Bioresource Technology 128 (2013) 513–517

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

High yield single stage conversion of glucose to hydrogen by photofermentation with continuous cultures of *Rhodobacter capsulatus* JP91

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HIGHLIGHTS

- ▶ Photofermentation of glucose was carried out in a single stage continuous culture.
- ▶ Yields varied with dilution rate (HRT).
- ▶ The highest yield, 9.0 ± 1.2 mol H_2/mol glucose was 75% of theoretical.
- ▶ There is room for improvement in light conversion efficiency.

ARTICLE INFO

Article history:
Received 11 August 2012
Received in revised form 22 October 2012
Accepted 22 October 2012
Available online 31 October 2012

Keywords:
Photofermentation
Rhodobacter capsulatus
Hydrogen production
One stage chemostat

ABSTRACT

Photofermentative hydrogen (H_2) production from glucose with the photosynthetic bacterium *Rhodobacter capsulatus* JP91 (hup$^-$) was examined using a photobioreactor operated in continuous mode. Stable and high hydrogen yields on glucose were obtained at three different retention times (HRTs; 24, 48 and 72 h). The H_2 production rates, varying between 0.57 and 0.81 mmol/h, and optical densities (OD_{600nm}) were similar for the different HRTs examined. However, the rate of glucose consumption was influenced by HRT being greater at HRT 24 h than HRTs 48 and 72 h. The highest hydrogen yield, 9.0 ± 1.2 mol H_2/mol glucose, was obtained at 48 h HRT. These results show that single stage photofermentative hydrogen production from glucose using photobioreactors operated in continuous culture mode gives high, nearly stoichiometric yields of hydrogen from glucose, and thus is considerably more promising than either two stage photofermentation or co-culture approaches.

1. Introduction

The purple non-sulfur photosynthetic bacteria are well known for their capacity to produce hydrogen from organic acids when grown under photoheterotrophic conditions with limiting nitrogen, a process called photofermentation. Hydrogen evolution under these conditions is catalyzed by nitrogenase, which normally functions to catalyze the reduction of dinitrogen to ammonia with the release of one H_2 per N_2 reduced. In the absence of other reducible substrates, nitrogenase continues to turnover reducing protons to hydrogen. Hydrogen production under these conditions is apparently a response to the metabolic need to maintain redox balance (Masepohl and Hallenbeck, 2010).

Light plays a key role in providing the required energy input, both high energy electrons and ATP (4 ATP/H_2), needed to drive substrate conversion to hydrogen to completion (Adessi and De Philippis, 2012; Hallenbeck, 2011; Keskin et al., 2011). Captured light energy is used to produce chemical energy (i.e. a proton gradient), which in turn is used both to drive reverse electron flow to nitrogenase, and for ATP synthesis. This energy allows in principle the complete irreversible oxidation of substrate.

Many studies have examined hydrogen production with *Rhodobacter* species growing on organic acids as substrate, converting typical fermentation products acetic, lactic, propionic, malic and butyric acids to H_2 and CO_2 under anaerobic conditions in the light. Dark, hydrogen producing fermentations convert sugars to hydrogen at maximum yields of only 33%, giving as byproducts acetate and butyrate (Abo-Hashesh and Hallenbeck, 2012a; Hallenbeck, 2009, 2012), compounds which can be completely oxidized to H_2 and CO_2 by photofermentation. Consequently, photofermentation is an attractive method for the total conversion of feedstocks that are only partially oxidized during dark fermentation and hence increases the yield of hydrogen from those substrates (Adessi et al., 2012; Hallenbeck, 2011, 2012; Hallenbeck et al., 2012; Keskin and Hallenbeck, 2012a).

Therefore several systems have been under investigation using photofermentation to extract additional hydrogen from the organic

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http://dx.doi.org/10.1016/j.biortech.2012.10.091

acids produced by dark fermentation, including co-culturing and two stage systems. For example, the Hyvolution project carried out studies of such a potential two stage process and showed that indeed additional hydrogen could be obtained with a second stage photofermentation, with a combined yield of 5–7 mol of hydrogen per mole of hexose (Claassen and de Vrije, 2006; Claassen et al., 2010; Özgür et al., 2010).

However, the photosynthetic bacteria have a versatile metabolism and can use a broad spectrum of reduced organic substrates as electron donors and carbon source while growing photoheterotrophically; some species can even grow photolithoautotrophically using H_2 as electron donor and CO_2 as the sole carbon source (Klemme, 1968). In fact, it has recently been shown that the photosynthetic bacterium, *Rhodobacter capsulatus*, is even capable of the microaerobic production of hydrogen from organic acids (Abo-Hashesh and Hallenbeck, 2012b). Early studies noted the capacity of some photosynthetic bacteria to grow photoheterotrophically on simple sugars, such as glucose, fructose and sucrose (van Niel, 1944). We have been exploring the direct conversion of sugars, glucose and sucrose, to hydrogen by photofermentation as an alternative to the co-culture or two step processes mentioned above. In an initial study using *R. capsulatus* in batch cultures, a maximum yield of 3.3 mol H_2/mol glucose was obtained (Abo-Hashesh et al., 2011), considerably higher than some previous studies where yields of 0.56 with *R. sphaeroides* (Fang et al., 2006) or 0.9 with *Rubrivivax gelatinosus* (Li and Fang, 2008) were obtained under similar conditions. We optimized the batch process using response surface methodology (RSM) and were able to increase the yield to 5.5 mol H_2/mol glucose (Ghosh et al., 2012). These studies have been extended to show that it is possible to obtain 4–5 mol H_2/mol hexose from sugar industry wastes in a batch process (Keskin and Hallenbeck, 2012b).

