**Zeolite addition to improve biohydrogen production from dark
fermentation of organic wastes**

Silva ARM^{1a}, Abreu AA^{1b}, Salvador AF^{1c}, Alves MM^{1d}, Neves IC,^{1,2e} Pereira MA^{1f*}

¹Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal.

²CQUM-Centre of Chemistry, University of Minho, 4710-057 Braga, Portugal

^arita.silva@ceb.uminho.pt, ^babreu.angela@gmail.com, ^casalvador@ceb.uminho.pt,
^dmadalena.alves@deb.uminho.pt, ^eineves@quimica.uminho.pt, ^falcina@deb.uminho.pt.

* Corresponding author

Address for correspondence:

Universidade do Minho

Departamento de Engenharia Biológica

Campus de Gualtar, 4710-057 Braga

Portugal

Tel: (+351) 253 604 401

Fax: (+351) 253 604 429

E-mail address: alcina@deb.uminho.pt

Abstract

Background

Hydrogen is a clean and renewable energy source that can be produced by biological processes, such as dark fermentation. However, hydrogen production yields are usually low.

Results

In this work, biohydrogen production from a mixture (1:1) of glucose and arabinose (4.4 g L^{-1} , in Chemical Oxygen Demand (COD) was improved about 1.3 times in batch reactors, and increased 3 times in a continuous reactor (from $143 \text{ mL H}_2 \text{ L}^{-1} \text{ d}^{-1}$ to $430 \text{ mL H}_2 \text{ L}^{-1} \text{ d}^{-1}$), when zeolite type-13X was added. The presence of zeolite led to the stimulation of different metabolic pathways and to changes in the microbial community composition, which seems to be linked to hydrogen production improvement. The zeolite effect in dark fermentation was also verified for more complex substrates. Hydrogen production yield from *Sargassum* sp., was improved 1.4 times by the presence of zeolite ($94.8 \text{ L H}_2 \text{ Kg}^{-1} \text{ Sargassum sp. Volatile Solids (VS))$).

Conclusions

The results show that zeolite is suitable to improve biohydrogen production by dark fermentation.

Keywords: Biohydrogen, Zeolite, Bioreactor, Dark fermentation.

Background

Hydrogen is considered an alternative to fossil fuels mainly due to its high efficiency of conversion to usable power, recyclability and non-polluting nature, making it a good energy carrier (Hallenbeck and Ghosh, 2009). Dark fermentation is a biological process where hydrogen and carbon dioxide are produced by anaerobic microorganisms (Rittmann and Herwig, 2012). This process is environmentally friendly and one of the most promising and sustainable processes for hydrogen production. However, the production of large quantities of soluble metabolic products during dark fermentation results in low hydrogen production efficiencies and low hydrogen yields (around 25 %) (Hallenbeck *et al.*, 2012). Biological processes efficiencies under anaerobic conditions may be improved by the addition of different types of materials (Martins *et al.*, 2018). The mechanisms of action depend on several properties as for example electric conductivity or surface area. For example, zeolites (an aluminosilicate material), can increase biogas production and methane yield, by reducing the ammonia concentration and thus preventing the inhibitory effect of this compound (Kotsopoulos *et al.*, 2008). Furthermore, zeolites can remove ammonia and heavy metals from industrial wastewaters, or be used as ion-exchanger (Karakurt *et al.*, 2010; Niaz *et al.*, 2015). Their effect in hydrogen production by dark fermentation was poorly explored. Cheng *et al.* (2012) show that the presence of a natural zeolite can significantly remove the ammonium in the residual solution obtained from dark fermentation, and the combination of dark- and photo-fermentation enhance the hydrogen yield. The removal of ammonium ions from dark fermentation was also studied using the zeolite clinoptilolite and the increase of the hydrogen production was observed (Androga *et al.*, 2012). Zeolites are frequently used in chemical processes as catalysts for hydrogen production by reforming of bioethanol and from water splitting (Chica, 2013). In this work, the zeolite effect by addition of type-13X, a molecular sieve characterised by the presence of *faujasite* structure, in biohydrogen

production by dark fermentation was evaluated in batch and continuous reactors treating glucose and arabinose (C6 and C5 sugars present in lignocellulosic hydrolysates), and a complex waste, *Sargassum* sp. *Sargassum* sp. is a macroalgae very abundant in the Portuguese coastline, composed by easily hydrolysable sugars (Nkemka and Murto, 2010). The utilization of *Sargassum* sp. for biohydrogen production can turn dark fermentation more competitive and economically sustainable (Costa *et al.*, 2015).

