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Enrichment of activated sludge for enhanced hydrogen production from crude glycerol

Cristiano Varrone [a,b,*], Silvia Rosa [a], Floriana Fiocchetti [a], Barbara Giussani [c], Giulio Izzo [a], Giulia Massini [a], Antonella Marone [a], Antonella Signorini [a], Aijie Wang [b,**]

[a] ENEA – Italian Agency for New Technologies, Energy and Sustainable Development, Italy
[b] State Key Lab of Urban Water Resource and Environment, Harbin Institute of Technology, China
[c] University of Insubria, Dep. of Science and High Technology, Como, Italy

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ABSTRACT

Enriched activated sludge that can effectively convert crude glycerol into bio-hydrogen was selected by an eco-biotechnological approach, in very strict conditions, using biodiesel-derived glycerol as the only carbon source. The thus obtained *functional consortium* was characterized by the genera *Klebsiella*, *Escherichia/Shigella* and *Cupriavidus*. During enrichment, the dominant metabolic end-product shifted from a 1,3 propanediol to ethanol, with a concomitant increase of the hydrogen yield from 0.18 ± 0.003 to 0.66 ± 0.06 mol/mol and an almost five-fold increase of the hydrogen production. Glycerol degradation efficiency showed an increase of around 50%. In optimized and upscaled conditions it was possible to obtain a hydrogen production rate of 2960 mL H_2/L/day ± 185 at a near stoichiometric yield (of 0.90 mol/mol ± 0.01), with a carbon recovery of almost 90%, both in sterile and non-sterile conditions. Glycerol was almost totally degraded (degradation efficiency of 97.42% ± 0.98), independently of the glycerol type used.

1. Introduction

Renewable resources, such as hydrogen, biodiesel or bio-ethanol are gaining importance as an alternative pollution-free fuel for the future [1]. For example, the conversion of lignocellulosic biomass to hydrogen or ethanol, or the use of oil accumulating algae in the production of biodiesel is being intensively investigated [2–5]. Industrial and living wastes, such as paper mill- and municipal solid waste, to produce hydrogen, have also been studied [6]. These approaches are very promising and might provide abundant nonfood feedstocks for the production of bioenergy and biofuels, with environmental benefits and large net energy gains [5]. So far, the production of chemicals and fuels via microbial fermentation has been largely based on the use of sugars (i.e. glucose, lactose, xylose, etc.) as carbon source. However, according to some authors this trend is likely to change in the near future, due to the large surplus of glycerol generated as byproduct in the production of biodiesel fuel [7,8].

* *Corresponding author*. ENEA Casaccia, Dep. UTRINN-BIO, Via Anguillarese 301, 00123 Rome, Italy. Tel.: +39 0630486882; fax: +39 0630484554.
** *Corresponding author*. Joint Center for Microbial Ecology in Engineered System, Harbin Institute of Technology, P.O. Box 2614, 202 Haihe Road, Harbin 150090, China. Tel./fax: +86 451 86282195.
E-mail addresses: cristiano.varrone@enea.it, cristiano.varrone@gmail.com (C. Varrone), waj0578@hit.edu.cn (A. Wang).
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The considerable growth of the biodiesel industry in the last decade has created a glycerol glut that resulted in a dramatic decrease in crude glycerol prices [5]. This decrease in prices poses a problem for the glycerol-producing and -refining industries, and the economic viability of the biodiesel industry itself has been greatly affected [7–9].

Therefore, the development of processes to convert low-priced glycerol into higher value products is expected to add value to the production of biodiesel and help the development of biorefineries [1]. However, crude glycerol from biodiesel production is usually contaminated with methanol, heavy metals, soap, oil and ashes from the process [10]. The chemical purification of these contaminants is becoming too costly (especially for small/medium-sized industries) and not feasible any more [11]. Therefore, the use of anaerobic fermentation, to directly convert abundant biodiesel-derived glycerol (crude glycerol) into higher value products might represent a promising route to achieve economic viability in the biofuels industry [12].

Clearly, the success of these kinds of technologies greatly depends on the use of suitable microorganisms [13]. Until now there have been several studies of microbial processes to convert glycerol, but still too little reports with crude glycerol, which would really reduce the production costs [14]. Usually the approach followed so far was to select and/or isolate suitable bacteria on a synthetic medium, with pure glycerol, and subsequently test the ability to degrade also crude glycerol, thus generally leading to a decreased yield and conversion efficiency (probably due to the contaminants contained in the crude glycerol). Moreover, most studies focused on the use of microbial pure strains as inoculum. The use of mixed microbial cultures for the conversion of a complex substrate (such as crude glycerol, containing several contaminants), would probably favor a higher efficiency, thanks to possible beneficial synergies. However, so far studies dealing with mixed cultures for hydrogen production (through dark fermentation) have been rather scarce [15].

In this study we applied an eco-biotechnological approach to select glycerol-fermenting bacteria, able to efficiently convert the substrate into hydrogen. Eco-biotechnology aims at working with processes employing mixed cultures and ecological selection principles [16]. More precisely, the approach relies on selection, cooperation and competition mechanisms, rather than on genetic or metabolic engineering, with a clear advantage in terms of costs and environmental safety. In our approach, the starting point is to explore the already existing biodiversity, obtaining the best candidates and finally identifying optimal niche/conditions which can enhance the performance of a particular metabolism/required process.

To our knowledge, the selection and acclimation of a highly specific mixed culture directly on crude glycerol as the only carbon source, without the use of any substrate supplementation, has not been investigated so far. Therefore, the main aim of this study was (1) to evaluate the possibility to select a suitable microbial mixed culture directly on the target substrate, by using a minimal medium without any nutrient supplements, in order to reduce operational costs, (2) to characterize the thus obtained microbial culture and (3) evaluate its effectiveness to perform the desired fermentation process on different glycerol types.

2. Material and methods

2.1. Culture medium

Minimal medium (MM) was used to dilute *crude glycerol*, which was supplied by ItalBiOil srl., a biodiesel factory from south Italy, which contained up to 90% of glycerol, while the impurities were mainly composed of salts (~7%), ashes (~2%), methanol (~1%) and moisture (less than 0.4%). Comparison tests were also performed (see Section 2.2.3), using *pure glycerol* (reagent grade) and a different kind of *crude glycerol*, supplied by a biodiesel industry located in north Italy (DII NOVAOL), which contained around 84% glycerol, 0.35% methanol, 0.25% of TFM and less than 10% water, but no salts.

Unless differently stated, the minimal medium contained per liter of distilled water: 20 g of glycerol, 3.4 g of $K_2HPO_4 \cdot 3H_2O$, 1.3 g of KH_2PO_4, 2 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 20 mg of $CaCl_2 \cdot 2H_2O$ and 5 mg $FeSO_4 \cdot 7H_20$. The use of a more complex medium was deliberately avoided, except for some specific experiments performed as comparison tests during the optimization step. Pre-boiled medium was added into the serum bottles and sealed with butyl rubber stoppers, prior to sterilization (15 min, at 121 °C), unless otherwise stated. After cooling down, pre-activated culture was inoculated (see the following paragraph) into the serum bottles. Subsequently the medium was flushed with argon for 3 min, using a hypodermic sterile syringe with a 0.22 μm filter, before being incubated at 37 °C with continuous stirring (120 rpm). Initial pH was 6.8 unless differently stated.

2.2. Experiments

2.2.1. Enrichment

During enrichment of activated sludge, the fermentation was performed in 60 mL serum bottles in batch tests with 30 mL working volume, under controlled temperature, initial pH and anaerobic condition. Sludge samples were collected from Harbin wastewater treatment plant, Heilongjiang province, China. We considered the sludge as a potentially suitable seed inoculum for glycerol-fermenting bacteria since, under anaerobic conditions, the lipids contained in a municipal treatment plant can be converted into glycerol [17]. In this case we would expect that some of the microbial organisms contained in the sludge would be able to exploit this resource and that they might be selected. Enrichment was performed according to Barbirato et al. [18], with the aim to select a pool of microorganisms suitable for crude glycerol digestion. The idea was to use very strict conditions, with crude glycerol as the only carbon source, without adding trace element- or vitamin solution, tryptone or yeast extract, in order to create very selective conditions and to reduce operation costs [19]. Besides, it should also be underlined that, according to some authors, yeast extract could affect hydrogen production [3,20,21], therefore its use should be evaluated case by cases, in order to avoid misleading yields (i.e. higher than the theoretical one). Moreover, to enhance hydrogen production we should try to reduce the 1,3 propanediol (1,3 PD) production, notably associated to low hydrogen production [22]. Since the formation of 1,3 PD from glycerol is vitamin B12-dipendent

[23], the use of a very simply synthetic medium, without additional nutrients and vitamins would probably favor the oxidative pathway for enhanced hydrogen production.

