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Isolation of Paraclostridium CR4 from sugarcane bagasse and its evaluation in the bioconversion of lignocellulosic feedstock into hydrogen by monitoring cellulase gene expression

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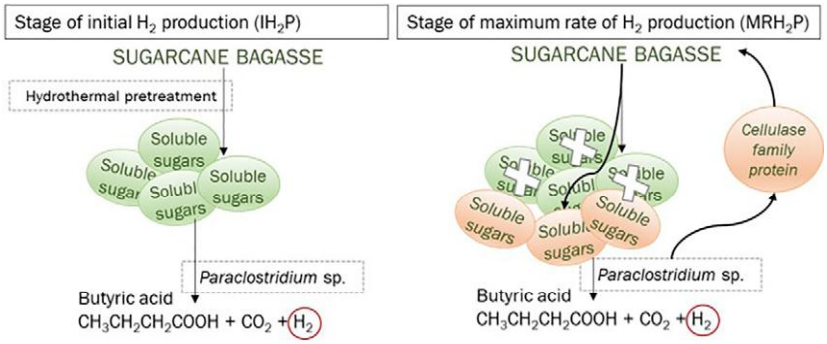
HIGHLIGHTS

- Paraclostridium was identified as autochthonous bacteria from sugarcane bagasse.
- Paraclostridium produced H_2 from glucose, cellulose and sugarcane bagasse.
- The substrate type regulated the formation of the final product (acetic/butyric).
- There was cell growth during all stages of H_2 production.
- The cellulase family protein expression was regulated by soluble sugars.

GRAPHICAL ABSTRACT



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abstract

Bioconversion of sugarcane bagasse (SCB) into hydrogen (H_2) and organic acids was evaluated using a biomolecular approach to monitor the quantity and expression of the cellulase (Cel) gene. Batch reactors at 37 °C were operated with Paraclostridium sp. (10% v/v) and different substrates (5 g/L): glucose, cellulose and SCB in natura and pre-heat treated and hydrothermally. H_2 production from glucose was 162.4 mL via acetic acid (2.9 g/L) and 78.4 mL from cellulose via butyric acid (2.9 g/L). H_2 production was higher in hydrothermally pretreated SCB reactors (92.0 mL), heat treated (62.5 mL), when compared to in natura SCB (51.4 mL). Butyric acid (5.8, 4.9 and 4.0 g/L) was the main acid observed in hydrothermally, thermally pretreated, and in natura SCB, respectively. In the reactors with cellulose and reactors with hydrothermally pretreated SCB, the Cel gene copy number 3 and 2 log were higher, respectively, during the stage of maximum H_2 production rate, when compared to the initial stage. Differences in Cel gene expression were observed according to the concentration of soluble sugars in the reaction medium. That is, there was no gene expression at the initial phase of the experiment using SCB with 2.6 g/L of sugars and increase of 2.2 log in gene expression during the phases with soluble sugars of b1.4 g/L.

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1. Introduction

Lignocellulosic material has been used as an alternative substrate for sustainable production of biofuels such as hydrogen (Ratti et al., 2015; Soares et al., 2017; Rabelo et al., 2018b), methane (Guo et al., 2014; Tantayotai et al., 2017) and ethanol (Kumar et al., 2008; Rabelo et al., 2011). Some of the main advantages of using these materials are their wide availability and high carbon content. In addition, the use of lignocellulosic material adds waste reduction to clean energy generation (Shrestha et al., 2017; Ahmad et al., 2018).

According to its origin, lignocellulosic material can be obtained from the processing of forest residues (mainly wood), agricultural residues (soybean, rice and sugarcane, among others) and urban and industrial residues (solid or liquid). Brazil is the largest producer of sugarcane in the world and 620.8 million tons of sugarcane were produced in the 2018/19 harvest, and later about 0.2 million tons of sugarcane bagasse (SCB) (UNICA, 2019). Although the traditional use of SCB is a substrate for boiler combustion within the plants themselves, there is surplus that can be made available for hydrogen production.

The effective degradation of lignocellulosic materials, requires synergistic stages of cellulolytic enzyme production, polysaccharide hydrolysis and sugar fermentation. In this process, the speed and yield of the end products are related to the initial hydrolysis (Saratale et al., 2008; Juturu and Wu, 2014). Hydrolysis is generally limited by the complex structure of materials, including cellulose crystallinity, particle size and the presence of associated materials such as hemicellulose and lignin (Binod et al., 2011; Galbe and Zacchi, 2012).

Cellulolytic anaerobic bacteria can be obtained from ruminal fluid (Deng et al., 2017), from invertebrates (Gupta et al., 2012) and mollusks (Muñoz et al., 2014), soil (Talia et al., 2012), decomposition of grass (Desvaux, 2005) and sugarcane bagasse (Ratti et al., 2015; Rabelo et al., 2018a). Ratti et al. (2015) and Rabelo et al. (2018a) identified autochthonous cellulolytic bacteria from sugarcane bagasse and similar to Clostridium, Tepidimicrobia, and Paenibacillus. Both authors used this source of autochthonous bacteria as an inoculum for the bioconversion of pre-treated sugarcane bagasse into hydrogen and organic acids.

Cellulolytic, anaerobic and gram-positive bacteria are included in the Firmicutes phylum and more particularly in the Clostridia class, Clostridiales order, most of them belonging to the Clostridiaceae family (Whitman, 2010). These bacteria have an enzymatic cellulolytic system consisting of at least 3 different types of cellulases: exoglucanases, endoglucanases and β-glycosidases, which synergistically provide complete cellulose hydrolysis (Juturu and Wu, 2014).

Zhang et al. (2015) obtained high hydrogen production yields with cellulose (772 mL), carboxymethylcellulose (646 mL) and corn stalk (1308 mL) using Clostridium sartagoforme, which was isolated from cow dung compost. Different bacterial cellulolytic strains have been isolated from self-fermentation of cellulosic material and used as inoculum in fermentative systems. Mazareli et al. (2019) isolated Bacillus sp. from banana residues and used this strain as inoculum for the fermentative production of hydrogen from pure and complex substrates. The authors reported hydrogen production of 36.3 and 59.6 mL using xylose and maltose, respectively, and when banana residues were used as substrate, hydrogen production was 106.5 mL.

When combined, qPCR and RT-qPCR analyses are useful tools for the specific detection and quantification and analysis of transcriptional cellulolytic activity (Dollhofer et al., 2016). These analyses have been widely applied in both environmental samples (Béra-maillet et al., 2009; Singh et al., 2014; Dollhofer et al., 2016) and in fermentative reactors (Lu and Lee, 2015; Salimi and Mahadevan, 2013) for monitoring bacterial activity (Béra-maillet et al., 2009; Lu and Lee, 2015; Salimi and Mahadevan, 2013) and cellulolytic fungi (Dollhofer et al., 2016).

Béra-maillet et al. (2009) evaluated expression of cellulolytic enzymes of the bacterium Fibrobacter succinogenes from sheep rumen microbiota by RT-qPCR analysis. The authors compared cellulase expression in sheep containing F. succinogenes as the only cellulolytic bacterium with the complex microbiota of a conventional sheep. The level of gene transcription in the monoculture was about two logs higher than that measured in the conventional animal, since F. succinogenes in monoculture does not compete with other species for the degradation of vegetal fibers.

The qPCR analysis was used by Dollhofer et al. (2016) for the quantification of anaerobic fungi in environmental samples. According to the results of these authors, the quantification of anaerobic fungi (in number of copies/mL) in biogas digesters was lower (1.78×10^8) than the amount reported in rumen fluid (1.69×10^{10}) and in cattle feces (1.88×10^9 to 6×10^9), since it does not represent their natural habitat. Based on the RT-qPCR analyses, there was no transcriptional cellulolytic fungal activity in biogas digesters. Bacterial cellulolytic activity in biogas digesters was evaluated by Wei et al. (2015) and, contrary to fungal activity, it was shown that bacterial endoglucanases were significant in these environments.

In the present study, the cellulolytic bacterium Paraclostridium sp. CR4 was isolated from self-fermentation of sugarcane bagasse. The bacterium was used as inoculum in fermentative reactors for the hydrolysis and fermentation of cellulose and also sugarcane bagasse for hydrogen production. Quantification and expression of the cellulase family protein gene were monitored by real-time polymerase chain analysis (qPCR) and reverse transcription followed by qPCR (RT-qPCR). These analyses were applied to samples of fermentative reactors with cellulose and sugarcane bagasse as substrates and Paraclostridium sp. CR4. The building stages of specific primers were necessary for this bacterial species, as well as for the study of reference genes to normalize gene expression.

2. Material and methods

2.1. Strain isolation

Sugarcane bagasse (SCB) used as a source of autochthonous bacteria was supplied by Usina São Martinho (Pradópolis, SP, Brazil).

SCB self-fermentation assays were performed using 500 mL Duran® flasks with 250 mL autoclaved PCS medium (1.0 g/L yeast extract, 5.0 g/L peptone, 2.0 g/L $CaCO_3$, and 5.0 g/L NaCl), and sugarcane bagasse (7.0 g/L). The reactors were subjected to N_2 atmosphere (100%) for 15 min, closed with butyl cap and plastic screw, and incubated at 37 °C for 10 days.

Samples of the liquid fractions of SCB self-fermentation reactors were seeded in Petri dishes with RCM (Reinforced Clostridium Medium - Oxoid®) and 1.5% nutrient agar (Difco®). The petri dishes were incubated in GasPack Jar with Anaerobic filter (Probac®) at 37 °C for 48 h.

Isolated colonies were collected aseptically with platinum needle and resuspended in Hungate tubes with 7.0 mL of PCS medium and a strip of filter paper (5 × 1 cm) as substrate. The flasks were subjected to N_2 atmosphere (100%), closed with butyl cap and plastic screw, and incubated at 37 °C for 10 days. Cell growth of CR4 cellulolytic culture was identified by the degradation of the filter paper. The culture was then transferred to 100 mL Duran® flasks with 50 mL PCS culture medium and glucose as carbon source (5 g/L). The flasks were subjected to N_2 atmosphere (100%), closed with butyl cap and plastic screw, and incubated at 37 °C.

Microscopic examinations with the CR4 isolate were performed under an Olympus BX60-FLA optical microscope using common light. The Gram staining technique was performed according to the procedure described in DSM (1991). After identification, CR4 bacterial culture samples were stored in glycerol (50%) at −80 °C.

