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The Role of Mathematical Modeling and Genetic Engineering for the Microalgae Based Technology

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Some recent achievements in the field of mathematical modeling and silmulation of CO_2 capture and biofuels production through microalgae are presented in this paper. Morevoer, the role of genetic engineering, with a focus on the research activity currently being performed and its future developments, is also discussed. Finally, recent results related to the genetic characterization of chloroplast and mitohondrial DNAs of *Chlorella sorokiniana*, are presented.

1. Mathematical modeling for the improvement of the microalgae based technology

Among the several feedstock's that are potentially useful for producing biofuels on the large scale, microalgae are today considered to be one of the most promising since, unlike first generation biomasses, their cultivation can be accomplished in non-agricultural lands without competition with the food market. Furthermore, when compared to first generation feedstocks, microalgae are characterized by higher bio-oil productivity and their cultivation might be coupled with the direct CO_2 capture from point sources of emission. In spite of these promising features, the existing microalgae-based technology for CO_2 sequestration and biofuels production is still not widespread since it is affected by economic and technical constraints that might limit the development of industrial scale production systems. Therefore, in view of industrial scaling-up, the current technology should be optimized in terms of oil productivities and CO_2 uptake capability. To this aim, mathematical models and process engineering techniques might be suitably exploited to identify the operating conditions of photobioreactors (i.e. light supply, mass transfer, growth media composition, etc.) that maximize growth rate, lipid accumulation and CO_2 fixation as well as the economic viability of the technique (Altimari et al., 2013).

1.1 Model aided investigation of CO_2 bio-fixation through *Chlorella vulgaris*

As an example, the possibility to exploit mathematical models to properly design photo-bioreactors capable to take advantage the CO_2 from flue gases as carbon source, is addressed in what follows. The biocapture of CO_2 from flue gases by microalgae represents in fact one of the main challenges in this field since it would lead to the reduction of green house gases emissions. However, the effects resulting from the direct feeding of CO_2 in the photobioreactors, i.e. low pH values and high dissolved concentration of CO_2, which might severely affect microalgae growth, should be investigated and simulated in order to properly control the main process parameter during the industrial scale operation of the photobioreactors. Along these lines, Concas et al. (2012) proposed a novel mathematical model of the growth of *Chlorella vulgaris* in semi-batch photobioreactors fed with pure CO_2 (100 % v/v) that simulates suitable gaseous streams which, in turn, might obtained from flue gases through the implementation of suitable CO_2 capture and concentration technologies. Specifically, the proposed model was capable to simulate temporal evolution of cells, light density and

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macronutrients concentration within the growth medium as well as carbon dioxide and oxygen concentration in liquid and gas phase. Moreover, by taking advantage of comprehensive kinetics and considering the ion speciation phenomena taking place in the growth medium, the model was able to quantitatively describe the dynamics of pH evolution and its effect on microalgae growth. Such aspects, often neglected by mathematical models available in the literature, are indeed critical in order to develop suitable control strategies for the optimization of photobioreactor's operation when using flue gas as carbon source for microalgae growth. As shown in Figure 1, model results were successfully compared with experimental data, thus confirming the capability of the proposed model to quantitatively describe the culture behaviour within semi-batch photobioreactors both in terms of biomass concentration and pH evolution during the cultivation.