Using the activated sludge as starting inoculum, repeated transfers (5% v/v) of the best hydrogen-producing batches in fresh liquid medium at short intervals (21 h) allowed the accumulation of desired microbial strains, since bacteria not able to ferment glycerol would be outcompeted by the faster growing glycerol-consuming bacteria. The effect of trasferring 5% (v/v) liquid inocula (also containing contaminants and fermentation products) rather than bacterial pellet (without the liquid) was also evaluated. In the latter case, liquid samples were centrifuged for 10 min at 10 000 rpm. Supernatant was washed away and pellets resuspended into fresh medium.

The enrichment phase was performed for several months, in order to verify how many transfers were necessary to obtain a stable and reproducible fermentation. The procedure was thus repeated in order to confirm the possibility to achieve the same results again, and we considered the enrichment phase concluded, once the same stable condition was reached. The thus obtained enriched activity sludge was used as inoculum in all the further tests. It was stored at −18 °C and refreshed monthly. For pre-activation, minimal medium was supplemented with 1 g/L of peptone.

2.2.2. *Optimization experiments*

After enrichment, 125 mL serum bottles were used in order to evaluate the effect of important operation parameters and conditions, such as inoculum%, headspace/medium ratio (H/M ratio, v/v), addition of yeast extract, as well as vitamin and mineral solution. Vitamin solution was prepared by adding 20.0 mg of biotin, 50.0 mg p-Aminobenzoic acid, 20.0 mg folic acid, 50.0 mg pantothenic acid calcium salt, 50.0 mg nicotinic acid, 1.0 mg vitamin B12, 5.0 mg thiamine·HCl, 100.0 mg pyridoxine hydrochloride, 50.0 mg thioctic acid and 5.0 mg riboflavin to 500 mL of distilled water. 0.25 mL of this solution were then added to the serum bottles with 50 mL working volume. Wolfe's Modified Mineral Elixir was prepared according to [24]. For 50 mL of medium, 0.25 mL of the mineral elixir was used. No $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ and $CaCl_2$ were added to the minimal medium when using the mineral elixir.

2.2.3. *Upscale tests*

Experiments were conducted using a BioFlo/CelliGen 115 Benchtop Fermentor & Bioreactor (New Brunswick Scientific, Edison, NJ, USA) of 3 L, with 1 L working volume kept at 37 °C with continuous agitation (120 rpm). Based on previous results, initial pH was set at 8, using KOH 6 M solution, and was not controlled during fermentation. Different glycerol types were tested (Itabiol, Novaol, pure glycerol and non-sterile Novaol) and diluted with the minimal medium, in order to reach an initial concentration of about 15 g/L. Anaerobic conditions were obtained by initially sparging the medium with pure nitrogen for 10 min, at a flow rate of 1 L/min. The vessel of the fermentor was equipped with a condenser (working with flowing water supply) in order to minimize medium evaporation. The bioreactor was inoculated with 10% v/v pre-activated bacterial seed culture, grown in 125 mL serum bottles with 50 mL culture medium for 21 h, at the same temperature. Unless for non-sterile Novaol experiments, the bioreactor was sterilized before inoculation with seed culture.

2.3. *Analytical methods*

In all experiments, gas products have been sampled and analyzed by GC with a thermal conductivity detector (TCD), while glycerol and liquid fermentation products have been analyzed by HPLC with UV detector and Refractive Index, as previously described [15]. Biogas production was determined by water displacement (followed by GC analysis), while mass balance equation was used to estimate hydrogen production [25]. A modified Gompertz equation was used to model cumulative hydrogen production [26] and to calculate production rate [27]. Bacterial growth during enrichment was estimated spectrophotometrically at 680 nm. Multivariate data analysis was performed using Unscrambler X 10.1 softare (by Camo), while Excel's patch "Solver" was used for the modified Gompertz equation.

2.4. *Molecular characterization of the enriched activated sludge*

2.4.1. *DNA extraction and PCR amplification*

Hundred mL of a fresh inoculum culture were used for DNA extraction, using GeneMATRIX™ Bacterial & Yeast Genomic DNA Purification Kit (EURx, Gdansk, Poland), according to the manufacturer's instructions. Universal bacterial primers 27F (AGAGTTTGATCCTGGCTCAG) and 1389R (ACGGGCGGTGTGTACAAG) were used to amplify near full-length 16S rDNA. Polymerase chain reaction (PCR) was performed using Perpetual Taq PCR Master Mix (EURx) containing 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates, 0.1 μM (each) primer, 1.25 U Perpetual Taq DNA Polymerase and 15 ng template DNA in 50 μl reaction volume. The amplification was performed using the 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with a touchdown program, conducted as follows: 94 °C for 2 min followed by 28 cycles of 95 °C for 40 s, 65 °C for 40 s (the annealing temperature decreased to 55 °C by 0.5 °C every cycle for the first 20 cycles and the remaining cycles at 55 °C), 72 °C for 90 s, and then the final extension at 72 °C for 10 min. The amplification products of four single PCR reactions were pooled for reducing the bias introduced with PCR.

2.4.2. *16S rDNA library construction and sequencing*

Two μl of the mixed PCR products were used for cloning into vector PCR®2.1-TOPO of the TOPO TA cloning® kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The clones were screened using Luria-Bertani (LB) medium agar plates containing 50 μg/ml kanamycin and 40 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). 102 white colonies were randomly picked up and regrown overnight in LB medium containing kanamycin for subsequent analysis. Plasmid DNA was extracted by means of GeneMATRIX Plasmid Miniprep DNA Purification kit (EURx). The plasmids were screened for 16S rDNA insert by performing PCR with the vector-located primers M13F (GTAAAACG ACGGCCAG) and M13R (CAGGAAACAGCTATGAC). The PCR reaction mixture was prepared like previously mentioned.

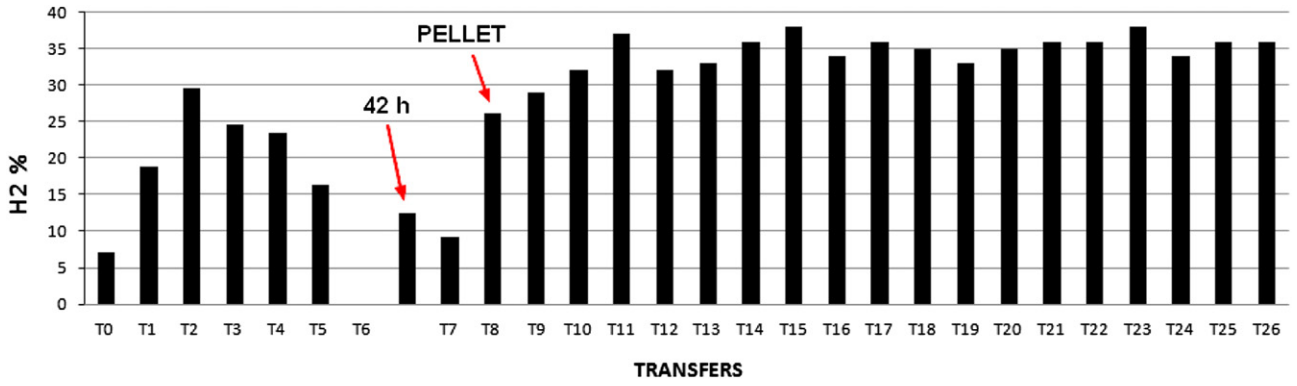


Fig. 1 – Hydrogen content in the biogas (expressed as H_2%) detected during the first enrichment phase. A clear inhibition was observed already after the third transfer, and only the use of bacterial pellets (starting from T8) allowed to prevent further inhibition and to carry on the enrichment process. Further transfers rapidly led to a stable hydrogen content in the biogas (around 35%). With the exception of T6 all the transfers occurred after 21 h fermentation.

Thermal cycling conditions were as follows: 95 °C for 10 min, followed by 25 cycles of 95 °C for 40 s, 46 °C for 40 s and 72 °C for 90 s. A final extension step of 10 min at 72 °C was applied.

The plasmid DNA of the selected clones was used to sequence 16S rDNA insert, using specific primers 27F and 1389R. DNA sequencing was performed on the ABI 3730 DNA analyzer by Genechron (C.R. ENEA Casaccia, Rome, Italy).