2.2. Strain identification and phylogenetic analysis

Extraction of genomic DNA from CR4 isolated culture was performed by Purilink Microbiome DNA Purification Kit (Invitrogen). For amplification of the 16S rRNA gene, the primer set 27F-1492R (Lane, 1991) and GoTaq® Master Mixes (Promega) were used according to the

manufacturer's instructions. The amplicons were sequenced by the Sanger method at the Animal Biotechnology Laboratory, Department of Zootechnics (ESALQ/USP Piracicaba). The nucleotide sequences were processed in SeqMan DNASTAR (Lasergene sequence analysis) to exclude low quality bases. Then, the sequences were compared with Genbank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for taxonomic identity. Genetic distance dendrogram was constructed by the Maximum Likelihood Method based on the Tamura-Nei model (Tamura and Nei, 1993). The percentage of replicas was calculated based on bootstrap values with 1000 replicas. The analysis was performed using MEGA7 (Kumar et al., 2016) using Bacteroides vulgatus as Outgroup.

The sequence data was deposited in NCBI Sequence Read Archive under accession number MN003025.

2.3. Batch fermentation from glucose and cellulose

Batch experiments with Paraclostridium sp. CR4, glucose and/or cellulose as substrates were carried out in triplicate, in 250 mL Duran® flasks (125 mL reaction volume and 125 mL headspace), autoclaved RCM modified medium (13.0 g/L yeast extract, 10.0 g/L peptone, 5.0 g/LNaCl, 3.0 g/L sodium acetate and 0.5 g/L cysteine), pH 6.8. A control assay with Paraclostridium sp. CR4 and autoclaved RCM modified medium without carbon source was also conducted.

To adapt the CR4 isolate in cellulose, the experiments were performed with the gradual increase of the cellulose concentration, and the organic substrate (glucose and/or cellulose) was added as described in Table 1.

CR4 was used as inoculum (10% v/v) for the initial experiment with 100% glucose (A). In successive assays, in the hydrogen production stabilization stage, cells were concentrated by centrifugation (8000 rpm/10 min, 4 °C) and used in subsequent experiments (A to B, B to C, C to D and D to E) in order to favor the adaptation of the isolate to the cellulose substrate. The cell suspension of experiment E was concentrated by centrifuge and preserved in glycerol at −80 °C for use as substrate in sugarcane bagasse experiments.

To monitor the experiments, analysis of hydrogen (H_2) and organic acids production (according to Rabelo et al., 2018a) and soluble sugars (according to DuBois et al., 1956 modified by Herbert et al., 1971) were carried out. Samples were taken according to the H_2 production stages, that is, at the initial stage of the experiment (Initial), initial H_2 production stage (IH_2P in 3 h), maximum H_2 production (MRH_2P in 48 h for A and C, 24 h for B, 78 h for D and 36 h for E) and H_2 production stabilization (SH_2P in 180 h for A, D and E, 48 h for B and 156 h for C).

2.4. Batch fermentation assays for H_2 production from sugarcane bagasse

Sugarcane bagasse (SCB) was supplied by São Martinho sugar mill (Pradópolis, SP, Brazil).

The experiments were carried out with in natura SCB; heat pretreated SCB (autoclaved, 121 °C, 1.5 kg/cm^2, for 15 min) and hydrothermally pretreated SCB (in hydrothermal reactor, 200 °C for 10 min at 16 bar, according to Soares et al., 2017).

The solid phase from the hydrothermal pretreatment was washed in running water until the complete removal of the dark color to eliminate inhibitory compounds (furfural and hydroxymethylfurfural) that may be present after the pretreatment.

The in natura and pretreated substrates were dried and prepared according to NREL procedure (Hames et al., 2008) and were subsequently used in the H_2 production assays in batch reactors.

Assays were performed in batch reactors (triplicates) using 250 mL Duran® flasks. 125 ml of modified RCM culture medium (pH 6.8), SCB (5.0 g/L) was added as the sole carbon source and cellulose-grown Paraclostridium sp. CR4 culture (experiment E) as inoculum (10% v/v). After inoculation, the reactors were subjected to N_2 atmosphere (100%) for 15 min for headspace gas exchange. After 15 min, the reactors were closed with a butyl cap and plastic screw and incubated at 37 °C until the stabilization of H_2 production.

Analyses of H_2 were performed using a gas chromatograph (Rabelo et al., 2018a). Organic acids, furfural and hydroxymethylfurfural were quantified by a high performance liquid chromatograph (Rabelo et al., 2018a). Soluble sugars were determined by the colorimetric method (DuBois et al., 1956, modified by Herbert et al., 1971). The samples were taken according to the H_2 production phases, that is, at the initial stage of the experiment (Initial), initial H_2 production stage (IH_2P in 3 h), maximum H_2 production rate stage (MRH_2P in 12 h) and H_2 production stabilization stage (SH_2P in 46 h).

2.5. Kinetic parameters

Cumulative H_2 production data were adjusted using the Gompertz equation, modified by Zwietering et al. (1990). This model has been used to describe the progress of cumulative H_2 production in batch experiments (Lay, 2001; Guo et al., 2010; Rabelo et al., 2017; Soares et al., 2017).

Based on the modified Gompertz equation (Eq. (1)), it was possible to estimate the maximum potential H_2 production (P), maximum H_2 production rate (Rm) and initial H_2 production stage (λ).

$$H \quad P \cdot \exp\left\{ - \exp\left[\qquad \right] \right\} \qquad 1$$

H is the cumulative H_2 production (mL), t is the operation time (hours) and e is 2.71.

The generation time was calculated according to Eq. (2) (Mazareli et al., 2019).

$$\ln N - \ln N_0 \quad (\qquad) \cdot \Delta t \qquad 2$$

where, N = maximum cell number (copy numbers); N_0 = initial cell number (copy numbers); Δt = period of MRH_2P (h) and Tg = generation time (h).

The consumption of soluble carbohydrates (%) was calculated using Eq. (3) (Rabelo et al., 2018b):

$$\%\ \text{Sugar consumed} \quad \frac{Ci - Cf}{Ci} \times 100 \qquad 3$$

where Ci is the concentration of soluble carbohydrates at the initial stage and Cf is the soluble carbohydrates concentrations in the H_2 production stabilization stage.

2.6. Quantification and gene expression analysis by q-PCR and RT-qPCR

2.6.1. Collection, storage and extraction of genetic material samples

Samples for the extraction of genetic material were collected from cellulose fermentative reactors as substrate (experiment E), according to the H_2 production stages. Samples were collected at the initial stage of the experiment (Initial), initial H_2 production stage (IH_2P in 3 h), H_2 production maximum rate stage (MRH_2P in 21 and 48 h) and H_2 production stabilization stage (SH_2P in 180 h).

Table 1
Concentration (g/L) and percentage (%) of glucose and/or cellulose in experiments A, B, C, D and E.

Experiments	Glucose		Celullose	
	g/L	%	g/L	%
A	5	100	Without	Without
B	3.75	75	1.25	25
C	2.5	50	2.5	50
D	1.25	25	3.75	75
E	Without	Without	5	100

Samples of the hydrothermally pretreated SCB fermentation reactors were also collected. Samples were collected at the initial stage of the experiment (Initial), initial H_2 production stage (IH_2P in 3 h), H_2 production maximum rate stage (MRH_2P in 6 and 12 h) and H_2 production stabilization stage (SH_2P in 46 h).

DNA extraction samples (2.0 mL) were collected with syringes directly from the reactors and stored in 2.0 mL microtubes at −20 °C. Genomic DNA extraction was performed using the Purilink Microbiome DNA Purification Kit (Invitrogen), according to the manufacturer's protocol.

RNA extraction samples (0.5 mL) were collected with syringes directly from the reactors and stored in 2.0 mL microtubes containing 1.0 mL of RNA Protect bacteria reagent (Qiagen®), according to the protocol described by the manufacturer, and stored at −20 °C. RNA extraction was preceded by a bacterial lysis stage that was performed using Proteinase K and Lisozime according to the Qiagen® RNA extraction protocol. RNA extraction and purification were performed with RNeasy Mini Kit (Qiagen®) according to the manufacturer's protocol.

2.6.2. DNAs treatment and reverse transcription

RNA samples were subjected to DNase treatment with DNase RQ1 rnase-free enzyme (Promega®) and the procedure was performed according to the manufacturer's protocol. Complementary DNA strand (cDNA) was synthesized from the RNA sample. The enzyme GoScript Reverse Transcription System (Promega®) and Random primers (IDT®) were used according to the manufacturer's protocol.

2.6.3. Design of specific primers

The oligonucleotide pairs for the cellulase (Cel) gene were designed according to cellulase family protein gene sequences from Paraclostridium sp. described in Genbank (https://www.ncbi.nlm.nih.gov/nuccore/AVNC01000015.1) and using the Primer3 computer program (http://bioinfo.ut.ee/primer3/).

Three reference genes (GyrB, Pfo and RecA) for Paraclostridium sp. were proposed, designed (http://bioinfo.ut.ee/primer3/) according to NCBI specific sequences (https://www.ncbi.nlm.nih.gov/nuccore/AVNC01000015.1). The stability of reference genes was analyzed with the BestKeeper tool according to Pfaffl et al. (2004).

2.6.4. Real time quantitative PCR

Reactions were performed on MicroAmp Optical 96-Well Reaction (Thermofisher) microplates, where 2.0 μL of each DNA or cDNA sample, 7.5 μL of QuantiNova SYBR® Green PCR Kit, 0.75 μL of Rox Reference Dye (Invitrogen) diluted 1:10 v/v, 0.3 μL of each set primer diluted to 10 μM (Results and discussion Table 5) and 4.15 μL of UltraPure DNase/RNase-Free Distilled Water (Invitrogen) were added. The plates were sealed with MicroAmp Clear Adhesive Film (Thermofisher) for optical reading. Assays were performed on a 7500 Real-time PCR System® thermal cycler (Applied Biosystems).

The thermal profile of the thermal cycler consisted of a first cycle at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. At the end of the cycles, the amplification products were dissociated between 60 °C and 95 °C, with readings every 0.5 °C for 5 s to evaluate the melting temperature (Tm). All samples were tested in duplicate and, for each gene at each Real Time Quantitative Polymerase Chain Reaction (qPCR) run, a template control (NTC) was included. After amplification, the results were expressed as Quantification Cycles (Cq).

2.6.5. Absolute quantification

For DNA quantification, a standard curve was constructed using synthetic DNA (GBlock® Gene fragments, IDT) with the specific sequence

CTATTGTTTACTGTAGAACTATTTATGCATTTAACCAACTCCTCAACTGTA
TTTATAGGTTTATCACTAAGCTCAAAATAAGCTGCAATAGATATAAAATC
TACTTTCGAAAACAATTTGTTATTTAATTTTTCATTATATAATTCTTTACTTT
TGAGATCCCATTTTGCAGTGTACCACCATGAAGTTTTATATGTTACAAGTCC
TTTGAATCTCTTTTTCGTAAAATCTATTATGTCACATTAAAAGAGTGTTC
AAATGTTGGTAAATGTGGGGTCTGTGCAGTAGAAGTTGAAGGAAAAAAGG
TTCTTTCTCTTGCATGTTTAACTAAAGTAGAAGAAGGTATGGTTGTAAGA
ACTAATACTGAGGCAACACAAGAGAGAGTAAAGATGAGAGTGGCAGCTTT
ACTTGACAAACATGAATTCAAATGTGGACCATGTCCTCGAAGAGAGACAT
GTGAATTCTTAAAATTAGTAATC.

