Bio-hydrogen production from glycerol by a strain of *Enterobacter aerogenes*

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Abstract

In this work, H_2 production by a strain of Enterobacter aerogenes using as substrate pure glycerol and glycerol-containing biodiesel wastes was compared. The effect of physico-chemical parameters such as temperature, initial substrate and biomass concentrations on the bio-hydrogen production efficiency was investigated. The influence of the simultaneous removal of gases produced was also evaluated
The results obtained showed that a decrease of the process temperature of 37 to 30 ºC leads to both, an increase of the bio-hydrogen production rate and a decrease of the equilibrium time of the process. Furthermore, it was also observed that using 10 g/dm^3 of pure glycerol or biodiesel wastes containing the same concentration of glycerol as substrate lead to very similar bio-hydrogen production yields (2.5dm^3 H_2/dm^3 fermentation medium). This proves that the performance of the strain of E. aerogenes used was not influenced by the presence of other components than glycerol in biodiesel residues, at least for the biodiesel wastes concentration studied.
Simultaneous removal of gaseous phase (mainly H_2 and CO_2), with its production, shows to be very efficient leading to an increase of the value of the H_2/CO_2 volumetric ratio, in the headspace, from 2 to 8, which is very promising regarding costs involved in the technologies for purification of H_2 produced.

Keywords: *hydrogen, Enterobacter aerogenes, glycerol, biodiesel residues, anaerobic process*

1 Introduction

The worldwide energy need has been increasing exponentially, the reserves of fossil fuels have been decreasing, and the combustion of fossil fuels has serious negative effects on environment because of CO_2 emissions. Therefore, the building of a sustainable energy world will require reduction of dependency of fossil fuels and lowering of the amount of pollution that is generated.
The "hydrogen economy", defined as the industrial system in which one of the universal energy carriers is hydrogen, has the potential to provide a sustainable and secure energy system [1, 2]. Hydrogen is the most promising in the succession of fuel evolution, with several technical, socio-economic and environmental benefits to its credit. It has the highest energy content per unit weight of any known fuel (142 kJ/g) and can be transported for domestic/industrial consumption through conventional means. [3, 4]. However, today, biological H_2 production processes are becoming important mainly due to two reasons: utilization of renewable energy resources, and usually operated at ambient temperature and atmospheric pressure [3, 5]. In fact, fermentative hydrogen production has the advantages of rapid hydrogen production rate and simple operation. Moreover, it can use various organic wastes as substrate for fermentative hydrogen production, what is of great significance because it can not only treat organic wastes, but also produce very clean energy. Therefore, fermentative hydrogen production has been received increasing attention in recent years [6, 7]. Bio-hydrogen is considered a clean and viable alternative fuel and "energy carrier" of future [5]. Regarding its use as biofuel in the transport sector, a target of 5% by 2020 is proposed in the European directive for biofuels - CE/30/2003.
In fact, many anaerobic organisms can produce hydrogen from carbohydrate containing organic wastes and the microbial conversion of agricultural and industrial residues to hydrogen is attracting increasing interest [8, 9]. For instance, the glycerol phase resulting from the biodiesel production can be an ideal feedstock for biohydrogen production since glycerol can be used as a carbon source in dark fermentation processes [10].
Usually, glycerol generated in biodiesel manufacturing is used in the cosmetic and pharmaceutical industries. However, the crude glycerol generated as by-product from biodiesel production holds very low value because of the impurities that contains. Thus, further refining is necessary, namely, for the use in food, cosmetics, and drugs. This purification is costly and generally compromised in terms of economic feasibility. An effective alternative is the use of such low-grade

quality glycerol as fermentation substrate, e.g. for the biological production of hydrogen.

