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Sustained hydrogen production using a semi-continuous system with immobilized and free cell cultures of *Scenedesmus* sp. and *Coelastrum* sp.

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Abstract:
Background*: Hydrogen gas provides clean energy with high energetic content (122 kJ/g). This contributes to it being considered as a promising substitute for fossil fuels. The biological production of hydrogen occurs through the photosynthetic activity of microorganisms. Particularly, microalgae stands out for its high photosynthetic efficiency of conversion of light. However, hydrogen production from microalgae depends on species and culture in anoxygenic conditions. In this regard, only a few microalgae species have been evaluated for hydrogen production, with variable yields.*
Materials and Methods*: Hydrogen production was evaluated from two different methods of culture, free and immobilized cell cultures in two genera of microalgae: Scenedesmus sp. and Coelastrum sp., in TAP medium by indirect biophotolysis. The free cell cultures were maintained for 4 consecutive days in anaerobiosis and sulfur limitation, while immobilized cell cultures were maintained during four cycles in a semi-continuous culture with cyclic two-phase operation of photosynthesis and hydrogen production. Both modes of culture were incubated under total darkness and continuous light and vice versa.*
Results*: From the free cell cultures, the hydrogen volume obtained was higher in total darkness (5-15 x 10^{-5}ml and 8.4-20.9 x 10^{-5}ml of H_2), than in continuous light (2-4.6 x 10^{-5}ml and 1-7.3 x 10^{-5}ml of H_2) for Scenedesmus sp. and Coelastrum sp., respectively. The immobilized cells produced a larger hydrogen volume in a semi-continuous process, by cyclic operation in two phases, with a total duration of more than 280 hrs. In the dark, 42-60 x10^{-5} ml and 96.7 -282 x 10^{-5} ml of H_2 were detected and with continuous light 8-21 x10^{-5} ml and 13-16 x 10^{-5} ml of H2 in Scenedesmus sp. and Coelastrum sp*
Conclusion: *The total darkness provided during incubation of immobilized and free cell cultures promoted hydrogen production from Scenedesmus sp. and Coelastrum sp. In this study Coelastrum sp. generated greater hydrogen production under the evaluated conditions in comparison with Scenedesmus sp. The hydrogen volume produced is promising, considering the volume of culture medium employed.*
Key Word*: Biohydrogen; Renewable energy; Bioenergy; Biofuels; Green microalgae; immobilization of cell.*

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I. Introduction

Hydrogen is a gas which provides clean energy, combustion of which produces only water. Its high energetic content (122 kJ/g) is 2.75 times higher than that obtained from fuels derived from hydrocarbons[1]. This contributes to hydrogen being considered as a substitute for fuels with a promising future.

Hydrogen is not naturally available on earth[2], and thus it has to be obtained from steam reforming (50% of total H2 produced) by the utilization of syngas, natural gas, coal and waste biomass[3]. However, these processes demand a great deal of energy because of the high temperatures required (970-1100°K), and release high quantities of CO_2, which means they are not recognized as eco-friendly processes on a commercial scale[4]. There are other process for H_2 production, such as gasification (carbon and biomass), electrolysis of water, partial oxidation, pyrolysis and biological methods[5].

The biological and renewable production of hydrogen occurs through the photosynthetic activity of microorganisms, cyanobacteria, microalgae, and photosynthetic bacteria[2]. Particularly, microalgae stands out for its high photosynthetic efficiency of conversion of light[6]. At low light intensities, microalgae can achieve values up to 80% of the theoretical maximum of photosynthetic efficiency of CO_2 fixed per mol photons absorbed[7]. In fact, South Korea has designed a plant that can produce ~330 tons of H_2/year, utilizing CO as feedstock in marine microorganisms[8].

Microalgae produce hydrogen in a process of two phases, known as indirect biophotolysis. Phase I occur in aerobiosis to capture and fix CO_2 during photosynthesis in the presence of light to store carbohydrates (glycogen or starch) in the form of biomass. In phase II the culture is transferred to an anaerobic condition without sulfur, to down-regulate the synthesis of protein D1, and limit the evolution of oxygen and activation of hydrogenase, a key enzyme for hydrogen generation[9]. In phase II, multi-enzymatic degradation of organic compounds synthesized in phase I occurs in anaerobiosis[10] under a series of complex biochemical reactions resulting in hydrogen[11].

