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EFFECTS OF HEAT TREATMENT ON MICROBIAL COMMUNITIES OF GRANULAR SLUDGE FOR BIOLOGICAL HYDROGEN PRODUCTION

--Manuscript Draft--

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Abstract:	Dark fermentation shares many features with anaerobic digestion with the exception that to maximize hydrogen production, methanogens and hydrogen consuming bacteria should be inhibited. Heat treatment is widely applied as inoculum pre-treatment due to its effectiveness in inhibiting methanogenic microflora but it may not exclusively select for hydrogen producing bacteria. This work evaluated the effects of heat treatment on microbial viability and structure of anaerobic granular sludge. Heat treatment was carried out on granular sludge at 100°C with four residence times (0.5, 1, 2 and 4 hours). Hydrogen production of treated sludges were studied from glucose by means of batch test at different pH values. Results indicated that each heat treatment strongly influenced the granular sludge resulting in microbial communities having different hydrogen productions. The highest hydrogen yields (2.14 mole of hydrogen per mole of glucose) were obtained at pH 5.5 using the sludge treated for 4 hour characterized by the lowest CFU concentration (2.3 x 103 CFU/g sludge). This study demonstrated that heat treatment should be carefully defined according to the structure of the sludge microbial community allowing the selection of highly efficient hydrogen producing microbes.

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DIPARTIMENTO DI AGRONOMIA ANIMALI ALIMENTI
RISORSE NATURALI E AMBIENTE
DAFNAE
DEPARTMENT OF AGRONOMY FOOD NATURAL RESOURCES ANIMALS AND ENVIRONMENT

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Ref.: **Ms. No. WST-WSTWS-EM111401**
EFFECTS OF HEAT TREATMENT ON MICROBIAL COMMUNITIES OF GRANULAR SLUDGE FOR BIOLOGICAL HYDROGEN PRODUCTION.
by Alibardi A. et al.

Dear Editor,

Thank you very much for the suggestions related to our manuscript No. WST-WSTWS-EM111401.

We proceeded to modify the text according to the indications of the Reviewers. A file with the answers, point by point, to the comments/questions of the Reviewers is contextually submitted with the revised version of the manuscript.

Please, note that the amended text contains several sections in red typing in order to make the changes more evident.

Thank you in advance for your consideration

Best regards

Sergio Casella

Reviewer #1:

The aim of the work as stated and as was made to understand to the reader in the introduction was to see whether heat treatment that aims in eliminating hydrogen consumers is selective enough to support all the hydrogen producers. The authors hypothesized that in this selection process the non spore forming hydrogen producers will also get inactivated and thus instead of higher hydrogen production, it might actually decrease the hydrogen production. However based on the methodology and the results that was presented in the manuscript, the authors have achieved higher hydrogen production with heat treatment and they could not provide a proper justification by their initial hypothesis failed. The 16S sequencing work that they have done was from only the cultivable microflora that does not give a proper representation of the actual microbial diversity of the sludge. This is a good piece with a good hypothesis. But the justification provided and the methodology used was not proper. Thus in my understanding the manuscript is not ready for publication.

Response: In this study, we demonstrated that heat treatment should be carefully defined according to the structure of the sludge microbial community allowing the selection of highly efficient hydrogen producing microbes. In other words, we wanted to define the most appropriate heat treatment time to achieve the highest hydrogen yields obtainable by culturable bacteria. The Reviewer states that "The authors hypothesized that in this selection process the non spore forming hydrogen producers will also get inactivated and thus instead of higher hydrogen production, it might actually decrease the hydrogen production.". However, we must clarify that our aim was a little different and the above hypothesis was only one among others. In this specific case, our initial hypothesis was "to verify if" the non-spore forming hydrogen producers will get inactivated by heat treatments, so affecting the final hydrogen production. Indeed, in the Introduction we reported that "Although heat treatment aims to kill methanogens, it does not select exclusively for hydrogen producing bacteria. Therefore, while spore-forming H_2-consuming bacteria can survive, non-spore forming hydrogen-producers may be inactivated." As a consequence, a sort of balance between "non-spore forming H_2 consumers" and "non-spore forming H_2 producers" that have been affected by the heat treatment is the crucial point making the real difference. The various time treatments used were specifically applied to find the optimal treatment to make this balance as positive as possible. The same concept can be applied also for the spore forming bacteria.
In the Results and Discussion, we have reported that, after a short heat treatment, non-spore forming hydrogen-producers were inactivated. As a result, rather than increasing H_2 production, 1 h heat treatment has actually negatively affected H_2 yield (Page 9, lines 284-300). However, as reported in Table 3, longer heat treatments greatly enhanced hydrogen productions.
We agree with the reviewer about the fact that 16S sequencing work done in this study only on the culturable microbes does not give a proper representation of the actual microbial diversity of the sludge. However, since this work was focused on the heat-treatment of granular sludge to start the development of microbial inoculants to be used for the conversion of organic wastes into hydrogen, we wanted to select for culturable bacteria.
To better explain why in this work we limited our research to the culturable bacterial populations, the following sentence has been added in the Introduction (Page 2, lines 90-92):

"This work evaluated the potential of heat treatment as enrichment strategy of granular sludge to select H_2-producing microbial consortia with promising yields, even in view of the future development of efficient microbial inoculants."

Nevertheless, to better understand the microbiological and biochemical aspects of using the heat-treated sludges to produce hydrogen, the study of the whole microbial community structure by means of DNA-based techniques is in progress and will be the matter of a future manuscript.

Reviewer #2

Effects of heat treatment on microbial community of granular sludge for biological hydrogen production

The present work is a well written and present interesting data about the application of "dark fermentations" and production of alternative energy sources.

The used methods are adequate and the results well-presented and discussed.

I would like to recommend the present work to be accepted for publication in Water Science and Technology after minor revisions.

I would like to recommend to the authors to check again entire manuscript for the style of the Water Science and Technology.

Response: The manuscript has been entirely checked for the style of the Water Science and Technology Journal.

However, I have some questions and remarks, which in my opinion will make the manuscript more clear.

1) Can you please explain better the statement from page 2, line 58-59. Why CO_2 from biogenic sources not contributing to the greenhouse effect?

Response: During the biological production of hydrogen by fermentation process, bacteria produce a mixture of gas composed by hydrogen and carbon dioxide as by-product of the metabolic process of degradation of organic substances. This carbon dioxide emission is considered carbon neutral (thus not contributing to the green house effect) because it is derived from organic substances produced by fixing atmospheric CO_2 from a photosynthetic process (biogenic), therefore with a very short carbon cycle (some month, if the growth of a crop is considered).

On the contrary the production of hydrogen based on the utilization of fossil fuel (steam reforming or thermal cracking of natural gas, coal gasification, electrolysis of water or other thermochemical processes based on the utilization of energy from fossil fuels) is not carbon neutral due to the emission into the atmosphere of carbon dioxide that was trapped millions of years ago as fossil carbon.

To better explain this concept, the sentence (Page 2, lines 58-60) has been reinforced as following:

"In this perspective, biological hydrogen production processes represent a sustainable opportunity. In fact, hydrogen producing bio-processes involve CO_2 emissions, but from biogenic sources, thus not contributing to the green house effect due to the brevity of the carbon cycle (Hellenbeck, 2009)".

2) Please, when you start sentences, avoid use of numbers or chemical formulas (see page 2, line 60, etc.).

Response: the text has been amended accordingly throughout the text.

3) On page 3, line 113, you can introduce the abbreviation for Nutrient Agar as "NA" and use later in the manuscript.

Response: the text has been amended accordingly throughout the text.

4) On page 3, line 115, please replace "were recorded" with "were determined". Word "recorded" is used for the directly obtained numbers, when you calculate (like in case of CFU), the correct word is "determined".

