Development of a Two-step Fermentative Biohydrogen Production Process Using Selectively Enriched Microbial Populations as Inoculum

IULIAN ZOLTAN BOBOESCU[1]*, VASILE DANIEL GHERMAN[1], ION MIREL[1], GERGELY MARÓTI[2], ADINA NEGREA[3], MIHAELA CIOPEC[3], MARILENA MOTOC[4]

Politehnica University of Timisoara, Hydrotechnical Engineering Department., 2 Vasile Parvan Blv., 300223, Timisoara, Romania
[2]Hungarian Academy of Sciences, Biological Research Centre, Institute of Biochemistry, Szeged, Hungary,5
[3] Politehnica University of Timisoara, Industrial Chemistry and Environmental Engineering Department, 6 Vasile Parvan Blv., 300223, Timisoara, Romania,
[4] "Victor Babes'" University of Medicine and Pharmacy Timisoara,2 Eftimie Murgu Sq., 300041, Timisoara, Romania,

Biohydrogen (H_2) production using two different types of substrates was studied in anaerobic small scale batch reactors. Different environments were sampled and sequentially subject to different types of pretreatment methods in order to obtain enriched bacteria populations used to start up the bioreactors. Experimental data demonstrated significant differences in H_2 production regarding the various substrates and pre-treatment methods used. The described process opens the way towards the dual benefits of renewable energy generation (H_2) with simultaneous wastewater treatment.

Keywords: biohydrogen, dark fermentation, microbial consortia, pretreatment

Because of the permanent growth in the energy demand and the non-renewable nature of today's fuels, novel and safe energy carriers have to be introduced. Hydrogen fulfills all the requirements for a clean, alternative fuel, resulting water as the sole byproduct upon combustion [1-3]. In addition to this, H_2 gas can be used directly in the internal combustion engines or in fuel cells to generate electricity. Its use in fuel cells is inherently more efficient than the combustion currently required for the conversion of other potential fuels to mechanical energy [4-6].

Among various hydrogen production processes, biological ways are known to be the least energy intensive. Moreover, some of these processes (e.g. dark fermentation) can utilize various organic wastes as substrates for fermentative hydrogen production. The complete oxidation of glucose would yield a stoichiometry of 12 mole H_2 per mole of glucose but in this case no energy is utilized to support growth and metabolism of the host organism. Thus, a theoretical maximum of 4 mole H_2 per mole of glucose can be produced by some obligate anaerobes and a theoretical maximum of 2 mole H_2 per mole of glucose can be produced in the case of some facultative anaerobes [7]. Dark H_2 production has the advantages of rapid hydrogen evolution rate and can be operated at ambient temperature (30–40°C) and pressure [8].

Bacteria and other microbes capable of hydrogen production widely exist in natural settings such as soil, wastewater sludge, compost, etc. [9-13]. Thus, well selected and concentrated derivatives of these sources can be used as inoculum for fermentative hydrogen production. Dark hydrogen production processes using mixed cultures are more efficient than those using pure cultures, because the former represent more simple systems to operate and easier to control, and may accept a broader source of feedstock [14]. However, hydrogen can be utilized by hydrogen-consuming bacteria, thus, restriction or termination of the methanogenic process is crucial to render H_2 to an end-product in the metabolic flow [15]. There are pretreatment possibilities to permit selective enrichment of specific groups of parent cultures by inhibiting H_2-consuming methanogenic bacteria [15, 16], which also prevents competitive growth and co-existence of further H_2-consuming bacteria [13, 17].

Reducing the cost of wastewater treatment and finding ways to produce useful products and energy from wastewater has been gaining importance in view of environmental sustainability [2]. One way to reduce the cost of wastewater treatment is to simultaneously generate bioenergy by utilizing the organic matter present in wastewater. Wastewaters generated by various industrial processes are considered to be the ideal substrates because they contain high levels of easily degradable organic material [15]. In the processes established so far, organic pollutants and wastes are converted into methane. Recently, developments of novel anaerobic processes aiming the conversion of organic pollutants into hydrogen, instead of methane gained more attention [18]. H_2 production by using wastewater as fermentative substrate connected to the simultaneous treatment of wastewater might be an effective way of tapping clean energy in a sustainable approach [19].

