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ORIGINAL PAPER

Hydrogen Production by *Clostridium cellulolyticum* a Cellulolytic and Hydrogen-Producing Bacteria Using Sugarcane Bagasse

Juliana K. Braga[1] · Angela A. Abreu[2] · Fabrício Motteran[1] · Maria Alcina Pereira[2] · Maria Madalena Alves[2] · Maria Bernadete A. Varesche[1]

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Abstract Hydrogen (H_2) production by *Clostridium cellulolyticum* was investigated. Anaerobic batch reactors were operated with cellobiose (2 g/L) and pretreated sugarcane bagasse (SCB) (2 g/L) using a hydrothermal system to observe the effects of carbon source on H_2 production. Salts (NH_4Cl, NaCl, $MgCl_2$ and $CaCl_2$) and vitamins (biotin, nicotinamide, p-aminobenzoic acid, thiamine, pantothenic acid, pyridoxamine, cyanocobalamin, riboflavin, folic and lipoic acid) were supplemented from stock solutions at different volumes percentages, ranging from 0 to 5%. The optimal concentration was 2.5% and the strain used both substrates and produced H_2 which was higher for cellobiose (14.9 ± 0.2 mmol/L) than for SCB (7.6 ± 0.2 mmol/L), although the λ phase was much smaller when SCB (59.9 h) was used in relation to the assay with cellobiose (164 h). H_2 was produced from SCB primarily through the fermentation of lactic and acetic acids.

Keywords Acetic acid · Cellobiose · Lactic acid · Lignocellulosic residue · SCB

✉ Juliana K. Braga
jukawanishi@gmail.com

Maria Bernadete A. Varesche
varesche@sc.usp.br

[1] Department of Hydraulics and Sanitation, School of Engineering of São Carlos, University of São Paulo, Av. Trabalhador Sãocarlense, São Carlos, SP, Brazil

[2] CEB – Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga, Portugal

Introduction

The production of hydrogen by dark fermentation processes is a promising alternative when compared to other biological or physical–chemical processes [1]. When selecting the substrate for H_2 production, the main criteria should be availability, cost, carbohydrate content, and biodegradability [2]. In this context, the use of carbohydrate-rich wastes, such as lignocellulosic residues, has being highlighted in H_2 production research [3].

According to Bayer and Lamed [4] despite its low density, cellulose is the most noteworthy, resistant and stable natural-organic compound, therefore, it tends to accumulate in the environment.

The biological hydrolysis of cellulose is performed by cellulolytic microorganisms or by their cellulolytic enzymes that provide the breakdown of cellulose and the availability of free carbohydrates for fermentation and hydrogen production [5]. Aerobic or anaerobic, mesophlic or thermophilic, microorganisms are widespread in nature and can produce these enzymes [5]. Examples include genera *Clostridium, Cellulomonas, Citrobacter; Bacillus, Thermomonospora, Ruminococcus, Bacteroides, Erwinia, Acetovibrio, Microbispora; Rhodopseudomonas* and *Streptomyces* [2, 6–10].

Endospore forming bacteria such as *Clostridium* sp. play a crucial role in fermentation to produce H_2. These microorganisms convert simple and complex carbohydrates such as glucose and cellulose into H_2 and organic acids during the exponential growth phase [11].

The production of hydrogen is regulated by the carbon-flow distribution between several metabolic pathways, which are significantly influenced by the medium environment. The nutrient medium composition has to be carefully planned as it controls the efficiency of a process [12].

Considerable research efforts have focused on the optimization of culture medium for hydrogen production [13–15], but none have involved direct fermentation of sugarcane bagasse (SCB). It is commonly known that Ca^{2+} increase the thermostability of proteins in *Clostridium cellulolyticum*. However, Lamed and Bayer [16] observed the lack of calcium enhancement in the cellulosome of *C. cellulolyticum*.

According to Sato et al. [17] *Clostridium thermocellum* needs at least 0.6% of yeast extract (YE) to competently convert 1% cellulose to ethanol. Yeast extract is a complex source of amino acids, nitrogen compounds and vitamins, important growth factors for many microorganisms [18]. The main functional constituents in YE required by some representatives of the genus *Clostridium* are *p*-aminobenzoic acid, vitamin B12, pyridoxine, and biotin [19]. Furthermore, these vitamins and other compounds found in the YE are also essential for the growth of *Clostridium thermopalmarium*, a hydrogen-producing bacteria [20].

The magnesium ions (Mg^{2+}) are the most abundant divalent cations in living cells acting as an indispensable cofactor for several enzymes involved in glycolysis and other reactions that depend on adenosine triphosphate (ATP) [21]. Dombek and Ingram [22] and Thanonkeo et al. [23] observed positive effects of high Mg concentrations on H_2 or ethanol production.

The mesophilic *C. cellulolyticum* is an anaerobe Gram-positive cellulosome-producing of the Family 4 of Clostridia [24] which was isolated from decayed grass compost [25]. This specie has become a model bacteria for the studies of mesophilic cellulose degradation [26]. Along with cellulose, members of this genus grow on a large variety of substrates (carbohydrates) including glucose, xylan, xylose, arabinose, fructose, galactose, mannose, ribose and soluble cellodextrins [25, 27, 28].

However, there are no studies in the literature regarding the use of *C. cellulolyticum* for hydrogen production from SCB, making this study a breakthrough for bioenergy production from lignocellulosic residues, thus reducing the impact caused by the inadequate disposal of those residues in the environment.

