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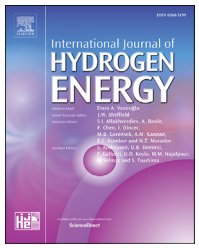

Effect of the nitrogen source on the hydrogen production metabolism and hydrogenases of *Clostridium butyricum* CWBI1009

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ARTICLE INFO

Article history:
Received 28 July 2017
Received in revised form
19 December 2017
Accepted 22 December 2017
Available online 20 February 2018

Keywords:
Biohydrogen
Clostridium
Nitrogen
Hydrogenase
Amino acid

ABSTRACT

Investigations were carried out to determine the effect of various concentrations of organic and ammonium nitrogen sources on fermentative hydrogen production by the strain *Clostridium butyricum* CWBI1009. The results indicated that the H_2-producing metabolism of the strain is favoured within the range (0.56–0.062 g_N/L) of peptone and $(NH_4)_2SO_4$. Optimal overall performance (*i.e.* 1.43 ± 0.08 mol H_2/mol glucose and 1.08 ± 0.03 mL_{H2}/h, respectively) was achieved with 0.062 g_N/L of casein peptone. The study of the amino acid uptake and the gene expression pattern for four [FeFe]-hydrogenases and the nitrogenase showed that nitrogen was in excess in all the experiments with a nitrogen concentration above 0.062 g_N/L and, at that optimal concentration, the expression of the *HydB2* gene would be responsible for the much higher H_2 yield.

Introduction

Hydrogen is regarded as an attractive energy vector for the future due to its clean and direct combustion characteristics. Moreover, a hydrogen-based economy could reduce our dependence on fossil fuels. However, hydrogen is mainly found as part of molecular compounds and currently its production is largely dependent on the use of fossil fuels such as natural gas. This results in the release of huge amounts of non renewable CO_2 and consequently to global warming and environmental degradation [1,2]. Alternatively, hydrogen produced by microorganisms from renewable biomass is promising since the whole process can be considered as carbon neutral [3].

Among the different microbiological routes to hydrogen, dark fermentation using facultative and/or strict anaerobic bacteria has received major attention. Most of the extensive

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https://doi.org/10.1016/j.ijhydene.2017.12.162
0360-3199/

research on this topic has focussed on the advantages of hydrogen production combined with waste treatment [4–7] since the major limiting factor in the scaling up of dark fermentation is the high cost of the main feedstock and some other nutrients to promote growth and metabolism.

Pure sugars are frequently used as the carbon source and casein peptone or yeast extract as the organic nitrogen source. For industrial-scale applications they should be replaced by readily available and cheap carbohydrate-rich residues and ammonia-based substitutes in order to decrease process costs [2,6,8].

Nitrogen-containing nutrients (*e.g.* ammonia, vitamins, and proteins) have a critical impact on fermentation performance since nitrogen is required for cell replication, maintenance, metabolism, and the production of carbohydrate-hydrolysing enzymes [9,10]. Moreover, the literature clearly indicates that acidogens such as *Clostridium* sp. and *Enterobacter* sp. can utilise not only organic nitrogen (*e.g.* proteins, amino acids), but also ammonia [9,11,12]. Therefore, the use of suitable pretreated residues containing ammonia (e.g. animal manure) should also be considered in biohydrogen production, since it is widely available and its disposal represents a significant cost if serious risks of air and water pollution are to be avoided [13]. As a consequence, there is a need to deeply investigate the nitrogen requirements for H_2-producing strains and their impact on bacterial metabolism and major enzymes.

The *Clostridium butyricum* CWBI1009 strain was selected for the study due to its high hydrogen production activity while fermenting different carbon sources (glucose, disaccharides or starch) [14–16]. Moreover, the strain achieved very high performance (up to 3.4 mol H_2/mol glucose) in immobilised cultures [17]. Hydrogen is produced via pyruvate-ferredoxin oxidoreductase (PFOR) and, at a lower extent, via NADH-ferredoxin oxidoreductase (NFOR) [18,19]. Both enzymatic complexes transfer electrons to [FeFe]-hydrogenases which generate hydrogen by a reversible reduction of protons accumulated during fermentation. This mechanism controls the electron flow within the cell and therefore determines the direction of by-product synthesis [20].