Results and Discussion

Effect of zeolite on biohydrogen production by dark fermentation

The zeolite choose for this work is type-13X, known as molecular sieves. This zeolite presents a typical *faujasite* structure, with supercages, 12-ring pore openings and 3-dimensional channel system, with high surface area, low Si/Al ratio, which confer higher cation exchange capacity and high thermal and chemical stability. In addition, the low Si/Al ratio, saturated in aluminium, offers excellent properties in terms of adsorption capacity (Flaningen, 1991.)

Figure 1 shows the effect of zeolite type-13X on biohydrogen production from a mixture of glucose and arabinose, evaluated in batch experiments. The highest hydrogen production was achieved for the zeolite/biomass (in VS) ratio of 0.26 g g⁻¹ (24.54 mmol L⁻¹), followed by the ratio of 0.065 g g⁻¹ (22.88 mmol L⁻¹), which were respectively, 1.3 and 1.2 times higher than the obtained for the control assay without zeolite (19.36 mmol L⁻¹) ($p < 0.05$) (Figure 1 A).

The zeolite ratio of 0.13 g g⁻¹ of biomass (in VS) reached only 20.88 mmol L⁻¹ ($p > 0.05$).

Glucose and arabinose were totally consumed during the experiment for all zeolite/biomass ratios tested and for the control (Figure 2 A). The main end-product formed was acetic acid (reaching a maximum of 54.7 mmol L⁻¹ for the zeolite type-13X ratio of 0.26 g g⁻¹ of biomass

(in VS)) (Figure 1 C). Lower amounts of lactic and formic acid were produced at similar concentrations for all the zeolite/biomass ratios tested and for the control (without zeolite) (Figure 1 B, D).

According to the results obtained, hydrogen was probably produced by two different pathways, i.e. the microorganisms could be performing the mixed-acid fermentation and the clostridial-type fermentation. In both cases, acetate can be produced from acetyl-CoA in order to maximize ATP production (Hallenbeck, 2009). The fact that formic acid was accumulating (approximately 13 mmol L⁻¹) during the first days of incubation suggest that bacteria belonging to the *Enterobacteriaceae* family (performing the mixed-acid fermentation (Sikora *et al.*, 2013)), were the most active in this period. Formic acid was afterwards degraded reaching residual concentrations (approximately 3 mmol L⁻¹) at the end of the assays, which was accompanied by the production of hydrogen. Formic acid was likely converted to hydrogen and carbon dioxide by the activity of the enzyme formate:hydrogen lyase which is activated at low pH (Hallenbeck, 2009). Indeed, at the end of the batch assays the pH dropped to 5.5.

(Figure 1)

The zeolite effect on biohydrogen production by dark fermentation was further evaluated in a continuous bioreactor fed with arabinose and glucose (Figure 2). The bioreactor operation started at an HRT of 12 h and reached a maximum hydrogen production rate of 1015.9 mL H₂ L⁻¹ d⁻¹ (Figure 2 A). Acetate and lactic acid were the main metabolic products and their concentration remained stable during this period of operation (approximately 8.8 and 6.4 mmol L⁻¹, respectively) (Figure 2 B).

(Figure 2)

Previous studies showed that the decrease of the hydraulic retention time (HRT) often causes instability on the bioreactor performance, leading to lower hydrogen yields (Abreu *et al.*, 2010). Therefore, in order to cause disturbance on the bioreactor performance and to study the zeolite effect on the system recovery, the HRT was decreased to 6 h. As expected, after lowering the HRT, the hydrogen production rate decreased, reaching a minimal value of 140 mL H₂ L⁻¹ d⁻¹ (after 49 days of operation) (Figure 2 A). At this point, an increase in the lactic acid concentration was observed, reaching a maximum of 17.8 mmol L⁻¹ (Figure 2 B). Abreu *et al.*, (2010) showed that the decreased of HRT led to lower hydrogen yields mainly due to the increase of lactic acid production. Lactic acid bacteria (LAB) are usually present in hydrogen producing consortium interfering in the dark fermentation process (Sikora *et al.*, 2013). In other studies, the development of LAB in bioreactors during dark fermentation was found to inhibit the hydrogen production (Sikora *et al.*, 2013). This interference in hydrogen generation may occur due to substrate competition (Jo *et al.*, 2007) and/or excretion of bacteriocins by LAB, which inhibit the growth of other bacteria (Noike, 2002), namely the hydrogen-producing bacteria.