In recent years, numerous products with economic and environmental interest have been achieved through biotechnological processes. The use of SCB as a source of substrates for biotechnological processes is an attractive and promising alternative, considering the high content of carbohydrates in this biomass.

It should be emphasized that the use of SCB for hydrogen production is still understudied, hence new research is needed to achieve an inoculum composed of cellulolytic microorganisms and hydrogen producers to reduce costs in the pre-treatment of these substrate.

Sugarcane bagasse are a complex of hemicellulose, lignin, and cellulose, along with other minor constituents [29]. Hemicellulose and lignin protect cellulose fibers from exposure to microbial enzymes, which are essential for conversion of the carbohydrates into fermentable glucose [30]. Hydrothermal pretreatment eliminates these barriers by solubilizing hemicellulose and displacing the lignin fraction. Furthermore, it decrystallizes cellulose and allows cellulase access to the microfibers. This kind of pretreatment, where fibers are heated in water, is of particular interest since the only solvent is water [31], which excludes catalyst costs and reduces process waste. According to da Cruz et al. [32] the main components from hydrothermal pretreatment in liquid fraction are xylose, arabinose, glucose, mannose, galactose, uronic acids, furfural and HMF. The pretreated solid fraction is enriched with cellulose and small fraction of lignin.

Mathematical models are important tools to understand and improve the processes, and the application of these models has enhanced hydrogen production [15]. According to Nath and Das [33] the modified Gompertz model has been used to study hydrogen production using the members of the genus *Clostridium*.

In this context, this study contributes to the innovation in H_2 production research using real lignocellulosic residue. For this, the optimum nutritional condition for the strain *C. cellulolyticum* to produce H_2 was evaluated, including SCB as a substrate since this residue is low-cost, has a large supply and availability of carbohydrates, therefore viable for H_2 production.

Materials and Methods

Culture and Media

In order to remove the dissolved oxygen, the medium was boiled, and then cooled in ice for 30 min, with the addition of N_2 (99.9%). The medium was distributed in serum bottles (60% volume for the gas phase) and capped with butyl stoppers and aluminum seals. The gaseous phase was replaced by a mixture of N_2/CO_2 (80/20% v/v) to a final pressure of 1.75 bar. The reactors containing the medium were then sterilized by autoclaving at 120 °C for 20 min. Prior to inoculation, the basal medium was supplemented with 5% bicarbonate solution (78 g/L sodium bicarbonate and 4.7 g/L sodium sulfide) and 5% salt/vitamins solution (5.8 g/L ammonium chloride, 5.8 g/L sodium chloride, 1.9 g/L magnesium chloride, 2.1 g/L calcium chloride and vitamins solution). The vitamin solution had the following composition: 0.4 mg/L biotin, 3.9 mg/L niacin, 9.8 mg/L pyridoxine, 1.9 mg/L riboflavin, 3.9 mg/L thiamine, 1.9 mg/L cyanocobalamin, 1.9 mg/L p-aminobenzoic acid, 1.9 mg/L pantothenic acid and 0.9 mg/L folic acid.

Batch Culture Experiments

H_2 production experiments were conducted in batch reactors of 120 mL in triplicate with 50 mL of reaction medium and 70 mL of headspace. The reactors were incubated at 37 °C under shaking (100 rpm). The experimental assays are outlined as follows: Assay 1 (353 h) was conducted to optimize the culture medium favoring better H_2 production. In this stage decreasing concentrations of salts and vitamin solution were tested (A-5%, B-2.5%, C-1.5%, D-0.5% and E-0%) using cellobiose (2 g/L) as carbon source.

In the second phase, the best nutritional condition of the previous stage was used employing 2 g/L of cellobiose (Assay 2) and 2 g/L of SCB (Assay 3), as carbon sources. In these stages the production of volatile fatty acids (VFA) and carbohydrate consumption were also analyzed.

The SCB employed in this study was provided by the São Martinho sugar mill (Pradópolis, SP, Brazil). The substrate was air-dried and stored in cardboard boxes at room temperature. The SCB (5 g) was pretreated using a hydrothermal system of 100 mL capacity, at a pressure of 20 bar, at 200 °C for 10 min [34]. *C. cellulolyticum* was pre-grown three days in basal medium supplemented with 2 g/L cellobiose and after for 20 days in basal medium supplemented with 2 g/L SCB and 1 g/L yeast extract in batch assays for a previous adaptation to the substrate.

Physicochemical and Chromatographic Analyses

Hydrogen content of the gas phase was determined by gas chromatography (GC) using a Molsieve column (80/100 mesh) and thermal conductivity detector (Bruker Scion 456 Chromatography - Bruker, Massachusetts, USA) with argon (30 mL/min) as the carrier gas. The injector, detector, and column temperatures were 100, 130 and 35 °C, respectively. The Shimadzu GC – 2010 was also used for Hydrogen detection, this GC was equipped with thermal conductivity detector using Carboxen® column (1010 plot 30 m X 0,53 mm), with argon (30 mL/min^{-1}) as the carrier gas. The injector, detector, and column temperatures were 220, 230 and 130 °C, respectively.

VFA and cellobiose were determined by high performance liquid chromatography using an HPLC (Jasco, Japan) with an Aminex® column (HPX-87H Ion 300 × 7.8 mm^2); sulfuric acid (0.01 N) at a flow rate of 0.7 ml/min was used as mobile phase. Column temperature was set at 60 °C. Detection of VFA and cellobiose was made sequentially with an UV detector at 210 nm and a RI detector, respectively. Carbohydrates were carried out indirectly by the phenol–sulfuric acid method using glucose as a standard according to Dubois et al. [35].