Since the first report of hydrogen production from the use of microalgae in 1938, only some genera have been studied, and thus it is necessary to evaluate more species under different culture conditions[12-14]. Hydrogen production is highly sensitive to culture conditions[15], such as carbon source and light energy[16]. In this respect, there are some authors who refer to hydrogen production as a photoregulated process[17], and thus different quality lights have been tested[14], or exposure to continuous light in sulfur deprivation, prior to total darkness, in order to consume remaining O_2 by respiration, and thus to promote anoxic conditions[18]. Others mention that in a dark anaerobic environment, hydrogenase was reactivated, and hydrogen gas evolved[10].

Although relatively new[19] and scarce[14], cell immobilization has been shown to promote hydrogen gas production in some species of microalgae[20], which is very convenient for culture in a semi-continuous system[10], because there is a more efficient alternation between oxygenic photosynthesis (growth) and hydrogen production[14]. However, this also depends on the adaptive capacity of microalgal species and the transition between oxygenic photosynthesis and anaerobiosis[21].

In this study, hydrogen production was evaluated in *Scenedesmus* sp. and *Coelastrum* sp. grown in TAP media under sulfur limitation, in two forms of culture: immobilized cells in a semi-continuous system and free cells. Both under continuous light and total darkness conditions and vice versa.

II. Material And Methods

Strains and growth conditions

In this study green freshwater microalgae: *Scenedesmus* sp. (SCRE-1) and *Coelastrum* sp. (COE-1) from the strain collection of CICY (Center of Scientific Research of Yucatan) were evaluated. The strains were donated in 2013 from the Collection of Marine and Freshwater Microalgae of the Yucatan Peninsula by the "Alfredo Barrera Marín" Herbarium. The precultures (in triplicate) of *Scenedesmus* sp. and *Coelastrum* sp. were created by inoculating 10% of their mother cultures in 100 ml of TAP medium (Tris-Acetate-Phosphate). The cell concentration was determined by daily counting using a hemocytometer and a light microscope (Nikon Eclipse E200). The culture conditions were continuous light with 51 µmol of photons m2/s, 25°C and 150 rpm (Excella Platform Shaker, Eppendorf).

Cell immobilization

Oxygenic phase (phase I)

The oxygenic phase initiated by inoculating 10% by volume of microalgal culture in logarithmic phase in 100 ml of TAP culture medium. The light intensity conditions were 95-97 µmol of photons m^2/s (LED) with photoperiod 16:8 h (light: dark), 25 °C and 150 rpm.

Anoxigenic phase (phase II)

This phase initiated with immobilization of cells collected in logarithmic phase: $1.49x10^7$ - $1.075\ x10^7$ cel/ml and $1.19x10^7$ – $1.82\ x10^7$ cel/ml from *Scenedesmus* sp. and *Coelastrum* sp., respectively. The collected cells were washed three times with distillated water, centrifuged at 6878 g during 15 min at 4ºC and finally resuspended in 10 ml of TAP medium. This TAP (Tris-Acetate-Phosphate) medium is suitable for producing hydrogen gas in *Chlamydomonas reinhardtii*, *Chlorella sorokiniana* and *Tetraspora* sp., among others[18, 22, 23].
This cell suspension was immobilized by adding 1.5% of agar. Then it was mixed until it homogenized and heated to 35°C. It was left to solidify in Petri dishes and then was cut into cubes of 0.7 cm × 0.7 cm × 0.7 cm (Figure 1).

Figure 1. Agar cubes with immobilized cells.

Approximately 40 g of immobilized cells were deposited in Erlenmeyer (250ml, in triplicate), 100 ml of TAP medium was added and it was incubated under aerobic conditions (Phase I) during 2 weeks for adaptation (Figure 2). The hydrogen production in anaerobiosis or phase II, initiated by replacing the medium with a culture medium limited in sulfur (TAP-S). The S-medium was prepared by replacing all sulfates with chloride salts at the same concentrations and the same molar concentration.

Figure 2. Agar cubes with immobilized cultures A) *Scenedesmus* sp. and B) *Coelastrum* sp.

