MICROALGAS VERDES INMOBILIZADAS EN ALGINATO DE SODIO PARA LA PRODUCCIÓN DE BIOHIDRÓGENO

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Resumen

La combinación de procesos de biotecnología algal y tratamiento de aguas residuales pueden contribuir a la producción de biocombustibles como el bioetanol, biodiésel y biohidrógeno, para remediar los retos que enfrenta la escasez de combustibles fósiles y los impactos ambientales. El hidrógeno como vector energético limpio, es una alternativa prometedora a los combustibles fósiles convencionales. Entre las diferentes tecnologías para producir hidrógeno, están las que utilizan microalgas, con sus características naturales para crecer, utilizando solo agua, nutrientes y luz solar. Dentro de las grandes oportunidades de las microalgas es que pueden cultivarse en aguas residuales urbanas, por los nutrientes que contienen, reduciendo costos de producción de biomasa y consumo de energía. Esta investigación propone la inmovilización de microalgas de las especies *Chlorella vulgaris* y *Scenedesmus obliquus* en alginato de calcio *y,* cultivadas en agua residual artificial para su crecimiento fotoautotrófico con tres distintas luces: blanca, azul y violeta. Posteriormente, se induce la producción de biohidrógeno bajo procesos de oscuridad (condición anaeróbica) a pH 7.5 y 30 °C. De acuerdo con los resultados, la luz azul induce un mayor crecimiento celular que la luz violeta, mientras que una mayor producción de hidrógeno en cultivos con crecimiento celular previo bajo luz violeta, con valores de 128 ml H_2 L^{-1} (productividad 204.8 ml H_2 L^{-1} d^{-1}) y 60.4 ml H_2 L^{-1} (productividad 39.18 mL H_2 L^{-1} d^{-1}) para *Scenedesmus obliquus* y *Chlorella vulgaris*, respectivamente. Una ventaja adicional es la alta remoción de carbono orgánico de los cultivos de *Scenedesmus obliquus* bajo luz incidente púrpura, en comparación con *Chlorella vulgaris*, ocurriendo doble beneficio: producción de hidrógeno y tratamiento de aguas residuales.

Palabras clave: *Chlorella vulgaris*, *Scenedesmus obliquus*, calidad de luz, células inmovilizadas, biohidrógeno.

GREEN MICROALGAE IMMOBILIZED IN SODIUM ALGINATE FOR THE PRODUCTION OF BIOHYDROGEN

Abstract

The combination of algal biotechnology processes and wastewater treatment can contribute to the production of biofuels such as bioethanol, biodiesel and biohydrogen, to remedy the

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challenges faced by the scarcity of fossil fuels and environmental impacts. Hydrogen as a clean energy source is a promising alternative to conventional fossil fuels. Among the different technologies to produce hydrogen, are microalgae, due to their natural characteristics to grow, using only water and sunlight. Among the great opportunities of microalgae is that they can be cultivated in urban wastewater, due to the nutrients they contain, reducing biomass and energy production costs. This research proposes the immobilization of microalgae of *Chlorella vulgaris* and *Scenedesmus obliquus* species in calcium alginate and, cultivated in artificial wastewater for their photoautotrophic growth with three different lights: white, blue and purple. Subsequently, the production of biohydrogen is induced under dark processes (anaerobic condition) at pH 7.5 and 30 ° C and under processes of reduction of sulfur. According to the results, blue light induces greater cell growth than purple light, while there was greater hydrogen production in cultures under violet light of 128 ml H_2 L^{-1} (productivity 204.8 ml H_2 L^{-1} d^{-1}) and 60.4 ml H_2 L^{-1} (productivity 39.18 mL H_2 L^{-1} d^{-1}) for *Senedesmus obliquus* and *Chlorella vulgaris*, respectively. An additional advantage is the high organic carbon removal of *Senedesmus obliquus* cultures under purple incident light compared to *Chlorella vulgaris*, with double benefit: energy production and wastewater treatment.

Keywords: *Chlorella vulgaris*, *Scenedesmus obliquus*, light quality, immobilized cells, biohydrogen.

