TÉCNICO LISBOA

Hydrogen production by *Clostridium butyricum*: improving production yields and assessing changes in the cell lipid profile during adaptation to furans and gallic acid

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ABSTRACT

With the current expansion of the energy sector, a constant demand for fossil fuels will continue, and an untenable future scenario will result from it. A potential alternative solution to fossil fuels might involve the use of hydrogen, which has a high energy content (120 MJ/kg) and produces a clean combustion. The aim of this work was to characterise the changes of the *Clostridium butyricum* DSM 10702 cells' lipid profile when exposed to the short- and long-term presence of selected inhibitor compounds - oxygen, gallic acid, 5-hydroxymethyl furfural (HMF), furfural - and to elucidate how do these changes correlate with cell growth and hydrogen production. Gallic acid, HMF and furfural are common constituents of lignocellulosic materials or are generated after acid pre-treatment of carbohydrate-containing biomass. Cells in the presence of 2.0 g/L of furfural presented a decrease in the optical density, after 12h of incubation, 70% lower than cells grown in similar concentrations of gallic acid and HMF. The cellular adaptation strategy to gallic acid and HMF/Furfural was successfully accomplished, since a higher hydrogen production yield was obtained for the best adapted cells in all cases. In the fermentation of carob pulp extract by cells adapted to gallic acid, a hydrogen yield of 3.63 mol/mol glucose eq. was achieved, whereas only 3.04 mol/mol glucose eq. was obtained from non-adapted cells. With the adaptation of *C. butyricum* cells to increased concentration of HMF/furfural, the hydrogen production yield from *Spirogyra* hydrolysate increased from 0.85 to 1.71 mol/mol glucose. In conclusion, the results showed that the cellular adaptation mechanisms to gallic acid and HMF/Furfural allowed a cellular adaptation to carob pulp extract and *Spirogyra* hydrolysate constituents, resulting in an improvement of the hydrogen production.

Keywords: Biohydrogen; Carob pulp; *Spirogyra* hydrolysate; Natural inhibitors; Cellular adaptation

Introduction

The term biofuel or biorenewable fuel is referred to as solid, liquid or gaseous fuels that are mainly produced from biomass. Some examples of biofuels are biodiesel, bioethanol, biogas and biohydrogen (Demirbas 2008). Hydrogen (H_2) is a promising alternative to fossil fuels with a high energy content (120 MJ/kg) associated to a clean combustion only producing water as by-product (Ferreira et al. 2013a; Batista et al. 2014). More specifically, biohydrogen, is produced from biorenewable feedstocks such as plants and microalgal by biological, biochemical and biophotolytical processes (Demirbas 2009). Biological production of H_2, using microorganisms, is an area of potential technological development, capable of using a variety of renewable resources (Levin 2004). *Clostridium* sp. are frequently associated to H_2 production, with reported yields of 0.73–3.1 mol H_2/mol of sugar (Ferreira et al. 2013a). In particular, *C. butyricum* is a good example of a well-known H_2 producer, together with butyric acid and acetate (Chong et al. 2009).

Concerning the fermentation conditions, the type and concentration of the organic substrate play an important role in H_2 production by *C. butyricum*, with limited conversion efficiency above 10 – 12 g/L of initial carbohydrate concentration already observed (Junghare et al. 2012; Hu et al. 2013). Oxygen also affects the fermentation performance, since strict anaerobes, like *C. butyricum,* are incapable of growing in aerated environments and sometimes are even intolerant to low amounts of oxygen (Kawasaki et al. 1998).

The choice of the type of substrate is mainly dependent on its availability and composition in terms of lipids, proteins, moisture, and carbohydrates in the soluble form (Ghimire et al. 2015). The carob pulp is an example of a substrate with great potential, since almost 50 % (w/w) of its content are sugars (mainly sucrose, glucose and fructose) (Batlle and Tous 1997). Therefore, carob pulp can be considered as an excellent raw material for ethanol production or fermentative H_2 production (Fountoulakis et al. 2014).