The GBlock® contained 500 ng mass (1691 fmol) and was eluted in 100 μL of TE solution. Serial dilutions of constant ratio of 10 were performed from this concentration. Diluted aliquots from 10^{-3} to 10^{-9} were subjected to the same qPCR quantitation conditions as the DNA samples.

The Cq (quantitation cycle) values obtained from the qPCR were used to calculate and plot a linear regression line by logging the template copy number (NC) and the corresponding Cq value.

The efficiency of qPCR (E) reaction was calculated according to Eq. (4) (Pfaffl, 2001), and the mathematical conversion of E to percentage according to Eq. (5).

$$E \quad 10^{-1/\text{slope}} \tag{4}$$

$$E\ \% \quad (\quad) * 100 \tag{5}$$

In the above equations, E represents the efficiency, and slope is the line derived from the calibration curve. 100% efficiency means that the target DNA is being duplicated at every cycle.

2.6.6. Relative quantification

The relative quantification of Cel gene expression was normalized to the Pfo gene, and calibrated according to the initial samples (zero time).

The quantification was performed according to the mathematical model proposed by Pfaffl (2001) which calculates the relative expression ratio based on qPCR efficiency and the crossing point deviation of the investigated transcripts (Eq. (6)).

$$\text{Relative Expression} \quad \frac{E_{cel}^{\ \Delta Cq_{cel}\ \text{sample control}}}{E_{Pfo}^{\ \Delta Cq_{Pfo}\ \text{sample control}}} \tag{6}$$

where, E_{cel} is the efficiency of the qPCR reaction with the Cel gene, E_{Pfo} is the efficiency of the qPCR reaction with the Pfo gene, and control is the initial sample (zero time).

Pfaffl's (2001) model was chosen because it considers the efficiency values of qPCR (E); since, for environmental samples and bacterial strains, the E value differs significantly (Brankatschk et al., 2012).

3. Results and discussion

3.1. CR4 identification and characterization

The isolated strain CR4 of SCB auto-fermentation grew on agar surface and formed circular, smooth and cream color colony. According to microscopic examination, the strain was rod-shaped, gram-positive, 1.2 × 3.3 μm, and forming terminal endospores. The occurrences of cells were single, in pairs or in chains (Fig. A1, Appendices document).

The nucleotide sequence of the isolated strain CR4 was similar (100%) to Paraclostridium genus. Fig. 1 shows the genetic distance dendrogram, the bootstrap values are shown at the nodes (%) and the scale bar indicates the phylogenetic distance (0.050).

Paraclostridium are obligate anaerobes can utilize D-Mannitol, cellobiose, cellulose, starch, D-mannose, D-sorbitol, D-mannitol, fumarate and benzoat for cell growth (Sasi Jyothsna et al., 2016). Paraclostridium sp. have already been identified from rat feces (Kutsuna et al., 2018) and in fermentative reactors inoculated with anaerobic digester sludge and glucose or gram as substrates for H_2 production (Hwang et al., 2018; Yang and Wang, 2018; Yang et al., 2019) have been reported to be efficient H_2 producers (Yang et al., 2019).

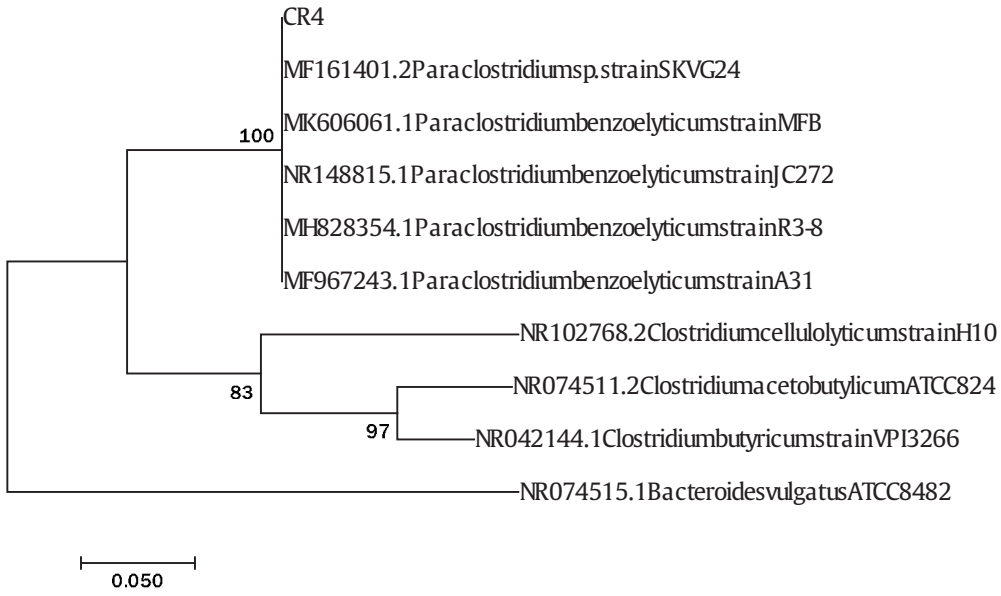

Fig. 1. Genetic distance dendrogram of the isolated strain CR4. Out-group: Bacteroides vulgatus.

Paraclostridium sp. CR4 was isolated from self-fermentation sugarcane bagasse, and this bacterium may have contributed to the degradation of SCB, as Sasi Jyothsna et al. (2016) verified cellulolytic activity of this bacterium isolated from marine sediments.

This strain was grown on cellulose (5.0 g/L) and hydrothermally pretreated SCB (5.0 g/L) at 37 °C, and the generation time (Tg) calculated according to the copy number of the Cel gene (NC) was 4.6 h and 5.1 h for the respective substrates.

Wang et al. (2008) analyzed sucrose fermentation by Clostridium butyricum and calculated the Tg of 4.5 h from the hydA gene copy number. Braga et al. (2019) studied the cellobiose fermentation by C. cellulolyticum and calculated the tg of 4.3 h, according to the optical density of the bacterial suspension at 600 nm. The lowest tg values reported by these authors were probably due to the less complex substrates than cellulose and SCB.

3.2. Effect of organic substrates on hydrogen and organic acids production by Paraclostridium sp. CR4

The gradual adaptation of the inoculum to the glucose for cellulose substrate was accomplished by increasing the cellulose proportion to 100% in order to stimulate cellulase production and, consequently, cellulose degradation. H_2 production experiments were performed by Paraclostridium sp. CR4 with 100% glucose (5 g/L) (A), 75% glucose (3.75 g/L) and 25% cellulose (1.25 g/L) (B), 50% glucose (2.5 g/L) and 50% cellulose (2.5 g/L) (C), 25% glucose (1.25 g/L) and 75% cellulose (3.75 g/L) (D) and finally 100% cellulose (5 g/L) (E), Fig. 2.

H_2 potential production (P) values differed in relation to the substrate, which were higher in the experiments with higher percentage of glucose (A and B), 162.4 ± 5.8 mL and 115.8 ± 7.6 mL, respectively. In experiments C, D and E, P was 97.9 ± 0 mL, 64.2 ± 14.8 mL, 78.4 ± 3.6 mL, respectively.

In a control assay, without carbon source, just 7.9 ± 0.6 mL of H_2 were produced, since Paraclostridium sp. CR4 not only grew in amino acids source present in RCM medium.

Glucose was the preferred carbon source over cellulose for Paraclostridium sp. CR4, since the highest H_2 production was in experiment A (162.4 ± 5.8 mL). Glucose has been the most commonly used carbon source for determining H_2 production potential either with mixed (Temudo et al., 2007; Mu et al., 2009; Masset et al., 2012) or pure cultures (Pan et al., 2008; Ren et al., 2008; Cai et al., 2010) and, as in the present study, it is the preferred carbon source when compared

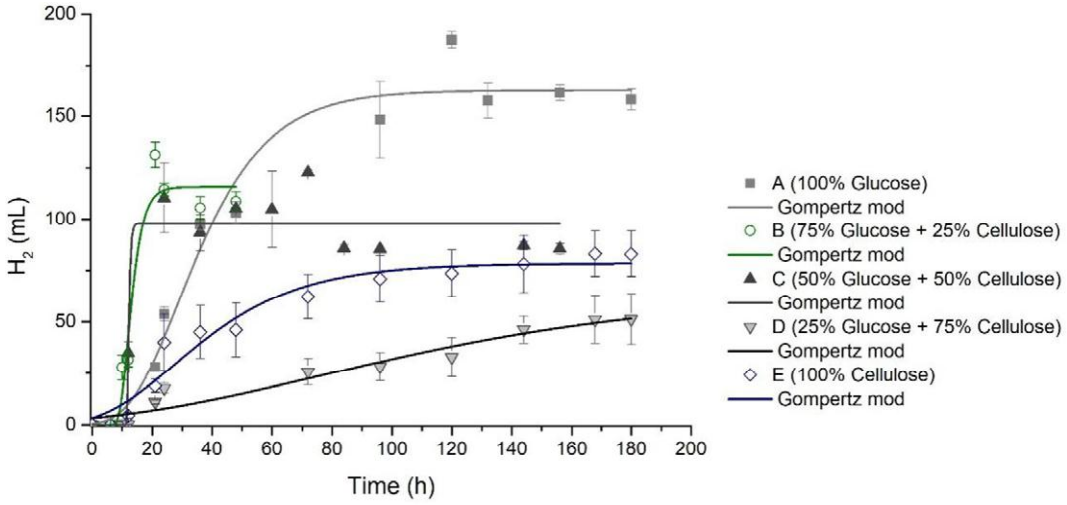

Fig. 2. H_2 production by Paraclostridium sp. CR4 in experiments A, B, C, D and E.

Table 2
Hydrogen production from cellulose as substrate.

Inoculum	Cellulose (g/L)	H_2 production (mL)	Reference
C. cellulolyticum	5.0	91.4	Ren et al. (2007)
C. cellobuioparum		35.4	
C. celerecrescens		44.6	
C. populeti		58.7	
C. phytofermentans		31.8	
C. termitidis	2.0	4.7	Ramachandran et al. (2008)
C. thermopalmarium and C. thermocellum	4.5	41.3	Geng et al. (2010)
C. thermocellum		5.3	
Paraclostridium sp. CR4	5.0	78.4	Present study

to other carbon sources for bacterial fermentation (Prakasham et al., 2009; Díaz and Willis, 2019).