No methane was detected during the experiment, which is in accordance with the low percentage of methanogenic archaea (0.02 % of the total microbial community) observed in the microbial community, meaning that inoculum pre-treatment at high temperature and low pH was efficient at inhibiting methanogens.

After the drop of the hydrogen production to a minimal value of 140 mL H₂ L⁻¹ d⁻¹, the zeolite type-13X was added to the bioreactor. The bioreactor performance was immediately recovered after zeolite addition (Figure 2 A) and hydrogen production was increased to approximately 430 mL H₂ L⁻¹ d⁻¹, which was approximately 3 times higher than the previous

condition. After zeolite addition, formic acid concentration increased from 2.6 mmol L⁻¹ to approximately 7.4 mmol L⁻¹ (Figure 2 B). Propionate and n-butyrate acid were also detected during the reactor's operation, however at concentrations lower than 1 mmol L⁻¹ (Figure 2 B). The metabolic profile observed is characteristic from a mixed-acid fermentation where a mixture of acids, such as acetic and formic acid, are produced along with hydrogen and carbon dioxide. In this pathway the pyruvate from glycolysis is cleaved by the enzyme pyruvate formate-lyase to form formic acid and acetyl-CoA (Sikora *et al.*, 2013). Then, acetyl- CoA can be converted in acetate while formic acid can be degraded into hydrogen and carbon dioxide by formate hydrogen-lyase (Sikora *et al.*, 2013). Glycolysis only occurs in the microbial cells if the cytoplasmic electron carrier nicotinamide adenine dinucleotide (NADH) is constantly reoxidized. This can occur by the formation of H₂ and/or reduced products such as lactate, ethanol, butyrate, succinate and propionate (Stams, 1994). When the organic load increased by reducing the HRT from 12 to 6 hours, there was an excess of reducing power overloading the H₂ producing pathway. Thus lactate production occurred as a way to cope with this excess protons. In this study, the observed enhancement of hydrogen production can be the result of one or more zeolites properties. As the process occurs at pH 5.5, the protons in the solution may be adsorbed by the zeolite. This protons sink may avoid the need for NAD⁺ regeneration that is usually associated to more reduced products such as lactate. In fact, it is worth to notice that zeolite addition triggered a dynamic fluctuation between formate (a precursor of H₂ production) and lactate, conducting ultimately to an increase in H₂ production from 140 mL H₂ L⁻¹ d⁻¹ to 430 mL H₂ L⁻¹ d⁻¹ between days 44 e 58. The stabilization of H₂ production thereafter, may be related to the limited adsorption capacity of the amount of zeolite used.

Microbial community analysis showed that *Klebsiella* sp. that belong to the *Enterobacteriaceae* family, was the most abundant microorganism (Figure 3), which can explain the metabolite profile obtained since *Klebsiella* sp. are known to produce hydrogen through the cleavage of formate (Lee *et al.*, 2009).

(Figure 3.)

Significant changes in the microbial community were observed after zeolite addition (Figure 3). *Klebsiella* sp. continued to be the most abundant microorganism although its relative abundance slightly decreased from 78 % to 52 %. On the other hand, the relative abundance of organisms closed related to *Bacillus coagulans* and *Clostridium beijerinckii* increased from 1.68 % to 14.13 % and from 0.33 % to 6.62 %, respectively. *Clostridium* and *Bacillus* genera are known to perform the clostridial-type fermentation (Sikora *et al.*, 2013) in which the end products are hydrogen and acetate or butyrate. The maximum theoretical hydrogen yield during dark fermentation is 4 moles of hydrogen per mole of glucose, considering total conversion of glucose to acetic acid (Saint-Amans *et al.*, 2001; Sikora *et al.*, 2013). However, hydrogen yields during dark fermentation are generally lower than the theoretical ones due to the production of butyrate, propionate and reduced end products, such as alcohols and lactic acid (Saint-Amans *et al.*, 2001). In this work, different metabolites and in particular lactic acid was detected in high concentration showing that several metabolic pathways were taking place simultaneously (Figure 2 B). The concentrations of glucose and arabinose measured in the bioreactor effluent show that arabinose was only partially consumed while glucose was almost completely consumed during the entire operation. Glucose was detected in residual amounts until day 49, and slightly increased to approximately 3 mmol L⁻¹ after lowering the HRT. Arabinose concentrations were maintained stable (around 10 mmol L⁻¹) along all the

