BIOHYDROGEN PRODUCTION FROM CRUDE GLYCEROL USING
IMMOBILIZED ACIDOGENIC CULTURES: EFFECT OF SUBSTRATE
CONCENTRATION ON CONVERSION EFFICIENCY AND YIELDS

DOUNAVIS A.,1,2 NTAIKOU I.1 and LYBERATOS G.1,3

1Institute of Chemical Engineering and Sciences, Foundation for Research & Technology
Hellas (ICEHT/FORTH), 10 Stadiou st., Platani, GR 26504, Patras, Greece.
2Department of Chemical Engineering, University of Patras, GR 26500, 1 Karathedori st.,
GR 26500, Patras, Greece.
3School of Chemical Engineering, National Technical University of Athens, Zografou
Campus, GR 15780, Athens, Greece
ntaikou@iceht.forth.gr

EXTENDED ABSTRACT

A continuous process for biohydrogen production from crude glycerol was developed,
using immobilized mixed acidogenic consortia in an anaerobic up-flow column bioreactor.
For the immobilization of bacterial cells cylindrical ceramic beads, with active surface
corresponding to 600 m²/L, were used, whereas for its start up the reactor was inoculated
with activated sludge from a Municipal Wastewater Treatment Plant and operated at
batch mode for 24h. Subsequently, the reactor was operated at continuous mode with
HRT, 36 h, feed pH value, 6 and three different initial glycerol concentrations, i.e. 10, 15
and 20 g waste/L. Although glycerol uptake was full only for the lowest organic loading, it
was shown that in terms of both volumetric hydrogen production and molecular hydrogen
yield better values were obtained with the higher organic load, leading to 825.30 ±
41.12 ml H₂/day and 0.246 ± 0.01 mol H₂/mol glycerol consumed, respectively. PDO was
the main metabolite detected in all cases, ranging from 48% to 52% of initial COD.
Ethanol and volatile fatty acids were also produced in different ratios. Acidification ratio
exhibited an increasing tendency for higher organic loadings, with butyrate and
hexanoate being the dominant acids.

Keywords: biohydrogen, waste glycerol, PDO, acidogenic cultures, immobilized bacteria.

1. INTRODUCTION

The biotransformation of wastes and wastewater towards hydrogen can be considered
quite appealing from both the environmental (pollution control, renewable energy) and the
economical (resources recovery, low total cost waste management) standpoint. The
criteria according to which a waste/wastewater would be characterized as efficient
feedstock for hydrogen generation are a high concentration of degradable organic
compounds, high proportion of readily fermentable compounds and low concentration of
inhibitory to microbiological activity compounds (Ntaikou et al., 2010).

A special type of waste that is attracting increased attention lately is crude glycerol,
coming from the biodiesel production industry. Glycerol is a valuable chemical that is
widely used in the cosmetics industry. The recent increase of the biodiesel production
from vegetable oils and fats nowadays has lead to the generation of large quantities that
have to be disposed somehow. In addition, the potential of glycerol utilization would help
improve the economics of biodiesel production. Although it is well established that the
maximum theoretical hydrogen yield that can be obtained from carbohydrates is 4
(Thauer et al., 1977) the maximum possible theoretical hydrogen yield from glycerol is
still under question. Akutsu et al. (2009) based on the study of Thauer et al. (1977) on
microbial metabolism, assume that the maximum theoretical yield is 3 mol H₂/mol glycerol, when acetate is the sole liquid fermentation product. On the other hand, according to Ito et al. (2005) and Sakai and Yagishita (2007) the maximum theoretical hydrogen yield from glycerol is assumed to be 1 mol H₂/mol glycerol, based on the reaction of glycerol conversion to H₂, CO₂, and ethanol. In reality glycerol anaerobic fermentation generates many different metabolites, especially when mixed microbial consortia are used- among which 1,3 propanediol (PDO) is of the most common.

The aim of the present study was to investigate the effect of organic loading on the bioconversion efficiency and yields of metabolites during dark fermentative hydrogen production from crude glycerol. Both continuous and batch cultures were performed using acclimatized mixed acidogenic consortia. A continuous process was developed, using immobilized mixed acidogenic consortia in an up-flow column bioreactor. Three different glycerol concentrations were tested i.e. 10g/L, 15g/L and 20g/L at the same HRT and pH.