In the present study, a two-step biohydrogen production process was investigated using different types of microbial communities as starting inocula. Prior to the inoculation, selective enrichments of the bacterial populations were achieved by various physical and chemical pretreatments. In the first experimental step, glucose-rich environment was applied, while in the second experimental step, defined synthetic wastewater was used as fermentation substrate. The model system was investigated in anaerobic small scale batch reactors. Our aim was to determine the factors involved in the desired shift from the traditional biogas forming communities to an ecosystem favouring hydrogen evolution rather than methane formation. Thus, our specific goal was the elaboration of a novel method suitable for the selective elimination of methanogenic archaea thereby creating the real possibility for the

* email: boboescu.iulian@yahoo.com; *Tel.: 0040752668993*

simultaneous biohydrogen production and wastewater treatment.

Experimental part

Seed inocula

In order to design, create and continuously control a microbial consortium capable of efficient biohydrogen generation along with wastewater treatment, different types of anaerobic ecosystems - all rich in organic matter - were sampled for parent inocula. These parent cultures were as follows: wastewater from beer brewing industry (S1), sludge from a heavily organic polluted watercourse (S2), sludge from a methane producing bioreactor (S3) and activated sludge from a municipal sewage wastewater treatment plant (S4). These ecosystems represent high biodiversity and are composed of naturally formed microflora suitable for biodegradation of complex organic substrates.

Identifying the optimum pretreatment methods for various seed inocula in relation to the substrates used

So as to enrich the hydrogen producing bacteria and inhibit H_2-consuming methanogenic bacteria, four pre-treatment methods plus a control, were used for the inocula. The following pre-treatment methods were used: heating of the inocula at 70°C for one hour, acid pre-treatment bringing the *p*H down to 3 for 24 h at room temperature using 1N HCl, ultrasonication of the samples for 30 min at a discontinues discharge of 24 KHz (0.5 seconds discharge followed by 0.5 seconds pause) and a combination of all of the pre-treatments.

Bioreactor design and operation

The experimental setup was conducted in two different phases, first using a complete medium and second using synthetic wastewater as fermentation substrates. The reasons for the two-step fermentation experimental plan were firstly to enrich the bacterial communities resulted after the pre-treatment before the inoculation of the synthetic wastewater, as well as for the acclimatisation of the microorganisms to the fermentation conditions. All of the batch experiments were performed in triplicate.

One liter of the DMI medium contained 3.54 g of NH_4Cl, 6.72 g of $NaHCO_3$, 0.125 g of K_2HPO_4, 0.21 g of $MgCl_2 . 6H_2O$, 0.017 g of $MnSO_4 . 1H_2O$, 0.61 g of $Na_2S . 1H_2O$, 0.018 g of $FeSO_4 . 7H_2O$, 0.01 g of resazurin and 17.8 g of carbon source (glucose) as a substrate in 1 L distilled water. The enrichment and adaptation phase was conducted in 30 mL serum vials with 20 mL of DMI medium and 4 mL of pretreated sediment samples added as inocula. The bottles were capped with rubber septum stoppers and aluminium rings under anaerobic conditions inside an COY type (Toepffer Lab Systems) anaerobic chamber. Incubation was done at 30°C for a period of seven days at 150 rpm mixing speed.

Designed synthetic wastewater (SW) [(g/L) glucose—3.0, NH_4Cl—0.5, KH_2PO_4—0.25, K_2HPO_4—0.25, $MgCl_2 . 6H_2O$—0.3, $FeCl_3 . 4H_2O$—0.039, $NiCl_2 . 6H_2O$—0.077, $CoCl_2$—0.025, $ZnCl_2$—0.0115, $CuCl_2 . 2H_2O$—0.0133, $CaCl_2 . 2H_2O$—0.006 and $MnCl_2$—0.015] was used as substrate for H_2 production in the second experimental phase. The *p*H was adjusted to 6 using 1N HCl. The pretreated and enriched anaerobic mixed microflora was used as inoculum in small scale bioreactors (100 mL serum vials) using synthetic wastewater as substrate. After the enrichment, 10 mL (20%) inoculum was used to inoculate 50 mL of synthetic wastewater in 100 mL serum vials. The bottles were capped with rubber septum stoppers and aluminium rings under anaerobic conditions, inside the anaerobic chamber. Incubation was done at 30°C at 150 rpm mixing speed for a period of 15 days.