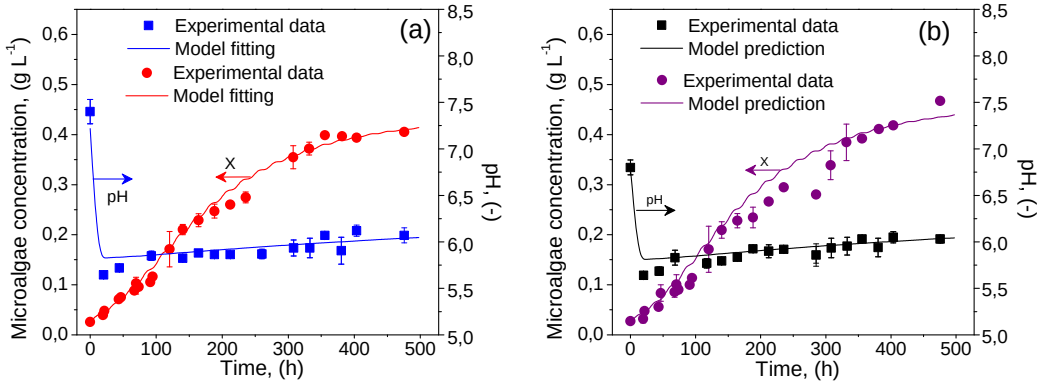

Figure 1. Comparison between model results and experimental data in terms of biomass concentration and pH as a function of time (a) fittings and (b) predictions. Adapted from Concas et al. (2012)

Ultimately, the experimental results confirm that *C. vulgaris* strain is capable to adapt to high levels of CO_2, probably by changing the carbon uptake mechanisms. The mathematical model might allow one to identify the operating conditions that maximize the CO_2 capture and the simultaneous biomass productivity in semi-continuous photobioreactors.

1.2 Model aided design and control of BIOCOIL photobioreactors cultured with *Spirulina platensis*.

A further challenge in the field of biofuels production and CO_2 sequestration through microalgae, is the identification of the geometry and the operating mode of photobioreactors that allows to optimize the distribution of photon flux, the CO_2 capture, the removal of photosynthetic oxygen and the nutrients utilization. To this aim, horizontal or helical tubular systems, as well as combinations of vertical flat panels and bubble columns appear to be the reactor configurations whose scale up may be relatively straightforward. In particular, the helical configuration named BIOCOIL (Figure 2-a) is characterized by very simple and relatively inexpensive design, easy to assemble and operate.

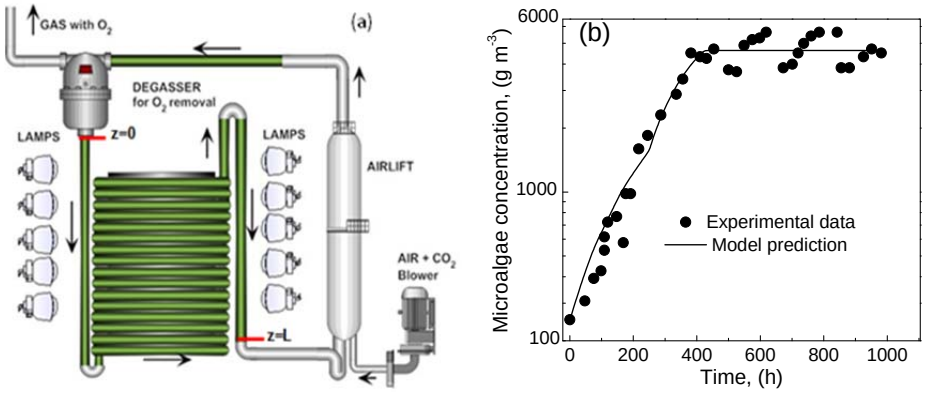

Figure 2. Scheme of the BIOCOIL (a) and comparison of model results (Concas et al., 2010) and experimental data (Travieso et al., 2001) in terms of biomass concentration in the final section (b).

However, only few mathematical models, aimed to the simulation, the optimization and the control of such photobioreactor typology have been reported in the literature.

Only recently, Concas et al. (2010) proposed a comprehensive model which was able to quantitatively describe the growth of *Spirulina platensis* in a re-circulating helical photobioreactor, i.e. the BIOCOIL. The model accounted for "mass structured" population balances which permitted to properly simulate cell growth, replication and the biomass distribution within the tubular-helical photobioreactor. Specifically, a novel mass-dependent growth kinetics was proposed. The latter one took into account cell size, light intensity, nutrients concentration and inhibitory effects of dissolved oxygen. Model results and literature experimental data (Travieso et al., 2001) in terms of dry biomass content and its distribution within the photobioreactor tube were successfully compared as shown in Figure 2-b, thus demonstrating the validity of the proposed model as well as its predictive capability. Therefore, the proposed model might be potentially useful for the optimization of design and process parameters of the BIOCOIL photobioreactor for the cultivation of *Spirulina platensis*.