The microbial conversion of glycerol to various compounds has been investigated recently with particular focus on the production of 1,3-propanediol, which can be applied as a basic ingredient of polyesters. The fermentation of glycerol to 1, 3-propanediol has been studied using microorganisms such as *Klebsiella pneumoniae*, *Citrobacter freundii*, *Clostridium butyricum* and *Enterobacter agglomerans*. However, the biological production of H_2 and ethanol from glycerol is also attractive because H_2 is expected to be a future clean energy source and ethanol can be used as a raw material and a supplement to gasoline [10]. Moreover, given the highly reduced nature of carbon in glycerol and the cost of anaerobic processes, fermentative metabolism of glycerol is of special interest concerning the economic viability [11].
Fermentative hydrogen production is a very complex process and influenced by many factors such as inoculum, substrate, reactor type, nitrogen, phosphate, metal ion, temperature and pH. And the effects of these factors on fermentative hydrogen production it is crucial [6].
The main goal of this work was the development of a microbiological process allowing producing bio-hydrogen from glycerol, with simultaneous valorization of that by-product of biodiesel production.

2 Material and Methods

Microorganism and culture conditions
The microorganism used in this study was E. aerogenes ATCC 13048. Cultures were maintained at 4 ºC in a solid medium CASO Agar.
The synthetic fermentation medium used in this study contained (per dm^3) 7.0g of K_2HPO_4, 5.5g of KH_2PO_4, 1.0g of $(NH_4)_2SO_4$, 0.25g of $MgSO_4.7H_2O$, 0.021g of $CaCl_2.2H_2O$, 0.12g of $Na_2MoO_4.2H_20$, 0.002g of nicotinic acid, 0.000172g of Na_2SeO_3, 0.00002g of $NiCl_2$, 5g of tryptone, 5g of yeast extract. The biodiesel residues containing glycerol were supplied from a biodiesel factory in Portugal. The residues contained 86% (w/w) glycerol and 6.2% (w/w) MONG. The impurities were mainly composed of ash (4.6% w/v) and methanol (0.03% w/w).

Bio-hydrogen production experiments
The effect of gaseous phase pressure decrease in the headspace and temperature on bio-H_2 production was carried out in 670 cm^3 flasks containing 290 cm^3 of medium (headspace= 380 cm^3), in a thermostatic water bath at 30 and 37 ºC, with magnetic shaking.
The optimization of biomass and glycerol concentrations, for bio-hydrogen production, was performed in 590 cm^3 erlenmeyer flasks containing 256 cm^3 of medium (headspace= 206 cm^3), in an orbital shaker of 150 rpm at 30 ºC with simultaneous removal of gases produced (mainly H_2 and CO_2) using a peristaltic pump (Ecoline) at a constant flow rate of 2.6 $cm^3.dm^{-3}$. Gaseous phase was harvested in a gas sampling bag (SKC-FlexFoil bags with stainless steel fitting 245 Series) (Fig. 1).
The fermentation media were bubbled with N_2 by 2 min. and inoculated with different concentrations of bacteria harvested from an exponentially growing 20g/dm^3 peptone media culture, according to the desired biomass concentration. Initial and final concentrations of pure glycerol and glycerol contained in biodiesel by-products were tested. Medium pH was about 6.50. Gas samples were taken periodically and analysed for H_2 and CO_2 content.

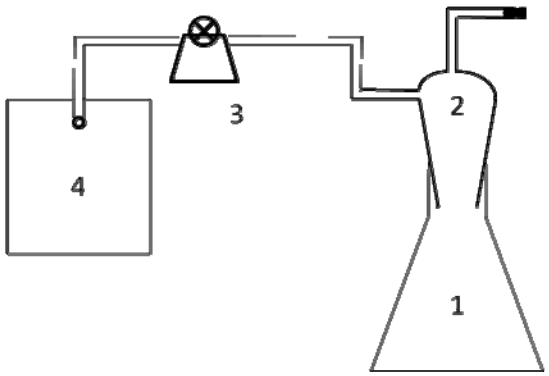

Fig. 1. Esquematic diagram of bio-hydrogen production setup: (1)-Reaction vessel; (2)- Glass head; (3)-Peristaltic pump; (4)-Gas sampling bag.

Analysis
Gas production was measured by the displacement of a saturated aqueous sodium chloride in a graduate cylinder and using a gas meter (Schlumberger), in the experiments carried out in the thermostatic water bath and in the orbital shaker, respectively.
The concentrations of CO_2 and H_2 were determined by gas chromatography (GC VARIAN model CP 3800, USA) with a thermal conductivity detector (TCD). The samples were injected in a 3m long, 1/8' Porapack S column.
Initial and final concentrations of glycerol were determined by the American Oil Chemist' Society official method Ea 6-51 (1997) and gas chromatography (GC HP model 5890, USA) with a flame ionization detector (FID). The samples were injected in a 2m long, 1/8' 4% CW (carbowax)-

20M, 1% trimesil acid 80-120 carbopack BDA column), respectively.

