Comparison of different pretreatments on mixed cultures to produce H2 from glycerol

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Comparison of different pretreatments on mixed cultures to produce H_2 from glycerol

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Abstract
Recent research has focused on finding new sources of renewable energy and the H_2 could be considered as renewable if produced from biomass. In that purpose, H_2 can be produced biologically by dark fermentation from a great variety of organic materials, including industrial waste such as glycerol. The aim of this study was to evaluate the effect of inoculum pretreatment on H_2 yield in a CSTR. Aerobic enrichment as well as thermal shock pretreatment were investigated on two types of mixed inoculums: aerobic and anaerobic sludge. H_2 yields showed no significant difference between the use of non pretreated aerobic sludge (control), and anaerobic or aerobic sludge pretreatment after heat shock or aerobic enrichment for 4 weeks. The best yield was around 0.55 mol_{H2} $mol^{-1}_{glycerol}$. The operational conditions of the continuous system had a higher influence on H_2 yields than inoculum pretreatments. It was observed a specific selection of hydrogen producers-microbial community and clearly distinct with non-producers- microbial community.

Keywords
Biohydrogen; Dark Fermentation; Glycerol; Microbial Community Structure; Mixed Inoculum

INTRODUCTION

Nowadays, energy crisis and environmental issues are two vital topics that sustain worldwide the development of alternative technologies to fossil fuels. In particular, bioH_2 as biofuels has gained in importance as an environmentally friendly solution for transportation, since its combustion generates only water and no greenhouse gas. Moreover, H_2 gas presents the highest amount of energy per unit mass, 142 kJ g^{-1}, more than any other known fuels [1]–[5]. H_2 can be produced biologically by dark fermentation from organic substrates, using pure or mixed cultures as microbial inoculum. The use of mixed cultures, eg. aerobic and anaerobic sludge, presents several advantages such as a high robustness, adaptability, physiological flexibility, with easily available inoculum at low cost [6], [7]. However, performing dark fermentation with mixed culture often requires a pretreatment of the inoculum both to eliminate H_2-consuming microorganisms, eg. methanogens, and concentrate in H_2 producers [5], [8]. Thermal shock is the most common pretreatment to remove methanogenic and acidogenic microorganisms that are not able to sporulate. Spore-forming fermentative bacteria, such as *Clostridium* sp., are specifically selected [5], [7]. Although most of the clostridia are producing H_2, some are well known homoacetogens, ie. *Clostridium aceticum* (hydrogen consumers). Besides several facultative anaerobic microorganisms from the *Enterobacteriaceae* family are able to produce H_2 and may be specifically selected by aeration that inhibits strict anaerobes such as clostridia. However, little is known about the impact of aerobic pretreatment [9]. Morever, when grown on glycerol, facultative anaerobes will prefer an oxidative pathway for glycerol degradation with higher H_2 production, unlike *Clostridium* sp. microorganisms which prefer the reductive pathway leading to higher 1,3-propanediol accumulation [10], [11]. The aim of this study was to evaluate the effect of inoculum pretreatment on H_2 production performances from glycerol in CSTR.

MATERIALS AND METHODS

Experimental design

Four experiments were carried out in a continuous reactor of 2 L of working volume at 37°C, pH 5.5 and HRT 12h with two mixed cultures as inoculum: anaerobic (**AnI**) and aerobic (**AI**) sludge. Controls corresponded to reactors inoculated with untreated sludge (**AnI-C** and **AI-C**). Two reactors were operated with heat-treated sludge (**AnI-HT** and **AI-HT**). Heat-shock pretreatment of the inoculum was performed at 105°C for 2 hours. Anaerobic and aerobic sludge were enriched during 1 week (**AnI-1w** and **AI-1w**) and 4 weeks (**AnI-4w** and **AI-4w**) of aeration and carbon source addition (glucose 10 g L^{-1})(Patent process, Application N°: 201402319). Experiments were fed with glycerol at 10 g·L^{-1} and trace elements as previously described by Tapia-Venegas et al. (2013). The steady state time was defined by the standard deviation of hydrogen flow (less than 30%). H_2 yields were calculated with steady state values (mole of hydrogen produced per mole of glycerol consumed). ANOVA was performed for to evaluate the statistically significant on the H_2 production with pretreatments.

