Hydrogenase activity monitoring in the fermentative hydrogen production using heat pretreated sludge: A useful approach to evaluate bacterial communities performance

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Abstract
The conversion of organic compounds into H₂ has received increasing attention. Enrichment of inocula by heat pretreatment eliminates non-spore-forming hydrogen consuming microorganisms and promotes spore germination in genus Clostridium, which is known as one of the key hydrogen producers. Useful information about metabolic pathways is provided by some intermediate metabolites, such as: acetic, propionic, butyric and formic acids. The increase of acetic/butyric acids ratio indicates H₂ production in heat pretreated inoculum when compared to untreated inoculum in the same cultivation conditions. The effect of heat pretreatment on inocula and consequently on the performance of bacterial communities responsible for H₂ production was monitored through the measurement of the level of hydrogenase gene expression, as well as through the content and distribution of volatile fatty acids. The acetic acid type fermentation was followed by the microorganisms presented in untreated and heat pretreated sludge. The medium containing untreated sludge presented a ratio of acetic/butyric acid of approximately 4, the same parameter was 7 when heat pretreated sludge was employed. The level of hydrogenase gene expression tripled when heat pretreated inoculum was used, indicating a higher production of H₂.
1. Introduction

Currently, global climate change and future supply of energy stimulates research on alternative fuels. From this perspective, the biological production of hydrogen, also known as biohydrogen, appears as a promising alternative [1]. The processes of biological H2 production are mostly operated at room temperature and pressure. These milder conditions are environmentally friendly and energy saving [2,3]. Biogas can be obtained by using different systems: direct biophotolysis, indirect biophotolysis, photofermentation and dark fermentation [4-7]. Among these systems, hydrogen production by dark fermentation has received increasing attention, mainly considering the higher H2 production when compared to other biological means [8-10]. Moreover, it has the additional advantage of using different types of residues and effluents as substrate (lignocellulosic or starch materials, glycerol, food and dairy wastes, crop residues, palm oil mill effluent, among others) [11-14]. Considering the worldwide increase of biofuels production, the integration of second generation ethanol residues and biodiesel byproducts with biogas production is a welcome idea [15-19].

During dark fermentation, substrates rich in organic matter are converted into several simpler compounds such as volatile fatty acids (acetic, propionic, butyric and isobutyric acids), alcohols (ethanol, butanol), hydrogen and carbon dioxide [20-24]. The identification of volatile fatty acids (VFAs) and alcohols formed during the dark fermentation process provides relevant information about the metabolic pathways followed by microorganisms. Besides, the ratio of acetic/butyric acids (HAc/HBu) obtained can be related to the production of H2. Previous studies showed that the increase of the ratio of acetic/butyric acids is accompanied by increased production of H2 [25-29]. This behavior can be explained by the main metabolic reactions involved in sucrose degradation for hydrogen gas production: (1) generation of acetic acid from the consumption of sucrose and (2) generation of butyric acid from the consumption of sucrose, as described in reactions (1) and (2) [25]:

\[
\text{C}_{12}\text{H}_{22}\text{O}_{11} + 3\text{H}_2\text{O} \rightarrow 4\text{CH}_3\text{COOH} + 4\text{CO}_2 + 8\text{H}_2 \quad \text{Reaction (1)}
\]

\[
\text{C}_{12}\text{H}_{22}\text{O}_{11} + \text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 4\text{CO}_2 + 4\text{H}_2 \quad \text{Reaction (2)}
\]

According to reaction (1), up to 8 mol H2/mol sucrose can be obtained in the conversion of sucrose into acetic acid. In the parallel reaction (2), the butyric acid was formed from sucrose and in this case the maximum yield of hydrogen is 4 mol H2/mol sucrose. The aforementioned reactions explained the importance of acetic/butyric acids ratio in the estimation of H2 production.

Reports in the literature showed that the microorganisms of the genus Clostridium are the main responsible for H2 production in dark fermentation [16,30-33]. These microorganisms dispose of hydrogenases enzymes that catalyze the reversible oxidation of 'hydrogen (2H+ + 2e- → H2)' [8,34-36]. Recently, the level of hydrogenase gene (hyd) expression has been used as an indicator of hydrogen production in different systems [27,32,37-39]. Hydrogenases can be grouped into two major families based on metal content of their respective catalytic center: those containing only Fe, called [FeFe]-hydrogenase and those with Ni, Fe and sometimes Se, [NiFe]-hydrogenase and [Ni-Fe-Se]-hydrogenase. The [FeFe]-hydrogenases normally occur in hydrogen-producing microorganisms Clostridia. [NiFe] and [Ni-Fe-Se]-hydrogenases are often found in hydrogen consuming microorganisms, such as methanogenic archaea, responsible for the conversion of H2 into CH4 [34,40-42].

