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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"Continuous effort – not strength or intelligence – is the key to unlock our potential"

Winston Churchill

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Resumo

Com a atual expansão do sector energético a constante dependência de combustíveis fósseis irá conduzir a um cenário de insustentabilidade. Uma possível alternativa aos combustíveis fósseis poderá passar pela utilização de hidrogénio, que apresenta um elevado conteúdo energético (120 MJ/kg) associado à capacidade de produzir uma combustão "limpa". O objetivo deste trabalho prende-se com a caracterização do perfil lipídico das células de *Clostridium butyricum* DSM 10702, quando expostas à presença de determinados compostos inibitórios, tais como oxigénio, ácido gálhico, 5- Hydroximetil furfural (HMF) e furfural. Tanto ácido gálhico, como HMF e furfural são constituintes comuns de materiais lignocelulósicos, ou são criados após pré-tratamentos em meio ácido de biomassa rica em carbohidratos. Para uma concentração de furfural de 2.0 g/L verificou-se um maior grau de inibição celular, quando comparado com o efeito de ácido gálhico e HMF, nas concentrações de 2.5 e 3.0 g/L, respetivamente. As células na presença de furfural com uma concentração de 2.0 g/L apresentaram um decréscimo na densidade ótica de 70 %, após 12h de incubação, relativamente ao efeito de ácido gálhico e HMF. Considerando a estratégia de adaptação celular ao ácido gálhico e à mistura de HMF/furfural, é possível afirmar que foi completada com relativo sucesso, uma vez que um maior rendimento de hidrogénio foi alcançado para as células melhor adaptadas, em ambos os casos. Da fermentação de extrato de polpa de alfarroba um rendimento de hidrogénio de 3.63 mol / mol eq. glucose foi alcançado, enquanto que somente 3.04 mol/mol eq. glucose foi obtido para células não adaptadas. Com a adaptação para concentrações crescentes de HMF/furfural, o rendimento de produção de hidrogénio a partir de hidrolisado de *Spirogyra* aumentou de 0.85 para 1.71 mol / mol eq. glucose. Relativamente à composição lipídica durante a adaptação, somente para as células adaptadas à concentração de 1.5 g/L de HMF/Furfural verificou-se um decréscimo no conteúdo de ácidos gordos monoinsaturados e um aumento nos ácidos gordos polinsaturados, em comparação com as células não adaptadas. Resumindo, os resultados obtidos sugerem que a adaptação celular aos constituintes tóxicos, presentes na polpa de alfarroba e no hidrolisado de *Spirogyra,* resultou num aumento de rendimento de hidrogénio produzido

Palavras-chave: Biohidrogénio; Fermentação escura; Polpa Alfarroba; Hidrolisado de *Spirogyra*; Inibidores naturais; Adaptação celular

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. A furfural concentration of 2.0 g/L inhibited cellular growth, whereas 2.5 and 3.0 g/L of gallic acid and HMF, respectively, were well tolerated by *C. butyricum*. 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 eq. Concerning the cellular lipid composition, only for the cells adapted to 1.5 g/L of HMF/Furfural, a decrease on MUFAs and an increase of PUFAs content was observed, in comparison to non-adapted cells. 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; Dark fermentation; Carob pulp; *Spirogyra* sp. hydrolysate; Natural inhibitors; Cellular adaptation

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Abbreviations

GHG: Green House Gases

NOx: Mono-nitrogen oxides

BioH_2: Biohydrogen

PNS: Purple non-sulfur

HMF: 5-(Hydroxymethyl)furfural

BM1: Basal Medium 1

RCM: Reinforced *Clostridium* Medium

OD: Optical Density

MetOH: Methanol

FAME: Fatty Acid Methyl Esters

MUFA: Monounsaturated Fatty Acid

MIC: Minimum Inhibitory Concentration

DMSO: Dimethyl sulfoxide

PUFA: Polyunsaturated Fatty Acid

1 State of the art

With the constant use of fossil fuels an untenable future scenario will arise, mainly due to the increment in greenhouse gases (GHG) emissions and their impact on global warming, the declining of petroleum reserves, and the political and economic instability [1, 2]. Currently, new energy sources are sought as a potential alternative to reduce fossil fuels dependence in the present and near future [3].

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 [2]. Biofuels offer a number of technical and environmental benefits over conventional fossil fuels. The benefits include GHG reductions encompassing reduced carbon dioxide (CO_2) emissions, diversification of the fuel sector, feedstock renewability, diminishing of the fossil fuel importations and energy external dependency [4]. Nevertheless, some disadvantages are still present on the use of certain liquid biofuels (*e.g.* biodiesel and bioethanol) like for instance the food *vs* fuel issue, for causing the rise of food prices, together with a lack of a cost efficient technology [5]. Additionally, some of these biofuels when combusted produce even higher pollutant emissions than fossil fuels, such as mono-nitrogen oxides (NO_x) species [6].

To overcome some of these disadvantages either from fossil fuels or liquid biofuels, it is mandatory to identify alternative renewable sources of fuels that are potentially carbon neutral [1]. Such potential future solution, might involve the use of hydrogen (H_2) that appears as an alternative fuel and energy carrier, with several technical, socio-economic and environmental benefits. It has the highest energy content per unit weight of any known fuel (120 MJ/kg) and its combustion only produces water as by-product. Therefore, H_2 is being studied to be used in combustion engines and fuel-cell electric vehicles, and so it is expected that its demand increases significantly in the near future [3, 7].

Biofuels derived from cheap carbon sources represent an enormous advantage over simple fermentable sugars, since several different waste resources and secondary products can be used as fermentative feedstock [8]. *Clostridium butyricum* is one microorganism described as capable of utilizing complex carbon sources, such as lignocellulosic materials and microalgae biomass, and at the same time producing H_2 [8]. Nevertheless, biohydrogen production processes are not yet economically profitable, demanding an investment in research and development in this field [9].

2 Theoretical background

2.1 Biohydrogen

Hydrogen (H_2) is the element of greatest abundance in the universe and is an alternative source of clean and renewable energy. No atmospheric pollutants such as carbon, nitrogen and sulfur oxides are produced when it is used as a fuel [10, 11]. H_2 can be produced through a wide number of conversion technologies that include thermochemical processes (e.g. gasification, steam methane reforming, partial oxidation of oil) from fossil or renewable feedstock, and electrolytical and splitting processes (water electrolysis and photolysis) [12]. H_2 can also be obtained through biological conversion by photosynthesis, photo-heterotrophic and dark-fermentation. Although electrolysis of water, and steam reformation are presently the main processes of H_2 production, there is an increase interest in the biological hydrogen production [2, 7, 13]. This is because current H_2 manufacturing processes are environmentally unfriendly with a considerable cost, due to high energetic demand and fossil fuels usage [12].

Biohydrogen refers to a renewable biofuel originated from biorenewable feedstock like plants and microorganisms by chemical, biological, biochemical and biophotolytical methods [10].Concerning the biohydrogen production processes, they are divided into bio-H_2, produced from chemical and thermochemical processes using renewable energy sources, and bioH_2 produced from microorganisms. [10]. Biological production of H_2, using microorganisms, is a potential area of technological development capable of using a variety of renewable resources [13]. Moreover, biological production processes are found to be more environmentally friendly and less energy intensive when compared with thermochemical and electrochemical processes [2]. BioH_2 can be generated by biophotolysis of water using microalgae, photodecomposition of organic matter by photosynthetic bacteria, and fermentation of organic compounds [10].

2.1.1 Direct biophotolysis

Biophotolysis is the action of light on biological systems that results in the dissociation of a substrate, usually water into molecular hydrogen and oxygen [10]. The general reaction (1) illustrates this process of hydrogen production [13].

$$2\,H_2O \xrightarrow{h.v} 2\,H_2 + O_2 \qquad (1)$$

Direct biophotolysis process is similar to the ones found in plants and algal during photosynthesis. In particular, green algae are capable of evolving H_2 under photosynthetic conditions, by using bidirectional hydrogenases as terminal proton reductases to equilibrate ATP production [14]. Light energy absorbed by photosystem II generates electrons which are transferred to ferredoxin, using light energy absorbed by photosystem I, and finally a reversible hydrogenase accepts electrons from ferredoxin to generate H_2 [13]. However, only under specific conditions hydrogen production is possible, since Fe-hydrogenase activity is highly sensitive to O_2 presence [15]. In specific culture conditions, the rate of H_2 production by the green algae *Chlamydomonas reinhardtii* was approximately 2.5 mL H_2 / L.h [16].

2.1.2 Indirect biophotolysis

Cyanobacteria, also known as blue-green algae, are a large group of photoautotrophic microorganisms that contain several photosynthetic pigments, and are able to perform oxygenic photosynthesis [13]. Moreover, many species of cyanobacteria possess several enzymes involved in hydrogen metabolism, including nitrogenases, uptake hydrogenases, and bi-directional hydrogenases, hence being capable of producing both O_2 and H_2 [14].

In indirect biophotolysis method, the problems associated with Fe-hydrogenase sensitivity to oxygen are surpassed by a spatial and/or temporal separation between O_2 and H_2 evolution [17]. Cyanobacteria have the capability of using atmospheric CO_2 as carbon source and solar energy as an energetic source (Equation 2). After taking up CO_2 and converting it into organic carbon source (glucose), this organic carbon is then used for hydrogen production (Equation 3) [15].

$$6\,H_2O + 6\,CO_2 \xrightarrow{h.v} C_6H_{12}O_6 + 6\,O_2 \qquad (2)$$

$$C_6H_{12}O_6 + 6\,H_2O \xrightarrow{h.v} 12\,H_2 + 6\,CO_2 \qquad (3)$$

According to several studies with cyanobacteria strains, the rates of H_2 production seems to be higher than in green algae [13]. In a study conducted with a mutant strain of *Anabaena variabilis*, the reported hydrogen production rate was 13 mL H_2/ L.h [18].

2.1.3 Photo-fermentation

Purple non-sulfur (PNS) bacteria produce molecular H_2 catalyzed by a nitrogenase under nitrogen-deficient conditions using light energy and reduced compounds (organic acids) (Equation 3) [13]. These photoheterotrophic bacteria have been investigated for their potential to convert light energy into H_2 using waste organic compounds as substrate [19]. Photo-fermentation is an attractive method for the total conversion of feedstock that is only partially oxidized during dark fermentation and hence increases the yield of hydrogen production from those substrates [20].

Hydrogen production occurs mainly in four species of PNS bacteria, which are *Rhodospirillum rubrum*, *Rhodopseudomonas palustris*, *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* [21]. Regarding the photo-fermentation process, PNS bacteria presents a more elaborated photosynthetic complex when compared to cyanobacteria and algae [19, 21]. In these bacteria, the nitrogenase enzyme plays a key role in hydrogen production and in nitrogen reduction to ammonia. Therefore, a set of specific conditions must be met for an optimum hydrogen production, since, for instance, stressful nitrogen concentrations induce an increase of hydrogen [22]. Some of the main advantages of this process include high substrate conversion rate, no oxygen inhibition, use of wider wavelength of light, and ability to use different organic substrates, including organic waste feedstock [19].

In the literature, hydrogen production rates differ according to the type of reactor, operation and strain, but for a *R. sphaeroides* cultivated in a photobioreactor, a production rate of 24 mL H_2/ L.h was reported [23].

2.1.4 Dark fermentation

Dark fermentation is the fermentative conversion of organic substrates, such as carbohydrates, to bioH_2, through the anaerobic metabolism pathway of pyruvate (Equation 4 and 5) [10, 24]. When hydrogen is produced, soluble metabolites such as volatile fatty acids and alcohols are produced simultaneously, together with a gaseous stream of carbon dioxide (CO_2) [25]. Depending on the fermentation pathway and end-product(s) it is possible to obtain different H_2 production yield per mole of glucose. When acetic acid is the end-product, a theoretical maximum of four mole of H_2 per mole of glucose is obtained. But when butyrate is the end-product, a theoretical maximum of two moles of H_2 per mole of glucose is obtained [13]. These remaining soluble metabolites can be further utilized via photo-fermentation resulting in more H_2 production at the expense of light energy. In fact, the combination of dark and photo-fermentation could increase hydrogen production yield up to a maximum of 12 moles of H_2 per mole of glucose. Therefore, a two-stage process combining dark and photo-H_2 fermentation has been largely investigated and considered as an effective and efficient system [26].

$$\text{Piruvate} + \text{CoA} + 2Fd_{(ox)} \rightarrow \text{Acetil-CoA} + 2Fd_{(red)} + CO_2 \quad (4)$$

$$2H^+ + Fd_{(red)} \rightarrow H_2 + Fd_{(ox)} \quad (5)$$

Dark fermentation has several advantages when compared to other biological production methods, including the capability to produce H_2 without a light source, with a higher production rate, process simplicity and lower energy input [27]. Nevertheless, several parameters have to be controlled to optimize the H_2 production, namely pH, organic substrate composition and concentration, process temperature and H_2 partial pressure [13, 21]. One of the most common and best H_2 producers are clostridial strains, in particular *C. butyricum* due to its capability to use several types of organic substrates, and achieve relevant H_2 yields [7, 28]. Many studies in different operational conditions are reported, but in a recent batch experiment using *C. butyricum* and glucose as substrate, a H_2 production rate of 126 mL H_2 / L.h was achieved [29].

2.2 *C. butyricum*

Clostridia are anaerobic, gram-positive bacteria capable of fermenting organic compounds, including many simple and complex carbohydrates [30]. *Clostridium* sp. are frequently found in H_2 producing bacterial consortia, reporting yields of 0.73 – 3.1 mol H_2/mol of sugar [3]. Due to the sensitivity of strict anaerobic bacteria to dissolved oxygen, they are often cultivated together with facultative anaerobes [27]. Still, some studies proved that pure *C. butyricum* cultures attain higher H_2 production yields than in bacterial consortia or in co-culture with other *Clostridium* strains (Table 1), as reported by Hiligsmann and co-workers (2011) [31].

Table 1. Comparison of hydrogen production yields from glucose fermentation by different facultative and strict anaerobic strains and bacterial consortia. Adapted from [31].