1. Introducción

Among the great challenges for the coming years, one is to use renewable, clean and environmentally friendly energy sources, avoiding dependence on fossil fuels. Most of the energy used is obtained from fossil sources, which are non-renewable energy sources that are harmful to the environment (Azwar et al. 2014). It is because of this situation that different works have been carried out to investigate other energy sources, clean and sustainable, so that they can gradually replace fossil fuels and avoid negative effects on the environment as much as possible. Among the best known biofuels is biodiesel obtained from microalgae biomass, since they produce saturated and polyunsaturated fatty acids, necessary in the production of this biofuel. In addition, different benefits of these microalgae can be indicated, such as the fixation of CO_2 during photosynthesis and removal of nutrients from wastewater. For these qualities, microalgae have high potential in the area of microalgal biotechnology (Ruiz-Marin et al. 2010; Lim et al. 2010; De Godos et al. 2010; Wang et al. 2010; Chinnasamy et al. 2010).

On the other hand, some of these photosynthetic microorganisms have the ability to produce molecular hydrogen (H_2), with a high energy content, 122 $kJ \cdot g^{-1}$, 2.75 times greater than the energy content of hydrocarbon fuels (Argun et al. 2008). Among the first investigations on the production of H_2 by microalgae, Gaffron and Rubin (1942) reported that the *Scenedesmus obliquus* strain can produce H_2 at low rates in dark conditions and by replacing the culture atmosphere with nitrogen gas. In the work carried out by Kessler (1974), it was reported that to produce H_2, microalgae need to adapt to the transition from anaerobic conditions of darkness and oxygenic photosynthesis, as a means to re-oxidize the electron transport pathway. Microalgae produces hydrogen by adopting a two-stage process (indirect biophotolysis). In stage 1, CO_2 is fixed in the presence of sunlight through

photosynthesis; that is, the microalgae produce O_2 and accumulate carbon in the form of biomass. In stage 2, the hydrogen produced by the degradation of stored organic compounds via anaerobic takes place in the absence of oxygen using multi-enzyme systems under a series of complex biochemical reactions (Argun et al. 2009; Kapdan and Kargi 2006).

Studies have reported that the use of immobilized cells for hydrogen production is more attractive than using free cells. The immobilized cells systems have advantages such as an increase in the cell retention time within bioreactors and higher metabolic activity than free cells (Tam and Wong, 2000). Additionally, immobilized cells help to avoid the settling during growth; this phenomenon inhibits growth due to limited gas diffusion and light penetration: therefore, immobilized cells show greater hydrogen production than free cell cultures (Rashid et al., 2013).

Several strategies have been implemented to improve hydrogen production such as the variation of light intensity, carbon source, pH, temperature and sulfur deprivation (Azwar et al., 2014; Rashid et al., 2013). The sulfur lack or deprivation in microalgae cultures is a key factor since it inhibits protein synthesis and consequently the production of oxygen declines which is hydrogenase enzyme inhibitor (Antal and Lindblad, 2005). For the production of H_2 in green microalgae, it is necessary that there exist anaerobic conditions in the absence of light, since this induces activation of enzymes that promote the metabolism of H_2. Hydrogenase sensitivity to oxygen is a big challenge for this method, so that further research is needed to develop engineered hydrogenase so that it is not sensitive to oxygen inactivation. Sulfure scarcity and anaerobic condition induce expression of [FeFe]- hydrogenases in algal cells, so that continuous hydrogen production can be achieved (Ghirardi et al., 2000). [FeFe]-hydrogenase is an enzyme which plays a vital role in anaerobic metabolism, which is produced by green algae and become more efficient among other catalyst hydrogenases. [FeFe]-hydrogenase is able to catalysis the reversible oxidation of molecular hydrogen (Florin et al., 2001; Azwar et al., 2014).

Then, hydrogen is produced by the degradation of stored compounds and is increased by adding some extra carbon source, hence the type of carbon source and concentration affect the costs of the process to produce biohydrogen. Within the alternatives, wastewater can be indicated as a source of carbon and other nutrients (N and P), which leads to another benefit because the wastewater treatment is carried out (Ruiz-Marin et al., 2010). According to Brennan and Owende (2010), the combination of these processes will be the most plausible commercial application in the short term and a sustainable way to produce bioenergy and bio-products (Batista et al., 2015). It can be indicated that in the production of hydrogen most of the investigations use cultivation methods with different qualities of light and sources of carbon, considered as important factors for the process. However, there is little information on the influence of light quality (wavelength) on the H_2 production process. It has been indicated that the light source contributes to the biochemical composition, in addition to the fact that blue light promotes cell growth and purple light to accumulate lipids (Chavez-Fuentes et al., 2018). Therefore, by manipulating the wavelength in the algal culture, it is feasible to regulate photosynthesis and biochemical composition. In this research, biohydrogen production was determined in immobilized

cells of two types of microalgae, *Chlorella vulgaris* and *Scendesmus obliquus*, grown in wastewater combining the effects of lighting, with three types of light: white, blue, and purple.

