Factors influencing the production of Hydrogen by fermentative processes

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Resumo - Fatores que influenciam a produção de hidrogênio por processos fermentativos. A crescente preocupação com a substituição de combustíveis fósseis tem incentivado a busca de novas formas de produção de energia limpa. O hidrogênio tem-se destacado como uma forma ideal de energia, pois não contribui para o aumento do efeito estufa além de possuir alto poder calorífico. A produção microbiológica deste gás é um processo que pode se tornar sustentável do ponto de vista social, ambiental e econômico, uma vez que microrganismos isolados do ambiente podem utilizar resíduos provenientes de processos industriais e agrícolas. Diversos microrganismos são descritos na literatura como produtores de hidrogênio, principalmente os gêneros Clostridium e Enterobacter, a partir de diversos tipos de substratos. Porém, diversos fatores e condições fermentativas que irão direcionar para a rota metabólica de produção de hidrogênio devem ser analisados. Dessa forma, o objetivo deste trabalho é contribuir com o estado da arte, fazendo uma revisão dos recentes trabalhos envolvendo a produção microbiológica de hidrogênio e os principais aspectos envolvidos no processo biotecnológico.


Abstract - Growing concern about finding suitable replacements for fossil fuels has encouraged the search for new clean energy production processes. Hydrogen has been highlighted as an ideal form of energy because this molecule does not contribute to the greenhouse effect and it possesses a high calorific value. The microbiological production of this gas is a process that can become sustainable from social, environmental and economic points of view once the microorganisms isolated from the environment can use agro-industrial wastes as substrate. Various microorganisms are described in the literature as producers of hydrogen from several types of substrates, especially species from the genera Clostridium and Enterobacter. However, various scientific factors and fermentation operating conditions that will steer the metabolic pathway to the production of hydrogen should be analyzed. The objective of this work is to contribute to the state of the art by reviewing recent studies involving the microbiological production of hydrogen and the main aspects involved in biotechnological process.


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1 INTRODUCTION

One of the major challenges in the coming years is will be to find alternative means for producing sustainable and clean energy to replace non-renewable fossil fuels, such as coal, petroleum and
natural gas. This need arises because the combustion of these fuels contributes to climate change, especially in relation to the greenhouse effect. In this regard, hydrogen (H₂) has been highlighted as an ideal form of energy that only forms water when combusted and no pollutants (Equation 1), in addition to being a renewable energy source (LIN et al., 2006; DAS & VEZIROGLU, 2008).

\[ \text{(1) } 2\text{H}_2 + \text{O}_2 \rightarrow 2\text{H}_2\text{O} \]

In this way, hydrogen fuel contributes to reducing the levels of atmospheric carbon dioxide (CO₂), minimizing the problems caused by fossil fuels (MARTINEZ-PEREZ et al., 2007).

Biological processes, such as bacterial photosynthesis (HALLENBECK et al., 2012) and/or fermentation (TUŠ’AK et al., 2007; PANDU & JOSEPH, 2012) produce H₂, and these processes are doubly sustainable because fermentable waste can be the substrate for the production of this gas by microorganisms isolated from soil and residual waters, among other environments, to generate a source of safe energy. H₂ is still a high cost energy source when compared with other conventional options, but the use of residues in organic production justifies the investments to make the H₂ gas more abundant and economically viable (CHÔNG et al., 2009; SALVI & SUBRAMANIAN, 2015).

A wide variety of pure or mixed bacterial cultures (MAINTINGUER et al., 2008; QIAN et al., 2011; Lee et al., 2011) have been used for the production of H₂ from different substrates, especially species from the genera Clostridium and Enterobacter. These bacteria are commonly used as inocula for the production of H₂ due to the significant output of these bacteria (OH et al., 2009; LAY et al., 2010; PANDU & JOSEPH, 2012). In addition, several factors such as the composition of the culture medium, the temperature, the pH and the partial pressure are important to improve and enhance this production.

This article reviews the main aspects involved in the organic in vitro production of hydrogen by fermenting microorganisms.

1.1 Fermentative Hydrogen Production

Hydrogen is the simplest and most abundant element in the universe, representing 3/4 of existing matter (KALINCI et al., 2009). The interest in hydrogen has increased due to technological advances in fuel cells for cars, homes and businesses, and also the potential of batteries in electronic products and internal combustion engines (DUNN, 2002). However, H₂ is not yet available in sufficient quantities and economically feasible production continues to be a challenge. Currently, 95% of the H₂ produced industrially is derived from fossil fuels (TANSKSALE et al., 2010).

H₂ can be produced biologically by microorganisms via photosynthesis (MATSU-NAGA et al., 2000; YASIN et al., 2013) or dark fermentation using carbohydrates for the formation of this molecule (FANG et al., 2002). Dark fermentation is the fermentative conversion of an organic substrate to H₂. This complex process is manifested by a diverse group of bacteria through a series of biochemical reactions. Fermentative microorganisms hydrolyze complex organic polymers to monomers, which are further converted to a mixture of lower molecular weight organic acids and alcohols by the necessary H₂ producing acidogenic bacteria (PANDU & JOSEPH, 2012; GHIMIRE et al.; 2015). Sources of fermentative substrate for the production of H₂ have been described and tested to determine the efficiencies of these processes, for example, starch (CHEN et al., 2007), cellulose (LEVIN et al., 2006; SIQUEIRA and Reginatto, 2015) and glucose (HERBERT et al., 2002; ABDALLAH et al., 2016), in addition to industrial and agricultural waste (WANG et al., 2010) such as glycerol (AMARAL et al., 2009; SUHAIMI et al., 2012) and vinasse (FERNANDES, 2008; LAZARO et al., 2014).