Microorganisms	H_2 production yield (mol /mol glucose)
C. butyricum DSM 2477	1.40
C. pasteurianum DSM 525	1.05
C. thermosulfurigenes DSM 2229	1.02
Consortia used for food waste valorization	1.10
Consortia used for wastewater treatment	0.75
Escherichia coli ATCC10536	0.80

Clostridia can obtain energy from several types of organic substrates using different metabolic pathways, that are influenced by specific culture conditions [29]. *C. butyricum* is a good example of a well-known butyric acid producer together with acetate, H_2 and CO_2 [32]. Although different metabolic pathways allow Clostridia to produce energy from carbohydrates, such as the acetate, butyrate, ethanol, lactate or formate routes, only acetate and butyrate pathways implicate H_2 release [29, 32, 33].

In theory, the maximum yield of H_2 by *C. butyricum* during glucose fermentation would be attained if all acetyl-CoA would enter the acetate pathway [13, 29]. However, *C. butyricum* produces butyrate and acetate simultaneously, with glucose fermentation stoichiometry defined by equation (6) which leads to the conclusion that, under unstressed conditions, the butyrate metabolic pathway is more used than the acetate route [33]. Nevertheless, working parameters such as pH, substrate concentration, temperature, H_2 partial pressure, acetate and butyrate concentrations can influence growth rate and product concentration [32, 33].

$$2\ C_6H_{12}O_6 \longrightarrow C_4H_8O_2 + 3\ C_2H_4O_2 + 2\ CO_2 + 2\ H_2 \quad (6)$$

Particularly in the case of low H_2 partial pressure, the NADH produced during glycolysis can be reoxidized by a [FeFe] hydrogenase, hence increasing the H_2 production yield. On the contrary, at higher H_2 partial pressures, the NADH reoxidation reaction by this complex is unfavourable since the redox

potential of hydrogen (-410 mV at neutral pH, STP) is lower than NAD/NADH couple (-320 mV), thus promoting the butyrate pathway (Figure 1) [34, 35].

Pyruvate is an important compound in the metabolic network of *C. butyricum* (Figure 1) because it controls flux distribution towards lactate or acetyl-CoA which is the origin for other end products. Since lactate is produced by a NAD-independent lactate dehydrogenase from pyruvate, reoxidizing NADH into NAD, its production is unfavourable for H_2 production [33, 36]. A metabolic engineering strategy to increase H_2 yield is focused on promoting acetate pathway over other routes. For that, overexpressing acetate kinase and phosphotransacetylase, and knockout phosphotransbutyrylase together with butyrate kinase, is an approach to enhance acetate production and simultaneously decrease butyrate formation, respectively [36].

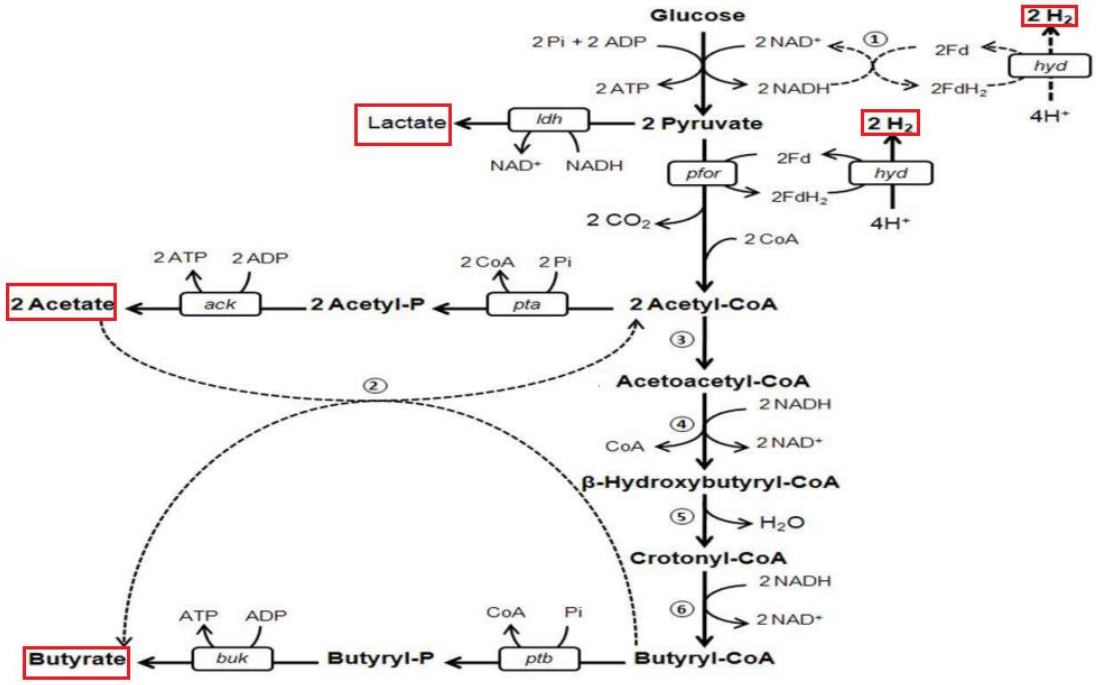


Figure 1. Clostridial-type fermentation in Clostridia. Inside red boxes are the possible final products. ***ldh***, lactate dehydrogenase; ***pfor***, pyruvate:ferredoxin oxireductase; ***pta***, phosphotransacetylase; ***ack***, acetate kinase; ***ptb***, phosphotransbutyrylase; ***buk***, butyrate kinase; ***hyd***, monomeric [FeFe]-H2ase; **1**, proposed enzyme NADH-ferredoxin oxireductase; **2**, proposed enzyme butyryl-CoA:acetate CoAtransferase; **3**, acetyl-CoA:acetyl-transferase; **4**, β-hydroxybutyryl-CoA dehydrogenase; **5**, crotonase; **6**, butyryl-CoA dehydrogenase; **Fd/FdH2**, oxidized/reduced ferredoxin; **NAD+/NADH**, oxidized/reduced nicotinamide adenine dinucleotide **ATP/ADP**, adenine triphosphate/adenine diphosphate; **Pi**, inorganic phosphorus; **CoA**, coenzyme A. Adapted from [33, 37].

2.2.1 *C. butyricum* and pH

The initial pH of the culture medium has an effect on enzymatic activity in microorganisms, since enzymes are active only in a specific pH range, and have maximum activity at an optimal pH value. pH has been reported as one of the key factors affecting H_2 production, profile of the organic acids produced, cell membrane transport behaviour, and cell lysis [33, 38].

In the case of *C. butyricum*, H_2, acetate and butyrate are commonly produced during the exponential growth phase, but when the population reaches the stationary growth phase, a major metabolic pathway switch takes place to convert excreted acetate into butyrate [33, 39]. The aim of recycling the acetate is to detoxify the medium by reducing total hydrogen ion concentration [33]. This specific enzyme (butyryl CoA-acetate transferase) responsible for acetate recycling, works at lower pH values (5.5 - 5) that are found at the end of the growth curve [33].

pH values higher than 6 are favourable for cell growth and butyric acid biosynthesis at the beginning of the exponential growth phase [40]. When pH starts to decrease, butyrate metabolic enzymes are shutdown, occurring a metabolic shift towards acetate and H_2 production at a pH range of 5.5 – 5.0 [39]. Therefore, pH control is important to influence the products of the fermentation. Moreover, the initial pH value also affects H_2 production potential and H_2 production rate. When using an initial pH value lower than 6, it was reported an increase in total H_2 production but a decrease in H_2 production rate, most likely due to the necessity of a longer cell adaptation period [39].

2.2.2 *C. butyricum* and substrate concentration

The type and the concentration of the organic substrate play an important role in H_2 production by *C. butyricum*. Previously published studies proved the significant effect of the initial substrate concentration on cellular growth and H_2 production [38]. For glucose assays, where substrate does not require hydrolysis, a similar effect is generally reported, namely for *C. butyricum*, limited conversion efficiency above 10 – 12 g/L of initial glucose concentration was observed [32]. Therefore, a decrease in the substrate conversion efficiency occurs with the increase of the initial glucose concentration [28, 38]. However, when glucose concentration is too low (5 g/L), the cumulative H_2 production is also low, because the fermentation process ceases at an early stage due to substrate depletion. On the other hand, for initial glucose concentrations higher than 12 g/L, the cumulative H_2 production tends to stabilize [28]. The excess of carbon source is mainly responsible for osmotic dehydration in microorganisms, substrate inhibition and product inhibition [38]. Hence, it is crucial to adjust the initial concentration of organic substrate before starting the fermentation [28, 33, 38].

2.2.3 *C. butyricum* and H_2 inhibition

As mentioned above, H_2 dissolved concentration has a significant influence on the fermentation process outcome. More specifically, H_2 partial pressure has a key role in the continuous H_2 synthesis. Thus, high H_2 partial pressure shifts cells to the production of more reduced compounds like lactate, and less acetate and H_2. On the contrary, H_2 production increases significantly with the decrease of H_2 partial pressure, pointing to an optimum partial pressure under 10.1 kPa [38].

Generally, biohydrogen production is limited by the thermodynamics of the hydrogenase catalysed reaction, involving transfer of electrons from NADH to protons mediated by a [FeFe] hydrogenase [41]. Besides thermodynamics, the disfavour of NADH reoxidation by H_2 presence also limits product production, as already explained [34, 41]. Therefore, some practical approaches to reduce H_2 partial pressure may rely on increasing the ratio between headspace and liquid in the batch culture, together with sparging the culture headspace with nitrogen [38, 41].

2.2.4 *C. butyricum* and oxygen

Strict anaerobes like *C. butyricum* are incapable of growing in aerated environments and sometimes are even intolerant to low amounts of oxygen. Molecular oxygen (O_2) reacts with Fe-S proteins, cytochromes and flavoproteins creating reactive oxygen species like hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet -}$). These strong oxidants are capable of destroying DNA, proteins and other essential cellular components [35]. Aerobes and facultative anaerobes possess superoxide dismutase, catalase and peroxidase, which are enzymes capable of detoxify them [35, 42]. And so a reason for strict anaerobes sensitivity to O_2 is explained in part by the lack of these enzymes. However, a specific group of strict anaerobes, including *C. butyricum*, evidence a small activity of superoxide dismutase and NADH/NADPH peroxidase when exposed to O_2 [35, 42].

For the specific case of *C. butyricum*, it was reported that for oxygen concentrations under 40 µM, the microorganism has the ability to convert the existing oxygen [42]. Afterwards, the cells can resume its normal growth at a similar rate to that observed under anaerobic conditions, indicating that no oxidative damage occurs to the cells [42]. Therefore, by proving that *C. butyricum* possesses active enzymes against reactive oxygen species, it is possible to conclude that these enzymes contribute to its capability to survive in microaerated environments [42].

2.2.5 *C. butyricum* and phenolic compounds

It is well known that polyphenolic compounds exert an antimicrobial activity, and for that reason they are used in new types of pharmaceutical products with the benefit of presenting low toxicity for the host cells [43]. The phenolic hydroxyl group in their structure acts as a H_2 donor generating reactive oxygen species, such as H_2O_2, causing inhibition of nucleic acid synthesis, dysfunction of cytoplasmic membrane, and disruption of the metabolism in different microorganisms [44].

Clostridium sp. are metabolically versatile and have the capacity to degrade a wide range of organic materials including to a certain extent some aromatic compounds [45, 46]. According to Tai and co-workers, (2010) the strain *C. butyricum* is capable of doing co-degradation of phenol up to a concentration of 600 mg/L, in the presence of another carbon source [45]. Mixed cultures in the presence of 1000 mg/L of phenol or vanillin the lag phase is considerably extended (4-5 times higher than a control without inhibitor), but the H_2 yield is not significantly affected (1.28 mol H_2/mol_{xylose} – phenol and 1.30 mol H_2/mol_{xylose} – vanillin, *versus* a control with solely xylose 1.67 mol H_2/mol_{xylose}) [46]. However, for concentrations of phenol higher than 1500 mg/L no cellular activity was reported, neither for mixed nor pure *C. butyricum* cultures. [45].

Gallic acid (3,4,5-trihydroxybenzoic acid) is another example of a naturally occurring phenolic compound in different fermentable substrates (*e.g.* lignocellulosic materials), hence it is commonly used in assays as a standard phenolic compound [44, 46, 47]. In this study, gallic acid was used as model compound to assess the potential effects of phenolic compounds in *C. butyricum* cells.

2.2.6 *C. butyricum* and sugar-degradation compounds

Furfural and HMF (5-hydroxymethyl furfural) are common furan aldehydes resultant from the dehydration process of pentoses and hexoses, respectively, during pre-treatment and hydrolysis of complex substrates like lignocellulosic and algal biomass [48, 49]. Their concentration levels are dependent on several factors such as the biomass origin, the type of pre-treatment process and the operating conditions, *i.e.*, pH, pressure, temperature, exposure time, concentrations and solid loading [50]. Notably, both furfural and HMF were reported to cause a deleterious effect to microorganisms, by inhibiting RNA synthesis, lowering cell membrane permeability, inducing reactive oxygen species, and reducing cell growth rate [51, 52]. As already mentioned the source of biomass and the pre-treatment can largely influence the furan aldehydes final concentration that can range from 0.5 to 11 g/L $_{\text{acid hydrolysate}}$ [49]. And although several processes for hydrolysate detoxification are described (*e.g.* evaporation, adsorption on active charcoal, adsorption on ion exchangers and solvent extraction), these methods would significantly increase the overall costs. Not only

in terms of capital (*e.g.* equipment) and chemical costs, but also concerning the substrate degradation, and loss of hydrolysate sugars decreasing the process yield [48].