The cubes of immobilized cells of *Scenedesmus* sp. and *Coelastrum* sp. were placed in serologic bottles (120 ml) and 55 ml of TAP-S medium was added (in triplicate). The serologic bottles were sealed with a crimper (EZ crimper, Wheaton) using septum of butyl rubber (20 mm) and aluminum aril. Then, the cultures were flushed during 10 min with N_2 to completely eliminate O_2 dissolved in the medium. Finally, the bottles were incubated under two different conditions: total darkness, at 25°C and 150 rpm and continuous light with 51 µmol of photons m^2/s (LED), 25 °C and without agitation during 96 hrs.

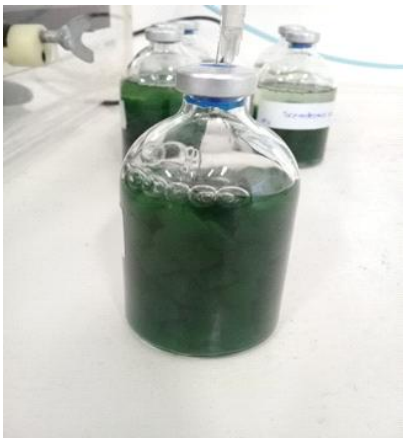

Figure 3. Flushed with N_2 in immobilized cultures.

Cycling of hydrogen production

Hydrogen production in semi-continuum was as follows. The immobilized cells in phase II were submitted to phase I during three consecutive days. That is, at the end of characterization of phase II (96 h) the limited culture medium was replaced with fresh TAP medium, to start up another cycle of hydrogen production. This cyclic procedure of phase II to phase 1 was carried out two more times on the same cell culture.

Free cell cultures

Hydrogen in free cell cultures was produced using oxygenic cultures, with a cell concentration of $1.68x10^7$ cell/ml and $1.05x10^7$ cell/ml for *Scenedesmus* sp. and *Coelastrum* sp. 1 ml was taken from each culture and centrifuged at 8,500 rpm for 15 min at 4°C, the supernatant was discarded and the cultures of each specie inoculated in triplicate, under anaerobiosis in serologic bottles (120ml) with 80ml of limited medium (TAP-S). Then they were incubated under two different conditions: continuous light with 51 µmol of photons m^2 s^{-1} (LED), 25 °C and without agitation for 96 hrs; and in total darkness at 25°C and 150 rpm during 96 hrs.

Hydrogen detection

The hydrogen concentration generated was determined by gas chromatography (Clarus 500, PerkinElmer) with a column packed with a molecular sieve of 30m x 0.53 mm (PerkinElmer), using nitrogen as carrier gas. The gas generated was collected with a 1-ml syringe (Hamilton Sample Lock). The heating ramp was 50°C for 4 min, the temperature of the thermic conductivity detector (TCD) was of 200°C and the injector temperature was 35°C. The standard time of retention of hydrogen was 0.57 minutes. The volume of the gas produced was calculated from its calibration curve.

III. **Result**

Strains and growth conditions

The different growth phases and the maximum cell concentration were determined after 24 hrs of inoculation and during nine days in oxygenic conditions (Figure 1). In both species, the initial phase was of two days and on the third day, the log phase began. *Scenedesmus* sp., showed a short log phase, with a cell concentration higher than *Coelastrum* sp. The maximum cell concentration registered in log phase was for *Scenedesmus* sp. and *Coelastrum* sp., of 1.49 x 107 cell·ml-1 and 1.19 x 107 cell·ml-1 for the fifth and fourth day of culture, respectively. *Scenedesmus* sp. and *Coelastrum* sp. showed a cell concentration of 2.396 x 107 cell·ml-1 and 1.29 x 107 cell·ml-1 on the 9th day of culture, respectively. The optimum cell concentration for initiation of anaerobiosis was 8 x 107 células·ml-1 (in log phase), for immobilized and free cell cultures.