operation time (Figure 2 B). The COD balance was closed showing that all metabolic products were identified (Table S1). Several other properties of zeolites can be responsible for the observed increase in H₂ production, namely, their ability of functioning as a support material for the immobilization of microorganisms or the presence of different catalytic sites (Brönsted and Lewis sites). They are also known as adsorption material of different types of molecules. Specifically Zeolites type-13X were described as capable of adsorption of CO₂, CH₄ and/or CO for hydrogen purification from gaseous mixtures (Brea *et al.*, 2019). In biological processes, it was already demonstrated that zeolites are capable of acting as a support material for the immobilization of microorganisms (Emami Moghaddam *et al.*, 2018). Also, in the anaerobic digestion process, it was observed that zeolites addition improved the process efficiency by reducing the ammonia concentration in the media (Montalvo *et al.*, 2012). Moreover, was also described that the addition of zeolites to anaerobic digestion process improved biogas production because this porous structure can function as a supplement of trace elements (i.e Ca, Na, K and Ba) and improve the activity of methanogens (Wang *et al.*, 2019). Therefore, it is possible that similar mechanisms may be also occurring when zeolite type-13X are added to the dark fermentation process.

Effect of zeolite in biohydrogen production from *Sargassum* sp.

Hydrogen production by dark fermentation of *Sargassum* sp. was also improved in the presence of zeolite type-13X (Figure 4). The highest hydrogen production was obtained for the zeolite\biomass (in VS) ratio of 0.13 g g⁻¹ (8.3 mmol L⁻¹) which was approximately 1.4 times higher than the control (6.10 mmol L⁻¹) (Figure 3). However, the observed increase in hydrogen production was not statistically significant ($p > 0.05$). The ratio 0.26 g of zeolite per g of biomass (in VS) achieved a hydrogen production of 7.0 mmol L⁻¹. The lower hydrogen production obtained for the highest zeolite concentration can be related to the

increase of the lactic acid concentration (reaching a maximum of 6.9 mmol L⁻¹), showing a shift in the metabolic pathways towards lactic acid production. As already mentioned above, the deviation of the metabolism for lactic acid production is associated with the decrease in hydrogen yields (Abreu *et al.*, 2010). No methane was detected during the assays in all conditions tested. In the blank assay, containing only anaerobic sludge, hydrogen was produced (2.8 mmol L⁻¹) which means that in the assay with *Sargassum* sp. part of the hydrogen produced is derived from the residual substrate associated with the inoculum sludge (Figure 4).

The VFA detected were acetate (up to 13 mmol L⁻¹), iso-butyric acid (up to 13 mmol L⁻¹), n-butyric acid (up to 13 mmol L⁻¹), formic acid (up to 13 mmol L⁻¹) and propionic acid (up to 13 mmol L⁻¹) (Figure S1). Similar concentrations were measured and no significant differences could be detected between the assays performed with and without zeolite.

(Figure 4)

Conclusion

The presence of zeolite increased hydrogen production by dark fermentation from carbohydrates and induced a shift in the soluble microbial products formed and in the microbial community. Several zeolite characteristics may be responsible for the enhancement observed in H₂ production, albeit the adsorption of H⁺, seems to be plausible to justify the dynamics of metabolites formed (formate versus lactate). These results suggest that the use of zeolites during biohydrogen production, can be a good strategy to make dark fermentation more efficient.

Methods

Batch experiments

Batch experiments were conducted in 120 mL serum bottles with a working volume of 50 mL, containing anaerobic medium with 0.6 mL g⁻¹ of Chemical Oxygen Demand (COD) of macronutrients (Abreu *et al.*, 2010), 10 mmol L⁻¹ of sodium bicarbonate and 15 mmol L⁻¹ of sodium 2-bromoethanesulfonate (BES) to inhibit the methanogenic activity. Anaerobic granular sludge (with a final concentration of 7.72 g L⁻¹ of volatile solids (VS)), collected from a brewery industry (Lisbon, Portugal), was used as inoculum and a mixture of L-arabinose and glucose monohydrated (25 mmol L⁻¹, 1:1) as substrate. Three different amounts of zeolite type-13X to biomass (in VS) ratios were tested: 0.26, 0.13 and 0.065 g g⁻¹. The bottles were sealed and the headspace flushed with N₂/CO₂ (80/20 %). Sodium sulfide (0.8 mmol L⁻¹), was used as reducing agent and the assays were performed at 70 °C, 150 rpm and at an initial pH of 7.0.