Different *Clostridium* sp. were identified as capable of converting furfural and HMF to less toxic compounds. Furfural is converted into furfuryl alcohol and furonic acid while HMF is either converted to 5-hydroxymethyl furfural alcohol or 2,5-bis-hydroxymethylfuran [49, 50]. However, despite *Clostridium* sp. capacity to metabolize these inhibitors, they still exert a strong inhibitory effect both on cellular growth and H_2 production. Regarding H_2 production, it is reported that at an initial concentration of 1 g/L of furan aldehydes, the H_2 production yield suffers a severe reduction of 68 % (furfural) and 76 % (HMF) [46]. This decrease in H_2 production might be associated to a shift to non-H_2 producing pathways, based on the decrease of acetate and butyrate production in detriment to an increase of ethanol concentration in the medium [50]. Concerning the effect on the cellular growth, a pulse addition of 1-2 g/L of HMF and furfural during the exponential growth phase has revealed to be much more lethal to *Clostridium* sp. than when added at the beginning of fermentation [49]. When added at the beginning of the fermentation these inhibitors demonstrated different outcomes: cells grown in the presence of 1g/L furfural exhibited a *lag* phase two times longer than the one observed with HMF [48]. This effect can be explained by the lower molecular weight of furfural (98 g/mol) when compared to HMF (126 g/mol). Since the higher is the molecular mass, the slower is the diffusion rate through the cell membrane, HMF with higher molecular mass tends to cause less toxicity to cells [46].

2.3 Alternative complex substrates – Carob pulp and microalgae biomass

The choice of the substrate plays an important role in the H_2 yield, H_2 production rate and on the global economic feasibility of the process. These outcomes are mainly dependent on the substrate availability, biodegradability potential, and its composition in terms of lipids, proteins, moisture, and in carbohydrates, preferably in the soluble form [53]. Carbohydrates such as glucose, sucrose and starch have been widely used in dark fermentation studies, however renewable bioH_2 production requires a renewable feedstock [54]. Second and third generation biomass sources are abundant and cheap, thus are capable of supporting a sustainable supply of fermentable substrate. These biomass resources can be grouped as wood and wood wastes, agricultural residues like lignocellulosic materials (*e.g.* rice straw, wheat straw and corn stalks), municipal solid waste, agro-industrial wastes such as waste from processing industries (*e.g.* Carob pulp), and macro and microalgae biomass (*e.g. Laminaria japonica* and *Spirogyra* sp. respectively) [55]. In this work, two biorenewable substrates - carob pulp and microalgae biomass - were used to assess the H_2 production yield by *C. butyricum*.

2.3.1 Carob pulp

The carob tree (*Ceratonia siliquia* L.) is a tree native to the Mediterranean region. This region is characteristic for its mild and dry areas with poor soil, with an average production of 2000-3500 kg of carob pod/ ha [56]. The carob tree is resistant to drought, requiring little maintenance and producing different products. The carob pod is mainly constituted by pulp (90% w/w) and seed (10% w/w), where the seeds are the most valuable product constituted by coat (30-33% w/w), endosperm (42-46% w/w) and embryo or germ (23-25% w/w). One of the main applications of the seeds is in the production of galactomannan, which is then used to produce food additives [57]. On the other hand, the carob pulp is mostly used as animal feed and human consumption although in a lower extent due to the presence of tannins (*ca.* 20% w/w) whose effect in human health is still unclear [56, 58]. Since almost 50 % (w/w) of the carob pulp content is constituted by sugars (mainly sucrose, glucose and fructose), it can be considered as an excellent raw material for ethanol production or fermentative H_2 production, although only few studies have been published regarding H_2 production [57, 59]. One of these studies by Fountoulakis and co-workers, (2014) reported a H_2 production yield of 0.43 mol H_2 / mol glucose in a continuously stirred tank reactor (CSTR), using an anaerobic sludge as inoculum [59]. Although the production yield is relatively low when compared to other substrates, it shows that there is potential to explore carob pulp for H_2 production.

2.3.2 Microalgae biomass

Research on anaerobic fermentation of algae biomass goes back to more than 50 years ago. But recently, the identification of microalgae strains with promising characteristics to be used as fermentation substrate and the progress in microalgae cultivation and harvesting techniques has raised the interest to use these organisms as feedstock for bioethanol and biohydrogen production [60]. Microalgae have been proposed as an alternative feedstock to the production of crop-based biofuels, due to higher biomass productivities, growth capability in non-arable land with non-potable water, and for not interfering with food crop purposes [1, 61, 62].

Some species of microalgae like *Chlorella vulgaris* accumulate relatively high amounts of carbohydrates: for example the glucose content can be up to 51% (g/g_{dw}) [63]. Therefore, *C. vulgaris* is a promising feedstock to use in dark fermentation by *C. butyricum*, with studies reporting a H_2 production yield of 81 mL H_2/g_{algae} [63]. In the present work, *Spirogyra* sp. a filamentous green algae, was used as carbon source. It contains about 11 - 21 % of lipids and 33 – 64 % of sugar [64]. The high growth rate together with its capacity to accumulate high amounts of sugar, make this microalgae an attractive biomass for dark fermentation [65]. However, almost no references exist related to bioH_2 production from this microalgae biomass.

Besides storage carbohydrates mainly present in the form of starch inside the microalgae cells, the cell wall polysaccharides may also contribute as a carbohydrate source for fermentation. Due to the lack of lignin, most components of the microalgae cell walls are more easily saccharified than conventional lignocellulosic biomass [66, 67]. This fact makes microalgae biomass prone to milder pre-treatments prior to fermentation [7, 66]. Nevertheless, the H_2 production yield using microalgae biomass without pre-treatment is lower (0.4 mol H_2 / mol $_{reduced\ sugar}$) than the one achieved by using acid pre-treated microalgae biomass (1 mol H_2 / mol $_{reduced\ sugar}$) [63].

2.4 Microorganisms' adaptation to stress conditions

In general, all bacterial cells present the following adaptation mechanisms to stress conditions: increase of DNA replication and repair, transcription and translation; toxic substrate transport and metabolism; signal transduction mechanisms; aggregation and biofilm formation; increase of transmembrane proteins for transportation and efflux of toxic substances; lipid metabolism and cellular membrane modification; and under more extreme conditions, physiological modifications to induce dormancy [68, 69]. By using these mechanisms, microorganisms have a quicker and generalised defensive strategy to face a wide series of different situations. The most frequent stress situations that trigger these adaptation mechanisms are heat, osmotic pressure, acid and alkaline shocks and the presence of reactive oxygen species and antibiotics [70]. Naturally, different microbial species have their own mechanisms to deal with different stress conditions, and after a general response they activate specific pathways according to the exact type of stress, in order to produce a more efficient response [69].

H_2 in its gaseous form, and at high concentrations (above 50% v/v), can cause inhibitory effects on the growth of some hydrogen-producing microorganisms, namely in *Clostridium* sp. [71]. An increase in the H_2 concentration leads to an increase of the lag phase and consequently to a decrease of the growth rate and final cell yield [71]. Furthermore, H_2 is both a product and a substrate for terminal electron-accepting processes such as methanogenesis and sulfate reduction. In pure cultures with *C. butyricum* cells, H_2, acetate and butyrate are the main metabolic products that accumulate in the fermentation medium. For mixed cultures that comprise H_2 producers and H_2 consumers, like methanogenic bacteria, the produced H_2 is subsequently used in electron accepting processes in the metabolic pathway of methanogens [72, 73]. Therefore, in the absence of H_2-consuming microorganisms, the H_2 partial pressure (P_{H_2}) rapidly reaches a level that thermodynamically inhibits further fermentation [74].

Under stressful conditions, microbial cells must be able to maintain the integrity of the cellular membrane to ensure survival and successful growth. In bacteria, this membrane performs such vital functions as the maintenance of the proton-motive force and nutrients uptake. It is also an interface between the external environment and the cellular cytoplasm, and so in response to external stimulus the membrane composition may be altered in order to maintain fluidity and functionality [75].

Cells are capable of modifying the lipids of the cellular membrane to guarantee a particular state of fluidity and stability. Under stress conditions some of these modifications include: modifications in the fatty acid composition of phospholipids and production of unsaturated fatty acids, that lead to changes in the degree of saturation of the membrane fatty acids; *cis/trans* isomerization that only occurs in specific strains (*e.g.* solvent-tolerant strains); and chain length modification of fatty acids linked to cell aggregation mechanism, cell surface hydrophobicity, and adhesion efficiency [76, 77]. Specific modifications may also be observed in certain strains, such as the production of unusual polyunsaturated fatty acids [78].

Another important defensive mechanism that microorganisms use to face stress conditions is the formation of cellular clusters and biofilms. When they grow in aggregated structures, they are able to increase the resistance against physical and chemical stresses, because inner cells are not directly exposed to the stress agents [76]. However to promote cell-to-cell adhesion, bacterial cells have first to increase surface hydrophobicity to stimulate and facilitate the interaction between cells [79].

2.5 Objectives

This work was an integration of two independent projects that were running separately at LNEG and iBB laboratories. The biohydrogen production by *C. butyricum* has been extensively studied using renewable feedstock, including lignocellulosic hydrolysates and microalgae biomass, and under different operating conditions. These studies have been mostly focused on the technological process and less on the physiology of hydrogen production by *C. butyricu*m. Therefore, in order to investigate the response of *C. butyricum* cells to different inhibitors naturally present in this type of substrates, an adaptation strategy to increasing concentrations of inhibitors and a simultaneous characterisation of the cellular lipids was performed. This later part was conducted at iBB, where similar studies have already been performed with aerobic bacteria.

The main purpose of the present study was to understand how physiological adaptive strategies developed by *C. butyricum* cells during H_2 production under inhibitory conditions could be used to increase the H_2 production yield from carob pulp and microalgal biomass.

To achieve that, the following working strategy was designed:

- The cellular lipid composition was determined along *C. butyricum* growth and biogas accumulation.
- The physiological response of *C. butyricum* cells to different concentrations of natural inhibitors, present on the tested substrates, was evaluated by studying the changes occurring in the lipid profile and along the growth curve.
- A cellular adaptation strategy to the studied natural inhibitors, together with the cellular lipid profile characterisation, were used to study and increase the H_2 production from carob pulp extract and *Spirogyra* hydrolysate.

3 Materials and Methods

3.1 Microorganism

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

3.2 Culture media

Basal Medium 1 (BM1), adapted from Patricia and co-workers (2007), was used as the fermentation medium for all the assays (Annex I) [80]. BM1 medium without glucose supplementation was prepared under non-sterile conditions and deoxygenated with N_2, with the pH adjusted at 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. Finally, the medium was autoclaved (Uniclave 88, AJC) at 121ºC for 20 minutes. The different carbon sources used in this work were the following: 25 % (w/v) glucose solution; carob pulp aqueous extract; and *Spirogyra* hydrolysate (the latter two were formerly prepared for other works) [65, 81]. The solutions were filter sterilised (Ø – 0.2 µm cellulose acetate filter, VWR) into a previously sterilised serum bottle, aseptically deoxygenated with N_2, and subsequently used to supplement the BM1 medium at specific/defined concentrations.

C. butyricum was pre-cultured in Reinforced Clostridial Medium (RCM, Oxoid) (Annex I), at an inoculum concentration of 1% (v/v), and cultured overnight at 37 ºC, in static conditions (G24 Envir. Incubator).

3.3 Fermentation assays

The BM1 supplemented with the respective carbon source at 10 g/L (glucose, carob pulp aqueous extract or *Spirogyra* hydrolysate) was inoculated at 2% (v/v) with the pre-cultured *C. butyricum*. The flasks were incubated at 37 ºC and 150 rpm (G24 Envir. Incubator Shaker). The fermentation was monitored between 0 and 144h with measurement of the final pH, biogas production and its composition, optical density (OD) at 600 nm (Genesys 20) and/or cell concentrations along fermentation. Different initial glucose concentrations (0, 10, 20, 30, 40, 50 and 60 g/L) were tested and the effect on H_2 production and cellular growth was assessed after a 24h incubation period. This assay was performed in triplicate.

3.3.1 Oxygen, gallic acid, furfural and HMF toxicity assays

A pulse of air, gallic acid, furfural and HMF was applied to mid exponential grown cells as determined by OD monitoring according to section 3.3. 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.

3.3.2 *C. butyricum* adaptation to inhibitors

The capacity of *C. butyricum* cells to resist to high concentrations of specific inhibitor compounds was induced through a stepwise adaptation strategy. Once cells were able to grow under a certain concentration of inhibitor, they were used to inoculate fresh fermentation medium with an increased concentration of the inhibitor. For example, once the cells were capable of growing at a gallic acid concentration of 0.5 g/L, exponentially growing cells were used to inoculate medium with a gallic acid concentration of 1.0 g/L. Cells were considered as grown under a specific concentration when the measured OD_{600nm} was at least 0.6.

One adaptation series started with 0.0 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 cellular passage, the gallic acid and HMF+ furfural adapted cells were also used to inoculate BM1 supplemented with carob pulp aqueous extract or *Spirogyra* hydrolysate (10 $g_{total\ sugars}$/L), respectively. This allowed to evaluate whether the adaptation of the cells to the inhibitor influenced the H_2 production yield.

3.4 Lipid extraction and analysis

The cells were collected at defined times along the assays by centrifugation at 13, 975 *g* (Biofuge 15, Heraeus Sepatech), the supernatant was discarded and the pellet was 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). The produced fatty acid methyl esters (FAMEs) were analysed by gas chromatograph analysis (according to section 3.5.2) and identified by the PLFAD1 method of Sherlock® software version 6.2 from MIDI, Inc and also by using qualitative

standards of bacterial FAMEs and polyunsaturated fatty acids, both from Supelco [78]. The saturation degree, which is defined by the ratio of total saturated fatty acids by the monounsaturated fatty acids, was calculated for each lipid analysis.

3.5 Analytical Methods

3.5.1 Gas Chromatography for 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. The injector and column were operated at 80ºC and the detector at 120ºC.

3.5.2 Gas chromatography for determination of fatty acids composition

The produced FAMEs (section 3.4) 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. The analytical method was controlled by the Sherlock® software (MIDI, Inc).

3.5.3 Total sugars quantification

Total sugars were determined by the phenol-sulfuric method as described in [82]. In glass test tubes, 1 mL of the sample was added together with 5 mL of sulfuric acid at 96 % (w/v) and 1 mL of phenol 5 % (w/v). The mixture was left unstirred for 10 minutes at room temperature, followed by a 5 minutes incubation at 30 ºC, in a thermostatic bath. Finally, 200 µL of each sample was transferred to a 96-well microplate and the absorbance was read at 490 nm in a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific).