Another substrate with great potential is microalgal biomass, which is being used as an alternative feedstock for the production of crop-based biofuels, due to higher biomass productivities, growth capability in non-arable land with non-potable water, and for not competing with food crops (Gouveia and Oliveira 2009; Pittman et al. 2011; Ferreira et al. 2013b). Nevertheless, phenolic and furanic compounds, which are commonly present in fermentation substrates from lignocellulosic origin and/or are produced after acid pretreatment of cellulosic and hemicellulosic biomass, are being described as toxic to cells (Monlau et al. 2014). Thus, the inhibitory effect of these compounds was also studied in this work. The main purpose of this study was to understand how physiological adaptive strategies developed by *C. butyricum* cells during H_2 production, simulated by the presence of inhibitory compounds, could be used to increase the H_2 production yield from carob and microalgal biomass.

Materials and methods

Microorganism: The strain used in this study was *Clostridium butyricum* DSM 10702 purchased from the Deutsche Sammlung von Mikroorganismen und Zelkuturen (DSMZ, Germany).

Culture media: C. butyricum was pre-cultured in Reinforced Clostridial Medium (RCM, Oxoid), at an inoculum concentration of 1% (v/v), and cultured overnight at 37 ºC, under static conditions (G24 Envir. Incubator). Afterwards, for the fermentation assays,

Basal Medium 1 (BM1) adapted from (Moura et al. 2007), was then used as the fermentation medium. BM1 medium without carbohydrate supplementation was deoxygenated with N_2, with the pH adjusted to 6.8. 20 mL of the medium was then distributed by 120 mL serum bottles, pre-flushed with N_2, which were sealed with rubber stoppers and aluminium crimp caps before autoclave sterilization at 121ºC for 20 minutes.

Fermentation assays: The BM1 was supplemented with 10 g/L of total sugars of glucose, carob pulp aqueous extract, and *Spirogyra* hydrolysate (the latter two were formerly prepared for other works) (Lima 2012; Pacheco et al. 2015). The flasks were incubated at 37 ºC and 150 rpm in the previously mentioned incubator. The fermentation was monitored between 0 and 144h with measurement of the final pH, biogas composition and production, and optical density (OD) at 600 nm (Genesys 20).

Toxicity assays: A pulse of air, gallic acid, furfural and HMF was applied to mid-exponentially grown cells. An aeration assay was first conducted with aeration volumes of 1 and 100 mL. Then the tested concentrations of the inhibitors compounds were the following: Gallic acid - 0.5, 1.0, 2.5 and 5.0 g/L; Furfural and HMF – 0.5, 1.0, 2.0 and 3.0 g/L. Two samples of 1 mL from each of cell suspension were taken 0.3, 1, 3 and 6 hours after the addition of the respective inhibitor compound, to characterise in terms of lipid profile.

Adaptation to inhibitors: A stepwise adaptation strategy was used to increase the natural tolerance of *C. butyricum* cells to the inhibitor compounds. Once cells were able to grow under a certain concentration of inhibitor, they were used to inoculate fresh fermentation medium with an increasing initial concentration of the inhibitor. One adaptation series started with 0 g/L of gallic acid and increased consecutively to 0.5, 1.0, 2.0, 2.5, 3.0 and 3.5 g/L. The other adaptation assay started with 0 g/L of HMF and furfural, and the subsequent concentrations were 0.25, 0.5, 0.75, 1.0, 1.5 g/L. Between each passage, the gallic acid and HMF+furfural adapted cells were also used to inoculate BM1 media supplemented with carob pulp aqueous extract or *Spirogyra* hydrolysate (10 $g_{total\ sugars}$/L), respectively.

Lipid extraction and analysis: The cells were collected at defined times along the assays and were washed twice with water and conserved at -16 ºC until lipid extraction. Bacterial lipids were extracted and fatty acids were methylated by an alkaline methylation process according to the instant FAME™ procedure from MIDI, Inc (USA).

Determination of fatty acids composition: The produced FAMEs were analysed on a 6890N gas chromatograph from Agilent Technologies, with a flame ionization detector and a 7683B series injector, equipped with a 25 m long Agilent J&W Ultra 2 capillary column from Agilent.