The zeolite used consisted in type-13X with a composition of 1 Na₂O: 1 Al₂O₃: 2.8 ± 0.2 SiO₂: xH₂O (Sigma Aldrich). The spherical beads of the zeolite have a size of 4-8 mesh with an average pore diameter of 10 Å and are composed essentially by the *faujasite* structure (Molecular Sieves - Technical Information Bulletin, Sigma-Aldrich, 2019, <http://www.sigmaaldrich.com/chemistry/chemical-synthesis/learning-center/technical-bulletins/al-1430/molecular-sieves.html>, accessed December 2019).

Consumption of arabinose and glucose and production of hydrogen and other soluble fermentation products (SFP) were monitored during the experiments.

A different set of batch assays was performed using *Sargassum* sp. as substrate, in the same conditions described for the batch assays with glucose and arabinose, with exception of the working volume (serum bottles and working volume of 160 and 60 mL, respectively).

265 *Sargassum* sp. was collected in the spring of 2013 from a location in the north coastline of
266 Portugal (Póvoa de Varzim). The macroalgae was dried at room temperature and then milled
267 into pieces with less than 0.5 cm, according to (Costa *et al.*, 2015). The *Sargassum* sp.
268 characterization can be found in (Costa *et al.*, 2015). To increase the soluble COD, the
269 macroalgae was autoclaved at 121 °C and 1 bar for 10 min (Costa *et al.*, 2015).
270 In this experiment a ratio of 1.54 g of biomass (in VS) per g of *Sargassum* sp. (in VS) was
271 used and the ratios 0.26 and 0.13 g zeolite per g of biomass (in VS) were tested.
272 For both experiments a blank assay without biomass and a control without zeolite were
273 included. In the assay with *Sargassum* sp. an additional blank assay with only biomass
274 without any substrate was also performed for quantification of the hydrogen produced from
275 the residual substrate present in the inoculum. For each condition four replicates were
276 performed. To avoid hydrogen leaks, gas composition was determined in two of the bottles
277 and liquid samples were collected from the remaining two bottles for VFA, lactic acid and
278 sugars quantification. pH was monitored in both experiments.

279

280 Continuous bioreactors operation

281 Anaerobic granular sludge from a brewery industry (Lisbon, Portugal) was previously
282 acclimated for two months on an expanded granular sludge bed bioreactor (EGSB) at extreme
283 thermophilic conditions (70 °C) and pH 5.5, to select for hydrogen producers and to inhibit
284 methanogens. This sludge (0.08 ± 0.01 g VS g⁻¹) was used as inoculum (300 mL) in a
285 stainless steel EGSB reactor with a working volume of 2.14 L. The reactor was fed with L-
286 arabinose and glucose monohydrated (1:1 (w/w)) at a concentration of 4.4 g L⁻¹(in COD) and
287 operated in continuous mode at 70 ± 2 °C, during 72 days. The feed was supplemented with
288 macronutrients and maintained at 4 °C to minimize acidification, as described elsewhere
289 (Abreu *et al.* 2010). The pH of the bioreactor was maintained at 5.5. The hydraulic retention

time (HRT) was kept at 12 h during the first month of operation and thereafter was decreased to 6 h. Zeolite type-13X was added to the bioreactor (in a ratio of 0.26 g of zeolite per g of biomass (in VS)) when the hydrogen production dropped, after 46 days of operation. Gas samples were collected for hydrogen and methane measurements. Volatile fatty acids (VFA), lactic acid, glucose and arabinose concentrations were determined in liquid samples collected from the effluent. Before and after zeolite addition, sludge samples (approximately 0.5 mL) were collected for microbial community taxonomic characterization. Samples were centrifuged, washed with PBS and stored at - 20 °C. Total genomic DNA was extracted by using the FastDNA SPIN kit for soil (MP Biomedicals LLC, Santa Ana, CA). 16S rRNA genes were sequenced by Illumina MiSeq, using the universal prokaryotic primer pair 515-f/806-r (Caporaso *et al.*, 2011). Sequencing and data analysis were performed by RTL (Research testing Laboratory, Texas, US) as described elsewhere (Salvador *et al.*, 2018). Sequencing reads were submitted to the European Nucleotide Archive (ENA) under the study accession number PRJEB23747 (sample SAMEA104420766, before zeolite addition; sample SAMEA104420767, after zeolite addition).