In order to inactivate the methanogenic archaea and maximize H2 production, chemical (acid, alkali or organic compounds addition) and physical (heat, aeration) pretreatments of inocula have been studied [44-50]. Among them, the heat pretreatment has been commonly used because of its simplicity and effectiveness. Heat pretreatment of consuming microorganisms, methanogens, are eliminated and the medium is enriched with spore forming hydrogen producers, Clostridium sp. [2,9,44,49-52]. This type of pretreatment has been widely used and molecular genetic techniques offer very important tools to evaluate and characterize the microbial population. Moreover, studies that correlate the effect of type of pretreatment of inocula with the level of hydrogenase gene expression could provide relevant information about the fermentative system. The purpose of this work is to investigate the effect of heat pretreatment of inocula and, consequently, the performance of bacterial communities responsible for H2 production, through the evaluation of the level of hydrogenase gene (hyd) expression.

The higher ratio of acetic acid/butyric acid also indicates an increase of H2 production, as presented in the stoichiometric equations (1) and (2). Therefore, the association of these two parameters is useful for the assessment of bacterial communities behavior.

2. Materials and methods

2.1. Seed sludge

The anaerobic sludge used in this study was obtained from a municipal sewage treatment plant located in Rio de Janeiro, Brazil. The concentration of total volatile solids presented in the anaerobic sludge was approximately 45 gL−1. In order to inhibit methanogenic archae and enrich H2-producing bacteria, the sludge was heated at 100°C for 1 h. Untreated anaerobic sludge was also used in control experiments.

2.2. Dark fermentation

Batch tests were performed for 24 h in 500 mL glass flasks sealed with rubber lids to avoid contact with air. The culture media were composed of 75 mL of heat pretreated anaerobic sludge or untreated anaerobic sludge, 75 mL of water and 500 µL of nutrients solution. The nutrients solution contained (mgL−1): sucrose 25,000; NaHCO3, 2005; K2HPO4, 3H2O 800; CaCl2, 50; MgCl2·6H2O 100; FeSO4·7H2O 25; NaCl 10; CoCl2·6H2O 5;
Technologies, CA, USA), 300 ng of DNA template and 0.5 µL of 0.5 U/mL recombinant Taq DNA polymerase (Invitrogen Life Technologies, CA, USA). The pH was adjusted to be optimum for H₂ production [23,50,52–54]. The pHi was not adjusted during the fermentation and the final pH varied from 5.5 to 4.6 ± 0.1. The fermentors were purged with H₂ gas to ensure the anaerobic environment. All experiments were carried out in rotatory shaker (Mascon), SP, Brazil at 35 °C and 100 rpm.

2.3. Analytical methods

Samples collected from the liquid phase were analyzed by high-performance liquid chromatography (HPLC) Shimadzu LC 10AT, Kyoto, Japan), for determining carbohydrates (sucrose, glucose and fructose), volatile fatty acids (formic, acetic, propionic, isobutyric and butyric) and ethanol. The liquid samples were centrifuged at 3000 rpm for 20 min, and then the supernatants were filtered through a 0.22 µm Millipore filter before the analysis in HPLC. The volatile fatty acids (VFAs) were determined by using Aminex HPX-87H column (Bio-rad, CA, USA) and refractive index detector (RID). Ultra pure water was used as mobile phase at a flow rate of 0.6 mL/min. The column temperature was 80 °C.

The concentration of total volatile solids was measured according to Standard Methods for Examination of Water and Wastewater [55].

2.4. DNA and RNA extraction

For the microbial community analysis, samples of untreated and heat pretreated sludge were taken before and after fermentation (24 h) and stored at –80 °C. The total genomic DNA was extracted and purified using DNeasy Blood & Tissue Kit (Qiagen Sciences, MD, USA). The procedures were performed in accordance with the manufacturer’s protocols.

2.5. Polymerase chain reaction (PCR)

The PCR mixture (50 µL total volume) contained 1× PCR buffer (20 mM Tris–HCl pH 8.4), 3.5 mM MgCl₂, 0.4 mM each dNTPs (Amersham Pharmacia Biotech, Buckinghamshire, England), 0.5 U/mL recombinant Tag DNA polymerase (Invitrogen Life Technologies, CA, USA), 300 ng of DNA template and of 0.5 µM of each primer (for Clostridium sp. 16S rRNA gene and hydrogenase gene), as showed in Table 1. The PCR was carried out in a thermocycler GeneAmp PCR System 9600 (Applied Biosystems, CA, USA). The amplification was performed with thermal cycling including initial denaturation at 95 °C for 10 min, 40 cycles of denaturation (95 °C, 15 s), annealing (55 °C, 1 min) and extension (72 °C, 30 s), and finally extension at 72 °C for 4 min.