1.3 Model aided development of iron-based strategies to increase lipid productivity of *C. vulgaris*

Another example of how mathematical modelling can be exploited to improve the current microalgae-based technology for biofuels production is reported in the work by Concas et al. (2014), where the possibility to exploit a suitable model to identify the operating conditions allowing microalgae to increase their lipid content while maintaining an high growth rates, is addressed. In this regard, it should be noted that even at the experimental level, the identification of cultivation conditions that result in a simultaneous increase of biomass growth rate and lipid content of algae represents one of the most crucial target to be achieved in order to make the microalgal technology feasible at the industrial scale. The most widespread technique adopted to trigger lipid accumulation in microalgae 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 thus thwarting the effects of the increased lipid content. For this reason the identification of suitable operating conditions that allow a concurrent increase of both lipid content and biomass growth rate is one of the main challenges in the field of biofuels production through microalgae. Along these lines, it was experimentally demonstrated by Concas et al. (2014) that the strain *C. vulgaris* is capable to simultaneously increase its growth rate and lipid content when cultured under suitable concentrations of iron. A comprehensive mathematical model describing the effect of iron on all the complex phenomena affecting growth rate and lipid accumulation of *C. vulgaris* was then proposed by Concas et al.(2014). The model took into account the effect of iron on the carbon specific photosynthesis rate, the nitrogen uptake rate as well as the chlorophyll and the lipid synthesis. In particular, the model was based on the idea that the oxidative conditions induced by high concentrations of iron in solution are somehow detected by *C. vulgaris* cells which activate lipid biosynthesis as a countermeasure to contrast possible phenomena of lipid oxidation induced by hydroxyl radicals produced as a result of the photo-induced Fenton-type reactions in solution. As it can be observed form Figure 3 the model was capable to quantitatively interpret the relevant experimental results obtained by Concas et al. (2014).

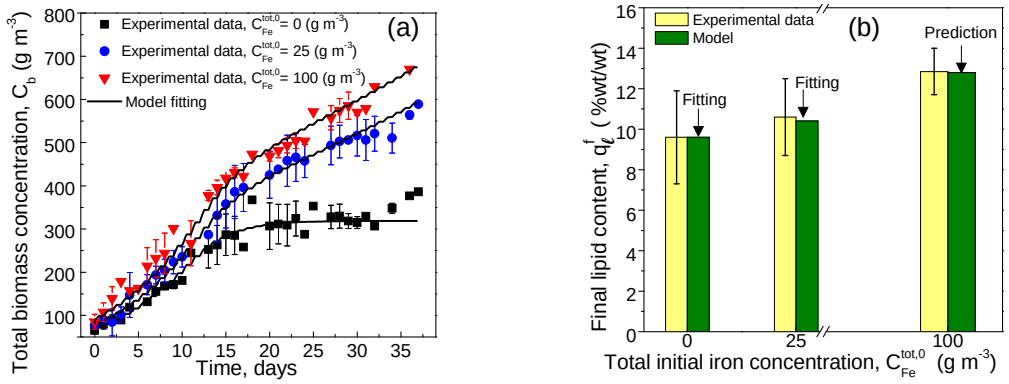

Figure 3 Comparison between model results and experimental data in terms of biomass concentration (a) and final lipid content of algae (b) when varying the initial concentration of total dissolved iron.

Therefore the proposed mathematical model is potentially useful to implement and optimize suitable iron-based strategies for the improvement of the current microalgal technology for the production of biofuels, since it might permit to identify the iron concentrations that optimize the lipid productivity of *C. vulgaris* in batch photo-bioreactors.