Analytical methods

Hydrogen and methane concentration in batch and continuous experiments were analyzed by gas chromatography using a BRUKER SCION 456 with a column molsieve (MS-13 x 80/100 mesh) and connected to a thermal conductivity detector. Argon was used as carrier gas at a rate of 30 ml min⁻¹ and the injector, detector and column temperatures were set at 100, 130, and 35 °C, respectively. VFA, lactic acid and sugars (i.e., arabinose, glucose) were determined by high performance liquid chromatography using a chromatograph (Jasco, Japan) with an Aminex ® column (HPX-87H Ion 300 mm x 7.8 mm²); Sulfuric acid (0.01 N)

at a flow rate of 0.7 mL min⁻¹ was used as mobile phase. Column temperature was set at 60 °C and was used a retention time of 50 min. Detection of VFA's and lactic acid was made through a UV detector at 210 nm, and the detection of glucose and arabinose was made by using an RI detector. pH was measured by using a benchtop metre inoLabVR pH 7110 (WTW, Weilheim, Germany).

Statistical Analysis

Significant differences between biological samples, collected from batch incubations with and without zeolite, were determined by applying a t-test using Microsoft Excel (unequal variance t-test was applied to batch experiments with *Sargassum* sp. and equal variance t-test to the batch experiments with sugars, as determined by the results of the variance analysis obtained with the F-test). The significant threshold was set at $P = 0.05$.

Abbreviations

ATP: adenosine triphosphate

BES: sodium 2-bromoethanesulfonate

COD: chemical oxygen demand

DNA: deoxyribonucleic acid

EGSB: expanded granular sludge bed bioreactor

HRT: hydraulic retention time

LAB: lactic acid bacteria

NADH: nicotinamide adenine dinucleotide

SFP: soluble fermentation products

338 VFA: volatile fatty acids

339 VS: volatile solids

340

341 **References**

342 Abreu, A.A., Alves, J.I., Pereira, M.A., Karakashev, D., Alves, M.M., Angelidaki, I., 2010.
343 Engineered heat treated methanogenic granules: A promising biotechnological approach for
344 extreme thermophilic biohydrogen production. *Bioresour. Technol.* 101, 9577–9586.
345 <https://doi.org/10.1016/j.biortech.2010.07.070>

346 Androga, D.D., Ozgur, E., Eroglu, I., Gunduz, U., Yucel, M., 2012. Amelioration of
347 photofermentative hydrogen production from molasses dark fermenter effluent by zeolite-
348 based removal of ammonium ion. *Int. J. Hydrog. Energy* 37, 16421–16429.
349 <http://doi.org/10.1016/j.ijhydene.2012.02.177>.

350 Brea, P., Delgado, J.A., Águeda, V.I., Gutiérrez, P., Uguina, M.A., 2019. Multicomponent
351 adsorption of H₂, CH₄, CO and CO₂ in zeolites NaX, CaX and MgX. Evaluation of
352 performance in PSA cycles for hydrogen purification. *Microporous Mesoporous Mater.* 286,
353 187–198. <https://doi.org/10.1016/j.micromeso.2019.05.021>

354 Cheng, J., Xia, A., Liu, Y., Lin, R., Zhou, J., Cen, K., 2012. Combination of dark- and photo-
355 fermentation to improve hydrogen production from *Arthrospira platensis* wet biomass with
356 ammonium removal by zeolite. *Int. J. Hydrog. Energy* 37, 13330–13337.
357 <http://dx.doi.org/10.1016/j.ijhydene.2012.06.071>.

358 Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh,
359 P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of
360 millions of sequences per sample. *Proc. Natl. Acad. Sci.* 108, 4516–4522.