H_2 quantification: The biogas samples were collected from the headspace of the serum bottles with a gas-tight syringe. H_2 and CO_2 content was analysed in a gas chromatograph (Varian 430-GC) equipped with a thermal conductivity detector and fused silica column (Select Permanent Gases/CO 2 -Molsieve 5A/Borabound Q Tandem #CP 7430) with helium as carrier gas.

Total sugars quantification: Total sugars were determined by the phenol-sulfuric method as described by Masuko et al. (2005). In summary, 1 mL of the sample was added to 5 mL of sulfuric acid at

96 % (w/v) and 1 mL of phenol 5 % (w/v) in glass test tubes. The absorbance of the mixture was read at 490 nm in a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific). Glucose was used as standard for the calibration curve.

Total phenols quantification: Total phenols were determined by the Folin-Ciocalteu method Alhakmani et al. (2013). In glass test tubes, a volume of 150 μL of each sample was mixed with 750 μL of the Folin-Ciocalteu reagent (diluted 1:10) and 600 μL of sodium carbonate at 7.5 % (w/v). The absorbance of the mixture was read at 765 nm in the microplate reader mentioned above. Gallic acid was used as standard for the calibration curve.

Results and discussion

C. butyricum growth and lipid profile characterization

In order to compare the lipid composition of the cells exposed to different conditions, it was first required to determine the lipid profile of *C. butyricum* DSM 10702 under optimal, non-stressed growth conditions. Significant changes in the lipid composition of the cells were only observed during the exponential growth phase (Figure 1). This is in accordance to previously published data (Goldfine and Panos 1971; Dürre 2005), since during the exponential growth phase the cells are metabolically more active and may perform *de novo* synthesis of fatty acids necessary to respond to the growth conditions. During the exponential phase (4-8 hours), the cells increased the percentage of monounsaturated fatty acids (MUFA) and decreased the saturated and cyclopropyl fatty acids.

C. butyricum cellular response to natural inhibitors

In order to investigate the potential effect of natural inhibitors on *C. butyricum* cells, the cellular viability and lipid profile were assessed following exposure. Four natural inhibitors were selected for this study: air, gallic acid, HMF and furfural. The last three compounds are commonly present in lignocellulosic materials and microalgae biomass, and are described as presenting toxicity for the cells.

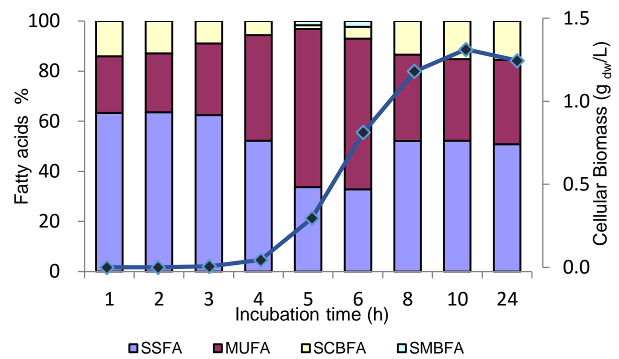

Figure 1. Fatty acid composition of C. butyricum cells at different growth stages (bars) and corresponding biomass concentration (diamonds). SSFAs straight-chain saturated fatty acids, MUFAs monounsaturated fatty acids, SCBFAs saturated cyclopropyl-branched fatty acids, SMBFAs saturated methyl-branched fatty acids

