

Hydrogen Photo-Production using *Chlorella* sp. through Sulfur-deprived and Hybrid System Strategy

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The world must find a way of producing fuel from renewable energy sources to replace the fossil fuels. The sustained production of hydrogen by *Chlorella* sp., a unicellular green alga, can be achieved in sulfur-deprived culture conditions, besides integrating dark and light bio-hydrogen production strategy. Moreover, dark fermentation carried out by bacterial consortium in anaerobic systems provides organic acids such as acetic, lactic, propionic, butyric. These compounds may be used by the algae as a carbon source to enhance the hydrogen production. In addition, acetate and citrate can be used by *Chlorella* sp. to increase the cell respiration rates due to inhibition of O₂ production during photosynthesis, and consequently, improving H₂ synthesis. This work evaluated the effect of the sulfur-deprived medium (WC) containing yeast extract and milk whey permeate, besides sugarcane molasses combined with milk whey permeate to bio-hydrogen production by *Chlorella* sp. Furthermore, it was evaluated the influence of a hybrid system in which a conditioned medium obtained from dark fermentation by bacterial consortium was used as substrate by algae on the hydrogen productivity and substrate conversion efficiency by green algae. The results showed that the hybrid systems and the use of complex carbon source can improved the biohydrogen production.

1. Introduction

Nowadays, worldwide demand for energy is growing at an alarming rate. The increased demand is being met largely by reserves of fossil fuel that emit both greenhouse gasses and other pollutants. Whereas fossil fuel reserves are limited, studies aimed at producing energy from renewable sources stand out. Hydrogen is one of the most efficient and clean desired energy sources (Milledge and Heaven, 2014). Biological production of hydrogen has been studied since it becomes an appropriate source due to the current energy demand and environmental issues (Bahadar and Khan, 2013). H₂ production may be mediated by hydrogenase or nitrogenase and ferredoxin systems and the agents may be pure or consortia of anaerobic bacteria, process known as dark fermentation (Cardoso et al., 2014), purple non-sulfur photosynthetic bacteria by photofermentation (Oliveira et al., 2014) and still photosynthetic microalgae as green algae and cyanobacteria by direct or indirect photolysis, respectively (Bahadar and Khan, 2013). Zhu et al. (2014) discussed that microalgae are able to use sunlight to metabolize carbon dioxide and split the water into oxygen and hydrogen ions and the production of H₂ is catalysed by hydrogenase. The species that have been indicated as potential hydrogen producers are *Chlamydomonas*, *Chlorella* and *Scenedesmus* (Milledge and Heaven, 2014). The limitations of H₂ production by microalgae are mainly the absence of large scale method, low yield and energy conversion efficiency and inhibition of hydrogenase by the oxygen, by-product of photolysis. Therefore, effort should be done to make biological hydrogen production a profitable and competitive process (Zhu et al., 2014). Sulfur deprivation is a key to avoid hydrogenase inhibition by oxygen. Under this condition, oxygen evolution is declined below respiration level and an anaerobic atmosphere is formed and hydrogenase may be kept active (Zhang et al., 2014).

In the current study, hydrogen production by *Chlorella* sp., a green unicellular alga, was investigated under different culture conditions, including sulphur deprivation, autotrophic and heterotrophic systems. Some species of *Chlorella* have ability to utilize heterotrophic substrate, for instance, sugars, organic acids and glycerol to grow (Samejima and Myers, 1958). In addition, the integration of more than one biological route, anaerobic consortium and phototrophic microorganisms, for hydrogen production utilizing residual organic substrates, in connection with wastewater treatment, is a competitive process. In this context, milk whey permeate and the fermentative effluent by dark fermentation were used as substrate to *Chlorella* sp. Milk whey is an agro-industrial waste that represents a promising feedstock to biofuels production, since both is quite common in Brazil.

2. Materials and Methods

2.1 Culture conditions

Chlorella sp. was grown in WC medium (g/L): 0.368 CaCl₂ 2H₂O, 0.37 MgSO₄ 7H₂O, 0.126 Na₂HCO₃, 0.114 K₂HPO₄ 3H₂O, 0.85 NaNO₃, 0.212 Na₂SiO₃ 5H₂O, 1 mL iron solution (3.15 mg/mL FeCl₃ H₂O and 4.36 mg/mL Na₂EDTA), 1 mL micronutrients solution (0.01 mg/mL CuSO₄ 5H₂O, 0.022 mg/mL ZnSO₄ 7H₂O, 0.01 mg/mL CoCl₂ H₂O, 0.18 mg/mL MnCl₂ 4H₂O, 0.006 mg/mL Na₂ MoO₄ 2H₂O and 1 mg/mL H₃BO₃) and 1 mL vitamin solution (0.1 mg/mL thiamin HCl and 0.0005 mg/mL Biotin). All solutions were prepared in distilled water.