Analytical methods

Bacterial cell mass in each individual culture was determined by measuring optical absorbance (OD) with a Jenway 6320D Spectrophotometer at 600 nm. *p*H measurements were performed in every 48 h using a Thermo Scientific Orion 3-star benchtop pH meter.

Quantity and composition of headspace gas of the cultures were directly measured by gas chromatography using an Agilent Technologies 7890A GC system equipped with thermal conductivity detector and argon as a carrier gas. The temperatures of the injector, detector and column were kept at 30, 200 and 230°C, respectively. HP MolSieve column (15 m x 530 mm x 40 mm) was used. Since a concentration gradient of H_2 gas can be formed in the headspace, gas samples (0.5 mL) were taken with gas-tight syringe after mixing of the headspace gas by sparging several times.

Metabolic products in liquid phase (remaining sugars and ethanol in the medium) were analyzed with a Hitachi LaChrom Elite HPLC (High Performance Liquid Chromatography) equipped with a L-2490 Refractive Index Detector (temperature was set to 41°C) and a L-2350 column thermostat. L-2200 Autosampler was used for the injection of 40 µL sample. For the separation of compounds a Transgenomic ICSep COREGEL-64H (7.8 x 300mm) organic acid analysis column was used. The temperature of the column was set at 50°C. The elution was performed by a 0.01M H_2SO_4 solution with the constant flow of 0.8 mL . min^{-1}.

Results and discussions

Enrichment of seed inocula

As a result of the culture enrichment and adaptation performed in DMI medium, clear differences were observed in the hydrogen evolution rate of the different inocula (fig. 1). The bacterial populations sampled from the heavily organic polluted water course (S2) showed the highest hydrogen production rate in this experimental phase, compared to the bacterial communities originated from the further three sampling sites.

The pretreatments used for the enrichment step showed different effects on the hydrogen production rate of the

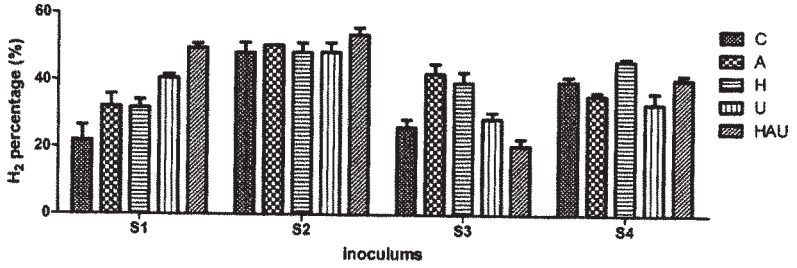

Fig. 1. Hydrogen ratio of the produced biogas at the end of the enrichment phase using various seed inocula (S1, S2, S3 and S4); **C**- control, **A**- acid pretreatment, **H**- heat pretreatment, **U**- ultrasonication pretreatment and **HAU**- combination of all the pretreatments