2. Metodología

Culture and acclimatization of the strains

Both microalgae, *Chlorella vulgaris* and *Scenedesmus obliquus* were obtained from the Biology Laboratory and Microalgae Culture Collection of the Center for Scientific Research and Higher Education of Ensenada, Baja California (CICESE), Mexico. Microalgae was cultured during acclimatization under laboratory conditions in a sterile artificial wastewater medium, with the following concentrations (mg L^{-1}): NaCl, 7; $CaCl_2$, 4; $MgSO_4.7H_2O$, 2; KH_2PO_4, 15; and NH_4Cl, 115 (Ruiz-Marin et al., 2010). Trace metals and vitamins were added by following guidelines for ‘‘f/2” medium preparation (Guillard and Ryther, 1962). The cultures were maintained under axenic /monospecific conditions in 250 mL flasks at (25 ± 1)°C, and at a continuous irradiance of 140 μE m^{-2} s^{-1} with fluorescent lamps (60 W) of cold white light; the photon flux rate was measured with a quantum sensor (Biospherical Instruments, QSL-100). The microalgae were transferred to fresh artificial wastewater every six days, maintaining the cultures in agitation through an orbital shaker (100 rpm).

Immobilization process of microalgae

The microalgae were cultivated in medium artificial wastewater at 90 mg L^{-1} of NH_4Cl to increase cell density. During the exponential growth phase (determined in a particle counter Automated Cell Counter T20), an inoculum with cell density of 2 x 10^6 cells mL^{-1} was harvested by centrifugation at 3500 rpm for 10 min. The cells were resuspended in 50 mL of distilled water to form a concentrated algal suspension with a cell density of 1 x 10^8 cells mL^{-1}. The algal suspension was then mixed with a 4% sodium alginate solution in 1:1 volume ratio to obtain a mixture of 2% algae-alginate suspension. The mixture was transferred to a 50 mL burette and drops were formed when “titrated” into a calcium chloride solution (2%). This method produced approximately 6500 uniform algal beads of approximately 2.5 mm diameter with an initial concentration of 3.2 x 10^5 cells $bead^{-1}$ for every 100 mL of the algae-alginate mixture. The beads were kept for hardening in the $CaCl_2$ solution for 4 hours at (25 +/- 2 °C), then rinsed with sterile distilled water (Ruiz-Marin et al., 2010).

Production and analysis of biohydrogen

For biohydrogen production, the two-stage method was used, where hydrogen and oxygen synthesis occur partially separated. In the first stage, the algae growth photosynthetically under normal cultivation conditions. During the second stage, the microalgae are exposed to anaerobic conditions and sulfur is limited. With this process system, no toxic products are generated, and compounds with high added value can be produced as a result of the microalgae cultivation (Costa and De Morais, 2011).

For the stage 1, the immobilized cultures (C. vulgaris and S. obliquus cells) were incubated by triplicate for a period of approximately 4 days with a nitrogen content of 30 mg NH_4 L^{-1} in photobioreactors glass flasks of 1.5 L with mechanical agitation (100 rpm) and containing artificial wastewater culture medium at 30 °C (Ruiz-Marin et al. 2010). Each culture was provided with a light source: white, blue and purple light at 140 µE m^{-2} s^{-1} (Figure 1).

Figura 1. Oxygen-free incubation chamber and hydrogen production monitoring

During the experiment, the manipulation of water samples and microalgae-alginate beads was avoided to prevent contamination of the culture medium; therefore, each reactor was confined in a chamber provided with fluorescent lamps (Osram 60 W) of cold white light, blue and purple.