The bacterial consortium was harvested from anaerobic sludge from an Upflow Anaerobic Sludge Blanket Reactor (UASB), gently donated by a dairy located in Uberlandia-MG (Brazil). The sludge (bacterial consortium) was initially cultured using (g/L): 20.0 lactose, 3.0 KH₂PO₄, 7.0 K₂HPO₄, 1.0 MgSO₄, 4.0 yeast extract; 1.0 (NH₄)₂SO₄ and 0.5 meat extract. Lactose was obtained from cheese whey permeate purchased from the Sooro Concentrado Indústria de Produtos Lácteos Ltda company (Brazil). The cheese whey permeate was composed mainly by lactose (92.97 %) and protein (1.42 %).

2.2 Hydrogen production assay

Dark fermentations by anaerobic consortia were carried out in 100 mL serum vials with a working volume of 75 mL and 25 mL of headspace. The reaction volume was composed by the inoculum (1.6% v/v) and the synthetic medium (98.4% v/v). The medium was purged with nitrogen gas for 1 min. Afterwards, the vials were sealed with rubber septum stoppers and aluminium rings. All experiments occurred in the dark at room temperature and in anaerobic condition during two days. Biogas and fermentative broth were analysed to determine hydrogen concentration and metabolites composition, respectively (Cardoso et al., 2014).

For the H₂ production by *Chlorella* sp, under autotrophic cell culture condition, WC medium and S-deprived WC medium were used. Under heterotrophic cell culture condition, WC medium and S-deprived WC medium, were supplemented with lactose from milk whey permeate (1.0 g/L) and yeast extract (1.0 g/L). Besides lactose, acetic acid or citric acid were tested as second carbon source in the medium.

In addition, the hydrogen production was also investigated using as substrate to green algae the fermentative effluent from dark fermentation of milk whey permeate by bacterial consortium.

The photosynthetic assays were carried out in two stages. At first, the algae cell cultures were inoculated photoautotrophically in WC medium at anaerobic conditions having an initial pH of 6.8 at 22 °C. Culture was kept in erlenmeyer flasks (500 mL) under fluorescent light (5000 lux) during the growth phase. After 10 to 15 days, cell culture was harvested and used in the second stage, that is, the hydrogen production step in penicillin flasks (50 mL), using 37.5 mL of work volume with initial algae concentration fixed at 0.1 g/L. Nitrogen (99.99 %) was purged to ensure the anaerobic conditions and the vials were sealed with rubber septum stoppers and aluminium rings. The vials were kept at 22 °C, under fluorescent light (5,000 lux) in photoperiod of 12 h.

2.3 Analysis

The growth of cells was measured via spectrophotometry (UVmini-1240, Shimadzu) and biomass dry weight. One milliliter of sample was appropriately diluted with deionized water and the absorbance of the sample was read at 570 nm. The biogas produced by cells was collected in graduated syringes (10 mL) coupled in the penicillin flasks. The hydrogen content in the biogas was determined by analyzing a gas sample (1 mL) using a gas chromatography (GC 17A Shimadzu, Japan) equipped with a CARBOXEN 1010 column, a thermal conductivity detector (TCD) and argon as a carrier gas. The temperature of both, the injector and the detector, were 230 °C. The column was maintained in 30 °C during the procedure. Sometimes, the peaks corresponding to nitrogen, carbon dioxide and oxygen also appeared with those of hydrogen, since they were already present in the system. To organic acids analysis a SUPELCOGEL C-610H column, an ultraviolet

detector and phosphoric acid (0.1%) as a carrier liquid at a rate of 0.5 mL/min were used. The oven temperature of 32 °C and sample injection volume 20 µL were also used in the procedure.

3. Results and Discussion

Many factors influence green algae growth including irradiance, culture temperature, algal biomass density, nutrient concentration, and culture age. Nutrients, for example, specifically nitrogen, phosphorus, and sulfur, are necessary for algae growth. Silica and iron, as well as several trace elements, are also considered important nutrients for growth. (Menetrez, 2012). In this present work, the influence of the medium composition on the hydrogen production by *Chlorella* sp was investigated, and the results are summarized in Table 1.

