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SOME BIOTECHNOLOGICAL APPLICATIONS OF CYANOBACTERIA AND GREEN MICROALGAE

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Abstract

In this paper we summarize some of the the biotechnological applications of oxygenic photosynthetic microorganisms (OPhM) , cyanobacteria and green microalgae, with special emphasis on the followings topics: i) epuration of domestic waste waters with simultaneous new biomass synthesis as source for dedicated chemicals (proteins, lipids, pigments, antioxidants etc.,); ii) gamma irradiation at non growth inhibitory doses in order to increase lipid content of the cells; iii) the synthesis of metal nanoparticles by these photosynthetic microorganisms,; iv) the use of living photosynthetic biomass as biocrusts to increase the chemical parameters of soil and physiological characteristics of plants; v) the study their bioelectrochemical properties for biotechnological applications such as the conversion of solar energy in electric energy. These topics are directly related to the experimental activities of the authors and cover partially the huge potential of these microorganisms.

Key words: *cyanobacteria, microalgae, non-lethal gamma irradiation, domestic waste waters, biomass, biocrusts, nanoparticles, biofuel cells.*

1. EPURATION OF DOMESTIC WASTE WATERS, INCLUDING AQUACULTURE WASTE WATERS, WITH SIMULTANEOUS NEW BIOMASS SYNTHESIS

The use of *oxygenic photosynthetic microorganisms* (OPhM) in wastewater treatment plants started almost 70 years ago with the pioneering work of Oswald and his colleagues (Ludwig et al., 1951; Oswald, 2003) and is still an on growing activity; (Pacheco et al., 2020; Solovchenko et al., 2020; Katam & Bhattacharyya, 2020). Although initially the goal of wastewater treatment was to protect downstream users from health risks, nowadays wastewater is perceived as a valuable resource of energy, fertilizers, other products, and clean water (for more details see Kehrein et al., 2020).
As reviewed by Wollmann et al., (2019), there are several big companies active in this field. Oswald Green Technologies has used a symbiotic bacterial algal consortium, known as *Advanced Integrated Wastewater Pond System* (AIWPSR), to take up both organic and inorganic pollutants (i.e., nutrients) from different types of wastewaters. The US company *AlgaeSystems* has developed a low-cost offshore floating bioreactor able to treat 50,000 gal day^{-1} of raw municipal wastewater with removal efficiencies of 75% (total N), 93% (total P), and 93% (BOD) whereas other approaches such as those of *HydroMentia*, *OneWater*, and *Gross-Wen Technologies* exploited microalgal biofilms, immobilized microalgae, or microalgae-bacteria co-cultures (Wollmann et al., 2019).
Mambo et al. (2014) reported processes which relies on the combined activity of methane fermentation and photosynthetic oxygenation by algae coupled with biological oxidation in the high-rate ponds to remediate domestic waste waters. In agreement with the authors,

the main advantages of their systems are: i) the sludge accumulation is extremely slow, so no sludge management is required; ii) carbon (C) is transformed through two important mechanisms: methane formation and C-biological assimilation by microalgae processes which provide the basis for primary, secondary, and tertiary treatment; iii) the molecular oxygen produced during oxygenic photosynthesis within the pond is 10-100 times more efficient in the oxygenation capacity as compared with mechanical aerators, which are also very expensive. Furthermore, the described system has been in continuous operation since 1996 and receives 75 m^3/d of raw sewage, being also an operational, passive, sequential, sewage treatment facility that functions virtually in perpetuity and without any need for fecal sludge handling (Mambo et al., 2014).

In the last five years there is a huge increase in research concerning the use of photosynthetic microorganisms for aquaculture wastewater (AQWW) treatment. This type of wastewater have high nutrient content (i.e., N and P compounds, dissolved organic C) and are considered an appropriate culture media for the growth of different microorganisms including microalgae and cyanobacteria (Ansari et al., 2019). This biomass is rich in proteins, lipids, carbohydrates, and other valuable products, which can subsequently be used to produce high quality aquaculture feed or biofuel (Ansari et al., 2017; Liu et al., 2018). By the utilization of this integrated process, it is not only possible to close the loop in the aquaculture industry, but also to make economical, sustainable and feasible aquaculture (Kehrein et al., 2020).