To maximize the potential energy contained in the substrates, the fermentative production of H₂ can be integrated with the production of CH₄, where an acidogenic step precedes a methanogenic process (HAWKES et al., 2007). This potential is due to the high concentration of volatile fatty acids and alcohols contained in the effluent, which represent organic pollutants of high-energy value. Producing power while protecting bodies of water requires a second stage to take advantage of the residual energy contained in the effluent (SHOW et al., 2012). However, this integration is still a challenge because the anaerobic process is quite complex and demands extensive research on the subject. Figure 1 presents a proposal for the processing of substrates to boost energy production.
Figure 1: Proposal for the processing of substrates aiming at maximum utilization.

One of the advantages of \( \text{H}_2 \) bioproduction is related to the ability of microorganisms to select a substrate even when this substrate is in a mixture of other compounds. This ability minimizes the need to purify the substrates fed to the process, thereby increasing the spectrum of raw materials that can be used, and reducing the need for chemical treatments (DRAPCHO et al., 2008). In addition, the employment of operating temperature and pressure similar to the environment would an advantage of the relevant microbiological process, providing a favorable energy balance to the process (DAS & VEZIROGLU, 2008).

A variety of possible advantages of the fermentative production of \( \text{H}_2 \) may be enumerated, such as the low demand for nutrients; the non-necessity of energy for aeration; the ease of constructing the processes compared to aerobic processes; the possibility of producing \( \text{H}_2 \) for 24 h without the need for light; the great variety of carbon sources that can be used; and the production of valuable metabolites, including butyric acid, acetic acid and various alcohols. The disadvantages are related to the susceptibility of the process to the possible presence of oxygen \((\text{O}_2)\), which is a strong inhibitor; the relatively low-income of \( \text{H}_2 \); the biogas produced by the process is not pure; and the technical difficulties associated with later processes aiming at the production of \( \text{CH}_4 \). As a consequence, the facultative anaerobic bacteria are, in general, preferred for the production of \( \text{H}_2 \) by fermentation (OH et al., 2002; DAS & VEZIROGLU, 2008).

However, the fermentative production of \( \text{H}_2 \) depends on a series of operational parameters and physico-chemical conditions that influence the process, as well as the type of substrate, the type of reactor and the presence and concentration of nutrients, in addition to the pH, the temperature and the species of microorganisms used (WANG & WAN, 2009; XIAO et al., 2010).

1.2 Main microorganisms involved in fermentative hydrogen production

This process used bacteria that are compulsory and voluntarily anaerobic. Facultative anaerobes produce a smaller quantity of \( \text{H}_2 \) per mol of substrate, while those microbes that are strictly anaerobic produce a higher amount (DAS & VEZIROGLU, 2008). Meanwhile, facultative anaerobic microbes are less sensitive to the presence of \( \text{O}_2 \) and have the ability to resume the production of \( \text{H}_2 \) in the case of depleted \( \text{O}_2 \). As a consequence, the facultative anaerobic bacteria are, in general, preferred for the production of \( \text{H}_2 \) by fermentation (OH et al., 2002; DAS & VEZIROGLU, 2008).

The microorganisms capable of producing \( \text{H}_2 \) can be isolated from natural environments such as soils and sludge treatment stations of domestic and industrial effluents (MAINTINGUER et al., 2015). Many bacteria have been described in the literature as producing \( \text{H}_2 \) from various types of substrates (WANG & WAN, 2009; KAPDAN & KARGI, 2006). This process can be performed by pure or mixed cultures of anaerobic fermentative bacteria.

The most common microorganisms used in \( \text{H}_2 \) bioproduction are Clostridium, Enterobacter and Klebsiella (ZHANG et al., 2006; LO et al., 2008; PAN et al., 2008; WANG & WAN 2009; LAZARO et al., 2014). Bacteria of the genus Clostridium are Gram-positive, strictly anaerobic and form endospores of environmental resistance (temperature, alkalinity and acidity) (Song and Dong, 2008). For this reason, bacteria of the genus Clostridium are easily found in a bacterial community (KUO et al., 2011), whereas Enterobacter and Klebsiella, are Gram-negative bacilli and facultative anaerobes (PODSCHUM et al.; 1998; ZHANG et al., 2011). According to Fang et al., (2002), the bacteria of the genus Clostridium represent approximately 65 to 70% of the total population of bacteria producing \( \text{H}_2 \) that are studied, and these bacteria are classified as proteolytic or saccharolytic, depending on the type of organic material that these
bacteria ferment. Bacteria from the genus *Clostridium* proteolytically degrade proteins or amino acids and saccharolytically ferment carbohydrates. A curious fact is that the largest production of H$_2$ reported in the literature was obtained in systems of immobilized cells (granules or biofilms) with the *Clostridium* genus represented prominently. Among the several *Clostridium* species, the largest producer was *C. pasteurianum* (LEE et al., 2011).

In relation to the Enterobacteriaceae: because these bacteria are facultative microorganisms, these bacteria are easier to manipulate than other microorganisms because cell growth can occur in the presence of O$_2$ (ZHANG et al., 2011), allowing inhibition of the fermentative process in an atmosphere of 100% H$_2$ to be investigated (NAKASHIMADA et al., 2002).

