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# Insights into the Effect of Carbon and Nitrogen Source on Hydrogen Production by Photosynthetic Bacteria

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Nowadays the growing interest in alternative fuels is motivated by several important considerations; since the most alternative fuels are not derived from finite fossil-fuel resources and generally produce fewer vehicle emissions that contribute to smog, air pollution or global warming. Within this context hydrogen production could highlight. It has been conventionally produced through thermochemical and electrochemical processes; although, photofermentation by Purple Non-Sulfur Bacteria (PNS) is a major field of research through which the overall yield for biological hydrogen production can be improved by optimization of culture conditions. Therefore, the purpose of this paper was to evaluate the influence of different carbon (organic acids and milk whey permeate) and nitrogen (glutamate, sulfate and yeast extract) sources to hydrogen production by *Rhodopseudomonas palustris* and *Rhodobacter capsulatus* strains. The cells were cultured in 50 mL penicilium flasks and incubated at 30°C in anaerobic conditions using initial pH of 6.8 and 2,200 lux. The best result to $H_2$ productivity was 110.46 µmol de $H_2$/Lh at 60 mM/L of malic acid and 6 g/L of milk whey permeate (corresponded at 5.58 g/L of lactose) to *Rhodopseudomonas palustris*.

## 1. Introduction

Conventional energy sources based on oil, coal, and natural gas have proven to be highly effective drivers of economic progress, but at the same time damaging to the environment and to human health. Therefore, green alternative fuel sources should be considered. Hydrogen ($H_2$) has been claimed to be a good alternative to replace fossil fuel since the 1970s, mainly due to its recyclability and non-polluting nature. As discussed by Basak and Das (2007), $H_2$ liberates large amounts of energy per unit weight by combustion (143GJ/t), and is easily converted to electricity by fuel cells.

Currently, $H_2$ can be produced by several methods highlighting steam reforming of natural gas, thermal cracking of natural gas, partial oxidation of heavier than naphtha hydrocarbons and coal gasification. In addition, methods of hydrogen production from biomass like pyrolysis or gasification are generally used. On the other hand, it is also possible to produce this target-product using water from electrolysis, photolysis, thermochemical process, direct thermal decomposition, thermolysis or by biological routes (Das and Veziroglu, 2001).

Biological hydrogen production processes are operated at ambient temperature and atmospheric pressure, thus are less energy intensive and more environmental friendly as compared to thermochemical and electrochemical processes. The biological processes can be broadly classified as: biophotolysis, photofermentation and dark fermentation. In general, hydrogen photoproduction by photosynthetic bacteria requires the use of a simple solar reactor or artificial illumination. There are many types of bacteria associated with $H_2$ production such as *Rhodobacter, Rhodopseudomonas, Rhodospirillum*, among others (Li and Fang, 2009). This process is catalyzed by nitrogenase and thus occurs when the ratio of fixed carbon to fixed nitrogen is high (Androga *et al*., 2011).

It is important to note that photofermentation by Purple Non-Sulfur bacteria (PNS) is a major field of research through which the overall yield for biological hydrogen production can be improved significantly

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by optimization of growth conditions. Then, the purpose of this paper was to evaluate the molecular $H_2$ production using PNS bacteria (*Rhodopseudomonas palustris* and *Rhodobacter capsulatus* strains). Suitable process parameters such as carbon (organic acids and lactose from milk whey permeate) and nitrogen (yeast extract, ammonium sulphate and sodium glutamate) sources were investigated.

## 2. Materials and Methods

### 2.1 Microorganism and culture conditions

*Rhodopseudomonas palustris* and *Rhodobacter capsulatus* were purchased from DSMZ German Collection of Microorganisms and Cell Culture. The strains were cultivated anaerobically in RCV medium (Table 1 and 2) as suggested by Weaver *et al*. (1975), at initial pH of 6.8, under photosynthetic conditions of 2,200 lux. In photofermentation, since the ammonium ion has inhibitory effect on the nitrogenase activity (Oh *et al*. 2004), sodium glutamate and yeast extract as a nitrogen source were evaluated. In addition, milk whey permeate (93% of lactose from Sooro Ltda, Brazil) and malic acid were used in the experiments as an effective carbon sources. The initial cell concentration was fixed at 0.1 g/L in all experiments.