2.4.3. *Phylogenetic analysis of clones*

Raw sequences from both strands were aligned using Clustal X version 2.0 and alignments were edited and manually corrected by ChromasPro version 1.5 (Technelysium Pty Ltd). All sequences were analyzed with the Mallard program (version 1.02) to detect the presence of possible chimeric artifacts. The sequences were checked and classified by the basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST) and the RDP-classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp) of the Ribosomal Database project (http://rdp.cme.msu.edu). Phylogenetic analysis was conducted using Molecular Evolutionary Genetics Analysis package, version 5 [28]. The closest 16S rDNA sequences of reference microorganisms were retrieved from the GenBank and were aligned to DNA sequences from library, using the Clustal W function. Phylogenetic tree was inferred, using the neighbor-joining method [29]. Evolutionary distance matrices were generated according to Kimura 2-parameter model [30]. Bootstrap re-sampling analysis with 1000 replicates was performed to check the robustness of the tree. Tree topology was confirmed by comparing three phylogenetic reconstruction methods: neighbor-joining, maximum likelihood and maximum parsimony.

The newly identified 16S rDNA sequences were deposited in the GenBank nucleotide sequence database, under accession number JX310739-JX310754 and JX416353-JX416361.

3. Results and discussion

3.1. *Enrichment*

Preliminary tests were performed to verify the possibility to grow activated sludge on crude glycerol as the only carbon

Table 1 – Main fermentation products and parameters monitored during the enrichment of activated sludge, using 60 mL serum bottles with 30 mL working volume. The values represent the average of three replicates. Lactate and other VFA with low concentration are not reported in the table.

Transfer	H_2 %	Total H_2 mL	Gly. Conv. %	Yield mol/mol	H_2/medium v/v	1,3 PD g/L	EtOH g/L	Acetic g/L	pH	OD 680
1	14.2 ± 0.78	10.7 ± 0.43	50.7 ± 1.55	0.18 ± 0.003	0.43 ± 0.02	4.01 ± 0.02	0.23 ± 0.002	1.89 ± 0.01	4.8 ± 0.0	0.56 ± 0.02
2	27.3 ± 2.07	33.8 ± 1.96	67.7 ± 3.14	0.43 ± 0.06	1.35 ± 0.08	2.88 ± 0.46	3.78 ± 0.21	0.77 ± 0.14	5.2 ± 0.17	1.07 ± 0.04
3	28.8 ± 0.19	44.5 ± 0.59	79.3 ± 1.99	0.49 ± 0.02	1.78 ± 0.02	1.16 ± 0.08	5.52 ± 0.20	0.04 ± 0.01	5.4 ± 0.0	1.14 ± 0.03
4	26.1 ± 0.55	26.2 ± 0.81	66.5 ± 3.72	0.34 ± 0.05	1.05 ± 0.03	3.23 ± 0.12	1.03 ± 0.05	1.34 ± 0.03	4.7 ± 0.23	0.78 ± 0.01
5	26.1 ± 5.03	30.0 ± 5.39	59.4 ± 4.96	0.37 ± 0.11	1.20 ± 0.22	1.87 ± 0.17	3.06 ± 0.33	0.95 ± 0.12	4.9 ± 0.29	0.97 ± 0.13
6	25.5 ± 0.17	25.8 ± 0.23	54.7 ± 1.16	0.39 ± 0.02	0.69 ± 0.6	4.18 ± 0.15	1.22 ± 0.37	2.46 ± 0.02	4.5 ± 0.14	0.88 ± 0.0
7	25.3 ± 1.19	25.4 ± 1.42	57.4 ± 5.05	0.41 ± 0.1	1.02 ± 0.06	3.74 ± 0.21	0.64 ± 0.07	2.34 ± 0.11	4.5 ± 0.12	0.93 ± 0.06
8	26.5 ± 0.30	24.2 ± 0.15	54.9 ± 3.49	0.32 ± 0.03	0.97 ± 0.01	3.74 ± 0.02	0.77 ± 0.07	2.08 ± 0.03	4.9 ± 0.29	0.94 ± 0.01
9	26.7 ± 1.84	25.1 ± 1.73	73.7 ± 8.15	0.36 ± 0.04	1.00 ± 0.07	3.16 ± 0.14	1.02 ± 0.13	1.88 ± 0.07	5.1 ± 0.0	0.85 ± 0.02
10	35.2 ± 3.74	47.4 ± 6.29	77.5 ± 5.32	0.66 ± 0.06	1.90 ± 0.25	0.95 ± 0.01	3.30 ± 0.31	1.01 ± 0.02	5.4 ± 0.0	0.90 ± 0.04

Gly. Conv. = glycerol conversion efficiency; 1,3 PD = 1,3, propanediol; EtOH = ethanol.

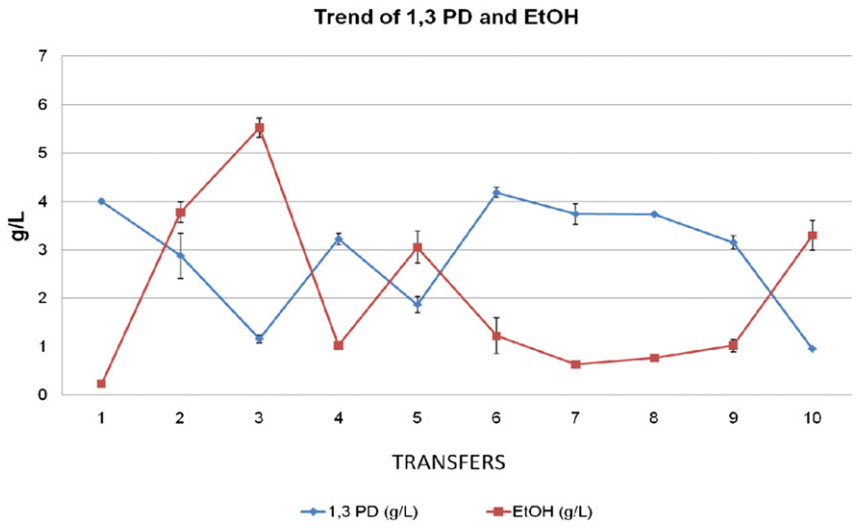


Fig. 2 – Trends of 1,3 propanediol (1,3 PD) and ethanol (EtOH) concentration during the enrichment.

source and to produce hydrogen. Subsequently, a first enrichment was performed for several months, in order to identify the maximum hydrogen production potential, and the possibility to obtain a stable microbial community and process. In our study this was obtained once the biogas composition reached a hydrogen content of around 35%, in 21 h fermentation. After that, further transfers didn't enhance the hydrogen production substantially (Fig. 1). It is worth noting that after an initial increase in hydrogen production, there was a clear inhibition of the fermentation process, probably due to accumulation of inhibitory fermentation products (Fig. 1). After six transfers (T6) there was no hydrogen production at all within the 21 h, and even after 42 h fermentation the detected hydrogen content in the biogas only reached around 12%. A further transfer into fresh medium didn't re-establish the previous conditions (T7). Therefore 5% v/v liquid inoculum was centrifuged (in order to wash away the liquid phase, containing high concentrations of soluble metabolites) and only the pellet was transferred in T8. Following to this procedure, a consequent rapid increase of H_2% was observed. Due to these findings, from that moment on all the transfers were performed with pellets only.

The enrichment process was thus repeated in order to evaluate the reproducibility and the minimum number of transfers necessary to obtain a stable fermentation. In this step, main fermentation products and parameters were monitored, in order to better characterize the process.

Data shown in Table 1 suggested that the enrichment process was not linear, but displayed a rather irregular pattern, with an initial rapid increase, followed by a decrease of the main parameters involved. At the end of the process, however, there was a clear enhancement in the ability of converting glycerol into hydrogen.

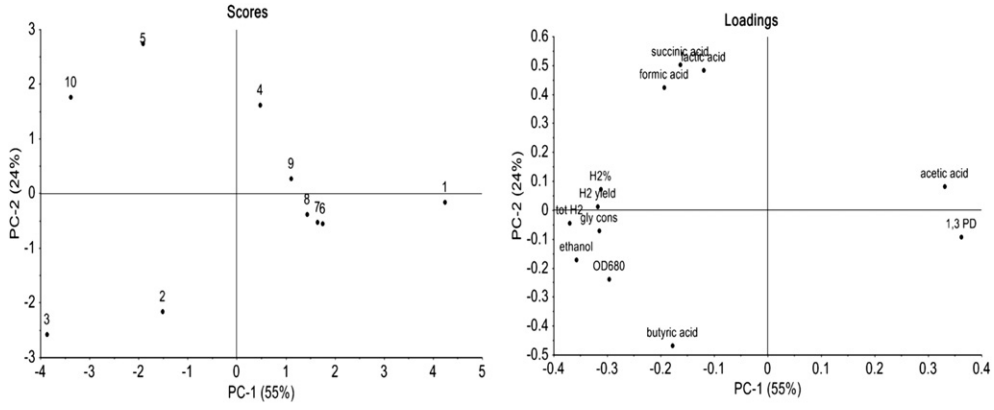


Fig. 3 – Principal Component Analysis, showing the most important fermentation products and parameters monitored during the enrichment. The score plot (left) shows the distribution of the 10 transfer experiments (scores). The loadings (right) show the main parameters influencing the distribution of the scores. Those laodings gathering together are positively correlated to each other, while those which are opposite are negatively correlated. The Principal Components (PC1 and PC2) explain 79% of the total variability. The score plot seems to display three different steps/directions during the enrichment: from transfer 1 to 3, then transfer 4 and 5, and finally from 6 to 10.