The cultures of immobilized microalgae previously cultivated under white, blue and purple light after 4 days, were transferred to similar reactors with agitation (Figure 1) under anaerobic conditions in sulfate free medium (replacing $MgSO_4.7H_2O$ with $MgCl_2$) of the following composition (mg L^{-1}): NaCl, 7; $CaCl_2$, 4; $MgCl_2$, 2; KH_2PO_4, 15; NH_4Cl, 115, and with the addition of 10 g L^{-1} glucose (1° Brix) as a carbon source, at pH 8. Each reactor was placed inside the chamber and N_2 gas was purged into the medium for 10 min to remove dissolved oxygen. The reactor was kept under mechanical stirring for 4 days. For the measurement of hydrogen flow (mL H_2 L^{-1}) a flow meter (H_2 Gas Flow Meter, series: 32908-51 Cole-Parmer Instrument Company) was installed for continuous recording of gas volume every second (mL H_2 L^{-1}) during the anaerobic period. For the statistical analysis, in relation to the biohydrogen production, analysis of variance (ANOVA) was performed using Statistic software (StatSoft Inc., Tulsa, OK, USA). On the other hand, when there were significant differences in the results, the Tukey test ($P \leq 0.05$) was applied.

Glucose removal analysis

An approximate analysis of glucose consumption at the end of the incubation period under anoxic conditions for each treatment was determined from the data of soluble solids, which corresponds to the total ratio of glucose dissolved in the solution, which is represented as ° Brix in a refractometer (10 g glucose L^{-1} equivalent to 1 ° Bx). The quantification of total reducing sugars was carried out under the method described in the Mexican standard NMX-F-312-1978.

3. Results

In the present study, during the first stage of photosynthetic growth, both immobilized microalgae, *C. vulgaris and S. obliquus*, showed no inhibitory effects on growth in relation to the quality of incident light (white, blue and purple). A cell count at the end of the culture period showed that during stage 1, microalgae *C. vulgaris* increased the number of cells from 3.0 x 10^5 cells beads^{-1} to 14 x 10^5 for white light, 17 x 10^5 under blue light, and 9 x10^5 cells beads^{-1} under purple light, while for *S. obliquus,* the increase in cell density was in the order from 3.0 x 10^5 cells beads^{-1} to 8 x 10^5, 10 x10^5, and 7 x 10^5 cells beads^{-1} for white, blue and purple light, respectively. During the second stage (dark anaerobic), the hydrogen production was measured until the maximum production was observed. As can be seen in **Table 1**, hydrogen production was proportional to the glucose consumption.

Table 1. Removal of glucose in dark-anaerobic and maximum productivity of H_2 in cultures of *C. vulgaris* and *S. obliquus* immobilized in alginate beads (data are shown as mean ± SD, n=3).

Microalgae	Light	Glucose removed (g L^{-1})	Maximum production H_2 (mL L^{-1})	Productivity (mL H_2 L^{-1} d^{-1})
C. vulgaris	White	from 10 to 6	2.02	$0.65^a \pm 0.25$
	Blue	from 10 to 8	0.91	$0.26^a \pm 0.19$
	Purple	from 10 to 3	60.4	$39.2^b \pm 23.1$
S. obliquus	White	from 10 to 5	7.6	$3.72^c \pm 1.60$
	Blue	from 10 to 9	1.13	$4.52^c \pm 2.70$
	Purple	from 10 to 0.5	128.0	$205^d \pm 61$

Means followed by similar letters showed not significantly different (Tukey; $p \leq 0.05$)

The glucose uptake showed significant differences ($p = 0.001$): for the cultures under purple light, it was observed a high glucose removed of 70% and 90%, for C. vulgaris and S. obliquus immobilized cells, respectively (see Table 1). This was related with the maximum production of H_2 for both microalgae, where the maximum hydrogen production for S. obliquus was of 128 mL H_2 L^{-1} (productivity of 204.8 mL H_2 L^{-1} d^{-1}); while for *C. vulgaris* the peak of maximum production was of 60.4 mL H_2 L^{-1} (productivity of 39.18 mL H_2 L^{-1} d^{-1}) (see Figure 2; Table 1).