Experimental design

A five-level-two-factor, central composite design (CCD) was employed in this optimization study. Biomass concentration (A) and glycerol concentration (B) were the independent variables selected to be optimized for the bio-hydrogen production by a strain of *Enterobacter aerogenes*. H_2/CO_2 volumetric ratio (Y) in the gas sampling bag was taken as the response of the design experiments. The coded and uncoded levels of the independent variables are given in Table 1. Eleven experiments were augmented with three replications were carried out at the center points to evaluate the pure error.

Table 1. Independent variables: coded and real value in CCD.

		Levels				
Variables	**Symbols**	**-1.414**	**-1**	**0**	**1**	**1.414**
[Biomass] g/dm^3)	**A**	0.1	0.4	1.1	1.7	2.0
[Glycerol] g/dm3)	**B**	10.0	12.9	20.0	27.1	30.0

3 Results and Discussion

Effect of the concentration of biodiesel residues on the fermentation and H_2 production

Firstly, H_2 production was performed in batch fermentations using synthetic medium and 10 g/dm^3 glycerol as carbon source. Similar experiments were carried out using biodiesel residues containing two different glycerol concentrations, 10 and 20 g/dm^3. The results were compared with the ones obtained for 10 g/dm^3 of pure glycerol (Table 2).

Table 2. H_2, CO_2 production and H_2/CO_2 volumetric ratio production for 10 g/dm^3 commercially available glycerol and 10 and 20 g/dm^3 glycerol in biodiesel residues, diluted with synthetic medium. Operational conditions: T=37ºC, magnetic shaking, [Biomass]=0.1 g/dm^3.

Substrate	**[Glycerol] (g/dm^3)**	**H_2 (cm^3)**	**CO_2 (cm^3)**	**H_2/CO_2 (cm^3/cm^3)**
Pure glycerol	10	608.7	292.2	2.1
Biodiesel residues containing glycerol	10	624.8	252.7	2.5
	20	710.0	345.0	2.1

The results obtained showed that biodiesel residues containing glycerol can be successfully utilized to produce hydrogen by *E. aerogenes*. When 10 g/L of pure glycerol or biodiesel residues containing the same concentration of glycerol were used as substrate similar values of H_2 production were observed. However, an increase of H_2 production from 624.8 to 710.0 cm^3 was observed, when biodiesel residues containing 20 g/L of glycerol were used as substrate. H_2/CO_2 volumetric ratio was similar for all experiments. This indicates that the performance of the strain of *E. aerogenes* used was not influenced by the presence of other components than glycerol in biodiesel residues at this concentration. Also the final medium pH was about 6.22 for all experiments without adjustment during the process.

Taking into account the results obtained, posterior studies were developed using 20 g/dm^3 glycerol in biodiesel residues, diluted with synthetic medium, as substrate.

Effect of temperature and bio-H_2 partial pressure decrease on the biological process

Temperature is one of the most important factors that influence the activities of hydrogen-producing bacteria and the fermentative hydrogen production. It has been demonstrated that in an appropriate range, increasing temperature could increase the ability of hydrogen-producing bacteria to produce hydrogen during fermentative hydrogen production, but temperature at much higher levels could decrease it with increasing levels [6].

In this work experiments were performed at 30 and 37 ºC for biological H_2 production, using as substrate 20 g/dm^3 glycerol in biodiesel residues diluted with synthetic medium (Table 3).

Table 3. Effect of temperature on bio-hydrogen production by a strain of *E. aerogenes* for 20 g/dm^3 glycerol in biodiesel residues, diluted with synthetic medium. Operational conditions: T=30 and 37ºC, magnetic shaking, [Biomass]=0.1 g/dm^3.