### 1.3 Pure Cultures

The capacity for the production of H$_2$ by pure cultures of *Clostridium* and *Enterobacter* has been the focus of many recent studies, as shown in Table 1. In addition to these bacteria, other microorganisms are also described in the literature as potential producers of H$_2$ such as the *Klebsiella* sp., bacteria of the genus *Citrobacter*, *Bacillus* (Poleto et al., 2016), *Escherichia coli* (facultative anaerobic), *Pseudomonas*, and *Thermoanaerobacterium* (from extreme environments). Other methods were used to study the production of H$_2$ from these microbes, including: glucose, glycerol, sucrose, xylose, cellobiose and agro industrial waste. In Table 1, the mode of operation in each batch that is the most widely used in fermentation processes is illustrated, however the maximum rate of production of H$_2$, 650 mL/g h, was in a continuous reactor for the conversion of glycerol by *E. aerogenes* (Table 1). However, the rates of hydrogen (Table 1 and 2) produced by the various biohydrogen systems are expressed in different units, making it difficult to assess and compare the rates and amounts of hydrogen synthesized by different biohydrogen technologies (LEVIN et al., 2004).

### 1.4 Mixed Cultures

However, the processes that use mixed cultures are more simple to handle and control, and the organisms in these cultures may even be capable of metabolizing a larger spectrum of substrates, demonstrating, in general, a higher yield of H$_2$ (Table 2) when compared to pure cultures. However, in these cases, attention must be paid to the presence of bacteria both as producers and consumers of H$_2$ in the microbial consortium, which would lead to a decrease in production (WANG & WAN, 2009). Maintinguer et al., (2008) highlighted the need for pre-treatment of the inoculums to eliminate hydrogenotrophic methanogenic archaea that metabolize H$_2$.

The pre-treatment heating step, together with the control of pH, has been applied for the selection of bacteria capable of producing H$_2$ such as those of the genus *Clostridium*, which favors the production of H$_2$ because these bacteria are tolerant to adverse environmental conditions and high temperatures (MAINTIGUER et al., 2008; KAWAGOSHI et al., 2005).

Other methods of pre-treatment of the inoculum such as acidic or basic treatment, aeration, freezing followed by thawing and chemical treatments with chloroform, sodium 2-bromoethanesulfonate and iodopropane can also be applied to select microorganisms (WANG & WAN 2009).

Studies in the literature (Table 2) have used the following carbon sources: purified sucrose, glucose and peptone, as well as sustainable pulp residual forms of manioc, vinasse, molasses, waste food and domestic effluents. Mixed cultures inoculum conducted in batch mode, were more commonly used, and, in general, mixed cultures were more efficient producers of H$_2$ than pure cultures (Table 2).

Microbial identification is essential to determining the composition of the community that effectively ferments substrates to produce H$_2$ and also to assess changes in the structure of mixed communities after the application of certain methods of pre-treatment of the inocula (WANG & WAN et al., 2008; KIM et al., 2008). Currently, in addition to the classic microbiology, microscopy and cultivation techniques in enriched culture media, other methods are being used for the determination of genus and species. These include qualitative and semi-quantitative molecular biology methods, such as PCR, PCR-RFLP, PCR-DGGE, Real Time PCR technique and gene sequencing (OLIVEIRA et al., 2014; MAINTIGUER et al, 2013; 2015).