Response: the text has been amended accordingly throughout the text.

5) On page 3, lime 117, please replace "picked" with "collected".

Response: the word "picked" has been changed with "collected" according to the reviewer's suggestion.

6) On page 3, line 128, please replace "checked" by "visualized"

Response: the word " checked " has been changed with "visualized" according to the reviewer's suggestion.

7) On page 3, line 131, please, specify if the 96% is sufficient for the taxonomic identification. Several authors use 98% as a breaking point for the identification purposes.

Response: For taxonomic identification, different similarity percentage values have been used in literature: from 95 to 100% (see Mazzon et al. (2008) Presence of specific symbiotic bacteria in flies of the subfamily Tephritinae (Diptera Tephritidae) and their phylogenetic relationships: proposal of '*Candidatus* Stammerula tephritidis'. Int. J. Syst. Evol. Microbiol., 58 (2008), pp. 1277–1287).
In this study, the similarity level considered for taxonomic attribution was always above 98% with the exception of two cases in which the similarity were found to be 96%. For this reason, we have reported in the Material and Methods that "A minimum sequence similarity level of 96% was considered for taxonomic attribution". However, the sentence has been modified as following (Page 3, lines 134-135) in order to improve the clarity:

"A minimum sequence similarity level of 96% was considered for taxonomic attribution, although the majority of the isolates showed a similarity level higher than 98%."

8) On page 3, lines 152-153. Last sentence needs to be rephrased.

Response: The sentence has been adjusted as following to improve the clarity (Page 4, Lines 154-157):

"*Biogas composition in terms of hydrogen, carbon dioxide and methane were measured by a gas chromatograph (HP5890) equipped with thermal conductivity detector (TCD), HP- MOLSIV and HP-PLOT U columns, nitrogen as carrier gas."*

9) On page 4, line 173, please replace "triplicate experiments" with "3 repetitions".

Response: the word " triplicate experiments" has been changed with "3 repetitions" according to the reviewer's suggestion.

10) On page 4, table 1. In my opinion will be better to present this table in logarithmic form.

Response: We have proposed Table 1 not in logarithmic form because, in our opinion, the reader can better appreciate the differences between the CFU values. As a consequence, we propose Table 1 to remain as it is.

11) On page 5, table 2. Please explain the values obtained for *Bacillus flexus* at 1h (3.8) and for *Brevibacterium brevis* at 1h (11.5). Why these bacteria are present after 1h treatment, but not after 0.5h? Is this related with an ecological interaction between species presented in this system? This is an interesting result and deserves a better discussion. Spelling of *Shinella* sp. is correct?

Response: The fact that *Bacillus flexus* and *Brevibacterium brevis* were not detected in the sludge heat-treated for 0.5 hour should not be considered surprising. The above bacterial species were not detected in both non-treated and 0.5 h heat-treated sludges because their population sizes were much lower than those detected at 10^{-6} dilution level (see Table 1), used for the plate counting method. Once the majority of the other bacterial species was killed by 1 h heat treatment, *Bacillus flexus* and *Brevibacterium brevis*, evidently able to tolerate the longer heat stress, can be easily detected at lower dilution levels (10^{-4} instead of 10^{-6}).

The spelling of the genus *Shinella* has been checked and it is correct.

Reviewer #3:

Review of manuscript number WST-WSTWS-EM111401, "Effects of heat treatment on microbial communities of granular sludge for biological hydrogen production".

The paper reports on the effect of the duration of heat treatment (100°C) on hydrogen production in batch culture and on the viability and microbial community structure. Different durations of treatments were used - 0.5, 1, 2, 3 and 4 hours. It was found that the best hydrogen production yield and the lowest diversity of the culture occurred when the granular sludge was treated for 4 hours. The paper makes a useful contribution with regard to the methodology needed to select for hydrogen formers. Before the paper is published I would recommend the following modifications:

1) In the abstract, it is important to mention that the best hydrogen yield was obtained when the sludge was heat treated for 4 hours. I believe it is erroneous to say , "the lowest amounts of microbes produced the highest hydrogen levels?". In the methods (page 3) the food to microorganism level was kept constant.

Response: We thank the reviewer for the suggestion about the abstract. Reviewer is right saying that the F/M ratio was kept constant for all the experiments. We would like to specify that the F/M ratio is based on the volatile solids of glucose and volatile solids of treated granular sludge. The heat treatment did not effect the Total Solids (TS) and Volatile Solids (VS) concentrations of the sludge as well as its chemical characterization. On the contrary, heat treatment strongly affected sludge microbial viability (see Table 1). Therefore, the sludges treated at different durations have the same value of VS (and therefore all experiments had the same F/M ratio) but different CFU concentrations. In other words, the same amount of VS brings to the batch different proportions of living microorganisms (those able to make hydrogen) and heat-killed bacteria.

To clarify this aspect in the paper, the paragraph regarding the brief presentation of the results in the abstract has been changed as following:

"Results indicated that each heat treatment strongly influenced the granular sludge resulting in microbial communities having different hydrogen productions. The highest hydrogen yields (2.14 mole of hydrogen per mole of glucose) were obtained at pH 5.5 using the sludge treated for 4 hour characterized by the lowest CFU concentration (1.5 x 10^2 CFU/g sludge)."

Moreover, in the section "Batch test for hydrogen production" of Material and Methods, the paragraph (Page 3, lines 148-150) has been modified as following:

"Food to microrganism ratio (F/M) was set at 1 gVS/gVS. Since heat treatment did not change the TS and VS of the sludge, every reactor was inoculated with 10 grams of treated or non-treated sludge."

2) In Table 3, indicate whether the hydrogen produced is normalised to glucose consumed or added. Also indicate the number of measurements that make up the average.

Response: The data about hydrogen production are normalised to added glucose. Each value reported in Table 3 is the average of 3 replicates. The caption of Table 3 has been revised accordingly as following:

*"**Table 3.** Average cumulative hydrogen productions (±SD, n=3), hydrogen yields and mathematical model results. Hydrogen productions are normalized to glucose added to batch tests."*

3) In Table 2, it is very surprising that no Clostridial species were found upon analysis. Could the authors confirm if all the data has been presented.

Response: Table 2 shows all the results we obtained from non-treated and treated sludge samples. We agree with the reviewer about the unexpected absence of *Clostridium* sp. strains in the granular sludge. In the literature, it is reported that the microbial species involved in bio-hydrogen production are mainly *Clostridium* sp. and, in lower extent, *Bacillus* sp. strains (Kapdan I.K. and Kargi F. 2006. Bio-hydrogen production from waste materials. Enzyme Microb. Technol., 38, 569-582.

However, in this study *Clostridium* sp. strains were not detectable in the anaerobically incubated plates resulting from the non-treated granular sludge. Since Clostridia are anaerobic, gram positive bacteria, able to resist to heat shocks by forming spores, the fact that after long heat-treatments, no *Clostridium* strains have been detected from the granular sludge seems to confirm that the sludge used in this study had no *Clostridium* species or alternatively the *Clostridium* sp. population size was always under the detection limit of the plate counting method (see response to the second reviewer). In future studies, DNA-based molecular techniques (i.e., DGGE-PCR) will be applied to confirm both hypothesis and to further study the peculiar microbial structure of the granular sludge used in this work.

Nevertheless, the microbial community of the non-treated sludge revealed to be quite similar to those of other previously studied granular sludges at least at phylum level (Narihiro *et al.*, 2009). This concept was already discussed at Page 4 (lines 201-204).

4) Is it possible to include pH as a variable in equation 1?