*Table 1: RCV medium formulation**

| ***Reagent*** | ***Concentration (g/L)*** |
|---|---|
| Malic acid | 4.02 |
| $KH_2PO_4$ | 0.60 |
| $K_2HPO_4$ | 0.90 |
| $MgSO_4.7H_2O$ | 0.12 |
| $CaCl_2.2H_2O$ | 0.075 |
| $Na_2EDTA.2H_2O$ | 0.02 |
| Thiamine | 0.001 |
| $(NH_4)_2SO_4$ | 1 |

*Micronutrient solution (1mL) was added

*Table 2: Micronutrient solution composition*

| ***Reagent*** | ***Concentration (g/L)*** |
|---|---|
| $H_3BO_3$ | 2.8 |
| $MnSO_4\ H_2O$ | 1.59 |
| $NaMoO_4\ 2H_2O$ | 0.75 |
| $ZnSO_4\ 7H_2O$ | 0.24 |
| $CaCl_2.2H_2O$ | 0.075 |
| $CuCl_2\ 2H_2O$ | 0.05 |

Batch experiments were performed in 50 mL penicillin flask (working volume of 37.5 mL) at 30°C, in duplicate. After inoculation, the bottle was flushed with Ar gas (99.999%) for 3 min to develop anaerobic conditions. Thereafter, the flasks were sealed with a thick butyl rubber septum and aluminium cap. The gas samples were collected after 7 days using 10 mL graduated syringes and stored in Gasometric ampoules (Construmaq LTDA, Brazil) until analysis.

### 2.2 Supplements evaluation on the hydrogen production

In the first batch of trials, seven formulations, using the basal RCV medium, were employed as culture media for the cells. In these assays, by fixing the sodium glutamate concentration at 2.54 g/L, the medium was supplemented with different lactose concentrations of 0.5, 1.0 or 2.0 g/L. Similar assays were performed by fixing the concentration of ammonium sulphate at 1.0 g/L. An assay using RCV medium (Table 1 and 2) without lactose supplementation was performed as a control. In addition, yeast extract at 1g/L was added in the experiments, except to the control assay.

Based on the findings obtained in the first step, 15 runs were carried out with the *Rhodopseudomonas palustris*. The influence of increasing milk whey permeate concentration from 2 to 6 g/L on hydrogen production was evaluated by varying malic acid concentration at 30, 60 or 90 mM. The effect of yeast extract supplementation on the hydrogen productivity was investigated at different yeast extract concentration (2 to 6 g/L) by fixing milk whey permeate at 2 g/L and varying malic acid concentration of 30 mM or 60 mM. The glutamate sodium concentration was 2.54 g/L.

Similar investigation was carried out using *Rhodobacter capsulatus*, with the exception of the experiment containing 90 mM/L of malic acid that was not tested. It is important to note that the malic acid was used as an essential organic acid (carbon source) as discussed in previous studies, in which acetic, lactic,

propionic and butyric acids were also investigated to hydrogen production (Oliveira *et al*., 2013; Lazaro *et al*, 2012; Shi and Yu, 2006).

**2.3 Analytical methodology**

Optical density (OD) was measured at 660 nm using a GENESYS spectrophotometer. The determination of total solids, fixed and volatiles was done by gravimetric method. An equation of correlation between OD and mass of volatile solids were determined (Clesceri *et al*., 1998). The biogas samples were collected using 10 mL syringes and the hydrogen content was determined by a gas chromatograph (GC) equipped with a thermal conductivity detector. A Carboxen 1010 capillary column (length 30 m, internal diameter 0.53 mm) was used with argon as a carrier gas at 15 mL/min. The oven, injector and detector temperatures were kept at 30°C; 230°C and 230°C, respectively. The concentrations of acids and lactose were analyzed by an HPLC (Shimadzu MODEL LC-20A Prominence, Supelcogel, column C-610H), where the components were detected by ultraviolet (UV-VIS). The column temperature was kept at 32°C and an aqueous solution of $H_3PO_4$ (0.1%) was used for elution at 0.5 mL/min.

## 3. Results and discussion

Table 3 and 4 summarize the results of hydrogen productivity (μmol $H_2$/L.h) for the first batch of trials. In general, for both strains *Rhodobacter capsulatus* and *Rhodopseudomonas palustris* higher hydrogen productivity was achieved in the presence of sodium glutamate than it was observed when ammonium sulphate was used as nitrogen source. These results showed that increases in the carbon source could provide expressive gain on the hydrogen productivity. Nevertheless, when ammonium sulphate was used as N source, a limit of milk whey permeate concentration (1.0 g/L) was observed. The best $H_2$ productivity of 89.73 μmol $H_2$/L.h was observed by *R. capsulatus* cultured in RCV medium with glutamate as nitrogen source (2.54 g/L) and milk whey permeate at 2.0 g/L (Assay 3). Increments in ammonium sulphate concentration also result in increases of $H_2$ productivity.