Even though these yields are higher than previously reported for single stage photofermentative conversion of sugars, and are close to those found with a much more complicated two stage system, they are still low, 50% or less than the chemically available hydrogen (12 mol H_2/mole glucose). However, in all cases photofermentation of glucose was carried out using a batch process, where growth rates and cell densities, which affect light availability, vary over the production period. Hence it was of interest to examine photofermentation of glucose with continuous cultures. Here we report on photofermentative hydrogen production from glucose by *R. capsulatus* in an illuminated chemostat operated at different retention times, 24, 48 and 72 h. We were able to define conditions under which 75% of the consumed glucose was converted to hydrogen, thus increasing the attractiveness of single stage sugar photofermentation.

2. Methods

2.1. Bacterial strain and pre-culture conditions

The purple non-sulfur photosynthetic bacterium *R. capsulatus* (JP91) hup$^-$ strain, kindly provided by Dr. John Willison, was routinely maintained in screw cap tubes (16 mm by 125 mm) completely filled with RCV medium (which contained 30 mM ammonium sulfate, 35.7 mM lactate, 19 mM phosphate buffer (pH 6.8) and incubated at 30 °C in an Biotronette Mark III environmental chamber (Lab-line Instruments) equipped with three 150 W incandescent bulbs. *R. capsulatus* JP91 was pre-cultured in RCV medium supplemented with 55.5 mM glucose as a carbon source and 7 mM sodium glutamate as N-source, which allows growth and nitrogenase derepression. Cells were cultured under anaerobic conditions with illumination overnight until inoculation into the bioreactor vessel containing RCV with the same composition.

2.2. Experimental setup for continuous photofermentation of glucose

Photofermentation experiments were carried out using an automated chemostat, BioFlo® C30 (500 ml vessel capacity, 7.5 cm diameter), New Brunswick Scientific Co. Inc. A pre-culture of *R. capsulatus* JP91 was used to inoculate (10% v/v) RCV medium that had been sterilized with the reactor vessel. Anaerobic conditions were achieved by flushing with argon at a flow rate of 5 ml/min. The culture was incubated with continuous agitation (250 rpm) under constant illumination using one 50 W incandescent bulb, while the temperature was maintained at 30 °C. RCV medium was supplied at the appropriate flow rates to give retention times (HRT) of 24, 48 and 72 h, respectively. The total culture volume in the bioreactor was maintained at 350 ml.

2.3. Analytical procedures

Gas and liquid sampling were carried out daily. Hydrogen was measured by injecting 50 µl of the effluent gas into a Shimadzu GC-8A chromatograph equipped with a thermal conductivity detector and a 2 m column packed with molecular sieve 5A with argon as carrier gas. The oven was maintained at 60 °C and the flow rate at 25 ml/min. Hydrogen production rates were calculated according to the argon sparging flow rate. Bacterial growth was determined by measuring the OD_{600nm} using a double-beam spectrophotometer (Shimadzu). Correlation of these readings with dry weight measurements (CDW) gave the relationship, CDW (mg/ml) = 0.55 × OD_{600nm}. The concentration of residual glucose was determined by the phenol–sulfuric acid assay (Masuko et al., 2005).

2.4. Statistical analyses

Data are presented in terms of mean and standard deviation using the data analysis tools of Microsoft Excel and SPSS version 17. One-Way ANOVA analysis was used to test the significance between variables at $P < 0.05$. P-value was set at <0.05 for significant results and <0.01 for highly significant results.

3. Results and discussion

3.1. Growth and operational stability

A late log phase culture of *R. capsulatus* JP91 was used to inoculate (10% v/v) the C30 bioreactor which was incubated with illumination under anaerobic conditions until growth reached a nearly constant value of 4–5 OD_{600nm} (corresponding to 2.2–2.8 mg CDW/ml). Three different retention times were used (24, 48 and 72 h) over 15 days (315 h, continuous operation) starting with 48 h HRT followed by 72 h HRT and then 24 h HRT. At steady state this process is governed by standard chemostat theory (Eq. (1)):

$$dC_c/dt = (\mu - D)C_c \tag{1}$$

where dC_c/dt is the change in cell concentration with time (=0 at steady state); Cc, cell cencentration; μ, specific growth rate; D, dilution rate.

Cell density (OD_{600nm}) did not show very significant variations throughout the entire process at each HRT and only varied slightly between different dilution rates (14.6, 7.3 and 4.9 ml/h) ($P = 0.043$) (Fig. 1A), showing that the cultures were at steady state and that therefore their growth rate was being controlled by the dilution rate as expected (Eq. (1)). Moreover, the reactor was in the range of stable operation; i.e. above the threshold for washout which oc-