Kinetic Analysis

The cumulative hydrogen production data were adjusted to Gompertz equation, modified by Zwietering (Eq. 1) [36] using Origin® 9.0 software. The H_2 concentration was calculated considering the injected volume (500 µL), the chromatographic area (promoted by calibration curve in mmol) and the volume of headspace (70 mL).

$$H = P \cdot \exp\left\{-\exp\left[\frac{R_m \cdot e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

where: P—Hydrogen Potential Production (mmol/L), Rm—Hydrogen Production Rate (mmol/h), t—incubation time of the reactors (hours), e—Euler number (2.71828) and λ—phase before H_2 production starts (hours).

The number of cell generations in the exponential growth period (n) (Eq. 2), exponential growth time (t), generation time—time required for a population to double the number of cells (g) (Eq. 3), specific growth rate (k) (Eq. 4), and growth rate - number of generations formed per unit of time of a culture in exponential growth (v) (Eq. 5) were calculated according to Pelczar [37] and Madigan et al. [38].

$$N = N_0 2^n \quad (2)$$

$$g = t/n \quad (3)$$

$$k = 0.301/g \quad (4)$$

$$v = 1/g \quad (5)$$

where: N—final cell number (OD_{600}), N_0—initial cell number (OD_{600}), n—the number of generations during the period of exponential growth, t—duration of exponential growth and g—generation time.

Results and Discussion

Optimization of Culture Medium for H_2 Production

In this study, the effect of salts and vitamins solutions on the cumulative H_2 production in the anaerobic mesophilic batch reactors was evaluated. The results from the batch reactor assays confirmed the importance of salts and vitamins solutions for *C. cellulolyticum* and that this strain could be used as inoculum for biohydrogen production from cellobiose. There were significant differences in H_2 production ($P < 0.05$) between all studied conditions.

The reactors were monitored for 352 h until H_2 production stabilized (Fig. 1). The cumulative H_2 production increased with an increasing concentration of salts and vitamins solution added to the reactor from 0 to 2.5%.

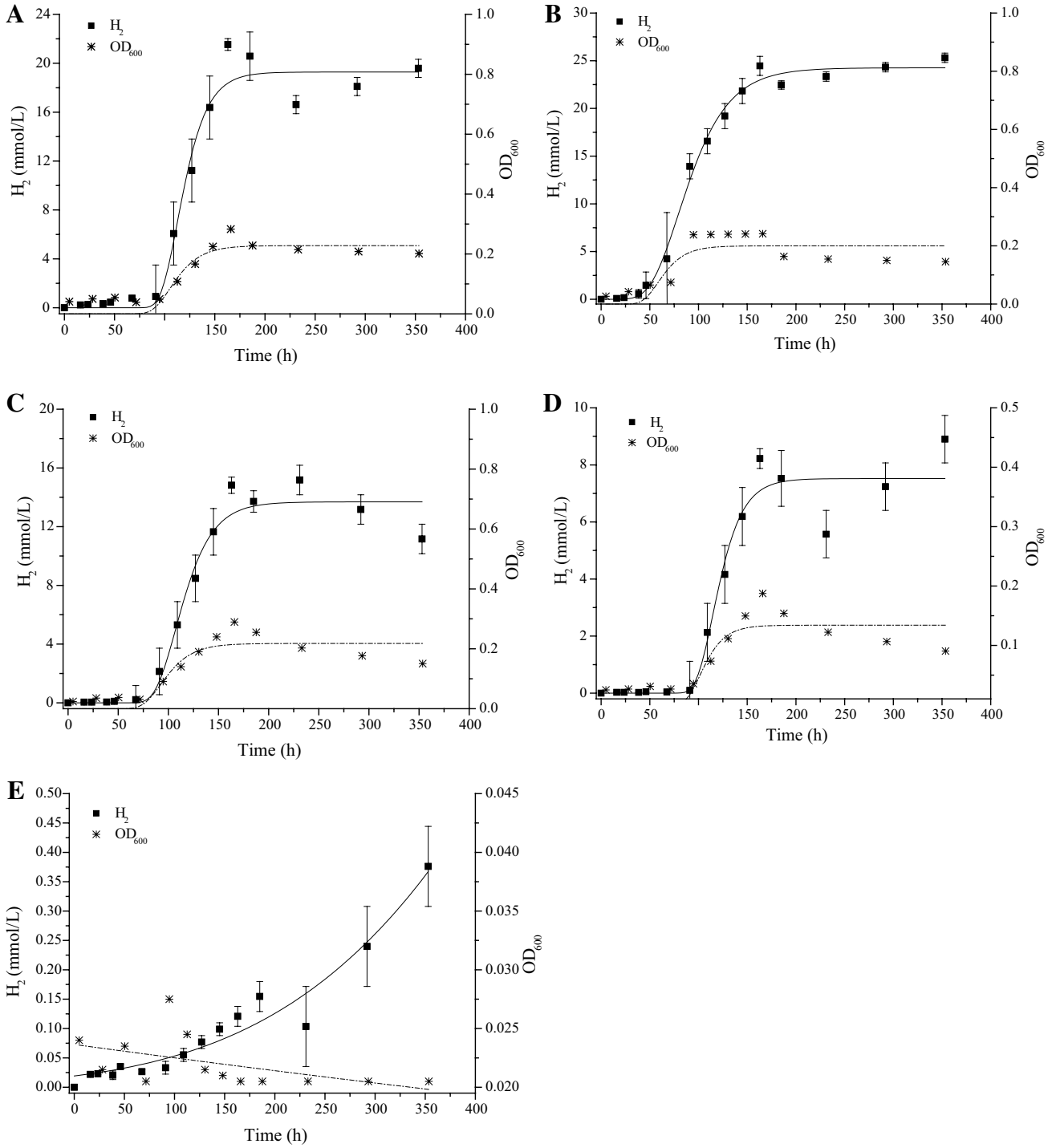

Fig. 1 H_2 production and cell growth (OD_{600}). **a** 5% salts and vitamins solution, **b** 2.5% salts and vitamins solution, **c** 1.5% of salts and vitamins solution, **d** 0.5% of salts and vitamins solution and **e** from 0% of salts and vitamins solution