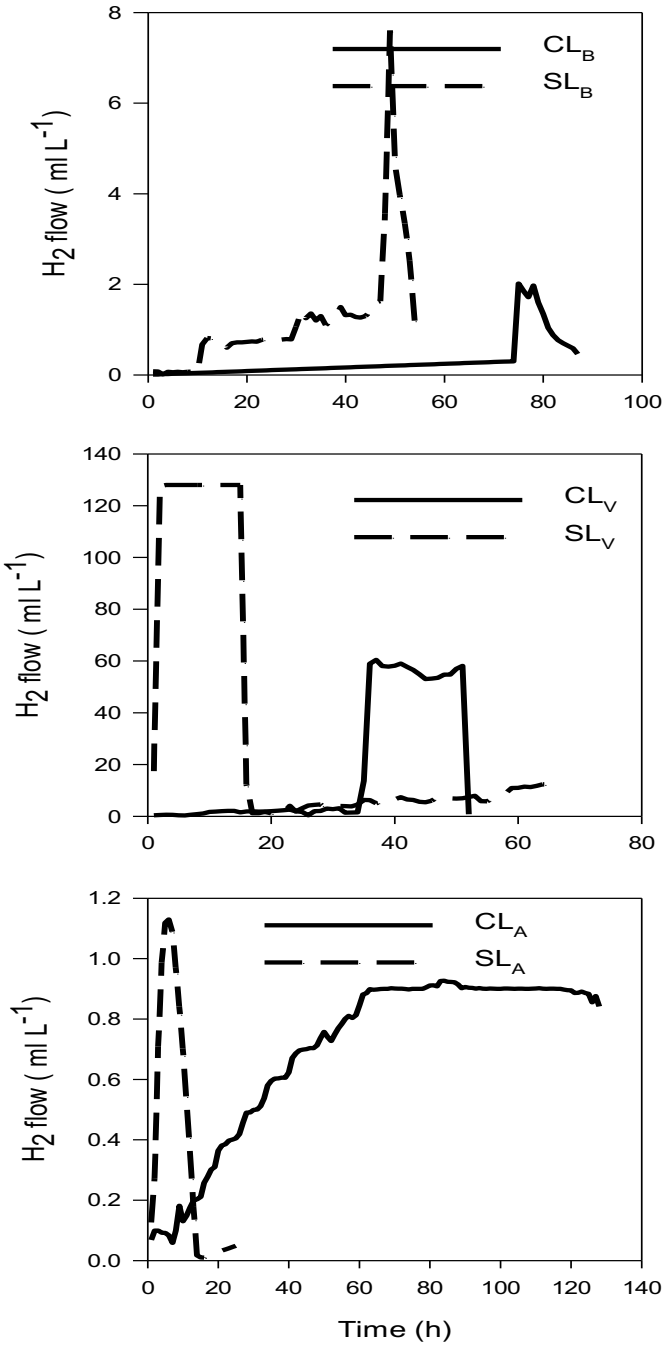

Figura 2. H_2 production by *C. vulgaris* and *S. obliquus* immobilized under different wavelength light: CLB and SLB: Culture of *C. vulgaris* and *S. obliquus*, respectively, previously cultured under white light; CLV and SLV: Culture of *C. vulgaris* and *S. obliquus*, respectively, previously cultivated under purple light; CLA and SLA: Culture of *C. vulgaris* and *S. obliquus*, respectively, previously cultivated under blue light. The continue lines are used for *C. vulgaris*, and dotted lines are used for *S. obliquus*.

Immobilized microalgae grown under anaerobic conditions showed the ability to change their metabolism and use sources of organic carbon (glucose) for growth. This is an opportunity to carry out integral microalgae cultures in wastewater, achieving significant energy savings in wastewater treatment systems and obtaining chemical products of high commercial value (Table 1). According to the results obtained, microalgae *S. obliquus* grown in artificial wastewater is proposed as a candidate for the production of hydrogen and to be able to participate in wastewater treatment systems using organic carbon sources.

4. Discussion

One alternative for the production of hydrogen by microalgae cultures is the use of cheap and available carbon sources with the aim of making hydrogen production profitable. Microalgae such as *C. vulgaris* and *S. obliquus* have shown the ability to develop in wastewater and efficiently remove nitrogen, phosphorus and organic carbon, as well as generate high-value chemicals such as lipids that are currently being investigated for biodiesel synthesis, and have the ability to change the metabolism from autotrophic to mixotrophic or heterotrophic. For this reason, currently urban wastewater has been considered as a profitable culture medium, providing nutrients and carbon sources to sustain the growth of *C. vulgaris* and *S. obliquus*.

In the present study, the maximum increase in cell density for the cultures under blue light for both immobilized microalgae were similar to the reported by Chavez-Fuentes et al. (2018) for free cells cultures of *C. vulgaris* and *S. obliquus*. Other studies suggest that blue light is more efficient for carrying out photosynthesis (Das et al., 2011; Korbee et al., 2005). While other authors report that the purple light, due to the high energy that can emit, cause negative effects on the growth of *C. vulgaris* and *S. obliquus* (Mohsenpour and Richards, 2012).