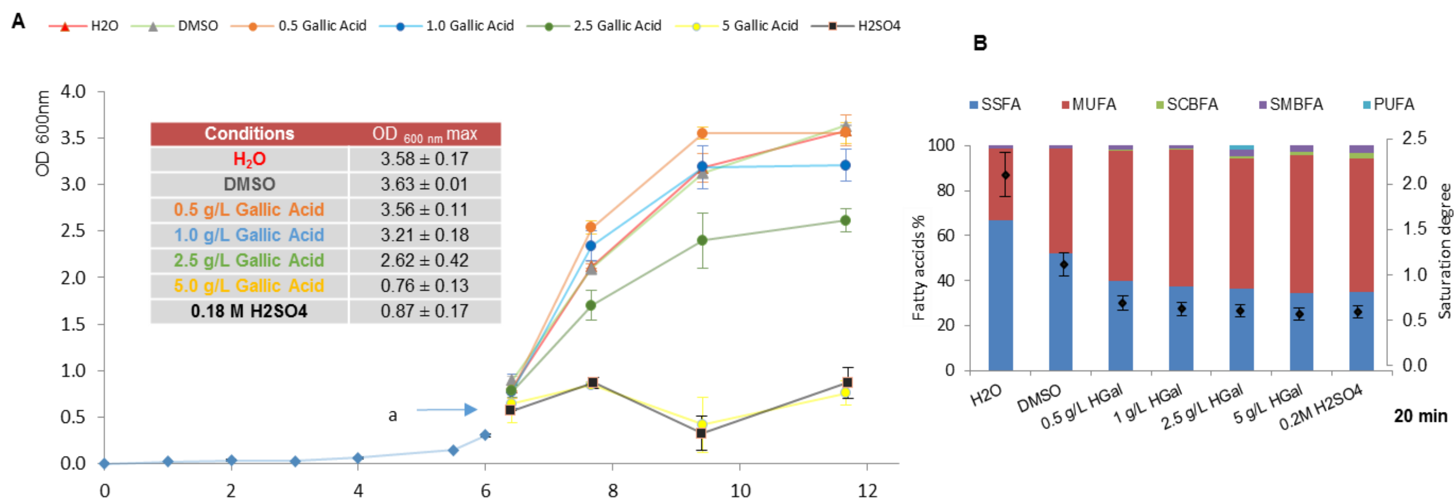

Figure 2. **A)** – *C. butyricum* growth curves under different tested conditions (lines). Cells were grown under optimal conditions until the beginning of the exponential phase (OD_{600nm} = 0.75) of growth, when a pulse of 1 mL of water, DMSO, gallic acid or H_2SO_4 solution was injected into the fermentation flask (a). **B)** Fatty acid composition of *C. butyricum* cells exposed to different conditions (bars) and corresponding calculated saturation degree (diamonds), after 20 minutes of exposure.

Inhibitor I – Oxygen assay: Obligatory anaerobic bacteria, like *Clostridium*, are incapable of growing in aerated environments and sometimes are even intolerant to low amounts of oxygen (Dürre 2005). In this study, the oxygen effect on the cellular growth of *C. butyricum* was assessed by promoting aeration of the culture at the beginning of the exponential growth phase. Globally, no growth inhibitory effect caused by oxygen was observed under the studied conditions. Since during the early exponential growth phase *C. butyricum* cultures have already attained a considerable cell density probably the volume of air injected into the fermentation flasks was insufficiently high to cause toxicity. This could be related to the low solubility of oxygen in water, associated with the increase of cellular biomass and reductant metabolites in the medium, hence decreasing even more the dissolution of oxygen in the medium.

Inhibitor II – Gallic acid assay: No substantial growth inhibition was observed for gallic acid at concentrations up to 1.0 g/L (Figure 2A). For a gallic acid concentration of 2.5 g/L, a decrease of 27% in the maximum OD_{600nm} was observed, compared to cells in the presence of H_2O and DMSO. Moreover, a decrease in the growth rate was observed with increasing gallic acid concentrations. The lipid profile was also characterised under different gallic acid concentrations (Figure 2B). The most evident alterations in the presence of gallic acid occurred after 20 minutes of exposure, with cells reducing the saturation degree by rapidly increasing the MUFAs content when compared to the cells exposed only to H_2O. This variation could be the result of a cellular adaptation mechanism to increase the membrane fluidity as response to the gallic acid and DMSO presence, since an increase of 47% in MUFAs for cells in the presence of DMSO was also noticed in relation to cells in the presence of H_2O.

