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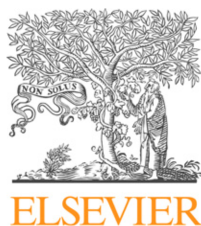

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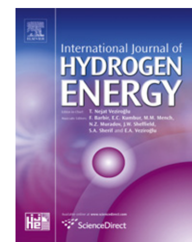

Inhibition of fermentative hydrogen production by lignocellulose-derived compounds in mixed cultures

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ABSTRACT

Dark fermentation using mixed cultures is an attractive biological process for producing hydrogen (H_2) from lignocellulosic biomass at a low cost. Physicochemical pretreatment is generally used to convert lignocellulosic materials into monosaccharides. However, the processes also involved release degradation byproducts which can, in turn, inhibit microbial growth and metabolism and, hence, impact substrate conversion. In this study, the impact on H_2 production of lignocellulose-derived compounds (*i.e.* furan derivatives, phenolic compounds and lignins) was assessed along with their effect on bacterial communities and metabolisms. Batch tests were carried out using xylose as model substrate (1.67 $mol_{H2}\,mol_{xylose}^{-1}$ in the control test). All the putative inhibitory compounds showed a significant negative impact on H_2 production performance (ranging from 0.34 to 1.39 $mol_{H2}\,mol_{xylose}^{-1}$). The H_2 yields were impacted more strongly by furan derivatives (0.40–0.51 $mol_{H2}\,mol_{xylose}^{-1}$) than by phenolic compounds (1.28–1.39 $mol_{H2}\,mol_{xylose}^{-1}$). Except for the batch tests supplemented with lignins, the lag phase was shorter for inhibitors having the highest molecular weight (8 days versus 22 days for the lowest MW). Variability of the lag phase was clearly related to a shift in bacterial community structure, as shown by multivariate ordination statistics. The decrease in H_2 yield was associated with a decrease in the relative abundance of several H_2-producing clostridial species. Interestingly, *Clostridium beijerinkii* was found to be more resistant to the inhibitors, making this bacterium an ideal candidate for H_2 production from hydrolyzates of lignocellulosic biomass.
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1. Introduction

Hydrogen (H_2) is one of the most promising energy carriers because it is energetically dense, clean-burning and is sustainable since it can be produced from renewable sources. Among the various kinds of H_2-producing biotechnology, dark fermentation using anaerobic microbial communities is particularly attractive for its ability to produce H_2 at high rates and at a low cost from complex and unsterilized substrates such as solid waste-derived carbon sources [1–3]. Despite

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significant advances in research on dark fermentation, its commercial development has been substantially limited by certain major challenges. These include the use of renewable sources for H_2 production, i.e. from pretreated biomass, and the improvement of H_2 production yields through the development of optimal control of mixed-culture fermentation [4–6].

Lignocellulosic biomass is an abundant renewable resource worldwide representing an attractive, low-cost feedstock for H_2 production. Lignocellulosic biomass contains variable amounts of cellulose (a glucose polymer), hemicellulose (a hexose/pentose heteropolymer with xylose as the major constituent) and lignin (an aromatic organic polymer intertwined amongst cellulosic and hemicellulosic compounds). Recently, H_2 production by dark fermentation from lignocellulosic residues has been shown in mesophilic and thermophilic mixed cultures [6–11]. Due to the complex structure of lignocellulosic materials, pretreatment processes are nevertheless required prior to dark fermentation. Such processes make the main constituents, cellulose and hemicellulose, more accessible to the enzymes that convert carbohydrate polymers into monosaccharides such as glucose or xylose [8,12,13]. The pretreatment methods used to improve dark fermentation of lignocellulosic biomass are classified into four groups: physical (e.g. mechanical disruption), chemical (e.g. alkali, dilute acid), thermal (e.g. steam explosion) and biological (e.g. lignin degradation by white rot fungi) [8,9,13,14].