The use of molecular techniques allows the fluctuations in the microbial communities and hydrogen production to be observed. Small changes in temperature, pH, substrate concentration and other parameters are very influential in shaping the consortium of bacteria that dominates the fermentation.
Table 1: Results described in the literature for the production of H₂ using pure cultures of bacteria from different types of substrates.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Substrate</th>
<th>Reactor type</th>
<th>Maximum hydrogen yield</th>
<th>Rate of hydrogen production</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. aerogenes</em></td>
<td>Glycerol</td>
<td>Continuous</td>
<td>0.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>650&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Markov et al., (2011)</td>
</tr>
<tr>
<td><em>E. aerogenes</em></td>
<td>Glycerol</td>
<td>Continuous</td>
<td>0.84&lt;sup&gt;c&lt;/sup&gt;</td>
<td>80&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Ito et al. (2005)</td>
</tr>
<tr>
<td><em>E. aerogenes</em></td>
<td>Paper and pulp industry effluent</td>
<td>Batch</td>
<td>2.03&lt;sup&gt;e&lt;/sup&gt;</td>
<td>225&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Lakshmidevi &amp; Muthukumar (2010)</td>
</tr>
<tr>
<td><em>E. aerogenes</em> ATCC 35029</td>
<td>Glycerol</td>
<td>Batch</td>
<td>0.85/0.95&lt;sup&gt;g&lt;/sup&gt;</td>
<td>-</td>
<td>Jitrwung et al., (2011)</td>
</tr>
<tr>
<td><em>E. aerogenes</em></td>
<td>Glycerol</td>
<td>Continuous</td>
<td>3.1&lt;sup&gt;h&lt;/sup&gt;</td>
<td>72.1&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Khanna et al., (2011)</td>
</tr>
<tr>
<td><em>E. cloacae</em> (WBK3)</td>
<td>Glucose</td>
<td>Continuous</td>
<td>1.8&lt;sup&gt;j&lt;/sup&gt;</td>
<td>180.7&lt;sup&gt;k&lt;/sup&gt;</td>
<td>Harun et al., (2012)</td>
</tr>
<tr>
<td><em>E. cloacae</em></td>
<td>Glucose</td>
<td>Batch</td>
<td>3.9&lt;sup&gt;l&lt;/sup&gt;</td>
<td>0.017&lt;sup&gt;m&lt;/sup&gt;</td>
<td>Mandal et al., (2006)</td>
</tr>
<tr>
<td><em>E. cloacae</em></td>
<td>Glucose</td>
<td>Batch</td>
<td>3.4&lt;sup&gt;n&lt;/sup&gt;</td>
<td>-</td>
<td>Kumar et al., (2001)</td>
</tr>
<tr>
<td><em>C. butyricum</em></td>
<td>Sugarcane bagasse</td>
<td>Batch</td>
<td>1.73&lt;sup&gt;o&lt;/sup&gt;</td>
<td>-</td>
<td>Patra et al., (2008)</td>
</tr>
<tr>
<td><em>C. acetobutylicum</em></td>
<td>Glucose</td>
<td>Batch</td>
<td>408&lt;sup&gt;p&lt;/sup&gt;</td>
<td>-</td>
<td>Alshiyab et al., (2008a)</td>
</tr>
<tr>
<td><em>C. acetobutylicum</em></td>
<td>Glucose</td>
<td>Batch</td>
<td>391&lt;sup&gt;q&lt;/sup&gt;</td>
<td>-</td>
<td>Alshiyab et al., (2008b)</td>
</tr>
<tr>
<td><em>Clostridium sp.</em></td>
<td>Molasses</td>
<td>Continuous</td>
<td>-</td>
<td>390&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Lay et al., (2010)</td>
</tr>
<tr>
<td><em>Clostridium sp. R1</em></td>
<td>Cellubiose</td>
<td>Batch</td>
<td>2.0&lt;sup&gt;s&lt;/sup&gt;</td>
<td>-</td>
<td>Ho &amp; Lee (2011)</td>
</tr>
<tr>
<td><em>C. perfringens</em> (W11)</td>
<td>Hexose</td>
<td>Batch</td>
<td>1.53&lt;sup&gt;t&lt;/sup&gt;</td>
<td>-</td>
<td>Wang et al., (2011)</td>
</tr>
<tr>
<td><em>Klebsiella pneumonia</em> ECU15</td>
<td>Glucose</td>
<td>Batch</td>
<td>2.07&lt;sup&gt;u&lt;/sup&gt;</td>
<td>482&lt;sup&gt;v&lt;/sup&gt;</td>
<td>Niu et al., (2010)</td>
</tr>
<tr>
<td><em>Klebsiella pneumonia</em> DSM2026</td>
<td>Glycerol</td>
<td>Batch</td>
<td>0.53&lt;sup&gt;w&lt;/sup&gt;</td>
<td>17.8&lt;sup&gt;x&lt;/sup&gt;</td>
<td>Liu &amp; Fang (2007)</td>
</tr>
<tr>
<td><em>Klebsiella sp. HE1</em></td>
<td>Sucrose</td>
<td>Batch</td>
<td>0.92&lt;sup&gt;y&lt;/sup&gt;</td>
<td>3.26&lt;sup&gt;z&lt;/sup&gt;</td>
<td>Wu et al., (2008)</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> TR17</td>
<td>Glycerol</td>
<td>Batch</td>
<td>0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>Chookaew et al. (2012)</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em> CWBI952</td>
<td>Sucrose/glucose</td>
<td>Batch</td>
<td>0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>Beckers et al., (2010)</td>
</tr>
<tr>
<td><em>Citrobacter amalonaticus</em> Y19</td>
<td>Glucose</td>
<td>Batch</td>
<td>8.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>Oh et al., (2008)</td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em> GZ1</td>
<td>Residual sludge</td>
<td>Batch</td>
<td>15.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
<td>Guo et al., (2008)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> S3</td>
<td>Glucose</td>
<td>Batch</td>
<td>16.07&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-</td>
<td>Junyapoon et al. (2011)</td>
</tr>
<tr>
<td><em>Thermotoga neapolitana</em></td>
<td>Glycerol</td>
<td>Batch</td>
<td>620&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-</td>
<td>Ngos &amp; Sim (2012)</td>
</tr>
<tr>
<td><em>Ethanoligenens harbinense</em> B49</td>
<td>Glucose</td>
<td>Batch</td>
<td>2.21&lt;sup&gt;g&lt;/sup&gt;</td>
<td>-</td>
<td>Guo et al., (2009)</td>
</tr>
<tr>
<td><em>Thermosanaerobacterium asteraeum</em></td>
<td>Glucose/syllose</td>
<td>Batch</td>
<td>2.71&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
<td>Li et al., (2010)</td>
</tr>
</tbody>
</table>

<sup>a</sup> mol H₂; <sup>b</sup> mol L−1; <sup>c</sup> g L−1; <sup>d</sup> mmol H₂; <sup>e</sup> mmol H₂; <sup>f</sup> mmol H₂; <sup>g</sup> mmol H₂; <sup>h</sup> mmol H₂; <sup>i</sup> mmol H₂; <sup>j</sup> mmol H₂; <sup>k</sup> mmol H₂; <sup>l</sup> mmol H₂; <sup>m</sup> mmol H₂; <sup>n</sup> mmol H₂; <sup)o</sup> mmol H₂; <sup>p</sup> mmol H₂; <sup>q</sup> mmol H₂; <sup>r</sup> mmol H₂; <sup>s</sup> mmol H₂; <sup>t</sup> mmol H₂; <sup>u</sup> mmol H₂; <sup>v</sup> mmol H₂; <sup>w</sup> mmol H₂; <sup>x</sup> mmol H₂; <sup>y</sup> mmol H₂; <sup>z</sup> mmol H₂.