Based on the increase in cellulose concentration in experiments B, C and D, there was a gradual decrease in H_2 production of 115.8 ± 7.6 mL (B), 97.9 mL (C) and 64.2 ± 14.8 mL (D), respectively. Quemeneur et al. (2011) observed H_2 production with glucose and cellobiose of 1120 mL and 1000 mL, respectively, in batch reactors with mixed culture as inoculum, and concluded that the increase in the substrate carbon chain had a significant effect on H_2 decrease. Braga et al. (2019) also evaluated H_2production with different substrates, cellobiose and pretreated SCB, from C. cellulolyticum. Higher H_2 production (23.4 mL) was obtained with cellobiose compared to SCB (11.9 mL), because cellobiose is a more readily biodegradable substrate. Decreased H_2 production with increasing cellulose concentration with Paraclostridium sp. CR4 may also have been due to the increased carbon chain and the need for previous substrate hydrolysis for subsequent fermentation.

In assay E, with 100% cellulose, H_2 production (78.4 ± 3.6 mL) was 22.1% higher than that observed in assay D with 25% glucose and 75% cellulose (64.2 ± 14.8 mL). According to Petitdemange et al. (1992), cellulase production in cellulolytic bacteria, such as Paraclostridium, is subject to low energy state-induced gene regulation within the cell, which occurs during balanced growth. In contrast, in easily metabolizable substrates such as glucose, the cellulase production is suppressed. Thus, the results obtained under condition E may have been due to the higher activity of the cellulolytic enzymes of Paraclostridium sp. CR4 in relation to condition D.

H_2 production (78.4 mL) by Paraclostridium sp. CR4 with 5.0 g/L cellulose at 37 °C was higher than that reported by Geng et al. (2010), who obtained 41.3 mL of H_2 using co-culture of C. thermocellum and C. thermopalmarium in batch reactors with 4.5 g/L cellulose at 55 °C. The production of H_2 with Paraclostridium sp. CR4 was also higher compared to the pure cultures C. cellobuioparum (35.4 mL), C. celerecrescens (44.6 mL), C. populeti (58.7 mL) and C. phytofermentans (31.8 mL) in batch reactors with 5.0 g/L of cellulose at 30 °C (Ren et al., 2007); and also compared to C. termitidis (4.7 mL) in batch reactors with 2.0 g/L cellulose at 37 °C (Ramachandran et al., 2008) (Table 2).

Paraclostridium belongs to the Clostridiaceae family, with the same physiological and morphological characteristics as Clostridium, such as fermentative H_2 production from organic substrates. However, with the exception of cellular fatty acid constitution, in which Paraclostridium has more $C_{16:0}$ (16-carbon fatty acid in saturated bonds), differentiating it from Clostridium (Sasi Jyothsna et al., 2016).

In all experiments (A E) with Paraclostridium CR4, the main organic acids observed were acetic and butyric (Fig. 3). One of the main physiological characteristics of bacteria belonging to the Clostridiaceae family (Rainey, 2009) refers to the production of these organic acids as observed for Paraclostridium sp. CR4. Acetic acid (2.9 g/L) and butyric acid (2.8 g/L) were produced more in glucose and cellulose, respectively.

The other acids produced (lactic, citric, formic, isovaleric, valeric and caproic acids), in lower concentrations, are available in Fig. B1 (Appendix document).

In experiments with higher glucose percentage (A, B and C), acetic and butyric acids were observed mainly in the initial H_2 production (IH_2P) stage, and maximum H_2 production rate stage (MRH_2P). In experiment A (100% glucose), acetic and butyric acids were observed in higher concentration in the IH_2P stage (2.9 and 1.3 g/L, respectively), while in experiments B (75% glucose and 25% cellulose) and C (50% glucose and 50% cellulose), mainly in the MRH_2 stage (2.8 and 1.5 g/L in B and 2.5 and 1.5 in C, respectively).

In contrast, in experiments D (25% glucose and 75% cellulose) and E (100% cellulose) with higher percentage of cellulose, acetic and butyric

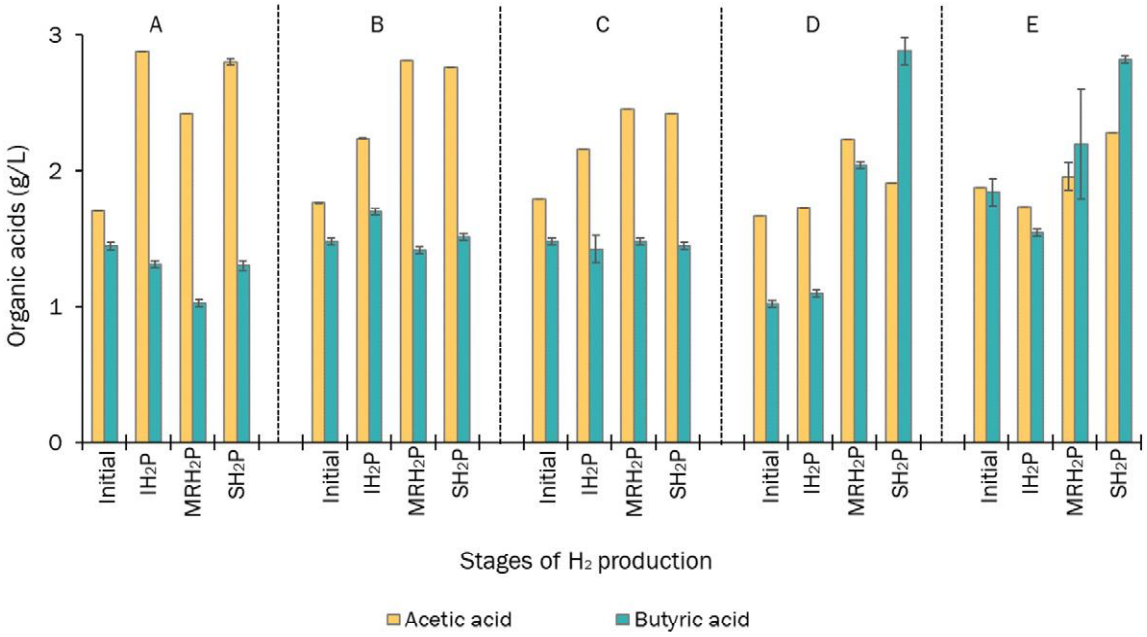

Fig. 3. The main organic acids in A, B, C, D and E experiments during the H_2 production stages: at the initial stage of the experiment (Initial), initial H_2 production (IH_2P in 3 h for all experiments), maximum H_2 production rate (MRH_2P, in 48 h for A and C, 24 h for B, 78 h for D and 36 h for E) and H_2 production stabilization (SH_2P, in 180 h for A, D and E, 48 h for B and 156 h for C).

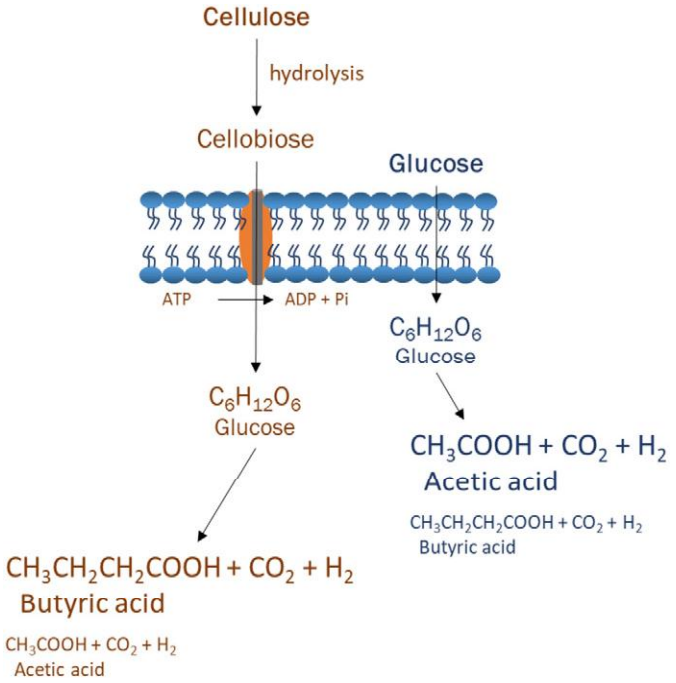

Fig. 4. Production of the main organic acids from glucose and cellulose by Paraclostridium sp. CR4.

acids were observed during the H_2 production stabilization stage (SH_2P); that is, 1.9 and 2.9 g/L in D and 2.3 and 2.8 g/L in E, respectively.

The effect of carbon source (glucose/cellulose) on the organic acids production period may have been due to bacterial growth in the respective substrates. According to Giallo et al. (1985), the growth of cellulolytic bacteria C. cellulolyticum in cellulose was lower compared to growth in glucose, with generation time of 24 and 10 h, respectively, due to the hydrolysis stage of cellulose in soluble sugars.

Thus, the formation of organic acids in experiments D and E was delayed (in the SH_2P stage) because of the slow growth of Paraclostridium sp. CR4 in cellulose, due to the substrate hydrolysis stage, when compared to the formation of acids in experiments A, B and C, with glucose as the main substrate (in the IH_2P and MRH_2P stages).

Table 3
Distribution of soluble carbon content of fermentative reactors.

Experiment	Stage of H_2 production	Organic acids (g/L)	Soluble sugars (g/L)	Total COD theoretical (g/L)
A (100% glucose)	Initial	7.3	7.7	15.0
	SH_2P	9.4	1.9	11.3
B (75% glucose and 25% cellulose)	Initial	7.0	3.6	10.6
	SH_2P	7.0	2.2	9.2
C (50% glucose and 50% cellulose)	Initial	8.1	2.7	10.8
	SH_2P	8.8	1.5	10.3
D (25% glucose and 75% cellulose)	Initial	7.5	1.2	8.7
	SH_2P	10.7	0.7	11.4
E (100% cellulose)	Initial	9.4	1.0	10.4
	SH_2P	10.3	1.2	11.5

The main organic acid formed during the experiments also differed between the experiments with glucose (A, B and C) and cellulose (D and E). In A (100% glucose), B (75% glucose and 25% cellulose) and C (50% glucose and 50% cellulose), there was a higher production of acetic acid (2.9, 2.8 and 2.8 g/L, respectively), while in D (25% glucose and 75% cellulose) and E (100% cellulose) there was a predominance of butyric acid (2.9 and 2.8 g/L, respectively), suggesting that bioconversion of glucose occurred from the acetic acid route, while cellulose bioconversion was from the butyric acid route.

According to Prakasham et al. (2009) and Gupta et al. (2014), the substrate type can regulate the formation of the final product. Gupta et al. (2014) evaluated H_2 production from different proportions of glucose, starch and cellulose with mixed inoculum in batch reactors, and found that acetic and butyric acids were the predominant products in the fermentation of glucose and starch, while propionic acid was obtained from cellulose. Quemeneur et al. (2011) also evaluated the production of H_2 by mixed culture in batch reactors from glucose, fructose, maltose and cellobiose. In this case, acetic and butyric acids were predominant in the fermentation of monosaccharides (glucose and fructose) and sucrose, while caproic and lactic acids were produced from cellobiose, maltose and maltotriose.