It is documented that both microalgae *C. vulgaris* and *S. obliquus* have the ability to change the metabolism from autotrophic to mixotrophic, but without a heterotrophic condition. In the present study, the phototrophic change during the first stage and followed by a dark cycle (second stage) makes it possible to evaluate the changes in growth and hydrogen production under a mixotrophic condition (light / dark cycles).

Although the available information is scarce on growth in immobilized systems under different light sources, it has been documented that, in free cell cultures, growth changes can occur when going from a phototrophic to a mixotrophic culture system in microalgae, as reported by Canedo-Lopez et al. (2016) in mixotrophic cultures (white light / dark) of *C. vulgaris* showed a low cell density in artificial wastewater medium and urban wastewater of 11.65 x 10^6 cells mL^{-1} and 10.76 x 10^6 cells mL^{-1}, respectively; compared with phototrophic culture of 17.66 x 10^6 cells mL^{-1} and 15.26 x 10^6 cells mL^{-1}, respectively. Concluding that lighting conditions (continuous light / photoperiods) influence algal growth. On the other hand, Papazi et al. (2012) reported a lower mixotrophic growth of *Scenedesmus obliquus* for 5 days with dichlorophenol from 4.5 x 10^5 cells mL^{-1} to 11.9 - 16.1 x 10^5 cells mL^{-1}; with the aim of increasing the rate of hydrogen production. Although the comparison is not absolutely correct between free and immobilized cells, because the conditions and the parameters used in the literature are totally different, it is a fact that mixotropic conditions tend to decrease cell density compared to phototrophic cultures.

In addition to the above, the mixotrophic culture under different light sources could also cause changes in algal growth such as those reported by Chavez-Fuentes et al. (2019) suggesting that the intensity and light source modifies the growth and biochemical composition, reporting the highest concentrations of biomass dry weight (g L^{-1}) and cellular density (cells mL^{-1}) for a white light source (140 µE m^{-2} s^{-1}) of 0.3 g L^{-1} and 4.9×10^6 cells mL^{-1}, respectively, and for blue light, of 0.4 g L^{-1} and 4.5×10^6 cells mL^{-1}, respectively; in contrast to the observed for purple light (0.23 g L^{-1} and 2.97×10^6 cells mL^{-1}, respectively) and yellow light (0.12 g L^{-1} and 3.13×10^6 cells mL^{-1}, respectively). This is congruent with the reported in the present study, cultivation of immobilized cells showed a low cell density (cell $beads^{-1}$) under purple light with respect to blue light, suggesting the light quality is a factor key that can modify the growth and, consequently, algal biochemical composition could be modifiable in cultures with artificial wastewater.

It is a fact that the immobilization of cells on substrates offers a greater advantage over free cells in suspension, since the immobilized cellular matter occupies less space, requires a smaller volume of growth medium, is easier to handle, and can be used repeatedly for products generation. In addition to photosynthetic bacteria, immobilized green algal cultures has also been employed to increase the yield and efficiency of H_2 production in these eukaryotic oxygenic photosynthesis systems. Immobilized systems have been found to be more efficient at switching between the oxygenic photosynthesis (growth) and the hydrogen production modes. Kosourov and Seibert (2009) reported for *Chlamydomonas reinhardtii* immobilized on alginate films in sulfur / phosphorus-deprived cultures, a high cell density (2000 µg Chl mL^{-1}) and high hydrogen production rates (12.5 µmol mg^{-1} Chl h^{-1}) . It is a fact that immobilization helps to improve the hypoxic environment in the vicinity of the cells, thus promoting conditions for H_2-production and making more efficient the use of the carbon sources contained in the culture media.