Response: We thank the reviewer for the question. It is not possible to include the pH in the equation [1]. The equation reported in the article was used only to compare the cumulative hydrogen productions from the different tests with a mathematical formula that represents the best fitting of the experimental data.

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On the contrary, pH and heat treatment duration were evaluated in the two ways factorial ANOVA. The interaction between pH and thermal duration was found to be statistically significant (Page 8, line 274).

5) A comparison of the best results in this study and results from other studies in the literature should be made.

Response: We thank the reviewer for the comment. In the original version of the paper, the references Fang et al., (2002) and Kapdan and Kargi, (2006) were used in the text to compare the data obtained in this study with other results reported in the scientific literature.
In the current version of the manuscript, Table 4 has been added according to the reviewer's suggestion and the following sentence has been inserted in the Results and Discussion section (Page 8, lines 275-277).

"The yield of 2.14 moles of hydrogen per mole of glucose obtained at pH 5.5 with the 4 hour treated sludge seems to be promising if compared to those previously reported from glucose (Table 4)."

Table 4. Comparison of hydrogen yields from different studies. All the data regard batch experiments using glucose as substrate and mixed culture as inoculum.

Reference	Inoculum	Inoculum pre-treatment	Fermentation temperature	Fermentation pH	Hydrogen yields (mol H_2/mol glucose)
This study	UASB granular sludge	heat treatment (100°C, 4 h)	35°C	5.5	2.14
This study	UASB granular sludge	heat treatment (100°C, 2 h)	35°C	5.5	1.82
Davila-Vazquez *et al.* (2008)	UASB granular sludge	heat treatment (100°C, 40 min)	37°C	7.5	1.46
Mu *et al.* (2006)	waste activated sludge	anaerobic storage for 6 month and heat treatment (85°C, 1h)	35°C	5.5	1.00
Mu *et al.* (2006)	waste activated sludge	anaerobic storage for 6 month and heat treatment (85°C, 1h)	41°C	5.5	1.67
Morimoto *et al.* (2004)	sludge compost	aerobic conditions (60°C, 3 days)	60°C	3.97*	2.1
Morimoto *et al.* (2004)	anaerobic pond sludge	-	50°C	4.02*	1.6
Morimoto *et al.* (2004)	compost	-	60°C	-	1.5
Logan *et al.* (2002)	soil used for tomato plants	heat treatment (104°C, 2 h)	26°C	6	0.92
Fang and Liu (2002)	anaerobic sludge from a CSTR reactor	-	36°C	5.5	2.1

* final pH

The following references have been added :

Morimoto M., Atsuko M., Atif A.A.Y., Ngan M.A., Fakhru'l-Razi A., Iyuke S.E., Bakir A.M. (2004). Biological production of hydrogen from glucose by natural anaerobic microflora. Int. J. Hydrogen Energy, 29, 709-713.

Mu Y., Zheng X.J., Yu H.Q., Zhu R.F. (2006). Biological hydrogen production by anaerobic sludge at various temperatures. Int. J. Hydrogen Energy, 31, 780-785.

Logan B.E., Oh S.E., Kim I.S., Van Ginkel S. (2002). Biological hydrogen production measured in batch anaerobic respirometers. Environ. Sci. Technol., 36, 2530-2535.

Fang H.H.P., Liu H. (2002). Effect of pH on hydrogen production from glucose by a mixed culture. Bioresour. Technol., 82, 87-93.

Davila-Vazquez G., Alatriste-Mondragon F., de Leon-Rodriguez A., Razo-Flores E. (2008). Fermentative hydrogen production in batch experiments using lactose, cheese whey and glucose: influence of initial substrate concentration and pH. Int. J. Hydrogen Energy, 33, 4989-4997.

6) Data on glucose consumption and fermentation metabolites should be provided. A COD balance should also be done.

Response: Residual glucose concentration of Hydrogen Production Medium (HPM) was measured after 8 days of incubation using the peroxidase-glucose oxidase method from the D-Glucose assay kit (Boehringer Mannheim). In all batch tests, glucose was not detectable (< 80 mg/l).
The manuscript has been revised according to the reviewer's suggestions. In the section of "Batch test for hydrogen production" of Material and Methods, the following sentence has been added (Page 4, lines 158-159):

"After 8 days of incubation, residual glucose in the HPM medium was measured using the peroxidase-glucose oxidase method from the D-Glucose assay kit (Boehringer Mannheim)."

Moreover, in the Results and Discussion (Page 6, lines 243-244), the following sentence has been inserted:

"After 8 days of incubation, glucose was completely depleted in all the batch tests"

Data about metabolites and COD balance were not reported because this paper aimed at the definition of a proper granular sludge heat treatment to obtain the highest hydrogen yields from the resulting microbial populations.
Results showed that heat treatment has reduced the size of microbial population of treated sludges, selecting the bacteria having the best hydrogen production performances at the pH of 5.5.
These strains (both as pure cultures and microbial consortium) will be evaluated in further studies to analyse their fermentative performances from glucose and other substrates. We believe that data about metabolites represent a more interesting information if they are related to a specific population, as different microbial populations can perform differently depending by the operational conditions.
We agree with the reviewer on the fact that the COD balance is a powerful tool to evaluate the

performances of fermentation process. We also believe that a COD balance is an essential tool whether complete modelling (biomass growth yields, hydrolysis and uptake rates, products and by-products formation) of biological processes is done using data from experimental tests. Therefore, in the present work, we decided to evaluate only cumulative hydrogen productions using the same equation (Equation [1]) to compare the different results. Moreover, no data about the biomass growth yields and consumption rates have been reported because the inoculum utilized for the batch test was not only composed of living (active) biomass due to the effect of heat treatment. In fact, as already answered to question n°1, the heat treated sludges had the same VS but not the same amount of viable organisms.

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Title: EFFECTS OF HEAT TREATMENT ON MICROBIAL COMMUNITIES OF GRANULAR SLUDGE FOR BIOLOGICAL HYDROGEN PRODUCTION

Authors: Luca Alibardi[1], Lorenzo Favaro[2], Maria Cristina Lavagnolo[1], Marina Basaglia[2], Sergio Casella[2*]

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Abstract

Dark fermentation shares many features with anaerobic digestion with the exception that to maximize hydrogen production, methanogens and hydrogen consuming bacteria should be inhibited. Heat treatment is widely applied as inoculum pre-treatment due to its effectiveness in inhibiting methanogenic microflora but it may not exclusively select for hydrogen producing bacteria. This work evaluated the effects of heat treatment on microbial viability and structure of anaerobic granular sludge. Heat treatment was carried out on granular sludge at 100°C with four residence times (0.5, 1, 2 and 4 hours). Hydrogen production of treated sludges were studied from glucose by means of batch test at different pH values.
Results indicated that each heat treatment strongly influenced the granular sludge resulting in microbial communities having different hydrogen productions. The highest hydrogen yields (2.14 mole of hydrogen per mole of glucose) were obtained at pH 5.5 using the sludge treated for 4 hour characterized by the lowest CFU concentration (2.3 x 10^3 CFU/g sludge). This study demonstrated that heat treatment should be carefully defined according to the structure of the sludge microbial community allowing the selection of highly efficient hydrogen producing microbes.

Keywords: bio-hydrogen; dark fermentation; granular sludge; heat treatment; microbial communities.

Introduction

Increasing attention is currently being focused on hydrogen, in an attempt to reduce the consumption of fossil fuels and their impact on climate changes. Hydrogen may be considered a truly clean energy source only if the emission of greenhouse gasses is avoided, even during the production phase. In this perspective, biological hydrogen production processes represent a sustainable opportunity. In fact, hydrogen producing bio-processes involve CO_2 emissions, but from biogenic sources, thus not contributing to the green house effect due to the brevity of the carbon cycle (Hellenbeck, 2009).