Table 2 – Optimization experiments. The best condition of H/M ratio (1.5) was used for the following experiments, while the best condition obtained in the inoculum experiments (10%) was then used in substrate supplementation tests.

	H_2 yield	H_2%
H/M ratio		
4.0	0.51 ± 0.05	33.7 ± 1.37
3.0	0.63 ± 0.02	34.3 ± 0.85
2.0	0.77 ± 0.01	39.6 ± 0.17
1.5	0.80 ± 0.01	40.9 ± 2.11
0.8	0.79 ± 0.03	42.6 ± 2.35
0.4	0.5 ± 0.07	41.9 ± 1.38
Inoculum		
0.50%	0.69 ± 0.12	38.6 ± 0.48
1%	0.73 ± 0.05	38.5 ± 0.32
2%	0.76 ± 0.02	39.4 ± 2.33
5%	0.80 ± 0.01	40.9 ± 2.11
10%	0.82 ± 0.01	41.3 ± 0.93
Substrate		
MM	0.82 ± 0.01	41.3 ± 0.93
Vitamins	0.79 ± 0.01	40.9 ± 0.067
Minerals	0.50 ± 0.02	22.3 ± 0.22
Yeast	0.80 ± 0.02.	40.5 ± 0.48
Complete	0.57 ± 0.07	21.8 ± 3.2

MM = minimal medium (no addition of any supplements); Complete = Vitamins, Mineral and Yeast extract together; H/M = headspace/medium ratio (v/v).

Interestingly, the concentration of 1,3 PD (1,3 propanediol) followed an opposite trend compared to the ethanol concentration (Fig. 2), probably because the 1,3 propanediol pathway is alternative to the ethanol/oxidative pathway. After step 10 the fermentation process achieved around 35% of hydrogen (in 21 h) and the microbial mixed pool was transferred in larger serum bottles (for optimization), in order to reduce inhibitory effects of increasing hydrogen partial pressure.

Principal Component Analysis (PCA) confirmed this irregular pattern (Fig. 3), as can be observed in the score plot. PC1, explaining 55% of the total variability, clearly showed that hydrogen (production and yield), glycerol consumption and ethanol concentration were positively correlated and showed higher values in step 3 and 10 of the enrichment (on the left side of the score plot), while 1,3 PD and acetic acid were negatively correlated with those parameters (loading plot), thus confirming the trend already observed in Fig. 2. This means that, moving along the PC1 from right to left, we can observe an increase of hydrogen production and yield (together with an increase of the glycerol conversion and ethanol production) and a correspondent decrease of the 1,3 PD and acetate production. The finding that 1,3 PD is associated to lower hydrogen (and ethanol) production is in accordance with a previous study by Zeng and colleagues [22].

The fact that ethanol is correlated with hydrogen production (and negatively correlated to acetate) probably depends on the effect of the hydrogen partial pressure in the reactor, which is known to greatly influence ethanol conversion rate. In fact, ethanol can accumulate only with high hydrogen partial pressure. More precisely, when hydrogen partial pressure is below 10^4 Pa, ethanol will be converted to acetic acid [31]. In our study, hydrogen partial pressure during enrichment usually reached values between 5 and $10 \cdot 10^4$ Pa, thus favoring ethanol accumulation.

3.2. *Optimization experiments*

Optimization of important fermentation parameters and conditions, such as headspace/medium ratio (H/M ratio, v/v), inoculum (v/v), as well as the use (or not) of specific supplements and nutrients, further enhanced the hydrogen (and ethanol) production, together with the glycerol conversion efficiency. As can be observed in Table 2, the best results were achieved with the use an H/M ratio of 1.5 (50 mL working volume in 125 mL) and 10% v/v inoculum, without the need of any substrate supplementation, thus reaching a hydrogen yield of around 0.82 mol/mol and a hydrogen content in the biogas of 41%. Glycerol was almost completely degraded in all the heasdspace/working volume experiments, but for H/M values of 0.4 and 0.8 it took more than twice the time (data not shown). Addition of vitamin solution or yeast extract to the enriched activated sludge didn't affect hydrogen yield significantly, while the use of mineral solution showed an inhibitory effect on hydrogen production, regardless if used together

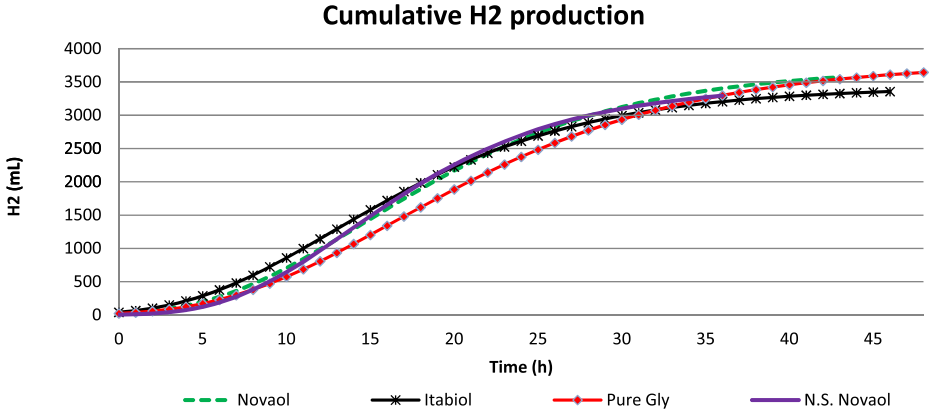


Fig. 4 – Comparison of cumulative hydrogen production using different glycerol types. Non sterilized Novaol experiments were performed in non sterilized bioreactor, without autoclaving minimum medium nor the crude glycerol. No difference in cumulative hydrogen production could be observed.

Table 3 – Modified Gompertz equation to describe the cumulative hydrogen production in the batch tests.

	P_{max} (mL)	R_{max} (mL/h)	λ (h)	R^2	H_2 rate (mL/L/d)	H_2 yield (mol/mol)
Novaol	3720.9	154.1	5.64	0.998	2997.7	0.90
Itabiol	3428.1	146.7	4.20	0.984	2983.4	0.88
Pure Gly	3808.1	138.8	6.34	0.998	2706.5	0.90
N.S. Novaol	3451.3	174.4	6.48	0.996	3152.4	0.91

P_{max} is the hydrogen production potential, R_{max} is the maximum hydrogen production rate and λ is the lag time. These parameters were used to calculate the hydrogen production rate (H_2 Rate), according to Wang and Wan [27].

with other supplements or not. Therefore we can affirm that the enrichment process allowed to successfully selected for a *functional consortium* (*sensu* Adav et al., [32]), which is able to effectively convert crude glycerol (as the only carbon source) into hydrogen, without any supplementation.

3.3. *Scale-up tests*

Scale-up tests using a 3 L bioreactor operated in optimized conditions, with different glycerol types, did not show significant differences of the main fermentation parameters. The modified Gompertz equation was used to fit cumulative hydrogen production and calculate production rate. As can be seen in Fig. 4, the use of Novaol or Itabiol crude glycerol, as well as non-sterile Novaol or pure glycerol, showed similar results in terms of cumulative hydrogen production. In all cases the substrate was (almost) totally degraded, with a glycerol degradation efficiency, expressed as the percent of the initial concentration, of 97.42% ± 0.98. At the end of the fermentation the hydrogen content in the biogas reached on average 54.05% ± 0.99.

The main parameters characterizing the modified Gompertz equation are shown in Table 3. Average (cumulative) hydrogen production potential (P_{max}) reached 3652 mL H_2 ± 199, with a production rate (H_2 Rate) of 2960 mL H_2/L/day ± 185 and a yield of 0.90 mol/mol ± 0.01. The high value of R^2 (>0.98) suggests that the modified Gompertz equation was able to describe the evolution of hydrogen production satisfactorily.

The quantification of fermentation products was also used to perform fermentation balance analysis (as reported by Murarka et al., [33]), and is presented in Table 4. The carbon recovery of fermentation products represented almost 90% (88.12% ± 1.93) of the consumed glycerol, thus indicating a good closure of the carbon balance. In accordance with Murarka et al. [33], 1,2 propanediol was undetectable via HPLC, possibly due to low concentration.