Kuo et al. (2016) used aquaculture wastewater supplemented with additional nutrients- including CO_2 form the boiler flue- to cultivate Chlorella sp., whereas Wuang et al. (2016) showed the ability of *S. platensis* to remove NH_4 and NO_3 from fish farming wastewater, the obtained biomass being applicable as agricultural fertilizer.

Guldhe et al. (2017) working on *C. sorokiniana* and Ansari et al. (2017) on *S. obliquus*, *C. sorokiniana* and *A. falcatus* showed that N, P and COD removal from AQWW reached values between 70-80% with simultaneous biomass synthesis (150 mg/L/day of biomass rich in lipids, carbohydrates and proteins).

In a large scale experiments (4,500 L), Nogueira et al. (2018) used the cyanobacterium *S. platensis* for the treatment of fish farming effluents from *O. niloticus* production, demonstrating that after 9 days of growth, *S. platensis* was capable of reducing the nitrite (NO_2), NO_3 and PO_4 levels by 100, 98.7, and 94.8%, respectively.

At laboratory level, Ardelean et al. (2019) used a selected consortium of OPhM, consisting of both cyanobacteria and microalgae, grown on artificial wastewater and obtained after 7 days of cultivation 0.562 g L^{-1} dry biomass, with a content of 58.25 mg lipids, 301.25 mg proteins and 2 mg carotenes g^{-1} dry weight (Ardelean et al., 2019). When it comes to water purification, after 5 days of cultivation the BOD decreased from 130 mg O_2 to zero, whereas total N and inorganic P decreased by 7% and 33%, respectively (Ardelean et al., unpublished but reported results).

In the next figure there are presented the functions of OPhM in waste water purification (Figure 1).

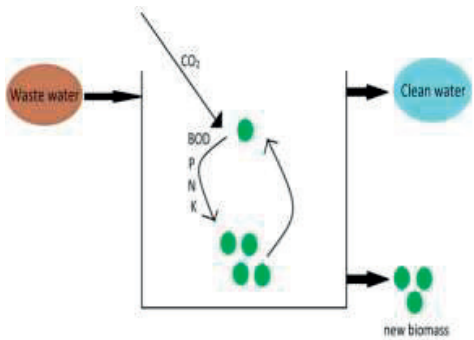

Figure 1. Schematic picture concerning the function of OphM in the consumption/use of organic substances (via heterotrophic or mixotrophic metabolism) as well as inorganic pollutants (N, P, K) from waste waters, and carbon dioxide from atmosphere for synthesis of new cells. This functioning results in simultaneous epuration of waste water and the generation of new biomass (the contribution of associated microbiota is not presented)

Apart of different types of bioreactors used, reviewed by Gao et al., 2016, there are several outdoor structures of interest in waste water epurations. Algal Turf Scrubbers (ATSs have been successfully used to treat multiple types of pollution, including agriculture runoff, excess nutrient accumulation in lakes, and manure effluents (Siville and Boeing, 2020).

The concept of algal turf scrubbers (ATSs) were first introduced simultaneously by Adey (1982) as well as Sladeckova et al. (1983) (cited by Siville and Boeing, 2020). Sladeckova et al. (1983) were studying the role of periphyton in waterworks pre-treatment for nutrient removal whereas Adey and Steneck (1985) descovered that the primary productivity on coral reefs was 5-10 times higher than that of most terrestrial forests. Their explanation of the increased productivity values was based on the filamentous turf algae growing on the surfaces of the reef and on the oscillating water motion caused by winds (Adey and Steneck, 1985).

Modern ATS provide a sloped surface for water to flow across, which promotes growth of benthic, filamentous macroalgae, periphytic microalgae, and bacteria, having the specificity of easy physical harvest, thus reduces the overall cost involved in biomass production. Siville and Boeing, 2020 published a study on an optimized harvest rates that can aid in increasing biomass production in management practices. Their hypothesized that selecting an optimized rate of harvest at anoptimum temperature would have positive efects on management goals, namely in nutrient reduction and algal biomass production. They concluded that ATS can play an important role in the remediation of high nitrate waste waters and in the production of commercially viable algal biomass. Harvest rates between 7 and 14 days were able to optimize ATS and maximize biomass production, nutrient absorption wbeing not impacted by harvest rate.