Table 1. Performance of *Chlorella* sp. maintained in several culture medium formulations

H ₂ production system	Parameter				
	Fermentation time (d)	Final cell density (g/L)	Biogas volume (mL)	Productivity (mmolH ₂ /L.d)	Y _{P/X} (mmolH ₂ /g _{cell})
<i>Dark fermentation (bacterial consortium)</i>	2	n.d.	16.5	0.57	n.d.
✓ WC	20	0.14	0.87	1.32 x 10 ⁻²	2.78x 10 ⁻¹
✓ S-deprived WC	20	0.54	1.13	6.62x 10 ⁻⁴	0.11 x 10 ⁻⁴
✓ WC + lactose + yeast extract	20	0.41	1.0	8.78 x 10 ⁻³	2.10 x 10 ⁻²
✓ S-deprived WC + lactose + yeast extract	20	0.38	1.2	0	0
✓ Fermentative broth	20	0.18	1.0	2.96 x 10 ⁻⁴	2.76 x 10 ⁻³

n.d.: not determined

Comparing the effect of sulfur in the medium, the results showed that the growth of green algae cells (0.54 g/L) in sulfur deprived WC medium was higher than in WC medium (0.14 g/L). Besides Rashid et al. (2013) reported that sulfur deprivation can increase hydrogen production, in this work, although the volume of biogas was lower (0.87 mL), hydrogen productivity (1.32 x 10⁻² mmolH₂/L.d) and the yield (2.78 x 10⁻¹ mmolH₂/ g_{cell}) was higher in the presence of sulfur. Likewise, Zhang et al. (2014) described that *Chlorella protothecoides* generated only traces of hydrogen under sulfur deprivation. On the other hand, these authors assessed that limitation of nitrogen source had a considerable influence on hydrogen evolution resulting in the formation of large amount of this fuel.

In respect to the influence of addition of lactose and yeast extract to basal WC medium and sulfur deprived WC medium, the effect was positive in the medium with sulfur, since the productivity was 8.78 x 10⁻³ mmolH₂/L.d and the yield was 2.10 x 10⁻² mmolH₂/ g_{cell}. In the basal WC medium with sulfur deprivation the supplementation of organic carbon and nitrogen sources affected negatively and no hydrogen was produced.

Further researches focused on study the effect of photoheterotrophic growth of *Chlorella*. Rai et al. (2013) investigated the growth of *Chlorella pyrenoidosa* in mixotrophic condition in presence of sodium acetate and glycerol. The biomass productivity and lipid productivity had an increment of six fold and thirty-two fold, respectively, in cultures grown with sodium acetate (0.01 g/L) in comparison to autotrophic culture. When glycerol (0.5 % by volume fraction) was the substrate, the biomass productivity and lipid productivity were three times and twenty times higher as compared to control. Rashid et al. (2013) investigated the effect of type of organic carbon source (glucose, fructose, sucrose and malt) on hydrogen production by *Chlorella* sp. as well as the influence of light and pH. The optimal pH was 8.0 and the optical fiber did not improve hydrogen evolution. Concerning the type of organic carbon source the optimum productivity was achieved in the presence of fructose (24 mL/L.h) and the highest production was 1.315 mL/L in the presence of sucrose. The maximum yield was attained for 5 g/L of each carbon source.

The fermentative broth of dark fermentation by bacterial consortium was also tested. The composition of the medium was rich in organic acids as Table 2 indicates.

Table 2. Fermentative broth composition of bacterial consortium after the two days of fermentation in the dark and under anaerobic conditions

Compound	Concentration (g/L)
Lactose	2.27
Ethanol	1.55
Lactic acid	9.24
Acetic acid	1.46
Butyric acid	0.27
Propionic acid	0.08

By analysing Table 1, the figures show that the production of hydrogen is possible using hybrid system. The productivity was lower (2.96×10^{-4} mmolH₂/L.d) than in basal WC medium, but it should be emphasized that the fermentative effluent was not supplemented with the minerals and metal traces that are part of the present in basal medium. Table 1 also indicates that lot of effort should be done to increase hydrogen production by green alga when the productivity is compared to dark fermentation stage (0.57 mmolH₂/L.d). Nevertheless, a large number of variables must to be evaluated, such as, photoperiod, photobioreactor configurations, composition medium, light intensity and dilution of dark fermentation effluent in order to improve hydrogen formation.