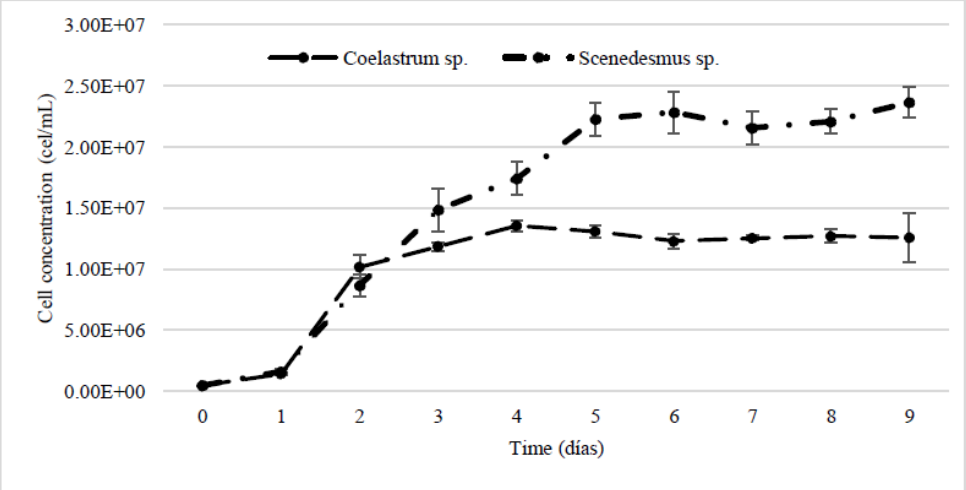

Figure 1. Cell concentration of *Scenedesmus* sp. and *Coelastrum* sp.

Cycling of hydrogen production under total darkness

The hydrogen production in Scenedesmus sp. and Coelastrum sp. (Figure 2) was stable during four cycles in a semi-continuous culture with cyclic two-phase operation of photosynthesis and hydrogen production. Under evaluated conditions the hydrogen production increased with respect to time. During the first cycle, a positive adaptation was observed in Scenedesmus sp. to anoxygenic conditions with hydrogen production (Figure 2 A). While Coelastrum sp. showed an adaptation phase with variable hydrogen production, although with a tendency towards stabilization in further cycles. Particularly, Scenedesmus sp., exhibited higher hydrogen production in the first cycle at 96 h, compared to the first cycle of Coelastrum sp., which showed less hydrogen production. In the second and third cycles, the hydrogen increased but with a tendency towards stabilization in Coelastrum sp., compared to Scenedesmus sp.

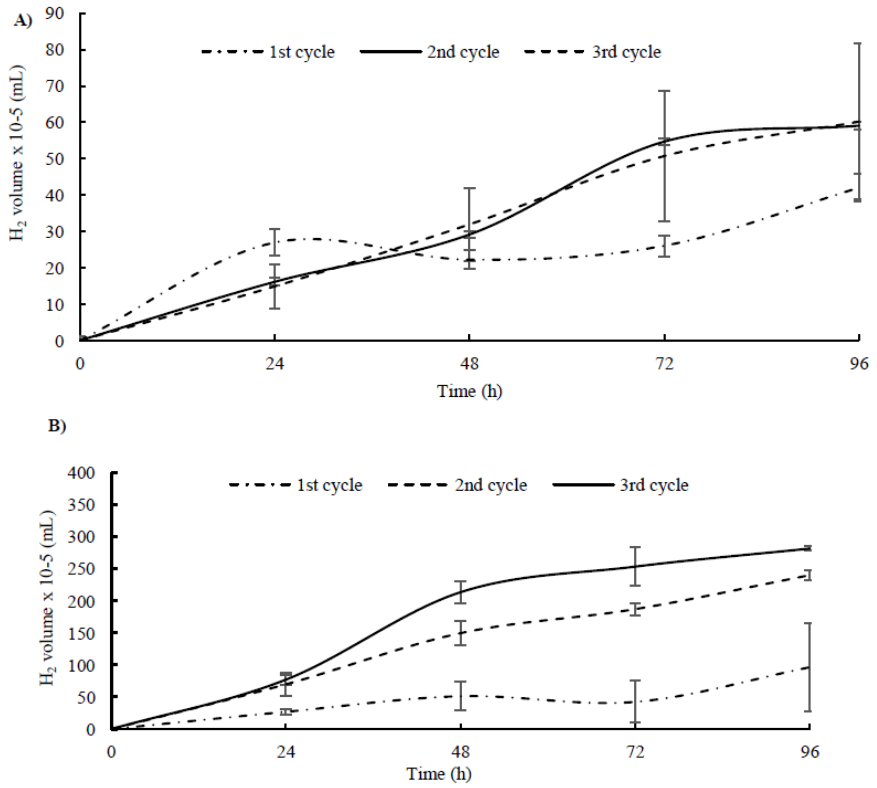

Figure 2. Hydrogen production in immobilized culture incubated in total darkness, *Scenedesmus* sp. (A) and *Coelastrum* sp. (B)