All the most important parameters describing the fermentation process (such as hydrogen production and yield, substrate degradation efficiency and carbon recovery) confirmed the ability of the selected enriched activated sludge to effectively convert different glycerol types into hydrogen, both in sterile and non-sterile conditions, without the necessity to previously select the bacteria on a synthetic substrate with pure glycerol.

The main studies conducted so far and dealing with mixed cultures grown on glycerol for hydrogen production (using dark fermentation) are summarized in Table 5 for comparison.

3.4. *Microbial community analysis*

Bacterial diversity of the selected microbial mixed culture (GCL) was investigated by cloning PCR-amplified (nearly complete) 16S rRNA genes. 90 clones, out of 102 totally isolated, produced useful sequences, showing high level of similarity (sequence identity ≥ 99%) with 16S rDNA sequences present in the NCBI database (Table 6).

No chimeric sequences were found among the clones analyzed. All sequences were phylogenetically allocated down to the order, family and genus level by the RDP Classification Algorithm, with bootstrap values ranging from 95 to 100% (Table 7). GCL clones related to the dominant group of

Table 4 – Fermentation balance analysis for the functional consortium grown on different glycerol types.

(No. of C atoms)		SA (4)	LA (3)	FA (1)	AA (2)	PA (3)	1,3 PD (3)	EtOH (2)	CO_2 (1)	Total C yield	C recovery[c] %
Novaol	mMol	3.58	4.65	5.71	1.08	2.68	11.89	127.0	97.70		
	Yield[a]	0.02	0.03	0.04	0.01	0.02	0.07	0.78	0.60		
	C yield[b]	0.09	0.09	0.04	0.01	0.05	0.22	1.57	0.60	2.66	88.8
Itabiol	mMol	5.43	9.33	4.98	3.29	2.69	12.70	127.03	94.53		
	Yield[a]	0.03	0.05	0.03	0.02	0.02	0.07	0.73	0.54		
	C yield[b]	0.13	0.17	0.03	0.04	0.05	0.23	1.51	0.55	2.61	87.0
Pure Gly	mMol	5.99	2.64	2.85	1.57	2.26	13.25	123.5	101.3		
	Yield[a]	0.04	0.02	0.02	0.01	0.01	0.08	0.74	0.61		
	C yield[b]	0.14	0.05	0.02	0.02	0.04	0.24	1.48	0.61	2.59	86.2
N.S. Novaol	mMol	2.48	1.04	1.98	2.03	3.07	17.59	116.5	80.19		
	Yield[a]	0.02	0.01	0.01	0.01	0.02	0.12	0.80	0.55		
	C yield[b]	0.07	0.02	0.01	0.03	0.06	0.36	1.61	0.55	2.72	90.5

Pure Gly = pure glycerol; N.S. Novaol = non-sterilized Novaol glycerol; SA = succinic acid; LA = lactic acid; FA = formic acid; AA = acetic acid; PA = propionic acid; 1.3 PD = 1.3 propanediol; EtOH = ethanol.

a Mol of product/mol of glycerol consumed.

b (Yield)·(number of carbon atoms in molecule).

c (Total C yield)/3·100 (being 3 the value of glycerol carbon yield).

Table 5 – Overview of the main results of recent studies dealing with glycerol fermentation to hydrogen, using mixed cultures.

	Inoculum type	Gly type	Gly Conc. g/L	Substrate consumpt.	Substrate supplem.	H_2 yield (mol/mol)	H_2 productivity	Ref.
Dark fermentation	Mesofilic	Crude	15	97.5%	No	0.90	2960 mL/L/day	Curr. st.
	Mesofilic	Crude	22.19	–	Yes	0.3	1.37 mmol/L	[34]
	Thermofilic	Crude	20.33	–	Yes	0.3	1502.84 mL/L	[35]
	Mesofilic	Crude	1	56%	Yes	1.1	16.1 mL	[36]
	Mesofilic	Crude	30	65%	Yes	–	34.19% mol	[9]
	Mesofilic	Crude	3	–	Yes	0.31	71 mL	[37]
	Mesofilic	Pure	1	~56%	Yes	–	14.8 mL	[36]
	Mesofilic	Pure	3	–	Yes	0.28	133 mL	[37]
	Mesofilic	Pure	10	100%	Yes	0.41	878 mL/L	[21]
	Various	Pure	5 g COD/L	Almost complete	Yes	–	58.2–195.8 mL/L	[38]

Gly = glycerol; Gly Conc. = initial glycerol concentration; Substrate Consumpt. = substrate consumption; Substrate Supplem. = substrate supplementation; Ref. = references; Curr. st. = current study.

Table 6 – Similarity between GCL library sequences and those retrieved from GenBank.

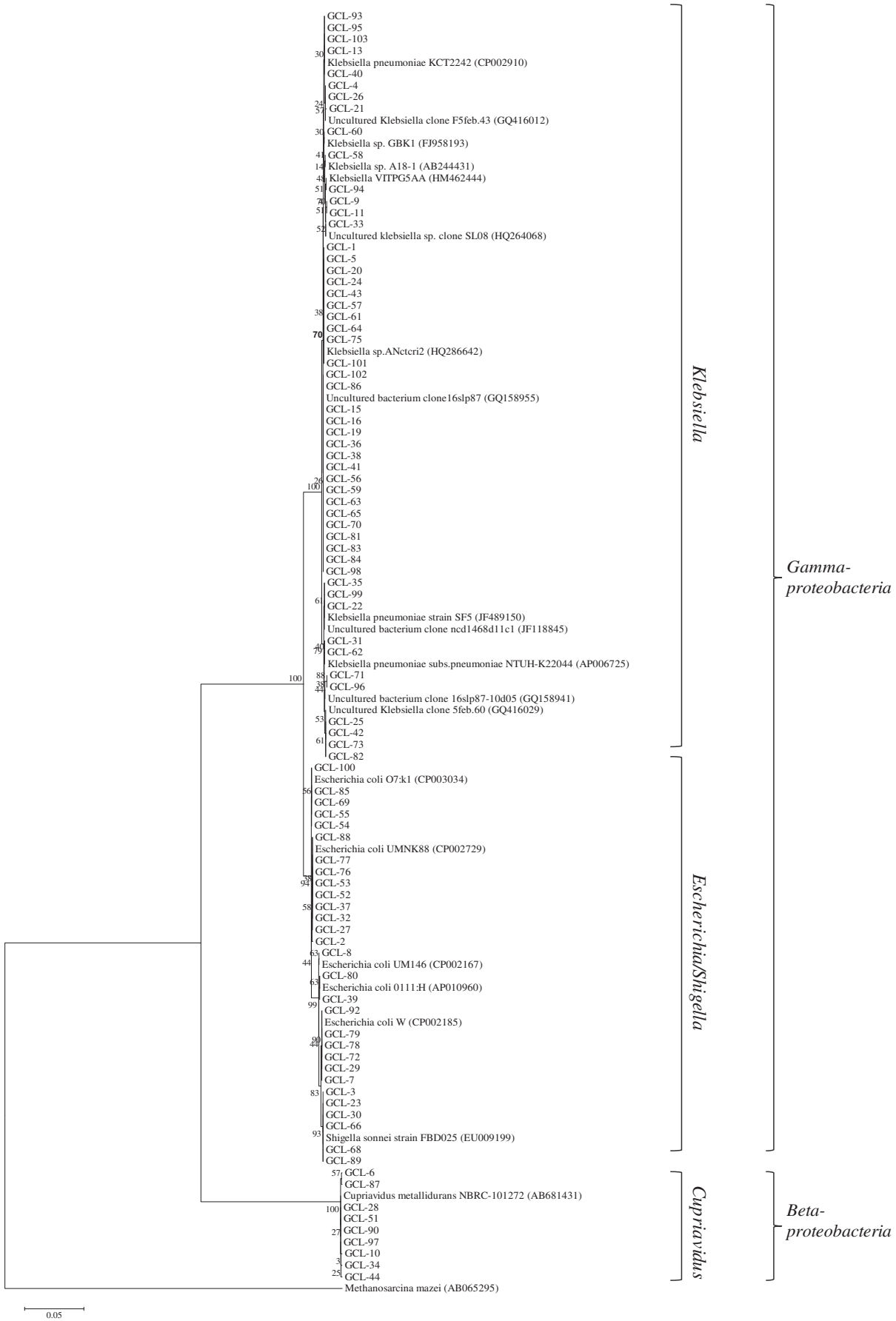
No. of clones	Abundance (%)	Closest relative	Accession no.	Similarity (%)
10	11.11	*Klebsiella* sp. ANctcri2	HQ286642	99–100
3	3.33	Uncultured *Klebsiella* sp. clone F5feb.43	GQ416012	99
3	3.33	Uncultured *Klebsiella* sp. clone SL08	HQ264068	99
5	5.56	*Klebsiella pneumoniae* KCTC2242	CP002910	99
17	18.89	Uncultured bacterium clone 16slp87-10f02.w2k	GQ158955	99–100
1	1.11	*Klebsiella* sp.A18-1 strain A18-1	AB244431	100
1	1.11	*Klebsiella pneumoniae* strain SF5	JF489150	99
4	4.44	Uncultured *Klebsiella* sp. Clone F5feb.60	GQ416029	99–100
2	2.22	*Klebsiella pneumoniae* subsp. pneumoniae NTUH-K2044	AP006725	99
2	2.22	Uncultured bacterium clone ncd1468d11c1	JF118845	99
1	1.11	Uncultured bacterium clone ncd1413a11c1	JF122956	99
1	1.11	*Klebsiella* sp. VITPGP5AA	HM462444	100
2	2.22	Uncultured bacterium clone 16slp87-10d05.p1k	GQ158941	99
5	5.56	*Escherichia coli* O7:K1 str. CE10	CP003034	99
9	10.00	*Escherichia coli* UMNK88	CP002729	99
2	2.22	*Escherichia coli* O111:H	AP010960	99–100
6	6.67	*Escherichia coli* W	CP002185	99–100
1	1.11	*Escherichia coli* UM146	CP002167	99
6	6.67	*Shigella sonnei* strain FBD025	EU009199	99
9	10.00	*Cupriavidus metallidurans* NBRC-101272	AB681431	99–100