As in the above studies, the metabolic route of Paraclostridium sp. CR4 was altered due to the added substrate chain length, changing the fermentation products. With simple substrates such as glucose, the main product was acetic acid, formed in the first hours of fermentation (IH_2P, 3 h). From cellulose, the butyric acid route was favored, however with delayed organic acid production (SH_2P, 180 h) (Fig. 4).

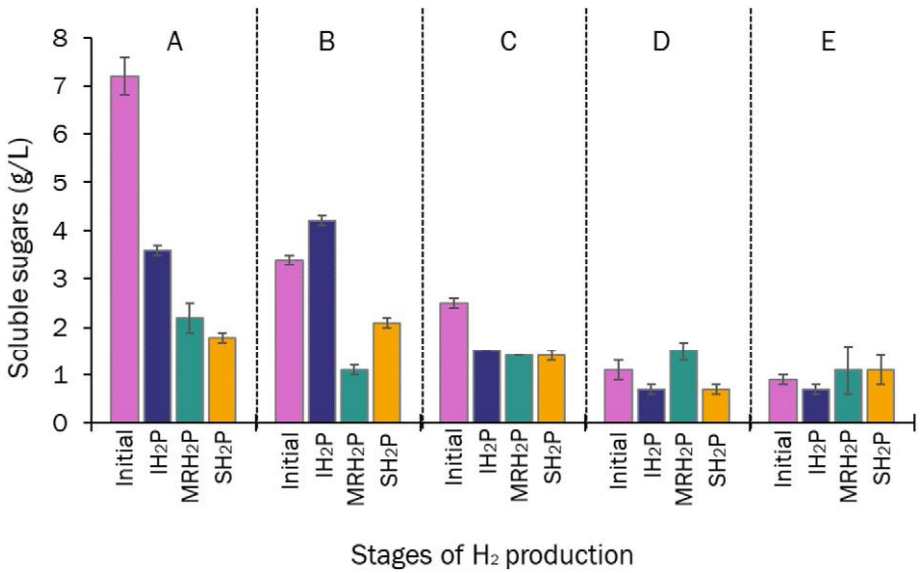

Fig. 5. Soluble sugars in experiments A, B, C, D and E, during the H_2 production stages, at the initial phase of the experiment (Initial), initial H_2 production (IH_2P in 3 h for all experiments), maximum H_2 production rate (MRH_2P, in 48 h for A and C, 24 h for B, 78 h for D and 36 h for E) and H_2 production stabilization (SH_2P, in 180 h for A, D and E, 48 h for B and 156 h for C).

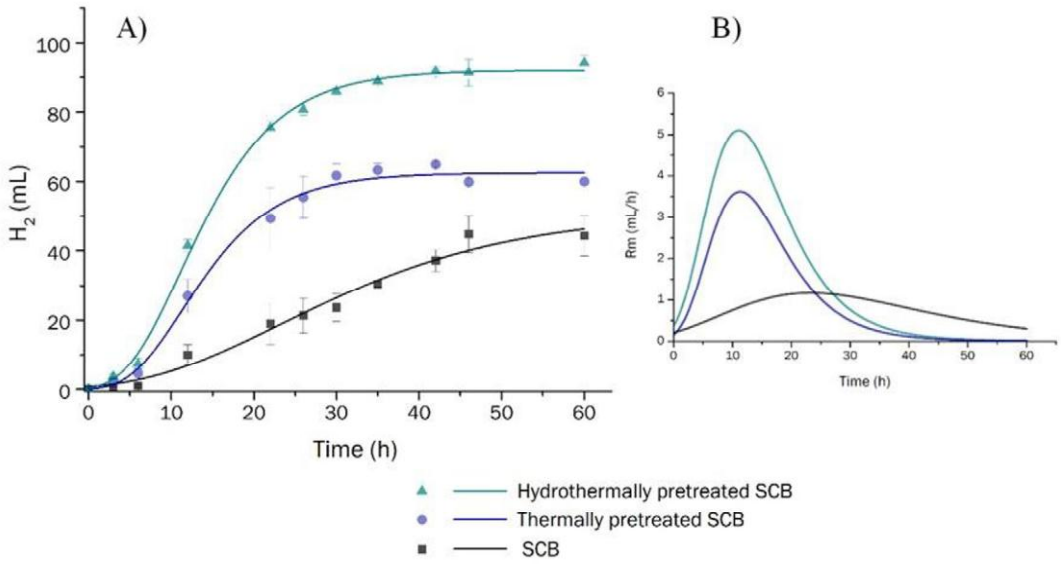

Fig. 6. A): Hydrogen production potential (P), and B): hydrogen production velocity (Rm) from SCB by Paraclostridium sp.

Total soluble sugars in these experiments (A E) were monitored during the H_2 production stages and are presented in Fig. 5.

In addition to the added substrate (glucose or cellulose), the culture medium (modified RCM medium with yeast extract, peptone, NaCl, sodium acetate and cysteine) and inoculum also contributed to the increase of soluble sugars in the medium. At the initial stage of experiment A (100% glucose), 7.2 ± 0.40 g/L of total soluble sugars was observed. Under such conditions only 5.0 g/L glucose was added. This fact can also be evidenced in experiment E, in which no glucose was added and yet 0.9 ± 0.20 g/L of total soluble sugars were obtained at the initial stage of the experiment.

In experiment A, only glucose showed a gradual decrease in soluble sugar concentration from 7.2 ± 0.4 g/L at the initial stage of the experiment (Initial) to 3.6 ± 0.15, 2.2 ± 0.35 and 1.8 ± 0.05 at theIH_2P, MRH_2P and SH_2Pstages, respectively. Thus, the carbohydrate conversion was 75.0%.

In experiment B, with 25% of cellulose, the concentration of soluble sugars increased from 3.4 ± 0.15 g/L (Initial) to 4.2 ± 0.05 g/L (IH_2P), decreased to 1.1 ± 0.05 g/L and later increased to 2.1 ± 0.15 g/L in MRH_2P and SH_2P, respectively.

In experiment C, with 50% cellulose and 2.5 ± 0.05 g/L (Initial) of soluble sugars, only a decrease of 1.5, 1.4 and 1.4 ± 0.15 g/L was observed in the IH_2P, MRH_2P and SH_2P stages, respectively, resulting in 44.0% of carbohydrate conversion.

In experiments D and E, with higher percentage of cellulose, a similar concentration of soluble sugars was observed during the H_2 production stage, probably due to the simultaneous release and consumption of sugars by Paraclostridium sp. CR4. In experiment D, with 75% cellulose and 1.1 g/L of soluble sugars (Initial), 0.7 ± 0.15 g/L (IH_2P), 1.5 ± 0.20 g/L (MRH_2P) and 0.7 ± 0.05 g/L (SH_2P) were observed, resulting in 36.4% of carbohydrate removal. In E, with 100% cellulose and 0.9 g/L of soluble sugars (Initial), 0.7 ± 0.05 g/L (IH_2P), 1.1 ± 0.45 (MRH_2P) and 1.1 ± 0.35 (SH_2P) were observed.

Rabelo et al. (2018b) also evaluated soluble sugars in cellulose fermentation and microbial consortium reactors. The authors observed higher carbohydrate conversion in reactors with higher initial cellulose concentration, i.e., 58, 66 and 74% of carbohydrate conversion in reactors with 2.0, 5.0 and 10.0 g/L of cellulose, respectively. At the initial stage of the experiments with 2.0, 5.0 and 10.0 g/L of cellulose, 13.9, 22.0 and 21.2 mg/L of soluble sugars were observed, respectively. During the highest H_2 production stage, the concentration of soluble sugars in the medium increased to 111.9, 105.0 and 60.5, respectively, and in the H_2 production stabilization stage, a decrease to 35.1, 27.3 and 25.4 mg/L was observed, respectively.

In the present study, the concomitant increase and decrease of soluble carbohydrates in the medium during the H_2 production stages were also observed, corroborating the importance of hydrolytic activity for the fermentative production of H_2.

The soluble carbon content (g $COD_{theoretical}$/L) of fermentative reactors at the initial stage of the experiment (Initial) and H_2 production stabilization (SH_2P) stage can be seen in Table 3.

In experiments A and C, a reduction in soluble carbon content from the Initial stage to SH_2P was observed (from 15.0 to 11.3 g/L and 10.8 to 10.3 g/L, respectively). This result was probably due to the consumption of carbon from soluble sugars (from 7.7 to 1.9 and 2.7 to 1.5 g/L, respectively), resulting in a conversion of 75 and 44%, respectively, into

Table 4
Biofuel production from pretreated lignocellulosic substrates.

Substrate	Pre-treatment	Results	Increase in production	Reference
Corn silage (1.3 g/L)	Hydrothermal	19.4 g/L of ethanol	61.6%	Xu et al. (2010)
Beet pulp (20 g COD/L)	Microwave and alkaline	111.7 mL of H_2	14.3%	Ozkan et al. (2011)
	Alkaline	134.0 mL of H_2	28.6%	
	Thermal and alkaline	148.5 mL of H_2	35.%	
SCB (0.5 and 2.0 g/L)	steam and alkaline explosion	64. mL of H_2 with 0.5 g SCB/L	43.1%	Ratti et al. (2015)
		157.7 mL of H_2 with 2.0 g SCB/L	72.4%	
SCB (2.0 g/L)	Hydrothermal	11.4 mL of H_2	15.8%	Soares et al. (2017)
SCB (5.0 g/L)	Thermal	62.5 mL of H_2	17.8%	Present study
	Hydrothermal	92.0 mL of H_2	44.1%	
	In natura	51.4 mL of H_2		

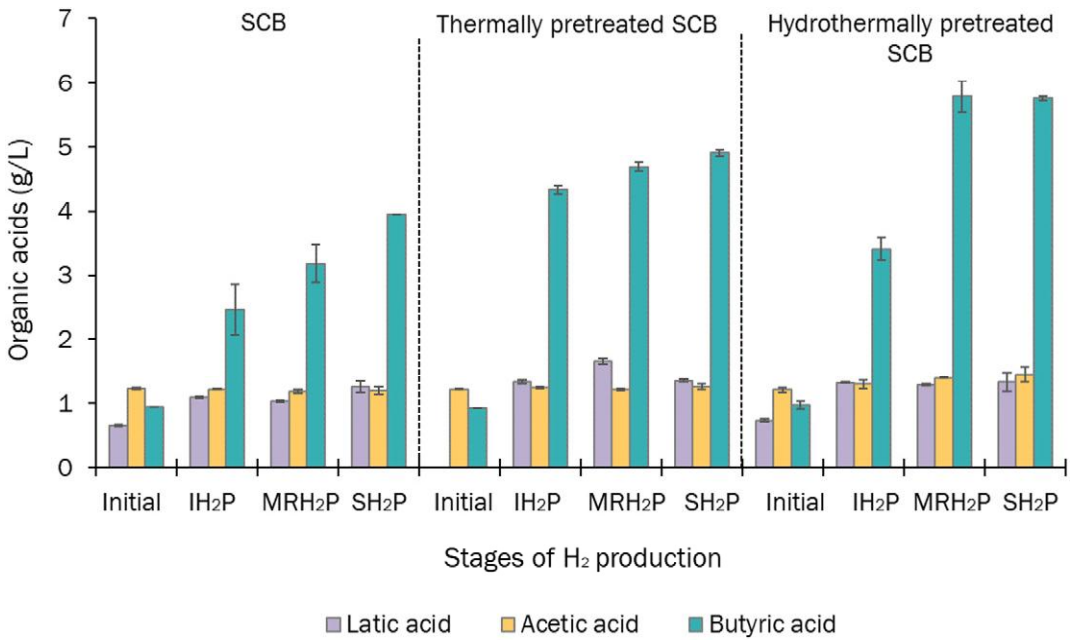

Fig. 7. Organic acids from SCB at the initial phase of the experiment (Initial, 0 h), initial H_2 production (IH_2P, 3 h), maximum H_2 production rate (MRH_2P, 12 h) and H_2 production stabilization (SH_2P, 46 h).

organic acids (9.4 and 8.8 g/L, respectively), carbon dioxide and cell growth.