The bacterial community that develops in a mixed culture influences the final production of products, such as acids and gases (Lazarro et al., 2014).

1.5 Main reactions involved in microbiological production of H₂

The fermentative production of H₂ is a phenomenon that occurs in anaerobic conditions, where the bacteria degrade organic compounds as a source of energy, especially glucose (Kumar et al., 2016). Because fermentation is less energetically efficient than aerobic oxidation, these microorganisms develop much more slowly than in an aerobic process.

The stoichiometry of the fermentation and oxidation reactions is vast and complex. These reactions rely on association syntrophy, i.e., when a species degrades a substrate and the generated product, is used by another species. The three main steps of the process are the anaerobic hydrolysis of organic matter, acidogenesis and methanogenesis (Levin et al., 2004; Valdez-Vazquez & POGGI-VARALDO, 2009; Grady et al., 2011). Before organic matter can be metabolized, the organic compound needs to be hydrolyzed, and the macromolecules must be reduced in size to facilitate transport through the cell membrane. A carbohydrate, for example, should be reduced until this sugar becomes a disaccharide (Sawyer et al., 1994). The reactions responsible for the hydrolysis and reduction in size of organic macromolecules are usually hydrolytic and are catalyzed by extracellular enzymes (amylase, cellulase, protease) produced by bacteria.
In acidogenesis, amino acids and sugars are degraded by fermentative reactions in which organic compounds serve as both donors and acceptors of electrons. Then, a simple sugar, for example, is converted to pyruvic acid and then pyruvic acid is converted to various products.

The formation of these products is dependent on the species involved in the fermentation. The main products of this step are intermediate products, such as propionic acid (Equation 3) and butyric acid (Equation 4), in addition to the formation of direct precursors of methane, acetic acid (Equation 5) and hydrogen. Depending on the microbial species involved, acetone (Equation 6) and ethanol (Equation 7) can also be formed.

In relation to the output of the different metabolic routes, when acetic acid is the end product, a theoretical maximum yield of 4 mols of H₂ per mol of glucose is obtained. When butyric acid is the end product, a theoretical maximum yield of 2 mols of H₂ per mol of glucose is obtained. The yield of H₂ is even smaller when more reduced organic compounds, such as lactic acid and ethanol, are produced.

\[
\begin{align*}
(3) \quad & \text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{COOH} + 2\text{H}_2 \\
(4) \quad & \text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{COOH} + 2\text{H}_2 + 2\text{CO}_2 \\
(5) \quad & \text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 4\text{H}_2 + 2\text{CO}_2 \\
(6) \quad & \text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COCH}_3 + 3\text{CO}_2 + 4\text{H}_2 \\
(7) \quad & \text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2\text{CH}_3\text{CH}_2\text{OH} + 2\text{CO}_2
\end{align*}
\]

One of the extensively studied species of *Clostridium*, *C. butyricum* (PATTRAT, 2008; SAINT-AMANS, 2001; SOLOMON et al., 1995), produces butyric acid as the main product of its fermentation process, along with the CO₂ and H₂. This metabolic pathway is found in approximately 50% of the...
microorganisms of the genus *Clostridium* (Khanal et al., 2004). The species of this genus produce H₂ during the exponential growth phase of cell growth because reaching the stationary phase requires changes in cellular metabolism, moving from the production of H₂ to the production of other compounds (Chong et al., 2009). However, the predominance of the two metabolic pathways that generate acetate acid and butyric acid depend not only on the type of microorganism but also on the growth conditions. For example, the pH, mixing process, hydraulic retention time, rate of organic load, partial pressure of H₂ inside the reactor and nutrients all influence this relationship (Khanal et al., 2004).

However, the production of H₂ by fermentation reactions is small. The greater part of the production of H₂ comes from the oxidation of long chain fatty acids (Equation 8) and volatile acids, such as propionic acid (Equation 9), butyric acid (Equation 10) and acetic acid, in a process called anaerobic oxidation.

\[
\text{(8)} \quad \text{CH}_3(\text{CH}_2)_n\text{COOH} + 14\text{H}_2\text{O} \rightarrow 8\text{CH}_3\text{COOH} + 14\text{H}_2
\]

\[
\text{(9)} \quad \text{CH}_2\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + 3\text{H}_2 + \text{CO}_2
\]

\[
\text{(10)} \quad \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 2\text{H}_2
\]

Equation 11 demonstrates that some H₂ can be combined with CO₂ by homoacetogenic bacteria to form acetic acid, representing a reduction in the efficiency of the production of H₂ (Kraemer and Bagley, 2007b).

\[
\text{(11)} \quad 4\text{H}_2 + 2\text{HCO}_3^- + \text{H}^+ \rightarrow \text{CH}_3\text{COO}^- + 4\text{H}_2\text{O}
\]

In the last step of the fermentation process, methanogenesis, the acetic acid and H₂ are converted by methanogenic acetoclastic and hydrogenotrophic bacteria to CH₄ and CO₂. Equation 12 shows the conversion to CH₄ by hydrogenotrophic bacteria.