2. Genetic engineering

While the mathematical models can be helpful to identify the operating conditions that optimize the microalgae cultivation in suitable photo-bioreactors, specific genetic engineering tools could be exploited to manipulate genome of existing strains with the aim of increasing their intrinsic photosynthetic efficiency and/or regulate their metabolism in order to achieve an abundant accumulation of lipids coupled with an high biomass productivity or an high capability of CO_2 uptake (Radakovits et al., 2010). Along these lines, an intense experimental activity is being carried out with the aim to the identify specific genes involved in the bio-synthesis of fatty acids and carbon dioxide uptake mechanisms of 5 strains belonging to the phylum of green algae, i.e. *Chlorella sorokiniana* (SAG 111-8k), *Pseudochloris wilhelmii* (SAG 55.87), *Monodus subterraneus* (SAG 848.1), *Scenedesmus obliquus* (SAG 276-1) and *Tetraspora* sp.(SCCA024). The activity consists of the extraction of the genomic DNA from the above mentioned 5 strains grown under typical operating conditions, and the "de novo" sequencing of such strains by a Next Generation Sequencing approach using the Illumina HiSeq2000 platform (Figure 4-a) by synthesis (SBS) technology (Imelfort and Edwards, 2009). This strategy returns enough high quality data to ensure a coverage of about 80-100X, over two libraries (300 – 800 bp) with different insert size, for each strain (Figure 4-b).

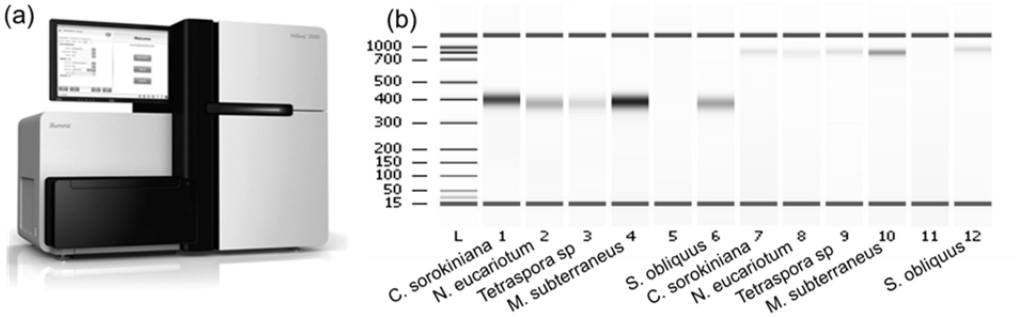

Figure 4. Illumina's HiSeq2000 sequencing system (a) and data quality over two libraries (300 – 800 bp) for each investigated strain

This approach will allow us to apply a bioinformatics workflow to optimize the assembly of both organelles and nuclear genomes (Naito et al, 2013). RNA-Seq analysis on Whole Transcriptomes is currently being performed on the above strains. Subsequently, the latter ones will be grown under specific operating conditions, i.e. for example low nitrogen or high iron concentration in the growth medium, which are capable to induce the corresponding bio-synthesis. Afterwards, the lipid-enriched strains will undergo the same transcriptomic characterization which was previously performed on the corresponding ones grown under typical operating conditions. By comparing the transcriptome profiles of such strains, the genes over/under expressed, when fatty acids synthesis is promoted, will be recognized as well as the key regulatory genes controlling the biosynthetic pathway. Such results will permit to implement suitable genetic engineering strategies to transfect the genes involved in lipid synthesis within the microalgal cells. If successful, such activity will permit to create microalgal strains characterized by an intrinsic high capacity to produce fatty acids and thus to make the current microalgae-based technology for producing biofuels economically viable.