Experiment B also showed a reduction in the soluble carbon content from the Initial stage to SH_2P (from 10.6 to 9.2 g/L), probably due to the conversion of substrates (cellulose and glucose) to carbon dioxide and cell growth.

In the experiments with higher amount of insoluble substrate, D and E (75 and 100% of cellulose, respectively), an increase in the initial soluble carbon content was observed for SH_2P (from 8.7 to 11.4 g/L and from 10.4 to 11.5 g/L, respectively), probably due to the conversion of the insoluble substrate to organic acids (10.7 and 10.3 g/L, respectively), and carbon dioxide and cell growth.

From these results one can infer about the hydrolytic-fermentative activity of Paraclostridium sp. CR4 in all experiments. Sasi Jyothsna et al. (2016) isolated Paraclostridium species from marine sediments, and also verified degradation of glucose and cellulose, as well as mannitol, starch, cellobiose, mannose, sorbiol, benzoate and fumarate.

3.3. Fermentation of in natura and pretreated SCB

pt?>The thermal and hydrothermal pretreatments were applied to SCB in order to increase the enzymatic accessibility and digestibility of cellulose and, consequently, the glycidic fermentation for H_2 production. The experiments with substrates subjected to pretreatment were compared with the control experiment (in natura SCB). The in natura and pretreated SCB were used by Paraclostridium sp. CR4 for H_2 production (Fig. 6).

Based on the heat-treated and hydrothermally pretreated BCA, a higher P value of 62.5 ± 1.3 and 92.0 ± 1.1 mL of H_2 was obtained,

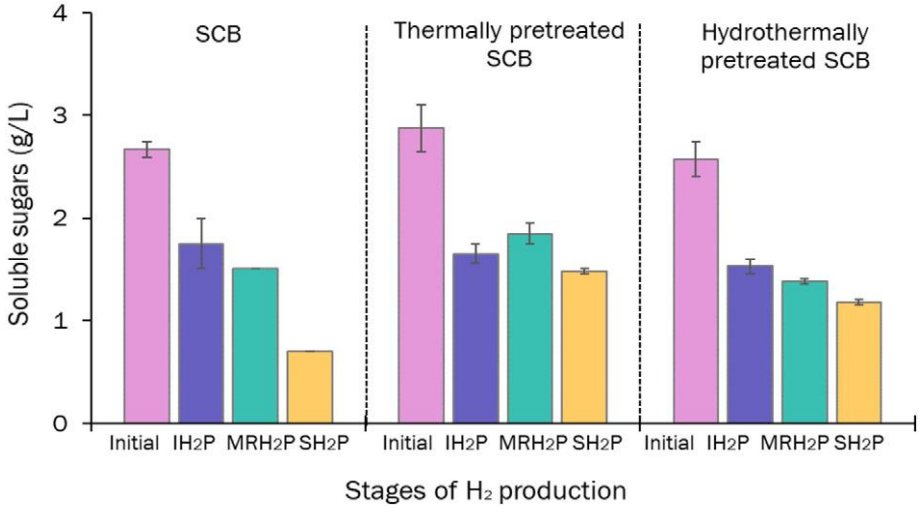

Fig. 8. Soluble sugars in batch reactors at the initial stage of the experiment (Initial), initial H_2 production (IH_2P in 3 h), maximum H_2 production rate (MRH_2P in 12 h) and H_2 production stabilization (SH_2P in 46 h).

Table 5
Sequence of primers designed for Cel, GyrB, Pfo, and RecA genes.

Gene	Primer sequence	Product size (bp)
Cel	F: TGCATTTAACCAACTCCTCAACTGT R: TGGTACACTGCAAAATGGGATCT	154
GyrB	F: GGCTATAGTGGTGGCTCATGGA R: AGGTTGTATGCTTTGCCTCACA	184
Pfo	F: CAGATCGTACATATCCAGGAGCTC R: CAACGGCCCCTGATACATTTGA	180
RecA	F: GCTGAGATAGAAGGGGACATGGG R: CCACCTGTTGTAGTTTCTGGGC	179

respectively, in relation to in natura SCB (51.4 ± 4.7 mL) and significantly different ($\alpha = 0.05$).

Higher H_2 production rate for hydrothermally pretreated SCB (5.1 ± 0.3 mL/h), compared to hydrothermally pretreated (3.6 ± 0.4 mL/h) and in natura (1.2 ± 0.1) was obtained and was significantly different ($\alpha = 0.05$). Under the conditions of hydrothermal pretreatment, disruption of hemicellulose and lignin and decreased crystallinity of cellulose may have occurred, resulting in greater enzymatic accessibility for fermentation.

In addition to hydrothermal pretreatment, other pretreatments have been applied to lignocellulosic biomass to increase H_2production (Ozkan et al., 2011; Ratti et al., 2015) and ethanol (Xu et al., 2010). Different pretreatment possibilities for lignocellulosic materials can be used favoring H_2production from 14.3% (Ozkan et al., 2011) to72.4% (Ratti et al., 2015). An increase of 17.8 and 44.1% in H_2 production was obtained from heat-treated and hydrothermally pretreated BCA, respectively, compared to in natura BCA (Table 4).

Butyric acid was the main organic acid formed from in natura and pre-heat treated SCB (at 121 °C, 1.5 kg/cm^2 for 15 min) and hydrothermally (at 200 °C, 16 bar for 10 min) by Paraclostridium sp. CR4 (Fig. 7).

The highest concentration of organic acids (8.5 g/L) occurred in the hydrothermally pretreated SCB assays, with 68.2% corresponding to butyric acid (5.8 g/L). 7.5 g/L and 6.4 g/L of total organic acids were obtained from the heat-treated pretreated and in natura SCB, respectively. Braga et al. (2018) obtained 10 g/L of total organic acids from hydrothermally pretreated SCB and consortium of cellulolytic and fermentative bacteria. Rabelo et al. (2018a) also reported organic acid production from 4.5 to 7.1 g/L using heat pretreated SCB as substrate and consortium of fermentative cellulolytic bacteria as inoculum.

Thus, with the fermentation of SCB by Paraclostridium sp. CR4, in addition to H_2, organic acids can be obtained, especially butyric acid as a product of added value.

Butyric acid is an important platform chemical, a precursor of many chemicals and materials and widely used in the chemical, food and pharmaceutical industries (Baroi et al., 2015). Butyric acid fermentative production, when compared to chemical production, has advantages such as lower production of environmental pollutants and the use of renewable resources as substrate, and this production mode is often necessary for some specific applications, such as for food and pharmaceutical industries (Luo et al., 2018).

Fermentative production of butyric acid can also be obtained from lignocellulosic substrates (Chi et al., 2018; Oh et al., 2019). Pretreated rice straw was used in batch reactors and cellulolytic bacterial consortium, as well as in batch reactors with cellulolytic bacterial consortium bioaugmented with C.tyrobutyricum. As a result, butyric acid production was increased from 5.53 g/L with cellulolytic consortium to 18.05 g/L with the addition of C. tyrobutyricum (Chi et al., 2018). Rabelo et al. (2018a) reported that response surface methodology and central composite design are effective statistical methodologies for the optimization of butyric acid production by bacterial consortium. The individual and mutual effects of substrate concentration (from 0.8 to 9.2 g/L) and pH (from 4.6 to 7.4) on butyric acid production from SCB were investigated in batch reactors. The authors observed an increase in butyric acid concentration from 14.6 to 33.8% of total acids produced (0.66 to 1.92 g butyric acid/L) with 2.0 g SCB/L at pH 5.0 and 9.2 g SCB/L at pH 6.0, respectively. In the present study, in the fermentation of SCB with Paraclostridium sp. CR4 was also able to obtain the simultaneous production of H_2 and butyric acid. The highest yield of butyric acid (5.8 g/L), as well as H_2 (92.0 mL) was obtained through hydrothermally pretreated BCA followed by heat pretreated BCA (4.9 g butyric acid/L and 62.5 mL H_2) and in natura BCA (4.0 g butyric acid/L and 51.4 mL H_2) at pH 6.8.