Hydrogen is biologically produced by means of two microbial processes: photosynthesis and anaerobic fermentation. Hydrogen production by fermentative bacteria, not light dependent, is known as "dark fermentation" and takes place during the fermentative phase of anaerobic digestion. Dark fermentation represents not only an energy production process but also a first stage of stabilization for organic substrates since it degrades complex organic matter to readily biodegradable compounds (volatile fatty acids and alcohols) suitable for methane production by anaerobic digestion (Kapdan and Kargi, 2006).

During anaerobic digestion, hydrogen is formed as an intermediate product with H_2 producers and H_2 consumers microbes working together to finally produce methane. In order to maximize hydrogen production via dark fermentation, methanogens and hydrogen consuming bacteria should be inhibited. Moreover, optimal process conditions, as type and pre-treatment of inoculum, pH, temperature and substrate characteristics, should be defined in order to promote the metabolic pathways resulting in hydrogen production (Liu and Fang, 2002).

Anaerobic sludges collected from full scale digesters are frequently reported to be used as inoculum for hydrogen production and their pre-treatments are essential for the inhibition of methanogenic microbial species. Several methods have been proposed to achieve this aim, including heat treatment, acidification, basification, freezing or dehydration (Van Ginkel and Sung, 2001; Ting *et al.*, 2004; Kawagoshi *et al.*, 2005; Tommasi *et al.*, 2008).

Heat treatment is widely applied due to its effectiveness on methanogenic inhibition. Different temperatures and residence times should be defined according to the structure of the microbial community in the sludge (Alibardi *et al.*, 2009). Temperature values reported in literature range from 50° to 105° C while residence times are described to be efficient from 30 to 300 minutes (Lay *et al.*, 1999; Kapdan and Kargi, 2006).

Although heat treatment aims to kill methanogens, it does not select exclusively for hydrogen producing bacteria. Therefore, while spore-forming H_2-consuming bacteria can survive, non-spore forming hydrogen-producers may be inactivated. Rather than increasing H_2 production, heat treatment may actually negatively affect H_2 yield (Kraemer and Bagley, 2007).

There is little information about the effects on microbial viability and hydrogen productivity of the inoculum due to increasing heat treatment durations and particularly on the use of temperature to enrich granular sludge of efficient H_2-producing bacteria. This work evaluated the potential of heat treatment as enrichment strategy of granular sludge to select H_2-producing microbial consortia with promising yields, even in view of the future development of efficient microbial inoculants. The effects of heat treatment were evaluated on the microbial viability and community structure of granular sludge. The hydrogen production performances of treated sludges were studied by means of batch test at different pH values. Hydrogen productions were assessed also using a mathematical model applied to cumulative hydrogen productions.

Materials and methods

Sludge pre-treatment

Granular sludge used as inoculum for batch test was collected from a full scale Upflow Anaerobic Sludge Blanket (UASB) anaerobic digester treating wastewater of a brewery factory located in

Padova, Italy. Total solids (TS) and volatile solids (VS) of granular sludge were 16% and 76%, respectively. Total organic carbon (TOC), total kjeldahl nitrogen (TKN) and total phosphorus were 30%, 3.8%, 0.1% refereed to TS, respectively. TS, VS, TKN and total phosphorous were analysed according to standard methods (APHA, 1999). Organic carbon was quantified using a Total Carbon Analyzer (TOC-V CSN, Shimadzu).
Heat treatment was carried out on granular sludge in a rotary water bath incubator at a fixed temperature of 100°C with four increasing residence times (0.5, 1, 2 and 4 hours). After each treatment, samples of sludge were used as inoculum of batch test for hydrogen production.

Microbiological analyses

To study the effects of heat treatment on the microbial viability of inoculum, samples of heat treated and non-treated sludges were dispersed into an aqueous suspension by vortexing in sterile 0.9% NaCl solution for 5 minutes. The suspensions were serially diluted, plated on Nutrient Agar (NA) medium (pH 7.0, Sigma) and incubated at 37°C under aerobic and anaerobic conditions for 5 days after which CFU (Colony Forming Units) were determined. Anaerobic conditions were obtained in anaerobic jars (OXOID) flushed with N_2 gas. Experiments were conducted in triplicate.
From each sampling, about 60 colonies were randomly collected from plates, purified by streaking twice and stored as stock cultures in 20% (v/v) glycerol at -80° C for their genetic identification as described below.

Genetic identification of microbial strains

Bacterial strains, aerobically and anaerobically isolated as reported before, were genetically identified by 16S rDNA sequencing. Genomic DNA was extracted as follows: a small colony of each strain, grown for 24 h on NA plates, were picked up with a sterile toothpick and resuspended in 50 μL of lysis solution (0.05 M NaOH, 0.25 % SDS). The suspension was heated at 94 °C (15 min) and then centrifuged (10,000 x g, 15 min).
Prokaryotic small rDNA subunits were amplified using bacterial universal primers 1389r and 63F as described in Hongoh *et al.* (2003). Amplification products were visualized by agarose gel electrophoresis and then subjected to sequencing. Species identification was done after BLASTN alignment (NCBI, 2011) of the obtained sequences with those present in the GenBank public database. A minimum sequence similarity level of 96% was considered for taxonomic attribution, although the majority of the isolates showed a similarity level higher than 98%.

Batch test for hydrogen production

Batch reactors, 0.5 litre Pyrex vessels, were filled with 250 ml of hydrogen production medium (HPM) contained glucose (5 g/l) and yeast extract (3 g/l) (Oh *et al.*, 2003). The pH of HPM was buffered at 5.5 and 7.0 using MES (2-N-Morpholino-EthaneSulfonic acid, Sigma). Phosphate buffer was used to adjust the pH at 8.5. Different pH values of the medium were used to evaluate the hydrogen production performances at optimal environmental conditions for hydrogen producing microbes (acid pH values: 4.5-6.0) as well as for methanogenic bacteria (neutral to basic pH values:7.0-8.0) (Cooney *et al.*, 2007).
After pH adjustment, HPM medium and the vessels were sterilized by autoclave (121° C, 20 minutes) in order to evaluate the performances of the bacteria introduced exclusively with the sludge that was aseptically transferred into the reactors as inoculum.
Food to microrganism ratio (F/M) was set at 1 gVS/gVS. Since heat treatment did not change the TS and VS of the sludge, every reactor was inoculated with 10 grams of treated or non-treated sludge.
After inoculation, the reactors were hermetically closed using a silicon plug. Once flushed with N_2 gas for 3 minutes, the vessels were incubated without stirring in a thermostatic chamber at 35° ± 2°C.

The amount of biogas produced was recorded daily, using the water displacement method. Biogas composition in terms of hydrogen, carbon dioxide and methane were measured by a gas chromatograph (HP5890) equipped with thermal conductivity detector (TCD), HP- MOLSIV and HP-PLOT U columns, nitrogen as carrier gas.
After 8 days of incubation, residual glucose in the HPM medium was measured using the peroxidase-glucose oxidase method from the d-Glucose assay kit (Boehringer Mannheim). All experiments were carried out in triplicate for each treated sludge and pH value. The obtained results were averaged.

Mathematical model of cumulative hydrogen production

Cumulative hydrogen productions from experimental tests were analysed using a mathematical model in order to compare the results. Hydrogen productions have been modelled using the following equation:

$$P(t) = P_{tot} \frac{t}{K_t + t} \qquad [1]$$

Where:
$P(t)$: cumulative hydrogen production at time t (Nml/g);
P_{tot}: maximum cumulative hydrogen production (Nml/g);
K_t: time at which the cumulative hydrogen production is half of the maximum (h);
t: time of fermentation from the beginning of the test (h).