\[
\text{(12)} \quad 4\text{H}_2 + \text{HCO}_3^- + \text{H}^+ \rightarrow \text{CH}_4 + 3\text{H}_2\text{O}
\]

When sulfates and nitrates are present, sulfate- and nitrate-reducing bacteria are able to use the H₂ as a donor of electrons generating sulfides and ammonia, respectively. Therefore, the H₂ produced in an anaerobic process can be consumed by archaean methanogenic bacteria, homoacetogenics bacteria (Equation 13) and sulfur- (Equation 14) and nitrate-reducing (Equation 15) bacteria.

\[
\text{(13)} \quad 4\text{H}_2 + 2\text{HCO}_3^- + \text{H}^+ \rightarrow \text{CH}_3\text{COO}^- + 4\text{H}_2\text{O}
\]

\[
\text{(14)} \quad 4\text{H}_2 + \text{SO}_4^{2-} \rightarrow \text{HS}^- + 3\text{H}_2\text{O} + 0\text{H}^-
\]

\[
\text{(15)} \quad 4\text{H}_2 + \text{NO}_3^- + 2\text{H}^+ \rightarrow \text{NH}_4^+ + 3\text{H}_2\text{O}
\]

To increase the efficiency of the production of H₂, several studies describe the bioengineering of strains of *Enterobacter aerogenes* to genetically manipulate specific metabolic pathways (Lu et al., 2009; Zhao et al., 2009; Lu et al., 2010; 2011) to direct metabolism towards only the production of H₂ and, thereby, increase the productivity of these modified organisms. Additionally, the addition of formate to the glucose culture (Kurokawa and Tanisho, 2005), the employment of substrates with different states of oxidation, or even the addition of external nicotinamide adenine dinucleotide in reduced form (NADH) (Zhang et al., 2009; Zhang et al., 2011), can also increase production of H₂ by cell cultures.

### 1.6 Parameters for the production of H₂

#### 1.6.1 Substrates

Proteins and carbohydrates can be used as substrates for the production of H₂, although the latter offer higher yields. Carbohydrates represent the preferred substrate of bacteria in fermentation processes aiming at the production of H₂. However, studies indicate that a combination of proteins and carbohydrates results in greater production of H₂. According to Ding et al. (2008), the presence of protein among the substrates not only increases the ability of the process to neutralize volatile acids due to the alkalinity of the process but also increases the production of H₂ by offering readily available organic nitrogen in the form of soluble protein and amino acids to the microorganisms (Ghimire et al., 2016).

Currently, the cost of H₂ generated from biological processes is very high. Several novel approaches have been proposed to the use of agro-industrial waste as substrate. As media composition significantly affects the production of organic acids and hydrogen by dark fermentation, the composition of complex media plays an important role (Sydney et al., 2014). Complex carbon sources, such as molasses (Lay et al., 2010), paper and pulp industry effluent (Lakshimdevi & Muthukumar,
Various authors differ with respect to the concentration of substrate to apply in anaerobic reactors to maximize the production of H₂ (WANG and WAN, 2009). According to LIN et al. (2012), there is no set optimal substrate concentration can be applied in anaerobic reactors, although in the literature several studies highlight the effects of the concentration of substrates and operating conditions on fermentative process. The majority of studies relate the organic production of H₂ from effluents and solid wastes at concentrations lower than 40 g COD (chemical oxygen demand)/L. Higher production of H₂ has also been obtained at low substrate concentrations (LIN et al., 2012).

The relationship between food/microorganism is also a factor that needs to be considered in the production of H₂ because this parameter allows the optimal concentration of inoculum and substrate present in a reactor to be determined. As an example, using a mixed culture in the degradation of sucrose, CHEN et al. (2006) obtained a maximum yield of H₂ for 7.3 g COD/g VSS (volatile suspended solids). Maximum yields were obtained when degraded waste from food and powdered milk were used at 7.8 g COD/g VSS and 14.7 g COD/g VSS, respectively.

1.6.2 Nutritional Solution

Bacteria can develop using very simple substrates, such as sugars; however, some essential elements must be present in the form of ions or inorganic salts.

Among the main nutrients that stand out are nitrogen and sulfur, due to the importance of these elements in bacterial growth. Phosphorus is also a very important element, however, only 1/5 as much phosphorous is required by bacteria compared to nitrogen (for example, 0.2 kg of phosphorus for each kg of nitrogen).

In addition to nutrients, trace elements (micronutrients) are also required for cellular metabolism. Many of these trace elements assist in enzymatic function or in other physiological capabilities. Among these micronutrients, Ca, Co, Cu, Fe, Mg, K, Mn, and Zn are prominent. For some bacteria, such as nitrogen-fixing bacteria, molybdenum is also necessary. Lastly, nickel is required by methanogenic bacteria (SAWYER et al., 1994).

Several studies have suggested the employment of a nutrient solution for the production of H₂; however, various combinations and concentrations are used. Clearly, successful fermentation processes depend on certain conditions, such as the substrate and consortium of bacteria used in the culture medium. PEIXOTO (2008) analyzed the production of H₂ in an anaerobic fixed bed reactor using the effluent from the refrigerant industry as substrate, and obtained a greater reduction in COD (23.1%) and a maximum production of H₂ (29.4 mL/min·L⁻¹) in an experiment conducted without the addition of nutrients. The author concluded that for the effluent used in the study, the addition of a medium containing macro and micronutrients was not required. On the contrary, according to the author the nutrient solution hampered the performance of the system.