Table 1 – Primers for PCR and real-time RT-PCR used in this study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5'-3')</th>
<th>Specificity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S-f</td>
<td>AGCGGTTG</td>
<td>Clostridium sp. 16S rRNA gene</td>
<td>[24,25]</td>
</tr>
<tr>
<td>16S-r</td>
<td>CGCAGTTTACTG</td>
<td>Clostridium sp. 16S rRNA gene</td>
<td>[38,39]</td>
</tr>
<tr>
<td>HG-f</td>
<td>GAAAGGTTTAA</td>
<td>Hydrogenase gene</td>
<td>[24,25]</td>
</tr>
<tr>
<td>HG-r</td>
<td>GGAAACATGAG</td>
<td>Hydrogenase gene</td>
<td>[24,25]</td>
</tr>
</tbody>
</table>

2.6. Synthesis of c-DNA

The synthesis of c-DNA was carried out using TaqMan Reverse Transcription Reagents (Applied Biosystems, USA), using random hexamer primer, in three stages: incubation starting at 25 °C for 10 min, activation of the enzyme Multiscribe RT at 48 °C during 30 min and inactivation at 95 °C for 5 min. Then, the c-DNA was used as template in the second stage, the Real-time PCR.

2.7. Real-time PCR

The expression of hydrogenase gene was analyzed using real-time PCR runs, through the use of the equipment ABI PRISM 7000 (Applied Biosystems, CA, USA). The reactions (25 µL total volume) were performed with SYBR Green PCR Master Mix (1×), 0.5 µM of each primer (constitutive and target), 5.0 mM MgCl₂, 200 µM of each dATP, dCTP, dGTP and dTTP, and 10 ng of each c-DNA as template. The amplification was performed with thermal cycling including initial denaturation at 95 °C for 10 min, 40 cycles of denaturation (95 °C, 15 s), annealing (58 °C, 1 min) and extension (72 °C, 30 s). The expression levels were determined by using the delta delta Ct method, calculated with Ct values from Clostridium sp. 16S rRNA gene (constitutive gene) and from the hydrogenase gene-hy (target gene) for each sample (SA1, SA2, SA3 and SA4). The calibrator was the SA1 sample. The dissociation curves plotted showed that the amplifications were performed as expected.

3. Results and discussion

In dark fermentation, the production of H₂ is usually accompanied by volatile fatty acids (VFAs) formation and coupled with solvent production. The formation of these intermediates indicates the metabolic pathway of the microorganisms involved in the bioprocess and provides a better understanding of the favorable conditions for H₂ production [23]. Therefore, before and after the fermentative process, liquid samples were collected and VFAs and solvents were analyzed by using HPLC.

Table 2 presents the concentration of individual VFAs formed after 24 h using untreated and heat pretreated anaerobic sludge. The content and distribution of VFAs suggested

MnCl₂·4H₂O 5; (NH₄)₂MoO₄·2H₂O 15; H₂BO₃ 5; NiCl₂·6H₂O 5; CuCl₂·2H₂O 10; ZnCl₂ 5. The pH was adjusted to 5.5 ± 0.1, which was reported to be optimum for H₂ production [23,50,52–54].
Table 2 - Intermediate metabolites concentrations after 24 h fermentation using untreated and heat pretreated anaerobic sludge.

<table>
<thead>
<tr>
<th>Inocula</th>
<th>Acetic acid</th>
<th>Propionic acid</th>
<th>Butyric acid</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>5.31±0.01</td>
<td>0.57±0.02</td>
<td>1.34±0.03</td>
<td>0</td>
</tr>
<tr>
<td>Pretreated</td>
<td>6.45±0.01</td>
<td>1.28±0.02</td>
<td>0.99±0.03</td>
<td>1.36±0.02</td>
</tr>
</tbody>
</table>

Concentration (mmol L⁻¹) ± standard deviation. n=3.

that acetic acid type fermentation was predominant for media containing either untreated or heat pretreated sludge, the acetic acid concentrations were 5.31 mmol L⁻¹ and 6.45 mmol L⁻¹, respectively. Medium containing untreated sludge also promoted the formation of butyric (1.34 mmol L⁻¹) and propionic (0.57 mmol L⁻¹) acids. It was possible to observe the formation of butyric (0.99 mmol L⁻¹) and propionic (1.28 mmol L⁻¹) acids and ethanol (1.36 mmol L⁻¹) in the medium containing heat pretreated sludge. The different intermediate metabolites produced could be associated with the groups of microorganisms selected during the heat pretreatment, as described by previous studies [12,22,56]. As mentioned before, this pretreatment of inocula eliminates non-spore forming microorganisms, while the spore forming microorganisms survive thermal shock [22]. Species of genus Clostridium such as Clostridium butyricum, Clostridium acetobutyricum, Clostridium beijerinckii and Clostridium thermohalitum are examples of spore forming hydrogen-producing microorganisms [12]. Depending on the type of dominant species in the bioreactor and the operating conditions after pretreatment, different intermediate metabolites can be formed [56]. In the majority of the studies for H₂ production by dark fermentation, acetic and butyric acids were the predominant metabolites produced, suggesting the presence of butyric bacteria in fermentation media [30,39,57]. Khanal et al. (2004) show that the high concentration of propionic acid obtained in the fermentation may be related to the presence of other species of microorganisms, such as Clostridium pectinovorum [25].