Average values of cumulative hydrogen production from each experimental condition were used to obtain the values of the parameters *Ptot* and *Kt*. These parameters were estimated by minimizing the sum square of errors between experimental data and results from the model. The estimations were carried out by using the 'Solver' function in Calc of OpenOffice.

Statistical analysis.

The results presented in this study are the average of a minimum of three repetitions. The bars in Figure 1 represent standard deviation. Statistical analysis of cumulative hydrogen productions was performed by two ways factorial ANOVA (ANalysis Of Variance) using Duncan test *post hoc* means differentiation. Heat treatment duration and pH medium were the factors considered in ANOVA.

Results and discussion

Effects of heat treatment on microbial viability and community structure of granular sludge

The non-treated sludge and the treated sludge (boiled at 100°C for 0.5, 1, 2 and 4 hours) were characterized for their microbial viability and results are reported in Table 1. All tested sludges, heat treated and non-treated, showed a higher number of microbial colonies when plates were anaerobically incubated. This evidence could be explained considering that the used granular sludge was collected from an industrial scale UASB anaerobic digester where most of microbial species had adapted to grow at very low oxygen concentration in the environment.
The non-treated granular sludge presented 1.3×10^7 CFU/g and 5.5×10^6 CFU/g after anaerobic and aerobic incubation of plates, respectively. As shown in Table 1, heat treatment strongly affected the sludge microbial viability. When determined in anaerobic condition, the viability of the granular sludge dropped from 1.3×10^7 CFU/g of the non-treated sludge to the 1.0×10^5 CFU/g of the sludge boiled only for 0.5 hours.
Heat treatment of granular sludge strongly influenced also its microbial community structure (Table 2). The bacterial community structure of the non-treated sludge was quite similar to those reported for twelve granular sludges collected from different types of full-scale UASB reactors

(Narihiro *et al.*, 2009). Bacterial 16S rDNA sequencing of microbial strains isolated from the non-treated sludge revealed that the most dominant phylum was *Proteobacteria* with about 59% of the identified isolates. The main species was *Sphingopyxis granuli* (12%), recently isolated from a brewery wastewater-treating UASB reactor (Kim *et al.*, 2005). The remaining bacterial isolates belong to the *Firmicutes* phylum and were mainly affiliated to the *Bacillaceae* family.

Table 1. Effect of the thermal treatments on granular sludge microbial viability.

Duration of heat treatment (100°C) on granular sludge	CFU/g sludge	
	Aerobic incubation	Anaerobic incubation
No heat treatment	$5.5 \pm 0.5 \times 10^6$	$1.3 \pm 0.2 \times 10^7$
0.5 hour	$3.5 \pm 0.4 \times 10^4$	$1.0 \pm 0.1 \times 10^5$
1 hour	$1.6 \pm 0.1 \times 10^4$	$4.5 \pm 0.5 \times 10^4$
2 hours	$6.5 \pm 0.1 \times 10^2$	$3.3 \pm 0.2 \times 10^3$
4 hours	$1.5 \pm 0.3 \times 10^2$	$2.3 \pm 0.6 \times 10^3$

Table 2. 16S rDNA sequencing of bacterial strains aerobically and anaerobically isolated from non-treated and 0.5, 1, 2, 4 hours treated granular sludge.

Phylum	**Closest species in GenBank**	**Number of isolates identified in granular sludge samples**				
		non-treated	0.5 h	1 h	2 h	4 h
Firmicutes	*Bacillus* sp.	5.9[a]	25.0	26.9	45.5	12.5
	Bacillus badius	5.9	25.0	23.1	-	-
	Bacillus beijingensis	-	9.4	7.7	-	-
	Bacillus farraginis	-	3.1	7.7	9.1	37.5
	Bacillus flexus	-	-	3.8	-	-
	Bacillus licheniformis	2.9	3.1	7.7	-	-
	Bacillus megaterium	2.9	-	-	-	-
	Brevibacillus sp.	-	6.3	-	9.1	12.5
	Brevibacillus brevis	-	-	11.5	-	-
	Brevibacillus parabrevis	2.9	-	-	-	12.5
	Paenibacillus sp.	2.9	6.3	3.8	18.2	25.0
	Paenibacillus cookii	-	-	3.8	9.1	-
	Planomicrobium sp.	-	3.1	-	-	-
	Sporosarcina sp.	2.9	6.3	3.8	9.1	-
	Staphylococcus saprophyticus	8.8	-	-	-	-
	Staphylococcus sp.	5.9	-	-	-	-
Proteobacteria	*Alcaligenes* sp.	8.8	-	-	-	-
	Alishewanella sp.	8.8	-	-	-	-
	Enterobacter sp.	8.8	6.3	-	-	-
	Enterobacter cloacae	-	3.1	-	-	-
	Pseudomonas sp.	8.8	-	-	-	-
	Shinella sp.	8.8	-	-	-	-
	Sphingopyxis granuli	11.8	-	-	-	-
	Uncultured beta proteobacterium	2.9	-	-	-	-

[a] Frequency of isolates assigned with a genus or species in percentage of the total number of isolates analyzed for each sample of sludge.

Once boiled for only 0.5 hour, the sludge exhibited a considerable reduction of the microbial diversity: *Firmicutes* became the predominant phylum, accounting for the 90% of the microbial strains. The remaining isolates, belonging to *Enterobacter* sp. and *E. cloacae*, were affiliated with *Proteobacteria* phylum. Therefore, the sludge was mainly enriched with spore-forming microbes, capable of withstand the thermal stress. However, few isolates of non-spore forming bacteria (*Planomicrobium* sp. and *Enterobacter* sp.) were detected. This finding may be explained considering that the complex structure of the granules in the sludge could have allowed them to tolerate the short thermal stress. Longer heat treatments, indeed, exclusively selected for spore-forming bacteria affiliated with *Firmicutes* phylum (Table 2). After 1 hour, the most abundant known species were *Bacillus badius* (23%) and *Brevibacillus brevis* (11%); however a significant amount of unknown *Bacillus* sp. was also present (27%). As a result, in the 1 hour heat treated sludge, the predominant genus was found to be *Bacillus* sp.. This genus represents strict or facultative aerobes having diverse metabolic activities potentially useful in the treatment of wastewater and in the production of hydrogen from several organic wastes (Kalia and Purohit, 2008).

Increasing the heat treatment times resulted in the reduction of both size and microbial structure of granular sludge (Table 1 and 2). The main genera of the 2 hours heat-treated sludge were *Bacillus* sp. and *Paenibacillus* sp. while, after 4 hours boiling time, the majority of the isolates belonged to *B. farraginis* and *Paenibacillus* sp..

Effects of heat treatment on hydrogen production of granular sludge

The average cumulative hydrogen productions obtained from tested experimental conditions are reported in Table 3. Equation [1] resulted the best fitting mathematical model of the experimental data of cumulative hydrogen productions. The parameters *Ptot* and *Kt* obtained from the Equation [1] are reported in Table 3. Cumulative hydrogen productions at different pH values of the heat-treated granular sludges are represented in Figure 1. After 8 days of incubation, glucose was completely depleted in all the batch tests.

Table 3. Average cumulative hydrogen productions (±SD, n=3), hydrogen yields and mathematical model results. Hydrogen productions are normalized to glucose added to batch tests.