In reactors supplemented with 5% of salts and vitamins solution (condition A) the H_2 production reached 19.3 ± 0.7 mmol/L in 163 h. When the concentration was decreased to 2.5% of salts and vitamins solution (condition B) the H_2 production was higher, reaching the maximum production of 24.3 ± 0.5 mmol/L, also in 163 h of reactors incubation.

The cumulative H_2 production obtained from conditions C and D (1.5% and 0.5% of salts and vitamins solution, respectively) was lower, reaching 13.7 ± 0.5 and 7.5 ± 0.4 mmol/L,

respectively. Comparing the data obtained in these last two conditions with that obtained in assays A and B, it can be observed that the addition of higher concentrations of salts and vitamins solution in the reactors facilitated cellobiose hydrolysis, as an increased production of H_2 was noted.

A huge amount of bacteria requires or is stimulated by water-soluble B vitamins and amino acids. Thiamine is the most commonly required vitamin, but biotin and vitamin B12 also are indispensable for a great number of bacteria [39]. Vitamins have an essential role in carbohydrate metabolism as co-factor and coenzymes to activate or inhibit numerous enzymes [13]. The addition of a suitable concentration of several vitamins such as cobalamin, vitamin C, riboflavin, citric acid, pyridoxine and folic acid in the fermentative medium improve the cell growth and accumulation of hydrogen from *Clostridium* sp. In this study a complex vitamins solution (biotin, nicotinamide, p-aminobenzoic acid, thiamine, pantothenic acid, pyridoxamine, cyanocobalamin, riboflavin, folic and lipoic acid) was used in the culture medium.

By contrast, in condition E (without salts and vitamins solution), almost no hydrogen production was observed during the entire operational period, the H_2 content was less than 0.1 mmol/L until 145 h, reaching maximum H_2 production of 0.15 mmol/L after 300 h incubation (Fig. 1). The absence of salts and vitamins solution in this condition negatively influenced the H_2 production, evidencing the dependence of *C. cellulolyticum* on these compounds for cell growth and H_2 production.

The batch reactors showed a stable hydrogen production rate according to the adjusted Gompertz equation (modified by Zwietering [36]) between 127 and 353 h and steady state was achieved in all conditions studied, except for condition E (0% salts and vitamins solution).

Table 1 summarizes the estimated kinetic parameters, H_2 potential production (*P*), maximum rate of H_2 production (*Rm*) and λ phase time. Higher H_2 potential production value was observed in condition B (2.5%), compared with condition A (5%), however this value did not correspond to the greater H_2 production rate which was 0.31 ± 0.02 and 0.42 ± 0.08 mmol/L h^{-1} for conditions B and A, respectively. Therefore, the Hydrogen production rate of the *C. cellulolyticum* culture reached the highest value with the addition of 5% of salts and vitamins solution in the culture medium (Table 1). Nevertheless, longer λ phase (96.1 ± 5.1 h) was observed for condition A, in contrast with condition B (50.7 ± 3.7 h).

Table 1 Parameters obtained from the modified Gompertz equation fitting

Condition	*P* (mmol/L)	R_m (mmol/L h^{-1})	λ (h)	R_2
A (5%)	19.3 ± 0.7	0.42 ± 0.08	96.3 ± 5.1	0.97
B (2.5%)	24.3 ± 0.5	0.31 ± 0.02	50.7 ± 3.7	0.99
C (1.5%)	13.7 ± 0.5	0.24 ± 0.05	86.9 ± 6.2	0.97
D (0.5%)	7.5 ± 0.4	0.16 ± 0.04	97.8 ± 7.2	0.95
E (0%)	0.4 ± 0.04	$0.003 \pm 1E\text{-}4$	78 ± 11.04	0.95

A 5% salts and vitamins solution, *B* 2.5% salts and vitamins solution, *C* 1.5% salts and vitamins solution, *D* 0.5% salts and vitamins solution and *E* with no salts and vitamins solution

As the concentration of the salts and vitamins solution decreases in the culture medium, the H_2 production rate also decreases. In conditions C (0.24 ± 0.05 mmol/L h^{-1}) and D (0.16 ± 0.04 mmol/L h^{-1}) lower rates were verified, compared to conditions A and B. In the last condition the *Rm* was the lowest among all the studied conditions (around 0.001 mmol/L h^{-1}), confirming the importance of salts (NH_4Cl, NaCl, $MgCl_2$ and $CaCl_2$) and vitamins (biotin, nicotinamide, p-aminobenzoic acid, thiamine, pantothenic acid, pyridoxamine, cyanocobalamin, riboflavin, folate and lipoic acid) for *C. cellulolyticum* strain in H_2 production from cellobiose. Furthermore, shorter H_2 potential production (*P*) and maximum rate of H_2 production (Table 1) were observed for the condition without solution salts and vitamins (E—0%).

