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Sub-dominant bacteria as keystone species in microbial communities producing bio-hydrogen

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ABSTRACT

Nowadays mixed cultures are considered as a serious alternative to pure cultures in biotechnological processes. Mixed cultures can use various organic substrates and be operated under unsterile and continuous conditions. Although most of studies dealing with fermentative hydrogen production have focused on dominant species, sub-dominant bacteria can also have a significant effect despite their low abundance. The determination of their exact ecological role is essential for better understanding microbial metabolic networks in mixed cultures.

In this work, the contribution of sub-dominant bacteria to fermentative H_2 production was investigated using chemostats continuously fed with a glucose-based medium. Interestingly, *Clostridium pasteurianum* was dominant in six assays on seven at steady state, and only bacterial populations in low abundance differed. Acting as keystone species, these bacteria impacted substantially the microbial metabolic network of the overall ecosystem despite their low abundance. While *Bacillus* spp. and *Lactobacillus* spp. lowered the H_2 yields by diverting a part of the H_2 potential to lactate production, the presence of *Escherichia coli* increased the H_2 yield by redirecting the metabolic network to acetate and butyrate hydrogen-producing pathways.

1. Introduction

Hydrogen (H_2) is considered as one of the most interesting energy carriers in a global context of exploring new sources of renewable energy and reducing greenhouse gases. H_2 has a high energetic yield (122 kJ g^{-1}), can be stored and its combustion produces only water. Nowadays, H_2 is mainly produced by chemical processes such as natural gas reforming or water electrolysis. These processes produce large amounts of H_2 but are high energy-consuming and release high amounts of CO_2 into the environment [1].

Hydrogen can also be produced in biological processes and becomes more environmental friendly. Especially, in anaerobic digestion bioprocesses, H_2 is a key metabolic intermediate which is produced by acetogenic bacteria during fermentation of organic compounds, and is then immediately consumed by microorganisms coupling the oxidation of H_2 to the reduction of more oxidized compounds [2]. Such H_2

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consumption can be avoided by using appropriate operating conditions [3,4]. In addition, fermentative processes operated with mixed bacterial cultures can use a large range of renewable, complex, non-sterile and inexpensive substrates to produce H_2 [5–7]. Compared to pure cultures, these bioprocesses are more robust and susceptible to adapt themselves more easily to changes of environmental conditions [4,7]. Finally, such complex microbial communities are able to provide useful combinations of metabolic pathways for the degradation and the transformation of complex substrates [8].

In counterpart, maximal conversion yields of carbohydrates are limited to 2.5 mol H_2 mol^{-1} hexose under experimental conditions [3], whereas the maximal theoretical yield is 4 mol H_2 mol^{-1} hexose through the acetate pathway. Instability problems of these processes are also often encountered. Instability is mainly due to either a shift of microbial metabolic pathways in response to environmental conditions, or the emergence competitive non-hydrogen-producing bacteria for carbohydrates or direct H_2-consuming bacteria [9].

To limit this instability and thus optimize bioprocesses, many operational parameters have already been tested, such as pH, temperature and hydraulic retention time [10,11]. It is recommended to maintain pH lower than 6.5, and more generally between 5.5 and 6.0 [8,12,13]. Hydrogen production occurs at mesophilic (around 37 °C), thermophilic (around 55 °C) or even hyperthermophilic (60–80 °C) conditions, although most of the studies found in the literature have been carried out under mesophilic conditions [12,14]. When reactors are operated in a continuous mode, especially with glucose as single substrate, a short hydraulic retention time ranging from 2 to 12 h is recommended to avoid the presence of methanogenic *Archaea* which consumes efficiently H_2 and fermentation end-products to produce methane and carbon dioxide [3,10,15]. Another way to limit the presence of H_2-consuming microorganisms is to apply several pre-treatment methods of the inoculum such as heat–-shock treatment, acidic and alkaline treatments and microaerobic treatment. These treatments, especially heat–shock, favor specifically the growth of spore-forming H_2-producing bacteria while H_2-consuming microorganisms are strongly inhibited, such as methanogens or homoacetogenic bacteria.