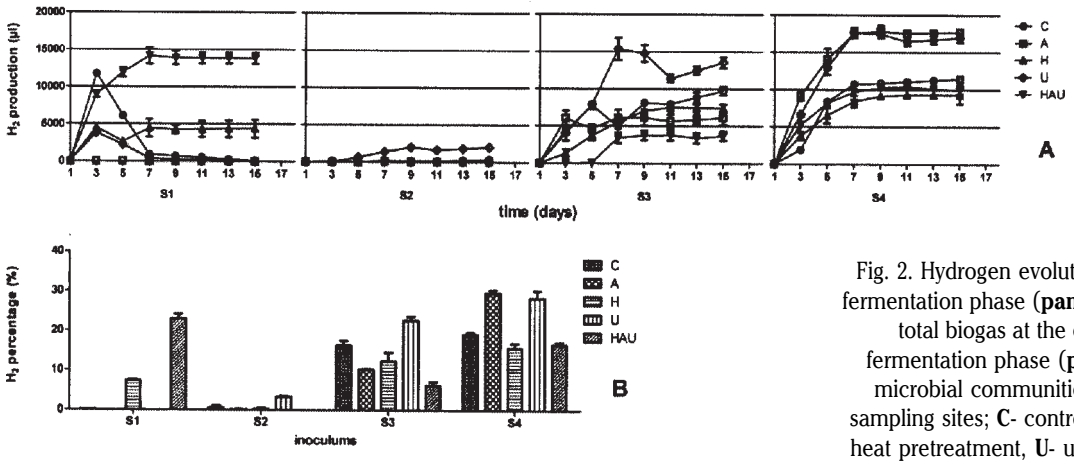

Fig. 2. Hydrogen evolution during the wastewater fermentation phase (**panel A**) and percentage of the total biogas at the end of the wastewater fermentation phase (**panel B**) produced by the microbial communities isolated from the four sampling sites; **C**- control, **A**- acid pretreatment, **H**- heat pretreatment, **U**- ultrasonication pretreatment and **HAU**- combination of all the pretreatments

various inocula used (fig.1). Generally, the pretreated inocula produced higher amount of hydrogen in comparison to the untreated controls. In the case of the bacterial communities sampled from S2, using a combination of all the pretreatments and only acid pretreatment generally resulted in a higher hydrogen concentration in the produced biogas (50.6 and 53.7% of the total biogas respectively). Applying a combination of all the pretreatments on the inoculum sampled from S1 generated similar hydrogen concentrations with a maximum of 49.6% hydrogen concentration of the total biogas produced. The bacterial populations of S3 subject to acid pretreatment showed the highest hydrogen concentration in the total biogas generated by this inoculum (42%), while in the case of the S4 inoculum heat pretreatment resulted in the highest hydrogen ratio in the total biogas (46%).

Fermentation and degradation of the synthetic wastewater

The microbial populations enriched in the first experimental phase using DMI medium as a fermentation substrate were used to inoculate the small scale bioreactors in the wastewater fermentation step (second phase). During this stage, clear differences were observed in the hydrogen evolution rate of the various inocula (fig. 2a). The enriched bacterial populations originated from S4 showed higher hydrogen production than microbial communities originated from S1, S2 and S3. The highest H_2 content of the produced biogas was observed in samples subject to acid pretreatment reaching a maximum of 29% H_2 (fig. 2b). In the case of the bacterial communities originated from S3 the overall H_2 content of the produced biogas is slightly lower with a maximum of 22% hydrogen of the total biogas produced, this value was observed after ultrasonication pretreatment. Regarding S1 and S2 inocula, only very low amounts of hydrogen were detected in the produced biogas, only the combination of all pretreatments on the S1 inoculum resulted in a hydrogen ratio of 23% of the total generated biogas.

Basic metabolites (glucose, succinate, lactic acid, formic acid, acetic acid, propionic acid, ethanol and butyric acid) were measured in the medium using HPLC during the batch experiments (fig. 3). The fermentative microenvironments of the small scale bioreactors showed significant differences depending on the inoculum types used to start up the bioreactors, as well as on the applied pretreatment methods. In the second phase the initial concentration of the glucose was 3 g/L. A high glucose consumption rate was associated with high hydrogen production yields in most of the cases. Both acid and ultrasonication pretreatments of S4 inoculum resulted in the highest hydrogen yields, the glucose was entirely consumed during the fermentation period (fig 3d).

Minor differences were detected in the ethanol concentration of the bioreactors inoculated with enriched bacterial communities originated from S1, S3 and S4, with an average of around 4 g/L ethanol concentration in the microenvironment (fig. 3a, 3c and 3d). Regarding the bioreactors containing inocula derived from S2 the ethanol concentration in the microenvironment fluctuated according to the different pretreatments used, ranging from a final concentration of 0.27g/L ethanol in the microenvironment in the case of heat pretreatment of S2 to a final concentration of 2.89 g/L of ethanol in the case of the control series of S2 (fig. 3b).

There were slight differences in the butyric acid levels between the four samples of different origins. The butyric acid levels were also dependent on the pretreatment methods. Generally, the butyric acid concentrations are higher in the bioreactors inoculated with S1, S3 and S4 inoculums (with a maximum butyric acid concentration of 1g/L in the case of ultrasonication pretreatment of S4 inoculum) compared to S2 inoculum, which showed a maximum butyric acid concentration of 0.54 g/L in the case of ultrasonication pretreatment (fig. 3).