A calibration curve was prepared with a glucose standard solution at different concentrations (0, 25, 50, 100, 150 and 200 mg/L – Annex II), which allowed the quantification of the total sugars present in the samples, expressed as glucose equivalents.

3.5.4 Total phenols quantification

Total phenols were determined by the Folin-Ciocalteu method [83]. Gallic acid was used as a reference standard for the calibration curve using the following concentrations: 0, 50, 75, 100, 150 and 175 mg/L (Annex II).

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 mixture was incubated at room temperature for two hours in the absence of light for colour development. Finally, 200 µL of each test tube were transferred to a 96-well microplate and the OD was measured at 765 nm in a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific).

Since the total phenolic contents were determined from the calibration curve prepared with gallic acid, the content of total phenolic compounds was expressed as gallic acid equivalents (GAE).

4 Results and Discussion

4.1 *C. butyricum* growth and lipid profile characterisation

4.1.1 Cellular lipid composition of *C. butyricum* cells during growth

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. Under these conditions, the total lipids of strain DSM 10702 were mainly constituted by the following fatty acids, at the end of the exponential phase: C14:0 (tetradecanoic acid, 6.6 %), C16:0 (hexadecanoic acid, 39.6 %), C18:1 w7c ((11Z)-11-Octadecenoic acid, 19.7%) and C19:0 *cyclo* w7c (*cis*-11,12-Methylene-Octadecanoic acid, 11.9%) (data not shown). This composition is similar to the ones described in the literature and used as taxonomic reference for the species [84]. For each culture condition (*e.g.* culture medium, carbon source and temperature) the cellular lipid composition may differ in the percentage of each fatty acid.

Significant changes in the lipid composition of the cells were only observed during the exponential growth phase (Figure 2). This is in accordance to previously published data [85, 86], since during the exponential growth phase the cells are metabolically more active, are more prone to external stress and may perform *de novo* synthesis of fatty acids necessary to respond to the growth conditions. During the *lag* phase (1-3 hours), the majority of the cell population is derived from pre-inoculum, which are well-adapted cells grown in RCM medium. For this reason, their lipid profile is similar to mature cells at 24 hours of incubation. As new cells start to appear and multiply, the lipid profile is completely rearranged (5 and 6 hours), the cells increase the percentage of monounsaturated fatty acids (MUFA) and decrease the saturated and cyclopropyl fatty acids. After cellular adaptation to the culture conditions, from the mid until the end of exponential phase/beginning of stationary phase (6-10 hours) cells tend to establish their lipid composition by increasing the saturation degree from 0.54 ± 0.01 to 1.55 ± 0.02 (saturated divided by monounsaturated fatty acids) and cyclopropyl fatty acids. According to the literature [85], a similar cellular behaviour was described in *C. butyricum*, ATCC 6015 starting with a decrease of saturated fatty acids at the beginning of the exponential phase. Then, when cells reached the end of the exponential growth phase, in order to stabilise the lipid membrane, both the content in saturated and cyclopropyl fatty acids increased [85, 87, 88].

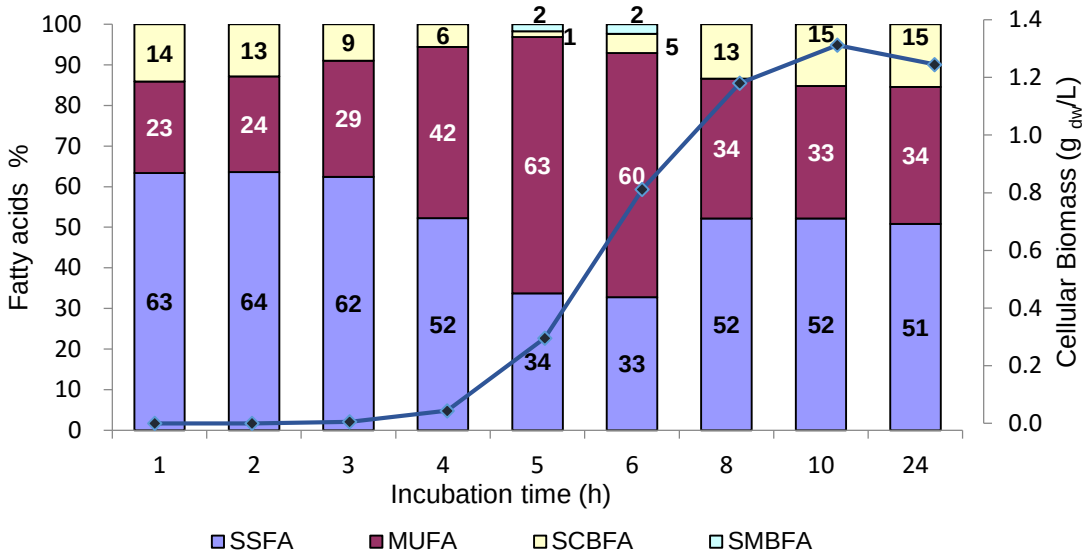


Figure 2. Fatty acid composition of *C. butyricum* cells at different growth stages (*bars*) and corresponding biomass concentration (*diamonds*). Numbers inside bars indicate the relative percentage of each class of fatty acids. *SSFAs* straight-chain saturated fatty acids, *MUFAs* monounsaturated fatty acids, *SCBFAs* saturated cyclopropyl-branched fatty acids, *SMBFAs* saturated methyl-branched fatty acids

4.1.2 Cellular lipid composition of *C. butyricum* during biogas production

Previously published work reported an inhibitory effect of hydrogen accumulation on different hydrogen-producing bacteria, resulting in decreased growth rates and final cell biomass [71]. With the aim of testing this possible effect on *C. butyricum*, the lipid profile was analyzed in order to help assessing a possible stress effect caused by accumulated biogas.

After 10 hours of incubation the biogas production stabilized at values close to 47 mL of accumulated biogas (Figure 3). Thus, the cells collected at 144 hours of incubation were in the presence of the maximum volume of cumulative biogas since the 10th hour of incubation. Regarding the lipid profile, the major alterations occurred at the beginning of fermentation, most likely related to cell adaptation to culture conditions as mentioned above (section 4.1.1.), since there was a minor accumulation of biogas (< 13 mL). At the end of the fermentation, when biogas accumulation was maximum, cells did not alter the fatty acid composition. Based on these results, it is possible to infer that under the studied conditions (e.g. medium composition, temperature, pH, and substrate) the produced volume of biogas was insufficient to induce changes in the fatty acid composition of the cells.

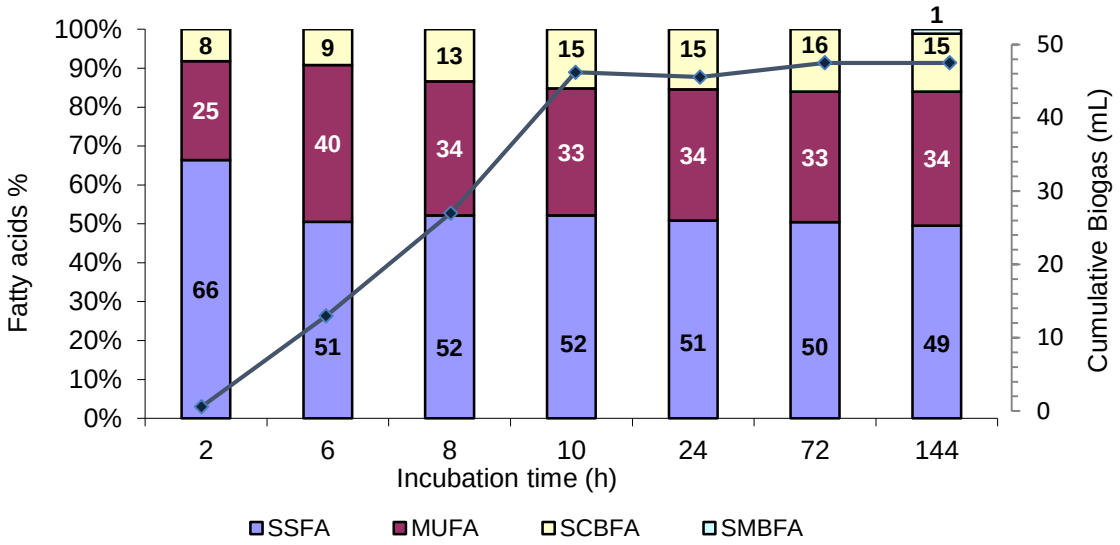


Figure 3. Fatty acid composition of *C. butyricum* cells at different growth stages (*bars*) and corresponding cumulative biogas volume (mL) (*diamonds*). Numbers inside bars indicate the relative percentage of each class of fatty acids. *SSFAs* straight-chain saturated fatty acids, *MUFAs* monounsaturated fatty acids, *SCBFAs* saturated cyclopropyl-branched fatty acids, *SMBFAs* saturated methyl-branched fatty acids.

4.2 *C. butyricum* cellular response to inhibitors

One of the main hurdles that dark fermentation by *C. butyricum* has to face is the presence of inhibitor compounds that affect the cellular integrity, hence reducing its capability to grow. In order to investigate the potential effect of some of these inhibitors on *C. butyricum* cells, the cellular viability and lipid profile were assessed following exposure. Four natural inhibitors were selected for this study: oxygen, which inhibits the growth or kills strict anaerobic microorganisms, gallic acid, HMF and furfural. The last three compounds are commonly present in fermentation substrates of lignocellulosic origin and are produced after acid pretreatment of cellulosic and hemicellulosic biomass, being described as toxic for cells.

4.2.1 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 [86]. In this study, the oxygen effect on the cellular growth of *C. butyricum* was assessed with the aeration of the culture at the beginning of the exponential growth phase with pulses of 1 and 100 mL of air.

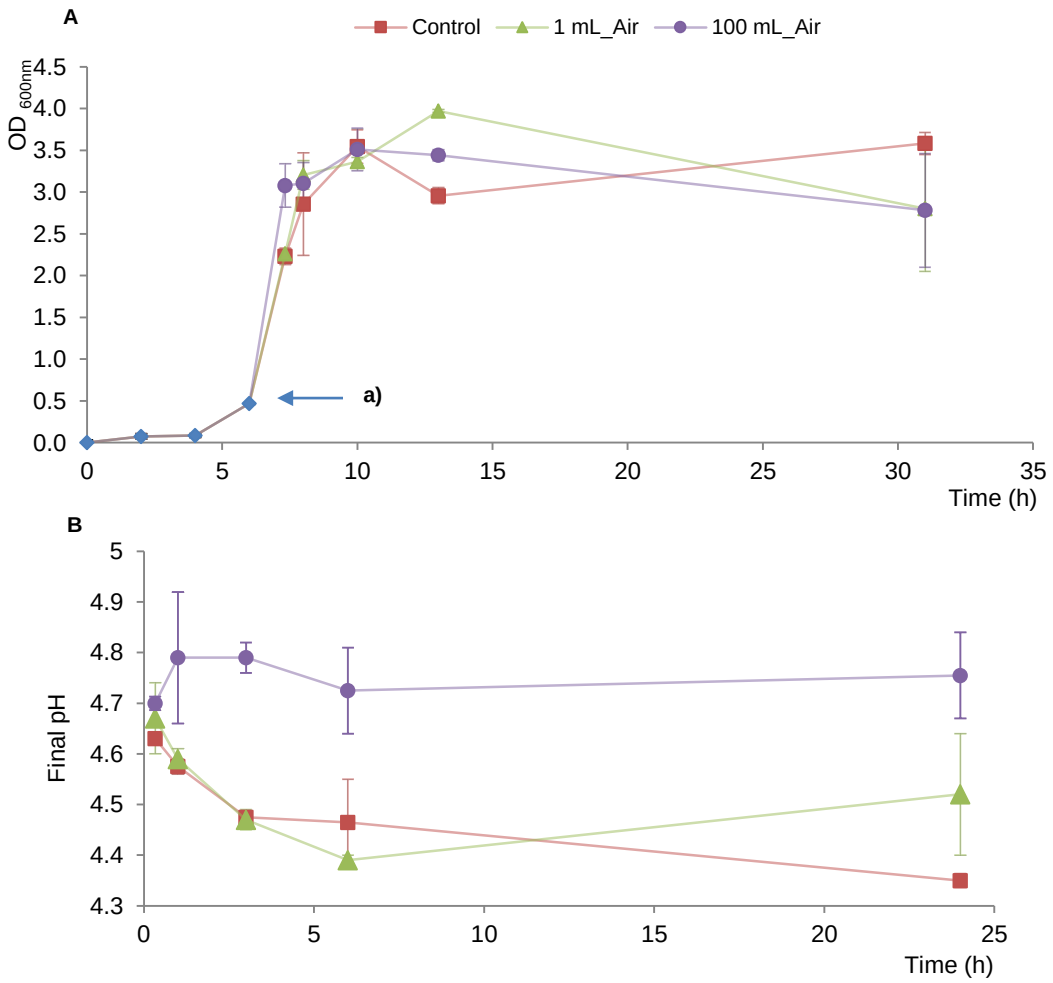


Figure 4. (A) – Optical density curves of *C. butyricum* growth under different aeration conditions. Cells were cultured under normal anaerobic conditions until the beginning of the exponential phase (OD_{600nm} = 0.6) of growth, when a pulse of filtered air was injected into the fermentation flask (a)). **(B)** – pH of the cultures after 0.3, 1, 3, 6 and 24 h of the aeration pulse.

Symbols: *Control* (red), the cumulative biogas was removed and no air was injected; *1 mL_Air* (green), the cumulative biogas was removed and 1 mL of filtered air was injected. *100 mL_Air* (purple), the cumulative biogas was removed and 100 mL of filtered air was injected.

No growth inhibitory effect caused by oxygen was observed under the studied conditions (Figure 4A). No significant differences occurred on *C. butyricum* growth curve profile after the pulse injection of either 1 mL or 100 mL of air, when compared to the control (no injection). It is described that *C. butyricum* is not capable of starting a fermentation in the presence of oxygen concentrations higher than 40 µM [42].

However, for cells in the exponential growth phase, no inhibitory concentrations are referred. 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. Moreover, according to Kawasaki and co-workers, 1998, *C. butyricum* was described as possessing the capability to reduce part of the dissolved oxygen, in amounts proportional to the size of the inoculum [42].