Inhibitor III – Furfural assay: For furfural concentrations of 2.0 and 3.0 g/L a clear inhibitory effect was observed, whereas for the concentrations of 0.5 and 1.0 g/L no significant growth inhibition occurred (Figure 3A). Concerning the lipid profile

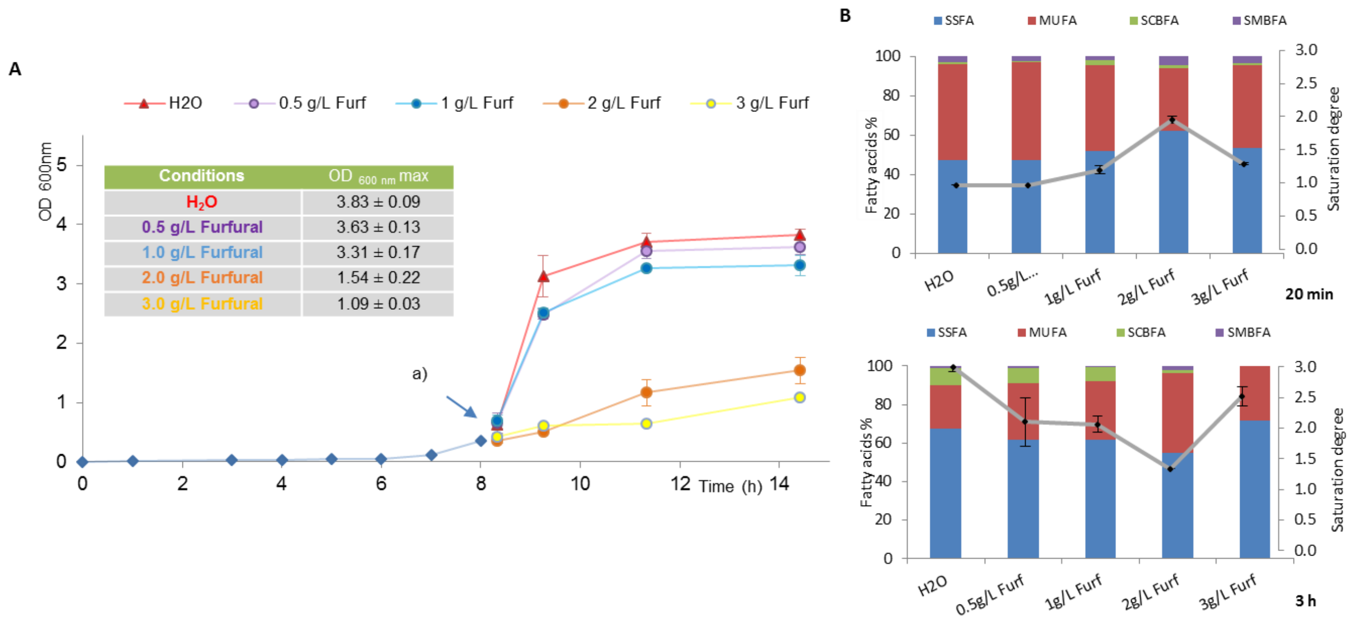

Figure 3. **A)** – *C. butyricum* growth under different tested conditions. Cells were grown under optimal conditions until the beginning of the exponential phase (OD_{600nm} = 0.75) of growth, when a pulse of 1 mL of water or furfural solution was injected into the fermentation flask (a). **B)** Fatty acid composition of *C. butyricum* cells exposed to different conditions (bars) and corresponding calculated saturation degree (line), after 0.3 and 3h of exposure.

modifications, after 20 minutes of exposure, cells doubled the saturation degree in response to furfural concentrations of 2.0 g/L (Figure 3B). When the exposure time increased to 3 h, the adaptation strategy apparently changed as well. Cells in this case decreased 55 % the saturation degree in the presence of a furfural concentration of 2.0 g/L, probably due to a *de novo* fatty acid synthesis in contrast to a possible enzymatic alteration at 20 minutes. For the furfural concentration of 3.0 g/L, only slight growth was observed, with a final OD_{600nm} of 1 after 14h of incubation.