Gammaproteobacteria (90% of the total number of clones) were affiliated to sequences of *Enterobacteriaceae* family and were identified at genus level as *Klebsiella* (57.78%), and *Escherichia/Shigella* (32.22%) (Table 7). The latter genus grouped together in *Escherichia/Shigella*, according to their genetic relationship, since strains of *Shigella* have arisen through multiple independent origins from within Escherichia *coli*, that is *Shigella* strains are actually clones of *E. coli* [39].

Table 7 – Phylogenetic affiliation of sequences from 16S rDNA library.

Phylogenetic affiliation[a]				Phylogenetic groups[b]
Class	Order	Family	Genus	%
Gammaproteobacteria 100%	*Enterobacteriales* 100%	*Enterobactericeae* 100%	*Klebsiella* 99–100%	57.78
			Escherichia 95–100%	25.56
			Shigella 100%	6.67
*Betaproteobacteria*100%	*Burkholderiales* 100%	*Burkholderiaceae* 100%	*Cupriavidus* 100%	10.0

a Identification performed using the RDP Classification Algorithm. Bootstrap confidence values are given in brackets (classification is well supported for confidence >80%).
b Percentage of GCL clones affiliated at genus level.


GCL-93
GCL-95
GCL-103
GCL-13
Klebsiella pneumoniae KCT2242 (CP002910)
GCL-40
GCL-4
GCL-26
GCL-21
Uncultured Klebsiella clone F5feb.43 (GQ416012)
GCL-60
Klebsiella sp. GBK1 (FJ958193)
GCL-58
Klebsiella sp. A18-1 (AB244431)
Klebsiella VITPG5AA (HM462444)
GCL-94
GCL-9
GCL-11
GCL-33
Uncultured klebsiella sp. clone SL08 (HQ264068)
GCL-1
GCL-5
GCL-20
GCL-24
GCL-43
GCL-57
GCL-61
GCL-64
GCL-75
Klebsiella sp.ANctcri2 (HQ286642)
GCL-101
GCL-102
GCL-86
Uncultured bacterium clone16slp87 (GQ158955)
GCL-15
GCL-16
GCL-19
GCL-36
GCL-38
GCL-41
GCL-56
GCL-59
GCL-63
GCL-65
GCL-70
GCL-81
GCL-83
GCL-84
GCL-98
GCL-35
GCL-99
GCL-22
Klebsiella pneumoniae strain SF5 (JF489150)
Uncultured bacterium clone ncd1468d11c1 (JF118845)
GCL-31
GCL-62
Klebsiella pneumoniae subs.pneumoniae NTUH-K22044 (AP006725)
GCL-71
GCL-96
Uncultured bacterium clone 16slp87-10d05 (GQ158941)
Uncultured Klebsiella clone 5feb.60 (GQ416029)
GCL-25
GCL-42
GCL-73
GCL-82
GCL-100
Escherichia coli O7:k1 (CP003034)
GCL-85
GCL-69
GCL-55
GCL-54
GCL-88
Escherichia coli UMNK88 (CP002729)
GCL-77
GCL-76
GCL-53
GCL-52
GCL-37
GCL-32
GCL-27
GCL-2
GCL-8
Escherichia coli UM146 (CP002167)
GCL-80
Escherichia coli 0111:H (AP010960)
GCL-39
GCL-92
Escherichia coli W (CP002185)
GCL-79
GCL-78
GCL-72
GCL-29
GCL-7
GCL-3
GCL-23
GCL-30
GCL-66
Shigella sonnei strain FBD025 (EU009199)
GCL-68
GCL-89
GCL-6
GCL-87
Cupriavidus metallidurans NBRC-101272 (AB681431)
GCL-28
GCL-51
GCL-90
GCL-97
GCL-10
GCL-34
GCL-44
Methanosarcina mazei (AB065295)
0.05
Klebsiella
Escherichia/Shigella
Cupriavidus
Gamma-proteobacteria
Beta-proteobacteria

The genus *Klebsiella* contained more species (Table 6) and up to thirty-one sequences were affiliated to uncultured relatives (59.61% of the total *Klebsiella*). Among *Klebsiella* spp., eight clone sequences (15.38%) were identified as *Klebsiella pneumoniae* and ten (19.23%) as *K. ANctcri2* respectively. The remaining sequences (1.73%/each) were affiliated to the species *K. GBK1*, *K. A18-1* and *K. VITPGP5AA*. Even though each of these sequences was represented by a unique GCL clone, they were still evaluated in the community composition, because the inferred assignment was robust (similarity: 99% and 100%) (Table 6).

Clones belonging to the group *Escherichia/Shigella* were affiliated to the species *E. coli* (79.31%) and *Shigella sonnei* (18.76%).

The betaproteobacteria-related clones (10% of the total number of clones) grouped into the unique *Cupriavidus* genus (formerly *Ralstonia*) within the *Burkholderiaceae* family. All sequences were affiliated to *Cupriavidus metallidurans*.

The phylogenetic tree established with a bootstrap neighbor-joining (NJ) method is shown in Fig. 5. The GCL clones fell into three clusters (*Klebsiella*, *Escherichia/Shigella* and *Cupriavidus*), each corresponding to the identified genera in the microbial community. The phylogenetic relationship between the dominant group (*gammaproteobacteria*) of GCL clones closely related at genus level (*Klebsiella* and *Escherichia/Shigella*) and the smaller and more distant group (*betaproteobacteria*), produced an NJ tree characterized by very short peripheral branches, while the longer external branches indicated the higher phylogenetic distances between the observed clusters.

Among all the identified microorganisms of the GCL community, *Klebsiella* spp. and *E. coli* sp. are probably playing the key role for hydrogen production. Previous studies showed that different strains of *K. pneumoniae* [40,41], and *Klebsiella oxytoca* [42], are able to metabolize both, crude and pure glycerol under anaerobic conditions, mainly producing 1,3 propanediol, ethanol and organic acids. *Klebsiella* sp. HE1 [43] was able to produce hydrogen from pure glycerol, while *K. pneumoniae* DSM 2006 and Blb01 were both used in optimization studies for hydrogen production from biodiesel waste [44,45]. More studies are available for *E. coli*, attesting the ability of different strains to anaerobically ferment glycerol, producing hydrogen and co-products [1,12,33,46]. In all these studies, pure cultures of genetically and metabolically engineered microbial strains were used for improving hydrogen or ethanol production.

Within the GCL community, a different and maybe less clear role has been played by *S. sonnei* and *C. metallidurans*. *S. sonnei* is a well known human pathogen and its presence in the community was not surprising, bearing in mind the origin of the inoculum. This bacterium is able to ferment sugars without gas production and *Shigella flexneri* strain G3, closely related to *S. sonnei*, was recently isolated and characterized as cellulolytic bacterium in anaerobic condition [47]. *S. sonnei* represented only a minor fraction (6.67%) of the microbial community and we may suppose that it was able to survive on crude glycerol, without performing any useful role, in terms of hydrogen production, in the microbial community.