2.1 Recent achievements in the genetic characterization of *Chlorella sorokiniana*

In the framework of the research activity above summarized, some relevant results have been achieved as far as the genetic characterization of the microalgal strain *C. sorokiniana* is concerned. The latter one is a non-motile unicellular alga which can grow phototrophically by exploiting wastewaters and flue gases as costless sources of inorganic nutrients and CO_2, respectively. Along with carbon dioxide, the *C. sorokiniana* strain is capable to take advantage also of organic carbon substrates to grow under mixotrophic conditions with higher biomass yields and lipid productivities than the corresponding ones observed under photoautotrophic conditions. Furthermore, in the same work, it was demonstrated that that different lipid compositions can be achieved by cultivating this strain under heterotrophic or mixotrophic conditions (Rosenberg et al. 2014). Ultimately, the desired lipid composition and content might be varied by suitably tuning the cultivation conditions. For these reasons, this strain might represent a promising feedstock for the production of nutritional oils or biofuels in a biorefinery framework. In spite of this, the commercial exploitation of *C. sorokiniana* is still not widespread since its large scale production process might be affected by technical constraints mainly arising from the still low lipid productivity achievable through the current cultivation

technologies. In this regard, the knowledge of *C. sorokiniana* genome represents the first step towards the identification of suitable genetic engineering strategies aimed to increase its lipid productivity and thus to overcome the limitations described above. Along these lines the complete chloroplast (Orsini et al., 2014a) and mitochondrial (Orsini et al., 2014b) genomes sequences of *C. sorokiniana* strain (SAG 111-8k) have been recently presented thus completing the knowledge of the organelles genomes of this organism. To this aim, whole-genome sequencing of *C. sorokiniana* strain was performed using the Illumina HiSeq2000 platform described above, after quality trimming reads were assembled by a de novo approach under the Orione framework (Cuccuru et al., 2014).
The obtained results highlighted that the chloroplast sequence (cpDNA) assembles as a circular map of 109,811 bp which encodes a total of 109 genes (Figure 5a). As shown in Table 1 this chloroplast gene repertoire includes 74 proteins, 3 rRNAs and 31 tRNAs.

Table 1. Attributes of the chloroplast (cpDNA) and mithocondrial (mtDNA) genomes of C. sorokiniana

Attribute	cpDNA	mtDNA
Genome size (bp)	109811	52528
DNA G + C content (%)	34.1 %	29.11 %
IR	0	0
Total genes	109	60
rRNA genes	3	3
tRNA genes	31	26
Protein coding genes	75	31