Concerning the pH variation, it remained practically unchanged after the replacement of the biogas in the headspace by a large volume of air (100 mL), whilst in the other two conditions (control and 1mL) the pH value decreased from 4.7 when the air pulse was applied to 4.4 - 4.5 after an additional period of 25 h of incubation (Figure 4B);it is known that *C. butyricum* is incapable of growing for pH values lower than 4.4 [42]. This absence of pH variation might be due to the replacement of the produced biogas for atmospheric air that contains a lower percentage of carbon dioxide. Consequently, there is a lower carbon dioxide dissolution in the culture medium and the pH value rapidly increases to 4.8. Although *C. butyricum* continued to grow, the production of acidic metabolites appears to be insufficient to drop the pH further below 4.7. Further assays must be conducted to elucidate this variation.

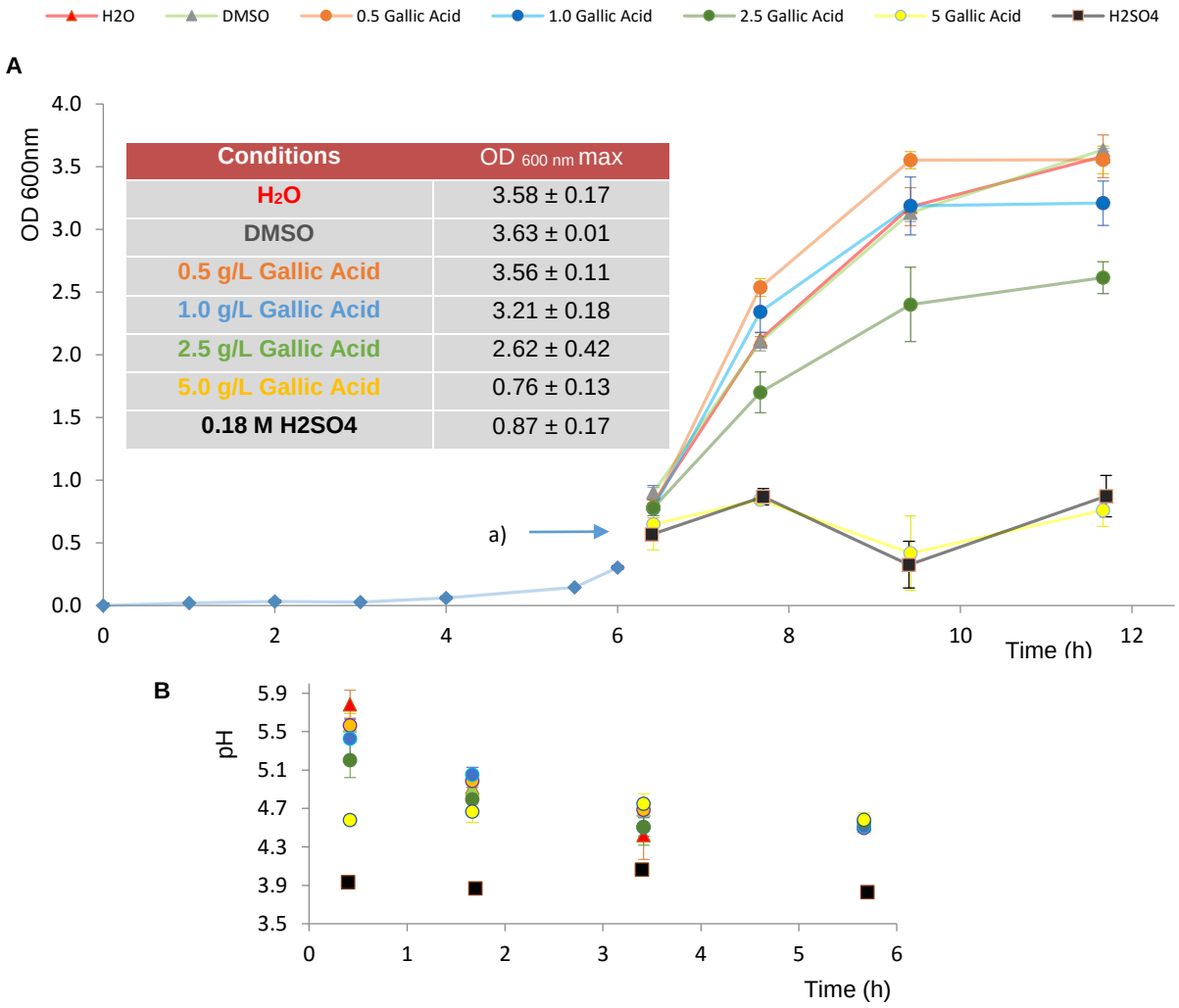
4.2.2 Inhibitor II – Gallic acid assay

Polyphenols are often found in the composition of renewable biomass like lignocellulosic materials. Since these compounds are described as antimicrobial agents, their presence in such fermentable materials might affect the growth and H_2 production yields [44]. Moreover, phenolic compounds are known for damaging microbial cells by altering selectively the membrane permeability, causing leakage of intracellular components and inactivation of essential enzymes [48].

The studied concentrations were selected based on phenol concentrations published on other studies and gallic acid minimum inhibitory concentration (MIC) for other microorganisms [43, 45]. Gallic acid solutions were prepared in 15% dimethyl sulfoxide (DMSO), so a pulse of 15 % DMSO was also tested as well as a control with water, together with a negative control of sulfuric acid used for the minimum pH value control.

No substantial growth inhibition was reported for gallic acid concentrations up to 1.0 g/L (Figure 5A). For a gallic acid concentration of 2.5 g/L a clear inhibitory effect was observed with a decrease of 27% in the maximum OD_{600nm}, compared to cells in the presence of H_2O and DMSO. Moreover, a decrease in the

growth curve slope was possible to be observed, with the increase of gallic acid concentration. For the highest gallic acid concentration tested (5.0 g/L) no growth was observed after the pulse, which was already expected since it is the MIC reported for other microorganisms [43]. This inhibitory effect might be due to the high phenolic concentration, nevertheless the acidity effect cannot be excluded because in the presence of 0.18 M sulfuric acid no growth was detected either. However, in the case of 2.5 g/L it was possible to conclude that the inhibitory effect was not caused by the pH, since the final pH stabilized at 4.7, just like it did for cells in the presence of H_2O (Figure 5B).



Conditions	OD 600 nm max
H_2O	3.58 ± 0.17
DMSO	3.63 ± 0.01
0.5 g/L Gallic Acid	3.56 ± 0.11
1.0 g/L Gallic Acid	3.21 ± 0.18
2.5 g/L Gallic Acid	2.62 ± 0.42
5.0 g/L Gallic Acid	0.76 ± 0.13
0.18 M H2SO4	0.87 ± 0.17

Figure 5. (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, DMSO, gallic acid or H_2SO_4 solution was injected into the fermentation flask (a). **(B)** - pH value of culture after 0.3h, 1h, 3h and 6h of each pulse. Symbols: Triangle (*red*), H_2O; Triangle (*green*), 15% (v/v) DMSO; Circle (*purple*), 0.5 g/L gallic acid ; Circle (*blue*), 1 g/L gallic acid; Circle (*orange*), 2.5 g/L gallic acid; Circle (*yellow*), 5 g/L gallic acid, all gallic acid solutions prepared in 15% (v/v) DMSO; Square (*black*), 0.18 M H_2SO_4.

To further study *C. butyricum* response, the lipid profile was characterized under different gallic acid concentrations and exposure times (Figure 6). 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 compared to the cells exposed only to H_2O. This variation could be a result of a cellular adaptation mechanism to increase the membrane fluidity to face 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. Moreover, the increase of MUFAs after 20 min of exposure, might be related to the activity of an enzymatic complex promoting this fast post *de novo* synthetic modification. In fact, it seemed that 43% of hexadecanoic acid (C16:0) disappeared, while 51% of (11Z)-11-Octadecenoic acid (C18:1w7c) was formed (data not shown). Thus, suggesting a possible conversion of C16:0 into C18:1w7c, by the action of two enzymes, first an C16/C18 elongase followed by a Δ11 desaturase (still unknown) reported in anaerobic microorganisms [89]. When the exposure time was increased, no major alterations were observed among the different conditions, with the exception for the increase of cyclopropyl fatty acids content over time. This increase is commonly associated with the stationary growth phase and cellular aging, as demonstrated

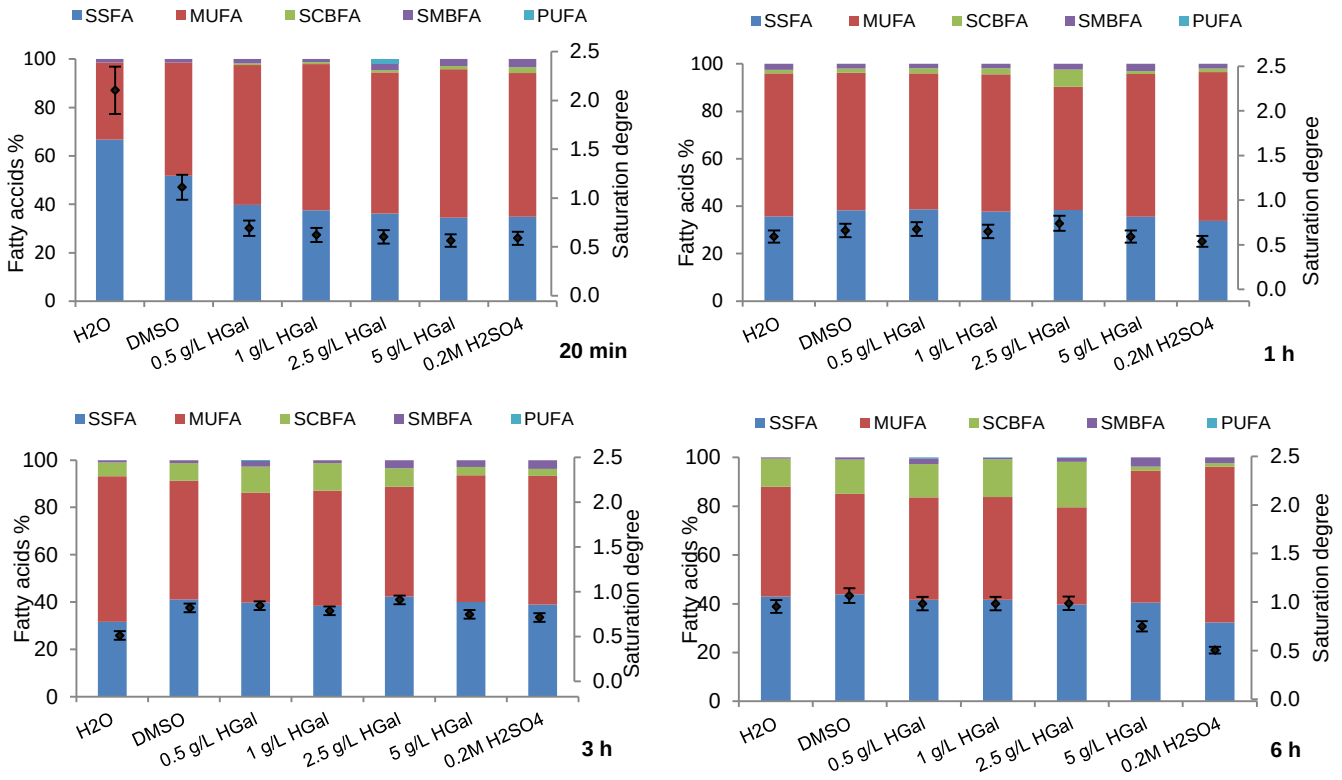
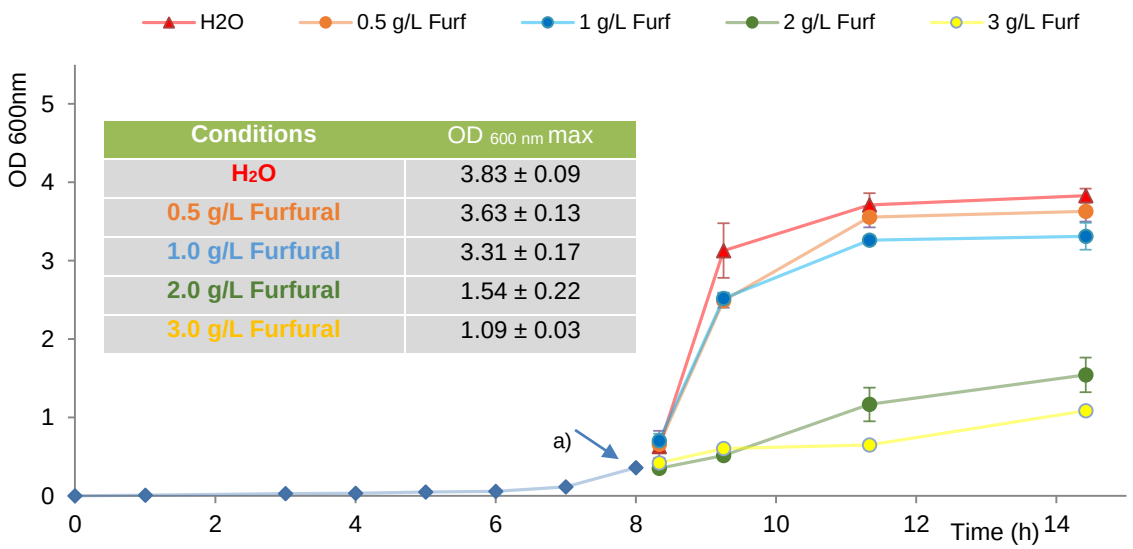


Figure 6. Fatty acid composition of *C. butyricum* cells exposed to different conditions (*bars*) and corresponding calculated saturation degree at four different exposure times – 0.3, 1, 3 and 6h. *SSFAs* straight-chain saturated fatty acids, *MUFAs* monounsaturated fatty acids, *SCBFAs* saturated cyclopropyl-branched fatty acids, *SMBFAs* saturated methyl-branched fatty acids, and *PUFAs* polyunsaturated fatty acids.

above (Section 4.1.1). No cyclopropyl fatty acids were observed for cells in the presence of 5.0 g/L gallic acid and sulphuric acid, where no growth occurred.