The concentration of succinate, lactic acid, formic acid, acetic acid and propionic acid greatly differed according to the various inoculum types and pretreatments applied (fig.3). The highest lactic acid concentration of 1.52 g/L was measured in the bioreactors inoculated with bacterial communities originated from S2 subject to a combination of all the pretreatments. The highest acetic acid concentration was measured in the case of the control series as well as the heat and ultrasonication pretreatments of S1 (with a value of 0.9 g/L). The propionic acid concentration in the bioreactors were hardly detectable, only the control series and acid pretreated S1 samples showed moderate concentrations (1.07 g/L and 0.62 g/L, respectively). The levels of succinate and formic acid were negligible in all of the bioreactor microenvironments.

The *p*H values were recorded throughout the wastewater experimental phase (fig.4). The starting *p*H value was 6. A permanent decrease in the *p*H was measured during the fermentation, regardless the pretreatment methods and various inocula used. However, slight differences could be observed in the final *p*H value between the samples. The highest *p*H values were measured in the microenvironments of the bioreactors

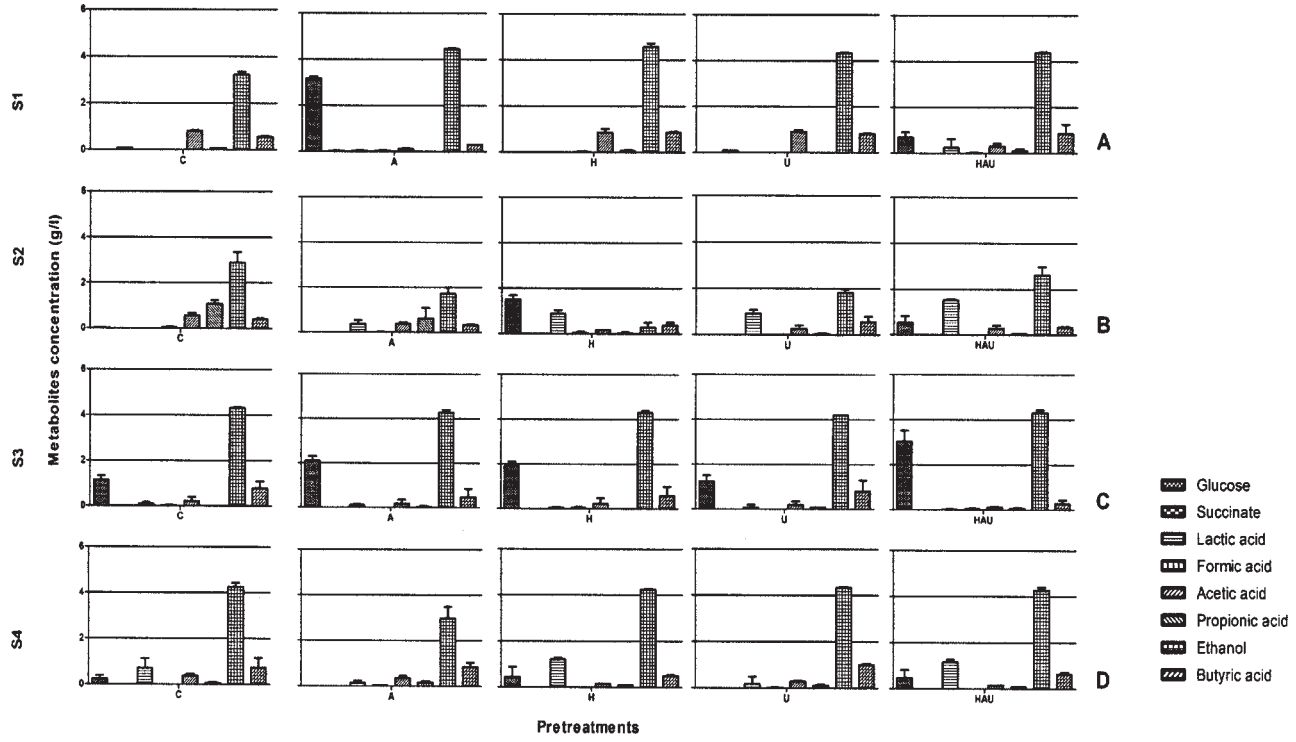

Fig. 3. Glucose and metabolites concentration measured at the end of the wastewater fermentation phase. Panel A- **S1**; panel B- **S2**; panel C- **S3** and panel D- **S4**. **C**- control, **A**- acid pretreatment, **H**- heat pretreatment, **U**- ultrasonication pretreatment and **HAU**- combination of all the pretreatments