C. metallidurans (formerly *Ralstonia metallidurans*) was previously found on industrial sites characterized by the presence of industrial wastes, soils or sludges rich in toxic heavy metals, often mixed with organic recalcitrant compounds and hydrocarbons [48]. Strain NBRC-101272, also retrieved in the GCL community, was first isolated from drainage water of a chemical laboratory [49]. Therefore, *C. metallidurans* strains are adapted to grow on metal contaminated environments and perform a strong resistance to environmental stressors. Moreover, this interesting bacterium is able to grow as a facultative hydrogen-oxidizing bacterium (acting as a sink for hydrogen), but it is also a methylotrophic bacterium, thus able to use C1 compounds such as methanol or methylamine (but not methane) as a carbon source for its growth [50]. There are no reports associating this bacterium to hydrogen production from glycerol. Therefore, its role in our microbial community may be related to detoxification functions. In fact, the methylotrophic ability of *C. metallidurans* might be useful for methanol degradation. On the other hand, being a chemolithoautotrophic bacterium [48], it may consume hydrogen and fermentation products, assuming the role of antagonist inside the community.

In the present study, the use of 16S rDNA cloning technique allowed to gain a deep insight in the community composition and heterogeneity, including the fraction of '*uncultured*' microbial populations.

The good performance in hydrogen production from crude glycerol, obtained with this consortium, may be related to the cooperative metabolic activities between microorganisms, that produced an increased flux of easily biodegradable substrates involved in hydrogen-generating pathways.

The structure of microbial community also pointed out an intra- as well an inter-species diversity among GCL clones (Fig. 5). The coexistence of such polymorphic populations (inter-species diversity) may be maintained by a so called cross-feeding mechanism, in which a strain, specializing on a primary resource, coexists with other cross-feeding strains, that specialize on a waste product resulting from consumption of the primary resource [51].

Clearly, mutualism such as syntrophic metabolic cooperation may also be active in the degradation of crude glycerol and its impurities. The main difference with the cross-feeding

Fig. 5 – Phylogenetic tree of species detected in GCL library, showing relationship of 16S rDNA sequences of isolated clones (GCL-) to the most closely related sequences from the Genbank (numbers in brackets represent accession number of sequences). The tree was developed using the neighbor-joining method with bootstrap consensus inferred from 1000 replicates. It is rooted using *Methanosarcina mazei* as an outgroup. Major phylogenetic groupings are indicated and bracketed on the right. The tree is drawn to scale, with branch lengths in the identical units as those of the evolutionary distances used to infer the phylogenetic tree. Kimura 2-parameter method was used to compute the evolutionary distances and is in the units of the number of base substitutions per site. The scale bar represents 0.05 substitutions per nucleotide position.

mechanism is that syntrophic metabolic cooperation is a strict relationship between two metabolically unrelated species of bacteria (inter-species diversity), which depend on each other for energetic reason and perform together a fermentation process that neither can perform alone [52]. Sarma et al., [53] pointed out that the use of suitable co-culture for impurities degradation (i.e. methanol and soap), that are known to have inhibitory effect on microbial growth, may play a significant role in improving hydrogen yield. Some species of *Klebsiella* and *Escherichia* are known to have soap degradation potential [54]. Moreover, *C. metallidurans* is probably contributing to a syntrophic metabolic cooperation, helping detoxifying crude glycerol through methanol degradation, while growing on the fermentation products (hydrogen and volatile fatty acids) of *Klebsiella* and *Escherichia* spp. Seemingly, intra- and inter-species metabolic cooperation (syntrophism and/or cross-feeding) was activated in our consortium as a consequence of the selective pressure applied during the enrichment of activated sludge, namely the use of crude glycerol as the only carbon source, without any supplementation of yeast extract, tryptone, nor vitamin- and mineral solution.

Clearly, successful biological hydrogen production depends on the overall performance (results of interactions) of the bacterial community (i.e. mixed cultures) in the reactor. Therefore, selected mixed cultures may provide useful combinations of metabolic pathways for the processing of complex waste material, thereby supporting a more efficient decomposition of biomass, compared with pure bacterial species [3,55]. Thus, the use of the enriched activated sludge, thanks to the establishment of positive mechanisms of metabolic cooperation (syntrophism and/or cross-feeding), probably favored the high conversion efficiency of the different glycerol types investigated.

4. Conclusions

In this study we applied enrichment of activated sludge to select glycerol-fermenting bacteria, able to efficiently convert the substrate into hydrogen. The microorganisms were directly selected and acclimatized on crude glycerol, without the use of substrate supplementation, in order to reduce operational costs. The enrichment displayed a non linear pattern, which however led to a significant improvement of glycerol conversion into hydrogen, also related to the increase in ethanol concentration and concomitant decrease of 1,3 PD, as shown by the PCA. The thus obtained enriched activated sludge was then used for further optimization of fermentation parameters, leading to a high hydrogen production rate (2960 mL H_2/L/d $\pm$ 185.5) and near stoichiometric yield (0.90 mol/mol $\pm$ 0.01), together with a (almost) complete substrate degradation (97.42% $\pm$ 0.98). At the end of the fermentation the hydrogen content in the biogas reached on average 54.05% $\pm$ 0.99.

16S rDNA cloning technique allowed to gain a deeper insight in the community composition and heterogeneity. The GCL community was characterized by a well-defined structure and was dominated by *Enterobacteriaceae* (90%), with the genera *Klebsiella* and *Escherichia/Shigella*, followed by *Burkholderiaceae* (10%) with genus *Cupriavidus*. The importance of metabolic cooperation mechanisms such as syntrophism and/or cross-feeding has been discussed.

The role of main hydrogen producers in the GCL community may be attributed to *Klebsiella* sp. and *E. coli* sp., while the exact contribution of *C. metallidurans* and *S. sonnei* still need to be clearly understood and will be further investigated.

In conclusion we can state that the eco-biotechnological approach turned out to be a valuable tool for selecting a suitable microbial pool, able to effectively convert different glycerol types into hydrogen. Moreover, the possibility to obtain a highly efficient conversion of biodiesel-derived crude glycerol in non-sterile conditions (with the same efficiency of pure glycerol, in sterile conditions) can also contribute to significantly reduce the operation costs of the process, especially on a larger/industrial scale.

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REFERENCES

[1] Dharmadi Y, Murarka A, Gonzalez R. Anaerobic fermentation of glycerol by *Escherichia coli*: a new platform for metabolic engineering. Biotechnol Bioeng 2006;94(5):821–9.
[2] Cofré O, Ramirez M, Gomez JM, Cantero D. Optimization of culture media for ethanol production from glycerol by *Escherichia coli*. Biomass Bioenergy 2012;37:275–81.
[3] Marone A, Massini G, Patriarca C, Signorini A, Varrone C, Izzo G. Hydrogen production from vegetable waste by bioaugmentation of indigenous fermentative communities. Int J Hydrogen Energy 2012;37(7):5612–22.
[4] Moazami N, Ashori A, Ranjbar R, Tangestani M, Eghtesadi R, Nejad AS. Large-scale biodiesel production using microalgae biomass of Nannochloropsis. Biomass and Bioenergy 2012;39:449–53.
[5] Yazdani SS, Gonzalez R. Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. Curr Opin Biotechnol 2007;18:213–9.
[6] Valdez-Vazquez I, Sparling R, Risbey D, Rinderknecht-Seijas N, Poggi-Varaldo HM. Hydrogen generation via anaerobic fermentation of paper mill wastes. Bioresour Technol 2005;96:1907–13.
[7] McCoy M. An unlikely impact. Chem Eng News 2005;83:24–6.
[8] McCoy M. Glycerin surplus. Chem Eng News 2006;84:7–8.
[9] Misturini Rossi D, Berne da Costa J, Aquino de Souza E, Do Carmo Ruaro Peralba M, Samios D, Zachia Ayub MA. Comparison of different pretreatment methods for hydrogen production using environmental microbial consortia on residual glycerol from biodiesel. Int J Hydrogen Energy 2011;36:4814–9.
[10] Johnson DT, Taconi KA. The glycerin glut: options for the value-added conversion of crude glycerol resulting from biodiesel production. Environ Prog 2007;26(4):338–48.
[11] Meyer PP, Pankaew S, Rukruang A and Tongurai C. Biohydrogen production from crude glycerol. In: Proceedings of the 17th World Hydrogen Energy Conference. Brisbane, Australia; 15–19 June 2008.