Microbial mats, either naturally occuring or artificially constructed, had been also used for different type of bioremediation, including for aquaculture wastewater treatment (Bender et al., 2004; Coban et al., 2018). Microbial mats are stratified microbial communities, composed of a complex of bacteria and dominated by photoautotrophic cyanobacteria, which can transform nitrogenous wastes into cellular protein and rapidly metabolize other fish wastes. Recent reviews on the subject of this paper are available (Wollmann et al., 2019; Dourou et al., 2020; Msanne et al., 2020; Pacheco et al., 2020; Solovchenko et al., 2020).

The newly synthesised biomass can be further used as feed in multitrophic systems or for conversion to methane (Angelidaki et al., 2009; Olsson et al., 2018) or other valuable compounds, could be an efficient and cheap solution for the treatment of AQWW, with a realistic chance of an economic viability in real conditions (Angelidaki et al., 2009; Olsson et al., 2018).

2. GAMMA IRRADIATION AT NON GROWTH INHIBITORY DOSES IN ORDER TO INCREASE LIPID CONTENT OF THE CELLS

In recent years, there has been an increasing interest in using relatively low doses of gamma radiation to stimulate biological processes in microalgae (Rivasseau et al., 2010; Tale et al., 2017; Ermavitalini et al., 2017a and b; Moisescu et al., 2019; Almarashi et al., 2020) as well as in other types of microorganisms (Ardelean et al., 2020a and b, and references herein).

Tale et al. (2017) used gamma irradiation as a stressor to induce lipid hyper-accumulation (up to 40% of biomass) in two strains of *Chlorella sorokiniana* (i.e. *C. sorokiniana* KMN2 and *C. sorokiniana* KMN3) whereas Jeong et al. (2017) have shown that chronic LDR-type irradiation leads to increased cell densities, specific growth rates, and biomass of the four species. Ermavitalini et al. (2017a), showed that *Botryococcus* sp. irradiated at low doses (2, 4, 6, 8 and 10 Gy), the highest biomass (0.833 g) and lipid content (41% total biomass) were found in the 10 Gy irradiated microalgae. Later, Ermavitalini et al. (2017b) analysed the fatty acid profile of *Botryococcus* sp. control cells and found only 6 types of fatty acids while in 10Gy irradiated microalgae cells found 12 types of fatty acids, with an increased proportion of long chain fatty acids and a low proportion of short chain fatty acids. Moisescu et al. (2019) demonstrated that the generation time of *Chlorella sorokiniana* UTEX 2130 decreases to 56% at 10 Gy, 60% at 50 Gy, and 77% at 100 Gy irradiation and the relative lipid content increases by 20% and 50% after 10Gy and 100Gy irradiation, respectively.

Apart from gamma irradiation there are other stressor effective in enhancing lipid synthesis in microalgae such as nitrogen starvation, phosphate limitation, magnesium supplementation, carbon source, iron content in the culture medium, high salinity, high light intensities, low oxygen pressure, and dehydration recent review Abo-State et al. (2019), phytohormones (Guldhe et al., 2019), a pre-treatment of inoculum with low doses of cold atmospheric-pressure plasma (Almarashi et al., 2020) and exogenous additions of reactive oxygen species Sivaramakrishnan & Incharoensakdi (2017).

Almarashi et al. (2020) in a very interesting paper showed that the biodiesel recovery from the green microalga *C. vulgaris* can be enhanced through a pre-treatment of inoculum with low doses of cold atmospheric-pressure plasma (CAPP). A treatment of 30s resulted in the highest biomass productivity of 0.193 g L^{-1} d^{-1}. Moreover, short exposure times (30 and 60 s) significantly increased the lipid content by 7.5% and 6.9%, respectively, over the control. Because 30 s pre-treatment enhanced both growth and lipid content, the volumetric lipid productivity (i.e., 40.7 mg L^{-1} d^{-1}) increased by 16.6% and 17.6% over the control and 60 s, respectively. Furthermore, the maximum volumetric fatty acid methyl esters (FAMEs) production (i.e., 998.1 mg L^{-1}) was recorded in the culture inoculated with 60 s exposed cells, which was 43.5% and 15.7% higher than that of the control and 30 s, respectively.

Furthermore, Sivaramakrishnan & Incharoensakdi (2017) showed that a UV pre-treatment followed by the application of H_2O_2 can increase the total lipid production in *Scenedesmus* sp. They reported that at 2 mM H_2O_2, the mutant had an increase in the lipid content of 55 to 60% of dry cell weight compared to the wild type grown under the same conditions. Importantly, these results also suggest that oxidative stress mediates lipid accumulation.