inoculated with microbial populations sampled from S1 and S3, reaching a final value of around 4 at the end of the experiments. In the case of inocula sampled from S2 and S4, the final *p*H values were detected around *p*H 3.5. Regarding the different pretreatments of the inocula, the utilization of a combination of all the pretreatments on the bacterial populations from S2 as well as from S4 resulted in the most acid microenvironment reaching a final *p*H value of 3.15 and 3.12, respectively. The most basic microenvironment was measured in the case of the S3 sample pretreated with the combination of all used methods reaching a final *p*H value of 4.65 at the end of the wastewater experimental phase.

Even though all of the four parent inocula used to start the bioreactors evolved hydrogen during both experimental phases, significant differences were observed in the amount and kinetics of hydrogen production. Differences could be detected between samples of different origins, different pretreatments and also between the two experimental phases.

During the enrichment and adaptation of the bacterial populations clear differences were noticed in the hydrogen yields generated by the various parent inocula. The highest hydrogen concentration in the total biogas was observed in samples containing S2 inoculum. This phenomenon might be explained by the fact that S2 represented a natural habitat with a highly diverse microbial community able to form complex ecological associations regardless the applications of various pretreatment methods. These complex microbial communities are able to degrade the organic substrates rapidly leading to a fast conversion of the carbon source to biogas. The bacterial populations enriched in samples originated from S1, S3 and S4 resulted in slightly lower hydrogen evolution rates, one reason for this might be the higher specificity of the sampling habitats possessing lower microbial biodiversity under the particular conditions. This lower microbial diversity enables slower adaptation to the changing ecological conditions provided by the various pretreatment approaches, by the fermentation substrates and physico-chemical growth parameters.

The pretreatment methods resulted in different responses provided by the different inocula. Again, this can be explained by the very different ecological conditions of each sampled habitat. It can be concluded that there is no general pretreatment, enrichment method suitable for the enrichment of hydrogen producing bacteria by the inhibition of the methanogens. Thus, all types of environments (microbial communities) must be handled in a unique way. In some cases the untreated inocula produced more hydrogen than the pretreated ones, indicating that in some cases fine adjustment of the microenvironment conditions might be enough for the required changes of the bacterial populations, for the transition of a predominantly methanogen community to a hydrogen evolving bacterial population. This can greatly decrease the operation costs of an industrial scale hydrogen producing bioreactor in the future.

In the second experimental phase, using synthetic wastewater as fermentation substrate, the hydrogen evolution behaviour was strikingly different compared to the first phase, when carbon rich DMI medium was used as fermentation substrate. The bioreactors inoculated with the enriched S2 bacterial communities showed the lowest hydrogen evolution rate, while the bioreactors inoculated with enriched S4 microbial communities generated the highest concentration of hydrogen in the total produced biogas (fig. 2). The pretreatment methods also resulted in very different hydrogen producing capabilities of the various inocula compared to the first experimental phase. All the differences can be explained by the characteristics of the synthetic wastewater, which represents a micro-environment offering a very strict and narrow ecological niche. This selectively affects the microbial biodiversity and the response of the various systems. Thus, the microbial populations sampled from S2, which generated

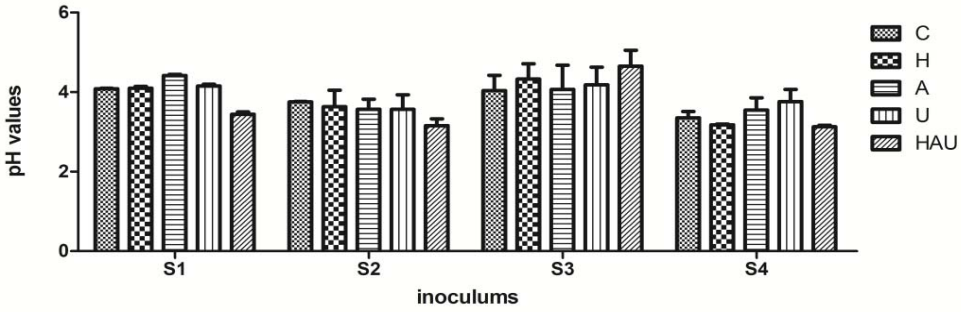

Fig. 4. pH values measured at the end of the wastewater fermentation phase; **C**- control, **A**- acid pretreatment, **H**- heat pretreatment, **U**- ultrasonication pretreatment and **HAU**- combination of all the pretreatments