*Table 3: Milk whey permeate and nitrogen source effects on the Rhodobacter capsulatus strain used to $H_2$ production*

| Assay | Milk whey Permeate (g/L) | Nitrogen source concentration (g/L) | Productivity (μmol $H_2$/L.h) |
|---|---|---|---|
| RCV medium supplemented (sodium glutamate as the nitrogen source)(1) | | | |
| 1 | 0.5 | | 58.61 |
| 2 | 1.0 | 2.54 | 80.96 |
| 3 | 2.0 | | 89.73 |
| RCV medium supplemented (ammonium sulfate as the nitrogen source)(1) | | | |
| 4 | 0.5 | | 18.11 |
| 5 | 1.0 | 1.00 | 71.30 |
| 6 | 2.0 | | 66.12 |
| RCV medium (control) | | | |
| 7 | 0 | 1.00 | 23.03 |

(1) Supplemented with yeast extract (1 g/L)

*Table 4: Lactose and nitrogen source effects on the Rhodopseudomonas palustris strain to $H_2$ production*

| Assay | Milk whey permeate *(g/L)* | Nitrogen source concentration (g/L) | Productivity (µmol $H_2$/L.h)[1] |
|---|---|---|---|
| RCV medium supplemented (sodium glutamate as the nitrogen source) | | | |
| 1 | 0.5 | 2.54 | 55.62 |
| 2 | 1.0 | | 78.07 |
| 3 | 2.0 | | 87.89 |
| RCV medium supplemented (ammonium sulfate as the nitrogen source) | | | |
| 4 | 0.5 | 1.00 | 50.23 |
| 5 | 1.0 | | 71.15 |
| 6 | 2.0 | | 77.12 |
| RCV medium (control) | | | |
| 7 | 0 | 1.00 | 17.58 |

Based on these results, in order to figure out the optimum medium formulation, the milk whey permeate and yeast extract concentrations were extended in the following assays. In addition, the medium was supplemented with different malic acid concentrations (30, 60 and 9 mM). The data obtained in the second batch of trials are summarized in Tables 5 and 6, and the findings are presented in terms of hydrogen productivity (µmol $H_2$/L.h) for *Rhodopseudomonas palustris* and *Rhodobacter capsulatus* strains, respectively.

*Table 5: Effect of milk whey permeate, yeast extract and malic acid concentration on the $H_2$ productivity by Rhodopseudomonas palustris*

| Assay | Malic Acid (mM) | Milk Whey Permeate (g/L) | Yeast Extract (g/L) | Productivity (µmol $H_2$/L.h) [1] |
|---|---|---|---|---|
| 1 | 30 | 2 | 0 | 90.47 |
| 2 | 30 | 4 | 0 | 94.02 |
| 3 | 30 | 6 | 0 | 96.33 |
| 4 | 60 | 2 | 0 | 94.34 |
| 5 | 60 | 4 | 0 | 99.98 |
| 6 | 60 | 6 | 0 | 110.46 |
| 7 | 90 | 2 | 0 | 62.04 |
| 8 | 90 | 4 | 0 | 68.72 |
| 9 | 90 | 6 | 0 | 70.24 |
| 10 | 30 | 2 | 2 | 44.37 |
| 11 | 30 | 2 | 4 | 38.56 |
| 12 | 30 | 2 | 6 | 27.57 |
| 13 | 60 | 2 | 2 | 50.62 |
| 14 | 60 | 2 | 4 | 46.38 |
| 15 | 60 | 2 | 6 | 32.25 |

By analyzing the data obtained using *Rhodopseudomonas palustris* and in the absence of yeast extract, it can be observed in assays 1 to 9 that, for each malic acid concentration, the $H_2$ productivity increased by increasing milk whey permeate concentration. In respect to malic acid concentration, there was a tendency to increase the $H_2$ productivity by varying the concentration from 30 to 60 mM. At 90 mM of malic acid, the $H_2$ productivity showed a significant decreased (assays 7 – 9) in comparison to the former concentrations

(assays 1 – 6). The optimum hydrogen productivity was 110.46 µmol of $H_2$/L.h (assay 6) using 60 mM/L of malic acid and 6 g/L of milk whey permeate.
In the study developed by Azbar and Dokgoz (2010), dark fermentation effluent from cheese whey wastewater was applying as substrate in $H_2$ production by photofermentation using *Rhodopseudomonas palustris* (DSM 127). Similarly to this work, these authors reported that mixing of the effluent with L-malic acid at increasing ratios had further positive effect and improved the hydrogen production significantly.
In the case of yeast extract supplementation, it was noted that lower productivity levels were achieved independently of the malic acid concentration, 30 mM and 60 mM) (assays 10 – 15) By comparing these results to data of $H_2$ productivity (87.89 µmol $H_2$/L.h) of assay 3 in Table 4 (2 g/L of milk whey permeate and 1 g/L of yeast extract) it is observed that yeast extract effects positively hydrogen production only in the absence of malic acid.