Figure 1 illustrates the *C. cellulolyticum* growth under 5% (A), 2.5% (B), 1.5% (C), 0.5% (D) and 0% (E) of salts and vitamins solution. In addition, the corresponding H_2 productions are also presented. While the *C. cellulolyticum* growth rate was similar at initial salts and vitamins solution concentrations of 1.5, 2.5 and 5%, the cell densities were significantly affected by the availability of this solution.

Clostridium cellulolyticum grew to a very low density, mainly for condition E (OD_{600} of 0.027 in 91 h) when a very low H_2 production was observed (0.37 ± 0.04 mmol/L). On the other hand, low concentration of 0.5% salts and vitamins solution supported cell-growth to OD_{600} of 0.18 in 163 h accompanied by H_2 production of 7.52 ± 0.4 mmol/L.

The increased concentration of salts and vitamins solution led to a gradual increase of OD_{600} values. Increased amount of NH_4 (present on salt solution) was available for the microorganism and therefore cell growth was also higher in these conditions. At high concentrations of salts and vitamins solution (5, 2.5 and 1.5%) cell-growth continued to a similar maximum OD_{600}, at 163 h, of approximately 0.25, 0.28 and 0.24 for conditions A, B and C, respectively. These values were lower than those observed by Gehin et al. [40], which detected OD_{600} of 1.05 (about 40 h); 0.6 (about 25 h) and 0.25 (about 20 h) for cultures of *C. cellulolyticum* submitted to 7, 1 and 0.5 g/L cellobiose, respectively. In this study, the stabilization of gas production correlated with the end of cell growth for all conditions studied, except for condition E.

Lin and Lay [41] evaluated the supplement of Mg, Na, Zn, Fe, K, I, Co, NH_4^+, Mn, Ni, Cu, Mo and Ca for H_2 production by anaerobic inoculum (dominated by *Clostridium pasteurianum*) using sucrose as substrate. According to these

authors, Mg, Na, Zn and Fe were the main trace metals that influenced H_2 production, with magnesium as the most important one. Those trace elements are all necessary for the metabolism of several anaerobic microorganisms which relate to the bacterial enzyme cofactor, transport processes and dehydrogenase.

Lin and Lay [41] observed that the H_2 production reached 3.52 mol/mol sucrose when the 120 mg/L $MgCl_2$ and 1000 mg/L NaCl were used. In this study a similar concentration of $MgCl_2$ (190 mg/L) was employed in condition A with 5% salts and vitamins solution. However, much lower concentration of NaCl (215 mg/L) was added in the same condition, which along with other substrate (cellobiose) used in the present study may have led to a lower H_2 production than that of the aforementioned authors.

Table 2 list values for the number of generations formed during the period of exponential growth (n); duration of exponential growth (t); generation time which represents the time required for a population to double the number of cells (g); specific growth rate (k); division rate (v).

The generation times (g) of *C. cellulolyticum* cultured on 2 g/L cellobiose were similar for all conditions studied, with the lowest value for condition B (10.54 h). Giallo et al. [42] observed a higher value of generation time (about 24 h) for *C. cellulolyticum* when the authors employed cellulose as substrate, probably due to the more complex substrate employed by these authors.

Moreover, number of generations (n), generation time (g) and duration of exponential growth (t) were lower for condition B (Table 2), even though this condition provided greater H_2 production (24.3 ± 0.5 mmol/L). This showed that the condition with 2.5% salts and vitamins solution promoted higher efficiency of that bacterial population using cellobiose for H_2 production. Reinforcing the choice of this nutritional condition as the most appropriate for H_2 production from SCB.

Although the concentration of salts and vitamins (0–5%) in the culture medium influenced the number of generations formed during the period of exponential growth (n) and the duration of exponential growth (t), a small variation was observed for parameters k (specific growth rate) and v (growth rate) (Table 2).

Gibson et al. [43] evaluated the effect of sodium chloride and temperature on the rate and degree of growth of *Clostridiun botulinicun* type A in pasteurized pork slurry. The authors observed generation times ranging from 1.2 h (1.5% NaCl and 20 °C) to 41.7 h (3.5% NaCl and 15 °C) and growth rates from 0.17 h^{-1} (3.5% NaCl and 15 °C) to 6.02 h^{-1} (1.5% NaCl and 20 °C). According to the authors the composition of the culture medium and the incubation temperature directly influence the microbial growth, which was also observed in our study using *C. cellulolyticum* and cellobiose as substrate.

Table 2 Parameters of microbial growth of *C. cellulolyticum* with different concentrations of salt and vitamins and cellobiose as substrate

Condition	n	t (h)	g (h)	k (h^{-1})	v (h^{-1})
A (5%)	4.99	54	10.82	0.0278	0.0924
B (2.5%)	4.27	45	10.54	0.0286	0.0949
C (1.5%)	6.75	77.5	11.48	0.0262	0.0871
D (0.5%)	4.74	54	11.38	0.0264	0.0879
E (0%) *	–	–	–	–	–

n number of generations formed during the period of exponential growth, *t* duration of exponential growth, *g* generation time (time required for a population doubles the number of cells), *k* specific growth rate, *v* growth rate (number of generations formed per unit of time of a culture in exponential growth), *no exponential phase, *A* 5% salts and vitamins solution, *B* 2.5% salts and vitamins solution, *C* 1.5% salts and vitamins solution, *D* 0.5% salts and vitamins solution and *E* with no salts and vitamins solution

Cellobiose and SCB for H_2 Production

In order to evaluate the efficiency of the *C. cellulolyticum* strain in H_2 production from a real lignocellulosic residue, in this study, the effect of cellobiose and pretreated SCB on the cumulative H_2 production and H_2 yield in anaerobic mesophilic batch reactors was evaluated with the best concentration of solution salts and vitamins (2.5%) tested previously. A strain of *C. cellulolyticum* was employed to identify possible synergetic effects on SCB degradation and biohydrogen production. The test results from the batch reactors establish that cellobiose and pretreated SCB could be used as substrate source for biohydrogen production by this strain. The presence of H_2 and CO_2 in the biogas was observed in both conditions studied and there were significant differences in H_2 production ($P < 0.05$).