So far, most of literature studies have focused on dominant hydrogen-producing species such as *Clostridium* spp. Nevertheless, the presence of low abundant bacterial species is always observed in reactors operated under continuous mode, but their exact role is still unknown [3]. These experimental observations are not consistent with the competitive exclusion principle stating that microbial diversity should not be maintained in a chemostat at steady-state, unless the microbial populations are subject to product inhibition, cross-feeding or other direct microbial interactions [16–18]. Thus, low abundant bacteria should participate significantly to ecosystem functioning despite their low abundance, and therefore some of them may be considered as keystone species as defined by Smee [19]. The influence of these keystone species occurs through interactions between microorganisms. These interactions are often trophic: a microorganism is dependent on another microorganism for the degradation of specific substrates or products [17,20], or different microorganisms outcompete for the same substrate [21]. In amensalism cases, a microorganism has an adverse effect on other microorganisms, for example by producing antibiotics or toxic compounds [22]. Other physical or chemical, intra and extracellular mechanisms can also occur, including cellular communication between microorganisms, so called "quorum sensing" or through direct exchange of molecules or electrons *via* cytoplasmic connections, pilis or nanotubes [23,24].

The aim of this study was to determine the ecological role of low abundant bacteria in mixed cultures producing H_2 by dark fermentation. Seven inocula originated from anaerobic sludge, caecotrophs, fermented cassava, or a mixture of them were tested. Heat shock treatment was also used to reduce the microbial diversity in initial inoculum. Hydrogen-producing microbial communities were characterized after 40 hydraulic retention times at steady state in continuous chemostats operated under same conditions.

2. Material and methods

2.1. Inoculum sources

Three different sources of anaerobic inoculum were selected: (i) an anaerobic sludge (AS) was sampled from the outlet of a lab-scale methanogenic reactor fed with wine distillery wastewater, (ii) cassava (Cas) was previously fermented in water and then homogenized with a sterile mixer, and (iii) cæcotrophs (Cæ) (*i.e.*, soft fæces of rabbits) were collected from the digestive system of rabbits and diluted in autoclaved physiological water solution (9 g NaCl L^{-1}). For each inoculum, a heat treatment shock (90 °C, 10 min) was additionally tested (named AS^{ht}, Cas^{ht} and $Cæ^{ht}$). Finally, a mixture of the three inoculum sources (Mix) was prepared in the same ratio of volatile solids (VS).

2.2. Feeding solution

A glucose solution of 10 g L^{-1} was used as sole carbon source in a feeding medium composed by the following nutrients (in mg L^{-1}): K_2HPO_4, 500; NH_4Cl, 2000; yeast extract, 200; HCl 37%, 55; $MgCl_2$, 55; $FeSO_4(NH_4)_2SO_4$, 7; $ZnCl_2$, 1; $MnCl_2$, 1.2; $CuSO_4$, 0.4; $CoSO_4$, 1.3; BO_3H_3, 0.1; $Mo_7O_{24}(NH_4)_6$, 1; $NiCl_2$, 0.05; Na_2SeO_3, 0.01; $CaCl_2$, 60.

The glucose and nutrient solutions were autoclaved separately at 121 °C for 20 min. The feeding solution was prepared daily under sterile conditions in an Esco Labculture Class II Type A2 Biological Safety Cabinet, sparged with nitrogen gas and stored at 4 °C prior to use.

2.3. Experimental set-up

Seven experiments were carried out using the different sources of inoculum (AS, Cas, Cæ, AS^{ht}, Cas^{ht}, $Cæ^{ht}$ and Mix). Continuous stirred tank reactors, with a working volume of 1.5 L, were used. Before each experiment, reactors and sampling tubes were autoclaved at 121 °C for 20 min. Each bioreactor was filled with the feeding solution, and was then inoculated with an inoculum to reach a final volatile solid content of 0.6 g VS L^{-1}. pH was then adjusted at 5.5 using HCl, and the reactor was flushed for 15 min with N_2 to achieve anaerobic conditions. Each assay started with a batch period