4.2.3 Inhibitor III – Furfural assay

Furans are found in biorenewable biomass as by-products of pre-treatment of lignocellulosic biomass. In this case furfural, derived from pentose dehydration, is described as having an impact in the microorganisms' metabolism [90]. Furfural is a known mutagen, interacting with double-stranded DNA, leading to the formation of non-functional enzymes [91]. To investigate the furfural effect on cell growth, *C. butyricum* was challenged with increasing concentrations of furfural. The range of studied furfural concentrations (0.5, 1.0, 2.0 and 3.0 g/L) was chosen based on its common composition reported for several acid hydrolysates of lignocellulosic materials [48]. 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 7).



Conditions	OD $_{600\ nm}$ max
H_2O	3.83 ± 0.09
0.5 g/L Furfural	3.63 ± 0.13
1.0 g/L Furfural	3.31 ± 0.17
2.0 g/L Furfural	1.54 ± 0.22
3.0 g/L Furfural	1.09 ± 0.03

Figure 7. *C. butyricum* growth with different furfural 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 furfural solution was injected into the fermentation flask (a).

Cells exposed to different concentrations of furfural responded by changing the lipid profile. After 20 minutes of exposure, cells doubled the saturation degree in response to furfural concentrations of 2.0 g/L (Figure 8). 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.

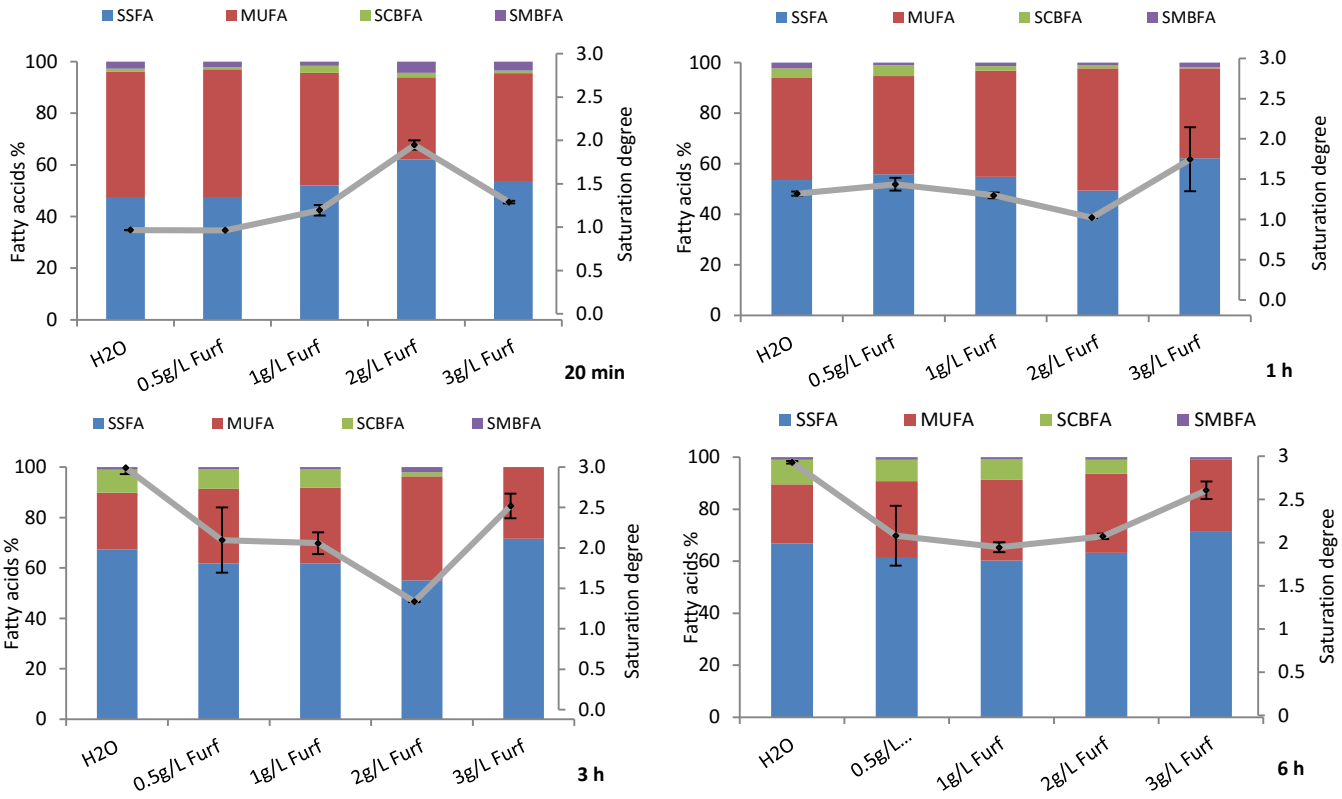


Figure 8. Fatty acid composition of *C. butyricum* cells exposed to different furfural concentrations (*bars*) and corresponding calculated saturation degree (*line*) at four different exposure times – 0.3, 1, 3 and 6h. *SSFAs* straight-chain saturated fatty acids, *MUFAs* monounsaturated fatty acids, *SCBFAs* saturated cyclopropyl-branched fatty acids, *SMBFAs* saturated methyl-branched fatty acids, and *PUFAs* polyunsaturated fatty acids.

4.2.4 Inhibitor IV – HMF assay

The other furaldehyde usually found on the same materials as furfural is HMF, which is formed from the dehydration of hexoses, mostly fructose and glucose. Although HMF is commonly present in lower concentrations than furfural, the same range of concentrations (0.5, 1.0, 2.0 and 3.0 g/L) were studied in order to compare the potential effect of both inhibitors on cells.

The inhibitory effect of HMF seemed to gradually increase with increasing concentrations of HMF, although without ceasing the cellular growth for the tested concentrations (Figure 9). Only for the concentration of 3.0 g/L a more significant decrease, of 34% in the maximum OD_{600nm}, was observed in comparison to cells grown in the presence of H_2O, together with an extended lag phase until 9h of incubation. Comparing both growth curves, HMF presented a lower growth inhibitory effect than furfural (Figure 7 and Figure 9), when present in the same conditions. This lower toxicity is visible for instance, through the comparison of the MIC for furfural, which is lower than for HMF. 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 [46].

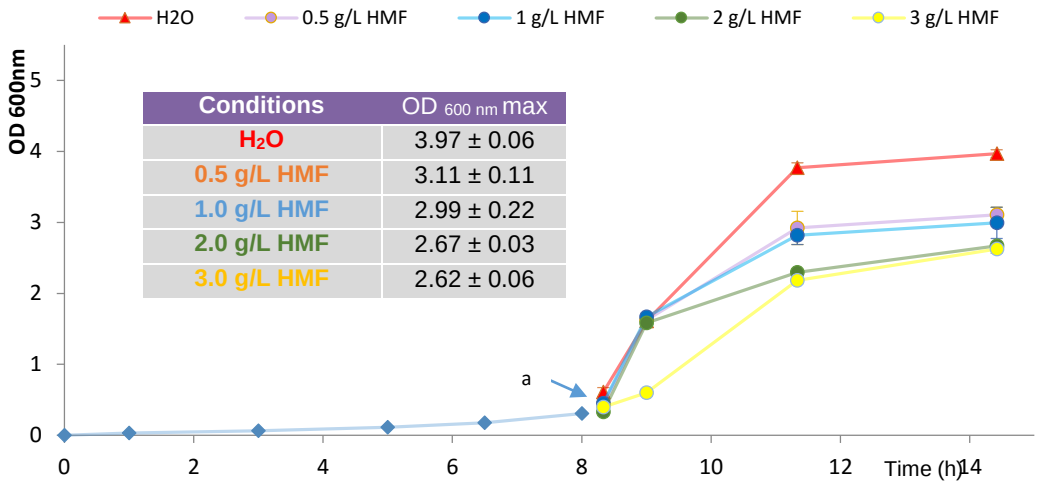


Figure 9. *C. butyricum* growth with different HMF concentrations. Cells were grown under optimal conditions until the beginning of the exponential phase (OD_{600nm} = 0.75) of growth, when a pulse of a 1 mL HMF solution was injected into the fermentation flask (a).

No significant variations in the *C. butyricum* lipid profile were observed in the presence of different concentrations of HMF (Figure 10). Generally, the fatty acid composition in the presence of H_2O (control) was similar to the composition in the presence of HMF, registering only the normal variations observed in *C. butyricum* growth (section 4.1.1), namely the increase of cyclopropyl saturated fatty acids. Nonetheless, the inhibitory effect caused by 3.0 g/L of HMF could still be detected through the lipid analysis, since the content in cyclopropyl saturated fatty acids was always lower than for the other conditions. This similarity among different concentrations of HMF might be related to the chemical properties of HMF in comparison to furfural [90]. Having a higher molecular mass and being more hydrophilic, due to the hydroxyl group, might make it less prone to interact with the cellular membrane than furfural.

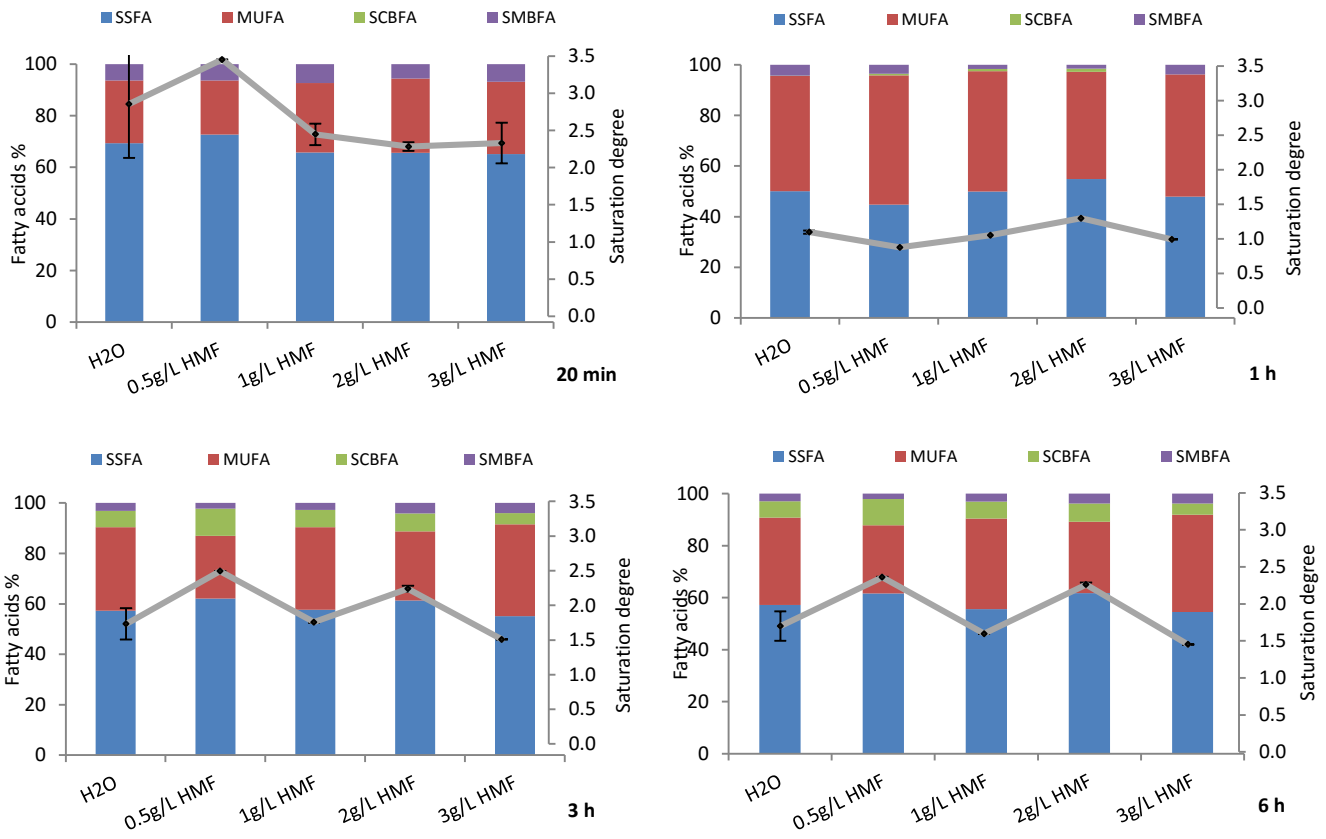


Figure 10. Fatty acid composition of *C. butyricum* cells exposed to different HMF concentrations (*bars*) and corresponding calculated saturation degree (*line*) at four different exposure times – 0.3, 1, 3 and 6h. *SSFAs* straight-chain saturated fatty acids, *MUFAs* monounsaturated fatty acids, *SCBFAs* saturated cyclopropyl-branched fatty acids, *SMBFAs* saturated methyl-branched fatty acids, and *PUFAs* polyunsaturated fatty acids.

4.3 H_2 production and adaptation to real substrates containing phenolic and furanic compounds

4.3.1 Determination of optimal carbohydrate concentration for H_2 production

Before testing a large fermentation scale with an uncharacterized fermentation substrate (e.g. microalgae biomass or carob pulp), a flask scale assay was performed, using glucose as standard carbon source. Thus, to determine the optimum H_2 production different glucose concentrations were tested.

The 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. The biogas produced presented a purity ratio (H_2 divided by CO_2 (mol/mol)) of 2.76 ± 0.03 (Table 2). For similar fermentation conditions, and using glucose as carbon and energy source, the values presented in the literature (1.2 – 2.4 mol H_2 / mol glucose) are comparable with the maximum yield obtained [28, 32, 92]. This maximum H_2 yield is explained by a high glucose conversion efficiency of 93 % and a considerable cumulative H_2 production of 66 mL (Figure 11). Since the H_2 molar yield is a ratio between the moles of H_2 produced and the moles of consumed glucose, it is directly affected by the outcome of these parameters. Consequently, when the glucose conversion efficiency decreases to values close to 80 % and the cumulative H_2 production is kept constant, the H_2 yield naturally decreases, like in the case for initial glucose concentrations of 20 and 30 g/L. However, it is also possible to have a low glucose conversion efficiency and an increase in H_2 production, hence increasing the fraction of mol of H_2/ mol of glucose, like observed at the concentration of 40 g/L. Concerning the cellular growth, apparently it was not affected by the variation of the initial glucose concentration neither by the volume of H_2 produced, since no significant variation in the cellular biomass was detected with the increase of glucose concentration (Figure 11).