The batch reactors were operated for 713 and 445 h for Assay 2 (2 g/L cellobiose) and Assay 3 (2 g/L pretreated SCB), respectively, until H_2 production stabilized (Fig. 2). Table 3 shows the values for the H_2 potential production, H_2 maximum rate production and λ phase. In both assays, the R_2 value was higher than 0.99. The cumulative H_2 production obtained from reactors fed with cellobiose and SCB reached 14.9 ± 0.25 and 7.6 ± 0.19 mmol/L, respectively (Fig. 2; Table 3).

Datar et al. [44] did not observe H_2 production from corn stover previously pretreated with steam explosion. H_2 production was observed only after the addition of the cellulase enzyme. Ratti et al. [45] evaluated H_2 production from cellulose using rumen fluid as inoculum source in batch experiments. These authors stated that positive results were observed only with the addition of commercial cellulase. These results differ from those observed in our study, which

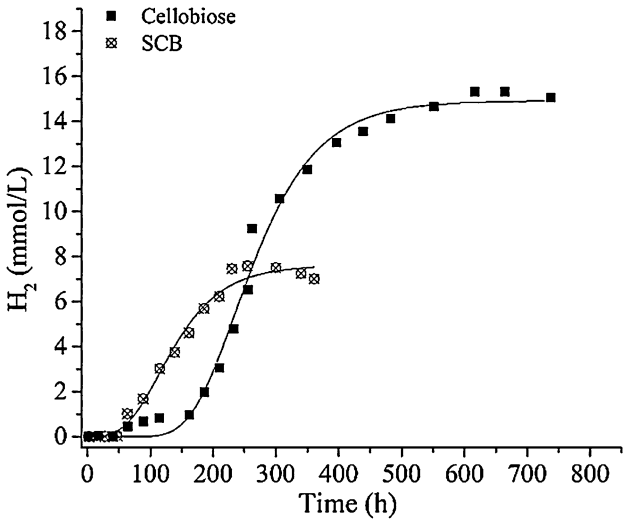

Fig. 2 Hydrogen production (mmol/L) from cellobiose and SCB by *C. cellulolyticum*

Table 3 Parameters obtained from Gompertz modified from Assays 2 (cellobiose) and Assay 3 (SCB)

Parameters	Assay 2 (cellobiose)	Assay 3 (SCB)
H_2 potential production (mmol/L)	14.9 ± 0.25	7.6 ± 0.19
H_2 production rate (mmol/L h^{-1})	0.07 ± 0.005	0.05 ± 0.003
λ phase (h)	164 ± 6.37	59.9 ± 5.12
R^2	0.99	0.99
HY (mmol H_2/g carbohydrate)	11.5	3.9

suggests that the use of pretreated SCB as substrate and *C. cellulolyticum* as inoculum provided the hydrolysis of cellulose with the consequent H_2 production. In this study, it was not necessary to add external cellulase enzyme, thus reducing process costs.

In this study the pH in the batch reactors was adjusted to 7. The addition of sodium bicarbonate in the reactors maintained the pH at 6.7 and 6.5 in Assays 2 and 3, respectively, at the end of the reactors operation, which favored the H_2 production. Hu et al. [46] evaluated the pH effect on cellulose degradation and concluded that the highest cellulose degradation (75%) was verified at pH values ranging between 6.8 and 7.3, whereas the cellulose degradation decreased significantly at lower pH values.

The pH alteration can inhibit the hydrogenase enzyme and change the metabolic pathway to solventogenesis, hence decreasing the H_2 production [47]. Fangkum and Reungsang [48] observed maximum H_2 production at pH 6.5 also using SCB as substrate and elephant dung as inoculum.

The kinetic parameters of the cellobiose and SCB conditions (Assay 2 and 3, respectively) were adjusted in the Gompertz equation with R^2 of 0.99. The H_2 maximum potential production (P) for Assay 2 was 14.9 ± 0.25 mmol/L. The maximum H_2 production rate (*Rm*) was 0.07 ± 0.005 mmol/L h^{-1} when the medium was supplemented with cellobiose and at the same condition the λ phase lasted 164 ± 6.37 h (Table 3).

For the condition with SCB (Assay 3) the H_2 maximum potential production (P) was 7.6 ± 0.19 mmol/L. The maximum H_2 production rate (Rm) was 0.05 ± 0.003 mmol/L h^{-1}, lower than Assay 2 when the medium was supplemented with cellobiose. On the other hand, the λ phase was much lower when SCB was applied as substrate, 59.9 ± 5.1 h (Table 3).

Prior to inoculation of *C. cellulolyticum* in the reactors with SCB, the bacteria was reactivated for three days in basal medium supplemented with 2 g/L cellobiose and after for 20 days in basal medium supplemented with 2 g/L SCB for a previous adaptation to the substrate, since it is more complex than cellobiose. It is possible this dual adaptation promoted a faster H_2 production (lower λ phase) when compared to reactors fed only with cellobiose but reaching lower values.