In both evaluated species, hydrogen production stabilized after 50 h, in a semi-continuous system during 4 cycles for a total time of 288 h, demonstrating that immobilization of *Scenedesmus* and *Coelastrum* cells is capable of producing H_2 in a semi-continuous manner via a two-stage cyclic operation. The total accumulated volume of hydrogen produced was 42-60 x 10^{-5} ml in *Scenedesmus* sp. (Figure 2A) and 96.7 -282 x 10^{-5} ml in *Coelastrum* sp. (Figure 2B).

Cycling of hydrogen production under continuum light

To evaluate continuous light during hydrogen production, immobilized cells were exposed to these incubation conditions. *Scenedesmus* sp. showed an adaptation phase during the first cycle with variable and low hydrogen production (Figure 3). Meanwhile, the adaptation phase was positive and on the rise in *Coelastrum* sp. under the conditions evaluated. In the first cycle *Scenedesmus* sp., showed slightly higher hydrogen production than *Coelastrum* sp. In the second cycle *Coelastrum* sp. exhibited higher hydrogen production than *Scenedesmus* sp. However, by the third cycle *Scenedesmus* sp. exhibited higher hydrogen production than *Coelastrum* sp. Evidently hydrogen production in *Scenedesmus* sp. was variable under the incubation conditions in comparison with *Coelastrum* sp.

Under the conditions mentioned, the total accumulated volume of hydrogen produced (Fig. 3) was of 8-21 x10^{-5} ml in *Scenedesmus* sp. and 13-16 x 10^{-5} ml de H_2 in *Coelastrum* sp.