3. THE USE OF LIVING PHOTOSYNTHETIC BIOMASS AS BIOCRUSTS TO INCREASE THE CHEMICAL PARAMETERS OF SOIL AND PHYSIOLOGICAL CHARACTERISTICS OF PLANTS

Many studies have shown the possibility of photosynthetic microorganisms, including eukaryotic microalgae, anoxygenic phototrophs and cyanobacteria, to stimulate soil fertility and increase crop yields (Li et al., 2017) forming so-called biological soil crusts or biocrusts (Weber et al., 2016).

Biostimulators are materials other than fertilizers, which, when applied in small amounts, promote the growth and quality of food crops/vegetables/fruits, stimulate the absorption of mineral nutrients and extend the tolerance of plants to abiotic stress. Moreover, they do not generate chemical residues and fully respect human health and the environment, which then makes them a sustainable alternative to synthetic plant protection products (Du Jardin, 2015).

They can increase seed germination, improve plant growth, crop yield, flower set and fruit production, as well as shelf life after harvest (Calvo et al., 2014).

Microalgae and cyanobacteria, such as phototrophs, can not only help replace chemical fertilizers with benefits for plant growth and crop yield, but can also contribute to CO_2 sequestration, as they add organic matter to the soil, thus improving soil structure (Maqubela et al., 2009). In addition, microalgal biomass is a rich source of metabolites in agriculture (Nirmal et al., 2018), it also produces extracellular polymeric substances (EPS). Freshwater microalgae, e.g. *Chlorella vulgaris*, have been shown to provide large amounts of macro- and micronutrients, carbohydrates and proteins (Elarroussia et al., 2016), as well as growth-promoting factors (e.g. cytokines (Stirk et al., 2002; Ördög et al., 2004).

In soil biocrust, cyanobacteria are found in close association with other organisms, such as bacteria, algae, lichens, and moss. As part of these communities, cyanobacteria play a key role in soil properties and functions. Filamentous cyanobacteria bind soil aggregates and create a stable surface layer that facilitates colonization by other organisms that form biocrusts such as lichens and mosses (Deng et al., 2020). Cyanobacteria fix CO_2 (Miralles et al., 2018) and some species are able to fix N_2, increasing the content of organic nitrogen and soil nutrients (Mager & Thomas, 2011). It also releases a wide range of substances into the soil, such as growth-promoting regulators, vitamins, amino acids, polypeptides, proteins and sugars that contribute to soil fertility and act as biocontrolling agents against plant pathogens, fungi and micro-algae (Singh et al., 2016). Cyanobacteria have received special attention as bioinoculants for the ecological restoration of degraded lands (Rossi et al., 2017). Inoculation of the soil with cyanobacteria has been shown to lead to soil improvements in desertified natural soils (Park et al., 2017).

Exopolysaccharides (EPS) are among the most important compounds synthesized by cyanobacteria that play a vital role in soil functions. The more soluble or poorly bound EPS fractions in the soil are considered to be an important source of energy for heterotrophic activity, while several condensed or closely related EPS fractions of soil are mainly involved in soil particle consolidation, contributing to soil stability (Chen et al., 2014; Chamizo et al., 2019).

4. SYNTHESIS OF METAL NANOPARTICLES BY OXYGENIC PHOTOSYNTHETIC MICROORGANISMS

Nanoparticles are materials with different shapes (spherical, triangular, rods etc) and dimensions between 1 nm and 100 nm. A recent major review on NP synthesis by oxygenic photosynthetic (micro) organisms (cyanobacteria, green algae, brown or red algae) clearly illustrates the state of the art in this field (Chaudhary et al., 2020).

The study of the synthesis of MNPs mediated by living matter is a relatively new scientific topic, focused on the use of bacteria, cyanobacteria and actinomycetes, fungi, lichens, algae and plants extracts in this process (Rai & Duran, 2011). Very interesting, these syntheses usually occur under normal conditions of temperature and pressure, with no toxic chemicals involved in the process, thus the protocol is friendly to the environment.

The first experiments on MNPs synthesis by cyanobacteria were done on *Plectonema boryanum* (Lengke et al., 2006) the reported results showing two important things about cyanobacterium *Plectonema boryanum* UTEX 485: i) interaction of cyanobacteria with the chemical environment is an important factor controlling the morphology of Au particles and ii) the reduction of Au(111) is actually two-step, involving an intermediate Au(I)-S phase, with the sulphur being of organic origin (Lengke et al., 2006).