Test conditions		Average experimental results		Mathematical model results	
Duration of heat treatment (h)	pH -	Cumulative hydrogen production per gram of glucose (Nml/g)	Hydrogen production per mole of glucose (mol H_2/mol)	Cumulative hydrogen production *Ptot* (Nml/g)	*Kt* value (h)
4	5.5	265 ± 10 ^{f}	2.14	272	4.3
	7.0	180 ± 5 ^{de}	1.44	184	3.1
	8.5	58 ± 14 ^{a}	0.47	59	3.0
2	5.5	226 ± 27 ^{ef}	1.82	244	12.0
	7.0	183 ± 17 ^{de}	1.47	194	8.6
	8.5	57 ± 16 ^{a}	0.47	59	7.4
1	5.5	161 ± 21 ^{d}	1.30	168	6.0
	7.0	110 ± 32 ^{bc}	0.88	115	6.5
	8.5	51 ± 20 ^{a}	0.41	52	4.8
0.5	5.5	171 ± 5 ^{d}	1.38	175	2.9
	7.0	150 ± 5 ^{cd}	1.21	156	5.8
	8.5	102 ± 5 ^{b}	0.82	104	3.9

Means with different superscript letters are significantly different (p≤0.01).

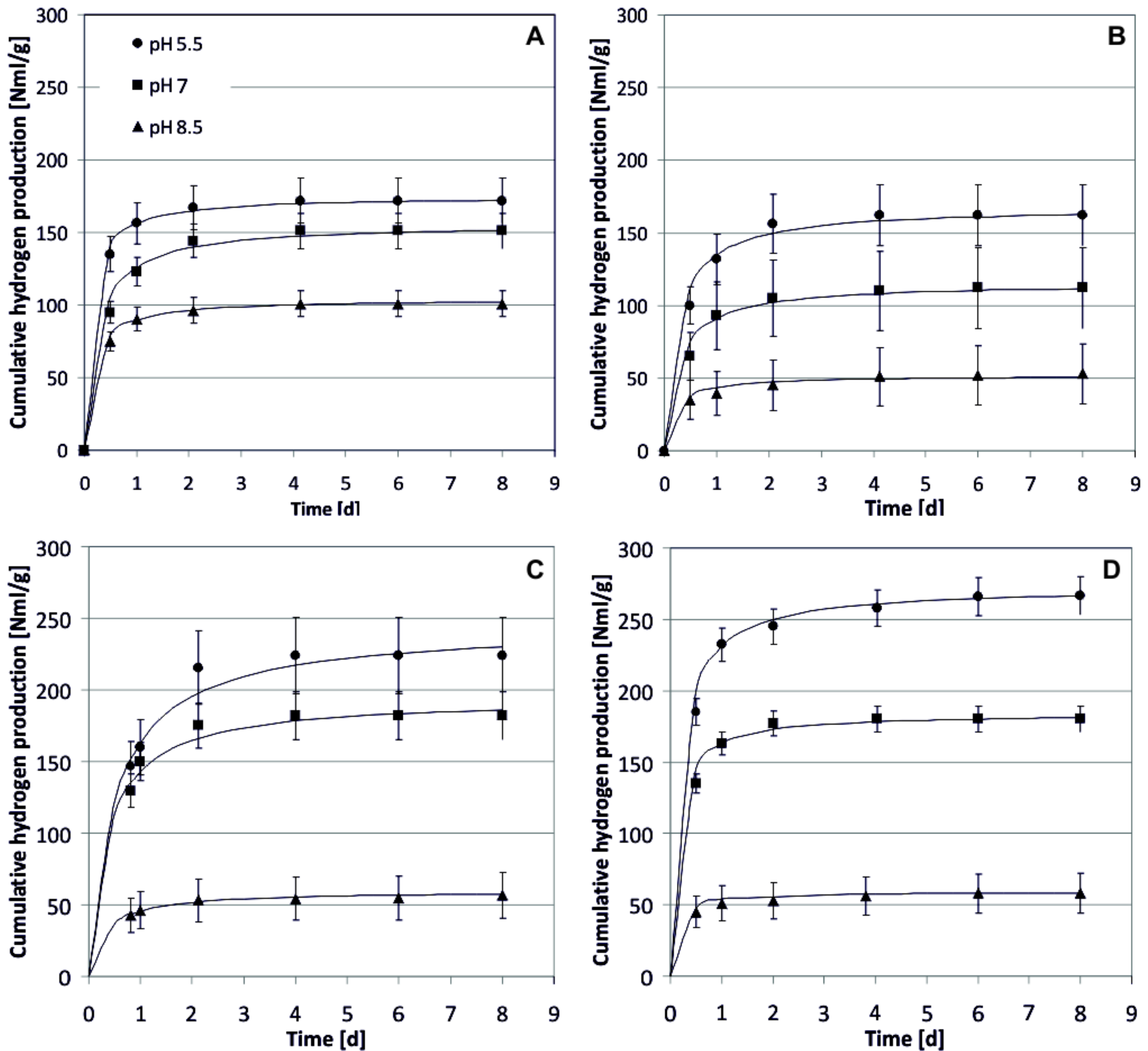

Figure 1. Cumulative hydrogen productions from average experimental data (symbols) and from the mathematical model (continuous line) at the three pH levels of the sludge heat-treated for 0.5 hour (A), 1 hour (B), 2 hours (C) and 4 hours (D). Error bars represent the standard deviation of experimental data.

During all hydrogen production batch tests no methane was detected, even at pH values favourable for methane producing microbes (7.0 and 8.5). On the contrary, when non-treated sludge was used as inoculum during batch tests, the system under study produced only methane and not hydrogen at all pH values (data not shown). Thermal pre-treatments were therefore efficient in the inhibition of the methanogenic bacteria.

It is evident from Figure 1 that pH had a clear effects on hydrogen production potentials. The higher the initial pH, the lower the total hydrogen production was ($p \leq 0.01$). In the tested conditions, the highest hydrogen levels were obtained with pH 5.5. This is in accordance with earlier works where this pH value was reported as optimal for hydrogen production (Lay *et al.*, 1999; Van Ginkel and Sung, 2001; Kapdan and Kargi, 2006).

As shown in Table 3, the heat treatment resulted in a marked effect on hydrogen production performances ($p \leq 0.01$). Increasing the treatment duration resulted in increasing hydrogen production levels. The tests inoculated with sludge treated for 0.5 and 1 h boiling time (Figure 1 A,B) converted glucose into H_2 at lower levels than those with the 2 and 4 hour treated

sludge (Figure 1 C,D). This indicates that inoculum thermal conditioning efficiently selected in the sludge the microbial species mainly involved in the hydrogen production: under the most favourable pH conditions, the higher levels of H_2 production were obtained with longer duration of sludge treatment. This finding is confirmed by ANOVA analysis showing that the interaction between pH and thermal duration was statistically significant ($p \leq 0.01$).

Highest hydrogen production was achieved using 4 hour heat-treated sludge. The yield of 2.14 moles of hydrogen per mole of glucose obtained at pH 5.5 with the 4 hour treated sludge seems to be promising if compared to those previously reported from glucose (Table 4).

Table 4. Comparison of hydrogen yields from different studies. All the data regard batch experiments using glucose as substrate and mixed culture as inoculum.