The existing pretreatment processes for lignocellulosic biomass have a major drawback: the lead to the formation of undesirable byproducts, other than monosaccharides, which reduce the fermentability of the hydrolyzates obtained. Indeed, a broad range of compounds generated during hydrolysis has been previously identified as inhibitors of microbial growth as well as their metabolism, i.e. ethanol fermentation [15–18]. Three groups of inhibitors have been distinguished: organic acids such as acetate generated from hemicellulose degradation; furan derivatives from sugar degradation such as furfural or 5-hydroxymethylfurfural (HMF); and phenolic monomers such as vanillin or syringaldehyde derived from lignin microbial alteration [6,15,19]. These inhibitors can affect microbial growth with three distinct modes of action: undissociated weak organic acids penetrate microbial cells and decrease the intracellular pH, furfural derivatives interfere with glycotic and/or fermentative enzymes, while macromolecules and phenolic compounds are damaging the microbial cellular membranes [15,19].

Clostridial species are generally found as the most abundant bacterial genus in H_2-producing mixed cultures [5,20–22]. In fact, the common use of heat-shock pretreatment of the inoculum leads not only to the removal of H_2-consumers, i.e. methanogens, but also to the selection of sporulating anaerobic H_2-producing bacteria, particularly *Clostridium* spp. Clostridial species are metabolically versatile and degrade a wide range of organic materials including carbohydrates, organic acids, alcohols and aromatic compounds [23]. In contrast to ethanologenic microorganisms (e.g. *Saccharomyces* spp.), carboxylic acids such as acetate have not been reported to significantly inhibit the growth of clostridial species and/or H_2 production [24,25]. To date, the impact of furan derivatives and phenolic compounds on mixed-culture fermentative H_2 production has not been investigated.

Inhibitory concentrations are quite specific to each microorganism, but, generally, 1 g L^{-1} can be considered as the concentration at which a significant impact can be observed on the growth of different types of microorganisms (i.e. yeast or bacteria) and on their H_2 or ethanol production [16,17,25]. Moreover, a concentration of 1 g L^{-1} corresponds to an intermediate concentration found in hydrolyzates generated from lignocellulosic biomass after standard pretreatment using dilute sulfuric acid and at high temperature. Furfural and HMF, for instance, have been retrieved at concentrations less than or equal to 0.1 g L^{-1} for wheat straw [26], about 1 g L^{-1} for corn stalks or cobs [26] or greater than 2 g L^{-1} for softwood [27,28].

The aim of this study was to evaluate the impact on the performance of fermentative H_2 production processes of seven different molecules: furan derivatives (i.e. furfural and HMF), phenolic compounds (i.e. phenol, syringaldehyde and vanillin) and lignins (i.e. kraft and organosolv lignins), presenting different molecular weights at a common concentration of 1 g L^{-1}. The metabolic routes and the bacterial communities involved were also assessed.

2. Materials and methods

2.1. Hydrogen production in batch tests

The H_2 production experiments were carried out in 500 mL glass bottles under batch conditions. An anaerobically digested sludge pretreated by heat shock (90 °C, 10 min) was used as inoculum. Two milliliters of this inoculum (final concentration of 250 mg-COD L^{-1}) were inoculated into the culture media (final working volume of 200 mL) containing 40 mM of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, 5 g L^{-1} of xylose and/or 1 g L^{-1} of the following individual lignocellulose-derived compounds (Table 1): furfural, 5-hydroxymethylfurfural (HMF), vanillin, syringaldehyde, phenol, kraft lignin, organosolv lignin. The initial pH was adjusted to 5.5 using NaOH (1 M). All batch tests were performed in triplicate. After inoculation, each bottle was flushed with nitrogen for 5 min to create anaerobic conditions. The bottles were then capped with a rubber stopper and incubated at 37 °C for 50 days. Two milliliters of the mixed cultures were periodically collected and then centrifuged (20,000*g*, 10 min). The supernatants and the pellets were stored at −20 °C, the supernatants for further chemical analysis and the pellets for DNA extraction.

2.2. Chemical data analysis (biogas, metabolic byproducts)

Biogas volume was periodically measured using an acidified water displacement method. Biogas composition (CH_4, CO_2, H_2 and N_2) was analyzed using a gas chromatograph (Clarus 280, Perkin Elmer) equipped with a HayeSep Q column and a molecular sieve. Operating conditions were as follows: the carrier gas was argon at a pressure of 102 kPa and a flow rate of

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