2. MATERIALS AND METHODS

2.1. Bioreactor, microbial cultures and media

Continuous experiments were performed in a PVC up-flow, packed bed column bioreactor. The reactor was double coated and temperature control (35±0.5 °C) was achieved via recirculation of warm water. For the immobilization of bacterial cells, cylindrical ceramic beads with porosity corresponding to 600 m²/L, were used. An acidogenic mixed culture derived from activated sludge, previously boiled for 20 min so to inhibit methanogens, was used as inoculum. The bioreactor was operated at batch mode for 24h and subsequently, operated at at mode with HRT 36 hand feed pH values 6. Crude glycerol was kindly supplied by the biodiesel production company PETTAS SA, and had the following characteristics: purity 92.2±0.3%, pH 5.2 and COD 1,28±0.00 gO₂/g waste. For the preparation of feed dilutions of crude glycerol were supplemented with 0.5 - 0.75g/L yeast extract, phosphate buffer (Na₂HPO₄, 4.16 g/L, NaH₂PO₄, 7.26g/L), FeSO₄.7H₂O, 0.07 g/L, and trace elements, 10 ml. Trace element solution was prepared separately and was of the following composition (g/L): (g/L): CaCl₂.2H₂O 22.5, NH₄Cl 35.9, MgCl₂.6H₂O 16.2, KCl 117, MnCl₂.4H₂O 1.8, CoCl₂.6H₂O 2.7, H₃BO₃ 0.51 , CuCl₂.6H₂O 0.24, Na₃MoO₄.2H₂O 0.23, ZnCl₂ g/L 0.19, NiCl₂.6H₂O 0.2, H₂WO₄ 0.01.

2.2. Analytical methods

VSS, TSS and d-COD were determined according to Standard Methods (APHA, 1995). For the quantification of VFAs (acetate, propionate, butyrate, valerate, isovalerate, isobutyrate) and alcohols (ethanol and butanol), acidified samples were analyzed by GC-FID (Varian CP-3800). Hydrogen was quantified by GC-TCD (SRI 8610c) and glycerol and PDO were quantified by HPLC (DIONEX GP50) equipped with RI detector (Shodex RI-101).

3. RESULTS AND DISCUSSION

In Figure 1 the performance of the continuous reactor is illustrated for the four different periods of operation, in terms of glycerol and COD consumption and suspended solids, gases and VFAs production. As shown, during the first days of its operation the reactor exhibited a rather unstable performance, for all parameters presented. This could be attributed to acclimatization of the bacteria (coming from an acidogenic environment) to glycerol, as well as to the time needed in order for the biomass to be settled onto the immobilization agent. At day 40 the system reached steady state, during which glycerol
consumption was full, and distribution of metabolites stable. Subsequently, the reactor was operated with increased organic loading, i.e. glycerol concentration in the feed 15 g/L and 20 g/L, with the same HRT.

The performance of the reactor during steady states is summarized in Table 1. Although relative glycerol uptake decreased for higher organic loadings, hydrogen productivity measured as volumetric hydrogen production per consumed glycerol had an increasing tendency. Organic loading rate can influence \( \text{H}_2 \) production in fermentative \( \text{H}_2 \)-producing continuous systems but there is controversy in the literature as to whether higher \( \text{H}_2 \) yields can be achieved with lower or higher OLRs, when using different types of reactors and different substrates (Kraemer and Bagley, 2007). In the case of glycerol, and immobilized cultures it seems that higher OLRs have an inhibitory effect on glycerol uptake, but enhance at the same time hydrogen generation. This could be attributed to either kinetic inhibition, or inhibitory effect of an accumulated metabolite, and has to be further investigated. It has to be noted though, that in the case of the higher organic loading tested i.e. for 20g/L glycerol in the feed, the increased concentration of nitrogen source enhanced glycerol uptake. Indeed glycerol relative uptake increase was 14% when extra yeast extract was added in the feed, whereas the relative increase in molecular hydrogen yield was only 4.8%. It seems that in that case metabolism is forwarded towards the production of metabolites that are not accompanied by hydrogen generation, such as PDO. As shown in Table 1, the increase of PDO concentration is indeed quite high in the latter steady state.

In general PDO was the major produced soluble metabolite in all cases, as shown before for anaerobic fermentation of glycerol (Han et al., 2012). In terms of VFAs production butyrate and or hexanoate were the dominant ones. Traces of propionate and valerate were also detected, as well as small amounts of ethanol. Taking into account that the theoretical \( \text{H}_2 \) production from glycerol during acitogenesis is 2 mol/mol butyrate and 3 mol/mol acetate, whereas during ethanol production 1mol/mol ethanol produced (Akutsu et al., 2009; Ito et al., 2005), the theoretical estimated \( \text{H}_2 \) accompanying the above measured metabolites, should be approximately 406ml \( \text{H}_2 \)/day, 523 ml \( \text{H}_2 \)/day, 670 ml \( \text{H}_2 \)/day and 705 ml \( \text{H}_2 \)/day for \( C_{\text{glyc}} \) 10g/L, 15g/L, 20g/L and 20g/L (+yeast) respectively. These values are in close agreement with the measured ones, as shown in Table 1, for \( C_{\text{glyc}} \) 10g/L, 15g/L. On the contrary, the theoretical estimated values seem lower than the measured ones for higher organic loadings, and especially when 20g/L glycerol with extra yeast extract was used as feed. These differences can be attributed to the increased concentration of hexanoate that is detected in the reactor.