Reference	Inoculum	Inoculum pre-treatment	Fermentation temperature	Fermentation pH	Hydrogen yields (mol H_2/mol glucose)
This study	UASB granular sludge	heat treatment (100°C, 4 h)	35°C	5.5	2.14
This study	UASB granular sludge	heat treatment (100°C, 2 h)	35°C	5.5	1.82
Davila-Vazquez *et al.* (2008)	UASB granular sludge	heat treatment (100°C, 40 min)	37°C	7.5	1.46
Mu *et al.* (2006)	waste activated sludge	anaerobic storage for 6 month and heat treatment (85°C, 1h)	35°C	5.5	1.00
Mu *et al.* (2006)	waste activated sludge	anaerobic storage for 6 month and heat treatment (85°C, 1h)	41°C	5.5	1.67
Morimoto *et al.* (2004)	sludge compost	aerobic conditions (60°C, 3 days)	60°C	3.97*	2.1
Morimoto *et al.* (2004)	anaerobic pond sludge	-	50°C	4.02*	1.6
Morimoto *et al.* (2004)	compost	-	60°C	-	1.5
Logan *et al.* (2002)	soil used for tomato plants	heat treatment (104°C, 2 h)	26°C	6	0.92
Fang and Liu (2002)	anaerobic sludge from a CSTR reactor	-	36°C	5.5	2.1

* final pH

Analysing the data reported in Table 3, the hydrogen productions at pH 7.0 and 8.5 obtained with 0.5 hour heat treatment were statistically higher ($p \leq 0.01$) than those achieved with 1 hour heat-treated sludge. On the contrary, at pH 5.5, the productions were similar. This observation could suggest that different species of hydrogen consuming and/or non hydrogen forming bacteria could be still present and may have negatively affected the net amount of produced H_2. Moreover, non spore-forming hydrogen producers, able to survive after 0.5 h of heat treatment, may be killed once exposed to longer treatment. In this case, rather than increasing H_2 production, heat treatment have actually reduced H_2 yield at the considered pH values. This is in accordance with Kraemer and Bagley (2007), reporting contradictory results on beneficial or negative effects of heat treatment compared to non-heat-treated systems and other inoculum conditioning methods (Zhu and Bèland, 2006).

These observations seem to be confirmed considering the results of the bacterial 16S rDNA sequencing (Table 2). The *Enterobacter* sp. and *E. cloacae* isolates, present in the 0.5 hour heat-treated sludge, may have enhanced hydrogen productions at level higher than those detected in the 1 hour treated sludge having only species affiliated with *Firmicutes* phylum. The *Enterobacter* genus, indeed, has been largely studied for H_2 production from glucose, sucrose and other substrates (Kalia and Purohit, 2008).

Half of the maximum cumulative hydrogen production (*Ptot*) was obtained within the first 12 hours as indicated by the *Kt* values (Table 3). No clear correlation was observed between the *Kt* values and the heat pre-treatment duration. Low *Kt* values were measured both for long (4 hours) and for short (0.5 hour) pre-treatments while higher *Kt* values were observed for tests at two hours of heat pre-treatment even though the cumulative hydrogen productions were not the lowest. These findings may be explained considering that each boiling duration have selectively enriched the sludge with mixed microbial consortia (Table 2) having different hydrogen production potentials. As a consequence, the most interesting hydrogen kinetics seem to be those obtained by the 4 hour treated sludge having both the lowest *Kt* values and the highest cumulative hydrogen production (*Ptot*).

The effects of heat-treatment on microbial community of sludge can be highlighted comparing the data reported in Table 1 and Table 3. In fact after heat-treatment, the size of the microbial population clearly decreased and the survived bacteria performed variable hydrogen yields at different pH. This finding suggests that the treatments themselves produced an evident selection within the entire microbial population.

Although the microbiological analyses reported that the 4 hour treatment strongly affected the sludge microbial viability (Table 1), the lowest concentration of bacteria (2.33×10^3 CFU/g of sludge in anaerobic conditions, corresponding to about 10^2 CFU per ml of suspension) exhibited the best hydrogen yield at pH 5.5 (Table 3). These results could suggest that the 4 hour boiling treatment resulted in the selection of the dominant microbial consortia of proficient hydrogen producing microbes. Therefore, the isolates able to survive after the longest heat treatment duration, mainly *B. farraginis* and *Paenibacillus* sp. (Table 2), may have the most promising hydrogen yields.

Conclusions

This study showed that the 4 hour treatment seems to be efficient for the selection of microbial populations exhibiting the best hydrogen performances. However, in order to maximize the hydrogen production yields, the optimal combination of heat treatment duration and pH value should be defined. As the highest hydrogen productions were obtained using the lowest microbial concentrations, it is presumable that microbial strains capable of surviving after 4 hour boiling time are very interesting as hydrogen producing bacteria. These isolates, belonging to the *Bacillaceae* family, will be studied as selected inoculants with outstanding ability to produce hydrogen from organic wastes and wastewater for industrial scale purposes.

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Figure
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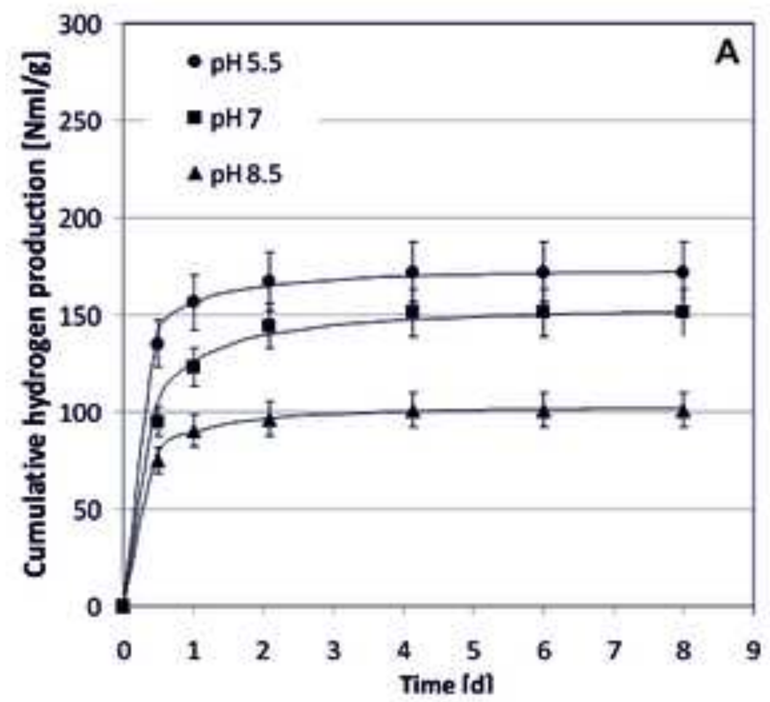