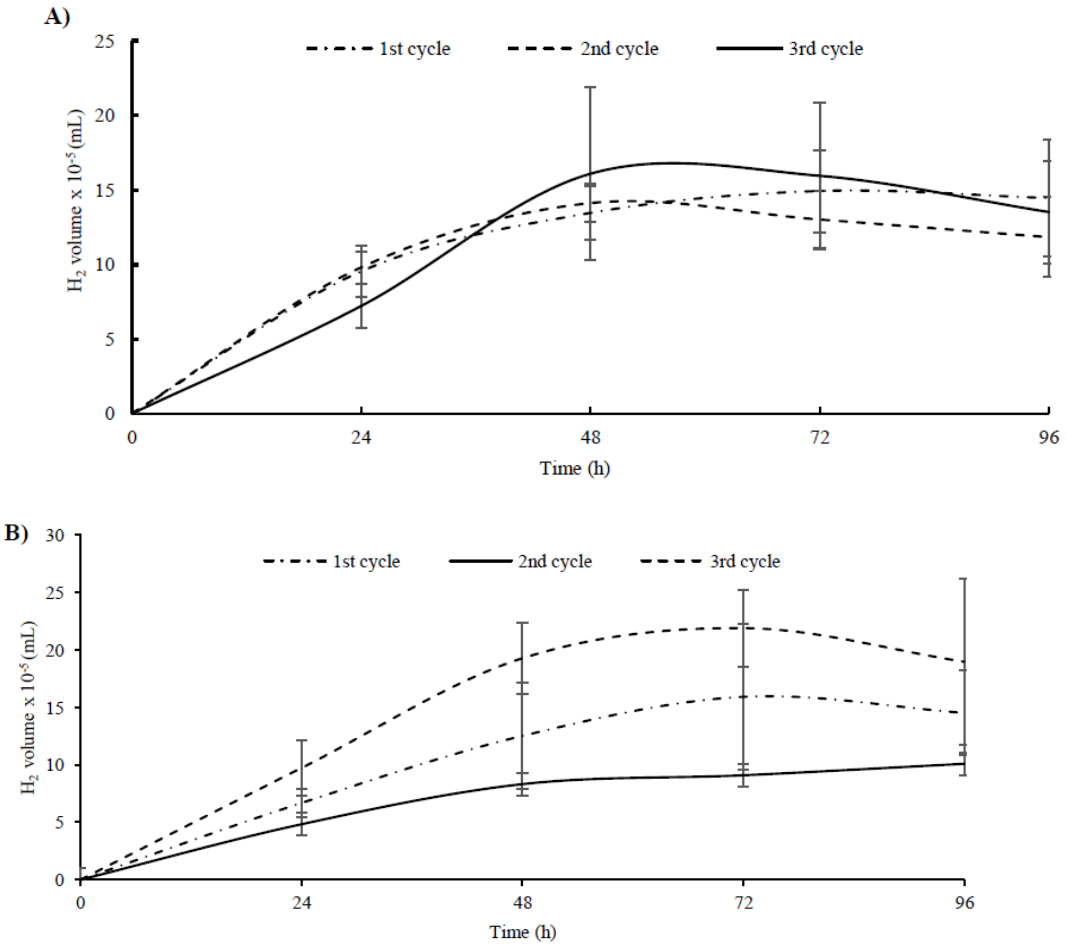

Figure 3. Hydrogen production in immobilized culture incubated in continuous light, Scenedesmus sp. (A) and Coelastrum sp. (B).

Hydrogen production in free cell cultures under total darkness and continuous light

In free cell cultures, hydrogen production increased with respect to time of culture in both species and conditions. Under total darkness, the adaptation phase to anoxygenic conditions in free cell culture was more positive and on the rise in *Coelastrum* sp. than *Scenedesmus* sp. The hydrogen volume detected in *Scenedesmus* sp. and *Coelastrum* sp. was 5-15 x 10^{-5}ml and 8.4-20.9 x 10^{-5}ml. Evidently *Coelastrum* sp. showed a greater volume of hydrogen in comparison with *Scenedesmus* sp. under evaluated conditions.