*Table 6: Effect of milk whey permeate, yeast extract and malic acid concentration on the $H_2$ productivity by Rhodobacter capsulatus*

| Assay | Malic Acid (mM) | Milk Whey Permeate (g/L) | Yeast Extract (g/L) | Productivity (µmol $H_2$/L.h)(1) |
|---|---|---|---|---|
| 1 | 30 | 2 | 0 | 88.36 |
| 2 | 30 | 4 | 0 | 93.58 |
| 3 | 30 | 6 | 0 | 105.37 |
| 4 | 60 | 2 | 0 | 80.24 |
| 5 | 60 | 4 | 0 | 85.77 |
| 6 | 60 | 6 | 0 | 93.78 |
| 7 | 30 | 2 | 1 | 87.94 |
| 8 | 30 | 2 | 2 | 50.37 |
| 9 | 30 | 2 | 4 | 42.24 |
| 10 | 30 | 2 | 6 | 32.44 |
| 11 | 60 | 2 | 1 | 77.22 |
| 12 | 60 | 2 | 2 | 48.55 |
| 13 | 60 | 2 | 4 | 39.30 |
| 14 | 60 | 2 | 6 | 28.85 |

Thereafter, similar experiments were performed by *Rhodobacter capsulatus*, except assays at malic acid concentration of 90 mM. It is also observed that hydrogen productivity reached a maximum content (105.37 µmol of $H_2$/L.h) in assay 3, at 30 mM/L of malic acid and 6 g/L of milk whey permeate. Furthermore, a downward trend was observed at higher malic acid concentrations of 60 mM ( assays 4-6), differently from the optimum concentration by *R. palustris*. Besides, similarly to the assays by *R. palustris,* the positive effect of increasing milk whey permeate concentration was verified in Assays 1 – 6 and the results of Assays 7-14 indicated that increase in yeast extract concentration was responsible to hydrogen productivity reduction. It should be pointed out that by comparing the data of both assays in the same yeast extract concentration (1 g/L), Assay 3 in Table 3 (89.73 µmol of $H_2$/L.h) and Assay 7 in Table 6 (87.94 µmol of $H_2$/L.h), it is possible that are the same. Therefore, the findings of this work indicated that the medium formulated at higher concentration of 6 g/L of milk whey concentration resulted in higher $H_2$ production. Furthermore, by supplementing the medium with the other C source, in this case, malic acid, produced better results than adding yeast extract as a second N source in addition to sodium glutamate.
By concerning on the potential of $H_2$ production of *Rhodobacter capsulatus* and *Rhodopseudomonas palustris*, this work indicated that the use of both strains in producing $H_2$ is promising. Both strains presented the same potential of $H_2$ production, although the medium formulation may be different.
Afsar *et al*. (2011) also investigated a hydrogen production by *Rhodopseudomonas palustris* and *Rhodobacter capsulatus* strains. However, the photofermentation was performed using as substrate the effluent from the dark fermentation. In this case, the medium was formulated with acetate (114 mM), lactic acid (6 mM), glucose (20 mM) and $NH_4Cl$ (1 mM). It was important to note that this effluent was diluted tree times. On the other hand, *Rhodobacter capsulatus* reached 430 µmol of $H_2$/Lh, while the authors observed that *Rhodopseudomonas palustris* was capable to reach 330 µmol of $H_2$/L.h. These findings are higher than those obtained in this study (110.46 µmol of $H_2$/Lh). This difference may have occurred since operating parameters, bioreactor design, pH, temperature and luminosity could strongly affect the target-product synthesis.

## 4. Conclusions

The best result to $H_2$ productivity was 110.46 μmol $H_2$/Lh at 60 mM/L of malic acid and 6 g/L of milk whey permeate (corresponded at 5.58 g/L of lactose) to *Rhodopseudomonas palustris*. These findings suggested that hydrogen productivity was biggest when sodium glutamate had been used as a nitrogen source, in contrast at ammonium sulphate. In addition, when yeast extract was supplemented to RCV medium together with sodium glutamate it was observed a reduction for both strains in terms of the target-product synthesis.

Finally, behaviour of strains for hydrogen production demonstrated similarity, since the highest content to hydrogen productivity (110.46 μmol of $H_2$/Lh) was observed to *Rhodopseudomonas palustris* (60 mM/L of malic acid and 6 g/L of milk whey permeate), whereas at similar culture conditions (30 mM/L of malic acid and 6 g/L of milk whey permeate) to *Rhodobacter capsulatus* was observed 105.37 μmol de $H_2$/Lh.

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