During the second stage, hydrogen production by *C. vulgaris* and *S. obliquus* immobilized cells was proportional to the glucose consumption (Table 1). These results also suggested that the maximum glucose uptake for the cultures of *C. vulgaris* and *S. obliquus* (70% and 90%, respectively) growth under purple light in stage 1, were related with the maximum production of H_2. The ability to remove organic carbon has been reported in numerous microalgae in mixotrophic and heterotrophic conditions, making this attractive for use in wastewater treatment systems. Canedo-López et al. (2016) reported a removal of total organic carbon (TOC) for *C. vulgaris* in mixotrophic free culture in artificial wastewater (70.5% - 86.0%) and urban wastewater (43.7% - 56.2%). Other studies in free cell culture suggest a high removal of chemical oxygen demand (COD) for *Chlorella sp* and *S. obliquus* from 63% to 88% (Lu et al., 2016; Gupta and Pawar, 2018). This suggests that both microalgae can change its metabolism from autotrophic to mixotrophic according to prevailing conditions, and continue to use inorganic and organic carbon (Ogbonna and Tanaka, 2000; Liang et al., 2009; Mandal and Mallick, 2011).

The high hydrogen production obtained for *S. obliquus* of 128 ml H_2 L^{-1} (productivity of 204.8 mL H_2 L^{-1} d^{-1}) and for *C. vulgaris* of 60.4 ml H_2 L^{-1} (productivity of 39.18 mL H_2 L^{-1} d^{-1}) (Figure 2; Table 1) were high, when

compared to the reported by Chader et al. (2009) for *C. sorokiniana* of 1.35 mL H_2 L^{-1} h^{-1} in free cell cultures, containing acetate as the only carbon source under optimal conditions of pH = 7.2 and light intensity of 120 μE m^{-2} s^{-1} at 30 ° C. Rashid et al. (2013) evaluated the production of hydrogen by immobilized *C. vulgaris* optimizing parameters such as: pH, carbon source (glucose, fructose, sucrose and malt extract) and light intensity. The authors reported a maximum production of 812, 874, 1315 and 1144 mL L^{-1} for the different carbon sources at pH = 8, respectively. These values were high compared to that obtained in the present study, but other factors could intervene in the production of H_2 when microalgae are cultivated in wastewater, such as organic load, carbon sources and competition and predation by other microorganisms. According to Das and Veziroglu (2001), a high concentration of carbon source modifies the metabolic pathway and leads to production of unwanted by-products, and, because of this, it is important to consider each of these factors during hydrogen production.

In cultures of *C. vulgaris* under white, purple and blue light, a prolonged lag phase was observed before hydrogen production of 70 h, 35 h and 10 h, respectively, suggesting this time as required to change the metabolism from autotrophic to heterotrophic to use the available carbon sources in the wastewater and be able to express the hydrogenase enzyme for subsequent hydrogen production. In contrast, S. *obliquus* only presented a lag phase in cultures under white light (Figure 2), compared with the cultures under purple and blue light suggesting a high capacity of the microalgae to adapt under these cultivation conditions and, to activate the enzyme hydrogenase for production of hydrogen in the first hours of dark anaerobic condition. In fact, microalgae *C. vulgaris* showed an insufficient ability to degrade glucose into protons, and consequently, during this period of prolong time lag, the hydrogenase enzyme was not active sufficiently to convert them into hydrogen.

Although the biochemistry of immobilized cells was not determined in the present study, some considerations may be mentioned. It is likely that a light source with a high level of energy (purple light) induces lower growth but with a high uptake of organic carbon and potentiate the production of hydrogen, while in the case of blue light (low energy level) induces growth but lower hydrogen production during the anaerobic stage. In this context, the results could suggest that the accumulation of lipids which is induced by light quality (purple light) contributes to a better use of external carbon sources, since cells under these conditions will have a lower content of carbohydrates-proteins, so it forces a metabolic change and quickly activates the hydrogenase enzyme under anaerobic conditions, which could be related to low energy uptake from purple light, compared to those cultures under white and blue light, where the carbohydrate and protein content assumes that they are high. However, more studies should be carried out to know which of these two conditions during anaerobic dark phase cultivation contributes to increased hydrogen production. In fact, microalgae *S. obliquus* represents a better proposal for the hydrogen production than *C. vulgaris* and is a candidate for the wastewater treatment with the ability to efficiently remove the carbon source from urban wastewater and obtain bio-hydrogen as an energy source.

5. Conclusions

The sulfur deprivation in immobilized microalgae cultures is a key factor for

hydrogen production, with the advantage of uptake external carbon source. The light source with a high level of energy (purple light) induces lower cell growth but with a high uptake of organic carbon and potentiate the production of hydrogen, while in the case of blue light (low energy level) cell growth is induced, but with lower hydrogen production during the anaerobic stage.

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