A maximum hydrogen yield (HY) was obtained with 2 g/L cellobiose (Assay 2) reaching 11.5 mmol H_2/g cellobiose equivalent to 2.82 mmol H_2/mmol cellobiose, differently from the theoretical maximum yield (4 mol H2/mol glucose) when the main route is relative to acetic acid. It is noteworthy that the substrate used in this study was cellobiose instead of glucose and the inoculum used was a pure culture of *C. cellulolyticum* submitted to nutritional restrictions. In Assay 3 with 2 g/L SCB, the HY obtained was 3.9 mmol H_2/g SCB (Table 3). These values were much lower than those observed by Ratti et al. [49], which verified 1.2 mol H_2/g substrate from cellulose hydrolysis of SCB (0.5 g/L) pretreated by steam explosion and alkaline delignification employing batch reactors inoculated with a cellulolytic-fermentative microbial consortium obtained from the SCB in nature (0.5 g/L) in thermophilic condition (55 °C).

It is possible that the aforementioned authors used a microbial consortium from the SCB also employed as residue, which improved the H_2 yield, compared with this study which used as inoculum pure culture of *C. cellulolyticum*. Furthermore, the higher temperature employed by Ratti and contributors may have favored the higher values they observed. According to Nissila et al. [50] H_2 yields may be enhanced by using thermophilic bacteria.

The substrate utilization rate varied from 16 mg/L h after 24 h of incubation to 0.2 mg/L h after 510 h of incubation for cellobiose assay. For SCB condition, the substrate utilization rate varied from 5 mg/h after 24 h of incubation to 0.7 mg/L h after 445 h of incubation.

Figure 3 shows that in both cellobiose and SCB experiments the hydrogen production is related to the growth of *C. cellulolyticum*. However, in the presence of the

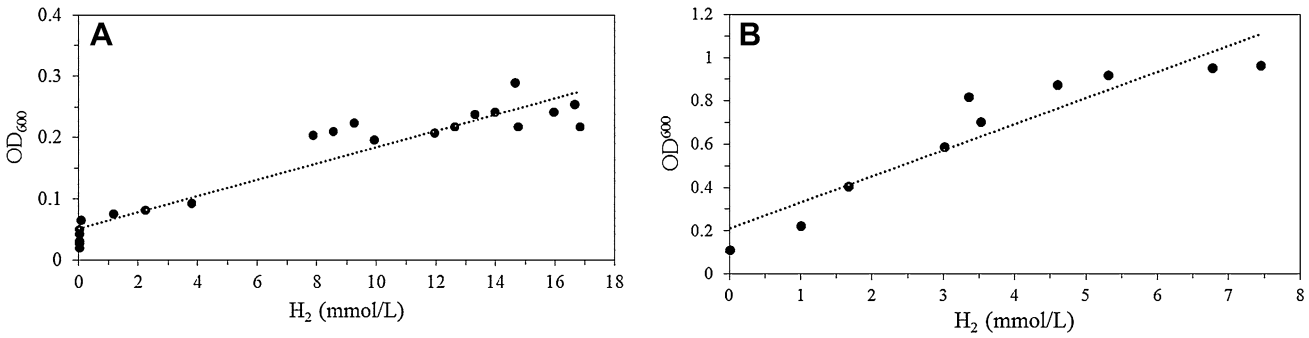

Fig. 3 OD_{600} and H_2 production data of the assay 2 with 2 g/L cellobiose (**a**) and the assay 3 with 2 g/L SCB (**b**) as carbon source

lignocellulosic residue (SCB – Assay 3) higher cell concentration (as OD_{600}) was required to produce smaller amount of H_2 compared with the more readily biodegradable substrate (cellobiose).

The pre-treatment (hydrothermal followed by autoclaving) of the SCB may have provided the release of easily assimilated sugars [49, 51, 52], thereby promoting greater microbial growth when this substrate was used. In addition, the inoculum (*C. cellulolyticum*) was previously acclimated in this complex substrate (SCB), thereby facilitating their adaptation and facilitating the production of H_2.

In this study, the addition of sodium bicarbonate in all reactors kept the pH at 6.6 ± 0.2, which favors the cellulose degradation and H_2 production. The pH modifications may inhibit iron-containing hydrogenase enzymes, thus decreasing the H_2 production [50].

Production of Soluble Microbial Products (SMP)

The fermentative production of H_2 is a complex process accompanied by the formation of organic acids and solvents, which may be influenced by several factors such as inoculum, substrate, pH, temperature and others [53]. Four organic acids were generated through the H_2 production in Assay 3: lactic acid (102.9–498.7 mg/L), acetic acid (54.9–125.6 mg/L), butyric acid (29.2–40.1 mg/L) and isobutyric acid (38.1–60 mg/L). However, in Assay 2 the values of organic acids were below the method detection limit, although higher H_2 production (14.9 ± 0.25 mmol/L) was observed for this condition.

Higher concentrations of organic acids observed in Assay 3 (SCB) was probably due to the intrinsic microbial community of SCB, as verified by Ratti et al. [49]. These authors evaluated the H_2 production using SCB as substrate and as inoculum source in thermophilic condition and observed the presence of 12 bacteria genera belonging to the phyla Firmicutes, Bacteroidetes and Actinobacteria. However, in Assay 2 of the present study cellobiose and the pure culture of *C. cellulolyticum* were used, thus providing lower concentrations of metabolites generated.