Another important group in this field demonstrated that three filamentous cyanobacteria strains *Anabaena, Calothrix* and *Leptolyngbya* have the capability to reduce Au, Ag, Pd and Pt ions to elemental metal organized as nanoparticles (Brayner et al., 2007). Very important, the authors put forward the hypothesis that nitrogenase is involved in nanoparticle production (Brayner et al., 2007).

Focsan and co-workers (2011) aimed to elucidate the interplay between biomineralization and metabolic activities in the case of the cyanobacterium *Synechocystis* sp. PCC 6803 exposed to gold ions. The authors demonstrated the ability of the cyanobacteria to reduce gold ions, the yield of GNPs synthesis being strongly dependent on the intensity of aerobic respiration and oxygenic photosynthesis. This is the first paper on cyanobacteria where surface-enhanced Raman scattering, SERS, uses biogenic MNP as reporter structures to analysis their own cellular localization, and where the evolution of respiratory oxygen consumption and photosynthetic oxygen production are quantified during (gold) nanoparticle synthesis, thus arguing the involvement of these catabolic and anabolic processes in MNP synthesis by cyanobacteria, under physiological conditions.

The ability of *Anabaena flos-aquae to form* (Dahoumane et al., 2012) represent further steps in elucidating this process in different strains, showing that before the addition of gold causes the inhibition of photosynthesis as measured by monitoring chlorophyll *a* fluorescence in *vivo*. Based on their original results, the authors (Dahoumane et al., 2012), in connection with other papers, conclude that the Au(III) species are first in contact with exopolissacharides network, where the reduction take place.

MNPs have great potential for applications in different domains such as the electronic, chemical, mechanical and life sciences industries. For example in biology and medicine the main applications are with respect to: fluorescent biological labels; drug and gene delivery; biodetection of pathogens, detection of proteins, probing of DNA structure, tissue engineering, tumor destruction *via* heating (hyperthermia), separation and purification of biological molecules and cells (Li et al., 2011) whereas in environmental protection MNPs with improved catalytic activity have become important for *in situ* destroying of organic pollutants (Hennebel et al., 2009).

5. BIOELECTROCHEMICAL PROPERTIES OF OPhM FOR BIOTECHNOLOGICAL APPLICATIONS

Historically, the first studies on bioelectrochemical properties of OPhM were done on biofuel cells (BFC). In the next figure (Figure 2), there is figurated the general structure of a BFC with, the anode compartment containing the OPhM which donate electrons to the anode either via an added electron carrier (hydrophylic artificial redox mediators (HARM) or lipophilic artificial redox mediators (LARM) either without the use of any added redox carrier (directly). The cathode chamber contains an electron acceptor (e.g. molecular oxygen which is reduced to water or ferrycianide which is reduced to ferrocyanide) which is reduced with electrons coming *via* the external circuit from the anode- the electroneutrality is maintained by protons passing through the semipermeable membrane from anode to cathode chamber (Bennetto, 1990; Greenman et al., 2019).

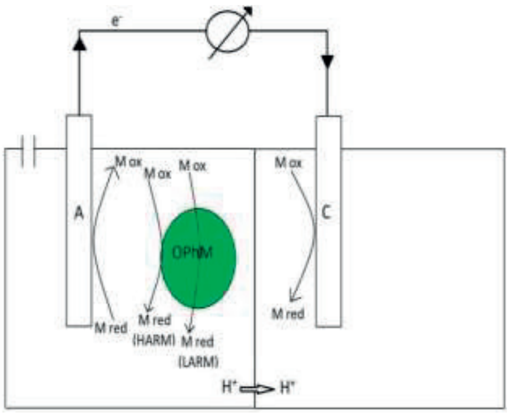

Figure 2. Schematic diagram of the structure of a dual chamber biofuel cell with photosynthetic oxygenic microorganisms (see the text for more explanation). A - the electrode (anode) in the anode chamber C - the electrode (cathode) in the cathode chamber. The protons pass through a semipermeable membrane from the anode compartment to the cathode compartment

There are plenty of reports concerning the use of OphM as biocatalyst in the anodic compartment to donate electrons to the anode, electron which come mainly from photosynthetic electron transport or from respiratory electron transport. Usually there are used added redox mediators but there are reports on direct electron transfer from cells to the anode (Apollon et al., 2021; Elshobary et al., 2021).