Table 2. 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)
10	2.28 ± 0.01	93	2.76 ± 0.03
20	1.32 ± 0.13	81	2.31 ± 0.18
30	0.84 ± 0.09	78	2.65 ± 0.26
40	1.42 ± 0.18	50	2.36 ± 0.21
50	1.06 ± 0.18	45	2.31 ± 0.12
60	0.87 ± 0.15	44	1.99 ± 0.09

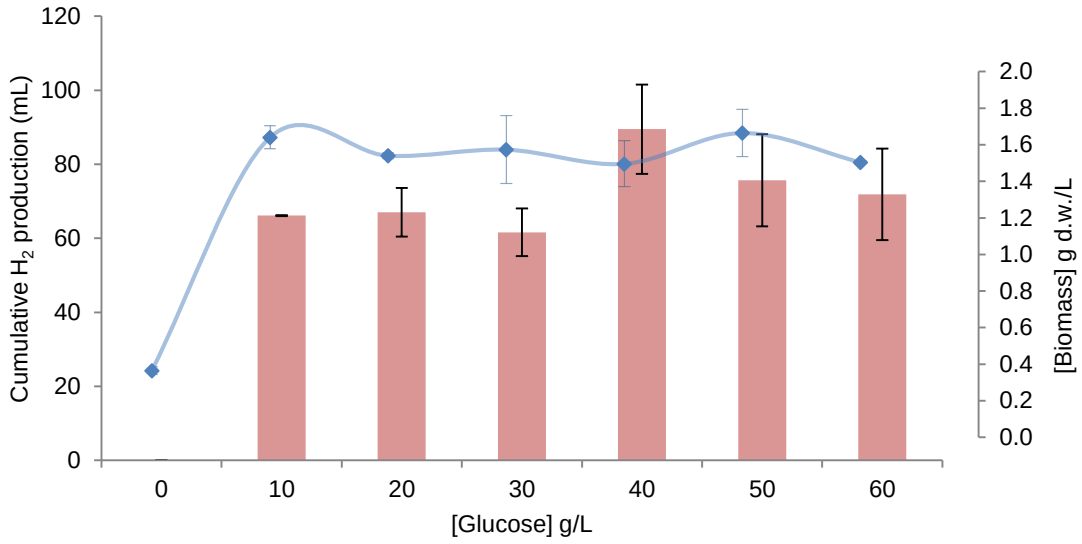


Figure 11. Effect of different initial glucose concentrations on H_2 production (*bars*) by *C. butyricum* and on final biomass concentration (*line*).

When comparing the H_2 yield from glucose in different volumes of culture medium (Table 3), it is possible to verify that for larger working volumes on batch mode, the H_2 yield tends to decrease. This is most likely influenced by the difficulty to maintain a complete gas tight vessel, leading to slight H_2 leakages [93]. From Table 3, it is also possible to compare H_2 yields from polysaccharides with glucose. Surprisingly, the highest H_2 yield was obtained from starch fermentation. Since starch $[(C_6H_{10}O_5)_n]$ is a glucose polymer, it first requires a biological hydrolysis step to transform the starch into simple sugars to be further used in the metabolism. Therefore starch presents a slower biodegradability than glucose [94]. Despite this biodegradability issue, it still presents a higher H_2 yield than from glucose fermentation. According to Masset, Julien and co-workers (2014) [94], this higher H_2 production, from starch degradation, is linked to the fermentation of alternative carbon sources, namely some metabolites such as lactate, activating alternative metabolic pathways that produce H_2.

Table 3. H_2 yields in batch fermentations by *C. butyricum* performed at different culture volumes using glucose, sucrose and starch as carbon and energy sources.

Substrate	Culture volume	Max. H_2 yield	Max theoretical H_2 yield [95]	Ref.
Glucose	60mL	2.40 mol H_2/mol glucose	4 mol H_2/mol glucose	[28]
Glucose	120 mL	2.28 mol H_2/mol glucose		This work
Glucose	2L Reactor	1.69 mol H_2/mol glucose		[29]
Sucrose	200 mL	1.20 mol H_2/mol sucrose	8 mol H_2/mol sucrose	[96]
Starch	3L Reactor	2.91 mol H_2/mol hexose	4 mol H_2/mol hexose	[94]

4.3.2 Adaptation of *C. butyricum* cells to inhibitors

The cells adaptation to higher concentrations of gallic acid, HMF and furfural and its effect on H_2 production and lipid profile of *C. butyricum* were studied. A stepwise adaptation strategy was used to grow cells in conditions far from optimum, in the presence of the natural inhibitors studied above (gallic acid, HMF and furfural). Once cells were able to grow in the presence of the inhibitor, they were used to inoculate fresh medium supplemented with carob pulp extract and *Spirogyra* hydrolysate containing phenolic and furanic compounds, respectively.

4.3.2.1 Cell adaptation to Gallic acid

Gallic acid was used as a standard phenolic compound representative of the total phenols, since is one of the main phenolic compounds present in carob pulp. *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. At lower concentrations, once the cells reached the mid-exponential growth phase, they were used as inoculum for a second fermentation medium supplemented

with carob pulp aqueous extract as carbon and energy source. The carob pulp aqueous extract was characterized in terms of total sugars concentration (221.4 g/L ± 4.2 glucose equivalents) and total phenols concentration (0.83 g/L ± 0.02 gallic acid equivalents, GAE).

Table 4. 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

Globally, the H_2 production yield was higher than what is commonly reported in other studies using biorenewable fermentable biomass, nevertheless this value is still lower than the theoretical H_2 yield from sucrose (8 mol H_2/mol sucrose) [96]. This high H_2 production yield might be related with the rich carob pulp composition in fermentable carbohydrates, which is mainly composed of a mixture of sucrose, fructose and glucose [47]. The highest H_2 production yield was attained after 24 h of fermentation with the cells adapted to 2.0 g/L of gallic acid (3.63 mol H_2 / mol eq. glucose) (Table 4). For cells adapted to a gallic acid concentration of 3.5 g/L, the H_2 production was significantly inhibited when compared to the control, probably due to an accumulated cellular stress caused by the successively increasing concentrations of gallic acid. 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 activating metabolic pathways that help to degrade carob pulp constituents. Concerning the substrate consumption, no improvements were obtained by culturing the cells in the presence of gallic acid, since the maximum

conversion percentage (75%) was attained with cells that were not adapted. Consequently, the cellular adaptation to gallic acid did not influenced the cellular efficiency on converting carob pulp carbohydrates.

Concerning the lipid profile characterization for the cellular adaptation to higher gallic acid concentrations, it was possible to observe that cells increased their saturation degree with increasing gallic acid concentrations until 2.5-3.0 g/L (Figure 12). This maximum saturation degree profile attained at the gallic acid concentrations of 2.0, 2.5 and 3.0 g/L is coincident with the concentrations where H_2 production yield was higher after 8h of fermentation (Table 4 and Figure 12). Therefore this result shows that the cellular adaptation mechanisms to gallic acid allowed a cellular adaptation to carob pulp constituents, resulting on a higher H_2 production yield. For the final concentration of 3.5 g/L the saturation degree decreased, possibly suggesting that cells were incapable of a proper growth under this condition. Nonetheless, an increase of polyunsaturated fatty acids (PUFAs) content was registered for cells cultivated under this condition. 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 [78, 97]. Moreover, the presence of PUFAs are also associated with the maintenance of cytoplasmic membrane fluidity, probably used by *C. butyricum* cells to maintain cellular integrity.

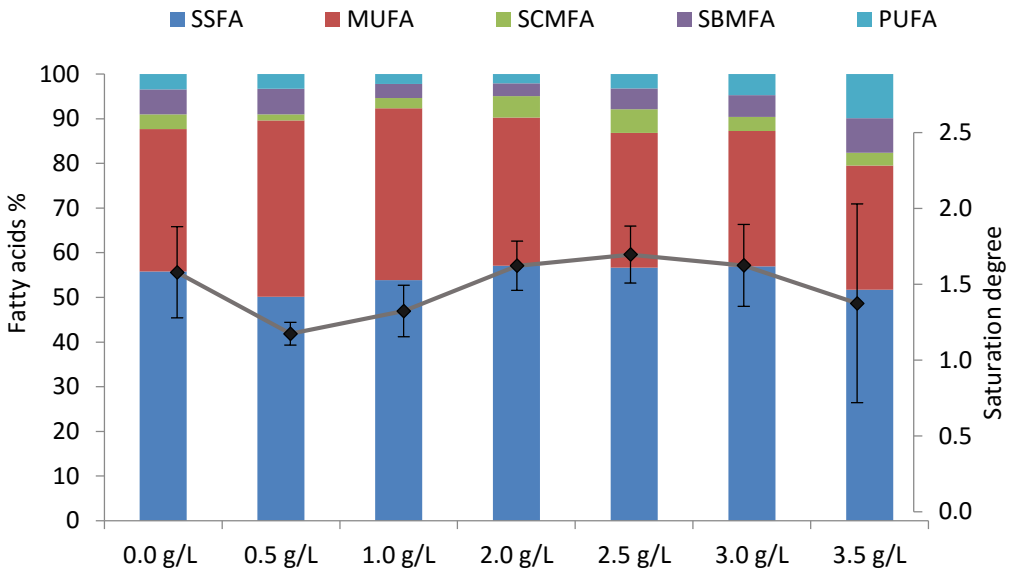


Figure 12. Fatty acid composition of mid-exponential grown *C. butyricum* cells adapted to different gallic concentrations (*bars*) and corresponding calculated saturation degree (*line*). *SSFAs* straight-chain saturated fatty acids, *MUFAs* monounsaturated fatty acids, *SCBFAs* saturated cyclopropyl-branched fatty acids, *SMBFAs* saturated methyl-branched fatty acids, and *PUFAs* polyunsaturated fatty acids.

4.3.2.2 Cell adaptation to HMF/Furfural

Based on the results described above for the cellular growth inhibition of HMF and furfural, the following concentrations of each inhibitor were tested together to study *C. butyricum* cells adaptation: 0.0, 0.25, 0.5, 0.75, 1.0 and 1.5 g/L. Moreover, according to the previous results for the effect of furfural on *C. butyricum*, no growth or cellular adaptation capacity was expected for concentrations near 2.0 g/L. Whereas HMF alone seemed to present low toxicity levels for these concentrations (Figure 9). A mixture of furfural and HMF were used in the adaptation since they are commonly found together in the majority of acid hydrolysates of lignocellulosic materials [50]. When cells reached the mid-exponential growth phase they were used as inoculum of the fermentation media supplemented with *Spirogyra* hydrolysate. The *Spirogyra* hydrolysate was characterized in terms of total sugars concentration (41.7 g/L ± 5.7 glucose equivalents), HMF (0.37 g/L) and furfural (0.12 g/L).

After 24h of fermentation, the highest H_2 production yield of 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 5). The maximum cumulative H_2 production was about 45% higher than the H_2 volume produced by cells grown under control conditions, and also than previous studies using *Spirogyra* hydrolysate [65]. Nevertheless, higher H_2 production yields are described from the fermentation of microalgae biomass, e.g. from *S. obliqus* which allowed a yield of 2.9 mol H_2 /mol $_{sugars}$ [3]. This discrepancy between the fermentation of different strains of microalgae might be due to different proportions of carbohydrates, lipids and proteins [48].

Table 5. Hydrogen production yield and cumulative production by *C. butyricum* using *Spirogyra* hydrolysate as substrate, after cellular adaptation to increasing concentrations 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

On the whole, the results show that the cellular adaptation mechanisms to HMF/Furfural lead to an adaptation to the *Spirogyra* hydrolysate constituents, resulting on 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. Concerning the substrate consumption, a maximum conversion percentage (61%) was obtained for cells adapted to lower HMF and furfural concentrations. Similarly to the cellular adaptation to gallic acid, no significant improvements were obtained in terms of substrate conversion, by adapting the cells to HMF/furfural.

Regarding the lipid profile characterisation for cellular adaptation to HMF and furfural, no significant lipid variation was registered in terms of the saturation degree associated with the increase of concentration (Figure 13). However, for the cells cultivated in the presence of the highest HMF and furfural concentration, an increase in the content of PUFA*s* was observed with a proportional decrease of MUFAs. This result was similar to what has been observed and described above for the *C. butyricum* cells adaptation to a gallic acid concentration of 3.5 g/L (Figure 12). Therefore, supporting the hypothesis of this increase of PUFAs being a cellular mechanism to overcome strict conditions. Furthermore, adapted cells were also analysed under the microscope (Figure 14), and it was possible to observe that *C. butyricum* cells under the studied conditions grew in cellular aggregates and formed brighter structures inside the cells.

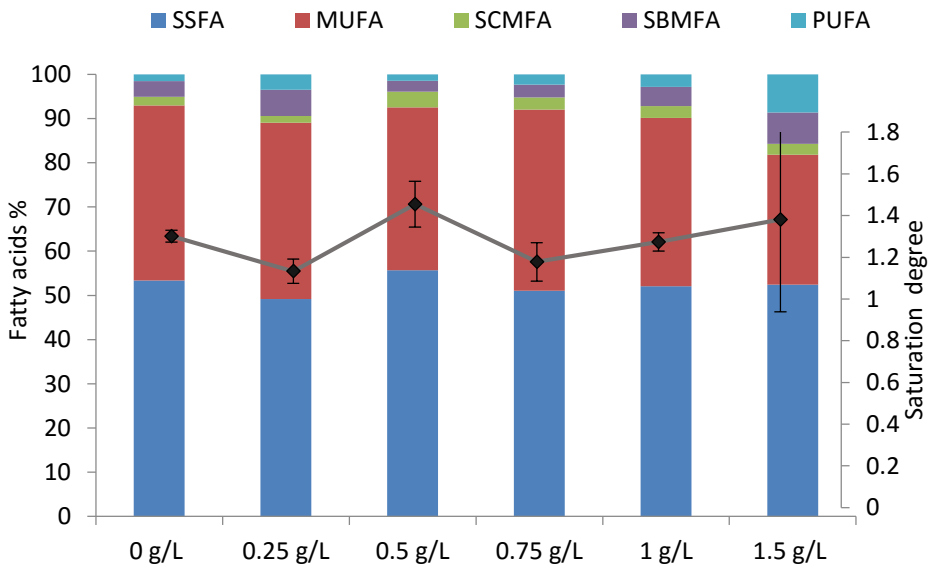


Figure 13. Fatty acid composition of mid-exponential grown *C. butyricum* cells adapted to different HMF/furfural mixture concentrations (*bars*) and corresponding saturation degree (*line*). *SSFAs* straight-chain saturated fatty acids, *MUFAs* monounsaturated fatty acids, *SCBFAs* saturated cyclopropyl-branched fatty acids, *SMBFAs* saturated methyl-branched fatty acids, and *PUFAs* polyunsaturated fatty acids.