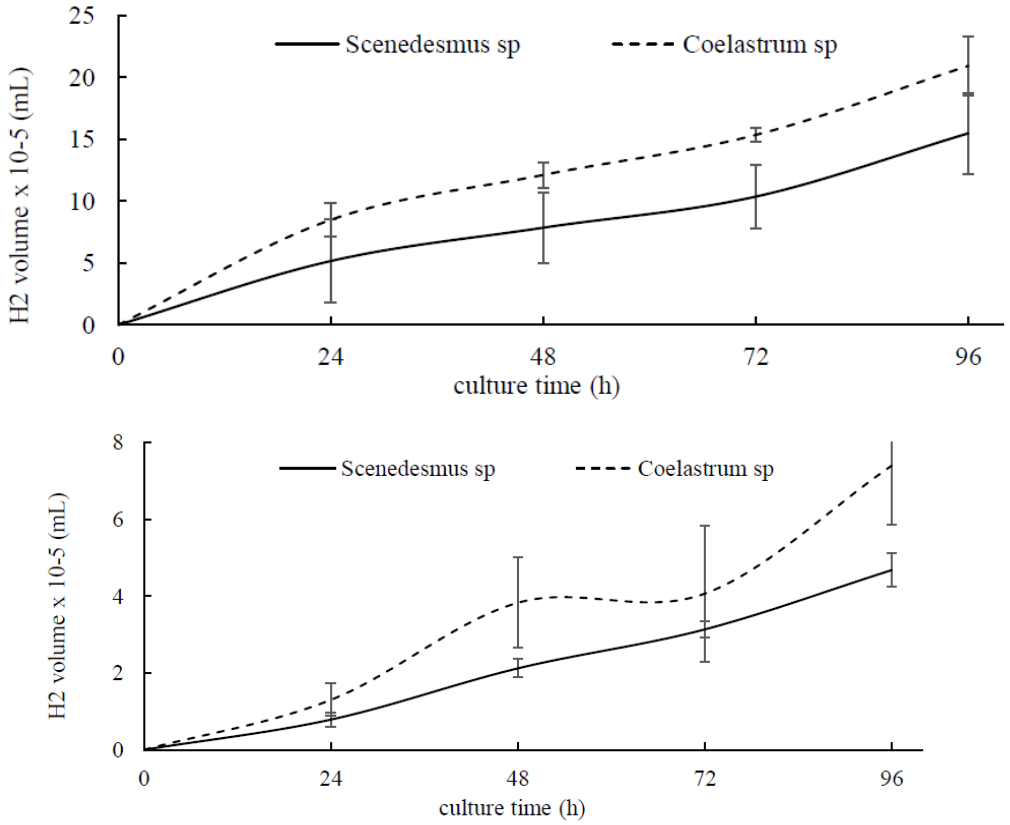

Figure 4. Hydrogen production each 24 hours in free cell culture of *Scenedesmus* sp. and *Coelastrum* sp. incubated under total darkness (A) continuous light (B).

For purposes of comparison in terms of hydrogen production free cell cultures of *Scenedesmus* sp. and *Coelastrum* sp. were exposed under continuous light. These conditions produced a delay in the time of hydrogen production in *Scenedesmus* sp. in comparison to *Coelastrum* sp., in which hydrogen production was variable and rising after 96 hrs, while *Scenedesmus* sp. exhibited sustained hydrogen production. Under the conditions evaluated, the hydrogen volume detected in *Scenedesmus* sp. and *Coelastrum* sp. was 2-4.6 x 10^{-5}ml and 1-7.3 x 10^{-5}ml, respectively. Therefore, hydrogen production in *Coelastrum* sp. was higher in comparison to *Scenedesmus* sp.

Another product of fermentation in anaerobic environment besides hydrogen is CO_2, detection of which in free cell culture increased with respect to H_2. Meanwhile, in immobilized cultures CO_2 production was lower, with respect to hydrogen.

IV. Discussion

Immobilized cultures of *Scenedesmus* sp. and *Coelastrum* sp. under dark conditions exhibit an adaptation phase in an anoxic and sulfur-deprived medium towards the production of hydrogen in the first 24 h. This adaptation phase of 25-35h under anoxic conditions is necessary for hydrogenase activation and further hydrogen evolution24. Hydrogen production was stable from 50 h. Maintenance was by immobilization of cells, which also allowed an acceleration of the changes in culture conditions for cyclic two-stage operation. This result coincides with those obtained by Rashid10, in that hydrogen production was sustained from 40h onwards, in each cycle during three repeated cycles of photosynthesis and hydrogen production in immobilized cultures of cyanobacterium *Microcystis aeruginosa*, under absence of light and sulfur, with a maximum evolution rate of 48 mL/h/L of hydrogen. Hydrogen production depends on the adaptive capacity of each species during transition from darkness to oxygenic photosynthesis, as measured by re-oxidising the route of electron transport21. Thus, the potential of *Scenedesmus* sp. and *Coelastrum* sp. for producing hydrogen is remarkable given the satisfactory viability for cell recovery during 3 days in which they are again exposed to photosynthesis or phase I. We reasoned that photosynthetic conditions provided between cycles in a semi-continuous system promoted anoxic conditions and hydrogenase activation, because as Melis18 mention, incubation in a sulfur-deprived medium under continuous light promotes O2 consumption by respiration before anoxygenic conditions. The

first scientific research about H2 evolution in microalgae was carried out on *Scenedesmus obliquus* under darkness, and produced low hydrogen production rates25. In this study, total darkness in immobilized cell cultures is a suitable incubation condition for hydrogen production in the species evaluated. Hydrogen production in immobilized cultures of *Coelastrum* sp. had not been reported before this study, only in free cell cultures and incubated under continuous light (54 μmol m-2s-1), with a low hydrogen production compared to *Scenedesmus acunae* G087 and *Scenedesmus obtusus* AARL G02013. Other incubation conditions, such as light qualities rendered in immobilized cultures of *Scenedesmus obliquus* and *Chlorella vulgaris* in sulfur-limited urban wastewater and exposed to purple light, produced 2.1 times more hydrogen (ml H2/L), the first compared to the second14.

