half of the reactor volume was removed (200 mL for the cylindrical and 425 mL for the flat panel PBR) and the same volume was refilled with fresh culture media. Measurements were taken every 24 hours from the onset of the culture. For the cultures grown in the dark media the absorbance of the media was subtracted from the measures. Vegetative cell division is the common way for reproduction for microalgae and it is exponential if there are no limitations to growth (Tomaselli, 2004). A common way to measure microbial growth is by specific growth rate, μ, in d^{-1}, describing the biomass growth related to the existing biomass. In our experimental trials, biomass growth was assessed through the optical density (OD) or absorbance of the culture, subtracting the absorbance of the medium. The specific growth rate can be determined by following relationship in batch cultivation assuming zero mortality:

$$\mu = \frac{\ln(OD690t1/OD690t0)}{(t1-t0)/24} \quad (1)$$

μ is the slope of a growth curve where the population size is expressed on a logarithmic scale.

2.3 Lambert-Beer's based light model and method

Literature models to illustrate microorganism growth kinetics dependent from light and substrates, have several limits, the most important is their empirical nature. When a model is formulated the main aim is looking for relations which guarantee a range of applicability. Lambert-Beer's was the first mathematical law used for light analysis in a media, it is the most used in literature and it has the simplest math form. The use of Lambert-Beer's in photometry is based on three assumptions: (1) the incident light direction does not change when it crosses the culture; (2) the radiation is monochromatic; (3) the scattering effects due to solid particles are negligible in respect of the absorbance. In the valued cases and in the exposed conditions the three assumptions are respected. Lambert-Beer's is not appropriate in the cases of high cell densities, but cell concentration was never above 1.1 g/l. Dye particle size, measured with the aid of a Nanosizer, were ~ 310 nm, the dimension of dye suspended particles cannot interfere with measured wavelength, so scattering phenomena were not taken in consideration. As a consequence of the Lambert-Beer law, the light intensity at depth x in a suspension is:

$$I = I_0 \cdot e^{-A \cdot x} \quad (2)$$

2.4 Modelling of Specific Growth Rate in varying light

In order to separate the nutritional/toxic effects and the energetic effect (deriving from different illumination) of media composition, residual light intensity in a cylindrical photobioreactor, containing a suspension of microalgae in an assigned culture medium with a specific absorbance spectrum, was expressed as a function of the radial position. Then, the instantaneous specific growth rate in the reactor volume was calculated by integration. The average specific growth rate during the whole run was calculated by substituting the instantaneous specific growth rate calculated at the average absorbance of the culture (medium + microalgae) to the integral over the culture lifetime of the instantaneous specific growth rate at the prevailing optical density. The synthetic culture medium was considered transparent. The reference wavelength for the calculation of absorbance and local specific growth rate was assumed to be 690 nm, which is very close to the absorbance peak of chlorophyll, and the wavelength at which experimental determinations are usually carried out. In order to cancel out unknown constant in the Monod form of the specific growth rate (μ_{max} and KI) the calculated average specific growth rate was normalised by dividing it by the value of the average specific growth rate, calculated for the culture developed on the synthetic medium (Cicci et al. 2014a). As explained in the mentioned paper, is it possible to express residual light intensity in a cylindrical PBR for a culture medium with a specific absorbance as a function of the radial position. The instantaneous specific growth rate could be written as:

$$\mu(r) = \mu max \frac{I(r)}{K+I(r)} \quad (3)$$

For values of irradiation fairly lower than the saturation value, as in this case, the following approximation could be done:

$$\mu(r) = \mu max \frac{I(r)}{K} \quad (4)$$

The relationship between the distribution of light intensity I along the distance from the centre, deriving from reported models is the difference in proposed methods. The function I (r) obtained can be substituted in the specific growth rate formula, obtaining growth rate in function of the r distance: μ(r).
The specific growth rate mediated through the volume, could be assessed with the integration:

$$\bar{\mu} = \frac{\int_0^R \mu(r)\cdot 2\pi r dr}{\int_0^R 2\pi r dr} \quad (5)$$

The obtained growth rate value contains an only quantity exclusively experimental: it is the value tied to the mean absorbance, calculated for every dilution. Non-transparent culture media dilution was expressed by the parameter D, the ratio between non-transparent media volume on solution total volume. In the specific experimental case, the only data indicating the dilution level (as the media transparency) is the absorbance, it was verified the relationship between the measures. It was highlighted a relation of linearity:
y=0,92x-0,01 with R^2 = 0,999.
Is it possible to define the growth rate calculated as semi-theoretical growth rate: $\mu_{ST}=\bar{\mu}$.