In cyanobacteria the situation is as follows. Hydrophilic artificial redox mediators/ carriers (HARM) can take electrons exclusively from cell membrane surface, being not able to penetrate hydrophobic membranes (in the absence of pores). In cyanobacteria, at the cell membrane occurs respiratory electron transport from which HARM can take electrons. However, due to the occurrence of common electron carriers functioning both in PhET and in RET and prokaryotic structure of cyanobacteria at the cell membrane can arrive also electrons originated in the PhET (Bennetto, 1990; Pisciotta et al., 2011). Lipophilic artificial redox mediators (LARM) being able to penetrate hydrophobic membranes can take electrons from inside the cell, mainly from thylakoids where both PhET and RET occurs as well as from the cytoplasm (where intermediary metabolic reaction occurs) and from cell membrane (Bennetto, 1990; Gadhamshetty et al., 2013; Greenman et al., 2019).

In microalgae the situation is as follows RET and PET occurs separately inside the eukaryotic cell, mithocondria and chloroplasts, respectively thus HARM cannot take electrons from these sites, whereas (LARM) cand take electrons from these processes as well as from intermediary metabolism occurring in the cytoplasm. It should be remembered that in both prokaryotes and eukaryotes these mediators may influence intracellular processes, dramatically shortening the lifetime of cell metabolism. This inhibitory effect drastically limited practical application for the conversion of solar energy to electricity (the initial goal of these studies) as well as other applications.

The biotechnological potential of BFC concerns the conversion of solar energy into electricity, the development of biosensors mainly for inhibitors of photosynthetic electron transport (as is the case with some herbicides) and, more recently, for waste water epuration. This rather new direction concerns the utilisation of OPhM together with non-photosynthetic microorganisms. Luo et al. (2020) have published a series of papers based on an integrated photo-bioelectrochemical system (IPB) that successfully combines a BFC and an algal bioreactor for bioremediation of wastewater. They reported associations of ammonia oxidizing bacterium (AOB), *Nitrosomonas europaea* and a nitrite oxidizing bacterium (NOB), *Nitrobacter winogradski* with and green alga *C. vulgaris*. This study, apart of developing the use of OPhM in IBS for wastewater treatment, is the first study to specifically test the effects of adding nitrifying bacteria (AOB/NOB) in the *C. vulgaris* culture, and their functions even under variable ammonium (NH_4) loading (Luo et al., 2020).

CONCLUSIONS

The conclusion of Grewe and Pulz (2012) that cyanobacterial (and algal) biotechnology must be considered to be still in its infancy is valid even today (Mutanda et al., 2020) an opinion we agree with. Since 2012 much work has been done and many excellent reviews have been published from which only few are summarized in the followings. Ruiz et al. (2016) conducted a market analysis taking into account the following biomass value pyramid (biofuels, chemicals, food, feed, specialties in food, cosmetics). Basically, their projections show a current cost per unit of dry biomass of 3.4 Euro/kg for microalgae, arguing that production of high-value products from microalgae could be profitable nowadays and commodities will become profitable within 10 years (Ruiz et al., 2016). However, the high costs associated with microalga bioprocessing for biofuel production are major constraints for the success of the algal biotechnology industry (Ruiz et al., 2016), Mutanda et al. (2020) explores the current status of the biorefinery approach, including genetic manipulation of microalgae for enhancement of product yield, focusing with lucidity on pros and cons (Mutanda et al., 2020). Urtubia et al (2016) stress on the fact that biotechnological application of cyanobacteria and microalgae will significantly benefit through increasing collection of genomes sequenced, together with the identification and characterization of new molecular elements within the cell (e.g., promoters, codon usage, terminators, plasmids, selection markers, and reporter genes, clearly arguing the importance of genetic modifications for biotechnologies. In agreement with Khan et al (2018), besides the potential of microalgae for a plethora of products and services there are still constraints which must be overcome to upgrade the technology from pilot-phase to industrial level, the most important being the growth rate and product synthesis, as well as dewatering of algae culture for biomass production, (more details Khan et al., 2018). Sharma and Sharma (2017) clearly stress on the fact that the need of water for microalgae cultivation is a major constraint for the development of biotechnologies at large level and in their review indicate some biotechnological companies and their already commercialized products (Sharma and Sharma 2017).

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