On the other hand, cell immobilization of *Scenedesmus* sp. and *Coelastrum* sp. under continuous light, generated less hydrogen production in comparison to total darkness. This contrasts with previous reports that indicate continuous light promotes hydrogen production17,18. In fact, it has been reported that light intensity significantly influences hydrogen yield during anaerobiosis, because activation of hydrogenase for catalytic reduction of protons and electrons generates molecular hydrogen26. The hydrogen volume produced under continuous light in immobilized cultures of *Scenedesmus* sp. and *Coelastrum* sp. was similar. Species of the same genera as those evaluated in this study, *Scenedesmus* sp. (*Scenedesmus acunae* AARL G087; *Scenedesmus obtusus* AARL G020) and *Coelastrum microporum* AARL G007, showed moderate to low capacity to produce hydrogen in free cell culture and continuous light (54 μmol $m^{-2}s^{-1}$), at 25°C and manual agitation each second day13.

Immobilized cultures incubated under total darkness and continuous light presented higher hydrogen production and better maintenance between cycles in *Coelastrum* sp., than *Scenedesmus* sp. Immobilized systems in algal cultures are a suitable cultivation option to increase the yield and efficiency of H_2 production, because improving the hypoxic environment in the vicinity of the cells promotes conditions for H_2-production and makes more efficient use of the carbon sources contained in the culture media27. On the other hand, it is important to highlight that those immobilized cultures incubated under continuous light produce more carbon dioxide in comparison to incubation in darkness. In particular, an increase of CO_2 was detected after 72 hrs in *Coelastrum* sp. and *Scenedesmus* sp., although less in *Scenedesmus* sp. than in *Coelastrum* sp. (data not shown).

In free cell cultures, incubation in total darkness promoted higher hydrogen production yield in comparison to continuous light conditions. As for species, *Coelastrum* sp. rendered higher hydrogen production than *Scenedesmus* sp. Despite the results obtained, comparatively, hydrogen production yield in free cell cultures gave inferior values to those obtained in immobilized cultures. These results are probably because, as has been reported, the light conversion efficiency in suspension cultures is low (0.24%)28.

In both incubation conditions of free cell culture, with microalgae cells suspended, it is difficult to cycle the microalgae cells between a sulfur-containing medium and a sulfur-depleted medium because this requires centrifuging to repeatedly wash the microalgae cells with sulfur-free medium. This is time-consuming, energetically inefficient, susceptible to contamination and has high possibility of losing the microalgae cells29. On the other hand, 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 product generation.

A previous study has reported that the different levels of hydrogen production using microalgae are affected by several cultivation factors. In this study *Scenedesmus* sp. and *Coelastrum* sp. exhibit under evaluated conditions, promising capability of hydrogen production, with *Coelastrum* sp. showing superior capability to produce hydrogen in semi-continuous system in darkness. This is remarkable because *Coelastrum* sp. appeared to be more promising than *Scenedesmus* sp. and perhaps more than *Chlorella* sp., because in a previous report *Chlorella* sp., one of the most promising strains of microalgae for hydrogen production, and *Scenedesmus*, showed differences in hydrogen production in cultures immobilized under purple light, with a maximum detected hydrogen production of 204.8 ml H_2/L/day and 39.18 ml H_2/L/day for *Scenedesmus* sp. and *Chlorella* sp. in a 1.5 L photobioreactor14.

Finally, it is worth mentioning that the gas composition in immobilized and free cell cultures included CO_2 in addition to H_2, although with notable differences in the immobilized cultures compared to free cell cultures. Anaerobic algal photofermentations are expected to produce CO_2 and small amounts of formate and ethanol30. In this study the accumulation of fermentation by-products such as formate and ethanol was not detected. Thus, in immobilized cultures H_2 was the main fermentation product in comparison with CO_2.

V. Conclusion

Hydrogen production from microalgae has advantages, because it only requires nutrients, anoxic conditions and total darkness, which reduce production costs in comparison with other technologies. In the present study, hydrogen was obtained from cultures of *Scenedesmus* sp. and *Coelastrum* sp. by indirect bio-photolysis in immobilized and free cell cultures. The total darkness provided during incubation of immobilized

and free cell cultures promoted hydrogen production from *Scenedesmus* sp. and *Coelastrum* sp. In this study *Coelastrum* sp. generated greater hydrogen production under the evaluated conditions in comparison with *Scenedesmus* sp. The hydrogen volume produced is promising, considering the volume of culture medium employed. Perhaps, increasing immobilized culture volume will lead to higher yields of hydrogen.

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