After 24 h sucrose was not detected in both media, either with pretreated or untreated inocula, and the presence of glucose and fructose indicated the total hydrolysis of sucrose (data not showed). The biohydrogen production using sucrose as substrate could be estimated through the ratio of acetic/ butyric acids (HAc/HBu) obtained during the fermentative process [25,26,28], as previously presented in reactions (1) and (2). In this study, the medium containing heat pretreated sludge presented a higher ratio of acetic and butyric acids (approximately 7) when compared to the medium where untreated sludge was employed (about 4), indicating the highest production of H₂. Ethanol production and the highest formation of propionic acid, after 24 h of fermentation, in the medium where heat pretreated sludge was employed (Table 2) could be associated with the increase of H₂ partial pressure in the liquid phase [21]. The production of ethanol (reaction (3)) and propionic acid (reaction (4)) from acetic acid and hydrogen are thermodynamically favorable [52,56]. Reactions (5) and (6) presented the metabolites acetate and butyrate and H₂ production from C₆ sugar (sucrose hydrolysis), respectively [12,58]. The formation of H₂ in the liquid medium from the C₆ sugar (reactions (5) and (6)) favors the occurrence of reactions (3) and (4). Thus, although the H₂ partial pressure has not been determined in the present study, the reactions (3)-(6) and also their respective Gibbs free energy (ΔG°) justify the previous explanation related to VFAs and solvent formation during hydrogen production.

![Fig. 1](url) - PCR products for genes amplifications: (A) Clostridia sp. 16S rRNA and (B) hydrogenase. S = standard low mass ladder; SA1 = untreated sludge before fermentation; SA2 = heat pretreated sludge before fermentation; SA3 = untreated sludge after 24 h of fermentation and SA4 = heat pretreated sludge after 24 h of fermentation.
Previous studies reported that the microorganisms of the genus Clostridium are the key responsible for $\text{H}_2$ production during anaerobic fermentation [16,30,31,33]. Clostridium sp. is a typical acidogenic bacteria and it has the capacity to sporulate when environmental conditions become hostile [2]. As expected, the spores are metabolically dormant and resistant to heat. The performance of Clostridium sp. in $\text{H}_2$ production is related to hydrogenase gene [27–32,37–39]. Within this context, a preliminary molecular genetic study by PCR using primers to detect the 16S ribosomal RNA gene and hydrogenase gene for Clostridium was performed. In Fig. 1, the amplification products for Clostridium sp. 16S rRNA gene (Fig.1A) and hydrogenase gene (Fig.1B), qualitatively showed the presence of microorganisms of genus Clostridium and hydrogenase activity, respectively, in all conditions studied. Based on these evidences, a subsequent study was performed to determine the level of hydrogenase gene ($\text{hyd}$) expression by real-time reverse transcriptase polymerase chain reaction (Real-time RT-PCR). In the present study, quantitative data presented in Fig. 2 show that the hydrogenase gene expression in the heat pretreated sludge after fermentation is about 3 times higher when compared with the untreated sludge after 24 h of fermentation. This result, combined with the ratio of acetic and butyric acids that was 7, obtained in the medium where heat pretreated sludge was employed, corroborates the direct relation between hydrogenase gene expression and $\text{H}_2$ production previously demonstrated in recent studies [27,37,38]. Therefore, the hydrogenase gene expression monitoring associated with the ratio of acetic/butyric acids ($H\text{Ac}/H\text{Bu}$), can be used as a practical index of hydrogen production in the bacterial communities.

4. Conclusions

The three fold increment on hydrogenase gene expression and the increase of acetic/butyric acids ratio (approximately 7, for the medium where heat pretreated sludge was employed) showed that pretreated inocula and untreated inocula had different behaviors. These findings indicate that the heat pretreated inocula favor the $\text{H}_2$ production.

The combination of these two parameters, $H\text{Ac}/H\text{Bu}$ and hydrogenase expression, could be a useful tool to evaluate bacterial performance for $\text{H}_2$ production.

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