The estimated growth rate relative at one determinate dilution of culture media, express the expected growth rate. The value is normalized using the ratio with the correspondent semi-theoretical growth rate calculated for the test containing only the synthetic transparent culture media, this could be reasonably considerate as reference value for an infinite dilution:

$$\mu_{STN} = \frac{\mu(D)_{ST}}{\mu(Ctr)_{ST}} \quad (6)$$

Similarly it is possible to individuate from the obtained experimental data, the correspondent value, for every dilution, of growth rate and call it experimental growth rate: μ_{EXP}. In the same way, the experimental value of growth rate in the media at certain dilution must be normalised relating it at the control value.

$$\mu_{EXP} = \frac{\mu(D)_{\exp}}{\mu(Ctr)_{\exp}} \quad (7)$$

From this model based on the Lambert-Beer's and on the monodian growth rate model is it possible to calculate the following specific normalized growth rate, calculating the integral (5) at the formula (4):

$$\mu_{STN} = \frac{A(Ctr)}{A(D)} \cdot \frac{[1-e^{-A(D)\cdot R}]}{[1-e^{-A(Ctr)\cdot R}]} \quad (8)$$

Subsequently to permit an assessment of the effective relevancy of the model on the experimental values (and to exclude discrepancies due to systematic errors) is it possible to effectuate a ratio between growth rate magnitudes. Defining the ratio μR, it could be written:

$$\mu R = \frac{\mu_{\exp}}{\mu_{STN}} \quad (9)$$

the two magnitudes express two evaluations of the only light limitation in relation to the media transparency. Their ratio is an indication of how and how much the theoretical model is far away from the experimental case.

3. Results and discussion

Data were analysed and outliers were eliminated. The replicas were mediated and were obtained *S. dimorphus* 1237 average growth rates at 24 °C in control and dyed culture media, with relevant standard deviations, average absorbance and normalised residual growth rates for cylindrical geometry (Table 1). Control dilution was arbitrarily adopted as 1/1000 (while it is theoretically 1/∞) to maintain the readability of the subsequent plots. For the cultures grown in flat panel geometry were calculated: average growth rates with standard deviations (Table 2).
In order to evaluate if growth rates differences were significant an ANOVA test was performed for the cultures grown in cylindrical PBRs and t test was performed for the cultures grown in the flat panel. The assumptions of normal distribution of the μ and homoscedasticity of variances for every data set were validated. Our null hypothesis was that there were no differences in average growth rates in the dyed cultures and the alternative hypothesis was that at least one average specific growth rate was different. There are no significative differences between control growth rate, 0.1 and 0.2 dyed cultures grown in cylindrical PBRs. There are differences in the growth rate comparing control with 0.4, 0.6 and 0.8 dyed cultures grown in the same PBR geometry. A significative reduction in the growth rate was confirmed by the statistical analysis. Conversely, in the flat, thin layer PBR irradiance remains higher throughout the reactor despite the higher concentration of dye (and consequently the higher absorbance) thanks to the limited absorption thickness. However, if the dye had other (either promotive, or demotive such as toxicity) effects on the microalga the observed specific growth rate of the control and dyed control would be different. The two specific growth rates were not found to exhibit a statistically significant difference, hence confirming that the dye is non toxic and its only effect upon the culture is light restriction, which applies in the cylindric PBR above A = 0.1 but does not apply to the thin

layer PBR even at A = 0.8. Therefore, the assumption that it can serve the purpose of validating the semi-theoretical model of light effects has been demonstrated.

Table 1: algal average growth rate, standard deviation, dilution and natural logarithm, average absorbance and μR ratio for cultures grown in cylindrical PBRs at different dye dilutions.

Culture	μ cyl PBR d^{-1}	Standard dev.	Dilution (D)	Ln(D)	$\bar{A}$	*μR*
Control	0.360	0.09	0.05	-3.00	1.596	1.00
0.1 Dyed	0.326	0.14	0.09	-2.41	1.550	0.88
0.2 Dyed	0.290	0.09	0.18	-1.69	1.753	0.88
0.4 Dyed	0.281	0.06	0.37	-1.00	1.793	0.88
0.6 Dyed	0.231	0.06	0.55	-0.59	1.763	0.71
0.8 Dyed	0.219	0.13	0.74	-0.31	1.663	0.63

Table 2: algal average growth rate standard deviation, dilution and natural logarithm for cultures grown in flat panel PBR at different dye dilutions.

Culture	μ cyl d^{-1}	St. dev.
Control	0.38	0.12
0.8 Dyed	0.30	0.09

To make a comparison and judge in base of the model, experimental growth rates were normalised following the Eq (5). OD_{690} measures were effectuated during every test and applying a pondered media on the times from refresh, are obtained average absorbance values ($\bar{A}$) reported in Table 1 following the Eq(10).

$$\bar{A} = A_{CM} + \frac{A(t=t_0)+A(t=t_f)}{2} \quad (10)$$

This value resulted from the sum of optical density of the media (A_{CM}) and average density for algae only. With the normalised data of semi-theoretical and experimental values, it has been valued the growth rate obtained from their ratio as in the Eq(9). Through this procedure are excluded effects due to the light limiting that are controlled in the theoretical model. *μR* obtained values from the implementation of model MLB (Lambert-Beer + Monod) are represented in Figure 1 in function of the logarithm of dilution.