T ºC	**H_2 (cm^3)**	**CO_2 (cm^3)**	**H_2/CO_2 (cm^3/cm^3)**
37	710.0	345.0	2.1
30	714.0	372.0	1.9

As can be verified in the Table 3, the decrease of temperature of the process does not affect H_2/CO_2 volumetric ratio. However, at 30ºC higher gases production rate and a lower time to reach equilibrium were observed. After 2 hours process 200 cm^3 of gases (mainly H_2 and CO_2) were produced at 30ºC while no gas production was observed at 37ºC, for the same time of reaction. This is an advantage because it can bring to a reduction of fermentation process energetic costs.

Also, the decrease of H_2 partial pressure in the headspace was considered as one of the approaches towards improvement of hydrogen productivity. Tests were performed in batch fermentations with simultaneous removal of produced gases to inside a gas sampling bag at a constant flowrate of 2.6 cm^3/min.. The results were compared with the ones obtained without gaseous phase removal (Table 4).
It was verified an increase of H_2/CO_2 volumetric ratio from 2 to 8 with the decreased of partial pressure of H_2 inside gas sampling bag by lowering the total pressure in the headspace of the reactor, what is very promising regarding costs involved in the technologies for purification of H_2 produced [4]. Others authors observed that lowering the total pressure in the reactor headspace from 760mmHg to 380mmHg, in a batch fermentation process containing *E. cloacae*, the molar yield of H_2 increased by 34% using glucose as a substrate. Also the lag period as well as total batch time of H_2 production decreased significantly using decreased partial pressure [3].

Table 4. Comparison of H_2, CO_2 production and H_2/CO_2 volumetric ratio, inside a gas sampling bag for 20 g/dm^3 glycerol in biodiesel residues, diluted with synthetic medium, with and without simultaneous gases removal. Operational conditions: T=30ºC, magnetic shaking of 150 rpm, [Biomass]=0,1 g/dm^3

	Gas sampling bag		
	H_2	CO_2	H_2/CO_2
	(cm^3)		(cm^3/cm^3)
Without gas removal	460.0	220.0	2.0
With gas removal	390.0	50.0	8.0

Basing in the results obtained above subsequent experiments were carried out with simultaneous gases removal with its production.

Optimization of bio-hydrogen production using response surface methodology (RSM)

To minimize the reactor size and running cost, it is desirable that the concentration of biodiesel residues is as high as possible. Therefore, batch fermentations were also carried out with biodiesel residues, diluted with the fermentation medium, to higher glycerol concentrations and biomass.
With this purpose, the response surface methodology (RSM) based on central composite design (CCD) was used to optimize these two important reaction variables for biological hydrogen production: initial biomass (between 0.1-2.0 g/dm^3) and glycerol in biodiesel residues (between 10.0-30.0 g/dm^3) concentrations.
The relationship between response (H_2/CO_2 volumetric) and both independent factors (biomass and glycerol concentrations) were studied. The experimental sequence was randomized in order to minimize the effects of the uncontrolled factors.
Regression analysis is the general approach to fit the empirical model with the collected response variable data. By using multiple regression analysis, the response obtained was correlated with the two independent factors using the polynomial equation as in Eq. (1).

$$Y = 0,25 + 3,39 \times C_{biomass} + 0.006 \times C_{Glicerol} - 0,12 \times$$

Eq.(1)

In which positive sign in front of the terms indicates synergistic effect, while negative sign indicates antagonistic effect.
The results obtained were than analyzed by ANOVA to assess the goodness of fit.
A significant test with 95% confidence interval was also done. The calculated value of F (S^2lof/S^2exp) was smaller (0.348) than the F critical tabulated (F_{crit}=19.164) [12] which means that the results are statistically significant for predicting the H_2/CO_2 volumetric ratio within the range of the variables studied.
Fig. 2 shows the changes of H_2/CO_2 volumetric ratio in the gas sampling bag with varying biomass and glycerol concentrations.