The concentration of soluble sugars in batch reactors with in natura, heat pretreated and hydrothermally pretreated SCB were also evaluated during the H_2 production stages (Fig. 8). The concentration of soluble sugars at the initial stage of the experiment was similar under the

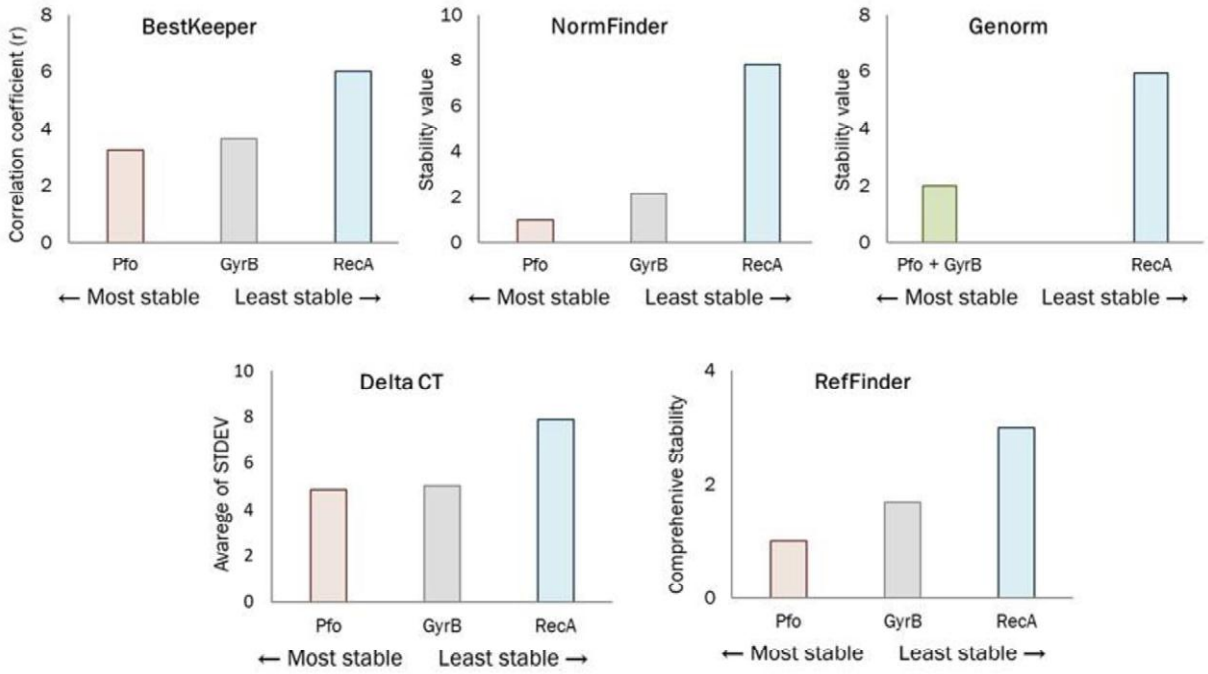

Fig. 9. Stability of reference genes according to DeltaCT, BestKeeper, NormFinder, Genorm and RefFinder.

three conditions (2.7, 2.9 and 2.6 g/L with in natura SCB, heat pretreated SCB and hydrothermally pretreated SCB, respectively). Lower concentrations of soluble sugars were observed during the H_2 production stages, resulting in a conversion of 73.8, 48.7 and 54.4% in the experiments with in natura, heat pretreated and hydrothermally pretreated SCB, respectively.

In the experiment with hydrothermally pretreated SCB, the conversion of sugars (54.4%) was lower when compared to the in natura SCB (73.8%) and, even so, in this experimental condition the highest H_2production (92.0 mL) was obtained. Probably, the higher H_2 production observed with hydrothermally pretreated SCB was due to the increase in available surface area of the substrate because of the hydrothermal pretreatment, which favored substrate hydrolysis in soluble sugars available for fermentation in H_2 and organic acids.

3.4. Primers to target-gene and reference gene candidates for Paraclostridium

In the experiment with cellulose (E) and hydrothermally pretreated SCB, the quantification of the celullase family protein gene (Cel) by qPCR was evaluated, and also for the quantification of Cel gene expression by RT-qPCR.

Primers for the target gene (Cel) as well as the reference genes (GyrB, Pfo and RecA) for Paraclostridium sp. have not yet been described in the literature. Thus, these primers were designed and the sequences and length of the amplified fragments are shown in Table 5.

Reference genes are genes whose expression remains stable in different situations, and are those which are essential to cellular metabolism (Pfaffl, 2001). The GyrB gene encodes the DNA gyrase enzyme subunit D (Peeters and Willems, 2011); the Pfo gene encodes the pyruvate ferredoxin oxidoreductase enzyme that catalyzes the conversion of pyruvate to acetyl CoA (Salimi and Mahadevan, 2013); and the RecA gene encodes the recombinase A enzyme (Liu et al., 2013).

Thus, the three chosen genes are essential for Paraclostridium metabolism and were tested for expression stability according to the RefFinder tool (Xie et al., 2012), which combines the geometric means of stability values calculated by the BestKeeper algorithms (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004), GeNorm (Vandesompele et al., 2002) and Delta CT comparative method (Silver et al., 2006), creating a comprehensive classification. According to Bestkeeper, NormFinder,

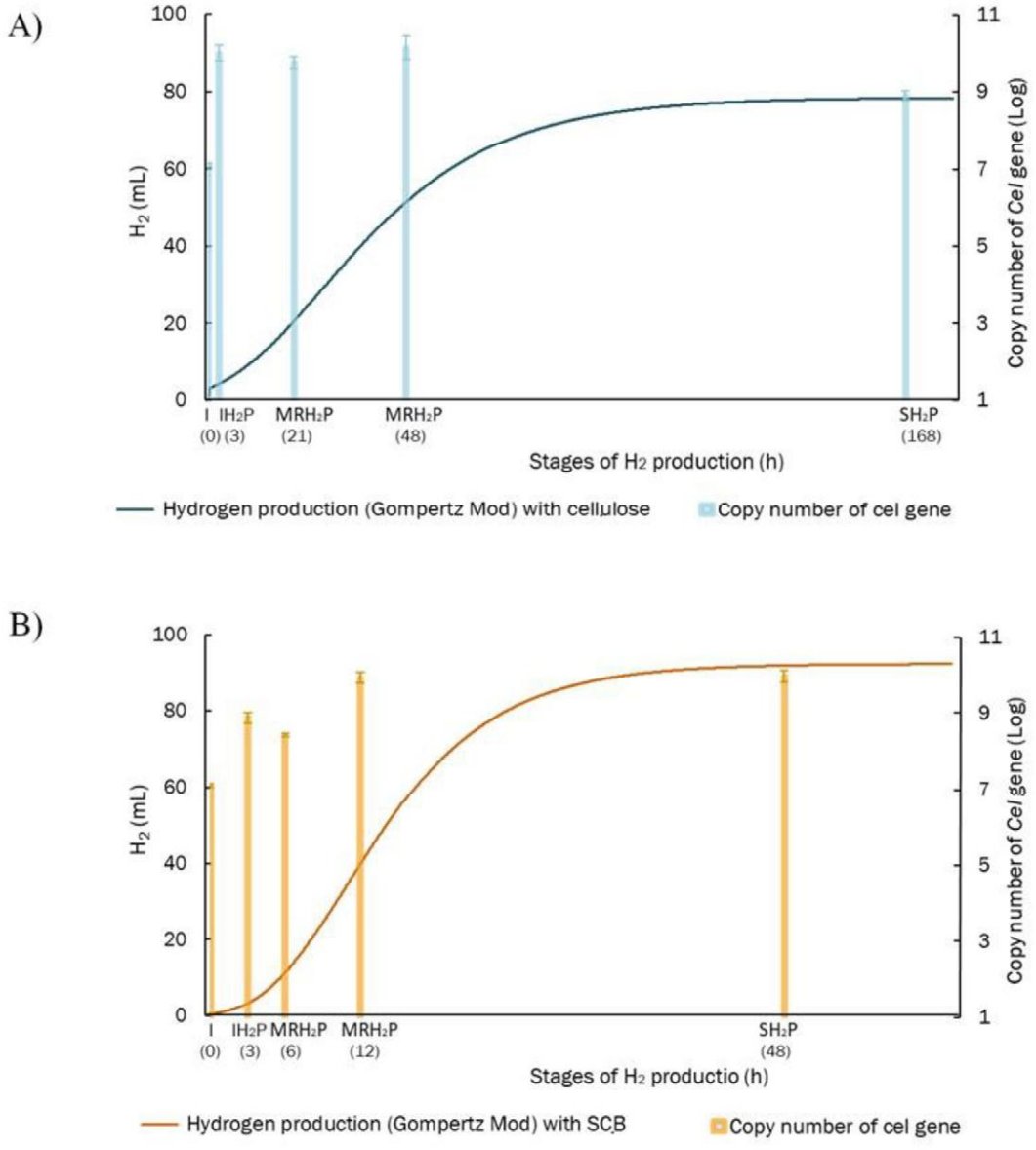

Fig. 10. Cumulative H_2production (mL) and copy number of the gene Cel of Paracolstridium sp. CR4 in the cellulose (A) and SCB (B) fermentation reactors, according to the H_2 production stages: Initial (I); initial H_2 production (IH_2P), maximum H_2 production rate (MRH_2P) and H_2 production stabilization (SH_2P).

Delta Ct and RefFinder analysis, Pfo was obtained as the most stable gene (Fig. 9), and was chosen as a reference for the relative quantification of the target Cel gene.

3.5. Absolute quantification

For absolute quantification, Eq. (7) was used, generated from the standard curve of the number of quantification cycles (Cq) as a function of the Log of the target gene copy number (CN), correlation coefficient (R^2) of 0.9942. The CN of Cel represents the cellular abundance of Paraclostridium sp. CR4.

$$CN \quad -0;2915Cq \quad 99;499 \qquad 7$$

The copy number of Paraclostridium sp. CR4 according to the H_2 production stages from SCB cellulose are shown in Fig. 10.

In reactors with cellulose and Paraclostridium sp. CR4 yielded 1.3×10^7 (Initial), 1.2×10^{10} (IH_2P) and 1.8×10^{10} CN at the highest H_2 production stage (MRH_2S), which had higher cell growth. However, in the H_2 production stabilization stage (SH_2P), a lower CN of 8.4×10^8 was observed. In SCB reactors, the increasing copy number of Cel gene was obtained throughout the experiment; i.e., CN of 1.3×10^7 (Initial), 7.9×10^8 (IH_2P), 8.4×10^9 in the highest H_2 production stage (MRH_2P) and 9.3×10^9during the H_2 production stabilization stage (SH_2P). Therefore, in both cellulose and SCB reactors, favorable bacterial community dynamics were observed by increasing the copy number.

Pugazhendhi et al. (2017) obtained CN from 10^9 to 10^{10} for C. butyricum in a fixed bed reactor with different hydraulic detention times (1.5 to 12 h) to produce H_2 from glucose. C. butyricum was the dominant species in all reactors examined, with over 91% of all bacteria. In the present study with pure culture of Paraclostridium sp. CR4, 10^9 to 10^{10} were obtained with cellulose and SCB.

