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Optimization of culture conditions for biological hydrogen production by *Citrobacter freundii* CWBI952 in batch, sequenced-batch and semicontinuous operating mode

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ABSTRACT

Investigations were carried out to determine the effect of the pH, the nitrogen source, iron and the dilution rate (h^{-1}) on fermentative hydrogen production from glucose by the newly isolated strain *Citrobacter freundii* CWBI952. The hydrogen production rate (HPR), hydrogen yield, biomass and soluble metabolites were monitored at 30 °C in 100 mL serum bottles and in a 2.3 L bioreactor operated in batch, sequenced-batch and semicontinuous mode. The results indicate that hydrogen production activity, formate biosynthesis and glucose intake rates are very sensitive to the culture pH, and that additional formate bioconversion and production of hydrogen with lower biomass yields can be obtained at pH 5.9. In a further series of cultures casein peptone was replaced by $(NH_4)_2SO_4$, a low cost alternative nitrogen source. The ammonia-based substitute was found to be suitable for H_2 production when a concentration of 0.045 g/L $FeSO_4$ was provided. Optimal overall performances (ca. an HPR of 33.2 mL H_2/L h and a yield of 0.83 $mol_{H_2}/mol_{glucose}$) were obtained in the semicontinuous culture applying the previously optimized parameters for pH, nitrogen, and iron with a dilution rate of 0.012 h^{-1} and degassing of biogas by N_2 at a 28 mL/min flow rate.

1. Introduction

In recent years policy makers have started looking for alternatives to fossil fuels, not only to counter the threat of global warming, but also to reduce the risk of overdependence on imported oil and gas supplies. The major alternative, nuclear energy, has an inherent problem of waste management. Hydropower is a mature technology, but is subject to site restrictions. Solar and wind power are well developed energy technologies, but are highly susceptible to climatic conditions. By contrast with fossil fuels hydrogen, whether burned directly or used in fuel cells, is intrinsically a clean energy vector with near zero carbon emissions. However the main current method of producing hydrogen, steam reforming of methane, involves the release of large quantities of greenhouse gases. So although hydrogen already accounts for around 2% of world consumption of energy, its more widespread adoption as a fuel is still limited by several challenges [1–3]. Consequently there has been increasing interest in recent years in the biological production of hydrogen using

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microorganisms: mainly algae and bacteria in which the generation of molecular hydrogen is an essential part of the energy metabolism, since it provides a way of eliminating excess electrons [4]. The two main exemplars of such biochemical pathways are photosynthetic microorganisms such as *Chlamydomonas reinhardtii* (oxygenic) or *Rhodobacter sphaeroides* (anoxygenic) and fermentative bacteria such as *Enterobacteriacea* (facultative anaerobe species) or *Clostridium* (obligate anaerobe species) [5–8]. The approach with the greatest commercial potential is fermentative hydrogen generation (dark fermentation) coupled with proton exchange membrane fuel cells (PEMFC). This type of system is very promising since it allows the production of hydrogen from a wide variety of renewable resources such as carbohydrate waste from the agricultural and agro-food industries or processed urban waste and sewage [9,10]. Hydrogen generation from such renewable biomass would reduce our dependence on fossil fuels and decrease carbon dioxide emissions [11,12]. Furthermore dark fermentation process units are feasible at mesophilic temperatures and at pressures requiring very little energy input.

The highest hydrogen production rates (HPR) have been obtained with *Enterobacteriacea,* which use formate – an intermediate in the glucose metabolism – to promote the formation of molecular hydrogen via the catalytic action of formate hydrogen lyase (FHL) [13]. The FHL complex consists of a formate dehydrogenase (FDH-H), a [FeFe]-hydrogenase (HYD-3) and electron transfer mediators such as 2[Fe_4S_4]-ferredoxin and NADH. The degradation of 1 mole of formate by FDH-H produces 2 H^+ mole which are subsequently reduced by the action of HYD-3, providing 1 mol H_2 ($HCOOH \rightarrow H_2 + CO_2$). Reoxidation of the NADH by NADH ferredoxin oxidoreductase followed by the interaction of high potential ferredoxin with HYD-3 produces another mol of H_2 ($NADH + H^+ \rightarrow NAD^+ + H_2$), resulting in a final theoretical conversion yield of 2 $mol_{H_2}/mol_{glucose}$ [14,15]. In practice the experimental yield ranges from 0.37 to 1.9 $mol_{H_2}/mol_{glucose}$ depending on key factors such as pH and temperature [16–19], nitrogen source [20,21], iron concentration [22,23] and, in semicontinuous and continuous cultures, the dilution rate of the medium in the bioreactor [24,25]. Higher yields can be obtained with pure *Clostridium* strains since their theoretical conversion yield is 4 $mol_{H_2}/mol_{glucose}$. However their HPRs are lower than those of *Enterobacteriacea* and the required culture conditions are more difficult to maintain. Furthermore, *Enterobacteriacea* can provide anaerobic conditions without the need for expensive reducing agents [26,27].