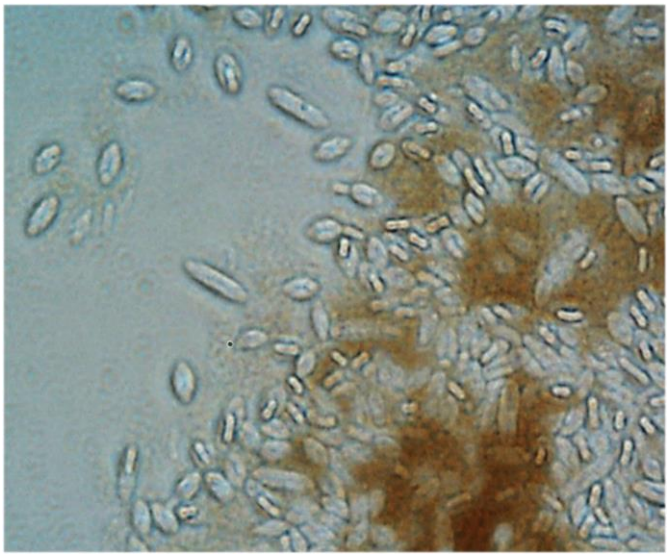


Figure 14. Light-microscopy photographs of *C. butyricum* cells cultured in the presence of HMF/furfural mixtures. Apparent sporulation process -bright structures inside cells (Olympus BX60 1000x magnification under bright field light).

4.3.2.3 C. butyricum *best adapted cells versus control cells*

Comparing the H_2 production yield of 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 6). 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 15). 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 6. 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 (2.0 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
Best adapted cells	1.71	3.63

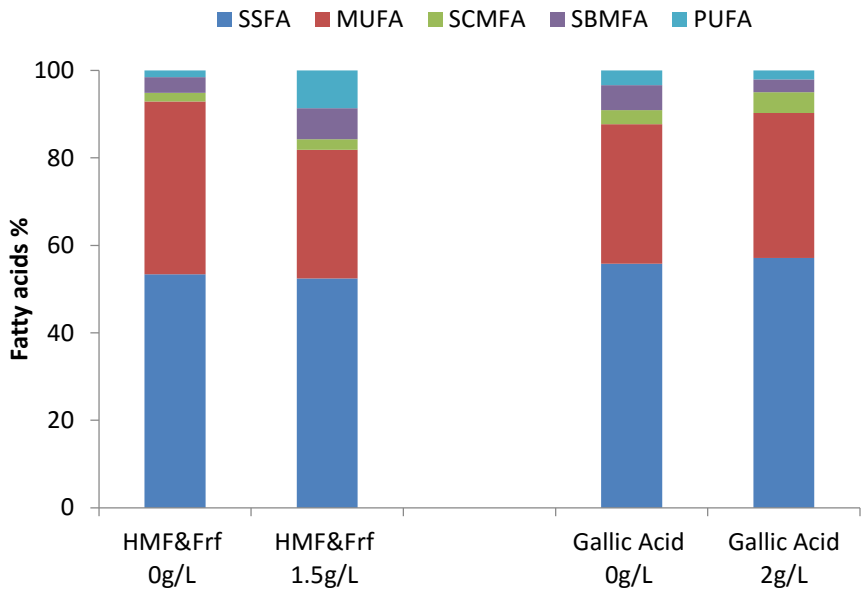


Figure 15. Comparison of the fatty acid composition between non-adapted and best adapted *C. butyricum* cells to HMF/Furfural (1.5 g/L) and gallic acid (2.0 g/L). *SSFAs* straight-chain saturated fatty acids, *MUFAs* monounsaturated fatty acids, *SCBFAs* saturated cyclopropyl-branched fatty acids, *SMBFAs* saturated methyl-branched fatty acids, and *PUFAs* polyunsaturated fatty acids.

5 Conclusions

In this work, two main aspects were studied: the physiologic response of *C. butyricum* cells to natural stress agents, and the H_2 production from different substrates. Some important conclusions were possible to be drawn from this work, since it is a completely novelty in terms of studying the physiologic response of *C. butyricum* cells to natural inhibitors, through the lipid profile characterisation, and in the adaptation and usage of *C. butyricum* to increase biohydrogen production.

From the cellular lipid characterisation during growth and biogas production, 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 more prone to suffer stress in the lipid membrane. Moreover, no significant changes in the lipid composition were observed with the increase of biogas production and its accumulation inside the fermentation flask. This indicates that the cells could theoretically produce higher amounts of biogas since, apparently, the concentration produced was not inhibitory.

Four different natural inhibitors were selected for this study: oxygen, which inhibits strictly anaerobic cells growth; 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. The agent which presented the highest growth inhibitory effect on *C. butyricum* was furfural, since cells in the presence of 2.0 g/L were incapable of a substantial growth. On the contrary, oxygen was the less inhibitory agent tested, in terms of affecting the cellular growth when introduced during the exponential growth phase. Concerning the modifications on *C. butyricum* lipid profile, in the presence of gallic acid, a decrease on the saturation degree was registered, after 20 minutes of exposure. This may suggest the existence of an enzymatic complex capable of promoting a lipid rearrangement, converting C16:0 into C18:1w7c, by the action of two enzymes, first an C16/C18 elongase followed by a Δ11 desaturase. Nonetheless, a possible *de novo* fatty acid synthesis was also observed as a cellular adaptation mechanism after 3 and 6 h of exposure to furfural. Comparing the effect of the two furaldehyde compounds (HMF and furfural), furfural clearly has a higher growth inhibitory effect than HMF, and it induced larger modifications in the fatty acid composition.

The fermentation of 10 g/L glucose by *C. butyricum* achieved a good H_2 production yield of 2.28 mol H_2 / mol glucose, close to 95 % of the maximum theoretical value. Moreover, a H_2 rich biogas was produced, in addition to a high glucose conversion efficiency of 93 %. Therefore, it was possible to conclude that the optimum carbohydrate concentration, which resulted on the highest H_2 production yield from a range of tested glucose concentrations, was of 10 g/L.

Finally, a stepwise adaptation strategy of *C. butyricum* cells to phenolic and furaldehyde compounds was used in the fermentation of carob pulp and microalgae biomass to evaluate the effect on H_2 production yield.

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 conclude that carob pulp aqueous extract is an excellent fermentable carbon source to produce biohydrogen, according to the high H_2 yields obtained from its fermentation. Globally, 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 on an improvement of the H_2 production yield.

In conclusion, the main objectives proposed for this work were fulfilled by compiling a physiological study of *C. butyricum* cells, and how to use adapted cells to increase biohydrogen production from carob pulp and *Spirogyra* hydrolysate. This biohydrogen production process is still far from being a feasible commercial source, notwithstanding important achievements from the physiological perspective of the *C. butyricum* cells were obtained, which can be further explored on the biohydrogen production research.

6 Future Work

Although this work introduced some novelty and a new scope of work by joining physiological advances and biohydrogen production, several bottlenecks remain to understand and further studies must be conducted to support the results here presented.

First, considering the inhibitors, other agents might be included by following the same procedure such as hydrogen, carbon dioxide, and other phenols besides gallic acid. Moreover, the lipid profile characterisation can also be complemented with an enzymatic assay to further detail if the lipid alterations at 20 minutes are in fact caused by an enzymatic complex.

Secondly, the adaptation process must be corroborated by further studies to support the results obtained, and for the HMF/Furfural adaptation, a separate assay for each compound should be conducted to detail the effect of each one individually. In addition, other complex fermentation substrates must be tested and compared, including lignocellulosic materials, since their content on HMF, furfural and phenols is considerably high.

Finally, to implement the optimizations here described, a scale-up fermentation must be addressed at a reactor level, including batch and continuous operation mode. And afterwards implement a life cycle analysis of the process to assess its global viability and potential.

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Annex I

Basal Medium I (BM 1)

Nutrient	Concentration
Trypticase Soya Broth w/o Dextrose	5.0 (g/L)
Yeast Nitrogen Base	5.0 (g/L)
Cysteine – HCl. H_2O	0.56 (g/L)
Salt Solution A	10.0 (mL/L)
Salt Solution B	2.0 (mL/L)
Salt Solution C	2.0 (mL/L)
Salt Solution D	10.0 (mL/L)

Salts Solution A:

Nutrient	Concentration (g/L)
NH_4Cl	1.0
$MgCl_2.6H_2O$	0.1
$CaCl_2.H_2O$	0.1

Salt Solution B:

Nutrient	Concentration (g/L)
$K_2HPO_4.3H_2O$	0.4

Oxygen indicator Solution C:

Nutrient	Concentration (mg/L)
Resazurin - $C_{12}H_7NO_4$	1.0

Salts Solution D:

Nutrient	Concentration (mg/L)
$Na_2.EDTA.2H_2O$	5.0
$CoCl_2.6H_2O$	1.5
$MnCl_2.4H_2O$	1.0
$FeSO_4.7H_2O$	1.0
$ZnCl_2$	1.0
$AlCl_3.6H_2O$	0.4
$Na_2WO_4.2H_2O$	0.3
$CuCl_2.2H_2O$	0.2
$NiSO_4.6H_2O$	0.2
H_2SeO_3	0.1
H_3BO_3	0.1
$Na_2MoO_4.2H_2O$	0.1

Reinforced *Clostridium* Medium (RCM, Oxoid) composition for pre-inoculum

Nutrient	Concentration (g/L)
Yeast Extract	3.0
'Lab-Lemco'	10.0
Peptone	10.0
Starch	1.0
Glucose	5.0
Cysteine Hydrochloride	0.5
Sodium Chloride	5.0
Sodium Acetate	3.0
Agar	0.5

Annex II

Total sugars quantification – Phenol sulfuric method

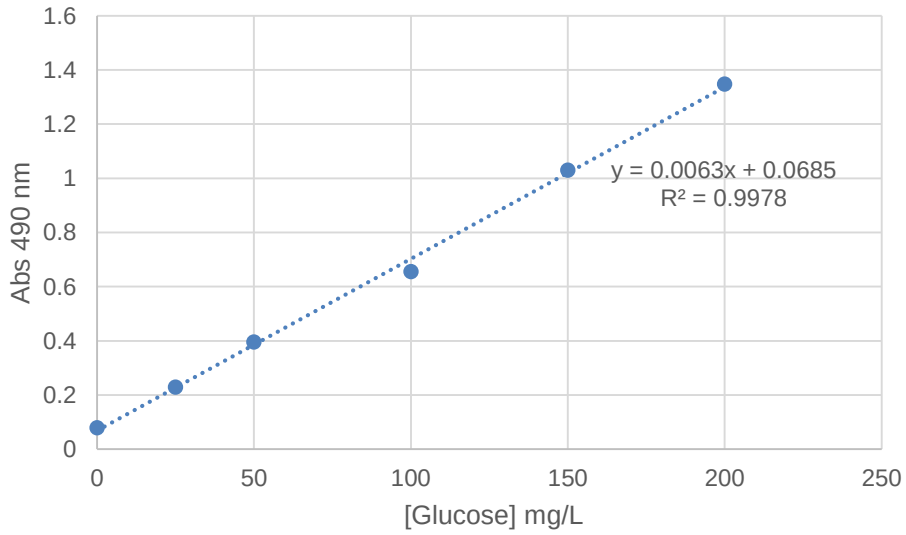

Figure 16. Phenol – sulfuric calibration curve with glucose for total sugar determination

Total phenols quantification - Folin-Ciocalteu method

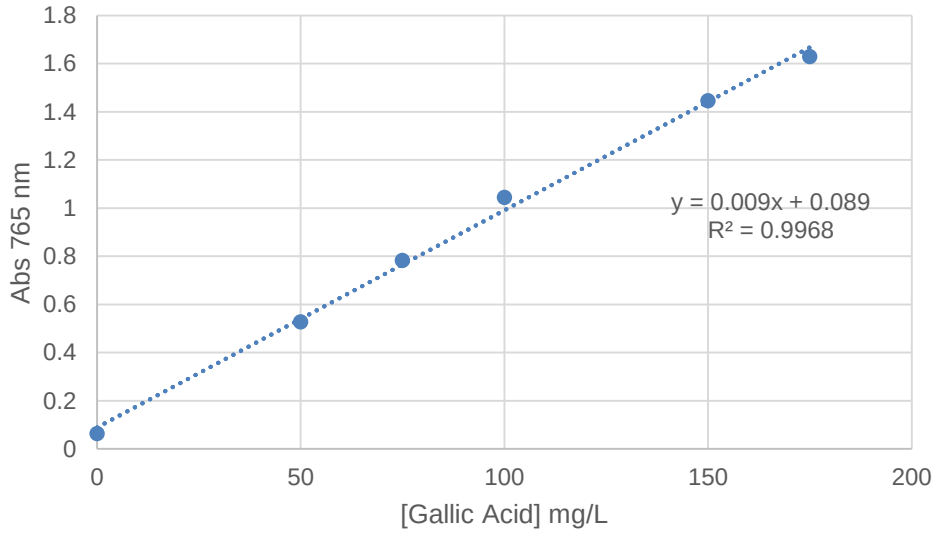

Figure 17. Folin – Ciocalteu calibration curve with gallic acid for total phenols determination