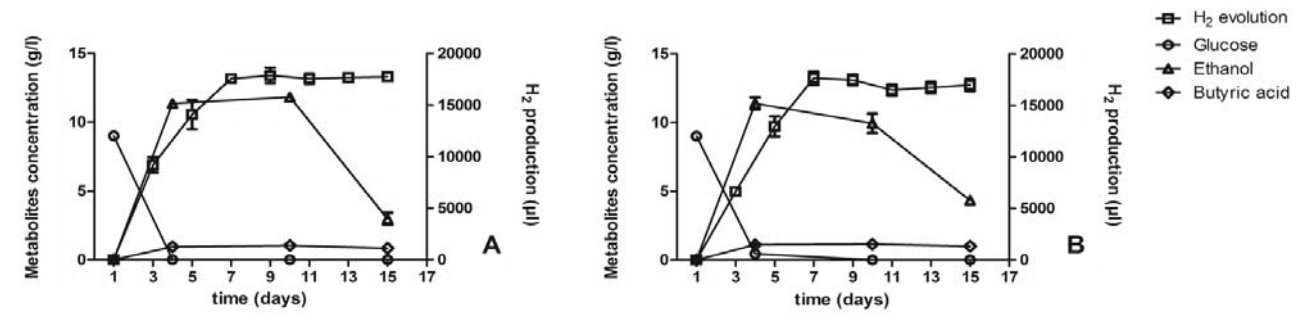

Fig. 5. Hydrogen evolution rate, glucose consumption,ethanol and butyric acid production during the wastewater fermentation phase, by the acid pretreatment (panel A) and ultrasonication pretreatment (panel B) of the microbial communities sampled from S4

high amount of hydrogen during the enrichment phase in the presence of a carbon-rich substrate, were not able to efficiently adapt to the strict parameters (low carbon source) offered by the synthetic wastewater used as sole substrate. On the other hand, the bacterial communities originated from S4, which generated moderate amounts of hydrogen during the enrichment phase, adapted very well to the new microenvironment in the synthetic wastewater phase (fig. 5). This might be explained by the higher similarity of the S4 samples original environmental conditions to the conditions presented by the synthetic wastewater.

As expected, glucose consumption values were directly correlated with hydrogen evolution rates. This implies that certain hydrogen evolving microbial communities are capable of fast and complete substrate degradation with simultaneous hydrogen production (fig. 5). These observations open the way for designing specifically constructed microbial communities for the dual purpose of wastewater treatment and renewable energy generation in the form of hydrogen. The butyric and acetic acid concentrations also directly correlated with hydrogen production in both experimental phases (fig. 5). The highest concentrations of these metabolites were measured in samples showing the highest hydrogen production indicating that the acetate and butyrate fermentations might be important metabolic pathways operated by the hydrogen evolving populations. Also as expected, lower hydrogen concentrations were associated with higher levels of propionate and reduced end-products such as ethanol and lactic acid. In these cases the metabolic pathways adopted by the microbial communities preferred the formation of these rather reduced metabolites. Monitoring these metabolites provides us useful hints on the various metabolic pathways possibly utilized by the microbes for hydrogen production. Thus, it can results in knowledge essential for designed interventions, adjustments and optimization of the fermentation process.

Conclusions

A two-phase method for obtaining enriched bacterial communities suitable for simultaneous biohydrogen production and efficient wastewater treatment was used. As a conclusion, each type of fermentation substrate used for biohydrogen production requires specific type of parent inoculum for effectively igniting the biohydrogen generation process. Thus, an increased attention has to be paid on the complex microbial composition and on the ecological relationships of the selected inoculum in order to obtain the most efficient substrate degradation combined with the highest possible hydrogen evolution rate.

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