In contrast, ITO et al. (2005) demonstrated that without the addition of a nutrient solution, the substrate was not completely degraded after 48 h and bacterial growth was not observed in this period for the conversion of glycerol to H₂ in batch mode operation and using the bacterium *E. aerogenes*. In the study, the degradation of glycerol required some additional nutrients. With the addition of a nutrient solution to the process, the glycerol was entirely consumed after 24 h, and a H₂ yield of 0.89 mol H₂/ mol of glycerol was obtained.

According to some studies, the compounds Mg, Na, Zn, and Fe are the trace elements that most affect the production of H₂, the first being the most important of all. In studies by Lin and Lay (2005), the concentrations (mg/dm⁻³) of these nutrients that led to maximum production H₂ were: 120 MgCl₂ · 6H₂O; 1000 NaCl; 0.5 ZnCl₂ and 3 FeSO₄ · 7H₂O. A 50% increase or decrease in magnesium resulted in a 40% reduction in the hydrogen production, while an increase or reduction of this same percentage for iron caused a 20% reduction in the production of hydrogen (LIN and LAY, 2005).

On the contrary, ALSHIYAB et al. (2008a) observed an inhibition of the production of H₂ when magnesium in the form of MgSO₄ · 7H₂O was added as a nutrient to a fermentative process in batch cultures containing 5 g/L of glucose. Concentrations ranging from 0 to 1000 mg/L MgSO₄ · 7H₂O
were investigated as the concentration of biomass was gradually increased in the reactors, reaching a maximum of 1466 g/L, but no significant increase in the production of H₂ was observed.

In the degradation of glycerol using the bacterium *Enterobacter aerogenes*, Jitrwung et al., (2011) suggested replacing the commonly used ammonium sulfate by ammonium nitrate and reducing the concentrations of some salts, such as FeSO₄ and Na₂HPO₄. The results showed a higher yield of H₂ compared to a study previously reported in the literature and even reduced the costs by 76% with the replacement and reduction of these reagents.

Specifically for nitrogen, studies in the literature emphasize the concentration of ammoniacal nitrogen as source for the production of H₂. We recommend new approaches to study the influence of nitrogen on the production of H₂, evaluating the implementation of other forms of this element in the fermentation process. A disagreement exists regarding the optimal concentration of ammoniacal nitrogen, varying from 0.01 g N/L up to 7.0 g N/L (Wang et al., 2009).

The metal ion Fe²⁺ is another important nutrient in the production of H₂, and this ion is essential in the formation of hydrogenase and other enzymes, and also determines the production of 1,3-propanediol, which is not desired in processes aiming at the production of H₂ (Dabrock et al., 1992; Lee et al., 2001; Alshiyab et al., 2008a).

The influence of phosphates and carbonates are also important to the production of H₂. These compounds are used as nutrients in biological processes and also provide buffering capacity that helps to control the variation in pH throughout the process. Lin & Lay (2004) evaluated the effects of the concentration of carbonates and phosphates such as NH₄HCO₃, NaHCO₃, Na₂CO₃, K₂HPO₄, KH₂PO₄ and Na₂HPO₄ in the production of H₂ using anaerobic sludge as an inoculum and sucrose as a substrate. The results suggest an advantage in replacing the carbonate by a phosphate in the form of Na₂HPO₄ as a buffering agent, and the optimal concentration is still 600 mg/L.

Another parameter commonly highlighted as important in anaerobic fermentation processes is the carbon/nitrogen ratio (C/N). In this respect, Lin & Lay (2004) evaluated the production of H₂ under different carbon/nitrogen ratios (ranging from 40 to 130) with carbon concentrations between 6.1-26.5 g COD/L. In these studies, the concentrations of carbon included both the concentrations of COD sewage sludge (5.2 g/L) as well as the added sucrose solution. The best results were obtained for the C/N ratio equal to 47, producing 4.8 mol H₂/mol sucrose in a continuous reactor with complete mixing using mixed cultures of domestic sewage sludge and the bacterium *Clostridium pasteurianum*.

### 1.6.3 pH

Changes in pH influence the metabolic activity of bacteria producing H₂ and the fermentative process in general because pH affects the activity of the hydrogenase enzyme as well as the metabolic routes. The metabolic pathways for the production of H₂ cause a decrease in pH during the exponential growth phase of the bacteria. (Ding et al., 2008; Fernandes, 2008; Pattra et al., 2008; Rossi et al., 2011) clearly indicating the acidogenic nature of microbiological activity. This drop in pH is important because the lower pH helps in the reduction of methanogenesis. The metabolism of microorganisms changes in the stationary phase. In the exponential phase, the carbohydrates and organic acids are converted to alcohols, such as 1-butanol. As a result of the consumption of acids, the pH of the medium becomes alkaline. Eventually, the concentrations of alcohols reach levels as high as possible, that can inhibit the activities of the bacteria and ceasing its metabolism.