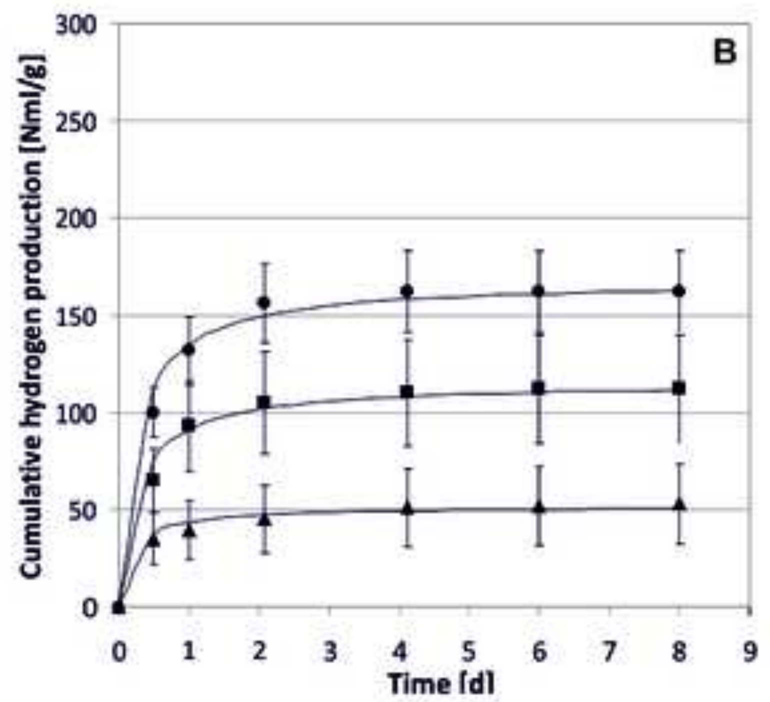

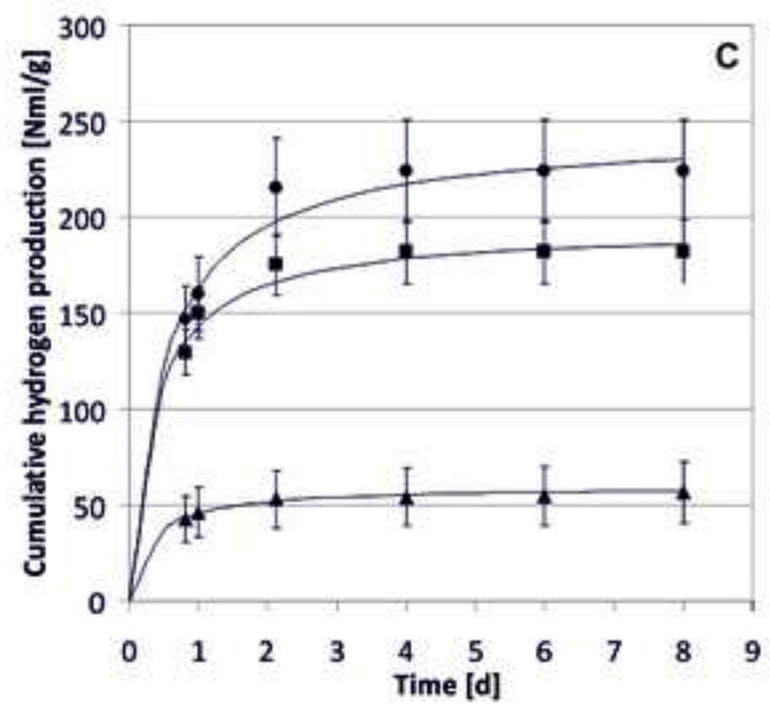

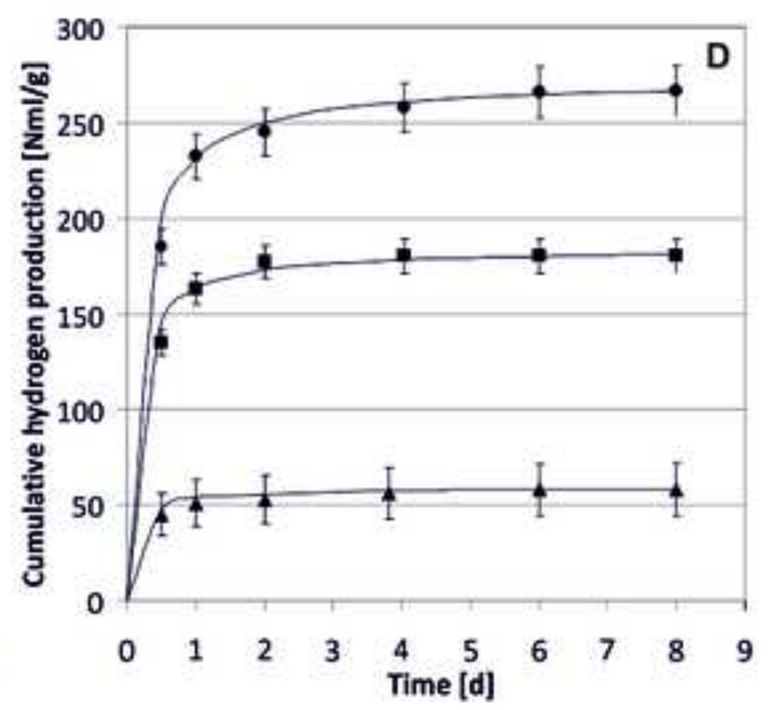

Table

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1 hour	$1.6 \pm 0.1 \times 10^4$	$4.5 \pm 0.5 \times 10^4$
2 hours	$6.5 \pm 0.1 \times 10^2$	$3.3 \pm 0.2 \times 10^3$
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	Bacillus flexus	-	-	3.8	-	-
	Bacillus licheniformis	2.9	3.1	7.7	-	-
	Bacillus megaterium	2.9	-	-	-	-
	Brevibacillus sp.	-	6.3	-	9.1	12.5
	Brevibacillus brevis	-	-	11.5	-	-
	Brevibacillus parabrevis	2.9	-	-	-	12.5
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	Planomicrobium sp.	-	3.1	-	-	-
	Sporosarcina sp.	2.9	6.3	3.8	9.1	-
	Staphylococcus saprophyticus	8.8	-	-	-	-
	Staphylococcus sp.	5.9	-	-	-	-
Proteobacteria	*Alcaligenes* sp.	8.8	-	-	-	-
	Alishewanella sp.	8.8	-	-	-	-
	Enterobacter sp.	8.8	6.3	-	-	-
	Enterobacter cloacae	-	3.1	-	-	-
	Pseudomonas sp.	8.8	-	-	-	-
	Shinella sp.	8.8	-	-	-	-
	Sphingopyxis granuli	11.8	-	-	-	-
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[a] Frequency of isolates assigned with a genus or species in percentage of the total number of isolates analyzed for each sample of sludge.

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Test conditions		Average experimental results		Mathematical model results	
Duration of heat treatment (h)	pH -	Cumulative hydrogen production per gram of glucose (Nml/g)	Hydrogen production per mole of glucose (mol H_2/mol)	Cumulative hydrogen production *Ptot* (Nml/g)	*Kt* value (h)
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	8.5	58 ± 14 ^{a}	0.47	59	3.0
2	5.5	226 ± 27 ^{ef}	1.82	244	12.0
	7.0	183 ± 17 ^{de}	1.47	194	8.6
	8.5	57 ± 16 ^{a}	0.47	59	7.4
1	5.5	161 ± 21 ^{d}	1.30	168	6.0
	7.0	110 ± 32 ^{bc}	0.88	115	6.5
	8.5	51 ± 20 ^{a}	0.41	52	4.8
0.5	5.5	171 ± 5 ^{d}	1.38	175	2.9
	7.0	150 ± 5 ^{cd}	1.21	156	5.8
	8.5	102 ± 5 ^{b}	0.82	104	3.9

Means with different superscript letters are significantly different ($p \leq 0.01$).

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Table 4. Comparison of hydrogen yields from different studies. All the data regard batch experiments using glucose as substrate and mixed culture as inoculum.

Reference	Inoculum	Inoculum pre-treatment	Fermentation temperature	Fermentation pH	Hydrogen yields (mol H_2/mol glucose)
This study	UASB granular sludge	heat treatment (100°C, 4 h)	35°C	5.5	2.14
This study	UASB granular sludge	heat treatment (100°C, 2 h)	35°C	5.5	1.82
Davila-Vazquez *et al.* (2008)	UASB granular sludge	heat treatment (100°C, 40 min)	37°C	7.5	1.46
Mu *et al.* (2006)	waste activated sludge	anaerobic storage for 6 month and heat treatment (85°C, 1h)	35°C	5.5	1.00
Mu *et al.* (2006)	waste activated sludge	anaerobic storage for 6 month and heat treatment (85°C, 1h)	41°C	5.5	1.67
Morimoto *et al.* (2004)	sludge compost	aerobic conditions (60°C, 3 days)	60°C	3.97*	2.1
Morimoto *et al.* (2004)	anaerobic pond sludge	-	50°C	4.02*	1.6
Morimoto *et al.* (2004)	compost	-	60°C	-	1.5
Logan *et al.* (2002)	soil used for tomato plants	heat treatment (104°C, 2 h)	26°C	6	0.92
Fang and Liu (2002)	anaerobic sludge from a CSTR reactor	-	36°C	5.5	2.1

* final pH