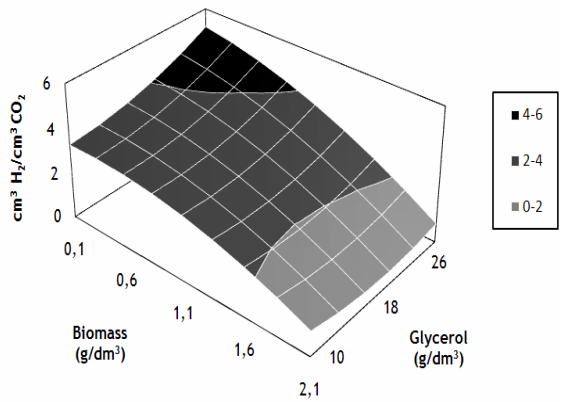

Fig. 2. Tree-dimension response surface contour plot for the effects of biomass and glycerol concentrations on bio-hydrogen production by *E. aerogenes*. [Biomass]=0.1-2 g/dm^3, [glycerol]=10-30 g/dm^3, T=30 ºC, orbital shaker=150 rpm.

As it can be observed, for all glycerol concentrations range the response factor decrease with the increase of biomass concentration. Small biomass and high glycerol concentrations allowed obtaining highest H_2/CO_2 volumetric ratios. The optimum condition for the biological proposed in

this work was as follows: [biomass]=0.38 g/dm^3 and [glycerol] =27.10 g/dm^3.

4 Conclusions

The results presented in this work showed that the use of anaerobic fermentation to convert abundant and low-priced glycerol streams generated in the production of biodiesel into higher value products represents a promising route to achieve economic viability in the biofuels industry.

The decrease of temperature of process the from 37 to 30 ºC and the decrease of partial pressure in the headspace of the reactor showed to be convenient leading to higher H_2 productivity and H_2/CO_2 volumetric ratio.

The statistical analysis between experimental and empirical model results shows that the RSM study based on CCD is adaptable for the anaerobic process studied in this work. The optimum condition for the hydrogen productions by *E. aerogenes* from glycerol in biodiesel residues, diluted with synthetic medium was as follows: [biomass]=0.38 g/dm^3 and [glycerol] =27.10 g/dm^3.

In order to improve the ability of *E. aerogenes* to increase hydrogen production as well as reducing costs, further studies about medium composition are needed.

References

[1] McDowall, W and Eames, M. Towards sustainable hydrogen: A multi-criteria sustainability appraisal of competing hydrogen futures. *International J. Hydrogen Energy* 32 (2007) 4611-4626.

[2] Penner SS. Steps toward the hydrogen economy. *Energy* 31 (2006) 33–43.

[3] Das D. Advances in biohydrogen production processes: An approach towards commercialization. International J. Hydrogen Energy (2009), doi:10.1016/j.ijhydene.2008.12.013

[4] Das D and Nejat Veziroğlu T. Hydrogen production by biological processes: a survey of literature. *International J. Hydrogen Energy* 26 (2001) 13-28.

[5] Kapdan, IK and Kargi, F. Bio-hydrogen production from waste materials. *Enz. Microb. Technol*. 38 (2006) 569-582.

[6] Wang, J and Wan, W. Factors influencing fermentative hydrogen production: A review. *International J. Hydrogen Energy* 34 (2009) 799-811.

[7] Das D and Nejat Veziroğlu T. Advances in biological hydrogen production processes. *International J. Hydrogen Energy* 33 (2008) 6046-6057.

[8] Angenent, LT, Karim, K, Al-Dahhan, MH, Wrenn, BA. and Domíguez-Espinosa, R. Production of bioenergy and biochemicals from industrial and agricultural wastewater. *TRENDS in Biotechnol*, 22 (9) (2004) 477-485.

[9] Fan, Y-T, Zhanga G-S, Guob, X-Y, Xinga, Y and Fan, M-H. Biohydrogen-production from beer less biomass by cow dung compost, *Biomass & Bionergy* 30 (2006) 493-496.

[10] Ito, T, Nakashimada, Y, Senba K, Matsui T and Nishio N. Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. *J. Biosc. Bioeng.* 100 (3) (2005) 260–265.

[11] Yazdani, SS and Gonzalez, R. Anaerobic fermentation of glycerol: a path to economic viability for biofuels industry. *Current Opinion in Biotechnology* 18 (2007) 213-219.

[12] Box, GEP, Hunter WG and Hunter JS. *in* Statistical for Experimenters. John Wiley and Sons, New York (1985).