Analytical methods

A volume counter MILLIGAS COUNTER® Type MGC-1 was used to determine the volume of biogas produced. Biogas composition (H_2, CO_2 and CH_4) was measured by gas chromatography coupled to a TCD detector. Ethanol, acetic acid, butyric acid and propionic acid were measured by gas chromatography and FID detection. Residual glycerol, succinic acid and formic acid were measured by HPLC equipped with an IR detector. A PCR-SSCP analysis was performed on DNA samples of inoculum, inoculum after pre-treatment and culture in steady state. The bacterial V3 region of the *16s rRNA* gene was amplified with the primers w49 (5'-ACGGTCCAGACTCCTACGGG-3') and w104 (5'-6FAM-TTACCGCGGCTGCTGGCAC-3'). PCA analysis of PCR-SSCP profiles were carried out by XSTAT software.

RESULTS AND DISCUSSION

H_2 yields in steady state are shown in Figure 1. H_2 was not produced when the anaerobic inoculums were treated with 1 or 4 weeks of aeration (AnI-1w and AnI-4w). Therefore, aeration treatment not only, disfavours anaerobic microorganisms, also favours non hydrogen producers in the anaerobic sludge. In contrast, when aerobic sludge was enriched under aeration, the hydrogen yields were 0.30±0.09 to 0.55±0.08 mol_{H2} $mol^{-1}_{glycerol}$ after 1 and 4 weeks of enrichment, respectively. However, the hydrogen yields with control (untreated), AI-HT (heat-treated), and AI-4w (aerobic enriched) and aerobic sludge were statistically equal (95% certainty) equivalent to 55% of the theoretical maximum yield with glycerol (1 mol_{H2} $mol^{-1}_{glycerol}$) (Figure 1). Interestingly, our results are consistent with previous data reported in continuous systems using a pure culture [12].

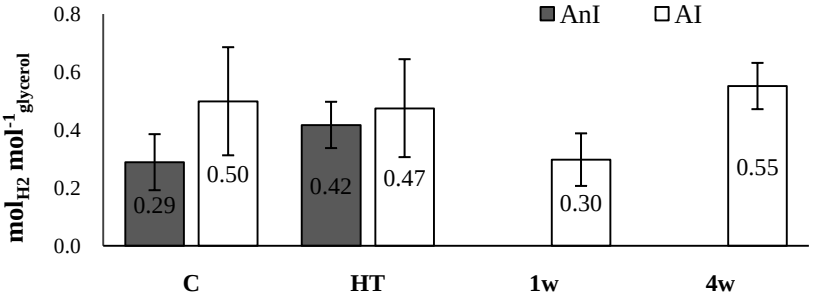

Figure 1. Hydrogen yields at steady-state of the continuous system. Inoculums: untreated (C), heat-shock treated (HT) and 1 week (1w) or 4 weeks (4w) aeration-enriched. Initial aerobic (AI) and anaerobic (AnI) sludge correspond to the white and black bars, respectively. Error bars represent the variation of steady-state data.

In addition, H_2 yield with anaerobic sludge heat shock treated (AnI-HT) was higher than the control (AnI-C) (approximately 45%). This is confirmed by literature where the increase of hydrogen yield, with a heat shock treated sludge has been largely reported [13]–[15]. Regarding metabolites produced, the major metabolite was butyrate in control systems (AnI-C and AI-C) while butyrate and succinate were in heat shock pretreated systems (AnI-HT and AI-HT).

CE-SSCP profiles and PCA analysis of CE-SSCP profiles are presented in Figure 2. The initial community of the anaerobic and aerobic sludge were substantially different and also, were different with the cultures at steady state. Similar ribotypes in cultures at steady state were found (Figure 2A) and this could be consistent with the similar H_2 yields measured. Consequently, the peak n°1 was dominant in the following profiles: AnI-C, AnI-HT, AI-HT, AI-1w and AI-4w while the peak n°2 was dominant in AI-HT, AI-4w, AnI-C and AnI-HT. Therefore, the relative abundance of ribotypes 1 and 2 (peak n°1 or n°2) were independent of H2 yields (statistically equals), however differences in metabolites produced, could suggest the importance of subdominant ribotypes present in each reactor, as previously observed by Rafrafi et al. (2013) in similar continuous systems. These authors reported that H_2 yields were dependant on subdominant species acting as keystone species in dark fermentative ecosystems. Interestingly, the ribotype 1 (peak n°1) was dominant in all reactors with H_2 production. Identification of the peaks is under progress and will provide new insights on the importance of the subdominant species in such systems.