In Assay 3 (SCB), the production of acetic acid substantially increased during the initial adaptation phase, (first 90 h) reaching a maximum value of 111.4 mg/L (Fig. 4). This production declined gradually during the exponential phase (79 mg/L), reaching lower values in the stationary phase (up to 50.2 mg/L) at about 400 h of incubation. Desvaux et al. [26] observed acetic acid as the main metabolite (3958%) together with a large quantity of H_2 when evaluating carbon flux distribution of *C. cellulolyticum* and cellulose (0.9–29.1 g/L) as substrate. These authors also observed that the increasing lactic acid production (about 450 mg/L in lower cellulose concentrations to 2350 mg/L in higher cellulose concentrations) corresponded to the decrease of acetic acid, ethanol, CO_2, and H_2 production.

The predominance of lactic acid was verified in this last condition, with maximum amount of 498.7 mg/L in 445 h of reactor operation, similar to that verified by Desvaux et al. [26]. The production of this acid initiates in the adaptation phase but a pronounced increase was observed

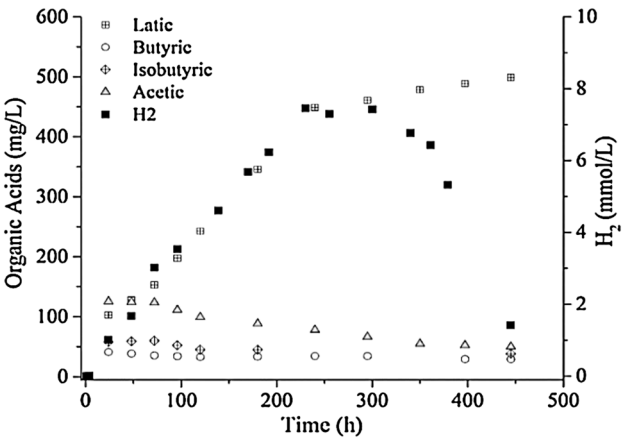

Fig. 4 Cumulative production of organic acids and hydrogen as a function of time for Assay 3 with 2 g/L SCB

mainly during the exponential phase (96 h at 240 h). After this, the lactic acid production rate decreased, maintaining the concentration of this metabolite constant over the stationary phase (Fig. 4). As previously discussed, this increase of lactic acid corresponded to the acetic acid decrease.

The presence of other bacteria derived from bagasse and which were not eliminated after the hydrothermal pretreatment followed by sterilization by autoclaving, may have contributed to the change in the end-metabolite, when compared with the cellobiose Assay.

SCB hydrolysate generally comprises fermentable sugars (glucose and xylose) which can be used for the production of lactic acid. Additionally, lactic acid bacteria (LAB) has been commonly found in sugar cane. Sobrun et al. [54] verified the presence of nine strains of LAB from fresh sugar cane juice. Gomes et al. [55] isolated and identified ten LAB (e.g. *Lactobacillus casei* and *L. plantarum*) related with a distilled beverage produced by the fermentation of sugar cane juice.

Giallo et al. [42] observed that the main metabolites of crystalline cellulose fermentation (2.5 g/L) by *C. cellulolyticum* were lactic acid, acetic acid and ethanol. The authors observed an increased production of acetic acid (about 1 g/L) when the substrate used was cellulose, compared to glucose and cellobiose (about 800 mg/L for both substrates). The authors observed that increasing the cellulose concentration (2.5–7.6 g/L) the production of acetic acid decreased, followed by an increase in lactic acid concentration (180–1800 mg/L). A similar trend was also observed in this study but using 2 g/L of SCB as a cellulosic substrate.

Lo et al. [56] used SCB pretreated by acid treatment to produce H_2. The authors verified a predominance of acetic acid (68%), followed by lactic acid (17%) and butyric acid (15%). Butyric and isobutyric acids were also detected in this assay with maximum amounts ranging between 41 and 60 mg/L, respectively. The percentage of butyric acid, and isobutyric acid were 4.5 and 5.9%, respectively, at the end of the assay.

From the end-metabolites observed during the reactors operation, it was evident that the substrate source (cellobiose or SCB) influenced the metabolic flux response of C. cellulolyticum. Furthermore, in this study it was concluded that C. cellulolyticum has considerable potential for efficient bioconversion of lignocellulosic feedstocks as SCB for sustainable production of biofuels (such as hydrogen) and other chemicals (e.g., acetic, formic, lactic acids). These data may be useful to enhance the process of bioreactor operation for continuous and upscale processes converting lignocellulosic residues into desired bio-products.

Conclusion

In this study, the optimal concentration of salts and vitamins solution was 2.5% as it provided higher H_2 production values. The strain *C. cellulolyticum* was able to use cellobiose and SCB as carbon source and produce H_2. The cumulative H_2 production obtained from the reactors was higher for cellobiose than for SCB, although phase λ was much smaller when SCB was used as substrate. H_2 was produced from SCB primarily through the fermentation of lactic and acetic acids, however, the increasing production of lactic acid corresponded to the decrease in acetic acid. The microbial kinetics of *C. cellulolyticum* can improve the production of biofuel engineering processes. Moreover, studies of lignocellulosic substrate consumption and microbial growth contribute to understanding microbial metabolism under SCB degradation and subsequent fermentation. However the present study could not provide more precise information about the metabolism of this strain. Therefore further studies are needed to optimize the biological process of hydrogen production using lignocellulosic residues.

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