361 <https://doi.org/10.1073/pnas.1000080107>

362 Chica, A., 2013. Zeolites: Promised Materials for the Sustainable Production of Hydrogen.

363 ISRN Chem. Eng. 2013, 1–19. <https://doi.org/10.1155/2013/907425>

364 Emami Moghaddam, S.A., Harun, R., Mokhtar, M.N., Zakaria, R., 2018. Potential of Zeolite

365 and Algae in Biomass Immobilization. *BioMed Res. Int.* 2018, 1–15.

366 <https://doi.org/10.1155/2018/6563196>

367 Flanigen, E.M., Zeolites and molecular sieves an historical perspective, in *Introduction to*

368 *zeolite science and practice*, Studies in Surface Science and Catalysis, Ed. H. van Bekkum,

369 E.M. Flanigen, J.C. Jansen, Elsevier, 1991, Chap. 2, pp. 13-34.

370 Hallenbeck, P.C., 2009. Fermentative hydrogen production: Principles, progress, and

371 prognosis. *Int. J. Hydrog. Energy* 34, 7379–7389.

372 <https://doi.org/10.1016/j.ijhydene.2008.12.080>

373 Hallenbeck, P.C., Abo-Hashesh, M., Ghosh, D., 2012. Strategies for improving biological

374 hydrogen production. *Bioresour. Technol.* 110, 1–9.

375 <https://doi.org/10.1016/j.biortech.2012.01.103>

376 Hallenbeck, P.C., Ghosh, D., 2009. Advances in fermentative biohydrogen production: the

377 way forward? *Trends Biotechnol.* 27, 287–297. <https://doi.org/10.1016/j.tibtech.2009.02.004>

378 Jo, J.H., Jeon, C.O., Lee, D.S., Park, J.M., 2007. Process stability and microbial community

379 structure in anaerobic hydrogen-producing microflora from food waste containing kimchi. *J.*

380 *Biotechnol.* 131, 300–308. <https://doi.org/10.1016/j.jbiotec.2007.07.492>

381 Karakurt, C., Kurama, H., Topçu, İ.B., 2010. Utilization of natural zeolite in aerated concrete

382 production. *Cem. Concr. Compos.* 32, 1–8.

383 <https://doi.org/10.1016/j.cemconcomp.2009.10.002>

384 Kotsopoulos, T.A., Karamanlis, X., Dots, D., Martzopoulos, G.G., 2008. The impact of
 385 different natural zeolite concentrations on the methane production in thermophilic anaerobic
 386 digestion of pig waste. *Biosyst. Eng.* 99, 105–111.
 387 <https://doi.org/10.1016/j.biosystemseng.2007.09.018>

388 Lee, H.-S., Krajmalinik-Brown, R., Zhang, H., Rittmann, B.E., 2009. An electron-flow model
 389 can predict complex redox reactions in mixed-culture fermentative BioH₂: Microbial
 390 ecology evidence. *Biotechnol. Bioeng.* n/a-n/a. <https://doi.org/10.1002/bit.22442>

391 Martins, G., Salvador, A.F., Pereira, L., Alves, M.M., 2018. Methane Production and
 392 Conductive Materials: A Critical Review. *Environ. Sci. Technol.* 52, 10241–10253.
 393 <https://doi.org/10.1021/acs.est.8b01913>

394 Montalvo, S., Guerrero, L., Borja, R., Sánchez, E., Milán, Z., Cortés, I., Angeles de la Rubia,
 395 M., 2012. Application of natural zeolites in anaerobic digestion processes: A review. *Appl.*
 396 *Clay Sci.* 58, 125–133. <https://doi.org/10.1016/j.clay.2012.01.013>

397 Niaz, S., Manzoor, T., Pandith, A.H., 2015. Hydrogen storage: Materials, methods and
 398 perspectives. *Renew. Sustain. Energy Rev.* 50, 457–469.
 399 <https://doi.org/10.1016/j.rser.2015.05.011>

400 Nkemka, V.N., Murto, M., 2010. Evaluation of biogas production from seaweed in batch
 401 tests and in UASB reactors combined with the removal of heavy metals. *J. Environ. Manage.*
 402 91, 1573–1579. <https://doi.org/10.1016/j.jenvman.2010.03.004>

403 Noike, T., 2002. Inhibition of hydrogen fermentation of organic wastes by lactic acid
 404 bacteria. *Int. J. Hydrog. Energy* 27, 1367–1371. [https://doi.org/10.1016/S0360-](https://doi.org/10.1016/S0360-3199(02)00120-9)
 405 [3199\(02\)00120-9](https://doi.org/10.1016/S0360-3199(02)00120-9)

406 Rittmann, S., Herwig, C., 2012. A comprehensive and quantitative review of dark
 407 fermentative biohydrogen production. *Microb. Cell Factories* 11, 115.