3.6. Relative quantification of gene expression

According to relative quantification, changes in target gene mRNA levels of Cel were determined, relative to the levels of another stable

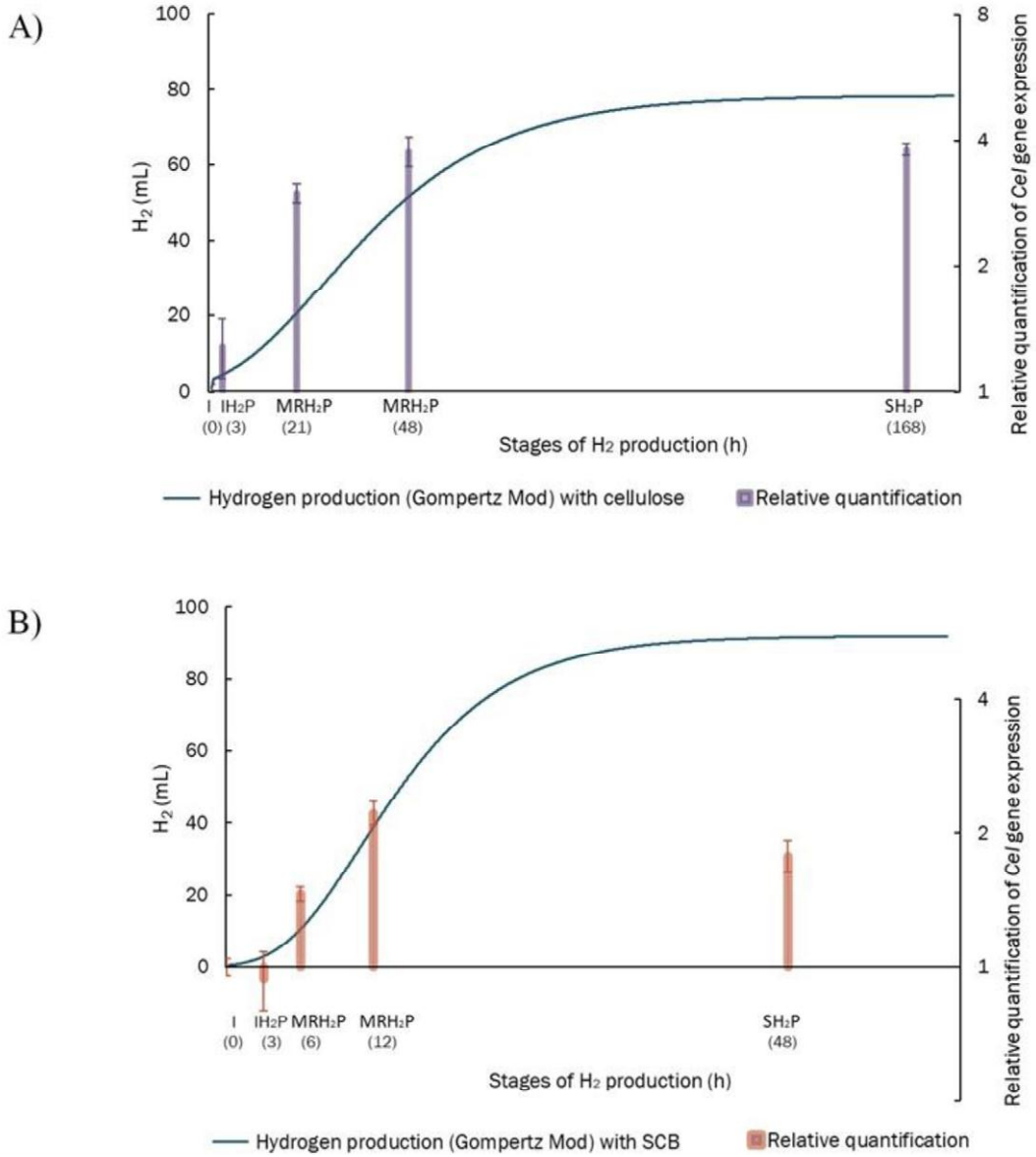

Fig. 11. Accumulated hydrogen production (mL) and relative quantification of the expression of the Cel gene of Paracolstridium sp. CR4 in the fermentative reactors with cellulose as substrate (A) and with SCB as substrate (B), according to the H_2 production stages: Initial (I); initial H_2 production (IH_2P), maximum H_2 production rate (MRH_2P) and H_2 production stabilization (SH_2P).

expression mRNA (reference gene, Pfo) and also normalized to a control group (time zero).

Thus, in reactors with cellulose substrate, the expression of the Cel gene increased during the H_2 production stages. In IH_2P the gene expression was 1.3 times higher than the Initial stage, and 3.0 and 3.8 times higher in MRH_2P and SH_2P, respectively (Fig. 11A).

Kuo et al. (2012) observed that the hydrolysis of cellulosic material was the limiting factor for the fermentative production of H_2 and methane in two-phase sequential reactors fed with kitchen waste and napier grass. Bio-augmentation with Clostridium sp. in the first stage favored substrate hydrolysis and H_2 production. The authors reported that Clostridium sp. inoculated in the first stage reactor was also transferred to the second stage and favored further substrate degradation and subsequent methane production. According to the quantitative analysis of the present study, gene expression may also favor second stage hydrolysis, since the Cel gene was more transcribed in the final stages of H_2 production (MRH_2P and SH_2P) than in the initial H_2 production stage (IH_2P).

In reactors with SCB as substrate, it was observed that even with increasing CN in IH_2P, lower gene expression was obtained in this stage (0.94) and higher during the MRH_2P (2.2) and SH_2P (1.78) stages when compared to the initial stage (1.0).

The initial concentration of soluble sugars in SCB reactors may have influenced Cel gene expression, since sugars released during hydrolysis may inhibit cellulase production (Petitdemange et al., 1992). Paraclostridium sp., as well as C. cellulolyticum studied by Petitdemange et al. (1992), may have a cellulolytic enzyme system subject to end-product regulation, in which soluble sugars may inhibit cellulase production.

The initial concentration of soluble sugars in the hydrothermally pretreated SCB reactors was 2.6 g/L, and lower concentrations were observed in the consecutive H_2 production stages (1.5, 1.4 and 1.2 g/L in IH_2P, MRH_2P and SH_2P, respectively). In the initial hydrogen production stage (IH_2P) there was no expression of the Cel gene, so the production of H_2 during this period may have been due to the fermentation of soluble sugars released from the hydrothermal pretreatment of SCB.

During the MRH_2P stage, with lower sugar concentration (1.4 g/L) the gene expression was higher (2.2 times) than in IH_2P, expressing more cellulases for substrate hydrolysis and fermentation of sugars in H_2 (92.0 mL) and organic acids (8.5 g/L) (Fig. 12).

4. Conclusion

Cellulolytic and fermentative bacteria similar to Paraclostridium sp. CR4 were isolated from sugarcane bagasse (SCB) and used as inoculum for the fermentative production of H_2 from glucose (162.4 mL), cellulose (78.4 mL) and SCB. The pretreatment applied to SCB favored hydrolysis and fermentation, as 92.0, 62.5 and 51.4 mL of H_2 were obtained from hydrothermally pretreated SCB, heat pretreated SCB and in natura SCB, respectively. Metabolic route change was observed according to the substrate, that is, was by the acetic route with glucose (N46%); and by the butyric route with cellulose (N44%), and SCB (N62%).

The copy numbers (CN) of Paraclostridium in cellulose reactors was higher during the initial stages of H_2 production, and lower during the stationary H_2 production stage, while the expression of the Cel gene increased in all these stages. In SCB reactors, even with increased CN of Paraclostridium in all H_2 production stages, the Cel gene expression was higher only during the maximum rate of H_2 production. The amount of soluble sugars in the medium was inhibitory for enzymatic expression, since there was gene expression only in the stages with sugars lower than 1.4 g/L. Thus, it is possible that Paraclostridium sp. CR4 has a cellulolytic enzyme system subject to end product regulation.

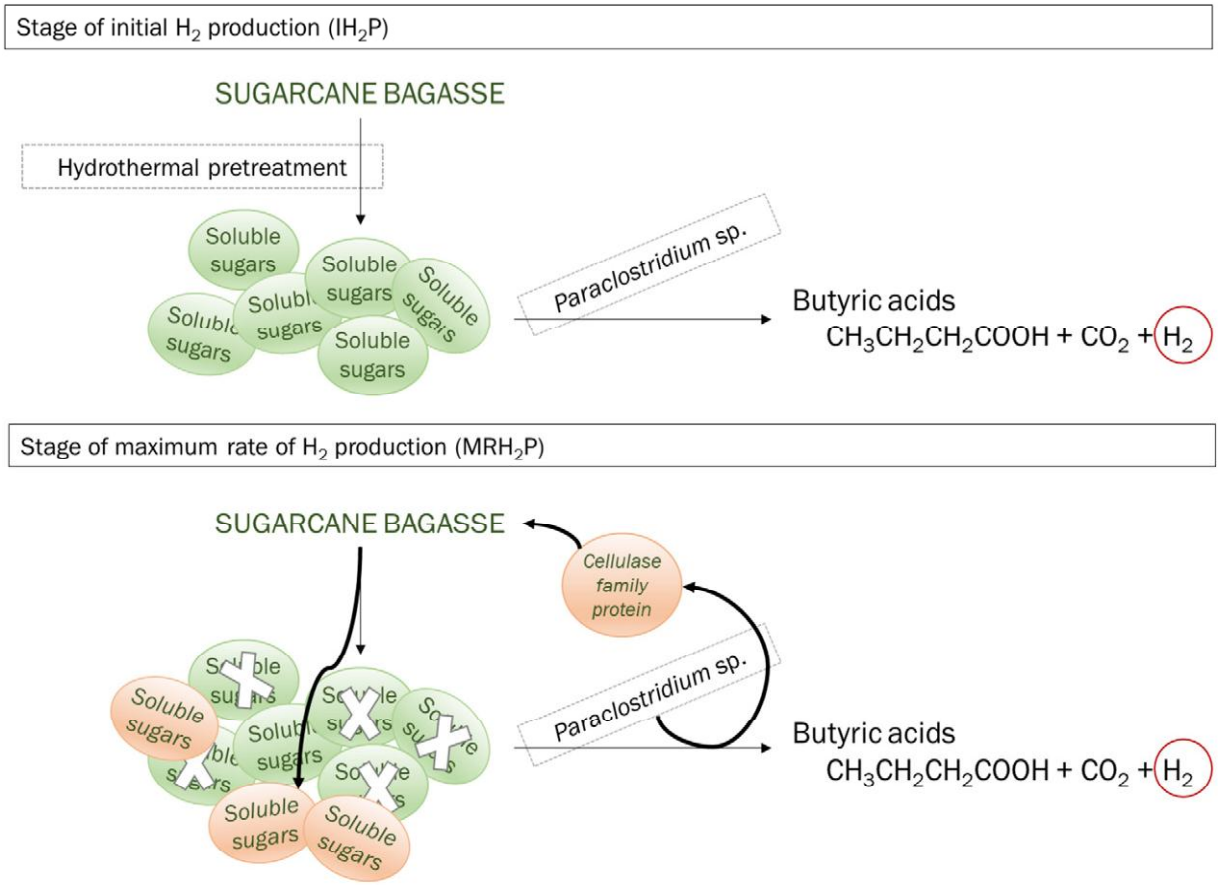

Fig. 12. Bioconversion of pretreated sugarcane bagasse into hydrogen and butyric acid by Paraclostridium sp. CR4.

Paraclostridium sp. CR4 hydrolyzes and ferments cellulose, and can be used as inoculum in fermentative reactors for the production of H_2 from different cellulosic residues, mainly SCB. In reactors with mixed inoculum, bioaugmentation with Paraclostridium sp. CR4 can increase the hydrolysis step of cellulosic waste digestion and favor conversion to other products of biotechnological interest, such as organic acids and biogas.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.136868.

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