It is reported that hexanoate can be generated from polyols either directly from pyruvate via the EMP pathway, or secondarily via consumption of acetate, ethanol and butyrate (Jeon et al., 2010). By assuming that hexanoate detected in the reactor was produced by consumption of acetate, butyrate and ethanol we could explain to the abovementioned contradiction between theoretically estimated and measured hydrogen productivity.
Figure 1. Glycerol consumption (a), TSS, VSS production (b), VFAs generation (c), d-COD removal (d), VFAs generation, daily gases production (e) and molecular H2 yields (f) throughout the operation of the UFCB with different glycerol concentrations in the feed. Regarding d-COD reduction, an increasing tendency is also noted for higher Cgl. d-COR removal can be attributed either to hydrogen generation or biomass formation. Adding the estimated CODs for measured H2 and suspended biomass (with empiric type C5H7O2N, i.e MW 113, corresponding to 1.42g O2/g biomass), the balance is incomplete. Thus it can be assumed that some amount of carbon is incorporated in fixed biomass, whereas some other soluble metabolite that was not detected could be produced.
Table 1. Performance of the up-flow continuous column bioreactor at steady states.

<table>
<thead>
<tr>
<th>C Glycerol in Feed</th>
<th>10 g/L</th>
<th>15 g/L</th>
<th>20 g/L</th>
<th>20 g/L, +yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.54 ± 0.04</td>
<td>5.42 ± 0.03</td>
<td>5.43 ± 0.05</td>
<td>5.22 ± 0.03</td>
</tr>
<tr>
<td>VSS, g/L</td>
<td>1.29 ± 0.03</td>
<td>1.09 ± 0.09</td>
<td>0.93 ± 0.08</td>
<td>1.08 ± 0.03</td>
</tr>
<tr>
<td>TSS, g/L</td>
<td>1.61 ± 0.03</td>
<td>1.37 ± 0.19</td>
<td>1.31 ± 0.20</td>
<td>1.59 ± 0.22</td>
</tr>
<tr>
<td>Glycerol reactor, g/L</td>
<td>0.38 ± 0.09</td>
<td>2.64 ± 0.36</td>
<td>6.51 ± 0.25</td>
<td>4.23 ± 0.24</td>
</tr>
<tr>
<td>% Glycerol removed</td>
<td>96.7 ± 1.8</td>
<td>82.3 ± 5.8</td>
<td>68.9 ± 5.7</td>
<td>78.6 ± 3.8</td>
</tr>
<tr>
<td>V biogas, ml</td>
<td>915.75 ± 37.80</td>
<td>1252.80 ± 90.92</td>
<td>1620.80 ± 63.84</td>
<td>1952.75 ± 151.04</td>
</tr>
<tr>
<td>V H₂, ml/d</td>
<td>403.79 ± 21.50</td>
<td>557.59 ± 37.79</td>
<td>772.78 ± 72.02</td>
<td>825.30 ± 41.12</td>
</tr>
<tr>
<td>Hexanoate, mg/L</td>
<td>516.54 ± 72.5</td>
<td>480.29 ± 149.52</td>
<td>507.48 ± 72.49</td>
<td>1848.66 ± 90.62</td>
</tr>
<tr>
<td>Butyrate, mg/L</td>
<td>302.30 ± 75.69</td>
<td>483.97 ± 62.19</td>
<td>635.53 ± 21.49</td>
<td>665.53 ± 32.04</td>
</tr>
<tr>
<td>Acetate, mg/L</td>
<td>172.29 ± 57.51</td>
<td>149.23 ± 18.42</td>
<td>143.31 ± 9.61</td>
<td>163.71 ± 10.12</td>
</tr>
<tr>
<td>PDO, g/L</td>
<td>3.68 ± 0.41</td>
<td>3.93 ± 0.43</td>
<td>3.43 ± 0.83</td>
<td>6.02 ± 0.48</td>
</tr>
<tr>
<td>Ethanol, mg/L</td>
<td>76.92 ± 10.46</td>
<td>174.18 ± 42.77</td>
<td>390.96 ± 52.19</td>
<td>400.46 ± 35.12</td>
</tr>
<tr>
<td>Yield H₂, mol/mol glyc conc</td>
<td>0.138 ± 0.02</td>
<td>0.164 ± 0.01</td>
<td>0.237 ± 0.01</td>
<td>0.246 ± 0.01</td>
</tr>
</tbody>
</table>

5. CONCLUSIONS

A continuous process for biohydrogen production from crude glycerol was developed, using immobilized mixed acidogenic consortia in an up-flow column bioreactor, in order to investigate the effect of organic loading on hydrogen yields and the distribution of metabolites. Although glycerol uptake was full only for the lowest organic loading, it was shown that in terms of both volumetric hydrogen production and molecular hydrogen yield, better values were obtained with the higher organic load. PDO was the main metabolite detected in all cases, whereas butyrate and hexanoate acids were the main acids produced.

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