The aim of the study described in this paper was to characterize the fermentative hydrogen production of pure *Citrobacter freundii* CWBI952 cultures and determine the optimum conditions for sustainable cost effective production. Initially the effect of pH on hydrogen yields, biomass and metabolite concentrations was investigated in order to find the optimum pH for H_2 production. Subsequent investigations examined outcomes when the casein peptone nitrogen source was replaced with a cheaper ammonia-based source. Finally the effect of the dilution rate was studied in a semicontinuous bioreactor using the ammonia-based nitrogen source and running the reactor vessel with all the previously optimized parameters.

2. Materials and methods

2.1. Isolation of the strain and identification test

The strain was isolated from a sample of cow manure cultured in a medium for isolating sulfate reducing bacteria (Postgate's medium E [28,29]) and it rapidly disrupted the agar. One mL of inoculum was successively diluted in 9 mL of sterile peptoned water (consisting of: 2 g/L Tween 80, 5 g/L NaCl and 1 g/L casein peptone). One mL of each dilution was then added to 25 mL sterile tubes and mixed with 24 mL of Postgate's medium E maintained in fluid state at 43 °C. After incubation at 30 °C the tube was broken at a convenient point; a white colony was withdrawn with a platinum loop and transferred successively on to agar plates prepared with PCA medium (containing 1 g/L glucose monohydrate, 5 g/L casein peptone, 2.5 g/L yeast extract and 15 g/L agar). Isolated colonies developed after 1 day of incubation and one of them was then transferred to 250 mL serum bottles for BHP tests (as described in Material and Methods, see Section 2.3.). Based on the fact that the volume and hydrogen content of the biogas produced by the different samples were similar (i.e. 80 ± 2 mL and $51 \pm 4\%$), the cultures were considered to contain pure strains. Identification was carried out by 16S rRNA gene amplification and sequencing. Bacterial cell lysates were used to amplify the 16S rRNA gene with universal bacterial primers 16S27F and 16S1492R in a 50 μL reaction volume under the following conditions: initial denaturation at 94 °C during 5 min, followed by 36 cycles with denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min and elongation at 72 °C for 2 min. The PCR products of the correct size obtained in this way were purified with the GeneJET™ PCR Purification Kit (Fermentas). Sequences were determined by GIGA (Genomic Facility, Liège, Belgium) using the 27F and 1492R primers.

2.2. Composition of the fermentation media

The growth of the strain was carried out in different modified synthetic media adapted from Ueno [30] and widely used for anaerobic bacterial growth and biological production of hydrogen by *Enterobacteriaceae* and *Clostridium*. The composition of the media was changed depending on the parameters being studied. The standard synthetic medium A was rich in organic nitrogen. This medium was used to determine the optimum pH and the fermentation profile for the strain and as a control test condition in the serum bottle experiments. It contained: 5 g/L glucose monohydrate, 5 g/L casein peptone, 0.5 g/L yeast extract, 2 g/L KH_2PO_4 and 0.5 g/L $MgSO_4 \cdot 7H_2O$. Medium B was used in serum bottles to study the effect of using an ammonia-based nitrogen source on hydrogen production (with an equivalent N content and replacing $MgSO_4$ with $MgCl_2$ to avoid higher SO_4 concentration compared to the former medium). It consisted of: 5 g/L glucose monohydrate, 6 g/L $(NH_4)_2SO_4$, 0.5 g/L yeast extract, 2 g/L KH_2PO_4 and 0.4 g/L $MgCl_2 \cdot 6H_2O$. Medium C, which was used to investigate the effect of iron when using an ammonia-based nitrogen source, had the same composition as medium B except for the addition of 0.125 g/L $FeSO_4$. The first batch sequence of the sequenced-batch cultures in the 2.3 L