Inhibitor IV – HMF assay: The inhibitory effect of HMF seemed to gradually increase with increasing concentrations, although without ceasing the cellular growth for the tested concentrations (Figure 4). For the concentration of 3.0 g/L, the highest (34%) decrease in the maximum OD_{600nm}, and an extended lag phase until 9h of incubation, was observed in comparison to cells grown in the presence of H_2O. Comparing the effect of HMF and furfural on cellular growth, the former presented a lower growth inhibitory effect than furfural. This lower toxicity of HMF has also been reported in other studies, and it is probably linked to its higher molecular mass (126.11 g/mol vs 96.08 g/mol from furfural), which makes the diffusion rate into the cells lower than for furfural (Quéméneur et al. 2012). Regarding the lipid profile characterisation, no significant variations were

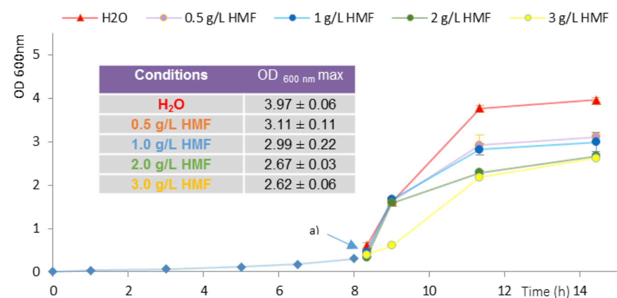

Figure 4. *C. butyricum* growth with different HMF concentrations. Cells were grown under optimal conditions until the beginning of the exponential phase (OD_{600nm} = 0.75) of growth, when a pulse of a 1 mL HMF solution was injected into the fermentation flask (a).

observed in the presence of different concentrations of HMF (data not shown). This might be related with the more hydrophilic properties, due to the hydroxyl group, making HMF less prone to interact with the cellular membrane than furfural (Almeida et al. 2009).

Determination of optimal carbohydrate concentration for H_2 production

H_2 production reached a maximum yield of 2.28 ± 0.01 mol H_2 / mol glucose for an initial glucose concentration of 10 g/L (Table 1). The values presented in the literature range from 1.2 to 2.4 mol H_2 / mol glucose and are comparable with the maximum yield obtained (Chong et al. 2009; Cai et al. 2010; Hu et al. 2013). The maximum H_2 yield obtained in this work is followed by a high glucose conversion efficiency of 93 % and a considerable cumulative H_2 production volume of 66 mL.

Adaptation of *C. butyricum* cells to inhibitors

A stepwise adaptation strategy to inhibitors was used by growing *C. butyricum* cells in the presence of increasing concentrations of gallic acid or HMF and furfural. Once cells were able to grow in a given concentration of inhibitor, the cells that reached the mid-exponential growth phase were used to inoculate fresh medium supplemented with carob pulp extract and *Spirogyra* hydrolysate naturally containing phenolic and furanic compounds, respectively.

Cell adaptation to gallic acid: C. butyricum was cultured in the presence of the following gallic acid concentrations: 0.0, 0.5, 1.0, 2.0, 2.5, 3.0 and 3.5 g/L. At gallic acid concentrations higher than 3.5 g/L, no growth was observed. In the fermentation medium supplemented with carob pulp extract, the highest H_2 production yield was attained with the cells adapted to 2.0 g/L of gallic acid (3.63 mol H_2 / mol eq. glucose) after 24 h of incubation (Table 2). This H_2 production yield was higher than what is commonly reported in other studies using biorenewable fermentable biomass, nevertheless it

Table 1. Cellular performance of H_2 production and substrate consumption by *C. butyricum* under different initial glucose concentrations.