408 <https://doi.org/10.1186/1475-2859-11-115>

409 Saint-Amans, S., Girbal, L., Andrade, J., Ahrens, K., Soucaille, P., 2001. Regulation of

410 Carbon and Electron Flow in *Clostridium butyricum* VPI 3266 Grown on Glucose-Glycerol

411 Mixtures. *J. Bacteriol.* 183, 1748–1754. <https://doi.org/10.1128/JB.183.5.1748-1754.2001>

412 Salvador, A.F., Cavaleiro, A.J., Paulo, A.M.S., Silva, S.A., Guedes, A.P., Pereira, M.A.,

413 Stams, A.J.M., Sousa, D.Z., Alves, M.M., 2018. Inhibition studies with 2-

414 bromoethanesulfonate reveal a novel syntrophic relationship in anaerobic oleate degradation.

415 *Appl. Environ. Microbiol.* <https://doi.org/10.1128/AEM.01733-18>

416 Sikora, A., Baszczyk, M., Jurkowski, M., Zielenkiewicz, U., 2013. Lactic Acid Bacteria in

417 Hydrogen-Producing Consortia: On Purpose or by Coincidence?, in: Kongo, J.M. (Ed.),

418 Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes. InTech.

419 <https://doi.org/10.5772/50364>

420 Wang, H., Xu, J., Sheng, L., Liu, X., Zong, M., Yao, D., 2019. Anaerobic Digestion

421 Technology for Methane Production Using Deer Manure Under Different Experimental

422 Conditions. *Energies* 12, 1819. <https://doi.org/10.3390/en12091819>

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424 Declaration

425 Ethics approval and consent to participate

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427 Consent for publication

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429 Competing interests

430 The authors declare that they have no competing interests.

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Authors' contributions

All authors contributed intellectually via scientific discussions during the work and have read and approved the final manuscript. Pereira MA and Silva ARM designed the study. Silva ARM executed the experimental work, interpreted the data and wrote the manuscript. Abreu AA and Salvador AF participated in the experimental work and in data interpretation. Alves MM, Neves IC and Pereira MA contributed to data interpretation and reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The authors confirm that all relevant data are included in the article and/or its additional information files.

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Figure captions:

Figure 1. Cumulative hydrogen production and fermentative products produced in the batch assay with different zeolite amounts to biomass (in VS) ratios (0.26, 0.13, 0.065 g g⁻¹) and for the control (without zeolite). (A) Glucose consumption: ■ 0.26 g g⁻¹; ▲ 0.13 g g⁻¹; ● 0.065 g g⁻¹; ✕ control. Arabinose consumption: □ 0.26 g g⁻¹; △ 0.13 g g⁻¹; ○ 0.065 g g⁻¹; □ control. Hydrogen production: □ 0.26 g g⁻¹; ▲ 0.13 g g⁻¹; ● 0.065 g g⁻¹; ✕ control. (B), (C) and (D) Lactic acid, Acetic acid and formic acid production, respectively: ■ 0.26 g g⁻¹; ▲ 0.13 g g⁻¹; ○ 0.065 g g⁻¹; ✕ control.

Figure 2. Effect of HRT and zeolite amounts in the bioreactor treating arabinose and glucose. (A) ● Hydrogen production rate and — HRT. (B) ■ Arabinose and ◆ glucose consumption and soluble fermentation products: ○ Formic acid; □ Acetic acid; ▲ Lactic acid; ● n-butyrate acid; ◇ Propionate acid.

Figure 3. Microbial community composition in the bioreactor treating arabinose and glucose. Only the class and specie of the microorganisms with relative abundance above 1% are represented.

Figure 4. Cumulative hydrogen production for the zeolite/biomass (in VS) ratio of □ 0.26 g g⁻¹, ▲ 0.13 g g⁻¹ and for the ● control, and respectively lactic acid production for the zeolite/biomass (in VS) ratio of ■ 0.26 g g⁻¹, ▲ 0.13 g g⁻¹, and for the ○ control. ✕ Blank assay without zeolite and *Sargassum* sp..

Figure 1.

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Figure 2.

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