It should be highlighted that the proposal of this work is a hybrid system in which hydrogen is produced in both stages, by bacterial consortium and *Chlorella* sp. In the literature, usually green algae is grown to provide biomass for the following stages. Xia et al. (2013) evaluated H₂ production in for five steps, including growth and pre-treatment of biomass saccharification followed by dark fermentation of *Chlorella pyrenoidosa* biomass, photofermentation and methanogenesis. Wiczorek et al. (2014) used *Chlorella vulgaris* as biomass in a two-stage combined process of H₂ evolution by and mixed anaerobic culture and methane from the residues of the dark fermentation step. Liu et al., (2013) evaluated the growth of *Chlorella vulgaris* ESP6 without considering H₂ formation. The authors used organic acids from dark fermentation effluent by *Clostridium butyricum* CGS5 as substrate and described that *Chlorella vulgaris* ESP6 could grow on the 4-fold diluted dark fermentation broth. Furthermore, green alga consumed efficiently acetate and cell growth was inhibited by lactate, butyrate for concentrations higher than 0.5 and 0.1 g/L, respectively.

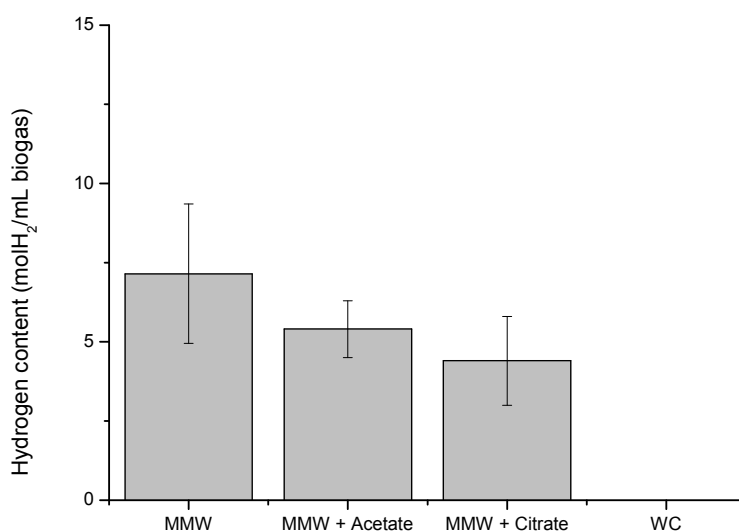


Figure 1. Hydrogen content observed to *Chlorella* sp. cultured in different medium formulation

In order to evaluate the enrichment of the medium with organic acid, acetate or citrate were used in a formulation of milk whey permeate. Kojima and Lin (2004) discussed that acetate could be use by algae cells as the carbon source that can be incorporated into the cells and as the substrate of respiration that can be

oxidated to carbon dioxide. Once anoxia has been reached to *Chlorella* sp. culture, acetate and citrate can contribute to the maintenance of a respiration rate sufficient for sustaining anoxic conditions.

Figure 1 show the concentration of hydrogen in the biogas for different formulations: sulfur deprived WC medium with lactose and yeast extract, milk whey permeate (MMW) and MMW with acetic acid (1 g/L) and MMW with citric acid (1 g/L).

As it can be observed in Figure 1, when acetate and citrate were added in MMW medium, the green alga cells produced, in the same period of time, 5.4×10^{-5} and 4.4×10^{-5} mol H₂/mL biogas, respectively. Probably, the reduction of H₂ synthesis when organic acid (acetate and citrate) was added in MMW medium occurred due to catabolic repression occurred, since the MMW medium has already a complex composition (sugar, fat, protein, minerals).

4. Conclusions

The present research aimed to investigate the influence of heterotrophic carbon source added in the culture medium in order to increase H₂ production by *Chlorella* sp. It also highlighted the use of a hybrid system, the dark fermentation by bacterial consortium and photofermentation by green algae. It can be concluded that the addition of lactose to the WC medium favoured hydrogen production. Moreover, the hybrid system is a promising alternative, since fermentative broth of dark fermentation, rich and organic acids, and allowed hydrogen evolution by green algae. Various culture parameters must still evaluated to substantially reduce cost and improve the microalgae potential to produce hydrogen, such as light intensity and photoperiod, composition medium, pH and photobioreactor configuration.

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