$[Glucose]_{initial}$ (g/L)	Molar Yield (mol H_2/mol Glucose)	Glucose conversion (%)	Biogas purity ratio (H_2 : CO_2)	Cumulative H_2 production (mL)
10	2.28 ± 0.01	93	2.76 ± 0.03	66.1 ± 0.2
20	1.32 ± 0.13	81	2.31 ± 0.18	67.0 ± 6.6
30	0.84 ± 0.09	78	2.65 ± 0.26	61.6 ± 6.5
40	1.42 ± 0.18	50	2.36 ± 0.21	89.5 ± 12.0
50	1.06 ± 0.18	45	2.31 ± 0.12	75.7 ± 12.5
60	0.87 ± 0.15	44	1.99 ± 0.09	71.8 ± 12.4

is still lower than the theoretical H_2 yield from sucrose (8 mol H_2/mol sucrose) (Lo et al. 2008). Moreover, carob pulp composition in fermentable carbohydrates is mainly composed of a mixture of sucrose, fructose and glucose (Carvalheiro et al. 2011). Generally, it seems that the cellular adaptation to a gallic acid concentration of 2.0 g/L might induce alterations at the membrane composition level, by facilitating the substrate transport through the membrane, or by

Table 2. Hydrogen production yield and cumulative production by *C. butyricum* using carob pulp as substrate, after cellular adaptation to increasing concentrations of gallic acid.

Gallic acid concentrations used for *C. butyricum* adaptation (g/L)	Molar Yield by adapted *C. butyricum* cells (mol H_2/mol eq. Glucose)		Cumulative H_2 production by adapted *C. butyricum* cells (mL)	
	8 h of incubation	24 h of incubation	8 h of incubation	24 h of incubation
Control (0)	1.60	3.04	15.6	71.6
0.5	1.64	2.87	30.5	58.3
1.0	1.67	3.32	34.6	70.0
2.0	2.36	3.63	42.1	70.4
2.5	1.99	3.12	34.0	56.9
3.0	1.94	2.77	35.0	44.3
3.5	1.45	2.28	15.9	47.7

activating metabolic pathways that help to degrade carob pulp constituents.

Cell adaptation to HMF and furfural: The following concentrations of each inhibitor, HMF and furfural, were tested together to study *C. butyricum* cells adaptation: 0.0, 0.25, 0.5, 0.75, 1.0 and 1.5 g/L. While HMF alone seemed to present low toxicity levels for these concentrations (Figure 4), a combination of furfural and HMF was used in the adaptation assays since they are commonly found together in the majority of the acid hydrolysates of lignocellulosic materials (Veeravalli et al. 2013). After 24h of fermentation, the highest H_2 production yield (1.71 mol H_2 / mol eq. glucose) was obtained for the cells adapted to the highest concentration of HMF and furfural tested (1.5 g/L; Table 3). The maximum cumulative H_2 volume was about 45% higher than the volume produced by control cells cultured in the absence of these inhibitors, and also higher than the one obtained in previous studies using *Spirogyra* hydrolysate (Pacheco et al. 2015). The results thus indicate that cellular adaptation mechanisms to HMF/Furfural lead to an adaptation to the *Spirogyra* hydrolysate constituents, resulting in a higher H_2 production yield. This higher H_2 production yield was observed for all the tested HMF/Furfural concentrations, indicating that cells grew and were successfully adapted to all the studied concentrations.

Table 3. Hydrogen production yield and cumulative production by *C. butyricum* using *Spirogyra* hydrolysate as substrate, after cellular adaptation to increasing concentrations of a combination of HMF and furfural.

Combination of HMF and furfural concentrations used for *C. butyricum* adaptation (g/L)	Molar Yield by adapted *C. butyricum* cells (mol H_2/mol eq. Glucose)	Cumulative H_2 production by adapted *C. butyricum* cells (mL)
	24 h of incubation	24 h of incubation
Control (0.0)	0.85	15.9
0.25	1.21	23.5
0.5	1.27	24.3
0.75	1.26	23.7
1.0	1.20	20.7
1.5	1.71	28.0