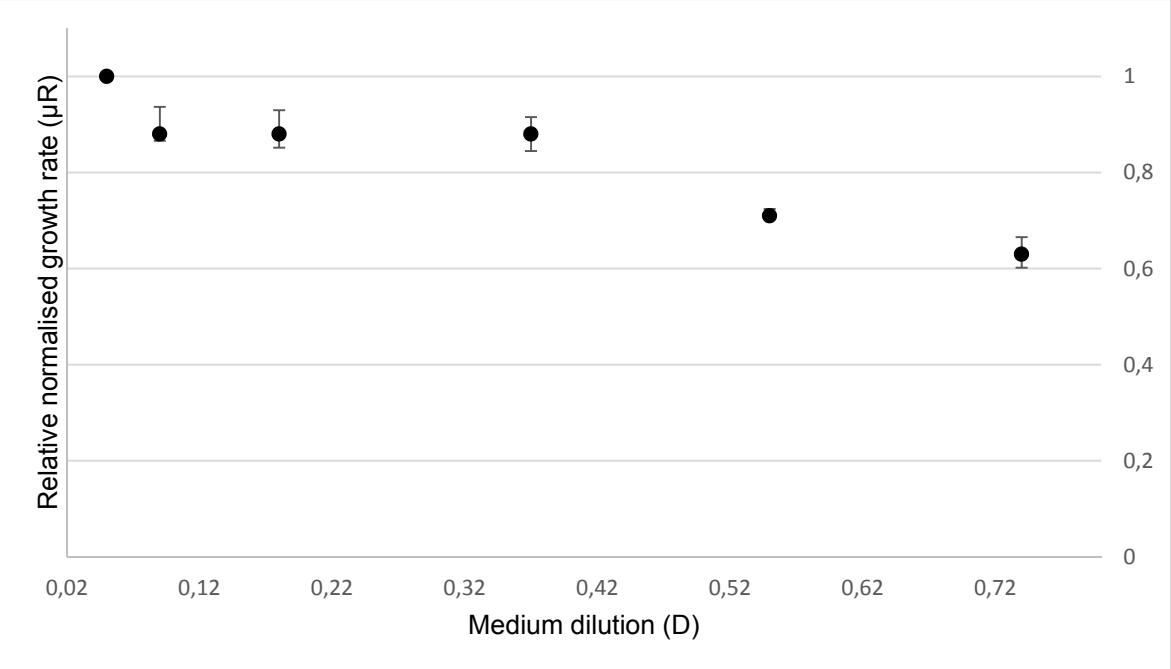

Figure 1: representation of ratio value of normalized growth rates obtained with MLB in function of the media dilution.

Experimental tests were made maintaining the cultures in conditions of just light limitation and if the model represent in the correct way the effects of residual light on microalgal growth in a non-transparent media, the μR ratio should evaluate to 1 to confirm that light restriction effects have been accurately masked and, by plotting the calculated ratio across the whole array of dilutions, a flat trend should result. The results shown by simple model were not up to expectation, thus revealing that simplifications in the physical and metabolic aspects, although useful in obtaining a very simple tool, were excessive.

4. Conclusions

The dependence of specific growth rate on residual light available in a dark medium has been quantified independently of other effects (toxic or promotion) in a medium-term sustained growth approach. This approach is not a redundant complicacy compared to simply reducing external impinging irradiance, since we aimed at duplicating the presence of an internal light intensity distribution without interfering effects from other inherent media components. In 6-cm cylindrical photobioreactors media with absorbance >0.4 induce a noticeable reduction of specific growth rate. A thin-layer photobioreactor is expected to preserve the growth-support potential of the optimised medium up to much higher concentrations of wastewater in the growth medium, thus potentially lowering the daily cost of culture operation. In future it could help us to potentially lowering the daily cost of microalgal culture. In the absence of any influence from the medium beyond light reduction the ratio between experimental and semi-theoretical specific growth rates would be expected to be a flat straight line. The cause of a residual decreasing trend has been traced back to the photosynthetic action spectrum of the microalga. The difficulty of the goal achievement proposed, is due to the numerable variables that influence the microalgae system. Biological systems thanks to their adaptation skills related to their previous lived conditions, need to be constantly verified and the models have to represent in the simplest way the innumerable variables.
A verifiable limit in the presented models could be the missing of corrective information. It is evident that description of microalgae biomass production in non-transparent culture media need to consider not only the light intensity, but also its affinity with catching and energetic conversion processes. These effects cannot be neglected during the whole biomass growth at low light incident rates.
A correction factor has been proposed to account for these factors and permit the use of the developed method as a diagnostic tool of the metabolic compliance of media obtained by dilution of dark wastewaters.

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