[12] Yazdani SS, Gonzalez R. Engineering *Escherichia coli* for the efficient conversion of glycerol to ethanol and co-products. Metab Eng 2008;10:340–51.
[13] Fountoulakis MS, Manios T. Enhanced methane and hydrogen production from municipal solid waste and agro-industrial by-products co-digested with crude glycerol. Bioresour Technol 2009;100:3043–7.
[14] Siles López JA, Martín Santos MA, Chica Pérez AF, Martín Martín A. Anaerobic digestion of glycerol derived from biodiesel manufacturing. Bioresour Technol 2009;100(23):5609–15.
[15] Varrone C, Giussani B, Izzo G, Massini G, Marone A, Signorini A, et al. Statistical optimization of biohydrogen and ethanol production from crude glycerol by microbial mixed culture. Int J Hydrogen Energy 2012;37:16479–88.
[16] Johnson K, Jiang Y, Kleerebezem R, Muyzer G, van Loosdrecht MCM. Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity. Biomacromolecules 2009;10:670–6.
[17] Yang Y, Tsukahara K, Sawayama S. Biodegradation and methane production from glycerol-containing synthetic wastes with fixed-bed bioreactor under mesophilic and thermophilic anaerobic conditions. Process Biochem 2008; 43(4):362–7.
[18] Barbirato F, Camarasa-Claret C, Grivet JP, Bories A. Glycerol fermentation by a new 1,3-propanediol-producing microorganism *Enterobacter agglomerans*. Appl Microbiol Biotechnol 1995;43:786–93.
[19] Ito T, Nakashimada Y, Senba K, Matsui T, Nishio N. Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. J Biosci Bioeng 2005;100(3):260–5.
[20] D'Ippolito G, Dipascuale L, Vella FM, Romano I, Gambacorta A, Cutignano A, et al. Hydrogen metabolism in the extreme thermophile *Thermotoga neapolitana*. Int J Hydrogen Energy 2010;35:2290–5.
[21] Seifert K, Waligorska M, Wojtowski M, Laniecki M. Hydrogen generation from glycerol in batch fermentation Process. Int J Hydrogen Energy 2009;34:3671–8.
[22] Zeng AP, Biebl H, Schlieker H, Deckwer WD. Pathway analysis of glycerol fermentation by *Klebsiella pneumoniae*: regulation of reducing equivalent balance and product formation. Enzym Microb Technol 1993;15:770–9.
[23] Zheng ZM, Hu QL, Hao J, Xu F, Guo NN, Sun Y, et al. Statistical optimization of culture conditions for 1,3-propanediol by *Klebsiella pneumoniae* AC 15 via central composite design. Bioresour Technol 2008;99:1052–6.
[24] Wolin EA, Wolin MJ, Wolfe RS. Formation of methane by bacterial extracts. J Biol Chem 1963;238:2332–86.
[25] Logan BE, Oh SE, Kim IS, Van Ginkel S. Biological hydrogen production measured in batch anaerobic respirometers. Environ Sci Technol 2002;36(11):2530–5.
[26] Chen WH, Chen SY, Khanal S, Sung S. Kinetic study of biological hydrogen production by anaerobic fermentation. Int J Hydrogen Energy 2006;31(15):2170–8.
[27] Wang J, Wan W. Optimization of fermentative hydrogen production process by response surface methodology. Int J Hydrogen Energy 2008;33:6976–84.
[28] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28: 2731–9.
[29] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406–25.
[30] Kimura MA. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 1980;16:111–20.
[31] Li YF, Ren NQ, Chen Y, Zheng GX. Ecological mechanism of fermentative hydrogen production by bacteria. Int J Hydrogen Energy 2007;32:755–60.
[32] Adav SS, Lee DJ, Wang AJ, Ren NQ. Functional consortium for hydrogen production from cellobiose: concentration-to-extinction approach. Bioresour Technol 2009;100:2546–50.
[33] Murarka A, Dharmadi Y, Yazdani SS, Gonzalez R. Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals. Appl Environ Microbiol 2008;74(4):1124–35.
[34] Sittijunda S, Reungsang A. Biohydrogen production from waste glycerol and sludge by anaerobic mixed cultures. Int J Hydrogen Energy 2012;37:13789–96.
[35] Sittijunda S, Reungsang A. Media optimization for biohydrogen production from waste glycerol by anaerobic thermophilic mixed cultures. Int J Hydrogen Energy 2012;37: 15473–82.
[36] Mangayil R, Karp M, Santala V. Bioconversion of crude glycerol from biodiesel production to hydrogen. Int J Hydrogen Energy 2012;37:12198–204.
[37] Priscilla AS, Joe MP, Wallis AL, Bruce EL. Enhanced hydrogen and 1,3-propanediol production from glycerol by fermentation using mixed cultures. Biotechnol Bioeng 2009; 104:1098–106.
[38] Yohei A, Dong- YL, Yu-You L, Tatsuya N. Hydrogen production potentials and fermentative characteristics of various substrates with different heat-pretreated natural microflora. Int J Hydrogen Energy 2009;34(13):5365–72.
[39] Pupo GM, Lan R, Reeves PR. Multiple independent origins of *Shigella* clones of *Escherichia coli* and convergent evolution of many of their characteristics. Proc Natl Acad Sci 2000;97: 10567–72.
[40] Oh BR, Seo JW, Heo SY, Hong WK, Luo LH, Joe MH, et al. Efficient production of ethanol from crude glycerol by *Klebsiella pneumoniae* mutant strain. Bioresour Technol 2011; 102:3918–22.
[41] Xiu ZL, Song BH, Wang TT, Sun LH, Feng EM, Zeng AP. Optimization of dissimilation of glycerol to 1,3-propanediol by *Klebsiella pneumoniae* in one- and two-stage anaerobic cultures. Biochem Eng J 2004;19:189–97.
[42] Metsoviti M, Paramithiotis S, Drosinos EH, Galiotou-Panayotou M, Nichas GJE, Zeng AP, et al. Screening of bacterial strains capable of converting biodiesel-derived raw glycerol into 1,3-propanedio, 2,3-butanediol and ethanol. Eng Life Sci 2012;12(1):57–68.
[43] Wu KJ, Lin YH, Lo YC, Chen CY, Chen WM, Chang JS. Converting glycerol into hydrogen, ethanol, and diols with *Klebsiella* sp. HE1 strain via anaerobic fermentation. J Taiwan Inst Chem Eng 2011;42:20–5.
[44] Liu F, Fang B. Optimization of bio-hydrogen production from biodiesel wastes by *Klebsiella pneumonia*. Biotechnol J 2007;2: 374–80.
[45] Costa JB, Rossi DM, De Souza EA, Samios D, Bregalda F, Do Carmo Ruaro Peralba M, et al. The optimization of biohydrogen production by bacteria using residuals glycerol from biodiesel synthesis. J Environ Sci Health A Tox Hazard Subst Environ Eng 2011;46(13):1461–8.
[46] Hu H, Wood TK. An evolved *Escherichia coli* strain for producing hydrogen and ethanol from glycerol. Biochem Biophys Res Commun 2010;391:1033–8.
[47] Wang AJ, Gao L, Ren NQ, Xu J, Liu C, Cao GL, et al. Isolation and characterization of *Shigella flexnery* G3, capable of effective cellulosic saccharifiaction under mesophilic conditions. Appl Environ Microbiol 2011;77:517–23.
[48] Diels L, Van Roy S, Taghavi S, Van Houdt R. From industrial sites to environmental applications with *Cupriavidus metallidurans*. Antonie van Leeuwenhoek 2009; 96:247–58.

[49] Miyake-Nakayama C, Masujima S, Ikatsu H, Shinoda S. Isolation and characterization of a new dicholorometane degradation bacterium, *Ralstonia metallidurans* PD11. Biocontrol Sci 2004;9:89–93.
[50] Miyake-Nakayama C, Ikatsu H, Kashihara M, Tanaka M, Arita M, Miyoshi SI, et al. Biodegradation of dichloromethane by the polyvinyl alcohol-immobilized methylotrophic bacterium *Ralstonia metallidurans* PD11. Environ Biotechnol 2006;70:625–30.
[51] Rosenzweigh FR, Sharp RR, Treves DS, Adams J. Microbial evolution in a simple unstructured environment: genetic differentiation in *Escherichia coli*. Genetics 1994;137:903–17.
[52] Schink B. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Bio Rev 1997;61:262–80.
[53] Sarma SJ, Brar SK, Sydney EB, Le Bihan Y, Buelna G, Soccol CR. Microbial hydrogen production by bioconversion of crude glycerol: a review. Int J Hydrogen Energy 2012;37: 6473–90.
[54] Ojo OA, Oso BA. Biodegradation of synthetic detergents in wastewater. Afr J Biotechnol 2009;8:1090–109.
[55] Hung CH, Chang YT, Chang YJ. Roles of microorganisms other than Clostridium and Enterobacter in anaerobic fermentative biohydrogen production systems: a review. Bioresour Technol 2011;102:8437–44.