C. butyricum *best adapted cells versus control cells:* By comparing the H_2 production yield for the best adapted and non-adapted *C. butyricum* cells, it is possible to conclude that for both substrates, carob pulp extract and *Spirogyra* hydrolysate, the adapted cells apparently achieved higher H_2 yields (Table 4). Furthermore, it was also observed that the H_2 production yield was higher when using carob pulp as substrate than *Spirogyra* hydrolysate. Concerning the cellular lipid composition, only for the cells adapted to HMF/Furfural a decrease on MUFAs and an increase of PUFAs content was observed, in comparison to non-adapted cells (Figure 5). The synthesis of PUFAs is not a general mechanism present in all type of cells, being associated with few bacteria, such as marine bacteria and *Rhodococcus erythropolis* under extreme conditions (de Carvalho 2012; de Carvalho et al. 2014). Moreover, the presence of PUFAs is also associated with the maintenance of cytoplasmic membrane fluidity, probably used by *C. butyricum* cells to maintain cellular integrity. For the cells adapted to 2.0 g/L of gallic acid only slight variations were observed in SCMFA and SBMFA content when compared to the non-adapted cells. This is indicative that the cellular adaptation mechanism to gallic acid, which resulted in an increase of the H_2 production yield, was not possible to be monitored through the lipid profile analysis conducted in this study.

Table 4. Comparison of the H_2 production yields between non-adapted and best adapted *C. butyricum* cells to the combination of HMF/Furfural (1.5 g/L) and gallic acid (2 g/L), after 24h of incubation.

	Molar Yield (mol H_2/mol eq. Glucose)	
	HMF/Furfural adaptation assay (*Spirogyra* hydrolysate)	Gallic acid adaptation assay (carob pulp)
Non-adapted cells	0.85	3.04
Adapted cells	1.71	3.63

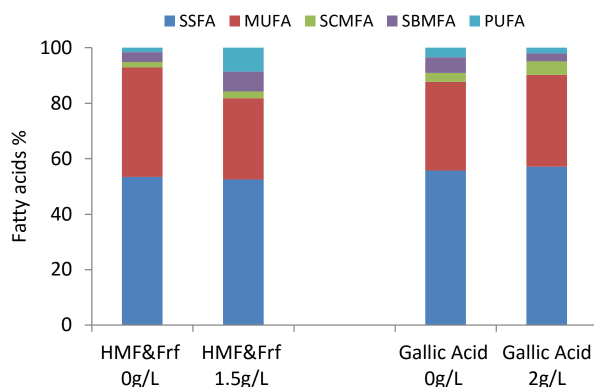

Figure 5. Comparison of the fatty acid composition between non-adapted and best adapted *C. butyricum* cells to HMF/Furfural (1.5 g/L) and gallic acid (2 g/L).

Conclusions

In this work, two main aspects were studied: the physiologic response of *C. butyricum* cells to stress agents, and the H_2 production from different substrates. From the cellular lipid characterisation during growth, it was possible to conclude that, as expected, the main alterations in fatty acid composition of the cells occurred during the exponential growth phase, when cells are metabolically more active, and also more exposed to stress on the cellular membrane.

Among the four natural inhibitors studied in this work, the agent which presented the highest growth inhibitory effect on *C. butyricum* cells was furfural, since cells in the presence of 2.0 g/L were incapable of a substantial growth.

From the range of the tested initial carbohydrate concentrations was possible to conclude that the concentration which resulted on the highest H_2 production yield was 10 g/L.

Finally, *C. butyricum* cells were submitted to a stepwise adaptation to phenolic and furaldehyde compounds and the effect on H_2 production from carob pulp and *Spirogyra* hydrolysate substrates fermentation was evaluated. A maximum H_2 production yield of 3.63 mol H_2 / mol eq. glucose was achieved by the cells adapted to 2.0 g/L of gallic acid. Concerning the cellular adaptation to HMF/Furfural, the maximum H_2 production yield almost duplicated from 0.85 to 1.71 mol H_2 / mol eq. glucose with cells adapted to a HMF/Furfural concentration of 1.5 g/L. Moreover, it was also possible to confirm that carob pulp is an excellent fermentable carbon source to produce biohydrogen, allowing high yields.

In conclusion, the main objectives proposed for this work were fulfilled by using physiological data of *C. butyricum* cells to increase biohydrogen production from carob pulp and *Spirogyra* hydrolysate.

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