Finally, on the ideal pH for the production of H₂, the majority of studies describe a range between 4 and 7 (Lin et al., 2012; Moncivais et al., 2007). This pH range depends on, for example, the type of culture used in the fermentation process. As an example, Trevisan (2014) developed an experiment with a mixed culture containing 2.5 g/L total volatile solids, and observed that the pH dramatically affected the production of H₂. The specific hydrogenic activity decreased 95% when the value of the initial pH (5.5) was adjusted to 6.0. In the same work, values lower than 5.5 or higher than 6.0 inhibited the action of microorganism producers of H₂ (Trevisan, 2014). However, Choi et al. (2014) showed that the hydrogen produced in this process should be released rapidly from the reactor before other biochemical reactions can consume the hydrogen, and substrates at a high pH level (≥9.0) can be used to produce hydrogen without needing to adjust the pH.
1.6.4 Partial Pressure

When the partial pressure of $H_2$ is relatively low ($10^4$ atm) (GRADY et al., 2011), NADH can be oxidized by hydrogenase, producing $H_2$, at a maximum yield of 4 mol/mol hexose consumed and a maximum yield of ATP. However, in normal conditions most NADH will be oxidized producing reduced final products such as butyrate, which represent a lower molar yield of hydrogen and low yields of ATP. In other studies, KRAEMER & BAGLEY (2006) and CLARK et al. (2012) affirmed that the reduction of the pressure of $H_2$ in the liquid medium, for example, by sprinkling of $N_2$, is important because the concentrations of dissolved $H_2$ and $CO_2$ decreased, reducing the substrate available for acetogenic, as well as methanogenic, bacteria to consume $H_2$. In this way, a drop in the production of $H_2$ is prevented.

In addition to sprinkling $N_2$, other ways to reduce the partial pressure of $H_2$ in liquid medium include: agitation of the liquid medium (CLARK et al., 2012), adoption of negative pressure in the system (MANDAL et al., 2006) and recirculation of effluent (SCHNITZHOFER et al., 2007).

1.6.5 Temperature

Temperature is another important factor in the production of $H_2$ and is related to the speed of biochemical reactions in anaerobic fermentation and, consequently, dissolution of the reagents in the culture medium. For effluent containing biodegraded organic matter, the effect of temperature is not large, however, when the effluent is composed of particulate organic matter, organic compounds or complexes, the effect of temperature on hydrolysis and acidogenesis becomes a concern. Low temperatures require a larger hydraulic detention time for the stabilization of solid biodegradable substances when compared to higher temperatures (GRADY et al., 2011).

An optimal temperature for the production of $H_2$ has not yet been defined, although some studies describe the range among 26 to 40°C (MONCIVAIS et al., 2007; MANGAYIL et al., 2012; GARCIA et al., 2012). The best temperature for the process, as well as the best pH, depends on the type of culture present in the reactor. It has been demonstrated that increasing temperature could increase the ability of hydrogen-producing bacteria to produce hydrogen during fermentative, but temperature at much higher levels could decrease it with increasing levels (WANG & WAN, 2009).

In accordance with the type of culture of microorganisms, the production of $H_2$ can be performed in four schemes: room temperature (15-30°C); mesophilic (30-39°C); thermophilic (50-64°C) and hyper-thermophilic (>65°C) (LEE et al., 2011).

1.6.6 Configurations of reactors and modes of operation

The fermentative process for obtaining $H_2$ can be carried out in a batch or continuous mode. According to Hallenbeck & Ghosh (2009), the continuous mode of operation tends to be more efficient in the production of $H_2$, which is the most commonly accepted mode in industry.

In relation to the reactors, several settings are applied in the production of $H_2$, among which several reactors stand out: simple batch reactors; FBR – fluidized bed reactor; MBR – membrane bioreactor; CSTR – continuous stirred tank reactor; ASBR - anaerobic sequencing batch reactor; UASB- upflow anaerobic sludge blanket, and each of these reactors has advantages and disadvantages. In addition to these reactors, three other types of reactors for high rates were developed: CSABR- Continuous stirred anaerobic bioreactor (WU et al., 2006), CIGSBR - Carrier induced granular sludge bed reactor (LEE et al., 2004; LEE et al., 2006) and the AGSBR - Agitated granular sludge bed reactor (LIN and LAY, 2010).

Reactors with suspended biomass, as in the CSTR, generally are not capable of operating with high dilution rates (hydraulic detention time < 2 h), due to the risk of “washing” the microorganisms from the system, compromising the efficiency of the production of $H_2$ (LIN and LAY, 2010). Lin and Lay (2010) recommend the use of the reactor type CSABR, which produced $H_2$ efficiently with a peak of 362 L.L⁻¹.d⁻¹ and yield of 3.5 mol H₂/mol sucrose with a hydraulic detention time of 0.5 h and a high concentration of sucrose (40 g COD/L).

2 CONCLUSION

The microbiological production of $H_2$ is a sustainable biotechnological technique that can be used agro-industrial wastes for the fermentation and ensures the production of low-cost energy. Although further studies are needed on the cost-benefit profile of this technique, the identification of microorganisms and the improvement of cultivation
conditions are the first steps for a program to optimize the production of H₂ through genetic approaches. In addition, due to its complexity, this process depends on several factors that confound the notation of optimum conditions, requiring the researcher to possess detailed knowledge of the metabolic pathways involved, the type and the concentration of microorganisms present in the fermentation, and the nutritional characteristics of each substrate, as well as the inter-relationships between each of the factors presented in this article. Thus, in practice the organic production of H₂ using different substrates and microbial cultures should require operating conditions that may vary significantly. However, the major concern in relation to the production of H₂ via microbiological techniques is obtaining processes that reliably transfer to industrial scale and generate a constant amount of H₂. For this reason, further studies need to investigate the organic production of H₂ from different substrates, wastes and microorganisms to find an ideal technique that can be used for commercial purposes.

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3 REFERENCES


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