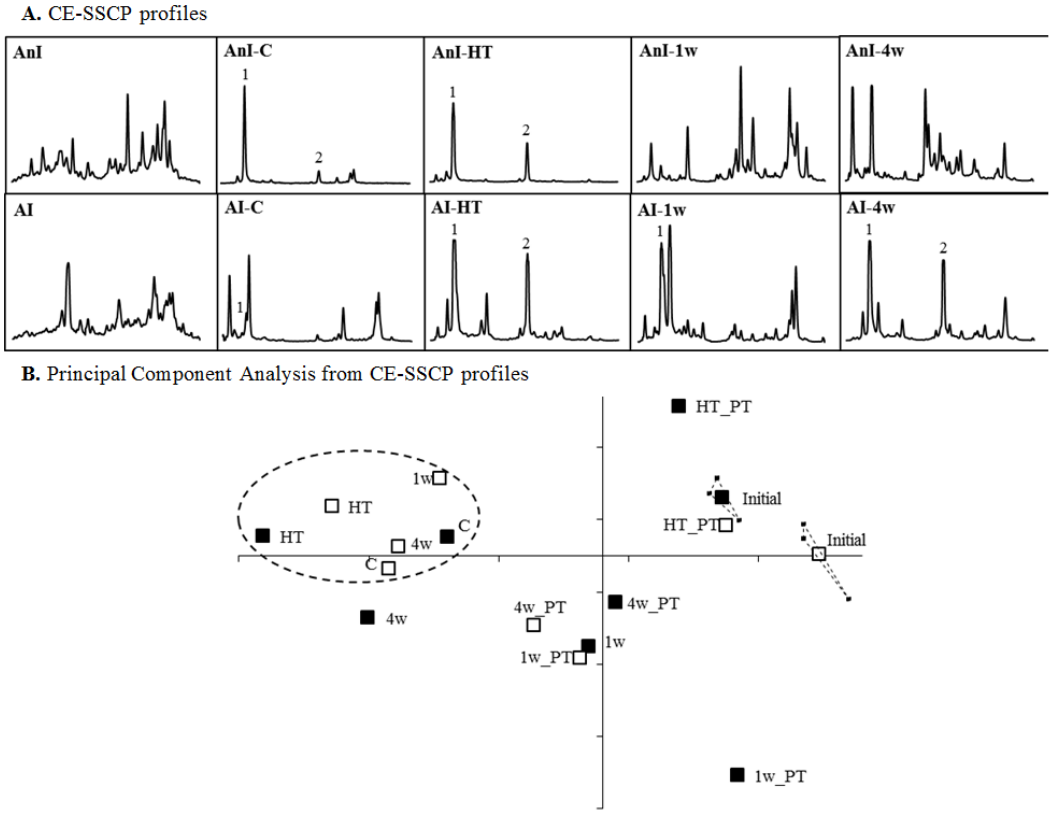

Figure 2. **A.** Molecular community CE-SSCP profiles of initial anaerobic (AnI) and aerobic (AI) sludge, and at steady state of reactors inoculated with untreated (C), heat-shock treated (HT) and 1 week (1w) or 4 weeks (4w) aeration-enriched inocula. Peaks were numbered for further identification. **B.** Principal Component Analysis performed from CE-SSCP profiles. PCA was calculated using the correlation coefficients of Pearson (n) representing the 40% of the data. Black squares: AnI; White squares: AI. PT represents profiles immediately after (pre)treatments.

Principal component analysis (Figure 2B) shows the relationship of the hydrogen producer cultures at steady state. Therefore continuous system has a greater power selection than pretreatments and suggests the importance of operation condition used.

CONCLUSION

The highest hydrogen yields from glycerol were obtained using aerobic sludge as inoculum but no statistical difference was observed with the different pretreatments performed. The aerobic enrichment in an anaerobic sludge was not adequate for hydrogen production.

Community structures of hydrogen producer systems were similar and differed of non-hydrogen producer systems.

The reactor operation as low pH and short HRT had a more effect on final microbial community structure than the inoculum pretreatments.

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