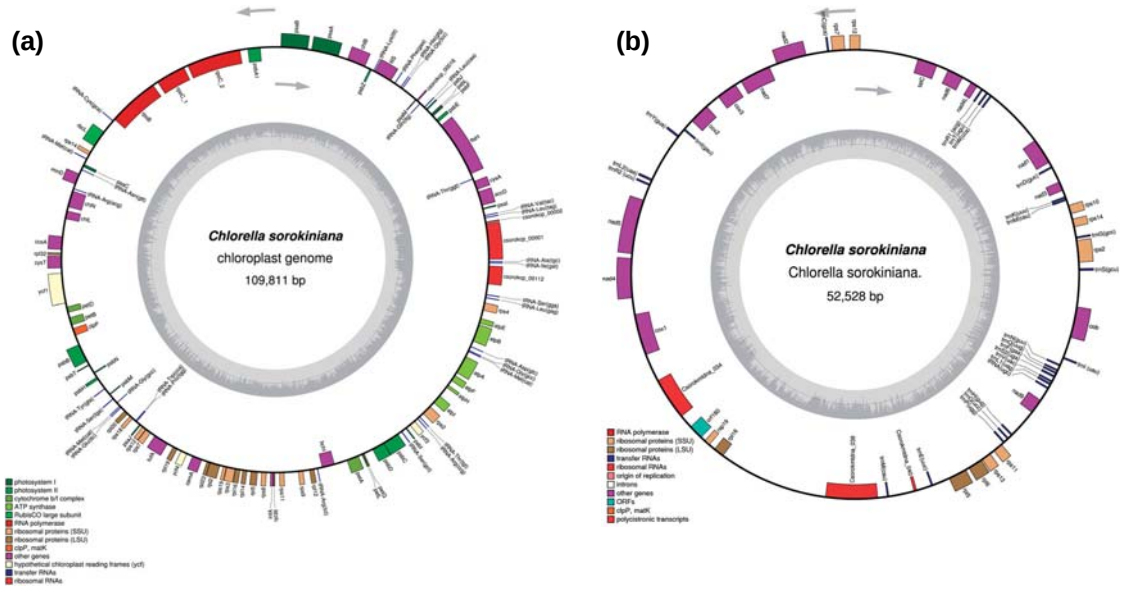

Figure 5. Gene map of (a) chloroplast genome and (b) mitochondrial genome of C. sorokiniana. Adapted from Orsini et al. 2014-a and Orsini et al., 2014-b, respectively.

The 74 proteins accounts for, in turn, 18 ribosomal proteins, an elongation factor and components of an RNA polymerase (rpo). In the genome, which doesn't show introns, all genes are present in single copy. Moreover, as well as for other Chlorella strains, the genome is lacking large inverted repeat (IR). C. sorokiniana belongs to the group that displays the standard architecture of the rRNA operon of Chlorphyta structure (16S rDNA – tRNA Ile – tRNA Ala – 23S rDNA). The overall AT content of *C. sorokiniana* cpDNA is 65.9 % and the coding sequence is 59.1 % (54279 bp). The sequence analysis, here reported, highlights that the plastid genome of *C. sorokiniana* does not display a quadripartite structure. Multiple sequence alignment compared to *C. variabilis* and *C. vulgaris* cpDNAs (NC_015359 and NC_001865, respectively) reveals a closer relationship to the former species with no particular rearrangements in the cpDNA sequence. On the contrary, a modest overall identity and large rearrangements are observed in the obtained chloroplast genome with respect to the *C. vulgaris* cpDNAs. On the other hand, the comparison among chloroplast encoded proteins showed a relevant similarity except for a small difference in two subunits of the *C. vulgaris* RNA polymerase.

As far as the mitochondrial DNA (mtDNA) sequence of *C. sorokiniana* is concerned, it can be observed from Figure 5b that it assembles as a circular map of 52528 bp (with an AT of 70.89 %) which encodes a total of 31 protein coding genes, 3 rRNAs and 26 tRNAs, while accounting for 97.4 % of the total genome. A synoptic prospect of the main attributes of the mtDNA of *C. sorokiniana* are shown in Table 1. The comparison among of the mtDNA of *C. sorokiniana* and the one of the Trebouxiophyceae revealed substantial rearrangements among strains including large inversion. In addition, a basic phylogenetic analysis placed the *C. sorokiniana* mtDNA in a specific clade distinct from that one of *Chlorella* sp. ArM0029B (which, in turn, resulted in a separate clade itself) and the other Trebouxiophyceae.
All the information above represent a first critical step towards the characterization of the whole genome, including the nuclear one, of *C. sorokiniana*, Once the whole genome will be characterized, specific activities aimed to identify the genes involved in the metabolic pathways related to lipid biosynthesis and CO_2 uptake may be performed. Finally, suitable strategies to over/under express the genes above will be investigated with the aim of increasing the lipid productivity and the CO_2 uptake rate by *C. sorokiniana*.

3. Concluding remarks

The possibility to improve the current microalgal technology for the production of biofuels and CO_2 capture by exploiting the potential of mathematical models has been discussed. To this aim, some literature models have been briefly discussed with a focus on their capability to aid the photobioreactor design and operation. In the second section of the paper, the strategies currently being adopted to pursue the final target of synthesizing an engineered strain characterized by high lipid productivities and high CO_2 uptake rate, have been briefly discussed. Finally, recent achievements related to the genetic characterization of chloroplast and mitochondrial DNAs of *Chlorella sorokiniana*, have been presented.

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