However, little is known about the physiological and biochemical properties of clostridial [FeFe]-hydrogenases and their role in the hydrogen metabolism [19,20] since most of the investigations focussed on the commonly known CpI (HydA) from *Clostridium pasteurianum* [21]. *Clostridium butyricum* CWBI1009 possesses four different [FeFe]-hydrogenases, three of which (*hydA2*, *hydB2*, *hydB3*) are monomeric intracellular enzymes and one (*hydA8*) is associated with the heterotrimeric hydrogenase cluster [19,20].

The objectives of this study were to further optimize the nitrogen source for biohydrogen production by the strain *Clostridium butyricum* CWBI1009. This was carried out by monitoring the H_2 yields (mole of H_2 produced per mole of glucose consumed), production rates (mole of H_2 produced per hour) and the formation of soluble by-products in 270 mL batch cultures at different ammonium and peptone concentrations. In addition, particularly relevant conditions were selected for amino acid fingerprinting (HPLC-UV) and expression pattern analysis (RT-qPCR) of the four [FeFe]-hydrogenase genes using specifically designed primers [22,23]. The same was done for the nitrogenase gene since its role in hydrogen production by *Clostridium butyricum* CWBI1009 appears to depend on the environmental conditions [19].

Materials and methods

Bacterial strain and culture media

The strain cultured in this work, *Clostridium butyricum* CWBI1009 (accession no. GU395290), was previously isolated from an anaerobic sludge [14]. The strain was grown in variously formulated buffered synthetic media previously used for growth and hydrogen production with *Clostridium* and *Enterobacteriaceae* [11,14,16]. The MDT culture medium (without the added variable mineral or organic nitrogen source) contained, per litre of deionised water, glucose monohydrate (5g), yeast extract (0.5g), Na_2HPO_4 (5.1g), KH_2PO_4 (1.2g), $MgSO_4{\cdot}7H_2O$ (0.5g), cysteine hydrochloride monohydrate (0.5g). L-cysteine hydrochloride was used to ensure strictly anaerobic conditions in the media and its effect was confirmed by measured redox potential ranging from −200 to −250 mV. To determine the impact on hydrogen production of an organic nitrogen source (casein peptone) compared to an ammonia-based substitute ($(NH_4)_2SO_4$), each of the two sources was used in a separate experiment at 10 concentrations ranging from 0.025 to 2.3 g of total nitrogen/L added to MDT medium (g_N/L). They supplement the basal nitrogen content of 0.055 and 0.034 g_N/L related to the yeast extract and cysteine initial concentration in MDT medium, respectively. The total amount of nitrogen in the medium compounds was calculated according to the supplier's product description and confirmed by measuring the total nitrogen and ammonia content using HACH kits for total nitrogen and ammonia and a HACH DR/2010 spectrometer. All the chemicals used were of analytical or extra pure quality and were supplied by Merck, VWR and Sigma. Casein peptone and yeast extract were supplied by Organotechnie (La Courneuve, France).

Experimental set-up

BHP (Biochemical Hydrogen Potential) tests were carried out to assess the influence of both nitrogen sources in 270 mL serum bottles (200 mL working volume). The sterile carbon source (glucose monohydrate in solution in deionised water) was added separately (to a final concentration of 5 g/L) to prevent Maillard reactions between the carbohydrates and the amino acids. The L-cysteine hydrochloride monohydrate was also added separately to obtain a final concentration of 0.5 g/L.

After the preparation of the MDT medium and adjustment of the pH to 7.3 the bottles were sterilised and cooled down before inoculation with 3 mL suspension from a fresh (incubated for 48 h) preculture tube. The bottles were then capped with a butyl stopper as described by Lin et al. [24] and flushed with nitrogen to remove the remaining oxygen in the gas phase before incubation at 30 °C. Each BHP test was conducted in triplicate at least and all the experimental conditions were monitored until biogas production had completely ceased (*i.e.* 96 h with casein peptone and 144 h with ammonium sulfate).